Non-nodulating Rhizobium-like ACO-34A fixes nitrogen in pure cultures and has a nif plasmid

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Abstract

Rhizobia fix nitrogen in plant nodules. Notably, *Rhizobium* sp. ACO-34A (which could be reclassified as *Paenirhizobium*), recovered from the rhizosphere of *Agave americana*, is capable of fixing nitrogen in a defined medium in microaerobic conditions and carries *nifHDKENBV* genes in a 213 kb plasmid. ACO-34A failed to induce nodules in several leguminous hosts and does not have *nod* genes. ACO-34A NifH mutant did not fix nitrogen in pure cultures and did not promote stem growth in *Lotus japonicum* plants as the wild strain did. The plasmid harboring the *nif* genes contains *repABC* replication genes, genes for homocitrate synthesis, for toxin-antitoxin production and for plant colonization. Comparative phylogenomic analyses revealed that strain ACO-34A is close to *Ciceribacter sichuanensis* S101, which was isolated from soybean nodules and should be reclassified. According to ANI, AAI and dDDH parameters, ACO-34A may represent a novel species within the Rhizobiacea family.

Introduction

Nitrogen fixation is an ancient and fundamental biochemical process (Pi et al., 2024). This process not only supports primary productivity in natural ecosystems but also underpins biogeochemical nitrogen cycling, particularly in nitrogen-limited soils (Mus et al., 2019; Pi et al., 2024). It may have evolved first in archaea and later transferred to bacteria (the archaea-first hypothesis) (Raymond et al., 2004; Mus et al., 2019; Koirala and Brözel 2021).

Nitrogen gas (N_2) is chemically inert, as the nitrogen are triply bonded to each other (Gastearena et al., 2025). Only the Haber Bosh industrial process and a few bacteria and archaea are capable of breaking the triple bond. However, biological nitrogen fixation is energetically costly in terms of ATPs and the nitrogenase enzyme converts the abundant and inert nitrogen from the atmosphere into ammonia. Nitrogen-fixing bacteria have been well studied because they are used to produce biofertilizers that may substitute for chemical fertilizers, contributing to sustainability in agriculture. Nitrogen-fixing bacteria together with other species could be used to constitute bacterial consortia for the production not only of biofertilizers but also of biopesticides and bio stimulants (Solomon et al., 2023). This could be the basis for an innovative, integral management strategy for plant inoculation.

The identification of diazotrophs has increased markedly, largely driven by the detection of nif genes in bacterial genomes, which has enabled the recognition of previously undescribed organisms with the capacity for nitrogen fixation. Comparative genomics approaches have been particularly useful for identifying conserved core nif operons and, simultaneously, for revealing lineage specific adaptive signatures within the broader nitrogen fixation gene repertoire, including regulatory and accessory components that modulate nif expression and activity beyond the essential structural genes (Tao et al., 2021; Zhang et al., 2024). Likewise, the integration of multiomics frameworks spanning genomics, proteomics, transcriptomics and metabolomics has improved mechanistic resolution of the complex processes that sustain nitrogen fixation and has clarified key layers of its regulation (Shantharam & Mattoo, 1997).

The core set of six nif genes (*nifHDKENB*) is used to identify nitrogen-fixing species (Dos Santos et al., 2012). However, some species with a five-gene set *nifHDKEB* or even a four-gene set *nifHDKB* have been found to be capable of fixing nitrogen (Chen et al., 2021; Ivanovsky et al., 2021). The mere presence of *nif* genes does not guarantee active nitrogen fixation, thus the acetylene reduction assays are needed to confirm nitrogen fixation in bacteria. However, few studies include this analysis. Besides the structural nitrogenase proteins, others are required for an efficient process. These include transporters for sugars or metals, gene regulators, hydrogen uptake dehydrogenases, and homocitrate synthase. For instance, metals that constitute the structural nitrogenase molecule need to be transported inside cells. In rhizobia the transport of sugars from root exudates is induced during colonization within the nodule, and dicarboxylate acid transporters are essential for supplying the energy required for nitrogen fixation in nodules (Yurgel and Kahn, 2004; Lindström & Mousavi, 2019). Similarly, homocitrate, an essential component of the iron-molybdenum cofactor of nitrogenase, is provided by the plant host in symbiotic relationships (Bellés-Sancho et al 2021). In contrast, free-living diazotrophs must synthesize their own homocitrate. Finally, uptake hydrogenases enhance efficiency by recycling the H_2 produced as a byproduct during the catalytic activity of the nitrogenase. For example, *Bradyrhizobium* strains containing uptake hydrogenases are more efficient symbiotic partners (Baginsky et al., 2002) and these enzymes are also found in free-living diazotrophs such as *Azospirillum* and *Azoarcus olearius* (Solaiyappan & Reinhold-Hurek, 2021).

Beijerinck was the first to isolate *Rhizobium* from nodules in 1888 (Fred et al., 1932). Afterwards, the commercialization of biofertilizers began in 1895 with the introduction of 'Nitragin' by Nobbe and Hiltner (Singh et al., 2019). Among nitrogen-fixing bacteria, rhizobia are preferred inoculants, as they are considered safe and have been in use for more than a hundred years (Sessitsch et al., 2002; Bhattacharjee et al., 2012; Glick, 2012). The main characteristic of *Rhizobium* is that the nodulation (*nod*) and nitrogen fixation (*nif*) genes are contained on plasmids that are part of the mobilome and considered accessory genes, as they can be lost without affecting bacterial growth (Wardell et al., 2021). These *nod* genes induce the formation of nodules that contain millions of nitrogen-fixing bacteria.

Typically, *Rhizobium* strains do not fix nitrogen in a free-living state. Instead, this process occurs within nodules, where rhizobia undergo a differentiation process to become bacteroids. Bacteroids are typically larger than their free-living counterparts and are pleomorphic in some cases (Solaiyappan & Reinhold-Hurek, 2021). These differentiated cells have distinct gene expression profiles and upregulate genes for microaerobic life (Uchiumi et al., 2004; Solaiyappan & Reinhold-Hurek, 2021).

Exceptionally, *Azorhizobium caulinodans* (Iki et al., 2007) and a few *Bradyrhizobium* strains can fix nitrogen in culture medium (Wongdee et al., 2018). In *A. caulinodans* two copies of *nifH* genes were found that differed in their promoter regions (Iki et al., 2007). Abundant population of non-nodulating bradyrhizobia exist in soil (VanInsberghe et al., 2015), and some contain *nif* genes that may be functional in free-living bacteria (Wongdee et al., 2018). When in plant nodules, rhizobia fix nitrogen and provide it to the plant as ammonium (Valentine et al., 2017) or alanine in some cases (Schulte et al., 2021). In contrast, free-living nitrogen-fixing bacteria retain the fixed nitrogen, using it for their own bacterial

growth and seemingly do not excrete it to the external media. Nevertheless, mutants and modified bacteria that excrete ammonium have been obtained for different bacterial species such as *Azospirillum, Kosakonia* and other plant-associated bacteria, and because they excrete ammonium, they are better stimulators of plant growth (Jiang et al., 2022).

