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ORIGINAL PAPER

Genetic diversity of rhizobia in a Brazilian oxisol nodulating Mesoamerican and Andean genotypes of common bean (*Phaseolus vulgaris* L.)

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Abstract Brazil is the largest producer and consumer of common bean worldwide, and the crop can benefit from its symbiosis with a variety of rhizobia by means of biological nitrogen fixation in root nodules. In this study, the role of Mesoamerican and Andean genotypes of common bean in trapping rhizobia directly from a Brazilian oxisol in the field or in pots in greenhouse conditions with unaltered or diluted soil solutions was investigated. Genetic diversity was evaluated by the profiles of BOX-PCR obtained, and by estimates of Shannon and Abundance-based Coverage Estimator (ACE) indices. Rhizobia trapped by Mesoamerican genotypes had greater diversity, reinforcing the hypothesis of an important and long-time contribution of this genetic center to the establishment of common bean in Brazil. Greater diversity was also seen in rhizobia trapped straight from the soil than from plants inoculated with diluted soil solutions, emphasizing a highly diverse and competitive rhizobial indigenous population. Studies on genetic diversity of common bean rhizobia are important not only for helping to understand the evolution of the legume-rhizobia symbiosis, but also to devise strategies to increase the contribution of the biological nitrogen-fixation process.

Keywords Biological nitrogen fixation · Brazil · Common bean · Genetic diversity · *Phaseolus vulgaris* · *Rhizobium*

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Introduction

Although Brazil is the largest producer (17.6%) and consumer of common bean (*Phaseolus vulgaris* L.) worldwide, persistent low yields are recorded, on average of only 850 kg ha^{-1}, attributed mainly to lack of inputs by poverty-affected farmers (CONAB 2010). However, farmers with access to modern technology and able to apply the most commonly limiting nutrients—nitrogen and phosphorus—can achieve grain yields as high as 4,000 kg ha^{-1} (e.g. Hungria et al. 2000, 2003; Vargas et al. 2000). Improving inputs of nitrogen via the biological process, i.e. by means of the legume's symbiosis with diazotrophic bacteria, commonly named as rhizobia, implies significant benefits at low cost to farmers, not only in Brazil, but also in other countries in South and Central America and in Africa, where common bean represents the most important source of protein for many rural populations.

Mesoamerica—including Colombia, Ecuador and north of Peru—and the Andean Region—from southern Peru to the north of Argentina—have been considered centers of domestication/diversity of common bean, and since wild relatives of common bean are not indigenous to Brazil, genotypes from both centers of origin have been cultivated there throughout recorded history (Gepts 1990; Kami et al. 1995; Freitas 2006). Common bean is promiscuous in its symbiotic relationships, being capable of nodulating with a

variety of rhizobial species (Jordan 1984; Martínez-Romero et al. 1991; Segovia et al. 1993; Amarger et al. 1997; Valverde et al. 2006), including several putative new species (e.g. Hungria et al. 1993; Herrera-Cervera et al. 1999; Grange and Hungria 2004). In both centers of domestication/diversity, *Rhizobium etli* has been shown to be the dominant species (Segovia et al. 1993; Souza et al. 1994; Bernal and Graham 2001; Martínez-Romero 2003; Aguilar et al. 2004).

Despite its socio-economic importance, few surveys have been carried out on diversity of common-bean rhizobia in Brazilian soils. *Rhizobium tropici* has been found to be the dominant species under field conditions, attributable to its tolerance of prevailing edaphic conditions, including high temperatures, acidity, and aluminum toxicity (Hungria and Vargas 2000; Hungria et al. 2000, 2003; Pinto et al. 2007). In contrast, when diluted soil solutions are used as inocula for plants grown under controlled conditions, *R. etli* and *R. leguminosarum* are the predominant isolates (Soares et al. 2006; Giongo et al. 2007; Grange et al. 2007; Stocco et al. 2008).

