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New Fungal Strains from Peat Soil in Malaysia: Morphological and Molecular Characteristics

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Abstract: Fungi have unique properties and are used in many areas of agriculture and industry because they can produce different enzymes. This study aims to study the fungal diversity in peat soil from Pontian in Johor, Malaysia. The fungal isolates were described on different culture media and on a new culture medium called EVA medium and were identified using the phenotypical characteristics and molecular properties of the D1/D2 domain of the 28S large subunit ribosomal RNA (28S rRNA) and ITS (ITS1-ITS4) rDNA regions. The results revealed that 14 fungal species (15 isolates) were identified, among them, 6 were categorized as newly isolated strains and recorded in Malaysia; these include *Aspergillus arenarioides* EAN603, *A. iizukae* EAN605, *Paraconiothyrium brasiliense* EAN202, *Parengyodontium album* EAN602, *Penicillium pedernalense* EAN604, and *Purpureocillium lilacinum* EAN601. The cultural, morphological, microstructure, and molecular characteristics of these new strains have been described in this study. It was noted that the EVA medium exhibited a moderate support for fungal growth and sporulation compared to other culture media. Furthermore, the efficiency of the new medium as an enrichment medium to isolate fungi from peat soils with high ligninolytic content was discussed.

Keywords: new recorded fungal strains; Malaysia; molecular analysis; chemical classification

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1. Introduction

Many fungal strains were isolated from the soil because they were available for the fungus growth needs, including the decomposition of plants [1]. Hawksworth and Lücking [2] estimated that there are 3.8 million species of fungal organisms on Earth, of which only 70,000 have been identified and described. Based on next-generation sequencing, the number of fungal species ranged between 3.5 and 5.1 million [3]. Gams [4] indicates that 3150 fungal strains were isolated from the soil, and 70% were cultivated in a culture medium. Many fungi species have been isolated from the peatland of the world. Similar studies have also been conducted in Malaysia including Perak, Pahang, and [5,6]. The abundance of fungi in wetlands has changed according to the plants that dominate the soil. Johor's peatlands account for 11% of Malaysia's total peatlands (26,000 km^2). However, there has been little research on this subject compared to the country's total peatland

area. Kin et al. [7] isolated *Isaria amoenerosea* and *Metarhizium anisopliae* from peat soil in Peninsular Malaysia. Omar et al. [8] isolated 20 fungal species belonging to *Aspergillus* and *Penicillium* from the peatland in Sarawak, Malaysia. However, these studies have been conducted in different locations. Peatlands are a rich source of fungal diversity, which play an important role in the element cycle. A wide variety of fungal species are associated with the high water and organic content of the peatland, which acts as a carbon source for fungal species growth. Exploring different types of grassland in different regions provides an opportunity to discover new fungal species of high economic and ecological value. Many fungal species, such as *Ceriporiopsis subvermispora*, *Ganoderma lucidum*, *Penicillium citrinum*, *Peniophora incarnate*, *Phanerochaete chrysosporium*, *Phlebia tremellosa*, *Physisporinus rivulosus*, *Pleurotus ostreatus*, *Pleurotuseryngii*, *Trametes versicolor*, and *Trichaptum abietinum*, have been isolated from soil and used in the environmental technology such as degradation of organic pollutants [9–13]. The high applicability of fungi in industrial and environmental applications such as fermentation and bioremediation processes are due to high extracellular enzyme production. Enzymes such as amylase, cellulase, chitinase, pectinase, lipase, protease, peroxidase, and laccase have high applicability to degrade complex compounds into simple substances.

Malaysia is one of the high-biodiversity countries in the world due to its rich natural and environmental resources [14]. The importance of flora as a valuable source of fungi needs to be studied in more depth. The information obtained could be used to activate agriculture and infrastructure. In fact, Malaysia is a subtropical country with a moderate and high-humidity climate and large forests, providing favourable conditions for fungus diversity [15]. The biodiversity of tropical forest decomposers is three times greater than that of other forest ecosystems [16]. Nevertheless, fungi isolation and screening in these places have not yet been systematically initiated [16,17]. The study aimed to explore the fungal diversity in Pontian's peatlands to develop a fungus load inventory and to serve as a database for future research, emphasising the originality of the present work. The study also focuses on recovering the fungal isolates on different culture media and suggests a new medium (EVA or EFAQ medium) in order to provide a more suitable medium for fungal strains isolated from peat soil with high ligninolytic compounds.

2. Materials and Methods

2.1. Peat Soil Sample

The peat soil samples were collected from Kampung Medan Sari (1°28′24.5″ N 103° 26′35.96″ E) located at Pontian, Johor (Figure 1). This study location was chosen because it is far from agricultural and development activities and is classified as a virgin peatland. The sampling point area is 100 m^2. The samples were obtained by scraping a freshly exposed surface at a depth of 1 m. Three samples (1 kg for each sample) were collected from each area in sterile zipper polythene bags, then immediately transferred to the laboratory of Universiti Tun Hussein Onn Malaysia (92 km from the study area) and stored at 4 °C until use within one month of collection [18].

2.2. Media Preparation

The morphology of the fungal isolates was observed on Potato Dextrose Agar (PDA, Oxoid, Hampshire, UK), V8 juice agar medium (V8A) (Himedia, Thane, India), Czapek-Dox Agar (CZ, R&M Marketing, Henfield, UK), Malt Extract Agar (MEA, Merck, Darmstadt, Germany), Czapek yeast extract Agar (CYA, Oxoid, UK), Sabouraud dextrose agar (SDA, HiMedia, India), and CZ medium with Rose bengal (CZR) (Himedia, India). The media were used to obtain an accurate description of the fungal isolates. According to the preparation protocol, the media were prepared by adding one litre of distilled water in a glass bottle, mixing well with magnetic stirrers, and adjusting pH (according to the preparation protocol for each media) using NaOH (0.1 M) and HCl (0.1 N).

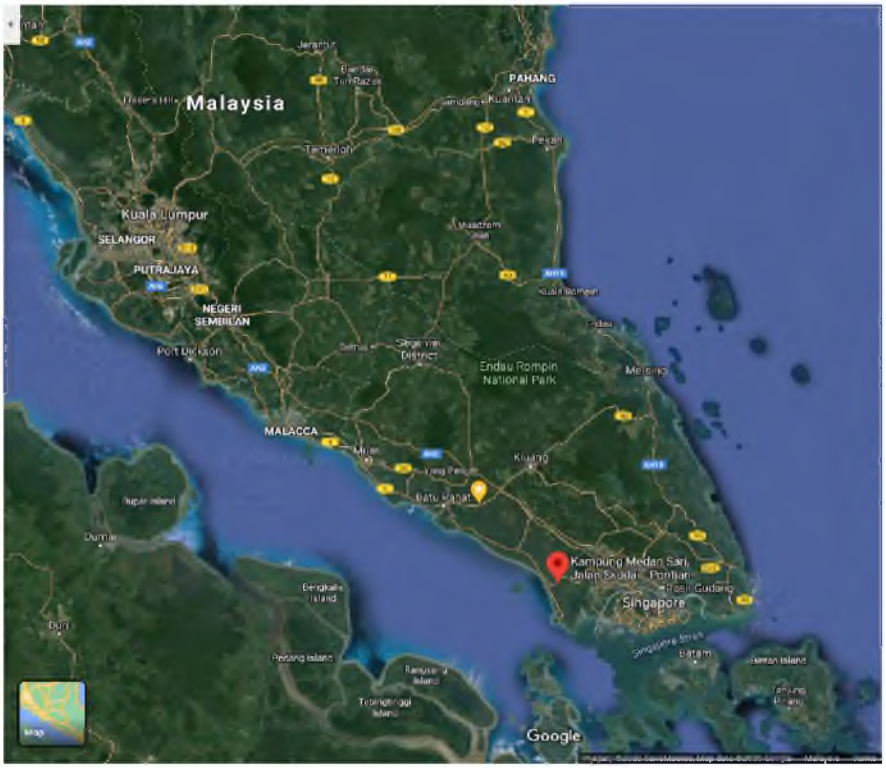

Figure 1. The map of the sampling location at Kampung Medan Sari, Pontian, Johor, Malaysia.

A new medium named Pumpkin Peel Medium (with the abbreviation of EVA) was used for the first time to describe the fungal growth and its applicability in isolating fungal species from peat soil with high contents of ligninolytic substances. Pumpkin peels were used as a substrate to confirm the ability of the fungal isolates to use the ligninolytic substances as a source of carbon for their growth. Since peat soil has high ligninolytic materials, this medium was suggested as a rich medium with ligninolytic materials to recover the fungi from peatlands. EVA medium was prepared by cutting 200 g of the Pumpkin peels into small pieces (1 cm) and then by crushing using a mortar and pestle. The crushed pumpkin peels were boiled for 10 min, and then the extract was harvested using white gauze. The extract was transferred to a 1 L conical flask and filled with distilled water. Then, 15 g of agar was added, and the mixture was homogenised using a magnetic stirrer (125 rpm) for 10 min, pH was adjusted to 5.5. All cultural media were automatically autoclaved for 20 min at 15 pounds per square inch (psi) and 121 °C prior to use.

After the culture media cooled down to a lukewarm temperature, 4 mL of chloramphenicol was added to each medium to prevent bacterial growth. The media were poured into the Petri plates in a laminated flow chamber and maintained within 3 days for use.

2.3. Fungal Strains Isolation and Purification from Soil Samples

The fungal isolates were isolated from the peat soil samples by culture-based method (the colony-forming unit, CFU) using the standard serial dilution spread plate method on Potato Dextrose Agar (PDA, Oxoid, UK) medium [19,20], as described in previous work [21]. The isolated fungi were purified following Noman et al. [22].

2.4. Phenotypic and Molecular Analysis of Fungi Isolated from Peatland Samples

The fungal isolates were identified based on morphological and molecular analysis as follows: The colony size (mm diameter) and surface were described on nine culture media after seven days of incubation at 28 °C. The primary identification of the fungal isolates was performed following the description in different references in the literature [23–29]. The microstructure of fungal conidiophore and spore shapes were observed using SEM.

The fungal isolates with pure culture were sub-cultured on new PDA medium, incubated at 28 °C for 2–3 days.

The molecular fungal identification was conducted based on the D1–D2 region of the 28S rRNA sequences using a PCR thermal cycler, "Veriti 96 Well Thermal Cycler", with a primer set, F63 and LR3, to amplify the D1–D2 region of the 28S rRNA at the Centre of Chemical Biology, USM (CCB, USM). The primers' sequences for D1/D2 region of 28S rRNA were as follows: forward primer F63 (5′ GCATATCAATAAGCGGAGGAAAAG) and reverse primer LR3 (5′ GGTCCGTGTTTCAAGACGG 3′) according to Fell et al. [30]. The internal transcribes spacer (ITS) primers, ITS 1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS 4 (5′ TCCTCCGCTTATTGATATGC 3′) were used to amplify the ITS rDNA region [31]. For B-tubulin, primers were Bt2a Forward 5′ GGT AAC CAA ATC GGT GCT GCT TTC 3′ and Bt2b Reverse 5′ ACC CTC AGT GTA GTG ACC CTT GGC 3′ [32].

To construct the phylogenetic tree, the sequences of each fungal strain which was obtained from PCR amplification were inserted into NCBI GenBank. Among several fungal strains, the sequences with 100% similarity at the website of NCBI GenBank were downloaded. The sequences obtained were subjected to multiple alignments using ClustalX2.1. The aligned sequences for the selected fungi were inserted into the PAUP4 software program and subjected to the rotating process. The phylogenetic tree was constructed based on a neighbour joining UPGMA algorithm.

2.5. Raman Spectroscopy

The chemical composition of the fungal mycelium cell walls was analysed using Raman spectroscopy. Each fungal strain was cultured for 5 days in a PDA medium containing 1% dimethyl sulphoxide (DMSO) to prevent the production of fungal pigment that could negatively affect the analysis. A small piece of fungal mycelium (1 × 1 cm) was placed on the surface of a glass slide and employed in Raman spectroscopy (Horiba, Kyoto, Japan) for analysis.

3. Results and Discussion

3.1. Fungal Isolates from Peat Soil

A total of 14 fungal species were isolated from 9 peat soil samples. The fungal strains were recognised in 9 genera and 15 strains and deposited in NCBI (Table 1).

Table 1. Fungal strains from peatlands and their accession number.

