Polyphasic characterization of Brazilian *Rhizobium tropici* strains effective in fixing N$_2$ with common bean (*Phaseolus vulgaris* L.)

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Abstract

Common bean (*Phaseolus vulgaris*) is native to the Americas, and *Rhizobium etli* is the dominant microsymbiont in both the Mesoamerican and the Andean centers of genetic diversification. Wild common beans are not found in Brazil, although the legume has been cropped in the country throughout time and all but one of the rhizobial species that nodulate it (*Rhizobium gallicum*) have been broadly detected in Brazilian soils. However, the majority of the effective rhizobial strains isolated so far from field-grown plants belong to *R. tropici*. This study describes the analysis of symbiotic and non-symbiotic genes of 15 effective *R. tropici* strains, isolated from four geographically distant regions in Brazil. With RFLP-PCR of the 16S and 23S rRNA genes and sequence analysis of 16S rRNA, two clusters were observed, one related to *R. tropici* type A and another to type B strains. Diversity in ribosomal genes was high, indicating that type A strains might represent a new species. High intraspecies diversity was also observed in the *rep*-PCR analysis with BOX, ERIC and REP primers. However, in the RFLP-PCR analysis of *nifH* and *nodC* genes, all *R. tropici* showed unique combinations of profiles, which might reflect an evolutionary strategy to maximize N$_2$ fixation.
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1. Introduction

Common bean (*Phaseolus vulgaris* L.) represents the most important source of protein for the poorer populations of Central and South America, with Brazil being the largest grower and consumer of the legume worldwide (Embrapa, 2006). *Phaseolus vulgaris* is considered native to the Americas, and domestication thought to have taken place separately in two major centers of genetic diversification: the Mesoamerican center or northern group (from Mexico to the northern region of South America), and the Andean center or southern group (from southern Peru to the north of Argentina); a third minor domestication center might exist in Colombia (Gepts, 1990; Kami et al., 1995). Archeological evidences indicate that common bean was spread by migration and trade among Indian populations throughout history (Freitas, 2006).

An important feature of the common bean plant is its ability to establish a symbiotic partnership with specific bacteria, setting up the biological N$_2$-fixation process in root nodules that may supply the plant's needs for N. Five species of *Rhizobium* have been described as capable of nodulating—and in most cases fixing N$_2$—with common bean: *R. leguminosarum* bv. phaseoli (Jordan, 1984), *R. tropici* (Martínez-Romero et al., 1991), *R. etli* bv. phaseoli (Segovia et al., 1993), *R. gallicum* (bv. gallicum and bv. phaseoli) and *R. giardinii* (bv. giardinii and bv. phaseoli) (Amarger et al., 1997). Furthermore, other genetically diverse isolates have been isolated from common bean nodules, possibly representing new species (Eardly et al.,

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1995; van Berkum et al., 1996; Straliotto et al., 1999; Grange and Hungria, 2004), while others can nodulate common bean but usually do not fix N_2 under laboratory conditions, and have not yet been isolated from field-grown plants (e.g., Michiels et al., 1998; Lok et al., 2006).

Studies have indicated that *R. etli* is the dominant microsymbiont in both the Mesoamerican and the Andean centers of genetic diversification (Segovia et al., 1993; Souza et al., 1994; Aguilar et al., 1998, 2004; Bernal and Graham, 2001). After colonization by Europeans, common bean was introduced into Europe and seeds may have carried *R. etli*, which transferred its symbiotic plasmid to indigenous *R. leguminosarum* (Segovia et al., 1993). Later, *R. leguminosarum* may have transferred the plasmid to *R. gallicum* bv. phaseoli and *R. giardinii* bv. phaseoli (Amarger et al., 1997). *R. tropici* was originally isolated from common bean nodules in Colombia and was then found to be abundant in Brazilian soils, trapped by either common bean or *Leucaena* sp. (Martínez-Romero et al., 1991; Mercante et al., 1998). However, *R. tropici* has also been isolated from nodules of common bean and other legumes in Europe (e.g., Amarger et al., 1994), Africa (e.g., Anyango et al., 1995; Odee et al., 2002), Australia (e.g., Lafay and Burdon, 1998, 2001), and Central America (e.g., Acosta-Durán and Martínez-Romero, 2002).

All described common bean rhizobial species have been detected in Brazilian soils, except for *R. gallicum* (Sá et al., 1993; Mercante et al., 1998; Straliotto et al., 1999; Oliveira et al., 2000; Mostasso et al., 2002; Grange and Hungria, 2004). In population studies, high percentages of *R. etli* (Grange and Hungria, 2004; Grange et al., 2007) and of *R. leguminosarum* (Straliotto et al., 1999; Andrade et al., 2002; Mostasso et al., 2002) were recovered when dilutions of Brazilian soils were used as inocula for common bean plants under axenic conditions. However, *R. tropici* was clearly dominant in field-grown plants, even when cultivars of the Mesoamerican group were used as trap hosts (Hungria et al., 1997, 2000, 2003; Oliveira et al., 2000; Mostasso et al., 2002). The dominance of *R. tropici* in nodules of field-grown plants has been attributed to the intrinsic properties of the species, particularly tolerance of acidic and high-temperature conditions (Martínez-Romero et al., 1991; Graham et al., 1994; Hungria et al., 2000, 2003), resulting in stronger competitiveness under the often-stressful tropical conditions in Brazil (Hungria and Stacey, 1997; Hungria et al., 1997, 2000, 2003; Mercante et al., 1998; Mostasso et al., 2002).

In light of these results, together with the important feature of higher symbiotic stability of *R. tropici* (Martínez-Romero et al., 1991), probably due to the presence of a unique copy of the *nifH* gene (Martínez-Romero et al., 1991; Geniaux et al., 1993), a strain-selection program was established in Brazil in 1994 aimed at selecting strains belonging to this species that are effective in fixing N_2. The first criterion considered in this program was the ability to nodulate both common bean and *Leucaena* spp. and to grow *in vitro* at elevated temperatures (Martínez-Romero

et al., 1991), although these properties may not be exclusive to *R. tropici* (e.g., Hernandez-Lucas et al., 1995). Three strains (CIAT 899, PRF 81 and H 12) have been identified in this selection program, capable of supplying sufficient N to support grain yields of $2500 \, kg \, ha^{-1}$ or more (Hungria et al., 2000, 2003); they have been officially recommended for the use in Brazilian commercial inoculants for common bean crops (MAPA, 2006). However, despite economic importance and the promising results achieved, Brazilian strains of high N_2-fixation capacity are still poorly characterized. Accordingly, the aim of this study was to characterize very effective putative *R. tropici* strains isolated from different Brazilian regions using a polyphasic approach that includes phenotypic properties and genetic analysis of ribosomal and symbiotic genes.

2. Material and methods

2.1. Rhizobial strains

Rhizobium tropici type A strain CFN 299 (= USDA 9039; = USDA 9029; = LMG 9517; = UMR1026; = CENA 183), and type B strain CIAT 899[T] (= USDA 9030; USDA 2744; = UMR1899; = TAL 1797; = HAMBI 1163; = CM01; = SEMIA 4077; = ATCC 49672; BR322), and *R. etli* bv. phaseoli *strain* CFN 42[T] (= USDA 9032) were supplied by Dr. Esperanza Martínez, Centro de Ciencias Genómicas, Cuernavaca, Mexico. *R. leguminosarum* bv. viciae USDA 2370[T] (= ATCC 10004), *R. leguminosarum* bv. phaseoli USDA 2671 (= RCR 3644), and *R. leguminosarum* bv. trifolii ATCC 14480 (= USDA 2145, = LMG 8820) were provided by Dr. Peter van Berkum (USDA, Beltsville, MD, USA). *R. giardinii* bv. giardinii strain H152[T] and *R. gallicum* bv. gallicum strain R602[T] were provided by Dr. Noelle Amarger, INRA, Dijon, France.

