RFLP analysis of the rRNA operon of a Brazilian collection of bradyrhizobial strains from 33 legume species

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Genetic diversity in tropical rhizobial species is still poorly known. With the aim of increasing this knowledge, three ribosomal regions of 119 strains belonging to the official Brazilian culture collection of rhizobia and classified as *Bradyrhizobium* based on morphological and physiological characteristics *in vitro* were analysed by RFLP-PCR. The strains were isolated from 33 legume species, representing nine tribes and all three subfamilies; they all form very effective N_2-fixing nodules and 43 of them are recommended for use in Brazilian commercial inoculants as the most effective for their hosts. For the 16S rRNA gene, type and reference strains of *Bradyrhizobium japonicum* fell into two major clusters, joined at a level of similarity of 50 %, which included 52 strains, 90 % of which were isolated from soybean. Two other clusters, joined at a similarity of 53 %, included reference strains of *Bradyrhizobium elkanii*, but not USDA 76^T; furthermore, two other major clusters were identified and all strains were clustered at a final level of similarity of only 28 %. For the intergenic spacer (IGS) between genes coding for the 16S and 23S rRNA, strains were clustered at a final level of similarity of 27 %. Reference strains of *B. japonicum* fell into a major group with 51 strains, 84 % isolated from soybean, with a similarity of 59 %, while strains of *B. elkanii* fell into another major group, with a similarity of 55 %, clustering 44 strains, 59 % of which were isolated from hosts other than soybean. New clusters were also observed for the IGS region. The largest number of differences was detected in the analysis of the 23S rRNA gene, and 16 groups and isolated strains were joined at a very low level of similarity (16 %). In a combined analysis with the three ribosomal regions, the majority of strains isolated from soybean clustered with a similarity of 54 % with type and reference strains of *B. japonicum*, while most strains isolated from Brazilian indigenous legume species grouped with *B. elkanii* at a level of similarity of 46 %. All strains were clustered at a very low level of similarity (27 %), and at least two new clusters were clearly defined. These new clusters might be related to intraspecific differences or to novel subspecies, or even to novel species; indeed, strains from one of these clusters show higher 16S rRNA gene sequence similarity to members of the genus *Burkholderia*. The results obtained in this study emphasize the high level of diversity of symbiotic diazotrophic bacteria in the tropics that still remains to be determined.

INTRODUCTION

The Leguminosae (known as the Fabaceae in the USA) is one of the largest families of plants, with over 18 000 species classified into around 650 genera, representing approximately one-twelfth of all known flowering plants and occupying nearly all terrestrial biomes (Polhill & Raven, 1981). Many species within this family are capable of establishing symbioses with a group of bacteria collectively called rhizobia, of which the most important feature is the capacity for fixing atmospheric nitrogen (N_2) (Allen & Allen, 1981).

Until 1982, all bacteria isolated from root nodules were classified in the genus *Rhizobium*, and speciation was based on the formation of nodules with certain host plants, establishing the 'cross-inoculation group' concept (Fred *et al.*, 1932; Jordan, 1982). Based on morphological and physiological patterns, the bacteria were then split into the genera *Bradyrhizobium*, which included relatively slow growers

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Abbreviation: IGS, intergenic spacer.

Individual cluster analysis of PCR-RFLP products from the 16S and 23S rRNA genes and the 16S–23S IGS and details of the origins of the strains used and classification of their host species are available as supplementary material in IJSEM Online.

that produced an alkaline reaction in culture medium with mannitol as carbon source, and *Rhizobium*, which contained fast-growing acid producers (Jordan, 1982, 1984). Initially, *Bradyrhizobium japonicum* was the only described species within the genus (Jordan, 1982, 1984), but reports of a large genetic and physiological variability among strains that nodulate soybean (*Glycine max*) led to the description of *Bradyrhizobium elkanii* a few years later (Kuykendall *et al.*, 1992). Thereafter, other species were described: *Bradyrhizobium liaoningense*, extra-slowly growing rhizobia that nodulate primitive and modern soybean genotypes (Xu *et al.*, 1995), *Bradyrhizobium yuanmingense*, the symbiont of *Lespedeza* spp., a legume that grows in the northern hemisphere (Yao *et al.*, 2002), *Bradyrhizobium betae*, an endophyte that causes gall-like deformities on sugar beet (*Beta vulgaris*) and does not nodulate legumes (Rivas *et al.*, 2004), and *Bradyrhizobium canariense*, a symbiont of genistoid legumes from the Canary Islands (Vinuesa *et al.*, 2005a).

Ribosomal sequences, with the emphasis on the region that encodes the 16S rRNA, have become the tool of choice in molecular taxonomy for tracing bacterial phylogenies (Woese, 1987; Weisburg *et al.*, 1991; Ludwig & Schleifer, 1994; Garrity & Holt, 2001). Partial or complete 16S rRNA gene sequences, which have also been used extensively for studying the phylogeny of rhizobia (e.g. Young *et al.*, 1991; Oyaizu *et al.*, 1992; Yanagi & Yamasato, 1993; van Rossum *et al.*, 1995; Urtz & Elkan, 1996; Moreira *et al.*, 1998; Vinuesa *et al.*, 1998; Wang *et al.*, 1999; Chen *et al.*, 2000; Jarabo-Lorenzo *et al.*, 2000), have contributed to the recent descriptions of four new genera and several rhizobial species. However, there are reports showing that, despite a high level of diversity in morphological, physiological and genetic properties, diversity is low in the 16S rRNA gene sequences of strains of *Bradyrhizobium* investigated so far (So *et al.*, 1994, Urtz & Elkan, 1996; Molouba *et al.*, 1999; Vinuesa *et al.*, 1998; Chen *et al.*, 2000; van Berkum & Fuhrmann, 2000; Willems *et al.*, 2001; Qian *et al.*, 2003).

The 23S rRNA is a long fragment of about 2·3 kb; it therefore contains more information than the 16S rRNA and has proven to be useful in the speciation of several genera of bacteria (Ludwig & Schleifer, 1994), including rhizobia (Tesfaye *et al.*, 1997; Terefework *et al.*, 1998; Tesfaye & Holl, 1998; Qian *et al.*, 2003). Furthermore, as the rate of sequence change seems to be faster in the 23S rRNA than in the 16S rRNA gene (Olsen & Woese, 1993), the former may be more valuable for delineating close relationships (Wang & Martínez-Romero, 2000).

When bacterial speciation is not clarified by 16S rRNA gene sequencing, analysis of sequences of the 16S–23S rRNA intergenic spacer (IGS) has also proven to be useful, as the usually long sequence and the greater variability make the region particularly interesting for phylogenetic studies (Laguerre *et al.*, 1996; Vinuesa *et al.*, 1998; Doignon-Bourcier *et al.*, 2000; van Berkum & Fuhrmann, 2000; Willems *et al.*, 2001).

Sequencing analysis of ribosomal genes of several strains can be very expensive; however, PCR-based locus-specific RFLP associated with ribosomal genes may be convenient for phylogenetic studies, generally showing high reproducibility and good agreement with partial or complete gene sequencing (Laguerre *et al.*, 1994, 1996; Vinuesa *et al.*, 1998; Wang *et al.*, 1999; Abaidoo *et al.*, 2000; Jarabo-Lorenzo *et al.*, 2000).

Although it has been suggested that *Bradyrhizobium* is the ancestor of all rhizobia (Norris, 1965; Provorov & Vorob'ev, 2000; Wang & Martínez-Romero, 2000) and strains have been isolated from a variety of legumes distributed worldwide, most studies on diversity and genetics have been performed with fast-growing rhizobia. Furthermore, as has been pointed out since the pioneering studies of ribosomal genes, it seems that there are many more varieties of rhizobia in the tropics and subtropics than in temperate regions (Oyaizu *et al.*, 1992; Vinuesa *et al.*, 1998). Indeed, bradyrhizobia seem to represent the majority of isolates from leguminous trees in Brazilian tropical forests (Moreira, 1991, 2000). In addition, a high level of diversity among strains has been reported in a few studies performed in South America (Moreira *et al.*, 1993; Urtz & Elkan, 1996; Chen *et al.*, 2000; Fernandes *et al.*, 2003; Hara, 2003; Menna, 2005). That diversity deserves more investigation; therefore, in this study, RFLP of PCR-amplified 16S and 23S rRNA genes and of the IGS was used to characterize the level of diversity among 119 strains, capable of effectively nodulating several leguminous species and most of tropical origin, from the Brazilian *Rhizobium* Culture Collection SEMIA (Seção de Microbiologia Agrícola) (IBP World Catalogue of *Rhizobium* Collections, no. 443 in the WFCC World Data Center on Micro-organisms).

METHODS

Strains. One hundred and five strains from the Brazilian *Rhizobium* Culture Collection SEMIA, classified as *Bradyrhizobium* in the catalogue of FEPAGRO-MIRCEN [Fundação Estadual de Pesquisa Agropecuária (Rio Grande do Sul, Brazil) Microbiological Resources Center] (FEPAGRO, 1999) were used in this study. Information about the original source of the strains, the origin of the strains and their numbers in other collections is available in Supplementary Table S1 available in IJSEM Online. The strains were classified in the catalogue of FEPAGRO (1999) as *Bradyrhizobium* based on their host specificity and ability to produce an alkaline reaction in YMB medium (Vincent, 1970). After receipt from FEPAGRO, they were characterized again in YMB after Vincent (1970) and stocks were prepared on YM agar and kept at −70 °C (in 30 % glycerol) and at 4 °C (working cultures). Three other strains from the north-eastern region of Brazil were included, R 17, R 35 and R 45, classified as *Bradyrhizobium* based on RFLP-PCR of the 16S and 23S rRNA and IGS and on the partial sequence of the 16S rRNA gene (Fernandes *et al.*, 2003). Five rhizobia from the Amazon region showing physiological properties of *Bradyrhizobium* (Hara, 2003) and four strains isolated from soybean nodules in Paraguay, with 16S rRNA gene sequences showing high similarity to *B. japonicum* (PRY 40 and PRY 42) and *B. elkanii* (PRY 52 and PRY 49) (Chen *et al.*, 2000), were also included. Finally, *B. elkanii* USDA 76^T and *Bradyrhizobium* sp. BTAi 1 were included, giving a total of 119 strains.

Description of type and reference strains. Type strains included were *B. japonicum* USDA 6^T (=SEMIA 5056^T), provided by FEPAGRO, and *B. elkanii* USDA 76^T, provided by the US Department of Agriculture (USDA). Strain BTAi 1 was included as an outgroup on the basis of its distinctive 16S RNA gene sequence (Willems *et al.*, 2001) and was also provided by the USDA. Other *B. japonicum* reference strains included SEMIA 566, SEMIA 586 (=CB 1809), their natural variants SEMIA 5079 (=CPAC 15) and SEMIA 5080 (=CPAC 7), respectively, both of which have been used in Brazilian commercial inoculants for soybean since 1992 (Nishi *et al.*, 1996; Chueire *et al.*, 2003), and SEMIA 5085, used in soybean commercial inoculants in Argentina, all of which had their taxonomic position confirmed by the sequencing of the whole 16S rRNA gene (Chueire *et al.*, 2003). *B. elkanii* reference strains included SEMIA 587 and SEMIA 5019 (=29w), both of which have also been used in Brazilian commercial inoculants for soybean since 1979 (Nishi *et al.*, 1996; Chueire *et al.*, 2003); their 16S rRNA genes have also been sequenced (Chueire *et al.*, 2003).

