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Phylogeny and taxonomy of a novel species of *Pseudocercospora* from India

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Abstract: A new species of *Pseudocercospora* is described causing leaf spot disease on *Crotalaria assamica* from India. The identity of isolate was based on asexual morphs, cultural characteristics and phylogenetic analyses of partial nuclear ribosomal 28S large subunit and complete internal transcribed spacer rDNA sequence data with high statistical support value. Morphologically this species has characters similar to allied species in genus *Pseudocercospora* but differs in having catenate and branched conidia. Phylogenetic analysis using LSU and ITS reveals it a new species of *Pseudocercospora* (Capnodiales, Mycosphaerellaceae).

Key words: *Pseudocercospora,* morphology, molecular phylogeny, Mycosphaerellaceae, taxonomy

1. Introduction

Crotalaria assamica Benth., (Family: Fabaceae) commonly known as 'Indian rattle box' is a perennial shrub growing wild through plains of Assam, Nagaland and Manipur and other parts of India. It is harvested from wild for local medicinal uses especially for treatment of snake bite and bladder stone (Jain 1991). During survey on cercosporoid fungi infecting medicinal plants during 2018–2019, a new pathogen was discovered infecting *Crotalaria assamica*, which on detailed morphological and phylogenetic analysis was found to be a new species of *Pseudocercospora*.

Pseudocercospora was established by Spegazini (1910) with *P. vitis* (Lev.) Speg. as the type species, a foliar pathogen of grape. Since the time of establishment, the generic circumscriptions have been revised many times on morphological bases (Deighton, 1976; Braun 1995) and on molecular phylogenetic study bases (Crous et al., 2000; Crous and Braun, 2003). Molecular phylogenetic study of DNA sequence of LSU gene of genera of Mycosphaerellaceae revealed that *Pseudocercospora* species cluster in two well defined clades i) *Pseudocercospora* senu stricto, the species clustering with type species *P. vitis* and ii) *Pseudocercospora* sensu lato, species with similar characters (Crous et al., 2009). Crous et al. (2013a) described the generic characters of the genus clustering in *Pseudocercospora* s. str. clade as conidiophores solitary, fasciculate, synnematal, or arranged in sporodochia, conidia colored, scars unthickened or slightly thickened. *Pseudocercospora* was believed to be an asexual morph of *Mycosphaerella* but at present it has been established as genus having *Mycosphaerella*-like sexual morphs (Hyde et al., 2013; Kirk et al., 2013; Wijayawardene et al., 2017). A new pathogen infecting leaves of *Pistachio* was discovered having pycnidial conidioma and pigmented conidia clustering *Pseudocercospora* s. str., suggesting, the present generic circumscription could still change (Crous et al., 2013b).

Pseudocercospora was considered to be host specific but later it was observed that few species occur on different host belonging to single plant family (Deighton, 1976). Therefore, to resolve host specificity in *Pseudocercospora* Crous et al. (2013a) generated a multigene analysis and concluded that majority of these species were host specific. Recently molecular studies performed on *Pseudocercospora griseola* on *Phaseolous vulgaris* has evaluated the diversity and confirmed the coevolution of host and parasite. (Serrato-Diaz et al., 2020; Chilagane et al., 2016). Three species of *Pseudocrcospora* have been earlier reported on the host genus from India. *Ps. luxurians* (Kar and Mandal) Deighton 1976 occuring on *Crotaraia albida*, *C. sericea* and *Crotalaria* sp. was reported from Darjeeling, West Bengal and Gorakhpur, Uttar Pradesh. *Ps. crotalariana* (Pavagi and Singh) Deighton 1976 has been reported on *Crotalaria medicagena* from Varanasi, U.P. and *Crotalaria*

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sp. from Hydrabad, Andhra Pradesh. *Ps. cotizensis* (Muller and Chupp) Deighton 1976 has been reported on many species of the host genus and is widely distributed throughout India (Kamal, 2010). Present novel species has been collected from Varanasi, Uttar Pradesh.

Consolidated species concept given by Quaedvlieg et al. (2014) is considered more authentic means to describe new species by workers which include morphology, culture characteristic, multigene analysis and ecology (Bakshi et al., 2014) and all these aspects are included in the study.

