*Journal of Applied and Natural Science*
10 (4): 1156-1161 (2018)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

# Identification of endophytic fungi from the medicinal plants of Biligirirangana hill, Karnataka

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**Abstract**
Four plants with medicinal properties *Carica papaya, Phyllanthus amarus, Tinospora cordifolia* and *Azadiracta indica* were selected as a host plant for the isolation of endophytic fungi collected from Biligirirangana Hill, ranges from 11° 40' to 12° 09' North and 77° 05' to 77° 01' East, the southern part of Eastern Ghats Chamarajanagar District, Karnataka. The leaves of these plants revealed two, four, six and three isolates of endophytic fungi which belonged to the family *Hypomycetes, Ascomycetes, Dothideomycetes, Coleomycetes* respectively. One of the unidentified strain from each of the above four medicinal plant isolate was subjected to molecular analysis followed by 18SrRNA sequence, internal transcribed spacer regions and phylogenetic analysis. Based on the gene sequencing and phylogenetic tree the clusters species of the fungal isolate was found to be *Stemphylium lycopersici, Epicoccum nigrum, Leptosphaerulina arachidicola* and *Phomopsis azadirachtae* reported in this paper.

**Keywords:** Biligirirangana Hill, Endophytic fungi, Medicinal plants, Phylogenetic analysis, Sequencing

***Article Info***
DOI:10.31018/jans.v10i4.1890
Received: September 10, 2018
Revised: October 12, 2018
Accepted: October 25, 2018

***How to Cite***
Sushma, K. S. *et al.* (2018). Identification of endophytic fungi from the medicinal plants of Biligirirangana hill, Karnataka. *Journal of Applied and Natural Science*, 10(4): 1156 -1161

## INTRODUCTION

Medicinal plants and their endophytes are the important resource of natural products. Traditionally herbal drugs are prescribed even today as they are effective, minimal side effects and relatively low cost (Valiathan, 1998). Microorganisms that lives inside the internal tissues of living plants colonizing inter and intracellular regions without any symptoms and causing no harm to the host plants is called endophyte (Schulz and Boyle, 2006). The endophytic interaction as not 'causing apparent harm' which refers to an absence of macroscopically visible symptoms. Endophytes form a symbiotic association with their host plant. The protection mechanism of the endophytes is exerted directly by releasing metabolites to attack any antagonists and promotes growth. It is believed in many cases that the microbes function as defense mechanism against phytopathogens. They play an essential role to provide protection to their host against other pathogens and environmental factors (Strobel, 2003).

Endophytic fungi are the organisms which spend whole or part of its life cycle inside the healthy tissues of the host plant (Tan and Zhou, 2001). Many researchers have isolated endophytic fungi from plants for the production of natural bioactive molecules like Equisetin (Pamoda *et al*., 2015), Camptothecin (Pu *et al*., 2013).Vincristine and Vinblastine (Palem *et al*., 2016). (El-Hawary *et al*., 2016) isolated Solamargine compound from the endophytic fungus, *Aspergillus flavus* from *Solanum nigrum* plant. One notable endophyte with medicinal benefits to human was reported by Gary Strobel isolated the endophytic fungus *Pestalotiopsis microspora*, from *Taxus wallachiana* (Himalayan Yew) was found to produce Taxol, an anticancer drug (Gary *et al*., 1996). In the present study endophytes were isolated and identified from four medicinal plants based on the 18SrRNA sequencing and phylogentic tree, the unknown

fungal strains have been identified.

**Medicinal plants:** *Carica papaya* commonly called papaya tree belongs to *Caricaceae* family. Papaya leaves contain powerful healing compounds that are very important for curing cancer and dengue fever (Paul, 2013). Hypoglycemic activity was seen in aqueous seed extract of *Carica papaya* in normal male wistar rats. It was found that crude extract significantly and progressively lowered fasting blood sugar, triglyceride, total cholesterol, low density lipoprotein cholesterol (LDL-c) and very low density lipoprotein cholesterol (VLDL-c) dose dependent (Adeneyea and Olagunjub, 2009).

