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Two new records of *Tricholoma* species from Pakistan based on morphological features and phylogenetic analysis

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Abstract. Two species of *Tricholoma*, *T. bonii* and *T. triste*, are reported as new records for Pakistan. Phylogenetic analysis based on the nuclear ribosomal internal transcribed spacer (nrITS) region clustered the Pakistani collections with *T. bonii* and *T. triste* sequences from previous worldwide collections. Detailed descriptions of macro- and microcharacters, habitat, general distribution, and diagnostic features are presented.

Key words: Chattar plain, Kumrat Valley, Mansehra, phylogeny, section *Terrea*, squamulose, taxonomy, woolly pileus

Introduction

Tricholoma, a classic and well-known representative of *Agaricales*, was raised to genus level by Staude (1857). Members of this genus are globally distributed, mostly in subtropical and north temperate regions (Tedersoo et al. 2010). They can form ectomycorrhizal relations with species of *Betulaceae*, *Fagaceae*, *Pinaceae*, and *Salicaceae*, but the genus also contains species that are associated with *Eucalyptus*, *Dryas* and *Helianthemum* (Christensen & Heilmann-Clausen 2013). Thus, they play an important role in forest ecosystems. Some species also form dual ectomycorrhizal and monotropoid associations linking trees and monotropoid plants (Leake et al. 2004). The genus also includes some valued edible mushrooms and some toadstools (Shanks 1996; Bidartondo & Bruns 2002; Leake et al. 2004; Smith & Read 2008; Christensen & Heilmann-Clausen 2013).

In the field, the genus *Tricholoma* can be recognized by fleshy basidiomata, white, cream to yellow emarginate lamellae and white spore deposits (Kibby 2012; Christensen & Heilmann-Clausen 2012). Species delimitation within the genus is sometimes difficult because of similar anatomical characteristics including oblong to subglobose, hyaline and smooth spores, simple pileipellis structures and lack of well-differentiated sterile elements, including cystidia (Singer 1986; Christensen & Heilmann-Clausen 2013).

The species of the section *Terrea* i.e., *T. terreum*, *T. triste* and *T. bonii* can be differentiated from the other members of the genus due to dry pale grey and brown-grey to black (rarely white) felty caps, unchanging flesh, indistinct smell and taste, spores with a comparatively high Q-value, pileipelles that do not stain yellow and the lack of clamp connections. Other species included in this section, *T. scalpturatum*, *T. argyraceum*, *T. inocybeoides*, *T. cingulatum*, share several morphological features like yellowish coloration in basidiomata when damaged, farinaceous odor and lack of a differentiated subpellis. *T. cingulatum* is well recognized by a prominent partial veil and its association with *Salix* species (Bon 1984; Christensen & Heilmann-Clausen 2008; Jargeat et al. 2010; Christensen & Heilmann-Clausen 2012, 2013).

Tricholoma triste and *T. bonii* have similar basidiocarps, but *T. triste* differs from *T. bonii* mainly by its white, woolly pileus margins (Galli 2003; Christensen & Heilmann-Clausen 2008).

Over 1374 members of *Tricholoma* have been recorded globally in Index Fungorum, but many were later shifted to other genera (Kirk et al. 2008; Tedersoo et al. 2010; Bessette et al. 2013; Christensen & Heilmann-Clausen 2013; Sánchez-García et al. 2014; Heilmann-Clausen et al. 2017; Ovrebo & Hughes 2018; Reschke et al. 2018; Ovrebo et al. 2019; Şen & Alli 2019; Xu et al. 2020; www.indexfungorum.org, access date 4 August 2022). However,

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only four species of *Tricholoma*, viz *T. aurantium*, *T. lascivum*, *T. terreum*, *T. vaccinum*, have been reported to date from Pakistan (Ahmad et al. 1997).

In this paper, we provide two new records of genus *Tricholoma* section *Terrea*, *T. bonii* and *T. triste*, from Pakistan based on morphological, anatomical and phylogenetic analyses.

Materials and methods

Collection sites

Specimens were collected from two separate locations of Khyber Pakhtunkhwa province, (KP) Pakistan during mycological surveys of these areas in September 2019. One of these sites was Chatter plains, located in the Mansehra district, (Coordinates: 34°36′53.2″N 73°07′4.8″E, 1088 m a.s.l) has high mountains, lakes, beautiful valleys, and more especially dominant conifers, *Pinus wallichana* and *P. roxburghii*. The environment of the district is moist temperate with high (129 mm) rainfall annually (Mustafa 2003).

