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Phylogenetic reconstruction of endophytic fungal isolates using internal transcribed spacer 2 (ITS2) region

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Abstract:
Endophytic fungi are inhabitants of plants, living most part of their lifecycle asymptomatically which mainly confer protection and ecological advantages to the host plant. In this present study, 48 endophytic fungi were isolated from the leaves of three medicinal plants and characterized based on ITS2 sequence – secondary structure analysis. ITS2 secondary structures were elucidated with minimum free energy method (MFOLD version 3.1) and consensus structure of each genus was generated by 4SALE. ProfDistS was used to generate ITS2 sequence structure based phylogenetic tree respectively. Our elucidated isolates were belonging to Ascomycetes family, representing 5 orders and 6 genera. *Colletotrichum/Glomerella* spp., *Diaporthae/Phomopsis* spp., and *Alternaria* spp., were predominantly observed while *Cochliobolus* sp., *Cladosporium* sp., and *Emericella* sp., were represented by singletons. The constructed phylogenetic tree has well resolved monophyletic groups with >50% bootstrap value support. Secondary structures based fungal systematics improves not only the stability; it also increases the precision of phylogenetic inference. Above ITS2 based phylogenetic analysis was performed for our 48 isolates along with sequences of known ex-types taken from GenBank which confirms the efficiency of the proposed method. Further, we propose it as superlative marker for reconstructing phylogenetic relationships at different taxonomic levels due to their lesser length.

Keywords: Endophytes, ITS, MEGA 5.1, ProfDistS, Sequence-secondary structure, 4SALE.

Background:
Endophytic fungi represents a group of diverse fungal lineages that live invisibly and symptomless within their host for at least part of their life time **[1]**. They have been isolated from almost all major groups of plant kingdom from varied ecosystems. They exhibit symbiogenic and mutualistic interactions **[2]** with the host thereby conferring ecological advantages and protection against pathogens and herbivores **[3, 4]**. Their potential to produce novel metabolites invites for rationale screening **[5, 6, 7]** of this group although their apparent diversity remains relatively less explored. Developing rapid methods of identification and classification is vital to tap this overwhelmingly diverse group **[8]**. Inadequacies in conventional morphotyping based fungal systematics owing to the intricate life styles and multiple origins of the discerning morphological characters had been resolved with the molecular identification and availability of easy to use phylogeny computational suites. Such a molecular phylogenetics based

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approach had strengthened our understanding of fungal evolution and systematics **[9]**; spurred up proposal for a single identity to an organism with anamorphic and telomorphic stages; uprooted and regrouped many synthetic taxa to erect evolutionarily supported taxon.

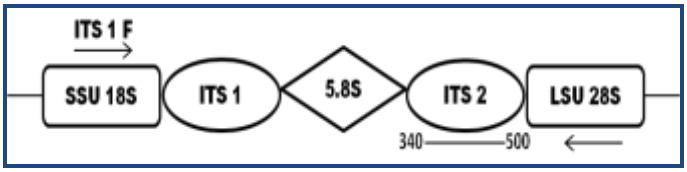

Figure 1: ITS regions flanked and interspersed by ribosomal unit coding sequence. Approximate ITS2 region has shown as line (340-500bp).

Internal transcribed region (ITS) rDNA has been the widely accepted standard molecular marker **[10, 11]** for fungal barcoding and features in many scientific literatures of the last two decades than the multilocus approach involving multiple markers such as Cytochrome oxidase c (cox), Tublin (tub), Translation elongation factor 1 subunit alpha (EF1a=tef1) and rpb2 **[12, 13]**. The internal transcribed spacer (ITS) region conventionally includes the entire ITS1, 5.8S gene and ITS2 portion of the nuclear rDNA cistron **(Figure 1)**. ITS based phylogenetic reconstructions provide more clarification at both genus and species level than other gene markers, also it corroborates with the relationship of organism as obtained from mating studies **[14]**. ITS2 a fast evolving sub-region (<200bp) of internal transcribed spacer; touted as the double edged tool **[14]** in phylogenetic analysis, has garnered much more attraction **[15]**. Incorporation of secondary structure data of this region significantly enhances the reliability of sequence alignments, stability of phylogenetic trees and provides finer resolution at both lower and higher taxa levels **[16, 17, 18]**. Phylogenetically useful information obtained from ITS2 secondary structure appears highly conserved in pan-eukaryotes **[19]**. Distinct hallmarks of ITS2 core secondary structure comprises: (1) four helices with (2) helix III as the longest and (3) containing an UGGU motif 5′ to the apex (deviations like UGGGU, UGG, or GGU have been described) as well as (4) a U-U mismatch in the second helix. Compensatory base changes (CBCs) were mutations observed at both the nucleotides of a paired site in the helical segments while the pairing itself is maintained. CBCs in the internal transcribed spacer 2 region (ITS2) of the nuclear rRNA cistron have been suggested as a possible marker for distinguishing species. They can be a sufficient but not a necessary criterion to differentiate between distinct species and the result of a CBC analysis may be used to estimate the minimal number of different species present in a multiple alignment **[20]**.

In the present study, endophytes from 3 medicinal plants - *Aegle marmelos*, *Coccinia indica* and *Moringa oleifera* were studied. These three medicinal plants are commonly found in south India. In addition to the well documented knowledge about their utility in traditional medicine and culinary uses, recently novel metabolites of higher therapeutic **[21, 22]** and nutraceutical values were being reported **[23, 24, 25]**. This study reports the diversity and phylogenetic relationship of endophytes from three medicinal plants.

