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Inocybe crenata sp. nov. (subsec. *Geophyllinae*, sect. *Tardae*) from Conifer Dominated Forests of Pakistan

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ABSTRACT

Inocybe crenata sp. nov. (subsec. *Geophyllinae*, sect. *Tardae*) has been described from conifer dominated forests of Pakistan. *Inocybe crenata* differs from other related species by having a pale-yellow pileus with crenate margins and a bumpy or cracked center, with a fibrillose stipe. Phylogenetic analysis based on nuclear ribosomal large subunit (LSU) and internal transcribed spacer (nrITS) sequence data supported the identity of *Inocybe crenata* as a distinct taxon. A detailed description of this novel species is provided.

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KEYWORDS

Phylogeny; Agaricales; *Inocybe*; morpho-anatomy

1. Introduction

Inocybaceae Jülich belongs to an ectomycorrhizal group of macrofungi in the Agaricales [1]. This diverse family of macrofungi is monophyletic, distributed worldwide, and makes associations with as many as 23 different families of vascular plants in mycorrhizal symbiosis [2, 3]. Matheny and Kudzma [2] divided this family into five clades: *Inocybe* (Fr.) Fr., *Inosperma* (Kühner) Matheny & Esteve-Rav., *Mallocybe* (Kuyper) Matheny, Vizzini & Esteve-Rav., *Nothocybe* Matheny & K.P.D. Latha and *Pseudosperma*. Furthermore, Matheny et al. [3] raised these five clades to the rank of genera, which are *Inocybe* (Fr.) Fr., *Inosperma* (Kühner) Matheny & Esteve-Rav., *Mallocybe* (Kuyper) Matheny, Vizzini, Esteve Rav., *Nothocybe* Matheny & K.P.D. Latha and *Pseudosperma* Matheny & Esteve-Rav. According to Matheny et al. [3], the family *Inocybaceae* now consists of total seven genera.

The genus *Inocybe* (Basidiomycota, Agaricales, *Inocybaceae*) is a diverse group of macrofungi with an estimated 1050 species worldwide. The number of *Inocybe* species is continuously increasing due to new discoveries [4–17]. Member of this family have some general common characters: brownish mature lamellae, non-adhesive pileus and pigmented spores; absence of a germ pore; presence of both cheilocystidia and pleurocystidia.


Morphologically, *Inocybe* can be recognized by their often small to medium-sized basidiomata with spermatic, earthy, bitter almond, pelargonium, or fruit-like smell (although many species do not have any distinct smell), radially fibrous to cracked and often brownish pileus, and smooth, spinose, nodulose, or angular yellowish brown basidiospores [18]. Thirty species of *Inocybe* have been previously reported from Pakistan [19–23]. Taxa within *Inocybe* subg. *Inocybe* [*Inocybe* s.str.] typically have angular/nodulose or smooth spores, thick-walled pleurocystidia, a cortina, and only slightly pruinose stipe apex. During fungal surveys in conifer dominated forests of Pakistan, a new species of *Inocybe* was collected and is described here on the basis of combined morphological and molecular methods.

2. Materials and methods

2.1. Taxon sampling

Collections were made during 2020–2021 (July–August) from Ayubia National Park (ANP) of Abbottabad district (34° 01' to 34° 38' North and 73° 22.8' to 73° 27.1' East), Shawar Valley of district Sawat, 2100 m a.s.l, and Bhurban town, Governor House of Rawalpindi district (33.9554° North, 73.4519° East). The areas are characterized by cool summers and mild winters, with a mean annual

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temperature of 10°C, a 3°C winter mean temperature during December and January and 26°C mean summer temperature during June to September; the mean annual rainfall is 1200 mm with 57% humidity [19, 24]. The vegetation is dominated by coniferous trees including deodar (*Cedrus deodara* [Roxb. Ex Lambert] G. Don), blue pine (*Pinus wallichiana* A. B. Jacks.), and chir pine (*Pinus roxburghii* Sarge.) with some mixed patches of deciduous trees [25–27].

