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Molecular diversity of native bradyrhizobia isolated from Lima bean (*Phaseolus lunatus* L.) in Peru

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Abstract

The diversity of a collection of 21 bradyrhizobial isolates from Lima bean (*Phaseolus lunatus* L.) was assayed by molecular methods. Moderately high to high genetic diversity was revealed by multilocus enzyme electrophoresis (MLEE) analysis of seven enzyme loci and genomic fingerprints with ERIC and BOX primers. Two groups with differences in growth rate were found among the isolates and their differentiation as two divergent bradyrhizobial lineages was supported by PCR-RFLP of the *rpoB* gene and sequence analysis of the 16S rDNA and *dnaK* genes. Isolates with slow growth (SG) were identified as *Bradyrhizobium yuanmingense*, while extra-slow growing isolates (ESG) constitute a new lineage different from all described *Bradyrhizobium* species. Three distinct symbiotic genotypes were detected among Lima bean bradyrhizobia by PCR-RFLP and sequence analysis of the *nifH* and *nodB* genes. One genotype was found in the ESG lineage and two in *B. yuanmingense*. Another symbiotic genotype was detected in *B. yuamingense* isolated from *Lespedeza* plants. The identified bradyrhizobial lineages constitute sympatric species effectively nodulating Lima bean on the coast of Peru.

Keywords: *Bradyrhizobium*; Phylogeny; *dnaK*; *nifH*; *nodB*; Symbiosis island

Introduction

The legume genus *Phaseolus* comprises around 50 species, all indigenous to the Americas. Among these, *P. lunatus* (Lima bean), *P. vulgaris* (common bean), *P. coccineus* (scarlet runner bean) and *P. acutifolius* (tepary bean) were domesticated by prehispanic civilizations and are widely used for human consumption. Beans, like other legume plants, can establish symbiosis with certain soil bacteria commonly known as rhizobia. These bacteria invade root tissues and induce the formation of specialized structures known as nodules where they differentiate and fix atmospheric nitrogen which is supplied to the plant. The agronomic implications of this symbiosis have promoted research on biological nitrogen fixation and on the characterization of rhizobia.

Lima bean is the second most economically important species of *Phaseolus* and one of the 12 primary grain legumes [4]; however, rhizobia associated with this crop

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have been scarcely studied. In the old host-based classification scheme, symbionts of *P. lunatus* were included in the slow growing "cowpea" rhizobia [1,3]. This group was a diverse assemblage of strains which were included later in the genus *Bradyrhizobium* [8]. To date, two species and several genospecies comprising "cowpea" bradyrhizobia have been described but none include Lima bean isolates [34,39,40].

Most of the studies on bradyrhizobia associated with Lima bean have included isolates obtained from areas where this legume is not native, and have focused only on the analysis of symbiotic characteristics, i.e. infectiveness and/or effectiveness [1,30]. Peru is one of the known centres of origin and diversity of *P. lunatus* [4]. In a previous study, we analysed rhizobia from Lima bean nodules collected on the coast of Peru and determined a high phenotypic diversity [16]. The aim of the present work was to study the molecular diversity of a collection of Lima bean symbionts isolated from the centre of diversification of their host and to establish their phylogenetic relationships with other *Bradyrhizobium* species.

Materials and methods

Isolation and cultural conditions

Nodules were collected on the north and central coast of Peru (Fig. 1) from Lima bean plants growing in soils with no history of inoculation. Collection sites located on the central coast had sandy or sandy loamy and neutral or slightly alkaline soils, and a subtropical arid climate. The site located on the north coast had a clayey and slightly alkaline soil, and a tropical arid climate. Bacteria were isolated from nodules as described by Vincent [33]. Colony size was determined on YEM agar [33]. Acid or alkali production was visually determined on the same medium supplemented with bromothymol blue as a pH indicator. Isolates were maintained in YEM or AG broth [22] containing 20% glycerol (vol/vol) at −80 °C.

Fig. 1. Map of Peru with sampling sites indicated with black stars. Names within parenthesis indicate the province where each site is located.