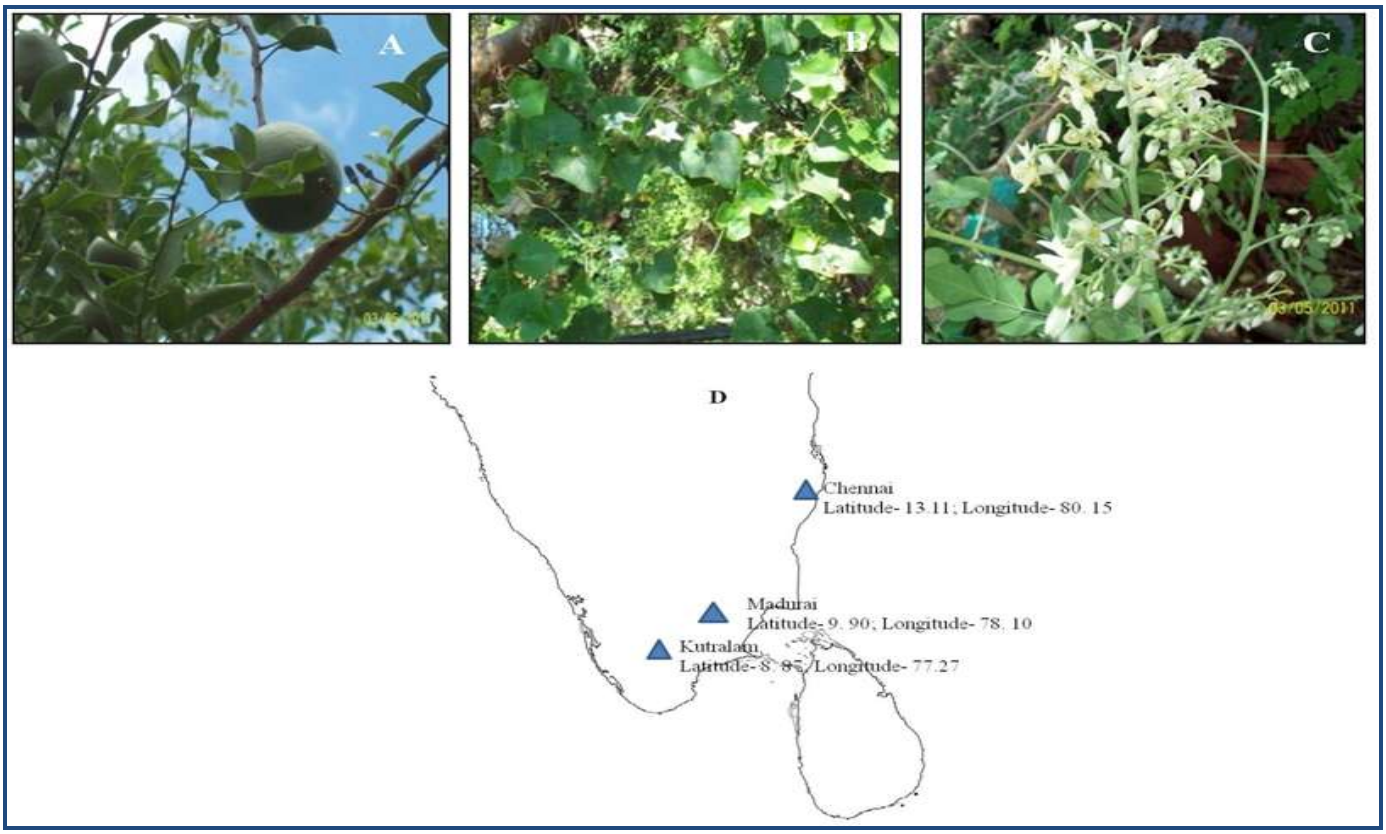

Figure 2: Plant photo of **A)** *A. marmelos*; **B)** *C. indica*; **C)** *M. oleifera*; **D)** Sample collection sites of three different medicinal plants with their corresponding latitude and longitudes.

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Methodology:

Collection of Samples

Plant samples of *A. marmelos*, *C. indica* and *M. oleifera* were collected from Chennai, Madurai and Courtallam (Kutralam) of Tamil Nadu, India **(Figures 2a & b)**. Samples were sealed and transported immediately to laboratory; asymptomatic leaves were separately processed within 24 hrs of collection for endophytic fungi isolation **[3, 26]**.

Isolation and identification of endophytic fungi from three different medicinal plants

Phylloplane fungal propagules adhering to the surface of the leaves were removed by surface sterilization using the modified method reported **[27]**: the leaves were washed with running tap water, sterilized with Ethanol (75% V/V) for 1 min and Sodium Hypochlorite (2.5% V/V) for 5 min, then rinsed in sterile water for three times and cut into 1 cm long segments. Plant segments were then transferred to Potato Dextrose Agar (PDA) plates supplemented with Ampicillin (200µg / ml) and Streptomycin (200µg /ml) emerging isolates were sub-cultured on PDA containing plates and incubated at 25°C for further studies **[28]**.

DNA extraction, amplification of ITS region and sequencing

Genomic DNA was extracted from the endophytic fungus using a modified CTAB method **[29]**.The partial nucleotide internal transcribed spacer (ITS) region was amplified from the genomic DNA using the polymerase chain reaction (PCR) by using the ITS1 forward primer (5′ TCC-GTA-GGT-GAA-CCT-GCG-G 3′) and ITS4 reverse primers (5′TCC-TCC-GCT-TAT-TGA-TAT-GC 3′). The PCR amplification was performed in an L196GGD Model Peltier Thermal Cycler Version-2.0 with a total 25 µl reaction that comprised of 20 ng of genomic DNA template, 10X buffer with 25mM $MgCl_2$, 10mM DNTP's, 2U of Taq DNA polymerase and 10 pmol of each primer (All molecular chemicals were purchased from Sigma Aldrich). The following reaction conditions were used: 4 min at 94°C for denaturation, 30 cycles each of 30 seconds at 94°C for denaturation, 1 min at 58.2°C for annealing, 2 min at 72°C for extension followed by the final extension at 72°C for 7min **[30].** The amplified DNA fragments were analyzed by 1% agarose gel electrophoresis with a 100bp ladder purchased from New England Biolabs (Catalogue No. 3231S) and the amplicons were visualized using a gel documentation system (Uvitech). A non-template control was included in each run. PCR products were purified using mini columns (PCR Preps DNA purification System, Sigma) according to the manufacturer's protocol. Further, the amplified products were sequenced by Eurofins Private Limited, Bangalore, India.

