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Diversity of endophytic bacteria in different tissues of sengon (*Falcataria moluccana*)

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Abstract. *Dewi M, Rahmawati N, Rumidatul A. 2023. Diversity of endophytic bacteria in different tissues of sengon (*Falcataria moluccana*). Biodiversitas 24: 5747-5756.* Endophytes are endosymbionts, usually bacteria or fungi, that live inside plants without causing disease. Endophytes are available in all plant species and play a role in enhancing plant resistance to insects, pathogens, and herbivores, increasing host growth, nutrient uptake, and increasing plant tolerance to abiotic stresses such as drought. The endophyte's ability to do all that is a result of the production of biological components or secondary metabolites, which may be the result of co-evolution or genetic transfer between endophytic microorganisms and host plants. In this study, the isolation, characterization and identification of endophytic bacteria from several different plant organs of sengon (*Falcataria moluccana* (Miq.) Barneby & J.W. Grimes) was carried out using the plant piece method in nutrient agar medium, and identification of selected isolates was carried out based on 16S rRNA. 16S rRNA sequencing results showed that 31 endophytic bacterial communities were successfully isolated from various parts of the sengon tree. Bacteria isolated from the stem were 3 strains, bark 7 strains, leaves 11 strains, and galls 10 strains. Molecular identification and phylogenetic analysis showed that the endophytes on the stem were *Curtobacterium citreum* and *C. luteum*, respectively. The endophytic bacteria on the bark are *C. herbarum, C. luteum, C. pusillum, C. citreum* and *Azotobacter chroococcum*. The endophytic bacteria on the leaves are *A. chroococcum, C. citreum, C. herbarum* and *Lysinibacillus sphaericus*. The endophytic bacteria in the gall are *Enterobacter ludwigii, Kosakonia radicincitans, Citrobacter gizzii* and *Erwinia endophytica*.

Keywords: Diversity, endophytes bacteria, *Falcataria moluccana*, molecular identification

INTRODUCTION

Endophytic microbes are naturally present in healthy plant organs, and approximately 300,000 plant species have been reported to host one or more endophytic microbes (Aleynova et al. 2023). Endophytic microbes can live in plant tissues at certain stages in their life cycle and can live in plant tissues by forming colonies without harming the host (Afzal et al. 2019). Endophytic bacteria are one of the endophytic microorganisms that live in the host plant tissue and do not cause disease symptoms (Indrawati et al. 2021). The presence of endophytic microbes, both fungi and endophytic bacteria, in host tissues, whether in roots, stems, leaves or tree bark, has been widely studied.

Rahmawati et al. (2016) has examined endophytic fungi in *Toona sinensis* plants on stems with various plant ages. In addition, Rumidatul et al. (2021) also revealed that there are endophytic fungi in the sengon (*Falcataria moluccana* (Miq.) Barneby & J.W. Grimes) tree gall that have antioxidant activity. Meanwhile, research on endophytic bacteria has also been widely reported in various studies. Wheat has a diverse population of endophytic bacteria, which varies according to the plant organ, reflecting its host-specific adaptations and interactions. Some organ-specific colonization and developmental stages of wheat endophytic bacteria may indicate a significant impact of plant physiological conditions on bacterial growth and reproduction (Pang et al. 2022).

Across plant organs and growth phases, the makeup of endophytic bacterial communities has been studied in several species. Peredo and Simmons (2018) found that certain bacterial taxa could only be isolated from the reproductive organs of grapes (*Vitis vinifera* L.) using fluorescence in situ hybridization (FISH) and microbial culture. Dadzie et al. (2022**)** isolated more bacterial taxa from seedlings of ginger (*Zingiber officinale* Rosc.) than from any other stage of plant development. According to Pang et al. (2022), the bacterial community associated with *Stellera chamaejasme* L. is determined by habitat (rhizosphere vs endosphere) and organ (leaf, stem, and root). Similar bacterial profiles between leaves and stems and plant rhizosphere from the endophytic microbial community of noni (*Morinda citrifolia* L.) has shown significant relationships (Liu et al. 2015).

The potential of endophytic microbes in improving host plant fitness and producing beneficial bioactive compounds has received much attention. Some types of endophytic bacteria are known to produce antibiotics (Martinez-Klimova et al. 2017), antimalarials (Baba et al. 2015), as well as antifungals (Al-Nadabi et al. 2021).

Each higher plant may contain several endophytic microorganisms capable of producing biological compounds or secondary metabolites, which are thought to be the result of co-evolution or genetic transfer of the host plant into the endophytic microorganisms (Rutkowska et al. 2023). Secondary metabolites produced by endophytic