Isolate No.	Species Name	Accession No.		The First Time Recorded in Malaysia
		28S rRNA	ITS	
EAN605	*Aspergillus iizukae*	MK518343	ND	√
EAN506	*Aspergillus aculeatus*	MK518351	MK518394	×
EAN603	*Aspergillus arenarioides*	MK518366	ND	√
EAN203	*Penicillium verruculosum*	MK518350	ND	×
EFAQ406	*Penicillium crustosum*	MK530088	ND	×
EAN604	*Penicillium pedernalense*	MK518385	ND	√
EFAQ405	*Penicillium crustosum*	MK530708	MK530709	×
102UTHM	*Trichoderma viride*	MK518057	ND	×
303UTHM	*Trichoderma asperellum*	MK518056	ND	×
EAN601	*Purpureocillium lilacinum*	MK518365	ND	√
EAN202	*Paraconiothyrium brasiliense*	MK517572	MK518445	√

Table 1. *Cont.*

Isolate No.	Species Name	Accession No.		The First Time Recorded in Malaysia
		28S rRNA	ITS	
EAN403	*Cochliobolus geniculatus*	MK517569	MK518444	×
104UTHM	*Schizophyllum commune*	MK517570	ND	×
EAN602	*Parengyodontium album*	MK518376	ND	√
EFAQ504	*Fusarium solani*	MK530101	ND	×

√ (*Yes*), × (No), ND (non-detected).

Six fungal strains were identified for the first time in Malaysia based on the comparison with the checklist of fungi in Malaysia, as reported by only one study conducted by Lee et al. [33]. Moreover, to confirm the list of fungi in Malaysia up to 2020, the Scopus database (877 documents) for fungi in Malaysia using specific key works ("Malaysia" AND "fungi" OR "macrofungi") was downloaded and subjected to bibliometric analysis based on the keywords using VOSviewer software. The 877 documents were compared with the list of all the fungi reported in Malaysia and compared to the fungal list identified in the current study (Figure 2). The 28S and ITS rDNA sequences of fungal isolates are illustrated in Table S1.

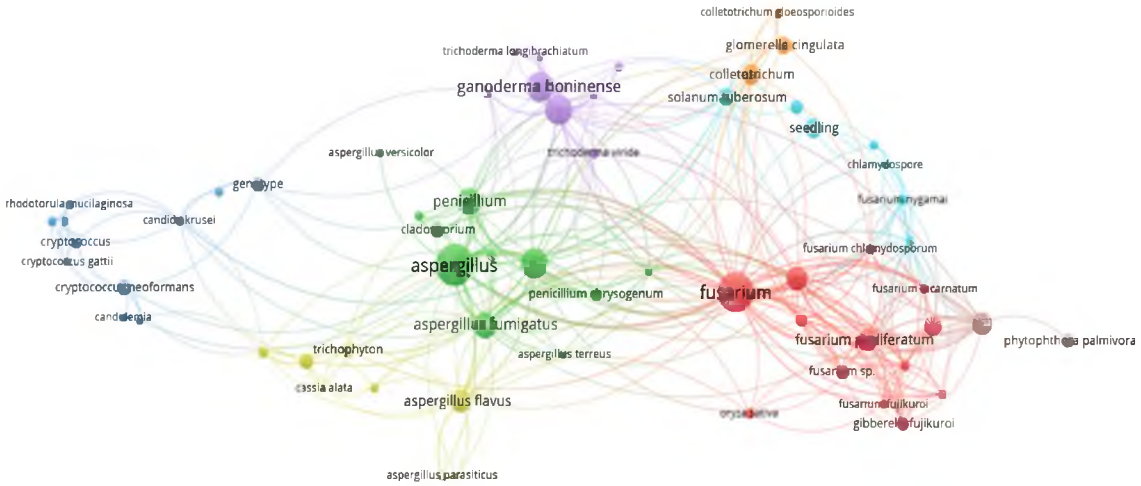

Figure 2. Bibliometric analysis for the fungi reported in Malaysia up to date (20 September 2020).

Each fungal species was searched in the Scopus database as "Malaysia" and fungal species name, for example, *Aspergillus arenarioides*, to confirm whether it has been reported before. It was noted that the fungal strains named *Aspergillus arenarioides* EAN603, *Parengyodontium album* EAN602, *Aspergillus iizukae* EAN605, *Paraconiothyrium brasiliense* EAN202, *Purpureocillium lilacinum* EAN601, and *Penicillium pedernalense* EAN604 were new strains, and this is the first report about them in Malaysia. According to the Scopus database record, the new fungal strains have very few studies; for example, it was noted that *A. arenarioides* has only been reported in two studies conducted in USA and Brazil [34,35], while *Penicillium pedernalense* has only been reported in one study conducted in Spain [36].

3.2. Culture Morphology of the Fungal Isolates from Peat Soil

3.2.1. *Aspergillus* spp.

The fungal strains were described on nine different culture media, including PDA, V8, MEA, CYA, CZ, SDA (4%), SDA, EVA, and DCZ. Three fungal strains were identified as species of *Aspergillus* and deposited in NCBI as *Aspergillus iizukae* EAN605 (MK518343), *Aspergillus aculeatus* EAN506 (MK518351), and *Aspergillus arenarioides* EAN603 (MK518366) (Figure 3). These strains exhibited differences in form, texture, colour, margin, growth

pattern, elevation, and colony size (Figure 3). The colonies of *A. aculeatus* were black with a diameter ranging from 29 ± 7.4 mm in SDA to 80 mm on V8A and CZ medium; the fungus grown well at 37 °C (the colonies ranged from 40 ± 3.9 mm on CYA to 25 ± 6.6 mm on SDA) (Table S1). In comparison, *A. arenarioides* were grow in light brown and white orchards with 17 ± 1.74 mm in CZRB and 45 ± 6.3 mm on MEA, and no growth was detected for this fungal strain at 37 °C (Figure 4). *A. aculeatus* produced high sporulation on different culture media compared to *A. arenarioides*. *A. iizukae EAN605* showed rapid growth in a single white wool texture and a brown reversal in different cultural media (Figure 3). Hubka et al. [37] mentioned that *A. iizukae* grows with a red or brownish soluble pigment. The fungus had high variable characteristics in the different culture media. The colonies of *A. iizukae* ranged from 23 ± 1.8 on DCZ to 33 ± 5.2 mm on SDA, considered the best for fungal growth. The fungus exhibited a weak growth at 37 °C, ranging from 10 ± 1.4 on CYA to 18 ± 0.3 on SDA (Table S1). Similar findings were also reported by Hubka et al. [37], where the colonies' diameters ranged from 16 to 32 mm on CYA at 25 °C to 18–21 mm at 37 °C. On EVA medium, *A. aculeatus* EAN506 exhibited similar growth to that noted on V8A and CZ with a colony size of 80 ± 0.0 mm. However, the colonies were black on EVA while brown on the V8A. *Aspergillus arenarioides* EAN603 had a weak growth (19 ± 1.4 mm) on EVA medium compared to MEA (21 ± 0.4 mm), but similar colonial morphology was noted on both media. *A. aculeatus* exhibited very high sporulation in SDA and EVA media; at 37 °C, the fungus colonies were 40 ± 3.9 mm on CYA and 25 ± 6.6 mm on SDA (Table S1). In comparison, *A. arenarioides* has low sporulation on CZ, CYA, PDA, EVA, and SDA media, while no sporulation was recorded on MEA and V8A media. No growth of the fungus was observed at 37 °C.

3.2.2. *Penicillium* spp.

Four fungal strains were classified within *Penicillium* spp., including *P verruculosum* EAN203 (MK518350), *P. crustosum* EFAQ406 (MK530088), *P. pedernalense* EAN604 (MK518385), and *P. crustosum* EFAQ405 (MK530708). It was observed that the strains EFAQ405 and EFAQ406 belonged to *P. crustosum*. However, the strains exhibited little difference in their culture characteristics (colony morphology and diameter) (Figure 4). The colonies of *P. crustosum* EFAQ405 ranged from 15 ± 1.9 on SDA (4% sucrose) to 25 ± 2.5 mm on PDA. In contrast, *P. crustosum* EFAQ406 was between 16 ± 0.6 on SDA (4% sucrose) and 30 ± 8.3 mm in V8A. In EVA medium, the average number of colonies of *P. crustosum* EFAQ405 was 23 ± 1.8 mm, while it was 12 ± 3.1 mm for *P. crustosum* EFAQ406. *P. verruculosum* EAN203 grows with black to white colonies and pink edges, while *P. pedernalense* EAN604 is a dark green colony with white edges (Figure 3). In EVA medium, the *P verruculosum* EAN203 colonies had a gelatine-like colour with an average size of 28 ± 2.8 mm, while *P. pedernalense* EAN604 was dark green in EVA medium with a size of 30 ± 1.8 mm. Among the *Penicillium* spp., only *P. verruculosum* EAN203 exhibited at 37 °C with a colony size of 17 ± 0.8 mm on CYA and 15 ± 0.6 mm on SDA medium.

3.2.3. Other Fungal Isolates

Trichoderma viride 102UTHM (MK518057) and *Trichoderma asperellum* 303UTHM (MK518056) (Figure 3) exhibited similar cultural characteristics. Both strains exhibited high growth (the colony size was 80 ± 0.0 mm on all media). The *Paraconiothyrium brasiliense* EAN202 strain grew well in all culture media and exhibited high differences in the colony's morphologies and sizes (Figure 5). It was noted that the strain was grown with a white colony on PDA (the diameter was 21 ± 2.2 mm) and yellow colonies on V8A (the diameter was 39 ± 4.9 mm) and that the strain exhibited good growth on the EVA medium (the colony size was 18 ± 1.4 mm). No fungal growth was observed at 37 °C.

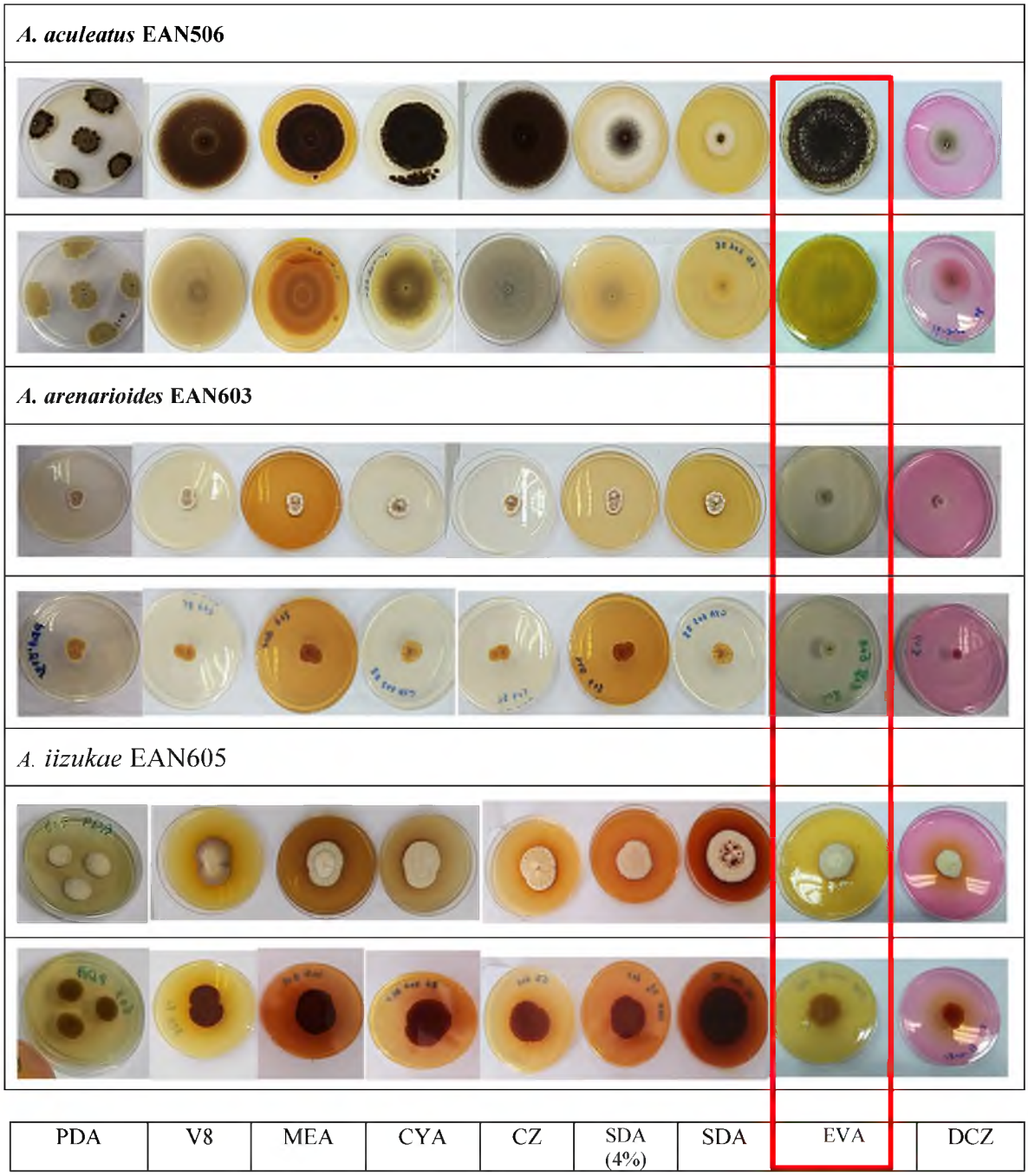

Figure 3. *Aspergillus iizukae* EAN605, *Aspergillus arenarioides* EAN603, and *Aspergillus aculeatus* EAN506, obtained from peat soil cultured on different culture media (at 28 °C for 7 days).

