Ann Microbiol (2015) 65:1803–1808
DOI 10.1007/s13213-015-1054-9

SHORT COMMUNICATION

Phenotypic and molecular differences among rhizobia that nodulate *Phaseolus lunatus* in the Supe valley in Peru

Minoru Matsubara · Doris Zúñiga-Dávila

Received: 2 December 2014 / Accepted: 30 January 2015 / Published online: 17 February 2015
© Springer-Verlag Berlin Heidelberg and the University of Milan 2015

Abstract A collection of 16 bacterial strains isolated from root nodules of Lima bean (*Phaseolus lunatus* L.) in the Supe valley of Peru were characterised using phenotypic and molecular methods. The isolates were clustered into fast-growing, alkalinising and extra-alkalinising slow-growing isolates with marked morphological differences, according to the rate of growth and alkalinisation of yeast extract mannitol (YEM) medium. Fast-growing isolates were salt tolerant and sensitive to 40 °C, while alkalinising slow-growing isolates behaved oppositely. Extra-alkalinising slow-growing isolates were sensitive to 8 °C and 40 °C and tolerated 1 % NaCl. Fast-growing isolates also showed higher indole-3-acetic acid (IAA) production and tri-calcium phosphate solubilisation than the alkalinising slow-growing isolates. Half of the isolates were able to nodulate *Phaseolus vulgaris* and *Vigna unguiculata*. Individually, BOX-PCR, ERIC-PCR, and REP-PCR fingerprints patterns resulted in ten profiles from 16 isolates and they were clustered into three profile groups that correspond to the clusters obtained by YEM medium alkalinisation. Analysis of 16S rRNA gene sequences revealed that fast-growing isolates showed 99.7 % sequence identity with *Rhizobium mesosinicum* CCBAU 25010^T and *Rhizobium alamii* GBV016^T. Alkalinising slow-growing isolates were related to both *Bradyrhizobium yuanmingense* CCBAU10071^T and *Bradyrhizobium liaoningense* 2281^T, with 99.8 % sequence identity, and extra-alkalinising slow-growing isolates had 100 % sequence identity to both *Bradyrhizobium paxllaeri* LMTR 21^T and *Bradyrhizobium icense* LMTR 13^T.

M. Matsubara · D. Zúñiga-Dávila (✉)
Laboratorio de Ecología Microbiana y Biotecnología "Marino Tabusso", Dpto. Biología, Facultad de Ciencias, Universidad Nacional Agraria La Molina - UNALM, Av. La Molina s/n, Lima, Perú
e-mail: dzuniga@lamolina.edu.pe

Keywords *Phaseolus lunatus* · Peru · *Bradyrhizobium* · *Rhizobium* · Phenotypic characterization · REP-PCR · 16S

Findings

Lima bean or pallar (*Phaseolus lunatus* L.) is the second most socioeconomically important crop among the *Phaseolus* species. Peru is one of the centers of Andean origin and diversity of the Lima bean, where this legume has been domesticated and cultivated since pre-Hispanic times. Cultivated Peruvian seed varieties are large, flat and white and belong to the Big Lima culti group (Fofana et al. 1997). It is known that Lima bean can also fix nitrogen in symbiosis with slow-growing rhizobial species from the *Bradyrhizobium* genus, like *B. yuanmingense*, *B. paxllaeri* and *B. icense* (Vinuesa et al. 2005; Ormeño-Orrillo et al. 2006; Durán et al. 2014), while *Ensifer meliloti* LMTR32 (formerly *Sinorhizobium meliloti*) is the only fast-growing rhizobia strain reported to nodulate *P. lunatus* (Ormeño et al. 2007).

Given the importance of *P. lunatus* in sustainable agriculture and the development of Peru, and the scarce knowledge about rhizobial specificity for this legume, we decided to study rhizobia naturally associated with this legume in local farms of the Supe valley, a recognized *P. lunatus* farming area with no records of rhizobia isolations.