Previously we reported *Rhizobium* sp. ACO-34A from the rhizosphere of *Agave americana* plants in Chiapas in Mexico and obtained its genome using the Pacific Biosciences (PacBio RSII) single-molecule real-time (SMRT) platform (Ruíz-Valdiviezo et al., 2017). ACO-34A does not produce nodules in several legumes tested such as *Phaseolus vulgaris*, *Leucaena* spp., and no *nod* genes were found. ACO-34A was found to be related to *Rhizobium daejeonense* L61^T (De La Torre-Ruiz et al., 2016). However, rhizobial taxonomy is being continuously modified (He et al., 2024) and some bacteria that were originally classified as *Rhizobium* were assigned to newly created genera, *e,g., Rhizobium daejeonense* recovered from a cyanide treatment bioreactor in Korea, was recently reclassified as *Ciceribacte*r (Rahi et al., 2021). Recent studies using phylogenomics, genome-to-genome distance, and digital DNA-DNA hybridization have proven effective in delineating novel genera within *Rhizobiaceae* (Ma et al., 2023; He et al., 2024). Considering novel parameters to define genera in rhizobia, *R. daejeonense* does not group within *Ciceribacter* and stands as an independent clade that could correspond to a new genus (Kuzmanović et al., 2022). Thus, ACO-34A taxonomic status should be further analyzed.

ACO-34A was recovered from *Agave* roots, similarly *Rhizobium* strains are successful root colonizers (Gutierrez-Zamora & Martínez-Romero, 2001). ACO-34A has a high capability to promote plant growth (De La Torre-Ruiz et al., 2016; García-Pérez et al., 2024) and is being used as a plant inoculant in agricultural fields. In this context, the aim of this work was to evaluate the ability of strain ACO-34A to fix nitrogen in microaerobic cultures, obtain a NifH mutant, establish the bacterium taxonomic position, describe characteristics of the *nif* plasmid and present an updated genome sequence of ACO-34A obtained with the Illumina and Nanopore platforms.

Materials and methods

Bacterial cultures and Acetylene Reduction Assay.

Bacteria were grown in PY medium (Toledo et al., 2003) and isolated pure colonies were obtained on plates in the same medium. For acetylene reduction assay (ARA), cultures were grown for 24 h in nitrogen-free, semisolid MM medium (Fåhraeus, 1957). Acetylene was injected into vials containing the ACO-34A cultures, followed by incubation for 24–48 h at 30 ºC. The conversion of acetylene to ethylene was quantified by gas chromatography following standard procedures. Ethylene and acetylene were measured using a Varian gas chromatograph as described by Martínez et al., (1985).

Genome assembly and ANI, AAI and dDDH Analyses

ACO-34A genome was re-sequenced using short reads (Illumina novas 6000 system) and long reads (Oxford Nanopore Technologies MinION system). Low-quality short-reads (Q < 30) were removed during

trim quality validation using trim-galore v0.6.10 and fastqc v0.12.1 (Krueger, 2015). Hybrid genome assembly was then performed using Unicycler v0.5.1 (Wick et al., 2017), and genome coverage was determined using Bowtie2 v2.5.4 (Langmead & Salzberg). CheckM v1.2.2 was used to verify the quality of the genome assembly (Parks et al., 2015). A circular map of the *nif* plasmid was visualized using the Proksee web server (https://proksee.ca/), and the annotation was performed using prokka v1.14.6 (Seemann, 2014), with default parameters, to identify coding sequences (CDS), tRNA and rRNA genes. Additional CDS prediction and functional annotation were performed with the RAST-tk server (Rapid Annotation Using Subsystem Technology) (Brettin et al., 2015) to ensure comprehensive identification of genetic elements. Circular plasmid map editing was performed with Inkscape software v1.4. The newly recovered sequence of a 213 kb plasmid from *Rhizobium* sp. ACO-34A was compared to the one in Genbank from the national center for biotechnology information (NCBI) database (accession number: CP021375.1).

Phylogenomic and phylogenetic analysis of ACO-34A

Phylogenomic comparisons were performed with genomes deposited in the NCBI-RefSeq database for the genera *Ciceribacter* and *Paenirhizobium*, deposited before December 2025. GET_HOMOLOGUES v14112024 was used to calculate the core genome and obtain shared single-copy genes (Contreras-Moreira & Vinuesa, 2013). Subsequently, GET_PHYLOMARKERS was run with the core-genome matrix to identify high-quality marker genes. The species tree was obtained using ASTRAL-IV, which accounts for genetic tree discordance and estimates branch lengths in substitutions per site, using the IQ-TREE implementation (Vinuesa et al., 2018). The phylogenies were visualized using FigTree v1.4.4. Average Nucleotide Identity (ANI) was calculated based on MUMmer alignments (ANIm) using pyani module v0.2.12 (Pritchard et al., 2016) and Average Amino Acid Identity (AAI) was computed with the CompareM toolkit v0.1.2, which integrates Prodigal v2.6.2 and DIAMOND v0.9.0 (https://github.com/dparks1134/CompareM/). On the other hand, *in silico* DNA–DNA hybridization (dDDH) analyses were performed with the closest strains using the TYGS online server (https://tygs.dsmz.de/).

For the phylogenetic analysis, homologous sequences were retrieved from the NCBI database for each gene family (e.g., nitrogen fixation, replication, transport, secretion systems, and stress response) and multiple sequence alignments were performed with MUSCLE v3.8.31 (Edgar, 2004) with default parameters. Poorly aligned regions were removed manually. Maximum Likelihood phylogenetic trees were constructed with IQ-TREE v2.1.2 using modelfinder for optimal model selection, with 1000 bootstrap replicates.

Construction of NifH mutants

The ACO-34A NifH single recombinant mutants were obtained through single-crossover homologous recombination via vector insertion mutagenesis (VIM) methodology. To achieve this, a ~ 390-bp intragenic region of the *nifH* gene was amplified by PCR using primers Up-nifHSR (5’ TGTGAATTCGGCTGTGCCGGTCGC 3’) and Lw-nifHSR (5’ TTGAATTCGTCGCGCGGCACGAAG 3’). This

PCR product was cloned into the pJET1.2/blunt vector (Thermo Scientific™) and subsequently sequenced. To construct a suicide plasmid for VIM, the *nifH* intragenic region was purified using the EcoRI sites in the primers and ligated to the EcoRI-restricted pK18mob plasmid (Schäfer et al., 1994). *E. coli* DH5α/pK18mob-nifHSR transformants were verified and used as donors in three independent triparental matings with the *Rhizobium sp*. ACO-34A strain, employing the DH5α/*pRK2013* plasmid as a helper. Transconjugants were selected on PY medium containing kanamycin and fosfomycin. To confirm that the expected single-crossover had occurred, PCR amplification of selected Km^R-Fm^R transconjugants was performed using a combination of primers corresponding to the insert and the universal primers M13 forward and reverse. An additional PCR was conducted using the primers nifHAcoFw (5' TTTGAATTCCTCGAACTCGAGGATGTCCTG 3') and nifHAcoRev (5' AGAGAATTC TCCAGCTCTTCCATGGTGAT 3'), whose specific sequence is outside the region utilized in the construction of pKmob-nifHSR; thus, the PCR product is only obtained in the wild-type strain.

Lotus japonicum inoculation assays

Lotus japonicum Gifu seeds were scarified with sandpaper, disinfected in 6% chlorine for 10 minutes, and rinsed thoroughly 3–5 times with distilled water. Seeds were then incubated at 21°C in a growth chamber under a photoperiod of 16:8 for germination (Montiel et al. 2024; García-Soto et al., 2024). Seven-day-old seedlings were transferred to square Petri dishes containing Noble Agar supplemented with 1/4 B&D medium (Broughton and Dilworth 1971) devoid of nitrogen. Seven-day-old seedlings were inoculated with *Rhizobium* sp. ACO34-A or its NifH mutant. For this, each Petri dish was supplemented with 1 mL of an aqueous solution containing the corresponding bacterium strain at OD 0.05, or with 1 mL of distilled water for control plants.

