A NOVEL SPECIES OF *MALLOCYBE* (INOCYBACEAE) FROM GILGIT-BALTISTAN, PAKISTAN

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ABSTRACT

The genus *Mallocybe* has been recently delineated within the family Inocybaceae. Its recognition is based on distinctive macro-anatomical features as well as molecular phylogenetic evidence. Despite this advancement, the genus remains globally understudied. In Pakistan in particular, *Mallocybe* is underexplored, and its species diversity has yet to be systematically assessed. In this study, a fungal specimen was collected from Fairy Meadows (35.38685°N, 74.58416°E) at an elevation of approximately 3,300 meters in Gilgit-Baltistan. Comprehensive morpho-anatomical examination was conducted, alongside molecular characterization targeting the internal trnscribed spacer (ITS) region of nrDNA. The ITS sequence was amplified, sequenced, and analyzed phylogenetically to determine its taxonomic affiliation. This integrative approach led to the identification of a novel species, *Mallocybe gilgitica*, characterized by a depressed pileus with orange-brown fibrillose surface and dextrinoid basidiospores. Phylogenetic inference revealed a close affinity with *Mallocybe crassivelata* and *Mallocybe leucoblema.* This study reports a new *Mallocybe* species from Pakistan, identified through integrated morphological and molecular analyses, and highlights its valuable contribution to the fungal diversity of Pakistan.

Key words: Fungal systematics, morphological traits, sequencing, South Asia.

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INTRODUCTION

Inocybaceae family (Agaricales, Basidimycota) is one of the most diverse families of ectomycorrhizal fungi, and more than 700 species are known worldwide at the moment. They are ecologically relevant, and these fungi develop mutualistic relationships with angiosperms and gymnosperms roots, hence supporting the exchange of nutrients and enhancing the health and robustness of forests (Matheny and Kudzma, 2019; Corrales *et al.*, 2022). Even though Inocybaceae are well published in unique temperate ecosystems, their distribution in tropical and mountainous ecosystems is under investigated especially in South Asia. The north of the highlands, including Gilgit Baltistan, Pakistan, is a special ecological environment that are part of the Himalaya and Hindu Kush ranges with Karakoram, which is, however, the habitat of fungal biodiversity, although a little-known scientific attention has been given to it (Hussain *et al.,* 2017).

Latest developments in molecular systematics have seen a significant taxonomic revision of Inocybaceae, with a series of distinct clades including *Mallocybe* being distinctly delimited previously as part of *Inocybe* s.l.t. (Tibpromma *et al.,* 2017). The species of *Mallocybe* are fibrous to scaly pilei, adnate gills, necropigmented basidia, and smooth basidiospores, but morphological variation frequently makes proper determination of species a difficult task (Saba *et al*., 2023). In order to solve these issues, the DNA-based methods have become inevitable to reveal the hidden diversity and clarification of phylogenetic connections in the genus by using the techniques of the internal transcribed spacer (ITS) region sequencing (Raja *et al.,* 2017; Asif *et al.,* 2024). Most of said species (64) are registered under Index Fungorum and continuing to be described in Asia as well as Europe and North America (Hyde *et al.,* 2020; Matheny *et al.*, 2023; Rähn *et al.,* 2024). New *Mallocybe* species have lately been described in Pakistan and this highlights the area as a hotspot to discover yet to be documented (Saba *et al.*, 2023; Rahn *et al.,* 2024).

In spite of it, the genus *Mallocybe* is not very well-investigated in Pakistan, and there are little comprehensive morphological and molecular studies that are combined into one survey. The current paper fills this gap by write-up of a

new species of *Mallocybe* that were found in Fairy Meadows in Gilgit-Baltistan. An integrative method was used to describe *Mallocybe gilgitica* by incorporating morphological studies with an inference method known as ITS. The study adds to the growing international collection of *Mallocybe*, has ecological relevance by using Gilgit-Baltistan as an example of a hot-spot of ectomycorrhizal fungi, and promotes the integrative studies in the fungal systematics field and biodiversity conservation efforts.

MATERIALS AND METHODS

Study site: The location of the fungal specimen collection study was Fairy Meadows, which is in the Diamer District of Gilgit-Baltistan, Pakistan. It lies at the base of Nanga Parbat in the north and it lies at an altitude of about 3, 300 m above the surface level (35.38685°N, 74.58416°E) and it belongs to the Western Himalayan range. Coniferous trees are prevalent in the area with *Pinus wallichiana*, and *Picea smithiana*. It has cold temperate climate with extended winters and short hot summer, with yearly rainfall of between 1000 and 1500mm. The loam and organic soil and a low level of anthropogenic disturbance make the Fairy Meadows an optimal environment to support ectomycorrhizal fungi hence a rare ecological niche to discover new taxa like the new taxon, *Mallocybe*.

