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Molecular phylogeny based on the 16S rRNA gene of elite rhizobial strains used in Brazilian commercial inoculants

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Abstract

Nitrogen is often a limiting nutrient, therefore the sustainability of food crops, forages and green manure legumes is mainly associated with their ability to establish symbiotic associations with stem and root-nodulating N_2-fixing rhizobia. The selection, identification and maintenance of elite strains for each host are critical. Decades of research in Brazil resulted in a list of strains officially recommended for several legumes, but their genetic diversity is poorly known. This study aimed at gaining a better understanding of phylogenetic relationships of 68 rhizobial strains recommended for 64 legumes, based on the sequencing of the 16S rRNA genes. The strains were isolated from a wide range of legumes, including all three subfamilies and 17 tribes. Nine main phylogenetic branches were defined, seven of them related to the rhizobial species: *Bradyrhizobium japonicum*, *B. elkanii*, *Rhizobium tropici*, *R. leguminosarum*, *Sinorhizobium meliloti*/*S. fredii*, *Mesorhizobium ciceri*/*M. loti*, and *Azorhizobium caulinodans*. However, some strains differed by up to 35 nucleotides from the type strains, which suggests that they may represent new species. Two other clusters included bacteria showing similarity with the genera *Methylobacterium* and *Burkholderia,* and amplification with primers for *nifH* and/or *nodC* regions was achieved with these strains. Host specificity of several strains was very low, as they were capable of nodulating legumes of different tribes and subfamilies. Furthermore, host specificity was not related to 16S rRNA, therefore evolution of ribosomal and symbiotic genes may have been diverse. Finally, the great diversity observed in this study emphasizes that tropics are an important reservoir of N_2-fixation genes.

Keywords: *Azorhizobium*; Biological nitrogen fixation; *Bradyrhizobium*; *Burkholderia*; *Methylobacterium*; *Rhizobium*; *Sinorhizobium*; 16S rRNA gene

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Introduction

Divergence within the family Leguminosae (Fabaceae in the USA) is estimated to have occurred over 65 million years ago, such that over 18,000 species are now classified into around 650 genera, occupying nearly all terrestrial biomes [18,34]. The wide use of legumes as food crops, forages and green manures is mainly associated with their ability to establish symbiotic associations with stem and root-nodulating N_2-fixing bacteria, collectively called rhizobia [2]. These bacteria are among the most intensely studied groups of microorganisms [38] mainly due to their potential to replace N-fertilizers, with emphasis on their key role in achieving sustainability of tropical N-poor soils.

Nodules generally occur in the subfamilies Mimosoideae and Papilionoideae, and are rare in the Caesalpinioideae; recent information indicates that about 3000 taxa are capable of nodulating and 400 taxa are not; however, information is lacking for nearly 40% of the genera [21]. Until the early 1980s, all bacteria isolated from root nodules were classified into the genus *Rhizobium*, and speciation was based on nodulation with certain host plants, establishing the "cross-inoculation group" concept [13]. After that, numerical taxonomy considering several properties led to the definition of a new genus, *Bradyrhizobium*, and the renaming of some other species [22]. Particularly in the last decade, ribosomal sequences with emphasis on the 16S rDNA have become the basis of bacterial molecular phylogeny and taxonomy [14] leading to the description of new rhizobial genera and species. Currently, rhizobia are positioned in four deep branches, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium-Sinorhizobium-Allorhizobium*, and non-symbiotic relatives within those branches may indicate common ancestry for rhizobial species and other parasitic or soil-borne bacteria [14,48]. However, in comparison to the number of nodulating-legume species, very few rhizobial species have been described.

In Brazil, economical and agronomic benefits have been achieved with several legumes due to research efforts focused on N_2 fixation. Since 1975, the government demands that inoculants commercialized in the country contain only strains recommended by Brazilian public research institutions. To enforce the recommendation, the RELARE (= Rede de Laboratórios para a Recomendação de Estirpes de *Rhizobium*) was created in 1985, by the initiative of the Microbial Resources Centre Network (MIRCEN), establishing a net of laboratories with the objective of identifying the most effective rhizobial strains for each legume species. Since then, the maintenance of the strains and their distribution to the inoculant industry has been a responsibility of the "*Rhizobium* Culture Collection SEMIA" (Seção de Microbiologia Agrícola) (IBP World Catalogue of *Rhizobium* Collections no. 443 in the WFCC World Data Center on Microorganisms), at the Fundação Estadual de Pesquisa Agropecuária [11].

Searching for the most effective rhizobial strains is a time-consuming process involving the production of thousands of rhizobial cultures, and many greenhouse experiments and field trials. In the case of soybean (*Glycine max*) alone, the benefits resulting from the use of inoculants with selected superior strains is equivalent to about US$ 3 billion per cropping season, that otherwise would be spent on the purchase, transportation and application of N-fertilizers [19]. However, despite the considerable effort expended in selecting effective strains for almost a hundred legumes, their genetic characterization and taxonomic position is still poor. In this context, this study aimed at gaining better understanding of phylogenetic relationships, based on the sequencing of the whole 16S rRNA gene, of 68 elite rhizobial strains recommended for 64 legumes in Brazil.

Material and methods

Strains used

Sixty-eight strains from the Brazilian (SEMIA) culture collection of rhizobia were selected. Table 1 provides information of the strains, as well as of the host plants from which they were isolated and for which they are recommended. In total the strains represent 94 recommendations for commercial inoculants. Strains were provided by FEPAGRO and their purity was verified on yeast extract-mannitol agar (YMA) medium [45] containing Congo red (0.00125%). Stocks were prepared on YMA and kept at −70 °C (under 30% of glycerol) for long-term storage and at 4 °C as source cultures.

Morpho-physiological characterization

Colony morphology (color, mucosity, diameter, transparency, borders, elevation) and acid/alkaline reaction were evaluated as described previously [45] after 7 days of growth in the dark, at 28 °C, on YMA containing either Congo red or bromothymol blue (0.00125%) as a pH-change indicator.

DNA extraction

Total genomic DNA of each strain was extracted from bacterial batch cultures grown in YM broth until late exponential phase (10^9 cells mL^{-1}). Extraction of DNA was performed as described before [12] and to obtain clean DNA samples the extraction procedure included the addition, for each 400 μL of bacteria

Table 1. Information about the strains recommended for the use in Brazilian commercial inoculants and which DNA was sequenced in this study

Plant species[a,b]	Some common names[c]	Subfamily[b]	Tribe[b]	Descriptor[c]	Main use in Brazil	Applications worldwide[c]	SEMIA number	Other designations[d,e]	Institution[f] recommendding[e]	Basis[g]
Acacia auriculiformis Benth.	Earpod wattle, blackwattle, acácia[h]	Mimosoideae	Acacieae	Perennial non-climbing tree	Tree	Environment, wood	6387	BR 3609, LMG 9961	Embrapa Agrobiologia	IV
							6391	BR 3624	Embrapa Agrobiologia	IV
Acacia decurrens Willd.	Green wattle, acácia da Austrália[h]	Mimosoideae	Acacieae	Perennial non-climbing tree	Tree	Environment, wood	6164	BR 3608, LMG 9960	Embrapa Agrobiologia	II
							6390	BR 3614	Embrapa Agrobiologia	II
Acacia mangium Willd.	Mangium, silk leaf acacia, akasia, acácia mangium[h]	Mimosoideae	Acacieae	Perennial non-climbing tree	Tree	Environment, wood	6387	BR 3609 LMG 9961	Embrapa Agrobiologia	IV
							6420	BR 3617, LMG 9965	Embrapa Agrobiologia	IV
Acacia mearnsii De Wild.	Black wattle, acacia negra[h]	Mimosoideae	Acacieae	Perennial non-climbing shrub or tree	Tree	Environment, chemical, wood	6163	BR 3607	Embrapa Agrobiologia	II
							6164	BR 3608	Embrapa Agrobiologia	II
Albizia lebbeck (L.) Benth.	Rain tree, woman's tongue, bois noir, baile de caballero, lengua de mujer, coração de negro[h], faveiro[h], pau preto[h], acácia[h]	Mimosoideae	Ingeae	Perennial non-climbing tree	Tree	Environment, chemical, toxins, wood	6160	BR 5610	Embrapa Agrobiologia	III
Arachis hypogaea L.	Peanut, groundnut, cacahoute, amendoim[h]	*Papilionoideae*	Aeschynomeneae	Annual non-climbing herb	Grain	Food, medicine, forage, environment	6144	SMS 400, USDA 3187, MAR 11	IAC	IV
Arachis pintoi Krapov. and W. Gregory	Forage peanut, amendoim forrageiro[h]	Papilionoideae	Aeschynomeneae	Perennial non-climbing herb	Forage (tropical),	Forage, environment	6440	MGAP 13	EPAMIG/Embrapa Cerrados	IV
Cajanus cajan (L.) Millsp.	Pigeonpea, Congo pea, pois cajan, frijol de arbol, lenteja, guandu[h]	Papilionoideae	Phaseoleae	Annual/perennial, non-climbing herb or shrub	Forage (tropical)	Forage environment, food, medicine, wood	6156	CPAC-IJ, DF F-2	Embrapa Cerrados	IV
							6157	BR 2801	Embrapa Agrobiologia	II
Calopogonium spp.	Calopo, calopogônio[h]	Papilionoideae	Phaseoleae	Perennial climbing or non-climbing herb	Green manure	Cover crop, green manure, forage	6152	BR 1602	Embrapa Agrobiologia	IV
Canavalia ensiformis (L.) DC.	Jackbean, swordbean, horsebean, pois de sabre, dolique, cocorico, haba criolla, feijão de porco[h]	Papilionoideae	Phaseoleae	Annual/perennial, climbing or non-climbing herb or shrub	Green manure	Cover crop, green manure, forage, food	6156	CPAC-IJ, DF F-2	Embrapa Cerrados	III
							6158	CPAC 42	Embrapa Cerrados	III
Centrosema spp.	Centrosema, fleur languette, pois piante, choncho, conchitas, centrosema[h]	Papilionoideae	Phaseoleae	Perennial climbing or non-climbing herb	Forage (tropical)	Forage, environment	6146	BR 1808	Embrapa Agrobiologia	III
							6424	CPAC L36	Embrapa Cerrados	IV
							6425	CIAT 2380	Embrapa Cerrados	IV