microorganisms can stimulate plant growth or repel plant pathogens. It has been recognized that these microorganisms have great potential as a source of new bioactive compounds in agriculture, pharmaceuticals and industry. The potential of endophytic bacteria and PGPR (Plant Growth Promoting Rhizosphere) to reduce or prevent the harmful effects of phytopathogenic organisms can be seen as an indirect way to improve plant growth. Plants treated with endophytic bacteria isolated from chickpea root nodules demonstrated features that increased plant growth, enhanced symbiotic performance of host plants with rhizobia, and increased crop output in high saline environments. Additionally, there was an increase in proline and a drop in H_2O_2 concentration, both of which pointed to a reduced amount of root rot infection (Egamberdieva et al. 2017). The potential of endophytic microbes, especially endophytic bacteria from sengon trees, has not been widely reported. Some higher plants that can be used as a source of endophytic microbes include forest trees that have not been widely utilized, such as sengon trees. The inhabitants of West Java frequently plant sengon trees in community forests that use agroforestry technologies. This is because they grow quickly, their open crowns allow sunlight to reach the forest, they can fix nitrogen, and they can boost productivity in community forests (Tsaniya et al. 2022). Previous research also showed that secondary metabolites of sengon and endophytic microbes found in sengon have antimicrobial and antioxidant activities (Rumidatul et al. 2021a; Rahmawati et al. 2023). In this study, endophytic microbes, especially endophytic bacteria, were isolated and identified to determine their diversity in various sengon plant tissues as a first step in utilizing endophytic microbes in forest plants.

MATERIAL AND METHODS

Isolation of endophytic bacteria

All endophytic bacteria were isolated from leaf, stem, bark and gall specimens of sengon (*Falcataria moluccana* (Miq.) Barneby & J.W. Grimes) collected from ITB Jatinangor Campus, Sumedang, West Java, Indonesia. The sengon trees selected were those with gall rust to make specimens that will be studied for endophytic bacterial diversity. Isolation of endophytic bacteria from plant specimens was carried out according to Bhore and Satisha (2010) with a little modification. The samples were washed with running water until clean, then cut at about 1-3 cm each. The cut samples were subjected to gradual surface sterilization. The sample pieces were immersed in 96% ethanol for 1 minute, followed by 5.25% Na-hypochlorite for 5 minutes, and then rinsed again in 96% ethanol three times. The sterilized samples were then inoculated in nutrient agar (NA) media containing nystatin (0.01% w/v) and then incubated at room temperature and observed until there was colony growth. Purification was carried out by transferring one loop of bacterial colonies into NA media. After obtaining a pure culture, the endophytic bacteria were transferred to NA slant agar.

Molecular identification

DNA isolation and purification genomic

DNA isolation from bacterial cultures was carried out using a modified Genomic DNA Mini Kit (Geneaid).

16S rRNA gene sequencing

The sequencing was carried out to determine the nucleotide sequence of the detected DNA fragments from the amplified DNA visualization in the PCR process using an auto sequencing machine. The sequencing process was carried out by sending samples to Macrogen Inc. in Singapore.

DNA sequence data processing

The sequencing process was carried out at Macrogen Inc., Singapore. Then the results were analyzed and edited using BioEdit software. The results of the sequences were compared with existing sequences using the Basic Local Alignment Search Tool program on the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov) to obtain homologies. The sequence obtained from the Basic Local Alignment Search Tool was then analyzed using MEGA 6.02 (Arizona State University) software to determine the level of kinship. Phylogenetic tree constructions are made using a character-based parameter model, maximum likelihood. A bootstrap method with 1000 replicates was used to evaluate the phylogenetic tree. The similarity value for each isolate was calculated manually using a scale generated by the software.

RESULTS AND DISCUSSION

Diversity of endophytic bacteria in sengon plants

The results showed that a total of 99 endophytic bacteria isolates were isolated from sengon trees, 35 isolates were obtained from leaves, 32 isolates from gall, 22 isolates from bark and 10 isolates from stem (Figure 1).

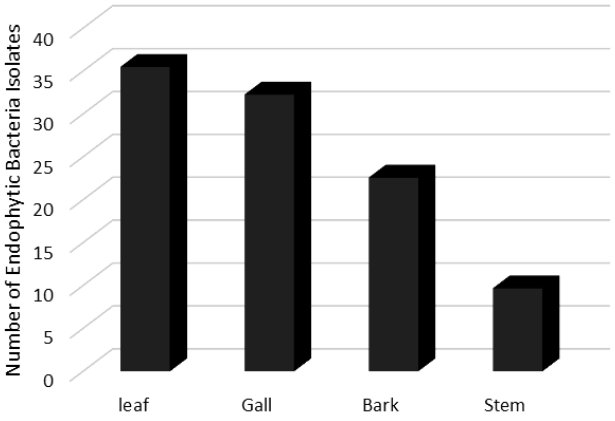

Figure 1. The distribution of endophytic bacteria isolated from sengon tree

Based on differences in morphological characteristics, 31 isolates were selected for molecular identification. Most of the endophytic bacterial isolates were beige in color, irregular in shape, entire edges, raised and convex elevations, and shiny/opaque surfaces. A total of 31 bacterial isolates were identified at the molecular level, out of which 11 isolates were isolated from leaves (35.4%), 7 isolates from bark (22.5%), 3 isolates from stems (9.7%) and 10 isolates were isolated from gall (32.2%) (Figure 1). In the selected isolates, both Gram-positive bacteria and Gram-negative bacteria were present (Table 1).

Molecular identification of endophytic bacteria on leaves

Based on the phylogenetic analysis of D01B_1, D01B_2, and D01B_3, molecular characterization of endophytic bacterial isolates found in leaves, showed similarities with *Azotobacter chroococcum* (Figure 2).

Molecular identification results of endophyte D02B based on phylogenetic analysis showed similarities with *Curtobacterium citreum*, while endophytes D05B and D06B had similarities with *C. herbarum* (Figure 3).

