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MOLECULAR IDENTIFICATION OF ENDOPHYTIC FUNGI FROM THE GRASSES OF KODAGU DISTRICT, KARNATAKA, INDIA

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Abstract: This study reports taxonomic characterization of seven species of *Curvularia,* viz. *C. kusanoi*, *C. trifolii*, C. *coatesiae*, *C. falsilunata*, *C. lunata*, *C. umbiliciformis* and *C. phaespora,* three species of *Epicoccum,* viz. *E. sorghinum*, *E. latusicollum* and *E. nigrum,* two species of *Nigrospora* viz. *N. sphaerica* and *N. oryzae* among others isolated as endophytic fungi, from grasses of Kodagu district in Karnataka state, India. These endophytes were identified by 18S rDNA sequencing protocol.

***Index Terms* - Grasses, endophytic fungi, molecular identification, 18S rDNA.**

I. INTRODUCTION

The grass family Poaceae of the plant kingdom consists around 12,000 species under 780 genera (Kellongg et al., 2020). The grasses are perennial or annual and usually terrestrial and free standing; rarely they are vines or aquatics. Their root system consists of fine, fibrous roots. The stems are called culms and are herbaceous or woody, and range from 2 cm to 40 m in height and 30 cm in diameter as in bamboos (Clay, 1988). The major part of grass biomass, the leaf blade, is usually long and narrow, with parallel margins.

Endophytes are microorganisms (bacteria and fungi) living in the intercellular spaces, such as cortex, epidermis, endodermis and vascular bundles of host plant tissues and also inside the cells (symplast), both of which causes no physical damage (Saikkonen et al., 1998). The endophytes are mutualists that live asymptomatically inside the plant tissues and are found in nearly whole plant kingdom.

The endophytes offer several advantages to plants such as fighting against plant pathogens and herbivores, and assisting plant growth by helping the production or promotion of growth hormones and secondary metabolites (Schulz and Boyle, 2006; Yim, et al., 2007).

Endophytic fungi in return receive shelter and nutrition from host plants. Besides, endophytic fungi provide tolerance to drought and promote growth in poor soil. Endophytes synthesizing bioactive compounds that can be used against numerous pathogens are poorly investigated amongst microorganisms.

Preliminary studies indicated that endophytes are possible sources of useful bioactive and chemically novel compounds that have proven to be useful in novel drug discovery (Baron and Rigobelo, 2022). The aim of this research work was molecular identification of endophytic fungi which colonize the leaves of grasses.

II. MATERIALS AND METHODS

2.1 Sample collection site:

Grass leaf samples for isolation of endophytic fungi were randomly selected from different locations of Kodagu. Kodagu is a hill district of Karnataka State in southern India and occupies about 4,100 Km^2 of land on the eastern slopes of the Western Ghats. The district lies between North latitude 11°56' to 12°56' and east longitude 75°22' to 76°11' (RangaPrasad, 2016).

2.2 Authentication:

18 grass species, along with its voucher specimens (Herbaria) listed in Table 1 were selected for isolation of endophytic fungi have been deposited at the Herbaria of Department of Studies in Botany, Mysore University, Manasagangotri, Mysore.

2.3 Isolation of endophytic fungi from grasses:

2.3a Surface disinfection of plant material:

Leaf samples collected were washed thoroughly in running tap water to remove surface dirt, air dried and excluded of visibly diseased/damaged parts. Leaf blade is cut into sections (4-5 mm^2) and surface sterilized by following the modified method of Araujo et al., 2001 and Muazzam et al., 2015 by dipping the mature leaf in 70% ethanol for 30-60 sec, followed by 1% NaOCl (sodium hypochlorite) for 3 min (Muazzam et al., 2015). The sample leaf was rinsed once again with 70% ethanol and finally twice in sterile distilled water.

The disinfection process is checked by pour plating the final rinse solution on to the PDA (Potato Dextrose Agar) media for Fungal and TSA (Tryptic Soya Agar) media for bacterial surface contaminants isolation.