Sampling and macro-microscopic analyses: Collected fungal specimens were examined for macroscopic features such as pileus, lamellae, stipe, and basidiocarps, with color references standardized using Munsell Soil Color Charts (Munsell Color, 1975). Specimens were dried for 7 to 9 hours at 39 °C, and microscopic structures (e.g., basidiospores, basidia, cystidia, pileipellis, stipitipellis) were examined after mounting in Congo red and 5% KOH by using microscope (Olympus BX40) and digital camera (XC50). Measurements were taken from 30–35 randomly selected elements per collection, with Q values and means calculated, and diagnostic features illustrated using a Leitz camera lucida.

Taxonomy- Molecular and Phylogenetic analyses: Genomic DNA was extracted from dried basidiocarps of *M. gilgitica* by using a slightly modified cetyltrimethylammonium bromide (CTAB) method (Gardes & Bruns, 1993). DNA quality was assessed via 1% agarose gel electrophoresis (Voytas, 2001). The internal transcribed spacer (ITS) region of ribosomal DNA was amplified using the primer pair ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes & Bruns, 1993) in a Bio-Rad T100™ thermocycler (Bio-Rad Laboratories, Singapore). PCR amplicons were sequenced by TsingKE Biotechnology (China), and consensus sequences were assembled using BioEdit v7.0.4.1 (Hall, 1999). The resulting ITS sequences were analyzed using BLAST in the NCBI (https://www.ncbi.nlm.nih.gov/) nucleotide database to identify closely related taxa. Reference sequences were obtained from GenBank and aligned with study-generated sequences using MUSCLE (EMBL-EBI) (http://www.ebi.ac.uk/). Phylogenetic relationships were reconstructed through Maximum Likelihood (ML) analysis with 1,000 bootstrap replicates in MEGA 11 (Tamura *et al*., 2021), which confirmed the placement of the novel taxon within the *Mallocybe* clade.

RESULTS

Taxonomy

***Mallocybe gilgitica* H. Ahmad, Ilyas. S & Razzaq. A, sp. nov.:** MycoBank No. 861350

Diagnosis.: *Mallocybe gilgitica* is morphologically similar to *M. crassivelata* and *M. leucoblema,* but it can be distinguished by its fibrillose orange-red pileus measuring 2.5–4.5 cm in diameter and its emarginated, grayish-red lamellae. The basidiospores of *M. gilgitica* are ellipsoid*,* slightly roughened, and dextrinoid, measuring (7.5–)8.0–10.5(–11.0) × (5.5–)6.0–6.5(–7.0) µm, which differ slightly from those of its close relatives. While all three species share clavate cheilocystidia and caulocystidia and possess tetrastrigmate basidia, *M. gilgitica* is phylogenetically distinct based on ITS sequence data, forming a well-supported clade separate from *M. crassivelata* and *M. leucoblema*, thereby confirming its recognition as a novel species.

Type. Holotype: PAKISTAN, Gilgit-Baltistan, Diamer District, Fairy Meadows (35.38685°N, 74.58416°E), under *P. wallichiana*, 15 August 2021, leg. H. Ahmad, S. Ilyas & A. Razzaq; AN#SR-63.

Etymology: The specific epithet *gilgitica* was chosen to honor the Gilgit-Baltistan region of Northern Pakistan, which is the location where the species was first found.



Fig. 1. Morphological features of *Mallocybe gilgitica*. (a) Basidiocarps showing pileus surface and stipe. (b) Basidiocarp showing hymenium with stipe. Scale bars: (a, b) = 1 cm.