The other collection site was Kumrat valley, which is a famous tourism spot, located at the edge of Dir Upper district (coordinates: 35°34′56″N 72°10′10″E). This area comes under the alpine zonation where elevation range is 2439–3048 m above sea level. The climate of this sampling site is dry and temperate with an average annual precipitation of 524 mm and mean annual temperature of 0.7–30°C. Coniferous forests and grasslands occur frequently throughout the valley (Ahmad & Nizami 2015).

Morpho-anatomical study

Basidiomata were collected and photographed onsite. Habit and habitat were recorded. The collected material was brought to the Fungal Biology and Systematics Research Laboratory at Institute of Botany, University of the Punjab, Lahore, and described macromorphologically, as well as microscopically, then deposited at the LAH Herbarium. Color codes refer to the Munsell's soil color chart (1975) and terminology follows Vellinga (2001).

For microscopic observations, slides were made with free-hand sections of the dried specimens rehydrated in 5% KOH and stained with Congo Red and Melzer's reagent. The microscopic features, i.e., shape and size of basidiospores, cheilocystidia, basidia, structure and hyphae of pileus covering and stipe covering, were observed using a light microscope (CXRII, Labomed Labo America Inc., Fremont, CA, USA) equipped with a HDCE-X5 microscopic camera under 400× and oil immersion 1000× magnification. Measurements were taken from 20 basidiospores from the basidiomata from each of the collections. The notations [n/b/p] describe the measurements that were made on 'n' spores from 'b' specimens from 'p' collections. The following abbreviations are used: 'l' for length, 'w' for width, 'avl' for average length, 'avw' for average width, 'Q' for quotient of length and width and 'Q_{av}' for average quotient.

DNA extraction, amplification and sequencing

DNA was extracted from 10 mg of dried material using the 2% CTAB method (Bruns 1995). The internal transcribed spacer (ITS) region of nrDNA was used for molecular analysis using the ITS1F/ITS4 primer pair for PCR amplification (White et al. 1990; Gardes & Bruns 1993). PCR cycling parameters were as follows: initial denaturation (94°C for 1 min), 35 cycles (94°C for 1 min, 53°C for 1 min, and 72°C for 1 min) and final extension at 72°C for 5 min. Amplified PCR products were purified and sequenced at TsingKe (China) using the same primer combination as used for PCR. The sequences generated in this study were deposited in GenBank under accession numbers OL376280–OL376282, OL306326 and OL306327.

Sequence alignment and phylogenetic analysis

The sequences generated in this study, and sequences retrieved from GenBank, were used for molecular phylogenetic analysis following Cai et al. (2018) and Liang et al. (2018). For rooting purpose, *Tricholoma bakamatsutake* Hongo (AF204807) was used as the outgroup taxon (Ayala-Vasquez et al. 2022). The consensus sequences were subjected to BLAST and compared with available GenBank sequences. The Final nrITS dataset was aligned with the closest matching GenBank sequences using the Multiple Sequence Alignment (MLA)search tool (MUSCLE) by EMBL- EBI (http://www.ebi.ac.uk/Tools/msa/muscle/) (Edgar 2004) and manually adjusted using BioEdit 7.0.5.3 (Hall 1999), where necessary. Maximum Likelihood (ML) analysis of the nrITS dataset was carried out using RAxML-HPC2 v 8.1.11 (Stamatakis 2014). In the MLA, 1000 bootstrap repetitions were obtained as statistical supports with rapid bootstrapping. Significant support was considered to be ≥70%. For phylogenetic analysis, CIPRES Portal v. 3.1. (Miller et al. 2010) was used. FigTree v 1.4.3 (Rambaut et al. 2014) was used for displaying the phylograms and then exported to Adobe Illustrator.

Results

Phylogenetic analyses

Our ITS based dataset comprises of 33 DNA sequences of *Tricholoma* section *Terrea*. The final alignment consisted of 652 characters: 504 conserved, 136 variable and 64 parsimony-informative. All newly generated sequences fit into *Tricholoma* section *Terrea*. Sequences of *T. triste* (OL306327) grouped with *T. triste* sequences retrieved from GenBank with 97% bootstrap support. The four different Pakistani samples of *T. bonii* clustered with the sequences of the same species which were downloaded from GenBank via strong bootstrap support (98%) (Fig. 1).