Sixteen bacterial strains were used in this work. They were isolated from independent nodules collected in three fields of *P. lunatus* culti group Big Lima var. Criollo, in the Supe valley at the north-central coast of Peru. The sampling sites were Tutumo-1 (S 10° 51' 13.4" WO 77° 38' 27.0"), Venturosa-1 (S 10° 51' 08.9" WO 77° 38' 20.4"), and Venturosa-3 (S 10° 51' 33.1" WO 77° 38' 20.6") (Table 1). Soils at collection sites had low macronutrient content and were sandy, slightly

Table 1 Geographical origin, symbiotic and main phenotypic characteristics of strains analysed in this study

	Isolate LMTR No.																*B. yuanmingense*		*B. elkanii*	*B. diazoef.*
	R3	R6	M2	M70	M7	M60	M29	M32	M47	M50	M53	M42	M56	M49	M62	M54	LMTR28	CCBAU 10071[T]	USDA76[T]	USDA110[T]
Geographical origin[a]	T1	T1	T1	T1	T1	T1	V1	V1	V3	V3	V3	V3	V3	V3	V3	V3	Peru	China	USA	USA
Days of colony appearance	2	2	5-7	5-7	5-7	5-7	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Alkalinisation of YEM/BTB[b]	Y	Y	B	B	B	B	GB	GB	GB	GB	GB	GB	GB	GB	GB	GB	GB	GB	GB	GB
Category at YEM/BTB[c]	F	F	XS	XS	XS	XS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS
Growth at[d]																				
8 °C	w	w	-	-	-	-	-	-	w	w	w	w	w	w	w	w	w	w	w	+
40 °C	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+
1 % NaCl	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-
2 % NaCl	w	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tri-calcium phosphate solubilization (mm)	2	4	0	0	0	0	0	0	0	2	0	2	2	0	2	2	2	1	3	0
IAA production (μg ml^{-1})	35	54	<0.5	<0.5	<0.5	<0.5	4	4	3	5	7	3	11	4	6	9	6	2	20	<0.5
Nodulation test of																				
Phaseolus vulgaris	-	-	+	+	-	-	-	-	+	+	-	+	-	-	+	+	+	-	+	-
Vigna unguiculata	nd	nd	-	+	+	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+
Phaseolus lunatus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-

[a] Sampling sites in Supe Valley-Peru were T1: Tutumo-1; V1: Venturosa-1; V3:Venturosa-3

[b] Alkalinisation of YEM/BTB: BTB change of colour from green to yellow (Y, acid), green-blue (GB, alkali), and blue (B, extra-alkali)

[c] Category at YEM: fast-growing (F), slow-growing alkalinising (AS), slow-growing extra-alkalinising (XS)

[d] All strains grew at 28 °C, 37 °C, 0.5 % NaCl, pH 4, 4.5, 8 and 8.8

saline and moderately alkaline. The typical weather was subtropical arid, with temperatures between 15-26 °C and precipitation levels between 0 and 3.2 mm. Reference strains of *Bradyrhizobium* were also included for analysis. All strains were maintained in 25 % (v/v) glycerol in yeast extract mannitol (YEM) broth at −80 °C.

Strains were Gram stained. Colony morphology and acid/alkaline reaction were evaluated on plates of yeast extract mannitol (YEM) solid medium containing 0.025 % (v/v) bromothymol blue (BTB) at 28 °C (Vincent 1970). Five microliters of bacterial cultures (10^8cell/ml) were inoculated onto plates of YEM solid medium with 0.5, 1 and 2 % (w/v) NaCl, or with pH adjusted to 4, 5, 8 and 8.8, respectively (Matos et al. 1998). In addition, cultures were inoculated onto YEM plates that were kept at 8, 37 and 40 °C, respectively. Most of the plates were incubated from 2 to7 days, and those that were kept at 8 °C were evaluated up to 30 days. Additionally, all the strains that were used were grown on YEM solid medium with pH 7 and 0.02 % (w/v) NaCl at 28 °C, and this condition was considered as a standard control (Vincent 1970).

Evaluation of phosphate solubilisation was conducted according to Nautiyal (1999) on NBRIP plates with bi-calcium or tri-calcium phosphate. Plates were inoculated with 5 μl of YEM bacterial cultures (10^8 cell/ml) and incubated at 28 °C. The production of a halo of phosphate solubilisation around the colonies was recorded for up to 30 days, and halo size was calculated according to Nautiyal (1999).

