

Research article

Candolleomyces asiaticus sp. nov. (Psathyrellaceae, Agaricales), a novel species from Punjab, Pakistan

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Abstract. During mycological surveys of different areas of Punjab, Pakistan, we collected a new species from the genus *Candolleomyces*. This is the first report of any species of this genus from Pakistan. *Candolleomyces asiaticus* M.Asif, A.Izhar, Niazi & Khalid sp. nov. is characterized by ellipsoid to oblong-ellipsoid ($8.2 \times 5 \mu\text{m}$) basidiospores with indistinct germ pores, lageniform to utriform cheilocystidia without adhering deposits at the apices, absence of pleuro-, pileo- and caulocystidia, pileipellis with spherical, subglobose to columnar cells, and presence of clamp connections. The morpho-anatomical and molecular phylogenetic analysis of nrITS revealed its distinct phylogenetic position, further supporting the recognition of a new species.

Keywords. Bahawalnagar, Basidiomycota, phylogeny, saprotrophic, Sheikhpura.

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Introduction

Psathyrellaceae Vilgalys, Moncalvo & Redhead is a small to large, dark-spored mushroom-forming family. Many of its species are difficult to recognize and are eminent for their ability to digest themselves using autodigestive chitinases (Kües 2000). All the members of *Psathyrellaceae* have brittle and fragile basidiomata. They are habitually saprotrophic and can grow on dead wood and plant debris, or more rarely live on dung, or parasitize other fungi (Smith 1972; Kits van Waveren 1985; Singer 1986; Büttner *et al.* 2020). Three new genera has recently been separated from *Psathyrella* (Fr.) Quél., resulting in four genera, including *Psathyrella*, *Britzelmayria* D.Wächt. & A.Melzer, *Candolleomyces* D.Wächt. & A.Melzer, and *Olotia* D.Wächt. & A.Melzer, based on comprehensive molecular phylogenetic analyses (Wächter & Melzer 2020).

Candolleomyces includes the taxa with small to large basidiomata, which can grow terrestrially, lignicolously, or rarely fimicolously (Wächter & Melzer 2020). *Candolleomyces* is differentiated

from other closely related genera, i.e., *Britzelmayria*, *Olotia* and *Psathyrella*, based on the absence of pleurocystidia (Örstadius *et al.* 2015; Büttner *et al.* 2020; Wächter & Melzer 2020). About 100 species of *Psathyrella* have been reported without pleurocystidia (Fries 1838; Smith 1972; Kits van Waveren 1985; Örstadius & Kundsén 2012; Battistin *et al.* 2014). Recently, 26 species have been transferred to the genus *Candolleomyces* (Wächter & Melzer 2020).

No species of *Candolleomyces* have yet been reported from Pakistan. In this paper, we present the first-ever species of *Candolleomyces* from Pakistan, that was collected from two different locations (Bahawalnagar and Sheikhpura) in Punjab. The collected specimens are described as new species based on micro- and macro-morphological as well as molecular phylogenetic analyses.

Material and methods

Sampling and morpho-anatomical observations

The holotype was collected from Haroonabad, District Bahawalnagar (Coordinates: 29°6081 N, 73°1468 E, 163 m), a semi-arid region, adjacent to Cholistan desert, Punjab, Pakistan. This region is characterized by a dry and hot climate, with temperatures ranging from 11°C to 50°C. Monsoons are the principal source of rainfall in the region, here mean annual precipitation is 99 mm (Ahmed *et al.* 2014a, 2014b).

Fresh basidiomata were collected from two different locations (Bahawalnagar and Sheikhpura) in Punjab, Pakistan. Habit, habitat, location, and soil type were noted and pictures were taken of the specimens with color codes given following Munsell's soil color chart (1975), and terminology following Vellinga (2001). Then the specimens were brought to the laboratory and characterized morphologically and microscopically. Specimens were dried with a fan heater at 40–50°C and deposited in the LAH Herbarium, Institute of Botany, University of the Punjab, Lahore, Pakistan.

