Diversity of endophytic fungi isolated from benzoin-producing tree *Styrax sumatrana*

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**Abstract.** *Styrax sumatrana*, native to Sumatra island, Indonesia, is distinctive for its production of benzoin resin. The resin is used for medicinal treatments and commercially valuable. Fungal endophytes are known to produce various bioactive metabolites and may contribute to host resin production or properties. However, the diversity of culturable endophytic fungi associated with *S. sumatrana* were still underexplored. This study was conducted to examine the distribution and diversity of culturable endophytic fungi associated with tree parts; bark, stem, and leaves of *S. sumatrana*. A total of 31 endophytic fungi isolates were obtained from *S. sumatrana* grown in North Sumatera Province, from which 10 genera and 17 species were identified by internal transcribed spacer (ITS) sequence analyses. Among 31 isolates, *Fusarium*, *Phyllostica*, *Neopestalotiopsis* and *Pestalotiopsis* were repeatedly isolated. The species richness \(S\) (10), Margalef index \(D'\) (3.323), Shannon–Wiener index \(H'\) (1.587), and Simpson diversity index \(D_s\) (0.756) and low dominant index \(\lambda\) (0.244) of endophytic fungi in the bark of *S. sumatrana* indicated its high diversity in comparison to other parts of the tree. This finding provides a knowledge of the diversity and organ-type-specificity of endophytic fungal that could be used for biotechnological application in the future.

1. **Introduction**

Fungal endophytes or endophytic fungi are fungi that reside within plant organs either temporarily during- or throughout their life cycle [1]. These endophytes can be found within roots, stems and/or leaves [2,3]. Unlike pathogenic fungi, fungal endophytes do not appear to be harming the host and may even improve the host defense towards pathogens [4]. Other functional roles range from improving host growth [5] and tolerance to stresses [6] to nutrient cycling [7]. These various roles in the ecosystem necessitate deeper investigation on their interaction with their respective host plants.

Certain fungal endophytes may even have distinctive relationship with their host plants and developed capacity to produce similar, if not the same, metabolites as their host [8]. It was even argued that bioactive compounds that were commonly extracted from medicinal plants were actually produced by the endophytes [9]. This finding suggests that the same premise may also be the case for other compounds which are usually extracted from certain plant species and inspire further studies on fungal endophytes residing in such plants.
Fungal endophytes are highly diverse and appear to associate with all plants in natural ecosystems [10], particularly tropical trees [11]. One of the tropical trees of interest is *Styrax*. Many species of this genus are sources for benzoin resin which is used as incense, making them economically valuable. Benzoin resin has also been used as a herbal remedy for its anti-inflammatory [12,13] and antibacterial properties [14]. The resin-producing trees were cultivated largely in North Sumatra, Indonesia, establishing the area as a well-known resin (locally known as *kemenyan*) producer [15]. One of the notable resin-producing *Styrax* species, *S. sumatrana*, is even endemic to this area. Kemenyan resin produced by *Styrax sumatrana* had an antioxidant activity and may be used as an antioxidant resource [12], which may add its value. *Styrax sumatrana* was one of the local plant species that was recommended for revegetation in a gold-mining tailing site [16]. Population of *S. sumatrana* in North Sumatra showed genetic differentiation among origin localities, urging conservation efforts to include all populations to maintain their genetic diversity [17].

Microbial endophytes are known to produce various bioactive metabolites such as those that enable antimicrobial activity [18,19], including the ones from endophytic bacteria isolated from benzoin-producing tree, *Styrax benzoin* [14], and may contribute to the host resin production or properties [9]. *Neopestalotiopsis*, which produced antimicrobial and antioxidant agents [20], and *Schizophyllum*, a wood-decaying fungus, which one of its species, *Schizophyllum commune*, was edible and showed anti-cancer properties [21] were found in *S. benzoin* [22]. Anti-inflammatory compounds, such as koninginins [23], were reported to be produced by *Phomopsis stipata*, endophytic fungus isolated from aromatic resin-producing *Styrax camporum* Pohl [24]. However, fungal endophytes associated with *S. sumatrana* were still largely underexplored. Therefore, in this study, distribution and diversity of culturable fungal endophytes associated with tree parts; bark, stem, and leaves of *S. sumatrana* were examined.

