



Phylogenetic placement of *Bahusandhika*, *Cancellidium* and *Pseudoepicoccum* (asexual Ascomycota)

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Abstract

Most hyphomycetous conidial fungi cannot be presently placed in a natural classification. They need recollecting and sequencing so that phylogenetic analysis can resolve their taxonomic affinities. The type species of the asexual genera, *Bahusandhika*, *Cancellidium* and *Pseudoepicoccum* were recollected, isolated in culture, and the ITS and LSU gene regions sequenced. The sequence data were analysed with reference data obtained through GenBank. The DNA sequence analyses shows that *Bahusandhika indica* has a close relationship with *Berkleasmiium* in the order Pleosporales and *Pseudoepicoccum cocos* with *Piedraia* in Capnodiales; both are members of Dothideomycetes. *Cancellidium applanatum* forms a distinct lineage in the Sordariomycetes.

Key words: anamorphic fungi, ITS, LSU, phylogeny

Introduction

Asexually reproducing ascomycetous fungi are ubiquitous in nature and worldwide in distribution, occurring from the tropics to the polar regions and from mountain tops to the deep oceans. These fungi colonize, multiply and survive in diverse habitats, such as water, soil, air, litter, dung, foam, live plants and animals, as saprobes, pathogens and mutualists. The asexual fungi were previously placed in hyphomycetes, coelomycetes and Blastomycetes, comprise about 20,000 species belonging to 1,700 genera. They can now be linked to their sexual relatives or states through molecular study (Hyde *et al.* 2011).

Only 10% of asexually reproducing species have been connected to their respective sexual states (Bärlocher 2009). Asexual fungi may lack or have unknown sexual stages, and were previously difficult to classify in a natural system. However, with molecular sequence data it is now possible to resolve their taxonomic hierarchy and phylogenetic affinity (Crous *et al.* 2009). In this paper, we report on the phylogenetic affinities of three asexually reproducing fungi, *Bahusandhika indica* (Subram.) Subram., *Cancellidium applanatum* Tubaki and *Pseudoepicoccum cocos* (F. Stevens) M.B. Ellis. Links to sexual states are not known for these taxa.

This is the first report of *Bahusandhika indica* and *Pseudoepicoccum cocos* in culture. *Cancellidium applanatum* has been often isolated from freshwater streams and was previously cultured (Webster & Davey 1980, Shaw 1994, Goh 1997, Fryar *et al.* 2004, Luo *et al.* 2004, Tsui & Hyde 2004). However, the type species has not been sequenced although another species, *C. pinicola*, has been sequenced and belongs to Hypocreales in Sordariomycetes (Yeung *et al.* 2006).

Materials and methods

Bahusandhika indica was isolated from floral litter and *Pseudoepicoccum cocos* from leaf spots of *Cocos nucifera*,

both collected from a plantation in Mashem, Canacona, Goa, India. *Cancellidium applanatum*, was isolated from submerged twigs of an unidentified plant from a freshwater stream in Netravali, Sanguem, Goa, India.

Samples were brought to the laboratory in Zip-lock polythene bags and examined under a stereoscope. The fungi were picked up with a sterile needle, mounted in lactophenol and observed under a light microscope. The cultures were obtained by single spore isolation (Bhat 2010). A drop of sterile distilled water was placed on a flame-sterilized slide and the sporulating fungal mass was aseptically transferred into the water and teased with flame-sterilised needle in order to obtain a spore suspension. The suspension was spread onto malt extract agar (MEA) or potato dextrose agar (PDA) plates containing antibiotics (20 mg/L each streptomycin and penicillin). The colonies developing from individual conidia were aseptically transferred into fresh plates (Choi *et al.* 1999). After confirming the identity of the culture, molecular sequencing was done at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India.

DNA isolation and PCR Analysis

Fresh fungal mycelia (20 mg), scraped from the growing culture incubated at 28°C for 7 days. DNA isolation and PCR Analysis was done according to Prabhugaonkar & Bhat (2011).

The 5.8S nuclear ribosomal gene with the two flanking internal transcribed spacers (ITS) and 28S nrDNA sequence (LSU) genes were amplified and sequenced using the primer pairs ITS-1F + ITS-4R (White *et al.* 1990) and LR5 + LROR (Crous *et al.* 2009), respectively. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al.* 2010).

Sequence alignment and phylogenetic analysis

The sequences were blasted in GenBank with Blastn. LSU and ITS data sets were analysed. Based on the blasts, further related sequences were assembled for each fungus. The combined data matrix was aligned using MAFFT v.7 (<http://mafft.cbrc.jp/alignment/software>) and manually adjusted using MEGA 5.2 to allow maximum alignment and maximum sequence similarity. A phylogenetic analysis was conducted using maximum likelihood (ML) in MEGA 5.2 (Kumar *et al.* 2008) with 1,000 bootstrap replicates. The most suitable substitution models for the respective datasets were selected by using MEGA5.2. General Time Reversible model with Gamma distribution was used in *Bahusandhika* and *Cancellidium* analysis and Tamura Nei model with Gamma distribution was used in *Pseudoepicoccum* analysis. Gaps were treated as a pairwise deletion and trees were viewed with Mega 5.2.

In addition, we performed a Bayesian Analysis (BA) using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) on the dataset to confirm the phylogenetic placement. The most suitable substitution models for the respective datasets were selected by using JModeltest. All newly generated ITS and LSU sequences used in this study are deposited in GenBank and the alignments in TreeBASE (S14827).

TABLE 1. Origin of DNA sequences in combined analyses of ITS and LSU. Newly deposited sequences are in bold.