Cochliobolus geniculatus EAN403 showed the highest growth on EVA, MEA, CYA, CZ and SDA medium (the colony size was 18 ± 1.4 mm) (Figure 5). On EVA medium, colonies appeared green in colour, while they were brownish on V8A. On average, the growth at 37 °C was 17 mm on both CYA and SDA. The *Fusarium solani* 504EFAQ strain has white colonies in all culture media, with sizes ranging from 56 ± 5.9 mm on SDA to 71 ± 7.4 mm on V8A (Figure 6). On EVA, the colony size was 68 ± 2.7 mm. The fungi exhibited weak growth at 37 °C with colony sizes of 5 ± 0.6 mm on CYA and 8 ± 0.6 mm on SDA. The fungus exhibited high sporulation of the different culture medium. The *Purpureocillium lilacinum* EAN601 strain had a similar colony morphology in the different culture media but with different colony sizes (Figure 6). The highest growth was observed in V8A (36 ± 7.8 mm), while the lowest growth appeared on DCZ (9 ± 0.65 mm), without

growth recorded at 37 °C. The *Parengyodontium album* EAN602 strain has a weak and slow growth on all culture media, with the colony sizes ranging from 9 ± 0.7 mm on SDA (4% sucrose) to 39 ± 4.9 mm on V8A medium.

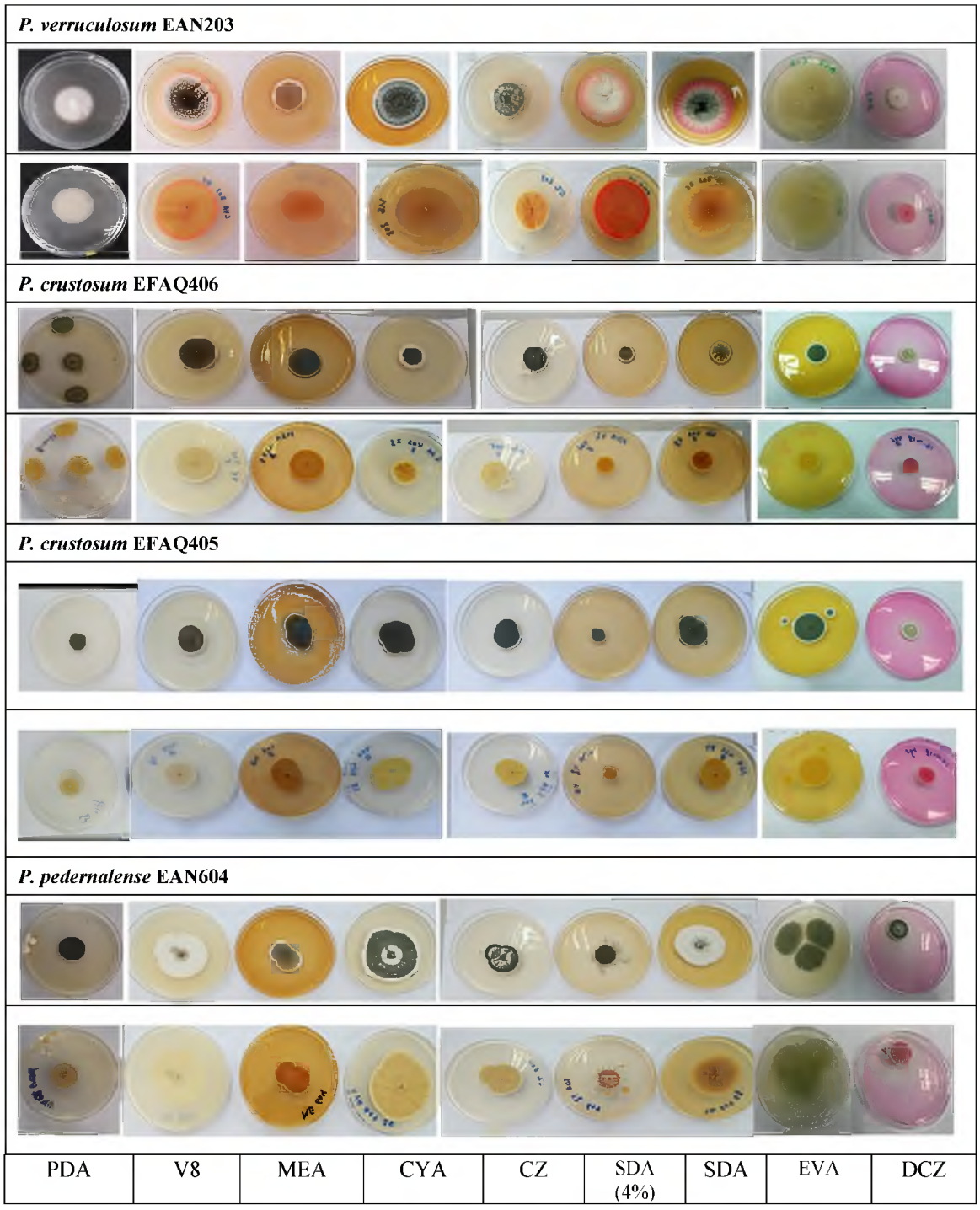

Figure 4. Culture morphology of *Penicillium crustosum* EFAQ406, *Penicillium verruculosum* EAN203, *Penicillium pedernalense* EAN604, and *Penicillium crustosum* EFAQ405, obtained from peat soil cultured on different culture media (at 28 °C for 7 days).

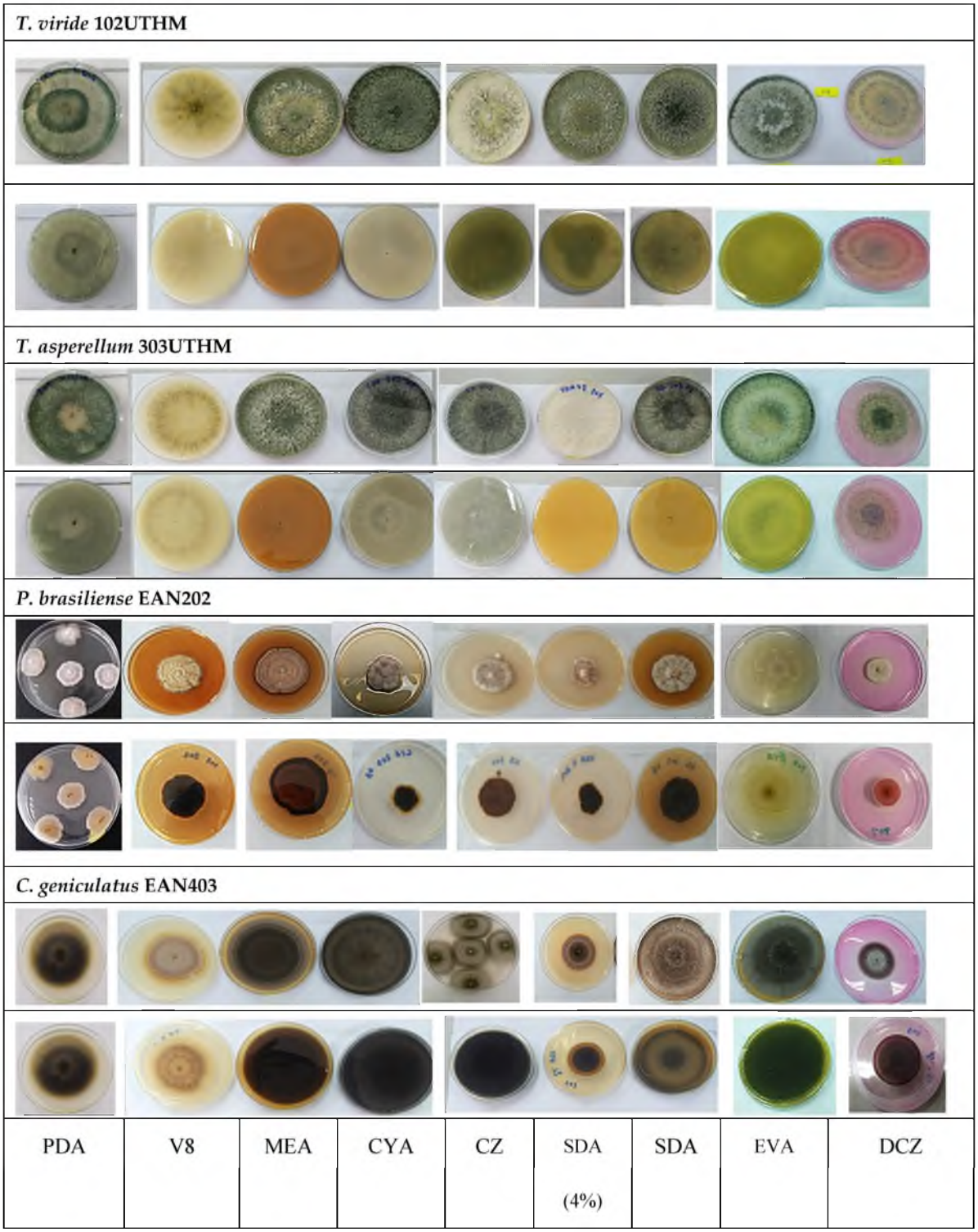

Figure 5. Culture morphology of *Trichoderma asperellum* 303UTHM, *Trichoderma viride* 102UTHM *Cochliobolus geniculatus* EAN403, and *Paraconiothyrium brasiliense* EAN202, obtained from peat soil cultured on different culture media (at 28 °C for 7 days).

The results of this study show that the PDA and V8A media support the growth of a variety of fungi; both are standard means of isolating fungi. These findings agree with those reported by Saha et al. [38]. However, V8A has been shown to be necessary to isolate fungi with complex growth and spore growth requirements [39].

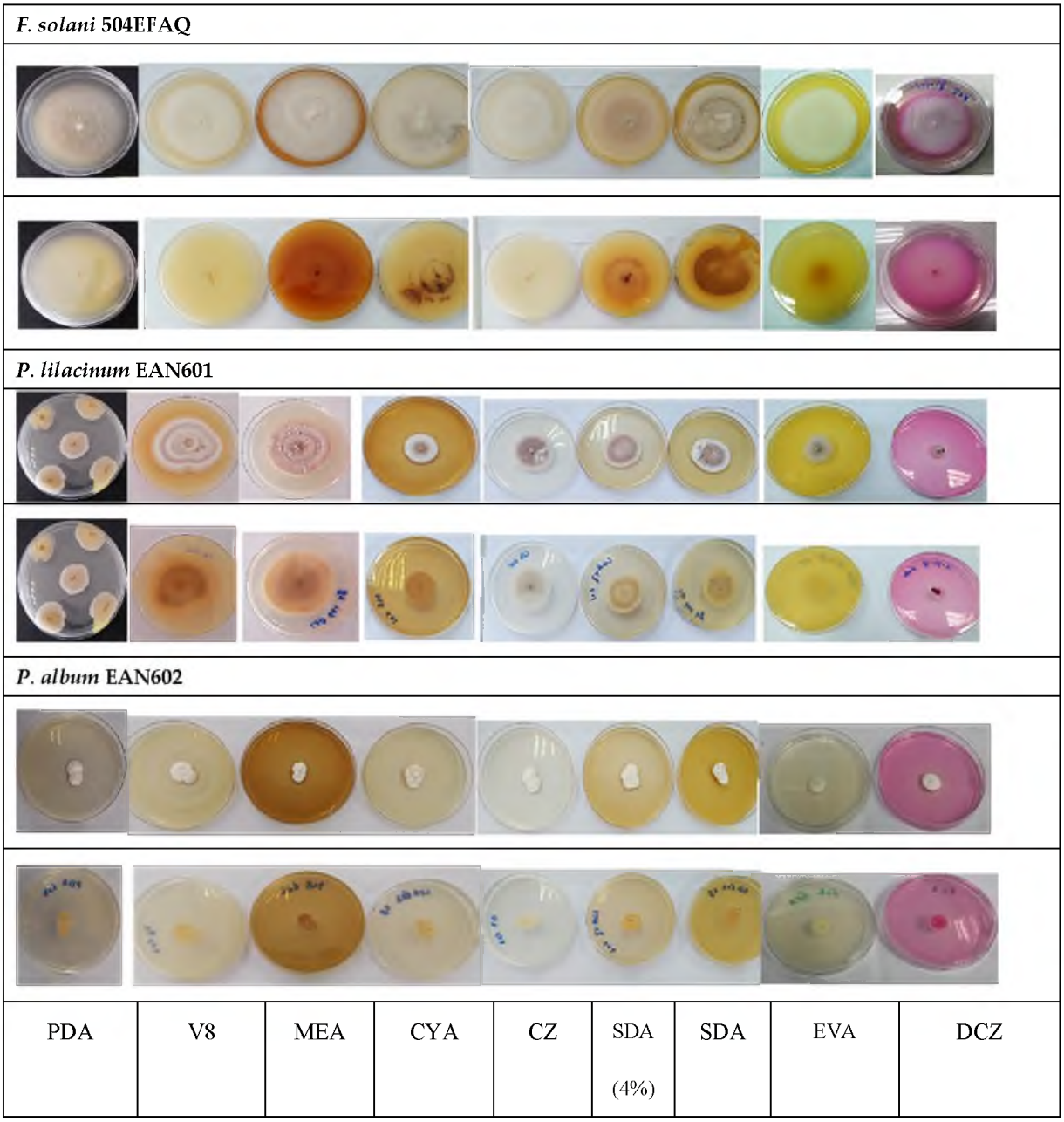

Figure 6. Culture morphology of *Purpureocillium lilacinum* EAN601, *Fusarium solani* 504EFAQ, *Schizophyllum commune* 104UTHM, and *Parengyodontium album* EAN602, obtained from peat soil cultured on different culture media (at 28 °C for 7 days).

