

Diversity of Culture dependent endophytic bacteria isolated from leguminous agroforestry trees in western Kenya

william omuketi Emitaro (✉ omuketiw@gmail.com)

Jaramogi Oginga Odinga University of Science and Technology <https://orcid.org/0000-0002-6736-252X>

Fanuel Kawaka

Jaramogi Oginga Odinga University of Science and Technology

David Musyimi

Maseno University

Asenath Adienge

Kenya Forestry Research Institute

Original article

Keywords: Niches, morphological, characterized, molecular, spherical, rod shaped

Posted Date: December 1st, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-2300842/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Plants have diverse and vast niches colonized by endophytic microorganisms that promote the well being of host plant. These microbes inhabit internal plant tissues with no signs of ill health. Bacterial endophytes from many plants have been isolated and characterized due to their beneficial roles however their diversity in leguminous plants still remain unexploited. Diversity of bacterial endophytes isolated from *L. diversifolia*, *S. sesban* and *C. Salothyrus* were assessed using morphological and molecular characteristics. A total of 27 pure isolates were recovered from *C. Calothyrsus*, *L. diversifolia* and *S. sesban* constituting 44.4%, 31.8% and 23.8% from the leaves, stems and roots respectively. The isolates differentiated into Gram positive and negative with rods and spherical shapes. Analysis of 16S rRNA gene sequence revealed 10 closely related bacterial genera that consisted of *Bacilli* (33.3%), *Staphylococcus* (22.2%), *Alcaligenes* (11.2%), *Xanthomonas* and *Sphingomonas* (47.4 %). Others included *Enterobacteria*, *Pantoea*, *Acinetobacter*, *Pseudomonas* and *Bacterium*. These results indicate the presence of high diversity of endophytic bacteria associated with the different parts of *L. diversifolia*, *S. sesban* and *C. salothyrsus* growing in western Kenya.

Introduction

Plant microbe interaction has been the subject of interest in current research due to its mutuality and biotechnological applications. Plants have diverse and vast niches which are colonized by microbes called endophytes that promote plant development and plant health [1,2]. Endophytes are heterotrophic microorganisms inhabiting the inner plant environment with no sign of ill health [3, 4]. Endophytes comprise bacteria, fungi and actinomycetes distributed in every tissue, organ and plant species worldwide [5, 6]. Endophytes get into different plant tissues via germinating radicals, natural openings such as stomata and secondary roots. They may also gain entry through mechanically damaged foliar or by use of hydrolytic enzymes they secrete to degrade cell wall that acts as a barrier to advancing microbial pathogen [7, 6]. Once inside the host plant, they may colonize the point of entry or may translocate to new sites and colonize intracellular or extracellular spaces of different parts of the plant parts to establish a mutual relationship with the plant [8, 7, 9, 10].

In the recent past, endophytes have received wide attention due to their protective and growth enhancement roles in host plants [11, 12]. They have shown unique intrinsic lifestyles and mechanisms to evade host defence reactions and bypass the host immune system to enable asymptomatic proliferation within the host [13]. Reports by Singh *et al.* [14] and Tidke *et al.* [15] show that endophytes can synthesize secondary metabolites such as peptides, quinolons, polyketones, alkaloids, phenolic compounds, steroids, flavonoids, terpenoids, azadirachtin and siderophores that have antimicrobial and insecticidal properties. Similarly, endophytes have unique enzyme systems that are responsible for synthesizing enzymes such as amylases, pectinases, laccases, cellulases, proteinases and lipases that arrest the proliferation and attack by microbial and insect pathogens [16, 17].

Endophytic bacteria establish a beneficial relationship with host plants after entry by being protected from adverse environmental conditions while promoting growth and tolerance of the plant to stresses due to abiotic and biotic factors [18, 19]. Bacterial endophytes improve health and growth of the host plant through solubilization of phosphates, synthesis of phytohormones, production of siderophores and enhancement of nitrogen fixation [20]. Moreover, endophytic bacteria exhibit antimicrobial properties that protect host plants from pathogenic microorganisms and their metabolites have been integrated into various biotechnological applications [21, 1]. Due to the beneficial roles played by bacterial endophytes in their host plants, they have been isolated and characterized [22, 23] from different plants including non-leguminous and leguminous plants but there are still many plants whose endophytes have not been identified. In leguminous plants, endophytic bacteria are dominated by *Bacillus*, *Pseudomonas*, *Burkholderia*, *Rhizobium*, and *Klebsiella* [24, 19]. Even though bacterial endophytes from some leguminous plants have been characterized [24, 19, 25, 26], more studies are still required to understand bacterial endophytes associated with *Sesbania sesbna*, *Leucaena diversifolia* and *Calliandra calothyrsus*. Knowledge of the bacterial endophytes colonizing these plants would be of great interest in understanding their role and application in crop production besides being used for nitrogen fixation. The present study assessed the diversity of endophytic bacteria colonizing *Sesbania sesban*, *Leucaena diversifolia* and *Calliandra calothyrsus* growing in western Kenya.

Materials And Methods

Study site, sampling and processing

Plant parts including roots, leaves and stems of *S. sesban*, *C. diversifolia* and *C. calothyrsus* were obtained separately from Maseno University farm in khaki bags. The University is located along Kisumu Busia road and lies at 0° 10' 0" South, 34° 36' 0" East. Plant materials collected were taken to the Microbiology laboratory of Jaramogi Oginga Odinga University of Science and Technology for processing. Plant materials were obtained in triplicates from demonstration plots and eventually pooled together before the isolation of endophytic bacteria.

Isolation of culturable bacterial endophytes

Plant roots, leaves and stems were first washed in running tap water to remove any soil or contaminant from the field before being washed in 70% ethanol for 5 minutes. They were transferred to 3% sodium hypochlorite for five minutes for complete surface sterilization and then rinsed several times in sterile distilled water [27]. The efficiency of surface sterilization was assessed by inoculating 100 µL aliquot of the last rinsing water on Nutrient agar plates and incubating for 48 hours at 28 ± 2 °C. Absence of any growth indicated complete surface sterilization. Surface sterilized plant parts were crushed in 5 ml distilled water and one milliliter serially diluted up to 10⁻⁴. Bacteria endophytes were isolated on nutrient agar using the pour plate method for each plant species and plant part. Triplicate plates were incubated for 48 hrs at 28 °C arranged in a completely randomized design. Colonies emerging from the plates were subcultured separately 2-3 times based on morphological differences to obtain pure cultures.