Taxonomy

Tricholoma triste (Scop.) Quél., Mém. Soc. Émul. Montbéliard, Sér. 2, 5: 79. 1872. (Figs 2–3)

Macroscopic description. Basidiomata small sized. Pileus 15–28 mm, light brown (7.5YR8/1), campanulate

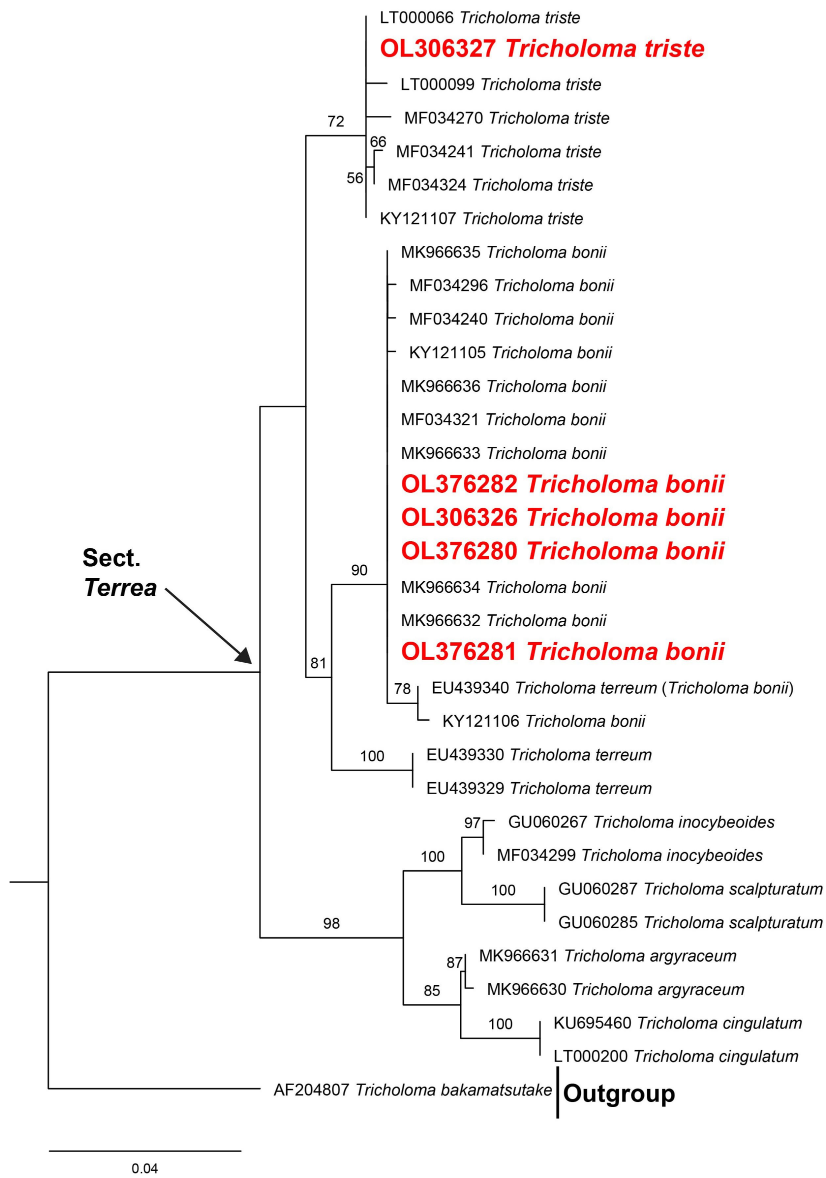

Figure 1. Molecular phylogenetic analysis of *Tricholoma* species resulted from the ML analysis based on nrITS sequences. Sequences generated during this study are represented in red and bold letters. Scale bar = nucleotide substitutions per site.

when young, becoming broadly convex to plane at maturity, umbonate, surface dry, woolly tomentose overall when young, remaining so until maturity, appressed radially fibrillose to minutely squamulse overall, the surface often splitting radially to reveal the whitish context, margin inrolled, heavily bearded when young, grayish brown (5YR 5/2), the margin often paler; context white (10YR8/1) to gray (10YR6/1) (Fig. 2A–C). Lamellae sinuate, pale gray (7.5Y8/1) to gray (10YR6/1), 2–5 mm broad, close to sub-distant, thin to slightly thick, crowded, edges serrulate, lamellulae present in a single tier, concolorous to lamellae. Stipe 21–29 × 7–10 mm, light gray (7.5 Y8/1), cylindrical, equal, slightly bulbous base with mycelial pads, surface dry, silky fibrillose, pruinose at apex, white (10YR8/1) to very pale gray (2.5YR7/1) (Fig. 2D). Cortina absent. Annulus and volva absent. Taste and odor not noticeable.

