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Genetic diversity of rhizobia nodulating *Arachis hypogaea* L. in Central Argentinean Soils

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Abstract

The diversity of thirty-nine isolates from peanut plants growing at fourteen different sites in the Argentinean province of Córdoba was examined by rep-PCR, RFLP of PCR amplified *16S rRNA* gene and complete sequencing of ribosomal genes. The genomic analysis of the peanut isolates indicated that each group encompasses heterogeneity among their members, having distinct rep fingerprints and *16S rRNA* alleles. Complete sequencing of *16S rRNA* demonstrated that native peanut rhizobia from Córdoba soils representative of the slow and fast growers are phylogenetically related to *Bradyrhizobium japonicum* and *Bradyrhizobium* sp. and *Rhizobium giardinii* and *R. tropici* species, respectively. The *nod*C gene sequence analysis showed phylogenetic similarity between fast grower peanut symbionts and *Rhizobium tropici*.

Introduction

Most of rhizobial species that induce nodule formation on leguminous plants belong to the alpha subgroup of the *Proteobacteria* which comprises at least six rhizobial genera: *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Azorhizobium, Mesorhizobium* and *Allorhizobium*. These all lie on quite phyllogenetically distinct branches, each of which also includes many bacterial species that are not rhizobia. In recent years, studies of indigenous rhizobia populations isolated from a variety of legume hosts around the world have revealed considerable genetic diversity and led definition of new genera and species such as *Mesorhizobium* and *Sinorhizobium* (Amarger et al., 1997; Chen et al., 1997; Jarvis et al., 1997; van Berkum et al., 1998). Furthermore, species from the beta proteobacteria subdivision, such as *Ralstonia* and *Burkholderia*, and from the gamma and α2-proteobacteria subclasses (genera *Hedysarum* sp. *and Ochrobactrum* sp.) are also able to nodulate legumes (Benhinzia et al., 2004; Chen et al., 2003; Moulin et al., 2001; Trujillo et al., 2005). These results may reflect the broader range of wild legumes examined and the use of improved technologies to examine genomes (Haukka, 1997) which altogether led to the definition and identification of new genotypes able to establish symbioses with legumes.

Polyphasic taxonomy analysis, including phenotypic and genotypic characterizations, is recommended to be used for classification of rhizobia (Graham et al., 1991). These studies have led to the description of several new species of *Rhizobium* (Amarger et al., 1997; Chen et al., 1997; van Berkum et al., 1998) and a new genus

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as *Mesorhizobium* (Jarvis et al., 1997). The precise taxonomic status of many *Bradyrhizobium* sp. strains isolated from various legumes is not clarified yet, and several authors have reported the lack of consistency between results that were obtained by the use of different taxonomic techniques (Doignon-Bourcier et al., 2000).

Arachis hypogaea L. (peanut or groundnut) is an important crop that provides food for direct human subsistence and other several food products. Argentina is one of the major peanut producers in the world, and about 94% of its production takes place in the province of Córdoba. It is generally accepted that peanut is native to the Americas; it was grown in Central and South America and had been proposed that domestication took place in a region located between the North of Argentina and the South of Bolivia and Paraguay (Kochert et al., 1996; Krapovickas, 1969).

Peanut has been reported to form effective root nodules with slow-growing rhizobia (Urtz and Elkan, 1996; van Rossum et al., 1995; Yang et al., 2005; Zhang et al., 1999) which in the current taxonomic status of the order Rhizobiales fall in the genera *Bradyrhizobium*. Moreover, bacteria that nodulate peanut in natural environments from all over the world have been classified as *Bradyrhizobium* (*Arachis*) sp. but species names have not yet been defined. Several reports describe the isolation and characterization of peanut nodulating rhizobia strains such as NC92 (Gillette and Elkan, 1996), MAR 411 (van Rossum et al., 1995) and Spr 3–7 (Urtz and Elkan, 1996; Zhang et al., 1999), that were assigned to the genus *Bradyrhizobium*. Fast growing peanut rhizobia were studied by Wong et al. (1988) but they found that they were ineffective. Moreover, the broad host range *Rhizobium* sp strain NGR234 which nodulates many diverse legumes can also form nodules on peanut albeit inefficiently (Pueppke and Broughton, 1999; Broughton, personal communication).

In an attempt to assess the diversity of peanut rhizobial populations in the peanut producing area of the province of Córdoba, Argentina, a collection of 39 authenticated isolates from this region was phenotypically characterized (Taurian et al., 2002). Our results revealed two very distinct populations which group within the slow and fast growing rhizobia, respectively. In this work, the analysis of this peanut rhizobial collection was extended in order to approach the phylogenetic position of representative members of the isolates. Rep-fingerprints and RFLP of PCR-amplified *16S rRNA* gene were used to identify representatives and to analyze their *16S rRNA* gene for species assignment. Our results demonstrated that peanut rhizobial isolates having *16S rRNA* alleles similar to species *R. giardinii* and *R. tropici* as well as *Bradyrhizobium* species were found among the peanut rhizobial populations in the environment of the cropping area of Argentina.

