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Molecular characterization of fungal endophytes associated with medicinal plant, *Ficus racemosa*

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Abstract

Fungal endophytes are a diverse group of microorganisms, heterotrophic in nature and associated with all types of healthy plants. All plant species have one or more fungal endophytes, of some associations providing potential advantage for both the host as well as fungi. The objective of the study was to isolate and characterize the fungal endophytes from *Ficus racemosa*. A total 150 leaf segments were screened for fungal isolation, from which 88 fungal colonies were recovered and 30 different morphotypes were identified based on the phenotypic appearance. Further, the thirty morphotypes were characterized by molecular methods using Internal Transcribed Spacer (ITS) and ITS2 sequence-secondary structures based analysis. Minimum Free Energy (MFE) method was followed to elucidate the secondary structure. The tools Mfold 3.1 (Structure modelled), 4SALE 1.7 (Alignment) and ProfDistS 0.9.9 (Tree construction) were utilized for the structural and phylogenetic analysis. On the basis of morphology and molecular analysis, all 30 morphotypes were classified into 18 different species, 10 genera and 7 orders (Botryosphaeriales, Capnodiales, Diaporthales, Glomerellales, Hypocreales, Pleosphorales and Xylariales) of Ascomycota. The utilization of ITS2 secondary structure information improved the accuracy and robustness of phylogenetic analysis for species level characterization.

Key words – Endophytic fungi – ITS region – ITS2 secondary structure – 4SALE – phylogenetic analysis

Introduction

Fungal endophytes are commonly associated with all species of plants in symptomless conditions and live within the plants (Petrini 1991). Fungal symbiotic relationship with host confers protection from insects, pests, pathogens, resistance to environmental stress, promote host growth, productivity improvement, etc. (Carroll 1991, Strobel et al. 2004, Gond et al. 2010, Singh et al. 2011, Lahrmann et al. 2013). Around the world, 1.5 million species of fungi are estimated to exist, of which 7 % are constituted by endophytic fungi (Chowdhary & Kaushik 2015). The fungi represent a significant resource for the field of biotechnology and they have been recognized as a source of novel bioactive metabolites which possess great economic importance such as in pharmaceutical, agricultural and industrial usages (Bills & Polishook 1992, Strobel & Daisy 2003, Arnold et al. 2007). Characterization of fungal endophytes was essential for diversity and

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ecological analysis. Traditional methods like comparative morphological features were used for fungal taxonomy in early stages (Lodge et al. 1996, Sette et al. 2006, Crous et al. 2007, Zhang et al. 2008). However identification of closely related species, similar morphotypes and non-sporulating isolates was challenging, as media composition could change the morphological characteristic features (Hyde & Soytong 2007). Nowadays molecular methods are habitually used for taxonomical classifications due to its sensitivity and specificity for identification of the organisms (Sette et al. 2006). Several recent studies successfully identified fungi based on molecular markers like Internal Transcribed Spacers (ITS), Actin (ACT), Calmodulin (CAL), Glutamine synthetase (GS), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-tubulin (Tub2), RNA polymerase II second largest subunit (RPB2), cytochrome c oxidase subunit 1 (COX1) Translation Elongation Factor 1-alpha (TEF1), etc. (Wang et al. 2005, Arnold & Lutzoni 2007, Ligrone et al. 2007, Silva et al. 2012, Weir et al. 2012). Among them, ITS has been predominantly used as a molecular marker and has been recommended as the universal fungal barcode sequence (Schoch et al. 2012).

The ITS region is found between ribosomal large subunit (LSU) and small subunit (SSU) with three sub regions: ITS1, 5.8S and ITS2 (Fig. 1). The complete length of this region contains ~ 0.45 Kb to 0.75 Kb. The universal primers ITS1 and ITS4 are widely used to amplify the ITS region using polymerase chain reaction (PCR). The primer will bind to the ribosomal small and large subunit regions and therefore will cover the complete length of the target region. The amplified ITS region is then sequenced and the similarity between the obtained sequences and those deposited in databases is often used for the identification of fungal species (Gardes & Bruns 1993, White et al. 1990).

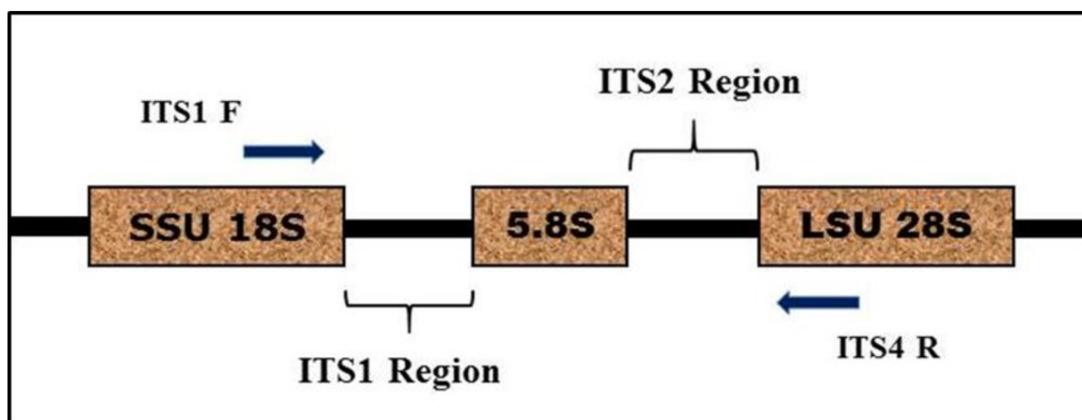

Fig. 1 – Schematic representation of Internal Transcribed Spacer (ITS) region with universal primer ITS1 and ITS4.

Recently, ITS2 has been proposed as a valuable barcode for fungal species level classifications and it is a mini barcode, easily amplified and sequenced (Keller et al. 2010, Yang et al. 2018). The ITS2 sequence-secondary structure data can improve the phylogenetic resolutions in closely related species. A number of studies have proved the potential applications of ITS2 for taxonomic classification and phylogenetic rebuilding at both the genus and species levels for eukaryotes, including animals, plants and fungi (Yao et al. 2010, Han et al. 2013). The sequence-structure based identification gives more information regarding variable characters i.e. Four helix structure, Helix III being longest compare to others, UGGU motif in 5' of the apex (deviations like UGGGU, UGG, or GGU have been described), as well as the U-U mismatch in the Helix II (Schultz & Wolf 2009). Compensatory base change (CBC) in the secondary structure is considered as a possible marker for delimitating species as well (Muller et al. 2007). The goal of this study is to explore the fungal endophytes in the leaves of *Ficus racemosa* from Azhagar hills, Madurai. The *Ficus racemosa* is a popular medicinal plant in India belonging to family Moracea and all parts of this plant are used to cure various ailments such as liver disorders, diarrhea, diabetes, hemorrhoids, respiratory and urinary diseases (Faiyaz & Asna 2010).

Materials & Methods

Sampling

Healthy leaves of *Ficus racemosa* were collected from Azhagar Hills, Madurai, Tamil Nadu, and India. Collected samples were sealed and transported to the laboratory using a sterile polythene bag and leaves were separately processed within 24 hrs of sample collection for fungal endophyte isolation.

