Research in Microbiology 164 (2013) 740–748

www.elsevier.com/locate/resmic

Novel *Rhizobium* lineages isolated from root nodules of the common bean (*Phaseolus vulgaris* L.) in Andean and Mesoamerican areas

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Received 13 February 2013; accepted 15 April 2013
Available online 11 June 2013

Abstract

The taxonomic affiliations of nineteen root-nodule bacteria isolated from the common bean (*Phaseolus vulgaris* L.) in Mexico, Ecuador and Brazil were investigated by analyses of 16S rRNA and of four protein-coding housekeeping genes. One strain from Mexico could be assigned to *Rhizobium etli* and two from Brazil to *Rhizobium leucaenae*, whereas another from Mexico corresponded to a recently described bean-nodulating species-level lineage related to *R. etli* and *Rhizobium phaseoli*. Ten strains isolated in Ecuador and Mexico corresponded to three novel *Rhizobium* lineages that fall into the *R. phaseoli*/*R. etli*/*Rhizobium leguminosarum* clade. One of those lineages, with representatives isolated mostly from Ecuador, seems to be dominant in beans from that Andean region. Only one of the Mexican strains clustered within the *Rhizobium tropici* clade, but as an independent lineage. Interestingly, four strains were affiliated with species within the *Rhizobium radiobacter* clade. The existence of yet non-described native *Rhizobium* lineages in both the Andean and Mesoamerican areas is discussed in relation to common-bean diversity and environmental conditions.

Keywords: Biological nitrogen fixation; MLSA; Phylogeny; Symbiosis; *Rhizobium*; 16S rRNA gene

1. Introduction

Legumes such as the common bean (*Phaseolus vulgaris* L.) are capable of forming root nodules when infected with symbiotic nitrogen-fixing bacteria known as rhizobia. *P. vulgaris*, domestication of which occurred several thousand years ago in Mesoamerica and in the Andean region (Rodiño et al., 2010), represents the most important grain legume in human diets, providing proteins and carbohydrates for more than 300 million people, especially in Latin America, the Caribbean and Africa (CGIAR, 2012). Diversity of the *P. vulgaris* nodule rhizobia has been extensively studied, showing that, at its sites of origin, there are preferred symbionts, but in introduced areas it is promiscuous and may function as a trap-host plant (Martínez et al., 1985; Michiels et al., 1998), forming nodules with diverse indigenous bacteria [reviewed in Martínez-Romero (2003)]. Additionally, in introduced areas, rhizobial species similar to those at the site of origin have also been found, probably introduced by carriage of rhizobia on seeds (Grange et al., 2007; Pérez-Ramírez et al., 1998).

In Mexico, *Rhizobium etli* seems to be the main *P. vulgaris* symbiont, but this needs further analysis in view of a recent taxonomy revision that proposed reclassification of some

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http://dx.doi.org/10.1016/j.resmic.2013.05.002

R. etli strains as *Rhizobium phaseoli*, and revealed the existence of other common-bean-nodulating species-level lineages previously considered to be *R. etli* (López-Guerrero et al., 2012b). Genomic-based data with average nucleotide identity (ANI) analysis supported the conclusion that such lineages represent different species, further evidenced by low recombination among the genomes of *R. etli, R. phaseoli* and the other lineages (Acosta et al., 2011). In countries of the Andean region, strains classified as *R. etli* and *Rhizobium leguminosarum*—based on phenotype or the 16S rRNA gene—have been described (Aguilar et al., 1998; Bernal and Graham, 2001). At least one of these Andean bean *Rhizobium* populations was shown to be different from those nodulating beans in Mexico (Bernal and Graham, 2001), raising the possibility that additional native lineages exist.

Rhizobium tropici, *Rhizobium leucaenae* and other related lineages (also called the "*R. tropici* group") are dominant in acid soils of Brazil where *P. vulgaris* has been cropped for centuries, a feature also reported in other tropical countries (Grange and Hungria, 2004; Martínez-Romero, 2003; Martínez-Romero et al., 1991; Ribeiro et al., 2009; Stocco et al., 2008). Nevertheless, *R. tropici* and related species seem to be natural symbionts of other legumes, such as *Leucaena leucocephala*, *Mimosa caesalpiniifolia* and *Gliricidia sepium* (Menna et al., 2006; Mercante et al., 1998).

