

## Research article

***Inocybe subhimalayanensis* (Agaricales, Inocybaceae),  
a new smooth spored *Inocybe* species from Pakistan**Annum RAZZAQ<sup>1,\*</sup>, Arooj NASEER<sup>1,2</sup> & Abdul Nasir KHALID<sup>1,3</sup><sup>1</sup>Fungal Biology and Systematics Research Laboratory, Institute of Botany,  
University of the Punjab, Lahore, Pakistan.\*Corresponding author: [annum.phd.botany@pu.edu.pk](mailto:annum.phd.botany@pu.edu.pk)<sup>2</sup>Email: [aroorj.hons@pu.edu.pk](mailto:aroorj.hons@pu.edu.pk)<sup>3</sup>Email: [drankhalid@gmail.com](mailto:drankhalid@gmail.com)

**Abstract.** During mycological explorations, a new smooth spored species, *Inocybe subhimalayanensis* Razzaq, Naseer & Khalid sp. nov. was collected from moist temperate sub-Himalayan region, Pakistan. Phylogeny of ITS and LSU regions of nrDNA, and morphoanatomical data make it distinct from other known species of the genus. The taxon is characterized by: a yellowish orange to brown pileus with prominent fibrillose, prominent umbo; ellipsoid to amygdaliform smooth larger basidiospores (8.4–)8.6–12.2(–12.6) × (4.9–)5.1–7.1(–7.3) μm; and lack of velipellis. Molecular phylogenetic analyses further support the recognition of the new species.

**Keywords.** Inocybaceae, ITS, LSU, Punjab, taxonomy.

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**Introduction**

The *Inocybaceae* is one of the most diverse families of *Agaricales* with approximately more than 1050 species worldwide (Matheny *et al.* 2020). Matheny *et al.* (2020) performed a six-loci phylogenetic analysis of the *Inocybaceae* and classified the family into seven genera; *Auritella* Matheny & Bougher ex Matheny & Bougher, *Inocybe* (Fr.) Fr., *Inosperma* (Kühner) Matheny & Esteve-Rav., *Mallocybe* (Kuyper) Matheny, Vizzini, Esteve-Rav., *Nothocybe* Matheny & K.P.D. Latha, *Pseudosperma* Matheny & Esteve-Rav., and *Tubariomyces* Esteve-Rav. & Matheny. Among these, *Inocybe* is the most speciose genus represented by 850 species worldwide (Matheny *et al.* 2020; Bandini *et al.* 2022) and has a cosmopolitan distribution. More than 200 new species of the genus have been reported during the last five years, the number is still growing as a result of new discoveries (Crous *et al.* 2017; Matheny & Bougher 2017; Bandini *et al.* 2021; Dovana *et al.* 2021; Mešić *et al.* 2021; Muñoz *et al.* 2022). *Inocybe* plays a significant role in ectomycorrhizal communities of temperate and boreal forests (Cripps *et al.* 2010; Kokkonen & Vauras 2012). This genus is characterized by the presence of cheilocystidia and generally also pleurocystidia with more or less thick walls, whereas this is not the case with the other six genera (Matheny *et al.* 2020).

The Himalayan moist temperate forests of Pakistan are included in one of the twenty-five hotspots for biodiversity (Myers *et al.* 2000). These forests are floristically rich, covered with evergreen vegetation and home to a number of plant species, therefore, an equally high diversity of fungal species is expected. However, only 33 species of this diverse genus have been reported from Pakistan (Naseer *et al.* 2019; Jabeen & Khalid 2020; Saba *et al.* 2020; Khan *et al.* 2022). During the course of a macrofungal survey from different parts of Himalayan forests of Pakistan, a novel species of *Inocybe* was discovered. In this paper, we present our collections as a new species with brief diagnosis, an extensive description, and phylogenetic analyses based on ITS and LSU sequences data.