Isolation of fungal endophytes

The disinfection of the healthy leaves was achieved by washing in running tap water to remove dust and external debris, then again thoroughly washed with sterile distilled water. Washed leaves were cut into small pieces ~1 cm in size using a sterile surgical blade. The segments were then treated with 70% ethanol for five seconds, immersed in 4% Sodium hypochlorite for ninety seconds, rinsed in autoclaved water for ten seconds and allowed to air dry for removing excess moisture present with the use of sterile tissue papers (Dobranic et al. 1995). Afterward, the sterile leaf sections were placed on Potato Dextrose Agar (PDA) plate with Streptomycin (200µg /ml) and the inoculated plates were allowed to incubate at 24 ± 2°C, 12 hrs. light: 12 hrs. dark cycles. Leaf imprinted control plates were also maintained to check the efficacy of surface sterilization. The petri dishes were checked for growth of endophytic fungi from the leaf segments. The hyphal tips emerging from the inoculated leaf segments were immediately sub-cultured on fresh PDA plates to obtain a single isolate. Further, the pure isolates were preserved in PDA slants at 4°C for further studies.

Morphology identification of fungi

The isolated endophytic fungi were grown on PDA plates at 24 ± 2°C for morphological study. The different morphological characters examined during the present study include: appearance of the colony color, growth, surface texture, margin and elevation. The conidial characteristics such as shape and size were observed in a light microscope (Olympus).

DNA Extraction

Genomic DNA was isolated from the seven day old pure fungal culture. One gram of fungal mycelia was scraped off and ground by mortar and pestle using 4ml of extraction buffer (200 mM Tris HCl, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) at pH 8.5. After that, 300µl of 3M Sodium acetate (pH 5.2) was added to 600 µl of the crushed sample, and tubes were placed at -20°C for 10 minutes. Then the tubes were spun at 12,000 rpm for 5 min and the supernatant was transferred to new sterile eppendorf tubes without disturbing the pellet. Then an equal volume of isopropanol was added to the supernatant and kept for 5 min at room temperature for precipitating the DNA. After incubation, the tube was centrifuged for 2 min at 12,000 rpm. Finally the pellet was collected and washed with 70% ethanol, air dried and suspended in 50 µl of sterile MilliQ water. The isolated fungal genomic DNA was confirmed by 0.7% of agarose gel electrophoresis (Cenis 1992).

Amplification of ITS region

The fungal ITS regions were amplified through Polymerase chain reaction (PCR) using the ITS1 and ITS4 universal primers. Bio-RAD instrument was used to perform PCR amplification with a total 25 µl reaction volume that included of 20 ng of genomic DNA, 10X PCR buffer with 25mM $MgCl_2$, 10mM dNTP's, 2U of Taq DNA polymerase and 10 pmol of ITS1 forward and ITS4 reverse primer. The following thermocycling parameters were used: Initial denaturation at 94°C for 4 min, followed by 30 cycles, each of 30 seconds at 94°C for denaturation, 1 min at 58.2°C for annealing, 2 mins at 72°C for extension with a final extension step at 72°C for 7 min (Sim et al. 2010). The amplified DNA fragments were analyzed by 1% agarose gel electrophoresis with a 100bp DNA ladder and the amplicons were visualized using a gel documentation system (Uvitech). A control (without DNA) was included in each run. Further, the amplified products were purified

(Gel Extraction Kit, Sigma) and sequenced by Eurofins Private Limited, Bangalore, Karnataka, India.

Phylogenetic analysis

The sequenced fungal ITS sequences were merged (both forward and reverse complemented sequences) by EMBOSS merger tool (http://www.bioinformatics.nl/cgi-bin/emboss/merger) to obtain the full length of the ITS sequence. Thereafter, the sequences were compared with National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST). The high similarity sequences were downloaded to analyze phylogenetic relationships. The downloaded sequences along with our query ITS sequences were subjected to alignment using CLUSTAL-W program implemented in MEGA 6. Phylogenetic analysis was carried out by the neighbor joining method using MEGA software version 6 with a bootstrap consensus of 1000 replicates to assess the reliable level of the nodes of the tree (Larkin et al. 2007, Tamura et al. 2011).

ITS2 RNA secondary structure analysis

The fungal ITS extractor (http://www.emerencia.org/FungalITSextractor.html) was used to extract the ITS2 region from the full length of ITS sequences (Nilsson et al. 2010). ITS2 RNA secondary structure of the query sequence and its closest matches in the ITS phylogenetic tree were predicted using Mfold server with a preset temperature of 37°C and following conditions: 1M NaCl (no divalent ions) ionic conditions, percentage sub-optimality number 5, upper bound number of the folding: 50, maximum asymmetry of an interior/bulge loop: 30, maximum interior/bulge loop size: 30, maximum distance between paired bases: no limit. The minimum free energy (MFE) methods followed to model the secondary structure and following the parameters, structural energy, length, base composition, GC content and common core based structure were selected. The structural data were downloaded in Vienna format from Mfold server (Zuker 2003, Rao & Satish 2016). 4SALE V 1.7 software used to align the ITS2 sequence-secondary structures and also generate the consensus structure. The resultant alignment was exported to ProfDistS 0.9.9 for phylogenetic analysis (Friedrich et al. 2005).

Results

Isolation and Identification of fungi

A total of 88 fungal endophytes were isolated from leaves of *Ficus racemosa* and were grouped into 30 morphotypes based on phenotypic characteristics such as colony growth, surface texture, colour, margin (Fig. 2), and conidial characteristics were observed using light microscope (Fig. 3). The characteristic features of the fungi were recorded and listed in (Table 1). Further, they were characterized at molecular level using ITS sequences. All the investigated isolates clearly separated into 7 clades belonging to the two classes Sordariomycetes and Dothideomycetes in seven orders (Botryosphaeriales, Capnodiales, Diaporthales, Glomerellales, Hypocreales, Pleosphorales and Xylariales,) of Ascomycota. All our isolates were denoted with strain number ranging FRAH01 – FRAH30. Sordariomycetes mainly included species in *Colletotrichum* (Clade I), *Fusarium* (Clade II), *Pestalotiopsis* and *Xylaria* (Clade III) and *Diaporthe/Phomopsis* (Clade IV). Dothideomycetes mainly includes *Cladosporium* (Clade V), *Guignardia/Phyllosticta* and *Lasiodiplodia* (Clade VI) and *Alternaria* and *Stagonoporopsis* (Clade VII) (Fig. 4). Further, the sequences were subjected to ITS2 secondary structure prediction to support the phylogenetic robustness of this study. The structure based analysis was more informative compared to sequence based analysis.

