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Using Sequencing Technique for Diagnostic Different Species of Genus *Rhizobium* Which Isolated from Legume Plants

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Abstract:

Samples of the root nodules were collected to isolate different species of the genus *Rhizobium* from several leguminous plants; *Trigonella foenum-graecum, Medicago sativa, Lens culinaris, Vigna mungo, Vicia faba, Phaseolus vulgaris, and Cicer arietinum,* and based on their morphological, cultural, and biochemical characteristics, in addition to the identification of each isolate at the species level by amplified polymerase chain reaction (PCR) and using the sequencing of the nitrogenous bases of the 16S rRNA gene, it was identified as *Sinrhizobium meliloti, Sinrhizobium meliloti, Bradyrhizobium elkanii, Rhizobium leguminosarium biovar viciae, Rhizobium leguminosarium biovar phaseoli and Mesorhizobium cicero*, respectively, (with the exception of bacteria isolated from *Lens culinaris*) it showed an agreement with the GenBank isolates with percentages ranging between (96-98) percent, as well as drawing the phylogenetic tree to determine the extent of evolution between these isolates and their convergence in the original with the international standard isolates.

Keywords: *Rhizobium*, Legume plants, 16S rRNA gene, PCR, Phylogenetic tree

استخدام تقنية تسلسل الحامض النووي لتشخيص الانواع المختلفة من جنس *Rhizobium* المعزولة من النباتات البقولية

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الخلاصة:

جمعت عينات من العقد الجذرية لعزل أنواع مختلفة من جنس *Rhizobium* من عدة نباتات بقولية الحلبة *Trigonella foenum-graecum* ، الجت *Medicago sativa* ، العدس *Lens culinaris* ، اللوبيا *Vigna mungo* ، الباقلاء *Vicia faba* ، الفاصوليا *Phaseolus vulgaris* والحمص *Cicer arietinum*، و بناءاً على خصائصها الشكلية والزرعية والفحوصات البايوكيمياوية ، بالإضافة إلى تشخيص كل عزلة على مستوى النوع عن طريق تفاعل البلمرة المتسلسل (PCR) المضخم وباستخدام تسلسل القواعد

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النتروجينية للجين 16S rRNA ، تم تشخيصها على انها *Sinrhizobium meliloti* ، *Sinrhizobium meliloti* ، *Bradyrhizobium elkanii*، *Rhizobium leguminosarium* biovar *viciae*، *Rhizobium leguminosarium* biovar *phaseoli* و*Mesorhizobium cicero* ، على التوالي، (باستثناء البكتيريا المعزولة من العدس) وقد اظهرت تطابقا مع عزلات بنك الجينات بنسب مئوية تراوحت بين (96–98) % فضلا عن رسم شجرة النشوء والتطور لتحديد مدى التطور الحاصل بين هذه العزلات وتقاربها في الاصل مع العزلات القياسية العالمية.

Introduction:

Soil contains multiple forms of microorganisms, including the rhizobium bacterium known for its specialized symbiotic relationship with leguminous plants, which leads to the fixation of atmospheric nitrogen to ammonia by the enzyme nitrogenase possessed by the bacterioid bacteria within the root nodules [1,2], in addition, they have the ability to exchange genetic information among themselves and with genetically close bacterial species in different ways[3], and to produce some enzymes such as chitinase, Pectinase, protease, and lipase enhanced their positive role in improving the growth of leguminous plants specialized in infecting them [4].

Molecular techniques have evolved to the extent that researchers have been able to address deficiencies in understanding the genetic traits encoded by the genes located on the DNA or RNA molecule[5], through the use of molecular methods that are characterized by their relative ease, and their ability to track genes located on chromosomes and p*Sym* plasmid in *Rhizobium* bacteria, which should lead to new insights into understanding the mechanisms of diversity and evolution in soils [6]. The polymerase chain reaction (PCR) is a method widely used in molecular biology, as it rapidly produces billions of copies of a specific sample of deoxyribonucleic acid, and this reaction includes DNA replication to determine the sequence, cloning and manipulating, mutating, and functional analysis of these genes [7], and agarose gel electrophoresis is the best way to separate DNA based on its sizes [8].

In a recent study, the dominance of *Bradyrhizobium* bacteria in tropical soils when diagnosed with the 16S rRNA gene, the study also suggested that the variable regions in this gene located between V5-V7 may be a suitable indicator to distinguish between the nodule-forming bacteria in the genus level [9].

