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Rescue and genetic assessment of soybean-nodulating *Bradyrhizobium* spp. strains from an experimental field thirty years after inoculation

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Abstract - As the long-term phenotype and genetic stability of bacteria used as inoculant are important parameters in their ecology and for agricultural purposes, this study aimed genotypically characterize several-rescued bradyrhizobia of an experimental field thirty years after the first inoculation. A high genetic diversity of 30 bradyrhizobia isolates was observed, either by AFLP (H = 4.87) or rep-PCR (H = 4.18). The results indicate that the *Bradyrhizobium* spp. population that persists in the Eldorado soil is genetically very diverse and different from the parental strains. All isolates were infective and trapped in IAS-5 soybean variety maintaining their nodulation and nitrogen fixation properties. Given that many rhizobia in a soil can lost the infective capacity and that the host genotype can affect the spectrum of rhizobial genotype selected from a soil, the genetic diversity of the complete bradyrhizobia population in Eldorado soil could be even higher than the identified in this work.

Keywords: Bradyrhizobium. Genetic diversity. AFLP. Trap host.

Resgate e avaliação genética de estirpes de *Bradyrhizobium* spp. noduladoras de soja de um campo experimental trinta anos após a inoculação

Resumo - Como o fenótipo de longo prazo e a estabilidade genética das bactérias usadas como inoculantes são parâmetros importantes em sua ecologia, e também para fins agrícolas, este estudo teve como objetivo caracterizar genotipicamente os isolados de *Bradyrhizobium* spp. resgatados de um campo experimental trinta anos após a primeira inoculação. Foi observada uma alta diversidade genética de 30 isolados de *Bradyrhizobium* spp., tanto por AFLP (H = 4,87) quanto por rep-PCR (H = 4,18). Os resultados indicam que a população de *Bradyrhizobium* spp. que persiste no solo Eldorado é geneticamente muito diversa e diferente das linhagens parentais. Todos os isolados foram infectantes e capturados com a variedade de soja IAS-5, mantendo suas propriedades de nodulação e fixação de nitrogênio. Dado que muitas rizóbios em um solo, ao longo do tempo, podem perder a capacidade infecciosa e que o genótipo hospedeiro pode afetar o espectro do genótipo rizobiano selecionado a partir de um solo, a diversidade genética da população completa de *Bradyrhizobium* spp. no solo de Eldorado pode ser ainda maior do que a identificada neste trabalho.

Palavras-chave: Bradyrhizobium. Diversidade genética. AFLP. Planta-isca.

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Introduction

Bacteria belonging to the *Bradyrhizobium* genus are of enormous agricultural value since they are able to fix atmospheric nitrogen in symbiosis with several leguminous plants, especially soybean [*Glycine max* (L.) Merrill]. In Brazil, *B. japonicum*, *B. elkanii* and *B. diazoefficiens* are the species reported as capable of nodulating soybean (FREIRE, 1977; PERES e VIDOR, 1980; andrade; SUGAWARA et al., 2017) and, since it is an exotic crop, Brazilian soils did not contain naturally such *Bradyrhizobium* species nodulating this legume. Probably, these bacteria came with seeds and inoculants from USA and its population in Brazilian soils increased due to the massive commercial inoculation during the last decades (FERREIRA; HUNGRIA, 2002).

Besides some intrinsic host or symbiont characteristics and the environmental stresses like high temperature, soil acidity and aluminum toxicity (HUNGRIA et al. 1993; HUNGRIA e VARGAS, 2000), bradyrhizobia introduced through seeds or inoculation usually survive in the soil for many years (REVELLIN et al., 1996). However, some experiments have shown that bradyrhizobia can naturalize and present morphology, physiology, genetic and symbiotic differences from the original introduced strains (BODDEY e HUNGRIA, 1997; FERREIRA e HUNGRIA, 2002) including a high percentage of nodules occupied by strains with unknown serological reaction after some years of soybean cropping (FREIRE et al., 1983; VARGAS et al., 1993; VARGAS e HUNGRIA, 1997; FERREIRA et al., 2000). Moreover, the diversity and the size of indigenous population in soil can vary with the presence of the host legume (PARKER, 1999; ANDRADE; MURPHY; GILLER, 2002). Coutinho et al. (1999) demonstrated that the diversity of rhizobia was reduced in plots cultivated with soybean compared with original uncultivated pasture. They also observed that the soybean crop did not increase the number of bradyrhizobia strains.

