World J Microbiol Biotechnol (2010) 26:1291–1302
DOI 10.1007/s11274-009-0300-3

ORIGINAL PAPER

Genetic diversity of elite rhizobial strains of subtropical and tropical legumes based on the 16S rRNA and *glnII* genes

Ilmara Varotto Roma Neto · Renan Augusto Ribeiro · Mariangela Hungria

Received: 3 August 2009 / Accepted: 29 December 2009 / Published online: 8 January 2010

Abstract Biodiversity of diazotrophic symbiotic bacteria in the tropics is a valuable but still poorly studied resource. The objective of this study was to determine if a second housekeeping gene, *glnII*, in addition to the 16S rRNA, can be employed to improve the knowledge about taxonomy and phylogeny of rhizobia. Twenty-three elite rhizobial strains, very effective in fixing nitrogen with twenty-one herbal and woody legumes (including species from fourteen tribes in the three subfamilies of the family *Leguminosae*) were selected for this study; all strains are used as commercial inoculants in Brazil. Complete sequences of the 16S rRNA and partial sequences (480 bp) of the *glnII* gene were obtained. The same primers and amplification conditions were successful for sequencing the *glnII* genes of bacteria belonging to five different rhizobial genera—*Bradyrhizobium*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium*, *Sinorhizobium*)—positioned in distantly related branches. The analysis of the concatenated genes (16S rRNA + *glnII*) considerably improved information about phylogeny and taxonomy of rhizobia in comparison to the single analysis of the 16S rRNA. Nine strains might belong to new species. The complementary analysis of the *glnII* gene was successful with all strains and improved the phylogenetic clustering and clarified the taxonomic position of several strains. The strategy of including the analysis of *glnII*, in addition to the 16S rRNA, is cost- and time- effective for the characterization of large rhizobial culture collections or in surveys of many isolates.

Keywords 16S rRNA · Biological nitrogen fixation · *glnII* · Inoculants · Leguminosae · Rhizobiales

I. V. Roma Neto · R. A. Ribeiro · M. Hungria (✉)
Embrapa Soja, Cx. Postal 231, Londrina, Paraná 86001-970, Brazil
e-mail: hungria@cnpso.embrapa.br; hungria@pq.cnpq.br

I. V. Roma Neto
e-mail: ilmaravarotto@yahoo.com.br

R. A. Ribeiro
e-mail: renanribeiro83@hotmail.com

I. V. Roma Neto · R. A. Ribeiro · M. Hungria
Department of Microbiology, Universidade Estadual de Londrina, Cx. Postal 60001, Londrina, Paraná 86051-990, Brazil

Introduction

Many bacteria collectively known as "rhizobia" form symbiotic associations with legumes, establishing the key process of biological nitrogen (N_2) fixation, which is responsible for the wide adoption of legumes as food crops, forages, green manures and in forestry (Allen and Allen 1981; Polhill and Raven 1981). An impressive number of studies on biological N_2 fixation were performed in the 1970s, but a relative ostracism was observed in the following decades. Recently, interest in the biological process is increasing and should expand in the coming years, due to higher costs of N fertilizers and concerns about environmental pollution (Binde et al. 2009).

Advances in the development of molecular tools have greatly contributed to improve knowledge about the symbioses between rhizobia and legumes. The profound changes in the taxonomy of rhizobia represent a good example. In 1984, rhizobial strains were classified in only two genera of the family Rhizobiaceae (Jordan 1984) and today, based on polyphasic analyses of phenetic and genetic properties, rhizobia are categorized in five genera of the order Rhizobiales—*Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Rhizobium* (Garrity and Holt 2001; Lloret and Martinez-Romero 2005; Willems 2006). In addition, as

Rhizobium, *Agrobacterium* and *Allorhizobium* are closely related, they were joined into the genus *Rhizobium* (Young et al. 2001); furthermore, *Sinorhizobium* was reclassified into the genus *Ensifer* (Young 2003), but this reclassification is still under debate (Lindström and Young 2009). Finally, new diazotrophic symbiotic bacteria have been described and classified in non-traditional rhizobial genera, belonging either to the *Alphaproteobacteria—Methylobacterium*, *Devosia*—or to the *Betaproteobacteria—Burkholderia*, *Cupriavidus* (former *Ralstonia* and *Wautersia*) (Garrity and Holt 2001; Lloret and Martinez-Romero 2005; Willems 2006).

Ribosomal sequences, with an emphasis on the 16S rRNA genes, have become the method of choice in molecular taxonomy for tracing bacterial phylogenies (e.g. Woese 1987; Woese et al. 1990; Weisburg et al. 1991; Garrity and Holt 2001). However, although precise for the definition of kingdoms and genera, 16S rRNA provides poor resolution at the species and subspecies levels (Woese 1987; Garrity and Holt 2001). Shortcomings reside mainly on the high level of conservation documented in the 16S rRNA (e.g. Gevers et al. 2005), but concerns were also raised after the reports that genetic recombination and horizontal gene transfer may also occur among 16S rRNA genes (van Berkum et al. 2003; Gevers et al. 2005). On the basis of these observations, as well as to minimize their effects, other genes with a faster evolution rate than the 16S rRNA, but conserved enough to retain genetic information, have been proposed as alternative phylogenetic markers (Stackebrandt et al. 2002; Stepkowski et al. 2003; Martens et al. 2007; Alexandre et al. 2008).

Other ribosomal genes, such as 23S rRNA and ITS improve species definition (e.g. Tesfaye et al. 1997; Vinuesa et al. 1998; van Berkum and Furhman 2000; Willems et al. 2001; Germano et al. 2006; Menna et al. 2009). However, as they are located in the same operon as the 16S rRNA, limitations due to horizontal gene transfer continue. Another strategy relies in a complementary analysis of housekeeping genes broadly distributed among taxa, present in single copies and dispersed throughout the genome (Stackebrandt et al. 2002; Zeigler 2003; Gevers et al. 2005). In phylogenetic studies of bacteria belonging to the order *Rhizobiales*, housekeeping genes used in this approach include *atpD*, *dnaK*, *dnaJ*, *gap*, *glnA*, *glnII*, *gltA*, *gyrB*, *pnp*, *recA*, *rpoA, rpoB* and *thrC* (e.g. Turner and Young 2000; Gaunt et al. 2001; Alexandre et al. 2008; Martens et al. 2008; Vinuesa et al. 2008; Ribeiro et al. 2009; Rivas et al. 2009; Menna et al. 2009). However, one problem with those studies has been that different sets of genes and primers are employed for each genus.

Biodiversity in the tropics is a valuable, important, but still poorly studied resource, and even though biological N_2 fixation is a key process for soil sustainability, genetic diversity of diazotrophic symbiotic bacteria has been scarcely investigated. In this study we have used the *glnII* gene, in addition to the 16S rRNA, aiming at improving the knowledge about taxonomy and phylogenetic relations of elite rhizobial strains previously studied by our group (Menna et al. 2006; Binde et al. 2009) and relevant for their economical importance as commercial inoculants in Brazil. This *glnII* gene was chosen after amplification tests with other housekeeping genes with a variety of rhizobial species, as we were searching for a gene that would be easily amplified with all species.

