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Inocybe kohistanensis, a new species from Swat, Pakistan

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Abstract: *Inocybe kohistanensis*, a new species, is described from Swat, Khyber Pakhtunkhwa, Pakistan, on the basis of morphological characters as well as molecular phylogenetic analyses. The new species is characterized by a fibrillose reddish brown pileus, pruinose stipe with a prominent marginate bulb, and nodular spores. Sequences from the internal transcribed spacer region suggest that *I. kohistanensis* is distinct from all other *Inocybe* species sampled.

Key words: Dry temperate forest, internal transcribed spacer, marginate bulb

1. Introduction

Inocybe (Fr.) Fr. (Agaricales, Inocybaceae) is a large genus with an estimated 735 species (Kirk et al., 2008; Kobayashi, 2009; Matheny et al., 2009; Kobayashi and Onishi, 2010; Kropp et al., 2010; Bougher and Matheny, 2011; Bougher et al., 2012; Kokkonen and Vauras, 2012; Matheny et al., 2012; Fan and Bau, 2013; Braaten et al., 2014; Fan and Bau, 2014; Esteve-Raventós et al., 2015) and the number will considerably increase as collections from unexplored regions are studied. Species of the genus are commonly found in temperate areas (Singer, 1962; Matheny et al., 2003) while occurring to a lesser extent throughout the tropics (Horak, 1979; Singer et al., 1983; Buyck and Eyssartier, 1999; Matheny et al., 2003). They are widespread and are found in all major biogeographic regions. Records have been found from Africa (Buyck and Eyssartier, 1999), Australia (Bougher and Matheny, 2011; Bougher et al., 2012; Braaten et al., 2014), Europe (Esteve-Raventós et al., 2003; Jacobsson, 2008; Larsson et al., 2009; Kokkonen and Vauras, 2012), North America (Kropp et al., 2010; Braaten et al., 2014), South America (Matheny et al., 2003; Cortez and Coelho, 2005; De Meijer, 2006; Wartchow et al., 2008; Matheny et al., 2012), New Zealand (Horak, 1977), and Indomalaya and Australasia (Horak, 1979, 1980; Pegler, 1986). Many reports of occurrences of *Inocybe* have been documented from Asia. Work on the genus has been done in China (Fan and Bau, 2013, 2014), India (Vrinda et al., 1996, 1997), Japan (Kobayashi and Courtecuisse, 1993, 2000; Kobayashi and Hungo, 1993; Kobayashi, 2009; Kobayashi and Onishi, 2010), and the Himalayas (Horak, 1981). From Pakistan 26 species of *Inocybe* have been reported to date (Ahmad et al., 1997; Sultana et al., 2011; Farooq et al., 2013; Ilyas et al., 2013).

Species within the genus are fairly small and inconspicuously brown, and they have a pruinose stipe. The genus has been divided into subgenera and sections mainly on the basis of spore morphology, the form and distribution of cystidia, and stipe morphology. The stipe may be of uniform thickness or have a distinctly bulbous base. The spores are variable in shape and may be ellipsoid, amygdaliform, or nodulose/angular. Many species have incrusted thick-walled metuloid pleurocystidia and cheilocystidia. Some groups completely lack metuloids but have numerous thin-walled cheilocystidia. Cheilocystidia and caulocystidia often provide important information for identification. A number of classifications combining these and other characters in various ways have been proposed (Heim, 1931; Kühner and Romagnesi, 1953; Kühner, 1980; Kuyper, 1986; Singer, 1986; Stangl, 1989; Kobayashi, 2002).

Inocybe is important because of its ectomycorrhizal mode of nutrition. It contributes a significant component of the ectomycorrhizal communities of temperate and boreal forests in alpine and arctic habitats (Cripps et al., 2010; Kokkonen and Vauras, 2012). It is also economically important as a potential contaminant in truffle production (Iotti et al., 2005). The aim of the present work is to propose a new taxon named *Inocybe kohistanensis* from the western mountains of Pakistan. The specimens were identified on the basis of morphological and anatomical characters as well as a molecular data set using the internal transcribed

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spacer (ITS) region of nuclear ribosomal nrDNA and placed within *Marginatae* subsection *Oblectabiles* following Bon's (1998) classification.