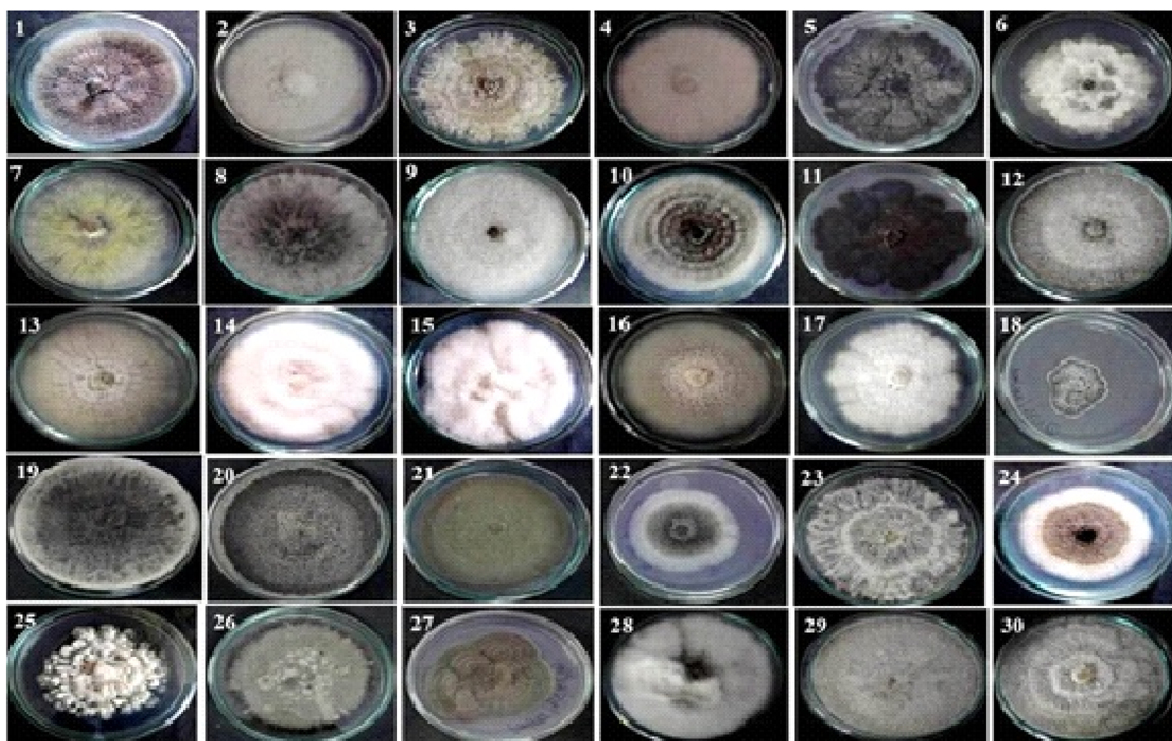

Fig. 2 – Colony morphology of fungal endophytes on PDA at 24°C. *Colletotrichum* sp. 1, 2, 4, 9, 12, 13, 16, 22, 28 and 29. *Phomopsis/Diaporthe* sp. 3, 7, 23 and 30. *Guignardia/Phyllosticta* sp. 5, 11 and 18. *Xylaria* sp. 6 and 25. *Lasiodiplodia* sp. 8 and 19. *Alternaria* sp. 10 and 20. *Fusarium* sp. 14 and 15. *Pestalotiopsis* sp. 17. *Cladosporium* sp. 21, 26 and 27. *Stagonosporopsis* sp. 24.

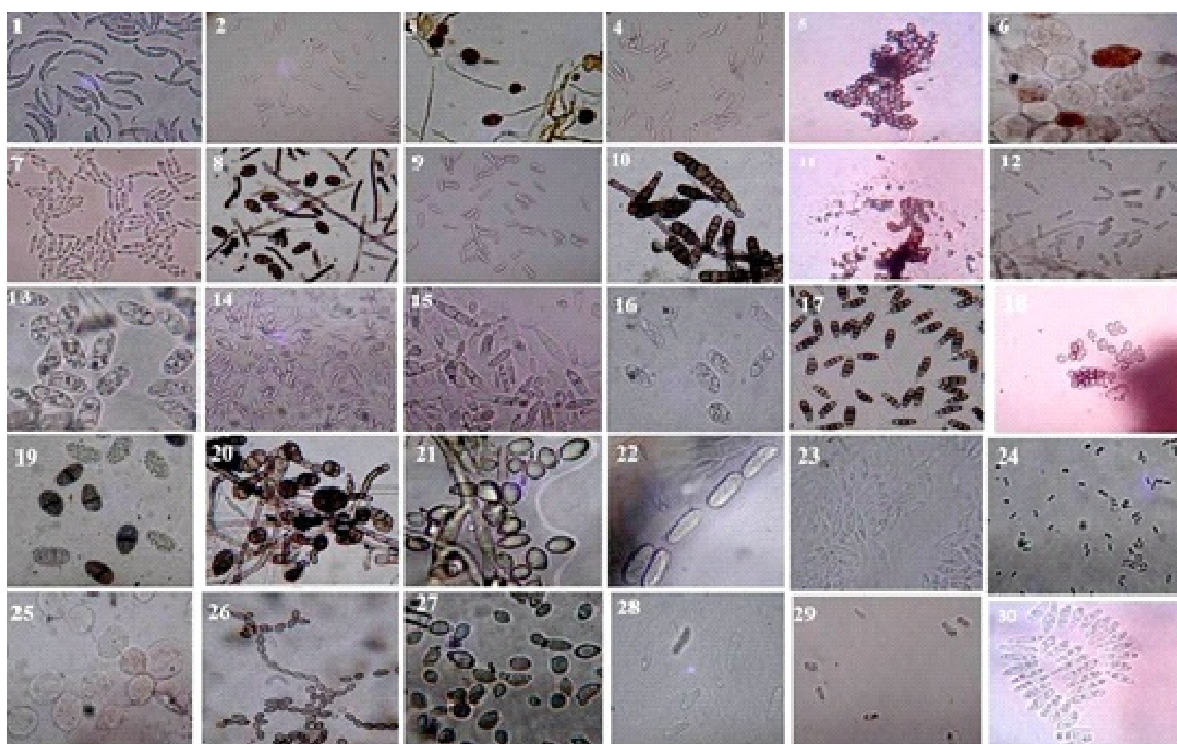

Fig. 3 – Microscopic (light) observation of fungal endophytes from *Ficus racemosa. Colletotrichum* sp. 1, 2, 4, 9, 12, 13, 16, 22, 28 and 29. *Phomopsis/Diaporthe* sp. 3, 7, 23 and 30. *Guignardia/Phyllosticta* sp. 5, 11 and 18. *Xylaria* sp. 6 and 25. *Lasiodiplodia* sp. 8 and 19. *Alternaria* sp. 10 and 20. *Fusarium* sp. 14 and 15. *Pestalotiopsis* sp. 17. *Cladosporium* sp. 21, 26 and 27. *Stagonosporopsis* sp. 24.

Table 1 Summary of cultural characteristics of the 30 fungal morphotypes from *Ficus racemosa* on PDA, at 24 °C.

