MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society For Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

ISOLATION AND IDENTIFICATION OF ENDOPHYTIC FUNGI FROM UiTM-PERHILITAN RESEARCH STATION, KUALA KENIAM, PAHANG, MALAYSIA

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History
Received: 26 September 2021
Accepted: 8 May 2022

Keywords:

Endophyte fungi; Identification; morphological characteristic; Molecular analysis; UiTM-Perhilitan Research Station, Kuala Keniam

Abstract

Endophytic fungi live inside plant tissues and do not degrade or produce chemicals that infect the host cell. Because of their species richness and diversity, endophytic fungi are an underutilised source of pharmacologically relevant chemicals. Hence, this study was conducted to isolate, identify and characterize endophytic fungi from the sampling site at UiTM-Perhilitan Research Station in Kuala Keniam, Pahang, Malaysia. In the present work, three different components (leaf, root, and stem) of four different plant species were randomly collected. The morphology of isolates was characterized based on mycelial texture, pigmentation, colony appearance, and spores, which were then subjected to molecular analysis. In total, 32 endophytic isolates were successfully obtained. Relatively, leaf samples were found to have greater colonization frequency in plant B (72.7%) as compared to root and stem parts. Morphological analysis clustered the isolates into 15 different groups and revealed that all the isolates belonged to Ascomycota and Basidiomycota phylum. Molecular identification by using ITS universal primer identified two isolates as *Candida metapsilosis* (C9) and *Clonostachys roses* (D13). However, 12 isolates were recorded as unidentified groups. The findings of this study point to the possibility of further research on bioactive substances produced by these two endophytic fungi.

INTRODUCTION

Endophytes are fungus or bacteria that spend all or part of their lives inside living plant tissue [1]. Depending on the type of contact they have with their host plant, they might be classed as useful, neutral, or harmful. Endophytic microbes are found on almost all plants in the wild. Within the plants, endophytes are usually found in the intercellular space as well as in the vascular tissues [2]. Endophytes are commonly found in the intercellular space as well as in the vascular tissues of plants [3,4]. A million endophytic fungal species have been discovered in the leaves, bark, wood, seeds, and roots of asymptomatic infections in forest trees, and fungal endophytes have been found to be ubiquitous. Glomeromycota, Ascomycota, and Basidiomycota are fungal kingdom divisions that constitute up 91 percent of plant-colonizing endophytic fungi. Glomeromyceta accounts for 40% of plant-colonizing endophytic fungus, while Ascomycetes, Basidiomycetes, and Zygomycetes account for 31%, 20%, and 0.1 percent, respectively. The rest of the endophytic fungi are classified as part of an unnamed phylum [5].

Endophytes can be isolated from stems, seeds, and leaves, among other plant parts. Endophyte isolation should be precise enough to remove epiphytic microorganisms from the plant's surface, yet sensitive enough to recover the complete number of endophytes colonized [6]. To reduce the presence of plant pathogenic and saprobic species, and to prevent the isolation of localized harmful endophytic bacteria, the plants collected for investigating endophytic communities should be disease free and apparently healthy. For identification, diagnostic features such as fungal sporulation structures were investigated. However, in addition to cultured properties, molecular methods were

necessary to identify specific non-sporulation strains. Endophytic fungi were identified using a combination of molecular phylogeny, cultural traits, and morphology. The spacer DNA between the big and small subunits of ribosomal RNA (rRNA) is known as the internal transcribed spacer (ITS) [7].