3.3. Microscopic Morphology of the Fungal Isolates from Peat Soil

Scanning electron micrographs of *A. aculeatus* reveal that this fungal strain has a long and smooth conidiophore with a round vein, a radiating head, and a biseriate phialid (Figure 7A). The spores are globular and ornamented with echinulate spiny texture (Figure 4), and the spore size ranges from 1.45 to 4.02 µm (Table S1, Figure S2). Moreover, *A. arenarioides* has a long, smooth conidiophore, a small column head, and a biseriate phialid (Figure 7B). The spores are globular and subglobular, and the texture of the spore is smooth/finely ripe (Figure 7B1). The spore size was between 1.6 and 4.3 µm (Table S1). *A. iizukae* EAN605 showed a spiny and long conidiophore with globose or pyriform vesicle, radiate head, and biseriate phialids (Figure 4). Furthermore, the fungal spores were globular in shape/smooth of texture. The spore size ranged from 2.6 to 4.1 µm (Figures 7 and S2, Table S1). A similar observation was also recognised in the previous identification of this

fungal strain in the Czech Republic [37]. The phylogenetic analysis of *A. iizukae* EAN605 recognised the strain with *A. flavipes*, *A. neo flavipes*, and *A. spelaeus*. According to Peterson [40], the analysis of large subunit rDNA sequences indicated that *A. iizukae* belongs to the *Aspergillus* section Flavipedes. Sect. Flavipedes members are distributed in subtropical and tropical soils [4,41]. Similarly, Varga and colleagues [42] found similar tree topology in the analysis of ITS rDNA. *A. iizukae*, *A. frequent*, and *A. mangaliensis* are species distributed worldwide, with sequence data deposited at GenBank. It has been isolated as herb and tree endophytes [43–46].

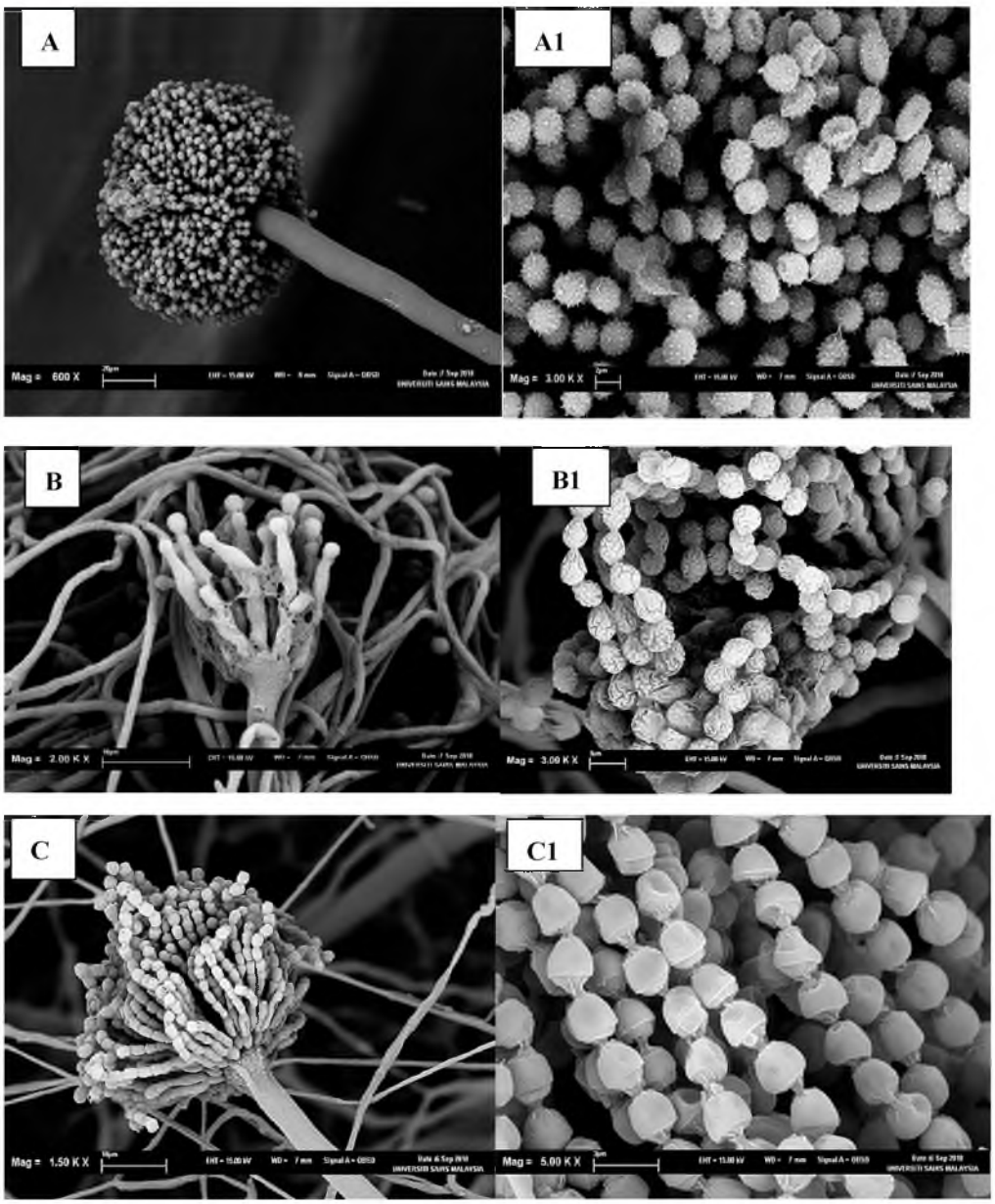

Figure 7. SEM images of fungi; (**A**) *A. aculeatus* EAN506 conidiophore (600×); (**A1**) spores (3000×); (**B**) *A. arenarioides* EAN603 conidiophore (2000×); (**B1**) spores (3000×); (**C**) *A. iizukae* EAN605 conidiophore (1500×); (**C1**) spores (500×).

Aspergillus iizukae Sugiy. 1967, J Fac Sci Univ Tokyo, Sect 3, Bot: 390; FIGS. 3, 7, Typification. JAPAN. GYMNA PREFECTURE: Fujioka, ex-soil from stratigraphic drilling core, 26 Jun 1969, J. Sugiyama (holotype TI 0007. Ex-holotype culture NRRL 3750 5 CBS 541.69 5 IMI 141, 552 5 CCF 4548).

Aspergillus iizukae is distributed worldwide in soil, including in the Czech Republic, Germany, Japan, and Romania, as well as in the USA [37]. However, it has not been reported in Malaysia. Moreover, very few studies have been conducted on *A. iizukae* based on the Scopus database search. The studies included the application of the fungus as potential antiviral xanthones [47], biological control agents of *Duponchelia fovealis* [48], Antioxidant aromatic butenolides [49], a source for diphenyl derivatives [50], and bioactive secondary metabolites [43,51].

P. crustosum 405EFAQ conidiophore appeared with a smooth surface, while *P. crustosum* 406EFAQ had a spiny conidiophore (Figure 8 and Figure S2). Furthermore, the spore size of the EFAQ405 strain ranged from 3.05 to 5.54 µm, while it ranged from 2.47 to 4.29 µm for the EFAQ406 strain. The *P. verruculosum* EAN203 and *P. pedernalense* EAN604 strains have a similar conidiophore structure and length, as well as the texture of the spore (Figures 9 and S4). However, the 28S rRNA sequences revealed that the strains belonged to different fungal species. The spores' sizes ranged from 1.65–3.25 µm for *P. verruculosum* EAN203 to 1.66–3.33 µm for *P. pedernalense* EAN604.

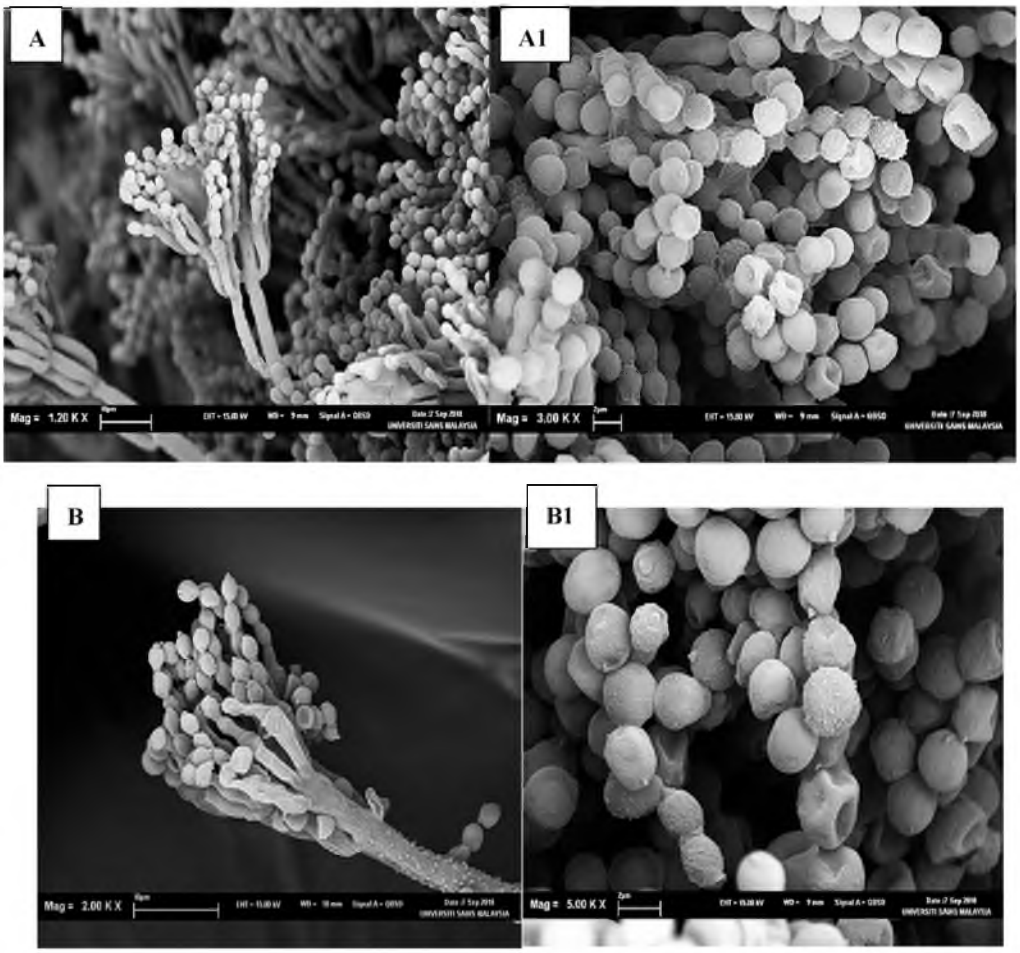

Figure 8. SEM images of fungi; (**A**) *P. crustosum* 405EFAQ conidiophore (1200×); (**A1**) spores (3000×); (**B**) *P. crustosum* 406EFAQ conidiophore (2000×); (**B1**) spores (5000×).

Trichoderma viride 102UTHM (MK518057) (Figure 10A and Figure S5) and *Trichoderma asperellum* 303UTHM (MK518056) (Figure 7B) also exhibited similar cultural characteristics. However, the main differences were shown in the spore texture, as determined by SEM analysis. The spore size of *Trichoderma viride* 102UTHM was between 2.0 and 4.4 µm, while *T. asperellum* 303UTHM had a spore size between 2.2 and 3.8 µm. *P. album* EAN602 strain (Figure 11A) has no spores, while *P. brasiliense* EAN202 (Figure 11B) exhibited very low sporulation on V8A, MEA, and SDA. The microstructures of both fungi have no clear

conidiophore and spores. In contrast, *C. geniculatus* had very high sporulation on all media, with a clear microstructure (Figure 11). *F. solani* EFAQ504 ranged from 7.95 to 26.17 µm, while *P. lilacinum* EAN601 has a spore size between 1.65 and 2.01 µm, and both fungal strains have long and smooth conidiophores (Figures 12A,B and S7).