*Phyllanthus amarus* belongs to *Phyllanthaceae* family. In India, *Phyllanthus amarus* is widely distributed as a weed. It has been used as a herbal remedy for kidney stones, viral infections, liver disorders, and many other ailments and has valuable compounds such as lignins, flavonoids, tannins, polyphenols, triterpenes, sterols and alkaloids (Itoro *et al*., 2013). The compounds isolated from *P. amarus* show a wide spectrum of pharmacological activities (Patel *et al*., 2011) including antiviral, anti-inflammatory, anticancer, antidiabetic, nephron protective and diurectic properties (Saranraj and Sivasakthivelan, 2012). Fresh leaf paste has wound healing capacity and used to cure white spots on skin and jaundice (Sonia *et al*, 2014).

*Tinospora cordifolia* commonly known as Amruthaballi belongs to *Menispermaceae* family and is highly potent herb used in Ayurveda for treating diabetes. The plant contains various secondary metabolites like tinosporine, tinosporide and β-sitosterol. Phenylpropanoids, diterpene furan glycosides, diterpene furon glycosides and plytoecdysones are present in methanolic extract of plant. The bitter principle of plant has various properties like antiperiodic, anti-inflammatory antispasmodic and antipyretic properties (Savitha *et al*., 2012). Tribals of Korkus (Melghat, Maharashtra, India) have been using the herb for polyuria, diabetes and fever (Tambekar *et al*., 2009).

*Azadirachta indica* commonly called as Neem tree which belongs to *Meliaceae* family. It is one of the most effective medicinal plant in natural therapy and Ayurveda in India. Its leaves, stem, bark and seeds possess hypoglycemic activity in increasing insulin secretion from beta cells of the pancreas. Leaves are characterized by the presence of high fiber content that is potent in diabetes management and controlling of post-prandial hyperglycemia through delaying gastric emptying (Tripathi *et al*., 2011).

## MATERIALS AND METHODS

Four medicinal plants were selected for its medicinal properties collected from the Biligirirangana Hill, located in Chamrajanagar district, South Eastern Karnataka, India. The hill latitude ranges from 11° 40' to 12° 09' North and 77° 05' to 77° 01' East and covers the area of 540 sqkm.

Plant samples were brought to laboratory in polythene bags within 24hrs. Surface sterilization was performed. The sample were washed under running tap water to remove dirt on the surface, then cut into small size segment and sequential rinsing with 70% ethanol for 30 seconds, and 0.5% sodium hypochlorite for 1 minute followed by sterile distilled water for 2-3 times and dried using sterile Whatman no. 1 filter paper The dried leaf segments were placed on petridish containing sabouraud dextrose agar (SDA) media and allowed to grow in room temperature (Schulz *et al*., 1993). After seven days, the emergence of fungal mycelium is examined by colony characteristics, macroscopic and microscopic observations. The unidentified fungal culture was subjected to 18SrRNA sequencing and phylogenetic analysis.

Mycelia is scraped from a 10-day-old culture grown on SDA media and placed in a sterile 2 ml Eppendorf tube. The genomic DNA is extracted from 0.5-1 g chilled mycelia in liquid nitrogen using SDS-CTAB method (Kim *et al*., 1990) and by using the InstaGene TM Matrix Genomic DNA isolation kit (Catalog # 732-6030).

Target gene fragment is amplified using Thermo Scientific Veriti Thermal Cycler. DNA fragments are amplified using 1$\mu\ell$ of template DNA in 20$\mu\ell$ of total PCR reaction mixture using ITS1/ITS4 primers (50 pmol) followed by 35 amplification cycles with denaturation at 94℃ for 45 second, primer annealing at 52℃ for 60 second, and extension or chain elongation at 72℃ for 60 second. PCR products were electrophoresed in 1.2% (w/v) agarose gels, stained with ethidium bromide and viewed under UV light to check the purity and size according to (Schoch *et al*., 2012). Primary PCR products showed the existence of multiple bands together. The unincorporated PCR primers and dNTPs is removed from the agarose gel with a sterile scalpel, and it is directly purified with PCR products by using Montage PCR Clean up kit (Millipore) according to the manufacturer's protocol.

The fungal isolates were identified by sequencing the translation elongation factor-1α (TEF-1α) gene and internal transcribed spacer (ITS) regions. The fungal ITS regions are amplified by PCR using the universal primers ITS1 and ITS4 primer pairs (White *et al*., 1990). Sequencing reactions is performed using ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq®DNA polymerase. Single-pass sequencing was performed on each template using16S rRNA gene universal primers. The fluorescent-labeled fragments are purified from the unincorporated terminators with an ethanol precipitation protocol. The samples are

re suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Bio systems).