Endophytes are microorganisms that help plants grow and diversify by producing growth-promoting secondary metabolites, insect and pest repellents, antimicrobials against plant infections, and protectors in stressful situations. Endophytic fungi can help plants grow by producing phytohormones and increasing nutrient availability ([8, 9, 10, 11, 12]), nitrogen fixation [13,14,15], by biocontrol of phytopathogens in the root zone (through the production of antibacterial or antifungal agents, nutrient competition, induction of systemic acquired host resistance or immunity), siderophore production or in the vascular system [16]. Despite this, only a small amount of research has been done on this important bacterium. Twelve species of endophytic fungi have been discovered in Indonesian tropical woods by [17] and colleagues. In Malaysia, a few studies has been reported including [18] who successfully isolated 148 fungal endophytes but only 134 of them (90.5%) exhibited inhibitory activity towards at least one test microorganisms. In addition, [19] has isolated endophytic fungi from juvenile *Aquilaria malaccensis* for their antimicrobial properties. Only a percentage of Malaysia's endophyte variety has likely been characterized thus far, and forest tree endophyte communities appear to be extraordinarily diverse. UiTM-PERHILITAN Research Station Kuala Keniam, Pahang is a research collaboration station between the Department of Wildlife and National Park (DWNP) Malaysia and Universiti Teknologi MARA (UiTM) aims to initiate a research program that would advance the scientific foundations of National Park in Malaysia. Lowland dipterocarp forest species are characterized by a high proportion of Dipterocarpaceae species, with Meranti (*Shorea spp*.) and Keruing (*Dipterocarpus spp.)* as the main species [20]. Ericaceous forests at higher elevations, montane oak, and humid rainforests in the lowlands are examples of other vegetation. In the present study, efforts have been made to isolate endophytes inhabiting the four selected plants in UiTM-Perhilitan Research Station, Kuala Keniam, Pahang, Malaysia. It is hoped that the isolated endophytic fungal will be a promising resource for prospecting new chemical entities as future drugs.

MATERIALS AND METHODS

Materials

Plant samples were collected from UiTM-Perhilitan Research Station, Kuala Keniam, Pahang, Malaysia. Potato Dextrose Agar were purchased from Oxoid, UK. Universal markers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCGTAGGTGAACCTGCGG-3') were purchased from Sigma-Aldrich Inc., USA.

Collection of Plant Sample

All four different components of plant species from the sampling site located within UiTM-Perhilitan Research Station, Kuala Keniam, Pahang, Malaysia was collected. The species of plant randomly collected based on three highest growth and within distribution 200m^2 sampling site with geocoordinate for the sampling site was 4^o 31' 07.17’ N, longitude 102^o 28' E with numerous morphological variations between the shape of the leaves and flowers. For the collection of all symptomless and seemingly stable leaves, pre-sterilized polythene bags were used. Via sampling, the three biological replicates were collected once made. Samples of three separate plants (stem, leaf and root) from four plants (A, B, C and D) were labelled (1 to 15), and then were analyzed within 24hours.

Isolation of Endophytic Fungi

Samples were washed initially with running tap water for 10 minutes and air-dried. The samples were soaked in 1% sodium hypochlorite for 3 - 4 minutes before being washed in 70% ethanol for 1 minute. Then finally the samples were rinsed with distilled water by the following method [21] with a slight adjustment. In order to ensure surface sterilizations, the rinsed water was streaked onto the potato dextrose agar (PDA) medium and sterilized segments were imprinted on the PDA medium. A sterile filter paper was blotted with the excess moisture. Sterilized segments were cut into pieces and placed in petri dishes containing PDA medium supplemented with chloramphenicol (100 mg/L) for a surface length approximately 1 cm × 1 cm length. The petri dishes were then sealed and incubated for three to five days at 28°C. All petri dishes were monitored by testing the production of fungal colonies every day. All Individual hyphal tips were separately transferred to the new PDA medium from the edges of each treated plant piece.

Morphological and Microscopic Detection of Endophytic Fungi

All morphological studies were performed on PDA by placing the fungi on it and incubating it for 7 days. Fungal identification was reported based on the culture’s morphology, the mechanisms of spore development, and the characteristics of the spores were recorded. The growth appearances were observed on both the top and bottom sides of the culture plates and tentative identification, microscopic slides of each fungal endophyte were tentatively described. For the preparation of the slide by tease mount staining, the process using lactophenol cotton blue was used and all observations were performed using a microscope [22].

