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CHARACTERIZATION OF RHIZOBIA THAT NODULATE *ARACHIS PINTOI* BY RAPD ANALYSIS

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ABSTRACT

The genetic relationships of 85 *Arachis pintoi* nodulating *Rhizobium* strains were determined using the random amplified polymorphic DNA (RAPD) methods. The analysis included 75 strains isolated from Cerrado soils and 10 other ones of different origins. The results indicated that there is a high level of similarity between these strains and that geographic distribution may affect their phylogenetic relationship. In addition, the results allowed the selection of the most suitable primers for characterisation of these *Rhizobium* strains which will be useful for implementation of competitiveness studies in Cerrado soils.

Key words: *Rhizobium*, *Arachis pintoi*, Cerrado soils.

INTRODUCTION

Pasture degradation in the Brazilian Savana (Cerrado) is mainly the result of predatory exploitation and/or inadequate management leading to loss of physical, chemical and biological soil properties (5). In an attempt to minimise this impact, several authors have discussed the beneficial contribution of forage legumes to soil fertility and to the increase in nutritive quality of forages (9,14). Among the forage legumes indicated for Cerrado soils, the species *Arachis pintoi* has been suggested as one of the best alternatives because of its ability to adapt to, and persist in, these soils (5,14). However, in spite of the positive contribution of this legume to the soil, the low number of rhizobial populations associated with *A. pintoi* in the soil (16) and the occurrence of inefficient native *Rhizobium* strains in the soils of tropical regions (1,19), including Cerrado soils (12,16), has limited the biological nitrogen fixation process in areas cultivated with this legume. Therefore, inoculation with selected *Rhizobium* strains is essential to increase the productivity of *A. pintoi*. In addition to the use of specific *Rhizobium* strains in the inoculation, it is fundamental to monitor the inoculated *Rhizobium* strains to ensure the success of the symbiosis process. Many methodologies used for this purpose have been described; however, the use of traditional methods such as serological studies or antibiotic resistance have been limited mainly by problems such as small size nodules, high frequency of antibiotic resistance and highly similar antigen structures (cross-reactivity) that usually occur among tropical *Rhizobium* strains (3,15).

More recently, molecular techniques have been developed to provide reliable information on the diversity of *Rhizobium* populations in soils (7,10,11). Among these techniques, genomic DNA fingerprinting using random amplification of polymorphic DNA (RAPD) has been shown to be useful to differentiate very closely related strains (4,8,21).

In this study, characterization and evaluation of the genetic diversity of *Rhizobium* strains from different regions of the Cerrado that are able to associate with *A. pintoi* were performed using genomic patterns obtained by RAPD. Afterwards the genetic groups (clusters) identified may give support to studies of persistence and competitiveness of these strains introduced into the field as inoculants.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Seventy five of the 85 *Rhizobium* strains used in this study were isolated from *A. pintoi* (BRA031143) nodules collected

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from non inoculated plants cultivated in Cerrado soils from field or green house conditions. These strains are listed together with their origin, fertility and historical characteristics of the clay soils from which they were obtained (Table 1). The other strains were supplied by the Centro Internacional de Agricultura Tropical (CIAT), Colombia (CIAT2138, CIAT3101, CIAT3806) and by the North Carolina State University collection-USA (NC229, NC70, NC230, NC502.3, NC656, TAL295), and the strain indicated for the genus *Arachis* BR1405 was from the Centro Nacional de Pesquisa em Agrobiologia-Empresa Brasileira de Pesquisa Agropecuária (CNPAB-EMBRAPA). These strains were previously characterised according to their level of effectiveness with the homologous host and physiological characteristics of both fast and slow growth (12,13). All the strains were grown in yeast mannitol medium (20) for three days at 29ºC in an orbital shaker (200 rpm).