In order to study the survival and competitiveness of bradyrhizobia strains for soybean nodule site, Freire et al. (1983) conducted a field experiment for six consecutive years beginning at agriculture year of 1974. The experimental area, located at Eldorado do Sul, Brazil, was inoculated with seven *Bradyrhizobium* spp. reference strains and has not been reinoculated or cropped with soybean since then. At the fifth year of analysis, besides the different ability of the strains to compete for nodule sites, it was observed a high percentage of nodules without serological reaction with any of the antisera used to identify the original strains. According to the authors (FREIRE et al., 1983) this result indicated that the nodules were formed by naturalized strain or strains of higher competitiveness. As the long-term phenotype and genetic stability of bacteria used as inoculant is an important parameter in their ecology as well as for agricultural purposes, the aim of this study was genotypically characterize several-rescued bradyrhizobia of that experimental field. The

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purpose was to investigate whether these bacteria were still able to persist for 30 years in the soil maintaining their nodulation and nitrogen fixation properties.

Material and methods

Bacterial isolates, reference strains and trap host

Soil samples were collected at the Estação Experimental Agronômica of the Universidade Federal do Rio Grande do Sul (UFRGS), located at Eldorado do Sul, Rio Grande do Sul, Brazil. Based on Soil Survey Staff (2010) the soil of the experimental area is a Paleudult with pH of 5.5, 34% of clay and 4.2% of organic matter. In 1974 this area was inoculated with seven strains of *Bradyrhizobium* spp. (SEMIA 509, 527, 531, 532, 566, 586 and 587) for a field experiment, which was carried out for six consecutive years. Each strain was inoculated as an individual inoculant only at the first year.

In this work we tried to use the same seven reference strains for comparison, however, strains SEMIA 531 and SEMIA 532 were replaced by strains SEMIA 5038 and SEMIA 5039. We also included in our analysis the currently recommended strains SEMIA 5019, SEMIA 5079, SEMIA 5080 and the type reference strain USDA 110. All reference strains were obtained from the Biological Nitrogen Fixation Laboratory/Department of Agricultural Diagnosis and Research, (DDPA), Brazil, and are described in Table 1.

The IAS-5 soybean cultivar was used as trap-host. The bradyrhizobia isolates were obtained from 50 fresh root nodules collected from plants that had grown in pots filled with the soil derived from the field cited above. Bacteria were isolated on yeast mannitol agar (YEM) (SOMASEGARAN; HOBEN 1994) supplemented with 0.025 g. l^{-1} of Congo red dye using standard procedures (SOMASEGARAN; HOBEN 1994). The isolates were purified by repeated streaking (VINCENT, 1970). To confirm their purity, all the isolates and reference strains were streaked on YEM agar supplemented with 0.1 g l^{-1} of bromothymol blue (SOMASEGARAN; HOBEN, 1994). The bacterial isolates were reinoculated in pouches with IAS-5 soybean cultivar according to standard procedures. Those that had confirmed their ability to nodulate soybean were selected for this study. Pure cultures were stored at -10°C in 25% glycerol-YEM broth (SOMASEGARAN; HOBEN, 1994).

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Table 1. *Bradyrhizobium japonicum* and *B. elkanii* reference strains utilized in this work.

