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Molecular Characterization and Metabolite Profiling of *Endomelanconiopsis endophytica* E.I. Rojas & Samuels, an Endolichenic Fungal Isolate

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Endolichenic fungi interact with their host mycobiont, photobiont, and other co-assembled microbial communities, by producing various chemical compounds. Identification of endolichenic fungi is challenging due to their high phenotypic plasticity, which results in most of them staying cryptic. Although the modern polyphasic approach to fungal identification deals with the morphology, molecular, and chemical profiles of the organism to delimit species, choosing this strategy have limitations. Endomelanconium-like fungi emerged from the rock inhabiting *Parmotrema* lichen collected from Shivamoga district. Since the isolate was typically non sporulating in nature, we utilized *ITS* and beta-tubulin (*Tub2*) gene genealogy to delineate species boundary. Both distance-based and coalescence-based phylogenetic analysis show that the isolate was *Endomelanconiopsis endophytica*. Secondary metabolite profiling using mass spectroscopy (OHR-LC-MS) analysis of ethyl acetate extract revealed the presence of 16 notable metabolites. Among this Tenuazonic acid, a mycotoxin produced in high concentration (90%), along with Fumonisin mycotoxin. The extract was further evaluated for radicle scavenging ability, indicating that metabolites produced by *E. endophytica* capable of neutralizing free radicles, may confer resistance against stress. Exploring the specific mechanisms by which these metabolites contribute to stress tolerance in lichen thalli could provide valuable insights into their adaptive strategies.

Keywords: Beta-tubulin 2 (*Tub2*), ITS,mass spectroscopy, non-sporulating fungi, polyphasic taxonomy, species delimitation

INTRODUCTION

Lichen is a symbiotic ecosystem of fungal and algal partners, dominated and shaped most of its portion by mycobionts. In addition to the primary fungal partner, lichen hosts a number of facultative microbes (Suryanarayana *et al.* 2005). Endolichenic fungi (ELF) are one of the non-obligate secondary fungal symbionts in lichen associations, distinct from mycobionts and lichenicolous fungi (Hawksworth and Grube 2020).

Unlike the lichenicolous fungi, which are associated with the various types of symptomatic growth on the thallus, ELFs are asymptomatic in nature and isolated from the interior of the apparently healthy thallus. (U'Ren *et al.* 2012). ELFs preferentially colonized in the medullary region of lichen thalli, specifically in close association with photobionts, to get nourishment and shelter. (U'Ren *et al.* 2014; Singh *et al. 2017*). In return, they produce secondary metabolites belonging to diverse chemical groups such as quinones, alkaloids, peptides, steroids, terpenes, simple aromatic compounds, and allylic compounds (Agrawal *et al.* 2019) to confer a multitude of benefits against stress conditions to their lichen host (Kellogg and Raza 2017). These secondary metabolites play a crucial role in protecting the lichen host from various stressors such as UV radiation, extreme temperatures, and desiccation. Due to their high phenotypic plasticity and frequent absence of reproductive structures, endophytic and lichen-associated fungi could not be discriminable, using morphology alone. As a consequence, most of them remain cryptic (Arnold *et al.* 2009). DNA based markers provide a large number of characters to delimit species boundaries and widely help to resolve complexity with species identification. The internal transcriber spacer (ITS) locus of the rDNA gene is regarded as a universal barcode region for fungal identification. Since there are no lineage-specific cutoff values exist for ITS to delimit species, its performance

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in highly diverse and poorly represented taxa was unpredictable. In such a circumstance, protein coding genes such as, Translation elongation factor 1- *á* (*Tef1-á)*, RNA polymerase *(RPB2),* and beta-tubulin (*Tub2)* serve as secondary barcodes. Introns in protein coding genes allow for easy recognition of homology and convergence as they contain less ambiguity due to codon constraints, and evolve at a faster rate than *ITS* (Raja*et al.* 2017). Phylogenetic analysis using one or more genes gives the best resolution for identification and inferring phylogenetic relationships.