2.3b Isolation of Endophytic Fungi:

Following Suresha and Jayashankar, 2018, leaf segments were placed onto PDA media (supplemented with 50 µg/ml chloramphenicol to suppress bacterial growth) at specific distance (2 cm from the centre) aseptically so that the freshly cut edges are in direct contact with the agar surface and incubated for 5-7 days at 25-28ºC or until the outgrowth of endophytic fungus was discerned. Hyphal tips originating from the plant segments are sub cultured and brought to pure culture in PDA slants without antibiotics and stored at 4ºC for further use.

2.4 Molecular identification of endophytic fungi using 18S rRNA method:

Genomic DNA isolation: DNA isolation from microbial samples was done using the Expure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt. Ltd.

1. **Lysis/homogenization:**

Fungal mycelium grown in monolayer was lysed by suspending 1-3 colonies aseptically with 500 µl of lysis buffer in a 2 ml micro centrifuge tube and by repeated pipetting. Added 4 µl of RNAse and 500 µl of neutralization buffer into it. Vortex the content and incubated the tubes for 30 minutes at 65˚C in water bath. DNA solutions were mixed by inversion to minimize shearing.

The micro-centritube was centrifuged for 10 minutes at 10,000 rpm and transferred the resulting viscous supernatant was transferred into a fresh 2 ml micro centrifuge tubewithout disturbing the pellet, following centrifugation. Six hundred µl of Chloroform Isoamly Alcohol was added to the centrifuge and hand mixed vigorously. The tubes are again centrifuged for 10 minutes at 10,000 rpm and 600 µl of aqueous phase was carefully transferred into a fresh2ml micro centrifuge tube.

2. **Binding:**

600 µl of binding buffer was added to the content, mixed thoroughly by pipetting and incubated at room temperature for 5 minutes. 600 µl of the contents was added to a spin column placed in 2 ml collection tube, centrifuged for 2 minutes at 10,000 rpm and the flow-through was discarded. The spin column was reassembled, the remaining 600µl of the lysate collection tube then transferred to the collection tube and centrifuged for 2 minutes at 10,000 rpm and the flow-through was discarded.

3. **Washing:**

500 µL washing buffer I was added to the spin column, centrifuged at 10,000 rpm for 2 mins and the flow- through was discarded. The spin column was reassembled, 500µl washing buffer II was added, centrifuged at 10,000 rpm for 2minsand flow-through was discarded. The tube was dry-spined for 5 minutes at 10,000 rpm and the spin column was transferred to a sterile 1.5-ml micro centrifuge tube.

4. **Elution:**

100 µl of elution buffer was added at the middle of spin column. Care was taken to avoid touch with the filtrate. The tubes were incubated for 2 minutes at room temperature and centrifuged at 10,000 rpm for 2 minutes. Thebuffer in the micro centrifuge tube contains the DNA. The DNA concentrations were measured by Qubit 3.0

PRIMER DETAILS:

Primer Name	Sequence Details	Number of Base
ITS1	5' TCCGTAGGTGAACCTGCGG 3'	19
ITS4	5' TCCTCCGCTTATTGATATGC 3'	20

5 µL of isolated DNA was added in 25 µL of PCR reaction solution (1.5 µL of Forward Primer and Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). The PCR was performed using the following thermal cycling conditions.

The DNA template was heated to 95°C, aiding the weak hydrogen bonds that hold DNAstrands together in a helix to separate creating single stranded DNA. The mixture is cooled to 55°C allowing the primers to bind (anneal) totheir complementary sequence in the template DNA.

The reaction is then heated to 72° C, the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner,using the target DNA as a template.

2.5 PCR amplification:

Fungal DNA was multiplied using 18s rRNA ITS1F (5' TCCGTAGGTGAACCTGCGG 3') and ITS4R (5' TCCTCCGCTTATTGATATGC 3') gene primers. The amplified genes were sequenced by commercial company Yaazh Xenomics Pvt. Ltd., Coimbatore, Tamil Nadu, India. The obtained sequence data were aligned by using the BLAST software (http://blast.ncbi.nlm.nih.gov) algorithm at NCBI.

Bioinformatics protocol:

1. The sequence was blast using NCBI blast similarity search tool. The phylogenyanalysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.
2. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0. 91b.This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.

3. PhyML was shown to be at least as accurate as other existing phylogeny programs usingsimulated data, while being one order of magnitude faster. PhyML was shown to be atleast as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering.(Dereeper et al., 2008).

