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**Research article**

# Molecular and morphological studies reveal a new species of *Panaeolus* (Agaricales, Basidiomycota) from Punjab, Pakistan

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**Abstract.** *Panaeolus punjabensis* M. Asif, Q. Firdous, A. Izhar, Niazi & Khalid sp. nov. was collected from three different localities (Bahawalnagar, Kasur, and Lahore) in Punjab, Pakistan. Morphological observations and phylogenetic analyses based on nuclear encoded internal transcribed spacers (ITS1-5.8S-ITS2 = ITS) and D1/D2 domain of large subunit (28S) rDNA confirmed the taxonomic distinctness of this species. The new species is potentially hallucinogenic and characterized by a parabolic pileus with a light brown center, broadly fusiform basidiospores, presence of cheilocystidia, pileocystidia, and caulocystidia, and absence of pleurocystidia and clamp connections. The DNA sequences of the species clustered together in a well-supported distinct clade. We present a detailed description, photographs, and line drawings, and elucidate and discuss the phylogenetic position of the new species. Morphological comparisons with phylogenetically and morphologically allied species are discussed.

**Keywords.** Agarics, hallucinogenic, molecular systematics, saprotrophic, taxonomy.

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## Introduction

The genus *Panaeolus* (Fr.) Quél (1872: 151) has been placed in different families, including Coprinaceae Overeem & Weese (Doveri 2011), Bolbitiaceae Singer (Tóth *et al.* 2013), and Psathyrellaceae Vilgalys, Moncalvo & Redhead (Amandeep *et al.* 2014), or has been treated as incertae sedis (He *et al.* 2019). More recently, *Panaeolus* along with *Copelandia* Bres., *Panaeolina* Pers., and *Panaeolopsis* Singer have been placed in a distinct family Galeropsidaceae Singer (Kalichman *et al.* 2020). These genera share several taxonomically important features such as the pileus covering, spore color, and coprophilous ecology (Singer 1986; Tóth *et al.* 2013; Kalichman *et al.* 2020). The members of the family Galeropsidaceae are mostly found growing on open lawns, in steppes and prairies, in mountain deserts and are characterized by a cutis that is rarely and slightly gelatinized, partly enclosed or ovoid pileus, lacunar hymenophore or in some cases regularly developed lamellae often connected by anastomoses, passive spore discharge, ochre-brown spores with germ pore, pileipellis with pileocystidia, the presence in some species of hymenial cystidia, and clamped hyphae (Zeller 1943; Singer & De Leon 1982; Malysheva *et al.* 2019). Species in the genus are characterized by their typically coprophilous or nitrophilous habitat, slender fruiting body with the hemispherical pileus, cartilaginous and relatively long stipe, bluing context, epithelial pileipellis, and a black spore print that does not fade in concentrated sulphuric acid (Watling & Gregory 1987; Gerhardt 1996; Stamets 1996; Strauss *et al.* 2022). *Panaeolus* includes some of the most hallucinogenic species after *Psilocybe* (Fr.) P.Kumm., for example, *P. subbalteatus* (Berk. & Broome) Sacc. and *P. cambodginiensis* Ola'h & R.Heim whose basidiomata contain two hallucinatory compounds, i.e., psilocybin and psilocin (Stamets 1996; Andersson *et al.* 2009).

There are sixteen species in the genus *Panaeolus* reported in literature (He *et al.* 2019; Hu *et al.* 2020) however, in *Index Fungorum*, 189 records are associated with this genus (accessed on 18 September 2022). Species of *Panaeolus* are mostly reported from Asia and Europe in tropical to sub-tropical and temperate habitats (Senn-Irlet *et al.* 1999; Halama *et al.* 2014; Kaur 2014; Wang & Tzean 2015; Desjardin & Perry 2017; Karunarathna *et al.* 2017; Akata *et al.* 2019). Some species of the genus have also been reported from North and South America and Africa in temperate habitats (Adeniyi *et al.* 2018; Silva-Filho *et al.* 2019; Teke *et al.* 2019).

To date, 1293 macrofungal species belonging to 411 genera, 115 families, and 24 orders have been reported from Pakistan (Aman *et al.* 2022). Out of these, 1117 species, 338 genera, 83 families, and 16 orders belong to Basidiomycota, and 176 species, 73 genera, 32 families, and eight orders belong to Ascomycota (Ahmad *et al.* 1997: Aman *et al.* 2022). So far, five species of *Panaeolus*, i.e., *P. fimicola* (Pers.) Gillet (1878: 621), *P. papilionaceus* (Bull.) Quél. (1872: 152), *P. sphinctrinus* (Fr.) Quél. (1872: 151), *P. semiovatus* (Sowerby) S.Lundell & Nannf. (1938: 537) and *P. rickenii* Hora (1960: 454) have been reported from Pakistan (Ahmad *et al.* 1997; Razaq *et al.* 2012).

During the past two decades, both morphological and phylogenetic analyses have been used in mycological research for the identification of new species of the genus *Panaeolus* (Drehmel *et al.* 1999; Zhang *et al.* 2004; Zhao *et al.* 2011; Razaq *et al.* 2012; Jayasiri *et al.* 2015; Wang & Tzean 2015; Li *et al.* 2016; Zhao *et al.* 2016). But, only a few species of *Panaeolus* have been reported based on both morphological and phylogenetic analyses (Ma 2014; Ediriweera *et al.* 2015; Wang & Tzean 2015; Undan 2016) and most *Panaeolus* species have been described based only on morphology (Gerhardt 1996; Amandeep *et al.* 2014; Halama *et al.* 2014; Silva-Filho *et al.* 2019).

In the present study, some interesting collections of *Panaeolus* were made from three different locations in Punjab, Pakistan. All the localities lie in the semi-arid region with a maximum average temperature of 45°C and a long rainy season, i.e., July to September (Ahmad *et al.* 2019). Both morphological characteristics and phylogenetic analyses of ITS and 28S sequence data were used to determine the taxonomic position of the new *Panaeolus* species which is subsequently described here in detail.