Results

Nitrogen fixation of *Paenirhizobium* sp. ACO-34A

Nitrogen fixation assays using multiple colonies in nitrogen-free medium consistently indicated acetylene reduction, providing solid evidence of nitrogenase activity in the clonal strains derived from ACO-34A. The strains recovered from the pellicles formed beneath the surface of the nitrogen-free medium exhibited a phenotypic profile identical to the original clone. These strains were subcultured in PY medium again and re-cultured in nitrogen-free medium to confirm the positive ARA assay. Additionally, the genotypic and antibiotic resistance profiles were evaluated, showing consistency with the original strain.

This indicates that the ACO-34A strain has the capacity to carry out biological nitrogen fixation in vitro. To substantiate a determinant relationship between *nif* gene function and acetylene reduction, independent NifH mutants were generated and functionally characterized. All NifH mutants did not produce ethylene in the acetylene reduction assay under the experimental conditions evaluated, whereas

the wild-type strain did. Genetic and molecular fingerprinting controls, including ERIC-PCR profiles, corroborated that the mutants were derived from ACO-34A (Supplementary Fig. 2).

Lotus japonicus is a model plant to study microbial symbiosis with the advantage of being small and fast-growing. In *Lotus*, ACO34-A promoted stem growth. Significant differences in stem height were observed in plants inoculated with the NifH mutant (Fig. 1). Plants inoculated with the NifH mutant allowed to determine the promoting effects due to nitrogen fixation and plants with the NifH mutants had slighty longer stems than control non-inoculated plants. This suggests that nitrogen fixation explains in part the growth promotion of ACO34-A.

Genome of *Paenirhizobium* sp. ACO-34A

ACO34-A deposited genome (GCF_002600635.1) was recently removed from the NCBI RefSeq database because it was argued that there was a potential contamination with *Ciceribacter* sequences. To address this issue about contamination, we re-sequenced its genome from a pure culture derived from an isolated colony after five subcultures, using the Illumina and Nanopore sequencing platforms. The comparison of the newly obtained genome assembly to the previously reported genome showed that they were identical in sequence and almost identical in replicon size (Table 1).

The data obtained for the reported replicons assembled with PacBio (Ruíz-Valdiviezo et al., 2017) were compared with the hybrid genome assembly using Illumina-Nanopore reads. As shown in Table 1, genomic analyses showed agreement between the two sequencing results. The lengths of the chromosomes (4.75 Mb) and plasmid replicons (pRACO-34Aa-d) showed insignificant differences (variation < 100 bp). From the genome analysis we found that *Rhizobium* ACO-34A has two plasmids and two chromids (one containing a copy of ribosomal genes). This was corroborated using the Eckhardt technique (Supplementary Fig. 1). The *nif* genes were found on the 213 kb plasmid that we define here as the *nif* plasmid. Like other rhizobia, ACO-34A possesses the genes that encode the dicarboxylate transporters. Notably, these are located in the ACO-34A chromosome. These observations confirm the structural integrity of the *Paenirhizobium* sp. ACO-34A genome. In addition, a completeness of 99.01% was estimated and it could be observed a contamination of 1.18%. These results, complemented by high sequencing depth (> 332X), support the quality of the assembly for further phylogenomic and taxonomic analyses.

Table 1
Comparative genome assembly metrics of strain ACO-34A derived from PacBio, and Illumina-Nanopore sequencing platforms.

	PacBio (Ruíz-Valdiviezo et al., 2017)	Illumina–Nanopore
Chromosome	**4,754,916**	**4,754,988**
pRACO34a	**213,273**	**213,280**
pRACO34b	**305,590**	**305,619**
pRACO34c	**494,144**	**494,163**
pRACO34d	**516,813**	**516,834**
Coverage	**347 X**	**332 X**

Phylogenomic and genomic relatedness indices

ACO-34A may be assigned to *Paenirhizobium* (that means almost *Rhizobium*, Ma et al., 2023) not yet officially accepted. *Rhizobium daejeonense* (Ma et al., 2023) is closely related to ACO-34A (Fig. 2). *R. daejeonense* was previously reclassified as *Ciceribacter* (Rahi et al., 2021) and now it is recognized as a distinct genus, *Paenirhizobium*. *Ciceribacter sichuanensis*, isolated from soybean nodules in China (Zhang et al., 2024), which was found to be close to ACO-34A likely also belongs to *Paenirhizobium*. We did not find *nod* genes in *Ciceribacter sichuanensis*, which is also the case for *Ciceribacter daejeonense*. All of these related strains with ACO-34A would appear to belong to a non-nodulating rhizobial genus.

The ANI, AAI, and dDDH values calculated from ACO34-A genome in comparison with the closest reference genomes were consistently below the accepted species-level thresholds. In Fig. 2A, ACO-34A forms a well-supported lineage, clearly separated from the representative strains of *Ciceribacter sichuanensis* S101. On the other hand, the topology of the species tree with respect to *Paenirhizobium* shows that the representative strain *Paenirhizobium daejeonense* KACC 13094 is the closest clade (Fig. 2B). According to this topology, ACO-34A occupies a single branch within this genus.

The representative strain closest to ACO-34A based on ANI was *Ciceribacter sichuanensis* S101 (92.17%), whereas *Paenirhizobium daejeonense* KACC_13094 showed an ANI of 92.06%. Similarly, the average AAI between ACO-34A and *C. sichuanensis* was 94.92%, and 94.58% with *P. daejeonense* KACC 13094. In addition to ANI analyses, we performed cAAI, which is now considered to be useful for delineating genera in rhizobia (Li et al., 2024; Fig. 2C). dDDH values against these strains were in both cases < 46%.

Key functional traits encoded in the *nif* plasmid

The complete set of nitrogen fixation genes in ACO-34A is located on a 213 kb plasmid (Fig. 3) in the newly sequenced genomes that we report here and in the previously reported genome (Ruíz-Valdiviezo et al., 2017). As evidenced by genome assembly analyses, this feature is consistently detected in both the

newly generated hybrid assembly and the previously reported PacBio-based genome (Fig. 3). In ACO-34A the *nif* plasmid contains all genes necessary for nitrogen fixation, namely *nifHDKENB* and in addition, it also has *nifA*, *nifB* and a *nifV* gene encoding homocitrate synthase that produces homocitrate (Fig. 3). This *nif* cluster is located in a plasmid region with high homology to the *nif* cluster annotated in the *C. sichuanensis* S101^T genome and is flanked by several transposase genes.

This plasmid harbors the structural and accessory *nif* genes required for nitrogenase activity, including *nifHDKENB*, and additionally contains key regulatory regions such as *nifA* and *nifB*.

Phylogenetic estimation of the *nifH* gene, together with additional genes encoded on the *nif* plasmid, revealed that the *nifH* sequence of strain ACO-34A clusters closely with that of *Ciceribacter sichuanensis* (Fig. 4). Likewise, it is positioned within a broader phylogeny-based taxonomic assemblage dominated by free-living, non-nodulating nitrogen fixers, including *Rhizobium cremeum* (Yang et al., 2022) as well as species of the genus *Martelella* (Li et al., 2021) and *Hartmannibacter diazotrophicus* (Suarez et al., 2015). The strong nodal support suggests that the phylogenetic affinity of ACO-34A *nifH* is robust to resampling and reflects a stable signal in the alignment. Notably, nodulation genes were not detected in the closest relatives examined; supporting the inference that ACO-34A belongs to a non-nodulating rhizobial lineage.

