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Diversity and the Molecular Identification of Some Ascomycetes Macrofungi Found in the Para Rubber Plantation, Thailand

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Abstract

This study aims to explore the diversity and to identify some ascomycetes macrofungal species found in a para rubber plantation, analysis of the internal transcribed spacer (ITS) region and nuclear large subunit rDNA sequences (LSU) morphology by Eastern Thailand. The species identification of the collection numbers RP2, RP3, RP4, and RP5 by both ITS and LSU sequences was consistent. The RP2, RP3, RP4, and RP5 were identified as *Daldinia eschscholtzii, Cookeina sulcipes, Cookeina garethjonesii,* and *Cookeina tricholoma,* respectively. Two unidentified species, *Trichoderma* sp. (RP1) and *Xylaria* sp.(RP6) maybe require additional molecular markers other than ITS and LSU. This study suggested that macrofungal identification required a combination of morphological and molecular biology approaches for specificity and accuracy. In some fungal taxa, sequence analysis of ITS and LSU could not discriminate fungal species. Although most of the ascomycetes in this study have previously been described in Thailand, this is the first report of ascomycetes macrofungi from para rubber plantations.

Keywords: Macrofungi, Ascomycetes, Identification, ITS, LSU, Para rubber plantation

Introduction

The agriculturist of Eastern Thailand, including Trat, Chantaburi, Rayong province, favor plated Para Rubber tree (*Hevea brasiliensis*) because this area is tropical but the summer rains are more plentiful that is suitable climate to do Para Rubber plantation. In this area, not only Para Rubber trees were growled but also found mushroom diversity. However, little is known about mushroom diversity in para rubber plantations.

Mushrooms are macrofungi that could form fruiting bodies in different shapes and sizes and usually could be observed by naked eyes. The macrofungi could be divided into ascomycetes and basidiomycetes due to the production of ascospore and basidiospore, respectively. The characteristic of ascomycetes macrofungi is the production of asci contained ascospore, usually producing 4-8 ascospore or more than per one ascus and some ascomycetes species could produce apothecia or perithecia. The Pezizaceae and Xylariaceae are ascomycetes fungal families that have been reported in species diversity and widespread. Several studies revealed that these ascomycetes macrofungi act as a decomposer in the ecosystem and are capable to produce different bioactive compounds (Sodngam *et al*., 2014; Adnan *et al.*, 2018; Noppawan *et al.*, 2020). The study of macrofungal diversity has increased the interest of a researcher to be discovered the species of macrofungi and to study the benefit of this fungus such as cultivation for commercial and their bioactive compound to be used in other applications such as pharmaceutical, cosmetic, and medical application.

However, the identification method of macrofungi by only morphology include the characteristic of fruiting body, ascus, basidium, and their spore are time-consuming and low accuracy, especially, the closely related macrofungal species is difficult to differentiate by this method. Therefore, molecular approaches such as Polymerase Chain Reaction (PCR) and sequence analysis were used to identification of macrofungi. The Internal Transcribed Spacer (ITS) of nuclear ribosomal DNA (rDNA) is the conserved region and usually has been targeted for fungal identification due to being highly conserved inside the same species and variable among species in this region (Kim *et al*., 2016; Raja *et al*., 2017). However, in several fungal taxa, molecular identification using ITS alone could not be certain. Particularly in complex genera such as *Trichoderma*, other molecular markers have been used alongside or instead of ITS, such as LSU (nuclear large subunit rDNA), SSU (nuclear small subunit rDNA), tef1-alpha (Translation elongation factor 1-alpha), and rpb2 (DNA-directed RNA polymerase II subunit 2) (Overton *et al*., 2006; Bissett *et al*., 2015; Jaklitsch & Voglmayr, 2015; Zhang & Zhuang, 2018). Therefore, this study aims to explore the diversity and to identify

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some ascomycetes macrofungal species found in a para rubber plantation of Thailand by morphological and analysis of the ITS and LSU region.