ELFs show multiple types of interaction with their host mycobiont, photobiont, and other co-assembled microbial communities by producing an array of chemical compounds. Unfortunately, fungi in endolichenic assemblages are rarely evaluated for their metabolite production. Result in the interaction type and the importance of endolichenic life style were still poorly understood. We have isolated one of the endomelanonium-like ELFs from *Parmotrema* lichen collected around the dry deciduous forest of Shivamoga district. As isolates were typically non- sporulating in culture conditions, their identity, species delimitation, and phylogenetic position have been tested based on distance and coalescence methods using *ITS* and *Tub2*. Further metabolite profiling was performed on the fungal extract using high resolution orbitrap liquid chromatography and mass spectrophotometric analysis. Also tested for the antioxidant potential of the extract.

MATERIALS AND METHODS

Lichen Sampling and isolation of Endolichenic fungi

Lichen sample was collected from moist deciduous forest, Shivamoga district lies on the central Western Ghats region of Karnataka, (latitude 14.0085 longitude 75.21079). Thallus were separated from their host with the help of razor blade, collected in paper bags, documented details of host, collection date and geological coordinates, and samples were brought to the laboratory identified as *Parmotrema praesorediosum.* Healthy thallus of *P. praesorediosum* was washed in running tap water performed surface sterilization according to protocol explained by Suryanarayanan and Thirunavukkarasu (2017) with slight modification. 10 surface-disinfected segments were aseptically placed on ciprofloxacin (100 mg L^{-1}) suspended Potato Dextrose agar (PDA) plates (HiMedia Laboratories, Mumbai) and incubated for 45 days at 25^0C temperature. Endomelanconiopsis characterized by black to grey color mycelia emergence from the lichen fragments; was carefully transferred to fresh Malt extract agar (MEA) plates, incubated at 35 ^{0}C temperature. Further the isolates were cultured on Oatmeal agar (OA) and Potato Dextrose agar media for characterization.

DNA isolation and gene Amplification

The total genomic DNA content was isolated using modified 2X CTAB method (Rogers &Bendich 1994) performed PCR for ITS and Tub2 gene using AMPLICON's ready to use Taq DNA polymerase 2x Master Mix RED, in Eppendorf Mastercycler. Using the primer pairs ITS1, ITS4 (White *et al*.1990) and Bt2a, Bt2b (Glass and Donaldson, 1995) The resulting PCR products were sequenced in Applied Biosystems 3730xL analyzer (Barcode Bioscience Private Limited Bengaluru, India). assured the sequence quality by inspecting the chromatograms, subjected to end trimming, ambiguity, and nucleotide composition analysis, created contigs of forward and reverse sequence with Sequencher V5.1 software. The consensus was subjected to BLAST against NCBI depositories for primary identification of the isolate, The sequences generated in this study have been in GenBank (http://www.ncbi.nlm.nih.gov)

Phylogenetic tree construction and analysis

Accessed the reference sequences of ITS, and Tub2 from NCBI GenBank, referring to recent publications (Slipper *et al.* 2013). NCBI retrieved sequences were aligned with Sequences generated in this study by performing MUSCLE sequence alignment in MEGA-X V.32. Model selection for each gene alignments was performed by running JModeltest, the best fit evolutionary models with the Akaike Information Criterion (AIC) was implemented in PAUP v.4. Analyzed genetic

distance for each gene alignments using MEGA-X V.32. Phylogenetic relat-ionships were inferred by reconstructions of individual and concatenated gene-trees. Individual Neighbor-joining and Maximum-likelihood tree were constructed using PAUP v.4. and RAxML 7.4.2 respectively with 1000 bootstrap replications, whereas, concatenated Bayesian inference tree of combined data was constructed in MrBayes-3.2.7-WIN. with 100000 generation MCMC runs (Zhang *et al.* 2013). The Fig Tree V.1.4 application was used to accomplish the rooting and topological depiction of all the resulting trees.

Sample preparation and metabolite extraction

In order to extract secondary metabolites, isolates are first cultured in PDA broth, then performed solvent extraction. Two 3 mm diameter culture plugs were transferred from MEA plates into 300 ml of potato dextrose broth (PDB), which was then incubated at 25±2 °C for 15 days with periodic shaking. To separate the culture's filtrate and mycelial mat, the culture was centrifuged at 800 rpm for five minutes. followed the filtration by triple layered Whatman filter paper no.1. Added ethyl acetate in 10:2 ratio to the extract in separating funnel and mixed well for 10 min, to obtain clear immiscible layers. Separated the upper layer containing compounds, allowed to evaporate excess ethyl acetate. Followed this separation tries for the same culture filtrate. Completely dried extract residues were dissolved in dimethyl sulfoxide (S. D. Fine Chem. Limited) to be used as a stock solution for further use (Nischitha and Shivanna 2021).