2. Materials and methods

2.1. Sampling site description

Basidiomata were collected from Kalam and Mashkun, Swat district (34°40′N to 35°N and 72′E to 74°6′E), Khyber Pakhtunkhwa Province, Pakistan. The area occupies the nexus of three great mountain ranges, i.e. the Himalayas, Hindu Kush, and Karakoram (Hamayun et al., 2003). Kalam Valley lies in the center of Swat Kohistan, which are offshoots of the Hindu Kush. The Swat River is formed by the joining of the Ushu and Utrot rivers in Kalam that run off from the Hindu Kush Mountains. Towards the west of Kalam, lush green mountains of the Mankyal at Mashkun are a forest reserve. These mountains are dominated by *Cedrus deodara* (Roxb. ex D.Don) G.Don forests together with *Pinus* spp. and *Quercus* sp. (Champion et al., 1965).

The area has a typical dry temperate climate. Some parts climatically resemble the Himalayan moist temperate zone. The valleys remain under snow cover during winter. July and August are the hottest months of the year. Rain is received in large amounts during March and April. The summer and autumn are relatively dry seasons. Mean annual maximum temperature is 17 °C while mean annual minimum temperature is 4 °C. Mean annual snowfall is 3310 mm and mean annual rainfall is 524.3 mm (Champion et al., 1965; Stucki and Khan, 1999).

2.2. Morphological observations

Macromorphology was recorded in the field. Color notations were indicated from the Munsell Color System (Munsell Color Co., 1975). Specimens were photographed using a Nikon D70S camera. The specimens were dried under a fan heater. Microscopic study was done with a light microscope (MX4300H, Meiji Techno Co., Ltd., Japan) after rehydrating and examining material in 5% KOH. Measurements were made using a Carl Zeiss Jena ocular micrometer. Spore measurements are given as averages and ranges while measurements of the other cells are given as ranges.

For basidiospores, the abbreviation "*n/m/p*" indicates *n* basidiospores measured from *m* fruit bodies of *p* collections. Dimensions for basidiospores are given using length × width (l × w), and extreme values are given in parentheses. The range contains a minimum of 90% of the values. *Q* indicates l/w ratio of the spore and avQ means average Q of all spores ± standard deviation. Line drawings were made using a Leitz Wetzlar Camera Lucida. Specimens were deposited at the LAH Herbarium, University of the Punjab, Lahore (LAH35001, LAH35002, LAH35003, LAH35024).

2.3. DNA extraction, amplification, and sequencing

DNA was extracted from the dried specimens with standard protocols (Bruns, 1995). Partial sequences were obtained from the ITS region of nrDNA under standard conditions (Gardes and Bruns, 1993). Agarose gel electrophoresis was performed to visualize PCR products in a gel documentation system (UVtec, Cambridge, UK) at default settings. The amplified products were sent to Macrogen Inc. (Korea) for sequencing using the same pair of primers. Sequences were submitted to GenBank (KP316243, KP316244, KP316245, KT897911).

2.4. Phylogenetic analyses

A consensus sequence was generated from the sequences obtained by both the primers from each specimen using BioEdit software. To search and retrieve nucleotide sequences from the GenBank database, NCBI BLAST was used (http://www.ncbi.nlm.nih.gov/). Sequences with the closest match were selected from BLAST. The sequences that showed less query cover and percentage identity as well as incomplete ITS regions were omitted. Closest relatives of the species were also included from the published literature in the final data set. ClustalW was used to align sequences in MEGA software. The sequences were trimmed with the conserved motifs. The alignment portion between 5'...GAT and CAAA...3' was included in phylogenetic analysis. Maximum likelihood (ML) analysis was performed using a general time-reversible model (Nei and Kumar, 2000) and nearest-neighbor interchange as the ML heuristic search method in MEGA6 software to test the phylogeny at 1000 bootstraps. Divergence in nrDNA-ITS was analyzed using MegAlign (DNASTAR).

