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Genetic diversity of *Rhizobium* present in nodules of *Phaseolus vulgaris* L. cultivated in two soils of the central region in Chile

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ABSTRACT

Although *Phaseolus vulgaris* L. is native from the Americas and is currently cultured in diverse areas, very little is known about the diversity of symbiotic nitrogen fixing *Rhizobium* (mycrosymbiont) in many of those cultures. Therefore, the aim of this study was to assess the genetic diversity of *Rhizobium* present in nodules of *P. vulgaris* in the central region of Chile. A method to extract DNA from surface-sterilized nodules was applied to two populations of the same seed variety grown in different fields. The 16S rRNA and *nifH* genes were amplified directly from the DNA extracted. DGGE analysis and clone libraries showed a restricted genetic diversity of the microsymbiotic populations that nodulate *P. vulgaris*. Both molecular markers revealed the presence of a microsymbiont closely related to *Rhizobium etli* in all the plants from the soils studied, indicating that the populations of *Rhizobium* sp. nodulating *P. vulgaris* in the central region of Chile displayed an extremely low genetic diversity. The level of genetic diversity in microsymbiont populations in plants grown in soils with different origin suggested that other factors rather than the indigenous soil rhizobial populations play a major role in the selection of the symbiotic partner in *P. vulgaris*.

1. Introduction

Phaseolus vulgaris L. (common bean) is the most important grain legume all over the world (Beebe et al., 2001). This plant, as well as other leguminous plants, benefits from a symbiotic interaction with rhizobial bacteria for fixation of atmospheric nitrogen as a direct source of this element. The symbiosis between legumes and rhizobia has been widely used to improve agricultural productivity. This is particularly relevant in developing countries where agriculture is prone to nitrogen losses and legumes can represent an alternative source of protein for human and animal consume (Peoples and Herridge, 1990; Kaneko et al., 2002).

Prior to the discovery of a wide diversity of non-rhizobial bacterial strains able to nodulate legumes including, for example, representatives of the genera *Methylobacterium* (Sy et al., 2001; Jourand et al., 2004), *Ochrobactrum* (Trujillo et al., 2005), *Devosia* (Rivas et al., 2002, 2003), *Ralstonia* (Chen et al., 2001, 2003) or *Burkholderia* (Moulin et al., 2001), nodulation of legume plants was believed to be restricted only to rhizobial species. Rhizobia are free-living soil bacteria belonging to the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Ensifer* (formerly classified as *Sinorhizobium*), and *Shinella*. These bacteria are characterized by their common ability to elicit the formation of nodules in their leguminous partners (van Rhijn and Vanderleyden, 1999; Gaunt et al., 2001; van Berkumm et al., 2003; Lin et al., 2008). The classification of rhizobial strains nodulating common bean (as well as the classification of rhizobia as a whole) is continuously under revision. *P. vulgaris* is a promiscuous legume that forms nodules with a variety of rhizobial partners (Michiels et al., 1998). Many different *Rhizobium* species have been recognized worldwide as symbiotic partners of common bean, including *Rhizobium etli*, *Rhizobium phaseoli* (previously considered as a biovar of *Rhizobium leguminosarum* bv *phaseoli*; (Ramirez-Bahena et al., 2008), *Rhizobium tropici*, *Rhizobium gallicum*, *Rhizobium leucaenae*, *Rhizobium lusitanum*, *Rhizobium pisi*, *Rhizobium freirei* and *Rhizobium giardinii* (Van Berkum et al., 1996; Herrera-Cervera et al., 1999; Silva et al., 2003; Dall'Agnol et al., 2013). *R. etli* is the predominant microsymbiont in countries where common bean is native such as Mexico, Colombia or the southern Andes (Souza et al., 1994; Aguilar et al., 2001). The same species also displays high prevalence in areas

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where beans have been introduced such as Spain, France, Gambia, Senegal and Tunisia (Souza et al., 1994; Diouf et al., 2000; Silva et al., 2003). A survey of the genetic diversity of *R. etli* nodulating wild and cultured *P. vulgaris* has suggested the co-evolution of these specific rhizobial partner in the centers of host diversification after geographical separation (Aguilar et al., 2004). However it is important to consider that a recent revision of the taxonomy of *R. etli* revealed that several strains from this species should be re-classified as *R. phaseoli* (Lopez-Guerrero et al., 2012). The emerging image of this and other studies (Aserse et al., 2012) is that both species are the result of a relatively recent speciation process, as seen by the high similarity of their 16S rRNA genes and the relatedness of some core genes in their genomes (Lopez-Guerrero et al., 2012).

