Ectomycorrhizal fungi inoculation on *Shorea balangeran* and *Tristaniopsis obovata* in peatland ecosystems

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**Abstract.** The majority of tropical forest plants form associations with Ectomycorrhizal fungi (EMF). These symbiotic fungi play important roles in promoting the survivability of their host, improving their productivity, and increasing microbial biodiversity in the underground. In order to promote the survivability and the growth of two peatlands tree species (*Shorea balangeran* and *Tristaniopsis obovata*) in Central Kalimantan and South Sumatra, their seedlings were inoculated with EMF. The aim of this study was to evaluate the effectiveness of EMF inoculation by identifying the EMF that colonized the root tips of *Shorea balangeran* and *Tristaniopsis obovata* using a molecular approach after 3 years of planting. The results show that eight molecular operational taxonomic units (MOTU) were successfully identified out of twelve EMF root tips. Most of these MOTU identified as Thelephoraceae family, which is a cosmopolitan fungus in Southeast Asia forests. However, none of the inoculated EMF were detected in the root tips, indicating the occurrence of natural succession process, where native EMF in the field replacing inoculated EMF in the nursery.

**1 Introduction**

Indonesia possesses the world’s largest tropical forests and is known for its remarkable biodiversity, securing a position among the top 3 tropical forest owners globally [1]. One of the pivotal topics discussed in climate change conferences and scientific forums pertaining to biodiversity, carbon measurement, and restoration techniques is the peatland ecosystem. Extensive peatlands stretch across Indonesia, spanning from Sumatra, Borneo, to Papua. The restoration of degraded peatland ecosystems, which frequently fall victim to fires, is of utmost importance and necessitates concerted efforts from stakeholders. Notably, the peatland ecosystem stands out as a significant carbon storage reservoir compared to forests situated on mineral soils and mountains [2].

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Growing various tree species in peatland ecosystems can be challenging, primarily due to
the difficulty in obtaining local sources of forest plant seeds. In some
cases, the original
peatland forests have been cleared or burnt [2], which poses a significant obstacle in mapping
native peatland tree species and conserving their biodiversity in designated peatland
conservation areas. Additionally, the complete status of native peatland tree species,
particularly their relationship with mycorrhizal fungi, remains largely unknown [3].
Mycorrhizal fungi have the ability to form symbiotic relationships with over 95% of forest
trees on Earth. However, information specifically regarding tropical peatland ecosystems is
still lacking.

Mycorrhizal fungi play vital roles in maintaining productivity and microbial biodiversity
in underground soils, particularly in peatlands that are often impermeable and oxygen-
deficient [3], [4]. Research reports suggest that nearly all forest plants in peatland ecosystems
form symbiotic relationships with mycorrhizal fungi. Consequently, it is recommended to
inoculate forest plant seedlings with mycorrhizal fungi to support their growth. In the context
of growing tree seedlings from peatland, nursery practices have incorporated the inoculation
of mycorrhizal fungi in forest plant seedlings during The Mushroom Initiative (TMI) project
activities in both Central Kalimantan and South Sumatra. Several tree species in the peatland
ecosystem have been found to establish symbiotic relationships with ectomycorrhizal fungi,
such as those from the Dipterocarpaceae family and some belonging to Myrtaceae. The
biodiversity of ectomycorrhizal fungi has been documented by Helbert et al. [5].

However, there are still many questions to be answered regarding the mechanisms of
mycorrhizal trees growing in peatland ecosystems, which are characterized by continuous
waterlogging, lack of oxygen, and acidic pH. One key question is whether ectomycorrhizal
fungi function normally in providing macro and micro nutrients required by forest plants in
peatland [6]. Moreover, it remains to be explored whether mycorrhizal fungi obtain a supply
of liquid carbohydrates to fulfill their daily energy needs while foraging and reproducing in
the challenging peat media. Additionally, the ability of mycorrhizal fungi to survive under
conditions of oxygen deficit or complete anoxia raises another intriguing question. The
underlying mechanisms that enable their survival in such environments, including the
potential involvement of vegetative development of mycelial fungi or the reproduction of
spores that remain dormant until favorable peat media conditions are met, warrant further
investigation. Furthermore, the possibility of specialized species of mycorrhizal fungi
adapting to the extreme and limited environmental conditions in the peatland ecosystem also
merits attention [6]. In conclusion, this scientific inquiry highlights the need for further
research to elucidate the functions and adaptations of mycorrhizal fungi in peatland
ecosystems. By answering these queries, we can improve our comprehension of the complex
ecological interactions and add to the body of information about the resilience and operation
of ecosystems.