Taxon	ITS	LSU
<i>Akanthomyces novoguineensis</i>	JN201872	JN201874
<i>Aliquandostipite khaoyaiensis</i>	JN819278	EF175650
<i>Amphiportha hranicensis</i>	DQ323525	DQ323521
<i>Anhellia nectandrae</i>	JQ071524	JQ071523
<i>Apiognomonia errabunda</i>	DQ313525	NG_027592
<i>Aplosporella prunicola</i>	EF564375	EF564377
<i>Aquaticola hongkongensis</i>	AF177156	AF132321
<i>Auerswaldia lignicola</i>	JX646798	JX646815
<i>Bagnisiella examinans</i>	EU167562	GU301803
<i>Bahusandhika indica</i>	KF460273	KF460274
<i>Batcheloromyces leucadendri</i>	JF499832	JF499852
<i>Berkleasium micronesticum</i>	DQ280262	DQ280272
<i>Berkleasium nigroapicale</i>	DQ280261	DQ280273

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TABLE 1. (continued)

Taxon	ITS	LSU
<i>Calosphaeria africana</i>	EU367444	EU367454
<i>Cancellidium applanatum</i>	KF460275	KF460276
<i>Cancellidium</i> sp.	NA	DQ144048
<i>Capnodium coffeae</i>	AJ244239	GU214400
<i>Capnodium coffeae</i>	AJ244239	GU214400
<i>Catenulostroma protearum</i>	GU214627	GU214401
<i>Ceratocystiopsis brevicomis</i>	EU913722	EU913683
<i>Cercophora caudata</i>	AY999135	AY999113
<i>Chaunopycnis alba</i>	HM595511	HM595579
<i>Chrysoportha</i> sp.	JN942341	JN940855
<i>Cladosporium silenes</i>	EF679354	JF770463
<i>Cochliobolus heterostrophus</i>	JX094779	JX094789
<i>Colletogloeopsis dimorpha</i>	DQ923529	NA
<i>Coniothyrium multiporum</i>	JF740187	JF740268
<i>Conlarium duplumascospora</i>	JN936997	JN936993
<i>Cordana inaequalis</i>	HE672146	HE672157
<i>Cordana</i> sp.	HE672151	HE672162
<i>Cryptosporella suffusa</i>	EU199184	EU199124
<i>Cucurbitaria berberidis</i>	JF740191	GQ387606
<i>Davidiella tassiana</i>	AF393705	AY342095
<i>Dissoconium aciculare</i>	JQ622083	JQ622091
<i>Ditopella ditopa</i>	EU199187	EU199126
<i>Dothidea sambuci</i>	AY883094	NG_027611
<i>Elsinoe verbenae</i>	JN943499	JN940391
<i>Endoxyla operculata</i>	JX460987	JX460992
<i>Epicoccum nigrum</i>	JN942899	JN938882
<i>Fissuroma maculans</i>	JN846710	JN846724
<i>Gnomoniella nana</i>	DQ323534	DQ323522
<i>Grosmania davidsonii</i>	GU134165	GU134181
<i>Guignardia citricarpa</i>	FJ538313	U301815
<i>Hortaea thailandica</i>	GU214637	GU214429
<i>Jahmula appendiculata</i>	JN819280	FJ743446
<i>Jattaea mookgoponga</i>	EU367450	EU367459
<i>Kirramyces viscidus</i>	FJ493186	FJ493204
<i>Lasiodiplodia theobromae</i>	JX646799	JX646816
<i>Lepidosphaeria nicotiae</i>	GQ203760	DQ384106
<i>Leptosphaeria pedicularis</i>	JF740224	JF740294
<i>Lophiostoma compressum</i>	JN942962	JN941379
<i>Lophiostoma macrostomum</i>	AB433276	AB433274
<i>Massarina</i> sp.	GQ141701	GQ141697
<i>Melanconis carthusiana</i>	EU199196	EU199131
<i>Montagnula aloes</i>	JX069863	JX069847
<i>Myriangium</i> sp.	EF464587	EF464576
<i>Neoastrorphaeriella krabiensis</i>	JN846715	JN846729
<i>Mycosphaerella brassicicola</i>	AY152554	AY152643

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TABLE 1. (continued)

Taxon	ITS	LSU
<i>Ophiognomonia melanostyla</i>	JF779850	JF779854
<i>Ophiostoma gemellus</i>	DQ821561	DQ821532
<i>Penidiella drakensbergensis</i>	KC005770	KC005792
<i>Periconia macrospinoso</i>	JN859364	JN859484
<i>Periconiella velutina</i>	EU041781	EU041838
<i>Phaeobotryosphaeria eucalypti</i>	JX646803	JX646820
<i>Phaeophleospora stonei</i>	EF394856	FJ493210
<i>Phaeosphaeria vagans</i>	KF251193	KF251696
<i>Phyllosticta vaccinii</i>	FJ603586	FJ588245
<i>Piedraia hortae</i>	GU214647	GU214466
<i>Plagiostoma</i> sp.	EU255059	EU255194
<i>Plenodomus wasabiae</i>	JF740257	JF740323
<i>Pleospora tarda</i>	KC584238	KC584345
<i>Polyposphaeria fusca</i>	AB524790	AB524605
<i>Preussia isomera</i>	GQ203763	GQ203723
<i>Preussia persica</i>	GQ292750	GQ292752
<i>Preussia similis</i>	DQ468028	DQ468048
<i>Pseudocercospora basiramifera</i>	GU269781	GU253802
<i>Pseudoepicoccum cocos</i>	KF460277	KF460278
<i>Pseudotaeniolina globosa</i>	KC311489	NA
<i>Pseudotetraploa longissima</i>	AB524796	AB524612
<i>Pseudotetraploa longissima</i>	AB524796	AB524612
<i>Pseudovalsaria ferruginea</i>	JX460988	JX460993
<i>Pyrenophora seminiperda</i>	JN943665	JN940088
<i>Quadricrura septentrionalis</i>	AB524800	AB524616
<i>Ramichloridium indicum</i>	EU041799	U041856
<i>Ramularia lamii</i>	KF251331	KF251835
<i>Readeriella considenianae</i>	JQ732899	JQ732948
<i>Rhodoveronaea varioseptata</i>	EU041813	EU041870
<i>Saccharata intermedia</i>	GU229888	GU229889
<i>Schizothecium carpinicola</i>	AY999118	AY999095
<i>Schizothyrium pomi</i>	EF134947	NA
<i>Septoria sigesbeckiae</i>	KF251547	KF252052
<i>Setosphaeria rostrata</i>	KC150019	KC150020
<i>Sporormiella pulchella</i>	GQ203789	GQ203747
<i>Stenella araguata</i>	EU019250	NA
<i>Sydowia polyspora</i>	AY152548	AY152616
<i>Teratosphaeria hortae</i>	FJ790276	FJ790299
<i>Teratosphaeria verrucosa</i>	AY725517	EU019293
<i>Tetraploosphaeria yakushimensis</i>	AB524808	AB524632
<i>Togniniella acerosa</i>	EU367452	AY761077
<i>Triploosphaeria maxima</i>	AB524812	AB524637
<i>Versicolorisporium triseptatum</i>	AB365596	AB330081
<i>Westerdykella cylindrica</i>	AY943056	NG_027595
<i>Westerdykella reniformis</i>	JX235700	JX235704
<i>Zopfiella erostrata</i>	AY999133	AY999110

