

## Isolation and Identification of Endophytic Fungi from UiTM Reserve Forest, Negeri Sembilan (Pemencilan dan Pengenalpastian Kulat Endofit dari Hutan Simpan UiTM, Negeri Sembilan)

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### ABSTRACT

*Endophytic fungi are those living inside the host plant without causing any apparent negative effect on the host plant. Two isolates endophytic fungi from leaves and two isolates from root at Universiti Teknologi MARA (UiTM) Reserve Forest, Negeri Sembilan were successfully isolated and identified by morphology and molecular characteristic. Samples were surface sterilized and sub-cultured to obtain a pure culture. Characteristics of the isolates such as colony appearance, mycelial texture, conidia/spores and pigmentation were studied to explore their morphology. Isolates were also subjected to a PCR-based genotyping test. There were noticeable differences in morphological characteristics among the four isolates. Microscopic analysis showed four isolates consist of septa and conidia/spores. The pigmentation result showed that colony in  $A1_{leaf}$  samples demonstrated an orange color on potato dextrose agar (PDA) media, colony in  $A1_{root}$  demonstrate a black texture in PDA media while hairy colonies in the others two isolates showed a white color on PDA media. Based on molecular analyses the fungal genera showed 99-100% similarity with the related fungi recorded in the GenBank. Both morphology and molecular sequencing of internal transcribed spacer (ITS) regions of endophytic fungi showed that three isolates ( $A1_{root}$ ,  $C2_{leaf}$  and  $C3_{root}$ ) were grouped in Basidiomycota while one isolate ( $A1_{leaf}$ ) belonged to Ascomycota. The endophyte funguses were identified as *Daldinia* sp. ( $A1_{leaf}$ ), *Polyporales* sp. ( $A1_{root}$ ), *Lentinus* sp. ( $C2_{leaf}$ ) and *Rigidoporus* sp. ( $C3_{root}$ ). Overall, the new discoveries of isolated endophyte fungal have dyeing potential of fungal pigments which offer a viable alternative to natural vegetable and harmful synthetic dyes.*

*Keyword: Endophyte fungi; identification; morphological characteristic*

### ABSTRAK

*Kulat endofit yang hidup di dalam tumbuhan perumah tanpa menyebabkan sebarang kesan negatif yang jelas terhadap tumbuhan perumah. Dua pencilan kulat endofit daripada daun dan dua pencilan daripada akar dari Hutan Simpan Universiti Teknologi MARA (UiTM), Negeri Sembilan telah berjaya dipencilkan dan dikenal pasti daripada segi morfologi dan ciri molekul. Sampel disterilkan pada permukaan dan di sub-kultur untuk memperoleh kultur tulen. Ciri-ciri pencilan seperti rupa koloni, tekstur miselium, conidia/spora dan pigmentasi dikaji untuk meneroka morfologi mereka. Pencilan juga dijalankan untuk ujian genotip berasaskan PCR. Terdapat perbezaan yang ketara di dalam ciri-ciri morfologi dalam kalangan empat pencilan. Analisis mikroskopik menunjukkan empat pencilan terdiri daripada septum dan conidia/spora. Keputusan pigmentasi menunjukkan bahawa koloni di dalam sampel  $A1_{daun}$  menunjukkan warna jingga pada media dekstrose agar (PDA), koloni di dalam  $A1_{akar}$  menunjukkan tekstur hitam di dalam media PDA manakala koloni berbulu di dua lagi pencilan menunjukkan media PDA berwarna putih. Berdasarkan analisis molekul, genus kulat menunjukkan persamaan 99-100% dengan kulat yang berkaitan yang direkodkan di dalam bank Gen. Kedua-dua morfologi dan jujukan molekul di kawasan mentranskripsi penjarak dalaman (ITS) kulat endofit mendedahkan bahawa tiga pencilan ( $A1_{akar}$ ,  $C2_{daun}$  dan  $C3_{akar}$ ) dikelompokkan di dalam Basidiomycota manakala satu pencilan ( $A1_{daun}$ ) tergolong di dalam Ascomycota. Kulat endofit dikenal pasti sebagai *Daldinia* sp. ( $A1_{daun}$ ), *Polyporales* sp. ( $A1_{akar}$ ), *Lentinus* sp. ( $C2_{daun}$ ) dan *Rigidoporus* sp. ( $C3_{akar}$ ). Secara keseluruhannya, penemuan baru pencilan kulat endofit mempunyai potensi pewarnaan pigmen daripada pigmen kulat yang menawarkan alternatif terkedepan kepada sayur-sayuran semula jadi dan pewarna sintetik yang merbahaya.*