Table 1. (*continued*)

Plant species[a,b]	Some common names[c]	Subfamily[b]	Tribe[b]	Descriptor[c]	Main use in Brazil	Applications worldwide[c]	SEMIA number	Other designations[d,e]	Institution[f] recommendding[e]	Basis[g]
Cicer arietinum L.	Chickpea, pois chiche, garbanzo, grão de bico[h]	Papilionoideae	Cicereae	Annual non-climbing herb	Grain	Food, forage, medicine	396	TAL 1148, USDA 3100, Nit 27A3	FEPAGRO/UFRGS	IV
Clitoria fairchildiana R. Howard	Butterfly pea tree, faveira, palheteira, palheiro[h], sombreiro[h]	Papilionoideae	Phaseoleae	Perennial non-climbing tree	Tree	Environment, medicine, wood	6412	BR 8005	Embrapa Agrobiologia	IV
Clitoria ternatea L.	Pigeon wings, pois sauvage, blue pea, azulejo, campanilla, conchita blanca o azul, zapatico de la reina, clitoria[h]	Papilionoideae	Phaseoleae	Perennial climbing herb or shrub	Tree	Environment, food, chemical, wood, forage, toxins	6053	TAL 827, UMKL I28	Embrapa Agrobiologia	II
Crotalaria juncea L.	Sunnhemp, cascavelle, grand sonnette, grand tcha-tcha, crotalaria[h]	Papilionoideae	Crotalarieae	Annual non-climbing herb	Green manure	Fibre, environment, firewood	6145	BR 2001	Embrapa Agrobiologia	II
Crotalaria juncea L.	Sunnhemp, cascavelle, grand sonnette, grand tcha-tcha, crotalaria[h]	Papilionoideae	Crotalarieae	Annual non-climbing herb	Green manure	Fibre, environment, firewood	6156	CPAC-IJ, DF F-2	Embrapa Cerrados	IV
Crotalaria spectabilis Roth	Rattle Box, showy rattlepod, cascavelle jaune, sonnette, maraquita, crotalaria[h]	Papilionoideae	Phaseoleae	Annual non-climbing herb or shrub	Green manure	Fibre, environment, firewood, toxins	6156	CPAC-IJ, DF F-2	Embrapa Cerrados	IV
							6158	CPAC 42	Embrapa Cerrados	IV
Cyamopsis tetragonoloba (L.) Taubert	Clusterbean, guar bean, guar gum, feijão guarda[h], guar[h]	Papilionoideae	Indigofereae	Annual non-climbing herb	Green manure	Environment, chemical, food, forage	6145	BR 2001	Embrapa Agrobiologia	II
							6319	NC 92, SMS 561	IAC	II
Dalbergia nigra (Vell. Conc.) Benth.	Brazilian rosewood, jacaranda, palisandre, cabiúna[h], jacarandá-da-Bahia[h]	Papilionoideae	Dalbergieae	Perennial non-climbing tree	Tree	Environment, chemical, wood	6101	BR 8404	Embrapa Agrobiologia	III
Desmodium incanum DC. *(D. canum)*	Spanish clover; wild peanut, colle-colle, herbe gallon, cepa de caballo, collant, pega pega; desmódio[h]	Papilionoideae	Desmodieae	Perennial non-climbing herb or shrub	Forage (tropical)	Environment, forage, medicine	6028	TAL 569, MAR 472	FEPAGRO/UFRGS	II
Desmodium intortum (Miller) Urban	Beggarlice, copal de coche, pega pega, zarza blanca, amor seco, desmódio[h]	Papilionoideae	Desmodieae	Perennial non-climbing herb or shrub	Forage (tropical)	Environment, forage	656	SEMIA original	FEPAGRO/UFRGS	II
Desmodium heterocarpon *(L.) DC. subsp.* Ovalifolium *(Prain) Ohashi*	*Desmodium ovalifolium*, desmodium, desmódio[h]	Papilionoideae	Desmodieae	Perennial non-climbing herb or shrub	Forage (tropical)	Environment, forage	6208	CIAT 2372	CEPEC	III
Desmodium uncinatum (Jacq.) DC.	Silverleaf desmodium, desmódio[h]	Papilionoideae	Desmodieae	Perennial non-climbing herb or shrub	Forage (tropical)	Environment, forage	696	CB 627	FEPAGRO/UFRGS	III
		Mimosoideae	Ingeae		Tree		6159	BR 4406		III

Enterolobium contortisiliquum (Vell. Conc.) Morong	Earpod tree, oreja de negro, timbó[h], tamboril do campo[h]; orelha de negro[h], orelha de macaco[h], timbaúba[h], timbaúva[h]			Perennial non-climbing tree		Environment, medicine, toxin, wood			Embrapa Agrobiologia	
Enterolobium cyclocarpum (Jacq.) Griseb.	Devil's ear, earpod tree, elephant's ear, monkey ear, bois tanniste rouge, oreille d'éléphant, guanacaste, orelha-de-elefante[h]	Mimosoideae	Ingeae	Perennial non-climbing tree	Tree	Environment, medicine, pasture, wood, food	6159	BR 4406	Embrapa Agrobiologia	IV
Enterolobium timbouva Martius	Timbor, tamboril[h], timbaúva[h], timboúva[h]	Mimosoideae	Ingeae	Perennial non-climbing tree	Tree	Environment,medicine, toxin, wood	6159	BR 4460	Embrapa Agrobiologia	IV
Erythrina verna Vell. Conc.	Mulungu, cristi galli, mulungu[h], suinã[h], suinã da serra[h]	Papilionoideae	Phaseoleae	Perennial, non-climbing tree	Tree	Environment, wood	6100	BR 5609	Embrapa Agrobiologia	II
Falcataria moluccana (Miq.) Barneby and Grimes	Albizia falcataria, molucca albizia, batai wood, sau, malesia, albizia[h]	Mimosoideae	Ingeae	Perennial non-climbing tree	Tree	Environment, wood, food	6100	BR 5609	Embrapa Agrobiologia	IV
							6169	BR 5612	Embrapa Agrobiologia	III
Galactia striata (Jacq.) Urban	Galactia, guatabito, frijolillo, galáxia[h], galáctia[h], amendoim de veado[h]	Papilionoideae	Phaseoleae	Perennial climbing herb	Forage (tropical)	Forage, environment	6149	CB 627, SMS 138	IAC	II
							6150	SMS 300	IAC	II
Gliricidia sepium (Jacq.) Walp.	Grow stick, cacahuananche, madricacao, gliricidia[h]	Papilionoideae	Robinieae	Perennial non-climbing tree	Tree	Environment, wood	6168	BR 8801, LMG 10132	Embrapa Agrobiologia	IV
Glycine max (L.) Merr.	Soybean, soya bean, Manchurian bean, soja[h]	Papilionoideae	Phaseoleae	Annual climbing or non-climbing herb	Grain	Food, medicine	587	BR 96	FEPAGRO/UFRGS	IV
							5019	29W, BR 29	FEPAGRO/UFRGS	IV
							5079	CPAC 15, DF 24	Embrapa Cerrados	IV
							5080	CPAC 7	Embrapa Cerrados	IV
Indigofera hirsuta L.	Hairy indigo, anil bravo[h], anileira[h], indigófera[h]	Papilionoideae	Indigofereae	Annual/perennial, non-climbing herb	Forage (tropical)	Forage, environment, medicine	6156	CPAC-IJ, DF F-2	Embrapa Cerrados	III
							6158	CPAC 42	Embrapa Cerrados	III
Lablab purpureus (L.) Sweet	Black bean antaque, pois antaque, bonavist, lablab[h]	Papilionoideae	Phaseoleae	Annual/pere-nnial, climbing or non-climbing herb	Forage (tropical)	Forage, chemical, environment, food, medicine, toxins	662	CB 188	FEPAGRO/UFRGS	II
							695	QA 922, E 85, SU 422, NA 630	FEPAGRO/UFRGS	II
Leucaena diversifolia (Schldl.) Benth.	Wild tamarind, guash, guache, guaje, guaje blanco, leucena[h]	Mimosoideae	Mimoseae	Perennial non-climbing shrub, tree	Tree	Environment, food	6168	BR 8801, LMG 10132	Embrapa Agrobiologia	III
							6169	BR 5612	Embrapa Agrobiologia	III
Leucaena leucocephala	Jumbie bean, lead tree, leucaena, cowbush, bois bourro,	Mimosoideae	Mimoseae	Perennial non-climbing shrub, tree	Tree	Environment, food	6069	DF 10, BR 414	Embrapa Cerrados	IV

