

MORPHO-MOLECULAR CHARACTERIZATION OF ENDOPHYTIC FUNGI FROM THREE TRADITIONAL MEDICINAL PLANTS

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Abstract

The present study focuses on the occurrence of endophytic fungi harboured in the healthy tissues of the three medicinal plants, namely, *Adhatoda vasica* Nees, *Cassia alata* L., and *Ocimum sanctum* L., during the monsoon and winter seasons. Using the "Tissue Planting" method, 19 endophytic fungi were isolated from the leaves and stem of those plants and subsequently identified through morphological characteristics and molecular approaches. From all plants employed in this investigation, *Aspergillus flavus*, *A. niger* (Type I), *A. niger* (Type II), *A. terreus*, *Colletotrichum* sp., *Penicillium* sp.1, *Pestalotiopsis mangiferae*, and white sterile mycelium were isolated. To delve deeper, ten of these isolates underwent characterization via internal transcribed spacer (ITS) region amplification, and the sequence results obtained utilizing the ITS1 and ITS4 primers were cross-referenced with NCBI GenBank database system through BLAST analysis. The phylogenetic tree generated by the UPGMA method grouped the endophytic fungi into different clades on the basis of genetic distances. *Cladosporium xanthochromaticum*, *Colletotrichum siamense*, *Fusarium incarnatum*, and *Talaromyces pinophilus* have been recorded for the first time from Bangladesh. The primary findings of this investigation concerning endophytic fungi established a valuable foundation for further research exploring their potential ecological and biotechnological roles in various ecosystems, most importantly in drug discovery.

Introduction

Endophytes are microscopic organisms that settle in healthy plant tissue intercellularly and/or intracellularly, remaining there for at least one cycle of their lives without causing any damage to the host plant through a symbiotic relationship (Nair and Padmavathy 2014). Of the nearly 300,000 species of higher plants existing on earth, each plant contains a diversity of endophytes, creating an enormous biodiversity among living microorganisms. Fungal taxa have been reported to be endophytic in different plant species, mostly Zygomycota, Ascomycota, and Basidiomycota (Strobel and Daisy 2003). The presence of fungal endophytes within a plant species can be influenced by various factors, such as the host plant type, tissue type, and the plant's phytochemical composition (Sun 2008 and Jia *et al.* 2016). Additionally, their distribution can be affected by geographical location, season, air moisture, and other environmental factors (Aly *et al.* 2010, Yuan *et al.* 2010). These endophytes contribute to plant growth promotion and bolster disease resistance, thus playing a pivotal role in the ecological process of plant succession (Singh *et al.* 2011, Grabka *et al.* 2022, Akram *et al.* 2023). Investigation of the endophytic fungi residing within medicinal plant species presents an opportunity to explore novel bioactive compounds (Wiyakrutta *et al.* 2004, Li *et al.* 2005, Huang *et al.* 2007).

Bangladesh is known to be home to around 6500 plant species, over 500 of which possess medicinal properties, with 250 being regularly employed in healthcare preparations (Ahmed *et al.* 2008), making them reliable host for endophytic fungi. *Adhatoda vasica*, *Cassia alata*, and *Ocimum sanctum* are especially important medicinal plants, frequently used globally in both Ayurvedic and modern medicine for their diverse healing properties (Ahmed *et al.* 2008, Cohen 2014, Oladeji *et al.* 2020, Gheware *et al.* 2021). So far, very limited research works has been

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focused on endophytes associated with medicinal plants in Bangladesh (Nasrin *et al.* 2021, Sharmin *et al.* 2022). The utilization of winter and monsoon seasons in Bangladesh for endophytic fungi research illuminates fungal community dynamics within plants. During the winter season, the weather tends to be cooler and drier, with lower levels of humidity and reduced plant growth activity, while the monsoon season is characterized by warmer temperatures, higher humidity, and frequent rainfall, promoting vigorous plant growth and creating favourable conditions for fungal proliferation. By comparing fungal diversity and abundance between these seasons can provide insights into seasonal variations in fungal populations and their potential ecological roles within plant ecosystems (Nessa *et al.* 2023, He *et al.* 2023). Moreover, molecular identification of endophytic fungi is of paramount importance for the discovery of novel species; understanding the association between the endophytic fungi and their plant hosts; bioprospecting efforts to discover novel bioactive compounds with pharmaceutical, agricultural, and industrial applications; as well as uncovering antagonistic fungi for disease management approach (Arnold and Lutzoni 2007, Rodriguez *et al.* 2009).

