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## Systematic and Applied Microbiology

journal homepage: [www.elsevier.de/syapm](http://www.elsevier.de/syapm)Native bradyrhizobia from Los Tuxtlas in Mexico are symbionts of *Phaseolus lunatus* (Lima bean)Aline López-López<sup>a</sup>, Simoneta Negrete-Yankelevich<sup>b</sup>, Marco A. Rogel<sup>a</sup>, Ernesto Ormeño-Orrillo<sup>a</sup>, Julio Martínez<sup>a</sup>, Esperanza Martínez-Romero<sup>a,\*</sup><sup>a</sup> Centro de Ciencias Genómicas, UNAM, Cuernavaca, Morelos CP 62210, Mexico<sup>b</sup> Red de Ecología Funcional, INECOL, Xalapa, Veracruz, Mexico

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## ABSTRACT

Los Tuxtlas is the northernmost rain forest in North America and is rich in *Bradyrhizobium* with an unprecedented number of novel lineages. ITS sequence analysis of legumes in polycultures from Los Tuxtlas led to the identification of *Phaseolus lunatus* and *Vigna unguiculata* in addition to *Phaseolus vulgaris* as legumes associated with maize in crops. Bacterial diversity of isolates from nitrogen-fixing nodules of *P. lunatus* and *V. unguiculata* was revealed using ERIC-PCR and PCR-RFLP of *rpoB* genes, and sequencing of *recA*, *nodZ* and *nifH* genes. *P. lunatus* and *V. unguiculata* nodule bacteria corresponded to bradyrhizobia closely related to certain native bradyrhizobia from the Los Tuxtlas forest and novel groups were found. This is the first report of nodule bacteria from *P. lunatus* in its Mesoamerican site of origin and domestication.

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## Introduction

Nitrogen (N)-fixing bacteria from different genera (collectively designated rhizobia) form nodules on legumes and allow plants to grow in N deficient soils, such as rain forest soil that becomes N deficient after a few years of tilling and cropping. Legumes and rhizobia coexist at their sites of origin and domestication, for example, there is a large diversity of bradyrhizobia and rhizobia nodulating soybean in Asia. In contrast, in Africa and Mexico there is a need to inoculate introduced soybeans with *Bradyrhizobium*, since soybeans are not native and there are no soybean nodulating bacteria. There has been an agricultural selection of soybeans that could form nodules with native African bradyrhizobia but this has had limited success [18]. In Mexico, there is a large diversity of *Phaseolus vulgaris* and *Phaseolus coccineus* nodulating bacteria [21,31] because Mexico is the center of origin and domestication of many *Phaseolus* species [3,6,10], including *Phaseolus lunatus* (Lima bean). *P. lunatus* is a widely consumed grain legume for human nutrition in the USA and other countries. There are many studies on *P. vulgaris* nodule bacteria [2,15], but very few on *P. lunatus* symbionts. *P. vulgaris* forms nodules with *Rhizobium* (reviewed in [15,21]) while *P. lunatus* forms them with

bradyrhizobia [27,35]. *P. lunatus* isolates had not been studied in its Mesoamerican site of origin and domestication [19,20,23], although *P. lunatus* nodule bacteria were described from Peru, which is another domestication center where new bradyrhizobial groups were found [27].

Legumes were domesticated in association with cereals in different geographical regions worldwide [1]. Simultaneous growth of crops in traditional agriculture had the practical value of providing complementary and diverse nutrition to sustain families and communities. Maize and *P. vulgaris* (bean) have been grown in association for several thousand years. This agricultural practice is still maintained in Mexico, Peru and North Spain where beans and maize were introduced several hundred years ago. In Mesoamerica, polycultures are called *milpa*, a slash and burn system, that commonly includes maize, squash, *P. vulgaris* (common bean), and sometimes perennials. It is estimated that agriculture started in Los Tuxtlas at least 4000 years ago [9]. Evidence suggests that *P. vulgaris* and maize were probably introduced there from their centers of origin in Central-West Mexico [11]. Rhizobia from Los Tuxtlas have been studied in relation to land use, with forest, secondary forest, pasture and maize crop soils sampled using *Macropitilium atropurpureum*, *P. vulgaris* and *Vigna unguiculata* as trap plants [25]. A large diversity of lineages seemingly corresponding to new *Bradyrhizobium* species were described in the sampled areas [25]. Polycultures were not studied then. Therefore, the aim of this work was to describe the nodule bacteria both from native *P. lunatus* and introduced *V. unguiculata* legumes in polycultures from previously unstudied Los Tuxtlas regions.