This study aims to characterize Rhizobium bacteria isolated from the root nodules of seven different leguminous plants from the local environment by culturing, morphological and biochemical tests, as well as Amplification and sequencing of 16Sr RNA, followed by establishing the phylogenetic tree of the, studied and reference isolates**.**

Materials and Methods:

Isolation of bacteria from the root nodules of leguminous plants:

The healthy mature and pink root nodules belong to the following leguminous plants: *Trigonella foenum-graecum, Medicago sativa, Lens culinaris, Vigna mugo, Vicia faba, Phaseolus vulgaris,* and *Cicer arietinum* were taken and used to isolate the rhizobium bacteria from them, and after they were washed with tap water several times, they were surface sterilized by washing for 3 minutes with 1% of sodium hypochlorite solution, after which they were washed seven times with sterile distilled water to remove any trace of disinfectant and followed by crushing them with glass rode under sterilized conditions with drops of [10]. A loop-full was transported from this suspension and streaked on the selective and solid Yeast Extract Mannitol (YEM) medium plates and incubated in the dark at 28°C for 2-3 days[11].

Bacterial isolates were preserved on slant YEM medium in sealed glass vials and kept in the refrigerator at 4°C.

Diagnostic tests for isolated bacteria understudy:

Morphological and cultural characteristics of isolated rhizobial bacteria: Macroscopically (colony's shape, color, texture, viscosity, and edge as mentioned by Shahzad *et al.*,[12] and microscopically (their susceptibility for uptake a gram stain, cells arrangement, spores).

Biochemical tests: Many biochemical tests were performed on all bacterial isolates such as Catalase, IMViC, Urease, Gelatinase, Motility, ONPG, and saccharides fermentation tests [13, 14, 15, 16]. Individual colonies of each species were re-subcultured by streaking it on solid YEM medium plates containing 25µg/ml of Bromothymol Blue (BTB) and Congo Red(CR) respectively and incubated in the dark at 28°C for 2-3 days [13].

Host specific test for *Rhizobium* isolates: The isolated bacteria it is called *Rhizobium* were re-inoculated with their plant hosts (*Trigonella foenum-graecum, Medicago sativa, Lens culinaris, Vigna mungo, Vicia faba, Phaseolus vulgaris, and Cicer arietinum*) from which they were isolated to ensure the purity of these isolates, according to the method described by Somasegaran and Hoben [17]; the sterilized seeds with 70% Ethyl Alcohol and after washed with sterile water, are germinated in water agar and inoculated after one week with 0.5ml of the bacterial suspension (should contain between 10^6 and 10^8 CFU/ml) by sterile syringe to over the roots of the seedlings, then transferred to the surface of Nitrogen Free (NF) agar medium in Petri dishes. Incubated them in the growth room by covering the roots with filter paper (to prevent the light from reaching it) under successive light and dark conditions; 8 hours dark / 16 hours light at a 25˚C, and examined periodically to observe the formation of nodules on them.

Molecular tests:

DNA extraction: The process of extracting DNA from the bacterial cells suspended in Yeast Extract Mannitol (YEM) broth, was carried out using the extraction kit equipment from the company PreMix, and according to the steps provided by the supplying company: The precipitated bacteria was transferred to an Eppendorf tube, 200 µl of Lysozyme enzyme (0.8 mg/20 ml) were added to it, and incubated at 37°C for 30 minutes, then 20 µl of Protease K enzyme was added to it and incubated at 60°C for 10 minutes, 200 µl of GB buffer prepared with the kit were added and incubated at 70°C, 200 µl of absolute ethanol was added to it. This mixture was transferred to the 2 ml collection tubes containing the GD filter column for DNA purification and equipped with the kit, centrifuged at a speed of 16,000 rpm for 30 seconds, then 600 µl of W1 Buffer washing solution prepared with the kit was added to the DNA and centrifuged at the same speed and time, the tubes returned to a centrifuge a second time to dry the columns at 16,000 rpm for 3 min. The DNA with 100 µl of the kit Elution Buffer was left for 3 minutes and then, refrigerated centrifuge at 16,000 rpm for 30 seconds to dissolve the DNA and kept at -20°C until later use.