It is generally accepted that *P. vulgaris* is native to the Americas. The analysis of natural bean populations has led to the proposal of two major centers of diversification: the Mesoamerican center (Mexico, Central America, and Colombia) and the Andean center (Ecuador, Peru, and Bolivia) (Beebe et al., 2001). Bean genotypes from Chile have been included into the Andean center forming a specific race (Race Chile), which has showed evidences of genetic introgression (Beebe et al., 2001).

The genetic diversity of rhizobia-nodulating beans in Chile is for the most part unknown. In a previous study, when the changes in diazotrophic rhizobial communities in soil were studied overtime, we have found that *P. vulgaris* specifically stimulates the populations of its microsymbiont in planted soil compared to unplanted controls. In this case, *R. etli* was found in the soil and nodules analyzed (Junier et al., 2009). The aim of the present study was to establish a more extensive survey of the genetic diversity of rhizobia nodulating *P. vulgaris* in the central region of Chile. For this bean plants from an experimental field and those from the same plant genotype grown in pots at the laboratory were compared. The genetic diversity of the rhizobial populations was assessed using DNA directly extracted from the nodules in a culture-independent approach. Although in a culture-independent approach the infectivity of the strains cannot be validated, now is largely acknowledge that cultivation studies can be biased. For example, in the case of soil, it does not bring information concerning the original distribution of bacteria in undisturbed samples (Bromfield et al., 1995) and it is unpractical in studies involving large amounts of samples (Santamaria et al., 1998). Therefore, in this study the genetic diversity of rhizobial nodulating populations was evaluated in a culture-independent manner using the 16S rRNA gene and the reductase nitrogenase gene (*nifH*). The first marker, 16S rRNA gene, was used to get insights on the phylogenetic affiliation of the nodulating strains. The second marker, *nifH* gene is one of the functional molecular markers traditionally used to characterize microbial communities of microorganisms able to fix atmospheric nitrogen (Zehr et al., 2003).

2. Material and methods

2.1. Plant sampling and soil characterization

Sixty individual nodules and 20 mixtures of nodules (5 combined nodules) were collected from 10 plants grown in a field traditionally used to culture beans at the La Platina Experimental Station of the Instituto de Investigaciones Agropecuarias (INIA), Chile. Nodule mixtures were included in order to increase to number of nodules analyzed (160 nodules), as well as the possibility for detecting variability between different plant individuals. Plants in the field were organized in rows with 1 m separation between each plant. The plants sampled were chosen randomly. Additional nodules were collected from plants bearing fruits (at 60 days) cultured at the laboratory. At this state, nitrogen fixing activity for the nodules has been verified in a previous study (Junier et al., 2009). Twenty-three individual nodules and two mixtures of 10 individual nodules were collected from 5 plants growing in the laboratory (43 nodules). The plants grown at the laboratory were cultured in individual pots with soil that was previously used in cropping of the legume *Medicago sativa*. Nodules from the plants grown at the laboratory were compared to the results previously obtained for the same soil (Junier et al., 2009). In total 203 nodules from 15 plants were analyzed (105 samples). The rationale behind selecting these two populations, although differing in the soil type that was used for culturing, was to maintain a unique plant genotype as the seeds used in each case corresponded to the same batch of seeds provided by the INIA.

