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Multilocus Phylogenetic Characterization of Mesorhizobium-like Nodule-associated Isolates from Root Nodules of *Genista acanthoclada*

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Abstract: The root nodule symbiosis between legumes and rhizobia plays a fundamental role in biological nitrogen fixation and ecosystem sustainability. *Genista acanthoclada*, a perennial woody legume native to arid and rocky slopes of Western Türkiye, has not previously been investigated regarding to its symbiotic microsymbionts. In this study, five bacterial isolates were obtained from root nodules of *G. acanthoclada* collected from Yamanlar Mountain (İzmir, Türkiye) on Yeast Extract Mannitol Agar (YEMA) and rhizobial strains (GA.7.1 and GA.9) were rapidly selected by colony PCR targeting the *nodC* gene. Both isolates exhibited typical rhizobial phenotypic characteristics, including Gram-negative rod-shaped morphology, positive catalase and oxidase activities, and weak Congo red absorption on YEMA medium. Taxonomic analyses based on 16S rRNA, *recA*, and *atpD* gene sequences revealed that both isolates belong to the genus *Mesorhizobium*. The incongruence between the phylogenetic analysis based on *nodC* amino acid sequences and the core gene phylogenies suggests that horizontal gene transfer may play a role in the evolution of nodulation-related genes. In contrast, the *nifH*-based phylogeny was largely congruent with housekeeping gene phylogenies, indicating a more conserved evolutionary history of nitrogen fixation genes. These findings provide the first data on the rhizobia associated with the nodules of *G. acanthoclada* and contribute to a better understanding of the evolutionary dynamics of legume–rhizobium interactions.

Keywords: Rhizobia, legume, symbiotic nitrogen fixation (SNF), nodC, nifH.

Genista acanthoclada'nın Kök Nodüllerinden Elde Edilen Mesorhizobium Benzeri Nodül Ilişkili Izolatların Çoklu Lokus Filogenetik Karakterizasyonu

Öz: Baklagiller ve Rhizobia arasındaki kök nodül simbiyozu, biyolojik azot fiksasyonunda ve ekosistem sürdürülebilirliğinde temel bir rol oynamaktadır. Batı Türkiye'de kurak ve kayalık yamaçlara özgü çok yıllık odunsu bir baklagil olan *Genista acanthoclada*, bugüne kadar simbiyotik mikrosimbiyontları açısından incelenmemiştir. Bu çalışmada, Yamanlar Dağı'ndan (İzmir, Türkiye) toplanan *G. acanthoclada* kök nodüllerinden Yeast Extract Mannitol Agar (YEMA) besiyerinde beş bakteri izolatı elde edilmiş ve *nodC* genine yönelik koloni PCR analizi ile rhizobiyal suşlar (GA.7.1 ve GA.9) hızlı biçimde seçilmiştir. Her iki izolat, Gram-negatif çubuk şekilli hücre morfolojisi, pozitif katalaz ve oksidaz aktiviteleri ve YEMA ortamında zayıf Kongo kırmızısı emilimi gibi tipik rhizobiyal fenotipik özellikler sergilemiştir. 16S rRNA, *recA* ve *atpD* gen dizilerine dayalı taksonomik analizler, her iki izolatın *Mesorhizobium* cinsine ait olduğunu ortaya koymuştur. *nodC* amino asit dizilerine dayalı filogenetik analiz ile çekirdek gen filogenileri arasındaki uyumsuzluk, nodülasyonla ilişkili genlerin evriminde yatay gen transferinin rol oynayabileceğini düşündürmektedir. Buna karşılık *nifH* genine dayalı filogeni, evrimsel kronometre olarak kabul edilen diğer genlerle büyük ölçüde uyumlu bulunmuş ve azot fiksasyon genlerinin daha korunmuş bir evrimsel geçmişe sahip olduğunu göstermiştir. Bu bulgular, *G. acanthoclada*'nın nodülleriyle ilişkili rhizobia hakkında ilk verileri sağlamakta ve baklagil-rizobiyum etkileşimlerinin evrimsel dinamiklerinin daha iyi anlaşılmasına katkıda bulunmaktadır.

Anahtar kelimeler: Rhizobia, baklagil, simbiyotik azot fiksasyonu (SNF), nodC, nifH.

1. Introduction

Soil microorganisms constitute a major reservoir of biodiversity on Earth and play a fundamental role in maintaining the sustainability of terrestrial ecosystems. This microbial diversity is particularly critical for the functioning of global carbon and nitrogen cycles. The biological reduction of atmospheric nitrogen into plant-available forms occurs through biological nitrogen fixation (BNF), a process exclusively carried out by microorganisms (do Vale et al., 2013). In this context, rhizobial bacteria are particularly important as they convert atmospheric nitrogen into ammonia by colonizing root nodules in symbiosis with legume plants (Kawaka, 2022). Understanding the evolutionary origins, diversity, and host–bacteria interactions in rhizobia–legume symbiosis is facilitated by examining the relationships between legume and rhizobial taxonomy and symbiotic traits as well as by comparing the organization of nodulation (*nod*) and nitrogen fixation (*nif*) genes across different rhizobial species (Arashida et al., 2022; Janczarek et al., 2024).

