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New insights into the origins and evolution of rhizobia that nodulate common bean (*Phaseolus vulgaris*) in Brazil

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Abstract

It is generally accepted that there are two major centers of genetic diversification of common beans (*Phaseolus vulgaris* L.): the Mesoamerican (Mexico, Colombia, Ecuador and north of Peru, probably the primary center), and the Andean (southern Peru to north of Argentina) centers. Wild common bean is not found in Brazil, but it has been grown in the country throughout recorded history. Common bean establishes symbiotic associations with a wide range of rhizobial strains and *Rhizobium etli* is the dominant microsymbiont at both centers of genetic diversification. In contrast, *R. tropici*, originally recovered from common bean in Colombia, has been found to be the dominant species nodulating field-grown common-bean plants in Brazil. However, a recent study using soil dilutions as inocula has shown surprisingly high counts of *R. etli* in two Brazilian ecosystems. In the present study, RFLP-PCR analyses of *nodABC* and *nifH* genes of 43 of those Brazilian *R. etli* strains revealed unexpected homogeneity in their banding patterns. The Brazilian *R. etli* strains were closely similar in 16S rRNA sequences and in *nodABC* and *nifH* RFLP-PCR profiles to the Mexican strain CFN 42^T, and were quite distinct from *R. etli* and *R. leguminosarum* strains of European origin, supporting the hypothesis that Brazilian common bean and their rhizobia are of Mesoamerican origin, and could have arrived in Brazil in pre-colonial times. *R. tropici* may have been introduced to Brazilian soils later, or it may be a symbiont of other indigenous legume species and, due to its tolerance to acidic soils and high temperature conditions became the predominant microsymbiont of common bean.
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1. Introduction

Domestication or semi-domestication of several plant species probably begun in the Americas some 8000–10,000 years ago (Harlan, 1971; Gepts and Debouck, 1991), however, new patterns were established from 1492, with European colonization. The Portuguese officially arrived in Brazil in 1500, and abruptly reduced an indigenous population estimated at 1–10 million people; as traditions were passed down orally, knowledge of the past was compromised. Written history began, but was recorded from the European point-of-view; nowadays, there are evidences that the agricultural resources of the remaining indigenous population poorly reflect pre-colonial patterns (Prous, 1997; FUNAI, 2005).

There is general agreement that common bean (*Phaseolus vulgaris* L.) was domesticated separately in two major centers of genetic diversification: the Mesoamerican center or northern group—and maybe it could represent the primary center—with alleles originating from Mexico to the northern region of South America (Colombia, Ecuador and north of Peru) and the Andean center of South America or southern group, with alleles from southern Peru to the north of Argentina; a third minor domestication may have taken place in Colombia (Kaplan, 1965, 1980; Harlan, 1971; Debouck et al., 1983; Debouck, 1986; Gepts, 1990; Gepts and Debouck, 1991).

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Common bean establishes symbiotic associations with a wide range of rhizobial bacteria forming specific structures—root nodules—in which N_2 fixation takes place. Five rhizobial species nodulating this legume have been described: *Rhizobium leguminosarum* bv. phaseoli, *R. etli* bv. phaseoli, *R. gallicum* (bv. phaseoli and bv. gallicum), *R. giardinii* (bv. phaseoli and bv. giardinii) and *R. tropici* (Jordan, 1984; Martínez Romero et al., 1991; Segovia et al., 1993; Amarger et al., 1997). While common bean is highly promiscuous in its relationship with rhizobia (Michiels et al., 1998; Herrera-Cervera et al., 1999; Martinez-Romero, 2003), *R. etli* is the dominant microsymbiont in both the Mesoamerican and the Andean centers of origin (Segovia et al., 1993; Souza et al., 1994; Aguilar et al., 1998, 2004; Bernal and Graham, 2001; Martinez-Romero, 2003). This is not always the case in other areas where the crop is now grown (Amarger et al., 1994, 1997; Herrera-Cervera et al., 1999; Bernal et al., 2004). Segovia et al. (1993) proposed that when seeds containing *R. etli* bv. phaseoli were introduced into Europe, the symbiotic plasmid could have been transferred to *R. leguminosarum*; later, the same process may have occurred from *R. leguminosarum* bv. phaseoli to *R. gallicum* bv. phaseoli and *R. giardinii* bv. phaseoli (Amarger et al., 1997). It should be noted, however, that common bean-nodulating *R. gallicum, R. etli* (Tlusty et al., 2005) and *R. giardinii* (Beyhaut et al., 2006) also occur as the natural microsymbionts of *Dalea* spp. and *Desmanthus illinoensis,* respectively, in the natural prairie regions of the Central USA. *R. tropici* is well adapted to acid soils and high temperatures and was originally recovered from common bean in Colombia (Graham et al., 1982; Martínez Romero et al., 1991); however, it has also been isolated in Europe (e.g., Amarger et al., 1994; Herrera-Cervera et al., 1999) and in Africa (e.g., Anyango et al., 1995).