## Materials and methods

### Study area

The basidiomata have been collected from Himalayan temperate forests of Bhurban during the rainy seasons (September 2020–2021). Bhurban is a small town of the Punjab province of Pakistan (33°56'26.24" N to 73°26'59.48" E). This area is a part of the sub-Himalayan tract with an approximate elevation of 1800–2200 m a.s.l. The area receives precipitation during the entire year with a mean annual rainfall of about 1800 mm (30 year period: World Weather Information Service 2018). The climate is humid and variable due to a broad range of elevation and the vegetation is mainly comprised of *Pinus wallichiana* A.B.Jacks., *Olea ferruginea* Royle, *Quercus leucotrichophora* A.Camus and *Cedrus deodara* (Roxb. ex D.Don) G.Don providing a perfect environment for the growth of mycorrhizal fungi (Hameed *et al.* 2012).

### Sampling and morphoanatomical studies

The specimens were photographed in the field using a Nikon D70s digital camera and morphological features of fresh basidiomata including size, shape and color of pileus, stipe, lamellae, presence or absence of annulus and volva were noted. Color codes were given according to Munsell (1975). The specimens were dried by using an electric fan heater (EcoStar GEH-800) and packed in labeled polythene bags. Samples were submitted in the Lahore (LAH) Herbarium, Institute of Botany, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan.

Anatomical structures were studied with a trinocular OLYMPUS CH30 microscope and measurements were taken with the help of calibrated Piximètre software attached to the microscope with a digital camera (HDCE-X5) under 400× and oil immersion 1000× magnification. For microscopic analyses, 5% KOH was used to rehydrate the tissues (pileus, gills and stipe) of the basidiomata and Congo Red (1%) was used as stain to clarify microscopic features. The abbreviation n/m/p specifies n basidiospores from m basidiomata of p collections. Measurements for basidiospores are taken as length × width (l × w). The average length and average width of all the microscopic characters were measured and given as avl × avw. Extreme values are given in parenthesis, Q value shows l/w ratio of the spore (Bas 1969; Yu *et al.* 2020) and avQ means average Q of all basidiospores (Liang & Yang 2011).

### Molecular and phylogenetic analyses

For molecular analyses, genomic DNA was extracted using modified CTAB method (Gardes & Bruns 1993). For the qualitative analysis of extracted DNA, Gel Electrophoresis was done by using 1% agarose gel (Voytas 2001). The extracted DNA was amplified using polymerase chain reaction by a thermocycler (Bio-Rad T100TM, Bio-Rad Laboratories Pte. Ltd., Jurong East, Singapore). Two genetic markers (ITS and LSU) were amplified in this study. In order to amplify ITS region, primer pairs ITS1F and ITS4 were used (Gardes & Bruns 1993) while LR0R and LR5 primer pairs were used for the amplification of LSU region (Moncalvo *et al.* 2000). Sequencing of the PCR products were done by TsingKE, China. A total of six ITS sequences and two LSU sequences were generated.

Consensus sequences were generated using BioEdit software ver. 7.0.4.1 (Hall 1999). These were BLAST searched at NCBI (<https://www.ncbi.nlm.nih.gov/>) and closely related *Inocybe* sequences were retrieved from GenBank. These sequences were aligned with the sequences generated in this study using the online MUSCLE tool at EMBL-EBI (<http://www.ebi.ac.uk/>). For phylogenetic analyses, Maximum likelihood (ML) bootstrap analyses were performed in MEGA ver. 11 (Tamura et al. 2021).

## Results

### *Molecular phylogenetic analyses*

The aligned ITS dataset, including the results of BLAST searching against GenBank and the published work of Bandini *et al.* 2022, consisted of 43 nucleotide sequences including *I. spuria* Jacobsson & E. Larss. as an outgroup (Bandini *et al.* 2022). 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 ML analysis was done using MEGA 11. In both ITS and LSU based phylograms, *I. subhimalayanensis* Razzaq, Naseer & Khalid sp. nov. get separated from other species with a strong bootstrap.

### *Taxonomy*

Phylum Basidiomycota R.T.Moore  
Class Agaricomycetes Doweld  
Order Agaricales Underw.  
Family Inocybaceae Jülich  
Genus *Inocybe* (Fr.) Fr.

*Inocybe subhimalayanensis* Razzaq, Naseer & Khalid sp. nov.  
Mycobank MB 845094  
Figs 1–2, Table 1

### **Diagnosis**

The new species differs from *Inocybe parvicystis* F.J.Rodr.-Campo & Esteve-Rav. by the combination of these characters: prominent umbo, larger spores up to 9.9 µm (vs 6.5 µm), hymenal cystidia with non-crystalliferous apex, non-crystalliferous caulocystidia and absence of velipellis.