The main chemical characteristics of the field soil were as follows: pH 8.0; NH_4^+ and NO_3^- content, 15.0 mg kg^{-1}; organic matter, 2.5%; phosphorus, 60.0 mg kg^{-1}; potassium, 120.0 mg kg^{-1}. The main chemical characteristics of the soil used in the laboratory were: pH 7.9; NH_4^+ and NO_3^- content, 7.0 mg kg^{-1}; organic matter, 4.5%; phosphorus, 47.0 mg kg^{-1}; potassium, 161.0 mg kg^{-1}.

2.2. Processing of nodules and DNA extraction

Once collected the nodules were kept at −20 °C until processing. For cleaning the external surface of the nodules each individual nodule or nodule mixtures were washed successively 30 times with sterile distilled water. The absence of free DNA on the nodule's surface was confirmed by assaying water samples from the last wash in a PCR with universal primers for the 16S rRNA gene. This PCR never rendered a positive amplification (data not shown). The following protocol was designed for extracting DNA directly from the nodules. Each nodule was macerated in 200 μl of sterile TES buffer (Tris–HCl 0.2 M; EDTA 5 mM; NaCl 100 mM; pH 8.0). Lysozyme (50 mM) and RNAse (25 mM) were added and then incubated for 30 min at 37 °C. Next, 200 μl of TES buffer were added together with Proteinase K (25 mM) and SDS (1%). Samples were incubated for 30 min at 37 °C. One volume of chloroform:isoamyl alcohol (24:1) was added, the samples were vortexed and incubated on ice for 30 min. Cell debris was removed by centrifugation at 10,000 × *g* for 20 min. DNA was recovered from the supernatant by precipitation overnight with 0.1 vol. of 3 M sodium acetate and 2 vol. of absolute ethanol. DNA was pelleted by centrifugation at 10,000 × *g* for 15 min, rinsed with 500 μl of 70% ethanol, and resuspended in 50 μl TE (Tris–HCl 10 mM; EDTA 1 mM; pH 7.4). Concentration and quality of the DNA was checked by electrophoresis in agarose gels and ethidium bromide staining.

2.3. PCR of 16S rRNA and nifH

The 16S rRNA was amplified using the bacterial primers Eub9_27 and Eub1542 (Brosius et al., 1978). PCR was set up in a total volume of 25 μl containing 1 to 2.5 ng DNA as template, 5 pmol of each primer, 1 × PCR buffer (10 mM Tris–HCl; 15 mM $MgCl_2$; 50 mM KCl, pH 8.3), 1% BSA, 1% formamide, 10 nmol of each dNTP and 1 U of *Taq* polymerase (Roche). The amplification consisted of: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 40 °C for 45 s and extension at 72 °C for 90 s. These PCR products were cleaned, diluted 100 times in HPLC water and used as templates in a second nested PCR with the primers P3 (GC-clamped) and P2 (Muyzer et al., 1993) for DGGE. For preparing PCR products for DGGE a touchdown temperature program was used (Muyzer et al., 1993). The *nifH* was amplified with the primers nifHF and nifHR (Rösch et al., 2002). PCR was set up in the same conditions used for the Eub primers above mentioned. The amplification consisted of: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 1 min, with a final extension at

72 °C for 7 min. A nested PCR using the previous products purified and diluted 100 times in HPLC water was used to obtain products suitable for DGGE. PCR was carried out using the reverse primer PolRR (5′-AGC GCC ATC ATC TCR CCG GA-3′) and the forward primer nifHF305gc (5′-GCG GGC GTC ATC ACC TCG ATC-3′) attached to the GC-clamped (Muyzer et al., 1993). Amplification of PCR products with the GC-clamp was carried out as mentioned for the 16S rRNA.

2.4. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed according to the manufacturer's guidelines with the DCode System (BioRad) in 7.5% polyacrylamide gels with a gradient of 30 to 60% of denaturants (100% denaturants contained 420 g l^{-1} urea and 400 ml l^{-1} deionized formamide in 0.5× TAE) during 6 h at 200 V and 60 °C. Gels were silver stained (Sanguinetti et al., 1994) and scanned using a HP scanjet 5470c.