Traditionally, classification based on 16S rRNA gene sequences has been widely used to assess the diversity of prokaryotic symbionts. However, the high sequence

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similarity of 16S rRNA among closely related species necessitates the use of additional genetic markers in rhizobial taxonomy (Rajkumari et al., 2022; Eren Eroğlu et al., 2024**)**. Accordingly, incorporating conserved evolutionary marker genes such as *recA* and *atpD* into multilocus sequence analyses enhances phylogenetic resolution (Kuzmanović et al., 2022). Genes determining host specificity and symbiotic performance are primarily represented by *nod* and *nif* loci. The *nodABCD* gene cluster is involved in the synthesis and regulation of nodulation factors, whereas *nif* genes encode the nitrogenase complex responsible for biological nitrogen fixation. The frequent localization of these genes on plasmids or mobile genomic islands reported in numerous studies indicates that horizontal gene transfer is a major driving force shaping rhizobial genetic diversity and host adaptation (Liu et al., 2023).

The tribe Genisteae, within the subfamily Faboideae of the family Fabaceae, comprises woody and herbaceous legume species widely distributed across Europe and Western Asia. The genus *Genista* is particularly prevalent in open steppe and pasture ecosystems where several species are utilized as forage crops and for pasture improvement. *Genista acanthoclada* (locally known as Kertikefen), native to Türkiye, is a perennial woody shrub that grows on arid, rocky slopes and is an important component of natural open shrublands and coniferous forest understories (Dinç and Bağcı, 2018). However, to date, no information is available regarding the nodule microsymbionts of this species.

In this study, we aimed to molecularly characterize rhizobial strains isolated from root nodules of *G. acanthoclada* and to determine their phylogenetic positions. To this end, multilocus phylogenetic analyses based on 16S rRNA, *recA*, and *atpD* evolutionary marker genes were performed and phylogenies inferred from *nodC* and *nifH* amino acid sequences were compared to specifically examine symbiotic and nitrogen fixation–related evolutionary relationships. The findings of this study provide new insights into the evolutionary dynamics of legume–rhizobia symbiosis.

2. Material and Method

2.1. Sample Collection and Isolation

Genista acanthoclada specimens were collected from Karşıyaka (Yamanlar Mountain), İzmir, Türkiye (altitude: 780 m) and taxonomically identified at the Ege University Herbarium Center (number: 44049) (Fig. 1). Healthy plants were carefully excavated with the surrounding root-associated soil to avoid damage to root nodules and transported to the laboratory. Actively nitrogen-fixing nodules were selected based on their pink–red coloration and larger size (Vincent, 1970). Nodules were thoroughly washed with distilled water to remove adhering soil particles, air-dried, and surface-sterilized by sequential immersion in 70% (v/v) ethanol for 1 min, 2% (w/v) sodium hypochlorite for 3 min, and 70% (v/v) ethanol for 30 s. Sterilized nodules were rinsed three times with sterile distilled water. Subsequently, nodules were aseptically crushed in sterile phosphate-buffered saline (PBS) to obtain homogeneous suspensions. Bacterial isolation was performed on Yeast Extract Mannitol Agar (YEMA) and homogenates were spread onto YEMA plates and incubated at 28°C for 2–10 days. Individual colonies were repeatedly subcultured on fresh YEMA plates until pure isolates were obtained.

Figure 1. *Genista acanthoclada* and active root nodules

2.2. Colony PCR for *nodC*

The *nodC* gene, which encodes the core structure of nod factors, is an important nodulation gene conserved across rhizobial species and plays a key role in establishing symbiosis with host plants. To screen for the presence of the *nodC* gene in symbiotic nitrogen-fixing isolates, colony PCR was performed using a *nodC*-specific primer pair

(Sarita et al., 2005). Isolates were spread onto YEMA plates and incubated at 28°C for 48–72 hours. Single colonies were collected with sterile toothpicks and suspended in 10 µL of sterile PBS (pH 7.4). Cell lysis was achieved by heating at 98°C for 15 minutes and the lysates were used directly as PCR templates. PCR reactions were performed in a total volume of 25 µL containing 12.5 µL of 2× Xpert Fast Hotstart Mastermix (GE45.5001), 1 µL each of forward and reverse primers, 7.5 µL of nuclease-free water, and 3 µL of template DNA (Table 1).

Table 1. Primer pairs and reaction conditions used in the study.

Genes	Primers	Reaction conditions	
16S rRNA	27F 5′AGAGTTTGATCCTGGCTCAG 3′ 1492R 5′GGTTACCTTGTTACGACTT 3′	95°C for 5 min, followed by 32 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 40 s, with a final extension at 72°C for 10 min	Koskey et al., 2018
atpD	atpD-771R 5′GCCGACACTTCCGAACCNGCCTG 3′ atpD-273F 5′ SCTGGGSCGYATCMTGAACGT 3′	95°C for 5 min; 32 cycles of 94°C for 30 s, 50°C for 60 s, and 72°C for 90 s; followed by a final extension at 72°C for 5 min.	Gaunt et al., 2001
recA	recAF 5′ATC GAG CGG TCG TTC GGC AAG GG 3′ recAR 5′ TTG CGC AGC GCC TGG CTC AT 3′	95°C for 5 min; 30 cycles of 94°C for 30 s, 50°C for 60 s, and 72°C for 90 s; followed by a final extension at 72°C for 5 min.	Gaunt et al., 2001
*n*odC	*nod*CF 5′ TGATYGAYATGGARTAYTGGCT 3 nodcR 5′ CGYGACARCCARTCGCTRTTG 3′	94°C for 5 min; 32 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; followed by a final extension at 72°C for 5 min.	Sarita et al., 2005
nifH	*NifHF 5′TTCTATGGAAAGGGCGGCATTGCCAAGCT′3* *NifHR 5′ATCTCGCCGGACATGACGATATAAATTTC′3*	95°C for 5 min; 33 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s; followed by a final extension at 72°C for 5 min.	Laguerre et al., 2001

