

Chapter 6

**BIODIVERSITY OF NITROGEN-FIXING
NODULE BACTERIA ASSOCIATED
WITH LIMA BEAN (*PHASEOLUS LUNATUS L.*)
IN ITS DOMESTICATION CENTERS**

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ABSTRACT

Like other leguminous plants, the Lima bean (*Phaseolus lunatus*) establishes a mutualistic symbiosis with nitrogen-fixing bacteria commonly known as rhizobia. These bacteria elicit the formation of nodule structures on the roots where they become intracellular and fix atmospheric nitrogen for the plant. The Lima bean shows a preference to form nodules with slow-growing bacteria of the genus *Bradyrhizobium* unlike its congeneric species, the common bean (*Phaseolus vulgaris*), which prefer fast-growing rhizobial strains. *P. lunatus* was independently domesticated in the Andes and Mesoamerica by pre Columbian societies.

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Knowledge of the genetic biodiversity of Lima bean symbionts has increased in recent years with data obtained in its domestication areas. In Peru, Lima beans host *Bradyrhizobium yuanmingense*, *Bradyrhizobium paxllaeri*, *Bradyrhizobium icense*, and a yet unnamed genospecies. At least seven *Bradyrhizobium* genospecies have been found in *P. lunatus* nodules in Mexico. This rhizobial richness indicates a relative promiscuity of Lima bean in contrast to previous statements that this plant was a restricted host for nodulation. Interestingly, Mexican and Peruvian bradyrhizobia are distinct, perhaps in relation to the different plant gene pools from which domestication occurred in those geographic areas. Besides *Bradyrhizobium*, other rhizobial genera such as *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* can be found in Lima bean nodules in its domestication centers and in other areas. Although effective nodulation with these genera has been reported, the ecological relevance of those associations is presently unknown.

Keywords: Pallar, ibes, Mexico, Peru, biological nitrogen fixation, biofertilizer, inoculant

INTRODUCTION

The Lima bean (*P. lunatus* L.) is one of five cultivated species of the leguminous genus *Phaseolus*. The other cultivated species are common bean (*Phaseolus vulgaris* L.), runner bean (*Phaseolus coccineus* L.), tepary bean (*Phaseolus acutifolius* A. Gray) and year bean (*Phaseolus polyanthus* Greenman). The Lima bean was independently domesticated from distinct wild gene pools in the Andean and Mesoamerican regions by pre Columbian societies (Gutiérrez Salgado et al., 1995). This species is the second most economically important species of the genus after common bean. In general, *Phaseolus* beans have been important sources of low-cost, high-quality proteins for human populations in the Americas for millennia, and for other people in places like Africa after their introduction from America.

As with other leguminous plants, Lima beans are able to establish a mutualistic symbiosis with nitrogen-fixing bacteria known as rhizobia (Santos et al. 2008). These bacteria inhabit soils and upon perception of flavonoids released by the roots activate the expression of nodulation (*nod*) genes, which are involved in the biosynthesis and secretion of molecules known as Nodulation (Nod) factors. Nod factors are perceived by the plant where they act as signals to trigger the morphogenesis of specialized root structures known as nodules. Inside nodules rhizobia express *nif* genes required for

nitrogen fixation. In exchange for fixed nitrogen, rhizobia receive carbon sources to sustain the nitrogen fixation process. Leguminous plants can fulfill most of their nitrogen needs through this symbiosis. Rhizobia can be grown in synthetic media and formulated in products known as biofertilizer inoculants that can be applied to seeds or soil in order to ensure the formation of the symbiosis. In agriculture, rhizobial inoculants represent cost effective and ecological friendly replacements for chemical nitrogen fertilizers (Zúñiga-Dávila, 2007).

Effective use of rhizobia requires knowledge on their biodiversity and symbiotic characteristics. Biodiversity of rhizobia has been, and still is, intensively studied around the globe. Several genera of alpha and beta Proteobacteria include rhizobial members. Most published work on rhizobia associated with *Phaseolus* beans centers on symbionts of *P. vulgaris* bean because of its economic importance and its worldwide distribution. In contrast, relatively few studies on Lima bean rhizobia are available. In the 1930s, it was shown that rhizobia associated with Lima bean belonged to the so called “cowpea miscellany group”, a heterogeneous assemblage of rhizobia able to establish symbiosis with cowpea (*Vigna unguiculata* (L.) Walp.) and other tropical leguminous species (Allen and Allen, 1939). Slow-growing rhizobial members of this group were later ascribed to the *Bradyrhizobium* genus of the alpha Proteobacteria (Jordan, 1984). Thies et al. (1991) reported that Lima bean was a relatively restricted host for nodulation when compared to other hosts like cowpea (*Vigna unguiculata*) and siratro (*Macroptilium atropurpureum*).