The *nif* cluster is embedded within a broader mobile genomic context (Fig. 5). In close proximity to the *nif* genes, the *hesB* gene was identified in the immediate vicinity of the nitrogen fixation locus (Fig. 3). This gene has been linked in multiple systems to nitrogen-related physiology (Böhme 1998) and is annotated as an iron-binding protein, which is biologically plausible given the high metalloprotein requirements during nitrogenase maturation.

The plasmid also encodes a *repABC* replication and maintenance system typical of large rhizobial replicons. (Cevallos et al., 2008; Wetzel et al., 2025). Phylogenetic patterns of *repABC* suggest affinity with *Agrobacterium* and *Ensifer* lineages, consistent with a rhizobial origin. In addition, conjugal transfer modules, including *tra* and *trb* genes and a type IV secretion related apparatus, indicate transfer competence and provide a mechanistic basis for dispersal among rhizosphere bacteria.

Most plasmids in *Rhizobium* have the *repABC* operon for replication (Mazur et al., 2011). A plasmid replication/maintenance system (the *repABC* operon) was identified and the analysis of the ACO-34A *repABC* gene sequences from the 213 kb plasmid showed that they resemble those from *Agrobacterium* and *Ensifer* (not shown) indicating a rhizobial origin of the replicon.

Multiple transport systems were also encoded in the plasmid, including glutathione transporters (Wang et al., 2024), bicarbonate and riboflavin transport systems. The dipeptide permease *DppA*, and the membrane trafficking protein EntH were encoded in the plasmid sequence. Dicarboxylate transporters, which are essential for nitrogen fixation in nodules, and sugar transporters involved in the utilization of root exudates were also found.

Genes conferring stress tolerance were also identified including those that encode for osmoregulatory proteins (OmpR, Rodriguez et al., 2020) and glycogen metabolism genes (*glgA* and *glgB*). Multiple proteins involved in the conjugal transfer (TraA, TraB and TraC) were also identified, Furthermore, these genes show high similarity to those identified in the plasmid pAt1D132b of *Agrobacterium fabrum* 1D132 (Fig. 5).

The *nif* plasmid contains many transposases, hypothetical genes as well as a secretion system corresponding to the Type I (Fig. 6A-b) transporters and Type IV system with genes similar to *virB* that does not match with the corresponding genes from rhizobia (Fig. 7). On the one hand, the annotation of multiple transposases (IS3, IS6 and IS481 families of transposases) that delimit regions with genes showing high percentages of similarity with different strains of the Rhizobiaceae family (depending on the region to be considered (Fig. 5) indicates a chimeric origin of the plasmid.

On the *nif* plasmid there were genes that resemble *emrA* and *emrB* which encode efflux pumps that confer resistance to antibiotics (Dalbanjan et al., 2024; Zack et al., 2024). The *emrA* and *emrB* genes found in ACO-34A genome were not found in the related *C. sichuanensis* or in *Rhizobium cremeum* genomes.

A gene encoding *BvgA*, which in rhizobia is part of a toxin-antitoxin system that could prevent plasmid loss, was found on the *nif* plasmid. In five serial subcultures of ACO-34A, the kanamycin resistance (located in the *nifH* gene on the *nif* plasmid) was conserved in bacteria (not shown) suggesting that curing the *nif* plasmid would be difficult with a toxin-antitoxin system present on the plasmid.

The *ompR* gene from ACO-34A *nif* plasmid is phylogenetically related to *Agrobacterium ompR* gene (Fig. 8). A hypothetical gene from the ACO-34A *nif* plasmid resembles a gene encoding a protein with an Ig domain (Chaterjee) from *Paraburkhoderia fungorum*. For biofilm formation, *glgA* and *glgB* gene products were identified. Notably, the *nif* plasmid contains several genes of unknown function or encoding hypothetical proteins. It is worth mentioning that no uptake-hydrogenase *(hup*) genes (Sotelo et al., 2021) were detected in the ACO34-A genome.

Discussion

In *Rhizobium* nitrogen fixation normally occurs in nodules, so ACO34-A is outstanding for being capable of fixing nitrogen in free living conditions and this characteristic is certainly encoded in *nif* genes as NifH mutants were uncapable of fixing nitrogen. Notably the NifH mutant did not promote stem growth in *Lotus japonicum* plants as the wild type did.

Rhizobium sp. ACO-34A isolated from the roots of *Agave americana* plants in Chiapas, Mexico showed plant growth promotion capabilities (De La Torre-Ruiz et al., 2016). Agave plants may select and promote growth of free-living diazotrophs due to its high photosynthetic capacity, which provides an abundant carbon supply to sustain bacterial populations, together with reduced nitrogen availability, thereby avoiding the inhibition of nitrogen fixation (Beltran-García et al., 2014). Similar processes may occur in

other cacti or in C4 plants such as maize (Roesch et al., 2008; Montanez et al., 2009; Van Deynze et al., 2018). Consequently, agaves may represent a potential niche for the isolation of nitrogen fixing bacteria, such as ACO-34A, which is currently being used successfully in agricultural fields (Manzano-Gómez et al., 2025). Furthermore, ACO-34A may be applied in mixed inoculants with the aim of establishing an integrated management strategy for plant biofertilizers.

ACO-34A consistently clustered with strains within the not officially recognized genus *Paenirhizobium*, including the lineage represented by *Paenirhizobium daejeonense.* Closely related genomes historically placed in *Ciceribacter* have been reclassified as new taxa, consistent with ongoing taxonomic revisions in this clade.

Taken together our results support that ACO-34A represents a previously undescribed species within *Paenirhizobium*. All genomic relatedness indices were significantly below the accepted species boundaries (95–96% for ANI-AAI and 70% for dDDH). In regard to the genome, clearly there was no contaminating DNA it the deposited genome of ACO34-A and the resemblance to *Ciceribacter* reflects the genetic similarity of *Ciceribacter* and *Paenirhizobium* and is therefore not actual evidence for contamination.

Nitrogen fixation in ACO-34A and perhaps in *C. sichuanensis* could be due to the origin of their *nif* genes, apparently from free-living nitrogen-fixing bacteria. The putatively transferred *nif* genes could have also carried as well the regulatory regions and mechanisms to be expressed under free-living conditions and not confined to nodules. Recent comparative analyses have shown that *nifH* phylogenies often reflect ecological rather than strictly taxonomic relationships, particularly in free-living diazotrophs inhabiting root-associated environments (Tao et al., 2021). In bradyrhizobia, few *nif* genes were found alone in small genomic islands in the chromosome, and they were not phylogenetically related to the *nif* genes linked to the *nod* genes from plant nodulating symbionts. Thus, they likely have an evolutionary origin different from the *nif* genes linked to *nod* genes (Tao et al., 2021). This is similar to what we discovered for the independent *Paenirhizobium nif* genes in absence of *nod* genes.

It has been reported that the modular architecture of *nif*-containing islands is often associated with the presence of transferable integrative elements and transcriptional promoters (Tao et al., 2021; Ma et al., 2023).

