Isolation and Characterisation of Filamentous Fungi from Animal Agricultural Farm Soil

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ABSTRACT

Aspergillus sp. is an extremely resilient species that can be found everywhere in the environment and is present abundantly in water and soil. The defining characteristic of Aspergillus sp. is their extensive hyphal network which enable them to survive anywhere, even in very harsh conditions. This study was carried out to isolate the filamentous fungi from peat soil of animal agricultural farm and characterise them based on their morphological and molecular characteristics. Growth rate of each isolated fungi was also evaluated in order to determine the period of maturity for each fungi. Soil samples were collected, weighed and then dissolved in sterile distilled water. The samples were serially diluted and spread onto potato dextrose agar (PDA) for isolation. Different isolated colonies that were morphologically different from each plate was purified and sub-cultured onto new media for macroscopic and microscopic identifications. For molecular identification, a conventional technique was used in genomic DNA extraction of filamentous fungi due to their thick cell wall and presence of surface proteins protecting the fungus. These characteristics make it difficult to harvest the genomic DNA. Polymerase chain reaction (PCR) was carried out using internal transcribed spacer primers; ITS1 (forward) and ITS4 (reverse).

The morphological identification and molecular technique showed that majority of these isolated fungi are Aspergillus sp.

Keywords: Aspergillus sp., filamentous fungi, internal transcribed spacer, macroscopic and molecular identifications, polymerase chain reaction
INTRODUCTION
Malaysia, comprising the regions of Peninsular Malaysia, Sabah and Sarawak, is covered with vast areas of peatland. Peatland is known as all lands on peat soil. This area plays an important role in preserving water supply, and providing nutrients for the trees and other resources to communities. Other than these, it also provides nutrients for soil micro flora. This study focuses on peatlands under crop or husbandry category in which the peatland areas are used for agricultural activities. In general, peat soil contains high carbon content which includes both organic and inorganic carbon. Organic carbon and water content in soil act as sources of nutrients for most soil fungi (Rudiyanto, Minasny, & Setiawan, 2016; Moore et al., 2013; Page et al., 2004; Drenovsky et al., 2004).

Animal agricultural farm is categorised under the crop and husbandry category. Most animal agricultural farm soils are contaminated with hazardous chemical compounds such as hormones and antibiotics (Kumar et al., 2005). These compounds are used on livestock to increase productivity rate and reduce diseases. Disposal of animal wastes on the soil contaminates the surrounding. Only around 30% of ingested compounds will remain in livestock’s system, while the remaining is excreted back into the environment (Kumar et al., 2005; Gersema & Helling, 1986; Gavalchin & Katz, 1994). The compounds in animal waste are scattered on animal agricultural farm area, thus affecting the soil composition (Gavalchin & Katz, 1994). These contaminations also cause the emergence of antimicrobial resistance in terrestrial environment. The usage of antimicrobial compounds has led bacteria to develop resistance towards antibiotics used (Nordenberg, 1998; Bebell & Muiru, 2014). The emergence of antibiotic-resistant bacteria in the environment will create problems for antibiotic therapy in human and animal in the future (Tollefson & Karp, 2004; Lees & Aliabadi, 2002).

Filamentous fungi are the major decomposers in peat soil environment where their extensive hyphal networks enable them to use available nutrients dispersed in vast areas of the land. Other characteristics that are advantageous for such fungi to thrive in these lands are their high growth rate and thick cell wall, which protect them from their predators. Some filamentous fungi produce antibacterial compounds and are able to degrade certain types of chemical (Shukri et al., 2015). This study was carried out to isolate the filamentous fungi from peat soil of animal agricultural farm and characterise them based on their morphological and molecular characteristics.

METHOD
Sample Collection
Soil samples were collected from animal agricultural farm at MJ Fatonah Sdn. Bhd., Kuala Selangor. The samples were collected from various locations with different livestock. The soil samples were taken from a depth of 1-10 cm from the surface and stored in biohazard plastic bags. Then, the samples were brought back to the laboratory for further study.
Isolation and Purification of Fungi

One gram of soil was collected from each sample and dissolved in 10 mL sterile distilled water. The mixture was serially diluted up to four times of tenfold dilution factor. 100 µL of each dilution was taken out and spread onto the potato dextrose agar (PDA) containing 50 mg/mL chloramphenicol to prevent any growth of bacteria and saprophytic fungi. Each PDA plate was incubated at 28°C. After three days, a colony of fungus was collected and transferred into new sterile PDA using a sterile needle in order to get a pure culture. Then, each plate was incubated for 3-14 days at 28°C. The growth rate of each fungal isolates was obtained by measuring the diameter of respective fungal colonies.

