ELSEVIER

Soil Biology & Biochemistry 36 (2004) 1389–1398

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Genetic diversity of indigenous common bean (*Phaseolus vulgaris*) rhizobia in two Brazilian ecosystems

Luciana Grange[1], Mariangela Hungria*

Embrapa Soja, Cx. Postal 231, 86001-970, Londrina, PR, Brazil

Received 25 March 2003; received in revised form 27 February 2004; accepted 1 March 2004

Abstract

Although rhizobia for common bean (*Phaseolus vulgaris* L.) are established in most Brazilian soils, understanding of their genetic diversity is very poor. This study characterized bean strains from two contrasting ecosystems in Brazil, the Northeast Region, with a semi-arid climate and neutral soils and the South Region, with a humid subtropical climate and acid soils. Seedlings of the cultivars Negro Argel and Aporé were used to trap 243 rhizobial isolates from 12 out of 14 sites. An analysis of ERIC-PCR products revealed enormous variability, with 81% of the isolates representing unique strains considering a level of 70% of similarity. In general, there was no effect of either the bean cultivar, or the ecosystem on rhizobial diversity. One-hundred and one strains showing genetic relatedness (ERIC-PCR) less than 70% were further analyzed using restriction fragment length polymorphism (RFLP) of the 16 S rDNA cleaved with five restriction enzymes. Twenty-five different profile combinations were obtained. *Rhizobium etli* was the predominant species, with 73 strains showing similar RFLP profiles, while 12 other strains differed only by the profile with one restriction enzyme. Fifty strains were submitted to sequencing of a 16 S rDNA fragment, and 34 clustered with *R. etli*, including strains with RFLP-PCR profiles similar to those species or differing by one restriction enzyme. However, other strains differing by one or two enzymes were genetically distant from *R. etli* and two strains with identical profiles showed higher similarity to *Sinorhizobium fredii*. Other strains showed higher similarity of bases with *R. tropici*, *R. leguminosarum* and *Mesorhizobium plurifarium*, but some strains were quite dissimilar and may represent new species. Great variability was also verified among the sequenced strains in relation to the ability to grow in YMA at 40 °C, in LB, to synthesize melanin in vitro, as well as in symbiotic performance, including differences in relation to the described species, e.g. many *R. etli* strains were able to grow in LB and in YMA at 40 °C, and not all *R. tropici* were able to nodulate *Leucaena*.

Keywords: ERIC-PCR; Nitrogen fixation; RFLP; *Rhizobium etli*; *Rhizobium tropici*; *Rhizobium leguminosarum*; 16 S rRNA

1. Introduction

Common bean (*Phaseolus vulgaris* L.), here referred to simply as bean, is widely cropped in Central and South America and in Africa, where it is an important source of protein for human consumption. Both Mesoamerica and the Andean region of South America are the centers of origin and/or domestication of *P. vulgaris* (Kaplan, 1965, 1980; Kaplan et al., 1973; Gepts, 1990), and an important secondary center of origin was identified in the southern Andes of Argentina, for the wild bean *P. vulgaris* var. aborigineus Burk (Baudet) (Gepts, 1990).

At least six rhizobia are able to nodulate and in most cases fix atmospheric nitrogen with beans, including *Rhizobium leguminosarum* bv. phaseoli (Jordan, 1984), *R. tropici* (Martínez-Romero et al., 1991), *R. etli* (Segovia et al., 1993), *R. gallicum* and *R. giardinii* (Amarger et al., 1997). *Bradyrhizobium*(Lange, 1961; Hungria et al., 1993), *Rhizobium* sp. OR 191 isolated from alfalfa nodules (Eardly et al., 1985), and *R. mongolense* (van Berkum et al., 1998) and other isolates with distinct phylogenic positions (Bromfield and Barran, 1990; Eardly et al., 1992, 1995) may well represent additional species. However, although *P. vulgaris* is able to perceive signals for nodulation from many rhizobia, most of the interactions are not effective (Michiels et al., 1998).

Bean rhizobia from the Americas are believed to have been disseminated to other continents on seeds.

* Corresponding author. Tel.: +55-43-3716206; fax: + 55-43-3716100.
E-mail address: hungria@cnpso.embrapa.br (M. Hungria).
[1] MSc at the Universidade Estadual de Londrina, Department of Genetics.

0038-0717/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2004.03.005

Predominance of *R. etli* has been reported in Mesoamerica (Segovia et al., 1993; Souza et al., 1994; van Berkum et al., 1996; Caballero-Mellado and Martinez-Romero, 1999) and in nodules of wild beans in Argentina (Aguilar et al., 1998). Initially, *R. leguminosarum* bv. phaseoli was believed to be the only microsymbiont of bean in Europe, probably arising from the transfer of the symbiotic plasmid of *R. etli* to a *R. leguminosarum* chromosome (Segovia et al., 1993). However, studies in France, Spain and Austria have detected the five *Rhizobium* mentioned above (Geniaux et al., 1993; Laguerre et al., 1993; Amarger et al., 1994; Sessitsch et al., 1997; Herrera-Cervera et al., 1999), while several strains of *R. leguminosarum* have been isolated in Brazil (Mercante et al., 1998; Straliotto et al., 1999; Mostasso et al., 2002). All bean rhizobia species except for *R. giardinii* have also been found in soils of Africa (Mhamdi et al., 1999), and in Brazil the only species so far not identified is *R. gallicum* (Mostasso et al., 2002). *R. tropici* appears highly promiscuous and reported hosts include *Leucaena* sp., *Macroptilium* sp. and several other hosts (Martinez Romero et al., 1991; Hernandez-Lucas et al., 1995), *Bolusanthus* and *Aspartium* in Africa (Dagutat and Steyn, 1995), and native shrubby legumes in Australia (Lafay and Burdon, 1998), while several rhizobia isolated from *Dalea* spp. in the USA show similar promiscuity and also nodulate and fix nitrogen with beans (Graham et al., 1999). The predominance of a bean rhizobia species is also affected by environmental conditions, e.g. in Kenya at a pH 4.5 *R. tropici* was the dominant species, changing to *R. etli* at pH 6.8 (Anyango et al., 1995).

