Microbial Ecology

Variability in *Bradyrhizobium japonicum* and *B. elkanii* Seven Years after Introduction of both the Exotic Microsymbiont and the Soybean Host in a Cerrados Soil

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Abstract

The plasticity of rhizobial genomes is far greater than previously thought, with complex genomic recombination events that may be accelerated by the often stressful environmental conditions of the tropics. This study aimed at evaluating changes in soybean rhizobia due to adaptation to inhospitable environmental conditions (high temperatures, drought, and acid soils) in the Brazilian Cerrados. Both the host plant and combinations of four strains of soybean *Bradyrhizobium* were introduced in an uncropped soil devoid of rhizobia capable of nodulating soybean. After the third year, seeds were not reinoculated. Two hundred and sixty-three isolates were obtained from nodules of field-grown soybean after the seventh year, and their morphological, physiological, serological, and symbiotic properties determined, followed by genetic analysis of conserved and symbiotic genes. *B. japonicum* strain CPAC 15 (same serogroup as USDA 123) was characterized as having high saprophytic capacity and competitiveness and by the seventh year represented up to 70% of the cultivable population, in contrast to the poor survival and competitiveness of *B. japonicum* strain CPAC 7 (same serogroup as CB 1809). In general, adapted strains had increased mucoidy, and up to 43% of the isolates showed no serological reaction. High variability, presumably resulting from the adaptation to the harsh environmental conditions, was verified in *rep*-PCR (polymerase chain reaction) profiles, being lower in strain CPAC 15, intermediate in *B. elkanii*, and higher in CPAC 7. Restriction fragment length polymorphism (RFLP)-PCR types of the 16S rDNA corresponded to the following: one type for *B. elkanii* species, two for *B. japonicum*, associated to CPAC 15 and CPAC 7, and unknown combinations of profiles. However, when *nodC* sequences and RFLP-PCR of the *nifH* region data were considered, only two clusters were observed having full congruence with *B. japonicum* and *B. elkanii* species. Combining the results, variability was such that even within a genetically more stable group (such as that of CPAC 15), only 6.4% of the isolates showed high similarity to the inoculant strain, whereas none was similar to CPAC 7. The genetic variability in our study seems to result from a variety and combination of events including strain dispersion, genomic recombination, and horizontal gene transfer. Furthermore, the genetic variability appears to be mainly associated with adaptation, saprophytic capacity, and competitiveness, and not with symbiotic effectiveness, as the similarity of symbiotic genes was higher than that of conserved regions of the DNA.

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Introduction

It is widely accepted that nitrogen (N_2) fixation is one of the most important biological processes on earth. The highest contribution in this process originates from the symbiosis of legumes with root-nodule bacteria collectively called "rhizobia", which represents the most efficient, well-developed, and intensively studied model of beneficial plant–microbe interaction. Although the biological process has been recognized almost 120 years ago, the past decade has been critical in breaking many

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established paradigms of rhizobial ecology. Improved understanding of relatedness between and within species has been achieved mainly as a result of the availability of new molecular tools, which have allowed the detection of a wide range of new N_2-fixing bacteria, and the realization that rhizobial genomes have an enormous plasticity with important implications in the soil microbial ecology [14, 61].

Examination of diazotrophic symbiotic bacteria especially in the still poorly documented soils of the tropics has led to the identification of unsuspected new genera not only within the α-Proteobacteria (e.g., *Methylobacterium* [60] and *Devosia* [46]), but also within the β-Proteobacteria, with a variety of *Burkholderia* capable of nodulating and fixing N_2 in association with several host legumes of the Mimosoideae [37] and Papilionoideae subfamilies [34].

The plasticity of rhizobial genomes is far greater than previously thought; complex genomic recombination events, including horizontal gene transfer, deletion, insertion, and integration of various DNA elements, mutations, and rearrangements, have led to extensive rhizobial variability [9, 35, 40, 41, 50, 59, 72]. This was emphasized with the complete sequencing of some rhizobial genomes and one major example was found in *Bradyrhizobium japonicum*, with a high number of transposases, repetitive RSα, and insertion sequences HRS1 [27], which may lead to insertions and deletions of genes, as well as transposition and genomic rearrangements [13, 27, 35]. In addition, the genes related to nodulation and N_2 fixation in both *B. japonicum* [13, 27] and *Mesorhizobium loti* [26] are located in the 500- to 600-kb regions of the chromosome called symbiotic islands, potentially transferable to other rhizobia [36, 57]. A major change in the paradigms came from reports that rhizobia can persist in soil in the absence of the host legumes [51, 57] and may acquire the symbiotic island from an inoculant strain upon legume reintroduction [57–59].

The extreme plasticity of rhizobia is also impacting the boundaries of phylogeny and taxonomy. First, although the conserved nature of the ribosomal genes, with emphasis on 16S rRNA, has led to its use for tracing bacterial phylogenies and defining taxonomy [10, 75], recombination in 16S [67] and 23S rRNA [40] genes of rhizobia has also been reported. Second, horizontal gene transfer resulting in strains with housekeeping and symbiotic genes from different species—which has been suggested, e.g., in *Rhizobium mongolense* [71], *Methylobacterium* [60], and *Burkholderia* [34]—might be more common than previously thought. As Thompson [64] noted, biologists often distinguish between evolutionary and ecological time, but for some evolutionary processes metapopulation structure can alter the genetic boundaries of species within mere decades.

Rhizobial diversity can also be affected by interactions with the host plant and agricultural practices (e.g., [7, 28, 44, 58]); furthermore, environmental stresses typical of the tropics, such as high temperatures, may accelerate genetic recombination (e.g., [53, 61]). Indeed, a high level of morphological, physiological, genetic, and symbiotic variability has been reported after several years of adaptation of exotic soybean rhizobia in the harsh environment of the Brazilian Cerrados, an edaphic type of tropical savanna occupying 207 million ha that is characterized by acid soils, aluminum toxicity, high temperatures, and drought conditions [1, 9, 18, 20, 38, 49]. Consistent with the recognition that adaptation to environmental and cropping conditions can affect the diversity of soybean rhizobia, the magnitude of the variability detected in the Brazilian Cerrados is greater than under less-severe environmental conditions, e.g., in *B. japonicum* in the United States [25] and in France [2, 39].

Our objective was to gain better understanding of the variability that occurs in conserved and symbiotic genes during adaptation of soybean rhizobia to the inhospitable conditions of the Brazilian Cerrados. To achieve this goal, isolates from field-grown soybean nodules were collected 7 years after the introduction of soybean and inoculants containing strains of *B. japonicum* and *B. elkanii* in an uncropped soil initially devoid of rhizobia capable of nodulating this host.