Colonization Frequency Analysis (CF)

The colonization frequency (CF) percentages of the endophytic fungi were formulated as described below [23].

$$\text{Colonization frequency (CF \%)} = \frac{\text{Number of segments colonized by endophytes}}{\text{Total number of segments analysed}} \text{X } 100$$

Molecular Identification of Fungal Isolates using ITS-PCR

Fungal DNA was extracted using the method described by [24]. A total of 0.05 gm of 5–8-day-old fungal mycelia was scraped from new PDA cultivation plates and homogenized with a sterile mortar and pestle in liquid nitrogen. 1 cc of extraction buffer was combined with powdered mycelium (100 mM Tris, 1.4 M NaCl, 20 mM EDTA, 2 percent CTAB). The homogenate is incubated for 10 minutes at 65°C before being centrifuged for 5 minutes at 12000 rpm. Both supernatants were transferred to a new Eppendorf tube and centrifuged at 12,000 rpm for 5 minutes with a mixture of chloroform and isoamyl alcohol (24:1). Two liters of ice-cold isopropanol were added to new tubes and incubated for 10 minutes at -20 degrees Celsius. The particle was washed in 500 mL of 70% ethanol and centrifuged at 10000 rpm for 5 minutes. Before being re-suspended in 30 L of TE buffer, the pellet was air-dried (10 mM Tris Cl pH 8.0, 1 mM EDTA). A total of 1 µL of RNase A (20 mg/ml) was added, and the mixture was incubated at 37°C for 1 hour before being processed at -20°C. The amount of DNA in 1 percent agarose was determined using electrophoresis (low EEO grade; HiMedia, India). A UV Transilluminator was used to visualize ethidium bromide added to the gel at a final concentration of 0.5 mg mL-1 and tris-acetate EDTA buffer (40 mM Tris; 2 mM EDTA; 20 mM glacial acetic acid, pH 8) for 2 hours at 50 UV Transilluminator.

Using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCGTAGGTGAACCTGCGG-3'), ribosomal DNA from the ITS region was amplified by PCR. PCR amplification of the ITS region was performed in a total reaction volume of 20 µL, using 10 µL of 2X PCR master mix, 1 µL of 0.5M forward and reverse primer, and 7 µL of sterile water. All PCR amplifications were performed on a Gradient thermal cycler, with an initial denaturation at 95°C for 3 minutes, followed by 35 amplification cycles at 92°C for 1 minute of denaturation, 50°C for 1 minute of annealing, 72°C for 2 minutes of extension, and 72°C for 10 minutes of final extension. As indicated, electrophoretic assessment of PCR products was performed using a 1 percent agarose gel supplemented with ethidium bromide and 100 bp DNA as a marker on a 1 percent agarose gel supplemented with ethidium bromide. The sequences were later submitted to GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov) after all PCR products were purified using a Quick gel extraction and PCR purification combo kit. To link all of the sequences described in the tests, the BLAST software with the G (http://www.ncbi.nlm.nih.gov/BLAST/) was employed.

RESULTS AND DISCUSSION

Endophytic fungi are the most understudied creature that contributes to a major source of biodiversity and biological products. Symbiotic relationships are created when endophytic fungi interact with plants to produce beneficial host chemicals. There have been several studies on biodiversity, taxonomy, ecology, and the symbiotic connection with the host. In this study, fifteen morphologically different endophytic fungi were successfully isolated from 4 different plants (Plant A, B, C and D) from UiTM-Perhilitan Research Station, Kuala Keniam, Pahang, Malaysia.

Table 1 shows the colonization frequencies recorded from fungal isolates of four different plants. Leaf samples showed the highest colonization frequency in plant sample B and D while plant sample A and C were dominated by endophytes isolated from stem samples. These findings were aligned with the study reported by researchers [25-27] which found the highest colonization rate at leaf samples. This is because of the surface area exposed to the environment and the existence of stomata on the leaf, which provide access for the entrance of fungal mycelia, the leaf segment had a larger diversity of endophytic fungi than the root and stem [28].