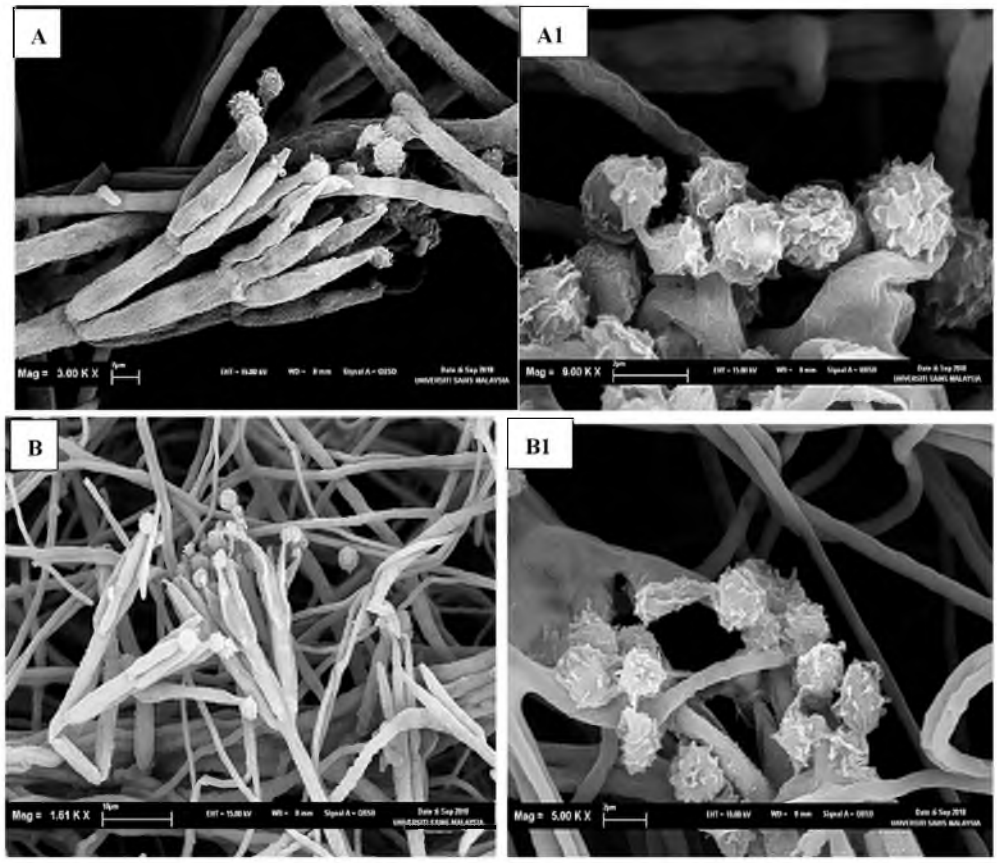

Figure 9. SEM images of fungi; (**A**) *P. verruculosum* EAN203 conidiophore (3000×); (**A1**) spores (9000×); (**B**) *P. pedernalense* EAN604 conidiophore (1610×); (**B1**) spores (5000×).

S. commune 104UTHM, having a hyaline, smooth, septate, and branched hyphae of two widths, included wider hyphae and narrower width hyphae, with clamp connections and spicules (Figure 13). Won et al. [52] and Chowdhary et al. [53] reported similar findings. *S. commune* 104UTHM exhibited dikaryotic (binucleate condition) and monokaryotic stages in the life cycle. Moreover, the microscopic morphology of *S. commune* 104UTHM shows binucleate in the hyphae as well as spicules and clamp connections on hyphae.

The microstructure characteristics, cultural characteristics, and molecular analysis are useful for identifying the fungal strains. However, Hubka et al. [37] reported that the significant variability in the shape and dimensions among the same *Aspergillus* spp. make these structures unsuitable for the identification process. The length, texture, shape, and orientation were also helpful in identifying the fungal strains. However, the conidiophores' lengths vary greatly. Many fungal species are longer than 1mm, so their characteristics are inaccurate and do not help with species identification [33]. Therefore, culture and morphological characteristics are insufficient to accurately fungal identification. Molecular analysis based on the D1/D2 of the 28S rRNA sequence was used to confirm the identification of fungi. Many studies have stated that sequencing of the D1/D2 region is the recognised gold standard for fungal identification [54]. However, the morphological findings and molecular data were compared to ensure consistency and identification of the fungus strains in the current work.

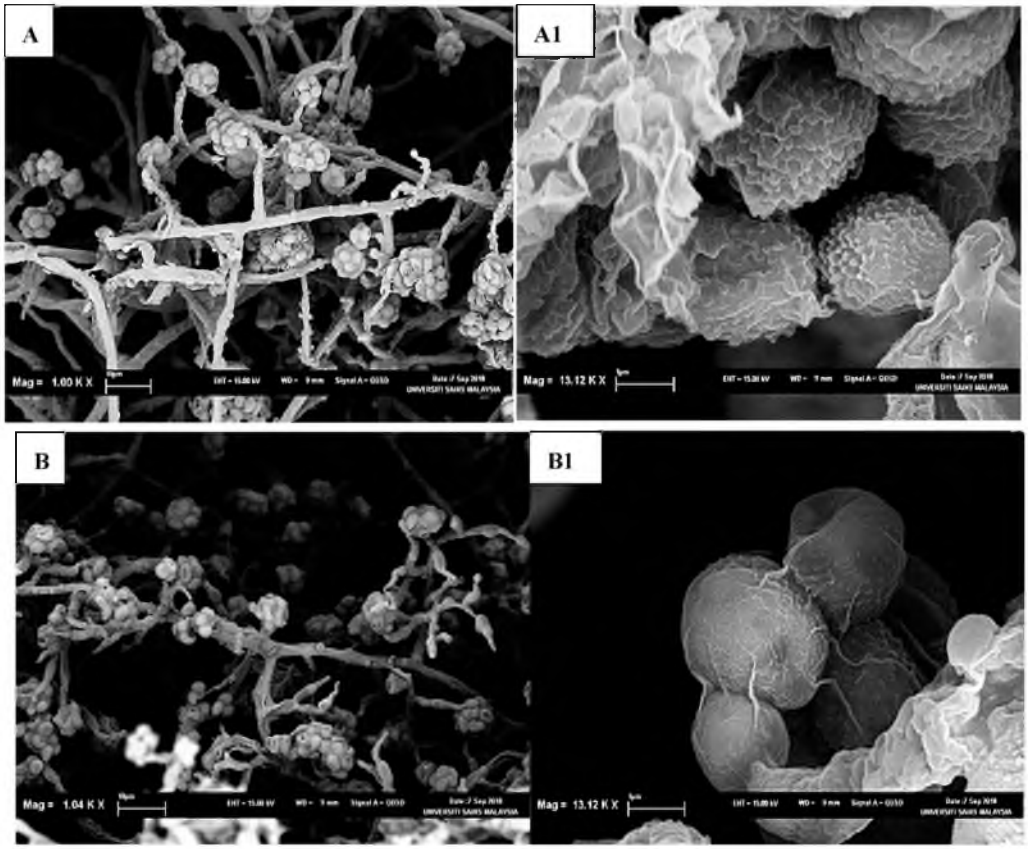

Figure 10. SEM images of fungi: (**A**) *T. viride* 102UTHM (1000×); (**A1**) spores (13,120×); (**B**) *T. asperellum* 303UTHM (1000×); (**B1**) spores (13,120×).

3.4. Molecular Characteristics of Fungal Isolates from Peat Soil

In comparison between the molecular analysis and morphological characteristics (culture and microstructure), it was noted that the morphological characteristics accomplished the identification process of the fungal isolates alongside the molecular analysis. The identification of the current fungal isolates based on ITS and 28S rRNA was not sufficient to identify and establish the fungal strains, while the morphological characteristics provided the details which helped to determine the fungal strains. For examples, *P. crustosum* (405EFAQ and 406EFAQ) shares the highest homology with *P. ulaiense*, *P. crustosum*, *P. resticulosum*, *P. granulatum*, *P. solitum*, *P. caseifulvum*, and *P. italicum* (Figure 14), while the morphological characteristics supported the identification of the fungal species as *P. crustosum*, according to Sang et al. [55]. However, the conidiophore of *P. crustosum* (405EFAQ) occurred with a smooth surface and size between 3.05 to 5.54 µm, while *P. crustosum* 406EFAQ has a spiny conidiophore, and size between 2.47 and 4.29 µm. Therefore, we considered that these isolates belong to same fungal species but different strains. *A. iizukae* EAN605 shows the highest homology with *A. flavipes* and *A. neo flavipes* (Figure 11). However, the culture morphology and microstructure were more similar to that reported by Hubka et al. [37]; therefore, it was recognized as *A. iizukae*. Further, *A. arenarioides* EAN603 shows the highest homology with *A. petersonii* (Figure 14); however, with the morphological characteristics and microstructure of the conidiospores and spores the fungal strain was more related to *A. arenarioides* EAN603, similar to the descriptions of Visagie et al. [56], since *A. petersonii has high spiny* conidiospores with a different ornament spore surface [34]. *A. aculeatus* EAN506 shows the highest homology with *A. violaceofuscus*, *A. niger*, *A. fijiensis*, *A. brunneoviolaceus*, and *A. japonicus* (Figure 14). However, based on the culture morphology and microstructure properties and compared to previous studies which described the fungal species [57,58], the fungal strain was identified as *A. aculeatus* EAN506. *P. pedernalense* EAN604 shows the highest homology with *P. mariae-crucis*, *P. brasilianum*, *P. simplicissimum*, *P. pulvillorum*,

and *T. biappendiculata* (Figure 11) However, *T. biappendiculata* was totally excluded since it has different spore morphology [59]; *P. brasilianum* was excluded based on the culture morphology reported by Freire et al. [60]; while *P. pulvillorum* was excluded based on the spore's morphology as reported by Mansouri et al. [61]. Together with the morphological characteristics in comparison with Laich and Andrade [36], the fungal strain was recognized as *P. pedernalense* EAN604. *P. verruculosum* EAN203 grew with black to white colonies and pink edges, while *P. pedernalense* EAN604 occurred as a dark green colony with a white edge. Both strains have a similar conidiophore structure and length as well as the spore texture. However, the 28S rRNA sequences revealed that the strains belonged to different fungal species. *P. verruculosum* EAN203 exhibited the highest homology with *P. flavus*, *P. marneffei*, *T. aculeatus*, T. *verruculosus*, *T. angelicus*, and *T. flavus* (Figure 14). The excluding of *T. flavus* was based on the morphology described by Dethoup et al. [62]; *T. aculeatus* and *T. verruculosus* were excluded according to the description by Yilmaz et al. [63]; *P. marneffei* was excluded based on the description by [64]; and *T. angelicus* was excluded according to the description by Sang et al. [65]; according to Shah et al., the fungal strains were closer to *P. verruculosum* EAN203 [66].

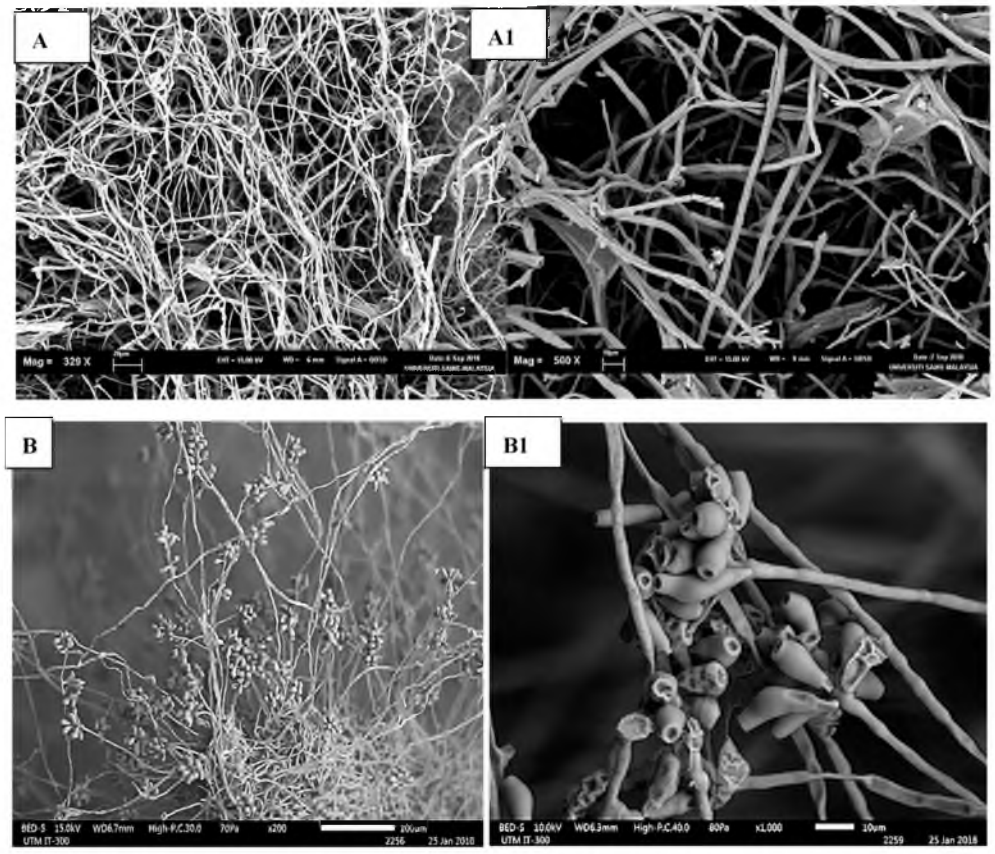

Figure 11. SEM images of fungi: (**A**) *P. album* EAN602 (329×); (**A1**) *P. brasiliense* EAN202 (500×); (**B**) C. *geniculatus* EAN403 (200 ×); (**B1**) spores (1000×).