Brazil is the first largest producer of beans (*P. vulgaris*) worldwide, and the grains represent the most important source of protein for the population. Indigenous bean rhizobia colonize both uncropped and cropped soils and the seeds usually carry high numbers of viable cells. Strain-selection programs aiming to increase nodulation and nitrogen fixation in Brazilian soils have shown that the most efficient, competitive and genetically stable strains belong to the species *R. tropici* (Hungria et al., 2000; Mostasso et al., 2002), but diversity was not taken into account in these studies. In other studies, a high level of diversity among strains was detected by the evaluation of morphological and physiological characteristics (Mercante et al., 1998; Straliotto et al., 1999). This study aimed to examine the genetic diversity of bean rhizobia in two contrasting Brazilian ecosystems, the Northeast Region, a semi-arid climate with neutral soils, and the South Region, with humid subtropical climate and acid soils.

2. Materials and methods

2.1. Bean rhizobia used as reference strains

Bean rhizobia reference strains included: *Rhizobium tropici* IIA CFN 299 (= USDA 9039, = LMG 9517), IIB CIAT 899^T (= UMR 1899, = USDA 9030, = TAL 1797, = HAMBI 1163, = SEMIA 4077, = ATCC 49672) and *R. etli* CFN 42^T (= USDA 9032) (CIFN, Cuernavaca, Mexico). *R. tropici* strain PRF 81 (= SEMIA 4080) came from the Embrapa Soja germplasm bank and *R. leguminosarum* bv. phaseoli USDA 2671 (= RCR 3644) from USDA, Beltsville, MD, USA. *R. giardinii* bv. giardinii strain H152^T and *R. gallicum* bv. gallicum strain R602^T were provided by INRA, Dijon, France.

2.2. Isolation of bean rhizobia

Two Brazilian states representing contrasting ecosystems were chosen for this study. Pernambuco in the Northeast Region has a semi-arid climate with long dry periods and soils of neutral pH, with samples collected from São Francisco, Santo Antonio, Caruaru and Serra Talhanda, in the Zona da Mata. Paraná, in the South Region, has a subtropical climate, good rainfall distribution and acid soils ((pH < 5.2), with samples collected from São João, Warta, Toledo and Francisco Alves. In total, samples were taken from 14 sites, including two undisturbed areas covered with native vegetation and 12 fields under various cropping managements, as shown in Table 1. Soil samples were collected at a depth of 0–20 cm with the soil corer cleaned with alcohol (95%) and flamed between samplings. Bean cultivars Aporé (meso-American colored seed) and Negro Argel (meso-American black seed), both previously reported as good N_2-fixing hosts (Hungria et al., 2000) were used as trap hosts. Seeds were surface sterilized and each seed was inoculated with 1 ml of a 10^{-2} dilution of soil in 0.85% (w/v) NaCl (Vincent, 1970). Three seeds were placed in each modified Leonard jar containing sterilized sand and vermiculite (1:2, v/v) and thinned to two seedlings per jar 3 days after emergence, and the experiment was performed with three replicates. Plants were grown under greenhouse conditions at 28/22 °C (day/night) and received N-free nutrient solution (Andrade and Hamakawa, 1994).

After 4 weeks, nodules were collected from 12 out of 14 soils. Number of nodules varied according to the treatment, ranging from 0 to 20 nodules per jar. Rhizobia were isolated using standard procedures (Vincent, 1970) from all nodules collected. The purity of the cultures was confirmed by repeatedly streaking the bacteria on yeast extract–mannitol agar (YMA) medium (Vincent, 1970) and verifying by colony morphology, absorption of Congo red (25 μg ml^{-1}) and Gram reaction. Each colony was transferred to YM liquid broth (YMB) and after growth was mixed with glycerol (1:1, v:v) and stored at −80 °C. Working cultures were maintained on YMA slants at 4 °C. Rhizobia were cultured routinely at 28 °C in YMB on a rotary shaker operating at 65 cycles per minute. A total of 243 isolates were obtained.

Table 1
Bean rhizobia isolates obtained from soils of the State of Pernambuco, in the Northeast Region and in the State of Paraná, South Region

Soil	State	District	Soil management
1	Pernambuco(PE)	São Francisco	Cropped with beans
2	PE	São Francisco	Cropped with beans three years before
3	PE	Santo Antônio	Undisturbed vegetation, with native
4	PE	Santo Antônio	Cropped with beans four years before
5	PE	Caruaru	Cropped with beans, an area that had
6	PE	Caruaru	Cropped with beans
7	PE	Serra Talhada	Undisturbed semi-arid vegetation
8	Paraná (PR)	São João	Beans cropped for 2 years between
9	PR	São João	Pasture, soybean and
10	PR	Warta	Cropped with soybean/wheat
11	PR	Toledo	Cropped with soybean/wheat
12	PR	Toledo	Cropped with soybean/wheat
13	PR	Francisco Alves	Beans for several years intercropped with maize
14	PR	Francisco Alves	Beans intercropped with maize

Isolates obtained from each area can be identified in Table 2.

2.3. Genetic characterization

2.3.1. Amplification with specific ERIC-primer

Total DNA was extracted from 210 out of the 243 isolates and 50 ng used for the amplification by PCR with primers ERIC1R and ERIC2R (de Bruijn, 1992), as described before (Santos et al., 1999). Cluster analysis was performed using the Bionumerics program (Applied Mathematics, Kortrijk, Belgium), with the UPGMA algorithm and the Jaccard coefficient (Sneath and Sokal, 1973). We have also analyzed the data using the Pearson coefficient, and there were no substantial differences in the grouping of the strains. However, as some of the gels showed some stains, we decided that the Jaccard coefficient would represent better the data.

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

PCR reaction of the DNA with primers Y1 and Y3, followed by individual digestion with five restriction endonucleases, *Cfo* I, *Msp* I, *Rsa* I, *Nde* II and *Hinf* I, was performed with 101 isolates, as described by Mostasso et al. (2002). Those isolates were randomly chosen within the groups formed considering a 70% level of similarity in the ERIC-PCR analysis.

2.3.3. rDNA sequence determination

Following ERIC-PCR and RFLP-PCR analyses, 50 isolates were selected based on the groups formed in the RFLP-PCR, and completed with strains showing the same profile as strain 29, the dominant profile. The strains were submitted to direct sequencing of PCR fragments obtained by amplification with primers Y1 and Y3 and intermediate primers, using the methodology described by Mostasso et al. (2002).