Results

Phylogenetic analyses

Bahusandhika indica

Forty-three taxa are included in the phylogenetic analysis (Table 1, Fig. 1). The result of preliminary phylogenetic analysis showed that *Bahusandhika* has close affinities with *Berkleasium* in the order Pleosporales, sub-class Pleosporomycetidae of Dothideomycetes.

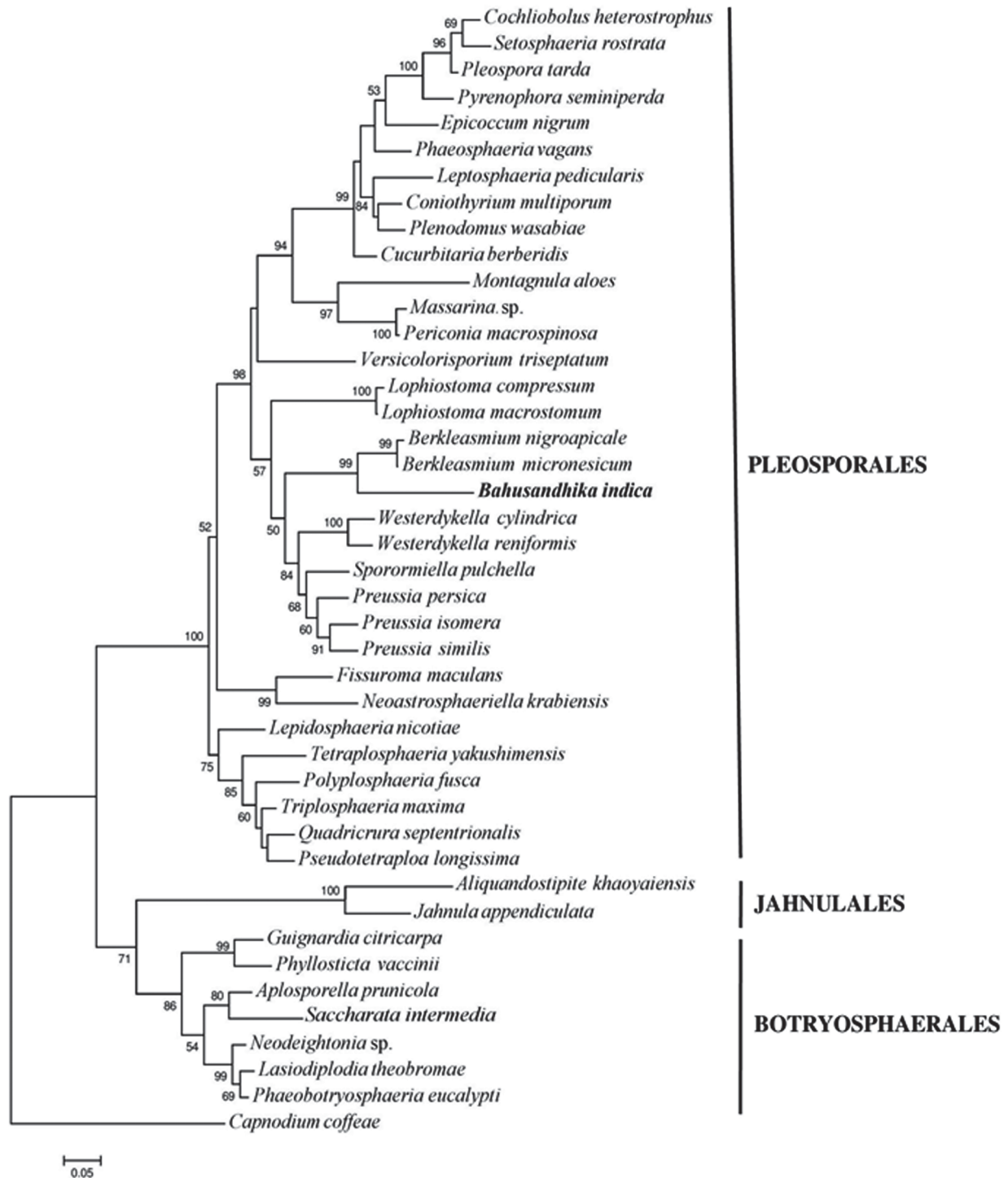


FIGURE 1. Maximum likelihood tree of *Bahusandhika indica* and related taxa based on combined analysis of ITS and LSU sequences. Species described in this study is in bold.

A dataset of three orders, Pleosporales, Botryosphaerales and Jahnuales from sub-class Pleosporomycetidae of the Dothideomycetes was assembled. *Capnodium coffeae* (Capnodiales, Dothideomycetidae, Dothideomycetes) was selected as the outgroup taxon. In the phylogenetic analysis *Bahusandhika indica* forms a distinct lineage, thus confirming the distinctiveness of the genus. *Bahusandhika*, has a close affinity with *Berkleasium nigroapicale*, *B. micronesicum* and *Westerdykella* which are members of family Sporormiaceae (Pinnoi *et al.* 2007). The sexual states of *Bahusandhika* and *Berkleasium* are unknown.

