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

PRATIBHA, J.[1], PRABHUGAONKAR, A.[1,2], HYDE, K.D.[3,4] & BHAT, D.J.[1]

[1] *Department of Botany, Goa University, Goa 403206, India*
[2] *Nurture Earth R&D Pvt Ltd, MIT Campus, Aurangabad-431028, India;*
email: ashishprabhugaonkar@yahoo.co.in
[3] *Institute of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand*
[4] *School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand*

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 *Berkleasmium* 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*,

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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, Thiruvananthpuram, 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
Akanthomyces novoguineensis	JN201872	JN201874
Aliquandostipite khaoyaiensis	JN819278	EF175650
Amphiporthe hranicensis	DQ323525	DQ323521
Anhellia nectandrae	JQ071524	JQ071523
Apiognomonia errabunda	DQ313525	NG_027592
Aplosporella prunicola	EF564375	EF564377
Aquaticola hongkongensis	AF177156	AF132321
Auerswaldia lignicola	JX646798	JX646815
Bagnisiella examinans	EU167562	GU301803
Bahusandhika indica	**KF460273**	**KF460274**
Batcheloromyces leucadendri	JF499832	JF499852
Berkleasmium micronesicum	DQ280262	DQ280272
Berkleasmium nigroapicale	DQ280261	DQ280273

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TABLE 1. (*continued*)

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

..... *continued on the next page*

TABLE 1. (*continued*)