For the anatomical study, slides were made with free-hand sections rehydrated in 5% KOH (w/v), stained in 1% aqueous Congo Red (w/v) and Melzer's reagent following the microscopic techniques of Liang & Yang (2011), Cai *et al.* (2018) and Liang *et al.* (2018). The anatomical characteristics, i.e., shape and size of basidiospores, cheilocystidia, basidia, structure, and hyphae of pileus covering and stipe covering, were examined using a light microscope (CXRII, Labomed Labo America Inc., Fremont, CA, USA) through an HDCE-X5 microscopic camera under 400× and oil immersion 1000× magnification. Data for morpho-anatomical features was based on at least 60 measurements each of basidia, cheilocystidia, and basidiospores. The notation 'n/b/p' indicates 'n' basidiospores measured from 'b' basidiomata from 'p' collections. In descriptions, the measurements are displayed as (a)b–c(d), where a is the lowest value, b–c covers at least 90% of values, and d is the highest value, while 'Q' is the length-width ratio of a spore (Bas 1969; Yu *et al.* 2020); and $av_l \times av_w$ designates average length and average width.

DNA extraction, PCR, and sequencing

Dried herbarium specimens (10 mg) were used for DNA extraction using a 2% CTAB protocol (Bruns 1995) with some modifications as proposed by Zhao *et al.* (2011). The Internal Transcribed Spacer (ITS) region of nrDNA was used for molecular analysis using ITS1F/ITS4 primer pairs for PCR (White *et al.* 1990; Gardes & Bruns 1993). PCR was carried out using a 25 µl reaction mixture in a thermocycler (Perkin-Elmer, Applied Biosystems). PCR amplification program was as follows: 5 min at 95°C, followed by 15 rounds of 1 min at 95°C, 30 s at 65°C (lowered by 1°C per cycle) and 1 min at 72°C, followed by 20 rounds of 1 min at 95°C, 30 s at 50°C and 1 min at 72°C, with a final extension of 10 min at 72°C (Yan & Bau 2018). Bidirectional sequencing was carried out at TsingKe (China) using the same primer pairs as used for PCR. The newly generated sequences were deposited in GenBank under accession numbers OK392606, OK392596, and OK392605.

Table 1 (continued on next page). Fungal species used for phylogenetic analyses of *Candolleomyces* D. Wächt. & A. Melzer including their current names, GenBank entry species name, locality, and GenBank accession numbers of the ITS regions. Sequences generated during this study are shown in bold.

Species (current name)	Species (GenBank entry)	Locality	GenBank No.
<i>Candolleomyces</i>	<i>Psathyrella</i>		
<i>C. aberdarensis</i>	<i>P. aberdarensis</i>	Kenya	MH880928
<i>C. aberdarensis</i>	<i>P. aberdarensis</i>	Kenya	MK421517
<i>C. albipes</i>	<i>P. albipes</i>	São Tomé	KX017209
<i>C. asiaticus</i> sp. nov. holotype	–	Pakistan	OK392605
<i>C. asiaticus</i> sp. nov.	–	Pakistan	OK392606
<i>C. asiaticus</i> sp. nov.	–	Pakistan	OK392596
<i>C. badhyzensis</i>	<i>P. badhyzensis</i>	Sweden	KC992883
<i>C. badiophylla</i>	<i>P. badiophylla</i>	Hungary	FN430699
<i>C. cacao</i>	<i>P. cacao</i>	São Tomé	NR148106
<i>C. cacao</i>	<i>P. cacao</i>	USA	KU847452
<i>C. cacao</i>	<i>P. cacao</i>	USA	KU847436
<i>C. candolleanus</i>	<i>P. candolleana</i>	China	MZ145108
<i>C. candolleanus</i>	<i>P. candolleana</i>	China	MZ145109
<i>C. cladii-marisci</i>	<i>P. cladii-marisci</i>	Thailand	MZ145228
<i>C. cladii-marisci</i>	<i>P. cladii-marisci</i>	Italy	MK080112
<i>C. efflorescens</i>	<i>P. efflorescens</i>	Sweden	KC992941
<i>C. eurysporus</i>	–	Vietnam	MT651560
<i>C. eurysporus</i>	–	Vietnam	NR172427
<i>C. luteopallida</i>	<i>P. luteopallida</i>	China	MG734736
<i>C. luteopallida</i>	<i>P. luteopallida</i>	Sweden	KC992884
<i>C. luteopallida</i>	<i>P. luteopallida</i>	Sweden	KC992885
<i>C. singer</i>	<i>P. singer</i>	China	MW301073
<i>C. singer</i>	<i>P. singer</i>	China	MG734718
<i>C. sp.</i>	<i>P. sp.</i>	India	KR154976
<i>C. sp.</i>	<i>P. sp.</i>	India	KR154977
<i>C. sp.</i>	<i>P. sp.</i>	India	KP686452
<i>C. sp.</i>	<i>P. sp.</i>	India	KP686449
<i>C. sp.</i>	<i>P. sp.</i>	Mexico	KR003281
<i>C. subcacao</i>	–	China	MW301064
<i>C. subcacao</i>	–	China	MW301065
<i>C. subcacao</i>	–	China	MW559219
<i>C. subcacao</i>	–	China	MW559218
<i>C. subcacao</i>	–	China	MW559220

