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Molecular identification of endophytic fungi associated with *Coleus forskohlii* (Willd.) Briq.

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ABSTRACT

Coleus forskohlii (Willd.) Briq. is a notable medicinal plant belonging to the family Lamiaceae. Understanding the diversity of endophytic fungi associated with this medicinally important plant species and screening them to yield bioactive compounds would be highly useful for the herbal drug industry. The present study was undertaken to isolate and identify the diversity of fungal endophytes associated with *C. forskohlii* (Willd.) Briq. The fungal endophytes were isolated following standard procedures and molecular identification was carried out by using the 18S rRNA gene; the amplified regions were sequenced and submitted to NCBI, GenBank. A total of 85 endophytic fungi were isolated from 280 leaf segments. Molecular identification revealed 34 fungal genera. Among these, species of *Cladosporium* sp., *Alternaria* sp., *Aspergillus niger*, *Aspergillus* sp., *Colletotrichum* sp., *Nigrospora oryzae*, *Penicillium* sp., and *Phyllosticta fallopiae* were found to be predominant genera. The percentage occurrence of members of Ascomycota was the highest, with 96.47% distribution and Basidiomycota members were distributed the least, with 3.53%. The study revealed the diversity of endophytic fungi associated with the leaves of *C. forskohlii* and the phylogenetic tree shows the relationships between the endophytic fungi.

1. INTRODUCTION

The phyllosphere and rhizosphere encompass a number of microorganisms. The microorganisms living inside the plant tissues are called endophytes. de Bary [1] introduced the term "endophytes" for the fungi residing inside the plant tissue and "epiphytes" for the fungi that reside on the surface of their host. Endophytic microorganisms such as bacteria, fungi, archaea, and protists live in the intercellular or intracellular regions of the plant tissues without causing any infectious symptoms to the plant. Such microorganisms exhibiting endophytic lifestyles play essential roles in the plant's growth, development, health and variation. Efficacious colonization of endophytes with the host plant depends upon many innate parameters such as plant genetic constitution, tissue type, microflora, and species type, as well as the external abiotic factors [2,3].

Almost all parts of the plants, for instance, the roots, stem, leaves, rhizome, inflorescence, seeds, etc. are occupied by endophytic fungi [4–6]. These endophytic fungi act as a reservoir for novel bioactive metabolites such as terpenes, phenolics, alkaloids, tannins, saponins, steroids, and quinones which aid as potential drugs against microbes, insects, cancers, and have many more curative roles. Thus, recently, the endophytes associated with the plants rather than the plants themselves have emerged as promising mines for novel drug discovery. Therefore, studies on endophytes are now of great significance, not only to encounter the microbial multiplicity but also to obtain novel chemical compounds that could be assuring drugs to treat several ailments [7,8].

The Mint family, known as Lamiaceae or Labiatae, encompasses 245 genera and 7,886 species. The major genera under Lamiaceae are *Salvia* (986 sp.), *Scutellaria* (486 sp.), *Plectranthus* (325 sp.),

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Leucas (111 sp.), *Pogostemon* (94 sp.), *Ocimum* (66 sp.), *Coleus* (56 sp.), etc. Most of the members of Lamiaceae are herbaceous, but some are shrubs. They are known for their aromatic nature as they yield essential oils from almost all parts of the plant, and hence are extensively cultivated [9,10].

Coleus forskohlii also known as *Plectranthus barbatus* or *Coleus barbatus*, vernacular names *Coleus*, Indian *Coleus*, false *boldo*, etc., is a medicinally important perennial plant which belongs to the Lamiaceae family and it grows to about 45–60 cm tall. It is found distributed in North, East, and Central Africa, Asia, and South America [11]. In India, *C. forskohlii* grows wild in the Himalayan region, from the Shimla hills extending through the Kumaon and Garhwal hills, at an altitudinal range of 600–2,300 m; it is even found growing in the Parasnath hills (Bihar), in Gujarat and in the Western Ghats [12]. *Coleus forskohlii* is characterized by its intensely aromatic plant parts and has fasciculate tuberous roots. It has been used in traditional ayurvedic medicine for over 3,000 years and is mentioned in the ancient Sanskrit texts as a tonic for a healthy heart and lungs. It is used to treat heart diseases, abdominal colic, respiratory disorder, insomnia, convulsions, asthma, bronchitis, intestinal disorders, burning sensation, constipation, epilepsy, and angina. *Coleus forskohlii* is the only source of diterpenoid forskolin [13–16]. The roots of *C. forskohlii* are exploited for forskolin. Recently, the cultivation of *C. forskohlii* has gained importance and is spreading to different parts of the country, like Maharashtra, Gujarat, Madhya Pradesh, Andhra Pradesh, Tamil Nadu, and Karnataka, for its pharmaceutical value [17].

Like other plants, the members of Lamiaceae also inhabit a wide array of fungal endophytes. Fungal endophytes have been reported from various members, such as *Coleus aromaticus*, *Ocimum sanctum*, *Ocimum basilicum*, *Leucas aspera*, and *Salvia miltiorrhiza* [18–22].