Homocitrate is a component of the nitrogenase FeMo-cofactor and is considered to be required for free-living nitrogen fixation (Nouwen et al., 2017; Bellés-Sancho et al., 2021; Warmack et al., 2023). Plants provide homocitrate but free-living bacteria must synthesize it to fix nitrogen. *nifV* is absent from a wide range of rhizobial genomes that fix nitrogen exclusively within nodules (Hakoyama et al., 2009). The presence of *nifV* gene in ACO34-A is congruent with its capacity to fix nitrogen under free-living conditions.

Conclusion

Overall, our results support the conclusion that ACO-34A is a free-living diazotroph.Nevertheless, additional experimental assays are required to determine whether, during nitrogen fixation, strain ACO-34A excretes ammonium to the plant.

This work confirms the ACO34-A genome sequence using Illumina and Nanopore sequencing. The hybrid assembly, generated from a meticulously purified lineage was identical to the previously obtained PacBio genome (Ruíz-Valdiviezo et al., 2017).

We surmise that ACO-34A represents a novel species within *Paenirhizobium*. In general terms, strain ACO-34A can be placed within a cluster dominated by non-nodulating diazotrophs. From an evolutionary perspective, strain ACO-34A shows a very close relationship with *C. sichuanensis* which should be reclassified as *Paenirhizobium sichuanensis*. Both taxa share the presence of *nif* genes which may be ancestral in this genus besides the supposition of their original acquisition by lateral transfer. Notably, *C. sichuanensis* was recovered from soybean yet lacks *nod* genes. In the case of ACO-34A, diazotrophic activity was confirmed using the acetylene reduction assay (ARA). In contrast, for *C. sichuanensis*, data on acetylene reduction have not yet been reported, nor has the replicon-level location of the *nif* gene cluster.

Agave plants may select for associated nitrogen-fixing bacteria because they have a large photosynthetic capability with abundant carbon supplies to feed bacteria, and limited nitrogen so as not to inhibit nitrogen fixation (Beltran-García et al., 2014). This may also occur in other cacti or in C4 plants as maize (Roesch et al., 2008; Montanez et al., 2009; Van Deynze et al., 2018). Seemingly, agaves may be an excellent niche from which to isolate nitrogen-fixing bacteria to be used as biofertilizers, such as ACO-34A, that is being successfully used as an inoculant in agricultural fields (Manzano-Gómez et al., 2025). ACO-34A may be used in mixed inoculants with the idea of integrated management of plant inoculants.

Declarations

Ethical Approval

not applicable

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Author Contribution

E.M-R wrote the main manuscript text, managed resources and directed the experimental work.L.G.G.P., M.A.R., and M.R-P performed the experiments and implemented the methodologyJ.M.R. and G.C.-F.

performed all phylogenetic analyses.L.G. designed primers and designed mutantJ.M.G. and R.P. performed experimentsM.G.G.R. performed genomic analyses.C.I.R.M. and R.R.R. proposed research activities.All authors reviewed the manuscript.

Data Availability

Genome will be available upon request, since NCBI is no longer accepting genome deposits.

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Figures

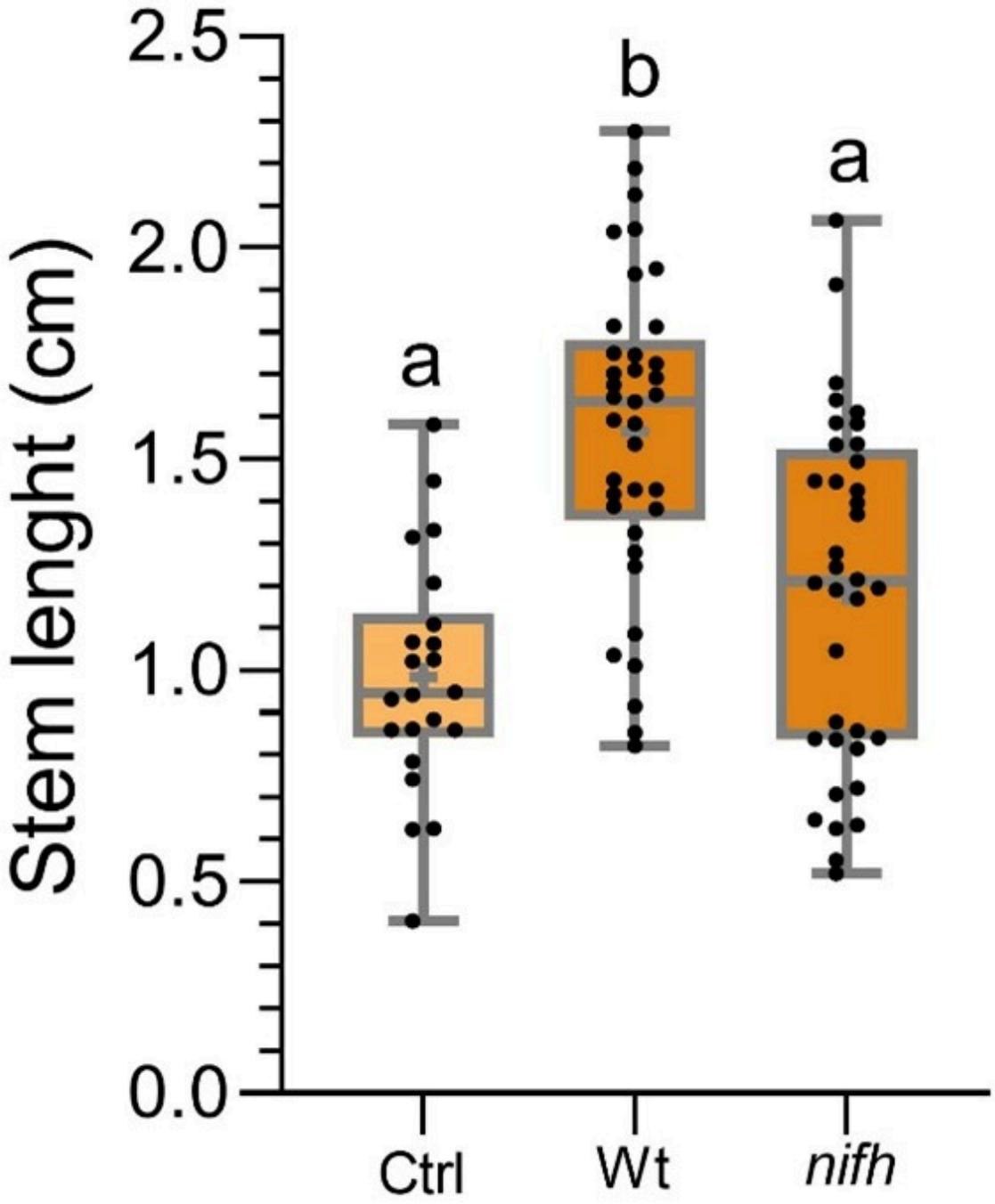

Figure 1

Stem length of *L. japonicus* plants inoculated with *Rhizobium* sp. ACO34-A.

Ctrl) non-inoculated plants, Wt) *Rhizobium* sp. ACO34-A inoculated plants, *nifh*) *nifH* mutant inoculated plants. Statistical significance was detected by a Tukey's multiple comparison test, different letters

represent significant difference. Black dots in the box plots indicate individual samples from three biological replicates.

