Taxonomy, Phylogeny and seasonal variation of endophytic fungi isolated from the traditional medicinal plant Madhuca neriifolia from the Western Ghats of Karnataka, India

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Research Article

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Abstract

Traditionally, the plant, *Madhuca neriifolia* is used for various disease treatments. Very few research reports about this plant from the South West of Western Ghats of Karnataka, India, are available. Further, no research reports about the possible existence of endophytic fungi inhabiting this plant are found in the literature. Compounds from endophytic fungi are crucial elements in curing diseases. Therefore, it is necessary to explore the endophytic fungi that may be inhabiting this plant. Hence, this study is conducted to isolate and identify the possible existence of endophytic fungi in this plant. The test results are found to be positive. Four different endophytic fungi strains with different morphologies are isolated and identified from the leaves and twigs of this medicinal plant. The isolated fungi are identified based on the morphology of the fungal colony, PC R-amplified fungal internal transcribed spacer sequences (ITS) and by the phylogenetic analysis with the MEGA X software. All four different endophytic fungi are identified at the level of their species. They are *Alternaria alternata, Trichoderma asperellum, Aspergillus stellatus* and *Cladosporium cladosporioides*.

Introduction

Endophytes are microorganisms present in different parts of a plant body and establish a mutual relationship with the host plant without causing any diseases in it (Bacon *et al*., 2000). Usually, endophytes can be found in different forms like fungi, bacteria or actinomycetes in the host plant's body (Gouda 2016). Based on the critical nature of the endophytes, it can be predicted that endophytes may be beneficial, neutral or detrimental to their host plant (Sikora 2007). In most of the cases, it was observed that the relationship between an endophyte is neither always pathogenic nor simply mutualistic (Wang 2007, Wilson 1995). Sometimes, the coexisting endophytes provide benefits to the host plant by protecting the plant from abiotic and biotic stresses (Gond 2010, Kharwar 2008). Fungal endophytes play a major role in the protection of the host plant from different pathogens through competition, antibiosis and mycoparasitism and it happens either directly or through proliferating within the host plant (Bailey 2006, Mejía 2008). Endophytic fungi can spend their life-cycle through intra or intercellular way to the whole or part of their life in the host plant's healthy tissues without causing any apparent symptoms of disease to the host plant (Li et al., 2008). It may be due to the enormous production of active novel compounds within it (Rodrigues et al., 2005). Different endophytic fungi show different biological activities, such as anticancer, antibacterial, antifungal, antitumor etc. (Rukachaisirikul et al., 2007). Therefore, it is necessary to study the endophytic fungi and the compounds existing in them and assess their effect in curing different diseases. Hence, a preliminary study has been conducted on the medicinal plant *Madhuca neriifolia* to isolate and identify the endophytic fungi existing in it.

Identifying endophytic fungi existing in a specific plant is a critical and difficult process. In earlier days, endophytic fungi were identified based on morphological characteristics of the genus and species. But it's quite difficult to identify all the species based only on morphology. Now, due to unprecedented advancement in molecular technology, it becomes comparatively easy and more reliable to identify the endophytic fungus from the fungal colony through a combined morphological study followed by

molecular analysis. Some fungal species cannot produce spores (sterile). In such types of fungi, the identification is solely based on molecular analysis using Internal Transcribed Spacer (ITS) region. ITS is normally used because of the presence of different variable regions in it (Guo et al., 2000). In addition to the ITS region, the large subunit (LSU), Small subunit (SSU), B tubulin, RNA polymerase-I (RPB1), RNA polymerase-II (RPB2), EF1alpha, Chitin(ech42), Calmodulin etc. regions are also normally used in the identification of exact endophytic fungus species (Raja *et al.*, 2017).

Material And Methods

Plant Materials Identification

The plant sample was collected in the month of November from Seethanadi, the South West of Western Ghats of Karnataka, (13 0 28 / 30 // N 75 0 2 / 0 // E). Elevation range is above 80 meters from Mean Sea Level of India. The botanical nomenclature of *Madhuca neriifolia* plant is

Kingdom → Plantae

Phylum → Tracheophyta

Class → Magnoliopsida

Order → Ericales

Family → Sapotaceae

Genus → *Madhuca*

Species → *neriifolia*
Common name of this plant is

Illipe Butter Tree → English language

Naanilu mara/Neeruhippe →Kannada language

Atta illupei →Tamil language

Attu-ilippa →Malayalam language
The *M. neriifolia* is a medicinal plant, which is found basically in river region of semi-ever green to evergreen forests of Western Ghats. This plant is found in Sri Lanka and India. In India, this is found in Bombay, Karnataka, and Kerala (Awasthi et al., 1975, Krishnan. *et al.*, 1985). It is a moderate-sized tree, growing about 10 metres tall. Leaves grow up to 24 x 6 cm in length, crowded at the ends of the branches, oblong-elliptic to narrowly oblong-lanceolate, acute to obtuse at apex, glabrous, lateral nerves 14–22 pairs; pedicles up to 1.3cm long. Young fruit oblong-lanceolate, glabrous. Flowers yellowish white,

4 to 10 in clusters (Bhatt 2014, Awasthi et al., 1975, Krishnan. *et al*., 1985). The morphological appearance of the plant is shown below in Fig: 1.

The morphological identification of the selected plant was carried out under the guidance of taxonomist, Prof. K.G. Bhatt (Udupi, Karnataka). After morphological identification of the plant, it was processed for molecular identification. The molecular identification was done through DNA barcoding method and the barcode matK gene sequence. The result was submitted to NCBI data base and it was published in NCBI with the Accession No. – MN841760.

