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AN ITS GENE-MEDIATED MOLECULAR DETECTION OF FUNGI ASSOCIATED WITH NATURAL AND ARTIFICIAL AGARWOOD FROM *Aquilaria malaccensis*

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ABSTRACT

The current study examined fungal diversity in manufactured and natural agarwood samples perceived from *A. malecensis* trees in a plantation and the East Malaysia rainforest. Isolated fungi were also subjected to molecular analysis. The fungal community composition of healthy and damaged tree samples from both plantation and the wild forest was identified using PCR amplification of the internal transcribed spacer (ITS) region from fungal isolates. Meanwhile, ten groups of fungi isolates are expected to be placed in both natural and inoculated based on 1000 bootstrap values. They validated earlier genomic identification; 55 fungal isolates were discovered in artificial and natural agarwood, including agarwood and healthy wood. Ten fungal groupings were developed based on morphological characterization similarities. Based on NCBI BLAST analysis, molecular identification revealed *Fusarium solani*, *Botryosphaeria theobromae*, *Polyporales spp*, *Schizophyllum commune*, *Aspergillus aculeatus*, and *Lasiodiplodia theobromae*. *Fusarium solani* excelled in *A. aculeatus* and *Polyporales* spp. in agarwood samples and healthy wood. The presence of more fungi species in natural agarwood than in artificial agarwood may be due to the favourable humid and shaded habitat for fungal growth. Nonetheless, synthetic agarwood was able to synthesize agarwood-related chemical compounds. Considering *F. solani* was often identified in both agarwood, agarwood inoculant may act as a booster for *F. solani* to begin pathogenicity in artificial agarwood. The current research shows that artificial agarwood may provide quality comparable to natural agarwood and is not influenced by fungus interacting with the tree.

Keywords: Agarwood; *Aquilaria malaccensis*; Inoculated; *Fusarium solani*

INTRODUCTION

In the global market, industries are prepared to pay any price to obtain a supply, despite the availability of synthetic sources at a cheaper cost. *Aquilaria* spp. is among the costliest agarwood species (**Rasool *et al.*, 2016; Chhipa & Kaushik, 2017; Faizal *et al.*, 2017**). The species would be worthless unless it included a resinous strip, often found along the inner section of the trunk. Some mechanism, which is currently unclear, contributes to resin formation in agarwood species. Regardless, nations having agarwood species seedlings have been practising agarwood plantations. While academics from around the globe compete to learn more about agarwood species' evolution, the species continue to decline with each passing year (**Turjaman *et al.*, 2016; Barden *et al.*, 2000; Bhuyar *et al.*, 2020a**). Agarwood (*Aquilaria malaccensis*) is a resinous fragrance-impregnated wood species found in tropical rainforests in South and Southeast Asia (**Jayachandran *et al.*, 2015; Ramli *et al.*, 2022a**). Among the Aquilaria species, *A. malaccensis* is the most common source. It goes by several names, including agar, agarwood/sandalwood, aloeswood, eaglewood, gaharu, and kalamabak (**Jayachandran *et al.*, 2015**). Its medicines, fragrances, incense, and religious ceremonies are in high demand across Asia and the Middle East. When burned, resinous agarwood has a distinct mellow aroma, although its perfume is discernible in high-quality agarwood. Agarwood is known differently in different nations, with various names and usages based on local culture. In Malaysia and Indonesia, it is known as 'gaharu' or 'karas.' While their vernacular names might be perplexing, their commercial names are generally known as agarwood, aloeswood, and eaglewood (**Chua, 2008**).

Aquilaria malaccensis is a tropical tree that naturally produces expensive resinous heartwood agarwood due to natural or artificial injury or microbial infection. Fungi are widely identified as the primary microbiological component responsible for the development of agarwood. Agarwood is not created in Aquilaria species' wood tissues; it is most likely developed after healthy tissues have been injured and infected by a fungus. The creation of volatile agarwood chemicals is primarily due to the agarwood tree's exposure to both biotic and abiotic (physically or chemically), which activates the defence mechanism (**Faizal *et al.*, 2017**). Several fungus species have been isolated from agarwood and used as inoculants, although the chemical compositions of volatile compounds are still of poor quality or sometimes variable (**Bhuiyan *et al.*, 2009**). The variations between naturally infected and artificially inoculated agarwood might be attributed to the variety of fungi in both agarwood trees.

