SYMBIOSIS (2009) 48, 120–130 ©2009 Balaban, Philadelphia/Rehovot ISSN 0334-5114

rep-PCR of tropical rhizobia for strain fingerprinting, biodiversity appraisal and as a taxonomic and phylogenetic tool

Pâmela Menna[1,2,3], Alan Alves Pereira[1,3], Eliane Villamil Bangel[4], and Mariangela Hungria[1,2,3*]

[1]Embrapa Soja, Cx. Postal 231, 86001-970, Londrina, Paraná, Brazil, Tel. +55-4333716206, Fax. +55-4333716100, Emails. pamela_menna@yahoo.com.br; alan_alvesp@yahoo.com.br, hungria@cnpso.embrapa.br;
[2]Universidade Estadual de Londrina, Department of Microbiology; Cx. Postal 60001, 86051-990, Londrina, Brazil;
[3]Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-MCT), Brasilia, Federal District, Brazil;
[4]FEPAGRO, Rua Gonçalves Dias 579, 90130-060, Porto Alegre, Rio Grande do Sul, Brazil,
Email. eliane-bangel@fepagro.rs.gov.br

(Received June 1, 2008; Accepted September 9, 2008)

Abstract
With more than 30 million doses of rhizobial inoculants marketed per year, it is probable that Brazilian agriculture benefits more than any other country from symbiotic N_2 fixation. As a result of strain-selection programs, 142 strains of rhizobia are officially recommended for use in commercial inoculants for ninety-six leguminous crops. In this study, sixty-eight of these elite strains were characterized by rep-PCR with the BOX-primer. Reproducibility of the DNA profiles was confirmed, suggesting efficacy of BOX-PCR both for control of quality of inoculants and for preliminary characterization of rhizobial culture collections. Strains of different species never showed similarity higher than 70% in the BOX-PCR analysis, however, some strains of the same species fit into more than one cluster, and correlation between BOX-PCR products and 16S rRNA sequences was low (7.6%). On the other hand, a polyphasic approach – 20%:80% of BOX-PCR:16S rRNA which correlated well with the 16S rRNA analysis (95%), and provided higher definition of the genotypes, resulting in clearer indications of the taxonomic groups – might expedite rhizobial diversity studies.

Keywords: Bacterial fingerprinting, bacterial taxonomy, biological nitrogen fixation, Rhizobiales, inoculants, 16S rRNA

1. Introduction

Many bacteria belonging to several genera, collectively known as "rhizobia," are capable of establishing symbiotic associations with thousands of plant species in the family Leguminosae. Rhizobia were first classified based on the capacity to nodulate certain host plants, in "cross inoculation" groups (Fred et al., 1932); 50 years later their taxonomy was redefined using numerical criteria, considering several morpho-physiological and genetic properties (Jordan, 1984). In the 1980s, several studies have demonstrated that the 16S rRNA gene is an especially useful molecular marker for assessing phylogeny and taxonomy of prokaryotes (Woese, 1987; Woese et al., 1990; Vandamme et al., 1996; Garrity and Holt, 2001), and when this criterion was applied to the rhizobia, it resulted in profound changes in taxonomy (Garrity and Holl, 2001; Young et al., 2001). However, although precise for the definition of kingdoms and genera, 16S rRNA provides poor resolution of species and subspecies (Woese, 1987; Garrity and Holt, 2001). Therefore, the classification of known species usually requires additional information, such as morpho-physiological and other genetic properties (Garrity and Holt, 2001), and the definition of new species usually involves labor- and time-consuming DNA-DNA hybridization (Vandamme et al., 1996; Coenye et al., 2005).

Other molecular techniques may be useful for prospecting bacterial biodiversity and fingerprinting strains, and rep-PCR – a technique consisting of the amplification of repetitive DNA elements dispersed in the genome and probably located in distinct, intergenic positions – has proven to be adequate with a variety of bacteria, including rhizobia (e.g., Versalovic et al., 1991, 1994; de Bruijn, 1992; Laguerre et al., 1997). Three main sets of repetitive

*The author to whom correspondence should be sent.

Presented at the 15th International Congress on Nitrogen Fixation, January 21–26, 2007, Cape Town, South Africa

DNA elements have been used for typing bacteria: the 35- to 40-pb repetitive extragenic palindromic (REP) sequences (Stern et al., 1984), the 124- to 127-bp enterobacterial repetitive intergenic consensus (ERIC) sequences (Hulton et al., 1991), and the BOX elements (Martin et al., 1992). This third class consists of differentially conserved subunits, named boxA, boxB, and boxC (Martin et al., 1992), though only the boxA-like subunit appears highly conserved across diverse bacteria (Koeuth et al., 1995).

Species biodiversity in the tropics, albeit poorly characterized, is often claimed as a major global treasure, of which the rhizobia are an important part (Oyaizu et al., 1992; Vinuesa et al., 1998; Germano et al., 2006; Menna et al., 2006). Indeed, indigenous and naturalized rhizobia in Brazilian soils have been found to exhibit high levels of genetic diversity using ERIC- (Hungria et al., 1998, 2000, 2006; Santos et al., 1999; Ferreira and Hungria, 2002; Mostasso et al., 2002; Grange and Hungria, 2004; Pinto et al., 2007), REP- (Hungria et al., 1998; Santos et al., 1999; Hungria et al., 2000, 2006; Mostasso et al., 2002; Barcellos et al., 2007; Pinto et al., 2007), and BOX-PCR (Fernandes et al., 2003; Galli-Terasawa et al., 2003; Alberton et al., 2006; Kaschuk et al., 2006; Barcellos et al., 2007; Batista et al., 2007; Pinto et al., 2007).

A polyphasic approach, combining ERIC-, REP- and BOX-PCR profiles, has been used to improve the precision of genetic discrimination (Louws et al., 1994; de Bruijn et al., 1996); it has also proven to be useful in surveys of diversity of tropical rhizobia (Hungria et al., 2000, 2006; Mostasso et al., 2002; Pinto et al., 2007). Although congruence among the three sets of rep-primers has rarely been evaluated, using *Xanthomonas* spp. as a model, Rademaker et al. (2000) showed high correlations between profiles obtained with the three sets of primers. Also among *Xanthomonas* spp., a high correlation between rep-PCR and DNA-DNA hybridization was found (Rademaker et al., 2000), but, apparently, there is poor congruence between rep-PCR and the taxonomy of rhizobial species (Laguerre et al., 1997; Mostasso et al., 2002; Fernandes et al., 2003; Grange and Hungria, 2004; Hungria et al., 2006).

Brazil may benefit from symbiotic N_2 fixation more than any other country; more than 30 million doses of rhizobial inoculants are applied to legume crops per year (Hungria and Campo, 2007). As a result of government-funded strain-selection programs, 142 strains are officially recommended for application in inoculants to ninety-six leguminous hosts. Maintenance of these bacteria as well as their distribution to the industry is the responsibility of the "*Rhizobium* Culture Collection SEMIA" (Seção de Microbiologia Agrícola) (IBP Catalogue of *Rhizobium* Collections nº. 443 in the WFCC World Data Center on Microorganisms). However, despite accrual over many years of a great deal of data on the symbiotic properties of those strains, evaluations of their genetic diversity and taxonomic position are just beginning (Germano et al., 2006; Menna et al., 2006). Furthermore, strain fingerprinting is not a component of quality control of the inoculants commercialized in Brazil (Hungria and Campo, 2007).

A first step towards the characterization of this valuable Brazilian collection consisted of determination of 16S rRNA sequences of sixty-eight strains (Menna et al., 2006). This set of strains represents valuable material to both confirm if rep-PCR has high discriminatory power and reproducibility for fingerprinting rhizobia, and to determine if the technique has taxonomic utility.