2.5. Cloning and sequencing

16S rRNA gene PCR products from the nodules N131 (laboratory plant, ribotype 1) and P2-N5 (field plant, ribotype 2) and *nifH* products from the nodules N213 (laboratory plant with genotype 1) and N133 (laboratory plant with genotype 2) were selected for cloning and sequencing. PCR was carried out in a total volume of 50 μl with the proofreading *Pfu* DNA polymerase (Promega) according to the manufacturer's guidelines. PCR was carried out using an annealing temperature of 60 °C for the 16S rRNA gene and 57 °C for *nifH*. For cloning the Zero Blunt PCR cloning kit (Invitrogen) was used according to the manufacturer's guidelines. From each sample 48 clones were selected and checked for inserts of the expected size by PCR with plasmid-specific primers M13f/M13r and agarose electrophoresis. For screening of these clones, one-shot sequencing using M13f primer was performed using the BigDye terminator v3.1 cycle sequencing kit, and analyzed with an ABI 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's recommendations. For complete sequencing, both strands were sequenced with overlap using M13f and M13r and additional internal primers (Lane, 1991).

2.6. Sequence analyses

The nucleotide sequences were compared with those in the GenBank using BLASTN (Altschul et al., 1997) (http://www.ncbi.nlm.nih.gov/blast/Blast). Phylogenetic analyses were carried out with the program ARB (http://www.arb-home.de) as described elsewhere (Purkhold et al., 2003). Briefly, 16S rRNA and *nifH* sequences were integrated and aligned into the respective database available for ARB, correcting the alignments by visual inspection. For *nifH*, the nucleotide sequences were translated into protein and re-aligned to correct the nucleotide alignment. For phylogenetic analysis no filter was applied for the calculation. The phylogenetic tree was constructed using the PHYLIP subroutine in ARB by the neighbor-joining algorithm using a distance matrix calculated with the Jones–Taylor–Thornton (JTT) as substitution model.

3. Results

3.1. DGGE and sequence analysis of 16S rRNA gene

Two different ribotypes were detected in the nodule populations (Fig. 1). In the case of the plants growth at the laboratory a single ribotype (type 1) in which two clearly distinguishable bands were observed appeared in all the nodules, whereas in the population from plants in the field station, a second ribotype composed of a unique band (also present in rybotype 1) was detected, as well. The ribotype 1 from nodules of plants growth at the laboratory was dominant in both populations appearing in 73% of the all nodules analyzed (Table 1).

In order to identify the microsymbiont associated with the bean plants, the sequence of the 16S rRNA gene from one of the nodules of each population was analyzed. The sequences were closely related to the 16S rRNA gene sequence from *R. leguminosarum* and *R. etli* (99% identity in both cases). In the phylogenetic analysis the sequences from the nodules were related to the clade of sequences from bean-nodulating species of *Rhizobium*, which includes *R. etli* and other species such as *R. tropici* and *R. leguminosarum* (Fig. 2).