The maximum likelihood resulted in a tree with largely the same topology and clades with the Bayesian analysis tree (TreeBASE study S14827).

Cancellidium applanatum

Twenty-seven taxa are included in the phylogenetic analysis (Table 1, Fig. 2). Preliminary phylogenetic analysis showed that *Cancellidium applanatum* has affinities with genus *Rhodoveronaea* and *Aquaticola* (Sordariomycetes). *Rhodoveronaea* was isolated from *Bertia moriformis* from Germany and remains Incertae Sedis at family level (Arzanlou *et al.* 2007). *Aquaticola* is placed in the Annulatascaceae based on morphological characteristics (Ho *et al.* 1999). The generic placement of *Aquaticola* is further supported by molecular studies indicating that *Aquaticola* is closely related to genera of the Annulatascaceae (Ranghoo *et al.* 1999). Both *Aquaticola* and *Cancellidium* are found in a similar habitat, i.e. on decaying wood submerged in freshwater streams (Ho *et al.* 1999, Tubaki 1975), but possible sexual connection remains uncertain because of low bootstrap values.

A dataset of six orders, Diaporthales, Hypocreales, Sordariales, Calosphaerales, Boliniales, Ophiostomatales and some species of Sordariomycetes genus, *incertae sedis* was assembled. *Dothidea sambuci* (Dothideomycetes) was selected as the outgroup taxon. *Cancellidium applanatum* forms a distinct lineage thus confirming the distinctiveness of the genus (Fig. 2). This analysis does not show any close relation between *C. applanatum* and *C. pinicola*, and lies in Sordariomycetes genus, Incertae Sedis. The taxonomic placement of *C. pinicola* in Hypocreales may be incorrect as the authors expressed fear of culture being contaminated (Yeung *et al.* 2006).

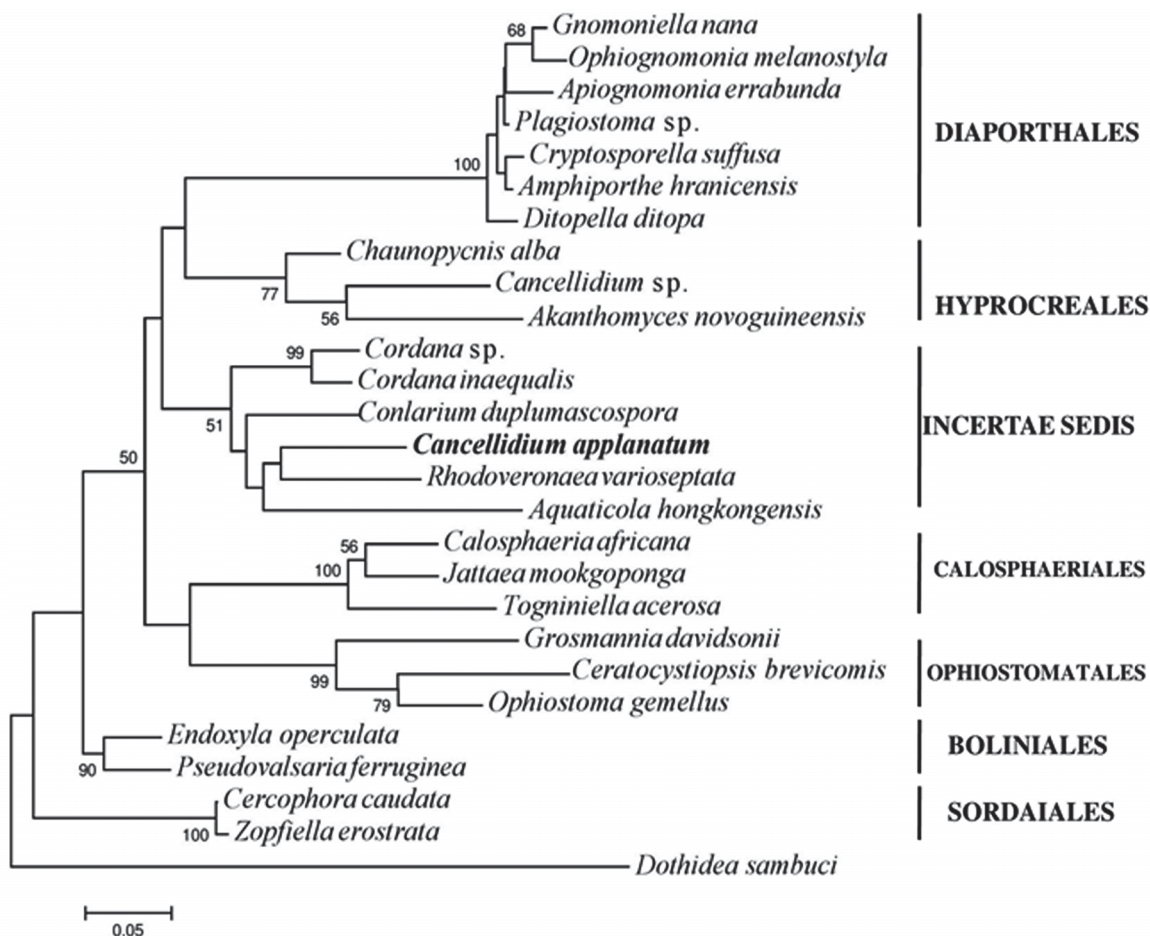


FIGURE 2. Maximum likelihood tree of *Cancellidium applanatum* and related taxa based on combined analysis of ITS and LSU sequences. Species described in this study is in bold.

