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Genomic Diversity of *Bradyrhizobium* from the Tree Legumes *Inga* and *Lysiloma* (Caesalpinioideae-Mimosoid Clade)

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Abstract: We identified diverse bradyrhizobia having distinct ERIC-PCR genomic fingerprints from native American trees *Inga vera* and *Lysiloma* spp. In addition, two nodule isolates recovered from *Lysiloma divaricatum* seedlings inoculated with soil from the tropical house of a UK botanical garden were also identified as *Bradyrhizobium*. Genomes were obtained (with sizes around 9 Mb each) from nine *Inga* and *Lysiloma* bradyrhizobial isolates; plasmids were detected in two of the *Inga* strains analyzed. Average Nucleotide Identity of whole genomes revealed five novel *Bradyrhizobium* genomospecies from Mexican trees, while the UK isolates were identified as *Bradyrhizobium cajani* and *Bradyrhizobium brasilense. Inga vera* and *Lysiloma* isolates, despite their genetic distances and different hosts, shared a common set of *nod* genes that suggested that *I. vera* and *Lysiloma* bradyrhizobia produce fucosylated, methylated and carbamylated lipochitooligosaccharides. Uptake hydrogenase *hup*, *hyp* and secretion system genes were found in some of the isolates. *Lysiloma* strains were found to be ineffective on *I. vera*. Some of the isolates may be used as plant inoculants.

Keywords: symbiosis; nitrogen fixation; *nod* genes; genomospecies; symbiosis island

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1. Introduction

Legume seeds and pods are protein rich and widely used in animal production and for human nutrition. In addition, legumes, especially trees, render an ecological service by fertilizing soils through their fixation of large amounts of nitrogen [1,2]. Nitrogen fixation is due to symbiotic bacteria in nodules, with different genera of rhizobia from both alpha- and beta- proteobacteria [3,4]. Nitrogen-fixing trees inhabit nitrogen-poor soils, are considered pioneer plants, have access to deep underground water and are tolerant to environmental stress [5] and are valuable resources in the face of climate change. Legume trees are recommended for restoration [6–8], and in arid areas legumes may nurse cacti [9]. Nitrogen fixation enhances plant fitness and extends plant habitat colonization so much that in some cases they have become pests in introduced areas [10–12].

Symbiosis in legume trees is less studied than that in crop legumes. There is the general view that trees are not specific in terms of their preferences for some rhizobial types [13]; however, there are cases of specific symbiotic associations in trees [14,15], in particular that of *Sesbania virgata* and *Azorhizobium doebereinerae* [16]. Host specificity seems to be related to the rhizobial symbiovar and symbiosis islands or plasmids that contain *nod* genes and other symbiosis genes [17,18]; *nod* genes and Nod factors are key to understand legume symbiosis [19–21]. Nod factors induce nodule formation in the absence of bacteria; however,

in a few cases (e.g., some species of the tropical wetland legume *Aeschynomene*), there is nodulation without Nod factors [22]. The LysR transcriptional regulator NodD induces the expression of *nod* genes that encode enzymes that produce Nod factors. Nodulation and legume-rhizobial interactions have been reviewed [20,21,23,24]. In addition, secretion systems have a role in symbiosis specificity [25,26].

Different rhizobial genera are found as symbionts of legume trees, and their presence in nodules might be related to abiotic conditions [27–32]. A global phylogenomic analysis of bradyrhizobia led us to predict that, in theory, there must exist over one thousand bradyrhizobial species [33]. Recently, many new *Bradyrhizobium* species and lineages have been described [34–38] and reviewed in [33]. *Lysiloma* isolates have not been studied, but *Inga* nodule bacteria from Brazil have been reported, revealing diverse symbionts [39], and *Bradyrhizobium ingae* was proposed for *Inga laurina* symbionts [40]. Previously, we studied nodule bacteria from soils with different agricultural use in Los Tuxtlas rain forest in Mexico and found several novel bradyrhizobial lineages, some capable of nodulating a native legume-tree *Inga* sp. in the laboratory [41] and some resembling Central American isolates [42].

Lysiloma and *Inga* belong to the Ingeae tribe from the Caesalpinoideae mimosoid clade. *Lysiloma* trees (2–8 m in height) are classified within eight species and may be found from Arizona and New Mexico to Costa Rica as well as in Florida, the Bahamas and Cuba. *Lysiloma acapulcense* is used as forage for cattle and deer. In Morelos, *Lysiloma* spp. grow in ravines in low-fertility soil; *Lysiloma divaricatum* is native to Mexico [43].

The genus *Inga* comprises around 300 species, which are found exclusively in the neotropics. It has diversified over the last 2–10 million years in three regions: Brazil, Central America and the South of Mexico, and in Western South America [44,45]. Fruits from *Inga* are edible [46], and the plants are used for firewood and animal food. *Inga vera* (and other *Inga* spp.) is used for shade in coffee plantations, especially in the states of Veracruz and Chiapas, Mexico [47]. *Inga* plants are fast growing, improve soil fertility and tolerate acid soils. *Inga* is nodulated by *Bradyrhizobium* [39,40,48,49], especially in its main center of diversification in Brazil. It was the aim of this work to study *Bradyrhizobium* symbionts isolated from *Lysiloma* sp. and from *I. vera* trees native to Mexico. In addition, a comparison to *Lysiloma divaricatum* isolates recovered in the UK was performed.

2. Materials and Methods

2.1. Bacterial Isolation, DNA Extraction, 16S rRNA and ERIC-PCR Analysis

Lysiloma sp. seeds were collected from trees in La Barranca del Tecolote, Cuernavaca, Morelos-Mexico (18°57′18″ N–99°16′36″ W) (Figure 1a). The almost complete chloroplast 16S rRNA gene sequence (1484 bp obtained with PCR using fD1 and rD1 primers [50]) from the *Lysiloma* trees sampled was 98.87% identical to that from *Lysiloma watsonii*.

Seeds were disinfected with ethanol and sodium hypochlorite as explained [51]. They were germinated on agar-water plates, and the seedlings were inoculated in 250 mL flasks with soil from the same location. *L. divaricatum* seeds were obtained from the Millenium Seedbank (Kew, London, UK), and seedlings of *L. divaricatum* were used to "trap" rhizobia from soil in the Royal Botanical Garden of Edinburgh (RBGE), UK, in which several tropical legumes were growing; this resulted in nodules that were red, indicating the presence of leghemoglobin, and hence they were likely to be fixing nitrogen. Other nodules from the same plants were used for microscopic observations, revealing that *Lysiloma* nodules have the anatomy typical of all the mimosoid legumes so far examined [52].

After two months, root nodule surfaces were disinfected as described [53] and crushed in YM media; strains were purified three times and were stored at −70 °C in YM with 30% (vol/vol) glycerol. DNA was extracted from all isolates with the High Pure FF-PET DNA Isolation kit (Roche). Isolates were analyzed by ERIC-PCR [54], and the patterns obtained were observed in an agarose gel. Then, 16S rRNA sequences from the strains that represented different ERIC-PCR patterns were amplified with primers fD1 and

rD1 [50], and they were sequenced at Macrogen, Korea, using universal primers, 518F (5′-CCAGCAGCCGCGGTAATACG-3′) and 800R (5′ TACCAGGGTATCTAATCC-3′).