*Kata kunci: Ciri morfologi; kulat endofit; pengenalpastian*

### INTRODUCTION

Endophytes are microorganisms that live within plants for at least a part of their life cycle without causing any visible display of disease (Jiao et al. 2016). Endophytes exist within plant tissues and may grow

within roots, stems and/or leaves, emerging to sporulate at plant or aged host-tissue (Rodriguez et al. 2009). Their biological diversity is enormous, especially in temperate and tropical rainforests. Endophytes often consist of bacterium or fungi.

The fungi are hosted in nearly 300,000 land plant species, with each plant hosting one or more of these fungi (Lu et al. 2012). Diverse group of endophytic fungi is a rich source of important and novel bioactive metabolites promising applications in food industry (Verma et al. 2009), agricultural applications and drug discovery (Porras-Alfaro & Bayman 2011). Many previous studies have reported that endophytic fungus have various potential in providing valuable resources such as high value bioactive molecule (Venugopalan & Srivastava 2015), source of antibiotic in their secondary metabolites (Martinez-Klimova et al. 2014) and as source of natural dyes (Anchana 2014).

Endophytes which are of interest in this study were focused on dye producers because of its biological advantage towards the environment. There is growing demand for eco-friendly/non-toxic colourant which specifically focuses on health effect such as the use of dye in food for children's textile (Velmurugan et al. 2010). Example of successfully isolated coloured pigments were quercetin glycoside, an orange pigment isolated from a fungal endophyte that belongs to *Penicillium* sp. (Liu et al. 2008) and red pigments from *Penicillium purpurogenum* SX01, identified from *Ginkgo biloba* L., which was used as a natural food coloring substances (Qiu et al. 2010). There is enormous distribution of fungal species and very few had been identified.

Therefore, there is a need for novel and rapid technique for detection and identification of fungi to explore the fungal diversity as a coherent whole (Saad et al. 2004). Molecular technique particularly the application of polymerase chain reaction (PCR) has revolutionized the molecular biology and the molecular diagnosis of fungi. The information from the molecular techniques helps in the differentiation of fungal species and varieties. Hence, the variable ribosomal-DNA (rDNA) internally transcribed spacer (ITS) regions had been applied to identify fungi to the species level as it provides greater taxonomic resolution (Anderson et al. 2003; Lord et al. 2002), highly sensitive assay, high copy number in the fungal genome and high variation in sequence between closely related species. Moreover, sequences in the ITS region are highly variable to potentially serve as markers for taxonomically distant groups. Therefore, the objective of this research was to identify the endophyte fungal isolation from three different plant species from Universiti Teknologi MARA (UiTM) Reserve Forest, Negeri Sembilan through morphological and molecular methods.

## MATERIALS AND METHODS

### FIELD SAMPLING

Three plant species were collected from the sampling site located within UiTM Reserve Forest, Kuala Pilah, Negeri Sembilan. Three plant species were randomly collected based on the three highest growth and distribution within 200 m<sup>2</sup> sampling site in the UiTM Reserve Forest,

Negeri Sembilan. The geocoordinate for the sampling site was 2°47'28.0"N 102°13'11.7"E. The plant samples were assumed from different plant species because of the morphological difference of the leaves shape and flowers among them. Plants with undamaged leaves and roots were collected in a sterile polythene bags and brought to the laboratory. Sampling occasion was made once, with three biological replicates collected. The samples from three different plants (A, B and C) were labelled as A1<sub>leaf</sub>, A1<sub>root</sub>, B1<sub>leaf</sub>, B1<sub>root</sub>, C2<sub>leaf</sub> and C3<sub>root</sub>. All samples were then processed within 24 h.