The present investigation reveals a dearth of studies on the appropriate identification of endophytic fungi inhabiting these traditional medicinal plants. Therefore, this study was conducted to delve into the both morphological and molecular characterization of the endophytic fungal community within these three traditional medicinal plants, aiming to characterize specific fungal isolates for uncovering novel drug candidates.

Materials and Methods

Mature and healthy plant parts such as the leaf and stem of *Adhatoda vasica*, *Cassia alata*, and *Ocimum sanctum* were collected from the Botanical Garden of Curzon Hall Campus, University of Dhaka. The collected samples were placed into sterile polythene bags, labelled, brought to the laboratory, and stored in the refrigerator at 4°C until isolation. Fungal isolation was done using the “Tissue Planting Method” (Anon. 1968) on Potato Dextrose Agar (PDA) medium. For the preparation of inocula, the preserved leaf and stem samples of experimental plants were rinsed in running tap water to remove dust and debris. The plant materials were cut into small segments about 5 mm in size with the help of a sterilized scissor under aseptic conditions and kept in sterilized Petri plates. Each of the leaf and stem samples were immersed in a 5% aqueous solution of sodium hypochlorite (NaOCl) for 1.5 minutes. Then the samples were rinsed out three times with sterile distilled water and allowed to surface dry on sterilized Petri plates with filter papers under aseptic conditions. The surface sterilized plant segments were inoculated on sterilized PDA medium with 1 drop of lactic acid (0.03 ml) and 3 inocula per plate. A total of 36 isolations with 1296 inocula from leaf and stem were performed for the three medicinal plants during the monsoon, and winter seasons. The inoculated Petri plates were incubated at 25 °C in an incubator for 7 days. After incubation, the fungal colonies were counted for individual species, and the total number was enumerated. Isolated fungi were sub-cultured in a test tube containing PDA medium and identified subsequently. Identification of the fungi was confirmed following relevant literatures (Barnett and Hunter 2000, Booth 1971, Ellis and Ellis 1976, Thom and Raper 1945, Raper and Thom 1949, Sutton 1980).

Genomic DNA extraction was done following the method described by Amer *et al.* (2011) with minor modifications. Molecular identification of the isolates was performed using the sequence of the internal transcribed spacer (ITS) region. For that, PCR amplification was conducted using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') as the forward primer and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as the reverse primer. The PCR was carried out in 0.2 ml PCR tube with 25 µl reaction volume containing 2.0 µl template DNA, 12.5 µl 2X master mix, 1.0

µl forward primer, 1.0 µl reverse primer, and 8.5 µl nuclease free H₂O. The reaction mixture was vortexed and centrifuged in a microcentrifuge. The PCR was initiated by an initial denaturation step at 94 °C for 5 minutes, following 35 cycles of 94, 54 and 72 °C each for 30 sec, with a final extension step of 5 min at 72 °C and ended with 4 °C. The PCR amplified products were resolved on 1% agarose gel. The gel was prepared using 1.0 g agarose powder containing ethidium bromide (0.5 µg/ml). Agarose gel electrophoresis was conducted in 1× TAE buffer at 90 volts and 300 mA for 40 minutes. 1kb DNA ladder was electrophoresed alongside the amplicons. DNA bands were photographed by using a gel documentation system (model: DI-HD, UK).

PCR- amplified products were purified by alcohol precipitation method (Islam and Mukherjee 2011) and sequenced through automated sequencer in Centre for Advanced Research in Sciences (CARS), University of Dhaka, Dhaka-1000. Obtained consensus sequences were compared with already available sequences in the National Center for Biotechnology Information (NCBI) using BLAST tool.

Results and Discussion

Nineteen endophytic fungi were isolated through the tissue planting method from the stem and leaf of three medicinal plants, viz., *A. vasica*, *C. alata*, and *O. sanctum*, during the monsoon and winter seasons (Table 1 and Fig. 1). The isolates belonging to diverse fungal groups are distributed within the Ascomycota phylum.

1. *Arthrinium* Kunze ex Fr., 1821; Kunze & Schmidt, Mykol Hefte 1: 9 (1817) (Fig. 1A)

Colonies white, floccose, fluffy, spreading, develops brown to black spore clusters with time. Conidiogenous cells integrated, terminal and intercalary, polyblastic, dentuculate; pegs usually very short, cylindrical, truncate. Conidia solitary, dark, smooth or verruculose.

