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***Inocybe quercicola* sp. nov. (Agaricales, Inocybaceae), from Pakistan**
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**ABSTRACT**

The new species *Inocybe quercicola* is described on the basis of morphological and genetic features. It is characterized by medium-sized basidiomata, radially fibrillose campanulate pileus, dark brown lamellae when mature, stipe that is pruinose along the whole length, nodulose, rather small basidiospores (av.  $8.8 \times 5.5 \mu\text{m}$ , Qav.=1.53) and pleurocystidia which are similar to cheilocystidia in shape but smaller in size ( $19\text{--}22 \times 47\text{--}65 \mu\text{m}$ ). Based on the phylogenetic analysis of nuclear ribosomal ITS and LSU nucleotide sequences *I. quercicola* belongs into *I.* sect. *Marginatae*, subsect. *Praetervisae*.

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**Introduction**

The family *Inocybaceae* Jülich is one of most diverse families of *Agaricales* with an estimated diversity of 1050 species globally (Matheny et al. 2020). Species of the family form ectomycorrhizal associations with numerous families of angiosperms and gymnosperms in tropical and temperate areas (Kirk et al. 2008). The family is characterized by the production of basidiomes with a stipe, non-glutinous pileus, brownish lamellae when mature, distinctive odors, pigmented basidiospores with a smooth exosporium and a lack of a germ pore, and presence of cheilocystidia and/or pleurocystidia. Recently Matheny et al. (2020) identified seven genera based on a six-gene phylogeny of the *Inocybaceae*. Several species of this family are reported from Asia (Kobayashi 2009, 2005, Kobayashi & Onishi 2010, Matheny et al. 2012, Fan & Bau 2013, 2014, Horak et al. 2015, Pradeep et al.

2016). From Pakistan, 32 species of the family have been reported (Ahmad et al. 1997, Ilyas et al. 2013, Saba et al. 2015, Jabeen et al. 2016, Farooqi et al. 2017, Naseer et al. 2017, Naseer et al. 2018, Ullah et al. 2018, Naseer et al. 2019, Jabeen & Khalid 2020, Saba et al. 2020).

The genus *Inocybe* (Fr.) Fr. (1863: 346) (*Agaricales*, *Inocybaceae*) is represented by 850 species globally (Kirk et al. 2008, Matheny et al. 2009, 2012; Kobayashi & Onishi 2010, Horak et al. 2015, Jabeen et al. 2016, Ullah et al. 2018 and Naseer et al. 2019, Matheny 2019). The genus *Inocybe* can be distinguished from the other members of the family *Inocybaceae* by amygdaliformellipsoid to subcylindrical, angular, nodulose, or spinose basidiospores with a distinct apiculus and presence of pleurocystidia (Matheny et al. 2020).

The aim of the present study is to describe a new species of *Inocybe*, based on morphological and molecular

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analysis of ITS & LSU. The newly described species falls in *I. sect. Marginatae* Kühner, subsect. *Praetervisae* Bon.

## Materials and Methods

### The sampling site

The holotype specimen was collected during a field investigation of ectomycorrhizal communities associated with oak forests near Alpurai forest. The collection area, Toa (34°54'N 72°39'E), lies on the boundary of Shangla and Swat districts (KP province, Pakistan). The region, which ranges in elevation from 850 to 2350 m, is characterized by a very prolonged cold winter, short summer, and annual rainfall of approximately 142 cm.

The second sampling site, Battagram is a northern district in Khyber Pakhtunkhwa Province, Pakistan. This district is bordered by Kohistan in the north, Torghar in the south, Mansehra in the east and south-east and Shangla in the west (Annon, 2010). District Battagram is located between 34°33' and 34°47' latitude and 72°55' and 73°14' longitude (Haq et al. 2010). The vegetation of this region is very diverse, including *Pinus roxburghii* Sarg mixed with *Quercus incana* Roxb, *Olea ferruginea* Royle, *Acacia modesta* Wall, and *Dodonaea viscosa* Benth (Barkatullah & Ibrar, 2011). The third locality, Murree, is an important hill station, part of outer Himalayas. It lies at an altitude of 2286 meters in the Himalayan foothills at 33.35° north latitude and 73.27° east longitude (murreehill.com/introduction.html). Precipitation is received during the entire year. The mean of total rainfall recorded annually is about 1800 mm (30year period: World Weather Information Service 2018).