Based on the results of phylogenetic analysis, endophytic bacteria D03B, D04B, D07B, and D09B were similar to *C. herbarum*, as shown in Figure 4. Endophytic bacterial isolate D08B was similar to *L. sphaericus* (Figure 5).

Molecular identification of endophytic bacteria from bark

The results of phylogenetic analysis showed that endophytic isolates K01B_1 and K01B_2 were similar to *C. herbarum*, while isolate K02B was similar to *C. luteum* (Figure 6).

Endophytic bacterial isolates K03B and K04B were found similar to *A. chroococcum* in tree-based phylogenetic analysis, as shown in Figure 7.

Table 1. Number and types of endophytic bacteria isolated from several parts of sengon trees

Plant tissues	Endophytic bacteria	Amount	Role on the host plant	Genus	Family/Gram
Leaves	*A. chroococcum*	3	PGPR, sintesis vitamin B, IAA, Giberelin (Ayesha et al. 2023). Increase N total, uptake N (Aasfaar et al. 2021)	*Azotobacter*	Pseudomonadota (Gram-negative bacteria)
	C. citreum	1	PGPR (Díez-Méndez and Rivas 2017)	*Curtobacterium*	Microbacteriaceae (Gram-positive bacteria)
	C. herbarum	6	PGPR (Díez-Méndez and Rivas 2017)	*Curtobacterium*	Microbacteriaceae (Gram-positive bacteria)
	L. sphaericus	1	Biological control agent (Santana-Martinez et al. 2019). Increase growth, and N nutrient uptake (Istifadah et al. 2017).	*Lysinibacillus*	Bacillaceae (Gram-positive bacteria)
	Sub Total	11			
Bark	*C. herbarum*	2	PGPR (Díez-Méndez and Rivas 2017)	*Curtobacterium*	Microbacteriaceae (Gram-positive bacteria)
	C. luteum	1	Phytoremediation (Suhandono et al. 2016).	*Curtobacterium*	Microbacteriaceae (Gram-positive bacteria)
	A. chrococcum	2	PGPR, sintesa vitamin B, IAA, Giberelin (Ayesha et al. 2023). Increase N total, uptake N (Aasfaar et al. 2021)		Pseudomonadota (Gram-negative bacteria)
	C. pusillum	1		*Curtobacterium*	Microbacteriaceae (Gram-positive bacteria)
	C. citreum	1	PGPR (Díez-Méndez and Rivas 2017)	*Curtobacterium*	Microbacteriaceae (Gram-positive bacteria)
	Sub Total	7			
Stem	*C. citreum*	2	PGPR (Kabir et al. 2023)	*Curtobacterium*	Microbacteriaceae (Gram-positive bacteria)
	C. luteum	1	Phytoremediation (Suhandono et al. 2016).	*Curtobacterium*	Microbacteriaceae (Gram-positive bacteria)
	Sub Total	3			
Gall	*E. ludwigii*	2	Pathogen in palm dates (Abedinzadeh et al. 2023)	*Enterobacter*	Enterobacteriaceae (Gram-negative bacteria)
	Kosakonia radicincitans	2	Increase the growth of Tomato (Berger et al. 2017). Nitrogen fixation, hormone production, phosphat solubilizer, Induce Plant Resistance (Romera et al. 2019). Nitrogen fixation, remediation (Chen et al. 2020).	*Kosakonia*	Enterobacterriaceae (Gram-negative bacteria)
	C. gillenii	3	Production of secondary metabolite of medicinal plant (Ahmad et al. 2019).	*Citrobakter*	Enterobacterriaceae (Gram-negative bacteria)
	E. endophytica	3	Plant pathogen	*Erwinia*	Erwiniaceae (Gram-negative bacteria)
	Sub Total	10			
	Total	31			

Endophytic bacterial isolate K05B had similarities with endophytic bacterial isolate *C. pusillum*, while endophytic bacterial isolate K06B, had similarities with *C. citreum* based on phylogenetic tree analysis, as shown in Figure 8.

Molecular identification of endophytic bacteria from stem

The results of molecular identification showed that only three endophytic bacterial isolates were found associated with sengon stem. Two of the three bacterial isolates, B01B_1 and B01B_2, were similar to *C. citreum*, while B02B was similar to *C. luteum* (Figure 9).

Molecular identification of endophytic bacteria from galls

The results of phylogenetic analysis revealed that bacterial isolates G01B and G03B were similar to *E. ludwigii*, and bacteria G02B and G05B were similar to *K. radicincitans* (Figure 10), while endophytic bacterial isolates G04B, B06B_1, and G06B_2 were similar to *C. gillenii*. Endophytic bacterial isolates G07B_1, G07B_2, and G07B_3 were found similar to *E. endophytica* (Figure 11).

Types and abundance of bacteria found in sengon plant tissues

There may be a relationship between bacterial endophytes and the health of sengon trees and other ecologically significant functions. Multiphasic analysis was used to determine the makeup of the endophytic bacteria inhabiting the various organ tissues of the sengon. Analysis of the 16S rRNA genes' terminal restriction fragment length polymorphism revealed the influence of various tissue parts on the structure of endophytic population. Endophytic bacteria were thoroughly analyzed using cultivation techniques in conjunction with the cloning of 16S rRNA genes amplified from plant tissue. A total of 99 samples were isolated from various organ tissues, resulting in 10 different species in 5 families, including Pseudomonadota, Microbacteriaceae, Bacillaceae, Enterobacteriaceae, and Erwiniaceae (Table 1). In terms of bacterial taxa present and their relative abundances in each tissue organ, the community structure clearly showed differences between them.