Strains	Relevant characteristics
Bradyrhizobium elkanii	
SEMIA 587	Brazilian (RS) reference strain. Commercially recommended as inoculant from 1968 to 1975 and since 1979. Isolated from Brazilian (RS) soybean fields
SEMIA 5019 (=29w; =BR 29)	Brazilian (RJ) reference strain. Commercially recommended as inoculant since 1979
Bradyrhizobium diazoefficiens	
USDA 110 (=TAL 102; SEMIA 5032)	Former *B. japonicum* Group Ia. Isolated from USA soybean fields.
SEMIA 5080 (=CPAC 7)	Former *B. japonicum* Group Ia. Brazilian reference strain. Natural variant of CB 1809. Commercially recommended as inoculant since 1992.
Bradyrhizobium japonicum	
SEMIA 586 (=CB 1809;=USDA 136b; =TAL 379)	Isolated from USA soybean fields
SEMIA 5079 (=CPAC 15)	Brazilian reference strain. Natural variant of SEMIA 566. Commercially recommended as inoculant since 1992.
SEMIA 566	Isolated from Brazilian (RS) soybean fields. Commercially recommended as inoculant from 1966 to 1978.
Undefined *Bradyrhizobium*	
SEMIA 509 (=UW 509; =USDA 487)	Isolated from USA soybean fields.
SEMIA 527	Isolated from Brazilian (RS) soybean fields.
SEMIA 531	
SEMIA 532	

DNA isolation

Bacterial cells were grown in YEM broth for seven days at 28ºC at 128 rpm. Cells were rinsed with TES buffer (50 mM Tris pH 8, 0.5 mM EDTA, 50 mM NaCl), and ressuspended in EDTA saline (150 mM NaCl, 10 mM EDTA pH 8). Cell lyses took place in 20% sodium dodecyl sulfate (SDS) warmed at 55ºC. DNA was extracted using chloroform/isoamylic alcohol (24:1) and precipitated with ethanol.

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rep-PCR

Rep-PCR reactions were carried out using enterobacterial repetitive intergenic consensus primers ERIC1-R (ATGTAAGCTCCTGGGGATTCAC) and ERIC-2 (AAGTAAGTGACTGGGGTGAGCG) (DE BRUIJN, 1992) and enterobacterial repetitive sequences (BOX A1) primer (CTACGGCAAGGCGACGCTGACG) (VERSALOVIC et al., 1994). The reactions were performed in a 25-µl volume, containing 50 ng of DNA template, 1 U *Taq* DNA polymerase, 1 X *Taq* DNA polymerase buffer, 15 mM $MgCl_2$, 200 mM dNTPs (Life Technologies) and 10 pmoles of each primer. A total of 32 cycles took place, as follows: one initial denaturation cycle at 95ºC for 7 min, 30 cycles of denaturation at 94ºC for 1 min, annealing at 52ºC for 1 min, and extension at 65ºC for 8 min, and one final extension cycle at 65ºC for 16 min. Reactions were performed in a PCR Express Temperature Cycling System (Thermo Hybaid) and fragments were visualized after electrophoresis at 100 V for 3 h on a 1% agarose gel stained with ethidium bromide. Molecular size marker was 1 Kb Plus DNA ladder (Gibco BRL).