2. Materials and methods

2.1. Isolates and morphology

The specimens with the disease symptoms on leaves, infected fruits and stem were collected in paper bags form collection site, and were brought to the laboratory**.** Symptoms were minutely observed and located on the samples. A scrape was taken from infection spot and mounted in lactic acid cotton blue and examined using an Olympus CH20i microscope. Thirty measurements of conidia and other fungal structure were taken for taxonomic diagnosis. Histological sections were prepared to observe the relationship of host plant and fungus. Scanning electron microscope photographs were taken by ZEISS-EVO-18-Research. Culture was prepared by single spore isolation. Spores were picked from the leaf spot and spore suspension was prepared. The spore suspension was spread over a thin film of agar in petri plates. Conidia were observed periodically and germinating conidia were transferred to the culture media (Choi et al., 1999). Subcultures were prepared on potato dextrose agar and incubated for three weeks at temperature 27 °C and colony diameter was measured.

The holotype specimen was deposited in Ajrekar Mycological Herbarium (AMH), MACS' Agharkar Research Institute, Pune, India under the accession number AMH: 10041. The ex-type culture was deposited in National Fungal Culture Collection (NFCCI) under accession no. NFCCI: 4441. The taxonomic novelties of the taxon were deposited in MycoBank[1] (Crous et al., 2004).

2.2. Phylogeny

DNA extraction was done according to Amir et al. (2015). Mycelia (2 mL) from seven day old culture was taken and crushed in lysis buffer (100 mM Tris HCl [pH8.0], 50 mM EDTA, 3% SDS) using sterile glass bead (425–600 µM, Sigma-Aldrich, St. Louis, MO, USA). Homogenization was done twice for 60 s at 6 M/S in a FastPrep-24 tissue homogenizer (MP Biomedicals, Irvine, CA, USA). The homogenate was centrifuged for 10 min at 13000 rpm and supernatant was transferred to a new microcentrifuge tube. 2 µl of RNAse A (10 mg/mL) was added to the supernatant and incubated at 37 °C for 15 min. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 13000 rpm for 10 min. The upper aqueous layer was taken and DNA was precipitated with 100% ethanol. The DNA pellet was washed with 70% ethanol and centrifuged at 12000 rpm for 5 min. The DNA pellet was air dried and dissolved in 1× TE buffer.

The ITS regions of rDNA were amplified by PCR using the primers ITS4 and ITS5 (White et al., 1990). The LSU regions of rDNA were amplified by PCR using the primers LROR and LR7 (Vilgalys and Hester, 1990). The PCR products were purified with Axygen PCR cleanup kit (Axygen Scientific Inc., Union City, CA, USA). Cycling of the PCR products was accomplished with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Beverly, MA, USA), using the PCR primers. The cycle sequencing products were run on an ABI Avant 3100 automated DNA sequencer (Applied Biosystems). DNA sequences were obtained manually and editing was done using Chromas Lite v. 2.01[2]. Obtained sequences were submitted to NCBI.

Sequences of the two gene markers including those of type strains were taken from gene bank and were aligned with muscle and two gene sequences were then concatenated using FABOX v.1.41. Molecular phylogenetic analysis was done by maximum likelihood method and Kimura 2-parameter, GTR+1 as the best model with 1000 bootstrap replications. The Bayesian analysis was performed using MrBayes v.3.2.6 (Ronquist et al., 2012). The analysis was performed for 10,000,000 generations till the standard deviation of split frequency was below 0.01. Burning tree samples of 25% was discarded. Presented tree was obtained with Bayesian approach. Tree reconstruction, visualization and editing were done with FIGTREE v1.4.4, TreeGraph_2.14.0 and MEGA 5.2

3. Results

3.1. Taxonomy

Pseudocercospora crotalariigena Archana Singh, Paras Nath Singh and Nawal Kishore Dubey sp.nov. Figures 1–5.

MycoBank: MB833805.

Etymology: The species epithet "*crotalariigena*" refers to *Crotalaria*, the host genus from which this fungus was isolated.

Culture characteristics: Conidia germinating on potato dextrose agar (PDA). Colonies very slow growing, velvety, grayish brown, reaching 2–5 mm diam. In 28 days at 27 ˚C, margin circular to irregular, reverse blackish brown, raising centrally with dense cottony mycelium and hard texture. Asexual and sexual spores were not formed.

[1] MycoBank (2021). MycoBank Database [online]. Website http://www.MycoBank.org [accessed 20 December 2019].

[2] Technelysium (2021). Chromas [online]. Website http:/www.technelysium.com.au [accessed 15 December 2019].

