Available online at www.garmian.edu.krd

Passer Journal

Passer 5 (Issue 2) (2023) 231-236

http://passer.garmian.edu.krd/

Molecular Identification and Phylogeny of Endophytic Bacterial Communities Isolated from Some Legumes Using 16SrDNA

Mohammed Amin Azeez Issa[1*], Shaymaa Hadi Ali[1]

[1]*Department of Biology, College of Science, University of Duhok, Duhok, Kurdistan Region, Iraq.*

Received 10 March 2023; revised 19 April 2023;
accepted 2 May 2023; available online 16 May 2023

DOI: 10.24271/PSR.2023.388837.1279

ABSTRACT

Bacterial endophytes are host-beneficial microbial symbionts inhabiting different plant tissues without causing damage to the host plant. To our knowledge, no previous data was available regarding the endophytic bacterial population colonizing root nodules of plant legumes in this region. Therefore, this study was conducted to identify and assess the biodiversity of the bacterial endophytes of four different legumes species of the Fabaceae family, including Chickpea, Faba bean, Common bean, and Lentil in Duhok province, using 16S rDNA sequencing. The results revealed that 69 different pure culture colonies were isolated based on the phenotypic characteristics of endophytes on YEM medium and Gram staining. Based on 16S rDNA sequencing, seventeen bacterial species have been identified, with the sequence identities ranging from 98.41-100%. They belonged to nine different bacterial genera. The identified bacterial genera were *Pantoea* sp. (35%), *Enterobacter* sp. (26%), *Clostridium* sp. (15%), *Kosakonia* sp. (9%), *Pseudomonas* sp. (4%), *Curtobacterium* sp. (4%), *Erwinia* sp. (3%), *Salmonella* sp. (3%), and *Rahnella* sp. (1%). These bacterial genera belonged to four Phyla, including *Pseudomonadota*, *Bacillota*, *Proteobacteria*, and *Actinobacteria*. The antibacterial sensitivity against six various antibiotics by disc diffusion method showed different resistant patterns (bacitracin 78%, amoxicillin 56%, rifampin 34%, erythromycin 11%, chloramphenicol and tetracycline 6%), respectively. The phylogenetic analysis clustered the identified bacterial endophytes into three major clades and 11 different sub-clusters (C1 to C11), with a high degree of similarity amongst bacteria belonging to the same species. In conclusion, the population densities, species richness, and frequency of isolated endophytes showed variations based on geographical locations and the type of plant legumes. Finally, the results of the current investigation might add significant knowledge regarding endophytic bacterial populations colonizing different plant legumes in this region.

Keywords: Endophytic Bacteria, 16s rDNA, PCR, Phylogenic analysis, Plant legume.

1. Introduction

Bacterial endophytes are symbiont microbes that live inside the host plant tissues and parts without causing any sign of disease[1]. The host plants in this symbiotic relationship could benefit from these endophytic bacteria as these natural symbionts produce a variety of beneficial compounds to improve plant growth, maximize crop productivity, and reduce the environmental impacts of agriculture[2].

Various microbial genera live endophytically in the roots and nodules of both legumes and non-legumes that are grown all over the world. Members of *Streptomyces*, *Azocareus*, *Serratia*, *Gluconobacter*, *Stenophomonas*, *Bacillus*, *Pseudomonas*, *Paenibacillus*, and *Enterobacter*, which belong to the three bacterial Phyla, including *Actinobacteria*, *Proteobacteria*, and *Firmicutes*, found as endophytes[3].

Legume plants play significant roles in agriculture and cropping systems, not only for their commercial value as food in providing vital nutrients such as protein, fiber, folate, vitamins, iron, phosphorus, calcium, potassium, and zinc, but also as a green manuring crop in improving soil fertility and crop yields by direct nitrogen transfer as well as residual fixed nitrogen through the symbiotic association with microorganisms and enhancement of other soil microbial activity[21]. Water availability is significantly affected by climate changes, which increases soil salinity and decreases many crops' production[7]. The presence of naturally occurring nitrogen-fixing bacteria in soil offers promising means of recovering depleted soil nutrients for food production to satisfy the demands of the world's expanding population while relieving farmers of the expense and excessive reliance on chemical fertilizers[5]. Endophytic microbial communities have received great interest in recent years, either in agriculture and medicine

* Corresponding author
E-mail address: muhammed.reben@gmail.com (Instructor).
Peer-reviewed under the responsibility of the University of Garmian.

or in controlling environmental contamination. These bacteria are used in agriculture as biofertilizers, biocontrol agents, or stress reducers[4, 5].