2.2. Morphology and microscopy

Macro-morphological descriptions were made from fresh samples. Micro-morphological characteristics of samples were described following Vellinga [28], and the Munsell color chart [29] was used for color codes. Basidiomata were photographed, tagged, dried with an electric dryer, and deposited in the Lahore Herbarium (LAH), Institute of Botany, University of the Punjab, Lahore, Pakistan. Spores, basidia, and cystidia were observed in squash preparations of small parts of the lamellae in 5% KOH and 1% Congo Red in distilled water; Melzer's reagent was used to test for any amyloid or dextrinoid reaction. The pileipellis was examined from a radial section of the pileus in Congo Red. Basidia were measured without sterigmata, and the spores without hilum. The structures were examined using a compound microscope (OLYMPUS CH30, Olympus, Hamburg, Germany) and measurements were done in 5% KOH and 1% Congo Red with the help of Scope Image 9.0 (5X) software. The formula [n/m/p] indicates that “n” basidiospores from “m” basidiomes from “p” collections were measured; at least 25 basidiospores, 20 basidia, cystidia, and pileipellis and stipitipellis elements were measured from each fruitbody. In the formula (a–) b–c (–d), “a” stands for minimum value, “b–c” indicate 90% of the calculated values and “d” for maximum value. “Q” indicates the individual spore length/width ratio while “Qav” presents the average of all Q values.

2.3. Molecular protocols and phylogenetic analysis

DNA was extracted from the dried specimens by using 2% CTAB method [30]. For amplification and then sequencing ITS1F (CTTGGTCATTTAGAGGA) and ITS4 (CCTCCGCTTATTGATATGC) primers for Internal Transcribed Spacer region (ITS), LR0R (ACCCGCTGAACTTAAGC) and LR5 (TCCTGAGG GAAACTTCG) for nuclear ribosomal large subunit, and bRPB2-6F (TGGGGYATGGTNTGYCCYGC) and bRPB2-7.1R (CCCATRGCYTGYYTMCCCATDGC) primers for RNA polymerase II (*rpb2*) were used,

under standard conditions [31–33]. Each 50 µl reaction contained 1 µl template DNA extraction, 2 µl of each primer (10 pmol/µl), 25 µl of 2 x PCRBIO Taq Red (PCR Biosystems Ltd., London, UK), 20 µl of RNase free water (Severn Biotech Ltd., Kidderminster, UK). Amplification was performed in a Dyad DNA Engine Peltier thermal cycler (Bio-Rad, Herts, UK) (95°C for 5 min, 35 x [95°C for 1 min 5°C (ITS and LSU) or 54°C (*rpb2*) for 1 min, 72°C for 1 min] increasing by 1 s/cycle, 72°C for 7 min). Sanger sequencing was performed by Eurofins Genomics (Ebersberg, Germany).

Forward and reverse sequences of ITS, LSU, and *rpb2* regions were obtained in FASTA format assembled and aligned using ClustalW in Bioedit. ver. 7.2.5 [34] and matched with other online DNA sequences available through BLAST NCBI database (<https://www.ncbi.nlm.nih.gov/guide/>). A comprehensive representation of currently available sequences, from GenBank with up to 82% identity and sequences from recent publications on *Inocybe* subsection *Geophylla* section *Tardae* were included in the phylogenetic trees (Table 1; Figures 1 and 2). Sequences of *Auritella foveate* were used as the outgroup. MEGA11 [35] was used for phylogenetic tree construction. A Maximum Likelihood (ML) tree of nrITS and LSU sequences was constructed using a General Time Reversible (GTR) model and nearest neighbor-interchange (NNI) as a ML heuristic search method. 1000 bootstrap replicates were performed. The newly generated sequences were deposited in GenBank (accessions highlighted in Figures 1 and 2).

3. Results

3.1. Phylogenetic analysis

A total of 46 ITS rDNA sequences were analyzed, including 40 obtained from the NCBI GenBank. The data matrix consisted of 740 unambiguously aligned nucleotide positions, of which 293 were conserved, 416 variable, 258 parsimony-informative and 154 were singleton variants. Figure 1 shows a cluster of six sequences of the taxon under investigation forming a separate branch within their clade. For concatenated genes ITS + LSU rDNA, a total of 43 sequences were analyzed, including 40 obtained from the NCBI GenBank. The data matrix consisted of 1832 unambiguously aligned nucleotide positions among which 1081 were conserved, 532 variable, 288 parsimony-informative and 227 were singleton variants. Figure S1 shows three sequences of the taxon under investigation forming a separate branch within their clade. For concatenated ITS + LSU + *rpb2* genes,

Table 1. Collection data, geographic origin, and DNA sequences of *Inocybe* species used in this study.