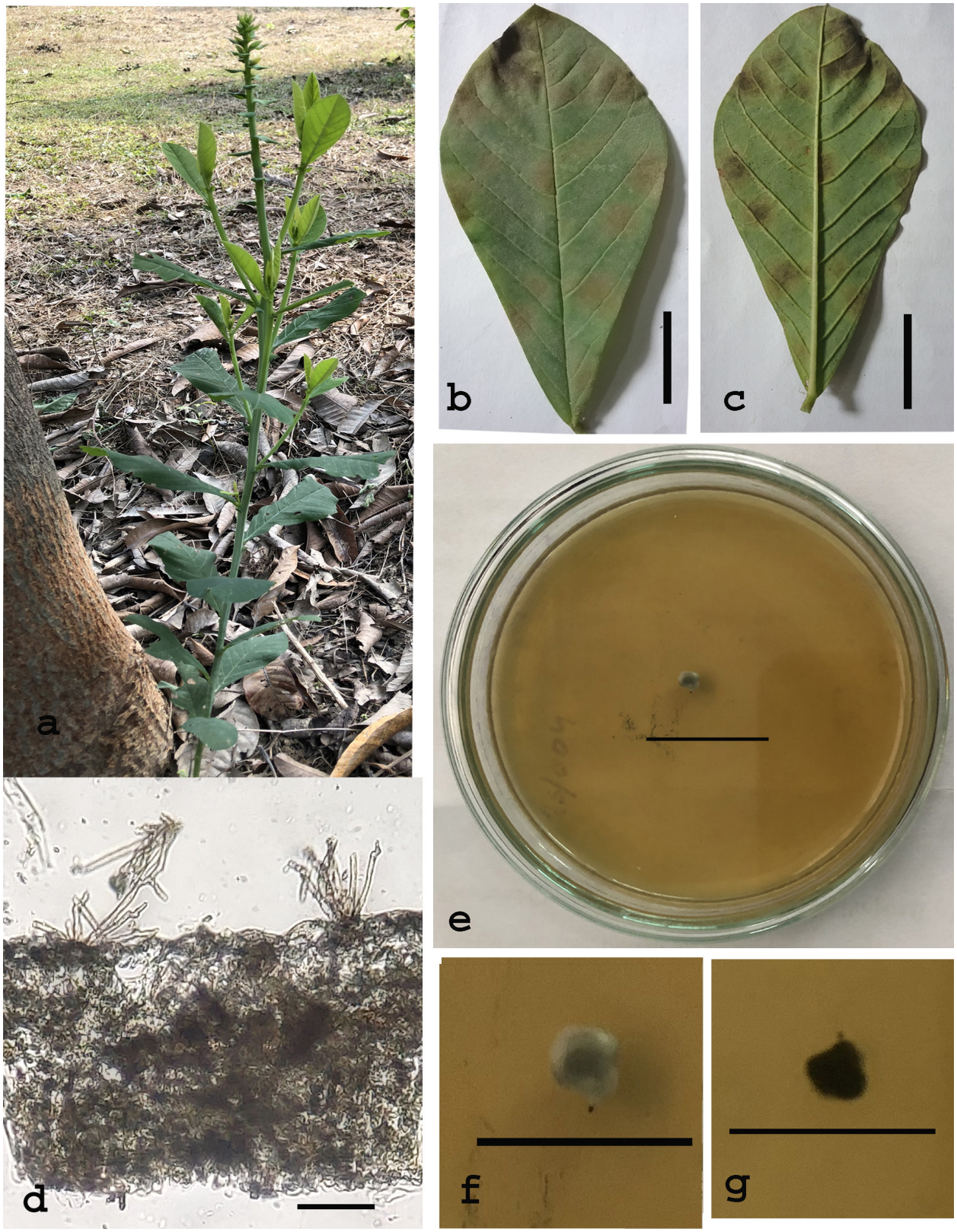

Figure 1. *Pseudocercospora crotalariigena* sp. nov. a. Habitat leaves of *Crotalaria assamica* Benth; b. Symptoms on leaf dorsal side; c. Symptoms on leaf ventral side; d. T.S. of infected leaf showing fascicles of conidiophores arising through stomata; e–f. Colonies on PDA after 30 days, front view; g. Reverse view of colony. Scale bar: b–c= 20 mm, d = 50 µm, e = 20 µm, f–g = 10 µm.