Table 1 (continued). Fungal species used for phylogenetic analyses of *Candolleomyces* D.Wächt. & A.Melzer including their current names, GenBank entry species name, locality, and GenBank accession numbers of the ITS regions. Sequences generated during this study are shown in bold.

Species (current name)	Species (GenBank entry)	Locality	GenBank No.
<i>Candolleomyces</i>	<i>Psathyrella</i>		
<i>C. subminutisporus</i>	–	China	MW301066
<i>C. subminutisporus</i>	–	China	MW301067
<i>C. subsingeri</i>	<i>P. subsingeri</i>	China	MG734725
<i>C. subsingeri</i>	<i>P. subsingeri</i>	China	MG734715
<i>C. sulcatotuberculosis</i>	<i>P. sulcatotuberculosis</i>	Italy	KJ138423
<i>C. sulcatotuberculosis</i>	<i>P. sulcatotuberculosis</i>	China	MW375696
<i>C. sulcatotuberculosis</i>	<i>P. sulcatotuberculosis</i>	Germany	KJ138422
<i>C. trinitatensis</i>	<i>P. trinitatensis</i>	Sweden	KC992882
<i>C. tuberculata</i>	<i>P. tuberculata</i>	Sweden	KC992886
<i>P. multipedata</i> (outgroup)	–	Sweden	KC992888

Phylogenetic analysis

Using the results of BLAST searching against GenBank and the published work of Bau & Yan (2021) and Büttner *et al.* (2020), we performed an analysis of a total of 43 ITS sequences, including 42 sequences of the genus *Candolleomyces* and an outgroup taxon, i.e., *Psathyrella multipedata* (Peck) A.H.Sm. (KC992888) (Table 1). MUSCLE ver. 3.8 (Edgar 2004) was used for the alignment of the sequences and BioEdit ver. 7.2.5. (Hall 1999) was used for manual adjustment. The ITS dataset's maximum likelihood (ML) analysis was done using RAxML-HPC2 ver. 8.1.11 (Stamatakis 2014) on CIPRES Portal ver. 3.1. (Miller *et al.* 2010). ML analyses were carried out by applying the ultrafast bootstrap approximation with 1000 replicates. FigTree ver. 1.4.3 (Rambaut 2014) was used for displaying the phylogenetic tree that was exported to Adobe Illustrator for final editing.