Taxon	Geographic area	Voucher	GenBank accession numbers		
			ITS	LSU	Rpb2
<i>Inocybe fuscicothurnata</i>	USA	PBM3980	MF487844.1	KY990485	—
<i>I. aff. geophylla</i>	Canada	040904av27 (TENN)	KY990538	KY990492	—
<i>I. aff. geophylla</i>	Canada	100823av02	KY990545	KY990499	—
<i>I. aff. geophylla</i>	Canada	110924av05	KY990542	KY990496	MF416415
<i>I. fuscicothurnata</i>	Canada	AU9919	NR_148184	NG_060354	—
<i>I. lilacina</i>	Sweden	EL12605	AM882875	—	—
<i>I. pudica</i>	Sweden	EL15905	AM882872	—	—
<i>I. geophylla</i> var. <i>lateritia</i>	France	EL24606	FN550916	—	—
<i>I. geophylla</i>	Sweden	EL8003	AM882877	—	—
<i>I. geophylla</i>	Sweden	EL9005	AM882870	—	—
<i>I. sp.</i>	Canada	HRL2223	KX897427	KY990518	—
<i>I. aff. geophylla</i>	USA	LG496	KY990534	KY990490	—
<i>I. pudica</i>	USA	MGW721	KY990557	KY990514	—
<i>I. insinuata</i>	USA	MGW783	KY990546	KY990500	MF416419
<i>I. ionocephala</i>	USA	MTS2488	KY990549	JN974950	—
<i>I. pallidicremea</i>	USA	PBM2448	HQ201357	—	MF416425
<i>I. aff. geophylla</i>	USA	PBM2456	KY990540	KY990494	MF416413
<i>I. aff. geophylla</i>	USA	PBM2457	KY990541	KY990495	MF416414
<i>I. aff. geophylla</i>	USA	PBM2732a	KY990543	KY990497	MF416416
<i>I. aff. geophylla</i>	USA	PBM3040	KY990532	KY990488	MF416409
<i>I. aff. geophylla</i>	USA	PBM3041	KY990533	KY990489	MF416410
<i>I. ionocephala</i>	USA	PBM3043	KY990550	KY990503	MF416421
<i>I. ionocephala</i>	USA	PBM3049	KY990551	KY990504	MF416422
<i>I. aff. geophylla</i>	USA	PBM546	KY990537	KY990491	—
<i>I. pallidicremea</i>	USA	PBM2448	KY990555	KY990508	—
<i>I. aff. geophylla</i>	Costa Rica	REH7879	KY990539	JN974953	—
<i>I. aff. geophylla</i>	USA	SAT0308001	KY990530	KY990486	—
<i>I. aff. geophylla</i>	USA	SAT0630802	KY990535	JN974952	MF416411
<i>I. pudica</i>	USA	SAT0630804	KY990559	KY990516	—
<i>I. pudica</i>	USA	SAT0732301	KY990560	KY990517	—
<i>I. whitei</i>	Sweden	SJ06012	FN550915	—	—
<i>I. armeniaca</i>	USA	SNH6	KY990524	KY990482	—
<i>I. fuscidula</i> var. <i>fuscidula</i>	USA	UBC: F19132	HQ604301	—	—
<i>I. aff. geophylla</i>	USA	CA1882	KY990536	JN974951	—
<i>I. pallidicremea</i>	Canada	ACAD11600	KY923033	KY923042	—
<i>I. aff. geophylla</i>	USA	TENN:068276	KY990544	KY990498	MF416417
<i>I. aff. geophylla</i>	USA	TENN:062544	KY990543	KY990485	—
<i>I. sambucella</i>	Netherlands	DB30-9-19-9	MW856442	—	—
<i>I. sambucella</i>	Netherlands	DB20-8-12-9	MW856441	—	—
<i>I. ionolepis</i>	UK	K(M):236689	NR_174908	—	—
<i>I. xantholeuca</i>	France	L:L-4370262	MW845940	—	—
<i>Inocybe crenata</i>	Pakistan	LAH37821	OQ826580	OQ826583	PQ593647
<i>Inocybe crenata</i>	Pakistan	LAH37822	OQ826579	OQ826582	PQ593648
<i>Inocybe crenata</i>	Pakistan	GB27	OQ826581	OQ826584	—
<i>Inocybe crenata</i>	Pakistan	KH440	OR534595	—	—
<i>Inocybe crenata</i>	Pakistan	KH422	OR534594	—	—
<i>Inocybe crenata</i>	Pakistan	LAH35292	OR543352	—	—
<i>I. aff. geophylla</i>	Canada	TENN:070836	KY990542	KY990496	—
<i>Auritella foveata</i>	India	TBGT9631	GU062740	GU062739	GU062738