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Materials and methods

Nodule bacteria isolation, PCR and sequencing

Composite samples

No specific permits were required for the described studies and no endangered or protected species were involved. Three soil samples from each of the 12 farmers from Ocotil Chico and Mazumapan municipalities (described in [24]) were mixed and used as substrate in small pots. Six different bean types commonly used in Los Tuxtlas, which were identified as distinct by farmers, were surface disinfected and grown. The surface of pots was covered with paper and cotton to prevent contamination. Irrigation was with sterile water. Plants were harvested 26 days after emergence.

Root nodules were surface disinfected with 1.5% sodium hypochlorite, and nodule extracts were grown on YM [37] and PY media [36]. Purified colonies were cultured on PY or YM at 28 °C. DNA extraction was with the DNA Isolation Kit for Cells and Tissues (Roche). 16S rRNA, *recA* and *rpoB* gene sequences were obtained after PCR, as previously described [17,29]. PCR-RFLP patterns were obtained by restriction of *rpoB* with the enzymes *Hind*III, *Msp*I, *Hha*I and *Rsa*I. Sequencing (Sanger) was performed by Macrogen. Alignments were performed with Clustal W [13] and manually revised. Phylogenetic trees were obtained with neighbor-joining [30] and maximum likelihood [8] analyses using MEGA5 [34].

