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Didymocrea leucaenae: A new record to Indian mycoflora

Rashmi Dubey

Botanical Survey of India, Western Regional Centre, Pune, Maharashtra, India
Email: dr.rashmidubey@gmail.com

Abstract
During a field survey of Sindhudurg district (Maharashtra, India), undertaken as a part of studying diversity of litter fungi of Northern Western Ghats of India, a species of *Didymocrea* was collected. Based on morphological and molecular data, the species was identified as *D. leucaenae.* From consultation of pertinent literature, it is found to be a new record to Indian mycoflora.

Keywords: Asexual morph, ITS, Litter, Sporodochia, Western Ghats

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Introduction

Didymocrea was established by Kowalski (1965) with *D. sadasivanii* [as '*sadasavanii*'] (Basionym - *Didymosphaeria sadasavanii* T.K.R. Reddy 1961) as com. nov. because of the presence of unique morphotaxonomic characters, such as 2-celled ascospores, unitunicate asci, pseudoparaphyse, subhymenial ascal development and absence of spermatia, which do not match with common features of *Didymosphaeria*. Aptroot (1995) suggested its placement under *Zopfiaceae* as it resembles with *Zopfia* in producing large ascocarps in culture. Kruys et al. (2006) confirmed *Didymocrea* lineage within this family, but Tanaka et al. (2015) placed it under *Didymospheriaceae*, based on multi-loci analysis of *SSU*, *LSU* and *tef1* gene regions. So far, only two species are established under *Didymocrea*. After *D. sadasivanii*, *D. leucaenae* was placed under the Didymosphaeriaceae (Jayasiri et al. 2019).

During a project on studying diversity of litter fungi of Northern Western Ghats of India, an interesting Dothideomycetous fungus was collected from the Amboli Ghats of Maharashtra, India (15°57'19.8"N and 73°59'31.7"E). Detailed morphological and molecular studies revealed that the new collection is *Didymocrea leucaenae*. Consultation of pertinent literature revealed that this is the first report of *D. leucaenae* from India.

Materials and methods

Fungal isolation and morphological characterization

The wood samples infested with the fungus were collected from Amboli, Sindhudurg district, Maharashtra, India. They were first observed using Nikon stereomicroscope SMZ745 (Nikon, Japan). One pure fungal culture SM14 (NFCCI 4842) was isolated from this sample and grown on potato dextrose agar (PDA) medium (Dextrose 20 g/l, Agar 20 g/l, 200 g/l infusion from

potatoes, pH 5.6±0.2) by direct culture method (Sanders 2012). Microscopic details were observed in lactophenol-cotton blue; fungal structures were measured, and photographs were taken using OLYMPUS microscope CX41 (aided with digital camera) (Olympus, Japan). Scanning electron microscopic images were taken using Zeiss Scanning Electron Microscope Model EVO 18-12 -97 (Zeiss, Germany) according to the manufacturer's protocol. Living culture (SM-14) was deposited at the National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute (ARI), Pune, India.

DNA extraction, PCR amplification, and DNA sequencing

Pure fungal isolate was grown on PDA medium for 15 days in dark at 25 °C. Genomic DNA was extracted from the growing mycelia using HiPurA Fungal DNA Purification Kit (HiMedia, India) as per manufacturer's instructions. Primer pair ITS4 and ITS5 was used to amplify the 5.8S rRNA gene and flanking internal transcribed spacer regions (ITS) (White et al. 1990) using SimpliAmp Thermal cycler (Applied Biosystems, USA). PCR was performed in a 40 µl reaction mixture containing 20 µl 2×Hi-Chrom PCR Mastermix, 2 µl 10 pmol primer, 16 µl 5× GC enhancer, 2 µl H_2O (Sterile Ultra-Pure Water, Sigma) and 2 µl template DNA. The thermal cycling program involved 5 minutes of initial denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 56 °C for 45 seconds and extension at 72 °C for 45 seconds, followed by a final extension at 72 °C for 10 minutes. During the amplification process a negative control using sterilized distilled water instead of template DNA was included. The amplified PCR products were examined by electrophoresis at 65 V in 0.8 % (W/V) agarose gel in 1X TAE (Tris-acetate-EDTA) buffer (0.4 M Tris, 10 mM EDTA, 50 mM NaOAc, pH 7.8) and visualized under UV light using E-Gel Doc Molecular Imager (Thermo Fischer Scientific, UK) after staining with ethidium bromide (0.5 µg ml^{-1}). The amplified PCR products were purified with HiPurA PCR Product Purification Kit (HiMedia, India) as per manufacturer's instructions. The purified PCR products were reassessed using agarose gel electrophoresis and submitted for sequencing to Avanira Biotech Pvt. Limited, Pune, India.