Multilocus enzyme electrophoresis (MLEE)

Cell extracts were prepared as described by Spoerke et al. [27], but the bacterial isolates were grown in AG broth until late log phase. Protein separation on 12.6% starch gels and staining of the enzymes were performed according to the procedures described by Selander et al. [23]. The following metabolic enzymes were evaluated three times for each isolate: indophenol oxidase, isocitrate dehydrogenase, malate dehydrogenase, malic enzyme, alcohol dehydrogenase, hexokinase, and α-esterases. Distinctive mobility variants of each enzyme were equated with alleles and each unique allele profile or electrophoretic type (ET) was equated with a multilocus genotype. Genetic diversity (h) for each enzyme locus was calculated as $h = [1 - \Sigma x_i^2][n/(n-1)]$, where x_i is the frequency of the ith allele and n is the number of ETs. Mean genetic diversity (H) is the arithmetic average of h-values across all the loci examined [23]. A strain richness index was calculated by dividing the number of ETs identified by the number of isolates recovered [17,26].

DNA isolation and hybridization

Genomic DNA was isolated and purified using the GenomicPrep™ kit (Amersham). DNA–DNA homologies were estimated by a filter hybridization method [15].

Genomic fingerprinting

Methods described by Versalovic et al. [32] were used to generate genomic fingerprints using primers ERIC1R and ERIC2, and BOX A1R. Reactions were carried out in 25 μL final volumes with 1 × polymerase buffer and 7.5 mM $MgCl_2$. The fingerprints were visually identified after separation of PCR products by electrophoresis in 1.5% agarose gels. A strain richness index was calculated by dividing the number of fingerprint patterns identified by the number of isolates recovered [17].

PCR amplification and gene sequencing

16S rDNA genes were amplified as described previously [37] using primers fD1 and rD1 [38]. Partial *dnaK* genes were amplified using primers dnaK1230F (5′-CACCACGATCCCGACCAA-3′) and dnaK1846R (5′-GGTGAACTCYGCGTCGAC-3′) with an annealing temperature of 62 °C. Partial *nifH* genes were amplified with primers *nifH*40F and *nifH*817R as described previously [35]. Partial *nodB* genes were amplified with primers nodB3F and nodCRR as described previously [24] or with primers nodB86F (5′-ATGGGCCMAAYCCVTTTTG-3′) and nodB737R (5′-GCYTGCRCGCTCTTRTAGA-3′) with an annealing temperature of 56 °C. Sequencing was performed with purified PCR amplified products as described previously [37].

PCR-restriction fragment length polymorphism (PCR-RFLP) of *rpoB*, *nifH* and *nodB* genes

Partial *rpoB* genes were amplified with primers Br3200F and Br3950R as described by Khamis et al. [9]. Aliquots (12 μL) of the PCR products of *rpoB*, *nifH* and *nodB* were digested with 5 U of the restriction enzymes indicated in Table 1, in 15 μL reaction volumes by using the buffers and temperatures recommended by the manufacturers. Patterns obtained were visually identified after restricted DNA was separated by electrophoresis in 2.5% agarose gels.

Phylogenetic analysis

Sequences were aligned using CLUSTAL W 1.83 [31] and manually corrected using BioEdit version 5 [6]. Phylogenetic trees were generated by the neighbor joining (NJ) and maximum likelihood (ML) methods using MEGA version 2.1 [10] or PhyML v2.4.1 [5], respectively. The Tamura–Nei model was used for all genes [29]. The gamma shape parameter of among site rate variation and the proportion of invariable sites were estimated with PhyML.

Genbank accession numbers

The accession numbers for the three 16S rDNA gene sequences determined in this study are AF485365, AY923030 and AY923031. The 16 *dnaK* gene sequences obtained have accession numbers AY923032 through AY923047. The 10 *nodB* gene sequences determined have been assigned accession numbers AF485366 through AF485370, AY923048 through AY923050, DQ085614 and DQ085615. The eight *nifH* gene sequences determined have accession numbers DQ085616 through DQ085623.

Results

Phenotypic diversity of *P. lunatus* isolates

Twenty-one alkali producing isolates from Lima bean nodules were obtained (Table 1). Nine (43%) were slow growers (SG) with their colonies reaching a size of 1–3 mm after 5–6 days of incubation on YEM medium, while the remaining 12 isolates (57%) were extra-slow growers (ESG) with punctiform colonies (<1 mm) visible only after 7–10 days of incubation. The ESG isolates had a stronger ability to produce alkali in comparison to the SG isolates as evidenced by the intense blue colour of YEM agar plates supplemented with bromothymol blue. Plasmids were not detected using a modified Eckhardt procedure [7]. All isolates induced effective nodulation in Lima bean plants. The nodules were pink and the leaves of the inoculated plants were dark-green, while the control non-inoculated plants had yellow-green leaves.

