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Symbiont shift towards *Rhizobium* nodulation in a group of phylogenetically related *Phaseolus* species

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ABSTRACT

Bean plants from the *Phaseolus* genus are widely consumed and represent a nitrogen source for human nutrition. They provide biological fertilization by establishing root nodule symbiosis with nitrogen-fixing bacteria. To establish a successful interaction, bean plants and their symbiotic bacteria need to synchronize a proper molecular crosstalk. Within the *Phaseolus* genus, *P. vulgaris* has been the prominent species to study nodulation with *Rhizobium* symbionts. However the *Phaseolus* genus comprises diverse species whose symbionts have not been analyzed. Here we identified and studied nodule bacteria from representative *Phaseolus* species not previously analyzed and from all the described wild species related to *P. vulgaris*. We found *Bradyrhizobium* in nodules from most species representing all *Phaseolus* clades except in five phylogenetically related species from the *P. vulgaris* clade. Therefore we propose that *Bradyrhizobium* nodulation is common in *Phaseolus* and that there was a symbiont preference shift to *Rhizobium* nodulation in few related species. This work sets the basis to further study the genetic basis of this symbiont substitution.

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1. Introduction

The *Phaseolus* genus is estimated to contain around 70 species and the majority of them are distributed from Northern Mexico to Central America (Freytag and Debouck, 2002; Delgado-Salinas et al., 2006); most of the wild species diversity is found in this area suggesting that the origin for the whole *Phaseolus* genus may reside within it. In Mexico, wild *Phaseolus* species thrive from rain forests to deserts and from lands close to the ocean to forests surrounding high mountains and volcanoes. *Phaseolus* diversity should be conserved as “we live in a period of rapid loss of biodiversity” (Ley et al., 2008). It is unfortunate that some wild species like *Phaseolus albescens* and *P. rotundatus* are endangered (Ramírez-Delgadillo and Delgado-Salinas, 1999; Salcedo-Castaño et al., 2009). The diversity of the natural *Phaseolus* symbionts may be at risk if their hosts are threatened.

Based on molecular studies, the genus *Phaseolus* comprises eight phylogenetic clades named after representative species from each group (*Phaseolus filiformis*, *P. leptostachyus*, *P. lunatus*, *P. pauciflorus*, *P. pedicellatus*, *P. polystachius*, *P. tuerckheimii* and *P. vulgaris*) and species with unclear phylogenetic relationships like *P. microcarpus*. The *Phaseolus vulgaris* clade is composed of seven species. In general, *P. vulgaris*, *P. coccineus*, *P. dumosus*, *P. costaricensis* and *P. albescens* prefer more temperate and cooler conditions and are commonly found in mountain ranges, valleys and volcanic hills from Mesoamerica and Central America. *P. vulgaris* is the only species from this clade that is naturally distributed in South America and its northern range even extends to Canada and the United States of America. The two outlier species, *P. parvifolius* and *P. acutifolius* are resistant to drought and warm conditions that are commonly found where these species thrive (Delgado-Salinas et al., 1999; Delgado-Salinas et al., 2006). Besides *P. vulgaris*, only a few species (*P. lunatus*, *P. coccineus*, *P. dumosus* and *P. acutifolius*) were domesticated for human consumption.

Knowledge of the specific symbionts for different *Phaseolus* species may be important for farmers as nitrogen-fixing bacteria

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can be used as natural fertilizers to increase crop yields. Wild and domesticated *P. vulgaris* beans are nodulated by *Rhizobium etli* and related species such as *Rhizobium phaseoli* (López-Guerrero et al., 2012), both in its sites of origin (Martínez-Romero et al., 1985; Segovia et al., 1991; Segovia et al., 1993; Souza et al., 1994; Caballero-Mellado and Martinez-Romero, 1999; Aguilar et al., 2004) and in some regions where beans have been introduced (Herrera-Cervera et al., 1999; Grange and Hungria, 2004; Tamimi and Young, 2004; Aserse et al., 2012). This is an indication of *Phaseolus* symbiont selection irrespective of the soil origin. Furthermore, when *P. vulgaris* was used as a trap plant with Los Tuxtlas forest soil that is rich in *Bradyrhizobium*, it did not trap any bradyrhizobial strain (Ormeño-Orrillo et al., 2012). Domesticated *P. coccineus* plants from milpa plots in Mexico have been reported to be nodulated by *Rhizobium gallicum* (Silva et al., 2003), which is uncommonly found in *P. vulgaris* nodules. The genome of *Rhizobium* strain CCGE 510 obtained from field nodules of *P. albescens* was recently sequenced (Servín-Garcidueñas et al., 2012).

Even though incomplete information still exists, most legumes reported in the tribe Phaseoleae (Leguminosae) are nodulated by *Bradyrhizobium* (Martínez-Romero, 2009) and this could also be the case in *Phaseolus*. In contrast to *P. vulgaris*, *P. acutifolius* and *P. lunatus* cultivars together with wild *P. parvulus* and *P. pauciflorus* are nodulated by *Bradyrhizobium* species (Somasegaran et al., 1991; Parker, 2002; Ormeño-Orrillo et al., 2006). *Bradyrhizobium paxllaeri* and *Bradyrhizobium icense* were recently characterized as novel bradyrhizobial species isolated from nodules of *P. lunatus* from Peru (Durán et al., 2014).