AFLP

All AFLP procedures were carried out as described by Dougnon-Bourcier et al. (2000). A 100 µg aliquot of genomic DNA of each isolate was overnight digested with 1.25 U of both *Apa*I and *Taq*I restriction enzymes (Invitrogen) at 37ºC and 65ºC, respectively, in a final volume of 25 µl. The restriction reaction was then ligated to the *Apa*I (TCGTAGACTGCGTACAGGCC and CATCTGACGCATGT) and *Taq*I adapters (GACGATGAGTCCTGAC and TACTCAGGACTGGC) (WONG et al. 1999; SINGH et al. 2001). The ligation mixture consisted of 25 µl of digested DNA, 10 pmol of each adapter and 1 U of T4 DNA ligase (Invitrogen) in correspondent buffer. Ligated DNA was 1:10 diluted in ultra pure water and heated at 80ºC for 10 min to inactivate T4 DNA ligase. Five □l of ligated DNA was then used as template for PCR DNA amplification. The reactions were performed in a 25 µl volume, containing 1 U *Taq* DNA polymerase (Invitrogen), 1 X *Taq* DNA polymerase reaction buffer, 15 mM $MgCl_2$, 200 mM dNTPs (Invitrogen) and 10 pmoles of each primer (*ApaI* GACTGCGTACAGGCCCE, where E means ATT/CCC/CTA/CTG/GAC/TTG and *TaqI* CGATGAGTCCTGACCGAE, where E means CCC/CTG/GAC/GTA). PCR amplification was performed using the *touchdown* protocol: 94°C for 3 min, followed by 10 cycles of 94°C for 30 s, 65°C to 56°C for 1 min and 72°C for 2 min, followed by 23 cycles of 94°C for 30 s, 56°C for 30 s, and a final extension of 72 °C for 1 min. Reactions were performed in a PCR Express Temperature Cycling System (Thermo Hybaid). The fragments were visualized after electrophoresis for 3 hours at 80 V in an 8% acrylamide-bisacrylamide gel stained with silver nitrate (SAMBROOK; RUSSEL, 2001). Molecular size marker (□ DNA digested with *Eco*RI/*Hind*III, Invitrogen) was run in all gels.

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Differentiation of *Bradyrhizobium japonicum/ B. diazoefficiens* and *B. elkanii* by PCR amplification of 16S rRNA gene sequences

The bradyrhizobia isolates were differentiated as strains of *B. japonicum/B. diazoefficiens* and *B. elkanii* using a protocol described by Giongo et al. (2007). The differentiation was based in a selective PCR amplification of 16S rRNA gene sequences using the forward primer sequence (Brady AMTKCCTTTGAKWYTKAAGATCTTG), and the reverse primers, specific for each *Bradyrhizobium* species [Bjap for *B. japonicum/B. diazoefficiens* (GTCACATCTCTGCGACCGGTC) and Belk for *B. elkanii* (AACTCCGTCTCTGGAGTCCGCGA)].

DNA fingerprint analysis

Fingerprint patterns were visualized under UV illumination. The size of the fragments obtained ranged from 650 to 5,000 bp. They were converted into a two-dimensional binary matrix (1, presence of a band; 0, absence of a band) and analyzed using Jaccard coefficient, which do not consider the negative similarities. The matrix was analyzed by NTSYS-PC package. UPGMA (Unweighted Pair Group Method with Arithmetic mean) algorithm was used to perform hierarchical cluster analysis and construct a dendrogram. Index of diversity (*H*) (SHANNON and WEAVER, 1949) was estimated based on the number of isolates belonging to each group of profiles in rep-PCR and AFLP dendrograms, considering a 70% of similarity in the cluster analysis (ALBERTON et al., 2006). The *H* index was calculated using the formula $H = C/N(N\log N - \Sigma n \log ni)$ (ATLAS; BARTHA, 1998), where *ni* corresponds to the number of isolates with the same rep-PCR/AFLP fingerprints, N corresponds to the total number of isolates and C is a constant that equals 23. The *H* values obtained were submitted to Student test (*t*) (MAGURRAN, 1987).

Results and Discussion

The assessment of long-term genetic stability of bacteria used as a seed inoculants is an important parameter in microbial ecology as well as for agricultural purposes. In 1974, a field experiment was carried out on Estação Experimental Agronômica of the Universidade Federal do Rio Grande do Sul (UFRGS), located at Eldorado do Sul, RS, Brazil (FREIRE et al., 1983). The objective of that experiment was to study the survival and competitiveness for nodule sites of seven *Bradyrhizobium* spp. strains of soybean for six consecutive years. It was reported that the strains showed different ability to compete for nodule sites. Moreover, at the fifth year of nodule analysis, it was observed a high percentage of nodules without serological reaction with any of the antisera used to identify the seven original strains. This result indicated that the nodules were formed by

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naturalized strain or strains of higher competitiveness, even higher than SEMIA 587, which had showed the high competitiveness and ability to colonize the soil in the previous years (FREIRE et al., 1983).