Pseudoepicoccum cocos

Thirty-four taxa are included in the phylogenetic analysis (Table 1, Fig. 3). *Pseudoepicoccum cocos* has affinities with genus *Piedraia* from family Piedraceae (Hyde *et al.* 2013) in the order Capnodiales (Dothideomycetes), but with very low bootstrap support. Therefore, a dataset of three different orders, Capnodiales, Dothideales and Myriangiales from sub-class Dothideomycetidae of Dothideomycetes was assembled. *Pseudotraploa longissima* was selected as the outgroup taxon. *Pseudoepicoccum cocos* formed a distinct lineage thus confirming the distinctiveness of the genus. This study further strengthens the proposition by Ellis (1971) who separated this taxon from *Epicoccum* based on morphological characters. The genus *Epicoccum* is currently placed in the Pleosporales (Fig. 1.) (Zhang *et al.* 2012). This study shows that *Pseudoepicoccum* can be best placed in order Capnodiales, but remains Incertae Sedis at family level. Further multigene phylogeny is required to resolve its placement in Capnodiales. Sexual state for *Pseudoepicoccum* is so far unknown.

The results of Bayesian analysis showed identical overall topology (data not shown; TreeBASE study S14827).

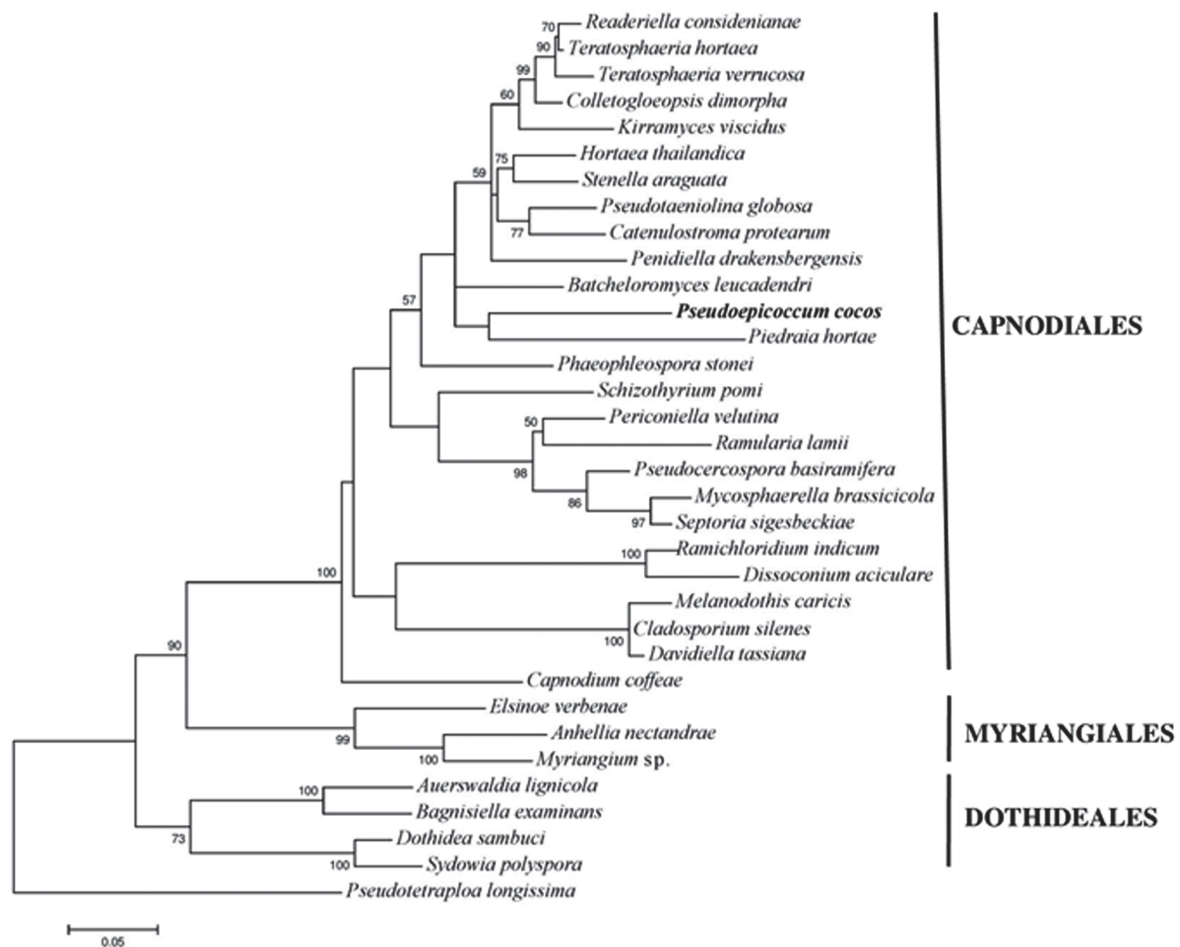


FIGURE 3. Maximum likelihood tree of *Pseudoepicoccum cocos* and related taxa based on combined analysis of ITS and LSU sequences. Species described in this study is in bold.

Taxonomy

Bahusandhika indica (Subram.) Subram., J. Indian bot. Soc. 35: 469 (1956) (Fig. 4)

Basionym: *Polydesmus indicus* Subram., J. Indian bot. Soc. 33: 33 (1954)

Colonies on natural substrate effuse, black, powdery. *Mycelium* partly immersed, partly superficial, composed of smooth, subhyaline, septate, branched, 2–3 µm wide hyphae. Sexual state: Unknown. Asexual state: *Conidiophores* 5–8 × 2–2.5 µm, micronematous, formed on lateral hyphae. *Conidigenous cells* 3–5 × 3–4 µm, monotretic,

integrated or discrete, terminal, formed on conidiophores as well as on conidia, light to pale brown, subspherical, smooth. *Conidia* 12–17 × 5–8 μm, catenate, in long, branched chains, dark brown, verrucose, thick-walled, ellipsoidal, 1–3-septate, with 1–2 connecting cells; connecting cells spherical, light brown, smooth, 3–4 μm. *Colonies* on MEA greyish brown, irregular in shape, cottony, attaining a diam. of 2.3 cm in 7 days; reverse dark brown.