The resin production method based on fungal group kinds determines the resin quality. *Alternaria* sp., *Clasdosporium* sp., *Fusarium* sp., *Curvularia* sp., *Clasdosporium* sp., and *Phaeacremonium* sp. were discovered in the resinous wood of *Aquilaria malaccensis* by **Premalatha and Kalra (2013)**. **Mohamed *et al.* (2010)** identified fungi diversity in agarwood among Cunninghamella, Curvularia, Fusarium, and Trichoderma. Although agarwood bacteria in commercial inoculants and nature may not be connected (**Monggoot *et al.*, 2018; Bhuyar *et al.*, 2020b**), some fungus groups isolated from resinous wood may impact agarwood fragrance quality. Given the marketability of high-quality agarwood, fungal identification based on their ability and capacity to induce Aquilaria wood into golden agarwood is critical to large-scale production to meet global demand (**Turjaman *et al.*, 2016; Nguyễn *et al.*, 2022**). Compared to the previous study, the focus is on the link between the fungal species group and biochemical content in resinous wood.

Prior research investigations are lacking in evaluating the fungal community composition in artificially inoculated and spontaneously infected agarwood. Thus, comparing the two agarwood samples regarding quality and fungal diversity is required to identify the active fungal group capable of inducing volatile agarwood chemicals. Furthermore, the comparison may reveal if the grade of artificially inoculated agarwood can match that of naturally infected agarwood in terms of fungal infection. The current study uses a fungal culture and molecular approach to identify fungi associated with agarwood formation from inoculants, natural agarwood, and the environment. The present study aimed to identify fungal species employing a molecular technique. The techniques include DNA extraction, Polymerase Chain Reaction (PCR) with a pair of universal fungus primers ITS1 and ITS4. The amplified DNA was submitted to a sequencing provider for the ITS sequence and phylogenetic analysis using bioinformatics tools.

MATERIAL AND METHODS

Agarwood sample collection

Both agarwood and healthy wood sample were taken from a forest in Rompin Pahang, where those trees are infected naturally. Identifying *A. malaccensis* in the forest was done with the guidance of native people. Six agarwood trees aged between 20 to 50 years old were identified. The agarwood sample was labelled as R1, R2, R3, R4, and R5 while healthy wood was labelled as HR1, HR2, HR3, HR4, and HR5. Meanwhile, artificial agarwood was obtained from Agarwood Plantation in Merchang, Terengganu, Malaysia, named M1 and M2. The healthy woods were labelled as HM1 and HM2. Artificial agarwood was harvested after 12 months of inducement from a 10-year-old agarwood tree. Healthy wood and agarwood samples were stored on paper and returned to the laboratory for fungal screening.

Fungal Identification

Fungi identification was made based on morphological and molecular studies. Morphological observation required fungi screening to produce one spore culture of each further growth of fungi. The matured colony will be observed under the microscope to examine the microscopic structure of the spore (conidia), conidiospore, or fruiting body. Molecular determination of fungi colony was done through DNA extraction and amplification using universal fungi primer forward ITS1 and reversed ITS4. After DNA quality and band gel of the expected size were detected in gel electrophoresis analysis, samples were purified and submitted for sequencing services. Finally, bioinformatic analysis was constructed to determine fungal species.

Fungal Molecular Identification

Similar isolates were grouped based on appearance and microscopic observation from identical batches. Samples were taken from each group to proceed with molecular identification.