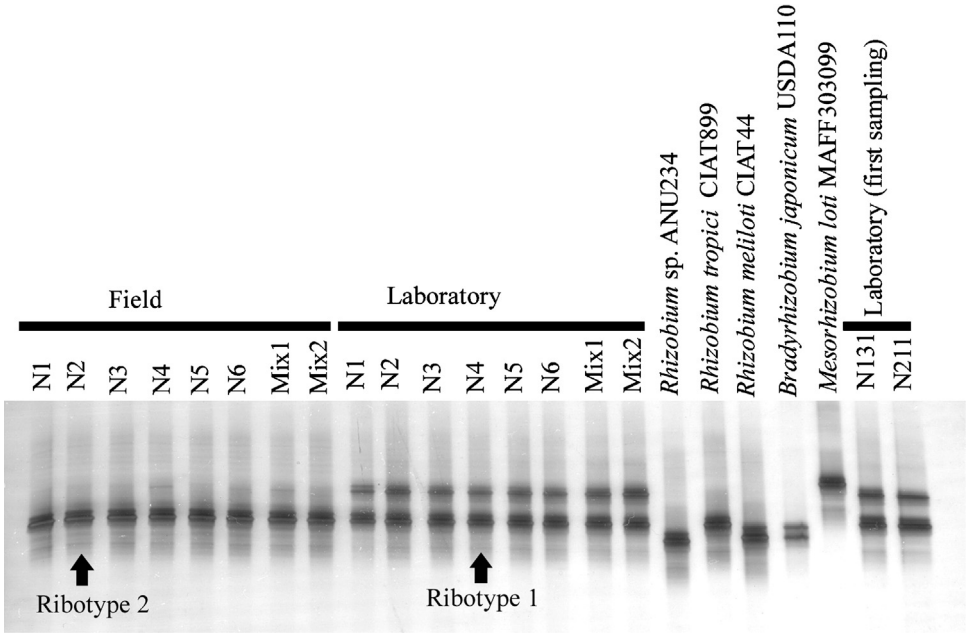

Fig. 1. Denaturing gradient gel electrophoresis of 16S rRNA gene PCR products from nodules in plants collected at the field and in the laboratory. The two ribotypes identified in the samples are indicated by arrows. 16S rRNA gene products from reference rhizobial strains as well as two previously characterized nodules are included as standards.

Table 1
Distribution and frequency of genotypes observed for the molecular markers 16S rRNA and *nifH* genes, in the nodules collected from plants grown in a field and at the laboratory.

Marker	Genotype	Field		Laboratory		Total	
		Total	Frequency	Total	Frequency	Total	Frequency
16S rRNA	1	52	65.0	25	100.0	77	73.3
	2	28	35.0	0	0.0	28	26.7
nifH	1	18	22.5	7	28.0	25	23.8
	2	62	77.5	18	72.0	80	76.2

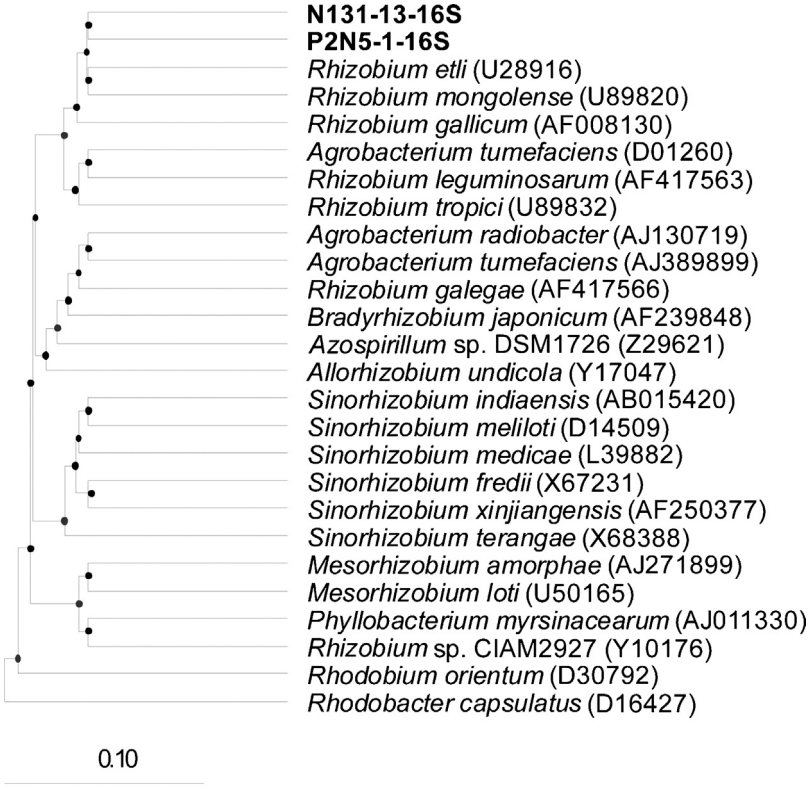

Fig. 2. Phylogenetic analysis of 16S rRNA gene sequences from the nodules and rhizobial references. The tree represents a consensus of trees obtained by maximum-likelihood and neighbor-joining methods. Sequences from this study are indicated in bold. 16S rRNA sequences representing the populations from the laboratory and the field are designed N131-13-16S and P2N5-1-16S, respectively. Bootstrap values are indicated as >90% black circles; 80–90% gray circles.