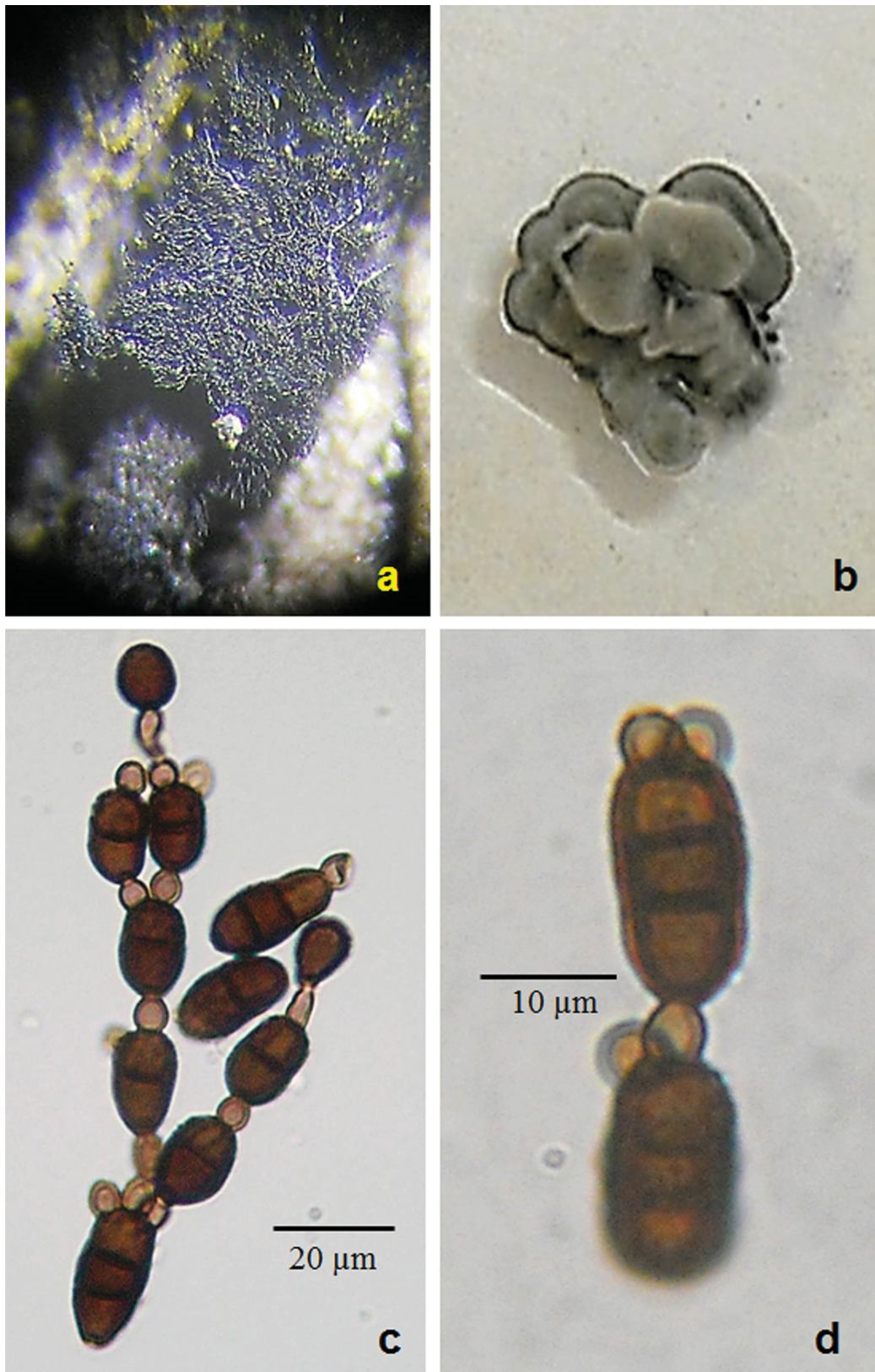


FIGURE 4. *Bahusandhika indica*. a. Colony on host. b. Culture on MEA. c–d. Conidial chains with conidiogenous cells.

Specimen examined—INDIA. Goa: Canacona, Mashem, on floral litter of *Cocos nucifera*, 19 October 2012, A. Prabhugaonkar (HCIO 51509). Extype living culture = MTCC 11761!

Notes:—The genus *Bahusandhika* was established with *B. Indica* (1956: 46) as the type species to accommodate *Polydesmus indicus* (1954: 33), isolated from coconut litter. At the same time he also transferred *Septonema intercalare* E.K. Cash & A.M.J. Watson (1955: 744), isolated from leaves, stems and pseudobulbs of orchids, to *Bahusandhika*, as *B. intercalaris* (E.K. Cash & A.M.J. Watson) Subram. Prasher & Verma (2012) described *B. indica* from the Himalaya region. *Bahusandhika caligans* Bat. & H.P. Upadhyay (1965: 321) was transferred to *Torula*, as *T. caligans* (Batista & Upadhyay) Ellis (1971: 337). *Bahusandhika sundara* Rao & D. Rao (1972: 289) was described from dead twigs of an unidentified plant from Andhra Pradesh, India, while *B. compacta* Rikhy, Mukerji & G. Malhotra (1975: 91) was later added to the genus. Four species are now accepted in the genus (Index Fungorum 2014).

Phylogenetic analysis shows that *Bahusandhika* has close affinities with *Berkleasium* in the order Pleosporales. *Berkleasium* is distinct in having sporodochia with macronematous conidiophores that are mostly unbranched and closely packed, producing monoblastic conidiogenous cells and conidia that are solitary, brown, muriform, clavate or oblong, with rounded or irregular ends and often with a protruding hilum (Ellis 1971).

