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Morphological and Molecular Characterization of Endophytic Fungi isolated from the leaves of *Bergenia ciliata*

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Abstract

Endophytic fungi are microorganisms that are present inside the healthy tissue of living plants. Endophytes existence inside the plants tissue enhances the growth and development of the bio-active compounds which increase the quality and quantity of crude drugs. The endophytic fungal assemblages from the medicinal plants are limited around the world. The present study was conducted for the morphological and molecular identification of the endophytic fungi isolated from medicinal plant leaves *Bergenia ciliata* collected from different mountain areas of Sikkim, India. In this study total of 130 leaves segment was selected for fungal isolation from which 75 fungal colonies were recovered among them 25 different endophytes were isolated and characterized based on the morphological appearance and colony characters. Further all 25 fungi were identified molecular level through Internal Transcribed Spacer (ITS) and ITS2 sequence-secondary structure based analysis. On the basis of morphological and molecular characterization the isolated fungi were belonging to 6 orders i.e. *Glomerellales, Trichosphaeriales, Diaporthales, Xylariales, Botryosphaeriales, Pleorotales* and 9 genera i.e. *Colletotrichum, Nigrospora, Phomopsis/ Diaporthe, Arthrinium, Neofusicoccum, Stagonosporopsis, Phomatodes, Pestalotiopsis, Guignardia* of the Ascomycetes class. Among the isolates *Colletotrichum* sp were dominant. This is the first report of endophytic fungal assemblage from *Bergenia ciliata.*

Key words: Endophytic Fungi, ITS region, ITS2 secondary structure, *Bergenia ciliata*.

Introduction

Endophytic fungi typically reside inside the tissues of the plant are unexplored by a community of microorganisms. Their symbiotic relationship protects their host from infectious agents and resists adverse conditions by discharging active metabolites (Petrini, 1991). Currently, 1.5 million fungi species have been identified so far, (Carroll, 1991; Strobel et al.,2004; Gond et al., 2010; Singh et al., 2011; Blackwell M., 2011; Lahrmann et al., 2013) to this 7% are occupied by endophytic fungi (Chowdhary& Kaushik, 2015). Endophytic fungi are the primary tools for scientific research. They are the origins of new bioactive compounds and have economical significance in various fields such as agriculture, pharmaceutical and industrial (Bills & Polishook, 1992; Strobel & Daisy, 2003; Arnold et al., 2007). The identification of fungal diversity and ecological analysis was expected to become essential in the field of fungal biology. Earlier, the fungal taxonomy was carried out by comparing their morphological features (Lodge et al., 1996; Sette et al., 2006; Crous et al., 2007; Zhang et al., 2008). However, it was difficult to distinguish closely related species, identical morphotypes and non-sporulating isolates. This was due to the essence of the media composition, which may impact the morphological characters (Hyde & Soytong,2007). Recently, many molecular techniques are employed for taxonomic classifications because of their sensitivity and specificity to classify species level (Sette et al. 2006). Different molecular markers such as Internal Transcribed Spacers (ITS), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Calmodulin (CAL), Actin (ACT), Glutamine synthetase (GS), ß-tubulin (Tub2), RNA polymerase II second largest subunit (RPB2), Translation

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Elongation Factor 1-alpha (TEF1), etc. have been successfully identified and used in several recent studies (Wang et al. 2005; Arnold &Lutzoni 2007; Ligrone et al., 2007; Silva et al., 2012; Weir et al., 2012; Sunderasan et al., 2019). The ITS region is located in three sub-regions between the ribosomal large subunit (LSU) and the small subunit (SSU): ITS1, 5.8S and ITS2. ITS was used primarily as a molecular marker and was proposed as the universal sequence of fungal barcodes (Schoch et al., 2012) (Fig.1).The full length of the region is between 0.45 Kb and 0.75 Kb. Primers ITS1 (forward) and ITS4 (reverse) are widely used to amplify the ITS region using polymerase chain reaction (PCR). The primer will bind to ribosomal regions of the small and large subunit and covers the complete length of the target region. Then the amplified ITS areas is sequenced. The similarity between the collected sequences and those contained in databases is often used to identify the fungal organism (Gardes & Bruns 1993; White et al., 1990)

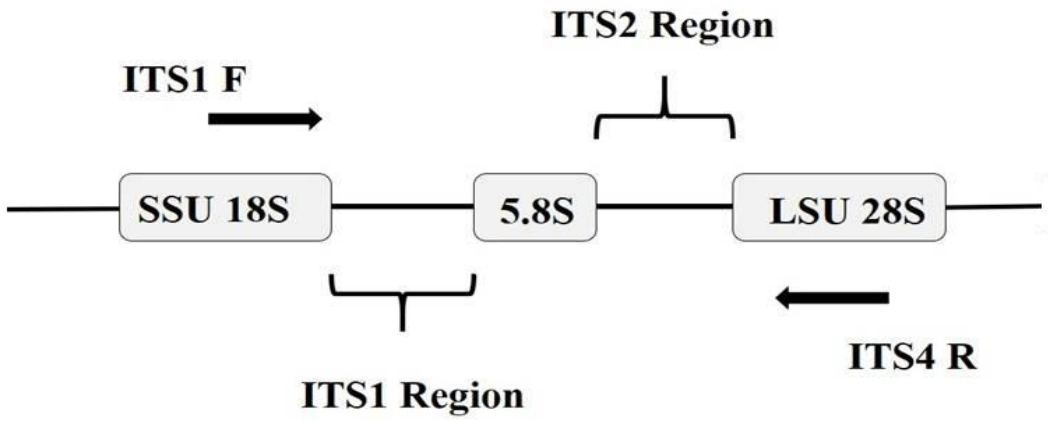

Fig. 1: Schematic representation of Internal Transcribe Spacer (ITS) region with universal primer ITS1 and ITS4.

In recent times, up to the species level, the ITS2 was used as an effective barcode for fungal classifications and is a mini barcode, simply amplified and sequenced (Keller et al., 2010; Yang et al., 2018). Phylogenetic resolutions in closely related species can be improved by the ITS2 sequence-secondary structure data. Several studies have shown the potential applications of ITS2 for taxonomic classification and phylogenetic reconstruction at both genus and species levels for eukaryotes, including plants, animals and fungi (Yao et al.,2010; Han et al., 2013).More information about variable characters are given in the identification based on the sequence structure, i.e. 5' apex UGGU motif (deviations such as UGGGU, UGG, or GGU have been described), as well as the Helix II-II U-U mismatch, four helix structure, Helix III being the longest compared to others (Schultz & Wolf 2009).Compensatory base change (CBC) has been used as a potential marker for species delimitation (Muller et al., 2007). The aim of this study is to search the fungal endophytes from ethno medicinal plant *Bergenia ciliata* collected from different mountain regions of the northeastern state of Sikkim, India. *Bergenia ciliata* is one of the most valuable medicinal plants of the Saxifragaceae family, found mostly in India's high-altitude mountain regions. The plant was used by indigenous people to treat various diseases such as urinary disorders, ear infections, coughs and colds, diarrhea, fevers. Pulmonary disorders, haemorrhoids, asthma, boils, skin disease, ophthalmia, cancer and dissolution of stones in the kidney (Asolkar et al.,1992; Sinha et al.,2001; Ahmad et al., 2018).

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Materials and Methods

Selection of Plant

Healthy leaves of *Bergenia ciliata* plants were collected from various mountain regions (altitude 5,410ft. Latitude 27.33 " N and longitude 88.60 " E) North Eastern State of Sikkim, India, used for the study. All the collected leaves were sealed and transported to the laboratory using a sterile ziplock polythene bag and stored at 4ºC for further use. Within 24 hours, the leaves are separately processed to extract noble endophytic fungi.