ITS2 secondary structure prediction, alignment, phylogenetic analysis

The ITS2 regions were extracted using fungal ITS extractor program. In this study, secondary structures of ITS2 were predicted for 48 query and 28 known isolates (downloaded from genbank NCBI) using Mfold programme (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) with default conditions (linear RNA sequence, folding temperature: 37°C, 1M NaCl (no divalent ions) ionic conditions, 5% sub-optimality, upper bound number of the folding: 50, maximum interior/bulge loop size: 30, maximum asymmetry of an interior/bulge loop: 30, maximum distance between paired bases: no limit). The selected secondary structures were downloaded in Vienna format from Mfold server **[31, 32]**. The consensus structure of each genus was generated using 4SALE **[33]**.

Phylogenetic analysis

ITS sequences of our isolates and control sequences were used for phylogenetic analysis (Neighbor-joining method with 1000 bootstrap replication) using MEGA 5.1. ITS2 sequences and secondary structures were synchronously aligned using 4SALE V 1.7 and resultant alignment was exported to ProfDistS **[34]** for tree construction.

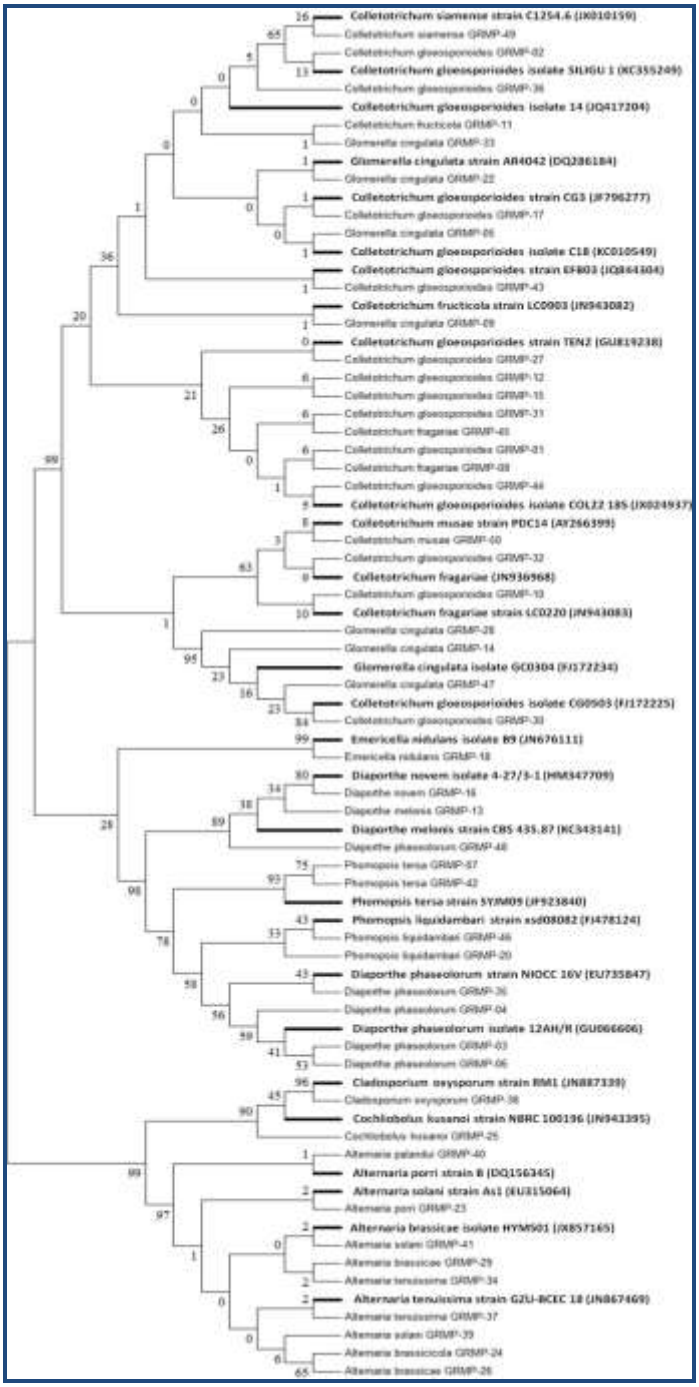

Figure 3: Phylogenetic tree inferred using Neighbor-Joining method for query (organism name with GRMPs) and control ITS sequence (organism written in bold and indicated by dark lines).

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Results:

Isolation of endophytes

A total of 166 isolates were obtained from the above three medicinal plants, among them 48 isolates were characterized based on molecular identification and their ITS sequences were submitted in Genbank (Genbank ID details were listed in **Table 1 (see supplementary material)**. Phylogenetic tree was constructed for our isolates and control isolates using MEGA 5.1 software **(Figure 3)**. Identified isolates belongs to 5 orders (Glomerales, Pleosporales, Diaporthales, Capnodiales and Eurotiales) and 6 genera (*Colletotrichum/Glomerella*, *Diaporthae/Phomopsis*, *Cochilobolus*, *Alternaria*, *Cladosporium*, *Emericella*) of Ascomycota. *Colletotrichum/Glomerella* genera showed maximum diversity (52%), while *Emericella*, *Cochliobolus* and *Cladosporium* showed minimum diversity (2.0%). Other genera such as *Alternaria* showed 19% and *Diaporthe/Phomopsis* had 23% diversity respectively **(Figure 4)**. *C. gloeosporioides* and *G. cingulata*, a telomorph of the former were commonly found in the leaves of all three medicinal plants. *C. kusanoi* and *E. nidulans* were present only in *C. indica* while *C. oxysporum* was found only from the leaves of *M. oleifera*. Distribution of other isolates was represented in **Table 2 (see supplementary material)**.