The scope of identification activities focused more on collecting samples of root tips with
ectomycorrhizal fungi on several tree species such as Shorea balangeran and Tristaniopsis
obovata. In previous research, it was reported that these two tree species can grow well after
being inoculated at the nursery level [3], [7], [8]. Specifically, ectomycorrhizal fungi were
identified through individual ‘root tips’ using molecular identification techniques. Some of
the obstacles faced are that there is no standard method for the molecular identification
process of ectomycorrhizal fungi in tropical forest ecosystems, especially in peatland
ecosystems.

The Mushroom Initiative (TMI) and the Ministry of Environment and Forestry had set up
demonstration plots in Central Kalimantan and South Sumatra provinces as effort to restore
native peatland tree species utilizing ectomycorrhizal fungi. However, there are knowledge
gap regarding mycorrhiza ecological dynamics and their effectiveness of restoration efforts
in flooded peatland environments. This information should provide insight into the
importance of mycorrhizal fungi in supporting the growth and survival of native peatland tree species, ultimately aiding conservation and restoration efforts for these ecologically valuable ecosystems.

The objective of this research was to conduct specific molecular identification of ectomycorrhizal fungi in dominant tree species adapted to flooded peatlands, including *Shorea balangeran* (Dipterocarpaceae) and *Tristaniopsis obovata* (Myrtaceae). This information is critical to determine whether ectomycorrhizal fungi form symbiotic relationships with native peatland tree species in demonstration plot areas that have undergone restoration efforts. This research outcome may have implications for future restoration projects and sustainable land management practices in similar ecosystems.

2 Materials and Methods

2.1 Location

Sampling location are located in The Mushroom Initiative (TMI) demo plots in South Sumatra and Central Kalimantan. These plots are forest areas that experienced severe forest fires in 2015. The South Sumatera plot is located in Pademaran, Ogan Komering Ilir, managed by the Forest Management Unit under the South Sumatra provincial government, specifically the Palembang Forestry Service. The Central Kalimantan Plot is managed as a Special Purpose Forest Area (KHDTK) by the Banjarbaru Instrument Standardization Institute (BSI) under the Ministry of Environment and Forestry (MoEF).

These demo plots are used as restoration activity that cover an area of 50 hectares each. The soil is very acidic peat soils, with natural vegetation consists mainly of pioneer shrubs (ferns and grasses) that can tolerate the extreme low pH levels. Both locations also experience extended periods of waterlogging throughout the year.

Restoration activity was carried out by infecting various types of forest plants, especially the Dipterocarpaceae (*Shorea* spp., *Dryobalanops* spp.) and Myrtaceae (*Tristaniopsis* sp.) with ectomycorrhizal fungi in the form of alginate beads and/or spore tablets (Figure 1.). This entire process started from the nursery, and then transplanted into the field, following the 4N concept (no burning, no fertilizer, no plastic, and native species). This restoration concept represents a TMI plot example that was first applied to peatland ecosystems in Indonesia.

![Fig 1.](image-url) The process flow of restoration activities in peatland ecosystems: Prepared Dipterocarpaceae and Myrtaceae seeds (a); Ectomycorrhizal fruiting body (b); propagated ectomycorrhiza hyphae trapped in alginate beads (c); The inoculated seed in TMI nursery (d); Inoculated Seedlings (e). Field plantation (f), maintenance and evaluation of trees growth (g); ECM root tips for identification (h).
2.2 Sampling Technique

In this study, *Shorea balangeran* and *Tristaniopsis obovata* plots that are 3 years old are the primary sampling areas (Figure 2). Each of the three treated trees had nine soil samples (5 cm × 5 cm x 10 cm depth) taken from three soil cores. To increase the likelihood of capturing the EMF roots of the tree, each sampling location was chosen at random close to it. To prevent gathering identical EMF fungal clones, trees have to be at least 5 m apart. A GPS (GPSMAP62SJ, Garmin, Olathe, KS, USA) was used to map out the locations of each sampling point. Before continuing, soil samples were packed individually in plastic bags and kept at a low temperature (4 °C).

Environmental conditions (pH and water level) were collected. For pH, we used direct measurement in the field using pH-indicator strips (Mquant® Merck; Germany). For water level, we made a hole in the soil, and the water table was measured. If the water table is below the soil, we give them a negative value.