For production of indole-3-acetic acid (IAA), strains were grown in YEM broth supplemented with 5 mM L-tryptophan at 28 °C for 2 to 7 days (Glickmann and Dessaux 1995). Then they were centrifuged (1,200 rpm, 5 min) and supernatants were mixed in a 1:5 ratio with Salkowski reagent (10 mM $FeCl_3$ in 36 % H_2SO_4) and left in the dark for 15 min at room temperature (Gordon and Weber 1951). Absorbance was determined on a spectrophotometer at 530 nm and concentration was calculated with an IAA standard curve. Non-inoculated broth was used as a negative control.

Seeds of *P. lunatus* cv-gr. Sieva var. UNALM-1, *Vigna unguiculata* var. Vaina-Blanca and *P. vulgaris* var. Canario-Centenario were used for experiments. Axenically grown surface-sterilized seedlings were transferred to 50-ml glass containers filled with polypropylene pellets and watered with N-free nutrient solution (Broughton and Dilworth 1971). Each plant was inoculated with one milliliter of a bacterial suspension (10^8 cell/ml) of each strain. Inoculated plants were placed in a growth chamber with controlled temperature (22–25 °C) and light cycle (13 h/day) for 30 days (*P. lunatus*) and 45 days (*P. vulgaris* and *V. unguiculata*) . Experiments were performed in triplicates per bacterial strain per plant. Results were recorded as presence or absence of nodulation per plant, and a control with no inoculation was used.

DNA was extracted from bacterial colonies. Colonies were suspended in 20 μl lysis buffer (0.05 M NaOH, 0.25 % SDS) and incubated for 10 min at 80 °C and 10 min in an ice bath. The lysate was diluted in 200 μl Milli-Q sterile water and spun down in a microcentrifuge for 5 minutes. Five microliters of the supernatant were used for PCR amplification of BOX-PCR, ERIC-PCR, and REP-PCR genomic fingerprints (Versalovic et al. 1991, 1994). DNA fragments were separated in a 1.5 % agarose gel. Genomic fingerprints were visually recorded, relationships were analysed by the UPGMA method, and distances were calculated with the Jaccard coeficcient using NTSYS-PC 2.0 software (Rohlf 1998).

Primers fD1 and rD1 were used to amplified 16S rRNA (Weisburg et al. 1991). Phylogenetic neighbors of the isolates were identified using the EzTaxon server (http://www.ezbiocloud.net/eztaxon; Kim et al. 2012) on the basis of 16S rRNA sequence data. Both studied and selected sequences were aligned by the Clustal X2 software (Larkin et al. 2007). Phylogenetic analysis was performed by the Neighbor-Joining (NJ) method and distances were calculated according to the Kimura-2 method using the MEGA 6 package (Tamura et al. 2013).

The 16 isolated strains were Gram-negative rods. Isolates grew in 2 days (fast-growing) and 5–7 days (slow-growing) (Vincent 1970). They were divided into two fast-growing isolates, ten alkalinising slow-growing (SG) isolates and four extra-alkalinising SG isolates, according to the colour change of the pH-indicator dye in YEM–BTB medium from green to yellow, green-blue and blue, respectively (Table 1). Additionally, fast-growing isolates had colonies of 4–6 mm with abundant and soft gum. Alkalinising SG isolates showed 1–2 mm colonies with regular and hard gum, while extra-alkalinising SG isolates had 0.5-1 mm colonies with no gum. The fast-growing and extra-alkalinising SG isolates shared the same sample site, in contrast to alkalinising SG isolates that were isolated from different sites.