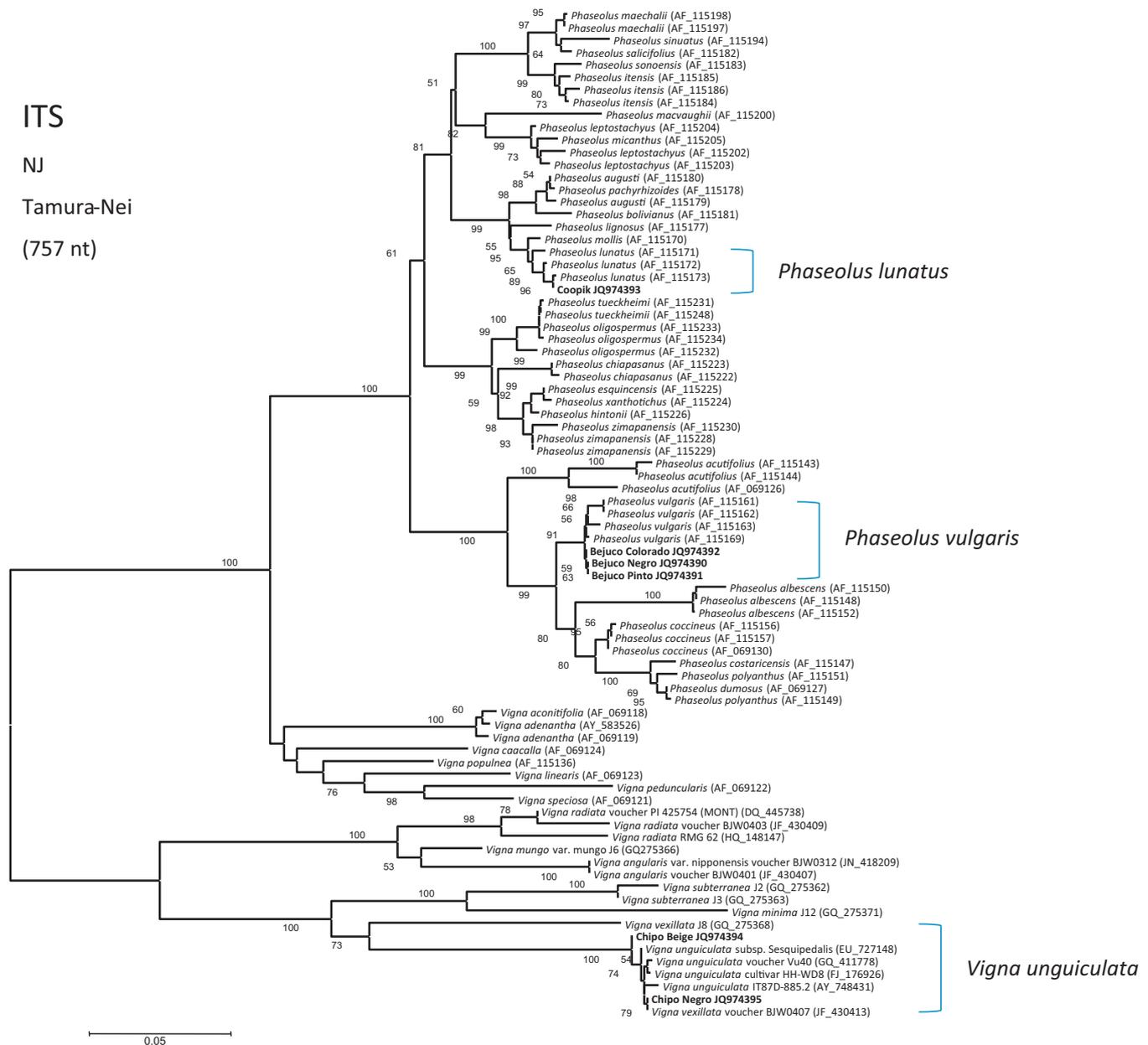


Fig. 1. Phylogenetic relationships of *Phaseolus* species based on ITS sequences. Sequences obtained in this work are shown bold. Bootstrap values >70% are indicated at the nodes. Bar, 5 nt substitutions per 100 nt.

### Plant DNA extraction and ITS analysis

To identify the *Phaseolus* species, a molecular approach was followed because only native seeds were available. Young leaves from the local bean plants germinated in the laboratory were macerated with liquid nitrogen and DNA was extracted with the same Roche DNA isolation kit. The internal transcribed spacer (ITS) was amplified with ITS primers PhaseoF and PhaseoR, and PCR conditions were as previously described [6]. PCR products were Sanger sequenced. Phylogenetic analyses were performed as described above.

### Accession numbers

The GenBank accession numbers for the *recA* gene sequences determined in this study are JQ966948–JQ966967 and JX943615–JX943617. The *nodZ* and *nifH* gene sequences have been assigned accession numbers JX645470–JX645478 and JX943618–JX943622, respectively.

### Results

Beans from Los Tuxtlas were classified into three different species, *P. vulgaris*, *P. lunatus* and *V. unguiculata*, according to ITS sequences (Fig. 1). Morphologically, *P. lunatus* seeds from Los Tuxtlas resembled the most common type of *P. lunatus* seeds grown in Southern Mexico [J. Martínez-Castillo, personal communication]. By plant trap experiments with local *P. lunatus* and *V. unguiculata* seeds, nodule bacteria were sampled from *milpa* soils from a region of Los Tuxtlas where a large diversity of crops have been preserved [4]. *P. lunatus* and *V. unguiculata* isolates were slow growing bacteria. A total of 157 bradyrhizobia isolates were obtained, 98 from *P. lunatus* and 59 from *Vigna* (Supplementary Table S1). Isolates from *Vigna* were grouped into eight ERIC profiles, with two of them representing almost 80% of the profiles. From *P. lunatus* isolates, 52 ERIC profiles were identified and most of them were represented only by a single strain, and only three profiles had five or more isolates. Only one ERIC profile was found in common among isolates from both legumes. PCR-RFLP patterns of *rpoB* genes, previously used to characterize bradyrhizobial isolates from *P. lunatus* in Peru [27], showed four distinct patterns in *Vigna* isolates, and two of them included 95% of the isolates. The dominant ERIC profiles were included in these *rpoB*-based genotypes. On the other hand, *P. lunatus* isolates corresponded to eight patterns by *rpoB* PCR-RFLP analysis, one of which had 91% of the isolates and most ERIC profiles. The remaining *rpoB* patterns had one or two isolates. It is known that ERIC profiles may distinguish different strains within a single species that may be represented by single *rpoB* gene sequences. The bradyrhizobial populations of *V. unguiculata* and *P. lunatus* were different and only one *rpoB* PCR-RFLP pattern was shared.