![Fig. 2.](image-url) (a-c) activities of collecting root tips from selected plots in which some were merged in water. (d) collected roots with root tips that were colonized by ectomycorrhizal fungi.
2.3 Ectomycorrhiza Identification

After being properly removed from each soil sample, all collected EMF roots were cleansed using tap water. The cleaned EMF root tips were inspected with a stereomicroscope to determine their morphological properties, such as surface texture, mantle color, radiating hyphae, and rhizomorphs, and they were then categorized into morphotypes [9]. When feasible, up to three EMF root tips were taken from each morphotype of soil sample and stored in separate 0.2-ml containers containing CTAB solution. After sampling, morphotyping was finished in 30 days.

One EMF tip per morphotype was subjected to DNA extraction separately. Genomic DNA was extracted using the Quick-DNA Magbead Plus Kit Zymo Research D4081, following the recommended instructions. The Internal Transcribed Spacer (ITS) regions (including ITS1, 5.8S, ITS2) of nuclear ribosomal DNA were amplified by the polymerase chain reaction (PCR) with the following reaction mix: 12.5 μl MyTaq HS Red Mix, 2X (Bioline, BIO-25048), 1 μl of 10 μM ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCTCCGCTTATTGATATG-3') primers each, 1 μl of DNA template, and 9.5 μl of ddH2O. The PCR condition started with a single 3-minute cycle at 95°C, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 45 seconds. A single cycle of final extension at 72°C for 3 minutes concluded the PCR cycle. The aforementioned primers were also used as sequence primers. Products from successful PCR were subjected to Bi-directional sequencing (PT. Genetika Science Indonesia), a partner company of 1st BASE Axil Scientific Pte Ltd, Singapore.

Based on the 97% similarity criteria, obtained sequences were organized into molecular operational taxonomic units (MOTUs). Based on BLAST results in the INSD (International Nucleotide Sequence Database), each MOTU's species identification was given.

3 Results

From total 164 soil cores collected from Central Kalimantan and South Sumatera; eighty-one cores (triplicate) were observed contain EMF roots. Soil cores from Central Kalimantan only contain 19 root tips originated from Tristaniopsis obovata, and 42 root tips from Shorea balangeran. Sample from South Sumatera contain 26 root tips from Tristaniopsis obovata, and 63 root tips from Shorea balangeran. From the collected root tips, seven root tips (11.47%) from Central Kalimantan and five root tips (5.61%) from South Sumatera were successfully sequenced (Table 1).

<table>
<thead>
<tr>
<th>Location</th>
<th>Soil collected</th>
<th>Soil with EMF</th>
<th>EMF tips in Tristaniopsis obovata</th>
<th>EMF tips in Shorea balangeran</th>
<th>Total DNA</th>
<th>Sequenced</th>
<th>% Success</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central Kalimantan</td>
<td>58</td>
<td>46</td>
<td>19</td>
<td>42</td>
<td>61</td>
<td>7</td>
<td>11.47%</td>
</tr>
<tr>
<td>South Sumatera</td>
<td>106</td>
<td>35</td>
<td>26</td>
<td>63</td>
<td>89</td>
<td>5</td>
<td>5.61%</td>
</tr>
</tbody>
</table>

From 12 sequences, 8 EMF species were identified, of which 4 species were collected from Tristaniopsis obovata. Shorea balangeran were associated with 5 EMF species. Identified EMF were dominated by Thelephroaceae sp.1, which was found in both host from both locations (Table 2).

None of the Inoculant were detected in sequence results. However, the majority of the EMF found in this investigation (>97%) did not share any sequences with records from INSD, indicating the presence of new species. With deposited sequences, only 2 EMF had
matches at >97% similarity. *Oidiodendron* sp. 1 which is similar to *Oidiodendron tokumasui* (99.42%) from Japan; and *Thelephoraceae* sp. 5 which is similar to *Thelephoraceae* sp. Ba23 (97.71%) originated from *Tristaniopsis* tree from Bangka Island.

Table 2. **Assigned identity of ectomycorrhizal fungi from collected root tips.**