Eleven (Table 1) strains used in this study, isolated by our group, were chosen as the most effective in fixing N_2 in previous work. They were isolated from the Federal District (FD), from the central state of Goiás (GO) (Mostasso et al., 2002), from the southern state of Paraná (PR) and from the northeastern state of Pernambuco (PE) (Grange and Hungria, 2004) and another set from PR (Hungria et al., 2000) (Fig. 1); some of their properties were described in those studies. Strains PRF 81 (Hungria et al., 2000) and H 12 (Mostasso et al., 2002; Hungria et al., 2003) have also received the denominations of SEMIA 4080 and SEMIA 4088, respectively.

Four other Brazilian strains (CPAO 2.11, CPAO 12.5, CPAO 29.8 and CPAO 1135) were isolated from the state of Mato Grosso do Sul (MS), in the central-western region (Fig. 1) and are described for the first time in this study. They were identified as the most effective strains in fixing N_2 among 527 isolates collected from 87 sites in 34 districts covering all common bean producing areas of the state.

Strains were maintained in yeast–mannitol (YM) liquid broth (YMB, Vincent, 1970) mixed with glycerol (25%)

Table 1
Growth *in vitro* of common bean rhizobial strains in LB, PY−Ca and in T4 at 40 °C or with pH 4.0

Strain	Species	Geographic origin	LB	PY	40 °C[a]	pH 4.0[a]
CFN 299	*Rhizobium tropici* type A	Brazil	N[b]	N	P	P
CIAT 899[T]	*R. tropici* type B	Colombia	Y	Y	Y	Y
CFN 42[T]	*R. etli* bv phaseoli	Mexico	N	N	N	N
USDA 2370[T]	*R. leguminosarum* bv. viciae	USA	N	N	N	N
USDA 2671	*R. leg.* bv. phaseoli	England	N	N	N	N
ATCC 14480	*R. leg.* bv. trifolii	Unknown	N	N	N	N
R602[T]	*R. gallicum* bv. gallicum	France	N	N	N	N
H152[T]	*R. giardinii* bv. giardinii	France	N	N	N	N
Brazilian strains						
H 12		Planaltina, DF	P	P	Y	Y
H 20		Planaltina, DF	P	P	Y	Y
H 52		Planaltina, DF	P	P	Y	Y
H 53		Planaltina, DF	P	P	Y	Y
77		Santo Antônio, PE	N	N	P	P
130		São João, PR	N	N	N	N
131		São João, PR	P	P	Y	Y
141		São João, PR	N	N	P	P
233		Francisco Alves, PR	P	P	Y	Y
PRF 35		Pitanga, PR	N	N	Y	Y
PRF 81		Londrina, PR	P	P	Y	Y
CPAO 2.11		Campo Grande, MS	N	N	Y	Y
CPAO 12.5		Taquarussu, MS	N	N	Y	Y
CPAO 29.8		Dois Irmãos do Buriti, MS	N	N	Y	Y
CPAO 1135		Dourados, MS	P	P	Y	Y

[a]In TY medium.

[b]All characteristics were confirmed in three replicates. Y, yes, normal growth; N, no growth; P, poor growth, of about 10% of that observed in the strains classified.
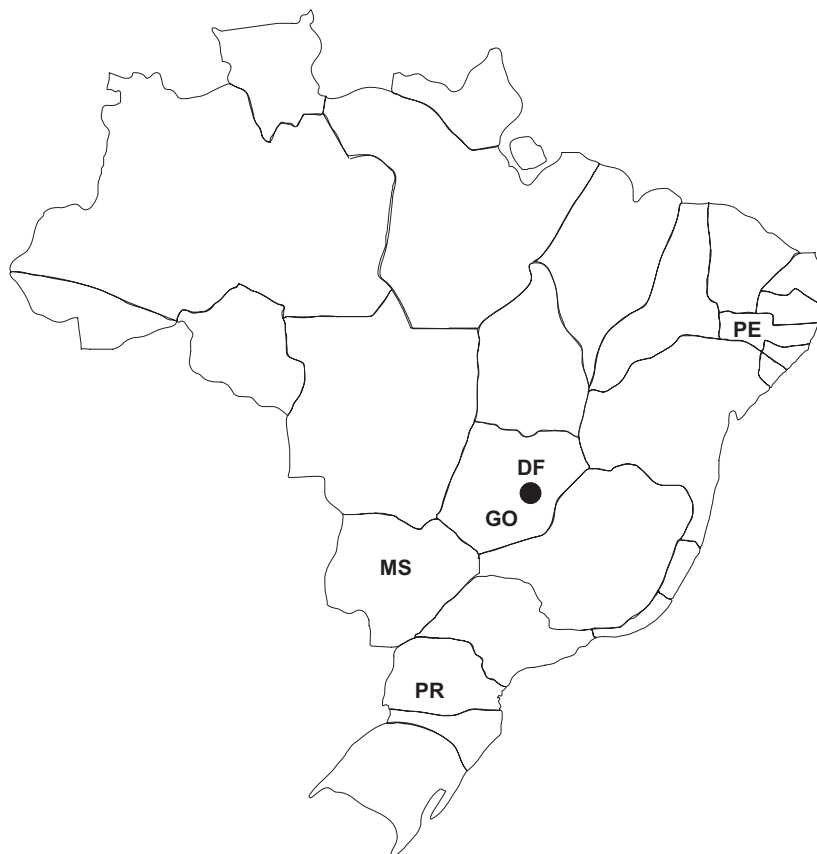

Fig. 1. Brazilian states in which the common bean rhizobial strains used in this study were isolated. Places are DF (Federal District) and States of Goiás (GO), Mato Grosso do Sul (MS) and Paraná (PR).

and stored at $-80\,^{\circ}$C, and working cultures were maintained on YM agar slants at $4\,^{\circ}$C. Rhizobia were cultured routinely at $28\,^{\circ}$C in YMB on a rotary shaker operating at 65 cycles min^{-1}. All strains have been deposited in the "Diazotrophic and PGPR Bacteria Culture Collection" of Embrapa Soja.

2.2. Morpho-physiological characterization

Colony morphology (size, color, mucosity, transparency, borders and elevation) and acid/alkaline reaction in vitro were evaluated as described by Vincent (1970), after 3 and 5 days of growth in the dark, at $28\,^{\circ}$C, on YM solid medium (YMA, containing 12 g of agar L^{-1}), with bromothymol blue (0.00125%) as a pH-change indicator. Growth in Luria broth (LB) and in peptone–yeast extract (PY) minus Ca medium (Martínez-Romero et al., 1991), as well as in tryptone–yeast extract medium (TY, Somasegaran and Hoben, 1994) at $40\,^{\circ}$C and in TY modified for a pH of 4.0 was verified as described before (Hungria et al., 2000).

2.3. Genetic characterization

2.3.1. Extraction of total DNA
Total DNA of the strains was extracted as described by Kaschuk et al. (2006). To obtain clean DNA, the extraction procedure included the addition, for each $400\,\mu$L of bacteria resuspended in TE 50/20, of $50\,\mu$L of 10% SDS, $5\,\mu$L of proteinase-K ($20\,mg\,mL^{-1}$), $10\,\mu$L of lysozyme ($5\,mg\,mL^{-1}$), and $2\,\mu$L of RNAse ($10\,mg\,mL^{-1}$). After two steps of purification with ethanol at 99.5% and at 70%, the pellet was resuspended in $50\,\mu$L of TE $\frac{10}{1}$ to estimate the concentration of the DNA. Samples were then diluted to 20 ng of DNA μL^{-1} and kept at $-20\,^{\circ}$C.