Results

Phylogeny

The fragment size of the target region was 645 bp. The ITS sequences of *Candolleomyces asiaticus* sp. nov. showed 99 % sequence similarity with *Candolleomyces* sp. (KP686449, KP686452, KR154976, and KR154977) and 98 % similarity with *Candolleomyces cacao* (Desjardin & B.A.Perry) D.Wächt. & A.Melzer (NR148106). The phylogenetic tree (Fig. 1) shows that the newly generated sequences of *Candolleomyces asiaticus* form a distinct lineage along with undescribed and unpublished Indian taxa of *Candolleomyces* (KP686449 and KP686452) with a high bootstrap support of 99.8%. The new species is sister to *Candolleomyces cacao* (KU847452). The next closest species in the tree is *Candolleomyces subcacao* T.Bau & J.Q.Yan (MW559220) with a high bootstrap value of 95%. When we compared nrITS sequences generated from the present study with the sequences of *C. cacao* and *C. subcacao*, 11 nucleotides difference in the sequences of *C. cacao* and 14 differences in nrITS sequences of *C. subcacao* were observed.

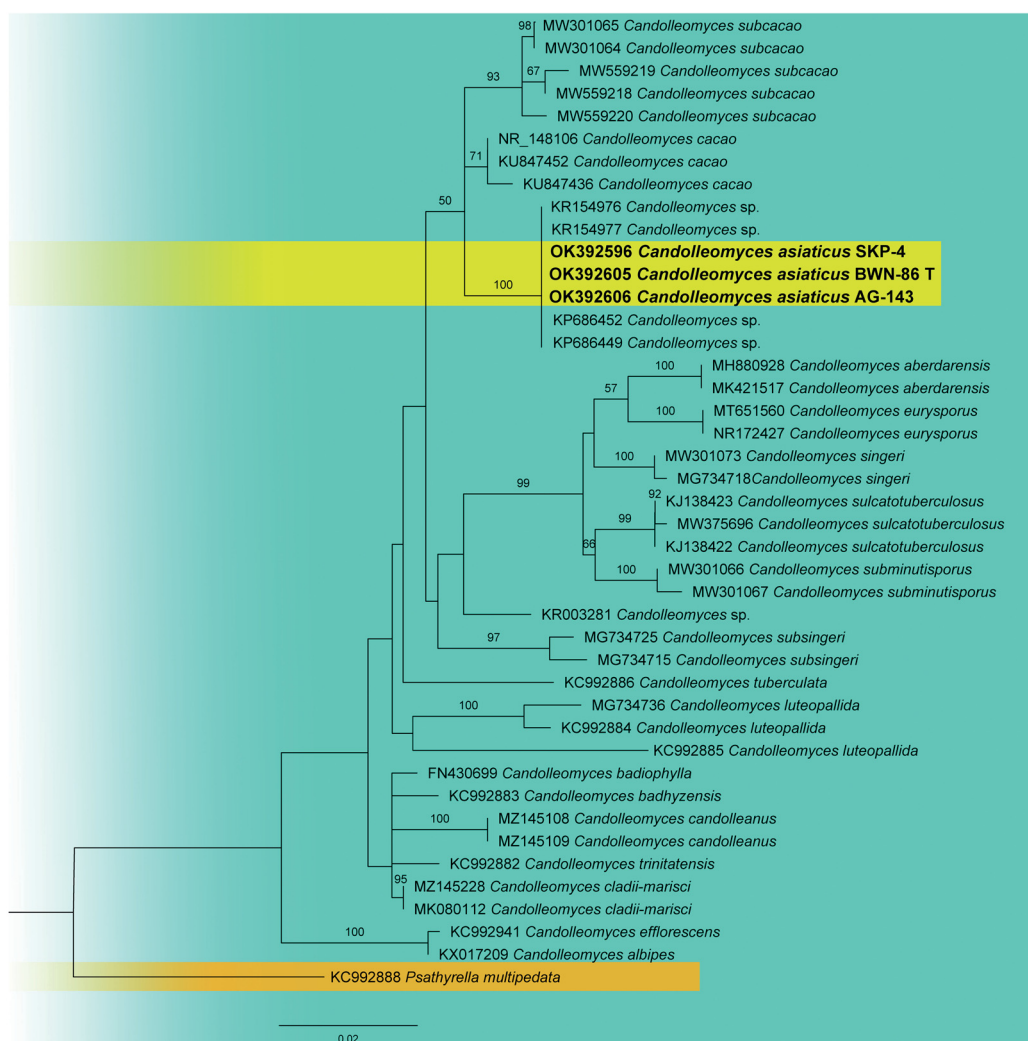


Fig. 1. Molecular phylogenetic placement of *Candolleomyces asiaticus* M.Asif, A.Izhar, Niazi & Khalid sp. nov. based on maximum likelihood (ML) method of nrITS sequences.

