International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 15-07-2021, Accepted: 30-07-2021, Published: 16-08-2021
Volume 6, Issue 4, 2021, Page No. 722-725

Molecular characterization and antibacterial activity of endophytic fungi *Calonectria indusiata* in *Lobelia nicotianifolia* roth ex Schult. of central western ghats of Chikkamagaluru

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Abstract
Endophytic fungi play an important role in the defense system of plants by secreting various antimicrobial agents. The present investigation reveals the isolation, molecular identification, phylogenetic analysis, and antibacterial studies of the endophytic fungi *Calonectria indusiata* in the leaves of *Lobelia nicotianifolia* Roth ex Schult. of Chikkamagaluru. The *Calonectria indusiata* was isolated and molecularly characterized by using ITS (ITS1 & ITS4) gene sequencing. The consensus sequence was submitted in NCBI GenBank and the analysis of the phylogenetic tree by using MEGA-X and Fig Tree software. The ethyl acetate extract of *C. indusiata* showing high antibacterial activity against all the tested bacteria.

Keywords: antibacterial, consensus, endophytic, gen bank, ITS, identification

Introduction
In recent years, the chemistry of fungal endophytes has attracted considerable interest as promising sources of novel antibiotics and other therapeutic agents to address new challenges in medicine and the environment (Nurunnabi *et al*., 2020) [12]. Fungal endophytes were reported that ubiquitous in the internal tissues of plants and the major producers of significant bioactive compounds (Techaoei *et al*., 2020[18]; Zheng *et al*., 2016 [19]). The association of host endophyte, many plants, and endophytic fungi were producing the same bioactive compounds (Tan and Zou 2001[17]; Cruz *et al*., 2020 [2]). Secondary metabolites that are co-produced by the hosts and their fungal endophytes have anticancer, antioxidant, anti-inflammatory agents (Chow *et al*., 2015[1]; Lakshmi *et al*., 2013) [8], antiviral, antimicrobial (Devi *et al*., 2012 [3]), antimycotics, immune suppressants (Nath *et al*.,2016) [11], immune modulatory, insecticidal, antiparasitic, antitubercular properties (Mahmud *et al*.,2020) [9].
Lobelia nicotianifolia Roth ex Schult. belongs to the family Campanulaceae, commonly known as wild tobacco. The plant has mainly used for ethnobotanical treatments (Kolap *et al*., 2020) [6]. The aims of this study were to isolation identification, phylogenetic analysis, and antibacterial activity of the endophytic fungi *Calonectria indusiata* in the leaves of *Lobelia nicotianifolia* from the central Western Ghats region of Chikkamagaluru, Karnataka.

Materials and Methods
Collection site
The healthy leaves of *L. nicotianaefolia* were collected from the central Western Ghats area of Chikkamagaluru during December 2020, situated at 13°25'19"N and 75°10'52" E. The samples were cultured and isolate endophytic fungus within 24 h of collection.

Isolation of endophytic fungus
The healthy leaves were sterilized and isolation of endophytic fungi was done by using the method (Rao *et al*., 2025) [13] with slight modifications. The endophytic fungus used in the present study is maintained at the Department of Applied Botany at the Kuvempu University Shivamogga, India.

Identification of Endophytic Fungi
Morphological and molecular identification
The endophytic fungi *C. indusiata* was identified based on colony characteristics and microscopic observations by the method (Manganyi *et al*., 2018) [10]. The genomic DNA was extracted by fresh mycelia using the CTAB method (Karthikeyan *et al*., 2010) [5] with modifications.
The PCR reactions were carried out in 0.2ml PCR tubes with 50µl reaction mixture containing, 25µl double distilled water, 8µl 10X PCR buffer A (Himedia), 2.5µl of each primer, 0.5µl of Taq DNA polymerase (3U/µl), 1.5µl d NTP's mixture (Himedia), and 10µl of DNA template. The primers of ITS 1 and ITS 4 were used. Thermal cycling for amplification; 4' 94°C, 32 cycles of 30" 94°C, 1' 52°C, 1' 72°C and a final extension step of 7' 72°C. The PCR product was observed on 1% Agarose gel, under a gel image documentation system. The sequences were trimmed using MEGA X and formed the consensus sequences using BioEdit software. The consensus sequence was analyzed nucleotide BLAST and deposited to Gen Bank (Kantharaja *et al*., 2010) [4].

