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GENETIC DIVERSITY AND PHYLOGENY OF RHIZOBIA ISOLATED FROM NODULES USING RFLP-PCR TECHNIQUE IN NINEVEH PROVINCE, IRAQ

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SUMMARY

Rhizobia are important bacteria, playing a vital role in atmospheric nitrogen fixation. In addition, it increases soil fertility, enhancing crop production and preserving the soil from pollution resulting from inorganic fertilizers' use. From 81 bacteria samples, nine isolates segregated from nine types of leguminous crops are economically valuable for cultivation within nine different locations in Nineveh Governorate, Iraq. The isolates underwent initial diagnosis by phenotypical and cultural characteristics and host specialization tests. Molecular characterization also ran on the selected bacterial isolates. Performing the *16S rRNA* region amplification applied forward primer 27F and a reverse primer 1541R using the polymerase chain reaction (PCR) technique. The results of restriction fragment length polymorphism (RFLP-PCR) employing three endonuclease enzymes showed that *Eco*RI had similar sequences among the isolates under study but significantly different for *Msp*I and *Hae*III. Based on the analysis of results using the statistical program MVSP version 3.22, the degree of similarity divided the isolates into three groups. Results revealed that there were genetic differences between the isolates and the suppressing enzymes, providing more accurate results in recognizing the similarity among the bacterial isolates as a result of the difference in the location and environmental conditions from which the bacteria were isolated, in addition to the different agricultural processes used for those regions.

Keywords: Leguminous crops, *rhizobium*, culture and biochemical tests, endonuclease enzymes, RFLP-PCR, *16S Rrna*

Key findings: The use of *16S rRNA*-RFLP technology enables the division of isolated bacteria by studying their genetic polymorphisms treated with restriction enzymes. Detecting three distinct groups from three different sections in Mosul City, Iraq, was possible. The differences were due to the influence of the environments and plants from which the bacteria were isolated and were evident using RFLP. It was possible to determine the phylogenetic tree that shows the degree of genetic affinity between the bacterial isolates.

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INTRODUCTION

In economic crop production, the importance of nitrogen fertilizers is increasing the products, and most countries have tended to search for inexpensive alternatives and less polluting the environment, especially nitrogen-fixing bacteria. These bacteria include the genera, i.e., *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Allorhizobium,* collectively called rhizobia and belonging to the family Rhizobiaceae that fix atmospheric nitrogen by forming nodules in the roots of leguminous crop plants (Pervin *et al*., 2017). Rhizobiaceae is Gram-negative, aerobic, and selectively organotrophic (Setu *et al*., 2019; Mansour *et al*., 2023; Smirnova *et al*., 2023). Bacillus, in shape, and non-spores forming, are usually found in soil individually or in pairs, and move with circumferential and polar flagella (Hisyan and Al-Shakarchi, 2021).

These bacteria grow as sticky, white colonies on solid media that contain yeast extract, mannitol sugar, and some inorganic salts. The sucrose served as an energy source, and urea, nitrates, and amino acids as nitrogen sources. The organism can also tolerate various degrees of salinity and pH, with the optimum temperature for their growth ranging between 28 °C–31 °C (Graham, 1992).

Rhizobia plays an imperative role in managing balanced agroecosystems due to its ability to form a symbiotic association with a wide range of leguminous plants, leading to biological nitrogen fixation (Orrell and Bennett, 2013). Some rhizobia strains have helped to enhance plant hormone production and mineral absorption and reduce the toxicity of heavy metals in soil, thus eventually promoting plant growth and development in contaminated soils. Modern agriculture focuses on using sustainable farming practices that are environment-friendly, efficient, and cost-effective for a less resource-consuming farming community (Karthik *et al*., 2017). The RFLP-PCR technique aided in classifying bacterial isolates and finding the level of similarity or difference between these bacterial strains belonging to one genus of different species of *Rhizobium*. Amplification of the *16S rRNA* gene (PCR/RFLP for 16S rDNA) began with four distinct RESTRACTION enzymes: *Msp*I, *Hinf*I, *Hha*I, and *Taq*I. The results showed varied profiles of the obtained fragments (Benselama *et al*., 2018).

The presented study aimed to: a) isolate the bacterial species belonging to the genus *Rhizobium* from some leguminous plants cultivated in various regions of Nineveh Governorate, Iraq, b) diagnose these isolated bacterial species using the phenotypic and biochemical characteristics via *16S rRNA* region, and c) determine the genetic polymorphism of these isolated bacterial species by restriction enzymes.