Diversity revealed by MLEE analysis and genomic fingerprinting

Isolates were analysed by MLEE for seven loci. Polymorphisms were detected at all loci with two to four alleles per locus. A total of eight distinct multilocus genotypes, or ETs, were identified (Table 1). SG and ESG isolates always displayed different ETs. Fingerprinting with ERIC and BOX primers distinguished nine and 12 genomic patterns, respectively, among isolates (Table 1). The mean genetic diversity (*H*) estimated from MLEE data was 0.4, and the strain richness indices were 0.38 and 0.57 based on MLEE and ERIC-BOX data, respectively.

Ribosomal DNA phylogenetic analysis

Almost complete 16S rDNA gene sequences (~1400 bp) were obtained for isolates LMTR 3, 21 and 28. The sequence of SG isolate LMTR 28 showed 99.7%, 99.6%, 99.2%, 99.1% and 98.8% identity with the corresponding genes of *Bradyrhizobium liaoningense* LMG18230^T, *B. yuanmingense* CCBAU10071^T, *B. canariense* BTA-1^T, *B. japonicum* DSM30131^T and *B. betae* PL7HG1^T, respectively. Phylogenetic analysis positioned the sequence within the *B. japonicum* 16S rDNA lineage (Fig. 2). The sequences of ESG isolates LMTR 3 and LMTR 21 were identical, showed 99.3% identity with the corresponding sequence of *B. elkanii* USDA 76^T, and grouped within the *B. elkanii* 16S rDNA lineage (Fig. 2).

Table 1. Relevant characteristics of the isolates and strains used in this study

Isolate or strain	Origin[a]	Host[b]	Phenotype[c]	ET[d]	Genomic fingerprinting[e]		PCR-RFLP[f]		
					ERIC	BOX	*rpoB*	*nifH*	*nodB*
Bradyrhizobium sp.									
LMTR 11, 12	SC	*P. lunatus*	ESG	6	A	A3	ABBAB	aaa	aaa
LMTR 15, 20	SC	*P. lunatus*	ESG	6	A	A1	ABBAB	aaa	aaa
LMTR 25	SA	*P. lunatus*	ESG	6	A	A1	ABBAB	aaa	aaa
LMTR 47	PC	*P. lunatus*	ESG	6	A	A1	ABBAB	aaa	aaa
LMTR 7, 21	SC	*P. lunatus*	ESG	7	A	A1	ABBAB	aaa	aaa
LMTR 17	SC	*P. lunatus*	ESG	7	A	A2	ABBAB	aaa	aaa
LMTR 14	SC	*P. lunatus*	ESG	4	A	A4	ABBAB	aaa	aaa
LMTR 19	SC	*P. lunatus*	ESG	5	B	B	ABBBB	aaa	aaa
LMTR 3	LM	*P. lunatus*	ESG	8	C	C	ABABC	aaa	aaa
B. yuanmingense									
LMTR 41	TG	*P. lunatus*	SG	2	G	D5	BACBA	bba	cca
LMTR 42	TG	*P. lunatus*	SG	1	F	D2	BACBA	bba	cca
LMTR 28	LP	*P. lunatus*	SG	1	D	D1	BACBA	dab	eec
LMTR 37, 38, 39	LP	*P. lunatus*	SG	1	E	D3	BACBA	dab	eec
LMTR 5	SC	*P. lunatus*	SG	3	H	D6	BACBA	dab	eec
LMTR 30	LP	*P. lunatus*	SG	3	H	D6	BACBA	dab	eec
LMTR 9	SC	*P. lunatus*	SG	3	I	D4	BACBA	dab	eec
TAL760	Mexico	*I. hirsuta*					BACBD	dcb	eec
CCBAU10071^T	China	*Le. cuneata*			J1	E1	BACBD	cba	afa
CCBAU10040	China	*Le. cuneata*			J2	E2	BACBD	cba	afa
B. japonicum									
DSM30131^T	Japan	*G. max*					BCDCD		
USDA 110	USA	*G. max*					BAFCE	abc	fga
USDA 122	USA	*G. max*					BAFCH		
FN 13	Mexico	*L. montanus*					BADCG		
B. liaoningense									
LMG18230^T	China	*G. max*					CACCA		
Spr3-7	China	*A. hypogaea*							
B. canariense									
BTA-1^T	Spain	*C. proliferus*					BAECF		
B. betae									
PL7HG1^T	Spain						BAFCD		
B. elkanii									
USDA76^T	USA	*G. max*					DCABC		
Bradyrhizobium sp.									
Km 50-90	Mexico	*L. campestris*					BAECD		ddb

[a]Two letter codes refer to localities in Peru: SC, San Camilo, Ica; SA, Santa Adela, Cañete; PC, Pachacamac, Lima; LP, Los Pobres, Ica; LM, La Molina, Lima; TG, Tambo Grande, Piura.