### Sampling and morpho-anatomical characterization

The photographing of the specimens and field notes such as texture, color, and shape of the basidiomata were done in the field. Colors were noted according to Munsell color system (1994). Anatomical studies were carried out after mounting tissues of the pileus, gills and stipe in 5% KOH solution and observing them under a compound microscope (LABOMED, Labo America, Inc. USA). Measurements of microscopic structures are based on calibrated Piximètre software (<http://www.piximetre.fr>). The specimens were deposited in the Lahore Herbarium, Department of Botany, University of Punjab Lahore, Pakistan and the University of Florida Herbarium, Gainesville FL, USA (FLAS).

### DNA extraction, amplification, and sequencing

Genomic DNA was extracted from dried specimens by CTAB method (Gardes & Bruns, 1993). ITS and LSU regions of nuclear rDNA were amplified using the pairs of primers ITS1F-ITS4B and LR0R-LR5 (Vilgalys and

Hester 1990, White et al. 1990, Gardes & Bruns 1993). Agarose gel electrophoresis was performed to visualize PCR products in a gel documentation system (UVtec, Cambridge, UK) with default settings. The amplified products were then sent to BGI, Shenzhen, China for sequencing. A total of four ITS sequences and three LSU sequences were produced.

### Analysis of sequence data

Consensus sequences were generated using BioEdit (Hall, 1999). A BLAST search tool (<https://blast.ncbi.nlm.nih.gov/>) was used to retrieve closely related sequences from the GenBank database. *Inosperma cookie* and *I. quietodor* were used as an outgroup. Sequences were aligned with MUSCLE (Edgar 2004).

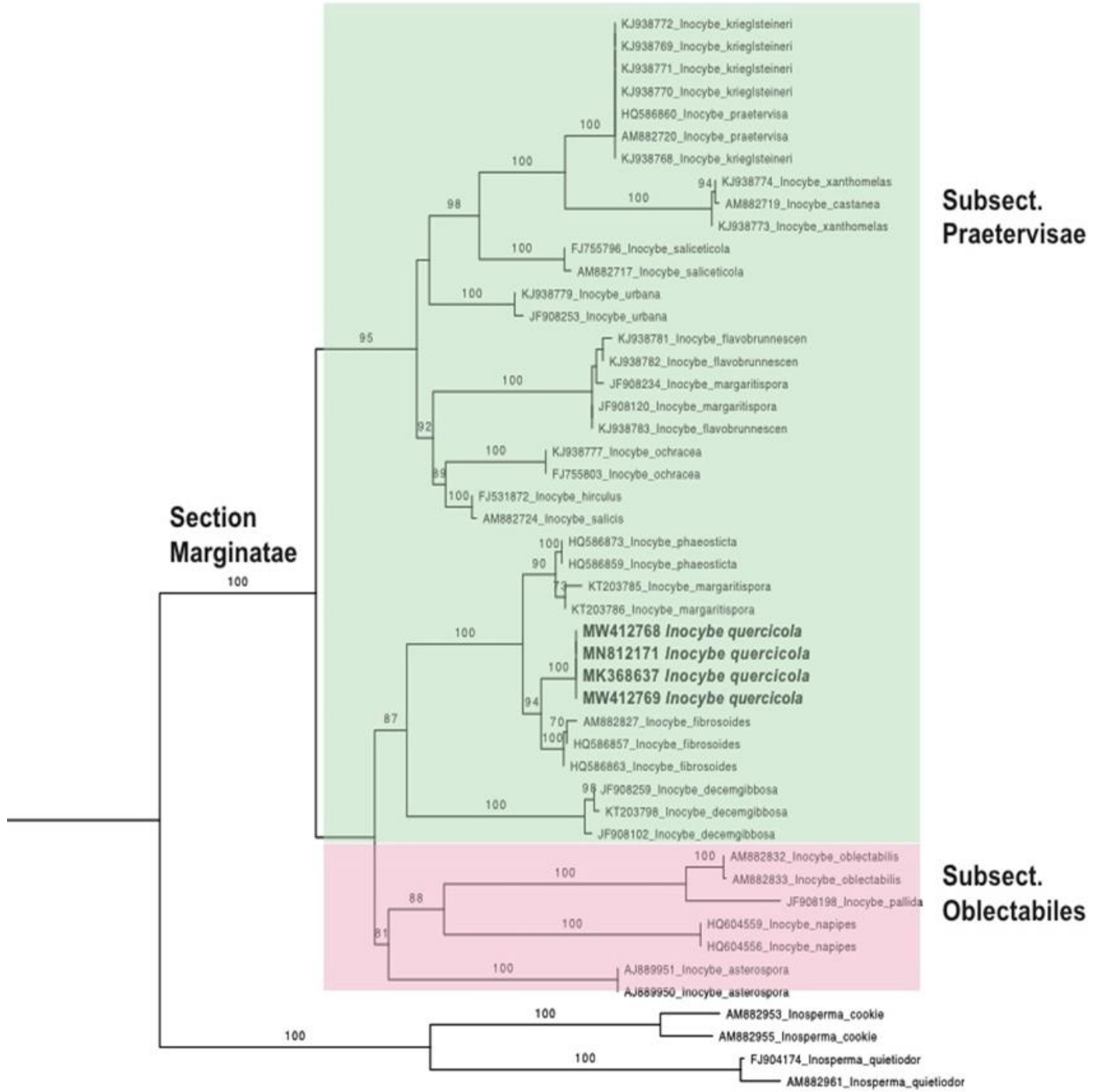
The aligned ITS dataset, including the sequences downloaded from GenBank, consisted of 48 nucleotide sequences for phylogenetic analysis and LSU consists of 22 nucleotide sequences. There were a total of 980 positions in the final ITS dataset after trimming the aligned sequences from both 5' and 3' ends at conserved sites. Of these 995 positions, 337 were conserved, 377 were variable and 333 were parsimony informative. The aligned LSU dataset had a total of 1808 positions after trimming the aligned sequences from both 5' and 3' ends. Of these 207 positions were conserved, 99 were variable and 63 were parsimony informative.

