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New Taxon of Fungal Endophytes from *Phrynium capitatum* Willd: A Promising Ethnomedicinal Plant in Northeast India and its Systematic and Phylogenetic Analysis

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Abstract—The findings of the current study deal with the first time report on the diversity of fungal endophyte from the ethnomedicinal plant- *Phrynium capitatum* Willd., **(**Marantaceae). The plant is growing wildly in the forest of Papum Pare, Arunachal Pradesh, India, and traditionally been used for anti-diabetic, antihyperglycemic or analgesic effects. During the investigation, samples of the plant parts, viz., leaves, stem, and roots were collected for isolation of endophytic spp. The endophytic spp were identified based on the morphological, cultural, and reproductive structures (hyaline, ellipsoidal, aseptate, pycnidia, beta conidia, perithecia, asci, and ascospores). Further, the phylogenetic analysis of the isolated species was made, using the sequences of 5.8S and 28S rDNA internal transcribed spacer sequence 1 and 4. The largest number of fungal endophytes (39%) were isolated from the leaves, followed by the roots (31%) and stems (30%). Overall 35 fungal species have been isolated, out of which, thirty-four belongs to the class Ascomycetes, and one from the class zygomycetes. The highest species richness and frequency of colonization were recorded in the leaf. The observations show that *Pestalotiopsis longiseta* was the most dominant endophytic species followed by *Diplodina* microsperma*, Nodulisporium hinnuleum, Aspergillus flavus* , *Diaporthe* sp., *Nigrospora oryzae* and *Lasiodiplodia viticola*. However, *Pestalotiopsis neglecta, Sordaria fimicola, Diplodina microsperma, Fusarium incarnatum, Preussia* sp., *Diaporthe* sp., *Aureobasidium* sp.were common fungal endophytes isolated from *P. capitatum*

Keywords: Biodiversity, Colonization Frequency, Endophyte, Internal Transcribed Spacer, Medicinal Plant, Arunanchal Pradesh

INTRODUCTION

De Barry (1866) coined the term 'endophyte' to detect fungi that live intercellulary and intracellularly in plants tissues causing no harm to the plant (Compant *et al.,* 2017; Jeewon *et al.,* 2017). Further, based on their interactions with host plants, fungal endophytes may be divided into three groups: commensalists, parasites, and mutualists (Jia *et al.,* 2016, Kirschner 2018)**.** In mutualism, definite fungal endophytes give tolerance to abiotic and biotic stresses on their host plants, improve their growth, and restrain diseases (Redman 2002, Rodriguez 2008). These Endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites (Nath *et al.,* 2012; Nongalleima *et al.,* 2013). Endophytic fungi were reported in almost all the plants spp. algae, ferns, mosses and mainly in gymnosperms, angiosperms reported from various parts of the world (Radic and Strukelj 2012; Doilom *et al.,* 2017). Many researchers have proven that endophytes are the potential source of novel natural products for exploitation in modern medicine, agriculture and industry (Kaul *et al.,* 2013). To date, it has been found that taxol can be produced by endophytic fungi *Metarhizium anisopliae* and *Cladosporium cladosporioides* (El-Maali *et al.,* 2018). However, fungal endophytes can be easily grown in labs under routine culture techniques, and hence the potential for discovering a virtually inexhaustible supply of metabolites is high. Fungal endophytes can

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produce compounds similar to their host plants and are capable of preserving the world's diminishing biodiversity (Bender *et al.,* 2016; Mane *et al.,* 2017).

Studies on fungal endophytes concentrated on medicinal plants have shown that the curative property of the medicinal plant is not only because of the chemicals present in the plant but also because of the fungal endophytes that present in the plant (Verma 2011; Suryanarayanan 2013). Thus there is a need to isolate and identify several fungal endophytes and explore the potency of their secondary metabolites. Although, number of studies have already been reported in the association of fungal endophytes with medicinal plants from India viz., *Clerodendron serratum, Pongamia, Ashwagandha, Taxus brevifolia, Azadirachta indica, Terminalia arjuna, Trigonella, foenum graecum, Labelia nicotinifolia, Adhatoda zeylanica, Bauhinia phoenicea,Catharanthus roseus, Parthenium hysterophorus, Ficus religiosa, Coffea Arabica, Crataeva magna, Silybum marianum, Allium sativum, Mamordica chaeantia, Azadarichata indica* and *Nothapodytes nimmoniana, (*Tejesvi *et al.,* 2005; Mane *et al.,* 2017; Mane and Vedamurthy 2018), but there is no such report on fungal endophytic association with *P. capitatum*.