2.3.4. GenBank accession numbers and phylogenetic analysis

The 16 S rDNA sequences generated from the selected strains were submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/blast) to seek significant 16 S rRNA alignments. Sequences confirmed in the 3′ and in 5′ directions ranged from 340 to 1470 bp length and received the accession numbers AY117620 to AY117669. The sequences were then aligned pairwise and compared to those of the following organisms (accession numbers of the GenBank Data Library in parentheses): *Agrobacterium radiobacter* LMG 383 (AJ130719); *A. radiobacter* LMG 196 (X67223) (now proposed as a new combination, *Rhizobium radiobacter*, Young et al., 2001); *Mesorhizobium plurifarium* LMG 10056 (Y14161); *R. etli* CFN 42^T (U28916), Olivia (M55235) and TAL 182 (U28939); *R. galegae* HAMBI 540^T (Y12355); *R. gallicum* bv. gallicum R602^T (U86343); *R. giardinii* bv. giardinii H152^T (U86344); *R. huautlense* S02^T (AF025852); *R. leguminosarum* bv. phaseoli ATCC 8002 (M55494); *R. leguminosarum* bv. viceae ATCC 10004^T (U29386); *R. mongolense* USDA 1844^T (U89817) and USDA 1877 (U89820); *R. tropici* IIB CIAT 899^T (U89832) and LMG 9517 (X67234); *R. tropici* IIA LMG 9518 (X67233); *R. tropici* strain PRF 81 (AF260274); *Rhizobium* genomic species Q strain BDV 5102 (Z94806); *Rhizobium* sp. OR 191 (X91211); *Rhizobium* sp. USDA 1920 (U89823) and WSM1583 (AF279266); *Sinorhizobium fredii* USDA 205^T (M74163), *Sinorhizobium* sp. C4 (AF227753). A phylogeny tree was inferred with the UPGMA algorithm using the Bionumerics program.

2.4. Morphological and physiological characterisation

The 50 strains used in the sequencing analysis were also characterized using morphological and physiological characteristics in vitro. An initial inoculum of

10^4 cells ml^{-1} was prepared in YM medium with a pH initially adjusted to 6.8. Growth in YMB at 40 °C, in Luria broth (LB) medium at 28 °C and synthesis of melanin in tryptophan-enriched medium were determined as described by Hungria et al. (2000). Symbiotic effectiveness on bean cv. Carioca and *Leucaena leucocephala* was evaluated under greenhouse conditions as described by Hungria et al. (2000).

3. Results and discussion

A total of 243 isolates were obtained, 132 from soils of Pernambuco and 111 from the Paraná State. Isolates were not obtained from one undisturbed soil in Pernambuco (soil 7) and from one soil of Paraná cropped with soybean and wheat (soil 10). Color-seeded cultivar Aporé trapped 138 isolates, while black-seeded cultivar Negro Argel trapped 105 isolates.

Two months after being isolated and maintained at − 80 °C, 33 out of the 243 isolates failed to grow, therefore the DNAs of 210 bacteria were amplified with the ERIC primer. The majority of the isolates that did not grow came from soil 11, and seven others from two soils of Pernambuco. Those isolates were trapped by both host cultivars and we still do not know why they stopped growing. Several major clusters of bean rhizobia were identified among the 210 isolates for which PCR banding patterns were obtained, and 81% of the isolates represented unique strains at the 70% similarity level (Fig. 1). Although a large diversity in morphological, physiological and genetic characteristics has been reported among bean rhizobia strains in Brazil (Mercante et al., 1998; Straliotto et al., 1999; Hungria et al., 2000; Mostasso et al., 2002), there are no reports of a variability as high as that observed in this study.

Considering a level of 50% of similarity in the ERIC-PCR analysis, the isolates were clustered in 34 groups (Table 2), and even though several strains occupied an isolated position (Table 2). For the discussion of the results, some clusters from Table 2 were highlighted on Fig. 1. From the analysis of Table 2 and Fig. 1 it appears that there was no recognizable effect of either the cultivar (thus of the different flavonoids released by color and black seeds, Hungria et al., 1991). There was also no evident effect of the ecosystem on rhizobial diversity, although many clusters included strains isolated from the same site (e.g. isolates 172, 177, 176, 166, 179, 178, 170, 169, 174 and 175, from a soil of Paraná grown with soybean and wheat, were grouped at 51% similarity in Group 1). Other clusters included isolates from the same soil and captured by the same host cultivar, e.g. 36, 38 and 40 (cv. Aporé and the state of Pernambuco), and isolates 51, 45 and 44 (cv. Negro Argel and the same soil), all in Group 8. Most of the isolates with identical ERIC-PCR profiles came from the same soil and host plants, e.g. isolates 7, 5, 10 and 8 (Group 7).