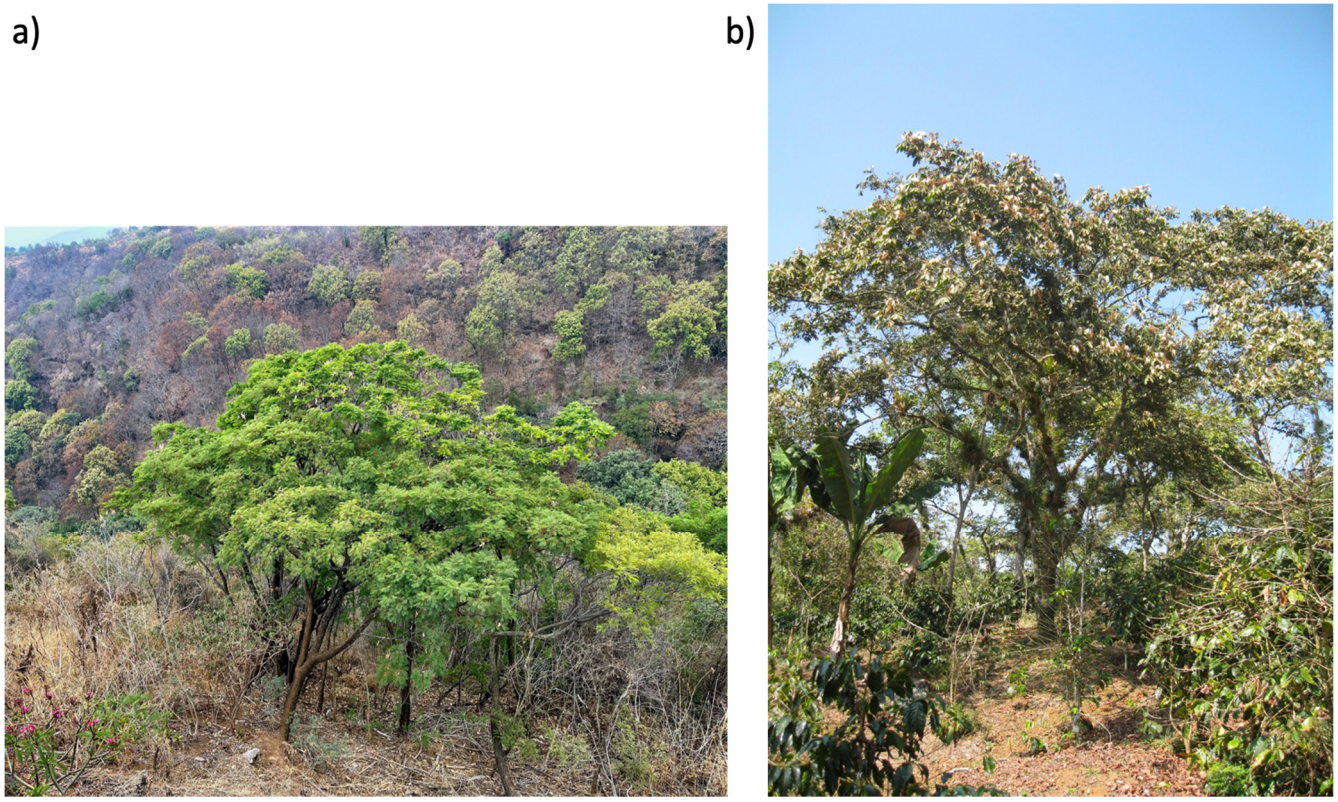

Figure 1. (**a**) *Lysiloma* sp. sampling site at Barranca del Tecolote Cuernavaca, Morelos, Mexico. (**b**) *Inga vera* sampling site in coffee plantations in San Marcos de León, Municipio de Xico, Veracruz, Mexico.

Nodules and soil were sampled from 21 *I. vera* plants that were cultivated as shade in coffee plantations in San Marcos de León, Municipio de Xico, in the center of Veracruz, Mexico (19°25′45″ N 96°58′22″ W) (Figure 1b). *I. vera* seeds are sensitive to desiccation, and their germinability declines within a few weeks after maturation [55]. Thus, we used as a trap plant *Macroptilium atropurpureum* (siratro), which has a broad symbiotic range [56,57]. Siratro seeds were disinfected and germinated in agar-water plates. Twenty siratro seedlings inoculated with *I. vera* nodule extracts and rhizospheric soils were grown in 250 mL agar flasks at 28 °C in the laboratory of Ecología Genómica in the Centro de Ciencias Genómicas, UNAM. As described for *Lysiloma*, after two months, siratro nodules were disinfected, crushed in YM medium, purified three times and stored at −70° C. Strains that were slow growers were further inoculated onto *I. vera* seedlings to confirm nodulation. The isolates were also analyzed by ERIC-PCR, and those with different patterns were used for amplification of their 16S rRNA gene with primers 27f and 1492r [58] and sequenced with the same primers. A partial *glnII* gene was also amplified from these isolates with primers glnII 12F and glnII 689R [59] and sequenced in Macrogen, Korea.

2.2. Genome Sequencing

Bradyrhizobial genomes were sequenced with Illumina NovaSeq 6000 (2 × 150 bp) at Macrogen, Korea, and with ONT MinION at the Mass Sequencing Unit of the Institute of Biotechnology at Universidad Nacional Autónoma de México. Adapter sequences were eliminated using Trim Galore v0.4.4 [60], and a de novo hybrid assembly was generated with the Unicycler pipeline v0.4.8 [61] and with SPAdes v3.13.1 [62]. A search for plasmids was done with RFPlasmid v0.0.18 [63] in all assemblies. Plasmid assembly was performed

with plasmidSPAdes v3.13 [64] for plasmid positive strains and for one plasmid negative strain as a control. Clean reads were mapped against the plasmid assembly using Bowtie2 v2.3.5.1 [65], Minimap2 v2.17 [66], Samtools v1.7 [67] and Bedtools v2.26.0 [68] to separate plasmid from chromosome, and a new assembly with no plasmid-matching reads was generated. Gene prediction and functional annotation were performed using servers RAST v2.0 with RASTtk toolkit [69] EggNogg-Mapper v2.17 [70] and Prokka v1.14.6 [71]. The search for secretion system genes was done with GhostKOALA [72]. To corroborate the presence of all coding genes for the T3SS apparatus, *B. vignae* ORS3257 genes were used as query, and a multiple alignment was done by Clinker [73]. The nine genomes generated in this work were deposited at NCBI under the following BioSample accession numbers: CCGUVB14 (SAMN28667582), CCGUVB1N3 (SAMN28667583), CCGUVB23 (SAMN28667584), CCGUVB4N (SAMN28667585), CCGB12 (SAMN28667586), CCGB20 (SAMN28667587), B51278 (SAMN28667588), B51279 (SAMN28667589) and CCGB01 (SAMN28667590). All raw sequences were deposited under the BioProject PRJNA842432. Genome visualization was done with Proksee of CGView [74].

2.3. Phylogenetic, Phylogenomic and Average Nucleotide Identity (ANI) Analyses

We used a total of 101 type and representative genomes of *Bradyrhizobium* species, some other strains of the superclade I [33], the nine strains analyzed in this study and seven *Nitrobacter* species as the outgroup (Table S1). Protein sequences were obtained with Prokka v1.14.6 [71] and 23,033 orthologous groups were identified by analyzing the proteomes using OrthoFinder v2.5.4 [75]. The phylogenomic species tree was generated using STAG [76] inferred from 1112 orthogroups, with 95% of species having single-copy genes in any orthogroup and the consensus species tree rooted with STRIDE [77]. The STAG support values are the fractions of orthogroup trees supporting each bipartition. The consensus species phylogenomic tree was edited with iTOL v5 [78].

A complementary analysis was done to estimate the Average Nucleotide Identity (ANIm) with the MUMmer algorithm. ANI was calculated using the pyany software [79] with default parameters. For this, we used 56 representative public genomes of the genus (updated April 2022), a representative genome of *Bosea thiooxidans* as an outgroup and the nine genomes generated in this study (Table S2).

