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ORIGINAL PAPER

Genetic diversity of indigenous tropical fast-growing rhizobia isolated from soybean nodules

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Abstract This study characterized genetically 30 fast-growing rhizobial strains isolated from nodules of Asian and modern soybean genotypes that had been inoculated with soils from disparate regions of Brazil. Analyses by *rep*-PCR (ERIC and REP) and RAPD indicated a high level of genetic diversity among the strains. The RFLP-PCR and sequencing analysis of the 16S rRNA genes indicated that none of the strains was related to *Sinorhizobium* (*Ensifer*) *fredii*, whereas most were related to *Rhizobium tropici* (although they were unable to nodulate *Phaseolus vulgaris*) and to *Rhizobium* genomic species Q. One strain was related to *Rhizobium* sp. OR 191, while two others were closely related to *Agrobacterium* (*Rhizobium*) spp.; furthermore, symbiotic effectiveness with soybean was maintained in those strains. Five strains were related to *Bradyrhizobium japonicum* and *B. elkanii*, with four of them being similar to strains carried in Brazilian inoculants, therefore modifications in physiological properties, as a shorter doubling time might have resulted from adaptation to local conditions. Phospholipid fatty acid analysis (PFLA) was less precise in delineating taxonomic relationships. The strains fit into eight Nod-factor profiles that were related to rhizobial species, but not to N_2-fixation capacity or competitiveness. The data obtained highlight the diversity and promiscuity of rhizobia in the tropics, being capable of nodulating exotic legumes and might reflect ecological strategies to survive in N-poor soils; in addition, the diversity could also represent an important source of efficient and competitive rhizobial strains for the tropics. Putative new rhizobial species were detected only in undisturbed soils. Three species (*R. tropici*, *B. japonicum* and *B. elkanii*) were found under the more sustainable management system known as no-till, while the only species isolated from soils under conventional till was *R. tropici.* Those results emphasize that from the moment that agriculture was introduced into undisturbed soils rhizobial diversity has changed, being drastically reduced when a less sustainable soil management system was adopted.

Keywords *Agrobacterium* · *Bradyrhizobium* · *Glycine max* · Molecular diversity · *Rhizobium* · Soil management systems · Tropical bacteria

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Introduction

Soybean [*Glycine max* (L.) Merrill] can establish effective N_2-fixing symbioses with *Bradyrhizobium japonicum* and *B. elkanii*, which are characterized by slow growth rate and alkaline reaction in media containing mannitol as carbon source (Jordan 1982; Kuykendall et al. 1992). In (1982) fast-growing rhizobial strains were also isolated from soybean nodules and from soil of the People's Republic of China, within the center of origin and diversity of this legume (Keyser et al. 1982). Later, fast-growing strains were isolated from other primary and secondary centers of soybean origin (e.g., Xu and Ge 1984; Dowdle and Bohlool 1985; Young et al. 1988; Rodriguez-Navarro et al. 1996). These fast growers were classified as the new species *Rhizobium fredii* (Scholla and Elkan 1984), later reclassified as *Sinorhizobium fredii* and *S. xinjiangensis* (Chen et al. 1988), and recently proposed to change to the genus *Ensifer* (Young 2003). Although it was originally thought that *S.* (*Ensifer*) *fredii* was specific for Asian soybean lines (Keyser et al. 1982; Stowers and Eaglesham 1984; Devine 1985), later it has been shown that several North American and Brazilian genotypes are capable of forming effective nodules with those bacteria (Balatti and Pueppke 1992; Chueire and Hungria 1997).

Soybean was introduced in Brazil in 1888, but commercial expansion of the crop did not take place until the 1960s. Brazilian soils are originally void of rhizobia able to effectively nodule soybean (e.g., Lopes et al. 1976; Peres 1979; Vargas and Suhet 1980; Ferreira and Hungria 2002; Hungria et al. 2006), therefore inoculants carrying *Bradyrhizobium* were brought mainly from the USA; *S. fredii* has never been used in Brazilian inoculants (Hungria et al. 2006). The presence of fast-growing rhizobial strains in soybean nodules in Brazil had never been reported, until in a survey carried out using one modern and six Asian soybean genotypes as trap hosts, thirty fast-growing isolates were obtained from twelve soils throughout Brazil; those strains were preliminary characterized in relation to morphological, physiological (Hungria et al. 2001b), and symbiotic (Hungria et al. 2001a) properties.

In this paper we evaluated several molecular approaches for assessing the rhizobial diversity in tropics, by using this population of indigenous fast-growing soybean strains as a model. The characterization of the indigenous fast-growers of soybean is important to understand better the ecological strategies that tropical rhizobia may take to survive after the introduction of an exotic host legume.

Materials and methods

Bacterial strains

Brazilian fast growing rhizobial strains

Thirty strains obtained from twelve Brazilian soils located in widely spread states, including undisturbed areas covered with native vegetation and fields traditionally cultivated with soybean and previously inoculated, were used in this study. The sites of isolation, soil chemical properties and preliminary morphological, physiological (Hungria et al. 2001b), and symbiotic (Hungria et al. 2001a) characteristics of the strains were described before.

Representative strains of rhizobial species

Sinorhizobium fredii CCBAU 114 (=RT 15) was received from Dr. E. T. Wang, Beijing Agricultural University, Beijing, China (as there are still discussions about the classification of *Sinorhizobium* as *Ensifer*, we will call the bacteria belonging to this genus as *Sinorhizobium*). The following strains were provided by Dr. P. van Berkum (USDA, Beltsville, MD, USA): *S. fredii* USDA 205^T (=ATCC 35423, =LMG 6217, =PRC 205); *S. meliloti* USDA 1002^T (=ATCC 9930, =LMG 6133, =3DOa2); *S. saheli* ORS 609^T (=LMG 7837, =USDA 4893); *S. teranga* ORS 1009^T (=USDA 4894); *Rhizobium leguminosarum* bv. trifolii USDA 2145 (=ATCC 14480, =LMG 8820, =3DIK22a); *R. leguminosarum* bv. viceae USDA 2370^T (=ATCC 10004); *R. leguminosarum* bv. phaseoli USDA (2671) (=RCR 3644); *Bradyrhizobium japonicum* USDA 6^T (=ATCC 10324, =3I1b6, =RCR

3425), USDA 110 (=3I1b110, =TAL 102, =RCR 3427, =61A89), USDA 122 and USDA 123; *B. elkanii* USDA 31, USDA 76^T and USDA 94; *Bradyrhizobium* sp. (*Aeschynomene indica*) BTAi1 (=USDA 4362); *Mesorhizobium huakuii* CCBAU 2609^T (=USDA 4779); *M. loti* NZP 2213^T (=ATCC 33669, =LMG 6125, =USDA 3471). Dr. E. Martínez, Cuernavaca, Mexico, sent the strains *R. tropici* type A CFN 299 (=USDA 9039, =LMG 9517); *R. tropici* type B CIAT 899^T (=ATCC 49672, =USDA 9030, =TAL 1797, =UMR 1899, =HAMBI 1163, =SEMIA 4077) and *R. etli* bv. phaseoli CFN 42^T (=USDA 9032). Dr. Noelle Amarger (INRA, Dijon, France) sent the strains H152^T of *R. giardinii* bv. giardinii, and R602^T, of *R. gallicum* bv. gallicum. *B. japonicum* strains SEMIA 5080 (=CPAC 7) and SEMIA 5079 (= CPAC 15) and *B. elkanii* strains SEMIA 587 and SEMIA 5019 (=29w, =BR 29), carried in Brazilian commercial inoculants for soybean, and *R. tropici* strains PRF 35, PRF 54 and PRF 81 (=SEMIA 4080), the last one recommended for commercial inoculants for common bean (*Phaseolus vulgaris* L.), came from the Embrapa Soja rhizobia germplasm bank.