Electrophoresis for DNA: We performed the electrophoresis of the DNA samples according to the following steps [18]: Adding the running buffer to the flask containing 1.5% agarose, then adding 0.5µg/ml EtBr and leaving the agarose to cool. Place comb into the gel mold to create the wells and removed it after hardening the agarose. Added a loading dye to the DNA samples to placed individually in gel wells, and added running buffer to cover the surface of the gel and put the lid of the gel box, then connected it to the power source with 5 vol/cm^2 for 1:15 hour. After completing the electrophoresis, expose the bands of DNA on the gel to UV

light at 336 nm by UV-Transillumination. Take a picture of these bands by the Gel Documentation apparatus.

PCR Reaction:

Molecular diagnostics of rhizobium based on 16S rRNA:

Primers used in the polymerization reaction: DNA extracted from the seven bacterial species was used as a template for 16S rRNA gene amplification using a primer as shown in Table 1, [19].

Table 1: Primer for 16S rRNA.

Primer	Sequence	Tm	GC	Size of product
Forward	5'- AGAGTTTGATCCTGGCTCAG- 3'	54.3°C	50.0%	1250 base pair
Reverse	5'- GGTTACCTTGTTACGACTT- 3'	49.4°C	42.1%	

The nitrogenous bases present in the genetic material of the rhizobium bacteria were detected by adding 1.5 µl (from the concentration of 100 ng/ µl) of template DNA and 1 µl of Forward primer and another 1 µl of Reverse primers (from the concentration of 10 pmol/ µl) to the Master-mix contents in special tubes (Table 2), according to the method of Edwardes *et al*., [20].

Table 2: The mixture of a specific interaction of diagnosis of the 16S rRNA gene.

.Components		Volume
Taq PCR PreMix		5µl
Primers	Forward	1 µl (10 pmol/µl)
	Reverse	1 µl (10 pmol/µl)
DNA		1.5µl
D.W.		16.5 µl
Volume (Final)		25µl

Then the tubes were inserted into a Thermocycler apparatus at the optimal conditions indicated in Table 3.

The DNA ladder (100-10000 bp) was used as a marker, and DNA samples were electrophoresed on 2% agarose gel for 1:30 hour, and 5vol/cm^2, bands were imaged with a digital camera as described above.

Table 3: The optimum condition for PCR reaction.

No.	Phase	Tm.	T.	Cycle No.
1	Initial Denaturation	95°C	5 min.	1
2	Denaturation -2	95°C	45sec	35
3	Annealing	52°C	1 min	
4	Extension-1	72°C	1 min	
5	Extension -2	72°C	7 min.	1

Gel Extraction DNA Protocol:

The protocol of Gel Extraction DNA is as the following [21]:

In a 1.5 ml Microcentrifuge Tube containing 300 mg of gel, 500 µl of DF Buffer was added and mixed well and incubated at 55-60˚C, and 800 µl of the mixture was transferred to a DF Column in a 2 ml Collection Tube and centrifugal at 16,000 rpm for 30 seconds, and 600 µl of wash buffer was added and centrifuged at 16,000 rpm for 30 sec, then the floating

part has been removed and centrifuged at 16,000 rpm for three minutes to dry the contents of the column. Add 20 µl of Ellution buffer to the column and centrifugation for two minutes at 16,000 rpm to dissolve the DNA.

Sequencing and Sequence Alignment:

To identify the DNA sequence in the isolates understudy, the DNA samples extracted from the gel by the analysis kit supplied by Geneaid company were sent to the Genome Center in Germany to perform DNA sequencing technology and determine the sequence of nitrogenous bases in the pure DNA segments of the 16S rRNA gene, which were read based on a device 3130 Genetic analysis supplied by the Japanese company/Hitachi and inserted into the DNA/Blast analysis program through the electronic link http://blast.ncbi.nlm.nib.gov/Blast.cgi, to determine the extent to which the special sequences of this gene match the strains documented in the National Center Biotechnology Information (NCBI).

The phylogenetic tree was prepared using the sequence of the nitrogenous bases of the 16S rRNA gene for the local isolates understudy that were obtained from the results of DNA sequencing and compared with the sequences of this gene in bacteria documented in the National Center for Biotechnology Information as the most closely related to the isolates of this study, using MEGA 6.0.

