Pertanika J. Trop. Agric. Sc. 42 (2): 735 - 743 (2019)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Short Communication

Identification of Microfungi Isolated from Belian Fruits

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ABSTRACT

Belian tree (*Eusideroxylon zwageri* Teijsm. & Binn.) is one of the highly demanded commercial timber tree indigenous to the Southeast Asian region. The tree is threatened with over exploitation; habitat destruction and slow regeneration. While vascular plants are known as major reservoirs of fungi species, there have been no studies to identify the microfungi isolated from fruits of this endemic tree. By using internal transcribed spacers (ITS) sequence analysis, five genera were isolated and identified as *Annulohypoxylon*, *Daldinia*, *Hypoxylon*, *Lasiodiplodia* and *Trichoderma*. This result will be primarily used as baseline data for further investigations on microfungi diversity associated with Belian tree.

Keywords: *Annulohypoxylon,* Borneo ironwood, *Daldinia*, *Eusideroxylon zwageri*, *Hypoxylon*, Lauraceae, *Lasiodiplodia*, *Trichoderma*

INTRODUCTION

Fungi plays an important role in the optimal functioning of the ecosystem. In general, they involved in the breakdown of nutrients on the forest floor, which for ready uptake by the plants but in most cases the role of individual fungi in nature is unknown (Mueller & Bill, 2004). Apart from being an important contributors to the diversity of organisms in the forest, microfungi have been consistently utilized in the drug production, food processing, bio-control agents as well as in enzyme biotechnolgy (Kumar et al., 2013; Snaddon et al., 2012).

A study on microfungi associated with endemic plants in Mauritius reported more than 200 taxa of microfungi with approximately 90% of being new genus (Dulymamode et al., 2001) while study in Kenya reported four fungal taxa were new to science detected from their endemic and rare

ARTICLE INFO

Article history:
Received: 29 October 2018
Accepted: 04 January 2019
Published: 30 May 2019

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ISSN: 1511-3701
e-ISSN: 2231-8542

plants (Siboe et al., 2000). This is shown that, many unknown microfungi are likely to be rare and threatened to the same degree as their plant host.

However, very little is known and reported on the microfungi diversity isolated from the endemic and endangered trees in Malaysia. Belian tree, scientifically known as *Eusideroxylon zwageri* Teijsm. & Binn. is one of the most important commercial timber trees indigenous to Southeast Asian forests. It is native to Malaysia (Sarawak and Sabah), Brunei, Indonesia and The Philippines. The tree is taxonomically classified in the Lauraceae family and listed as a vulnerable species by the IUCN Global Red List of Threatened Species (version 2017.3) (International Union for Conservation of Nature [IUCN], 2017) resulting from the over exploitation and habitat destruction. To date, only one study of the microfungal diversity on the green and leaf litters of Belian tree from Kubah National Park, Sarawak has been reported (Lateef et al., 2015).

This study therefore, is aimed to identify microfungi community isolated from Belian fruits which will add value to the latest knowledge on Belian tree especially related to the diversity of microfungal. This information will aid further study on their numerous roles with respect to Belian tree.

MATERIALS AND METHODS

Materials

Ten available matured fruits were collected randomly under the adult Belian tree in May 2017 from Semenggoh Reserved Forest in Sarawak, Malaysia. The fruit samples were collected on the forest floor near to the mother tree and were kept in brown paper bags, labeled and transported to the laboratory for fungi isolation.

Isolation of Microfungi

The fruits were washed under tap water and were surface sterilized with 70% ethanol solution for 5 minutes, and then, the fruits were rinsed with sterilized distilled water for three times and blot-dried using sterilized filter paper (Kouame et al., 2010). The pericarp layer of the fruit samples was divided into three parts: tip, middle and bottom of the fruits to get an overall estimation of the fungi reside in the pericarp part of the fruits. Then it was aseptically cut into 1 cm^2 using sterile surgical blades and carefully transferred onto potato dextrose agar (PDA) plate supplemented with Streptomycin (20 mL/L of medium).

The plates were sealed and labeled by fruits sample part, initial culturing date and incubated at 28±2^oC. Observation and isolation of the growing microfungi on the surface of the media plates, colonies that differ in time of appearance, size, color, and elevation were recorded. A loop of each original pure fungi colony was picked up using plating technique, and re-plated onto a solid pure culture agar plates containing the same culture media but without antibiotics. The plates were sealed, labeled and incubated until the fungi grew to half of the plate.