Taxonomy

Phylum Basidiomycota R.T.Moore
 Class Agaricomycetes Doweld
 Order Agaricales Underw.
 Family Psathyrellaceae Vilgalys, Moncalvo & Redhead
 Genus *Candolleomyces* D.Wächt. & A.Melzer

Candolleomyces asiaticus M.Asif, A.Izhar, Niazi & Khalid sp. nov.

Mycobank: MB841351

Figs 2–3

Diagnosis

Differs from *Candolleomyces cacao* by the combination of the following characters: broad spores (up to 6.3 µm) with indistinct germ pore, absence of caulocystidia, and whitish evanescent veil.

Etymology

The specific epithet '*asiaticus*' refers to the continent from where it was collected.

Type material

Holotype

PAKISTAN • Punjab Province, Haroonabad City, District Bahawalnagar; 29°60'81" N, 73°14'67" E; alt. 163 m a.s.l.; on nutrient-rich loamy soil; 4 Oct. 2019; *Muhammad Asif*, *BWN-86*; GenBank No. OK392605 (nrITS); LAH[36809].

Additional material examined

PAKISTAN – **Punjab Province** • Sheikhpura City; 31°42'40" N, 73°59'16" E; 236 m a.s.l.; on muddy soil; 23 Jul. 2017; *Aiman Izhar*, *SKP-04*; GenBank No. OK392596 (nrITS); LAH[35718] • Haroonabad City, District Bahawalnagar; 29°60'81" N, 73°14'67" E; 163 m a.s.l.; on nutrient-rich loamy soil; 8 Sep. 2020; *Muhammad Asif*, *AG-143*; GenBank No. OK392606 (nrITS); LAH[36975].

Description

Pileus 3–6.3 cm, plano-convex to applanate, hygrophanous, light grey (10YR8/1) to brownish grey (10YR4/1), subumbonate, some slightly depressed at the center, disc dull brown (7.5YR5/4), surface shiny, silky fibrillose, fibrils crowded at the center, radially translucent striate, striations prominent near margins, margins irregular, splitting on full maturity. Pileal veil white, fragile, powdery evanescent. Lamellae about 0.1–0.2 cm broad, moderately close, adnate to adnexed, in shades of dull orange (7.5YR7/4) to brown (10YR4/3), edges even, serrate, lamellulae present in 2–3 different lengths. Stipe 5.5–7 cm long, 0.3–0.5 cm diameter, equal, cylindrical with a small grainy bulb at the base, hollow, fragile, off-white (7.5Y9/2), apex pruinose, covered with white to greyish, evanescent fibrils.

Basidiospores [60/3/3] (6.2–)7.2–7.6(–9.1) × (4–)4.5–6(–6.3) μm , $\text{avl} \times \text{avw} = 7.5 \times 5 \mu\text{m}$, $Q = 1.4\text{--}1.5$, ellipsoid to oblong-ellipsoid, few elongated in face view, slightly flattened in side view, dull orange in water (5YR 6/4), greyish red (10R6/2) in 5% KOH, inamyloid, smooth, thick-walled, multi-guttulate, germ pore indistinct. Basidia 19.3–22.5 × 9.4–10.5 μm , broadly clavate, hyaline, thick-walled, weakly granular, mostly 4-spored, a few 2-spored. Cheilocystidia 21–38 × 9.6–16 μm , hyaline, moderately thick-walled, mostly lageniform to utriform, few fusiform, base with a tapered short to long stipe, abundant, smooth, no adhering deposits or crystals at apex. Pleurocystidia absent. Trama of gills irregular, consisting of septate hyphae, up to 6 μm . Pileipellis consists of thin-walled, hyaline, 2–3 cells deep layers of spherical, subglobose to columnar cells, 17.3–34.6 μm broad interspersed with very few septate 3–5 μm broad hyphae. Stipitipellis cuticle made up of 4.5–7 μm broad parallel, septate hyphae, rarely branched. Caulocystidia absent. Clamps are present throughout.