Microscopic description. Basidiospores [60/3/3] (6–)7–8.5(–9) × 3.8–5 µm, Q = 1.5–2.3 µm, avl × avw = 7.6 × 4.4 µm, Q = 1.62–2.3, Q_{av} = 1.9, ellipsoid to narrowly ellipsoid, hyaline in 5% KOH, inamyloid, smooth, apiculus prominent (Fig. 3A). Basidia 17–32 × 4.8–7.8 µm, avl × avw = 27.5 × 6.4 µm, thin-walled, hyaline in 5% KOH, clavate, 4-spored (Fig. 3B). Cheilocystidia 26–34 × 5.3–7.3 µm, clavate, ovoid or utriform, hyaline in 5% KOH (Fig. 3C). Pileipellis a cutis changing to trichoderm, hyphae width 3.6–7.9 µm, hyaline in 5% KOH, thin walled, branched, septate, subpellis not well differentiated, some elements slightly inflated than pilei trama, 4–10 µm, yellowish brown pigment incrusting pileipellis, clamp connections absent (Fig. 3D). Stipitipellis a cutis, consisting of cylindrical hyphae 2.5–8 µm, branched, septate, parallel, thin walled, hyaline in 5% KOH, caulocystidia 7–16 × 5–7 µm, few, narrowly clavate to narrowly conical, clamp connections not observed (Fig. 3E).

Known distribution. Known from all over Europe, Asia (China), United states of America and now South

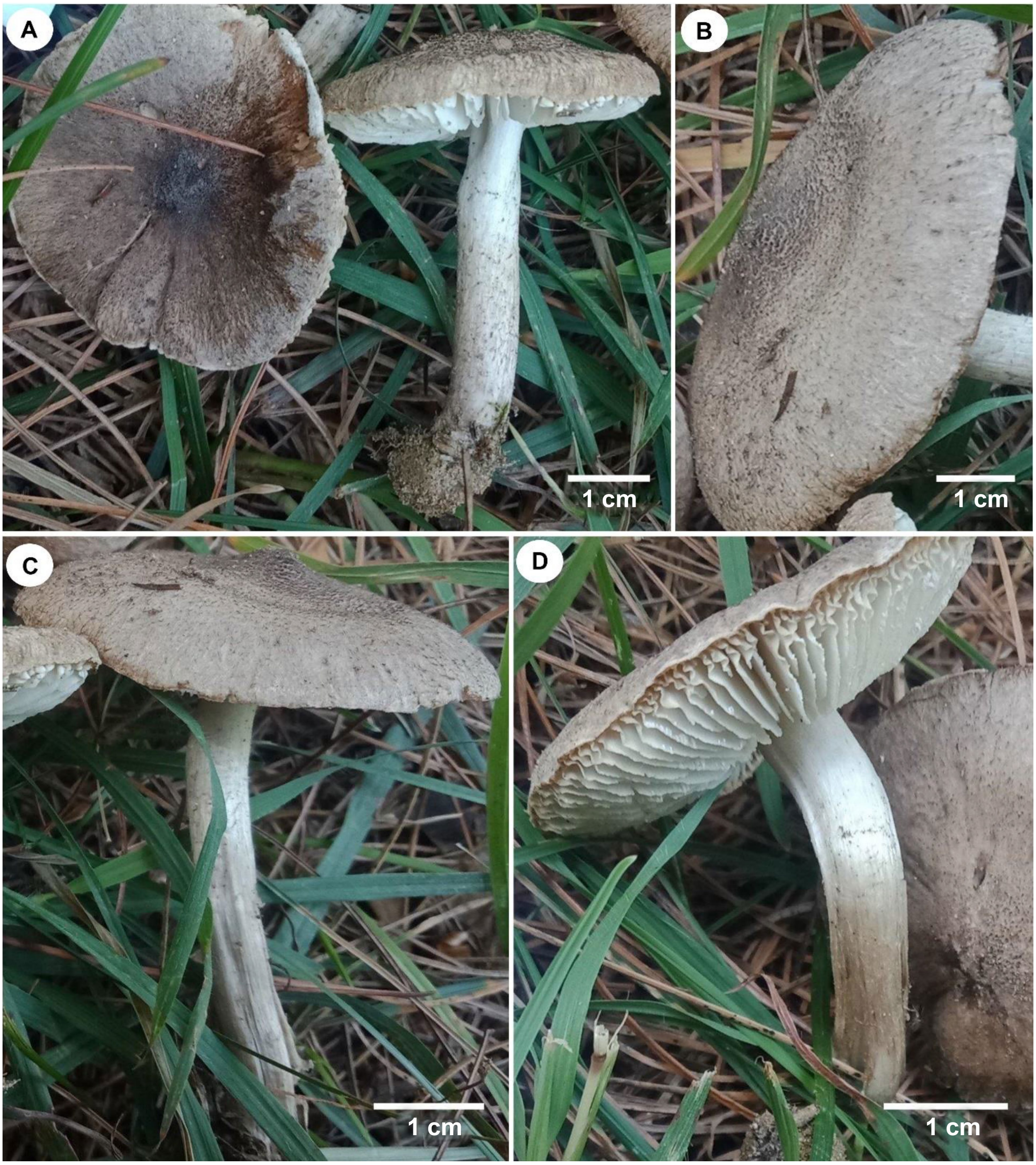

Figure 2. Basidiomata of *Tricholoma triste*. A–D – CHP-07. Photos by Abdul Rehman Niazi.