[b]Abbreviations for host genera are: *P*, *Phaseolus*; *G*, *Glycine*; *L*, *Lupinus*; *Le*, *Lespedeza*; *I*, *Indigofera*; *A*, *Arachis*; *C*, *Chamaecytisus*.

[c]SG, slow growth; ESG, extra-slow growth (see Results).

[d]Electrophoretic type representing different combinations of six enzyme patterns distinguishable by starch gel electrophoresis.

[e]Letters were arbitrarily assigned to represent specific genomic fingerprints obtained with ERIC or BOX primers.

[f]Letters were arbitrarily assigned to represent specific restriction patterns obtained after digestion with the following endonucleases: *Taq*I, *Hin*fI, *Rsa*I, *Hin*dIII, *Hha*I for *rpoB*; *Rsa*I, *Xho*I, *Sac*I for *nifH*; and *Rsa*I, *Taq*I, *Dde*I for *nodB*.

PCR-RFLP analysis of the *rpoB* gene

A highly variable fragment (~750 bp) [9] of the *rpoB* gene encoding the β subunit of RNA polymerase was amplified for all Lima bean isolates and reference bradyrhizobia, and digested with five endonucleases. Four restriction patterns were observed among Lima bean isolates (Table 1). All SG isolates displayed the same *rpoB*

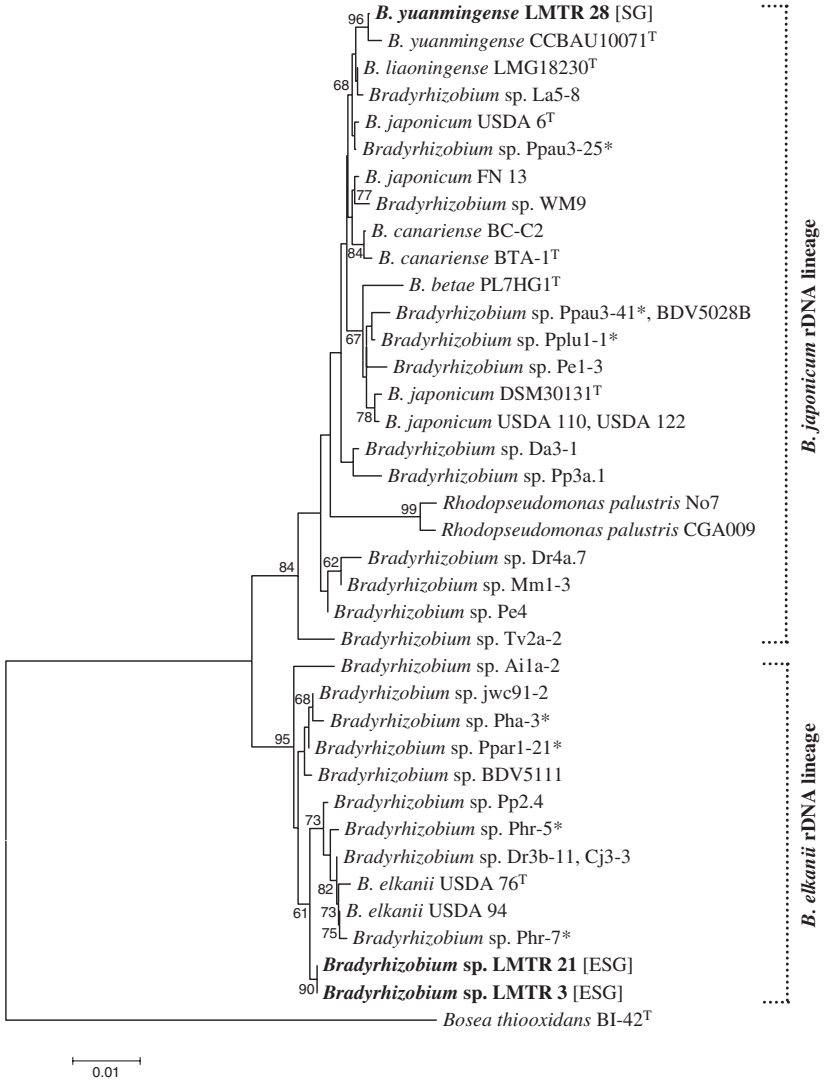

Fig. 2. Neighbour joining phylogenetic tree of 16S rDNA gene sequences. Values at branching points indicate bootstrap support higher than 60% (1000 pseudoreplicates). Lima bean isolates are shown in bold with their growth phenotype (see Results) indicated within brackets. Strains with an asterisk were isolated elsewhere from other *Phaseolus* spp. Phylogenetic rDNA lineages are indicated at right.