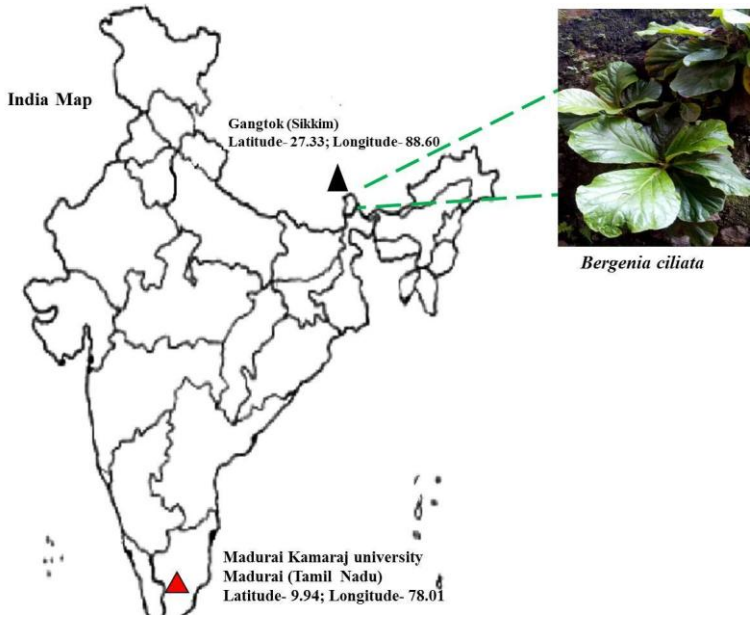

Fig. 2: Location map of sample collection sites of the medicinal plant (*Bergenia ciliata*) with their corresponding latitude and longitude.

Isolation of Endophytic fungi

The leaves are thoroughly washed by running tape water for five minutes to remove all the imprinted dust and debris on the leaf surface. The cleaned leaves were again washed by sterile distilled water and with the aid of a sterile blade, the washed leaves were cut into tiny pieces ~ 0.5 – 1cm in size. The cut part of the leaves was treated for five seconds with 70 % ethanol and then dipped again for ninety seconds in 4 % sodium hypochlorite, rinsed by autoclaved water for ten seconds and allowed to air dry with the use of sterile tissue papers to remove excess water present on the leaf pieces (Dobranic et al., 1995). The sterile leaf parts were then placed on the Streptomycin (200µg/mL) amended Potato Dextrose Agar (PDA) plate. The inoculated plates were allowed to incubate at 24 ± 1ºC, 12 h light and 12 h dark cycles. To verify the efficacy of surface sterilization, leaf imprinted control plates were also maintained. For the growth of endophytic fungi from the leaf segments, the plates were periodically examined. To obtain a single isolate, the hyphal tips emerging from the inoculated leaf segments were immediately sub-cultured on fresh PDA plates. For further studies, the pure isolates were maintained in PDA slants at 4°C.

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Morphological identification of fungi

The isolated endophytic fungi used for the morphological identification were grown on PDA plate at 24°C±1° C. Different morphological characters were used for the study. The major morphological appearance are front and reverse of the colonies' colours on culture plates, colony's surface margin, texture, elevation, growth patterns, hyphae, mycelium, spore shape and size.

DNA Extraction

One-week-old pure fungal culture was selected to isolate the genomic DNA. One gram of fungal mycelia was collected from the culture plate and uniformly ground by mortar and pestle using 4mL of extraction buffer (200mMTrisHCl, 250mMNaCl, 25 mM EDTA, 0.5 percent SDS) at pH 8.5. After that, 600 µL of the crushed sample was taken in centrifuge tubes to this a 300µL of 3M Sodium acetate (pH 5.2) was added and allows for incubation at -20ºC for 10 minutes. After the tubes were centrifuged for 5 minutes at 12,000 rpm. The supernatant was transferred to fresh eppendrof tubes without disrupting the pellet. An equivalent amount of isopropanol was applied to the supernatant and kept at room temperature for 5 minutes for DNA precipitation. The tube was centrifuged again for 2 min at 12,000 rpm. Finally, with 70 % ethanol, the pellet was washed, air-dried and suspended in 50 µL of sterile milliQ water. 0.7 % of agarose gel electrophoresis confirmed the isolated fungal genomic DNA (Cenis 1992).

Amplification of ITS region

The universal primers ITS1 and ITS4 were used by Polymerase chain reaction (PCR) to amplify the fungal ITS region. The Bio-RAD instrument was used for PCR amplification with a total reaction volume of 25 µL comprising 20 ng genomic DNA, 10X PCR buffer with 25mM MgCl2, 10mM dNTP, 2U Taq DNA polymerase and 10 pmol ITS1(forward) and ITS4 (reverse) primers. The following parameters were used for thermocycling: initial denaturation at 94ºC for 4 min, followed by 30 cycles, every 30 seconds at 94ºC for denaturation, 1 min at 58.2ºC for annealing, 2 minutes at 72ºC for an extension with a final extension at 72ºC for 7 minutes (Sim et al., 2010). By using 1% agarose gel electrophoresis with a 100bp DNA marker analyzed the amplified DNA fragments. The amplified products were visualized using gel documentation (Uvitech). A non-template control was included in each run. Then the amplified DNA was sequenced by Eurofins Private Limited Bangalore, Karnataka, India.

Phylogenetic analysis

The EMBOSS merger tool (http://www.bioinformatics.nl/cgi-bin/emboss/merger) was used to get full-length ITS sequences. The fungal ITS sequence was compared using the Basic Local Alignment Search Tools (BLAST) from the National Center for Biotechnology Information (NCBI) database. For the study of phylogenetic relationships, more identical sequences were download and aligned by using the CLUSTAL-W program implemented in MEGA 6. The multiple sequence alignment (MSA) generated was used for phylogenetic tree construction. A neighbor-joining tree was reconstructed using MEGA software version 6 with a bootstrap

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consensus of 1000 replicates to determine the reliability of the generated nodes (Larkin et al., 2007; Tamura et al., 2011).

ITS2 RNA secondary structure analysis

To extract the ITS2 region from the complete length of ITS sequences, the fungal ITS extractor (http://www.emerencia.org/FungalITSextractor.html) was used (Nilsson et al., 2010). Using the Mfold server with a preset temperature of 37^{o}C, the secondary ITS2 RNA structure of the query sequence and its closest matches in the ITS phylogenetic tree was predicted using the following conditions:- 1M NaCl (no divalent ions) ionic conditions, percentage sub-optimality number 5, upper folding number: 50, maximum interior/bulge loop asymmetry: 30, maximum interior/bulge loop size: 30, maximum interior/bulge loop size. The various parameters, such as structural energy, length, base composition, GC content and standard core-based structure, minimum free energy (MFE) were chosen for the construction of the secondary structure. Structural data were downloaded from the Mfold server in Vienna format (Zuker 2003; Rao & Satish 2016). 4SALE V 1.7 software was used to align and create the consensus structure with the ITS2 sequence-secondary structures. For phylogenetic analysis, the resultant alignment was exported to ProfDistS 0.9.9 (Friedrich et al., 2005).

Result

Isolation and identification of fungi

A total of 75 fungal endophytes were isolated from *Bergenia ciliata* leaves. They were grouped into 25 morphotypes based on phenotypic features such as colony colour, growth patterns, surface texture (Fig.3) and by using light microscope conidial features, spore dimensions were measured (Fig.4). The morphological features of the fungi have been explained and listed on table - 1. Also, The fungal isolates were also characterized at the molecular level using ITS sequences. All the isolated fungi were examined and arranged on six clades divided into two classes -Sordariomycetes and Dothideomycetes. Sordariomycetes include *Colletotrichum* (Clade 6), *Diaporthe / Phomopsis* (Clade 5), *Nigrospora, Pestalotiopsis, and Arthrinium* (Clade 4). Dothideomycetes include *Guignardia, Neofusicoccum* (Clade 2), *Stagonosporopsis, Phomatodes* (Clade 3), and out-group genera *Aureobasidium melanogenum* on clade 1 (Clade 1 to 6 counted from lower to upward direction on the phylogenetic tree shown on Fig.5). All the isolates came under six orders (Botryosphaeriales, Diaporthales, Glomerellales, Pleosporales, Xylariales and Trichosphaeriales). All the isolated fungus strain was indicated as JPSK1 to JPSK25. Using ITS based identification, the organism (isolated endophytic fungi) can be identified only at the genus level, which can be further identified up to the species level using ITS2 sequence-structure base identification (Schultz and Wolf 2009).

