

MORPHO-MOLECULAR CHARACTERIZATION OF ENDOPHYTIC FUNGI ASSOCIATED WITH *AQUILARIA MALACCENSIS* LAM.

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Keywords: Endophytic fungi; Agarwood; Morphological identification; ITS sequencing.

Abstract

A total of 26 fungal isolates were identified from *Aquilaria malaccensis* Lam. (Agarwood). Among them *Aspergillus flavus* Link type-1, *Aspergillus flavus* Link type-2, *Aspergillus niger* Tiegh. type. 1, *Aspergillus niger* Tiegh. type. 2, *Aspergillus* sp. 1, *Aspergillus* sp. 2, *Alternaria alternata* (Fr.) keissl., *Curvularia lunata* (Wakker) Boedijn, *Penicillium digitatum* (Pers.) Sacc., *Penicillium commune* Thom, *Penicillium italicum* Wehmer, *Penicillium* sp. 1, *Penicillium* sp. 2, *Penicillium* sp. 3, *Penicillium* sp. 4, *Eupenicillium* sp. 1, *Eupenicillium* sp. 2, *Sphaeropsis* sp. Sacc. and *Harknessia* sp. Cooke. were identified by morphological analysis and *Alternaria tenuissima* (Kunze) Wiltshire, *Alternaria palandui* Ayyangar, *Fusarium sporotrichioides* Sherb., *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Lasiodiplodia pseudotheobromae* A.J.L. Phillips, A. Alves & Cronus, *Diaporthe hongkongensis* R.R. Gomes, Glienke & Cronus and *Diaporthe perseae* (Zerova) R.R. Gomes, Glienke & Cronus were identified upto genus level by morphological analysis, which were later on identified and confirmed at species level by molecular analysis. Among these isolated fungal species- *Alternaria palandui*, *Diaporthe hongkongensis*, *Diaporthe perseae* and *Lasiodiplodia pseudotheobromae* have been reported as newly recorded species and *Harknessia* sp. and *Sphaeropsis* sp. were reported as new generic records for Bangladesh.

Introduction

Endophytes are organisms that live their entire lives or for a specific period of time during their life cycles inside their host tissues without causing visible harm or morphological changes. These organisms include bacteria, actinomycetes, mycoplasma and fungus. Most endophytes are capable of synthesizing bioactive secondary compounds that may provide plants with a defense against pathogens and some of these compounds may be useful for novel drug discovery (Guo *et al.*, 2008; Yan *et al.*, 2011). Endophytic fungi have been found to be present in almost all plants, including those that have colonized the Arctic and Antarctic, deserts, oceans, and tropical rainforests (Ding *et al.*, 2015; Jin *et al.*, 2021). Agar tree (*Aquilaria malaccensis* Lam.) belonging to Thymelaeaceae family, is widely known for the production of aromatic, dark and resinous heartwood that is commonly named agarwood, eaglewood, aloeswood and gaharuwood (aguru in Bengali) (Bouvierie, 1885). This aromatic heartwood or agarwood is originated from the natural defence mechanism of the plant against the fungal infections on different parts of agar tree caused by various endophytic fungi (Hartono *et al.*, 2019). Agarwood has a very high economic value as the essential oil obtained from agarwood is considered as the most expensive (Islam and Chowdhary, 2017). It is used in luxury perfume production and also in manufacturing soap and shampoos (Chakrabarty *et al.*, 1994). By the investigation of interrelated studies, agarwood has

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significantly high anticancer activities (Gunasekera *et al.*, 1981), analgesic and anti-inflammatory activities (Zhou *et al.*, 2008), and anti-depression activities (Okugawa *et al.*, 1993; 1996). It is used for treating diseases of female genital organ (Chakrabarty *et al.*, 1994), asthma (Anon, 1995), jaundice (Chakrabarty *et al.*, 1994), rheumatism and other body pain (Burkill, 1966). High grade agarwood powder is used in production of pharmaceutical tinctures (Beek and Phillips, 1999) and Agar dust is used in making incense sticks or coils for indoor fragrance and also used for religious ceremonies (Yaacob, 1999). Endophytic fungi isolated from different parts of agar plant may have significant potential in the production of the aromatic and resinous agarwood used for therapeutic, industrial and religious purposes. The present study describes the isolation and identification of (morphologically and molecularly) endophytic fungi from *Aquilaria malaccensis* 4 different locations of Dhaka and Sylhet district.

Materials and Methods

Sample collection

Mature and healthy leaf, stem and bark samples of *Aquilaria malaccensis* Lam. were collected from 4 different locations- a. Jagadishpur tea estate, post office: Itakhola, police station: Madhabpur, district: Habiganj; b. Chundeechara tea estate, post office: Chandpu bagan, police station: Chunarughat, district: Habiganj; c. Botanical garden of Curzon Hall Campus, University of Dhaka; d. Botanical Garden, Mirpur, Dhaka.

Isolation of fungi

Endophytes associated with selected samples were isolated using the "Tissue planting method" (CAB, 1968) on potato dextrose agar medium. The preserved leaf, stem and bark samples of *Aquilaria malaccensis* Lam. were cleaned under running tap water to get rid of the dust and debris before being preparation of inocula.. Under aseptic conditions, 5×5 mm² sized inocula were prepared using sterilized scissor and placed in sterilize Petri plates.