MATERIALS AND METHODS

Bacterial isolation

Eighty-one bacterial samples collected from the root nodules of nine different leguminous crop plants included alfalfa, clover, lentils, fenugreek, peas, chickpeas, beans, , and cowpea, grown within nine locations. The selection of nine bacterial isolates transpired in the crop season 2020 at the Nineveh Governorate, Iraq. The studies ran at the greenhouse of the Department of Life Sciences, University of Mosul, Mosul, Iraq, and at farms in the Shamsiyat area, the Western Yarmjeh region, and two locations each at the Ba'shiqah town, Sadaa, Bauezeh, and Rashidiya areas, Iraq.

The nodes formed on the roots of leguminous plants underwent small cuttings, then washed with tap water to remove soil residues, immersed in 70% ethyl alcohol for 3 min, washed with sterile distilled water three times, and transferred to a 3:1 v/v NaOCl solution water/sodium hypochlorite for 15 min, and finally washed with pure distilled water. The transfer of the specimen to a Petri dish

ensued with a sanitized filter paper to dry. Their next transfer was onto a Yeast Extract Mannitol Agar (YEMA) (yeast extract: 0.4, mannitol: 10, NaCl: 0.1, $MgSO4.7H_2O$: 0.2, K_2HPO4: 0.5, and Agar: 15) g/L, undergoing incubation at 28 °C ± 1 °C for 24 to 48 h to ensure the sterilization of the root nodes (Talaro and Talaro, 1999).

Later, the transfer of nodes continued into sterile tubes containing 2 mL liquid YEMB (yeast extract mannitol) and crushed using a sterile glass rod. The streaking method progressed, taking loopful amounts spread on a Petri dish containing YEMA medium, followed by inoculation at 28 °C ± 1 °C for 24 to 48 h (Marga *et al*., 2021). Examining morphological characteristics of the developing bacterial colonies used a compound light microscope with a 1000× magnification (Sarah *et al*., 2015). Nine different bacterial isolates selected from nine studied plants totaled 81 samples collected for studies on legume-rhizobium symbiosis, morphological, and cultural tests plus biochemical tests.

A specific family test conducted for each isolate took isolated colonies, inoculated them in 50 ml of liquid YEM medium using a conical flask for 48 h, and then centrifuged them to obtain a wave of bacteria at the concentration of 3×10^8 cells/ml. The roots of the growing plants inoculated on the nitrogen-free medium (NF) had a pH of 6.6 ± 0.2. A previous step, preparing the inoculated bacterial isolates, ensured the ability of isolated bacteria to form root nodes on its host plant (Singh *et al*., 2008).

Biochemical tests

The biochemical tests ran on isolated bacteria under study, i.e., gelatin liquefaction test, spread the fresh colonies of isolated bacteria on Kings Medium and BTB (bromothymol blue) medium containing 0.0025% BTB (Singh *et al*., 2008). The other tests conducted were the urease test (Deora and Singhal, 2010), the catalase test (Deka *et al.*, 2019), the citrate utilization test (Wadhwa *et al*., 2017), the cytochrome oxidase test (revealing the ability of the bacteria to produce the enzyme oxidase) (Wadhwa *et al*., 2017), and the Voges-Proskuar test (Cappuccino and Sherman, 1999). The methyl red test, i.e., inoculating a set of test tubes containing peptone, glucose, and phosphate water medium with new colonies of pure *Rhizobium* bacteria isolates, then incubated at 28 °C ± 1 °C for 48 h. A change in media color to red/pink indicates the ability of bacteria to ferment sugar, which reduces the pH to 4.5 (Benson, 2002). The Indol production test occurred according to Baron *et al*. (1997). The motility and production of H_2S hydrogen sulfide gas went on by the semi-solid SIM inoculation (Atlas *et al*., 1995).

Molecular diagnosis of *Rhizobium* based on *16S rRNA* region

Extraction of* Rhizobium *DNA

DNA extraction used the Geneaid Biotech Ltd Kit., to extract DNA from bacterial isolates belonging to the genus *Rhizobium*. DNA concentration and purity determination employed nanodrops.

Specific PCR reaction

Running the polymerase chain reaction comprised 27 universal primers Forward (AGAGTTTGATCCTGGCTCAG) and Reverse Primers 1541R (AAGGAGGTGATCCAGCCG CA) using Eppendorf tube 0.2 ml. Preparing the Master Reaction mixture for each PCR reaction contained 4 µl (100 nanograms) of template DNA, 1 µl (10 picomols) from each primer, 12.5 µl Master Mix solution, with the final result volume completed to 25 µl by adding distilled water (Heuer *et al*., 1997). The reaction tubes' placement into the thermocycler device performed the rejoinder using the specific program for each reaction (Table 1).

Three restriction enzymes (*Eco*RI, *Hae*III, and *Msp*I) helped to digest the PCR products to distinguish the cutting regions between bacterial isolates and to find out the similarity among them in the digestion regions of *16S rRNA* using Eppendorf tube containing 0.5 µl of restriction enzyme and 25 µl of PCR product, placed for several seconds in a centrifuge and incubated at 37 °C for 2 h, then

Table 1. PCR reaction conditions.