DNA Extraction

Fungi isolate for DNA extraction was prepared in Potato Dextrose Broth which enhanced fungal growth and sporulation (**Ramli *et al*., 2022b**). The culture was incubated under 27±2 °C for 7 to 10 days with intervals of 12 h in daylight and 12 h under darkness. Mycelia were extracted using sterile forceps and air-dried for about 30 minutes. Excessive liquid from the mycelia sample was dabbed with the tissue before being transferred into the mortar. Liquid nitrogen was poured onto the mycelia and ground to a fine powder using the pestle. Fine mycelia powder was then scoped with a sterile spatula dipped in liquid nitrogen into a 1.5 ml Eppendorf tube. Mycelia powder weighed 50 mg to 100 mg before DNA extraction using i-genomic BYF DNA Extraction Mini Kit (Intron, Belgique).
Lysis buffer with a volume of 200 µL was added into mycelia powder, followed by 20 µL Proteinase K and 5 µL RNase A. Lysate was mixed vigorously before being incubated under 65 °C for 30 minutes in the water bath. During the incubation, the lysate was mixed by inverting the tube a few times to assist in complete lysis. After the lysis, 250 µL binding buffer was added, gently mixed, and spun down to remove the lysate drop from inside the lid. 250 µL 80% ethanol was added to the lysate and incorporated by pipetting until the lysate was mixed evenly. 750 µL mixture was transferred into a spin column inside a 2.0 ml collection tube and centrifuged at 13000 rpm for 1 min. The collection tube and flow-through were discarded and replaced with a new collection tube. 700 µL washing buffer was added to the spin column and centrifuge for 1 min at 13000 rpm. The flow-through was discarded, but the same collection tube was kept. The spin column was dried by centrifuge for another minute. Finally, DNA elution was obtained by adding 30 µL to 50 µL elution buffer to the spin column in a new 1.5 ml tube. The elution step was incubated for 1 min at room temperature before centrifuging for 1 min at 13000 rpm to elute. The DNA solution was collected and stored under -20°C.

Gel Electrophoresis Analysis

The gel was prepared using analytical grade agarose with a concentration of 1.2% immersed in 1X TAE buffer. The solution was heated in the microwave until the gel wholly dissolved, and the solution was cooled down under running tap water. The precaution was taken to remain the solution in liquid form. In a 50 ml solution, 0.5 µL gel red stain was added and poured carefully into an electrophoresis tray to avoid bubble formation. When the gel solidified, the comb was inserted into a liquid solution to produce a well. After the gel hardened, the comb was removed slowly from the gel and left empty wells loaded with a ladder and sample. The gel was transferred into a gel electrophoresis system and submerged with 1X TAE buffer. The first row was loaded with a 2 µL 1 kb ladder followed by a sample and ended with a 2 µL 100 bp ladder. Samples loads were mixed of 1 µL loading dye, 8 µL water and 1 µL DNA sample. Gel electrophoresis was run according to 70 V for 40 minutes. After gel electrophoresis, the gel was documented using BIORAD Gel Doc™ EZ Imager.

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was achieved in 25 µL of the reaction mixture, which contained 4 µL of 5x Green GoTaq Flexi Buffer with 4 µL 15 mM MgCl2, 0.5 µL of dNTP mix (10 mM, Applied Biosystem), 1 µL of each ITS1 (5′TCCGTAGGTGAACCTGCGG3′) and ITS4 (5′TCCTCCGCTTATTGATATGC3′) primers (10 picomole/µL), 1 µL of DNA template, and 0.5 µL of GoTaq Flexi DNA Polymerase (5 U/µL). All PCR components used were from Promega (USA). The PCR was done using the following programs (Table 1): initial denaturation at 95°C for 3 min; 30 cycles of denaturation, annealing, and elongation at 95°C for 15 s; 58°C for 15s, and 72°C for 1 min 30 s followed by a final extension at 72◦C for 3 min.

Table 1 PCR amplification of ITS region

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	95	3 min	1
Denaturation	95	15 s	
Annealing	58	15 s	30
Extension	72	1 min 30 s	
Final extension	72	3 min	1