Isolation and Sub-culturing of Endophytic fungi

Healthy leaves and twigs of the selected medicinal plant were collected, packed with the transparent sterilized plastic bag and carried from the location of the plant species to the laboratory on the same day. Leaves and small twigs were processed for the next step. The process was done with the help of the standard method as described by Santos, (2003) with a minor modification at the level of the sterilising agent and its concentration. 70% ethanol was used for 5 sec followed by 0.01% mercuric chloride for 30 Sec. to remove the epiphytic microorganism as well as other bacterial microorganisms.

Initially, the samples were washed with running tap water to remove the dust and debris which was attached to the outer part of the samples; it was done for 10 minutes. After cleaning the samples, it was soaked with 70% ethanol (v/v) for 5 sec. followed by 0.01% mercuric chloride ($HgCl_2$) for 30 sec. After 10 sec, finally, samples were rinsed with sterilized distilled water three times and dried with blotting paper and the samples were cut into 1 cm in length with the help of a sterilized blade for twigs and paper cutter for leaves. The samples were placed in 90mm diameter Petri-dish containing Potato Dextrose Agar media (PDA). The PDA was made by using 200gm peeled potato cut into small pieces and boiled initially with 500ml distilled water, followed by 200ml distilled water. The potato liquid collected was by filtering the sample with the help of muslin cloth and made the final volume up to 1000ml by adding 200ml of distilled water. Then 20g of Dextrose was added followed by pouring and mixing agar (20g) in the medium. The medium was autoclaved at 121^0C for 15 minutes. Antibiotic streptomycin (150mg/l) was added to the medium to prevent bacterial contamination. After aseptic inoculation of the samples in the PDA medium, the Petri- dishes were sealed with parafilm (Size-M) and incubated for 12 hours in daylight and 12 hours at night at room temperature. After three to five days of inoculation, different fungal colonies were observed in the inoculated Petri plates. Each colony was transferred aseptically from the master plate to the newly prepared PDA plate; it took nearly two weeks. After the full maturity of each colony, it was again picked up from the edge of the individual colony with the fine-tipped needle and transferred into newly prepared different media like Malt Extract Agar (MEA), Czapek's Agar (CZA), Potato Dextrose Agar (PDA) containing Petri plates. Some endophytic fungal cultures were kept in the refrigerator for future reference while the remaining cultures were processed for morphological analysis and molecular analysis followed by genomic DNA extraction of those endophytic fungal cultures.

Microscopic observation of isolated endophytic fungi

The morphological identification was done through microscopic analysis. Newly grown endophytic fungal colony plates of MEA, CZA and PDA media culture plates were processed for microscopic analysis. Lactophenol cotton blue stain was used for the preparation of slides of the cultures. The endophytic fungi were picked up from the edge of the colony and transferred onto the lactophenol cotton blue stain containing sterilized glass slide and spread the endophytic fungi thoroughly and covered with slide and coverslip. After preparing the slides, the fungus morphology was observed under 10X, 40X and 100X magnification. Taxonomic identification was done based on the following characteristics as the morphology of the colony, growth pattern of the colony, hyphae, septa with spore morphology, colour of the colony on media, surface texture, margin characters, aerial mycelium, mechanism of spore production and conidial characteristics (Barnet 1972, Kirk *et al.*, 1943).

Extraction and PCR (ITS sequence) amplification of the endophytic fungal genomic DNA

According to the instruction of DNeasy Plant Mini Kit (QIAGEN), the endophytic fungal mycelial genomic DNA was extracted from the culture plates. The ITS-rDNA partial gene sequence was done for all endophytic fungal samples. Each endophytic fungal mycelial genomic DNA was extracted and amplified with the primer pair ITS4, ITS5 [(ITS4 R-5´TCCTCCGCTTATTGATATGC3´) and ITS5(5 ´GGAAGTAAAAGTCGTAACAAGG3´)]. The PCR amplification was carried out with total volume of 25ul solution which contained following components; 10X buffer (2.5ul), dNTPs (2ul), ITS4 (1ul), ITS5 (1ul), Taq polymerase (0.25ul), sterile dH_2O (19ul). The DNA amplification was carried for 30 cycles which is as follows: 5minute initial Denaturation for 94^0 C followed by 1min Denaturation at 94^0 C, 30 sec primer annealing at 52^0 C, 1minute extension for 72^0 C and final 10-minute extension at 72^0 C. With the help of gel documentation system, the PCR amplified product was visualized. After visualizing the PCR product, the quality and quantity of the PCR product was checked through purification.

The purified PCR product was processed for sequencing by ABI Prism® Big Dye® Terminator v3.1 cycle sequencing Kit (Applied Biosystem) 3100 Genetic Analyzer. The obtained sequences from the sequencer were edited by using Chromas Lite software for inconsistencies.

Phylogenetic Analysis

The obtained sequences were aligned and FASTA format was made and checked the identity of the sequences through BLAST in National Centre for Biotechnology Information (NCBI) website. The relevant target DNA sequences were retrieved from the BLAST search. The phylogenetic analysis was carried out based on the closely related sequences and the newly identified sequence comparison (Identity, query coverage) from the blast search in NCBI. BLAST search results were processed for construction of phylogenetic tree with the help of MEGA X software followed by multiple sequence alignment using MUSCLE, a maximum likelihood method by a bootstrap of 1000 replicates.

Results

Morphological identification of isolated endophytic fungi

After three to four days of culturing the endophytic fungi in PDA media, potential different fungal colonies were observed on the media. Each colony was transferred from the master plate to a new PDA containing plate. There was no contamination found in the pure culture plates after transferring the endophytic fungi from the master culture plate. Total four different endophytic fungi were isolated in pure form and their morphology was observed and analysed microscopically.