Materials and methods

Sampling sites and rhizobial isolates

Rhizobia were isolated from peanut plants or field soil samples in the laboratory by using peanut as trapping host. Fourteen different sites located in the center and south region of the Argentinean province of Córdoba (latitude, 32°–34°; longitude, 63°–65°), which have no history of *Arachis hypogaea* L. inoculation, were sampled and therefore it was assumed that isolates represent the native populations. The rhizobia isolates and reference strains used in this study are listed in Table 1.

Rhizobia were isolated from fresh nodules as previously described (Taurian et al., 2002) by the standard method on YEMA medium (Vincent, 1970). The nodulation on the original host plant of each isolate was verified by plant test using the method of Vincent (1970). The seedlings, growing in plastic pots containing sterilized vermiculite, were inoculated with 3–5 mL of the appropriate rhizobial broth culture (YEM) in stationary growth phase ($1–4\times10^9$ cells mL^{-1}). A negative control (uninoculated seedling) and a positive control (inoculated with the strain recommended as peanut inoculant) were included. Plants were grown under controlled conditions and harvested 5 weeks after inoculation for root nodule observation. To make sure that nodules were formed by the inoculated isolates, the ERIC-PCR profiles obtained from the bacteria from inside the nodules and the original inoculant were compared each other. Authenticated

Table 1. Isolates of *Arachis hypogaea* L. from peanut cropping area of Argentina and reference strains

Isolates	Geographical origin		Original host plant	Source or Reference
	Locality	*Department*		
NHOL2, NHOL16	Holmberg	Río Cuarto	*Arachis hypogaea* L.	Taurian et al., 2002
NOD31, *TT001*, TT002	Río Cuarto	Río Cuarto	*Arachis hypogaea* L.	Taurian et al., 2002
NLH24, NLH25, NLH30, *NLH22, NLH27*	Las Higueras	Río Cuarto	*Arachis hypogaea* L.	Taurian et al., 2002
NMAL12	Malena	Río Cuarto	*Arachis hypogaea* L.	Taurian et al., 2002
NCAR2B	Carnerillo	Río Cuarto	*Arachis hypogaea* L.	Taurian et al., 2002
NALE	Alejandro	Juárez Celman	*Arachis hypogaea* L.	Taurian et al., 2002
NCHAX, NCHA30, NCHA31, *NCHA22, NCHA32, NCHA33, NCHA35*, NCHA42	Charras	Juárez Celman	*Arachis hypogaea* L.	Taurian et al., 2002
NDEHE, *NDE11*	General Deheza	Juárez Celman	*Arachis hypogaea* L.	Taurian et al., 2002
NMAN5, NMAN6, *NMAN10, NMAN11*	Manfredi	Río Segundo	*Arachis hypogaea* L.	Taurian et al., 2002
NONC1, NONC4, NONC5, NONC8, NONC9, NONC10, *NONC11, NONC13*	Oncativo	Río Segundo	*Arachis hypogaea* L.	Taurian et al., 2002
NTI31	Ticino	San Martín	*Arachis hypogaea* L.	Taurian et al., 2002
NVAM24	Villa María	San Martín	*Arachis hypogaea* L.	Taurian et al., 2002
NCHOO4	Chazón	San Martín	*Arachis hypogaea* L.	Taurian et al., 2002
NET30	Etruria	San Martín	*Arachis hypogaea* L.	Taurian et al., 2002
Reference strains				
Bradyrhizobium SEMIA 6144			*Arachis hypogaea* L.	IPAGRO, Brasil
Bradyrhizobium sp TAL1000			*Arachis hypogaea* L.	NifTAL, USA
Bradyrhizobium japonicum USDA 110			*Glycine max*	Lab. Dr. Aguilar
Bradyrhizobium elkanii USDA 76			*Glycine max*	Lab. Dr. Aguilar
Rhizobium etli CFN42			*Phaseolus vulgaris*	Lab. Dr. Aguilar
Rhizobium etli SC15			*Phaseolus vulgaris*	Lab. Dr. Aguilar
Rhizobium tropici CIAT 899			*Phaseolus vulgaris*	Lab. Dr. Aguilar
Rhizobium leguminosarum 2671			*Phaseolus vulgaris*	Lab. Dr. Aguilar
Rhizobium giardinii H152			*Phaseolus vulgaris*	Lab. Dr. Aguilar
Rhizobium giardinii RO17			*Phaseolus vulgaris*	Lab. Dr. Aguilar
Sinorhizobium fredii 191			*Glycine max*	Lab. Dr. Aguilar
Sinorhizobium meliloti 116			*Medicago sativa*	IPAGRO, Brasil
Mesorhizobium loti 3471			*Lotus* spp.	Lab. Dr. Aguilar

Isolates indicated by italics characters are fast growers.

cultures were stored in 20% (v/v) glycerol at −80 °C.