Maximum likelihood analyses for individual gene regions were performed via CIPRES Science Gateway (Miller et al. 2010) employing RAxML-HPC v.8. Rapid bootstrap analysis for the best-scoring ML tree was configured for each dataset. For the bootstrapping phase, the GTRCAT model was selected. One thousand rapid bootstrap replicates were run. A bootstrap proportion of  $\geq 70\%$  was considered significant (Figs 3, 4).

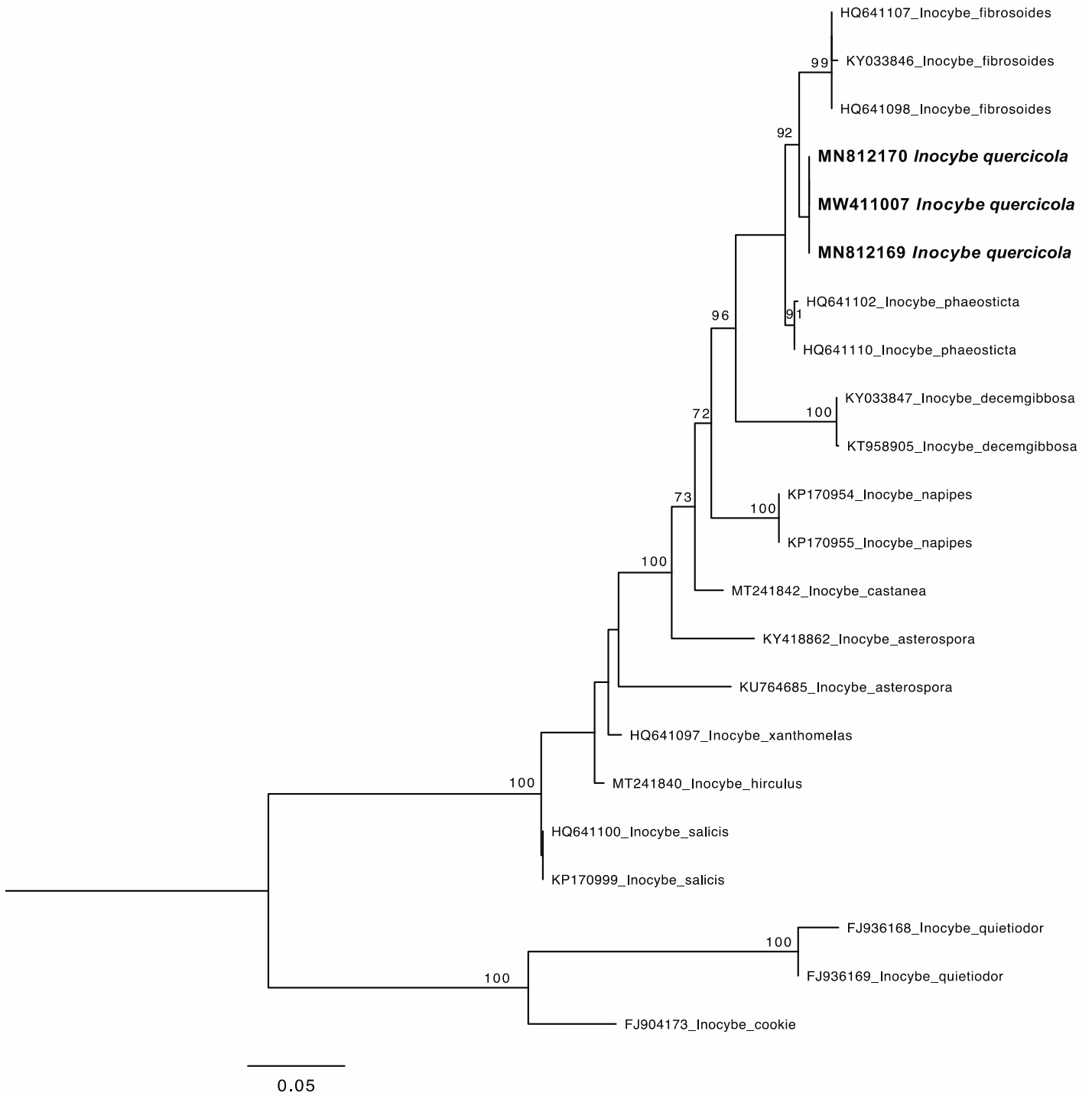
## Results

### Molecular phylogenetic characterization

The ITS sequences generated from Pakistani collections showed 91% similarity to *I. pheaostricta* (HQ586859 & HQ586873) from France. They also showed 93% similarity with sequences of *I. margritispora* (KT203786 & KT203785). Similarly, the LSU sequences of Pakistani collections showed 96% similarity with *I. pheaostricta* (HQ641102) from USA, *I. fibrosoides* (HQ641098) from Switzerland and *I. pheaostrictathe* (HQ641110) from France. In ITS and LSU based phylograms, *I. quercicola* get separated from these species forming a sister lineage within the same clade (Fig. 3 & 4).



**Fig 1.** Maximum likelihood phylogenetic tree based on nrDNA ITS as generated with RAxML with 1000 bootstrap iterations showing the placement of *Inocybe quercicola* within I. subject. Praetervisae. Bolded lettering refers to sequences generated in this study.



**Fig 2.** Maximum likelihood phylogram based on nrLSU as generated with RAxML with 1000 bootstrap iterations showing the placement of *Inocybe quercicola* within I. subsect. Praetervisae. Bolded lettering refers to sequences generated in this study.



**Fig 3.** A–B: Basidiomata of *Inocybe quercicola*; **A & B:** View of pileus and lamellae **C.** Stipe and pileus; **D.** Stipe and lamellae.