3.2. DGGE and sequence analysis of the nifH gene

In the case of the gene *nifH*, two genotypes were also detected in the nodules (Fig. 3). Differences between both genotypes consisted in the presence of an additional band located at a lower denaturant concentration that was observed in genotype 2. Both genotypes were detected in the nodules collected from plants grown at the laboratory and in the field. Similar to the results of the 16S rRNA gene, one of the genotypes was dominant in both nodule populations, with frequencies of 72 and 77.5% in plants from the laboratory and field, respectively (Table 1).

The *nifH* gene PCR products from two of the nodules from the laboratory population were also cloned and sequenced. In the BLAST search the highest identity was obtained with the nitrogenase reductase gene of different strains of *R. etli* (99% identity). The phylogenetical analysis showed the relatedness of the sequences obtained in the nodules with *nifH* sequences from *R. etli* (Fig. 4).

4. Discussion

The results obtained in this study showed a very restricted genetic diversity of *Rhizobium* present in nodules of common bean growing in two different soils in the central part of Chile. Although the microsymbiont was not isolated, nor its infectivity tested in vivo, evidence for additional rhizobial strains was not obtained from the nodules. Previous assays had shown nitrogen-fixing activity in the nodules from the populations grown at the laboratory (Junier et al., 2009), suggesting that the microsymbiotic populations identified here could be involved in nodulation and nitrogen fixation in the host plant. These soils consisted of a soil traditionally used for the culture of beans and a soil previously used to culture another leguminous species. Sequence analyses of partial *nifH* gene identified *R. etli* as the microsymbiont in both populations. In contrast, the results of the 16S rRNA gene analysis were not conclusive, since the comparison of 16S rRNA gene sequences obtained from the bean nodules showed an identity of 99% with sequences from either *R. etli* or *R. leguminosarum*. Although this appears contradictory, it can be explained by the fact that *R. etli* isolates have been shown in certain cases to contain a 16S rRNA gene sequence highly related to *R. leguminosarum* (Eardly et al., 1995). A recent analysis of rhizobial bacteria nodulating beans in Ethiopia found the opposite, in this case *R. leguminosarum* strains carried an ancestral 16S rRNA gene sequence reminiscent of *R. etli* (Beyene et al., 2004). Eardly et al. (1995) concluded that the most plausible explanation