Materials and Methods

Macrofungi Sample Collection

The macrofungi samples were collected from soil, decaying wood, and para rubber tree from 40 para rubber plantation farms in Trat province, Thailand (12°27'16" N to 12°18'49" N and 102°24'40" E to 102°22'27" E) between July 2019 to September 2019. The macrofungi specimens were kept in a plastic box and some pieces of macrofungi were kept in absolute ethanol and stored at -20°C until use. The remaining specimens were preserved by drying in an oven at 50°C

Morphological Study of Ascomycetes Macrofungi

The macrofungi were identified by morphological examination as previously described by fruiting body characteristics and microscopic features (Weinstein *et al*., 2002; Jaklitsch *et al*., 2008; Jaklitsch, 2009, 2011; Ekanayaka *et al*., 2016; Wongkanoun *et al*., 2019; Wongkanoun *et al*., 2020).

DNA Extraction and PCR Amplification

DNA extraction was performed by methods according to the manufacturer's protocol (Flavogen, Taiwan). Both ITS and LSU were used as a target for fungal identification in this study. The ITS and LSU region was amplified with specific primer as follows: ITS1 5'-TCCGTAGGTGAACCTGCGG-3', ITS4 5'-TCCTCCGCTTATTGATATGC-3' and LR5 5'-TCCTGAGGGAAACTTCG-3', LROR 5'-ACCCGCTGAACTTAAGC-3', respectively.

A PCR reaction mixture contained 1x PCR master mix (Apsalagen, Thailand), distilled water, 0.5 µM of each primer, and DNA template at a final volume of 20 µl. Subsequently, the ITS and LSU regions were amplified in the thermal cycler. The ITS amplification was performed by initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The LSU amplification was performed as described for ITS except the annealing step was done at 55°C for 30 sec. The 2% agarose gel electrophoresis was used to analyses PCR products at 100V for 30 min with RedSafe (iNtRONbiotechnology, Korea) staining. The PCR products were purified using PureDirex PCR Clean-up & gel extraction kit according to manufacturing instruction (Bio-Helix, Taiwan)

DNA Sequence Analysis and Molecular Identification

The purified PCR products were sent to ATGC company (Pathum Thani, Thailand) to perform DNA sequencing. The BLAST in GenBank was used to analyze the percent similarity of the specimen sequence compared within the sequence in the database. The Neighbor-Joining method was used for tree construction (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2500 replicates for ITS, 10000 replicates for LSU) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004). Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

Results and Discussion

This is the first evidence of ascomycetes macrofungal species found in a para rubber plantation of Eastern Thailand. The macrofungi were harvested for 52 samples, among these samples, six specimens were classified in ascomycetes macrofungi due to the production of ascospore in asci **(Figure 1)**. They designated in the collection number as RP1, RP2, RP3, RP4, RP5, RP6 and could be identified as *Trichoderma* sp. (RP1), *Daldinia* sp. (RP2), *Cookeina* sp. (RP3-5), and *Xylaria* sp. (RP6), respectively **(Figures 1 and 3)**. The morphological studies of these specimens were described as follows.

Trichoderma sp. (RP1): Stromata 1–9 mm diam, and 0.5–1.5 mm thick, later thick and rounded, surface smooth and colour orange, margin free and centrally attached. Perithecia 180–250 × 120–225µm, flask-shaped, ellipsoidal to globose. Asci cylindrical, 80–121 µm long, including a stipe of 9–19 µm, 6–8 µm wide. Ascospores hyaline, subglobose to oval.

Daldinia sp. (RP2): Stomata 3.5-5.5 cm diam × 1.5-2.5 cm high, surface brown vinaceous. Stomata with internal concentric zones below the perithecial layer and without a stipe. Perithecia tubular, 0.3-0.4 mm diam × 1-1.3 mm high. Asci 166-180 × 7-9 µm. Ascospores dark brown, 11-12 × 5.5-6 µm.

Cookeina sp. (RP3): Apothecia 1–3 × 4–8 cm arising singly, stipitate and pink. Hairs 13–14 × 2.5–3 µm on flanks and margins. Stipe 1–2.5 mm long. Hymenium pinkish orange. Paraphyses 2.5–3 µm wide with filiform. Asci 290–360 × 16–20 µm, non-amyloid. Ascospores 23–29 × 11–16 µm, 1-celled, ovoid, hyaline.