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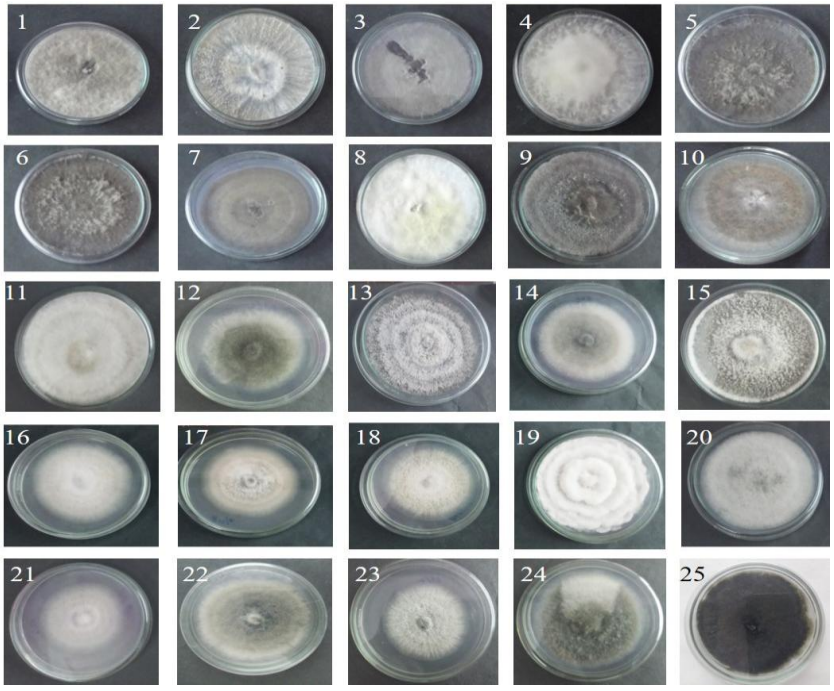

Fig. 3. Colony morphology of fungal endophytes on PDA at 24°C. isolated from *Bergeni*a *ciliata. Colletotrichum* sp. 3, 9, 12, 14, 16, 17,18, 20, 21, 22, 23and 24. *Nigrospora* sp. 10, 11 and 15.*Arthrinium* sp. 4 and 8. *Neofusicoccum* sp. 5 and 6. *Diaporthe* sp. 13. *Stagonosporopsis* sp. 1.*Phomopsis* sp. 2. *Phomatodes* sp. 7. *Pestalotiopsis* sp. 19. *Guignardia* sp. 25.

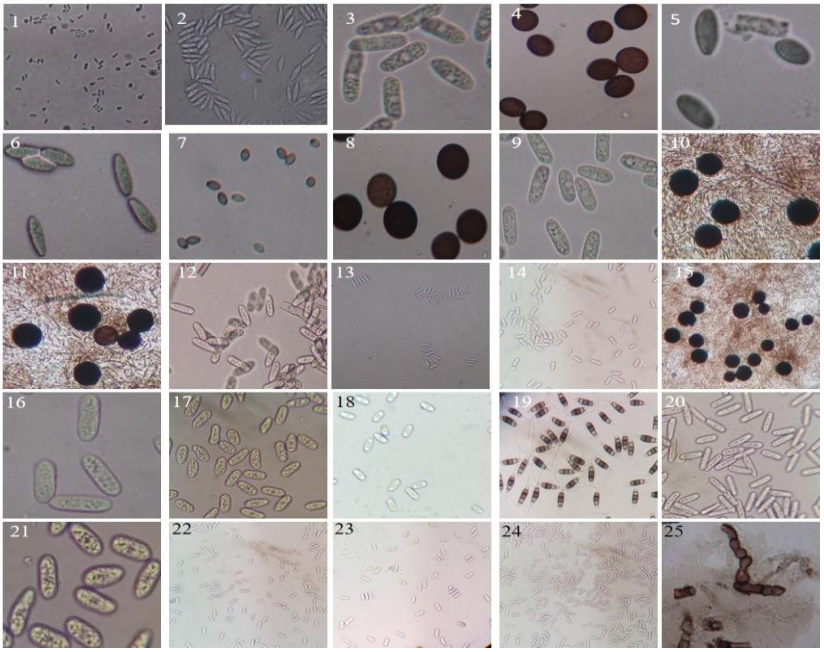

Fig. 4. Light Microscopic observation of fungal endophytes isolated from *Bergenia ciliata*. *Colletotrichum sp.* 3,9,12,14,16,17,18,20,21,22,23 and 24. *Nigrospora* sp. 10, 11 and 15. *Arthrinium* sp. 4 and 8. *Neofusicoccum* sp. 5 and 6. *Diaporthe* sp. 13, *Stagonosporopsis* sp. 1. *Phomopsis* sp.2. *Phomatodes* sp.7. *Pestalotiopsis* sp.19. *Guignardia* sp. 25.

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Table. 1: Summary of cultural characteristics of the 25 fungal morphotypes from *Bergenia ciliata* on PDA, at 24ºC.

Morpho - Type	Colour (Front)	Colour (Reverse)	Shape	Mycelium	Margin	Hyphae	Spore Shape	Spore Size (µm)
JPSK1	White to Dark brown	Dark gray	Irregular	Flat	Irregular	Septate	Cylindrical	4.2 - 8.3 x 2.6 - 3.1
JPSK2	White	White to Brown	Circular	Aerial	Entire	Septate	Cylindrical	8.1 - 15.5 x 1.4 - 2.7
JPSK3	White to pale gray	Dark to white	Circular	Aerial	Entire	Septate	Ellipsoidal	9.4 - 21.3 x 3.1 - 4.1
JPSK4	White brown	White to pale yellow	Irregular	Flat	Undulated	Septate	Cylindrical	6.5 - 12.5 x 3.5 - 4.2
JPSK5	White to grey	Pale grey to greenish black	Irregular	Aerial	Irregular	Septate	Ellipsoidal	9.2 -18.1 x 4.8 - 5.1
JPSK6	White to grey	Pale grey to greenish black	Irregular	Aerial	Irregular	Septate	Ellipsoidal	14.1 - 17.5 x 3.4 -3.5
JPSK7	White to Pale Green	Light green	Circular	Aerial	Entire	septate	Cylindrical	5.1 - 10.0 x 2.1 - 4.0
JPSK8	White brown	White to pale yellow	Irregular	Flat	Undulated	Septate	Spherical	9.8 - 12.2 x 3.1 - 5.4
JPSK9	White or pale gray	Dark white & pale gray	Circular	Aerial	Entire	Septate	Cylindrical	9.1 - 21.1 x 3.3 - 4.1
JPSK10	Gray to Dark Brown	Dark gray	Irregular	Aerial	Irregular	Aseptate	Globose	8.5 - 18.1 x 9.0 -14.9
JPSK11	White	White	Circular	Aerial	Entire	Aseptate	Globose	12.0 - 18.1 x 9.1 - 14.5
JPSK12	White to dark brown	Dark blue	Circular	Aerial	Entire	Septate	Cylindrical	10.1 - 20.5 x 3.5 - 6.2
JPSK13	White to dark gray	Dark gray	Irregular	Aerial	undulated	Septate	Cylindrical	9.5 - 16.2 x 1.4 - 2.5
JPSK14	Grey to light brown	yellow to Pale grey	Circular	Aerial	Entire	Septate	Cylindrical	9.3 - 21.1 x 3.3 - 4.1
JPSK15	White	White to light black	Circular	Aerial	Irregular	Septate	Globose	18.1 - 21.4 x 14.1 -15.3
JPSK16	White	Orange yellow to white	Circular	Aerial	Circular	Septate	Cylindrical	16.5 - 22.5 x 4.5 - 6.5
JPSK17	White to pale yellow	White to pale yellow	Circular	Aerial	Entire	Septate	Cylindrical	13.3 - 15.3 x 4.6 - 5.2
JPSK18	White to light dark	White to yellow	Circular	Aerial	Entire	Septate	Cylindrical	21.1 - 28.5 x 2.1 - 3.2
JPSK19	White	White to light yellow	Irregular	Aerial	Irregular	Septate	Globose	14.8 - 21.2 x 5.4 - 6.6
JPSK20	White	Dark blue	Circular	Aerial	Circular	Septate	Cylindrical	10.5 - 21.3 x 3.0 - 4.3
JPSK21	White to light dark	Brick red to pale yellow	Circular	Aerial	Entire	Septate	Cylindrical	13.5 -15.5 x 4.6 -5.1
JPSK22	Dark gray or black	Dark blue	Circular	Aerial	Entire	Septate	Cylindrical	8.3 - 18.5 x 3.2 - 5.1
JPSK23	White to light gray	Light orange to yellow	irregular	Aerial	Entire	Septate,	Cylindrical	8..3 - 28.5 x 2.4 - 3.3
JPSK24	White to dark gray	Gray to black	irregular	Aerial	Irregular	Septate	Spherical	10.5 - 21 x 3.1 - 4.3
JPSK25	Dark blue to green	Dark blue	irregular	Aerial	Entire	Septate	Cylindrical	6.5 - 11.3 x 5.5 - 6.2