Specimen examined: Isolated from fresh and healthy stem of *C. alata* and leaf of *O. sanctum* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 01, 25 July 2019.

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

Colonies effuse, greenish. Mycelia well developed, septate, profusely branched and hyaline. Cells are multinucleate. Conidiophores long. Vesicles subglobose, 22-46 µm. Conidia green, catenulate, dry, usually globose, smooth, 3-5 µm in diameter.

Specimen examined: Isolated from fresh and healthy leaves and stems of *A. vasica*, *C. alata* and *O. sanctum* from botanical garden of Curzon Hall Campus, University of Dhaka, Z. Maria 02, 11 July 2019.

3. *Aspergillus fumigatus* Fresen., Beiträge zur Mykologie 3: 81 (1863) (Fig. 1C)

Colonies grayish green. Mycelium well developed, septate. Conidiophores short, smooth-walled. Vesicles broadly clavate, 25-32 µm in diameter. Phialides directly borne on the vesicle, often greenish pigmented, 6-8 x 2-3 µm. Conidia globose to subglobose, 2.5-3.0 µm in diameter.

Specimen examined: Isolated from fresh and healthy leaves and stems of *A. vasica* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 03, 11 July 2019.

4. *Aspergillus niger* van Tieg. (Type I), Annls sci. nat. (Bot.), Ser. 5(8): 240 (1867) (Fig. 1D)

Colonies black powdery with conidial production. Conidiophores arise from long, broad, thick-walled, mostly brownish, branched foot cells present. Conidia in large, radiating heads, mostly globose, irregularly roughened, 4.0-6.0 µm in diameter, uninucleate.

Specimen examined: Isolated from fresh and healthy leaves and stems of *A. vasica*, *C. alata* and *O. sanctum* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 04, 11 July 2019.

5. *Aspergillus niger* van Tieg. (Type II), *Annls sci. nat. (Bot.)*, Ser. **5**(8): 240 (1867) **(Fig. 1E)**

Colonies effuse, brownish black. Mycelium well developed, septate, profusely branched and hyaline. Cells are multinucleate. Conidiophores are very long, often with a foot cell, straight or flexuous, swollen at the apex in to a spherical vesicle. Surface of vesicle covered by closely packed more or less clavate sterigmata. Conidia catenulate, dry, usually globose, echinulate, dark brown, 5-6 μm .

Specimen examined: Isolated from fresh and healthy leaves and stems of *A. vasica*, *C. alata* and *O. sanctum* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 05, 18 July 2019.

6. *Aspergillus ochraceus* K. Wilh., *Beitr. Kenntn. Aspergillus*: 66 (1877) **(Fig. 1F)**

Colony yellow, up to 1 mm in diameter, reverse pale brownish. Conidiophore brown. Vesicles globose with thin walls, 35 \times 50 μm in diameter, produce sterigmata over the entire surface in culture. The primary sterigmata measures 15-25 \times 5-6 μm , while the secondaries are 7-11 \times 2-3.3 μm . Conidia in chains, hyaline, 3-2-4 μm in diameter.

7. *Aspergillus terreus* Thom, *Am. J. Bot.* **5**: 85 (1918) **(Fig. 1G)**

Colony brownish. Conidial head compact, biserial and densely columnar, 30-50 μm diameter. Conidia small, about 2 μm in diameter, globose, smooth-walled, light yellow.

Specimen examined: Isolated from fresh and healthy leaves of *C. alata* and *O. sanctum* and stems of *A. vasica* and *O. sanctum* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 07, 25 July 2019.

8. *Cladosporium* Link, *Mag. Ges. Naturf. Freunde Berlin* **7**: 37 (1816) **(Fig. 1H)**

Colonies effuse, greyish brown, thinly hairy, Conidiophore solitary or in fascicles, straight or slightly flexuous, distinctly nobose, pale to mid brown, smooth with terminal and intercalary swelling. Conidia arising in simple or branched chains, cylindrical, ellipsoidal, limoniform or sub spherical, sub hyaline or pale olivaceous brown, smooth.

Specimen examined: Isolated from fresh and healthy leaves and stems of *A. vasica* and *O. sanctum* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 08, 10 October 2019.

9. *Colletotrichum* Corda, *Deut. Flora, Abt. III. Die Pilze Deut.* **3**(12): 41 (1831) **(Fig. 1I)**

Colony cottony white, flat, front side white with yellowish in the middle, reverse blackish. Conidiophores hyaline, septate, branched, smooth. Conidia hyaline, aseptate, straight to falcate, thin walled.

