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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 lebbbeck* (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 stiptipellis. 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  $\geq 80\%$ . 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.

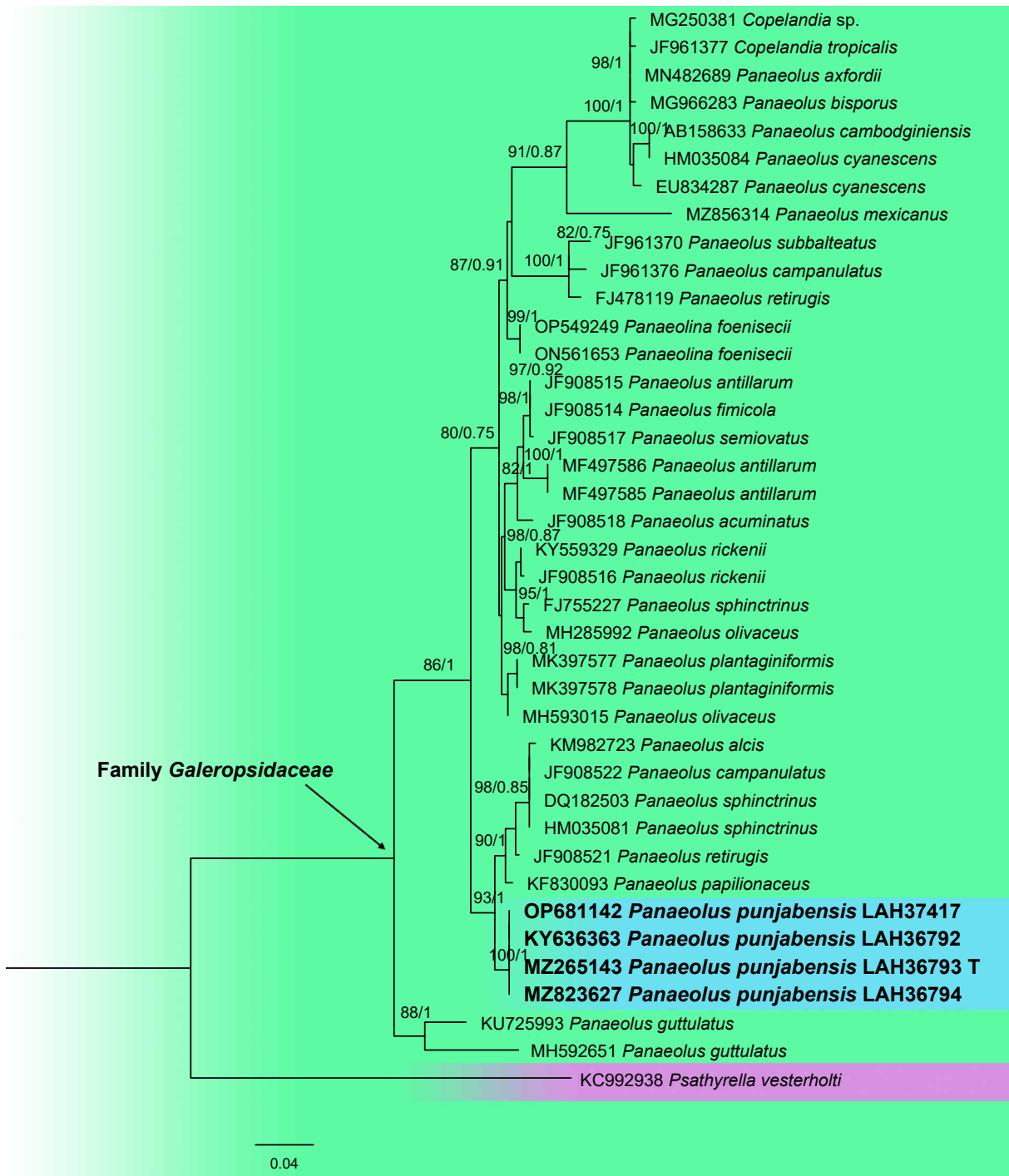
## **Results**

### **Phylogenetic analysis of ITS dataset**

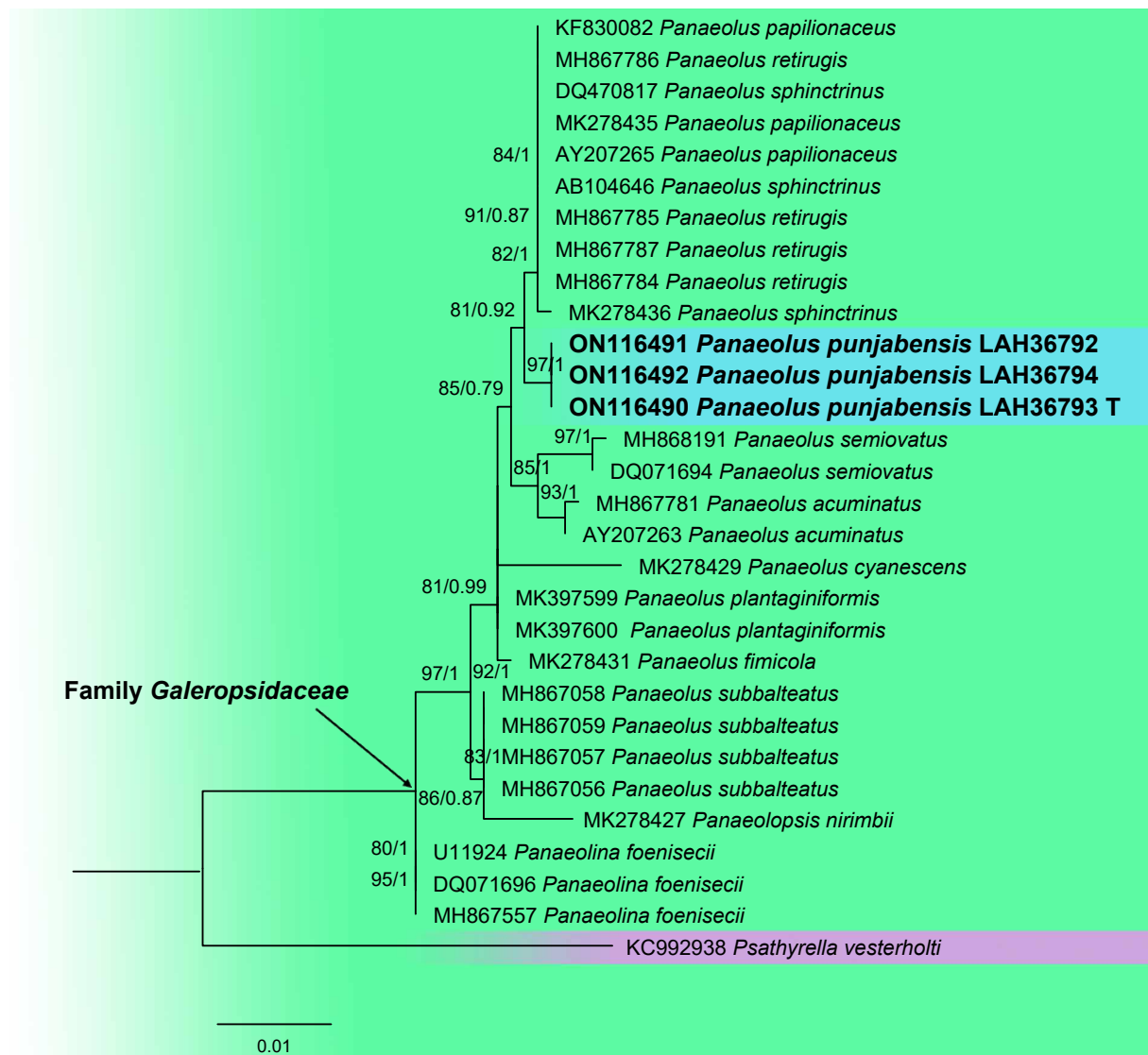
The fragment size of the target region was 758 bp long. From BLAST results, the ITS sequences of *Panaeolus punjabensis* sp. nov. show 99% similarity with *P. papilionaceus*. In the ITS phylogenetic tree, the four sequences of the new species formed a separate lineage with strong statistical support. 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		
<i>Copelandia</i> sp.	294130	MG250381	No data	USA	Unpublished
<i>C. tropicalus</i>	–	JF961377	No data	China	Unpublished
<i>Panaeolina foeniseccii</i>	FO 46609	No data	DQ071696	–	Garnica <i>et al.</i> 2007