Characteristics of endophytic fungi isolate-1 from MEA media

Endophytic fungus was transferred from PDA media to Malt Extract Agar media (MEA). After seven days of inoculation, fungal colony appeared on MEA media was mouse grey, floccose forming cushions on surface of colony, reverse slate black. Hyphae variable in width, smooth to rough walled, sub hyaline to olivaceous brown. Chlamydospores variable in shape and size, intercalary to lateral to terminal, simple to muri form, light olivaceous to sub hyaline, globose. Conidiophore produced from superficial hyphae, simple to branched, up to 81.6 × 4.88µm in dimension. Conidia variable in shape and size, dark brown, 2–3 with transverse and longitudinal septa, up to 42.5 × 22.7µm in dimension. (Fig: 1). These morphological features of isolate-1 are the key characteristics of fungal species *Alternaria*, thus, tentatively identified as *Alternaria alternata* (Keissler 1912, Woudenberg et al., 2013)

Endophytic fungi isolate-2 from PDA media

Endophytic fungi colony was observed on Potato Dextrose Agar (PDA) media after five days of inoculation. Morphology of colony on PDA media looks dull yellowish green, postulate, fast growing, reverse pale yellow. Conidiophores are sparingly branched. Phialides are elongated with long neck, two to three in groups, variable in shape and size, 2–5 in number, measuring 11.6–9.1×3.1–2.5 µm. Conidia produced on phialides forming conidial heads, oval globose to sub-globose, wall thickened and darkened, smooth walled, measuring 3.7–5.1× 2.7–3.6 µm (Fig:2). Above identified isolate-2 characteristics are the key features of the fungal species *Trichoderma*, thus, tentatively identified as *Trichoderma asperellum* (Samuels *et al*., 1999, Nagamoni et al., 2002).

Endophytic fungi isolate-3 from CZA media

Endophytic fungus was transferred aseptically from the PDA media to Czapeks Agar (CZA) media. After four days of inoculation on Czapeks Agar CZA media, fungal colony was observed. The colony grown faster at 25^0C. Its appearance was olivaceous green, velvety, reverse light olivaceous. Hulles cells hyaline were seen abundantly, produced in compact mass, smooth walled and measuring 21.8 × 21.2 µm in size. In ascomata, teleomorph is not seen, but hulle cells are present there abundantly. Conidiophores were long, olivaceous, smooth walled, up to 510×6.2µm in size. Vesicles were globose to sub-globose, olivaceous in form, smooth walled and up to 121.5 ×20.9 µm in size. Sterigmata of the fungus was biseriate, primaries smooth walled, hyaline, up to 7.2×3.9µm in size. Secondary-ampulliform, hyaline, up to 4.5×2.5 µm in size. Conidia appearance was globose to sub globose, olivaceous, smooth walled and 2.5–2.9µm in size (Fig:3). Above observed characteristics of the isolate-3 are the key feature of the fungal species *Aspergillus*, thus tentatively identified as *Aspergillus stellatus* (Curzi 1934, Raper *et al*.,1965).

Endophytic fungi isolate-4 from MEA media

Endophytic fungus was transferred from PDA media to Malt Extract Agar (MEA) media. After five days of inoculation, fine colony was observed in the MEA media. Appearance of the colony is olivaceous grey velvety, margin olivaceous green and reverse slate black. Hyphae are olivaceous brown, branched, septate, smooth walled to rough walled. Conidiophores are branched, septate, smooth to rough walled, fertile on terminal part of conidiophore, variable in size. Conidia are catenate, branched, base thickened and darkened, cylindrical, globose to pyriform to fusoid, olivaceous brown to sub hyaline, variable in shape and size − 29.65 × 4.95–15 − 7 × 4–3 µm (Fig: 4). The isolate 4 morphological characteristics are the key features of the fungal species *Cladosporium*, thus, isolate-4 was tentatively identified as *Cladosporium cladosporioides*. (Domsch *et al*., 1980, Crous 2017,).

ITS identification of the endophytic fungi

The ITS region of the extracted isolates was amplified with the help of ITS4 and ITS5 primers and Blast aligned to identify the corresponding fungal ITS sequences in GenBank. Blast alignment was done in FASTA format. The fungi are identified at the level of species by taking the percentage identity. For all the four isolates, 100% query coverage and 100% identity at the species level are observed. The isolate-1 is identified as *Alternaria alternata*, Isolate-2 is identified as *Trichoderma asperellum*, Isolate-3 as *Aspergillus stellatus* and Isolate-4 as *Cladosporium cladosporioides* (Table-1, 2, 3 & 4).

The phylogenetic tree was constructed based on the isolated endophytic fungi ITS sequences. These newly identified sequences were submitted to the Gen Bank and the obtained accession numbers are given in the table-5.

Discussion

The present study has been conducted to isolate and identify the endophytic fungi (Taxonomy, Phylogeny) from the medicinal plant *M. neriifolia* and to observe their seasonal variation in three different seasons, namely Summar, rainy and winter. In this study, only the leaves and twigs of this plant have been selected. However, different endophytic fungi can be present in various parts of the plant body, such as flowers, fruits, leaves, twigs, stems, inner bark, roots, and shoots.

M. neriifolia belongs to the family *Sapotaceae*; plants of this family are known for high medicinal value (Deshmukh *et al*., 2009, Awasthi et al., 1975, Jayaweera 1982, Krishnana *et al*., 1985), and hence, there is a high possibility of the presence of valuable compounds with medicinal properties. During the literature review, a few reports have been found suggesting the availability of endophytic fungi in the genus *Madhuca* from India (Deshmukh *et al*., 2009, Verma *et al*., 2014, Kuralarasi and Lingakumar, 2018).

However, there are no reports found about the presence of endophytic fungi in the plant *M. neriifolia.* Hence, to check the availability of endophytic fungi, the plant *M. neriifolia* has been collected from Seethanadi, South West of Western Ghats, Karnataka. Since, Western Ghats region is known for its

richness in medicinal plants, a study from this specific region may help us in future to understand the regional differences of endophytic fungi ecology that this plant can host in different regions of the world. Further, extraction of endophytic compounds and their applications in curing different diseases may confirm the medicinal value of this plant as well as the endophytic fungi.