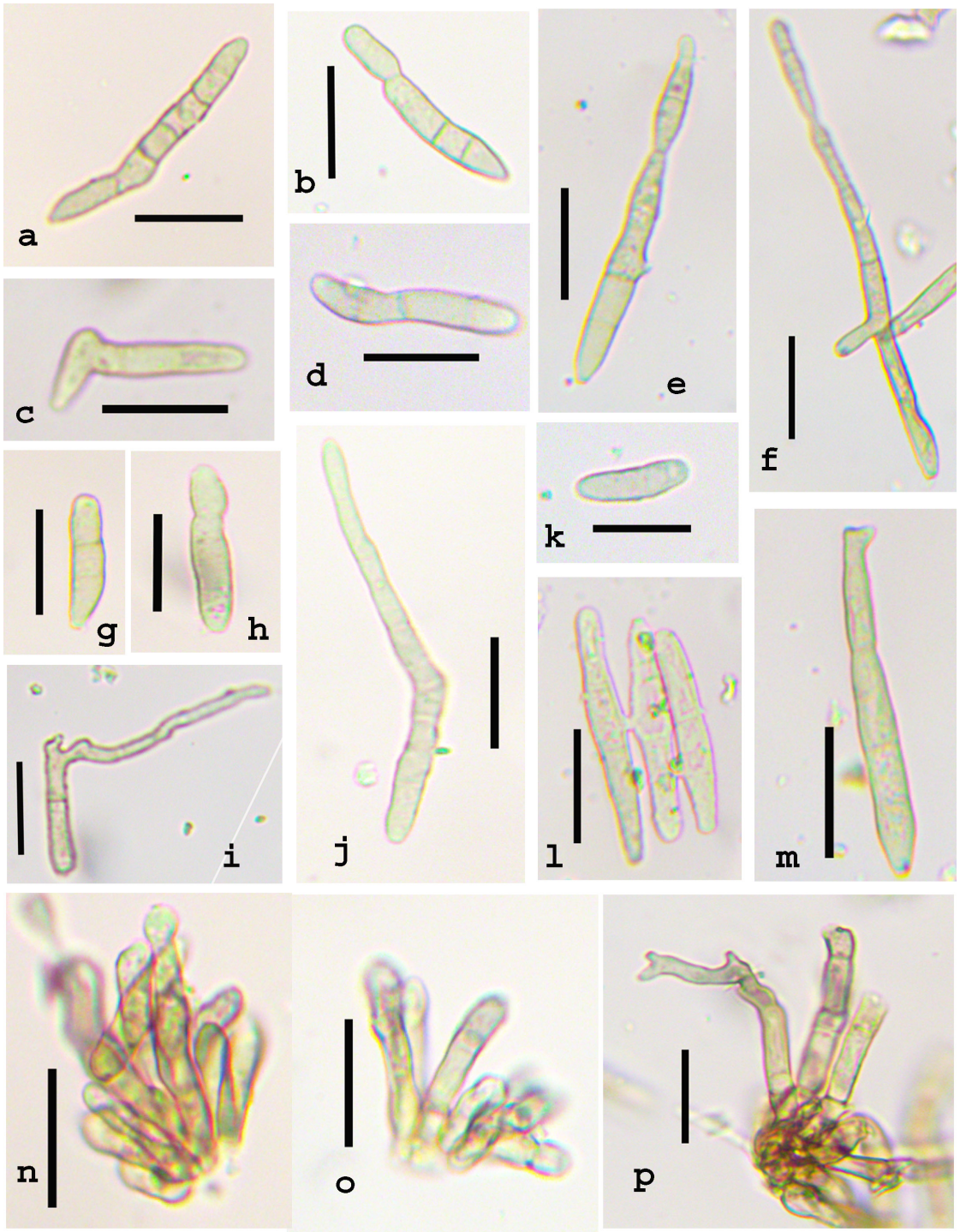

Figure 2. *Pseudocercospora crotalariigena* sp. nov. a–d. Conidia with narrowly truncate base; e–f. Catenate and branched conidia; g–h. Conidia showing obtuse tip and broad truncate base; i. A Germinating conidium; j. A curved long conidium; k. A broadly fusoid conidium; l. Conjugating conidia; m. An obclavate conidium; n–o. Fascicle of conidiophores; p. Fascicle of conidiophores and a conidiophore bearing cynlidrical to dentate terminal and lateral conidiogenous cells. Scale bar: a–p = 20 µm.