The 16S rRNA sequences were edited manually with Bioedit v7.0.5 [80] and aligned with the program Infernal v1.1 [81] through the online platform Ribosomal Database Project v11.5 [82], and they were trimmed manually with MEGA X [83]. The *glnII* sequences and *rpoB, recA, glnII* and *gyrB* concatenated sequences were aligned with the online server RevTrans v2.0 [84]. Nucleotide alignments were carried out with the program MUSCLE v3.8.425 [85]; the alignments were also trimmed with MEGA X, and the Maximum likelihood phylogenies were constructed with IQ-Tree v1.6.11 [86,87] using 1000 ultrafast-bootstrap pseudoreplicates and with the best fit model according to the Akaike information criterion (AIC). For 16S rRNA and *glnII* phylogenies, the model used was GTR+I+G4, with 1196 nt for 16S and 523 for *glnII*. In the concatenated *rpoB*, *recA*, *glnII* and *gyrB* phylogeny, the analysis included 2103 nucleotides and the model used was GTR+F+I+G4 and the ANI values were calculated. Sequences from other *Inga* spp. isolates were also included, as well as *Bosea thiooxidans* or *B. elkanii* as an outgroup. All phylogenetic trees were edited with iTOL v5 [78].

2.4. Nodulation Assays and Nitrogenase Activity Assay

Lysiloma sp. seedlings were inoculated with each strain that was selected as being representative of the different ERIC-PCR profiles, with three replications for each strain in agar flasks with Fahraeus solution [88]. Acetylene reduction activity (ARA) was measured as previously described using a gas chromatograph [53]. Ten *I. vera* isolates, each with a different *glnII* sequence, were used to inoculate *I. vera* seedlings in the Instituto de Biotecnología y Ecología Aplicada (INBIOTECA) of the Universidad Veracruzana, Mexico. Inoculation assays were performed in vermiculite contained in 250 mL bottles watered

with Fahraeus medium, with two plants per bottle and four repetitions. After three months in greenhouse conditions (21.22 ± 5.3 °C and 76.74 ± 14.43% relative humidity), the plants were harvested, fresh and dry weights obtained (plants and nodules) and ARA was measured as described [53]. ANOVA analyses with Tukey tests were performed.

3. Results and Discussion

3.1. Rhizobial Diversity in Lysiloma and I. vera Nodules

3.1.1. Nodule Isolates, Growth and Genomic Fingerprints

Sixty-nine isolates were obtained from nodules of six *Lysiloma* plants. All the isolates were slow-growing rhizobia forming colonies after seven days. All isolates formed nodules on *Lysiloma* in subsequent reinoculation assays. Ten different ERIC-PCR profiles (Figure S1a) were obtained from 25 isolates tested. Three different strains from three different plants representing distinct ERIC patterns were selected for whole genome sequencing; one of these, CCGB01, corresponded to the most abundant pattern of ERIC-PCRs, while CCGB12 and CCGB20 exhibited the highest ARA activities with *Lysiloma* sp. plants.

I. vera bradyrhizobial isolates were obtained with a trap-plant approach, except for isolates CCGUVB23 and CCGUVB40 (that have the same ERIC pattern), which were isolated directly from *I. vera* nodules on two individual trees (6.2 m and 7.0 m in height, respectively, and both with a 31.2 cm trunk diameter) in a Veracruz field with a soil pH of 3.82. Seventeen isolates were obtained from siratro inoculated with nodule extracts or with soil from the rhizosphere of *I. vera* from San Marcos de León, Xico, Veracruz, Mexico. All isolates grew in YM medium and were slow-growing rhizobia forming visible colonies at around 9 to 12 days. All of them were capable of forming nodules on *I. vera* in further inoculation assays with different levels of ARA activity (data not shown). Fingerprints obtained by ERIC-PCR showed ten distinct patterns (Figure S1b). Isolates that performed better when inoculated onto *I. vera* plants (i.e., resulted in significantly higher plant biomass Figure S2), were chosen for genome sequencing.

3.1.2. Phylogenies of Several Nodule Isolates with Selected Genes

Phylogenies were obtained with 16S rRNA (Figure 2a) and *glnII* (Figure 2b) gene sequences, and both reconstructions showed that all isolated strains belonged to the genus *Bradyrhizobium* and allowed a comparison to several reported bradyrhizobial strains with sequences from these gene markers available. In these phylogenies, *I. vera* isolates clustered into three phylogenetic groups; some had gene sequences that resembled those previously reported from Los Tuxtlas soils [41] in South of Veracruz. Some isolates related to CCGUVB1N3 were close to BRUESC1066 from *Inga ingoides* isolated in Brazil [39]. Isolates related to CCGUVB23 had identical *glnII* and 16S rRNA sequences to TUX-10, which was close to BRUESC644 from Brazil. CCGUVB14 and CCGUVB4N were related to isolates from diverse plants as well as some others isolated from diverse *Inga* species, such as TUX-15, BRUESC1084 and BRUESC441 [39]. Isolate CCGB01 was close to isolate CCGB20 and TUX-7 and CCGB12 was closer to TUX-4; TUX strains were isolated from Los Tuxtlas soils in Veracruz [41].