<i>P. foeniseccii</i>	CBS 142.40	No data	MH867557	–	Vu <i>et al.</i> 2018
<i>P. foeniseccii</i>	–	No data	U11924		Chapela <i>et al.</i> 1994
<i>P. foeniseccii</i>	PUL00038201	ON561653	No data	USA	Unpublished
<i>P. foeniseccii</i>	–	OP549249	No data	USA	Unpublished
<i>Panaeolopsis nirimbii</i>	PERTH 7680368	No data	MK278427	Australia	Varga <i>et al.</i> 2019
<i>Panaeolus acuminatus</i>	4084	JF908518	No data	Italy	Osmundson <i>et al.</i> 2013
<i>P. acuminatus</i>	GLM 45986	No data	AY207263	Germany	Walther <i>et al.</i> 2005
<i>P. acuminatus</i>	CBS 268	No data	MH867781	Hungary	Varga <i>et al.</i> 2019
<i>P. alcis</i>	88085	KM982723	No data	Canada	Moser 1984
<i>P. antilarum</i>	748	JF908515	No data	–	–
<i>P. antilarum</i>	CORT:013830	MF497586	No data	Dominican Republic	Desjardin & Perry 2017
<i>P. antilarum</i>	SFSU:DED 7874	MF497585	No data	Thailand	Desjardin & Perry 2017
<i>P. axfordii</i>	MFLU 19-2367	MN482689	No data	China	Hu <i>et al.</i> 2020
<i>P. bisporus</i>	188954	MG966283	No data	USA	Ediriweera <i>et al.</i> 2015