Based on 16S rRNA gene sequences, *R. etli*, *R. phaseoli* and related lineages constitute a single group with little differentiation (López-Guerrero et al., 2012b), while several other lineages group with *R. tropici* (Menna et al., 2006; Pinto et al., 2007). Indeed, it has been shown that only phylogenies based on protein-coding housekeeping genes in a multilocus sequence analysis (MLSA) scheme can reveal the distinctiveness of these related lineages (López-Guerrero et al., 2012b; Ribeiro et al., 2009, 2012). Therefore, the goal of this study was to determine the taxonomic affiliations of a collection of nodule isolates from *P. vulgaris* grown in Mexico and Ecuador, obtained in Mesoamerican and Andean bean cultivation areas, by using MLSA. The collection was obtained by the late Peter Graham who devoted his professional life to research in nitrogen fixation, with special emphasis on bean symbionts.

2. Materials and methods

2.1. Strains and characteristics

Nineteen isolates from field-grown *P. vulgaris* collected by Dr. Peter H. Graham were analyzed in this study (Table 1).

After isolation, Koch's postulates for each strain were verified both at the University of Minnesota and at Embrapa Soja. For this study, the capacity to nodulate and fix nitrogen with common beans was verified again, in an experiment performed at Embrapa Soja in Leonard jars (Vincent, 1970). All strains were able to nodulate common beans of both Andean (cultivar Goiano Precoce) and Mesoamerican (cultivar Diamante Negro) origin. For 15 strains the nodules were effective in fixing nitrogen, showing the typical red color inside and green leaves when grown in jars containing sterile sand and vermiculite and receiving N-free nutrient solution. The exceptions were the four strains that were related to the *Rhizobium radiobacter* clade, that were able to nodulate beans, but were ineffective in fixing nitrogen, with nodules with white color inside and yellow leaves.

Strains are deposited at the "Culture Collection of Diazotrophic and PGPR Bacteria" of Embrapa Soja (http://www.bmrc.lncc.br). Isolates were grown on yeast extract-mannitol (YM) agar medium (Vincent, 1970) as described before (Menna et al., 2006).

Table 1
Rhizobium strains isolated from the common bean (*Phaseolus vulgaris* L.) and used in this study.

CNPSo strain name	Other strain designations	Geographical origin	Species or lineage determined in this study
655	UMR 1239, CP 32	Mexico	*Rhizobium* sp.
657	UMR 1265, IAPAR 80B	Brazil	*Rhizobium leucaenae*
659	UMR 1271,023-85 EF	Mexico	*Rhizobium* sp. PEL4
660	UMR 1283, CNPAF 180	Brazil	*Rhizobium leucaenae*
661	UMR 1286, Z31-4	México	*Rhizobium* sp. PEL5
662	UMR 1287, Z113-10	Mexico	*Rhizobium pusense*
664	UMR 1298, Z146-15	Mexico	*Rhizobium etli*
665	UMR 1315, Z78A-6	Mexico	*Rhizobium pusense*
666	UMR 1317, Z95B-8	Mexico	*Rhizobium* sp. PEL5
668	UMR 1320, Z87-8	Mexico	*Rhizobium* sp. PEL5
669	UMR 1322, Z52-4	Mexico	*Rhizobium* sp. PEL1
670[a]	UMR 1449, PIMAMPIRS I 4	San Jose/Imbaburra/Ecuador	*Rhizobium* sp. PEL4
671[a]	UMR 1450, PIMAMPIRS I 5	Pimampiro/Imbaburra/Ecuador	*Rhizobium* sp. PEL4
672[a]	UMR 1452, Los Olivos I 7	La Olivos/Imbaburra/Ecuador	*Rhizobium* sp. PEL4
673	UMR 1454, Chaltura 2 I 9	Chaltura/Imbaburra/Ecuador	*Rhizobium radiobacter*
675	UMR 1457, CHIMBORAZO pallatanga 1 Ch2	Pallatanga/Chimborazo/Ecuador	*Rhizobium* sp.
676[a]	UMR 1462, Salsipuedes Ch 5	Salsipuedes/Chimborazo/Ecuador	*Rhizobium* sp. PEL4
679	UMR 1471, Chuquipata L2 A4	Chuquipata/Azuay/Ecuador	*Rhizobium* sp. PEL6
683	UMR 1490, Salapa L17	Salapa/Loja/Ecuador	*Rhizobium* sp. PEL4

[a] Strains collected and used in a previous study (Bernal and Graham, 2001).

2.2. DNA isolation and gene sequencing

The 19 strains, in addition to eight reference/type strains needed to complete the phylogenetic study (Table S1), were submitted to sequencing analysis. Total genomic DNA was extracted as described before (Kaschuk et al., 2006). Primers and amplification conditions of partial sequences of the 16S rRNA (Menna et al., 2006), *recA* (Gaunt et al., 2001), *glnII* (Stepkowski et al., 2005), *gyrB* (Martens et al., 2008) and *rpoA* (Ribeiro et al., 2009) genes were obtained from the literature. After PCR product purification, sequencing was performed as described by Ribeiro et al. (2009) on a MEGA BACE 1000 (Amersham Biosciences) capillary sequencer.

