



Isolation and identification of microfungi from soils in Serdang, Selangor, Malaysia

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Abstract

Microfungi are commonly inhabited soil with various roles. The present study was conducted in order to isolate and identify microfungi from soil samples in Serdang, Selangor, Malaysia. In this study, the soil microfungi were isolated using serial dilution technique and spread plate method. A total of 25 isolates were identified into ten genera based on internal transcribed spacer region (ITS) sequence analysis, namely *Aspergillus*, *Clonostachys*, *Colletotrichum*, *Curvularia*, *Gliocladiopsis*, *Metarhizium*, *Myrmecridium*, *Penicillium*, *Scedosporium* and *Trichoderma* consisting 18 fungi species. *Aspergillus* and *Penicillium* species were claimed as predominant microfungi inhabiting the soil. Findings from this study can be used as a checklist for future studies related to fungi distribution in tropical lands. For improving further study, factors including the physicochemical properties of soil and anthropogenic activities in the sampling area should be included.

Key words – *Aspergillus* – diversity – fungi – *Penicillium* – *Trichoderma*

Introduction

The soil serves as a reservoir for many microbial communities of various plants which can be a part of CO₂ and the nitrogen cycle. The microorganisms include fungi that play a significant role in the soil ecosystem. According to Zifcakova et al. (2016) and Maron et al. (2018), fungi have the ability to break down all kinds of organic matter, decomposing the components of soil and regulating the balance of carbon and nutrients. In daily human life, microfungi have been used in both industrial and commercial purposes such as food, medicine, and chemical. For instance, various cheeses in the food industry are produced from *Penicillium roquefortii* which can enrich the cheese with a pungent and robust flavor (Ropars et al. 2017). *Aspergillus* species have been utilized in making sodas and soft drinks as an alternative way of producing citric acid (Oladele et al. 2014). Microfungi are also contributing to the medicinal industry as *Penicillium* species naturally produce antibiotics (penicillin) that contributed to the discovery of highly effective antibiotics (Ziemons et al. 2017). Those beneficial microfungi species can be obtained by isolating them from various habitats including soil (Tedersoo et al. 2014).

However, populations of soil microfungi are being threatened and need to be conserved as part of biodiversity. According to Tedersoo et al. (2014), the richness of fungal species is declining

as they found a huge gap between known and described species and the actual numbers of different fungi in the world of soils. Nowadays, the study on the fungi in Asian countries including Malaysia is limited (Hyde 2003, Lee et al. 2012). Based on various publication resources, about 1141 species of Ascomycota fungi have been documented for Malaysia, but still, there are numerous undiscovered species (Lee et al. 2012).

This study contributes additional information for the related organization about the source of beneficial microfungi. Hence, the objectives of this study are to examine morphological characteristics of microfungi isolated from soil samples throughout Serdang, Selangor, and to identify the isolated microfungi into species level based on internal transcribed spacer (ITS) sequence analysis.

Materials & Methods

Sampling

Four sampling sites in Serdang, Selangor were selected as the study areas (Table 1). The temperature in this area reaches a maximum of 33°C and minimum 23°C with 27°C as an average temperature. Average humidity is 70% (80%, maximum and 66%, minimum), whereas the annual average rainfall is 187 mm (<http://www.met.gov.my/>). The atmosphere pressure is 1008.7 to 1011.5 mb (<https://www.worldweatheronline.com/>). The samples were collected in 2018 with five plots per site as replicates. Quadrat sampling was conducted in the sampling areas, where a rectangle marked out with area 20 × 20 m was measured on the ground by measuring tape (Mueller et al. 2004). All plots were randomly selected for soil sampling. The soil samples were collected from each plot near roots of tree. The soil was dug for 8 to 10 cm deep by using a soil corer and was collected with a clean polythene bags using a clean scoop. The collected samples were brought to the Laboratory of Mycology for further analysis.