### ISOLATION OF ENDOPHYTES FUNGAL FROM LEAVES AND ROOTS

The leaves and roots were washed thoroughly in running tap water for 10 min and air dried. Tissue segments (5 cm<sup>2</sup>) were cut from the midrib and surface sterilized. This sterilization method was described by Guo et al. (2000) and Parthibhan et al. (2017) with some modification. The segments were first rinsed with 70% ethanol for 1 min. Then, the segments were soaked in commercial chlorox for 60 s (3.25% aqueous sodium hypochlorite) and later with another 30 s in 75% ethanol. Finally rinsed with distilled water for three times. Under laminar flow, the sterilized segments were cut in 2 mm pieces. These smaller segments were then placed on potato dextrose agar (PDA) medium aided with antibiotic consist of 1 mg mL<sup>-1</sup> streptomycin sulphate (Guo et al. 2000). The petri dishes were then sealed and incubated for three to five days with 28°C.

### MORPHOLOGICAL IDENTIFICATION

Small fragments of endophytes fungus were removed using inoculating needles and placed on a clean glass slide. The funguses were stained using Lactophenol cotton blue (Hardy Diagnostics, California, USA), a fungal stain. The slides were then observed under a light fluorescence microscope (Olympus Bx51, Tokyo, Japan). The morphological identification was done based on culture characteristic such as colony shape, the presence of septa, shape of conidia/spores and colony pigmentation.

### MOLECULAR CHARACTERIZATION

Morphologically scrutinized fungal samples were further subjected to genome sequencing, for further confirmation. Fungal isolates were cultured on PDA medium. The plates were incubated at 30° approximately 3 days. The fungal isolates were used for DNA isolation. DNA was extracted by 1st BASE Laboratories Sdn Bhd (Seri Kembangan, Selangor, Malaysia). The fungal ITS gene was amplified using universal primers ITS1(5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGTTGATATGC-3'). The total reaction volume of 25 µL contained gDNA purified using in-house extraction method or commercial kit followed manufacturer's protocol, 0.5 pmol of each primer, deoxynucleotides triphosphates (dNTPs, 200 µM each),

0.5u DNAPolymerase, supplied PCR buffer and water. The PCR was performed as follow: 1 cycle (98°C for 2 min) for initial denaturation; 25 cycles (98°C for 15 s; 60°C for 30 s; 72°C for 30 s) for annealing and extension and 1 cycle (72°C for 10 min) for extension of the amplified DNA. The PCR products were purified by standard method and directly sequenced using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequence results were subsequently submitted for construction to dendrogram tree by using NCBI Blast Tree Method.

## RESULTS AND DISCUSSION

The endophytes displayed diverse taxonomic positions and bioactive potential in different aspect including pharmacology, agriculture and natural dyes. In this study, six endophytic fungi were successfully isolated from 3 different plants (Plant A, B, and C) from Reserve Forest UiTM Negeri Sembilan. ITS sequence and culture morphology illustrate that the fungi isolates were classified into two different phyla, which are Ascomycota and Basidiomycota (Table 1). However, two other isolates were designated as ‘unknown fungi’ with BLAST hits sequences lower than 95% identity. This might be due to the mixed isolated species in culture media and the PCR products could not be determined.

To identify the endophytic fungal isolates, pure cultures from single spore isolation were used. The fungal isolates were identified using microscopic and macroscopic characters (Ellis 1971; Leslie & Summerell 2007). The main purpose of morphological identification is to group the fungal isolates according to similar morphological characteristics. The fungal isolates were considered as belonging to the same group or genus if their morphological characteristics matched the morphological descriptions previously described or reported. Potato dextrose agar (PDA) was used to isolate the endophytic fungi. According to classical mycology, most species of endophytic fungi can be differentiate based on their morphological features such as ascospore and peridium morphology, colour, odour and other organoleptic characteristics (Barseghyan & Wasser 2010). The results showed differences in growth of colony, morphology and color between fungi.

Observation on A1<sub>leaf</sub> isolate demonstrates whitish colour colonising the agar from the inoculum point at the centre by non-differentiated hyphae with fairly rapid

growth. After one week growth, a black colour colonising the agar and the colony were densely packed (Figure 1(A)). Microscopic morphology of A1<sub>leaf</sub> isolate showed branch hyphae (Figure 1(B)) with septate (Figure 1(C)) surrounded with elliptical spores (Figure 1(D)). Based on morphological characteristic, A1<sub>leaf</sub> belongs to *Daldinia* sp. and classified under Ascomycota. Cultures of *Daldinia* sp. are characterised by certain macromorphological features that can sometimes facilitate recognition of the species or species group. However, these features may also be highly dependent on the culture medium and the age of the cultures (Stadler et al. 2014). Visualization of endophytic colonization of A1<sub>root</sub> isolate clearly showed an orange pigmentation colonising the agar with rapid growth (Figure 1(E)). Mycelia were scarcely found in the culture due to abundance formation of conidia, hence, suppressing the mycelia growth. Microscopic morphology of A1<sub>root</sub> isolate demonstrates spores with uniseriate phialides (Figure 1(F)) with septate (Figure 1(G)). Conidia/spore were profusely produced with smooth surface and mostly globose (Figure 1(H)). Based on morphological characteristic, A1<sub>root</sub> isolate is belonged to Polyporales sp. and classified under Basidiomycota.