MATERIALS & METHODS

Study Area

The study area Papum Pare is located at the N 26°56′11″ to 27°35°44″ and E 93°12′45″ to 94°13′30″ is one of the important hotspots in the world, ranked 25th (Chowdhery 1999), and among the 200 globally important eco-regions (Olson and Dinerstein 1998). It covers an area of 2875 sq. km, having an annual rainfall of 2694 mm. Major part (75%) of this district is covered by thick forest which has a sub-tropical, deciduous and humid type of vegetation (Fig.1-a).

Plant & Sample Collection

The selected plant, *P. capitatum* is a semi-evergreen herb with creeping rootstock and is wildly growing in forest area (Papum Pare) in Arunanchal Pradesh (Fig. 1-b). Leaves oblong, acuminate, base rounded or obtuse, to 30 x 18 cm; petiole to 60 cm long, spike globose, sessile on the petiole, 4-6 cm across**.** Inner bracts ovate**-**oblong, fimbriate at the apex, corolla yellow, outer petaloid staminodes orange-red, lip with a pendulous appendage, ovary tomentose (Anon 2019).

Samples of leaves, stems, and roots were collected during (Dec 2016- Dec 2018) from the healthy plant of *P. capitatum* in sterile bags and brought to the laboratory for processing within 24 hours after sampling.

Surface Sterilization and Isolation

The samples thus collected were washed gently in running tap water to remove the soil and debris. Further, isolation of fungal endophytes was determined, using the method of Suryanarayanan *et al.,* (1998). Samples were cut into small pieces of (0.5-1.0 cm). The pieces were then surface-sterilized by dipping them serially in 70% ethanol for 5 sec and 4% NaOCl for 90 sec; finally rinsed in sterile distilled water for 10 sec. Now, 150 segments of the samples were selected randomly and plated on Potato Dextrose Agar (PDA) medium (supplemented with 150 mg/L chloramphenicol) contained in Petri dishes (7.5 cm diam). Petri dishes were incubated (12 h dark: 12 h light cycle) for 25 days at 25± 2°C, to observe the growth of endophytes, following the method of Suryanarayanan (1992). The hyphal tips which grew out from the segments were isolated and subcultured on PDA medium and preserved at 4°C for further investigation.

Further, the colonization frequency (CF) was calculated, using the following formula (Suryanarayanan *et al.,* 2003):

$$CF\% = \frac{\text{Number of segments colonized by fungal endophytes}}{\text{Total number of segment observed}} \times 100$$

However, the density of colonization (rD%) of a single endophyte species was calculated, using the method of Fisher and Petrini (1987).

$$rD\% = (Ncol/Nt) \times 100$$

Where,

Ncol = Number of segments colonized by each fungus

Nt = Total number of segments inoculated

Morphological Identification

We identified the endophytes thus isolated based on their macroscopic and microscopic characteristics viz., the morphology of fruiting structures and spore morphology (Sutton 1980; Subramanin 1983 and Nagmani 2005).

MOLECULAR IDENTIFICATION

DNA Extraction and PCR Amplification of rDNA

Genomic DNA was extracted from fresh fungal mycelia growing on PDA plates (at 25± 2°C and 5 to 8 days old), using a Qiagen microbial extraction kit. Fragments of 18S rRNA gene were amplified by PCR, using forward ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer (White *et al.,* 1990). Each PCR amplification reaction was performed in a final volume of 25 µL containing 25 ng template DNA, 10X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM MgCl2, 200 µM of each dNTP, 50 pM of each primer, 1 unit of Taq polymerase (Genei, Banglore) and distilled water (Sigma, USA). The conditions of the PCR was initial denaturation at 95°C for 5 minutes followed by 35 cycles for 45 sec, annealing at 56 °C for 1 minute (35 cycles) and primer extension at 72 °C for 1 minute (again 35 cycles), final extension at 72 °C for 7 minutes (1 cycle) and hold (cooling) at 4 °C. PCR products were checked on 1.2 % Agarose gel in Tris–acetate-EDTA buffer (TAE) at pH 8.0, stained with ethidium bromide (0.3 g/mL) and visualized under UV light by using Gel documentation system. Sequencing was done by GeNei Labs Private Limited, Bengaluru using PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase, as per the manufacturer instructions. Obtained Sequences were subjected to BLAST (Basic Local Alignment Search Tool). All obtained sequences were submitted and awarded access numbers in GenBank of NCBI (Table 1).