Morphological characterization of endophytic bacteria

Bacterial endophytes were characterized using colony characteristics such as colour, cell shape, type of edge, opacity and appearance of cells after Gram staining [28]. The shape of the cell and Gram's reaction were determined by observation under a light microscope (Leica DM 500) at ×100 [29].

Molecular Characterization

Genomic DNA extraction

Zymo Research DNA Mini Prep™ kit (ZR, South Africa) was used for DNA extraction. Nanodrop™ Lite Spectrophotometer (Thermo Scientific Inc, USA) was used to estimate the concentration of DNA at 260-280 nm wavelengths. Horizontal gel electrophoresis (Thistle Scientific Ltd, USA) was used to estimate the purity on a 1% (w/v) agarose gel at 100V for 40min. The gel was stained with SYBR Safe dye (Invitrogen 10,000x concentrate in DMSO) and visualized under UV according to Adienge *et al.* [30].

16S rRNA gene amplification

The identification of the bacterial endophyte isolates by 16S rRNA gene partial sequencing was performed using universal primers 1492R (5'TACCTTGTACGACTT-3') and 27F (5'AGAGTTTGATYMTGGCTCAG-3') [18]. Amplification was carried out in a 20 µL PCR tubes each containing 1.4 µl MgCl₂, 2 µl DNA, 2 µl Taq buffer, Taq DNA Polymerase 0.4 µl, dNTPs 0.4 µl, Primers 2 µl and Nuclease free water 11.8 µl. The mixtures were transferred to a 96 well thermocycler (Applied Biosystems). Thermocycler was optimized to run at the following temperatures; initial denaturation for 5 minutes at 94 °C, denaturation for 30 seconds at 94 °C, annealing for 30 seconds at 47°C, elongation at 72°C for 2 minutes and a final elongation for 10 minutes at 72°C. The cycles for denaturation, annealing and elongation were repeated 35 times. Products of amplification were separated on 2% (w/v) agarose gel in 1X TAE buffer, stained with SYBR Safe dye (Invitrogen 10,000 x concentrate in DMSO), and visualized under UV illumination table (ATTA E-Graph).

DNA sequencing and phylogenetic analysis

The PCR products recovered were sent to **Macrogen Europe B.V.** (Meibergdreef 311105 AZ, Amsterdam, Netherlands) for sequencing. Forward and reverse gene sequences obtained were imported to Geneious Prime® 2020.0.4 (www.geneious.com) and contigs generated through De Novo assembly. Sequences were analyzed using BLASTn tool at the National Centre for Biotechnology Information database (NCBI) GenBank using the Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) [18]. A similarity search of 16S rRNA sequences was performed to identify closely related sequences available in the GenBank. Assembled multiple sequences of approximately 500 bp were transferred to MEGA Version 6.0 software and aligned using Clustal W method according to Tamura *et al.* [31]. Sequences with greater than 97% similarity were

retrieved for phylogenetic analysis. Evolutionary histories and diversity of the isolates were determined using the Neighbour-Joining method and distances computed using Maximum Composite Likelihood [32]. A bootstrap test (1000 replicates) was used to cluster associated taxa and replicate trees with above 50% likelihoods indicated on the branches.

Results

A total of 27 different colonies of bacteria were isolated from leaves, stems and roots of the three agroforestry trees. More bacterial endophytes were recovered from the leaves compared to the stems while roots had the least percentage recovery (Table 1). Most bacteria were recovered from *C. calothyrsus* followed by *L. diversifolia*.

Phenotypic characterization of the bacterial isolates

Based on phenotypic characteristics, the isolates were characterized using colony appearance, morphology such as elevation, type of margin, opacity and appearance after Gram staining (Table 2). Yellow raised colonies with entire margins, opaque, cocci in cell shape and Gram negative were recovered from all the three plant parts. White colonies lying flat on the media with an entire margin, translucent, rod shaped and Gram negative colonized the roots, leaves and stems of *L. diversifolia*, roots and leaves of *C. Calothyrsus* and leaves of *S. sesban*. Filamentous white colonies with irregular margins, opaque, rod shaped and Gram negative were present in the leaves of all the plants. Cream colonies that were raised with entire margins, opaque and Gram negative bacilli were found to colonize leaves, stem and roots of *S. Sesban*, *C. Calothyrsus* and stem of *L. diversifolia*. White colonies, raised with undulated margins, opaque, rod shaped which stained purple, were recovered from the three parts of *C. Calothyrsus*. Raised yellow light colonies with entire margin, opaque in opacity, rods in shape and Gram negative were found in the leaves and stems of *L. diversifolia* and *C. Calothyrsus* while white colonies that are flat on the media surface with entire margins, translucent, cocci in shape and Gram positive were recovered from stems of *S. sesban* and leaves of *L. diversifolia*.

Molecular characterization

A total of 27 pure bacterial isolates were successfully amplified and sequenced using 16S rRNA primers. Analysis of 16S rRNA gene sequence revealed closely related bacterial species belonging to 10 genera. Genus *Bacilli* (33.3%) was dominant compared to genus *Staphylococcus* (22.2%) and *Alcaligenes* (11.2%). Genus *Xanthomonas* and *Sphingomonas* each had 2 isolates. Others with 1 isolate each included *Pseudomonas*, *Pantoea*, *Bacterium*, *Enterobacteria* and *Acinetobacter*. Isolates (BLS1, BLS2, BLS3, BRC3, BRC5, BSS3, BLS5, BRS1, BSC2, BSC5, BSL3 and BRL5) constituted 66% of the isolates and belonged to phylum proteobacteria. A total of 44% of the isolates (BLL4, BLL6, BSL1, BLC4, BLC5, BLC6, BSS1, BSS2, BRS3, BLC1, BLC3, BLL5, BRC1, BRC3 and BSC1) belonged to phylum firmicutes (Table 3). All isolates had sequences with 97.00% identity match with gene bank sequences apart from isolate *BLS2* and *BLS1* whose match identity was 87.08% and 91.07% respectively. Sequences of the isolates were registered in the NCBI Bankit with accession numbers ranging from MW251519.1 to MW251545.1 (Table 3).