The study pertaining to the identification and diversity of endophytic fungi of *C. forskohlii* and their medicinal properties is still an unexplored area of research. Therefore, an attempt was made to isolate and identify the fungal endophytes associated with *C. forskohlii*.

2. MATERIALS AND METHODS

2.1. Sampling Site and Collection of Plant Material

Coleus forskohlii leaves were collected from medicinal plant garden of St. Joseph's College Campus, Bengaluru (Latitude: 12°57′45.72″N; Longitude: 77°35′49.56″E; altitude above sea level: 900 m/ 3,020 ft), Karnataka, India. The plant herbarium specimen was submitted to Foundation for Revitalisation of Local Health Traditions for identification and authentication, and the voucher number is FRLH Coll. No. 124372. About 3–4 months old, apparently healthy, mature, undamaged leaves were collected from the mature plants during the month of June 2019. The collected leaves (Fig. 1) were hermetically sealed in an aseptic plastic bag, carried to the laboratory, and were processed immediately.

2.2. Isolation of Endophytic Fungi

The samples were thoroughly washed under running tap water for 10 minutes to remove the debris and washed with sterile distilled water to wash off the epiphytic microbes. Three disks along the midvein and three disks from one or both sides of the midvein were cut into small disks of approximately 8 × 8 mm size under aseptic conditions inside a laminar air flow cabinet. In total, 280 leaf disks were obtained from about 50 mature leaves. These bits of leaves were surface sterilized by immersing in 70% ethanol for 30 seconds, followed by 4% sodium hypochlorite for 30 seconds and dipping in 70% ethanol for 5–10 seconds and washed thoroughly (thrice) with sterilized distilled water [23]. Earlier studies have shown that the potato dextrose agar (PDA) medium was suitable to isolate fungal endophytes and hence PDA medium was chosen. After surface sterilization, the leaf material was dry blotted on sterile blotting paper and plated onto a 90 mm Petri plate containing PDA media supplemented with chloramphenicol (200 mg/l) to suppress the growth of bacteria [24]. Validation of surface sterilization was carried out by imprinting the surface sterilized plant tissue onto the PDA media. About five to six pieces of the plant material were carefully placed onto the PDA media. After 3–7 days of incubation with 12 hours of natural light and 12 hours of darkness, the mycelia emerging out from the plant material (Fig. 2) were subcultured onto fresh PDA media as and when the endophytic fungi were emerging, to obtain pure cultures (Figs. 3 and 4). Pure cultures of all the isolates were maintained by subculturing every month and also preserving the cultures using sterile distilled water [25]. The pure cultures were then subjected to molecular identification by using 18S rRNA sequencing method.

Figure 1: Healthy, fresh & undamaged leaves of *C. forskohlii* (Willd.) Briq.

2.3. Identification of Fungal Endophytes

The molecular identification of endophytic fungi was carried out using 18S rRNA gene sequencing method which is described below. The identification was confirmed by observing the morphological characteristics by conducting microscopic studies of the spores, hyphae, and colony.

2.4. DNA Extraction and polymerase chain reaction (PCR) Amplification

The DNA extraction was carried out by employing the cetyltrimethylammonium bromide (CTAB) method. 1 g of fresh fungal hyphae was obtained from 3 to 5 days old pure cultures grown on the PDA media. The fungal culture with CTAB extraction buffer was taken in a 2 ml Eppendorf tube. The fungal sample was crushed in a chilled mortar pestle and volume was made up to 1 ml. To remove the RNA, DNAse-free RNAse A (10 mg/ml) was added to the tubes. The tubes were incubated at 37°C for 15 minutes then at 65°C for 30 minutes. The tubes were inverted at regular intervals. 1 ml of phenol:chloroform:isoamylalcohol (25:24:1) was added to the tubes and centrifuged at 10,000 rpm for 10 minutes. The upper aqueous layer from each tube was transferred to a fresh 2 ml Eppendorf tube containing an equal volume of chloroform:isoamylalcohol (24:1). These tubes were centrifuged at 10,000 rpm for 10 minutes. The upper aqueous layer from each tube was transferred to a fresh 2 ml Eppendorf tube containing an equal volume of 100% isopropyl alcohol and one-tenth volume 3 M sodium acetate for DNA precipitation. The tubes were centrifuged at 10,000 rpm for 10 minutes after incubation period of 30 minutes at room temperature. The DNA pellet was washed with 1 ml of 70% ethanol by centrifuging at 5,000 rpm for 5 minutes and then air-dried. The DNA pellet was suspended in 1× Tris-EDTA buffer (Tris base 100 mM, ethylenediamine tetraacetic acid salt 1 mM). The pure DNA extracted was amplified by PCR using universal and degenerate primers. The PCR was carried out for 35 cycles with the following conditions: initial denaturation at 94°C for 5 minutes; denaturation at 94°C for 45 seconds; annealing at 57°C for 45 seconds; extension at 72°C for 2 minutes; and final extension at 72°C for 7 minutes. The PCR product was then subjected to gel electrophoresis to check the purity of the DNA, followed by which the sequence was analyzed using Sanger's dideoxy method to obtain a DNA sequence [26–28]. The forward and reverse DNA sequences of every sample were subjected to National Centre for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) algorithm for identification and the phylogenetic tree was also constructed using NCBI BLAST. All the identified 18S rRNA sequences were submitted to the GenBank in fast-all format and accession numbers for the same were obtained.