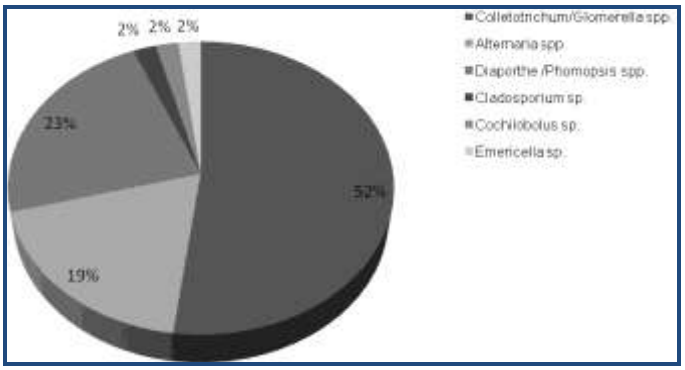

Figure 4: Diversity percentage of different genus of endophytes isolated in this study.

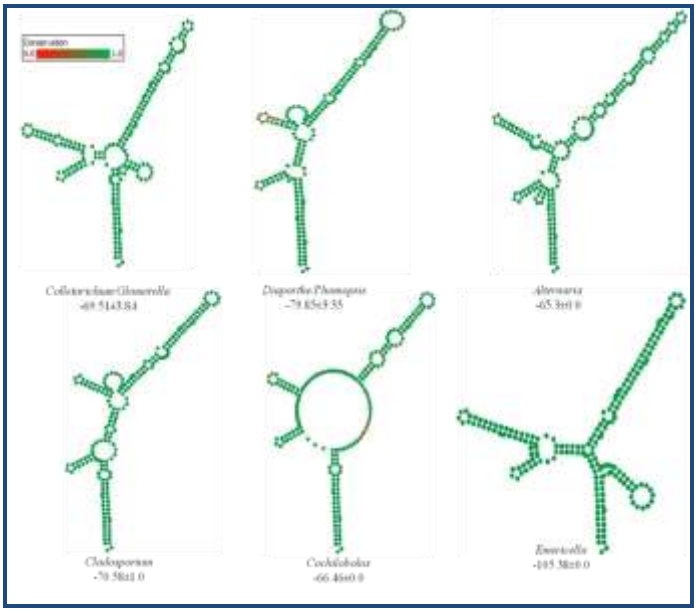

Figure 5: Consensus ITS2 secondary structure of endophytes genera observed in this study. *Colletotrichum/Glomerella* and *Emericella* shared the four helix loop regions, as helices II and III are recognizable. *Alternaria, Cladosporium, Diaporthe/ Phomopsis* and *Cochilobolus* possessed three helixes and third helix is the longest in all genuses.

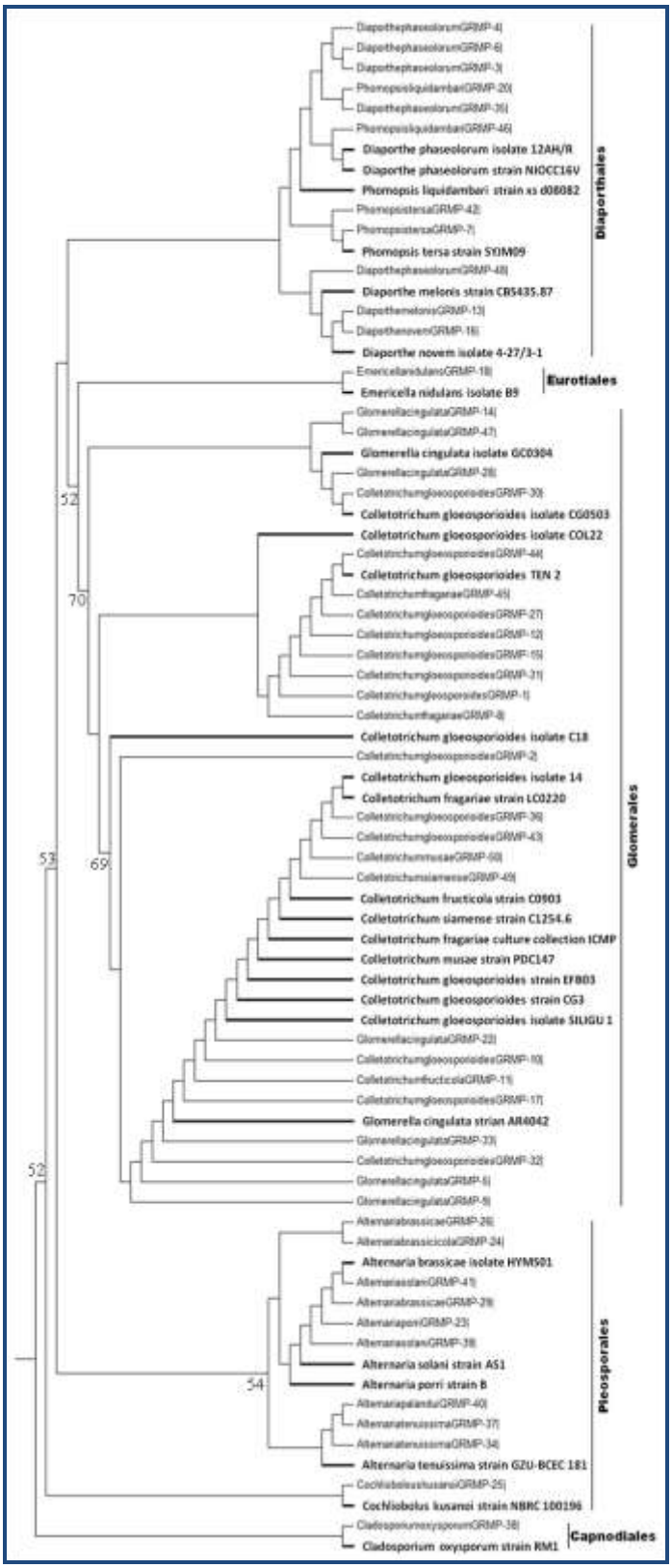

Figure 6: Molecular phylogenetic analysis of query (organism name with GRMPs) and control ITS2 sequence (organism writeen in bold and indicated by dark lines) by neighbour joining method.