### **Etymology**

The epithet 'subhimalayanensis' is referring to the sub-Himalayan region where the type specimen was collected.

### **Type material**

#### **Holotype**

PAKISTAN • Punjab province, Rawalpindi district, Bhurban town; 33.9554° N, 73.4519° E; alt. 1828 m a.s.l.; on soil below *Pinus*; 7 Sep. 2020; *Annum Razzaq*, GB-18; GenBank nos ON810645 (nrITS), ON810652 (LSU); LAH[37437].

#### **Additional material examined**

PAKISTAN • Punjab province, Rawalpindi district, Bhurban town; 33.9554° N, 73.4519° E; alt. 1828 m a.s.l.; on soil below *Pinus*; 7 Sep. 2020; *Annum Razzaq*, GB-26; GenBank nos ON810643 (nrITS), ON810653 (LSU); LAH[37438] • same data as for preceding; 10 Sep. 2021; *Annum Razzaq*, GC-35; GenBank no ON810644 (nrITS); LAH[37439] • Kashmiri bazar; 10 Sep. 2021; *Annum Razzaq*, BR-88; GenBank no ON810646 (nrITS); LAH[37440] • same data as for preceding; 4 Sep. 2021; *Annum*



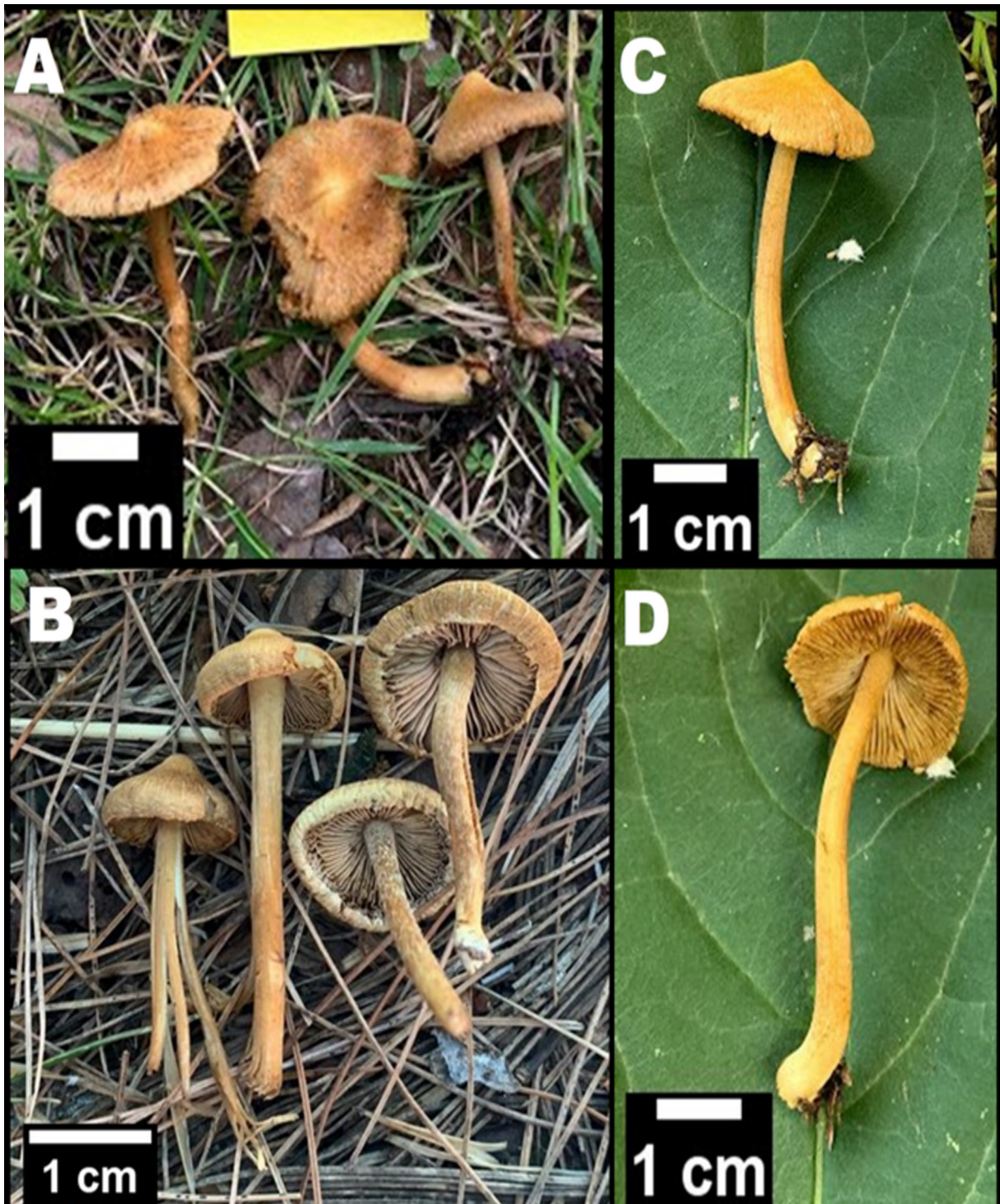
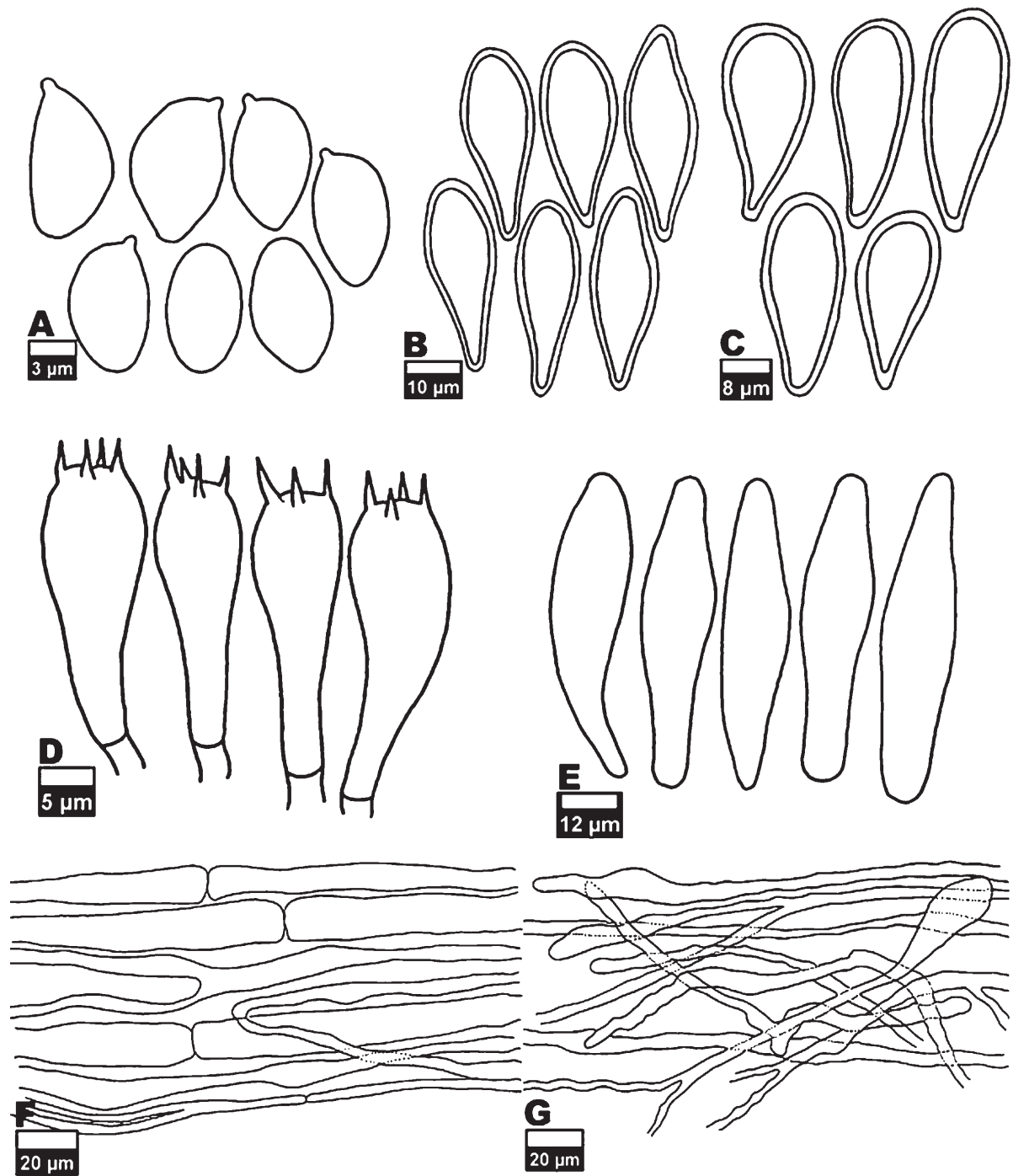