Cookeina sp. (RP4): Apothecia 1–2 × 2.5–3 cm, stipitate and yellow to orange. Hymenium glabrous, bright yellow to orange. Stipe 1–1.5 × 0.2–0.3 cm. Hairs 50–60 × 12–14 µm length and arranged around the margin. Hymenium hyaline. Paraphyses 2.5–3.6 µm wide with filiform. Asci 290–300 × 19–22 µm, non-amyloid. Ascospores 27–29 × 16–17 µm, 1-celled, ovoid, hyaline.

Cookeina sp. (RP5): Apothecia 1.5–2.5 × 5–7 cm arising singly, stipitate, and orange. Stipe 1-1.5 cm long, 0.3-0.4cm broad. Spines 3–6.5 × 0.5–1 mm cylindrical. Hairs 75–80 × 9–11 µm on flanks and margins, cylindrical, hyaline. Hymenium orange to hyaline. Paraphyses 2.5–3.5 µm wide, filiform, septate, highly branched. Asci 225–310 × 9–20 µm, non-amyloid. Ascospores 12–23 × 7.5–11.5 µm, 1-celled, ovoid, hyaline to pinkish.

Xylaria sp. (RP6): Stroma 2–6 cm tall, 1–1.5 cm thick, club shape, with a rounded tip and black. Perithecia in stroma about 0.5-1 mm across, spherical, just below the surface. Asci 8-spored. Ascospore

13–15 x 5.5–6.5 µm, fusiform, smooth, brown, spiraling germ slit that runs the length of the spore.

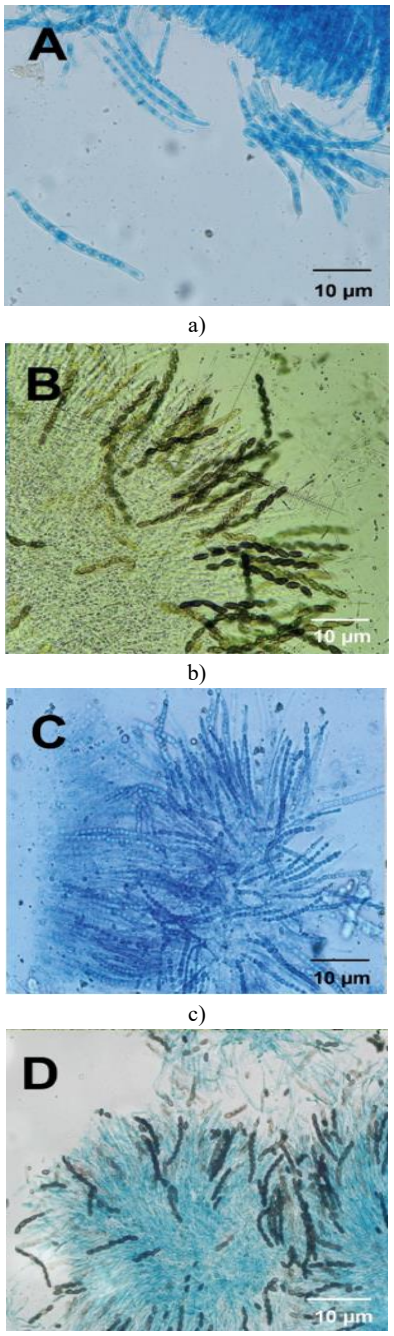


Figure 1. Asci contained ascospore of some ascomycetes mushrooms from a rubber plantation. a) *Cookeina* sp.; b) *Daldinia* sp.; c) *Trichoderma* sp.; d) *Xylaria* sp.