17 sequences were analyzed, as limited *rpb2* sequences were available in GenBank. The data matrix contained 2546 unambiguously aligned nucleotide positions: out of these, 1690 were conserved, 643 were variable, 258 were parsimony-informative and 367 were singletons. Figure 2 shows the two *I. crenata* sequences making a distinct branch within the phylogram. An initial blast search using sequences of the proposed new species *Inocybe crenata* against GenBank sequences of the ITS, LSU and *rpb2* regions showed 88% similarity with *Inocybe aff. geophylla* (KY990544), 98% similarity with *Inocybe aff. geophylla* (KY990497) and 97% *Inocybe aff. geophylla* (MF416415) respectively. These sequences and all other closely related sequences are included in the final dataset/phylogram represented in this research article (Figures 1 and 2).

The specimens investigated in the present study constituted a discrete branch in phylogenetic trees (Figures 3 and 4).

4. Taxonomy

Inocybe crenata Ashraf, Afshan, Razzaq, Ali, Niazi, Nasser & Khalid sp. nov.

Mycobank MB850056.

Etymology:—“*crenata*” (Latin) refers to the crenate pileus margin.

Diagnosis:—It differs from *Inocybe geophylla* by having a pale-yellow pileus, crenated margins, bumpy or cracked center of pileus, stipe is uneven in color and texture, entire stipe is fibrillose, oblong to ellipsoid basidiospores, suprahilar depression is absent, ectomycorrhizal association with *Pinus roxburghii*.