*recA* gene phylogenies have been used to describe bradyrhizobial diversity [38], and the current study showed that, on this basis, most *P. lunatus* symbionts were closely related to previously described isolates from Los Tuxtlas (Fig. 2). Bradyrhizobial isolates from *P. lunatus* corresponded to reported genospecies TUXTLAS-17, TUXTLAS-4, TUXTLAS-20 and TUXTLAS-2, to three novel genospecies (TUXTLAS-33, -34 and -35) and to *Bradyrhizobium japonicum* (Fig. 2). *P. lunatus* isolate Pop235 was closely related to *Inga* isolate Inga3-1a, while Pop363 was related to Inga3-2d. *Inga* species are native trees in Los Tuxtlas forest and their nodule bacteria were isolated and characterized previously [25]. Mexican isolates from *P. lunatus* were different to those from Peru (Fig. 2). Most isolates from *Vigna* grouped with *Bradyrhizobium*

*elkanii*, whereas others grouped with genospecies TUXTLAS-17 and TUXTLAS-21.

Representatives of all *rpoB* restriction patterns from *Vigna* and *P. lunatus* isolates were tested in the laboratory for nodulation of both legumes (Supplementary Table S3). All *P. lunatus* isolates could nodulate and fix nitrogen in *Vigna* and *P. lunatus*. *Vigna* isolates were capable of nodulating both legumes but only one (Pop352) could fix nitrogen in *P. lunatus*, which indicated that *P. lunatus* is a more restricted host while *Vigna* is considered promiscuous [14,28]. *B. japonicum* USDA 110 and *B. elkanii* USDA 76 nodulated and fixed nitrogen in *Vigna* but failed to nodulate in *P. lunatus*. *B. elkanii* USDA 76 formed only root bumps in *P. lunatus*. Additionally some bradyrhizobial strains previously described, representing native TUXTLAS genospecies [25] were tested in nodulation assays in the laboratory. All tested strains were found to nodulate *P. lunatus* with some of them fixing nitrogen (Supplementary Table S2).