Specimen examined: Isolated from fresh and healthy leaves of *A. vasica*, *C. alata* and *O. sanctum* and stems of *C. alata* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 09, 15 July 2019.

10. *Curvularia lunata* (Wakker) Boedijn (1933) **(Fig. 1J)**

Colonies effuse greenish black. Conidiophores solitary, mostly unbranched, straight or slightly undulating, brown, septate up to 37-64 μm long, 9-14 μm thick. Conidia mostly three

septate, brown, slightly curved, third cell from the base is broader and darker than others, smooth, $24\text{--}29 \times 9\text{--}12 \mu\text{m}$.

Specimen examined: Isolated from fresh and healthy leaves of *A. vasica* and stems of *O. sanctum* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 10, 5 August 2019.

11. *Curvularia soli* Y. Marin & Crous (2017) (Fig. 1K)

Colonies effuse grayish black, radiating like star, hairy, flat, reverse side grayish. Conidiophores solitary, mostly unbranched, straight or slightly undulating, brown. Conidia curved, dark to light brown, length of conidia $20.72 \mu\text{m}$ and width $9.36 \mu\text{m}$.

Specimen examined: Isolated from fresh and healthy leaves and stems of *A. vasica*, *C. alata* and *O. sanctum* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 11, 12 November 2019.

12. *Fusarium* Link, Mag. Ges. Naturf. Freunde Berlin 3 (1): 10 (1809) (Fig. 1L)

Colony pinkish yellow, cottony. Conidiophores variable, slender and simple or stout, short, branched irregularly or bearing a whorl of phialides. Conidia hyaline, variable, principally of two kinds, macroconidia several-celled, canoe shaped; microconidia 1-celled, ovoid or oblong, borne singly or in chains; some conidia intermediate, 2 or 3-celled, oblong or slightly curved.

Specimen examined: Isolated from healthy stem of *C. alata* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 12, 15 September 2019.

13. *Nigrospora* Zimm., Centralbl. Bakteriolog. Parasitenk. 8: 220 (1902) (Fig. 1M)

Colonies bluish, hairy, cottony, reverse side grayish black. Conidiophores light brown, septate. Conidia dark black, round to lanceolate, diameter of conidia $15 \times 11 \mu\text{m}$.

Specimen examined: Isolated from healthy stem of *A. vasica* and *C. alata* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 13, 19 August 2019.

14. *Pestalotiopsis mangiferae* (Henn.) Steyaert, Bull. du Jardin Bot. de l'État à Bru. 19 (3): 320 (1949) (Fig. 1N)

Colonies white, cottony, reverse white. Hyphae septate, branched, hyaline. Acervuli black, small, shining. Conidiophores septate, branched, dark brown, cylindrical or lageniform. Conidia fusiform, straight or slightly curved, mostly 3 euseptate: basal cells hyaline, truncate, with an endogenous, cellular, appendage: apical cell conic, hyaline, with 2 or more apical, simple or branched, spatulate or spatulate appendages: median cells brown, sometimes versicolored, thicker-walled, smooth, $24\text{--}31 \times 6\text{--}9 \mu\text{m}$.

Specimen examined: Isolated from fresh and healthy leaves of *A. vasica*, *C. alata* and leaf and stem of *O. sanctum* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 14, 20 October 2019.

15. *Penicillium* Link, Mag. Ges. Naturf. Freunde Berlin 3(1): 16 (1809) (Fig. 1O)

Colony typically exhibits certain striking characteristics. These include color and color change; texture which may be varied, floccose, funiculose or fasciculose or corremiform. Conidiophores mostly arising from the mycelium singly, branched near the apex to form a brush like conidia bearing apparatus; ending in phialides which pinch off conidia in dry chains. Conidia hyaline or brightly colored in mass, one celled, mostly globose or ovoid, produce basipetally.

Specimen examined: Isolated from fresh and healthy leaves and stems of *A. vasica*, *C. alata* and *O. sanctum* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 15, 31 October 2019.

16. *Rhizopus stolonifer* (Ehrenb.) Vuill., Rev. Mycol. (Toulouse) 24: 54 (1902) (Fig. 1P)

Mycelium coenocytic, well developed, branched and fluffy. Mycelium produces many aerial stolons that develop rhizoids at certain points. Directly above the rhizoids one or more sporangiospores are produced. Sporangiospores grow between 210-2500 µm in length and 5-18 µm in diameter, becomes swollen as the latter reaches maturity and a sporangium is developed. Columella present. The average diameter growth ranges from 30-110 µm. Sporangium produces non-motile sporangiospores, pale brown, smooth, 58 µm in diameter.