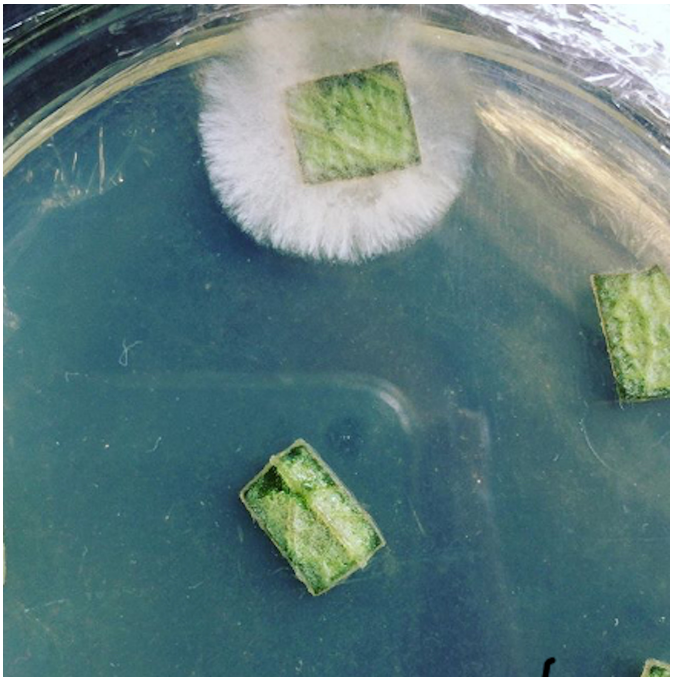

Figure 2: Fungal endophyte, *A. flavus* emerging from *C. forskohlii* leaf bit after 24 hours of incubation.

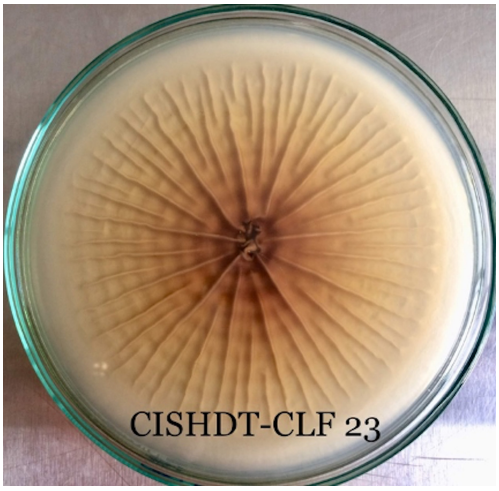

Figure 3: Lower surface view of fugal endophyte *A. ochraceus*.

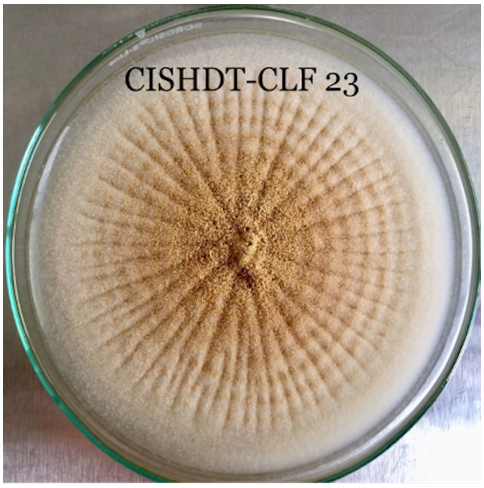

Figure 4: Upper surface view of fugal endophyte *A. ochraceus*.

2.5. Phylogenetic Affiliation

A phylogenetic tree is essential to recognize the relationship between the fungal endophytes associated with *C. forskohlii*; hence, molecular evolutionary genetics analysis (MEGA X) was used for depicting the same [29].

2.6. Statistical Analysis

In order to understand the results, colonization frequency, endophyte isolation rate, and relative percentage occurrence were calculated using the below-mentioned formula.