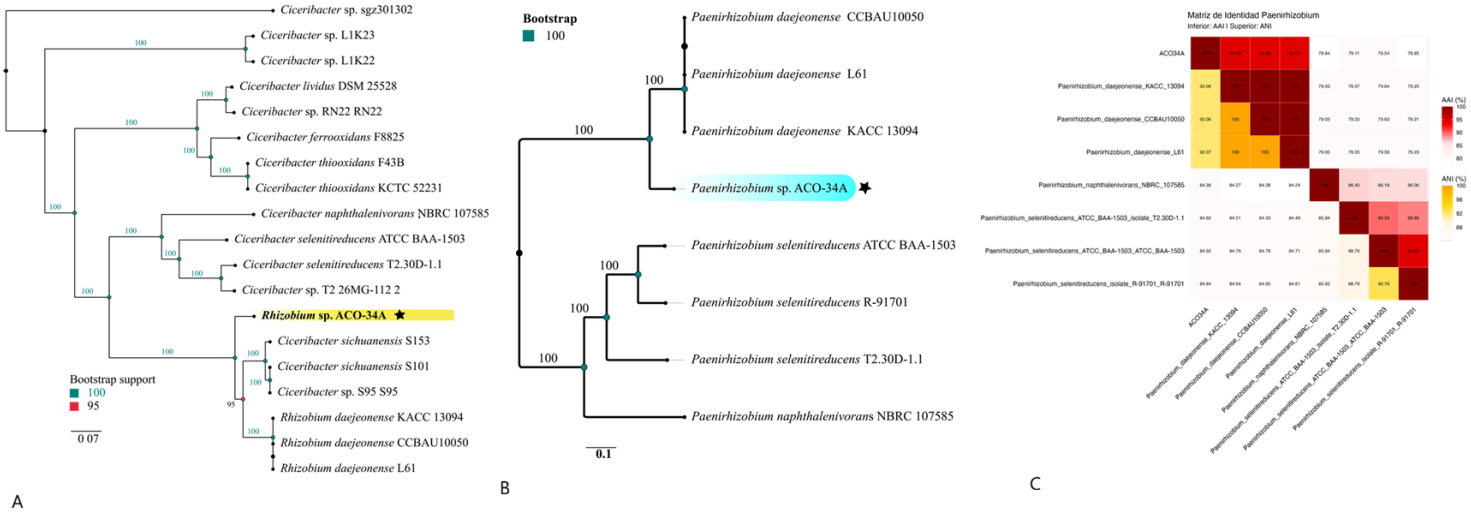

Figure 2

A. Phylogenomic tree based on 284 single-copy marker genes from 15 strains of the *Ciceribacter* genus and 4 strains of *Rhizobium* (including *R. daejeonense* and *Rhizobium*sp. ACO-34A). The tree was constructed using the GTR+F+ASC+R3 evolutionary model with 1000 bootstrap replicates to assess branch support.

B. Phylogenomic tree reconstruction bases on 1416 single copy gene markers from 8 strains of *Paeniirhizobium*genes. The tree was inferred using the GTR+F+ASC+R4 model, with 1000 bootstrap to asses brach support.

C. Average nucleotide identity and average aminoacid identity with the closest strains of *Paenirhizobium genus*.

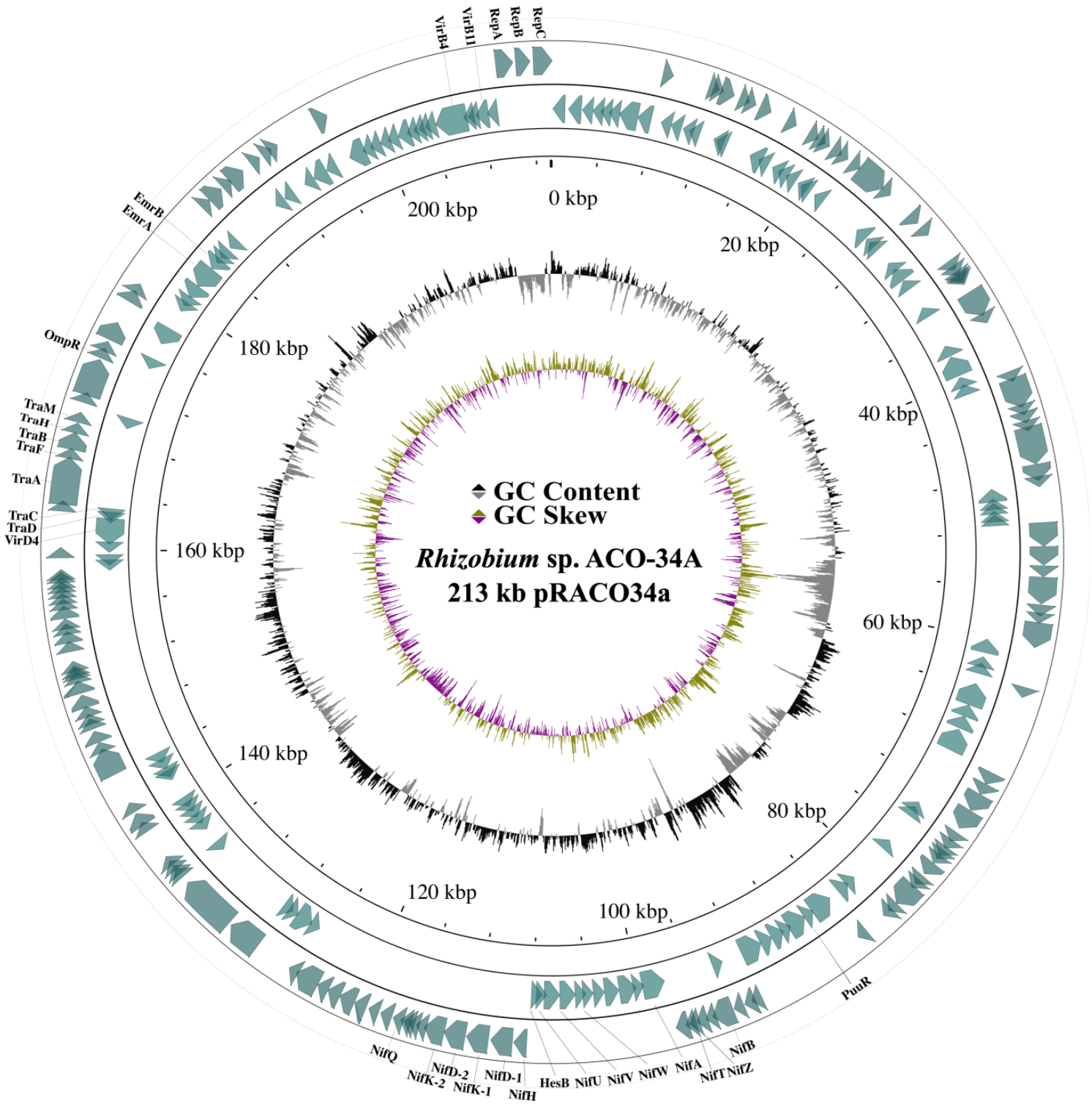

Figure 3

Circular map of the 213 kb plasmid of *Rhizobium*sp. ACO-34A. Selected genetic elements are shown on the plasmid.

