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Short title: Five *Leucoagaricus* species

Underexplored regions of Pakistan yield five new species of

Leucoagaricus

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ABSTRACT

The large genus *Leucoagaricus* (Basidiomycota) is poorly studied in Pakistan, where the northern parts of the country are considered hotspots for biodiversity. Based on morphological and molecular data, five new species are described: *Leucoagaricus badius*, *L. lahorensiformis*, *L. pakistaniensis*, *L. sultanii* and *L. umbonatus*. Descriptions and illustrations are given for the new taxa. A phylogeny based on morphology and four DNA regions, including the internal transcribed spacers (ITS1-5.8S-ITS2 = ITS) and D1-D2 domains of the 28S gene of the nuclear rDNA), the gene for RNA polymerase II second largest subunit (*RPB2*) and the translation

elongation factor 1 α gene (*TEF1- α*), show that the new taxa are clustered in a clade representing *Leucoagaricus* section *Rubrotincti* subgenus *Sericeomyces*.

KEY WORDS: Agaricales, biodiversity, *Leucocoprinus*, Malakand, phylogenetic analysis, taxonomy

INTRODUCTION

Pakistan is situated in a region where major floras intersect. The richness and diversity of its flora suggest that fungal biodiversity studies will be particularly productive. There are a wide diversity of habitats from coastal areas along the Arabian Sea to arid scrubland in Punjab and Sindh to high elevation forests and tundra of the western Himalaya in Khyber Pakhtunkhwa province of Pakistan. This ecological diversity promotes a wide variety of fungi from those of grasslands to those occurring in forests and deserts (Yousaf et al. 2012). The diversity of the region has begun to be revealed by recent studies focusing on various fungal groups (Ahmad et al. 1997; Niazi et al. 2006, 2009; Razaq et al. 2012, 2013; Sarwar and Khalid 2012; Nawaz et al. 2013; Fiaz et al. 2014; Thongklang et al. 2014; Hussain et al. 2016; Jabeen et al. 2016).-This paper, concerning the genus *Leucoagaricus* Locq. ex Singer, is one of several studies documenting this mycota.

The genus *Leucoagaricus* includes approximately 150 species based on our estimates from Indexfungorum and MycoBank. The recent description of several new species, particularly from Pakistan and adjacent regions, suggests that the genus is more diverse and speciose in this area than previously assumed (Liang et al. 2010; Vellinga 2010; Malysheva et al. 2013; Justo et al. 2015; Ge et al. 2015; Qasim et al. 2015). Species of *Leucoagaricus* have small to medium-sized, thin or fleshy basidiomata ranging in stature from slender to sturdy; pileus surfaces are radially fibrillose, floccose, squamulose to fibrillose-scaly and rarely granulose; margins that are

entire or very short striate; a context that may stain when bruised; and central, equal to bulbous stipes that have membranous, sometimes moveable annuli; basidiospores generally lack well-defined germ pores and are thin-walled and smooth; and the pileipellis is either a trichoderm or a cutis of repent and radially arranged hyphae lacking sphaerocysts. Pleurocystidia are mostly absent but rarely may be present and cheilocystidia are frequent. Clamp connections are absent (Singer 1986; Vellinga 2001).

Species of *Leucoagaricus* are saprotrophic, occurring on soil, wood chips and sawdust in a wide range of habitats. Species are found in both the northern and southern hemispheres and are more diverse in the tropics than in cool temperate regions. No species are reported in arctic and alpine regions (Vellinga 2001). Regional treatments and reports include Europe (Candusso and Lanzoni 1990; Bon 1996; Vellinga 2001), North America (Kauffman 1924; Smith 1954; Sundberg 1967 (all under *Lepiota*), Akers 1997; Vellinga 2007; Vellinga and Davis 2007; Vellinga 2010; Vellinga et al. 2010; Justo et al. 2015), South America (Dennis 1970) and Asia (Berkeley and Broome 1871; Pegler 1972, 1986; Ahmad et al. 1997; Kumar and Manimohan 2009; Ge 2010; Liang et al. 2010; Malysheva et al. 2013; Yuan et al. 2014; Ge et al. 2015; Qasim et al. 2015).

Phylogenetic studies reveal that species of *Leucoagaricus* and *Leucocoprinus* Pat. intermix within a single clade (Vellinga 2003, 2004; Vellinga et al. 2011). Taxonomic and phylogenetic relationships and indeed the circumscriptions of species of *Leucoagaricus* and *Leucocoprinus* are unresolved because molecular data for previously described species are limited and morphological characters intergrade to some extent. To distinguish species of *Leucoagaricus* from those of *Leucocoprinus*, several morphological characters are used. *Leucocoprinus* species have plicate pilei, spores with or without apical germ pores and hymenia

with pseudoparaphyses (Singer 1986). Should a single genus be adopted for this group, *Leucocoprinus* is the older name and would have priority. Because the distinction between these two genera has not been resolved conclusively, we describe our species in *Leucoagaricus* because the taxa we studied show morphological characters that ally them with the prevailing concept of *Leucoagaricus*. Ultimately, the several elements that make put this group may be segregated as several genera. To avoid confusion, we use the abbreviation *La.* for *Leucoagaricus* and *Lc.* for *Leucocoprinus*.

Previously, only four species of *Leucoagaricus* [*La. lahorensis* Qasim, Amir & Nawaz; *La. asiaticus* Qasim, Nawaz & Khalid; *La. leucothites* (Vittad.) Wasser and *La. serenus* (Fr.) Bon & Boiffard)] were reported from Pakistan (Ahmad et al. 1997; Ge et al. 2015; Qasim et al. 2015). During our explorations of basidiomycetous fungi of Pakistan during 2013 to 2014, several collections of *Leucoagaricus* were encountered. These included five new species. The descriptions of these new species are based on morphological characters and molecular phylogenetic analyses of four DNA regions, including the internal transcribed spacers (ITS1-5.8S-ITS2 = ITS) and D1-D2 domains of the 28S gene of the nuc rDNA (28S), the gene for the second largest subunit RNA polymerase II (*RPB2*) and the translation elongation factor 1- α gene (*TEF1- α*). These markers have proven to be reliable in phylogenetic studies of the mushrooms (Matheny et al. 2007; Ge et al. 2015).

MATERIALS AND METHODS

Sampling and morphological characterization.—Specimens were collected from Malakand and the Swat districts of Khyber Pakhtunkhwa and the Lahore district Punjab, Pakistan in 2013 and 2014. Basidiomata were photographed, tagged and field data were recorded on material in fresh condition. Munsell (1975) was used for color determination, as indicated in descriptions by capitalized color names and alphanumeric codes. The specimens were air-dried and kept in zip-lock bags. Specimens are deposited in the mycological collection of the University of the Punjab, Lahore (LAH) Pakistan and the Farlow Herbarium (FH) Harvard University, Cambridge Massachusetts, USA.