Materials and methods

Strains

Twenty-three strains from the Brazilian "*Rhizobium* Culture Collection SEMIA" (Seção de Microbiologia Agrícola) (IBP World Catalogue of *Rhizobium* Collections—SEMIA; WFCC—World Federation for Culture Collections # 443) were selected from previous studies (Menna et al. 2006; Binde et al. 2009). 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 as inoculants. Strains were provided by FEPAGRO (Fundação Estadual de Pesquisa Agropecuária, Porto Alegre, Rio Grande do Sul, Brazil), 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°C (under 30% glycerol) for long-term storage and at 4°C as source cultures. Strains are also currently maintained at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection" of Embrapa Soja.

DNA extraction, amplification and sequencing

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, purification and maintenance of the DNA were performed as described before (Menna et al. 2006).

To obtain the complete sequence of the 16S rRNA gene, reactions with five pairs of primers were carried out, as described before (Menna et al. 2006), and resulted in readings of about 1,422 bp. For the *glnII* gene, two reactions were performed, using the primers [(TSglnIIf (5′-AAGCTC GAGTACATCTGGCTCGACGG-3′) and TSglnIIr (5′-SG AGCCGTTCCAGTCGGTGTCG-3′)], under the amplification conditions described by Stepkowski et al. (2005), resulting in fragments of about 600 bp. All PCR reactions were carried out on an MJ Research Inc. PTC 200 thermocycler. The PCR products were purified with the PureLink™

Table 1 Information about the strains used in this study and their host legumes

SEMIA strain	Other designations[a]	Source of the strain[b]	Country (source)	Rhizobial species[c]	Plant host species[d]	Tribe[d]	Subfamily[d]	Some common names[f]	Main use in Brazil
384	SEMIA original	FEPAGRO/ UFRGS	Brazil	*Rhizobium etli*[1]	*Vicia sativa* L.	Vicieae	Papilionoideae	Common vetch, pois France, arveja, ervilhaca	Forage (subtropical)
396	TAL 1148, USDA 3100, Nit 27A3	Niftal	Unknown	*Mesorhizobium ciceri*[1]	*Cicer arietinum* L.	Cicereae	Papilionoideae	Chickpea, pois chiche, garbanzo, grão de bico	Grain
696	CB 627	CSIRO	Australia	*Bradyrhizobium elkanii*[1]	*Desmodium uncinatum* (Jacq.) DC.	Desmodieae	Papilionoideae	Silverleaf desmodium, desmódio	Forage (tropical)
816	SEMIA original	FEPAGRO/ UFRGS	Brazil	*Mesorhizobium* sp.[1]	*Lotus corniculatus* L.	Loteae	Papilionoideae	Cat's clover, birds′ foot trefoil, broadleaf trefoil, cornichão	Forage (subtropical)
830	Unknown	Hansen inoculant (USA)	Unknown	*Mesorhizobium* sp.[1]	*Lotus glaber* Miller *(L. tenuis* Willd.)	Loteae	Papilionoideae	Narrow trefoil, birds′ foot trefoil, cornichão	Forage (subtropical)
2051	SEMIA original	FEPAGRO/ UFRGS	Brazil	*Rhizobium leguminosarum*[1]	*Trifolium vesiculosum* Savi	Trifolieae	Papilionoideae	Arrowleaf clover, trevo yuchi, trevo vesiculoso	Forage (subtropical)
2082	EEL 8,186	EPAGRI	Brazil	*Rhizobium leguminosarum*[1]	*Trifolium pratense* L.	Trifolieae	Papilionoideae	Red clover, trebol de los prados, trebol rojo, trevo vermelho	Forage (subtropical)
3007	B 11A	IPN	Mexico	*Rhizobium leguminosarum*[1]	*Pisum sativum* L.	Vicieae	Papilionoideae	Field pea, petit pois, alverja, guisante, ervilha	Grain
3012	CPAC EV6	Embrapa Cerrados	Brazil	*Mesorhizobium tianshanense*[2]	*Pisum sativum* L.	Vicieae	Papilionoideae	Field pea, petit pois, alverja, guisante, ervilha	Grain
3026	CPAC L12	Embrapa Cerrados	Brazil	*Rhizobium leguminosarum*[2]	*Lens culinaris* Medik. subsp. culinaris	Vicieae	Papilionoideae	Adi Merchi, burchak, chachavitza zvychainaya, chechevitza kulturnaya, common lentil, edamas lecas, lenteja, lenticchia	Grain
4088	PRF 81	Embrapa Soja/ IAPAR	Brazil	*Rhizobium tropici*[2]	*Phaseolus vulgaris* L.	Phaseoleae	Papilionoideae	Frijol, common Bean, feijão, poroto	Grain
6053	TAL 827, UMKL I28	Malásia	Malasya	*Bradyrhizobium elkanii*[1]	*Clitoria ternatea* L.	Phaseoleae	Papilionoideae	Pigeon wings, pois sauvage, blue pea, azulejo, campanilla, conchita blanca o azul, zapatico de la reina, clitoria	Tree
6153	BR 827	Embrapa Agrobiologia	Brazil	*Bradyrhizobium japonicum*[2]	*Leucaena leucocephala* (Lam.) De Wit vK72, v.K8, v. Peru	Mimoseae	Mimosoideae	Aroma blanca,'bois bourro, cassie blanc, cowbush, jumbie bean, leucena, tamarindillo	Tree
6154	BR 446	Embrapa Agrobiologia	Brazil	*Bradyrhizobium japonicum*[2]	*Stylosanthes* sp.	Aeschynomeneae	Papilionoideae	Stylo, Brazilian lucerne, stylosanthes, estilosantes	Forage (tropical)
6161	BR 4002, PRJ B4	Embrapa Agrobiologia	Brazil	*Sinorhizobium* sp.[1]	*Prosopis juliflora* (Sw.) DC.	Mimoseae	Mimosoideae	Mesquite, cashaw, bayarone, epinard bayahonda blanca, algaroba	Tree
6392	BR 3804	Embrapa Agrobiologia	Brazil	*Mesorhizobium amorphae*[2]	*Acacia salicina* Lindl[e]	Acacieae	Mimosoideae	Broughton willow, cooba, doolan, native wattle, native willow, willow acacia	Tree
					Chamaecrista ensiformis (Vell.) H.S. Irwin & Barneby[e]	Cassieae	Caesalpinioideae	Jaúna, coração-de-negr	Tree
6396	BR 6815	Embrapa Agrobiologia	Brazil	*Bradyrhizobium japonicum*[2]	*Albizia pedicellaris* (Dc.) L. Rico	Ingeae	Mimosoideae	Juerana-branca, Galinazo	Tree