All isolates grew at 28 °C and 37 °C, 0.5 % NaCl and at an acid pH (4 and 5) and an alkaline pH (8 and 8.8). All fast-growing and extra-alkalinising SG isolates failed to grow at 40 °C. No extra-alkalinising SG isolates grew at 8 °C and the others isolates grew weakly. All isolates grew with 0.5 % (w/v) NaCl, while fast-growing and extra-alkalinising SG isolates grew with 1 % (w/v) NaCl. In the presence of 2 % (w/v) NaCl, fast-growing isolates showed poor growth (Table 1). Salt tolerance varies among rhizobial species, and it is still a useful characteristic for distinguishing related species. Some bradyrhizobia related to *P. lunatus*, like *B. yuanmingense*, do not tolerate 1 % (w/v) NaCl (Yao et al. 2002), while others, such as *B. paxllaeri and B. icense*, can (Durán et al. 2014).

Fast-growing isolates produced 35–54 μg IAA/ml, which is approximately six times more IAA than the amount produced by alkalinising SG isolates (Table 1). Extra-alkalizing SG isolates produced less than 0.5 μg/ml of this auxine. IAA

production has been observed in many strains of rhizobial species (Boiero et al. 2007). Solubilisation of phosphate was only detected when tri-calcium phosphate was used as substrate. Solubilisation halos were produced by fast-growing isolates after 10 days of incubation (Table 1), whilst some alkalinising SG isolates produced a halo only after 30 days of incubation. Nonetheless, phosphate solubilisation detection based on halo formation on agar plates is not an infallible technique, since many isolates that do not show any halo can solubilise insoluble phosphates in liquid medium (Nautiyal 1999). In addition, reference strain *Bradyrhizobium elkanii* USDA76 produced higher levels of IAA (20 μg/ml) and solubilised both bi-calcium and tri-calcium phosphate, but its halos were larger in bi-calcium-containing media, which has not been reported.

All the isolates nodulated *P. lunatus* as was expected, since this is the original host (Table 1). Both *P. vulgaris* and *V. unguiculata* were nodulated with just over the half of the *P. lunatus* isolates, but there was no evidence of nitrogen fixation on *P. vulgaris* nodules since they were small and did not show the typical leghaemoglobin reddish colour. Also, soybean-derived reference strains *B. elkanii* USDA76^T and *Bradyrhizobium diazoefficiens* USDA110^T (formerly *B. japonicum*) did not nodulate *P. lunatus* (Table 1). *Bradyrhizobium yuanmingense* CCBAU10071^T nodulated *P. lunatus*, despite that it was isolated from *Lespedeza cuneata* (Yao et al. 2002). This species was also reported to nodulate *Pachyrhizus erosus* (Rodríguez-Navarro et al. 2004), *V. unguiculata* and *V. radiata* (Zhang et al. 2008). Further studies of nodulation are required with *P. lunatus*, *L. cuneata* and *Glycine max* to confirm nodulation specificity, since *B. yuanmingense* CCBAU10071^T was not able to nodulate *Glycine max* (Yao et al. 2002). These differences could imply modifications to bradyrhizobia cross-inoculation groups and the existence of symbiovars among *Bradyrhizobium* spp.

Genomic fingerprints analysis of the 16 root-nodule isolates resulted in ten different profiles for each primers set; nonetheless, ERIC-PCR and REP-PCR clustered the isolates in the same groups, differing slightly from BOX-PCR results (Fig. 1). The combined analysis of the profiles of the three techniques showed, at 9 % of similarity, three major clusters of profiles that correspond to the same classifications obtained with the YEM–BTB medium: fast-growing isolates (I and II), extra-alkalinising SG (III) and alkalinising SG (IV to XI). Most of REP-PCR groups were restricted to their nodular origin and sampling sites but REP-PCR group III isolates (extra-alkalinising SG) came from different nodules from the same sampling site. Despite the low sample size, the profiles from the three PCR amplifications revealed that there appears to be more genetic diversity within alkalinising SG isolates than within extra-alkalinising ones, although all the isolates were obtained from independent nodules collected from different plants.