Specimen examined: Isolated from healthy stem of *A. vasica* and leaf and stem of *C. alata* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 16, 25 August 2019.

17. *Talaromyces* C.R. Benj., Mycologia 47: 681 (1955) (Fig. 1Q)

Colonies olive green, cottony, slightly raised, reverse dark brownish, secreted brown color pigmentation. Conidiophore erect, straight, aseptate, hyaline. Conidia round to oval.

Specimen examined: Isolated from fresh and healthy leaf of *C. alata* and stem of *O. sanctum* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 17, 28 August 2019.

Table 1. Morphologically identified endophytic fungi associated with leaf and stem of three medicinal plants

| Sl. No. | Fungal species | <i>Adhatoda vasica</i> | | <i>Cassia alata</i> | | <i>Ocimum sanctum</i> | |
|---------|----------------------------------|------------------------|------|---------------------|------|-----------------------|------|
| | | Leaf | Stem | Leaf | Stem | Leaf | Stem |
| 1 | <i>Arthrimum</i> sp. | - | - | - | + | + | - |
| 2 | <i>Aspergillus flavus</i> | - | + | + | + | + | + |
| 3 | <i>A. fumigatus</i> | - | + | - | - | - | - |
| 4 | <i>A. niger</i> (Type I) | + | + | + | + | + | + |
| 5 | <i>A. niger</i> (Type II) | + | + | + | - | + | + |
| 6 | <i>A. ochraceus</i> | - | + | - | - | - | - |
| 7 | <i>A. terreus</i> | - | + | + | - | + | + |
| 8 | <i>Cladosporium</i> sp. | - | + | - | - | + | - |
| 9 | <i>Colletotrichum</i> sp. | + | - | + | + | + | - |
| 10 | <i>Curvularia lunata</i> | + | - | - | - | + | - |
| 11 | <i>C. soli</i> | - | - | + | + | + | - |
| 12 | <i>Fusarium</i> sp. | - | - | - | + | - | - |
| 13 | <i>Nigrospora</i> sp. | - | + | - | + | - | - |
| 14 | <i>Penicillium</i> sp. | + | + | + | + | + | + |
| 15 | <i>Pestalotiopsis mangiferae</i> | + | - | + | - | + | + |
| 16 | <i>Rhizopus stolonifer</i> | - | - | + | + | - | - |
| 17 | <i>Talaromyces</i> sp. | - | - | + | + | - | + |
| 18 | <i>T. trachyspermus</i> | - | + | - | - | - | - |
| 19 | White sterile mycelia | + | + | + | + | + | + |

'+' and '-' represent the presence and absence of fungi, respectively.

18. *Talaromyces trachyspermus* (Shear) Stolck & Sam., Stud. Mycol. 2: 32 (1972) (Fig. 1R)

Colony white, reverse light brown, texture floccose, sporulation moderately dense, colony grew slowly, reaching a diameter of 25~27 mm after 7 days at 25°C. Phialides lanceolate, metulae in small verticils of 2~3 range 12~14 × 2~3 μm. Conidia ellipsoidal to ovoid, 6-8 μm in diameter.

Specimen examined: Isolated from healthy stem of *A. vasica* and *C. alata* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 18, 09 December 2019.

19. White sterile mycelia (Fig. 1S)

Colony white, fluppy, reverse white. Mycelia hyaline, septate, profusely branched. Conidia absent. No fructification.

Specimen examined: Isolated from fresh and healthy leaves and stems of *A. vasica*, *C. alata* and *O. sanctum* from botanical garden of Curzon Hall Campus, University of Dhaka, Z Maria 18, 09 December 2019.