2. Material and Methods

Strains

Sixty-eight elite strains from the "Brazilian *Rhizobium* Culture Collection SEMIA", of the FEPAGRO-MIRCEN [Fundação Estadual de Pesquisa Agropecuária (Rio Grande do Sul, Brazil)-Microbiological Resources Center] recommended for use in commercial inoculants in Brazil are listed on Table 1, including the host legumes of origin and the legumes for which the strains are recommended. Taxonomic classification of the strains, based on the sequence analysis of the 16S rRNA gene, previously determined by Menna et al. (2006), is also included in Table 1. Other information about the strains and their host legumes is available elsewhere (Menna et al., 2006).

Fourteen reference strains were used as follows: *Bradyrhizobium japonicum* USDA 6^T, *Bradyrhizobium liaoningense* USDA 3622^T, *Bradyrhizobium elkanii* USDA 76^T, *B. japonicum* USDA 110, *Rhizobium tropici* type A CFN 299, *R. tropici* type B CIAT 899^T, *Rhizobium leguminosarum* USDA 2370^T, *Rhizobium giardini* $H152^T$, *Sinorhizobium (Ensifer) fredii* USDA 205^T, *Sinorhizobium (Ensifer) meliloti* USDA 1002^T, *Mesorhizobium loti* USDA 3471^T, *Mesorhizobium ciceri* USDA 3383^T, *Azorhizobium caulinodans* USDA 4892^T and *Rhizobium etli* CFN 42^T. They were provided by USDA, Beltsville, USA, and by the Centro de Ciéncias Genómicas, Cuernavaca, Mexico, and are deposited at the ''Diazotrophic and PGPR Culture Collection'' of Embrapa Soja. Preparation of stock cultures and strains maintenance were as described before (Menna et al., 2006).

DNA extraction and rep-PCR (BOX) genomic fingerprinting

Total genomic DNA of each strain was extracted as described by Kaschuk et al. (2006), and amplified by PCR with the primer BOX A1R (Versalovic et al., 1994; Koeuth et al., 1995). Amplification procedures were performed as described by Kaschuk et al. (2006), and the 1-kb DNA marker (Invitrogen™) was included on the left, right and in

Table 1. Strains used in this study.

SEMIA strain	Original host	Host for which the strain is recommended	Source of of the strain	Taxonomy based 16S rRNA[a]	Gene Bank access no.[a]
103	*Medicago polymorpha*	*Medicago polymorpha*	Brazil	*Sinorhizobium meliloti*	AY904726
134	*Medicago sativa*	*Medicago sativa*	Brazil	*Sinorhizobium meliloti*	AY904727
135	*Medicago sativa*	*Medicago sativa*	Brazil	*Sinorhizobium meliloti*	AY904728
222	*Trifolium subterraneum*	*Trifolium pratense; Trifolium repens; Trifolium subterraneum*	Australia	*Rhizobium leguminosarum*	AY904729
384	*Vicia* sp.	*Vicia sativa*	Brazil	*Rhizobium etli*	AY904730
396	Not known	*Cicer arietinum*	USA	*Mesorhizobium ciceri*	AY904731
587	*Glycine max*	*Glycine max*	Brazil	*Bradyrhizobium elkanii*	AF234890
656	*Neonotonia wightii*	*Desmodium intortum; Macroptilium atropurpureum; Neonotonia wightii*	Brazil	*Bradyrhizobium* sp.	AY904732
658	*Lotononis bainesii*	*Lotononis bainesii*	South Africa	*Methylobacterium* sp.	AY904733
662	*Vigna unguiculata*	*Lablab purpureus; Vigna unguiculata*	Australia	*Bradyrhizobium elkanii*	AY904734
695	*Neonotonia wightii*	*Lablab purpureus; Neonotonia wightii*	Australia	*Bradyrhizobium elkanii*	AY904735
696	*Desmodium uncinatum*	*Desmodium uncinatum*	Australia	*Bradyrhizobium elkanii*	AY904736
816	*Lotus corniculatus*	*Lotus corniculatus*	Brazil	*Mesorhizobium* sp.	AY904737
830	*Lotus corniculatus*	*Lotus glaber*	USA	*Mesorhizobium* sp.	AY904738
938	*Lupinus albus*	*Lupinus albus*	USA	*Bradyrhizobium elkanii*	AY904739
2051	*Trifolium vesiculosum*	*Trifolium vesiculosum*	Brazil	*Rhizobium leguminosarum*	AY904740
2081	*Trifolium pratense*	*Trifolium pratense*	Brazil	*Rhizobium leguminosarum*	AY904741
3007	*Pisum sativum*	*Pisum sativum*	Mexico	*Rhizobium leguminosarum*	AY904742
5019	*Glycine max*	*Glycine max*	Brazil	*Bradyrhizobium elkanii*	AF237422
5079	*Glycine max*	*Glycine max*	Brazil	*Bradyrhizobium japonicum*	AF234888
5080	*Glycine max*	*Glycine max*	Brazil	*Bradyrhizobium japonicum.*	AF234889
6002	*Vigna unguiculata*	*Vigna unguiculata*	Zimbabwe	*Bradyrhizobium japonicum*	AY904743
6028	*Desmodium uncinatum*	*Desmodium incanum*	Zimbabwe	*Bradyrhizobium elkanii*	AY904744
6053	*Clitoria ternatea*	*Clitoria ternatea*	Malaysia	*Bradyrhizobium elkanii*	AY904745
6069	*Leucaena leucocephala*	*Leucaena leucocephala*	Brazil	*Bradyrhizobium elkanii*	AY904746
6070	*Leucaena leucocephala*	*Leucaena leucocephala*	Brazil	*Rhizobium* sp.	AY904747
6100	*Falcataria moluccana*	*Erythrina verna; Falcataria moluccana*	Brazil	*Bradyrhizobium elkanii*	AY904748
6101	*Dalbergia nigra*	*Dalbergia nigra*	Brazil	*Bradyrhizobium elkanii*	AY904749
6144	*Arachis hypogaea*	*Arachis hypogaea*	Zimbabwe	*Bradyrhizobium* sp.	AY904750
6145	*Arachis hypogaea*	*Crotalaria juncea; Cyamopsis tetragonoloba; Vigna unguiculata*	Libya	*Bradyrhizobium* sp.	AY904751
6146	*Centrosema* sp.	*Centrosema* spp.	Brazil	*Bradyrhizobium elkanii*	AY904752
6148	*Neonotonia wightii*	*Neonotonia wightii*	Brazil	*Bradyrhizobium* sp.	AY904753
6149	*Galactia striata*	*Galactia striata; Macrotyloma axillare*	Australia	*Bradyrhizobium elkanii*	AY904754
6150	*Acacia mearnsii*	*Galactia striata*	Brazil	*Bradyrhizobium elkanii*	AY904755
6152	*Calopogonium* sp.	*Calopogonium* spp.	Brazil	*Bradyrhizobium japonicum*	AY904756
6155	*Stylosanthes* sp.	*Stylosanthes* spp.	Brazil	*Bradyrhizobium japonicum*	AY904757
6156	*Crotalaria spectabilis*	*Cajanus cajan; Canavalia ensiformis; Crotalaria juncea; Crotalaria spectabilis; Indigofera hirsuta*	Brazil	*Bradyrhizobium* sp.	AY904758
6157	*Cajanus cajan*	*Cajanus cajan*	Brazil	*Bradyrhizobium elkanii*	AY904759
6158	*Crotalaria spectabilis*	*Canavalia ensiformis; Crotalaria spectabilis; Indigofera hirsuta; Stizolobium aterrimum*	Brazil	*Bradyrhizobium elkanii*	AY904760
6159	*Enterolobium ellipticum*	*Enterolobium contortisiliquum; Enterolobiumcyclocarpum; Enterolobium timbouva*	Brazil	*Bradyrhizobium elkanii*	AY904761