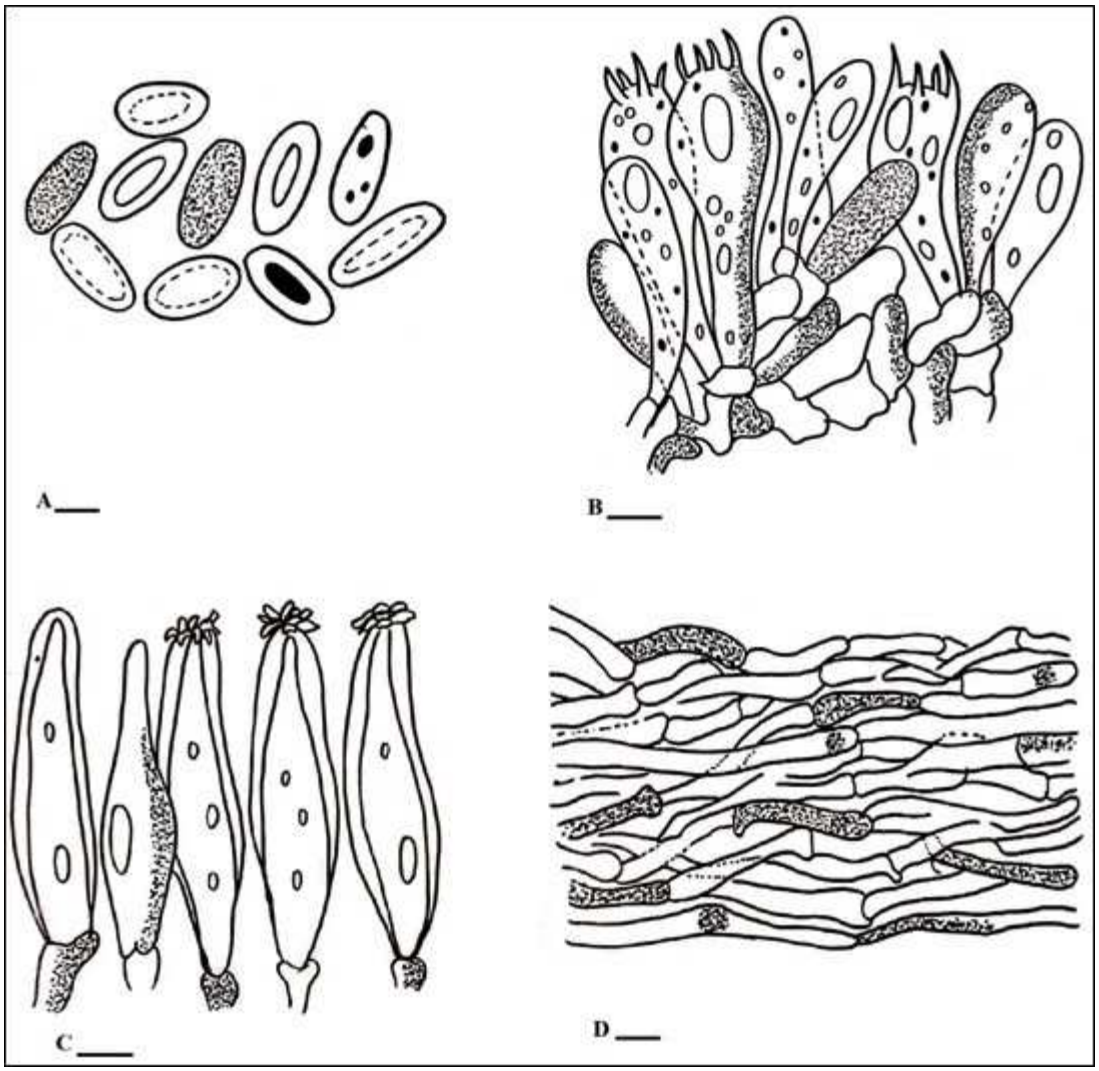


Fig 2. Line drawings of *Mallocybe gilgitica*. (a) Basidiospores. (b) Basidia. (c). Caulocystidia. (d) Pileipellis. Scale bars: (a) = 5 µm; (b) = 10 µm; (c) = 10 µm; (d) = 10 µm.