Table 1 continued

SEMIA strain	Other designations[a]	Source of the strain[b]	Country (source)	Rhizobial species[c]	Plant host species[d]	Tribe[d]	Subfamily[d]	Some common names[f]	Main use in Brazil
6407	BR 6813	Embrapa Agrobiologia	Brazil	*Methylobacterium mesophilicum*[2]	*Pithecellobium tortum* Mart	Ingeae	Mimosoideae	Tataré, Jacaré, Angico-branco, Jurema, Vinhático-de-espinho	Tree
6423	BR 4302	Embrapa Agrobiologia	Brazil	*Rhizobium rhizogenes*[2]	*Calliandra houstoniana* (Mill.) Standl. var. calothyrsus (Meissner)Barneby	Ingeae	Mimosoideae	Calliandra, caliandra, salsa pompon de marin	Tree
6428	BR 3628	Embrapa Agrobiologia	Brazil	*Bradyrhizobium elkanii*[2]	*Acacia saligna* (Labill.) Wendl	Acacieae	Mimosoideae	Blue-leafed wattle, golden wreath wattle, golden wreath wattle, orange wattle, Port Jackson willow, Western Australian golden watt	Tree
6435	BR 8802	Embrapa Agrobiologia	Brazil	*Rhizobium* sp.[2]	*Gliricidia sepium* (Jacq.) Walp.	Robinieae	Caesalpinioideae	Grow stick, cacahuananche, madricacao, gliricidiah Gliciridia ingá	Tree
6437	EEL 15,084	EPAGRI/ Embrapa Trigo	Brazil	*Rhizobium* sp.[2]	*Adesmia latifolia* (Spreng.) Vogel	Adesmieae	Papilionoideae	Adesmia	Forage (subtropical)
6439	MGAP 13	EPAMIG/ Embrapa Cerrados	Brazil	*Bradyrhizobium japonicum*[2]	*Arachis pintoi* Krapov. and W. Gregory	Aeschynomeneae	Papilionoideae	Forage peanut, amendoim forrageiro	Forage (tropical)

[a] Culture collections: B (I.P.N.); BR (Brazil, Embrapa Agrobiologia, Seropédica, Brazil); CB (Commonwealth Scientific and Industrial Research Organization—CSIRO, Canberra, Australia); CPAC (Embrapa Cerrados, Planaltina, Brazil); EEL (Empresa de Pesquisa de Santa Catarina, Lages, Santa Caterina, Brazil); IPN (Instituto Politecnico Nacional, Centro de Investigación de Estudos Avanzados, Mexico, Mexico); MGAP (Ministerio de Ganadería, Agricultura y Pesca, Laboratorio de Microbiologia y Suelos, Montevideo, Uruguay); NC (North Caroline, University of North Caroline, Raleigh, USA); Nit (Nitragin, Inc., Brookfield, USA); PRF (Paraná Feijão, Embrapa Soja/IAPAR, Londrina, Brazil); SEMIA (Seção de Microbiologia Agrícola, FEPAGRO, Porto Alegre, Brazil); TAL (NifTAL, Nitrogen Fixation by Tropical Agricultural Legumes Project, University of Hawaii, Paia, USA); UMKL (University of Malaya—Kuala Lumpur, Department of Genetics and Cellular Biology, Kuala Lumpur, Malasya); USDA (United States Department of Agriculture, Beltsville, USA)

[b] CSIRO (Commonwealth Scientific and Industrial Research Organization, Canberra, Australia); Embrapa Agrobiologia (Empresa Brasileira de Pesquisa Agropecuária, Agrobiologia, Seropédica, Rio de Janeiro, Brazil); Embrapa Cerrados (Planaltina, Distrito Federal, Brazil); Embrapa Soja (Londrina, Paraná, Brazil); EPAGRI (Empresa de Pesquisa Agropecuária e Extensão Rural, Estação Experimental de Lages Santa Catarina, Brazil); EPAMIG (Empresa de Pesquisa Agropecuária de Minas Gerais); FEPAGRO (Fundação Estadual de Pesquisa Agropecuária, Porto Alegre, Rio Grande do Sul, Brazil); IAPAR (Instituto Agronômico do Paraná, Londrina, Paraná, Brazil); IPN (Instituto Politecnico Nacional, Centro de Investigación de Estudos Avanzados, Mexico, Mexico); Niftal (Nitrogen Fixation by Tropical Agricultural Legumes, University of Hawaii, Paia, USA); UFRGS (Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil)

[c] Classification [1]after Menna et al. (2006) and [2]after Binde et al. (2009)

[d] Taxonomy after www.ildis.org

[e] Strain recommended for more than one legume

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

PCR Purification kit (Invitrogen), according to the manufacturer's instructions. Sample concentration was verified by electrophoresis of 2 μL PCR products on 1% agarose gel and staining with ethidium bromide.

The sequencing reactions were carried out in ninety-six-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. The same program was used for all primers, as follows: denaturation at 95°C for 2 min; thirty 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. The sequencing was performed on a MEGA BACE 1000 (Amersham Biosciences) capillary sequencer, according to the manufacturer's instructions.

High-quality sequences obtained for each strain were assembled into contigs using the programs phred (Ewing et al. 1998), phrap version 0.990722 (www.phrap.org) and Consed (Gordon et al. 1998). 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. For the 16S rRNA, sequences were in full agreement with those deposited by Menna et al. (2006) and Binde et al. (2009). Accession numbers obtained for the *glnII* genes are listed on Table 2.

Phylogenetic data analysis

Multiple alignments for each gene were performed with ClustalX version 1.83 (Thompson et al. 1997). Sequences of type/reference strains were included in the analyses and the accession numbers of the GenBank/EMBL/DDBJ Data Libraries are listed in parentheses for the 16S rRNA and *glnII* genes, respectively, as follows: *Mesorhizobium amorphae* strain ACCC 19665^T (DQ02832, EU518372); *M. tianshanense* USDA 3592^T (AF041447, AF169579); *M. loti* USDA 3451^T (X67229, not available—meaning that the sequences are not available at the Genbank database); *M. ciceri* USDA 3383^T (U07934, AF169580); *Rhizobium tropici* CIAT 899^T (U89832, EU488791); *R. etli* CFN 42^T (U28916, NC007 761); *R. leguminosarum* USDA 2370^T (U29386, EU1550 89); *R. mongolense* USDA 1844^T (U89817, AY929453); *R. rhizogenes* ATCC 11325^T (AY945955, not available); *R. lusitanum* P1-7^T (AY738130, AY738130); *B. elkanii* USDA 76^T (U35000, AY599117.1); *B. betae* PL7HG^T (AY372184, AB353733); *B. yuamingense* CCBAU 10071^T (AF193818, AY386780); *B. canariense* BCC2^T (AY577 427, AY386762.1); *B. japonicum* USDA 6^T (U69638, AF169582); *Methylobacterium nodulans* ORS 2060^T (AF2 20763, not available). *Caulobacter crescentus* strain CB15 (AE005673) was used as outgroup.

Phylogenetic trees were generated using MEGA version 4.0 (Kumar et al. 2004) with default parameters, K2P distance model (Kimura 1980), and the Neighbor-Joining algorithm (Saitou and Nei 1987). Statistic support for tree nodes was evaluated by bootstrap analyses (Felsenstein 1985) with 1,000 samplings.

For the alignment of the sequences aiming at verifying differences in the number of nucleotides among pairs of strains we have used ClustalW program (Thompson et al. 1994), version ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Results

Five main phylogenetic branches or groups were observed in the 16S rRNA tree (Fig. 1) and they were split in ten subgroups, in addition to several strains forming distinct phylogenetic lineages. In the tree built after the alignment and analysis of 480 bp of *glnII* there were also five main groups, with eight subgroups in addition to isolated lineages (Fig. 2). The concatenation of the 16S rRNA and *glnII* genes provided a much better definition of the clusters of the strains, generating four main groups and twelve subgroups (Fig. 3), most with higher bootstrap support than in the single trees.

We have also analysed the differences in the number and percentage of nucleotides of the 16S rRNA, *glnII* and of the concatenated genes, comparing the sequences of each strain with the closest type strain after alignment with ClustalW (Table 2). To the results obtained from this comparison, we applied a value previously established in our laboratory (Menna et al. 2006), in which 1.03% of different nucleotides in the 16S rRNA sequences might indicate new species. As studies with *glnII* or with the concatenated sequences are still scarce, we have proposed 5 and 3% nucleotide differences, respectively, as indicative of putative new species. The results obtained are shown on Table 2.

