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Genotypic analysis of isolated peanut-nodulating rhizobial strains reveals differences among populations obtained from soils with different cropping histories

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ABSTRACT

Peanut (*Arachis hypogaea*) is one of the most important crops of Córdoba province, Argentina; however, knowledge of the genetic diversity of peanut-nodulating rhizobial populations is fragmentary. In view of the importance of information on native rhizobial populations in relation to improved agricultural management practices, we performed genetic characterization of rhizobia isolated from root nodules of plants grown on soils with previous peanut cropping history (PH: Río Cuarto and Cabrera sites) vs. soils with no previous peanut cropping history (NPH: La Aguada and Chaján sites). Ten different *16S rRNA* restriction fragment length polymorphism (RFLP) genotypes were obtained through combination of restriction patterns obtained with four endonucleases. Cluster analysis of genotypes gave two major groups at 80% similarity. Each group contained strains from mostly unrelated origins, indicating a genetic relationship among peanut-nodulating populations isolated from different peanut cropping systems. Furthermore, sequence analysis of the *16S rRNA* gene demonstrated identity of isolates with strains of the genus *Bradyrhizobium*, and phylogenetic association among rhizobial populations from sites with different peanut cropping histories. Diversity indexes estimated for RFLP genotypes showed that populations obtained from PH soils were less diverse than those from NPH soils, suggesting that the presence of the legume results in selection of particular rhizobial strains. Higher polymorphism and diversity were revealed by enterobacterial repetitive intergenic consensus (ERIC)-PCR analysis. Peanut strains clustered at very low levels of similarity (55%). Populations of different origins clustered together, although at elevated genetic distance. ERIC-PCR results indicated the presence of different rhizobial populations in soils of Córdoba province.

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1. Introduction

Members of the family Leguminosae are an important group of plants because of their richness, diversity, and agroindustrial production as food for humans and animals (Graham and Vance, 2003). A distinctive characteristic of legumes is their capacity to establish a symbiotic relationship with rhizobia (Gage, 2004). The symbiosis leads to the development of new organs, nodules, on the roots of the legumes. Inside root nodules, differentiated bacteria (bacteroids) reduce atmospheric N_2 to ammonia for plant nutrition, a process termed biological nitrogen fixation (Spaink et al., 1998).

Peanut (*Arachis hypogaea* L.) is a legume that originated in the Bolivian and northwest Argentinean Andean region. It is currently cultivated worldwide in tropical, subtropical and warm temperate regions. Peanuts are a major part in the economic development of the central region of Cordoba province, Argentina, which is responsible for more than 90% of its production in this country. A main genus of rhizobia that nodulates peanut is *Bradyrhizobium* (van Rossum et al., 1995) although recently peanut nodulation has been described with strains assigned to the genus *Rhizobium* (Ibañez et al., 2008). Several reports have shown that populations of indigenous rhizobia are widely distributed in the soils of diverse geographical areas around the world (Peng et al., 2002; Aoki et al., 2010; Appunu et al., 2008; Cardinale et al., 2008; Estrella et al., 2009). In particular, high diversity and heterogeneity have been reported for peanut-nodulating strains isolated from different geographic regions (Saleena et al., 2001; Zhang et al., 1999). This heterogeneity can be partially explained by the promiscuous way in which rhizobia infect peanut, a process termed *crack entry*. According to this mechanism, rhizobia invade peanut through little injuries between epidermal cells at the base of emerging lateral roots, and subsequently proliferate in intercellular spaces

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Table 1
Sites of strain isolation, soil characteristics, and previous cultivation history.

	Sites			
	Cabrera[a]	Río Cuarto[a]	La Aguada[b]	Chaján[b]
Location				
Latitude	32°47′28″ S	33°6′29″S	32°56′52″S	33°35′16″S
Longitude	63°18′54″W	64°18′01″W	64°38′49″W	64°57′30″W
Soil characteristics				
Texture	Loam	Loam	Sandy loam	Sandy loam
Organic matter (%)	1.98	2.04	1.58	1.93
Nitrate (ppm)	56.0	28.0	25.4	97.3
Phosphorus (ppm)	41.5	17.5	23.9	26.5
pH (water)	6.1	6.2	6.8	6.3
Cropping history (seasons)				
1999–2000	Soybean (*Glycine max*)	Peanut (*Arachis hypogea*)	–	–
2000–2001	Peanut (*Arachis hypogea*)	Alfalfa (*Medicago sativa*)	Weeping Lovegrass (*Eragrostis curvula*)	–
2001–2002	Soybean (*Glycine max*)			–
2002–2003	Maize (*Zea mays*)			–
2003–2004	Soybean (*Glycine max*)	Triticale (*Triticosecale*)		Maize (*Zea mays*)
2004–2005	Peanut (*Arachis hypogea*)[c]	Peanut (*Arachis hypogea*)[c]		Sunflower (*Helianthus annuus*)
2005–2006	–	–	Peanut (*Arachis hypogea*)[c]	Soybean (*Glycine max*)
2006–2007	–	–	–	Peanut (*Arachis hypogea*)[c]

[a] Typical peanut cropping area.
[b] Non-typical peanut cropping area.
[c] Cropping season when strains were isolated.

before invading the cortical cells and differentiating into bacteroids (Boogerd and van Rossum, 1997).

