

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

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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

Introduction

Many bacteria collectively known as “rhizobia” form symbiotic associations with legumes, establishing the key process of biological nitrogen (N₂) 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 N₂ 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

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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 N₂ 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 [(TSglnIIIf (5'-AAGCTC GAGTACATCTGGCTCGACGG-3') and TSglnIIr (5'-SG AGCCGTTCAGTCGGTGTGCG-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	<i>Rhizobium etli</i> ¹	<i>Vicia sativa</i> L.	Vicieae	Papilionoideae	Common vetch, pois France, arveja, ervilhaca	Forage (subtropical)
396	TAL 1148, USDA 3100, Nit 27A3	Niflta	Unknown	<i>Mesorhizobium ciceri</i> ¹	<i>Cicer arietinum</i> L.	Cicereae	Papilionoideae	Chickpea, pois chiche, garbanzo, grão de bico	Grain
696	CB 627	CSIRO	Australia	<i>Bradyrhizobium elkanii</i> ¹	<i>Desmodium uncinatum</i> (Jacq.) DC.	Desmodieae	Papilionoideae	Silverleaf desmodium, desmódio	Forage (tropical)
816	SEMIA original	FEPAGRO/UFRGS	Brazil	<i>Mesorhizobium</i> sp. ¹	<i>Lotus corniculatus</i> L.	Loteae	Papilionoideae	Cat's clover, birds' foot trefoil, broadleaf trefoil, cornichão	Forage (subtropical)
830	Unknown	Hansen inoculant (USA)	Unknown	<i>Mesorhizobium</i> sp. ¹	<i>Lotus glaber</i> Miller (<i>L. tenuis</i> Willd.)	Loteae	Papilionoideae	Narrow trefoil, birds' foot trefoil, cornichão	Forage (subtropical)
2051	SEMIA original	FEPAGRO/UFRGS	Brazil	<i>Rhizobium leguminosarum</i> ¹	<i>Trifolium vesiculosum</i> Savi	Trifolieae	Papilionoideae	Arrowleaf clover, trevo yuchi, trevo vesiculososo	Forage (subtropical)
2082	EEL 8,186	EPAGRI	Brazil	<i>Rhizobium leguminosarum</i> ¹	<i>Trifolium pratense</i> L.	Trifolieae	Papilionoideae	Red clover, trebol de los prados, trebol rojo, trevo vermelho	Forage (subtropical)
3007	B 11A	IPN	Mexico	<i>Rhizobium leguminosarum</i> ¹	<i>Pisum sativum</i> L.	Vicieae	Papilionoideae	Field pea, petit pois, alverja, guisante, ervilha	Grain
3012	CPAC EV6	Embrapa Cerrados	Brazil	<i>Mesorhizobium tianshanense</i> ²	<i>Pisum sativum</i> L.	Vicieae	Papilionoideae	Field pea, petit pois, alverja, guisante, ervilha	Grain
3026	CPAC L12	Embrapa Cerrados	Brazil	<i>Rhizobium leguminosarum</i> ²	<i>Lens culinaris</i> Medik. subsp. culinaris	Vicieae	Papilionoideae	Adi Merchi, burchak, chachavitza zychainaya, chechevitza kultumaya, common lentil, edamas lecas, lenteja, lenticchia	Grain
4088	PRF 81	Embrapa Soja/IAPAR	Brazil	<i>Rhizobium tropici</i> ²	<i>Phaseolus vulgaris</i> L.	Phaseoleae	Papilionoideae	Frijol, common Bean, feijão, poroto	Grain
6053	TAL 827, UMKL 128	Malásia	Malasya	<i>Bradyrhizobium elkanii</i> ¹	<i>Clitoria ternatea</i> L.	Phaseoleae	Papilionoideae	Pigeon wings, pois sauvage, blue pea, azulajo, campanilla, conchita blanca o azul, zapatico de la reina, clitoria	Tree
6153	BR 827	Embrapa Agrobiologia	Brazil	<i>Bradyrhizobium japonicum</i> ²	<i>Leucaena leucocephala</i> (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	<i>Bradyrhizobium japonicum</i> ²	<i>Stylosanthes</i> sp.	Aeschynomeneae	Papilionoideae	Stylo, Brazilian lucerne, stylosanthes, estilosantes	Forage (tropical)
6161	BR 4002, PRJ B4	Embrapa Agrobiologia	Brazil	<i>Sinorhizobium</i> sp. ¹	<i>Prosopis juliflora</i> (Sw.) DC.	Mimoseae	Mimosoideae	Mesquite, cashaw, bayarone, epinard bayahonda blanca, algaroba	Tree
6392	BR 3804	Embrapa Agrobiologia	Brazil	<i>Mesorhizobium amorphae</i> ²	<i>Acacia salicina</i> Lindl ^e	Acacieae	Mimosoideae	Broughton willow, cooba, doolan, native wattle, native willow, willow acacia	Tree
6396	BR 6815	Embrapa Agrobiologia	Brazil	<i>Bradyrhizobium japonicum</i> ²	<i>Chamaecrista ensiformis</i> (Vell.) H.S. Irwin & Barneby ^f <i>Albizia pedicellaris</i> (Dc.) L. Rico	Cassieae	Caesalpinioideae	Jatna, coração-de-negr	Tree
6396	BR 6815	Embrapa Agrobiologia	Brazil	<i>Bradyrhizobium japonicum</i> ²	<i>Albizia pedicellaris</i> (Dc.) L. Rico	Ingeae	Mimosoideae	Juerana-branca, Galinazo	Tree