Table 1 Four sampling sites located in Serdang, Selangor

Sampling site	Name of the sampling site	GPS	Type of land use
1.	Oil palm plantation	2°59'18.8" N, 101°43'20.7" E	Agricultural
2.	Bukit Ekspo	2°59'21.6" N, 101°42'38.7" E	Recreational
3.	Lawn of Rumah Muzik	3°00'01.7" N, 101°42'10.8" E	Residential
4.	Lawn of Pangung Percubaan	3°00'01.5" N, 101°42'26.4" E	Residential

Fungal isolation and purification

Fungal isolation was performed using serial dilution method (Apinis 1963) and cultured on Potato dextrose agar (PDA) and Rose Bengal agar (RBA). The diluents from each dilution were pipetted about 1 ml onto a different surface of PDA and were spread evenly over the surface of agar using a sterile glass rod L-shaped. The diluents from test tubes were then pipetted 1 ml into an empty plate using a sterile pipette. The molten RBA was poured into the plates and the plates were swirled until the agar sets. Each dilution factor for PDA and RBA media were conducted in three replicates and were incubated in an incubator at 28°C for 48 to 72 hours. All fungal cultures were purified using streaking dilution technique. The inoculum was spread by making a wider stroke on the surface of new fresh PDA. The plate was sealed and was replaced in the incubator for 28°C for 24 hours to allow the growth of the colony. A pure and visible growth of fungi was obtained using hyphal tip technique on the PDA plates.

The pure isolated microfungi were preserved in the short term for working cultures, and long-term for stock cultures. Filter paper technique (Fong et al. 2000) and 25% glycerol complete medium with xylose (CMX) conidial suspension stock solution was utilized for long-term preservation of microfungi, while half PDA media were used for working cultures.

Morphological observation

The fungal characteristics were observed based on the macromorphological characteristics on PDA including the colony color, pigmentation of the colony and the texture of the colony. Micromorphological characteristics such as the types of conidiophores, the presence of chlamydospores, the arrangements of conidia, the shape of microconidia and the shape of macroconidia were observed by using slide cultures. The slide was observed using a compound light microscope (CX31RBSFA, Olympus Corporation). The images were captured using Dino Capture 2.0 (ANMO Electronics Corporation, Taiwan).

DNA extraction

All isolates were preceded to molecular identification using ITS sequence analysis. The isolates were cultured on PDA and incubated for 5 days. DNA of the isolates was extracted using UltraClean® Microbial DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) according to manufacturer's instructions.

PCR amplification

The microfungi DNA was amplified by using 8.0 µL of 5× Green GoTaq buffer, dNTP stock mixture which is 4.0 µL (2 mM), 4.0 µL of MgCl₂ (25 mM), 0.2 µL of Taq DNA polymerase, 2.0 µL of each primer (10 mM), 17.8 µL of nuclease-free water, and 2.0 µL of genomic DNA with an overall volume of 40 µL. The ITS primers used are ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') to amplify the ITS sequences (White et al. 1990). The PCR protocol were set as follows: initial denaturation for 30 seconds at 95°C, followed by 35 cycles of denaturation (95°C for 10 seconds), annealing (59°C for 15 seconds) and extension (72°C for 30 seconds), and was completed by a final extension step at 72°C for 5 minutes.

Gel electrophoresis

The PCR products were gel-electrophoresed using 1.5% agarose gel. The mixture of 2.5 µL of 6× loading dye and 2.5 µL of 100 bp DNA marker were used as a ladder. The DNA and ladder were pipetted with 5 µL in volume into the holes by using a micropipette and electrophoresed. The amplicon size was visualized under a UV trans-illuminator.

Sequence analysis

The PCR products were purified using a QIAquick gel extraction kit (QIAGEN, USA), following the manufacturer's procedure. The purified PCR products were sequenced by using an Applied Biosystem 3730xl DNA Analyzer (MyTACG Bioscience Company, MY). Sequences of ITS were analysed using Molecular evolutionary genetics analysis (MEGA) 7.0 software to obtain alignment sequences (Kumar et al. 2016). The BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to find homologous sequences from the NCBI GenBank (<https://www.ncbi.nlm.nih.gov/>).

Phylogenetic tree

Phylogenetic tree was constructed using Neighbor-Joining (NJ) method in MEGA 7.0. Tamura-Nei model used to as a substitution model with 1000 bootstrap replicates to test of phylogenetic tree (Tamura et al. 2013). *Saccharomyces boulardii* was chosen as the out-group. All new sequences were deposited at the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>).