## Material and methods

### *Type locality*

The type specimen was collected from Haroonabad, Bahawalnagar District, Punjab, Pakistan (29°60′81″ N, 73°14′68″ E, 163 m a.s.l.) during the monsoon rainy season of August 2019. The temperature of the collection site varies from a minimum of 11°C to a maximum of 50°C and the average annual rainfall is 99 mm (Ahmed *et al.* 2014a, 2014b). The main vegetation of the area includes *Dalbergia sissoo* Roxb., *Vachellia nilotica* (L.) P.J.H.Hurter & Mabb., *Eucalyptus camaldulensis* Dehnh., *Azadirachta indica* A.Juss. and *Albizia lebbeck* (L.) Benth. (Ahmed *et al.* 2014b). The region falls under a hot semi-arid climate (BSh) following the climate map and classification (Peel *et al*. 2007; Belda *et al.* 2014).

The second collection site is Kasur, Punjab, Pakistan (31°12′79″ N, 74°44′08″ E, 218 m a.s.l.). Climatic conditions are tremendously variable and described as scorching hot summers and cold winters. Monsoons occur towards the end of June with the rainy season lasting 2–3 months. The common woody flora of the district includes *Capparis decidua* Edgew. (Forssk.), *Dalbergia sissoo* Roxb., *Prosopis cineraria* (L.) Druce, *Senegalia modesta* (Wall.) P.J.H.Hurter, and *Vachellia nilotica* (L.) P.J.H.Hurter & Mabb. (Nasir *et al.* 1995; Zabihullah *et al.* 2006; Lateef *et al*. 2008; Durrani & Shakoori 2009; Anwar *et al.* 2012; Waheed *et al.* 2020).

The third collection site is Lahore, the capital of Punjab, Pakistan (32°52′04″ N, 74°35′87″ E, 217 m a.s.l.). It has a hot semi-arid climate (Köppen climate classification BSH) with long, wet, and exceptionally hot summers, dry and warm winters, annual monsoons and dust storms. The monthly mean temperature ranges between 10 and 38°C during the year in Lahore (https://rmcpunjab.pmd.gov.pk/). It has a long rainy season (from the end of June to mid-September), with annual mean rainfall of 838 mm, which increases the humidity of the area (Siddiqui *et al.* 2020; Tanveer *et al.* 2020).

***Morphoanatomical study***

Basidiomata were photographed in the field, and morphological features such as size, shape, and color of basidiomata were recorded at the time of collection. Munsell's soil color chart (1975) was used for color notations and for morphological terminologies, Vellinga (2001) was followed. Specimens were air-dried, kept in zipper bags, and deposited in the Herbarium of the Institute of Botany, University of the Punjab, Lahore (LAH).

For the microscopic study, slides were prepared (lamellae, pileus, stipe) using free-hand sections of the dried materials rehydrated in 5% aqueous KOH (percentage weight/volume (w/v)) and stained with Congo Red (2%) and Melzer's reagent following the microscopic procedures of Liang *et al.* (2011, 2018) and Cai *et al.* (2018) and observed under the light microscope (CXRII, Labomed Labo America Inc., Fremont, CA, USA) equipped with a camera to examine the following microscopic characteristics under 400× and oil immersion 1000× magnification: size and shape of basidiospores, basidia, cheilocystidia, pileipellis, and stipitipellis. Data for morphoanatomical characteristics was based on at least 25 measurements each of basidia, cheilocystidia, and basidiospores. Length and width ratios of basidiospores is designated as 'Q', while the average length and average width ratios of all the basidiospores measured is given as 'avQ'. The notation 'n/b/p' is given, where 'n' is the number of basidiospores measured, from 'b' basidiomata and from 'p' collections (Bas 1969; Yu *et al.* 2020).

***DNA extraction, PCR amplification, and sequencing***

Genomic DNA was extracted using the CTAB method (Porebski *et al.* 1997). We amplified the ITS and 28S regions of nuclear ribosomal DNA, using the primer combination ITS1F/ITS4 and LR0R/LR5 for ITS and 28S, respectively (White *et al.* 1990; Vilgalys & Hester 1990). Polymerase chain reaction (PCR) was performed in a 25 μL reaction volume following the protocol given by Warnke (2020). The (PCR) products were sequenced with the same primers in both directions at ©Macrogen Inc. (238, Teheran-ro, Gangnam-gu, Seoul, Republic of Korea). The newly generated sequences of *P. punjabensis* M. Asif, Q. Firdous, A. Izhar, Niazi & Khalid sp. nov. were submitted to GenBank: KY636363, MZ265142, MZ823627, OP681142 (ITS) and ON116490, ON116491, ON116492 (28S).

### *Phylogenetic analyses*

For phylogenetic analysis, ITS and 28S sequences of nrDNA, generated from the Pakistani collections were compared with sequences in GenBank using the BLAST tool, priority was given to those sequences which showed high bootstrap value in phylogenetic analyses (Altschul *et al.* 1990). The datasets were created by adding newly generated sequences of *P. punjabensis* sp. nov. plus the highest-scored BLAST hits were chosen from GenBank and the other sequences of the genus from previous studies (Malysheva *et al.* 2019; Hu *et al.* 2020; Voto & Angelina 2021). Four ITS and three 28S sequences were newly generated during this study, and 39 ITS sequences of family Galeropsidaceae including *Psathyrella vesterholti* Örstadius & E.Larss. (Örstadius *et al.* 2015: 29) (KC992938) as an outgroup and 30 LSU sequences of family Galeropsidaceae including *Psathyrella vesterholti* as an outgroup, were used for phylogenetic analyses. The sequence alignment of both datasets was done separately using MUSCLE ver. 3.8 (Edgar 2004), and initial phylogenetic analyses were performed using MEGA-X software (Tamura *et al.* 2011) using the Maximum Likelihood (ML) method. To calculate the appropriate model of nucleotide evolution, the nrITS dataset was segmented into three partitions, ITS1, 5.8S, and ITS2. The best fit model of nucleotides substitution based on the lowest BIC (Bayesian information criterion) values for each partition and for 28S based dataset was chosen with jModelTest2 on XSEDE via CIPRES science gateway (Darriba *et al.* 2012).