Phylum Zygomycota has recorded the highest colonization frequency (72.7%) in leaf samples of plant B. Meanwhile, the lowest colonization frequency recorded in stem samples of plant B (9%) belongs to Ascomycete's phylum. Results in this study were aligned with findings reported by [28] where only two phylum of endophytic fungus (Zygomycota and Ascomycota) were identified in *Melastoma malabatrichum* species. Secretion of phytochemicals containing some antifungal may cause the low colonization rate [28]. In addition, the number of endophytic fungal species is highly related to the sampling range of the plant age, the chance of colonization by endophytic fungi changes depending on the climate, resulting in an endless variety of species and numbers of endophytic fungi. To facilitate the isolation of new endophytic fungi, it is proposed that the sample collection be expanded by increasing the sampling region and collecting plant samples of various ages [25].

Table 1. Endophytic fungi colonization frequency (%) from different plant samples at UiTM-Perhilitan Research Station, Kuala Keniam, Pahang, Malaysia

Sample number	Plant sample	Parts of plant	Number of isolated fungi	Phylum	Colonization frequency (%)
1	A	root	3	Ascomycota	37.5
2		stem	3	Zygomycota	37.5
3		leaf	2		25.0
4	B	stem	1	Ascomycota	9.0
5			2	Zygomycota	18.0
6		leaf	3	Zygomycota	72.7
7			3		
8	C	stem	2	Zygomycota	62.5
9			2		
10			3		
11		leaf	1	Ascomycota	12.5
12			2	Zygomycota	25.0
13	D	stem	1	Zygomycota	20.0
14		leaf	1	Ascomycota	20.0
15			3	Zygomycota	60.0

Next, for isolation purposes, single spore isolation was used to identify the endophytic fungal isolates from pure cultures. Microscopic and macroscopic characters were used to identify fungal isolates [29]. The main aim of morphological characteristics is based on similar morphological identification used to group the fungal isolates. If their morphological characteristics matched the morphological descriptions previously described or documented, the fungal isolates were grouped into the same group or genus. Isolation of the endophytic fungi was done using Potato dextrose agar (PDA). Most species of endophytic fungi can be differentiated by classical mycology based on their morphological characteristics, such as color, odor, ascospore and peridium, and other organoleptic characteristics [30]. The results showed differences in colony growth, morphology and color between fungi (Table 2).

Generally, the endophyte fungal morphology consists of mycelia, hyphae and spore. Out of thirty-two isolates, only fifteen different morphologies of endophytic fungal cultures were further analyzed as shown in Figure 1. Observation on fifteen isolates demonstrates that 4 isolates demonstrated septate hyphae (A, C, K, M) and the rest showed non-septate hyphae. Sample F and L showed yellow color colonies; sample A showed brown color colonies with filamentous form. Black color colonies were observed in sample D, E and N while sample I, K and N showed a black and white color of colonies. Fungal species identification by using morphological variation were challenging because endophyte fungus did not showed significant morphological variations. [30] stated that a restricted collection of morphological and homoplastic characteristics caused the use of morphological features to be difficult in the evolutionary systematics of hypogenous ascomycetes. Findings made by [31] also recorded that some of the isolated endophytic fungi were unable to be identified since the colony lacked many of its features and did not produce spores on PDA medium. Therefore, phylogenetic studies based on rDNA sequences can also be used to define species that cannot be identified using morphological features alone. Furthermore, growth on artificial media prevents the isolation of some endophytic fungi, and accurate, quick identification technology for these fungi is required since some fungal isolates were resistant to PDA medium culture [28].

The advent of molecular methods for identifying endophytic fungus has offered a fresh viewpoint on taxonomic characterization and connections between complicated groupings of species. ITS sequencing was applied to characterize endophytic fungal isolates on a molecular level. Internal transcribed spacer polymerase chain reaction was used to recover genomic DNA from endophytic fungal cells prior to amplification (ITS PCR). The primers amplified the entire ITS area of interest. The PCR product appeared in all lanes after electrophoresis as single 600-bp band. ITS sequencing was used to validate the identification of the 15 fungal isolates (Figure 2). The sequences were then uploaded to the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) and BLAST was used to search for similar species (standard nucleotide-nucleotide). The ITS sequence is a conserved rDNA sequence that has been frequently utilised to identify, describe, and perform phylogenetic analysis of fungal isolates, both alone and in

conjunction with other universal sequences like tubulin and actin [32].