3. Results

3.1. Taxonomy

Inocybe kohistanensis S. Jabeen, I. Ahmad and A.N. Khalid, sp. nov.

MycoBank no.: MB 812275.

Diagnosis: Pileus umbonate, fibrillose, reddish brown. Lamellae regular, adnate to adnexed, edges even, cream to grayish and fimbriate when immature, dark brown or fulvous at maturity. Stipe central, cylindrical, pruinose towards the base and apex, with a distinct marginate bulb. Basidiospores (8.7) 9.4–12.5 (13.3) × (6.1) 6.6–9.2 (9.4) µm, nodulose. Basidia clavate, cheilocystidia and pleurocystidia crystalliferous, paracystidia catenate, caulocystidia mucronate, clamp connections observed at the base of basidia and cystidia. Pileipellis a cutis, hyphae branched, with fusiform terminal. Clamp connections frequent. Stipitipellis filamentous, rarely branched, infrequently septate, clamp connections not observed.

Type: Pakistan, Khyber Pakhtunkhwa Province, Swat district, Mashkun, under *Cedrus deodara* (Roxb. ex D.Don) G.Don, 5 September 2013, S. Jabeen (holotype SJ16;

K4-37; LAH35001; GenBank KP316243) (isotype SJ20; K4-36; LAH35002; GenBank KP316244) (isotype SJ24; K4-C; LAH35024; GenBank KT897911), Kalam, under *Cedrus deodara* (Roxb. ex D.Don) G.Don, 4 September 2013, I. Ahmad (isotype IS190P42; LAH35003; GenBank KP316245).

Etymology: "*kohistanensis***"** refers to 'Kohistan'. Swat Kohistan is the type locality.

3.2. Morphological description

Basidiomata (Figures 1a–1f) medium to large. Pileus 3.1–4.8 cm in diam., hemispherical to spherical, obtusely conical when young, becoming subcampanulate, more or less convex upon expansion, umbonate; surface dry, fibrillose to appressed fibrillose; margin incurved, radially rimose to rimulose, dark brown (2.5YR4/10) or reddish brown (10R3/8); velipellis absent. Lamellae regular, adnate to adnexed, edges even to slightly fimbriate, moderately close, up to 4 mm broad, cream (2.5Y9/2) to grayish (5Y8/2) and pruinose when immature to dark brown or fulvous (2.5YR/10) in age, not changing color when bruised. Lamellulae short, 2/3 in length of lamellae, alternating with lamellae; stipe 3.5–10 cm × 4–9 mm,

Figure 1. *Inocybe kohistanensis*: A–E) Photographs of basidiomata. F) Line drawing of basidiomata. (A, B, and F: Holotype SJ16; C: Isotype SJ20; D and E: Isotype IS190P42). Bars: A–C, 1.5 cm; D–F, 1 cm.

central, cylindrical, swollen up to 1.5 cm in diam. at base. Surface smooth, reflective, white to cream (2.5Y9/2) and pruinose towards the base and apex, brownish (5YR6/8) at center.