Molecular analyses

Amplification with specific (ERIC and REP) and arbitrary (RAPD) primers

DNA was extracted from all strains as described before (Kaschuk et al. 2006). Total DNA was extracted from each strain and 50 ng were used in each amplification by *rep*-PCR with ERIC and REP primers (de Bruijn 1992), as described previously (Santos et al. 1999), in an MJ Research Inc. PT 100 thermocycler. Amplified fragments were separated by horizontal electrophoresis on a 1.5% agarose (low EEO, type I-A, GibcoBRL) gel (17 × 18 cm) at 100 V for 6 h.

For the Random Amplified Polymorphic DNA (RAPD) analysis, 50 ng of DNA were amplified with three 10-bp long primers of kit-S of Operon (OPS-17, 5′ TGGGGACCAC 3′; OPS-18, 5′ CTGGCGAACT 3′ and OPS-19, 5′ GAGTCAGCAG 3′), previously identified as good markers for rhizobial isolates (Ferreira et al. 2000). Amplification was performed as described before (Ferreira et al. 2000) and fragments were separated on a 1.5% agarose gel (17 × 11 cm) at 100 V for 4 h.

Cluster analyses were performed with the PCR products using the Bionumerics program (Applied Mathematics, Kortrijk, Belgium, version 1.50), with the algorithm UPGMA (unweighted pair-group method, with arithmetic mean, Sneath and Sokal, 1973) and the coefficient of Jaccard (Jaccard, 1912).

Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rDNA

Five replicates of total DNA were amplified by PCR with primers Y1 (5′–TGGCTCAGAACGAACGCTGGCGGC-3′) and Y3 (5′–CTGACCCCACTTCAGCATTGTTCCAT–3′), as described before (Chen et al. 2000), and all strains produced a single PCR product with approximately 1,500 bp. The PCR products were then digested with restriction endonucleases *Msp*I, *Cfo*I, *Hinf*I, *Rsa*I and *Nde*II (Invitrogen^{TM}), as recommended by the manufacturers. The fragments obtained were analyzed by horizontal electrophoresis in a gel (17 × 11 cm) with 3% agarose, at 100 V for 4 h. The cluster analysis was performed as described in the previous section.

16S rRNA sequence determination and GenBank accession numbers

Twenty-two isolates were submitted to the direct sequencing of PCR fragments obtained by amplification of the DNA region coding for the 16S rRNA region, as described before (Chen et al. 2000), in an ABI 377 (PE-Applied Biosystems) sequencer analyzer. The generated rDNA sequences were confirmed forward and backward and were submitted to the GenBank database to seek for significant 16S rRNA alignments. Strains 1, 2, 3, 5, 7, 8, 9, 11, 14, 15, 16, 18, 20, 22, 23, 24, 25, 26, 27, 28, 29 and 30 were named as ''PRSFG'' (*Pa*R*aná* *S*oybean *F*ast *G*rower) and received accession numbers AF260276 to AF260297, respectively.

Cluster analysis

The sequences of the rhizobial strains from this study were aligned pairwise and compared to those of the following organisms (accession numbers of the GenBank Data Library in parentheses): *Agrobacterium* biovar I strain LMG 11936 (AJ130721); *Agrobacterium* sp. MSMC211 (AJ004859) [*Agrobacterium* has been reclassified as *Rhizobium* (Young et al. 2001), but as there is still controversy about the nomenclature we will call the bacteria as *Agrobacterium*]; *B. elkanii* USDA 76^T (U35000), USDA 94 (D13429), SEMIA 587 (AF 234890) and SEMIA 5019 (AF237422); *B. japonicum* strains USDA 6^T (U69638), USDA 110 (Z35330), SEMIA 5079 (AF234888) and SEMIA 5080 (AF234889); *Rhizobium* sp. OR 191 (X91211); *M. loti* NZP 2213^T (X63825); *R. galegae* HAMBI 540^T (Y12355); *Rhizobium* genomic species Q strain BDV 5102 (Z94806); *R. huautlense* S02^T (AF025852); *R. leguminosarum* bv. phaseoli ATCC 8002 (M55494); *R. tropici* type B CIAT 899^T (U89832); *R. tropici* IAM 14206 (D12798); *R. tropici* type A LMG 9518 (X67233); *S. fredii* USDA 205^T (M74163); *S. saheli* OR 609^T (X68390). A dendogram was inferred with the UPGMA algorithm, using the Bionumerics program.

Phospholipid fatty acid (PLFA) composition

Fatty acid analyses were carried out with duplicate washed plates containing bacteria that had been grown on yeast mannitol agar (Vincent 1970) for 48 h at 28°C, as described previously (Hungria et al. 2000), on a Hewlett Packard (6890) gas chromatograph equipped with a flame ionization detector and a 60-m HP 5 capillary column. The percent of the PLFA values were $\log_{10}$ transformed before being subjected to principal component analysis (PCA; Hartman 1967) to elucidate major variation and covariation patterns. The multivariate calculations were performed using the computer program SYSTAT v 5.0, for Windows™.

Nod factor profiles

Thin layer chromatographic analysis (TLC) of lipo-chitin oligosaccharide Nod signals (Nod factors) was performed as described previously (Hungria et al. 2000) after induction of *nod* genes with 2 μM naringenin. Cells incubated in the absence of any *nod*-gene inducer served as controls, and 2 μl of D-[^{14}C]-glucosamine HCl (50–60 mCi mmol^{-1}, Amersham, Int.) were used as radiotracer. TLC plates (pre-coated plates of silica gel with 100% octadesylsilanization, Sigma) were exposed to Kodak Biomax MR-Kodak film for 15 d, then developed with Kodak reagents.

Results

Molecular analyses

Amplification with specific (ERIC and REP) and arbitrary (RAPD) primers

DNA amplification by PCR with specific primers (ERIC and REP) resulted in up to thirty products per strain. However, some strains produced only one or two bands (strains 4, 6, 10 and 27) and no bands were obtained for strains 3, 9, 12 and 13. Different DNA extractions and lysis procedures did not improve the results therefore strains producing only few bands (up to three) were not included in the cluster analysis. The bands of twenty-two strains from this study and of eighteen reference strains were combined to produce a dendrogram (Fig. 1). At the top of the dendrogram, strain 19 was linked to *B. japonicum* USDA 110 at a level of similarity of 43%. Strains 18, 21, 26, 28, 30, 23 and 17 were clustered at a level of similarity of 38%. Another cluster (46% of similarity) included strains 5, 1, 2, 25, 14 and 7. Following, strain 20 showed low relatedness (35%) with *S. fredii* USDA 205 and with *R. leguminosarum* bv. phaseoli USDA 2671, strains 15 and 29 were joined at a low level of similarity (37%) and strain 24 was poorly related to all other strains. *B. japonicum* SEMIA 5080 was linked with strain 11 at a 66% level of similarity, and with strains 16 and 8 at 41%, while *B. elkanii* SEMIA 587 was related to strain 22 at a 42% level of similarity (Fig. 1).