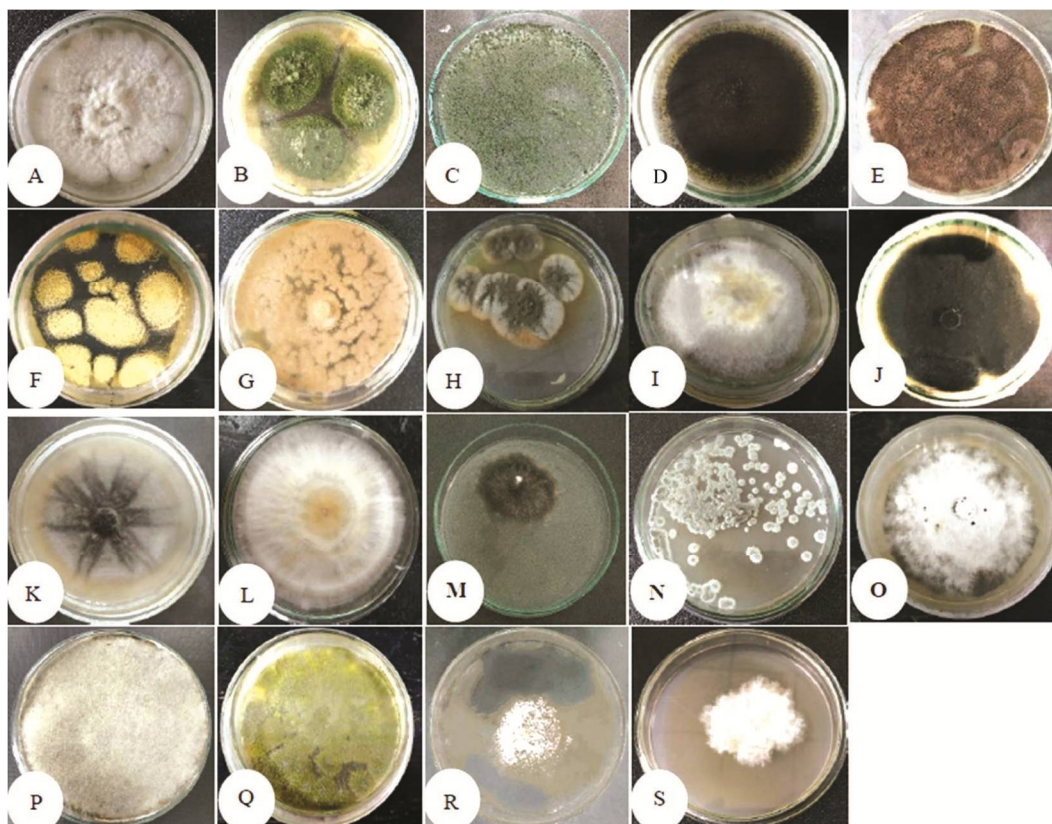


Fig. 1. Colonies of endophytic fungi on PDA medium. A. *Arthrinium* sp., B. *Aspergillus flavus*, C. *A. fumigatus*, D. *A. niger* (Type I), E. *A. niger* (Type II), F. *A. ochraceus*, G. *A. terreus*, H. *Cladosporium* sp., I. *Colletotrichum* sp., J. *Curvularia lunata*, K. *C. soli*, L. *Fusarium* sp., M. *Nigrospora* sp., N. *Penicillium* sp. O. *Pestalotiopsis mangiferae*, P. *Rhizopus stolonifer*, Q. *Talaromyces* sp. R. *T. trachyspermus* and S. White sterile mycelium.

Molecular characterization of fungal endophytes provides evidence about the separation of morphologically identical fungi, which may vary enough genetically to be considered separate species (Munshi *et al.* 2021). Among the 19 fungi, some were unable to be identified up to species level based on their morphological features alone. Therefore, molecular characterization of the fungal isolates was conducted for proper identification using ITS sequence analysis. Out of the 19 fungi, 10 were confirmed up to species level through ITS sequence based molecular analysis (Table 2). Polymerase chain reaction (PCR) of genomic DNA was successfully conducted using ITS1 as forward and ITS4 as reverse primers, and ~550 bp of DNA band was amplified (Fig. 2). Sequence analysis of the amplified DNA through BLAST search in GenBank was conducted and found 90 to 99.82% similarity with the partial sequence of the 18S ribosomal RNA gene; complete sequence of internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, and the partial sequence 28S ribosomal RNA gene of different isolates (Table 2).