Results

Ten genera were determined in the present study including *Aspergillus*, *Clonostachys*, *Colletotrichum*, *Curvularia*, *Gliocladiopsis*, *Metarhizium*, *Myrmecridium*, *Penicillium*, *Scedosporium* and *Trichoderma*. In results, 18 species were successfully identified with percentage similarity ranged from 91% to 100%, where four species from *Aspergillus* (*A. aculeatinus*, *A. aculeatus*, *A. nomius* and *A. tamaritii*), five from *Penicillium* (*P. javanicum*, *P. oxalicum*, *P. panissanguineum*, *P. rolfsii* and *P. singaporense*) and 1 species belongs to *Clonostachys* (*C. rosea* f.

catenulata), *Colletotrichum* (*C. gloeosporioides*), *Curvularia* (*C. lunata*), *Gliocladiopsis* (*G. peggii*), *Metarhizium* (*M. anisopliae*), *Myrmecridium* (*M. schulzeri*), *Scedosporium* (*S. boydii*) and *Trichoderma* (*T. koningiopsis*). The identified species name, size of deposited sequences and GenBank accession number for 25 selected microfungi are listed in Table 2.

Table 2 GenBank accession numbers of deposited microfungal isolates.

No.	Isolates	Species	Sampling site	Size (bp)	GenBank accession number
1.	B2819	<i>Aspergillus aculeatinus</i>	Lawn of Rumah Muzik	522	MK204521
2.	B3017	<i>Aspergillus aculeatus</i>	Bukit Ekspo	552	MK204506
3.	B3046	<i>Aspergillus aculeatus</i>	Lawn of Panggung Percubaan	522	MK204507
4.	B2786	<i>Aspergillus nomius</i>	Oil palm plantation	551	MK204524
5.	B2990	<i>Aspergillus tamari</i>	Bukit Ekspo	619	MK204509
6.	B3042	<i>Clonostachys rosea</i> f. <i>catenulata</i>	Lawn of Rumah Muzik	569	MK204505
7.	B2853	<i>Colletotrichum gloeosporioides</i>	Bukit Ekspo	573	MK204517
8.	B3068	<i>Colletotrichum gloeosporioides</i>	Lawn of Panggung Percubaan	577	MK204518
9.	B2836	<i>Curvularia lunata</i>	Bukit Ekspo	684	MK204512
10.	B2985	<i>Curvularia lunata</i>	Bukit Ekspo	679	MK204511
11.	B2852	<i>Gliocladiopsis peggii</i>	Bukit Ekspo	550	MK204519
12.	B2968	<i>Metarhizium anisopliae</i>	Oil palm plantation	561	MK204515
13.	B2988	<i>Myrmecridium schulzeri</i>	Bukit Ekspo	542	MK204510
14.	B2972	<i>Penicillium javanicum</i>	Bukit Ekspo	547	MK204513
15.	B3049	<i>Penicillium javanicum</i>	Lawn of Panggung Percubaan	547	MK204514
16.	B3056	<i>Penicillium javanicum</i>	Lawn of Panggung Percubaan	560	MK226178
17.	B2781	<i>Penicillium oxalicum</i>	Oil palm plantation	571	MK204525
18.	B2982	<i>Penicillium oxalicum</i>	Lawn of Rumah Muzik	571	MK204526
19.	B3030	<i>Penicillium oxalicum</i>	Lawn of Rumah Muzik	571	MK204527
20.	B3087	<i>Penicillium panissanguineum</i>	Lawn of Panggung Percubaan	587	MK204503
21.	B3014	<i>Penicillium rolfsii</i>	Bukit Ekspo	597	MK204508
22.	B2851	<i>Penicillium singorense</i>	Bukit Ekspo	560	MK204520
23.	B2773	<i>Scedosporium boydii</i>	Oil palm plantation	606	MK204523
24.	B2811	<i>Scedosporium boydii</i>	Oil palm plantation	607	MK204522
25.	B2862	<i>Trichoderma koningiopsis</i>	Bukit Ekspo	563	MK204516

Aspergillus species growth on PDA showed various color on the upper surface including black, brown, white and green (may green to earth green). The pigmentation of the colony on the reverse plate ranged from white, cream and ivory to light yellow (Fig. 1). Microscopic features are shown by *Aspergillus* species have swollen part known as vesicle at the end of the conidiophore. Vesicle holds phialide only on uniseriate conidiophore, while it holding metulae and phialide on biseriate conidiophore. Most of the species from genera *Aspergillus* produced globose to sub-globose and smooth or rough wall conidia in chain arrangement.