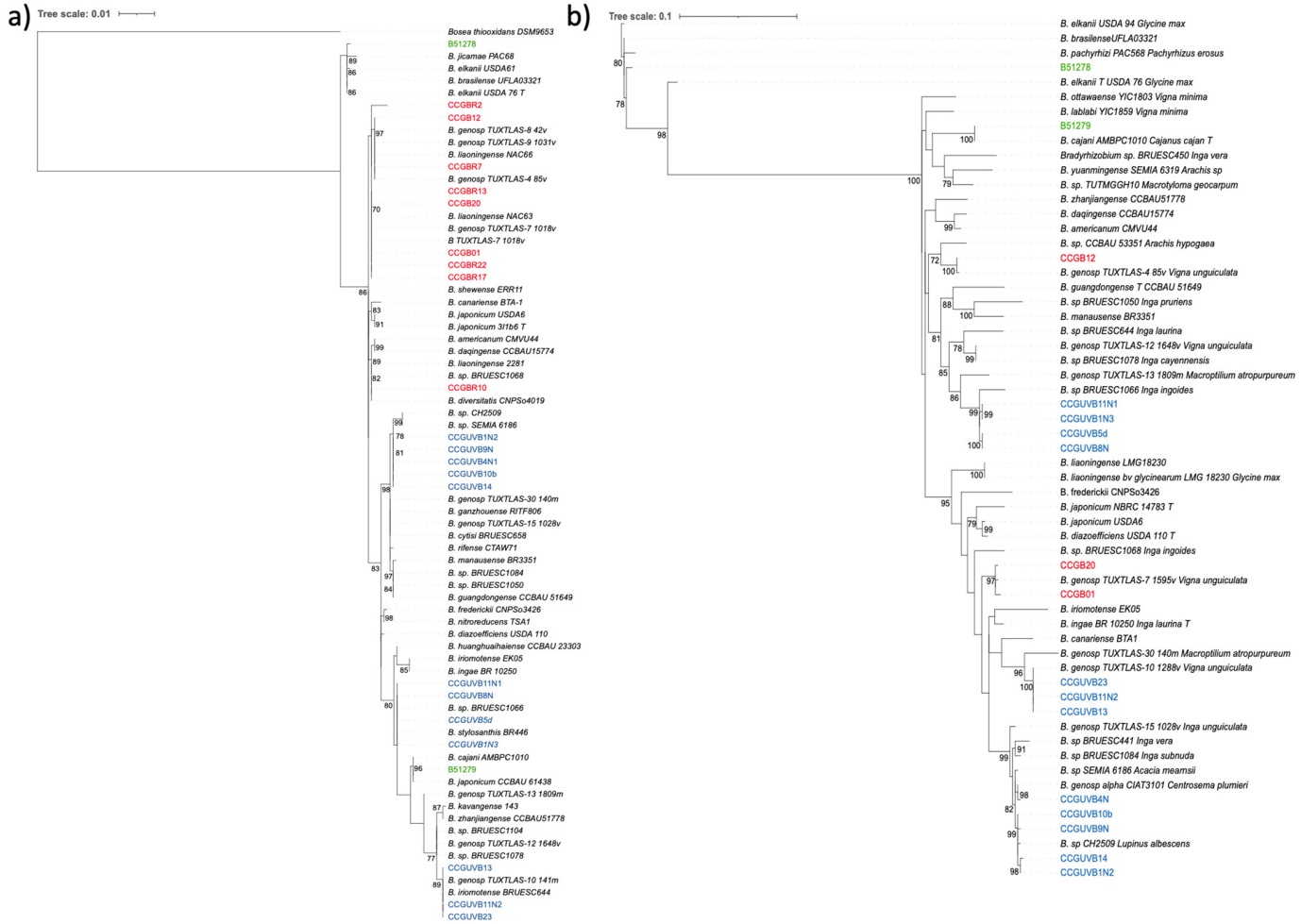

Figure 2. Maximum-likelihood phylogenetic trees based on (**a**) 16S rRNA and (**b**) *glnII* gene sequences showing relationships between *Lysiloma* sp., *L. divaricatum*, *Inga vera* isolates and other strains from genus *Bradyrhizobium*. Alignment lengths: 1196 bp 16S rRNA; 523 bp *glnII*. Substitution model: GTR+I+G for both phylogenies. Bootstrap values lower than 70% are not shown. Blue for *I. vera* isolates, red for *Lysiloma* sp. isolates, green for *L. divaricatum* isolates. *Bosea thiooxidans* DSM9653(T) was used as an external group on 16S rRNA phylogeny. T, type strain.

To compare to *Bradyrhizobium ingae,* for which there is no genome available but only the sequence of marker genes, we performed a phylogenetic tree of *glnII*, *gyrB*, *recA* and *rpoB* concatenated gene sequences (Figure 3). None of the newly found genomospecies corresponded to *B. ingae.* In all trees, the *Lysiloma* strain B51278 clustered within the elkanii superclade, whereas all the other strains clustered within the japonicum superclade [33].

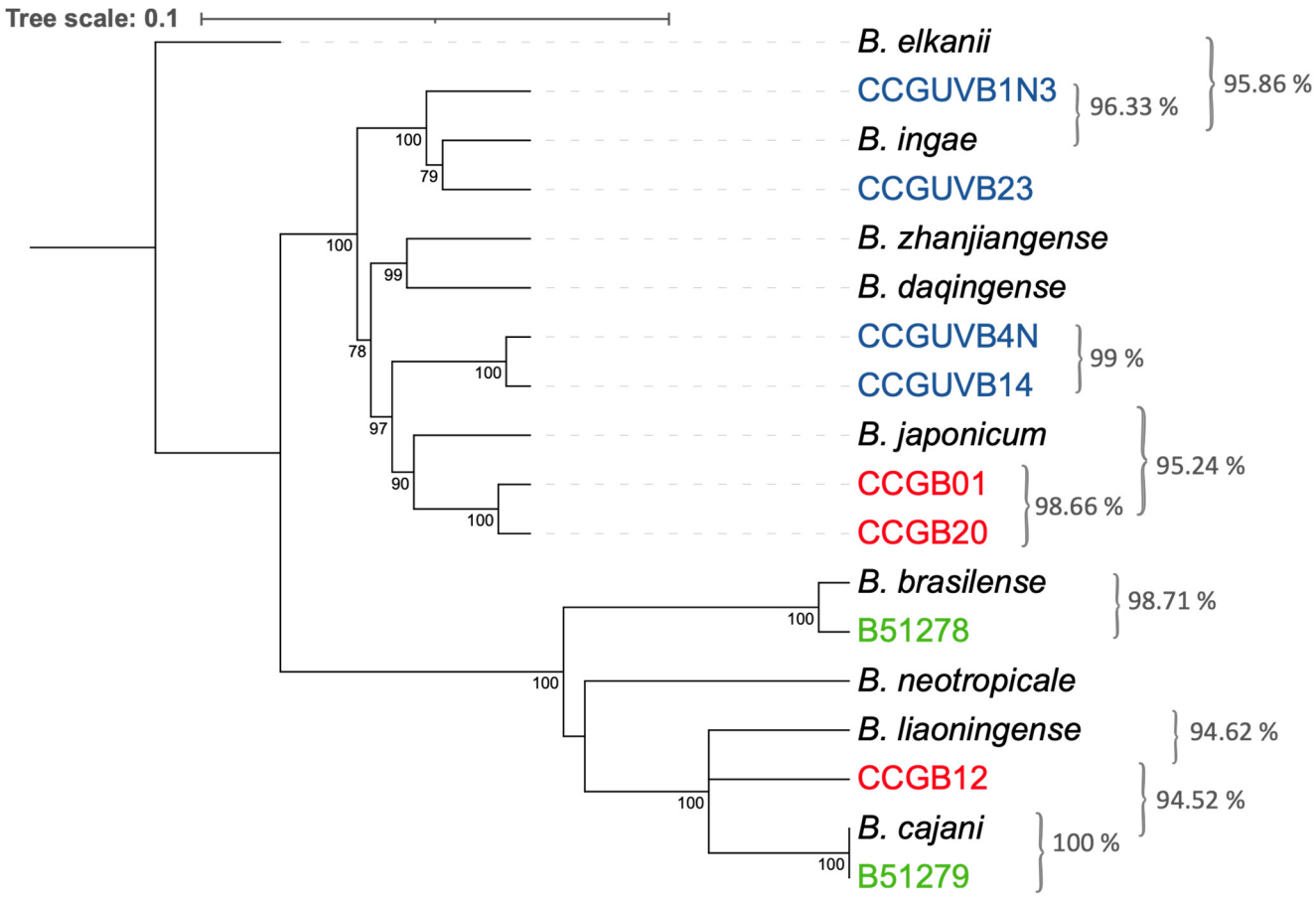

Figure 3. Maximum-likelihood phylogenetic tree based on *glnII*, *rpoB*, *recA* and *gyrB* gene sequences of Mexican *Inga vera* and *Lysiloma* isolates. Concatenate alignment length 2013 bp: *glnII* (537 bp), *gyrB* (621 bp), *recA* (420 bp) and *gyrB* (525 bp). Substitution model was GTR+F+I+G4. Bootstrap values lower than 70% are not shown. *B. elkanii* USDA76(T) was used as an external group. Bar, substitutions per site. Blue for *I. vera* isolates, red for *Lysiloma* sp. isolates, green for *L. divaricatum* isolates. The average nucleotide identity (ANI) of these genes (%), within and between groups is indicated.

The species tree was inferred from 1133 orthogroups, with 95% of species having single-copy genes in any orthogroup, and the consensus tree was created from 21,907 gene trees (Figure 4). The orthologous species consensus tree showed that B51278 clustered with *B. brasilense* and strain B51279 with *B. cajani*; this clustering was also seen in the 16S rRNA and *glnII* phylogenetic trees. *Lysiloma* CCGB01 and CCGB20 strains clustered together, and the *Inga* CCGUVB14 and CCGUVB4N were also grouped.