Using the IAS-5 soybean cultivar as trap host and the soil deriving from the same field used in the former experiment, we obtained 30 bradyrhizobia isolates. These bacteria were genotypic and symbiotically characterized according to standard procedures used to isolate *Bradyrhizobium* species (SOMASEGARAN; HOBEN, 1994). All the isolates failed to absorb Congo red and their colonies were well visualized after 5-7 days of incubation at 28ºC in plates containing YEMA medium. All isolates also alkalinized YEM medium supplemented with bromothymol blue, which is a typical characteristic of *Bradyrhizobium* genus (BATISTA et al. 2007). Although Hungria et al. (2001) and Galli-Terasawa et al. (2003) reported that fast growing rhizobia represent 17 to 29% of soybean rhizobia population in Brazil, this kind of bacteria did not appear among our isolates.

Genetic diversity of the bradyrhizobia isolates was evaluated by electrophoretic profiles of rep-PCR and AFLP amplification products. It was possible to distinguish well-defined groups of bradyrhizobia using ERIC1-R, ERIC-2 and BOX primers (data not shown). These groups were confirmed by AFLP analysis, although a higher diversity among isolates was observed within AFLP-groups. Figure 1 shows the AFLP-relationships among all the strains analyzed. According to the dendrogram, two large groups (I and II) were obtained with a similarity index of 28%. Group I was subdivided in two other groups, IA and IB. The group IB was again subdivided in IB1 and IB2. Group IA was composed by 15 isolates, from which five where identified as *B. japonicum* and ten as *B. elkanii*. Reference strains were clustered within groups IB1 and IB2. Group IB1 was composed by four reference strains: *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 566, and SEMIA 5038, which belong to a group of still not defined bacteria. Group IB2 was composed by seven reference strains: SEMIA 5080 and USDA 110, belonging to *B. diazoefficiens* species; SEMIA 5079 and SEMIA 586, belonging to *B. japonicum* species; and SEMIA 509, SEMIA 527 and SEMIA 5039, which also belong to a group of undefined bacteria. Group II was composed by another set of isolates, as occurred with group IA, being eight identified as strains of *B. japonicum* and seven as strains of *B. elkanii.*

When used in combination, rep-PCR and AFLP can provide accurate information of strains relationship as well as to show the extent of genetic diversity among field population. The Shannon index (*H*) was used in order to assess the diversity among our isolates. The rep-PCR and AFLP fingerprints were compared on the basis of a 70% similarity. The results revealed a remarkably high genetic diversity among the isolates either by AFLP (H = 4.87) or rep-PCR (H = 4.18) techniques. These results do not differ statistically (test *t*). Similar results were obtained by Vargas et al. (2007) that found a *H* of 4.3 studying the genetic diversity of black wattle nodulating rhizobia in Rio Grande do Sul, Brazil. Andrade et al. (2002) also found a similar

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diversity index (*H* = *3*.93) analyzing common bean rhizobia in Brazilian acid soils altered by liming. Lõhmus et al. (2006) obtained Shannon indexes of 4.63 and 4.56 among cultivable bacterial communities extracted from soil–root interface and rhizosphere bulk soil, respectively. A high level of diversity within a sample of 100 soybean rhizobia isolated from an uncropped area in Cerrados, Brazil, was also reported by Galli-Terasawa et al. (2003). Although this area had received inoculants 15 years before, nowadays it lacks rhizobia able to nodulate soybean. Chen et al. (2000) also confirmed the high level of genetic diversity among the Paraguayan soybean rhizobia. They showed that DNA profiles of the slow growers differed from the reference strains used in inoculants. Soybean rhizobia from field sites in Croatia were also genotypically analyzed using RFLP, rep-PCR and RAPD and most of the isolates significantly diverged from inoculant strains (SIKORA; REDZEPOVIC, 2003). The authors suggested that even with inoculation, indigenous rhizobia remained predominant in these areas, as could be the case of the bradyrhizobia population analyzed in the present work.