2. Materials and methods

2.1. Chemicals and plant materials
Alcohol, sodium hypochlorite (NaOCl) 4%, Potassium hydroxide (KOH) 10%, hydrogen chloride (HCl), Glycerol 20% were purchased from Merck chemical (Germany). Potato Dextrose Agar (PDA), and Malt Extract Agar (MEA) was purchased from Himedia (India) and all other chemicals were provided at the highest purity possible. DNA Wizard (Promega, USA) was used for DNA extraction. Go Taq® Green Master Mix was used for polymerase chain reaction (PCR) (Promega, USA) with primer pair ITS1 and ITS4 [25]. Eleven trees of *S. sumatrana* were selected from local forest community in Simalungun (1 tree), Dolok Humbang Hasundutan (6 trees), and North Tapanuli (4 trees) Regency, North Sumatera Province - Indonesia (Figure 1). The trees’ breast-high diameter ranged from 15 to 25cm. From each tree, three healthy segments (sample) of at least two types of tissue (stem and bark) and leaves when available were randomly sampled. The plant organs were kept in polyethylene bags and brought back directly to be processed immediately.
2.2. Culturable endophytic fungi isolation

The plant organs were surface-sterilized according to the protocol suggested by Hidayat et al. [26]. The plant organs were washed under running tap water, followed by soaking in alcohol 70% for 120 s, in NaOCl for 120 s (4% Cl active), rinsed three times in sterile distilled water, and then dried on a sterile filter paper. Subsequently, outer layer of samples was removed to expose the inner part which then was excised 5 x 5 mm from the edge and placed on respective agar medium. To select the most suitable medium in the isolation process, several agar media were used as follows PDA, yeast dextrose agar (YDA), yeast malt extract agar (YMA) and Pachlewksi (P5) media [27]. Chloramphenicol (100 mg/L) was added to media for suppressing bacterial growth. For control, the final sterile water used for rinsing was plated, incubated, and observed. Absence of fungal growth in control plates after 7 d would indicate the surface-sterilization was successful, and fungal growth found in plates with inner part of plant organs were subjected as endophytic fungi. The plates with plant parts were incubated at 25 °C for up to 7 days, during which the development of different colonies was periodically observed and verified. Each colony of different fungi that appeared from tissue fragment were cut out and purified on the same agar medium used for isolation. All obtained pure cultures were used for further investigation.

2.3. DNA extraction and identification

DNA was obtained by extraction of mycelia cultured in 7 d grown on potato dextrose broth (PBD) using DNA Wizard Kit (Promega, USA) according to manufacturer’s instruction. The nuclear ribosomal DNA ITS of the fungal isolates were amplified with Go Taq® Green Master Mix (Promega, USA) according to manufacturer’s instruction with primer pair ITS1 and ITS4 [25]. Prior to sequencing, PCR products were confirmed on 1.2% m/v agarose gel. PCR products were then purified and Sanger-sequenced (First Base Sequencing Service, Singapore). Similarity searches were conducted by using the BLASTn program in the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/).
2.4. Data analysis
The important parameters of species richness; species richness index (S) and Margalef index (D') were evaluated [28]. Species richness index (S) was obtained by counting the number of endophytic fungal species in each plant organ. The Margalef index (D') was determined according to equation (1). Endophytic fungi species diversity was evaluated according to Shannon-Wiener index (H'), Simpson diversity index (DS) and Simpson's dominant index (λ) [28]. Shannon-Wiener index (H'), Simpson diversity index (DS) and Simpson's dominant index (λ) were calculated by equation (2-4). The isolation frequency (IF) for each endophytic fungal species in different plant organs were calculated using equation (5). The uniformity index (E) was determined by equation (6) according to Li et al. [28].

\[
D' = \frac{(S-1)}{\ln N_t} \quad (1)
\]
\[
H' = \sum P_i \ln P_i, \quad P_i = \frac{N_i}{N_t} \quad (2)
\]
\[
DS = 1 - \lambda \quad (3)
\]
\[
\lambda = \sum P_i^2 \quad (4)
\]
\[
IF(\%) = P_i \times 100\% \quad (5)
\]
\[
E = \frac{H'}{\ln S} \quad (6)
\]

N\(_t\) represents the total number of isolates of all genera obtained by separation, N\(_i\) is the number of isolates belonging to the i-th species.