Microscope slide preparations were made using 5% aqueous KOH and 1% Congo red in ammonia (w/v). Characters such as size and shape of spores, basidia, cheilocystidia and pileipellis were studied under a light microscope (MX4300H, Meiji Techo Co., Ltd., Japan) with at least 20 structures measured for each. The abbreviations [$n/m/p$] denote the following: n basidiospores measured from m fruit bodies of p collections. At least 20 basidiospores from two collections were measured at 1000 \times . Measurements were rounded to the nearest 0.5 μm . Dimensions of basidiospores are presented as (a)b–c(d), where b–c includes a minimum of 90% of the measured values. Extreme values are given in parentheses (a and d). The Q was calculated as length and width ratio, avQ is the mean length/width of all basidiospores.

DNA extraction, PCR and sequencing.—DNA was extracted from 5–15 mg of dried specimens using the DNeasy Plant Mini Kit (Qiagen, Valencia, California) according to the manufacturer's instructions. We amplified four DNA regions using the primer combinations ITS1F/ITS4 for ITS (White et al. 1990); LR0R/LR5 for 28S (Hopple and Vilgalys 1990); gRPB2-6F/gRPB2-7R for *RPB2* (Liu et al. 1999) and EF1-983F/EF1-1567R for *TEF1- α* (Rehner and Buckley 2005). 25

μL volume PCR reactions were performed, containing 2.5 μL 10× Econo *Taq* Buffer (Lucigen, Middleton, Wisconsin, USA), 0.5 μL dNTPs, 1.25 μL of each primer (10 μM/μL), 0.125 μL of Econo *Taq*® DNA Polymerase (Lucigen), 14.375 μL H₂O and 5 μL of DNA template.

Amplification protocols and PCR conditions were the same as those described in Ge et al. (2014), except that annealing temperatures were optimized for each gene region: 54 C for ITS, 52 C for 28S, and 60 C for *RPB2* and *TEF1-α*. PCR products were purified using the QIAquick PCR Purification kit (Qiagen). Sequencing was performed with the same PCR primers using the Big Dye Sequencing Kit 3.1 on an ABI-3730-XL DNA Analyzer (Applied Biosystems, Foster City, California). Sequences produced for this study have been deposited in GenBank (TABLE 1).

Phylogenetic analyses.—Consensus sequences were generated from both forward and reverse primer reads in BIOEDIT 7.2.5 (Hall 1999) and then homology searches were performed at NCBI using BLAST. These BLAST results, along with the sequences employed in recent phylogenetic studies of *Leucoagaricus* in neighboring regions (Liang et al. 2010; Vellinga et al. 2010; Ge et al. 2015), were used in the phylogenetic analyses. DNA sequences were aligned and assembled in CLUSTAL X 2.1 (Larkin et al. 2007). Ambiguous positions and regions were excluded from the matrix. Gaps were treated as missing data. *Coprinus comatus* (O.F. Müll.) Pers. was selected as outgroup. The resulting alignments were deposited in TreeBase (study no. S20910).

Phylogenetic inference was conducted using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI) methods. Maximum parsimony analyses were performed in PAUP4.0b10 (Swofford 2004) with a heuristic search of 1000 replicates with random stepwise addition using tree-bisection-reconnection (TBR) branch swapping and starting from trees obtained by the stepwise addition of sequences.

ML tree generation and bootstrap analyses were performed for individual gene regions and also for the combined ITS, 28S, *RPB2* and *TEF1- α* dataset, on the CIPRES Science Gateway (Miller et al. 2010) employing RAxML-HPC 8. Rapid bootstrap analysis/search for best-scoring ML tree was performed for each dataset. For the bootstrapping phase, the GTRGAMMA model was selected. One thousand rapid bootstrap replicates were run. A bootstrap proportion of $\geq 70\%$ was considered significant.

BI analyses were done with a Markov chain Monte Carlo (MCMC) coalescent approach implemented in BEAST 1.6.2 (Drummond and Rambaut 2007). A Bayesian skyline coalescent tree prior was used in all simulations, and the starting tree was randomly generated. Four independent runs were undertaken. Chain length was 10 million generations, with a sampling frequency of 1000. Tracer 1.5 (Rambaut and Drummond 2007) was used to check the effective sample size (ESS), and burn in values were adjusted to achieve an overall ESS of ≥ 200 . A maximum clade credibility tree (20% burn in value) was generated using TreeAnnotator 1.6.2 (Drummond and Rambaut 2007). Posterior probabilities (PP) of ≥ 0.95 were considered significant.

RESULTS

Phylogenetic analyses.—The ITS dataset consisted of 89 sequences and 889 characters. After exclusion of ambiguous sites, the final analysis of the ITS region consisted of 620 distinct aligned sites. The optimal ML tree was found with a log likelihood score of -11792.300875 (FIG. 1). The new taxa were recovered in a well-supported clade with ML bootstrap, MP bootstrap and BI posterior probabilities (MLB 89%, MPB 81%, PP 1). This clade, consisting of species with both white and colored basidiomata, is formed by the members of *Leucoagaricus* sect. *Rubrotincti* Singer, typified by *La. rubrotinctus* (Peck) Singer, with colored pilei and subgenus

Sericeomyces (Heinem.) Bon, typified by *La. serenus* (Fr.) Bon & Boiffard, which typically have white basidiomata. The new species *La. sultanii*, with colored pilei, belongs in a well-supported clade with *La. asiaticus*, *La. subcrystallifer* Ge & Yang, the white species *La. crystallifer* Vellinga, *La. subvolvatus* (Malençon & Bertault) Bon and *La. menieri* (Sacc.) Singer. *Leucoagaricus badius* was recovered with excellent bootstrap support together with an undescribed species from India (GenBank KR154966). Similarly, *La. pakistaniensis* forms a sister clade with an undescribed species from China (JN907015). *Leucoagaricus lahorensiformis* forms part of the clade to which *La. lahorensis* belongs. One species with white basidiomata, *La. umbonatus*, was recovered with high bootstrap support with a sister clade with an undescribed species from California (AY176430).

ML analysis of the 28S dataset (67 sequences, 965 characters) yielded an optimal tree with a log likelihood score of -4975.974705 (SUPPLEMENTARY FIG. 1). Bootstrap support for the 28S dataset was generally weak compared to the ITS dataset, but individual taxa and our new species were recovered with strong bootstrap support.

The *RPB2* dataset included 69 sequences and the resulting alignment had 829 characters. After excluding ambiguously aligned sites, the final analysis of the *RPB2* region contained 508 clearly aligned sites. The analysis resulted in an optimal ML tree with log score -11131.079781. The new taxa are nested within a strongly supported clade (MLB 97%) with other members of *La. sect. Rubrotincti* and subgen. *Sericeomyces* (SUPPLEMENTARY FIG. 2). Species within this strongly supported clade share diagnostic morphological characters such as a cutis-like pileipellis, ovoid to amygdaliform basidiospores and cylindrical to fusiform cheilocystidia.

The *TEF1- α* dataset consisted of 36 sequences and 557 sites; after exclusion of ambiguously aligned sites, it consisted of 365 distinctly aligned positions and produced a ML

tree with optimized log likelihood value -5695.633034 (SUPPLEMENTARY FIG. 3). The new species were recovered with excellent bootstrap support of 98%.