Figure 2 shows that molecular techniques were used to confirm the identification of 15 isolated endophytes. All 15 isolates were successfully amplified using primers ITS1 and ITS4, and BLAST searches revealed their identities, with just three species being recognised out of the total of 15 (Table 3). ITS sequences identified the sample A2 as *Acarapis dorsalis*, sample C9 as *Candida metapsilosis*, and sample D13 as *Clonostachys roses*. *Candida metapsilosis* has been reported as endophytic fungus in [33] studied for its potential as biocontrol agents and plant growth stimulating hormone. [34] has reported the *Clonostachys roses* as plant fungal endophytes isolated from roses and cucumbers. However, *Acarapis dorsalis* found in this study was considered as a phytophagous mite which is commonly interacting and possibly impacting endophytic fungus or population dynamics; nevertheless, these interactions are poorly understood and generally unexplored [35].

Table 2. Endophytic fungi morphological and microscopic identification

Number	Endophytic		Macroscopic characteristics	Microscopic characteristics
1	A	A1	Brown color colonies, flat elevation and filamentous form	Septate hyphae, sterile mycelia surrounded elliptical spores
2	B	B1	White colonies later turned in to cream colour, flat elevation and filamentous form	Non-septate hyphae, sterile mycelia
3	C	C1	White cotton colonies, Flat elevation and filamentous form	Non-septate hyphae, sterile mycelia
4	D	D1	Black colonies	Septate hyphae, sterile mycelia
5	E	E1	Black colonies	Non-septate hyphae, sterile mycelia
6	F	F1	Yellow colour colonies, flat elevation and circular form	Non-septate hyphae, sterile mycelia
7	G	G1	White colonies later turned in to cream colour	Non-septate hyphae, sterile mycelia
8	H	H1	White colonies later turned in to cream colour	Non-septate hyphae, sterile mycelia
9	I	I1	Black color colonies	Non-septate hyphae, sterile mycelia with the clump of sub globose spore
10	J	J1	White cotton colonies. black colour on back side	Non-septate hyphae, sterile mycelia
11	K	K1	Black color colonies	Septate hyphae, sterile mycelia
12	L	L1	Yellow colour colonies, flat elevation and circular form	Branched non-septate hyphae surrounded elliptical yellow spores
13	M	M1	White cotton colonies. black colour on back side	Non-septate hyphae, sterile mycelia
14	N	N1	White cotton colonies. black colour on back side	Septate hyphae, sterile mycelia
15	O	O1	White colour colonies with a yellow colour back view. Flat elevation and undulate margin	Non-septate hyphae, sterile mycelia