Basidiospores (Figure 2a) [60/4/3] (8.7) 9.4–12.5 (13.3) × (6.1) 6.6–9.2 (9.4) µm, *Q* = (1.15) 1.21–1.70 (1.88), avQ = 1.39 ± 1.42, globose to elongated heterodiametrical, angular, nodulose, with 8 or 9 nodules, yellowish brown

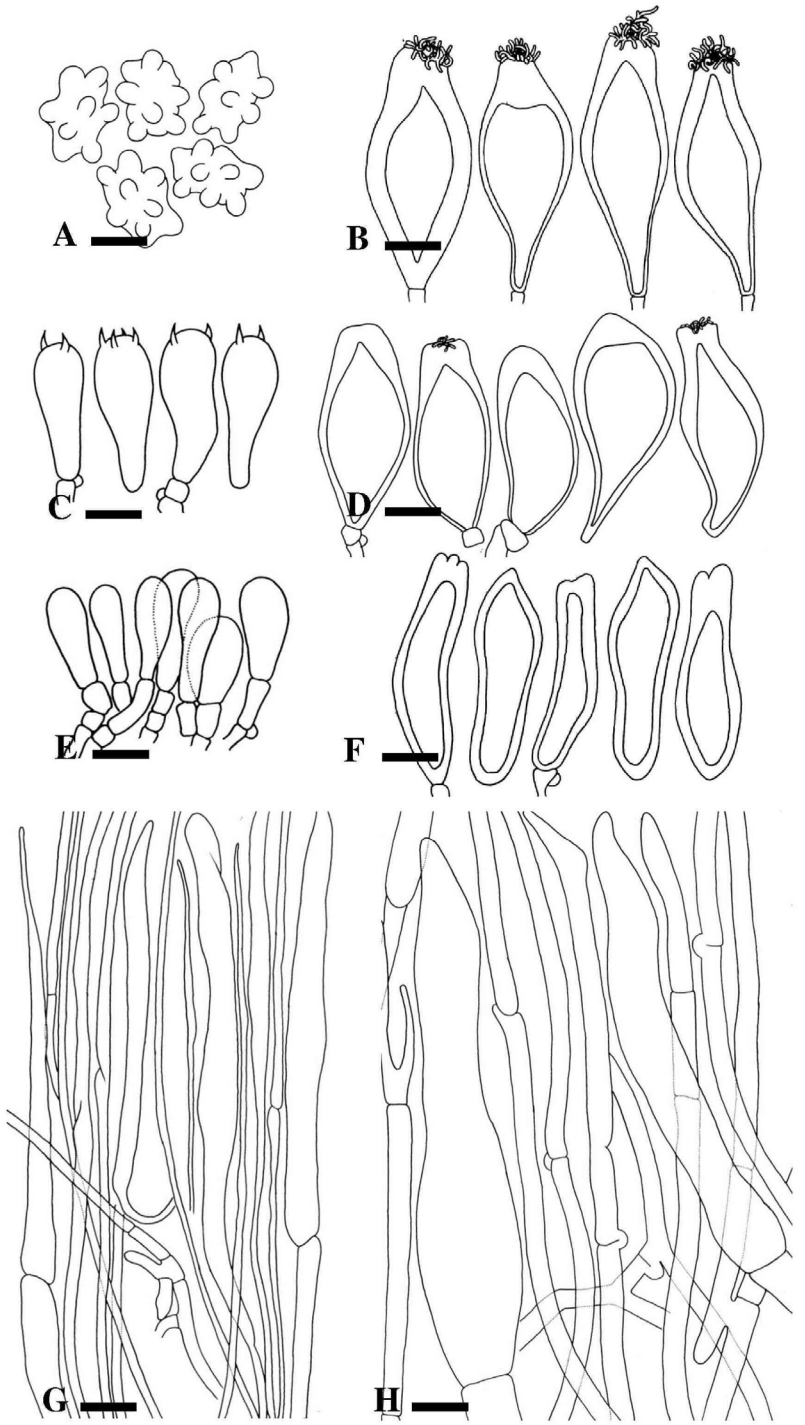

Figure 2. Anatomy of *Inocybe kohistanensis*: A) Basidiospores. B) Cheilocystidia. C) Basidia. D) Pleurocystidia. E) Paracystidia. F) Caulocystidia. G) Hyphae from stipitipellis. H) Hyphae from pileipellis. Bars: A, 5.5 µm; B, 17 µm; C, 10 µm; D, 15 µm; E, 7 µm; F, 12 µm; G, 14 µm; H, 7 µm. A–H from Holotype SJ16.