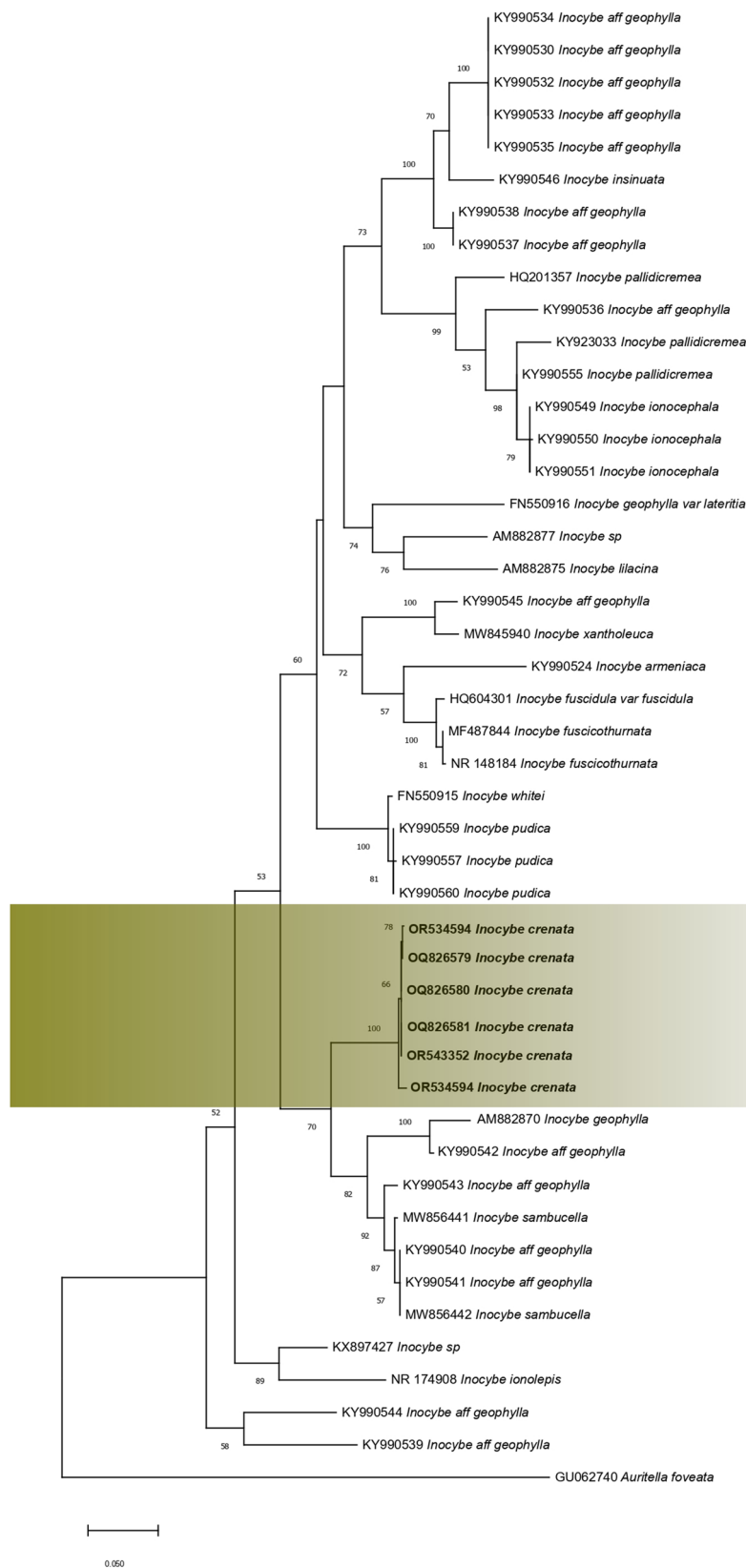


Figure 1. Molecular phylogenetic placement of *Inocybe crenata* based on Maximum Likelihood method of ITS sequences. Sequences representing the proposed new species are highlighted in bold. *Auritella foveata* was selected as an outgroup.

Typification:—PAKISTAN. Khyber Pakhtunkhwa, Abbottabad, district, Ayubia National Park (34°01' to 34° 38' North and 73° 22.8' to 73° 27.1' East) near *Pinus roxburghii* Sarge., soil rich in organic matter, 28 July 2019, Urooj Ashraf, Najam ul Sehar Afshan and Abdul Nasir Khalid, FBSR-54, (Holotype

LAH37822). GenBank: ITS = OQ826579; LSU = OQ826582.

Further specimens examined: PAKISTAN. Khyber Pakhtunkhwa, Abbottabad, district, Ayubia National Park (34°01' to 34° 38' North and 73° 22.8' to 73° 27.1' East) near *Pinus roxburghii* Sarge., soil rich in