DNA extraction. Total genomic DNA of each strain was extracted from bacterial batch cultures grown in YMB until late exponential phase (10^9 cells ml^{-1}). Extraction of DNA was performed as described previously (Fernandes *et al.*, 2003). To obtain clean DNA samples, the extraction procedure included the addition, for each 400 μl bacterial culture resuspended in TE 50/20, of 50 μl 10 % SDS, 5 μl proteinase K (20 mg ml^{-1}), 10 μl lysozyme (5 mg ml^{-1}) and 2 μl RNase (10 mg ml^{-1}).

RFLP of PCR-amplified DNA region encoding the 16S rRNA. Three replicates of DNA from each bacterium were used for amplification with the universal primers described by Weisburg *et al.* (1991), fD1 (5′-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3′) and rD1 (3′-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-5′). Each replicate contained, in a volume of 50 μl (final concentration in parentheses), dNTPs (300 μM of each), 5·0 μl 10× buffer, $MgCl_2$ (1·5 mM), primers (15 pmol of each), *Taq* DNA polymerase (1·25 U), DNA (20 ng), DMSO (5 %) and sterile Milli-Q water to complete the final volume. The reaction was carried out in a PTC 200 thermocycler (MJ Research Inc.), using an initial cycle of denaturation at 95 °C for 2 min, 30 cycles of denaturation at 94 °C for 15 s and 93 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 2 min and a final extension cycle at 72 °C for 5 min, with a final soak at 4 °C.

The PCR products were then digested with three restriction endonucleases, *Cfo*I, *Msp*I and *Dde*I (Invitrogen Life Technologies), as recommended by the manufacturer. The fragments obtained were analysed by electrophoresis in a gel (17 × 11 cm) with 3 % agarose and carried out at 100 V for 4 h. Gels were stained with ethidium bromide and photographed under UV light. The profiles obtained were confirmed in triplicate.

RFLP-PCR of the 23S rRNA region. Three replicates of the DNA of each strain were amplified with primers P3 (5′-CCGTGCGGGA-AAGGTCAAAAGTAC-3′) and P4 (5′-CCCGCTTAGATGCTTTCA-GC-3′), described by Terefework *et al.* (1998), with the same PCR program described above. The PCR products were digested with three restriction endonucleases, *Hha*I (=*Cfo*I), *Hae*III and *Hin*fI, as recommended by the manufacturer (Invitrogen Life Technologies). The fragments were visualized as described above and the profiles obtained were confirmed in triplicate.

RFLP-PCR of the 16S–23S rRNA IGS. Three replicates of the DNA of each strain were amplified with primers FGPS1490 (5′-TGCGGCTGGATCACCTCCTT-3′) and FGPS132 (5′-CCGGG-TTTCCCCATTCGG-3′), described by Laguerre *et al.* (1996). The reaction mixture contained, in a volume of 50 μl (final concentration in parentheses): dNTPs (200 μM of each); 5·0 μl 10× buffer, $MgCl_2$ (1·5 mM), primers (12·5 pmol of each), *Taq* DNA polymerase (1·0 U), DNA (40 ng) and sterile Milli-Q water to complete the final volume. The PCR program was as follows: denaturation at 95 °C for 3 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min followed by an extension cycle at 72 °C for 3 min and a final soak at 4 °C. The PCR products were then digested with the restriction enzymes *Msp*I, *Dde*I and *Hae*III (Invitrogen Life Technologies) as recommended by the manufacturer. The fragments were visualized as described above and the profiles were confirmed in triplicate.

Cluster analyses. The sizes of the fragments in all analyses were normalized according to the size of the DNA markers, which were included on the left and right and in the centre of each gel. For the combined analysis of the three ribosomal regions with the respective enzymes, a combined profile of all the individual sets of restriction patterns was obtained. Cluster analyses were performed with the Bionumerics program version 1.50 (Applied Mathematics), using the UPGMA algorithm (unweighted pair-group method with arithmetic means) (Sneath & Sokal, 1973) and the Jaccard coefficient. Analyses of each ribosomal region with three restriction enzymes and of the three combined ribosomal regions were performed in the Bionumerics program setting the position tolerance of the bands to 1, 3 and 5 %, as well as utilizing the optimum values indicated for optimization and position tolerance in the Bionumerics program for each restriction enzyme. Increasing the position tolerance sometimes resulted in a smaller number of subclusters, and often bands that were different in one gel at 1 or 3 % were similar at 5 %; however, the main clusters were always confirmed. We have thus decided to consider the optimum values indicated by the program for each region and enzyme, as follows (in parentheses are values of optimization; tolerance): 16S rRNA with *Cfo*I (0; 2·25), *Msp*I (0·52; 0·87) and *Dde*I (0; 0·87); IGS with *Msp*I (0·44; 5·67), *Dde*I (0·47; 2·25) and *Hae*III (0·67; 2·25); 23S rRNA with *Hae*III (0·43; 0·37), *Hin*fI (0; 3·26) and *Hha*I (0·43; 0·72).

RESULTS

The properties of all 119 strains were consistent with those of the genus *Bradyrhizobium* (Jordan, 1982, 1984). None of the strains showed the extra-slow growth of *B. liaoningense* (Xu *et al.*, 1995) or the characteristics of either *B. yuanmingense* isolated from *Lespedeza* spp. (Yao *et al.*, 2002) or *B. canariense* isolated from *Chamaecystisus proliferus* (Vinuesa *et al.*, 2005a); therefore, type strains for those species were not included in this study. Strains used in this study were isolated from 33 legume species (Table 1 and Supplementary Table S1), representing nine tribes and all three subfamilies (Supplementary Table S2).

Amplification of the 16S rRNA gene always produced a single PCR product of about 1500 bp (data not shown). A high level of diversity was observed and bacteria were clustered in seven 'great groups' (GG) and 19 subgroups (SG) (Table 1; Supplementary Fig. S1). GG I clustered strains at 36·6 % similarity and could be split into two subgroups and one isolated strain (SEMIA 658). Strains PRY 40 and 42, within SG I, have previously been shown to exhibit 16S rRNA gene sequence similarity to *B. japonicum*, whereas PRY 52 was genetically closer to *B. elkanii* (Chen *et al.*, 2000). The other strains within GG I were isolated from various tropical legumes (*Prosopis*, *Mimosa*, *Vigna* and *Lotononis*). GG II included five subgroups (III–VII), at a

Table 1. Strains included in this study and their positions in 16S rRNA, IGS and 23S rRNA RFLP-PCR cluster analysis and the combined analysis

Great groups (GG), groups (G) and subgroups (SG) in which each strain fell in each cluster analysis are indicated. Details of type and reference strains are given in Methods, and details of the origins of the strains are given in Supplementary Table S1. i, Isolated strain; NA, not amplified.

Strain	Host plant	16S rRNA SG (GG)	IGS SG (GG)	23S rRNA G	Combined SG (GG)
SEMIA 6169*	*Albizia falcataria*	XIX (GG V)	VIII (GG II)	V	I (GG I)
SEMIA 6175*	*Pueraria phaseoloides*	XIX (GG V)	VIII (GG II)	III	I (GG I)
SEMIA 6208*	*Desmodium ovalifolium*	XII (GG IV)	VIII (GG II)	III	I (GG I)
SEMIA 6387*	*Acacia auriculiformis*	XIV (GG IV)	i (GG II)	III	I (GG I)
SEMIA 6425*	*Centrosema pubescens*	XIX (GG V)	VII (GG II)	XIII	II (GG I)
SEMIA 549	*Glycine max*	XVIII (GG V)	VII (GG II)	XIII	II (GG I)
B. elkanii SEMIA 5019*	*Glycine max*	XV (GG V)	VI (GG II)	VII	II (GG I)
R 35	*Cajanus cajan*	XVII (GG V)	VII (GG II)	V	III (GG I)
R 17	*Vigna unguiculata*	XVII (GG V)	VII (GG II)	V	III (GG I)
SEMIA 5069	*Glycine max*	XVII (GG V)	VII (GG II)	V	III (GG I)
SEMIA 6053*	*Clitoria ternatea*	XVII (GG V)	VII (GG II)	VII	III (GG I)
SEMIA 6028*	*Desmodium uncinatum*	XVIII (GG V)	VII (GG II)	VII	III (GG I)
SEMIA 590	*Glycine max*	XVI (GG V)	VII (GG II)	V	IV (GG I)
SEMIA 598	*Glycine max*	XVI (GG V)	VII (GG II)	V	IV (GG I)
B. elkanii SEMIA 587*	*Glycine max*	XVI (GG V)	VII (GG II)	V	IV (GG I)
SEMIA 696*	*Desmodium uncinatum*	XV (GG V)	VII (GG II)	VI	IV (GG I)
SEMIA 5011	*Glycine max*	XVI (GG V)	VII (GG II)	VI	IV (GG I)
SEMIA 5012	*Glycine max*	XVI (GG V)	VII (GG II)	V	IV (GG I)
SEMIA 5016	*Glycine max*	XVI (GG V)	VII (GG II)	VII	IV (GG I)
SEMIA 662*	*Vigna unguiculata*	XVI (GG V)	VII (GG II)	VI	IV (GG I)
SEMIA 695*	*Neonotonia wightii*	XV (GG V)	VII (GG II)	VI	IV (GG I)
SEMIA 5027	*Glycine max*	XV (GG V)	VII (GG II)	VI	IV (GG I)
SEMIA 5026	*Glycine max*	XV (GG V)	VII (GG II)	VI	IV (GG I)
SEMIA 5070	*Glycine max*	XVII (GG V)	VII (GG II)	VI	i (GG I)
SEMIA 6150*	*Acacia mearnsii*	XVII (GG V)	VII (GG II)	III	V (GG I)
SEMIA 6157*	*Cajanus cajans*	XII (GG IV)	VII (GG II)	VI	V (GG I)
SEMIA 6159*	*Enterolobium ellipticum*	XII (GG IV)	VII (GG II)	III	V (GG I)
SEMIA 6158*	*Crotalaria spectabilis*	XII (GG IV)	VII (GG II)	II	V (GG I)
SEMIA 6149*	*Galactia striata*	XII (GG IV)	VII (GG II)	III	V (GG I)
SEMIA 6093	*Aeschynomene americana*	XIII (GG IV)	VII (GG II)	VI	V (GG I)
SEMIA 6099	*Dimorphandra exaltata*	XIII (GG IV)	VII (GG II)	VI	V (GG I)
SEMIA 6101*	*Dalbergia nigra*	XIII (GG IV)	VII (GG II)	VI	V (GG I)
SEMIA 6145*	*Crotalaria juncea*	XVI (GG V)	VII (GG II)	VI	V (GG I)
SEMIA 6146*	*Centrosema* sp.	XIII (GG IV)	i (GG II)	III	V (GG I)
SEMIA 6148*	*Neonotonia wightii*	XIII (GG IV)	i (GG II)	III	V (GG I)
SEMIA 6160*	*Albizia lebbek*	XVII (GG V)	VII (GG II)	II	V (GG I)
AM-01-517	*Stryphnodendron pulcherrimum*	XVI (GG V)	VIII (GG II)	V	VI (GG I)
AM-2-855	*Vigna unguiculata*	XVI (GG V)	VIII (GG II)	XIII	VI (GG I)
SEMIA 538	*Glycine max*	XIII (GG IV)	VII (GG II)	XIII	VII (GG I)
SEMIA 542	*Glycine max*	X (GG III)	VI (GG II)	XIII	VII (GG I)
SEMIA 577	*Glycine max*	XVIII (GG V)	VII (GG II)	V	VII (GG I)
SEMIA 501	*Glycine max*	XVIII (GG V)	VII (GG II)	V	VII (GG I)
R 45	*Cajanus cajan*	XIV (GG IV)	VI (GG II)	XIII	i (GG I)
SEMIA 562	*Glycine max*	X (GG III)	V (GG I)	XIV	VIII (GG I)
SEMIA 565	*Glycine max*	X (GG III)	VII (GG II)	XIV	VIII (GG I)
SEMIA 6424*	*Centrosema pubescens*	XIX (GG V)	VII (GG II)	XIII	i (GG I)
B. elkanii USDA 76^T	*Glycine max*	i	VII (GG II)	XV	i (GG I)
SEMIA 6163*	*Acacia mearnsii*	VI (GG II)	IV (GG I)	XII	IX (GG II)

Table 1. cont.