2.3.2. rep-PCR fingerprinting with BOX, ERIC and REP primers
PCR amplification of repetitive regions of the DNA (rep-PCR) was carried out with BOX-A1R (5′-CTACGG-CAAGGCGACGCTGACG-3′) (Versalovic et al., 1994), enterobacterial repetitive intergenic consensus (ERIC) (ERIC1R, 3′-CACTTAGGGGTCCTCGAATGTA-5′; and ERIC2, 5′-AAGTAAGTGACTGGGGTGAGCG-3′), and repetitive extragenic palindromic (REP) (REP1R, 3′-CGGIC-TACIGCIGCIIII-5′; and REP2-I, 5′-CGICTTATCI GGCCTAC-3′) (de Bruijn, 1992) primers. DNA fingerprintings with the BOX primer were obtained following the procedure described by Kaschuk et al. (2006), while the amplification with ERIC and REP primers was performed as described by Santos et al. (1999). The reactions were performed in an MJ Research Inc. PT 200 thermocycler. The amplified fragments were separated by electrophoresis on 1.5% agarose (low EEO, type I-A) gels (20×25 cm), at 120 V for 7 h. The 1 kb Plus DNA Ladder (Invitrogen^{TM}) was used as a molecular size marker on the right, left and central lanes of each gel. The gels were stained with ethidium bromide and photographed under UV radiation with a Kodak Digital

Science 120 apparatus. The profiles obtained were confirmed in triplicate.

2.3.3. Restriction fragment length polymorphism (RFLP) of the PCR-amplified DNA region coding for the 16S rRNA gene
The DNA of each strain was amplified with the universal primers rD1 (3′-AAGGAGGTGATCCAGCC-5′) and fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′), which correspond to positions 8–27 and 1524–1540, respectively, of Escherichia coli strain K12 (Weisburg et al., 1991). Volumes and cycles used in the reaction were as described before (Germano et al., 2006). Six μL aliquots of the PCR products were then digested separately with each of the following restriction enzymes: HpaII (= MspI) (5′-C/CGG-3′), HhaI (= CfoI) (5′-GCG/C-3′), DdeI (5′-C/TNAG-3′) and HaeIII (5′-GG/CC-3′) (Invitrogen^{TM}), as recommended by the manufacturers. The fragments obtained were analyzed by electrophoresis in a 3% agarose gel (17×11 cm) at 120 V for 4 h. The 1 kb Plus DNA Ladder (Invitrogen^{TM}) was used as a molecular marker in the right, left and central lanes of each gel. Gels were stained with ethidium bromide and photographed under UV radiation with a Kodak Digital Science 120 apparatus. The profiles obtained were confirmed twice.

2.3.4. RFLP-PCR of the DNA region coding for the 23S rRNA gene
The DNA of each strain was amplified with primers P3 (5′-CCGTGCGGGAAAGGTCAAAAGTAC-3′) and P4 (5′-CCCGCTTAGTGCTTTCAGC-3′) (Terefework et al., 1998), and the volumes and the cycles were performed as described by Germano et al. (2006). Six μL aliquots of the PCR products were digested with three restriction enzymes, HhaI, HaeIII and HinfI (5′-GANT/C-3′) (Invitrogen^{TM}), as recommended by the manufacturers. The fragments obtained were visualized and photographed as described in item 2.3.3, and the profiles obtained were confirmed twice.

2.3.5. RFLP-PCR of the DNA region coding for the nifH and nodC genes
For the nifH region, the DNA of each strain was amplified with primers nifHF (5′-TACGGNAARGGS-GGNATCGGCAA-3′) and nifHI (5′-AGCATGTCYTC-SAGYTCNTCCA-3′) (Laguerre et al., 2001), as described before (Grange et al., 2007), resulting in a product of approximately 780 bp. Amplification of the nodC region was achieved using nodCF (5′-AYGTHGTYGAYGA-CGGTTC-3′) and nodCI (5′-CGYGACAGCCANTCKC-TATTG-3′) primers (Laguerre et al., 2001), also as described by Grange et al. (2007), and resulting in a fragment of about 930 bp. After amplification, $6\,\mu$L aliquots of the PCR products were digested separately with the following restriction enzymes (Invitrogen^{TM}): HpaII, HaeIII and MboII (= NdeI) for the nifH DNA region and HpaII, HaeIII, MboII, RsaI and HinfI for the nodC DNA region, as recommended by the manufacturers.

The fragments obtained were visualized and photographed as described in item 2.3.3, and the profiles obtained were confirmed twice.

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

To obtain the full-length portion of the 16S rDNA (approximately 1500 bp), five reactions were carried out with the primers described by Menna et al. (2006) (fD1 (Weisburg et al., 1991, item 2.3.3); Y2 (5′-CCCACTGCTGCCTCCCG-TAGGAGT-3′, Young et al., 1991); 362f (target region 339–362) (5′-CTCCTACGGGAGGCAGCAGTGGGG-3′); 786f (target region 764–786) (5′-CGAAAGCGTGGGGAG-CAAACAGG-3′); and 1203f (target 1179–1203) (5′-GAGGTGGGGATGACGTCAAGTCCTC-3′)). Reactions and sequencing analyses were carried out using the same procedures as described before (Menna et al., 2006). Sequencing was performed in a MegaBACE 1000 DNA Analysis System (Amersham Biosciences), with the dye terminator chemistry.

2.3.7. Plasmid profiles

The plasmid profiles were obtained as described previously by Eckhardt (1978), with modifications. Cells were grown in 2 mL of PY liquid medium at 28 °C to an optical density of 0.3 at 600 nm and 200 μL were pelleted by centrifugation (12,000 rev min^{-1}, 4 °C, 1 min). After that, 900 μL of N-lauroylsarcosine 0.3% were added and centrifuged again. The cells were lyzed by adding 20 μL of saccarose solution 10% (with RNAse 0.4 mg/mL; lysozyme 2 μg/mL). The plasmids were separated by electrophoresis on 0.8% agarose (low EEO, type I-A) gels (12 × 25 cm), at 10 V for 12 h. R. etli strain CFN 42 was used as a molecular size reference.

2.4. Data analyses

2.4.1. DNA profiles obtained in the rep-PCR and RFLP-PCR analyses

The sizes of the fragments in each analysis were normalized according to the MW of the DNA markers. The fingerprintings obtained in the RFLP-PCR and in the rep-PCR analyses were analyzed using BioNumerics software (Applied Mathematics, Kortrijk, Belgium, Version 1.50), setting up a position tolerance of 2%. In the RFLP-PCR analysis, the analysis was performed first with the profiles obtained with each restriction enzyme and then with the combined profiles obtained with all restriction enzymes. The analyses were performed using the un-weighted pair-grouping method with arithmetic mean (UPGMA) algorithm (Sneath and Sokal, 1973) with the coefficient of Jaccard (1912).

2.4.2. Analyses of the complete sequences of the 16S rRNA genes

The high-quality sequences obtained for each strain were assembled into contigs using the phred, phrap and consed

programs as described before (Menna et al., 2006). 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.

Of the Brazilian strains, the following have been already sequenced: R. tropici strains PRF 81 (AF260274), PRF 35 (AF260298) (Chueire et al., 2003), and PRF 77 (AY117667) (Grange and Hungria, 2004). Strains H 12, H 20, H 52, H 53 have been partially sequenced before, but have not been deposited in the GenBank database. Therefore, the full sequences for the strains were obtained, submitted to the GenBank database and received the accession numbers EF054889, EF054890, EF054891 and EF054892, respectively. A partial sequence of strain 233 (AY117622) was known (Grange and Hungria, 2004); in this study, sequencing of the entire gene was achieved and the database was updated. Sequences coding for the entire 16S rRNA genes of the strains 130, 131 and 141 were obtained and when submitted to the GenBank database received the accession numbers EF054884, EF054885 and EF054886, respectively. Finally, accession numbers EF054887, EF054883, EF054888 and EF054882 were given to strains CPAO 2.11, CPAO 12.5, CPAO 29.8 and CPAO 1135, respectively.

Multiple alignments were performed with ClustalX version 1.83 (Thompson et al., 1997). Phylogenetic trees were generated using MEGA version 3.1 (Kumar et al., 2004) with default parameters, K2P distance model (Kimura, 1980) and the Neighbor-Joining algorithm (Saitou and Nei, 1987), using Azospirillum brasilense strain A154 (DQ104848) as an outgroup reference for the 16S rRNA phylogeny. Statistic supports for tree nodes were evaluated by bootstrap analysis (Felsenstein, 1985) with 2000 samplings (Hedges, 1992).