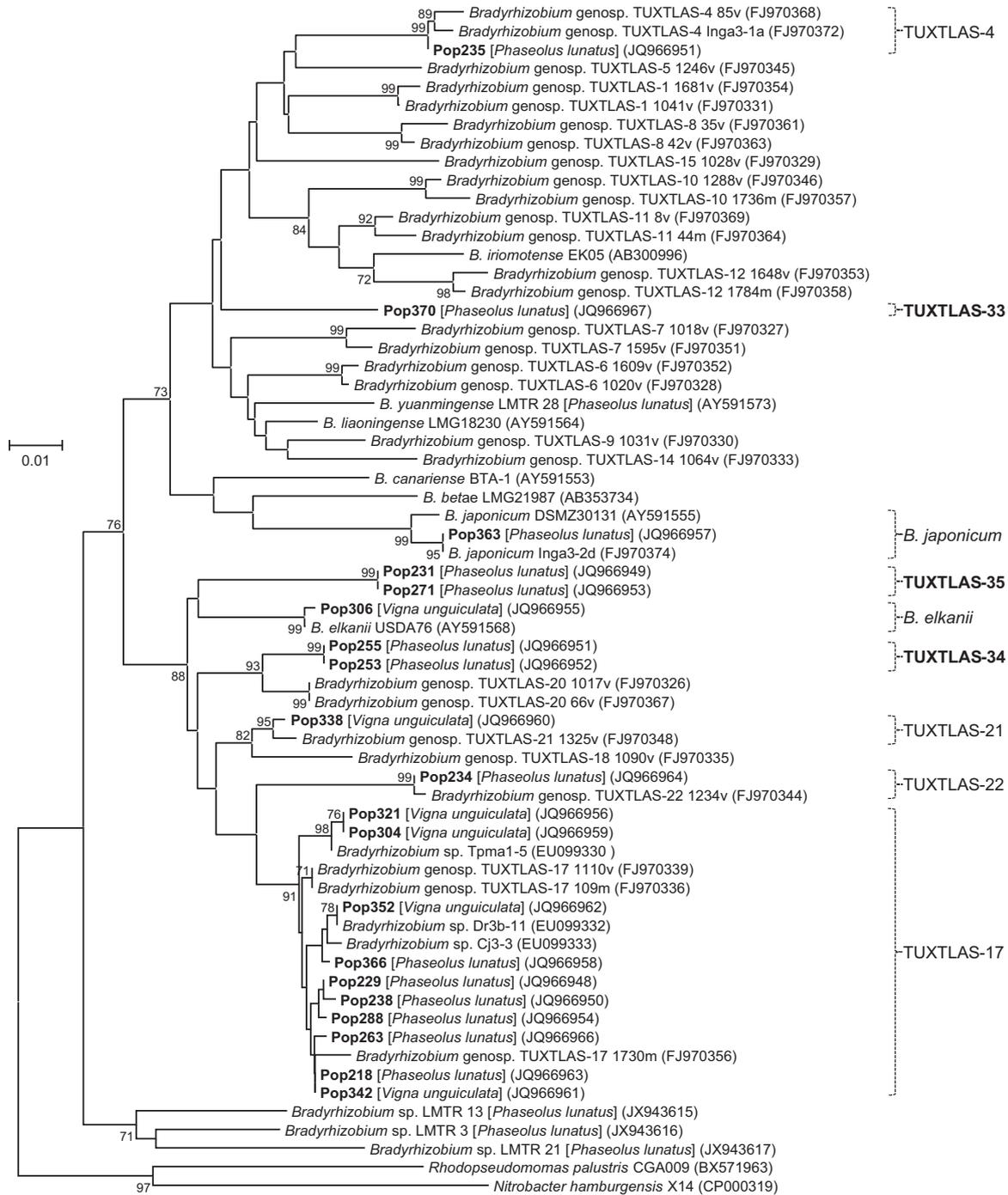
Based on *nodZ* sequences, most *V. unguiculata* isolates corresponded to *nodZ* gene group III (Fig. 3), according to a classification proposed earlier [33]. Group III is considered a pantropical ancient group of bradyrhizobial nodulation genes and most *Vigna* bradyrhizobia from Africa possess these genes [33]. Pop352, the only *Vigna* isolate able to fix nitrogen with *P. lunatus*, clustered in *nodZ* clade V together with Pop367 from *P. lunatus* and other bradyrhizobial isolates previously isolated from Los Tuxtlas (Fig. 3). Group V was proposed earlier based on a single sequence from a Brazilian *Lupinus* bradyrhizobia [33]. In this study, it was found that bradyrhizobia from Los Tuxtlas, as well as Brazilian strains isolated from different plants, had *nodZ* genes corresponding to group V, which seems to have an American origin. *nifH* gene phylogeny also separated isolates from groups III and V (Supplementary Fig. S1) and showed the same close relationship between Brazilian group V isolates and bradyrhizobia from Los Tuxtlas.