Clonostachys species produced pale yellow with the white color of the colony on the upper surface on PDA. They produced light yellow pigmentation on the reverse plate (Fig. 1). The conidiophore bears the conidia at tips. Ellipsoidal conidia are formed and can be observed in a cluster arrangement. *Colletotrichum* species often produced numerous black mass conidia that slowly grow on PDA. The colony pigmentation ranged from white and cream to black, where some of the species produced the pigment in the concentric ring (Fig. 1). Most of the formation of conidia for *Colletotrichum* species occurred in a false head arrangement. At the tips of simple and short conidiophore, cylindrical and falcate conidia were formed. This genus can be recognized from the formation of appressoria at the end of hyphal branches. In addition, *Curvularia* species were

isolated and identified in the present study.

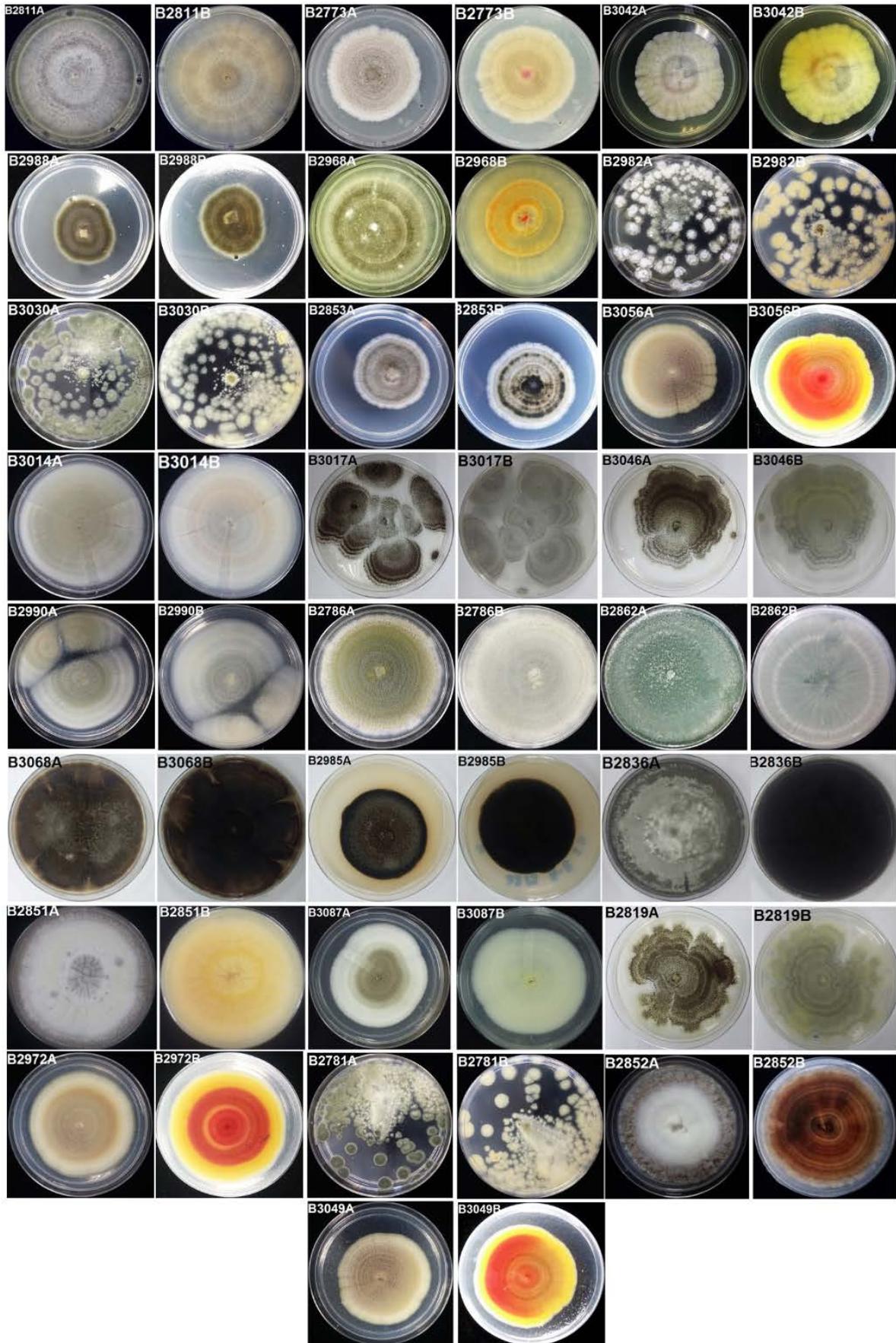


Fig. 1 – Colony features and pigmentation produced by all 25 isolates of microfungi on PDA.