Table 1. Strains used in this study. *Continued*

SEMIA strain	Original host	Host for which the strain is recommended	Source of of the strain	Taxonomy based 16S rRNA[a]	Gene Bank access no.[a]
6160	*Albizia lebbeck*	*Albizia lebbeck; Sclerolobium paniculatum*	Brazil	*Bradyrhizobium* sp.	AY904762
6161	*Prosopis juliflora*	*Prosopis juliflora*	Brazil	*Sinorhizobium* sp.	AY904763
6163	*Acacia mearnsii*	*Acacia mearnsii*	Brazil	*Bradyrhizobium* sp.	AY904764
6164	*Acacia mearnsii*	*Acacia decurrens; Acacia mearnsii*	Brazil	*Bradyrhizobium* sp.	AY904765
6165	*Mimosa scabrella*	*Mimosa scabrella*	Brazil	*Rhizobium* sp.	AY904766
6166	*Mimosa caesalpiniifolia*	*Mimosa caesalpiniifolia*	Brazil	*Burkholderia* sp.	AY904767
6167	*Mimosa caesalpiniifolia*	*Mimosa caesalpiniifolia*	Brazil	*Burkholderia* sp.	AY904768
6168	*Gliricidia sepium*	*Gliricidia sepium; Leucaena diversifolia*	Brazil	*Rhizobium* sp.	AY904769
6169	*Falcataria moluccana*	*Falcataria moluccana; Leucaena diversifolia*	Brazil	*Bradyrhizobium elkanii*	AY904770
6175	*Pueraria phaseoloides*	*Pueraria phaseoloides*	Brazil	*Bradyrhizobium elkanii*	AY904771
6192	*Tipuana tipu*	*Tipuana tipu*	Brazil	*Bradyrhizobium* sp.	AY904772
6208	*Desmodium heterocarpon*	*Desmodium heterocarpon* subsp. *ovalifolium*	Colombia	*Bradyrhizobium elkanii*	AY904773
6319	*Arachis* sp.	*Cyamopsis tetragonoloba*	Bolivia	*Bradyrhizobium* sp.	AY904774
6382	*Mimosa caesalpiniifolia*	*Mimosa caesalpiniifolia*	Brazil	*Burkholderia* sp.	AY904775
6383	*Mimosa caesalpiniifolia*	*Mimosa acutistipula*	Brazil	*Rhizobium* sp.	AY904776
6384	*Mimosa obovata*	*Mimosa acutistipula*	Brazil	*Bradyrhizobium elkanii*	AY904777
6387	*Acacia auriculiformis*	*Acacia auriculiformis; Acacia mangium*	Brazil	*Bradyrhizobium elkanii*	AY904778
6390	*Acacia decurrens*	*Acacia decurrens*	Brazil	*Burkholderia cepacia*	AY904779
6391	*Acacia auriculiformis*	*Acácia auriculiformis*	Brazil	*Bradyrhizobium* sp.	AY904780
6394	*Ormosia nitida*	*Ormosia nitida*	Brazil	*Burkholderia cepacia*	AY904781
6398	*Piptadenia stipulacea*	*Piptadenia gonoacantha; Piptadenia stipulacea*	Brazil	*Burkholderia* sp.	AY904782
6401	*Sesbania virgata*	*Sesbania virgata*	Brazil	*Azorhizobium* sp.	AY904783
6402	*Sesbania virgata*	*Sesbania virgata*	Brazil	*Azorhizobium* sp.	AY904784
6412	*Clitoria fairchildiana*	*Clitoria fairchildiana*	Brazil	*Burkholderia* sp.	AY904785
6420	*Acacia mangium*	*Acacia mangium; Sclerolobium paniculatum*	Brazil	*Bradyrhizobium* sp.	AY904786
6424	*Centrosema pubescens*	*Centrosema* spp.	Brazil	*Bradyrhizobium elkanii*	AY904787
6425	*Centrosema pubescens*	*Centrosema* spp.	Brazil	*Bradyrhizobium elkanii*	AY904788
6440	*Arachis pintoi*	*Arachis pintoi*	Brazil	*Bradyrhizobium* sp.	AY904789

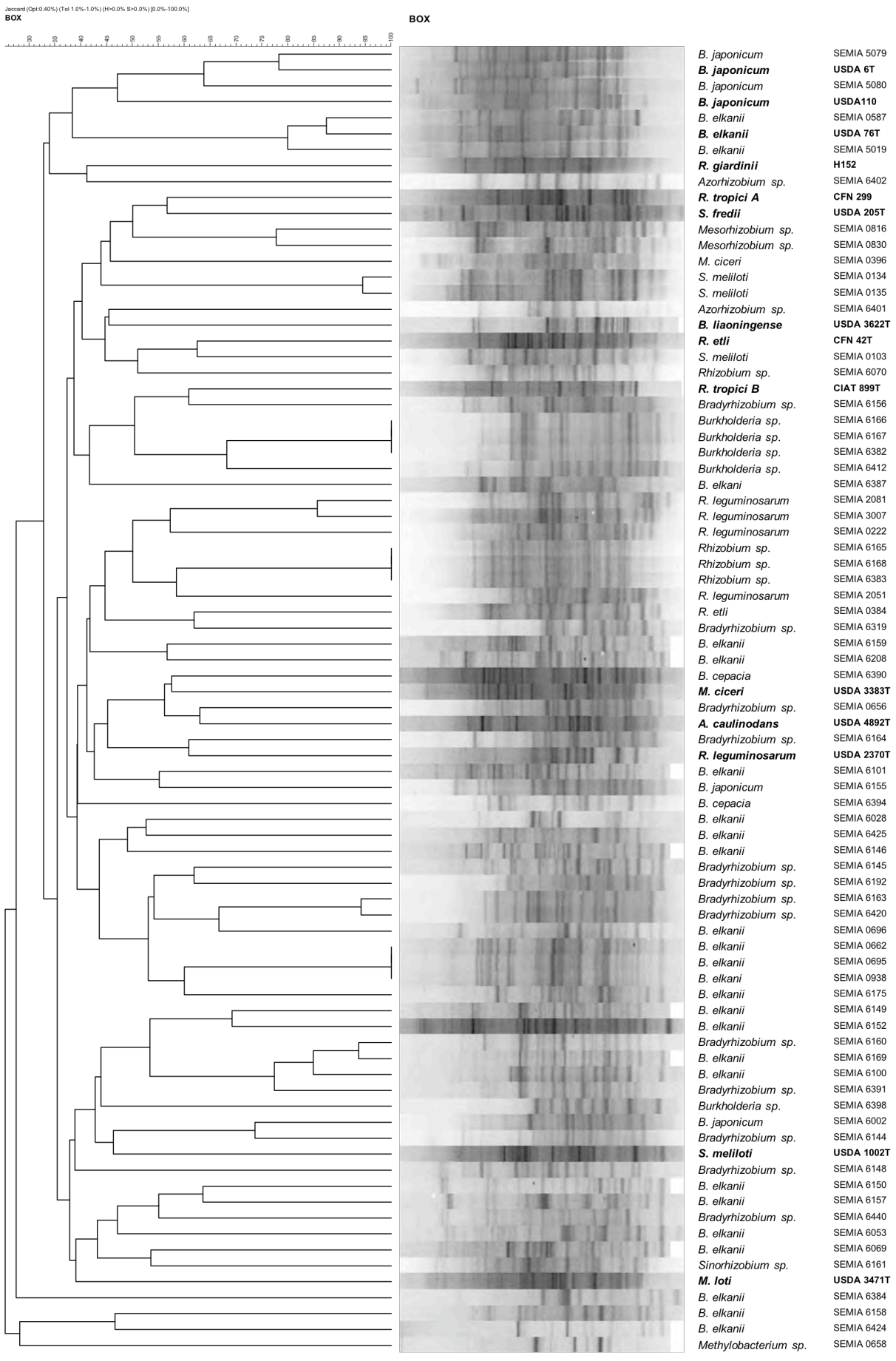
[a]After Menna et al. (2006).

the centre of each gel. The amplified fragments were separated by horizontal electrophoresis on 1.5% agarose, as described before (Kaschuk et al., 2006). Gels were stained with ethidium bromide, visualized under UV radiation and photographed.