The final phylogenetic analyses of the ITS and 28S datasets were carried out separately using RAxML-HPC2 ver. 8.1.11 under the CIPRES Science Gateway (Miller *et al.* 2010). In the ML analysis, 1000 bootstrap repetitions were obtained as statistical support with rapid bootstrapping for both datasets. Phylogenetic trees generated by Bayesian Inference (BI) analyses were performed with a Markov chain Monte Carlo (MCMC) coalescent approach implemented in BEAST ver. 1.8.2 (Drummond & Rambaut 2007). Both analyses resulted in a similar topology. Significant support was considered to be ≥ 80%. In the resulting trees, bootstrap values obtained from maximum likelihood analyses and values of Bayesian posterior probabilities > 0.7 were reported. FigTree ver. 1.4.3 (Rambaut 2014) was used for displaying the phylogenetic trees, and both trees were annotated using Adobe Illustrator 2020 ver. 24.1.2.408.

## Results

### *Phylogenetic analysis of ITS dataset*

The fragment size of the target region was 758 bp long. From BLAST results, the ITS sequences of *Panaeolus punjabensis* sp. nov. show 99% similarity with *P. papilionaceus*. In the ITS phylogenetic tree, the four sequences of the new species formed a separate lineage with strong statistical support. It formed a sister clade with these species of *Panaeolus*: *P. papilionaceus*, *P. campanulatus* (Bull.) Quél., and *P. sphinctrinus.* In this analysis, *P. guttulatus* Bres. (KU725993) is also closely related to the new species (Fig. 1; Table 1).

### *Phylogenetic analysis of 28S dataset*

The 28S alignment contained 892 total characters of which 815 were conserved and 73 were variable. In the 28S phylogenetic tree, the three sequences of the new species also formed a separate lineaged with good bootstrap support. It also formed a sister clade with these species of *Panaeolus*: *P. papilionaceus*, *P. campanulatus*, and *P. sphinctrinus.* In this analysis, *P. semiovatus* (MH868191) is also closely related to the new species (Fig. 2; Table 1).

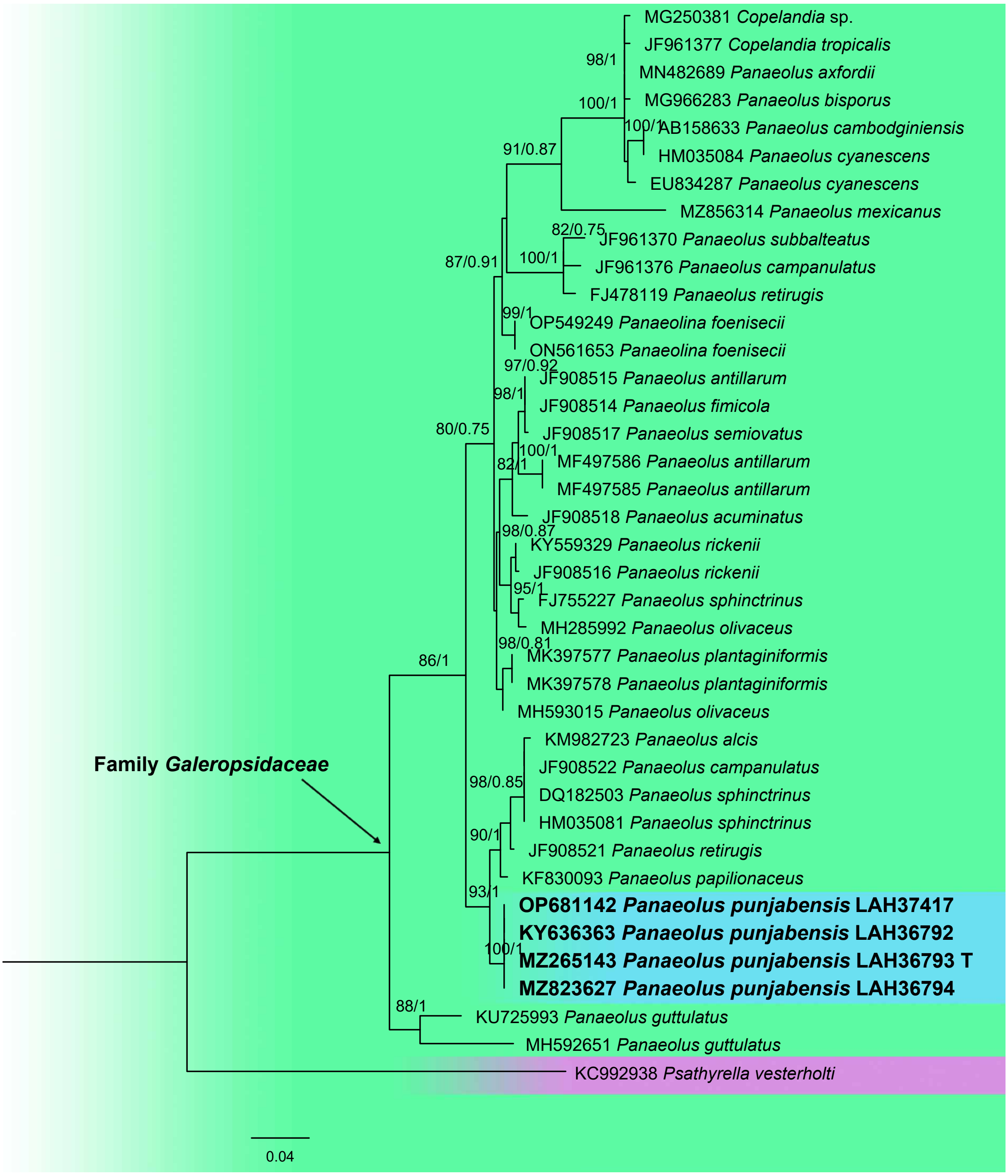


**Fig. 1.** Molecular phylogenetic placement of *Panaeolus punjabensis* M. Asif, Q. Firdous, A. Izhar, Niazi & Khalid sp. nov. based on Maximum Likelihood (ML) method of ITS sequences. Newly generated sequences are in **bold.** *Panaeolus punjabensis* M. Asif, Q. Firdous, A. Izhar, Niazi & Khalid sp. nov. (LAH36793, T = Type specimen) is referring to the holotype. Bootstrap values > 80% and Bayesian posterior probabilities > 0.7 are shown above the branches.