### Discussion

As polycultures in Mexico commonly include *P. vulgaris* it was surprising to find *P. lunatus* and *V. unguiculata* in milpas from Los Tuxtlas. In comparison to *P. vulgaris* and *V. unguiculata*, *P. lunatus*-nodulated roots with Los Tuxtlas rhizobia exhibited the highest levels of acetylene reduction activity (not shown) and highest symbiont diversity. Of all *Phaseolus* species, *P. lunatus* seems to have the broadest geographical distribution. It is widely cultivated in the tropics and subtropics, possibly in relation to its capacity to form nodules with bradyrhizobia that are abundant in tropical soils [22,25].

Wild *P. lunatus* are found in Mexico [19,20,23] and have been described in Los Tuxtlas, but wild *P. vulgaris* have not been found in this area [Jaime Martínez-Castillo, personal communication]. Mexico, together with Peru, is a *P. lunatus* domestication site [10,23,27] but *P. lunatus* cultivars grown in Peru and Mexico are different and their bradyrhizobial symbionts seem to be specific for each domestication site ([27] and this work). Supposedly, there is a domestication origin of *P. lunatus* in Southern Mexico but it has not been located yet.

Most *P. lunatus* isolates were found to correspond to genospecies TUXTLAS-17 that was reported as abundant in crop fields [25,26]. Genospecies TUXTLAS-20 and TUXTLAS-22 were less frequently isolated from *P. lunatus*, which corresponds to the observation in previous studies that they were less abundant in crop fields [25,26]. *P. lunatus* forms nodules with native bradyrhizobia in tropical Los Tuxtlas soils, whereas *P. vulgaris* symbionts are exotic and have been introduced to Los Tuxtlas [25,26]. Therefore, it is concluded that *P. lunatus* is a more adequate crop legume for Los Tuxtlas than *P. vulgaris*.