Common-bean varieties available commercially in Brazil are derived both from Mesoamerican and Andean germplasms; however, differences in the diversity of rhizobia trapped by these genotypes have not been investigated. In this study the rhizobial diversity—by means of BOX-PCR profiles—in a Brazilian oxisol, with rhizobia trapped by various common-bean genotypes grown under field conditions or receiving diluted soil solutions as inocula was investigated.

Materials and methods

Study area and experiments conduction

The field experiment was performed at the Experimental Station of the "Genetics and Breeding Enterprise Semilla", in an oxisol located in Campo Largo, Paraná, Southern Brazil (25°37′S and 49°52′W; altitude of 956 m; climate Cfb in the Koeppen's classification). The main chemical characteristics of the soil were: pH in $CaCl_2$, 4.7; C (g dm^{-3}) 27.5; P, Ca, Mg and K ($cmol_c$ dm^{-3}), 20.7, 4.6, 2.6 and 0.43, respectively. The population of common bean rhizobia was evaluated by the plant infection method using the most probable number (MPN) counting technique (Vincent 1970), and estimated at 10^2 cells g^{-1} soil.

The field experiment considered the diversity of rhizobia isolated from nodules collected from four common bean cultivars, two from each center of diversity, as follows: Mesoamerica—Diamante Negro and Ouro Negro— and Andean—Jalo Precoce and Goiano Precoce. Before sowing, seeds were surface sterilized (Vincent 1970) and sown in four lines of 4.0 m for each cultivar, spaced by 20 cm, resulting in plant density of 20 plants $line^{-1}$. Forty days after germination plants were harvested and nodules were collected from ten central plants per treatment. Nodulation varied with the cultivar, such that one-hundred nodules were collected from Ouro Negro and Goiano Precoce, and fifty from Diamante Negro and Jalo Precoce.

The greenhouse experiment considered the diversity of rhizobia isolated from nodules collected from the same cultivars used in the field experiment. First, twenty randomly soil subsamples (from the 0–10 cm soil layer) representative of the same oxisol were taken and joined to compose the soil sample. The experiment was performed in 500-ml pots and consisted of three treatments: (1) pots filled with soil taken directly from the field; (2) pots filled with sand:vermiculite (1/2, v/v), sterilized and receiving as inoculant diluted soil solutions at the 10^{-1} concentration; (3) the same as (2), but receiving a 10^{-2} dilution as inoculant. Each treatment had ten replicates. Surface-sterilized seeds (Vincent 1970) were previously germinated for 4 days at 25°C, in the dark, and seedlings were transferred to the pots (1 plant per pot). Diluted soil solutions were prepared with 0.85% NaCl (Vincent 1970) and the 10^{-1} and the 10^{-2} dilutions were used as inoculants for the seedlings (1 ml per seedling). Plants received sterilized N-free nutrient solution and were grown under controlled greenhouse conditions, with mean temperatures of 25°C/21°C (day/night). Four weeks after sowing plants were collected and fifty nodules were randomly chosen per cultivar.

Rhizobia isolation and morpho-physiological characterization of the isolates

Bacteria were isolated from the nodules using standard procedures (Vincent 1970). Purity of the cultures was confirmed by repeatedly streaking the bacteria on yeast extract-mannitol agar (YMA) medium (Vincent 1970) and verifying a single type of colony morphology, absorption of Congo red (0.00125 mg kg^{-1}) and uniform Gram-stain reaction. Colony morphology (color, mucosity, transparency, borders and elevation) and acid/alkaline reaction were evaluated on YMA containing bromothymol blue (0.00125 mg kg^{-1}) as indicator, after incubation of bacteria in the dark at 28°C.