2.3. Phenotypic and Molecular Identification

2.3.1. Phenotypic Identification

Phenotypic validation of the isolates was performed by Gram staining, catalase and oxidase tests, and growth assays on YEMA medium supplemented with Congo red. Catalase activity was assessed using 3% (v/v) H_2O_2 solution and oxidase activity was determined using tetramethyl-p-phenylenediamine reagent (Makkar and Jangra, 2017). For selective differentiation of rhizobial isolates, cultures were incubated on YEMA medium containing Congo red (0.025 g L^{-1}) at 28°C for 48–72 h. Pale pink to cream-colored, mucoid colonies showing weak dye absorption were recorded as typical rhizobial characteristics (Hamza and Alebejo, 2017).

2.3.2. DNA isolation

Pure cultures of the isolates were activated in Luria Bertani (LB) Broth medium at 28°C for 48 hours. 200 µl of the activated liquid culture was transferred to a sterile eppendorf tube and centrifuged at 8,000 rpm for 5 minutes. The cell pellets were washed with sterile PBS. Genomic DNA was extracted from isolates according to the Roche High Pure PCR Preparation Kit protocol. The obtained DNA samples were checked by visualization on a 1% agarose gel electrophoresis.

2.3.3. PCR amplification of 16S rRNA, atpD, recA, and nifH

Taxonomic characterization of the bacterial isolates was conducted through PCR amplification of conserved housekeeping genes (16S rRNA, *recA* and *atpD*). Furthermore, the *nifH* gene was targeted as a functional marker to assess nitrogen-fixation potential. The primer pairs used and corresponding thermal cycling conditions are summarized in Table 1. Reactions were performed in 25 µL volumes containing 12.5 µL 2× Xpert Fast Hotstart Master Mix (GE45.5001), 1 µL of each primer, 7.5 µL nuclease-free water, and 3 µL of genomic DNA.

2.4. Phylogenetic Analyses of Housekeeping and Symbiotic Genes

PCR amplification products of the 16S rRNA, *atpD*, *recA*, *nodC*, and *nifH* genes obtained from the bacterial isolates were purified and sequenced using the Sanger method with the corresponding amplification primers (Letgen Biotechnology, Bornova, İzmir, Türkiye). The resulting nucleotide sequences were compared with reference sequences retrieved from public databases. Homologous sequences of closely related taxa were retrieved from the GenBank database and included in the analysis (Table 2). Multiple sequence alignments were performed separately for each gene using the CLUSTALW algorithm implemented in MEGA 11. For the housekeeping genes (16S rRNA, *atpD*, and *recA*), nucleotide sequences were aligned and analyzed, whereas for the functional genes (*nodC* and *nifH*) analyses were conducted based on deduced amino acid sequences. The alignments were manually inspected and trimmed to remove poorly aligned regions and ambiguous positions. Positions containing gaps and missing data were treated using the pairwise deletion option. Phylogenetic trees were constructed in MEGA 11 using the Neighbor-Joining method, with evolutionary distances calculated under the Kimura 2-parameter model. The robustness of tree topologies was assessed by bootstrap analysis with 1000 replicates (Tamura et al., 2021).

Table 2. List of bacterial strains used in this study and corresponding GenBank accession numbers.

Strains	**Accession number**	**Gene sites**
M. oliveresii CPS 13	NR_149815.1	16S rRNA
M. norvegicum 10.2.2	NR_170463.1	16S rRNA
M.ciceri UPM-Ca7	NR_025953.1	16S rRNA
M.comanense 3P27G6	NR_181255.1	16S rRNA
M.neociceri CCANP35	NR_178425.1	16S rRNA
M.meditarraneum UPM-Ca36	NR_118684.1	16S rRNA
P. leguminum ORS 1419	NR_042396.1	16S rRNA
P.ifriqiense STM 370	AY785325.1	16S rRNA
P. brassicacearum STM 169	NR_043190.1	16S rRNA
Sinorhizobium sp. MZ6	MT956581.1	16S rRNA
Sinorhizobium sp. Sin 15	OM935981.1	16S rRNA
R. leguminosarum MNF23	OP458393.1	16S rRNA