Phylogenic analysis

The phylogenetic tree established using the neighbour joining method clustered the isolates into 6 clades (Figure 1). The clades represented orders which included Bacillales, Xanthomonadales Sphingomonadales, Burkholderiales, Pseudomonadales and Enterobacterales. Bacillales comprised of isolates belonging to two genera; *Bacilli* and *Staphylococci*. Genus *Bacilli* had 9 sequences compared to *Staphylococci* that had 6 sequences clustering at 100% bootstrap. Isolates in the order Bacillales colonized all 3 plants (*S. Sesban*, *C. Calothyrsus* and *L. Diversifolia*). Order Xanthomonadales and Sphingomonadales comprised of 2 isolates each with 100% bootstrap support. Order Enterobacterales comprised of genus *Bacterium*, *Pantoea* and *Enterobacteriaceae* with 100% bootstrap support. Order pseudomonadales comprised of genus *Pseudomonas* and *Acinetobacter* while genus *Alcaligenes* belonged to order Burkholderiales. Bacterial endophytes in the order Pseudomonadales were isolated from *S. Sesban* and *C. Calothyrsus* while endophytes in the order Burkholderiales were recovered from *L. diversifolia* and *C. calothyrsus*.

Discussion

The recovery of 27 pure bacterial isolates in this study is an indication of occurrence of diverse endophytes in different parts of *S. sesban*, *C. calothyrsus*, and *L. diversifolia*. Similar results were reported by Bind and Nema [18] and Benjelloun *et al.*, [33] who

isolated endophytic bacteria from pigeon pea and Chickpea plants using the same protocol. The presence of higher bacterial isolates in the leaves compared to the other plant parts could be attributed to the availability of nutrients due to photosynthesis. Chowdhary and Kaushik [23] and Katoch and Pull [34] reported that leaves have a high diversity of bacterial endophytes than any other plant part. Bacterial endophytes often colonize the intercellular spaces of the plant parts because these areas are endowed with an abundance of amino acids, carbohydrates and inorganic nutrients [35, 36] especially the leaves where photosynthesis takes place. Bacterial endophytes recovered from roots, leaves and stems of *L. diversifolia*, *C. calothyrsus* and *S. sesban* exhibited varied morphological features based on elevation, colour, opacity, shape, opacity and Gram staining.

Nhu and Diep [37] recorded similar results after recovering bacterial endophytes with different phenotypic characteristics from Soybean (*Glycine max*). Morphological variation in the colonies of bacterial isolates could be due to the ability of different bacterial species to metabolize different constituents of culture media for colonies to have different shades, shapes and elevations.

Bacterial endophytes exhibit wide variations in their phenotypic characteristics even when they are isolated from the same plant tissue, organ or plant species [38, 39]. According to Sinha *et al.* [40], bacteria synthesize pigments as secondary metabolites by utilizing different nutrients in the media hence the variation in colony colour. Pigments protect bacterial cells from toxicity that results from exposure to visible and ultraviolet light rays which could have brought about variation in pigmentation amongst the bacterial isolates. Bacterial isolates were divided into two groups based on the Gram's reaction and cell shape as Gram negative bacilli and cocci, Gram positive bacilli and cocci. These results are in line with the report of Bhagya *et al.* [1] that the legume Green gram (*Vigna radiata* L.) is colonized by both Gram positive cells and Gram negative cells of bacterial endophytes. The variation in colour of bacterial cells after staining is due to the difference in the structural composition of their cell walls. The cell wall of Gram-negative bacteria has a lipid layer called lipopolysaccharide that dissolves when treated with alcohol hence losing the primary stain crystal violet and taking up secondary stain to appear red. Cell walls of Gram positive bacteria contain teichoic acid and thick peptidoglycan layers that retain the primary stain crystal violet on decolourization hence appearing purple [40].

Researchers [41, 42, and 40] have used phenotypic features to characterize bacterial endophytes but they are inadequate for complete identification because of the existence of intermediate forms within a subgroup.

Conclusive identification of bacteria requires polyphasic taxonomic approach that puts emphasis the use of classical methods in combination with modern genetic/molecular techniques [43]. Based on the 16S rRNA gene sequence, the majority of the isolates belonged to the genus *Staphylococci* and *Bacilli*. This may be due to their vital role in the growth of the plant which includes protection against invading plant pathogens and synthesis of hormones that promote plant growth. Ek-Ramos *et al.* [44] observed that bacterial endophytes belonging to the genus *Bacilli* enable the host plant to tolerate biotic and abiotic stress. This is achieved by stimulation of immune response, niche competition, and metabolism of phenylpropanoid to produce plant defence through structural support and activation of survival molecule. Brígido *et al.* [19] reported similar results during the identification of bacterial endophytes of Chickpea (*Cicer arietinum* L.). Leguminous plants harbour the majority of bacteria belonging to genus *Bacilli* and *Pseudomonas* because of their symbiotic association. Members of the genus *Bacilli* such as *Bacillus amy-loliquefaciens* have been reported to be responsible for the solubilization of zinc, potassium and phosphorous. They are also involved in the production of plant hormone (IAA), nitrogen fixation and synthesis of bio-control agents [45] hence their dominance as endophytes of *S. sesban*, *C. calothyrsus* and *L. diversifolia*.