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ITS2 secondary structure

The results of the ITS2 extraction for 76 sequences had been summarised in **Table 2 & 3 (see supplementary material)**. ITS2 sequences varied from 157 to 167 base pairs and GC content 48.73 – 68.86%. Further they were used to model a consensus structure for each of the respective genera **(Figure 5)**. In *Colletotrichum/Glomerella* genus, secondary structures had been modelled for 44 sequences whose minimum free energy (MFE) was -69.51±3.84 (mean ± standard deviation (SD). Similarly, secondary structure predicted for 17 sequences of *Diaporthae/Phomopsis* had -79.85±3.35 MFE, 13 sequences of *Alternaria* had -65.3±0.0 MFE and 2 sequences each of *Cladosporium*, *Cochilobolus*, *Emericella* possessed -70.58±1.0, -66.46±0.0 and -105.38±0.0 MFE respectively.

The putative secondary structures of all isolates from the 6 genera and 4 orders could be characterized into 2 patterns: pattern I, the benchmark 4-domain model; pattern II, a 3-domain model. The 20 bp of the 5.8S and 28S rDNA that was present flanking to the 5′-end and 3′-end of the ITS2 apparently forms canonical bonds with each other. Consensus structure of genus *Colletotrichum/Glomerella* and *Emericella* were of 4 helices while three helixes were observed in genus *Diaporthe/Phomopsis*, *Alternaria, Cladosporium* and *Cochilobolus*. Third helix being the longest-extremely conserved region in all genuses studied where as helix I show variation among different taxa; helix IV is not always occurs. *Colletotrichum/glomerella* and *Diaporthe/Phomopsis* isolates had CBCs whereas in *Alternaria* none could be detected. Hemi - CBCs. were observed in loop I, II, III of all genera studied. Loop I and II has single hemi-CBCs (G-U) in all observed genera except in GRMP-13 and 48 of *Diaporthe* genus; While in loop III, in addition to G-U hemi-CBC (2-5) other hemi-CBCs (A-C and C-A) were observed Table 4 (Available with authors). To the overall structure we observed a conserved motif like an UGGC sequence preceding the apex of the third helix in all class involved in this study. Further the UGGUUU motif was observed in the loop III of order Capnodiales, Glomerales and Pleosporales, whereas AGGA and CGGA motif was only observed in Diaporthales and Glomerales. Likewise CGGC motif was present in Capnodiales, Eurotiales and Pleosporales.

Phylogenetic reconstruction

Phylogenetic tree was constructed based on ITS2 sequence-structure using our isolates and control sequences **(Table 3)** have yielded well resolved clades with higher bootstrap support. Monophyletic clades formed were supported with a bootstrap value >50% were represented at their respective nodes **(Figure 6)**.

Discussion:

A surge in the molecular phylogeny supported research has greatly illuminated the fungal systematics the fungal systematic, ecological and diversity studies as robust computational algorithms with high statistical support and advancements in sequencing technology continue to evolve. ITS2 had been suggested as a standard marker for fungi and integrating ITS2 based phylogenetic analysis with the morphological features of their primary sequences has been the recent trend, which has significantly enhanced the resolution and stability of the clades **[35, 36, 37, 38]**. ITS2 secondary structure of the sequences analysed in our study were modelled with optimum and sub-optimal free energy in RNAfold program from MFOLD server at default folding conditions **[31]**. We have employed this approach due to its wide spread use especially in modelling ITS2 secondary structure.

Structures sharing similarities (3-4 helixes) to pan-eukaryotic ITS2 model were chosen from the predicted set of suboptimal structures. On further evaluation of the chosen structures, several conserved motifs were observed. Similar versions of a conserved UGGU motif, preceding the apex of the III helix were observed in our ITS2 structures. A UGGC motif in the 5′ side to the apex of the III helix was highly conserved across the investigated genera barring *Cochilobolus* and *Emericella*. Variations in this conserved motif were common among fungi **[17, 37, 39, 40]** while their occurrence at similar position has been maintained. Another conserved UGGUUU motif was observed in III helix of all genera except in *Diaporthe* genus. Conserved pyrimidine – pyrimidine bulge in the second helix had been reported to occur in most fungi **[17]** while NS1, an environmental sequence of fungal origin had been reported to lack this motif **[41]**.

In our study, *Alternaria* and *Colletotrichum* genera possessed Pyr-Pyr motif while the others lacked. Also the single stranded region connecting the II and III helices appeared to be conserved among each genus **[14, 19, 42, 43]**. Sequence – structure alignment in 4SALE program **[33, 44]** that allows synchronous editing and visualization was further analysed for the presence of CBCs. *Diaporthe* and *Colletotrichum* isolates possessed CBCs in the basal regions of II helix. Occurrence of even one CBC in ITS2 region has been regarded as a significant evolutionary diversification event that distinguishes two closely related organisms. He vividly demonstrated CBC as a classifier with 93.11% reliability score in distinguishing species albeit the fact a lack of CBCs is not an indicator of two organisms belonging to the same species **[45]**.

A profile neighbour joining tree construct based on our sequence-structure alignment resulted in well separated clades. All the investigated isolates belonged to 5 orders and 6 genera of Ascomycota. Distinct clades in *Diaporthe* and *Colletotrichum* genera were formed based on the presence of CBCs. Several sub-clades with less bootstrap value were also formed within *Diaporthe* and *Colletotrichum* genera that were not supported by CBCs. At species level, several incongruences were observed mainly due to the conventional practices of naming nucleotide sequences. Similar anomalies had been reported earlier **[46, 47, 48]** and this mandates revisiting the erroneous Genbank entries and naming after the maximum identical Genbank entries with minimum E-value. Overall resolution of our phylotree at genera level had a bootstrap support >50%. The consensus tree depicted Diaporthales closest to the root and all other genera examined in this studied have to be derived. Glomerales, the other Sorodoriomycetes order studied and Eurotiales of Eurotiomycetes formed sister clades with Dothidiomycetes clade that hosted 2 pleosporales genus (*Alternaria* and *Cochilobolus*) and *Cladosporium* of Capnodiales in subclades, but contradicted with the multigene based phylotree of Ascomycota **[49]**. The foremost reason for this variation may be due to the random mutation (insertion and deletion) which happens rapidly during the evolution and it depends upon mistakes associated in DNA replication.

