The Diversity of Endophytic Fungi in Kemaitan (*Lunasia amara* Blanco)

Y Istikorini*, A P P Hartoyo

Department of Silviculture, Faculty of Forestry, IPB University (Bogor Agricultural University), Bogor, Indonesia.

*Corresponding email: yunik.istikorini@gmail.com

Abstract. All plants in natural ecosystems appear to be symbiotic with fungal endophytes. Endophytes are especially little known in tropical forest trees, which their abundance and diversity are thought to be greatest. This research aims to identify the diversity endophytes fungal in the shoots, leaves and stem of Kemaitan (*L. amara*). There are 180 endophytic fungi were isolated using a modified Photita's method, by isolating in their shoots (34 isolates), leaves (70 isolates), and stems (76 isolates), which categorized in 37 morphospecies. The number of morphospecies endophytic fungi found on stem was higher (24 morphospecies) than on shoot and leaf (22 morphospecies). The index value of endophytic fungi on Kemaitan’s stem (3.050) was considered as high diversity, while on shoots (2.966) and leaves (2.911) were classified as moderate diversity. The index value dominance of endophytic fungi was categorized as low criterion. The evenness levels on stem, leaf, and shoot of Kemaitan were categorized into high level. The level similarity of endophytic fungi is categorized as low similarity.

1. Introduction

Indonesia’s landscape is covered with dense tropical rainforests which has rich biodiversity and the high of potency of biological resources. One of the potential resources that contain bioactive compound is Kemaitan (*Lunasia amara* Blanco), a genus in Rutaceae family. *Lunasia amara* is small tree or shrub that could be found around Indonesia’s tropical forest. Part of Kemaitan which used for common traditional medicine are leafs and dried bark.

Every vascular plants has various endophytic microbes which could produce secondary metabolism as a result of co-evolution or genetic transfer from its host. Endophyte is microbe that lives inside plant tissues, like leaf, flower, branch or root without any presence of disease symptoms [1, 2].

Endophyte microbes from forest vegetations and medicinal plants are now succesfully isolated [1, 2, 3]. Endophyte microbes from vegetation’s plant tissues that grow in tropical rainforest are known to have higher biological activity [3]. Endophyte could be found in every families of plant [6, 7]. But the researchs about endophyte’s diversity in Indonesia’s forest vegetations are limited in numbers and therefore need to be studied more.

Endophytic fungi’s *Thievalia polygonopera* that isolated from akar kuning (*Fibraurea chloroleuca*) in Kalimantan forest has powerful antibacterial [8]. The role of endophyte is largely known, especially in improving plants’s tolerance to drought stress, preventing herbivore insects, patogen fungi, virus, nematode [9]. Endophyte microbes could produce bioactive compounds as their secondary metabolism such as alkaloid, terpen, steroid, flavonoid, quinone, phenol etc. [5, 10].
Bioactive compounds also used as antimicrobial, anticancer, anti malaria, enzyme, and antibiotic [3, 11]. Endophytic fungi’s isolation and exploration on Kemaitan has a great prospect in order to discover new bioactive compounds, especially as biocontrol agent that could boost growth and endurance of forest vegetation. The objective of this research is to find out the diversity of endophytic fungi of Kemaitan. The research is done with exploration methods and comparing endophytic fungi on shoots, leaves and stems.

2. Method

2.1. Time and Place

This research was conducted between February until August 2018. The location of collecting sample of Kemaitan was in Faculty of Forestry, IPB University. Endophytic fungi isolation was done in Pathology Laboratory, Department of Silviculture, Faculty of Forestry, IPB University.

2.2. Endophytic Fungi Isolation

Endophytic fungi were isolated from shoots, leaves, and stems of Kemaitan. The method to isolate the endophytic fungi and was done adopting the methodology of Rodrigues (1994). Shoots, leaf, and stem of Kemaitan are washed thoroughly with tap water, then had surface treatment in stages by immersing the tissues in 96% alcohol for 30 seconds, NaOCl for 1 minute, 70% alcohol for 1 minute, and 30% alcohol for 30 seconds. Then rinsed with sterile water twice and dried with sterile paper. The shoots, leaf, old leaf, and stem of Kemaitan were then cut into small pieces of segment and placed in petridishes containing potato dextrose agar (PDA) medium complemented with Chloramphenicol to color PDA. Each petri dish has 4 segment of each part of the plant. Mycelium that grows on plant segments were then purified and kept in PDA media. The similar shape and color fungi will be assumed as one genus. Identification of endophytic fungi is based on color and colony characteristic and microscopic shape.