The hyphae tip technique was performed to obtain pure culture isolate (Leslie & Summerell, 2006). Then the pure fungi isolates were transferred to PDA slants for short-term storage or in complete media xylose (CMX) 25% glycerol at -80°C for long-term preservation.

DNA Extraction and PCR Amplification

Total genomic DNA were extracted from ten successful pure cultured isolates using an Ultra Clean ® Microbial DNA isolation kit (MO-BIO, Carlsbad, CA, USA) according to manufacturer's instructions. Oligonucleotide primers ITS1 (5'-TCCGTAGGTGAACCTGCGG- 3') and ITS4 (5'-TCCTCCGCTTATTGATA TGC-3') (White et al., 1990) were used to amplify internal transcribed spacer (ITS) region of the nuclear ribosomal DNA. Each polymerase chain reaction (PCR) contained 5 uL of 5X PCR buffer, 4 uL of 25 mM $MgCl_2$, 0.5 uL of 10 mM dNTPs (Promega), 0.5 uL of each 10uM primer, 0.1uL of 5 units/uL GoTaq® DNA polymerase, 0.5 uL of 10X BSA and 2.0 uL of template DNA adjusted with nuclease free water to a final volume of 50uL. Amplification reactions were carried out in a thermal cycler (Eppendorf AG 22331, Hamburg). PCR cycling protocol was as follows: an initial pre-denaturation for 2 min at 95ºC, followed by 35 cycles of denaturation at 95ºC for 20 s, annealing at 50ºC for 30 s, and extension at 72ºC for 30 s; with a single cycle of final extension at 72ºC for 5 min. The PCR amplification products were separated by electrophoresis in 1.5% agarose gel in 1.0x Tris-boric acid-EDTA (TBE) buffer with Etb "Out" Staining Solution (YEASTERN BIOTECH Co. Ltd) and photographed under UV light. The images were captured with DOC PRINT system (Vilber Lourmat, USA). A good quality of amplified PCR products was sequenced in both directions using an Applied Biosystems 3730xl DNA Analyzer by MyTACG Bioscience Company, MY.

Data Analysis

ClustalW in Molecular Evolutionary Genetic Analysis (MEGA 7) was used to generate and aligned the consensus sequences. The consensus sequences were search against sequences in GenBank using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.gov) to determine the closest matched ITS sequence from the database. All the assembled DNA sequences were deposited in GenBank.

A Maximum Likelihood analysis was conducted using MEGA 7 with 1000 bootstrap replication value based on Tamura-Nei Model (Kumar et al., 2016). Branches corresponding to partitions reproduced in less than 50% bootstrap replicated were collapsed. The representative sequences of each species were obtained from GenBank and were included in the tree. The tree was rooted with *Puccinia graminis* (HM131358) as an out-group for this phylogenetic tree. The tree was constructed to comprehend the placement of each isolates and the reference sequences in the lineage.

RESULTS

The phylogentic tree was constructed to understand the placement among the isolates and the reference sequence (Figure 1) and colony features for each isolates were shown in Figure 2. All fungi isolates were identified belong to the phylum Ascomycota, which comprised nine taxa from five genera; *Annulohypoxylon* (2 species); *Daldinia* (1 species); *Hypoxylon* (1 species); *Lasiodiplodia* (1 species); and *Trichoderma* (4 species) (Table 1). The molecular fragment size of ITS region amplified was between 500 – 600 bp except for ITS fragment of *Annulohypoxylon* species which was > 800 bp. Based on BLAST search, the ITS sequences showed high percentages similarities (99% and 100%) with sequences in the GenBank database (http://www.ncbi.nlm.gov). All ITS sequences of the isolates were deposited into GenBank and the accession number are listed in Table 1.

The phylogenetic tree was divided into two main clades; I and II (Figure 1). Main clade I clustered all species under Class Sordariomycetes while main clade II grouped Class Dothideomycetes. Main clade I was further divided into subclades A and B which clustered the species into their specific order. Subclade A nested all species under Xylariales order and subclade B grouped Hypocreales species.