Cancellidium applanatum Tubaki, Trans. Mycol. Soc. Japan 16(4): 368 (1975) (Fig. 5)

Colonies on natural substrate, effuse, black, velvety. *Mycelium* partly immersed, partly superficial, composed of septate, hyaline, smooth, 2–3 µm wide hyphae. Sexual state: Unknown. Asexual state: *Conidiophores* mononematous, micronematous. *Conidiogenous cells* monoblastic, integrated, terminal, determinate. *Conidia* 105–190 × 60–158 µm, solitary, dictyoseptate, muriform, flattened, dark brown, thick-walled, smooth, internally containing branched chains of blastoconidia, cicatrized, 8–10 × 4–9 µm monilioid cells, developing from the base. *Colonies* on MEA light green, irregular in shape, powdery, attaining 1 cm diam. in 7 days; reverse dark green. *Mycelium* composed of filamentous, septate, branched, smooth hyaline hyphae producing branched chains of blastoconidia, cicatrized, 7.5–12 × 4–8 µm monilioid cells.

Specimen examined:—INDIA. Goa: Sanguem, Netravali, on dead twig of unidentified plant, 19 October 2012, J. Pratibha (MTCC 11763!)

Notes:—*Cancellidium* Tubaki (1975: 358), with *C. applanatum* as the type species, was described as an aero-aquatic hyphomycete, originally from Japan. It has subsequently been isolated from a variety of substrates in different parts of the world, both tropical and temperate (Webster & Davey 1980, Shaw 1994, Goh 1997, Fryar *et al.* 2004, Luo *et al.* 2004, Tsui & Hyde 2004). The genus is characterised by dictyosporous, flattened, dark brown, obclavate to oval conidia formed singly on small conidiophores (Tubaki 1975). Shaw (1994) described the presence of chains of monilioid cells inside the conidia. Yeung *et al.* (2006) added another species *C. pinicola* from leaf litter of *Pinus massoniana*, collected from Hong Kong.

Pseudoepicoccum cocos (F. Stevens) M.B. Ellis, Dematiaceous Hyphomycetes: 270 (1971) (Fig. 6)

Basionym: *Epicoccum cocos* F. Stevens, Philipp. Agric. 21: 81 (1932)

Colonies on leaf spots brown, with circular zones, with blackened elevated centres and yellow margins, up to 1 cm diam. *Stromata* superficial, hemispherical, brown, pseudoparenchymatous. Sexual state: Unknown. Asexual state: *Sporodochia* 90–110 × 50–75 µm, punctiform, dark brown. *Conidiophores* 13–18 × 3–4 µm, macronematous, mononematous, straight or slightly flexuous, septate, unbranched, pale brown, smooth-walled. *Conidiogenous cells* 3–5 × 3–4 µm, integrated, terminal, sympodial, cicatrized. *Conidia* 3–5.5 × 2.5–3 µm, solitary, dry, aseptate, simple, acropleurogenous, subspherical, pale brown, minutely verrucose. *Colonies* on MEA dark green, irregular, powdery, attaining 0.8 cm diam. in 7 days; reverse dark green to black.

Specimen examined:—INDIA. Goa: Canacona, Mashem, on living leaves of *Cocos nucifera*, 19 October 2012, A. Prabhugaonkar (MTCC 11762!). PHILIPPINES. Entomological Exp. Field, Laguna, on leaves of *Cocos nucifera*, 29 Jul 1930, F.L. Stevens (MICH15584, isotype).