Habitat

Found from nutrient-rich loamy soil, along the canal bank under *Vachellia nilotica* (L.) P.J.H.Hurter & Mabb. (Fabaceae), and on rotten wood debris in muddy soil.

Distribution

This species is reported for the first time from two different locations in Punjab, Pakistan.

Discussion

In this study we identified a new fungal species of *Candolleomyces* collected from Punjab, Pakistan based on morpho-anatomical features and its phylogenetic placement. The combination of microscopic features, dull orange to greyish red basidiospores with indistinct germ pore, polymorphic, varying

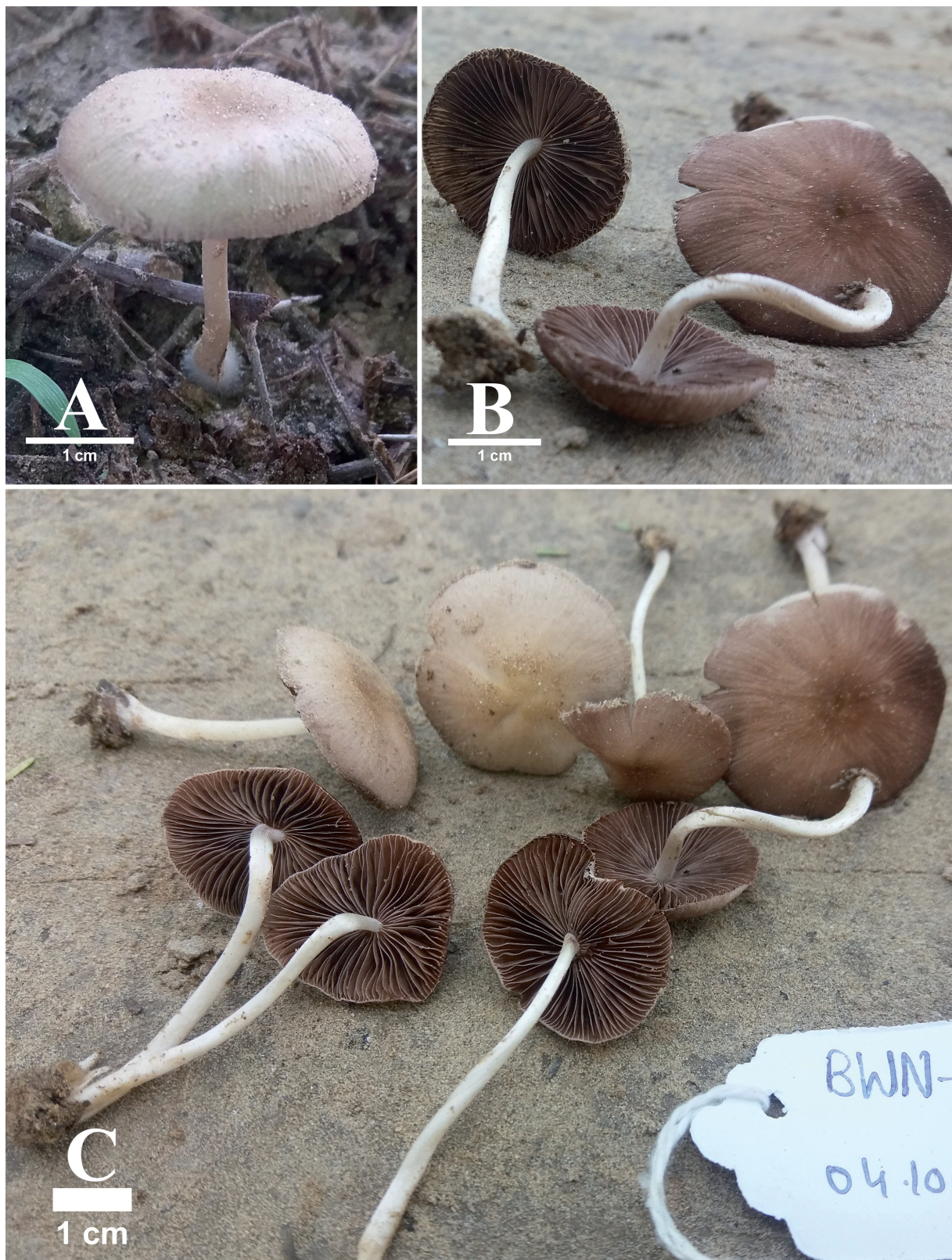


Fig. 2. *Candolleomyces asiaticus* M.Asif, A.Izhar, Niazi & Khalid sp. nov., holotype (LAH36809). Basidiomata. Photos by Muhammad Asif & Aiman Izhar.