In the RAPD analysis, two strains (7 and 29) did not amplify with at least two primers, and therefore were not included in the study. Most

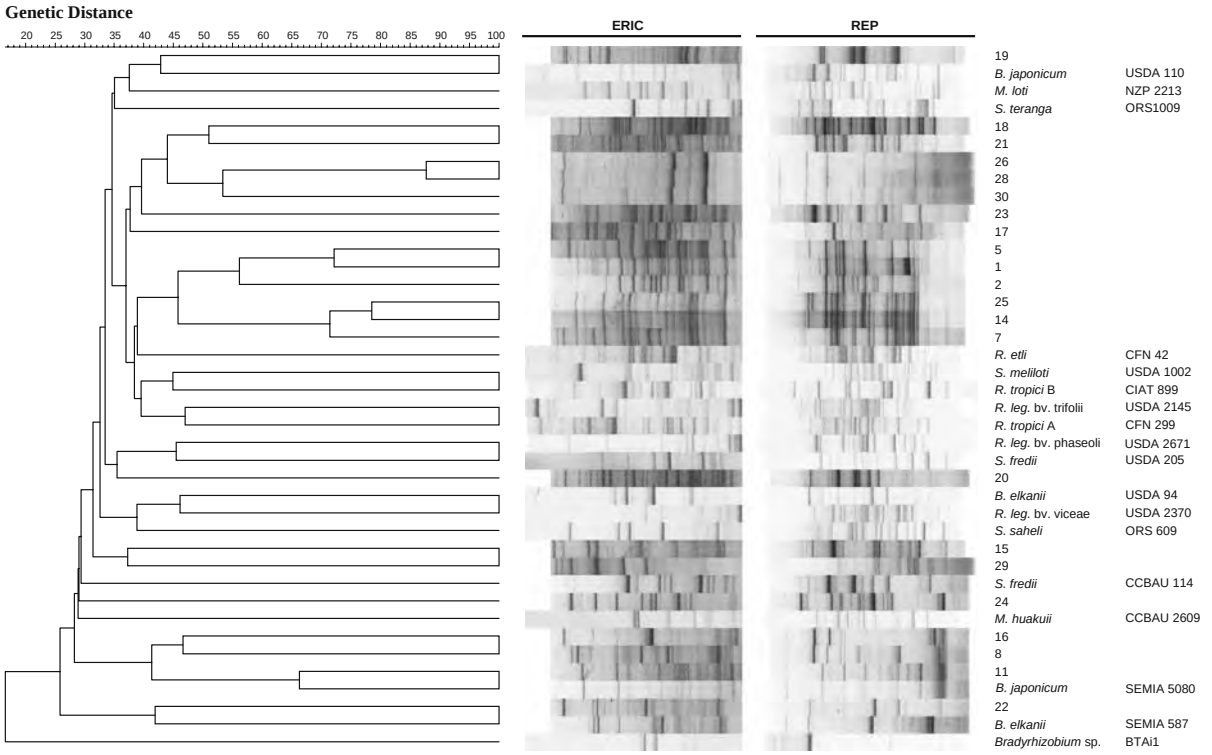

Fig. 1 Genetic dendrogram showing the Brazilian and representative strains of several rhizobial species after cluster analysis of PCR products obtained by DNA amplification with ERIC and REP primers. Cluster analysis using the UPGMA method and the Jaccard coefficient

RAPD groupings of Brazilian strains (data not shown) resembled those obtained with the ERIC-REP-PCR products (Fig. 1), e.g., strains 25, 14 and 5 were joined with 31% of similarity and strains 11, 8 and 16 with 37% of similarity. However, several clusters differed in relation to the reference strains used, e.g., in RAPD analysis *S. fredii* USDA 205 was joined to strain 19 at a 36% level of similarity and then to strains 23 and 24, at 31%. Diversity was high, as all strains were clustered at a final level of similarity of only 14% (data not shown).

Restriction fragment analysis of PCR amplified 16S rDNA

When amplified with primers Y1 and Y3, all strains produced a single band of about 1,500 bp and were submitted to RFLP analysis. Thirty Brazilian strains and twenty-eight reference strains were grouped into four great clusters, joined with a similarity of 16% (data not shown). Cluster I included reference strains of *B. japonicum*, *B. elkanii* and *Bradyrhizobium* sp. BTAi1 with strains 8, 10, 11 and 30. Strains 13, 17, 19 and 28 were grouped in cluster II, which did not include any of the reference strains and were linked to cluster I with a very low similarity (19%). Cluster III included only reference strains of *Sinorhizobium* and *Mesorhizobium* species (28% of similarity). Finally, all reference strains of bean rhizobial species, as well as *R. leguminosarum* bv. viceae and *S. fredii* CCBAU 114 were positioned in cluster IV, together with twenty two of the thirty Brazilian strains (data not shown).

Partial 16S rRNA sequencing analysis

After fifteen colony passes, eight strains (strains 4, 6, 10, 12, 13, 17, 19, 21), all grouped in cluster IV of RFLP-PCR analysis lost the capacity of nodulating soybean. Although the procedure of testing nodulation stability under laboratory conditions might not be representative of the environmental

conditions, the strains could have lost the symbiotic plasmid or could represent opportunistic microsymbionts. Therefore more detailed studies are being performed with those eight strains and they were not considered in the 16S rRNA partial sequences analysis in this study. Sequences confirmed forward and backward varied from 391 to 650 bp. Fourteen strains showed high identities (99% to 100%) with the following strains: *Rhizobium* genomic species Q strain BDV 5102 (Z94806), *R. tropici* strain IAM 14206 (D12798), *Agrobacterium tumefaciens* K-Ag-3 (D14504), and *R. leguminosarum* LMG 9518 (X67233), which is now classified as *R. tropici* type A. Within this group, strains 2, 7, 9, 22 and 23 had complete identity of bases, similarly to the pair of strains 26 and 27 and to the strains 1 and 3; the other strains (5, 14, 15, 25 and 29) differed in some nucleotides. Following, both strains 24 and 20 were related with *Agrobacterium* sp. strains MSMC211 (AJ004859) and LMG 11936 (AJ130721) and with *A. radiobacter* LMG 383 (AJ130791), all three isolated from nodules of tropical legumes. These two strains were preliminary named as *Rhizobium* sp., as they might represent a new species. Two strains, 8 and 30, showed complete identity with *B. japonicum* USDA 6 (U69638), USDA 123 (AF236088), as well as with *B. japonicum* Brazilian strains SEMIA 566 (AF236086) and SEMIA 5079 (AF234888). Considering the partial sequences obtained, strains 11 and 16 also showed complete identity of bases with *B. japonicum* USDA 110 (Z35330) and USDA 136 (L23331), and with the Brazilian strains SEMIA 586 (AF236087) and SEMIA 5080 (AF234889). Strain 28 was 99% related to *B. elkanii* USDA 31 (AF236089) and strain 18 showed a 98% identity with *Rhizobium* sp. OR 191 (X91211).

A dendrogram was built with one Brazilian strain from each group showing similar 16S rRNA sequences and with reference strains (Fig. 2). *Agrobacterium* reference strains were clustered with a 98.7% identity, and the cluster was linked to strain 20 at a 98.6% level, and finally to strain 24 at 96.2% of similarity. Seven Brazilian strains were clustered with *R. tropici* type A and type B and with *Rhizobium* genomic species Q with 97.2% identity. Strain 18 showed 98.6% identity with *Rhizobium* sp. OR 191. Strains 8 and 16 were clustered with *B. japonicum* strains and joined at a level of 98.5% and *B. elkanii* and strain 28 were clustered with 98.8% of similarity; the two groups were linked with a final level of similarity of 97.4%. *Agrobacterium*, *Rhizobium*, *Mesorhizobium* and *Sinorhizobium* strains were linked at a 92.9% level of similarity and then linked to the bradyrhizobia group with a similarity of 86.1% (Fig. 2).

Phospholipid fatty acid composition

In general, the composition of phospholipid and fatty acids (data not shown) was quite similar to that found by Jarvis and Tighe (1994) for different rhizobial species. Figure 3 shows a two dimensional plot of the principal component analysis (PCA) carried out with thirteen PFLAs considered for the thirty strains studied and for *S. fredii* USDA 205 and CCBAU 114, *B. japonicum* SEMIA 5080 and *B. elkanii* SEMIA 587. The two first axis explain 67% of the analysis (36% axis I and 31% axis II), and three groups were identified. Fatty acids (loadings) with more weight at positive values of axis I were: cy17, 17:1ω8, 18:1ω9, and 16:1ω7c (related mainly with samples of groups II and III); fatty acids 18:1ω7, i17 and cy19 were related to group I.