3. Results and discussion
3.1. Identification of culturable endophytic fungi
A total of 31 isolates were successfully recovered from 576 different part organs of S. sumatrana, with the majority of isolates were isolated from barks (15 isolates). The morphological characteristics (colony form, mycelium color and reverse media color) of 17 species showed in Figure 2. Based on the rDNA-ITS sequence analysis, all of isolates of endophytic fungi were identified into 2 classes, 6 families, 10 genera and 17 species (Table 1). All of fungi species classified as member of Ascomycota. Ascomycetes are the most common representatives of endophytic fungal communities isolated using standard separation protocols. Sordariomycetes and Dothideomycetes were the classes of Ascomycota that were found in this study.

Additionally (data not shown), certain genera were isolated using respective agar medium. Trichoderma and Daldinia were isolated with PDA medium, whereas Pithomyces, Botryosphaeria, and Diaporthe with YDA medium. Acremonium were successfully isolated on P5 medium, whereas Neopestalotiopsis were found on both P5 and PDA media. Pestalotiopsis was observed and isolated on PDA, YDA, and P5 media. Fusarium and Phyllosticta could be isolated from all media.

The species and the isolation frequency of endophytic fungi isolated from S. Sumatrana varied between organs. Community of endophytic fungi depends on the interaction of other endophytic microbes and/or pathogen [29]. The existence of endophytic fungi is affected by season [30], environmental factors [31], and their host tissue/organ type [10]. A total of 31 endophytic fungal isolates from 10 genera and 17 species were found in all respective part of organ of S. sumatrana (Table 1). Out of 31 individual fungal isolates, 15 were found in barks and each 8 isolates were observed from stem and leave parts. The fungal isolation frequency from each organ is presented in Figure 3. The highest total isolation frequency was for Fusarium genus which was found in 14 specimens (45%) followed by Phyllosticta, Pestalotiopsis, Neopestalotiopsis, and Trichoderma in 4, 3, 3, and 2 specimens (6%), respectively.
Six genera were found in barks (Figure 4), those were Trichoderma, Fusarium, Neoepestalotiopsis, Acremonium, Pestalotiopsis and Daldinia. The genus with highest isolation frequency from barks was Fusarium with 6 specimens (40%), whereas the genera with lowest value were Acremonium and Daldinia with each one specimen (7%). From stem, five genera were isolated, with Phyllosticta as the most frequent with 4 specimens (50%), followed by Pithomyces, Botryosphaeria, Diaporthe, and Fusarium with each 1 specimen. For leaves, two genera were found; Fusarium with the higher isolation frequency of 88% (7 specimens) and Neoepestalotiopsis which were found in only 1 specimen (13%) (Figure 4). This finding is rather different from previous study which could only find F. solani in leaves of S. Sumatrana from Aek Nauli [22]. The assembly of endophytic fungi from previous study was Phomopsis and Lasiodiplodia from stem, and F. solani, Phyllosticta, and Phomopsis from leaves [22]. This study has added more genus that were found in stem, newly discovered genus from bark and a different assembly of genus from leaves. The genus Fusarium has been known as endophytes and also pathogens in various species and cultivar of vascular plants. Genus Fusarium is known as
parasitic, causing wilt and rot in various plant species. However, the endophytic *Fusarium* sp. were reported to have resistance against virulent pathogens [10], as well as anticancer properties, anti-inflammatory, antitussive (to prevent or relieve a cough) and anti-allergic properties [32].

![Figure 2](image_url)

**Figure 2.** Representative of fungal isolates from bark, stem and leaves of *S. sumatrana* based on distinctive colony morphology. The observed isolates were cultivated on respective agar mediums for 7 days at 25 °C.

![Figure 3](image_url)

**Figure 3.** Isolation frequency of endophytic fungi in bark, leaves, and total (bark + stem + leaves) of *S. sumatrana*. 
3.2. Diversity analyses

Endophytic fungal species showed varying distribution in respective S. sumatrana plant organs; 10, 6, and 2 species in bark, stem, and leaves, respectively (Table 1 & 2). *Fusarium* was the only genus that could be observed in all organs, particularly *F. solani*, *F. striatum* and *Fusarium* sp. Furthermore, *Fusarium* was the most common endophytic fungi isolated in this study, with isolation frequency of 45% (Figure 3). This result indicated that endophytic fungal species varied between plant organs. Isolation frequency of each genus or species also depended on the colonized plant organ.