Rhizobia reference strains

The following strains were included in this study: *R. tropici* strain type A CFN 299 and type B CIAT 899^T (=USDA 9030; =ATCC 49672; =UMR1899; =TAL 1797; =HAMBI 1163; =CM01; =SEMIA 4077; =DSM 11418; =BR 322), and *R. etli* bv. phaseoli strain CFN 42^T (=USDA 9032; =ATCC 51251; =DSM 11541) were supplied by Dr. Esperanza Martínez-Romero (Centro de Ciencias Genómicas, Cuernavaca,

México). *R. leguminosarum* bv. phaseoli USDA 2671 (=RCR 3644) was provided by Dr. Peter van Berkum (USDA, Beltsville, Maryland, USA). *R. giardinii* bv. giardinii strain H152^T (=USDA 2914) and *R. gallicum* bv. gallicum strain R602sp^T (=USDA 2918) were provided by Dr. Noelle Amarger (INRA, Dijon, France). *R. tropici* PRF 81 (=SEMIA 4080) is from the culture collection of Embrapa Soja. Except when specified, all strains from this study were grown on yeast extract-mannitol agar (YMA) medium (Vincent 1970), in the dark, at 28°C. Stocks were prepared on YMA and kept at −70°C (under 30% of glycerol) for long-term storage and at 4°C as source cultures.

Genetic characterization

Total genomic DNA of each strain was extracted and amplified by PCR with the primer BOX A1R (5′-CTACG GCAAGGCGACGCTGACG-3′, Invitrogen^{TM}) (Versalovic et al. 1994), as described before (Kaschuk et al. 2006). The amplified fragments were separated by horizontal electrophoresis on 1.5% agarose, as described before (Kaschuk et al. 2006), with the 1-kb DNA marker (Invitrogen^{TM}) being included on the left, right and in the centre of each gel for normalization of the bands. Gels were stained with ethidium bromide, visualized under UV radiation and photographed.

Cluster analysis and genetic diversity indices

First, the sizes of the fragments were normalized according to the sizes of the DNA marker. Cluster analysis of the BOX-PCR profiles was performed using the Bionumerics program (Applied Mathematics, Kortrijh, Belgium, version 4.6), with the UPGMA algorithm (Unweighted Pair-Group Method, with Arithmetic mean) (Sneath and Sokal 1973) and the Jaccard coefficient (Jaccard 1912), with a tolerance of 3% established in the Bionumerics program.

Abundances of BOX-PCR profiles were analyzed using the SPADE (Species Prediction And Diversity Estimation; Chao and Shen 2003–2005) program. Diversity was estimated by the traditional Shannon index and richness was estimated by ACE (Abundance-based Coverage Estimator, Chao and Shen 2003), a nonparametric estimator based on the separation of observed species into rare or abundant groups with only the rare groups used to estimate the number of missing species. More details about the indices utilized are given elsewhere (Loureiro et al. 2007). BOX-PCR profiles were considered similar when showing a level of similarity equal or higher than 70%, as defined before (Grange and Hungria 2004; Alberton et al. 2006; Kaschuk et al. 2006; Loureiro et al. 2007). Indices were estimated for the comparison of cultivars derived from Mesoamerican and Andean genotypes and for different nodule collection procedures.

Results

Morpho-physiological characterization

All isolates obtained from the field and greenhouse experiments were characterized by fast growth, acid reaction and typical morphology of rhizobia after 2–5 days of growth on YMA medium (Vincent 1970). The majority of the rhizobial colonies were white in color, opaque, circular, convex, creamy, with smooth borders and with moderate production of exopolysaccharides (data not shown).

Rhizobia trapped by field-grown Mesoamerican and Andean genotypes

As described in the "Materials and Methods" section, in the field experiment one-hundred nodules were removed from the roots of cultivars Diamante Negro and Jalo Precoce and fifty from Ouro Negro and Goiano Precoce; after isolation, purification and confirmation of typical rhizobial properties, the number of isolates with successful amplification obtained is shown in Table 1. Considering the level of similarity of 70%, a high number of different BOX-PCR profiles was obtained for each cultivar; in addition, the strains from each group were clustered at very low final levels of similarity, ranging from only 7.29 to a maximum of 12.05% (Table 1).

Also noteworthy was the observation that, in addition to the high diversity within each group of rhizobia, the strains collected by each cultivar were very different. This occurred even in genotypes derived from the same center of origin, as shown in Fig. 1 for the Mesoamerican cultivars Diamante Negro and Ouro Negro, and in Fig. 2 for the Andean cultivars Jalo Precoce and Goiano Precoce. Furthermore, there was no consistent grouping of strains for any cultivar (Figs. 1, 2).