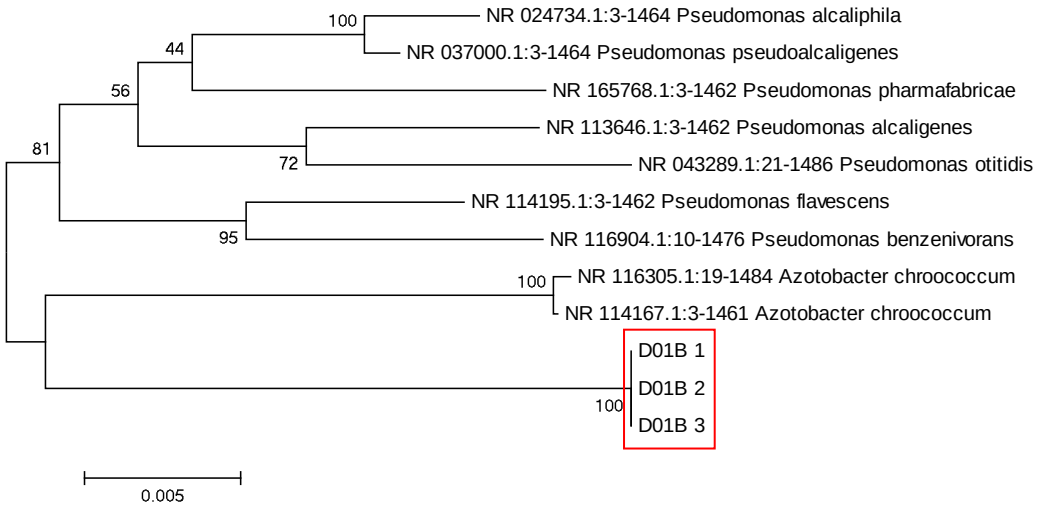

Figure 2. Dendrogram of phylogenetic analysis of endophytic bacteria (D01B_1, D01B_2, D01B_3) based on 16rRNA sequences using the neighbor joining method with 1000x bootstrap

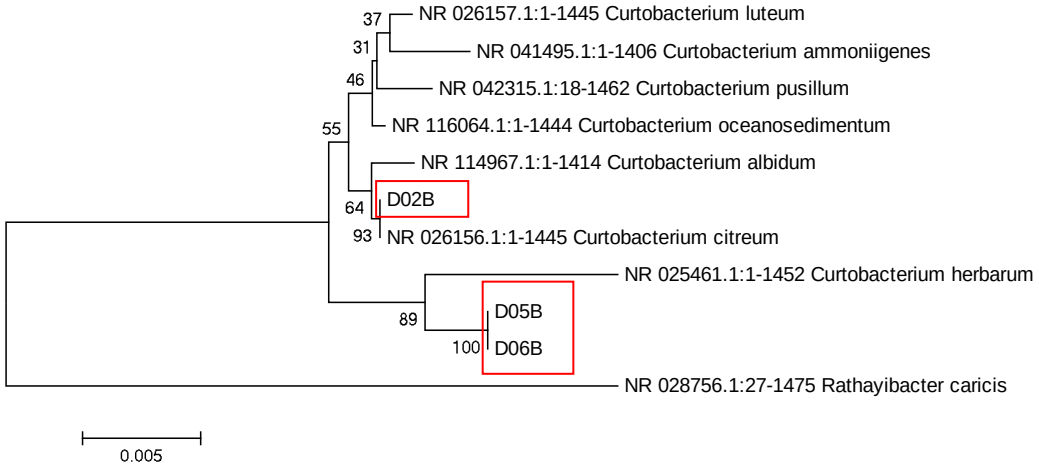

Figure 3. Dendrogram of phylogenetic analysis of endophytic bacteria (D02B, D05B, D06B) based on 16rRNA sequences using the neighbor joining method with 1000x bootstrap