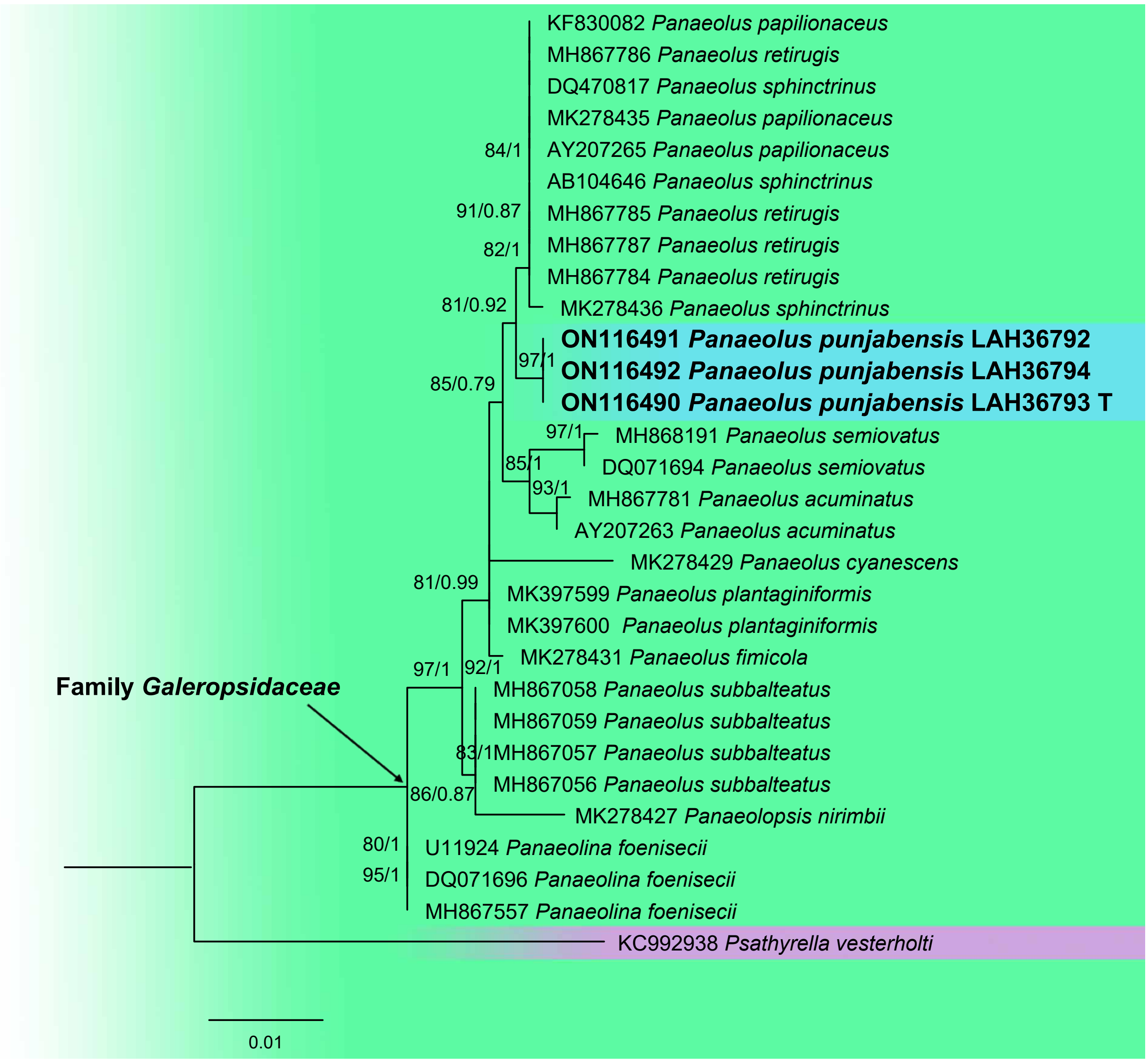


**Fig. 2.** Molecular phylogenetic placement of *Panaeolus punjabensis* M. Asif, Q. Firdous, A. Izhar, Niazi & Khalid sp. nov. based on Maximum Likelihood (ML) method of 28S sequences. Newly generated sequences are in **bold.** *Panaeolus punjabensis* M. Asif, Q. Firdous, A. Izhar, Niazi & Khalid sp. nov. (LAH36793, T = Type specimen) is referring to the holotype. Bootstrap values > 80% and Bayesian posterior probabilities > 0.7 are shown above the branches.

**Table 1.** (continued on next page) Taxa information and GenBank accession numbers of nrITS and LSU sequences of *Panaeolus* (Fr.) Quél. used in the molecular phylogenetic analyses. Sequences generated for this study are shown in bold.

| Species | Voucher No. | GenBank Accession No. | | Origin | Reference |
|---|---|---|---|---|---|
| | | ITS | LSU | | |
| *Copelandia* sp. | 294130 | MG250381 | No data | USA | Unpublished |
| *C. tropicalus* | – | JF961377 | No data | China | Unpublished |
| *Panaeolina foenisecii* | FO 46609 | No data | DQ071696 | – | Garnica *et al.* 2007 |
| *P. foenisecii* | CBS 142.40 | No data | MH867557 | – | Vu *et al.* 2018 |
| *P. foenisecii* | – | No data | U11924 | | Chapela *et al.* 1994 |
| *P. foenisecii* | PUL00038201 | ON561653 | No data | USA | Unpublished |
| *P. foenisecii* | – | OP549249 | No data | USA | Unpublished |
| *Panaeolopsis nirimbii* | PERTH 7680368 | No data | MK278427 | Australia | Varga *et al.* 2019 |
| *Panaeolus acuminatus* | 4084 | JF908518 | No data | Italy | Osmundson *et al.* 2013 |
| *P. acuminatus* | GLM 45986 | No data | AY207263 | Germany | Walther *et al.* 2005 |
| *P. acuminatus* | CBS 268 | No data | MH867781 | Hungary | Varga *et al.* 2019 |
| *P. alcis* | 88085 | KM982723 | No data | Canada | Moser 1984 |
| *P. antilarum* | 748 | JF908515 | No data | – | – |
| *P. antilarum* | CORT:013830 | MF497586 | No data | Dominican Republic | Desjardin & Perry 2017 |
| *P. antilarum* | SFSU:DED 7874 | MF497585 | No data | Thailand | Desjardin & Perry 2017 |
| *P. axfordii* | MFLU 19-2367 | MN482689 | No data | China | Hu *et al.* 2020 |
| *P. bisporus* | 188954 | MG966283 | No data | USA | Ediriweera *et al.* 2015 |
| *P. cambodginiensis* | NBRC30222 | AB158633 | No data | – | Maruyama *et al.* 2006 |
| *P. campanulatus* | 10141 | JF908522 | No data | Italy | Osmundson *et al.* 2013 |
| *P. campanulatus* | No data | JF961376 | No data | China | Ma 2014 |
| *P. cyanescens* | No data | HM035084 | No data | – | Broussal & Dumesny 2015 |
| *P. cyanescens* | 6576 AQUI | EU834287 | No data | Italy | Han *et al.* 2010 |
| *P. cyanescens* | NL-0429 | No data | MK278429 | Hungary | Varga *et al.* 2019 |
| *P. fimicola* | 474 | JF908514 | No data | Italy | Wang & Tzean 2015 |
| *P. fimicola* | NL-0232 | No data | MK278431 | Hungary | Varga *et al.* 2019 |
| *P. guttulatus* | 137 | MH592651 | No data | – | Unpublished |
| *P. guttulatus* | AMB n. 18101 | KU725993 | No data | – | Unpublished |
| *P. mexicanus* | ANGE1557 | MZ856314 | No data | Dominican Republic | Voto & Angelini 2021 |
| *P. olivaceus* | 89608 | MH285992 | No data | USA | Unpublished |
| *P. olivaceus* | – | MH593015 | No data | Iran | Unpublished |
| *P. plantaginiformis* | LE 2864 | MK397578 | MK397600 | Uzbekistan | Malysheva *et al.* 2019 |