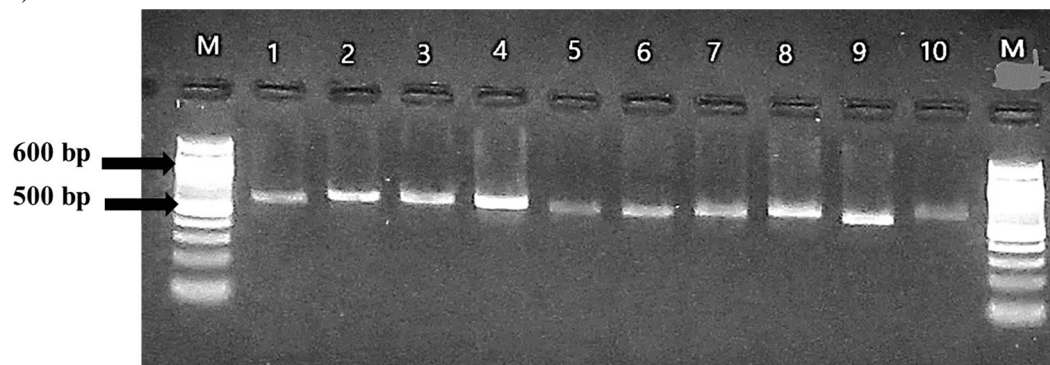


Fig. 2. Gel electrophoresis of the PCR product of 10 fungal isolates performed by ITS1 (F) and ITS4 (R) primers showing ~550 bp amplification. (M represents 100 bp DNA ladder).

Results obtained from the BLAST analysis indicated 97.31% nucleotide identities with *Aspergillus flavus*; 99.27% nucleotide identities with *Aspergillus ochraceus*; 99.60% nucleotide identities with *Cladosporium xanthochromaticum*; 90.00% nucleotide identities with *Colletotrichum siamense*; 99.82% nucleotide identities with *Curvularia lunata*; 99.29% nucleotide identities with *C. soli*; 99.61% nucleotide identities with *Fusarium incarnatum*; 99.42% nucleotide identities with *Pestalotiopsis mangiferae*; 99.63% nucleotide identities with *Talaromyces pinophilus* and 98.57% nucleotide identities with *T. trachyspermus*.

The amplified sequences of ten endophytes based on ITS region were also used to construct the phylogenetic tree using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method to observe the phylogenetic relationship among them (Fig. 3). The dendrogram generated from the analysis indicated that the isolates from various fungal groups are spread across the Ascomycota phylum. The branches and nodes in the tree indicate the degree of genetic similarity among the isolates, with different classes, such as Eurotiomycetes, Sordariomycetes and Dothideomycetes, highlighted as major clusters. This tree provides insights into the evolutionary relationships and classification of the fungi identified by molecular analysis.

From the comparison between morphological and molecular identification, it is clear that out of 10 fungal isolates, morphological identification of one fungal isolates did not match with molecular identification (Table 2). Morphologically identified *Curvularia soli* fungi was matched with *C. geniculata* after ITS sequence analysis. Moreover, *Colletotrichum* sp., *Cladosporium* sp.,

Fusarium sp. and *Talaromyces* sp. were identified up to the species level as *Colletotrichum siamense*, *Cladosporium xanthochromaticum*, *F. incarnatum*, and *Talaromyces pinophilus*, respectively.

Table 2. Identification of endophytic fungal isolates by comparing ITS sequences with GenBank data using a BLAST search.

| Isolates No. | Morphologically identified fungi | Details of molecular identification | | | | | | |
|-----------------|----------------------------------|-------------------------------------|---|-----------|-------------|--------------------|---------|--------------|
| | | Accession No. | Identified isolates | Max score | Total score | Query coverage (%) | E-value | Identity (%) |
| M ₁ | <i>Talaromyces</i> sp. | MT152340.1 | <i>Talaromyces pinophilus</i> strain SC57B01 | 977 | 1900 | 92 | 0.0 | 99.63 |
| M ₂ | <i>Talaromyces trachyspermus</i> | KJ510654.1 | <i>Talaromyces trachyspermus</i> strain MV9 18S | 983 | 983 | 94 | 0.0 | 98.57 |
| M ₃ | <i>Curvularia lunata</i> | KM510420.1 | <i>Curvularia lunata</i> strain MACA8 | 1024 | 1024 | 94 | 0.0 | 99.82 |
| M ₄ | <i>Curvularia soli</i> | MT565489.1 | <i>Curvularia geniculata</i> strain C4 | 1014 | 1014 | 97 | 0.0 | 99.29 |
| M ₅ | <i>Cladosporium</i> sp. | LC775700.1 | <i>Cladosporium xanthochromaticum</i> EAN4 | 905 | 905 | 95 | 0.0 | 98.64 |
| M ₆ | <i>Pestalotiopsis mangiferae</i> | KM510404.1 | <i>Pestalotiopsis mangiferae</i> strain BPHD3 | 931 | 931 | 95 | 0.0 | 99.42 |
| M ₇ | <i>Colletotrichum</i> sp. | MT571330.2 | <i>Colletotrichum siamense</i> isolate C | 665 | 665 | 95 | 0.0 | 90.00 |
| M ₈ | <i>Aspergillus ochraceus</i> | KX610750.1 | <i>Aspergillus ochraceus</i> isolate TN-26 | 985 | 985 | 94 | 0.0 | 99.27 |
| M ₉ | <i>Fusarium</i> sp. | LC388383.1 | <i>Fusarium incarnatum</i> SKA06131 | 939 | 939 | 95 | 0.0 | 99.61 |
| M ₁₀ | <i>Aspergillus flavus</i> | MG518443.1 | <i>Aspergillus flavus</i> isolate OR2 | 937 | 937 | 93 | 0.0 | 97.31 |