The sequences obtained were aligned and compared to those of the following type/reference strains (accession numbers of the GenBank Data Library in parentheses): R. etli CFN 42^T (U28916); R. tropici (type A) CFN 299 (X98514); R. tropici (type B) CIAT 899^T (U89832); R. leguminosarum bv. phaseoli USDA 2671 (U29388); R. leguminosarum bv. viciae USDA 2370^T (U29386); R. leguminosarum bv. trifolii ATCC 14480 (AY509900); R. giardinii bv. giardinii strain H152^T (U86344); R. gallicum bv. gallicum strain R602^T (U86343); and R. rhizogenes (formally Agrobacterium rhizogenes) ATCC 11325 (AY945955).

3. Results

3.1. Morpho-physiological characterization

All Brazilian and reference/type common bean rhizobial strains used in this study formed circular colonies with regular borders, flat in elevation, opaque in color, showing intermediate to high production of mucus. After 3 to 5 days of growth on YMA at 28 °C, all strains acidified the medium (as indicated by the bromothymol blue) and colony diameter ranged from 2 to 5 mm (data not shown).

Three out of the four strains from the state of Mato Grosso do Sul (MS) (CPAO 2.11, CPAO 12.5 and CPAO 29.8), as well as strains 77 (from PE), 130, 141 and PRF 35 (from PR) were unable to grow in LB or PY minus Ca media (Table 1), typical characteristics of all common bean rhizobial species except for the great majority of *R. tropici* type B strains (Martínez-Romero et al., 1991). Three other strains from PR (PRF 81, 131, 233) and all strains from the FD grew poorly on both media, giving colony sizes of only approximately 10% of those observed for CIAT 899[T]. In relation to the growth at 40 °C and in medium of pH 4.0, the great majority of the Brazilian strains grew as well as *R. tropici* type B strain CIAT 899[T]. The exceptions were poorer growth of strains 77 and 141, similar to *R. tropici* type A strain CFN 299, and the absence of growth of strain 130, as observed for all other common bean rhizobial species (Table 1).

3.2. Analysis of combined BOX, ERIC and REP-PCR genomic fingerprints

A high level of genetic diversity was observed in the *rep*-PCR fingerprintings (Fig. 2). When the combined profiles obtained with the three sets of primers (BOX, ERIC and REP) were submitted to cluster analysis using the UPGMA algorithm and the coefficient of Jaccard, five groups emerged, joined at a final level of similarity of 46%, with

each strain identified by a unique combination of profiles. Group I clustered with a similarity of 52%, containing *R. tropici* type B strain CIAT 899[T] with seven Brazilian strains from the central (DF), central-western (MS) and southern regions (PR). The second cluster joined the three reference strains of *R. leguminosarum* with strain 130 (PR), with a similarity of 67%. Type strains CFN 42[T] and R602[T] had a low similarity of 48% in the third group. The fourth cluster included strain CFN 299 of *R. tropici* with the three CPAO strains from MS and with strains 77 (from PE) and 141 (from PR), with a similarity of 57%. Finally, strains 131 and 233, both from PR, were linked to *R. giardinii* H152[T] in cluster V, also with a similarity of 57% (Fig. 2).

3.3. RFLP-PCR of the 16S rDNA and 23rDNA regions

The DNA amplification with the primers for the 16S rDNA region resulted in a single product of about 1500 bp for 17 strains, while the PCR product obtained with five Brazilian strains (77, 141, CPAO 2.11, CPAO 12.5 and CPAO 29.8), as well as with *R. tropici* type A CFN 299 had about 1600 bp (data not shown). Five combinations of profiles were obtained when the products were digested with four restriction enzymes (Table 2, Fig. 3). The five Brazilian strains mentioned above also shared similar profiles as strain CFN 299, while nine others were similar to *R. tropici* type B CIAT 899[T]. The only Brazilian
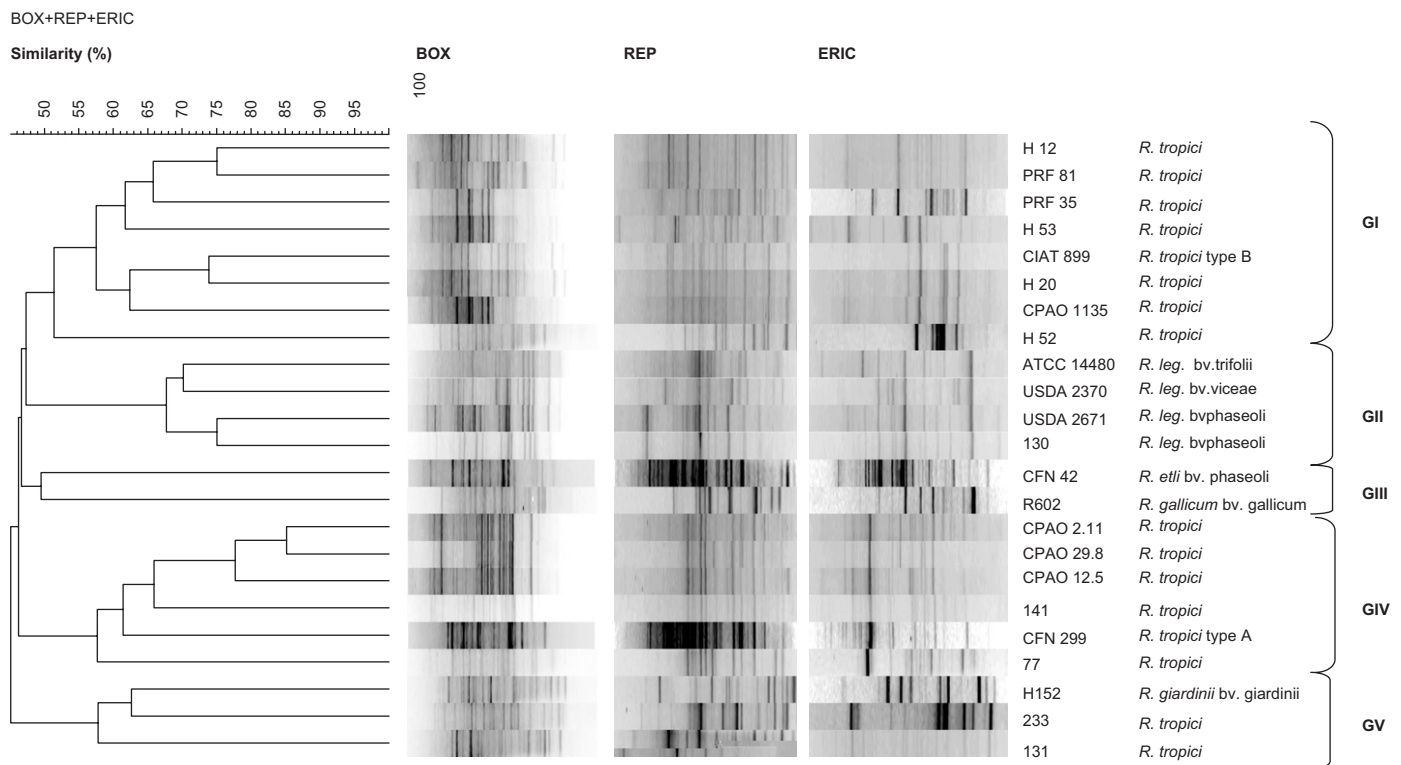

Fig. 2. Cluster analysis (UPGMA algorithm with the Jaccard coefficient, converted to percentage similarity) of products obtained by *rep*-PCR (primers BOX, REP, ERIC) analysis of selected Brazilian strains effective in fixing N$_2$ with common bean and of reference/type strains of described rhizobial species that are microsymbionts of this legume.