Colony growth of *Curvularia* species are rapid on PDA and produced cold grey or black color from the upper surface (Fig. 1). The pigmentation color from the reverse plate is black. Simple or geniculate conidiophore is possessed by *Curvularia* species, where they hold conidia at the tips of conidiophore. The conidia formed in ovoid and fusiform shape, where some of the conidia are slightly curved at the second cell with three septate.

Gliocladiopsis species produced white cottony morphology on the upper surface on PDA. The color of the colony pigmentation is grayish brown intermediate with walnut brown in concentric rings (Fig. 1). The penicillate and mononematous conidiophore holds conidia at the tips. The cylindrical conidia clump in a cluster when detached from conidiophore.

Metarhizium species produced grey and white color colony in a ring on the upper surface of PDA media (Fig. 1). The pigmentation on the reverse plate is orange glaze intermediate with naples yellow and white in a concentric ring. The branches conidiophore bears conidia at the ends in a false head arrangement. The conidia produced are in a cylindrical shape. *Myrmecridium* species, the colony grows moderately rapid on PDA with dark sepia and grey color on the upper surface due to compact submerged mycelium. The reverse plate is raw umber in color (Fig. 1). *Myrmecridium* species possess straight, unbranched conidiophores that hold conidia at the end of the conidiophore. The conidia form is in fusiform shape with smooth-walled.

Penicillium species, the colony produced a variety of color on the upper surface and the reverse plate of PDA (Fig. 1). Some of the colony produced concentric ring or limited aggregation, where the color of the pigmentation changed at mature. *Penicillium* species showed distinct microscopic characteristics from other genera, where they have various types of conidiophore. The conidiophore holds phialide that produced conidia at the end in chain arrangement. They possess monoverticillate, biverticillate, divaricate and quaterverticillate conidiophore that differs in term of their branching metulae and phialide from a main conidiophore. The conidia produced are globose and ellipsoidal in shape.

The colony of *Scedosporium* species is moderately grown on PDA with grey to white color on the upper surface of culture. The pigmentation color of the colony ranged from raw umber, white and warm grey with concentric rings on the reverse plate (Fig. 1). The microscopic features showed that the branches conidiophore bears the conidia in a single or small cluster. The branch or solitary phialide conidiophore bears the conidia in single or small cluster. Ovoid conidia with truncate bases are produced by *Scedosporium* species at the end of the conidiophore.

Trichoderma species grow rapidly on PDA, where they produced white, earth green to permanent green compact mycelial mass in the concentric ring. The colony produced colorless, white or cream pigmentation on the reverse plate (Fig. 1). The conidia arrangement of this genus is in clump formation known as a cluster. The conidiophore bears ellipsoidal to globose conidia at the tips of phialides. The isolates of *Trichoderma* species in this study showed irregularly verticillate conidiophore that branched repeatedly.

The size of ITS fragment was at 500–700 bp (Fig. 2). Based on the constructed phylogenetic analysis, the tree was divided into two branches which are clade I and clade II with one outgroup (*Saccharomyces boulardii*) clade (Fig. 3). Clade I was grouped into two subclades (subclade A and subclade B), where it showed 96% similarities between those two subclades. Subclade A is divided into two branches with 100% similarities between subclades A1 and A2, which placed genus *Penicillium* and *Aspergillus*. However, there are mixed of sequences in the branch that placed *Penicillium* species with *A. nomius* (B2786) and *A. tamarii* (B2990) in subclade A1, whereas *A. aculeatinus* (B2819) and *A. aculeatus* (B3017 and B3046) are closely related to 100% similarities in subclade A2. Subclade B showed 87% bootstrap value between subclade B1 and B2. *Myrmecridium schulzeri* was placed in subclade B1 with 100% similarities between isolated microfungi (*M. schulzeri* B2988) with GenBank reference (CBS 188.96). In subclade B2, isolates from *Colletotrichum gloeosporioides* (B3068), *Scedosporium boydii* (B2811 and B2773), *Trichoderma koningiopsis* (B2862), *Clonostachys rosea* (B3042), *Gliocladiopsis peggii* (B2852) and *Metarhizium anisopliae* (B2968) are classified together and closely related to each other with 89% similarities. Clade II consists of two isolates of *Curvularia lunata* (B2836 and B2985) from