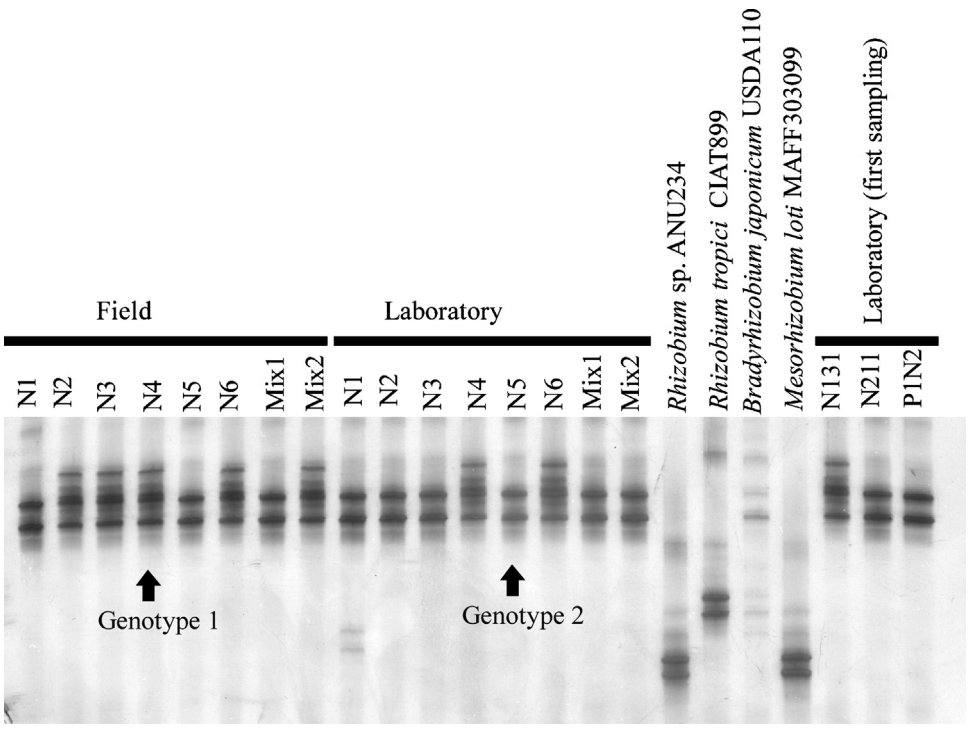

Fig. 3. DGGE of *nifH* PCR products from the same nodules presented in Fig. 1. The genotypes are indicated by arrows. *nifH* products from rhizobial strains as well as previously characterized nodules are included as standards.

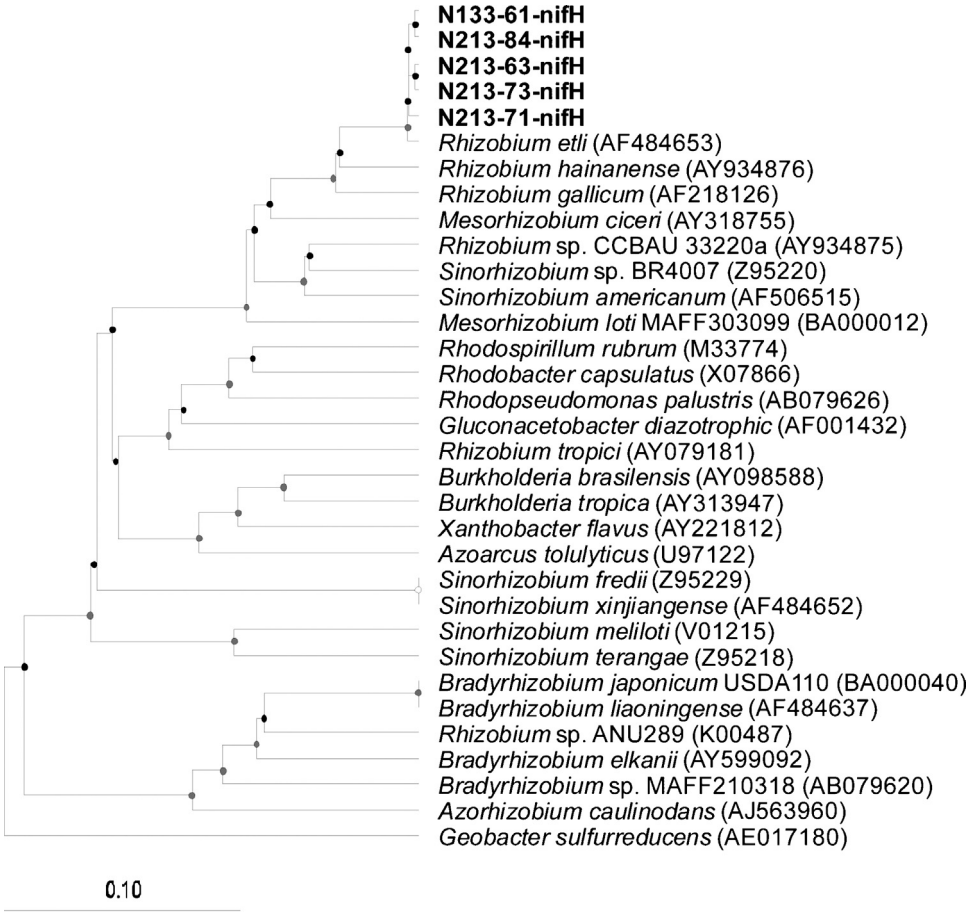

Fig. 4. Phylogenetic analysis of *nifH* sequences from the nodules and rhizobial references. The tree represents a consensus of trees obtained by different maximum-likelihood and neighbor-joining methods. Sequences from this study are indicated in bold. Bootstrap values are indicated as >90% black circles; 80–90% gray circles.

for these observations is the transfer and recombination occurring between divergent 16S rRNA alleles in rhizobia.