Fig. 1. Macromorphological characters of *Inocybe subhimalayanensis* Razzaq, Naseer & Khalid sp. nov. A. LAH37438. B. LAH37439. C–D. LAH37437 (holotype).



**Fig. 2.** *Inocybe subhimalayanensis* Razzaq, Naseer & Khalid sp. nov. holotype (LAH37437). A. Basidiospores. B. Pleurocystidia. C. Cheilocystidia. D. Basidia. E. Caulocystidia. F. Stipitipellis. G. Pileipellis.

Razzaq, BR-22; GenBank no ON810647 (nrITS); LAH[37441] • same data as for preceding; 12 Sep. 2020; Annum Razzaq, BR-57; GenBank no ON810648 (nrITS); LAH[37442] • Murree; 33.9070° N, 73.3943° E; alt. 2291 m a.s.l.; 18 Aug. 2019; Arooj Naseer AN-36; GenBank no ON911331 (nrITS); LAH[151676].

### Description

*Pileus* 15–25 mm wide, (sub)campanulate to subumbonate when immature becoming broadly convex or expanded at maturity, with prominent large umbo; margin decurved at young stage later becoming straight, incised to eroded, concolorous, pileus depressed near the umbo; prominent dark brown radial striations at pileal surface extended up to the central umbonate region; color yellowish orange (10YR 7/8) to orange (7.5YR 8/8) when fresh becoming yellowish brown (7.5YR 6/8) to brown hue (7.5YR 5/8) when old. *Hymenophore* regular, close, adnexed, ventricose, whitish at first becoming brownish with age; edge entire. *Stipe* 21–35 × 2–4 mm, cylindrical or curved, fine whitish tomentum when young, later becoming longitudinally striate or glabrous, yellowish orange (10YR 7/8) to brown (7.5YR 5/8) colored. *Annulus* and *Volva* absent. *Context* thin. *Habit* pluteoid. *Odor* unspecific. *Taste* not recorded.

*Basidiospores* [100/3/3] (8.4–)8.6–12.2(–12.6) × (4.9–)5.1–7.1(–7.3) μm, avl × avw = 9.9 × 5.9 μm, Q = 1.62–1.73, avQ = 1.69, smooth, (sub)amygdaloid with rounded base and rounded to acute apex in side view, oblong to ovoid with rounded to (sub)acute apex in frontal view, often with explicit suprahilar depression, prominent hilar appendix, moderately thick-walled, guttulated, pale to dark brown in 5% KOH and water. *Basidia* (24.8–)26.2–35.2(–38.7) × (12.2–)13.3–19.2(–19.7) μm, avl × avw = 29.8 × 8.4 μm, thin-walled, hyaline in 5% KOH, tetrasterigmate, broadly clavate, content present. *Pleurocystidia* (35.2–)37.2–49.4(–49.6) × (7.7–)8.1–12.6(–14.2) μm, avl × avw = 42.6 × 16.6 μm, clavate or (sub)clavate to broadly clavate or (sub)cylindrical or fusiform, metuloidal, thick walled, at apex generally wide, apex non crystalliferous. *Cheilocystidia* (28.3–)32.3–48.3(–49.4) × (13.1–)14.2–22.4(–23.6) μm, avl × avw = 37.2 × 16.9 μm, similar to pleurocystidia in size and shape. *Pileipellis* a cutis of parallel hyphae 3.5–11.5 μm wide, subcutis with wider and paler to colorless hyphae. *Stipitipellis* a cutis of parallel hyphae, 1.73–16.2 μm thick (av = 7.6 μm), septate, branched. *Caulocystidia* only near the apex of the stipe, (56.2–)58.4–97.8(–101.1) × (17.7–)19.2–35.7(–37.6) μm, avl × avw = 78.1 × 23.4 μm, long and narrow subcylindrical to (sub)utriform; intermixed.