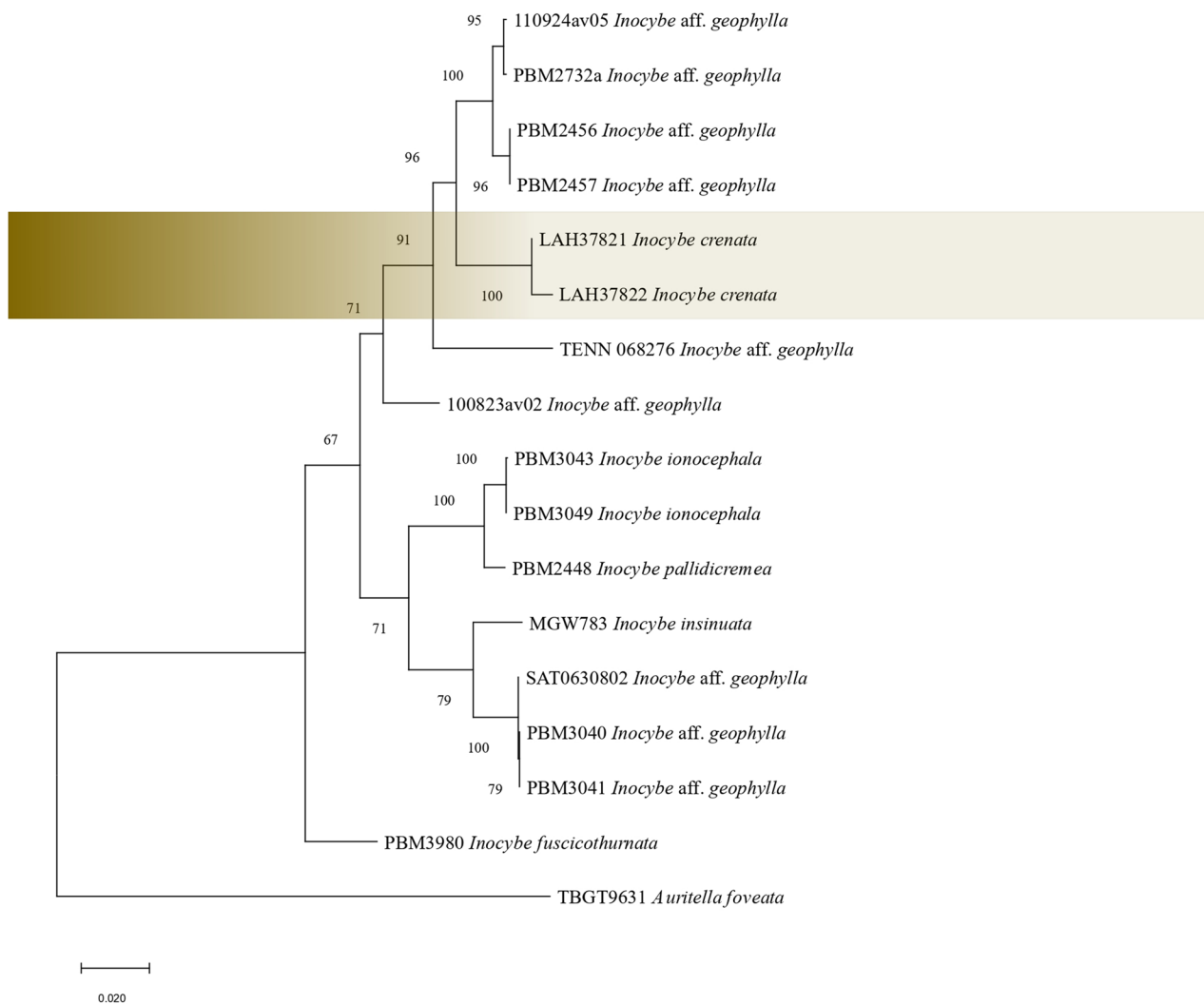


Figure 2. Maximum Likelihood tree inferred from three concatenated genes (ITS, LSU and *rpb2*) of *Inocybe creneta*. The new species is indicated in bold. *Auritella foveata* was again selected as an outgroup.

organic matter, 28 July 2019, Urooj Ashraf, Najam ul Sehar Afshan and Abdul Nasir Khalid, FBSR-71, (LAH37821). GenBank: ITS=OQ826580; LSU=OQ826583. PAKISTAN. Punjab province, Rawalpindi district, Bhurban town, Governor House; 33.9554° N, 73.4519° E; alt. 1828.8m asl, September 07, 2020; Annum Razzaq, GB27, (LAH37940), GenBank: ITS=OQ826581; LSU=OQ826584. PAKISTAN, Khyber Pakhtunkhwa province, Swat, Shawar Valley, 2100m a.s.l, solitary on soil under *Quercus incana*, 14 July 2019, Arooj Naseer and Abdul Nasir Khalid, SWT127, (LAH35292), GenBank: ITS=OR543352. PAKISTAN. Khyber Pakhtunkhwa, Abbottabad, district, Ayubia National Park, on humid soil near *Cedrus deodara*, 15 July 2020, Muhammad Ali and A. R. Niazi, Kh440, (440 PUL000446), GenBank: ITS=OR534595. PAKISTAN. Khyber Pakhtunkhwa, Abbottabad, district, Ayubia National Park, on humid soil near *Abies pindrow*, 30 July 2021, Muhammad Ali and A. R. Niazi, Kh422, (PUL00044680), GenBank: ITS=OR534594.

Description: Basidiomata small sized. PILEUS 1.6-3.5 cm in diameter, pale yellow (25YR 8/3) in center becoming yellowish (5Y 8/3) toward margins, entire pileus is uneven in color and texture, conical or subglobose when young, later it becomes campanulate to umbonate, texture or surface is uneven bumpy in central, later fibrose, margins crack or split, margin at first incurved, later decurved to straight or even uplifted, and then pileus depressed around the umbo. Lamellae dull orange (7.5YR 7/4), regular, adnexed, alternate with lamellae, edges even, slightly close to crowded, 2–3 mm in width. Stipe 2.6–7.2 cm long, 0.5 cm in diameter, white at apex becoming light grey (7.5Y 8/2) in middle and base is (2.4YR 3.1/9.3) central cylindrical, curved flexuous, with fibrillose, pruinose, densely at apex, longitudinally striate; slightly bulbous toward base concolorous with pileus; surface entirely pruinose; solid; context moderately thick; whitish creamy at apex and pale brownish toward base. Annulus absent. Odor not detectable.