Subclade A contained three genera from *Xylariaceae* family; *Annulohypoxylon, Daldinia* and *Hypoxylon*. All the isolates are highly similar to their respective reference sequences from GenBank and well supported with 100% bootstrap value. Isolate Q2687 was identified as *Annulohypoxylon nitens* (KU684021) while isolate Q2688 was identified as *Annulohypoxylon viridistratum* (KX376325) both with 99% similarity. Colony of *A. nitens* was observed with filamentous white mycelium. The reverse side of the plate showed changes in

Table 1
Fungi species isolated from endocarp layer of Belian fruits which were characterized based on sequencing of the ITS region

Isolate No.	Sequence based identification	Deposited GenBank Accession No.	Percentage of similarities (%)	Accession No. of fungi closest match to GenBank database
Q2680	*Lasiodiplodia theobromae*	MG711820	100 %	KY473061
Q2681	*Trichoderma spirale*	MG711821	100 %	NR_077177
Q2682	*Trichoderma virens*	MG711822	100 %	KR296891
Q2683	*Trichoderma asperellum*	MG711823	100 %	HQ857121
Q2684	Trichoderma crassum	MG711824	100%	NR_134370
Q2685	Daldinia eschscholtzii	MG711825	99%	KU304335
Q2686	Hypoxylon investiens	MG711826	99 %	KC968925
Q2687	Annulohypoxylon nitens	MG711827	99 %	KU684021
Q2688	Annulohypoxylon viridistratum	MG711828	99 %	KX376325
Q2689	Daldinia eschscholtzii	MG711829	99 %	AB284189

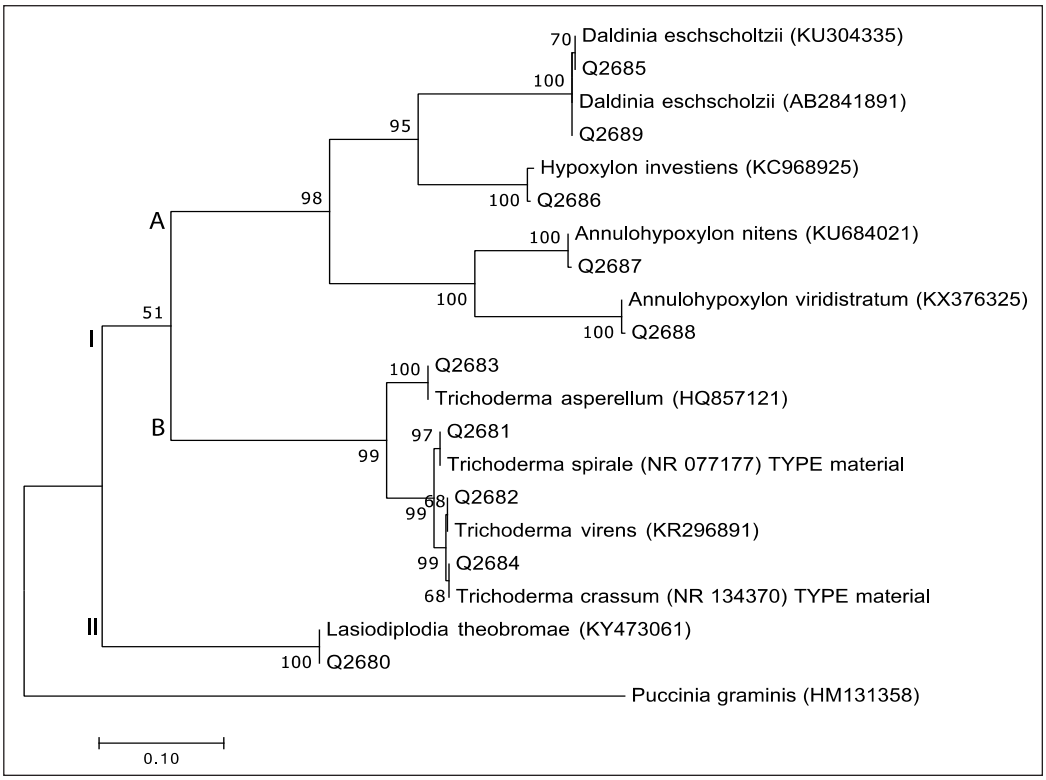

Figure 1. Maximum Likelihood (ML) tree, showing the relationship of fungi isolated from Belian fruits inferred from ITS region. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. *Puccinia graminis* was chosen as the out-group

pigmentation to orange brownish when aging. This differs from the colony feature of *A. viridistratum* which showed a reverse side of yellow pigmentation when aging (Figure 2).