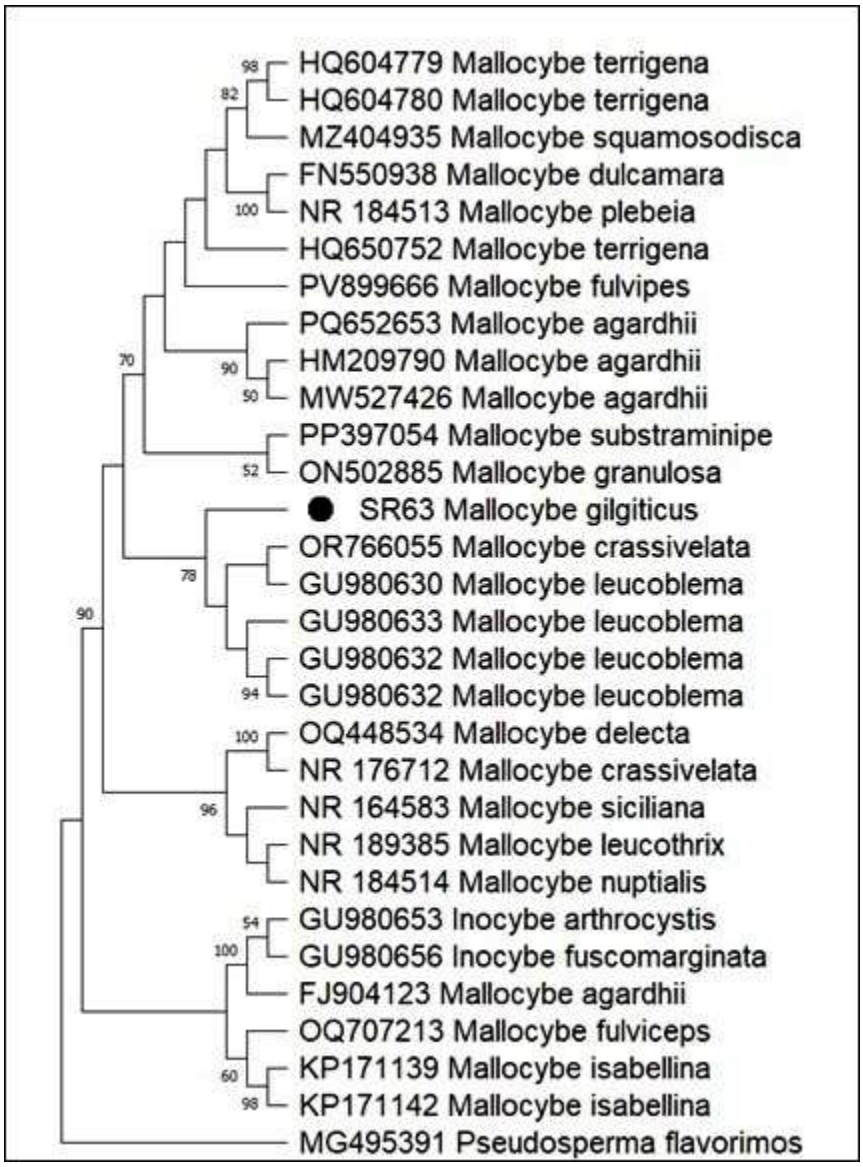


Fig 3. Molecular phylogenetic analysis of ITS sequences of *Mallocybe gilgitica* sp. nov. inferred by using the maximum likelihood method. Sequences generated from local collection are marked with bullets.

Description. Pileus 25–45 mm in diameter, wide convex shape to a flat surface, with a noticeable central depression in maturity; dry surface, densely fibrillose, orange-red to reddish-orange (approx. 2.5YR 5/8–6/8, Munsell), often with a slightly darker disc; margin incurved when young, becoming uplifted and irregular with age, not splitting. **Lamellae** emarginated, medium-spaced, grayish-red to cinnamon-brown (approx. 5YR 4/6), with entire margins; one to two tiers of lamellulae present. **Stipe** 40–60 × 4–7 mm, central, cylindrical, slightly curved in young basidiomata, dry, fibrillose, concolorous with pileus or slightly paler; apex often with violet tinges; annulus absent; cortina not observed. **Context** light brown, firm, thickness up to 2 mm. **Odor** neutral to slightly mushroom-like. **Taste** not recorded.

Basidiospore 7.5-10.5 × 5.5–6.5 µm, (9.0 ± 0.6 × 6.0 ± 0.3 µm), Q= 1.3-1.75, Qm=1.5; elliptical to subglobose, slightly roughened, dextrinoid, pale brown with 5% KOH. **Basidia** 20–25 × 10–12 µm, (22.4 ± 1.6 × 11.1 ± 0.6 µm), Q = 1.8–2.3, Qm = 2.0; clavate to cylindrical, thin-walled, tetrasterigmate, hyaline to pale brown, often with yellowish necropigment. **Pleurocystidia** absent. **Cheilocystidia** 14–28 × 8–14 µm (21.3 ± 3.2 × 10.6 ± 1.4 µm), Q = 1.5-2.6, Qm= 2.0; clavate to subcylindrical, smooth-walled, hyaline, occasionally in chains. **Caulocystidia** 35–45 × 6–9 µm, clavate to sublageniform, abundant at stipe apex, yellowish brown when treated with KOH, thin-walled, with clamp connections that are present at the base. **Pileipellis** composed of repent, cylindrical hyphae that are 5.4- 20.7µm wide, thin-walled and show a light to pale brown reaction with KOH. **Stipitipellis** composed of cylindrical hyphae with a width of 4.9-12.5µm.