Morpho-types	Colour (Front)	Colour (Reverse)	Shape	Mycelium	Margin	Hyphae	Spore shape	Spore size (µm)
1	Olive-grey	Dark grey	Circular	Flat	Entire	Septate	Falcate	22.4-29.6x2.3-4.2
2	White	Pale white	Circular	Arial	Entire	Septate	Cylindrical	10-21×3-4-4.3
3	Brownish	Dark Brown	Filamentous	Flat	Filamentous	Aseptate	Globose	-
4	Cream	Cream	Circular	Arial	Entire	Septate	Cylindrical	9.8-20×3.2-4.0
5	Dark grey	Dark grey	Irregular	Flat	Undulate	Septate	Ellipsoidal	6.5-11x5.5-6.2
6	White	White	Irregular	Arial	Undulate	Aseptate	Globose	-
7	Greenish yellow	Brown	Circular	Flat	Filamentous	Aseptate	Cylindrical	9.5-16.2x1.4-2.5
8	Pale white	Dark	Circular	Arial	Entire	Septate	Ellipsoidal	19-22x12.8-15.4
9	White	White	Circular	Arial	Entire	Septate	Cylindrical	10.2-21.2x3.1-4.2
10	White to brown	Dark brown	Circular	Arial	Entire	Septate	Club-shaped	26-32x3.8-6.2
11	Dark grey	Dark grey	Irregular	Flat	Undulate	Septate	Ellipsoidal	7-10.5x5-6.3
12	White	White	Circular	Arial	Entire	Septate	Cylindrical	10.1-20.5x3.2-4.1
13	White to orange	White to orange	Circular	Arial	Entire	Septate	Cylindrical	14-16x5.6-6.8
14	White to light pink	White to light pink	Circular	Arial	Entire	Septate	Elongate	15-40x2.8-4
15	White to light pink	White to light pink	Circular	Arial	Entire	Septate	Elongate	19-42x3.4-4.0
16	Off white to orange	Off-white to orange	Circular	Arial	Entire	Septate	Cylindrical	14.2-16.3x5.8-6.4
17	White	White	Circular	Arial	Undulate	Septate	Fusiform	15-21.2x4.4-6.6
18	Grey	Grey	Irregular	Flat	Undulate	Septate	Ellipsoidal	7.2-10.8x4.6-6
19	Pale white	Dark	Circular	Arial	Entire	Septate	Ellipsoidal	20-24.4x12.5-16.8
20	Grey	Dark grey	Circular	Arial	Entire	Septate	Club-shaped	15.5-19x3.8-6.2
21	Olivaceous green	Olivaceous grey	Circular	Flat	Entire	Septate	Globose	2.4–6.2 × 2.2–3
22	White to greenish	Greenish	Circular	Arial	Entire	Septate	Cylindrical	12-25x7.5-10.4
23	Pale white	Pale white	Filamentous	Flat	Curled	Aseptate	Cylindrical	8–15x1.4-2.5
24	Brownish	Brownish	Circular	Flat	Entire	Septate	Cylindrical	7.2-8x2.6-3.2
25	White	White	Irregular	Arial	Undulate	Septate	Globose	-
26	Olivaceous grey	Olivaceous grey	Circular	Arial	Undulate	Aseptate	Globose	1.5-2x3.6-4.2
27	Olivaceous grey	Olivaceous grey	Irregular	Arial	Curled	Septate	Globose	2.4-5.8x2.2-3.1
28	White	White	Circular	Arial	Entire	Septate	Cylindrical	15-20x5.5-8.5
29	White	White	Circular	Arial	Entire	Septate	Cylindrical	10-20.5x3.2-4
30	White	White	Circular	Flat	Curled	Aseptate	Cylindrical	7-15.5x1.4-2.7

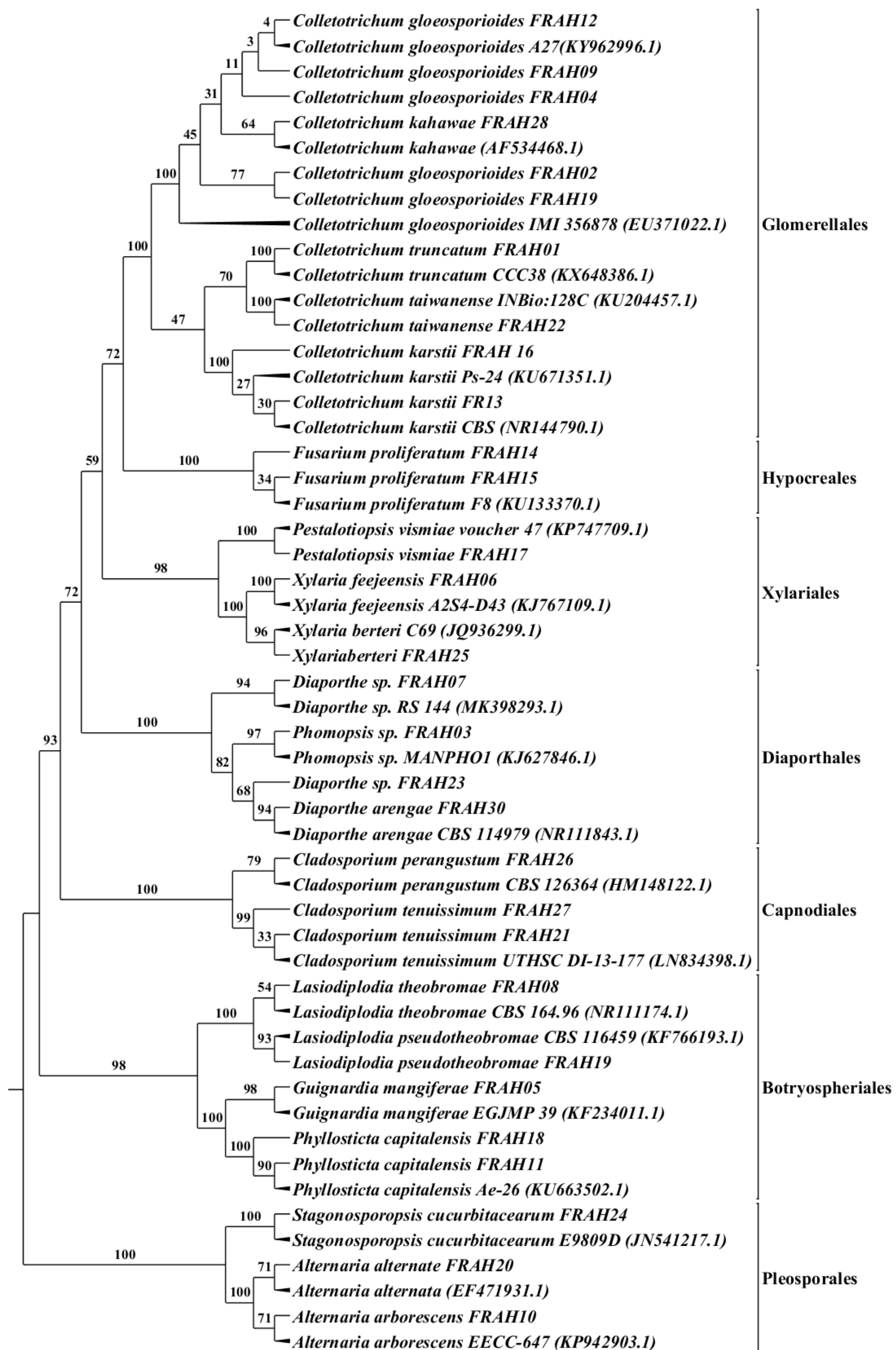

Fig. 4 – Neighbor Joining (NJ) tree showing phylogenetic relationships of endophytic fungi from Ascomycota phylum, based on the ITS rDNA sequences (Bold line represents reference sequence from NCBI and the strains FRAH01 – FRAH30 indicate our isolates).