Material and Methods

Reference Strains. Reference strains of soybean [*Glycine max* (L.) Merrill] rhizobia were as follows: *B. japonicum* strains SEMIA 566 (very competitive, belongs to the same serogroup as USDA 123; used in Brazilian commercial inoculants from 1966 to 1978); CPAC 15 (=SEMIA 5079, natural variant of SEMIA 566 selected after adaptation to the Cerrados; used in commercial inoculants since 1992); CB 1809 (=SEMIA 586, very effective in fixing N_2, but shows poor competitiveness; used in commercial inoculants in 1977); CPAC 7 (=SEMIA 5080, natural variant of CB 1809, selected for adaptability to the Cerrados soils and shows greater competitiveness than CB 1809; used in commercial inoculants since 1992); and *B. elkanii* strains SEMIA 587 (used in commercial inoculants from 1968 to 1975 and since 1979) and 29W (=SEMIA 5019, used in commercial inoculants since 1979). More information about the strains is available elsewhere [19, 49]. CPAC 15 and CPAC 7 represent most of the strains applied to areas of the Cerrados cropped to soybean [19]. Type strains included in the study, *B. japonicum* USDA 6 and *B. elkanii* USDA 76, were provided by the United States Department of Agriculture (USDA, Beltsville, MD). It is noteworthy that all commercial inoculants in Brazil can carry exclusively the officially recommended strains and only four strains

(SEMIA 587, 29W, CPAC 7 and CPAC 15) have been used since the earlier 1980s; additionally, there are strict rules for inoculant quality control.

Rhizobial Isolation. For this study rhizobial isolates were obtained from nodules of field-grown soybean in an experiment that has been in progress since 1993 at the Experimental Station of the Brazilian Cerrados Research Center (Embrapa Cerrados), in Planaltina, Federal District, Brazil. The experiment is arranged in a completely randomized block design, in plots of 4 × 17 m, with 15 treatments, each with three replicates. The experiment has been described in detail by Mendes *et al.* [33]. The soil is classified as a clay dark-red oxisol—initially covered with the indigenous Cerrados vegetation—that had never been inoculated with rhizobia. At the start of the experiment, tests showed that the soil was devoid of indigenous rhizobia capable of establishing an effective symbiosis with soybean. In the first year, each plot was inoculated with one of four serologically distinct strains: *B. japonicum* strains CPAC 15 and CPAC 7 and *B. elkanii* strains 29W and SEMIA 587. In the second year, each plot was subdivided, and was either not inoculated, or inoculated with CPAC 7 or CPAC 15. In the third year, the entire area was inoculated with CPAC 7, and subsequently none of the plots was inoculated, although cropping was continued. Every year, the plots were cropped with soybean in the summer season (November–December to April–May), except for the fifth year when maize (*Zea mays* L.) was planted. Seeds were always surface-disinfected before sowing [33]. In this study, we focused only on the nine treatments that had been inoculated with *B. japonicum* strains CPAC 15 and CPAC 7, as shown in Table 1.

In the seventh year of the experiment, 40 nodules per treatment were randomly collected from field-grown soybean plants of cultivar Doko and rhizobia were isolated by using standard procedures [70]. Purity of the cultures was confirmed by repeatedly streaking the bacteria on yeast extract–mannitol–agar (YMA) medium [70] and verifying a single type of colony morphology, and uniform absorption of Congo red and Gram-stain reaction. Because isolates stopped growing after isolation, 21 to 39 isolates were obtained from each treatment, reaching a total of 263 rhizobia (Table 1). Single colonies were individually transferred to YM liquid broth (YMB) and after growth were mixed with glycerol (25%) and stored at −80°C. Working cultures were maintained on YMA slants at 4°C. Rhizobia were routinely cultured at 28°C in YMB on a rotary shaker operating at 65 cycles/min.

Colonies were characterized in relation to size, color, mucoidy, and acid/alkaline reaction as previously described [70], after 7 days of growth in the dark at 28°C on YMA containing either Congo red or bromothymol blue as a pH-change indicator.

Serotyping of Rhizobial Isolates. Serotyping was done by immunoagglutination [70]. Polyclonal antisera were prepared against the somatic thermostable antigens of strains 29W (serogroup 29W), SEMIA 587 (serogroup 587), CPAC 15 (same serogroup as SEMIA 566), and CPAC 7 (same serogroup as CB 1809), as described by Somasegaran and Hoben [54].

Extraction of DNA. DNA was extracted from reference strains and rhizobial isolates as previously described [28]. To obtain clean DNA, the extraction procedure included the addition, for each 400 μL of bacteria resuspended in TE 50/20, of 50 μL of 10% SDS, 5 μL of proteinase K (20 mg mL^{-1}), 10 μL of lysozyme (5 mg mL^{-1}), and 2 μL of RNase (10 mg mL^{-1}). After two steps of purification with ethanol at 99.5% and at 70%, the pellet was resuspended in 50 μL of TE 10/1 to estimate the concentration of the DNA. Samples were then diluted to 20 ng of DNA μL^{-1} and were kept at −20°C.

Restriction Fragment Length Polymorphism (RFLP) of the PCR-Amplified DNA Region Coding for the 16S rRNA Gene. A nearly full-length portion of the 16S rDNA (1500 bp) was amplified from each strain and isolate

Table 1. Treatments for the field experiment performed in a Brazilian Cerrados soil from which the rhizobial isolates were obtained

	Year[a]			
Treatments	*First*[a]	*Second*	*Third*	*No. of isolates*[b]
1	CPAC 7	Noninoculated	CPAC 7	22
2		CPAC 7	CPAC 7	28
3		CPAC 15	CPAC 7	37
4	CPAC 15	Noninoculated	CPAC 7	25
5		CPAC 7	CPAC 7	39
6		CPAC 15	CPAC 7	28
7	Noninoculated	Noninoculated	CPAC 7	22
8		CPAC 7	CPAC 7	29
9		CPAC 15	CPAC 7	33

[a]Soybean was cropped every year except for the fifth year, cropped with maize. In the fourth, fifth, sixth, and seventh years, the plots were not inoculated.
[b]Obtained in the seventh year of the experiment.

by PCR with rD1 (3′-AAGGAGGTGATCCAGCC-5′) and fD1 (5′-AGAGTTTGATCCTGGCTCAG -3′) primers, which correspond to positions 8–27 and 1524–1540, respectively, of *Escherichia coli* 16S rRNA gene [73]. Volumes and cycles used in the reaction were as previously described [11], except for a decreased concentration of *Taq* DNA polymerase (1.0 U), and the reaction was carried out in an MJ Research Inc. PTC 200 thermocycler. Aliquots (6 μL) of polymerase chain reaction (PCR) products were separately digested with each of the following restriction endonucleases: *Hpa*II, *Hha*I, and *Dde*I (Invitrogen™), as recommended by the manufacturers. The fragments obtained were analyzed by gel electrophoresis (17 × 11 cm) with 3% agarose, and carried out at 90 V for 4 h. The 1-kb Plus DNA Ladder (Invitrogen™) was used as a molecular size marker at the right, left, and central lanes of each gel. The gels were then stained with ethidium bromide and photographed under UV radiation with a Kodak Digital Science 120 apparatus. The profiles obtained were confirmed in triplicates.

rep-PCR Fingerprinting with BOX Primer. PCR amplification of repetitive regions of the DNA (*rep*-PCR) was carried out with BOX-A1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′, Invitrogen™) [69]. Volumes and amplification cycles were performed as previously described [28], and the reaction was performed in an MJ Research Inc. PT 200 thermocycler. The amplified fragments were separated by electrophoresis on 1.5% agarose (low EEO, type I-A) gels (20 × 25 cm), at 120 V, for 7 h. The 1-kb Plus DNA Ladder (Invitrogen™) was used as a molecular marker at the right, left, and central lanes of each gel. Gels were stained with ethidium bromide, visualized under UV radiation and photographed.