Cluster analyses

First, the sizes of the fragments in all analyses were normalized according to the sizes of the DNA marker. Cluster analyses of the BOX-PCR profiles and of the 16S rRNA sequences were performed using the Bionumerics program (Applied Mathematics, Kortrijh, Belgium, version 4.6), with the UPGMA algorithm (unweighted pair-group method, with arithmetic mean) (Sneath and Sokal, 1973). In the BOX-PCR, the Jaccard coefficient (Jaccard, 1912) was used, considering the optimum values indicated by the Bionumerics program for the tolerance and the optimization parameters.

After cluster analyses of each experiment (16S rRNA and BOX-PCR) and of the polyphasic analysis (16S rRNA + BOX-PCR), matrices were constructed. In the polyphasic analysis, different ratios (5:5; 6:4; 7:3; 8:2 and 9:1; 16S rRNA:BOX-PCR) were used. Congruence of each experiment was calculated according to the Bionumerics program.


Jaccard (Opt:0.40%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
BOX
30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
BOX
B. japonicum SEMIA 5079
B. japonicum USDA 6T
B. japonicum SEMIA 5080
B. japonicum USDA110
B. elkanii SEMIA 0587
B. elkanii USDA 76T
B. elkanii SEMIA 5019
R. giardinii H152
Azorhizobium sp. SEMIA 6402
R. tropici A CFN 299
S. fredii USDA 205T
Mesorhizobium sp. SEMIA 0816
Mesorhizobium sp. SEMIA 0830
M. ciceri SEMIA 0396
S. meliloti SEMIA 0134
S. meliloti SEMIA 0135
Azorhizobium sp. SEMIA 6401
B. liaoningense USDA 3622T
R. etli CFN 42T
S. meliloti SEMIA 0103
Rhizobium sp. SEMIA 6070
R. tropici B CIAT 899T
Bradyrhizobium sp. SEMIA 6156
Burkholderia sp. SEMIA 6166
Burkholderia sp. SEMIA 6167
Burkholderia sp. SEMIA 6382
Burkholderia sp. SEMIA 6412
B. elkani SEMIA 6387
R. leguminosarum SEMIA 2081
R. leguminosarum SEMIA 3007
R. leguminosarum SEMIA 0222
Rhizobium sp. SEMIA 6165
Rhizobium sp. SEMIA 6168
Rhizobium sp. SEMIA 6383
R. leguminosarum SEMIA 2051
R. etli SEMIA 0384
Bradyrhizobium sp. SEMIA 6319
B. elkanii SEMIA 6159
B. elkanii SEMIA 6208
B. cepacia SEMIA 6390
M. ciceri USDA 3383T
Bradyrhizobium sp. SEMIA 0656
A. caulinodans USDA 4892T
Bradyrhizobium sp. SEMIA 6164
R. leguminosarum USDA 2370T
B. elkanii SEMIA 6101
B. japonicum SEMIA 6155
B. cepacia SEMIA 6394
B. elkanii SEMIA 6028
B. elkanii SEMIA 6425
B. elkanii SEMIA 6146
Bradyrhizobium sp. SEMIA 6145
Bradyrhizobium sp. SEMIA 6192
Bradyrhizobium sp. SEMIA 6163
Bradyrhizobium sp. SEMIA 6420
B. elkanii SEMIA 0696
B. elkanii SEMIA 0662
B. elkanii SEMIA 0695
B. elkani SEMIA 0938
B. elkanii SEMIA 6175
B. elkanii SEMIA 6149
B. elkanii SEMIA 6152
Bradyrhizobium sp. SEMIA 6160
B. elkanii SEMIA 6169
B. elkanii SEMIA 6100
Bradyrhizobium sp. SEMIA 6391
Burkholderia sp. SEMIA 6398
B. japonicum SEMIA 6002
Bradyrhizobium sp. SEMIA 6144
S. meliloti USDA 1002T
Bradyrhizobium sp. SEMIA 6148
B. elkanii SEMIA 6150
B. elkanii SEMIA 6157
Bradyrhizobium sp. SEMIA 6440
B. elkanii SEMIA 6053
B. elkanii SEMIA 6069
Sinorhizobium sp. SEMIA 6161
M. loti USDA 3471T
B. elkanii SEMIA 6384
B. elkanii SEMIA 6158
B. elkanii SEMIA 6424
Methylobacterium sp. SEMIA 0658

3. Results

The sixty-eight elite strains used in this study had been isolated from forty-seven leguminous hosts; no information was available on the original host of one strain (SEMIA 396). They are recommended for use in commercial inoculants in Brazil, for application to sixty-six legumes (Table 1). Complex fingerprinting patterns with multiple distinct bands of various intensities were obtained for the strains in the BOX-PCR analysis, with the smallest number of bands (seven) observed for SEMIA 658, a *Methylobacterium* sp. isolated from *Lotononis bainesii*, whereas the others showed an average of twenty bands (Fig. 1).

To confirm the reproducibility of the BOX-PCR fingerprinting protocol, the strains were analyzed three times, starting from the DNA extraction. Visual comparisons of the band profiles revealed good agreement, and coefficients of similarity for profiles of the same strain were estimated at 94% or higher.

Although we usually adopt a tolerance of 2% in clustering analyses of BOX-PCR products (e.g., Alberton et al., 2006; Kaschuk et al., 2006), in this study we considered the levels of tolerance and optimization indicated by the Bionumerics program, which were more strict, of 1.0% and 0.4, respectively. The resulting dendrogram identified sixty-two distinct profiles for the sixty-eight strains analyzed, and, together with the type and reference strains, were clustered at a very low level of similarity, only 25% (Fig. 1). Interestingly, some strains that had been selected based exclusively on their symbiotic properties showed identical or very similar profiles. In this category are strains isolated from and recommended for the same host legume, such as *Sinorhizobium meliloti* strains SEMIAs 134 and 135, isolated from alfalfa (*Medicago sativa*), and *Burkholderia* sp. SEMIAs 6166, 6167 and 6383, isolated from *Mimosa caesalpiniifolia*. In addition, highly similar profiles were obtained from strains isolated from hosts belonging to the same genus, such as *Bradyrhizobium* sp. SEMIAs 6163 (*Acacia mearnsii*) and 6420 (*Acacia mangium*). However, other strains showing identical or almost identical profiles had been isolated from hosts belonging to different genera: *Rhizobium* sp. SEMIAs 6165 (*Mimosa scabrella*), 6168 (*Gliricidia sepium*) and 6383 (*M. caesalpiniifolia*); *Bradyrhizobium elkanii* SEMIAs 662 (*Vigna unguiculata*), 695 (*Neonotonia wightii*) and 938 (*Lupinus albus*); and *Bradyrhizobium* sp. SEMIAs 6160 (*Albizia lebbeck*) and *B. elkanii* 6169 (*Falcataria moluccana*) (Fig. 1). Strains sharing similar BOX-PCR profiles also showed strong similarity – 99% or higher – in the sequences of the ribosomal gene 16S RNA. In general, strains positioned in the same BOX-PCR group were not related in terms of the original host of derivation.