<i>P. cambodginiensis</i>	NBRC30222	AB158633	No data	–	Maruyama <i>et al.</i> 2006
<i>P. campanulatus</i>	10141	JF908522	No data	Italy	Osmundson <i>et al.</i> 2013
<i>P. campanulatus</i>	No data	JF961376	No data	China	Ma 2014
<i>P. cyanescens</i>	No data	HM035084	No data	–	Broussal & Dumesny 2015
<i>P. cyanescens</i>	6576 AQUI	EU834287	No data	Italy	Han <i>et al.</i> 2010
<i>P. cyanescens</i>	NL-0429	No data	MK278429	Hungary	Varga <i>et al.</i> 2019
<i>P. fimicola</i>	474	JF908514	No data	Italy	Wang & Tzean 2015
<i>P. fimicola</i>	NL-0232	No data	MK278431	Hungary	Varga <i>et al.</i> 2019
<i>P. guttulatus</i>	137	MH592651	No data	–	Unpublished
<i>P. guttulatus</i>	AMB n. 18101	KU725993	No data	–	Unpublished
<i>P. mexicanus</i>	ANGE1557	MZ856314	No data	Dominican Republic	Voto & Angelini 2021
<i>P. olivaceus</i>	89608	MH285992	No data	USA	Unpublished
<i>P. olivaceus</i>	–	MH593015	No data	Iran	Unpublished
<i>P. plantaginiformis</i>	LE 2864	MK397578	MK397600	Uzbekistan	Malysheva <i>et al.</i> 2019