restriction pattern (BACBA), which differed for only one enzyme from the pattern obtained for *B. yuanmingense* (BACBD). On the other hand, ESG isolates showed three restriction patterns distinct for at least two enzymes from all reference bradyrhizobia (Table 1).

dnaK gene relationships

Nearly 600 bp of the *dnaK* gene, which encodes for a conserved Hsp70 class chaperone, were sequenced in seven Lima bean isolates and nine reference strains and compared with the corresponding sequences from other bradyrhizobia. All bradyrhizobial sequences form a monophyletic clade with 100% bootstrap support in the NJ (not shown) and ML (Fig. 3) phylogenetic trees. This clade splits into three main lineages with high bootstrap support. The first lineage included sequences of all the bradyrhizobial species and non-classified strains that grouped within the *B. japonicum* 16S rDNA lineage (Figs. 2 and 3). Sequences of *B. japonicum*, *B. canariense*, *B. liaoningense* and *B. yuanmingense* form a highly supported sublineage (Ia in Fig. 3). The sequences of SG isolates were intermingled with those of *B. yuanmingense*. A second lineage included sequences of *B. elkanii* and other strains possessing a *B. elkanii*-like 16S rDNA allele. The third lineage was formed only by sequences of ESG Lima bean isolates.

Symbiotic loci analysis

A fragment (~333 bp) of the *nifH* gene encoding the nitrogenase iron protein from seven Lima bean isolates