Considering the strains from this study, SEMIA 6423, isolated from *Calliandra houstoniana* (subfamily Mimosoideae) clustered with *R. lusitanum*—a symbiont of *Phaseolus vulgaris* (Papilionoideae), also effective for *Macroptilium atropurpureum* and *Leucaena leucocephala* (Valverde et al. 2006)—in subgroup I.I of the 16S rRNA, with a bootstrap support of 75% (Fig. 1). However, in the trees built with the *glnII* (Fig. 2) and with the concatenated (Fig. 3) genes, the strain was positioned in an isolated cluster with SEMIA 6435, symbiont of *Gliricidia sepium* (Caesalpinoideae). According to the aligned sequences with ClustalW, SEMIA 6423 is fully conserved in the 16S rRNA gene, but has a 6.66% nucleotide difference in the *glnII* gene in comparison to the closest type strain, and a

Table 2 Information about the gene sequences of the strains used in this study

SEMIA strain	16S rRNA			*glnII*			16S rRNA + *glnII*		Proposed taxonomic position
	Gene bank access #	Identities with the closest type strain	Differences in bp (%) from the closest type strain	Gene bank access #	Identities with the closest type strain	Differences in bp (%) from the closest type strain	Identities with the closest type strain	Differences in bp (%) from the closest type strain	
384	AY904730	1433/1439	6 (0.41)	GQ160496	457/480	23 (4.79)	1890/1919	29 (1.51)	*Rhizobium etli*
396	AY904731	1441/1450	9 (0.62)	GQ160515	462/480	18 (3.75)	1903/1930	27 (1.39)	*Mesorhizobium ciceri*
696	AY904736	1442/1449	7 (0.48)	GQ160506	477/481	4 (0.83)	1889/1930	22 (2.12)	*Bradyrhizobium elkanii*
816	AY904737	1436/1443	7 (0.48)	GQ160514	423/480	57 (11.87)	1859/1923	64 (3.32)	*Mesorhizobium* sp
830	AY904738	1432/1445	13 (0.89)	GQ160504	422/480	58 (12.08)	1854/1925	71 (3.68)	*Mesorhizobium* sp.
2051	AY904740	1436/1441	5 (0.34)	GQ16013	446/480	34 (7.08)	1882/1921	39 (2.03)	*Rhizobium leguminosarum*
2082	FJ025094	1431/1439	8 (0.55)	GQ160494	480/480	0 (0.0)	1911/1919	8 (0.41)	*Rhizobium leguminosarum*
3007	AY904742	1429/1441	12 (0.83)	GQ160503	476/480	4 (0.83)	1905/1921	16 (0.83)	*Rhizobium leguminosarum*
3012	FJ025121	1435/1443	8 (0.55)	GQ160512	472/480	8 (1.66)	1907/1923	16 (0.83)	*Mesorhizobium tianshanense*
3026	FJ025093	1436/1441	5 (0.34)	GQ160502	474/480	6 (1.25)	1910/1921	11 (0.57)	*Rhizobium leguminosarum*
4088	EF054889	1427/1439	12 (0.83)	GQ160511	480/480	0 (0.0)	1907/1919	12 (0.62)	*Rhizobium tropici*
6053	AY904745	1443/1447	4 (0.27)	GQ160501	464/480	16 (3.33)	1907/1927	20 (1.03)	*Bradyrhizobium elkanii*
6153	FJ025097	1447/1447	0 (0.0)	GQ160510	454/480	26 (5.41)	1901/1927	26 (1.34)	*Bradyrhizobium liaoningense*[*]
6154	FJ025100	1431/1446	15 (1.03)	GQ160500	427/479	52 (10.85)	1858/1925	67 (3.48)	*Bradyrhizobium* sp.[*]
6161	AY904763	1428/1444	16 (1.10)	GQ160509	428/480	52 (10.83)	1856/1924	68 (3.53)	*Ensifer* sp.
6392	FJ025126	1438/1444	6 (0.41)	GQ160499	435/480	45 (9.37)	1873/1924	51 (2.65)	*Mesorhizobium* sp.[*]
6396	FJ025099	1432/1446	14 (0.96)	GQ160495	453/480	27 (5.62)	1885/1926	41 (2.12)	*Bradyrhizobium canariense*[*]
6407	FJ025133	1361/1441	80 (5.55)	GQ160508	449/480	31 (6.45)	1810/1921	111 (5.77)	*Methylobacterium* sp.[*]
6423	FJ025132	1439/1439	0 (0.0)	GQ160507	428/480	32 (6.66)	1867/1919	52 (2.79)	*Rhizobium* sp.*, **
6428	FJ025106	1441/1448	7 (0.48)	GQ160498	459/480	21 (4.37)	1900/1928	28 (1.45)	*Bradyrhizobium elkanii*
6435	FJ025130	1434/1439	5 (0.35)	GQ160505	449/480	31 (6.45)	1883/1919	36 (1.87)	*Rhizobium* sp.**
6437	FJ025118	1416/1439	23 (1.6)	GQ160497	412/480	68 (14.16)	1828/1919	91 (4.74)	*Rhizobium* sp.
6439	FJ025098	1423/1447	24 (1.65)	GQ160516	448/479	31 (6.47)	1871/1926	55 (2.85)	*Bradyrhizobium japonicum*

[*] Denotes different classification when compared to that based exclusively on the 16S rRNA (Menna et al. 2006; Binde et al. 2009); [**] closest species is *Rhizobium rhizogenes*