Table 1 continued

SEMIA strain designations ^a	Other strain designations ^b	Source of the strain	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	<i>Methylobacterium mesophilicum</i> ²	<i>Pithecellobium tortum</i> Mart	Ingeae	Mimosoideae	Tataré, Jacaré, Angico-branco, Jurema, Vinhático-de-espinho	Tree
6423	BR 4302	Embrapa Agrobiologia	Brazil	<i>Rhizobium rhizogenes</i> ²	<i>Calliandra houstoniana</i> (Mill.) Standl. var. <i>calothyrsus</i> (Meissner) Bameby	Ingeae	Mimosoideae	Calliandra, calliandra, salsa pompon de marin	Tree
6428	BR 3628	Embrapa Agrobiologia	Brazil	<i>Bradyrhizobium elkanii</i> ²	<i>Acacia saligna</i> (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	<i>Rhizobium</i> sp. ²	<i>Gliricidia sepium</i> (Jacq.) Walp.	Robinieae	Caesalpinioideae	Grow stick, cacahuananche, madricacao, gliricidiah	Tree
6437	EEL 15,084	EPAGRI/Embrapa Trigo	Brazil	<i>Rhizobium</i> sp. ²	<i>Adesmia latifolia</i> (Spreng.) Vogel	Adesmieae	Papilionoideae	Adesmia	Forage (subtropical)
6439	MGAP 13	EPAMIG/Embrapa Cerrados	Brazil	<i>Bradyrhizobium japonicum</i> ²	<i>Arachis pintoi</i> 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 Catarina, Brazil); IPN (Instituto Politécnico Nacional, Centro de Investigación de Estudios Avanzados, Mexico, Mexico); MGAP (Ministerio de Ganadería, Agricultura y Pesca, Laboratorio de Microbiología y Suelos, Montevideo, Uruguay); NC (North Carolina, University of North Carolina, 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, Malaysia); 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 Politécnico Nacional, Centro de Investigación de Estudios 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 μ L 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 μ L 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, NC007761); *R. leguminosarum* USDA 2370^T (U29386, EU155089); *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 (AY577427, AY386762.1); *B. japonicum* USDA 6^T (U69638, AF169582); *Methylobacterium nodulans* ORS 2060^T (AF220763, 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		<i>glnI</i>		16S rRNA + <i>glnI</i>		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		
384	AY904730	1433/1439	6 (0.41)	GQ160496	457/480	23 (4.79)	1890/1919	<i>Rhizobium etli</i>