Strain	Host plant	16S rRNA SG (GG)	IGS SG (GG)	23S rRNA G	Combined SG (GG)
SEMIA 6192*	*Tipuana tipa*	VI (GG II)	IV (GG I)	VIII	IX (GG II)
SEMIA 6420*	*Acacia mangium*	(GG VI)	i (GG I)	X	IX (GG II)
SEMIA 6164*	*Acacia mearnsii*	(GG VI)	VI (GG II)	XII	IX (GG II)
SEMIA 5005	*Glycine max*	III (GG II)	II (GG I)	IX	X (GG II)
SEMIA 5000	*Glycine max*	III (GG II)	III (GG I)	IX	X (GG II)
SEMIA 5068	*Glycine max*	IV (GG II)	III (GG I)	IX	X (GG II)
SEMIA 5075	*Glycine max*	IV (GG II)	III (GG I)	IX	X (GG II)
SEMIA 5055	*Glycine max*	IV (GG II)	III (GG I)	X	X (GG II)
SEMIA 5038	*Glycine max*	IV (GG II)	III (GG I)	i	X (GG II)
SEMIA 5042	*Glycine max*	IV (GG II)	II (GG I)	II	X (GG II)
SEMIA 5063	*Glycine max*	IV (GG II)	II (GG I)	II	X (GG II)
B. japonicum USDA 6^T	*Glycine max*	IV (GG II)	II (GG I)	X	X (GG II)
SEMIA 5048	*Glycine max*	V (GG II)	I (GG I)	II	X (GG II)
SEMIA 5045	*Glycine max*	V (GG II)	I (GG I)	II	X (GG II)
SEMIA 5062	*Glycine max*	V (GG II)	V (GG I)	X	XI (GG II)
SEMIA 5003	*Glycine max*	V (GG II)	V (GG I)	XII	XI (GG II)
SEMIA 656*	*Neonotonia wightii*	VII (GG II)	V (GG I)	VIII	XI (GG II)
SEMIA 560	*Glycine max*	VIII (GG III)	V (GG I)	X	XI (GG II)
SEMIA 583	*Glycine max*	IX (GG III)	V (GG I)	X	XI (GG II)
SEMIA 5006	*Glycine max*	V (GG II)	i (GG I)	VII	XI (GG II)
SEMIA 5029	*Glycine max*	III (GG II)	III (GG I)	VII	XI (GG II)
SEMIA 5022	*Glycine max*	III (GG II)	I (GG I)	XII	XI (GG II)
SEMIA 5023	*Glycine max*	XVIII (GG V)	I (GG I)	VII	XI (GG II)
SEMIA 5030	*Glycine max*	V (GG II)	I (GG I)	X	XI (GG II)
SEMIA 5036	*Glycine max*	V (GG II)	I (GG I)	VII	XII (GG II)
SEMIA 5021	*Glycine max*	III (GG II)	I (GG I)	VII	XII (GG II)
SEMIA 5024	*Glycine max*	IX (GG III)	I (GG I)	VII	XII (GG II)
SEMIA 5084	*Glycine max*	VI (GG II)	I (GG I)	IV	XII (GG II)
SEMIA 5060	*Glycine max*	V (GG II)	I (GG I)	II	XII (GG II)
SEMIA 5059	*Glycine max*	V (GG II)	I (GG I)	II	XII (GG II)
SEMIA 581	*Glycine max*	IX (GG III)	I (GG I)	X	XII (GG II)
B. japonicum SEMIA 586	*Glycine max*	IX (GG III)	I (GG I)	V	XII (GG II)
B. japonicum SEMIA 5080*	*Glycine max*	IV (GG II)	I (GG I)	V	XII (GG II)
B. japonicum SEMIA 5085	*Glycine max*	XI (GG III)	III (GG I)	IX	XIII (GG II)
SEMIA 580	*Glycine max*	VIII (GG III)	III (GG I)	IX	XIII (GG II)
SEMIA 5082	*Glycine max*	IV (GG II)	II (GG I)	XII	XIII (GG II)
B. japonicum SEMIA 566	*Glycine max*	VIII (GG III)	II (GG I)	XII	XIII (GG II)
SEMIA 571	*Glycine max*	VIII (GG III)	II (GG I)	XII	XIII (GG II)
SEMIA 568	*Glycine max*	VIII (GG III)	II (GG I)	IX	XIII (GG II)
SEMIA 567	*Glycine max*	VIII (GG III)	II (GG I)	IX	XIII (GG II)
SEMIA 576	*Glycine max*	VIII (GG III)	II (GG I)	IX	XIII (GG II)
B. japonicum SEMIA 5079*	*Glycine max*	IX (GG III)	II (GG I)	X	XIII (GG II)
SEMIA 509	*Glycine max*	XI (GG III)	II (GG I)	IX	XIV (GG II)
SEMIA 511	*Glycine max*	XI (GG III)	II (GG I)	X	XIV (GG II)
SEMIA 518	*Glycine max*	XI (GG III)	I (GG I)	X	XIV (GG II)
SEMIA 515	*Glycine max*	XI (GG III)	III (GG I)	IX	XIV (GG II)
SEMIA 528	*Glycine max*	XI (GG III)	III (GG I)	XI	XIV (GG II)
SEMIA 6144*	*Arachis hypogaea*	VII (GG II)	V (GG I)	IV	XV (GG II)
SEMIA 6156*	*Crotalaria spectabilis*	(GG VI)	V (GG I)	VI	XV (GG II)
SEMIA 6155*	*Stylosanthes* sp.	(GG VI)	II (GG I)	XII	XV (GG II)
SEMIA 6152*	*Calopogonium* sp.	XII (GG IV)	I (GG I)	III	XV (GG II)
SEMIA 6002*	*Vigna unguiculata*	VIII (GG III)	V (GG I)	VII	XV (GG II)
SEMIA 543	*Glycine max*	X (GG III)	i (GG I)	XI	i (GG II)

Table 1. cont.

Strain	Host plant	16S rRNA SG (GG)	IGS SG (GG)	23S rRNA G	Combined SG (GG)
SEMIA 6319*	*Arachis* sp.	(GG VI)	X	II	i
SEMIA 535	*Glycine max*	i (GG III)	i (GG I)	I	i
SEMIA 569	*Glycine max*	i	i (GG I)	I	i
SEMIA 658*	*Lotononis bainesii*	i (GG I)	i (GG II)	i	i
SEMIA 6161*	*Prosopis juliflora*	II (GG I)	i (GG II)	I	(GG III)
SEMIA 6165*	*Mimosa scrabella*	II (GG I)	i (GG II)	I	(GG III)
PRY 52	*Glycine max*	II (GG I)	NA	I	(GG III)
AM-Cp 17	*Vigna unguiculata*	II (GG I)	i (GG II)	I	(GG III)
PRY 42	*Glycine max*	I (GG I)	NA	I	(GG III)
PRY 40	*Glycine max*	I (GG I)	i (GG I)	I	(GG III)
Bradyrhizobium sp. BTAi 1	*Aechynomene indica*	i	i (GG II)	XV	i
SEMIA 6154*	*Stylosanthes* sp.	i	i (GG II)	i	i
SEMIA 6167*	*Mimosa caesalpiniifolia*	(GG VII)	IX	XVI	(GG IV)
SEMIA 6166*	*Mimosa caesalpiniifolia*	(GG VII)	IX	XVI	(GG IV)
SEMIA 6382*	*Mimosa caesalpiniifolia*	(GG VII)	IX	XVI	(GG IV)
PRY 49	*Glycine max*	(GG VII)	IX	XVI	(GG IV)
AM-P5 Abac	*Vigna unguiculata*	(GG VII)	X	XVI	(GG IV)
AM-P2 Lima	*Vigna unguiculata*	(GG VII)	IX	XVI	(GG IV)

*Strain recommended for use in Brazilian commercial inoculants for that legume species.

level of similarity of 58·7 %. Several strains within these subgroups showed complete profile similarity and all strains within SG III, IV and V, representing 82 % of GG II, were isolated from soybean. *B. japonicum* strains SEMIA 5080 and 5056^T (=USDA 6^T) clustered into SG IV at 82·6 % similarity, while SG VI and VII included strains isolated from soybean, *Tipuana*, *Acacia*, *Arachis* and *Neonotonia*. GG III was split into four subgroups (VIII–XI) at a 59·0 % level of similarity and included 24 strains, all isolated from soybean except for SEMIA 6002 (from *Vigna*). GG III also included *B. japonicum* reference strains SEMIA 566, 586, 5079 and 5085 (Table 1; Supplementary Fig. S1). Therefore GG II and III clustered 52 strains, 90 % of which were from soybean, including type and reference strains, with a similarity of 50 %. The great majority of strains isolated from tropical legume trees was positioned in GG IV, V and VI. GG IV contained 14 strains, only one (SEMIA 538) from soybean. The large GG V clustered 31 strains, 15 from soybean, including the *B. elkanii* reference strains SEMIA 5019 and SEMIA 587, which were positioned in SG XV and XVI, respectively. Within GG V, some strains showed complete similarity, e.g. four strains within SG XVI isolated from crotalaria, soybean and cowpea were similar to the commercial soybean strain SEMIA 587. GG IV and V grouped two reference *B. elkanii* strains with a similarity of 53·1 %, but not USDA 76^T. Five strains were grouped at 51·8 % in GG VI, none of which were from soybean. Three quite dissimilar strains were joined to GG IV, V and VI, including *B. elkanii* USDA 76^T, at 32·9 % similarity (Table 1; Supplementary Fig. S1). GG VII included six strains clustered at 45·2 % similarity, none of which were from soybean. Finally, strain BTAi 1 was quite distinct from the other groups and joined them at a level of 27·9 % similarity (Table 1; Supplementary Fig. S1).