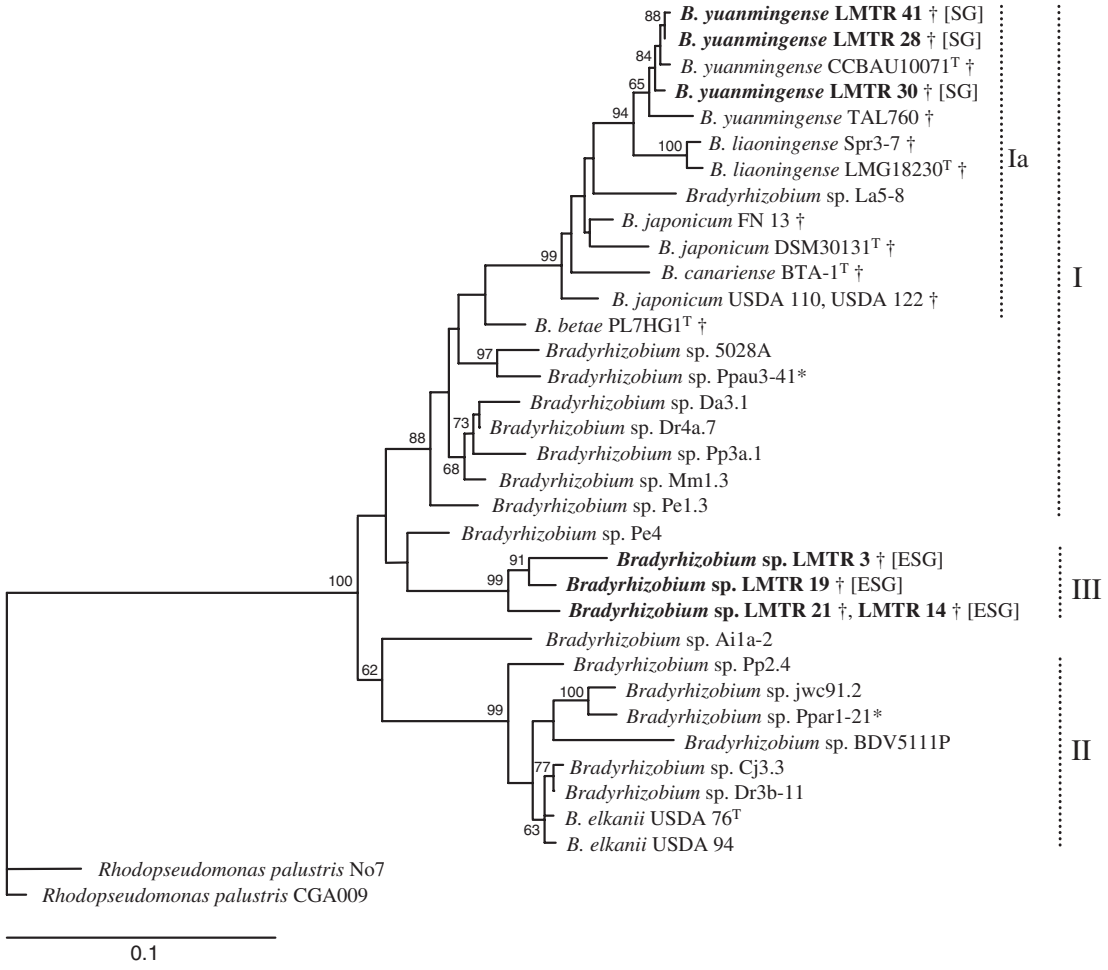

Fig. 3. Maximum likelihood phylogenetic tree of *dnaK* gene sequences. Values at branching points indicate bootstrap support higher than 60% (100 pseudoreplicates). Lima bean isolates are shown in bold with their growth phenotype (see Results) indicated within brackets. Strains with an asterisk were isolated elsewhere from other *Phaseolus* spp. Bradyrhizobial *dnaK* lineages are indicated by Roman numerals. The sequences determined in this study are indicated with a cross (†).

was compared to different rhizobial homologues. Phylogenetic analysis revealed that the sequences of Lima bean isolates were dispersed among three distinct clades (Fig. 4). The *nifH* gene of SG isolate LMTR 28 was highly similar to the corresponding gene of the *Indigofera hirsuta* strain TAL 760 [35] (99.4% identity), while the sequences from SG isolates LMTR 41 and LMTR 42 showed 97.2% identity between each other and clustered apart as an independent clade. The ESG isolates showed greater than 94% identity in their *nifH* genes which formed a third well-supported clade.

Another symbiotic locus, the nodulation gene *nodB*, was partially sequenced (~550 bp) and analysed in seven *P. lunatus* isolates. With the exception of a photosynthetic stem nodulating strain, all bradyrhizobial *nodB* sequences cluster together, although without a high bootstrap support. The sequences of Lima bean isolates were subdivided in the same three clades observed with the *nifH* gene. The SG isolates LMTR 41 and LMTR 42 had identical sequences which cluster with the *nodB* gene of the *Lupinus campestris* strain Km 50-90 [2], although they showed only 89.8% identity. The sequences of SG isolates LMTR 28 and LMTR 30 and that of the TAL 760 strain were greater than 99.4% identical and they formed another clade. The ESG isolates LMTR 21 and LMTR 25 had the same *nodB* sequence which was 95.8% identical to the gene of ESG isolate LMTR 3, together they cluster apart as a well-supported clade.

To determine the symbiotic genotypes of the non-sequenced isolates, PCR-RFLP of *nifH* and *nodB* were performed with three endonucleases. As shown in Table 1, six restriction patterns corresponding to the clades uncovered by the sequence analysis were obtained for all Lima bean isolates.

Distribution of lineages and strains

At the San Camilo site, both SG and ESG bradyrhizobia were recovered but nine out of 11 isolates (82%)