**Table 1.** (continued).

| Species | Voucher No. | GenBank Accession No. | | Origin | Reference |
|---|---|---|---|---|---|
| | | ITS | LSU | | |
| *P. plantaginiformis* | LE 2862 | MK397577 | MK397599 | Russia | Malysheva *et al.* 2019 |
| *P. papilionaceus* | DNA1940 | KF830093 | KF830082 | USA | Ediriweera *et al.* 2015 |
| *P. papilionaceus* | DNA1940 | KF830093 | KF830082 | USA | Ediriweera *et al.* 2015 |
| *P. papilionaceus* | DB 4552 | No data | MK278435 | Austria | Varga *et al*. 2019 |
| *P. papilionaceus* | GLM 45989 | No data | AY207265 | Germany | Walther *et al.* 2005 |
| ***P. punjabensis*** | **LAH36792** | **KY636363** | **ON116491** | **Pakistan** | **This study** |
| ***P. punjabensis*** | **LAH36794** | **MZ823627** | **ON116492** | **Pakistan** | **This study** |
| ***P. punjabensis* T** | **LAH36793** | **MZ265143** | **ON116490** | **Pakistan** | **This study** |
| ***P. punjabensis*** | **LAH37417** | **OP681142** | **No data** | **Pakistan** | **This study** |
| *P. reckenii* | TENN:054965 | KY559329 | No data | Argentina | Unpublished |
| *P. retirugis* | 7070 | JF908521 | No data | Italy | Osmundson *et al.* 2013 |
| *P. retirugis* | xsd08077 | FJ478119 | No data | China | Undan 2016 |
| *P. retirugis* | CBS 271 | No data | MH867784 | France | Vu *et al.* 2019 |
| *P. retirugis* | CBS 273 | No data | MH867786 | France | Vu *et al.* 2019 |
| *P. retirugis* | CBS 274 | No data | MH867787 | France | Vu *et al.* 2019 |
| *P. retirugis* | CBS 272 | No data | MH867785 | France | Vu *et al.* 2019 |
| *P. rickenii* | 749 | JF908516 | No data | Italy | Osmundson *et al.* 2013 |
| *P. semiovatus* | 4083 | JF908517 | No data | Italy | Osmundson *et al.* 2013 |
| *P. semiovatus* | GLM 51235 | No data | DQ071694 | – | Garnica *et al.* 2007 |
| *P. semiovatus* | CBS 388 | No data | MH868191 | France | Vu *et al.* 2019 |
| *P. sphinctrinus* | CBS 582 | HM035081 | No data | Pakistan | Razaq *et al.* 2012 |
| *P. sphinctrinus* | PBM 2009 | DQ182503 | DQ470817 | Pakistan | Razaq *et al.* 2012 |
| *P. sphinctrinus* | CZ519-3 | FJ755227 | No data | Pakistan | Razaq *et al.* 2012 |
| *P. sphinctrinus* | KY7130 | No data | AB104646 | Japan | Maruyama *et al.* 2003 |
| *P. sphinctrinus* | NL-3955 | No data | MK278436 | Slovakia | Varga *et al*. 2019 |
| *P. subbalteatus* | No data | JF961370 | No data | China | Sette *et al.* 2010 |
| *P. subbalteatus* | CBS 331 | No data | MH867059 | France | Vu *et al.* 2019 |
| *P. subbalteatus* | CBS 329 | No data | MH867058 | France | Vu *et al.* 2019 |
| *P. subbalteatus* | CBS 327 | No data | MH867056 | France | Vu *et al.* 2019 |
| *P. subbalteatus* | CBS 328 | No data | MH867057 | France | Vu *et al.* 2019 |
| **Outgroup** | | | | | |
| *Psathyrella vesterholtii* | JHP10.086 | KC992938 | KC992938 | Denmark | Örstadius *et al.* 2015 |

## *Taxonomy*

Phylum Basidiomycota R.T.Moore
Class Agaricomycetes Doweld
Order Agaricales Underw.
Family Galeropsidaceae Singer
Genus *Panaeolus* (Fr.) Quél.

***Panaeolus punjabensis*** M. Asif, Q. Firdous, A. Izhar, Niazi & Khalid sp. nov.
MycoBank MB 840898
Figs 3–4

### Diagnosis

The new species turns bluish on handling so it is hallucinogenic and can be distinguished by its broadly fusiform basidiospores, claviform cheilocystidia with rounded tips, and clavate caulocystidia.

### Etymology

Specific epithet '*punjabensis*' refers to the type locality, Punjab Province, Pakistan.

### 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 Aug. 2019; *Muhammad Asif*, *BWN-45*; GenBank nos MZ265143 (nrITS); ON116490 (28S); LAH[36793].