The dispersion of the microsymbiont associated with the seeds seems to be a likely mechanism to explain the introduction of *R. etli* in areas where common bean is not native (Perez-Ramirez et al., 1998). Evidences for the attachment and long-term viability of *R. etli* to the coat of bean seeds have been obtained before (Perez-Ramirez et al., 1998). This probably explains the worldwide distribution and dominance of this microsymbiont, as well as the occurrence of *R. etli* as the microsymbiont from all the plants analyzed here despite the differences in the initial soil used to grow the host. However, considering that recent evidence show that several strains previously thought to be *R. etli* should be re-classified as *R. phaseoli* (Lopez-Guerrero et al., 2012), the global and biogeographical distribution patterns of both species should be re-examined.

Dispersion associated to the seeds does not explain completely the success of a particular microsymbiont in the environment. Prior to competition for root infection, rhizobia-infecting bacteria should also be able to survive and grow in the soil in the absence of the host plants (Laguerre et al., 2003). In *Frankia* it was shown that the phylogenetic groups associated with plant in non-native areas probably corresponded to those that have a greater ability to survive independently of the host (Simonet et al., 1999). Therefore, a higher competence for saprophytic growth should be expected for *R. etli*, and indeed we have obtained evidence for the saprophytic growth of *R. etli* populations, which were stimulated by the presence of host plant (Junier et al., 2009).

It has been suggested that rhizobial communities in soil are generally diverse. However, the role that this diverse genetic background plays on the microsymbiont selection has not been clearly established. If the selection is mainly due to factors associated to the plant, it is expected that symbiotic communities from genetically related plants growing in different soils would be similar, whereas if the selection is determined by the initial composition of soil rhizobial communities, the symbiotic populations from plants grown in different soils must differ. Evidence supporting both scenarios has been recompiled. In some cases, the dominance of a particular rhizobial genotype in nodules results from a higher abundance in soil (Bromfield et al., 1995; Hartmann et al., 1998; Velazquez et al., 1999) whereas in other cases, the abundance of different genotypes in soil did not coincide with their frequency in the microsymbiont population (Leung et al., 1994).

Our comparison of microsymbiontic populations from two soils that have harbored different species of leguminous plants in the past suggested that the plant plays a more relevant role in the selection of the microsymbiont than rhizobial free-living populations in soil. Analysis of bean-nodulating rhizobia in the northern part of Argentina have come to similar conclusions by showing that despite the diversity of potentially-nodulating species in the soil, rhizobial populations in common bean nodules were dominated by *R. etli* (Aguilar et al., 2001). Similar results have been obtained in other models of rhizobial bacteria. Studies in *R. meliloti* isolated from soil and nodules of two host plants (*M. sativa* and *Melilotus alba*), have also shown that nodule occupancy is not representative of soil distribution (Bromfield et al., 1995).

The results found here and in other studies indicated that although *P. vulgaris* as a whole is a very promiscuous species that forms nodules with a large variety of rhizobial partners (Michiels et al., 1998), even beyond the genus *Rhizobium* (e.g. *Ensifer* sp. (Chamber and Iruthayathas, 1988), the plants we studied here were highly restrictive for the microsymbiont selection. Previous studies of genetic diversity in other plant-bacteria nitrogen-fixing symbiotic models such as the cyanobacteria *Nostoc* in interaction with the angiosperm *Gunnera*, showed a reduced genetic variability of the cyanobacterial populations colonizing a single host plant (Guevara et al., 2000). In *Frankia*, the microsymbiont populations have shown to be dominated by a particular genotype (Simonet et al., 1999; Chavez, 2003). Therefore, the dominance of few strains might be a common phenomenon in plant-bacteria symbiosis interactions with diazotrophic microorganisms.

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