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Nodule bacteria from the cultured legume *Phaseolus dumosus* (belonging to the *Phaseolus vulgaris* cross-inoculation group) with common tropici phenotypic characteristics and symbiovar but distinctive phylogenomic position and chromid

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ABSTRACT

Phaseolus dumosus is an endemic species from mountain tops in Mexico that was found in traditional agriculture areas in Veracruz, Mexico. *P. dumosus* plants were identified by ITS sequences and their nodules were collected from agricultural fields or from trap plant experiments in the laboratory. Bacteria from *P. dumosus* nodules were identified as belonging to the phaseoli-etli-leguminosarum (PEL) or to the tropici group by 16S rRNA gene sequences. We obtained complete closed genomes from two *P. dumosus* isolates CCGE531 and CCGE532 that were phylogenetically placed within the tropici group but with a distinctive phylogenomic position and low average nucleotide identity (ANI). CCGE531 and CCGE532 had common phenotypic characteristics with tropici type B rhizobial symbionts. Genome synteny analysis and ANI showed that *P. dumosus* isolates had different chromids and our analysis suggests that chromids have independently evolved in different lineages of the *Rhizobium* genus. Finally, we considered that *P. dumosus* and *Phaseolus vulgaris* plants belong to the same cross-inoculation group since they have conserved symbiotic affinites for rhizobia.

Introduction

Rhizobia are the oldest bacterial products used in agriculture and have been commercially produced for over one hundred years. Even now, biotechnology companies have a strong preference for *Rhizobium* strains because they are considered safe and are not potential human pathogens. Crop inoculants produced with rhizobia constitute one of the best examples of the successful use of bacteria to avoid the use of chemical fertilizers [31], which are a source of pollution [71] and represent a large cost for farmers. Additionally, the high nitrogen content of legume seeds may be related to the nitrogen input from nitrogen-fixing rhizobia [39].

Phaseolus vulgaris (common bean) is the most widely used legume for human nutrition in the world, its site of origin is the Balsas region in Mexico [7] and domestication regions have been recognized in Mesoamerica and South America [15]. *P. vulgaris* symbionts have been studied from many geographical regions [4,5,10,12,18,20,22,30,38,42,45–47,57,58,61,62,68–70,76,83,84,86,87]. In contrast, nodule bacteria from other *Phaseolus* species have been less studied, except those from other cultivated species such as *Phaseolus lunatus* [19,37,50,51,53,55] and *Phaseolus coccineus* [76].

Among the many rhizobial species that nodulate *P. vulgaris*, *Rhizobium tropici*, *Rhizobium freirei* [14] and *Rhizobium etli* selected

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strains are used commercially as bean inoculants [31,36]. Our description of *R. tropici* highlighted an outstanding bacterial species [44] that became the preferred inoculant for *P. vulgaris* in Brazil [31] and illustrates the value of rhizobial diversity studies. Several species related to *R. tropici* have subsequently been described commonly isolated from tropical areas [11,13–14,26,28,63,80] and here they are collectively referred to as the tropici group.

In Mexico, besides *P. vulgaris*, there are several native *Phaseolus* species. For example, *Phaseolus albescens* which is endemic and related to *P. vulgaris*, climbs over pine trees with vines that are over 10 m long. It is a wild non-cultivated species, and its rhizobial isolates resembled *Rhizobium leguminosarum*. *P. albescens* rhizobia had a symbiosis plasmid that was clearly related to the phaseoli plasmid (for *P. vulgaris* nodulation), however, it formed ineffective nodules in *P. vulgaris* reflecting its adaptation to *P. albescens* instead of to *P. vulgaris* [73]. Other *Phaseolus* with studied symbionts are *Phaseolus microcarpus* [74], *Phaseolus acutifolius* and *Phaseolus* spp. [56]. *Phaseolus dumosus*, is a high mountain endemic species whose symbionts have scarcely been studied. Mountain tops may be considered as islands in evolutionary ecology analyses. In a phylogenomic profile *P. dumosus* plants are closely related to *Phaseolus costarricense* and related to *P. coccineus* and *P. vulgaris* [60]. Previously, wild *P. dumosus Rhizobium* symbionts were described but were not assigned to any known species [75]. *P. dumosus* was among the *Phaseolus* species that were identified as those with a symbiont shift to form nodules with *Rhizobium* instead of bradyrhizobia [75].

Traditional agriculture in Mexico dates back thousands of years [24] and nowadays it is frequently found in low resource areas where farmers depend on small pieces of land. The site of study in Ocotepec, Ayahualulco, Veracruz is a marginal area of extreme poverty with traditional agricultural practices. In a previous study from Veracruz we obtained rhizobia of the tropici group from *Gliricidia sepium*, the alfalfa from the tropics [1], and from *P. vulgaris* [52] and bradyrhizobia from *P. lunatus* [37]. The interest in studying *Phaseolus* symbionts was to stimulate the cultivation of these crops, to try to motivate the inhabitants to return to traditional products that are more nutritional than the junk food that is currently consumed in Mexico, and to obtain knowledge of *Phaseolus* nitrogen-fixing symbionts that could help to provide regional and efficient inoculants.

Materials and methods

Study site and plant collection

Plants were collected in June 2016 in the rural community of Ocotepec, Ayahualulco in the State of Veracruz, Mexico (19°21′N, 97°9′W), which is close to the region known as Cofre de Perote. The altitude is 2280 ma.s.l. with annual ranges of temperature and rainfall (in Ayahualulco) of 2–18 °C and 300–1600 mm, respectively.