Figure 3. Fresh basidiomes of the newly described species. A. *Inocybe crenata* (LAH37822, holotype). B & D. *I. crenata* (LAH37821). C. *I. crenata* (LAH35292).

Basidiospores [60/3/3] (7.0–)9.1–10.5(–11.7) × (4.1–)4.4–4.8(–5.6) μm , $\text{avl} \times \text{avw} = 10.6 \times 5.6 \mu\text{m}$, $Q = (1.5\text{--})1.7\text{--}2.2(\text{--}2.3)$, $\text{av}Q = 1.9$, smooth, oblong to ellipsoidal and amygdaliform; mostly uniguttulate light brown in KOH; light brown in water; distinct apiculus; inamyloid. Basidia (31.4–)32.7–36.2(–37.7) × (6.9–)7.2–9.2(–10.5) μm , $\text{avL} \times \text{avW} = 35.3 \times 10.5 \mu\text{m}$; hyaline in KOH; clavate; thin walled; 4 spored. Cheilocystidia (36.4–)38.7–56.1(–57.5) × (13.7)13.9–22.7(–23.4) μm , $\text{avL} \times \text{avW} = 47.5 \times 17.3 \mu\text{m}$; hyaline in KOH; variable in shape; metuloids with crystalliferous apex, spheropedunculate to broadly fusiform, pedicellate, utriform; thick walled. Pleurocystidia (29.6–) 35.3– 50.9 (–53.1) × (12.4) 13.8–23.1(–28.4) μm , $\text{avL} \times \text{avW} = 45.5 \times 15.6 \mu\text{m}$; variable; similar in shape to cheilocystidia; crystalliferous apex; hyaline in KOH. Pileipellis cutis made up of filamentous hyphae, 2.1–10.3 μm in diameter, $\text{avW} = 6.8 \mu\text{m}$; thin walled; hyaline in KOH; septate; branched; clamp connection present. Stipitipellis filamentous hyphae, 4.1–9.18 μm in diameter, $\text{avW} = 6.6 \mu\text{m}$; thin walled; hyaline in KOH; septate; branched, clamp connection present. Caulocystidia (42.7–)47.4–64.2(–64.7) × (8.5–)

9.0–19.1(–20.2) μm , $\text{avL} \times \text{avW} = 56.3 \times 13.6 \mu\text{m}$; variable in shape and size; numerous at stipe apex; utriform to flexuose.

5. Discussion

In the present study, *Inocybe crenata* is described as a new species based on combined morphological and molecular phylogenetic analysis. *Inocybe crenata* sp. nov. shows some morphological similarities with *I. geophylla*, the closest morphological and phylogenetic species, in terms of their small-sized basidiocarps, smooth ellipsoidal spores, and cheilocystidia and pleurocystidia that are similar in appearance and size. Both species also have abundant clamp connections in all tissues.

However, morphological studies revealed that *Inocybe crenata* sp. nov. differs from *I. geophylla* due to the presence of cracks or bumpy texture in center of basidiocarps with crenated margins whereas *I. geophylla* has a smooth pileus and entire margins. Our taxon appears distinctive by having variable colors on pileus; pale yellow (25YR 8/3) in center and