No.	Stage	Temperature °C	Time	Cycle number
1	Initial denaturation	95	6 min.	1
2	Denaturation	95	45 s	
3	Annealing	55	1 min.	35
4	Extension	72	1 min.	
5	Final extension	72	5 min.	1

ran in an agarose gel at 2% (Al-Shakarchi, 2013).

The* 16S rRNA *analysis

Using the Multivariate Statistical Package (MVSP) version 3.22 analyzed restriction patterns of RFLP-PCR. A simple matching coefficient formulated helped construct a similarity matrix, with the UPGMA algorithms performing hierarchical cluster analysis to construct a phylogenetic tree.

RESULTS AND DISCUSSION

Family specialization test for isolated *rhizobium* bacteria

Figure 1 shows the observed root hairs of leguminous plants that began to deform after three to five days of incubation, forming coil shapes known as the shepherd's stick, then producing and developing the primary and viable nodules after 10 to 21 days of infection. These results were consistent with the findings of Geurts and Basseling (2002) while studying the formation of mature nodules on the roots of inoculated plants. Results also revealed different shapes between the group of rhizobia bacteria obtained from the infected plants of alfalfa and fenugreek. The nodules were spherical and rectangular in form, regular or lobed on the roots and secondary branches of plants and those previously inoculated with *rhizobium* bacteria to obtain root nodes that confirm infection. These findings were also in analogy with the previous observations regarding the deformation of root hairs and the forms of root nodules in crop plants (Geurts and Basseling, 2002; Hisyan and Al-Shakarchi, 2021).

Figure 1. Stages of formation of root nodules. A) Deformities on the root hairs of plants, B) The primary nodule, and C, D) The nodules in the root plants.

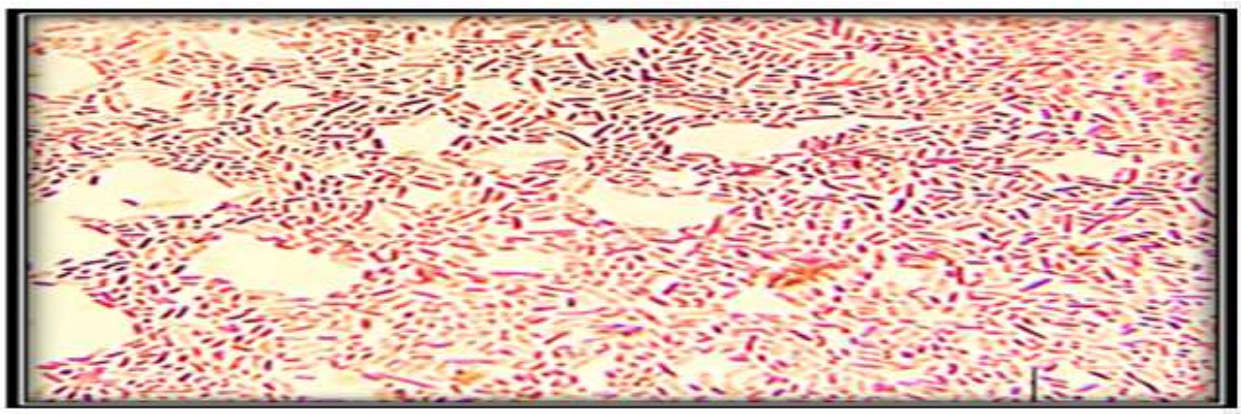

Figure 2. *Rhizobium* bacteria under microscope (100×).

Morphological and cultural characters for isolated rhizobial bacteria

Root bacteria colonies growing on YEMA medium appeared with various characters from cream to ivory, with smooth rounded edges and mucoid (Figure 2). The presented findings also showed colonies recorded with more extracellular polysaccharides in a carbon-rich medium within a few days, causing precipitation of large amounts of polysaccharides on the cover of the Petri dishes. Colonies that appeared were gram-negative and rod-shaped. Such results agree with the previous studies on rhizobia characteristics of six strains isolated from alfalfa (Hussain *et al*., 2002). Likewise, findings corresponded with those after isolating and studying 10 varieties of the rhizobia bacteria in lentil plant that grows in different locations in Punjab Province, Pakistan (Sajjad *et al*., 2008).

Biochemical characterization

Culturing the bacterial isolates on a medium (SIM) by the stabbing method showed that the bacteria proliferated throughout the culture medium, signifying the ability of the bacteria to move. The results also confirmed the bacteria's ability to produce the enzyme *catalase*, which decomposes H_2O_2 and releases oxygen. These results were consistent with previous findings while studying *Mesorhizobium* isolates secluded from the nodules of the chickpea plants (Wadhwa *et al*., 2017). The appearance of the filter paper, wetted with drops of the oxidase reagent in violet color after immersing it in the liquid culture, also indicates the ability of the bacteria to produce cytochrome oxidase.