All *Bradyrhizobium* strains from this study were in group I, which included also other *R. tropici* strains (Fig. 3). The second group (II) included reference strains of *S. fredii*, *B. elkanii* SEMIA 587, six strains classified as *R. tropici*, strain 20, genetically related to *Agrobacterium*, and strain 18, showing similarity with OR 191. The last group III included seven strains and reference *B. japonicum* SEMIA 5080. Another PCA was carried out with the same data, excluding SEMIA 5080 and SEMIA 587 and ordination results were not very different from previously described PCA, appearing also as three groups, with the same relative position and composition (data not shown). Although PFLAs (Fig. 3) were not related to the rhizobial species, several similarities were detected with strain grouping by *rep*-PCR (Fig. 1), RAPD (data not shown) and PCR-RFLP (data not shown). For

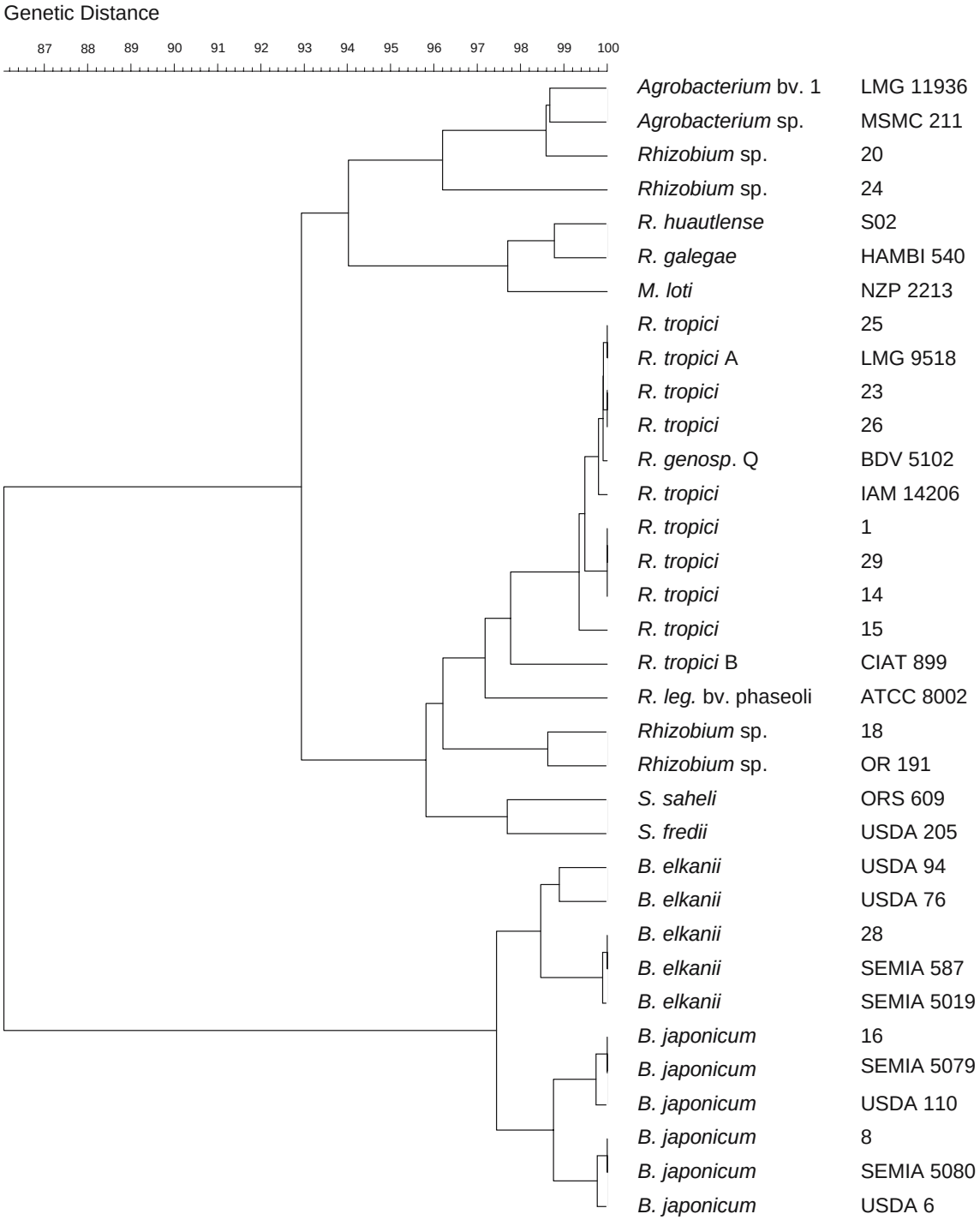

Fig. 2 Dendrogram (UPGMA) of genetic relationships among 16S rRNA genes of Brazilian and reference strains of several rhizobial and agrobacterial species. The GenBank accession numbers for the reference strains are listed in the material and methods section

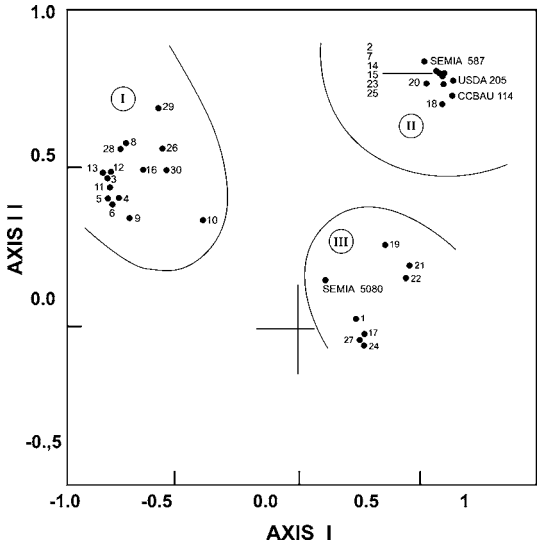

Fig. 3 Principal component analysis (two dimensional plot) obtained with seventeen fatty acids for the Brazilian rhizobial strains and the following reference strains: *B. elkanii* SEMIA 587, *B. japonicum* SEMIA (5080) and *S. fredii* USDA 205 and CCBAU 114

example, strains 2, 7, 14, 15 and 23 positioned in group II of PFLA were in cluster IV of PCR-RFLP, with a relatedness of 59%; strains 8, 11, 30 and 10 in group I of PFLA were in cluster I of PCR-RFLP. The exceptions were in group III of PFLA, with strains in different groups in the *rep*-PCR, RAPD and PCR-RFLP clusters; five of the seven strains within this PFLA group came from uncropped areas in widely spread sites of Brazil.

Nod factor profiles

The Brazilian strains were grouped into eight Nod-factor profiles (Fig. 4) and a relationship between Nod factor profiles and rhizobial species was observed. *R. tropici* strains showed three different profiles, I, IV and VI; strain 18, with similarity with *Rhizobium* sp. OR 191, was also in group IV. Groups II, VII and VIII included exclusively strains showing identity with *Bradyrhizobium*. Rhizobial strains 20 and 24, showing high relatedness with *Agrobacterium*, were in group V. All bacteria from Group III lost their effectiveness, and others that showed this feature were within groups, I, IV and V (Fig. 4).