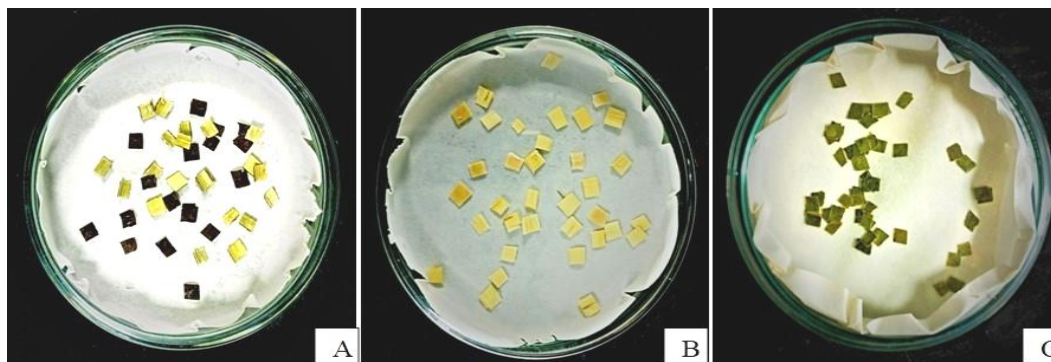


Fig. 1. Surface sterilized inocula of *Aquilaria malaccensis* – A. roots, B. stems and C. leaves.

Each of leaf, stem and root samples in the Petri plates was washed with distilled water for 2 minutes submerged in a 2–4% aqueous Clorox solution for one and a half minutes. Sterilized inocula were rinsing, the samples were placed within Petri plates under aseptic conditions on sterilized filter sheets to surface dry (Fig. 1). On sterilized Petri plates with potato dextrose agar medium (PDA), the surface-sterilized inocula were inoculated on patri plates with PDA medium. Each Petri dish was contained 15 ml of PDA, 1 drop (0.03 ml) of lactic acid, and three inocula were placed in each petridish. For each sample, a total of 9 Petri plates with 27 inocula (with

replications R1, R2, and R3) were employed. From June 2022 to January 2023, a total of 48 isolations of the leaf, stem, and bark of the Agar plant were completed. The isolation method was done 4 times for Agar plant of each location (4 times for leaf, 4 times for stem and 4 times for bark). Lactic acid was used to stop bacterial growth while PDA was utilized as a growth medium. Petri plates with PDA medium and inocula were incubated in the incubation chamber at a temperature of 28°C. After 5 days, using sterilized needles viable hyphae at the edge of fungal colonies were selected and transferred to a new PDA plate to obtain pure cultures.

Morphological identification of fungi

Morphological identification of the isolated fungi were determined following the standard literature (Thom *et al.*, 1945; Booth, 1971; Ellis, 1971, 1976; Barnett *et al.*, 1972; Benoit *et al.*, 1970; Sutton, 1980).

Molecular characterization of fungi

In case of molecular identification of the fungi, protocol was used following Noman *et al.* (2021).

DNA extraction

On PDA medium, fungi were cultivated for 10 days at 28°C. By using a sterile spatula to scrape the surface of cultures that were 10 days old from the Petri plates, fungal mycelium was obtained. Each isolate's fungal mycelium weighed one gram, and it was placed in a 1.5 ml sterile Eppendorf tube. In each Eppendorf tube, 400 µl of sterile extraction buffer (200 mM Tris-HCl, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added before the mycelium was promptly homogenized. Next, 6 µl of RNase (20 mg/ml) was added to each Eppendorf. The mixture was homogenized by using a vortex mixer. The tubes were placed in a water bath that had been prepared to 65°C for 10 minutes. After being removed from the water bath, the samples were cooled at room temperature. Each tube received 130 µl of 3M sodium acetate (pH 5.2). Tubes were vortexed at their highest speed for 30 seconds and then incubated at -20°C for 10 minutes. The samples were centrifuged for 15 minutes at 13,000 rpm. The supernatants were transferred to new tubes and an equivalent volume of a 24:1 chloroform: isoamyl alcohol combination was added, mixed gently and centrifuged at 12000 rpm for 5 minutes. After discarding the supernatant, the pellet underwent two washings in 700 µL of 70% ethanol. The DNA pellets were then dried by air. The final DNA pellet was then resuspended in 100 µl of 1x TE buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA). At 4°C, the DNA was allowed to breakdown for the entire night. It was then kept at -20°C for later testing.

PCR amplification and sequencing

The internal transcribed spacer (ITS) regions were used for the isolates' molecular identification. The ITS sections were amplified by PCR using the ITS1 (5'-TCCGTAG-GTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers. In a 0.2 ml PCR tube, a 25 µl reaction volume containing 2.0 µl of template DNA, 12.5 µl of master mix, 1.0 µl of forward primer, 1.0 µl of reverse primer, and 8.5 µl of nucleus-free water was used for the PCR. A micro centrifuge was used to vortex and centrifuges the reaction mixture. The first phase of the PCR was a denaturation step at 94°C for 5 minutes, followed by 30 cycles of 94, 54, and 72°C for 30 seconds each, a final extension step at 72°C for 5 minutes, and it was finished at 4°C. PCR-amplified products were kept in a freezer at -20°C until they could be resolved on a 1% agarose gel for examination. Ethidium bromide-containing 1.0 g of agarose powder was used to make the gel. In 1xTAE buffer, agarose gel electrophoresis was carried out at 90 volts and 300 mA for 40 minutes. Using a gel documentation device (Model: DI-HD, UK), DNA bands were

captured on camera. Using an automated sequencer, CARS (The Centre for Advanced Research in Sciences, University of Dhaka, 1000) sequenced the pure DNA samples. Using the BioEdit Sequence Alignment program, sequences were aligned, edited, and compared to sequences already present in the databases using the BLASTn program (<http://www.ncbi.nlm.nih.gov/BLAST>).