2.3. Phylogenetic analysis

Multiple sequence alignments for each gene were performed with MUSCLE (Edgar, 2004) using sequences obtained in this work and sequences retrieved from the GenBank database. Phylogenetic trees were generated using MEGA version 5 (Tamura et al., 2011) with the neighbor joining (NJ) (Saitou and Nei, 1987) and maximum likelihood (ML) (Felsenstein, 1981) methods, using the TN93 and GTR + I + G models, respectively. Support for tree nodes was evaluated by bootstrap analyses (Felsenstein, 1985) with 500 pseudoreplicates. Conserved, variable and parsimony-informative characters were determined with the MEGA program. Only NJ phylograms are presented as they showed similar topologies to ML-inferred trees.

2.4. Sequence accession numbers

Nineteen 16S rRNA, 20 *recA*, 19 *glnII*, 24 *gyrB* and 22 *rpoA* gene sequences determined in this study were deposited in the GenBank database. Accession numbers are shown in Table 1S.

3. Results and discussion

3.1. 16S rRNA gene phylogeny

Over 50% of the strains under study (12 out of 19) isolated in Ecuador and Mexico were positioned in the *R. etli*/*R. phaseoli*/*R. leguminosarum* clade (from now on denominated as the *R. etli* group) based on 16S rRNA gene sequences (Fig. 1). Two strains from Brazil and one from Mexico were grouped within the *R. tropici* and allied species group. The remaining four isolates were placed in the *R. radiobacter* phylogenetic branch [formerly *Agrobacterium tumefaciens* (Young et al., 2001)], with one from Ecuador closely related to the *R. radiobacter*-type strain, two from Mexico related to *Rhizobium pusense* (Panday et al., 2011) and one from Ecuador close to *Rhizobium nepotum* (Pulawska et al., 2012). As expected, the low resolving power of the 16S rRNA gene—due to its high conservation—did not allow clear species definition for the majority of strains. This was especially the case for related species, such as those of the *R. etli* group, some of which shared more than 97% sequence identity, primarily defined as the border limit of species definition (Stackebrandt and Goebel, 1994). In further reviews, it has been proposed that this value should be raised to 97%–99% (Stackebrandt and Ebers, 2006), and more specifically, a value of around 98.5% may be defined at which DNA–DNA hybridization (DDH) experiments should be obligatory for testing genomic uniqueness (Stackebrandt, 2011).

3.2. Single gene housekeeping phylogenies

Phylogenetic affiliations were further investigated with the analysis of the core genes *recA*, *glnII*, *gyrB* and *rpoA*, which encode recombinase A protein, glutamine synthase II, DNA gyrase B subunit and RNA polymerase alpha subunit, respectively. The amount of phylogenetic information associated with each gene was assessed by calculating the number and percentage of variable and parsimony-informative sites (Table 2). Gene *gyrB* had the highest number and percentage of parsimony-informative sites, with 233 characters (40%), whereas *rpoA* had the lowest with 74 characters (18%) (Table 2). All four phylogenies showed three major clusters, corresponding to the *R. etli*, *R. tropici* and *R. radiobacter* groups (Figs. S1A to S1D), which mimicked the major clusters observed in 16S rRNA gene analysis (Fig. 1). Nonetheless, a far greater resolution than with the 16S rRNA gene was obtained, with an emphasis on the *R. etli* group. An exception was the *rpoA* phylogeny where most clades did not show significant bootstrap support.

Not all single-gene-based phylogenies were congruent, but similar species-level clusters were recovered within each major group in most phylograms, as previously observed in other studies with *Rhizobium* (Hou et al., 2009; Ribeiro et al., 2009). Coalescent theory (Barraclough et al., 2009) may account for this observation, and Castillo-Ramirez and Gonzalez (2008) suggested that single gene phylogenetic tree incongruences may be due to the close relatedness of bacterial groups that show uneven segregation of different gene alleles from an ancestral polymorphic population. The 19 strains under study were distributed in 10 or 11 groups in the single gene phylogenies (Figs. S1A to S1D). Exceptions were strain CNPSo 659, which grouped with the same five strains in all phylogenies except in *rpoA*, and strain CNPSo 666, which grouped with two strains in all phylogenies, except in *recA*.