2.3. Data Analysis

2.3.1. Frequency of colonization

The frequency of colonization is calculated based on the Hatta and Futai’s method [12, 13]:

\[
CF = \frac{N_{col}}{N_t} \times 100\% 
\]

where:

- \( CF \) : Relative frequency of colonization
- \( N_{col} \) : number of segments of plant tissue colonized by each fungus
- \( N_t \) : total number of segments of plant tissue studied.

2.3.2. Evenness Index (E)[14]

Evenness index is used to determine the balance of the fungal community. It is based on the size of the similarity of the number of individuals among species within a community. The formula for calculation of evenness is as follows:

\[
E = \frac{H'}{\ln s}
\]

Where

- \( E \) : Evenness index
- \( H' \) : Shannon diversity index
- \( s \) : Number of genus/species

Evenness index was determined as follows [15]:

1.00 <\( E \) <0.50 : Low evenness, depressed community
0.50 < E < 0.75 : Moderate evenness, community I
0.75 < E < 1.00 : High evenness, stable community

2.3.3. Diversity Index (H’)
Calculation of species diversity is carried out using the Shannon-Wiener formula [16].

\[ H' = - \sum Pi \ln(Pi), \quad Pi = \frac{n_i}{N} \]

Where:
H’ = Shannon-Wiener diversity index
ni = Number of i-type individuals
N = The number of individuals of all types
The criteria for the Shannon-Wiener (H’) diversity index are as following:
H’ < 1 : low diversity, distribution of individuals per species is low
1 < H’ ≤ 3 : moderate diversity, distribution of individuals per species is medium
H’ > 3 : high diversity, distribution of individuals per species is type is high

2.3.4 The dominant index (C) [17]
The dominant type index is used to determine the dominance of endophytic fungus species in a community. The Dominance index was calculated using the following formula:

\[ C = -\sum \left( \frac{n_i}{N} \right)^2 \]

Where,
C : Simpson dominance index
ni : Number of individual types I
N : Total number of individuals
The value of dominance index ranged from 0-1 [18, 19]:
0.00 < C < 0.50 : Low
0.50 < C < 0.75 : Medium
0.75 < C < 1.00 : High.

3. Result and discussion
Endophytic fungi colonize several parts of the plant. Plant tissue or organs is a good reservoir for endophytic fungi. According to Petrini et al. [20], plant organs are microhabitat for endophytic infections. Results of isolation of endophytic fungi Kemaitan’s shoot, leaves and stem were found 180 isolates consisting of 34 isolates on shoots, 70 isolates on leaves, and 76 isolates on stems. The results of the isolation of endophytic fungi in leaves are more than those of shoots. The endophytic fungi in leaf tissues has an relatively equal numbers to endophytic fungi in stem. According to Stone et al. [21], infection frequency and diversity of endophytic fungi increases as the tissues or host aged. As older leaf has much more endophyte than younger leaf [22, 23].

Species composition and colonization frequency on stem tissues were higher. Five morphospecies of endophytic fungi (C12, C19, C24, C27 and C28) were found in stem which were absent on shoots and stem (Figure 1). The result suggested that the endophytic fungi only infected stem. Whereas three morphospecies of endophytic fungi (C21, C22 and C37) were only found in shoots. Isolate C26, C29, C35 and C36 were only found in leaves (Figure 1).

Species composition and frequency of endophyte are known to be varied in host or plant tissues [24]. Several factors that may affect the variety of endophyte on plant are environmental factor, vegetation type, microcosmos spatiotemporal (root) pattern, and interaction with various microbes [25].
Figure 1. Abundance of endophytic fungi morphospecies isolated from shoots, leaf, and stem of Kemaitan.

There were 22 morphospecies on shoots and leaves and 24 morphospecies on stems. Among 37 morphospecies, there’s at least 15 morphospecies detected that have singleton trait (C12, C19, C21, C22, C24, C26, C27, C28, C29, C30, C31, C33, C35, C36, C37) and dominant trait (C36). Singleton trait was endophyte fungi which isolated in singular number from the segment.

