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Conlarium indicum: A novel fungus from Western Ghats of India

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Abstract

The present paper describes and illustrates *Conlarium indicum*, a new species in the family *Conlariaceae* (Ascomycota). This taxon was isolated as an epiphyte from decaying *Bamboo* collected from Western Ghats of India. The isolate was identified based on asexual-morphs, cultural characteristics and phylogenetic analyses of partial nuclear ribosomal 28S large subunit (LSU) and complete internal transcribed spacer (ITS) rDNA. Phylogenetic reconstructions based on ITS+LSU sequences show that the new species form independent monophyletic groups and are well separated from previously known seven species of *Conlarium* viz. *C. dupliciascosporum* (as *duplumascospora*), *C. aquaticum*, *C. thailandense*, *C. sacchari*, *C. baiseense*, *C. nanningense* and *C. subglobosum*. This supports the erection of *C. indicum* as a new species. A brief description of the morphology of *C. indicum* with morphological and molecular data is provided. To our understanding, the present taxon has turned out to be hitherto unreported.

Keywords – Ascomycota – Asexual morph – ITS – LSU – Novel species – Taxonomy

Introduction

The genus *Conlarium* is described by Liu et al. (2012), which belongs to the family of freshwater ascomycetes; Conlariaceae (Zhang et al. 2017). This genus includes seven species *C. dupliciascosporum* (as *duplumascospora*), *C. aquaticum*, *C. thailandense*, *C. sacchari*, *C. baiseense*, *C. nanningense* and *C. subglobosum*. Out of these species, *C. dupliciascosporum* (as *duplumascospora*), *C. aquaticum and C. subglobosum* were isolated from the submerged woody samples in streams (Liu et al. 2012, Zhang et al. 2017) and *C. thailandense* was isolated from dead wood (Phookamsak et al. 2019). Three species viz. *C. baiseense*, *C. nanningense* and *C. sacchari* were isolated from sugarcane rhizosphere (Xie et al. 2019).

The fungus described in this paper was collected from Sawantwadi area of Maharashtra as part of the research project. Our study identified the identity of the present taxon by a comparative analysis of morphological, *in-vitro* cultural characteristics and phylogenetic analysis of ITS and LSU combined sequence data. Asexual morph was confirmed by the molecular evidence. Hence, present taxon *C. indicum*, isolated from a dead bamboo is described as species new to science.

Materials & Methods

Morphological study

The samples were collected from Sawantwadi, (15°54'22.7"N 73°49'23.5"E) Sindhudurg district, Maharashtra, India. The decaying bamboo sample infested with the fungus was first

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observed under the stereomicroscope. The raised pure fungi were then grown on different culture media, viz. Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), and Potato Carrot Agar (PCA). Photographs and microscopic details were observed in lactophenol-cotton blue using (OLYMPUS CX41 aided with Digi-CAM) microscope. Measurements of the fungal structures were taken from the microscope.