Inoculation of legumes with selected, highly efficient rhizobia is the way by which symbiotic nitrogen fixation can be improved in agroecosystems. It represents a major strategy for the development of sustainable input of nitrogen into agricultural soils (Lindström et al., 2010). However, the occurrence of native rhizobial populations in soil often leads to inoculation failure due to the large size of the populations, their positional advantage, or their superior adaptation to local environmental conditions, facts that determine a superior competitive ability of native strains (Dowling and Broughton, 1986; Streeter, 1994; Bogino et al., 2008). Application of inoculation technology for improvement of peanut crop is not a common practice because it is frequently assumed that nodulation by native strains is satisfactory (Bohlool et al., 1992). However, selection of efficient strains based on their local ecologic adaptation could lead to increased grain production of crops (Lindström et al., 2010; Denton et al., 2003). In recent years there has been increasing movement of peanut cropping towards non-typical areas such as the south of Córdoba province, and other provinces such as San Luis and La Pampa (Fernandez and Giayetto, 2006). Studies of phenotypic and genotypic characteristics of native nodulating strains are therefore crucial for selection and application of superior strains, and for development of successful inoculation strategies (McInnes et al., 2004). In particular, molecular methods leading to identification and characterization of isolates are a valuable tool for management and application of suitable agricultural practices, *e.g.*, fertilizer or inoculation application, crop rotation, and use of selective host legume.

Despite the importance of peanut crop in Córdoba province, knowledge about the genetic properties of native rhizobia nodulating peanut in Argentinean soils is still sparse. In particular, the degree of genetic diversity of peanut rhizobia has not been fully described. Previous studies of strains able to nodulate peanut in central Argentina showed that there is considerable diversity (Taurian et al., 2006; Bogino et al., 2010). However, detailed information regarding the degree of genetic diversity among peanut-nodulating populations from different agrogeographical origins in this region is not available. The present study was designed to test the hypothesis that peanut-nodulating populations differ depending on cropping history of soils, by genetically characterizing rhizobia isolated from nodules of plants grown on soils with vs. without previous peanut cropping history, utilizing RFLP analysis of *16S rRNA* gene, nucleotide sequence analysis of this gene, and ERIC-PCR fingerprinting.

2. Materials and methods

2.1. Field locations and soil properties

Strains used in the present study were isolated from peanut plants grown in four different sites located in the south region of Córdoba province, Argentina. The distance between sampled sites was at least 30 km. Río Cuarto and General Cabrera are sites with previous peanut cropping history (PH), located within the typical peanut cropping area, whereas La Aguada and Chaján are sites with no previous peanut cropping history (NPH), located outside the typical peanut cropping area. Site locations, cropping history, and physicochemical characteristics of soils are summarized in Table 1.

2.2. Isolation of bacterial strains and growth conditions

A runner Virginia-type peanut (Manigran) cultivar from 'Criadero El Carmen', Córdoba, Argentina, was sown in each sampling site. We obtained our collection of peanut-nodulating strains from untreated (non-inoculated) soils. A total of ten peanut plants at R_1 growth (flowering, 31 days after planting) were randomly selected from 1 ha within each site. The distance between individual sampled plants was ≥30 m. Five nodules per plant were processed using standard procedures (Vincent, 1970), and bacteria were isolated by plating on yeast extract mannitol agar (YEMA) supplemented with congo red and bromothymol blue (Somasegaran and Hoben, 1994). Bacteria isolated were confirmed as peanut-nodulating strains based on nodulation testing of plants grown in sterilized vermiculite. By this procedure, a total of 40 peanut-nodulating native strains were isolated. Four representative strains of *Bradyrhizobium* sp. which are used for peanut inoculation were also included. The native and recommended strains studied are listed in Table 2. Bacteria were grown and maintained on yeast extract mannitol broth (YBM) at 28 °C for 4–6 days depending on the strain.

Table 2
Strains used and *16S rRNA* gene analysis.