Wild common beans are not found in Brazil (Debouck, 1986), but the legume has been cultivated in the country throughout recorded history. Nowadays, the legume is a major component of the Brazilian diet, and the country is the largest producer and consumer worldwide (CONAB, 2006). All described common bean rhizobial species except for *R. gallicum* have been already isolated from host plants in Brazil (Mercante et al., 1998; Straliotto et al., 1999; Andrade et al., 2002; Mostasso et al., 2002; Grange and Hungria, 2004), in addition to other rhizobial genera and species (Hungria et al., 1993; Straliotto et al., 1999; Grange and Hungria, 2004). However, *R. tropici* seems clearly dominant under field conditions, even when cultivars of the Mesoamerican group are used as trap hosts (Hungria et al., 1997, 2000, 2003; Mercante et al., 1998; Mostasso et al., 2002). Nevertheless, in a recent study, when soil dilutions were used as inocula under axenic conditions, Grange and Hungria (2004) identified *R. etli* as the predominant symbiont of common bean in two Brazilian ecosystems. Our objective in this work was to study *nod* and *nif* gene diversity among *R. etli* strains isolated in the latter study, to further illuminate possible migration routes of this legume and its rhizobia.

2. Materials and methods

2.1. Strains

Common bean rhizobia used as reference strains, including type strains, are shown in Table 1. Forty-three *R. etli* strains from the study of Grange and Hungria (2004) were further considered in this study. The taxonomy of these strains was determined using RFLP-PCR and 16S ribosomal DNA gene-sequence analysis (Grange and Hungria, 2004), with information on these strains shown in Table 2. All strains isolated by Grange and Hungria (2004) were originally designated as "PRF" strains, but are shown here without the PRF designation. From unpublished data collected at the time of isolation of the strains, the *R. etli* population in the soils of the state of Pernambuco was estimated at 10^2 cells g^{-1} of soil, while in Paraná state it was estimated at 10^3 cells g^{-1}.

Strains were maintained in yeast–mannitol (YM) liquid broth (YMB, Vincent, 1970) mixed with glycerol (25%) and stored at −80 °C; working cultures were maintained on YM agar slants at 4 °C. Rhizobia were cultured routinely at 28 °C in YMB on a rotary shaker operating at 65 cycles min^{-1}. All strains are deposited at the "Diazotrophic and PGPR Bacteria Collection Culture" of Embrapa Soja.

2.2. RFLP-PCR of DNA regions encoding nodA, nodB, nodC and nifH genes

Rhizobial DNA was extracted as described by Kaschuk et al. (2006) and amplified by PCR reaction using the following primers: (1) *nodA*-nodA1 (5′-TGCRGTGGAAR NTRNNCTGGGAAA-3′), and nodA2 (5′-GGNCCGTC RTCRAAWGTCARGTA-3′) (Haukka et al., 1998), nodA3 (5′-TCATAGCTCYGRACCGTTCCG-3′) and nodA4 (5′-ATCATCKYNCCGGNNGGCCA-3′) (Zhang et al., 2000). For the strains that did not amplify with primers nodA1 and nodA2, PCR was performed with primers nodA1 and nodA3, followed by a second reaction with primers nodA1 and nodA4, and using the PCR product of the first reaction, as has been suggested by Haukka et al. (1998); (2) *nodB*-nodB3F (5′-TACCT-GACSTTYGACGACGGTCC-3′) (Wernegreen and Riley, 1999) and nodCRR (5′-GAGACGGCGRCRCTGGTTG-3′) (Silva et al., 2003); (3) *nodC*–nodCF (5′-AYGTHGTY-GAYGACGGTTC-3′) and nodCI (5′-CGYGACAGC-CANTCKCTATTG-3′) (Laguerre et al., 2001); (4) *nifH*-nifHF (5′-TACGGNAARGGSGGNATCGGCAA-3′) and nifHI (5′-AGCATGTCYTCSAGYTCNTCCA-3′) (Laguerre et al., 2001). The final volume of PCR reaction was 50 μL and cycles for amplification were performed as follows: *nifH*—30 s at 95 °C; 35 cycles of 1 min at 94 °C, 30 s at 63 °C, 2 min at 72 °C; followed by 3 min at 72 °C; *nodA*—2 min at 97 °C; 25 cycles of 2 min at 97 °C, 40 s at 92 °C,