Meanwhile, isolates Q2685 and Q2689 resulted with 99% similarity to two *Daldinia eschscholtzii* strains (KU304335 and AB2841891). Colony features of Q2685 were found to have a dusted white mycelium with yellow tint while colony of Q2689 only formed a dusted white mycelium. The reverse side of Q2689 was seen to form more blackish green pigmentation than Q2685 (Figure 2). Isolate Q2686 was observed with 99% similarity to *Hypoxylon investiens* (KC968925). The colony was found to form circular sparse and thin mycelium and brown ring pigmentation can be seen forming from the front and reverse side of the plate (Figure 2).

Subclade B, order Hypocreales strongly grouped four isolates (Q2681, Q2682, Q2683 and Q2684) to their respective reference sequences as *Trichoderma* species in *Hypocreaceae* family. Nucleotide BLAST for sequences of isolate Q2682 resulted with 99% similarity to *T. virens* (KR296891) while Q2681 resulted as *T. spirale* (NR_077177); Q2683 as *T. asperellum* (HQ857121)

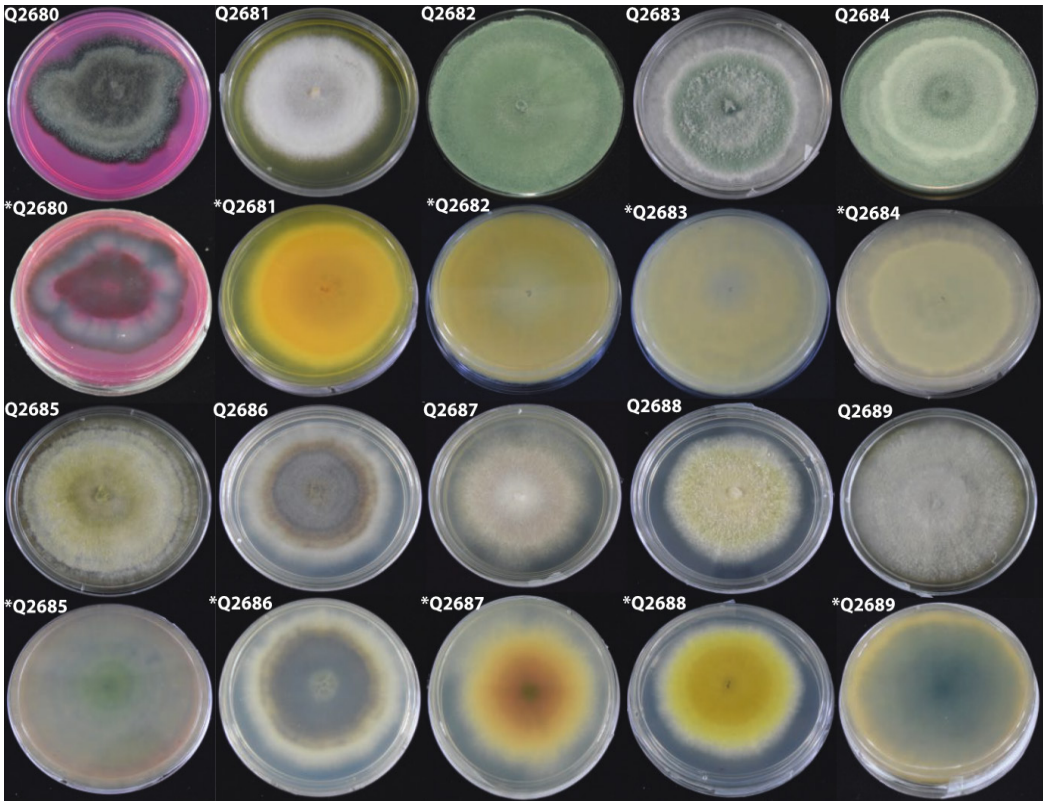

Figure 2. Colony features of the fungi isolated on Potato Dextrose Agar plate
* = pigmentation on reverse plate

and Q2684 as *T. crassum* (NR_134370) resulted with 100% similarity. Colony of *T. asperellum* were observed forming filamentous white floccose aerial mycelium while colony of *T. spirale* formed a thick circular cottony mycelium with green tint that changes the PDA media to yellowsih tan. While both colony of *T. virens* and *T. crassum* have similar colony characteristics of green dusted mycelium except that colony of *T. crassum* was also observed to form white dusted circular on the front features.