Genetic diversity indices were estimated for the two groups of cultivars grown under field conditions, and when considering the traditional Shannon index, no statistically significant differences were detected between the Mesoamerican and Andean groups (Table 1). However, when richness was estimated (ACE), the Mesoamerican cultivars trapped rhizobia with significantly higher richness than did the Andean genotypes (Table 1).

Genetic diversity of rhizobia trapped by plants grown under greenhouse conditions, in pots filled with soil or inoculated with diluted soil solutions

The greenhouse experiment consisted of the comparison of Mesoamerican and Andean genotypes growing in pots filled with soil, or filled with sand/vermiculite and inoculated with soil at two dilutions (10^{-1} and 10^{-2}). With both types of genotypes, genetic diversity estimated by the traditional

Table 1 Number of rhizobial isolates obtained from field-grown plants and used in the BOX-PCR analysis. DNA profiles were considered different when showing similarity lower than 70% and genetic diversity indices were based on the number of BOX-PCR profiles obtained

Cultivar	Origin	No. isolates	BOX-PCR analysis			Genetic diversity indices		
			No. isolates amplified	No. different profiles	Final level of similarity (%)	Traditional Shannon index	Richness (ACE)	Estimated coverage
Diamante Negro	Mesoamerican	93	45	25	10.22	3.658 ± 0.078	121.3 ± 21.1	0.582
Ouro Negro	Mesoamerican	36	27	18	7.29			
Jalo Precoce	Andean	71	30	21	12.05	3.660 ± 0.055	90.5 ± 19.6	0.464
Goiano Precoce	Andean	46	19	16	9.53			

Shannon index was higher for rhizobia trapped by plants growing in pots filled with unaltered soil than for those inoculated with diluted soil solutions (Table 2). In addition, the Shannon index was higher for the Mesoamerican than for the Andean genotypes (Table 2). Finally, in the cluster analyses using UPGMA and the Jaccard coefficient, the final level of similarity in each group was again very low, ranging from 5.65% to a maximum of 27.99 (Table 2).

Discussion

The promiscuity of common bean, i.e. its ability to symbiose with a broad range of rhizobia, has been broadly reported (e.g. Michiels et al. 1998; Martínez-Romero 2003), and although previous studies reported large diversity of rhizobia trapped by this legume in Brazil (e.g. Straliotto et al. 1999; Mostasso et al. 2002; Grange and Hungria 2004; Alberton et al. 2006; Grange et al. 2007; Kaschuk et al. 2006; Pinto et al. 2007; Stocco et al. 2008), the diversity detected in this study is the greatest reported so far. The biochemical basis of this promiscuity is poorly understood, but it may be associated with the variety of flavonoid *nod*-gene inducers released by the legume (Hungria et al. 1991a, b; Bolanõs-Vásquez and Werner 1997), or with the variety of Nod factors released by the rhizobial microsymbionts (Morón et al. 2005; Estévez et al. 2009).

The diversity of common bean rhizobia may be affected by abiotic factors as soil acidity (e.g. Anyango et al. 1995; Andrade et al. 2002) and temperature (Raposeiras et al. 2002), by the trapping host species (e.g. *Leucaena* X common bean, Mercante et al. 1998; Straliotto et al. 1999), and the sampling method (e.g. Alberton et al. 2006), among others. The results from this study indicate that the diversity is dependent also on the origin of the common bean cultivar used as trapping host; the Mesoamerican genotypes captured a higher diversity than did the Andean-derived genotypes. Indications for that come from the estimates of richness (ACE) in the field experiment (Table 1) and from the traditional Shannon index in the pot experiment (Table 2). Noteworthy was also the surprising degree of diversity of isolates captured by each cultivar, such that a variety of different BOX-PCR profiles were observed for each genotype. Even within the same group of Mesoamerican and Andean cultivars, most isolates were unique (Figs. 1, 2), consistent with common bean's promiscuous nature.