2.6.1. Colonization frequency (CF)

The CF% of the fungal endophytes was calculated using the following formula given by Hata and Futai (1995) [30]:

$$CF\ (\%) = \frac{\text{Number of segments colonized by each endophyte}}{\text{Total number of segments examined}} \times 100$$

2.6.2. Endophytic isolation rate (EIR)

The EIR was calculated using the following formula [31]:

$$EIR = \frac{\text{Number of isolates obtained from plant segments}}{\text{Total number of segments incubated}}$$

2.6.3. *Relative percentage occurrence (RPO)*

The RPO of different fungal groups was calculated using the following formula [32]:

$$RPO\ (\%) = \frac{\text{Number of segments colonized by a group of fungi}}{\text{Total number of segments colonized by all the groups of fungi}} \times 100$$

3. RESULTS

3.1. Isolation and Identification of Fugal Endophytes

A total of 85 endophytic fungi were isolated from 280 bits of leaves of *C. forskohlii*. Molecular identification using 18S rRNA sequencing revealed 34 genera of fungal endophytes, which are as follows: *Albifimbria verrucaria*, *Alternaria alternata*, *Alternaria* sp., *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus* sp., *Aspergillus versicolor*, *Byssochlamys spectabilis*, *Cercospora sojina*, *Chaetomium globosum*, *Cladosporium* sp., *Cochliobolus* sp., *Colletotrichum coccodes*, *Colletotrichum* sp., *Colletotrichum truncatum*, *Coprinopsis atramentaria*, *Davidiellaceae* sp., *Diaporthe* sp., *Didymosphaeria variabile*, *Hebeloma angustilamellatum*, *Lecanicillium* sp., *Nigrospora oryzae*, *Ochraceocephala foeniculi*, *Penicillium citrinum*, *Penicillium limosum*, *Penicillium oxalicum*, *Penicillium* sp., *Phyllosticta fallopiae*, *Phyllosticta neopyrolae*, *Psathyrella gracilis*, *Pseudocercospora fuligena*, *Pseudocercospora pallida*, and *Scopulariopsis brevicaulis*. The frequency of occurrence of these 34 endophytic fungi is shown in Figure 5. Figures 6–8 show the morphology of the colonies of different fungi that were isolated. Among the 85 endophytic fungi, 83 belonged to Ascomycota and 2 belonged to Basidiomycota. To understand the phylogenetic affiliations between the endophytic fungi, a phylogenetic tree was constructed using MEGA X software. The DNA sequences were submitted to GenBank and the accession numbers were obtained for the same.

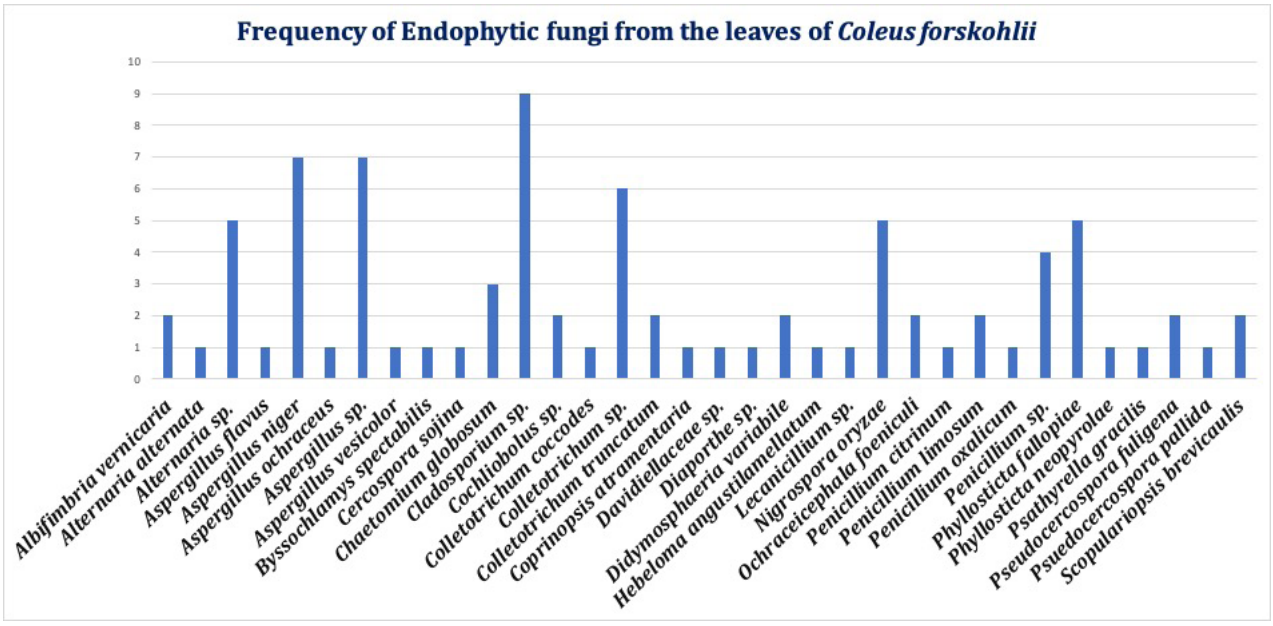

Figure 5: Frequency of occurrence of fungal endophytes of *C. forskohlii*.

Figure 6: Pure cultures of the fungal endophytes isolated from *C. forskohlii* CISHDT-CLF01- CISHDT-CLF36.

Figure 7: Pure cultures of the fungal endophytes isolated from *C. forskohlii* CISHDT-CLF37- CISHDT-CLF75.