S. memiloti NBRC 14782	NR_113670.1	16S rRNA
Rhizobium sp. S95	ON342880.1	16S rRNA
R. leguminosarum MNF23	OP458393.1	16S rRNA
R.aquaticum SA-276	NR_178732.1	16S rRNA
Rhizobium sp. 26bp3	OK384638.1	16S rRNA
R.sopharae CCBAU 03386	NR_178709.1	16S rRNA
R. tropici CIAT 899	NR_102511.1	16S rRNA
B. ripae WR4	NR_179732.1	16S rRNA
Bradyrhizobium sp. SNB120A	OP268341.1	16S rRNA
B.japonicum DSM 30131	NR_119191.1	16S rRNA
E. coli O1:KI:H7	NR_024570.1	16S rRNA
R. pisi SDM 30132	MK753111.1	*atpD*
R.leguminosarum bv. Trifolii ATCC 14480	EF113150.1	*atpD*
R.phaseoli ATCC 14482	EF113151.1	*atpD*
S. fredii CCBAU 25509	HQ231660.1	*atpD*
S. meliloti RMOF18	EU290595.1	*atpD*
S. medicae RMP05	EU290594.1	*atpD*
P. endophyticum PEPV15	JN848780.1	*atpD*
P. loti S658	KC577467.1	*atpD*
P. brassicacearum STM 196	AY785335.1	*atpD*
P. leguminum ORS 1419	AY785339.1	*atpD*
M. loti 75-Elvas	DQ659504.1	*atpD*
M. waitakense ICMP 14330	AY493461.1	*atpD*
M. robiniae CCNWYC 115	GQ856506.1	*atpD*
M. temperatum HAMBI 2583	DQ659499.1	*atpD*
Bradyrhizobium sp Ba-Z45	MK359481.1	*atpD*
B. elkanii ICMP 13638	AY493446.1	*atpD*
Bradyrhizobium sp. ORS 400	FJ347487.1	*atpD*
B. huanghuaihaiense CCBAU 23303	HQ231682.1	*atpD*
Burkholderia cenocapacia ba-110	MH744774.1	*atpD*
Phyllobacterium sp. CNXE1	JF440639.1	*recA*
P. endophyticum PEPV 15	MG652920.1	*recA*
Phyllobacterium sp. T1018	FJ010175.1	*recA*
R. rosettiformans W3	GU562963.1	*recA*
R. phaseoli ATCC 14482	EF113136.1	*recA*
R. mongolense USDA 1844	AY907358.1	*recA*
R. leguminosarum bv. Viciae WBAV recA 81	KP754843.1	*recA*
Sinorhizobium sp. YH13	KU904582.1	*recA*
S. fredii CCBAU 25509	HQ231585.1	*recA*
S. meliloti RTM 17	EU290600.1	*recA*
S. medicae RMP05	EU290598.1	*recA*
M. terimanse CCBAU 83306	EF549482.1	*recA*
Mesorhizobium sp. RM567	MG704209.1	*recA*
M. robiniae CCNWYC 124	GQ856503.1	*recA*
M. ciceri R30	MT237340.1	*recA*
Brdyrhizobium sp. ORS 375	FJ347481.1	*recA*
B. japonicum CCBAU 83623	EU169223.1	*recA*
Bradyrhizobium sp. STM 8097	KJ653441.1	*recA*
E. coli E1140	HQ660620.1	*recA*
Bradyrhizobium sp. TUTGMeS33	UTM72507.1	*nodC (aa)*
Bradyrhizobium sp. CI137	AVK51756.1	*nodC (aa)*
Bradyrhizobium sp. LmiT14	APF29155.1	*nodC (aa)*
Bradyrhizobium sp. TUTGMeS4	UTM72500.1	*nodC (aa)*
S. sojae CCBAU 05638	ADG63646.1	*nodC (aa)*
R. leguminosarum bv. Viciae 1079	QDP42311.1	*nodC (aa)*
R. pisi DSM 30132	AFK94222.1	*nodC (aa)*
M. temperatum SDW018	ACT34097.1	*nodC (aa)*
Phyllobacterium sp. CCNWNX0150	ACI47342.1	*nodC (aa)*
Mesorhizobium sp. N. Ca. ET. 138. 01. 1	RUW49666.1	*nodC (aa)*
M. ciceri UPM-Ca7	ABD94164.1	*nodC (aa)*
R. phaseoli ATCC 14482	ADN27529.1	*nodC (aa)*
Rhizobium sp. TUTpvES30(1)	WBY50013.1	*nodC (aa)*
M. robiniae CCNWYC 115	ACH91245.1	*nodC (aa)*
Phyllobacterium sp. R-45798	CCA94535.1	*nodC (aa)*
S.medicae USDA 1037	ABN09217.1	*nodC (aa)*
S. meliloti MS25-3	AAU11354.1	*nodC (aa)*
Sinorhizobium sp. T3	UBY12737.1	*nodC (aa)*
B. japonicum CCBAU 25435	ADZ23532.1	*nifH (aa)*
B. elkanii S 127	ABG74604.1	*nifH (aa)*
Bradyrhizobium sp. Lalb5.2	UPQ41499.1	*nifH (aa)*
Phyllobacterium sp. ORS 1402	CAI91558.1	*nifH (aa)*
S.trangae ORS 1009	CAB08522.1	*nifH (aa)*
S.sojae CCBAU 05606	ADG63613.1	*nifH (aa)*
R. leguminosarum CCNWSX0509	ACO90394.1	*nifH (aa)*
S. meliloti CCNWNX0117	ACI47351.1	*nifH (aa)*
R. tropici CFN ESh25	ABZ89804.1	*nifH (aa)*
Rhizobium sp. TUTpvES57(3)	WBY50009.1	*nifH (aa)*
Sinorhizobium sp. ITTG S8	ABZ89811.1	*nifH (aa)*
Rhizobium sp. CFN ESH34	ABZ89805.1	*nifH (aa)*
P. sophorae CCBAU 03422	AIF28016.1	*nifH (aa)*
Mesorhizobium sp N. Ca. ET. 092.01.1	TKC02180.1	*nifH (aa)*
M.temperatum SDW018	ACT67928.1	*nifH (aa)*
M. neociceri CCANP35	CEK41207.1	*nifH (aa)*
M. septentrionale SDW014	ACT67925.1	*nifH (aa)*
Phyllobacterrium sp. BIHB 4156	AHY83147.1	*nifH (aa)*

3. Results

3.1. Rapid Screening and Selection of Rhizobial Isolates

Root nodules of *G. acanthoclada* were used to isolate symbiotic bacteria. Five bacterial isolates were obtained by cultivation on YEMA. To verify their rhizobial identity, colony PCR targeting the *nodC* gene was performed. Agarose gel electrophoresis of the PCR products revealed the presence of the expected *nodC* amplicons in isolates GA.7.1 and GA.9, indicating that they are putative nodule-associated rhizobia (Fig. 2).