Strains used			*16S rRNA* gene						
Denomination	Origin	Source	*16S rRNA* genotype[a]	*16S rRNA* PCR-RFLP patterns[b]				*16S rRNA* sequence analysis	
				*Alu*I	*Msp*I	*Hae*III	*Hinf*I	GenBank accession	Most closely related sequence (% identity)
USDA 4438	Recommended	USDA, ARS, USA	A	A	A	A	A	FJ418928	*Bradyrhizobium japonicum* SEMIA 5056 (FJ390913) (99%)
USDA 3180	Recommended	USDA, ARS, USA	A	A	A	A	A	FJ418916	*Bradyrhizobium japonicum* SEMIA 5045 (FJ390924) (99%)
P8A	Río Cuarto	Bogino et al. (2006)	A	A	A	A	A	FJ418919	*Bradyrhizobium* sp. ORS199 (AF514794.1) (99%)
P8B	Río Cuarto	Bogino et al. (2010)	A	A	A	A	A		
61LA	La Aguada	This work	A	A	A	A	A	JF317682	*Bradyrhizobium japonicum* HMS-02 16S (DQ499019) (99%)
65LA	La Aguada	This work	A	A	A	A	A		
Ch25	Chaján	This work	A	A	A	A	A	JF317685	*Bradyrhizobium japonicum* SEMIA 5046 (FJ390926) (99%)
SEMIA 6144	Recommended	IPAGRO, Brasil	B	A	A	B	A		
6H	Río Cuarto	This work	B	A	A	B	A		
PC3	Cabrera	Bogino et al. (2010)	B	A	A	B	A		
PC4	Cabrera	Bogino et al. (2010)	B	A	A	B	A		
PC27	Cabrera	Bogino et al. (2010)	B	A	A	B	A	FJ418927	*Bradyrhizobium* sp. SEMIA 929 (FJ390938) (99%)
PC29	Cabrera	Bogino et al. (2010)	B	A	A	B	A		
PC31	Cabrera	Bogino et al. (2010)	B	A	A	B	A		
PC33	Cabrera	Bogino et al. (2010)	B	A	A	B	A		
PC34	Cabrera	Bogino et al. (2010)	B	A	A	B	A	JF317681	*Bradyrhizobium* sp. SEMIA 905 (FJ959100) (98%)
26LA	La Aguada	Bogino et al. (2010)	B	A	A	B	A	FJ418912	*Bradyrhizobium japonicum* USDA 127 (AF208508) (100%)
27LA	La Aguada	Bogino et al. (2010)	B	A	A	B	A	FJ418913	*Bradyrhizobium* sp. LAR-20 (EF638789) (99%)
62AG	La Aguada	Bogino et al. (2010)	B	A	A	B	A	FJ418925	*Bradyrhizobium* sp. ASL-2 (EF629392) (99%)
45AG	La Aguada	Bogino et al. (2010)	B	A	A	B	A	FJ418914	*Bradyrhizobium* sp. H13-CR (AB601652) (99%)
51LA	La Aguada	This work	B	A	A	B	A		
P1	Río Cuarto	Bogino et al. (2006)	C	A	B	A	B	FJ418917	*Bradyrhizobium elkanii* SEMIA 5002 (FJ390895) (99%)
P5	Río Cuarto	Bogino et al. (2010)	C	A	B	A	B	FJ418922	*Bradyrhizobium elkanii* M 14 (AB601669) (99%)
P7	Río Cuarto	Bogino et al. (2006)	C	A	B	A	B	FJ418918	*Bradyrhizobium elkanii* SEMIA 938 (AY904739) (99%)
PR1	Río Cuarto	Bogino et al. (2006)	C	A	B	A	B	FJ418926	*Bradyrhizobium elkanii* M 13 (AB601668) (100%)
PC32	Cabrera	Bogino et al. (2010)	C	A	B	A	B		
8A	La Aguada	This work	C	A	B	A	B		
40A	La Aguada	This work	C	A	B	A	B		
35LA	La Aguada	This work	C	A	B	A	B		
Ch1	Chaján	This work	C	A	B	A	B		
Ch4	Chaján	This work	C	A	B	A	B	JF317683	*Bradyrhizobium elkanii* (AB601670) (99%)
Ch10	Chaján	This work	C	A	B	A	B		
Ch12	Chaján	This work	C	A	B	A	B		
Ch19	Chaján	This work	C	A	B	A	B		
Ch21	Chaján	This work	C	A	B	A	B		
Ch13	Chaján	This work	D	A	A	A	B	JF317684	*Bradyrhizobium* sp. C14-2660 (AB513458) (97%)
Ch17	Chaján	This work	D	A	A	A	B		
C-145	Recommended	NifTAL, USA	E	A	E	A	C	FJ418929	*Bradyrhizobium* sp. PRNB-21 (HM125059) (99%)
4A	La Aguada	This work	F	A	D	A	B		
Ch9	Chaján	This work	F	A	D	A	B		
9A	La Aguada	This work	G	A	C	A	A		
20AG	La Aguada	Bogino et al. (2010)	H	A	C	B	A	FJ418911	*Bradyrhizobium japonicum* SEMIA 6154 (FJ025100) (99%)
55AG	La Aguada	Bogino et al. (2010)	I	A	E	B	C	FJ418915	*Bradyrhizobium* sp. SEMIA 637 (FJ390899) (99%)
Ch8	Chaján	This work	J	A	C	A	C		

[a] The *16S rRNA* genotype represents the combination of RFLP patterns obtained from four restriction enzymes.
[b] Strains with the same letter have the same RFLP patterns obtained from one endonuclease.

2.3. 16S rRNA polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Total genomic DNA was isolated as described by Ausubel et al. (1994). The *16S rRNA* gene amplification was carried out by using fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) primers (Weisburg et al., 1991) as described by van Berkum et al. (1996). PCR products, of about 1500 bp, were incubated with endonucleases *Alu*I, *Hinf*I, *Hae*III, and *Msp*I (Laguerre et al., 1994). Restriction of the *16S rRNA* gene was carried out in a final volume of 30 μl containing 18 μl nuclease-free water, 2 μl 10X buffer, 10 μl amplified product, and 2 μl of each enzyme. The reactants were incubated for 16 h at 37 °C. DNA digestion fragments were separated electrophoretically on a 3% (w/v) agarose gel, stained with ethidium bromide, visualized, and photographed under UV illumination.

2.4. 16S rRNA gene nucleotide sequence analysis

Nucleotide sequence of *16S rRNA* gene was analyzed for 20 peanut-nodulating strains (Table 2). Direct PCR was performed utilizing 1 μl of DNA template in a 20 μl PCR reaction mixture containing the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) (Willems et al., 2001). Purified PCR products of ∼1400 bp were sequenced with an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) by Macrogen Inc. Laboratories (Korea). Identities among these sequences were determined using the BLAST search program (National Center for Biotechnology Information) (Altschul et al., 1997) (Table 2). The nucleotide sequence of the *16S rRNA* gene of Ch4, Ch13, Ch25, 61LA and PC34 strains were deposited in GenBank under accession numbers JF317683, JF317684, JF317685, JF317682, and JF317681, respectively.

To produce a phylogenetic tree reflecting evolutionary relationships among peanut-nodulating strains from different origins, a phylogenetic analysis was conducted using *MEGA* version 4 (Tamura et al., 2007). Multiple alignments were made with Clustal W software (Higgins et al., 1994). Aligned sequences were used to construct a phylogenetic tree based on the neighbor-joining method (Saitou and Nei, 1987), using the Kimura 2-parameter model (Kimura, 1980).