III. RESULTS AND DISCUSSION

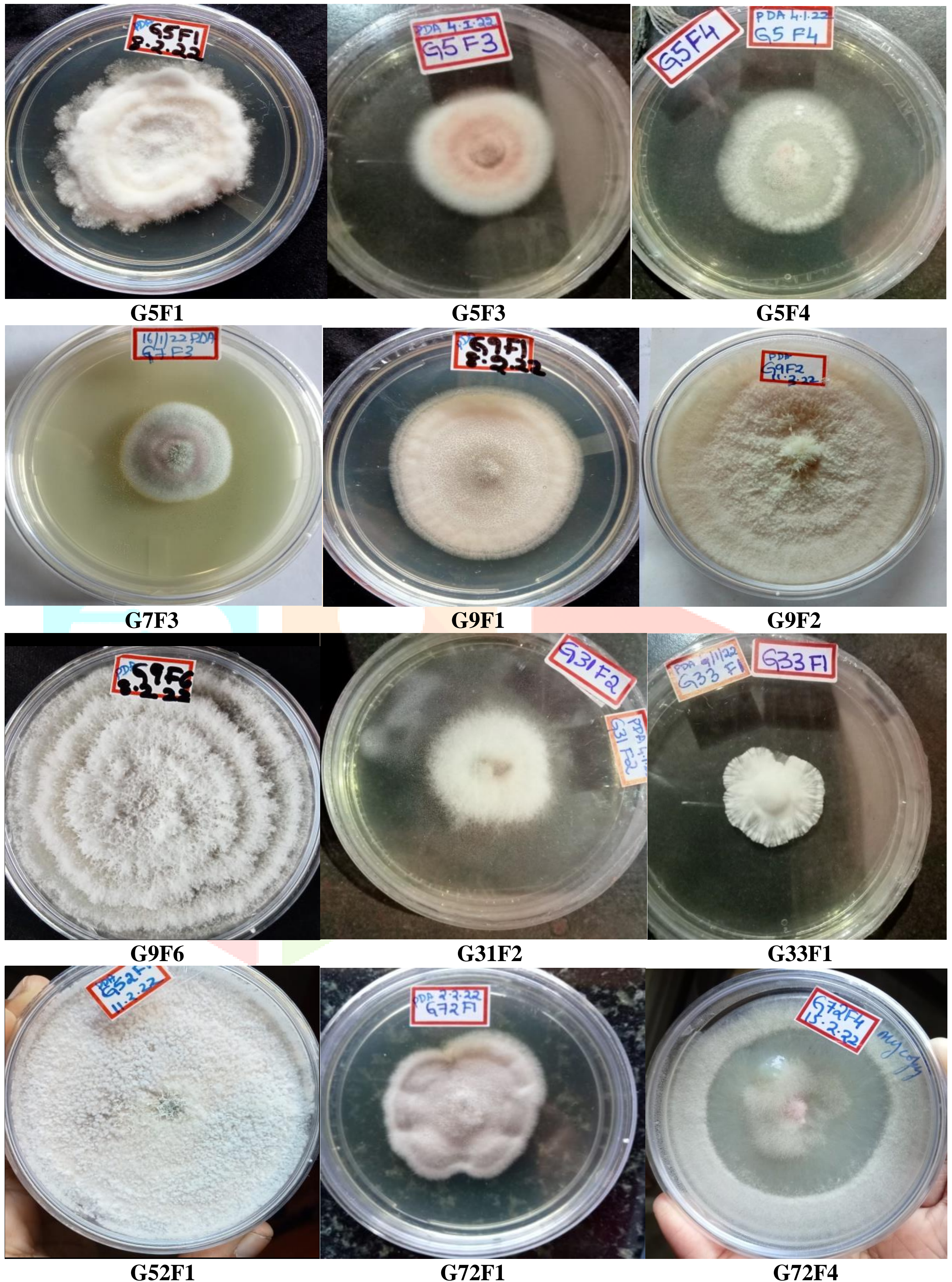


Figure 1: Above plates showing pure culture colonies of isolated endophytic fungi which were molecularly identified as: G5F1 - *Neopestalotiopsis clavispora.* G5F3 - *Epicoccum sorghinum.* G5F4 - *Colletotrichum gloeosporioides.* G7F3 - *Curvularia kusanoi.* G9F1 - *Curvularia trifolii.* G9F2 - *Curvularia coatesiae.* G9F6 - *Macrophomina phaseolina.* G31F2 - *Annulohypoxylon atroroseum.* G33F1 - *Xylaria grammica* G52F1 - *Curvularia falsilunata.* G72F1 - *Bipolaris bicolor.* G72F4 - *Nigrospora sphaerica*