PHYLOGENETIC ANALYSIS

The primer pairs ITS1/ITS4 (White *et al.,* 1990) were used to amplify ITS-rDNA and partial. The reaction mixture and thermal cycling conditions were the same as described by Karimi *et al.* (2016). The data thus sequenced was subjected to BLAST (Basic Local Alignment Search Tool), for homology search and multiple sequence alignments were made by ClustalW. Phylogenetic relationship of all fungal species; drawn their phylogenetic relationship. Further, it was analyzed using Neighbor-Joining methods of MEGA 5 (Tamura *et al.,* 2011); and the robustness of the inferred phylogeny was assessed using bootstrap value at 1,000 replications.

RESULTS

The findings of the present investigation deal with the isolation of endophytic fungi, from *P.capitatum*; an ethnomedicinal plant commonly used to treat various ailments in human beings. The samples of the plants were collected randomly from the study area (Papum Pare). Out of total of 15 plant materials of *P. capitatum*, 150 segments were processed (including root, stem and leaves plant parts); for the isolation of endophytic fungi. The observations recorded total of 127 isolates of endophytic fungi; which belong to 35 fungal spp. The maximum number of fungal isolates were recorded from leaves (39%), followed by roots (31%) and stems (30%) [Fig. 1-c], respectively; however, the colonization frequency rate was recorded highest in leaves (80%) followed by roots (70%) and stem (59.61%). Total of 35 fungal endophytes were isolated, which belongs to 25genera.The observations show that *Pestalotiopsis longiseta* was the most dominant endophytic fungi, isolated from the selected plant (*P.capitatum*); followed by *Diplodina microsperma, Nodulisporium hinnuleum, Aspergillus flavus*, etc. However, *Pestalotiopsis neglecta, Sordaria fimicola, Diplodina microsperma, Fusarium incarnatum, Preussia* sp., *Diaporthe* sp. and *Aureobasidium* sp. were the common fungal endophytes recorded from all the plant parts viz., leaf, stem, and roots. While, isolating the organ-based fungal endophytes; it was recorded highest in leaves viz., *Biscogniauxia mediterranea, Nodulisporium hinnuleum, Corynespora cassiicola, Scopulariopsis brevicaulis, Aspergillus nidulans* and *A. fischeri*; followed by *Curvularia borreriae, Nigrospora oryzae, Lasiodiplodia pseudotheobromae, L. viticola* and *Chaetomium globosum* from the stem as well as *Mucor hiemalis, Bipolaris bicolor* and fungal Endophyte sp., from the roots. Besides, the density of colonization (rD%) was recorded in the chronology of *P. longiseta* (14%) < *D. microsperma* (6%) < *N. hinnuleum* (5.33%) < *S. fimicola* (4%), and 0.66% to 4.00% for the remaining endophytes (Table 1; Fig. 2).

The analysis of fungal taxonomy of all the isolated 35 fungal endophytes shows that 14 endophytic fungi belong to class- Sordariomycetes; 13 belongs to the class Dothideomycetes; 06 belongs to the class Eurotiomycetes; 01 belong to the class Zygomycetes and 01 could not identify properly. While categorizing up to the order level; it was observed that *C. borreriae, C. spicifera, C. cassiicola, Alternaria* sp. *B.bicolour, P. chartarum, Preussia* sp., *Phoma* sp. and *P. brabeji* belongs to the order Pleosporales;