isolates used and one isolates from BLAST search with 100% similarities.

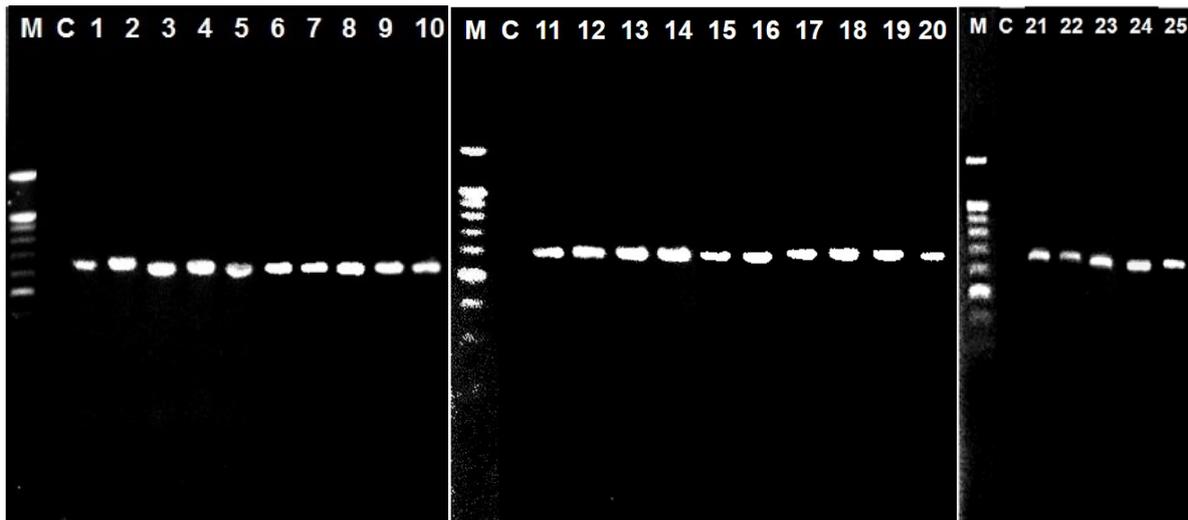


Fig. 2 – Banding patterns generated by primer ITS 1 and ITS 4 for each selected isolated microfungi. Lanes 1) *Scedosporium boydii* B2811, 2) *Curvularia lunata* B2985, 3) *Clonostachys rosea* B3042, 4) *Gliocladiopsis peggii* B2852, 5) *Aspergillus aculeatinus* B2819, 6) *Colletotrichum gloeosporioides* B2853, 7) *Aspergillus aculeatus* B3017, 8) *Colletotrichum gloeosporioides* B3068, 9) *Aspergillus aculeatus* B3046, 10) *Penicillium singorense* B2851, 11) *Penicillium oxalicum* B2781, 12) *Penicillium javanicum* B2972, 13) *Curvularia lunata* B2836, 14) *Aspergillus nomius* B2786, 15) *Metarhizium anisopliae* B2968, 16) *Aspergillus tamarii* B2990, 17) *Penicillium rolsii* B3014, 18) *Trichoderma koningiopsis* B2862, 19) *Myrmecridium schulzeri* B2988, 20) *Scedosporium boydii* B2773, 21) *Penicillium panissanguineum* B3087, 22) *Penicillium oxalicum* B2982, 23) *Penicillium oxalicum* B3030, 24) *Penicillium javanicum* B3056, and 25) *Penicillium javanicum* B3049. M=100 bp ladder. C=Control.

Discussion

The highest occurrence species of microfungi in recreational area (Bukit Ekspo) is supported with a study by Góralaska et al. (2014) in which they found that the microfungi frequently obtained from the soil in playgrounds. This might be due to the high human activities in that area that can change the intensity of nutrients since it might rich in organic matter that can be a better reservoir which affected the populations of fungal.