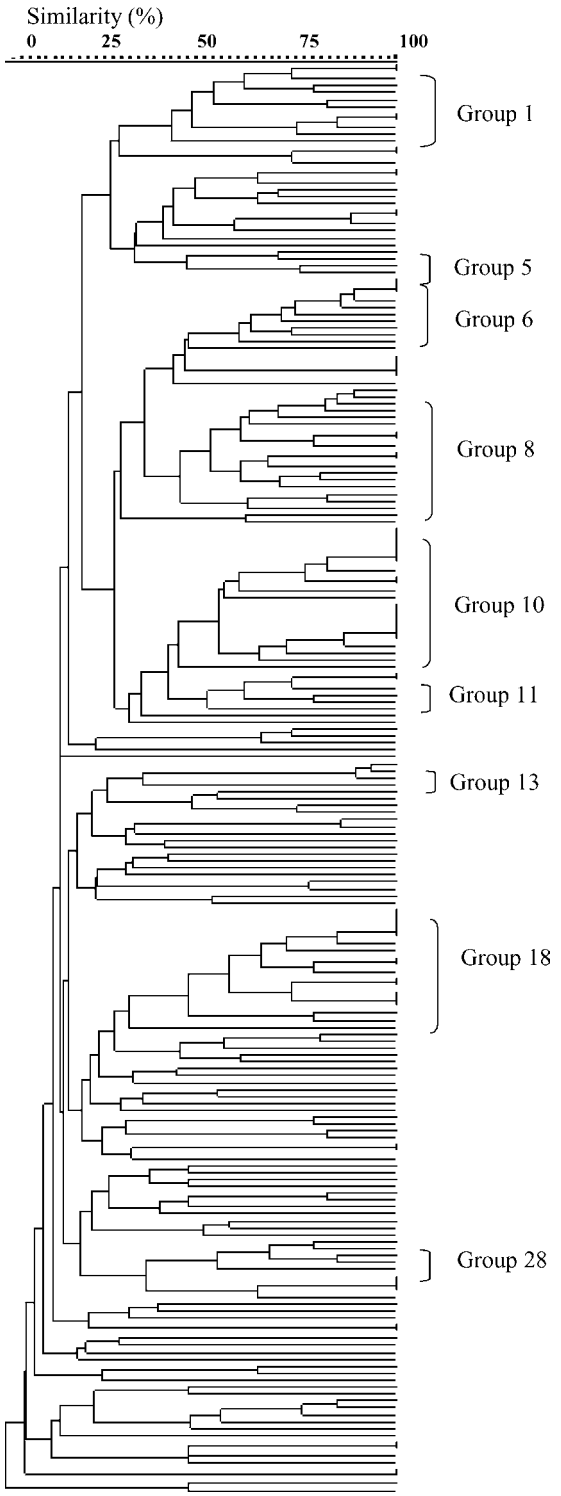

Fig. 1. Dendrogram showing the Brazilian bean rhizobia isolates, after cluster analysis of ERIC-PCR products using the UPGMA algorithm and the Jaccard coefficient. The isolates of this dendrogram are specified in Table 2.

Isolates from different soils in the same ecosystem were only rarely similar (e.g. isolates 53, 57, from soil 2 and isolate 119, from soil 5, all in PE and within Group 6). Similar isolates recovered from different ecosystems

Table 2
Clusters and isolates included within each cluster in the analysis of the ERIC -PCR products considering a level of similarity of 50%. Some of the clusters are highlighted in Fig. 1, others are classified as ungrouped (U) and the order of the isolates is the same obtained in the dendrogram. After the number of each isolate it follows the number of each soil and the bean cultivar from which the isolate was obtained, A (Aporé) or N (Negro Argel)

Clusters	Number of isolates	Isolate number, followed by the soil and the host plant
1	11	172(12A), 177(12N), 176(12N), 166(12A), 179(12N), 178(12N), 224(14A), 170(12A), 169(12A), 174(12N), 175(12A)
U	1	73(4A)
2	3	76(4A), 187(13A), 202(14A)
3	6	191(13N), 189(13A), 181(12N), 183(12N), 197(13N), 173(12A)
4	4	184(12N), 185(12N), 186(12N), 188(13A)
U	1	182(12N)
5	5	190(13A), 193(13N), 192(13N), 195(13N), 194(13N)
6	11	53(2N), 57(2N), 119(5A), 39(2A), 121(5A), 220(14A), 120(5N), 31(2A), 70(4A), 69(4A), 42(2A)
7	4	7(1N), 5(1N), 10(1N), 8(1N)
U	1	35(2A)
8	18	36(2A), 38(2A), 40(2A), 227(14A), 9(1N), 75(4A), 51(2N), 45(2N), 44(2N), 214(14A)), 247(14N), 218(14A), 217(14A), 215(14A), 216(14A), 29(2A), 50(2A), 41(2A)
9	2	74(4 A), 82(4N)
10	20	12(1N), 49(1N), 17(1A), 13(1N), 19(1A),18(1A), 225(14A), 16(1A), 231(14A), 30(2A), 226(14A), 200(13N), 208(14A), 210(14A), 209(14A), 211(14A), 196(13N), 212(14A), 207(14A), 205(14A)
U	1	228(A)
11	6	153(9A), 232(14 A), 230(15A), 206 (14A), 223(14A), 168(12A)
U	2	22(1A), 198(13N)
12	3	64(3N), 61(3A), 222(14A)
U	2	62(3A), 84(4N)
13	3	155(9A), 156(9A), 237(9N)
U	1	33(1A)
14	5	3(1N), 1(1N), 2(1N), 4(1N)
15	2	96(5A), 99(5 A)
U	7	112(6N), 128(8A), 148(6N), 48(2N), 123(5N), 236(6N), 79(4A)
16	2	92(5A), 93(5A)
17	2	246 (5A), 101(6A)
18	17	131(8A), 134(8A), 135(8A), 133(8A), 136(8A), 235(6N), 130(8A), 249(6N), 116(6N), 125(6N), 149(9A), 150(9A), 141(6N), 142(8N), 143(8N), 152(9A), 151(9A)
U	1	140(8N)
19	2	48(2N), 151(9A)
U	1	140(8N)
20	3	46(2N), 58(2N), 180(12N)
21	2	43(2N), 86(5N0
U	3	52(2N), 122(5A), 91(5N)
22	2	67(4A), 72(4A)
U	2	94(5A), 87(5N)
23	2	132(8N), 137(8A)
24	2	63(3A), 65(3N)
25	2	95(5A), 124(5A)
U	5	154(9A), 118(5A), 221(14A), 54(2N), 219(14N)
26	2	89(5N), 90(5N)
U	2	244(4N), 68(4A)
27	3	77(4A), 83(4N), 80(4A)
28	5	158(9 A), 243(9N), 159(9 A), 160(9A), 238(9N)
29	4	241(9N), 163(12A), 164(12A), 240(12N)
U	3	34(2A), 171(12A), 248(6A)
30	2	98(5A), 100(5A)
U	4	115(6N), 233(14A), 199(13N), 37(2A)
31	2	71(4A), 203(14A)
U	3	129(8A), 126(5N), 229(14A)
32	5	106(6A), 110(6N), 108(6A), 113(6A), 245(4N)
U	1	234(13A)
33	4	60(3A), 109(6N), 138(8A), 107(6A)
U	2	14(1N), 239(9N)
34	2	127(8A), 204(14A)

(e.g. isolate 76, from soil 4, in PE and isolate 187, from soil 13, PR, within Group 2) were ever rare (Fig. 1 and Table 2). Although there is a possibility that strains showing the same ERIC-PCR profile may differ in other genomic regions, from this step each isolate showing a unique profile was called as strain.