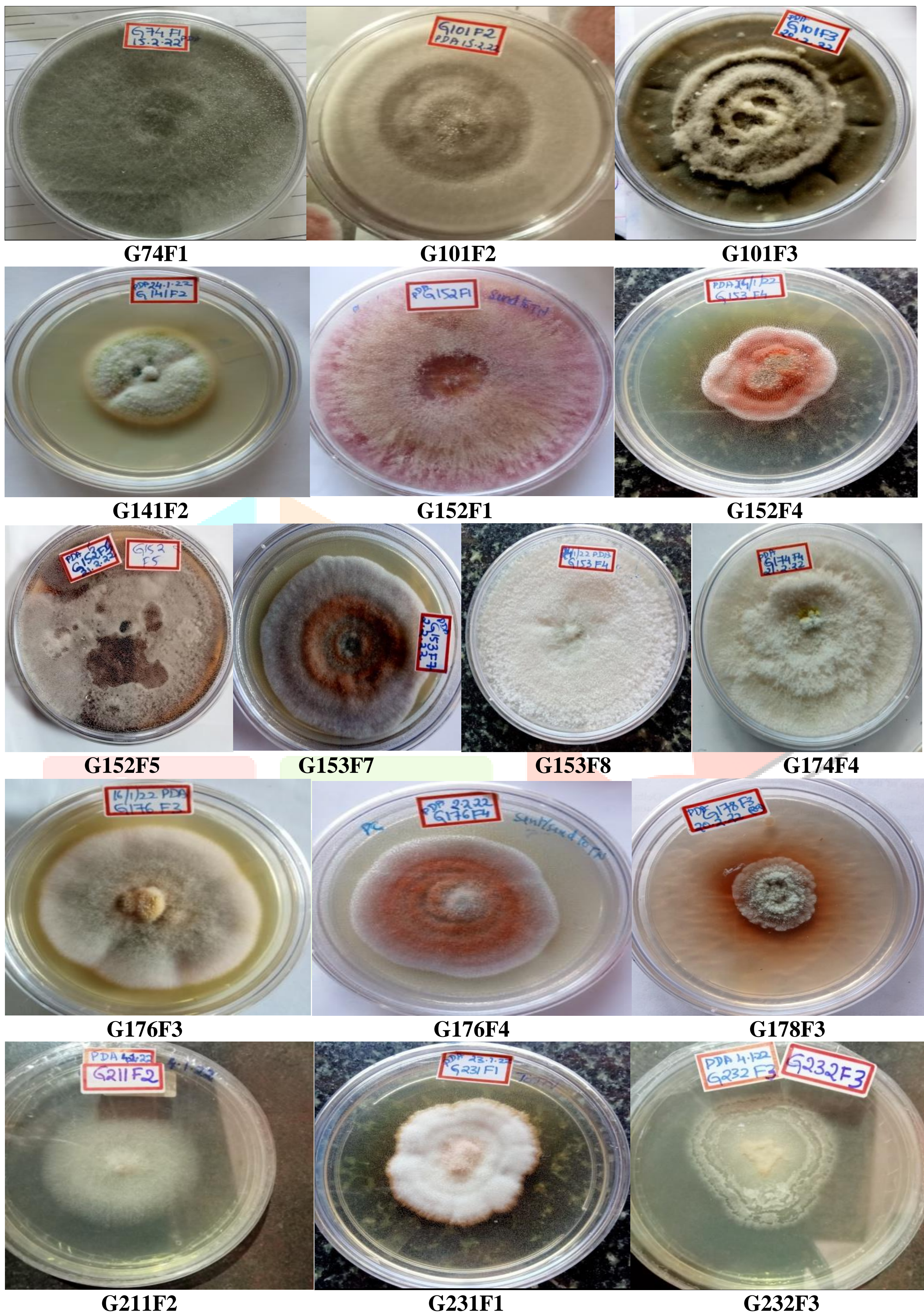


Figure 2: Above plates of endophytic fungi were molecularly identified as: G74F1 - *Curvularia kusanoi.* G101F2 - *Nigrospora oryzae.* G101F3 - *Diaporthe eres.* G141F2 - *Talaromyces amestolkiae.* G152F1 - *Fusarium graminearum.* G152F4 - *Curvulari kusanoi.* G152F5 - *Curvularia lunata.* G153F7 - *Ascochyta phacae.* G153F8 - *Nigrospora sphaerica.* G174F4 – *Curvularia umbiliciformis.* G176F3 - *Epicoccum latusicollum.* G176F4 - *Cochliobolus kusanoi.* G178F3 – *Curvularia phaespora*. G211F2 – *Arthrinium marii*. G231F1 – *Epicoccum nigrum*. G232F3 – *Phoma insulana*.

Table 1: List of Endophytic fungi isolated from its respective grass species.

SL. NO	CULTURE ID	ENDOPHYTIC FUNGAL SPECIES	GRASS SPECIES CODE	GRASS SPECIES	ACCESSION NUMBER
1	G5F1	*Neopestalotiopsis clavispora* (G.F. Atk.) Maharachch., K.D. Hyde & Crous, 2014	G5 UOMBOT22G04	*Arundinella ciliata*	ON970005
2	G5F3	*Epicoccum sorghinum*(Sacc.) Aveskamp, Gruyter & Verkley			ON970009
3	G5F4	*Colletotrichum gloeosporioides*(Penz.) Penz. & Sacc., 1884			ON970010
4	G7F3	*Curvularia kusanoi* (Y. Nisik.) Manamgoda, Rossman & K.D Hyde, 2014	G7 UOMBOT22G06	*Arundinella pumila*	ON969999
5	G9F1	*Curvularia trifolii* (Kauffman) Boedijn, (1933)	G9 UOMBOT22G08	*Axonopus compressus*	MG029439.1
6	G9F2	*Curvularia coatesiae* Y.P.Tan & R.G. Shivas, 2018			LC605635.1