Molecular Phylogenetic Analysis by Maximum Likelihood Method
The phylogenetic evolutionary study was conducted by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992) [16]. The phylogenetic tree with the highest log likelihood (-1309.66) is shown. The

total percentage of the tree is the associated taxa grouped next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Tamura 3 parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.7459). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 45.62% sites). The tree is drawn to scale, with the lengths of the branches being measured in terms of the number of substitutions per location (next to the branches). This analysis involved 15 nucleotide sequences. There was a total of 707 positions in the final dataset. The Phylogenetic analysis was conducted in MEGA X (Kumar *et al*., 2018) [7].

Culture and extraction

The pure culture of endophytic fungi *C. indusiata* was cultivated in 1000 ml of potato dextrose broth and incubated for 4 weeks at 28°C in a shaker at 160 rpm, the culture filtrate was filtered and extracted with ethyl acetate by separating funnel (solvent-solvent extraction). The extract was dissolved in dimethyl sulfoxide (DMSO)and evaluated the antibacterial activity (Rustamova *et al*., 2020) [14].

Antibacterial activity

The evaluate the antibacterial activity against three human bacterial pathogens namely, (*Escherichia coli*, MTCC-1599), (*Klebsiella pneumoniae*, MTCC-7028), (*Staphylococcus aureus*, MTCC-4734), and one plant pathogen – (*Xanthomonas campestris*, MTCC-228) by agar well diffusion method. The ethyl acetate extract was diluted in dimethyl sulfoxide. The 6 mm wells are made by sterile borer and loaded with 40 µL of the extract (1 mg/ml of extract).

The antibiotic drug Amoxicillin and DMSO served as positive and negative controls. The plates were incubated at 37 ° C overnight and the zone of inhibition (mm) was recorded (Suryavamshi *et al*., 2020) [15].

Results

Morphological and molecular identification

The colony morphology of the endophytic fungi was studied based on morphological characteristics. The molecular identification of the ITS sequence was edited by BIO edit software and the consensus sequence was formed and submitted in NCBI-GenBank (Accession number-MT072215).

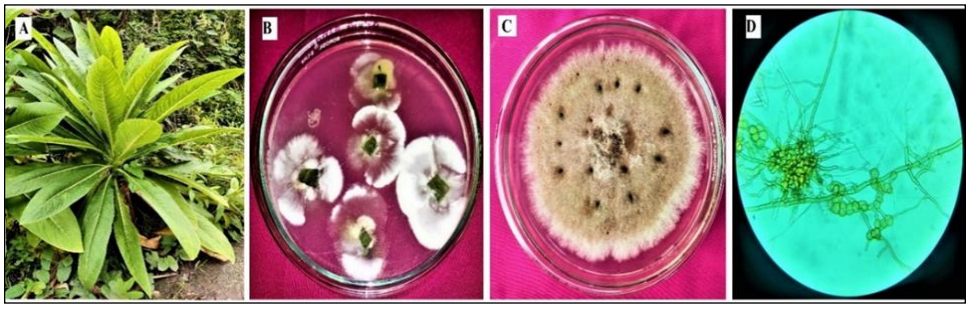

Fig 1: Isolation, culture, and Identification of *Calonectria indusiata* A) *Lobelia nicotianifolia* Roth ex Schult. B) Isolation C) Pure culture, D) Microscopic observation.