Table 1. (continued).

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

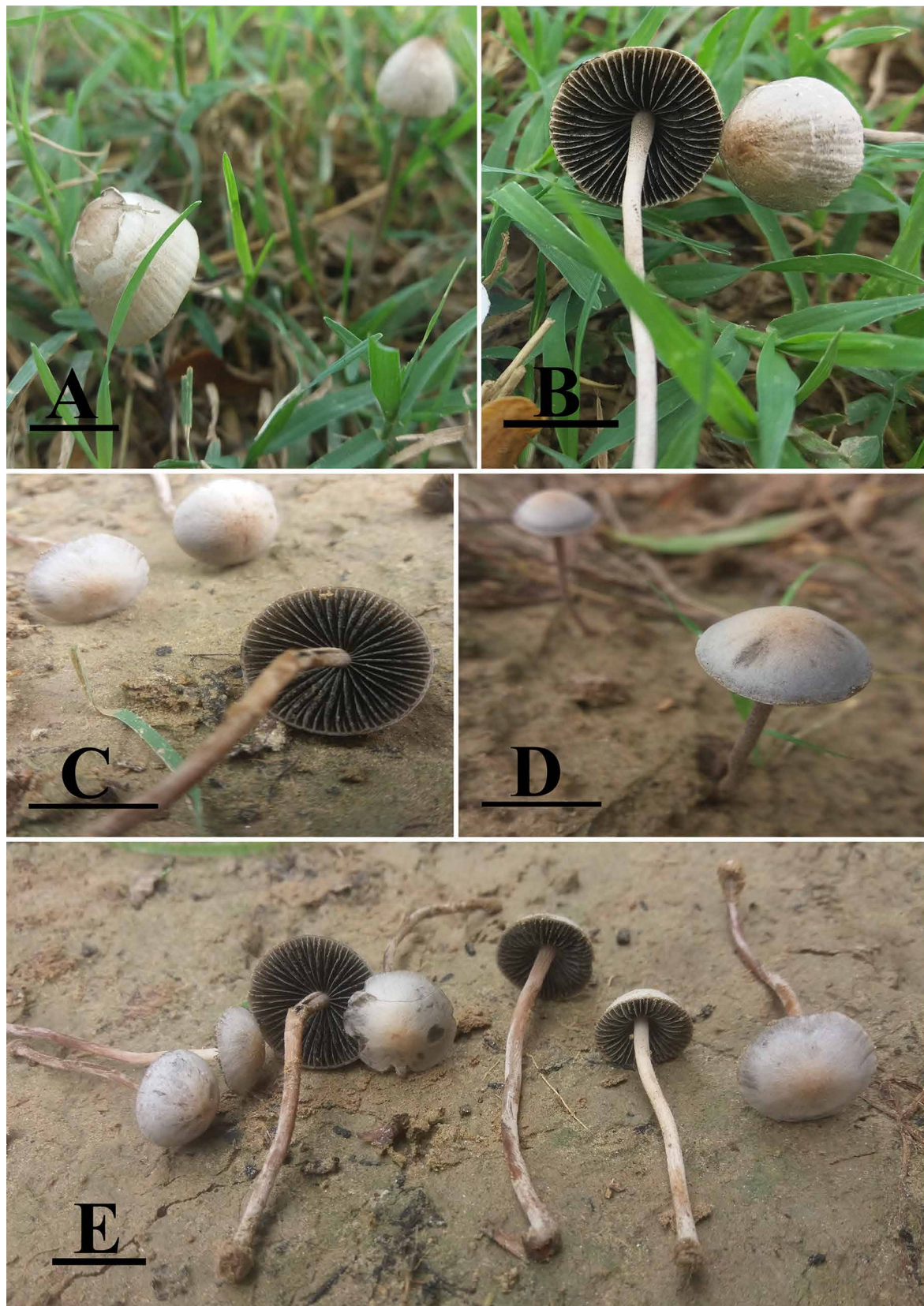
### Additional material examined

PAKISTAN • Punjab Province, Lahore, 32°52'04" N, 74°35'87" E; alt. 217 m a.s.l.; on loamy soil; 10 Jul. 2015; *Qudisia Firdous, BRB S-01*; GenBank nos KY636363 (nrITS); ON116491 (LSU); LAH[36792] • same collection data as for preceding; 28 Jul. 2016; *Qudisia 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].