Results and Discussion:

Bacterial Isolation and Diagnosis:

All isolates growth on YEM solid medium appeared, creamy white, circular in shape, with a high surface and mucous texture, Gram-negative rod, and devoid of spores, these results are consistent with Vincent's description[22]. It's evident from Table 4, that the seven isolates were positive for the following (Indole, Catalase, Motility, ONPG, and can ferment the following of these sugars: Glucose, Mannitol, Galactose, mannose, and Xylose), and negative (Methyl red, Voges Proskauer, Citrate test, Urease and Gelatinase, all these results were identical to what was mentioned by Shahzad *et al.*, [12] and Al-Shakarchi [23].

Table 4: Biochemical Characteristics of Rhizoial Isolates from Differentes Kindes of Legume Plants.

Tests		Number of Isolates						
		1	**2**	**3**	**4**	**5**	**6**	**7**
Catalase		+	+	+	+	+	+	+
IMViC	I	+	+	+	+	+	+	+
	M	-	-	-	-	-	-	-
	V	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-
Urease		-	-	-	-	-	-	-
Gelatinase		-	-	-	-	-	-	-
Motility		+	+	+	+	+	+	+
ONPG		+	+	+	+	+	+	+
Glucose		+	+	+	+	+	+	+
Mannitol		+	+	+	+	+	+	+
Galactose		+	+	+	+	+	+	+
Mannose		+	+	+	+	+	+	+
Xylose		+	+	+	+	+	+	+
BTB		Yellow	Yellow	yellow	Yellow	Yellow	Yellow	Yellow
CR		Pink	Pink	Pink	Pink	Pink	Pink	Pink

Number of Isolates from:1; *Trigonella foenum-graecum*,2; *Medicago sativa*,3; *Lens culinaris*,4; *Vigna mugo*,5; *Vicia faba*,6; *Phaseolus vulgaris*,7;*Cicer arietinum*, (+);Positive test(-); Negative test, I:Indole, M:Methyl Red, V:Voges Proskauer, C:Citrate test.
Each *Rhizobium* inoculated with a leguminous plant isolated from its root nodules. All isolates succeeded in infecting their leguminous hosts and forming root nodules on them in different shapes according to the type of leguminous plant (Figure 1) and can be seen three weeks after inoculation, are relatively small sizes, they grow over time to start fixing atmospheric nitrogen to ammonia, as in the recent study completed by Khaled *et al*., [24].

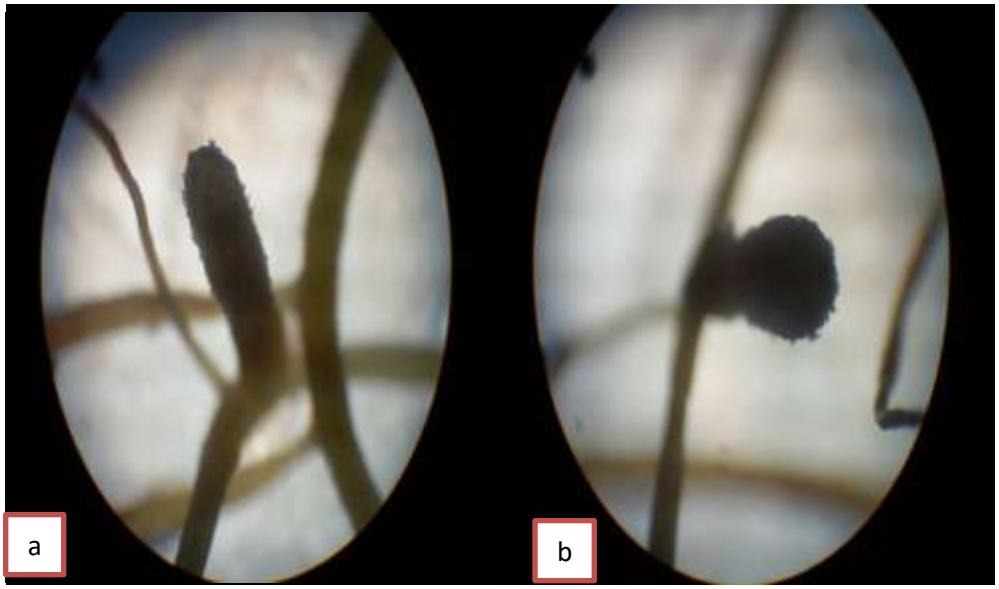

Figure 1: Root nodules formed on seedlings of *Trigonella foenum-graecum* (a) and *Cicer arietinum* (b) *in vitro* using the 40X lens of the compound microscope.