The bacterial endophytes interact with the host plants in mutualistic, neutral, or hostile ways. They can raise the nitrogen pool in the soil to improve plant nutrition for improved productivity. Additionally, the endophytic bacteria can promote the growth of legumes and biologically control plant pathogens[5].

Endophytes living inside a plant may be responsible for many significant characteristics and therapeutic capabilities. The physiological changes in the plant cell are triggered either actively or passively by endophytes[20]. It has been reported that bacterial endophytes can produce the phytohormones such as IAA, gibberellins, and cytokinins to directly stimulate the growth of the host plant, phosphate solubilization, N2 fixation, or indirectly by the production of antibiotics, siderophores, and lytic enzymes against the pathogens[6]. Many studies have also shown that endophytes produce bioactive substances that promote plant growth and boost plant resistance to diseases[9].

Recently, using endophytic bacteria to improve the production of legumes has gained increasing attention. Endophytic bacteria play a fascinating role in increasing soil fertility and minimizing the usage of chemical fertilizers[8]. Recently, 16S rDNA sequencing has become a vital tool for precise bacterial identification, particularly for bacteria with odd phenotypic profiles, rare bacteria, slow-growing bacteria, non-cultivable bacteria, and illnesses with a negative culture response[10]. Applying this molecular technique has led to the discovery of many novel bacteria in the last two decades (2001 –2022)[11].

It has suggested that the root nodules of legumes establish an ecological niche for the growth and survival of diverse bacterial endophytes, however to the best of our knowledge; no previous data were available reporting the endophytic bacterial population in root nodules of plant legumes in the Kurdistan region and Iraq. This study aimed to identify the endophytic bacteria of the root nodules of some common legumes of the Fabaceae family in different geographical locations of Duhok province at the molecular level and verify the biodiversity and phylogenetic analysis of the endophytic bacterial community of the studied legumes.

2. Materials and Methods

2.1 Sample collection and study site

Legumes samples, including Common bean (*Phaseolus vulgaris*), Chickpea (*Cicer arietinum*), Lentil (*Lens culinaris*), and Faba bean (*Vicia faba*), have been collected from seven districts (Duhok, Sumeal, Zakho, Amedy, Sheikhan, Bardarash, and Akre).

Duhok city lies in the western part of the Kurdistan region, northwest of Iraq, about 470km north of Baghdad and 430-450m above sea level. For each plant species, four samples were randomly selected per location (district).

2.2 Isolation and culturing of the endophytic bacteria

Roots and nodules of the collected samples were cleaned with tap water to dispose of the soil particles and remove any exterior debris, and then washed with distilled water. Under aseptic conditions, the surface of roots and nodules was well disinfected with (1/3) of NaClO for 5 minutes, being cut into small pieces and mashed one at a time in sterile mortars and pestles, using sterilized distilled water. The homogenized mixtures had streaked on yeast extract-mannitol agar (YEM) media. The inoculated Petri dishes had incubated at 28 °C for 24 to 48 hours in an incubator. The plates had checked for bacterial growth 48 hours later. Entophytic bacterial colonies had collected with an inoculation needle and then further subcultured on a new yeast extract-mannitol (YEM) medium to produce pure single-culture colonies. The purified colonies were stored in 40% (v/v) glycerol at -20 C as stocks before being used in another experimental technique.

2.3 Phenotypic Characterization of the bacterial isolates

2.3.1 Colony Characteristics

Microbiological characterization of the isolated bacterial endophytes had performed using colony morphology and its characteristics on the YEM agar[12].

2.3.2 Gram stain test

This step had performed as follows:-

One drop of overnight bacterial culture had spread on a clean slide to make a smear and has fixed by a heat stream. A few drops of crystal violet (to stain gram-positive bacteria) have added to the bacteria for 60 seconds. Then, 2-4 drops of iodine have added to the bacterial smear for 45-60 seconds. A few drops of ethanol have added for 10 seconds to wash out the excess stain and decolorize gram-negative bacteria. Then, a few drops of safranin have added for 1 minute to counterstain gram-negative bacteria. The slide was then gently washed with tap water and observed under the (1000X) using a light microscope, following standard protocol[13].