7	G9F6	*Macrophomina phaseolina* (Tassi) Goid. (1947)			HG938366.1
8	G31F2	*Annulohypoxylon atroroseum*(J. D. Rogers) Y. M. Ju, J. D.Rogers & H. M. Hsieh 2005	G31 UOMBOT22G12	*Chloris barbata*	ON970011
9	G33F1	*Xylaria grammica* (Mont.) Mont., 1851	G33 UOMBOT22G66	*Chrysopogon hackelii*	ON970012
10	G52F1	*Curvularia falsilunata* M. Raza, K.D. Hyde & L. Cai, 2019	G52 UOMBOT22G19	*Digitaria ciliaris*	MN215660.1
11	G72F1	*Bipolaris bicolor Helminthosporium bicolor* Mitra, 1931	G72 UOMBOT22G23	*Eleusine indica*	ON970001
12	G72F4	*Nigrospora spherica* (Sacc.) E.W. Mason (1927)			ON970006
13	G74F1	*Curvularia kusanoi* (Y. Nisik.) Manamgoda, Rossman & K.D Hyde, 2014	G74 UOMBOT22G25	*Eragrostis tenuifolia*	ON970007
14	G101F2	*Nigrospora oryzae* (Berk. & Broome) Petch, 1924	G101 UOMBOT22G30	*Heteropogon contortus*	ON970008
15	G101F3	*Diaporthe eres* Nitschke, 1870			ON970019
16	G141F2	*Talaromyces amestolkiae* N. Yilmaz, Houbraken, Frishvad & Samson 2012	G141 UOMBOT22G35	*Melinis minutiflora*	ON970016
17	G152F1	*Fusarium graminearum* Schwabe, 1839	G152 UOMBOT22G38	*Oplismenus compositus*	ON970002
18	G152F4	*Curvularia kusanoi* (Y. Nisik.) Manamgoda, Rossman & K.D Hyde, 2014			ON970015
19	G152F5	*Curvularia lunata* (Wakker) Boedijn, (1993)			KF897859.1
20	G153F7	*Aschochyta phacae* (Corbaz) Q. Chen & L. Cai, 2015	G153 UOMBOT22G39	*Oryza sativa*	ON970003
21	G153F8	*Nigrospora sphaerica* (Sacc.) E.W. Mason (1927)			ON970017