A.niger, A. flavus, A. nidulans, A. fischeri, A. fumigates and *P. chrysogenum* belongs to the order Eurotiales; *D. microsperma, F. merismoides* and *F. incarnatum* belongs to the order Hypocreales; *P. longiseta, B. mediterranea, N. hinnuleum* and *Eutypa* sp.belongs to the order Xylariales; *S. fimicola* and *C. globosum* belongs to order *Sordariales; L. pseudotheobromae* and *L. viticola* belongs to order Botryosphaerial; *C. fructicola* and *C. gloeosporioides* belongs to the order Glomerellales; *Dothidea* sp., *Aureobasidium* sp. belongs to the order Dothideales; Besides this, 02 fungal endophytes belong to order Mucorales viz., *M. hiemalis, S. brevicaulis;* 01 fungal endophyte *N. oryzae* belongs to the orders Trichosphaeriales, and one *Diaporthe* sp. belongs to the orders Diaporthales, respectively.

Samples of the fungal endophytes thus isolated (35 samples) were used for extraction of the DNA and its molecular characterization and submitted to the NCBI and got the accession number (Table 1). Further, phylogenetic analyses based on the ITS1-ITS4 sequencing data of the isolated fungal endophytes were also recorded. The observations of the neighbor-joining tree as constructed based on the sequence-structure alignment, and recorded in figure two; shows the structure alignment clearly in five well-separated groups. Group 'A' consisted of *L. viticola, Alternaria* sp., *P.brabeji, P.chrysogenum, F.incarnatum, Aureobasidium* sp., *P. longiseta*, and *C. cassiicola* where only *P. brabeji* and *P.chrysogenum* show strong bootstrap value. Similarly, Group 'B' consisted of *C. globosum, C. spicifera, S.brevicaulis*, *A. fumigates* and *Phoma* sp. Group 'C' contains *F. merismoides, Preussia* sp., *B. bicolor, L. pseudotheobromae A.fischeri*, *C.gloeosporioides, B. mediterranea.* Group 'D' contains *S. fimicola*, *A.niger, Eutypa* sp., *Diaporthe* sp., *C. fructicola.* .However, group 'E' consisted *P. longiseta, Dothidea* sp., Fungal Endophyte sp., *D. microsperma*, *C.borreriae, N.hinnuleum, M. hiemalis*, *P. chartarum, A. flavus, N. oryzae* (Fig. 3).

The findings also show that there is not any such type of report on the isolation of endophytic fungi from *P. capitatum* as investigated in the current study; it indicates itself the uniqueness of the work as the first report.

DISCUSSION

Literature reveals that diversity of fungal endophytes have already been reported from several medicinal plants viz., *Aspergillus candidus, Nigrospora oryzae, Cladosporium cladosporioides* and *Rhizoctonia solani* reported from *Ananas comosus* (Krishnamurthy *et al.,* 2008); *Fomitopsis* sp. *Penicillium* sp., *Diaporthe* sp., *Arthrinium* sp. *Phomopsis* sp. and *Schizophyllum* sp. have been reported from *Garcinia mangostana* and *G .parvifolia* (Sim *et al.,* 2010); *Mortierella minutissima, N. sphaerica, Acremonium strictum, Humicola Grisea, Mortierella hyaline, Oidiodendron echinulatum, O. griseum, Humicola fuscoatra* and *Arthroderma tuberculatum* reported from *Elaeocarpus sphaericus* (Shukla *et al.,* 2012); *Phoma tropica, Cladosporium sphaerospermum, Xylaria* sp., *Phomopsis archeri,* and *Tetraploa aristata* from *Opuntia ficus-indica* (Bezerra *et al.,* 2012); *Phomopsis* spp, *Diaporthe* spp, *Schizophyllum* spp, *Penicillium* spp, *Fomitopsis* spp and *Arthrinium* spp from *Cinchona* spp. (Maehara *et al.,* 2013); *A. alternata, Phomopsis* sp. and *Fomitopsis* sp. from *Miquelia dentate* (Shweta *et al.,* 2013); *F. oxysporum,Talaromyces radicus* and *Eutypella* spp. From *Catharanthus roseus* (Kuriakose *et al.,* 2016); *Altenaria alternata, C. capsici, and C. taiwanense from Passiflora incarnate* (Seetharaman *et al.,* 2017); *Penicillium sp., P. griseofulvum, A. flavus, Mycoleptodiscus terrestris, Trichoderma* sp., *C. gloeosporioides* and *Shiraia* sp. from *Huperzia serrata* (Su *et al.,* 2017); *P.citrinum, A. alternate, A. niger, Cladosporium* sp., *Rhizopus* sp., *C.vermiformis* reported from *Helicteres isora* (Gayathri and Chandra 2017); *Periconia hispidula, Allomyces arbuscula, N. sphaerica, A. falciforme, P. chrysogenum Aureobasidium sp. Chaetomium sp. from Litsea cubeba (Deepanwita and Dhruva 2018); and Fusarium spp*, *Aspergillus sp.*,, *Chaetomium sp.*,, *Penicillium sp., Setosphaeria rostrata*, *F.solani*, *Bipolaris maydis, D.phaseolorum, Rhizoctonia bataticola,* and *Macrophomina phaseolina* reported from *Chlorophytum borivilianum* (de Carvalho *et al.,* 2019) as well as *A. alternata, A. terreus, Alpestrisphaeria reported from Vitex rotundifolia* (Yu-Hung Yeh and Roland Kirschner 2019). However, there are no such reports of the fungal endophytes on *P. capitatum*; as recorded in the present investigation.