Until 2006, reports on Lima bean rhizobia focused mainly on their performance as inoculant strains. Furthermore, most of those rhizobia were isolated in regions where the Lima bean was not native and none of them were characterized by molecular methods so their assignment to *Bradyrhizobium* or other rhizobial genera was based only on growth rate. In this chapter we will summarize the studies on biodiversity of the Lima bean rhizobia in the domestication sites of *P. lunatus*.

BIODIVERSITY OF LIMA BEAN RHIZOBIA IN THE ANDEAN REGION

Archeological and molecular evidence suggest that Lima bean was domesticated in Western South America (Northern Peru and Ecuador) from

the Andean gene pool of *P. lunatus* (Gutiérrez Salgado et al., 1995). Nowadays, this crop is cultivated in several countries in South America. In Peru, Lima bean is cultivated mainly in the central and south subtropical coastal regions.

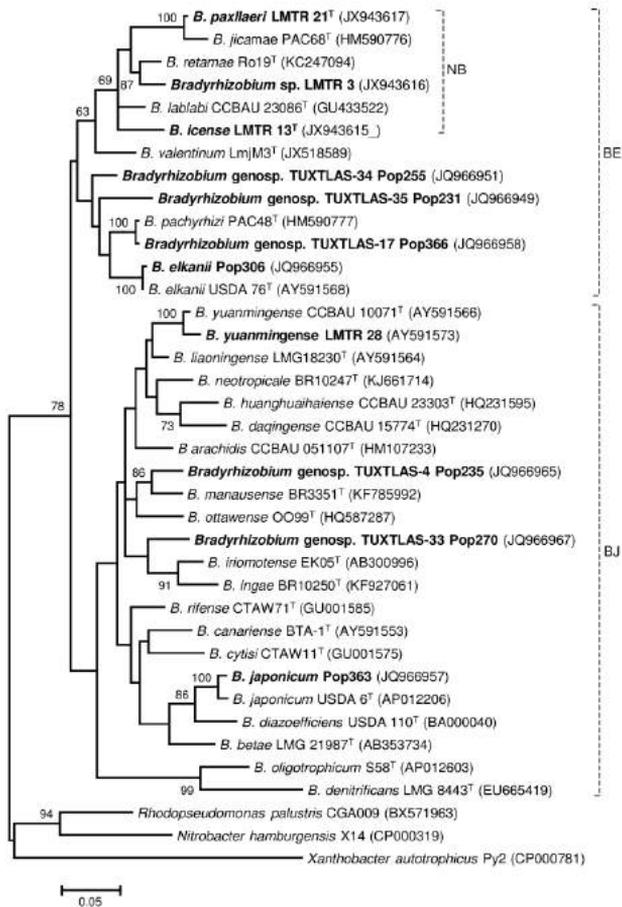


Figure 1. Phylogenetic tree showing the relationships between Lima bean bradyrhizobial symbionts (shown in bold) and all currently described *Bradyrhizobium* species based on *recA* gene sequences. Type strains are indicated with a superscript letter T. Phylogenetic branches indicated with dotted brackets: NB, branch first described by Ormeño-Orrillo et al. (2006); BE, *B. elkanii* 16S rRNA phylogenetic superclade; BJ, *B. japonicum* 16S rRNA phylogenetic superclade. The tree was constructed with the maximum likelihood method using GTR+I+G as evolutionary model. Bootstrap support values $\geq 60\%$ are indicated at tree nodes. Bar, 5 substitutions per 100 nucleotides.