22	G174F4	*Curvularia umbiliciformis* M. Raza, K.D. Hyde & L. Cai, 2019	G174 UOMBOT22G43	*Paspalum conjugatum*	MN215713.1
23	G176F3	*Epicoccum latusicollum* Q. Chen, Crous & L. Cai, 2017	G176 UOMBOT22G45	*Pennisetum pedicellatum*	ON970000
24	G176F4	*Cochilobolus kusanoi* (Y. Nisik.) Manamgoda, Rossman & K.D Hyde, 2014			ON970004
25	G178F3	*Curvularia phaespora* M. Raza, K.D. Hyde & L. Cai, 2019	G178 UOMBOT22G67	*Pennisetum purpureum*	MN215686.1
26	G211F2	*Arthrinium marii*(Larrondo & Calvo, 1990) Pintos & P. Alvarado, 2021	G211 UOMBOT22G49	*Saccharum officinarum*	ON970013
27	G231F1	*Epicoccum nigrum* Link 1816	G231 UOMBOT22G58	*Themeda tremula*	ON970018
28	G232F3	*Phoma insulana* (Mont.) Boerema & Malathr., 1982.	G232 UOMBOT22G59	*Themeda triandra*	ON970014

The above table shows that a total number of 25 different endophytic fungal species were isolated from the leaves of randomly selected 18 grass species.

Majority of identified endophytic fungal species were from genera *Curvularia*, including seven species, namely *Curvularia kusanoi*, *C. trifolii*, C. *coatesiae*, *C. falsilunata*, *C. lunata*, *C. umbiliciformis* and *C. phaespora*. Three species were from *Epicoccum* genera, namely *Epicoccum sorghinum*, *E. latusicollum* and *E. nigrum*. Two species were from *Nigrospora* genera, namely *Nigrospora sphaerica* and *N. oryzae*. Remaining species belonged to 13 different genera, namely, *Neopestalotiopsis, Colletotrichum, Macrophomina, Annulohypoxylon, Xylaria, Bipolaris, Diaporthe, Talaromyces, Fusarium, Aschochyta, Cochilobolus, Arthrinium,*and *Phoma*.

It was noticeable that *Curvularia kusanoi* was commonly present in three grass species namely, *Arundinella pumila, Eragrostis tenuifolia, and Oplismenus composites. Nigrospora sphaerica* was commonly present in two grass species namely, *Eleusine indica, and Oryza sativa.*

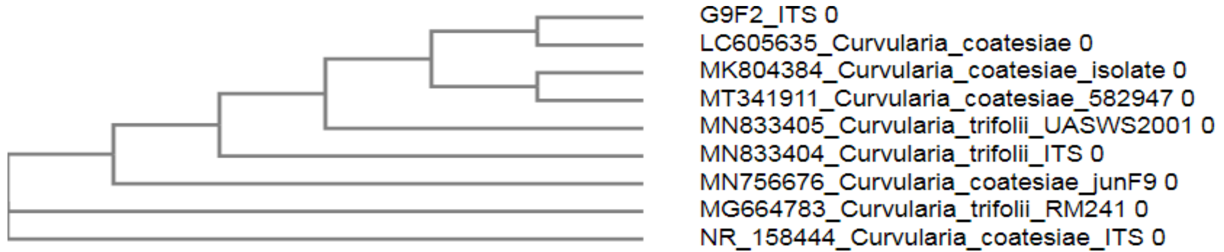
G7F3 - *Curvularia kusanoi*

MT557514.1:13-498_Phoma_sp.
EU082106.1:55-541_Phoma_herbarum
Contig_G7F3_Cochliobolus_kusanoi
JN943393.1:76-561_Cochliobolus_kusan
MT322734.1:31-516_Ascochyta_phacae
MT557354.1:19-504_Curvularia_kusanoi