Leaf spots indistinct with yellowish circular to subcircular area on upper leaf surface and dark greyish brown on lower surface, later coalescing to form irregular patches. Caespituli chiefly hypophyllous but amphiphyllous later. Circular to subcircular 8–26 mm in diam, brownish grey with yellow margin, velvety,

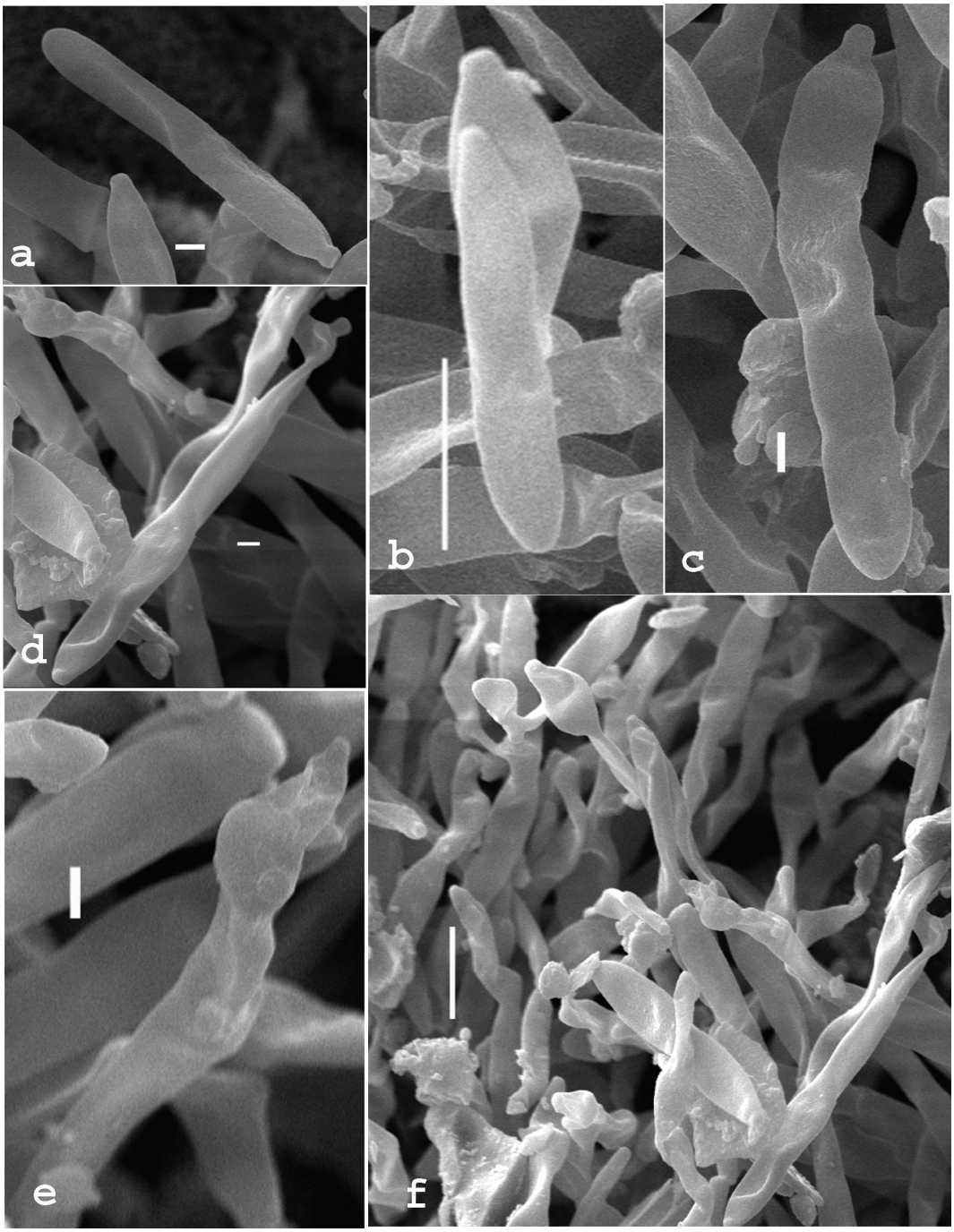

Figure 3. *Pseudocercospora crotalariigena* SEM photomicrographs. a–d. Conidia; e. Conidiophores with denticles; f. Conidiophores with cylindrical and dentate conidigenous cells and numerous conidia. Scale bar: a, c, d, e = 2 µm and b and f = 10 µm.

erumpent, discrete. *Mycelium* internal branched. Stromata substomatal, pseudoparenchymatous, light brown to brown, globular, feebly developed up to 22 µm diam. Conidiophores macronematous, mononematous, fasciculate in divergent fascicle (up to 17 in numbers) smooth walled, 0–5 septate, unbranched to branched, straight to curved, geniculate, subcylindrical, olivaceous brown to brown, paler towards the tip, smooth walled, 17.5–66.5 × 2.0–3.0 µm. Conidiogenous cells integrated, terminal to intercalary, denticulate, polyblastic, pale olivaceous, smooth walled, geniculate, scar unthickened. Conidia unbranched to branched, solitary or in chains,