### Additional material examined

PAKISTAN • Punjab Province, Lahore, 32°52′04″ N, 74°35′87″ E; alt. 217 m a.s.l.; on loamy soil; 10 Jul. 2015; *Qudsia Firdous*, *BRB S-01*; GenBank nos KY636363 (nrITS); ON116491 (LSU); LAH[36792] • same collection data as for preceding; 28 Jul. 2016; *Qudsia Firdous*, *BRB S-22*; GenBank nos MZ823627 (nrITS); ON116492 (LSU); LAH[36794] • Punjab Province, Kasur District, 31°12′79″ N, 74°44′08″ E; alt. 218 m a.s.l.; on fallen plant debris; 6 Sep. 2020; *Aiman Izhar*, *KS-0018*; GenBank no. OP681142 (nrITS); LAH[37417].

### Description

*Basidiomata* 4.4–5.8 cm tall. *Pileus* 1–1.5 cm diam, conic to parabolic when young, becoming convex with maturity, dry; surface light brown at the center (7.5YR8/4), light grayish green (7.5GY8/1) toward margins, smooth when young becoming rugulose at maturity; margin straight in young stage, striate at maturity (Fig. 3A, D). *Lamellae* free, olive black (5GY2/1), even margins, distantly placed, two tiers of regularly arranged lamellulae (Fig. 3B–C). *Stipe* 3.8–5.3 × 0.3–0.6 cm, surface light grayish-green (10GY8/1), central, equal, surface smooth and glabrous, dry, slightly bulbous base, bruising blue on handling (Fig. 3E). *Annulus* and *volva* absent. *Odor* is indistinct.

*Basidiospores* [75/3/3] (13.2–)13.4–16.4(–16.7) × (7.5–)8.2–9.6(–11.4) µm, on average 15 × 9.5 µm, Q = 1.4–1.6, $Q_{av}$ = 1.5, broadly fusiform, smooth, apiculus absent, thick-walled, hyaline in KOH, no colour change in Melzer's reagent, non-guttulate, germ pore obvious (Fig. 4B). *Basidia* (24.8–)24.9–27.9 (–28.1) × (14.3–)14.4–15.6(–15.9) µm, on average 26.2 × 15.1 µm, broadly clavate, mostly bi-spored, rarely tri- or tetra-spored, thick-walled, hyaline in KOH, non-guttulate (Fig. 4A). *Cheilocystidia* (30.5–) 32.1–41.4(–44.3) × (6–)6.1–9.1(–9.5) µm, on average 37.4 × 7.8 µm, claviform with flexuous neck and rounded apices, thin-walled, hyaline in KOH, non-guttulate (Fig. 4C). *Pileocystidia* (18–)19.2–32

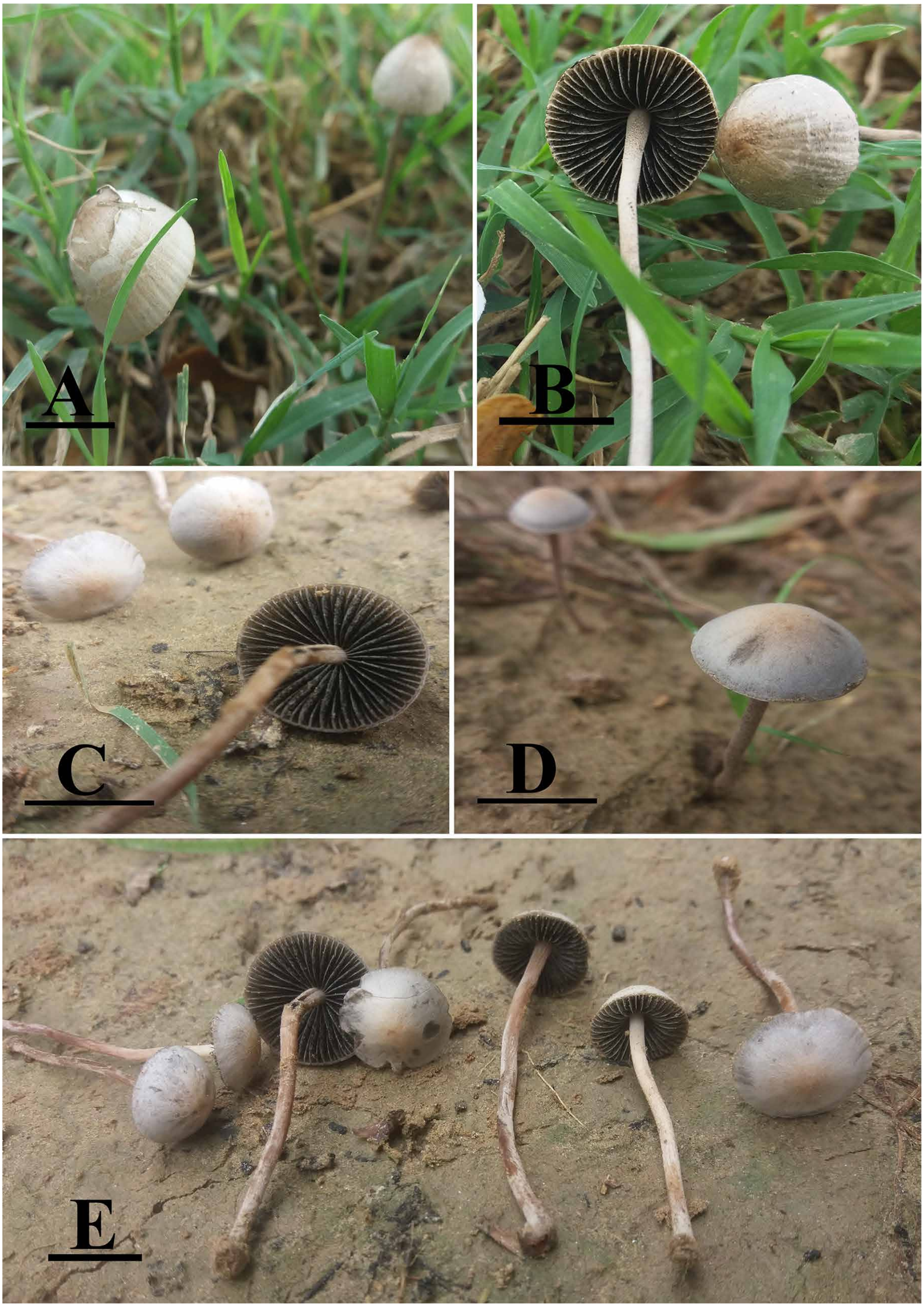


**Fig. 3.** Morphology of *Panaeolus punjabensis* M. Asif, Q. Firdous, A. Izhar, Niazi & Khalid sp. nov. (LAH36793, A–B holotype). Scale bars: A–D = 1 cm, E = 10 cm. Photos by Muhammad Asif.