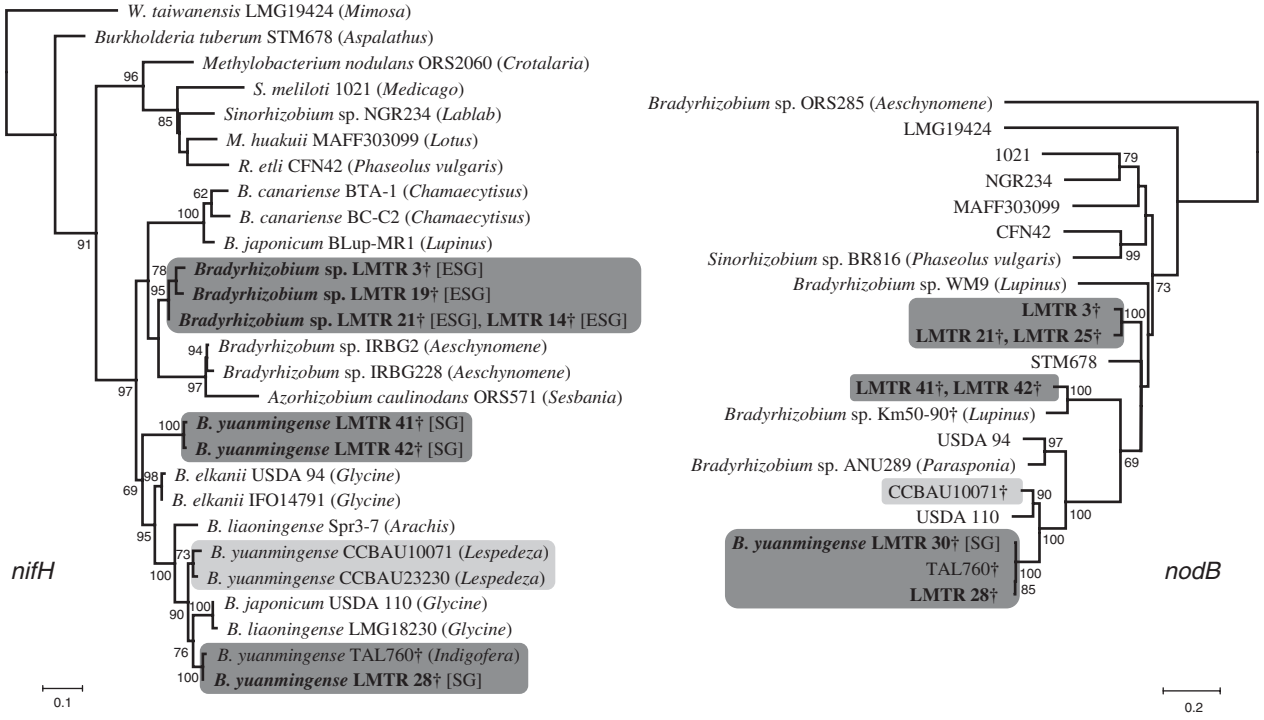

Fig. 4. Maximum likelihood phylogenetic trees of *nifH* and *nodB* gene sequences. Values at branching points indicate bootstrap support higher than 60% (100 pseudoreplicates). Lima bean isolates are shown in bold with their growth phenotype (see Results) indicated within brackets. Hosts genera are indicated in parenthesis. The symbiotic types uncovered in this study are highlighted (those in dark grey include Lima bean isolates). The sequences determined in this study are indicated with a cross (†).

belonged to the ESG bradyrhizobial lineage. Only one of the lineages was represented at the other sites where more than two isolates were recovered (Table 1). Most sites had similar soil types and climate; therefore, this apparent differential geographical dominance is most likely due to a sampling bias. Some strains, identified on the basis of ET and genomic fingerprinting, were isolated from sites as far as 300 km apart (Table 1 and Fig. 1). This result contrasts with the very restricted distribution of strains reported in other studies of rhizobia [35] and is probably the result of human mediated dispersal. There was not a correlation between lineages and cultivars, since both lineages were isolated from small and large seeded Lima bean cultivars (data not shown).

Discussion

Most of the knowledge about rhizobia associated with the legume genus *Phaseolus* refers to symbionts isolated from the common bean (*P. vulgaris*) which belong to several species within the *Rhizobium* genus [14,15,24,26]. Lima bean (*P. lunatus*) associates with different rhizobia. Its symbionts are mostly slow growing bradyrhizobia that have been scarcely studied [1,30]. Accordingly, our Lima bean isolates alkalized the growth medium, did not have plasmids and grew like *Bradyrhizobium* species [8].

Lima bean has a long cultivation history in Peru, which is one of the known centres of origin and diversification of this legume [4]. Lie et al. [13] proposed that the centres of legume diversity coincide with those of their symbionts. Accordingly, the genetic diversity observed among our isolates was high and comparable to that determined for rhizobia isolated from legumes in their sites of origin [26,27,35]. The strain richness index values also indicated a moderately high to high diversity in comparison with other studies [17].

Previously we have shown that SG isolate LMTR 28 grouped with *B. yuanmingense* in ML phylogenies inferred from *atpD*, *recA* and *glnII* gene sequences as well as in a maximum parsimony phylogeny reconstructed from ITS sequences [34,35]. In the present study, PCR-RFLP of *rpoB*, and *dnaK* sequence analysis of a larger sample of SG Lima bean isolates evidenced the same close relationship with *B. yuanmingense*. Total DNA–DNA hybridization experiments established values of 70.9 between SG isolates LMTR 28 and LMTR 41, and 60.9 between LMTR 28 and *B. yuanmingense* CCBAU10071^{T}. Taken together, these results support the classification of all SG Lima bean isolates as *B. yuanmingense*. This species seems to have a naturally broad intercontinental distribution. It has been recovered from China associated with *Lespedeza cuneata*, a

native tree [40], and here we report its isolation from South America nodulating a native legume growing in soils with no history of inoculation. A similar conclusion of non-human-mediated wide geographical distribution has been reported previously for *B. canariense* [35] and *Mesorhizobium plurifarium* [35a,36].