Table 1
Bean rhizobia used as reference strains

Strain	Name	Synonym	Place of isolation	Origin of the strain and main study of its description
Rhizobium etli bv. phaseoli	CFN 42^T	ATCC 51251, DSM 11541, USDA 9032	Mexico	CCG, México, E. Martinez (Segovia et al., 1993)
Rhizobium tropici type A	CFN 299	USDA 9039, LMG 9517	Mexico	CCG, México, E. Martinez (Martínez Romero et al., 1991)
Rhizobium tropici type B	CIAT 899^T	ATCC 49672, DSM 11418, USDA 9030, UMR 1899, SEMIA 4077	Colombia	CCG, México, E. Martinez (Martínez Romero et al., 1991)
Rhizobium tropici	PRF 35		Paraná, Brazil	Embrapa Soja (Hungria et al., 2000)
Rhizobium tropici	PRF 54		Paraná, Brazil l	Embrapa Soja (Hungria et al., 2000)
Rhizobium tropici	PRF 81	SEMIA 4080	Paraná, Brazil	Embrapa Soja (Hungria et al., 2000)
Rhizobium tropici type B	LMG 9518			USDA, USA, P. van Berkum
Rhizobium leguminosarum bv. phaseoli	USDA 2671	RCR 3644	England	USDA, USA, P. van Berkum
Rhizobium leguminosarum bv. trifolii	ATCC 14480^T	DSM 30141, 3D1k22a		USDA, USA, P. van Berkum (Jordan, 1984)
Rhizobium leguminosarum bv. viciae	USDA 2370^T		USA	USDA, USA, P. van Berkum (Jordan, 1984)
Rhizobium etli bv. phaseoli	ATCC 8002		England	USDA, USA, P. van Berkum (originally *R. leg.* bv. phaseoli, Young et al., 1991, now *R. etli*, Münchbach et al., 1999)
Rhizobium galicum bv. gallicum	R602sp^T	USDA 2918	France	INRA, France, N. Amarger (Amarger et al., 1997)
Rhizobium giardinii bv. giardinii	H152^T	USDA 2914	France	INRA, France, N. Amarger (Amarger et al., 1997)
Rhizobium tropici type A	77		Pernambuco, Brazil	Embrapa Soja (soil 4, Grange and Hungria, 2004)
Rhizobium sp.	84[a]		Pernambuco, Brazil	Embrapa Soja (soil 4, Grange and Hungria, 2004)
Rhizobium sp.	100[a]		Pernambuco, Brazil	Embrapa Soja (soil 5, Grange and Hungria, 2004)
Rhizobium sp.	H 129[a]		D. Federal, Brazil	Embrapa Soja
Rhizobium sp.	129[b]		Paraná, Brazil	Embrapa Soja (soil 8, Grange and Hungria, 2004)
Rhizobium sp.	215[b]		Paraná, Brazil	Embrapa Soja (soil 14, Grange and Hungria, 2004)
Rhizobium sp.	233[a]		Paraná, Brazil	Embrapa Soja (soil 14, Grange and Hungria, 2004)
Rhizobium tropici	H 12	SEMIA 4088	D. Federal, Brazil	Embrapa Soja (Mostasso et al., 2002)
Rhizobium tropici	H 20		D. Federal, Brazil	Embrapa Soja (Mostasso et al., 2002)
Rhizobium tropici	H 52		D. Federal, Brazil	Embrapa Soja (Mostasso et al., 2002)
Rhizobium etli bv. phaseoli	ISP 34		Spain	Univ. Sevilla, Spain,.M. Megías
Rhizobium etli bv. phaseoli	ISP 23		Spain	Univ. Sevilla, Spain, M. Megías
Rhizobium etli bv. phaseoli	ISP 19		Spain	Univ. Sevilla, Spain,.M. Megías

[a]Partial 16S rRNA sequences.
[b]Whole 16S rRNA sequences.

1 min at 55 °C, 1 min and 30 s at 72 °C, followed by 5 min at 72 °C; *nodB*—1 min and 30 s at 96 °C; 30 cycles of 1 min and 30 s at 96 °C, 30 s at 6 °C, 30 s at 66 °C, 30 s 72 °C; followed by 3 min at 72 °C; *nodC*—30 s a 94 °C, 40 cycles of 30 s at 96 °C, 1 min at 53 °C, 30 s at 72 °C; followed by 5 min a 72 °C. The PCR products obtained were analyzed by horizontal gel electrophoresis (17 × 11 cm) with 1.5% agarose and carried out at 100 V for 4 h. Gels were stained with ethidium bromide and visualized under UV radiation.

The PCR products were digested with four restriction endonucleases (Invitrogen™): *Hae*III (5′-GGCC-3′; 3′-CCGG-5′), *Hha*I (5′-GCG/C-3′; 3′-C/GCG-5′), *Mbo*II (5-GAAGA(N)$_8$/-3′, 3′-CTTCT(N)$_7$/-5′) and *Msp*I (5′-C/CGG-3′; 3′-GGC/C-5′). In addition, two other endonucleases were used for the three *nod* regions: *Hinf*I (5′-G/ANTC-3′; 3′-CTNA/G-5′) and *Rsa*I (5′-GT/AC-3′; 3′-CA/TG-5′). Digestion was performed as recommended by the manufacturers, and the fragments obtained were then analyzed by electrophoresis in a 17 × 11 cm gel containing 3% agarose, and carried out at 100 V for 4 h. A 100-kb DNA Ladder™ (Invitrogen™) was used as a molecular marker, included in three positions in each gel. Gels were stained with ethidium bromide and photographed under UV radiation with a Kodak Digital

Table 2
Information about the forty-three *R. etli* strains obtained from soils of the States of Pernambuco (PE) and Paraná (PR)[a] and used in this study