The results were negative for the Voges-Proskauer tests, citrate consumption, gelatinase enzyme production, and H_2S production. These results aligned with the findings of Deka and Azad (2006). The tests for the production of indole and urease were positive, as well as, the production of acid from the sugars (glucose, rhamnose, maltose, mannose, and lactose). With the ability of these bacteria to ferment the sugars and lower the pH of the medium, the color of the medium changed from yellow to red. The presented outcomes further agree with the study of Deka and Azad (2006). Elzanaty *et al*. (2015) studied pea plants in different regions of Egypt and observed urease production. The findings were also in close conformity with bacteria characterization (*Rhizobium*) in chickpea (*Cicer arietinum*) (Roychowdhury *et al*., 2015) and classification of rhizobia from rhizosphere and root nodules of cowpea, elephant, and lablab plants (Hamza and Alebejo, 2017).

In Figure 3, the bacteria grown on King Media resulted fluorescent by exposing to ultraviolet rays using a UV light transilluminator at 320 nm. All the bacterial isolates grown on the BTB medium turned from dark green to yellow upon growth, indicating that the isolates were acid-producing and rapidly spreading. These results were in analogy with the findings of Koskey *et al*. (2018) while studying the *Rhizobium* strains

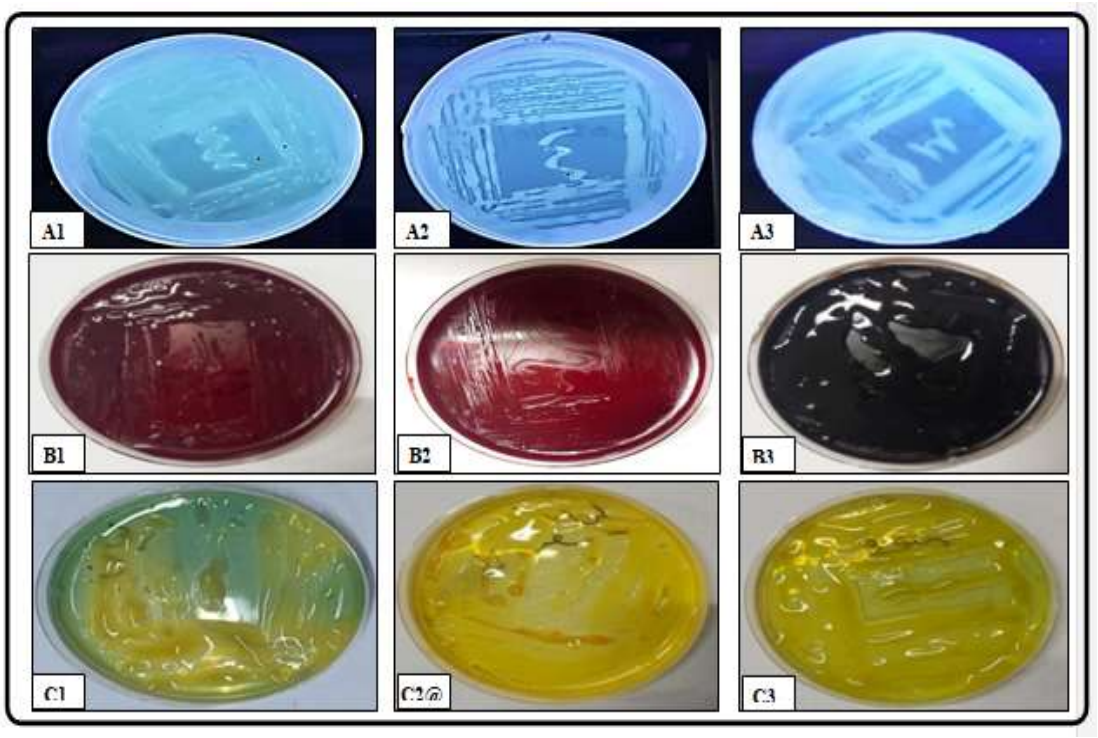

Figure 3. Colonies of rhizobia bacterial isolates grown on (A1-3) YEMA media, (B1-3) King media, and (C1-3) BTB medium.

isolated from the root nodes of the *Vigna radiata* plant. Study results also showed that all the rhizobia isolates, when grown on MacConkey Agar medium, occurred positive for this test and in agreement with the previous studies (Kumari *et al*., 2017; Al-Mujahidy *et al*., 2013).

Genomic DNA extraction

The DNA extraction and detection of nine selected bacterial isolates ran in agarose gel electrophoresis. The appeared bands were in one size, indicating that these isolates belong to the same genus. The DNA purity measured used a Nanodrop at 1.7–1.8.