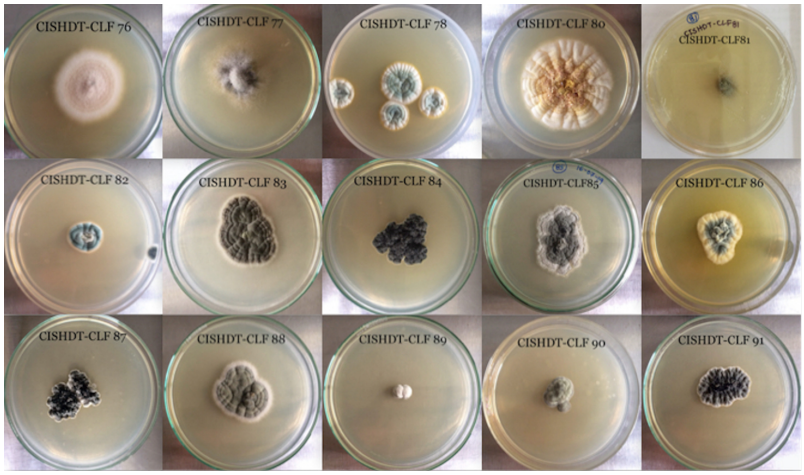

Figure 8: Pure cultures of the fungal endophytes isolated from *C. forskohlii* CISHDT-CLF76-CISHDT-CLF91.

3.2. Phylogenetic Affiliation

The phylogenetic affiliation of fungal endophytes associated with *C. forskohlii* is shown in Figure 9.

3.3. Statistical Analysis

3.3.1. Colonization frequency (CF)

The CF% of the fungal endophytes was calculated using the following formula given by Hata and Futai (1995):

$$CF\ (\%) = (85/280) \times 100$$

$$= 30.35\%$$

3.3.2. Endophytic isolation rate (EIR)

The EIR was calculated as follows:

$$EIR = 85/280$$

$$= 0.303$$

3.3.3. Relative percentage occurrence (RPO)

The RPO of different fungal groups were calculated as follows:

RPO of Ascomycota group (%) = 96.47%

RPO of Basidiomycota group (%) = 3.53%

4. DISCUSSION

As plants evolved to the terrestrial locale during the Ordovician period (470 million years), their interaction and association with the microbial communities have occurred instantaneously. The microorganisms have evolved along with the plants to shape their new niches into the host plant tissues to benefit themselves. At the same time, this colonization has benefitted the plant's growth, fitness, and adaptability to nature [33]. The association of fungal endophytes with plants has been well understood in most plant groups, from the primitive groups like lichens and mosses, as well as some of the angiosperms [34,35]. The colonization of endophytic fungi with the host plant could enhance the ability of the host plant to survive in nature by secreting some bioactive compounds which could help the host plant defend itself against a wide range of pathogens and other abiotic stresses [36–38].

Although understanding the different kinds of secondary metabolites produced by the host plant and the endophytic fungi is essential, it is also necessary for us to understand the diversity and phylogenetic relationships of the endophytic fungi associated with the host plant as this will help us to fish out some host-specific fungal endophytes and their bioactive molecules. Studies on *C. forskohlii* (Willd.) Briq. have shown that endophytes regulate and enhance the production of secondary metabolites such as forskolin [39], but understanding the diversity of endophytes associated with *C. forskohlii* is least understood; therefore, the results of the present study have elaborated on the diversity of the fungal endophytes associated with *C. forskohlii* and also revealed their phylogenetic relations.

In the present study, 85 endophytic fungi were isolated from 280 leaf segments of *C. forskohlii*. Molecular identification using the 18S rRNA gene sequencing has revealed 34 different genera of endophytic fungi namely *A. verrucaria*, *A. alternata*, *Alternaria* sp., *A. flavus*, *A. niger*, *A. ochraceus*, *Aspergillus* sp., *A. versicolor*, *B. spectabilis*, *C. sojina*, *C. globosum*, *Cladosporium* sp., *Cochliobolus* sp., *C. coccodes*, *Colletotrichum* sp., *C. truncatum*, *C. atramentaria*, *Davidiellaceae* sp., *Diaporthe* sp., *D. variabile*, *H. angustilamellatum*, *Lecanicillium* sp., *N. oryzae*, *O. foeniculi*, *P. citrinum*, *P. limosum*, *P. oxalicum*, *Penicillium* sp., *P. fallopiae*, *P. neopyrolae*, *P. gracilis*, *P. fuligena*, *P. pallida*, and *S. brevicaulis* which showed >97.1% match with the existing fungi in the NCBI database (Table 1). This is the first study to report the diversity of fungal endophytes from *C. forskohlii* except for one study which has reported the existence of endophytic fungi—*Rhizactonia bataticola*—and a sterile hypha [40]. Although many fungi reported are commonly isolated ones, with reference to "A worldwide list of endophytic fungi with notes on ecology

Table 1: Maximum nucleotide identity matches for 85 endophytic fungi based on 18S rRNA sequencing using the BLAST analysis.