The presence of some species of microfungi in agricultural areas (oil palm plantation) might be due to the differences in the level of gas, trace elements or heavy metal presence in the soil compared to other sampling locations. Kaltseis et al. (2009) reported that the level of ammonium from fertilizers affected the distributions of *Scedosporium* species, in which they are more abundant in the area with the highest level of ammonium. In this study, *Metarhizium* species only found in an oil palm plantation, where this is supported by Meyling & Eilenberg (2006) which discovered a higher abundance of *Metarhizium anisopliae* in soils from the cultivated areas.

The residential area showed the least species of microfungi present in the soil. This might be due to the lower richness of plant presence in the surrounding area, where it leads to the lower soil fungal diversity in that area (Cline et al. 2017). However, the differences of the distribution patterns of genera in the four sites might be due to the differences level of chemical inputs and nutrient availability, pH level and moisture content in the soil, which should be considered for future study.

The presence of *Aspergillus*, *Colletotrichum*, *Curvularia*, *Penicillium* and *Trichoderma* species in the soil are parallel with the previous studies by Wuczkowski et al. (2004), Ilhan et al. (2006) and Raja et al. (2017). *Colletotrichum* species were obtained in the present study, which is parallel to the previous study conducted by Raja et al. (2017) from the soil in Chennai, India. Ashok et al. (2015) recorded three species of *Curvularia* from the soil in India. According to

Latiffah et al. (2011), *Curvularia* species are rarely recorded in high occurrence in soil. This is supported by the low percentage of isolates *Curvularia* species found in crop fields at Salur Mandar (Gaddeyya et al. 2012).

