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rep-PCR fingerprinting and taxonomy based on the sequencing of the 16S rRNA gene of 54 elite commercial rhizobial strains

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Abstract In tropical soils, diversity and biotechnological potential of symbiotic diazotrophic bacteria are high. However, the phylogenetic relationships of prominent strains are still poorly understood. In addition, in countries such as Brazil, despite the broad use of rhizobial inoculants, molecular methods are rarely used in the analysis of strains or determination of inoculant performance. In this study, both rep-PCR (BOX) fingerprintings and the DNA sequences of the 16S rRNA gene were obtained for 54 rhizobial strains officially authorized for the production of commercial inoculants in Brazil. BOX-PCR has proven to be a reliable fingerprinting tool, reinforcing the suggestion of its applicability to track rhizobial strains in culture collections and for quality control of commercial inoculants. On the other hand, the method is not adequate for grouping or defining species or even genera. Nine strains differed in more than 1.03% (15) nucleotides of the 16S rRNA gene in relation to the closest type strain, strongly indicative of new species. Those strains were distributed across the genera *Burkholderia*, *Rhizobium*, and *Bradyrhizobium*.

Keywords Bacterial fingerprinting · Bacterial taxonomy · Biological nitrogen fixation · Culture collections · Inoculants · 16S rRNA

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Introduction

Within the large family of plants Leguminosae (Fabaceae in the USA), there are many species capable of establishing symbiotic associations with bacteria which results in biological nitrogen (N_2) fixation, a process responsible for the wide adoption of legumes as food crops, forages, green manures, and in forestry (Allen and Allen 1981; Polhill and Raven 1981). From the 1970s, the green revolution resulted in an increased use of N-fertilizers in agriculture, and applied research on N_2 fixation went through some decades of relative ostracism. Exceptions were found in countries where N-fertilizers have always been very expensive and were usually related to the economical pressure for large-scale production of cash crops, as is the case of soybean in South America (Hungria et al. 2005, 2006a; Hungria and

Campo 2007). However, an increased interest in biological N_2 fixation is restarting and should increase in the coming years, due to concerns about greater water pollution by nitrate, ideals of a more sustainable agriculture, and especially, their increasing higher cost of N fertilizers. The use of N_2-fixing legumes is also expected to increase, not only as cash crops or biofuel but also in the recovery and improvement of soil fertility; therefore, it is timely to obtain new information regarding legumes and diazotrophic symbiotic bacteria.

Searching for the most effective rhizobial strains for each legume is a labor- and time-consuming process involving the production of dozens of rhizobial cultures, greenhouse experiments, and field trials. In Brazil, considerable efforts have been expended for more than 40 years in selecting effective strains for several legumes. Efforts have also been made to create strong legislation to control the use of rhizobial strains in commercial inoculants, aiming at avoiding the spread of noneffective strains. Since 1975, inoculants commercialized in the country can only contain strains recommended by Brazilian public research institutions (Hungria and Campo 2007). To enforce the strain recommendation, a network of microbiology laboratories and inoculant industries was created in 1985, 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 Catalog of *Rhizobium* Collections #443 in the WFCC World Data Center on Microorganisms), at the Fundação Estadual de Pesquisa Agropecuária (FEPAGRO), Porto Alegre, Brazil.

Presently, there are in the SEMIA collection 142 elite rhizobial strains officially recommended for inoculant production in Brazil (MAPA 2006), but despite the economical importance, their genetic characterization is still very poor. The strains are classified only as *Rhizobium* and *Bradyrhizobium*, based on the host legume specificity and on the acid/alkaline reaction and fast/slow growth in medium-containing mannitol as carbon source (FEPAGRO 1999). In a first effort to characterize this collection, the 16S rRNA gene of 68 rhizobial strains was sequenced, and 49 of them were reclassified at the genus and/or species level (Menna et al. 2006).

In this study, 54 of the remaining elite strains contained in the SEMIA collection, officially authorized for the use in commercial inoculants for 47 legumes used as forages, grains, and trees were investigated, with two main objectives. The first was to confirm if the rep-PCR technique shows discriminatory power and reproducibility for fingerprinting rhizobia, helping in the program of quality control of culture collections and of inoculants. The second objective was to investigate the phylogeny and taxonomic position of those elite strains based on the sequencing of the 16S rRNA gene.

Materials and methods

Strains

Fifty-four strains from the Brazilian (SEMIA) culture collection of rhizobia were selected. Table 1 provides information about both the strains, and the 47 legume hosts for which they are recommended. Strains were provided by FEPAGRO, and their purity was verified on yeast extract–mannitol agar (YMA) medium (Vincent 1970) containing Congo red (0.00125%). Stocks were prepared on YMA and kept at −70 (under 30% glycerol) for long-term storage and at 4 as source cultures.

In the rep-PCR analysis, 14 reference strains were included, as follows: *Bradyrhizobium japonicum* strains USDA 6^T (= LMG 6138, = NZP 5549, = ATCC 10324, = DSM 30131, = 3I1b6, = SEMIA 5052, = RCR 3425, = ACCC 15032) and USDA 110; *Bradyrhizobium liaoningense* LMG 18230^T (= 2281, = USDA 3622); *Bradyrhizobium elkanii* USDA 76^T (= LMG 6134, = NZP 5531, = ATCC 49852, = DSM 11554); *Rhizobium tropici* type A CFN 299 (= USDA 9039, = LMG 9517, = UMR1026); *R. tropici* type B CIAT 899^T (= USDA 9030, = SEMIA 4077, = UMR1899, = TAL 1797, = HAMBI 1163, = CM01, = ATCC 49672, = BR322); *R. leguminosarum* strains USDA 2370^T (= ATCC 10004, = LMG 14904), and USDA 2671 (= RCR 3644); *Rhizobium etli* CFN 42^T (= USDA 9032, = ATCC 51251, = DSM 11541); *Ensifer* (= *Sinorhizobium*) *fredii* USDA 205^T (= ATCC 35423, = DSM 5851, = PRC 205); *Ensifer meliloti* USDA 1002^T (= ATCC 9930, = DSM 30135); *Mesorhizobium loti* USDA 3471^T (= ATCC 33669, = ATCC 700743, = DSM 2626, = NZP 2213); *M. ciceri* USDA 3383^T (= ATCC 51585, = DSM 11540, = LMG 14989, = UPM-Ca7), and *Azorhizobium caulinodans* ORS 571^T (= USDA 4892, = DSM 5975, = LMG 6465, = ORS 571). They were provided by USDA, Beltsville, USA and by the Centro de Ciencias Genómicas, Cuernavaca, Mexico. All strains from this study are deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection" of Embrapa Soja (http://bmrc.lncc.br) and also at the "*Rhizobium* Culture Collection SEMIA" (IBP World Catalog of *Rhizobium* Collections #443 in the WFCC World Data Center on Microorganisms) at the FEPAGRO.