Metabolites profiling by OHR LC-MS analysis

The metabolite fraction was subjected to orbitrap nnnhigh resolution or OHR LC–MS analysis (Q-Exactive Plus Biopharma, Thermo Scientific). The compound separation was achieved by using the Syncronis 1.7 microns C18 100 x 2.1 mm (Thermo Scientific). The mobile phase consists of 0.1% formic acid in Milli-Q water (solvent A), methanol (solvent B), and acetonitrile (solvent D) run over 35 min. compounds are detected in quarterly UHPLC with a variable wavelength detector mass photometer attached to Thermo Scientific Xcalibur, V.4.2.28.14. data Acquisition Software. Both negative and positive ionization modes, with mass (m/z) ranges of 50 to 8000 amu, were used for the direct infusion of ions. Resulting data were processed and analyzed in Compound Discoverer 3.2 SP1. analyzed at the Sophisticated Analytical Instrument Facility (SAIF), IIT, Bombay.

Radical scavenging ability by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method

DPPH radicle scavenging assay was performed to asses free radical neutralization potential of the extract, using flat bottom 96-well microtiter plate, according to the method described by Blois (1958) with slight modifications. Different doses of extract and standard antioxidant gallic acid (100.00, 200.00, 400.00, 800.00 and 1000 μg/mL) was added to 100 μL of 0.1mM methanolic DPPH solution in the 96-well plate. All reagents were mixed by pipetting and incubated for 30 min at room temperature under dark conditions. The experiment was carried in triplicates. The % inhibition was estimated by measuring the absorbance at 517 nm with a Multiskan FC Microplate Photometer (Thermo Scientific). Plotted a linear regression graph to get the half maximum effective concentration (EC50).

RESULTS AND DISCUSSION

The *Endomelanconiopsis* colonies appears as white, hyaline, immersed hyphae that gradually turn olivaceous in the center with irregularly shaped concentric rings. At optimum growth temperature of 30–35 °C the average colony diameter on PDA and OA media was 6±0.5 cm and 2±0.5 cm respectively. After 10 days, the aerial mycelium becomes dark olivaceous grey to black in color with dark dematiaceous hyphae (Rojas *et al.* 2008). Since, no observable conidiophore and conidiospores are produced under both PDA and OA agar media the isolate has been considered as mycelial sterile morphotype.

Phylogenetic analysis and species recognition

The query sequence shows the highest percent similarity to the species *E. endophytica* after

Table 1 : Detail of metabolite profile of ethyl actetate extract of *E.endophytica*

Name of the compound	Formula	Molecular weight	retention time	Nature of the compound
NP-019811	C6 H7 N O2	125.0477	1.36	5 hydroxy-2-pyridinemethanol
Hypoxanthine	C5 H4 N4 O	136.0385	2.29	Endogenous metabolite
Pantothenic acid	C9 H17 N O5	219.1107	2.73	Vitamin
Veratrole	C8 H10 O2	138.0681	5.3	Isomer of dimethoxybenzene an Natura insect attractant
NP-007065	C8 H10 O3	154.063	7.02	Natural compound
N-Acetylvaline	C7 H13 N O3	159.0895	8.369	Endogenous metabolite
Acetanisole	C9 H10 O2	150.0681	9.46	Aromatic compound
N-(2,4-Dimethylphenyl)formamide	C9 H11 N O	149.0841	10.16	Environmental transformed product of insecticide
3-Hydroxypicolinic acid	C6 H5 N O3	139.0269	10.658	No significant records
(2E)-3-(3,4-dimethoxyphenyl)prop-2-enoic acid	C11 H12 O4	208.0736	11.27	Propanoic acid derivative
Tenuazonic acid	C10 H15 N O3	197.1052	12.186	Mycotoxin
NP-011228	C34 H59 N O14	705.3936	13.47	Fumonisin mycotoxin
Butylparaben	C11 H14 O3	194.0943	14.292	Paraben
Sakuranetin	C16 H14 O5	286.0841	16.52	flavanone
(2S,3S,4R,5R)-4-hydroxy-2,5-bis(hydroxymethyl)-2-{[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}oxolan-3-yl (2E) -3-(4-hydroxy-3,5- dimethoxyphenyl)prop-2-enoate	C23 H32 O15	548.1741	18.35	Natural product from plant and some microorganism
NP-006255	C17 H26 O4	294.1831	20.1	Natural compound