Characterization of isolates of *Rhizobium* bacteria depending on the 16s rRNA:
1-Isolation of genomic DNA :

After electrophoresis was carried out using 1.5% agarose gel of seven samples of genomic DNA of seven local isolates of the *Rhizobium* genus understudy, the gel was shown to ultraviolet radiation at wavelength 336nm in the UV trans illuminator, and seven clear DNA bands with large sizes because they moved for short distances as well as they spread over equal distances, and this indicates that they are of similar sizes [25].

2- PCR of genomic DNA chain:

The specific PCR of genomic DNA samples extracted from different isolates of rhizobium bacteria was carried out with the use of a specialized primer (Figure 2), which indicates that there are seven amplified bands for a specific location in the genomic DNA samples, whose size stabilized at 1250bp in terms of its measurement in Ladder DNA. This result can be explained by the presence of a common similarity in the sequence of nitrogenous bases present in the genome of these bacterial groups and complementary to the nitrogenous bases in the specialized primers, which led to the completion of the specialized replication reaction and the production of DNA bands of large sizes and at one level.

Several studies indicated that the size of the amplified bands reached to1200 bp and 1500 bp of genomic DNA isolated from different species of *Rhizobium* [24, 25, 26], this slight difference in size between this study and the results of previous studies may inevitably be due to the existence of large differences between isolates and indicates that the soil may contain groups of highly diverse strains that led to the emergence of this variation [26,27].

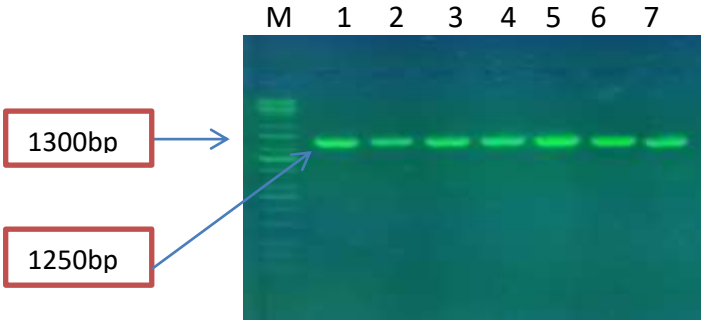

Figure 2: PCR product of pure genomic DNA samples with the use of a primer of 16S rRNA gene was electrophoresis on 2% agarose at 5 volt/cm^2 for 1:30 hour. M: DNA ladder, lane 1-7: band size 1250bp.

Sequencing of PCR Products:

The sequencing of the 16S rRNA gene (1250 bp), showed that the isolated bacteria were closely related to the *Sinorhizobium meliloti* strain S8, *Bradyrhizobium elkanii* strain CUR, *Rhizobium leguminosarum* biovar *viciae* strain ICMP 5943, *Rhizobium leguminosarum* bv. *phaseoli* strain SWD27-2 and *Mesorhizobium ciceri* strain 11683778, which are registered in Gene Bank with number KU230302.1, MK228880.1, MK382447.1, KJ634556.1, and KF981519.1, and the similarity ratio reached to 97%, 98%, 96%, 97%, 98%, and 98%, respectively, as shown in Figure 3 and Table 5, therefore [28] indicated that if the similarity ratio is 95 to 99%, this explained that the studied isolate belongs to a similar species to it in the gene bank, while if the similarity ratio is > 99%, it refers to the same bacteria. Therefore, the difference in the sequence of some nitrogenous bases may be due to the Substation or Transition mutations [29].