3.3. MLSA analysis reveals novel species-level lineages among common bean rhizobia

By combining the phylogenetic information of several genes, the analysis of concatenated genes in the MLSA approach allows a better resolution in species definition in comparison to single gene analysis; in addition, it can also alleviate the distorting effects of horizontal gene transfer (Gevers et al., 2005; Martens et al., 2008). Concatenated sequences of the four housekeeping genes resulted in a 1846-nt-long alignment comprising 593 (32.1%) and 486 (26.3%) variable and parsimony informative sites, respectively (Table 2). In the resulting MLSA phylogeny (Fig. 2), strain CNPSo 664 from Mexico grouped tightly with *R. etli* CFN 42^T (Fig. 2). Strain CNPSo 669, also from Mexico, grouped with strain IE4771, previously designated as *R. etli*, but

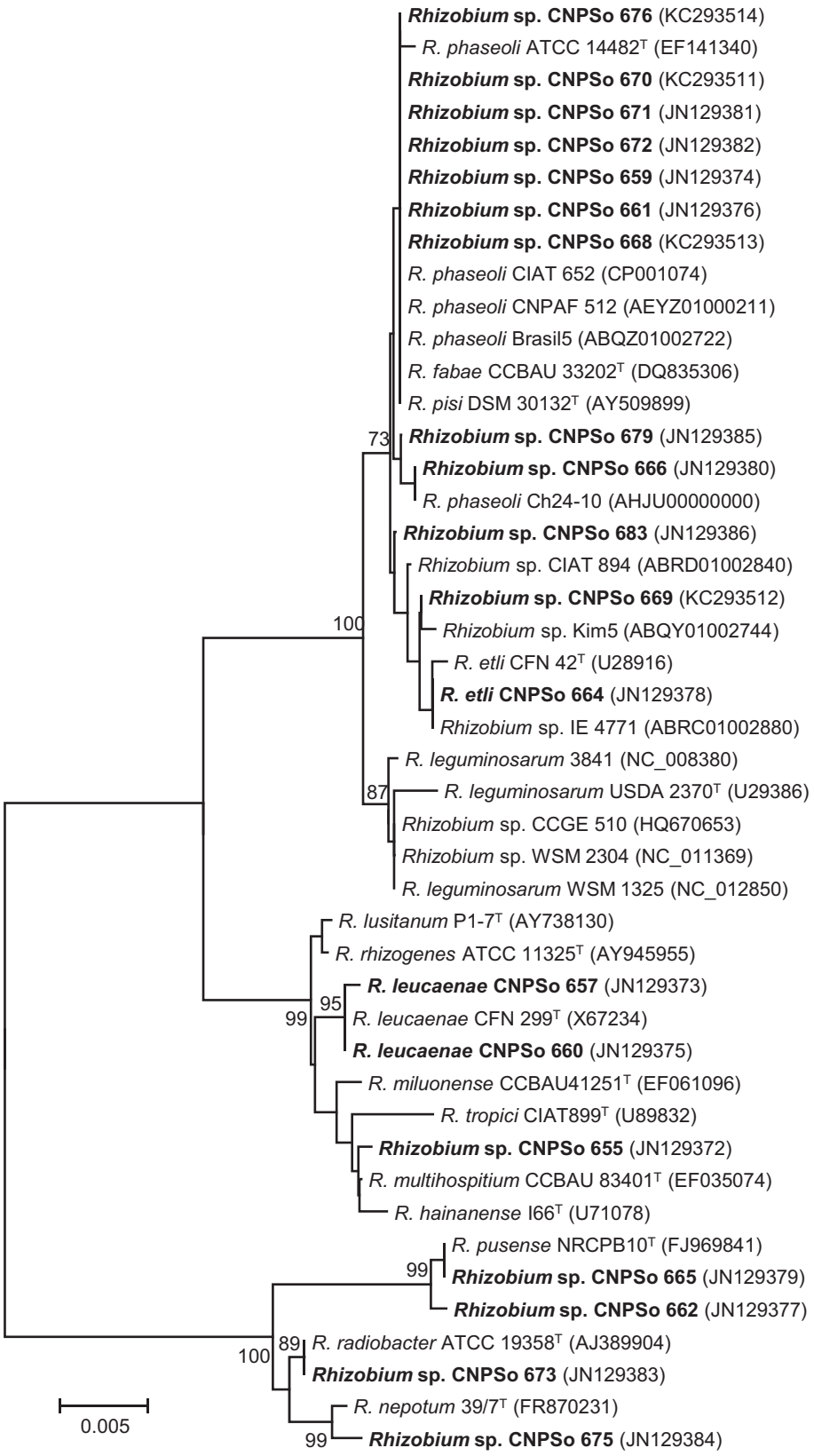

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA sequences (1226 nt) of *Rhizobium* strains from this study (shown in bold) and type/reference strains. Bootstrap support values ≥70% are shown at tree nodes. GenBank accession numbers are provided within parenthesis. Bar, 5 nt substitutions per 1000 nt.