In this research, endophytic fungi were classified based on similarity of the species (morphospecies), which were color morphology, miselium pattern. From the classification, there were 37 different morphospecies (Table 1).

Table 1. Colony characteristic of morphospecies endophyte fungi on Kemaitan

| Isolate Code | Surface | Reverse | Colony characteristic | Isolate Code | Surface | Reverse | Colony characteristic |
|--------------|---------|---------|-----------------------|--------------|---------|---------|-----------------------|
| C1           | ![Image](image1.png) | ![Image](image2.png) | Pink fungi on the surface, concentric dot from pink to white on the reverse. Hyphae did not grow vertically. Colony’s edges smooth. It produces pink pigment. | C3           | ![Image](image3.png) | ![Image](image4.png) | White fungi on surface, orange on the reverse with concentric circle. Irregular shape of edges. Absence of pigment and has flowery pattern. |
| C2           | ![Image](image5.png) | ![Image](image6.png) | White fungi on surface with concentric circle, mycelia grow on the circle aerial. Irregular shape of edges. Absence of pigment and has flowery pattern. | C4           | ![Image](image7.png) | ![Image](image8.png) | White fungi with tiny spots of black and orange on concentric zone. Smooth edges, zonate pattern, and absence of pigment. |
| C5           | ![Image](image9.png) | ![Image](image10.png) | White fungi with orange dots along in the concentric zone, smooth edges, zonate pattern, with orange color on the bottom. | C24          | ![Image](image11.png) | ![Image](image12.png) | White fungi, texture-wise resupinate. Irregulate edges with radiate pattern. |
| C6           | ![Image](image13.png) | ![Image](image14.png) | White fungi with a slight of orange and black on the reverse. Formed concentric zone, irregular edges, arachnoid pattern, texture-wise velvety. | C25          | ![Image](image15.png) | ![Image](image16.png) | White fungi with chocolate and orange on the reverse. Formed a cleavage like rose flower. Mycelia spreading like moss. |
| C7           | ![Image](image17.png) | ![Image](image18.png) | White fungi with concentric circle, ivory white and brown on the bottom. Texture-wise powdery, mycelia spreading and did not grown vertically, zonate pattern. | C26          | ![Image](image19.png) | ![Image](image20.png) | White fungi, formed a translucent zone around the starting growth point, then formed concentric circle with mycelia grown vertically, flowery pattern. |
A community will have high diversity if the community has a high number of species. The diversity index ($H'$) used is the Shannon-Wiener diversity index [16]. If the value of $H'$ is smaller than 1, diversity is classified as low. If the value of $H'$ ranges between 1 and 3, diversity is classified as moderate. If $H'$ is greater than 3, diversity is high. Based on Table 2, the level of diversity of