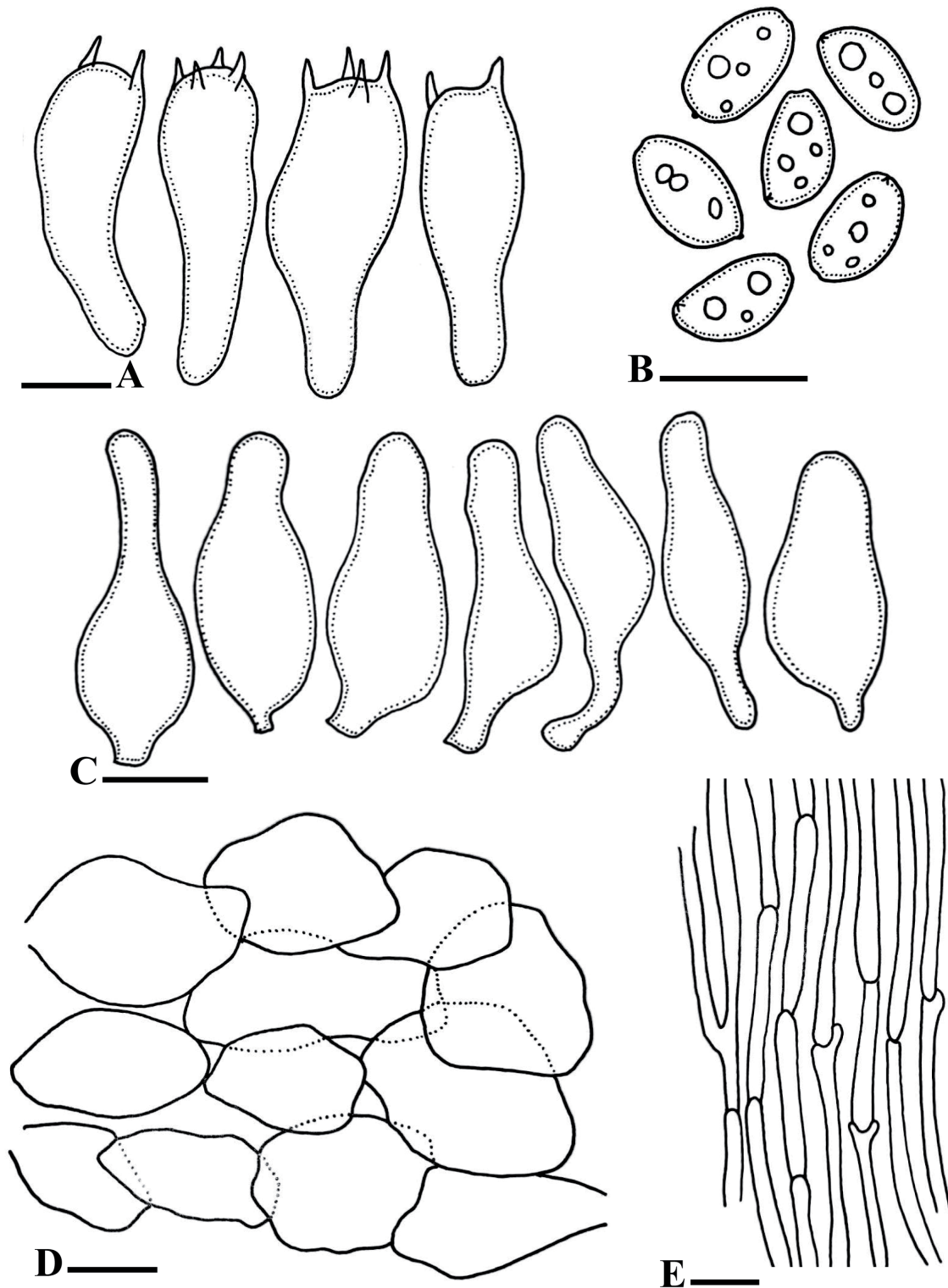


Fig. 3. *Candolleomyces asiaticus* M.Asif, A.Izhar, Niazi & Khalid sp. nov., holotype (LAH36809), line drawings. **A.** Basidia. **B.** Basidiospores. **C.** Cheilocystidia. **D.** Pileipellis. **E.** Stipitipellis hyphae. Scale bars: A–C = 5 µm; D–E = 20 µm. Drawings by Aiman Izhar.

Table 2. Comparison of the diagnostic characters of *Candolleomyces asiaticus* M.Asif, A.Izhar, Niazi & Khalid sp. nov. with phylogenetically close species.

Character	<i>C. asiaticus</i>	<i>C. cacao</i>	<i>C. subcacao</i>	<i>C. sulcatotuberculosis</i>
Basidiospores	6.3–9.1 × 4–6.3 µm	5.7–6.8 × 3.5–4.5 µm	6.8–8.0 × 3.9–4.9 µm	6.2–8.7 × 3.6–5 µm
Q Value	Q = 1.4–1.5	Q = 1.3–1.6	Q = 1.4–1.8	Q = 1.3–1.7
Germ pore	Indistinct	Absent	Absent	Indistinct to absent
Caulocystidia	Absent	Cylindrical to clavate or lageniform	Present at the apex, clavate	Present, polymorphic
Veil	Whitish evanescent	Absent	Whitish evanescent	White velar remnants

from lageniform to utriform, smooth cheilocystidia, lack of pleuro- and caulocystidia, and presence of clamp connections assigns this interesting species to the genus *Candolleomyces* which make it easy to recognize in the laboratory (Fig. 3).

According to the taxonomy of Smith (1972), *C. asiaticus* sp. nov. belongs to *Psathyrella* section *Subatratae* (Romagn.) ex Sing, emended in having an evanescent veil, small basidiospores (5–10 µm long), and absence of pleurocystidia.

