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Molecular identification and antibacterial activity of endophytic fungi *Curvularia lunata* in *Wendlandia thyrsoidea* (Roth) Steud. of central western Ghats region of Chikkamagaluru, Karnataka

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Abstract

Endophytic fungi are the mutualistic microbial organisms that exist in every part of the Plants. The present study was focused on isolation, molecular identification, phylogenetic and antibacterial studies of the endophytic fungi *Curvulaia lunata* in the leaves of *Wendlandia thyrsoidea* (Roth) Steud. of Chikkamagaluru. The *C. lunata* was isolated and molecularly characterized by using ITS (ITS1 & ITS4) gene sequencing. The consensus sequence was submitted in NCBI GenBank and to study the phylogenetic evolutionary analysis by using MEGA-X and Fig Tree software. The ethyl acetate extract of *C. lunata* showing high antibacterial activity against all the tested pathogenic bacteria.

Keywords: evolutionary, genbank, ITS, mutualistic, NCBI, phylogenetic, sequence

Introduction

Endophytic fungi are the great novel microorganisms that live in different parts of plants and without causing any symptoms or injury to the host (Vinu *et al.*, 2021 [19]; An *et al.*, 2020 [2]; Sofiya *et al.*, 2021) [19] [2] [14]. The study showed that there is a mutualistic interaction and beneficial relationship between the host and the endophytic fungi, in which the host provides shelter and nutrition, while endophytes act as chemical guards (Ababutain *et al.*, 2021) [1], promotes plant growth, develop medicinal ingredients (Du *et al.*, 2020) [4] and they produce many unique natural compounds includes alkaloids, terpenoids, various polyketides, isocoumarins, flavonoids, lactones (Rustamova *et al.*, 2020) [13], antimicrobial metabolites (Liang *et al.*, 2012) [9], anticancer, antibacterial, antifungal, anti-diabetic and immunosuppressant compounds(Rai *et al.*, 2014) [11]. The renewable source of bioactive and chemically new compounds with potential for exploitation in a huge variety of medical, agricultural, and industrial uses (Treasure *et al.*, 2020) [17].

Wendlandia thyrsoidea (Roth) Steud. belongs to the family Rubiaceae, the plant is an ethnomedicinal small tree and commonly called mountain Wendlandia. It is used in ayurvedic treatments, antimicrobial and analgesic properties are shown in the leaf extracts (Bodke *et al.*, 2015; Vagdevi *et al.*, 2009) [3, 18]. The study reveals the isolation, identification, and phylogenetic study of the endophytic fungi *Curvularia lunata* in the leaves of *W. thyrsoidea* of Central Western Ghats region of Chikkamagaluru, Karnataka.

Materials and Methods

1. Study area and collection of samples

The fresh leaves of *W. thyrsoidea* were collected from the central Western Ghats area of Chikkamagaluru during December 2020 situated at 13° 33’ 16.92” N and 75° 45’ 50.45” E.

2. Isolation of endophytic fungi

The healthy matured leaves were sterilized and isolation of endophytic fungi was done by using the method (Ghimire *et al.*, 2011) [5] with slight modifications and examined regularly for emerging fungal colonies.

3. Identification of endophytic fungi

3.1 Morphological and molecular Identification

The endophytic fungi *C. lunata* was identified based on colony characteristics and microscopic observations by using a standard manual (Nagamani *et al.*, 2006) [10]. The fungal genomic DNA was extracted from the cultured mycelia using the CTAB method (Karthikeyan *et al.*, 2010) [7] 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 dNTP’s mixture (Himedia), and 10µl of DNA template. The primer pair ITS 1 and ITS 4 were used for the ITS region. 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 followed by purification and sequencing. The sequences were trimmed using MEGA X and formed the consensus sequences using BioEdit. The BLAST search in the GenBank and the consensus sequence was deposited to GenBank (Kantharaja *et al.*, 2010) [6].

4. Phylogenetic analysis by Maximum Likelihood method

The evolutionary record was inferred by utilizing the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992) [16]. The tree with the best log-likelihood (-1037.22) has appeared. Initial tree(s) for the heuristic search were acquired consequently by applying Neighbor-Join and BioNJ calculations to a matrix of pairwise distances assessed utilizing the Tamura 3 parameter model, and afterward choosing the topology with predominant log-likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4596)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 46.32% sites), and the tree was drawn to scale, with branch lengths measured in the number of replacements per site (next to the branches). This analysis involved 15 nucleotide sequences. There was a total of 585 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018) [8] and the final tree was modified using Fig tree software.

5. Preparation of fungal extract

The pure culture of *C. lunata* was cultivated in 1000ml of Potato Dextrose Broth and incubated at 28°C on a shaker at 160 rpm for 4 weeks. The culture filtrate was filtered and extracted with ethyl acetate using a separatory funnel (Solvent–Solvent extraction). The extract was dissolved in dimethyl sulfoxide (DMSO), then evaluated for antibacterial activity (Rustamova *et al*., 2020) [12].