Purification of PCR Product

Proven PCR product form after gel electrophoresis was determined to be the targeted DNA fragment based on gel vision from Gel Documentation System. Purification started with a targeted DNA band cut using a sterile scalpel or razor blade. The gel was weighed and transferred into a 1.5 ml tube. The reagent and buffer for the purification process used MegaQuick-spin Total Fragment DNA Purification Kit (Intron Biotechnology, South Korea). BNL buffer was added to the gel slice three times the volume of the gel weight. The mixture was vortexed for a few minutes and incubated at 55 °C for 10 minutes or more to let the gel dissolve completely. The mix with dissolved gel was transferred into a column tube in a collection tube and centrifuged for 1 min. The collection column tube with flow-through was discarded and replaced with a 2.0 ml tube. 700 µL of washing buffer was added to the mixture and centrifuged at 13 000 rpm for 1 min. Flow-through was discarded and centrifuged again using the same tube to dry the spin membrane. The column was transferred into a fresh 1.5 ml tube. Approximately 30 to 100 µL elution buffer was pipetted into the column membrane and left for 1 min at room temperature. Next, the eluted product was obtained after centrifuge at 13 000 rpm for 1 min. Eluted DNA was stored under -20°C before submitting to the sequencing service.

Phylogenetic Analysis

Sequence alignment was edited manually using MEGA 7.0 to obtain a consensus sequence. The edited sequences were then copied into the Basic Local Alignment Search Tool (BLAST) to find similarities to available sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). Therefore, species identification of respected isolates should be known or predicted based on the Genbank library. The analysis of isolate variation compared to known species in the Genbank library was done using phylogenetic tree analysis. The phylogenetic tree was constructed using the Neighbour-Joining statistical method coupled with the Jukes-Cantor Model and bootstrap of 1000 replication.

RESULTS AND DISCUSSION

Fungal molecular identification

Fungal DNA identification was executed to obtain further information about fungal genera and species based on a comparison with the collected fungal sequences in the NCBI database. Morphological observation alone could be erroneous as fungi characteristics vary depending on media, temperature, and environment (**Hawksworth, 2004; Hibbett *et al*., 2011; Krull *et al*., 2013**). In addition to that, the biological variation of fungi from vast genera and species could be confusing. Especially to the non-expert, even though a fungi atlas is available and becomes handy to certain species. Thus, fungal DNA identification has become a favourable approach to support fungal identification with or without prior morphological examination, especially for fungi recalcitrant to laboratory culture (**Raja *et al*., 2017; Schoch *et al*., 2012**).

DNA Extraction

Fungal DNA must be obtained from crude fungal samples from spore or mycelium after ten days of incubation. In this study, fungal mycelium was grown in a broth medium of PDB to prepare a generous amount of mycelium, which can be further easily separated from the media. This method is not commonly used in fungal identification but is helpful in fungal secondary metabolite studies. Researchers usually scrape mycelium from agar culture before proceeding to the DNA

extraction method (**Mohamed *et al*., 2010; Premalatha & Kalra, 2013; Patil *et al*., 2022**). However, fungi from Groups 5, 6, 8, 9 and 10 produced less mycelium in agar medium; thus, growing this fungus in broth culture seems to be the right solution. Mycelium samples harvested from the culture broth were then air-dried in a biosafety cabinet to remove excess liquid media. All precautions suggested by the manufacturer were followed to produce high-quality DNA extract during the extraction process. The purity of DNA was analyzed under the absorption wavelength of A260/A280, with the high-quality DNA being within an absorption value of 1.8 to 2.0 (**Kasem *et al*., 2008; Lorenz, 2012**). High intact DNA could be detected under wavelength A260 at a concentration of 50 ng/µl (**Demeke & Jenkins, 2010**). However, obtaining the ideal result with contaminants such as protein, phenol, RNA, and other reagents residues is almost impossible. The quality of template DNA contributes to successful PCR product rather than concentration.

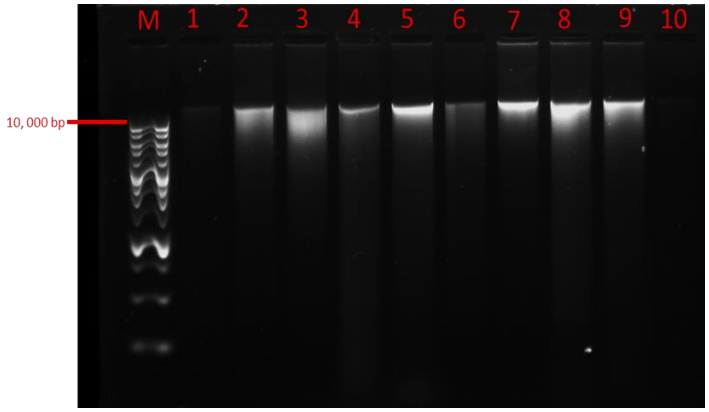

Figure 1 DNA detection of fungi Group 1 to Group 10 through gel electrophoresis. Based on band formation, fungal DNA was successfully extracted at over 10000 bp sizes. M: 1kb DNA Ladder (Promega); Lane 1 to Lane 10 represent group 1 to group 10 fungi isolate.