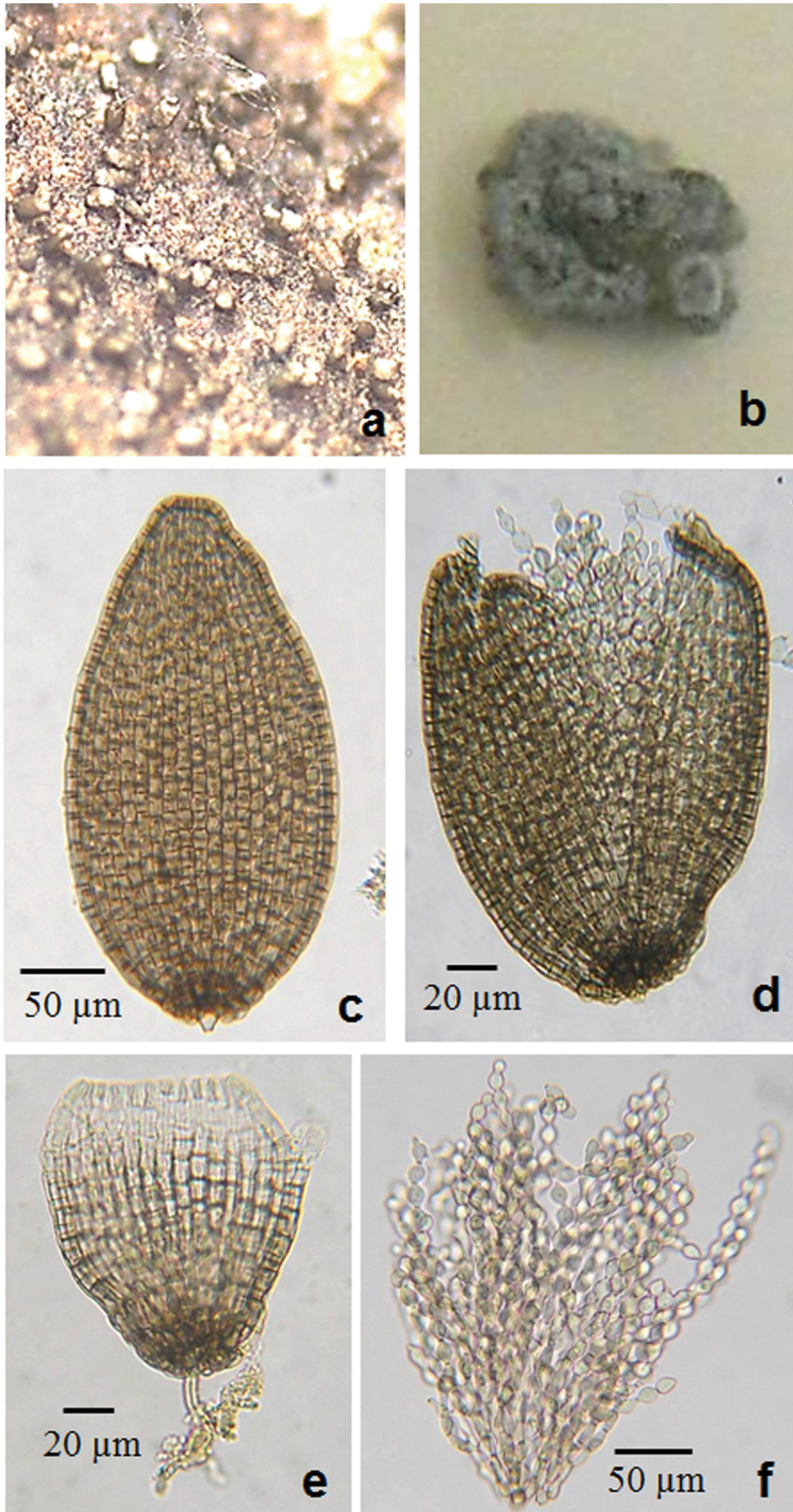


FIGURE 5. *Cancellidium applanatum*. a. Colony on host. b. Culture on MEA. c–e Conidia. f. Chains of moniloid cells.

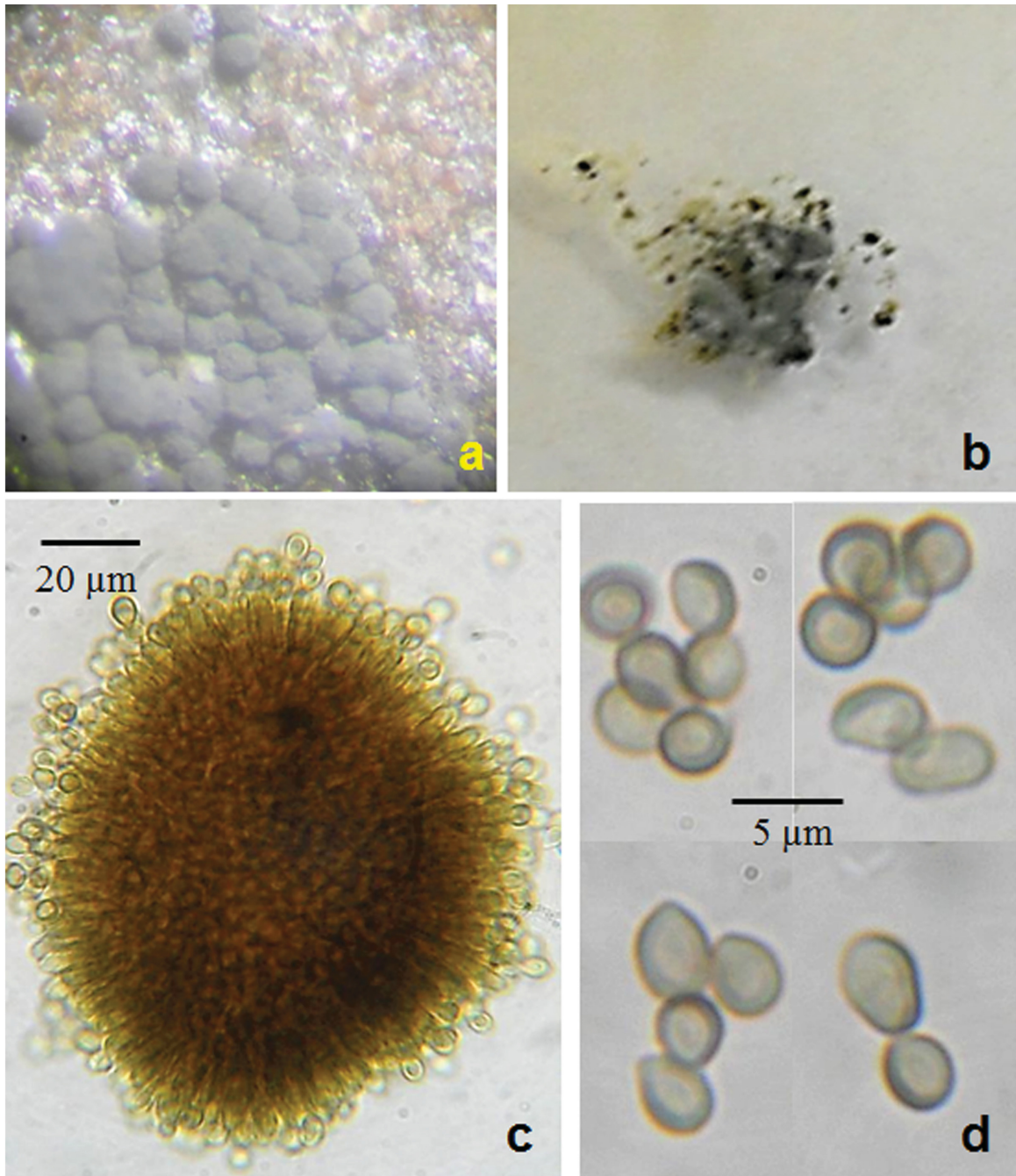


FIGURE 6. *Pseudoepicoccum cocos*. a. Colonies on leaf spots. b. Culture on MEA. c. Sporodochia. d. Conidia.

Notes:—Ellis (1971) established the genus *Pseudoepicoccum* to accommodate *Epicoccum cocos* Stevens (1932: 81), isolated from coconut leaves in Philippines, with *P. cocos* as type species. Sharma *et al.* (1985) added *P. tectonae* Sharma & Mohanan isolated from leaves of *Tectona grandis* from Kerala, India, as a second species in the genus. We observed the type specimen of *Epicoccum cocos*, which proved to be identical to our specimen.

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