RFLP-PCR of the DNA Region Coding for the nifH Gene. Primers nifHF and nifHI [31] were used to amplify a region of the DNA of approximately 800 bp of the *nifH* gene. Volumes and amplification cycles were performed as described by Laguerre *et al.* [31]. Aliquots (6 μL) of PCR products were separately digested with each of the three restriction endonucleases: *Hpa*II, *Hin*dIII, and *Hae*III (Invitrogen™), as recommended by the manufacturers. The fragments obtained were analyzed as described in the item of RFLP-PCR of the 16S rDNA.

Sequencing of nodC Gene. DNA amplification of *nodC*-gene region was carried out by using nodCIF (5′-GTCGATTGCMRGTCAAGACTACG-3′) and nodCp8 (5′-GCCAGGTCTIGTTGCGATTGCTC-3′) primers [55]. The amplification consisted of an initial cycle of denaturation at 94°C for 1 min and 10 s; 35 cycles of denaturation at 94°C for 20 s; of annealing at 59°C for 50 s; of extension at 72°C for 1 min and 20 s; and a final extension step at 72°C for 5 min. A PCR product of about 300 bp was obtained and was purified as previously described [34]. Sequencing of the fragments was performed by using the same primers (nodCIF and nodCp8) and the procedure described by Menna *et al.* [34], in a MegaBACE Amersham Biosciences automated sequencing system with dye terminator chemistry.

Data Analyses. The sizes of the fragments in each analysis were normalized according to the MW of the DNA markers. The fingerprints obtained by both the RFLP-PCR of the 16S rDNA and *nifH* genes and the *rep*-PCR were analyzed via BioNumerics software (Applied Biosystems, Kortrijk, Belgium, version 1.50). In the RFLP-PCR analysis, the pattern with each restriction enzyme was first obtained, and then the combined analysis of the three enzymes was performed. A tolerance of 3% was established in the Bionumerics for the BOX analysis. The unweighted pair-grouping method with arithmetic mean (UPGMA) algorithm [52] with the coefficient of Jaccard [22] were used in both RFLP-PCR and *rep*-PCR analyses.

For the *nodC* region, sequences confirmed in the 3′ and 5′ directions were submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/blast) to seek significant alignments. The sequences obtained were deposited in the GenBank database under accession numbers DQ376585 to DQ376617. Multiple alignments were performed with ClustalX version 1.83 [63]. Phylogenetic trees were generated by using MEGA version 3.1 [30] with default parameters, K2P distance model [29], and the neighbor-joining algorithm [47]. The sequences obtained were aligned and compared to those of the following type/reference strains (accession numbers of the GenBank Data Library in parentheses): *B. japonicum* USDA 6 (D28962), *Rhizobium tropici* CFN 299 (X98514), *B. elkanii* USDA 46 (D28963), *Rhizobium* sp. NGR234 (NC000914), *Sinorhizobium* (*Ensifer*) *meliloti* 1021 (AE007237). *Azorhizobium caulinodans* ORS571 (L18897) was used as an outgroup reference. Statistic support for tree nodes was evaluated by bootstrap analysis [5] with 2000 samplings [16].

Results

Morphological Characterization and Serotyping. After 7 days of cultivation, all but one of the 263 isolates alkalinized the medium containing mannitol as carbon source, showing typical properties of *B. japonicum/B. elkanii in vitro*. Large variability was detected in mucoidy, ranging from dry colonies to copious production of mucus, and isolates varied also in relation to the mucus viscosity, from aqueous to sticky. Size of the colonies

varied from 0.2 to 2.5 mm in CPAC 15, from 0.2 to 2.0 mm in CPAC 7, and from 0.2 to 1.5 mm in *B. elkanii* SEMIA 587 and 29W. Size and mucoidy were not associated with any of the treatments from which the isolates were obtained (data not shown).

In relation to the serotype CPAC 15, by the seventh year 40.0% to 69.7% (treatments inoculated with CPAC 15 in the first and in the second year, respectively) of the isolates reacted with the antiserum of CPAC 15 (Table 2). It is also noteworthy that treatments 7, 8, and 9 showed the highest percentages of isolates belonging to serogroup CPAC 15, although the treatments 7 and 8 had never been inoculated with that strain. In contrast, only a few isolates from two treatments reacted with the antiserum of CPAC 7, representing 7.1% and 5.1% of the isolates from treatments 2 and 5, respectively (Table 2).

Although none of the plots had been inoculated with *B. elkanii* strains, isolates belonging to serogroup SEMIA 587 represented up to 8.0% of the isolates in treatment 4, but were absent in the plots inoculated with CPAC 15 in the second year (treatments 3, 6 and 9). Serogroup 29W was detected in all treatments except those inoculated with CPAC 7 for 3 consecutive years, with the highest percentages occurring in plots inoculated with CPAC 15 in the first year—24.0% and 17.9%—for treatments 4 and 5, respectively. Isolates with unknown serological reaction were detected in all treatments and represented 13.6% (treatment 7) to 42.9% (treatment 2) of the total number of isolates. In addition, unknown serogroups occurred mainly in treatments 1, 2, and 3, inoculated with strain CPAC 7 in the first year. Double reactions occurred with very few isolates (Table 2).

Low, medium, and high production of mucus as well as variability in colony size were observed in isolates from all four serogroups; however, higher mucoidy was more often verified in the CPAC 15 serotype (data not shown).

RFLP-PCR of the 16S rDNA. When the DNA of all of the isolates and reference strains were analyzed by RFLP-PCR of the 16S rDNA, two different profiles were obtained with the *Hha*I and *Hpa*II restriction enzymes, and three were obtained with *Dde*I (Fig. 1). Both strains belonging to serogroup of CPAC 15 (parental SEMIA 566 and variant CPAC 15) showed similar profiles to *B. japonicum* USDA 6. However, serogroup of CPAC 7 (parental SEMIA 586 and CPAC 7) was different in the fingerprinting obtained with enzyme *Dde*I. The three *B. elkanii* strains (SEMIA 587, 29W, and USDA 76) showed similar profiles with all three enzymes (Table 3).