DNA preparation

Bacterial DNA template was obtained by applying the two following procedures: (a) Total DNA was isolated using a modified procedure of Meade et al. (1982). Isolates were cultured on YEM for 24–72 h at 30 °C in aerobic conditions. Cells were concentrated by centrifugation and suspended in 1 mL of 1 *M* NaCl and centrifuged at 14,000 rpm for 4 min. The supernatant was discarded and the pellet was washed with 1 mL TES (10 m*M* Tris pH 8; 25 m*M* EDTA; 150 m*M* NaCl), resuspended and centrifuged at 14,000 rpm for 2 min. The cellular pellet was suspended in 500 μl TE buffer (10 m*M* Tris pH 8; 25 m*M* EDTA) and treated with lysozyme (2 mg ml^{-1} of TE buffer) for 30 min at 37 °C. The cells were lysed by the addition of 100 μL of sarcosyl-pronase (19% sarcosyl + 5 mg mL^{-1} pronase in TE) and incubated at 37 °C for 1 h. The DNA solution was extracted with phenol according to standard procedures, DNA precipitated with ethanol, air dried and finally dissolved in 100 μL double distilled sterile water. (b) Bacteria were grown on YEMA plates and colonies were collected, suspended in 300 μL of 1 *M* NaCl (picked up by using a plastic disposable loop), mixed thoroughly and centrifuged at 14,000 rpm for 4 min. The supernatant was discarded and the pellet was suspended in 300 μL double distilled sterile water. After the sample was mixed and centrifuged as described before, the supernatant was removed and the pellet was suspended in 150 μL of 6% (aqueous suspension) resin Chelex 100® (Bio Rad). This suspension was incubated at 56 °C for 20 min, followed by mixing and further incubation at 99 °C for 8 min (Walsh et al., 1991). DNA concentration of the samples was approximately 5 ng ml^{-1}.

ERIC-PCR analysis

The DNA sequences of enterobacterial repetitive intergeneric consensus (ERIC) primers used in this study E_1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′)/E_2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) have been reported by Versalovic et al. (1994). Polymerase chain reaction (PCR) was performed in 15 μL reaction mixture containing 1× PCR buffer, 1.5 m*M* $MgCl_2$, 200 μM each nucleotide (Promega), 0.308 μM of each primer, 1 U of *Taq* DNA polymerase (Promega) and 3.6 μl of template DNA solution. The temperature profile was as follows: initial denaturation at 95 °C for 1 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 65 °C for 8 min; and a final extension step at 68 °C for 16 min.

PCR-amplifications were performed in a Biometra UNO-thermo block, Göttingen, Germany. The ERIC amplification products in 5 μL sub-samples were separated according to molecular size by horizontal electrophoresis on 1.2% agarose gels and stained with ethidium bromide. Computer-assisted analysis of the fingerprints was carried out using GelCompar system software (Applied Maths, Kortrijk, Belgium).

16S Amplified rDNA restriction analysis (ARDRA)

Nearly full-length *16S rRNA* gene was PCR-amplified by using primers fD1 (5′-AAGGAGGTGATCCAGCC-3′) and rD1 (5′-AGAGTTTGATCCTgGCTCAG-3′) (Weisburg et al., 1991). PCR was performed in 20 μL reaction mixture containing 1× PCR buffer, 1.5 m*M* $MgCl_2$, 200 μM each nucleotide (Promega), 1 μM of each primer, 1 U of *Taq* polymerase and 5 μL of template DNA solution. The temperature profile was as follows: initial denaturation at 95 °C for 3 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min; and a final extension step consisting of 72 °C for 10 min. Three to five μL aliquots of PCR products were incubated respectively with endonucleases *Msp*I, *Hinf*I, *Hae*III, *Cfo*I, *Rsa*I and *Alu*I as it was described by Laguerre et al. (1994). Restricted fragments were separated by horizontal electrophoresis on 2.5% agarose gels and were visualized by ethidium bromide staining.

16S rRNA gene sequencing and data analysis

The slow growing isolate NLH25 and two fast growing isolates NET30 and NCHA22 were used in 16S rDNA sequence determination. The nearly full-length of *16S rRNA* gene was PCR-amplified as described above. The DNA-amplified

fragments were precipitated by adding 1 volume of isopropanol and 0.1 volume of sodium acetate (3 *M*) and collected by centrifugation for 10 min in a microcentrifuge. The resulting DNA pellet was washed with 70% ethanol, air-dried and suspended in double distilled water (Sambrook et al., 1989). The nucleotide sequence of purified PCR products was determined by Oswell Sequencing (Lab 3145, Medical and Biological Science Building University of Southampton, Boldrewood, Southampton, United Kingdom).

DNA sequencing of partial nodC gene fragment

A 930 bp *nodC* gene fragment from isolates NET30 and NCHA22 was amplified by using primers NodCF and NodCR designed by Laguerre et al. (2001). The PCR products were purified from the gel using GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The gel-purified PCR products were cloned into the pCR® 2.1-TOPO® vector by a TOPO TA Cloning® Kit (Invitrogen). The fragments were sequenced with M13F and M13R sequencing primers by Macrogen Inc. Laboratories (Korea).

Analysis of sequence data

Sequence analyses of *16S rRNA* and *nod*C genes were performed by using the algorithm BLASTN (Altschul et al., 1997) to identify similarities and to perform alignments. Multiple alignments and NJ trees were constructed by using CLUSTALX 1.64 b software (Thompson et al., 1997).

Nucleotide sequence accession numbers

The nucleotide sequence of the *16S rRNA* gene of isolates NLH25, NET30 and NCHA22 have been deposited in GenBank data bank under accession no. AY427207, AY427206 and AY728040, respectively.