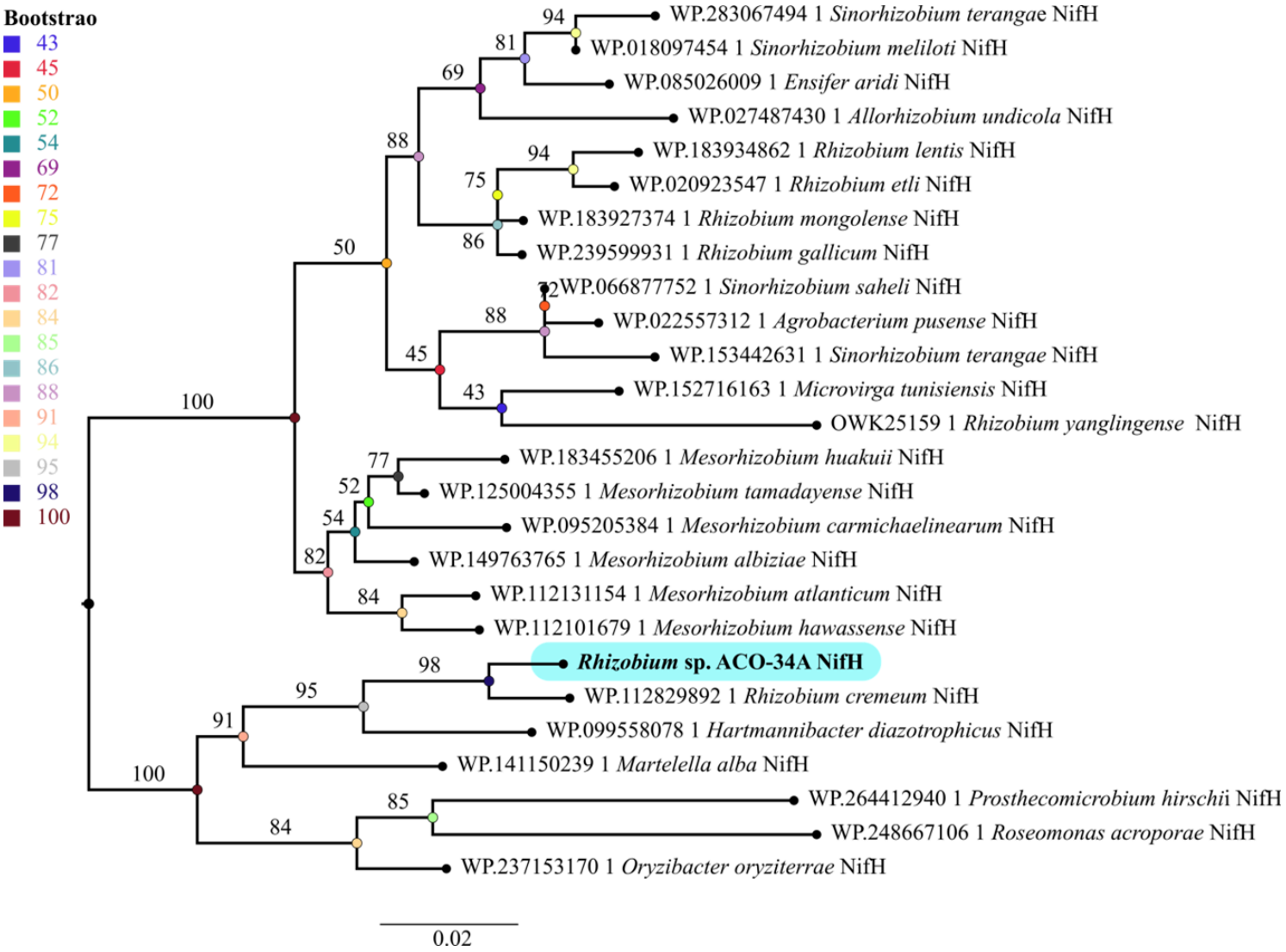

Figure 4

Phylogenetic analysis of the NifH protein, showing the position of *Rhizobium* sp. ACO34-A (starred) and its closest homologs with *Ciceribacter sichuanensis* S101^{T}.

Figure 5

Linear representation (from bottom to top) of plasmid pRACO-34A (CP021375.1) from the strain *Rhizobium* sp. ACO-34A, compared by BLAST with the sequences of *Agrobacterium fabrum*1D132 plasmid pAt1D132b (CP033025.1), *Ciceribacter sichuanensis* S101T (JAMXLX010000001.1), *Rhizobium cremeum* strain S302 plasmid 2(CP180219.1) and *Hartmannibacter diazotrophicus* strain E19T (LT960614.1). Genes related to horizontal gene transfer (HGT) are annotated at the top.