Phylogenetic analysis

The phylogenetic evolutionary examination was done by NCBI-Blastn and MEGA-X software. Blastn search was made to choose the nearest 14 taxa and selected as one outgroup (*Fusarium proliferatum*) of the evaluation (Table.1). The evolutionary study inferred the use of the ITS (ITS1 & ITS4) sequences with the alignment of ClustalW, to create the consensus sequence and to develop a Maximum likelihood phylogenetic tree (Kumar *et al.*, 2018), by the use of MEGA-X and Fig tree software program (Figure.2). The recorded *Calonectria indusiata* (GenBank Accession number MT072215) was highlighted in blue colour.

Table 1: List of *Calonectria* species, origin, and GenBank accession numbers of the ITS sequences used in phylogenetic analysis. A newly generated sequence is in bold.

Sl no.	Species	Gen bank accession number	Strain	Origin and year
1	*Calonectria indusiata*	MT072215	VKL003	India, 2020
2	*Calonectria indusiata*	MH863822	CBS 125933	Netherland, 2019
3	*Calonectria uxmalensis*	MT359852	CBS 110919	China,2020
4	*Calonectria uxmalensis*	MT359851	CBS 110925	China,2020
5	*Calonectria amazonica*	MT359649	CBS 116242	China,2020
6	*Calonectria amazonica*	MT359648	CBS 116305	China,2020
7	*Calonectria brasiliensis*	MT359662	CMW 32949	China,2020
8	*Calonectria brasiliensis*	MT359661	CBS 230.51	China,2020
9	*Calonectria gracilipes*	MT359714	CBS 111141	China,2020
10	*Calonectria gracilipes*	MT359713	CBS 115674	China,2020
11	*Calonectria brachiatica*	MT359657	CMW 25302	China,2020
12	*Calonectria brachiatica*	MT359656	CMW 25298	China,2020
13	*Calonectria madagascariensis*	GQ280593	CBS 114571	South Africa, 2009
14	*Calonectria madagascariensis*	GQ280592	CBS 114572	South Africa, 2009
15	*Fusarium proliferatum*	MH858428	CBS 240.64	Netherland, 2017