DNA extraction and RAPD amplification

Bacterial genomic DNA from each strain was extracted according to the methodology described by Sá *et al*. (15). Amplification reactions were performed with a Perkin-Elmer 9600 thermocycler. These reactions consisted of 40 cycles with each cycle including the following steps: denaturation at 94ºC for 15 sec, annealing at 35ºC for 30 sec and elongation at 72ºC for one min. An extra elongation step at 72ºC for 7 min was performed after the last cycle. Each reaction mixture consisted of 25 mL, with the following composition: 1X PCR buffer, 10 mM of each dNTP (dATP, dTTP, dCTP and dGTP), 4 mM of primer (Operon

Table 1. *Rhizobium* strains grouped according to their site of origin and chemical soil analysis of the isolation sites.

Rhizobium strains	Historical characteristics	Site/ Subsite	Soil characteristics: pH	Al	Ca	Mg	K	P	Organic Matter dag / Kg
			cmolc / dm						
MGAC1, MGAC2, MGAC3, MGAC4, MGAC5, MGAC6, MGAC13, MGAC15, MGAC18, MGAC20, MGAC22, MGAC24, MGAC27, MGAC30, ALS8, ALS9, ALS15, ALS16, ALS18, ALS20, ALS23, ALS24, ALS25, ALS26, ALS31, ALS35, ALS41	Cerrado under *A. pintoi* cultivation for 3 years, previously under corn cultivation	Sete Lagoas 1	6.1	0.0	6.9	1.1	108	34	4.2
MGAP6, MGAP8, MGAP11, MGAP12, MGAP13, MGAP14, MGAPCS11, MGAPCS14	Cerrado cultivated for more than 15 years with different leguminous forage included *A. pintoi*	Sete Lagoas 2 (subsite 1)	5.4	0.1	2.6	0.4	69	9.0	2.7
MGAV14, MGAV19, MGIIV2, MGAPAV2		(subsite 2)	6.0	0.0	6.1	0.5	42	14	2.3
MGAPCA3, MGAPCA20		(subsite 3	5.8	0.0	3.1	0.4	59	10	3.0
CPAC1, CPAC8, CPAC25, CPAC26, CPAC28	Cerrado cultivated for more than 10 years with several leguminous plants included *A. pintoi*	Brasília (subsite 1)	5.5	0.1	3.1	...	80	11.9	2.6
		(subsite 2)	5.8	0.1	3.4	...	23	1.7	2.6
DFA3.1		(subsite 3)	5.7	0.1	3.6	...	13	7.6	2.4
DFA5.1, DFA5.3, DFA5.8, DFA6.3, DFA6.6, DFA6.10, DFA6.12, DFA7.1									
DFA8.1, DFA8.8, DFA 8.9, DFA8.13, DFA9.1, DFA9.6, DFA10.3, DFA10.8, DFA11.1, DFA11.12, DFA12.3, DFA12.10, DFA14.3, DFA16.5, DFA16.10, DFA16.11		(subsite 4)	5.5	0.1	3.0	...	80	11.9	2.6
NC229, NC70, NC230, NC502.3, NC656.1, TAL295	...	Different origin in South America	...	...	...	...	...	...	...
CIAT2138, CIAT3806, CIAT3101	...	Colombia	...	...	...	...	...	...	...
BR1405	...	Brazil (Unknown)	...	...	...	...	...	...	...