Amplification of the IGS resulted in one fragment with sizes ranging from 1200 to 1800 bp; five strains (SEMIA 695, 5060, 5075, 6146, 6159) produced an extra fragment of 800–1500 bp (data not shown), while amplification failed with two others (PRY 42 and PRY 52). The fragments produced with the primers were larger than those obtained by Willems *et al.* (2001) and Doignon-Bourcier *et al.* (2000), but we have used different primers. Cluster analysis of the RFLP products of the IGS resulted in two main great groups and isolated strains that were joined at a final level of similarity of 26·7 % (Table 1; Supplementary Fig. S2). GG I was divided into five main subgroups, joined at a level of similarity of 58·6 %, and included type and reference strains of *B. japonicum*; six other strains were joined to this great group at a level of similarity of 37·4 %. SG I included the *B. japonicum* reference strain SEMIA 5080 and its putative parent SEMIA 586, while SG II included *B. japonicum* SEMIA 5079 and its putative parent SEMIA 566, as well as the type strain, SEMIA 5056^T. Of the 40 strains in SG I, II and III, all but one from SG I (SEMIA 6152, from *Calopogonium*) and one from SG II (SEMIA 6155, from *Stylosanthes*) were isolated from soybean, while the two strains comprising SG IV and four of the nine strains of SG V came from other hosts. It is also noteworthy that three strains within SG V, SEMIA 6002, 6144 and 6156, showed complete similarity of profiles although isolated from different genera and tribes, *Vigna* (Phaseoleae), *Arachis* (Aeschynomeneae) and *Crotalaria* (Crotolarieae), respectively, all belonging to the subfamily Papilionoideae. All

but one (SEMIA 6420) of the six isolated strains that were joined to GG I were isolated from soybean (Table 1; Supplementary Fig. S2). Type and reference strains of *B. elkanii* were positioned in SG VI, VII and VIII of GG II with a final level of similarity of 55·1 %. Of the 44 strains within this group, 26 were isolated from legumes other than soybean. Nine other strains were joined to this group at a final level of similarity of only 44·5 %, all isolated from legumes other than soybean, including the reference strain BTAi 1. GG I and II were joined at a level of similarity of 36 %. Finally, two other small subgroups clustered seven strains. Within SG IX, three strains isolated from *Mimosa* showed similarity of 89·2 % and were joined to soybean strain *B. elkanii* PRY 49 and to a cowpea strain at 36·8 % similarity. SG X included two strains isolated from *Arachis* and *Vigna* with a very low level of similarity (33·1 %) (Table 1; Supplementary Fig. S2).

One fragment of about 2·3 kb was obtained for all strains for the 23S rRNA region (data not shown). Analysis of the 23S rRNA region resulted in the highest level of diversity observed in this study. Great groups were not observed in the cluster analysis; however, 16 groups (G) were observed, as well as some isolated strains (Table 1; Supplementary Fig. S3). G I of the 23S rRNA region joined eight strains at a very low level of similarity (23·6 %). Four subclusters of pairs of strains were shown in G I: PRY 52 (*Glycine*)/AM-Cp 17 (*Vigna*) (65·5 %), PRY 42/PRY 40 (*Glycine*) (78·3 %), SEMIA 6161 (*Prosopis*)/6165 (*Mimosa*) (49·8 %) and SEMIA 535/569 (*Glycine*) (50·0 % similarity). Nine strains were clustered in G II with a similarity of 50·6 %, six from soybean and three from species of the subfamilies Papilionoideae and Mimosoideae. Another nine strains were placed in G III at a level of similarity of 61·5 %, isolated from a wide range of host legumes. G IV included only two strains, from soybean and peanut, with a similarity of 72·2 %. Although showing a higher level of similarity than many other groups (78·5 %), G V was unusual, as it clustered reference strains of both *B. elkanii* (SEMIA 587) and *B. japonicum* (SEMIA 5080 and its putative parent SEMIA 586), together with ten other strains isolated from both soybean and other legumes. G VI joined 13 strains at a similarity of 80·2 %, five of which were completely similar, although isolated from completely different hosts. Eleven strains were clustered in G VII at a level of similarity of 71·8 %; all but three had been isolated from soybean, including *B. elkanii* reference strain SEMIA 5019. G VIII joined only two strains (65·5 % similarity), isolated from *Tipuana tipa* and *Neonotonia wightii*, both from the Papilionoideae. Groups IX–XI were joined at 64·5 % similarity and included 24 strains, all but one (SEMIA 6420) of which had been isolated from soybean, including *B. japonicum* reference strains SEMIA 5085, 5079 and 5056^{T}, which were positioned in G IX and G X. G XII clustered (65·2 %) five strains isolated from soybean, including reference strain *B. japonicum* SEMIA 566, as well as three strains isolated from other legumes. In addition, SEMIA 5038 showed high variability in the 23S rRNA region and occupied an isolated position. Seven strains were clustered in G XIII with a similarity of 51·1 %, isolated from four genera of the subfamily Papilionoideae (*Glycine*, *Cajanus*, *Vigna* and *Centrosema*), while G XIV included two soybean rhizobia (78 % similarity). SEMIA 658 from *Lotonis bainesii* occupied an isolated position, while *B. elkanii* USDA 76^{T} and BTAi 1 were clustered into G XV with a very low level of similarity (33·9 %). The last group, G XVI, clustered six strains, three isolated from *Mimosa*, two isolated from *Vigna* in the Amazon region and PRY 49 from soybean nodules in Paraguay. Finally, SEMIA 6154, symbiont of *Stylosanthes*, joined the other clusters at a very low level of similarity, 15·5 % (Table 1; Supplementary Fig. S3). Analysis of the 23S rRNA region therefore resulted in the largest number of differences in comparison with the 16S rRNA and IGS regions. When the 16S rRNA and IGS regions were compared with the 23S rRNA region, in a few clusters the majority of the strains showed the properties of either *B. japonicum* (G IV, IX, X, XI) or *B. elkanii* (G III), but a predominance of strains showed mixed properties (G II, V, VII, XII, XIII, XIV). Furthermore, at least two groups (I and XV) were quite different from the others in the 23S rRNA region and might represent novel species (Supplementary Fig. S3).

A dendrogram was built considering all three ribosomal regions, each with three restriction enzymes (Fig. 1). Furthermore, to help the comparison of the results obtained in the analysis of the 16S rRNA (Supplementary Fig. S1), IGS (Supplementary Fig. S2) and 23S rRNA (Supplementary Fig. S3), Table 1 was constructed and will be used here for description of the results. Analysis considering the three ribosomal regions resulted in four main great groups and six isolated strains, joined at a final level of similarity of 26·9 % (Fig. 1).

Forty-seven strains were joined in GG I of the combined analysis with a similarity of 46·5 % and included all reference and type strains of *B. elkanii*; GG I could be split into eight subgroups and four isolated strains (Fig. 1). Table 1 shows that 30 strains of GG I of the combined analysis had been clustered into GG V (composed of 31 strains) and 13 into GG IV (composed of 14 strains) in the 16S rRNA analysis; in addition, all strains within GG I of the combined analysis fell into GG II of the IGS analysis. However, the strains occupied different groups in the 23S rRNA analysis, as follows (number of strains in the combined analysis followed by the percentage in relation to all strains within that group in the 23S rRNA analysis): G II (2, 22 %), III (8, 89 %), V (11, 85 %), VI (12, 92 %), VII (4, 36 %), XIII (7, 100 %), XIV (2, 100 %) and XV (1, 50 %). Furthermore, strains representing a large percentage of some groups of the 23S rRNA analysis (as those of G III, V, VI, XIII and XIV) were often positioned in different subgroups of the 16S and IGS analyses. Reference strains of *B. elkanii*, SEMIA 587 and 5019, were positioned in GG V of the 16S rRNA analysis, GG II of the IGS analysis and G V and VII, respectively, of the 23S rRNA analysis, while the type strain USDA 76^{T} occupied an isolated position in the

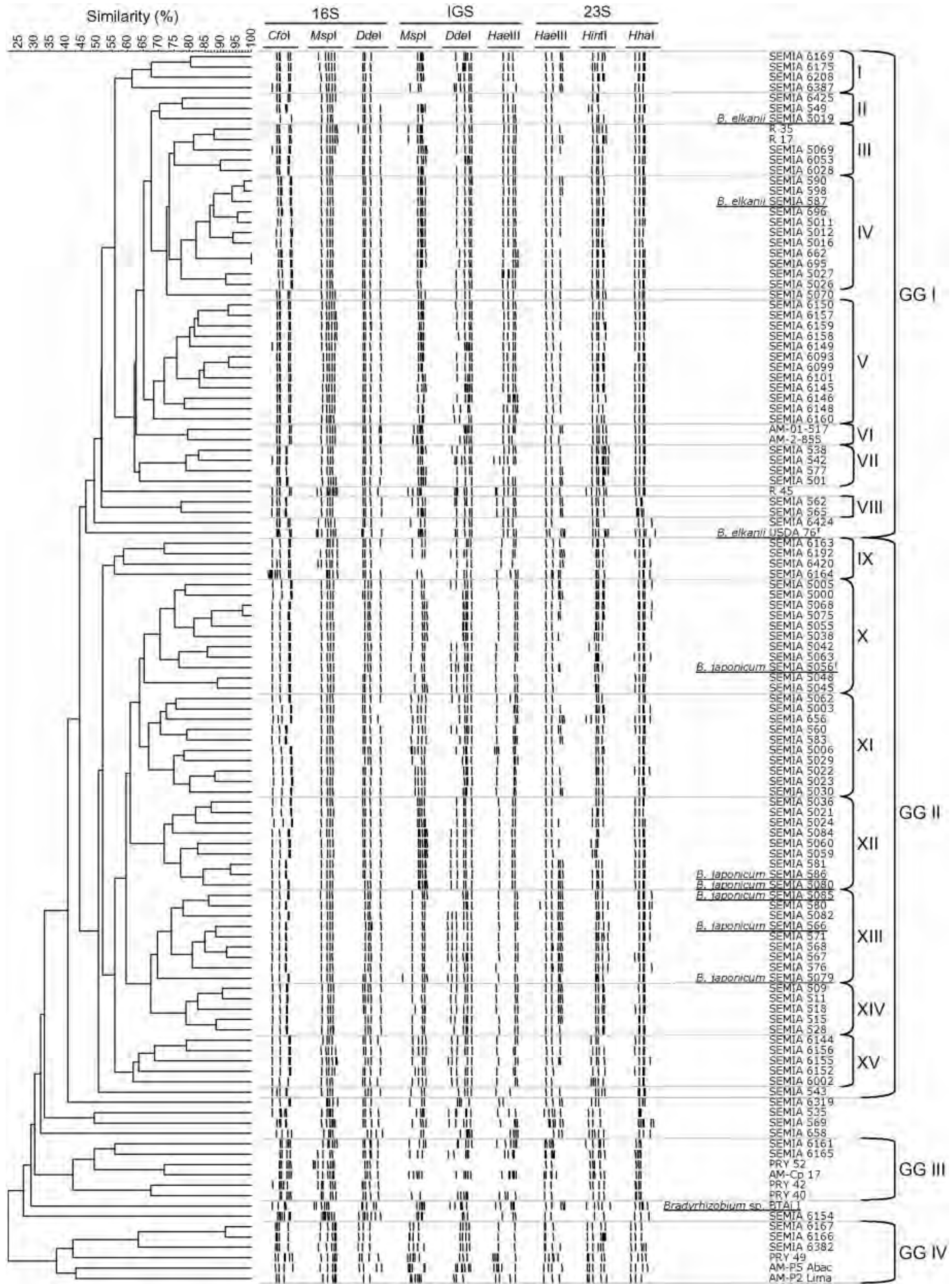

Fig. 1. Cluster analysis (UPGMA with the Jaccard coefficient, converted to percentage similarity) of products obtained by PCR-RFLP analysis of the 16S rRNA gene, IGS and 23S rRNA gene of bradyrhizobial strains, each with three restriction enzymes. Reference and type strains (underlined) are labelled and classified according to the sequencing analysis of the 16S rRNA gene, as described in Methods.