Results and Discussion

Morphological identification

From the present study, *Alternaria alternata*, *Aspergillus flavus* type. 1, *Aspergillus flavus* type. 2, *Aspergillus niger* type. 1, *Aspergillus niger* type. 2, *Aspergillus* sp. 1, *Aspergillus* sp. 2, *Curvularia lunata*, *Eupenicillium* sp. 1, *Eupenicillium* sp. 2, *Harknessia* sp. *Penicillium digitatum*, *Penicillium commune*, *Penicillium italicum*, *Penicillium* sp. 1, *Penicillium* sp. 2, *Penicillium* sp. 3, *Penicillium* sp. 4 and *Sphaeropsis* sp. were identified from bark, stem and leaf tissues of *Aquilaria malaccensis* by morphological analysis and *Alternaria tenuissima*, *Alternaria palandui*, *Fusarium sporotrichioides*, *Lasiodiplodia theobromae*, *Lasiodiplodia pseudotheobromae*, *Diaporthe hongkongensis* and *Diaporthe perseae* were identified upto genus level by morphological analysis, which were later on identified and confirmed at species level by molecular study.

Key morphological features of the isolated fungi

1. *Alternaria alternata* (Fr.) Keissl., Beih. Bot. Centralbl. 29: 433 (1912) (Fig. 2a)

Colony grey, dark brown to black in colour. Conidiophores and conidia are typically a light golden brown colour. Typically, conidiophores are straightforward, straight or curved, 1-3-septate, up to 50 µm long, 3-6 µm wide, and they have one or more apical conidial pores. Conidial chains profusely branched. Conidia obclavate to ellipsoidal, with a short and cylindrical beak, medium brown, rugulose with muriform septation, 18-63 × 7-18 µm.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 1. 18 September, 2022. M. Zafrin 09.

2. *Alternaria palandui* Ayyangar, Agricultural Research Institute Pusa Bulletin 179: 14 (1928) (Fig. 2b)

Brown to dark brown coloured colony, growth of which is radial without obvious rings of sporulation. Primary conidiophores are erect and simple in young growth, up to 40-100 × 3.5-5.0 µm. Each produces a long chain of narrow conidia. Mature conidia may range within 35-55 × 7-11 µm.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 4. 26 January, 2023. M. Zafrin 26.

3. *Alternaria tenuissima* (Kunze) Wiltshire, Trans. Brit. Mycol. Soc. 18 (2): 157 (1933) (Fig. 2c)

Brown to blackish colony on PDA media in seven days. Conidiophores up to 115 µm long and 4-6 µm thick, pale or medium pale brown in colour. Conidia obclavate or ellipsoidal, 22-95 × 8-19 µm.

Specimen examined: Isolated from fresh and healthy stem tissue of *Aquilaria malaccensis* plants from location: 2. 21 December, 2022. M. Zafrin 21.

4. *Aspergillus flavus* Link, Mag. Ges. Naturf. Freunde Berlin 3 (1): 16 (1809), type 1 (Fig. 2d)

Yellowish-green colonies, usually flat at border but rose at middle. Vesicles are globose to sub-globose, 25-45 µm in diameter. Phialides are borne directly on the vesicle or on the metulae,

6-10 × 4.0-5.5 µm. Metulae 6.5-10 × 3-5 µm. Conidia globose to sub-globose, 3.6 µm in diameter, pale green.

Specimen examined: Isolated from fresh and healthy stem tissue of *Aquilaria malaccensis* plant from location: 1. 21 June, 2022. M. Zafrin 01.

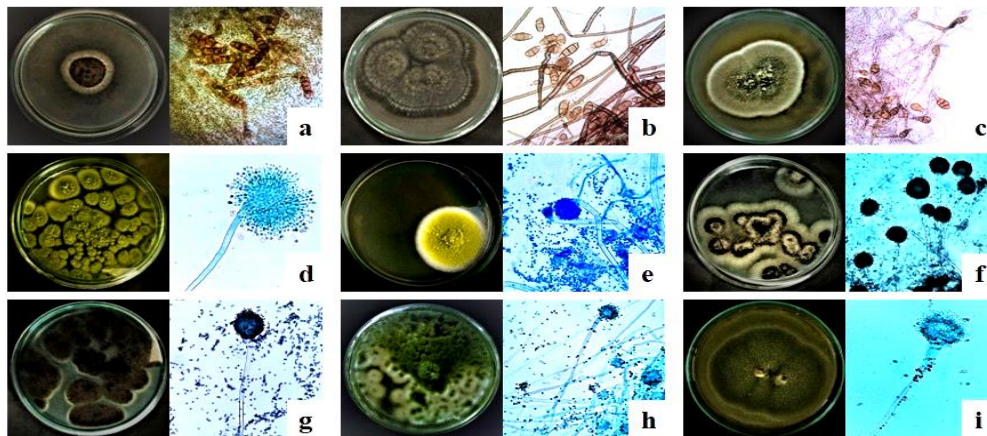


Fig. 2. Colony on PDA medium and conidia under microscope a. *Alternaria alternata*, b. *A. palandui*, c. *A. tenuissima*, d. *Aspergillus flavus* type -1, e. *A. flavus* type -2, f. *A. niger* type -1, g. *A. niger* type -2, h. *Aspergillus* sp. 1, i. *Aspergillus* sp. 2. (Bar = 50 µm).