16S rRNA gene sequencing revealed that, at 99.7 % sequence identity, the fast-growing isolate LMTR-R3 was phylogenetically related to *Rhizobium mesosinicum* CCBAU

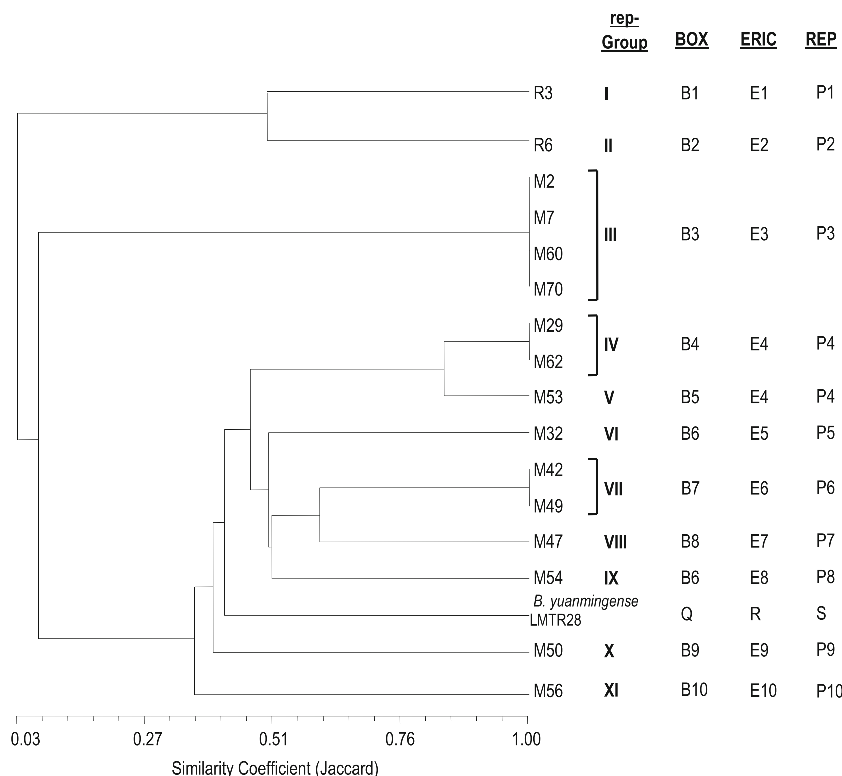

Fig. 1 Dendrogram generated from the combined REP-PCR, ERIC-PCR and BOX-PCR fingerprints patterns of Lima bean isolates and *B. yuanmingense* LMTR28 (reference strain) grouped by UPGMA and Jaccard coefficient. The same pattern code within columns corresponds to isolates with similar banding patterns