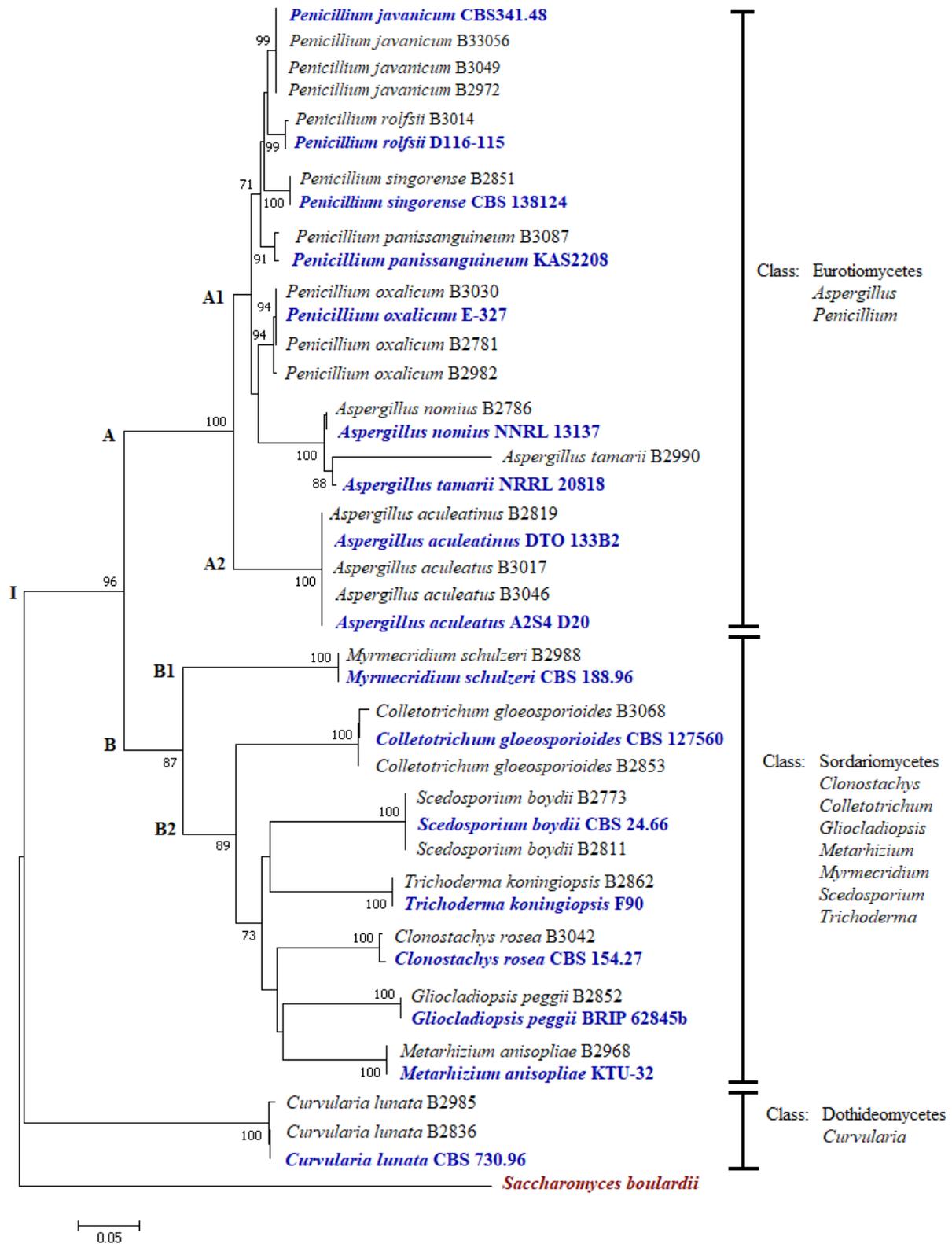


Fig. 3 – The phylogenetic tree was constructed by using Neighbor-Joining (NJ) method for 43 isolates (25 isolates used and 18 isolates control) of microfungi based on ITS sequence analysis. *Saccharomyces boulardii* is used as the outgroup.

Clonostachys species were found and the identification of this genus is referring to the branching patterns of primary and secondary conidiophores (Schroers 2001). Previously, Afshari & Hemmati (2017) reported that *Clonostachys* species were isolated from soil and plant materials such as decaying leaves and tree as saprotrophs. The distribution of genus *Clonostachys* in the present study is low may be due to the less existence of organic matter at sampling sites since they depend on the organic matter for carbon sources (Ravnskov et al. 2006).

Gliocladiopsis species also present in Serdang soil community. The penicillate and mononematous conidiophore is produced by the *Mymecridium* species isolates of the present study, where this is supported by Lombard & Crous (2012). Fatima et al. (2008) discovered that the findings of this genus from the soil are rare with the usage of soil dilution plate method. *Mymecridium* species found in the present study and formed fusiform conidia with no septum, where this is supported by Peintner et al. (2016). Previously, Romão-Dumaresq et al. (2016) found only one isolate of this genus from both rhizosphere and root around sugarcane.

Scedosporium species is distinguished by having more irregular hyphae with multiple branching at acute angles (Kimura et al. 2010). Harun et al. (2010) stated that the colonies of *Scedosporium* species are often undetected due to the prodigious growth of rapid-growing filamentous fungi in soil including *Aspergillus* spp. According to Rougeron et al. (2017), genus *Scedosporium* acts as soil saprophytes. However, they are less frequently experienced in the tropics including Asia but frequently encountered in temperate climates. Cortez et al. (2008) mentioned that *Scedosporium* species can withstand high temperature and have the ability to live in a habitat that provided very low oxygen pressure. In addition, anthropogenic activity on the ecology involved does influences the distribution of *Scedosporium* species, where some of the species present in high human activity area while the other exists in low human activity area (Rougeron et al. 2017).

This study revealed that various microfungi were isolated from the soil samples collected from Serdang, Selangor. The 18 species were successfully identified are *Aspergillus aculeatinus*, *A. aculeatus*, *A. nomius*, *A. tamari*, *Clonostachys rosea* f. *catenulata*, *Colletotrichum gloeosporioides*, *Curvularia lunata*, *Gliocladiopsis peggii*, *Metarhizium anisopliae*, *Myrmecridium schulzeri*, *Penicillium javanicum*, *P. oxalicum*, *P. panissanguineum*, *P. rolfsii*, *P. singorense*, *Scedosporium boydii* and *Trichoderma koningiopsis*. The information obtained can be used as a baseline for future studies on the occurrence and species diversity of microfungi isolated from soil. The distribution of the microfungi assemblages are mainly affected by anthropogenic activities. However, many parameters could affect the distribution and need to be considered for further study.

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