16S rRNA analysis but clustered in GG II in the IGS analysis and G XV in the 23S rRNA analysis (Table 1).

Although the results obtained in the combined analysis might indicate a clustering of the *B. elkanii* species, the detection of several subclusters, the low level of similarity joining the strains and the low similarity of the type strain to the other strains clearly show that the genetic variability detected in this study deserves further investigation. For example, at 61·8 % similarity, SG I clustered four strains, SEMIA 6169, 6175, 6208 and 6387, which were isolated from four different genera, *Falcataria*, *Pueraria*, *Desmodium* and *Acacia*, and were quite distinct from the other strains within GG I. SG IV clustered 11 strains, eight isolated from soybean, including reference strain SEMIA 587, while SG V included 12 strains, none from soybean, ten of them indigenous to Brazilian soils (Fig. 1).

GG II of the combined analysis could be split into seven subgroups and clustered 54 strains, including the type strain (SEMIA 5056^T) and reference strains (SEMIA 586, 5080, 5085, 566 and 5079) of *B. japonicum*, at a level of similarity of 51·4 % (Fig. 1). Only ten were isolated from soybean, nine of which clustered into two subgroups, SG IX and XV. SG IX clustered four strains, three from *Acacia* (Mimosoideae) and one from *Tipuana* (Papilionoideae) (Fig. 1, Table 1); these strains fell into two great groups in the 16S (GG II and VI) and IGS (GG I and II) analyses and three groups (G VIII, X, XII) in the 23S analysis (Table 1). SG X of the combined analysis clustered 11 symbionts of soybean, including the type strain of *B. japonicum*; all fell into GG II of the 16S analysis and GG I of the IGS analysis, but were positioned in different groups in the 23S analysis (II, IX, X and isolated). In SG XI, all but one (SEMIA 658) of the ten strains were symbionts of soybean and fell into GG I of the IGS analysis, but occupied different great groups of the 16S analysis (GG II, III and V) and four different groups of the 23S rRNA analysis (G VII, VIII, X and XII). All strains within SG XII of the combined analysis were isolated from soybean, including *B. japonicum* SEMIA 5080 and its putative parent SEMIA 586, and, again, they fell into different groups of each isolated ribosomal region; a similar situation was observed with the nine strains of SG XIII, which clustered the pair of *B. japonicum* strains SEMIA 566 and SEMIA 5079, as well as the strain recommended as soybean inoculant in Argentina, *B. japonicum* SEMIA 5085. SG IV included four strains from soybean that grouped into GG III of the 16S, GG I of the IGS and G IX, X and XI of the 23S analysis. Finally, five strains isolated from five different genera, but all belonging to the subfamily Papilionoideae, composed SG XV of the combined analysis, while strain SEMIA 543 occupied an isolated position. Again, in this second great group of the combined analysis, strains could be placed within the species *B. japonicum*, but several subgroups were clearly shown and deserve further investigation. Finally, four strains quite diverse in all ribosomal regions, SEMIA 6319, 535, 569 and 658, were clustered to GG I and II of the combined analysis at a very low level of similarity (31·9 %).

GG III in the combined analysis joined six strains, three isolated from soybean in Paraguay and showing a similarity of 42·8 %, all of which were placed in GG I of the 16S analysis and G I of the 23S analysis, showing greater differences in the IGS region. Finally, GG IV of the combined analysis joined six strains with a similarity of 31·9 %, also showing high similarity in the 23S (XVI), 16S (GG VII) and IGS (SG IX except for AM-P5, which was placed in SG X) analyses (Fig. 1, Table 1).

DISCUSSION

Large diversity among *Bradyrhizobium* strains from the tropics has been reported in several studies when properties such as colony morphology, exopolysaccharides, serological reactions, intrinsic resistance to antibiotics, substrate utilization, protein profiles, presence of enzymes such as hydrogenases and multilocus enzyme electrophoresis (MLEE) profiles were considered (e.g. van Rossum *et al.*, 1995; Moreira, 1991, 2000; Moreira *et al.*, 1993; Boddey & Hungria, 1997; Doignon-Bourcier *et al.*, 1999; Chen *et al.*, 2002). However, the diversity observed among bradyrhizobial strains is usually not reflected in 16S rRNA gene sequence analysis; therefore, few *Bradyrhizobium* species have been described so far. As bradyrhizobia seem to constitute a major part of the rhizobial isolates in the tropics (Norris, 1965; Moreira, 2000) and as rhizobia might be more diverse in the tropics (Oyaizu *et al.*, 1992; Moreira *et al.*, 1993; Urtz & Elkan, 1996; Vinuesa *et al.*, 1998, 2005a), a large collection of *Bradyrhizobium* strains would add valuable information about the diversity of ribosomal genes within the genus.

The genetic characterization in this study was performed with 119 strains from the Brazilian official culture collection SEMIA of rhizobial strains. The strains had been isolated from 33 legume species (Table 1), most from the tropics, representing nine tribes and including all three subfamilies (Supplementary Table S2). It is recognized that root nodules are generally found in members of the subfamilies Mimosoideae and Papilionoideae and are rare in the Caesalpinioideae (Allen & Allen, 1981); indeed, in our collection, just one strain, SEMIA 6099, was isolated from a member of the Caesalpinioideae, *Dimorphandra exaltata*.

In previous studies, RFLP-PCR of 16S rRNA genes clearly differentiated *Bradyrhizobium* from *Rhizobium*, and often *B. japonicum* from *B. elkanii* (Laguerre *et al.*, 1996; Wang *et al.*, 1999), as well as resolving a group of *Bradyrhizobium* isolates from the Canary Islands that included strain BTAi 1 (Jarabo-Lorenzo *et al.*, 2000). In our study, 52 strains fell into two great groups, at a level of similarity of 50 %, including all reference strains of *B. japonicum*, 90 % of them isolated from soybean. However, soybean strains were also present in other groups, and isolates from Paraguay, which may represent indigenous bradyrhizobia (Chen *et al.*, 2000, 2002), were quite dissimilar from reference and type strains.

In relation to *B. elkanii*, two other clusters, joined at a similarity of 53 %, included reference strains, but not USDA

76^T. In addition, several strains were placed in three great groups (GG I, VI and VII) and were quite dissimilar from all type and reference strains (Table 1; Supplementary Fig. S1). The great majority of strains isolated from tropical legume species were placed in clusters related to *B. elkanii*, or within the new clusters. Indeed, in a previous study in which the protein profiles of isolates from the Brazilian Amazon and Atlantic forests were analysed, most (92 of 120) were clustered in a large group ($r=0{\cdot}86$) with *B. elkanii* (Moreira *et al.*, 1993). In contrast, bradyrhizobia isolated from the canopy tree *Platypodium elegans* and the lianas *Machaerium milleflorum* and *M. arboretum* in Panama showed higher similarity (98–99 %) to *B. japonicum* (Parker & Lunk, 2000). Also, in an analysis of a large collection of indigenous rhizobia in China, no *B. elkanii* strains were detected (Dr Wen-Xin Chen, personal communication). In conclusion, despite the low variability reported for the 16S rRNA region of *Bradyrhizobium* in some studies (So *et al.*, 1994; Urtz & Elkan, 1996; Moreira *et al.*, 1998; Molouba *et al.*, 1999; van Berkum & Fuhrmann, 2000; Chen *et al.*, 2000; Willems *et al.*, 2001), and despite the coefficients used in some of the analyses being different, the strains from our study were joined at a very low level of similarity, 28 %, showing greater variability than any previous report. For example, an analysis of African indigenous soybean bradyrhizobia by RFLP-PCR with five restriction enzymes grouped the strains with a similarity of 70 % (Abaidoo *et al.*, 2000), while in strains from native leguminous species of Senegal, the similarity with five restriction enzymes was approximately 74 %, decreasing to 55 % when a strain from *Aeschynomene* was included (Doignon-Bourcier *et al.*, 1999).

The great resolving power of RFLP-PCR of the IGS has been pointed out in some studies with rhizobia (e.g. Laguerre *et al.*, 1996) and, in a study with *Bradyrhizobium* strains, the region provided taxonomic information similar, but not always identical, to that obtained by DNA–DNA hybridization (Willems *et al.*, 2001). Furthermore, the results also indicated that the genus consists of a group of four highly related genospecies (*B. japonicum*, *B. liaoningense* and two other genospecies) and at least three other genospecies, one of which is *B. elkanii* (Willems *et al.*, 2001). In our study, type and reference strains of *B. japonicum* fell into a major group that included 54 strains, 59 % isolated from soybean, at a final level of similarity of 55 %. Type and reference strains of *B. elkanii* fell into another major group that clustered 53 strains, 64 % isolated from hosts other than soybean, at 44 % similarity. Once again, subclusters were observed within each major group, and seven other strains resulted in a final grouping of the IGS region at a level of similarity of 27 % (Table 1, Supplementary Fig. S2). As a comparison, when bradyrhizobia nodulating Senegalese legumes were analysed by PCR-RFLP of the IGS with eight enzymes, the strains were clustered at a final level of similarity of 36 % (Doignon-Bourcier *et al.*, 2000).

When variability in the 16S rRNA gene sequence is low, it has also been suggested that analysis of the 23S rRNA region can be useful, as the longer fragment contains more information with a faster rate of sequence change (Olsen & Woese, 1993; Ludwig & Schleifer, 1994; Wang & Martínez-Romero, 2000). Indeed, in our study, analysis of this region resulted in the largest number of differences and the strains were grouped at a very low level of similarity (16 %). At least two groups (I and XV) were quite different from the others and might represent novel species. However, in the other clusters, speciation was not clear, including strains with mixed properties of both *B. japonicum* and *B. elkanii* when the 23S rRNA was compared with the 16S rRNA and IGS regions. Therefore, the results from our study confirm that phylogenies of rhizobia based on 16S rRNA and 23S rRNA genes may be discordant (van Berkum *et al.*, 2003).