### Table 1. Colony characteristic of morphospecies endophyte fungi on Kemaitan (continuation)

| Isolate Code | Surface | Reverse | Colony characteristic | Isolate Code | Surface | Reverse | Colony characteristic |
|--------------|---------|---------|-----------------------|--------------|---------|---------|-----------------------|
| C8           | ![Image](image1.png) | ![Image](image2.png) | White fungi, brownish on the reverse, texture-wise powdery, spreading mycelia, radiate pattern. | C27          | ![Image](image3.png) | ![Image](image4.png) | White fungi, formed rose-like circle on the reverse. Distinctive concentric line. Mycelia did not grown vertically, spreading edges, flowery pattern. |
| C9           | ![Image](image5.png) | ![Image](image6.png) | White fungi, concentric circle, white and brown on the reverse. Texture-wise shrunken, mycelia spreading, zonate pattern. | C28          | ![Image](image7.png) | ![Image](image8.png) | White fungi, concentric pattern like flower, on concentric circle mycelia grow vertically, edges of colony rugged, spreading, flowery pattern. |
| C10          | ![Image](image9.png) | ![Image](image10.png) | White fungi, orange on the reverse. Texture-wise powdery, edges shaped like rose's petal, mycelia grown irregularly, flowery pattern. | C29          | ![Image](image11.png) | ![Image](image12.png) | White fungi, orange to brown on the reverse, texture-wise powdery, flowery pattern. |
| C11          | ![Image](image13.png) | ![Image](image14.png) | White fungi, rose-like pattern, powdery texture, flowery pattern. | C30          | ![Image](image15.png) | ![Image](image16.png) | White fungi, orange to brown on the reverse. Mycelia like soft cotton, smooth edges, flowery pattern. |
| C20          | ![Image](image17.png) | ![Image](image18.png) | White fungi, wrinkles, and watery. | C31          | ![Image](image19.png) | ![Image](image20.png) | White fungi, mycelia spreading like lined cotton. Formed a concentric circle. |
| C21          | ![Image](image21.png) | ![Image](image22.png) | White fungi with black edges surrounding. Mycelia did not grow vertically. Texture-wise like moss, spreading. | C32          | ![Image](image23.png) | ![Image](image24.png) | White fungi, black on the center, mycelia grown vertically only a little. Towel-like texture. |
| C22          | ![Image](image25.png) | ![Image](image26.png) | White fungi that formed concentric circle with vertically-grown mycelia along the circle. Black spots were present along the circle. Flowery pattern. | C33          | ![Image](image27.png) | ![Image](image28.png) | Dark green fungi, slow growth, grows on media, smooth edges. |
| C23          | ![Image](image29.png) | ![Image](image30.png) | White fungi with black spots, no concentric circle, cotton-like mycelia, spreading pattern. | C34          | ![Image](image31.png) | ![Image](image32.png) | Dark grey fungi, slow grown. |
| C35          | ![Image](image33.png) | ![Image](image34.png) | Black fungi like moss, slow grown. | C37          | ![Image](image35.png) | ![Image](image36.png) | White fungi. Black on the reverse, slow growth. |
| C36          | ![Image](image37.png) | ![Image](image38.png) | Black fungi, slow growth. | | | | |
endophytic fungi was higher on stem as compared to that on shoot and stem. The index value of endophytic fungi on stem (3.050) was considered as high diversity ($H' > 3$), while on shoots (2.966) and leaves (2.911) were classified as moderate diversity ($1 < H' \leq 3$). According to Stone et al. [21], the frequency of infections and the diversity of endophytic fungi increases with increasing age of the organ or host tissue.

Table 2. Index of diversity, dominance, evenness and morphospecies richness of Kemaitan’s endophytic fungi

| No | Organs | $H'$  | $C$   | $E$   | $R$ |
|----|--------|-------|-------|-------|-----|
| 1  | Shoot  | 2.966 (m) | 0.059 (l) | 0.959 (h) | 22  |
| 2  | Leaf   | 2.911(m)  | 0.064 (l) | 0.942 (h) | 22  |
| 3  | Stem   | 3.050 (h)  | 0.052 (l) | 0.960 (h) | 24  |

Note: $H'$: diversity index, $C$: dominance index, $R$ = Species Richness, $E$ = Evenness index; $l$= low, $m$= moderate, $h$= high

Dominance is used to find out which species dominates other species. The dominance index used is the Simpson’s dominance index [17]. Table 2 showed that the level of dominance of endophytic fungi on leaf, stem and shoot were categorized into low criteria. It means there were no endophytic fungi dominating the Kemaitan’s tissue.

Based on Table 2, the evenness levels on stem, leaf, and shoot of Kemaitan were categorized into high level. This value was opposite with the dominance value. It means that endophytic fungi was distributed equally. The number of morphospecies endophytic fungi found on stem was higher (24 morphospecies) than on shoot and leaf. While, the number of morphospecies found on shoot and leaf of Kemaitan were same, specifically 22 morphospecies.

A similarity index is needed to determine the level of similarity between several endophytic fungi in Kemaitan’s shoots, leaves and stem. The similarity index used is the index of equality in Odum [16].

Table 3. Index of similarity of isolated endophytic fungi on Kemaitan

| Plant organs | Shoot | Leaf | Stem |
|--------------|-------|------|------|
| Shoot        | 1.000 |      |      |
| Leaf         | 0.545 | 1.000|      |
| Stem         | 0.522 | 0.565| 1.000|

Based on the result shown in Table 3, the level similarity if endophytic fungi on stem-leaf plant tissue was higher than that of leaf-shoot and stem-shoot. This finding is based on the total index similarity of endophytic fungi on leaf-shoot and stem-shoot as of 0.545 and 0.522, whereas the index similarity of endophytic fungi on stem-leaf was 0.565