Sinorhizobium meliloti strain S8 16S rRNA gene, partial sequence

Sequence ID: KU230302.1 /Similarity : 273/281 (97%)

```
Query  1
GGGCGGCGGCTTACACATGCAAGTCGAGCGCCCCGCAAGGGGAGCGGCAGACGGGTGAGT
60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6
GGGCGGCGGCTTACACATGCAAGTCGAGCGCCCCGCAAGGGGAGCGGCAGACGGGTGAGT
65
Query  61
AACGCGTGGGAATCTACCCATCCCTACGGAACAACTCCGGGAAACTGGAGCTAATACCGT
120
|||||||||||||||||| |||| |||||||| || | | |||||||| | ||||||||||
Sbjct  66
AACGCGTGGGAATCTACCCTTTTCTACGGAATAACGCAGGGAAACTTGTGCTAATACCGT
125

Query  121
ATGAGCCCTTCGGGGGAAAGATTTATCGGGAAAGGATGAGCCCGCGTTGGATTAGCTAGT
180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  126
ATGAGCCCTTCGGGGGAAAGATTTATCGGGAAAGGATGAGCCCGCGTTGGATTAGCTAGT
185
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Query  181
TGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCC
240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  186
TGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCC
245
Query  241  ACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCA  281
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  246  ACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCA  286
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Figure 3: Results of comparison of nitrogenous base sequences between local isolate No. 1 and the *Sinorhizobium meliloti* strain S8 recorded in the GenBank No. KU230302.1**.**

Table 5: Sequence Identities (%) and Accession numbers for 16S rRNA gene of *Rhizobium* isolates.

Isolates No.	Identities (%)	DNA GeneBank Accession Numbers	16S rRNA Gene Identification
1	97	KU230302.1	*Sinorhizobium meliloti* strain S8
2	98	KU230302.1	*Sinorhizobium meliloti* strain S8
3*	-----	-----	-----
4	96	MK228880.1	*Bradyrhizobium elkanii* strain CUR_S25#2
5	97	MK382447.1	*Rhizobium leguminosarum* biovar *viciae* strain ICMP 5943
6	98	KJ634556.1	*Rhizobium leguminosarum* bv. *phaseoli* strain SWD27-2
7	98	KF981519.1	*Mesorhizobium ciceri* strain 11683778

* Isolation Number 3 occurred in which a mistake or contamination led to the failure of sequencing

These results show that the bacteria isolated in this study are the same as infecting their hosts of leguminous plants. The first and second isolates were diagnosed as *Sinorhizobium meliloti*, which is specialized in infecting *Trigonella foenum-graecum*[30] and *Medicago Sativa* [31], while *Bradyrhizobium elkanii* infect *Vigna mungo*[32], *Rhizobium leguminosarum* biovar *viciae* infect *Vicia faba* [33], *Rhizobium leguminosarum* bv. *phaseoli* infect *Phaseolus vulgaris* [34] and *Mesorhizobium cicero* infects *Cicer arietinum* [35].

The results of this study show that there is a difference in some nitrogenous bases between isolates of *Rhizobium* bacteria and similar to the standard strains in the Gene Bank at specific sites. When comparing the sequences of nitrogen bases of isolates of *Rhizobium* bacteria and the standard strain, this difference is considered as point mutations that occur in the sequence of nucleotides of the DNA of isolates of Rhizobium bacteria, represented by replacing a single nucleotide with another in the nitrogen base sequence, adding a base to the DNA sequence, or removing a base from the sequence. Our results can explain that the errors that occurred during DNA replication were caused by the addition of false codons or deletions from template codons[36], while the data provided by researchers [37] indicate that the incidence of mutations is relatively low, so the exchange of nucleotides takes place by recombination, and horizontal transfer of homologous DNA segments between closely related organisms is involved in genetic diversification because single nucleotide polymorphisms