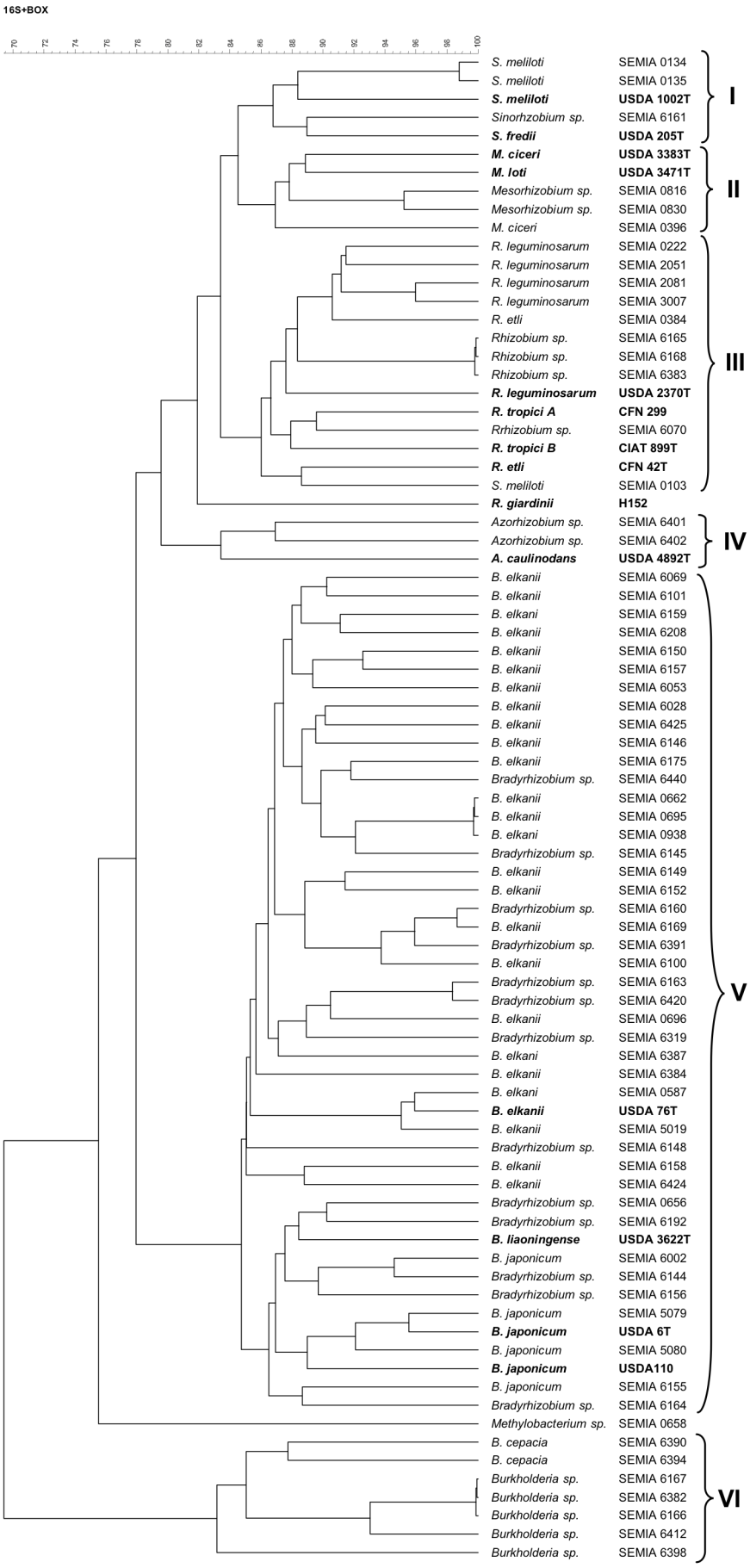
In the BOX-PCR dendrogram, some strains belonging to the same species were positioned in more than one cluster, e.g. *B. japonicum* and *S. meliloti* (Fig. 1). However, considering a cut off of 70%, as suggested for diversity studies of tropical rhizobia using rep-PCR (Grange and Hungria, 2004; Alberton et al., 2006; Kaschuk et al., 2006), although a species might be found in more than one cluster, strains belonging to different species never showed similarity higher than 70% (Fig. 1).

The BOX-PCR technique was thus confirmed to be powerful both in fingerprinting and in detecting high genetic diversity among rhizobial strains. However, the results obtained also illustrate difficulties of using the profiles for grouping or defining species and even genera. On the other hand, the dendrogram resulting from the analysis of the 16S rRNA (Menna et al., 2006) was probably very conservative in showing putative new species. Therefore, in this study we also investigated the possibility of using a combined analysis of the sequences of the 16S rRNA gene and of the BOX-PCR products for a more accurate taxonomic classification of the strains.

After transforming the 16S rRNA sequencing data into a similarity matrix, it was possible to perform a polyphasic analysis and to determine the correlations between the dendrograms built with the BOX-PCR profiles, with the 16S rRNA sequences and with the combination of 16S rRNA + BOX-PCR. First, the correlation between BOX-PCR products and 16S rRNA sequences was very low, 7.6%. Then, several ratio values were given to the matrices and, as expected, the correlation with the 16S rRNA clustering analysis obtained by Menna et al. (2006) decreased as the weight of the BOX-PCR analysis increased (Table 2). In contrast, when the weight of the BOX-PCR analysis was decreased, genetic diversity was lower in comparison to the BOX-PCR dendrogram (data not shown). We found that, starting from a proportion of 6:4 (60% 16S rRNA:40% BOX-PCR), good correlations were obtained between the combined dendrogram and the cluster analysis of the 16S rRNA (Table 2), which allowed detection of higher genetic diversity in comparison to the clustering based on the 16S rRNA sequences.

Although few differences were detected in the clusters formed in the dendrograms obtained at BOX-PCR ratios of 6:4, 7:3, 8:2 and 9:1% of 16S rRNA:BOX, we chose the combined analysis at a ratio of 8:2, which showed a high

Figure 1. *See figure on opposite page.* Cluster analysis (UPGMA with the coefficient of Jaccard) of the products obtained by BOX-PCR analysis. Reference and type strains are labeled and SEMIA strains are classified according to the sequencing analysis of the 16S rRNA gene, as described by Menna et al. (2006). More information about the strains is given on Table 1.