Table 2
Profiles of plasmids and of RFLP-PCR[a] of the ribosomal 16S rDNA and 23S rDNA regions and of the symbiotic *nifH* and *nodC* rDNA regions

Strain	Species	Plasmid profile	16S rDNA				23S rDNA			NifH			NodC				
			*Dde*I	HhaI	HpaII	HaeIII	HaeIII	*Hha*I	HinfI	HaeIII	*Hpa*II	MboI	HaeIII	*Mbo*I	RsaI	HinfI	HpaII
CFN 299	*R. tropici* type A	P1	A[a]	A	A	A	A	A	A	A	A	A	A	A	A	A	A
77	*R. tropici*	P2	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
141	*R. tropici*	P3	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
CPAO 2.11	*R. tropici*	P2	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
CPAO 12.5	*R. tropici*	P2	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
CPAO 29.8	*R. tropici*	P2	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
CIAT 899[T]	*R. tropici* type B	P2	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A
CPAO 1135	*R. tropici*	P6	B	**B**	B	B	A	B	A	A	A	A	A	A	A	A	A
H 12	*R. tropici*	P2	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A
H 20	*R. tropici*	P2	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A
131	*R. tropici*	P2	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A
233	*R. tropici*	P2	B	B	B	B	A	B	A	A	A	A	A	A	A	A	A
H 52	*R. tropici*	P5	B	B	B	B	A	B	A	A	A	A	A	A	A	A	A
H 53	*R. tropici*	P5	B	B	B	B	A	B	A	A	A	A	A	A	A	A	A
PRF 35	*R. tropici*	P3	B	B	B	B	A	B	A	A	A	A	A	A	A	A	A
PRF 81	*R. tropici*	P4	B	B	B	B	A	B	A	A	A	A	A	A	A	A	A
130	*R. leg.* bv. phaseoli	P7	B	C	C	B	C	A	B	B	B	B	D	D	D	D	D
USDA 2671	*R. leg.* bv. phaseoli	P7	B	C	C	B	C	A	B	B	B	B	D	D	D	D	D
USDA 2370[T]	*R. leg.* bv. viciae	P8	B	C	C	B	C	A	B	D	D	D	B	B	B	B	B
ATCC 14480	*R. leg.* bv. trifolii	P10	B	C	C	B	C	A	B	C	C	C	C	C	C	C	C
CFN 42[T]	*R. etli* bv. phaseoli	P11	B	C	C	C	D	C	B	B	B	B	D	D	D	D	D
R602[T]	*R. gallicum* bv. gallicum	P9	B	C	C	C	A	A	B	E	E	E	E	E	E	E	E
H152[T]	*R. giardinii* bv. giardinii	P4	C	C	B	B	A	C	C	no *nifH*			F	F	F	F	F

The analyses were performed with Brazilian selected strains very effective in fixing N$_2$ with common bean and reference/type strains of the described species microsymbionts of this legume.

[a]Identical patterns are designated by the same letter for each restriction enzyme. Profiles for the RFLP-PCR of 16S and 23S rRNA genes are shown in Fig. 3, of *nodC* and *nifH* genes in Fig. 6, and of the plasmid profiles in Fig. 7.

rhizobium that did not share similar profiles with *R. tropici* was strain 130, with profiles similar to *R. leguminosarum*. Enzyme *Hha*I allowed the identification of all *R. tropici*, and the combination of only three restriction enzymes (*Dde*I, *Hae*III and *Hha*I or *Hpa*II) allowed the identification of four out of the five rhizobial species. However, four restriction enzymes did not allow differentiation between *R. etli* and *R. gallicum* (Table 2).

A unique fragment of ~2.3 kb was obtained for all common bean rhizobial strains when the DNAs were amplified with the primers for the 23S rDNA region (data not shown). After the digestion of the PCR fragments with three restriction enzymes, seven combinations of profiles were obtained (Table 2, Fig. 3). All five rhizobial species were identified by unique combinations of profiles. Similar to what has been observed for the RFLP-PCR of the 16S rDNA, *R. tropici* type A resulted in a different combination of profiles than type B CIAT 899[T]. However, for the 23S rRNA region, restriction enzyme *Hae*III resulted in different profiles for type B strains, one including strain CIAT 899[T] and three Brazilian strains (H 12, H 20 and 131) and another with six other Brazilian strains (CPAO 1135, 233, H 52, H 53, PRF 35 and PRF 81) (Table 2). The cluster analysis of the RFLP-PCR products of the 16S rDNA and 23S rDNA regions indicates high variability of the ribosomal genes within the species of *R. tropici* (Fig. 4).

3.4. Phylogeny based on the 16S rRNA gene

Almost full sequences of the 16S rRNA genes were obtained for all strains and 1450 bp were considered in the multiple alignment analysis to build a phylogenetic tree. All strains that shared similar RFLP-PCR profiles with *R. tropici* were joined in one cluster, strongly supported by the bootstrap analysis (99%), that also included *R. rhizogenes* strain ATCC 11325 (Fig. 5). However, within this cluster, *R. tropici* type A strains were positioned in a different subcluster with a bootstrap support of 100%. The subcluster with the other *R. tropici* strains was more diverse and four Brazilian strains (PRF 35, H 52, H 53 and PRF 81) were split into another group. Sequencing analysis confirmed the similarity of nucleotides of strain 130 with *R. leguminosarum*, and none of the Brazilian strains was clustered with *R. gallicum* or *R. etli* (Fig. 5). Finally, the sequencing analysis strongly supported the results obtained in the RFLP-PCR analysis (Table 2).

3.5. RFLP-PCR of the DNA region coding for the nifH and nodC genes

The amplification reaction with *nifH* primers produced a fragment of about 780 bp for all strains except for *R. giardinii* bv. *giardinii* H152[T] (data not shown), as expected, since this biovar probably lacks *nifKDH* genes
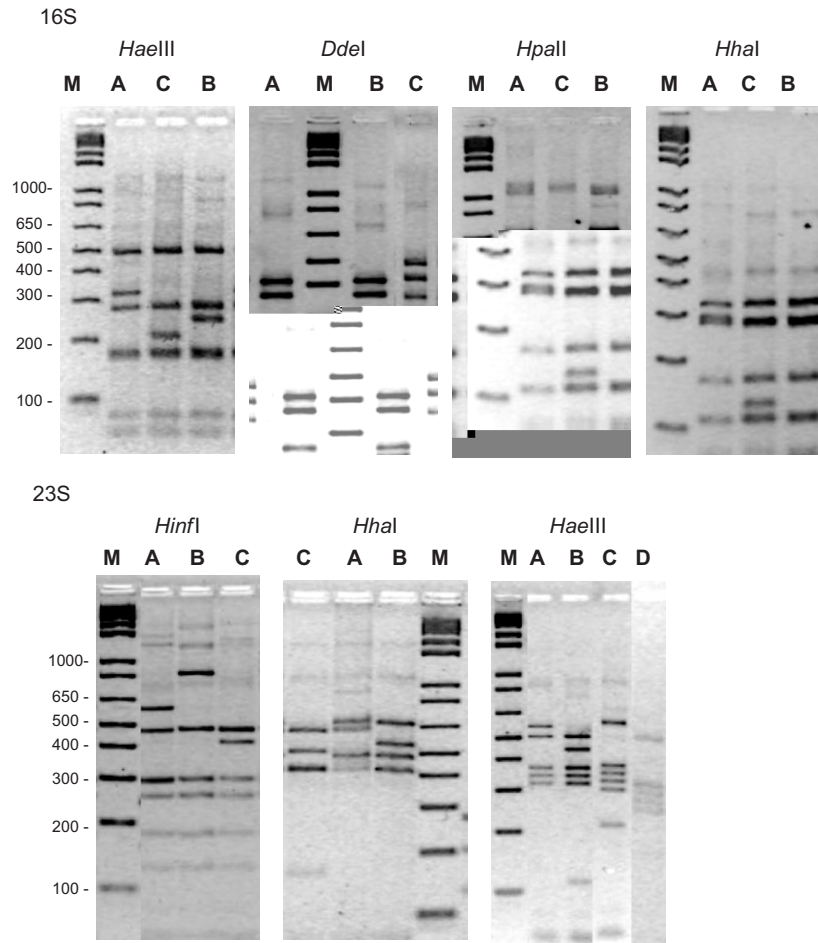
Fig. 3. RFLP-PCR profiles of the 16S and 23S rDNA regions of common bean rhizobial strains. (M) represents the 1 kb Plus DNA Ladder (Invitrogen™) and the letters represent the profiles obtained with each restriction enzyme, as described in Table 2.