Based on Table 2, DNA extract quality from all fungal groups except for groups 3, 9 and 10 showed acceptable quality at absorption value range from 1.6 to 2.0; thus, the process can proceed with PCR amplification. Groups 3, 9 and 10 have offered some DNA extract contamination that shows A260/A280 absorption of less than 1.3, as shown in Table 2. A lower absorption ratio may indicate the presence of contaminants such as protein, phenol, and reagents, which can inhibit PCR operation and produce a non-specific product or no product at all (**Demeke & Jenkins, 2010; Lorenz, 2012**). The abundance could cause protein contents in the sample that Proteinase K may not be sufficient to hydrolyze protein through extraction fully. Phenol contaminant may exist in residue as it was not entirely removed from DNA through DNA precipitation. Phenol is a standard reagent to separate cellular debris and organic from DNA. Although the DNA quality of Groups 3, 9 and 10 was not applicable for the actual procedure, under specific A260 wavelength, the DNA concentration of group 3, 9 and 10 isolates are sufficient for PCR amplification and comparable with other isolates represented in Table 2 and Figure 1. Therefore, all DNA samples were subjected to PCR amplification analysis for internally transcribed spacer rRNA.

Table 2 DNA quality and concentration of extracted samples from fungi group 1-10

Fungi Group	DNA Quality (A260/ 280)	Concentration (ng/µl)
1	1.77	25.0
2	1.66	19.8
3	1.27	26.0
4	1.60	30.8
5	1.71	19.9
6	1.74	31.5
7	2.04	22.9
8	1.69	31.0
9	1.24	21.5
10	1.26	29.9

Polymerase Chain Reaction (PCR)

During PCR amplification, the employment of fungal-specific primers is crucial in fungal identification studies that previous research may use the protein-coding marker and ribosomal RNA. Internal transcribed spacer (ITS) usage has become favourable in fungal molecular identification compared to the 18S and 28S rRNA amplification approach due to its success rate (**Schoch *et al*., 2012**). Moreover, ITS employment showed lower discrimination among genera that it can identify a wide range of fungi (**Liu *et al*., 2015; Schoch *et al*., 2012**), including lichen, basidiomycota, ascomycota and yeast, (**Badotti *et al*., 2017; Raja *et al*., 2017**). Since the proposal of ITS as primary fungal barcoding was accepted by the Consortium for the Barcode of Life (CBOL) (**Schoch *et al*., 2012**), ITS has become a preferred approach. The application of ITS in fungal identification has increased over the years (**Raja *et al*., 2017**). Primer pair ITS1-F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4-B (5'CAG GAG ACT TGT ACA CGG TCC AG-3') were employed in this study, which would produce PCR product of size range between 500 bp to 750 bp **(Porter & Brian Golding, 2011**). The same pair of primers were used in fungi study related to *A. malaccensis* by **Mohamed *et al*. (2010)** and **Premalatha and Kalra (2013)**, identifying fungi *Fusarium solani*, Cunninghamela bainieri, Hypocrea lixii, Alternaria sp. and *Lasiodiplodia theobromae*. **White *et al*. (1990)** originally suggested the ITS1-F and ITS4-B genes, which were known as ITS1 and ITS4. In 1993, **Gardes & Bruns, (1993)** modified the primer sequence by **White *et al*. (1990)** to produce a more specific and efficient product for fungi and basiodiomycota identification named ITS-F and ITS4-B. The region of primer pair amplification of ITS-F and ITS4-B is slightly shorter than primer pair ITS1 and ITS4 (**Bellemain *et al*., 2010**). Therefore, primer pairs ITS1-F and ITS4-B are more effective. Nevertheless, the capacity of primer ITS1-F and ITS4-B can be indicated by the performance of ITS1 and ITS4. Among primers within small subunit ribosomal DNA region, ITS1 seem to show a higher rate of coverage of broad genus and species, with lower discrimination and more accurate result (**Bokulich & Mills, 2013**). However, it wasn't proven very objective with the basiodiomycota group (**Bellemain *et al*., 2010**). Meanwhile, the best reverse primer represented ITS4 from a large subunit ribosomal DNA region, demonstrating high fungal taxon coverage and low discrimination (**Bellemain *et al*., 2010; Toju *et al*., 2012**). Hence, ITS1 and ITS4 primer pairs were the most effective at characterizing the most dominant fungal communities (**Bellemain *et al*., 2010**).