Partial *nod*C gene sequence of isolates NET30 and NCHA22 have been deposited in GenBank data bank under accession no. AY919866 and AY919865, respectively.

Results and discussion

ERIC fingerprinting and ARDRA analysis

ERIC-PCR fingerprinting on total genomic DNA prepared from 39 peanut rhizobial isolates was performed, in order to compare their chromosomal backgrounds. PCR reaction was optimized in order to obtain consistency in the fingerprint profiles. The sample of strains examined were those isolates that represent the two rhizobial groups that we found in nature associated to peanut in the Argentinean peanut producing area of Córdoba, namely fast-growing and slow-growing groups. As shown in Figure 1, different electrophoretic patterns were produced with ERIC primers in a way that almost each isolate exhibited its own profile. These data indicated that the native population of peanut nodulating rhizobia is highly diverse, and that there is not obvious predominant subpopulation within the collection examined. The result is not unexpected to us, since it is accepted that peanut is a promiscuous host (Alwi et al., 1989).

Further analysis of the isolates that represented the different ERIC-PCR profiles (14 slow growers and 13 fast growers) was carried on by ARDRA. The DNA product of the PCR-amplified *16S rRNA* gene, of about 1500 bp, was used for restriction analysis. Results of the ARDRA analysis are summarized in Tables 2 and 3. The resulting fingerprint patterns were compared as well as with those from reference strains. Ten patterns among the 14 representatives of the slow growing isolates (Table 2) and 12 patterns among the 13 representatives of the fast growing isolates (Table 3) were identified. The patterns displayed by both fast and slow peanut rhizobia were different within each group (Figure 2). Endonuclease *Rsa*I was found to be the most discriminative among members of the slow growing rhizobia group, in which eight different patterns were found. On the other hand, enzyme *Alu*I was the most discriminative among the fast growers generating seven distinct patterns.

Overall, a high number of *16S rRNA* types, as defined by combination of restriction patterns, was found in the slow and fast- growing group, respectively. These results support those obtained by using the rep-fingerprinting procedure and

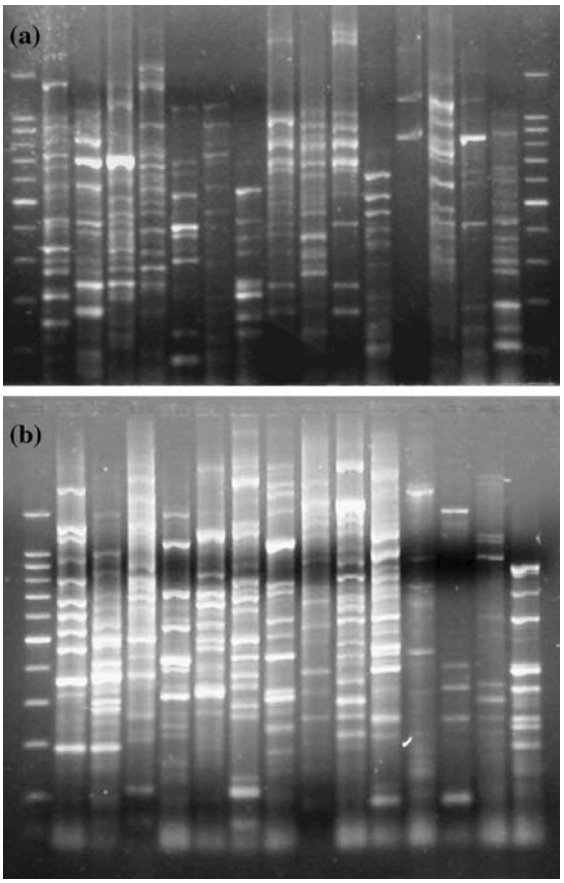

Figure 1. ERIC-PCR fingerprints of peanut isolates from Córdoba, Argentina. (a). Fingerprints obtained from 14 slow-growing isolates From left to right: 100 bp ladder (100–1500 bp, Promega), NONC1, NMAN6, NCHOO4, NHOL16, NLH24, NOD31, TT002, NMAN5, NDEHE, NONC4, NONC9, NCHA31, NLH25, NONC10, *Bradyrhizobium* sp SEMIA 6144, ladder 100 bp (100–1500 bp, Promega). (b). Fingerprints obtained from 13 fast-growing isolates, from left to right: 100 bp ladder (100–1500 bp, Promega), *Bradyrhizobium* sp. TAL1000, NLH27, NVAM24, NCHA32, NCHA33, NCHA35, NCHA22, NTI31, NET30, NHOL2, NMAN10, NONC13, NMAL12, TT001.

confirm the chromosomal heterogeneity among the strains from each major group.

The ARDRA fingerprint pattern that was found to be predominant among slow growing rhizobia (genotype I, see Table 2) was identical to that of the reference strain *Bradyrhizobium japonicum* USDA 110 whereas other patterns were distinct among them as well as to other reference bradyrhizobia species examined.

Taken these results altogether we concluded that the two major rhizobia populations found associated to peanut in nature, encompass diverse *16S rRNA* alleles. These results differ from those found in other symbiotic association showing preference for the microsymbiont either at the genera or species level. For instance, studies on peanut rhizobia done by several authors confirm that bradyrhizobia is the predominant bacteria isolated from peanut nodules (Alwi et al., 1989; van Rossum et al., 1995; Yang et al., 2005; Zhang et al., 1999).

