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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.

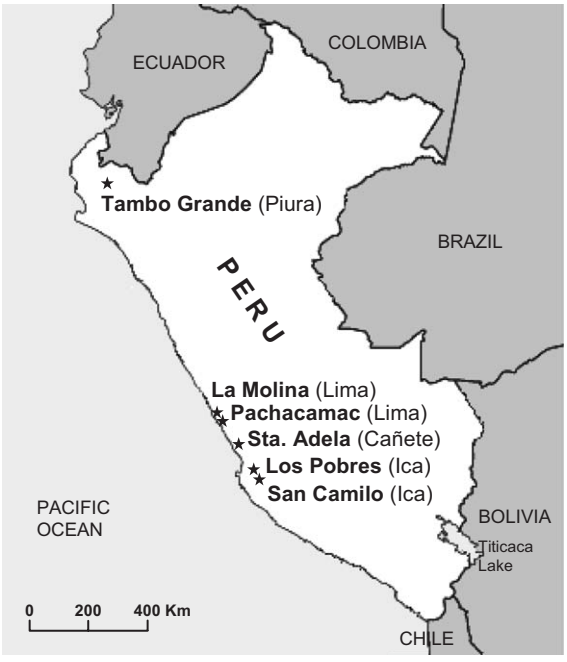

Fig. 1. Map of Peru with sampling sites indicated with black stars. Names within parenthesis indicate the province where each site is located.

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].