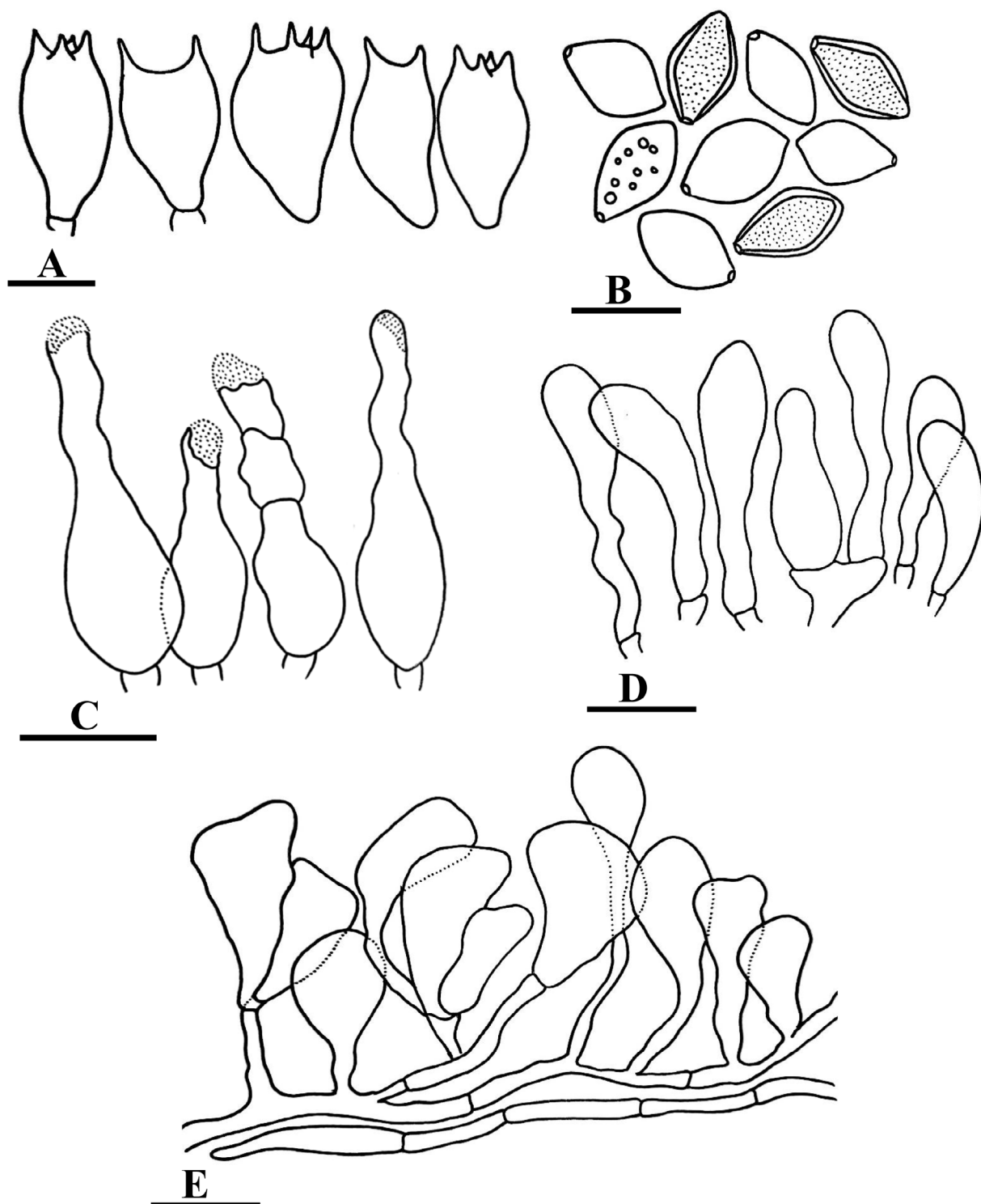
## Description

*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<sub>av</sub> = 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  $\mu$ m, E = 20  $\mu$ m. 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
<i>P. punjabensis</i> 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
<i>P. acuminatus</i>	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 cylindrical	Kaur <i>et al.</i> 2014; Osmundson <i>et al.</i> 2013
<i>P. alcis</i>	4–10	complanate to conical	pale gray	dark gray	20–90 × 0.5–1.5	pex ochraceous gray, base brownish	18–21 × 9.5–12	ellipsoid	25–35 × 4–6	versiform	Moser 1984
<i>P. axfordii</i>	16–21	hemispherical 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	limoniform to ellipsoid	24.6–42.7 × 5.9–10.7	narrowly utri-form	Hu <i>et al.</i> 2020
<i>P. cambodginiensis</i>	12–25	convex to broadly convex	chocolate brown becoming yellowish 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 <i>et al.</i> 1979
<i>P. cyanescens</i>	10–15	hemispheric to convex	pure white	dark grayish brown	50 × 2–3	uniformly white	11–18 × 9–13.7	limoniform to ellipsoid	absent	absent	Wartchow <i>et al.</i> 2010
<i>P. fimicola</i>	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-amygdaliform	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	Cheilocystidia L × W (µm)	Cheilocystidia shape	Reference
<i>P. guttulatus</i>	10	convex	dark olive brown	black	40 × 2	snuff brownish	7.5–10 × 4–6	elliptical	25 × 5	cylindrical	Seidmoham-madi <i>et al.</i> 2019; Sultia-man 2019
<i>P. papilionaceus</i>	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	citriiform	35–53 × 7–12.5	vermiform, urn-shaped or tubular	Abraham 2007; Kuo 2007; Aman-deep <i>et al.</i> 2014; Ediri-weera <i>et al.</i> 2015
<i>P. rickenii</i>	18–20	campanulate to convex	dark brown	mottled gray-ish 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
<i>P. semiovatus</i>	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 <i>et al.</i> 2013
<i>P. sphincrinus</i>	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	citriiform to lemon-shaped	11.0–16.5 × 5.5–9.2	polymorphic	Ediriweera <i>et al.</i> 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 closely 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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