Both GA.7.1 and GA.9 were Gram-negative, rod-shaped bacteria and exhibited positive catalase and oxidase activities. On YEMA medium supplemented with Congo red and incubated at 28°C for 72 h, GA.7.1 formed pale pink colonies, whereas GA.9 produced cream-white colonies with weak dye absorption. These phenotypic characteristics are consistent with typical rhizobial colony morphology (Fig. 3).

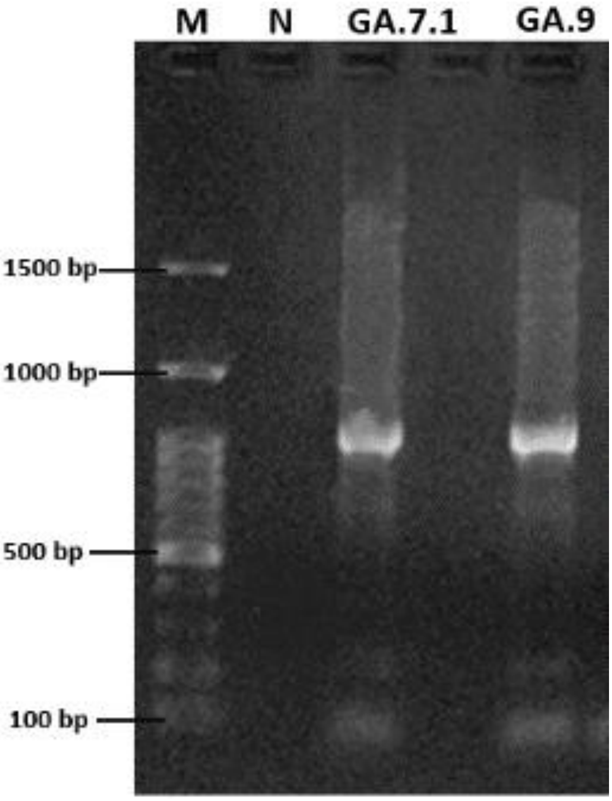

Figure 2. Detection of *nodC* amplicons in isolates GA.7.1 and GA.9 by colony PCR. M: DNA ladder; N: negative control.

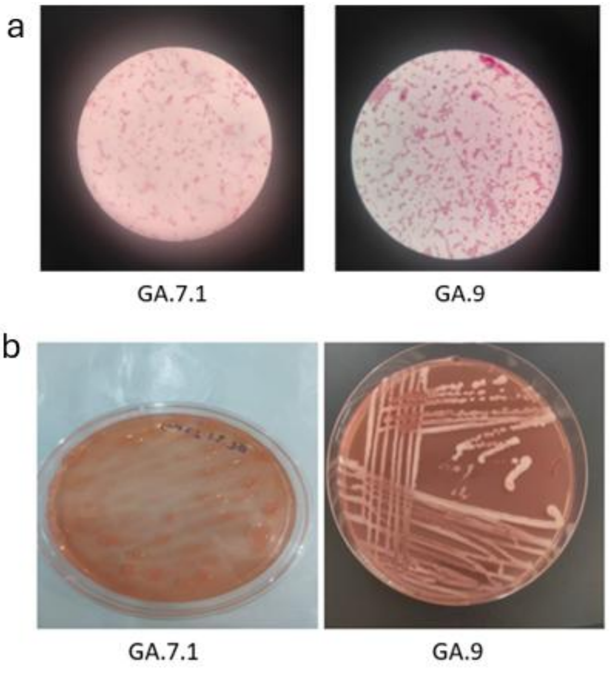

Figure 3. Phenotypic characterization of isolates GA.7.1 and GA.9. (a) Light microscopy images of Gram-stained cells; (b) colonies on Congo red–supplemented YEMA medium after 72 h incubation at 28°C

3.2. Phylogenetic Analyses Based on Housekeeping Genes

For 16S rRNA-based identification, amplicons of approximately 1500 bp were successfully sequenced. BLAST analysis indicated that isolates GA.7.1 and GA.9 exhibited the highest sequence similarity to members of the genus *Mesorhizobium*, with identities of 98.93% and 98.23%, respectively. In the 16S rRNA phylogenetic tree including representatives of *Mesorhizobium*, *Phyllobacterium*, *Sinorhizobium*, *Rhizobium*, and *Bradyrhizobium*, both isolates clustered within the *Mesorhizobium* clade and formed a monophyletic group closely related to *M. olivaresii* strain CPS13 (Fig. 4).

Amplification of the *atpD* gene yielded products of approximately 450 bp. BLAST analysis revealed sequence identities of 96.47% (GA.7.1) and 97.51% (GA.9) with *Mesorhizobium* species. In the *atpD*-based phylogeny, GA.7.1 grouped most closely with *M. temperatum,* whereas GA.9 clustered with *M. robiniae* (Fig. 5a).

Subsequently, ~500 bp *recA* amplicons were obtained. Sequence comparisons showed that GA.7.1 and GA.9 shared 94.98% and 94.68% similarity, respectively, with reference to the *Mesorhizobium recA* sequences. In the *recA* based phylogenetic tree, both isolates were positioned within the *Mesorhizobium* lineage but formed a distinct and well-supported monophyletic branch (Fig. 5b).