Table 2
Number of sequences and phylogenetic information of analyzed loci.

Locus	Strains analyzed	Number of nucleotides (percentage)			
		Conserved	Variable	Parsimony-informative	Total
16S rRNA	45	1143 (93.2)	83 (6.8)	69 (5.6)	1226
glnII	53	312 (69.3)	138 (30.7)	118 (26.2)	450
gyrB	51	320 (55)	262 (45)	233 (40)	582
recA	50	250 (66.1)	128 (33.9)	111 (29.4)	378
rpoA	42	321 (79.7)	82 (20.3)	74 (18.4)	403
Concatenated genes[a]	38	1253 (67.9)	593 (32.1)	486 (26.3)	1846

[a] Concatenated alignment of *glnII* (467 nt), *gyrB* (583 nt), *recA* (390 nt) and *rpoA* (406 nt).

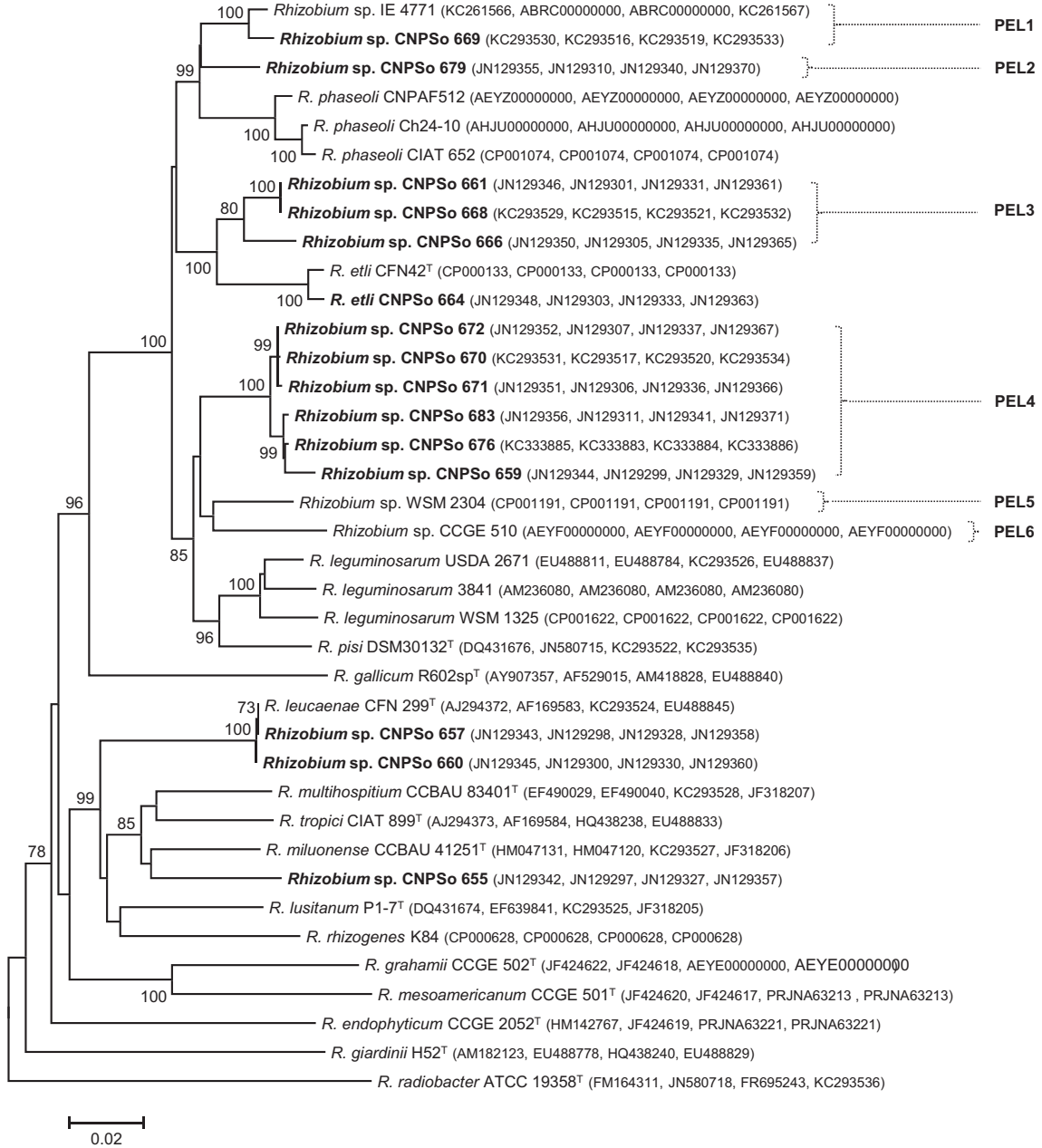

Fig. 2. Neighbor joining phylogenetic tree based on a concatenated alignment of *recA* (390 nt), *glnII* (467 nt), *gyrB* (583 nt) and *rpoA* (406 nt) sequences of *Rhizobium* strains from this study (shown in bold) and type/reference strains. Bootstrap support values ≥70% are shown at tree nodes. Novel lineages named in this study are indicated at the right. GenBank accession numbers are provided in parenthesis. Bar, 2 nt substitutions per 100 nt.