The combined ITS-28S-*RPB2-TEF1*- α dataset included 57 sequences and 2262 characters. The analysis produced an optimal ML tree with log score -33058.711364 (FIG. 2). The new species of *Leucoagaricus* are nested in a clade (*Rubrotincti*), well supported in ML, MP and BI analyses (MLB 94%, MPB 85% and PP 1).

TAXONOMY

Leucoagaricus badius Hussain, Pfister, Afshan & Khalid, sp. nov.

FIGS. 3A–B, 4A–D, 5A.

Mycobank: MB815374

Diagnosis: Basidiomata fragile with dark red pileus, covered with radially arranged, dark red, fibrillose squamules and a deep red umbo; basidiospores amygdaliform to ovoid, slightly thick-walled, without germ pore; cheilocystidia smooth, fusiform; pileipellis cutis of radially arranged elements.

Typification: PAKISTAN. KHYBER PAKHTUNKHWA: Qaldara, Malakand, 490 m elev, 13 Aug 2014, *S. Hussain SH-148* (**holotype** LAH). GenBank: ITS = KU647736; 28S = KU900518; *RPB2* = KU900506; *TEF1*- α = KU900510.

Etymology: *badius* (Latin), referring to the dark red color of the pileus.

Macroscopic characters: Pileus 20–40 mm diam, at first ovoid to ellipsoid, expanding to applanate to convex with conspicuous umbo and deflexed margin, Moderate Red (5R 4/8–5R 4/10) to Dark Red (5R 3/6–5R 3/8) fibrillose squamules on a white background; umbo Deep Reddish (5R 3/10). Lamellae free, crowded, white, 5 mm wide, with 0–2 series of lamellulae; edge finely scalloped, white. Stipe 50–70 × 1.5–2.5 mm, subclavate, thickening toward base,

white in the upper-half, brownish in the lower-half, smooth, shiny, hollow; annulus white, membranous, persistent midway on the stipe. Context white, no color changes on handling, not tasted.

Microscopic characters: Basidiospores [50/4/3] (6–)6.5–7.5(–8.5) × (3.5–)4–5(–5.5) μm, (Q = 1.35–1.55, avQ = 1.46), amygdaliform to oblong ovoid to ellipsoid in the side view, ovoid in face view, slightly thick-walled, hyaline in KOH, without germ pore, smooth, strongly dextrinoid, metachromatic in Cresyl blue. Basidia 14–17 × 6–8 μm, hyaline, 4-spored. Cheilocystidia 21–31 × 9–13 μm, hyaline, arranged in clusters, clavate to fusiform, with no crystals. Pleurocystidia absent. Pileipellis is a cutis, with radially arranged cylindrical hyphae, reddish-brown, 4–8 μm diam. Clamp connections absent.

Habitat and distribution: Saprotrophic and solitary to scattered on nutrient-rich loamy soil under *Acacia modesta* and *A. indica*. It is so far only known from lowlands of northern Pakistan.

Additional specimen examined: PAKISTAN. KHYBER PAKHTUNKHWA: Kharkai, Malakand, 450 m elev, scattered in humus rich soil, 30 Sep 2014, *S. Hussain SH-210* (FH, LAH).

Notes: The main distinguishing features of *La. badius* are: (i) fragile basidiomata with dark red pileus, with radially arranged dark red fibrillose squamules and a deep red umbo; (ii) amygdaliform to ovoid basidiospores that are slightly thick-walled, and lack a germ pore; (iii) smooth, fusiform cheilocystidia; and (iv) a pileipellis cutis of radially arranged elements.

In the ML phylogeny based on ITS sequences, *La. badius* forms a separate clade within the section *Rubrotincti*. The close relatives are *La. vassiljevae* E. F. Malysheva, T. Yu,

Svetasheva & E. M. Bulakh, *La. sublittoralis* (Kühner ex Hora) Singer and the newly described species, *La. pakistaniensis*.

Leucoagaricus vassiljevae, described from Russian Far East, differs from *La. badius* by: (i) its medium-size pileus with appressed red brown or crimson brown fibrils, deep brown umbo; (ii) longer stipe (50–130 mm), larger spores ($8\text{--}11 \times 4\text{--}5 \mu\text{m}$) that are somewhat similar in shape and have an apical papilla; and (iii) broadly to narrowly clavate to subutriform or cylindrical cheilocystidia (Malysheva et al. 2013). *Leucoagaricus sublittoralis*, also known from the Russian Far East, differs in its pale pink-brown pileus and cheilocystidia that are more variable in shape, from clavate to narrowly clavate. The new species differs from *La. rubrotinctus* mostly by the shape of spores (Vellinga 2001). A species recently described from Pakistan, *La. lahorensis*, has a brownish pileus covering, mostly ellipsoidal to amygdaliform basidiospores, $8\text{--}10 \times 6\text{--}7 \mu\text{m}$, and narrowly clavate to subcylindrical cheilocystidia (Qasim et al. 2015). *Leucoagaricus subpurpureolilacinus* Z.W. Ge & Zhu L. Yang from southwestern China, has similarly shaped basidiospores but differs in its broad, brown to dark ruby umbo and clavate cheilocystidia with gelatinized covering intermixed with crystals (Ge et al. 2015).

Leucoagaricus lahorensiformis Hussain, Ahmad, Afshan & Khalid, sp. nov.

FIGS. 3D, 4E–H, 5B.

Mycobank: MB815378

Diagnosis: Basidiomata medium-sized, convex to plane pileus with orange fibrils on white background, inconspicuous orange umbo, slightly clavate stipe, ellipsoidal to amygdaliform basidiospores, clavate cheilocystidia with sparse apical crystals and a cuticular pileipellis.

Typification: PAKISTAN. PUNJAB: Lahore, Hostel Area, University of the Punjab, 217 m elev, 8 Sep 2014, *S. Hussain SH-L2* (**holotype** LAH). GenBank: ITS = KU647729; 28S = KU900517; *RPB2* = KU900509; *TEF1- α* = KU900511.

Etymology: “-formis” (Latin), meaning similar, and *lahorensis*, referring to the epithet of the species that this species closely resembles.

Macroscopic characters: Pileus 10–30 mm diam, initially ovoid to umbonate, becoming convex to plane at maturity with inflexed margin, with Light Orange (2.5YR 8/8–5YR 8/8) to Moderate Orange (2.5YR 7/8–5YR 7/8) fibrils on white background; umbo inconspicuous, Strong Orange (2.5YR 7/12–5YR 7/12). Lamellae free, crowded, white, with 1–3 series of lamellulae, with finely scalloped white edges. Stipe 30–50 × 3–7 mm, central, white, smooth, shiny, hollow, broader toward the bulbous base, base 7 mm wide. Annulus membranous, white and persistent. Context white, no color changes on handling, flavor not tasted.

Microscopic characters: Basidiospores [48/3/2] (6–)6.5–7.5(–8) × (3–)3.5–4(–4.5) μm , ($Q = 1.15–1.28$, $\text{av}Q = 1.22$), fusiform to amygdaliform or ovoid in side view, ellipsoidal in face view, moderately thick-walled, without germ pore, smooth, dextrinoid, metachromatic in Cresyl blue. Basidia 18–22 × 6–9 μm , clavate, hyaline, 4-spored, rarely 2-spored. Cheilocystidia 20–26 × 8–11 μm , clavate to cylindrical, abundant and with crystals usually at the apex of the cystidium. Pleurocystidia absent. Pileipellis cutis of radially arranged elements, 5–9 μm diam, light orange. Clamp connections absent.