in 5% KOH, guttulate. Cheilocystidia (Figure 2b) (56.6) 67.2–72 (75.5) × (19.6) 23.4–26.2 (28.8) µm, wall up to 3 µm, pale yellow to hyaline, with crystalliferous apex. Basidia (Figure 2c) 4-spored, (24.5) 25.2–30 (35.4) × (9.2) 9.4–12.5 (16.5) µm, clavate, thin-walled, yellowish, densely guttulate, clamp connection observed at the base. Pleurocystidia (Figure 2d) (50.7) 65–76 (79) × (20.8) 21–28 (31.9) µm, wall up to 3 µm, pale yellow to hyaline, with densely crystalliferous apex. Paracystidia (Figure 2e) 10–15.3 × 5–6.8 µm, broadly clavate, thin-walled, pale yellow to hyaline, catenate, clamp connections observed at the base. Caulocystidia (Figure 2f) 45–50.3 × 9.4–16 µm, often in clusters, mucronate**,** thin-walled, pale yellow to hyaline, clamp connections observed at the base. Stipitipellis (Figure 2g) a cutis made up of filamentous, (2.1) 2.6–7 (9.7) µm wide, rarely branched, pale yellow, rarely septate hyphae, clamp connections not observed. Pileipellis (Figure 2h) filamentous, hyphae (3.3) 4.5–8.5 (13.2) µm wide, branched, fusiform terminals, yellowish brown, sometimes incrusting of pigments observed on hyphal wall, clamp connections frequent.

Ecology: Growing on loamy soil under *Cedrus deodara* in dry temperate forest .

3.3. Molecular phylogeny

Sequencing of the PCR products of the nrITS region of *Inocybe kohistanensis* yielded 658–698 base pairs by using ITS1F and ITS4 primers. The consensus sequences of 584 base pairs were subjected to BLAST. It was revealed that these sequences were 85% identical to *I. dunensis* P.D. Orton (JF908092) from Italy with 100% query cover. The sequences of all three specimens were aligned with ITS sequences of the other related taxa. The final data set included 51 nucleotide sequences to reconstruct a phylogeny. The analysis revealed two major clades within *Inocybe* section *Marginatae* (Figure 3). The new species clustered with *I. dunensis*, *I. oblectabilis* (Britzelm.) Sacc., and *I. pallida* Velen. with strong bootstrap support within subsection *Oblectabiles*.