(SNPs) are responsible for most of the DNA variations found in bacteria, there are no Calculate how much of this difference comes from mutation versus recombination [38].

Phylogenetic tree:

The phylogenetic tree is one of the genetic achievements to understand the genetic changes in bacteria that may occur during their evolution.

A phylogenetic tree from the isolates of the 16S rRNA gene is established (Figure 4), the results showed that the isolates under study were from different genera of Rhizobium bacteria, depending on the sequence that got from the sequencing analysis and to show their similarity with the sequencing of nitrogenous bases of the 16S rRNA gene with global standard isolates (Figure 5 and Table 6).

The sequence of isolates (1, 2, 4, 5, 6, 7) is shown within one major group except for the sequence of isolate (4) *Bradyrhizobium elkanii* 16S ribosomal RNA gene, which appeared as a single outgroup. In this context, Khalid *et al*., [24], mentioned that the bacterium *Bradyrhizobium japonicum* (USDA 6^T) appeared as an out-group among eight bacterial isolates that were isolated from the Peanut plant.

It is noted from these results, that the longest genetic distance is 0.0622 for *Bradyrhizobium elkanii* 16S ribosomal RNA gene isolated from *Vigna mungo* plant, while the shortest genetic distance is 0.0088 for *Sinorhizobium meliloti* 16S ribosomal RNA gene isolated from plants *Trigonella foenum-graecum* and *Medicago sativa*, this means the bacterial species that infects Alfalfa and Fenugreek plants have an equal genetic distance and are shorter than those distances between bacterial species isolated from the root nodules of different hosts [23].

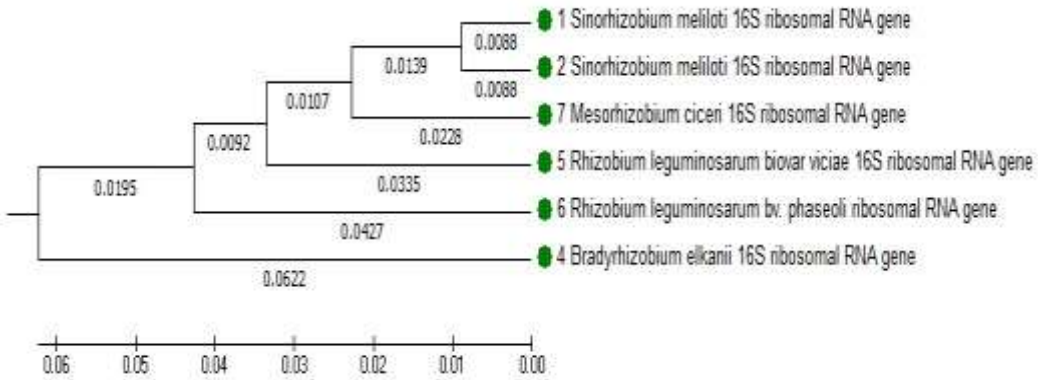

Figure 4: Phylogenetic tree based on the sequences of nitrogenous bases of the 16S rRNA gene of isolated *Rhizobium* strains, and recognized species of the genus *Rhizobium*.

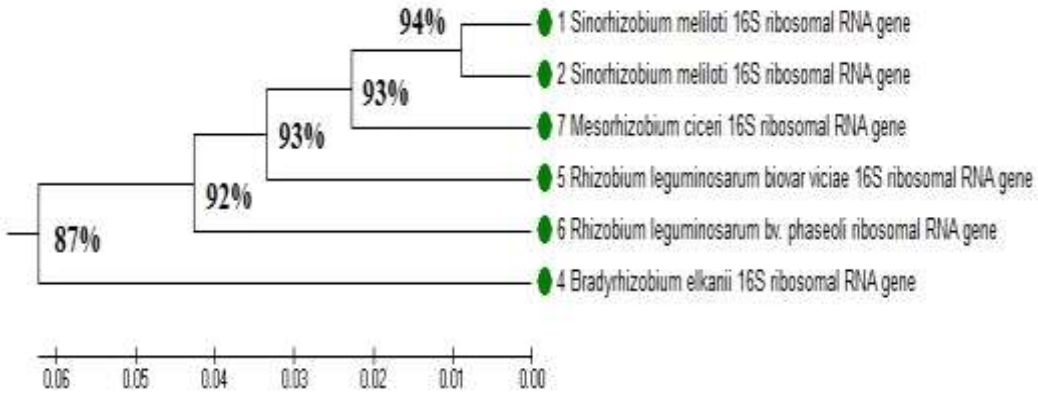

Figure 5: Phylogenetic trees to show the percentage of similarity of the studied rhizobia isolates to standard geneBank isolates.

Table 6: Matrix values of genetic distances between the different species of Rhizobia under study.

	1	2	3	4	5	6
1. 1 Sinorhizobium meliloti 16S ribosomal RNA gene						
2. 2 Sinorhizobium meliloti 16S ribosomal RNA gene	0.0177					
3. 4 Bradyrhizobium elkanii 16S ribosomal RNA gene	0.1442	0.1684				
4. 5 Rhizobium leguminosarum biovar viciae 16S ribosomal RNA gene	0.0540	0.0739	0.0827			
5. 6 Rhizobium leguminosarum bv. phaseoli ribosomal RNA gene	0.0725	0.0931	0.0931	0.1120		
6. 7 Mesorhizobium ciceri 16S ribosomal RNA gene	0.0358	0.0552	0.1338	0.0728	0.0639	

Conclusion:

At present, it is recommended to use polymorphic classification methods that include phenotypic and genetic characteristics as a tool for diagnosing and classifying *Rhizobium* bacteria, because it enables the researcher to validate the various characteristics of *Rhizobium* species, which indicates the possibility of identifying many new species from them. Therefore, the matching of phenotypic and genetic characteristics indicates that the isolated bacteria belong to different species of the genus *Rhizobium*, according to the leguminous plant from which it was isolated.

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