DNA extraction and rep-PCR (BOX) genomic fingerprinting

Total genomic DNA of each strain was extracted as described by Kaschuk et al. (2006) and amplified by PCR with the primer BOX A1R (5′-CTACGGCAAGGCG ACGCTGACG-3′; Versalovic et al. 1994). Amplification procedures were performed as described by Kaschuk et al.

(2006), except for the reduction from 35 to 30 cycles of amplification. The 1-kb DNA marker (Invitrogen™) was included on the left, right, and in the center of each gel. The amplified fragments were separated by horizontal electrophoresis on 1.5% agarose gel, stained with ethidium bromide, visualized under UV radiation, and photographed.

Sequencing analysis of the DNA region coding for the 16S rRNA gene

The procedure for amplification and sequencing analysis of the 16S rDNA was performed as previously described (Menna et al. 2006). The sequencing analysis was performed on a MegaBACE 1,000 DNA Analysis System (Amersham Biosciences).

The high-quality sequences obtained for each strain were assembled into contigs as described before (Menna et al. 2006) and 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 given to the 16S rRNA sequences of the fifty-four strains are listed in Table 2.

Cluster analyses

For the rep-PCR analysis, first, the sizes of the fragments in all analyses were normalized according to the sizes of the DNA markers. Cluster analyses of the BOX-PCR profiles were performed using the Bionumerics program (Applied Mathematics, Kortrijh, Belgium, version 4.6), with the UPGMA algorithm (unweighted pair-group method, with arithmetic mean; Sneath and Sokal 1973) and the Jaccard coefficient (Jaccard 1912), considering the optimum values indicated by the Bionumerics program for the tolerance and the optimization parameters.

For the 16S rRNA analysis, the sequences obtained were aligned pairwise and compared to those of the following type/reference strains (accession numbers of the GenBank Data Library in parentheses): *B. elkanii* USDA 76^T (U35000); *B. japonicum* USDA 6^T (U69638); *B. liaoningense* LGM 18230^T (AF208513); *B. canariense* BC-C2^T (AY577427); *B. yuanmingense* CCBAU 10,071^T (= CFNEB 101, = B071) (AF193818); *B. betae* PL7HG1^T (= LMG 21,987, = CECT 5,829; AY372184); *Burkholderia cepacia* ATCC 53867^T (AY741356); *Mesorhizobium amorphae* ACC 19665^T (= RCAN13; DQ022832); *M. ciceri* USDA 3383^T (U07934); *M. loti* USDA 3471^T (X67229); *M. tianshanense* USDA 3592^T (= A-1BS; AF041447); *Methylobacterium nodulans* ORS 2060^T (= CNCM I 2,342, = LMG 21,967; AF220763); *R. leguminosarum* USDA 2370^T (U29386); *R. mongolense* USDA 1844^T (U89817); *Rhizobium* (= *Agrobacterium*) *rhizogenes* ATCC 11325^T (= 163C, = DSM 30,148, = IFO 13,257, = IMET 11,180; AY945955.1); *R. tropici* CIAT 899^T (U89832); *R. lusitanum* P1-7^T (= CECT 7,016, = LMG 22,705; AY738130); *E. fredii* USDA 205^T (X67231), *E. meliloti* USDA 1002^T (X67222). *Methanococcus maripaludis* strain C6 (U38487) was used as an outgroup strain.

Multiple alignments were performed with ClustalX version 1.83 (Thompson et al. 1997). Phylogenetic trees were generated using MEGA version 3.1 (Kumar et al. 2004) with default parameters, K2P distance model (Kimura 1980), and neighbor-joining algorithm (Saitou and Nei 1987). *Methanococcus maripaludis* strain C6 was used as an outgroup for 16S rDNA phylogenies. Statistic support for tree nodes was evaluated by bootstrap (Felsenstein 1985) analyses with 1,000 samplings (Hedges 1992).

Results

Complex fingerprinting patterns with multiple distinct bands of various intensities were obtained in the BOX-PCR analysis of the 54 elite rhizobial strains and allowed the identification of numerous well-defined groups with a high level of inter- and intraspecific diversity (Fig. 1). Furthermore, it is worth mentioning that the strains were analyzed three times, starting from the DNA extraction and resulted in band profiles showing good agreement, confirming the robustness of the method.

Among the high diversity detected in the BOX-PCR analysis in this study, only two pairs of strains showed identical (100% similarity) fingerprints: (1) *B. elkanii* SEMIAs 6,389, recommended for *Acacia podalyriifolia* (subfamily Mimosoideae) and 6,403, recommended for four hosts, *Enterolobium cyclocarpum*, *Pithecellobium guachapele*, *Samanea saman* (Mimosoideae), and *Poecilanthe parviflora* (Papilionoideae) and (2) *Rhizobium* sp. SEMIAs 6,436 (host, *Acacia farnesiana*, Mimosoideae) and 6,438 (*Adesmia latifolia,* Papilionoideae). In addition, highly similar profiles (95% of similarity or higher) were obtained for SEMIA 6,437 (*Adesmia latifolia*) and SEMIAs 6,436 and 6,438, as well as for *B. cepacia* strains 6,417 and 6,422, both recommended for *Mimosa flocculosa* (Fig. 1).

In the cluster analysis of BOX-PCR profiles, all strains were grouped at a very low level of similarity, of about 20% (Fig. 1), highlighting the high genetic diversity of our rhizobial collection. The assigned genera and species in Fig. 1 were based on the 16S rRNA genes, as shown in Table 2. The comparison of BOX-PCR profiles (Fig. 1) with the classification based on the 16S rRNA (Table 2) shows a poor correlation between both sets of data. It is noteworthy that even strains belonging to the same genus showed high genetic variability in BOX-PCR profiles such that some clusters included strains occupying completely different taxonomic positions.