The cultivation fields are distributed among the families in the community and each one takes charge of their own crop field. In order to eliminate the use of agrochemicals, the addition of compost has been implemented since 2009. However, there are differences in crop management. *Phaseolus* are co-cultured with maize (*Zea mays*) and other vegetables like swiss chard (*Beta vulgaris*), peas (*Pisum sativum*), and broad bean (*Vicia faba*). Bulk density of soil is 0.74–0.90 g cm^{-3}, and pH 4.3–5.1. In June 2016 five crop fields were selected for root sampling and soils were collected from twelve different fields for the trap plant experiments. *Phaseolus* plants growing in close proximity to maize were randomly selected from each crop field and taken with a clean garden shovel being careful not to cut the roots, placed in a clean plastic bag and transported to the lab in a clean container. A total of 30 plants were taken for DNA extraction and for nodule sampling. Bacteria were isolated from nodules as described below.

Trap plant assays

For the trap plant assay, *P. dumosus* seeds were surface sterilized with 70% ethanol and washed with sterile water, placed in 10% bleach for 2 min and washed again with sterile water 10 times. For germination, approximately 150 g of bulk soil collected from each of 12 crop fields were pooled. Surface-sterilized seeds were taken with sterile forceps and placed in approximately 200 g of soil and grown under controlled conditions of light/dark cycles (12:12 h) at 25 °C for 30 days to allow the plants to form nodules. Subsequently, bacteria were isolated.

Bacteria isolation

Bacteria were isolated from surface disinfected nodules. Roots were rinsed in sterile water to remove soil excess. Nodules were separated from the roots using sterile forceps, rinsed with 70% ethanol and washed with sterile water, placed in 10% bleach for 2 min and washed again with sterile water 10 times. Using sterile forceps, nodules were squashed on plates with PY medium (5 g l^{-1} peptone; 3 g l^{-1} yeast extract, 0.6 g l^{-1} calcium chloride) or YM medium (2.87 mM K_2HPO_4; 1.66 mM $MgSO_4$; 1.71 mM NaCl; 10 g l^{-1} mannitol; 400 mg l^{-1} yeast extract) and incubated for 48–72 h at 30 °C. Individual colonies were streaked on plates and incubated for 48–72 h at 30 °C. Pure isolates were tested for nodulation of *P. dumosus, P. vulgaris* or *Leucaena leucocephala* in flasks with Fahraeus solution under gnotobiotic conditions in the laboratory. Non-inoculated controls were incubated under the same conditions as inoculated plants. Strains were stored in 30% glycerol stocks at −80 °C.

Plant ITS analyses

For plant DNA extraction, one leaf from the same plant from which the nodules were isolated was used. DNA was extracted using DNeasy Blood and tissue kit (QIAGEN) following the manufacturer's instructions. ITS primers used were from Delgado-Salinas et al. [17] and the PCR reaction was one cycle at 95 °C for 3 min followed by 35 cycles of denaturation (95 °C for 45 s), annealing (55 °C for 45 s), extension (72 °C for 1 min) and a final cycle at 72 °C for 10 min. PCR products were sequenced at Unidad de Síntesis y Secuenciación de ADN, IBt, UNAM.

Bacteria DNA extraction, PCR amplicon analysis, plasmid patterns and genome sequencing

DNA was extracted from 3 ml of liquid cultures with a DNA isolation kit for cells and tissues (ROCHE). For 16S rRNA gene sequences DNA was used as template for PCR reactions using primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) [85]. Plasmids were visualized with ethidium bromide in gel electrophoresis following the modified Eckhardt procedure [32].

Genomes were sequenced by Pacific Biosciences (PacBio RSII) single-molecule real-time (SMRT) platform. One SMRT cell of a 15- to 20-kb insert library was sequenced for each strain.

Sequences were used for the *de novo* genome assembly using "Canu release v1.5" program [33]. Genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

Phylogenetic, phylogenomic and average nucleotide identity (ANI) analyses

Gene and genome sequences were retrieved from GenBank database. Gene sequence edition and multiple sequence alignments were performed with BioEdit [27]. Maximum likelihood phylogenies were reconstructed with Mega 7 [34,77] using 1000 bootstrap pseudoreplicates.

Phylogenomic analysis from 26 sequenced *Rhizobium* strains using maximum likelihood phylogeny was reconstructed with FastTree 2 [59] using a concatenated alignment of the 3847 most discriminative amino acid positions of 373 proteins conserved in all genomes which were identified with PhyloPhlAn [72]. Shimodaira–Hasegawa (SH)-like local support values were calculated. All proteins used in the PhyloPhlAn analysis were encoded in chromosomes in CCGE531 and CCGE532.

Average nucleotide identity using MUMMER for genome alignment (ANIm) was calculated with JSpecies [64] for whole genomes, selected genes, for chromids and for symbiosis plasmids [16].