### Habitat

Found from nutrient-rich soil, under *Pinus wallichiana* (Pinaceae).

### Distribution

This species is reported for the first time from sub-Himalayan forests of Punjab, Pakistan.

### Discussion

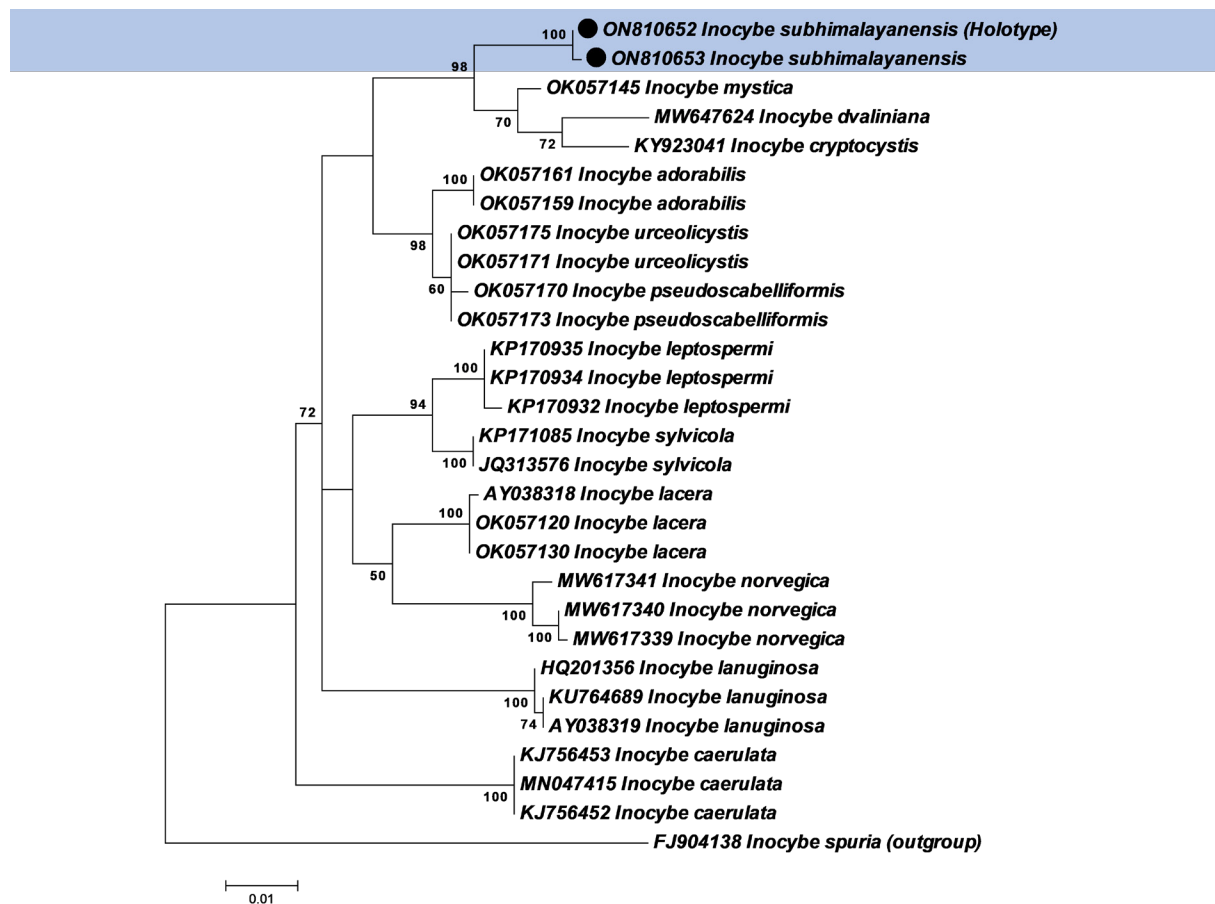
In this study, a new species of *Inocybe* collected from Punjab, Pakistan, is identified on the bases of its morpho-anatomical features and phylogenetic analyses of ITS and LSU sequences dataset (Fig. 3). The small sized basidiomata, campanulate to convex yellowish orange to brown pileus with radial striations, prominent large umbo, smooth and amygdaloid to oblong basidiospores, presence of pleurocystidia and caulocystidia and presence of clamp connections allocate this species in the genus *Inocybe* (Figs 1–2).