Habitat and distribution: Saprotrophic and solitary to scattered on loamy soil under planted trees of *Dalbergia sissoo*. So far only known from lowland northeastern Pakistan.

Additional specimen examined: PAKISTAN. PUNJAB: Lahore, University of the Punjab, 217 m alt, scattered in humus rich soil, 23 Sep 2014, *S. Hussain SH-L5* (FH SHL5).

Notes: Leucoagaricus lahorensiformis is characterized by medium-sized basidiomata with a convex to plane pileus covered with orange fibrils on a white background, inconspicuous strong orange umbo, slightly clavate stipe, ellipsoidal to amygdaliform basidiospores, clavate cheilocystidia with sparse apical crystals and cuticular pileipellis.

Leucoagaricus lahorensiformis is similar to *La. lahorensis* in habitat and both species occur in the same area around Lahore. Morphologically the basidiomata and shape of the basidiospores and the cheilocystidia of these species are similar. However, in the latter the pileus is reddish brown, and basidiospores are larger, $8\text{--}10 \times 6\text{--}7 \mu\text{m}$, as are the cheilocystidia (Qasim et al. 2015). Another colored species *La. rubrotinctus*, is an eastern North American species with a distinct orange-red radially fibrillose pileus; the name has been widely misapplied. The description of *La. rubrotinctus* from Brazil by Ferreira and Cortez (2012) does not match the one from the USA and is almost certainly another species (EC Vellinga, pers. comm.).

Leucoagaricus vassiljevae differs in having red brown appressed fibrils on the pileus and larger basidiospores, $8.5\text{--}11.5 \times 5\text{--}6 \mu\text{m}$, with an apical papilla (Malysheva et al. 2013).

Leucoagaricus pakistaniensis Jabeen & Khalid, sp. nov.

FIGS. 3C, 4I–L, 5C.

MycoBank: MB815375

Diagnosis: Pileus surface creamy white, smooth and shiny, with white fibrils and a dark brown umbo; ovoid to ellipsoidal basidiospores; clavate cheilocystidia and a cuticular pileipellis.

Typification: PAKISTAN. KHYBER PAKHTUNKHWA: Swat, Mankial, 2500 m alt, 6 Sep 2013, S. Jabeen SJF13 (**holotype** LAH). GenBank: ITS = KU647727; 28S = KU900515; *RPB2* = KY965808; *TEF1- α* = KU900512.

Etymology: *pakistaniensis*, refers to the country in which this species was collected.

Macroscopic characters: Pileus 20–40 mm diam, convex to plano-convex at maturity with incurved margin, Creamy White (10Y9/2) fibrils on white background; umbo Dark Brown (7.5YR2/4); texture smooth, shiny and dry; margin incurved. Lamellae Creamy White (10Y9/2), sub-distant to close, 0–3 series of lamellulae, with entire white edges. Stipe 50–70 × 6 mm, Creamy White (10Y9/2), hollow, fibrillose, narrower towards the pileus. Annulus prominent, ascending, white. Context white, no color change on handling, not tasted.

Microscopic characters: Basidiospores [53/3/2] (7–)7.5–8(–8.5) × (4–)4.5–5 μm, (Q = 1.22–1.33, avQ = 1.26), ellipsoidal to rarely amygdaliform in side view, ovoid to ellipsoid in face view, smooth, slightly thick-walled, lacking a germ pore, dextrinoid, metachromatic in Cresyl blue. Basidia 28–31 × 8–10 μm, clavate, smooth, hyaline, mostly 4-spored, rarely 2-spored. Cheilocystidia 15–20 × 6–9 μm, clavate to subclavate, hyaline, without crystals. Pleurocystidia absent. Pileipellis a cutis with clavate to cylindrical elements, 5–11 μm diam, hyaline. Clamp connections absent.

Habitat and distribution: Saprotrophic and solitary on humus-rich dry soil under *Cedrus deodara*, at 2500 m elevation, in cold temperate coniferous vegetation. So far only known from upper Swat region of northern Pakistan.

Additional specimen examined: Pakistan. KHYBER PAKHTUNKHWA: Swat, Mankial, 2500 m elev, solitary in humus-rich dry soil under *Cedrus deodara*, 6 Sep 2013, S. Jabeen SJF23 (LAH).

Notes: *Leucoagaricus pakistaniensis* is distinguished from other species by its creamy white, smooth and shiny pileus surface with a dark brown umbo, ovoid to ellipsoidal basidiospores, clavate cystidia and a cuticular pileipellis.

In our molecular analyses *La. pakistaniensis* is shown to be related to *La. vassiljevae* and allies. This species shares pileus hyphal characters with *La. vassiljevae* and spores shape with *La. lahorensis*. *Leucoagaricus pakistaniensis* can be separated from these taxa by its smooth and shiny pileus covered with white fibrils; the pilei are reddish-brown in *La. vassiljevae* and *La. lahorensis*. The ovoid to ellipsoidal basidiospores of *La. pakistaniensis* also distinguish it from *La. vassiljevae* and *La. lahorensiformis*, which have amygdaliform basidiospores (Malysheva et al. 2013). Pileus hyphal characteristics of *La. pakistaniensis* are more or less similar to *La. vassiljevae* and *La. lahorensis*. In *La. lahorensiformis* the pileipellis is a cutis of radially arranged elements. The clavate cheilocystidia of *La. pakistaniensis* also distinguish it from *La. vassiljevae* and *La. lahorensiformis*, in which clavate to subutriform or cylindrical cystidia and fusiform cystidia are found (Malysheva et al. 2013).

Leucoagaricus sultanii Hussain, Ahmad & Khalid, sp. nov.

FIGS. 3F, 4M–P, 5D.

Mycobank: MB815376

Diagnosis: Basidiomata medium in size, pileus depressed with dark yellowish umbo, pileus surface slightly fibrillose squamules; basidiospores amygdaliform, cheilocystidia utriform with crystals mostly at the apex of cystidia.

Typification: PAKISTAN. KHYBER PAKHTUNKHWA: Qaldara, Malakand, 510 m elev, 06 Aug 2014, S. Hussain SH115 (**holotype** LAH). GenBank: ITS = KU647733; 28S = KU900519; *RPB2* = KU900523; *TEF1- α* = KU900513.

Etymology: *sultanii*, in honor of the great mycologist of Pakistan, the late Sultan Ahmad (1910-1983), former Professor and Head of the Department of Botany, Government College Lahore, Fellow of the Pakistan Academy of Sciences.

Macroscopic characters: Pileus 40–60 mm diam, initially broadly ovate to conical, later plane, at maturity depressed; with Moderate Yellowish Pink (2.5YR 7/4–5YR 7/4) to Dark Yellowish Pink (7.5R 6/6) umbo; short, slightly fibrillose to rimose, squamules on white background, squamules usually with light yellowish tinge, with slightly uplifted margin. Lamellae free, crowded with 1–3 series of lamellulae, nearly white, with even whitish edges. Stipe 30–70 × 3–7 mm, smooth, central, white, shiny, almost equal with bulbous base. Annulus double-edged uplifted, concolorous with the stipe. Context white, not changing color on handling, not tasted.