5. *Aspergillus flavus* Link, Mag. Ges. Naturf. Freunde Berlin 3 (1): 16 (1809), type 2 (Fig. 2e)

Yellow-green colonies are usually flat at border but raised at middle, colonies have narrow white margin. Conidiophores hyaline. Vesicles are globose to sub-globose, 25-45 µm in diameter. Phialides are borne directly on the vesicle or on the metulae, 6-10 × 4.0-5.5 µm. Metulae 6.5-10 × 3-5 µm. Conidia globose to sub-globose, 3.6 µm in diameter, pale green.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 3. 28 June, 2022. M. Zafrin 07.

6. *Aspergillus niger* Tiegh., Ann. Sci. Nat., Bot. 8: 240 (1867), type 1 (Fig. 2f)

Colonies are typically black, powdery except narrow growing white margin. The reverse colony is usually colourless. Conidia mostly globose, irregularly roughened, 4.0-5.0 µm diameter.

Specimen examined: Isolated from fresh and healthy leaf tissue of *Aquilaria malaccensis* plants from location: 1. 21 June, 2022. M. Zafrin 02.

7. *Aspergillus niger* Tiegh., Ann. Sci. Nat., Bot. 8: 240 (1867), type 2 (Fig. 2g)

Colonies are typically black, powdery sometimes. The reverse colony is usually colourless, sometimes branched foot cells. Conidia mostly globose, irregularly roughened, 4.0-5.0 µm diameter.

Specimen examined: Isolated from fresh and healthy leaf tissue of *Aquilaria malaccensis* plants from location: 1. 21 June, 2022. M. Zafrin 02.

8. *Aspergillus* sp. 1**(Fig. 2h)**

Greyish green colony. Mycelium is aseptate and well-developed. Long conidiophores. Conidia are globose, smooth, dry, gray-green to brown in colour; size ranging from $1.2-3.2 \times 1.5-2.7 \mu\text{m}$ in diameter.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 2. 19 September, 2022. M. Zafrin 17.

9. *Aspergillus* sp. 2**(Fig. 2i)**

Colonies are grayish green to brown in color. Mycelium is septate and well-developed. Long conidiophores. Conidia are globose, smooth, dry, grey-green to brown, size ranging from $2.4-3.6 \times 2.4-3.10 \mu\text{m}$ in diameter.

Specimen examined: Isolated from fresh and healthy leaf tissue of *Aquilaria malaccensis* plants from location: 2. 19 September, 2022. M. Zafrin 18.

10. *Curvularia lunata* (Wakker) Boedijn, Bull. Jard. Bot. Buitenzorg 13 (1): 127 (1933) (Fig. 3a)

Colonies are hairy, greenish black in colour. Colonies on PDA markedly zonate, Conidiophores are mostly unbranched, straight or slightly undulating, brown, septate. Conidia mostly three septate, smooth-walled, $24.4-29.2 \times 9.1-12.4 \mu\text{m}$ in diameter.

Specimen examined: Isolated from fresh and healthy stem tissue of *Aquilaria malaccensis* plants from location: 1. M. Zafrin 25.

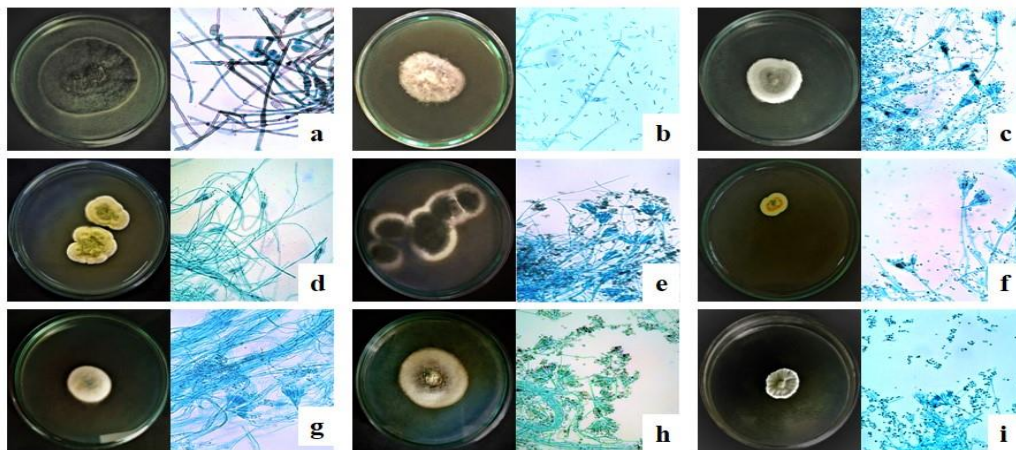


Fig. 3. Colony on PDA medium and conidia under microscope a. *Curvularia lunata*, b. *Fusarium sporotrichioides*, c. *Penicillium commune*, d. *P. digitatum*, e. *P. italicum*, f. *Penicillium* sp. 1, g. *Penicillium* sp. 2, h. *Penicillium* sp. 3, i. *Penicillium* sp. 4. (Bar = $50 \mu\text{m}$).