396	AY904731	1441/1450	9 (0.62)	GQ160515	462/480	18 (3.75)	1903/1930	<i>Mesorhizobium ciceri</i>
696	AY904736	1442/1449	7 (0.48)	GQ160506	477/481	4 (0.83)	1889/1930	<i>Bradyrhizobium elkanii</i>
816	AY904737	1436/1443	7 (0.48)	GQ160514	423/480	57 (11.87)	1859/1923	<i>Mesorhizobium</i> sp.
830	AY904738	1432/1445	13 (0.89)	GQ160504	422/480	58 (12.08)	1854/1925	<i>Mesorhizobium</i> sp.
2051	AY904740	1436/1441	5 (0.34)	GQ16013	446/480	34 (7.08)	1882/1921	<i>Rhizobium leguminosarum</i>
2082	F1025094	1431/1439	8 (0.55)	GQ160494	480/480	0 (0.0)	1911/1919	<i>Rhizobium leguminosarum</i>
3007	AY904742	1429/1441	12 (0.83)	GQ160503	476/480	4 (0.83)	1905/1921	<i>Rhizobium leguminosarum</i>
3012	F1025121	1435/1443	8 (0.55)	GQ160512	472/480	8 (1.66)	1907/1923	<i>Mesorhizobium tianshanense</i>
3026	F1025093	1436/1441	5 (0.34)	GQ160502	474/480	6 (1.25)	1910/1921	<i>Rhizobium leguminosarum</i>
4088	EF054889	1427/1439	12 (0.83)	GQ160511	480/480	0 (0.0)	1907/1919	<i>Rhizobium tropici</i>
6053	AY904745	1443/1447	4 (0.27)	GQ160501	464/480	16 (3.33)	1907/1927	<i>Bradyrhizobium elkanii</i>
6153	F1025097	1447/1447	0 (0.0)	GQ160510	454/480	26 (5.41)	1901/1927	<i>Bradyrhizobium liaoningense</i> *
6154	F1025100	1431/1446	15 (1.03)	GQ160500	427/479	52 (10.85)	1858/1925	<i>Bradyrhizobium</i> sp.*
6161	AY904763	1428/1444	16 (1.10)	GQ160509	428/480	52 (10.83)	1856/1924	<i>Ensifer</i> sp.
6392	F1025126	1438/1444	6 (0.41)	GQ160499	435/480	45 (9.37)	1873/1924	<i>Mesorhizobium</i> sp.*
6396	F1025099	1432/1446	14 (0.96)	GQ160495	453/480	27 (5.62)	1885/1926	<i>Bradyrhizobium canariense</i> *
6407	F1025133	1361/1441	80 (5.55)	GQ160508	449/480	31 (6.45)	1810/1921	<i>Methylobacterium</i> sp.*
6423	F1025132	1439/1439	0 (0.0)	GQ160507	428/480	32 (6.66)	1867/1919	<i>Rhizobium</i> sp.**
6428	F1025106	1441/1448	7 (0.48)	GQ160498	459/480	21 (4.37)	1900/1928	<i>Bradyrhizobium elkanii</i>
6435	F1025130	1434/1439	5 (0.35)	GQ160505	449/480	31 (6.45)	1883/1919	<i>Rhizobium</i> sp.**
6437	F1025118	1416/1439	23 (1.6)	GQ160497	412/480	68 (14.16)	1828/1919	<i>Rhizobium</i> sp.
6439	F1025098	1423/1447	24 (1.65)	GQ160516	448/479	31 (6.47)	1871/1926	<i>Bradyrhizobium japonicum</i>

* Denotes different classification when compared to that based exclusively on the 16S rRNA (Memma 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.