Table 1. (*continued*)

Plant species[a,b]	Some common names[c]	Subfamily[b]	Tribe[b]	Descriptor[c]	Main use in Brazil	Applications worldwide[c]	SEMIA number	Other designations[d,e]	Institution[f] recommendding[e]	Basis[g]
(Lam.) De Wit v. Cunningham	cassie blanc, graines de lin, granadillo bobo, leucena[h]									
							6070	DF 15	Embrapa Cerrados	IV
Lotononis bainesii Baker	Miles lotononis, lotononis[h]	Papilionoideae	Crotalarieae	Annual non- climbing herb	Forage (tropical)	Forage	658	CB 376	FEPAGRO/UFRGS	III
Lotus corniculatus L.	Cat's clover, birds′ foot trefoil, broadleaf trefoil, cornichão[h]	Papilionoideae	Loteae	Perennial non-climbing herb, shrub	Forage (subtropical)	Forage, environment	816	SEMIA original	FEPAGRO/UFRGS	IV
Lotus glaber Miller *(L. tenuis* Willd.)	*Lotus tenuis*, narrow trefoil, birds′ foot trefoil, cornichão[h]	Papilionoideae	Loteae	Perennial non-climbing herb, shrub	Forage (subtropical)	Forage, environment	830	Hansen inoculant	FEPAGRO/UFRGS	II
Lupinus albus L.	White lupine, Egyptian lupine, chochos, lupino blanco, tremozo, tremoço[h]	Papilionoideae	Cytiseae	Annual non- climbing herb	Green manure	Forage, environment, green manure, medicine, toxin, food	938	Hansen inoculant	FEPAGRO/UFRGS	III
Macroptilium atropurpureum (DC.) Urban	Purple bean conchito, siratro[h]	Papilionoideae	Phaseoleae	Perennial climbing or non- climbing, herb	Forage (tropical)	Forage, environment, medicine, food	656	SEMIA original	FEPAGRO/UFRGS	II
Macrotyloma axillare (E. Meyer) Verdc.	Macrotyloma, perennial horse, macrotiloma[h]	Papilionoideae	Phaseoleae	Perennial climbing or non-climbing herb	Forage (tropical)	Forage, environment	6149	CB 627, SMS 138	IAC	II
Medicago polymorpha L.	Bur medic, purple medick, California burclover, luzerne, clover, trevo carretilha[h]	Papilionoideae	Trifolieae	Annual non-climbing herb	Forage (subtropical)	Forage, environment, medicine, food	103	SEMIA original	FEPAGRO/UFRGS	II
Medicago sativa L.	Alfalfa, lucerne, luzerne, alfafa[h]	Papilionoideae	Trifolieae	Perennial non-climbing herb	Forage (subtropical)	Forage, environment, medicine, food	134	SEMIA original	FEPAGRO/UFRGS	IV
							135	SEMIA original	FEPAGRO/UFRGS	IV
Mimosa acutistipula Benth.	Mimosa, jurema preta[h], espinheiro[h]	Mimosoideae	Mimoseae	Perennial non-climbing shrub or tree	Tree	Environment, wood	6383	BR 3407	Embrapa Agrobiologia	III
							6384[i]	BR 3446[i]	Embrapa Agrobiologia	III
Mimosa caesalpiniifolia Benth.	Mimosa, jupinuan, mimosa[h], unha de gata[h], sabiá[h], sansão do campo[h]	Mimosoideae	Mimoseae	Perennial non-climbing shrub or tree	Tree	Environment, wood	6166[i]	BR 3446[i]	Embrapa Agrobiologia	II
							6167	BR 3452	Embrapa Agrobiologia	II
							6382	BR 3405	Embrapa Agrobiologia	IV
Mimosa scabrella Benth.	Abaracaatinga, bracaatinga, paracaatinga, bracatinga[h]	Mimosoideae	Mimoseae	Perennial non-climbing shrub or tree	Tree	Environment, wood	6165	BR 3454	Embrapa Agrobiologia	III

Neonotonia wightii (Wight and Arn.) Lackey	Glycine, perennial soybean, soja perene[h]	Papilionoideae	Phaseoleae	Perennial climbing herb	Forage (tropical)	Forage, environment, food	656	SEMIA original	FEPAGRO/UFRGS	III
							695	E85, QA 922, SU 422, NA 630	FEPAGRO/UFRGS	II
							6148	SMS 303	IAC	II
Ormosia nitida Vogel	Ormosia[h], tento macanaíba[h]	Papilionoideae	Sophoreae	Perennial non-climbing shrub or tree	Tree	Environment, wood	6394	BR 4103	Embrapa Agrobiologia	II
Piptadenia gonoacantha (Martius) Macbr.	Piptadenia, pau jacaré[h], angico jacaré[h], caniveteiro[h], icarapé[h]	Mimosoideae	Mimoseae	Perennial non-climbing tree	Tree	Wood, environment, chemical	6398	BR 4812, UFC 832.55	UFC	III
Piptadenia stipulacea (Benth.) Ducke	Piptadenia, jurema branca[h]	Mimosoideae	Mimoseae	Perennial non-climbing tree	Tree	Wood, environment	6398	BR 4812 UFC 832.59	UFC	II
Pisum sativum L.	Field pea, petit pois, alverja, guisante, ervilha[h]	Papilionoideae	Vicieae	Annual, climbing, herb	Grain	Food, forage	3007	B 11A	FEPAGRO/UFRGS	IV
Prosopis juliflora (Sw.) DC.	Mesquite, cashaw, bayarone, epinard bayahonda blanca, algaroba[h]	Mimosoideae	Mimoseae	Perennial non-climbing, tree, shrub	Tree	Environment, wood	6161	BR 4002, PRJ B4	Embrapa Agrobiologia	IV
Pueraria phaseoloides (Roxb.) Benth.	Tropical kudzu, grande feuille, puero, cudzu tropical[h], kudzu[h]	Papilionoideae	Phaseoleae	Perennial climbing herb	Green manure	Green manure, forage, environment	6175	DF Q-1	Embrapa Cerrados	III
Sclerolobium paniculatum Vogel	Velame, veludo, taxi-branco[h], ajusta contas[h], angá[h], arapaçu[h], carvoeiro[h]	Caesalpinioideae	Caesalpinieae	Perennial non-climbing tree	Tree	Environment, wood	6160	BR 5610	Embrapa Agrobiologia	II
							6420	BR 3617 LMG 9965	Embrapa Agrobiologia	II
Sesbania virgata (Cav.) Pers.	Acacia, cibil, cumand, sesbania[h]	Papilionoideae	Robinieae	Perennial non-climbing shrub, tree	Tree	Environment, wood, forage, medicine	6401	BR 5401, LMG 9993	Embrapa Agrobiologia	II
							6402	BR 5404 LMG 9994	Embrapa Agrobiologia	II
Stizolobium aterrimum Piper and Tracy	Velvet-, Bengal bean, Portuguese coffee, black mu-cuna, café Brazilii, pois mascate, feijão-mucuna[h], mucuna-preta[h]	Papilionoideae	Phaseoleae	Perennial non-climbing, shrub	Green manure	Environment, green manure, forage	6158	CPAC 42	Embrapa Cerrados	IV
Stylosanthes spp.	Stylo, Brazilian lucerne, stylosanthes, estilosantes[h]	Papilionoideae	Aeschynomeneae	Perennial non-climbing herb or shrub	Forage (tropical),	Forage, environment	6155	BR 502	Embrapa Agrobiologia	III
Tipuana tipu (Benth.) Kuntze	Tipa, palo mortero, tipa blanca, tipuana[h], tipa- branca[h], tipu[h]	Papilionoideae	Dalbergieae	Perennial non-climbing tree	Tree	Environment, wood, chemical, forage	6192	SEMIA original	FEPAGRO/UFRGS	II
Trifolium pratense L.	Red clover, trebol de los prados, trebol rojo, trevo vermelho[h]	Papilionoideae	Trifolieae	Perennial non-climbing, herb	Forage (subtropical)	Forage, environment, medicine	222	SU 329, TA-1, NA 14	FEPAGRO/UFRGS	IV
							2081	EEL 1285	EPAGRI	IV
Trifolium repens L.	Dutch, ladino, whiteclover, trebol blanco, trevo branco[h]	Papilionoideae	Trifolieae	Perennial non-climbing herb	Forage (subtropical)	Forage, environment, medicine	222	SU 329, TA-1, NA 14	FEPAGRO/UFRGS	IV
Trifolium subterraneum L.	Subclover, subterranean clover, trevo subterrâneo[h]	Papilionoideae	Trifolieae	Annual non-climbing, herb	Forage (subtropical)	Forage, environment, medicine	222	TA 1, CB 1165	FEPAGRO/UFRGS	IV