3.3. *nodC* and *nifH* Based Phylogenetic Analyses

PCR products of the *nodC* and *nifH* gene fragments from strains GA.7.1 and GA.9 were sequenced using the Sanger method. Raw sequence reads were assembled using the CAP3 sequence assembly program and consensus nucleotide sequences were translated into amino acid sequences using EMBOSS Transeq. The resulting *nodC* and *nifH* sequences, approximately 180 bp and 142 bp, respectively, were analyzed using BLASTp. The *nodC* amino acid sequence of GA.7.1 showed 98.72% identity to *Mesorhizobium* N-acetylglucosaminyltransferase (*NodC*), while GA.9 showed 98.87% identity. Similarly, the *nifH* amino acid sequences of GA.7.1 and GA.9 exhibited 99.61% and 99.11% identity, respectively, to *Mesorhizobium* nitrogenase iron protein (*NifH*) sequences deposited in GenBank. Phylogenetic trees constructed based on *nodC* and *nifH* amino acid sequences placed both isolates within the *Mesorhizobium* lineage. However, consistent with the housekeeping-gene phylogenies, the isolates formed distinct branches, suggesting divergence from currently described species within the genus (Fig. 6).

4. Discussion

To rapidly screen nodule-associated rhizobia from *G. acanthoclada*, colony PCR targeting the *nodC* gene was employed. Among five isolates purified on YEMA medium, two (GA.7.1 and GA.9) yielded *nodC* specific amplicons. It is well established that legume root nodules harbor not only rhizobia but also diverse non-rhizobial endophytic bacteria, predominantly belonging to the genera *Bacillus* and *Pseudomonas* (Hnini and Aurag, 2024). Nodulation genes, including *nodC*, function as key bacterial determinants in the signal exchange between rhizobia and legume hosts and are therefore widely used as molecular markers of symbiotic potential (Laguerre et al., 2001; Kalita and Małek, 2020; Lopez et al., 2022). However, this screening approach based solely on targeting the *nodC* gene has certain limitations. The failure to amplify *nodC* does not necessarily indicate that the corresponding isolates are non-rhizobial.

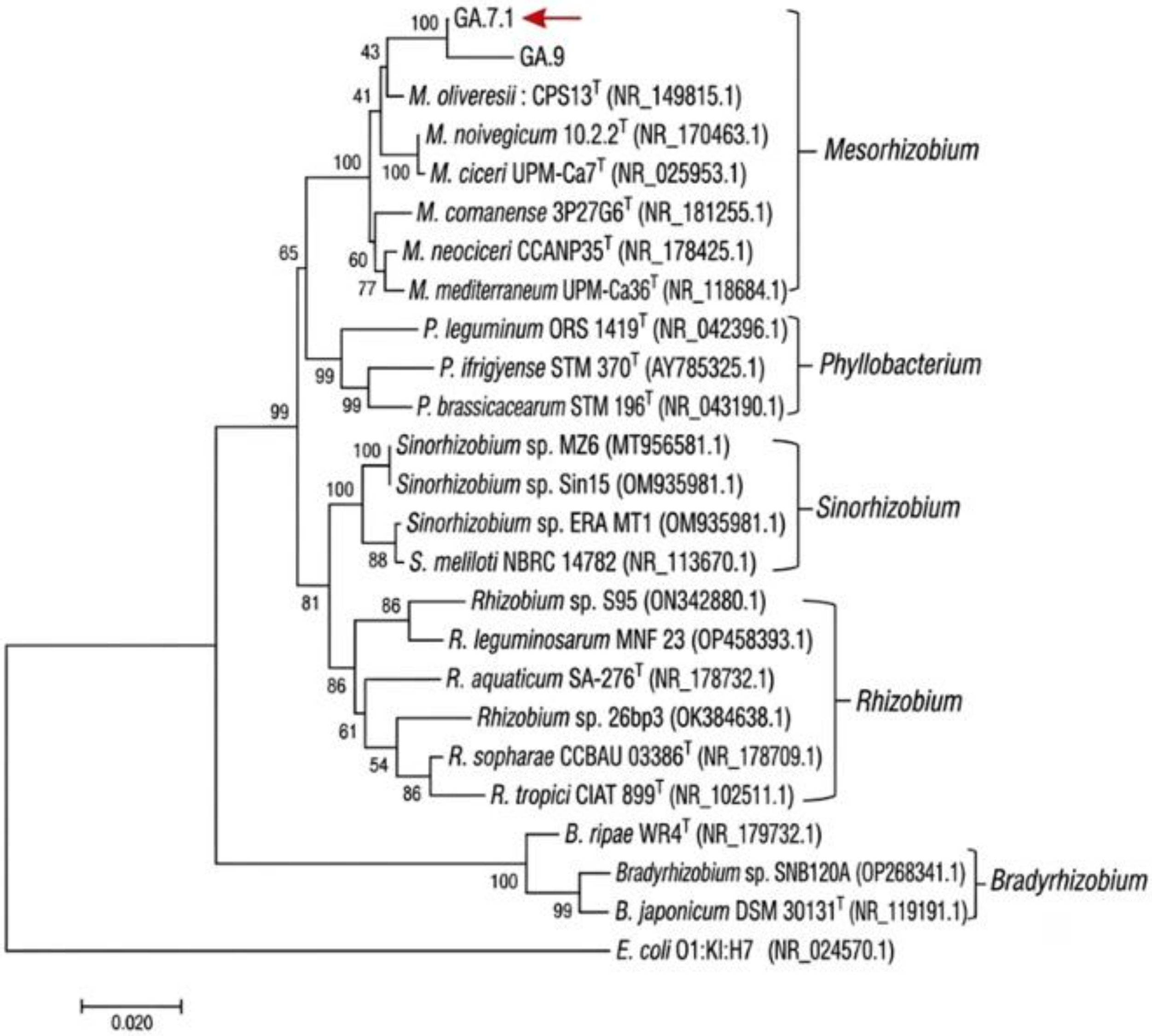

Figure 4. 16S rRNA gene–based Neighbor-Joining phylogeny of isolates GA.7.1 and GA.9. *Escherichia coli* O1:K1:H7 served as the outgroup.