Microscopic characters: Basidiospores [70/5/3] (5–)5.5–7(–7.5) × (3–)3.5–4.5(–5) μm, (Q = 1.27–1.57, avQ = 1.44), amygdaliform to ellipsoid in side view, ellipsoid to ovoid in face view, smooth, thin-walled, without a germ pore, dextrinoid, metachromatic in Cresyl blue. Basidia 21–27 × 7–10 μm, subcylindrical to clavate, mostly 4-spored, rarely 2-spored, hyaline. Cheilocystidia 33–41 × 8–11 μm, abundant, utriform, hyaline with crystals usually at the apex of cystidium. Pleurocystidia absent. Pileipellis a cutis with radially arranged hyphae, 3–6 μm wide, yellowish, thin-walled, with acute apex. Clamp connections absent.

Habitat and distribution: Saprotrophic and abundant on nutrient-rich loamy soil under *Acacia modesta*, and *A. indica*. So far only known from lowland northern Pakistan.

Additional specimens examined: PAKISTAN. KHYBER PAKHTUNKHWA: Kharkai, Malakand, 450 m elev, scattered in humus rich soil, 12 Aug 2014, *S. Hussain* SH115b (FH); location as above, 30 Aug 2014, *S. Hussain* SH115c (LAH).

Notes: *Leucoagaricus sultanii* can be distinguished from other species by its medium-sized, white basidiomata, depressed pileus with dark yellowish umbo, slightly fibrillose squamules, amygdaliform basidiospores and utriform cheilocystidia with crystals at the apex.

The taxa closely related to *La. sultanii* are *La. asiaticus*, *La. crystallifer*, *La. menieri* (Sacc.) Singer, *La. subvolvatus* and *La. subcrystallifer*. *Leucoagaricus sultanii* resembles *La. asiaticus* in some morphological features; for example, both species have medium-sized basidiomata, bulbous stipes and amygdaliform basidiospores, but the latter has a dark brown umbo with grayish-brown squamules and comparatively larger basidiospores, $7\text{--}10 \times 5\text{--}6.5 \mu\text{m}$ (Ge et al. 2015).

Leucoagaricus crystallifer has a cream to ocher umbo, crystal-bearing cheilocystidia and longer spores, $5.5\text{--}9.0 \times 3.5\text{--}4.5 \mu\text{m}$ (Vellinga 2001). Similarly, the two other white species *La. menieri* and *La. subvolvatus*, have basidiospores of similar shape and cheilocystidia with crystals at the apex. However, *La. menieri* has fragile, shining, sericeous basidiomata and *La. subvolvatus* has stout, fleshy basidiomata with a white to cream pileus, and roundish stipe base that is somewhat volvate (Candusso and Lanzoni 1990; Bon 1996). *Leucoagaricus subcrystallifer* is a white species with greenish-gray to yellow gray cutis with olive gray to dark gray umbo. It also has larger basidiospores, $7.5\text{--}8.5 \times 5\text{--}5.5 \mu\text{m}$ (Ge et al. 2015).

Leucoagaricus umbonatus Hussain, Ahmad & Afshan, sp. nov.

FIGS. 3E, 4Q–T, 5E.

MycoBank: MB815377

Diagnosis: Basidiomata white, medium-sized; pileus applanate to convex, cutis of white fibrils radially arranged, umbo conspicuous and yellowish-pink, with reflexed margin; stipe almost equal, annulus membranous, stipe with swollen base; basidiospores $5.5\text{--}6.5 \times 3.5\text{--}4 \mu\text{m}$, with ventricose cheilocystidia.

Typification: PAKISTAN. PUNJAB: Lahore, University of the Punjab, 217 m elev, 08 Sep 2014, S. Hussain SH-L1 (**holotype** LAH). GenBank: ITS = KU647737; 28S = KU900521.

Etymology: *umbonatus* (Latin), having a rounded projection or umbo.

Macroscopic characters: Pileus 20–30 mm diam, ovoid to ellipsoid when closed, expanding on opening, becoming plane to depressed, umbonate, with slightly reflexed margin, with white fibrillose squamules; umbo prominent, smooth, Light Yellowish Pink (2.5YR 9/4–7.5R 9/4) to Moderate Yellowish Pink (2.5YR 7/4–5YR 7/4). Lamellae free, crowded, white, 1–3 series of lamellulae with finely scalloped white edges. Stipe 30–50 × 1–1.5 mm, equal, smooth, white, shiny, hollow with slightly bulbous base up to 2 mm wide. Annulus membranous, white, ascending, persistent at the middle part of the stipe. Context white, no color change on handling, not tasted.

Microscopic characters: Basidiospores [50/2/3] (5–)5.5–6.5(–7) × (3–)3.5–4(–4.5) μm, (Q = 1.54–1.62, avQ = 1.59), amygdaliform to ellipsoidal in side view, ovoid in face view, thin-walled, smooth, without germ pore, dextrinoid, metachromatic in Cresyl blue. Basidia 18–20 × 7–9 μm, 4-spored, rarely 2-spored, hyaline. Cheilocystidia 24–29 × 8–12 μm, hyaline, rarely in clusters, mostly single, ventricose to fusiform. Pleurocystidia absent. Pileipellis a cutis, with radially arranged cells, terminal cells, 4–7 μm diam, hyaline. Clamp connections absent.

Habitat and distribution: Saprotrophic and solitary to scattered on loamy soil under planted trees of *Dalbergia sissoo*. So far only known from lowland northeastern Pakistan.

Additional specimen examined: PAKISTAN. PUNJAB: Lahore, University of the Punjab, 217 m alt, scattered in humus rich soil, 18 Sep 2014, S. Hussain SH-L8 (FH, LAH).

Notes: *Leucoagaricus umbonatus* is a white species characterized by medium-sized basidiomata, applanate to convex pileus, with radially arranged white fibrils, yellowish-pink umbo, with reflexed margin; stipe almost equal with swollen base, annulus white and membranous; basidiospores 5.5–6.5 × 3.5–4 μm, with ventricose cheilocystidia. Only two

markers where available for analysis because we were unable to amplify the *RPB2* and *TEF1- α* regions after several attempts.

On the basis of phylogenetic analyses, the species closely related to *La. umbonatus* are: *La. rubrobrunneus* E.F. Malysheva, T.Yu. Svetasheva & E.M. Bulakh, *La. proximus* E.F. Malysheva, T.Yu. Svetasheva & E.M. Bulakh and *La. lateritiopurpureus* (Lj.N. Vassiljeva) E.F. Malysheva, T.Yu. Svetasheva & Bulakh. and an undescribed species, *Leucoagaricus* sp. ecy2561 (GenBank AY176430).