Information about the structure of the indigenous rhizobial population and the co-evolution with the host plant is important for a variety of reasons, ranging from the basic need to understand the evolution of nitrogen-fixing symbioses to practical issues of predicting responses of crops to inoculation. In this context, the results from this study showing higher diversity of rhizobia trapped by Mesoamerican genotypes also help to shed some light on Brazil's still poorly documented cropping history. The Portuguese officially arrived in Brazil in 1500, finding a high indigenous population that was abruptly reduced, compromising knowledge of the past. Written history began, but was recorded from the European point-of-view, such that it has been suggested that the agricultural resources of indigenous populations today poorly reflect pre-colonial patterns (Prous 1997; FUNAI 2010). Therefore, the idea that common bean in Brazil is predominantly of Andean origin may not be correct, and a recent analysis of seeds from an archaeological site verified that the legume had a stronger influence from the Mesoamerican than from the Andean center (Freitas 2006). The Mesoamerican contribution was also discussed in terms of *R. etli* populations in two Brazilian states, showing high similarity with Mexican rhizobia (Grange et al. 2007). Therefore, the results from this study certainly indicate high promiscuity of common bean in its symbiotic relationship, but might

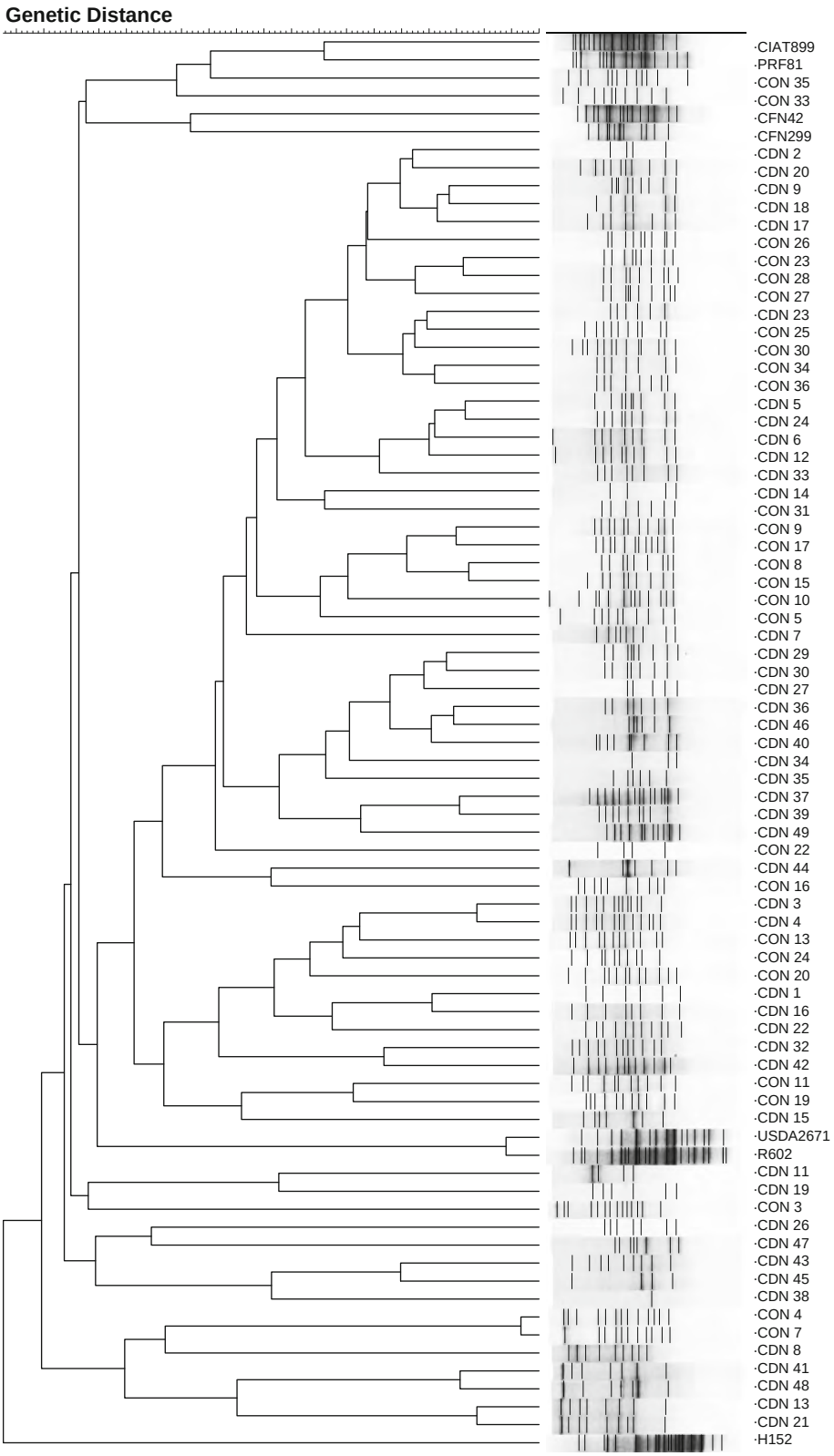

Fig. 1 Dendrogram, based on the cluster analysis of BOX-PCR products using the UPGMA algorithm and the Jaccard coefficient, of common bean rhizobial strains trapped under field conditions by Mesoamerican genotypes Diamante (DN) and Ouro Negro (ON). Reference strains included in the dendrogram are *R. leguminosarum* USDA 2671, *R. tropici* CFN 299, CIAT 899^{T} and PRF81, *R. etli* CFN 42^{T}, *R. giardinii* H152^{T} and *R. gallicum* R602^{T}

also point out that a long-time exchange of seeds (carrying rhizobia, as discussed by Pérez-Ramírez et al. 1998) and agronomic practices between Brazilian and Mesoamerican Indians took place.

Genetic diversity was also higher when trapped straight from the soil, confirming results in another Brazilian ecosystem (Alberton et al. 2006) and highlighting the high competitiveness of several indigenous strains. This result