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

Plant species [a,b]	Some common names [c,d]	Subfamily[c]	Tribe [c]	Description [c]	Main use in Brazil	Applications worldwide [c]	SEMIA number	Other designations[e]
Acacia angustissima (Mill.) Kuntze	Carboncillo, Timbe, Timbre, acácia	Mimosoideae	Acacieae	Perennial non-climbing tree; not threatened	Tree	Environment, wood	6430	BR 3630
Acacia farnesiana (L.) Willd	Acacia Jaune, Acacia Odorant, Aroma, Amarilla, Aromo, Ban Baburi, Bayahonda, Carambomba, Cashia, Cassie,Espino Blanco	Mimosoideae	Acacieae	Perennial non-climbing tree;not threatened	Tree	Medicine, chemical products, domestic, environment, forage	6436 6430	BR 9002, CVRD-6-II BR 3630
Acacia podalyriifolia G. Don	Mt Morgan Wattle; Queensland Silver Wattle	Mimosoideae	Acacieae	Perennial non-climbing tree; not threatened	Tree	Environment	6389	BR 3612
Acacia saligna (Labill.) Wendl	Blue-leafed Wattle,Golden Wreath Wattle, Golden Wreath Wattle, Orange Wattle, Port Jackson Willow, Western Australian Golden Watt	Mimosoideae	Acacieae	Perennial non-climbing tree; not threatened	Tree	Chemical products, domestic, environment, forage, wood	6096 6428	BR 8601 BR 3628
Acacia salicina Lindl	Broughton Willow, Cooba, Doolan, Native Wattle, Native Willow, Willow Acacia	Mimosoideae	Acacieae	Perennial non-climbing tree	Tree	Environment	6392 6400	BR 3804 BR 5005
Adesmia latifolia (Spreng.) Vogel	Adesmia	Papilionoideae	Adesmieae	Perennial, non-climbing herb	Forage	Forage, environment	6437 6438	EEL 15084 ET 226
Albizia lebbeck (L.) Benth.	Rain tree, woman's tongue, bois noir, baile de caballero, lengua de mujer,coração de negro, faveiroh, pau preto,acácia	Mimosoideae	Ingeae	Perennial nonclimbing tree	Tree	Environment, chemical, toxins, wood	6432	BR 5611
Arachis pintoi Krapov. and W. Gregory	Forage peanut,amendoim forrageiro	Papilionoideae	Aeschynomeneae	Perennial nonclimbing herb	Forage	Forage, environment	6439	MGAP 13
Albizia pedicellaris (Dc.) L. Rico	juerana-branca, Gallinazo	Mimosoideae	Ingeae	Perennial non-climbing tree	Tree	Environment, wood	6396 6408	BR 6815 BR 6816
Bowdichia virgilioides Kunth	Alcornoque, Alcornoque, Sapupira Sebipira Guaz, Sepibira, Sucupira, Sucupirucu-parda	Papilionoideae	Sophoreae	Perennial non-climbing tree, not threatened	Tree	Environment	6414 6096	BR 8602 BR 8601
Calliandra houstoniana (Mill.) Standl. var. calothyrsus (Meissner)Barneby	caliandra	Mimosoideae	Ingeae.	Perennial non-climbing tree	Forage	Forage, wood	6395 6423	BR 4301 BR 4302
Calliandra surinamensis Benth	Calliandra, Pompon de Marin, Salsa	Mimosoideae	Ingeae	Perennial non-climbing tree	Forage	Environment	6395 6,423	BR 4301 BR 4302
Centrosema spp.	Centrosema, fleurlanguette, pois piante, choncho, conchitas, centrosemah	Papilionoideae	Phaseoleae	Perennial climbing or non-climbing herb	Forage	Forage, environment	690	C 100a
Chamaecrista ensiformis (Vell.) H.S. Irwin & Barneby	Jaúna, coração-de-negro	Caesalpinioideae	Cassieae	Perennial	Tree	Environmental	6392	BR 3804
Clitoria fairchildiana R. Howard	Butterfly pea tree, faveira, palheteira, palheiroh, sombreiro	Papilionoideae	Phaseoleae	Perennial non climbing, tree	Tree	Environmental, medicine, wood	6411	BR 8003
Dalbergia nigra (Vell. Conc.) Benth.	Brazilian rosewood, jacaranda, palisandre, cabiúnah, jacarandá-da-Bahia	Papilionoideae	Dalbergieae	Perennial nonclimbing tree	Tree	Environment, chemical, wood	6413	BR 8402
Dimorphandra jorgei M.F. Silva	Angelim Vermelho, Pau-Pratudo	Caesalpinioideae	Caesalpinieae	Perennial non-climbing tree; not threatened	Tree	Environment, wood	6400	BR 5005