Potato Dextrose Agar media, Malt Extract Agar and Czapeks Agar media have been used for the culturing of the fungal samples extracted from leaves and twigs of the plant *M. neriifolia*. After pure culturing of the extracted samples, it was sub-cultured for further analysis. For isolation and separation of endophytes, preventive measures have been under taken for unwanted epiphytic fungi contamination (Singh *et al.*, 2016). Sterilized media was kept as the control to observe the possible contamination in the media containing plate every time. No contamination was observed in control and inoculated culture plates. Therefore, it is confirmed that the four endophytic fungi identified in the present study are pure in nature without any contamination.

Morphologically, the extracted fungi showed different fungal growth rates and spore production rates for various media. The production of spores is dependent on the types of media (Van Wyk *et al.*, 2007) and the nature of the fungi-whether sporulating or non-sporulating fungi (Duong et al. 2008). The production of spores were more prominent for isolate-1 and isolate-4 in the Malt Extract Agar (MEA) media than the Potato Dextrose Agar (PDA) media. Contrary to it, for isolate-2, the sporulation was found to be better in Potato Dextrose Agar (PDA) media. In the case of isolate-3, the sporulation was good in Czapeks Agar (CZA) media. The identification of the extracted fungus was carried out based on the morphological and molecular analyse data. This combined approach gives accurate results in the identifying process of a species. The morphological identification is based on the morphology of the colony, mycelia structure, conidiophores, and size, shape, colour and texture of the conidia (Barnet, 1972). Molecular analysis was carried out with the ITS4, ITS5 sequence analysis. ITS sequencing plays a major role in identifying an endophytic fungal species. Due to the advances of having different variable regions, the ITS4 and ITS5 primers have been selected for molecular identification (Peay 2008). The phylogenetic tree has been constructed based on the similarity of the sequences. MEGA X software has been used to construct the phylogenetic tree for all the four endophytic fungi. The combined identification process confirms that the four endophytic fungi identified are *Alternaria alternata* (family-*Pleosporaceae*), *Trichoderma asperellum* (family *Glomerellaceae*), *Aspergillus stallatus* (family *Trichocomaceae*), *Cladosporium cladosporides* (family *Cladosporiaceae*).

In this study, based on the morphological and preliminary molecular analysis, it is also confirmed that the isolates are 100% pure at species level and hence no further sequencing with reference to LSU, SSU, B-tubulin, RPBI, RPBII, EF1 alpha, ech42, and Calmodulin (Raja *et.al.*, 2017) was carried out.

The extracted fungal samples were sent “National Fungal Culture Collection of India” (NFCCI) for further authentication. The NFCCI results are in agreement with our results. The molecular sequence analysis data were deposited in NCBI and the fungal cultures were deposited in NFCCI for further references. The accession numbers of NCBI and NFCCI are listed below in Table 5.

There are several reports on the isolation and identification of endophytic fungi from various plants. Deshmukh *et al*., (2009) reported that *A. alternata*, and *C. cladosporoides* were isolated from the inner bark of the medicinal plant *Mimusops elengi* which belongs to the family *Sapotaceae*. Further, the anticancer compound ergoflavin ($C_{30}H_{26}O_{14}$) was extracted from the leaf of this plant The plant *Madhuca indica*, belonging to the family *Sapotaceae*, also showed the endophytic fungi such as *A. alternata, Alternaria sp., Aspergillus sp., Trichoderma sp., Cladosporium cladosporoides* and many other hyphomycetes, coelomycetes and ascomycetes from the leaves, stems and bark of the plant from different regions of India (Verma *et al*., 2014). *Madhuca longifolia* plant bark showed the existence of endophytes such as: *Alternaria sp., Colletotrichum sp., Diaporthea sp., Phomopsis sp., Mycosperellacea sp., Fusarium sp*., and *Pestolopsis sp.* (Kuralarasi and Lingakumar, 2018). These results clearly indicate that irrespective of the regional differences, different plant species of family *Sapotaceae* found across India hosts similar type of endophytic fungi.

Seasonal variation in terms of inhabiting endophytic fungal diversity was observed in different medicinal plant species. In this study, it is found that all the four fungi are not seen in all the seasons (summer, rainy and winter). *Alternaria* and *Trichoderma* fungi are found in the twigs of the plant in rainy season and it is not found in the leaves as well as not in any other season of the plant. Similarly, *Aspergillus* sp. are found in winter season in the leaves of the plant, while it is not found in the twigs as well as in any other season of the plant. But *Cladosporium* fungi are found in both the leaves and twigs of the plant in all the three seasons (summer, rainy and winter).

From the literature, it is observed that the colonization of different endophytic fungi is dependent on the presence of bioactive compounds of the selected materials, their rates of growth (Arnold *et al*., 2001), leaf-age and the seasonal variation (Osono and Masuya 2012).

In this study also, seasonal variation in terms of colonization of different endophytic fungi in the selected materials is observed. The variation might be influenced by the factors like geography of the selected plant species (mean see level), environmental condition (temperature, humidity percentage) (Strobel and Daisy, 2003), growth pattern of the leaves (Arnold *et al*., 2001), age of the selected leaves, dominant behaviour of the microbes present inside the plant body, samples collection time, host and microbes interaction and their seasonal priority. Aforementioned factors might also influence the colonization of different endophytic fungi in this plant species. Available literature shows the presence of seasonal variation in *Madhuca longifolia* (Kuralarasi and Lingakumar, 2018), *Madhuca elengi* (Verekar, 2017), *Madhuca indica* (Verma *et., al* 2014) etc. which belongs to the same family *Sapotaceae.* Similarly, different plant species like *Camellia japonica* (Osono 2008), *Heisteria concinna and Ouratea lucens* (Arnold, 2000), *Theobroma cacao* (Arnold & Herre, 2003), *Kigelia pinnata* (Maheswari, and Rajagopal, 2013) also showed seasonal variation.