(Geniaux et al., 1993; Amarger et al., 1997). When the PCR products were digested with three restriction enzymes, a combination of six profiles was obtained, one for each species or biovar included in the study (Table 2, Fig. 6); furthermore, the *nifH* pattern of each species was recognized by the use of only one restriction enzyme (Table 2).

The DNA amplification with primers for the *nodC* gene resulted in a single product of about 930 bp for all strains including *R. giardinii*. When the PCR products were digested with five restriction enzymes, six different combinations of profiles were obtained (Fig. 6). Once more all *R. tropici* strains shared similar profiles, and contrarily to the results obtained with *nifH*, *R. etli* and *R. leguminosarum* bv. phaseoli showed similar profiles of RFLP-PCR of the *nodC* gene (Table 2).

3.6. Plasmid profiling

Eleven plasmid profiles were detected (Fig. 7), consisting of three to four plasmids for each strain, except for *R. etli* strain CFN 42[T], characterized by six plasmids. Six plasmid profiles could be discerned among the 15 bean strains

(Table 2; Fig. 7). The most abundant profile (P2) was shared by *R. tropici* type B CIAT 899[T], together with the four Brazilian strains (77, CPAO 2.11, CPAO 12.5, CPAO 29.8) that have shown similar ribosomal genes as CFN 299 and with four (H 12, H 20, 131, 233) of the nine strains that shared similarity with CIAT 899[T] (Table 2). Similar plasmid profiles were observed for strain 130 and *R. leguminosarum* bv. phaseoli USDA 2671 (Table 2).

4. Discussion

Common bean is native to the Americas (Gepts, 1990; Kami et al., 1995), and despite being one of the most promiscuous hosts in its symbiotic associations with rhizobia (Michiels et al., 1998; Herrera-Cervera et al., 1999), there is evidence that *R. etli* bv. phaseoli is the dominant microsymbiont in both the Mesoamerican and the Andean centers of genetic diversification (Kipe-Nolt et al., 1992; Segovia et al., 1993; Souza et al., 1994; Aguilar et al., 1998, 2004; Bernal and Graham, 2001). However, there is archeological evidence that for centuries seeds were exchanged among American Indian populations, resulting from trade and migration (e.g., Prous, 1986; Freitas, 2006),
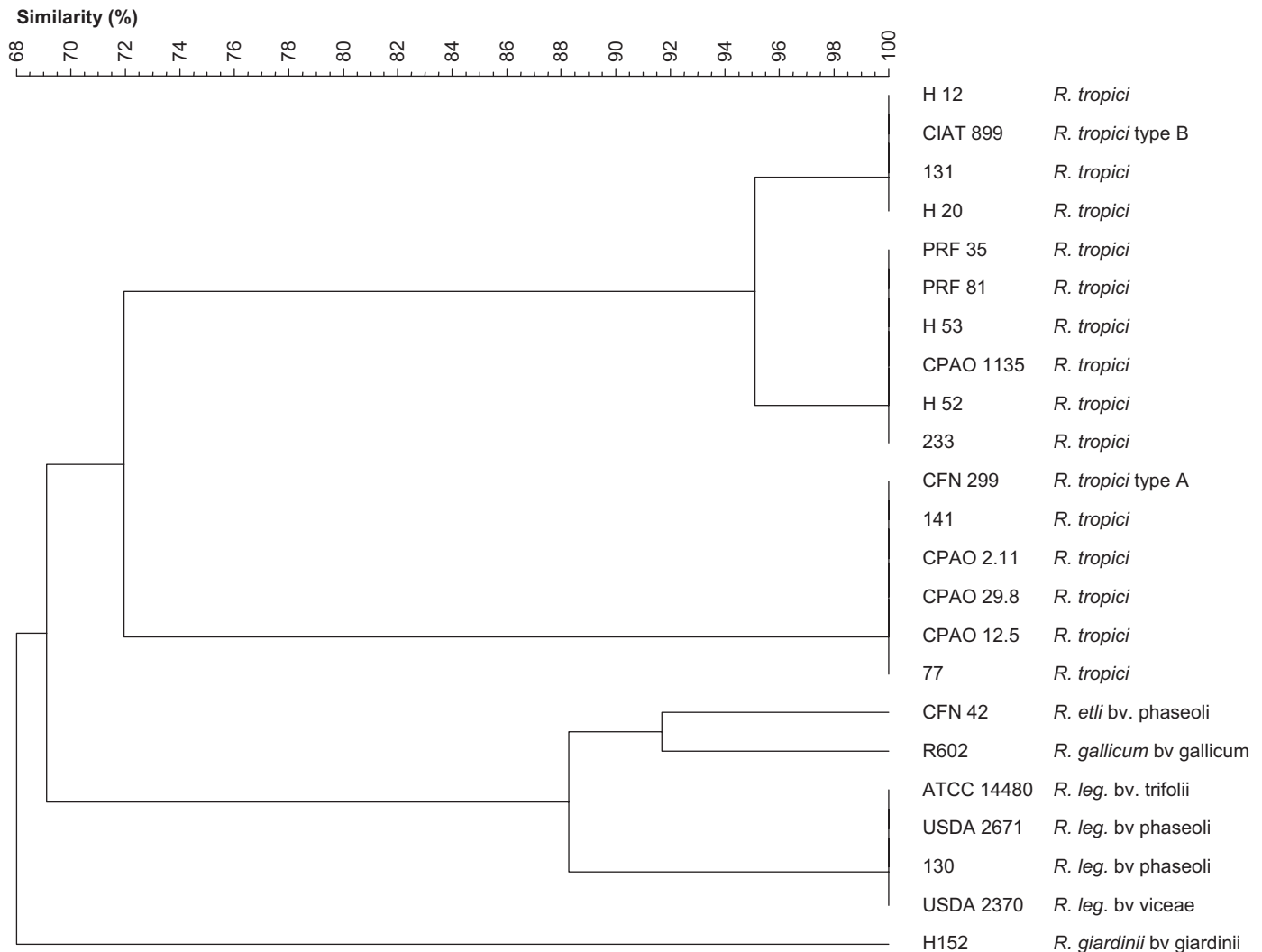
Similarity (%)

Strain	Species
H 12	*R. tropici*
CIAT 899	*R. tropici* type B
131	*R. tropici*
H 20	*R. tropici*
PRF 35	*R. tropici*
PRF 81	*R. tropici*
H 53	*R. tropici*
CPAO 1135	*R. tropici*
H 52	*R. tropici*
233	*R. tropici*
CFN 299	*R. tropici* type A
141	*R. tropici*
CPAO 2.11	*R. tropici*
CPAO 29.8	*R. tropici*
CPAO 12.5	*R. tropici*
77	*R. tropici*
CFN 42	*R. etli* bv. phaseoli
R602	*R. gallicum* bv gallicum
ATCC 14480	*R. leg.* bv. trifolii
USDA 2671	*R. leg.* bv phaseoli
130	*R. leg.* bv phaseoli
USDA 2370	*R. leg.* bv viceae
H152	*R. giardinii* bv giardinii

Fig. 4. Cluster analysis (UPGMA algorithm with the Jaccard coefficient, converted to percentage similarity) of products obtained by RFLP-PCR analysis of the 16S rRNA and 23S rRNA genes with four and three restriction enzymes, respectively. The dendrogram includes Brazilian selected strains very effective in fixing N_2 with common bean and reference/type strains of described rhizobial species that are microsymbionts of this legume.

likely carrying viable cells of *R. etli* (Pérez-Ramírez et al., 1998; Andrade and Hungria, 2002). Later, with colonization by Europeans, seeds of common bean—probably carrying *R. etli*—were introduced into Europe, and the symbiotic plasmid transferred to local *R. leguminosarum* bv. phaseoli (Segovia et al., 1993), and then to *R. gallicum* bv. phaseoli (Amarger et al., 1997).