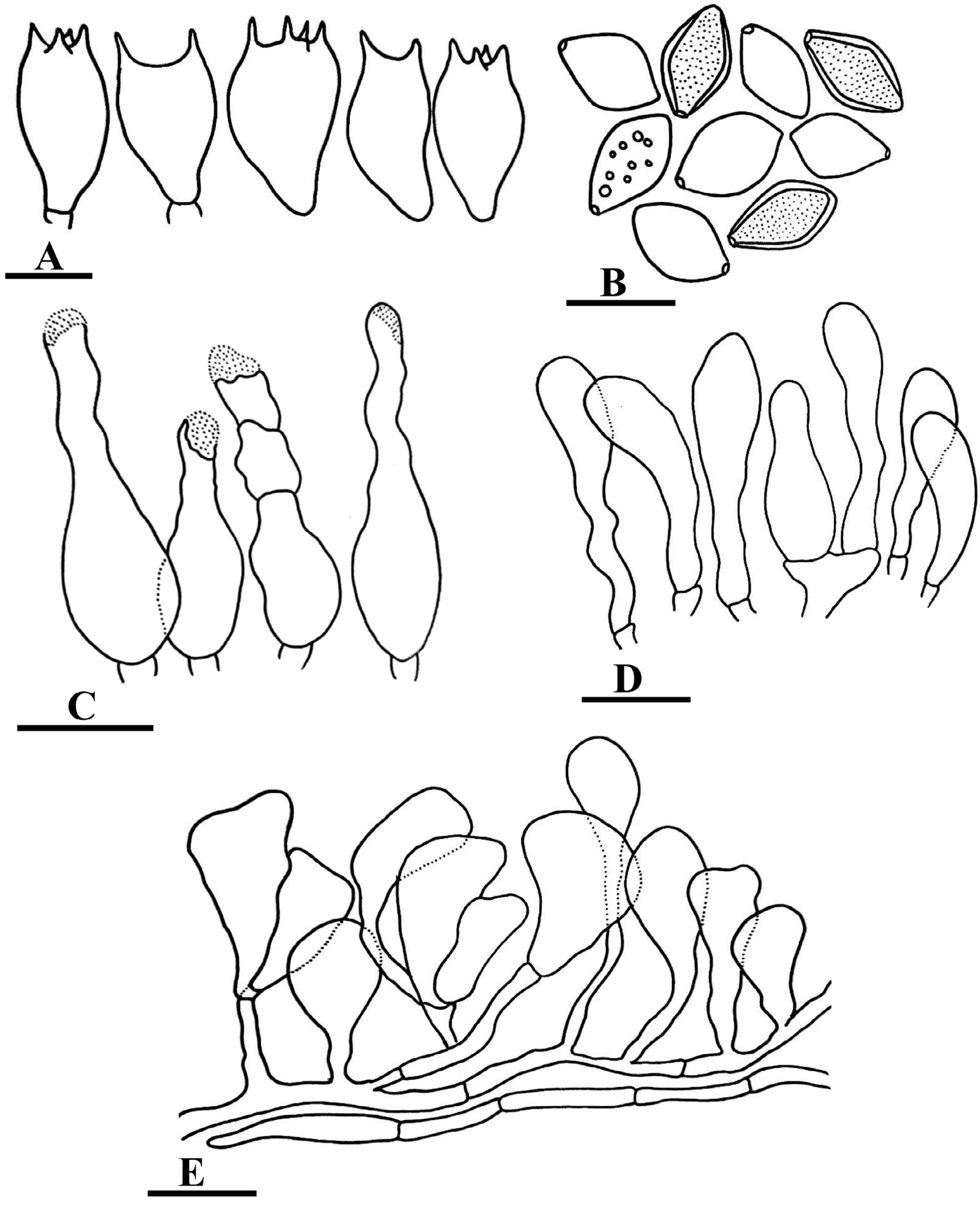


**Fig. 4.** Microscopic characters of *Panaeolus punjabensis* M. Asif, Q. Firdous, A. Izhar, Niazi & Khalid sp. nov. (LAH36793, holotype). **A**. Basidia. **B**. Basidiospores. **C**. Cheilocystidia. **D**. Caulocystidia. **E**. Pileocystidia. Scale bars: A–D = 10 µm, E = 20 µm. Drawings by Aiman Izhar.

**Table 2.** (continued on next page) Comparison of macro- and micro-characteristics of *Panaeolus punjabensis* M. Asif, Q. Firdous, A. Izhar, Niazi & Khalid sp. nov., and its closely related taxa. Abbreviations: L = Length; W = Width.