Discussion

Fast-growing strains capable of nodulating Asian genotypes have been isolated from the primary and secondary centers of origin of soybean in Asia (Keyser et al. 1982; Xu and Ge 1984; Dowdle and Bohlool 1985; Young et al. 1988; Rodriguez-Navarro et al. 1996) and it has been suggested that they have coevolved with the soybean plant (Devine 1985). However, *S. fredii* may also show broad host range, being capable of nodulating several species of the ''cowpea cross-inoculation group'', such as *Cajanus cajan*, *Macroptilium atropurpureum*, *Psophocarpus tetragonolobus*, *Vigna radiata* and *V. unguiculata* (Broughton et al. 1984; Stowers and Eaglesham 1984; Chen et al. 1988; Buendía-Clavería et al. 1989; Romero et al. 1993; Rodriguez-Navarro et al. 1996). Therefore *S. fredii* may exist in Brazilian soils as a symbiont of native leguminous trees. Indeed, Moreira et al. (1993) and de Lajudie et al. (1994), using electrophoretic protein patterns, classified strains BR 811 and BR 817 isolated from *Leucaena leucocephala* in Brazil as *S. fredii* and Straliotto et al. (1999), based on morphological and physiological properties, classified some common bean (*Phaseolus vulgaris*) rhizobia into the same species. In addition, based on 16S rRNA sequencing analysis, rhizobial strains isolated from both common bean (Grange and Hungria 2004), and from the leguminous tree *Prosopis juliflora* (common name: mesquite, cashew) (Menna et al. 2006) fit into the genus *Sinorhizobium*.

In this study thirty fast-growing and acid-producing strains previously isolated from Asian and modern soybean genotypes inoculated with Brazilian soils (Hungria et al., 2001a, b) were genetically characterized, but none was related to *S. fredii.* Based on a comparison of rRNA sequences, a group of fourteen strains showed high identity with *R. tropici* and with *Rhizobium* genomic species Q strain BDV 5102, isolated from native shrubs in Australia (Lafay and Burdon 1998). Martínez-Romero et al. (1991) isolated *R. tropici* as a common bean (*Phaseolus vulgaris* L.) symbiont native to tropical regions of South America. Indeed, *R. tropici* represented most of the rhizobia capable of nodulating

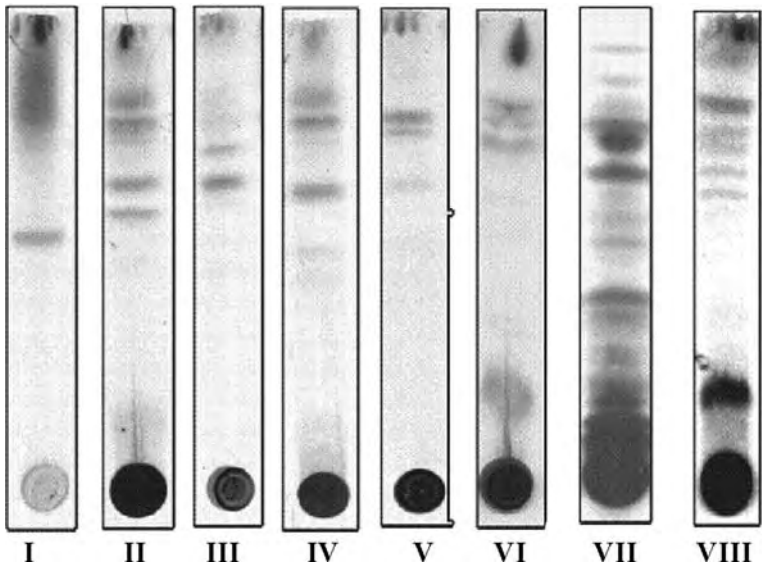

Fig. 4 Nod-factor profiles of the Brazilian rhizobial strains obtained by thin layer chromatography (TLC) of radiolabeled Nod metabolites after induction with genistein. Strains showing each profile were: Group I (1, 2, 3, 4, 5, 6, 7, 9, 10, 23); II (8, 11, 16); III (12, 13, 21); IV (14, 15, 17, 18, 22); V (19, 20, 24); VI (25, 26, 27, 29); VII (28); VIII (30)

common bean and *Leucaena* sp. when isolated under field conditions using as trapping hosts both plants (Hungria and Stacey 1997; Hungria et al. 1997; Mercante et al. 1998; Hungria et al. 2000), but not when using soil dilutions (Grange and Hungria 2004). In addition, recently Alberton et al. (2006) confirmed that the populations isolated from common bean nodules collected straight from the field or after inoculation with soil dilutions may be considerably different. However, it has also been hypothesized that *Phaseolus* might not be the original or unique host of *R. tropici*, as the species has been found on other continents and nodulating other hosts, for example, it was isolated from *Bolusanthus* and *Aspartium* nodules in Africa (Dagutat and Stein 1995), from African soils with no history of bean cultivation (Anyango et al. 1995), from nodules of native shrubby legumes in Australia (Lafay and Burdon 1998), from sand dune plant species *Calystegia soldanella* and *Elymus mollis* (Park et al. 2005), and was described as a natural microsymbiont of the leguminous tree *Gliricidia sepium* in Mexico (Acosta-Durán and Martínez-Romero 2002). In addition, *R. tropici* is capable of nodulating several other host legumes (Hernandez-Lucas et al., 1995). All together, the results reported in our paper reinforce that *R. tropici* is a competitive species under the acidic and high-temperature conditions of Brazil; the species may also be microsymbiont of several other legumes and/or acquire the capacity of nodulating new legumes. However, it should be mentioned that the strains from our study showing high relatedness with *R. tropici* could nodulate and fix N_2 with soybean but did not nodulate common bean.

Strain 18 showed 98% identity of nucleotides with the promiscuous *Rhizobium* sp. OR 191 and was designated as *Rhizobium* sp.; the differences in nucleotides might indicate a new rhizobial species. Strains 20 and 24 showed relatedness with *Agrobacterium* bv. 1 and other *Agrobacterium* sp. strains isolated from root nodules of tropical legumes (Khbaya et al. 1998), as well as with rhizobia resembling agrobacteria isolated from soybean nodules in Paraguay (Chen et al. 2000). However, *R. tropici*, *Rhizobium* genomic species Q, *R. galegae*, and *R. huautlense* share several characteristics and are genetically closely related to *Agrobacterium* spp. (Martínez-Romero et al. 1991; Lafay and Burdon 1998; Terefework et al. 1998; Wang et al. 1998). Furthermore, at least under laboratory conditions rhizobia symbiotic plasmids may be transferred to related genera, such as *Agrobacterium* (Martínez et al. 1987; Novikova and Safronova 1992), but natural transfer in natural environments has not been reported yet. An increasing number of reports on horizontal genetic transfer in prokaryotes has highlighted the importance of the process in

genomic evolution, speciation and adaptation (Gogarten and Townsend 2005). In rhizobia, the transfer of the whole symbiotic island between inoculant and non-symbiotic *Mesorhizobium* has been demonstrated under field conditions in New Zealand (Sullivan and Ronson 1995), and evidences of massive horizontal transfer of symbiotic genes between inoculant and indigenous bradyrhizobia strains under the stressful conditions of the Brazilian Cerrados has been recently shown (Batista et al. 2006). Maintenance of symbiotic properties was not observed in rhizobia resembling agrobacteria isolated from *Acacia* spp. by Khbaya et al. (1998), and from common bean in Tunisia (Mhamdi et al. 1999), and in our study some strains have also lost their symbiotic effectiveness. However, as previously observed with some strains from Paraguay (Chen et al. 2000), both strains 20 and 24 maintained symbiotic effectiveness and might represent indigenous rhizobia (or agrobacteria) still not described, or they might have received the symbiotic plasmid from another rhizobia that gave to the recipient bacterium an increased adaptive capacity and improved fitness to environmental changes. We are now performing detailed genetic studies with those strains. Finally, it is worthwhile mentioning that one main reason claimed by Young et al. (2003) for a unique species including *Rhizobium* and *Agrobacterium* is that tumorigenic and rhizogenic populations cannot be circumscribed as species in formal nomenclature because the different pathogenic characters are borne on transmissible Ti or Ri plasmids, therefore the acquisition, exchange or loss of one of these plasmids by a bacterial strain would lead to a change in its species identity.