Sequence analysis of 16S rRNA gene

Representative isolates of the slow and fast growing groups were chosen for 16S rDNA sequence analysis. Isolate NLH25 was selected because it is an efficient nitrogen fixer in peanut and represents the predominant pattern observed in the slow grower group (genotype I, see Table 2). The fast growing isolates NET30 and NCHA22 were chosen because of their high symbiotic effectiveness with peanut (Taurian et al., 2002). The nucleotide sequence of nearly full length of the PCR-amplified *16S rRNA* gene was determined and compared to sequences of known rhizobia species available in data banks.

Alignment of 1388 bp of the 16S rDNA sequence of isolate NLH25 showed to be very similar to *Bradyrhizobium* species (Figure 3), which is not unexpected since very low allelic variability have been detected among *16S rRNA* genes of bradyrhizobia (van Berkum and Fuhrman, 2000). The minimum and maximum number of nucleotide differences was five and seven, which represented a level of identity of about 99% with the sequences of *Bradyrhizobium japonicum* USDA110, *B. japonicum* USDA62, *B.* sp *Pplu*1–1, *B. liaoningense*, etc. Alignment with *16S rRNA* gene of *B. elkanii* showed less identity (89%) which corresponds to 17 number of nucleotide differences. Since full length *16S rRNA* gene sequence of peanut bradyrhizobia is not available in the public data base, we were unable to perform a more precise comparison between them. However, we compared about 255 bp of the *16S rRNA* gene from several peanut isolates described by van Rossum et al. (1995). Alignment of the partial sequence of NLH25 *16S rRNA* gene showed that it was 100% identical to that of strains included in van Rossums' homology group A (*Bradyrhizobium* sp MAR strains 471, 1576, 1510, 1505, 967, 411, *B. japonicum* MAR

Table 2. Restriction patterns of *16S rRNA* gene revealed by PCR-RFLP of representative isolates from the slow-growing group and reference strains

	*Hin*fI	*Msp*I	*Hae*III	*Cfo*I	*Rsa*I	ARDRA Genotypes
NLH25	A	A	A	A	A	**I** (AAAAA)
NLH24	C	D	D	C	E	**V** (CDDCE)
TT002	C	D	D	A	A	**VII** (CDDAA)
NDEHE	A	A	A	A	B	**II** (AAAAB)
NOD31	B	B	B	A	F	**VIII** (BBBAF)
NCHA31	A	A	A	A	A	**I** (AAAAA)
NMAN5	A	A	A	A	A	**I** (AAAAA)
NMAN6	B	C	C	B	C	**III** (BCCBC)
NONC1	D	E	B	E	B	**IV** (DEBEB)
NONC4	A	A	A	A	A	**I** (AAAAA)
NONC9	E	E	F	F	G	**IX** (EEFFG)
NONC10	A	A	A	A	A	**I** (AAAAA)
NCHOO4	C	C	E	D	D	**VI** (CCEDD)
NHOL16	F	F	F	G	H	**X** (FFFGH)
B. sp SEMIA 6144	A	A	A	A	A	**I** (AAAAA)
B. sp USDA 110	A	A	A	A	A	**I** (AAAAA)
B. elkanii USDA 76	G	G	G	H	I	**XI** (FFFGH)

Table 3. Restriction patterns of *16S rRNA* gene revealed by PCR-RFLP of representative isolates from the fast-growing group and reference strains

	*Hin*fI	*Msp*I	*Hae*III	*Cfo*I	*Alu*I	*ARDRA Genotypes*
NLH27	A	A	A	A	A	**I** (AAAAA)
NTI31	A	C	A	C	A	**III** (ACACA)
NET30	C	E	D	F	D	**VI** (CEDFD)
NCHA22	C	F	D	F	B	VIII (CFDFB)
NVAM24	A	B	A	B	B	**II** (ABABB)
NCHA32	C	G	C	D	C	**V** (CGCDC)
NCHA33	B	C	C	D	E	**IV** (BCCDE)
NCHA35	D	C	C	D	C	**IX**(DCCDC)
TT001	F	A	C	D	C	**X** (FACDC)
NONC13	A	D	B	ND	ND	**XII** (ADB–)
NMAN10	B	G	ND	E	F	**XI** (BG-EF)
NMAL12	C	F	C	F	G	**VII** (CFCFG)
NHOL2	A	A	A	A	A	**I** (AAAAA)
R. leguminosarum 2671	C	H	D	F	G	**XIII** (CHDFG)
R. etli CFN42	G	H	E	F	G	**XIV** (GHEFG)
R. tropici CFN 299	I	J	G	G	H	**XV** (IJGGH)
S. fredii 191	H	I	D	F	G	**XVI** (HIDFG)
M. loti 3471	J	G	D	H	F	**XVI** (JGDHF)

strains 1526 and 1491, and *B.* sp 32H1 and BTAil) (data not shown). In conclusion, partial sequence analysis of 16S rDNA of isolate NLH25 supports its inclusion within the genus *Bradyrhizobium*. In general, this genus includes slow-growing bacteria that were isolated from diverse host legumes and were not assigned to any particular species, which are referred as *Bradyrhizobium* sp. It is noteworthy, that at the level of 16S rDNA sequence, some of these strains, as it is also the case of the isolate NLH25, are phylogenetically closer to *Bradyrhizobium japonicum* strains than