4. Conclusion

Isolated endophytic fungi on Kemaitan resulted on 180 isolat which consist of 34 shoot isolates, 74 leaf isolates and 76 stem isolates. The number of morphospecies endophytic fungi found ohamsia stem was higher (24 morphospecies) than on shoot and leaf (22 morphospecies). The index value of endophytic fungi on Kemaitan’s stem (3.050) was considered as high diversity, while on shoots (2.966) and leaves (2.911) were classified as moderate diversity. The evenness levels on stem, leaf, and shoot of Kemaitan were categorized into high level. The index value dominance of endophytic fungi categorized as low criterion. The level similarity if endophytic fungi on stem-leaf, leaf-shoot and stem-shoot as categorized as low level of similarity.

References
[1] Clay K 1988 J Ecol, 69(1), 10-16.
[2] Petrin O 1991 *Fungal endophytes of tree leaves*. In: *Microbial ecology of the leaves* (eds. N.J. Fokkema and I. van den Heuvel). (Cambridge Cambridge University Press)
[3] Strobel G A 2003 *Microbes and Infection*, 5, 535-544.
[4] Suryanarayanan T S, Venkatesan G, Murali, T S 2003 Endophytic fungal communities in leaves of tropical forest trees: Diversity and distribution patterns. https://www.researchgate.net/publication/255654960_Endophytic_fungal_communities_in_leaves_of_tropical_forest_trees_Diversity_and_distribution_patterns/
[5] Tan R X, and Zou W X 2001 *Natural Products Reports*, 4.
[6] Faeth S H 2002 *Oikos*, 98, 25-36.
[7] Strobel G A 1996 *Pharmaceutical News*, 3, 7-9.
[8] Prihatiningtias W 2005 Bioactive compounds Yellow Root Endophytic Fungi (*Fibraurea chloroleuca* Miers) as antimicrobial compounds [Thesis]. (Yogyakarta: Gadjah Mada University)
[9] Ranzluebbers A J, Nazih N, Stuedemann J A, Fuhrmann J J, Schomberg H H, Hartel P G 1999 *Soil Sci. AM. J.*, 63, 1687-1694.
[10] Gunatilaka A A L 2006 *J. Nat. Prod.*, 69, 509-526.
[11] Carroll G C 1988 *Ecology*, 69, 2-9.
[12] Hata K and Futai K 1995 *Canadian Journal of Botany*, 73, 384-390
[13] Suryanarayanan T S, Kumaresan V, and Johnson J A 2001 *Fungal endophytes: the tropical dimension*. In: *Trichomycetes and other Fungal Groups* (New Hampshire: Science Publishers)
[14] Ludwig J A and Reynolds J F 1988 *Statistical Ecology* (New York: John Wiley & Sons.).
[15] Brower J E and Zar J H 1977 *Field and Laboratory Methods for General Ecology* (Dubuque: Wm. C. Brown Publishers).
[16] Parsons T R, Takahashi M, Hargrave B 1977 *Biological oceanographic processes* (Oxford: Oxford University).
[17] Odum E P 1996 *Basics of Ecology. Third edition* (Yogyakarta: Gajah Mada Universitas Press).
[18] Hamsiah 2006 *Jurnal Protein*, 13(2), 172-180.
[19] Hamsiah, Herawati E Y , Mahmudi M, Sartimbul A 2016 AACL *Bioflux*, 9, 775-784.
[20] Petrin O, Sieber T N, Toti L, Viret O 1992 *Natural Toxins*, 1, 185-196.
[21] Stone J K, Polishook, J D, and White Jr J F 2004 Endophytic fungi In: Mueller G M, Bills G F and Foster M S, (eds.), *Biodiversity of Fungi: Inventory and Monitoring Methods*. (New York: Elsevier Academic Press)
[22] Taylor I E, Hyde K D, and Jones E B G 1999 *New Phytologist*, 142, 335-346.
[23] Rajagopal K and Suryanarayanan T S 2000 *Current Science*, 78, 1375-1378.
[24] Rodrigues K F 1996 *Fungal endophytes of palms*. In: *Endophytic Fungi in Grasses and Woody Plants. Systematics, Ecology and Evolution* (St Louis: APS Press)
[25] Sieber T N and Grünig C R 2006 *Microbial Root Endophytes*, 9, 134-137.