The first study to apply molecular characterization methods to Lima bean rhizobia in one center of domestication was reported by Ormeño-Orrillo et al. (2006). Twenty one rhizobial strains were isolated from nodules collected in the major cultivated area of this crop in Peru. All isolates were identified as *Bradyrhizobium* based on 16S rRNA and *dnaK* gene sequencing. Two groups were recognized: one could be assigned to the species *Bradyrhizobium yuanmingense* while the other corresponded to a new phylogenetic branch within *Bradyrhizobium* (marked as NB in Figure 1). Genetic heterogeneity within the new lineage was evidenced by sequence analysis of several house-keeping genes (Durán et al., 2014; Ormeño-Orrillo et al., 2006). Two subgroups within this lineage were recently described as the novel species *Bradyrhizobium paxllaeri* and *Bradyrhizobium icense* (Figure 1) (Durán et al., 2014). Bradyrhizobia phenotypically similar to the novel species have been isolated from Lima bean nodules collected in other cultivated areas of Peru (Matsubara Bautista et al., 2005; Matsubara and Zúñiga-Dávila, 2015).

Analysis of symbiotic genes *nodB*, *nodC* and *nifH* revealed that Lima bean bradyrhizobia in Peru did not share a unique symbiotic genotype. Two genotypes were observed in *B. yuanmingense*, while *B. paxllaeri* and *B. icense* each had unique, although related, genotypes. In the *Bradyrhizobium* genus, symbiosis genes are linked and contained in mobile genetic elements known as symbiosis islands (Kaneko et al., 2002). On this basis, it has been speculated that the symbiotic genotypes observed in Lima bean bradyrhizobia could correspond to four distinct symbiosis islands (Ormeño-Orrillo et al., 2006). *B. yuanmingense* strains from Lima bean have nodulation genes belonging to clade III, which is present in pantropical rhizobia (Steenkamp et al., 2008). Nodulation genes of *B. paxllaeri* and *B. icense* belong to clade IV, which is found in rhizobia isolated mostly in subtropical regions from a wide range of legume plants growing in the Mediterranean region, Australia and China (Cardinale et al., 2008; Durán et al., 2013; Ormeño-Orrillo et al., 2013; Stepkowski et al., 2012).

It is now recognized that most species of *Phaseolus* associate preferentially with *Bradyrhizobium* and only a minority, like *P. vulgaris* and *P. coccineus*, prefer to enter in symbiosis with fast growing rhizobial genera like *Rhizobium* or *Sinorhizobium* (Martínez-Romero, 2003; Servín-Garcidueñas et al., 2014). Occasionally, fast-growing rhizobia have been isolated from Lima bean in Peru (Matos-Cuzcano et al., 1998; Ormeño et al., 2007; Zúñiga-Dávila, 2007). Strain LMTR 32, the only strain of the fast-growing rhizobia characterized by molecular methods, was identified as *Sinorhizobium meliloti* and induced effective nodules on Lima bean in

laboratory experiments, although it had low nitrogen fixing ability (Ormeño et al., 2007) and tended to lose its symbiotic genes (Ormeño-Orrillo et al., unpublished).

BIODIVERSITY IN THE MESOAMERICAN REGION

Two domestication events in Mesoamerica have been inferred for Lima bean, one occurring in Western central Mexico and the other between Guatemala and Costa Rica (Andueza-Noh et al., 2013). Nowadays, major production areas of Lima bean in Mexico are located in regions bordering the Gulf of Mexico in states like Yucatan and Veracruz.

López-López et al. (2013) isolated Lima bean rhizobia from soils collected in Los Tuxtlas, Veracruz where Mexican farmers grow this crop in association with maize, common bean and cowpea. All ninety eight isolates were ascribed to *Bradyrhizobium* by molecular characterization methods. ERIC-PCR genomic fingerprints revealed that Lima bean bradyrhizobia were more diverse than isolates obtained from cowpea from the same soil samples, probably because cowpea is an exotic crop in the area while Lima bean is native. Seven distinct *Bradyrhizobium* genospecies were present among Lima bean nodule bacteria. Three genospecies, TUXTLAS-33, 34 and 35, were novel while the remaining corresponded to *Bradyrhizobium japonicum*, and *Bradyrhizobium* genospecies TUXTLAS-4, 17 and 22 which were previously reported from forest soils of the same area (Figure 1) (Ormeño-Orrillo et al., 2012). As Lima beans form nodules with bradyrhizobia from tropical forests, they may be well suited for cultivation in other tropical regions. Lima bean nodule bacteria from Los Tuxtlas possessed nodulation genes that belong to clade V, which until now has been found only in rhizobia of American origin.