#### **Taxonomy**

*Inocybe quercicola* A. Naseer, M.B. Khan & F. Aqdu

**Mycobank** NO.838386

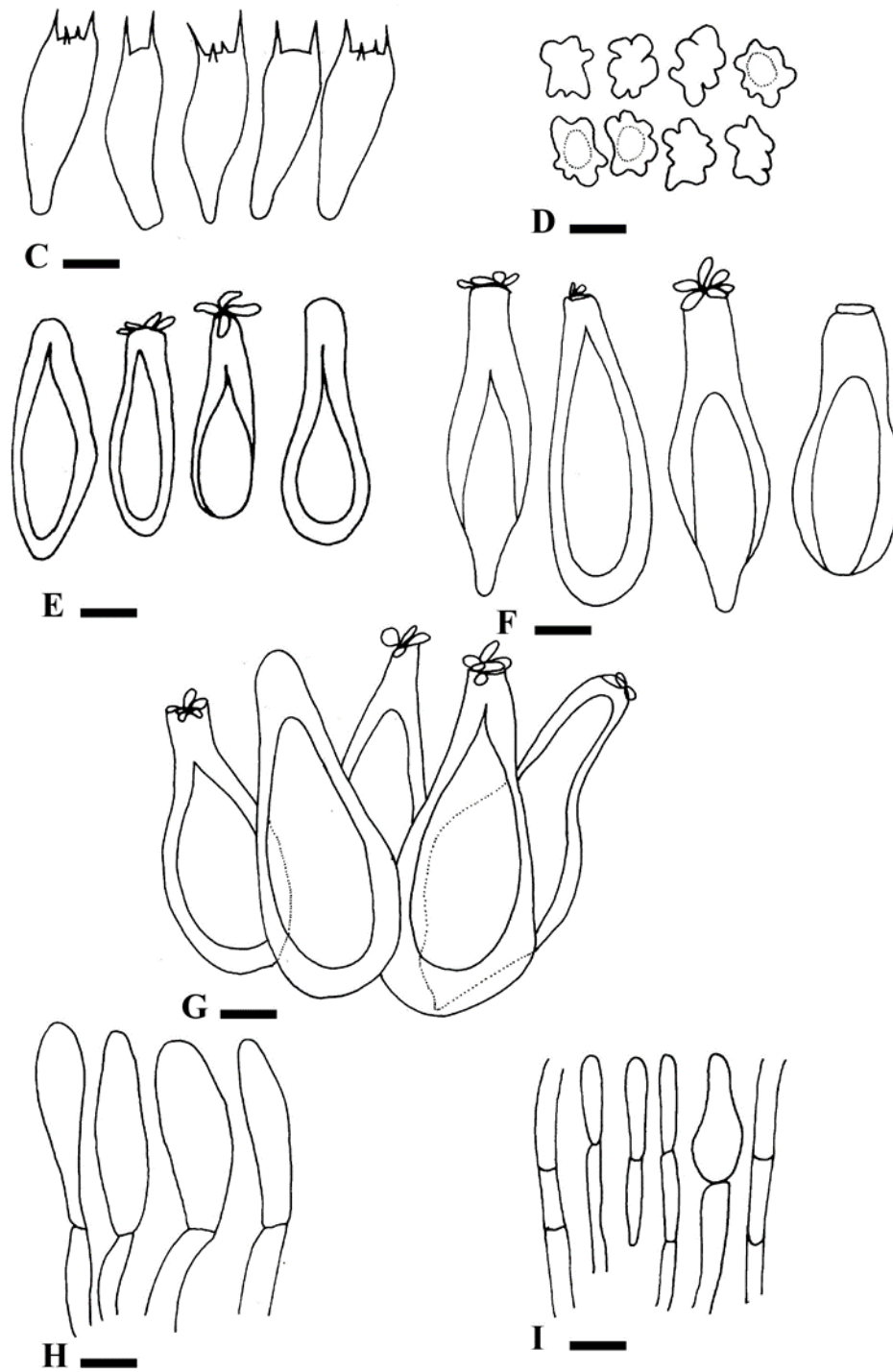
**Etymology** - Specific epithet *quercicola* (L.) refers to association with *Quercus* spp.

**Diagnosis**- Basidiomata medium-sized/rather small. Pileus campanulate, radially fibrillose, lamellae dark brown when mature, stipe that is pruinose along entire length, nodulose basidiospores ( $8.8 \times 5.5 \mu\text{m}$ ) and pleurocystidia which are similar to cheilocystidia in shape but smaller in size ( $19\text{--}22 \times 47\text{--}65 \mu\text{m}$ ).

**Holotype**- PAKISTAN, Khyber Pakhtunkhwa province, Swat, Toa, 2800 m a.s.l, on soil under *Quercus* spp., 15 July 2015, Arooj Naseer & Abdul Nasir Khalid, AST52 (FLAS-F59415; LAH35270).

**Distribution**- Currently known from Battagram district, Khyber Pakhtunkhwa, Pakistan, in forests of *Quercus incana* Roxb and from Punjab, Murree, on soil in forests of *Quercus* spp.