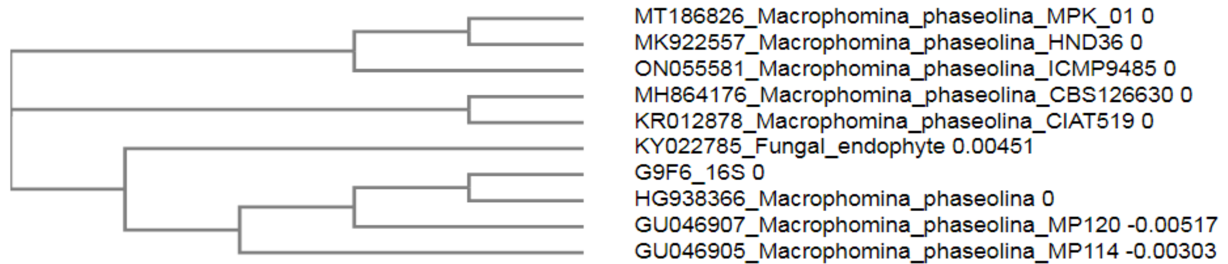
G9F1 - *Curvularia trifolii*

G9F1_ITS 0.01322
MG029439_Curvularia_trifolii 0.00166
KM507765_Curvularia_sp._325c 0.00023
MT341911_Curvularia_coatesiae 0
NR_158444_Curvularia_coatesiae_BRIP 0
JN712458_Curvularia_trifolii_strain 0
MT849336_Curvularia_sp. 0
AF212310_Curvularia_trifolii_ITS 0
AF455446_Curvularia_trifolii_wb403 0

G9F2 - *Curvularia coatesiae.*

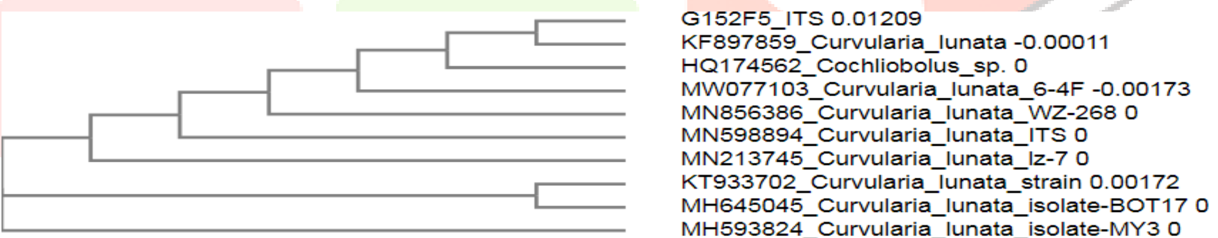


G9F6 - *Macrophomina phaseolina.*

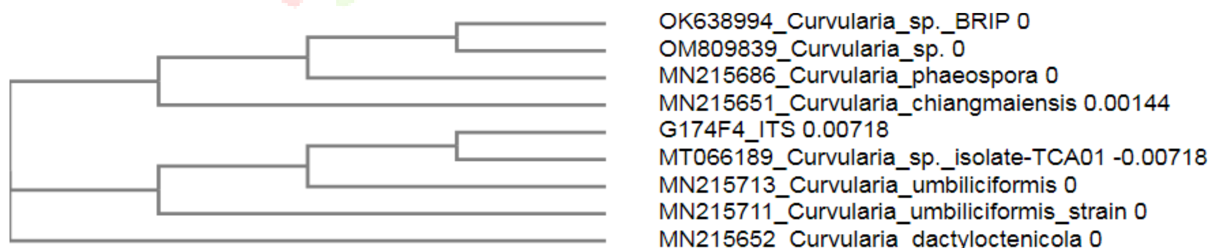


G52F1 - *Curvularia falsilunata.*

G152F5 - *Curvularia lunata.*



G174F4 – *Curvularia umbiliciformis*.



G178F3 – *Curvularia phaespora*.