It seems that "symbiosis with *Bradyrhizobium* represents the ancestral condition in the genus *Phaseolus* and that utilization of *Rhizobium* is a recent innovation that may be restricted to *P. vulgaris* and some of its close relatives, such as *P. coccineus*" (Parker, 2002). To support this assumption, other species should be analyzed as only a few *Phaseolus* species had been sampled for their nodule bacteria. We hypothesized that wild and domesticated *P. coccineus* and *P. dumosus* together with the wild species *P. costaricensis* and *P. albescens* may be naturally nodulated by *Rhizobium* strains due to their close phylogenetic affinities with *P. vulgaris*. The aim of this work was to study the symbiotic nodule bacteria from wild *Phaseolus* plants from representative species belonging to different clades and from all of the seven species that group within the *P. vulgaris* clade. We found that only species closely related to *P. vulgaris* were nodulated by *Rhizobium*.

Nod factors are produced by rhizobia during the early development of nodules upon perception of flavonoid molecules secreted by legume roots. The structure of Nod factors can vary between species, and chemical substitutions are commonly added that may affect legume specificity (D'Haeze and Holsters, 2002; Geurts and Bisseling, 2002). We compared potential *nod* genes encoded in the genomes of *Rhizobium* sp. CCGE 510 and *Bradyrhizobium* sp. CCGE-LA001 to predict Nod factors structures from two phylogenetically distinct strains isolated from field nodules of wild *Phaseolus* species.

2. Materials and methods

2.1. Wild Phaseolus nodule and soil samplings

P. vulgaris and *P. microcarpus* field nodules were collected from the town of Oaxtepec 1500 masl in the state of Morelos, Mexico. *P. vulgaris* and *P. leptostachyus* field-collected nodules were obtained in a pine and oak forest located in Cuernavaca City. *P. albescens* nodules were collected from the roots of a large vine (~12 m) growing in a mountain forest in the state of Jalisco, Municipio de Tecalitlán, Sierra del Halo. *P. coccineus* field collected nodules were recovered from two plants growing in the pine forest of Tetela del Bosque close to Cuernavaca City. Soils from sampling areas contained both rhizobial and bradyrhizobial symbionts and were mixed and used for subsequent trapping experiments.

2.2. Trapping experiments

Seeds were collected either from wild plants during field expeditions or provided by colleagues. Seeds were surface sterilized with serial washes of 70% ethanol, 1.5% sodium hypochlorite and several rinses with distilled water as described before (López-López et al., 2010). Surface sterilization was checked by rubbing the seeds on YEM medium (Vincent, 1970) in plates. After germination, seedlings were directly transferred to soils collected from our sampling areas. Plants were irrigated with sterile water every three days and were maintained in a temperature controlled room at 28 °C with a twelve hour light cycle. Nodules were collected from secondary roots after one month.

2.3. Phaseolus species identification

DNA was extracted from all the collected *Phaseolus* plants and seeds and the internal transcribed spacer marker (ITS) was amplified as described previously (Delgado-Salinas et al., 1999). DNA was extracted from leaves or nodules with the Genomic DNA Purification Kit (Fermentas) using a modified protocol. Briefly, leaves were chopped and mixed with 400 μl of lysis solution, incubated at 65 °C for five min and then 600 ml of chloroform was added. The mixed sample was then centrifuged at 11000*g* for 3 min. The supernatant was then purified using the High Pure PCR Product Purification Kit (Roche Applied Science). The amplified ITS regions were Sanger sequenced at Macrogen, Korea. *Phaseolus* ITS sequences of around 750 bp were searched against the GenBank database using BLASTN in order to validate their species identity and were used for phylogenetic analyses.

2.4. Bacterial isolation and identification

P. albescens field nodules were processed 4 days after collection and for all the other wild plants their nodules were processed within the same sampling day. In the laboratory, bacteria were recovered after nodule surface sterilization with 70% ethanol and then with 1.5% sodium hypochlorite followed by serial washes with sterile water as described previously (Ormeño-Orrillo et al., 2012). Surface sterilization was checked by rubbing the nodules on YEM medium (Vincent, 1970) in plates. Nodules were then crushed and their extracts were rubbed on YEM medium and on PY medium (Toledo et al., 2003). Plates were incubated at 28 °C for one week. One bacterial isolate was retained from each nodule. The same processing was performed for nodules retrieved from plant assays. DNA was extracted from each isolate and PCR amplifications were performed with 16S rRNA gene primers as described (Weisburg et al., 1991). PCR products were purified and sequenced. 16S rRNA gene sequences of 650–700 bp were searched against the GenBank database using BLASTN and their genus identities were verified.

2.5. Phylogenetic analyses

Sequences from related species were retrieved from GenBank. *rpoB*, *nodB* and *nifH* marker gene sequences were obtained from *R. phaseoli* strain ATCC 14482, *R. fabae* strain CCBAU 33202 and *R. pisi* strain DSM 30132 (Khamis et al., 2003; Silva et al., 2003; Zehr and McReynolds, 1989). Sequences were aligned with MUSCLE v3.8.31 (Edgar, 2004). Sequence alignments were inspected using MEGA5 software (Tamura et al., 2011). Phylogenetic

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