Soil	State	District	Crop management	Common bean cultivar used as trap host	
				Aporé[b]	Negro Argel[b]
1	PE	São Francisco	Beans	5	14, 28[c]
2	PE	São Francisco	Beans 3 yr before	29, 31, 39	48, 51, 54
4	PE	Santo Antonio	Beans 4 yr before	68, 69, 72, 73, 74, 76, 79	82, 244
5	PE	Caruaru	Beans without fertilizer for 10 yr[3]	96, 118, 124, 230[c], 246	123, 126
6	PE	Caruaru	Beans	108, 248	109, 115, 116, 236
8	PR	São João	Beans for 2 years between coffee (*Coffea arabica*) lanes[3]	127, 133	
9	PR	São João	Pasture, soybean and beans for the past 2 yr[3]	160	
12	PR	Toledo	Soybean and wheat (*Triticum aestivum*)	164	178
13	PR	Francisco Alves	Bean intercropped with maize (*Zea mays*) for several years	189, 190	199
14	PR	Francisco Alves	Bean intercropped with maize	204, 206, 209, 222	

[a]Adapted from Grange and Hungria (2004).
[b]Aporé has colored and Negro Argel has black seeds. Both cultivars belong to the Mesoamerican group.
[c]Modified from Grange and Hungria (2004).

Science 120 apparatus. The profiles obtained were confirmed twice.

2.3. Cluster analysis

Sizes of RFLP-PCR fragments were normalized according to the MW markers included in each gel, with cluster analyses then performed using Bionumerics version 1.50 (Applied Mathematics, Kortrijk, Belgium), and the UPGMA (unweighted pair-group method, with arithmetic mean) algorithm (Sneath and Sokal, 1973) and the coefficient of Jaccard (Jaccard, 1912). Analyses of each region with all restriction enzymes were performed setting the position tolerance of the bands to 3%.

3. Results

Sizes obtained for the PCR products were: *nodA,* ~660 bp; *nodB,* ~600 bp; *nodC*, ~930 bp; and *nifH* between 780 and 890 bp (data not shown).

The dendrogram obtained by RFLP-PCR of the *nodA* region with six restriction enzymes showed a high level of diversity, with three great groups distinguishable (data not shown). The first great group (GG I) clustered fifteen strains at a 37% level of similarity and included only reference strains belonging to species other than *R. etli* bv. phaseoli (designated hereafter as "*R. etli*"). Within this group, nine strains were joined to *R. tropici* CIAT 899^T with a similarity of 60%. The second great group (GG II), including all but one of the 43 Brazilian *R. etli* strains recovered by Grange and Hungria (2004), clustered at a similarity of 66%, but was split into two subgroups. The first of these included five strains from Paraná and five from Pernambuco, together with *R. etli* strains CFN 42^T and ATCC 8002, with a similarity of 68%. The other clustered the remaining 32 Brazilian *R. etli* strains at a 69% level of similarity. There was no obvious correlation in these groups with strain origin, or with the common bean trapping host cultivar. The final great group clustered three *R. etli* strains from Spain at a similarity of 59%, while *Rhizobium* sp. strain H 129, isolated from the Brazilian Cerrados, was joined to all others at a final level of similarity of only 33%.

Considerable diversity was evident also in the dendrogram obtained by amplification of the *nodB* region followed by restriction with six endonucleases; five major groups were identified, joined at a final level of similarity of 35% (data not shown). All but one strain (204) of the Brazilian *R. etli* behaved similarly, clustering into two subgroups, and joined with a similarity of 67%. This group also included *R. etli* strain CFN 42^T and *R. leguminosarum* bv. phaseoli strain USDA 2671. Again, there was no relation between the site of origin of the Brazilian *R. etli* and their position in the dendrogram. The Brazilian *R. etli* clustered completely separately from *R. etli* strains ATCC 8002 and 204 (GG III) and from all *R. etli* strains from Spain.

The dendrogram of the *nodC* region showed three major groups joined at the very low level of similarity of 27% (data not shown). GG II included a diversity of strains clustered at 37% of similarity and could be split into two SGs. SG IIA clustered all *R. tropici* reference strains and two *Rhizobium* sp. strains at a level of similarity of 51%; *R etli* ATCC 8002 and *R. giardinii* H152 were joined to SG IIA at a similarity of 38%. SG IIB clustered, with a similarity of 50%, three *Rhizobium* sp. and two *R. tropici* strains, one of them (PRF 35) showing close similarity to *R. leguminosarum* (91%). Once more, the 43 Brazilian *R. etli* strains were joined in a single large cluster with CFN 42^T, though five subgroups were evident. This cluster did not include the three Spanish *R. etli* strains, *R. leguminosarum* bv. phaseoli strain USDA 2671, or *R. etli* ATCC 8002.