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Conclusion:
The present result provides novel support from immense analysis of ITS2 sequences and CBC estimation for different endophyte complex. CBC can be used as primary molecular indicator to confirm no genetic exchange between two populations is happened which widens the identification and classification of endophytic species further. The proposed ITS2 based phylogenetics with the fungal isolates from our own study (GRMP) with the reference sequences (ex-types) has clearly distinguish the isolates with greater precision than any other existing methods. This is the first report from India on ITS2 sequence-structure analysis of endophytic fungi from the medicinal plants of *A. marmelos*, *C. indica* and *M. oleifera*.

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References:
[1] Wilson D, *Oikos.* 1995 **73**: 274
[2] Rodriguez RJ *et al. New phytol*. 2008 **182:** 314 [PMID: 19236579]
[3] Strobel G & Daisy B, *Microbiol Mol Biol Rev*. 2003 **67**: 491 [PMID: 14665674]
[4] Selim KA *et al. Mycosphere*. 2011 **2:** 669
[5] Saikkonen K *et al. Ann Rev Eco Syste*. 1998 **29**: 319
[6] Suryanarayanan TS *et al. Can J Bot*. 2002 **20**: 819
[7] Pandi M *et al. Afr J Biotechnol*. 2011 **10**: 1428
[8] Arnold AE & Lutzoni F, *Ecology.* 2007 **88**: 541 [PMID: 17503580]
[9] Blackwell M *et al. Mycologia.* 2006 **6**: 829 [PMID: 17486960]
[10] Troitsky AV *et al. Biochemistry (Mosc)*. 2007 **72**: 1368 [PMID: 18205621]
[11] Bellemain E *et al. BMC Microbiol*. 2010 **10:** 189 [PMID: 20618939]
[12] Won H & Renner SS, *Mol Phylog Evol*. 2005 **36:** 581 [PMID: 16099382]
[13] Sajeewa SN *et al. Mycol Prog*. 2013
[14] Coleman AW, *Trends Genet*. 2003 **19:** 370 [PMID: 12850441]
[15] Han J *et al. BioMed Res Int*. 2013 2013: 741476 [PMID: 23484151]
[16] Kruger D & Gargas A, *Mycol Res.* 2008 **112: 316** [PMID: 18342242]
[17] Koetschan C *et al. Nucleic Acids Res*. 2010 **38**: D275 [PMID: 19920122]
[18] Hoshina R, *Microbes Environ*. 2010 **25**: 313 [PMID: 21576888]
[19] Coleman AW, *Nucleic Acids Res.* 2007 **35:** 3322 [PMID: 17459886]
[20] Schultz J & Wolf M, *Mol Phylogenet Evol.* 2009 **52:** 520 [PMID: 19489124]
[21] Karmase A *et al. Phytomedicine*. 2013 **20**: 805 [PMID: 23632084]
[22] Nugroho AE *et al. Bioinformation* 2013 **9**: 383 [PMID: 23750086]
[23] Fahey JW, *Trees for Life Journal*. 2005 **1:** 5
[24] Shaheen SZ *et al. Afr J Biotechnol*. 2009 **8:** 7073
[25] Dhankhar S *et al. J Medicinal Plants Res*. 2011 **9:** 1497
[26] Strobel GA, *Can J Phy.* 2002 **24:** 14
[27] Dobranic JK *et al. Can J Microbiol*. 1995 **41:** 194
[28] Liu JY, *J Biotechnol.* 2004 **114:** 279 [PMID: 15522437]
[29] Doyle JJ & Doyle JL, *Proc Natl Acad Sci U S A.* 1990 **87:** 714 [PMID: 11607059]
[30] Sim JH *et al. J Microbiol Biotechnol*. 2010 **20**: 651 [PMID: 20467234]
[31] Zuker M, *Nucleic Acids Res.* 2003 **31**: 3406 [PMID: 12824337]
[32] Meyer IM & Miklos I, *PLoS Comput Biol.* 2007 **3:** 149 [PMID: 17696604]
[33] Seibel PN *et al. BMC Res Notes*. 2008 **1:** 91 [PMID: 18854023]
[34] Friedrich J *et al. Bioinformatics* 2005 **21:** 2108 [PMID: 15677706]
[35] Wolf M *et al. In Silico Biol.* 2005 **5:** 291 [PMID: 15996120]
[36] Miao M *et al. Protist.* 2008 **159**: 519 [PMID: 18675585]
[37] Schultz J *et al. RNA*. 2005 **11:** 361 [PMID: 15769870]
[38] Selig C *et al. Nucl Acids Res*. 2008 **36**: D377 [PMID: 17933769]
[39] Oliveiro M *et al. Mol Ecol Resour*. 2009 **9:** 94 [PMID: 21564571]
[40] Salvi D *et al. Mol Phylog Evol.* 2010 **56:** 1059 [PMID: 20416386]
[41] Glass DJ *et al. Mol Phylogenet Evol*. 2013 b67: 234 [PMID: 23403224]
[42] Coleman AW, *Mol Phylogemet Evol.* 2009 **50:** 197 [PMID: 18992828]
[43] Coleman AW & Vacquier VD, *J Mol Evol.* 2002 **54:** 246 [PMID: 11821917]
[44] Seibel PN *et al. BMC Bioinformatics*. 2006 **7:** 498 [PMID: 17101042]
[45] Muller T *et al. RNA*. 2007 **13**: 1469 [PMID: 17652131]
[46] Ahvenniemi P *et al. J Mol Evol*. 2009 **69:** 150 [PMID: 19609478]
[47] Cai L *et al. Fungal Diversity*. 2009 **39:** 183
[48] Linnakoski R, *Insects.* 2012 **3**: 200
[49] Schoch CL *et al. Syst Biol.* 2009 **58**: 224 [PMID: 20525580]

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Supplementary material:

Table 1: Summary of our fungal isolates' ITS2 RNA secondary structure modelled with RNA folding form 3.0