Phenotypic assays

Bacterial liquid cultures were prepared from glycerol stocks. Phenotypic characteristics were assessed in minimal medium, PY and YM liquid cultures. Initial cultures were adjusted at 0.1 O.D. (600 nm) and evaluated in MM media with only one carbon and nitrogen source, PY with no calcium added, variable salt concentration (0.25 M NaCl; 0.5 M NaCl), and variable temperature (30, 37 and 42 °C). The MM culture contained per liter: 21.82 mM K_2HPO_4; 21.91 mM KH_2PO_4; 5 ml sucrose (200 mg ml^{-1}); 5 ml KNO_3 (5% solution); 5 ml $MgSO_4$ (2% solution); 1 ml of microelements solution [H_3BO_3 (2.86 g l^{-1}); $MnSO_4{\cdot}4H_2O$ (2.03 g l^{-1}); $ZnSO_4{\cdot}7H_2O$ (220 mg l^{-1}); $CuSO_4{\cdot}5H_2O$ (80 mg l^{-1}); $Na_2MoO_4{\cdot}H_2O$ (80 mg l^{-1})]; 5 ml ferric citrate (10 mg ml^{-1}). Three replicates for each strain were included. Cultures were incubated at 30 °C unless otherwise indicated.

Nitrogenase activity assay

Seeds of *P. vulgaris*, *P. dumosus* and *L. leucocephala* were superficially disinfected as described [78] and germinated on agar-water plates (6.8 g l^{-1}). Three day seedlings were transplanted into 200 ml flasks containing Fahraeus medium, per liter: 10 ml $Na_2HPO_4{\cdot}12H_2O$ (15 g l^{-1}); 10 ml $MgSO_4{\cdot}7H_2O$ (12 g l^{-1}); 10 ml KH_2PO_4 (10 g l^{-1}); 10 ml ferric citrate (500 mg l^{-1}); 10 ml $CaCl_2$ (10 g l^{-1}); 1 ml microelements [H_3BO_3 (2.86 g l^{-1}); $MnSO_4{\cdot}4H_2O$ (2.03 g l^{-1}); $ZnSO_4{\cdot}7H_2O$ (220 mg l^{-1}); $CuSO_4{\cdot}5H_2O$ (80 mg l^{-1}); $Na_2MoO_4{\cdot}H_2O$ (80 mg l^{-1})] and 7.5 g agar. Seedlings were incubated at 30 °C overnight. Cells in sterile water were adjusted to 0.1 D.O. (600 nm). Plants were inoculated with 500 μl of this suspension and placed under controlled conditions for light/dark cycles (12:12 h) at 25 °C for 17 days. Roots were removed from the plants, placed in 20 ml flasks which were hermetically sealed. Using sterile syringes, 400 μl of air in the flask was substituted by 400 μl of acetylene and incubated for 60 min. Nitrogenase activity was evaluated in a gas chromatograph by injecting 400 μl of the gas contained in the flasks and measuring the ethylene produced, compared to a standard ethylene sample.

Data availability

The *Phaseolus* ITS and rhizobial 16S ribosomal gene sequences reported in this study have been deposited in the GenBank database under accession numbers MH999282–MH999290 and MK128456–MK128484, respectively. Genome sequences of *Rhizobium* sp. CCGE531 and *Rhizobium* sp. CCGE532 have been deposited in GenBank database under accession numbers CP032694–CP032697 and CP032684–CP032688, respectively and were deposited as NCBI bioprojects under accession numbers PRJNA492910 and PRJNA492914.

Results and discussion

Phaseolus *plant identification*

Intergenic plant ribosomal sequences (ITS) were used to identify the *Phaseolus* species grown by farmers in Ocotepec and revealed that cultured bean plants were *P. dumosus* and *P. vulgaris* (data not shown). It was unknown that farmers therein cultivated *P. dumosus*. Other cultured *Phaseolus* identified only by morphology with no ITS sequence determined were designated here as *Phaseolus* sp.

Phaseolus *nodule-bacteria identification*

From agricultural fields and plant-trap assays, *Phaseolus* nodule isolates were identified by the sequence of 16S rRNA genes as belonging to the phaseoli-etli-leguminosarum (PEL) [62] or tropici groups (Fig. 1). *P. vulgaris* isolates were not further analyzed as they have been extensively studied (see Introduction). From the plant-trap assays using surface-disinfected *P. dumosus* seeds and a composite soil sample from Ocotepec, we found that seven isolates belonged to the tropici group and one to the PEL group. PEL rhizobia have been found to be prevalent in *P. vulgaris* crop fields in many areas of Mexico including Veracruz [52] and also in cultivated *P. coccineus*, so it was not unexpected to find them as *Phaseolus* symbionts, thus we focused here on the less studied rhizobia from the tropici group. In total, thirteen isolates belonging to the tropici group (including CCGE531 from the laboratory trap plant assay and CCGE532 from an agricultural field) were obtained from *P. dumosus, P. vulgaris* and *Phaseolus* sp. and were found related to *Rhizobium jaguaris*, *Rhizobium paranaense* and *Rhizobium vallis* by 16S rRNA gene phylogenies. *R. jaguaris* CCGE525^{T} was described from *Calliandra* spp. nodules from a rain forest area in Chiapas, on the other (Pacific) coast of Mexico [65]. Notably 16S rRNA gene phylogenies provide inaccurate phylogenetic relationships in the tropici group and this has been observed in *R. freirei* [14], *Rhizobium hainanese* [11] and *Rhizobium rhizogenes.* Similarly, we found here that CCGE531 and CCGE532 were not related to *R. jaguaris* as shown in 16S rRNA gene phylogenies but were more related to *Rhizobium lusitanum* than to *R. jaguaris* in a phylogenetic tree using concatenated *recA*, *atpD* and *rpoB* (Fig. 2). *R. lusitanum* isolates were originally obtained from north–west Portugal from *P. vulgaris* [80]. However, on a genome-based phylogeny, *R. lusitanum* was not found closely related to CCGE531 or to CCGE532 (see below). Thus we recommend that genome sequences should become the gold standard to identify and classify rhizobia.