6. Antibacterial activity

The antibacterial activity against three human bacterial pathogens (*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 fungal extract was prepared in dimethyl sulfoxide. The 6 mm wells are made by using a sterile borer and loaded with 40 µL of the extract (1 mg ml of extract). The antimicrobial drug Amoxicillin and DMSO served as positive and negative controls. The inoculated plates were incubated overnight at 37 °C and the zone of inhibition (mm) was recorded (Suryavamshi *et al*., 2020) [12].

Results

1. Morphological and Molecular Identification

The colony morphology of the endophytic fungi *C. lunata* showing dark grey mycelia, usually zonate form, regular stromata, mycelium branched, septate, conidiophores long, conidia elliptic in shape, curved, septate 2-3 middle cells broad and darker than other cells, (21-30×9-15µm.). The molecular identification of the ITS sequence was edited using BIO edit software and the consensus sequences were formed and the sequence was submitted in NCBI-GenBank (Accession number- MZ368890).

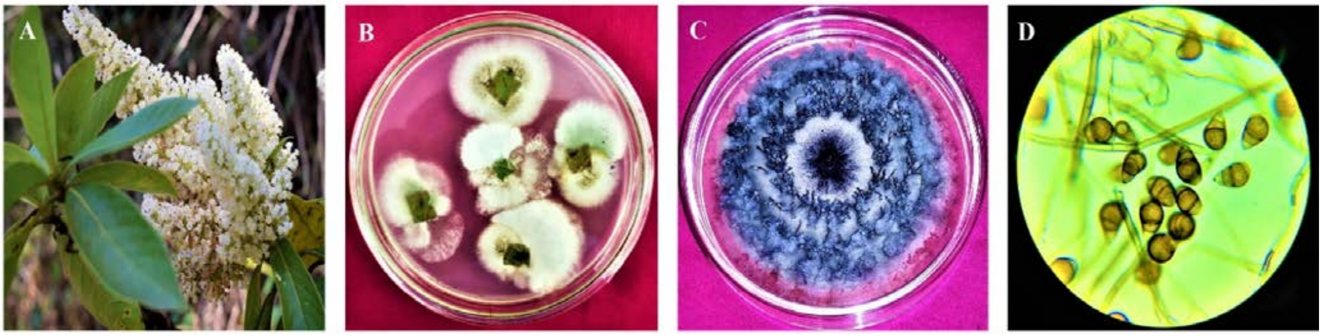

Fig 1: Isolation, culture, and Identification of *Curvularia lunata* A) *Wendlandia thyrsoidea* (Roth) Steud. B) Isolation of fungal endophytes, C) Pure culture, D) Conidia (21-30×9-15µm).

2. Phylogenetic Analysis

The phylogenetic evolutionary study was conducted by NCBI-Blastn and MEGA-X software. Blastn search was made to choose the nearest 14 taxa and selected as one outgroup (*Alternaria alternata* MN615420) of the evaluation (Table.1). The evolutionary examine inferred the use of the ITS (ITS1 & ITS4) sequences with the alignment of ClustalW, to create the consensus sequence and to determine the most appropriate model 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 *Curvularia lunata* (GenBank Accession number MZ368890) was highlighted in blue colour.

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

Sl No	Species	GenBank Accession number	Strain	Origin and Year
1	***Curvularia lunata***	**MZ368890**	**WTE01**	**India, 2020**
2	*Curvularia lunata*	KU221488	CU 406	India, 2015
3	*Curvularia lunata*	MN180825	K2	India, 2019
4	*Curvularia lunata*	MN173127	CEL25	India, 2019
5	*Curvularia aeria*	KT933656	858	Malaysia, 2015
6	*Curvularia aeria*	KT933655	855	Malaysia, 2015
7	*Curvularia chlamydospora*	MN197597	UAGro-3	Mexico, 2019
8	*Curvularia chlamydospora*	MG250429	AY988	Germany, 2017
9	*Curvularia hawaiiensis*	LN482468	TUHT72	Saudi Arabia, 2014
10	*Curvularia hawaiiensis*	MZ045513	KSUD64	Saudi Arabia, 2021

11	*Curvularia australiensis*	KC999933	SZMC13041	Hungary, 2013
12	*Curvularia australiensis*	KC424607	BRIP 12112a	China, 2012
13	*Curvularia plantarum*	MN215689	LC11985	China, 2019
14	*Curvularia plantarum*	MN215688	CGMCC3.19342	China, 2019
15	*Alternaria alternata*	MN615420	YZU 191238	China, 2019