The three colored taxa described from the Russian Far East, *La. rubrobrunneus*, *La. proximus* and *La. lateritiopurpureus*, have similarly shaped basidiospores (Malysheva et al. 2013). However, *La. rubrobrunneus* has a smaller pileus, 7–17 mm, with red brown to brick red squamules, without a distinct umbo and with incurved margins, with somewhat similar basidiospores, 5–6 \times 3.5–4.5 μ m, and *La. proximus* has pink brownish to bright orange-brown squamules, with darker red-brown umbo and larger basidiospores, 7–9 \times 3.5–4.5 μ m. *Leucoagaricus lateritiopurpureus* has reddish-brown squamules, dark reddish-brown umbo and broad, basidiospores 5.5–8 \times 3.0–4.5 μ m.

DISCUSSION

In this study, five new species of *Leucoagaricus* were studied in morphological and anatomical detail and sequences of four gene regions were analyzed. Morphological and anatomical data are useful to identify taxa at the genus level, but it is difficult to precisely identify a species in this difficult complex without molecular tools. Along with the traditionally employed fungal markers, nuc rDNA (ITS and 28S), we employed protein-coding genes *RPB2* and *TEF1- α* for this study. These protein-coding genes are useful in phylogenetic analyses because they are conserved at the amino acid level and are easy to align, and because primers sites have been

identified (Liu et al., 1999; Rehner and Buckley, 2005; Matheny et al. 2007). It is necessary to study the protein-coding genes along with the nuc rDNA regions for accurate characterization of lepiotaceous genera such as *Leucoagaricus*, *Leucocoprinus*, *Lepiota* and *Macrolepiota*.

From the present study we demonstrated that low altitude mountains and grasslands in this area of Pakistan are rich in species of *Leucoagaricus*. Of the 150 of the described species more than half have been published since the year 2000; of these, 28 are based on material from Asia. Further exploration of the diversity hotspots of Asia will certainly yield more new species. Further phylogenetic studies will also inform the generic circumscriptions within this clade.

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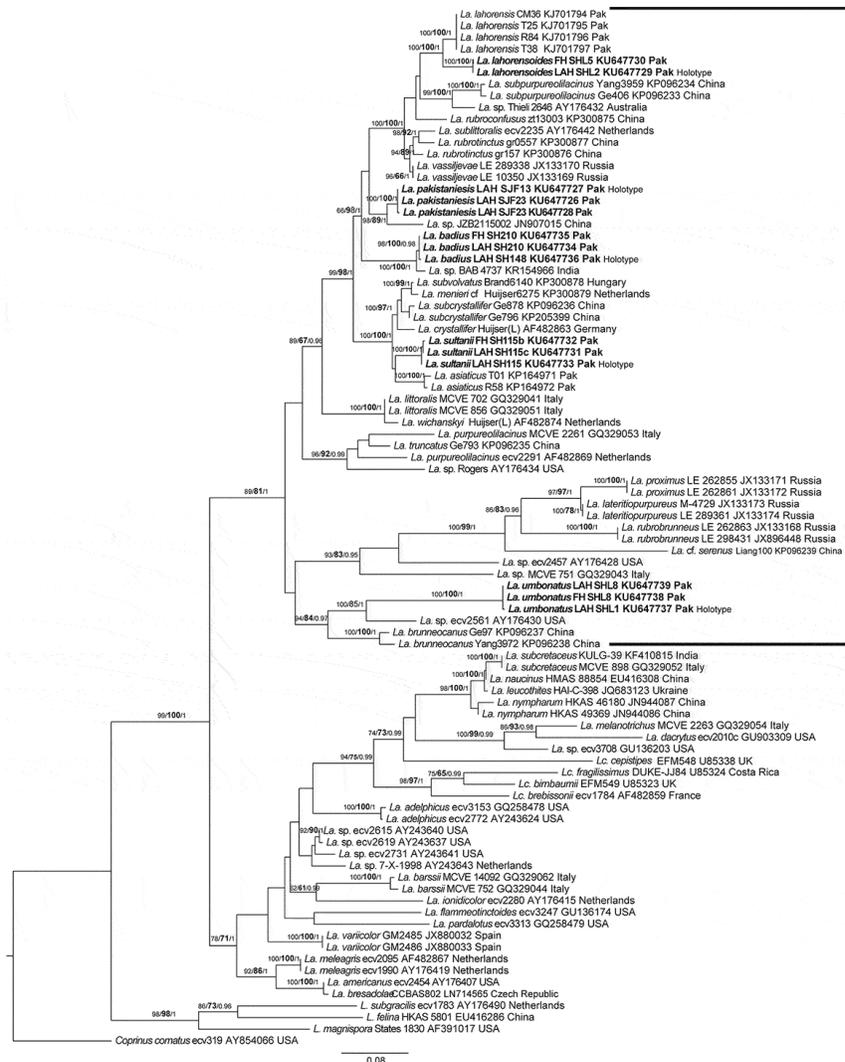
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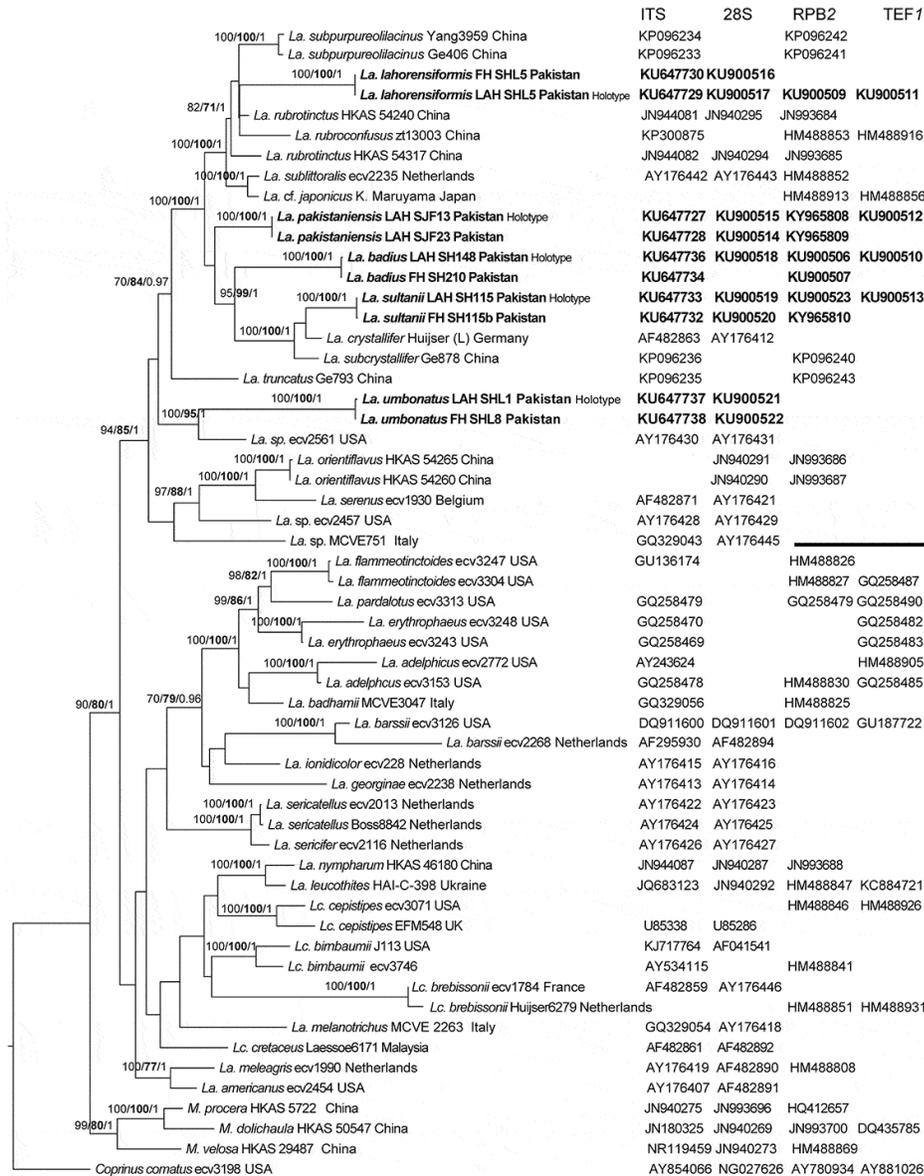
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Figure 1. ML Maximum likelihood phylogeny of *Leucoagaricus* based on ITS sequences, bootstrap support values ($\geq 70\%$ significant) above branches are the maximum likelihood bootstrap (MLB), maximum parsimony bootstrap (MPB) in boldface, and Bayesian posterior probabilities (PP; ≥ 0.95). The new species represented in bold fonts nest in a well-supported clade.