Morphological Characteristics of Fungi

At day 3 of incubation, macroscopic identification was carried out by observing the colour and texture of each fungal colony through the naked eyes. For microscopic identification, adhesive tape method was used to observe the reproductive system of each fungal isolate. A drop of methylene blue was added onto the glass slide. The sample was collected using transparent adhesive tape on the culture and transferred onto glass slide for observation under light microscope (Olympus CX21) by using 400× magnification.

Genomic DNA Extraction of Isolated Fungi

The technique for DNA extraction was performed using a conventional method. This method was designed to disrupt fungal cell wall through heating and rapid cooling. About 100 µL of 1 x 10^6 cfu/mL fungal spores suspension was inoculated onto PDA, followed by two days of incubation at 28°C. After the incubation period, approximately 5 mL of sterile distilled water was added onto the plate, followed by a collection of fungal spores and mycelia. This step was carried out gently using an inoculating loop or a sterile pipette tip to prevent any agar debris in the fungal and mycelia collection. About 1 mL of the spores and mycelia collected was respectively transferred into the microcentrifuge tube, followed by centrifugation at 14,000 rpm for 1 minute. The supernatant was removed carefully to avoid any dislodged to the pellet. After that, 1 mL of phosphate buffer saline (PBS) was mixed with the pellet. 25 µL of spore suspension containing PBS was transferred into a PCR tube. Then, 25 µL of sterile distilled water was added to dilute the spore suspension to 1:1 dilution.

Thermocycler MJ Mini Personal Thermal Cycler from BIO RAD was set at certain conditions: initial denaturation (95°C, 5 minutes), denaturation (95°C, 5 minutes) and rapid cooling (4°C, 10 minutes). Next, the nucleic acid was either stored at -20°C, or directly used for PCR amplification.
**Molecular identification of isolated fungi.** Briefly, ITS1 primer (5´-TCC GTA GGT GAA CCT TGC GG-3´) and ITS4 primer (5´-TCC TCC GCT TAT TGA TAT GC-3´) were used for a total of 25 μL reaction. The PCR reaction was carried out by using MJ Mini Personal Thermal Cycler from BIO RAD. The PCR mix contained template DNA, 10 μM of each primer (AIT biotech), 10x standard buffer (New England BioLabs), 10 mM dNTP mix (New England BioLabs) and 1.25U of *taq* DNA polymerase (New England BioLabs). The PCR conditions were set at: i) initial denaturation (95°C, 30 seconds), (ii) 35 cycles of denaturation(95°C, 30 seconds), (iii) annealing (45°C, 60 seconds), (iv) extension (68°C, 60 seconds), and followed by (v) another final extension (68°C, 60 seconds).

About 2 μl of each PCR product was electrophoresised in 1% agarose gel (BIO RAD). The samples were sent to AIT Biotech, Singapore, for sequencing. By using BioEdit software, the sequences were cleaned and sequence alignment was carried out using BLAST, NCBI.

**RESULTS AND DISCUSSION**

**Morphological Identification of Fungal Isolates**

A total of seven filamentous fungi were isolated from the peat soil of animal agricultural farms. Based on the morphological and molecular identification, only one sample was identified as *Eupenicillium* sp., while the remaining samples were identified as *Aspergillus* sp. The low pH level of peat soil, which ranged from 2 to 4, and the non-selective characteristics of *Aspergillus* sp. are the reasons for the abundance of *Aspergillus* sp. (Sarmah, Meyer, & Boxall, 2006). *Aspergillus* sp. can survive in a variety of substrates including animal remains which may contain excess contaminants from animal agricultural activities such as antibiotics and hormones. It can also survive in a very large range of temperatures and humidity (Krijgsheld et al., 2013).