Based on the bidirectional sequencing of 18SrRNA gene of 1400 bp the microbial communities are examined using eukaryotic and prokaryotic specific primers separately. The two samples are used to obtain 16S amplicons by using prokaryotic universal primers Ribosomal RNA. ITS Region Forward Primer ITS1 (TCCGTAGGTGAACCTGCGG) and Reverse Primer ITS4 (TCCTCCGCTTATTGATATGC) ~550bp.

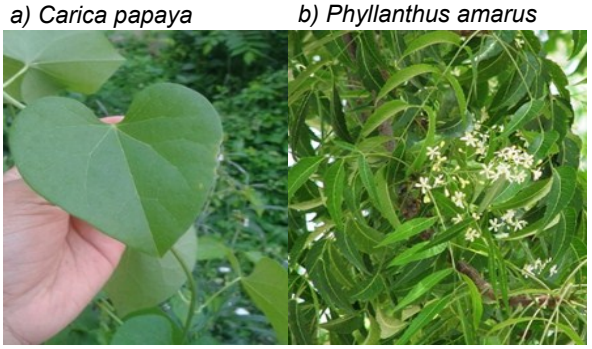
## RESULTS

Medicinal plants *Carica papaya, Phyllanthus amarus, Tinospora cordifolia, Azadirachta indica* were collected from Biligirirangana hills for the isolation of endophytic fungi (Fig 1). In the present study, total fifteen endophytic fungi were isolated from the leaves of four medicinal plants and identified by colony morphology, macroscopic and microscopic observations. It was found to be *Curvularia sp, Fusarium sp, Penicilluium sp, Colletotrichum sp, Aspergillus sp, Alternaria sp* respectively. Among the isolated fungal colony, the unidentified strain from each of the sample was subjected to molecular sequencing and phylogenetic analysis (fig 2). The identified strain were *Stemphylium lycopersici, Epicoccum nigrum, Leptosphaerulina arachidicola, Phomopsis azadirachtae***.** (Table 1). The sequences were subjected for mega BLAST analysis (http://www.ncbi.nlm.nih.gov/), Homology was calculated by pair wise comparison of sequence from NCBI database and the sequences were deposited to the gene bank with the Accession number *S. lycopersici*- MH375406, *E. nigrum* - MH375409, *L. arachidicola*- MH250053 and *P. azadirachtae*- MH375411. (Table 2). The Internal Transcribed Spacer region is regarded as a DNA barcode marker for the identification of fungi, ITS region can be amplified in single PCR of 400 to 700bp in length 543bp. The ITS region contains ITS1 and ITS4 that are located between the 18S and 28S ribosomal subunits and the ITS1 and ITS4 regions are separated by the 5.8S ribosomal RNA. 5.8S rRNA is a non-coding RNA component of the large subunit of the eukaryotic ribosome which plays an important role in protein translation.

## DISCUSSION

*Phomopsis sp.* is an endophytic fungi that belongs to *Diaporthaceae* family and consists of approximately 900 species from a wide range of hosts (Udayanga *et al.,* 2011). Several species of *Phomopsis* have been reported as plant endophytes. (Murali *et al.,* 2006; Zhang *et al.,* 2014) studied antioxidant, antifungal and cytotoxic activity of *Phomopsidone* and isobenzofuranones isolated

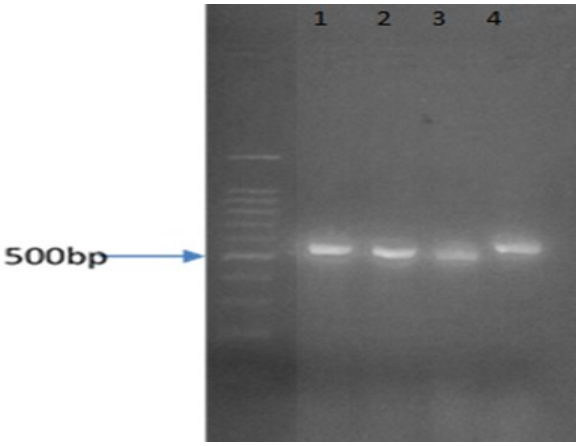
*a) Carica papaya* *b) Phyllanthus amarus*