The ITS sequences of *I. subhimalayanensis* sp. nov. showed 99.8% similarity with an unpublished soil ECM sequence from Pakistan (GenBank accession numbers KF679813) (Fig. 4). In phylogenetic analyses of the ITS region, the sequence *I. subhimalayanensis* sp. nov. form a clade in strong supported sister relationship to *I. parvicystis* (KY349121, KY349127, KY349122, KY349120, KY349126), a species described from Comunidad de Madrid (Spain), but separated with strong bootstrap value



**Table 1** Comparison of the diagnostic characters of *I. subhimalayanensis* Razzaq, Naseer & Khalid sp. nov., with phylogenetically closed species.

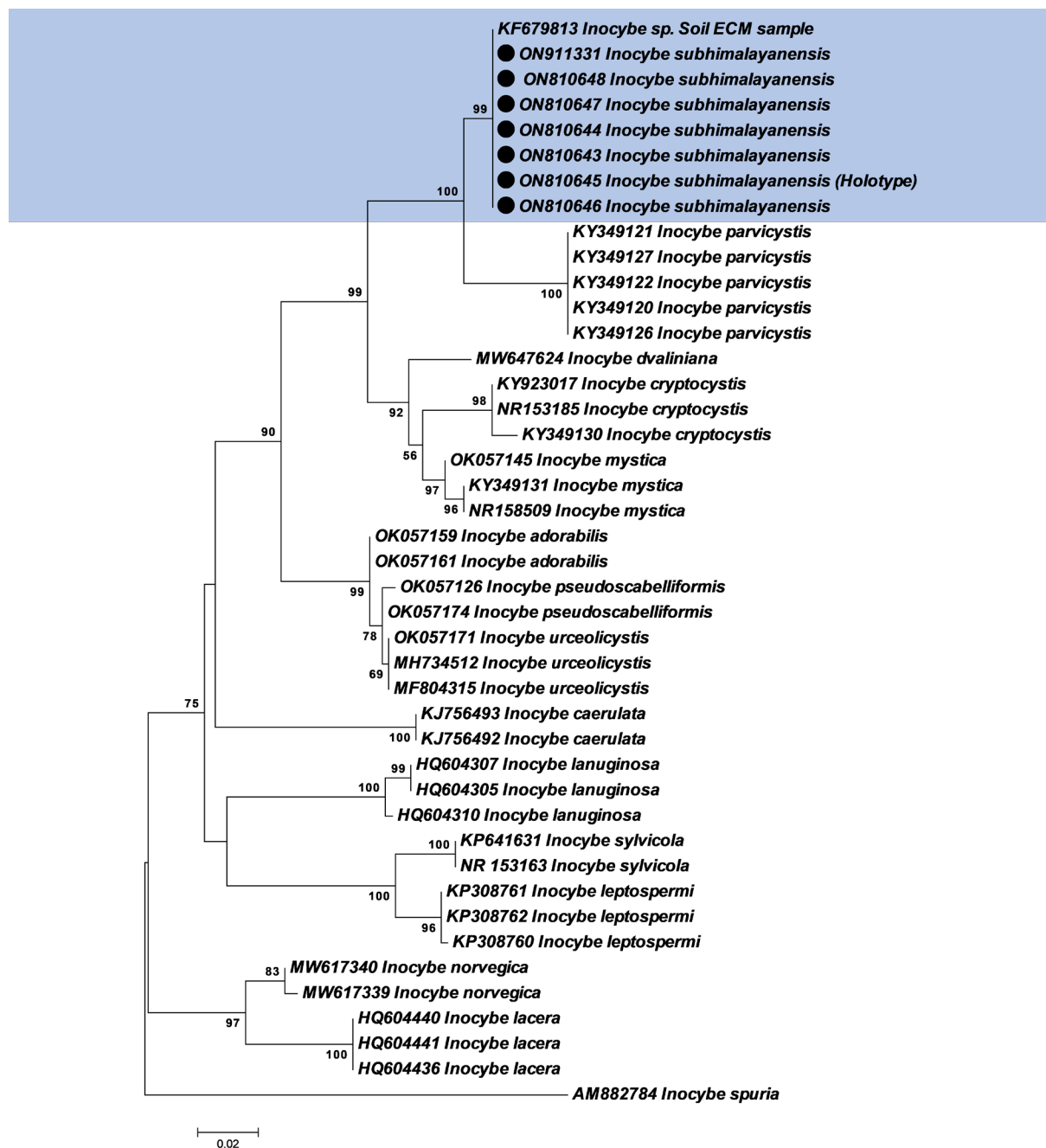
Characters	<i>I. subhimalayanensis</i>	<i>I. parvicystis</i>	<i>I. mystica</i>	<i>I. dvaliniana</i>	<i>I. cryptocystis</i>
Pileus size (mm)	15–25	15–40	Up to 30	Up to 20	Up to 35
Pileus surface	Fibrillose	Surface smooth, radially fibrillose at margin	Finely fibrous	Smooth to finely felted and fibrillose	Smooth and compact with finely scaly
Umbo	Prominent large	Not or hardly present	Present at early stages	Prominent	Prominent
Velipellis	Absent	Present	–	Present	Present (only in young basidiomata)
Spores	8.4–12.6 × 4.9–7.3 μm	7.5–11.5 × 4.5–6. μm	Up to about 9(–9.5) μm	7.3–10.4 × 4.7–5.9 μm	7–8 × 5 μm
Caulocystidia	Non-crystalliferous, at apex of stipe	Crystalliferous, accompanied by paracystidia	–	–	Numerous at the top