The presence of *Aspergillus* sp. in the peat soil acts as a natural decomposer of animal remains in the farm (Song et al., 2010). Some of them are categorised under mycotoxin-producing fungi such as *A. fumigatus* (gliotoxin, fumagilin), *Aspargellus terreus* (citreoviridin) and *A. versicolor* (cyclopiazonic acid) (Manshor et al., 2012). Even though the use of these mycotoxins in decomposition process is hazardous to organic materials, it does not show any significant effect on humans and animals (Tamiya et al., 2015). As a saprotroph, *A. terreus* is widely found on decaying organic matter as it has important roles in carbon and nitrogen cycles (Haines, 1995; Pitt, 1994). The surface colour is cinnamon brown and the reverse area is tan or yellow (Latge, 1999). The microscopic observation of hyphae shows them in terms of hypha to be hyaline.
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*Figure 1.* The macroscopic and microscopic observations of fungal isolates on the third day of incubation under 400× magnification using light microscope: (a) *A. fumigatus*; (b) *A. terreus* strain PTN 45; (c) *Aspergillus flavipes* strain ATCC1030; (d) *Eupenicillium* sp.; (e) *A. terreus* strain CO1; (f) *Aspergillus nomius* strain Tur4; and (g) *Aspergillus niveus* strain IHEM 5804.
Figure 1 shows the macroscopic and microscopic views of fungal isolates. *Aspergillus fumigatus* has green coloured surface with the formation of white ring around it. The texture was flat and it did not change colour of the media even after 14\textsuperscript{th} day of incubation. The vesicle and conidiosphores have round shape, and no hyphae were observed. For *A. terreus* strain PTN 45, the colony colour was slightly green with the formation of white ring border. The texture was flat and the formation of segments was observed on the colony. *A. terreus* strain PTN 45 does not change the colour of the media after 14\textsuperscript{th} day of incubation. For the microscopic observation, the vesicle and conidiosphores are round. There were long septate hyphae observed connected to conidiophores.

The colony of *A. flavipes* strain ATCC1030 appeared white colour with a flat surface. There was also formation of segments observed on the colony. The colony changed the colour of media to brick red. From the microscopic observation, the vesicle and conidiophores were round and there was no hyphae observed connected to conidiophores.

*Eupenicillium* sp. was different from the other isolates. The colony of *Eupenicillium* sp. was observed to be white to yellow with a flat surface. No observable change in the colour of the media observed after 14\textsuperscript{th} day of incubation. *Eupenicillium* sp. has round conidiosphores. The aseptate hyphae is connected to conidiophores and attached to the metulae. The spread-like structure called phialides is also present. *Eupenicillium* sp. an anamorph of *Penicillium* sp. usually sporulates on the head of *Aspergillus* species (Horn & Peterson, 2008). Both *Penicillium* sp. and *Aspergillus* sp. are found abundantly in plant decaying matter, such as in the peat soil. The growth rate of *Eupenicillium* sp. was low even when cultured onto Czapek agar and it took 14 days for this fungal isolate to grow to approximately 3 cm in diameter (Stolk & Scott, 1967).

*A. terreus* strain CO1, *A. nomius* strain Tur4, and *A. niveus* strain IHEM 5804 had brown, green and white colour surfaces, respectively. The microscopic observations for these species were also found to be alike. The conidiosphores and vesicle are round with flat surfaces, and septate hyphae with long conidiophores were also present. There were no colour changes in the media for all the three species observed after 14 days of incubation.

![Growth curve of isolated fungi](image)

*Figure 2. Growth rate of the fungal isolates from day zero to day 14. Strain K2 (*A. fumigatus*); strain L1 (*A. terreus* strain PTN 45); strain L2 (*A. flavipes* strain ATCC1030); strain M (*Eupenicillium* sp.); strain N1 (*A. terreus* strain CO1); strain N2 (*A. nomius* strain Tur4); strain N3 (*A. niveus* strain IHEM 5804)*
In this study, the growth rate of each fungal isolate was measured. A colony of each fungal isolate was selected before they were measured. Figure 2 shows the growth curve of all the fungal isolates. The results show that most fungal isolates have exponential rate from day 2 to day 8. The lag phase started from day 0 to day 2 when the fungal isolates were initially introduced to the medium (PDA). At this phase, the fungal isolates took some time to adjust to their new environment and the cellular metabolism started to accelerate.

From day 2 to day 8, the growth rate increased exponentially for all the fungal isolates, except for *A. flavipes* and *Eupenicillium* sp. Both the fungal isolates’ growth rates increase proportionally to the graph. During the exponential phase, the fungal isolates grew rapidly as the medium was rich in nutrients. From day 8 to day 14, the growth rates reached stationary phase where the growth started to slow down as the nutrients gradually depleted. On the other hand, the amount of toxic metabolites, waste materials and other compounds caused pH value to change. These would impair the growth of fungal isolates and result in slow growth and death.