A combined analysis performed using the RFLP-PCR products of *nodA*, *nodB* and *nodC* with all restriction enzymes resulted in five great groups joined at a final level

of similarity of 30% (Fig. 1). GG I included three *Rhizobium* sp. strains with a similarity of 43%. All other *R. tropici* reference strains were clustered into GG II with a similarity of 59%; *Rhizobium* sp. strains H 129 and 100 were joined to GG II with a final similarity of 39%. All Brazilian *R. etli* strains were grouped with 61% of similarity in GG III and *R. etli* CFN 42^T was joined to this group with a similarity of 58%, followed by *R. etli* ATCC 8002 at 46%. GG IV contained only two strains, *R. giardinii* H152^T and *R. leguminosarum* bv. viciae USDA 2671, at 42% similarity. Finally, the three *R. etli* from Spain were clustered with a 62% similarity and, together with *Rhizobium* sp. strain 215, formed the final GG V with a similarity of 38% (Fig. 1).

Three distinct major groups were observed in the dendrogram produced with the RFLP-PCR products of the *nifH* region, with all strains clustered at a final level of similarity of 30% (Fig. 2). GG I could be split into two SGs, the first one joining four Brazilian *Rhizobium* sp. strains with a similarity of 53%. Seven *R. tropici*, including CIAT 899^T, were clustered into SG IB (63% similarity), and *R. giardinii* occupied an isolated position with a final similarity of 36%. In relation to *R. etli* reference strains, CFN 42^T from Mexico and ATCC 8002 from Europe were quite similar (95%), and the three strains from Spain were grouped with a similarity of 67%. GG II also included two *Rhizobium* sp. and one *R. tropici* from Brazil, but the final level of similarity was relatively low (46%). All Brazilian *R. etli* strains were joined in GG III with a similarity of 62%, but SGs and divisions within them were clear. Contrary to the clusters obtained with *nod* RFLP-PCR, none of the Brazilian *R. etli* strains clustered with CFN 42^T or *R. leguminosarum* bv phaseoli ATCC 8002 in the *nifH* analysis (Fig. 2).

4. Discussion

Despite being the largest producer of common bean in the world, Brazil's yields per unit area are low (789 kg ha^{-1} average) (CONAB, 2006). Constraints include poor technology and cropping in acid soils that are low organic matter content and deficient in N. Increased availability of N through the root-nodule symbiosis with efficient rhizobial strains might increase yields at a low cost and with environmental benefits. However, poor nodulation and low N_2 fixation rates and lack of responses to inoculation have been reported in this crop worldwide, which is often attributed to inefficient indigenous rhizobia (e.g., Graham, 1981; Hardarson, 1993; Michiels et al., 1998). In Brazil, although a few experiments with selected strains of *R. tropici* have shown increases in grain yield (Hungria et al., 2000, 2003; Mostasso et al., 2002), there are still reports of erratic responses to inoculation. Information about the structure of the indigenous rhizobial population and the co-evolution with the host plant could greatly contribute to understand the frequent reports of nodulation failure. Furthermore, this information might shed some light on Brazil's still poorly documented cropping history.

Two major centers of genetic diversification of the common bean are often cited, the Mesoamerican and the Andean; a likely minor third center of origin—Colombia—has also been suggested (Harlan, 1971; Kaplan, 1965, 1980; Debouck et al., 1983; Debouck, 1986; Gepts, 1990; Gepts and Debouck, 1991). Freitas (2006) verified that the phaseolin content of seeds from subterranean silos in archaeological sites in the State of Minas Gerais, Brazil, had a larger influence on the Mesoamerican than on the Andean center, and the same origin was confirmed in maize (*Zea mays* L) when the alcohol-dehydrogenase-2 gene was analyzed. Those results are strongly indicative that common bean and maize seeds were introduced in the country, by trade or migration, between the populations of Mesoamerica and Brazil, in pre-colonial times. Nevertheless, although *R. etli* is the dominant microsymbiont in both the Mesoamerican and the Andean centers (Segovia et al., 1993; Souza et al., 1994; Piñero et al., 1988; Aguilar et al., 1998, 2004; Bernal and Graham, 2001; Martinez-Romero, 2003), it is usually not isolated from nodules of field-grown common bean plants in Brazil, what has been attributed to stressful conditions as the acidic nature of the soils and high temperature (Hungria et al., 1997, 2000, 2003).

Recently, using soil dilutions as inocula under axenic conditions, populations of *R. etli* were isolated from two Brazilian ecosystems (Grange and Hungria, 2004), and estimated at 10^2–10^3 cells g^{-1} of soil. Although they showed diversity in their *rep*-PCR profiles (Grange and Hungria, 2004), these strains of *R. etli* were relatively homogenous in this study, when the banding patterns produced with RFLP-PCR of their *nodA*, *nodB*, *nodC* and *nifH* gene regions were considered. Grange and Hungria (2004) also showed similarity among these strains in the 16S rRNA gene sequence analysis. The highest similarity of these organisms to reference strains used in this study was to *R. etli* CFN 42^T, though *R. etli* ATCC 8002 and *R. leguminosarum* bv. phaseoli USDA 2671, both from Europe, also clustered with the Brazilian *R. etli* in the *nodA* and *nodB* dendrograms (data not shown). The combined analysis for all *nod* regions clearly indicates that the Brazilian strains were closely related to CFN 42^T, but relatively distant from *R. etli* strains obtained in Spain (Fig. 1). However, when the *nifH* gene region was considered, the Brazilian *R. etli* formed a distinct cluster in relation to all other reference strains (Fig. 2).