2.5. ERIC-PCR fingerprint

The DNA sequences of enterobacterial repetitive intergenic consensus (ERIC) primers E1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and E2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) were used for PCR amplification as described by Versalovic et al. (1991). PCR was performed as described by de Bruijn (1992) for rhizobia and other rhizobacteria. Amplification products were separated according to molecular size by electrophoresis on 1.5% agarose gel, stained with ethidium bromide, visualized, and recorded under UV illumination.

2.6. Clustering analysis

Fingerprints generated by various enzymes on the amplified *16S rRNA* gene (RFLP), and banding patterns of ERIC-PCR, were carefully manually analyzed and converted into a two-dimensional binary matrix through a binary scoring system: 1 for presence and 0 for absence of a given band. The genetic distance of peanut rhizobia was evaluated by a Jaccard coefficient. Dendrograms were constructed with Infogen software, using the UPGMA algorithm (Balzarini and Di Rienzo, 2003). Distances among geographical sites were similarly evaluated considering all molecular markers obtained for each location, and dendrograms were constructed accordingly.

2.7. Diversity indexes

We estimated indexes of Shannon (H', diversity), Margalef (R_1, richness) and Pielou (E_1, evenness) based on the number of isolates belonging to each RFLP of *16S rRNA* genotype and clusters at 55% similarity of ERIC. Shannon index was calculated by the equation $H' = -\sum [(n_1/n) \ln (n_1/n)]$ (Shannon and Weaver, 1949), where n_1 is the number of isolates in each group and n is the number of isolates in all groups. For Margalef index $R_1 = (S-1)/\ln(n)$ was used (Margalef, 1958), where S is the number of groups and n is the number of isolates in all groups. Pielou index was estimated with $E_1 = H'/\ln(S)$ (Pielou, 1977), where H' is the Shannon index and S is the number of groups.

3. Results

3.1. PCR-RFLP analysis of 16S rRNA gene

RFLP of *16S rRNA* gene methodology is a widely used tool for carrying out genetic studies of collections of bacterial strains. In particular, rhizobial strains can be analyzed using appropriate restriction endonucleases (Laguerre et al., 1996). We used the restriction endonucleases *Alu*I, *Msp*I, *Hae*III, and *Hinf*I in this study because they have shown good ability to discriminate among peanut-nodulating strains (Taurian et al., 2006; Yang et al., 2005). These restriction endonucleases produced 29 molecular markers, of which only 10 (35%) were variable (polymorphic) across all observations. *Msp*I endonuclease was the enzyme that revealed the highest polymorphism on restriction of *16S rRNA* gene sequence; it produced five different fingerprints or restriction patterns. *Hae*III, *Hinf*I, and *Alu*I produced two, three, and one restriction patterns, respectively, among the strains tested (Table 2).

As a result of the analysis, ten genotypes from eleven RFLP patterns were detected in 44 bacterial strains able to nodulate peanut (Table 2). The dominant RFLP genotypes were A, B, and C, which grouped 35 of the 44 peanut-nodulating strains. These genotypes were notable for their heterogeneity according to origin of grouped strains.

In order to test distance or similarity among genotypes, we generated a dendrogram from the genotypes or combined RFLP patterns. The dendrogram obtained shows two clusters (I and II) at 55% similarity (45% distance) (Fig. 1A). Cluster I was formed by genotypes C, D, and F, which included 18 strains; cluster II was larger and was formed by the remaining seven genotypes, which included 26 strains. At higher similarity (about 82%), 6 clusters were formed (Ia, Ib, IIa, IIb, IIc, IId). Interestingly, cluster IIc grouped genotypes A and B with a total of 21 strains, of which 14 were from non-related sites according to cropping history: Cabrera (7) and La Aguada (7). Similarly, cluster Ib grouped genotypes C and D with 16 strains, which included most of the strains from Chaján (8 of 11) and Río Cuarto (4 of 7), suggesting genetic similarity among strains from these origins.

To assess relationships among the different locations according to RFLP analysis, all molecular markers were compared in terms of their origin. In this sense, and considering the genetic background of each location, the algorithm showed two clusters among zones not related in terms of peanut cropping history: Chaján/Río Cuarto, and La Aguada/Cabrera (Fig. 1B).

To further estimate the degree of genetic difference among strains of each geographical origin we estimated the relation among RFLP genotypes and number of strains (G/S, Table 3). Higher numbers of genotypes and values for G/S were found in the non-typical peanut cropping area (NPH; Chaján and La Aguada). Similarly as in a previous study of bean rhizobial populations (Kaschuk et al., 2006), we calculated indexes of diversity (Shannon index), richness