<table>
<thead>
<tr>
<th>No</th>
<th>DNA No</th>
<th>Sample No.</th>
<th>Location</th>
<th>Host</th>
<th>Inoculant</th>
<th>Sequence Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>PLW-K-3C</td>
<td>Central Kalimantan</td>
<td><em>Tristaniopsis</em></td>
<td>Control</td>
<td><em>Thelephoraceae</em> sp. 1</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>BL-Ca-3A</td>
<td>Central Kalimantan</td>
<td><em>Shorea balangeran</em></td>
<td>Cantharellus</td>
<td><em>Thelephoraceae</em> sp. 2</td>
</tr>
<tr>
<td>3</td>
<td>49</td>
<td>BK-K-3C</td>
<td>Central Kalimantan</td>
<td><em>Shorea balangeran</em></td>
<td>Control</td>
<td><em>Thelephoraceae</em> sp. 3</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>BK-K-2A</td>
<td>Central Kalimantan</td>
<td><em>Shorea balangeran</em></td>
<td>Control</td>
<td><em>Thelephoraceae</em> sp. 4</td>
</tr>
<tr>
<td>5</td>
<td>255</td>
<td>BL-TH-3C</td>
<td>Central Kalimantan</td>
<td><em>Shorea balangeran</em></td>
<td>Termitomyces</td>
<td><em>Thelephoraceae</em> sp. 5</td>
</tr>
<tr>
<td>6</td>
<td>261</td>
<td>BL-TH-3A</td>
<td>Central Kalimantan</td>
<td><em>Shorea balangeran</em></td>
<td>Termitomyces</td>
<td>Oidiodendron sp.1</td>
</tr>
<tr>
<td>7</td>
<td>279</td>
<td>PLW-CH-3A</td>
<td>Central Kalimantan</td>
<td><em>Tristaniopsis</em> sp.</td>
<td>Scleroderma</td>
<td><em>Thelephoraceae</em> sp. 6</td>
</tr>
<tr>
<td>8</td>
<td>94</td>
<td>3.3.1</td>
<td>South Sumatera</td>
<td><em>Tristaniopsis</em> sp.</td>
<td><em>Heimioporus</em> sp.</td>
<td><em>Thelephoraceae</em> sp. 7</td>
</tr>
<tr>
<td>9</td>
<td>146</td>
<td>6.3.1</td>
<td>South Sumatera</td>
<td><em>Shorea balangeran</em></td>
<td>Cantharellus</td>
<td><em>Thelephoraceae</em> sp. 8</td>
</tr>
<tr>
<td>10</td>
<td>438</td>
<td>15.3.2</td>
<td>South Sumatera</td>
<td><em>Tristaniopsis</em></td>
<td>Cantharellus</td>
<td><em>Thelephoraceae</em> sp. 9</td>
</tr>
<tr>
<td>11</td>
<td>444</td>
<td>2.2.3</td>
<td>South Sumatera</td>
<td><em>Shorea balangeran</em></td>
<td>Cantharellus</td>
<td><em>Thelephoraceae</em> sp. 1</td>
</tr>
<tr>
<td>12</td>
<td>471</td>
<td>2.1.1</td>
<td>South Sumatera</td>
<td><em>Shorea balangeran</em></td>
<td>Cantharellus</td>
<td><em>Thelephoraceae</em> sp. 1</td>
</tr>
</tbody>
</table>

4 Discussion

In this study, a total of 8 EMF fungal species were found in 164 peat soil samples collected from Central Kalimantan and South Sumatera, with 81 of these samples containing EMF roots. Out of 150 root tips examined, only 12 were successfully sequenced. This resulted in a low success rate of 8%, which is significantly lower compared to other research on tropical secondary forests, where the success rate is usually over 50% [5], [9]. Several factors were identified as potential contributors to this low success rate, including water stress and peat soil pH.

Peat soils are well-known for their water-logged and acidic pH conditions (Table 3). Most of the identified EMF in this study originated from dry (non-waterlogged or seasonally submerged) soil cores. It has been reported that many EMF are highly sensitive to flooding and fail to colonize roots when subjected to frequent flooding, except for *Thelephora terrestris, Laccaria laccata,* and *Hebeloma crustuliniforme* in *Pinus sylvestris* [10].

Another factor that may have contributed to the low identification of EMF is the pH of the peat soil. Although most EMF prefer acidic conditions for growth, excessively low pH levels can inhibit the formation of EMF root associations[11]. The pH tolerance range of
EMF varies from 5.5 to 10 [12]. In our study sites, the peat soil pH was found to be less than 4, which is far from optimal for EMF growth. Additionally, this low pH could disrupt the DNA extraction process through depurination [13], further interfering with PCR and sequencing.

**Table 3.**

<table>
<thead>
<tr>
<th>Location</th>
<th>Avg. pH H₂O</th>
<th>Avg. Water level (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central Kalimantan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Sumatera</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In terms of species richness and frequency, Thelephoraceae emerged as the dominant family. This dominance in Southeast Asian forests has also been documented by Helbert et al. [5] and Phosri et al. [9]. Thelephoraceae is widely distributed in temperate forests [14–16] and associated with various host lineages [17]. Among the 8 identified species, only 2 had sequences with >97% identity in the INSDC. This is likely due to the limited availability of tropical sequence records, as molecular studies of EMF in the tropics have been relatively scarce. The fewer matches to previous records may also suggest the endemism of tropical EMF [18]. Furthermore, these 2 species were detected in both Japan and Bangka Island, indicating the ubiquitous distribution of Oidiodendron and Thelephoraceae as EMF species.