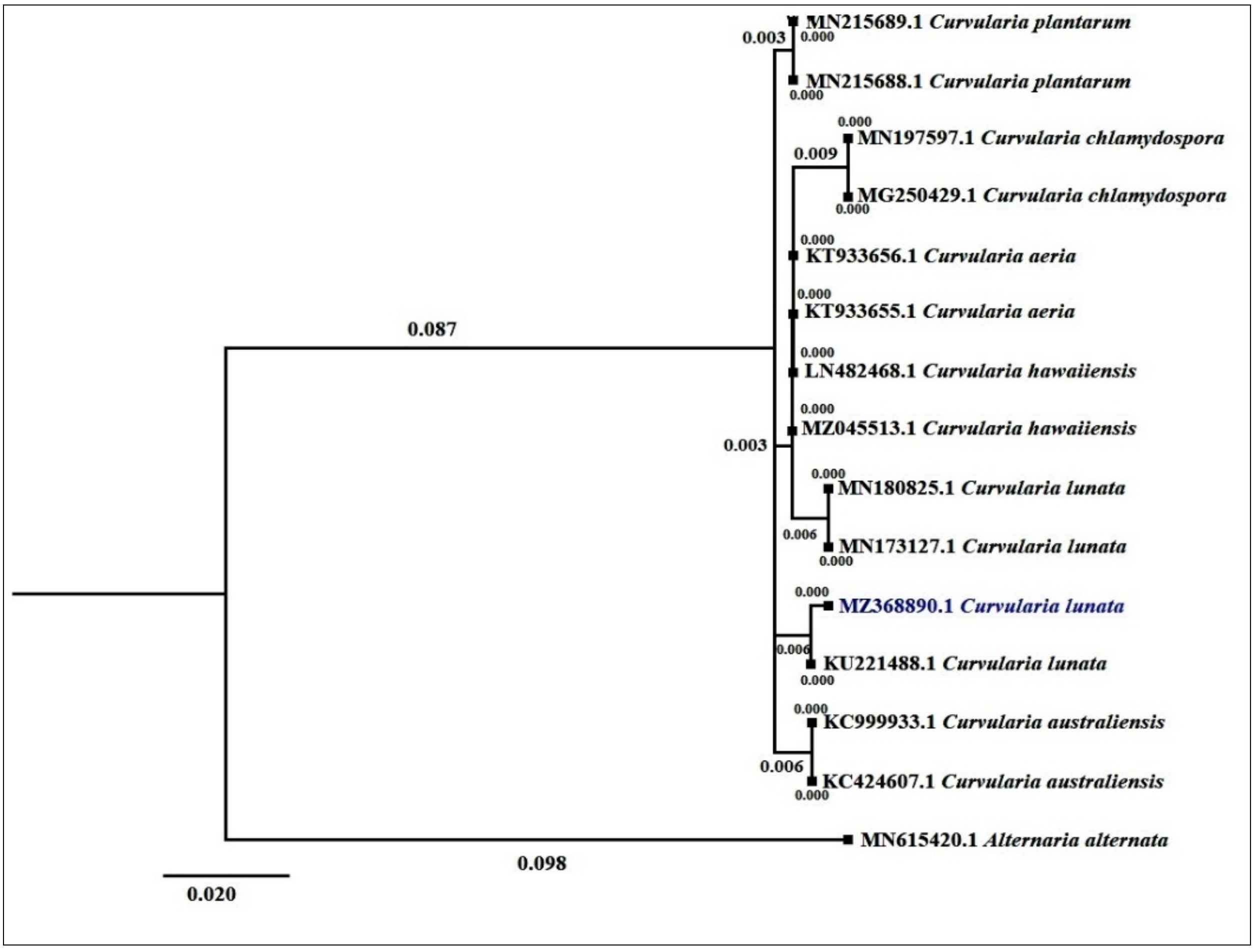

Fig 2: Maximum likelihood Phylogenetic analyses by ITS sequence data of *Curvularia lunata.* It illustrates the relationships between the *Curvularia lunata* (MZ368890) with other *Curvularia* species and *Alternaria alternata* selected as outgroup.

3. Antibacterial activity

The present study reveals the ethyl acetate extract of *C. lunata* expressed high antibacterial activity against *E. coli, S. aureus*, *K. pneumoniae*, *X. campestris* compared to Amoxicillin. *E. coli* showed a high zone of inhibition among the tested bacterial strains.

Table 2: Zone of inhibition of endophytic fungi *C. lunata* isolated from *W. thyrsoidea* against different tested strains in mm

Sl.No	**Organism**	**Inhibition Zone (In cm)**				
		100%	**50%**	**25%**	**Standard (Amoxicillin)**	**Control (DMSO)**
1	*Escherichia coli*	9.66±0.33	4.6±0.33	2±0.57	10.66±0.33	0
2	*Staphylococcus aureus*	6.66±0.66	5±0.57	3.33±0.33	11.33±0.66	0
3	*Klebsiella pneumoniae*	8±0.57	7.66±0.33	2.33±0.33	10±0.57	0
4	*Xanthomonas citri*	7.66±0.88	5.66±0.33	3.66±0.88	12.66±0.88	0

Conclusion

Fungal endophytes are the most beneficial microbes that exist in all plants, and they produce bioactive compounds used in pharmaceutical and agricultural fields. The present study reveals the isolation, molecular identification, Phylogenetic analysis, and antibacterial activity of the endophytic fungi *C. lunata associated with the W. thyrsoidea* of the central Western Ghats region of the Chikkamagaluru. The ethyl acetate extract shows significant antibacterial activity and *E. coli* showing high inhibition zone when compared to the other tested pathogenic bacteria at 100% concentration.

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Conflict of Interest

The authors declare no conflicts of interest.

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