2.3.3 Antibiotic sensitivity test

The antimicrobial activity of the isolated endophytes had assayed against six antibiotics, including Amoxicillin (30µg), Bacitracin (30µg), Chloramphenicol (30µg), Erythromycin (30µg), Rifampin (30µg), and Tetracycline (30µg), employing the Kirby Bauer disc-diffusion assay technique. Organisms classified as resistant (R) or sensitive (S) according to the inhibition zone as described in the DIFCO manual, 10th edition [1]

2.4 Molecular identification of the isolates

2.4.1 DNA extraction

Genomic DNA was extracted from all isolated endophytic bacteria using AddPrep Bacterial genomic DNA extraction kit following the manufacturer's protocol. The quality and quantity of the extracted DNA had checked by a NanoDrop spectrophotometer.

2.4.2 PCR amplification of 16S rDNA gene

Amplification of 16SrDNA gene fragments (1400bp) was carried out by PCR in a thermal cycler (Eppendorf AG / USA), using primers 16Sa (5'- CGCTGGCGGCAGGCTTAACA-3') and 16Sb (5'-CCAGCCGCAGGTTCCCCT-3') according to Taoufiq and others (2018) with few modifications. The PCR conditions were as follows: one cycle at 94°C for five minutes served as the initial denaturation, followed by 30 cycles beginning with denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, and amplification at 72°C for 90 seconds, followed by a final extension at 72°C for seven minutes. Amplified products had resolved by 1.2% agarose gel electrophoresis, visualized using a UV-transilluminator, and a clear picture had captured using a Nikon digital camera.

2.4.3 Sequencing of 16S rDNA

MinElute PCR Purification Kit (50) (Qiagen) had used to purify PCR products following the supplier's instructions. The purified PCR products have been sequenced by Macrogen Incorporation (Seoul, South Korea), implementing an ABI3730 XL automatic DNA analyzer and the primer pair 16Sa and 16Sb.

2.4.4 Analysis of 16S rDNA sequences and species identification

Geneious, version R8.1, Biomatters[14], and BioEdit version 7.2 software programs have been applied to analyze, edit, trim, and verify the sequenced fragments of 16S rDNA for each forward and reverse strand and saved in Fasta format. The sequenced fragments (1400 bp) of 16S rDNA amplicons had compared with the available sequences at NCBI (National Center for Biotechnology Information). The nucleotide sequences showed ≥ 99 % similarities retrieved by using the Basic Local Alignment Search Tool (BLAST) against 16S rDNA sequences of type strains (www.ncbi.nlm.nih.gov/ BLAST).

2.4.5 Phylogenetic analysis

Geneious program version R8.1, Biomatters[14], has been used for the phylogenetic analysis and nucleotide sequence alignment. The ClustalW algorithm, using the default parameters, has been used to align sequences. The UPGMA method has been applied to construct a phylogenetic tree.

3. Results and Discussion

In the current study, legumes of Common bean (Phaseolus vulgaris), Chickpea (Cicer arietinum), Lentil (Lens culinaris), and Faba bean (Vicia faba) from seven different districts of Duhok governorate, including Duhok, Sumeal, Zakho, Amedy, Sheikhan, Bardarash, and Akre have selected to identify the endophytic bacteria at the molecular level and to address their biodiversity. The colony characteristic results of the endophytic bacteria on the YEM medium revealed that 69 distinct pure culture colonies have obtained, of which 28 colonies were from Faba bean, 16 from Chickpea, 13 from Common bean, and 12 from Lentil. The isolated endophytes had characterized using colony morphology and appearance, including shape, size, color, type of margin, opacity, elevation, texture, and their appearance after Gram staining. Bacterial isolates produced different colony morphology and characteristics on the YEM. For instance, white colonies with entire margins, translucent, and rod-shaped gram-negative bacteria had characterized from each of the studied legumes. Further, Rod-shaped gram-negative white colonies with opaque, uneven edges had also observed in most study sites. The endophytes that colonized some of the investigated legumes had recognized as being creamy in color with elevated, whole edges and opaque with gram-negative rods. In addition, gram-negative bacteria produced pale yellow, opaque colonies with full borders and bacilli in the cell shape of bacteria observed in all studied plant legumes. In addition, small to medium-sized, white, and flattened periphery colonies with gram-positive rods have been observed in some studied legumes. Moreover, smooth, translucent, convex, with entire margins, pale reddish yellow colonies with gram-negative rods had also been found in some of the studied legumes. These results might reflect the biodiversity and richness of the endophytic bacteria that colonized the studied legumes.

The antibacterial properties of the isolated bacterial endophytes have screened against six different antibiotics using the disc diffusion method. Results showed that the highest resistance rate (78%) was for bacitracin, followed by 56% for amoxicillin, 34% for rifampin, and 11% for erythromycin. In contrast, the lowest resistance rate (6%) was for each chloramphenicol and tetracycline, respectively. Resistance to antibiotics among endophytic bacteria might be due to the increasing use and applications of antibiotics over the last decades. This led to the increasing release of antibiotic-resistant genes into the environment and soil through resistant environmental strains and the significant accumulation of these antibiotic-resistant genes in agricultural soils that horizontally transferred to other microorganisms, including endophytic bacteria[16].