Specific PCR

The results of the PCR using universal primers showed the presence of nine bands ranging between 1500–1517 bp in size (Figure 4). The appearance of these DNA bands resulted from similar sequences in the purified genomic DNA of the local isolates, which complemented the nitrogenous bases in the primers and completed the reaction. Thus, the presence of matching size of DNA bands means that all local isolates belong to the same species, and the morphological and biochemical tests carried out in this study also support these results. Research outcomes were consistent with the findings in terms of *16S rRNA* gene size of 1500 pb (Koskey *et al*., 2018; Sijilmassi *et al*., 2021).

RFLP-PCR

Digestion of PCR products occurred with the restriction enzymes, i.e., *Eco*RI, *Hae*III, and *Msp*I. These results were consistent with the previous study isolating 27 bacterial isolates from *Vicia*, *Lathyrus,* and *Pisum* from different agroecological regions in Middle and Southern Italy using RFLP-PCR of the 16S rDNA region with four restrictions enzymes, showing the studied isolates had the same band size (1532 bp) (Moschetti *et al*., 2005). The results of

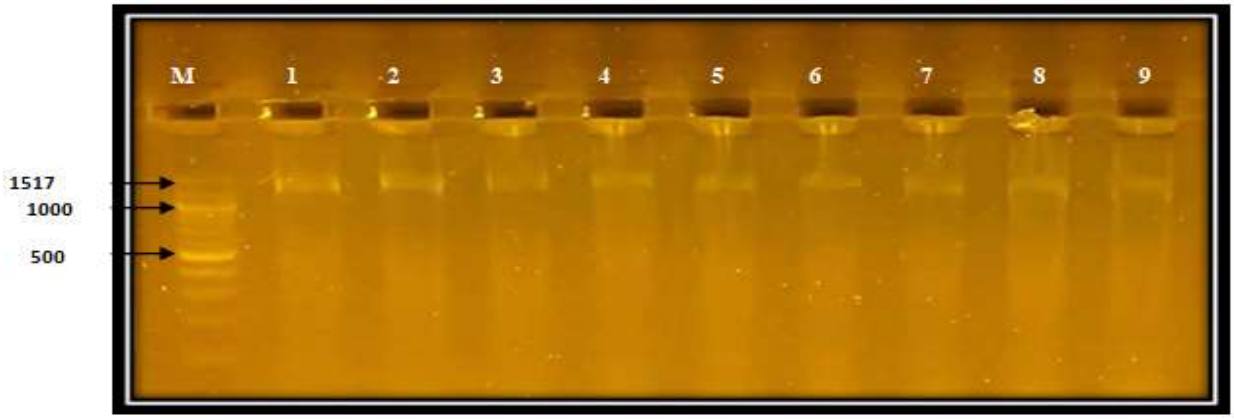

Figure 4. The PCR product of *Rhizobium* DNA samples shows the *16S rRNA* region and the reaction product of 1500–1517 bp.

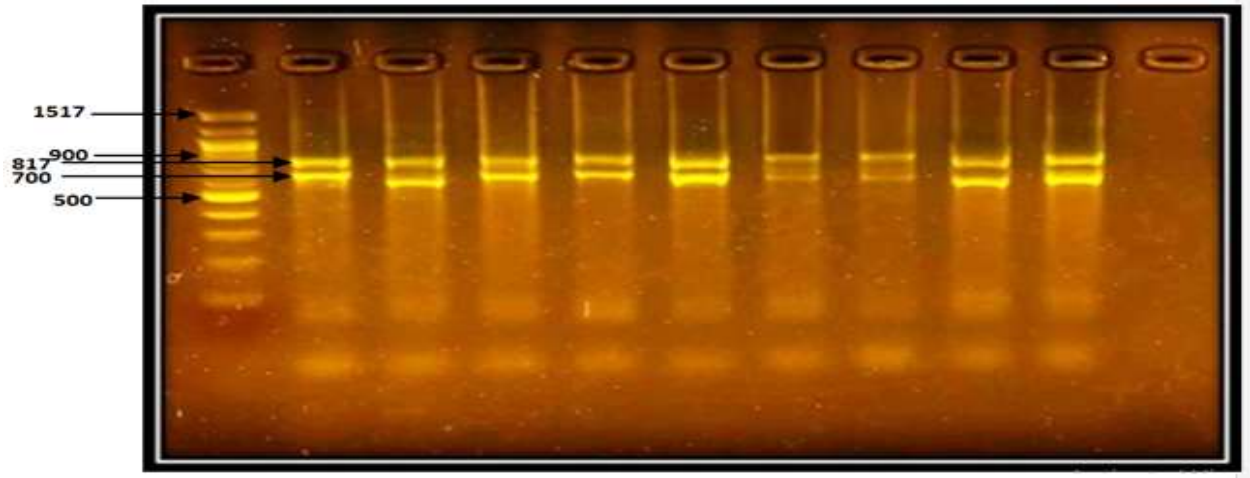

Figure 5. The PCR products of *Rhizobium* DNA samples treated by the restriction enzyme ***EcoR*I.**