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

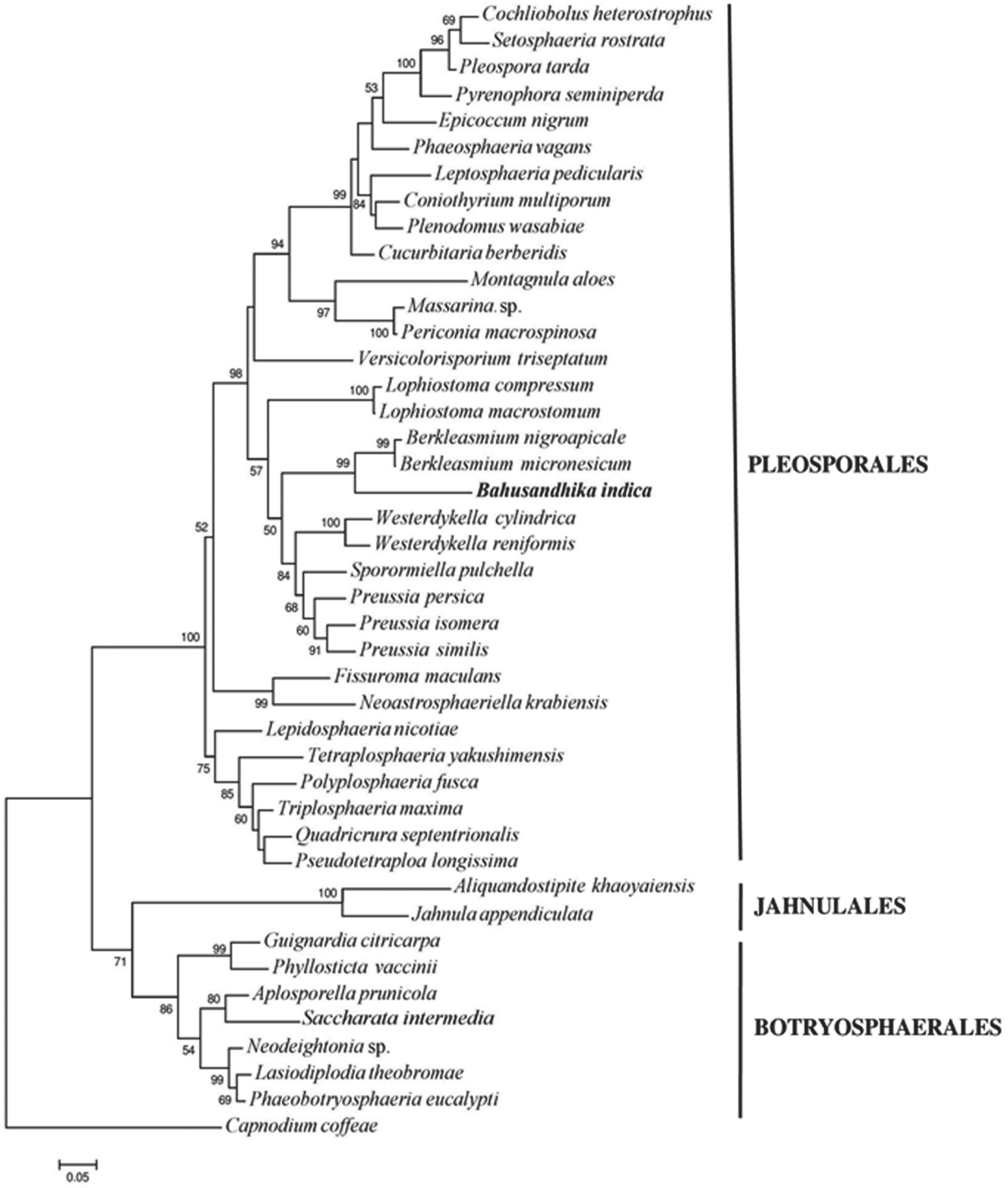

FIGURE 1. Maximum likelihood tree of *Bahusandhika indica* and related taxa based on combined analysis of ITS and LSU sequences. Species described in this study is in bold.

A dataset of three orders, Pleosporales, Botryosphaeriales 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 *Berkleasmium nigroapicale*, *B. micronesicum* and *Westerdykella* which are members of family Sporormiaceae (Pinnoi *et al.* 2007). The sexual states of *Bahusandhika* and *Berkleasmium* 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, ie. 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, Calosphaeriales, 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).