Sl. no.	Isolate ID (GenBank accession no.)	Endophytic fungal species	GenBank accession number	Percentage match (%)
1	Centre for Innovative Studies in Herbal Drug Technology – Coleus Leaf Fungi (CISHDT-CLF 01) (MT740749)	*A. flavus*	CP044617.1	99.82
2	CISHDT-CLF 02 (MT764264)	*D. variabile*	NG_064914.1	99.40
3	CISHDT-CLF 03 (MT749388)	*N. oryzae*	AB220233.1	100
4	CISHDT-CLF 04 (MT749387)	*B. spectabilis*	KT031992.1	98.10
5	CISHDT-CLF 05 (MT749670)	*P. gracilis*	DQ851582.1	99.23
6	CISHDT-CLF 06 (MT764263)	*H. angustilamellatum*	NG_070875.1	98.56
7	CISHDT-CLF 07 (MT750014)	*C. coccodes*	MF376146.1	99.67
8	CISHDT-CLF 08 (MT750219)	*P. fuligena*	GU214675.1	98.70
9	CISHDT-CLF 09 (MT763960)	*Aspergillus* sp.	MF185177.1	99.48
10	CISHDT-CLF 10 (MT750290)	*A. niger*	MN420840.1	99.52

Continued

Sl. no.	Isolate ID (GenBank accession no.)	Endophytic fungal species	GenBank accession number	Percentage match (%)
11	CISHDT-CLF 11 (MT750289)	*N. oryzae*	AB220233.1	99.87
12	CISHDT-CLF 12 (MT762337)	*A. versicolor*	AB002064.1	99.21
13	CISHDT-CLF 13 (MT762297)	*P. fuligena*	GU214675.1	100
14	CISHDT-CLF 14 (MT762340)	*C. truncatum*	AJ301945.1	99.88
15	CISHDT-CLF 15 (MT762341)	*A. niger*	MG889595.1	99.61
16	CISHDT-CLF 16 (MT762356)	*Aspergillus* sp.	KP872521.1	99.88
17	CISHDT-CLF 17 (MT762359)	*Cochliobolus* sp.	KX852423.1	99.94
18	CISHDT-CLF 18 (MT762367)	*C. sojina*	CP036216.1	100
19	CISHDT-CLF 19 (MT801133)	*Cochliobolus* sp.	KX852423.1	99.94
20	CISHDT-CLF 20 (MT762369)	*Penicillium* sp.	KP872503.1	98.84
21	CISHDT-CLF 21 (MT762399)	*A. niger*	MN420840.1	99.80
22	CISHDT-CLF 22 (MT762400)	*P. fallopiae*	NG_064828.1	99.76
23	CISHDT-CLF 23 (MT762907)	*A. ochraceus*	AF548065.1	100
24	CISHDT-CLF 24 (MT762814)	*Colletotrichum* sp.	AB076801.1	99.88
25	CISHDT-CLF 25 (MT762908)	*N. oryzae*	AB220233.1	99.93
26	CISHDT-CLF 26 (MT762909)	*N. oryzae*	MH014997.1	99.80
27	CISHDT-CLF 27 (MT763199)	*Diaporthe* sp.	MK299422.1	100
28	CISHDT-CLF 28 (MT776313)	*O. foeniculi*	MN516743.1	97.10
29	CISHDT-CLF 29 (MT776176)	*O. foeniculi*	MN516743.1	99.58
30	CISHDT-CLF 30 (MT763390)	*Alternaria* sp.	KT192438.1	99.94
31	CISHDT-CLF 32 (MT763957)	*Colletotrichum* sp.	MK299420.1	99.64
32	CISHDT-CLF 33 (MT763958)	*Alternaria* sp.	KT192438.1	99.35
33	CISHDT-CLF 34 (MT763959)	*Lecanicillium* sp.	LT992875.1	99.88
34	CISHDT-CLF 35 (MT763960)	*Aspergillus* sp.	MF185177.1	99.48
35	CISHDT-CLF 36 (MT763961)	*C. globosum*	JQ964323.1	99.47
36	CISHDT-CLF 37 (MT763962)	*A. niger*	MN420840.1	99.93
37	CISHDT-CLF 38 (MT767108)	*Cladosporium* sp.	KP997210.1	99.76
38	CISHDT-CLF 39 (MT767111)	*Colletotrichum* sp.	AB076801.1	99.41
39	CISHDT-CLF 40 (MT767107)	*Colletotrichum* sp.	AB076801.1	99.81
40	CISHDT-CLF 41 (MT770956)	*P. fallopiae*	NG_064828.1	99.16
41	CISHDT-CLF 42 (MT767113)	*A. niger*	MG889595.1	99.93
42	CISHDT-CLF 43 (MT767172)	*N. oryzae*	AB220233.1	99.74
43	CISHDT-CLF 44 (MT767173)	*C. atramentaria*	DQ115781.1	99.35
44	CISHDT-CLF 45 (MT767174)	*Cladosporium* sp.	LT860211.1	99.87
45	CISHDT-CLF 46 (MT767175)	*Cladosporium* sp.	MH07202.1	99.82
46	CISHDT-CLF 47 (MT770925)	*Cladosporium* sp.	MH015002.1	99.80
47	CISHDT-CLF 48 (MT770926)	*P. limosum*	NG_062729.1	98.89
48	CISHDT-CLF 49 (MT770927)	*Cladosporium* sp.	MH047202.1	99.88
49	CISHDT-CLF 50 (MT770928)	*Aspergillus* sp.	MN326853.1	100
50	CISHDT-CLF 51 (MT770929)	*Cladosporium* sp.	KP997210.1	99.67
51	CISHDT-CLF 52 (MT770930)	*A. alternata*	HM165489.1	99.94