Phylogenetic analysis of the isolates clustered them into six orders each supported by 98% bootstrap with the majority coming from the phylum Proteobacteria. Bacterial endophytes that clustered together in any given order had high similarity in gene structure and nucleotide arrangement enabling their sequences to align close to each other during analysis [46, 47]. These findings concur with the report of Chimwamurombe *et al.* [48] which indicated that endophytic bacteria in leguminous plants are dominated by members of phylum Proteobacteria while a few belong to phylum Firmicutes. Diverse species of bacteria belonging to phylum proteobacteria are endophytes probably because they have different strategies of overcoming plant defence mechanisms to gain entry and systemically move and lodge into different parts of the host plant. Once inside, they improve plant nutrient uptake and stimulate the synthesis of growth promoting as well as stress tolerance hormones. [49]. Endophytic bacteria also synthesize secondary metabolites with antimicrobial and anti-insect activities thus enabling the host plant to resist pathogenic attack [50, 49].

In this study, bacterial endophytes of the genus *Staphylococcus*, *Bacilli*, and *Alcaligenes* were isolated from more than one plant species and plant organ while some were specific to the plant species and organ of colonization. Colonization of more than one plant species could be because the plants belong to the family leguminosaea and secrete exudates with similar nutritional and chemical composition that attracted similar bacterial endophytes. According to [51], bacterial endophytes tend to disregard the theory of host specificity thereby becoming naturally promiscuous to interact with different host plants which supports the findings of our study. On the other hand, *Acinetobacteria johnsonii*, *Pantoea agglomerans* and *Alcaligenes spp* were specific to the plant and organ of origin. Different plants and organs have varied chemical compositions due to genetic variability that determines the selection and preference of colonizing bacterial endophytes which could be the case in this study. According to Maggini *et al.* [52] the presence of different bioactive compounds in different plant species and organs dictates the species of bacteria that colonize as endophytes. Some of the bioactive compounds that control and dictate endophyte colonization include alkenes, acid derivatives, alkamides, polysaccharides and caffeine. Endophytic bacteria are attracted to their host rhizosphere by exudates rich in different phenolic compounds, amino acids and sugars before penetrating to lodge within the plant [53, 37, 52]. Once they are in the rhizosphere, they use different mechanisms to gain entry into the host plant where they will spend either part or whole of their lifecycle [54].

List Of Abbreviations

ZR	Zymo Research
DNA	deoxyribonucleic acid
SYBR	Synergy Brands
DMSO	Dimethyl sulfoxide
rRNA	Ribosomal ribonucleic acid
dNTP	Deoxynucleoside triphosphate
Taq	Thermus aquaticus
TAE	Tris base, acetic acid and EDTA
NCBI	National Centre for Biotechnology Information database
BLAST	Basic Local Alignment Search Tool
MEGA	Molecular Evolutionary Genetic Analysis

Declarations

Ethics approval and consent to participate

Not applicable as there was no involvement of animal or human specimens

Consent for publication

Not applicable

Data Availability

The data used in writing the results of this study are part of this article.

Competing interests

The authors declares that there are no competing interests

Funding statement

There was no support from any of the funding agencies during the conceptualization of the idea as well as in the entire process of this study.

Authors' contributions

William Emitaro was involved in the laboratory isolation, characterization of the isolates and writing the manuscript while David Musyimin was involved in the design of the research. Asenath Adienge carried molecular analysis while Fanuel Kawaka edited the manuscript.

Acknowledgments

We acknowledge the Department of Biological Sciences, Jaramogi Oginga University of Science and Technology for providing the laboratory space for the study. We also acknowledge Maseno University for allowing collection of plant material from their farms.

References

1. M. Bhagya, K. Nagaraju, B. J. Praveen, G.P. Santhosh, and R.C. Gundappagol, "Isolation and Characterization of Endophytic Bacteria from Nodule, Root and Seeds of Greengram (*Vigna radiata* L.)". *Indian Journal of Pure and Applied Bioscience*, vol. 7, no. 4, pp. 319-328, 2019.
2. W.O. Emitaro, D.M. Musyimin and G.T. Opande, "Bioactivity of Endophytes from *Calliandra calothyrsus*, *Leucaena diversifolia* and *Sesbania sesban* Against *Cercospora zea-maydis*". *International Journal of Research and Scientific Innovation*, vol. 7, no. 6, pp. 117-121, 2020.
3. J-L. Li, X. Sun, Y. Zheng, P-P. Lü, Y-L. Wang, L-D. Guo, "Diversity and Community of Culturable Endophytic Fungi from Stems and Roots of Desert Halophytes in Northwest China". *MycologyKeys*, vol. 62, pp. 75–95, 2020.
4. J.M. Niem, R. Billones-Baaijens, B. Stodart and S. Savocchia, "Diversity Profiling of Grapevine Microbial Endosphere and Antagonistic Potential of Endophytic Pseudomonas Against Grapevine Trunk Diseases". *Frontiers in Microbiology*, vol. 11, no. 477. Pp.1-19, 2020.
5. R.O. Anyasi and H.I. Atagana, "Endophyte: Understanding the Microbes and its Applications". *Pakistan Journal of Biological Sciences*, vol. 22, no. 4, pp.154-167, 2019.
6. B. El-Deeb, K. Fayed and Y. Gherbawy, "Isolation and Characterization of Endophytic Bacteria from *Plectranthus tenuiflorus* Medicinal Plant in Saudi Arabia Desert and their Antimicrobial Activities". *Journal of Plant Interactions*, vol. 8, no. 1, pp. 56-64, 2013.
7. M.S. Dashed, C.G. Sangeetha, V. Appanna, G.K. Halesh and V. Devappa, "Isolation and Morphological Characterization of Endophytic Fungi Isolated from Ten Different Varieties of Mango". *International Journal of Current Microbiology and Applied Sciences*, vol. 8, no. 3, pp. 717-726, 2019.
8. M. M. Coêlho, M. S. Ferreira-Nozawa, S. R. Nozawa and A. L. W. Santos, "Isolation of Endophytic Bacteria from Arboreal Species of the Amazon and Identification by Sequencing of the 16S rRNA Encoding Gene". *Genetics and Molecular Biology*, vol. 34, no. 4, pp. 676-680, 2011.
9. E. Khare, J. Mishra and N.K. Arora, "Multifaceted Interactions Between Endophytes and Plant: Developments and Prospects". *Frontiers in Microbiology*. Vol. 9, pp. 1-12, 2018.
10. A. Suman, N.Y. Ajar and V. Priyanka, "Endophytic Microbes in Crops: Diversity and Beneficial Impact for Sustainable Agriculture". *Microbial Inoculants in Sustainable Agricultural Productivity*, vol. 1, pp. 117-143, 2011.
11. R. Abdennabi, M.A., Triki, R.B. Salah and N. Gharsallah, "Antifungal Activity of Endophytic Fungi Isolated from Date Palm Sap (*Phoenix dactylifera* L.)". *Ecronicon Microbiology* vol.13, no. 4, 123-131, 2017.
12. M. E. Constantin, F. J. De Lamo, B. V. Vlioger, M. Rep and F. L. Takken, "Endophyte-mediated Resistance in Tomato to *Fusarium oxysporum* is Independent of ET, JA, and SA". *Frontiers in plant science*, vol. 10, pp. 979, 2019.