Phylogenetic analysis

The ITS sequence was used to confirm the identification of the fungal isolate SM14 (NFCCI 4842). Resulting chromatograms were checked with BioEdit v.5 (Hall 1999) to confirm sequence quality. The sequence chromatograms were analyzed using MegaBLAST search algorithm and related reference sequences of already known taxa of *Didymocrea* were retrieved from National Center for Biotechnology Information (NCBI) for phylogenetic analysis. *Pleospora herbarum* KP334719 was selected as an outgroup taxon (Jayasiri et al. 2019). The sequence generated in this study was aligned with sequences downloaded from GenBank using Clustal W2 and manually adjusted using BioEdit v.5 wherever necessary. Ambiguous regions in the alignment were excluded from further analyses, and gaps were treated as missing data. The concatenated file contained sequence data of 18 taxa. Model K2+G (Kimura 2 parameter + Gamma distribution) was found to be the best-fit model of 32 models tested and was chosen on the basis of the Bayesian information criterion (BIC). The phylogeny was inferred by using the maximum likelihood method. Tree branches were tested based on 1000 ultrafast bootstrap (UFBoot) support replicates as well as with SH-like approximate likelihood ratio test (SH-like aLRT) with 1000 replicates. The newly generated 593 bp sequence from this study was deposited in NCBI GenBank.

Results

Phylogenetic study

Phylogenetic analysis using the ITS region (Fig. 1) showed the similarities between the study sample *Didymocrea leucaenae* NFCCI-4842, *D. leucaenae* MFLUCC 17-0896 [NR_164298] and *D. leucaenae* [MK347721]. Based on a MegaBLAST search on NCBI GenBank nucleotide database, the closest hit using the ITS gene sequence was with *D. leucaenae* MFLUCC 17-0896 (the type material) showing 100 % (567/567) identity and no gaps (0 %).