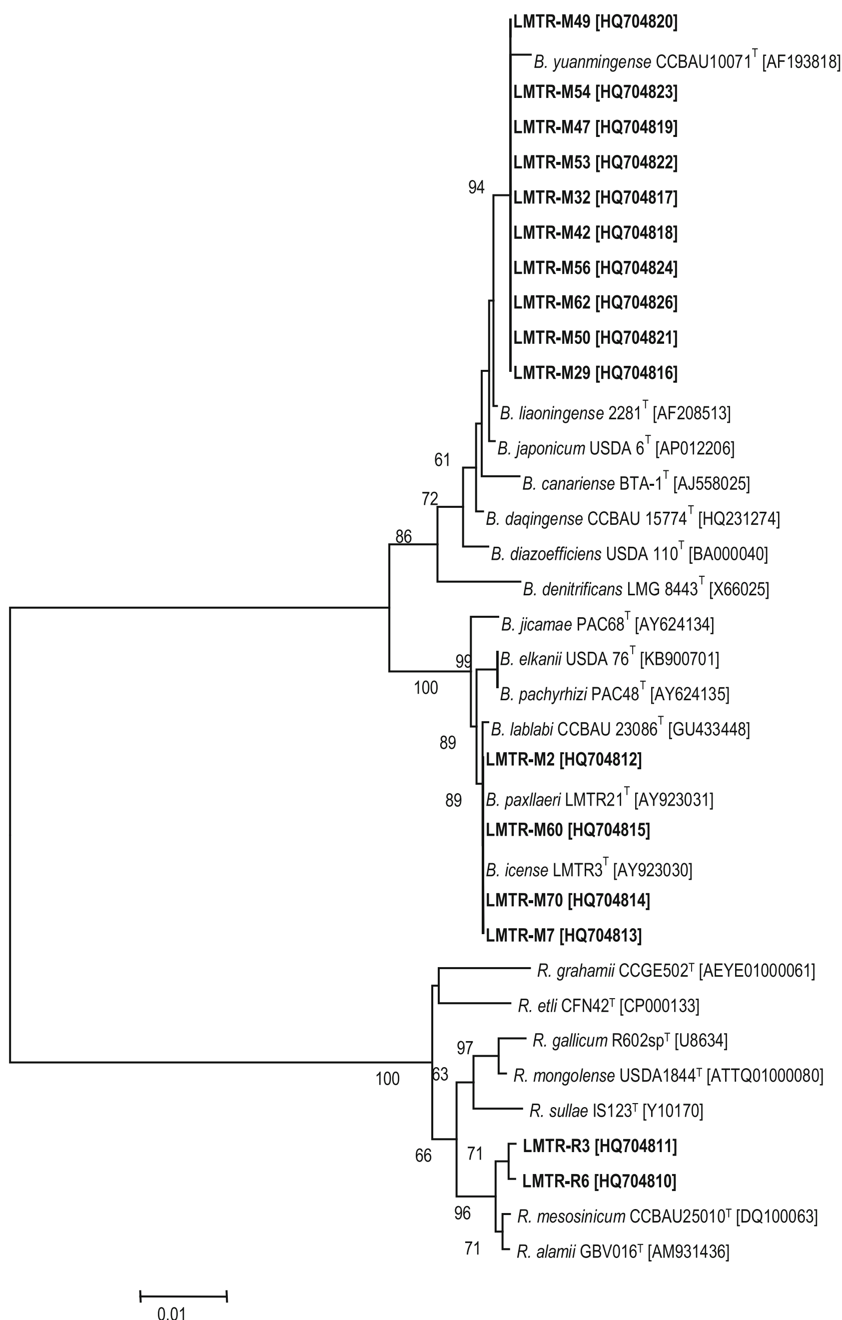

Fig. 2 Phylogenetic analysis of 16S rRNA sequences from isolates obtained in this study. Type strains were included for analysis with the Neighbor joining method. Strains having the same sequence are shown in the same terminal branch. Lima bean isolates are indicated in bold and sequence accession numbers are given within brackets. Only bootstrap values greater than 60 % are shown (1,000 pseudoreplicates)

25010^{T,} and LMTR-R6 was related to *Rhizobium alamii* GBV016^{T} (Fig. 2). Alkalinising SG isolates were related to both *B. yuanmingense* CCBAU10071^{T} and *B. liaoningense* 2281^{T,} with 99.8 % sequence identity, and extra-alkalinising SG isolates had 100 % sequence identity to both *B. paxllaeri* LMTR 21^{T} and *B. icense* LMTR 13^{T}. These results suggest that fast-growing and slow-growing isolates belong to the *Rhizobium* and *Bradyrhizobium* genus, respectively. The fact that most of the isolates are from the Supe valley and from others areas of the Peruvian coast, supports the idea that *P. lunatus* specifically selects bradyrhizobial genotypes as its predominant microsymbionts (Ormeño-Orrillo et al. 2006).

Despite 16S rRNA gene sequence analysis providing important information about bacteria identity, it is necessary to conduct a sequence analysis of other house-keeping genes or ITS sequences for establishing an accurate identity of the isolate. Some species have identical *16S* rRNA gene sequences but vary in their *recA*, *atpD*, *glnII*, *dnaK* and *gyrB* genes, e.g., *B. paxllaeri* LMTR21^{T} and *B. icense* LMTR13^{T} (Durán et al. 2014).

The results we obtained herein highlight the diversity of fast-growing and slow-growing rhizobial isolates that nodulate *P. lunatus*, and suggest phenotypic differences among *P. lunatus* strains as selection tools of bacterial

candidates to be used as biofertilizer. Additional phenotypic data were provided in comparison to previous molecular-oriented studies, especially in the case of rhizobia and extra-alkalinising bradyrhizobia. Our study points out the need for further studies on the diversity and taxonomy of fast-growing rhizobia associated with *P. lunatus*, since it seems to be a numerically less abundant group than bradyrhizobia. This work offers basic information for further studies on the diversity of and complex interactions among the root-nodule bacteria, the host plant, and environmental factors. This research was supported by Consejo Nacional de Ciencia y Tecnología del Perú (CONCYTEC) PROCYT N°229-2005-CONCYTEC-P and FDA biol-111/UNALM.