13. B. Basumatary, D. Das, B. N. Choudhury, P. Dutta² and A. Bhattacharyya, "Isolation and Characterization of Endophytic Bacteria from Tomato Foliage and Their in vitro Efficacy". *Journal of Nematology*, vol. 53, pp. 1-16, 2021.
14. S. Sinha, S. Choubey, K.A. Ajay, P. Bhosale, "Identification, Characterization of Pigment Producing Bacteria from Soil and Water and Testing of Antimicrobial Activity Bacterial Pigments". *International Journal of Pharmaceutical Sciences Review and Research*, vol. 42, no. 2, pp. 119-124, 2017.
15. S.A. Tidke, R.K.L. Kumar, D. Ramakrishna, S. Kiran, G. Kosturkova and R.A.Goka, "Current Understanding of Endophytes: Their Relevance , Importance , and Industrial Potentials". *IOSR Journal of Biotechnology and Biochemistry*, vol. 3, no. 3, pp. 43–59, 2017.
16. A.H. Fouda, S.E. Hassan, A.M. Eid and E.E. Ewais, "Biotechnological Applications of Fungal Endophytes Associated with Medicinal Plant *Asclepias sinaica* (Bioss.)". *Annals of Agricultural Sciences*, vol. 1, pp. 95-104, 2015.
17. D. Handayani, R. Harrizul, M. Rizka, S. Netty, R. Roslaili and H. Triana, "Antimicrobial and Cytotoxic Activities of Endophytic Fungi Isolated from Mangrove Plant *Sonneratia alba* Sm". *Journal of Applied Pharmaceutical Science*, vol. 8, no. 2, pp. 049-053, 2018.
18. M. Bind and S. Nema, "Isolation and Molecular Characterization of Endophytic Bacteria from Pigeon Pea Along With Antimicrobial Evaluation against *Fusarium udum*". *Applied Microbiology Open Access*, vol. 5, no. 2, pp.1-12, 2019.
19. C. Brígido, E. Menéndez, A. Paço, R.G. Bernard, B. Anabela, R.F. Maria, O. Solange and C. Mário, "Mediterranean Native Leguminous Plants: A Reservoir of Endophytic Bacteria with Potential to Enhance Chickpea Growth under Stress Conditions". *Microorganisms*, vol. 7, no. 392, pp.1-23, 2019.
20. E. Gamalero, E. Favale, E. Bona, G. Novello, P. Cesaro, N. Massa, B. R. Glick, M.C. Orozco-Mosqueda, G. Berta and G. Lingua, "Screening of Bacterial Endophytes Able to Promote Plant Growth and Increase Salinity Tolerance". *Applied Sciences*, vol. 10, no. 5767, pp. 1-21, 2020. <https://doi:10.3390/app10175767>
21. M. D. F. ALKahtani, A. Fouda, K. A. Attia, F. Al-Otaibi, A. M. Eid, E.E. Ewais, M. Hijri, M. St-Arnaud, S.E. Hassan, N. Khan, Y. M. Hafez and K. A. A. Abdelaal, "Isolation and Characterization of Plant Growth Promoting Endophytic Bacteria from Desert Plants and Their Application as Bioinoculants for Sustainable Agriculture". *Agronomy*, vol. 10, no. 1325, pp. 1-18, 2020. <https://doi:10.3390/agronomy10091325>
22. M. Mahadevamurthy, H. Puttaswamy, T. M. Channappa, M. Sidappa, P. Madegowda, J.S Chikkamanche Gowda and A.K. Nagaraj, "Antibacterial Potential of Fungal Endophytes Isolated from *Boerhaavia diffusa* L". *Journal of Applied Pharmaceutical Science*, vol. 6, no. 10, pp. 216-221, 2016.
23. K. Chowdhary and N. Kaushik, "Fungal Endophyte Diversity and Bioactivity in the Indian Medicinal Plant *Ocimum sanctum* Linn." *PLoS ONE*, vol. 10, no, 11, pp. 1–25, 2015.
24. G. Webster, J.M. Alex, E. Cunningham-Oakes, R. Arun, B.A. Jamuna, M. Eshwar and R.V. Ravishankar, "Culturable diversity of bacterial endophytes associated with medicinal plants of the Western Ghats, India". *FEMS Microbiology Ecology*, vol. 96, no. 9, pp.1-17, 2020.
25. A. Domka, P. Rozpądek. R. Ważny and K. Turnau, "*Mucor* Sp . - An Endophyte of Brassicaceae Capable of Surviving in Toxic Metal-Rich Sites". *Journal of Basic Microbiology*, vol. 18, pp. 24–37, 2019.
26. P. Rozpądek, A. Domka, R. Ważny, M. Nosek, R. Jędrzejczyk, K. Tokarz and K. Turnau, "How Does the Endophytic Fungus *Mucor* sp. Improve *Arabidopsis arenosa* Vegetation in the Degraded Environment of a Mine Dump?". *Environmental and Experimental Botany*, 147: 31–42, 2018.
27. H.N. Yousefi, K.B. Hassanzadeh and F.B. Firouzjahi, "Identification and Determination of Characteristics of Endophytes from Rice Plants and Their Role in Biocontrol of Bacterial Blight Caused by *Xanthomonas Oryzae* Pv . *oryzae*". *Hellenic Plant Protection Journal*, vol. 11, pp.19 -33, 2018.
28. D.N.T. Thi and C.N. Diep, "Isolation , Characterization and Identification of Endophytic Bacteria in Maize (*Zea Mays* L .) Cultivated on Acrisols of the Southeast of Vietnam". *American Journal of Life Sciences*. Vol. 2, no. 4, pp. 224–33, 2014.
29. M.P. Prasad and S. Dagar, "Identification and Characterization of Endophytic Bacteria from Fruits Like Avacado and Black grapes". *International Journal of Current Microbiology and Applied Science*, vol. 3, no. 8, pp. 937-947, 2014.
30. A. Adienge, G. Muturi, S. Nadir, J. Gicheru, J. Kinyua and J. Ngaira, "Genetic diversity and population structure of three commercial indigenous Aloe species in selected ASALs of Kenya". *Journal of Plant Science & Molecular Breeding*, vol. 8, no. 1,