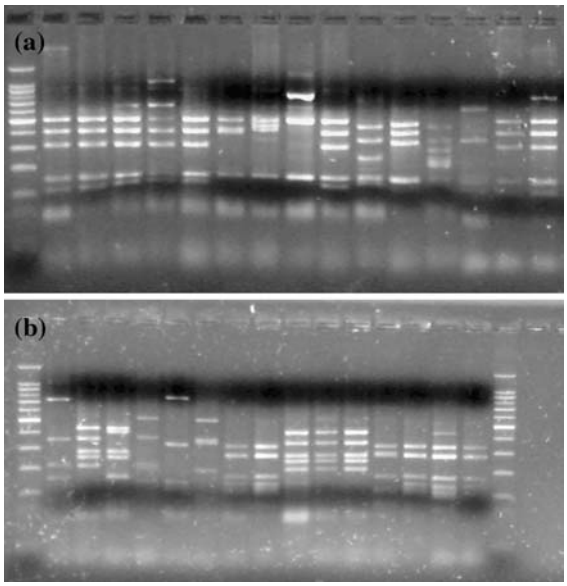

Figure 2. Gel electrophoresis of 16S *rRNA*-RFLP of peanut isolates. (a). Fingerprints obtained with *Rsa*I in the slow-growing group, from left to right: 100 bp ladder (100–1500 bp, Promega), *Bradyrhizobium* sp. SEMIA 6144, NLH25, NONC10, NCHA31, NMAN5, NDEHE, NOD31, NMAN6, TT002, NLH24, NONC1, NCHOO4, NONC9, *B. japonicum* USDA 110, *B. japonicum* USDA76. (b). Fingerprints obtained with *CfoI* in the fast-growing group, from left to right: 100 bp ladder (100–1500 bp, Promega), *Bradyrhizobium* sp. TAL1000, NCHA33, NMAN10, NLH27, NVAM24, NTI31, NET30, NMAL12, NCHA32, NCHA35, TT001, *R. tropici* CFN299, *R. etli* CFN42, *M. loti* 3471, *S. fredii* 191, ladder 100 bp (100–1500 bp, Promega).

these strains to each other. In spite of this similarity, strain NLH25 formed no nodules on soybean roots (Taurian et al., 2002). Therefore, molecular analysis based on markers other than the *16S rRNA* gene is needed to identify relationships with the symbiotic ability of slow growing rhizobia.

The sequences of NET30 and NCHA22 *16S rRNA* genes were different each other. Isolate NET30 sequence showed 99% identity with that of *Rhizobium giardinii* H152, which corresponds to six different nucleotides and isolate NCHA22 sequence showed 99% identity with that of *Rhizobium tropici* type IIB corresponding to four different base pairs. When alignment of both fast grower isolates' sequences was analyzed the number of nucleotide differences was 23, which represented a level of identity of about 97%. Therefore, the phylogenetic analysis based on the 16S rDNA sequences shown in Figure 3, places isolates NET30 and NCHA22 within the genus *Rhizobium* in branches closely related to *R. giardinii* H152 and *R. tropici* type II, respectively. Species *R. giardinii* was described by Amarger et al. (1997), to assign taxonomic positions to isolates from *Phaseolus vulgaris* collected from French soils. After that, biovars have been created within this specie to account for the differences in symbiotic specificity. On the other hand, species *R. tropici* type II is a broad host range rhizobia that forms nodules on *Leucaena leucocephala* and *P. vulgaris* among other host legumes.

The sequence analysis of *16S rRNA* gene of peanut rhizobia from Argentinean soils demonstrated that *Arachis hypogaea L.* is nodulated by *Bradyrhizobium* species, confirming previous reports (Urtz and Elkan, 1996; van Rossum et al., 1995; Yang et al., 2005; Zhang et al., 1999), and also by fast-growing rhizobia closely related to *Rhizobium giardinii* and *Rhizobium tropici* species. From this point of view, other legumes such as *Lotus subbiflorus, Glycine max, Acacia saligna, A. caven* and *Sesbania sesban* are similar to *Arachis hypogaea* since all these legumes are promiscuous, being nodulated by both slow and fast growing rhizobia (Chueire and Hungría, 1997; Frioni et al., 2001; Irisarri et al., 1996; Marsudi et al., 1999; Odee et al., 1997).

Sequencing and phylogeny of nodC gene

The *nod*C gene fragment of about 760 bp was amplified for the fast growing isolates NET30 and NCHA22 by using the pair of primers NodCF and NodCR. The complete sequence of these fragments was obtained and compared with those from databanks. High level of similarity was found between isolates NET30 and NCHA22 (99%, five base pairs different) and both of them were most similar to the *nod*C sequence segment of species *R. tropici* showing also 99% of identity.