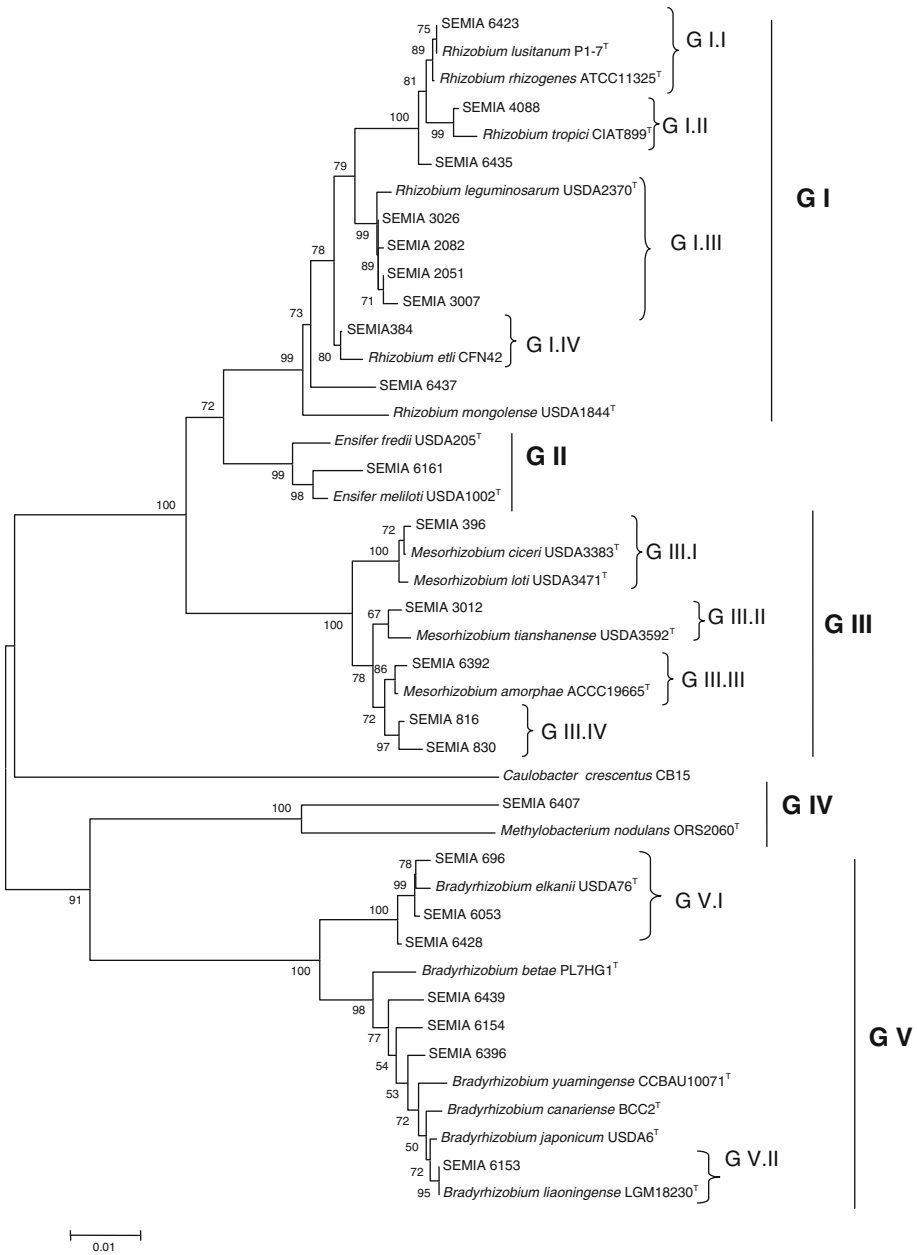

Fig. 1 Phylogenetic tree of the 16S rRNA genes of twenty-three rhizobial strains from this study and of other rhizobial taxa. Strains and accession numbers are described in the "Materials and methods" section. The tree was generated using MEGA version 4.0 with default parameters, K2P distance model and the Neighbor-Joining algorithm

2.79% nucleotide difference on the concatenated tree (Table 2). Interestingly, the cluster included strains capable of nodulating species from the three subfamilies of legumes.

Strain SEMIA 4088 clustered in subgroup I.II of the 16S rRNA with *R. tropici* type strain, with a bootstrap support of 99%. Clustering was confirmed in the analysis of both the *glnII* (Fig. 2) and the concatenated (Fig. 3) genes, with bootstrap supports of 100%, confirming the phylogenetic grouping of these two symbionts of *P. vulgaris.*

Four SEMIAs—3026, 2082, 2051 and 3007—were positioned in subgroup I.III of the 16S rRNA, together with *R. leguminosarum* (Fig. 1). Except for SEMIA 2051, clusters were confirmed with high bootstrap supports in both the *glnII* (Fig. 2) and on the concatenated (Fig. 3) trees. Considering the three strains, the maximum difference in the number of nucleotides for the *glnII* gene relative to *R. leguminosarum* was of 1.25% for SEMIA 3026 (Table 2), strongly indicating their taxonomic position as *R. leguminosarum*. On the other hand, SEMIA 2051 showed a 7.08% nucleotide difference in the *glnII* gene, and of 2.03% on the concatenated genes (Table 2), deserving further studies; for now the strain will continue to be classified as *R. leguminosarum*.

Strain SEMIA 384, symbiont of *Vicia sativa*, was positioned in subgroup I.IV of the 16S rRNA tree and clustered with *R. etli* CFN 42^T with a bootstrap support of 80% (Fig. 1); the clustering was confirmed considering the

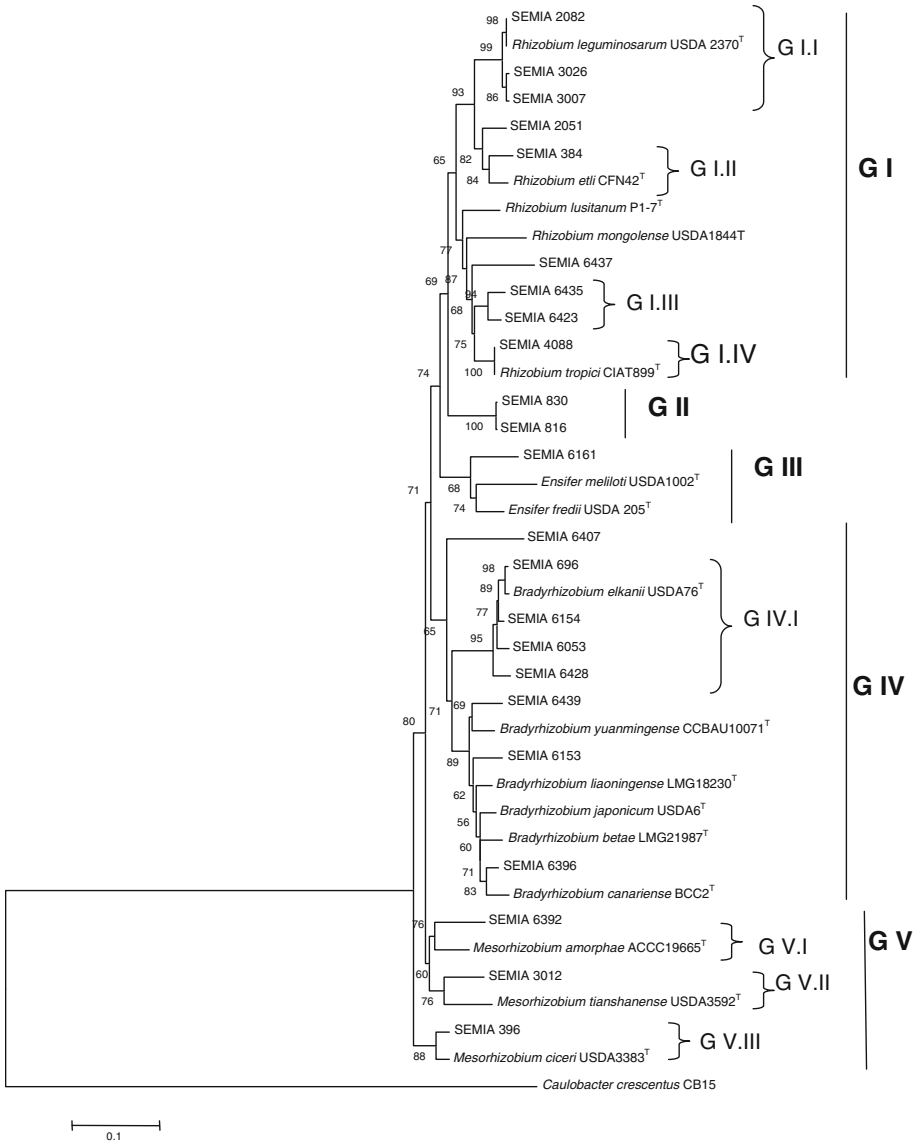

Fig. 2 Phylogenetic tree of the *glnII* genes of twenty-three rhizobial strains from this study and of other rhizobial taxa. Strains and accession numbers are described in the "Materials and methods" section. The tree was generated using MEGA version 4.0 with default parameters, K2P distance model and the Neighbor-Joining algorithm

glnII and the concatenated genes, with bootstrap supports of 84 and 99%, respectively. Considering the *glnII* and the concatenated genes, 4.79 and 1.51% of the nucleotides, respectively, were different from *R. etli*, therefore, the strain resembles *R. etli*, but other housekeeping genes should be investigated to confirm its precise taxonomic position (Table 2).