In this study, the molecular identification of ascomycetes macrofungi was performed by using ITS and LSU region as the molecular target for PCR amplification. The agarose gel electrophoresis presents the PCR product size between 600-700 bp for ITS **(Figure 2a)** and 900 bp for the LSU region **(Figure 2b)**. The PCR product size of LSU was correlated with other studies but the ITS differs from several studies that use the same primer. PCR product was found between the size 400-850 bp (Appiah *et al*., 2017; Adeniyi *et al*., 2018) and 350-880 bp (Fujita *et al*., 2001). The difference in the size of the ITS region in fungal species and variability of the DNA quality may be affected by this variation. (Lorenz, 2012; Krimitzas *et al*., 2013).

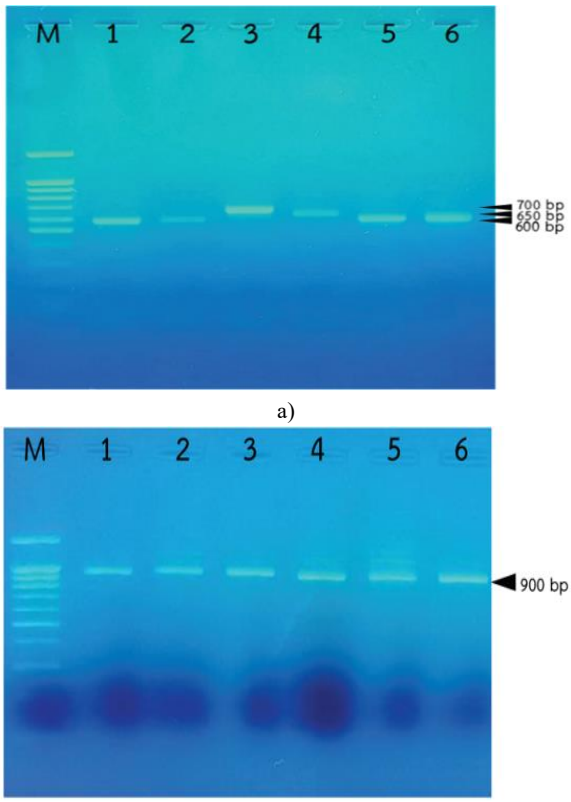


Figure 2. Agarose gel electrophoresis of ITS region (a) and LSU (b) amplification in ascomycetes macrofungi. Lane M: DNA ladder (100 bp) Lane 1: *Trichoderma* sp. (RP1); Lane 2 *Daldinia eschscholtzii* (RP2); Lane 3: *Cookeina sulcipes* (RP3); Lane 4: *Cookeina garethjonesii* (RP4); Lane 5: *Cookeina tricholoma* (RP5); Lane 6: *Xylaria* sp. (RP6)

The percent similarity of ITS and LSU region among ascomycetes macrofungi were analyzed in the sequence database by BLAST as presented in **Table 1**. The species identification of the collection numbers RP2, RP3, RP4, and RP5 by both ITS and LSU sequences was consistent. The RP2, RP3, RP4, and RP5 were identified as *Daldinia eschscholtzii*, *Cookeina sulcipes*, *Cookeina garethjonesii*, and *Cookeina tricholoma* respectively. However, two unidentified species, RP1, showed the best match of ITS and LSU sequences with *Trichoderma pezizoides* (98.79 % similarity) and *Trichoderma leguminosarum* (98.69 % similarity), respectively. The RP6 showed the best match of ITS and LSU sequences with *Xylaria terricola* (88.42 % similarity) and Xylariaceae sp. (100 % similarity). Therefore, RP1 and RP6, maybe require additional molecular markers other than ITS and LSU especially in complex genera such as *Trichoderma* (Overton

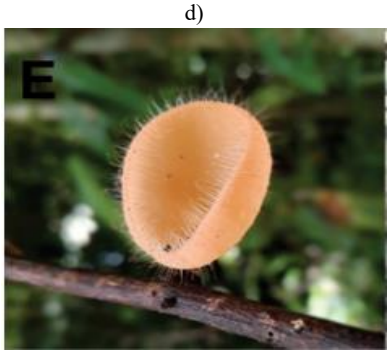
et al., 2006; Bissett *et al*., 2015; Jaklitsch & Voglmayr, 2015; Zhang & Zhuang, 2018). Moreover, the phylogenetic tree of ascomycetes macrofungi showed high genetic relatedness with reference strains **(Figure 4)**.