Furthermore, the quantification and qualification results showed that the concentration of the isolated DNA ranged from (27.5ng/µl) to (154.9ng/µl) with a purity between 1.7 and 2. The findings of PCR amplification of the 16S rDNA fragment, using a primer pair 16Sa and 16Sb, showed distinct, single amplified DNA bands of about 1400 bp (Figure 1).

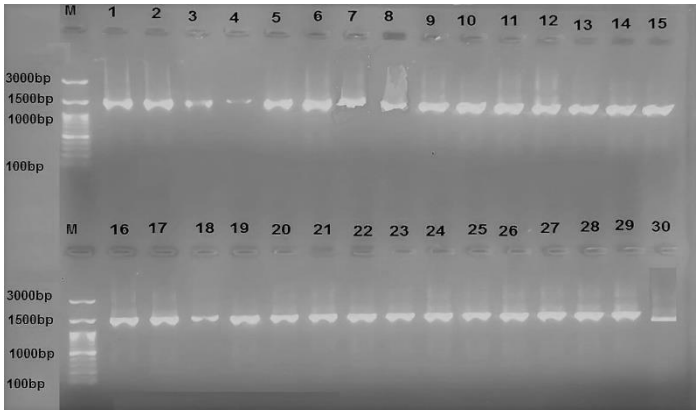

Figure 1: Represents an example of the PCR amplification products of 16SrDNA for tested endophytic isolates, electrophoresed in 1.2% agarose gel at 5-8 v/cm. Lane M represents the molecular weight marker (1500bp). Numbers from 1 to 30 represent the tested sample.

The nucleotide sequences of 16S rDNA for both forward and reverse strands had determined for all 69 endophytic bacterial isolates. The obtained nucleotide sequences for each isolate searched for their identity and molecular identification of the

bacterial endophytes implementing the BLAST algorithm of the GenBank database against 16S rDNA sequences of type strains (www.ncbi.nlm.nih.gov/ BLAST) at the National Center for Biotechnology Information (NCBI). The results revealed that seventeen bacterial species identified with nucleotide sequence identities of 98.41-100% compared with the bacterial sequences in the NCBI database. The identified bacterial species belonged to nine different bacterial genera, including *Clostridium* sp., *Pantoea* sp., *Enterobacter* sp., *Rahnella* sp., *Pseudomonas* sp., *Erwinia* sp., *Kosakonia* sp., *Salmonella* sp., and *Curtobacterium* sp., belonging to four different bacterial phyla including; *Pseudomonadota*, *Bacillota*, *Proteobacteria*, and *Actinobacteria*.

The most abundant bacterial endophytes identified from all legume plants, regardless of the legume type and their study sites, were *Pantoea agglomerans* (n=23), followed by *Clostridium sporogenes* (n=9), *Enterobacter mori* (n=9), *Enterobacter ludwigii* (n=5), *Enterobacter asburiae* (n=4), and *Kosakonia sacchari* (n=4), respectively. Other endophytic bacterial species such as *Kosakonia pseudosacchari*, *Pseudomonas reidholzensis*, *Salmonella enterica*, *Pantoea vagans*, *Curtobacterium plantarum*, *Erwinia endophytica*, *Erwinia aphidicola*, *Clostridium butyricum*, *Pseudomonas azotoformans*, *Pseudomonas koreensis*, and *Rahnella aceris* were found to be less abundant and diagnosed from one or two legume plants regardless their study sites (Figure 2).