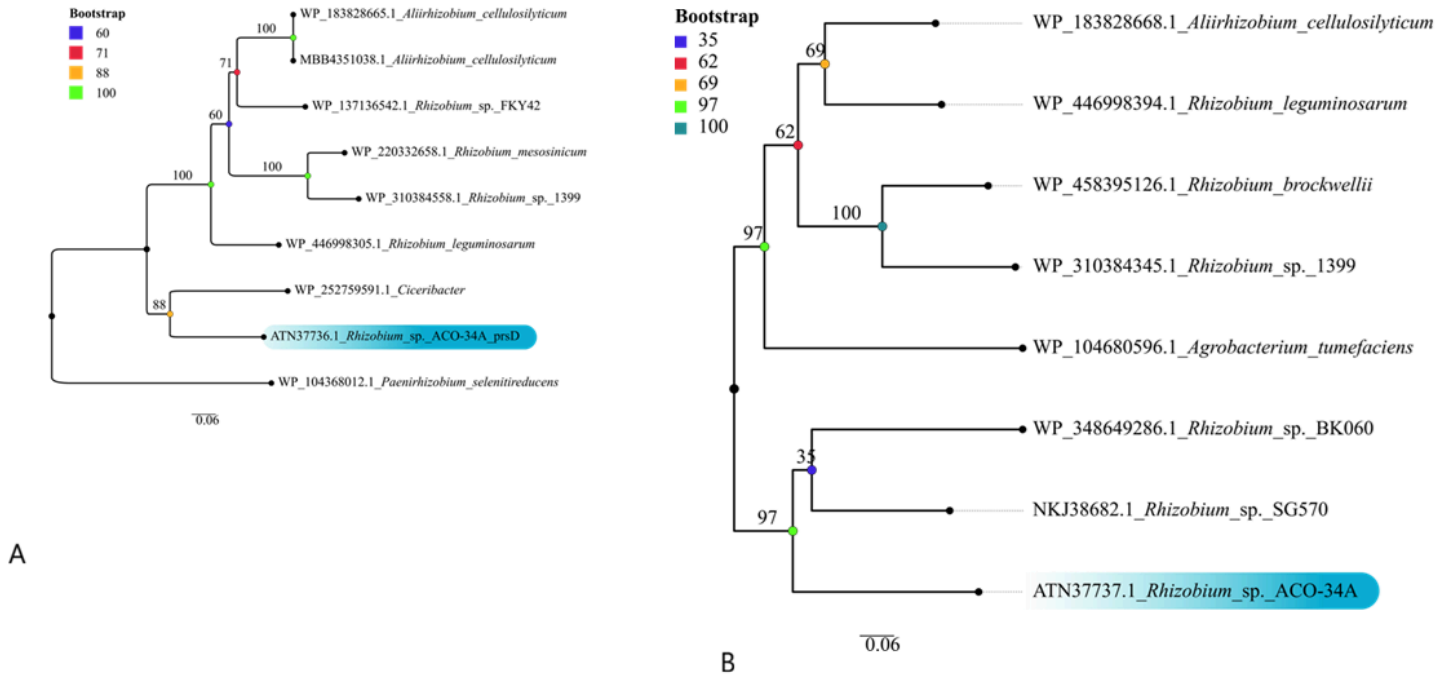

Figure 6

A. Phylogenomic tree *prsD* gene showing the placement of strain ACO-34A among reference species

B. Phylogenomic tree *prsE* (I) gene showing the placement of strain ACO-34A among reference species

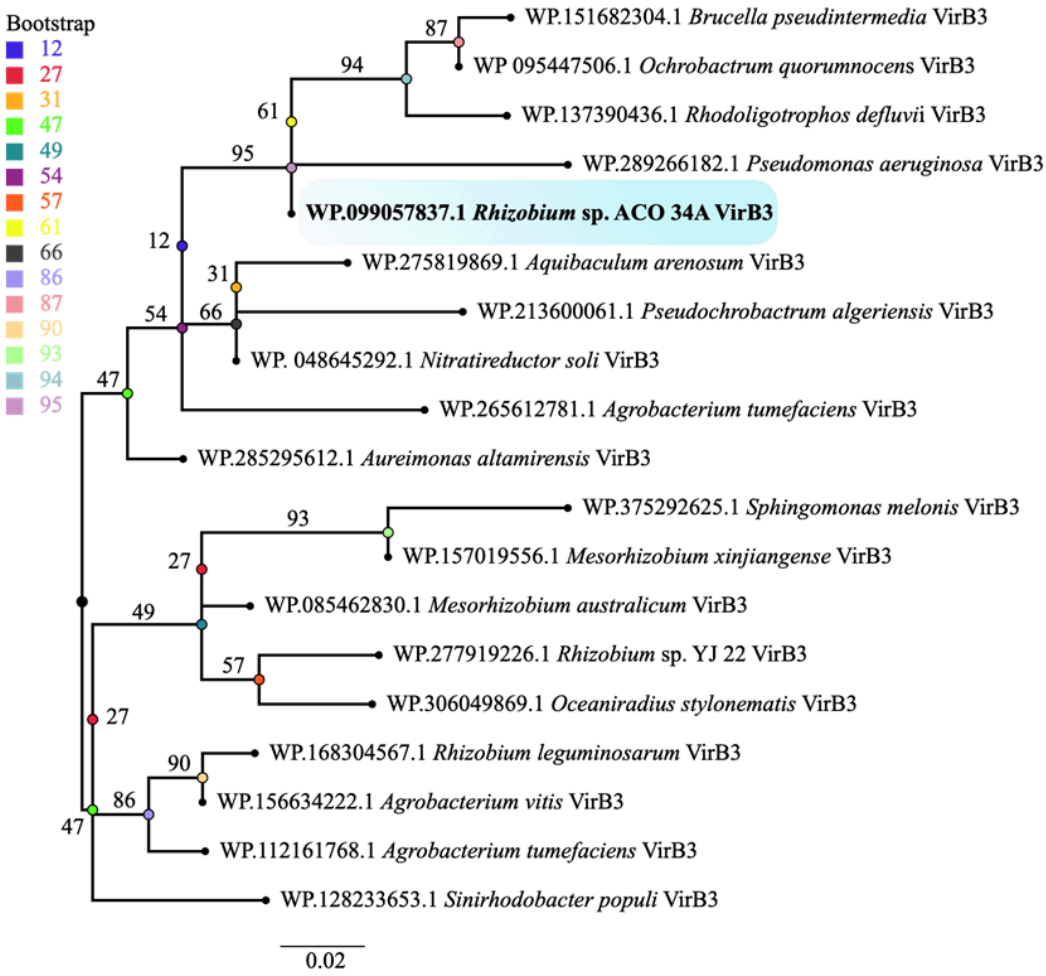

Figure 7

Phylogenomic tree *virB* (IV) gene showing the placement of strain ACO-34A among reference species

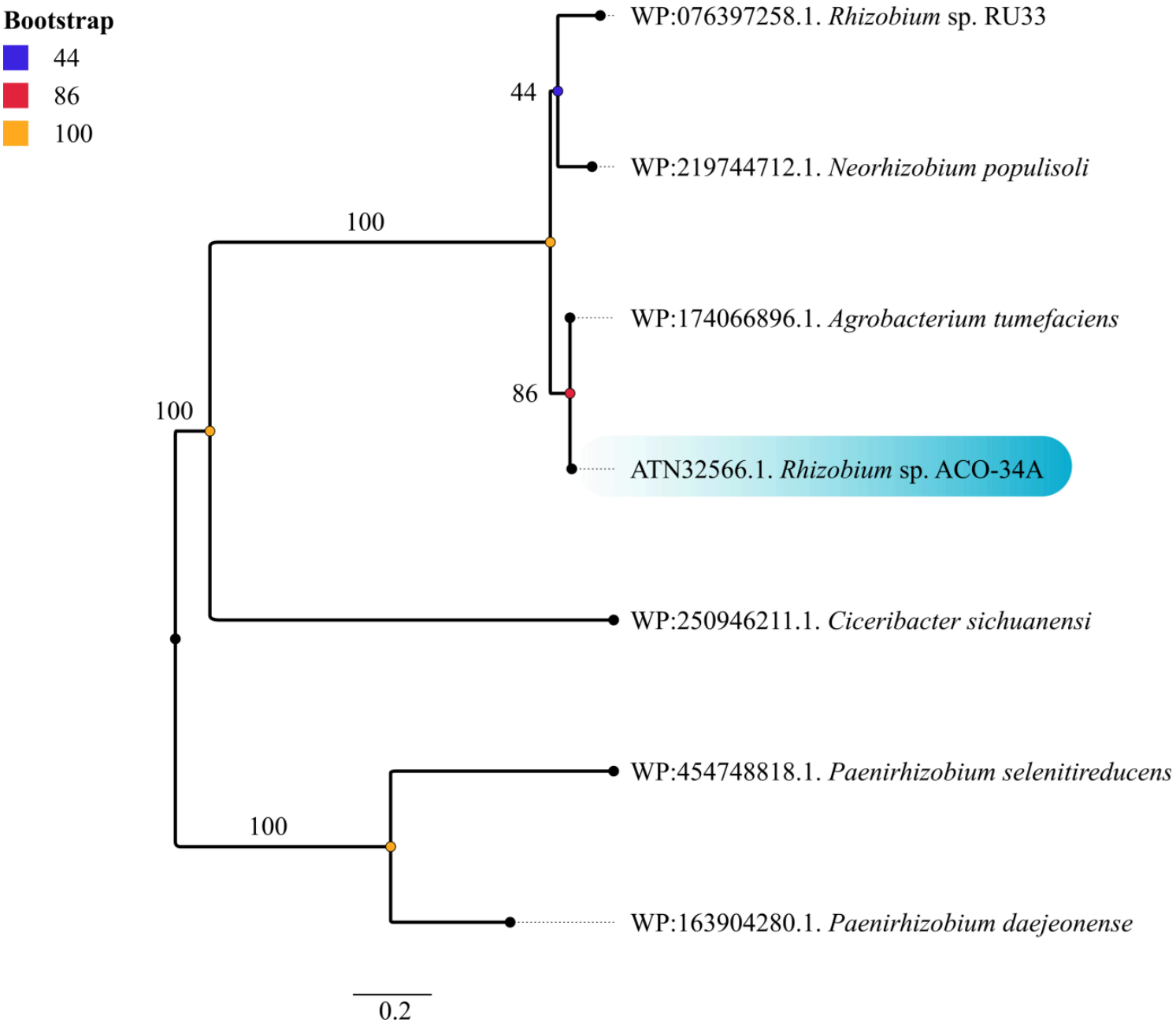

Figure 8

Phylogenomic tree *ompR* gene showing the placement of strain ACO-34A among reference species

Supplementary Files

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- SUPPLEMENTARYFIGURES.docx