Asia (Pakistan) (Heilmann-Clausen et al. 2017; Reschke et al. 2018).

Material examined. PAKISTAN. Khyber Pakhtunkhwa, Mansehra district, Chattar plain, 1627 m, a.s.l., gregarious under *Quercus robur* 18 September 2020, CHP-07, Abdul Rehman Niazi (LAH36962; GenBank accession for ITS: OL306327).

Tricholoma bonii Basso & Candusso, Docums Mycol. 27(107): 64. 1997. (Figs 4–5)

Macroscopic description. Pileus 25–30 mm diam., first bell-shaped, later convex to flat, umbonate, light brownish gray (5YR7/1), surface felty to tomentose scaly, paler towards the margin, margins ruptured (Fig. 4A). Lamellae emerginate, white (2.5YR5/12) to pale gray (5YR7/1), free, broad, closed, crenate margins, 1 tier of lamellulae. Stipe 20–25 × 7–10 mm, cylindrical, off white (2.5YR5/12), central, equal, fibrillose, solid (Fig. 4B, C). Annulus and volva absent. Taste and odor not noticeable.

Microscopic description. Basidiospores [60/3/3] (4.2–)4.8–6.7(–8) × (3–)3.6–4.3(–4.5) µm, avl × avw 5.9– 3.8 µm, Q = 1.4–1.8, Q_{av} = 1.6, obovoid, inamyloid, apiculus prominent, hyaline in 5% KOH (Fig. 5A). Basidia 23–42 × 5–9 µm, avl × avw = 32 × 7 µm, thin walled, clavate, 2, 3 or 4 spored, hyaline in 5% KOH (Fig. 5B). Cheilocystidia 21–54 × 7–10 µm, clavate, hyaline in 5% KOH (Fig. 5C). Pileipellis a cutis of hyphae