The combined RFLP-PCR profiles of all isolates were compared with those of reference strains and grouped according to treatment and serotyping (Table 2). In

Table 2. Percent distribution[a] of soybean rhizobial isolates considering the serogroups and the RFLP-PCR profiles of the 16S rDNA

		Treatment								
Serogroup	*RFLP-PCR*	1	2	3[b]	4[b]	5	6	7	8	9[b]
CPAC 15	CPAC 15	31.8	32.1	43.2	20.0	43.6	50.0	59.1	51.7	60.6
	CPAC 7	13.6	3.6	–	12.0	–	–	9.1	6.9	3.0
	B. elkanii	4.5	7.1	2.7	8.0	2.6	–	–	3.4	6.1
	unknown	–	–	2.7	–	–	3.6	–	–	–
Total		50.0	42.9	48.6	40.0	46.2	53.6	68.2	62.1	69.7
CPAC 7	CPAC 15	–	–	–	–	–	–	–	–	–
	CPAC 7	–	3.6	–	–	2.6	–	–	–	–
	B. elkanii	–	–	–	–	–	–	–	–	–
	unknown	–	3.6	–	–	2.6	–	–	–	–
Total		–	7.1	–	–	5.1	–	–	–	–
SEMIA 587	CPAC 15	–	3.6	–	–	–	–	–	–	–
	CPAC 7	–	–	–	–	–	–	–	3.4	–
	B. elkanii	4.5	3.6	–	8.0	5.1	–	4.5	–	–
	Unknown	–	–	–	–	–	–	–	–	–
Total		4.5	7.1	–	8.0	5.1	–	4.5	3.4	–
29W	CPAC 15	–	–	5.4	–	2.6	–	–	3.4	–
	CPAC 7	–	–	–	4.0	–	–	–	3.4	–
	B. elkanii	4.5	–	5.4	16.0	15.4	7.1	13.6	–	6.1
	unknown	–	–	–	4.0	–	3.6	–	–	–
Total		4.5	–	10.8	24.0	17.9	10.7	13.6	6.9	6.1
Unknown reaction	CPAC 15	13.6	21.4	18.9	20.0	10.3	25.0	4.5	20.7	15.2
	CPAC 7	4.5	7.1	8.1	4.0	5.1	7.1	4.5	–	–
	B. elkanii	22.7	14.3	10.8	–	10.3	3.6	4.5	3.4	–
	unknown	–	–	–	–	–	–	–	3.4	–
Total		40.9	42.9	37.8	24.0	25.6	35.7	13.6	27.6	15.2

[a]RFLP-PCR profile not detected is represented by "–".
[b]Treatments 3, 4, and 9 had also one isolate with a 29W/587 double reaction, one with a 29W/CPAC 15 double reaction, and three with a 29W/587 double reaction, respectively, and all with RFLP profiles of *B. elkanii*.

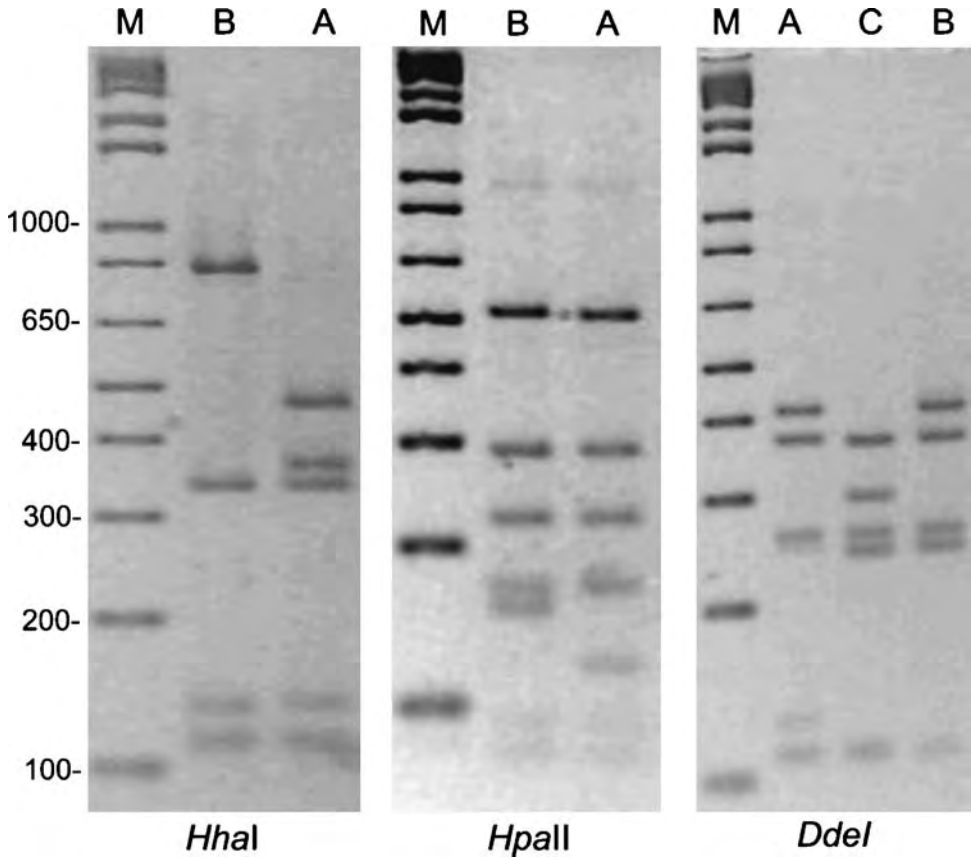


Figure 1. RFLP-PCR profiles of the 16S rDNA region of reference strains of *B. japonicum* and *B. elkanii* obtained with three restriction enzymes. M: the 1-kb Plus DNA Ladder (Invitrogen™); A, B, and C: profiles obtained with each restriction enzyme, as described in Table 3.

relation to the dominant serotype CPAC 15, the great majority of the isolates showed congruence between the serological properties and the RFLP profiles. In the first three treatments—all inoculated with CPAC 7 in the first year—there were increases in the percentage of isolates showing similarity to the CPAC 15-serogroup-RFLP type from treatments 1 (noninoculated) to 3 (inoculated with CPAC 15 in the second year), representing from 63.6% to 88.9% of all isolates belonging to this serogroup. In treatments not inoculated in the first year (7, 8, and 9), CPAC 15-serogroup-RFLP type represented the majority of the isolates, probably due to high saprophytic capacity of a few contaminants in the first year (Table 2).

Isolates of the CPAC 7-serogroup-RFLP type were exclusively present in treatments 2 and 5, the first inoculated with CPAC 7 for 3 consecutive years and the second inoculated with this strain in the second and third years. The CPAC 7-RFLP type represented 50% of all isolates belonging to this serogroup, the other 50% being represented by isolates of the *B. elkanii*-RFLP type (Table 2).