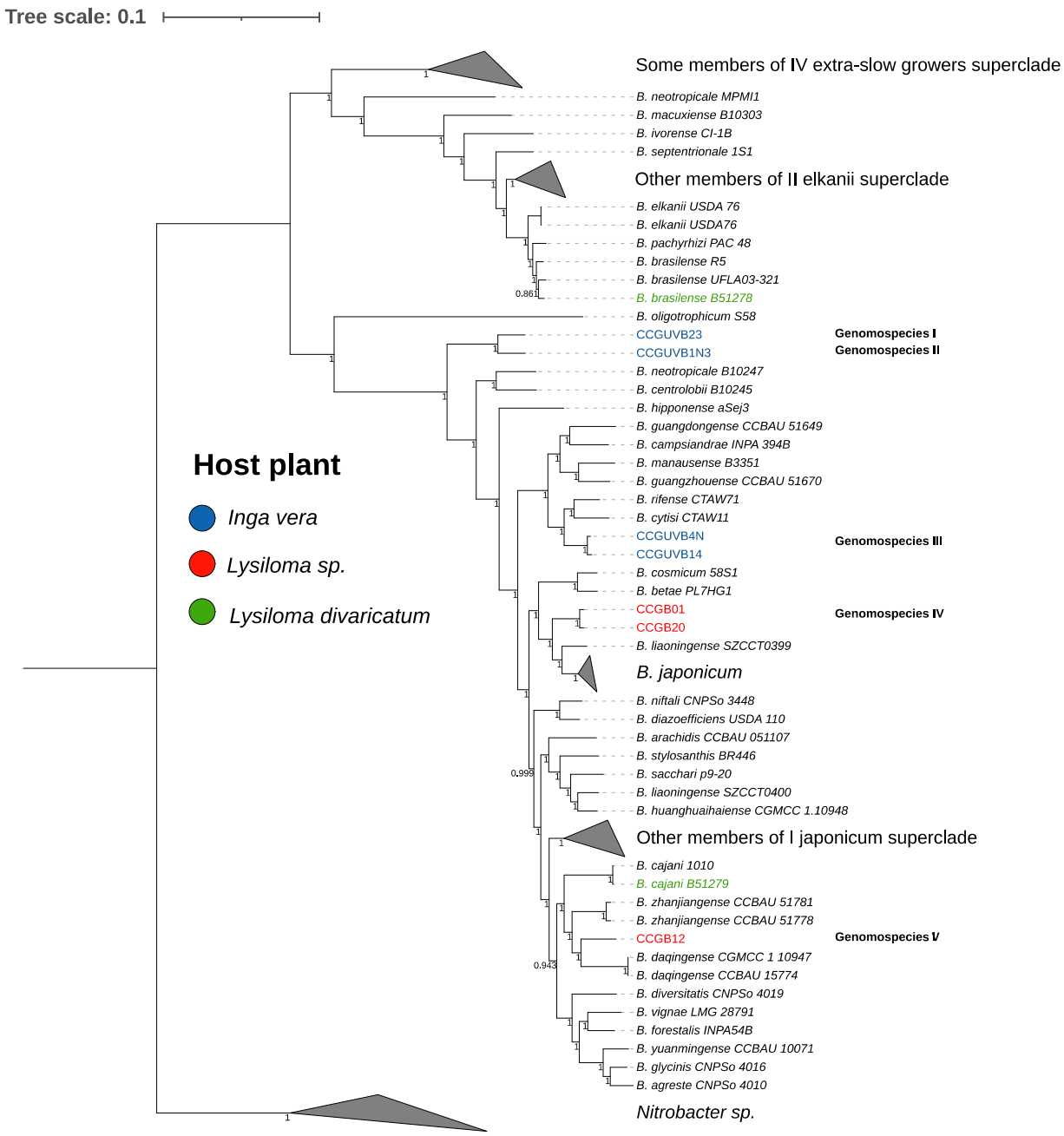

Figure 4. Species consensus phylogenomic tree based on 1,113 orthogroups of 101 genomes (Table S1). Support values correlate to the proportion of times that the bipartition is seen in each of the individual trees used to create the consensus tree. The scale represents substitutions per site. Seven *Nitrobacter* strains were used as an external group. Blue for *Inga vera* isolates, red for *Lysiloma* sp. isolates, green for *L. divaricatum* isolates.

3.1.3. Genome Sizes, Phylogenomics and ANI of Genomes

Genome sequences are very useful to define bacterial diversity, with ANI and *in silico* DDH as parameters for comparison to large numbers of bacteria [89]. To complement the phylogenetic and phylogenomic results, we performed an ANI analysis with representative genomes from *Bradyrhizobium* and the obtained genomes. From ANI and DDH results, *Lysiloma* and *I. vera* isolates were distantly related to other species. According to ANI results, the closest species to CCGB01 and CCGB20 was *B. lupini* (91.05%); to CCGB12, *B. zhanjiangense* (90.62%); to CCGUVB14 and CCGUVB4N, *B. rifense* (91.83%) and to CCGUVB1N3 and CCGUVB23, *B. centrolobii* (87.60%) (Table 1). These values are

below the 95/96% established limit for bacterial species classification; thus, the *Lysiloma* sp. and *I. vera* isolates could represent novel species. ANI results were consistent with the species consensus tree, confirming that B51278 and B51279 isolated from *L. divaricatum* corresponded to *B. brasilense* and *B. cajani*, respectively. In addition, the percentage of ANI among the isolates that showed a close relationship in the phylogenetic analysis was more than 98%, confirming their belonging to the same species (Table S3).

Table 1. Features of *Bradyrhizobium* sequenced genomes.

Bradyrhizobium Strains	Host	Chromosome (pb)	GC (%)	ANI to the Closest Species (%)	Plasmid
B51278	*Lysiloma divaricatum*	8,566,773	63.98	97.14 with *B. brasilense*	no
B51279	*Lysiloma divaricatum*	8,361,602	63.97	99.99 with *B. cajani*	no
CCGB12	*Lysiloma* sp.	9,426,670	63.11	90.61 with *B. zhanjiangense*	no
CCGB01	*Lysiloma* sp.	9,317,328	63.79	91.05 with *B. lupini*	no
CCGB20	*Lysiloma* sp.	9,227,337	63.84	91.07 with *B. lupini*	no
CCGBUVB14	*Inga vera*	9,769,664	63.30	91.80 with *B. rifense*	no
CCGUVB4N	*Inga vera*	9,212,720	63.56	91.83 with *B. rifense*	no
CCGUVB1N3	*Inga vera*	9,882,385	62.98	87.58 with *B. centrolobii*	yes
CCGUVB23	*Inga vera*	10,272,334	62.69	87.51 with *B. centrolobii*	yes

3.2. Genomic Diversity

All the bradyrhizobial isolates from the studied *Inga* and *Lysiloma* species have very large genomes and a similar GC content (Table 1, Figure 5). We found non-symbiotic plasmids in only two strains from *I. vera* and, accordingly, three and two copies of *repB* genes (for plasmid replication) were found in the *I. vera* CCGUVB23 and CCGUVB1N3 genomes, respectively. Plasmids are not frequently found in *Bradyrhizobium* [48]. All the genomes of the isolates showed a putative symbiotic island, with the exception of strain B51278 although it has the nodulation genes that are found in the symbiosis islands in other isolates.

3.3. nod Genes in I. vera and Lysiloma Bradyrhizobia

Irrespective of their host or site of isolation, bradyrhizobia from *Inga vera* and *Lysiloma* spp. share a common core of *nod* genes (Figure 6) that provide hints on the molecular structure of their Nod factors, which may be fucosylated, methylated and carbamylated due to the presence of *nodZ*, *nodS*, *nodU*, *nolO* and *noeI*. Fucosylation of Nod factors seems to be quite common in bradyrhizobia. Besides fucosyation, sulfation of Nod factors may occur in one of the *I. vera* isolates, which could enlarge the host range.

Gene transfer has been reported for *B. japonicum* introduced strains and native *B. elkanii* in soybean in Brazil [90]. The search of *nod* genes in the *Lysiloma* isolates from the UK *B. cajani* isolate B51279 from *L. divaricatum* added evidence for horizontal gene transfer events in *Bradyrhizobium* because the *B. cajani* B51279 isolate from the japonicum group showed *nod*, *nol* and *nifH* genes similar to those from B51278 from the elkanii group.