In KOH, they appear hyaline to pale yellow or olivaceous. All tissues are inamyloid. **Clamp connections** found throughout the tissues.

Habitat. The species occurs individually or in small clusters within subalpine coniferous forest floors characterized by rich humus.

Known Distribution. To date, *M. gilgitica* is endemic to the western Himalayan range in northern Pakistan, specifically from Fairy Meadows in Gilgit-Baltistan. The species was found growing scattered on humus-rich soil in subalpine coniferous forests dominated by *P. wallichiana.*

DISCUSSION

The research registers *M. gilgitica* as the new Pakistan species of ectomycorrhizal fungi, thus, pointing to Gilgit-Baltistan as a poorly studied hotspot of ectomycorrhizal fungi. This finding neutralizes the worth of integrative taxonomic method in not only biodiversity conservation but also systematization of fungi. Another indication of the richness of the fungal diversity in the region is recent mycological surveys in Pakistan which have resulted in the establishment of several new taxa within the Agaricales, Boletales, and Russulales (Razaq *et al*., 2012: Nawaz *et al*., 2013: Qasim *et al*., 2015a, 2015b; Saba *et al*., 2019a, 2019b; Kiran *et al*., 2025). *Inocybe* sensu lato is approximated to have 35 species of the genus of those suggested, and the genus *Mallocybe* is increasingly better known (Saba *et al*., 2023; Saba and Khalid, 2020). *Mallocybe leucoblema, Mallocybe velutina, Mallocybe pakistanica*, and *Mallocybe pinicola* are some of the seven known species of *Mallocybe* in the country, which have been already reported in Pakistan before this study (Ahmad *et al*., 1997; Saba and Khalid, 2020; Saba *et al*., 2023; Kiran *et al.*, 2025).

Mallocybe gilgitica, which is gathered in subalpine coniferous forests of Gilgit Baltistan, morphologically differs with its close relatives *(M. crassivelata* and *M. leucoblema*). It is characterized by presence of orange-red fibrillose pileus and notched gray red gills. It has a slender stipe which can be faintly violet colored, and gives rise to somewhat scaly, dextrinoid, ellipsoid spores having a reaction to the Melzerian reagent. Microscopically it has clavate basidia with a lot of caulocystidia and cheilocystidia as chains in few cases. In comparison, the temperature-sensitive temperate variants of *M. crassivelata* are characterised by a stipe which is more robust and thicker, a darker brown hue to its pileus, and somewhat wider basidiospores, and do not possess the reddish hueing or violet stipe colour of *M. gilgitica* (Kiran *et al.,* 2025). *M. leucoblema* is found in Europe and North America and is characterized even further by a brownish fulvous to ochraceous pileus that tends to have traces of whitish veils, slender adnate pale yellow to olivebrown gills, and small basidiospores with no reddish pigmentation or violet stain of the stipe as with *M. gilgitica* (Cripps *et al.,* 2010).

The ecological distribution of *M. gilgitica* is restricted to subalpine coniferous forests with *P. wallichiana* as the dominant herb and *M. crassivelata* is a characteristic of the deciduous woodland habitat and *M. leucoblema* of the mixed hardwood habitat. The distinctness of *M. gilgitica* is also confirmed by molecular phylogenetic studies, which have detected minimum bootstrap support of 99% using ITS sequence to differentiate *M. gilgitica* clade (99 percent) as an independent and distinct monophyletic lineage compared to *M. crassivelata* and *M. leucoblema* (Saba *et al*., 2023; Kiran *et al*., 2025). These comparisons have demonstrated the morphological peculiarities, niche, and molecular distinctiveness of *M. gilgitica* that allows accepting it as a new species and enrich the official diversification of *Mallocybe* in the South Asian regions.