Trichoderma viride 102UTHM and *Trichoderma asperellum* 303UTHM share the highest homology in the 28S rRNA sequences with *T. viride*, *T. paucispinum*, *T. asperellum*, *T. atroviride*, *T. hamatum*, and *T. pubescens* (Figure 15), as well as the cultural characteristics on the culture media. Together with the morphological identification, we determined the strain 303UTHM to be *T. asperellum*, as described by Wu et al. [67]. However, *T. viride* 102UTHM also exhibited similar culture characteristics. The main differences appeared in the spore texture determined by SEM analysis. *T. viride* 102UTHM spores were between 2.0 and 4.4 µm with strong warted surface, while *T. asperellum* 303UTHM spores were between 2.2 and 3.8 µm, with a less strongly warted surface. These observations were also reported by

Meyer and Plaskowitz [68], who revealed that *T. viride* had warted more strongly, while *T. asperellum* had fewer conidia or weak warted conidia [69,70].

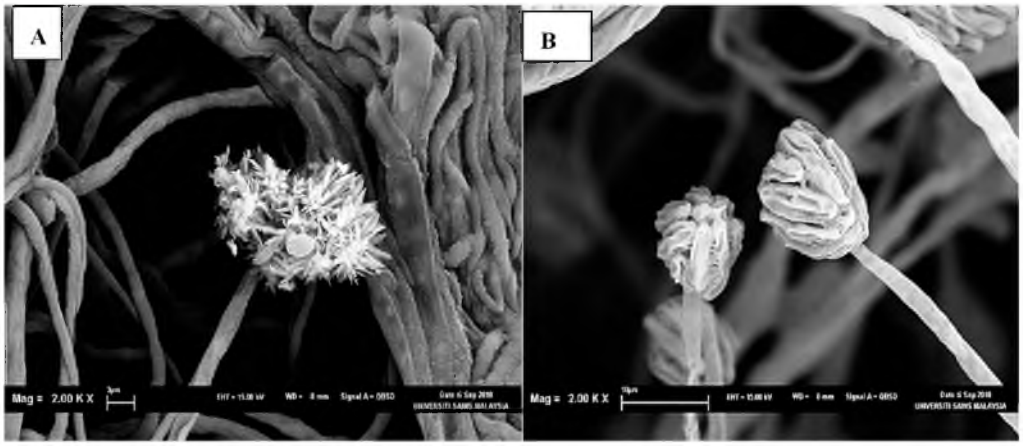

Figure 12. Scanning electron micrographs of fungal strains from peatland: (**A**) *F. solani* EFAQ504 (2000×); (**B**) *P. lilacinum* EAN601 (2000×).

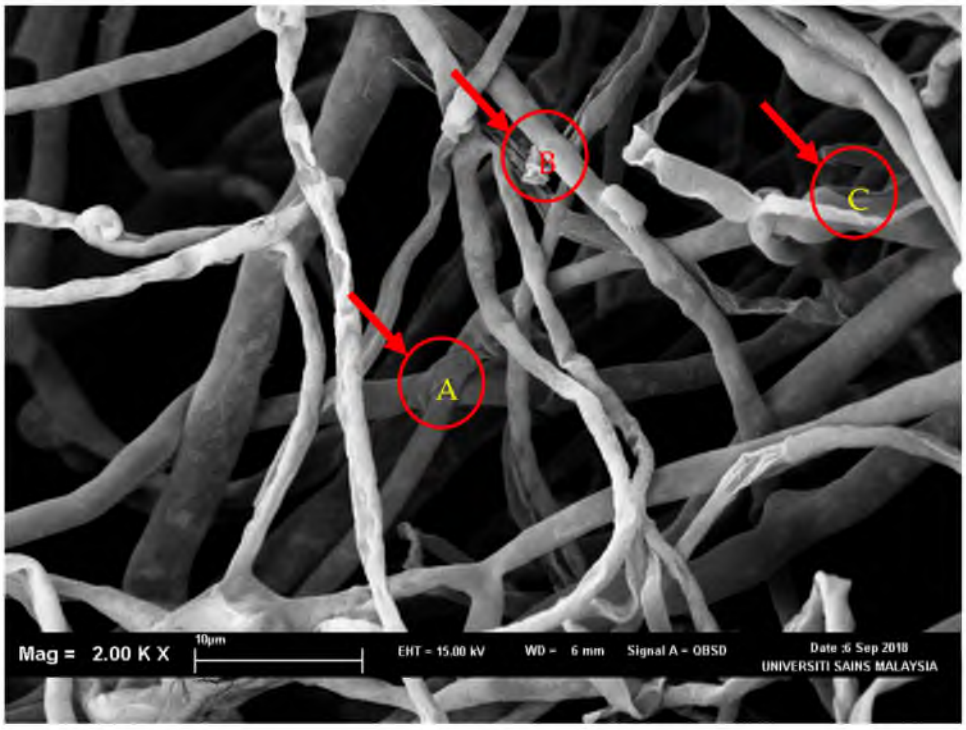

Figure 13. SEM images of *S. commune* 104UTHM (A) a wider hypha; (B) a narrower width hypha; (C) the clamp connections and spicules (2000×).

Parengyodontium album EAN602 shows the highest homology with *Verticillium* sp. (Figure 16). However, according to previous studies for both species [71–73], the fungal isolate is closer to *P. album* EAN602. *P. lilacinum* EAN601 shows the highest homology with *Aschersonia* sp. (Figure 17), which was excluded according to the spore's morphology as reported by Sudiarta et al. [74], and recognized as closer to *P. lilacinum* according to Deng et al. [75] and Perdomo et al. [76].

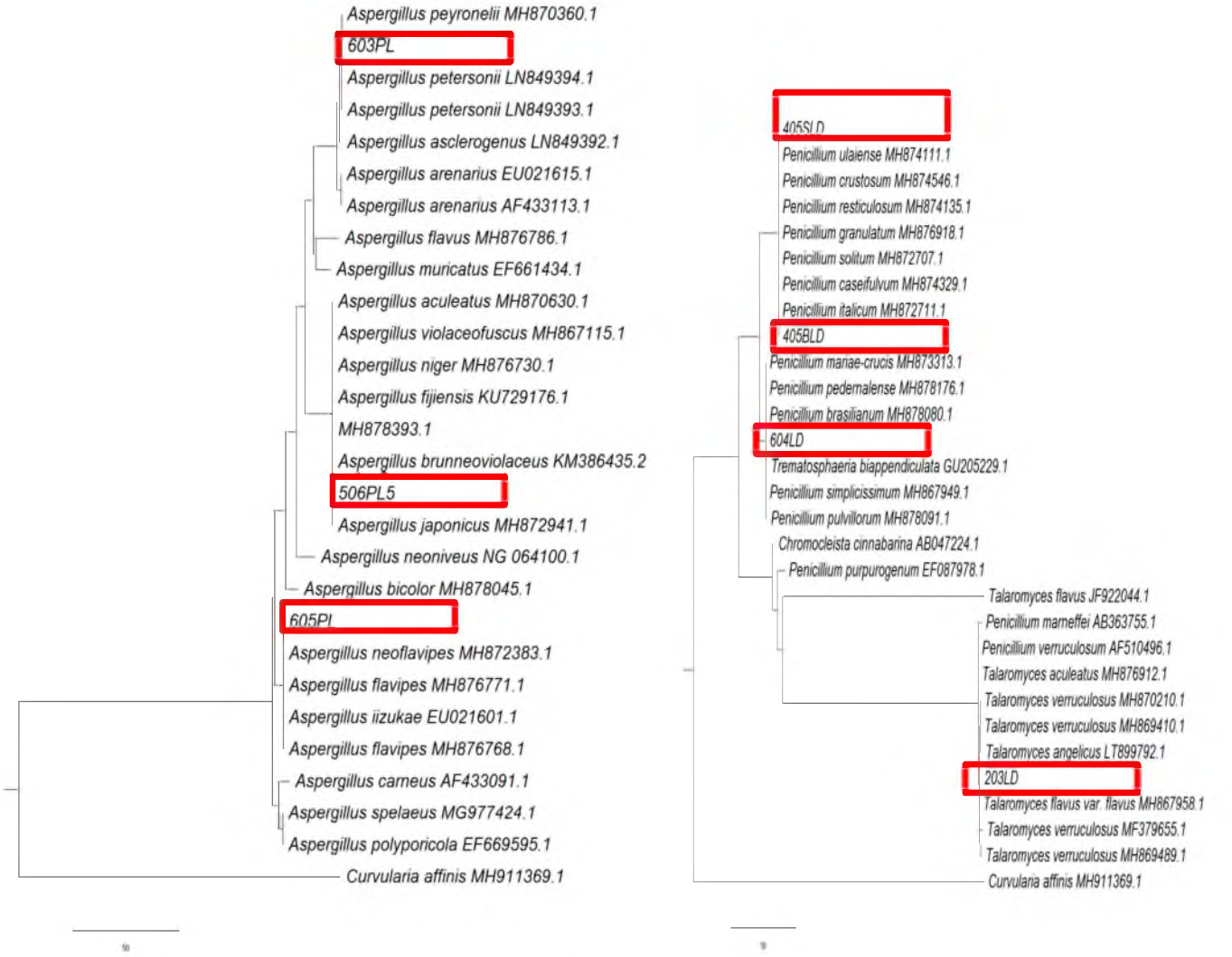

Figure 14. Phylogenetic tree showing the relationship between *Aspergillus* spp. and *Penicillium* spp. and the relevant selected sequences based on 28S rRNA sequence comparisons.

Fungal isolate no, 403UTHM shows the highest homology with *C. geniculatus* and is recognized as *C. geniculatus* 403UTHM, along with the morphological and culture characteristics as described by Kusai et al. [77] (Figure 18). Fungal isolate no. 104UTHM shows the highest homology with *S. commune* and was recognized as *S. commune* 104UTHM according to the description provided by Won et al. [52] and Chowdhary et al. [53] (Figure 19). Fungal isolate EAN202 exhibited the highest homology with *Paraconiothyrium brasiliense* (Figure 20).

It must be mentioned that it was very difficult to identify the isolates affiliated with *Aspergillus* and *Penicillium* into the taxonomical level of species in the absence of more sensitive gene sequences. Therefore, ITS and beta-tublin was used to confirm the fungal strain identification. *A. aculeatus* ITS sequences exhibited a similar homology to that reported using 28S rRNA and was identified as *A. aculeatus* EAN506, as mentioned above, while fungal isolates EFAQ405 and EAN403 ITS sequences exhibited the highest homology with only *P. crustosum* and *C. geniculatus*, respectively (Figure 21). In contrast, beta-tublin exhibited more accuracy and a homology closer to fungal strains such as *Parengyodontium album* EAN602, *Aspergillus arenarioides* EAN603, and *Penicillium pedernalense* EAN604, which facilitated and confirmed the identification process (Figure 22). However, it must be mentioned that the sequences of these strains have not been deposited in NCBI yet. Since, in many samples, the non-specific bands appeared, we used a gel extraction kit to purify

the PCR products. More work is currently being conducted on these species for further studies.