nucleotide BLAST. In light of this, both genes are subjected to phylogenetic analysis using both distance-based (Neighbor joining) and coalescent-based (Maximum-likelihood and Bayesian) phylogenetic analysis. The best-fitting evolutionary models for both *ITS* and *Tub2* was TIM3ef+G4 under the AICc criterion. Divergence values at different hierarchical levels for the *ITS* and *Tub2* genes are: intraspecies genetic distances of 0.00096 and 0.0291, interspecies genetic distances of 0.02 and 0.06, and intergeneric distances of 0.145 and 0.209, respectively. Further beta tubulin gene possess more parsimony informative site than *ITS*. The query sequence forms a reciprocally monophyletic clade for the species *E. endophytica*, with bootstrap support greater than 70% in trees generated using Neighbour-joining, maximum likelihood (Fig.1A-D), and > 95% posterior probability value for the concatenated Bayesian inference tree (Fig.2).

Metabolites profiling extraction and screening

The OHR-LC–MS is highly sensitive and has yielded the separation of a wide range of compounds. From the ethyl acetate extract of *E. endophytica* about 20 and 399 compound were separated and annotated in positive and negative-ionization modes respectively. Compounds showing well-established peaks were considered, identified and their properties have been recognized as per the literature search (Table1). The ion chromatograms (Fig.3) contain prominent peaks for 16 compounds. Among them, compounds 11 (RT value 12.186) and 12 (RT 13.4) were detected in both positive and negative ion modes and identified as tenuazonic and NP-011228, respectively. Tenuazonic acid is a natural tetrameric acid and mycotoxin produced by *Alternaria alternata*,whereas NP-011228 is natural product structurally similar to Fumonisin, a group of mycotoxins produced by *Fusarium*

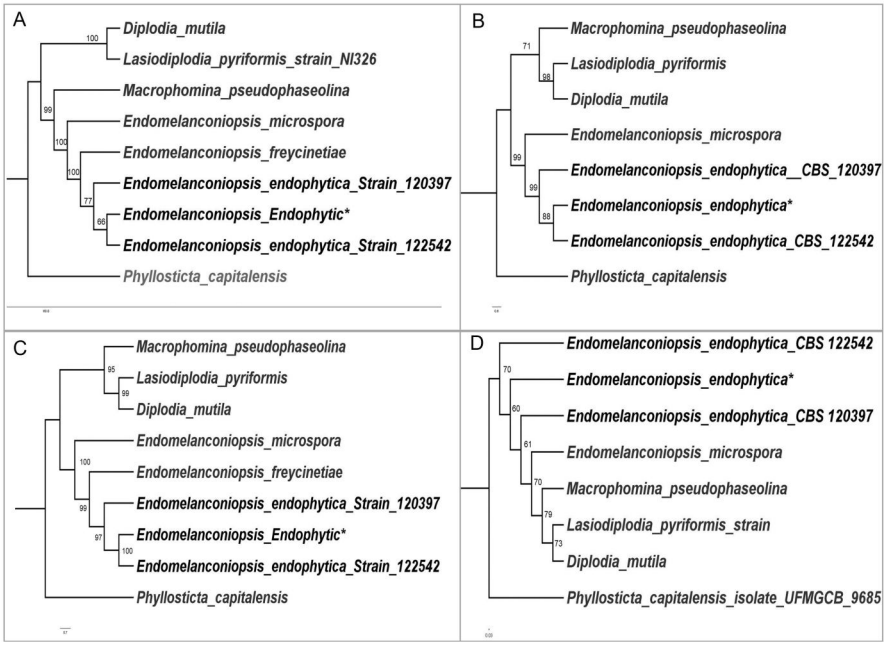

Fig.1(A-D): Neighbor joining and Maximum likelihood 50% majority rule consensus tree, with 1000 bootstrap replicates, all the tree rooted at *Phyllosticta cacpitalensis*.; A. ITS Neighbor joining, B. beta- tubulin Neighbor joining tree: C. ITS Maximum likelihood tree, D. Beta-tubulin maximum likelihood tree (Length of the nodes reciprocal to the divergence rate)