The origin of the symbiotic association of common bean with *R. tropici* is not as clear as that with *R. etli*. One hypothesis is that *R. tropici* is indigenous to South America (Martínez-Romero et al., 1991), and Brazil is a strong candidate, representing the source of the great majority of the strains isolated so far (Hungria et al., 1993, 1997, 2000; Mercante et al., 1998; Straliotto et al., 1999; Andrade et al., 2002; Mostasso et al., 2002; Grange and Hungria, 2004; this study). However, in view of the fact that wild common beans are not found in Brazil (Debouck, 1986), *R. tropici* could be a microsymbiont of other indigenous host legumes, and possible candidates are species of the genera

Mimosa and *Gliricidia*, reported to establish very effective symbioses with *R. tropici* under field conditions (Menna et al., 2006). It is noteworthy that *R. tropici* has also been reported as a microsymbiont of indigenous legumes in Mexico, *Gliricidia* (Acosta-Durán and Martínez-Romero, 2002) and in Africa, *Bolusanthus* and *Spartium* (Dagutat and Steyn, 1995). It should also be considered that the presence of *R. tropici* as the microsymbiont of common bean plants in other countries (e.g., Amarger et al., 1994; Anyango et al., 1995) could also be explained by trade or migration, e.g., common bean seeds may have carried from Brazil to Africa in the 16th century. Finally it should be mentioned that the former genus *Agrobacterium* (now *Rhizobium*) comprises well known plant-pathogenic bacterial species closely related to symbiotic *Rhizobium*, more specifically to *R. tropici* (Gaunt et al., 2001; Young et al., 2001); furthermore, there are reports of N_2-fixing isolates from soybean nodules in South America resembling agrobacteria (Chen et al., 2000; Hungria et al., 2006).
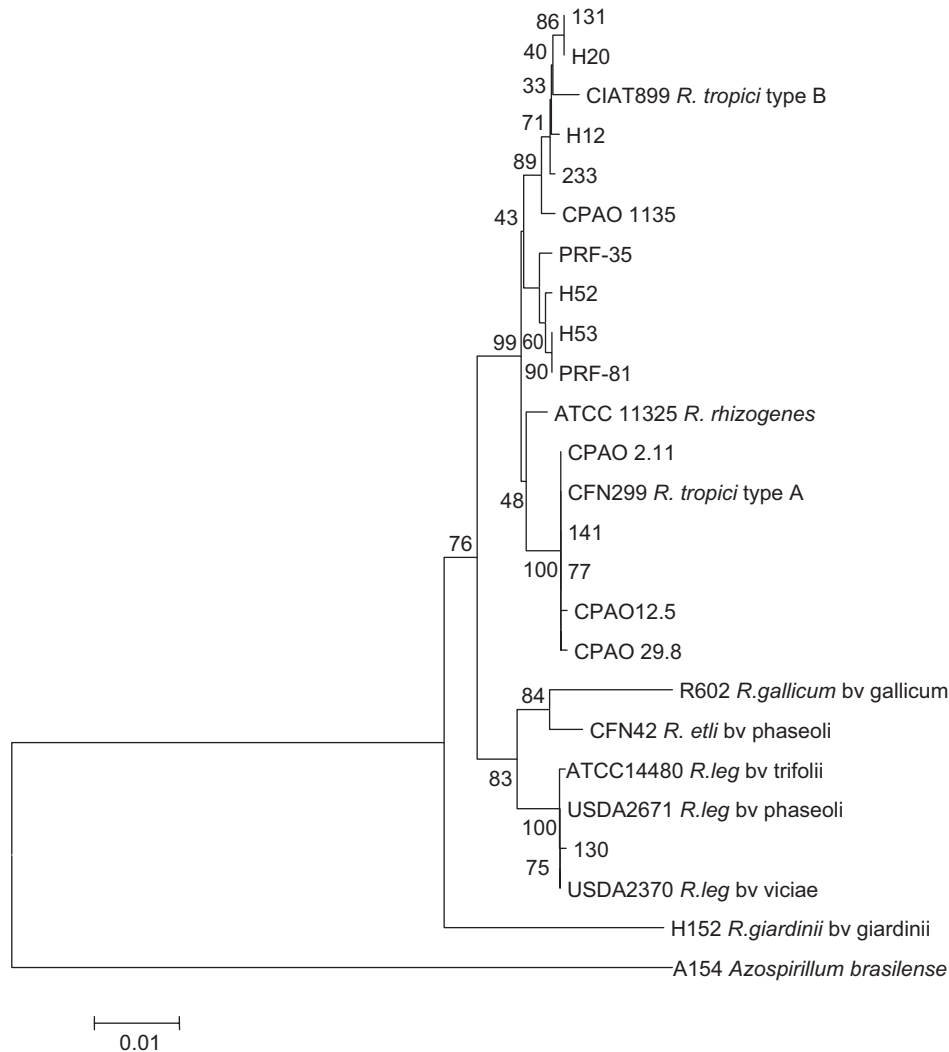
Fig. 5. Phylogenetic tree based on the 16S rRNA sequences of the Brazilian strains and of the reference/type strains of described rhizobial species that are microsymbionts of common bean. *Rhizobium rhizogenes* (former classified as *Agrobacterium*) was included in the analysis and *Azospirillum brasilense* was used as an outgroup strain. GenBank accession numbers are given in Section 2. The tree was generated using MEGA version 3.1 with default parameters, K2P distance model and the Neighbor-Joining algorithm.

Therefore, the genetic similarity of conserved genes between *R. tropici* and the former *A. rhizogenes* is noteworthy in studies of evolution towards pathogenesis or symbiosis.

In our study, 15 common bean rhizobia very effective in fixing N_2, isolated in four surveys performed in different regions of Brazil (Hungria et al., 2000; Mostasso et al., 2002; Grange and Hungria, 2004; this study), were genetically characterized in relation to conserved and symbiotic genes. Based on the RFLP-PCR analysis of the 16S and 23S rRNA genes, and on the sequencing of the 16S, all strains but one (130) were classified as *R. tropici*. A first conclusion is that the results confirm that the species is capable of establishing effective symbioses with common bean in a variety of Brazilian ecosystems, from the humid subtropics in the southern region, through the savannah-type climate of the central and central-western regions, to the semi-arid climate of the northeast. Two factors may

have contributed to the broad association of *R. tropici* with common bean in Brazil. First, the broadly infective nature of *R. tropici*, and its high capacity to establish effective symbioses even with exotic newly introduced crops, e.g., leucaena (Mercante et al., 1998) and soybean (Hungria et al., 2006). Second, the intrinsic properties of *R. tropici* of high competitiveness and genetic stability under the acidic-soil (Hungria and Stacey, 1997) and high-temperature (Hungria and Franco, 1993; Hungria et al., 1993; Sá et al., 1993; Pinto et al., 1998; Amaral and Baldani, 1999; Raposeiras et al., 2002) conditions that predominate in Brazil.