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Figure 2. Four-locus (ITS, 28S, *RPB2* and *TEF1- α*) maximum likelihood (ML) phylogeny, bootstrap support values ($\geq 70\%$) are presented in order MLB, MPB in boldface (based on 1000 bootstrap replicates, respectively) and Bayesian posterior probabilities (PP) ≥ 0.95 .



Rubroticti

Figure 3. Basidiomata of species of *Leucoagaricus*. A, B. *Leucoagaricus badius* (holotype). C. *Leucoagaricus pakistaniensis* (holotype). D. *Leucoagaricus lahorensiformis* (LAH SHL2). E. *Leucoagaricus umbonatus* (holotype). F. *Leucoagaricus sultanii* (holotype).

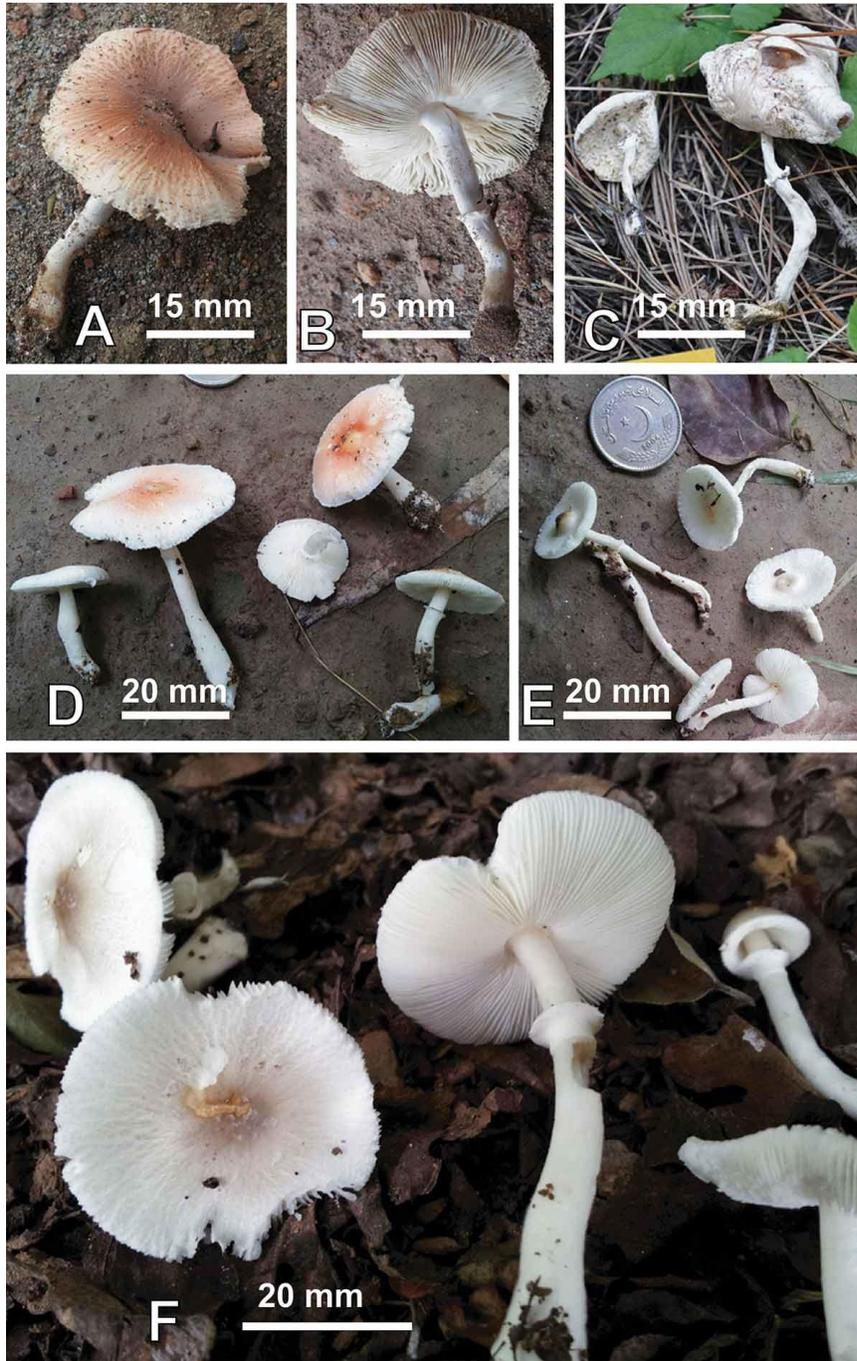


Figure 4. Anatomical features of species of *Leucoagaricus*. A–D *Leucoagaricus badius* (LAH SH148; FH 210). A. Basidiospore. B. Basidia. C. Cheilocystidia. D. Pileipellis. E–H *Leucoagaricus lahorensiformis* (LAH SHL2). E. Basidiospores. F. Basidia. G. Cheilocystidia. H. Pileipellis. I–L *Leucoagaricus pakistaniensis* (LAH SJF13). I. Basidiospores. J. Basidia. K. Cheilocystidia. L. Pileipellis. M–P *Leucoagaricus sultanii* (LAH SH115; FH SH115c). M. Spores. N. Basidia. O. Cheilocystidia. P. Pileipellis. Q–T *Leucoagaricus umbonatus* (LAH SHL1; FH SHL8). Q. Basidiospore. R. Basidia. S. Cheilocystidia. T. Pileipellis. Bars: A, E, I, M, Q = 5 μm ; B–E, F–H, J–L, N–P, R–T = 10 μm .