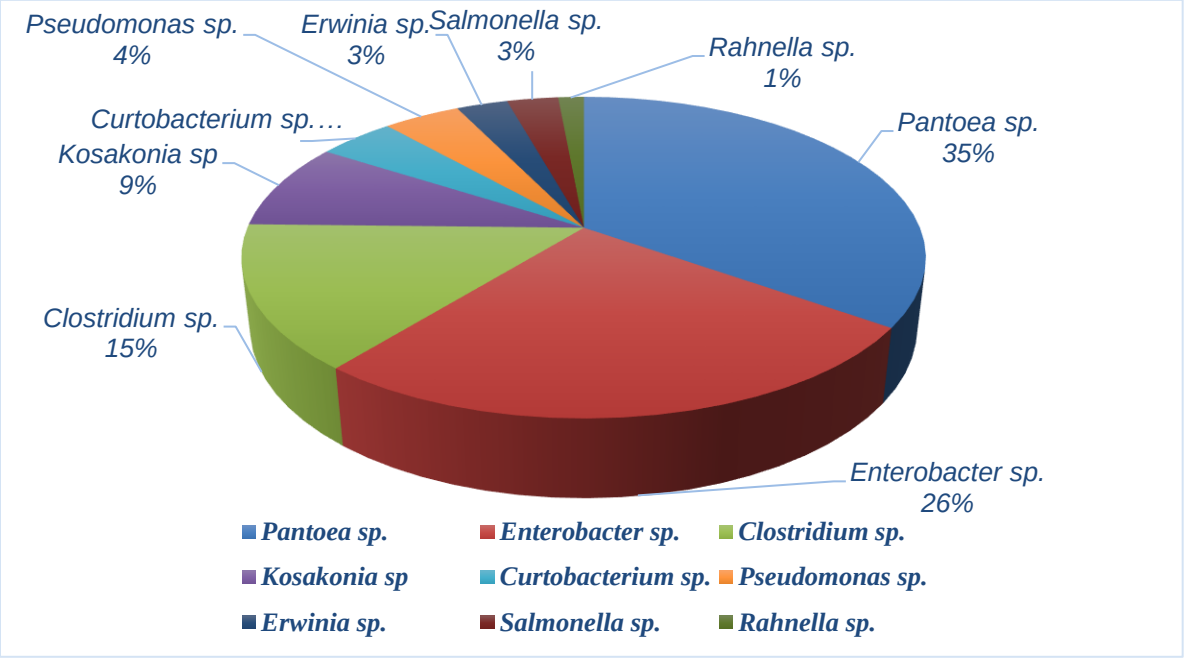

Figure 2: represents the pie chart of the abundance and percentage of each bacterial genus.

Furthermore, based on the legume type, the overall Faba bean bacterial community had found to be the most diverse one represented by nine different species, including *Clostridium sporogenes*, *Pantoea agglomerans*, *Enterobacter mori*, *Erwinia aphidicola*, *Pseudomonas koreensis*, *Pseudomonas azotoformans*, *Enterobacter ludwigii*, *Rahnella aceris*, and *Enterobacter asburiae*. In addition, the second most diverse endophytic bacterial community had observed in legumes of Chickpea plants represented by eight different bacterial species, including *Enterobacter mori*, *Clostridium sporogenes*, *Pantoea agglomerans*, *Enterobacter asburiae*, *Curtobacterium plantarum*, *Clostridium butyricum*, *Erwinia endophytica*, and *Pantoea vagans*. In addition, seven bacterial species had identified from common bean legume, including *Enterobacter mori*, *Enterobacter ludwigii*, *Pseudomonas reidholzensis*, *Pantoea agglomerans*, *Clostridium sporogenes*, *Kosakonia pseudosacchari*, *Salmonella enterica*. In contrast, the less diverse bacterial endophyte community observed in Lentil plant legumes had represented by four different bacterial species, including *Enterobacter asburiae*, *Pantoea agglomerans*, *Kosakonia sacchari*, and *Kosakonia pseudosacchari*.

The 16S rRNA gene sequence, used in the current investigation, for molecular identification of bacteria at the genus and species levels, was an accurate and robust molecular and bioinformatics tool, particularly for isolates that might not fit any established cultural or biochemical profiles. In the current investigation, *Pantoea* sp. was the most prevalent species (35%), followed by *Enterobacter* sp. (26%), *Clostridium* sp. (15%), and *Kosakonia* sp. (9%), respectively. *Pantoea* is a Gram-negative bacterial genus of the family *Erwiniaceae*, which includes 20 species, recently separated from the genus of *Enterobacter*. *Pantoea agglomerans*, a gammaproteobacterium of plant origin, was the most frequent endophytic species (33.3% of all isolates) recovered from all plant legumes screened in this study. This bacterium is known to possess many beneficial properties for the prevention of diseases, and fighting plant pathogens, as it produces a range of antibacterial substances, including agglomerins, pantocins, herbicolin, andrimid, microcin, phenazine, and others, which can combat human, animal, and plant pathogens. Besides, it promotes plant growth, as well as the bioremediation of the environment[17]. Other endophytic species such as *Pantoea vagans*, *Enterobacter mori*, *Enterobacter ludwigii*, *Enterobacter asburiae*, *Clostridium sporogenes*,