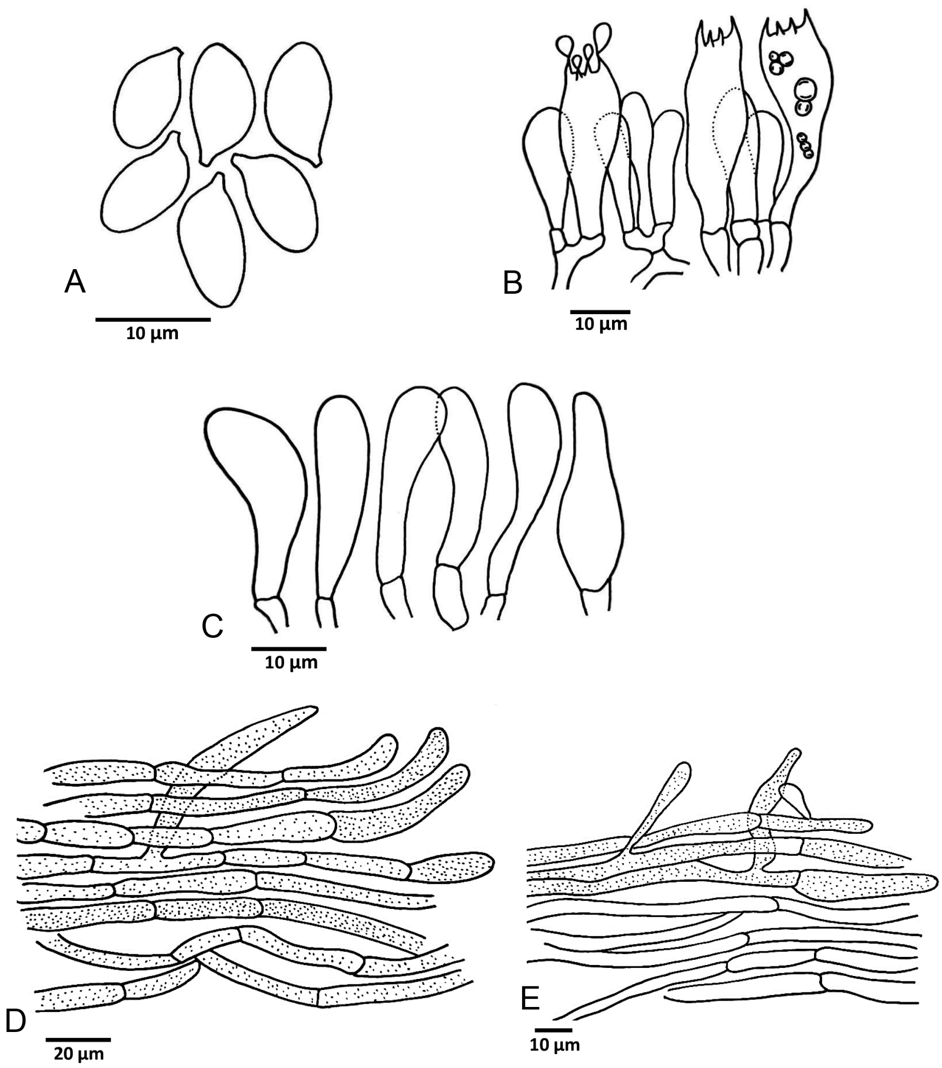

Figure 3. Line drawings of *Tricholoma triste* (LAH36962). A – basidiospores; B – basidia; C – cheilocystidia; D – pileipellis; E – stipitipellis. Drawings by Aiman Izhar.

with 4.1–16 µm diam., hyaline in 5% KOH, thin walled, branched, septate, epicutis with inflated cells up to 16 µm diam., with brownish incrustations, subpellis hyphae narrower, upto 7 µm diam., no pigmentations observed (Fig. 5D). Clamp connections absent. Stipitipellis a cutis of hyphae with 3.9–7.9 µm diam., hyaline in 5% KOH, thin walled, branched, septate, parallel, clamp connections absent (Fig. 5E), caulocystidia not observed.

Known distribution. Widely distributed, known from Europe (Denmark, Estonia, Norway, Austria, Germany), East Asia (China, Province), and now South Asia (Pakistan, China, Province) (Basso & Candusso 1997; Heilmann-Clausen et al. 2017; Reschke et al. 2018; Şen et al. 2018).

Material examined. PAKISTAN. Khyber Pakhtunkhwa province, Dir Upper district, Kumrat, 2232 m a.s.l., on soil under *Cedrus deodara*, 25 August 2019; K-0011, K-15, KU-92, Abdul Nasir Khalid (LAH36989, LAH36990, LAH36991, GenBank accessions for ITS: OL376281, OL376282, OL376280), Mansehra district, Chattar plain, 1627m, a.s.l., gregarious on forest floor 18 September 2020, CHP-20, Abdul Rehman Niazi (LAH36963; GenBank accessions for ITS: OL306326).

Discussion

Our Pakistani collection of *Tricholoma bonii* has a close resemblance to *T. bonii* reported from Europe, China and Turkey (Basso & Candusso 1997; Heilmann-Clausen et al. 2017; Reschke et al. 2018; Sen et al. 2018). Our species differs from holotype of *T. bonii* reported from Europe by its light brownish gray pileus, pale gray lamellae, short stipes (20–25 mm), smooth basidiospores, and presence of cheilocystidia. Basidiomata of European collections produced white pilei, hygrophanous lamellae, much longer stipes (40–60 mm) and no cheilocystidia (Basso & Candusso 1997). The similar characteristics of Pakistani specimen with Turkish specimen are convex to flat pileus, felty to tomentose scaly surface, emarginate lamellae, cylindrical stipes, smooth and oblong basidiospores, but Pakistani collections of *T. bonii* produced smaller basidiospores than reported from Turkey (Heilmann-Clausen et al. 2017; Sen et al. 2018).

Tricholoma scalpturatum differs from *T. bonii,* having dark grey-brown, conico-convex pilei with grey-brown, small, appressed fibrillose-squamules, broadly ellipsoid to oblong spores, clavate to cylindrical cheilocystidia,