On the other hand, some authors have reported lower diversity indexes when studying rhizobial communities in different situations (MARILLEY et al., 1998; PALMER e YOUNG, 2000; ZILLI et al., 2004). Gibson et al. (1991) found stability with no exchange of RFLP pattern among strains of *B. japonicum* that have survived for 9 years in the soil. Brunel et al. (1988) reported that a population of *Bradyrhizobium* strains did not show any significant genomic alteration after 8-13 years of release in a soybean field. Soybean was not grown in that field after the year of bacterial inoculation. Besides DNA hybridization using *nifD* gene as a probe the authors confirmed the stability of the bradyrhizobia population regarding morphology, serology, sugar utilization, antibiotic resistance, and enzymatic activity properties. They concluded that once introduced into a suitable soil without indigenous *B. japonicum* populations, *B. japonicum* inoculants would integrate into the indigenous soil communities without significant modifications. Ferreira; Hungria (2002) also have found genetic similarity among most of the isolates obtained from a Cerrado field (an uncropped area) and the strains used in commercial inoculants. In this case, the similarity found was probably due to a high level of dispersion of bacteria from neighbor cropped areas. Similar result was obtained by PCR-RAPD for 48 isolates from two French regions, Dijon and Toulouse, where none obvious genomic rearrangement was observed (Obaton et al. 2002). The authors attributed this result to a low or absent saprophytic competition and/or to a low probability of contact between colonies.

Variability in rhizobial population has been attributed to several factors, including mutation and recombination in isolated strains and lateral gene transfer to local strains (SULLIVAN; RONSON, 1998); furthermore, these processes can be affected by the interaction with the host plant, by agricultural practices (MARTINEZ-ROMERO e CABALLERO-MELLADO, 1996; PROVOROV e VOROB'EV, 2000; GALLI-TERASAWA et al., 2003; SILVA et al., 2003) or environmental stress condition, common in tropical regions

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(SANTOS; VARGAS e HUNGRIA, 1999; HUNGRIA e VARGAS, 2000). Some authors have assumed that nodule populations are more sensitive to environmental disturbances and may be a more valuable biological indicator of ambient variation that free-living populations (DEPRET et al., 2004). It was also suggested that the acquisition of the symbiosis island by another rhizobia can convert saprophyte bacteria into a symbiont one (SULLIVAN; RONSON, 1998). Therefore, as well as encoding genes required for nodule formation and symbiotic nitrogen fixation, the island is likely to contain other genes that contribute specifically to the success of the plant-microbe interaction.