The ESG isolates showed a *B. elkanii*-like 16S rDNA allele. However, analysis of the *rpoB* gene distinguished them from *B. elkanii* and sequence analysis of *dnaK* clearly separated them as an independent group (Fig. 3). These results indicate once more the limited usefulness of ribosomal genes for phylogenetic analysis since strains possessing very similar or almost identical 16S rDNA gene sequences could belong to different species [34,40]. Previous studies have found three main *dnaK* lineages within *Bradyrhizobium* sequences: one related to *B. japonicum*, another one allied to *B. elkanii* and one including photosynthetic bradyrhizobia [20,28]. The first two correspond to lineages I and II from our analysis (Fig. 3). The sequences of ESG isolates showed only 88.8–89.5% identity with a shorter sequence of the photosynthetic strain ORS278 [28] and thus constitute a new *dnaK* lineage.

Tepary bean (*P. acutifolius*) and other wild species of *Phaseolus* are naturally nodulated by bradyrhizobia [11,19,25]. 16S rDNA sequences are available for some of these strains and they are different from those of the *P. lunatus* isolates (Fig. 2). More informative are two *dnaK* sequences from *Bradyrhizobium* strains isolated from wild beans in Mexico: a *P. pauciflorus* strain sequence grouped within lineage I but is not related to *B. yuanmingense* and a *P. parvulus* strain sequence is included in lineage II with *B. elkanii* (Fig. 3) [20]. These results indicate that a wide diversity of bradyrhizobial lineages nodulate *Phaseolus* spp. in nature.

Despite the chromosomal diversity found among Lima bean bradyrhizobia, it was unexpected to find divergent *nodB* and *nifH* alleles among them, since previous studies have shown that Lima beans have a high specificity for both nodulation and nitrogen fixation [1,30]. Our results may indicate that this legume is more promiscuous than previously thought. Isolates possessing these different symbiotic genes were effective symbionts of *P. lunatus*; however, it would be interesting to establish if there is a differential competitiveness for nodule occupancy in order to make recommendations for future programs of inoculant production.

The phylogeny of *nodB* did not correlate with those of housekeeping chromosomal genes as has been previously reported for other symbiotic loci [18,34]. With the exception of the sequence from a stem nodulating photosynthetic strain, bradyrhizobal *nodB* genes appeared to be monophyletic. This is consistent with the results of Moulin et al. [18] who found evidence for the monophyly of bradyrhizobial *nodA* genes but pointed out the possible exception of the sequences from photosynthetic strains. These results may reflect a special adaptation of the common *nod* genes to stem nodulation since all bradyrhizobia constitute a cluster by sequence analysis of other symbiotic loci, like *nodZ*, *noeI* [18] and *nifH*. Interestingly, the common *nod* genes of *Azorhizobium caulinodans*, which induces stem nodules in *Sesbania rostrata*, also show a higher rate of divergence compared to those from other rhizobia [12,18].

The analysis of symbiotic loci evidenced three distinct *nodB* genotypes linked to three *nifH* genotypes within *B. yuanmingense* (Fig. 4). These symbiotic genotypes could belong to three different putative symbiosis islands. Cross-inoculation experiments are needed to determine if the symbiosis islands are related to plant specific nodulation often designated as biovarieties. However, the presence of an unrelated putative symbiosis island in the ESG lineage from Lima bean (Fig. 4) and the lack of correspondence reported between bradyrhizobial *nodA* phylogenetic clades and legume host systematics [18] raise doubts about the possible assignment of clear biovarieties among rhizobia nodulating promiscuous tropical legumes [21].

In addition to establishing *B. yuamingense* as a natural symbiont of Lima bean, we found a new *Bradyrhizobium* lineage not described before. These bradyrhizobial lineages were isolated from nodules of the same plant; therefore, they constitute sympatric species. Future studies will aim to describe the ESG lineage as a new species of *Bradyrhizobium* on the basis of sequence analysis of other metabolic and informational genes.

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