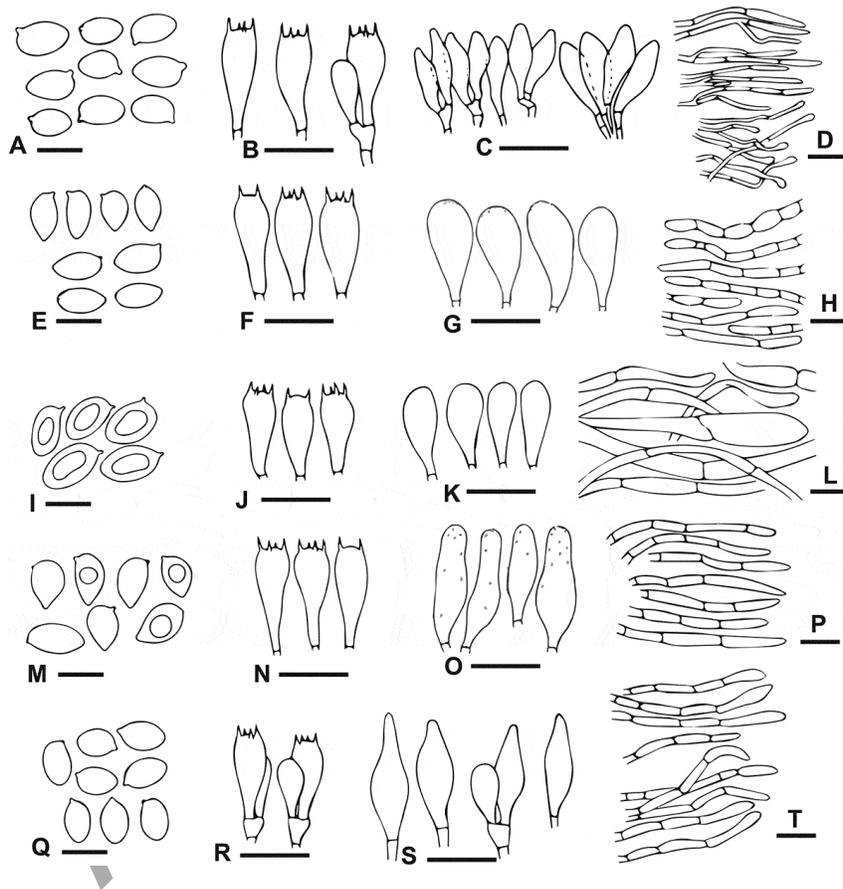
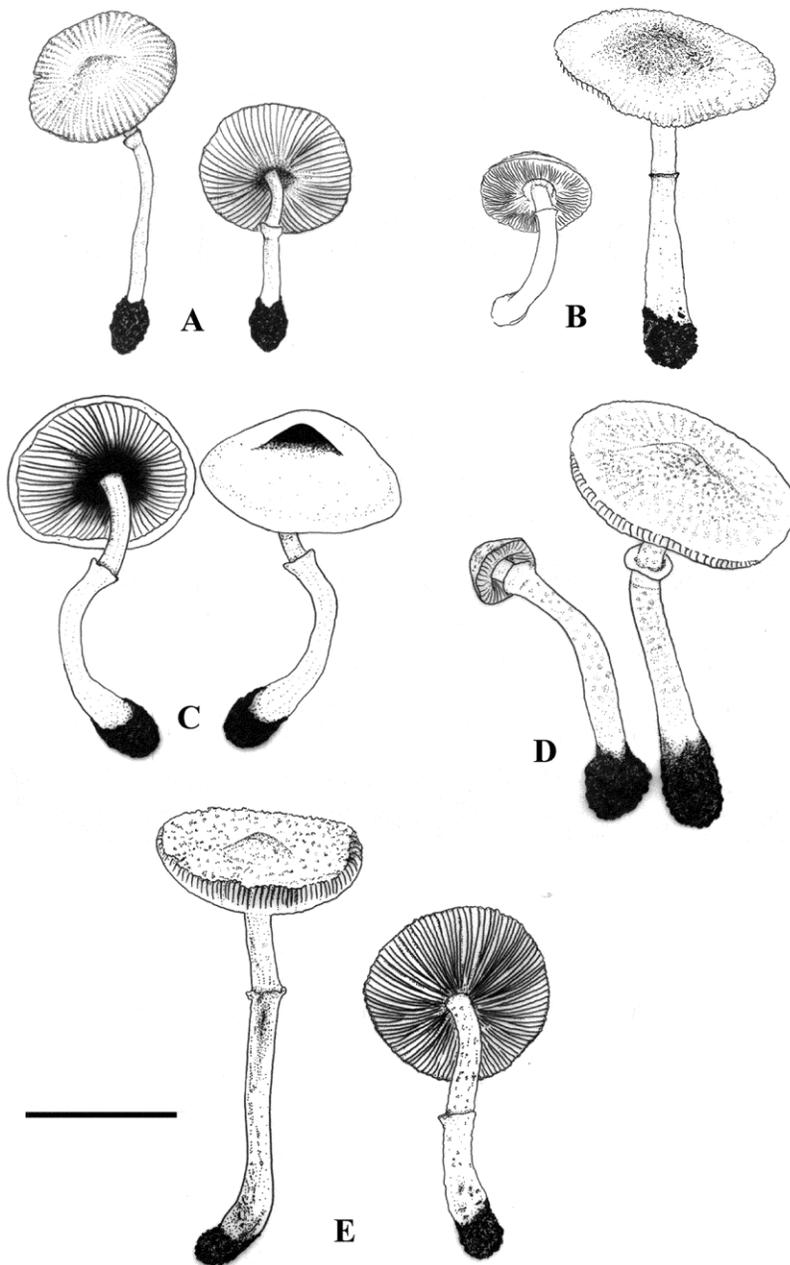


Figure 5. Line drawings of species of *Leucoagaricus*. A. *Leucoagaricus badius*. B. *Leucoagaricus lahorensiformis*. C. *Leucoagaricus pakistaniensis*. D. *Leucoagaricus sultanii*. E. *Leucoagaricus umbonatus*.

Bar: 20 mm.



FOOTNOTES

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Table 1. Sequences generated during this study. All specimens were collected in Pakistan.

Species	Herbarium voucher	GenBank			
		ITS	LSU	<i>RPB2</i>	<i>TEF1-α</i>
<i>Leucoagaricus badius</i>	LAH SH 148 Holotype	KU647736	KU900518	KU900506	KU900510
	LAH SH 210	KU647734		KU900507	
	FH SH 210	KU647735		KU900508	
<i>La. lahorensiformis</i>	LAH SH L2 Holotype	KU647729	KU900517	KU900509	KU900511
	FH SHL 5	KU647730	KU900516		
<i>La. pakistaniensis</i>	LAH SJF13 Holotype	KU647727	KU900515	KY965808	KU900512
	LAH SJF23	KU647728	KU900514	KY965809	
	LAH SJF23	KU647726			
<i>La. sultanii</i>	LAH SH 115 Holotype	KU647733	KU900519	KU900523	KU900513
	FH SH 115b	KU647732	KU900520	KY965810	
	LAH SH 115c	KU647731		KY965811	
<i>La. umbonatus</i>	LAH SH L1 Holotype	KU647737	KU900521		
	FH SH L8	KU647738	KU900522		
	LAH SH L8	KU647739			