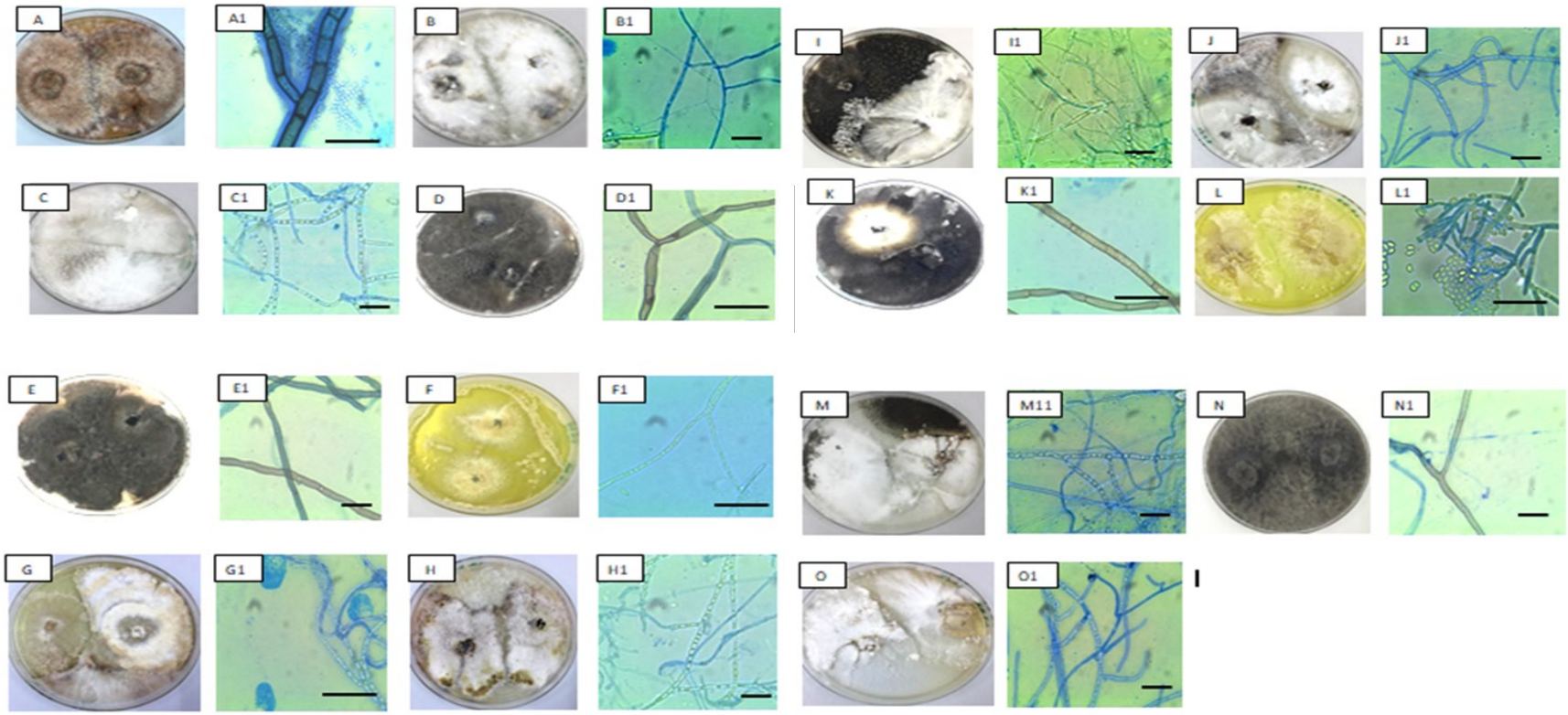

Figure 1. Endophytic fungi morphology and microscopic view isolated from UiTM-Perhilitan Research Station, Kuala Keniam, Pahang, Malaysia. (Scale bars = 5)

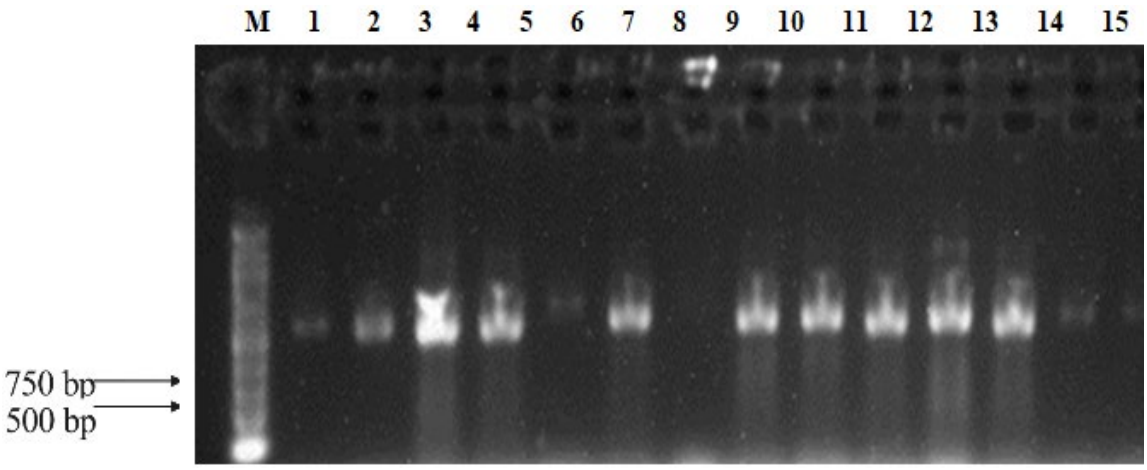

Figure 2. PCR product amplification of endophytic fungus isolates with primers ITS-1 and ITS-4. Lane M: 100-bp marker, Lanes 1–15: 600-bp PCR amplified product