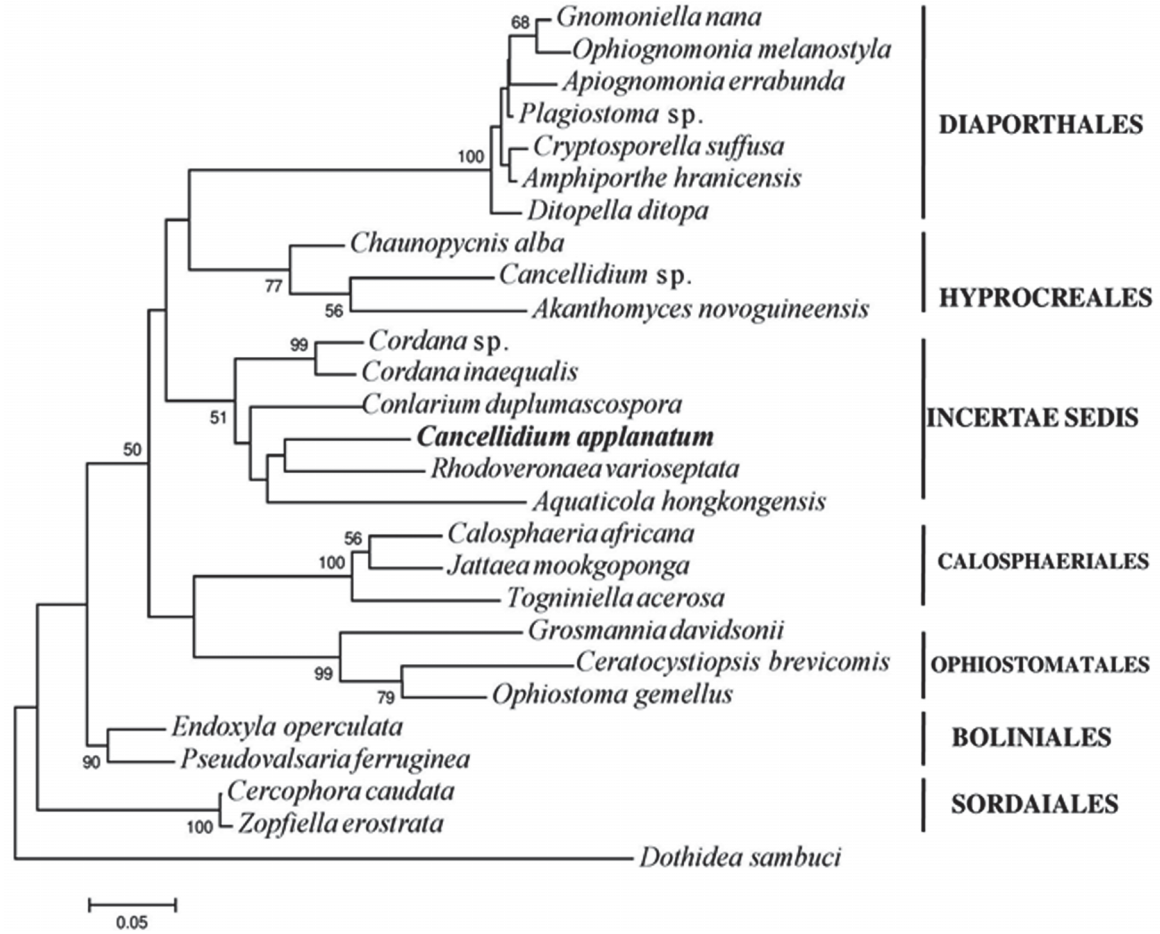

FIGURE 2. Maximum likelihood tree of *Cancellidium applanatum* and related taxa based on combined analysis of ITS and LSU sequences. Species described in this study is in bold.

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 Myringiales from sub-class Dothideomycetidae of Dothideomycetes was assembled. *Pseudotetraploa 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 Baysian analysis showed identical overall topology (data not shown; TreeBASE study S14827).

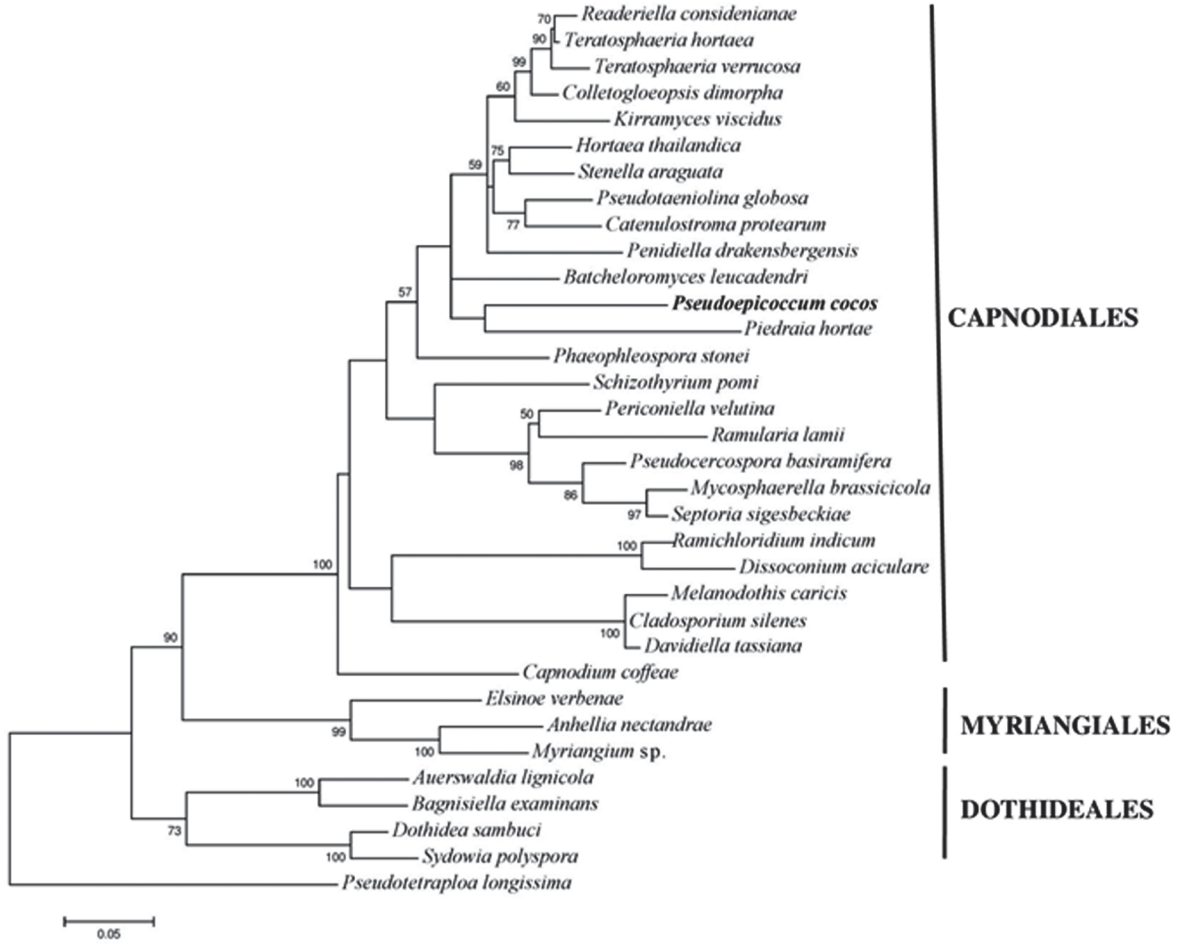

FIGURE 3. Maximum likelihood tree of *Pseudoepicoccum cocos* and related taxa based on combined analysis of ITS and LSU sequences. Species described in this study is in bold.