The ITS sequences of *C. asiaticus* sp. nov. showed 99.8% similarity with sequences from Indian specimens (GenBank accession numbers KP686449, KP686452, KR154976, and KR154977) (Fig. 1). These specimens formed a well-supported clade with the Pakistani specimens analyzed in this study, clearly distinguishable from other species of *Candolleomyces*. The sequences KR154976 and KR154977 correspond to the vouchers BAB-4747 and BAB-4748 respectively, and KP686449 and KP686452 correspond to the vouchers BAB-4772 and BAB-4775 named as *Psathyrella* species in GenBank. These Indian collections should be also recognized as *C. asiaticus* based on our phylogenetic study, but their morpho-anatomical characteristics need to be studied and confirmed in future studies.

In phylogenetic analysis, *Candolleomyces asiaticus* sp. nov. reveals to be close relative of *C. cacao*, a species described from São Tomé (Africa), but the latter has significant morphological differences such as convex to broadly convex pileus, smaller in diameter (0.5–1.5 cm), lack of partial veil, smaller basidiospores (5.7–6.8 × 3.5–4.8 µm) and frequent occurrence of caulocystidia (Desjardin & Perry 2016). *Candolleomyces asiaticus* sp. nov. is also phylogenetically related to *C. subcacao* but differs from it by its bigger basidiospores (6.3–9.1 × 4–6.3 µm) (vs 6.8–8.8 × 3.9–4.9 µm in *C. subcacao*; Bau & Yan 2021). A comparison of diagnostic characters of phylogenetically close species is also given in Table 2.

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