Nucleotide information and ITS2 RNA secondary structure analysis

ITS2 secondary structures were predicted using Mfold program for our dataset, which included 30 query and 22 reference sequences. The length of ITS2 sequences ranged from 150 to 165 nucleotide bases and the percentage of GC content were about 46.66–60.60 (Table 3). Further, a genus-wise consensus secondary structure was modeled. For *Colletotrichum/Glomerella* secondary structures were modeled for 17 sequences whose minimum free energy (MFE) was -73.32 kcal/mol (mean value). Similarly, the secondary structure predicted for 7 sequences of *Diaporthe/Phomopsis* had -77.06 MFE, 5 sequences of *Cladosporium* and *Phyllosticta/Guignardia* had -72.93 and 82.50 MFE, 4 sequences of *Lasiodiplodia*, *Alternaria* and *Xylaria* each possessed -70.82, -65.25 and -66.72 MFE respectively, 3 sequences in *Fusarium* had -83.35 MFE and 2 sequences of *Pestalotiopsis* and *Stagonoporopsis* possessed -66.69 and -60.55 MFE respectively. Generally, the secondary structure of ITS2 contains two forms: (i) 4-helix domain (ii) 3- helix domains model with longest third helix and fourth helix not always present. In the observed study *Guignardia* sp./*Phyllosticta* sp., *Lasiodiplodia* sp. and *Xylaria* sp. shared four helixes with a longer third helix. While *Alternaria* sp., *Colletotrichum* sp., *Cladosporium* sp., *Diaporthe/Phomopsis* sp., *Fusarium* sp., and *Stagonoporopsis* sp. contained three helixes with the third helix being longest in all genera except for *Pestalotiopsis* sp. In *Pestalotiopsis* sp. only two helixes were found, the first helix not recognizable but second and third helixes were recognized (Fig. 5).

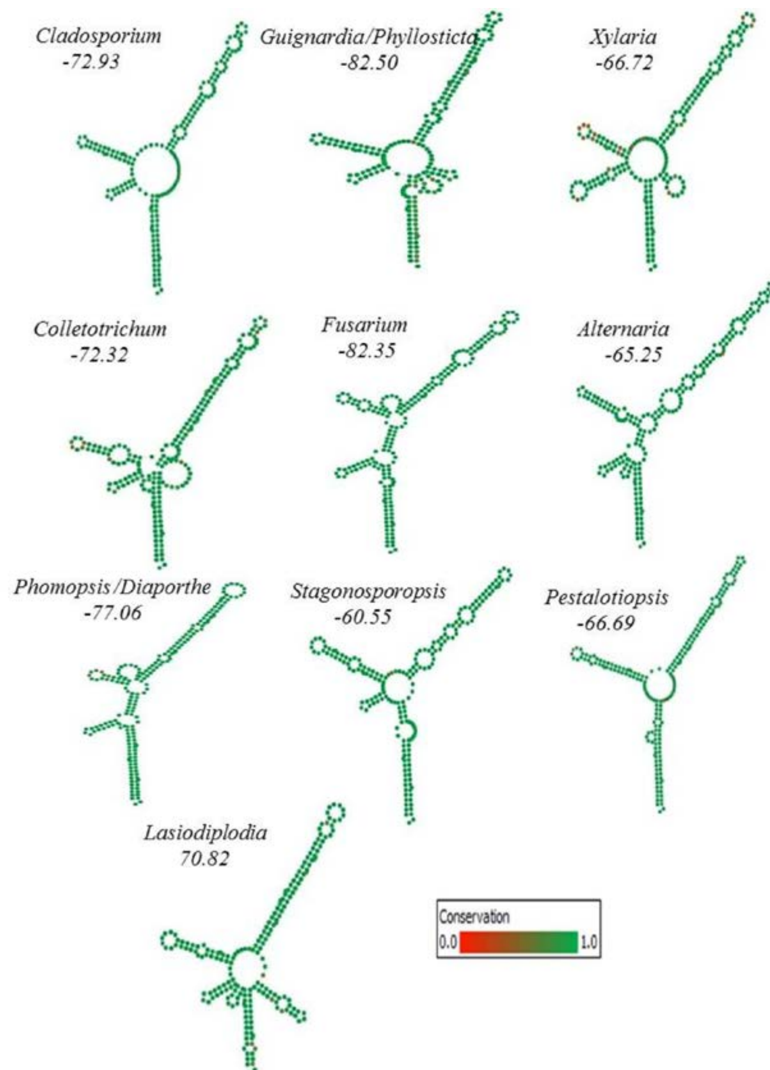

Fig. 5 – Consensus ITS2 secondary structure of fungal genera, *Cladosporium Guignardia/Phyllosticta, Xylaria, Colletotrichum, Fusarium, Alternaria, Diaporthe/ Phomopsis, Stagonoporopsis, Pestalotiopsis, Lasiodiplodia* and their Minimum Free Energy (MFE) Value.

CBC analysis

The compensatory base change (CBC) observed in a pair of structures were documented and analyzed using 4SALE software. Observance of one or more CBCs between a pair of secondary structures renders the two structures under comparison to be distinct and belong to different species. Colored regions indicate lack of CBC between the pair of sequences; which may belong to same species or different species. In the study, *Colletotrichum gloeosporioides* and *Colletotrichum kahawae* differentiated from *Colletotrichum karstii, Colletotrichum truncatum* and *Colletotrichum taiwanense.* Similarly *Xylaria feejeensis* differentiated from *Xylaria berteri* by presence of CBC (Fig. 6). Despite *Alternaria arborescens* and *Alternaria alternate, Cladosporium tenuissimum* and *Cladosporium perangustum* showed no CBC but the nucleotide variations occured between their sequences (Table 3).

Phylogenetic analysis

Sequence-structure based alignment performed in 4SALE was subsequently exported to ProfDistS for phylogenetic analysis. The phylogenetic clade formation was clearly separated. Based on the analysis, 18 different species were observed, which included *A. alternata, A. arborescens, C. truncatum, C. gloeosporioides, C. karstii, C. kahawae. C. taiwanense, C. tenuissimum C. perangustum, D. arengae, F. proliferatum, L. theobromae, L. pseudotheobromae, P. capitalensis, P. vismiae, S. cucurbitacearum, X. feejeensis* and *X. berteri*. They belong to 10 genera in seven orders of Ascomycota. Three isolates were classified belonging to the genus *Phomopsis/Diaporthe* (Fig. 7). Among the 30 morphotypes *Colletotrichum* was the dominant group with isolates belonging to five different species, namely *C. gloeosporioides* (5)*, C. karstii* (2), *C. kahawae* (1)*, C. truncatum* (1), *C. taiwanense* (1). *P. vismiae* and *S. cucurbitacearum* were least frequent, having one isolates each (Table 2). Finally all the identified fungal rDNA-ITS sequences were submitted in GenBank (www.ncbi.nlm.nih.gov) and their accession numbers are listed in (Table 3).