One major feature of the Brazilian *R. tropici* strains in our study was their high variability in ribosomal genes. Based on the 16S rRNA sequence, on the rRNA operon, and on the DNA–DNA hybridization (values lower than 36%), it has been suggested that *R. tropici* species could be separated in two subgroups, namely types A and B
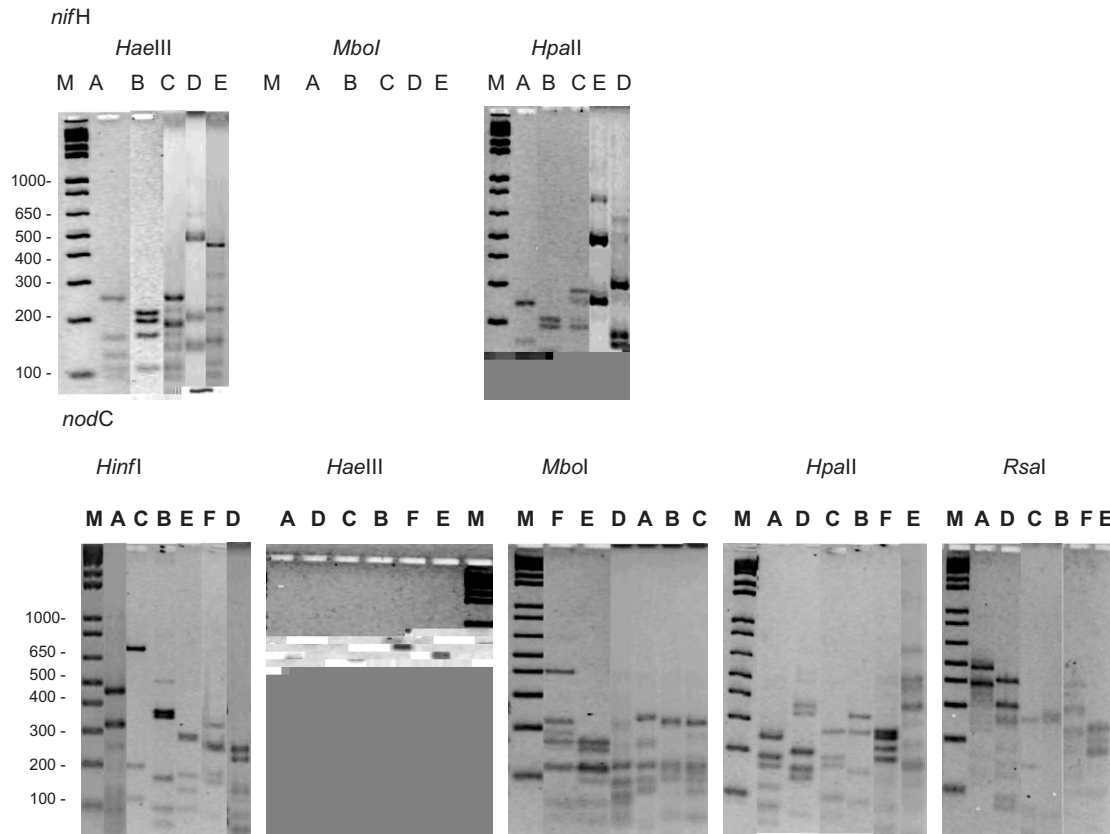
Fig. 6. RFLP-PCR profiles of the DNA regions coding for *nif*H and *nod*C genes of common bean rhizobial strains. (M) represents the 1 kb Plus DNA Ladder (Invitrogen[TM]) and the letters represent the profiles obtained with each restriction enzyme, as described in Table 2.
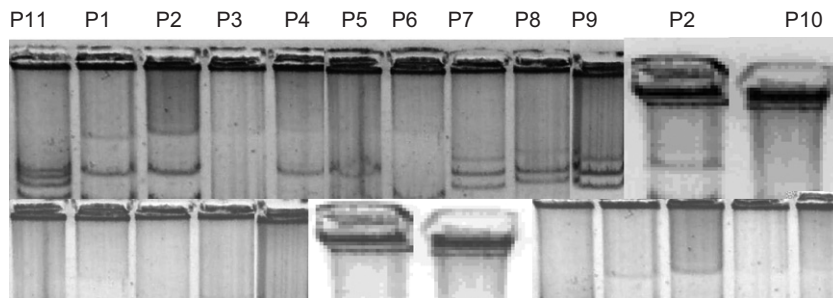

Fig. 7. Plasmid profiles of Brazilian selected strains effective in fixing N$_2$ with common bean and of reference/type strains of described rhizobial species that are microsymbionts of this legume. The numbers represent the profiles obtained, as described in Table 2.

(Martínez-Romero et al, 1991; Laguerre et al. 1994; Geniaux et al., 1995). Among several intrinsic characteristics of type A group, Willems and Collins (1993) and van Berkum et al. (1994) reported an insertion of 72 nucleotides in the 16S rRNA genes, although later Laguerre et al. (1994) found that not all strains shared this characteristic. In our study, five strains, from the states of PE, PR and MS were clustered together in the RFLP-PCR analysis (Fig. 4) and showed complete identity of bases of the 16S rRNA gene (Fig. 5) with type A strain CFN 299—apparently also a Brazilian strain (Martínez et al., 1987; Navarro et al., 1993; van Berkum et al., 1996)—and also had the inserted nucleotides. Furthermore, the cluster of the 16S rRNA

sequences of type A strains was dissimilar to that including type B strain CIAT 899[T].

The variability in the ribosomal genes observed in the type B strains was very high. In both the RFLP-PCR of the 16S and of the 23S rRNA and in the sequencing analysis of the 16S rRNA gene, strain CIAT 899[T] was positioned in a first subcluster, together with two strains from the state of PR, one from MS and two from the Federal District (DF). Those strains shared similar profiles in RFLP-PCR of both 16S and 23S rRNA genes and differed by 0.5% to 0.63% of nucleotides in comparison to CIAT 899[T]. Additionally, a second subcluster was observed, which included two strains from the state of PR and two from the Federal District,

very dissimilar from subcluster I, with strains differing in up to 0.89% of nucleotides in comparison to CIAT 899[T]. Therefore, one major conclusion is that our work reinforces the concept that type A strains represent a new species. However, the intrinsic properties of types A and B strains should be re-evaluated, as at least the Brazilian strains showed mixed morphological and physiological characteristics of both types (Hungria et al., 2000; this study). Additionally, our work also showed that all but one of the Brazilian strains share similarity of ribosomal genes with type A strain CFN 299 and had plasmid profiles similar to that of type B CIAT 899[T] (Table 2).

High intraspecies diversity among the Brazilian R. tropici strains was also revealed by the analysis with the consensus sequences BOX, ERIC and REP (Fig. 2), capable of amplifying repetitive and conservative elements diffused in DNA. rep-PCR profiles may be very useful for strain identification, although often not for phylogenetic characterization (Laguerre et al., 1996; Mostasso et al., 2002; Grange and Hungria, 2004; Kaschuk et al., 2006), and in our study each strain was characterized by a unique combination of rep-PCR profiles.

In contrast to the variability observed in conserved genomic regions, all Brazilian R. tropici strains shared similar RFLP-PCR nifH and nodC profiles (Fig. 6), indicating that the genes might be closely related. nifH genes may be strongly conserved, and less variability than with the ribosomal genes has been observed before, e.g., for Bradyrhizobium japonicum (Batista et al., 2007); the similarity could be related, for example, to the conserved structure of the nitrogenase iron protein. In relation to the similarity in nodC genes, it is possible that rhizobia showing closely related nod genes produce similar Nod factors more effective for specific common bean receptors, enabling nodulation. Finally, similarity of nifH and nodC genes might indicate horizontal gene transfer of ancestral common bean rhizobia to indigenous R. tropici, a subject that deserves further investigation.

Although both R. etli and R. leguminosarum are broadly present in Brazilian soils (Straliotto et al., 1999; Andrade et al., 2002; Mostasso et al., 2002; Grange and Hungria, 2004; Grange et al., 2007), when very effective rhizobia were isolated from common bean field-grown plants, R. tropici represented the majority of the strains (e.g., Hungria et al., 2000, 2003; Mostasso et al., 2002; this study). Strong competitiveness, tolerance of stressful environmental conditions and genetic stability of symbiotic genes in R. tropici make it a good choice for use in inoculants for application to common bean in Brazil. Two R. tropici strains (PRF 81 and H 12) have already been recommended for use in commercial inoculants, supporting high grain yields (Hungria et al., 2000, 2003). It is noteworthy that in Brazilian studies, even in soils with high population of indigenous common bean rhizobia, composed of several species and strains, increases in nodulation and N₂ fixation by the inoculation with selected R. tropici strains is still obtained. That strategy might be considered in other countries showing similar soil and environmental conditions, e.g., in Africa. The relevance of our study lies in the addition of genetic information on Brazilian R. tropici very effective in fixing N₂. Our results highlight an intriguing characteristic of the strains: high variability in ribosomal genes supports the theory that the current R. tropici comprises two species, and high similarity in nifH and nodC genes may reflect an evolutionary advantage maximizing N₂ fixation.

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