The recent evidences of the larger influence of Mesoamerican center in archeological seeds in Brazil (Freitas, 2006), in addition to the results from this study, showing greater similarity of Brazilian *R. etli* to the Mexican isolate CFN 42^T might indicate migration from the Mesoamerican region. An additional factor is that Pérez-Ramírez et al. (1998) have shown that *R. etli* may be carried on bean-seed testa, possibly the means by which the species was distributed worldwide. Furthermore, maize may also be a

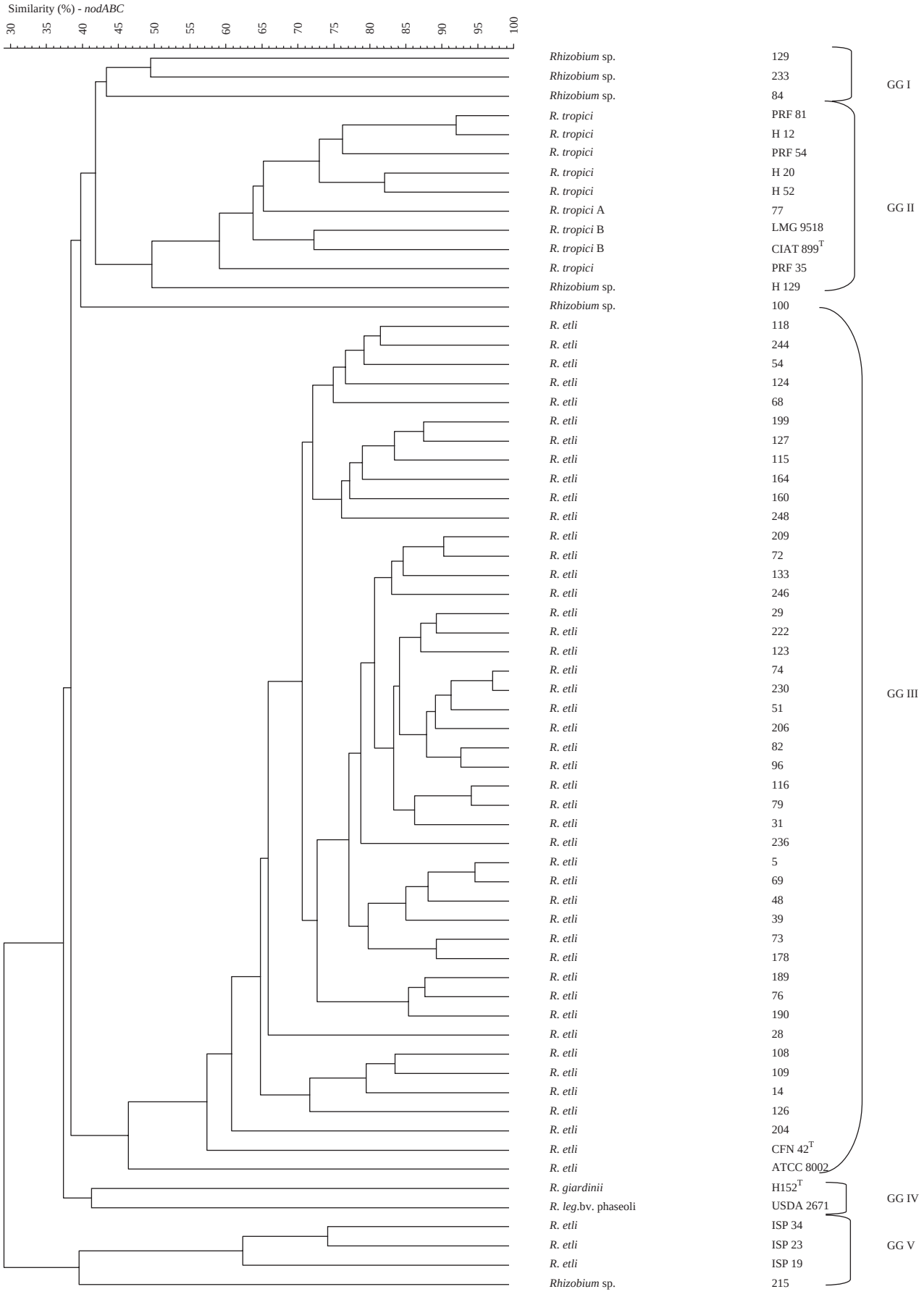

Fig. 1. Cluster analysis (UPGMA with the coefficient of Jaccard) of the products obtained by PCR-RFLP analysis of the *nodA*, *nodB* and *nodC* genes of Brazilian *R. etli* and of reference common bean rhizobial strains.