enzyme digestion with *EcoR*I showed two bands with similar sizes (700 and 900 pb) for all *Rhizobium* isolates (Figure 5). These results agree with the previous study on the variation in size and number of bands produced using the *EcoR*I enzyme on six types of *Rhizobium* bacteria isolated from different regions in Mosul city (Al-Shakarchi, 2013).

The variability in the number and size of DNA bundles generated by electrophoresis of PCR products using the *Hae*III restriction enzyme, indicated that the first isolate showed four bands ranging from 200 to 600 bp (Figure 6). The second isolate showed four bands (200, 300, 350 and 400 bp), the third isolate also with four bands of DNA (200, 470, 550, and 600 bp), and the fourth has three bands of DNA (400 bp). The fifth isolate contains five DNA bands (150, 200, 250, 400, and 500 bp), and the sixth has five (200, 240, 300, 400, and 450 bp). However, a similarity emerged in the size of the bands produced in isolates seven, eight, and nine, which resulted in five bands (150, 200, 300, 350, and 400 bp). These results aligned with earlier findings where using RFLP-PCR identified *Rhizobium* by three restriction enzymes (Koziel *et al*., 2022).

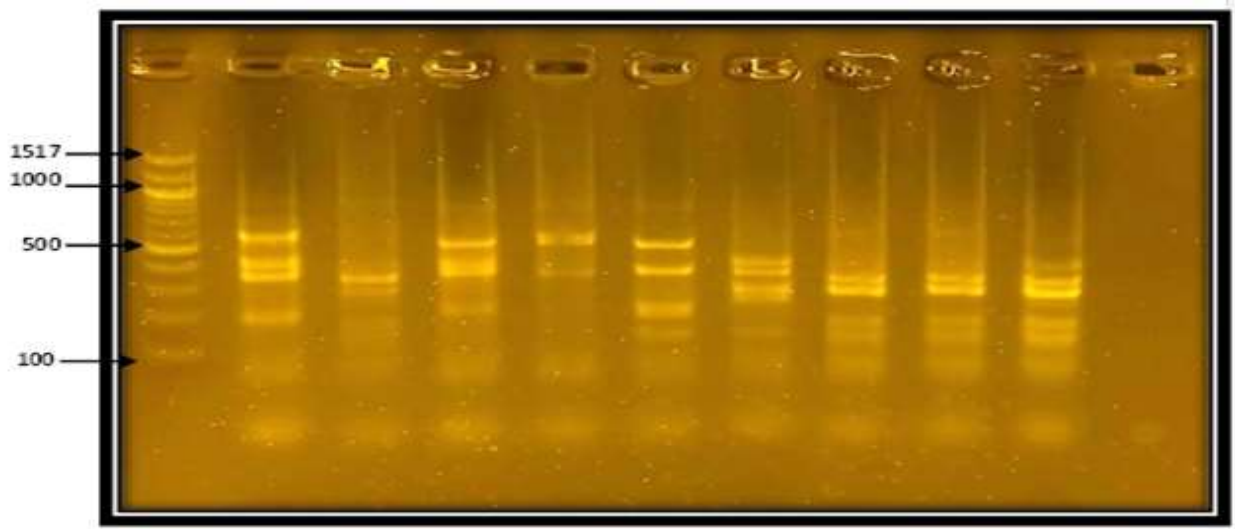

Figure 6. The PCR products of *Rhizobium* DNA samples treated by the restriction enzyme *Hae*III.