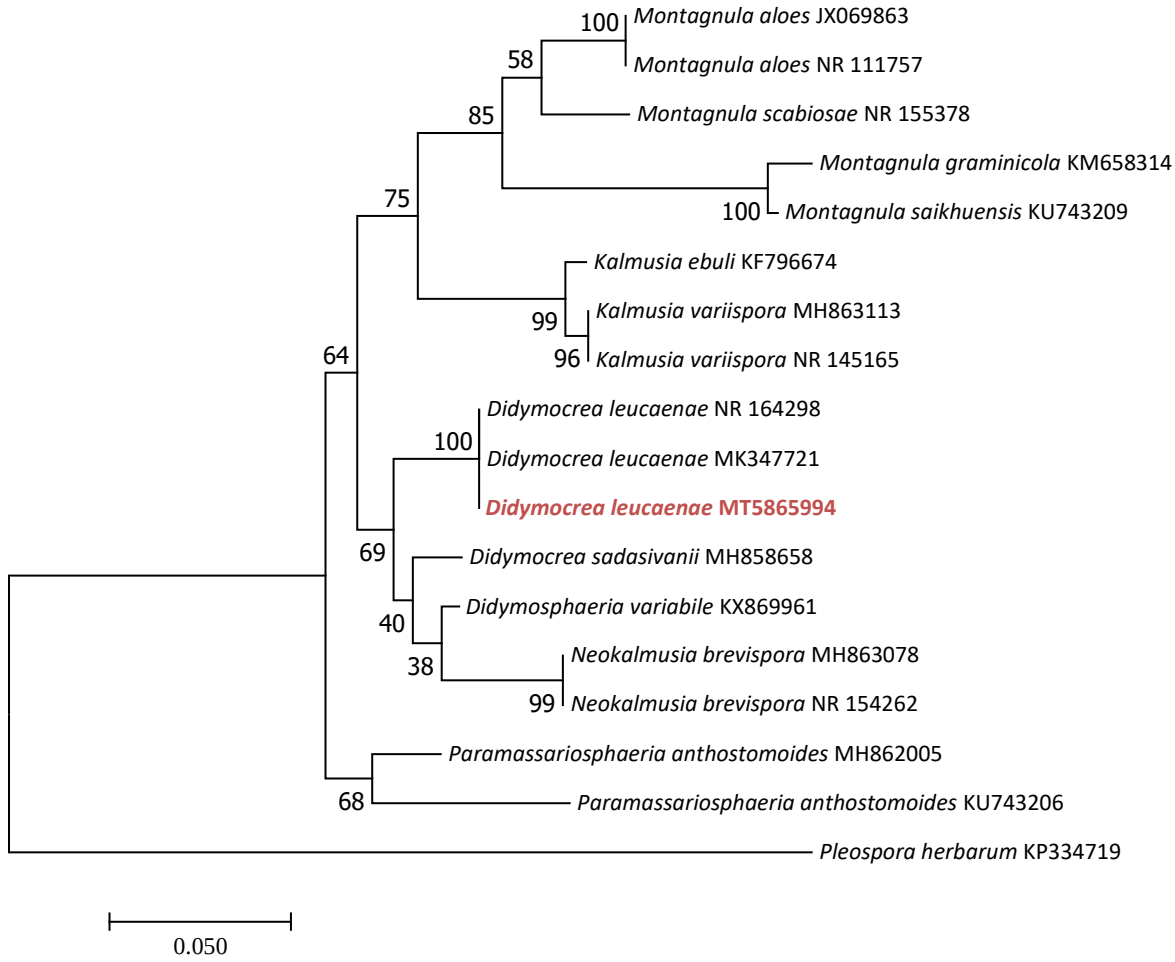

Figure 1. Simplified phylogram generated from Maximum Likelihood analysis based on ITS nucleotide sequences of 18 taxa.

The MEGA output file read as follows: “The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [Kimura 1980]. The tree with the highest log likelihood (-1521.96) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+G, parameter = 0.0500)). The tree is drawn to scale, with branch lengths measured in the

number of substitutions per site. The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 387 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [Kumar et al. 2016.]"

Taxonomy
Didymocrea leucaenae Jayasiri, E.B.G. Jones & K.D. Hyde, Mycosphere 10(1):69 (2019) Figure 2

Sexual morph: Not observed.

Asexual morph – Hyphomycetous. *Sporodochia* on host substrate pulvinate, punctiform, granular, black and shining. *Mycelium* immersed in the substrate, composed of branched, septate, smooth, subhyaline to pale brown, hyphae. *Conidiophores* 29–42 × 2.4–3.5 μm ($\bar{x}$ = 35 × 3 μm, n=10) micronematous or semi-macronematous, mononematous, fasciculate, simple or sometimes branched. *Conidiogenous cells* 11–15 × 11–15 μm ($\bar{x}$ = 12.97 × 13 μm, n= 10) integrated, holoblastic, terminal, determinate. *Conidia* 14–16 × 15–19 μm ($\bar{x}$ = 15.2 x 15.6, n=15) acrogenous, solitary, reddish brown, fusiform to obclavate in lateral view, broadly ellipsoidal to obovoid in surface view, flattened, muriform, 2–3 rows of transverse septa, constricted at the septa, dark and thickly banded at the septa, canals in the septa obscured by dark pigmentation in face view and visible inside view, thin and smooth-walled. The number of cells per conidium varied from 7 to 9. Basal cell subhyaline to pale brown, cuneiform.

Culture characteristics – Conidia germinating on PDA within 3–4 days. Germtubes produced at the end of conidia. Colony circular, edge entire, raised, fluffy, dense, convex or dome-shaped with white surface, to superficial at the center, flat or effuse at the edge, greyish brown from above, dark brown from below.

Material examined – India, Maharashtra, Sindhudurg District, Amboli Ghat, on decaying bark of unidentified plant, 14 Feb. 2018, Rashmi Dubey, BSI-209202, living culture NFCCI 4842. GenBank number – ITS: MT586599.

Notes: The isolate (NFCCI 4842, GenBank Accession no. ITS: MT586599) from this study clustered with the ex-type of *Didymocrea leucaenae* MFLUCC 17-0896 (Fig. 1), which is the second species reported in *Didymocrea* (Jayasiri et al. 2019). However, *D. sadasivanii*, the type species was reported as a sexual morph. The strain reported in this study (NFCCI-4842) is an asexual morph. It is, therefore, not possible to make a morphological comparison of the two species. A comparison of the ITS sequences of the three strains viz., MFLUCC 17-0896 (NR_164298), MK347721.1 (C150) and MT586599 reveals 100 % similarity with *D. leucaenae*. The review of literature (Index Fungorum, 2021) reveals that earlier *D. sadasivanii* was reported from India (Reddy 1961) and *D. leucaenae* has been reported from Thailand (Jayasiri et al. 2019). Accordingly, this is the first report of *D. leucaenae* from India and is a new addition to the mycoflora of India.