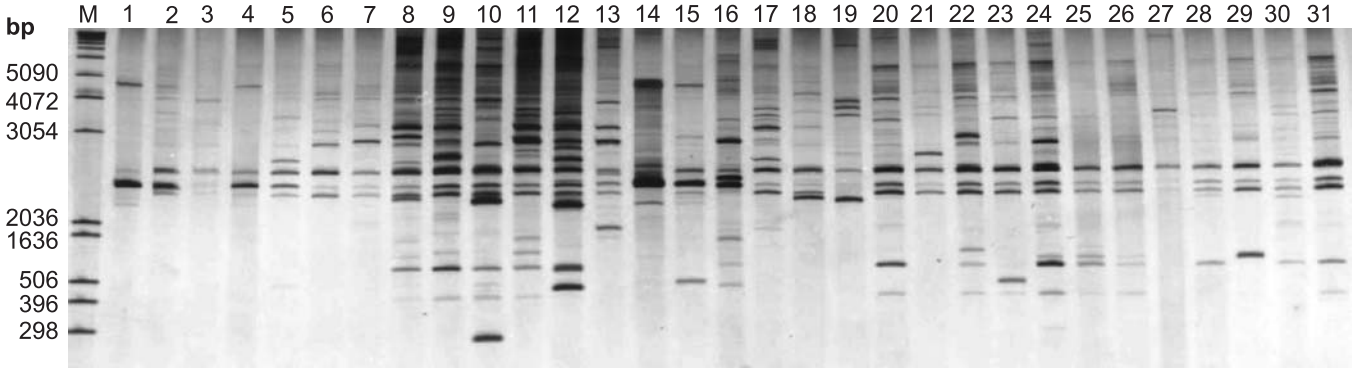
Technologies, Inc., Alameda, CA, USA), 1U Taq DNA polymerase and 25 ng DNA sample. The samples were previously tested using 36 different primers, from which 14 that showed higher polymorphism were selected: OPC03 (CACTGGCCCA), OPC04 (ACGGGACCTG), OPC10 (AGCAGCGAGG), OPC19 (AGT CC GCCTG), OPC20 (ACGGAAGTGG), OPA01 (AGA CG GCTCC), OPA04 (AGGACTGCTC), OPA06 (GTGGGT GCCA), OPA09 (AGATGGGCAG), OPA10 (TGGTCGGGTG), OPE07 (GTG TCAGTGG), OPE10 (CTGAAGCGCA), OPE11 (AAGA CCGGGA) and OPE15 (TGCCTGGACC). The amplification products were separated electrophoretically on 5% acrylamide gels. The DNA bands were silver stained according to a methodology described by Santos *et al*. (17) and photographed.

Data analysis

Each analysis was a consensus of at least two replicates runs. The DNA bands were scored as 1 (present) or 0 (absent) and all intense and reproducible bands were considered. These data were used to determine the genetic distance between strains. Average linkage (UPGMA – unweighted pair group method with averages) dendrograms were produced using the Statistica program (Copyright\STASOFT Inc. 1993 – UNMN 06/74). The percentage of polymorphic products was calculated for every *Rhizobium* strain tested (8). The diversity index was calculated according to the equation: number of profiles/total number of strains described by Handley *et al*. (6) for each site, using the higher polymorphic primers (OPA10, OPA04, OPC03 and OPC10).

RESULTS AND DISCUSSION

The different primers used in this study yielded reproducible and polymorphic RAPD patterns for all the *Rhizobium* strains tested. Among these primers, OPA10, OPC10, OPA04 and OPC03 showed the highest polymorphism, with the percentage of polymorphic products ranging from 67% to 100%, depending on the primer used and the site of origin of the *Rhizobium*. Most of the genotypes could be identified using one or two primers. Fig. 1 shows an example of the banding patterns obtained for some strains tested using the OPA10 primer. The amplification products varied in length from approximately 5090 to 298 bp. Based on variations in number and site of bands it was possible to identify individual strains. Despite the polymorphism observed with some primers, the diversity index was low, ranging from 0.19 to 0.37 according to the primer used and region of origin of the *Rhizobium* strains tested. The Sete Lagoas site showed the lowest index (Table 2). Analyis of RAPD fingerprinting data generated a dendrogram (Fig. 2) which suggested divisions based on genetic relationships between all of the strains evaluated. Low degrees of variation (9% to 30%) shown by linkage distance confirmed the trend in the diversity index obtained. In addition, it was possible to divide the strains into three clusters according to their site of origin . Two groups were formed with native *Rhizobium* strains from Sete Lagoas (1 and 2) and one with native *Rhizobium* strains from Brasília. The commercial strains from CIAT and the other strains of different origins were grouped within the Sete Lagoas 1 and Sete Lagoas 2 clusters, respectively. Analysis of physiological characteristics of the strains, such as growth rate and effectiveness previously determined (12,13) were related with the groups generated by dendrograms. The majority of strains (75%) were slow-growing (alcali producers) as a typical *Bradyrhizobium* strains and the remaining were fast-growing (acid producers). However this differential growth characteristic was not sufficient to distinguish groups in the generated dendrograms. Probably this behaviour