Table 2 Number of fungal endophytes identified from *Ficus racemosa.*

S. No	Taxon name	Number of strains
1	*Colletotrichum gloeosporioides*	5
2	*Guignardia mangiferae/Phyllosticta capitalensis*	3
3	*Phomopsis* sp./*Diaporthe* sp.	3
4	*Colletotrichum karstii*	2
5	*Cladosporium tenuissimum*	2
6	*Fusarium proliferatum*	2
7	*Alternaria alternata*	1
8	*Alternaria arborescens*	1
9	*Colletotrichum truncatum*	1
10	*Colletotrichum kahawae*	1
11	*Colletotrichum taiwanense*	1
12	*Cladosporium perangustum*	1
13	*Diaporthe arengae*	1
14	*Lasiodiplodia theobromae*	1
15	*Lasiodiplodia pseudotheobromae*	1
16	*Pestalotiopsis vismiae*	1
17	*Stagonosporopsis cucurbitacearum*	1
18	*Xylaria feejeensis*	1
19	*Xylaria berteri*	1

Organism names	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52
01 Colletotrichum gloeosporioides FRAH02	0																																																			
02 Colletotrichum gloeosporioides FRAH04	0	0																																																		
03 Colletotrichum gloeosporioides FRAH09	0	0	0																																																	
04 Colletotrichum gloeosporioides FRAH12	0	0	0	0																																																
05 Colletotrichum gloeosporioides FRAH29	0	0	0	0	0																																															
06 Colletotrichum gloeosporioides A27	0	0	0	0	0	0																																														
07 Colletotrichum gloeosporioides *IMI 356878*	0	0	0	0	0	0	0																																													
08 Colletotrichum kahawae FRAH28	0	0	0	0	0	0	0	0																																												
09 Colletotrichum kahawae CIFC	0	0	0	0	0	0	0	0	0																																											
10 Colletotrichum truncatum FRAH01	1	1	1	1	1	1	1	1	1	0																																										
11 Colletotrichum truncatum *CCC38*	1	1	1	1	1	1	1	1	1	0	0																																									
12 Colletotrichum taiwanense *FRAH22*	1	1	1	1	1	1	1	1	1	0	0	0																																								
13 *Colletotrichum taiwanense INBio:128C*	1	1	1	1	1	1	1	1	1	0	0	0	0																																							
14 *Colletotrichum karstii FRAH13*	1	1	1	1	1	1	1	1	1	0	0	0	0	0																																						
15 *Colletotrichum karstii FRAH16*	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0																																					
16 *Colletotrichum karstii Ps-24*	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0																																				
17 *Xylaria feejeensis FRAH06*	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	0																																			
18 *Xylaria feejeensis A2S4-D43*	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	0	0																																		
19 *Xylaria berteri FRAH25*	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	2	2	0																																	
20 *Xylaria berteri C69*	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	2	2	0	0																																
21 *Pestalotiopsis vismiae FRAH17*	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	2	2	1	1	0																															
22 *Pestalotiopsis vismiae 47*	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	2	2	1	1	0	0																														
23 *Fusarium proliferatum FRAH14*	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	1	1	1	2	2	0																													
24 *Fusarium proliferatum FRAH15*	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	1	1	1	2	2	0	0																												
25 *Fusarium proliferatum F8*	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	1	1	1	2	2	0	0	0																											
26 *Phyllosticta capitalensis FRAH11*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	1	1	1	2	2	2	3	3	3	0																										
27 *Phyllosticta capitalensis FRAH18*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	1	1	1	2	2	2	3	3	3	0	0																									
28 *Phyllosticta capitalensis Ae-26*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	1	1	1	2	2	3	3	3	3	0	0	0																								
29 *Guignardia mangiferae FRAH05*	3	3	3	3	3	3	3	3	3	3	3	4	4	3	3	3	1	1	1	2	2	2	5	5	5	2	2	2	0																							
30 *Guignardia mangiferae EGJMP 39*	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	1	1	2	1	2	4	4	4	2	2	2	0	0																						
31 *Cladosporium perangustum FRAH26*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	2	2	2	1	1	0																					
32 *Cladosporium perangustum CBS 126364*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	2	1	1	0	0																				
33 *Cladosporium tenuissimum FRAH21*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	2	1	1	0	0	0																			
34 *Cladosporium tenuissimum FRAH27*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	2	1	1	0	0	0	0																		
35 *Cladosporium tenuissimum UTHSC DI-13-177*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	2	1	1	0	0	0	0	0																	
36 *Alternaria alternate FRAH20*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	2	2	2	2	2	2	3	3	3	4	4	4	4	3	3	3	3	3	3	0																
37 *Alternaria alternate IMI 393883*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	2	2	2	2	2	2	3	3	3	4	4	4	4	3	3	3	3	3	3	0	0															
38 *Alternaria arborescens FRAH10*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	2	2	2	2	2	2	3	3	3	4	4	4	4	3	3	3	3	3	3	0	0	0														
39 *Alternaria arborescens EECC-647*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	2	2	2	2	2	2	3	3	3	4	4	4	4	3	3	3	3	3	3	0	0	0	0													
40 *Stagonosporopsis cucurbitacearum FRAH24*	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	1	1	2		1	1	3	3	3	4	4	4	4	3	3	3	3	3	1	1	1	1	0												
41 *Stagonosporopsis cucurbitacearum E9809D*	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	1	1	2	2	2	2	2	2	4	4	4	4	4	3	3	3	3	3	1	1	1	1	0	0											
42 *Lasiodiplodia theobromae CBS 164.96*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	2	2	2	2	2	2	4	4	4	1	1	1	1	1	1	1	4	4	4	4	2	2	0										
43 *Lasiodiplodia theobromae FRAH08*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	2	2	2	2	2	2	4	4	4	1	1	1	1	1	1	1	3	3	3	3	2	2	0	0									
44 *Lasiodiplodia pseudotheobromae FRAH19*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	2	2	2	2	2	2	4	4	4	1	1	1	1	1	1	1	4	4	4	4	2	2	0	0	0								
45 *Lasiodiplodia pseudotheobromae CBS 116459*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	2	2	2	3	3	3	2	2	2	1	1	1	1	1	1	1	4	4	4	4	2	2	0	0	0	0							
46 *Diaporthe arengae FRAH30*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	1	1	1	1	2	2	3	3	3	2	2	2	4	3	1	1	1	1	1	1	1	1	1	3	3	2	2	2	2	0						
47 *Diaporthe arengae CBS 114979*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	1	1	1	1	2	2	3	3	3	2	2	2	3	3	1	1	1	1	1	1	1	1	1	3	3	2	2	2	2	0	0					
48 *Diaporthe sp. FRAH07*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	1	1	1	1	2	2	3	3	3	2	2	2	3	3	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	1	1	0				
49 *Diaporthe sp. RS_144*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	1	1	1	1	2	2	3	3	3	2	2	2	3	3	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	1	1	0	0			
50 *Diaporthe sp. FRAH23*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	3	3	3	2	2	2	2	2	2	2	2	4	3	1	1	1	1	1	1	1	1	1	3	3	1	1	1	1	0	0	0	0	0		
51 *Phomopsis sp. FRAH03*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	1	1	1	1	2	2	3	3	3	2	2	2	4	3	1	1	1	1	1	1	1	1	1	3	3	2	2	2	2	0	0	0	0	0	0	
52 *Phomopsis sp. MANPHO1*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	1	1	1	1	2	2	3	3	3	2	2	2	4	3	1	1	1	1	1	1	1	1	1	3	3	2	2	2	2	0	0	1	1	0	0	0

Fig. 6 – Compensatory base change (CBC) analyses of our isolated fungi and reference sequences. Block colored region indicate lack of CBC and remains are presence of CBC.