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

(100) from *I. parvicystis* (Fig. 4). *Inocybe subhimalayanensis* sp. nov. differs morphoanatomically in having smaller pileus, 15–25 mm wide (vs 15–40 mm wide) with prominent dark brown fibrillose (vs surface smooth, radially fibrillose at margin), prominent large umbo (vs absent or hardly present), and absence of velipellis (vs present), caulocystidia with non-crystalliferous apex (vs crystalliferous apex caulocystidia) (Esteve-Raventós *et al.* 2017).

Another closely related taxon is *I. dvaliniana* Bandini & B.Oertel, which also has the cystidia with non-crystalliferous apex but differs from the new taxon by mottled straw-colored to honey-colored pileus,



**Fig. 4.** Molecular phylogenetic analysis of ITS sequences of *Inocybe subhimalayanensis* sp. nov. inferred by using the maximum likelihood method. Sequences generated from local collection are marked with bullets.



presence of whitish velipellis and relatively short hymenial cystidia ( $17\text{--}42 \times 5\text{--}11 \mu\text{m}$ , av.  $28 \times 7 \mu\text{m}$ ) (<https://www.inocybe.org>) whereas our new taxon has prominent dark brown radial striations on the pileus, absence of velipellis and large sized cheilocystidia ( $28\text{--}49 \times 13\text{--}23 \mu\text{m}$ , av.  $37 \times 16 \mu\text{m}$ ).

The two lineage forming taxa *I. mystica* Stangl & Glowinski and *I. cryptocystis* D.E.Stuntz are close to the new taxon. *Inocybe mystica* differs in having relatively large size basidiospores (up to  $9\text{--}9.5 \mu\text{m}$ ), short cheilocystidia with crystalliferous apex (<https://www.inocybe.org>) and *I. cryptocystis* shows clear morphoanatomical differences with *I. subhimalayanensis* sp. nov. in having smooth and compact pileal surface, and a partial white veil at young stage (Murrill *et al.* 1932). A comparison of diagnostic characters of phylogenetically close species is given in Table 1.

Himalayan forests of Pakistan exhibit large altitudinal variation, with climatic conditions and a diverse vegetation that supports a diverse and conspicuous lichen biota. The nature reserves have abundant biological resources, it is expected that more new species of macrofungi may be discovered in the future.

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