**Additional specimens examined**- Pakistan, Khyber Pakhtunkhwa province, Battagram district, 1800 m a.s.l., on soil under *Quercus incana* Roxb, 15 July 2017. Muhammad Binyamin Khan, IM0059! GenBank



**Fig 4.** Anatomical description of *Inocybe quercicola*. C–I. LAH35270 (holotype). C. Basidia; D. Basidiospore; E. Cheilocystidia; F. Pleurocystidia; G. Caulocystidia; H. Pileipellis. Scale bars. C = 6.8  $\mu\text{m}$ ; D = 8.13  $\mu\text{m}$ ; E = 17.68  $\mu\text{m}$ ; F = 3.51  $\mu\text{m}$ ; G = 1.50  $\mu\text{m}$ ; H = 1.47  $\mu\text{m}$ ; I = 10.4  $\mu\text{m}$ .

for ITS MK368637 and LSU MN812170. Murree, 2290 m a.s.l., on soil in forests of *Quercus* spp., 12 August 2018, Fauzia Aqduş, FA167!; Genbank for ITS MN812171 and LSU MN812169.

**Description-** Basidiomata medium-sized. Pileus 2.5–3 cm in diameter, creamish white (4.6Y 6.1/5.2) in centre becoming dark brown (7.2YR 2.6/5), campanulate at young to plano convex when mature, umbonate, fibrillose, margins incurved, entire, smooth, in a ring of dark brown (7.4YR 2.6/6). Lamellae light brown to blackish brown (5.7Y 0.3/0.2), close, regular, adnate, edges serrate. Lamellulae short, not regular. Stipe 5.5–6 cm long, 0.4–1.4 cm broad, base white (5.1GY 8.1/1.6) becoming light brown (5.7Y 6.6/3/1) towards the apex, pruinose, central, cylindrical, tapering towards the apex, slightly marginate bulbous at the base. Basidiospores [60/4/3] (7.5–) 8.0–10.0 × (4.8–) 5.0–7.5 µm, avL × avW = 8.8 × 5.5, Q = (1.1–) 1.2–1.8 (–2.0), avQ = 1.53, subglobose to ellipsoid, nodulose, yellowish brown or pale yellow in 5% KOH, guttulated, inamyloid. Basidia 21–23.5 × 8.0–11.0 µm, clavate, thin walled, pale yellow in 5% KOH, 4-spored, guttulated. Cheilocystidia 48–57 × 16–20 µm, narrowly utriform or short neck lageniform, olive green in 5% KOH, thick-walled, metuloid with crystalliferous apex. Pleurocystidia 19–22 × 47–65 µm, shape like cheilocystidia, with crystalliferous apex, light brown in 5% KOH. Caulocystidia present all over on stipe, 6.0–7.0 × 21.5–25.0 µm, clavate, rather thick-walled, yellowish brown in 5% KOH, often with septa. Stipitipellis hyphae 4.0–6.0 µm wide, a cutis of parallel filaments, pale yellow in color, septate. Pileipellis hyphae 5.5–6.0 µm wide pale yellow in 5% KOH, filamentous, unbranched, hyphal cell walls and terminal cells incrustated.

## Discussion

Species in the section *Marginatae* Kühner (1933) are characterized by nodulose basidiospores, marginate to submarginate bulbous, and pruinose stipe. Section *Marginatae* is divided into two subsections on the basis of morphological characteristics, named *Praetervisae* and *Oblectabiles*. The species included in subsection *Praetervisae* have mostly white, buff, or yellowish stipe that lack any pinkish or reddish colors or tinges in the stipe.

*Inocybe quercicola* belongs to sect. *Marginatae*, subsect. *Praetervisae* and is characterized by relatively small, radially fibrillose campanulate pileus, dark brown lamellae when mature, stipe that is pruinose along the entire length, nodulose basidiospores (av. 8.8×5.5 µm, Qav.=1.53 ) and pleurocystidia which are similar to cheilocystidia in shape but smaller in size. Our new

species is similar to its sister species *I. fibrosoides* but separated with strong bootstrap value from *I. fibrosoides* (AM882827, HQ586857, HQ586863), with which it forms a sister clade, but can be distinguished from it based on morphological and molecular data. *Inocybe fibrosoides* has a larger pileus (30–40 mm), grayish white to yellow gills and larger basidiospores ((9.5–)10.0–13(–13.5) × (7.0–) 7.5–9.5 (–10.0) µm, with avQ = 1.65). (Pegler & Young, 1972; Fan et al. 2018).

The two other sister species, *I. phaeosticta* and *I. margaritispota*, are also morphologically different from *I. quercicola*. *Inocybe margaritispota* is distinguished by its larger pileus (30–50 mm), broader basidiospores (7–8.5 µm) and cheilocystidia that are identical to pleurocystidia (Akata et al. 2011).

## Declaration of competing interest

The authors declare that they have no competing interests.

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