The *nodH* gene is not commonly found in bradyrhizobia, but it is found in some species of *Rhizobium* and *Sinorhizobium*. In the complete genome of the *I. vera* isolate CCGUVB4N, we found a *nodH* gene in the symbiosis island, suggesting the sulfation of Nod factors besides fucosylation, which could enlarge the host range of the strain. A *nodH* phylogeny showed that it is related to the corresponding gene from *Methylobacterium nodulans*, which produces sulfated Nod factors [91] (data not shown). We also found a *nodX* gene in the symbiosis island in the complete genome of the *I. vera* isolate CCGUVB14 (Figure S5). The *nodX* gene encodes an acetyl transferase that mediates the O-acetylation of the Nod factor at the acetylglucosamine residue in the reducing end [92]. Originally, *nodX* was discovered in *Rhizobium leguminosarum* nodulating Middle East peas and a relic legume *Vavilovia formosa*, and an Oriental origin of this gene was proposed [93]. Most European and American *R. leguminosarum* strains do not contain *nodX*. Phylogenies of the *nodX* gene from the *Inga*

isolate CCGUVB14 showed that it is related to acyltransferases from bradyrhizobia (data not shown).

NolR and SyrM control the level of Nod factor production in a *Sinorhizobium* strain with a broad host range [94,95] and have effects on nodulation specificity in plants [94–96]. SyrM regulates the expression of a large number of genes and has not been reported in *Bradyrhizobium;* however, we detected the *syrM* gene in most of the *Bradyrhizobium* genomes we sequenced (Figure 6), and their phylogenies revealed their relationships to the corresponding genes in several bradyrhizobial strains (not shown).

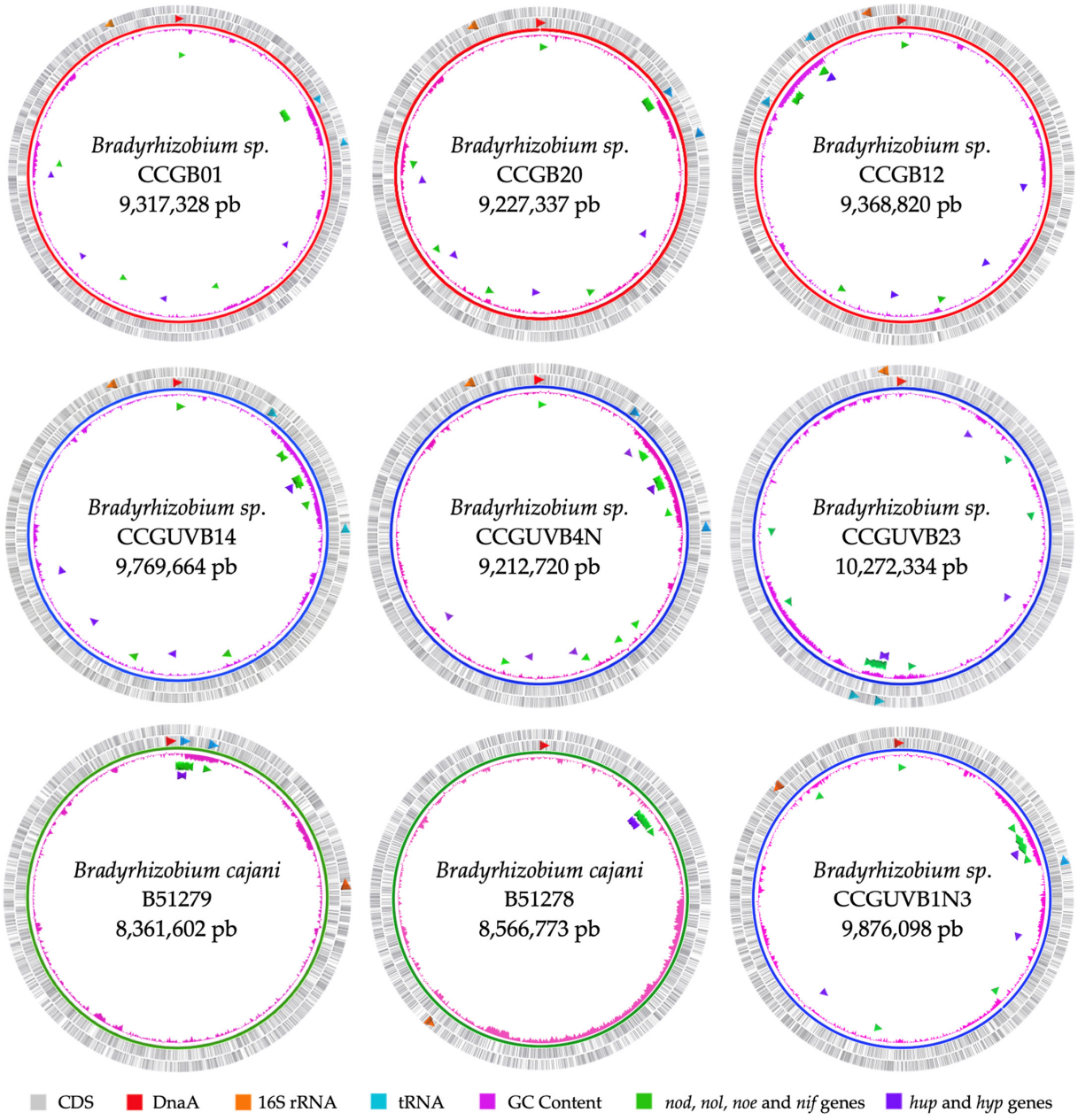

Figure 5. Circular representation of the chromosome of the *Lysiloma* spp. and *Inga vera Bradyrhizobium* isolates. The rings from outside to inside indicate: (1) the chromosome and the coding sequences (CDS); (2) the genome sequence in blue color for *Inga vera* isolates, red for *Lysiloma* sp. isolates, green for *L. divaricatum* isolates; (3) the GC percentage across the chromosome; (4) the *nod, nol, noe* and *nif* genes predicted with EggNogg Mapper; (5) the *hup* and *hyp* genes predicted with EggNogg Mapper. The name of the strain and its genome size are indicated in the center of the circle. The probable symbiotic islands are flanked by a t-RNA, highlighted in the outermost ring.