Conclusion

Endophytic fungi have the potential to help not only the plant body (host) in different ways but also can provide effective secondary metabolites or active novel compounds for treating diseases. Numerous medicines have been isolated from different endophytic compounds. Therefore, identification of different endophytic fungi living in different medicinal plants which are having a history of usages as medicine is required. It can confirm the medicinal value of such plants. Study of the endophytic fungi available in a traditionally used medicinal plant and the influences of the active compounds in curing different diseases found in such endophytic fungi may lead to the discovery of novel drugs. Study of the effectiveness of endophytes in curing diseases may give a better understanding of the working pattern of a traditional medicinal plant in disease treatment. The present study is able to confirm the presence of four endophytic fungi viz., *Alternaria alternata, Trichoderma asperellum, Cladosporium cladosporoides*, and *Aspergillus stellatus*. These fungi are known for their medicinal values. Therefore, further pharmacological studies are required to know the potential and effectiveness of these fungi in treating different diseases which may give a better an insight in understanding how this plant helps in treating diseases.

Declarations

Authors contribution

Mamukan Boruah wrote the manuscript text, tables, figures, collected the samples, processed and analysed the samples, and reviews the different articles which are related to the present research work and finally prepared the final draft for publication.

The research supervisor (Prof. Riaz Mahmood) evaluated the complete research work which was done by me and approved for publication.

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Conflict of Interest

There is no conflict of interest.

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Tables

Table: 1 Top five sequence from BLASTn analysis

Gene Bank Accession No.	Description	Max score	Query cover	Query coverage	E value	Identity (%)
MT487794.1	*Alternaria alternate* KU20017.1	949	949	100%	0.0	100%
MT420650.1	*Alternaria* sp. isolate R9	949	949	100%	0.0	100%
MT640581.1	*Alternaria* sp. voucher HQU PS16	949	949	100%	0.0	100%
MT336603.1	*Alternaria alternate* strain Y. H. Yeh I0828	949	949	100%	0.0	100%
MT336602.1	*Alternaria alternate* strain Y. H. Yeh I0827	949	949	100%	0.0	100%

Table: 2 Top five sequence from BLASTn analysis

Gene Bank Accession No.	Description	Max score	Query cover	Query coverage	E value	Identity (%)
MT529846.1	*Trichoderma asperellum* clone SF_570	1000	1000	100%	0.0	100%
MT529837.1	*Trichoderma asperellum* clone SF_570	1000	1000	100%	0.0	100%
MT529422.1	*Trichoderma asperellum* clone SF_570	1000	1000	100%	0.0	100%
MT529370.1	*Trichoderma asperellum* clone SF_570	1000	1000	100%	0.0	100%
MT367901.1	*Trichoderma asperellum* isolate UGM-LHAF	1000	1000	100%	0.0	100%

Table: 3 Top five sequences from BLASTn analysis

Gene Bank Accession No.	Description	Max score	Query cover	Query coverage	E value	Identity (%)
MT122792.1	*Aspergillus stellatus* isolate white 3	937	937	100%	0.0	100%
MN796091.1	*Aspergillus stellatus* isolate FMF5	937	937	100%	0.0	100%
MN686298.1	*Aspergillus stellatus* strain CFE-76	937	937	100%	0.0	100%
MK376947.1	*Aspergillus* sp. strain 582PDA13	937	937	100%	0.0	100%
KU866665.1	*Aspergillus stellatus* strain DTO 325-A9	937	937	100%	0.0	100%

Table: 4 Top five sequences from BLASTn analysis

Gene Bank Accession No.	Description	Max score	Query cover	Query coverage	E value	Identity (%)
MT573533.1	*Cladosporium cladosporioides* strain BFMY-2	915	915	100%	0.0	100%
MT497437.1	*Cladosporium tenuissimum* isolate SS_69	915	915	100%	0.0	100%
MT497434.1	*Cladosporium tenuissimum* isolate SS_25	915	915	100%	0.0	100%
MT483945.1	*Cladosporium tenuissimum*	915	915	100%	0.0	100%
MT367253.1	*Cladosporium cladosporioides* strain HSX11#-10-1	915	915	100%	0.0	100%

Table: 5 Gen Bank and NFCCI Accession Number

Strain name	Parts of isolation	Species name	Family name	Gen Bank Accession no	NFCCI Accession no
Isolate 1, MNMB1	Twigs	*Alternaria alternata* (Fr.) Keissl.	*Pleosporaceae*	OK326856	5054
Isolate 2, MB2	Twigs	*Trichoderma asperellum* Samuels, Lieckf. & Nirenberg	*Glomerellaceae*	MN880489	5055
Isolate 3, MNMB3	Leaves	*Aspergillus stallatus* Curzi	*Trichocomaceae*	OK326861	5056
Isolate 4, MNMB5	Leaves, Twigs	*Cladopsorium cladosporides*, (Fresen.) G.A. de Vries	*Cladosporiaceae*	OK326878	5057