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. *Conidiogenous 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.

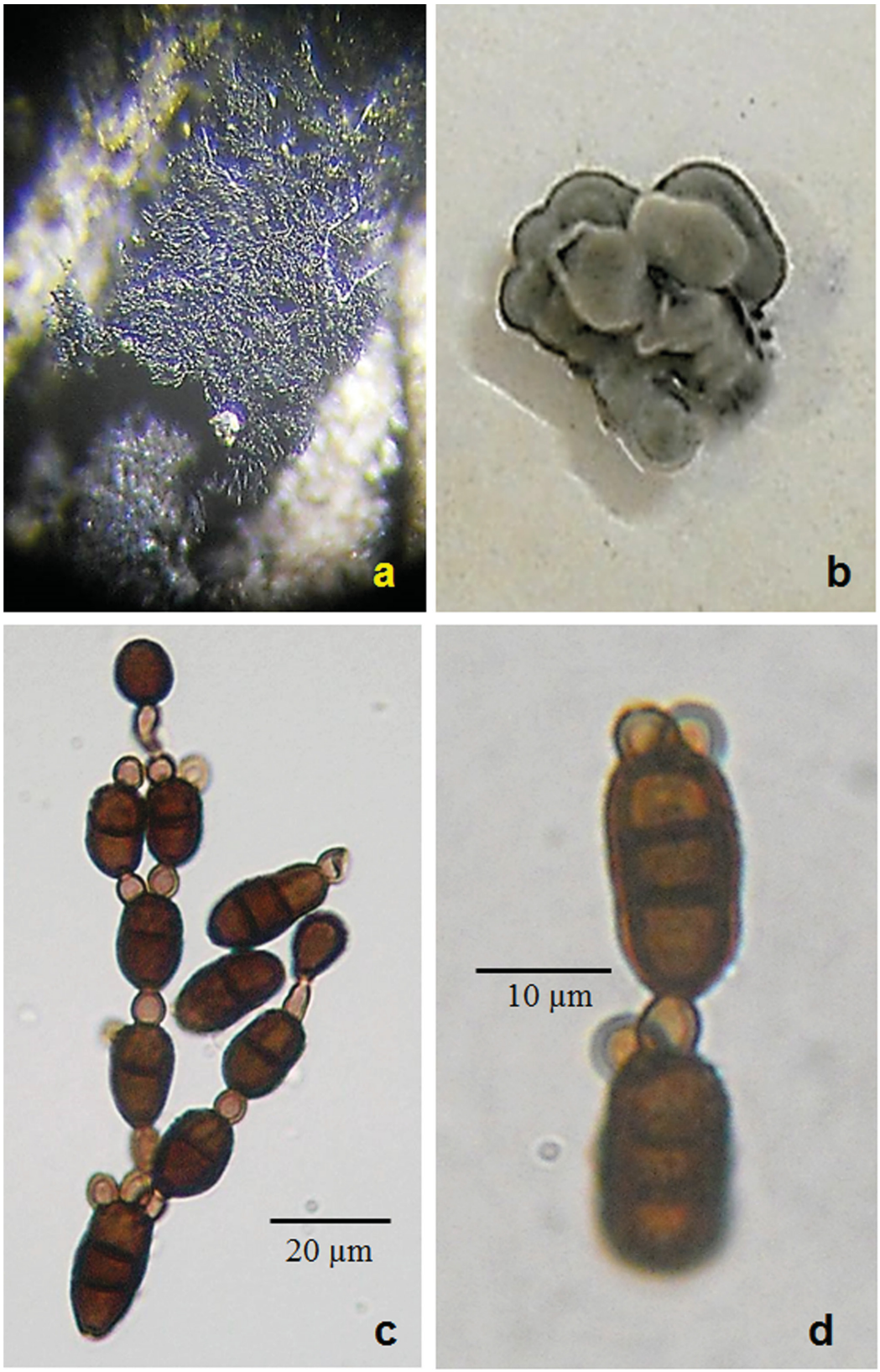

FIGURE 4. *Bahusandhika indica.* a. Colony on host. b. Culture on MEA. c–d. Conidial chains with conidiogenous cells.