Genome-based analysis

The complete genome sequences of isolates CCGE531 and CCGE532 were obtained (Table 1). In Eckhardt gels different chromid sizes and plasmids (Fig. 3) matched the replicon sizes defined from the genome analysis (Table 1). A phylogenomic analysis of the tropici group is presented here that showed that CCGE531 and CCGE532 constitute an independent lineage within this group (Fig. 4).

ANI metric was used to compare CCGE531 and CCGE532 genomes to those from other tropici group species. ANI values were found to be below 90% with all compared species for which genome sequences were available (Table 2). The highest ANI (89%) was with *R. tropici* CIAT 899^{T}. Besides the global genome-based ANI, we calculated an ANI using few genes, the same concatenated genes (*recA*,

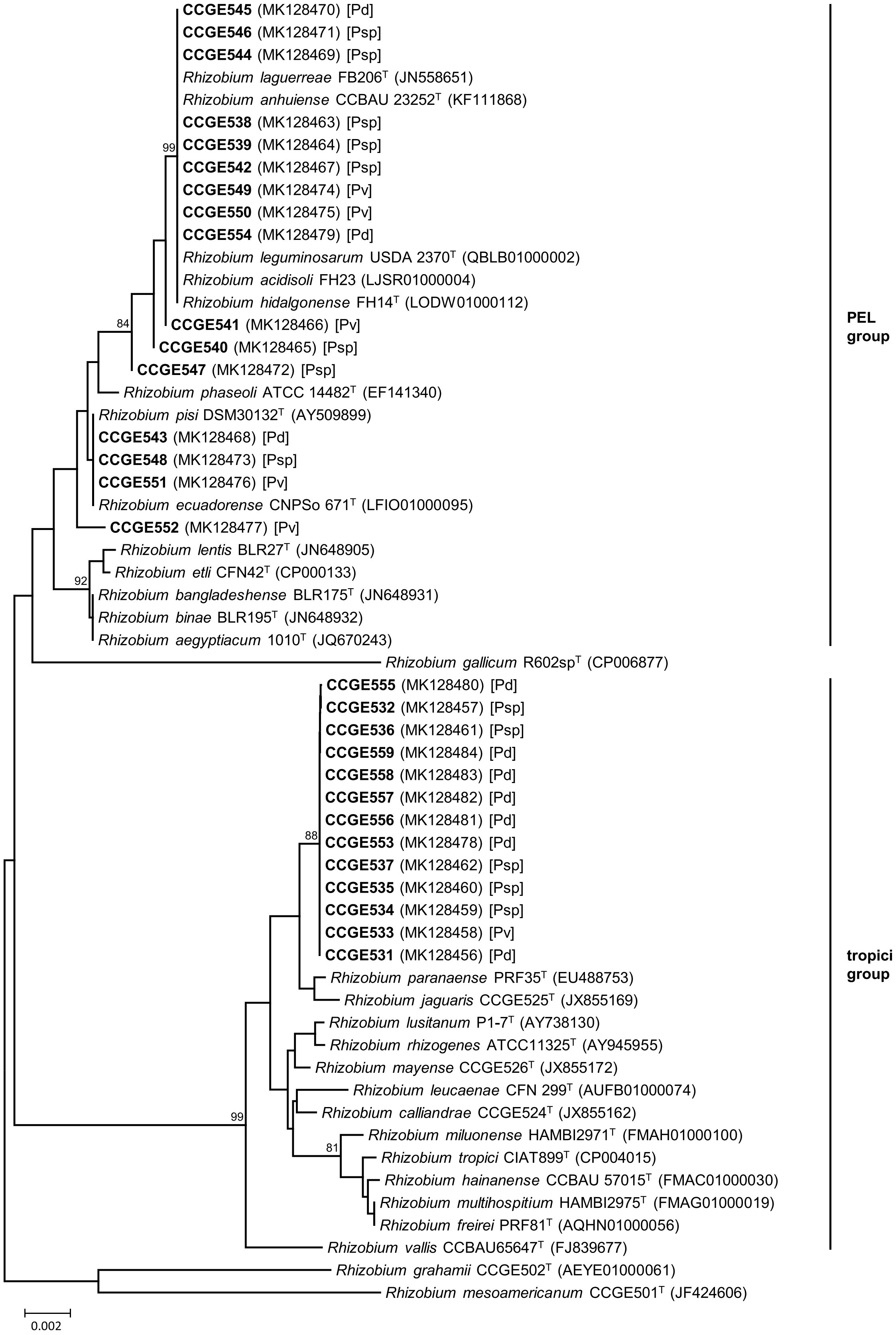


Fig. 1. Maximum likelihood phylogenetic tree of 16S rRNA gene sequences from rhizobia type strains and isolates obtained from nodules of *P. dumosus* [Pd], *P. vulgaris* [Pv] or *Phaseolus* sp. [Psp] shown in bold. Partial 16S rRNA gene sequences were used (1308 bp). Bootstrap support values higher than 70% are shown at nodes. The bar indicates 2 substitutions per 1000 nucleotide positions.