Figures

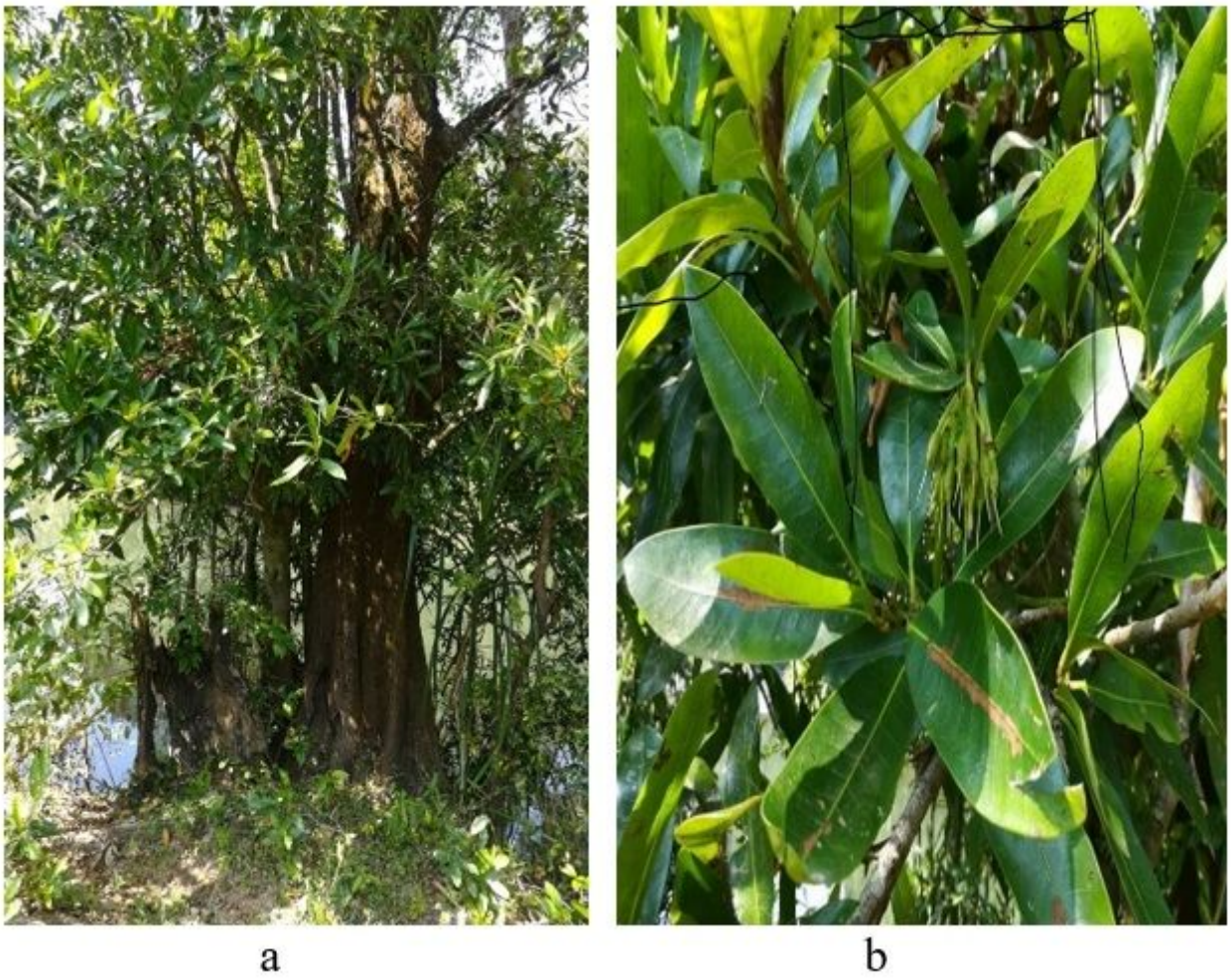

Figure 1

1. a) *M. neriifolia,* b) Flowering body of the *M. neriifolia* plant.

Figure 2

1. a) Pure culture of endophytic fungi *Alternaria alternata*, b) Hypha, conidiophore with conidia (10µm), c) Single, mature and branching conidia (20 µm). d) Branching chain of conidia with terminal conidia (10µm).

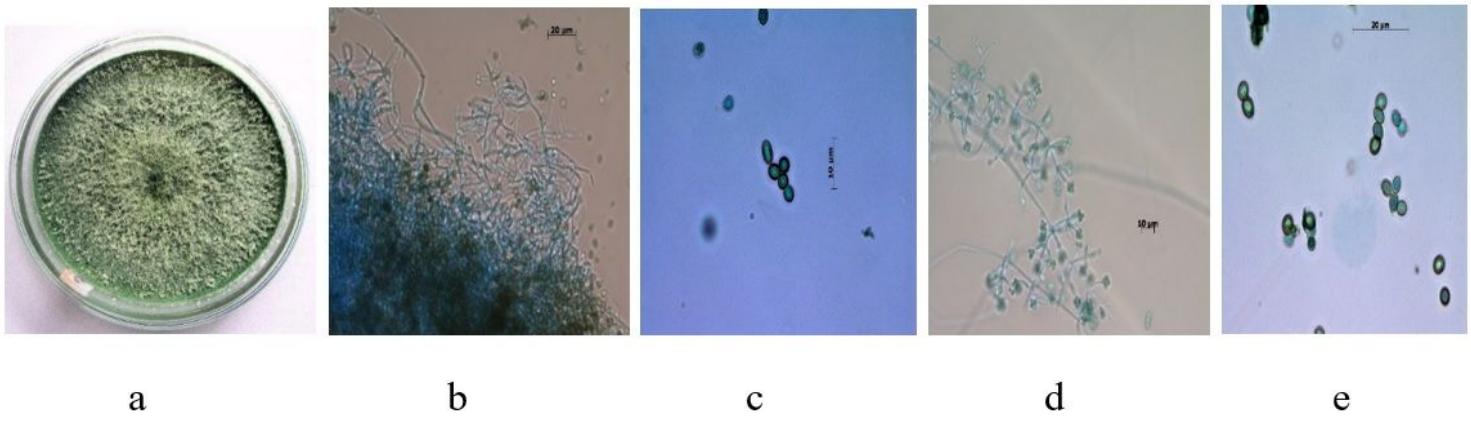

Figure 3

2. a) Pure culture of *Trichoderma asperellum* b) Sparingly branched conidiophores. c) Conidia of *T. asperellum* (10µm). d) Phialides were elongated with long neck, two to three groups, variable in shape and size with conidia (10µm) e) Conidia of *T. asperellum* (20 µm).