Combined analysis with the results of several ribosomal regions has been applied to phylogeny (e.g. Ludwig & Schleifer, 1994; Vinuesa *et al.*, 1998; Willems *et al.*, 2001). When Vinuesa *et al.* (1998) analysed the products of RFLP-PCR of the 16S rRNA and IGS regions of *Bradyrhizobium* strains with two enzymes per region, five genotypes were obtained, at a level of similarity of 85 % or higher. In our study, when the three ribosomal regions were considered, the diversity observed was considerably greater, and great groups clustered reference strains of *B. japonicum* and *B. elkanii* at similarity values of 54 and 46 %, respectively (Fig. 1). However, subclusters within the main groups were clearly observed and one priority is now to investigate whether they are related to subspecies, representing genetically determined clusters of strains within the species (Staley & Krieg, 1984), whether they represent intraspecific differences or even whether they represent novel species. Several other strains were quite dissimilar, including BTAi 1, confirming previous suggestions that this strain might belong to a separate *Bradyrhizobium* species (So *et al.*, 1994; Willems *et al.*, 2001; Vinuesa *et al.*, 1998; Jarabo-Lorenzo *et al.*, 2000). It is interesting to remember that BTAi 1 has mixed physiological properties of *Rhizobium* and *Bradyrhizobium*, e.g. it has a fast growth rate but produces an alkaline reaction with most carbon sources (Stowers & Eaglesham, 1983). The majority of soybean strains fell into the species *B. japonicum*, and most strains isolated from Brazilian legume species fell into *B. elkanii* or separate clusters. Combined analysis of the three ribosomal regions confirmed at least two new clusters of bacteria showing a very low level of similarity in relation to *B. japonicum* and *B. elkanii*. These clusters are not related to the other described species of *Bradyrhizobium*, since none of the strains showed extra-slow growth, like *B. liaoningense* (Xu *et al.*, 1995), or the characteristics of *B. yuanmingense*, isolated from *Lespedeza* spp. (Yao *et al.*, 2002), or of *B. canariense* (Vinuesa *et al.*, 2005a).

The groups defined in our study for all three ribosomal regions were confirmed in another computational study aiming at investigating the stability of the clusters by introducing perturbations using subsampling techniques. The analysis was performed for 511 experiments, where each

experiment was a combination of possibilities between restriction enzymes and ribosomal regions. The main groups as well the new clusters were confirmed using this new approach (Milagre, 2003). Our main goal is now to characterize the new clusters, and we have already started by sequencing the 16S rRNA region of the strains from this study. Our first results have confirmed relatedness of the strains with the genus *Bradyrhizobium*, although several of them differ in many nucleotides from other bradyrhizobia (Menna, 2005). However, combined analysis of other conserved and housekeeping genes may be necessary to define their exact taxonomic position, as has been recently shown by Vinuesa *et al.* (2005b) for other *Bradyrhizobium* strains. The strains clustered into GG IV of the combined analysis represented one exception and, although classified as *Bradyrhizobium* in the Brazilian rhizobial collection SEMIA, they show greater sequence similarity in the 16S rRNA gene to members of *Burkholderia* (Menna, 2005).

In Brazil, commercial inoculants must contain the strains recommended by an official committee of rhizobiologists, and decisions on these strains are taken based on their agronomic performance in trials with several strains (Hungria *et al.*, 2005). One important feature of the strains from this study is that they are all very effective in fixing N_2 and, indeed, 43 of them are recommended as the most effective for 31 of the 33 host legumes in this study (Table 1). Furthermore, many of the strains are effective and recommended for more than one legume species, e.g. SEMIA 6156 and SEMIA 6158 are officially recommended for *Crotalaria spectabilis* and *Canavalia ensiformis*, while SEMIA 6156 is also recommended for *Crotalaria juncea* and SEMIA 6158 for *Stizolobium atterrimum*. Another interesting observation is that some strains with identical RFLP profiles of the ribosomal regions were isolated and recognized as very effective for hosts from various legume species and tribes, e.g. SEMIA 6002, 6144 and 6156 gave identical profiles for the IGS region, but were isolated from different genera and tribes, *Vigna* (Phaseoleae), *Arachis* (Aechynomeneae) and *Crotalaria* (Crotolarieae), respectively. The only strain isolated from a species of the subfamily Caesalpinioideae, SEMIA 6099, was also quite similar in all ribosomal regions to SEMIA 6093, isolated from *Aeschynomene americana*, in the subfamily Papilionoideae. Thus, our data confirm previous results obtained in the analysis of protein profiles of 171 strains belonging to 14 legume species (Moreira *et al.*, 1993), since no evolutionary correlation was observed between bradyrhizobial clusters and host plants.

In conclusion, three ribosomal regions were analysed from a collection of strains classified as *Bradyrhizobium* based on morphological and physiological properties *in vitro* (FEPAGRO, 1999). All strains were characterized by high efficiency of N_2 fixation and had been isolated from a wide range of host tropical legumes. A degree of variability never reported before has been detected, emphasizing the high level of diversity of symbiotic diazotrophic bacteria in the tropics that still remains to be determined.

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Supplementary Table S1. Strains used in this study

Strain	Host plant	Source	Origin*	Other designation(s)†
SEMIA strains				
501	*Glycine max*	Brazil	FEPAGRO	
509	*Glycine max*	USA	Univ. Wisconsin	UW 509, USDA 487
511	*Glycine max*	USA	Univ. Wisconsin	UW 511, USDA 500
515	*Glycine max*	Brazil	FEPAGRO	
518	*Glycine max*	USA	Univ. Wisconsin	UW 518, USDA 10, Nit 3I1b10
528	*Glycine max*	Brazil	FEPAGRO	
535	*Glycine max*	Brazil	IAC	S 89
538	*Glycine max*	USA	Dixie (commercial)	NK
542	*Glycine max*	Brazil	IPA	R 1
543	*Glycine max*	Brazil	IPA	R 2
549	*Glycine max*	USA	Nitragin (commercial)	NK
560	*Glycine max*	Brazil	FEPAGRO	No synonyms
562	*Glycine max*	Brazil	FEPAGRO	No synonyms
565	*Glycine max*	Brazil	FEPAGRO	No synonyms
566	*Glycine max*	USA	Dixie (commercial)	BR 40
567	*Glycine max*	USA	Dixie (commercial)	No synonyms
568	*Glycine max*	USA	Dixie (commercial)	No synonyms
569	*Glycine max*	USA	Dixie (commercial)	No synonyms
571	*Glycine max*	USA	Dixie (commercial)	No synonyms
576	*Glycine max*	Brazil	FEPAGRO	
577	*Glycine max*	Brazil	FEPAGRO	
580	*Glycine max*	Brazil	FEPAGRO	
581	*Glycine max*	Brazil	FEPAGRO	
583	*Glycine max*	Thailand	CSIRO	CB 1786
586	*Glycine max*	USA	CSIRO	CB 1809, USDA 136b, TAL 379, BR 33
587‡	*Glycine max*	Brazil	FEPAGRO	BR 96
590	*Glycine max*	Brazil	IPEACS	RT-2a
598	*Glycine max*	Brazil	IPEACS	R-5a
656‡	*Neonotonia wightii*	Brazil	FEPAGRO	
658‡	*Lotononis bainesii*	South Africa	CSIRO	CB 376
662‡	*Vigna unguiculata*	Australia	CSIRO	CB 188
695‡	*Neonotonia wightii*	Australia	INTA	E 85, QA 922, SU 422, NA 630
696‡	*Desmodium uncinatum*	Australia	CSIRO	CB 627
5000	*Glycine max*	Brazil	FEPAGRO	
5003	*Glycine max*	Brazil	FEPAGRO	
5005	*Glycine max*	Brazil	FEPAGRO	
5006	*Glycine max*	Brazil	FEPAGRO	
5011	*Glycine max*	Brazil	FEPAGRO	
5012	*Glycine max*	Brazil	FEPAGRO	No synonyms
5016	*Glycine max*	Brazil	FEPAGRO	
5019‡	*Glycine max*	Brazil	Embrapa Agrobiologia	29w, 29W, BR 29
5021	*Glycine max*	Brazil	FEPAGRO	
5022	*Glycine max*	Thailand	NIFTAL	TAL 205
5023	*Glycine max*	Thailand	NIFTAL	TAL 211, THA 136
5024	*Glycine max*	NK	NIFTAL	TAL 378, CC 709
5026	*Glycine max*	Thailand	NIFTAL	TAL 415, THA 9
5027	*Glycine max*	USA	NIFTAL	TAL 183, 61a76
5029	*Glycine max*	USA	NIFTAL	TAL 103
5030	*Glycine max*	Thailand	NIFTAL	TAL 216, THA 106
5036	*Glycine max*	NK	Hansen (commercial)	No synonyms
5038	*Glycine max*	NK	FEPAGRO	No synonyms
5042	*Glycine max*	Brazil	IAC	S 89, 535Re
5045	*Glycine max*	NK	FEPAGRO	Nit 123P35
5048	*Glycine max*	USA	FEPAGRO	Nit 123P67
5055	*Glycine max*	Brazil	FEPAGRO	532Re
5056^{T}	*Glycine max*	NK	USDA	USDA 6^{T}, $3I1B6^{T}$, ATCC 10324^{T}, RCR 3425^{T}
5059	*Glycine max*	Indonesia	USDA	USDA 143, 3I1B143, ACCC 15039
5060	*Glycine max*	Japan	USDA	J 507
5062	*Glycine max*	Brazil	INPA	SVJ-04
5063	*Glycine max*	Uruguay	MGAP	U 284
5068	*Glycine max*	Nigeria	IITA	IRC 2180a
5069	*Glycine max*	Brazil	Embrapa Cerrados	CPAC BW
5070	*Glycine max*	Brazil	Embrapa Cerrados	CPAC 74K
5075	*Glycine max*	Finland	Univ. Helsinki	HAMBI 1135, ATCC 1032
5079‡	*Glycine max*	Brazil	Embrapa Cerrados	CPAC 15, DF 24
5080‡	*Glycine max*	Brazil	Embrapa Cerrados	CPAC 7
5082	*Glycine max*	Brazil	Embrapa Cerrados	CPAC 42
5084	*Glycine max*	Brazil	Embrapa Cerrados	CPAC 45
5085	*Glycine max*	Argentina	INTA	E 109
6002‡	*Vigna unguiculata*	Zimbabwe	CSIRO	CB 756, TAL 309, RCR 3824
6028‡	*Desmodium uncinatum*	Zimbabwe	NIFTAL	TAL 569, SPRL 472, MAR 472
6053‡	*Clitoria ternatea*	Malaysia	NIFTAL	TAL 827, UMKI 28