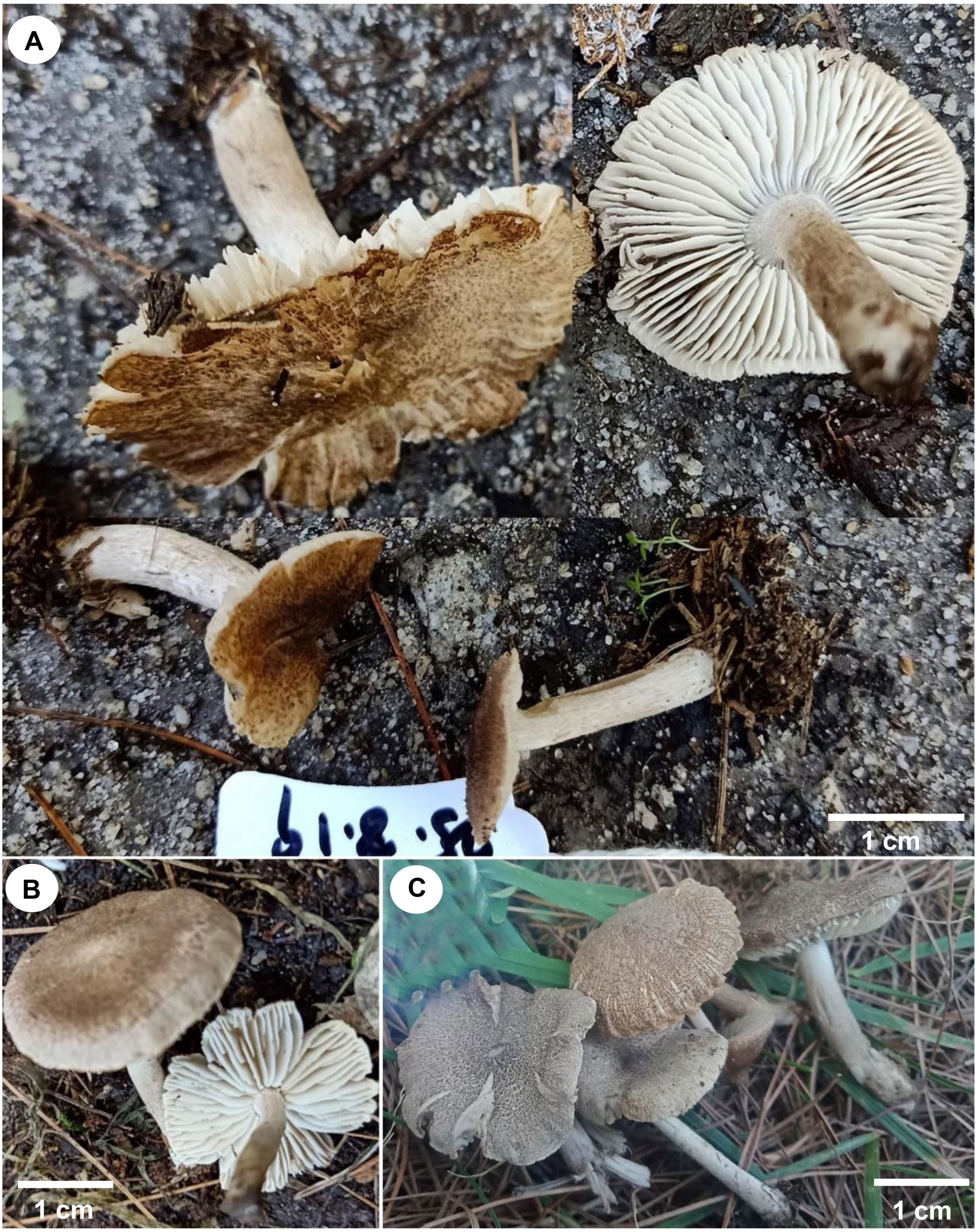

Figure 4. Basidiomata of *Tricholoma bonii*. A – K-15; B – KU-92; C – CHP-20. Photos by Abdul Nasir Khalid & Abdul Rehman Niazi.

pileipelles composed of cylindrical elements with brown pigmentations, and presence of caulocystidia. Clamp connections are absent in both species (Christensen & Noordeloos 1999).

The second Pakistani species matched to *T. triste* reported from Europe and Germany (Christensen & Noordeloos 1999; Heilmann-Clausen et al. 2017). Both species, i.e., Pakistani collection and European collection, share similarities in producing conical to conico-convex pilei, subentire to eroded edged lamellae, stipes that are cylindrical or slightly broadened towards base, and oblong basidiospores (Christensen & Noordeloos 1999). The German collections of *T. triste* differ solely by their brown pilei and emerginate grayish white lamellae and relatively smaller basidiospores (4.5–7 × 3–4.5 µm) (Quélet 1872). The European collections differ by broader diameter pilei