Genbank Numbers	Organism Name	Total[a] length	ITS2[b] region	A's	U/T's	G's	C's	%[c]GC	Free energy[d] (37°C)
JQ433884	*Colletotrichum gloeosporioides* GRMP-1	543	345-502	31	36	41	50	57.59	-68.03
JQ585643	*Colletotrichum gloeosporioides* GRMP-2	535	340-496	31	38	41	47	56.05	-67.73
JQ585644	*Diaporthe phaseolorum* GRMP-3	534	340-497	35	32	43	48	57.59	-80.42
JQ585645	*Diaporthe phaseolorum* GRMP-4	543	345-502	34	32	44	48	58.23	-86.72
JQ585646	*Glomerella cingulata* GRMP-5	539	342-499	31	37	41	49	56.96	-68.03
JQ585647	*Diaporthe phaseolorum* GRMP-6	539	343-500	35	32	43	48	57.59	-80.42
JQ585648	*Phomopsis tersa* GRMP-7	545	344-506	32	35	44	52	58.90	-82.65
JQ585649	*Colletotrichum fragariae* GRMP-8	538	342-499	31	36	41	50	57.59	-68.03
JQ585650	*Glomerella cingulata* GRMP-9	538	342-499	31	37	41	49	56.96	-68.03
JQ585651	*Colletotrichum gloeosporioides* GRMP-10	538	343-499	31	37	41	48	56.96	-67.73
JQ585652	*Colletotrichum fructicola* GRMP-11	535	340-496	31	37	41	48	56.96	-67.73
JQ782660	*Colletotrichum gloeosporioides* GRMP-12	546	346-503	31	36	41	50	57.59	-68.03
JQ782661	*Diaporthe melonis* GRMP-13	543	346-504	31	32	45	51	60.38	-82.85
JQ796654	*Glomerella cingulata* GRMP-14	546	347-504	33	29	39	57	60.76	-78.49
JQ796655	*Colletotrichum gloeosporioides* GRMP-15	541	345-502	31	36	41	50	57.59	-68.03
JQ796656	*Diaporthe novem* GRMP-16	538	343-499	33	33	44	47	57.96	-81.45
JQ796657	*Colletotrichum gloeosporioides* GRMP-17	510	310-467	31	37	41	49	56.96	-68.03
JQ796658	*Emericella nidulans* GRMP-18	478	271-437	18	34	57	58	68.86	-105.3
JQ818172	*Colletotrichum gloeosporioides* GRMP-19	351	342-end	-	-	-	-	-	-
JQ818173	*Phomopsis liquidambari* GRMP-20	540	344-501	34	34	42	48	56.96	-78.42
JQ818174	*Colletotrichum fragariae* GRMP-21	471	340-end	-	-	-	-	-	-
JQ818175	*Glomerella cingulata* GRMP-22	542	344-501	31	37	41	49	56.96	-68.03
JQ818176	*Alternaria porri* GRMP-23	528	332-490	31	49	37	42	49.69	-65.25
JQ818177	*Alternaria brassicicola* GRMP-24	533	333-491	30	49	37	43	50.31	-65.25
JQ818178	*Cochliobolous kusanoi* GRMP-25	533	332-494	30	51	37	45	50.31	-71.28
JQ818179	*Alternaria brassicae* GRMP-26	503	303-461	30	49	37	43	50.31	-65.25
JQ818180	*Colletotrichum gloeosporioides* GRMP-27	538	344-501	31	36	41	50	57.59	-68.03
JQ818181	*Glomerella cingulata* GRMP-28	541	342-498	33	29	39	56	60.51	-78.55
JQ818182	*Alternaria brassicae* GRMP-29	532	335-493	31	49	37	42	49.69	-65.25
JQ818183	*Colletotrichum gloeosporioides* GRMP-30	533	341-498	32	29	40	57	61.39	-80.55
JQ818184	*Colletotrichum gloeosporioides* GRMP-31	540	344-501	31	36	41	50	57.59	-68.03
JQ818185	*Colletotrichum gloeosporioides* GRMP-32	537	343-499	31	37	41	48	56.69	-67.73
JQ818186	*Glomerella cingulata* GRMP-33	538	339-496	31	37	41	49	56.96	-68.03
JQ818187	*Alternaria tenuissima* GRMP-34	531	334-493	31	50	37	42	49.38	-63.27
JQ818188	*Diaporthe phaseolorum* GRMP-35	543	344-501	35	34	41	48	56.33	-78.42
JQ818189	*Colletotrichum gloeosporioides* GRMP-36	497	298-455	31	37	41	49	56.96	-68.03
JQ818190	*Alternaria tenuissima* GRMP-37	534	335-494	31	50	37	42	49.38	-63.27
JQ818191	*Cladosporium oxysporum* GRMP-38	559	356-518	34	47	40	42	50.31	-66.46
JQ818192	*Alternaria solani* GRMP-39	535	335-493	31	49	37	42	49.69	-65.25
JQ818193	*Alternaria palandui* GRMP-40	536	335-494	31	50	37	42	49.38	-63.27
JQ818194	*Alternaria solani* GRMP-41	534	334-492	31	49	37	42	49.69	-65.25
JQ818195	*Phomopsis tersa* GRMP-42	553	349-511	32	35	44	52	58.90	-82.65
JQ818196	*Colletotrichum gloeosporioides* GRMP-43	537	344-501	31	37	41	49	56.96	-68.03

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JQ818197	*Colletotrichum gloeosporioides* GRMP-44	540	344-501	31	36	41	50	57.59	-68.03
JQ818198	*Colletotrichum fragariae* GRMP-45	545	344-501	31	36	41	50	57.59	-68.03
JQ818199	*Phomopsis liquidambari* GRMP-46	539	340-497	33	35	41	49	56.96	-74.70
JQ818200	*Glomerella cingulata* GRMP-47	545	346-503	33	29	39	57	60.76	-78.49
JQ818201	*Diaporthe phaseolorum* GRMP-48	545	344-502	31	35	45	48	58.49	-78.37
JQ818202	*Colletotrichum siamense* GRMP-49	537	340-496	31	38	41	47	56.05	-67.73
JQ818203	*Colletotrichum musae* GRMP-50	541	345-501	31	37	41	48	56.69	-67.73

[a]ITS region obtained by sequencing , [b]ITS2 region extracted using fungal ITS extractor, [c] and [d] GC% and free energy of secondary structure.