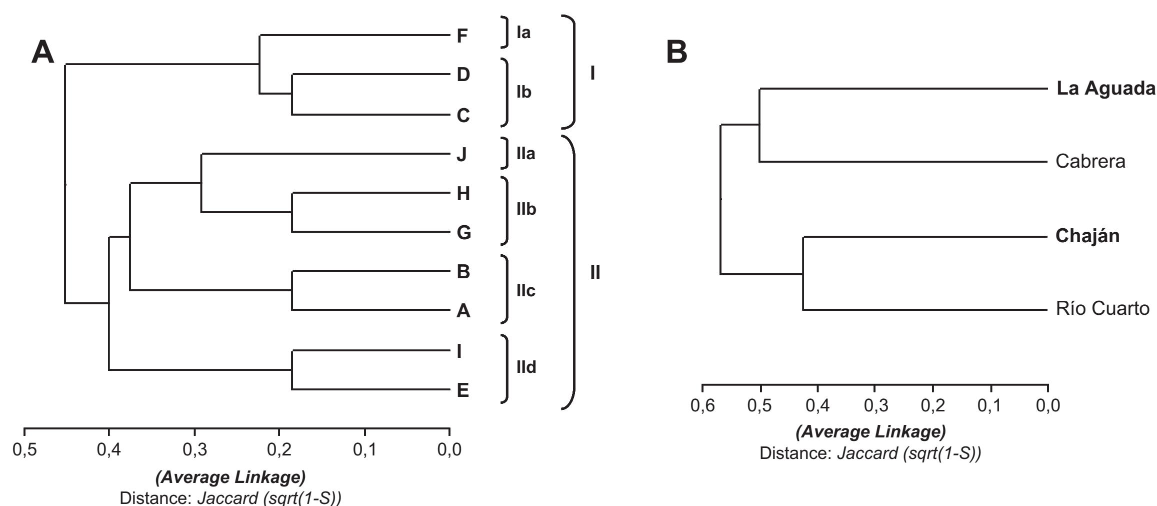

Fig. 1. Dendrograms based on RFLP of *16S rRNA* gene analysis using the UPGMA algorithm and Jaccard coefficient. (A) Dendrogram generated from RFLP genotypes of peanut-nodulating strains. (B) Dendrogram generated from origins of peanut-nodulating strains, according to their RFLP of *16S rRNA* patterns. Sites without previous peanut cropping history (NPH) are indicated by boldface.

(Margalef index), and evenness (Pielou index). Values of diversity indexes (H') for NPH areas were almost twice those for PH areas, whereas richness index (R_1) was three-fold higher for NPH areas. Evenness indexes (E_1) were similar for PH vs. NPH areas.

3.2. 16S rRNA gene nucleotide sequence analysis for peanut-nodulating strains

We determined the nucleotide sequence of *16S rRNA* gene in recommended strains and in several native strains with different origins, in order to establish their relationships and taxonomic positions. Sizes of amplified products with specific primers for *16S rRNA* gene were uniform and near 1400 bp. Table 2 shows accession numbers and identities of the strains studied. As expected on the basis of growth and culture properties, all strains tested were assignable to the genus *Bradyrhizobium*.

To estimate evolutionary distance and phylogenetic relationships among peanut-nodulating strains with different geographical origins, a phylogenetic tree was constructed. This tree showed two major branches in which bradyrhizobial strains were divided according to origin. Most strains from Río Cuarto and Chaján were grouped together, whereas all strains from Cabrera and La Aguada were grouped into a cluster which also included the recommended strains (Fig. 2). Separation of isolates indicated clearly that strains able to establish a symbiotic relationship with peanut are closely related to two major species of *Bradyrhizobium*, *Bradyrhizobium japonicum* or *Bradyrhizobium elkanii* (Table 2 and Fig. 2).

3.3. ERIC-PCR fingerprint

To further test the genetic relationship among peanut rhizobia, we employed another widely used molecular tool, based on analysis of amplified fragments of DNA located between ERIC elements on the entire bacterial genome (Versalovic et al., 1991). According to ERIC analysis, an elevated numbers of markers were detected for each strain tested. 112 molecular markers were obtained for the 44 strains studied, of which 98% were variable through all observations (*i.e.*, showed polymorphism).

Analysis of ERIC markers from each strain shows that the dendrogram obtained is remarkably complex (Fig. 3A). We found four main groups that clustered at ~55% similarity (45% distance), grouping strains from all origins. Clusters I and II were each formed by 4 strains from Chaján, with grouping at ~58% similarity. Cluster III included 8 strains from different origins, but mainly from La Aguada (5 strains). Cluster IV was larger, and included 13 strains isolated from all four sites, as well as recommended strains C-154 and SEMIA 6144. Based on lower level of similarity (<55%), the rest of the strains analyzed (19) were not grouped into the four clusters as above. The dendrogram shows clustering of small groups of strains of common origin, at varying levels of similarity. PC3, PC33, and PC34 grouped at ~52% similarity, PC31 and PC32 at ~55%, and

Table 3
Diversity indexes.

Origin	No of strains	RFLP Genotypes[c]	G/S[d]	Diversity indexes					
				RFLP *16S rRNA* gene[a]			ERIC-PCR[b]		
				H'	R_1	E_1	H'	R_1	E_1
Río Cuarto	7	A, B and C (**3**)	0.43	0.95	1.03	0.86	1.25	1.54	0.90
Cabrera	8	B and C (**2**)	0.25	0.37	0.48	0.54	1.55	1.92	0.96
Chaján	11	A, C, D, F and J (**5**)	0.45	1.29	1.67	0.80	1.38	1.67	0.86
La Aguada	14	A, B, C, F, G, H and I (**7**)	0.5	1.71	2.27	0.88	1.29	1.51	0.80
PH	15	A, B and C (**3**)	0.2	0.95	0.74	0.86	1.92	2.58	0.92
NPH	25	A, B, C, D, F, G, H, I and J (**9**)	0.36	1.86	2.45	0.84	1.73	1.86	0.89

PH: sites with previous peanut cropping history; NPH: sites with no previous peanut cropping history; H': Shannon index; R_1: Margalef index; E_1: Pielou index.
[a] Indexes calculated according to number of strains belonging to each RFLP genotype.
[b] Indexes calculated according to number of strains clustered at 55% similarity.
[c] Number of RFLP genotypes for each site is given in brackets and highlighted in bold.
[d] RFLP genotypes and number of related strains.