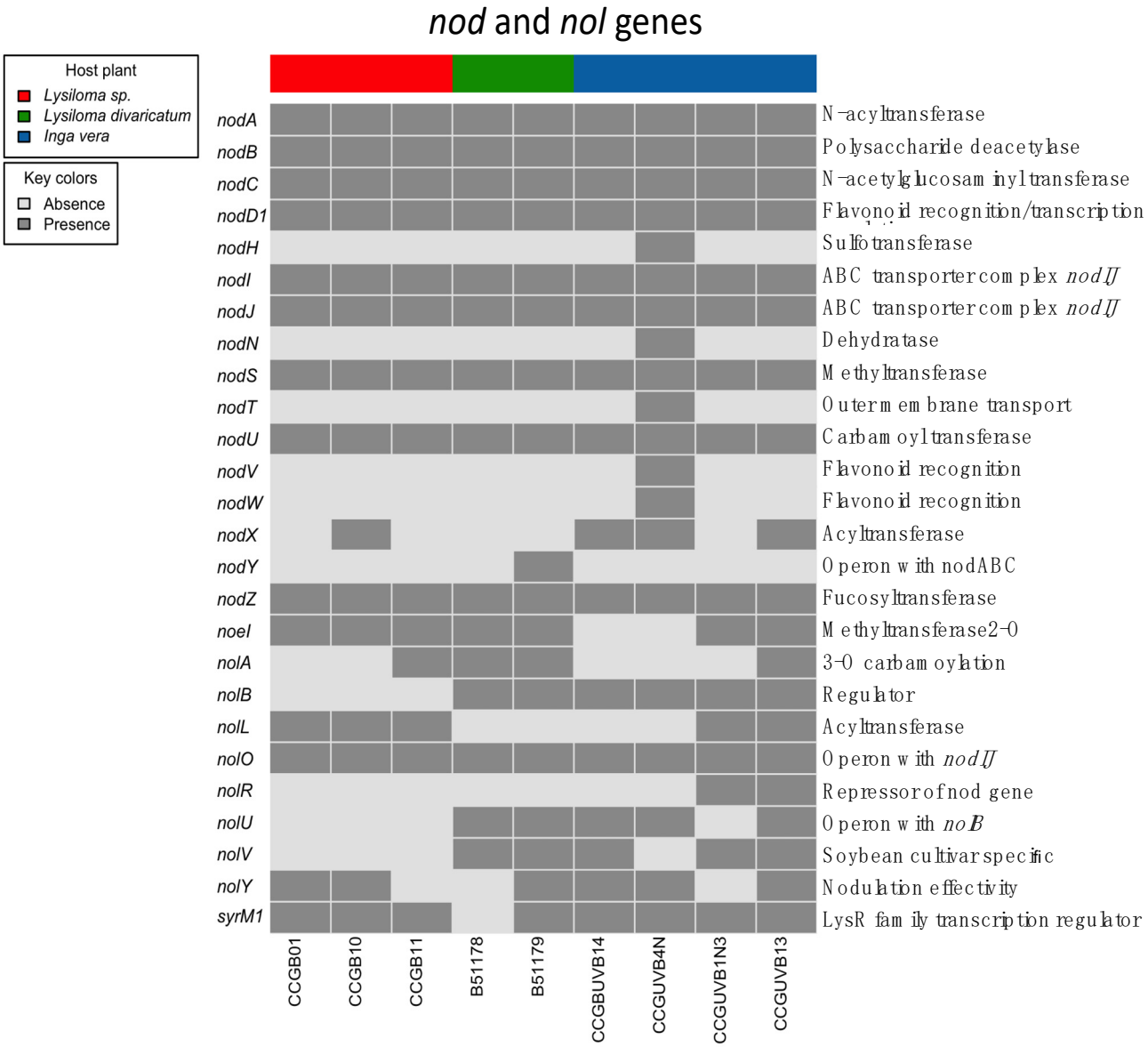

Figure 6. Comparative analysis of nodulation genes in the *Lysiloma* spp. and *Inga vera Bradyrhizobium* isolates. The top sidebar indicates the legume host: red for *Lysiloma* sp., green for *L. divaricatum* and blue for *I. vera*.

3.4. Hydrogenases

Increased plant dry weight, yield and nitrogen fixation have been reported in soybean plants inoculated with bradyrhizobia that harbor uptake hydrogenases (encoded by *hup* and *hyp* genes), which capture the hydrogen produced by nitrogenases [97]. Plants may provide nickel for hydrogenase structure and function [98]. The *I. vera* isolates CCGUVBIN3, CCGUVB14 and CCGUVB23 (with high nitrogen-fixing capability) as well as B55278 and B55279 have the structural hydrogenase *hupSL* genes, which were not found in the *Lysiloma* sp. isolates from Mexico despite having few other *hup* genes (Figure S3). The high nickel concentrations in Los Tuxtlas soils [99] may favor the existence of uptake hydrogenases in native bradyrhizobia, such as those from *I. vera*.

3.5. Secretion Systems

Secretion systems transport molecules from bacterial cells into host cells and may modulate plant defense responses. Distinct secretion systems are known to be in Gram-

negative bacteria and have been extensively studied in pathogens, but they may also play a role in nodulation and host specificity in symbionts [26,100–102]. Genes for different types of secretion systems were found (Figure 7a). The type 3 secretion system (T3SS) has a determinant role in some legume rhizobial symbioses [26,100–104]. The alignment to the *B. vignae* T3SS gene region [105] showed that the isolates of *L. divaricatum* possess all these genes, and the *I. vera* isolates had all the genes but a *y4yS* homolog (Figure 7b). A *y4yS* of unknown function is required for the formation of the *Mesorhizobium loti* T3SS secretin (RhcC2) complex [106]. However, we found the *rhc1* and *rhc2* (secretin, for adhesion) T3SS genes in *I. vera* isolates. T3SS may enlarge the bacterial host range by inhibiting plant innate defense reactions [26,103,104]. T3SS genes were not found in *Lysiloma* sp. isolates. Thus, we may speculate that *Inga vera* isolates have a broader host range than *Lysiloma* isolates and coincidently *nodX* and *nodH* genes, which could contribute to the enlargement of the host range, were also found in *I. vera* isolates. The bradyrhizobial isolates from *Lysiloma* sp., although capable of forming nodules in *I. vera,* did not promote plant growth (Figure S4).

The *nolBTUV* operon was found in *S. fredii* USDA257 to be expressed in the presence of flavonoids. The *nolB* gene constitutes part of the type 3 secretion system, and it is specifically required to form nodules on the *Erythrina costaricensis* tree [107]. The genomes from all our *Inga* and *Lysiloma* isolates studied here had the *nolB* gene (Figure 6), but none had the *ernA* gene that encodes a T3SS effector, which induces nodule formation in the absence of Nod factors [108].

Type 6 secretion systems are found in different bacteria [109], including symbionts. The *vasA* (tssF) gene from the type 6 secretion system (T6SS) was found in the *I. vera* isolates CCGUVB1N3 and CCGUVB23. VasA is a structural component and key for T6SS. In *Rhizobium etli* symbiovar mimosa, T6SS promoted tree nodulation [110].

Lysiloma and *Inga* trees both belong to the Ingeae tribe but are from different geographical regions with different soil conditions and temperatures. A large plant diversity, such as that of *Inga* with 300 species, could promote rhizobial diversity, which is generated by different genetic mechanisms in bacteria [111]. Different tree legumes in a site may converge and maintain a selection of a group of symbionts that are not identical but would be suitable to form nodules in various sympatric legumes. This would have practical advantages in sites where a specific legume density is low, such as in the rain forests in Veracruz. Specificity seems not to be related to bacterial phylogenies and would depend on accessory genes that may be transferred between bacteria [90,112–114].

3.6. Novel Genomospecies

A novel metric to define species arose with genomics, i.e., ANI with a limit of 95/96% between species. Here we discovered upon inspection of ANI results that *Bradyrhizobium liaoningense* [115] and *Bradyrhizobium diversitatis* [116] share a large ANI (98.65%), suggesting that they are synonymous species. The ANI of B51278 was 97% in comparison to *Bradyrhizobium brasilense,* and that of B51279 was 99.99% compared to *B. cajani*; thus, they were assigned to their corresponding species. ANI (98%) showed that CCGUVB4N and CCGUVB14 belong to the same species. They exhibited different ERIC-PCR fingerprints (Figure S1, Table S3), indicating that they are not siblings.

Some of the *Inga* isolates from Mexico resembled *Bradyrhizobium ingae,* which was proposed from *Inga laurina* in Brazil using the phylogenetic analysis *of glnII*, *gyrB*, *recA* and *rpoB* and *dnak*, in addition to phenotypic characteristics [40]. A concatenated five gene phylogeny and the corresponding ANI showed that CCGUVB23 was related to *Bradyrhizobium ingae,* but the ANI value obtained was below the limit to define species (97%), with few concatenated genes. The phylogenetic relationship to this species needs to be further clarified by comparison with the *B. ingae* genome. Finally, we identified five novel genomospecies among the isolates: three for *I. vera* and two for *Lysiloma* sp. *Inga vera* isolate CCGUVB23 and TUX-10 belong to genomospecies I; *Inga vera* isolate CCGUVB1N3 belongs to genomospecies II; *Inga vera* isolates CCGBUVB14 and CCGBUVB4N correspond to genomospecies III; *Lysiloma* sp. isolates CCGB20, CCGB01 and TUX7 belong to genomospecies IV; and

Lysiloma sp. isolate CCGB12 and TUX-4 belong to genomospecies V. CCGB20 and CCGB01 had an ANI of 98.29, showing that they belong to the same species but are not identical, with distinct genome sizes and ERIC-PCR fingerprints.