Table 1. (*continued*)

Plant species[a,b]	Some common names[c]	Subfamily[b]	Tribe[b]	Descriptor[c]	Main use in Brazil	Applications worldwide[c]	SEMIA number	Other designations[d,e]	Institution[f] recommendding[e]	Basis[g]
Trifolium vesiculosum Savi	Arrowleaf clover, trevo yuchi, trevo vesiculoso[h]	Papilionoideae	Trifolieae	Annual non- climbing herb	Forage (subtropical)	Forage, environment, medicine	2051	SEMIA original	FEPAGRO/UFRGS	II
Vicia sativa L.	Common vetch, pois France, arveja, ervilhaca[h]	Papilionoideae	Vicieae	Annual climbing or non- climbing herb	Forage (subtropical)	Forage, environment, medicine	384	SEMIA original	FEPAGRO/UFRGS	II
Vigna unguiculata (L.) Walp.	Cowpea, black eye pea, barbati, boeme, pois manger cochon, caupi[h], feijão-de-corda[h], feijão miúdo[h]	Papilionoideae	Phaseoleae	Annual/pere-nnial, climbing, non-climbing herb	Grain	Grain, forage	662	CB 188	FEPAGRO/UFRGS	III
							6002	CB 756, TAL 309, RCR 3824	FEPAGRO/UFRGS	III
							6145	BR 2001	Embrapa Agrobiologia	IV

[a]Plant species for which the strain is commercially recommended.

[b]Taxonomy based on ILDIS [21].

[c]Information obtained from the following sites: juazeiro.cnip.org.br/edalcin/arvores/; umbuzeiro.cnip.org.br/db/forrag/vernac.shtml; www.arboretos.cnpm.embrapa.br/faz_sm/especies.html; www.arvores.brasil.nom.br/listacient.htm; www.fao.org/ag/AGA/AGAP/FRG/AFRIS/Data/215.HTM; www.fao.org/ag/agp/agpc/doc/Gbase/latinsearch.htm; www.hear.org/pier/species/; www.ildis.org/LegumeWeb/6.00/taxa/1621.shtml; www.ipef.br/identificacao/nativas/detalhes.asp?codigo = 65; www.newcrops.uq.edu.au/listing/clitoriaternatea.htm; www.rain-tree.com/plants.htm.

[d]Culture collections: ATCC (The American Type Culture Collection, Manassas, USA); BR (Brazil, Embrapa Agrobiologia, Seropédica, Brazil); CB (Commonwealth Scientific and Industrial Research Organization – CSIRO, Canberra, Australia); CIAT (Centro Internacional de Agricultura Tropical, Cali, Colombia); DF (Distrito Federal, Embrapa Cerrados, Planaltina, Brazil); E (Instituto Nacional de Tecnología Agropecuaria – INTA – Castelar, Argentina); H (Embrapa Cerrados, Planaltina, Brazil); LMG (Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium); MAR (Marondera, Grasslands *Rhizobium* Collection, Soil Productivity Research Laboratory, Marondera, Zimbabwe; also called SPRL); MGAP (Ministerio de Ganadería, Agricultura y Pesca, Laboratorio de Microbiologia y Suelos, Montevideo, Uruguay) CPAC (Centro de Pesquisa Agropecuária dos Cerrados. Embrapa Cerrados, Planaltina, Brazil); NA (New South Wales Dept. of Agriculture, NSW Dept. of Primary Industries – Agriculture, Victoria, Australia); NC (North Caroline, University of North Caroline, Raleigh, USA); Nit (Nitragin, Inc., Brookfield, USA); PRF (Paraná Feijão, Embrapa Soja, Londrina, Brazil); QA (Queensland Austrália, University of Queensalnd, St. Lucia, Australia); RCR (Rothamsted *Rhizobium* Collection, Harpenden, UK); SEMIA (Seção de Microbiologia Agrícola, FEPAGRO, Porto Alegre, Brazil); SMS (Seção de Microbiologia do Solo, IAC, Campinas, Brazil); SU (The University of Sydney, Sidney, Australia); TA (Tasmania Dept. of Agriculture, The Department of Primary Industries, Water and Environment, Tasmanian State Government Agency, Tasmania, Australia); TAL (NifTAL, Nitrogen Fixation by Tropical Agricultural Legumes Project, University of Hawaii, Paia, USA); UMKL (University of Malaya – Kuala Lumpur, Dept. of Genetics and Cellular Biology, Kuala Lumpur, Malasya); UMR (University of Minnesota *Rhizobium*, St. Paul, USA); USDA (United States Department of Agriculture, Beltsville, USA).

[e]After Hungria and Araujo (1995); FEPAGRO [11] and actas of RELARE (unpublished).

[f]Embrapa (Empresa Brasileira de Pesquisa Agropecuária); EPAMIG (Empresa de Pesquisa Agropecuária de Minas Gerais); IAC (Instituto Agronômico de Campinas); FEPAGRO (Fundação Estadual de Pesquisa Agropecuária); UFRGS (Universidade Federal do Rio Grande do Sul); CEPEC (Centro de Pesquisas do Cacau); IAPAR (Instituto Agronômico do Paraná); UFC (Universidade Federal do Ceará).

[g]Basis for strain recommendation: (I) selection in glass tubes or bags under axenic conditions; (II) selection in jars under axenic conditions; (III) selection in non-sterile soil; (IV) field experiments.

[h]Common names used in Brazil. Information obtained from the sites cited on ([3]).

[i]Different numbers in FEPAGRO [11].

resuspended in TE 50/20, of 50 μL of 10% SDS, 5 μL of proteinase-K (20 mg mL^{-1}), 10 μL of lysozyme (5 mg mL^{-1}), and 1 μL of RNAse (20 mg mL^{-1}). After two steps of purification with ethanol at 99.5% and at 70%, the pellet was resuspended in 50 μL of TE 10/1 to estimate the concentration of the DNA. Samples were then diluted to 20 ng of DNA μL^{-1} and were kept at −20 °C.

Amplification of the DNA region coding for the 16S rRNA gene and purification of the PCR-products

The DNA of each bacterial strain was amplified with the universal primers fD1 and rD1 described previously [49] (target gene regions in *Escherichia coli* strain K12), fD1 (8-27) and rD1 (1525–1541). Each replicate contained, in a volume of 50 μL: dNTPs (300 μM of each); PCR-buffer (Tris-base 20 mM pH 8.4 and KCl 50 mM); $MgCl_2$ (1.5 mM); primers (15 pmol of each); Taq DNA polymerase (1.2 U); DNA (20 ng) and DMSO (5%). The reaction was carried out in an MJ Research Inc. PTC 200 thermocycler, using an initial cycle of denaturation at 95 °C for 2 min; 30 cycles of denaturation at 94 °C for 15 s, 93 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 2 min; a final extension cycle of 72 °C for 5 min.

For the purification of the PCR-products, 48 μL of each PCR reaction were added to each 300 μL-capacity well of a Nunc U96 MicroWell™ Plate. Each well also received 5 μL of sterile ammonium acetate (7.5 M) and 165 μL of 99.5% ethanol (room temperature). The plate was sealed, homogenized and the mixture was centrifuged at 4000 rpm for 45 min. The supernatant was discarded and the plate was inverted on absorbent paper to dry. After drying, the pellet received 150 μL of freshly prepared 70% ethanol, the plate was sealed and the suspension was homogenized and centrifuged again at 4000 rpm for 10 min. The supernatant was discarded as described previously and the plate was inverted on absorbent paper and centrifuged at 300 rpm for 25 s. The pellet was dried at room temperature for 30 min, or at 37 °C for 15 min, followed by the addition of 15 μL of milli-Q water, homogenized, and kept at −4 °C. After 24 h, the concentration of the samples was verified in 1.5% agarose gels, adjusted to 40 ng DNA μL^{-1} and kept at −20 °C.