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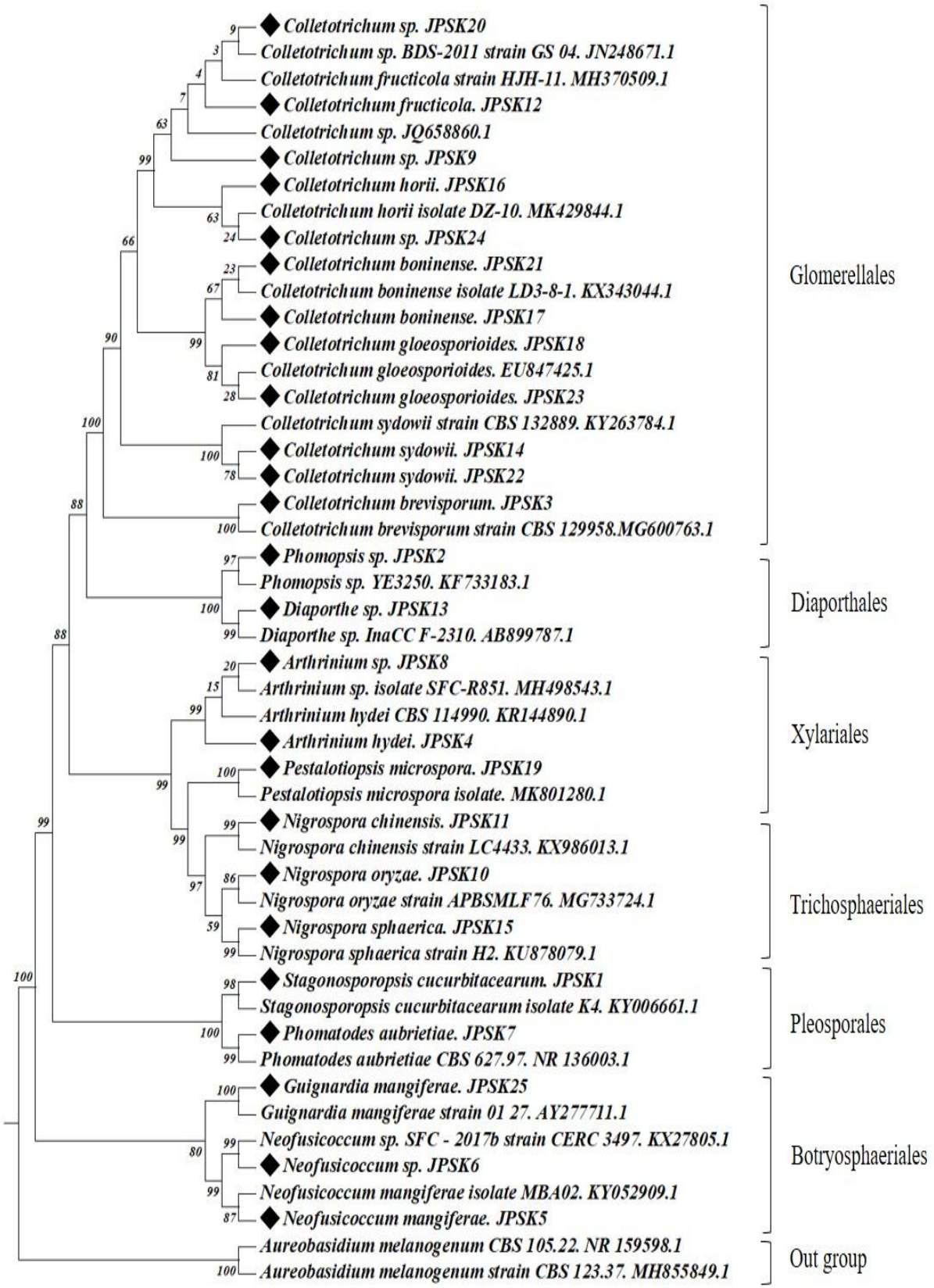

Fig. 5: Neighbor-Joining (NJ) Phylogenetic tree with ITS rDNA sequences indicating the phylogenetic relationship of endophytic fungi. The endophytic fungi isolated from the selected plant in the current study are labeled in black.

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Nucleotide information and ITS2 RNA secondary structure analysis

The Mfold software was used to predict the ITS2 secondary structures. The ITS2 sequence length ranged from 132 to 162 nucleotide bases, and the GC content ratio was approximately 52.22 – 63.63 (Table 3). Besides, consensus secondary structure was constructed genus-wise. For *Colletotrichum* 20 sequences whose minimum free energy (MFE) was -67.85 kcal/mol (mean value). Likewise, the *Arthrinium, Diaporthe/Phomopsis, Nigrospora* and *Neofusicoccum* secondary structure was constructed with 4 sequences whose MEF -68.97, -73.00, -61.12 and -73.08 respectively. The next series 2 sequences of *Pestalotiopsis, Guignardia, Phomatodes* and *Stagonosporopsis* had -53.48,-76.83,-61.58 and -62.06 MFE respectively. The secondary structure of ITS2 containing two forms they are (i) 4-helix domain (ii) 3 helix domains with the most extended third helix and the fourth helix not always present. In the present study, a genus called *Arthrinium, Diaporthe / Phomopsis, Nigrospora*, and *Neofusicoccum* contain four helixes with a more extended third helix. While *Colletotrichum, Pestalotiopsis, Phomatodes, Stagonosporopsis* and *Guignardia* having three helixes with the longest third helix was found in all genera.

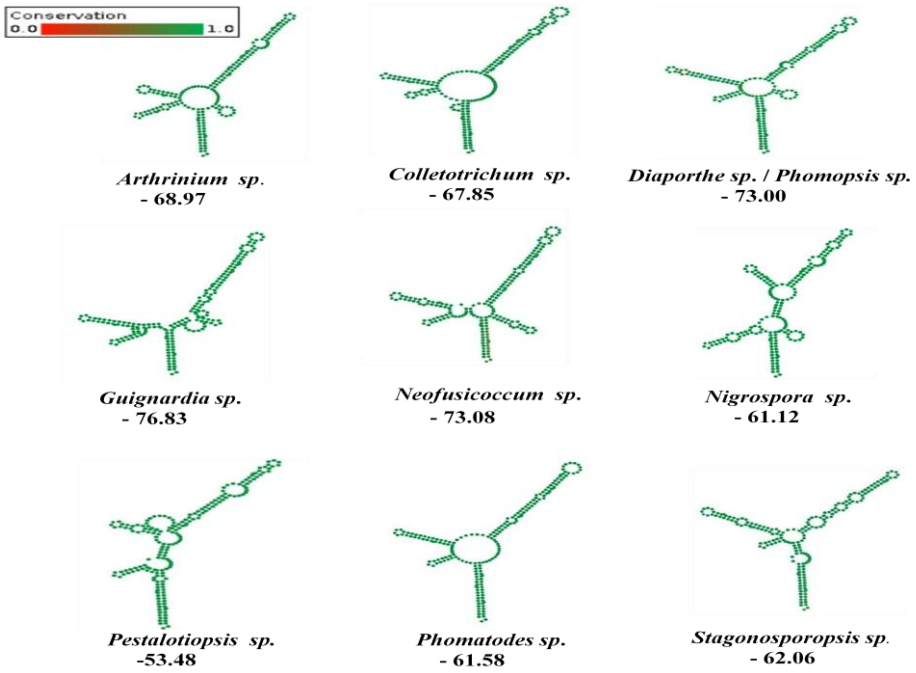

Fig. 6: Consensus ITS2 secondary structure of endophytic fungal genera (*Arthrinium, Colletotrichum, Phomopsis / Diaporthe, Guignardia Neofusicoccum, Nigrospora, Pestalotiopsis, Phomatodes* and *Stagonosporopsis*) and their Minimum Free Energy (MEF) value.