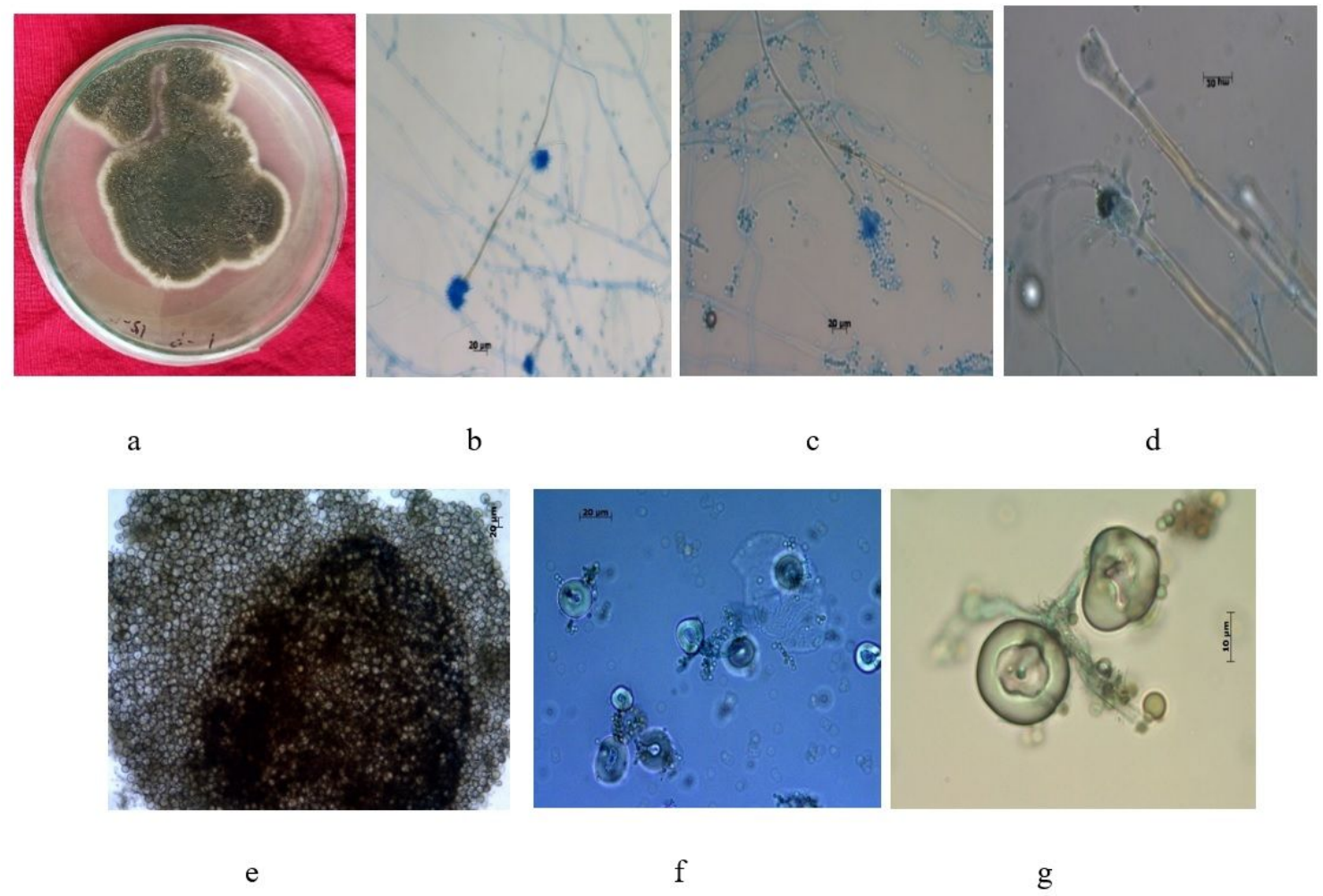

Figure 4

3. a) Pure culture of *Aspergillus stellatus*. b) Foot cell, conidiophore with compact conidia. c) long conidiophore, biseriate sterigmata with conidia. d) Vesicles of *A. stellatus*. e) Compact mass of hulles cells. f) Single hulle cell (20 µm). g) Hulle cells (10 µm).

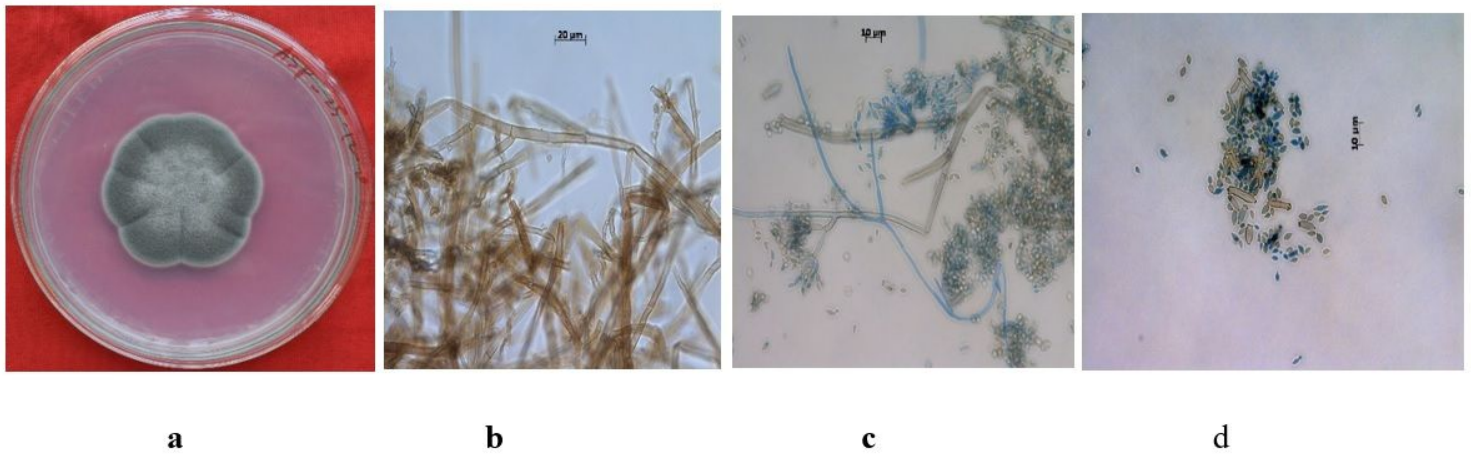

Figure 5

4. a) Pure culture of *Cladosporium cladosporioides*. b) Olivaceous brown, branched, septate, smooth to rough walled hyphae. c) Branched, septate, smooth to rough conidiophore with conidia. d) Branched, catenate, cylindrical and variable shape conidia.