Continued

Sl. no.	Isolate ID (GenBank accession no.)	Endophytic fungal species	GenBank accession number	Percentage match (%)
52	CISHDT-CLF 53 (MT770931)	*Penicillium* sp.	KP256500.1	99.38
53	CISHDT-CLF 54 (MT770948)	*Aspergillus* sp.	EU853156.1	99.55
54	CISHDT-CLF 55 (MT770931)	*C. globosum*	JQ964323.1	99.54
55	CISHDT-CLF 56 (MT770933)	*C. truncatum*	AJ301937.1	97.45
56	CISHDT-CLF 57 (MT770934)	*P. citrinum*	LC127087.1	99.93
57	CISHDT-CLF 61 (MT770935)	*S. brevicaulis*	KJ443074.1	99.60
58	CISHDT-CLF 62 (MT770936)	*C. globosum*	JQ964323.1	98.69
59	CISHDT-CLF 63 (MT770937)	*Alternaria* sp.	KT192438.1	99.30
60	CISHDT-CLF 65 (MT770938)	*Alternaria* sp.	KT192438.1	99.94
61	CISHDT-CLF 66 (MT770939)	*Colletotrichum* sp.	AB076801.1	99.20
62	CISHDT-CLF 67 (MT770940)	*Aspergillus* sp.	EU853156.1	100
63	CISHDT-CLF 68 (MT770941)	*Davidiellaceae* sp.	GU250343.1	97.12
64	CISHDT-CLF 69 (MT770942)	*A. niger*	MN420840.1	99.87
65	CISHDT-CLF 70 (MT770943)	*A. verrucaria*	NG_061023.1	99.61
66	CISHDT-CLF 71 (MT770944)	*Cladosporium* sp.	MH015002.1	99.87
67	CISHDT-CLF 72 (MT770945)	*A. niger*	MG889595.1	99.74
68	CISHDT-CLF 73 (MT770946)	*Penicillium* sp.	KC143067.1	99.27
69	CISHDT-CLF 74 (MT770947)	*Colletotrichum* sp.	AB076801.1	99.21
70	CISHDT-CLF 75 (MT770948)	*Aspergillus* sp.	EU853156.1	99.55
71	CISHDT-CLF 76 (MT770949)	*D. variabile*	MK123327.1	99.78
72	CISHDT-CLF 77 (MT770950)	*Colletotrichum* sp.	MK299420.1	99.46
73	CISHDT-CLF 78 (MT770951)	*P. oxalicum*	KF152942.1	99.75
74	CISHDT-CLF 80 (MT770952)	*A. verrucaria*	NG_061023.1	98.27
75	CISHDT-CLF 81 (MT770953)	*Alternaria* sp.	KT192438.1	99.33
76	CISHDT-CLF 82 (MT770954)	*P. limosum*	NG_062729.1	99.61
77	CISHDT-CLF 83 (MT770955)	*Cladosporium* sp.	MH047202.1	99.76
78	CISHDT-CLF 84 (MT770956)	*P. fallopiae*	NG_064828.1	99.16
79	CISHDT-CLF 85 (MT770957)	*P. fallopiae*	NG_064828.1	100
80	CISHDT-CLF 86 (MT770958)	*Penicillium* sp.	KP872503.1	99.94
81	CISHDT-CLF 87 (MT770959)	*P. neopyrolae*	NG_064830.1	99.58
82	CISHDT-CLF 88 (MT770960)	*Cladosporium* sp.	MH047202.1	99.74
83	CISHDT-CLF 89 (MT770961)	*P. pallida*	GU214680.1	99.94
84	CISHDT-CLF 90 (MT770962)	*S. brevicaulis*	KJ443074.1	100
85	CISHDT-CLF 91 (MT770963)	*P. fallopiae*	NG_064828.1	99.80

and diversity" [41], *A. verrucaria*, *C. sojina*, *C. truncatum*, *H. angustilamellatum*, *O. foeniculi*, *P. fallopiae*, *P. neopyrolae*, *P. gracilis*, *P. fuligena*, and *P. pallida* are among the rarely/not very commonly reported endophytic fungi which are newly reported in this study.