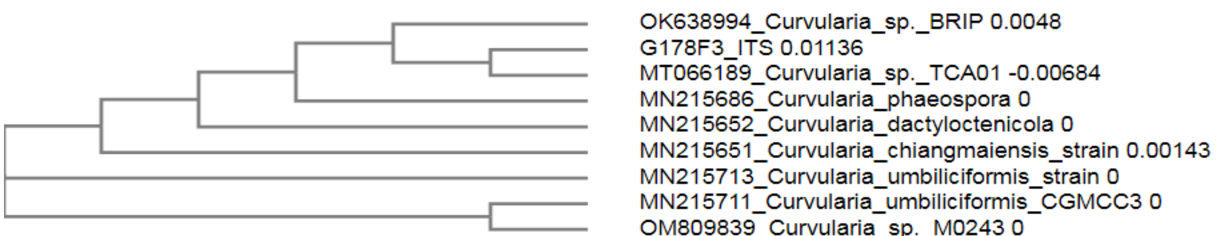


Figure 4: Evolutionary relationships of few taxa of fungal endophytes (includes all *Curvularia* species) isolated from the grasses of Kodagu district are represented here.,

Table 2: Relative abundance of Endophytic fungi isolated from grass specimens of Kodagu.

Sl. No	Genera of Endophytic fungi	No. of Species	Relative abundance %	Sl. No	Genera of Endophytic fungi	No. of Species	Relative abundance %
1	Curvularia	7	28	9	Colletotrichum	1	4
2	Epicoccum	3	12	10	Diaporthe	1	4
3	Nigrospora	2	8	11	Fusarium	1	4
4	Annulohyposylon	1	4	12	Macrophomina	1	4
5	Arthrinium	1	4	13	Neopestalotiopsis	1	4
6	Aschochyta	1	4	14	Phoma	1	4
7	Bipolaris	1	4	15	Talaromyces	1	4
8	Cochilobolus	1	4	16	Xylaria	1	4
	Total					25	100

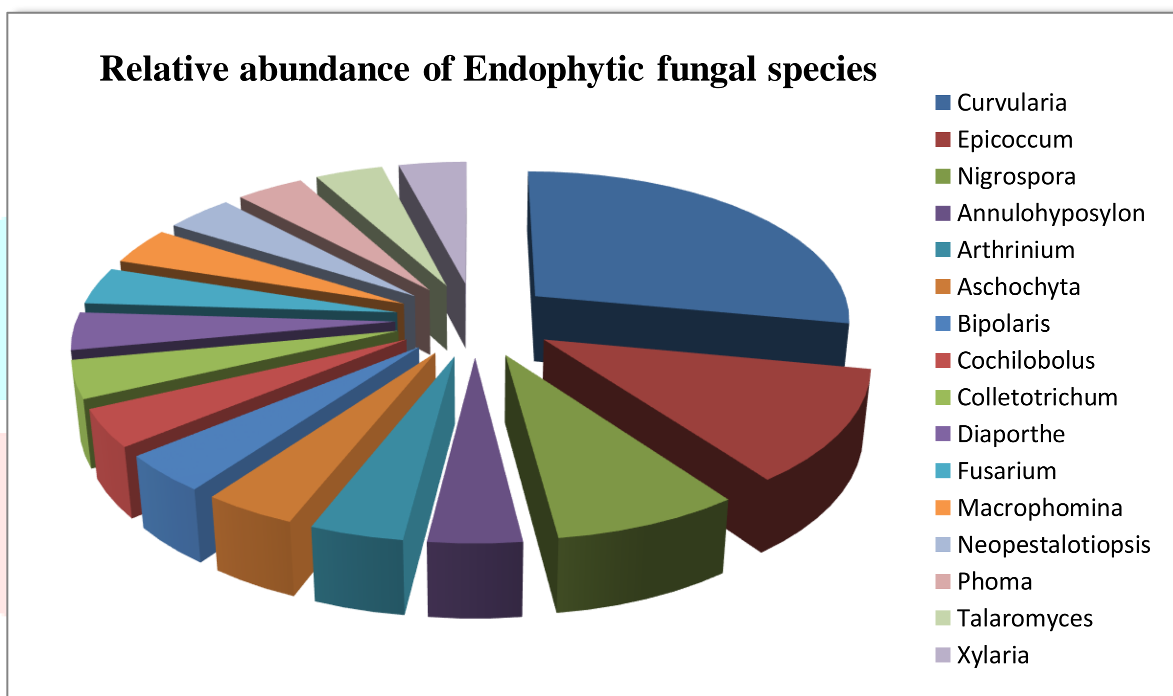


Figure 5: Relative abundance of Endophytic fungi with respect to its genera isolated from the leaves of different grass species.

IV. ACKNOWLEDGMENT

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