In the current study, overall 127 species of endophytic fungi; which belong to 35 fungal spp have been isolated from the plant parts including leaves (39%), followed by roots (31%) and stems (30%), respectively [Fig. 1c]. Further, it was observed that *Pestalotiopsis longiseta* was the most dominant endophytic fungi followed by *Diplodina microsperma, Nodulisporium hinnuleum, Aspergillus flavus* and others. However, *Pestalotiopsis neglecta, Sordaria fimicola, Diplodina microsperma, Fusarium incarnatum, Preussia* sp., *Diaporthe* sp. and *Aureobasidium* sp.were the common fungal endophytes recorded from all the plant parts viz., leaf, stem and roots as well as the density of colonization (rD%) was recorded for *P. longiseta* (14%) < *D. microsperma* (6%) < *N. hinnuleum* (5.33%) < *S. fimicola* (4%), and 0.66% to 4.00% for the remaining endophytes

(Table 1; Fig. 2). Furthermore, the fungal taxonomy of all the isolated 35 fungal endophytes was also recorded. Sordariomycetes was the class represented the maximum endophytes (14), however, Zygomycetes was the class with the least representation (01). Besides this, extraction of the DNA and its molecular characterization as well as phylogenetic analyses based on the ITS1-ITS4 sequencing data of the isolated thirty-five fungal endophytes, have also been investigated and recorded in Table -1.

Furthermore, literature also reveals that fungal endophytes mimic the plants behavior and function and also are capable to produce bioactive compound/ secondary metabolites as novel compounds having antimicrobial, anticancer, antioxidants, insecticidal, anti-malaria, anti-tuberculosis, anti-diabetes mellitus and cure cancer and cytotoxic activity (Das *et al.,* 2012; Desale 2016; Kumar and Mongolla, 2018). Biology of fungal endophytes has useful in several clinical applications with molecular approaches and fungal endophytes may assist to improve the activity of drug research area. Fungal endophytes nanoparticles may be used to improve plant growth, as bio- fertilizers, enhance crop yield and soil fertility (Netala *et al.,*, 2016; Mohamed *et al.,* 2019). Secondary metabolites produced by fungal endophytes used various field of food, and pharmaceutical, agricultural and other related industries (Das *et al.,* 2012; Desale 2016; Netala *et al.,* 2016; Kumar and Mongolla, 2018; Mohamed *et al.,* 2019). Similarly, in the present study, some of the endophytic fungi have also been recorded to have antimicrobial efficacy.