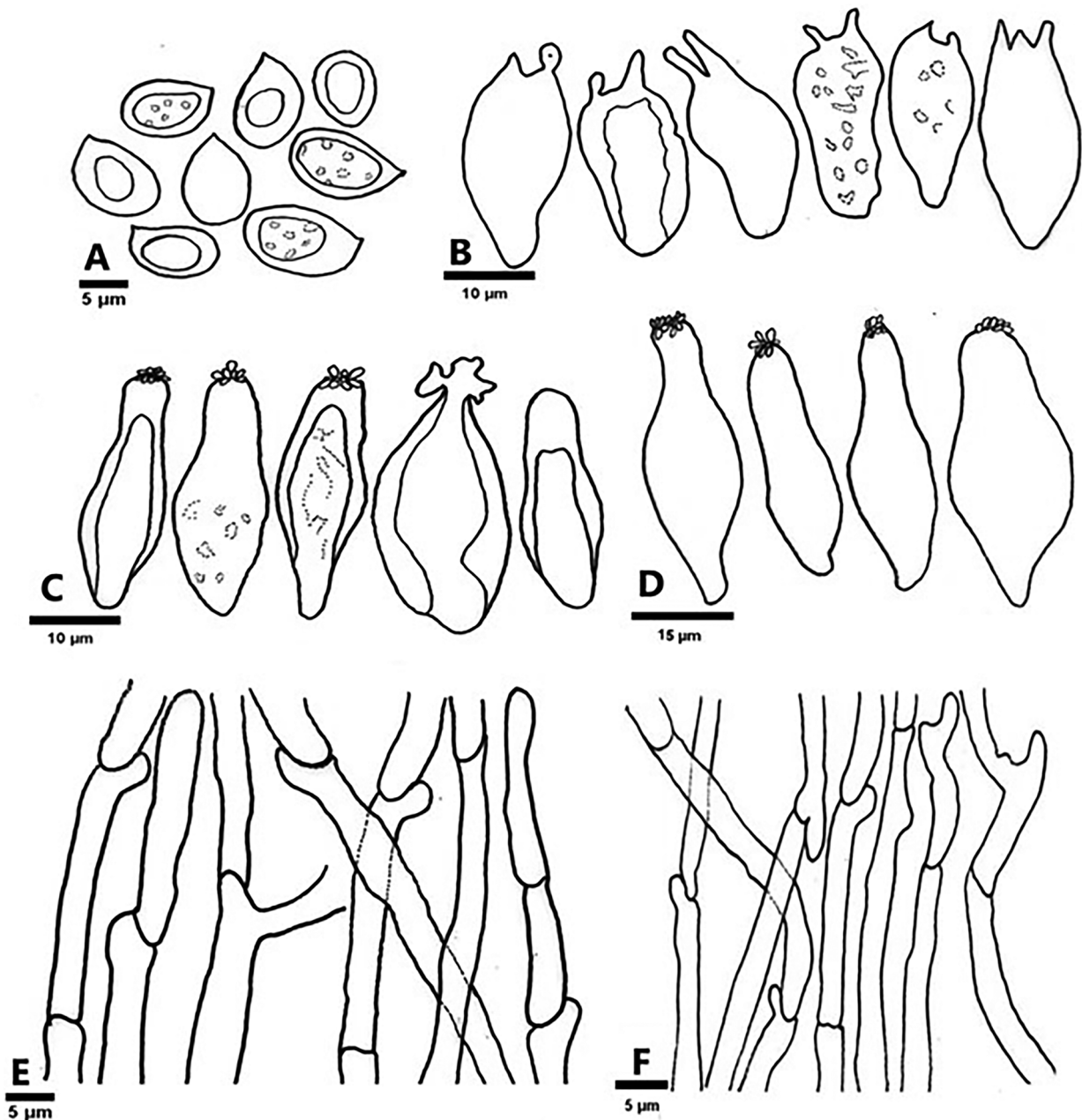


Figure 4. Micromorphological features of *Inocybe crenata* (LAH37822, holotype). A. Basidiospores. B. Basidia. C. Cheilocystidia, D. Pleurocystidia, E. Pileipellis, F. Stipitipellis.

then becoming yellow (5Y 8/3) toward margins while the comparable taxon *I. geophylla* has a whitish creamy pileus.

Furthermore, *I. crenata* sp. nov. has a longer stipe (26–76 mm) than its sister clade species *Inocybe geophylla* (15–50 mm). Our newly described taxon has an entirely pruinose stipe as compared to *I. geophylla* that has pruinose stipe texture near to the apex only.

The habitat of *I. crenata* sp. nov. is also different as it grows near *Pinus* trees while *I. geophylla* preferably grows on calcareous soil, often with *Picea abies* and broadleaf trees [17].

Molecular analysis generated from rDNA sequencing of the ITS, LSU and *rpb2* regions showed that

specimens of *I. crenata* sp. nov. form a separate clade within subsec. *Geophyllinae*, sect. *Tardae* of the genus *Inocybe*. The data from GenBank included many “aff. *geophylla*” sequences: specimens with affinity to *I. geophylla* but lacking definitive identification. These were scattered throughout the trees, reflecting the poorly resolved molecular and taxonomic diversity within the genus *Inocybe* [3, 36] and the need for characterization of previously undescribed *Inocybe* species such as *I. crenata*.

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