which has been shown to constitute an independent lineage different from *R. etli* and *R. phaseoli* (López-Guerrero et al., 2012b) and which will be referred to here as phaseoli-etli-leguminosarum lineage 1 (PEL1). Surprisingly, all remaining strains assigned to the *R. etli* group in the analysis of the 16S rRNA gene represented novel lineages. Ecuadorean strain 679 was positioned independently as novel lineage PEL2, whereas Mexican strains CNPSo 661, 666 and 668 constituted another lineage, PEL3. Strains CNPSo 676, 683, 670, 671 and 672 from Ecuador and 659 from Mexico grouped with 100% bootstrap support and formed the novel lineage PEL4. Indeed, we performed DDH experiments with PEL4 lineage strains and closely related rhizobial species, and the values obtained were below 70%, indicating that the lineage should represent a novel species (data not shown). It is also noteworthy that PEL4 strains were previously shown to be abundant in northern and central Ecuador (Bernal and Graham, 2001). Interestingly, of the *R. leguminosarum* strains with sequenced genomes, only 3841 (sv. viciae, isolated from *Pisum sativum* in the United Kingdom) and WSM1325 (sv. trifolii, Mediterranean origin, isolated from *Trifolium pratense),* may be ascribed to the species *R. leguminosarum*. In contrast, strain WSM2304 (sv. trifolii, isolated from *Trifolium polymorphum* in Uruguay) represented an independent lineage, designated here as PEL5. Similarly, strain CCGE510, isolated in Mexico from the non-domesticated species *Phaseolus albescens* and having a *R. leguminosarum*-like 16S rRNA gene (Servín-Garcidueñas et al., 2012), was ascribed as an independent lineage, for which the designation PEL6 was given. Two previously identified distinct *Rhizobium* lineages (López-Guerrero et al., 2012b), named here as PEL7 (for strain GR56, Figs. S1A to S1C) and PEL8 (for strain CIAT 894, Figs. S1B to S1D) could be recognized in single-gene phylogenies, but were not included in the MLSA analysis, as no sequences for the four loci were available. Phylogenies of *recA* and *glnII* (Figs. S1A and S1B, respectively) indicated that all PEL lineages proposed here are distinct from Ethiopian bean-nodulating strains from *Rhizobium* groups A and C defined by Aserse et al. (2012).

The range of nucleotide identities calculated for the concatenated alignment (including strain IE4771, representing a new lineage in a previous study by López-Guerrero et al., 2012b), was of 82.8–95.8% between recognized species, and of 97–100% within species. Thus, for this particular set of *recA* + *glnII* + *gyrB* + *rpoA* concatenated genes (Table 2) and strains analyzed (Fig. 2), 97% seems to represent a threshold that circumscribes species. Identity within the novel PEL lineages was always higher than 97%, and the maximum identity between PEL lineages, and any other species or lineage, was of 96.8%. If we exclude the comparison between the closely related lineages PEL1 and PEL2 (Fig. 2), the maximum identity was of 96%. These results indicate that most novel PEL lineages identified here might represent distinct species, as 95–97% of ANI (average nucleotide identity) of core genes of the genome or concatenated housekeeping genes in the MLSA approach seems to correspond to the 70% DNA–DNA hybridization cut-off (Konstantinidis et al., 2006; Martens et al., 2008; Stackebrandt, 2011).

The phylogenetic position of the three strains belonging to the *R. tropici* group by 16S rRNA gene sequences was also analyzed by MLSA. Brazilian strains CNPSo 657 and 660 grouped tightly with *R. leucaenae* CFN 299^T (99.9–100% identity) and possessed the 72-bp insertion in the 16S rRNA gene that is characteristic of *R. leucaenae* (Ribeiro et al., 2012). Previously, we reported that *R. leucaenae* isolates are commonly found in Brazil (Mercante et al., 1998; Ribeiro et al., 2012). On the other hand, Mexican strain CNPSo 655 did not group with any of the described species within the *R. tropici* group and seemed to represent an independent lineage as it displayed a maximum identity of only 93.8% with the other species. Phylogenies of the *recA*, *glnII* and *rpoA* genes showed that strain CNPSo 655 can also be differentiated from other subgroups within the *R. tropici* group previously reported (Ribeiro et al., 2009) and represented by strains PRF 35, 233 and PRF 81 (data not shown).