PCR results were analyzed using UV Gel Documentation (Bio-Rad, US), Figure 2. All DNA extracts were amplified at an expected size between 500 to 750 bp. All PCR samples were approximately the same size except for G5 and G6, indicating a longer base-pair near the maximum length primer pair ITS1-F and ITS4-B can amplify. PCR amplification is more efficient if the length of the targeted sequence is closer to the maximum length of the primer pair expected sequence. The efficiency of the PCR process is influenced by several factors, including primer pair selection, GC content, DNA template concentration, and thermocycling condition (**Pinto & Raskin, 2012**). The unknown variable is DNA templates' GC content, which has been proven to affect PCR products. Localized GC content in templates contributes to successful PCR where it was statistically shown based on the high predictive value of the index, Norm AUCGC (**Benita *et al*., 2003**). The findings were supported by (**Mamedov *et al*., 2008**), where the best annealing duration for GC-rich gene is within three to six seconds to assist with more efficient PCR was confirmed. GC content and template size are vital dependent factors to ensure the efficiency of the PCR process (**Benita *et al*., 2003**). Hence, Group 5 and Group 6 DNA templates probably had higher GC content to allow more extended sequence PCR amplification, considering the DNA template size was as sound as other fungal groups.

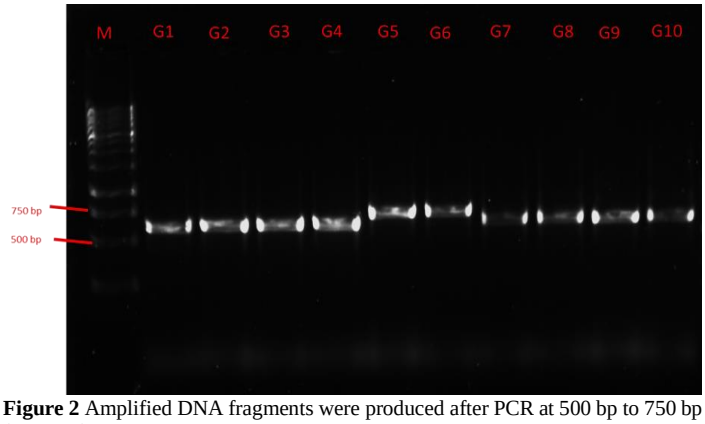

Figure 2 Amplified DNA fragments were produced after PCR at 500 bp to 750 bp from all fungal group samples; M: 1 kb ladder; Lane 2: Group 1; Lane 3: Group 2, and so on.

Target DNA fragments obtained from PCR amplification were purified to remove PCR reagents and possible contaminants interrupting DNA sequencing. The quality and concentration of purified PCR products in Table 3 ranged between 21.5 to 45.6 ng/µl. DNA concentration after purification was lower than crude DNA extract but considered sufficient to proceed with DNA sequencing.

Table 3 DNA quality and concentration after DNA purification of all fungal groups

Fungi Group	DNA Quality (A260/ 280)	Concentration (ng/µl)
1	1.93	45.6
2	1.59	29.7
3	1.35	29.9
4	1.53	31.7
5	1.28	40.6
6	1.37	25.6
7	1.54	22.6
8	1.43	33.0
9	1.24	21.5
10	1.26	29.9

Fungal Sequencing Analysis

A molecular approach using ITS identification preceded the sequence to double-confirm the morphological identification. Sequence results compared to NCBI databases showed similarity from 94% and above to a related known fungi sequence collection in the database (Table 4).