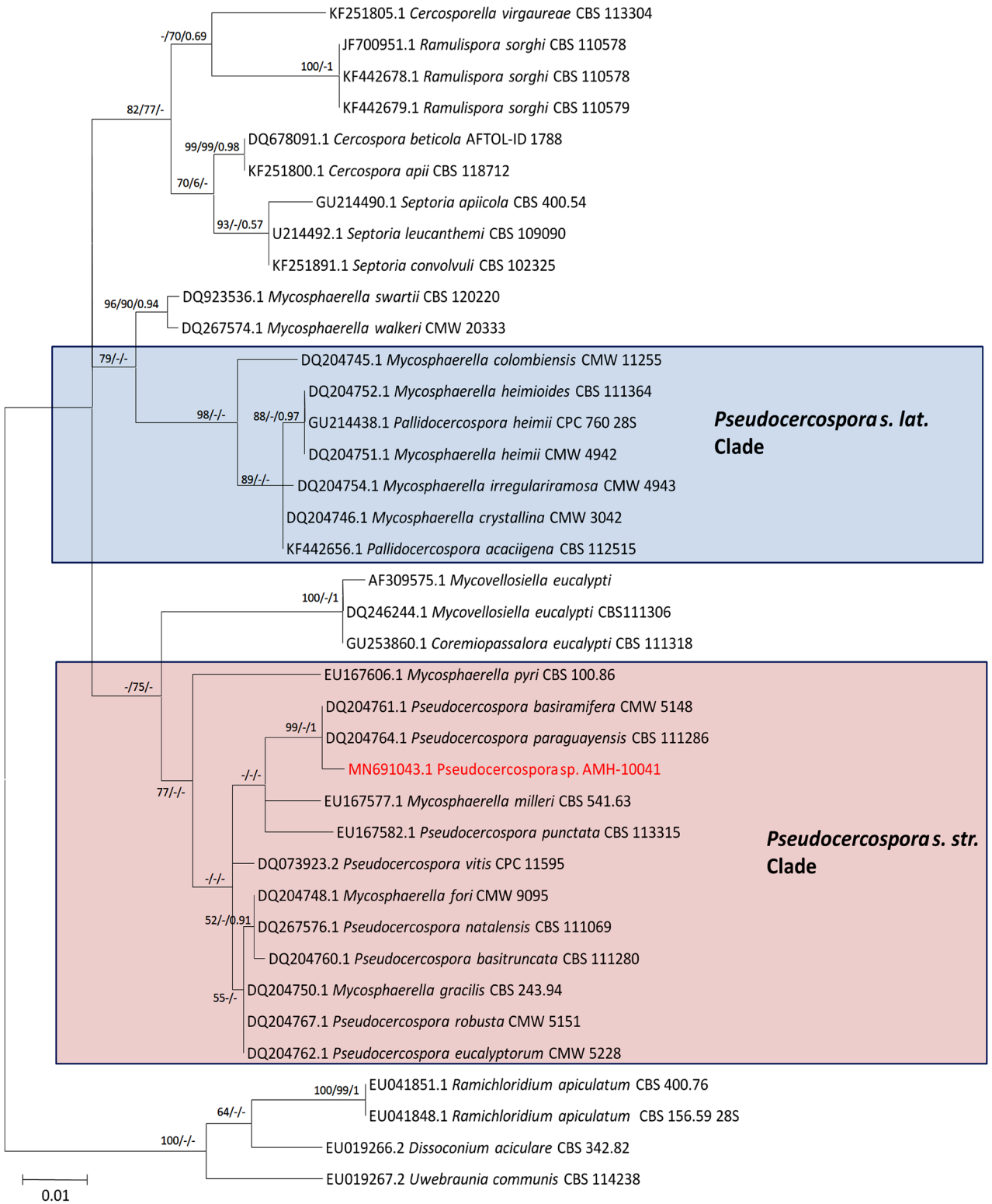

Figure 4. Molecular phylogenetic analysis of *Pseudocercospora crotalariigena* sp. nov. (AMH: 10041 Holoytpe; NFCCI: 4441 ex-type): Bayesian tree from LSU. Numbers on the branches are bootstrap values for Mega 5 maximum likelihood (ML) and Mega 5 maximum parsimony and Bayesian posterior probability (PP).