C2<sub>leaf</sub> (Figure 1(I)) and C3<sub>root</sub> (Figure 1(K)) isolates exhibited similar growth and morphology with whitish colony in PDA media. Both hyphae demonstrate a hairy structure with septate (Figure J & L). However, although under microscopic observation, the C2<sub>leaf</sub> spores were unable to observed. Figure 1(K) illustrates the C3<sub>root</sub> isolates characteristic with branch hypha (Figure 1(L)) and subglobose spores (Figure 1(M)). Based on morphological characteristic, C2<sub>leaf</sub> and C3<sub>root</sub> were classified and grouped under Basidiomycota and identified as *Lentinus* sp. (C2<sub>leaf</sub>) and *Rigidoporus* sp. (C3<sub>root</sub>). Although endophytes fungi had major differences between morphology, however, these fungi were difficult to identify at the species level. The use of morphological features was problematic for phylogenetic systematics of hypogeous ascomycetes due to a small set of morphological characteristics and homoplasy.

Identification of the four fungal isolates species were further confirmed by ITS sequencing where sequence search was performed using the BLAST standard nucleotide-nucleotide basic local alignment search tool (National Centre for Biotechnology Information sequence-based identification).

TABLE 1. Identification of fungal isolates from three different plant based on ITS region sequences

Plant	Isolates	Phylum	Species	Sequence ID	%	
A	Leaf	A1 <sub>leaf</sub>	Ascomycota	<i>Daldinia</i> sp.	KU571495.1	100
	Root	A1 <sub>root</sub>	Basidiomycota	Polyporales sp.	LC133884.1	99
B	Leaf	B1 <sub>leaf</sub>	Unknwon	Unknown	-	-
	Root	B1 <sub>root</sub>	Unknown	Unknown	-	-
C	Leaf	C2 <sub>leaf</sub>	Basidiomycota	<i>Lentinus</i> sp.	KT956127.1	99
	Root	C3 <sub>root</sub>	Basidiomycota	<i>Rigidoporus</i> sp.	HQ400710.1	100