Sequencing analysis of the 16S rRNA gene

The PCR-reactions were carried out in 96-well-full-skirt-PCR microplates. Purified PCR products of each bacterium culture (80 ng per reaction) received a mixture of 3 μL of dye (DYEnamic ET terminator reagent premix for the MegaBACE, Amersham Biosciences), and 3 pmol of each primer. To obtain the complete sequence of the 16S rRNA gene, five reactions were carried out, with the following primers: fD1, Y2 [54] (target gene region in *E. coli* K12 362-339) and primers designed by Prof. Leonardo M. Cruz (Dept. of Biochemistry, UFPR, Curitiba, PR, Brazil): 362f (target region 339–362) (5′-CTCCTACGGGAGGCAGCAGT-GGGG-3′), 786f (target region 764-786) (5′-CGAAA-GCGTGGGGAGCAAACAGG-3′) and 1203f (target region 1179-1203) (5′-GAGGTGGGGATGACGTCA-AGTCCTC-3′). The same program was used with all primers, as follows: denaturation at 95 °C for 2 min; 30 cycles of denaturation at 95 °C for 10 s, 50 °C for 4 s, and extension at 60 °C for 4 min; final soak at 4 °C.

After amplification, to 20 μL of each sample (7 μL of each PCR reaction + 13 μL of milli-Q water) were added 2 μL of sterile ammonium acetate (7.5 M) and 65 μL of 99.5% ethanol (room temperature). The plate was sealed, homogenized and centrifuged at 4000 rpm for 45 min. The supernatant was discarded and the plate was inverted on absorbent paper to dry. After drying, the pellet received 165 μL of freshly prepared 70% ethanol, the plate was sealed, homogenized, centrifuged again at 4000 rpm for 10 min, and the supernatant was discarded. The plate was inverted on absorbent paper and centrifuged at 300 rpm for 25 s. The pellet was dried at room temperature for 30 min, or at 37 °C for 15 min, resuspended in 7 μl of milli-Q water or in buffer (70% formamide, 1 mM EDTA), and submitted for sequencing analysis in a MegaBACE 1000 DNA Analysis System (Amersham Biosciences). In general, the electrophoresis parameters used were: sample injection voltage, 1 kV; sample injection time, 40 s; run voltage, 5 kV; run time, 240 min.

The high-quality sequences obtained for each strain were assembled into contigs using the programs phred [8,9] phrap (www.phrap.org) and Consed [15]. Sequences confirmed in the 3′ and 5′ directions were submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/blast) to seek significant alignments. Accession numbers from AY904726 to AY904789 were given to the 16S rRNA sequences of 64 strains (Table 2). The sequences of the four strains recommended for the soybean crop were reported before [7] and were also confirmed and used in this study: *B. japonicum* strains SEMIA 5079 (AF234888) and SEMIA 5080 (AF234889), and *B. elkanii* strains SEMIA 587 (AF234890) and SEMIA 5019 (AF237422).

Phylogeny based on the 16S rRNA gene and diversity of rhizobia

The sequences obtained were aligned and compared to those of the following type/reference strains

Table 2. Acid/alkaline reaction in yeast extract-mannitol agar (YMA) medium, accession number of the 16S rRNA sequence, original host and proposed taxonomic position of 68 strains officially recommended for the use in inoculants for 64 legume species in Brazil

SEMIA strain	Reaction in YMA	Original host	Source of the strain	Proposed taxonomic position	Gene Bank Access no.
103	Neutral	*Medicago polymorpha*	Brazil	*Sinorhizobium meliloti*	AY904726
134	Acid	*Medicago sativa*	*Brazil*	*Sinorhizobium meliloti*	AY904727
135	Acid	*Medicago sativa*	*Brazil*	*Sinorhizobium meliloti*	AY904728
222	Acid	*Trifolium subterraneum*	Australia	*Rhizobium leguminosarum*	AY904729
384	Acid	*Vicia* sp.	*Brazil*	*Rhizobium etli*	AY904730
396	Acid	Not known	*USA*	*Mesorhizobium ciceri*	AY904731
587	Alkaline	*Glycine max*	*Brazil*	*Bradyrhizobium elkanii*	AF234890 [a]
656	Alkaline	*Neonotonia wightii*	*Brazil*	*Bradyrhizobium sp.*	AY904732
658	Alkaline	*Lotononis bainesii*	*South Africa*	Methylobacterium sp.	AY904733
662	Alkaline	*Vigna unguiculata*	Australia	*Bradyrhizobium elkanii*	AY904734
695	Alkaline	*Neonotonia wightii*	*Australia*	*Bradyrhizobium elkanii*	AY904735
696	Alkaline	*Desmodium uncinatum*	Austrália	*Bradyrhizobium elkanii*	AY904736
816	Acid	*Lotus corniculatus*	*Brazil*	Mesorhizobium sp.	AY904737
830	Acid	Not known	*USA*	Mesorhizobium sp.	AY904738
938	Alkaline	*Lupinus albus*	USA	*Bradyrhizobium elkanii*	AY904739
2051	Acid	*Trifolium vesiculosum*	*Brazil*	*Rhizobium leguminosarum*	AY904740
2081	Acid	*Trifolium pratense*	*Brazil*	*Rhizobium leguminosarum*	AY904741
3007	Acid	*Pisum sativum*	*Mexico*	*Rhizobium leguminosarum*	AY904742
5019	Alkaline	*Glycine max*	*Brazil*	*Bradyrhizobium elkanii*	AF237422 [a]
5079	Alkaline	*Glycine max*	*Brazil*	*Bradyrhizobium japonicum*	AF234888 [a]
5080	Alkaline	*Glycine max*	*Brazil*	*Bradyrhizobium japonicum.*	AF234889 [a]
6002	Alkaline	*Vigna unguiculata*	*Zimbabwe*	*Bradyrhizobium japonicum*	AY904743
6028	Alkaline	*Desmodium uncinatum*	Zimbabwe	*Bradyrhizobium elkanii*	AY904744
6053	Alkaline	*Clitoria ternatea*	Malaysia	*Bradyrhizobium elkanii*	AY904745
6069	Alkaline	*Leucaena leucocephala*	*Brazil*	*Bradyrhizobium elkanii*	AY904746
6070	Acid	*Leucaena leucocephala*	*Brazil*	*Rhizobium sp.*	AY904747
6100	Alkaline	*Falcataria moluccana*	*Brazil*	*Bradyrhizobium elkanii*	AY904748
6101	Alkaline	*Dalbergia nigra*	*Brazil*	*Bradyrhizobium elkanii*	AY904749
6144	Alkaline	*Arachis hypogaea*	Zimbabwe	*Bradyrhizobium* sp.	AY904750
6145	Alkaline	*Arachis hypogaea*	*Libia*	Bradyrhizobium sp.	AY904751
6146	Alkaline	*Centrosema* sp.	*Brazil*	*Bradyrhizobium elkanii*	AY904752
6148	Alkaline	*Neonotonia wightii*	*Brazil*	Bradyrhizobium sp.	AY904753
6149	Alkaline	*Galactia striata*	Australia	*Bradyrhizobium elkanii*	AY904754

Table 2. (*continued*)