Table 1. *Mallocybe* species and related taxa included in the ITS-based phylogenetic analysis, showing comparison with the newly described *M. gilgitica.*

Species	Specimen voucher	Country	ITS (accession number)	Reference
Inocybe arthrocystis	CLC1141	Sweden	GU980653	Larsson *et al.* (2009)
I. fuscomarginata	BJ980718	Sweden	GU980656	Larsson *et al.* (2009)
Mallocybe agardhii	AB980912	Sweden	HM209790	Larsson *et al.* (2009)
M. agardhii	F-2389	Sweden	PQ652653	Esteve-Raventós *et al.* (2024)
M. agardhii	DB2-10-12-16	Greece	MW527426	Daskalopoulos *et al.* (2021)
M.agardhii	EL88-04	Sweden	FJ904123	Larsson *et al.* (2009)
M.crassivelata	M41	China	OR766055	Hu *et al.* (2023)
M.crassivelata	MCVE:29561	Italy	NR176712	Bandini *et al*. (2019)

M.delecta	JV21393	Sweden	OQ448534	Matheny *et al.* (2023)
M.dulcamara	EL21206	Sweden	FN550938	Ryberg *et al.* (2010)
M.fulvipes	RR24.278	Sweden	PV899666	Cripps *et al.* (2010)
M.fulviceps	DPL13914	USA	OQ707213	Matheny *et al*. (2023)
M.gilgitica	LCW123	**Pakistan**	PP468336	Sequence generated during this study
M.granulosa	HMUT M851	China	ON502885	Mao *et al.* (2022)
M.isabellina	NLB420	USA	KP171139	Matheny *et al.* (2020)
M.isabellina	NLB491	USA	KP171142	Matheny *et al.* (2020)
M.leucoblema	SM2324	Sweden	GU980630	Cripps *et al.* (2010)
M.leucoblema	TAA128324	Sweden	GU980633	Cripps *et al.* (2010)
M.leucoblema	CLC1721	Sweden	GU980632	Cripps *et al.* (2010)
M.leucoblema	CLC1721	Sweden	GU980632	Cripps *et al.* (2010)
M.leucothrix	TENN 075564	USA	NR189385	Matheny *et al*. (2020)
M.nuptialis	STU:SMNS-STU-F-0901661	USA	NR184514	Daskalopoulos *et al.* (2021)
M.plebeia	STU:SMNS-STU-F-0901665	USA	NR184513	Daskalopoulos *et al.* (2021)
M.siciliana	AMB 18274	USA	NR164583	Brugaletta *et al.* (2017)
M.substraminipe	0645	USA	PP397054	Cripps *et al.* (2010)
M.squamosodisca	LVK20133	USA	MZ404935	Matheny *et al.* (2020)
M.terrigena	2050622-012	USA	HQ604779	Matheny *et al.* (2020)
M.terrigena	2070612-002	USA	HQ604780	Matheny *et al.* (2020)
M.terrigena	DAVFP 28178	Sweden	HQ650752	Ryberg *et al*. (2008)
Pseudosperma flavorimos	LAH35042	Pakistan	MG495391	Jabeen & Khalid (2020)

Table 2. Main differences among *M. gilgitica* and its closely related species.

Characters	***Mallocybe gilgitica* sp. nov.**	***Mallocybe crassivelata***	***Mallocybe leucoblema***
Pileus	Fibrillose, orange-red, 2.5–4.5 cm	Dark brown to fulvous, thicker, robust	Brownish fulvous to ochraceous, often with whitish veil remnants
Lamellae	Emarginated, grayish-red	Adnate to slightly decurrent, pale brown	Narrowly adnate, pale yellow to olive brown
Stipe	Slender, often curved, violet tinges at apex	Thick, robust, without violet tones	Cylindrical, pale to brown, lacks violet pigmentation
Basidiospores (µm)	7.5–10.5 × 5.5–6.5; ellipsoid, slightly roughened, dextrinoid	Slightly broader, smooth to faintly roughened, dextrinoid	9–11.5 × 5–6; smaller, globose to ellipsoid, dextrinoid
Basidia (µm)	Clavate, 20–25 × 10–12; Qm = 2.0	Clavate, slightly larger	Clavate, similar in size
Cheilocystidia	Clavate, occasionally in chains	Clavate, solitary	Clavate, solitary
Caulocystidia	Abundant	Present	Present
Distribution	Gilgit-Baltistan, Pakistan	Temperate regions (Europe/Asia)	Europe and North America

Conclusion: *M. gilgitica* introduces the current diversity of *Mallocybe* in Pakistan and implicates Gilgit-Baltistan as a nexus of new taxa of fungi. This paper provides support to the ecological importance of ectomycorrhizal fungi with morphological and molecular data, and indicates the necessity of further surveys and phylogenetic studies within the underrepresented habitats in South Asia.

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Declaration of competing interest: The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

Data availability statement: The data-set generated and analyzes during this study is openly available in GenBank and MycoBank for further evaluation.

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