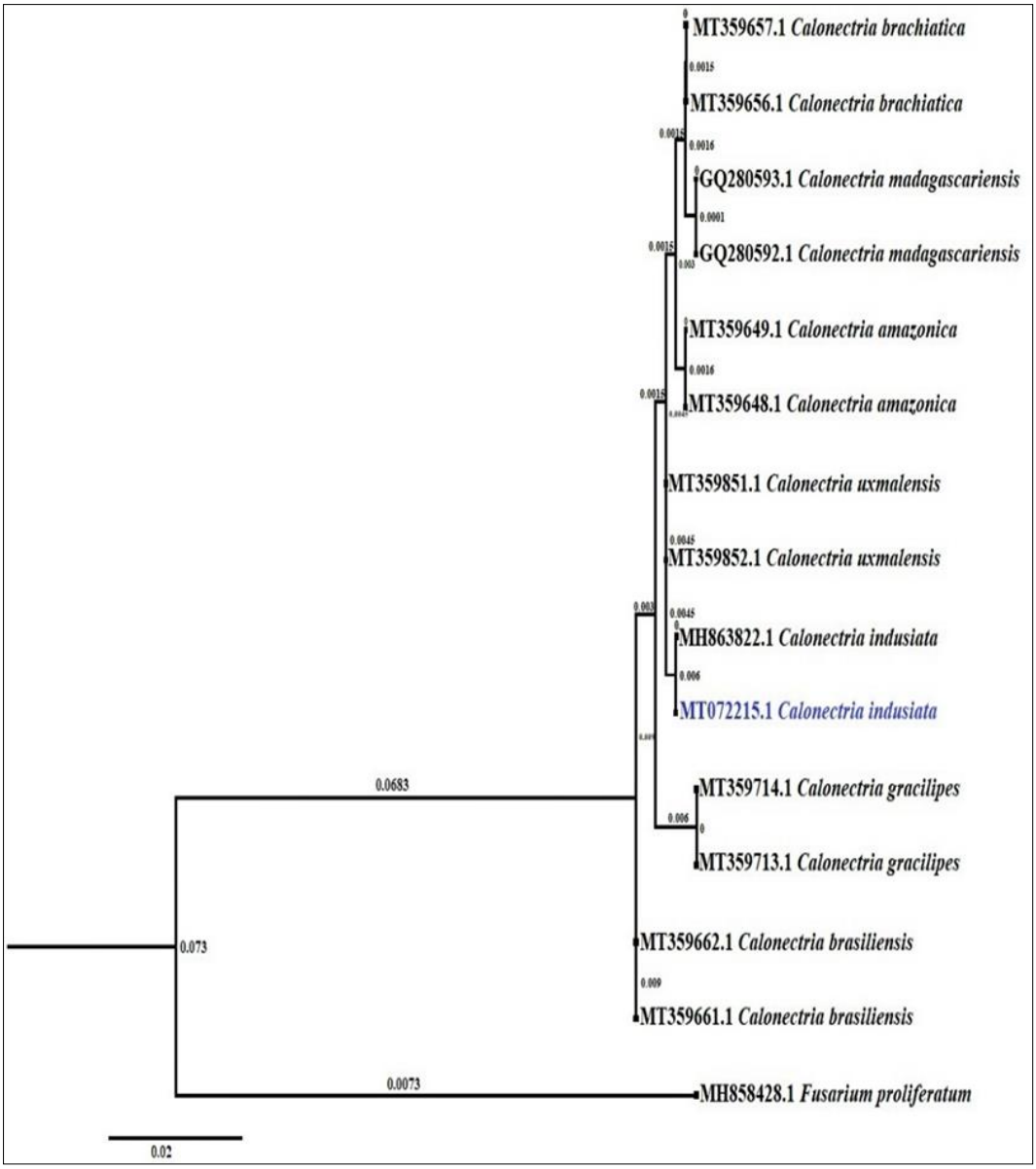

Fig 2: Maximum likelihood Phylogenetic analyses by ITS sequence data of *C. indusiata.* It illustrates the relationships between the *C. indusiata* (MT072215) with other *Calonectria* species and *Fusarium proliferatum* (MH858428) selected as an outgroup.

Antibacterial activity

The antibacterial studies of the ethyl acetate extract of *C. indusiata* showed high activity against *E. coli,* S. *aureus*, *K. pneumoniae*, *X. campestris* compared to the standard Amoxicillin. *E. coli* showed a high zone of inhibition in 100% among the tested bacterial strains.

Table 2: Zone of inhibition of endophytic fungi *C. indusiata* isolated from *L. nicotianifolia* against different tested strains in mm.

Sl. no	Organism	Inhibition Zone (In mm)				
		100%	50%	25%	Standard (Amoxicillin)	Control (DMSO)
1	*Escherichia coli*	6.33±0.33	2.66±0.33	1.33±0.33	7.66±0.33	0
2	*Staphylococcus aureus*	5±0.57	3±0	1.66±0.33	8.66±0.66	0
3	*Klebsiella pneumoniae*	4.33±0.33	3.66±0.66	2.33±0.33	8±0.57	0
4	*Xanthomonas citri*	6±0.57	3±0.57	1.33±0.33	9.66±0.33	0

Conclusion

The present investigation reveals the isolation, molecular identification, maximum likelihood evolutionary analysis, and antibacterial activity of the *C. indusiata in L. nicotianifolia* of the central western ghats of Chikkamagaluru.

The ethyl acetate extract of the endophytic fungi *C. indusiata* shows significant antibacterial activity than standard drug. *E. coli* showing more inhibition zone in 100% concentration when compared to the other tested pathogenic bacteria.

Acknowledgments

The authors are thankful to the Department of Applied Botany, Kuempu University, Shankaraghatta. This work was financially supported by grants from the BUILDERS-PROJECT Department of Biotechnology, New Delhi, Government of India.

Conflict of Interest

The authors declare no conflicts of interest.

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