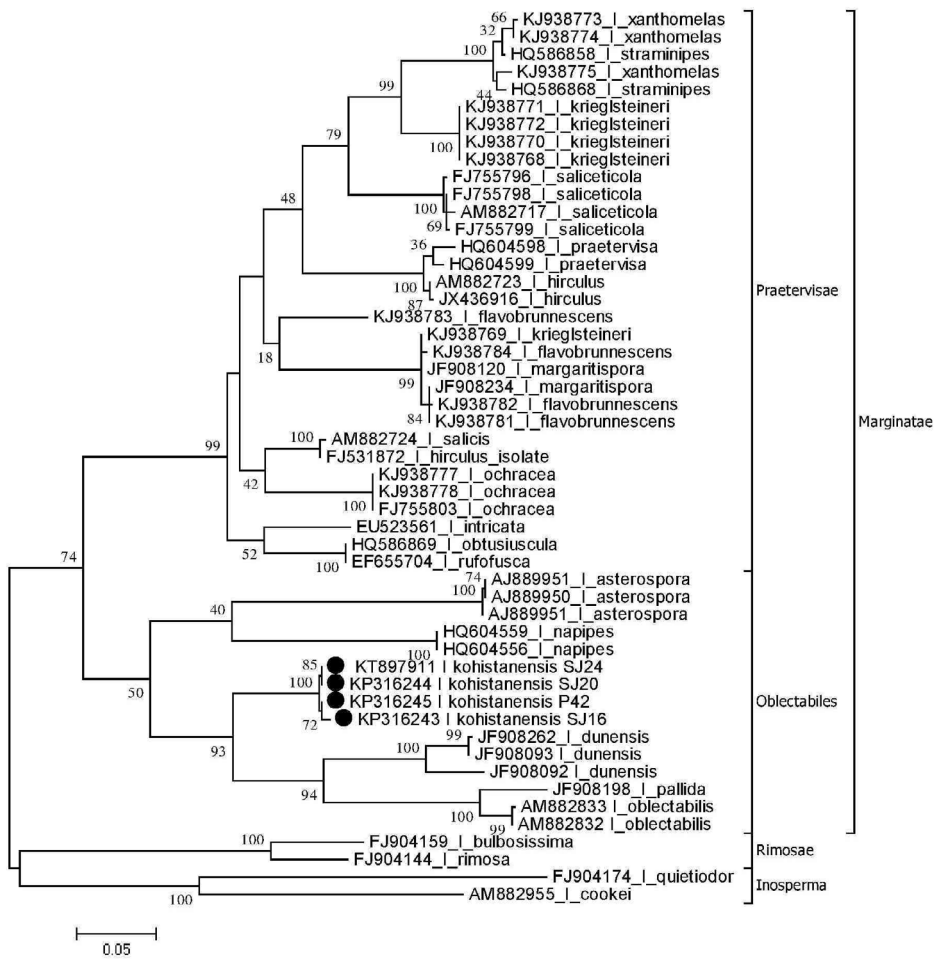

Figure 3. Molecular phylogenetic analysis inferred by using the maximum likelihood method. Sequences generated from Pakistan are marked with . GenBank accession numbers of all the taxa are given. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 51 nucleotide sequences. There were a total of 745 positions in the final data set.

4. Discussion

Inocybe kohistanensis is characterized by its reddish brown pileus surface, nodulose spores, metuloid cystidia, and prominent marginate bulb. It is found closest in morphology to *I. dunensis* and *I. oblectabilis* and clustered with these taxa in subsection *Oblectabiles* within *Marginatae*. Spore morphology and presence of a marginate bulb at the base of the stipe is an important feature separating *Marginatae* from other sections, namely *Rimosae* and *Inosperma*, within *Inocybe*.

In *Marginatae*, two major subgroups were separated on a molecular basis. This separation is supported by morphological characters. *Praetervisae* includes taxa bearing white, buff, or yellowish stipe when young, lacking any pinkish or reddish coloration, while in *Oblectabiles*, the taxa show more or less pinkish to reddish coloration on the stipe, at least on the upper surface of the stipe, following the pattern proposed by Bon (1988). In *Marginatae* most of the species bear angular spores having nodulose spore wall. Some species bear smooth spores while some have an intermediate state between smooth and nodular spores.

In the phylogenetic tree (Figure 3) *Oblectabiles* is subdivided into two clades. *Inocybe kohistanensis* is separated along with *I. dunensis* and *I. oblectabilis* from the other taxa due to the fact that in all other species the spores are between smooth and nodular. *Inocybe kohistanensis* was separated with 93% bootstrap value from *I. dunensis* (JF908092, JF908262, JF908093), *I. oblectabilis* (AM882833, AM882832), and *I. pallida* (JF908198) forming a sister clade. Morphologically these taxa are also distinct from *I. kohistanensis*. *Inocybe oblectabilis* has a dull reddish brown pileal surface and pale reddish brown surface of the stipe, fusiform pleurocystidia with short pedicel, and spores with small nodules. These characters make it distinct from *I. kohistanensis*. In the comparison of *I. kohistanensis* with *I. dunensis*, wavy margins of the pileus, rarely submarginate, spores subrectangular, and intermediate state between Leiosporae and Goniosporae of *I. dunensis* separate it from *I. kohistanensis*.

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