6093	*Aeschynomene americana*	USA	USDA	USDA 3331
6099	*Dimorphandra exaltata*	Brazil	Embrapa Agrobiologia	BR 5004, LMG 9989
6101‡	*Dalbergia nigra*	Brazil	Embrapa Agrobiologia	BR 8404
6144‡	*Arachis hypogaea*	Zimbabwe	IAC	SMS 400, USDA 3187, MAR 11
6145‡	*Crotalaria juncea*	Brazil	Embrapa Agrobiologia	BR 2001
6146‡	*Centrosema* sp.	Brazil	Embrapa Agrobiologia	BR 1808
6148‡	*Neonotonia wightii*	Brazil	IAC	SMS 303
6149‡	*Galactia striata*	Australia	IAC	CB 627, SMS 138
6150‡	*Acacia mearnsii*	Brazil	IAC	SMS 300
6152‡	*Calopogonium* sp.	Brazil	Embrapa Agrobiologia	BR-1602
6154‡	*Stylosanthes* sp.	Brazil	Embrapa Agrobiologia	BR 446
6155‡	*Stylosanthes* sp.	Brazil	Embrapa Agrobiologia	BR 502
6156‡	*Crotalaria spectabilis*	Brazil	Embrapa Cerrados	CPAC IJ
6157‡	*Cajanus cajan*	Brazil	Embrapa Agrobiologia	BR 2801
6158‡	*Crotalaria spectabilis*	Brazil	Embrapa Cerrados	CPAC 42
6159‡	*Enterolobium ellipticum*	Brazil	Embrapa Agrobiologia	BR 4406, LMG 9980
6160‡	*Albizia lebbek*	Brazil	Embrapa Agrobiologia	BR 5610
6161‡	*Prosopis juliflora*	Brazil	Embrapa Agrobiologia	BR 4002
6163‡	*Acacia mearnsii*	Brazil	Embrapa Agrobiologia	BR 3607
6164‡	*Acacia mearnsii*	Brazil	Embrapa Agrobiologia	BR 3608, LMG 9960
6165‡	*Mimosa scrabella*	Brazil	Embrapa Agrobiologia	BR 3454
6166‡	*Mimosa caesalpiniifolia*	Brazil	Embrapa Agrobiologia	BR 3446
6167‡	*Mimosa caesalpiniifolia*	Brazil	Embrapa Agrobiologia	BR 3452
6169‡	*Albizia falcataria*	Brazil	Embrapa Agrobiologia	BR 5612
6175‡	*Pueraria phaseoloides*	Brazil	Embrapa Cerrados	DF Q-1
6192‡	*Tipuana tipa*	Brazil	FEPAGRO	
6208‡	*Desmodium ovalifolium*	Colombia	CIAT	CIAT 2372
6319‡	*Arachis* sp.	Bolivia	Univ. North Carolina	NC 92
6382‡	*Mimosa caesalpiniifolia*	Brazil	Embrapa Agrobiologia	BR 3405
6387‡	*Acacia auriculiformis*	Brazil	Embrapa Agrobiologia	BR 3609, LMG 9961
6420‡	*Acacia mangium*	Brazil	Embrapa Agrobiologia	BR 3617, LMG 9965
6424‡	*Centrosema pubescens*	Brazil	Embrapa Cerrados	CPAC 36
6425‡	*Centrosema pubescens*	Colombia	Embrapa Cerrados	CIAT 2380
Other strains				
PRY 40	*Glycine max*	Paraguay	Embrapa Soja	
PRY 42	*Glycine max*	Paraguay	Embrapa Soja	
PRY 49	*Glycine max*	Paraguay	Embrapa Soja	
PRY 52	*Glycine max*	Paraguay	Embrapa Soja	
R 17	*Vigna unguiculata*	Brazil	Embrapa Tab. Costeiros	
R 35	*Cajanus cajan*	Brazil	Embrapa Tab. Costeiros	
R 45	*Cajanus cajan*	Brazil	Embrapa Tab. Costeiros	
AM-Cp 17	*Vigna unguiculata*	Brazil	INPA	
AM-P2 Lima	*Vigna unguiculata*	Brazil	INPA	
AM-P5 Abac	*Vigna unguiculata*	Brazil	INPA	
AM-2-855	*Vigna unguiculata*	Brazil	INPA	
AM-01-517	*Stryphnodendron pulcherrimum*	Brazil	INPA	
USDA 76^T	*Glycine max*	USA	USDA	
BTAi 1	*Aechynomene indica*	USA	USDA	

NK, Not known.

*CIAT, Centro Internacional de Agricultura Tropical, Cali, Colombia; CSIRO, Commonwealth Scientific and Industrial Research Organization, Canberra, Australia; Embrapa Agrobiologia, former IPEACS, SNLCS, UAPNPBS, RJ, Brazil; Embrapa Cerrados, former EMBRAPA CPAC, Planaltina, DF, Brazil; Embrapa Tabuleiros Costeiros, SE, Brazil; FEPAGRO, Fundação de Pesquisa Agropecuária, RS, Brazil; IAC, Instituto Agronômico de Campinas, SP, Brazil; IITA, International Institute of Tropical Agriculture, Ibadan, Nigeria; INPA, Instituto Nacional de Pesquisa da Amazônia, AM, Brazil; INTA, Instituto Nacional de Tecnologia Agropecuaria, Castelar, Argentina; IPA, Empresa Pernambucana de Pesquisa Agropecuária, PE, Brazil; IPEACS, later SNLCS, EMBRAPA-UAPNPBS, Embrapa Agrobiologia, RJ, Brazil; MGAP, Lab. Microbiología y Suelos, Montevideo, Uruguay; NIFTAL, Nitrogen Fixation in Tropical Agricultural Legumes Project, Hawaii, USA; USDA, US Department of Agriculture, Beltsville, MD, USA.

†ACCC, Center for Agricultural Culture Collection, Beijing, China; AM, INPA, Brazil; ATCC, American Type Culture Collection, Manassas, VA, USA; BR, from Embrapa Agrobiologia, Brazil; CB and CC, from CSIRO, Australia; CPAC, Embrapa Cerrados, Brazil; DF, Embrapa Cerrados, Brazil; E, INTA, Argentina; HAMBI, University of Helsinki, Finland; J, Institute of Agricultural Sciences, Tokyo, Japan; IRC, IITA, Nigeria; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Belgium; MAR, Grasslands *Rhizobium* Collection, Marandellas, Zimbabwe; NA, Gosford, Australia; NC, University of North Carolina, Durham, NC, USA; Nit, Nitragin Company Inc., Whitewater, WI, USA; PRY, Paraguay; QA, DPI, Queensland, Australia; R, Embrapa Tabuleiros Costeiros, Brazil; R- and RT-, IPEACS, now Embrapa Agrobiologia, Brazil; RCR, Rothamsted *Rhizobium* Collection, Harpenden, UK; S and SMS, Seção de Microbiologia do Solo, IAC, Brazil; SEMIA, from FEPAGRO-MIRCEN, Brazil; SPRL, Soil Productivity Research Laboratory, Zimbabwe; SU, University of Sydney, Australia; SVJ, INPA, AM, Brazil; TAL, NIFTAL, USA; THA, Dept of Agriculture, Bangkok, Thailand; U, MGAP, Uruguay; UMKI, Dept of Genetics and Cellular Biology, Kuala Lumpur, Malaysia; UW, University of Wisconsin, Madison, WI, USA.

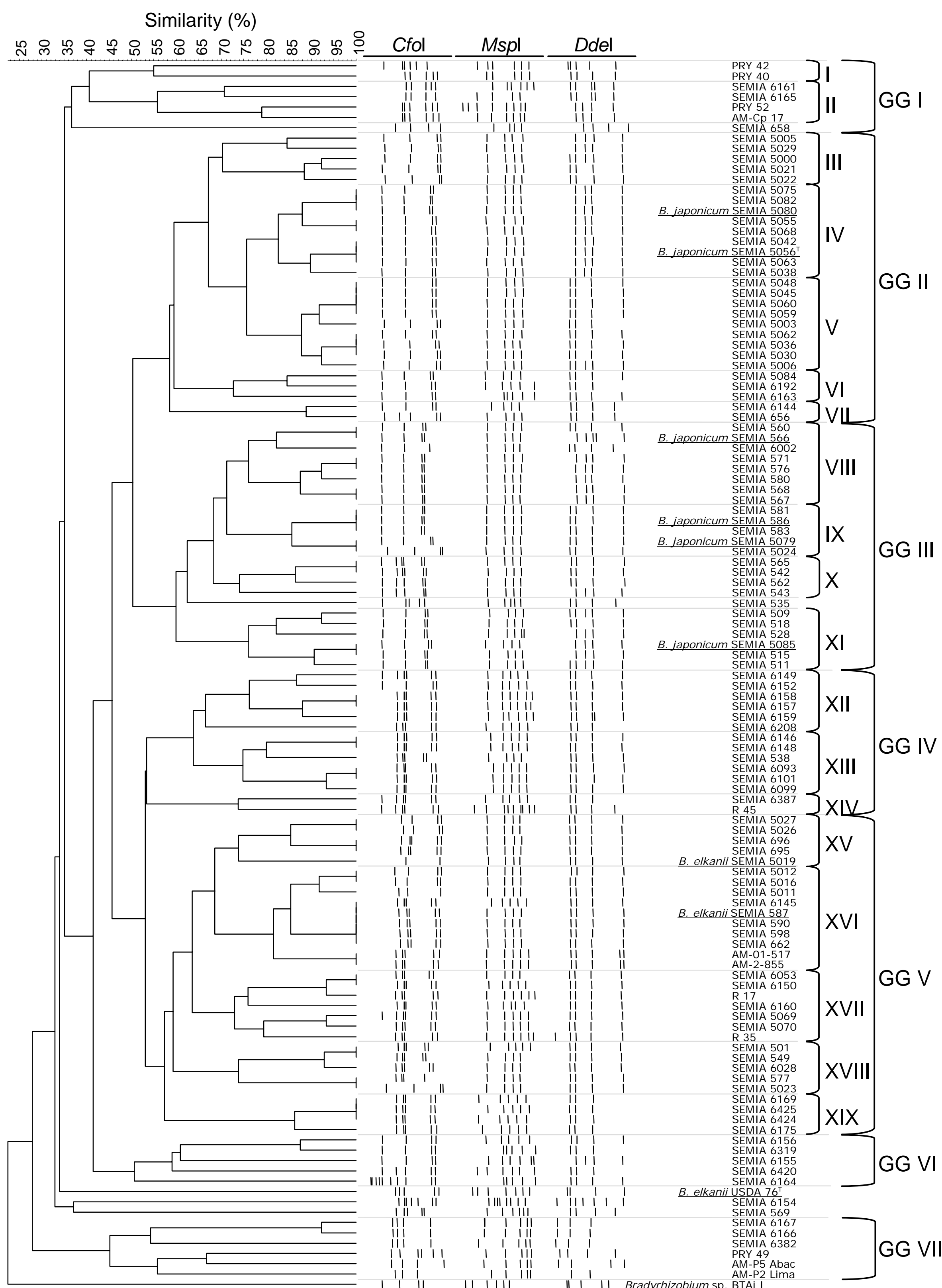
‡Strains recommended for use in Brazilian commercial inoculants for that host legume.

Gomes Germano, M., Menna, P., Mostasso, F. L. & Hungria, M. (2006). RFLP analysis of the RNA operon of a Brazilian collection of bradyrhizobial strains from 33 legume species. *Int J Syst Evol Microbiol* **56**, 217–229.

Supplementary Table S2. Species and subfamilies of host species of rhizobial strains characterized in this study

Plant species names and classification follows ILDIS (International Legume Database & Information Service; http://www.ildis.org), except for *Desmodium ovalifolium*.