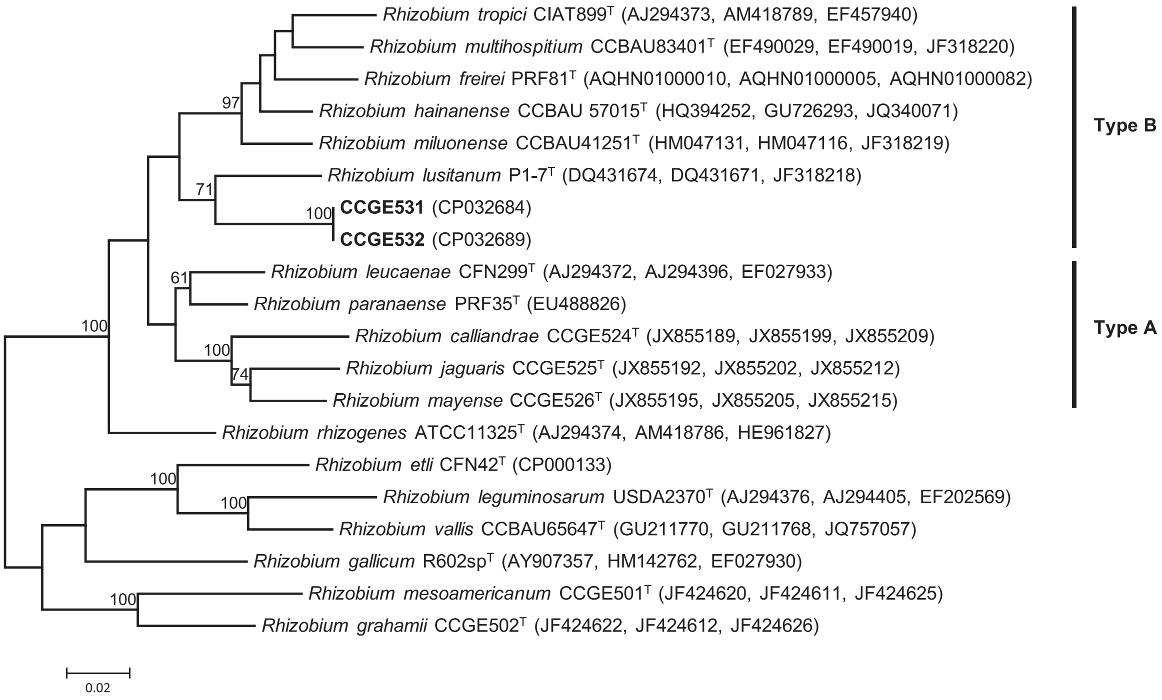


Fig. 2. Maximum-likelihood phylogeny based on concatenated alignment of partial sequences of the marker genes *recA* (398 bp), *atpD* (438 bp) and *rpoB* (650 bp). Bootstrap values higher than 70% are shown at nodes. The bar indicates 2 substitutions per 100 nucleotide positions.

Table 1
Replicon size in bp and GC content.

Replicon	*Rhizobium* sp. CCGE531	*Rhizobium* sp. CCGE532	*R. tropici* CIAT899^{T} [a]
Chromosome	4,041,163	3,907,717	3,387,060
Chromid	1,460,465	1,460,443	2,083,197
Plasmid pC	756,635	747,941	
Symbiotic plasmid pB	577,596	506,160	549,467
Plasmid pA	304,634	300,963	216,610
Total	7,140,493	6,923,224	6,686,334
GC content	59.08	59.12	59.5

^{T}: Type strain.
[a] Data taken from Ref. [50].

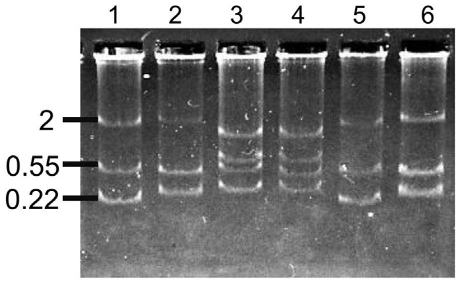


Fig. 3. Plasmid patterns in electrophoresis gel using the Eckhardt procedure. Lanes 1 & 5 *Rhizobium tropici* CIAT 899^{T}; 2 & 6 *R. jaguaris* CCGE525^{T}; 3 & 4, *Rhizobium* spp. CCGE531 and CCGE532, respectively. Genome size in Mb.

atpD, *rpoB*) from the phylogenetic analysis (Fig. 2). In all cases an ANI lower than 94–95% was obtained between CCGE531 and CCGE532 and the tropici group species. Previously, ANI estimated with few selected genes resulted in good correlation to other taxonomic estimates [5].

Phenotypic tests and group A and B congruence to phylogenetic trees

We used here the phenotypic tests that distinguished the originally described *R. tropici* strains [42,44]. CCGE531 and CCGE532 grew in LB medium and in medium with high sodium concentrations (0.25 M and 0.5 M NaCl), and also at high temperatures (37 and 42 °C), in PY medium with no calcium added and were resistant to antibiotics like *R. tropici* CIAT 899^{T} (Table 3). As other bacteria in the tropici group, CCGE531 and CCGE532 were acid tolerant (pH 4.5). It is remarkable that the phenotypic characteristics, which separated the originally described type A and type B strains in *R. tropici* [44], seem to follow phylogenetic relationships

Table 2
Average nucleotide identity based on MUMMER alignments (ANIm) between genome sequences of strains CCGE531 and CCGE532 and type strains of the tropici group.