DNA extraction, PCR amplification and DNA sequencing

Genomic DNA was isolated from pure colony grown on PDA & PCA media plate for 5 days. Approximately 2 square centimeters of mycelium, taken from the growth front of the cultures was scraped off the surface of the medium and was transferred to an extraction tube, following a rapid and simple DNA extraction protocol (*HiPurA™ Fungal DNA Purification* Kit). The ITS gene region, internal transcribed spacer region 1, 5.8 ribosomal RNA gene, and internal transcribed spacer region 2 were amplified using the primers ITS4/ITS5 (White et al. 1990, Liu et al. 1999). Ribosomal nuclear, large subunit (nucLSU) (partial) was amplified using primers LROR/LR7 (Vilgalys & Hester 1990) with the help of PCR System using (Applied Biosystems ProFlex).PCR was performed in a 40 µl reaction using 20 µl 2X Hi-Chrom PCR Mastermix, 2 µl 10 pmol primer, 16 µl 5X GC enhancer H_2O (Sterile Ultra-Pure Water, Sigma) and 2 µl template DNA (10–20 ng). The thermal cycling program for ITS gene region involved 5 min initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C, annealing at 56°C, extension at 72°C and a final 10 min extension at 72°C. In case of partial nucLSU, conditions were 5 min denaturation step at 94ºC, 35 cycles of 1 min at 94°C, 50 s at 52°C, and 1.2 min at 72°C with a final 8 min extension step at 72°C. Negative control using sterilized distilled water instead of template DNA was also included in the amplification process. The amplified products were examined by electrophoresis at 65 V in 0.8% (W/V) agarose gel in 1×TAE buffer (0.4 M Tris, 10 mM EDTA, 50 mM NaOAc with pH 7.8) and visualized using GelDoc Imager after staining with ethidium bromide (0.5 µg ml^{-1}).The amplified PCR products were purified with *HiPurA™ PCR Product Purification* Kits as per manufacturer's instructions. Purified PCR products of these marker genes were checked on 1.2% agarose electrophoresis gel stained with ethidium bromide (0.5 µg ml^{-1}). They were directed to sequencing using BigDye®Terminator v3.1 Cycle Sequencing Kit and ABI 3100 DNA analyzer (Perkin Elmer, Applied Biosystems, Foster City, CA, USA). Sequences obtained were submitted to NCBI GenBank accession numbers MT586317 (LSU), and MT586318 (ITS). The sequences were deposited in GenBank. The chosen strains used in the construction of the phylogenetic tree and their accession numbers and other related details are listed in Table 1.

Table 1 Species, specimens and GenBank accession number of sequences used in this study

Species	Culture accession no.	Gene bank no.	
		ITS	LSU
Conlarium aquaticum	MFLUCC 150992	MF374354	MF374363
Conlarium baiseense	TD2	MF083157	MF083158
Conlarium baiseense	TD17	MK164653	MK164655
Conlarium duplumascospora	CGMCC 14938	JN936995	JN936991
Conlarium duplumascospora	CGMCC 14939	JN936996	JN936992
Conlarium duplumascospora	CGMCC 14940	JN936997	JN936993
Conlarium indicum	**NFCCI 4841**	**MT586318**	**MT586317**
Conlarium nanningense	M1	KX886204	KX886202
Conlarium nanningense	M8	MK164654	MK164656
Conlarium sacchari	NN1	MF083160	MF083161
Conlarium sacchari	LA3	MF083163	MF083164
Conlarium sacchari	DX4	MF083166	MF083167
Conlarium subglobosum	MFLU 17 -1728	MW286494	MW287768
Conlarium thailandense	MFLUCC 172349	MH624129	MH624127
Aquidictyomyces appendiculatus	KUMCC 19-0061	-	MW287756

New species is in **bold**

Results

Phylogenetic Analyses

The sequences obtained in the form of chromatograms were analyzed using megablast search algorithm, and sequences of related strains were retrieved from NCBI to compare with the already known taxa of *Conlarium*. *Aquidictyomyces appendiculatus* MW287756 was selected as the out group. The sequences were deposited in Gene Bank. The chosen strains used in the construction of the phylogenetic tree and their accession numbers and other related details are listed in Table 1.

The ITS sequences were used to confirm the resolution of the present isolate. The ITS sequences were used to confirm the resolution of the present isolate. The file contained sequence data of 13 taxa (Table 1) 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 based on the model mentioned above. 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. Combined phylogenetic analysis using nuclear ribosomal DNA (ITS and 28S partial) nested based on a Mega BLAST search on NCBI GenBank nucleotide database, the closest hit using the ITS gene sequence was with *Conlarium* sp. (subglobosum) MW286494 showing 95.63% (503/526) identity and (0%) gap, *Conlarium sacchari* (MF083166) showing 93.67% (488/521bp) identity and having (1%) gap; 93.69% (490/523) identity and (2%) gap with *C. nanningense* (KX886204) and 93.06% (496/533) identity and (2%) gap with *C. duplumascospora* (NR138382). For the LSU gene region, *Conlarium aquaticum* (NG067554) was the closest hit with 98.46% (832/845bp) identity and (0%) gap, 98.23% (833/848) identity and (0%) gap with *C. thailandense* (NG068567).