CBC analysis

By using 4SALE tools the compensatory base change (CBC) was observed in a pair of structures. Observing one or more CBCs between a pair of secondary structures signifies the two structures under comparison to being distinct belong to different species (Fig.7). The black mark region indicates a lack of CBC between the pair of sequences (Fig.7). It may belong to the same or different species. In this study *Colletotrichum horii*, and *Colletotrichum fructicola* were distinguished from *Colletotrichum gloeosporioides, Colletotrichum brevisporum, Colletotrichum sydowii* because of presence of CBC in the isolates. Whereas

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Nigrospora oryzae, N. chinensis and N. sphaerica show no CBC but the nucleotide variation found between their sequences (Details of CBC were mentioned in Table.3).

Phylogenetic Analysis

For the phylogenetic analysis, Sequence-structure based alignment performed in 4SALE was subsequently exported to ProfDistS. The phylogenetic clade formation was separated. Based on the analysis, 15 different species were identified, which included *Stagonosporopsis cucurbitacearum, Phomatodes aubrietiae, Arthrinium hydei, Neofusicoccum mangifera, Pestalotiopsis microspora, Guignardia mangiferae, Nigrospora oryzae, N. chinensis, N. sphaerica, Colletotrichum brevisporum, C. horii, C. fructicola, C. sydowii, C. boninense, C. gloeosporioides.* Seven isolates were classified up to the genus level that is *Arthrinium* sp. (1), *Neofusicoccum* sp (1), *Phomopsis / Diaporthe* sp (2), *Colletotrichum* sp (3). In this study, *Colletotrichum* was most dominant among the twenty-five isolates. They are *Colletotrichum sp.* (3) *Colletotrichum sydowii* (2), *Colletotrichum boninense* (2), *Colletotrichum gloeosporioides* (2), *Colletotrichum brevisporum* (1), *Colletotrichum horii.* (1), and *Colletotrichum fructicola* (1). The remaining isolates are *Nigrospora oryzae, N. chinensis, N. sphaerica, Stagonosporopsis cucurbitacearum, Phomatodes aubrietiae, Arthrinium hydei, Neofusicoccum mangiferae, Pestalotiopsis microspora, Guignardia mangiferae*. All the isolates of the present study belong to nine genera and six orders of Ascomycetes (Table 2). All these identified fungal rDNA-ITS sequences were submitted to GenBank (www.ncbi.nlm.nih.gov), and their accession numbers are listed in (Table 3).

Table. 2. Number of fungal endophytes identified from *Bergenia ciliata.*

Sl. no.	Number of Genus	Number of species	Number of strains
1	*Colletotrichum* sp.	*Colletotrichum* sp.	3
		Colletotrichum sydowii.	2
		Colletotrichum boninense.	2
		Colletotrichum gloeosporioides.	2
		Colletotrichum brevisporum.	1
		Colletotrichum horii.	1
		Colletotrichum fructicola.	1
2	*Nigrospora* sp.	*Nigrospora oryzae.*	1
		Nigrospora chinensis.	1
		Nigrospora sphaerica.	1
3	*Phomopsis* sp./*Diaporthe* sp.	*Phomopsis* sp./ *Diaporthe* sp.	2
4	*Arthrinium sp.*	*Arthrinium hydei.*	1
		Arthrinium sp.	1
5	*Neofusicoccum* sp.	*Neofusicoccum* sp.	1
		Neofusicoccum mangiferae	1
6	*Stagonosporopsis* sp.	*Stagonosporopsis cucurbitacearum.*	1
7	*Phomatodes* sp.	*Phomatodes aubrietiae.*	1
8	*Pestalotiopsis* sp.	*Pestalotiopsis microspora.*	1
9	*Guignardia* sp.	*Guignardia mangiferae.*	1

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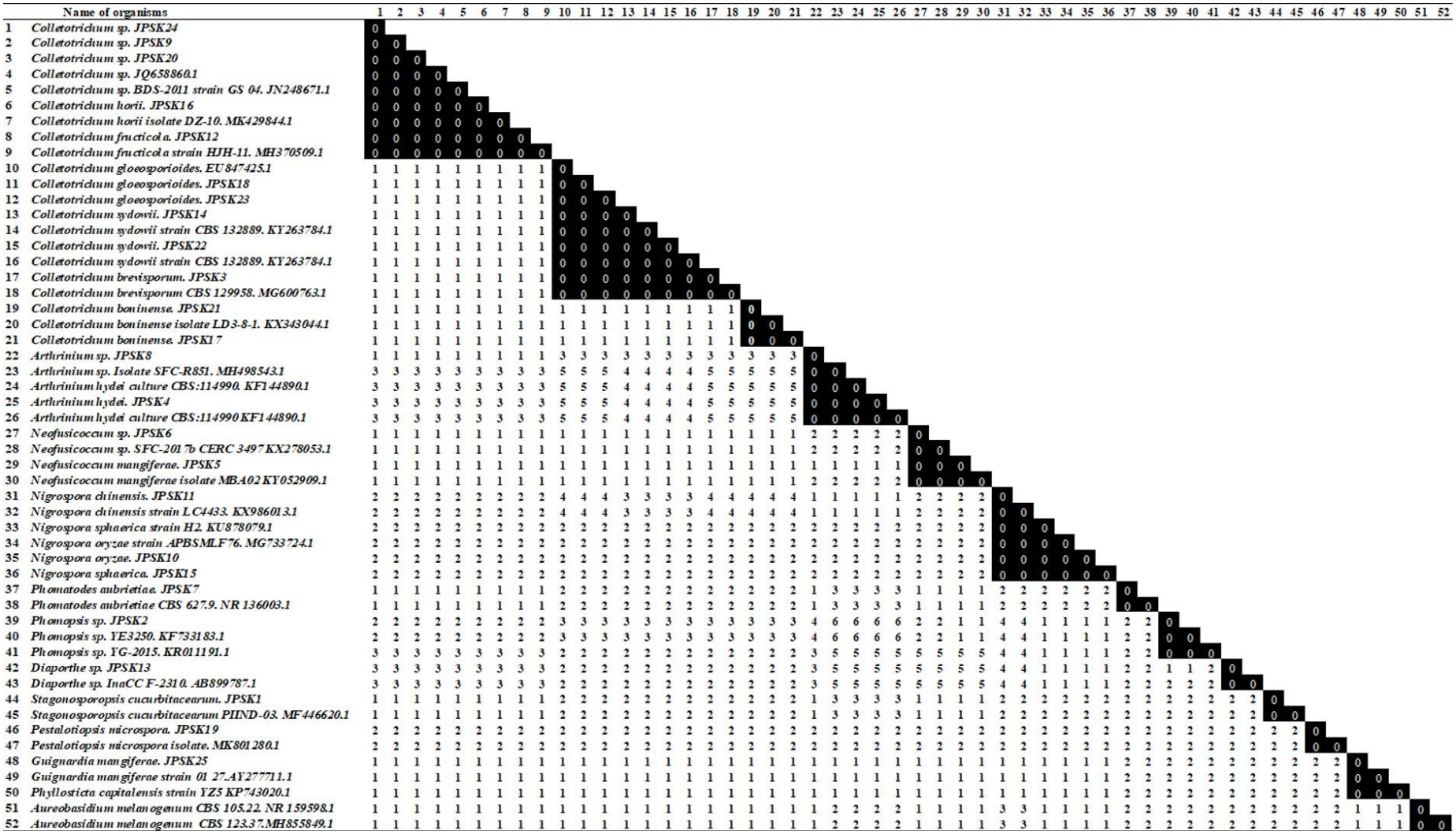