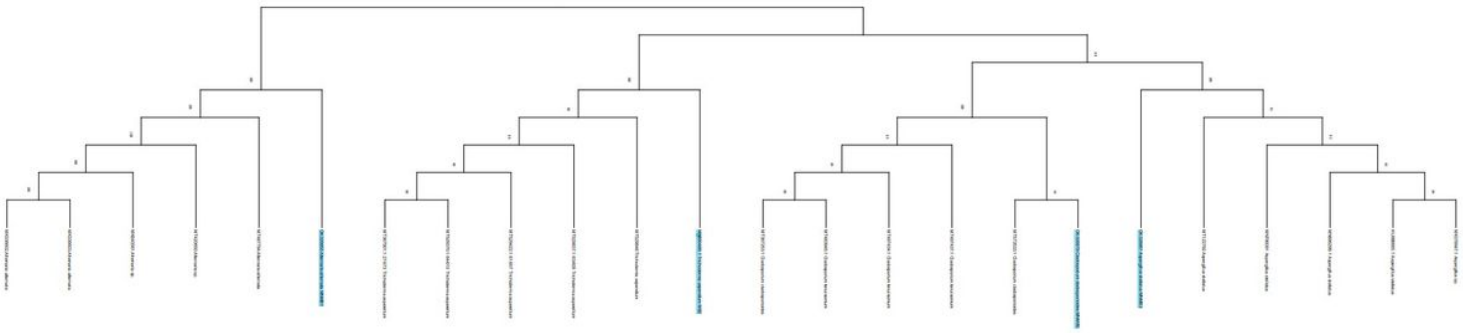

Figure 6

5. Phylogenetic tree of isolated four endophytic fungi

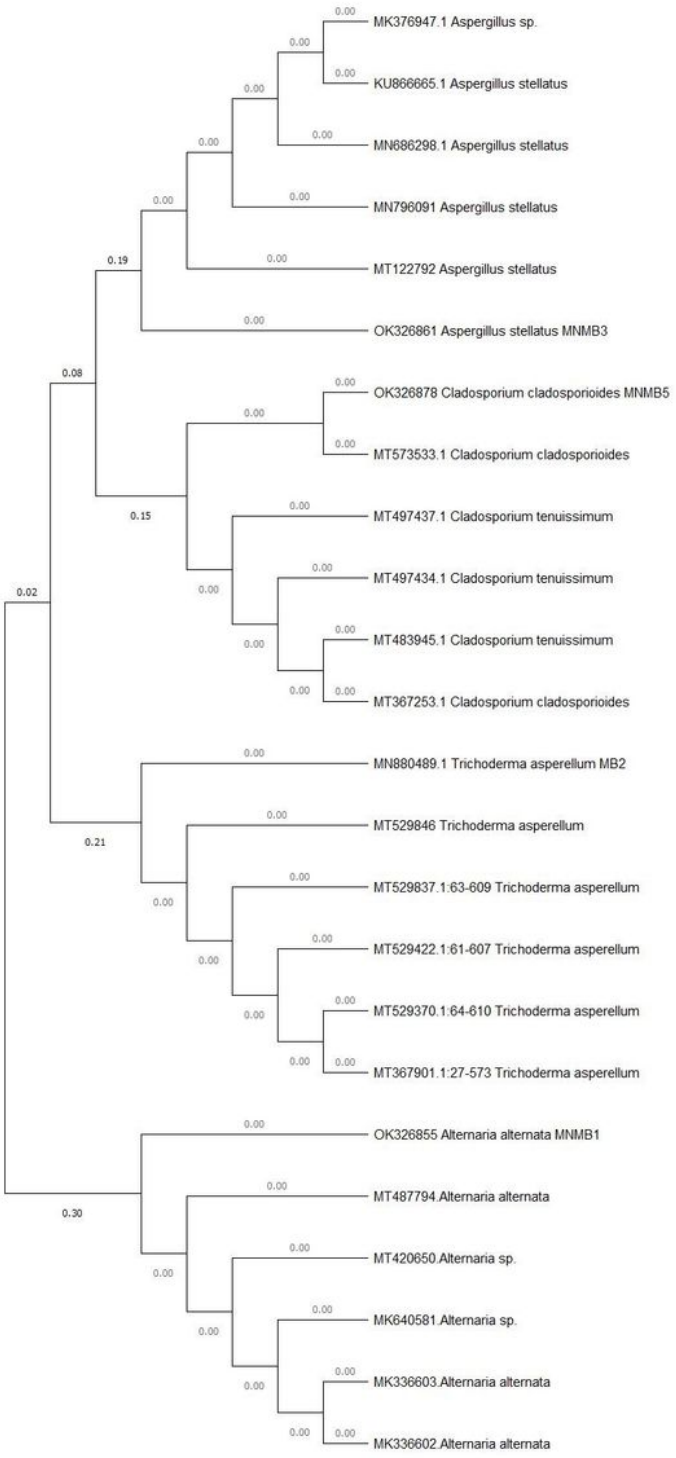

Figure 7

5. Phylogenetic tree of isolated four endophytic fungi (high Resolution)