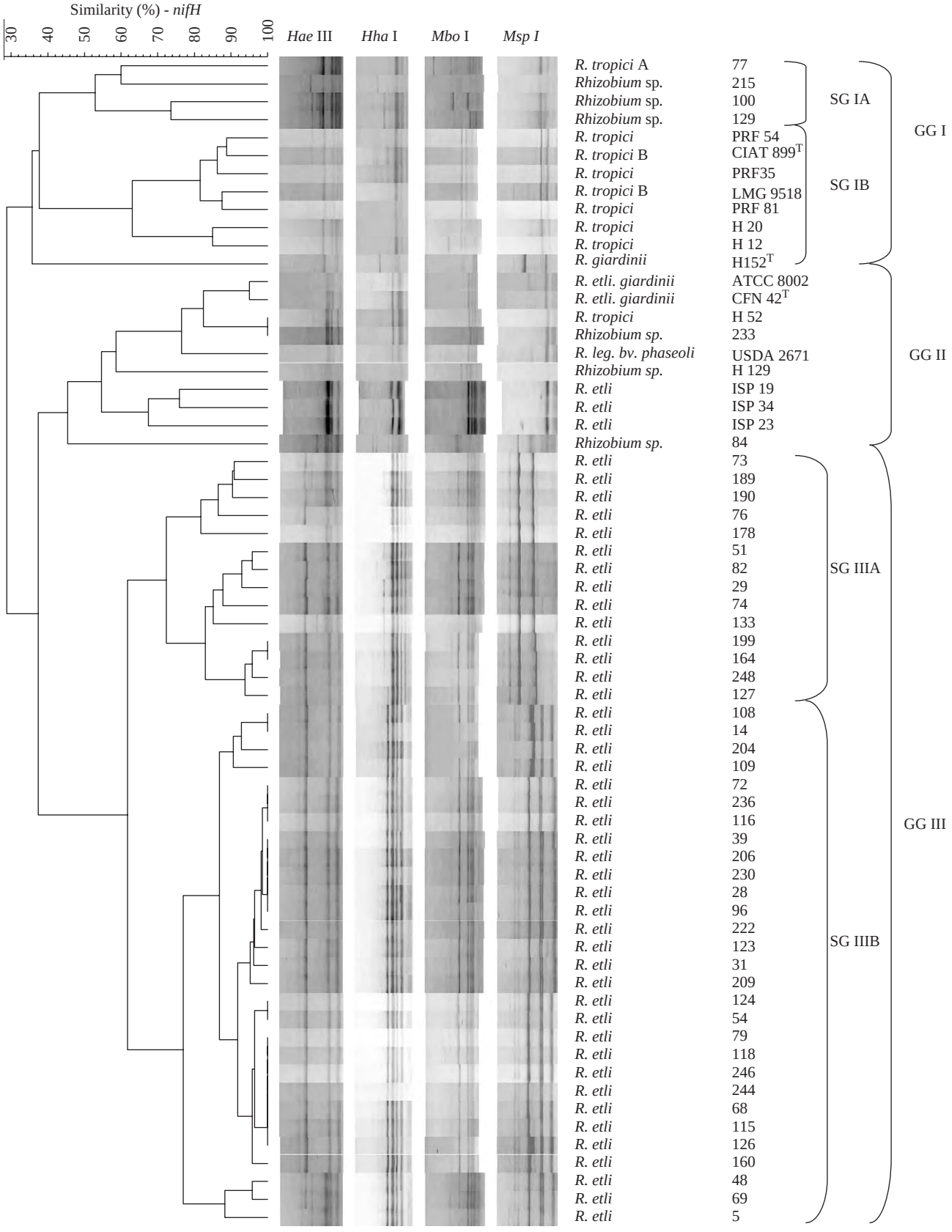

Fig. 2. Cluster analysis (UPGMA with the coefficient of Jaccard) of the products obtained by PCR-RFLP analysis of the *nifH* genes of Brazilian *R. etli* and of reference common bean rhizobial strains.

host of *R. etli* (Gutiérrez-Zamora and Martínez-Romero, 2001), and transfer from maize—also from Mesoamerica—to beans may have occurred when the two species were grown in association in Brazil. It is also important to remember that, after 1492, a trade barrier was established in South America by the domains of Spain and Portugal, and isolation might have prevented the spread of other genotypes of *R. etli* to Brazil. Considering the historical aspects and the high similarity of ribosomal 16S and *nod* genes between the Brazilian *R. etli* and the Mexican strain CFN 42^T we hypothesize that *R. etli* was introduced to Brazil through common bean seeds or as a maize endophyte from Central America in pre-colonial times, and has been present in Brazilian soils since then. It is noteworthy that *R. etli* strains from Europe may have different origins, as shown by ATCC 8002, closely related to CFN 42^T, and the three ISP strains from Spain, all being considerably different.