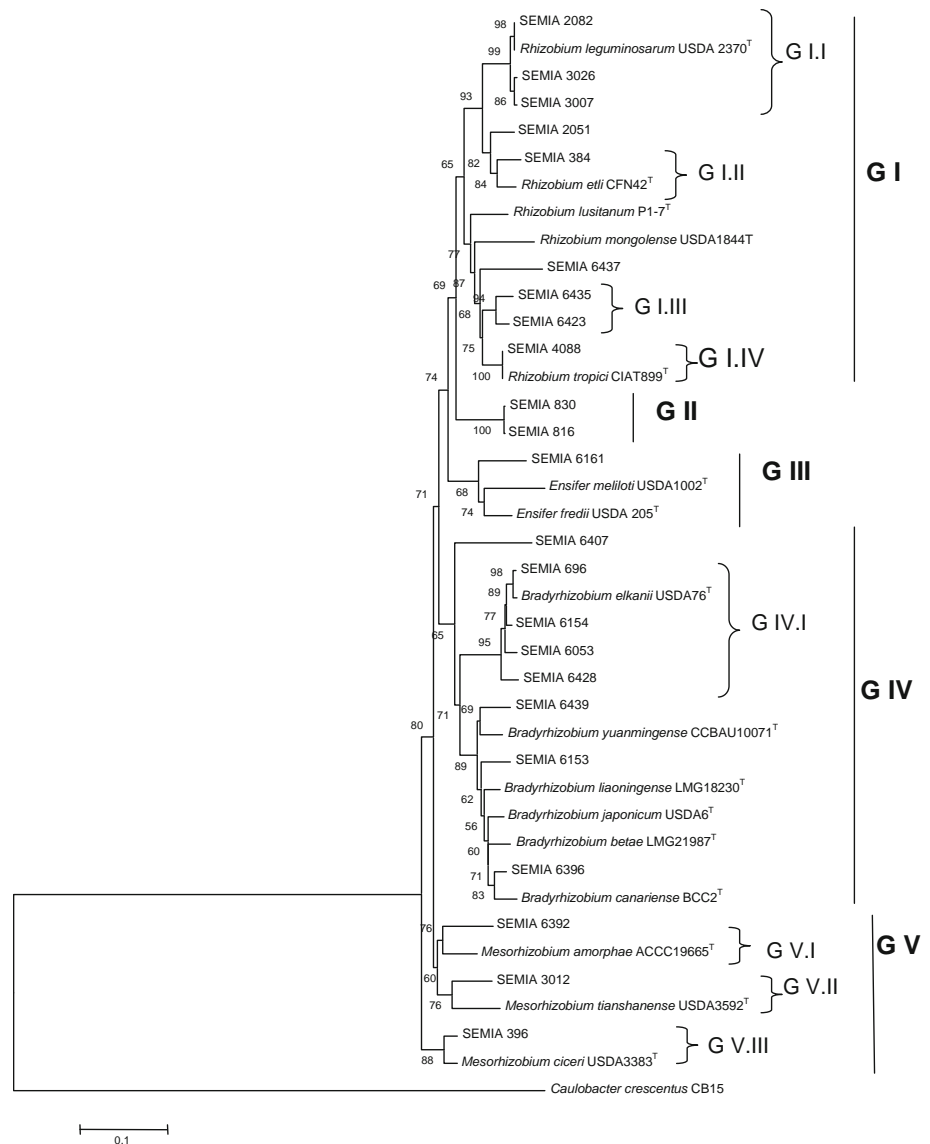
Strain SEMIA 4088 clustered in subgroup I.II of the 16S rRNA with *R. tropici* type strain, with a bootstrap support of 99%. Clustering was confirmed in the analysis of both the *glnII* (Fig. 2) and the concatenated (Fig. 3) genes, with bootstrap supports of 100%, confirming the phylogenetic grouping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