Plant species	Subfamily	Tribe
Acacia auriculiformis Benth.	Mimosoideae	Acacieae
Acacia mangium Willd	Mimosoideae	Acacieae
Acacia mearnsii De Wild	Mimosoideae	Acacieae
Aeschynomene americana L.	Papilionoideae	Aeschynomeneae
Aechynomene indica L.	Papilionoideae	Aeschynomeneae
Albizia falcataria [synonym of *Falcataria moluccana* (Miq.) Barneby & Grimes]	Mimosoideae	Ingeae
Albizia lebbek (L.) Benth.	Mimosoideae	Ingeae
Arachis hypogaea L.	Papilionoideae	Aeschynomeneae
Arachis sp.	Papilionoideae	Aeschynomeneae
Cajanus cajan (L.) Millsp.	Papilionoideae	Phaseoleae
Calopogonium sp.	Papilionoideae	Phaseoleae
Centrosema pubescens Benth.	Papilionoideae	Phaseoleae
Centrosema sp.	Papilionoideae	Phaseoleae
Clitoria ternatea L.	Papilionoideae	Phaseoleae
Crotalaria juncea L.	Papilionoideae	Crotalarieae
Crotalaria spectabilis Roth	Papilionoideae	Crotalarieae
Dalbergia nigra (Vell.Conc.) Benth.	Papilionoideae	Dalbergieae
Desmodium ovalifolium	Papilionoideae	Desmodieae
Desmodium uncinatum (Jacq.) DC.	Papilionoideae	Desmodieae
Dimorphandra exaltata Schott	Caesalpinioideae	Caesalpinieae
Enterolobium ellipticum Benth.	Mimosoideae	Ingeae
Galactia striata (Jacq.) Urban	Papilionoideae	Phaseoleae
Glycine max(L.) Merr.	Papilionoideae	Phaseoleae
Lotononis bainesii Baker	Papilionoideae	Crotalarieae
Mimosa caesalpiniifolia Benth.	Mimosoideae	Mimoseae
Mimosa scabrella Benth.	Mimosoideae	Mimoseae
Neonotonia wightii (Wight & Arn.) Lackey	Papilionoideae	Phaseoleae
Prosopis juliflora (Sw.) DC.	Mimosoideae	Mimoseae
Pueraria phaseoloides (Roxb.) Benth.	Papilionoideae	Phaseoleae
Stylosanthes sp.	Papilionoideae	Aeschynomeneae
Stryphnodendro pulcherrimum	Mimosoideae	Mimoseae
Tipuana tipa (Benth.) Kuntze	Papilionoideae	Dalbergieae
Vigna unguiculata (L.) Walp.	Papilionoideae	Phaseoleae



Supplementary Fig. S1. Cluster analysis (UPGMA with the Jaccard coefficient, converted to percentage similarity) of products obtained by PCR-RFLP analysis of the 16S rRNA gene of bradyrhizobial strains with three restriction enzymes. Reference and type strains (underlined) are labelled and classified according to sequencing analysis of the 16S rRNA gene, as described in Methods.

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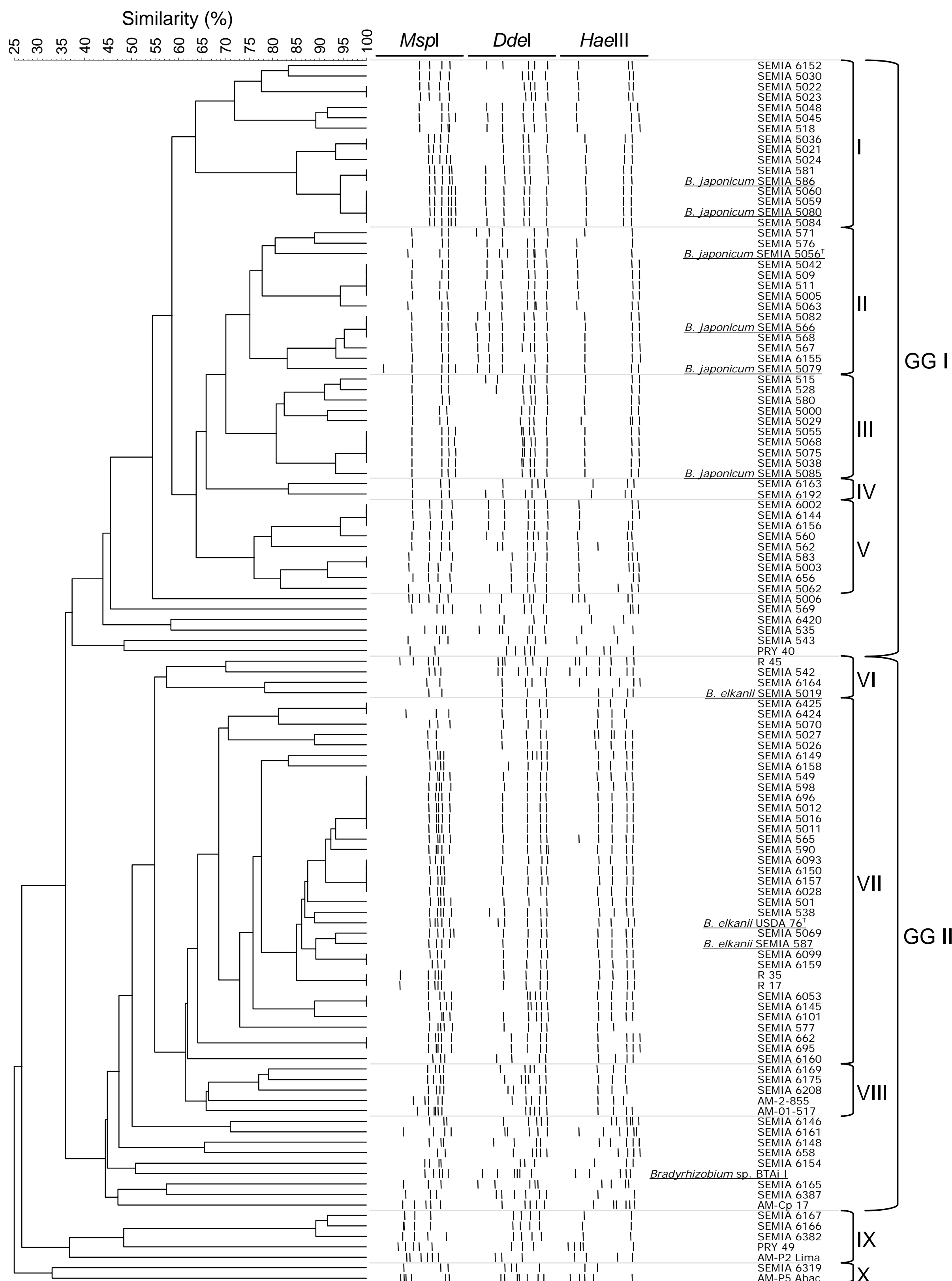
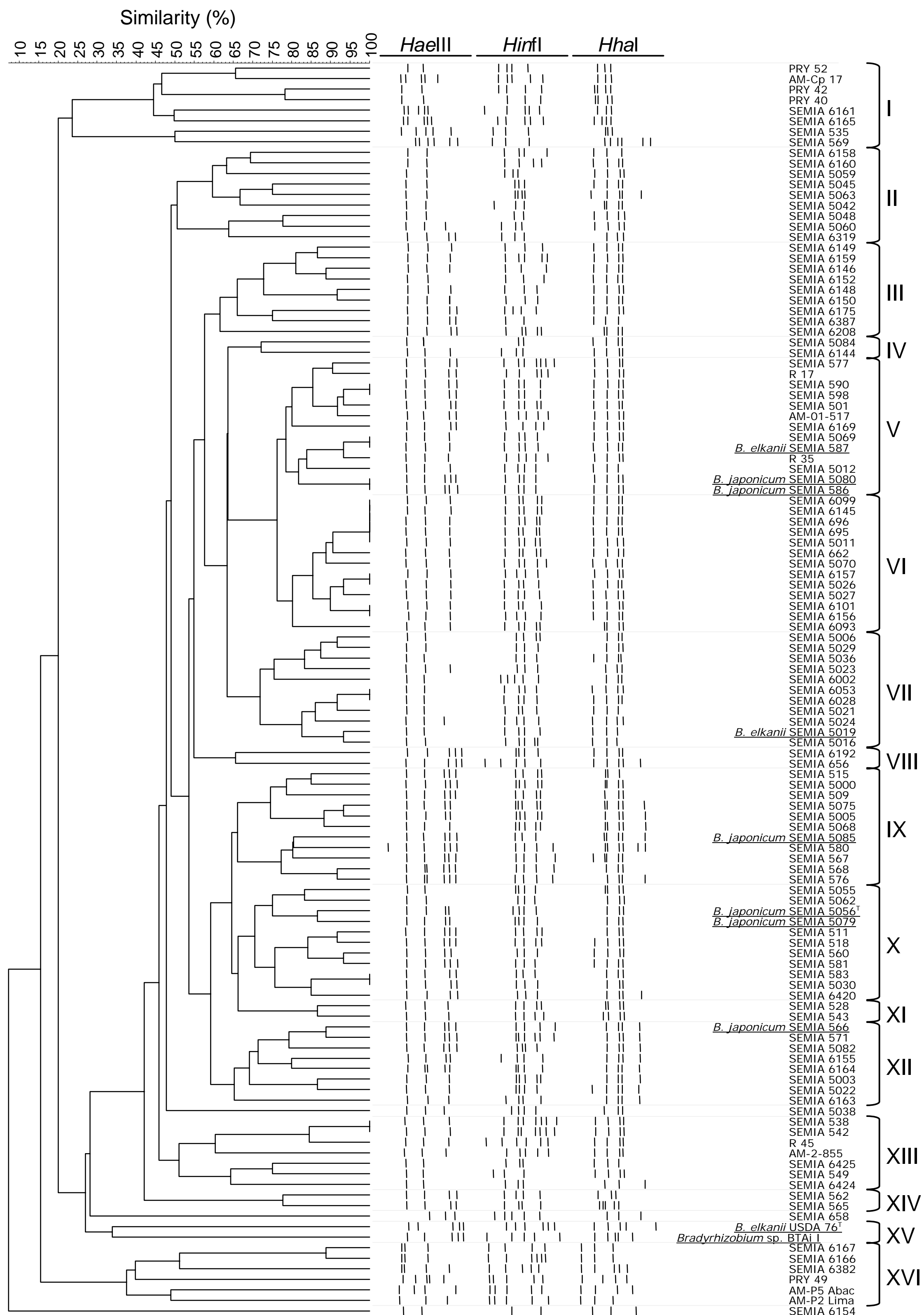


Supplementary Fig. S2. Cluster analysis of products obtained by PCR-RFLP analysis of the 16S–23S rRNA IGS of bradyrhizobial strains. Other details are given in the legend to Supplementary Fig. S1.

Gomes Germano, M., Menna, P., Mostasso, F. L. & Hungria, M. (2006). RFLP analysis of the RNA operon of a Brazilian collection of bradyrhizobial strains from 33 legume species. *Int J Syst Evol Microbiol* **56**, 217–229.



Supplementary Fig. S3. Cluster analysis of products obtained by PCR-RFLP analysis of the 23S rRNA gene of bradyrhizobial strains. Other details are given in the legend to Supplementary Fig. S1.

Gomes Germano, M., Menna, P., Mostasso, F. L. & Hungria, M. (2006). RFLP analysis of the RNA operon of a Brazilian collection of bradyrhizobial strains from 33 legume species. *Int J Syst Evol Microbiol* **56**, 217–229.