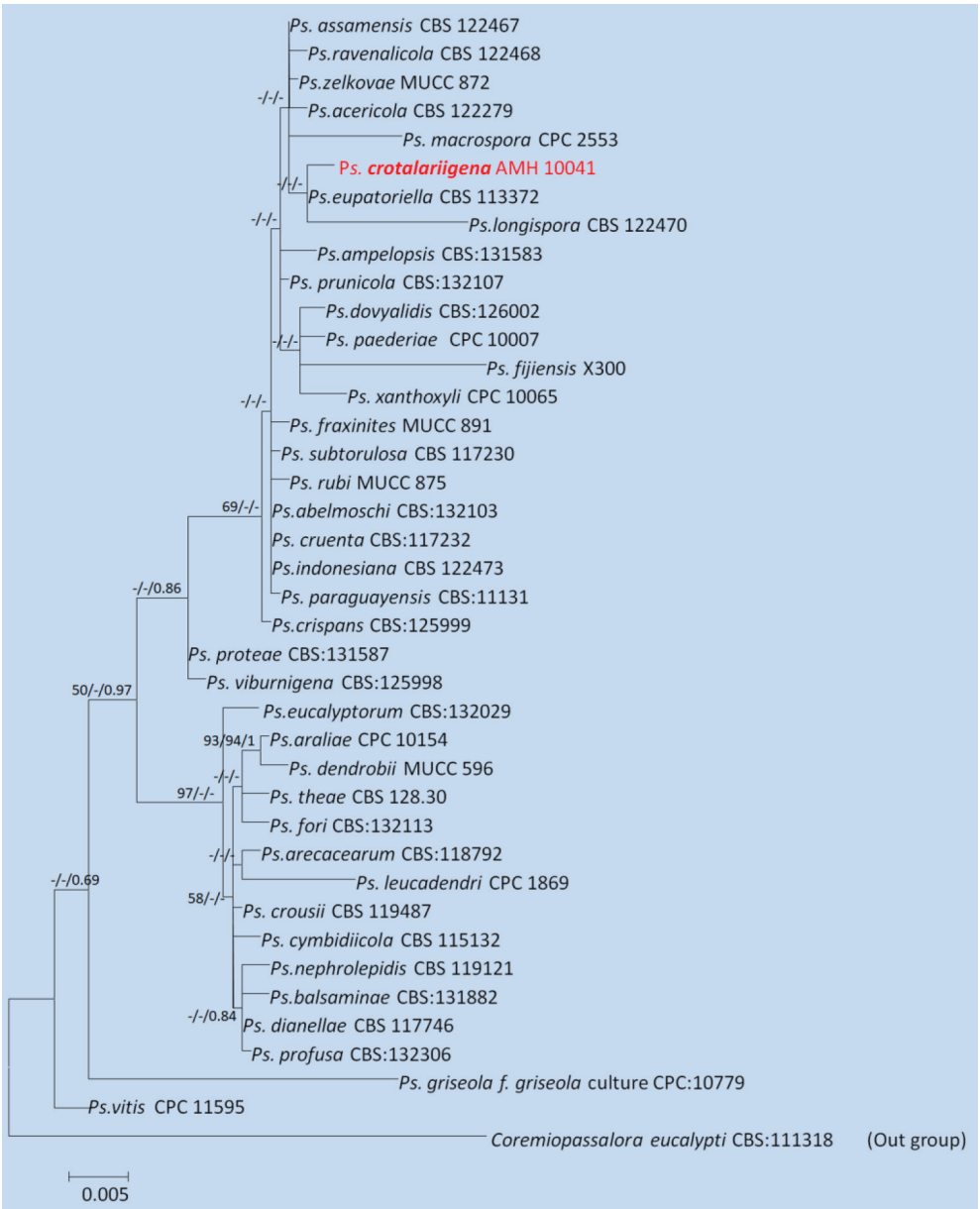

Figure 5. Molecular phylogenetic analysis of *Pseudocercospora crotalariigena* sp. nov. (AMH: 10041 Holoytpe; NFCCI: 4441 ex-type): Bayesian tree from concatenated LSU and ITS. Numbers on the branches are bootstrap values for maximum likelihood (ML) and Mega 5 maximum parsimony and Bayesian posterior probability.

acropleurogenous, holoblastic, pale brown, subcylindrical to obclavate, apex obtuse to subacute, base obconically narrowly truncate sometimes broadly truncate, tip subobtuse to obtuse, straight to curved, 1–9 transversally septate, smooth walled, light olivaceous, 14–122 × 2–3 µm, hilum unthickened, sometimes with refractive thickening.

Type: On living leaves of *Crotalaria assamica* Benth. (Fabaceae) Varanasi, UP, India, Dec. 2017, Isotype BHU Herb no. AS/17/23, Holotype AMH-10041, ex-type living culture, NFCCI: 4441, GenBank LSU: MN691043, ITS: MN654924.