The analysis of the 16S rRNA gene has also indicated that five strains fit into the genetic group of *Bradyrhizobium*, although differing in several characteristics from that genus, e.g., a faster growth rate (205–240 min) and absence of alkaline reaction in yeast mannitol medium (Hungria et al. 2001b). These strains did not react serologically with and showed different protein and lipopolysaccharide profiles from strains used in Brazilian commercial inoculants for the last several decades, although partial 16S rRNA sequences were identical to those of Brazilian commercial strains. This could be attributed to both physiological modifications resulting from adaptation to local conditions, as has been extensively reported in Brazilian isolates (e.g., Hungria et al. 1996, 1998; Nishi et al. 1996; Boddey and Hungria 1997; Santos et al., 1999; Hungria and Vargas 2000; Galli-Terasawa et al. 2003), or to horizontal gene transfer to originally nonsymbiotic rhizobia indigenous to the Brazilian soils (Batista et al. 2006). One strain, 28, might represent a native bradyrhizobium with mixed characteristics of *Bradyrhizobium* and *Rhizobium*, as has been shown for strain BTAi1 (Stowers and Eaglesham 1984).

The partial sequencing of the 16S rRNA gene (391–650 bp), corresponding to two reads (back and forward) has allowed a preliminary classification into genus and the detection of high relatedness with known rhizobial species. To obtain the complete sequencing of the 16S rRNA it would be necessary to perform at least six runs in the sequencing analyzer (Menna et al. 2006), with triplicate costs. It might be thus considered that the partial sequencing of the 16S rRNA represents a powerful technique to assess a large and still poorly known diversity in less explored ecosystems, while full sequences should be required in studies aiming at describing new species. In this study, some strains would fit into at least two putative new species: strain 18, showing higher relatedness with OR 191 but differing in several base pairs, and strains 20 an 24, resembling agrobacteria. It is noteworthy that all three strains were isolated from undisturbed areas (Hungria et al. 2001b). In addition, rhizobia resembling agrobacteria were not exclusive from a unique ecosystem, as strain 20 was isolated from the sourthern state of Rio Grande do Sul, while strain 24 was isolated from Amazon (Hungria et al. 2001b). *R. tropici* was also broadly detected in undisturbed soils (strains 9, 22, 23, 25). Three species were isolated from soils under no-till: *R. tropici* (strains 15, 26, 27 and 29), *B. japonicum* (strains 8, 11, 16 and 22) and *B. elkanii* (strain 28), while the only species isolated from soils under conventional till management was *R. tropici* (strains 1, 2, 3, 5, 7 and 14).

Assessment of genetic diversity of rhizobia has been reported using both RAPD (e.g., Harrison

et al. 1992; Richardson et al., 1995; Selenska-Pobell et al. 1995) and consensus sequences (e.g., de Bruijn 1992; Judd et al. 1993; Madrzak et al. 1995; Selenska-Pobell et al. 1995; van Rossum et al. 1995; Laguerre et al. 1997; Hungria et al. 1998, 2000; Santos et al. 1999; Alberton et al. 2006; Kaschuk et al. 2006) and in this study both methods were also adequate to assess the diversity of tropical rhizobia. The PCR products obtained with specific and arbitrary primers confirmed that each isolate from this study was a unique strain, however, neither one of the techniques has allowed the grouping at genus or species level. Therefore the results confirm previous reports that amplification with specific and arbitrary primers may be quite useful to detect genetic diversity at the strain level, but not for phylogenetic characterization (Laguerre et al. 1997). However, it is noteworthy that congruence of several morphological and physiological parameters, including the utilization of carbon sources (Hungria et al. 2001b), with the genetic analyses performed in this study was observed for several groups of strains, e.g., 1, 2, 5, 7, 14; 8, 11, 16; 28 and 30.

A comparison of PLFA data with the genetic methods showed that the former is less accurate for delineating phylogenetic relationships, as was also observed by Hollaway et al. (1997) with strains of a *Pseudomonas* species. However, there were similarities between phenotypic ordination (Hungria et al. 2001b) and PLFAs for groups I and II, but not for group III, which contained most of the isolates from uncropped areas.

Several Nod-factor profiles were detected among the strains, but none was similar to previously reported profiles for Brazilian strains of *B. japonicum*, *B. elkanii* (Hungria et al. 1996) or *R. tropici* (Hungria et al. 2000). In general the profiles were not related to the N_2-fixation capacity or to the competitiveness of those strains (2001a).

In conclusion, all methods used in this study were effective in assessing rhizobial diversity in the tropics. *rep*-PCR technique could be chosen as the best technique to detect diversity among strains. In addition, partial sequencing of 16S rRNA was required to assess a preliminar taxonomic position of each strain. The results emphasize the high diversity and promiscuity of rhizobia in tropics, being capable of nodulating exotic legumes and may reflect ecological strategies to survive in tropical N-poor soils. Furthermore, the high promiscuity should also be considered when introducing new exotic legumes, as searching for efficient and competitive rhizobial strains within the indigenous diversity might represent an attractive agronomical approach.

Finally, an important issue was highlighted in this study. It has been shown that agricultural practices as tillage (Ferreira et al. 2000), soil fertilization (Caballero-Mellado and Martínez-Romero 1999), and crop rotation (Hungria and Vargas 2000) may affect rhizobial diversity. In this study, the strains isolated from undisturbed soils covered with native vegetation indicated the highest diversity of fast-growing rhizobia, showing relatedness with *R. tropici*, *Rhizobium* sp. OR 191, and *Rhizobium* resembling *Agrobacterium*. *R. tropici* was also broadly detected in undisturbed soils, but the strains were unable to effectively nodulate common beans. The introduction of agriculture in uncropped areas has drastically changed the diversity assessed. With the adoption of a more sustainable agriculture system, as the no-tillage system, three species were detected, *R. tropici*, *B. japonicum* and *B. elkanii*. However, isolates from cropped soils under conventional tillage, characterized by stressful environmental conditions such as high temperature and low soil moisture, consisted only of *R. tropici*, known as very tolerant to environmental stressful conditions (Martínez-Romero et al. 1991; Hungria et al. 2000). Those results emphasize that from the moment that agriculture was introduced into undisturbed soils, rhizobial diversity has changed and was drastically reduced when a less sustainable soil management system was adopted.