	Name of organisms	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52
1	*Colletotrichum sp. JPSK24*	0																																																			
2	*Colletotrichum sp. JPSK9*	0	0																																																	
3	*Colletotrichum sp. JPSK20*	0	0	0																																																
4	*Colletotrichum sp. JQ658860.1*	0	0	0	0																																														
5	*Colletotrichum sp. BDS-2011 strain GS 04. JN248671.1*	0	0	0	0	0																																												
6	*Colletotrichum horii. JPSK16*	0	0	0	0	0	0																																											
7	*Colletotrichum horii isolate DZ-10. MK429844.1*	0	0	0	0	0	0	0																																										
8	*Colletotrichum fructicola. JPSK12*	0	0	0	0	0	0	0	0																																									
9	*Colletotrichum fructicola strain HJH-11. MH370509.1*	0	0	0	0	0	0	0	0	0																																								
10	*Colletotrichum gloeosporioides. EU847425.1*	1	1	1	1	1	1	1	1	1	0																																							
11	*Colletotrichum gloeosporioides. JPSK18*	1	1	1	1	1	1	1	1	1	0	0																																						
12	*Colletotrichum gloeosporioides. JPSK23*	1	1	1	1	1	1	1	1	1	0	0	0																																					
13	*Colletotrichum sydowii. JPSK14*	1	1	1	1	1	1	1	1	1	0	0	0	0																																				
14	*Colletotrichum sydowii strain CBS 132889. KY263784.1*	1	1	1	1	1	1	1	1	1	0	0	0	0	0																																		
15	*Colletotrichum sydowii. JPSK22*	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0																																	
16	*Colletotrichum sydowii strain CBS 132889. KY263784.1*	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0																																
17	*Colletotrichum brevisporum. JPSK3*	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0																															
18	*Colletotrichum brevisporum CBS 129958. MG600763.1*	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0																														
19	*Colletotrichum boninense. JPSK21*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0																													
20	*Colletotrichum boninense isolate LD3-8-1. KX343044.1*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0																											
21	*Colletotrichum boninense. JPSK17*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0																											
22	*Arthrinium sp. JPSK8*	1	1	1	1	1	1	1	1	1	3	3	3	3	3	3	3	3	3	3	3	3	0																											
23	*Arthrinium sp. Isolate SFC-R851. MH498543.1*	3	3	3	3	3	3	3	3	3	5	5	5	4	4	4	4	5	5	5	5	5	0	0																									
24	*Arthrinium hydei culture CBS:114990. KF144890.1*	3	3	3	3	3	3	3	3	3	5	5	5	4	4	4	4	5	5	5	5	5	0	0	0																								
25	*Arthrinium hydei. JPSK4*	3	3	3	3	3	3	3	3	3	5	5	5	4	4	4	4	5	5	5	5	5	0	0	0	0																							
26	*Arthrinium hydei culture CBS:114990 KF144890.1*	3	3	3	3	3	3	3	3	3	5	5	5	4	4	4	4	5	5	5	5	5	0	0	0	0	0																						
27	*Neofusicoccum sp. JPSK6*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	0																					
28	*Neofusicoccum sp. SFC-2017b CERC 3497 KX278053.1*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	0	0																				
29	*Neofusicoccum mangiferae. JPSK5*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0																			
30	*Neofusicoccum mangiferae isolate MBA02 KY052909.1*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	0	0	0	0																		
31	*Nigrospora chinensis. JPSK11*	2	2	2	2	2	2	2	2	4	4	4	3	3	3	3	4	4	4	4	4	4	1	1	1	1	1	2	2	2	2	0																	
32	*Nigrospora chinensis strain LC4433. KX986013.1*	2	2	2	2	2	2	2	2	4	4	4	3	3	3	3	4	4	4	4	4	4	1	1	1	1	1	2	2	2	2	0	0																
33	*Nigrospora sphaerica strain H2. KU878079.1*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0	0															
34	*Nigrospora oryzae strain APBSMLF76. MG733724.1*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0	0	0														
35	*Nigrospora oryzae. JPSK10*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0	0	0	0													
36	*Nigrospora sphaerica. JPSK15*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0	0	0	0	0												
37	*Phomatodes nebrietiae. JPSK7*	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	1	3	3	3	3	1	1	1	1	2	2	2	2	2	2	0											
38	*Phomatodes nebrietiae CBS 627.9. NR 136003.1*	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	1	3	3	3	3	1	1	1	1	2	2	2	2	2	2	0	0										
39	*Phomopsis sp. JPSK2*	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3	4	6	6	6	6	2	2	1	1	4	4	1	1	1	1	2	2	0									
40	*Phomopsis sp. YE3250. KF733183.1*	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3	4	6	6	6	6	2	2	1	1	4	4	1	1	1	1	2	2	0	0								
41	*Phomopsis sp. YG-2015. KR011191.1*	3	3	3	3	3	3	3	3	2	2	2	2	2	2	2	2	2	2	2	2	2	3	5	5	5	5	5	5	5	5	4	4	1	1	1	1	2	2	0	0	0							
42	*Diaporthe sp. JPSK13*	3	3	3	3	3	3	3	3	2	2	2	2	2	2	2	2	2	2	2	2	2	3	5	5	5	5	5	5	5	5	4	4	1	1	1	1	2	2	1	1	2	0						
43	*Diaporthe sp. InaCC F-2310. AB899787.1*	3	3	3	3	3	3	3	3	2	2	2	2	2	2	2	2	2	2	2	2	2	3	5	5	5	5	5	5	5	5	4	4	1	1	1	1	2	2	2	2	2	0	0					
44	*Stagonosporopsis cucurbitacearum. JPSK1*	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	1	3	3	3	3	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	0				
45	*Stagonosporopsis cucurbitacearum PIIND-03. MF446620.1*	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	1	3	3	3	3	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0			
46	*Pestalotiopsis microspora. JPSK19*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0		
47	*Pestalotiopsis microspora isolate. MK801280.1*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0	
48	*Guignardia mangiferae. JPSK25*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	0	
49	*Guignardia mangiferae strain 01 27. AY277711.1*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	0	0
50	*Phyllosticta capitalensis strain YZ5 KP743020.1*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	0	0
51	*Aureobasidium melanogenum CBS 105.22. NR 159598.1*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	1	1	1	1	3	3	1	1	1	1	2	2	2	2	2	2	2	2	2	2	1	1
52	*Aureobasidium melanogenum CBS 123.37. MH855849.1*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	1	1	1	1	3	3	1	1	1	1	2	2	2	2	2	2	2	2	2	2	1	1

Fig.7. Compensatory base change (CBC) analysis of the isolated fungi and reference sequences. The black mark region indicates lack of CBC and remains are presence of CBC.