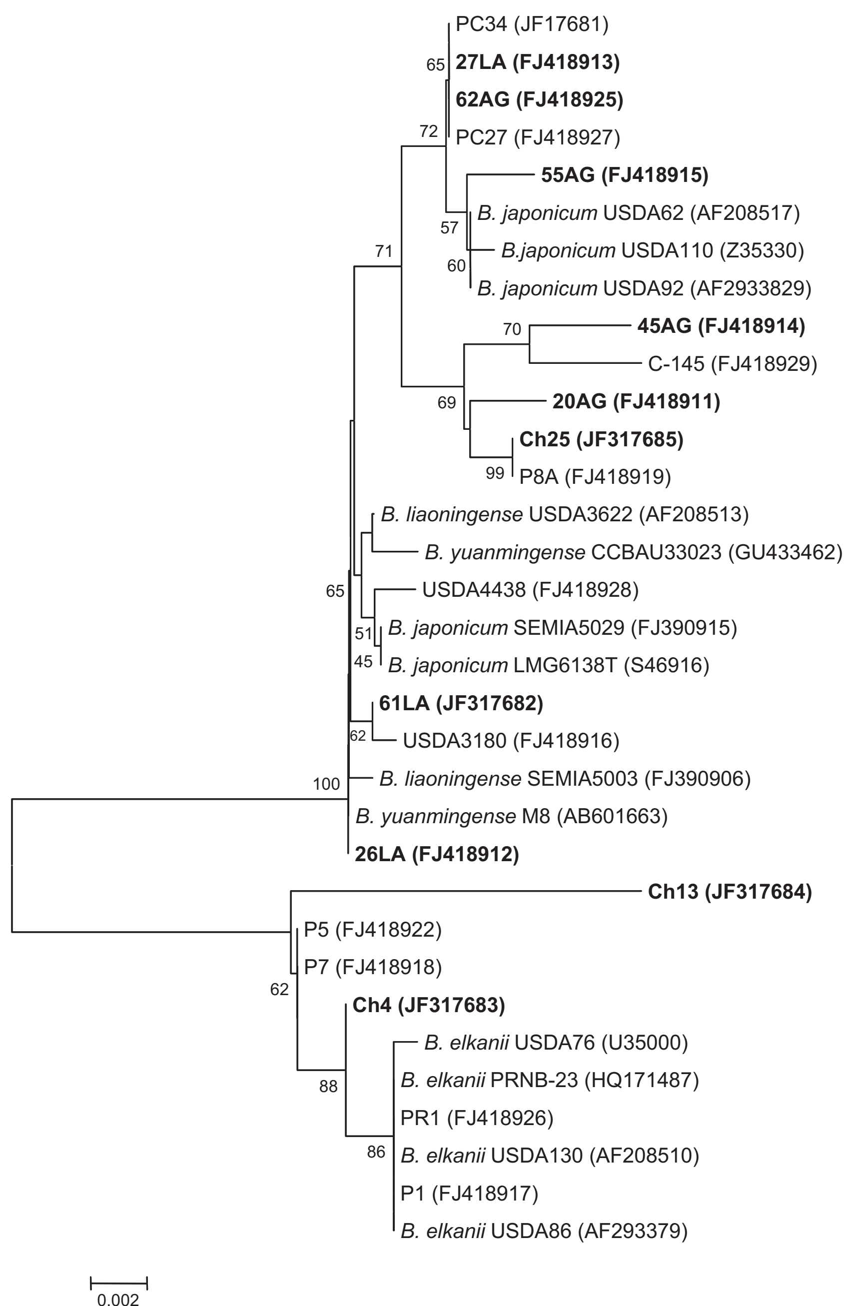

Fig. 2. Phylogenetic tree of bradyrhizobial strains isolated from peanut, and representative members of genus *Bradyrhizobium*, based on aligned *16S rRNA* gene sequences. Peanut-nodulating strains from locations without previous peanut cropping history (NPH) are indicated by boldface. Multiple alignments and neighbor-joining method were used to construct the trees, using MEGA 4.0.2 software. Bootstrap values, expressed as a percentage of 1000 replications, are given at the branch point. Sequence accession numbers are given in parentheses. Scale bar: 2 nt substitutions per 1000 nt.

PC27 and PC29 at ~66%, suggesting some linkage among strains from Cabrera, as was suggested by RFLP analysis of *16S rRNA*. ERIC analysis showed the presence of isolates from a common origin having identical fingerprinting (100% similarity; Fig. 3A), indicating that these isolates (PC3-PC33 from La Aguada, and P8A and P8B from Río Cuarto) are probably a unique strain. This must be confirmed by another type of PCR analysis, *e.g.*, REP-PCR or BOX-PCR.

We also assessed relationships among the different locations according to ERIC markers from peanut-nodulating rhizobia, and a different dendrogram of geographical origins was generated. This dendrogram showed a cluster between two zones with different peanut cropping history, La Aguada and Cabrera, although at a very low level of similarity (~30%) (Fig. 3B).