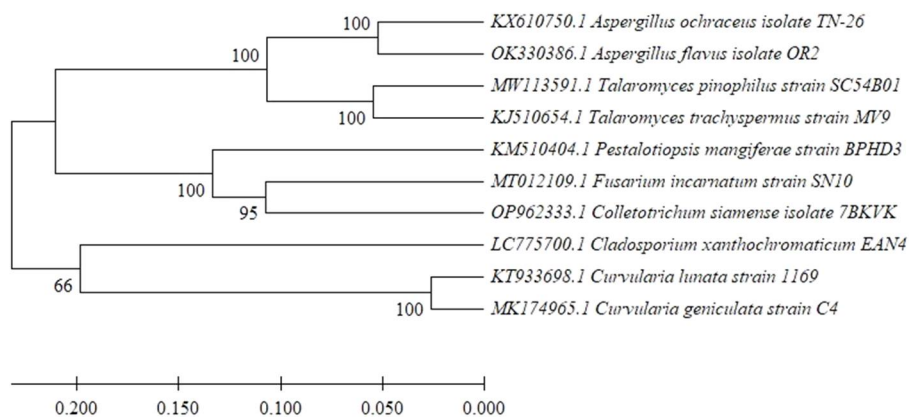


Fig. 3. Phylogenetic tree of ten endophytic fungal isolates. The UPGMA phylogenetic tree illustrates the genetic relationships among fungal isolates based on sequence data. The tree is constructed using the UPGMA method by MEGA 7 software.

A complete review of the available literature reveals that there have been no documented reports of *Colletotrichum siamense*, *Cladosporium xanthochromaticum*, *Fusarium incarnatum*, *Talaromyces pinophilus*, and *C. xanthochromaticum* in any relevant publications pertaining to Bangladesh (Talukder 1974, Siddiqui *et al.* 2007, Kibria *et al.* 2016, Shamsi *et al.* 2017, 2018 2019, Hosen *et al.* 2023, and Nessa *et al.* 2023). Therefore, *C. siamense*, *C. xanthochromaticum*, *F. incarnatum* and *T. pinophilus* are being reported here for the first time in the context of Bangladesh.

The molecular characterization of endophytic fungi holds significant value in unlocking their diverse and intricate roles. Through DNA sequence analysis, researchers can precisely categorize and differentiate fungal species, shedding light on intricate plant-fungus relationships (Azuddin *et al.* 2023, González-Teuber *et al.* 2017 and Singh *et al.* 2020). In this research work, the endophytic fungus *Curvularia geniculata* was also able to differentiate from the morphologically identified *C. soli* clearly through the ITS based sequence analysis with 99.29% sequence similarity, query coverage 97% and E = 0.0. Prasai *et al.* (2021) identified 25 endophytic fungal isolates at the morphological and molecular level from the leaves of *Bergenia ciliata* where *Colletotrichum* sp. were dominant isolates. Two species of *Fusarium* (*F. oxysporum* and *F. solani*) and one species of *Clonostachys* sp. isolated from *Ceriops decandra* were identified through molecular approach and subsequently performed the bioactivity and phytochemical screening (Munshi *et al.* 2021). Bhagat *et al.* (2011) isolated 63 endophytic fungi from *O. sanctum* and *Sapindus detergens* of which 16 isolates were characterized at molecular level by sequencing the amplified ITS1-5.8-ITSII region of rDNA. They observed that out of the sixteen isolates selected on the basis of antibacterial activity, eight exhibited anti-cancerous activity. The dendrogram they produced for sixteen endophytic fungi also reinforces the findings found in this investigation.

Overall, the present investigation suggests that molecular identification based on the ITS region holds potential as a crucial alternative to the traditional mycological approach to precisely identifying fungi. The knowledge generated in this study will help in better understanding of endophytic mycoflora harboured in the studied medically important plants.

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