Main clade II segregated isolate Q2680 from the rest of the isolates. It was identified as *Lasiodiplodia theobromae* (KY473061) with 100% similarity, which belonged to *Botryosphaeriaceae* family in Dothideomycetes clade. The placement was well supported with 100% bootstrap value. The colony was observed forming irregular black velvety mycelium surface when cultured on PDA media (Figure 2). It formed white to maroon pigmentation when aging in the reverse side of the plate. It was observed that the colony also changed the PDA media to pink coloration when incubated at room temperature.

Puccinia graminis (HM131358) which was chosen as an out-group control for this tree belongs to Basidiomycota phylum, a sister phylum to Ascomycota phylum and distinctly separated from their main clade.

DISCUSSIONS

In this study, we used molecular application techniques to identify the isolated microfungi up to their species level. The use of molecular approaches in this study is more reliable, exhibited higher sensitivity and more time saving compared to morphological characterization techniques. This is due to their cryptic and often ephemeral character that make them difficult to identify with only using classical morphological identification especially when there is no basic information about the fungi diversity on the infected host of Belian fruits. We used the internal transcribed spacers, ITS 1 and 4 regions to amplify and identify the isolated microfungi. The ITS have a convenient target region for molecular identification of fungi due to the variability of length and nucleotide content among different species and amplify highly variable ITS1, ITS2 and the 5.8S-coding sequences (Martin & Rygiewicz, 2005).

It was observed that the size of ITS region for *Annulohypoxylon* species is more than 800 bp as compared to other genera. This was in agreement with a similar study conducted on the fungi isolation from Thailand, which revealed large differences in size from 479 to 936 bp in *Annulohypoxylon* species. A further sequencing analysis using alpha-actin and beta-tubulin was suggestive of the presence cryptic species (Suwannasai et al., 2013) This also supports the need to subject the species of *Annulohypoxylon* identified in this study for further identification processes using other markers. *A. viridistratum* identified in this study was recently reported as new tropical and subtropical species (Kuhnert et al., 2014). *Annulohypoxylon* species usually associated with dead dicotyledonous wood (Fournier & Lechat, 2016) and also frequently found as endophytes in seed plants (Ikeda et al., 2014).

Little is known on the diversity of fungi species associated with Belian tree especially related with their fruits, which will generally affect their seed germination and survival. Study on *Cecropia insignis* has shown that seed-associated fungi are highly diverse (U'ren et al., 2009). It is noted that the most common seed-associated fungi in tropical forest are host-generalists however; they still have profound effects on seed germination for a particular tree species as well as site-specific effects on seed survival. It is desirable to learn more about this understudied topic as fungi are one of the major causes of seed mortality in soil for a variety of tropical trees (U'ren et al., 2009) and it is of more concern when allied with one of the vulnerable endemic tree.

Furthermore, many scientist have acknowledged that various fungi species are associated with many endangered plant species (Buchanan at al., 2002; Fuchs & Haselwandter, 2008; Zubek et al., 2011). This fungi species has the potential for novel utilization in many scientific undertakings. Their association with endangered plant species put them at the risk of going into extinction with their host plants (Fuchs & Haselwandter, 2008). As a threatened species, this scenario is foreseen in Belian tree because of the diversity of their microfungi not yet well studied. It is hoped

that the findings reported in this study will serve as a baseline for further investigations that will enhance the conservation status of this economic tree and its associated microfungi.

CONCLUSION

Since this is a first attempt on studying the microfungi diversity of Belian fruits, data from this study will be used for future understanding of the biology of *E. zwageri* and can also be used to strengthen its conservation importance. It is hoped that the data presented here will lend an insight into further investigation of fungi diversity involved with rooted seed in Belian and as well serving as the starting point for potential novel fast seed germination processes derived from the seed-associated fungi. The outcomes is believed to be far reaching in the understanding, protection and conservation of Belian tree as well as in the conservation of the fungi species associated with endangered plant species which are proned to extinction in the nearest future.

ACKNOWLEDGEMENTS

We are grateful to the Forest Department of Sarawak (FDS); Sarawak Forestry Co-operation (SFC) and Semenggoh Seed Bank for the permit to conduct the study and permission to collect samples from the National Park. The authors are thankful to the Ministry of Higher Education Malaysia for the award of the Fundamental Research Grants Scheme project no. FRGS/1/2015/WAB13/UPM/02/4 made available to Universiti Putra Malaysia. Study on molecular characterization was supported through UPM/700-1/3/GeranPutra; vot. No. 9504300, to Nur Ain Izzati M. Z. We are thankful to Mohd. Akmal Mohd. Raffi for his assistance during the fieldwork.

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