(M) marker 1 Kb Ladder; (1) CIAT3806; (2) CIAT3101; (3) CIAT2138; (4) BR105; (5) MGAC27; (6) MGAC15 (7) MGAC30; (8) MGAC22; (9) MGAC2; (10) MGAC13; (11) MGAC4; (12) MGAC5; (13) MGAC1; (14) MGAC6; (15) MGAC20; (16) MGAC18; (17) MGAC3; (18) MGAC24; (19) ALS18; (20) ALS16; (21) ALS8; (22) ALS43; (23) ALS25; (24) ALS9; (25) ALS15; (26) ALS20; (27) ALS31; (28) ALS24; (29) ALS35; (30) ALS23; (31) ALS26.

Figure 1. Example of RAPD banding patterns obtained for 31 *Rhizobium* strains associated with *Arachis pintoi* with primer OPA10.

Table 2. Genetic diversity index (Handley *et al*., (6)) of the *Rhizobium* strains associated with *A. pintoi* from Sete Lagoas 1, Sete Lagoas 2 and Brasília sites, according to four different primers.

Primer	Origin site		
	Sete Lagoas 1	Sete Lagoas 2	Brasília
OPA10	0.37	0.29	0.36
OPC10	0.35	0.29	0.36
OPA04	0.32	0.29	0.36
OPC03	0.32	0.19	0.36

represents more an adaptation of these strains to specific environmental conditions like micro niches with higher pH than of general soil conditions. Nevertheless these physiological differences may explain the polymorphism found among the analysed patterns. Similar results were found obtained by Mathan *et al*. (8) with strains isolated from *Arachis hypogaea* when studying these and other phenotypic characteristics. The relationship of strains effectiveness with groups generated by dendrogram showed higher number of effective strains in Sete Lagoas 1 cluster. However these differences may be explained by the presence of *A. pintoi* species in these site. According to many authors (3,6,21) the specific host legumes species may favor the increase of rhizobia number, select the more effective and genetically more similar strains (2,18) for each legume. The genetic variation within the groups Sete Lagoas 1 and Sete Lagoas 2 ranged from 11.6% to 25.5% and from 14% to 25.5%, respectively, and within the Brasília group, from 9% to 28.5% (Fig. 2). The high similarity (approximately 70%) found among the strains that are able to associate with *A. pintoi* is in accordance with several studies on genetic diversity of *Bradyrhizobium* populations which showed an elevated genetic homogeneity in this rhizobia group. Young and Cheng (21) compared similarities between *Bradyrhizobium* and *Rhizobium* from soybean cultivated in different tropical soils and found lower genetic variation in *Bradyrhizobium* than in *Rhizobium*. Strains isolated from *Arachis hypogaea* in Indian soils were also found to be a homogeneous *Rhizobium* group (8). In addition to that, the presence of *A. pintoi* in origin sites of the strains and the use of only one ecotype (BRA031143) as trap host may favor the selection of genetic similar strains.

The three clusters established by the analysed strains indicated the influence of environmental conditions or soil characteristics on the genetic distribution of these rhizobia. The physical-chemical characteristics of the soils are shown in Table 1. In Sete Lagoas 1 higher levels of organic matter, nutrients and pH values were observed. In contrast, the soils from Sete Lagoas 2 and Brasilia subsites had more homogeneous characteristics and lower organic matter and nutrient levels and pH values. These discriminating characteristics (organic matter and nutrients) seem to be acting as