Table 4 Identical value of fungal DNA sequence compared to related species database in NCBI BLAST

Fungi Group	Suggested Species	Identical value (%)
1	*Fusarium solani*	99
2	*Fusarium solani*	97
3	*Botryosphaeria rhodina*	99
4	*Aspergillus aculeatus*	100
5	*Schizophyllum commune*	99
6	*Phanerochaete chrysosporum*	94
7	*Lasiodiplodia theobromae*	100
8	*Polyporales* sp	95
9	*Polyporales* sp	100
10	*Ceriporia* sp	100

Sequences from Group 1 and Group 2 were identified as *Fusarium solani* with 99% of identical identities (Table 4 and 5). *Fusarium solani* from Group 1 was accurate considering morphological observation of isolate matched *Fusarium solani* description. However, Group 2 identification of *Fusarium solani* seems faulty due to the appearance of pigmentation in Group 2, which should not be found in *Fusarium solani* (**Chehri *et al*., 2011; Leslie & Summerell, 2006; Shahnazi *et al*., 2012**). Group 3 showed 99% identity with *Botryosphaeria rhodina* and concordance with morphological (microscopic and macroscopic) observation analysis. As expected from Group 4, it was confirmed from the Aspergillus genus, and sequence analysis discovered it was explicitly similar to *Aspergillus aculeatus* (100% identity). Group 5 isolate was identical to *Schizophyllum commune* (99% identity) and supported with morphological observation. Isolate from Group 6 related to *Phanerochaete chrysosporum* with 94% identity, and Group 7 was analyzed specifically as *Lasiodiplodia theobromae* with 100% identity. *Polyporales* sp was a genus closer to Group 9 with a 100% identical ITS sequence than Group 8, with only 95% identity. Group 10 was found to be similar to the genus *Ceriporia* sp. *Polyporales* sp is one of the large orders under the Basidiomycota phylum. *Polyporales* cover wide species, including *Ceriporia* sp and *Phanerochaete chrysosporum*; some were unknown to species level. Unfortunately, correct identification to species level may require a more extended sequence, approximately 600 bp and above, compared with readily NCBI data (**Min & Hickey, 2007**). However, unavailable information for related species may hinder studied species identification at the species level.

Phylogenetic analysis

The sequencing service results were analyzed using bioinformatic software MEGA6 before edited sequences were compared to the sequence from the online gene bank at https://www.ncbi.nlm.nih.gov/blast.

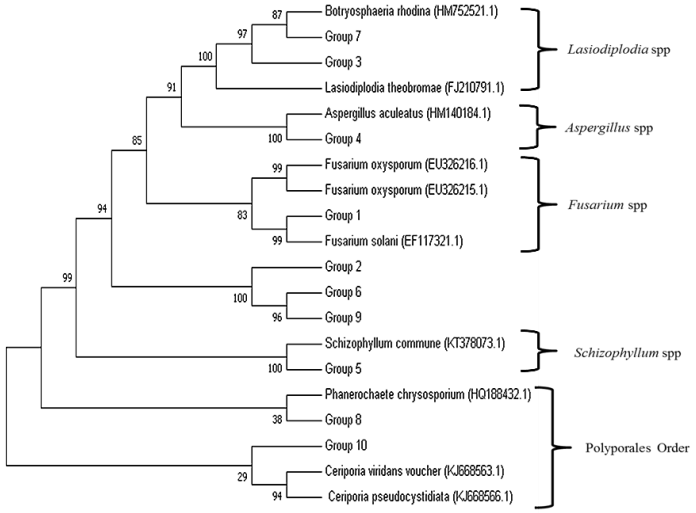

Figure 3 Phylogram (Neighbor-joining method) shows the genetic relationship between fungal groups and related reference fungi based on the ITS region sequence analysis. The numbers at branching points or nodes refer to bootstrap values based on 1000 re-sampling.