Considering the level of similarity of 70% (ERIC-PCR), 101 strains representing the clusters were chosen and analyzed by the PCR-RFLP of the 16 S rDNA region, and a high level of polymorphism was evident. The number of bands obtained for each restriction enzyme varied as follows: *Nde* II, 2–4 bands; *Rsa* I, 2–7 bands; *Hinf* I, 2–5 bands; *Cfo* I, 2–5 bands; *Msp* I, 3–6 bands (data not shown). Twenty-five different combinations of profiles were obtained (Table 3). Despite the high diversity, 73 strains (72%) showed similar profiles to *R. etli*, and 12 others were only different from *R. etli* in the profile generated by only one restriction enzyme (*Hinf* I, or *Cfo* I, or *Msp* I). Strains showing RFLP-PCR profiles similar to *R. etli* (Table 3) often occurred in quite dissimilar ERIC-PCR clusters (Fig. 1, Table 2) (e.g. 248, 5 and 14), supporting the value of ERIC-PCR methods in the evaluation of diversity of rhizobial strains, but not of rhizobial species, as clearly they were less valuable for taxonomic purposes (see also Laguerre et al., 1997; Mostasso et al., 2002). None of the other 28 strains showed combinations of profiles similar to those for other bean rhizobia (Table 3).

When the DNA fragment corresponding to the 16 S rRNA region of 50 strains, (representing the RFLP-PCR groups, and several isolates showing the dominant profile of strain 29) was sequenced, 15 of the 17 strains having RFLP-PCR profiles with very high similarity to that of *R. etli* CFN 42 were clustered with *R. etli* strain Olivia; exceptions were strains 129 and 215. This cluster linked to CFN 42 at a 94% level of similarity (Fig. 2). *R. mongolense* strain USDA 1877 (van Berkum et al., 1998) also clustered in this group. However, since neither the DNA relatedness nor the SSU rRNA sequence comparisons support the grouping of USDA 1877 with the type strain USDA 1844 (van Berkum et al., 1998), and based on the phylogenetic analyses of this paper, this strain would be better classified as *R. etli*. The two strains 129 and 215 probably differed in the 16 S rRNA region in other sites than those analysed by the RFLP-PCR and in the sequencing analysis they clustered with *Sinorhizobium fredii* USDA 205, although the lowered similarity of 129 may indicate a new species. In addition, 14 strains that had not been analyzed for RFLP-PCR, but were in ERIC-PCR clusters close to *R. etli*, strains were also sequenced (1, 7, 30, 35, 42, 49, 82, 127, 150, 164, 173, 227, 229, 228, Fig. 1 and Table 2) and they were positioned as *R. etli* (Fig. 2). Finally, four strains different from CFN 42 in the profile with restriction enzyme *Msp* I (79, 82, 116 and 230), or *Cfo* I (68) were also clustered with *R. etli*, whereas one strain differing with *Msp* I (126) and another with *Cfo* I and *Msp* I enzymes (246) were clustered with a lower level of similarity (92%).

Table 3
Profiles of RFLP-PCR of the 16 S rDNA region with five restriction enzymes obtained with the bean rhizobia isolated from the States of Pernambuco and Paraná and with type strains

Strains[a]	Enzymes				
	Nde II	*Rsa* I	*Hinf* I	*Cfo* I	*Msp* I
R. etli	a	a	a	a	a
29[b]	a	a	a	a	a
126	a	a	a	a	c
79	a	a	a	a	e
82	a	a	a	a	e
230	a	a	a	a	k
116	a	a	a	d	a
246	a	a	a	d	d
52	a	a	a	e	a
68	a	a	a	e	a
71	a	a	a	h	a
168	a	a	d	a	a
169	a	a	d	a	a
241	a	a	d	a	a
164	a	a	k	a	a
R. gallicum	b	b	a	a	a
R. tropici IB	b	b	a	b	b
R. tropici IIA	b	b	a	b	d
R. legum.bv.phaseoli	b	f	a	a	j
R. giardinii	f	b	a	a	b
77	a	b	a	b	a
34	a	b	a	j	b
233	b	a	a	b	o
93	b	b	a	b	a
100	b	e	a	a	a
62	b	g	b	a	c
43	c	c	b	c	d
180	c	c	b	i	d
86	c	i	b	a	c
84	d	b	a	a	a
61	d	d	c	a	c
247	e	l	h	g	a
112	g	k	f	k	n
234	i	a	e	c	g
171	j	e	g	l	f

[a] Strains used as reference are described in Section 2.
[b] Strains 5, 14, 31, 38, 39, 41, 48, 51, 53, 54, 63, 69, 72, 73, 74, 75, 76, 83, 89, 90, 96, 101, 107, 108, 109, 115, 118, 122, 123, 124 128, 129, 133, 156, 158, 160, 166, 172, 175, 177, 178, 181, 182, 183, 185, 186, 188, 189, 190, 192, 194, 195, 199, 202, 203, 204, 205, 206, 209, 212, 215, 216, 218, 219, 221, 222, 236, 238, 239, 244, 245 and 248 showed the same profile as that strain.

Strains with 16 S rRNA sequences similar to *R. etli* were spread throughout the ERIC-PCR dendrogram, e.g. strains 29 and 51 were positioned in the first cluster, in the upper part of the dendrogram, while in the lower cluster were positioned strains 127 and 79 (Fig. 1 and Table 2). Two strains, 86 and 62 were clustered with a 99% similarity of bases with *Mesorhizobium plurifarium* LMG 10056 (de Lajudie et al., 1998), three others (233, 34 and 92) were grouped with the *R. tropici* strain PRF 81 used in Brazilian commercial inoculants, while strain 100 showed 88% of similarity with *R. leguminosarum* (Fig. 2). Finally, strain 77 showed higher similarity with *R. tropici* IIB, but two other