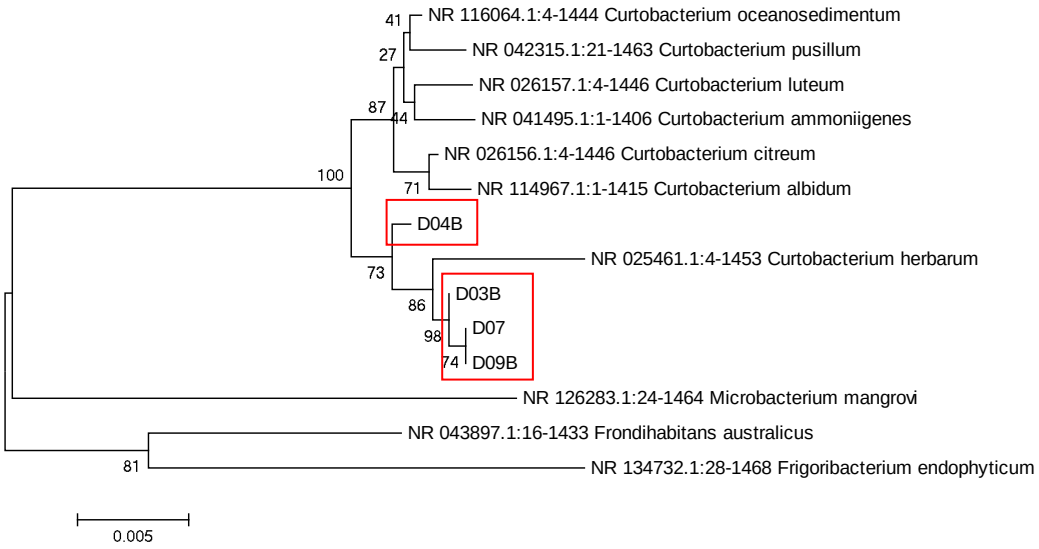

Figure 4. Dendrogram of phylogenetic analysis of endophytes bacteria (D03B, D04B, D07B, D09B) based on 16rRNA sequences using the neighbor joining method with 1000x bootstrap

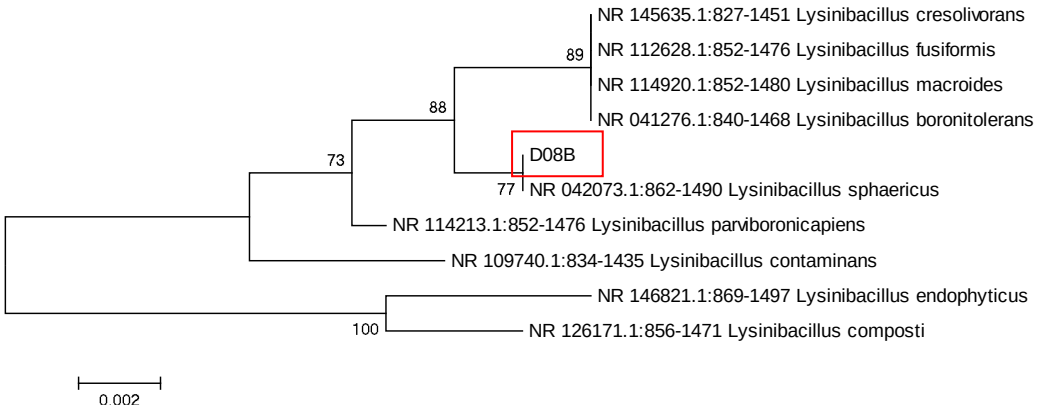

Figure 5. Dendrogram of phylogenetic analysis of endophytes bacteria (D08B) based on 16rRNA sequences using the neighbor joining method with 1000x bootstrap

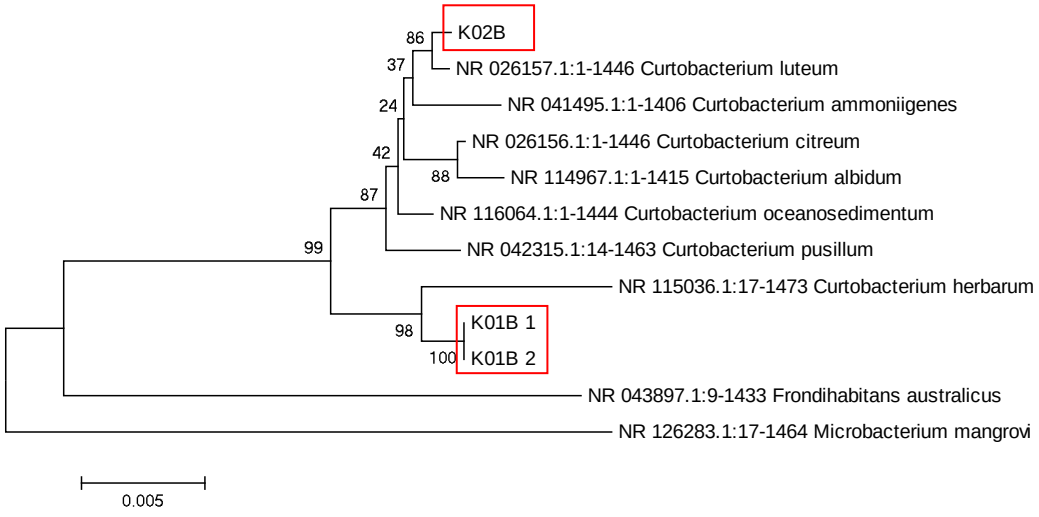

Figure 6. Dendrogram of phylogenetic analysis of endophytes bacteria (K01B_1, K01B_2) based on 16rRNA sequences using the neighbor joining method with 1000x bootstrap