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 caligens* 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 *Berkleasmium* in the order Pleosporales. *Berkleasmium* 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 blastic, 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 blastic, 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 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).

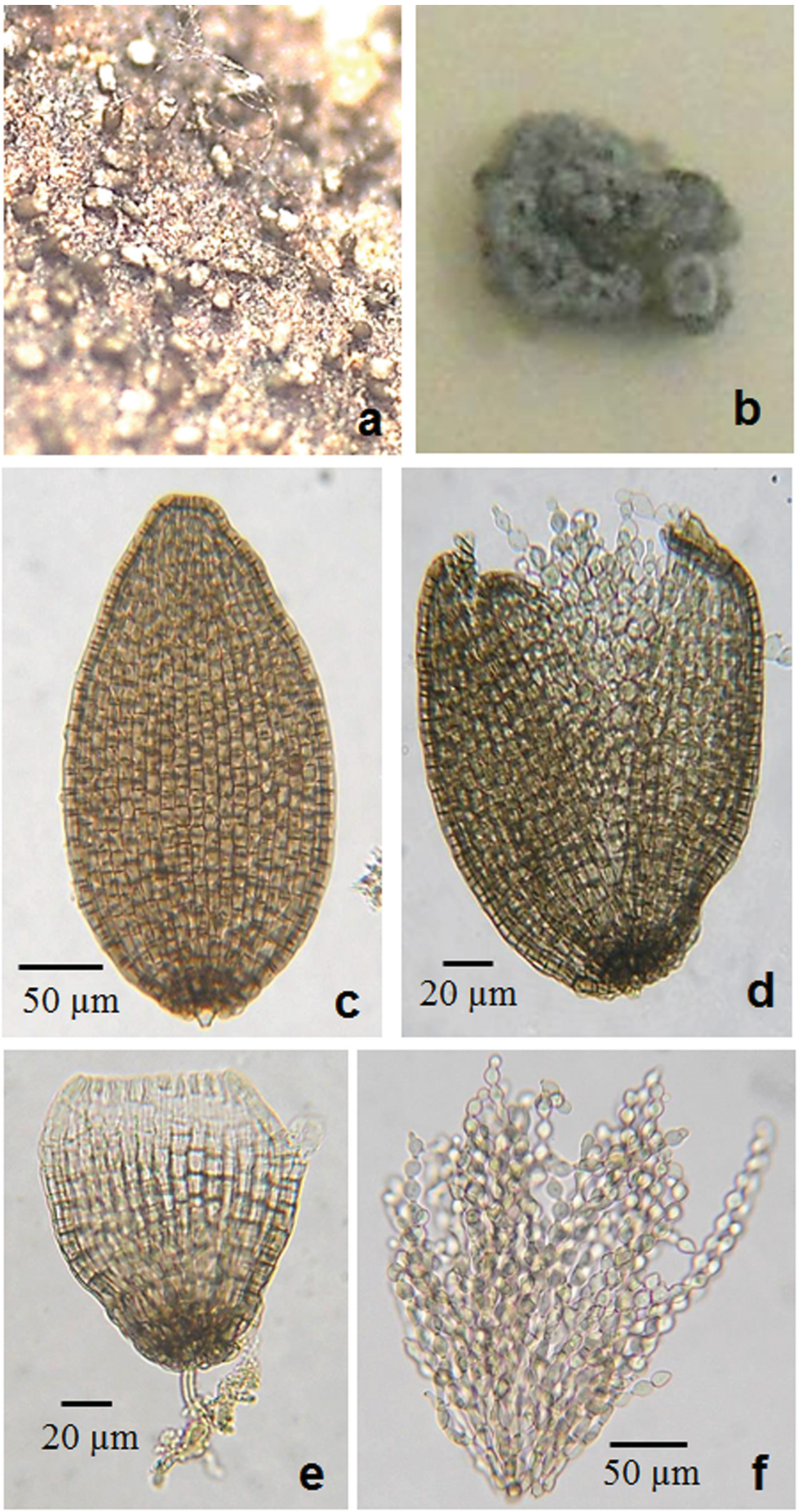

FIGURE 5. *Cancellidium applanatum.* a. Colony on host. b. Culture on MEA. c–e Conidia. f. Chains of monilioid cells.