Wild Lima beans can be found in several regions of Mexico (Andueza-Noh et al., 2013). Two studies have reported the identification of their symbionts based on 16S rRNA gene sequencing. Bacteria from nodules collected in two localities in Oaxaca had sequences similar either to *Bradyrhizobium elkanii* or to *Rhizobium radiobacter* (synonym *Agrobacterium radiobacter*) (Eilmus, 2009). Although closely related to plant pathogenic species, the *R. radiobacter* strain was able to nodulate and fix nitrogen under controlled conditions (Thamer et al., 2011). Four rhizobial nodule isolates from *P. lunatus* obtained in the state of Morelos were identified as *Bradyrhizobium* (Servín-Garcidueñas et al., 2014). Sequence from one isolate

grouped within the *B. japonicum* 16S rRNA superclade while the remaining four had sequences which grouped within the *B. elkanii* superclade.

BIODIVERSITY IN OTHER AMERICAN REGIONS

Brazil is not considered as part of the South American center of origin of Lima bean, nevertheless, Northeast Brazilian states are important production areas for this crop (Santos, 2008). The characterization of rhizobia trapped from soils collected in the state of Piauí was reported by Santos et al. (2011). Of fifty isolates obtained, twenty one were fast-growing bacteria while the remaining were classified as intermediate- or slow-growers. Restriction fragment analysis of 16S PCR-amplified genes revealed high diversity among the isolates, which were assigned to *Bradyrhizobium*, *Rhizobium* and *Mesorhizobium*. This was the first time that mesorhizobia were reported as Lima bean nodule bacteria.

Lima beans are also cultivated and widely consumed in the United States (Santamaria, 2007). Based on 16S-23S intergenic spacer sequence analysis, strains USDA 3259 and USDA 3426 obtained in the state of Illinois were assigned to the *B. elkanii* superclade (van Berkum and Fuhrmann, 2009).

CONCLUSION

Despite being described as a restricted host for nodulation (Thies et al., 1991), *P. lunatus* is able to associate with many rhizobial symbionts. At least eleven *Bradyrhizobium* species or genospecies have been found in Lima bean nodules in its centers of domestication (Figure 1). Bradyrhizobia from Andean and Mesoamerican domestication sites are different probably due to differences in their hosts, which were domesticated from distinct gene pools. A higher number of species are reported as being associated with Lima bean crops in Mexico than in Peru. Sites sampled in Peru had monocultures while polycultures with other leguminous plants growing along the Lima bean were sampled in Mexico. The dissimilarities between sites may have contributed to the observed differences in bradyrhizobial species richness between both countries. When summed across countries and sites, most bradyrhizobia associated with the Lima bean belong to the *B. elkanii* 16S rRNA superclade (Figure 1), suggesting a preference to nodulate with species within this

lineage. Other *Phaseolus* species do not seem to have this preference (Parker, 2002; Servín-Garcidueñas et al., 2014).

The Lima bean bradyrhizobia from Mexico and Peru are different not only at the species or genospecies level but also in their symbiotic genes. Although this observation may not be unexpected given that *P. lunatus* seems to be a relatively promiscuous host, it raises the question as to whether hosts from one domestication center may preferentially nodulate with rhizobia from the same origin as has been reported for *P. vulgaris* (Aguilar et al., 2004).

Although *Bradyrhizobium* strains are its predominant symbionts, Lima bean is able to effectively nodulate with strains of other rhizobial genera like *Rhizobium*, *Sinorhizobium* and *Mesorhizobium*. It can be speculated that these latter rhizobial genera can gain access to Lima bean nodules in certain edaphic and/or climatic conditions. For example, soybean forms nodules with *Bradyrhizobium* in acid soils and with *Sinorhizobium* in alkaline soils (Tian et al., 2012).

The promiscuity of Lima bean and the high biodiversity of its rhizobial symbionts pose a challenge for programs aimed at using biofertilizers inoculants for this crop, since inoculant strains will have to compete with a relatively high number of other soil rhizobia. Nevertheless, we have observed that certain *Bradyrhizobium* species or genospecies are more common than others in nodules (López-López et al., 2013; Zúñiga-Dávila, 2007) and thus constitute excellent candidates for developing inoculants for Lima bean.

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