Strain	ANIm (%)	
	CCGE531	CCGE532
CCGE531	100	99.78
CCGE532	99.76	100
R. tropici CIAT899^{T}	89.15	89.06
R. freirei PRF 81^{T}	88.93	88.82
R. lusitanum P1-7^{T}	88.28	88.2
R. hainanense CCBAU 57015^{T}	88.2	88.2
R. multihospitium HAMBI 2975^{T}	88.11	88.1
R. leucaenae CFN 299^{T}	87.88	87.69
R. miluonense HAMBI 2971^{T}	87.64	87.64
R. paranaense PRF 35^{T}	86.22	86.17
R. jaguaris CCGE525^{T}	85.74	85.73
R. rhizogenes NBRC 13257^{T}	85.75	85.74

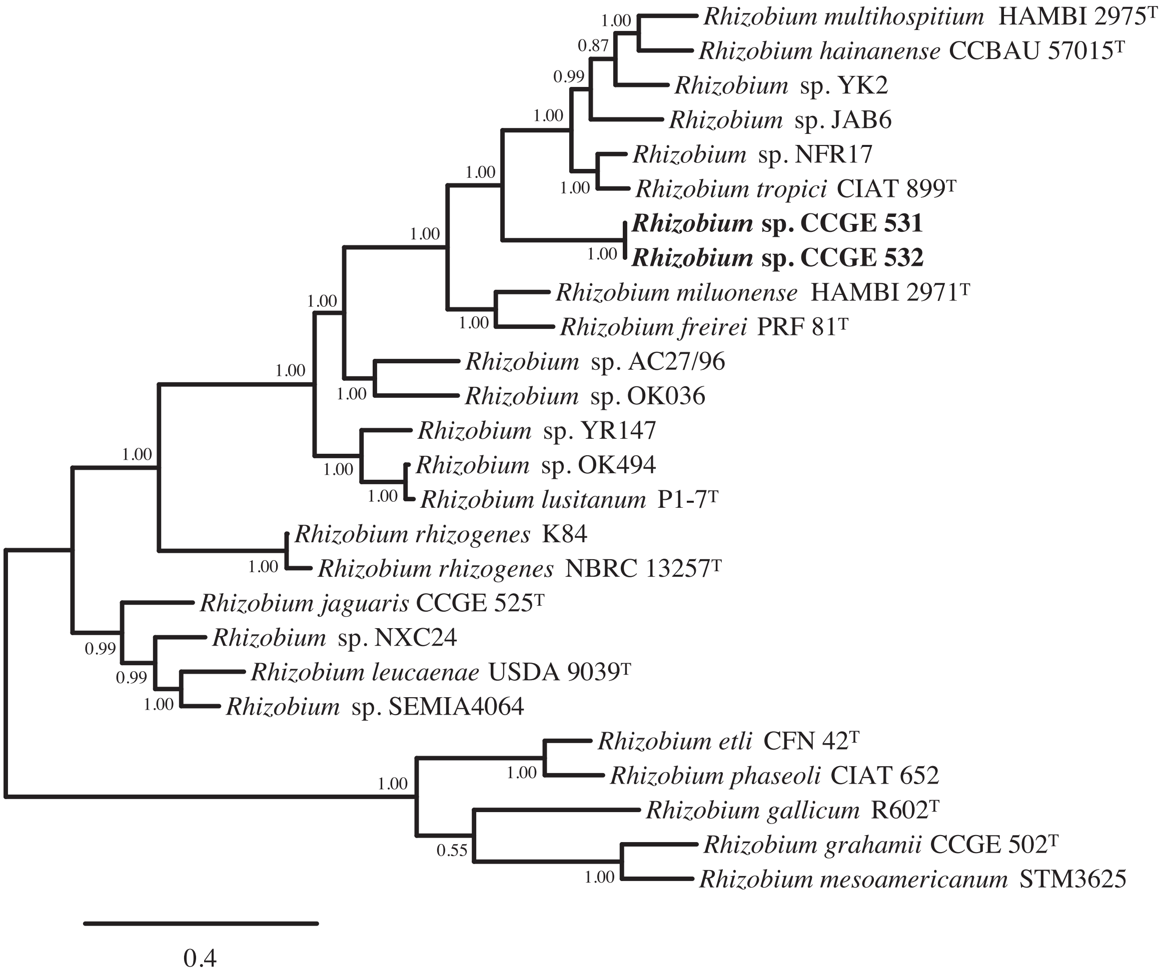


Fig. 4. Phylogenomic analysis from 26 sequenced *Rhizobium* strains using maximum likelihood reconstructed with FastTree 2. Type strains are indicated with a superscript T letter. Isolates obtained from nodules of *P. dumosus* are indicated in bold. Shimodaira–Hasegawa (SH)-like local support values for all nodes are indicated. Scale bar represents the estimated number of amino acid changes per site for a unit of branch length.

Table 3
Rhizobium phenotypic characteristics.