Table 2: List of fungi isolated from selected medicinal plants

S. No.		Organism Name	*A. marmelos*	*M. oleifera*	*C. indica*
1.		Glomerales (*Colletotrichum/Glomerella*)	+[3]	+[2]	+[8]
	a.	*C. gloeosporioides*	+[1]	-	+[1]
	b.	*C. fragariae*	+[1]	-	-
	c.	*C. siamense*	-	-	+[1]
	d.	*C. musae*	-	+[1]	-
	e.	*C. fructicola*	+[4]	+[1]	+[2]
	f.	*G. cingulata*			
2.		Diaporthales (*Diaporthe/Phomopsis*)	+[1]	+[4]	-
	a.	*D. phaseolorum*	-	+[1]	-
	b.	*D. melonis*	-	-	+[1]
	c.	*D. novem*	+[1]	+[1]	-
	d.	*P. tersa*	+[1]	-	+[1]
	e.	*P. liquidambari*			
3.		Eurotiales (Emericella)			
	a.	*Emericella nidulans*	-	-	+[1]
4.		Pleosporales (*Alternaria/Cochilobolus*)	+[1]	-	-
	a.	*A. porri*	-	+[1]	-
	b.	*A. brassicicola*	-	+[1]	+[1]
	c.	*A. brassicae*	-	-	+[1]
	d.	*A. palandui*	+[1]	-	+[1]
	e.	*A. tenuissima*	-	+[2]	-
	f.	*A. solani*			
	a.	*Cochlioboluskusanoi*	-	-	+[1]
5.		Capnodiales (*Claodosporium*)			
	a.	*Cladosporium oxysporum*	-	+[1]	-

Table 3: Summary of control (NCBI) sequences' ITS2 secondary structures modelled with RNA folding form 3.0

GenBank Number	Organism	Total[a] length	ITS2[b] region	A's	U/T's	G's	C's	% GC[c]	Free energy[d] (37°C)
JX010159.1	*Colletotrichum siamense* strain C1254.6	593	388-544	31	38	41	47	56.05	-67.73
DQ286184.1	*Glomerella cingulata* strain AR4042	587	384-541	31	37	41	49	56.96	-68.03
JN943082.1	*Colletotrichum fructicola* strain LC0903	549	346-503	31	37	41	49	56.96	-68.03
JQ844304.1	*Colletotrichum gloeosporioides* strain EFB03	593	377-534	31	37	41	49	56.96	-68.03
AY266399.1	*Colletotrichum musae* strain PDC147	599	384-541	31	37	41	49	56.96	-68.03
JF796277.1	*Colletotrichum gloeosporioides* strain CG3	544	345-502	31	37	41	49	56.96	-68.03
FJ172225.1	*Colletotrichum gloeosporioides* isolate CG0503	564	362-519	34	29	39	56	60.13	-77.08
HM347709.1	*Diaporthe novem* isolate 4-27/3-1	536	342-498	34	32	44	47	57.96	-81.45

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KC343141.1	*Diaporthe melonis* strain CBS 435.87	573	372-530	30	32	45	52	61.01	-81.77
JF923840.1	*Phomopsis tersa* strain SYJM09	562	341-502	32	34	44	52	59.26	-76.32
FJ478124.1	*Phomopsis liquidambari* strain xsd08082	600	385-542	35	36	41	46	55.06	-73.60
EU735847.1	*Diaporthe phaseolorum* strain NIOCC 16V	535	327-484	35	34	41	48	56.33	-76.32
JN676111.1	*Emericella nidulans* isolate B9	543	318-484	18	34	57	58	68.86	-105.38
JN887339.1	*Cladosporium oxysporum* strain RM1	592	368-530	34	47	40	42	50.31	-66.46
JN943395.1	*Cochliobolus kusanoi* strain NBRC 100196	1120	335-492	33	48	35	42	48.73	-69.87
JX857165.1	*Alternaria brassicae* isolate HYMS01	571	354-512	31	49	37	42	49.69	-65.25
JN867469.1	*Alternaria tenuissima* strain GZU-BCEC181	581	366-525	31	50	37	42	49.38	-63.27
JX024937.1	*Colletotrichum gloeosporioides* isolate COL22	551	351-508	31	37	41	49	56.96	-68.03
KC355249.1	*Colletotrichum gloeosporioides* isolate SILIGU1	580	365-521	31	38	41	47	56.05	-67.73
GU066606.1	*Diaporthe phaseolorum* isolate 12AH/R	565	351-508	35	33	43	47	56.96	-80.90
JN936968.1	*Colletotrichum fragariae* ICMP:17927	549	347-504	31	37	41	49	56.96	-68.03
JQ417204.1	*Colletotrichum gloeosporioides* isolate 14	570	369-526	31	37	41	49	56.96	-68.03
KC492494.1	*Colletotrichum gloeosporioides* strain MGEF51	536	373-end	-	-	-	-	-	-
DQ156345.1	*Alternaria porri* strain B	569	353-511	31	49	37	42	49.69	-65.25
KC010549.1	*Colletotrichum gloeosporioides* isolate C18	583	360-516	31	37	41	48	56.69	-67.73
EU315064.1	*Alternaria solani* strain As1	570	354-512	31	49	37	42	49.69	-65.25
GU819238.1	*Colletotrichum gloeosporioides* strain TEN2	551	351-508	31	36	41	50	57.59	-68.03
JN943083.1	*Colletotrichum fragariae* strain LC0220	552	350-507	31	37	41	49	56.96	-68.03
FJ172234.1	*Glomerella cingulata* isolate GC0304	565	363-520	33	29	39	57	60.76	-78.49

[a]Known ITS sequence downloaded from NCBI – genbank database, [b]ITS2 region extracted using fungal ITS extractor, [c] and [d]GC% and free energy of secondary structure.

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