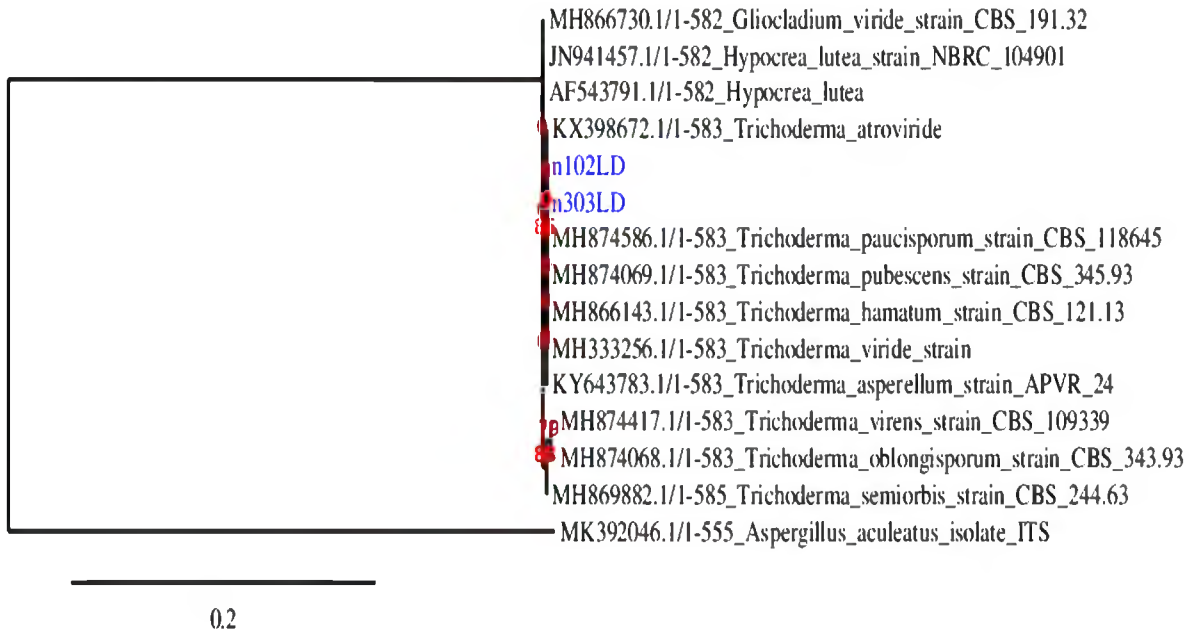

Figure 15. Phylogenetic tree showing the relationship between *Trichoderma* spp. and the relevant selected sequences based on 28S rRNA sequence comparisons.

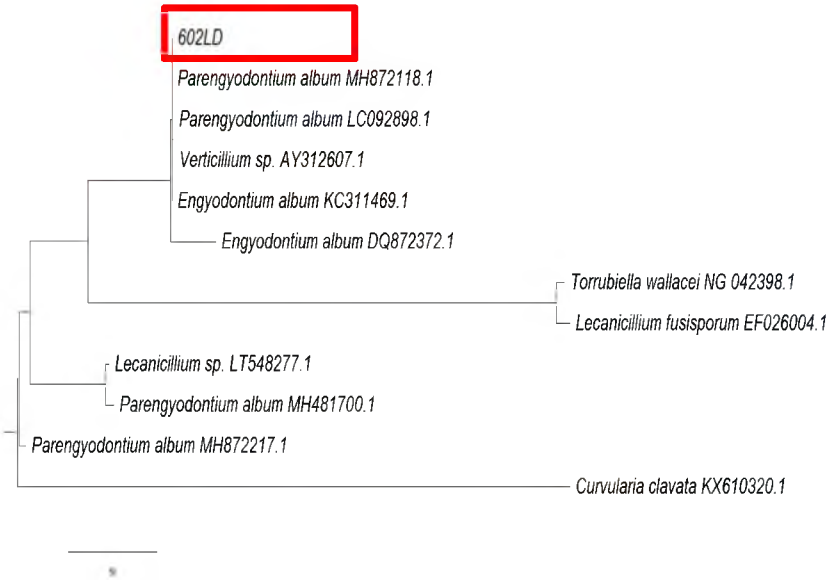

Figure 16. Phylogenetic tree showing the relationship between *Parengyodontium album* EAN602 and the relevant selected sequences based on 28S rRNA sequence comparisons.

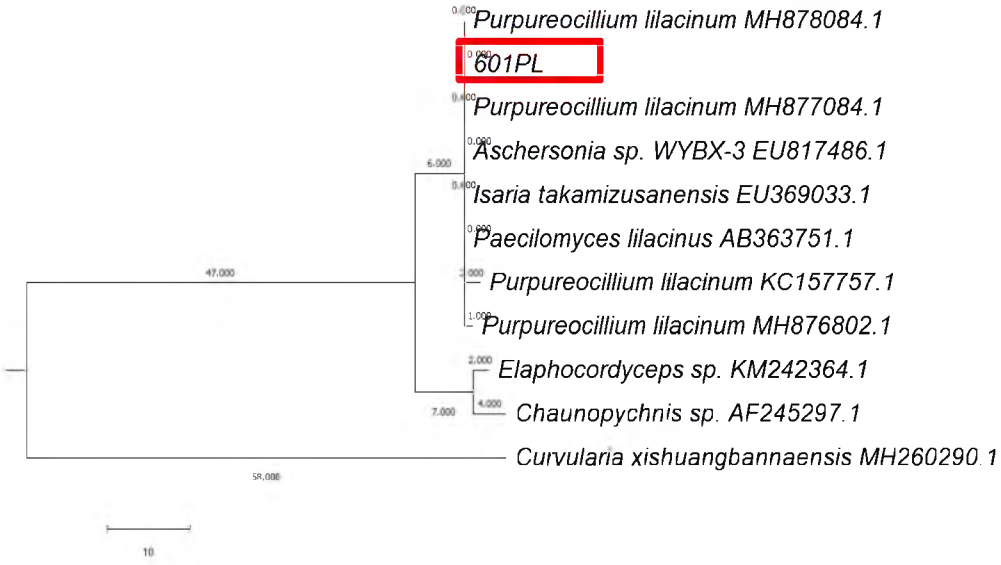

Figure 17. Phylogenetic tree showing the relationship between *Purpureocillium lilacinum* EAN601 and the relevant selected sequences based on 28S rRNA sequence comparisons.

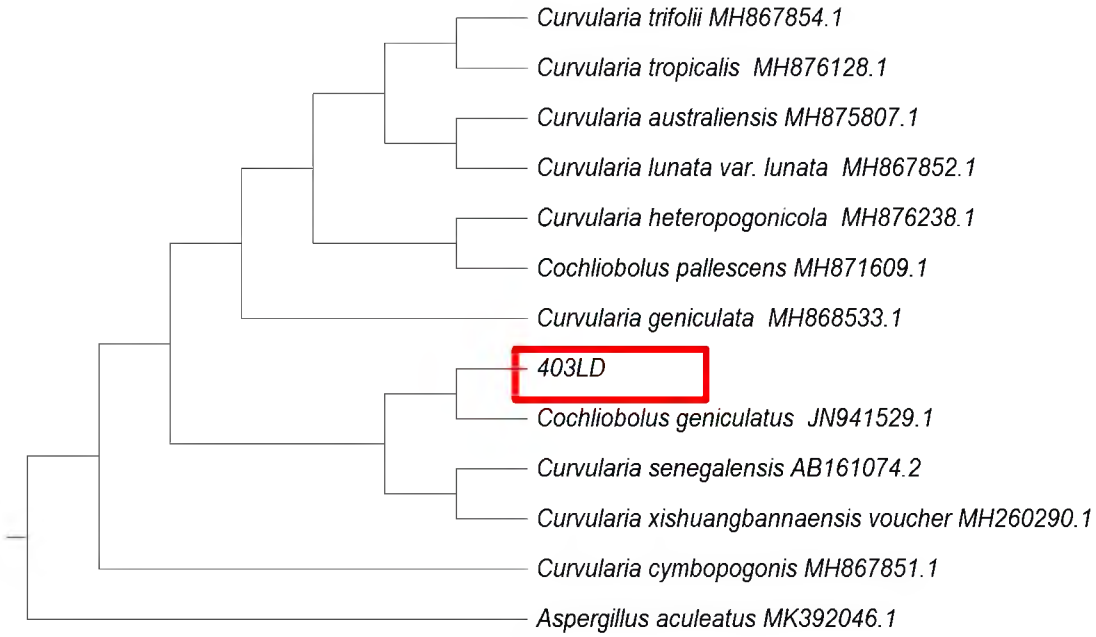

Figure 18. Phylogenetic tree showing the relationship between *Cochliobolus geniculatus* 403UTHM and the relevant selected sequences based on 28S rRNA sequence comparisons.

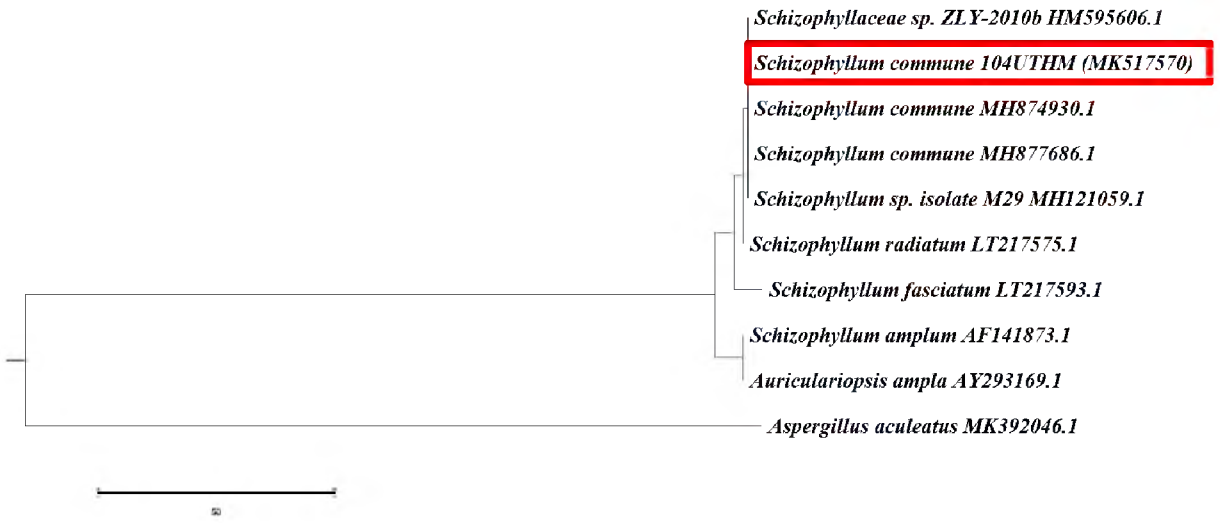

Figure 19. Phylogenetic tree showing the relationship between *Schizophyllum commune* 104UTHM and the relevant selected sequences based on 28S rRNA sequence comparisons.

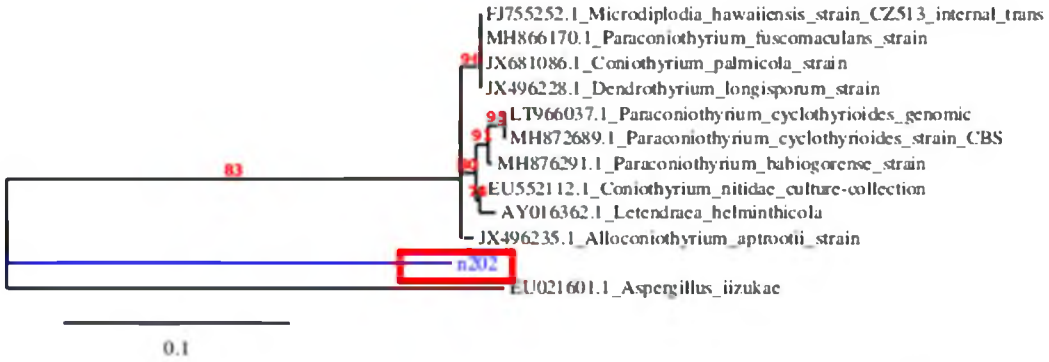

Figure 20. Phylogenetic tree showing the relationship between *Paraconiothyrium* sp. EAN202 and the relevant selected sequences based on 28S rRNA sequence comparisons.

3.5. Chemical Structure of the Fungal Mycelium

To explore the chemical structure of the mycelium cell wall, the mycelium for each fungal strain was analysed using Raman spectroscopy (Figure 23). The results showed that bands between 1300 and 1600 cm^{-1} indicate the presence of CH_2 and CH_3, and lipid deformation occurs on the surface of mycelium. In contrast, peaks from 1000 to 1050 cm^{-1} can be linked to the presence of lipids, fatty acids, and phenylalanine, as indicated by Fazio et al. [78]. The 1600 cm^{-1} peaks indicate an unsaturated fatty acid group attributed to the C=C expansion vibration [79]. These groups might have been contributed during the decolourisation of the dye and might have improved the adsorption of the dye on the surface of the fungal mycelium. *P. pedernalense* EAN604 and *C. geniculatus* EAN403 have a high peak between 2850 and 2950 $cm^{-1,}$ indicating cyclohexane compounds, such as symmetric CH_2 stretching and asymmetric CH_3 stretching, as well as polyethylene, polypropylene, and methine in these fungal strains.