16S+BOX
70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100
S. meliloti SEMIA 0134
S. meliloti SEMIA 0135
S. meliloti USDA 1002T
Sinorhzobium sp. SEMIA 6161
S. fredii USDA 205T
I
M. ciceri USDA 3383T
M. loti USDA 3471T
Mesorhizobium sp. SEMIA 0816
Mesorhizobium sp. SEMIA 0830
M. ciceri SEMIA 0396
II
R. leguminosarum SEMIA 0222
R. leguminosarum SEMIA 2051
R. leguminosarum SEMIA 2081
R. leguminosarum SEMIA 3007
R. etli SEMIA 0384
Rhizobium sp. SEMIA 6165
Rhizobium sp. SEMIA 6168
Rhizobium sp. SEMIA 6383
R. leguminosarum USDA 2370T
R. tropici A CFN 299
Rrhizobium sp. SEMIA 6070
R. tropici B CIAT 899T
R. etli CFN 42T
S. meliloti SEMIA 0103
III
R. giardinii H152
Azorhizobium sp. SEMIA 6401
Azorhizobium sp. SEMIA 6402
A. caulinodans USDA 4892T
IV
B. elkanii SEMIA 6069
B. elkanii SEMIA 6101
B. elkani SEMIA 6159
B. elkanii SEMIA 6208
B. elkanii SEMIA 6150
B. elkanii SEMIA 6157
B. elkanii SEMIA 6053
B. elkanii SEMIA 6028
B. elkanii SEMIA 6425
B. elkanii SEMIA 6146
B. elkanii SEMIA 6175
Bradyrhizobium sp. SEMIA 6440
B. elkanii SEMIA 0662
B. elkanii SEMIA 0695
B. elkani SEMIA 0938
Bradyrhizobium sp. SEMIA 6145
B. elkanii SEMIA 6149
B. elkanii SEMIA 6152
Bradyrhizobium sp. SEMIA 6160
B. elkanii SEMIA 6169
Bradyrhizobium sp. SEMIA 6391
B. elkanii SEMIA 6100
Bradyrhizobium sp. SEMIA 6163
Bradyrhizobium sp. SEMIA 6420
B. elkanii SEMIA 0696
Bradyrhizobium sp. SEMIA 6319
B. elkani SEMIA 6387
B. elkanii SEMIA 6384
B. elkani SEMIA 0587
B. elkanii USDA 76T
B. elkanii SEMIA 5019
Bradyrhizobium sp. SEMIA 6148
B. elkanii SEMIA 6158
B. elkanii SEMIA 6424
Bradyrhizobium sp. SEMIA 0656
Bradyrhizobium sp. SEMIA 6192
B. liaoningense USDA 3622T
B. japonicum SEMIA 6002
Bradyrhizobium sp. SEMIA 6144
Bradyrhizobium sp. SEMIA 6156
B. japonicum SEMIA 5079
B. japonicum USDA 6T
B. japonicum SEMIA 5080
B. japonicum USDA110
B. japonicum SEMIA 6155
Bradyrhizobium sp. SEMIA 6164
V
Methylobacterium sp. SEMIA 0658
B. cepacia SEMIA 6390
B. cepacia SEMIA 6394
Burkholderia sp. SEMIA 6167
Burkholderia sp. SEMIA 6382
Burkholderia sp. SEMIA 6166
Burkholderia sp. SEMIA 6412
Burkholderia sp. SEMIA 6398
VI

correlation, almost 95%, with the 16S rRNA genes (Table 2). Considering a cutoff of 90% in the combined analysis, fifty-one distinct profiles were defined (Fig. 2), therefore showing considerably higher genetic diversity than the 16S rRNA analysis alone (Menna et al., 2006).

The polyphasic analysis of BOX-PCR profiles and 16S rRNA sequences also defined some major clusters (Fig. 2), and, in general, they were similar to those observed in the phylogenetic tree of the 16S rRNA (Menna et al., 2006). The two first clusters included strains belonging to the genera *Sinorhizobium* (I) and *Mesorhizobium* (II). Fourteen strains were linked in cluster III, thirteen of them of the genus *Rhizobium*; the exception was strain SEMIA 103 of *S. meliloti*. The *R. giardinii* type strain occupied an isolated position and was not included in cluster III. High genetic diversity was observed among the three *Azorhizobium* strains, which were linked in cluster IV with relatively low similarity, 84%. Forty-six *Bradyrhizobium* strains were linked in the major cluster V, but several subclusters were also observed. In the upper part of cluster V, the first subclusters included thirty-four strains showing higher 16S rRNA gene similarity with *B. elkanii* or *Bradyrhizobium* sp. In the lower part of cluster V, twelve strains had stronger 16S rRNA similarity with *B. japonicum*, except for the type strain of *B. liaoningense*, clustered with *Bradyrhizobium* sp. strains SEMIAs 656 and 6192, and three other *Bradyrhizobium* sp. strains, SEMIAs 6144, 6156, and 6164. The only strain of genus *Methylobacterium* was linked to all previous strains at a low level of similarity, of 76%. Finally, cluster VI linked seven strains belonging to the genus *Burkholderia*, and the cluster was joined to the others at a level of similarity of 70%. When compared to the phylogenetic tree based on the 16S rRNA (Menna et al., 2006), the clusters and subclusters obtained in the combined analysis of BOX and 16S rRNA resulted in better definition of the genotypes.

Table 2. Correlation between the 16S rRNA sequencing analysis and the polyphasic analysis of 16S rRNA+ BOX-PCR considering different ratios of each one.

Polyphasic dendrogram 16S rRNA:BOX-PCR	16S rRNA dendrogram
50%:50%	64.7%
60%:40%	76.9%
70%:30%	87.3%
80%:20%	94.8%
90%:10%	98.8%

4. Discussion

Particularly in the last 15 years, DNA-typing methods employed in diversity studies have greatly improved our understanding of bacterial phylogeny and taxonomy. Among those methods, rep-PCR analysis might be highlighted for being relatively quick, easy and inexpensive, therefore useful in the screening of large numbers of isolates (Hulton et al., 1991; Versalovic et al., 1991, 1994; de Bruijn, 1992; Martin et al., 1992; Koeuth et al., 1995). rep-PCR is also useful for fingerprinting rhizobia (e.g., de Bruijn, 1992; Laguerre et al., 1997); it has been used in our laboratory largely for typing indigenous or naturalized strains (Hungria et al., 1998, 2000; 2006; Santos et al., 1999; Chen et al., 2000; Ferreira and Hungria, 2002; Fernandes et al., 2003; Galli-Terasawa et al., 2003; Grange and Hungria, 2004; Alberton et al., 2006; Kaschuk et al., 2006; Barcellos et al., 2007; Batista et al., 2007; Pinto et al., 2007).

In this study, profiles of BOX-PCR for sixty-eight rhizobial strains were obtained, and their reproducibility confirmed, corroborating previous reports (Schneider and de Bruijn, 1996). In contrast with diversity studies, the SEMIA strains from this study represent elite diazotrophic symbiotic bacteria identified in selection programs performed in the last 40 years in Brazil. The strains had been isolated from forty-seven leguminous species, including hosts belonging to all three subfamilies of the Leguminosae, and, therefore, were expected to be diverse. Among the strains from this study collectively called rhizobia, were eight belonging to two newly described symbiotic genera, *Methylobacterium* and *Burkholderia*. These sixty-eight elite strains are officially recommended for the production of inoculants for sixty-six legumes, and as reliable BOX-PCR profiles were easily obtained for all of them, we suggest that the method should be included as an important tool for control of inoculant quality, not only in Brazil, but also in other countries where rhizobial inoculants are produced commercially.

Another critical issue in the determination of diversity and its biotechnological potential is the taxonomy of elite microorganisms. Since the 1970s, when Carl R. Woese and coworkers began sequencing 16S rRNA genes, our knowledge of phylogeny and taxonomy of bacteria has greatly increased, resulting in profound changes (Woese, 1987; Woese et al., 1990). Nevertheless, as highlighted by Garrity and Holt (2001), only Phylum, Class, Order, Family and Genera can be defined by sequencing 16S rRNA genes.