SEMIA strain	Reaction in YMA	Original host	Source of the strain	Proposed taxonomic position	Gene Bank Access no.
6150	Alkaline	*Acacia mearnsii*	*Brazil*	*Bradyrhizobium elkanii*	AY904755
6152	Alkaline	*Calopogonium* sp.	*Brazil*	*Bradyrhizobium japonicum*	AY904756
6155	Alkaline	*Stylosanthes* sp.	*Brazil*	*Bradyrhizobium japonicum*	AY904757
6156	Alkaline	*Crotalaria spectabilis*	*Brazil*	Bradyrhizobium sp.	AY904758
6157	Alkaline	*Cajanus cajan*	*Brazil*	*Bradyrhizobium elkanii*	AY904759
6158	Alkaline	*Crotalaria spectabilis*	*Brazil*	*Bradyrhizobium elkanii*	AY904760
6159	Alkaline	*Enterolobium ellipticum*	Brazil	*Bradyrhizobium elkanii*	AY904761
6160	Alkaline	*Albizia lebbeck*	*Brazil*	Bradyrhizobium sp.	AY904762
6161	Acid	*Prosopis juliflora*	*Brazil*	Sinorhizobium sp.	AY904763
6163	Alkaline	*Acacia mearnsii*	*Brazil*	*Bradyrhizobium sp.*	AY904764
6164	Alkaline	*Acacia mearnsii*	*Brazil*	*Bradyrhizobium sp.*	AY904765
6165	Acid	*Mimosa scabrella*	Brazil	*Rhizobium* sp.	AY904766
6166	Alkaline	*Mimosa caesalpiniifolia*	*Brazil*	Burkholderia sp.	AY904767
6167	Alkaline	*Mimosa caesalpiniifolia*	*Brazil*	Burkholderia sp.	AY904768
6168	Acid	*Gliricidia sepium*	*Brazil*	Rhizobium sp.	AY904769
6169	Alkaline	*Falcataria moluccana*	*Brazil*	*Bradyrhizobium elkanii*	AY904770
6175	Alkaline	*Pueraria phaseoloides*	*Brazil*	*Bradyrhizobium elkanii*	AY904771
6192	Alkaline	*Tipuana tipu*	*Brazil*	*Bradyrhizobium sp.*	AY904772
6208	Alkaline	*Desmodium heterocarpon*	Colombia	*Bradyrhizobium elkanii*	AY904773
6319	Alkaline	*Arachis* sp.	Bolivia	*Bradyrhizobium* sp.	AY904774
6382	Neutral	*Mimosa caesalpiniifolia*	*Brazil*	Burkholderia sp.	AY904775
6383	Acid	*Mimosa caesalpiniifolia*	*Brazil*	Rhizobium sp.	AY904776
6384	Alkaline	*Mimosa obovata*	Brazil	*Bradyrhizobium elkanii*	AY904777
6387	Alkaline	*Acacia auriculiformis*	*Brazil*	*Bradyrhizobium elkanii*	AY904778
6390	Acid	*Acacia decurrens*	*Brazil*	*Burkholderia cepacia*	AY904779
6391	Alkaline	*Acacia auriculiformis*	*Brazil*	*Bradyrhizobium sp.*	AY904780
6394	Alkaline	*Ormosia nitida*	*Brazil*	*Burkholderia cepacia*	AY904781
6398	Acid	*Piptadenia stipulacea*	*Brazil*	*Burkholderia sp.*	AY904782
6401	Alkaline	*Sesbania virgata*	*Brazil*	Azorhizobium sp.	AY904783
6402	Alkaline	*Sesbania virgata*	*Brazil*	Azorhizobium sp.	AY904784
6412	Neutral	*Clitoria fairchildiana*	*Brazil*	Burkholderia sp.	AY904785
6420	Alkaline	*Acacia mangium*	*Brazil*	*Bradyrhizobium sp.*	AY904786
6424	Alkaline	*Centrosema pubescens*	Brazil	*Bradyrhizobium elkanii*	AY904787
6425	Alkaline	*Centrosema pubescens*	*Brazil*	*Bradyrhizobium elkanii*	AY904788
6440	Alkaline	*Arachis pintoi*	Brazil	*Bradyrhizobium* sp.	AY904789

[a]The nucleotides confirmed a previous submission of our group [7].

(accession numbers of the GenBank Data Library in parentheses): *Azorhizobium caulinodans* USDA 4892^T (X67221); *Bradyrhizobium betae* PL74H1^T (AY372184); *Bradyrhizobium canariense* BC-C2^T (AY577427); *Bradyrhizobium elkanii* USDA 76^T (U35000); *Bradyrhizobium japonicum* USDA 6^T (U69638); *Bradyrhizobium liaoningense* USDA 3622^T (AF208513); *Burkholderia cepacia* ATCC 53867^T (AY741356); *Burkholderia graminis* C4D1 M^T (U96939); *Burkholderia* sp. TJ182 (AJ505301); *Burkholderia* sp. BR 3405 (AY773186); *Burkholderia* sp. BR 3407 (AY773186); *Burkholderia* sp. tpig4.4 (AY691396); *Burkholderia* sp hpud10.4 (AY691394); *Mesorhizobium ciceri* USDA 3383^T (U07934); *Mesorhizobium loti* USDA 3471^T (X67229); *Methylobacterium nodulans* ORS 2060^T (AF220763); *Rhizobium etli* CFN 42^T (U28916); *Rhizobium leguminosarum* USDA 2370^T (U29386); *Rhizobium rhizogenes* ATCC 11325^T (D14501.1); *Rhizobium tropici* CIAT 899^T (U89832); *Sinorhizobium fredii* USDA 205^T (X67231); *Sinorhizobium meliloti* USDA 1002^T (X67222). *Azospirillum brasilense* strain A154 (DQ104848) was used as an outgroup reference.

Multiple alignments were performed with ClustalX version 1.83 [43]. Phylogenetic trees were generated using MEGA version 3.1 [25] with default parameters, K2P distance model [24] and the Neighbor-Joining algorithm [35]. *Azospirillum brasilense* strain A154 was used as an outgroup for 16S rDNA phylogenies. Statistic support for tree nodes was evaluated by bootstrap [10] analyses with 2000 samplings [17].

PCR-amplification of the DNA region coding for the *nodB*, *nodC* and *nifH* genes

The DNAs of the bacteria showing similarity with the genera *Methylobacterium* and *Burkholderia* were used for amplification of the regions coding for the genes *nodB*, *nodC* and *nifH*.

For the amplification of the *nodB* gene region, the primers used were nodB3f [50] and nodCRR [39]. Each replicate contained, in a volume of 50 μL: dNTPs (200 μM of each); PCR-buffer (Tris-base 20 mM pH 8.4 and KCl 50 mM); $MgCl_2$, (2.4 mM); primers (30 pmol of each); Taq DNA polymerase (1.5 U); DNA (40 ng). The reaction was carried out with 30 cycles of denaturation at 94 °C for 90 s, annealing at 67 °C for 30 s, and extension at 72 °C for 80 s; and a final extension cycle of 72 °C for 3 min.

The *nodC* amplification was performed with primers nodCIf and nodCp8, used for *Bradyrhizobium*, as described previously [40]. For the DNA region coding for *nifH*, amplification was performed with primers nifHF and nifHI [26].

Results

Morpho-physiological characterization of SEMIA strains

Seventeen strains showed acid reactions on YMA medium (Table 2), and in general they were characterized by medium to high production of mucus (data not shown). Forty-eight strains showed alkaline reactions on YMA, most with low to medium production of mucus (data not shown). In relation to the other morphological parameters evaluated (color, transparency, borders, elevation), no consistent patterns emerged within the acid- or alkali-producing group (data not shown). Only three strains, SEMIA 103, SEMIA 6382 and SEMIA 6412 showed neutral reaction on YMA (Table 2), all of which with medium production of mucus and colonies of 2–3 mm in diameter after 7 days.

Phylogeny based on the 16S rRNA gene and diversity of rhizobia

Fig. 1 shows the phylogenetic tree obtained with the 16S rRNA aligned sequences of the 68 rhizobial strains, as well as of the type and reference strains used in this study; *A. brasiliense* strain A154 as used as an outgroup reference. Strains were grouped into nine phylogenetic branches or well-supported main clusters, with some subclusters. We have defined that strains differing in more than 1.03% (15) nucleotides from the closest type strain could represent new species and were therefore designated in this paper as "sp." (Table 2).

In the first phylogenetic branch (I) strain SEMIA 658 was clustered with *Metylobacterium nodulans* ORS 2060^T with a bootstrap support of 99% (Fig. 1). The second phylogenetic branch (II) clustered 42 *Bradyrhizobium* strains with a bootstrap support of 99%, and two main clusters (II.1 and II.2) were observed (Fig. 1). Cluster II.1 included 28 SEMIA strains together with *B. elkanii* type strain USDA 76^T and reference strain SEMIA 587. Within this cluster were strains isolated from legumes of the following subfamilies and tribes: Papilionoideae (Aechynomeneae, Crotalarieae, Cytiseae, Dalbergieae, Desmodieae, Phaseoleae) and Mimosoideae (Acacieae, Ingeae, Mimoseae). Three out of the four strains from this study that had been isolated from tribe Desmodieae, as well as one from the Dalbergieae, and the only strain from the Cytiseae were within this branch. The SEMIA strains positioned in the cluster II.1 are officially recommended for 34 host legume species, with some (SEMIAs 6160, 6100, 6169, 6387 and 6149) being the most effective for two different legume species, SEMIA 6145 and 6159 the most effective for three and SEMIA 6158 the most effective for four different host species. Strains from this phylogenetic branch differing