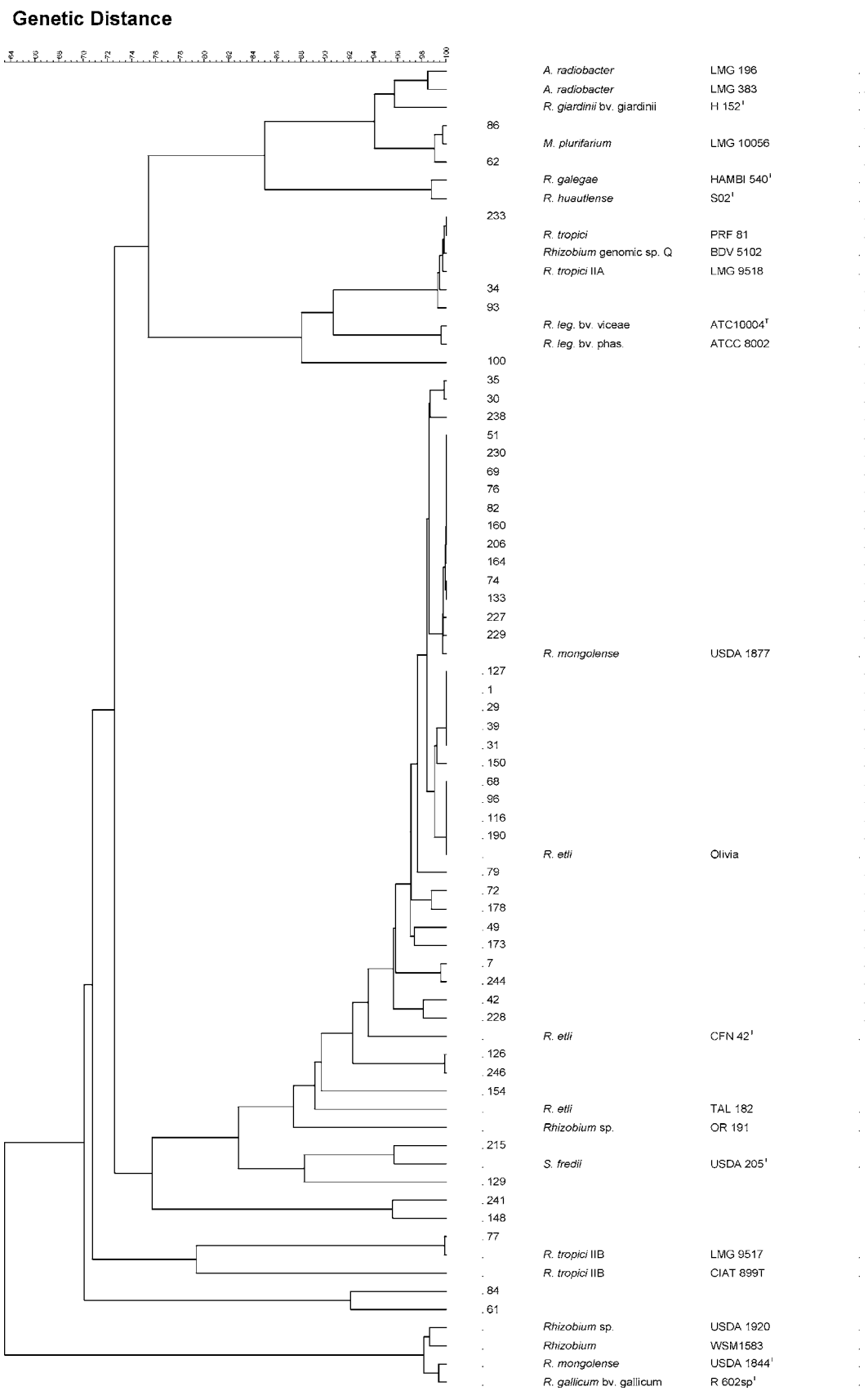

Fig. 2. Dendrogram built with the UPGMA algorithm with the aligned 16 S rRNA sequences of 50 Brazilian bean rhizobia isolates and of reference strains. Accession numbers are cited in Section 2.

Table 4
Characterization in vitro (growth in YMA at 40 °C, growth in LB and synthesis of melanin in tryptophan-enriched medium) and symbiotic effectiveness with *Phaseolus vulgaris* and *Leucaena leucephala* inoculated with fifty bean rhizobia isolated from two different ecosystems in Brazil.

Strain	Species[a]	40 °C	LB	Melanin	Leucaena[b]
1[c]	R. etli	−	−	+	−
30[d]	*R. etli*	−	+	−	−
39[e]	*R. etli*	+	+	−	−
49[f]	*R. etli*	−	−	−	−
69	*R. etli*	+	+	+	−
96	*R. etli*				
61	*Rhizobium* sp.	−	−	−	−
84	*Rhizobium* sp.	+	+	−	+
100	*Rhizobium* sp.	−	−	−	−
129	*Rhizobium* sp.	+	+	−	−
148, 241	*Rhizobium* sp.	+	+	−	−
215	*Rhizobium* sp.	+	+	+	+
34	R. tropici	+	+	−	+
77	*R. tropici*	−	−	−	−
93	*R. tropici*	−	−	+	−
233	*R. tropici*	+	+	−	+
62	*Mesorhizobium* sp.	−	−	−	−
86	Mesorhizobium *sp.*	−	−	−	−

[a] Classification according to the 16 S rRNA sequencing analysis (Fig. 1).
[b] All strains nodulated and showed symbiotic effectiveness with beans, and all strains labelled (+) nodulated and were effective with *Leucaena*.
[c] Strains with similar characteristics: 7, 29, 51, 127, 164, 244.
[d] Strains with similar characteristics: 31, 35, 74, 76, 206, 230, 238.
[e] Strains with similar characteristics: 42, 82, 126, 154, 227, 228, 229.
[f] Strains with similar characteristics: 72, 79, 116, 133, 173, 178, 150, 160, 190.

strains, 84 and 61 showed very low relatedness with the other clusters and may also represent other species. None of the strains from this study was clustered with *R. gallicum* or *R. giardinii*.

The sequenced strains were also evaluated in relation to some morphological and physiological characteristics in vitro and great variability was verified (Table 4). None of the *R. etli* strains was able to effectively nodulate *Leucaena*, however, many were able to grow in LB and in YMA at 40 °C, contrary to the description of this species (Segovia et al., 1993) and not all *R. tropici* strains were able to effectively nodulate *Leucaena* (Table 3). Furthermore, recent studies found in archeological sites in Brazil bean seeds genetically related to those from Mesoamerica (F.O. Freitas, unpublished).