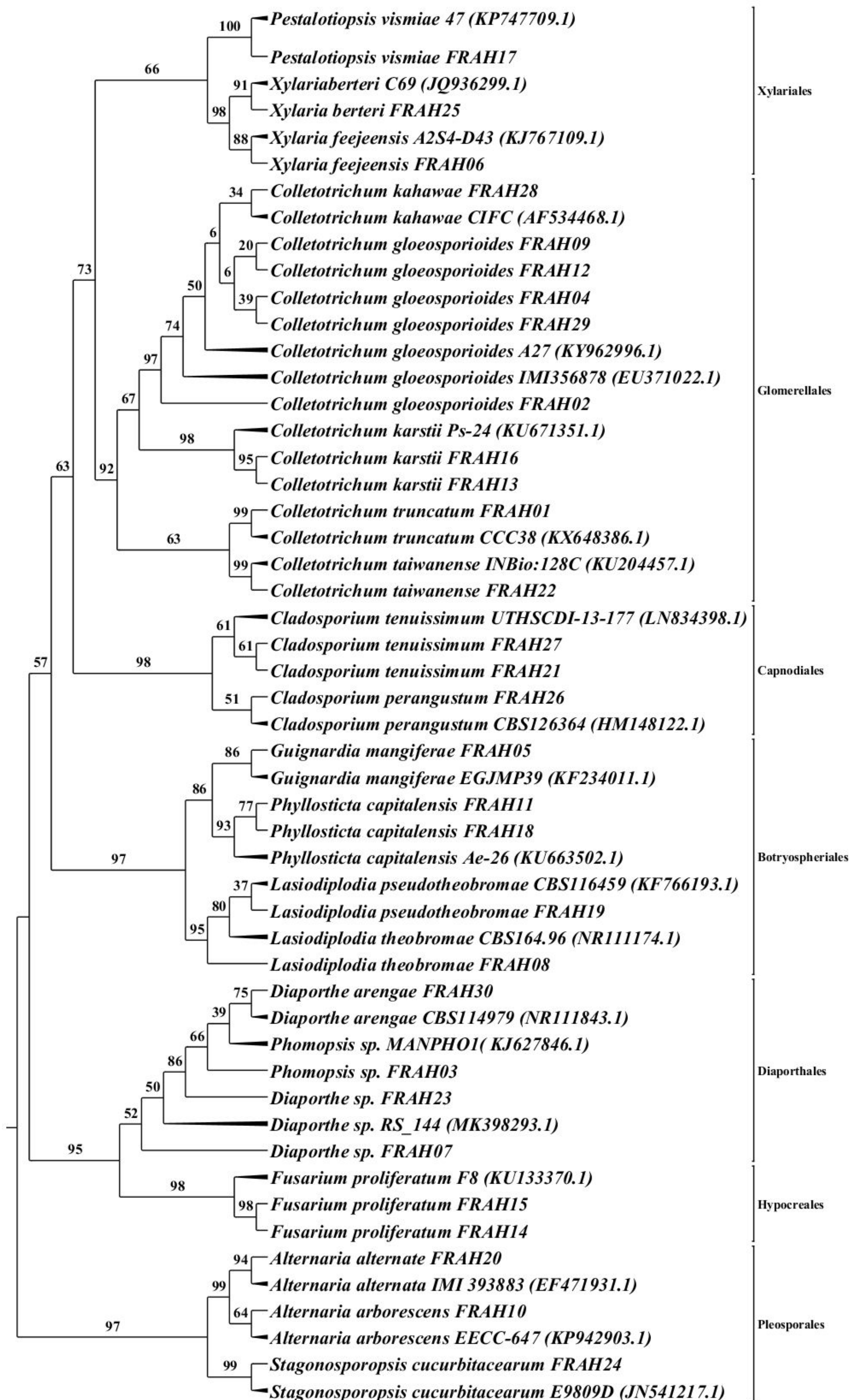

Fig. 7 – ITS2 secondary structure based phylogenetic analysis of query (organism name with FRAH01 – FRAH30) and Reference ITS2 sequence (organism indicated by dark lines) by neighbor joining method. Phylogenetic tree construct with ProfDistS software.

Table 3 Nucleotide information of the ITS Region. a-Full length of ITS sequences, b-ITS2 sequence and Nucleotide compositions (A, U/T, G and C), percentage of GC content and Minimum Free Energy of secondary structure (MFE). 1–30 our isolates (FRAHs) and 31–52 reference sequences from NCBI.

S. no	Organism name	Accession No	[a]Total length	[b]ITS2 region	A's	U/T's	G's	C's	% GC	Free energy 37°c
1	*Colletotrichum truncatum* FRAH01	MK089846	563	361-517	31	42	42	42	53.50	-71.93
2	*Colletotrichum gloeosporioides* FRAH02	MK111073	549	337-494	31	37	40	50	56.96	-68.33
3	*Phomopsis* sp. FRAH03	MK530217	550	347-504	33	35	41	49	56.96	-77.72
4	*Colletotrichum gloeosporioides* FRAH04	MK111074	551	353-510	31	37	41	49	56.96	-74.03
5	*Guignardia mangiferae* FRAH05	MK105811	566	371-533	30	37	47	49	58.59	-82.85
6	*Xylaria feejeensis* FRAH06	MK426764	565	355-515	35	48	37	41	48.44	-66.17
7	*Diaporthe* sp. FRAH07	MK530214	583	391-550	37	29	39	55	58.75	-67.34
8	*Lasiodiplodia theobromae* FRAH08	MK507848	500	305-464	30	34	44	52	60.00	-65.53
9	*Colletotrichum gloeosporioides* FRAH09	MK426765	559	352-509	31	37	41	49	60.58	-74.03
10	*Alternaria arborescens* FRAH10	MK507847	501	301-459	31	49	37	42	49.68	-65.25
11	*Phyllosticta capitalensis* FRAH11	MK443377	610	401-563	30	38	47	48	58.28	-81.78
12	*Colletotrichum gloeosporioides* FRAH12	MK467538	544	345-502	31	37	41	49	56.96	-74.03
13	*Colletotrichum karstii* FRAH13	MK426762	540	346-503	30	37	42	49	57.59	-73.43
14	*Fusarium proliferatum* FRAH14	MK443375	491	281-445	32	33	47	53	60.60	-82.35
15	*Fusarium proliferatum* FRAH15	MK443376	528	319-483	32	33	47	53	60.60	-82.35
16	*Colletotrichum karstii* FRAH16	MK426763	544	346-503	30	37	42	49	57.59	-73.43
17	*Pestalotiopsis vismiae* FRAH17	MK530079	579	370-534	35	53	36	41	46.66	-66.69
18	*Phyllosticta capitalensis* FRAH18	MK497043	521	365-528	30	39	47	48	57.92	-81.65
19	*Lasiodiplodia pseudotheobromae* FRAH19	MK497042	508	305-464	30	36	43	51	58.75	-74.68
20	*Alternaria alternata* FRAH20	MK467537	538	334-492	30	49	37	43	50.31	-65.25
21	*Cladosporium tenuissimum* FRAH21	MK497038	502	309-458	32	33	40	45	56.66	-72.93
22	*Colletotrichum taiwanense* FRAH22	MK507849	535	333-488	31	41	40	44	53.84	-64.51
23	*Diaporthe* sp. FRAH23	MK530215	576	359-519	37	34	40	50	55.90	-76.92
24	*Stagonosporopsis cucurbitacearum* FRAH24	MK467536	516	315-472	32	45	37	44	51.26	-61.58
25	*Xylaria berteri* FRAH25	MK530219	551	351-504	35	45	34	42	48.71	-62.32

Table 3 Continued.