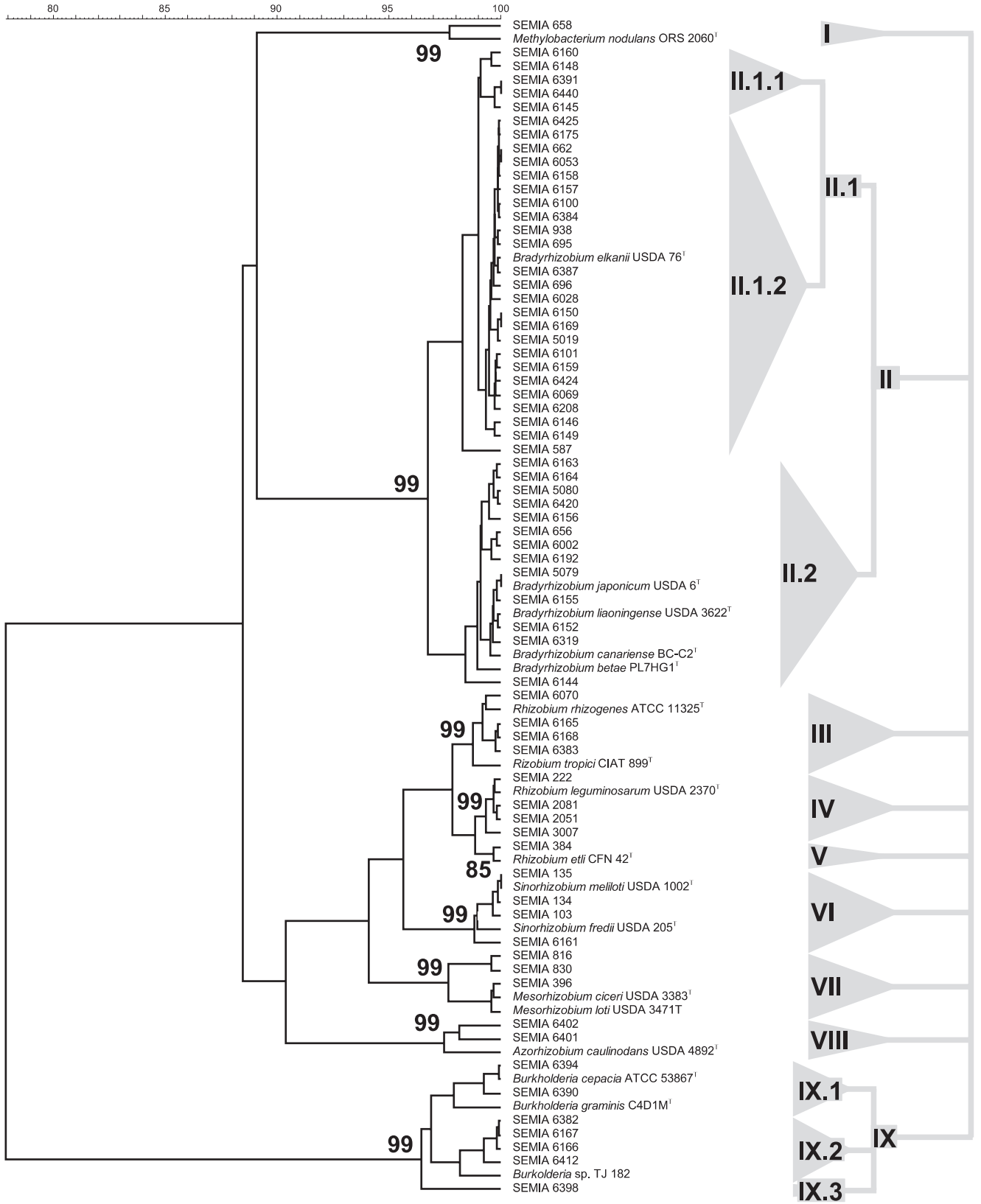

Fig. 1. Phylogenetic tree based on the 16S rRNA sequences of 68 strains, N_2-fixing symbionts isolated from 46 legumes and officially recommended for the use in Brazilian commercial inoculants. GeneBank accession numbers of SEMIA strains (Table 2) and of type strains (material and methods) are given in the text. Phylogenetic trees were generated using MEGA version 3.1 with default parameters, K2P distance model and the Neighbor-Joining algorithm.

in more than 15 bases from *B. elkanii* USDA 76^T were nominated as *Bradyrhizobium* sp. (Table 2).

Phylogenetic branch II.2 grouped 13 SEMIA with type strains of *B. japonicum*, *B. liaoningense*, *B. canariense* and *B. betae* with a bootstrap support of 99% (Fig. 1). The majority (38%) was isolated from legumes of the Papilionoideae tribe Phaseoleae, followed by 23% from the Mimosoideae tribe Acacieae; promiscuity of host species was also observed within this group (Fig. 1, Table 1). The strains differing in more than 1.03% of bases of *Bradyrhizobium* species positioned in this cluster were nominated as *Bradyrhizobium* sp.

Four SEMIA strains fit into phylogenetic branch III, of *Rhizobium tropici-R. rhizogenes* (*Agrobacterium*), with a bootstrap support of 99% (Fig. 1). The comparison of the four SEMIA with the type strains showed higher similarity of bases with *R. rhizogenes* ATCC 11325^T, but differing in 0.76–1.34% nucleotides. The highest blast was with another strain (163C) of *R. rhizogenes* (AY206687), isolated from tumors of *Prunus persica*, and high similarity was also observed with strain p1-7 (AY206687), isolated from nodules of common beans (*Phaseolus vulgaris*) and classified as *R. lusitanum.* Finally, a lower but still high similarity of nucleotides was observed with several *R. tropici* strains isolated from common bean, some of them from Brazil.

The *R. leguminosarum* type strain was positioned in phylogenetic branch IV, together with four SEMIA strains, with a bootstrap support of 99% (Fig. 1). Strain SEMIA 384, symbiont of *Vicia sativa* and isolated in Brazil, was highly related to *R. etli* type strain in phylogenetic branch V, but with the lowest bootstrap support from this study (85%) (Fig. 1, Table 1); due to the symbiotic properties, further investigation of the plasmids and other genes of SEMIA 384 should be performed to confirm its taxonomic position.

Sinorhizobium species were positioned in cluster VI (Fig. 1). *Medicago* is the host genus of SEMIA strains 135, 134 and 103, and the strains were highly related to *S. meliloti* USDA 1002^T (Fig. 1, Table 1). Contrarily, strain SEMIA 6161 from *Prosopis* was distinct from type strains of *S. meliloti* (1.17% nucleotides) and from *S. fredii* (1.72% nucleotides); it might represent another species, thus was nominated as *Sinorhizobium* sp. (Table 2).

Three SEMIA strains were positioned in *Mesorhizobium* phylogenetic branch VII, with a bootstrap support of 99%. Strains SEMIA 816 and 830 are highly related (99.5% of similarity of bases); but differed considerably from type strain of *M. loti* and might represent another species (Table 2). SEMIA 396 is a symbiont of *Cicer arietinum* and was highly related (99.6%) to the *M. ciceri* type strain (Fig. 1, Tables 1 and 2).

Strains SEMIA 6402 and 6401, isolated from stem nodules of *Sesbania virgata* (Table 1), were clustered with *Azorhizobium caulinodans* type strain in phylogenetic branch VIII (Fig. 1). However, both SEMIAs differed in more than 2.07% nucleotides from the type strain, therefore at this moment they are nominated as *Azorhizobium* sp., as they might represent another species (Table 2).

Finally, the most divergent group of strains fit into phylogenetic branch IX, also with bootstrap support of 99% (Fig. 1). This branch included seven SEMIA strains, all isolated in Brazil, with type and reference strains belonging to the genus *Burkholderia*. Two subclusters were defined, and one isolated strain, SEMIA 6398. Subcluster IX.1 included strain SEMIA 6390 and SEMIA 6394 with *Burkholderia cepacia* ATCC53867^T, while strains SEMIAs 6167, 6382, 6166, and SEMIA 6412 were joined to *Burkholderia* sp. strain TJ 182 with a bootstrap support of 92% (Fig. 1, Tables 1 and 2). At the SEMIA collection, strains SEMIA 6382 and SEMIA 6383 should be the same as BR 3405 and BR 3407, respectively, however, differences in some nucleotides were observed when compared with previously deposited sequences (AY773185 and AY773186, respectively). We still have to compare the strains in relation to other characteristics.

Amplification of the DNA of *Methylobacterium* and *Burkholderia* strains with primers for the *nod* and *nif* gene regions

The DNA of eight SEMIA strains classified as *Burkholderia* and *Methylobacterium* were used for the amplification with primers for the *nod* and *nif* regions. Using the primers reported to amplify *nodB* genes of *Rhizobium,* a PCR-product of about 300 bp was obtained exclusively with *Burkholderia* sp. SEMIA 6398 (Table 3). For the *nodC* gene, using a set of primers that resulted in a product of 243 bp in *Bradyrhizobium* strains [40], amplification was obtained with *Burkholderia* sp. strains SEMIA 6398 and 6412, with *B. cepacia* strains SEMIA 6390 and SEMIA 6394 and with *Methylobacterium* sp. SEMIA 658 (Table 3). The primers used to detect *nifH* resulted in products between 780 and 890 bp in analyses of several rhizobial strains [26] and the PCR-products of *Methylobacterium* and both *B. cepacia* had approximately 700 bp, while the products of *Burkholderia* sp. SEMIAs 6166, 6167 and 6382, all isolated from *Mimosa caesalpiniifolia*, had about 1500 bp (Table 3); amplification with those primers was not observed with strains SEMIA 6398 and 6412 (Table 3). Our goal is now to sequence the PCR-products obtained aiming at better understand the evolutionary relationships of symbiotic genes in tropical rhizobia.