Other studies in Brazil have identified the majority of bean strains recovered as *R. tropici* (Mercante et al., 1998; Straliotto et al., 1999; Hungria et al., 2000; Mostasso et al., 2002). However, the isolates from those studies were either selected using *Leucaena* as a trap host (Mercante et al., 1998), or the objective was the selection of more efficient and competitive strains for the tropics (Hungria et al., 2000; Mostasso et al., 2002). Although the previous studies have also used jars containing nutrient solution with neutral pH (Mercante et al., 1998; Hungria et al., 2000; Mostasso et al., 2002), in our study the characterization of all strains trapped by two bean cultivars indicated a predominance of *R. etli*, in a situation similar to that reported for isolates of wild beans in Argentina (Aguilar et al., 1998). Most important was the wide diversity within *R. etli* species, especially in relation to morphological, physiological and symbiotic properties, giving emphasis to the need for a better definition of the characteristics of this species.

In this paper at least three genera, *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* were isolated from bean nodules, confirming, along with other reports (Lange, 1961; Bromfield and Barran, 1990; Eardly et al., 1992, 1995; Hungria et al., 1993, 2000; Martinez Romero et al., 1991; Segovia et al., 1993; Amarger et al., 1997; Mercante et al., 1998; Straliotto et al., 1999; Mostasso et al., 2002) the promiscuous nature of bean. A lack of response to inoculation with superior strains has often been reported for bean crops, although a search within the established population for efficient and competitive strains is feasible (Hungria et al., 2000, 2003). Probably is one of the highest promiscuity reported, therefore understanding the relationship between the bean rhizobia diversity and the contribution of nitrogen fixation to the bean crop could be the key to improving yields of this crop.

Acknowledgements

The authors thank Ligia Maria O. Chueire, Luciano de Souza, Rinaldo B. Conceição and Belquis Renê dos Santos for helping this study, Dr Rubens J. Campo, for discussion and suggestions and Dr Allan R.J. Eaglesham, for reviewing the manuscript. Research described herein was partially financed by CNPq (PRONEX-41.96.0884.00 and 520396/96-0). L. Grange acknowledges the MSc fellowship from CAPES and M. Hungria a research fellowship from CNPq (520396/96-0).

References

Aguilar, O.M., Lópes, M.V., Riccillo, P.M., González, R.A., Pagano, M., Grasso, D.H., Pühler, A., Favelukes, G., 1998. Prevalence of the *Rhizobium etli* like allele in genes coding for 16 S rRNA among the indigenous rhizobial populations found associated with wild beans from the Southern Andes in Argentina. Applied and Environmental Microbiology 64, 3520–3524.

Amarger, N., Bours, M., Revoy, F., Allard, M.R., Laguerre, G., 1994. *Rhizobium tropici* nodulates field-grown *Phaseolus vulgaris* in France. Plant and Soil 161, 147–156.

Amarger, N., Macheret, V., Laguerre, G., 1997. *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. International Journal of Systematic Bacteriology 47, 996–1006.

Andrade, D.S., Hamakawa, P.J., 1994. Estimativa do número de células viáveis de rizóbio no solo e em inoculantes por infecção em plantas. In: Hungria, M., Araujo, R.S. (Eds.), Manual de Métodos Empregados em

Estudos de Microbiologia Agrícola, EMBRAPA-SPI, Brasília, pp. 63–94.

Anyango, B., Wilson, K.J., Beynon, J.L., Giller, K.E., 1995. Diversity of rhizobia nodulating *Phaseolus vulgaris* L. in two Kenyan soils with contrasting pHs. Applied and Environmental Microbiology 61, 4016–4021.

van Berkum, P., Beyene, D., Eardly, B.D., 1996. Phylogenetic relationships among *Rhizobium* species nodulating the common bean (*Phaseolus vulgaris* L.). International Journal of Systematic Bacteriology 46, 240–244.

van Berkum, P., Beyene, D., Bao, G., Campbell, T.A., Eardly, B.D., 1998. *Rhizobium mongolense* sp. nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) Ledebour]. International Journal of Systematic Bacteriology 48, 13–22.

Bromfield, E.S.P., Barran, L.R., 1990. Promiscuous nodulation of *Phaseolus vulgaris*, *Macroptilium atropurpureum*, and *Leucaena leucocephala* by indigenous *Rhizobium meliloti*. Canadian Journal of Microbiology 36, 369–372.

de Bruijn, F.J., 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 soils bacteria. Applied and Environmental Microbiology 58, 2180–2187.

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

Dagutat, H., Steyn, P.L., 1995. Taxonomy and distribution of rhizobia indigenous to South African soils. In: Tikhnovich, I.A., Provorov, N.A., Romanov, V.I., Newton, W.E. (Eds.), Nitrogen Fixation: Fundamentals and Applications, Kluwer, Boston, pp. 683–686.

Eardly, B.D., Hannaway, D.B., Bottomley, P.J., 1985. Characterization of rhizobia from ineffective alfalfa nodules: ability to nodulate bean plants (*Phaseolus vulgaris* (L.) Savi). Applied and Environmental Microbiology 50, 1422–1427.

Eardly, B.D., Young, J.P.W., Selander, R.K., 1992. Phylogenetic position of *Rhizobium* sp. strain OR 191, a symbiont of both *Medicago sativa* and *Phaseolus vulgaris*, based on partial sequences of the 16 S rRNA and *nifH* genes. Applied and Environmental Microbiology 58, 1809–1815.

Eardly, B.D., Wang, F.S., Whittam, T.S., Selander, R.K., 1995. Species limits in *Rhizobium* populations that nodulate the common bean (*Phaseolus vulgaris*). Applied and Environmental Microbiology 61, 507–512.

Geniaux, E., Laguerre, G., Amarger, N., 1993. Comparison of geographically distant populations of *Rhizobium* isolated from root nodules of *Phaseolus vulgaris*. Molecular Ecology 2, 295–302.

Gepts, P., 1990. Biochemical evidence bearing on the domestication of *Phaseolus* (Fabaceae) beans. Economic Botany 44, 28–38.

Graham, P.H., Ballen, K.G., Montealegre, C., Jones, R.K., Fischer, B., Luque, E., 1999. Characterization of rhizobia associated with *Dalea* spp. in natural prairies and revegetation areas in Minesota. In: Martinez, E., Hernandez, G. (Eds.), Highlights of Nitrogen Fixation Research, Kluwer Academic/Plenum, New York, pp. 69–75.