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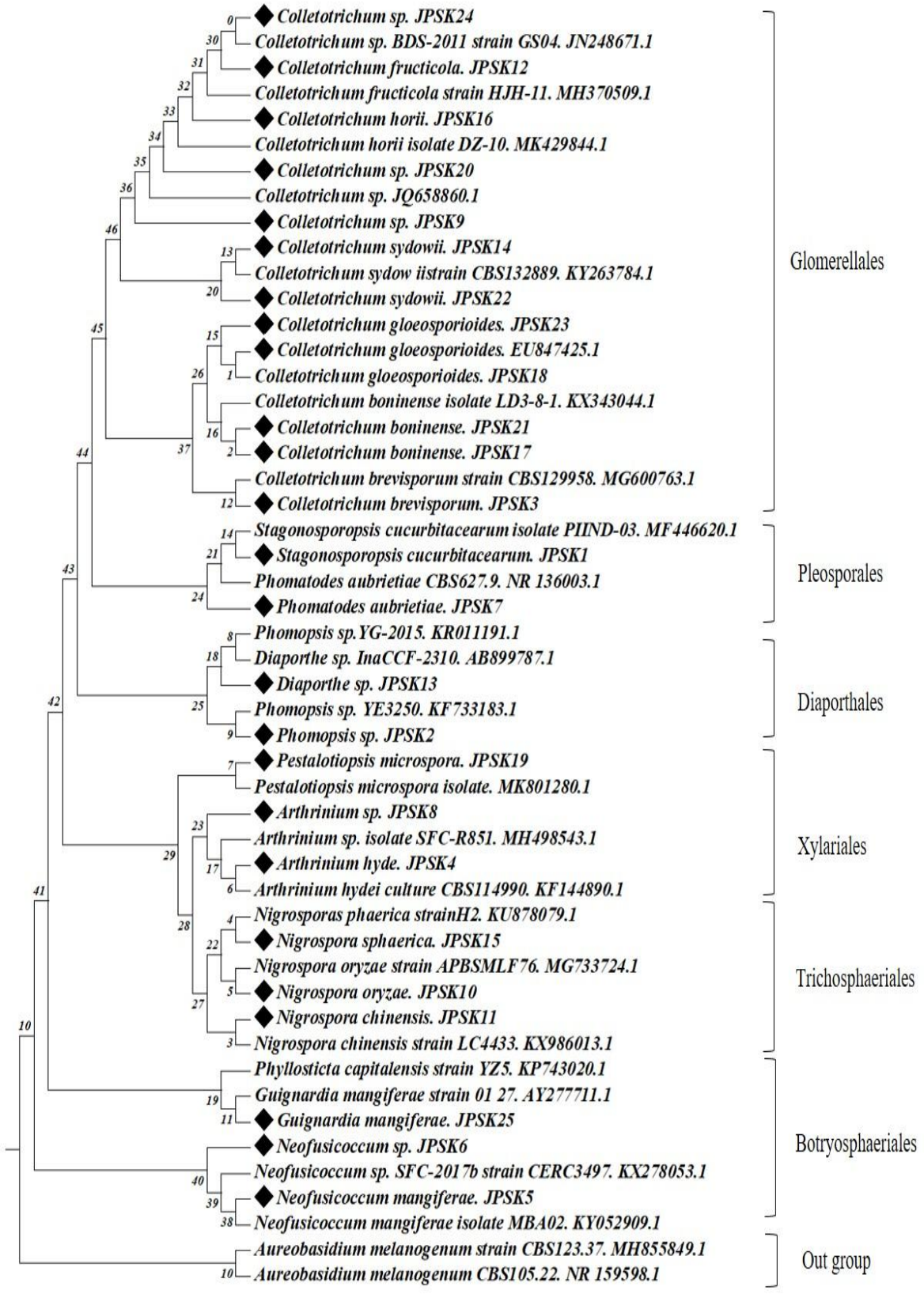

Fig. 8: Secondary structure-based (ITS2) phylogenetic analysis of query (organisms name ranging from JPSK1 to JPSK25 suggesting black mark) reference sequence by neighbor joining process. Constructing the phylogenetic tree by ProDistS software.

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Table. 3. ITS region nucleotide information a- maximum length of ITS sequences, b-ITS2 sequence and nucleotide compositions (A, U/T, G and C), percentage of secondary structure GC content and minimum free energy (MFE). 1 to 25 is our isolates (JPSK) and 26 to 52 reference sequences from NCBI. The last number 51 & 52 are out group ref. sequence from NCBI.

Sl. no.	Name of the Organism	Accession No	[a]Total length	[b]ITS2 region	A's	U/T's	G's	C's	% GC	Free Energy (kcal / mol)
1	*Stagonosporopsis cucurbitacearum*. JPSK1	MT555754	485	314 -470	32	43	37	45	52.22	-62.58
2	*Phomopsis* sp. JPSK2	MT560320	550	349-508	33	28	47	51	61.63	-84.34
3	*Colletotrichum brevisporum*. JPSK3	MT560321	612	354-510	33	29	39	56	60.50	-80.32
4	*Arthrinium hydei*. JPSK4	MT672781	582	386-539	32	47	36	39	48.70	-69.48
5	*Neofusicoccum mangiferae*. JPSK5	MT798866	533	402 -533	23	25	37	47	63. 63	-66.70
6	*Neofusicoccum* sp. JPSK6	MT560383	642	399-554	28	35	42	51	59.61	-76.86
7	*Phomatodes aubrietiae*. JPSK7	MT560384	506	312-467	32	45	37	42	50.64	-62.33
8	*Arthrinium* sp. JPSK8	MT560587	587	388-541	32	47	36	39	48.70	-69.48
9	*Colletotrichum* sp. JPSK9	MT560588	573	358-515	31	37	41	49	56.96	-74.03
10	*Nigrospora oryzae*. JPSK10	MT561421	526	327-481	30	44	35	46	52.25	-64.68
11	*Nigrospora chinensis*. JPSK11	MT561422	520	320-472	30	48	35	40	49.01	-60.79
12	*Colletotrichum fructicola*. JPSK12	MT708144	728	401-558	31	37	41	49	56.96	-65.84
13	*Diaporthe* sp. JPSK13	MT672790	573	352-513	35	35	43	50	57.05	-73.30
14	*Colletotrichum sydowii*. JPSK14	MT561432	516	310-467	32	36	41	49	56.96	-67.25
15	*Nigrospora sphaerica*. JPSK15	MT561433	559	344-498	30	45	36	44	51.61	-57.89
16	*Colletotrichum horii*. JPSK16	MT568591	578	362-518	31	37	41	48	56.68	-65.94
17	*Colletotrichum boninense*. JPSK17	MT568592	581	379-536	30	37	42	49	57.59	-67.43
18	*Colletotrichum gloeosporioides*. JPSK18	MT568594	595	386-542	29	38	42	48	57.32	-63.42
19	*Pestalotiopsis microspora*. JPSK19	MT568595	524	319-480	35	58	33	36	42.59	-53.48
20	*Colletotrichum* sp. JPSK20	MT568596	554	349-505	31	37	41	48	56.68	-65.94
21	*Colletotrichum boninense*. JPSK21	MT568597	596	376-533	30	37	42	49	57.59	-67.43
22	*Colletotrichum sydowii*. JPSK22	MT568598	519	305-462	32	36	41	49	56.96	-67.25
23	*Colletotrichum gloeosporioides*. JPSK23	MT568599	682	370-526	29	38	42	48	57.32	-63.42
24	*Colletotrichum* sp. JPSK24	MT568600	554	345-501	31	37	41	48	56.68	-65.94
25	*Guignardia mangiferae*. JPSK25	MT568601	641	421-583	30	38	47	48	58.28	-76.83