Clostridium butyricum, *Kosakonia pseudosacchari*, *Kosakonia sacchari*, *Salmonella enterica*, *Pseudomonas azotofarmans*, *Pseudomonas koreeusis*, *Pseudomonas reidholzensis*, *Rahnella aceris*, *Erwinia aphidicola*, *Erwinia endophytica*, and *Curtobacterium plantarum* have already been identified as endophytic bacteria in numerous earlier studies and isolated from rhizospheric soil. Certain cultivars of legumes contain members of specific genera, which may indicate that those members are better suited than others to survive as endophytic bacteria in legumes[18]. Variations in the make-up of the endophytic population according to cultivar or the plant clone had been reported previously in citrus plants, poplar trees, potatoes, salix, and legumes. It is unclear whether endophytic bacterial species confer any benefits to the host plant or if they may use the host as a survival strategy in the environment to reach plants on which they can develop the disease. Some bacterial species considered pathogenic for certain plant species have been isolated as endophytic in other species[19]. Understanding how endophytic microbes interact with their host plants requires the study of these species.

Among the isolated endophytes, nine were from Faba bean, eight from Chickpea plants, seven from Common bean, and four from Lentil plant legumes, suggesting that legumes harbor a diverse array of bacterial endophytes. Although the endophytic bacterial species had been isolated successfully from all plant legumes used in the current study, the population densities, species richness, and frequency of the isolated endophytic bacteria varied based on the geographical locations and the type of plant legume. The fluctuations in the endophytic bacterial population might be due to seasonal variations and environmental conditions like temperature, humidity, and rainfall, which might not be favorable for all identified bacterial endophyte colonization. In addition, differences in soil texture and variations in legume species might influence the pools of resident endophytic bacteria in plants[15].

The phylogenetic analysis, based on the 16S rDNA nucleotide sequences, clustered the identified bacterial endophytes into three major clades and 11 different sub-clusters (C1 to C11), as seen in Figure 3. with a significant degree of similarity amongst bacteria belonging to the same species, which aided in the correlation of the identified endophytes based on the 16S rDNA sequences and demonstrated that the bacterial endophytes colonized the studied legumes were diverse and belonged to different phylogenetic groups. These findings may offer crucial insights into endophytic bacterial population analyses for potential and promising agricultural, medical, and biotechnological applications of endophytes for crop improvement, technological innovation, and combating or controlling the pathogenic microorganism by endophytes.