Figure 2. *See figure on opposite page.* Polyphasic cluster analysis considering a ratio of 80%:20%, 16S rRNA sequences:BOX-PCR products. Reference and type strains are labeled and SEMIA strains are classified according to the sequencing analysis of the 16S rRNA gene, as described by Menna et al. (2006). More information about the strains is given on Table 1.

Species determined still depended on a polyphasic approach that includes both phenotypic (e.g., biochemical properties, fatty-acid composition) and genotypic (e.g., rRNA gene sequences, DNA fingerprints) data. Isolates differing by 3% or more of the 16S rRNA bases are probably different species, but DNA-DNA-hybridization experiments are still required for definition of new species (Garrity and Holt, 2001). In the DNA-DNA hybridization method, isolates with more than 70% DNA-DNA-binding values and less than 5% difference in their melting temperature (ΔTm) should belong to the same species, whereas those sharing less than 50% of DNA-DNA-binding values belong to different species (Vandamme et al., 1996; Stackebrandt, et al., 2002; Coenye et al., 2005; Gevers et al., 2005). However, few laboratories routinely perform time-consuming, complex DNA-DNA hybridization analyses, representing a major limitation to microbial diversity studies (Rademaker et al., 2000).

The apparent evolutionary conservation of the repetitive elements used in rep-PCR methods is the main property enabling their use for fingerprinting bacteria (de Bruijn et al., 1996; Koeuth et al., 1995), but the application of rep-PCR in phylogeny or taxonomy studies remains to be confirmed. An exhaustive study was performed with a *Xanthomonas* collection, in which rep-PCR profiles were compared to DNA-DNA homology groups (Rademaker et al., 2000). Interestingly, the authors found high correlation between both methods, such that ERIC-REP-PCR fingerprint similarity values above 0.5 correlated with values exceeding 70% of DNA-DNA homology, and above 0.8 correlated with values exceeding 90% (Rademaker et al., 2000).

In our study, we opted for BOX-PCR analysis because in several studies we found that it results in more bands than with ERIC and REP primers. In the cluster analysis, strains belonging to different species were never grouped at a level of similarity higher than 70%; however, some strains belonging to the same species were positioned in more than one cluster. Additionally, the correlation between the BOX-PCR and the 16S rRNA was very low, 7.6%. Previous reports have mentioned lack of cohesion between rep-PCR grouping and taxonomic classification (Laguerre et al., 1997; Mostasso et al., 2002; Fernandes et al., 2003; Grange and Hungria, 2004; Hungria et al., 2006), but those studies did not include detailed comparisons. Our results indicate that the use of rep-PCR for taxonomy of rhizobia is indeed very limited. Differences between BOX-PCR and 16S rRNA can be attributed to several issues; for example, high rates of horizontal gene transfer that have been reported in rhizobia (e.g., Sullivan et al., 1995; Barcellos et al., 2007; Batista et al., 2007), potentially affecting the rep-PCR profiles.

When the 16S rRNA sequences of the sixty-eight elite strains were determined, despite a high level of genetic diversity and strong indications of several new species, there were indications that diversity was underestimated in some genera (Menna et al., 2006), for example *Bradyrhizobium* (Menna et al., 2006), consistent with previous indications of its low diversity in terms of the 16S rRNA gene (e.g., Vinuesa et al., 1998; Chen et al., 2000; Willems et al., 2001). The analysis of other ribosomal regions, such as the 23S rRNA and the intergenic space between the 16S and the 23S rRNA can greatly improve speciation, at least in *Bradyrhizobium* (Germano et al., 2006), and other conserved genes might also be useful. Besides using rep-PCR profiles for fingerprinting strains, we have investigated if it might also be helpful in taxonomy and phylogeny studies.

When adopting a polyphasic approach, the topology of the dendrograms resulting from the combined analysis of 6:4, 7:3, 8:2, 9:1 (16S rRNA:BOX-PCR) correlated well with the 16S rRNA analysis (from 77 to 99%), and also showed higher definition of the genotypes, resulting in clearer indication of the taxonomic groups. One major example was observed in the genus *Bradyrhizobium*: much higher genetic diversity was obtained in the polyphasic approach in comparison to the 16S rRNA analysis. Therefore we suggest the use of this polyphasic analysis – good results were obtained with the ratio 8:2 (16S rRNA:BOX-PCR) – as a rapid and more precise method of determining taxonomic diversity and phylogenetic structure of large collections of rhizobial isolates.

In conclusion, most of the large collections of rhizobia that exist worldwide are poorly characterized. Interest in biological nitrogen fixation should increase in the next decade, not only due to concerns about the indiscriminate use of N-fertilizers and their greenhouse effects, but also because prices of these fertilizers are expected to increase considerably. Therefore, we suggest, as an initial step in comparing strains from rhizobial collections, that they be fingerprinted with BOX-PCR. Later, in addition to sequencing of the 16S rRNA of representative or elite strains, polyphasic analyses of BOX-PCR and 16S rRNA may improve our understanding of the phylogeny and taxonomy of rhizobia.

Acknowledgments

The work was partially supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil), projects "Coleção de Culturas" (552393/2005-3), "Edital Universal" 021/2005, and "Instituto do Milênio". The authors thank Ligia Maria Oliveira Chueire (Embrapa Soja) for help in several steps of this work and Dr. Fernando G. Barcellos and Allan R.J. Eaglesham for helpful discussion.

REFERENCES

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

Barcellos, F.G., Menna, P., Batista, J.S.S., and Hungria, M. 2007. Evidence of horizontal transfer of symbiotic genes from a *Bradyrhizobium japonicum* inoculant strain to indigenous *Sinorhizobium (Ensifer) fredii* and *Bradyrhizobium elkanii* in a Brazilian savannah soil. *Applied and Environmental Microbiology* **73**: 2635–2643.

Batista, J.S.S., Hungria, M., Barcellos, F.G., Ferreira, M.C., and Mendes, I.C. 2007. 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 Ecology* **53**: 270–284.

Chen, L.S., Figueredo, A., Pedrosa, F.O., and Hungria, M. 2000. Genetic characterization of soybean rhizobia in Paraguay. *Applied and Environmental Microbiology* **66**: 5099–5103.

Coenye, T., Gevers, D., Peer, Y.V., Vandamme, P., and Swings, J. 2005. Towards a prokaryotic genomic taxonomy. *FEMS Microbiology Reviews* **29**: 147–167.

de Bruijin, F.J. 1992. Use of repetitive (repetitive extragenic palindormic and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacterial. *Applied Environmental Microbiology* **58**: 2180–2187.

de Bruijn, F.J., Rademaker, J., Schneider, M., Rossbach, U., and Louws, F.J. 1996. Rep-PCR genomic fingerprinting of plant-associated bacteria and computer-assisted phylogenetic analyses. In: *Biology of Plant-Microbe Interaction, Proceedings of the 8th International Congress of Molecular Plant-Microbe Interactions*. Stacey, G., Mullin, B., and Gresshoff, P., eds. Knoxville, APS Press, pp. 497–502.

Fernandes, M.F., Fernandes, R.P.M., and Hungria, M. 2003. Caracterização genética de rizóbios nativos dos tabuleiros costeiros eficientes em culturas do guandu e caupi. *Pesquisa Agropecuária Brasileira* **38**: 911–920.

Ferreira, M.C. and Hungria, M. 2002. Recovery of soybean inoculant strains from uncropped soils in Brazil. *Field Crops Research* **79**: 139–152.

Fred, E.B., Baldwin, I.L., and McCoy, E. 1932. *Root Nodule Bacteria of Leguminous Plants*. University of Wisconsin Press, Madison, USA.