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Table 3 Continued

Sl. no.	Name Organism	Accession No	[a]Total length	[b]ITS2 region	A's	U/T's	G's	C's	% GC	Free Energy (kcal /mol)
26	*Stagonosporopsis cucurbitacearum* isolate PIIND-03	MF446620.1	539	333-489	32	43	37	45	52.22	-61.55
27	*Phomopsis* sp. YE3250	KF733183.1	558	339-498	33	29	47	51	61.25	-84.35
28	*Colletotrichum brevisporum* strain. CBS129958	MG600763.1	558	344-500	33	29	39	56	60.50	-81-90
29	*Arthrinium hydei* culture CBS 114990.	KF144890.1	699	469-622	32	47	36	39	48.70	-69.40
30	*Neofusicoccum mangiferae* isolate MBA02.	KY052909.1	514	348-502	27	34	43	51	60.64	-69.95
31	*Neofusicoccum* sp. SFC-2017b strain CERC3497	KX278053.1	562	356-514	29	36	42	52	59.11	-78.82
32	*Phomatodes aubrietiae*. CBS627.97	NR136003.1	485	305-459	32	46	36	41	49.67	-60.83
33	*Arthrinium* sp. isolate SFC-R851	MH498543.1	591	393-547	32	48	36	39	48.38	-67.46
34	*Colletotrichum* sp.	MH156052.1	568	353-510	31	37	41	49	56.96	-74.03
35	*Nigrospora oryzae.* strain APBSMLF76.	MG733724.1	555	342-496	30	44	35	46	48.33	-64.68
36	*Nigrospora chinensis.* strain LC4433	KX986013.1	522	321- 473	30	48	35	40	49.01	-60.79
37	*Colletotrichum fructicola.* strain HJH-11.	MH370509.1	566	360-517	31	37	41	49	56.96	-65.84
38	*Diaporthe* sp. Ina CC F-2310.	AB899787.1	606	387-548	36	33	42	51	57.40	-72.70
39	*Colletotrichum sydowii* strain CBS 132889.	KY263784.1	530	335-492	32	36	41	49	56.96	-67.25
40	*Nigrospora sphaerica* strain H2.	KU878079.1	558	345-499	30	45	36	44	51.61	-57.89
41	*Colletotrichum horii* isolate DZ-10.	MK429844.1	538	362-518	31	37	41	48	56.68	-72.70
42	*Colletotrichum boninense* isolate LD3-8-1.	KX343044.1	599	381-538	30	37	42	49	57.59	-67.43
43	*Colletotrichum gloeosporioides*.	EU847425.1	594	379-535	29	38	42	48	57.32	-63.42
44	*Pestalotiopsis microspora* isolate.	MK801280.1	551	332-493	35	58	33	36	42.59	-53.48
45	*Colletotrichum* sp.	JQ658860.1	554	350-506	31	37	41	48	56.68	-65.94
46	*Colletotrichum boninense* isolate LD3-8-1	KX343044.1	599	381-538	30	37	42	49	57.59	-67.43
47	*Colletotrichum sydowii* strain CBS 132889.	KY263784.1	530	335-492	32	36	41	49	56.96	-67.25
48	*Colletotrichum gloeosporioides*.	EU847425.1	594	379-535	29	38	42	48	57.32	-63.42
49	*Colletotrichum* sp. BDS-2011 strain GS 04.	JN248671.1	579	373- 529	31	37	41	48	56.68	-65.94
50	*Guignardia mangiferae* strain 01 27.	AY277711.1	630	416 -578	30	37	47	49	58.89	-76.83
51	*Aureobasidium melanogenum* CBS 105.22	NR 159598.1	588	379 - 533	34	42	41	38	50.96	-69.47
52	*Aureobasidium melanogenum* strain CBS 123.37	MH855849.1	589	378 -534	34	44	41	38	50.31	-70.39

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Discussion

Fungi are a group of species with the largest variety of organisms and are the world's leading components of the tropical ecosystem. To understand the role of endophytic fungal species and diversity in the ecosystem, more focus should be given to fungal research. Endophytic fungi stand as an important genetic resource in the search for novel bioactive compounds (Guimaraes et al. 2008). The endophytic fungi isolated from ethno medicinal plant *Bergenia ciliate* collected from a different region of high altitude mountains of north-eastern hilly state Sikkim, India were used in this study. The plant was selected due to its multiple ethno medicinal uses to dealing and cures various forms of human infection such as urinary disorders, ear infections, coughs and colds, diarrhea, fevers, pulmonary disorders, hemorrhoids, asthma, boils, skin disease, ophthalmia, cancer, and dissolution of stones in the kidney (Asolkar et al.,1992; Sinha et al.,2001; Ahmad et al., 2018). On the other side, endophytic fungi play a major role in plant tissue physiology and protect plants from various pathogens (insects, pests, nematodes, etc.) and also provide resistance to various environmental factors causing the disease (Clay & Holah, 1999., Omacini et al., 2001, Brundrett., 2006). In recent years, endophytic fungal research has been used up on cataloging the species to clarify the endophytes' existence with specific emphases in researching the novel compound residing in medicinal plants. Mostly endophytic fungi that multiply when temperature and humidity occur in a favorable condition have been documented and more different bioactive compounds are produced, particularly those isolated from medicinal plants that grow in such type of healthy climatic condition (Blackwell M., 2011; Mishra et.al., 2012). The colonization of fungal endophytes may provide additional benefits to their host by generating many substances that provide defense and increase the hosts' fitness (Redman et al.,2002, Arnold et al., 2003; Tejesvi et al., 2007). The most significant steps in the isolation process are choosing healthy plant components and removing dust debris and epiphytes that adhere to the plant's parts. Their ecological and medicinal importance does not distinguish fungal endophytes (Sette et al. 2006., Tao et al. 2008). Therefore, it is very essential to classify and characterized the fungal endophytes to know their phylogenetic as well as molecular relationship. The endophytes that exhibited characteristic colony morphology and microscopic features such as colonies, shape, size, margin, the colour of the fungal culture, hyphal and spore arrangement were putatively identified at genus level but morphological features only were difficult to identify up to the species level (Larone, 2002., G. S. Barseghyan and S. P. Wasser, 2010). The endophytes which were difficult to identify morphologically were subjected to molecular based on sequencing the ITS1 - 5.8S - ITS2 region as well as the relation of these strain to their close related isolates using neighboring joining plot. Differences between *Candida* and *Penicillium* species have previously been used to identify using the amplified ITS1 - 5.8S - ITS2 region (Skouboe et al. 1999; Korabecna et al. 2003; Morakotkam et al. 2007). The ITS region has been frequently used to classify fungi as a phylogenetic marker (Schoch et al., 2012; Sun & Guo 2012). Through molecular characterization, many studies have been done on the identification of endophytic fungi isolates from several different medicinal plants (Chen et al., 2011; Yoo & Eom, 2012; Bhagat et al., 2012). For the delimitation of species levels between closely related organisms, ITS2 sequence-secondary structure-based analysis is now used as a third-dimensional approach to characterizing fungi and other eukaryotic organisms, including plants, animals, insects, etc. (Zhang et al. 2015).

Sequence-secondary structure-based study of ITS2 is more descriptive than structural evidence can provide additional support for organism characterization, as the secondary RNA structure was stronger and more detailed, and well suggested in phylogenetic reconstruction (Coleman

2003, 2009). In particular, ITS data has enhanced molecular-based identification since 2010 in comparison with other approaches (Raja et al., 2017). This has recently helped to classify fungi by providing additional information (GokulRaj et al., 2014; Sundaresan et al., 2019). Another additional supporting technique for distinguishing structural pairs in the series of CBC (Compensatory Base Change). The existence of CBC among pairs of the structure shows that the two distinct species and their likelihood were ~93%. If that there is no CBC in the gap between the structural pairs in that case, with a likelihood of ~ 76 percent, which indicates that they may belong to the same or different species. (Muller et al., 2007; Wolf et al., 2013). Due to its very shorter length with a lower GC gap, the ITS sequence - secondary structure is used as a suitable for fungal species level recognition, and the structural characters have greater phylogenetic utility.

ITS2 sequences and neighboring joining method further analyzed structural details that differed from other strategies based on our sequence-structure-based alignment, resulting in more accurately segregated clades. Based on these sequences, the phylogenetic tree was built along with aligned reference sequences NCBI databases. The dendrogram generated 25 isolates that were arranged on six clades which include nine genera and six orders. The generated isolates were divided into two classes i.e. Sordariomycetes and Dothideomycetes belonging to the diverse group of Ascomycota. Among all, *Colletotrichum* was the highly dominant genera.

Conclusion:

On the study of morphological and molecular information, the 25 fungal morphotypes were isolated from *Bergenia ciliata*. The 25 isolates were arranged on six different orders they are Botryosphaeriales, Diaporthales, Glomerellales, Pleosporales, Xylariales, and Trichosphaeriales and nine genera are Stagonosporopsis, *Phomopsis / Diaporthe, Guignardia ,Phomatodes, Pestalotiopsis, Neofusicoccum, Arthrinium, Nigrospora* and *Colletotrichum.* Among the 25 morphotypes, 15 isolates were identified to species level and the remaining seven were at the genus level. Of all the 25 isolates, *Colletotrichum* was found more dominant in this study. The ITS2 sequence secondary structure could be the most essential and useful tool for distinguishing between closely related species. Further investigation is now being focused on extract, and purifies the bioactive compounds for their antimicrobial, antioxidant, and anticancer activities.

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