11. *Diaporthe hongkongensis* R.R. Gomes, Glienke & Crous, Persoonia 31: 23 (2013) (Fig. 6a)

White colored, circular colonies with cottony surface and entire edge are formed on PDA media that reaches 70 mm in diameter after 7 days at 25°C . White aerial mycelia, conidiophores long and hyaline. Reverse colony yellowish white.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 1. 21 June, 2022. M. Zafrin 03.

12. *Diaporthe perseae* (Zerova) R.R. Gomes, Glienke & Crous, *Persoonia* 31: 29 (2013) **(Fig. 6b)**

Dirty white to iron grey colored, circular colonies with entire edge are formed on PDA media. White aerial mycelia, conidiophores long and hyaline. Reverse colony iron-grey with concentric patches of umber.

Specimen examined: Isolated from fresh and healthy stem tissue of *Aquilaria malaccensis* plants from location: 4. 26 December, 2022. M. Zafrin 23.

13. *Fusarium sporotrichioides* Sherb., *Mem. Cornell Univ. Agric. Exp. Sta.* 6: 183 (1915)**(Fig. 3b)**

Colonies are initially white to yellowish pink or pale pink. Mature colonies maybe greyish red or purplish red shades near the centre and pale yellowish pink or pale pink at the margins. Reverse colonies usually vary from medium to dark red or purplish red or reddish brown. Floccose aerial mycelium, conidiophores unbranched or more or less abundantly branched, irregular or verticillate. Conidia variable in size, shape and septation. Sporodochial macro conidia may be 3 to 5 septate, but predominantly 3-septate, 23-43 x 3-5 μm , falcate, pedicillate or apedicillate; widest in the upper half of the conidia with the apical cell more strongly curved than the rest of the conidia.

Specimen examined: Isolated from fresh and healthy stem tissue of *Aquilaria malaccensis* plants from location: 3. 19 September, 2022. M. Zafrin 19.

14. *Harknessia* sp. Cooke.**(Fig. 4a)**

White to pale brown colony with cottony mycelium. Pycnidia globose, conical, thin, white, porous-lacerate at the apex, ranging from 24-33 x 10-14 μm in diameter, bursting out through the leaf tissue; conidiophores filiform; conidia dark, 1-celled, ellipsoid to ovoid, ranges from 8-17 x 5-9 μm in diameter, drawn out into a hyaline pedicel (conidiophores).

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 3. 28 June, 2022. M. Zafrin 08.



Fig. 4. Colony on PDA medium and pycnidia with conidia under microscope a. *Harknessia* sp., b. *Sphaeropsis* sp. (Bar = 50 μm).

15. *Lasiodiplodia pseudotheobromae* A.J.L. Phillips, A. Alves & Crous, *Fungal Diversity* 28: 8 (2008). **(Fig. 6c)**

Fluffy white to grey, circular colonies forms on PDA medium with entire margin and rough surface. Reverse colony is fuscous black to dark black. Aerial mycelium white to whitish grey as the colony matures.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 1. 21 June, 2022. M. Zafrin 04.

16. *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., Bull. Soc. Mycol. France 25: 57 (1909).

(Fig. 6d)

Colonies are usually greyish sepia to mouse grey to black in colour, fluffy with abundant aerial mycelium; reverse colony is fuscous black to black.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 1. 21 June, 2022. M. Zafrin 05.

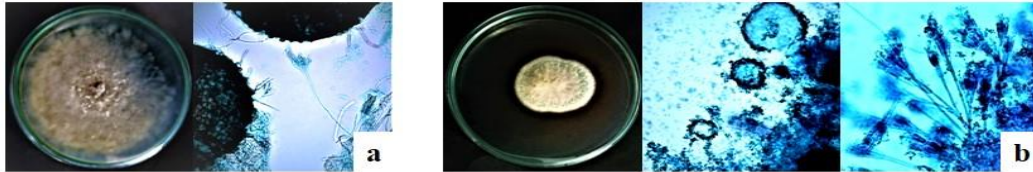


Fig. 5. Colony on PDA medium and cleistothecia with perfect stage under microscope a. *Penicillium* sp. with perfect stage- *Eupenicillium* sp. type -1, b. *Eupenicillium* sp. type - 2. (Bar = 50 μ m).