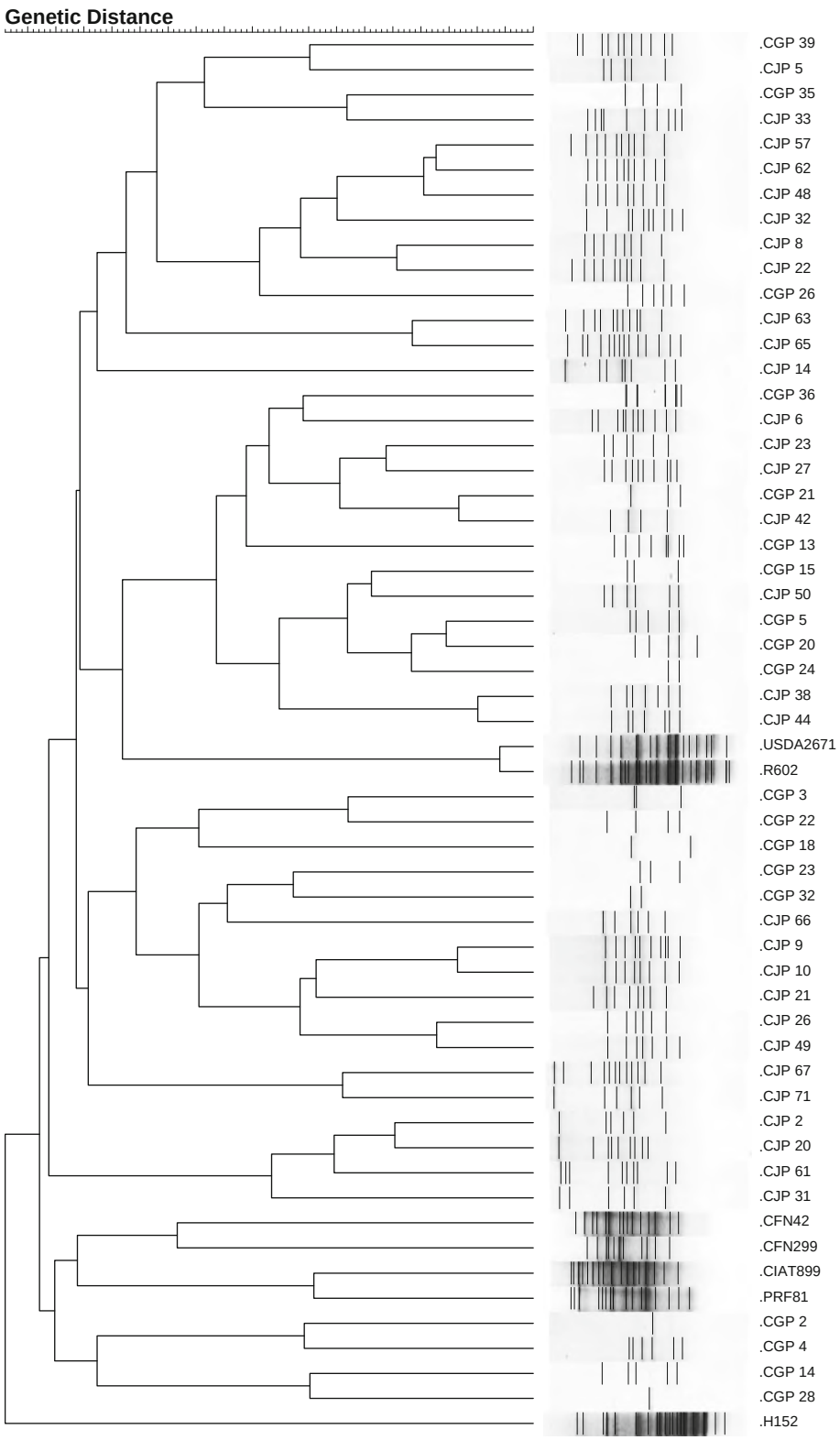

Fig. 2 Dendrogram, based on the cluster analysis of BOX-PCR products using the UPGMA algorithm and the Jaccard coefficient, of common bean rhizobial strains trapped under field conditions by Andean genotypes cultivars Jalo Precoce (JP) and Goiano Precoce (GP). Reference strains are described in the legend of Fig. 1

may be connected with the general belief that poor nodulation, low nitrogen-fixation rates and lack of responses to inoculation is attributable to inefficiency of competitive indigenous rhizobia (e.g., Graham 1981; Hardarson 1993; Michiels et al. 1998). However, most important is that within this high diversity it is possible to identify individuals with high capacity of fixing nitrogen that can be selected for use in commercial inoculants, potentially

Table 2 Genetic diversity indices obtained when considering BOX-PCR profiles of rhizobial isolates trapped from field-grown plants or from greenhouse-grown plants inoculated with diluted soil solutions (10^{-1} and 10^{-2})

Genetic diversity indices	Mesoamerican genotypes[a]			Andean genotypes[a]		
	Soil	10^{-1}	10^{-2}	Soil	10^{-1}	10^{-2}
Traditional Shannon index	2.483 ± 0.133	2.303 ± 0.117	2.247 ± 0.154	2.232 ± 0.180	1.673 ± 0.252	1.764 ± 0.222
Richness (ACE)	33.5 ± 11.4	25.7 ± 13.6	35.3 ± 16.4	42.2 ± 22.8	46.0 ± 44.1	36.5 ± 19.5
No. genotypes	41	15	33	24	22	39
Estimated coverage	0.780	0.467	0.727	0.625	0.636	0.744
Final similarity[b]	9.19%	27.99%	5.65%	11.93%	18.96%	14.45%

[a] Mesoamerican (Diamante Negro and Ouro Negro) and Andean (Jalo Precoce and Goiano Precoce) genotypes

[b] Final level of similarity in the cluster analysis of BOX-PCR profiles considering the UPGMA algorithm and the coefficient of Jaccard

bringing enormous benefits to farmers (Hungria and Vargas 2000; Hungria et al. 2000, 2003; Mostasso et al. 2002).

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