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Table 1: Fungal Endophytes Isolated from *P. capitatum*, its Frequency (CF %), Density (rD%) and Accession Number

S. N.	Isolated of Fungal Endophytes	Plant parts			CF (%)	rD (%)	NCBI Accession No.
		Leaf	Stem	Root			
1	*Pestalotiopsis longiseta*	Leaf	Stem	Root	42	14.0	MK299129
2	*Sordaria fimicola*	Leaf	Stem	Root	12	4.00	MK299157
3	*Curvularia borreriae*	---	Stem	---	10	3.33	MK299132
4	*Biscogniauxia mediterranea*	Leaf	---	---	4	1.33	MK398255
5	*Colletotrichum fructicola*	---	Stem	Root	10	3.33	MK299145
6	*Diplodina microsperma*	Leaf	Stem	Root	18	6.00	MK332492
7	*Nodulisporium hinnuleum*	Leaf	---	---	16	5.33	MK299151
8	*Mucor hiemalis*	---	---	Root	6	2.00	MK299138
9	*Aspergillus niger*	Leaf	---	Root	12	4.00	MK248610
10	*Nigrospora oryzae*	---	Stem	---	14	4.66	MK299154
11	*Corynespora cassiicola*	Leaf	---	---	6	2.00	MK299155
12	*Dothidea sp.*	Leaf	Stem	---	8	2.66	MK299156
13	*Penicillium chrysogenum*	Leaf	---	Root	6	2.00	MK299147
14	*Fusarium merismoides*	Leaf	Stem	---	10	3.33	MK299158
15	*Alternaria sp.*	Leaf	Stem	---	8	2.66	MK332475
16	*Aspergillus flavus*	Leaf	---	Root	16	5.33	MK332476
17	*Bipolaris bicolor*	---	---	Root	2	0.66	MK332477
18	*Curvularia spicifera*	Leaf	---	Root	8	2.66	MK332478
19	*Pithomyces chartarum*	Leaf	Stem	---	2	0.66	MK299154
20	*Lasiodiplodia pseudotheobromae*	---	Stem	---	8	2.66	MK248599
21	*Scopulariopsis brevicaulis*	Leaf	---	---	10	3.33	MK299149
22	*Colletotrichum gloeosporioides*	---	Stem	---	6	2.00	MH752465
23	*Eutypa sp.*	Leaf	Stem	---	8	2.66	MK299142
24	*Aspergillus nidulans*	Leaf	---	---	12	4.00	MK299125
25	*Fungal Endophyte sp*	---	---	Root	4	1.33	MK248607
26	*Fusarium incarnatum*	Leaf	Stem	Root	8	2.66	MK299134
27	*Lasiodiplodia viticola*	---	Stem	---	14	4.66	MK398249
28	*Aspergillus fischeri*	Leaf	---	---	10	3.33	MH748593
29	*Preussia sp.*	Leaf	Stem	Root	4	1.33	MK398250
30	*Phoma sp.*	---	Stem	---	6	2.00	MK398251
31	*Pseudocamarosporium brabeji*	Leaf	---	Root	2	0.66	MK398252
32	*Aspergillus fumigatus*	---	Stem	root	8	2.66	MK398253
33	*Diaporthe sp.*	Leaf	Stem	Root	16	5.33	MK398254
34	*Aureobasidium sp.*	Leaf	Stem	Root	10	3.33	MK398283
35	*Chaetomium globosum*	---	Stem	---	2	0.66	MK398256

--- indicates absences of the fungal endophytes.

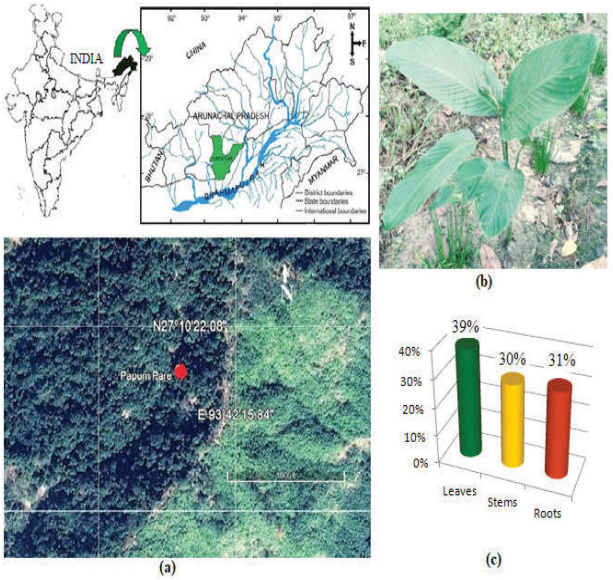

Fig. 1: Sample Collection Site of *P. capitatum*, in Arunachal Pradesh, India (Satellite Image), where, Red Circle Indicates Collection Site (b) Ethnomedicinal Plant *P. capitatum*, an Overview (c) Fungal Endophytes Isolated from Different Parts of *P. capitatum*