Among the 34 genera, the frequency of occurrence of *Cladosporium* sp. was found to be highest, and the other predominant genera were found to be *Alternaria* sp., *A. niger*, *Aspergillus* sp., *Colletotrichum* sp., *N. oryzae*, *Penicillium* sp., and *P. fallopiae* (Fig. 5). Colonization frequency of the endophytic fungi associated with the leaves of *C. forskohlii* (Willd.) Briq. was found to be 30.35% and the endophyte isolation rate from leaf tissue was found to be 0.303. Many studies have revealed ascomycetes to be highest associated fungal endophytic group [42,43], likewise the fungal group highest associated with the leaves of *C. forskohlii* was also found to be Ascomycota with 96.47% RPO and Basidiomycota to be the least associated with 3.53% RPO. The percentage occurrence of members of Ascomycota was the highest, with 96.47% distribution and Basidiomycota members were distributed the least, with 3.53%.

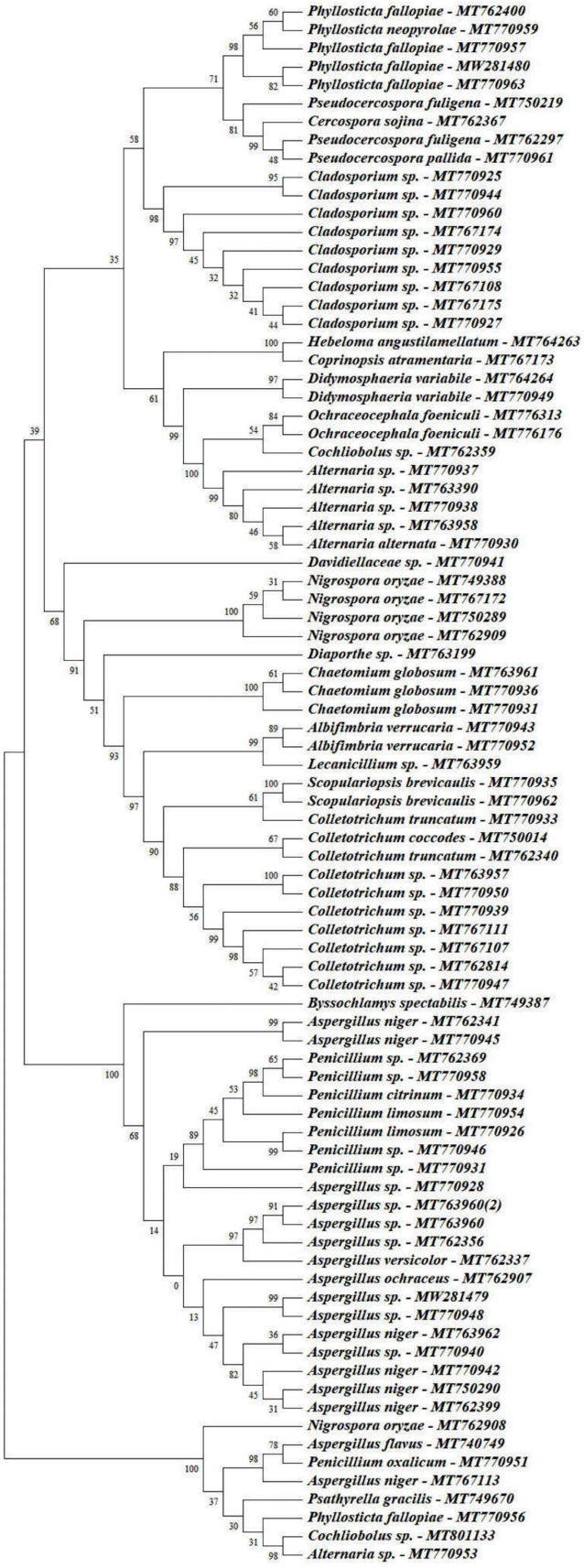

Figure 9: Phylogenetic affiliation of fungal endophytes from *C. forskohlii*.

5. CONCLUSION

Coleus forskohlii is an essential pharmaceutical crop which is a rich source of secondary metabolites. About 68 medicinally important compounds are isolated from different parts of the plant, among which forskolin is the principal compound which is found only in *C. forskohlii* [44]. The root tubers are the main source of forskolin. Harvesting of tubers results in the death of plants, thus harvesting of forskolin is a plant destructive harvest process. Thus, there is a need for alternative non-destructive, eco-friendly process for obtaining forskolin.

Fungal endophytes associated with medicinal plants represent an abundant and dependable source of novel bioactive compounds. Fungal endophytes of *C. forskohlii* are unexplored. Understanding the diversity of fungal endophytes associated with *C. forskohlii* is the first step toward exploitation of these endophytes to obtain different medicinally active compounds. The present investigation is first report of the diversity of fungal endophytes (34) associated with *C. forskohlii* leading to the future lines of research of screening these frequently occurring endophytes for medicinally active bioactive compounds.

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7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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9. CONFLICT OF INTEREST

The authors have declared no conflicts of interest.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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