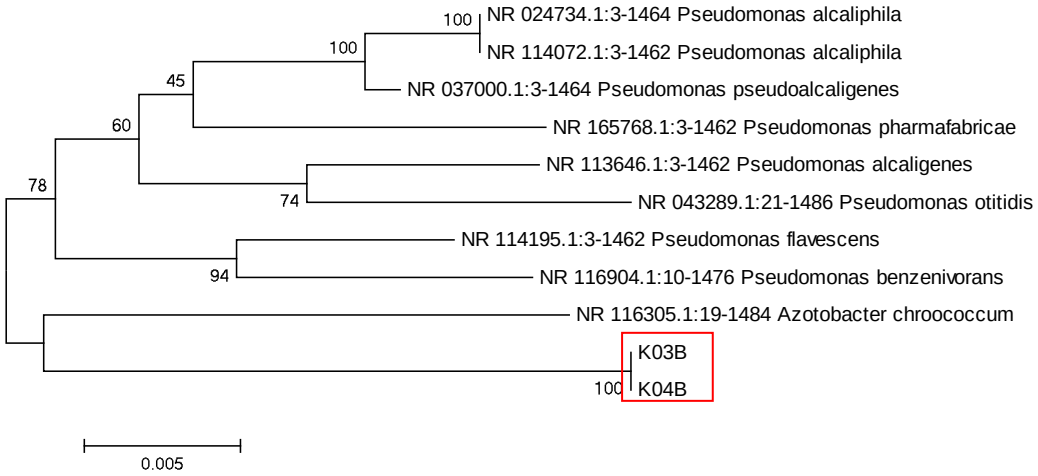

Figure 7. Dendrogram of phylogenetic analysis of endophytes bacteria (K03B, K04B) based on 16rRNA sequences using the neighbor joining method with 1000x bootstrap

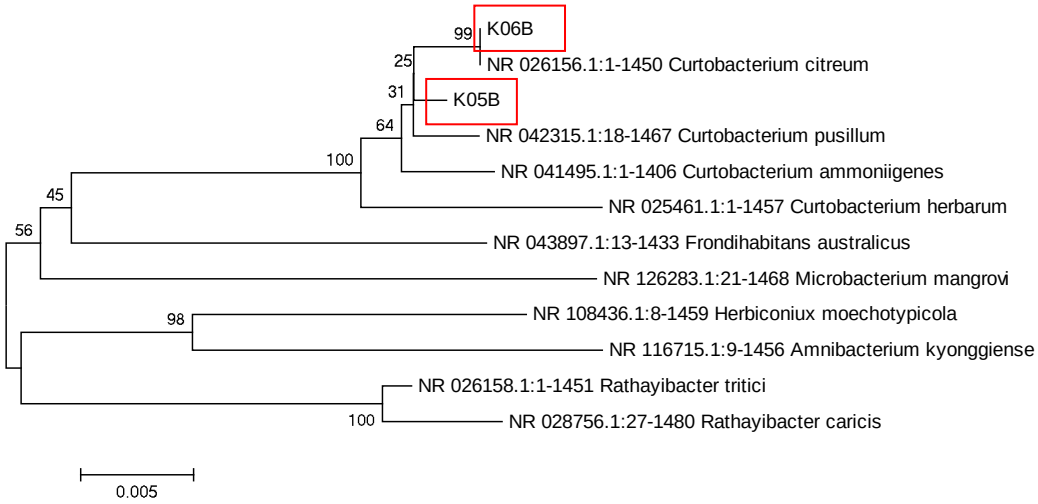

Figure 8. Dendrogram of phylogenetic analysis of endophytes bacteria (K05B, K06B) based on 16rRNA sequences using the neighbor joining method with 1000x bootstrap

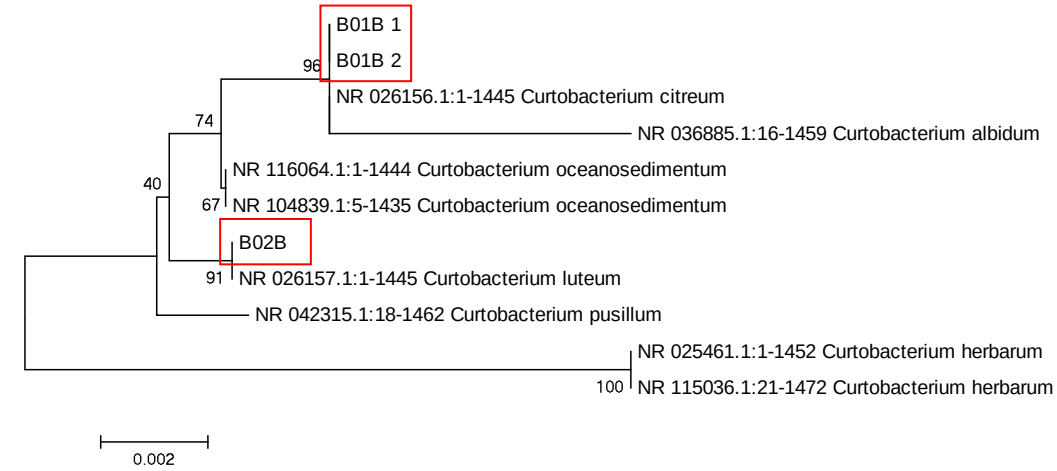

Figure 9. Dendrogram of phylogenetic analysis of endophytes bacteria (B01B_1, B01B_2, B02B) based on 16rRNA sequences using the neighbor joining method with 1000x bootstrap