Still in Group I of the 16S rRNA tree, strains SEMIA 6435, symbiont of *Gliricidia sepium* and SEMIA 6437, symbiont of *Adesmia latifolia*, occupied isolated positions. In both the *glnII* (Fig. 2) and on the concatenated (Fig. 3) trees, strain SEMIA 6437 also occupied an isolated position, while SEMIA 6435, as discussed before, clustered with SEMIA 6423. In addition to a high percentage of different bases when compared to the closest type strain (Table 2), the results indicate that both SEMIA 6435 and 6437 might represent new species.

Bacteria belonging to the genus *Sinorhizobium* (= *Ensifer*) were coherent in all three trees, and the cluster included SEMIA 6161, symbiont of the tropical tree *Prosopis juliflora* (Figs. 1, 2, 3). Although clustered with *Methylobacterium nodulans* in the 16S rRNA tree, strain SEMIA 6407 differs in 6.45% of the nucleotides (Table 2) and unfortunately, no *glnII* sequences were available for *M. nodulans*. However, as pointed out before (Binde et al. 2009), SEMIA 6407 shows higher similarity of bases with the 16S rRNA of the non-diazotrophic *M. mesophilicum*, formerly classified as *Pseudomonas mesophilica*; therefore it will be interesting to proceed with the investigation of both housekeeping and symbiotic genes of this strain.

Interesting results were also obtained with SEMIAs 830 and 816, positioned in the great group of *Mesorhizobium* in the 16S rRNA tree (Fig. 1), but showing a high percentage of different nucleotides in comparison to the closest type

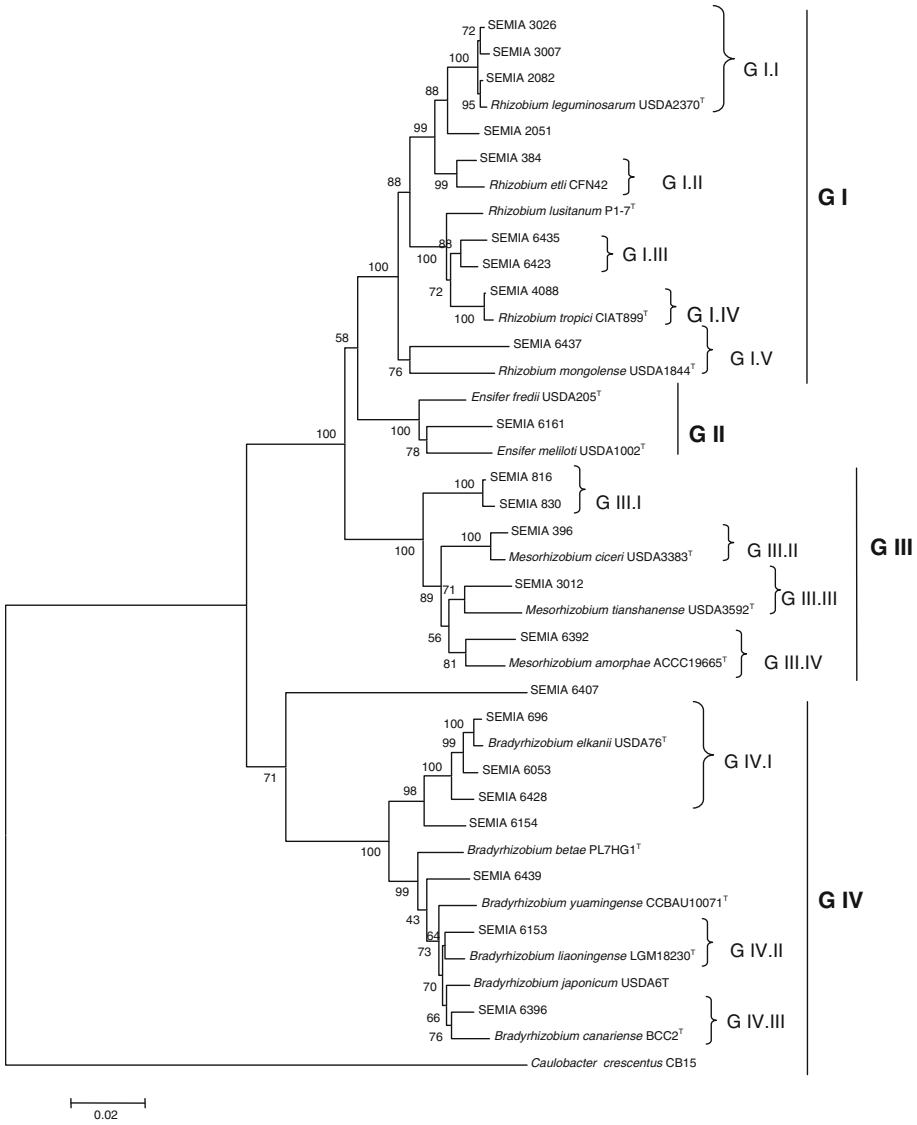

Fig. 3 Phylogenetic tree of concatenated genes (*glnII* + 16S rRNA) of twenty-three rhizobial strains from this study and other rhizobial taxa. Strains and accession numbers are described in the "Materials and methods" section. The tree was generated using MEGA version 4.0 with default parameters, K2P distance model and the Neighbor-Joining algorithm

strain (Table 2). Both strains formed a separated cluster in the tree built with the *glnII* gene (Fig. 2), resulting in a different subgroup of the genus *Mesorhizobium* on the concatenated tree (Fig. 3). The phylogenetic topology of all other strains fitting into the *Mesorhizobium* great group in the 16S rRNA tree (Fig. 1) was confirmed in both the *glnII* (Fig. 2) and on the concatenated (Fig. 3) trees; furthermore, the clustering of SEMIA strains with specific *Mesorhizobium* species was greatly improved with the complementary analysis of the second gene.

The last great group in the 16S rRNA tree included strains belonging to the genus *Bradyrhizobium* (Fig. 1), and species definition was very poor when using exclusively this gene, but improvement was achieved with the analysis of the *glnII* gene (Figs. 2, 3 and Table 2). Better certainty in the classification of strains SEMIA 696, 6053 and 6428 as *B. elkanii* was obtained, and a clustering of SEMIA 6153 with *B. lianingense* was also demonstrated; based exclusively on the 16S rRNA, this strain had been previously classified as *B. japonicum* (Binde et al. 2009). Strain SEMIA 6396, which did not occupy a clear position in the 16S rRNA tree, clustered with *B. canariense* in the *glnII* and on the concatenated trees. Finally, the high diversity (5.62–10.85% of different bases in comparison to the closest type strain) of the *glnII* genes of SEMIA strains 6154, 6392, 6396, 6407 and 6423 is noteworthy (Table 2).

On an overall basis, the additional analysis of the *glnII* for the twenty-three strains of this study resulted in changes in the nomenclature previously proposed (Menna et al. 2006; Binde et al. 2009) for six strains, SEMIAs 6153, 6154, 6392, 6396, 6407 and 6423 (Table 2). The observation that the clusters formed in the concatenated tree (Fig. 3) were far more defined than in the single analysis of the 16S rRNA gene (Fig. 1) is also very important. Finally,

it should be noted that nine strains are denominated as "sp." in Table 2, as this study strongly indicates that they may represent new species (Table 2).