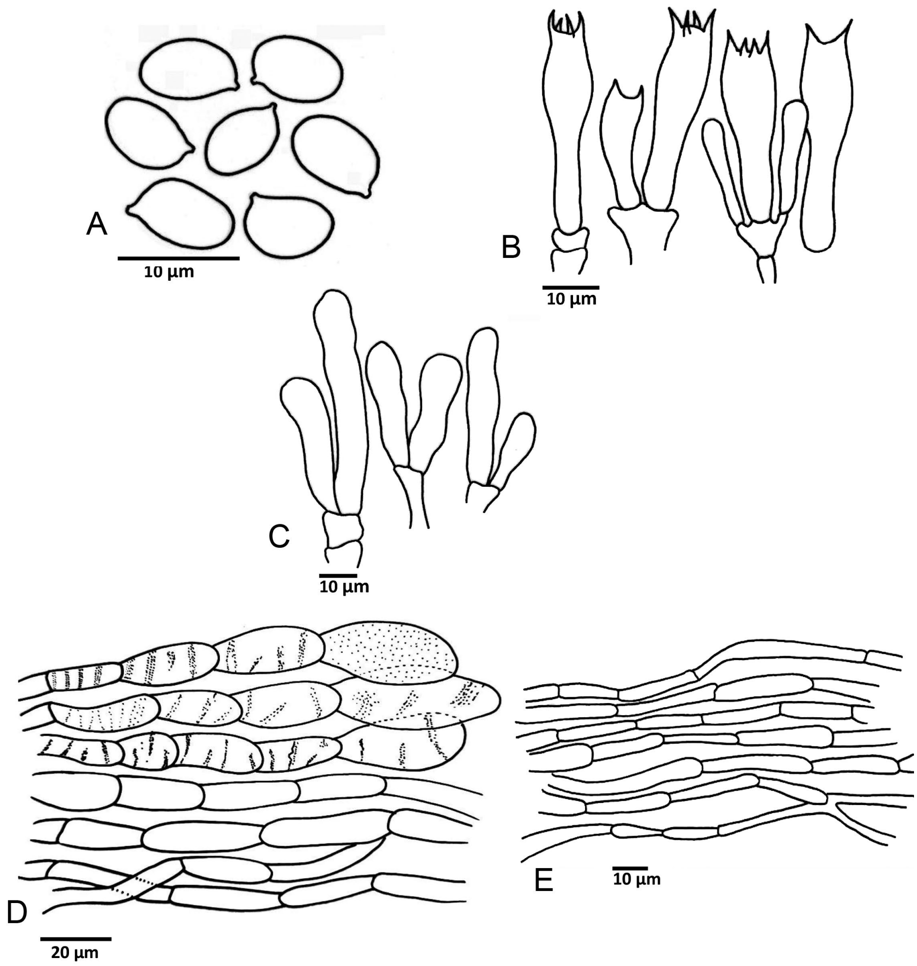

Figure 5. Line drawings of *Tricholoma bonii*. A – basidiospores; B – basidia; C – cheilocystidia; D – pileipellis; E – stipitipellis. Drawings by Aiman Izhar.

(reaching up to 50 mm), reaching to wholly black color at full maturity, lamellae having black dots, long but narrow (25–40 × 4–7 mm), glabrescent stipes, no cheilocystidia, inflated elements in subpellis, and clusters of much longer (14–40 µm) caulocystidia (Christensen & Noordeloos 1999). In BLAST analysis, *T. triste* (CHP-07) had 98% similarity with *T. triste* (LT000194). When the nrITS region of Pakistani collection and German collection were compared, a difference of only one nucleotide was observed.

Tricholoma argyraceum lies close to *T. triste* during phylogenetic analysis and differs from the latter due to its very pale cream colored, conico-convex pilei, presence of veil, very pale grey crowded lamellae that bruised yellow, ellipsoid to oblong basidiospores, sub-cylindrical to clavate or lageniform cheilocystidia and cylindrical pileipellis hyphae with pale yellow pigmentation. Lack of clamp connections and caulocystidia was found in both (Christensen & Noordeloos 1999).

Tricholoma cingulatum, another closer species to *T. bonii* and *T. triste*, can be differentiated from both of the Pakistani collections due to its conico-convex to plano-convex pilei with conical umbo and dark grey squamules, pale grey lamellae, ellipsoid, oblong to subcylindrical spores, absence of cheilocystidia, pileipellis having trichodermal bundles with yellow pigmentation. Absence of clamp connections and caulocystidia are the only characters that it shares with both Pakistani species (Christensen & Noordeloos 1999).

Our phylogenetic analysis supports the presence of seven well-differentiated species in *Tricholoma* section *Terrea*, viz. *T. argyraceum, T. cingulatum, T. scalpturatum, T. inocybeoides, T. terreum, T. bonii* and *T. triste*, previously recorded from Europe, Turkey and China. In this section, albinism seems to be rather common, with albinistic forms and varieties described also in *T. cingulatum* and *T. scalpturatum* (Hermosilla & Sánchez 1994; Bidaud & Thévenard 2003). *T. bonii* was initially described as a species with whitish colors (Basso & Candusso 1997), but the type displays 100% ITS sequence resemblance with collections with greyish pileus colors. Jargeat et al. (2010) studied the taxonomy and phylogeny of the species that group around *T. argyraceum* with the help of three molecular markers. The study proved to have consistent phylogenies for all markers that support the strong delimitation of *T. argyraceum, T. inocybeoides, T. cingulatum* and *T. scalpturatum* as biological species (Heilmann-Clausen et al. 2017). From all the above discussion, we may conclude that high bootstrap values in the phylogenetic tree generated based on nrITS sequences confirm the morphological distinctions of the species. These species are easily identifiable by nrITS gene sequence homologies.

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