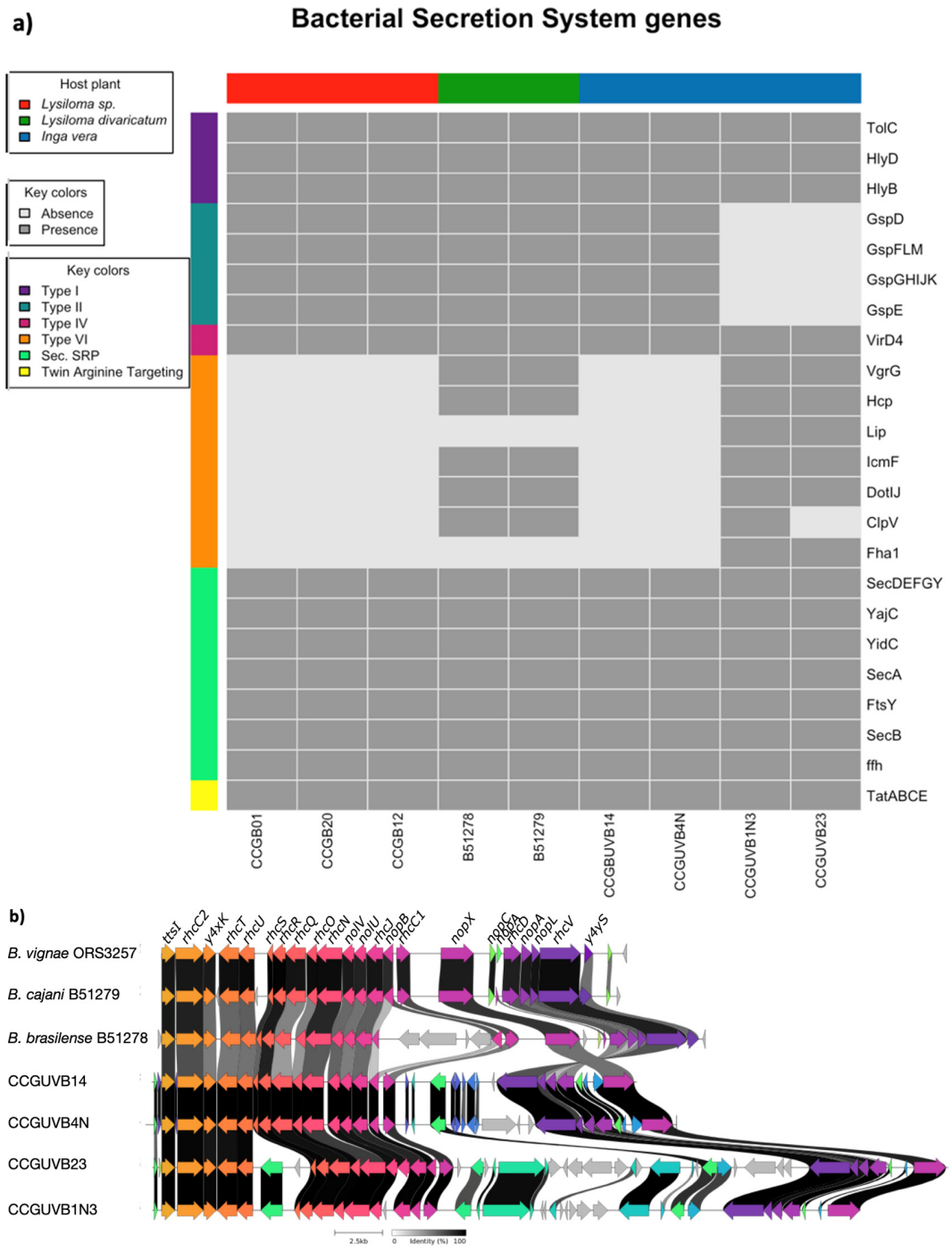

Figure 7. Comparative analysis of Secretion System genes present in *Lysiloma* spp. and *Inga vera Bradyrhizobium* isolates. (**a**) Presence/absence table of coding genes for different types of secretion system, predicted with GhostKOALA. The top sidebar indicates the legume host: red for *Lysiloma* sp., green for *L. divaricatum* and blue for *I. vera*. The left sidebar indicates the Secretion System Type. (**b**) Alignment of the coding region for the T3SS apparatus of *B. vignae* against the isolates of *L. divaricatum* and *I. vera*, made with Clinker. The connecting lines indicate the identity percentage of each gene according to the intensity bar below the figure. Only >35% of identity homologies are displayed.

4. Concluding Remarks

Most reported studies on bradyrhizobial diversity have made use of PCR products (which in many cases are incomplete genes) and subsequent sequencing [41,59,112,117–120]. Here, the bacterial diversity and *nod* gene content of nodule isolates from the legume trees *Lysiloma* spp. and *I. vera* was studied on a genome basis. The ANI values obtained from genome comparisons allowed us to identify five novel genomospecies that could represent new *Bradyrhizobium* species for *Inga* and *Lysiloma*.

L. divaricatum nodule bacteria are not known in its native site, but we suppose they are not the same encountered in the soil from the RBGE. Rhizobia capable of forming nodules in a legume species generally co-exist with the host plant; however, legumes may pick up new symbionts in new environments [121–123].

The search for *nod* genes in *Bradyrhizobium* genomes is particularly valuable because these bacteria are not easily studied by genetic approaches, and the knowledge of the symbiosis genes in bradyrhizobia from native legumes is very poor. Common and host specificity nodulation genes that are responsible for Nod factor synthesis were identified in all isolates, suggesting that *Bradyrhizobium* from *Inga* and *Lysiloma* produce Nod factors with a similar chemical structure; however, a few isolates may produce a wider repertoire of Nod factors. Other symbiosis genes, such as those for uptake hydrogenases and for secretion systems, were differentially distributed among the isolates. The results presented provide the basis to further study tree symbioses and the host selection of the best adapted strains that would lead to rhizobial specificity [124,125].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14070518/s1, Link to the GitHub repository with all bioinformatic analysis. Figure S1: ERIC-PCR profiles; Figure S2: Fresh weight of the above ground portion of the plant of *Inga vera* inoculated with the different *Bradyrhizobium* isolates; Table S1: Accession numbers of representative genomes of *Bradyrhizobium* species used in the phylogenomic analysis; Table S2: Accession numbers of representative genomes of *Bradyrhizobium* species used to perform ANI analysis; Table S3: Average Nucleotide Identity of representative *Bradyrhizobium* species. Figure S3. *Bradyrhizobium hup* and *hyp* genes; Figure S4: Effect of *Bradyrhizobium* on the growth of *Inga vera* plant; Figure S5: Zoom view of the putative symbiotic island of CCGUVB14.

Author Contributions: D.H.-O. was responsible for the assemblies, annotation and analyses of genomes, phylogenomic analysis and analysis of symbiotic genes, and wrote part of the paper. K.L.C.-M. performed the ERIC-PCR and DNA extraction of *Lysiloma* sp. strains, analysis of CCGB01 and its symbiotic island, part of the phylogenomic analysis, and phylogenies. M.A.R. isolated the *Lysiloma* sp. strains and performed ERIC-PCR, acetylene reduction, and extraction of DNA. M.R. isolated the *I. vera* strains and performed nodulation assays, PCRs, and phylogenies. J.A.V.-T. isolated *I. vera* strains, provided the seeds and soil from the *I. vera* fields, and performed nodulation assays of *I. vera*. E.A.-G. and J.A.G.-P. contributed to discussions and the design of nodulation experiments. J.M.-R. was responsible for phylogenetic trees and references. E.K.J. provided the Illumina raw genomes of an isolate of *L. divaricatum* from nodules grown in soil from the RBGE. E.M.-R. performed the writing of the paper and research coordination. E.M.-R., E.K.J., M.R., D.H.-O. and J.M.-R. reviewed and edited the paper. All authors have read and agreed to the published version of the manuscript.

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