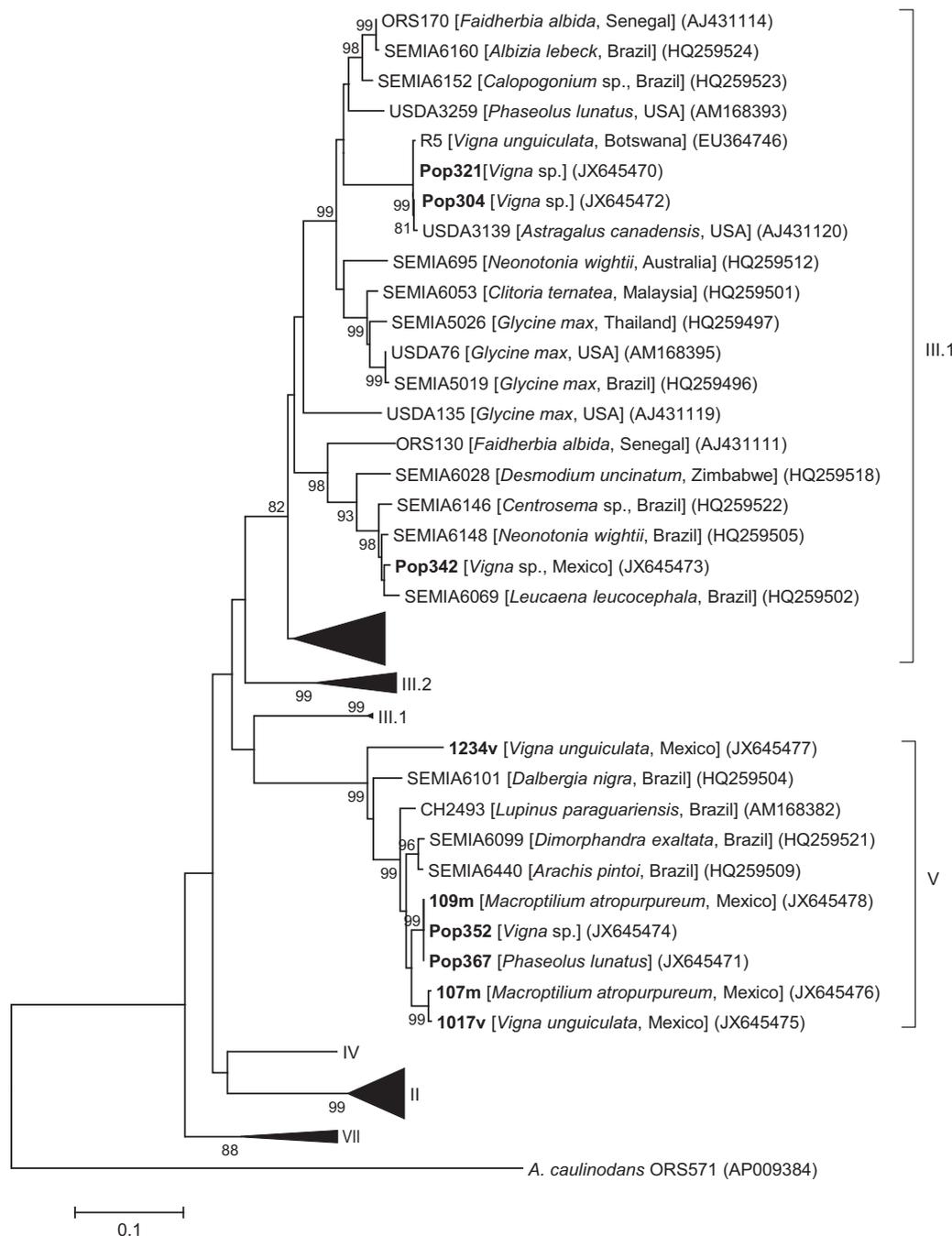


**Fig. 2.** *recA* gene phylogenetic relationships of *Bradyrhizobium* from *Phaseolus lunatus* and *Vigna unguiculata* isolated in this work (Pop strains). Geno(species) affiliations of Pop strains are indicated on the right-hand side. Novel genospecies are shown in bold. Bootstrap values >70% are indicated at the nodes. Bar, 1 nt substitution per 100 nt.

*Vigna* includes more than 200 species distributed throughout the tropics, some of them were domesticated and are widely used in tropical agriculture. The geographical origin of cowpea (*V. unguiculata*) is Africa and the bacteria associated with this legume have been reported as bradyrhizobia and some fast growing rhizobia. Cowpea bradyrhizobia isolated in Africa [33,39], China [40,41] and Brazil [42] were identified as *B. elkanii*, *B. japonicum*, *Bradyrhizobium liaoningense*, *Bradyrhizobium yuanmingense* and other unnamed *Bradyrhizobium* genospecies. In this work, the symbionts of *Vigna* were identified as *B. elkanii* and two previously reported genospecies (TUXTLAS-17 and TUXTLAS-21) [25,26]. Symbionts of *V. unguiculata* had already been studied using *Vigna* as

a trap plant from soils of another region in Los Tuxtlas [25]. *B. elkanii* and the three novel genospecies found here were not previously reported in that study [25,26]. This indicates that there is geographical heterogeneity of soil bacteria, which was not unexpected in view of the different landscape and environmental conditions therein [5,12]. However, it is remarkable that rhizobia similar to those found in Los Tuxtlas also exist in Central America and a microbiological corridor comprising such regions has been identified [16].

Approximately 40% of the forest cover has been lost in Los Tuxtlas [32] and soils are nutrient depleted [7]. Knowledge of the legume species grown in the Los Tuxtlas milpas is essential for



**Fig. 3.** Maximum likelihood phylogeny of *nodZ* gene sequences. Sequences of strains isolated from Los Tuxtlas are shown in bold. Those obtained in this study have the Pop prefix. *nodZ* clades are indicated with Roman numerals. Bootstrap values >70% are indicated at the nodes. Bar, 1 nt substitution per 10 nt.

determining which rhizobia could be used as inoculants for fulfilling our agro-ecological project aimed at maintaining soil fertility through symbiosis with native microorganisms.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.syapm.2012.10.006>.

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