pp. 1-10, 2019.

31. K. Tamura, D. Peterson, N. Perterson, G. Stecher, M. Nei and S. Kumar, "MEGA 6.0: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods". *Journal of Molecular Biology and Evolution*, vol. 28, no. 10, pp. 2731-2739, 2013.
32. K. Tamura, D. Peterson, N. Perterson, G. Stecher, M. Nei and S. Kumar, "MEGA 5.0: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods". *Journal of Molecular Biology and Evolution*, vol. 28, no.10, pp. 2731-2739, 2011.
33. I. Benjelloun, A.I. Thami, A. Douira and S.M. Udupa, "Phenotypic and Genotypic Diversity Among Symbiotic and Non-symbiotic Bacteria Present in Chickpea Nodules in Morocco". *Frontiers in Microbiology*, vol. 10, no. 1885, pp.1-19, 2019. doi: <https://10.3389/fmicb.2019.01885>
34. M. Katoch and S. Pull, "Endophytic Fungi Associated with *Monarda citriodora*, an Aromatic and Medicinal Plant and Their Biocontrol Potential." *Pharmaceutical Biology*, vol. 55, no. 1, pp. 1528-1535, 2017.
35. L.S. Kandel, M.P. Joubert and S.L. Doty, "Bacterial Endophyte Colonization and Distribution within Plants". *Microorganisms*, vol. 5, no. 77, pp.1-26 <https://doi:10.3390/microorganisms5040077>
36. H. Liu, L.C. Carvalhais, M. Crawford, E. Singh, P.G. Dennis, C.M.J. Pieterse and P.M. Schenk, "Inner Plant Values: Diversity, Colonization and Benefits from Endophytic Bacteria". *Frontiers in Microbiology*, vol. 8, no. 2552, pp. 1–17, 2017.
37. V.T.P. Nhu and C.N. Diep, "Isolation and Characterization of Endophytic Bacteria in Soybean (*Glycine max* L. (Merrill) Cultivated on Alluvial Soil of Can Tho city, Vietnam". *International Journal of Innovations in Engineering and Technology*, 8:208-221, 2017.
38. Y. Sondang, K. Anty and R. Siregar, "Identification of Endophytic and Rhizosphere Bacteria in Maize (*Zea mays* L.) in Limapuluh Kota Region, West Sumatra, Indonesia. 6th International Conference on Sustainable Agriculture, Food and Energy. IOP Conf. Series". *Earth and Environmental Science*, vol. 347, pp.1-8, 2019.
39. S.A. Padder, G.H. Dar, Z.A. Bhat and K. Verma, "Morphological Metabolic and Biochemical Characterization of Bacterial Root Endophytes Associated with Brown Sarson (*Brassica rapa* L.)". *Journal of Pharmacognosy and Phytochemistry*, vol. 6, no. 2, pp. 226-232, 2017.
40. S. Sinha, S. Choubey, K.A. Ajay, P. Bhosale, "Identification, Characterization of Pigment Producing Bacteria from Soil and Water and Testing of Antimicrobial Activity of Bacterial Pigments". *International Journal of Pharmaceutical Sciences Review and Research*, vol. 42, no. 2, pp.119-124, 2017.
41. R. Singh, K.D. Pandey, M. Singh, S.K. Singh, A. Hashem, A.-B.F. Al-Arjani, E.F. Abd Allah, P.K. Singh and A. Kumar, "Isolation and Characterization of Endophytes Bacterial Strains of *Momordica charantia* L. and Their Possible Approach in Stress Management". *Microorganisms*, vol.10, pp.1-14, 2022. <https://doi.org/10.3390/microorganisms10020290>
42. B. I. G. Maulani, D. A. C. Rasmi and L. Zulkifli, "Isolation and Characterization of Endophytic Bacteria from Mangrove *Rhizophora mucronata* Lam. and Antibacterial Activity Test Against Some Pathogenic Bacteria". *Journal of Physics: Conference Series*, pp.1-7, 2019. <https://doi:10.1088/1742-6596/1402/3/033038>
43. F. Kawaka, "Characterization of symbiotic and nitrogen fixing bacteria". *AMB Expr* **12**, 99 (2022). <https://doi.org/10.1186/s13568-022-01441-7>
44. M.J. Ek-Ramos, R. Gomez-Flores, A.A. Orozco-Flores, C. Rodríguez-Padilla, G. González-Ochoa and P. Tamez-Guerra, "Bioactive Products From Plant-Endophytic Gram-Positive Bacteria". *Frontiers In Microbiology*. Vol. 10, pp.1-12, 2019.
45. K.L. Rana, D. Kour, T. Kaur, R. Devi, A.N. Yadav, N. Yadav, H.S. Dhaliwal and A.S.Saxena "Endophytic Microbes: Biodiversity , Plant Growth-Promoting Mechanisms and Potential Applications for Agricultural Sustainability". *Springer International Publishing, Antonie van Leeuwenhoek*, vol. 113, pp. 1075–1107, 2020.
46. T. Horiike, "An Introduction To Molecular Phylogenetic Analysis. *Reviews in Agricultural Science*, vol. 4, pp. 36- 45, 2016.
47. G. Munjal, S. Sangeet and H. Madasu, "Phylogenetic Methods and its Applications". *Open Access Biostatistics and Bioinformatics*, vol. 1, no. 10, pp. 1-3, 2018.
48. P.M. Chimwamurombe, L.G. Jann and B. Reinhold-Hurek, "Isolation and characterization of culturable seed-associated bacterial endophytes from gnotobiotically grown Marama bean seedlings". *FEMS Microbiology Ecology*, vol. 92, no. 6, pp. 1- 11, 2016.