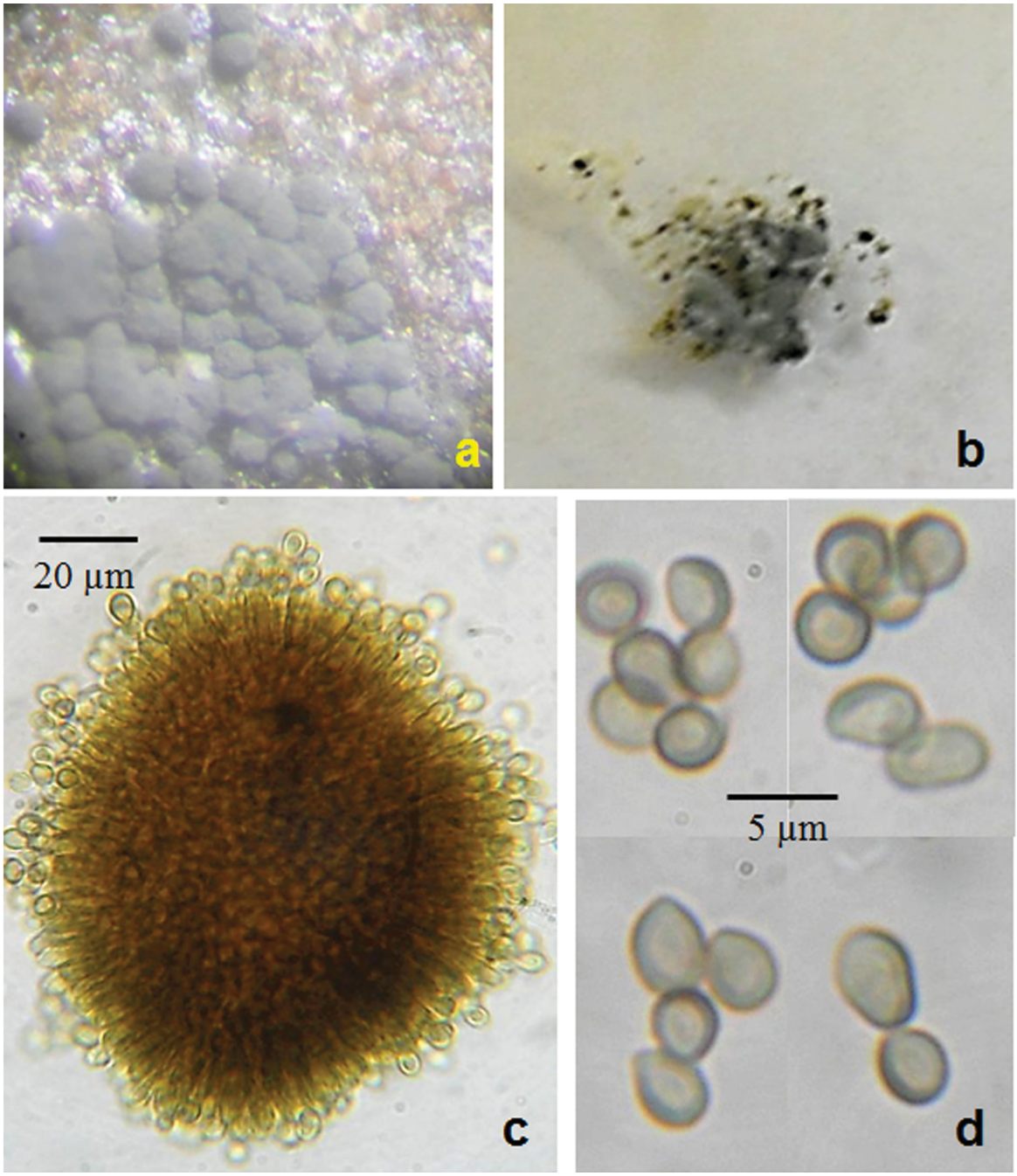

FIGURE 6. *Pseudoepicoccum cocos*. a. Colonies on leaf spots. b. Culture on MEA. c. Sporodochia. d. Conidia.

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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