3.2. Phylogeny

Ps. crotalariigena has rDNA LSU sequence of 1164 bp with G+C content 50.7% and ITS sequence of 522 bp with G+C content 51.5%. Selected *Pseudocercospora* sequences including the two ribosomal genes LSU and ITS were retrieved from GenBank. Phylogenetic analysis of LSU data set reveals it clustering in *Pseudocercospora sensu stricto*

Table. Morphotaxonomic comparative study of *Ps. crotalariigena* sp nov. with allied species of *Pseudocercospora* spp. reported on the *Crotalaria* species.

SN	Fungus	Mycelium	Stromata	Conidiophores	Conidia
1.	*Pseudocercospora crotalariana* (Pavgi and Singh) Deighton 1976	Internal	Well developed substomatal, 13.4–44.0 µm	Fasiculate,1 –3 septate, 27.5–46.2 × 3.3–5.5 µm	Solitary, 1–7 septate, 19.8–52 × 4.4–5.5 µm
2	*Ps. crotolariicola* (Yen) Yen 1978	Secondary mycelium present 2.4–4.0 µm	Absent	2–8 in a fascicle, 1–5 septate, 36–149 × 4.8–7.8 µm	Solitary, 5–12 septate, 10–134 × 3.5–5.0 µm
3	*Ps. cotizensis* (Muller and Chupp) Deighton 1976	Secondary mycelium present, 1–2.5 µm wide	Stromata small, filling stomatal opening	Up to 15 in divergent fascicle, 15–75 × 3.5–6 µm	Solitary, 3–9 septate, 20–80 × 3–5 µm
4	*Ps. leguminum* Deighton 1976	Mycelium internal	Not developed	Up to 12 in fascicle, up to 300 µm long, 3.5–5.5 µm	Solitary, 1–3 septate, 24–44 × 5.5–7 µm
5	*Ps. luxurians* (Kar and Mandal) Deighton 1976	Mycelium internal	Substomatal, brown, 6.5–25.0 µm	3.33–6.0 x 16.5–240.0 µm	Solitary, 1–7 septate, 3.4–6.3 × 19.8-66.3 µm
6	*Ps. crotalariigena* sp nov.	Mycelium internal	Substomatal, few cells to 21.8 µm.	Up to 17 in fascicle, 0–5 septate, 17.6–66.7 × 2–3 µm	Branched in chains, 1–9 septate, 14.–122.7 × 2–3 µm

clade with *Ps. vitis* the type species of the genus (Figure 4). The phylogenetic tree created with concatenated LSU and ITS sequences data set showed that *Ps. crotalariigena* was separated from other *Pseudocercospora* species, with *Ps. eupatoriella* CBS: 113372 as closely related species (Figure 5).

4. Discussion

A search in MycoBank data reveals that there are only five species of *Pseudocercospora* have been described on the host genus i.e. *Ps. crotalariana* (Pavagi and Singh) Deighton 1976, *Ps. crotalariicola* (Yen) Yen 1979, *Ps. cotizensis* (Muller and Chupp) Deighton 1976, *Ps. leguminum* (Chupp and Linder) Deighton 1976 and *Ps. luxurians* (Kar and Mandal) Deighton 1976. Sequences of these species are not available and they were not included in phylogenetic study. However, based on published description of the symptoms and micromorphology these species are clearly different from *Ps. crotalariigena*. To justify the distinct identity of the present collection, a comparison of the morphotaxonomic features of the present fungus with those of the earlier described species is presented in Table 1.

It is evident from the table that the present collection is different from previously described species having hypogenous fruiting which later become amphigenous, stromata filling stomatal opening, conidiophores and conidia vary in size and unique feature is branched and catenate conidia.

A key to *Pseudocercospora* spp. occurring on *Crotalaria*:

1a. Mycelium internal and external secondary mycelium present 2

2a. Stromata absent, secondary hyphae producing conidiophores 2–8 in a fascicle ..... *Ps. crotolariicola*

2b. Stromata present, substomatal ..... *Ps. cotizensis*

1b. Mycelium internal, external secondary mycelium absent 3

3a. Stroma well developed up to ..... *Ps. crotalariana*

3b. Stromata not developed ..... *Ps. leguminum*

3c. Stromata, substomatal upto 25 µm 4

4a. Conidia solitary ..... *Ps. luxurians*

4b.Conidia catenate and branched ..... ***Ps. crotalariigena* sp. nov.**

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Conflict of interest

The authors declare that they have no conflicts of interest.

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