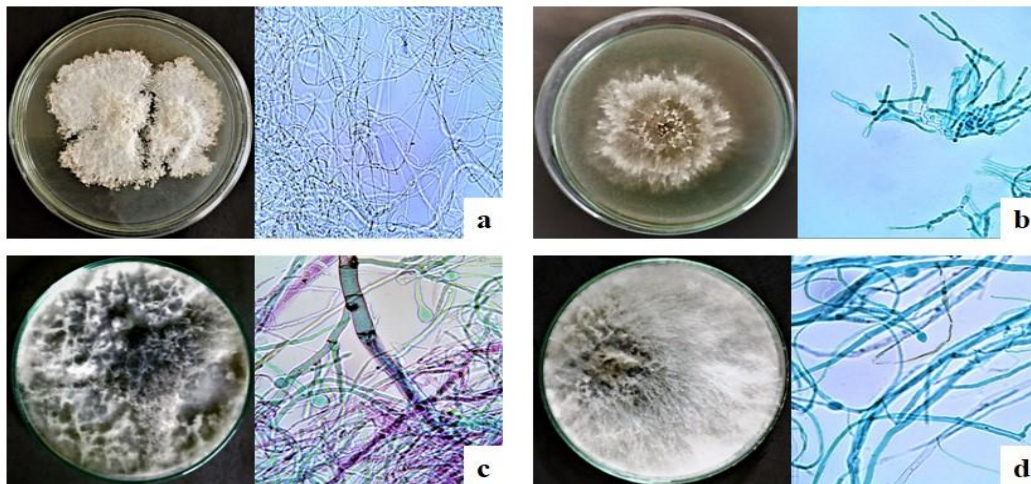


Fig. 6. Colony on PDA medium and conidiophores under microscope a. *Diaporthe hongkongensis*, b. *D. perseae*, c. *Lasiodiplodia pseudotheobromae*, d. *L. theobromae*. (Bar = 50 μ m).

17. *Penicillium commune* Thom, U.S.D.A. Bureau of Animal Industry Bulletin 118: 56 (1910).

(Fig. 3c)

Colonies with moderate growth, velutinous to floccose; conidial mass dull gray. Conidiophores stipes rough-walled, 100-200 μ m long; penicilli terverticillate. Metulae 8-15 μ m long, in whorls of 2-5. Phialides flask-shaped, tapering into a narrow neck, 7-9 μ m long. Conidia sub-globose to ellipsoidal, smooth-walled, grey green to greyish turquoise, 3.5-5 μ m diameter. Reverse colony cream coloured to beige or cream-yellow.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 1. 18 September, 2022. M. Zafrin 10.

18. *Penicillium digitatum* (Pers.) Sacc., Fungi Italici Autographice Delineati. Fasc. 17-28: tab. 894 (1881). **(Fig. 3d)**

Colonies typically have a velvety, yellow to brown-green colour. Conidiophores with irregular branching, smooth-walled whorls with 3-6 phialides at the end of short stipes with few metulae. Phialides are frequently solitary, cylindrical, with a short neck, and a range of sizes between 15 and 30 by 3.5 and 5.0 μm . Conidia are olive-green in colour, smooth, ellipsoidal to cylindrical, and range in size from 3.5 to 8.0 by 3.0 to 4.0 μm . A colony matures when it develops a yellow back and stops secreting.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 1. 18 September, 2022. M. Zafrin 11.

19. *Penicillium italicum* Wehmer, Hedwigia 33: 211 (1894) **(Fig. 3e)**

Colonies are usually grey-green in colour. Exudate is largely absent, and when it is, it congregates in hyaline drops. Uncoloured to yellow-brown in reverse. Conidiophores are normally terverticillate, smooth-walled, hyaline, and occasionally mononematous. 100-250 x 3.5-5.0 μm stipes. More or less cylindrical metulae with smooth walls, measuring 15- 20 x 3.5- 4.0 μm , and containing 3 to 6 phialides each. Phialides are slim, cylindrical, 8-15 x 2.0-5.0 μm , with short, distinct necks. Conidia are smooth-walled, greenish cylinders that are occasionally ellipsoidal to subglobose in shape and measure 4.0-5.0 x 2.5-3.5 μm .

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 1. 18 September, 2022. M. Zafrin 12.

20. *Penicillium* sp. 1 **(Fig. 3f)**

Colonies typically have a velvety, yellow to brown-green colour with light yellow to brown margin. Conidiophores, which emerge from the mycelium individually or less frequently in synnemata, branch near the tip to produce a brush-like conidia-bearing apparatus. Conidia are one-celled, typically globose or ovoid, size ranges from 2.2-2.7 x 2-2.5 μm in diameter, hyaline or brilliantly colored masses that form basipetally. When a colony is mature, it turns yellow on the back and lacks secretion.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 3. 18 September, 2022. M. Zafrin 13.

21. *Penicillium* sp. 2 **(Fig. 3g)**

Pinkish white to orangish white, circular, velvety colony. Conidiophores, which emerge from the mycelium individually or less frequently in synnemata, branch near the apex to produce a brush-like conidia-bearing apparatus. They end in phialides, which pinch off conidia in dry chains. Conidia are one-celled, size ranges from 2.1-3.6 x 1.7-2.8 μm in diameter, typically globose or ovoid, hyaline or brilliantly colored masses that secrete a pinkish-red fluid basipetally.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 2. 19 September, 2022. M. Zafrin 20.

22. *Penicillium* sp. 3 **(Fig. 3h)**

Green to grey-green colonies with thick margin that expands towards the centre as the colony matures. Conidiophores end in phialides, which pin conidia off in dry chains. One-celled, typically globose or ovoid, size ranges from 2.2-3.6 x 2.1-3.1 μm in diameter, basipetally

produced conidia that are either hyaline or vividly colored. As the colony matures, it turns dark brown to red on the rear and secretes a pinkish red fluid.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 1. 18 September, 2022. M. Zafrin 14.