Therefore, unlike the *16S rRNA* gene, analysis of *nod*C gene indicated that fast growing isolates are closely related to each other (Figure 4). In addition, these results suggest a common origin for symbiotic replicons of our peanut rhizobia and *R. tropici*, which illustrate combinatorial possibilities granted by lateral transfer of genetic material mediated by plasmids. Although plasmid localization of the symbiotic genes of species

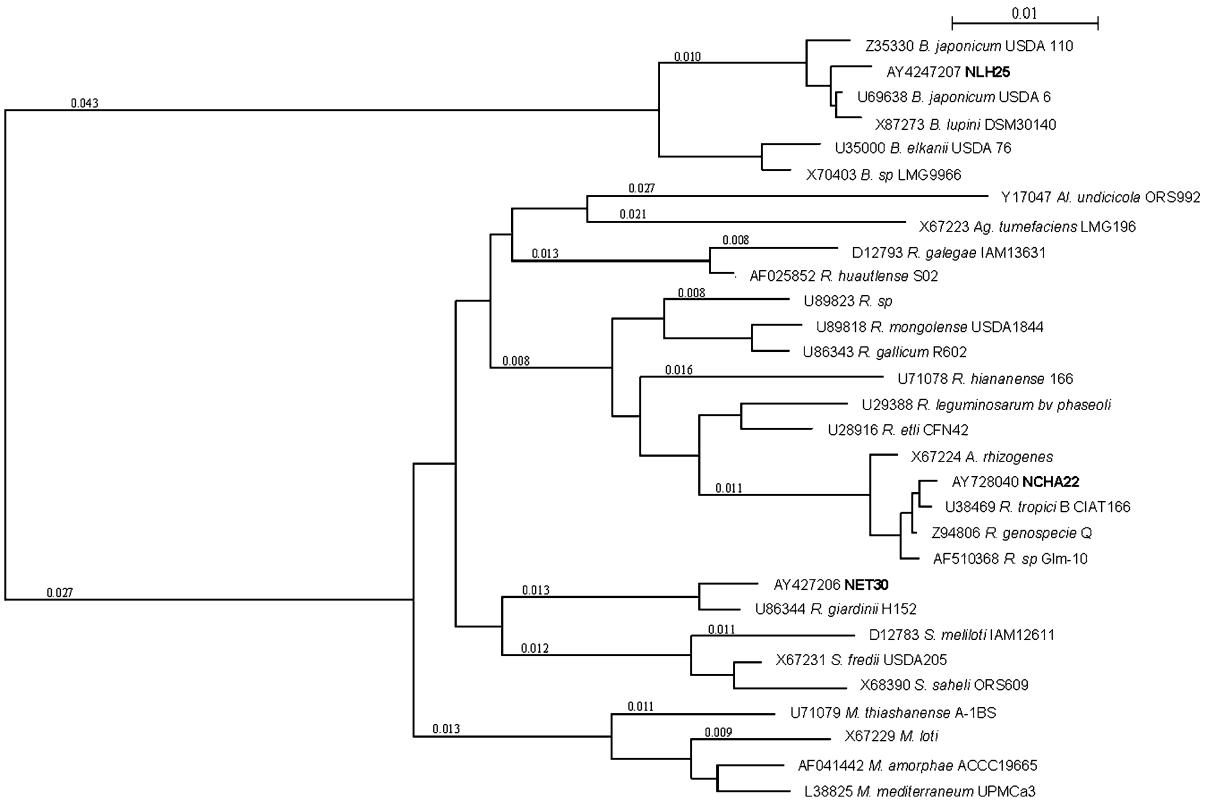

Figure 3. Phylogenetic tree, based on aligned sequences of *16S rRNA* genes, showing relationship among the isolates NET30, NCHA22 and NLH25 and known rhizobia species. Abbreviations: *Ag. Agrobacterium; Al. Allorhizobium; B., Bradyrhizobium; M., Mesorhizobium; R., Rhizobium* and *S., Sinorhizobium*. Multiple alignments and NJ trees were constructed by using CLUSTALX 1.64 b software.

R. tropici CIAT 899 has been shown, we have no evidence yet that the symbiotic genes of our isolates are carried on the indigenous plasmids we have detected (data not shown).

Occurrence of genetic exchange has been argued by several authors. Young and Johnston (1989) reported that the phylogenetic tree for NodD protein does not correlate with the *16S rRNA* phylogeny and suggested lateral gene transfer of Sym plasmid. Lateral gene transfer in rhizobia was also suggested by Oyaizu et al. (1993) since the phylogeny of *16S rRNA* and the host specificity does not correlate each other. Lateral gene transfer of the symbiotic genes appears to be the most plausible hypothesis to explain cases of phylogenetic incongruence between Sym and housekeeping genes (Martínez-Romero and Caballero-Mellado, 1996; Willems et al., 2003).

In the work of Laguerre et al (2001), the *nod*C gene of strain *R. giardinii* bv. phaseoli H251 was phylogenetically examined and found to cluster with other species that also nodulate common beans. The *nod*C of isolate NET30 clustered with the *R. tropici nod*C gene, thus NET30 may represent a novel biovar having different host specificity.