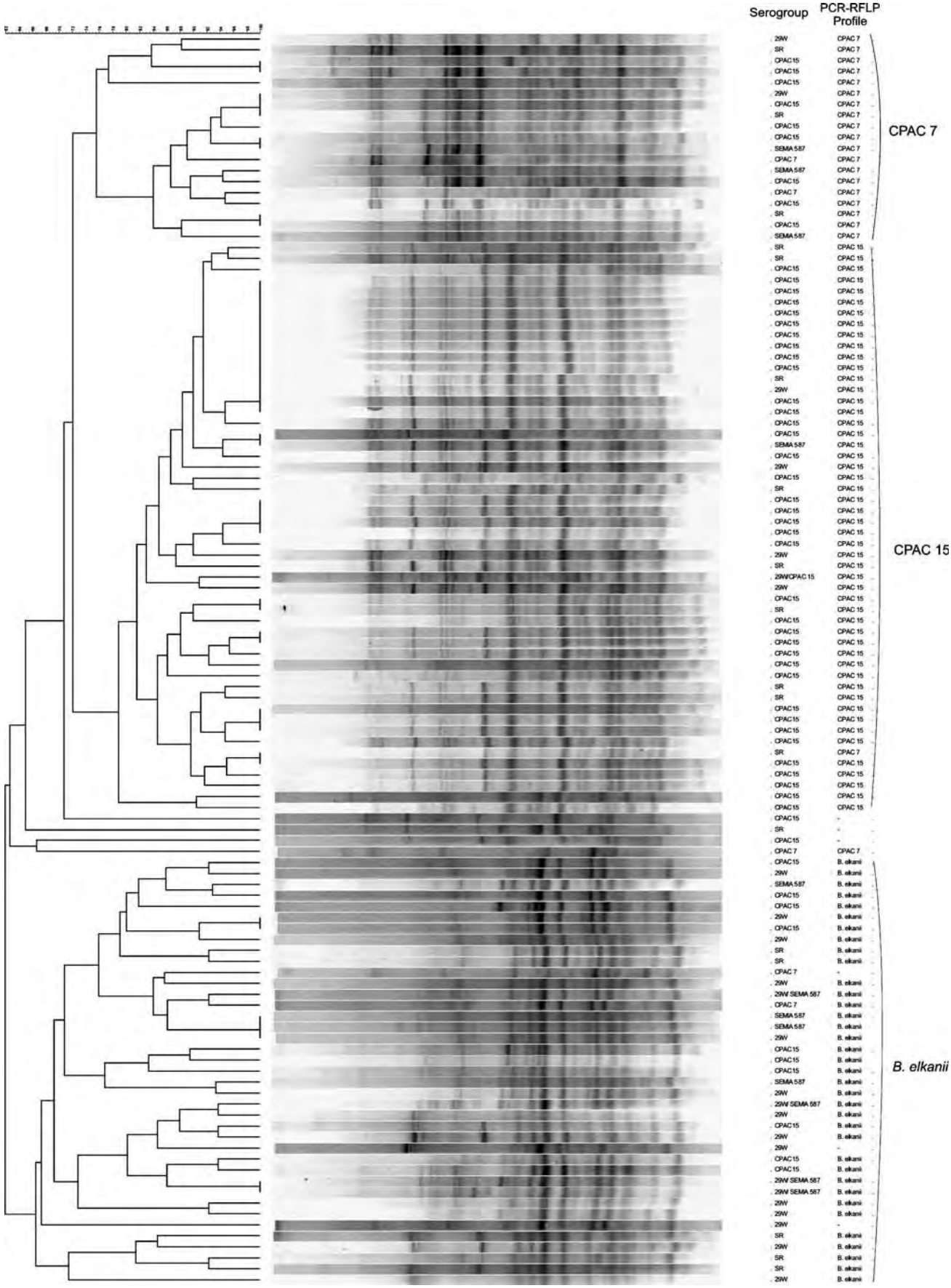
Although SEMIA 587 and 29W were not used as inoculants in the plots in this study, strains showing similar serogroup-RFLP types of both strains were detected in all treatments, although generally at low percentages, and probably represent contamination from other plots. In addition, the *B. elkanii*-RFLP type was detected in the CPAC 15 but not in the CPAC 7 serogroup (Table 2).

In relation to the isolates with unknown serological reaction that represented up to 42.9% of all isolates, the great majority was mainly of the CPAC 15-RFLP type and of *B. elkanii*; however, the CPAC 7 type was also detected in all but treatments 8 and 9. Unknown RFLP profiles were observed in six isolates; five others that showed cross-serological reactions were all *B. elkanii*-RFLP type. There was no indication of a relationship between treatment and the number of isolates with different RFLP types (Table 2).

Table 3. Profiles[a] of RFLP-PCR of the 16S rDNA region with three restriction enzymes obtained for each reference strain

	Restriction enzyme			
Strain	*Hha*I	*Hpa*II	*Dde*I	*Serotype*
CPAC 7	b	b	b	CPAC 7
CB 1809	b	b	b	CPAC 7
CPAC 15	b	b	c	CPAC 15
SEMIA 566	b	b	c	CPAC 15
29W	a	a	a	29W
SEMIA 587	a	a	a	SEMIA 587
USDA 6^T	b	b	c	USDA 6
USDA 76^T	a	a	a	USDA 76

[a]Profiles as shown in Fig. 1.


Serogroup
PCR-RFLP Profile
CPAC 7
CPAC 15
B. elkanii

rep-PCR Fingerprinting. Eight to 15 isolates were taken from each treatment and were used for the *rep*-PCR fingerprinting, resulting in 109 profiles and a dendrogram was built with those isolates as well as with the four inoculant strains. Three main clusters were observed, related to the CPAC 7, CPAC 15, and SEMIA 587-29W inoculant strains, respectively (Fig. 2).

The first cluster joined the isolates resembling CPAC 7 at a final level of similarity of 75.5% and clearly showed high variability; moreover, none of the isolates showed a profile exactly the same as the inoculant strain. Within this cluster, both the disappearance of bands and the appearance of new bands were observed in comparison to inoculant strain CPAC 7. Additionally, the first five isolates were positioned in a subcluster considerably different from the parental strain (Fig. 2).

The second and largest group included isolates identical or resembling the inoculant strain CPAC 15 at a final level of similarity of 78.8%. Within this cluster, a first group included 17 isolates showing high similarity (91.5%) with CPAC 15, whereas the last 20 strains were grouped in two subclusters mainly characterized by the absence of several bands in comparison to CPAC 15. Four strains occupied an intermediate position between CPAC 15 and *B. elkanii* clusters, showing bands of both types (Fig. 2).

Inoculant strains SEMIA 587 and 29W showed identical *rep*-PCR profiles and 37 isolates were grouped in a third cluster with those two strains at a final level of similarity of 66.5%. Interestingly, the variability of the isolates within this group was mainly associated with the appearance of new bands (Fig. 2).

RFLP-PCR of the DNA Region Coding for the nifH Gene. The DNA region containing approximately 800 bp of the *nifH* gene was amplified in 29 isolates, chosen to represent all treatments, and submitted to RFLP with three restriction enzymes. Only two patterns were obtained, one related to *B. japonicum*—including strains CPAC 7 and CPAC 15—and the other related to *B. elkanii* (Fig. 3).

nodC Partial Sequence Analysis. Partial sequences (280 bp) of the PCR-amplified *nodC* fragments were also determined for the 29 isolates used in the analysis of RFLP-PCR of the *nifH* region. The dendrogram obtained with the partial sequences of isolates and reference strains clearly showed two distinct clusters, each with a bootstrap support of 99% (Fig. 4). Again, one cluster included both RFLP-16S rDNA types of *B. japonicum* and the second included the RFLP-16S rDNA type of *B. elkanii*. Furthermore, the same cluster included isolates showing variability in the *rep*-PCR analysis. For example, isolates from the *rep*-PCR cluster showing similarities of 69.6% [70] to 100% with CPAC 15 (Fig. 2) showed complete similarity of bases of the *nodC* sequences (Fig. 4). Additionally, for strains belonging to noncongruent RFLP and serological groups, including unknown serotypes, *nodC* group was correlated with RFLP type.

Combining the Serotyping, RFLP-PCR of the 16S rDNA, and rep-PCR Data. Serological reaction, RFLP-PCR of the 16S rDNA, and *rep*-PCR profiles of the 109 isolates were analyzed together to investigate possible mechanisms of variability occurring after the introduction of inoculant rhizobial strains and the host plant in the soil. DNA regions encoding *nodC* and *nifH* were not considered because of the full congruence with the 16S rDNA and the lower level of information, as only two types were observed, corresponding to *B. japonicum* and *B. elkanii* species.