4. Discussion

RFLP data for peanut-nodulating strains showed the presence of a high number of genotypes obtained from the RFLP patterns yielded by four endonucleases. This analysis also revealed that ~85% of peanut-nodulating strains studied (37 of 44), were grouped in clusters Ib and IIc (Fig. 1A), while the remaining ~15% (7 strains) were distributed among 6 genotypes and 4 clusters. These may include rhizobial strains having low genetic relationships with the majority of strains analyzed, and among themselves. Our results are consistent with a previous report of elevated numbers of genotypes for slow-growing strains isolated from peanut nodules in typical peanut cropping areas of Argentina (Taurian et al., 2006). In

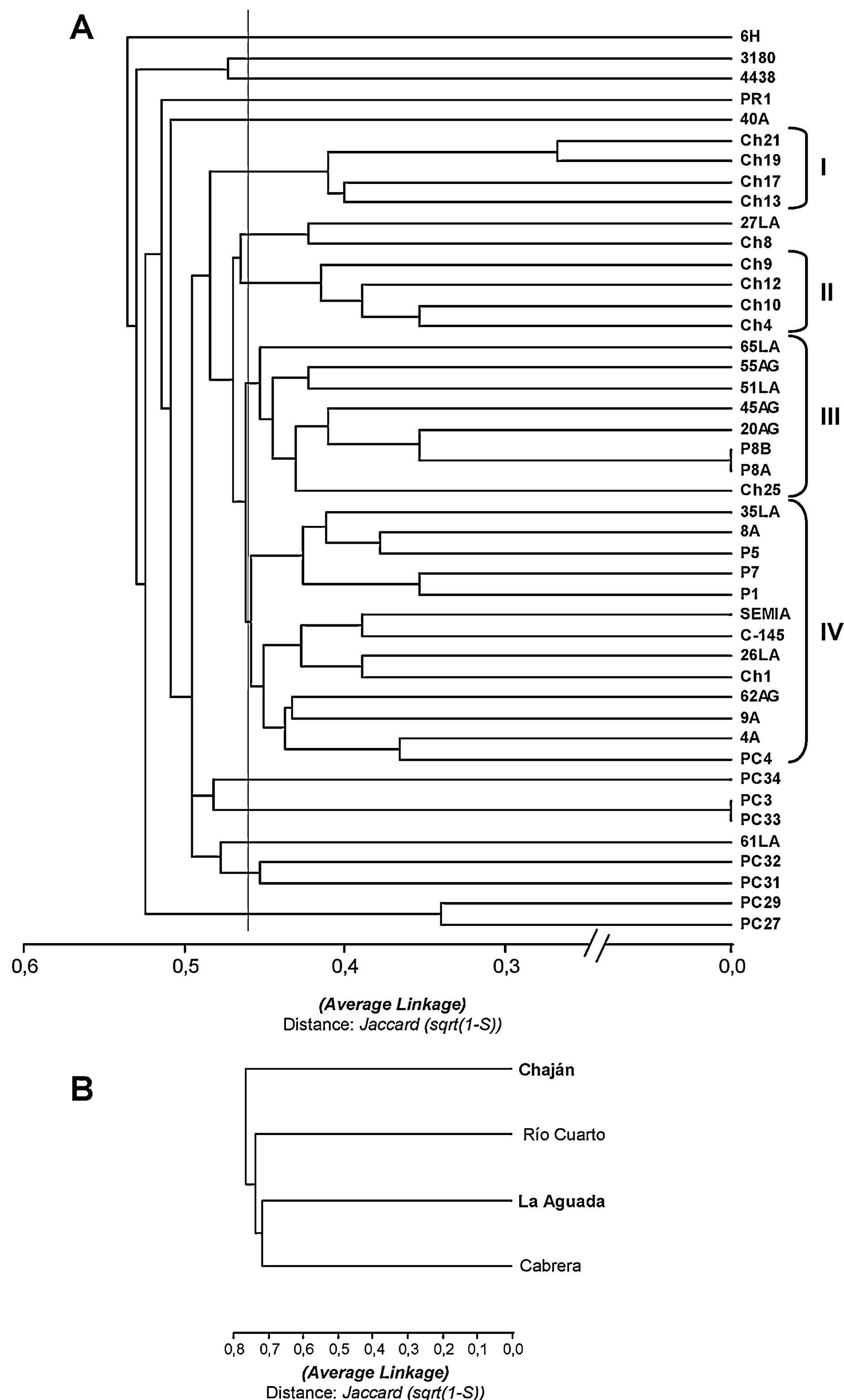

Fig. 3. Dendrograms based on ERIC-PCR fingerprint analysis, using the UPGMA algorithm and Jaccard coefficient. (A) Dendrogram generated from fingerprints of peanut-nodulating strains. (B) Dendrogram generated from origins of peanut-nodulating strains, according to their ERIC fingerprints. Sites without previous peanut cropping history (NPH) are indicated by boldface.

contrast, in a similar study of peanut populations isolated in China, only three RFLP genotypes were found using seven endonucleases (Yang et al., 2005).

By RFLP analysis, strains from different agrogeographical origins were grouped into major genotypes as described above and in Table 2. Genotypes A, B and C included isolates from all sites, indicating a lack of strong correlation between geographical origin and genotype, and that strains capable of establishing a symbiotic relationship with peanut plants form genetically diverse populations independently of their geographical origin. On the

other hand, cluster analysis of genotypes revealed linkages among strains from locations with different peanut cropping history. This observation was supported by cluster analysis of origins (Fig. 1B). Although associations were detected between zones unrelated in terms of peanut cropping history, clusters formed at low levels of similarity (50–60%), suggesting major differences among peanut-nodulating populations from different locations. Since isolates from typical peanut cropping areas were obtained during a single cropping season, whereas isolates from non-typical areas were obtained in successive years, one might argue that various seasonal factors affected the edaphic communities differently for different years that strains were isolated. Extension of this study to several growing seasons is necessary to strengthen our conclusions.

The peanut-nodulating rhizobial strains isolated in the present study showed high similarity with previously described strains of *B. japonicum* and *B. elkanii* strains (Table 2), consistent with results of previous studies (Bogino et al., 2010; Yang et al., 2005; Willems et al., 2001). Strains isolated from peanut plants growing in Chaján and Río Cuarto showed high identity with strains assigned to *B. elkanii*, whereas strains isolated from plants growing in Cabrera and La Aguada showed identity with strains assigned to *B. japonicum*, *B. liaoningense*, and *Bradyrhizobium yuanmingense* (Fig. 2). Analysis of *16S rRNA* gene sequence suggests that populations isolated from sites with different peanut cropping systems showed similarities according to their strain identities.