Table 3. Amplification[a] of *nif* and *nod* genes of SEMIA strains classified in the genera *Methylobacterium* and *Burkholderia*

SEMIA strain	*nifH*	*nodB*	*nodC*
658	+	−	+
6390	+	−	+
6394	+	−	+
6166	+	−	−
6167	+	−	−
6382	+	−	−
6398	−	+	+
6412	−	−	+

[a](+) amplified and (−) not amplified with the primers described in the material and methods section.

Discussion

Nitrogen is often the most limiting nutrient for plant growth worldwide. The situation is especially critical in the tropics, where the usually low levels of soil organic matter have resulted in the depletion of this nutrient. In addition, the high cost of N-fertilizers in countries like Brazil has resulted in the need for actions emphasizing biological nitrogen fixation (BNF) [19,20]. Successful approaches begin with long-term programs of rhizobia selection, and the identification of elite strains for each legume host of interest. Countries differ in their policies concerning the commercialization of rhizobial inoculants, and in Brazil they must contain elite strains evaluated and recommended by an official committee of rhizobiologists [20]. The Brazilian *Rhizobium* Culture Collection (SEMIA) was created in 1985 by the Microbial Resources Centre Network (MIRCEN), with the purpose of maintain the recommended rhizobial strains and distribute the cultures to the inoculant industry [20]. Nowadays, SEMIA strains are classified as *R. meliloti*, *R. leguminosarum*, *B. japonicum*, *Bradyrhizobium* sp., *R. fredii*, or *R. loti* [11] based exclusively on their ability to produce alkaline or acid reaction in YM medium and on the cross-inoculation group [13,45]. Therefore, although the SEMIA collection is a reservoir of rhizobia resulting from decades of selection, the genetic knowledge about the strains in the collection is very poor.

Phylogeny of the 68 SEMIA strains, the great majority from Brazil, was based on the sequencing of the 16S rRNA, as this gene has become the method of choice for tracing bacterial phylogenies and defining taxonomy [14,49]. Many SEMIA strains have a broad host range, and apparently we found no evidence of evolutionary correlation with the host plants, similarly to other results obtained with tropical rhizobia [31]. Some of the strains were very effective in fixing N_2 with legumes of distinct tribes and even subfamilies, e.g., *B. elkanii* SEMIA 6160, recognized as the most effective for both *Albizia lebbeck* (Mimosoideae, Ingeae) and *Sclerolobium paniculatum* (Caesalpinioideae, Caesalpinieae), SEMIA 6156, isolated from *Crotalaria spectabilis*, and identified as the most effective for five species (*C. spectabilis*, *C. juncea*, *Cajanus cajan*, *Canavalia ensiformis*, *Indigofera hirsuta*), and *B. japonicum* SEMIA 656, recommended for plants of the subfamily Papilionoideae: *Desmodium* (Desmodieae), *Macroptilium* (Phaseoleae), and *Neonotonia* (Phaseoleae).

R. etli species has been reported as the main symbiont of common beans in the centers of origin of this legume: Mesoamerica [28,37] and Northern [4] and Southern [1] Andean South America. However, recent reports indicate a wide distribution of *R. etli* associated with common bean in Brazil [16] and this study extends the host specificity including the Brazilian strain SEMIA 384 from *Vicia sativa*. Symbionts of *Medicago* isolated from subtropical Brazil were confirmed as *S. meliloti*, but SEMIA 6161 from tropical *Prosopis juliflora* differed considerably from both *S. meliloti* and *S. fredii* type strains, which suggests that it may represent a new *Sinorhizobium* species.

Four SEMIA strains were clustered with plant-pathogenic non-N_2-fixing agrobacteria. It has been known that *Agrobacterium* spp. share several characteristics and are genetically closely related to some rhizobial species (*R. tropici*, *Rhizobium* genomic species Q, *R. galegae*, *R. huautlense*, and *Allorhizobium undicola*) [27,42,48,53,55]. Consequently, based on 16S rRNA gene sequences, agrobacteria were recently reclassified into the genus *Rhizobium* [53]. N_2-fixing rhizobia resembling agrobacteria were isolated from root nodules of *Acacia* spp. [23] and common bean [29] in Africa, but the isolates were not able to maintain the symbiotic effectiveness. However, isolates from soybean nodules in Paraguay [5] and the isolates obtained from *Mimosa scabrella*, *Gliricida sepium,* and *Leucaena leucocephala* in this study showed effectiveness and genetic stability of symbiotic properties. In the future, efforts should focus in understanding the evolution and ecological importance of these effective tropical rhizobia closely related to agrobacteria.

Putative new species were also observed in two other genera. Strains SEMIAs 816 and 830, symbionts of *Lotus corniculatus*, isolated in Brazil and USA, respectively, differed from type strain of *Mesorhizobium loti* by 1.65% and 2.07% nucleotides, respectively. Also two strains isolated from stem nodules of *Sesbania virgata* in Brazil, SEMIAs 6401 and 6402, differed by more than 30 nucleotides from type strain of *Azorhizobium caulinodans*.

The majority of the strains from this study (42) were classified into the genus *Bradyrhizobium* and two major subclusters were observed. The first one included *B. elkanii* USDA 76^T and symbionts of all three

Leguminosae subfamilies and several tribes. Type strains of *B. japonicum*, *B. liaoningense* and *B. canariense* were clustered into the second subcluster, and the similarity of the sequences of these three species has been previously reported [47,51,55]. Although high diversity in morphological, physiological, and genetic properties within *Bradyrhizobium* strains has been reported, the differences are not reflected in diversity of the 16S rRNA genes [3,5,30,44,46,51,52]. However, the results obtained in our study show novel variability in the 16S rRNA genes within *Bradyrhizobium*.

Strain SEMIA 658 isolated from *Lotononis bainesii* in South Africa and effective under Brazilian conditions, was clustered with the type strain of the α-proteobacterium *Methylobacterium nodulans*, isolated from *Crolalaria podocarpa* in Senegal [36,41]. The strains studied by Sy et al. [41] showed similarity of *nodA* genes with *Bradyrhizobium*, which was suggestive of horizontal gene transfer. SEMIA 658 should be the same as CB 376, cited as belonging to the genus *Methylobacterium* [41] and in our study amplification was achieved with the primers designed for the *nodC* region of *Bradyrhizobium*.

Seven strains from Brazil were grouped with β-Proteobacteria of the genus *Burkholderia*. N_2-fixing symbiotic associations of burkholderia with legume plants, preferentially from the Mimosoideae, have been reported [6,32]. In our study, SEMIA 6394 isolated from *Ormosia nitida* (Papilionoideae), and SEMIA 6390, isolated from *Acacia decurrens* (Mimosoideae), showed higher similarity of nucleotides with *Burkholderia cepacia* ATCC53867^T. Three other strains (SEMIA 6382, 6167, 6166) isolated from *Mimosa caesalpiniifolia* were highly similar with each other in the 16S rRNA analysis, but we were unable to get amplification of those strains with the *nodB* or *nodC* primers. However, DNA amplification with primers for the *nifH* region of these three strains produced PCR-products of similar size (~1500 bp), but differed from the products obtained with the other SEMIA classified as *Burkholderia* (~700 bp). Strain SEMIA 6412 isolated from *Clitoria fairchildiana* (Papilionoideae) was grouped in the same cluster with these three strains and amplified with the *nodC* primers. The grouping of these four strains was genetically closer to the diazotrophic *Burkholderia* strain TJ 182, isolated from *Mimosa diplotricha* in Taiwan [6]. Finally, SEMIA 6398 from *Piptadenia gonoacantha* (Mimosoideae) occupied an isolated position in the cluster and the strain did not amplify with *nifH* primers, but PCR-products were obtained with both *nodB* primers of common bean rhizobia and *nodC* primers of soybean bradyrhizobia. It is also noteworthy that PCR-products obtained with *nodC* primers varied in size among the strains, and were different from those obtained with *Bradyrhizobium* by Sterner and Parker [40]. The results obtained in our study indicate higher variability in relation to the host plant, to the ribosomal 16S gene, and probably to the *nif* and *nod* genes among burkholderia capable of nodulating legumes than previously thought [6,32]. Further sequencing of *nod* and *nif* PCR-products may help to clarify the origin of those genes in burkholderia.

It has been suggested that tropical rhizobia are poorly documented [55] although reports indicate that rhizobia diversity may be greater in tropical than in temperate regions [33]. The strains from this study have been selected for higher efficiency of N_2 fixation with several legume hosts and are recognized as the most effective for their host legumes in Brazil. Host specificity of several strains was very low, as they were capable of nodulating legumes of different tribes and subfamilies. Host specificity was not related to 16S rRNA, therefore evolution of ribosomal and symbiotic genes may have been diverse. The promiscuous nature of some strains, the wide-range of symbiotic associations with α- and β-proteobacteria, and the detection of several putative new species emphasize the great diversity of rhizobial strains that still remains to be discovered in the tropics.

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