*c) Tinospora cordifolia* *d) Azadirachta indica*

**Fig. 1.** *Medicinal plants collected from Biligirirangana hill.*


**Fig. 2.** *Polymerase Chain Reaction.*




**1-** *Stemphylium lycopersici*, **2-** *Epicoccum nigrum*, **3-** *Leptosphaerulina arachidicola*, **4-** *Phomopsis azadirachtae***.**

**Fig. 3.** *1% Agarose gel data showing the band of amplified DNA.*

from *Phomopsis sp*. (Kumaresan *et al.,* 2014) studied the Biological activity spectrum of ethyl acetate extract of the *Phomopsis sp* isolated from *Andrographis paniculata* and was analyzed in GC-MS. *Epicoccum sp.* is a fungi belongs to *Pleosporaceae* family. (Fa´varo *et al*., 2012) demonstrated

**Table 1.** Ribosomal Gene organization and Target region amplified.

| ITS Primer for Fungi | Sequence Details | Amplicon size (bp) |
|---|---|---|
| Forward PrimerITS1 | TCCGTAGGTGAACCTGCGG | |
| Reverse Primer ITS4 | TCCTCCGCTTATTGATATGC | **~550bp** |

**Table 2.** Strains identified based on the BLAST search of NCBI Gene database.

| Sl. No | Fungal Isolate | Isolated from medicinal plants | Strain identification | Family | Gene bank accession no. | Phylogenetic tree Reference |
|---|---|---|---|---|---|---|
| 1. | F1 | *Carica papaya* | *Stemphylium lycopersici* | Pleosporaceae | MH375406 | Fig. 4 |
| 2. | F2 | *Phyllanthus amarus* | *Epicoccum nigrum* | Pleosporaceae | MH375409 | Fig. 5 |
| 3. | F3 | *Tinospora cordifolia* | *Leptosphaerulina arachidicola* | Pleosporaceae | MH250053 | Fig. 6 |
| 4. | F4 | *Azadirachta indica* | *Phomopsis azadirachtae* | Diaporthaceae | MH375411 | Fig. 7 |