| Species name | Pileus diameter (mm) | Pileus shape | Pileus color | Lamellae color | Stipe L × W (mm) | Stipe color | Spore L × W (µm) | Spore shape | Cheilo-cystidia L × W (µm) | Cheilo-cystidia shape | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. punjabensis* sp. nov. | 10–15 | parabolic to convex | light brown at the center, light grayish green toward margins | olive black | 38–53 × 3–6 | light grayish green | 13.2–16.7 × 7.5–11.4 | broadly fusiform | 30.5–44.3 × 6–9.5 | claviform with flexuous neck and rounded apices | This study |
| *P. acuminatus* | 17–20 | broadly conic to broadly bell-shaped | dark reddish brown to grayish brown | grayish black | 62–75 × 2 | reddish brown | 11.4–15 × 7.8–11 | lemon-shaped | 15.5–25.5 × 5.5–8.5 | fusoid-ventricose to irregularly cylindric | Kaur *et al.* 2014; Os-mundson *et al.* 2013 |
| *P. alcis* | 4–10 | companulate to conical | pale gray | dark gray | 20–90 × 0.5–1.5 | pex pale ochraceous gray, base brownish | 18–21 × 9.5–12 | ellipsoid | 25–35 × 4–6 | versiform | Moser 1984 |
| *P. axfordii* | 16–21 | hemi-spherical to campanulate | reddish brown to grayish white | mottled dark gray | 42–51 × 1.5–2.5 | grayish orange to light brown | 8.8–11.4 × 6.3–9 | limoni-form to ellipsoid | 24.6–42.7 × 5.9–10.7 | narrowly utri-form | Hu *et al.* 2020 |
| *P. cambodginiensis* | 12–25 | convex to broadly convex | chocolate brown be-coming yel-lowish brown | pallid, then grayish black to black | 55–95 × 3.5–5 | whitish to cream, brown near the base | 10.5–12 × 6.5–9 | lemon-shaped | 26–39 × 10–12.2 | polymorphic | Stamets 1996; Weeks *et al.* 1979 |
| *P. cyanescens* | 10–15 | hemispheric to convex | pure white | dark grayish brown | 50 × 2–3 | uniformly white | 11–18 × 9–13.7 | limoni-form to ellipsoid | absent | absent | Wartchow *et al.* 2010 |
| *P. fimicola* | 10–20 | campanulate to convex | dingy gray to black, slight reddish | mottled gray | 60–100 × 1–2 | dingy pale to whitish | 11–14 × 7–9.5 | sub-amyg-daliform | 22.7–27 × 5.7–10 | cylindric to sublageniform | Stamets 1996; Wang & Tzean 2015 |

**Table 2.** (continued).

| Species name | Pileus diameter (mm) | Pileus shape | Pileus color | Lamellae color | Stipe L × W (mm) | Stipe color | Spore L × W (µm) | Spore shape | Cheilo-cystidia L × W (µm) | Cheilo-cystidia shape | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. guttulatus* | 10 | convex | dark olive brown | black | 40 × 2 | snuff brownish | 7.5–10 × 4–6 | elliptical | 25 × 5 | cylindrical | Seidmohammadi *et al.* 2019; Suliaman 2019 |
| *P. papilionaceus* | 10–50 | conical to campulanate | brownish to grayish brown | gray to entirely black | 60–120 × 2–4 | whitish to snuff brown | 12–14 × 7–8 | citriform | 35–53 × 7–12.5 | vermiform, urn-shaped or tubular | Abraham 2007; Kuo 2007; Amandeep *et al.* 2014; Ediriweera *et al.* 2015 |
| *P. rickenii* | 18–20 | campanulate to convex | dark brown | mottled grayish brown with whitish margin | 15–140 × 1–2 | buff, late dark brown to reddish brown | 9–12 × 7–9.5 | ellipsoid to broadly ellipsoid | 19.5–37 × 7–13 | clavate to lageniform | Ma 2014 |
| *P. semiovatus* | 30–60 | egg-shaped to conico-convex | cinnamon buff to pinkish buff and fading to whitish | pale brown, later mottled blackish | 100–160 × 6–10 | whitish to pallid buff | 18.5–21 × 10–11.5 | elliptical | absent | absent | Stamets 1996; Osmundson *et al.* 2013 |
| *P. sphinctrinus* | 10–20 | bell shaped to umbonate | brownish when immature, grayish brown when mature | gray and mottled black, later entire black | 25–90 × 1–2 | snuff brown | 0.4–11.3 × 6.6–7.5 | citriform to lemon-shaped | 11.0–16.5 × 5.5–9.2 | polymorphic | Ediriweera *et al.* 2015 |

(–34.4) × (10.4–)11.9–13.9(–17.4) µm, on average 25.5 × 13.4 µm, clavate to vesiculose, thin-walled, hyaline (Fig. 4E). *Caulocystidia* (25.3–)26.7–36.9(–37.5) × (6–)8.1–9.7(–10.5) µm, on average 31.4 × 8.7 µm), clavate, thick-walled (Fig. 4D). *Clamp connections* are absent in all tissues.

### Habitat

Solitary or in small groups on loamy soil containing herbivore (cattle) dung.

### Known distribution

Known only from three localities, Bahawalnagar, Kasur, and Lahore, Punjab, Pakistan.

## Discussion

The genus *Panaeolus* is quite similar in appearance to *Panaeolina* in the field (Kalichman *et al.* 2020). The two genera can be differentiated on the basis of basidiospores morphology and lamellae color. Lamellae in *Panaeolus* are grayish-black and basidiospores are smooth, in *Panaeolina* spores are ornamented and lamellae are dark brown (Kaur *et al.* 2014). All previously reported species of *Panaeolus* from Pakistan are not hallucinogenic, but our new species turns bluish on handling which indicates that it is a hallucinogenic species. Several different tests can be performed to test the hallucinogenic various mushrooms including *Panaeolus*, such as Amplified Fragment Length Polymorphism (AFLP) and High-Resolution Melting essays (HRM) (Lee *et al.* 2000; Zhang *et al.* 2022). A detailed comparison of macro- and micro-characteristics of all the closely related species of *Panaeolus* is given in Table 2, and from the molecular phylogenetic analyses of ITS and 28S and morphoanatomical comparison given in Table 2, we conclude that *Panaeolus punjabensis* sp. nov. is a new species.

In the ITS-based phylogenetic analysis, *P. papilionaceus* (KF830093), *P. retirugis* (Fr.) Gillet (JF908521), *P. sphinctrinus* (HM03581, DQ182503), *P. companulatus* (L.) Quél. (JF908522), and *P. alcis* M.M.Moser (KM982723) lie in the same clade and are closly related to the newly described species, while in the 28S-based phylogenetic analysis, *P. semiovatus* (MH868191, DQ071694), *P. acuminatus* (P.Kumm.) Quél. (MH867781, AY207263), *P. sphinctrinus* (MK278436, AB104646), *P. papilionaceus* (KF830082, MK278435, AY207265), and *P. retirugis* (MH867784, MH867785, MH867786, MH867787) are close relatives of our new species. In both phylogenetic analyses, different sequences of the same species appear on different positions such as *P. retirugis* (Gillet 1878: 621) (current name, *P. papilionaceus*), *P. sphinctrinus*, and *P. campanulatus* (Quélet 1872: 151), and this could result to misidentification of species owing to close similarities among the species in the genus *Panaeolus* (Razaq *et al.* 2012).

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