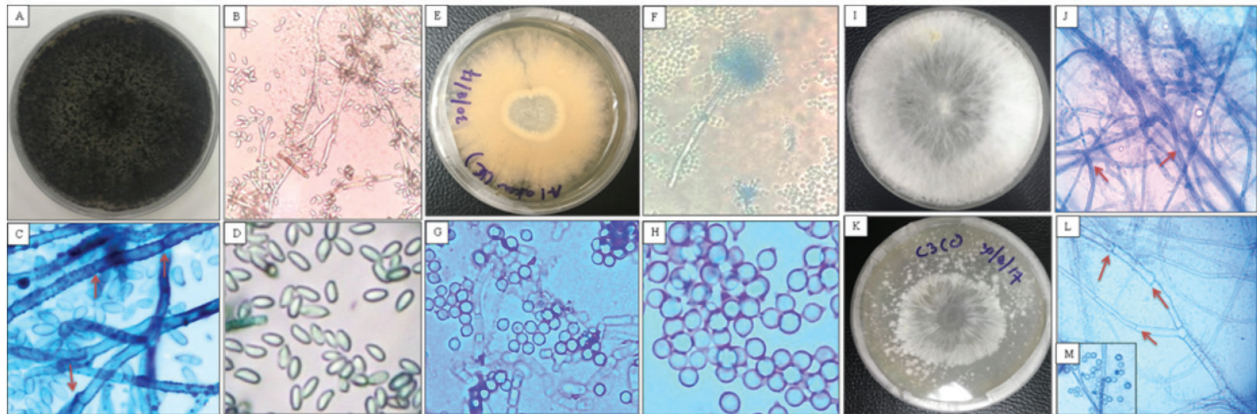


FIGURE 1. (A - D) *Daldinia* sp isolated from A1<sub>leaf</sub>. (A) Colony appearance and black pigmentation of *Daldinia* sp., (B) branch hyphae (magnification 40×), (C) hyphae with septate (magnification 100×), (D) elliptical spores (magnification 100×), (E-H) Polyporales sp. isolated from A1<sub>root</sub>. (E) colony appearance and orange pigmentation of Polyporales sp., (F) globose spores with uniseriate phialides, (magnification 40×), (G & H) globose spores (magnification 100×), (I) colony appearance with white hairy mycelium of *Lentinus* sp. isolated from C2<sub>leaf</sub>. (J) mycelium of *Lentinus* sp., (magnification 40×). (K-M) *Rigidoporus* sp. isolated from C3<sub>root</sub>. (K) colony appearance with white hairy mycelium, (L) branched hyphae (magnification 100×), (M) subglobose spores (magnification 100×); red arrow shows septate

Identification of the four fungal isolates species were further confirmed by ITS sequencing (Figure 2). Subsequently, the sequences were submitted to National Centre for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>) and sequence search was performed using BLAST (standard nucleotide-nucleotide). The ITS sequence is a conserved rDNA sequence that has been widely used both alone and in combination with other universal sequences, such as tubulin, and actin to identify, characterize and perform phylogenetic analysis of fungal isolates (Bałazy et al. 2008).

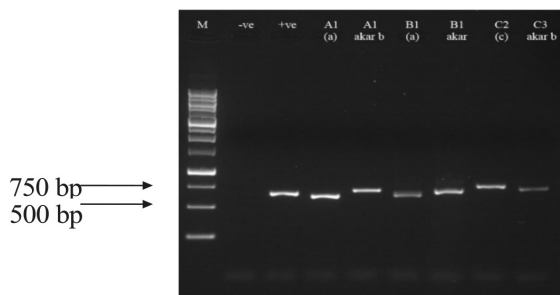


FIGURE 2. PCR product for fungal isolate. M = 100-bp ladder; -ve: PCR no-template control (water to replace DNA template); +ve= DNA extracted from *Candida hellenica*

Based on the ITS sequence obtained, phylogenetic tree of all four isolates (A1<sub>leaf</sub>, A1<sub>root</sub>, C2<sub>leaf</sub> and C3<sub>root</sub>) were constructed by NCBI Blast Tree method (Figure 3). Sequences blast against the NCBI nucleotide database showed 99-100% homolog to *Megnaporthe oryzae* with expect value (E) of zero (data not shown). E-value defines as a parameter that indicated by chance similarity when browsing a database subject of interest. Lower number of E-value or closer to zero describe that the match is

significant. The dendrogram obtained for A1<sub>leaf</sub>, (573 bp) consists of 2 clusters, *Daldinia* sp. BAB-4902 and *Daldinia* sp. 119CA/T (100% similarity). Another isolate from leaf, C2<sub>leaf</sub> (685 bp) showed the relationships of 4 taxa. The tree was rooted with *Lentinus* sp. (99% similarity). Endophytic fungi from root, A1<sub>root</sub> (636 bp) showed the relationships of 4 taxa. The tree was rooted with Polyporales sp. or *Perenniporia* sp. (99% similarity). Another isolates from root, C3<sub>root</sub> (646 bp) also showed the relationships of 4 taxa, rooted with *Rigidoporus vinctus* (100% similarity).

Greater than 98% homology with referenced culture is required to confirm the preliminary identification of the test sequence. However, all four fungal showed 99% similarity with reference strain: *Daldinia* sp. (Sequence ID gbl KU571495.1), *Lentinus* sp. Sequence ID gbl KT956127.1), Polyporales sp. Sequence ID gbl LC133884.1), and *Rigidoporus* sp. Sequence ID gbl HQ400710.1). The reference strain was previously submitted to the Gen Bank database.

The ITS sequencing result confirms the isolated fungal strain under the phylum Basidiomycota and Ascomycota. The sequence analysis confirmed the microscopic analysis, which showed that all the fungal isolates have septate hyphae, an important characteristic of fungi under the phylum Basidiomycota and Ascomycota.