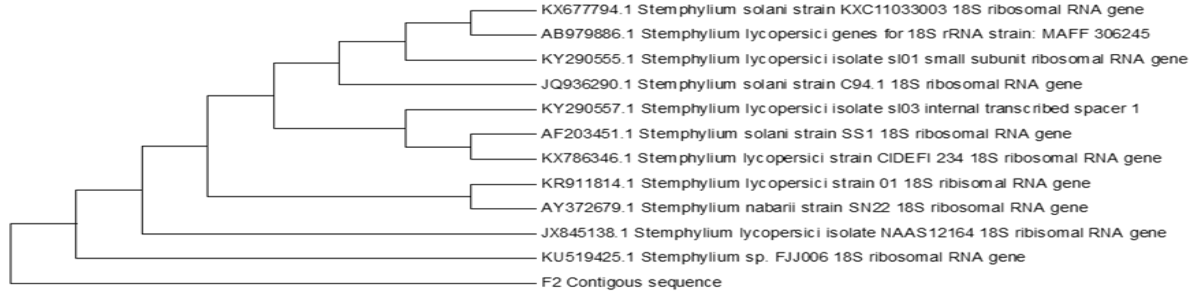



**Fig. 4.** *F1 Fungal isolate (Stemphylium lycopersici) phylogenetic tree*

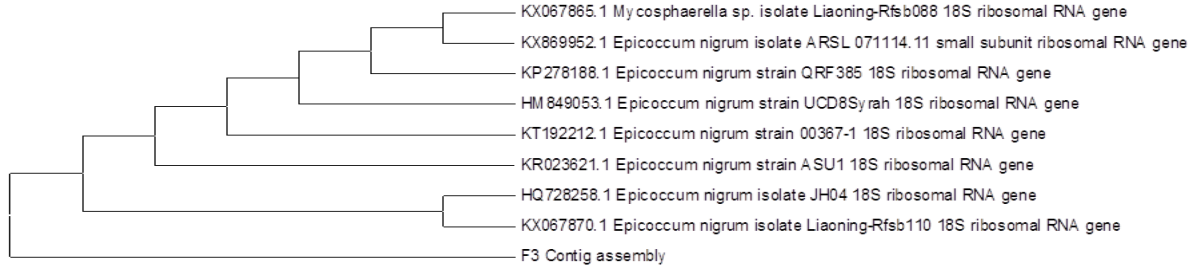



**Fig. 5.** *F2 Fungal isolate (Epicoccum nigrum) phylogenetic tree*

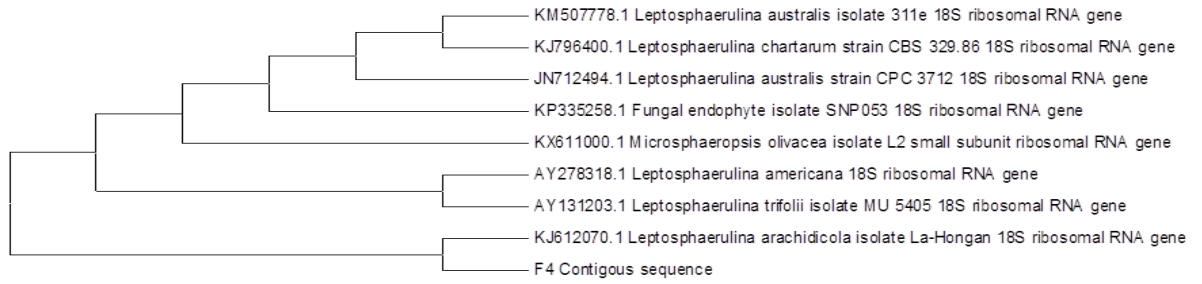



**Fig. 6.** *F3 Fungal isolate (Leptosphaerulina arachidicola) Phylogenetic tree.*

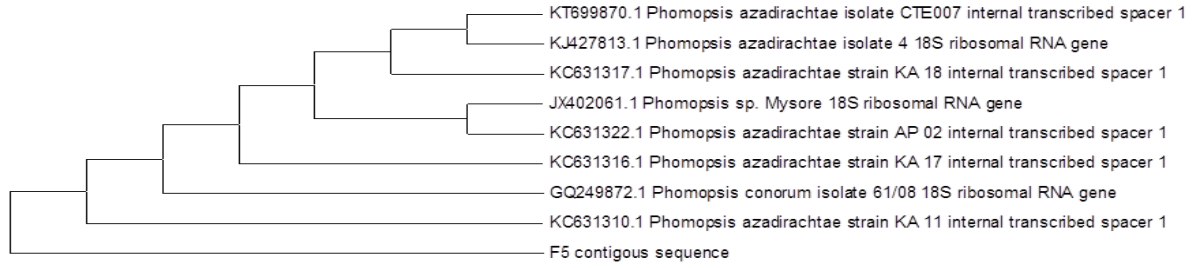



**Fig. 7.** *F4 Fungal isolate (Phomopsis azadirachtae) Phylogenetic tree.*

**Figs. 4-7.** *Represents phylogenetic tree, reference Nucleic Acids Research Vol. 18 Supplement. Determined sequence based on the 18SrRNA.*

that *E. nigrum* has great potential for sugarcane crop application because it is capable of increasing the root system biomass and controlling pathogens. They studied the basic aspects of the interaction of ubiquitous endophyte with the host plant *E. nigrum* with sugarcane as a facultative endophytism for the phylloplane environment and also to a better use of microbial endophytes in agriculture.

## Conclusion

Medicinal plants are one of the important natural source for curing diseases. Endophytic fungi are the found as the symbionts present internally colonizing internal tissues of the cell. Manual sequencing of 18S rRNA genes is one of the most commonly used method for phylogenetic analysis to identify at species level. Phylogenic tree based on ITS region gene sequences showing relationships and the most close type strain species of four organisms based on sequence alignment and Phylogenetic analysis the identification of fungal isolates has been summarized. With this required parameters it has proposed that ITS region along with 5.8S gene sequence is the standard barcode for fungal genome identification. Therefore ITS sequencing is fast and sensitive method widely used for the molecular phylogeny of the fungal genome study in a short period of time. Hence it is the most useful method for identification of fungi at species level. The organism is related species reported in this paper as a fungal endophyte, future work to be carried out for the extraction of bioactive compounds.

## ACKNOWLEDGEMENTS

The authors are thankful to Mangalore University for providing the fellowship and facilities to carry out this work.

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