Table 2. Diversity analyses of endophytic fungi from S. sumatrana.

| Part of tree | Shannon–Wiener index (H') | Simpson diversity index (D's) | Uniformity index (E) | Dominant index (λ) | Margalef index (D') | Species richness (S) | Number of isolates |
|--------------|---------------------------|-------------------------------|---------------------|--------------------|---------------------|---------------------|-------------------|
| Bark         | 1.587                     | 0.756                         | 0.689               | 0.244              | 3.323               | 10                  | 15                |
| Stem         | 1.386                     | 0.688                         | 0.774               | 0.313              | 2.404               | 6                   | 8                 |
| Leaves       | 0.377                     | 0.219                         | 0.544               | 0.781              | 0.481               | 2                   | 8                 |

The level of richness (S), abundance (number of isolates) and Margalef index (D') varied between the observed plant organs. The species richness (S) and Margalef index (D') reflects the richness of endophytic fungal species [28]. Bark has the richest fungal species and most abundant in terms of fungal isolates among the observed organs (Table 2) (S = 10 vs. 6 and 2, isolates = 15 vs. 8, D' = 3.323 vs. 2.404 and 0.481). The diversity indices, Shannon–Wiener (H') and Simpson’s (D's), and dominance index (λ) were determined. The value of H' index of endophytic fungi was highest in bark (1.587), which was followed closely by stem (1.386), whereas leaves had the lowest (0.377). The similar results were also observed for D's index, where bark had the highest value (0.756), and stem and leaves had 0.688 and 0.219 values, respectively. In general, the higher of the H' index value, commonly in the range 1.5 to 4.5, and the closer of the D's index value to 1, indicate more abundance heritable variation and stronger adaptive capacity to micro-environmental changes because they tend to spread the distribution range and adaptive to new environments [28]. The H' index values were categorized as

![Figure 4](image-url)
follow: <1, 1-3, and >3 indicated in low, medium, and high diversity with the spread of the number of individuals of each species [33]. Thus, diversity of endophytic fungi in bark and stem are considered as medium diversity. Species richness and diversity indices reflect a certain degree of heterogeneity due to considerable differences in diversity caused by the microenvironment. Thus, the observed organ specificity of endophytic fungi in S. sumatrana in this study may be influenced by variations in the microenvironment of plant tissues. Plant characteristics and tissue/organ sampling is likely to affect the composition and dynamics of an endophytic fungal community. A study on the microecological distribution of endophytes in different tissues of Pteroceltis tatarinowit [34] and Zanthoxylum bungeanum [28] produced similar results, which demonstrated the tissue specificity of endophytes.

Furthermore, the λ index showed the lowest value in bark (0.244), compared to stem (0.313) and leaves (0.781). It could be seen that the λ value showed negative correlation with the Ds. The high λ value in the community indicates the endophytic fungal community may have less diversity and a rather balanced abundance among species. The λ index values were categorized as follow: <0.5, 0.5-0.75, and >0.75 indicated in low, medium and high dominance species, respectively [35]. Thus, dominance of endophytic fungi in leaves was greater than in bark and stem, which considered as high dominance, with just 2 genera of Fusarium and Neopestalotiopsis. The uniformity index (E) was used to determine the balance of fungal community. It is based on the size of the similarity of the number of individuals among species within a community. The E index is classified into small uniformity if the value was 0.00 < E < 0.50, moderate if 0.50 < E < 0.75 and high if 0.75 < E < 1.00 [33]. Based on this classification, the E index in leaves classified into small, whereas in the bark classified into moderate and the stem classified into high uniformity.

In general, endophytic fungal communities from different isolated parts indicated the structure, richness, diversity, and dominance of different species. Endophytic fungal community composition was also affected by plant organs (stem, bark, and leaves). Previous studies suggested that morphology and chemical substance in host tissues also affected the endophytic fungal community composition in plant roots and stems [36,37]. Endophytic fungi in this study were isolated from three different organs and thus may represent endophytic fungal community in S. sumatrana shoots. Further investigations which include non-culturable endophytic fungi is necessary to obtain more inclusive fungal assemblages.

4. Conclusion
A total of 31 culturable endophytic fungal isolates were successfully obtained from different parts of Styrax sumatrana grown in North Sumatera, Indonesia. High diversity and significant endophytic fungal from plant organ specificity were observed in S. sumatrana. Bark was the most diverse and had the highest isolation frequency among observed organs. The current study provides initial information on endophytic fungal diversity, community composition, and a hint of organ-specificity in shoot organs of kemenyan-producing S. sumatrana. Bioactive compounds produced by these fungi as well as its relation to resin production are to be further investigated.

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