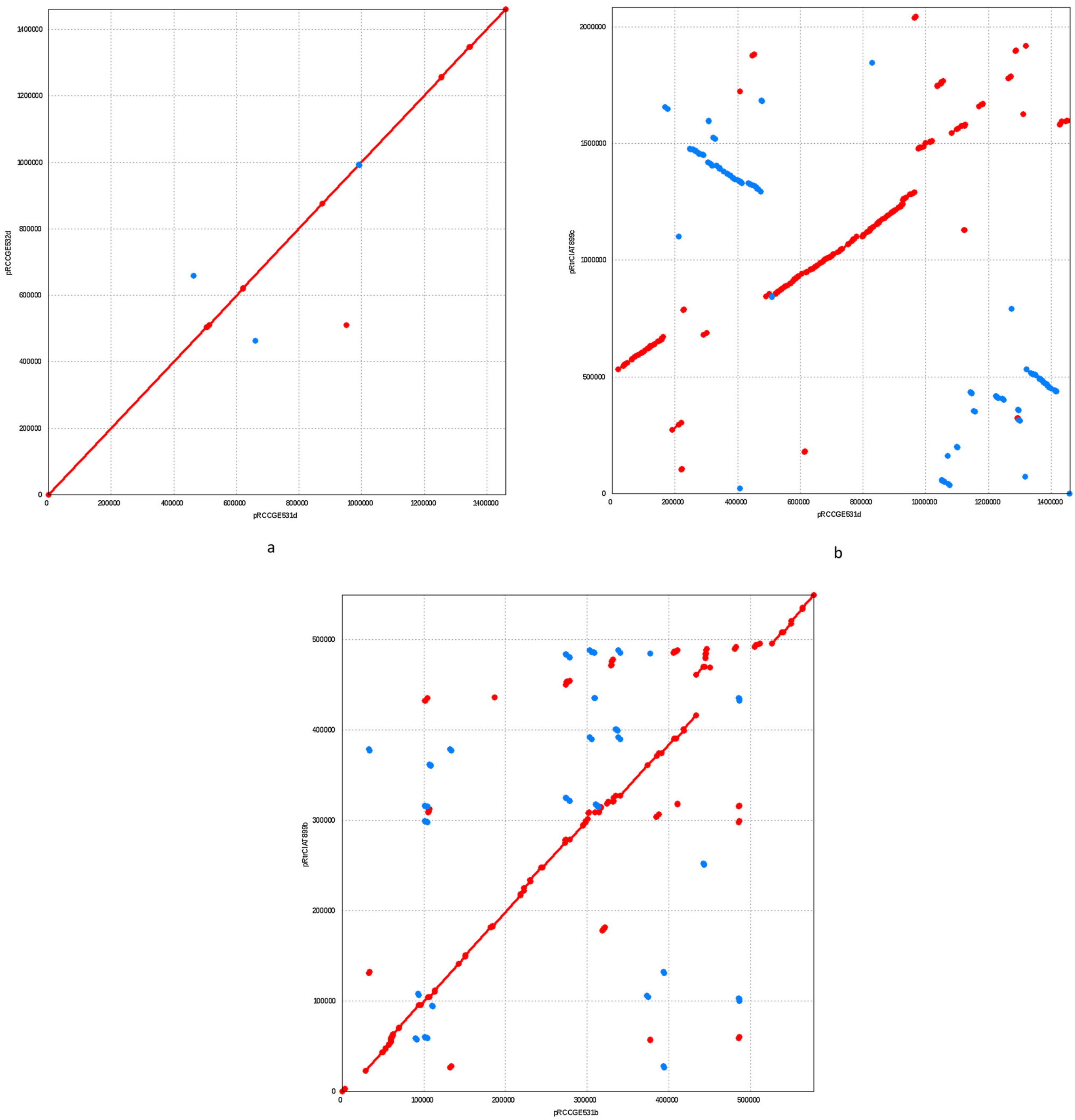
	Rhizobium sp. CCGE531	*R. tropici* CIAT899^{T}	*R. jaguaris* CCGE525^{T}
Acid tolerance (pH 4.5)	+	+	+
Colony morphology in YM medium	Opaque	Opaque	Translucent
Colony morphology in PY medium	White creamy	White creamy	Pearly
Growth in LB	+	+	-
Growth at 42 °C	+	+	-
Tolerance to high salt concentration (see text)	+	+	-
Antibiotic resistance[a]	+	+	-
ARA in *P. vulgaris* nodules[b]	5.5	8.0	ND

ND = not determined. ^{T}:Type strain.

[a] Resistance to carbenicillin and penicillin (30 μg/ml), nalidixic acid (20 μg/ml) and chloramphenicol (10 μg/ml).

[b] Average acetylene reduction activity (ARA) in ethylene μmol per g per h from 3 plants each. Ethylene (in parenthesis) was also quantified from *P. vulgaris* Negro Jamapa nodules with PEL strains CCGE541 (7.1); CCG543 (7.5); CCG545 (14.2); CCG549 (5.8); CCGE551 (6.1); CCGE552 (3.1) and with tropici strains CCGE532 (1.8); CCGE533 (2); CCGE534 (5.3); CCGE556 (4.5).

(Fig. 2). For example, the recently described species, *Rhizobium calliandrae*, *Rhizobium mayense* and *R. jaguaris* genetically related to the tropici A group, which includes *R. leucaenae*, have common tropici type A phenotypic characteristics [65]. Furthermore, the new linage that we describe here from *P. dumosus* nodules has common phenotypic characteristics with the type B group (Table 3) in which it is phylogenetically placed (Fig. 2). With variation in few phenotypic traits, in general type B strains were more resistant in comparison to type A strains. Some of these characteristics could be explained by differences in LPS structure or extrusion pumps [49]. Common fatty acids (with slight differences) found among tropici type B strains may also be related to stress resistance [82]. We have emphatically and critically considered that phenotypes are not useful to describe rhizobial species [48] since carbon and nitrogen use genes may be plasmid borne in rhizobium [6] and thus are disqualified as taxonomy markers. However the acid tolerance phenotype seems to be a general characteristic of the tropici group and in the type B strain CIAT899^{T} it



a

b

c

Fig. 5. Synteny graph comparing (a) CCGE531 and CCGE532 chromids, (b) CCGE531 and *R. tropici* chromids, (c) CCGE531 and CIAT 899^T symbiosis plasmids.

is chromosomally encoded [25,49,54,67]. Several different genes encoding beta lactamase and chloramphenicol acetyltransferase were found in the CCGE531 chromosome or chromid and could account for the antibiotic resistances described (Table 3). The genetic basis and replicon location of other phenotypic characters found in type B strains, such as growth in LB, growth in PY without Ca, growth at high temperatures or with salt are not known. Agrobacteria related to the tropici group had intermediate phenotypes [41,42] and could not be clearly assigned to type A or type B. Those agrobacteria were later reclassified as *R. rhizogenes* [82].