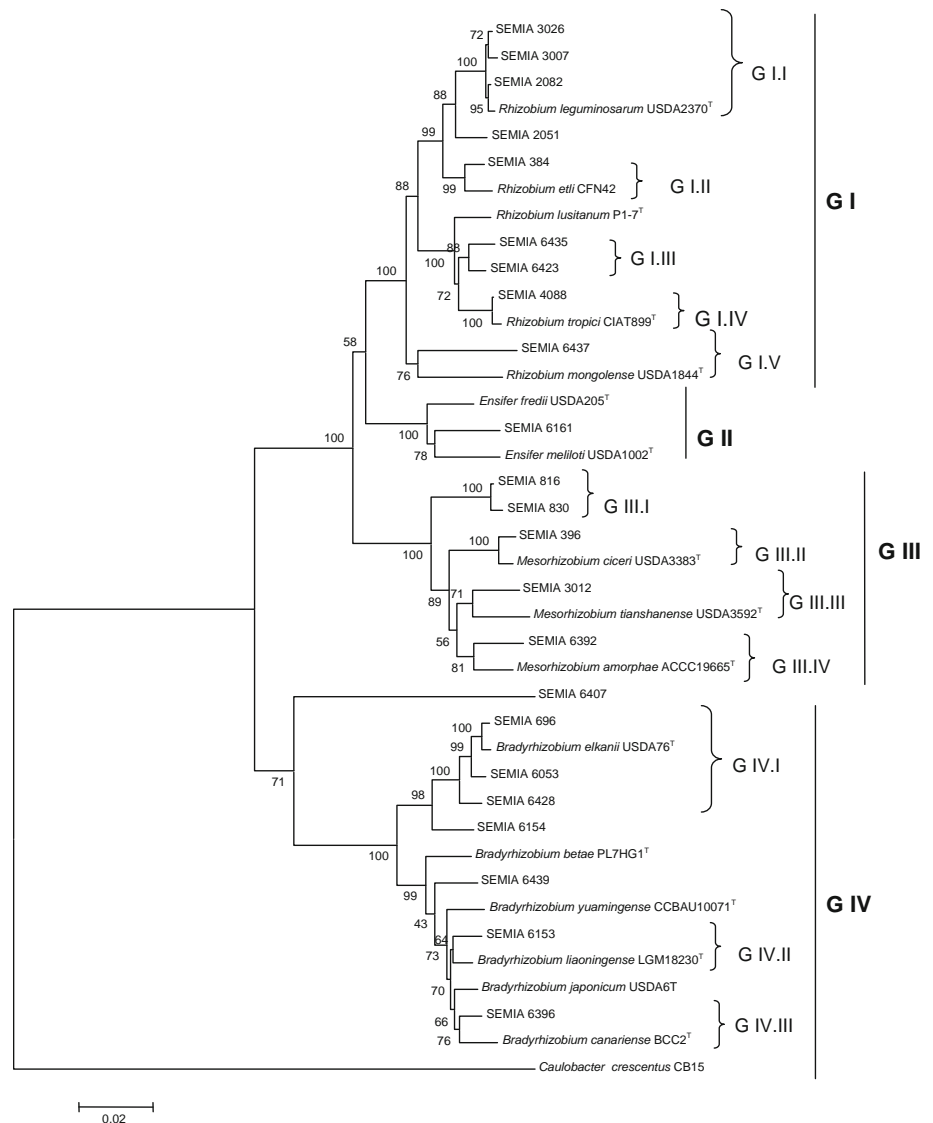
glnII and the concatenated genes, with bootstrap supports of 84 and 99%, respectively. Considering the *glnII* and the concatenated genes, 4.79 and 1.51% of the nucleotides, respectively, were different from *R. etli*, therefore, the strain resembles *R. etli*, but other housekeeping genes should be 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 mesophilyca*; 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. liaoningense* 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 N₂ 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.

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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 bradyrhizobial 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, Martínez-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, Kämpfer 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, Krzyżńska A, McInnes A, Law JJ, 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 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 palindromic 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 ribosomal 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