Enterolobium cyclocarpum (Jacq.) Griseb.	Devil's ear, earpod tree, elephant's ear, monkey ear, bois tanniste rouge, Oreille d'e´ le´ phant, guanacaste, orelhade- elefante	Mimosoideae	Ingeae	Perennial nonclimbing tree	Tree	Environment, medicine, pasture, wood, food	6403	BR 6205
Enterolobium timbouva Martius	Timbor, tamborilh, timbau´ vah, timboú vah	Mimosoideae	Ingeae	Perennial nonclimbing tree	Tree	Environment,medicine, toxin, wood	6397	BR 3407
Falcataria moluccana (Miq.) Barneby and Grimes (=Albizia falcataria)	Albizia falcataria, molucca albizia, batai wood, sau, malesia, albiziah	Mimosoideae	Ingeae	Perennial nonclimbing tree	Tree	Environment, wood, food	6432	BR 5611
Gliricidia sepium (Jacq.) Walp.	Grow stick, cacahuananche, madricacao, gliricidiah	Caesalpinioideae	Robinieae	Perennial nonclimbing tree	Tree	Environment, wood	6435	BR 8802
Inga semialata (Vell.) C. Mart	ingá	Mimosoideae	Ingeae	Perennial non-climbing tree, not threatened	Tree	Environment	6433	BR 6609
Lathyrus odoratus L	Bunte Wicke, China Dushistaya, Chyna Pakhuchaya; Duftende Platterbse; Duftwicke; Dushistyi Goroshek; Ervilha de Cheiro; Etirli Kululche; Frijol de Olor, Sweet Pea	Papilionoideae	Vicieae	Annual climbing herb, not threatened	Tree	Environment; chemical products; domestic	388 3018	TAL 364
Lens culinaris Medik. subsp. culinaris	Adi Merchi, Burchak , Chachavitza Zvychainaya, Chechevitza Kulturnaya, Common Lentil, Edamas Lecas, Lenteja, Lenticchia	Papilionoideae	Vicieae	Annual non-climbing her, cultigen not known in the wild	Grain	Food and drink; chemical products, forage; medicine	344 3026 3025	 CPAC L12 CPAC L3
Leucaena diversifolia (Schldl.) Benth.	Wild tamarind, guash, guache, guaje, guaje blanco, leucenah	Mimosoideae	Mimoseae	Perennial nonclimbing shrub, tree	Tree	Environment, food	6162	UFC 933.52
Leucaena Leucocephala (Lam.) De Wit v. Cunningham	Jumbie bean, lead tree, leucaena, cowbush, bois bourro, cassie blanc, graines de lin, granadillo bobo, leucena	Mimosoideae	Mimoseae	Perennial nonclimbing shrub, tree	Tree	Environment, food	6153	BR 827
Lonchocarpus costatus Benth	No common name	Papilionoideae	Millettieae	Perennial nonclimbing	tree	Environmental	6399 6404	BR 6010 BR 6009
Lotus corniculatus L.	Cat's clover, birds foot trefoil, broadleaf trefoil, cornichão	Papilionoideae	Loteae	Perennial nonclimbing herb, shrub	Forage	Forage, environment	806	
Lotus subbiflorus Lag	Hairy Birdsfoot Trefoil, el rincón	Papilionoideae	Loteae	Annual non-climbing herb	Forage	Forage, environment	848 849	D226, 510 D281, 512
Medicago sativa L.	Alfalfa, lucerne, luzerne, alfafah	Papilionoideae	Trifolieae	Perennial nonclimbing herb	Forage	Forage, environment, medicine, food	116	USDA 1088
Mimosa caesalpiniifolia Benth.	Mimosa, jupinuan, mimosah, unha de gatah, sabiá, sansão do campo	Mimosoideae	Mimoseae	Perennial nonclimbing shrub or tree	Tree	Environment, wood	6410	BR 3451
Mimosa flocculosa Burkart	Bracatinga-rósea, bracaatinga-rósea, bracatinga-rosa, Bracatinga-de-campo-mourão, jurema	Mimosoideae	Mimoseae	Perennial nonclimbing tree	Tree	Environment, wood	6417 6422	BR 3462 BR 3463
Parapiptadenia rigida (Benth.) Brenan	Angico, Angico-cedro, Angico-do-banhado, Angico-dos-montes, Angico-verdadeiro, Angico-vermelho, Guarucaia, Paric	Mimosoideae	Mimoseae	Perennial non-climbing tree; not threatened	Tree	chemical, medicinal, wood	6416	BR 9004
Phaseolus vulgaris L	Frijol, Common Bean, feijão	Papilionoideae	Phaseoleae	Annual non-climbing herb; not threatened	Grain	Food	4088	PRF 81
Piptadenia gonoacantha (Martius) Macbr.	Piptadenia, pau jacare, angico jacaré, caniveteiroh, icarapé	Mimosoideae	Mimoseae	Perennial nonclimbing tree	Tree	Wood, environment, chemical	6385	BR 3452
Pisum sativum L.	Field pea, petit pois, alverja, guisante, ervilha	Papilionoideae	Fabeae	Annual, climbing, Herb	Grain	Food, forage	3012	CPAC EV6
Pithecellobium tortum Mart	Tataré, Jacaré, Angico-branco, Jurema, Vinhático-de-espinho	Mimosoideae	Ingeae	Perennial non-climbing tree	Forage	Forage	6407 6406	BR 6813 BR 6812
Poecilanthe parviflora Benth	coração de negro, canela do brejo,	Papilionoideae	Brongniartieae	Perennial non-climbing	Tree	Wood, chemical	6403	BR 8205

Table 1 (continued)

Plant species [a,b]	Some common names [c,d]	Subfamily[c]	Tribe [c]	Description [c]	Main use in Brazil	Applications worldwide [c]	SEMIA number	Other designations[e]
	coração negro, ipê coração, jacarandá de mato grosso, pau ferro, pau jantar			tree, not threatened				
Prosopis juliflora (Sw.) DC.	algaroba Mesquite, cashaw, bayarone, epinard bayahonda blanca, algarobah	Mimosoideae	Mimoseae	Perennial nonclimbing, tree, shrub	Tree	Environment, wood	6162	UFC 933.52
Pithecellobium guachapele (Kunth) J.F. Macbr	Cadeno, Carreto, zaro Macho, Guachapeli, Guaramillo, Higo Amarillo, Lara Blanca, Sanaguaro	Mimosoideae	Ingeae	Perennial non-climbing tree insufficiently known	Tree	Wood	6409 6403	BR 6821 BR 6205
Samanea saman (Jacq.) Merr (==Mimosa, Pithecellobium, Enterolobium, Inga e Calliandra saman)	Acacia Preta, Algarrobo, Arbor de Lluvia, Arbre a la Pluie, Campano, Carabeli, Rain Tree, Saman	Mimosoideae	Ingeae	Perennial non-climbing tree; not threatened	Tree	Environment, forage, wood, chemical products, food, medicine, Wood, domestic	6405 6403	BR 6212 BR 6205
Stylosanthes spp.	Stylo, Brazilian lucerne, stylosanthes, estilosantesh	Papilionoideae	Aeschynomeneae	Perennial nonclimbing herb or shrub	Forage	Forage, environment	6154	BR 446
Trifolium pratense L.	Red clover, trebol de los prados, trebol rojo, trevo vermelhoh	Papilionoideae	Trifolieae	Perennial nonclimbing, herb	Forage	Forage, environment, medicine	265 2082	U-26 EEL 8186
Trifolium repens L.	Dutch, ladino, whiteclover, trebol blanco, trevo brancoh	Papilionoideae	Trifolieae	Perennial nonclimbing herb	Forage	Forage, environment, medicine	235 2082 2083	UNZ-29 EEL 8186 EEL 7782
Trifolium semipilosum Fresen	Kenya Clover, Kenya White Clover	Papilionaceae	Trifolieae	Perennial non-climbing herb; not threatened	forage	Forage	2002	CB 782
Trifolium subterraneum L.	Subclover, subterranean clover, trevo subterrâneoh	Papilionaceae	Trifolieae	Annual nonclimbing, herb	Forage	Forage, environment, medicine	265	U-26
Trifolium vesiculosum Savi	Arrowleaf clover, trevo yuchi, trevo vesiculosoh	Papilionaceae	Trifolieae	Annual nonclimbing herb	Forage	Forage, environment, medicine	2050	

ATCC 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* Tropical Agricultural Legumes Project, *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)

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

[b] Taxonomy based on ILDIS (2005)

[c] Information obtained from: www.ildis.org and www.biodiversityexplorer.org/plants

[d] Common names used in worldwide. Information obtained from the site cited on ([c])

[e] Different numbers in FEPAGRO (1999)