a)

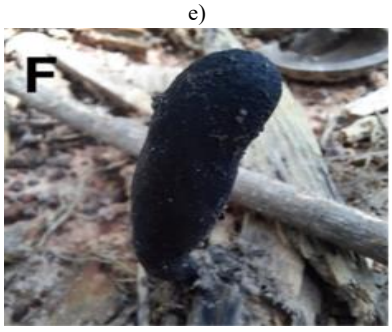
b)

c)

d)



e)

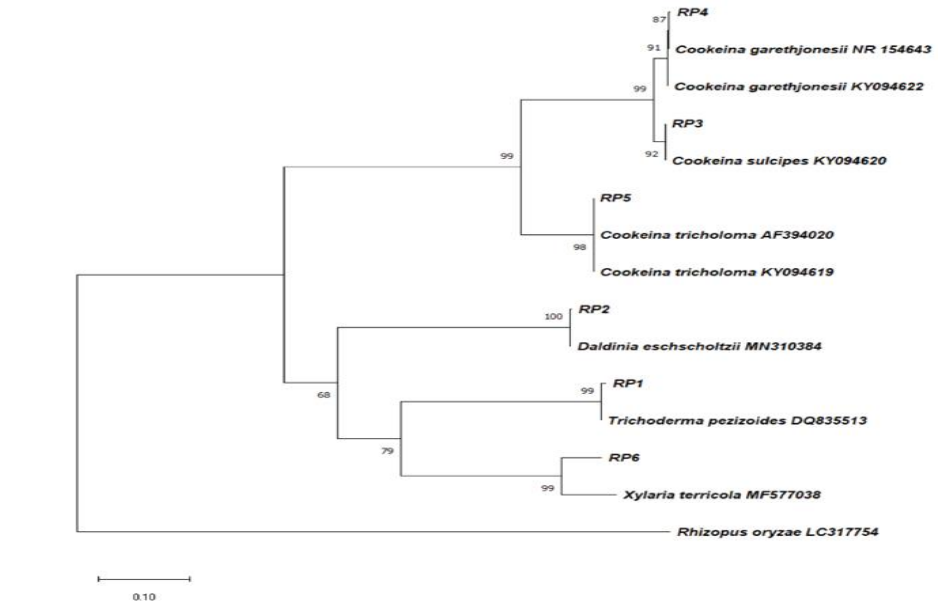


f)

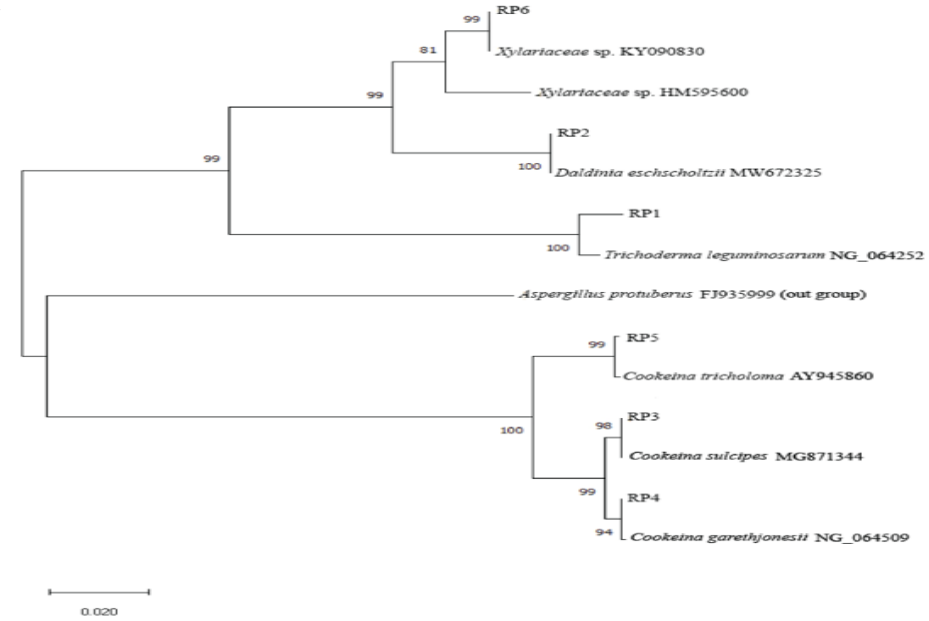
Figure 3. Ascomycetes macrofungi were identified in this study. a) *Trichoderma* sp. (RP1); b) *Daldinia eschscholtzii* (RP2); c) *Cookeina sulcipes* (RP3); d) *Cookeina garethjonesii* (RP4); e) *Cookeina tricholoma* (RP5); f) *Xylaria* sp. (RP)