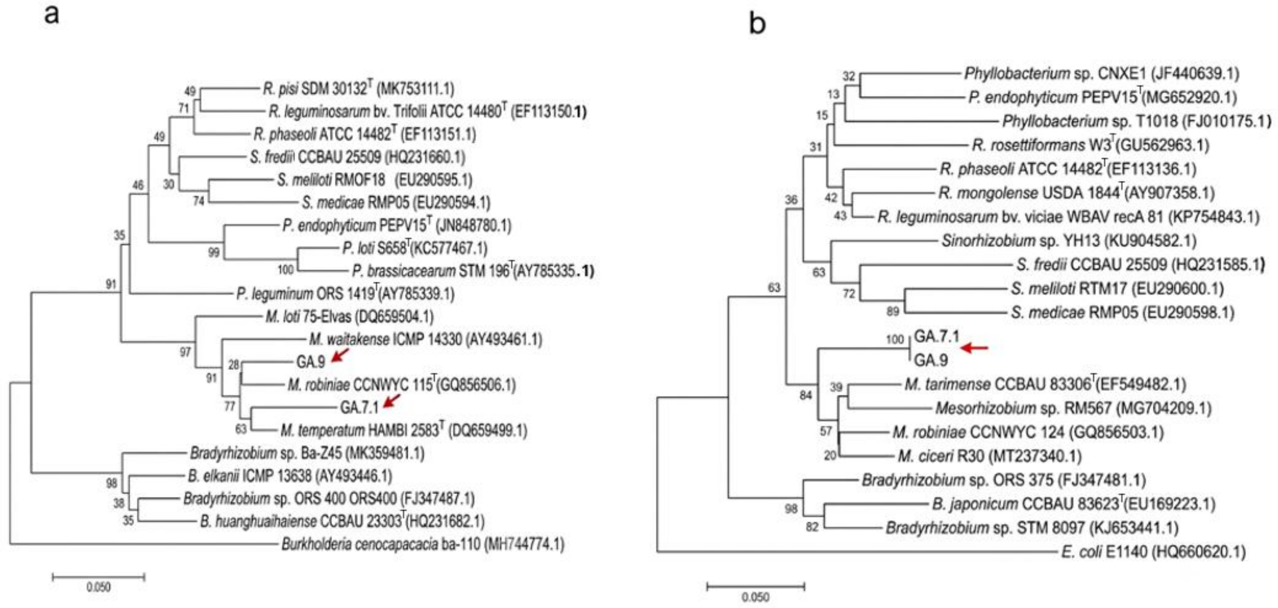

Figure 5. Neighbor-Joining phylogenetic trees based on (a) *atpD* and (b) r*ecA* gene sequences showing the phylogenetic positions of isolates GA.7.1 and GA.9. *Burkholderia cenocepacia* BA-110 and *Escherichia coli* E1140 were used as outgroups for the *atpD* and *recA* trees, respectively.

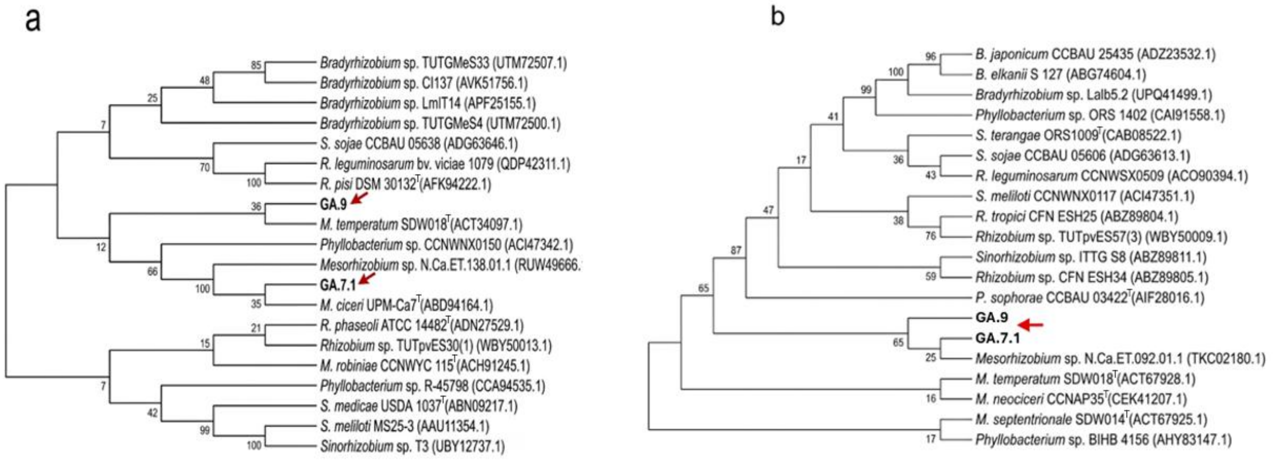

Figure 6. Neighbor-Joining phylogenetic trees based on (a) nodC and (b) nifH amino acid sequences showing the phylogenetic positions of isolates GA.7.1 and GA.9.