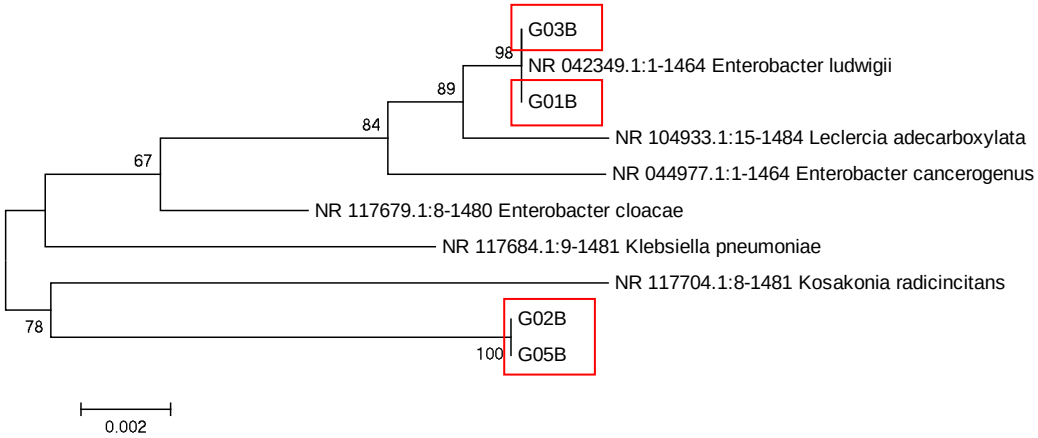

Figure 10. Dendrogram of phylogenetic analysis of endophytes bacteria (G01B, B02B, G03B,G05B) based on 16rRNA sequences using the neighbor joining method with 1000x bootstrap

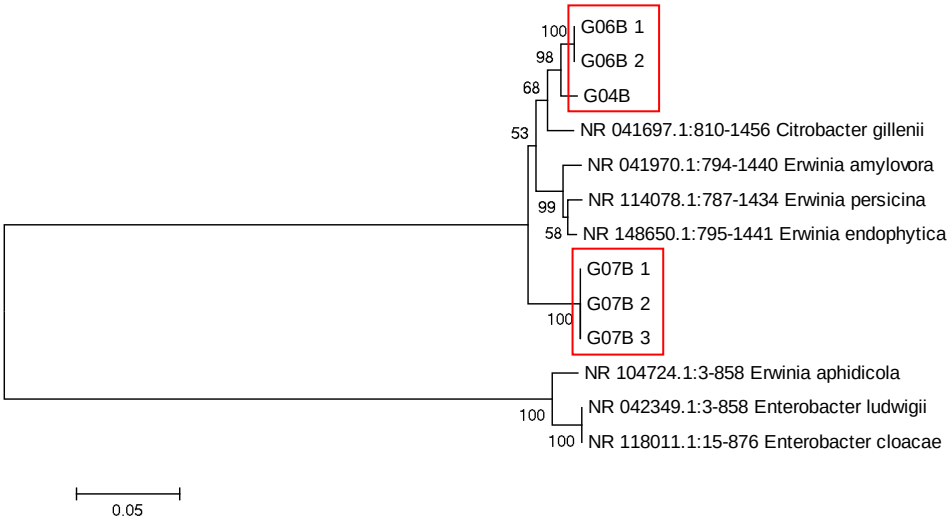

Figure 11. Dendrogram of phylogenetic analysis of endophytes bacteria (G04B, B06B_1, G06B_2, G07B_1, G07B_2,G07B_3) based on 16rRNA sequences using the neighbor joining method with 1000x bootstrap

Discussion

Based on the number of endophytic bacteria obtained, the highest number of endophytic bacteria isolates were obtained from leaves, followed by gall, bark and stem. This is thought to be due to the ability of each endophytic strain to occupy their respective niches or habitats, so that the endophytic population between plant parts is different. The variation in endophytic microbes found indicates that the habitat of endophytes determines the abundance of endophytic populations in plant tissues.

Identification of endophytes can be done by observing the morphological characteristics of endophytic colonies or molecularly. Endophytic bacterial isolates usually had rod-shaped cells, but some were round. This is similar to the research of Singh et al. (2021) that endophytes isolated from sugarcane plants (cv. SP80-1842**)** are generally rod-shaped. Gram staining method is used to distinguish gram-positive and gram-negative bacteria. The difference is related to the chemical composition of the cell wall. Gram-positive cells have a thick layer of peptidoglycan covering their walls, while gram-negative cells are thinner and covered by an outer membrane layer consisting of lipids. Morphological observations of bacterial isolates included color, shape, height, edge shape, and surface texture. The isolates obtained were generally white, yellow-white, yellow, round, prominent, with flat edges and shiny surface texture. This indicates the diversity of endophytic bacteria on the bark, stem, leaves, and stump of sengon plants. Some researchers reported that endophytic bacteria are known to grow and develop in plant tissues (xylem, phloem) of leaves, roots, and healthy stems at a certain time without causing damage to the host (Adeleke et al. 2021).

Endophytic microbes could be found in every plant because no studies had shown the presence of plant species without endophytes. High species diversity is another characteristic of endophytic microbes (Anand et al. 2023). The highest number of endophytic bacteria were

discovered in maize plants at the flowering stage, followed by the vegetative stage and the maturity stage. Based on plant organs, the highest number of bacteria were discovered in roots, followed by stems and leaves (Marag and Suman 2018). The results of research on the variation of endophytic bacteria in orchid plants both above and below ground found that most of the endophytic bacteria found belonged to the Proteobacteria and Actinobacteria groups with the structure of the bacterial community largely determined by plant organs. In addition, it was also found that in a selective and systematic way there was an increase in the bacterial community from vegetative organs to reproductive organs (Alibrandi et al. 2020).