49. Q. Zhang, J.A. Jacqueline, G.I. Nitz, L.M. María, R. Sergio, J.S. Michael and A.J. Milko, "Endophytic Bacterial Communities Associated with Roots and Leaves of Plants Growing in Chilean Extreme Environments". *Scientific Reports*, vol. 9, pp.1–12, 2019.
50. A.A. Elmagzob, M.M. Ibrahim and G.Zhang, "Seasonal Diversity of Endophytic Bacteria Associated with *Cinnamomum camphora* (L.) Presl". *Diversity*, vol. 11, no. 112, pp 1-15, 2019.
51. S. Card, L. Johnson, S. Teasdale and J. Caradus, "Deciphering Endophyte Behaviour: The Link between Endophyte Biology and Efficacious Biological Control Agents". *FEMS Microbiology Ecology*, vol. 92, no. 16, pp. 1–19, 2016.
52. V. Maggini, A. Mengoni, E.R. Gallo, S. Biffi, R. Fani, F. Firenzuoli and P.Bogani, "Tissue Specificity and Differential Effects on in Vitro Plant Growth of Single Bacterial Endophytes Isolated from the Roots, Leaves and Rhizospheric Soil of *Echinacea purpurea*". *BioMed Central Plant Biology*, vol. 19, no. 284, pp.1–9, 2019.
53. B. Iyer and S. Rajkumar, "Host Specificity and Plant Growth Promotion by Bacterial Endophytes". *Current Research in Microbiology and Biotechnology*, vol. 5, no. 2, pp. 1018-1030, 2017.
54. W. O. Emitaro, "Diversity, Antagonistic Potential of Endophytes, Phytochemicals and Antimicrobial Activity of Selected Agroforestry Trees Against *Xanthomonas Campestris* Pv. *Musacearum* and *Cercospora Zeae-Maydis* (Doctoral Thesis, Maseno University)". [http https:// repository.maseno.ac.ke](https://repository.maseno.ac.ke)

Tables

Table 1. Percentage bacterial endophytes recovered from roots, stems and leaves, of *L. Diversifolia*, *C. Calothyrsus* and *S. sesban*

Plant species	% Bacterial recovery per plant part			
	leaf	stem	root	Total
<i>C. calothyrsus</i>	15.2	12.8	11.9	39.9
<i>L. diversifolia</i>	15.2	11.9	7.1	34.2
<i>S. sesban</i>	14.0	7.1	4.8	25.9
Total (%)	44.4	31.8	23.8	

Table 2. Morphological characteristics of bacterial isolates from *C. calothyrsus*, *L. diversifolia* and *S. sesban*

Bacterial Isolates	Colony Characteristics				<i>C. calothyrsus</i>			<i>S. sesban</i>			<i>L. diversifolia</i>			G. stain	shape
	colour	elevation	margin	opacity	L	S	R	L	S	R	L	S	R		
<i>BLL4, BLS5</i>	White	flat	entire	translucent	-	-	-	-	+		+			+ve	cocci
<i>BRC1, BSL1, BSC1, BLS1, BSS1, BRS1, BLC1,</i>	Yellow	raised	entire	opaque	+	+	-	+	+	+	+	+	+	-ve	cocci
<i>BSL3, BLL3, BSS2</i>	White	raised	entire	opaque	+	+	+	+	+	+	+	+	+	-ve	cocci
<i>BLC3, BRL3, BLS2</i>	White	flat	entire	Translucent	+	-	+	+	-	-	+	+	+	-ve	bacilli
<i>BLC4</i>	White	filamentous	irregular	opaque	+	-	-	+	-	-	+	-	-	-ve	bacilli
<i>BLS3, BRC, BSS3, BLC5, BRS3, BSC2, 3</i>	Cream	raised	entire	opaque	+	+	+	+	+	+	-	+	-	-ve	bacilli
<i>BRC5, BSC5, BLC6</i>	White	raised	undulated	opaque	+	+	-	-	-	-	-	-	-	+ve	bacilli
<i>BLL6, BSC3</i>	Light yellow	raised	entire	opaque	-	+	-	-	-	-	+	-	-	-ve	bacilli

Key: L- leaves, S-stem, R-roots, +-present, -absent

Table 3: Maximum nucleotide identity matches of bacterial isolates based on 16S rRNA sequences