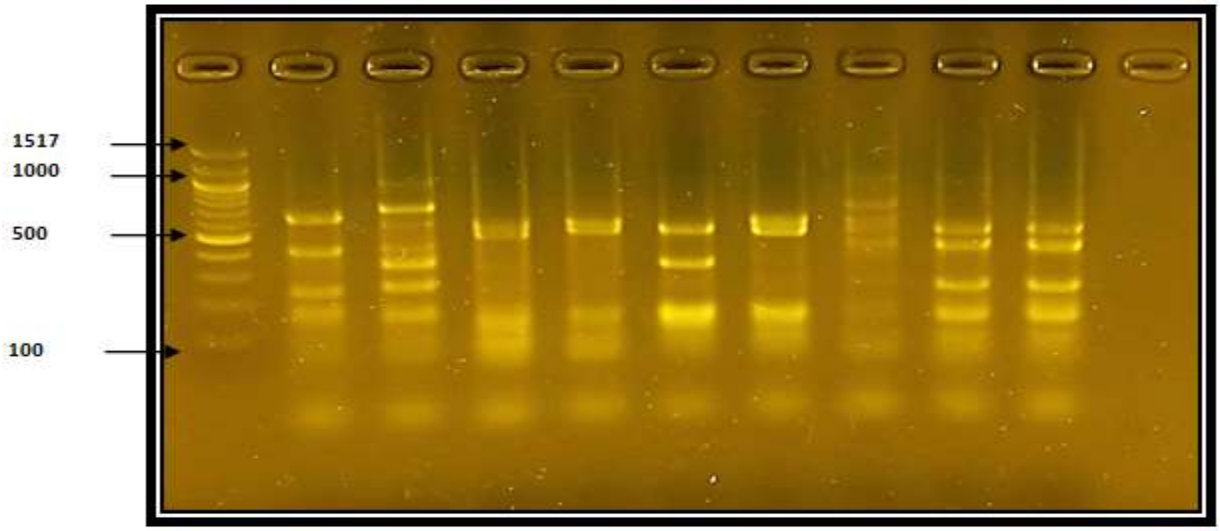

Figure 7. The PCR products of *Rhizobium* DNA samples treated by the restriction enzyme M*sp*I.

The results of the RFLP-PCR using the restriction enzyme *MspI* showed that the electrophoresis of the PCR products resulted in diversity in the number and size of the DNA bands. The first isolate had four bands (700, 400, 250, and 180 bp), while the second isolate showed seven bands of DNA (1000, 800, 600, 450, 350, 250, and 180 bp). Concerning third, fourth, and sixth isolates, more DNA bands appeared resulting from their cleavage by *Msp*I restriction enzyme and were 600, 500, 180, and 100 bp. The fifth isolate also contained band of 600, 400, 200, and 180 bp, but in the seventh isolate, resulting DNA bands varied with seven (800, 700, 600, 500, 300, 200, and 100 bp). The eighth and ninth isolates matched with equal sizes of DNA bands (600, 450, 250, 200, and 100 bp). (Figure 7). These results were similar with previous observations that amplification of a 16S rDNA region from 18 field isolates and six standard strains of *bradyrhizobia* using RFLP PCR by *Msp*I endonucleases, *Hinf*I, *Cfo*I, *Nde*II, *Rsa*I, and *Dde*I, produced more distinct

isoforms than others, with others obtained from *Msp*I, *Hinf*I, and *Cfo*I (Sikora and Redzepovic, 2003).

The presented study disclosed that amplification of the 16S rDNA cutting regions, through which distinguishing the types of bacterial isolates came about by analyzing the bands resulting from the cuts with *Eco*RI, *Hae*III, and *Msp*I enzymes, showed discrimination among the bacterial isolates under study due to the variation in the bacterial sampling regions and the different species. The bacteria analysis within the study used the MVSP ver. 3.22 analysis program, whereas the tree showed genetic differences among the bacterial isolates. By conducting the process of analyzing the nitrogen base sequences of the isolates under study, it was possible to make a matching process of 100% identical from the results obtained and the sequences of the standard strains in NCBI (Alhayale and Al-Shakarchi, 2021; Hisyan and Al-Shakarchi, 2021).

The genetic tree indicates RFLP-PCR shows genetic polymorphisms among the isolates treated with the three restriction enzymes (Figure 8). The first group includes isolates 1 and 2, having 52% similarity, while the second group consists of isolates 3, 4, and 5 with two groups. The first one (4,5) has similarities of 85.5%, with 89% similarity to isolate 3. The last isolates, 6, 7, 8, and 9, have the widest similarity of 64%. However, isolates 7 and 8 were 100% similar. Dividing the bacterial isolates is also possible, sharing similar results in a previous study that the rapid diagnosis of rhizobia using restriction segment length variation (RFLP) analysis of PCR amplified products of its *16S rRNA* genes and the group of 48 strains of rhizobia bacteria represented by eight types of several plant hosts (Laguerre *et al*., 1994). The comparison of *16S rRNA* sequences allows differentiation between *Rhizobium* species when studying the RFLP values of *16S rRNA* genes amplified to diagnose *Rhizobium* species (Vinuesa *et al*., 1998). Also, the estimated differences in *16S rRNA* genes could result from using restriction fragment length polymorphism (RFLP) analysis after being amplified in the process of differentiating species of laboratory bacteria (Al-Shakarchi, 2013).