Phylogenetic reconstructions based on ITS+LSU sequences show that the new species form independent monophyletic groups and are well separated from previously known species of *Conlarium* viz. *C. dupliciascosporum, C. aquaticum, C. thailandense, C. sacchari, C. baiseense, C. nanningense* and *C. subglobosum* (Fig. 1). This supports the erection of the new species. Therefore, we introduce *C. indicum* as a new species, following Jeewon & Hyde 2016.

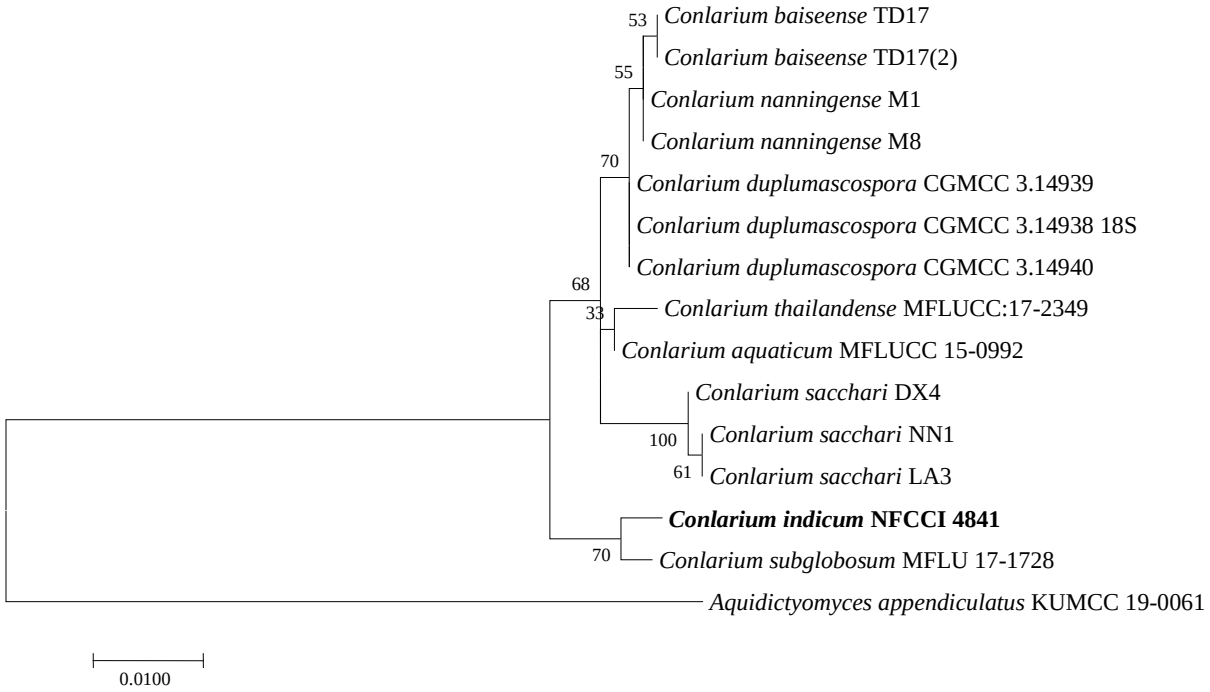

Fig. 1 – Molecular phylogenetic analysis by maximum-likelihood (ML) method based on combined LSU and ITS sequence data. The novel sequence of *Conlarium indicum* is highlighted in **black bold** (Kimura 1980).

Taxonomy

Conlarium indicum R. Dubey, S. Manikpuri sp.nov. Figs 2, 3

Facesoffungi Number: FoF09310; Mycobank: MB835757

Etymology – *Indicum*, referring to the name of the country (India).