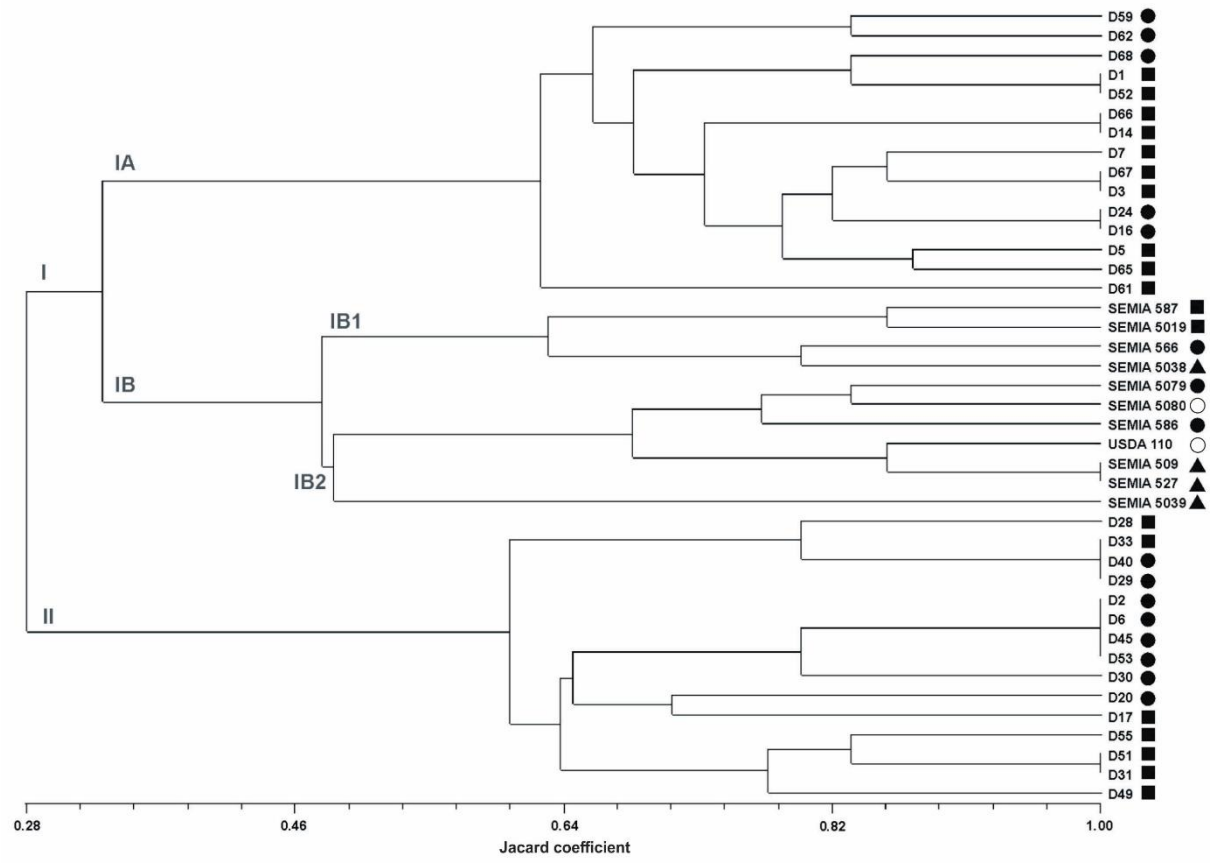

Figure 1. UPGMA-dendrogram derived from AFLP fingerprints of reference strains and soybean bradyrhizobia. Symbols represent: ○ *B. diazoefficiens* reference strains; ● *B japonicum* isolates and reference strains; ■ *B elkanii* isolates and reference strains; ▲ *Bradyrhizobium* undefined strains.

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Another possibility is that insertion sequences elements in rhizobia can promote genetic diversification through genomic rearrangements and recombination (LABERGE; MIDDLETON e WHEATCROFT, 1995; FREIBERG et al., 1997; PROVOROV e VOROB'EV, 2000). One example was provided by *B. japonicum* that presented a high number of transposases, repetitive RSα elements and insertion sequences (GÖTTFERT et al., 2001; KANEKO et al., 2002). The occurrence of spontaneous mutation or recombination in bradyrhizobia is a preoccupying point in culture collections once this variability represents a potential change in maintenance and indication of strains to taxonomy and inoculant production (CARVALHO; SELBACH; BIZARRO, 2005).

The present study indicates that the *Bradyrhizobium* spp. population that was able to persist in the Eldorado soil for more than 30 years after inoculation is genetically very diverse and different from the parental strains. It should be recognized that only a small fraction of strains from the total soil population was sampled here. All isolates analyzed were infective in IAS-5 soybean cultivar and were trapped using the same cultivar maintaining their nodulation and nitrogen fixation properties. Given that many rhizobia in a soil can lost the infective capacity and that the host genotype can affect the spectrum of rhizobial genotype selected from a soil (LAGUERRE et al., 2003), it is likely that genetic diversity of the complete bradyrhizobia population in Eldorado do Sul soil could be even higher than the identified in this work.

Conclusions

Soybean-nodulating *Bradyrhizobium* spp. population was able to persist in the soil of Eldorado do Sul, maintaining their nodulation and nitrogen fixation properties, for more than 30 years without the host plant. This is genetically *Bradyrhizobium* spp. population is different from the parental strains and has high genetic diversity.

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