Endophytic microbes can be found on every plant as no study has shown any plant species without endophytes. High species diversity is another characteristic of endophytic microbes. *Azotobacter chroococcum*, which belongs to the proteobacteria phylum, was successfully isolated from sengon leaves and bark. It is known that *Azotobacter* is one of the bacteria that acts as a plant growth-promoting bacteria that can help increase plant growth through nitrogen fixation. In addition, it is reported that *Azotobacter* is able to synthesise vitamin B, indole acetic acid, and gibberellins, Zhu et al. (2021), which makes plants quickly absorb NO_3 and NH_4^+. The results of present study are in line with the research of Aasfar et al. (2021) which states that *Azotobacter* sp. can increase total N in soil and plant N uptake. Soil conditions contaminated with Cd up to 20 mg kg1 did not prevent *A. chroococcum* from facilitating plant nitrogen uptake. In this study, the genus *Curtobacterium* were isolated from the leaves, bark, and trunk of sengon trees. *Curtobacterium* is a member of the phylum Actinobacteria. It was reported that some *Curtobacterium* species, such as *Curtobacterium flaccumfaciens*, have been shown to act as biocontrol agents against pathogens by stimulating plant resistance systems and antibiosis mechanisms (Koscak et al. 2023).

Several researchers reported that *Curtobacterium* has found as endophytic bacteria on maize (Wallace 2023), soybean (Dimkić et al. 2021), grapevine (Pacifico et al. 2019), potato (Shi et al. 2021), and red clover (Tshikhudo et al. 2023). Endophytic bacteria, *Curtobacterium*, was also found in Rambutan fruits (*Nephelium lappaceum* L.), cultivar Binjai, which are suspected to play a role as plant growth-promoting bacteria. Suhandono et al. (2016) reported that *Curtobacterium luteum* was found as endophytic bacteria in a phytoremediation plant, the poplar tree, but there was not enough information about the role of these bacteria in plants. *Lysinibacillus sphaericus* is also found in *Oryza sativa* (Shabanamol et al. 2020). The genus *Lysinibacillus* belongs to the phylum Firmicutes. *L. sphaericus*, known previously as *Bacillus sphaericus*, is usually found in soil. These bacteria are reported to be used as biological control agents. In addition, *L. sphaericus* can control *Aedes aegypti* larvae effectively. Santana-Martinez et al. (2019) reported that endophytic diazotrophic bacteria *Lysinibacillus sphaericus* was studied to know the effect of its bacteria on growing a rice crop under greenhouse conditions. *Lysinibacillus sphaericus* inoculation on rice crops resulted in higher yield and nutrient uptake than the unisolated control Istifadah et al. (2017). Endophytic bacteria *E. ludwigii* was isolated from sengon tree gall. The genus *Enterobacter* belongs to the phylum Proteobacteria. Some Enterobacter species can be potential plant pathogens, and some Enterobacter species appear to have favorable relationships with plant hosts. *E. ludwigii* is found in soil, water, and plants (Wei et al. 2022). *Erwinia endophytica* sp. nov., isolated from potato (*Solanum tuberosum* L.) stems (Ji et al. 2019).

Kosakonia radicincitans, which was isolated from the gall of the Sengon tree, belongs to the phylum Proteobacteria. *K. radicincitans* can increase the growth of tomato plants (*Solanum lycopersicum*) (Berger et al. 2017). It is reported that *K. radicincitans* can biologically fix atmospheric nitrogen, produce hormones, solubilize rock phosphates, and induce plant resistance (Andrade et al. 2023). It was observed that endophytic bacteria *K. radicincitans*, a free-living strain of nitrogen-fixing bacteria (NF) isolated from corn roots (*Zea mays* L.), can degrade aromatic hydrocarbons (Chen et al. 2020). *Erwinia endophytica* is one of the endophytic bacteria that belong to the phylum Proteobacteria. The genus *Erwinia* is found in association with plants as pathogens, saprophytes, or epiphytes. Sharma and Mallubhotla (2022) found that the highest bacterial densities are usually observed in the roots and decrease progressively from the stem to the leaves. The genus *Curtobacterium* and *Enterobacter* are among the 17 genus of endophytic bacteria found in *Curcuma Heyneana* plants (Sulistiyani and Lisdiyanti 2016).

In conclusion, a total of 99 endophytic bacterial species were isolated from various parts of sengon plant tissues, of which 31 isolates were identified based on morphological and molecular analysis. 11 isolates of endophytic bacteria were found on leaves, 7 isolates on bark, 3 isolates on stem and 10 isolates on gall. The results of molecular identification and phylogenetic analysis showed that endophytic bacteria isolated from all parts of sengon plant consisted of 10 species, namely *Azotobacter chroococcum, Curtobacterium citreum, C. herbarum, Lysinibacillus sphaericus, C. luteum, C. pusillum, Enterobacter ludwigii, Kosakonia radicincitans, Citrobacter gillenii*, and *Erwinia endophytica*. The types of bacterial species inhabiting stem were *C. citreum and C. luteum*, whereas on the bark were *C. herbarum, C. luteum, C. pusillum, C. citreum*, and *A. chroococcum*. Endophytic bacteria found on leaves were *A. chroococcum, C. citreum, C. herbarum*, and *Lysinibacillus sphaericus*, while endophytic bacteria associated with galls were *E. ludwigii, K. radicincitans, C. gillenii*, and *E. endophytica*.

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