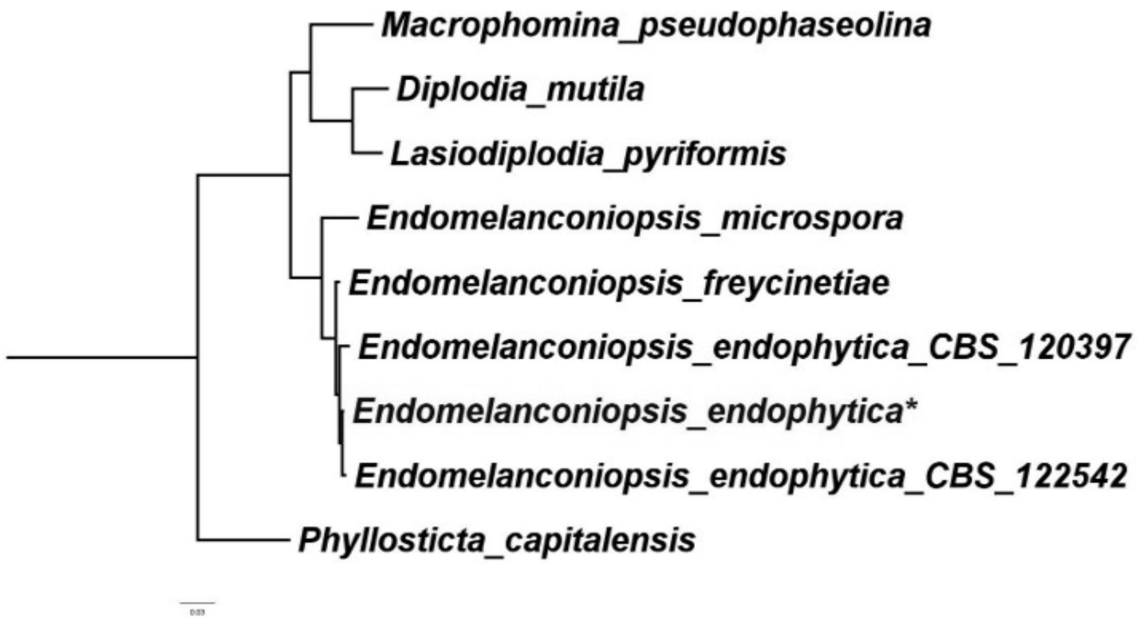

Fig. 2: Bayesian inference tree based on the combined ITS and beta-tubulin sequences. With MCMC runs for 10000 generation Outgroup: Phyllostictacapitalensis. nodes labeled with posterior probability percentage

molds (Kaur *et al*. 2023). Compounds 1–10, 13, 15, and 16 are natural products and endogenous metabolites known to be produced by several fungi and other organisms. Compound 14 (RT: 16.52) is identified as Sakuranetin, a phytoalexin known to be produced in response to UV stress in several plant-microbe interactions.

DPPH radicle scavenging activity (total antioxidant potential)

Spectrophotometric evaluation of the radical scavenging potential is ability of compounds to react with the stable 2,2-Diphenyl-1-Picrylhydrazyl Radical (Csepregi *et al*. 2016).