3.4. Common bean endophytes within the Rhizobium radiobacter group

Analysis of *recA* and *gyrB* gene sequences (Figs. S1A and S1C) confirmed that Ecuadorean strain CNPSo 673 grouped tightly with *R. radiobacter* LMG 140^T, whereas Mexican strains CNPSo 662 and 665 were strongly related to *R. pusense* NRCPB10^T. The Ecuadorean strain CNPSo 675 clustered with *R. nepotum* 39/7^T, but seemed to constitute a different species. We were unable to amplify *nodB* and *nifH* fragments from any of these *P. vulgaris* strains. As we described before, these strains were able to nodulate common beans but were not effective in fixing nitrogen. Amplification of *nodB* of common bean rhizobia has shown to be a difficult task, due to the high diversity of the gene (e.g. Grange et al., 2007), which could explain the lack of amplification with these novel strains. In relation to *nifH*, in association with the Fix- phenotype this suggests that the strains have unstable symbiotic plasmid, as recently reported for other common bean symbionts (López-Guerrero et al., 2012a; Ormeño-Orrillo et al., 2012).

R. radiobacter, *R. nepotum* and *R. pusense* belong to a phylogenetic clade formerly known as agrobacteria and considered as non-symbiotic. *R. pusense* was described as a non-symbiotic bacterium isolated from the rhizosphere of chickpea (*Cicer arietinum* L.) and here we report that two strains—CNPSo 662 and 665—clustering with this species in *recA* and *gyrB* trees can also nodulate common bean roots. Other studies have reported isolation from nodules of common bean of agrobacteria that lost effectiveness (Mhamdi et al., 1999). In addition, agrobacterial isolates able to nodulate *Acaciella angustissima* in Mexico were found to have low efficiency for N_2 fixation (Rincón-Rosales et al., 2009). However, strains resembling agrobacteria, obtained from nodules of primitive and modern genotypes of soybean grown in South America (Chen et al., 2000; Hungria et al., 2006), were effective in fixing N_2 with the modern soybean cultivars (Chen et al., 2002; Hungria et al., 2001). Interestingly, transfer of the symbiotic plasmid from *Rhizobium* to *Agrobacterium* under laboratory conditions led to some transconjugants acquiring nodulation and N_2-fixing capabilities albeit with reduced efficiency (Martínez et al., 1987; Novikova and Safronova, 1992). Certainly, this group of strains resembling agrobacteria deserves further study.

3.5. Rhizobium *lineages naturally associated with* P. vulgaris

Rhizobia that nodulate the common bean have been assigned to the species *R. leguminosarum* (Jordan, 1984), *R. tropici* (Martínez-Romero et al., 1991), *R. etli* (Segovia et al., 1993), *Rhizobium gallicum*, *Rhizobium giardinii* (Amarger et al., 1997), *Rhizobium lusitanum* (Valverde et al., 2006), *Rhizobium multihospitium* (Han et al., 2008), *R. phaseoli* (Ramirez-Bahena et al., 2008), *Rhizobium vallis* (Wang et al., 2011), *R. leucaenae* (Ribeiro et al., 2012), *Rhizobium grahamii* and *Rhizobium mesoamericanum* (López-López et al., 2012). Some other un-named, species-level *Rhizobium* lineages have been described as well, in America where the common bean is native (López-Guerrero et al., 2012b) and in areas where the common bean is exotic, such as Ethiopia (Aserse et al., 2012) and Brazil (Ribeiro et al., 2009; Stocco et al., 2008). Although the small sample analyzed here does not represent the diversity of bean rhizobia in Mexico and Ecuador, the results highlight the existence of additional native lineages naturally nodulating this crop in these two areas of cultivation. How many other *Rhizobium* lineages are naturally associated with the common bean? Undoubtedly, MLSA analysis of bean rhizobia from other cropping areas such as Peru, Bolivia, Argentina and Brazil will shed light on this question.

Related bean-rhizobial lineages seem to coexist and, at a single site, multiple species may simultaneously nodulate the common bean. This raises the interesting question of what drives this lineage abundance. Although lineages may be a consequence of low recombination and clonality in some rhizobial groups (Acosta et al., 2011), it is clear that there is a parallel diversity in *P. vulgaris* plants that may select different rhizobial lineages and species. For example, Mesoamerican bean cultivar RAB39 (Montealegre et al., 1995), but not wild *P. vulgaris* (Kipe-Nolt et al., 1992), nodulates preferentially with *R. tropici*. Ecuadorian beans were preferentially nodulated by local strains (Bernal and Graham, 2001). Wild *P. vulgaris* also shows preferences for local rhizobial strains (Aguilar et al., 2004).