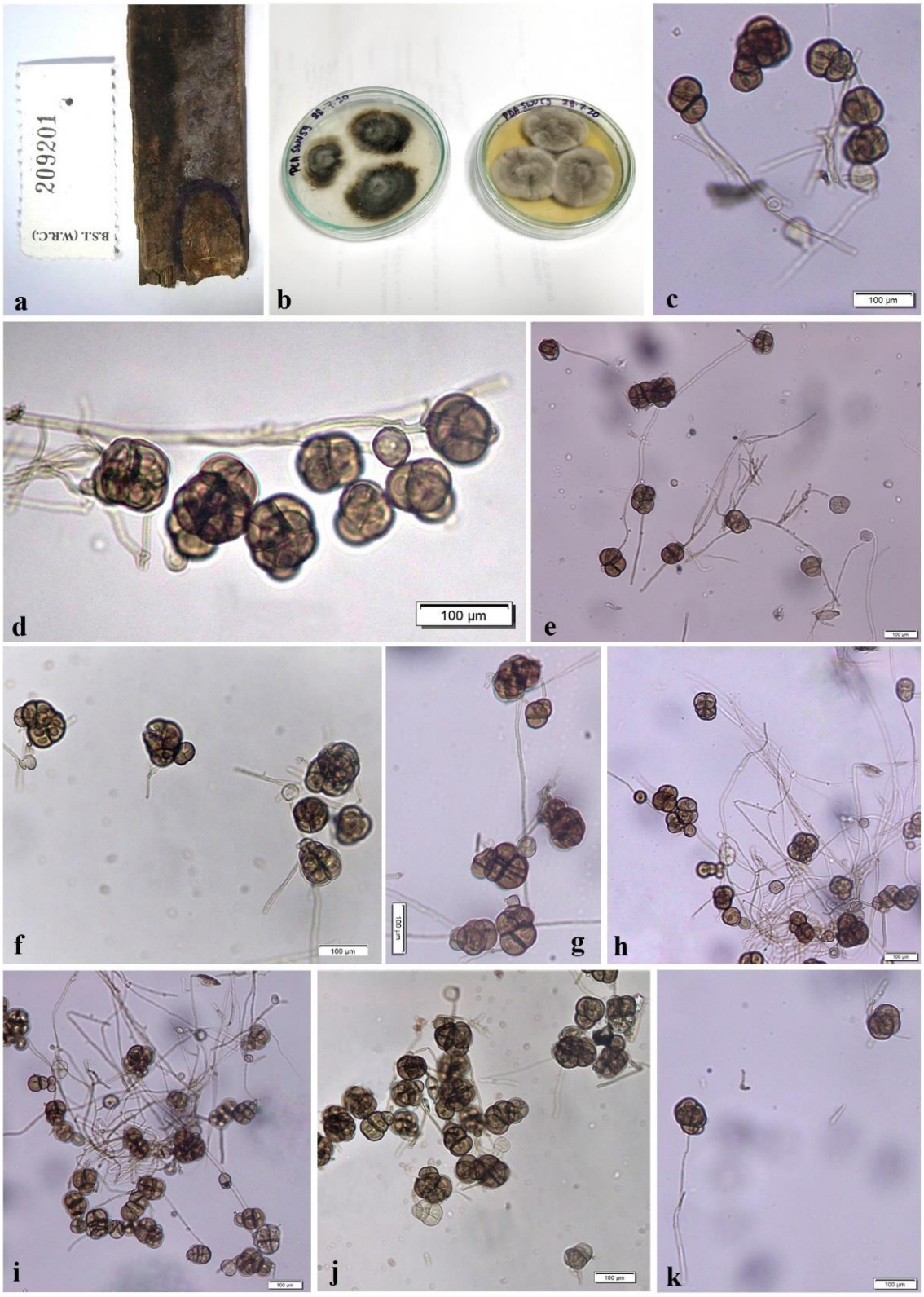

Fig. 2 – *Conlarium indicum* (NFCCI 4841). A Colonies on host surface. B Colony morphology on PCA and PDA. C-K Mature conidia. Scale bars: C-K=100 µm.