Table 2 Information about the 16S RNAr sequence of the strains

SEMIA strain	Identities with the closest type strain	Percentage (%)[a]	Number of distinct nucleotides[b]	Length[c]	Gene Bank access #	Proposed taxonomy position[d]
116	1464/1464	0	0	1464	FJ025128	*Ensifer meliloti*
235	1457/1462	0.34	5	1460	FJ025090	*Rhizobium leguminosarum*
265	1457/1462	0.34	5	1460	FJ025088	*Rhizobium leguminosarum*
344	1450/1463	0.89	13	1461	FJ025087	*Rhizobium leguminosarum*
388	1456/1462	0.41	6	1462	FJ025090	*Rhizobium leguminosarum*
690	1461/1468	0.47	7	1468	FJ025108	*Bradyrhizobium elkanii*
806	1463/1464	0.06	1	1464	FJ025125	*Mesorhizobium amorphae*
848	1458/1464	0.40	6	1464	FJ025122	*Mesorhizobium amorphae*
849	1457/1465	0.54	8	1464	FJ025123	*Mesorhizobium amorphae*
2002	1457/1462	0.34	5	1460	FJ025086	*Rhizobium leguminosarum*
2050	1457/1462	0.34	5	1460	FJ025096	*Rhizobium leguminosarum*
2082	1455/1463	0.54	8	1460	FJ025094	*Rhizobium leguminosarum*
2083	1457/1462	0.34	5	1460	FJ025096	*Rhizobium leguminosarum*
3012	1456/1464	0.54	8	1464	FJ025121	*Mesorhizobium tianshanense*
3018	1457/1463	0.41	6	1461	FJ025093	*Rhizobium leguminosarum*
3025	1455/1462	0.48	7	1460	FJ025095	*Rhizobium leguminosarum*
3026	1457/1462	0.34	5	1460	FJ025093	*Rhizobium leguminosarum*
4088	1451/1464	0.89	13	1460	EF054889	*Rhizobium tropici*
6,096	1465/1468	0.20	3	1468	FJ025115	*Bradyrhizobium elkanii*
6153	1465/1468	0.20	3	1468	FJ025097	*Bradyrhizobium japonicum*
6154	1455/1468	0.88	13	1467	FJ025100	*Bradyrhizobium japonicum*
6162	1450/1464	0.95	14	1464	FJ025127	*Ensifer meliloti*
6385	1467/1502	2.33	35	1500	FJ025136	*Burkholderia* sp.
6389	1464/1468	0.27	4	1467	FJ025113	*Bradyrhizobium elkanii*
6392	1459/1465	0.40	6	1465	FJ025126	*Mesorhizobium amorphae*
6395	1454/1470	1.08	16	1468	FJ025101	*Bradyrhizobium* sp.
6396	1457/1469	0.81	12	1467	FJ025099	*Bradyrhizobium japonicum*
6397	1459/1466	0.47	7	1468	FJ025139	*Bradyrhizobium elkanii*
6399	1461/1468	0.47	7	1468	FJ025102	*Bradyrhizobium elkanii*
6400	1464/1468	0.27	4	1468	FJ025114	*Bradyrhizobium elkanii*
6403	1465/1469	0.27	4	1469	FJ025112	*Bradyrhizobium elkanii*
6404	1461/1468	0.47	7	1468	FJ025104	*Bradyrhizobium elkanii*
6405	1465/1468	0.20	3	1468	FJ025109	*Bradyrhizobium elkanii*
6406	1456/1461	0.34	5	1460	FJ025116	*Rhizobium etli*
6407	1382/1463	5.54	4	1462	FJ025133	*Methylobacterium mesophilicum*
6408	1461/1468	0.47	7	1468	FJ025103	*Bradyrhizobium elkanii*
6409	1456/1461	0.34	5	1460	FJ025117	*Rhizobium etli*
6410	1467/1502	2.33	35	1500	FJ025137	*Burkholderia* sp.
6411	1429/1461	2.19	32	1461	FJ025129	*Rhizobium* sp.
6413	1467/1502	2.33	35	1500	FJ025138	*Burkholderia* sp.
6414	1465/1468	0.20	3	1468	FJ025111	*Bradyrhizobium elkanii*
6416	1465/1468	0.20	3	1468	FJ025108	*Bradyrhizobium elkanii*
6417	1502/1502	0	0	1502	FJ025134	*Burkholderia cepacia*
6422	1499/1503	0.26	4	1502	FJ025135	*Burkholderia cepacia*
6423	1458/1460	0.13	2	1460	FJ025132	*Rhizobium rhizogenes*[e]
6428	1461/1469	0.54	8	1469	FJ025106	*Bradyrhizobium elkanii*
6430	1457/1466	0.61	9	1466	FJ025124	*Mesorhizobium amorphae*
6432	1463/1468	0.34	5	1467	FJ025110	*Bradyrhizobium elkanii*

Table 2 (continued)

SEMIA strain	Identities with the closest type strain	Percentage (%)[a]	Number of distinct nucleotides[b]	Length[c]	Gene Bank access #	Proposed taxonomy position[d]
6433	1461/1468	0.47	7	1468	FJ025105	*Bradyrhizobium elkanii*
6435	1441/1464	1.57	23	1460	FJ025130	*Rhizobium* sp.
6436	1438/1461	1.57	23	1460	FJ025119	*Rhizobium* sp.
6437	1438/1461	1.57	23	1460	FJ025118	*Rhizobium* sp.
6438	1438/1461	1.57	23	1460	FJ025120	*Rhizobium* sp.
6439	1454/1468	0.95	14	1468	FJ025098	*Bradyrhizobium japonicum*

[a] Percentage of distinct nucleotides in relation to the closest type strain

[b] Number of distinct nucleotides in the sequence in relation of the closest type strain

[c] Length of the sequence used in the comparison and the building of the phylogenetic tree

[d] The taxonomy was proposed based on the discrepancy of the 15 nucleotides in relation of the type strain (Menna et al. 2006)

[e] Showing high similarity with *Rhizobium rhizogenes* and *Rhizobium tropici*, as observed in Fig. 2

Similar to our previous definition that strains differing by more than 1.03% (15) nucleotides from the closest type strain could represent new species (Menna et al. 2006), nine strains from this study were denominated as "sp." and some of them have differed by up to 35 nucleotides in relation to the closest type strain (Table 2).