Discussion

We have studied a collection of twenty-three elite rhizobial strains, chosen from previous studies (Menna et al. 2006; Binde et al. 2009) in which their precise taxonomic position—based on the 16S rRNA genes—was not clearly defined. All strains have been selected as the most effective in fixing N_2 with twenty-one legume hosts and they are officially authorized for the production of commercial inoculants for these legumes in Brazil. The strains studied nodulate a wide-range of legumes, positioned in fourteen different tribes in all three subfamilies of the family *Leguminosae* (= *Fabaceae* in USA), including species grown in both tropical and subtropical regions (denominated here as tropics) (Table 1) and represent a valuable resource of symbiotic rhizobia with biotechnological potential for the tropics. A high level of diversity was clearly shown, reinforcing previous statements that there are many more varieties of rhizobia in tropical and subtropical than in temperate regions (Oyaizu et al. 1992).

Complete sequences of the 16S rRNA and partial sequences (480 bp) of the *glnII* genes were obtained. The same primers and amplification conditions were successful for the sequencing of the *glnII* genes of bacteria belonging to five different rhizobial genera—*Bradyrhizobium*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium*, *Sinorhizobium*)—positioned in distantly related branches. For the great majority of the strains there was a good agreement between clustering with the 16S rRNA and with the *glnII* genes. However, as expected due to a higher number of informative sites, analysis of the *glnII* detected a higher level of genetic diversity than the 16S rRNA. Most important, the analysis of the concatenated genes (16S rRNA + *glnII*) considerably improved the information about phylogeny and taxonomy of rhizobia in comparison to the single analysis of the 16S rRNA, with most groups showing higher bootstrap support than in the single trees. The improvements were particularly important for bacteria belonging to the genera *Mesorhizobium* and *Bradyrhizobium*. One good example was achieved with *Bradyrhizobium*, as the poor species resolution within this genus based on the analysis of the 16S rRNA gene has been pointed out (e.g. Vinuesa et al. 1998; van Berkum and Fuhrmann 2000; Willems et al. 2001; Germano et al. 2006; Menna et al. 2006). In our study, in few cases the clustering position was not confirmed, and further analyses with other housekeeping genes will be performed, trying to define the correct taxonomic position and if horizontal gene transfer events have occurred.

In studies aiming at improving knowledge about diversity and taxonomy of prokaryotes, including rhizobia, at least five housekeeping genes have been included, in addition to the 16S rRNA. However, different genes and amplification conditions have been used for each genus (e.g. Stackebrandt et al. 2002; Zeigler 2003; Gevers et al. 2005; Vinuesa et al. 2008; Menna et al. 2009; Ribeiro et al. 2009). This strategy is thus expensive and time-demanding for the characterization of large culture collections, or in surveys of many rhizobial isolates.

In this study we have tried to determine if a single gene capable of improving information about taxonomy and phylogeny of rhizobia could be used in addition to the 16S rRNA. The gene should be easily amplified in a wide range of rhizobia using the same primers and amplification conditions. A similar approach has been previously used with *gltA* gene, coding for the citrate synthase; however, sequencing was not applicable to all rhizobial species studied (Hernández-Lucas et al. 2004). We have then proposed the broadly distributed and well conserved *glnII* gene, coding for the glutamine synthetase 2 (EC = 6.3.1.2). Genes coding for the glutamine synthetase are amongst the oldest ones on earth and thus may be very useful for tracing phylogeny (Tateno 1994) or to improve taxonomy. Two forms of glutamine synthetase (GSI and GSII) can be found in nitrogen-fixing bacteria: GSI, a typical prokaryotic glutamine synthetase and GSII, similar to the eukaryotic enzyme; a third GS isozyme (GSIII) can be found in *S. meliloti* and *R. etli* (Patriarca et al. 1992; Espín et al. 1994). Shatters and Kahn (1989) have found 83.6% of identity when comparing GSII proteins of *S. meliloti* and *B. japonicum*. In addition, the comparison with several GSII has proven that the gene has not been transferred among large taxonomic groups (symbiotic bacteria, plants and mammalian) (Shatters and Kahn 1989); the gene has also been successfully applied in studies with *Bradyrhizobium* (Vinuesa et al. 2008). In our study, the same primers and amplification conditions resulted in *glnII* sequences for symbiotic bacteria belonging to five genera (*Bradyrhizobium*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium* and *Sinorhizobium*) and positioned in considerably different phylogenetic branches. The higher number of parsimony informative sites of *glnII* in comparison to the 16S rRNA has allowed to detect higher diversity among the strains; furthermore, the analysis of the concatenated genes (*glnII* + 16S rRNA) has greatly improved phylogeny, clarified the taxonomic position of many strains, and indicated that others might represent new species, deserving further studies.

Acknowledgments The work was partially supported by CNPq/MCT/MAPA (577933/2008), CNPq-Universal (470162/2009-0) and CPNq/MCT/PROTAX (563960/05-1). I. V. Roma Neto acknowledges

a M.Sc. fellowship from CNPq, R. A. Ribeiro a PhD fellowship from Fundação Araucária and M. Hungria a researcher fellowship from CNPq. The authors thank Ligia M. O. Chueire, Pâmela Menna, Fernando G. Barcellos and Ricardo S. Araujo (Embrapa Soja) for help in several steps of this work.

References

Alexandre A, Laranjo M, Young JPW, Oliveira S (2008) *dnaJ* is a useful phylogenetic marker for alphaproteobacteria. Int J Syst Evol Microbiol 58:2839–2849

Allen ON, Allen EK (1981) The leguminosae: a source book of characteristics, uses, and nodulation. University Wisconsin Press, Madison, U.S.A

Binde DR, Menna P, Bangel EV, Barcellos FG, Hungria M (2009) rep-PCR fingerprinting and taxonomy based on the sequencing of the 16S rRNA gene of fifty-four elite commercial rhizobial strains. Appl Microbiol Biotechnol 83:897–908

Espín G, Moreno S, Guzman J (1994) Molecular genetics of the glutamine synthetases in *Rhizobium* species. Crit Rev Microbiol 20:117–123

Ewing B, Hillier L, Wendl MC, Green P (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res 8:175–185

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Garrity GM, Holt JG (2001) The road map to the manual. In: Boone DR, Castenholz RW (eds) Manual of systematic bacteriology, the archaea, the deeply branching, phototrophic bacteria. Springer-Verlag, New York, U.S.A., pp 119–166

Gaunt MW, Turner SL, Rigottier-Gois L, Lloyd-Macgilp SA, Young JP (2001) Phylogenies of *atpD* and *recA* support the small subunit rRNA based classification of rhizobia. Int J Syst Evol Microbiol 51:2037–2048

Germano MG, Menna P, Mostasso FL, Hungria M (2006) RFLP analysis of the rRNA operon of a Brazilian collection of bradyhizobial strains from 33 legumes species. Int J Syst Evol Microbiol 56:217–229

Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, Stackebrandt E, de Peer YV, Vandamme P, Thompson FL, Swings J (2005) Re-evaluating prokaryotic species. Nat Rev Microbiol 3:733–739