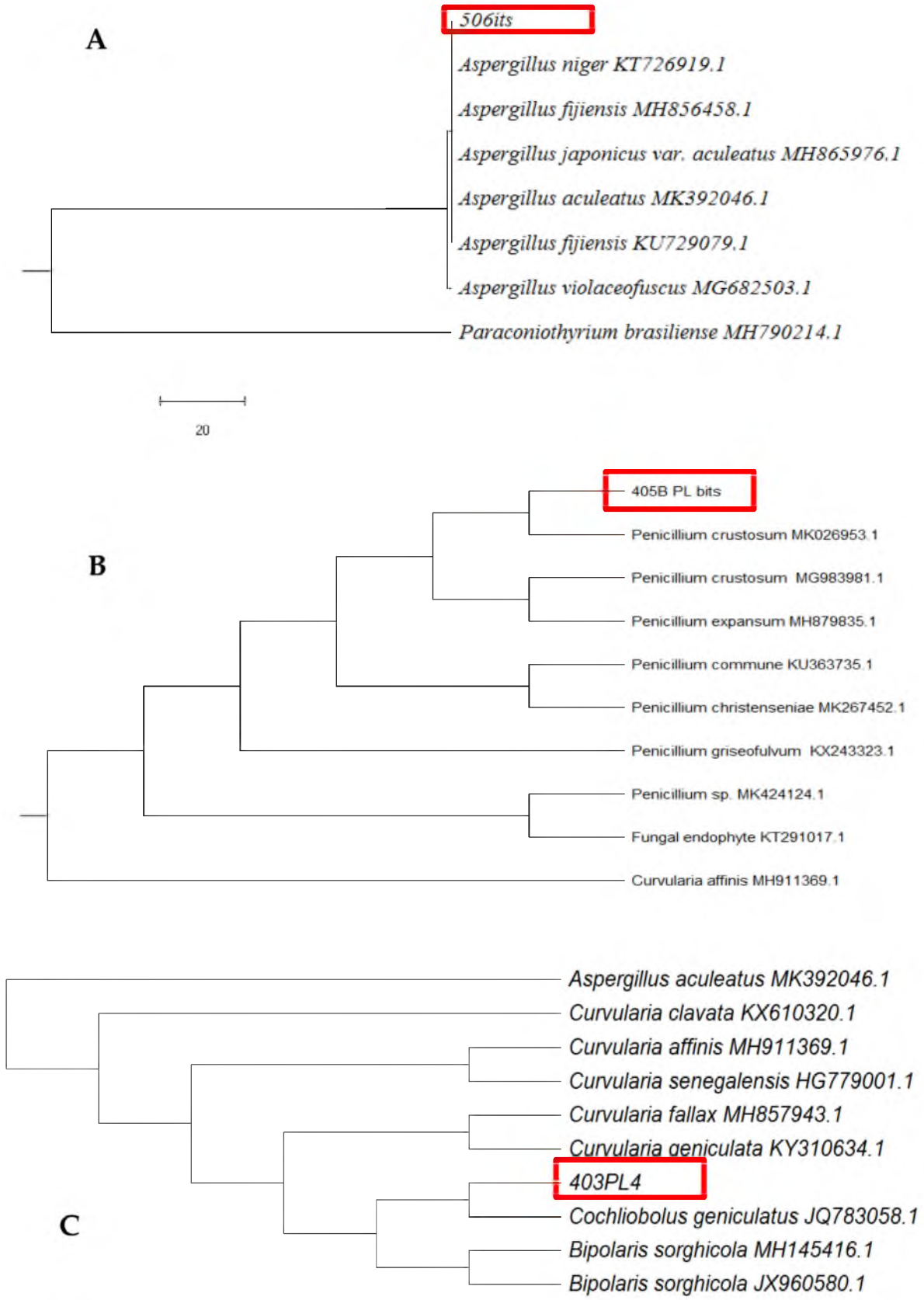

Figure 21. Phylogenetic tree showing the relationship between *Aspergillus aculeatus* EAN506 (**A**), *Penicillium crustosum* EFAQ405 (**B**), *Cochliobolus geniculatus* EAN403 (**C**), and the relevant selected sequences based on ITS sequence comparisons.

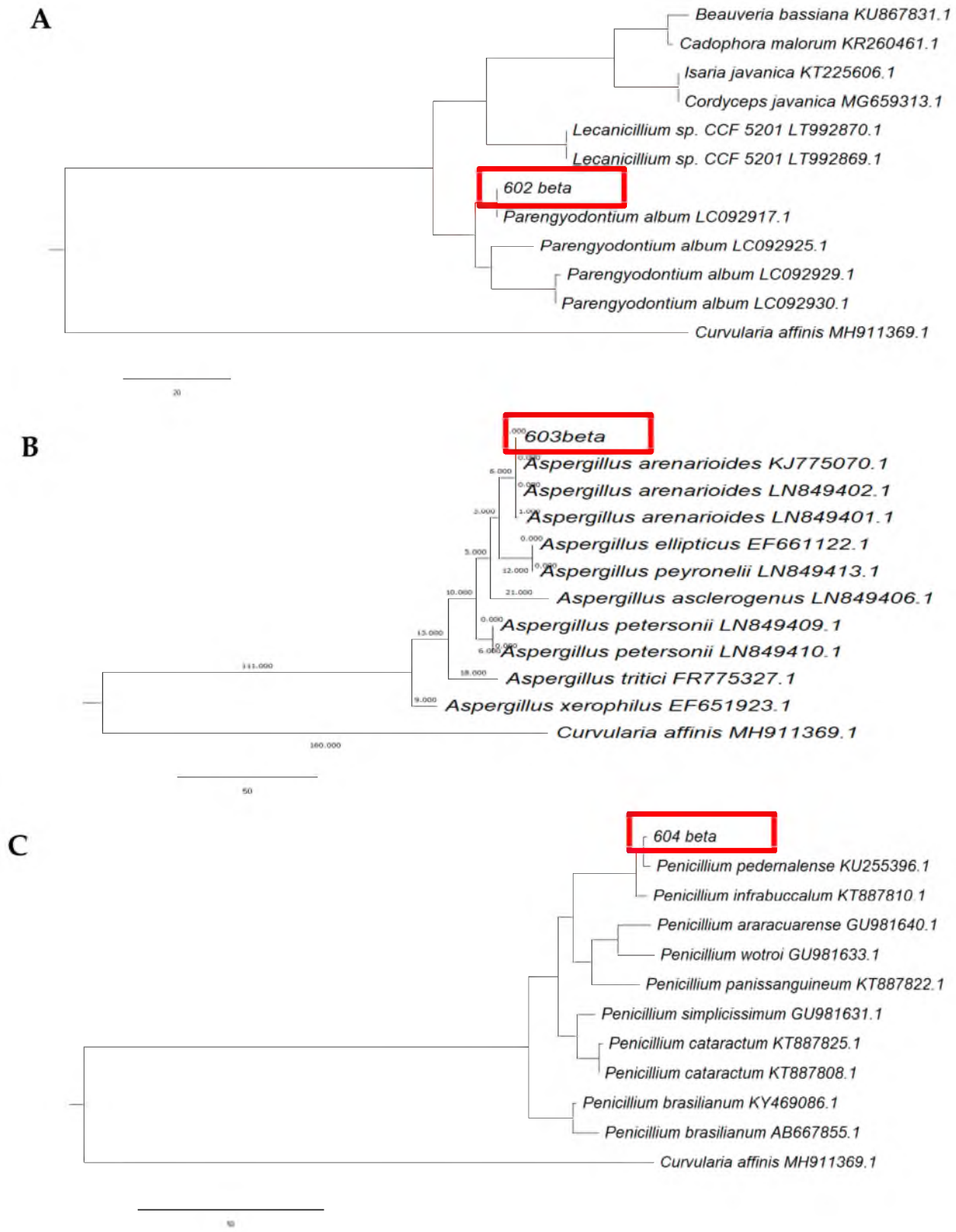

Figure 22. Phylogenetic tree showing the relationship between *Parengyodontium album* EAN602 (**A**), *Aspergillus arenarioides* EAN603 (**B**), and *Penicillium pedernalense* EAN604 (**C**), and the relevant selected sequences based on ITS sequence comparisons.

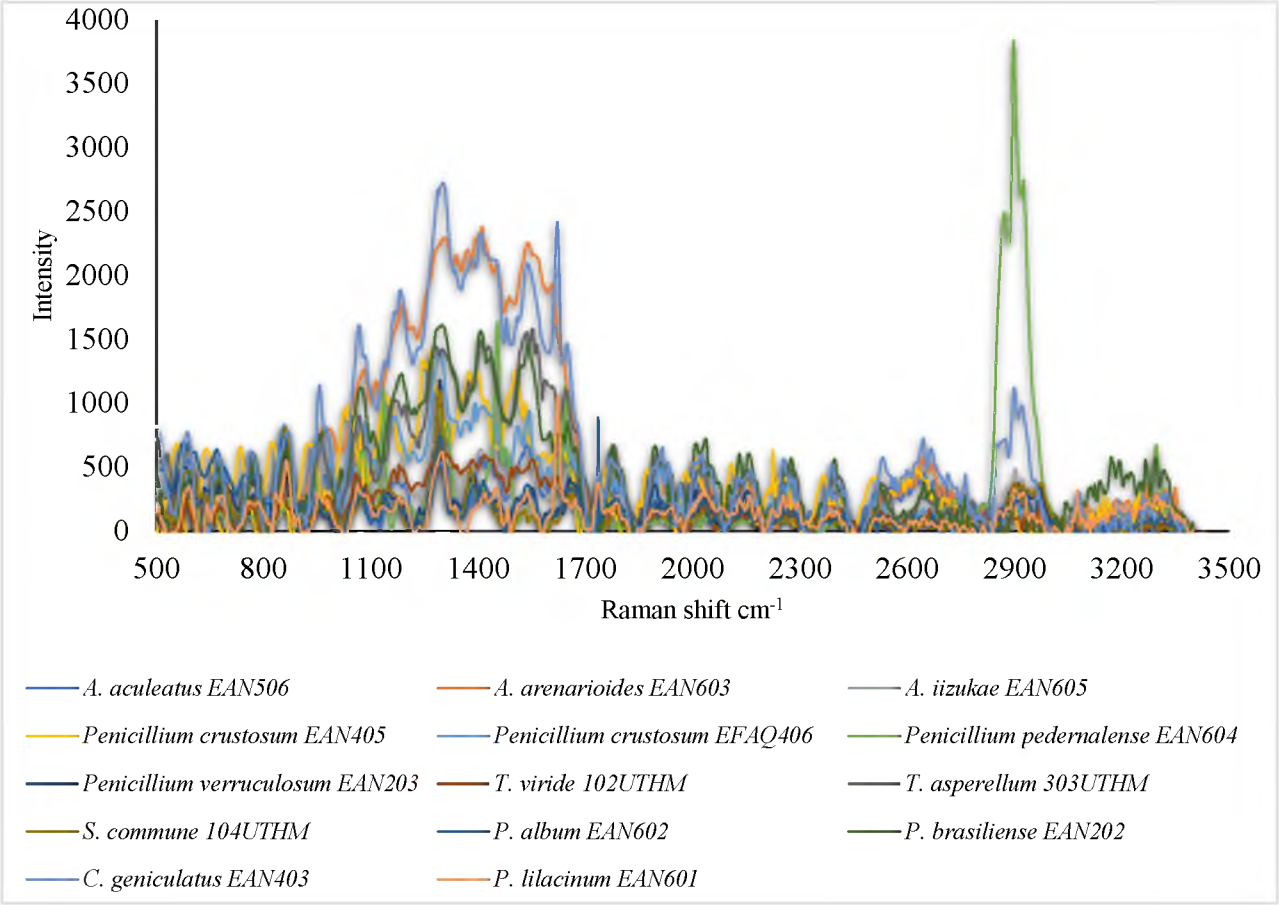

Figure 23. Raman spectroscopy analysis of fungal strains' mycelium cells.

4. Conclusions

Peat soil has a high fungal diversity with unique characteristics. This study has identified six fungal strains that are categorized as newly isolated strains and recorded in Malaysia. Different culture media differ in their ability to support fungal growth and sporulation. The EVA medium demonstrated good efficiency in supporting the fungal strains isolated from the soil due to its similar contents, such as the presence of the lignin. The chemical composition of the fungal strain, as determined using Raman spectroscopy, includes a variety of chemical compounds on the fungal surface (spores). However, since there is no database for fungal surface chemical composition, the Raman spectroscopy analysis is not applicable. Therefore, molecular analysis is used to confirm fungal identification.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/su15075902/s1.

Author Contributions: Conceptualisation, E.A.N. and A.A.A.-G.; methodology, E.A.N.; software, A.A.A.-G. and R.A.; validation, B.A.T., R.M.S.R.M., N.A.-S. and H.A.E.E.; formal analysis, R.M.S.R.M. and R.A.; investigation, E.A.N.; resources, R.M.S.R.M.; data curation, E.A.N.; writing—original draft preparation, F.A.A.-W. and E.A.N.; writing—review and editing, N.A.-S., A.A.A.-G., B.A.T., R.M.S.R.M. and H.A.E.E.; visualisation, B.A.T.; supervision, B.A.T. and R.M.S.R.M.; project administration, B.A.T. and A.A.A.-G.; funding acquisition, B.A.T. and H.A.E.E. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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