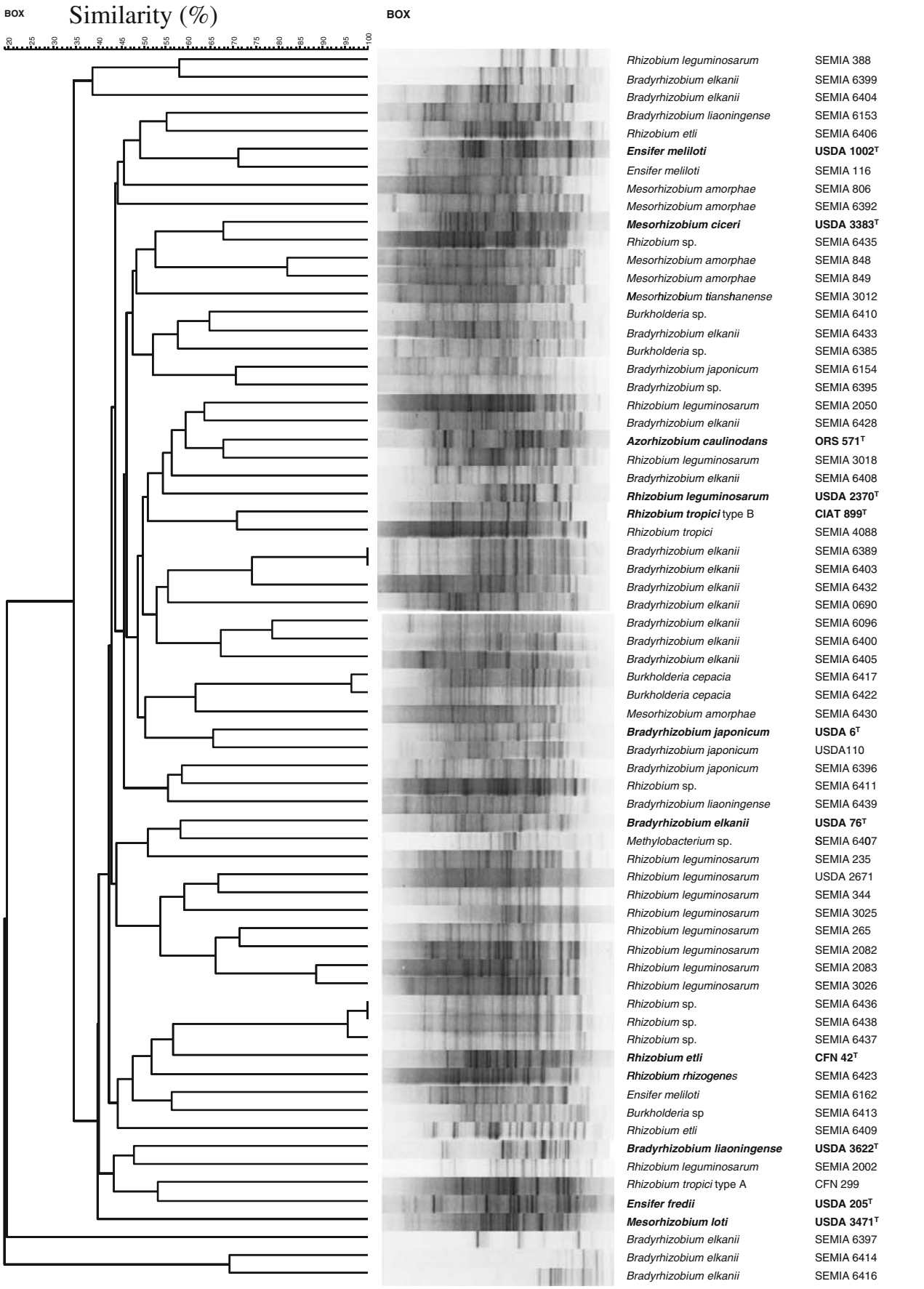
The phylogenetic tree obtained with the 16S rRNA-aligned sequences of the 54 rhizobial strains, as well as of the type and reference strains used in this study, is shown in Fig. 2. The strains were clustered into six main phylogenetic branches or well-defined main clusters (I–VI), with bootstrap support of 99%. In addition, there were some strongly bootstrap-supported subclusters and also a few isolated strains. Evidence of a new *Rhizobium* species was confirmed in the subcluster III.4, a cluster including strains SEMIAs 6,436, 6,437, and 6,438. In addition, *Rhizobium* sp. strains SEMIAs 6,411 and 6,435 were found to be closer to the *R. tropici* branch, showing differences of 32 and 23 bp in relation to the type strain, respectively. Cluster I grouped *Methylobacterium* strains, but clearly SEMIA 6,407 is highly different from *M. nodulans* type strain, a bacterium capable of fixing N_2. However, strain SEMIA 6,407 has shown 99% similarity of bases with *Methylobacterium mesophilicum* (BLASTN), formerly classified as *Pseudomonas mesophilica* and not reported as being a diazotrophic bacterium; therefore, pathogenic and symbiotic genes of these strains should be investigated in more detail. Another interesting group of strains deserving further study is that formed by SEMIAs 6,396, 6,154, 6,395, and 6,439, positioned within subcluster II.2 (Fig. 1). These strains showed differences in relation to the closest type strains ranging from 12 to 16 bp, corresponding to 0.81% to 1.08% of the nucleotides (Table 2) and may well represent a new *Bradyrhizobium* species. Finally, *Burkholderia* sp. SEMIAs 6,410, 6,385, and 6,413 were grouped with strong bootstrap support (99%) as a subcluster of cluster VI also showing differences in relation to the closest type strain (Fig. 2).