## CONCLUSION

Four fungal endophytes successfully isolated from leaves and roots tissue of plant. *Daldinia* sp. and *Lentinus* sp. were present in leaf tissue while *Rigidoporus* sp. and Polyporales sp. from root tissue. All four species showed pigmentation and this is the first report of fungal endophytes pigmentation from the UiTM Reserve Forest, Kuala Pilah, Negeri Sembilan. Further research work is required to investigate the role of these fungal endophytes

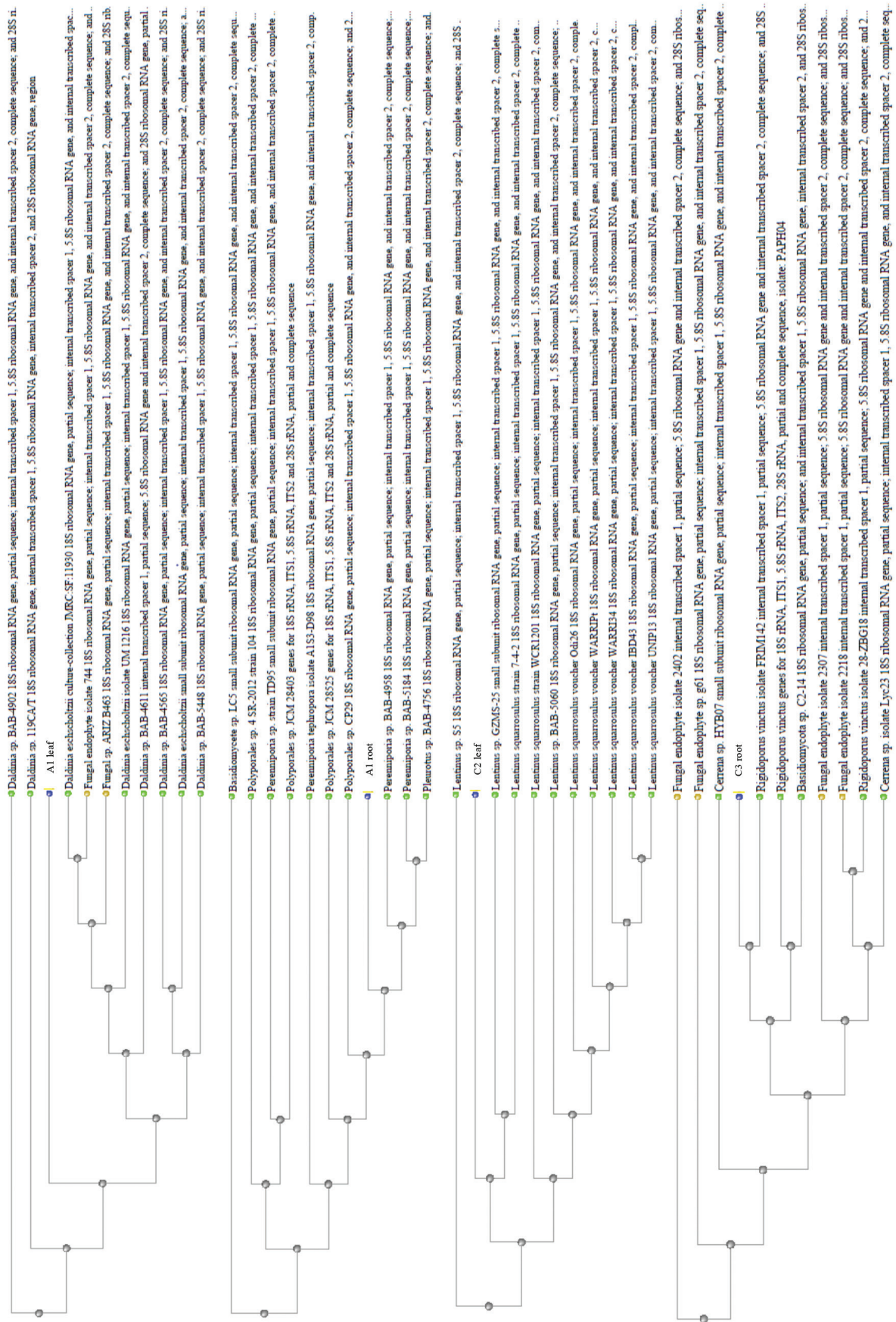


FIGURE 3. Phylogenetic tree of four isolates (A1<sub>leaf</sub>, A1<sub>root</sub>, C2<sub>leaf</sub> and C3<sub>root</sub>) by NCBI Blast Tree method

in the metabolism of the plant and their promising potential as natural dyes.

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