Interestingly, the identified EMF species were completely different from the inoculated EMF. None of the inoculated EMF were detected in the root tips, indicating that the inoculated EMF had already been replaced by native EMF existing in the peat soil. In natural conditions, the succession of EMF is usually initiated by one or two first-stage fungi, with additional species recruited as the host grows [19]. Therefore, while EMF inoculation is important during the nursery stage, it appears that naturally existing EMF in the field eventually will take over.

**Fig. 3.** Fruiting body appearance of unidentified Thelephoraceae from Sorong, West Papua (source: Helbert, personal documentation, 2023)

Genera *Thelephora* and *Tomentella* represent a cosmopolitan group of ectomycorrhizal fungi, exhibiting a diverse array of basidiocarp morphologies, with species found worldwide in a wide range of sub-tropical and tropical ecosystems, spanning from upland to lowland forests [20] (Figure 3). These genera have also been found to form ectomycorrhizal symbiosis...
with various tree species across the globe. Notable tree families reported to be symbiotic with these two genera include Conifers, Dipterocarpaceae, Fabaceae, and others [21–23].

For instance, *Thelephora terrestris*, when found in mineral soils, has been observed to form hydrophobic hyphae, thereby enhancing water transfer among plants, whereas *Suillus granulatus*, an edible mushroom belonging to the Boletaceae family, lacks this water transport capacity [21]. It is plausible that tree species planted in flooded peatland areas might establish a symbiotic relationship with the Thelephoraceae family, as these fungi may be adept at sustaining growth and obtaining nutrients from peatlands. Moreover, they may even aid forest plants in oxygen-deficient conditions. However, the exact role and mechanisms of ectomycorrhizal fungi from Thelephoraceae must be scientifically established and proven.

Their ability to transfer water (H\textsubscript{2}O) likely provides a simultaneous supply of oxygen (O\textsubscript{2}) to forest plants in peatlands, where the roots of these plants remain inundated with water for periods ranging from 1 to 4 months each year.

In a notable national publication, Ulfa et al. [24] successfully identified molecular diversity in the secondary forest of the Dipterocarpaceae family, particularly within the *Tomentella* genera. Several species were identified, including *Tomentella* sp., *T. subilacina*, and *T. beaverae*, all of which exhibit multi-host symbiosis with tree species such as *Shorea leprosula*, *S. mecistopteryx*, *S. stenoptera*, *Anisoptera* sp., *Cotylelobium* sp., and *Dipterocarpus* sp. Additionally, *Thelephora* spp. were also identified and found to be symbiotic with Dipterocarpus sp., *Cotylelobium* sp., and *S. leprosula*. This research was conducted in Jambi province, which overlaps with the TMI plots in South Sumatra, on the expansive island of Sumatra.

The findings suggest that the Thelephoraceae family might represent a group of ectomycorrhizal fungi that can adapt and truly dominate the underground ecosystems of tropical forests in Indonesia. These fungi interact with various mycorrhizal groups and soil microbial networks, playing diverse roles in maintaining microbial diversity in Indonesian peatland ecosystems.

5 Conclusion

Ectomycorrhizal fungi are essential for the growth of *Shorea balangeran* (Dipterocarpaceae) and *Tristaniopsis obovata* (Myrtaceae) species within peatland ecosystems at the two TMI demo plot in South Sumatra and Central Kalimantan. However, it was observed that the ectomycorrhizal fungi, which were inoculated on the two forest plant species during the nursery stage, only successfully colonized and demonstrated their effectiveness in that controlled environment. Surprisingly, the molecular identification of ectomycorrhizal root tip samples did not indicate the presence of the introduced ectomycorrhizal fungi from the nursery. Instead, the dominant presence of the cosmopolitan Thelephoraceae family of ectomycorrhizal fungi was observed, which efficiently established mycorrhizal networks, facilitating nutrient uptake for forest plant roots in highly acidic (pH < 4) and waterlogged peatland conditions in both TMI plots. These particular species of EMF have the remarkable ability to thrive in the challenging conditions of degraded peatland ecosystems. As the native peatland trees progress towards maturity and their biodiversity expands, the diversity of EMF also increases in a sequential manner. This leads to a balanced and stable interaction of various mutually beneficial associations in the climax conditions of peatland ecosystems in the tropical forests of Indonesia.
6 Acknowledgement

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