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References

Acosta-Durán C, Martínez-Romero E (2002) Diversity of rhizobia from nodules of the leguminous tree Gliricidia sepium, a natural host of Rhizobium tropici. Arch Microbiol 178: 161–164

Alberton O, Kaschuk G, Hungria M (2006) Sampling effects on the assessment of genetic diversity of rhizobia associated with soybean and common bean. Soil Biol Biochem 38: 1298–1307

Anyango B, Wilson KL, Beynon JL, Giller KE (1995) Diversity of rhizobia nodulating *Phaseolus vulgaris* L. in two Kenyan soils with contrasting pHs. Appl Environ Microbiol 61: 4016–4021

Balatti PA, Pueppke SG (1992) Identification of North American soybean lines that form nitrogen-fixing nodules with *Rhizobium fredii* USDA 257. Can J Plant Sci 72: 49–55

Batista JS, Hungria M, Barcellos FG, Ferreira, MC, Mendes IC (2006) Variability in *Bradyrhizobium japonicum* and *B. elkanii* seven years after introduction of both the exotic microsymbiont and the soybean host in a Cerrados soil. Microbial Ecol (in press)

Boddey LH, Hungria M (1997) Phenotypic grouping of Brazilian *Bradyrhizobium* strains which nodulated soybean. Biol Fert Soils 25: 407–415

Broughton WJ, Heycke N, Meyer HZA, Pankhurst CE (1984) Plasmid-linked *nif* and *nod* genes in fast-growing rhizobia that nodulate *Glycine max*, *Psophocarpus tetragonolobus* and *Vigna unguiculata*. Proc Natl Acad Sci USA 81: 3093–3097

Buendía-Clavería AM, Chamber M, Ruíz-Saínz JE (1989) A comparative study of the physiological characteristics, plasmid content and symbiotic properties of different *Rhizobium fredii* strains. Syst Appl Microbiol 12: 203–209

Caballero-Mellado J, Martínez-Romero E (1999) Soil fertilization limits the genetic diversity of *Rhizobium* in bean nodules. Symbiosis 26: 111–121

Chen LS, Figueredo A, Pedrosa FO, Hungria M (2000) Genetic characterization of soybean rhizobia in Paraguay. Appl Environ Microbiol 66: 5099–5103

Chen WX, Yan GH, Li JL (1988) Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. Int J Syst Bacteriol 38: 393–397

Chueire LMO, Hungria M (1997) N_2-fixation ability of Brazilian soybean cultivars with *Sinorhizobium fredii* and *Sinorhizobium xinjiangensis*. Plant Soil 196: 1–5

Dagutat H, Stein PL (1995) Taxonomy and distribution of rhizobia indigenous to South African soils. In: Tikonovich IA, Provoporv NA, Romanov VI, Newton WE (eds) Nitrogen fixation: fundamentals, applications. Kluwer Academic Publishers, Dordrecht, pp 683

de Bruijn FJ (1992) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of Rhizobium meliloti isolates and other soil bacteria. Appl Environ Microbiol 58: 2180–2187

de Lajudie PD, Willems A, Pot B, Dewettinck D, Maestrojuan G, Neyra M, Collins MD, Dreyfus B, Kersters K, Gillis M (1994) Polyphasic taxonomy of rhizobia: Emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov. *and Sinorhizobium teranga* sp. nov. Int J Syst Bacteriol 44: 715–733

Devine TE (1985) Nodulation of soybean plant introduction lines with the fast-growing rhizobial strain USDA 205. Crop Sci 25: 354–356

Dowdle SF, Bohlool BB (1985) Predominance of fast-growing *Rhizobium japonicum* in a soybean field in the People's Republic of China. Appl Environ Microbiol 50: 1171–1176

Ferreira MC, Andrade DS, Chueire LMO, Takemura SM, Hungria M (2000) Tillage method and crop rotation effects on the population sizes and diversity of bradyrhizobia nodulating soybean. Soil Biol Biochem 32: 627–637

Ferreira MC, Hungria M (2002) Recovery of soybean inoculant strains from uncropped soils in Brazil. Field Crops Res 79: 139–152

Galli-Terasawa LV, Glienke-Blanco C, Hungria M (2003) Diversity of soybean rhizobial population adapted to a Cerrados soil. World J Microbiol Biotechnol 19: 933–939

Gogarten JP, Townsend JP (2005) Horizontal gene transfer, genome innovation and evolution. Nature Rev Microbiol 3: 679–687

Grange L, Hungria M (2004) Genetic diversity of indigenous common bean (*Phaseolus vulgaris*) rhizobia in two Brazilian ecosystems. Soil Biol Biochem 36: 1389–1398

Harrison SP, Mytton LR, Skøt L, Dye M, Cresswell A (1992) Characterization of *Rhizobium* isolates by amplification of DNA polymorphisms using random primers. Can J Microbiol 38: 1009–1015

Hartman JH (1967) Modern factor analysis. University Press, Chicago, IL

Hernandez-Lucas I, Segovia L, Martínez-Romero E, Pueppke SG (1995) Phylogenetic relationships and host range of *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. Appl Environ Microbiol 61: 2775–2779

Hollaway GJ, Gillings MR, Fahy PC (1997) Use of fatty acid profiles and repetitive element polymerase chain reaction (PCR) to assess the genetic diversity of *Pseudomonas syringae* pv. *pisi* and *Pseudomonas syringae* pv. *syringae* isolated from field peas in Australia. Aust J Plant Pathol 26: 98–108

Hungria M, Andrade DS, Chueire LMO, Probanza A, Guttierrez-Mañero FJ, Megías M (2000) Isolation and characterization of new efficient and competitive bean (*Phaseolus vulgaris* L.) rhizobia from Brazil. Soil Biol Biochem 32: 1515–1528

Hungria M, Andrade DS, Colozzi-Filho A, Balota EL (1997) Interação entre microrganismos do solo, feijoeiro e milho em monocultura e consórcio. Pesq Agropec Bras 32: 807–818

Hungria M, Boddey LH, Santos MA, Vargas MAT (1998) Nitrogen fixation capacity and nodule occupancy by

Bradyrhizobium japonicum and *B. elkanii* strains. Biol Fert Soils 27: 393–399

Hungria M, Campo RJ, Chueire LM, Grange L, Megias M (2001a) Symbiotic effectiveness of fast-growing rhizobial strains isolated from soybean nodules in Brazil. Biol Fert Soils 33: 387–394

Hungria M, Campo RJ, Mendes IC, Graham PH (2006) Contribution of biological nitrogen fixation to the nitrogen nutrition of grain crops in the tropics: the success of soybean (Glycine max L. Merr.) in South America. In: Singh RP, Shankar N, Jaiwal PK (Eds.), Nitrogen nutrition in plant productivity. Studium Press, Houston, Texas, pp 43–93

Hungria M, Chueire LMO, Coca RG, Megías M (2001b) Preliminary characterization of fast growing rhizobial strains isolated from soyabean nodules in Brazil. Soil Biol Biochem 33: 1349–1361

Hungria M, Nishi CYM, Cohn J, Stacey G (1996) Comparison between parental and variant soybean *Bradyrhizobium* strains with regard to the production of lipo-chitin nodulation signals, early stages of root infection, nodule occupancy, and N_2 fixation. Plant Soil 186: 331–341

Hungria M, Stacey G (1997) Molecular signals exchanged between host plants and rhizobia: Basic aspects and potential application in agriculture. Soil Biol Biochem 29: 819–830

Hungria M, Vargas MAT (2000) Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. Field Crops Res 65: 151–164

Jaccard P (1912) The distribution of flora in the alpine zone. New Phytol 11: 37–50

Jarvis BDW, Tighe SW (1994) Rapid identification of *Rhizobium* species based on cellular fatty acid analysis. Plant Soil 161: 31–44

Jordan DC (1982) Transfer of *Rhizobium japonicum* Buchanan (1980) to *Bradyrhizobium* gen. nov., a genus of slow growing root-nodule bacteria from leguminous plants. Int J Syst Bacteriol 32: 136–139

Judd AK, Schneider M, Sadowsky MJ, de Bruijn FJ (1993) Use of repetitive sequences and the polymerase technique to classify genetically related *Brayrhizobium japonicum* serocluster 123 strains. Appl Environ Microbiol 59: 1702–1708

Kaschuk G, Hungria M, Santos JC, Berton-Junior JF (2006) Differences in common bean rhizobial populations associated with soil tillage management in southern Brazil. Soil Till Res 87: 205–207

Keyser HH, Bohlool BB, Hu TS, Weber DF (1982) Fast-growing rhizobia isolated from root nodules of soybeans. Science 215: 1631–1632