RFLP analysis also showed that in locations such as Río Cuarto and Cabrera, where peanut cropping is a common practice, diversity indexes were smaller than in Chaján and La Aguada (Table 3), suggesting that the plants select particular rhizobial strains, *i.e.*, in areas where peanut cropping is common, genotypes tend to decrease in number, and the presence of peanut reduces the degree of diversity and richness in rhizobial populations. Similarly, previous reports showed decreased rhizobial diversity in the presence of legume hosts such as cowpea (Zilli et al., 2004) and peanut (Ngo Nkot et al., 2008). Thus, cropping of a particular legume for several seasons may promote selection of more homogeneous rhizobial taxa.

Our ERIC-PCR fingerprinting results demonstrate high genetic diversity of peanut-nodulating rhizobia, and are consistent with several previous reports of elevated genetic diversity using this technique (Taurian et al., 2006; Yang et al., 2005). In cluster analysis, the main clusters of strains grouped at low genetic similarity (55%) (Fig. 3A). Similarly, analysis of genomic bacterial fingerprints for bradyrhizobia isolated from peanut nodules in Botswana and South Africa showed several clusters of strains with $<60\%$ similarity (Law et al., 2007).

Cluster analysis of origins for ERIC markers showed minimal or no linkage among areas with similar peanut cropping history, and formation of clusters at high levels of genetic distance (Fig. 3B), suggesting that each soil contains unique peanut-nodulating rhizobial populations with high genetic diversity. This conclusion was supported by diversity indexes obtained at 55% similarity for ERIC analysis (Table 2). Indexes of Shannon (H'), Margalef (R_1), and Pielou (E_1) were similar in typical and non-typical peanut cropping areas, as well as in each location analyzed (Table 3). Analyses of indexes and marker descriptions (data not shown) also demonstrated differences between the two molecular tools employed here, ERIC-PCR and RFLP of *16S rRNA*. ERIC-PCR produced a high number of markers from the whole genome, whereas RFLP analysis was restricted to markers from a unique gene. Markers obtained from ERIC-PCR were more polymorphic than those from RFLP analysis. We consider that these two methodologies complement each other. The conclusions obtained, reflecting the properties of each molecular tool, provide a more integrated and global view of genetic diversity of rhizobial populations.

Based on our observations, we hypothesize that both PH and NPH soils in Córdoba province contain populations of peanut-nodulating rhizobia which are stable members of the soil community, and whose composition and genotype have been determined over time by edaphic, climatic, anthropogenic, and other environmental factors. The continuous, long-term presence of peanut plants as a result of cropping, together with the above factors, models the structure of these nodulating populations by selecting their members in a manner optimal for both symbionts: rhizobia and legume.

The success of inoculation strategies depends on the structure of populations into which inoculant strains are introduced (McInnes et al., 2004). The strains most likely to be selected by a cultivated legume would be those most competitive for reaching nodulation sites. Thus, long-term adaptation of native rhizobia to their environment results in a high degree of saprophytic competence, and their population size tends to increase in sites where compatible legumes are cultivated, and the soil is fertile (Zengeni et al., 2006). For this reason, inoculation strategies are unlikely to be successful in PH soils of Río Cuarto and Cabrera. On the other hand, in spite of the higher rhizobial diversity found in NPH areas, inoculation strategies have a better chance of success in NPH soils of Chaján and La Aguada. In this case, an introduced strain would face less competition because population sizes of native strains would be smaller, and highly competitive native genotypes would not be predominant. This concept is supported by our previous findings of higher nodulation by native strains in PH soils (Bogino et al., 2006), and reduced nodulation and competitive effect by native strains in NPH sites (Bogino et al., 2008).

Knowledge of genotypic properties of rhizobial populations is also important for improved crop management in terms of use of cultivars that exhibit high strain selectivity. Given the variation in genetic diversity of rhizobial populations among sites, more effective agricultural strategies will involve use of host legume varieties that are selectively nodulated by native strain populations, or management practices that favor nodule occupancy of selected host varieties by inoculated rhizobial strains (McInnes and Haq, 2003). Further studies will determine the best “fit” between host legume varieties and native or introduced rhizobial populations, and how this knowledge can be applied to optimize crop yield.

5. Conclusions

Genotypic analysis as described here demonstrates the complex genetic structure of peanut-nodulating rhizobial populations. The molecular data, taken together, indicate the high genetic diversity of the rhizobia, and provide the first quantitative analysis of diversity of *Bradyrhizobium* strains isolated from PH vs. NPH sites in Córdoba province, Argentina. Global study of genotypic characteristics illustrates the high diversity of peanut-nodulating populations independent of their agrogeographical origin, and the effect on soil biology by presence of a particular legume crop, through its influence on nodulating rhizobial populations.

Knowledge of genotypic characteristics of these rhizobial populations will help improve agricultural peanut production worldwide, through application for inoculation strategies and other sustainable management practices. Given the economic importance of peanut production in Córdoba province, and the steadily increasing interest in expansion to non-traditional cropping areas, it is essential to better understand the factors affecting growth of this legume, including its associated nodulating rhizobial populations. Further detailed studies on genotypic composition, seasonal evolution of populations, and effects of rhizobia on different varieties of host legume, in combination with

biogeographic approaches, will clarify the behavior of local rhizobial populations, and have direct application for agricultural purposes.

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