Table 5 Internal Transcribed Spacer (ITS) identification of fungal groups in agarwood sample

Fungi species	Identity (%)	Agarwood	Healthy wood	Accession Numbers
Fusarium solani	99	R1 R2 R3 R4 R5 M1 M2	HR3 HR5	EF117321.1
Fusarium solani	99	R1 R4	HR3 HR4	EF117321.1
Botryosphaeria rhodina	99	R2 R3	None	HM752521.1
Aspergillus aculeatus	100	R2 R5	HR3 HR4 HM1	HM140184.1
Schizophyllum commune	99	R3 R4	None	KT378073.1
Phanerochaete chrysosporum	94	R5 M1	None	HQ188432.1
Lasiodiplodia theobromae	100	R1 R3	None	FJ210791.1
Polyporales sp.	95	R1 R4	None	None
Polyporales sp.	100	M2	None	None
Ceriporia sp.	100	R1	HM1	KJ668563.1

Note: Naturally infected agarwood: R1, R2, R3, R4, R5
Healthy natural woods: HR1, HR2, HR3, HR4, HR5
Artificial agarwood: MI, M2
Healthy artificial woods: HM1, HM2

Based on phylogenetic analysis (Figure 3), Group 1 was genetically related to *Fusarium* spp with 99% bootstrap support with the reference sequence of *F. solani* and *F. oxysporum*. Group 2 was unexpectedly the outgroup of *Fusarium* spp. after showing similarity to *F. solani* with more than 90% identification value. Group 2 was obviously under *Fusarium* spp. class based on morphology and reference with NCBI sequence regardless of the phylogenetic finding. Instead, Group 2 appeared to be a 100% bootstrap value similarity to the monophyletic clade of Group 6 and Group 9 without closely related to any reference sequence. Group 3 and Group 7 were genetically proven under the Lasiodiplodia spp. group with 100% bootstrap value support of monophyletic clade. Group 4 and Group 5 formed a monophyletic clade with the reference sequence of A. aculeatus, and S. commune with 100% bootstrap value, confirming its species as identified by BLAST. Group 8 showed a relationship with *P. chrysosporium* with only 38% bootstrap value compared to Group 6. Initially, Group 6 was identified as *P. chrysosporium* with 94% identification.

On the contrary, the sequence was an outgroup from the reference sequence of *P. chrysosporium* and turned out to be a monophyletic clade with Group 9 with 96% bootstrap value. Group 9 was previously detected under *Polyporales* Order.

However, both Group 6 and Group 9 were totally out of the *Polyporales* Order group, where Group 8 and Group 10 were clustered. Group 10 was identified under the genus *Ceriporia* spp, proving a relationship with the genus but lowly related to the reference sequence. It was suggested that Group 10 might be closer to other species under the Ceriporia genus. As expected, most groups were clustered correctly except for Group 2, Group 6 and Group 9. Erroneous in the phylogenetic tree may be caused by deletion or duplication of sequence, causing the targeted sample sequences to be out group from suggested species in BLAST (**Philippe *et al*., 2011**).

CONCLUSION

The discovery of the relationship between fungus and the quality of agarwood began with no foundation of fungi involved with agarwood inoculant. On the other hand, 55 fungal isolates were found in artificial and natural agarwood, including agarwood and healthy wood. Ten fungal groups were formed after considering the similarity in morphological characterization. According to ITS region, molecular identification disclosed *Fusarium solani*, *Botryosphaeria theobromae*, *Polyporales* spp, *Schizophyllum commune*, *Aspergillus aculeatus* and *Lasiodiplodia theobromae* based on NCBI BLAST analysis. *Fusarium solani* was dominant in agarwood samples and healthy wood, A. aculeatus and *Polyporales* spp. Fungi species found in nature agarwood were more than in artificial agarwood may be caused by the conducive moist and shady environment for fungal growth. Nevertheless, artificial agarwood was able to produce similar agarwood-related compounds. Since *F. solani* were frequently found in both agarwood, agarwood inoculant may function as a booster to *F. solani* to start its pathogenicity in artificial agarwood. The fungus *F. solani* can be developed as a booster for artificial inoculation in *Aquilaria malaccensis* trees for high-quality agarwood oil production.

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