Khbaya B, Neyra M, Normand P, Zerhari Z, Filali-Maltouf A (1998) Genetic diversity and phylogeny of rhizobia that nodulate *Acacia* spp. in Morocco assessed by analysis of rRNA genes. Appl Environ Microbiol 64: 4912–4917

Kuykendall LD, Saxena B, Devine TE, Udell SE (1992) Genetic diversity in *Bradyrhizobium japonicum* Jordan (1982) and a proposal for *Bradyrhizobium elkanii* sp. nov. Can J Microbiol 38: 501–505

Lafay B, Burdon JJ (1998) Molecular diversity of rhizobia occurring on native shrubby legumes in southeastern Australia. Appl Environ Microbiol 64: 3989–3997

Laguerre G, van Berkum P, Amarger N, Prevost D (1997) Genetic diversity of rhizobial symbionts isolated from legume species within the genera *Astragalus*, *Oxytropis*, and *Onobrychis*. Appl Environ Microbiol 63: 4748–4758

Lopes ES, Giardini AR, Kiihl RAS (1976) Presença e eficiência de *Rhizobium japonicum* em solos cultivados ou não com soja, no Estado de São Paulo. Bragantia 35: 389–396

Madrzak CJ, Golinska B, Króliczak J, Pudelko K, Lazewska D, Lampka B, Sadowsky M (1995) Diversity among field populations of *Bradyrhizobium japonicum* in Poland. Appl Environ Microbiol 61: 1194–1200

Martinez E, Palácios R, Sáchez F (1987) Nitrogen-fixing nodules induced by *Agrobacterium tumefaciens* harboring *Rhizobium phaseoli* plasmids. J Bacteriol 169: 2828–2834

Martínez-Romero E, Segovia E, Mercante FM, Franco AA, Graham PH, Pardo MA (1991) *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int J Syst Bacteriol 41: 417–426

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

Mercante FM, Cunha CO, Straliotto R, Ribeiro-Junior W, Vanderleyden J, Franco AA (1998) *Leucaena leucocephala* as a trap-host for *Rhizobium tropici* strains from the Brazilian ''Cerrado'' region. R Microbiol 29: 49–58

Mhamdi R, Jebara M, Aouni ME, Ghir R, Mars M (1999) Genotypic diversity and symbiotic effectiveness of *Phaseolus vulgaris* L. grown in Tunisian soils. Biol Fert Soils 28: 313–320

Moreira FMS, Gillis M, Pot B, Kersters K, Franco AA (1993) Characterization of rhizobia isolated from different divergence groups of tropical *Leguminosae* by comparative polyacrylamide gel electrophoresis of their total proteins. Syst Appl Microbiol 16: 135–136

Nishi CYM, Boddey LH, Vargas MAT, Hungria M (1996) Morphological, physiological and genetic characterization of two new *Bradyrhizobium* strains recently recommended as Brazilian commercial inoculants for soybean. Symbiosis 20: 147–162

Novikova NY, Safronova V (1992) Transconjugants of *Agrobacterium radiobacter* harbouring sym genes of *R. galegae* can form an effective symbiosis with *Medicago sativa*. FEMS Microbiol Lett 93: 262–268

Park MS, Jung SR, Lee MS, Kim KO, Do JO, Lee KH, Kim SB, Bae KS (2005) Isolation and characterization of bacteria associated with two sand dune plant species, *Calystegia soldanella* and *Elymus mollis*. J Microbiol 43: 219–227

Peres JRR (1979) Seleção de estirpes de *Rhizobium japonicum* e competitividade por sítios de infecção

nodular em cultivares de soja (*Glycine max* (L.) Merrill). UFRGS-FA, Porto Alegre, Brazil. (M.Sc. Thesis)

Richardson AE, Viccars LAA, Watson JM, Gibson AH (1995) Differentiation of *Rhizobium* strains using the polymerase chain reaction with random and directed primers. Soil Biol Biochem 27: 515–524

Rodriguez-Navarro DN, Ruiz-Sainz JE, Buendía-Clavería A, Santamaria C, Balatti PA, Krishnan HB, Pueppke SG (1996) Characterization of fast-growing rhizobia from nodulated soybean [*Glycine max* (L.) Merr.] in Vietnam. Syst Appl Microbiol 9: 240–248

Romero F, Buendia-Claveria A, Ruíz-Saínz JE (1993) Broad host-range effective mutants of *Rhizobium fredii* strains. J Appl Bacteriol 74: 610–619

Santos MA, Vargas MAT, Hungria M (1999) Characterization of soybean bradyrhizobia strains adapted to the Brazilian Cerrados Region. FEMS Microbiol Ecol 30: 261–272

Scholla MH, Elkan GH (1984) Rhizobium fredii sp. nov., a fast-growing species that effectively nodulates soybeans. Int J Syst Bacteriol 34: 484–486

Selenska-Pobell S, Gigova L, Petrova N (1995) Strain-specific fingerprints of *Rhizobium galegae* generated by PCR with arbitrary and repetitive primers. J Appl Bacteriol 79: 425–431

Sneath PBA, Sokal RR (1973) Numerical taxonomy. W.H. Freeman & Co, San Francisco, USA

Stowers MD, Eaglesham AR (1984) Physiological and symbiotic characteristics of fast-growing *Rhizobium japonicum*. Plant Soil 77: 3–14

Straliotto R, Cunha CO, Mercante FM, Franco AA, Rumjanek NG (1999) Diversity of rhizobia nodulating common beans (*Phaseolus vulgaris* L.) isolated from Brazilian tropical soils. An Acad Bras Ciênc 71: 531–543

Sullivan JT, Ronson CW (1995) Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. Proc Natl Acad Sci USA 95: 5145–5149

Terefework Z, Nick G, Suomalaine S, Paulin L, Lindström K (1998) Phylogeny of *Rhizobium galegae* with respect to other rhizobia and agrobacteria. Int J Syst Bacteriol 48: 349–356

van Rossum D, Schuurmans FP, Gillis M, Muyotcha A, van Verseveld HW, Stouthamer AH, Boogerd FC (1995) Genetic and phenetic analyses of *Bradyrhizobium* strains nodulating peanut (*Arachis hypogaea* L.) roots. Appl Environ Microbiol 61: 1599–1609

Vargas MA, Suhet AR (1980) Efeito de tipos e níveis de inoculantes na soja cultivada em um solo de cerrado. Pesq Agropec Bras 15: 343–347

Vincent JM (1970) Manual for the practical study of root nodule bacteria. Blackwell, Oxford, UK

Wang ET, van Berkum P, Beyene D, Sui XH, Dorado O, Chen WX, Martinez-Romero E (1998) *Rhizobium huautlense* sp. nov., a symbiont of *Sesbania herbacae* that has a close phylogenetic relationship with *Rhizobium galegae*. Int J Syst Bacteriol 48:687–699

Xu LM, Ge C (1984) Physiological-biochemical characteristics and symbiotic responses of the fast-growing Rhizobium japonicum. Soybean Sci 3: 102–109

Young CC, Chang JY, Chao CC (1988) Physiological and symbiotic characteristics of *Rhizobium fredii* isolated from subtropical-tropical soils. Biol Fert Soils 5: 350–354

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: 2107–2110

Young JPW, Downer H, Eardly BD (1991) Phylogeny of the phototrophic *Rhizobium* strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. J Bacteriol 173: 2271–2277

Young JM, Kuykendall LD, Martínez-Romero E, Kerr A, 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

Young JM, Kuykendall LD, Martínez-Romero E, Kerr A, Sawada H (2003) Classification and nomenclature of *Agrobacterium* and *Rhizobium* – a reply to Farrand *et al.* (2003). Int J Syst Evol Microbiol 53: 1689–1695