Table 3: Molecular identification of fungal endophyte isolates from Forest UiTM Kuala Keniam based on blastN queries in NCBI

Isolate codes	Representative isolate	16s rRNA identification	Accession no.	Phylum, Class, Family
Plant A	A1	Unidentified	-	Ascomycota
	A2	*Acarapis dorsalis*	JF817296.1	Zygomycota
	A3	Unidentified	-	Zygomycota
Plant B	B4	Unidentified	-	Ascomycota
	B5	Unidentified	-	Zygomycota
	B6	Unidentified	-	Zygomycota
	B7	Unidentified	-	Zygomycota
	B8	Unidentified	-	Zygomycota
Plant C	C9	*Candida metapsilosis*	MW775502.1	Zygomycota
	C10	Unidentified	-	Zygomycota
	C11	Unidentified	-	Ascomycota
	C12	Unidentified	-	Zygomycota
Plant D	D13	*Clonostachys roses*	MG925324.1	Zygomycota
	D14	Unidentified	-	Ascomycota
	D15	Unidentified	-	Zygomycota

However, 12 additional isolates were labelled as 'unidentified fungus' since they had BLAST matches with sequences that were less than 95 percent identical (Table 3). One possible reason for this phenomenon might be because the culture medium included mixed isolated species, and the PCR products could not be identified as mentioned by [31]. In addition, this might also be attributed to differences in the resolution of barcoding loci, particularly ITS, across various populations [36]. In specific loop sections of the ITS, there are little constraints on variation in other species, resulting in a variety of sequences that might be misconstrued as distinct species.

Despite the fact that the ITS region works effectively as a fungal barcoding identifier, it has been a source of contention. Because several species, such as *Aspergillus, Cladosporium, Fusarium, Penicillium*, and *Trichoderma,* have tiny or no barcode gaps in their ITS regions, the ITS region does not operate effectively. This is a crucial issue for natural product research since these genera produce several important secondary metabolites, the most notable of which is *penicillin*. Furthermore, intragenomic ITS variation is seen in several families of fungi, however current research suggests that it is not widely widespread in fungus (occurring in 3–5% of 127 Ascomycota and Basidiomycota species).

Thus, Protein-coding genes are used for species identification via barcoding because they contain intron sections, which develop at a faster pace than ITS and are used in phylogenetic studies because they have a greater resolution at higher taxonomic levels than rRNA genes. Furthermore, because they are thought to exist as a single copy in fungus, these genes allow for simple detection of homology and convergence, are less variable in length due to fewer mutations in exons and are easier to align over rRNA genes due to codon restrictions [37].

CONCLUSION

Fifteen fungal endophytes were isolated from different parts of leaves, stem and root tissue of four plants examined. Molecular analysis identified those fungal endophyte*s as Candida metapsilosis* (C9), *Clonostachys roses* (D13). Further research work will be required to investigate and focus on the role of these fungal endophytes in producing bioactive compounds, antimicrobial compounds, and others potential as natural dyes.

ACKNOWLEDGEMENTS

The work was performed at UiTM Cawangan Negeri Sembilan. The authors would like to thank UiTM for providing all the necessary facilities for successfully completing the research work. Nor'Aishah Hasan, Nur Intan Hasbullah and Mohd Zaini Nawahwi are registered members under Special Interest Group (SIG), BioMECs, Universiti Teknologi MARA, Cawangan Negeri Sembilan, Kampus Kuala Pilah.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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