23. *Penicillium* sp. 4 (Fig. 3i)

Colonies entire or slightly polygonal in outline, velvety; mycelium white, conidia dull green or greyish dull green. Conidiophores which emerge from the mycelium individually or less frequently in synnemata, branch near the tip to produce a brush-like conidia-bearing apparatus. These conidiophores terminate in phialides, which pin conidia off in dry chains. Conidia are one-celled, typically globose, smooth, 2.5–3.5 µm in diameter, soluble pigments absent; reverse colony brown.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 1. 18 September, 2022. M. Zafrin 15.

24. *Penicillium* sp. with perfect stage- *Eupenicillium* sp. 1 (Fig. 5a)

Yellow to brown, circular, velvety colony. Reverse colony light yellow. In combination with a *Penicillium* anamorph, *Eupenicillium* produces macroscopic (100–500 µm diameter), smooth-walled, frequently vividly colored cleistothecia. Cleistothecia mature into a rock-hard state in many species and may stay that way for weeks or months before finally maturing from the center to produce a large number of eight-spored asci.

Specimen examined: Isolated from fresh and healthy stem tissue of *Aquilaria malaccensis* plants from location: 2. 21 December, 2022. M. Zafrin 22.

25. *Penicillium* sp. with perfect stage- *Eupenicillium* sp. 2 (Fig. 5b)

Colony pale bluish green, Reverse colony light pink to yellow. In combination with a *Penicillium* anamorph, *Eupenicillium* produces macroscopic (100–500 µm diameter), smooth-walled, frequently vividly colored cleistothecia. Cleistothecia mature into a rock-hard state in many species and may stay that way for weeks or months before finally maturing from the center to produce a large number of eight-spored asci.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 4. 26 December, 2022. M. Zafrin 24.

26. *Sphaeropsis* sp. Sacc. (Fig. 4b)

Yellow to brown colony. Pycnidia black, separate or grouped globose, erumpent, ostiolate, size ranging from 96-163 x 90-120 µm in diameter ; conidiophores short; conidia large, dark, 1 celled, ovoid, elongate or somewhat irregular, ranges from 8-13 x 3-5 µm in diameter.

Specimen examined: Isolated from fresh and healthy bark tissue of *Aquilaria malaccensis* plants from location: 2. 21 June, 2022. M. Zafrin 06.

Molecular identification

Utilizing sequence analysis of the internal transcribed spacer (ITS) region, molecular characterization of the fungi species was carried out for accurate identification. By employing the ITS1 as the forward primer and the ITS4 as the reverse primer to analyze ITS regions sequences, seven isolates were discovered. PCR generated bands (~550 bp) from seven samples were subjected to automated sequencing, followed by BLAST analysis, to confirm at the genomic

sequence level (Table 1). The sequence similarity of the ITS region was used to identify the endophytic fungi in this investigation. Internal transcribed spacer (ITS) PCR amplification produced a distinct band of about 550 bp in 1% agarose, indicating that the targeted area was present in each strain.

Table 1. BLAST analysis of the amplified sequences from the isolated DNA of endophytic fungi.

Sample ID	Name of fungi	Max score	Total score	Query coverage	E value	Identity (%)	NCBI gene bank accession no.
M4	<i>Alternaria palandui</i>	955	955	96%	0.0	98.88	KF852593.1
M6	<i>Alternaria tenuissima</i>	891	891	99%	0.0	95.70	MF405157.1
M2	<i>Diaporthe hongkongensis</i>	933	933	94%	0.0	97.98	JF317194.1
M7	<i>Diaporthe perseae</i>	918	918	93%	0.0	97.76	MZ266635.1
M5	<i>Fusarium sporotrichioides</i>	905	905	95%	0.0	98.82	MN644696.1
M3	<i>Lasiodiplodia pseudotheobromae</i>	907	907	95%	0.0	99.01	MF536297.1
M1	<i>Lasiodiplodia theobromae</i>	887	887	94%	0.0	98.42	MK530072.1

The acquired DNA sequences of the isolated endophytic fungi were compared with the sequences already present in the National Center for Biotechnology Information database in order to confirm the identity of the fungal isolates. The obtained DNA sequences showed 98.88% identity with *Alternaria palandui*, 95.70% identity with *Alternaria tenuissima*, 97.98% identity with *Diaporthe hongkongensis*, 97.76% identity with *Diaporthe perseae*, 98.82% identity with *Fusarium sporotrichioides*, 99.01% identity with *Lasiodiplodia pseudotheobromae* and 98.42% identity with *Lasiodiplodia theobromae* (Table 1). Molecular analysis showed species identification of all the fungal genera studied morphologically (Table 2).

Table 2. Comparison between morphological and molecular identification of seven fungal isolates.