The results of Grange and Hungria (2004)—indicating that *R. etli* represented the predominant population when diluted soil was used as inoculum—are apparently in conflict with previous reports showing that the majority of isolates effective in fixing N_2 with common bean (isolated from nodules of field-grown common bean or leucaena) belong to *R. tropici* species (Hungria et al., 1997, 2000; Mercante et al., 1998). Furthermore, with suspensions of soils from Paraná State, Andrade et al. (2002) identified both *R. tropici* type B and *R. leguminosarum* bv. phaseoli as the predominant species. However, a recent report by Alberton et al. (2006) confirmed clear differences in rhizobial population data according to the sampling method; diversity was much higher with soil dilution, indicating that poorly competitive genotype, as *R. etli*, are not detected by isolation from nodules of field-grown plants.

Altogether, we may conclude that although *R. etli* is established in Brazilian soils, it usually fails to nodulate common bean, or it forms inefficient white nodules often observed under field conditions. *nifH* genes are multicopy in *R. etli* and the differences between Brazilian *R. etli* and Mexican CFN 42^T might explain the losses in N_2-fixing capacity in Brazilian strains, as *nifH* is susceptible to gene conversion and multiple recombination (Segovia et al., 1993; Martinez-Romero, 2003). The large diversity of *nod* and mainly *nif* genes of *R. etli* in this study might also indicate that the species has been present in Brazilian soils for a long time and that reiteration under local conditions has been taken place. Finally, given the variation among *R. etli* bv. phaseoli strains, together with the recovery of organisms similar to *R. etli* from species as different as *Mimosa* and *Dalea* (Wang et al., 1999; Tlusty et al., 2005), additional studies are needed to determine taxonomic relationships within this species.

The next question relates to the origin of the symbiosis with *R. tropici* in Brazil, considering that the species has not yet been isolated from common bean nodules in the Mesoamerican and Andean centers of genetic diversification. Kipe-Nolt et al. (1992) reported that wild *P. vulgaris* accessions were restricted for nodulation with some *R. tropici* strains, however, Andean and Mesoamerican cultivars in Brazil were capable of nodulating and fixing N_2 with both *R. etli* and *R. tropici* under axenic conditions (Nodari et al., 1993; Franco et al., 2002). It was first suggested that *R. tropici* is indigenous to South America including Brazil; the type strain was isolated in Colombia (Martínez Romero et al., 1991), likely a third center of the host common bean (Debouck et al., 1983). A first hypothesis would be that *R. tropici* was introduced to Brazil from Colombia or nearby and it became the predominant species due to its tolerance of acidity, high temperatures and other stress factors (Martínez Romero et al., 1991; Hungria et al., 1993, 1997; Pinto et al., 1998; Graham et al., 1982, 1994). *R. tropici* might have became a strong competitor in the Brazilian soils characterized by high acidity, as has been shown in Kenya, where at pH 4.5 *R. tropici* was the dominant species, changing to *R. etli* at pH 6.8 (Anyango et al., 1995). In addition, most cultivars in Brazil are of Mesoamerican origin, including landraces (Emygdio et al., 2003), and Martínez-Romero et al. (1998) have demonstrated that *R. tropici* may block nodulation by *R. etli* in those beans; this blocking could also represent a source of lack of efficacy in N_2 fixation often reported in tropics. The hypothesis would be that although *R. etli* remained living saprophitically in the soils, it has been outcompeted by *R. tropici* under the usual acidic conditions.

An alternative hypothesis for the origin of *R. tropici* in Brazil would be that common bean is not the natural host of this species. It is well known that *R. tropici* is broadly infective and reported hosts include *Leucaena* sp. and *Macroptilium* sp., among several others (Martínez Romero et al., 1991; Hernandez-Lucas et al., 1995; Mercante et al., 1998; Hungria et al., 2000). In addition, *R. tropici* has been isolated from *Bolusanthus* and *Aspartium* in Africa (Dagutat and Steyn, 1995), from native shrubby legumes in Australia (Lafay and Burdon, 1998), and as a natural symbiont of *Gliricidia sepium* in Mexico (Acosta-Durán and Martínez-Romero, 2002). Two possible candidates to host *R. tropici* in Brazil would be species of the genera *Mimosa* and *Gliricidia*, as three very effective rhizobia showing high similarity with *R. tropici* have been isolated from *Mimosa caesalpinifolia* (SEMIA 6383), *Mimosa scabrella* (SEMIA 6165) and *G. sepium* (SEMIA 6168) (Menna et al., 2006).

In conclusion, large populations of *R. etli* were found in Brazilian soils showing higher similarity of 16S rRNA and *nodABC* genes with the Mexican strain CFN 42^T. The results contribute to the still-modest knowledge about the agricultural history of Brazil, bringing support to the theory that trade and migrations might have occurred between indigenous Brazilian and Central America populations in pre-colonial times. Despite its presence in high numbers in the soil and its capability in fixing N_2 under axenic conditions, *R. etli* is rarely detected in nodules of

field-grown common bean plants, being out-competed by *R. tropici*, probably because most Brazilian soils are very acidic. More studies are needed to investigate whether interactions within common bean rhizobial communities may be responsible for the failures in nodulation and N_2 fixation often reported in the tropics.

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