References

Boiero L, Perrig D, Masciarelli O, Penna C, Cassán F, Luna V (2007) Phytohormone production by three strains of *Bradyrhizobium japonicum* and possible physiological and technological implications. Appl Microbiol Biotechnol 74:874–880

Broughton WJ, Dilworth MJ (1971) Control of leghaemoglobin synthesis in snake beans. Biochem J 125:1075–1080

Durán D, Rey L, Mayo J, Zúñiga-Dávila D, Imperial J, Ruiz-Argüeso T, Martínez-Romero E, Ormeño-Orrillo E (2014) *Bradyrhizobium paxllaeri* sp. nov. and *Bradyrhizobium icense* sp. nov., nitrogen-fixing rhizobial symbionts of Lima bean (*Phaseolus lunatus* L.). Int J Syst Evol Microbiol 64:2072–2078

Fofana B, Vekemans X, du Jardin P, Baudoin JP (1997) Genetic diversity in Lima bean (*Phaseolus lunatus* L.) as revealed by RAPD markers. Euphytica 95:157–165

Glickmann E, Dessaux Y (1995) A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. Appl Environ Microbiol 61:793–796

Gordon SA, Weber RP (1951) Colorimetric estimation of indoleacetic acid. Plant Physiol 26:192–195

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J (2012) Introducing EzTaxon: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62:716–721

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948

Matos G, Ormeño E, Zúñiga D (1998) Diversidad de los rizobios que nodulan el cultivo de pallar (*Phaseolus lunatus* L.) en la costa central del Perú. Ecología 1:42–46

Nautiyal SC (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:265–270

Ormeño E, Torres R, Mayo J, Rivas R, Peix A, Velásquez E, Zúñiga D (2007) *Phaseolus lunatus* is nodulated by a phosphate solubilizing strain of *Sinorhizobium meliloti* in a Peruvian soil. In: Velázquez E, Rodríguez-Barrueco C (eds) First International Meeting on Microbial Phosphate Solubilization. Dev Plant Soil Sci 102:143–147

Ormeño-Orrillo E, Vinuesa P, Zúñiga-Dávila D, Martínez-Romero E (2006) Molecular diversity of native bradyrhizobia isolated from Lima bean (*Phaseolus lunatus* L.) in Peru. Syst Appl Microbiol 29:253–262

Rodríguez-Navarro DN, Camacho M, Leidi EO, Rivas R, Velázquez E (2004) Phenotypic and genotypic characterization of rhizobia from diverse geographical origin that nodulate *Pachyrhizus* species. Syst Appl Microbiol 27:737–745

Rohlf FJ (1998) NTSYSpc: Numerical taxonomy and multivariate analysis system version 2.02. Exeter Software, Setauket

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 30:2725–2729

Versalovic J, Koeuth T, Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19:6823–6831

Versalovic J, Schneider M, de Bruijn FJ, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol 5:25–40

Vincent JM (1970) A Manual for the Practical Study of Root-Nodule Bacteria. Blackwell Scientific Publications, Oxford

Vinuesa P, Silva C, Werner D, Martínez-Romero E (2005) Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in *Bradyrhizobium* species cohesion and delineation. Mol Phylogenet Evol 34:29–54

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703

Yao Z, Kan F, Wang E, Wei G (2002) Characterization of rhizobia that nodulate legume species of the genus *Lespedeza* and description of *Bradyrhizobium yuanmingense* sp. nov. Int J Syst Evol Microbiol 52:2219–2230

Zhang YF, Wang ET, Tian CF, Wang FQ, Han LL, Chen WF, Chen WX (2008) *Bradyrhizobium elkanii*, *Bradyrhizobium yuanmingense* and *Bradyrhizobium japonicum* are the main rhizobia associated with *Vigna unguiculata* and *Vigna radiata* in the subtropical region of China. FEMS Microbiol Lett 285:146–154