**Molecular Identification of the Isolated Fungi**

Filamentous fungi have high rigidity of cell wall compared to bacteria. It consists of four main components such as α-glucan, β-glucan, chitin, and monoprotein (Gooday, 1995). The rigidity of fungal cell wall increased due to the formation of covalent cross-link between β-glucan and chitin chains (Ruiz-Herrera et al., 1994). Genomic DNA extraction was conducted using the conventional method. It is the best method for Genomic DNA extraction because it is fast, easy and cost saving. By using the boiling method principle, PCR thermo cycler was used as it provides specific temperature ranges which are suitable for disruption of fungal cell wall. It also provides a longer period of incubation so that the cell wall could be completely disrupted. Genomic DNA from all the isolated fungi was successfully harvested using this method. However, this method does not enable the harvest of high quality of fungal genomic DNA due to the absence of purification steps. The high amount of genomic DNA harvested using this method was sufficient to precede for polymerase chain reaction (PCR).

Internal transcribe spacer (ITS) regions were used in molecular identification of fungi because they were closely found between other species, closely related species or even among other populations of the same species (Korabecna, Liška, & Fajfrlik, 2003). Since these regions are known for heterogeneity, ITS1 and ITS4 primers were used as the universal primers to identify filamentous fungi isolated. The sequence variation of ITS regions were able to distinguish between *Aspergillus* sp. from other species and a small variation between *Aspergillus* and *Penicillium* within ITS 2 region was found (Gaskell et al., 1997). Thus, ITS 1 region is sufficient for the identification of fungal isolates (Gaskell et al., 1997). In this study, ITS1 and ITS4 primers cover both ITS 1 and ITS 2 regions, along with 18srRNA and 5.8srRNA with amplicons size ranging from 500bp to 700bp. A BLAST search was carried out on all Genbank sequences and the sequence similarities of all the samples were 99% with a query cover of 98% for both *Aspergillus* sp. and 97% for *Eupenicillium* sp.. All the samples were identified by using the highest bit score in BLAST search.
able to distinguish between Aspergillus sp. from other species and a small variation between Aspergillus and Penicillium within ITS 2 region was found (Gaskell et al., 1997). Thus, ITS 1 region is sufficient for the identification of fungal isolates (Gaskell et al., 1997). In this study, ITS1 and ITS4 primers cover both ITS 1 and ITS 2 regions, along with 18srRNA and 5.8srRNA with amplicons size ranging from 500bp to 700bp. A BLAST search was carried out on all Genbank sequences and the sequence similarities of all the samples were 99% with a query cover of 98% for both Aspergillus sp. and 97% for Eupenicillium sp.. All the samples were identified by using the highest bit score in BLAST search. Using DNA sequencing information, a phylogenetic tree was constructed from the isolated fungi, except for Eupenicillium sp. According to the phylogram above, a line bar represents the evolutionary lineage change over time. The longer the branch is in the horizontal dimension, the larger the amount of genetic variability. In this case, the line segment shows a genetic variability from 0.00 to 0.05. A clade was formed from A. terreus PTN-45, A. terreus CO1, A. nevius IHEM5804, A. flavipes ATCC1030 and A. fumigatus, indicating that they share a common ancestor. A clade or branch is defined as a group of an ancestor and all descendants. From the major clade, nested clades were formed as it is nested within one another. For example in this case, a clade of A. terreus PTN-45 and A. terreus CO1 was nested within A. terreus PTN-45, A. terreus CO1 and A. nevius IHEM5804 clade. Furthermore, A. terreus PTN-45 and A. terreus CO1 share a common ancestor, and thus are known as the sister group. This shows that these species have a lot of common evolutionary history but very little unique evolutionary history to either one or two sister species. Apart from that, A. nomius Tur4 was observed as an out group of all others Aspergillus sp. isolates. This is because all the members are more closely related to each other than A. nomius Tur4.

CONCLUSION
A total of seven filamentous fungi were successfully isolated and identified based on the morphological observation and sequence alignment of the gene using the NCBI BLAST system. Those species were Aspergillus fumigatus, A. terreus strain PTN 45, A. flavipes strain ATCC1030, Eupenicillium sp., A. terreus strain CO1, A. nomius strain Tur4 and A. niveus strain IHEM 5804. From this study, it was found that peat soil from animal agricultural farm has a large quantity of Aspergillus sp. compared to other types of fungi. Thus, this work supports previous studies on soil micro flora which states that Aspergillus sp. is one of the most abundant filamentous fungi in peat soil.
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