Table 1. The number of ascomycetes macro-fungi found in the Para rubber plantation because of BLAST and accession.

Species	Collection No.	Best match (Accession No.)		GenBank Accession number ITS	Best match)Accession No(.		GenBank Accession number LSU
		ITS	Similarity (%)		LSU	Similarity (%)	
Trichoderma sp.	RP1	*Trichoderma pezizoides* (DQ835513)	98.79	MW659098	*Trichoderma leguminosarum* (NG_064252)	98.69	OL441089
Daldinia eschscholtzii	RP2	*Daldinia eschscholtzii* (MN310384)	100	MW659100	*Daldinia eschscholtzii* (MW672325)	100	OL441048
Cookeina sulcipes	RP3	*Cookeina sulcipes* (KY094620)	98.44	MW659101	*Cookeina sulcipes* (MG871344)	99.11	OL441046
Cookeina garethjonesii	RP4	*Cookeina garethjonesii* (KY094622)	99.06	MW680773	*Cookeina garethjonesii* (NG_064509)	99.88	OL441043
Cookeina tricholoma	RP5	*Cookeina tricholoma* (KY094619)	100	MW680771	*Cookeina tricholoma* (AY945860)	99.76	OL441044
Xylaria sp.	RP6	*Xylaria terricola* (MF577038)	88.42	MW659104	.Xylariaceae sp (KY090830)	100	OL441091



a)



b)

Figure 4. Phylogenetic tree based on ITS (A) and LSU (B) sequences of 6 ascomycetes macrofungi and other reference sequences from the GenBank

Diversity of ascomycetes macrofungi was investigated in Thailand, several reports revealed some ascomycetes fungi in the family Xylariaceae and provided the data of their bioactive compound (Velmurugan *et al*., 2013; Srihanant *et al*., 2015; Noppawan *et al*., 2020; Wongkanoun *et al*., 2020). Interestingly, some species of ascomycetes macrofungi were reported as a new species in Thailand such as *Xylaria thailandica* (Srihanant *et al*., 2015) and *D. chiangdaoensis* (Wongkanoun *et al*., 2020). This evidence suggested that the diversity of ascomycetes macrofungi in Thailand remains to be explored. Although in the present study, no new species were discovered and the species of ascomycetes macrofungi that were found in a para rubber plantation have previously been reported in Thailand (Weinstein *et al*., 2002; Jaklitsch *et al*., 2008; Jaklitsch, 2009, 2011; Ekanayaka *et al*.,

2016; Wongkanoun *et al*., 2019, 2020). However, this is the first report of ascomycetes macrofungi that found in the para rubber plantation of Eastern Thailand.

Conclusion

The diversity of ascomycetes macrofungi has been explored in a para rubber plantation, Eastern Thailand. The macrofungal specimens were identified by morphological and sequences analysis of ITS and LSU region. The ascomycetes fungal species were identified as *Daldinia eschscholtzii, Cookeina sulcipes, Cookeina garethjonesii, Cookeina tricholoma,* and two unidentified species, *Trichoderma* sp. and *Xylaria* sp. This study suggested that macrofungal identification required a combination of morphological and molecular biology approaches for specificity and accuracy. In some fungal taxa, sequence analysis of ITS and LSU could not discriminate fungal species, additional other molecular markers may be required.

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Conflict of interest: None

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Ethics statement: None

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