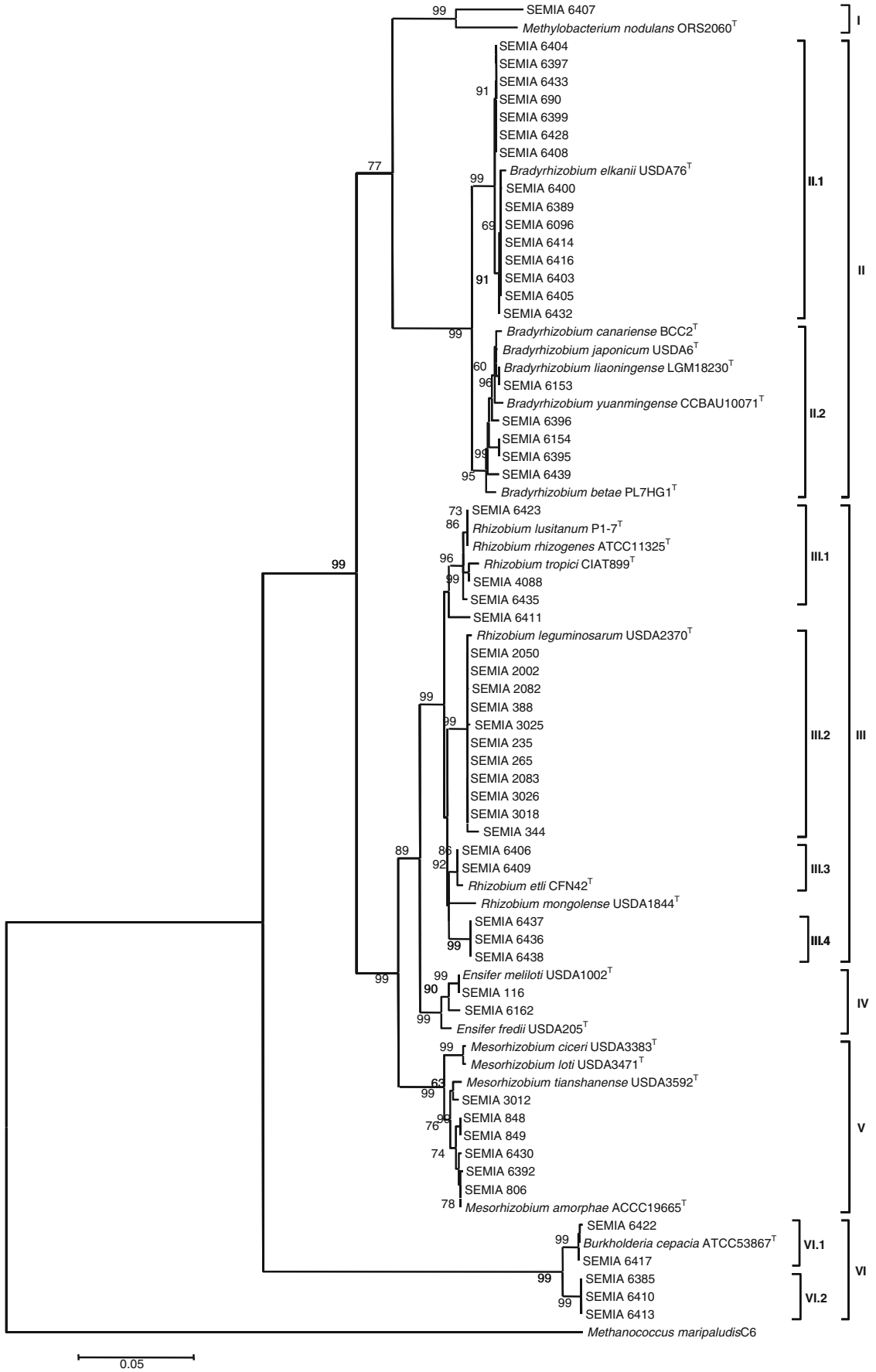
Discussion

In this study, we investigated a collection of 54 elite rhizobial strains, selected as the most effective in fixing N_2 with 47 legume hosts. Some of the strains are characterized by high rates of N_2 fixation with more than one legume; thus, our collection represents a total of 69 recommendations for the production of commercial inoculants in Brazil and in some other countries of South America.

The BOX-PCR technique has been broadly used (e.g., de Bruijn 1992; Alberton et al. 2006; Kaschuk et al. 2006; Barcellos et al. 2007; Batista et al. 2007; Menna et al. 2009) as a powerful fingerprinting tool, revealing strong genetic diversity among rhizobial strains. Indeed, also in this study, a very high level of diversity among the strains was detected in the BOX-PCR analysis, such that they were all joined at a final level of similarity of only 20%. In addition, high reproducibility of the BOX-PCR fingerprinting protocol was recently confirmed using a variety of rhizobial strains (Menna et al. 2009) and also confirmed in this study. Therefore, we validate a previous suggestion (Menna et al. 2009) that the method is reliable and represents an important tool for purposes other than detecting genetic diversity, including tracking of rhizobial stains in culture collections and programs of quality control of commercial inoculants. Indeed, based on the results from our group, including this study, the Brazilian government has decided to adopt BOX-PCR fingerprinting for such purposes.

Fig. 1 Cluster analysis (UPGMA with the coefficient of Jaccard) of the products obtained by BOX-PCR analysis. Type strains are labeled and SEMIA strains are classified according to the sequencing analysis of the 16S rRNA gene, as described on Table 2 ▶


BOX
Similarity (%)
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
BOX
Rhizobium leguminosarum SEMIA 388
Bradyrhizobium elkanii SEMIA 6399
Bradyrhizobium elkanii SEMIA 6404
Bradyrhizobium liaoningense SEMIA 6153
Rhizobium etli SEMIA 6406
Ensifer meliloti USDA 1002T
Ensifer meliloti SEMIA 116
Mesorhizobium amorphae SEMIA 806
Mesorhizobium amorphae SEMIA 6392
Mesorhizobium ciceri USDA 3383T
Rhizobium sp. SEMIA 6435
Mesorhizobium amorphae SEMIA 848
Mesorhizobium amorphae SEMIA 849
Mesorhizobium tianshanense SEMIA 3012
Burkholderia sp. SEMIA 6410
Bradyrhizobium elkanii SEMIA 6433
Burkholderia sp. SEMIA 6385
Bradyrhizobium japonicum SEMIA 6154
Bradyrhizobium sp. SEMIA 6395
Rhizobium leguminosarum SEMIA 2050
Bradyrhizobium elkanii SEMIA 6428
Azorhizobium caulinodans ORS 571T
Rhizobium leguminosarum SEMIA 3018
Bradyrhizobium elkanii SEMIA 6408
Rhizobium leguminosarum USDA 2370T
Rhizobium tropici type B CIAT 899T
Rhizobium tropici SEMIA 4088
Bradyrhizobium elkanii SEMIA 6389
Bradyrhizobium elkanii SEMIA 6403
Bradyrhizobium elkanii SEMIA 6432
Bradyrhizobium elkanii SEMIA 0690
Bradyrhizobium elkanii SEMIA 6096
Bradyrhizobium elkanii SEMIA 6400
Bradyrhizobium elkanii SEMIA 6405
Burkholderia cepacia SEMIA 6417
Burkholderia cepacia SEMIA 6422
Mesorhizobium amorphae SEMIA 6430
Bradyrhizobium japonicum USDA 6T
Bradyrhizobium japonicum USDA110
Bradyrhizobium japonicum SEMIA 6396
Rhizobium sp. SEMIA 6411
Bradyrhizobium liaoningense SEMIA 6439
Bradyrhizobium elkanii USDA 76T
Methylobacterium sp. SEMIA 6407
Rhizobium leguminosarum SEMIA 235
Rhizobium leguminosarum USDA 2671
Rhizobium leguminosarum SEMIA 344
Rhizobium leguminosarum SEMIA 3025
Rhizobium leguminosarum SEMIA 265
Rhizobium leguminosarum SEMIA 2082
Rhizobium leguminosarum SEMIA 2083
Rhizobium leguminosarum SEMIA 3026
Rhizobium sp. SEMIA 6436
Rhizobium sp. SEMIA 6438
Rhizobium sp. SEMIA 6437
Rhizobium etli CFN 42T
Rhizobium rhizogenes SEMIA 6423
Ensifer meliloti SEMIA 6162
Burkholderia sp SEMIA 6413
Rhizobium etli SEMIA 6409
Bradyrhizobium liaoningense USDA 3622T
Rhizobium leguminosarum SEMIA 2002
Rhizobium tropici type A CFN 299
Ensifer fredii USDA 205T
Mesorhizobium loti USDA 3471T
Bradyrhizobium elkanii SEMIA 6397
Bradyrhizobium elkanii SEMIA 6414
Bradyrhizobium elkanii SEMIA 6416