Gordon D, Abajian C, Green P (1998) Consed: a graphical tool for sequence finishing. Genome Res 8:195–202

Hernández-Lucas I, Rogel-Hernández MA, Segovia L, Rojas-Jiménez K, Martínez-Romero E (2004) Phylogenetic relationships of rhizobia based on citrate synthase gene sequences. Syst Appl Microbiol 27:703–706

Jordan DC (1984) Rhizobiaceae. In: Krieg NR, Holt JR (eds) Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore, U.S.A., pp 234–244

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform 5:150–163

Lindström K, Young JPW (2009) International committee on systematics of prokaryotes, subcommittee on the taxonomy of *Agrobacterium* and *Rhizobium*–minutes of the meeting, 31 Aug 2008, Gent, Belgium. Int J Syst Evol Microbiol 59:921–922

Lloret L, Martinez-Romero E (2005) Evolución y filogenia de *Rhizobium*. Rev Latinoam Microbiol 47:43–60

Martens M, Delaere M, Coopman R, de Vos P, Gillis M, Willems A (2007) Multilocus sequence analysis of *Ensifer* and related taxa. Int J Syst Evol Microbiol 57:489–503

Martens M, Dawyndt P, Coopman R, Gillis M, De Vos P, Willems A (2008) Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus *Ensifer* (including former *Sinorhizobium*). Int J Syst Evol Microbiol 58:200–214

Menna P, Hungria M, Barcellos FG, Bangel EV, Hess PN, Martínez-Romero E (2006) Molecular phylogeny based on the 16S rRNA gene of elite rhizobial strains used in Brazilian commercial inoculants. Syst Appl Microbiol 29:315–332

Menna P, Barcellos FG, Hungria M (2009) Phylogeny and taxonomy of a diverse collection of *Bradyrhizobium* strains based on multilocus sequence analysis of 16S rRNA, ITS, *glnII*, *recA*, *atpD* and *dnaK* genes. Int J Syst Evol Microbiol 59:2934–2950

Oyaizu H, Naruhashi N, Gamou T (1992) Molecular methods of analyzing bacterial diversity: the case of rhizobia. Biodivers Conserv 1:237–249

Patriarca EJ, Chiurazzi M, Manco G, Riccio A, Lamberti A, Paolis A, Rossi M, Defez R, Iaccarino M (1992) Activation of the *Rhizobium leguminosarum glnII* gene by NtrC is dependent on upstream DNA sequences. Mol Gen Genet 234:337–345

Polhill RM, Raven PH (1981) Advances in legume systematics. Royal Botanic Gardens, Kew, UK

Ribeiro RA, Barcellos FG, Thompson FL, Hungria M (2009) Multilocus sequence analysis of Brazilian *Rhizobium* strains microsymbionts of common beans (*Phaseolus vulgaris*) reveals unexpected taxonomic diversity. Res Microbiol 160:297–306

Rivas R, Martens M, de Lajudie P, Willems A (2009) Multilocus sequence analysis of the genus Bradyrhizobium. Syst Appl Microbiol 32:101–110

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Shatters RG, Kahn ML (1989) Glutamine synthetase II in *Rhizobium*: reexamination of the proposed horizontal transfer of DNA from eukaryotes to prokaryotes. J Mol Evol 129:422–428

Stackebrandt E, Frederiksen W, Garrity GM, Grimont PAD, Kampfer P, Maiden MCJ, Nesme X, Rossello-Mora R, Swings J, Truper HG, Vauterin L, Ward AC, Whitman WB (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol 52:1043–1047

Stepkowski T, Czaplinska M, Miedzinska K, Moulin L (2003) The variable part of the *dnaK* gene as an alternative marker for phylogenetic studies of rhizobia and related alpha *Proteobacteria*. Syst Appl Microbiol 26:483–494

Stepkowski T, Moulin L, Krzyżańska A, McInnes A, Law IJ, Howieson J (2005) European origin of *Bradyrhizobium* populations infecting lupins and serradella in soils of western Australia and south Africa. Appl Environ Microbiol 71:7041–7052

Tateno Y (1994) Evolution of glutamine synthetase genes is in accordance with the neutral theory of molecular evolution. Jpn J Genet 69:489–502

Tesfaye M, Petersen DJ, Holl FB (1997) Comparison of partial 23S rDNA sequences from *Rhizobium* species. Can J Microbiol 43:526–533

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Turner SL, Young JPW (2000) The glutamine synthetase of rhizobia: Phylogenetics and evolutionary implications. Mol Biol Evol 17:309–319

Valverde A, Igual JM, Peix A, Cervantes E, Velázquez E (2006) *Rhizobium lusitanum* sp. nov. a bacterium that nodulates *Phaseolus vulgaris*. Int J Syst Evol Microbiol 56:2631–2637

van Berkum P, Fuhrmann JJ (2000) Evolutionary relationships among the soybean bradyrhizobia reconstructed from 16S rRNA gene and internally transcribed spacer region sequence divergence. Int J Syst Bacteriol 50:2165–2172

van Berkum P, Terefework Z, Paulin L, Suomalainen S, Lindström K, Eardly BD (2003) Discordant phylogenies within the rrn loci of rhizobia. J Bacteriol 185:2988–2998

Vincent JM (1970) Manual for the practical Study of Root Nodule Bacteria. Blackwell, Oxford

Vinuesa P, Rademaker JLW, de Bruijn FJ, Werner D (1998) Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary Islands by PCR-Restriction Fragment Length Polymorphism analysis of genes encoding 16S rRNA (16S rDNA) and 16S-23S rRNA intergenic spacers, repetitive extragenic palindomic PCR genomic fingerprinting, and partial 16S rRNA sequencing. Appl Environ Microbiol 64:2096–2104

Vinuesa P, Rojas-Jiménez K, Contreras-Moreira B, Mahna SK, Prasad BN, Moe H, Selvaraju SB, Thierfelder H, Werner D (2008) Multilocus sequence analysis for assessment of the biogeography and evolutionary genetics of four *Bradyrhizobium* species that nodulate soybeans on the Asiatic Continent. Appl Environ Microbiol 74:6987–6996

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribossomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703

Willems A (2006) The taxonomy of rhizobia: an overview. Plant Soil 287:3–14

Willems A, Coopman R, Gillis M (2001) Comparison of sequence analysis of 16S–23S rDNA spacer regions, AFLP analysis and DNA-DNA hybridizations in Bradyrhizobium. Int J Syst Evol Microbiol 51:623–632

Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains archaea, bacteria, and eucarya. Proc Natl Acad Sci USA 87:4576–4579

Young JM (2003) The genus name *Ensifer* Casida 1982 takes priority over *Sinorhizobium* Chen et al. 1988, and *Sinorhizobium morelense* Wang et al. 2002 is a later synonym of *Ensifer adhaerens* Casida 1982. Is the combination '*Sinorhizobium adhaerens*' (Casida 1982) Willems et al. 2003 legitimate? Request for an Opinion. Int J Syst Evol Microbiol 53: 2107–10

Young JM, Kuykendall LD, Martínez-Romero E, Kerr A and Sawada H (2001) A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie et al. 1998 as new combinations: *Rhizobium radiobacter, R. rhizogenes, R. rubi, R. undicola* and *R. vitis*. Int J Syst Evol Microbiol 51: 89–103

Zeigler DR (2003) Gene sequences useful for predicting relatedness of whole genomes in bacteria. Int J Syst Evol Microbiol 53:1893–1900