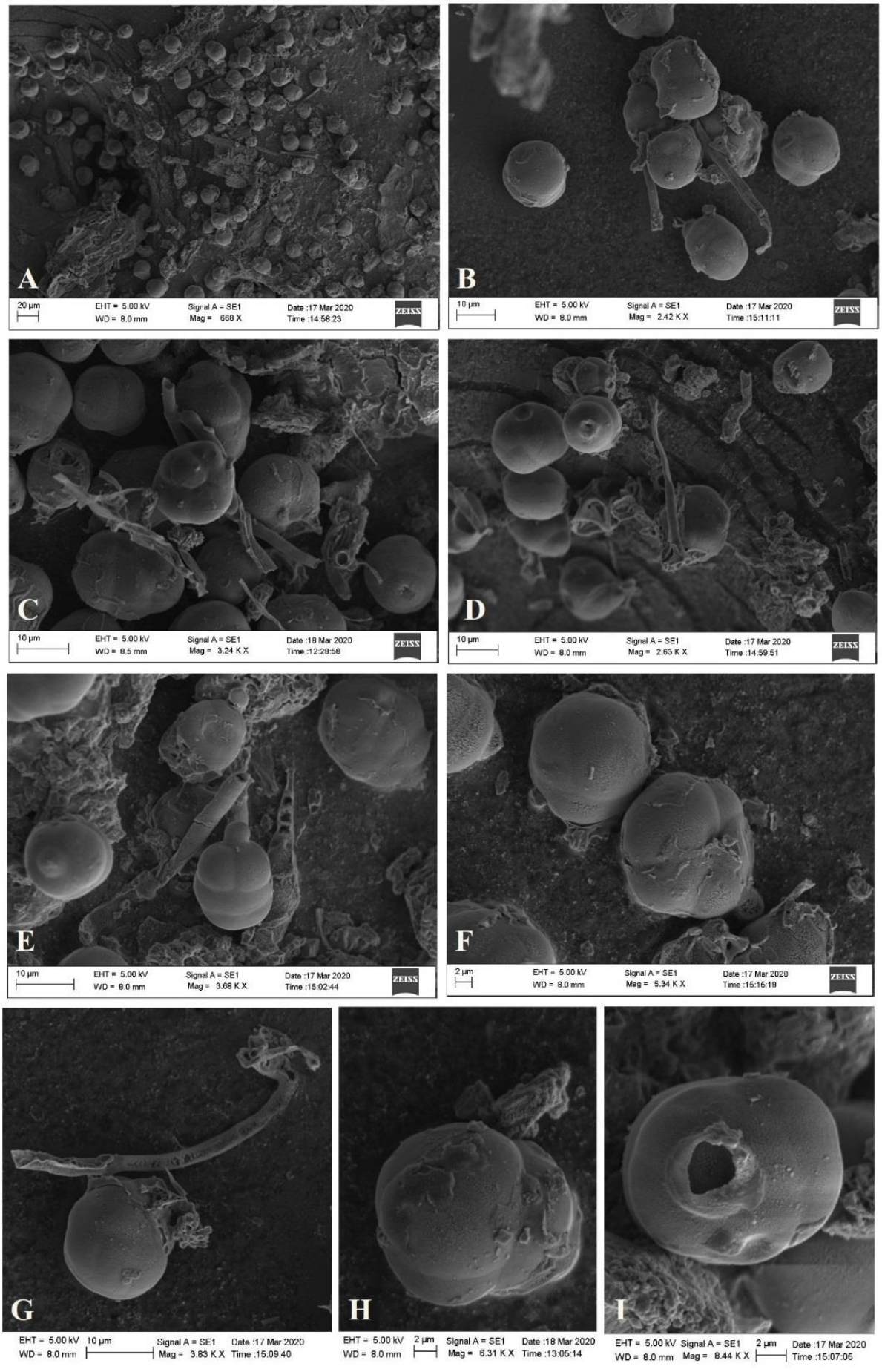

Fig. 3 – *Conlarium indicum* (NFCCI 4841). A-I Scanning Electron Microscopic Images.