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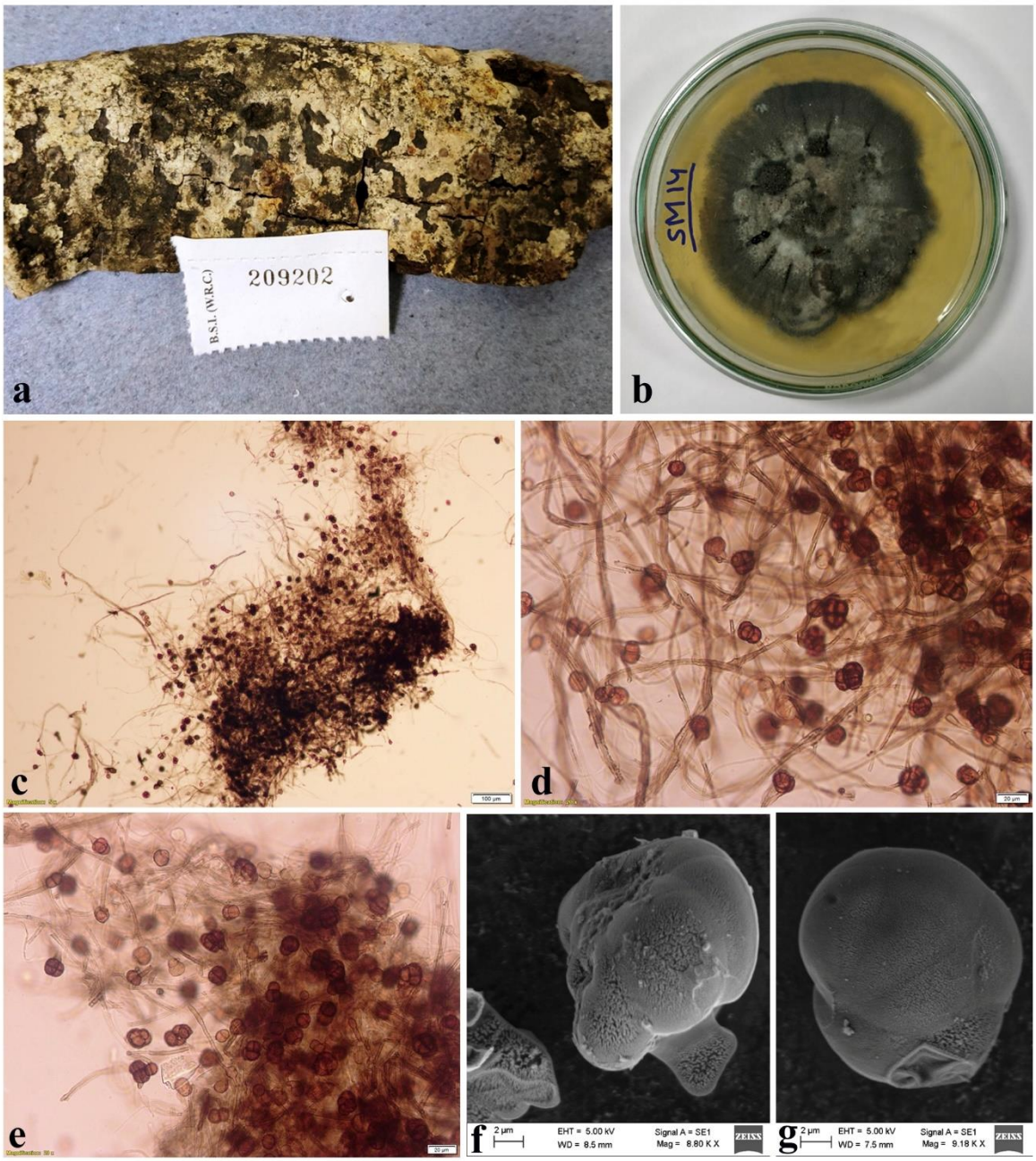

Figure 2. *Didymocrea leucaenae* (MT586599). a Colonies on host surface. b Colonies on PDA medium. c Hyphal coils and anastomosing hyphae with conidiophores and conidia. d & e conidia f-g Scanning Electron microscopic images. (Bars c = 100 µm; d, & e = 20 µm.)

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Statement on conflict of interest-
The author declares no competing interests.

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