Rhizobial variability observed 7 years after the introduction in a Cerrados soils was very high; for example, isolates obtained from plots inoculated with CPAC 15 and showing high similarity in all properties to the inoculant strain (6.4%) represented as much as the isolates from plots not inoculated with CPAC 15, but with dispersion of that strain from other areas and also showing high variability in the *rep*-PCR profiles (Table 4).

Discussion

In our study, rhizobial isolates were obtained from a soil initially devoid of indigenous soybean rhizobia to which *B. japonicum* strains CPAC 15 (natural variant of SEMIA 566) and CPAC 7 (natural variant of CB 1809) were added as inoculants in different combinations for 3 years, with no further inoculation. SEMIA 566 was one of the first strains "selected" in Brazil. Isolated from a soybean nodule in the early 1960s, it effectively nodulated the cultivars released by that time; therefore, it was commercially recommended from 1966 to 1978, and greatly contributed to the successful establishment of the crop in the southern region of Brazil. More than a decade later, increased N demands of newer and more productive soybean genotypes dictated the selection of strains with

◀**Figure 2.** Cluster analysis (UPGMA with the coefficient of Jaccard) of the *rep*-PCR (primer BOX) products of soybean rhizobial isolates obtained 7 years after the introduction of inoculant strains (*B. japonicum* CPAC 15 and CPAC 7 and *B. elkanii* SEMIA 587 and 29W) in a Cerrados soil. At the right side, the first column presents the serological characterization and the second column the profile obtained by RFLP-PCR of the 16S rRNA for each isolate.

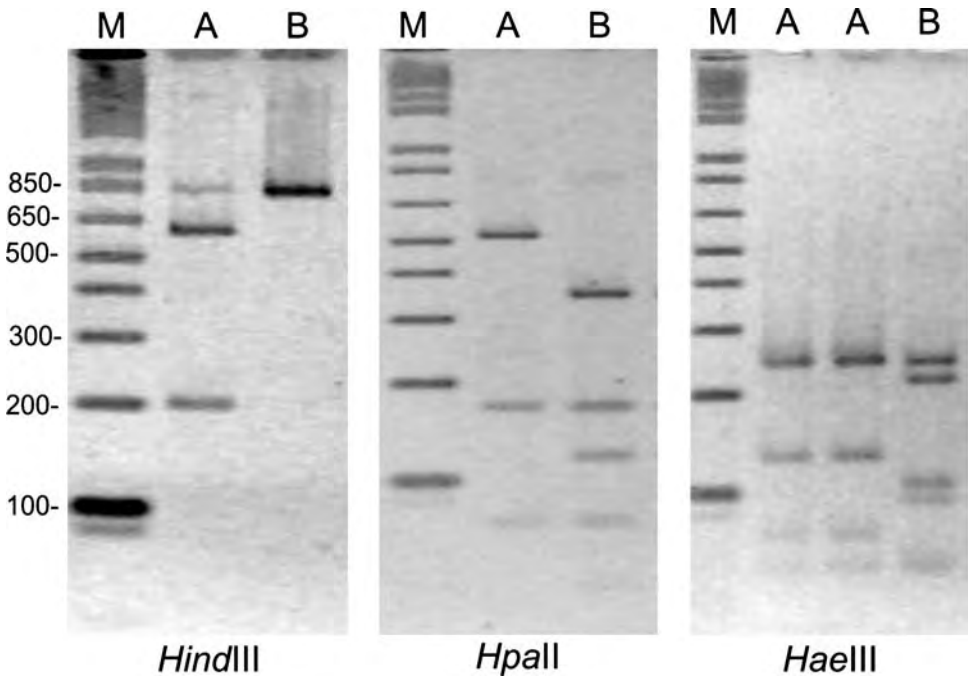


Figure 3. RFLP-PCR profiles of the DNA region coding for *nifH* gene of reference strains after digestion with three restriction enzymes. M: the 1-kb Plus DNA Ladder (Invitrogen™); A: profiles with *B. japonicum* strains CPAC 7 and CPAC 15; B: profiles with *B. elkanii* strains SEMIA 587 and 29W.

higher N_2 fixation capacity. The more effective variant of SEMIA 566, CPAC 15, has been used in Brazilian inoculants since 1992 [7, 18, 19, 21, 38, 43]. Since 1986, there have been reports showing that strains related to this serogroup may occur in up to 70% of the soybean nodules growing in the Brazilian Cerrados, even in areas that had never been inoculated before. This has been attributed to dissemination on seeds and on agricultural machinery from other soybean-growing areas in southern Brazil [7, 68], as well as by wind [7]. SEMIA 566 and CPAC 15 differ from USDA 123 in *rep*-PCR (ERIC primer), lipopolysaccharide, and protein profiles [7], but they are serologically related to USDA 123 [33], considered as the most competitive of *B. japonicum* strains in the midwestern soils of the United States (e.g., [15, 5]). In contrast, strain CB 1809 is recognized as very effective in fixing N_2 but poorly competitive in relation to many soybean rhizobial strains. Therefore, again, in the late 1980s, a natural variant with improved competitiveness (CPAC 7) was obtained and has been commercially recommended since 1992 [7, 18, 19, 21, 38, 43]; however, its competitiveness is poor when compared to CPAC 15 [33].

Differences among isolates in our study were first seen in colony size and mucus production (extracellular polysaccharides, EPSs); in particular in serogroup CPAC 15, mucus production tended to increase with adaptation, similar to prior observations by Boddey and Hungria [1]. A previous study with soybean rhizobia from the Brazilian Cerrados, employing pyrolysis mass spectrometry both of cells and their polysaccharides, showed that nodule isolates differed from the parental strains, indicating that adaptation had affected polysaccharide composition [4]. Indeed, Pellock *et al.* [42] suggested that quantitative and qualitative differences in EPSs might represent a selective advantage, allowing the strain to interact as efficiently as possible under a variety of conditions and with many cultivars or ecotypes of legumes. In addition, in *S. (Ensifer) meliloti*, three symbiotically important EPSs (succinoglycan, EPS II, and K antigen) are produced and for at least with one of them (EPS II) the production is associated with the excision of an insertion sequence [43]. As those DNA elements are often related to genetic recombination and are broadly detected in the genome of *B. japonicum* [27], they might also be related to the changes in mucoidy of adapted isolates.