S. no	Organism name	Accession No	[a]Total length	[b]ITS2 region	A's	U/T's	G's	C's	% GC	Free energy 37°c
26	*Cladosporium perangustum* FRAH26	MK497041	520	329-478	31	33	40	46	57.33	-72.93
27	*Cladosporium tenuissimum* FRAH27	MK497039	525	328-477	32	33	40	45	56.66	-72.93
28	*Colletotrichum kahawae* FRAH28	MK497040	521	320-476	31	36	41	49	57.32	-74.09
29	*Colletotrichum gloeosporioides* FRAH29	MK467539	545	339-496	31	37	41	49	56.96	-67.53
30	*Diaporthe arengae* FRAH30	MK507846	547	348-505	35	33	41	49	56.96	-77.50
31	*Colletotrichum karstii* Ps-24	KU671351.1	559	362-519	30	37	42	49	57.59	-73.43
32	*Colletotrichum truncatum* CCC38	KX648386.1	585	368-524	31	42	42	42	53.50	-71.93
33	*Fusarium proliferatum strain* F8	KU133370.1	553	335-499	32	33	47	53	60.60	-82.35
34	*Alternaria alternata* IMI 393883	EF471931.1	569	453-511	30	49	37	43	50.31	-65.25
35	*Alternaria arborescens* EECC-647	KP942903.1	576	375-533	31	49	37	42	49.68	-65.25
36	*Phyllosticta capitalensis* Ae-26	KU663502.1	613	407-569	30	38	47	48	58.28	-81.78
37	*Cladosporium tenuissimum* UTHSCDI13-177	LN834398.1	569	370-519	32	33	40	45	56.66	-72.93
38	*Cladosporium perangustum* CBS 126364	HM148122.1	639	454-603	31	33	40	46	57.33	-72.93
39	*Colletotrichum gloeosporioides*A27	KY962996.1	575	360-537	31	37	41	49	56.96	-74.03
40	*Colletotrichum kahawae* CIFC	AF534468.1	575	361-517	31	37	44	48	57.50	-74.09
41	*Colletotrichum gloeosporioides* IMI 356878	EU371022.1	582	376-533	33	36	41	50	56.87	-74.09
42	*Colletotrichum taiwanense* INBio:128C	KU204457.1	547	351-506	31	41	40	44	53.84	-70.61
43	*Diaporthe* sp. RS_144	MK398293.1	546	374-532	33	32	43	51	59.11	-79.66
44	*Diaporthe arengae* CBS 114979	NR111843.1	571	371-528	35	32	41	50	57.58	-81.90
45	*Stagonosporopsis cucurbitacearum* E9809	JN541217.1	532	325-481	31	45	37	44	51.59	-51.59
46	*Guignardia mangiferae* EGJMP39	KF234011.1	570	400-562	29	36	49	49	60.12	-84.46
47	*Lasiodiplodia pseudotheobromae* CBS 1164	KF766193.1	551	334-493	32	36	42	51	57.76	-68.48
48	*Xylaria feejeensis* A2S4-D43	KJ767109.1	586	368-528	35	47	37	42	49.06	-70.97
49	*Xylaria berteri* C69	JQ936299.1	553	354-508	34	46	34	41	48.38	-67.42
50	*Lasiodiplodia theobromae* CBS 164.96	NR111174.1	542	325-485	31	35	43	52	59.00	-74.62
51	*Phomopsis* sp. MANPHO1	KJ627846.1	579	364-521	35	34	41	48	56.32	-78.42
52	*Pestalotiopsis vismiae voucher* 47	KP747709.1	615	388-552	35	53	36	41	46.66	-66.69

Discussion

Fungi are ubiquitous organisms that are able to grow rapidly depending on favorable conditions like moisture and temperature (Abdin et al. 2010). Worldwide, more than one million species of fungal endophytes were identified and they could produce several bioactive secondary metabolites, especially those isolated from medicinal plants. The colonization of fungal endophytes may provide advantages to their host plants by producing a plethora of substances that offer protection or increase the fitness of the hosts (Redman et al. 2002, Arnold et al. 2003, Tejesvi et al. 2007). The healthy plant part is the most important step in the isolation process and also in eliminating the epiphytes. The ecological and biomedical importance of the fungal endophytes remains not characterized (Sette et al. 2006, Tao et al. 2008). Molecular investigation could help to identify the fungal endophytes from host plants and in examining their phylogenetic relationships. The ITS region has been commonly used as a phylogenetic marker for identifying the fungi (Schoch et al. 2012, Sun & Guo 2012). During 2010–2016 molecular based identification has increased especially by using ITS data, compared to other methods (Raja et al 2017). Several studies have been done based on molecular basis to identify the fungal endophytes from various medicinal plant species (Chen et al. 2011, Yoo & Eom 2012, Bhagat et al. 2012, González-Teuber et al. 2017). *Alternaria, Colletotrichum, Phomopsis,* and *Xylaria* species were reported as dominant fungal endophytes from *Artemisia* based on morphological and molecular methods (Huang et al. 2009). Nowadays ITS2 sequence-secondary structure based analysis is used for species level delimitation among closely related organisms and it is a third dimensional approach to characterize the fungi as well as other eukaryotic organisms including animals, plants, insects etc., (Yao et al. 2010, Han et al. 2013, Zhang et al. 2015).

ITS2 sequence-secondary structure based analysis is more informative than sequence data. Structural information can produce additional data for species level characterization, as RNA secondary structures improve the accuracy and robustness in phylogenetic reconstruction (Coleman 2003, 2009). This has been proved during recent studies for identifying fungi (GokulRaj et al. 2014, Sundaresan et al. 2019). Compensatory base change (CBC) is additional supporting data for analyzing the variations between structural pair in the sequences.

The presence of CBC between the pair of structures denotes that the two are different species and that their probability was ~93%. If there is no CBC between the pairs of structures, it denotes that they may belong to the same or different species with a probability ~76% (Muller et al. 2007, Wolf et al. 2013). The ITS2 sequence-secondary structure is a most suitable marker for fungal species level classification because of its very shorter length with lower GC variation and the structural characters render greater phylogenetic utility. In our analysis of ITS2 sequences, structural data differed from other methods and the neighbour joining analysis based on our sequence – structure based alignment resulted in more accurately separated clades. All the investigated isolates belonged to seven orders and ten genera of Ascomycota.

Conclusion

This study reports 30 fungal morphotypes isolated from *Ficus racemosa,* identified by morphology and molecular data. They belong to the ten genera *Alternaria* sp., *Colletotrichum* sp., *Cladosporium* sp., *Diaporthe/Phomopsis* sp., *Fusarium* sp., *Guignardia/Phyllosticta* sp., *Lasiodiplodia* sp., *Pestalotiopsis* sp., *Stagonoporopsis* sp. and *Xylaria* sp. of in the orders Botryosphaeriales, Capnodiales, Diaporthales, Glomerellales, Hypocreales, Pleosphorales and Xylariales. Among the 30 morphotypes 27 were identified up to species level. In that the remaining three isolates were identified up to the genus level. *Colletotrichum* sp. were the most dominant among our isolates. ITS2 sequence-secondary structure based characterization could be a valuable tool for distinguishing closely related species. The next step in our research program is to extract and purify the bioactive compounds from those promising fungal endophytes to evaluate their antimicrobial and anticancer potentials.

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