Description – Saprobic on bamboo stick. Colony 40 mm in diameter, grey-white on PDA medium and brown to blackish brown on PCA after 3 weeks at 28 °C, circular, flat growth, less aerial hyphae, regular edge of colony. Hyphae light brown, septate. Conidiophores light brown, mostly stubby, 0–2-branched, 0–4 septate, straight or flexuous, 35-40 × 6–8 μm. Conidiogenous cells determinate, doliiform, yellow brown to brown, 3–8 × 5–12 μm. Muriform conidia yellow-brown to brown, irregularly globose or subglobose, smooth, constricted at the separation, 1-3 transversely septa, 1–3 longitudinal or oblique septa, 60-103 × 39-88 μm. Sexual morph: undetermined.

Habitat and Distribution – In Western Ghats of India

Holotype – India, Maharashtra, Sindhudurg District, Sawantwadi, on decaying Bamboo, 16.2.2018, Holotype (BSI-139548) deposited in Botanical Survey of India, Western Regional Centre, Pune, Maharashtra, India and ex-type living culture, NFCCI 4841, deposited in National Fungal Culture Collection of India, Agarkar Research Institute, Pune, Maharashtra, India.

Discussion

Conlarium is unique among genera in Conlariaceae, for its unusual combination of several morphological characters, for instance, the teleomorphic form has gregarious, dark brown to black ascomata with a long neck; biseriate ascospores with or without globose or papillary appendages at one or each end, and anamorph form is with muriform conidia. (Liu et al. 2012). They have subglobose or irregular, brown, clathrate, muriform conidia (Zhang et al. 2017). This genus comprises of seven species *C. dupliciascosporum* (as *duplumascospora*), *C. aquaticum*, *C. thailandense, C. sacchari, C. baiseense, C. nanningense* and *C. subglobosum* and hyphomycetous asexual morph taxon *C. aquaticum* and there is no record of *Conlarium* from India.

Conlarium indicum is morphologically compared with *C. dupliciascosporum* (as *duplumascospora*), *C. thailandense*, *C. aquatcium, C. sacchari, C. baiseense, C. nanningense* and *C. subglobosum.* The present taxa is similar to the asexual morph of all these taxa. They all have monoblastic, holoblastic conidiogenous cells and mostly irregular, brown, clathrate, muriform conidia (Liu et al. 2012).

However, *C. indicum* can be easily distinguished from *C. aquaticum*, *C. dupliciascosporum* (as *duplumascospora*), *C. thailandense, C. sacchari, C. baiseense, C. nanningense* and *C. subglobosum* by the number of conidial septa (2–4-transversely septate, 1–3-longitudinally septate in *C. duplumascospora*; 6–12-transverse septa, 4–10-longitudinal septa in *C. aquaticum*; 4–8-transverse septa, 4–6-longitudinal septa in *C. thailandense,* 0–1 transversely septa, 0–4 longitudinal septa in *C. baiseense*, 0–1 transversely septa, 0–4 longitudinal septa in *C. nanningense*, 0–1 transverse septa, 0–3 longitudinal septa in *C. sacchari*, 1-2 transverse septa,1-2 longitudinal septa vs 1-3 transversely septa, 1-3 longitudinal/Obliquely septa in *C. indicum*) and conidial size (15.5–35 × 11–26.5 μm in *C. duplumascosporum*, 45–70 × 20–57 μm in *C. aquaticum*, 25–45 × 17–33 μm in *C. thailandense*, 21–35 × 7–12 μm in *C. baiseense,* 11–21 × 9–21 μm in *C. nanningense,* 14–19×13–22 μm in *C. sacchari,* 14.5–24 μm in *C. subglobosum* and 60–103 × 39–88 μm in *C. indicum*) (Liu et al. 2012, Zhang et al. 2017, Phookamsak et al. 2019, Dong et al. 2021). The large size of conidia separates it as a new species. *C. indicum* is established on the basis of morphological characters and molecular evidence inferred from ITS and LSU rDNA sequences.

Phylogenetic reconstructions based on the combined result of ITS+LSU sequences show that independent monophyletic group is formed by the new species and is well separated from *C. duplumascophora*, *C. thailandense*, and *C. aquatcium, C. sacchari, C. baiseense, C. nanningense* and *C. subglobosum*, this further supports the erection of the new taxon.

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