Polyclonal antibodies of each strain were used in the somatic agglutination reaction. The main limitation of this methodology in ecology studies is cross-reaction with indigenous rhizobia [61]; however, that did not occur with the four inoculant strains first used as inoculants [33]. By the sixth year, serological reaction of 20 nodules collected from six plants of each replicate

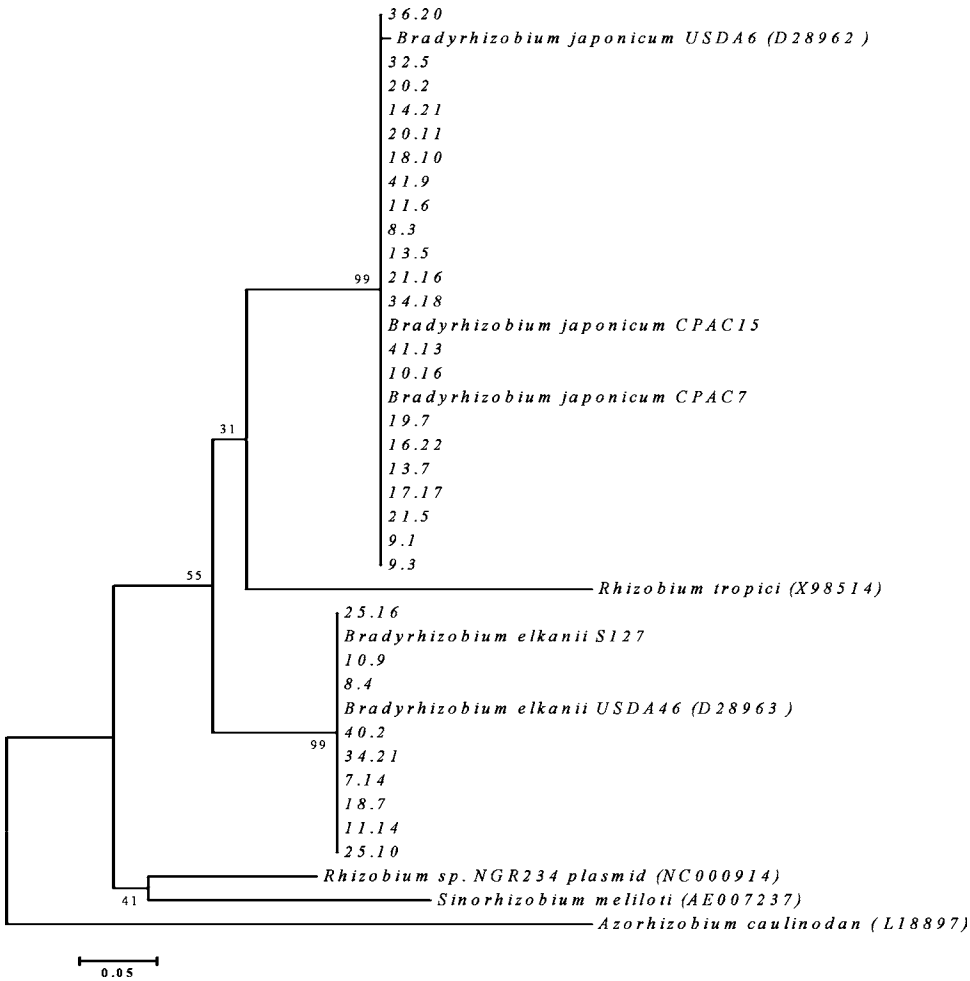


Figure 4. Phylogenetic tree based on the *nodC* nucleotide sequences of soybean rhizobial isolates and of reference strains of *B. japonicum* and *B. elkanii*. GeneBank accession numbers are given in the text. Analyses were conducted using MEGA version 3.1 and numbers in the main branches indicate bootstrap values obtained with 2000 replicates.

indicated that 48–74% of the nodules were occupied by strains serologically related to CPAC 15, whereas only 2–9% were occupied by strains related to CPAC 7 [33]. High saprophytic capacity and competitiveness, as well as dispersion of serogroup CPAC 15 from other plots, were confirmed in the seventh year, representing 40–70% of the isolates. The results showing that serogroup CPAC 15–USDA 123 became dominant in soils over time are consistent with previous reports both in the United States [56] and Brazil [33, 68], and emphasize the high saprophytic capacity of strains belonging to this serogroup. Interestingly, the studies of Freire *et al.* [8] and Mendes *et al.* [33] indicate that CPAC 15 prevailed in soybean nodules after the third year of introduction, and similar reports were obtained for serogroup 123 in the United States [56]. Those observations might imply that strains belonging to serogroup CPAC 15–USDA 123 need a certain length of time in soils before becoming dominant. In contrast, the presence of CPAC 7 in nodules dramatically declined in the year after introduction, occupying only 2–9% of the nodules by the sixth year [33] and being undetectable in the seventh year.

In the United States, it has been often shown that in a soil harboring rhizobia the introduction of a new strain may fail or that the strain persists only in the first year (e.g., [32, 62]). The situation is more critical if the established population belongs to a competitive serogroup such as USDA 123; inoculant rhizobia usually produce only 5–20% of the nodules after the first year [15, 45, 56]. Our study highlights the importance of continued inoculation with strains like CPAC 7, which are more effective in fixing N_2 but less competitive, to guarantee some nodule occupancy and N_2-fixation benefit while avoiding dominance of less effective but very competitive strains such as CPAC 15. Currently, massive annual inoculation using superior strains is the only strategy available to displace other established rhizobia in the tropics, where the continuous cropping

Table 4. Percent distribution of one hundred and nine soybean rhizobial isolates based on the combined analyses of serological reaction, RFLP-PCR of the 16S rDNA and *rep*-PCR fingerprintings and on the inoculation history of the plot from which the isolates were obtained

Characteristics of the isolates	*Isolates (%)*
Highly similar to inoculant strains[a]	
CPAC 15	6.4
CPAC 7	0.0
Showing variability[b] in relation to the inoculant strains	
CPAC 15	9.2
CPAC 7	0.9
Dispersion from other areas[c]	
CPAC 15	10.1
B. elkanii[d]	0.9
Dispersion[c] and variability[b] in relation to the inoculant strains	
CPAC 15	6.4
B. elkanii[d]	14.7
Mixed characteristics of the inoculant strains	
CPAC 15 and CPAC 7	
plots inoculated with both strains	2.8
plots inoculated only with CPAC 7	7.2
CPAC 15 and *B. elkanii*[d]	13.8
CPAC 7 and *B. elkanii*[d]	5.5
Mixed characteristics of the inoculant strains and unknown[e] rhizobia	
CPAC 15	9.2
CPAC 7	2.8
B. elkanii[d]	8.3
Mixed properties of more than one inoculant strain and unknown[e] rhizobia	
CPAC 15 and *B. elkanii*[d]	0.9

[a]To be considered similar to the inoculant strains, isolates should belong exclusively to the same serogroup, and show the same RFLP-PCR and *rep*-PCR profiles with similarity higher than 90% in comparison to the inoculant strain.
[b]Variability in *rep*-PCR profiles, with similarity lower than 90% in comparison to the inoculant strain.
[c]Strain not used as inoculant in that plot.
[d]*B. elkanii* includes both strains SEMIA 587 and 29W as they shared similar RFLP-PCR and *rep*-PCR profiles.
[e]Showing RFLP-PCR profiles and/or serological reaction distinct from any of the inoculant strains.

year-round favors rhizobial persistence. Fortunately, in contrast to many reports from other countries, such as the United States (e.g., [56, 62]), soybean reinoculation in Brazil guarantees benefits in N_2 fixation and yield, probably because the inoculant rhizobia in a better physiological condition when applied to the seed [19].