Although the *nodC* gene is generally conserved and plays a central role in Nod factor synthesis and symbiosis, some strains may lack this gene entirely or carry non-functional variants (Laguerre et al., 2001; Giraud et al., 2007).

In the present study, *nodC* screening was performed for the first time using a colony PCR-based approach. This technique enables direct PCR amplification from bacterial colonies without prior DNA extraction, providing a rapid, simple, and cost-effective alternative for screening large numbers of isolates. Colony PCR has been successfully applied in the diagnosis of plant pathogens, including the detection of Ralstonia solanacearum in tomatoes and tumor-forming Agrobacterium strains in apples (Suzaki et al., 2004; Umesha et al., 2012).

Cultural characterization of rhizobial isolates commonly relies on colony morphology and Congo red absorption on YEMA medium as standard diagnostic criteria. In the present study, isolates GA.7.1 and GA.9 formed light-colored, glossy, and raised colonies on Congo red–supplemented YEMA, consistent with typical phenotypic traits described for *Rhizobium* spp. in previous reports (Wang et al., 2019; Kawaka and Muoma, 2020). Moreover, the appearance of visible colonies within 72 h indicates that these isolates belong to fast-growing rhizobial groups, in agreement with the growth characteristics reported by Hungria et al. (2001).

In the 16S rRNA gene-based phylogenetic tree, isolates GA.7.1 and GA.9 clustered within the same clade as *M. olivaresii* strain CPS13, forming a monophyletic group. The 16S rRNA gene sequence similarity values of GA.7.1 (98.93%) and GA.9 (98.23%) indicate a close phylogenetic relationship with *M. olivaresii*. However, because one isolate exhibited a similarity value below the commonly accepted 98.7% threshold for species delineation and 16S rRNA gene sequences often show limited discriminatory power among rhizobial species, species-level assignment cannot be established based solely on this marker (Meier-Kolthoff and Göker, 2019). Phylogenetic analysis based on *atpD* sequences revealed that GA.7.1 grouped closely with *M. robiniae*, whereas GA.9 showed affinity to *M. temperatum*. In contrast, *recA* based phylogeny indicated that both isolates formed a distinct monophyletic lineage, clearly separated from other *Mesorhizobium* species. The *recA* gene has been proposed as a robust phylogenetic marker with high discriminatory power at both genus and species levels in rhizobia (Jiao et al., 2015; Yan et al., 2014). Accordingly, multilocus sequence analysis using two or more conserved housekeeping genes (e.g., *atpD*, *recA*, *glnII*, *gyrB*, *rpoB*) is considered a reliable approach for accurate rhizobial taxonomy (Martens et al., 2008). Previous studies have also demonstrated that phylogenies inferred from *recA* and *atpD* are congruent with large-scale bacterial evolutionary relationships (Gaunt et al., 2001).

In *nodC* based analyses, the isolates clustered even with distinct rhizobial genera, a pattern that is consistent with scenarios in which horizontal gene transfer of nodulation genes occurs, potentially due to their localization on plasmids or mobile genomic islands. It has previously been reported that nod gene phylogenies may evolve independently of 16S rRNA and other housekeeping gene phylogenies (Laguerre et al., 2001). In contrast, phylogenetic reconstruction based on *nifH* amino acid sequences was largely consistent with housekeeping gene–based trees. This observation suggests that nitrogenase genes may possess a more stable evolutionary history in most rhizobial groups. Similarly, Drew et al. (2021) reported parallel evolutionary signals between *nifH* and 16S rRNA phylogenies across several rhizobial lineages. Nevertheless, evidence for horizontal transfer of *nif* genes has also been documented, supporting the mosaic architecture of rhizobial genomes (Kawaka, 2022).

Previous studies from North Africa and Europe have shown that *Genista* species mayestablish symbioses with diverse rhizobial genera and harbor high levels of genetic diversity among their microsymbionts (Chaïch et al., 2017; Boudehouche et al., 2020; Kalita and Małek, 2020). However, to date, no study has reported the molecular characterization of rhizobial strains isolated from root nodules of *G. acanthoclada*. In this regard, the present work provides the first molecular insight into nodule-associated rhizobia linked to this host species.

It is now well recognized that genome-based approaches have become the standard in rhizobial taxonomy (Ormeno-Orrillo et al., 2015). Average Nucleotide Identity (ANI) and in silico DNA–DNA hybridization (dDDH) analyses have largely replaced classical DNA–DNA hybridization and are considered the gold standard for species delineation (Ferraz et al., 2022; Eren Eroğlu et al., 2025). Based on the multilocus phylogenetic evidence obtained in this study, isolates GA.7.1 and GA.9 can be assigned to the genus *Mesorhizobium*; however, their precise taxonomic status at the species level remains unresolved. Although these isolates appear to form distinct phylogenetic lineages, the available data are insufficient to support their designation as novel species. Nevertheless, their phylogenetic distinctiveness and separation from currently described Mesorhizobium species suggest that they may represent previously unrecognized taxonomic entities within the genus (Chun et al., 2018). Therefore, further genome-based analyses, including whole-genome sequencing and comparative genomic approaches, will be necessary to clarify their taxonomic position and to robustly assess their potential novelty.

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