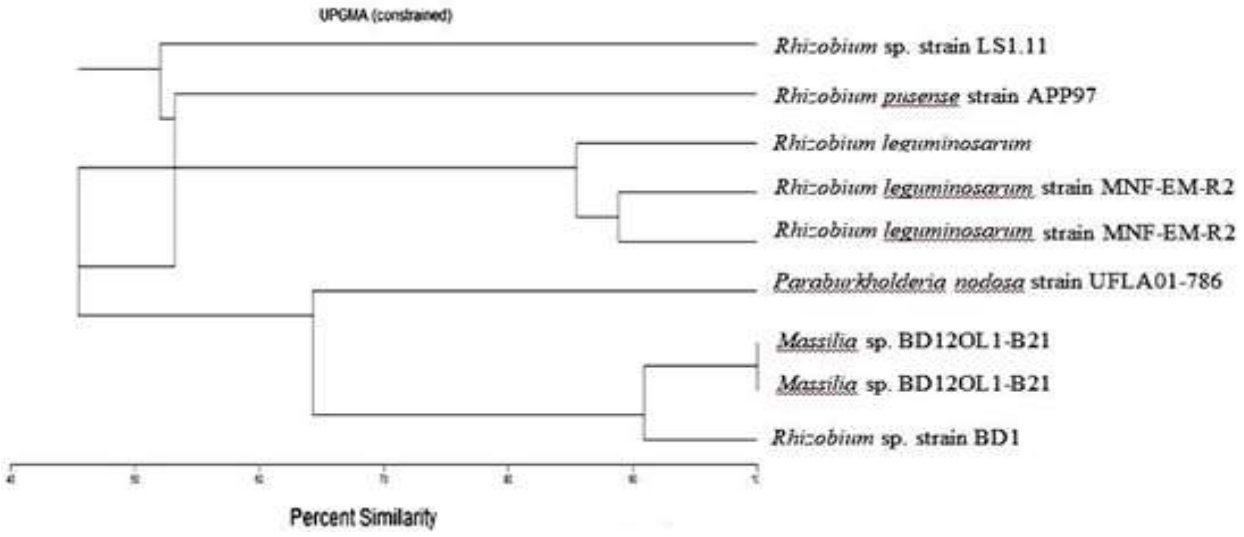

Figure 8. The phylogenetic tree shows the genetic affinity between isolates based on RFLP patterns for *16s rRNA* gene polymerase chain reaction amplification. Cluster analysis was performed using the percent similarity method between the estimated genetic distances from fragments shared by all bacterial isolates for three groups, developing in Nineveh Governorate, according to RFLP patterns. Isolates within each group share the same RFLP patterns when digested with *Eco*RI, *Hae*III, and *Msp*I enzymes**.**

The comparison of genomic diversity among bacterial isolates using the PCR-RFLP technique for *16S rRNA* has high discriminatory power because it generates specific patterns that allow the study of precise differentiation, with a greater diversity found in the studied bacterial isolates proving this. Additionally, the genetic diversity was much higher for the strains from the temperate region than those in the cold ones, as the low temperature harms the genetic diversity and bacterial strains associated with leguminous plants. In addition to the geographical areas and environmental conditions, the results indicated the distinct grouping of the vast majority of strains according to the geographical regions to which they belong. Researchers have noted that environmental factors influence genetic diversity, and previous studies revealed that variation in the genetic structure of a group of *R. leguminosarum* sv. emerged from a difference in soil pH and geographic location (Van-Cauwenberghe *et al.,* 2014). Higher genetic diversity for *R. leguminosarum* sv. affected the soil diversity from where the isolates came from, suggesting that long-term pea (*Pisum sativum*) cultivation could positively alter soil bacterial diversity (Palmer and Young, 2000). It indicates that root diversity could gain influence from variations among various agriculture systems.

Low temperatures adversely impact genetic diversity and lineage structure associated with alfalfa plants from the subarctic region. However, ruling out other factors is improbable, such as geographic distance and local environmental conditions, as these may also influence the genetic diversity of bacterial strains. Previous studies suggested that environmental factors also influence the variety and composition of root bacterial groups (Koziel *et al*., 2022). The RFLP-PCR is an essential technique with a high discriminatory ability among the bacterial species used to diagnose and identify bacteria at the species level and not only at the genus level, as in usual diagnostic tests. It aids in diagnosing and identifying bacteria at the species and genus levels, which include phenotypic, sensitivity, and antibiotic resistance tests, earlier conducted on 15 types of bacteria *Rhizobium* and *Bradyrhizobium* (Abdel-lateif and Abd El-ghany, 2023).

CONCLUSIONS

Results revealed distinction among the bacterial isolates under study by using the endonuclease enzymes, *Hae*III and *Msp*I, for the *16S rRNA* gene amplified by RFLP-PCR technology, while the enzyme *Eco*RI produced less discriminated bands among the bacterial isolates. Vast differences occurred in the sizes and numbers of bands among the bacterial isolates, in addition to the differences in family specialization in nodule formation on the roots of the studied plants, showing their appearance as three groups in the phylogenetic tree.

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