Hernandez-Lucas, I., Segovia, L., Martínez-Romero, E., Pueppke, S.G., 1995. Phylogenetic relationships and host range of *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. Applied and Environmental Microbiology 61, 2775–2779.

Herrera-Cervera, J.A., Caballero-Mellado, J., Laguerre, G., Tichy, H.V., Requena, N., Amarger, N., Martínez-Romero, E., Olivares, J., Sanjuan, J., 1999. At least five rhizobial species nodulate *Phaseolus vulgaris* in a Spanish soil. FEMS Microbiology Ecology 30, 87–97.

Hungria, M., Joseph, C.M., Phillips, D.A., 1991. Anthocyanidins and flavonols, major nod gene inducers from seeds of a black-seeded common bean (*Phaseolus vulgaris* L.). Plant Physiology 97, 751–758.

Hungria, M., Franco, A.A., Sprent, J.I., 1993. New sources of high-temperature tolerant rhizobia for *Phaseolus vulgaris* L. Plant and Soil 149, 103–109.

Hungria, M., Andrade, D.S., Chueire, L.M.O., Probanza, A., Guttierrez-Mañero, F.J., Megías, M., 2000. Isolation and characterization of new efficient and competitive bean (*Phaseolus vulgaris* L.) rhizobia from Brazil. Soil Biology and Biochemistry 32, 1515–1528.

Hungria, M., Campo, R.J., Mendes, I.C., 2003. Benefits of inoculation of common bean (*Phaseolus vulgaris*) crop with efficient and competitive *Rhizobium tropici* strains. Biology and Fertility of Soils 39, 88–93.

Jordan, D.C., 1984. *Rhizobiaceae* Conn 1938. In: Kriegn, N.R., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, London, pp. 235–244.

Kaplan, L., 1965. Archeology and domestication in American *Phaseolus* (Beans). Economic Botanic 19, 358–368.

Kaplan, L., 1980. What is the origin of the common bean? Economic Botanic 35, 240–254.

Kaplan, L., Lynch, T., Smith, C., 1973. Early cultivated beans (Phaseolus vulgaris L.) from an intermontane Peruvian valley. Science 179, 76–77.

Lafay, B., Burdon, J.J., 1998. Molecular diversity of rhizobia occurring on native shrubby legumes in southeastern Australia. Applied and Environmental Microbiology 64, 3989–3997.

Laguerre, G., Fernandez, M.P., Edel, V., Normand, P., Amarger, N., 1993. Genomic heterogeneity among French *Rhizobium* strains isolated from *Phaseolus vulgaris* L. International Journal of Systematic Bacteriology 43, 761–767.

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*. Applied and Environmental Microbiology 63, 4748–4758.

de Lajudie, P., Willems, A., Nick, G., Moreira, F., Molouba, F., Hoste, B., Torck, U., Neyra, M., Collins, M.D., Lindström, K., Dreyfus, B., Gillis, M., 1998. Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. International Journal of Systematic Bacteriology 48, 369–382.

Lange, R.T., 1961. Nodule bacteria associated with the indigenous Leguminosae of south-western Australia. Journal of General Microbiology 26, 351–359.

Martinez Romero, E., Segovia, L., Mercante, F.M., Franco, A.A., Graham, P., Pardo, M.A., 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. International Journal of Systematic Bacteriology 41, 417–426.

Mercante, F.M., Cunha, C.O., Straliotto, R., Ribeiro-Junior, W.Q., Vanderleyden, J., Franco, A.A., 1998. *Leucaena leucocephala* as a trap-host for *Rhizobium tropici* strains from the Brazilian cerrado region. Revista de Microbiologia 29, 49–58.

Mhamdi, R., Jebara, M., Aouani, M.E., Ghrir, R., Mars, M., 1999. Genotypic diversity and symbiotic effectiveness of rhizobia isolated from root nodules of *Phaseolus vulgaris* L. in Tunisian soils. Biology and Fertility of Soils 28, 313–320.

Michiels, J., Dombrecht, B., Vermeiren, N., Xi, C., Luyten, E., Vanderleyden, J., 1998. *Phaseolus vulgaris* is a non-selective host for nodulation. FEMS Microbiology Ecology 26, 193–205.

Mostasso, L., Mostasso, F.L., Dias, B.G., Vargas, M.A.T., Hungria, M., 2002. Selection of bean (*Phaseolus vulgaris* L.) rhizobial strains for the Brazilian Cerrados. Field Crops Research 73, 121–132.

Santos, M.A., Vargas, M.A.T., Hungria, M., 1999. Characterization of soybean bradyrhizobia strains adapted to the Brazilian Cerrados Region. FEMS Microbiology Ecology 30, 261–272.

Segovia, L., Young, J.P.W., Martínez-Romero, E., 1993. Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strains as *Rhizobium etli* sp. nov. International Journal of Systematic Bacteriology 43, 374–377.

Sessitsch, A., Hardarson, G., Akkermans, A.D.L., Vos, W.M.de, 1997. Characterization of *Rhizobium etli* and other *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. in an Australian soil. Molecular Ecology 6, 601–608.

Sneath, P.H.A., Sokal, R.R., 1973. Numerical taxonomy, W.H Freeman and Co, San Francisco.

Souza, V., Eguiarte, L., Avila, G., Capello, R., Gallardo, C., Montoya, J., Piñero, D., 1994. Genetic structure of *Rhizobium etli* biovar phaseoli associated with wild and cultivated bean plants (*Phaseolus vulgaris* and *Phaseolus coccineus*) in Morelos, México. Applied and Environmental Microbiology 60, 1260–1268.

Straliotto, R., Cunha, C.O., Mercante, F.M., Franco, A.A., Rumjanek, N.G., 1999. Diversity of rhizobia nodulating common beans (*Phaseolus vulgaris* L.) isolated from Brazilian tropical soils. Anais da Academia Brasileira de Ciências 71, 531–543.

Vincent, J.M., 1970. A Manual for the Practical Study of Root Nodule Bacteria, IBP Handbook No. 15, Blackwell, Oxford.

Young, J.M., Kuykendall, L.D., Martínez-Romero, E., Kerr, A., Sawada, H., et al., 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.1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. International Journal of Systematic and Evolutionary Microbiology 51, 89–103.