SEMIA 6407
Methylobacterium nodulans ORS2060T
SEMIA 6404
SEMIA 6397
SEMIA 6433
SEMIA 690
SEMIA 6399
SEMIA 6428
SEMIA 6408
Bradyrhizobium elkanii USDA76T
SEMIA 6400
SEMIA 6389
SEMIA 6096
SEMIA 6414
SEMIA 6416
SEMIA 6403
SEMIA 6405
SEMIA 6432
Bradyrhizobium canariense BCC2T
Bradyrhizobium japonicum USDA6T
Bradyrhizobium liaoningense LGM18230T
SEMIA 6153
Bradyrhizobium yuanmingense CCBAU10071T
SEMIA 6396
SEMIA 6154
SEMIA 6395
SEMIA 6439
Bradyrhizobium betae PL7HG1T
SEMIA 6423
Rhizobium lusitanum P1-7T
Rhizobium rhizogenes ATCC11325T
Rhizobium tropici CIAT899T
SEMIA 4088
SEMIA 6435
SEMIA 6411
Rhizobium leguminosarum USDA2370T
SEMIA 2050
SEMIA 2002
SEMIA 2082
SEMIA 388
SEMIA 3025
SEMIA 235
SEMIA 265
SEMIA 2083
SEMIA 3026
SEMIA 3018
SEMIA 344
SEMIA 6406
SEMIA 6409
Rhizobium etli CFN42T
Rhizobium mongolense USDA1844T
SEMIA 6437
SEMIA 6436
SEMIA 6438
Ensifer meliloti USDA1002T
SEMIA 116
SEMIA 6162
Ensifer fredii USDA205T
Mesorhizobium ciceri USDA3383T
Mesorhizobium loti USDA3471T
Mesorhizobium tianshanense USDA3592T
SEMIA 3012
SEMIA 848
SEMIA 849
SEMIA 6430
SEMIA 6392
SEMIA 806
Mesorhizobium amorphae ACCC19665T
SEMIA 6422
Burkholderia cepacia ATCC53867T
SEMIA 6417
SEMIA 6385
SEMIA 6410
SEMIA 6413
Methanococcus maripaludisC6
0.05
I
II
II.1
II.2
III
III.1
III.2
III.3
III.4
IV
V
VI
VI.1
VI.2

◀ **Fig. 2** Phylogenetic tree based on the 16S rRNA sequences of 54 strains, N_2-fixing symbionts isolated from 47 legumes and officially recommended for the use in Brazilian commercial inoculants. GeneBank accession numbers of SEMIA strains (Table 2) and of type strains ("Materials and methods") are given in the text

Most rhizobial strain-selection programs worldwide have been performed based exclusively on symbiotic properties, partially because genetic methods for characterization of elite strains are rarely available. It is noteworthy that although some strains showing identical or very similar BOX-PCR profiles in this study were identified as the most very effective in fixing N_2 for dissimilar legumes, some belong to different subfamilies of the Leguminosae family. In addition, the cluster analysis of BOX-PCR products showed no correlation with the host legumes, in accordance with previous reports from our group (Germano et al. 2006; Menna et al. 2006, 2009). Differences in the symbiotic plasmids or islands should be further investigated, but apparently, many tropical rhizobia and legumes are highly promiscuous.

Although the BOX-PCR analysis was highly effective in detecting genetic diversity, the correlation with the classification based on the 16S rRNA sequences was very poor. Therefore, the results from this study confirm previous reports (Laguerre et al. 1997; Mostasso et al. 2002; Grange and Hungria 2004; Hungria et al. 2006b; Menna et al. 2009) that BOX-PCR is a powerful means of fingerprinting and detecting high genetic diversity among rhizobial strains, but it is inadequate for grouping or defining species or even genera. However, we should mention that for other genera and species, that assumption might not be true, and one example was shown with *Yersinia* species, in which 16S rRNA sequencing and BOX-PCR were not efficient in differentiating *Yersinia pseudotuerculosis* from *Yersinia pestis*, while a clearer definition was achieved with REP- and ERIC-PCR (Kim et al. 2003). Finally, a recent suggestion for improving definition of the rhizobial genotypes and taxonomic groups that includes a polyphasic approach of BOX-PCR/16S rRNA (20/80%; Menna et al. 2009) deserves further consideration and could also apply to the definition of other genera and species.

Considering that strains differing by more than 1.03% (15) nucleotides from the closest type strain could represent new species (Menna et al. 2006), nine strains from this study might represent new species, including three *Burkholderia* sp., five *Rhizobium* sp., and one *Bradyrhizobium* sp. strain. The phylogenetic tree built with the 16S rRNA sequences highlights these putative new species.

It has long been suggested that rhizobia are more diverse in tropical than in temperate soils (e.g., Oyaizu et al. 1992; Urtz and Elkan 1996; Vinuesa et al. 1998, 2005; Doignon-Bourcier et al. 1999; Germano et al. 2006; Menna et al. 2006), and the fingerprinting and 16S rRNA results obtained in this study suggest that much diversity in symbiotic tropical diazotrophic bacteria remains to be revealed. However, in practical terms, the results from our study also point out the feasibility of recognizing and tracking rhizobial strains—including those commercially used in inoculants—by using BOX-PCR fingerprinting. In addition, the sequencing analysis of the 16S rRNA genes of the 54 strains contributed to understanding of the phylogenetic relationships among tropical rhizobia and revealed putative new species that will be subject to further study. The broad distribution of both rhizobia and legume hosts from this study across phylogenetic branches and the lack of relationship among micro and macrosymbiont clusters confirm previous observations (Germano et al. 2006; Menna et al. 2006) that the evolution of ribosomal and symbiotic genes might have occurred independently at least for the strains from this study.

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