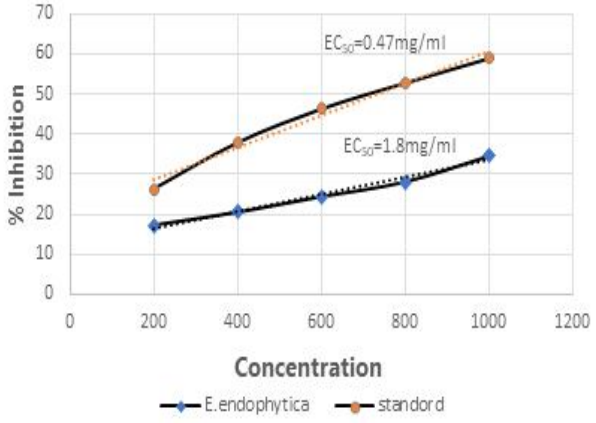

Fig. 4 : Scattered plot of concentration v/s inhibition rate of E. endophytica and standard (Gallic acid) EC50 concentration 1.4 mg/ml and 0.47 mg/ml

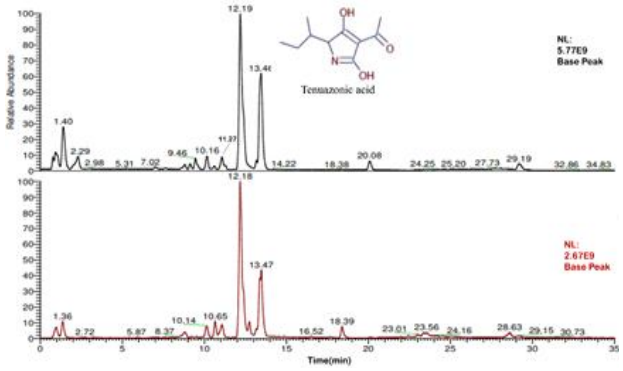

Fig.3: OHR LCMS chromatogram of ELF Endomelanconiopsisendophytica included those with prominent peaks and high retention values as well as those with insignificant peaks represented in the chromatogram

Radical scavenging ability of the extract was expressed in terms of EC_{50} value. Extracts of *E. endophytica* successfully scavenge DPPH radicles, with around 1.8 mg/ml EC_{50} concentration (Fig.4). Sakuranetin a methoxy flavanone present in the extract may associate with radicle scavenging potential of the extract (Alam *et al.* 2017).

Although the modern polyphasic approach to fungal identification deals with the morphology, molecular, and chemical profiles of the organism to delimit species boundaries, choosing this strategy is challenging since most of the known species of today are described based on morphology and lack chemical repositories to compare. So, the identification of non-sporulating sterile groups is remains cornered. Utilising a sequence based phylogenetic species delimitation tool is considered the best method to delimit mycelia-sterile fungi and fungi that show taxonomically insignificant features at present. (Maharachchikumbura *et al.* 2021). Endomelanconium-like fungi are exclusive endophytes of angiosperm plants (Dissanayake *et al.* 2016; Gagana and Shivanna, 2020) and belong to the order Botryosphaeriales. *E. endophytica* is a newly described anamorphic species in the family Endomelanconiaceae, which comprises single genera and three species (Azuddin *et al,* 2021). The existence of *E. endophytica* in lichen thallus has been reported recently (Maduranga *et al.* 2018; Weerasinghe *et al.*2021). Since they frequently lack sporulating structure, the use of genetic information is crucial for identification.

Further, this fungus is known to produce an array of secondary metabolites with great bioactive potential and industrial importance. Sun *et al*. (2016) reported two new Endomeketal and Xyloketal compounds in the ethyl acetate extract of *E. endophytica* isolated from *Ficus hirta* which are analogous to Alboatrin, *O*-methylalboatrin, and Methyl-xyloketal B and possesses cytotoxic potential suggests its potential use in cancer treatment or drug development (Sun *et al.* 2016; Parambayi*et al.* 2020; Matias *et al,* 2022). In this study, *E. endophytica* isolated from the lichen produces Tanazonic acid, and Fumonisin mycotoxin, along with other minor compounds have potential to neutralize DPPH radicles. Indicating that, these metabolites may play a role in protecting the lichen from environmental stressors and enhancing its survival. These findings highlight the diverse chemical composition of *E. endophytica* and expands our knowledge about the secondary metabolite of *E. endophytica*. Further studies are required to investigate the potential ecological implications of these mycotoxins and their role in the overall fitness of E. endophytica. Additionally, exploring the specific mechanisms by which these metabolites contribute to stress tolerance in lichen thalli could provide valuable insights into their adaptive strategies.

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