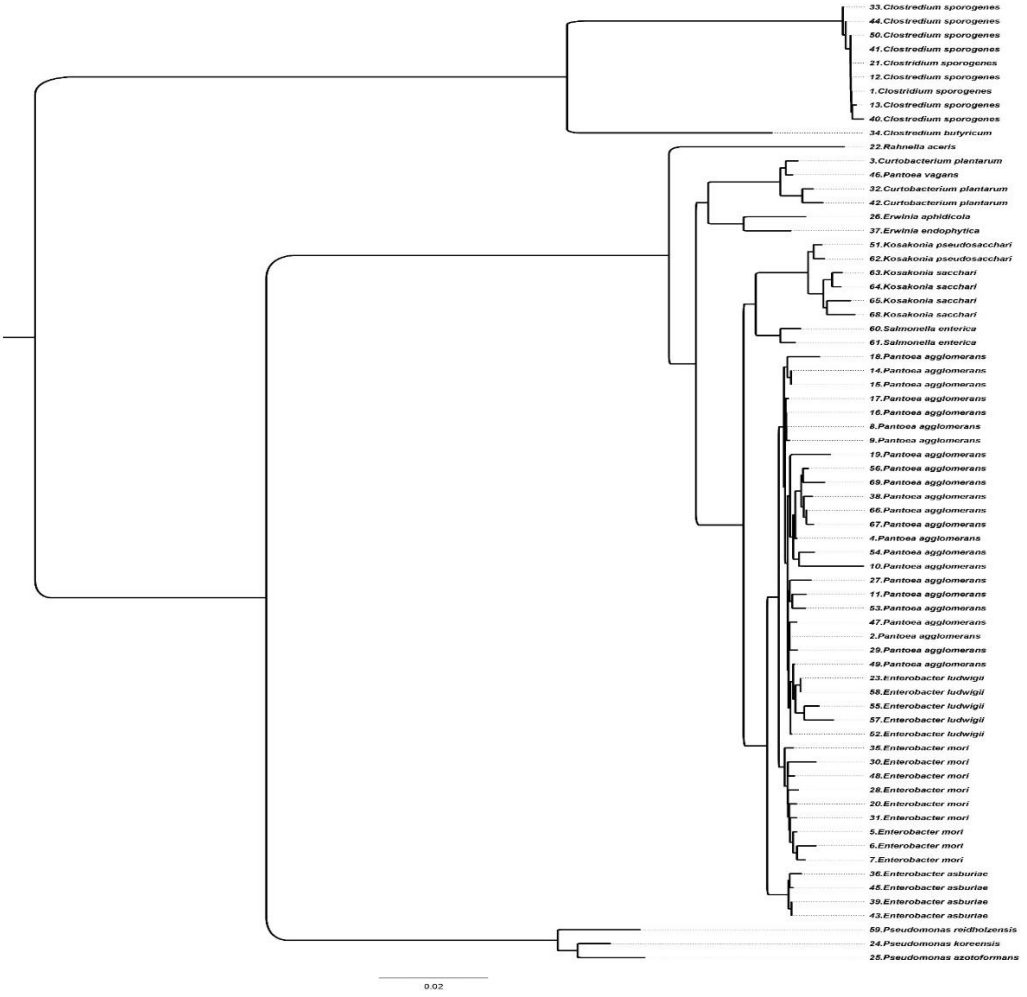

Figure 3: Represents the phylogenetic tree of the isolated bacterial endophytes.

Conclusion

Overall, it concluded that the endophytic bacterial populations varied depending on the legume type and geographical distribution. Further, the molecular identification of the bacterial endophytes based on 16S rDNA sequencing was an accurate and straightforward tool for bacterial identification at genus and species level with high discriminatory power and produced a robust phylogenetic tree. Finally, the current investigation will add additional knowledge to the literature regarding the endophytic bacterial populations colonizing different plant legumes in this region. These might give insights into potential and promising agricultural and biotechnological applications of endophytes for crop improvement.

Conflict of interests

The authors declare that they have no conflict of interests.

Author Contribution and Funding Information

Authors confirm that this manuscript is original and it hasn't been published or under consideration for publication elsewhere.

Funding

Kurdistan Regional Government, Ministry of Higher Education and Scientific Research, University of Duhok, College of Science supported and funded this work.