Isolates No.	Morphological identification	Molecular identification
M4	<i>Alternaria</i> sp. 1	<i>Alternaria palandui</i>
M6	<i>Alternaria</i> sp. 2	<i>Alternaria tenuissima</i>
M2	<i>Diaporthe</i> sp. 1	<i>Diaporthe hongkongensis</i>
M7	<i>Diaporthe</i> sp. 2	<i>Diaporthe perseae</i>
M5	<i>Fusarium</i> sp.	<i>Fusarium sporotrichioides</i>
M3	<i>Lasiodiplodia</i> sp. 1	<i>Lasiodiplodia pseudotheobromae</i>
M1	<i>Lasiodiplodia</i> sp. 2	<i>Lasiodiplodia theobromae</i>

The endophytic fungi associated with agar plant (*Aquilaria malaccensis*) are quite diverse and a rich source of important bioactive natural products. These products carry high economic and medicinal values. Due to this reason researchers from different countries have worked on agar plant in quest of isolating and identifying these diverse group of endophytic fungi (Hartono *et al.*, 2019), their diversity and biosynthetic activities (Du *et al.*, 2022), their chemical compounds (Zhang *et al.*, 2022), molecular phylogenetic identification (Premalatha *et al.*, 2013), antioxidant and antifungal activity of endophytic fungi of agar plant (Hidayat *et al.*, 2019).

The purpose of this study was to isolate and characterize the endophytic fungi from bark, stem and leaf tissue of Agar plant (*Aquilaria malaccensis* Lam.). From this study a total of 26

fungal isolates were identified. Morphological and molecular analysis was done. These were- *Alternaria alternata*, *Alternaria palandui*, *Alternaria tenuissima*, *Aspergillus flavus* type. 1, *Aspergillus flavus* type. 2, *Aspergillus niger* type. 1, *Aspergillus niger* type. 2, *Aspergillus* sp. 1, *Aspergillus* sp. 2, *Curvularia lunata*, *Diaporthe hongkongensis*, *Diaporthe perseae*, *Fusarium sporotrichioides*, *Harknessia* sp., *Lasiodiplodia theobromae*, *Lasiodiplodia pseudotheobromae*, *Penicillium digitatum*, *Penicillium commune*, *Penicillium italicum*, *Penicillium* sp. 1, *Penicillium* sp. 2, *Penicillium* sp. 3, *Penicillium* sp. 4, *Eupenicillium* sp. 1, *Eupenicillium* sp. 2 and *Sphaeropsis* sp. Among these isolated fungal species- *Alternaria palandui*, *Diaporthe hongkongensis*, *Diaporthe perseae*, *Lasiodiplodia pseudotheobromae* have been reported as new species and *Harknessia* sp., *Sphaeropsis* sp. were reported as new generic records for Bangladesh as these were not documented in relevant literature (Siddiqui *et al.*, 2007; Shamsi S, 2017; Nahar *et al.*, 2019; Khatun *et al.*, 2022; Nessa *et al.*, 2023).

Up to this point, no comprehensive research had been conducted on the endophytic fungi associated to the agar plant in Bangladesh. The endophytic fungi isolated from the bark, stem, and leaf of the agar plant (*Aquilaria malaccensis* Lam.) as well as their relationship with the plant from Bangladesh are thus the subject of this research, which may be useful in evaluating and comparing the endophytic fungal isolates reported from the agar plant in other countries.

Several endophytic fungi such as *Alternaria* sp., *Curvularia* sp., *Fusarium* sp., *Sterilia* sp., *Cladosporium* sp., *Rhizopus* and *Penicillium* sp. were isolated and identified from juvenile *Aquilaria malaccensis* from India (Mochahari *et al.*, 2020). Premalatha *et al.* (2013) reported *Alternaria* sp., *Cladosporium* sp., *Curvularia* sp., *Davidiella* sp., *Fusarium* sp., *Hypocrea* sp., *Massarina* sp., *Phaeoacremonium* sp., *Pichia* sp. as endophytes of *Aquilaria malaccensis* from India. Hartono *et al.* (2019) reported *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Trichoderma* sp., *Curvularia* sp. and *Peniophora* sp. as endophytic fungi from Indonesia. *Fusarium* sp., *Hypocrea* sp., *Lasiodiplodia* sp., *Cochliobolus lunata*, *Cunninghamella bainieri*, *Curvularia* sp. and *Trichoderma* sp. were identified from Malaysia (Mohamed *et al.*, 2010). *Acremonium* sp., *Alternaria* sp., *Cladosporium* sp., *Fusarium* sp., *Mucor* sp., *Nigrospora* sp., *Scopulariopsis* sp. and *Scytalidium* sp. were isolated and identified from the stem of *Aquilaria malaccensis* by Lisdayani *et al.* (2015).

Species belonging to fungal genera *Aspergillus*, *Fusarium*, *Lasiodiplodia*, and *Penicillium* have been reported to show potential for use in the production of agarwood as well as synthesize important secondary metabolites in other nations. (Subasinghe *et al.*, 2019; Mohamed *et al.*, 2010; Tian *et al.*, 2013; Chen *et al.*, 2017; Faizal *et al.*, 2017; Sen *et al.*, 2017; Huang *et al.*, 2017). In the future, secondary metabolites produced by the endophytic fungal isolates reported from this study should be isolated and analyzed in Bangladesh. The species isolated from these genera should be used for inoculating *Aquilaria malaccensis* trees in order to see if they can induce the production of agarwood.

Acknowledgement

The first author expresses her gratitude for the financial assistance provided to her work through the "NST fellowship" by the People's Republic of Bangladesh's "Ministry of Science and Technology".

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