Galli-Terasawa, L.V., Glienke-Blanco, C., and Hungria M. 2003. Diversity of a soybean rhizobial population adapted to a Cerrados soil. *World Journal of Microbiology and Biotechnology* **19**: 933–939.

Garrity, G.M. and Holt, J.G., 2001. The road map to the manual. In: *Bergey's Manual of Systematic Bacteriology*. Garrity, G.M., Boone, D.R., and Castenholz, R.W., eds. Springer-Verlag, New York. pp. 119–154.

Germano, M.G., Menna, P., Mostasso, F.L., and Hungria, M. 2006. RFLP analysis of the rRNA operon of a Brasilian collection of bradyhizobial strains from 33 legumes species. *International Journal of Systematic Evolutionary Microbiology* **56**: 217–229.

Gevers, D., Cohan, F.M., Lawrence J.G., Spratt, B.G., Coenye, T., Feil, E.J., Stackebrandt, E., de Peer, Y.V., Vandamme, P., Thompson, F.L., and Swings, J. 2005. Re-evaluating prokaryotic species. *Nature Reviews Microbiology* **3**: 733–739.

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

Hulton, C.S.J., Higgins, C.F., and Sharp, P.M. 1991. ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella typhimurium* and other enterobacteria. *Molecular Microbiology* **5**: 825–762.

Hungria, M. and Campo, R.J. 2007. Inoculantes microbianos: situação no Brasil. In: *Biofertilizantes en Iberoamérica: Visión Técnica, Científica y Empresarial*. Izaguirre-Mayoral, M.L., Labandera, C. and Sanjuan, J., eds. Cyted/Biofag, Montevideo. pp. 22–31.

Hungria, M., Chueire, L.M.O., Megías, M., Lamrabet, Y., Probanza, A., Guttierrez-Mañero, F.J., and Campo, R.J. 2006. Genetic diversity of indigenous tropical fast-growing rhizobia isolated from soybean nodules. *Plant and Soil* **288**: 343–356.

Hungria, M., Andrade, D.S., Chueire, L.M.O., Probanza, A., Guttierrez-Mañero, F.J., and 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., Boddey, L.H., Santos, M.A., and Vargas, M.A.T. 1998. Nitrogen fixation capacity and nodule occupancy by *Bradyrhizobium japonicum* and *B. elkanii* strains. *Biology and Fertility of Soils* **27**: 393–399.

Jaccard, P. 1912. The distribution of flora in the alpine zone. *New Phytologist* **11**: 37–50.

Jordan, D.C. 1984. Rhizobiaceae. In: *Bergey's Manual of Systematic Bacteriology*. Krieg, N.R. and Holt, J.R., eds. Williams & Wilkens Co, Baltimore, USA, v. 1, pp. 234–244.

Kaschuk, G., Hungria, M., Andrade, D.S., and Campo, R.J. 2006. Genetic diversity of rhizobia associated with common bean (*Phaseolus vulgaris* L.) grown under no-tillage and conventional systems in Southern Brazil. *Applied Soil Ecology* **32**: 210–220.

Koeuth, T., Versalovic, J., and Lupski, J.R. 1995. Differential subsequence conservation of interspersed repetitive *Streptococcus pneumoniae* BOX elements in diverse bacteria. *Genome Research* **5**: 408–418.

Laguerre, G., van Berkum, P., Amarger, N., and 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.

Louws, F.J., Fulbright, D.W., Stephens, C.T., and de Bruijn, F.J. 1994. Specific genomic fingerprinting of phytopathogenic *Xanthomonas* and *Pseudomonas pathovars* and strains generated with repetitive sequence and PCR. *Applied and Environmental Microbiology* **60**: 2286–2295.

Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M., Alloing, G., Hakenbeck, R., Morrison, D.A., Boulnois, G.J., and Claverys, J.P. 1992. A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. *Nucleic Acids Research* **20**: 3479–3483.

Menna, P., Hungria, M., Barcellos, F.G., Bangel, E.V., Hess, P.N., and Martinez-Romero, E. 2006. Molecular phylogeny based on the 16S rRNA gene of elite rhizobial strains used in Brazilian commercial inoculants. *Systematic and Applied Microbiology* **29**: 315–332.

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

Oyaizu, H., Naruhashi, N., and Gamou, T. 1992. Molecular methods of analysing bacterial diversity: The case of rhizobia. *Biodiversity and Conservation* **1**: 237–249.

Pinto, F.G.S., Hungria, M., and Mercante, F.M. 2007. Polyphasic characterization of Brazilian *Rhizobium tropici* strains effective in fixing N_2 with common bean (*Phaseolus vulgaris* L.). *Soil Biology and Biochemistry* **39**: 1851–1864.

Rademaker, J.L.W., Hoste, B., Louws, F.J., Kersters, K., Swings, J., Vauterin, L., Vauterin, P., and de Bruijin, F.J. 2000. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. *International Journal of Systematic and Evolutionary Microbiology* **50**: 665–677.

Santos, M.A., Vargas, M.A.T., and Hungria, M. 1999. Characterization of soybean bradyrhizobia strains adapted to the Brazilian cerrados region. *FEMS Microbiology Ecology* **30**: 261–272.

Schneider, M. and de Bruijn, F.J. 1996. Rep-PCR mediated genomic fingerprinting of rhizobia and computer-assisted phylogenetic pattern analysis. *World Journal of Microbiology and Biotechnology* **12**: 163–174.

Sneath, P.B.A. and Sokal, R.R. 1973. *Numerical Taxonomy.* W.H. Freeman & Co, San Francisco, USA.

Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A.D., Kampfer, P., Maiden, M.C.J., Nesme, X., Rossello-Mora, R., Swings, J., Truper, H.G., Vauterin, L., Ward, A.C., and Whitman, W.B. 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology* **52**: 1043–1047

Stern, M.J., Ames, G.F.L., Smith, N.H., Robinson, E.C., and Higgins, C.F. 1984. Repetitive extragenic palindromic sequences: a major component of the bacterial genome. *Cell* **37**: 1015–1026.

Sullivan, J.T., Patrick, H.N., Lowther, W.L., Scott, D.B., and Ronson, C.W. 1995. Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. *Proceedings of the National Academy of Sciences of USA* **92**: 8985–8989.

Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K., and Swings, J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiological Review* **60**: 407–438.

Versalovic, J., Koeuth, T., and Lupski, J.R. 1991. Distribution of repetitive DNA sequence in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* **19**: 6823–6831.

Versalovic, J., Schneider, M., de Bruijn, F.J., and Lupski, J.R.. 1994. Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). *Methods in Molecular and Cellular Biology* **5**: 25–40.

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

Willems, A., Coopman, R., and Gillis, M. 2001. Comparison of sequence analysis of 16S-23S rDNA spacer regions, AFLP analysis and DNA-DNA hybridizations in *Bradyrhizobium. International Journal of Systematic and Evolutionary Microbiology* **51**: 623–632.

Woese, C.R. 1987. Bacterial evolution. *Microbiological Reviews* **51**: 221–271.

Woese, C.R., Kandler, O., and Wheelis, M.L. 1990. Towards a natural system of organisms: proposal for the Domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences USA* **87**: 4576–4579

Young, J.M., Kuykendall, L.D., Martínez-Romero, E., Kerr, A., and Sawada, H. 2001. A revision of *Rhizobium* Frank 1889, with an emended description of the genus and inclusion of all species of Agrobacterium Conn, 1942 and *Allorhizobium undicola* de Lajudie et al., 1998, A new combination: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis. International Journal of Systematic and Evolutionary Microbiology* **51**: 89–103.