In our study, by the seventh year many isolates showed unknown serological reactions. Recovery of a high percentage of unknown serogroups after some years of soybean cropping has been previously shown both in southern Brazil [8, 6] and in the Cerrados [68, 9]. Also, in Australia, differences in serological patterns were found in variant strains 9 years after the introduction of *B. japonicum* strain CB 1809 [12]. This may be a function of changes in cell surface, in one or more components of the outer cell membrane, including lipopolysaccharides and/or other polysaccharides. Indeed, this hypothesis is supported by the results of Johnsson *et al.* [24], who showed that bacterial cell-surface markers may change over time. However, horizontal transfer of the symbiotic island from inoculant strains to indigenous rhizobia [58, 59] may also help to explain the appearance of unknown serogroups nodulating soybean. Although Cerrados isolates belonging to unknown serogroups have mostly shown RFLP-16S rDNA types similar to CPAC 7, CPAC 15, and to *B. elkanii*, variability in the 16S ribosomal genes may be low in *B. japonicum* and *B. elkanii* (e.g., [66, 74]); therefore, indigenous rhizobia capable of receiving the symbiotic island may share similar profiles.

A high level of variability in *rep*-PCR profiles was observed within all three clusters, related to CPAC 7, CPAC 15, and *B. elkanii* SEMIA 587-29W. Dispersed DNA repeats, as BOX elements, differ extensively in number and location among prokaryote chromosomes and evolve at higher frequencies than insertion or deletions events; apparently, they are related to a high degree of genomic flexibility, acting in specific adaptive evolution [65] and phenotypic variation [48]. Interestingly, apparently in the process of adaptation to the Cerrados soils BOX elements decreased in *B. japonicum* CPAC 15 and increased in *B. elkanii*, with both processes occurring in *B. japonicum* CPAC 7. In addition, higher variability occurred with the CPAC 7 serogroup and the lowest with most of the CPAC 15 isolates, serogroup that become dominant with time. Interestingly, an intermediate group with mixed profiles of CPAC 15 and SEMIA

587 was found, indicative of an intensive exchange of genetic material. The magnitude of the variability detected in *rep*-PCR profiles in our study supports previous reports on other isolates from the same region [9, 18, 49] and contrasts with the results obtained in less stressful conditions of soils of midwestern United States [25].

Isolates from our study fit into different RFLP-16S rDNA types. First, six isolates produced unknown patterns when compared to the reference and type strains. Second, *B. elkanii* strains SEMIA 587, 29W, and USDA 76 produced the same patterns with all three enzymes. Lastly, *B. japonicum* differed from *B. elkanii* with all three enzymes; however, serogroup CPAC 15 was similar to USDA 6 but differed from serogroup CPAC 7 with enzyme *Dde*I. In fact, the analysis of the whole sequences of the 16S rRNA genes of CPAC 7 and CPAC 15 [3] indicates that fragments with different sizes should be obtained with *Dde*I. As already noted, diversity in the 16S rRNA sequences of *Bradyrhizobium* has been found to be low in most strains investigated so far (e.g., [66, 74]). However, recent RFLP-PCR analysis of a collection of Brazilian *Bradyrhizobium* strains revealed differences not only in the 16S- but also in the 23S- and 16-23S-intergenic ribosomal regions, with CPAC 7 and CPAC 15 also fitting into different clusters [11]; results were further confirmed with the complete sequencing of the 16S rRNA gene [34]. Besides, CPAC 15 and CPAC 7 are remarkably different in several phenotypic and symbiotic properties, as well as in protein, lipopolysaccharide, random amplification polymorphic DNA, and *rep*-PCR profiles with ERIC and REP primers [1, 18–21, 38, 49]. Sawada *et al.* [50] emphasized that *B. japonicum* strains are too diverse to be grouped into one species; we also plan to investigate the genetic similarity of other genes in these two strains. In addition, in studies by Germano *et al.* [11] and Menna *et al.* [34] even higher variability was confirmed within Brazilian *B. elkanii* strains; however, most of these strains were isolated from nodules of indigenous legumes species and not from soybean. Finally, it should be mentioned that in other bacterial species it has also been shown that strains showing identical 16S rRNA gene sequences may be very different in other ribosomal regions, *rep*-PCR and even DNA–DNA hybridization, also showing differences in physiological properties. As Jaspers and Overmann [23] concluded, there is an increasing perception that natural diversity goes beyond the level of 16S rRNA.

To investigate the symbiotic genes, we chose one gene related to nodulation (*nodC*) and another one to N_2 fixation (*nifH*). These genes are relatively distant on the genome of *B. japonicum* [27], facilitating examination for possible horizontal transfer of the symbiotic island. Considering both the partial sequencing of *nodC* and the RFLP-PCR of *nifH*, CPAC 7 and CPAC 15 shared the same patterns, but differed from the profiles obtained for *B. elkanii*. Parker *et al.* [41], analyzing 38 *Bradyrhizobium* strains from different continents, observed that all strains fitted either *B. japonicum* or *B. elkanii* when the 16S rRNA gene was considered. However, when the *nifD* gene of those same strains was analyzed, clustering occurred according to geographic region. In addition, phylogenies of both genes were statistically incongruent, suggesting that each area was initially colonized by several diverse 16S rRNA lineages, followed by horizontal gene transfer of *nifD* within each area [41]. Likewise, results from analysis of ribosomal and *nod* and *nifH* genes in the *Astragalus sinicus–S.* (*Ensifer*) *meliloti* [76] and *Bradyrhizobium*–several legume hosts symbioses supported the hypothesis of horizontal transfer among diverse bacteria. [72]. Results from our study differ from those of Parker *et al.* [41] in the sense that we see full congruence between the conserved 16S rRNA genes and symbiotic genes (*nodC* and *nifH*) of *B. japonicum* and B. elkanii strains. However, the variability detected in the 16S rRNA gene of *B. japonicum* was higher than in the symbiotic genes, suggesting horizontal transfer between strains CPAC 7 and CPAC 15. Greater conservation of symbiotic genes in *B. japonicum*, in addition to the transferable nature of the symbiotic island [13, 27, 36], might be indicative of an ecological advantage related to the symbiotic effectiveness.

In conclusion, variability in several morphological, physiological, serological, genetic, and symbiotic properties of isolates adapted to the Brazilian Cerrados in relation to the parental inoculant strains has been previously shown [1, 7, 49, 9]. In our study, emphasis was given to conserved and symbiotic genes and high variability was confirmed after 7 years from introduction in a soil of the Brazilian Cerrados. The magnitude of rhizobial variability detected in isolates adapted to the harsh conditions of the Cerrados resembles differences obtained in RFLP and serological patterns after 9 years in an Australian soil [12], and is much higher than that reported under less stressful environmental conditions in the United States [25] and France [2, 39]. The high genetic variability detected in our study is certainly related to the plasticity of the *Bradyrhizobium* genome [27]. However, without a doubt, it was accelerated by interaction with the host plant [17, 44], adaptation to the environment [21, 44, 59, 58], and agricultural practices [6, 21, 28]. Variability in our study appears to have resulted from a variety of events including strain dispersion, genomic recombination, and horizontal gene transfer. It is also reasonable to infer that variability was associated with adaptation, saprophytic capacity, and competitiveness, and not with symbiotic effectiveness, as *nodC* and *nifH* genes were more conserved. Certainly, the phenotypic and genotypic variability associated with the adaptation process has profound ecological implications that warrant further detailed study.

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