Other lineages may be selected by the environment. Mexican strain IE4771 of lineage PEL1 is a representative of a group previously shown to be the dominant common bean rhizobia in San Miguel Acuexcomac, Puebla, a site characterized by alkaline soil (pH 8.4) (Silva et al., 2003). In contrast, in acid soils, there was a predominance of *R. tropici* in Brazil (Stocco et al., 2008) and in Africa (Anyango et al., 1995). Interestingly, another study showed that common bean rhizobial populations isolated directly from soil were very different from those "trapped" in root nodules of plants inoculated with the same soil under greenhouse conditions (Alberton et al., 2006). Therefore, collecting information on the environment, sampling procedure and cultivar in future studies of common-bean-nodulating rhizobia, will surely be aimed at elucidating the distribution patterns of these bacteria.

Acknowledgments

The study was partially supported by the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil), Project Repensa (562008/2010-1). The authors acknowledge Ligia Maria O. Chueire and Jakeline R. M. Delamuta for help in several steps of this work, and Allan R.J. Eaglesham for suggestions on the manuscript. R.A. Ribeiro acknowledges a PhD fellowship from the Fundação Araucária (Paraná, Brazil), and M. Hungria a research fellow from the CNPq (300547/2010-2). The laboratories acknowledge a bilateral collaboration project CNPq/CONACYT (490048/2009-9). Approved for publication by the Editorial Board of Embrapa Soja as manuscript 01/2013.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2013.05.002.

Supplementary Table 1
GenBank accession numbers for the sequences obtained in this study.

Strain	16S rRNA	*recA*	*glnII*	*gyrB*	*rpoA*
Rhizobium sp. CNPSo 655	JN129372	JN129342	JN129297	JN129327	JN129357
Rhizobium sp. CNPSo 657	JN129373	JN129343	JN129298	JN129328	JN129358
Rhizobium sp. CNPSo 659	JN129374	JN129344	JN129299	JN129329	JN129359
Rhizobium sp. CNPSo 660	JN129375	JN129345	JN129300	JN129330	JN129360
Rhizobium sp. CNPSo 661	JN129376	JN129346	JN129301	JN129331	JN129361
Rhizobium sp. CNPSo 662	JN129377	JN129347	JN129302	JN129332	JN129362
Rhizobium etli CNPSo 664	JN129378	JN129348	JN129303	JN129333	JN129363
Rhizobium sp. CNPSo 665	JN129379	JN129349	JN129304	JN129334	JN129364
Rhizobium sp. CNPSo 666	JN129380	JN129350	JN129305	JN129335	JN129365
Rhizobium sp. CNPSo 668	KC293513	KC293529	KC293515	KC293521	KC293532
Rhizobium sp. CNPSo 669	KC293512	KC293530	KC293516	KC293519	KC293533
Rhizobium sp. CNPSo 670	KC293511	KC293531	KC293517	KC293520	KC293534
Rhizobium sp. CNPSo 671	JN129381	JN129351	JN129306	JN129336	JN129366
Rhizobium sp. CNPSo 672	JN129382	JN129352	JN129307	JN129337	JN129367
Rhizobium sp. CNPSo 673	JN129383	JN129353	JN129308	JN129338	JN129368
Rhizobium sp. CNPSo 675	JN129384	JN129354	JN129309	JN129339	JN129369
Rhizobium sp. CNPSo 676	KC293514	KC333885	KC333883	KC333884	KC333886
Rhizobium sp. CNPSo 679	JN129385	JN129355	JN129310	JN129340	JN129370
Rhizobium sp. CNPSo 683	JN129386	JN129356	JN129311	JN129341	JN129371

Supplementary Table 1 (*continued*)

Strain	16S rRNA	*recA*	*glnII*	*gyrB*	*rpoA*
Rhizobium sp. IE4771	–	KC261566	–	–	KC261567
R. leucaenae CFN 299^T	–	–	–	KC293524	–
R. multihospitium CCBAU 83401^T	–	–	–	KC293528	–
R. miluonense CCBAU 41251^T	–	–	–	KC293527	–
R. lusitanum P1-7^T	–	–	–	KC293525	–
R. leguminosarum USDA 2671	–	–	–	KC293526	–
R. phaseoli ATCC 14482^T	–	–	–	KC293518	–
R. pisi DSM 30132^T	–	–	–	KC293522	KC293535
R. fabae CCBAU 33202^T	–	–	–	KC293523	–
R. radiobacter ATCC 19358^T	–	–	–	–	KC293536

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