References

1. Singh, R., Pandey, K. D., Singh, M., Singh, S. K., Hashem, A., Al-Arjani, A. B. F., & Kumar, A. Isolation and characterization of endophytes bacterial strains of Momordica charantia L. and their possible approach in stress management. Microorganisms, 10(2), 290. https://doi.org/10.3390/microorganisms10020290 (2022)
2. Sharma, A., Malhotra, B., Kharkwal, H., Kulkarni, G. T., & Kaushik, N.. Therapeutic agents from endophytes harbored in Asian medicinal plants. Phytochemistry Reviews, 19(3), 691-720. https://doi.org/10.1007/s11101-020-09683-8 (2020)
3. Bhutani, N., Maheshwari, R., Kumar, P., & Suneja, P.. Bioprospecting of endophytic bacteria from nodules and roots of Vigna radiata, Vigna unguiculata, and Cajanus cajan for their potential use as bioinoculants. Plant Gene, 28, 100326. https://doi.org/10.1016/j.plgene.2021.100326. (2021)
4. Dubey, A., Malla, M. A., Kumar, A., Dayanandan, S., & Khan, M. L.. Plants endophytes: unveiling hidden agenda for bioprospecting toward sustainable agriculture. Critical reviews in biotechnology, 40(8), 1210-1231. https://doi.org/10.1080/07388551.2020.1808584. (2020)
5. Kumar, A.; Zhimo, Y.; Biasi, A.; Salim, S.; Feygenberg, O.; Wisniewski, M.; Droby, S. Endophytic Microbiome in the Carposphere and Its Importance in Fruit Physiology and Pathology. In Postharvest Pathology; Springer: Cham, Switzerland, pp. 73–88. https://doi.org/10.1007/978-3-030-56530-5_5. (2021)
6. Kumar A., Singh R., Akhilesh Yadav A., Giri D.D., Singh P.K, and Pandey K.D. Isolation and characterization of bacterial endophytes of Curcuma longa L. Biotech 6:60. (2016)
7. Walters, S. A., Abdelaziz, M., & Bouharroud, R.. Local melon and watermelon crop populations to moderate yield responses to climate change in North Africa. Climate, 9(8), 129. https://doi.org/10.1007/s13205-016-0393-y. (2021)
8. Meena, V. S., Meena, S. K., Verma, J. P., Kumar, A., Aeron, A., Mishra, P. K., ... & Dotaniya, M. L.. Plant beneficial rhizospheric microorganism (PBRM) strategies to improve nutrients use efficiency: a review. Ecological Engineering, 107, 8-32. https://doi.org/10.1016/j.ecoleng.2017.06.058. (2017)
9. Syed C. S., Mounika P.P.N., Mounika Y., Kumar S.S., Bai V.T. and Audipudi A.V. Evaluation of Antimicrobial and Antibiotic Sensitivity of Chilli Root Endophytic Bacteria for Eco-friendly Biofertilizer. Int. J. Curr.Microbiol.App.Sci Special Issue-5: 45-53. (2017)
10. Church, D. L., Cerutti, L., Gürtler, A., Griener, T., Zelazny, A., & Emler, S.. Performance and application of 16S rRNA gene cycle sequencing for routine identification of bacteria in the clinical microbiology laboratory. Clinical Microbiology Reviews, 33(4), e00053-19. https://doi.org/10.1128/CMR.00053-19. (2020)
11. Gofton, A. W., Oskam, C. L., Lo, N., Beninati, T., Wei, H., McCarl, V., & Irwin, P.. Inhibition of the endosymbiont "Candidatus Midichloria mitochondrii" during 16S rRNA gene profiling reveals potential pathogens in Ixodes ticks from Australia. Parasites & Vectors, 8(1), 1-11. https://doi.org/10.1186/s13071-015-0958-3. (2015)
12. Sarjono, P. R., Hazrina, Q. H., Saputra, A., Mulyani, N. S., Aminin, A. L. N., Ngadiwiyana, & Prasetya, N. B. A., June). Isolation, characterization, and identification of endophytic bacteria by 16S rRNA partial sequencing technique from leaves of Carica papaya and its potential as an antioxidant. In AIP Conference Proceedings (Vol. 2237, No. 1, p. 020053). AIP Publishing LLC. https://doi.org/10.1063/5.0005715. (2020)
13. Tripathi, N., & Sapra, A. Gram staining. PMID: 32965827. (2020)
14. Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., & Drummond, A. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics, 28(12), 1647-1649.2012))
15. 15. Martínez Alcántara V, Medina R, Gauna JM, Balatti PA. Bacterial endophytes diversity of tree legumes from Argentina. Agrociencia Uruguay, Volume 24, Number NE2, Article 411. DOI: 10.31285/AGRO.24.411. (2020)
16. Sharma M and Mallubhotla S Diversity, Antimicrobial Activity, and Antibiotic Susceptibility Pattern of Endophytic Bacteria Sourced from Cordia dichotoma L. Front. Microbiol. 13:879386. https://doi: 10.3389/fmicb.2022.879386. (2022)
17. Dutkiewicz, J., Mackiewicz, B., Lemieszek, M. K., Golec, M., Milanowski, J.. Pantoea agglomerans: a mysterious bacterium of evil and good. Part IV. Beneficial effects. Ann Agric Environ Med., 23(2), 206-222. https://doi.org/10.5604/12321966.1203879. (2016)
18. Costa L. E.; Queiroz M. V.; Borges A. C.; Moraes C. A.; and Araújo E. F.. Isolation and characterization of endophytic bacteria isolated from the leaves of the common bean (Phaseolus vulgaris). Environmental Microbiology. Braz. J. Microbiol. 43 (4). https://doi.org/10.1590/S1517-8382201200040004. (2012)
19. Omomowo, O. I., & Babalola, O. O. Bacterial, and fungal endophytes: tiny giants with immense beneficial potential for plant growth and sustainable agricultural productivity. Microorganisms, 7(11), 481. https://doi.org/10.3390/microorganisms7110481. (2019)
20. Conrath, U., Beckers, G. J., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., & Mauch-Mani, B. (2006). Priming: getting ready for battle. Molecular plant-microbe interactions, 19(10), 1062-1071. https://doi.org/10.1094/MPMI-19-1062.
21. Kebede E. Contribution, Utilization, and Improvement of Legumes-Driven Biological Nitrogen Fixation in Agricultural Systems. Front. Sustain. Food Syst. 5:767998. https://doi: 10.3389/fsufs.2021.767998. (2021)
22. Taoufiq K., Faghire M, Tahrouch S., Nejmeddine M., Göttfert M., Oufdou K. and Hatimi A. (2018). Screening and Molecular Identification of Endophytic Bacteria Isolated From Legumes Nodules and Roots Cultivated in Acacia Rhizosphere Soils Collected in an Arid Region, Tata-Akka in South of Morocco. Indian Journal of Natural Sciences. Vol. 9 / Issue 50.