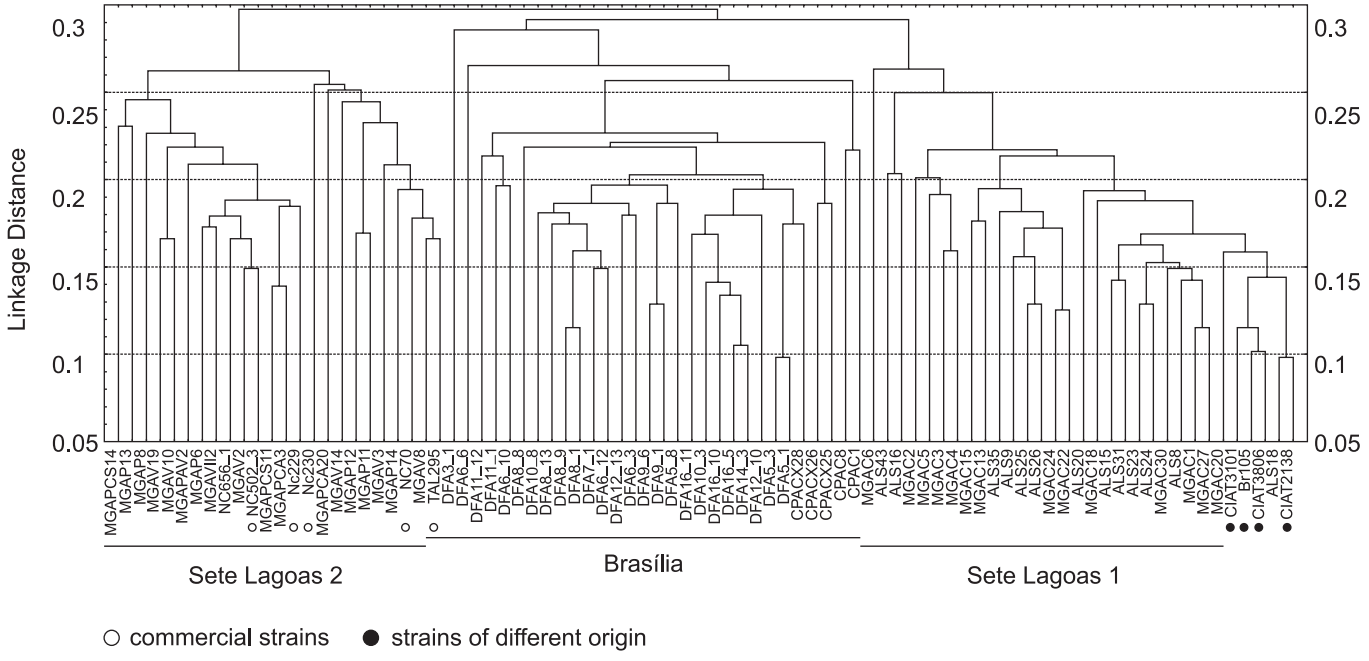


Figure 2. Dendrogram showing the genetic relationship between 85 *Rhizobium* strains associated with *A. pintoi*, by RAPD analysis, using 14 different primers.

a selective pressure for grouping closely related strains, but did not appear to influence the diversity index of rhizobia. Similar results were obtained in studies conducted on different species of rhizobia (4,6,8,21). These reports indicated the direct influence of the site on the grouping of the strains investigated.

In spite of the high genetic homogeneity observed among the rhizobia strains associated with *A. pintoi* in Cerrado soils, RAPD analyses identified adequate primers for the characterisation of strains as demonstrated with OPA10 (Fig. 1). In addition, the results of this study confirm that PCR is a useful tool for phylogenetic and ecological investigations of rhizobial communities and will also permit to conduct competitiveness and persistence studies on these rhizobia strains when inoculated in Cerrado soil as inoculants.

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RESUMO

Caracterização de rizóbios capazes de nodular *Arachis pintoi* via análise de "RAPD"

As relações genéticas de 85 estirpes de *Rhizobium* capazes de nodular *Arachis pintoi* foram determinadas usando o método de "RAPD" (Random Amplified Polymorphic DNA). As análises incluíram 75 estirpes isoladas de solos de Cerrado e 10 de diferentes origens. Os resultados indicaram que existe um alto grau de similaridade entre estas estirpes e que a distribuição geográfica pode afetar suas relações filogenéticas. Além disso, os resultados permitiram a seleção de "primers" mais adequados para a caracterização dessas estirpes de *Rhizobium*, os quais serão úteis para a implementação de estudos de competitividade nos solos de Cerrado.

Palavras-chave: *Rhizobium*, *Arachis pintoi*, solos de Cerrado.

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