Promiscuity exists among diverse legumes widely dispersed in nature. So far, all the isolates from peanut were found to be slow growing rhizobia (Urtz and Elkan, 1996; van Rossum et al., 1995; Yang et al., 2005; Zhang et al., 1999) indicating that promiscuity of peanut could be restricted to putative genotypes within the genus *Bradyrhizobium*, which species remain to be defined. However, our findings have revealed that promiscuity is extended to include symbionts phylogenetically distant such as those that belong to the genus *Rhizobium*. It is interesting to find that in the natural environment of Córdoba, peanut establishes symbiotic association with both

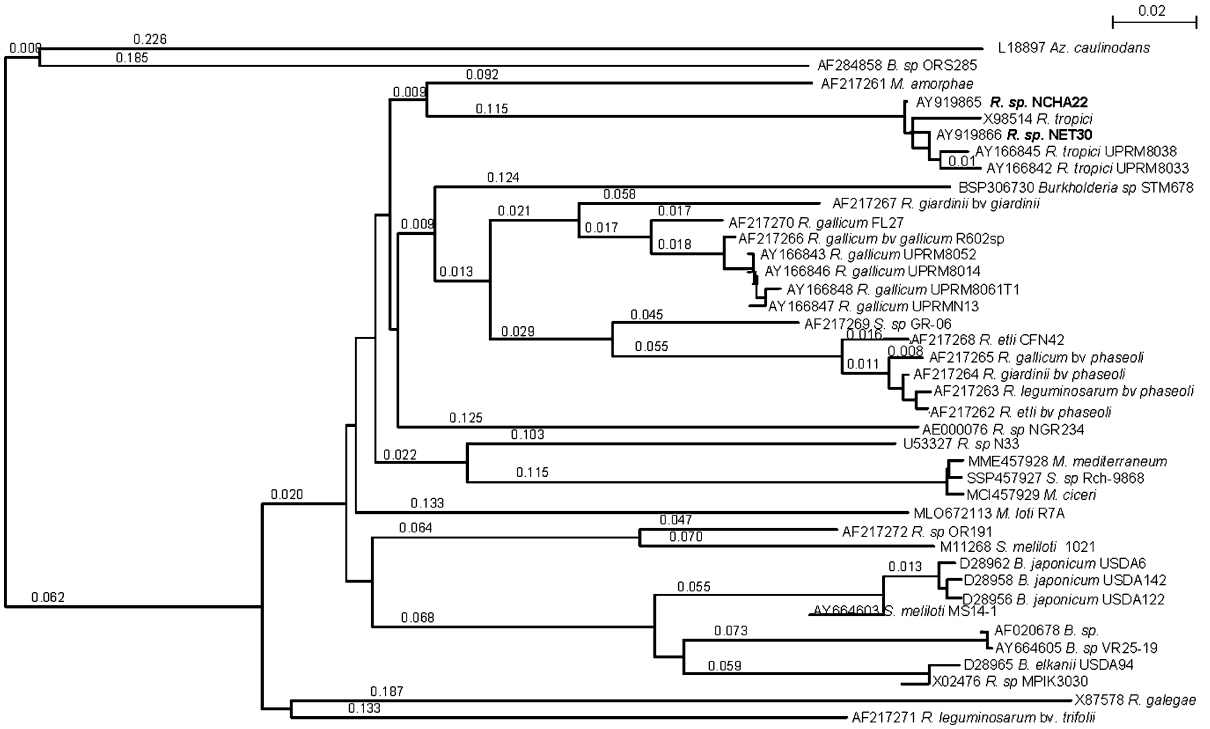

Figure 4. Phylogenetic tree based on the *nod*C sequences of fast-growing isolates NET30 and NCHA22 and that of known rhizobia species. Abbreviations: *Az., Azorhizobium; B., Bradyrhizobium; Br., Burkholderia; M., Mesorhizobium; R., Rhizobium* and *S., Sinorhizobium*. Multiple alignments and NJ trees were constructed by using CLUSTALX 1.64b software.

slow and fast growing rhizobia (Taurian et al., 2002). By contrast, *Phaseolus vulgaris*, a host considered to be promiscuous, enter in symbiotic association only with species of genus *Rhizobium* (Aguilar et al., 1998; Amarger, 2001; Jordan, 1984; Martínez-Romero et al., 1991).

The diversity of nodulating peanut rhizobia detected in this study was much greater than the reported for other countries (Urtz and Elkan, 1996). As it was reported (Santamaria et al., 1997) the diversity of natural populations of rhizobia deserves more attention than received until now because they could be a source of valuables genotypes to improve strains of agricultural importance. Our results are emphasizing the importance of analyze the soil area where the legume is believed to have originated, in rhizobial biodiversity studies.

It is generally accepted that peanut was domesticated in the lowlands of Bolivia and in Paraguay (Krapovickas, 1969), and northern Argentina (Kochert et al., 1996). From this region the domesticated peanut spread throughout the Andean zone and into other areas such as that of province of Córdoba. It is possible that during this process of adaptation to new environments, the interaction between peanut and naturally existing rhizobia have developed novel lineages and some specificity in the region. Several reports suggest that the host plant is the main factor which determines structure of rhizobial populations (Bromfield et al., 1995; Hirsch, 1996; Wernegreen et al., 1997).

Our result invites to the speculation that some of the isolates found in the peanut centre of cropping in Córdoba, may have evolved from more than one *Rhizobium* background which gained the ability to nodulate peanut by lateral gene transfer.

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