NO	Isolate ID (GeneBank Accession)	Match identity (%)	Species	Genus % dominance	Phylum % dominance	
1	BLL4 (MW251519.1)	99.86	<i>Staphylococcus pasteurii</i>	Staphylococcus 22.2%	Firmicutes 44%	
2	BLL6 (MW251521.1)	99.41	<i>Staphylococcus epidermidis</i>			
3	BSL1 (MW251522.1)	99.93	<i>Staphylococcus warneri</i>			
4	BLC4 (MW251536.1)	100	<i>Staphylococcus epidermidis</i>			
5	BLC5 (MW251537.1)	99.49	<i>Staphylococcus sp</i>			
6	BLC6 (MW251538.1)	100	<i>Staphylococcus pasteurii</i>			
7	BSS1 (MW251529.1)	99.78	<i>Bacillus tequilensis</i>	Bacillus 33.3%		
8	BSS2 (MW251530.1)	99.93	<i>Bacillus sp.</i>			
9	BRS3 (MW251533.1)	99.29	<i>Bacillus toyonensis</i>			
10	BLC1 (MW251534.1)	100	<i>Bacillus altitudinis</i>			
11	BLC3 (MW251535.1)	99.48	<i>Bacillus toyonensis</i>			
12	BLL5 (MW251520.1)	99.35	<i>Bacillus toyonensis</i>			
13	BSC1 (MW251539.1)	99.08	<i>Bacillus toyonensis</i>			
14	BSC3 (MW251541.1)	100	<i>Bacillus cereus</i>			
16	BRC1 (MW251543.1)	99.33	<i>Bacillus toyonensis</i>	Alcaligenes 11.2%		
16	BLS1 (MW251525.1)	91.07	<i>Alcaligenes aquatilis</i>			
17	BLS2 (MW251526.1)	87.08	<i>Alcaligenes faecalis</i>			
18	BLS3 (MW251527.1)	98.91	<i>Alcaligenes faecalis</i>			
19	BRC3 (MW251544.1)	99.37	<i>Sphingomonas echinoides</i>		Sphingomonas 7.4%	
20	BRC5 (MW251545.1)	99.22	<i>Sphingomonas echinoides</i>			
21	BSS3 (MW251531.1)	98.19	<i>Acinetobacter johnsonii</i>		Acinetobacter 3.7%	Proteobacteria 66%
22	BLS5 (MW251528.1)	97.81	<i>Enterobacteriaceae bacterium</i>		Enterobacteriaceae 3.7%	
23	BRS1 (MW251532.1)	94.84	<i>Bacterium strain</i>		Bacterium 3.7%	
24	BSC2 (MW251540.1)	99.9	<i>Pantoea agglomerans</i>		Pantoea 3.7%	
25	BSC5 (MW251542.1)	99.03	<i>Pseudomonas plecoglossicida</i>	Pseudomonas 3.7%		
26	BSL3 (MW251523.1)	99.93	<i>X.campestris pv. campestris</i>	Xanthomonas 7.4%		
27	BRL3 (MW251524.1)	99.85	<i>X.campestris pv. campestris</i>			

Figures

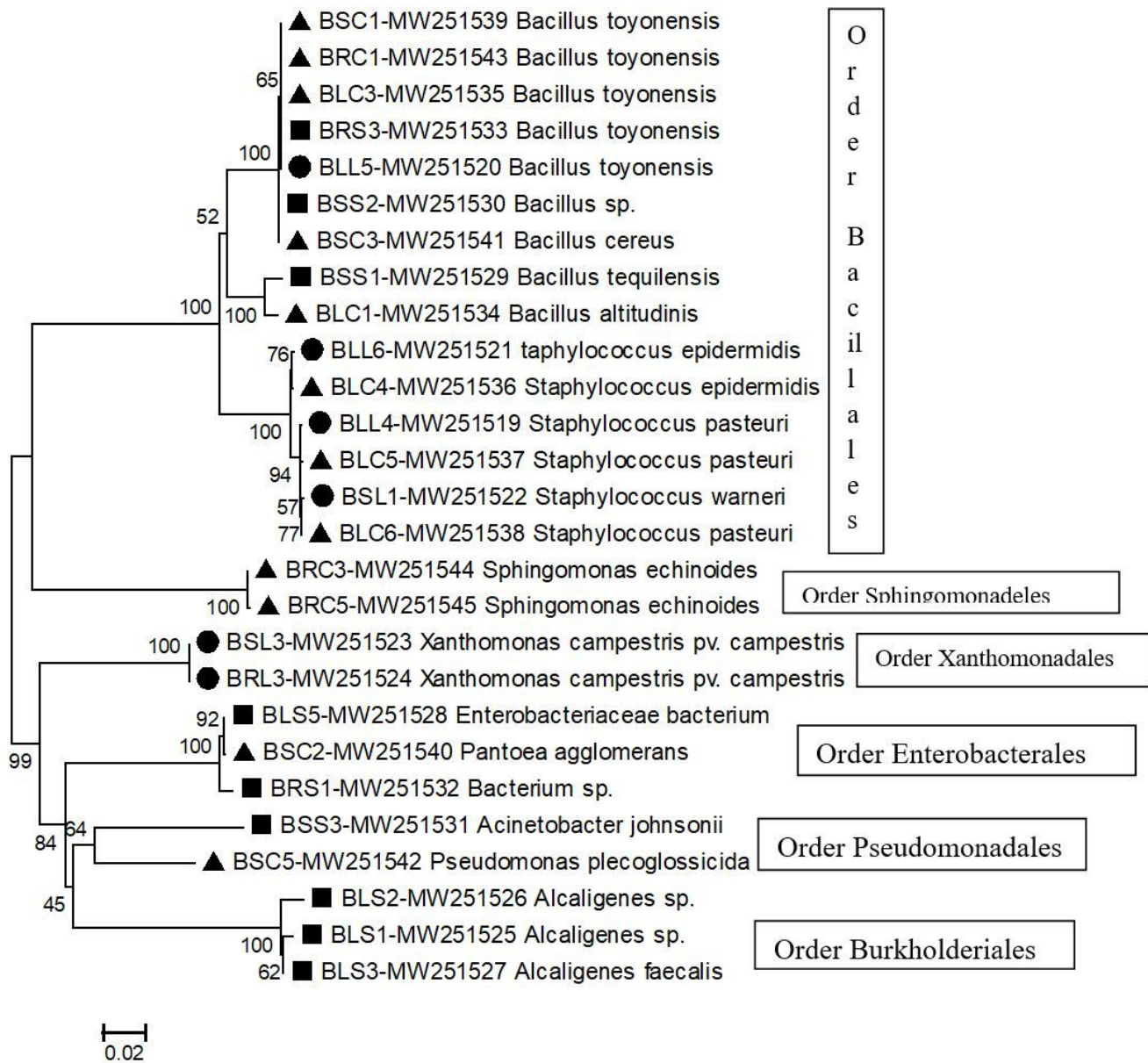


Figure 1

Neighbour joining phylogenetic tree of bacterial isolates of *S. sesban*, *C. calothyrsus*, and *L. diversifolia* isolates.

Key: ●- *L. diversifolia* isolates, ■-*S. sesban* isolates,▲-*C. calothyrsus* isolates