Chromids in rhizobia

Chromids are extracellular replicons that resemble chromosomes in GC content and by carrying essential genes [29]. Megaplasmids in the tropici group fall into the chromid category and CCGE531 and CCGE532 had almost identical chromids (Fig. 5a) with 99% gene content conservation, but 87.2% with the *R. tropici* CIAT 899^T megaplasmid with which there is no overall order conservation (Fig. 5b). Chromids are not conserved neither between CCGE531 and *R. lusitanum* (not shown). In *R. tropici*, *R. freirei* and *R. leucaenae* species specific chromids were identified [23,49], as occurs in other rhizobial groups [2]. Considering all these results, we suggest that chromids have independently evolved in distinct *Rhizobium* species.

Trap plant assay and rhizobial competitiveness for nodule formation

Nodulation capabilities are among the outstanding rhizobium characteristic and competition assays are used to detect subtle nodulation capabilities [79]. Under laboratory conditions at neutral pH *R. tropici* CIAT 889^{T} strain was found to be less competitive for nodule formation in the presence of *R. etli* strains [43]. The trap-plant assay performed here using a mixture of 12 soils from the region rendered mostly tropici group symbionts from *P. dumosus* nodules indicating that these isolates are competitive for nodule formation in acid soils. As mentioned above tropici group strains are acid tolerant [44] and consequently these bacteria would have an advantage under acid conditions.

Symbiovar designation

Symbiovars reflect rhizobial specificity for host plants in relation to symbiosis plasmids [66]. The presence of conserved symbiosis plasmids among distinct species seems to be due to lateral transfer [8,9,40]. Among several tropici group species, a common symbiosis plasmid (around 500 kb) has been found and thus the symbiovar tropici (with a broad host range including *P. vulgaris* beans) has been assigned to them. Notably, genes from this symbiosis plasmid are found to be expressed in free-living conditions [21]. *R. lusitanum nod* and *nif* genes resembled those from *R. tropici* CIAT 899^{T}, thus they were assigned to the symbiovar tropici [80]; *R. lusitanum* nodulation genes seem to have originated from the Americas [81]. Besides forming nitrogen-fixing nodules in *P. vulgaris* (Table 3), CCGE531 and CCGE532 also formed nodules in *L. leucocephala* roots (not shown), as other strains corresponding to symbiovar tropici. CCGE531 and CCGE532 isolates had *nifH* gene sequences identical or almost identical to those from *R. tropici* CIAT 899^{T} and showed three copies of *nodA* like other symbiovar tropici strains. We found *nodPQH* genes in CCGE531 and CCGE532, thus we may infer that they produce sulfated Nod factors as other tropici group strains [35]. ANI for the symbiosis plasmids from CCGE531 was 98% and 97% with the respective plasmids from *R. tropici* CIAT 899^{T} and *R. leucaenae* CFN299^{T}. Overall sequence order conservation was found between the pSym of CCGE531 with that of CIAT 899^{T} in a synteny analysis (Fig. 5c).

Other rhizobia from P. dumosus *nodules*

Besides the tropici group nodule bacteria, we also found rhizobia from *P. dumosus* similar to a group that we previously designated PEL group [62]. These bacteria were recovered as well from *P. vulgaris* (Fig. 1) and were capable of forming-nitrogen fixing nodules in *P. vulgaris* (Table 3). *R. leguminosarum*, *Rhizobium laguerreae*, *Rhizobium fabae*, *Rhizobium pisi* are common symbionts of legumes such as peas and fava beans which are also grown in the agricultural fields in Ocotepec. Co-occurring *R. leguminosarum* sv. phaseoli and *R. leucaenae* strains (from the tropici group) were reported in acid sandy soils in France [3]. Previously in the UK, related rhizobial lineages were found within *R. leguminosarum* that showed limited recombination and diverging symbiosis plasmids, notably there were no clear distinguishing phenotypes among them, thus they could not be defined as distinct species according to classic taxonomy rules. Maybe PEL isolates from Ocotepec, Veracruz (Fig. 1) represent novel rhizobial lineages that should be further analyzed to define their taxonomic status.

A common Phaseolus *cross-inoculation group*

In contrast to other *P. vulgaris* relatives such as *P. coccineus* [76] or *P. albescens* [73] which have different nodulation specificities, *P. dumosus* seem to have conserved symbiotic affinities with *P. vulgaris*. The fact that PEL and tropici group rhizobia were obtained from field nodules both from *P. vulgaris* and *P. dumosus* and form nitrogen-fixing nodules in *P. vulgaris* in nodulation assays (Table 3) support that both *P. vulgaris* and *P. dumosus* belong to the same cross inoculation group.

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