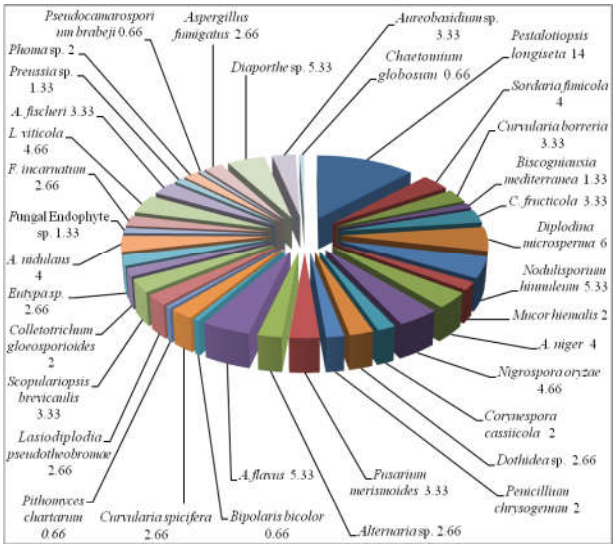

Fig. 2: Isolated Fungal Endophytes and its rD (%) from *P. capitatum*

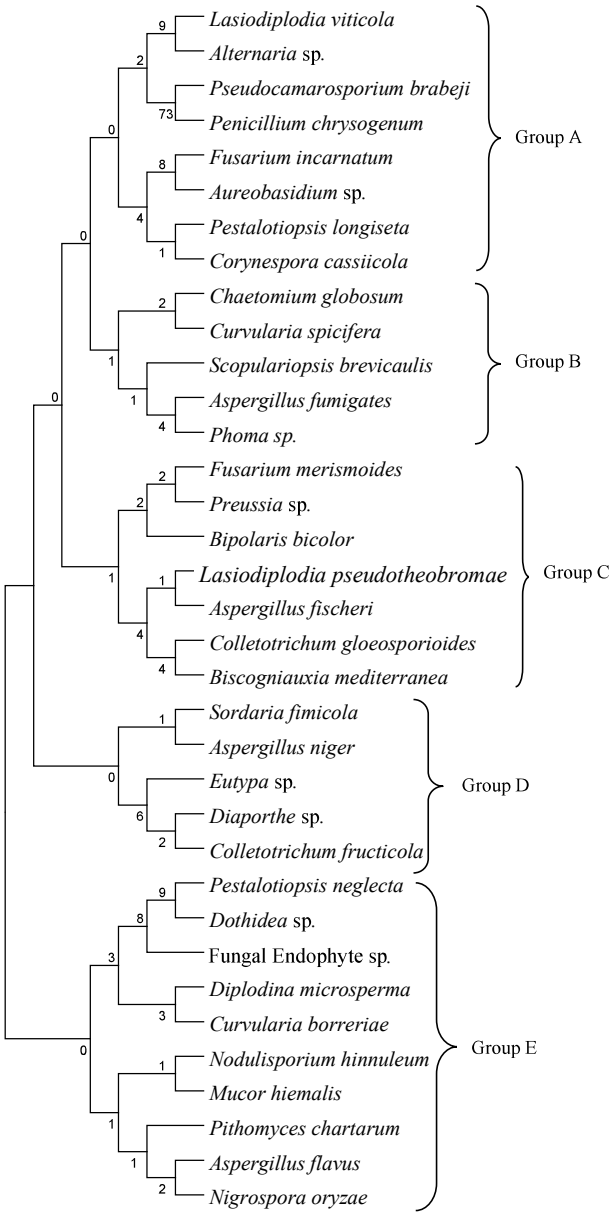

Fig. 3: Phylogenetic Analysis based on Neighbor-joining Tree based on ITS rDNA Sequences of the Isolated 35 Fungal Endophytes. Numerical Values Indicate Bootstrap Percentile from 1000 Iterations