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Leucocoprinus Flavoaurantiacus (Agaricaceae), a New Species from Buner District Pakistan

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ABSTRACT

A new species *Leucocoprinus flavoaurantiacus* is collected and described from Buner District, Pakistan, based on morphological characteristics and molecular evidence. The characteristics of fresh basidiomata and phylogenetic placement using the rDNA (ITS) barcode region confirm the new species. Description with comprehensive discussions, illustrations, and phylogenetic analysis results are provided for the novel species.

Keywords

Basidiomycota
ITS
Molecular phylogeny
Taxonomy

INTRODUCTION

The genus *Leucocoprinus* Pat. is a genus of lepiotaceous fungi within the Agaricaceae Chevall., and is characterized by the combination of white dextrinoid, metachromatic basidiospores, and absence of clamp connections [1,2]. The members of this genus are saprotrophic in nature, widespread in the tropics and subtropics and also found growing in potting soils, greenhouses on rich organic matter [2,1,3].

The genus *Leucocoprinus* was traditionally restricted to species with a plicate pileus, dextrinoid, metachromatic spores with a germ pore, and the absence of clamp connections (e.g., Singer 1986). The genus *Leucoagaricus* Locq. ex-Singer could be distinguished from *Leucocoprinus* by the non-plicate pileus [4], whereas the spores in general lack a germ pore, but several species, such as *La. leucothites* (Vittad.) Wasser do have one. However, morphologically the distinctions between the two genera are not very sharp, as species, such as *Lc. heinemannii* Migl. and *Lc. straminellus* (Bagl.) Narducci & Caroti with a plicate pileus but lacking a germ pore in the spores, were described in *Leucocoprinus*, and species in the *Lc. badhamii* group were moved back and forth between the two genera [2].

Molecular phylogenetic and taxonomic studies so far have revealed 27 species of *Leucoagaricus* and five species of *Leucocoprinus* from Pakistan [1]. There is a high myco-diversity found in the northern area of Pakistan especially district Buner due to availability of litter and favorable climatic conditions. Species identification based on morphological methods is not reliable, as some poisonous species are

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confused with edible species. Recently, several individuals have lost their lives mistakenly eating poisonous mushrooms which are morphologically similar to edible mushrooms. The present study focuses on the morphological and molecular characterization of a *Leucocoprinus* species collected in a conifer forest in Buner District, Khyber Pakhtunkhwa, Pakistan. This region is known for its high biodiversity, with only five taxa of fungi reported to date [5].

MATERIALS AND METHODS

Collection and morphological studies

Specimens were collected from 2018 to 2020 in the mountains of Buner District, Khyber Pakhtunkhwa Province, Pakistan. Basidiomata were dug out using a sharp knife and photographed in their natural habitat. Pileus diameter was measured along with observation of the pileus surface [6]. The Munsell Color System (1975) [7] was followed for colors. The basidiomata were dried with the help of a fan heater at 30–40°C [8]. For microscopic studies, tissues of pileus, gills, annulus, and stipes were mounted in 5% KOH and stained with 1% aqueous Congo red (w/v). They were observed under a light microscope (LABOMED, Labo America, Inc. USA). Measurements of the microscopic structures are based on calibrated images in Piximètre software (http:// www.piximetre.fr/). Melzer's reagent was used to check the amyloid nature of basidiospores. The abbreviation [n/b/p] indicates the 'n' number of basidiospores measured from the 'b' number of basidiomata from the 'p' number of collections. For basidiospores, L is the average length of the measured basidiospores. Similarly, W is the average width of the measured basidiospores, Q is the range of the L/W ratio of all the measured basidiospores, and Qav is the average L/W ratio of all measured basidiospores. The shape of the basidiospores is quoted according to [9]. Measurements of other microscopic features, like basidia (without sterigmata), contain a range between the extreme values calculated in length and width. The collections are vouchered and deposited in the Lahore Herbarium (LAH), Department of Botany, University of Punjab, Quaid-e-Azam Campus, Lahore, Pakistan, and the Herbarium of Department of Botany (HUP), Hazara University Mansehra, KP, Pakistan.

DNA extraction, PCR amplification, and sequencing

A modified CTAB method was used for DNA extraction, following [10]. Amplification of the nuclear ribosomal internal transcribed spacer (ITS) locus was done with the fungal-specific ITS1F primer (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes & Bruns 1993) and the eukaryotic ITS4 primer (5′-TCCTCCGCTTATTGATATGC-3′) [11]. PCR procedure for ITS region had an initial 4 min denaturation at 94°C, 40 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and a final extension of 10 min at 72°C. PCR products were visualized using 1 % agarose gel stained with 3 µl ethidium bromide. The sequencing of the amplified products (ITS) was done from Macrogen (38, Teheran-ro, Gangnam-gu, Seoul, Republic of Korea).

Phylogenetic analysis

The newly generated ITS sequences were BLAST searched against the NCBI GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and closely related sequences were retrieved from GenBank. Additional sequences used in the ITS analysis were taken from recent molecular studies [1]. Sequences were assembled and aligned in BioEdit [12] and ClustalW [13] All aligned ITS sequences were trimmed with conserved motifs 5′– (…GAT) CATTA–and –GACCT (CAAA…)–3′ [14].

A maximum likelihood tree was inferred for the ITS data set using RAxML-HPC2 v.8.1.11 [15] with a GTRCAT model of nucleotide substitution. Rapid bootstrapping was performed with 1000 bootstrap iterations. A bootstrap proportion of ≥50 was considered significant. The phylogenetic analysis was performed on the CIPRES Science Gateway v.3.1 [16]. The phylogenetic tree from ML and analyses was visualized using FigTree v.1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and then exported to Adobe Illustrator for editing.

RESULT

Phylogenetic analysis

The ITS sequences obtained from Pakistani collections were subjected to BLAST searches. The novel taxon *Lc. flavoaurantiacus* showed 98% identity with *Leucocoprinus rubrobrunneus* E.F. Malysheva, Svetash. & Bulakh sequences (MG719773, OM974303) from China.

Our final ITS dataset is composed of 200 sequences including two newly generated sequences of our new species and outgroup taxon *Agaricus bisporus* (J.E. Lange) Imbach. The resulting alignment was 963 bp in length including gaps. In the ITS phylogram, the Pakistani species, two new sequences of *Lc. flavoaurantiacus* formed a well-supported monophyletic clade, sister to *Lc. rubrobrunneus* with 100 bootstrap support (Figure 1).

Taxonomy

Leucocoprinus flavoaurantiacus M. Ishaq, M. Fiaz & Khalid, **sp. nov**.

MycoBank No. MB856736

Figure 1: Phylogenetic tree of *Leucocoprinus flavoaurantiacus* based on Maximum-likelihood analysis of ITS region. Maximum likelihood (ML) bootstrap value of ≥ 50 are shown for each node above the branch leading to that node. The newly described species is indicated in bold and different color.

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Etymology: The "*flavoaurantiacus*" refer to yellow-orange pileus color.

Holotype: PAKISTAN, Khyber Pakhtunkhwa Province, Buner District, Chagharzai, 1526 m asl, under *Pinus roxburghii* Sarg., 5 August 2017, Muhammad Ishaq HUP-11545.

Diagnosis: Pileus pilose-fibrillose squamules, yellow-orange disc, becoming light yellow-orange to yellow-white toward margin, radially arranged and dense at center, white background, lamellae white to pale cream, close to crowded, free, broadly ellipsoid to amygdaliform, smooth, thin-walled, dextrinoid, apiculated basidiospores.

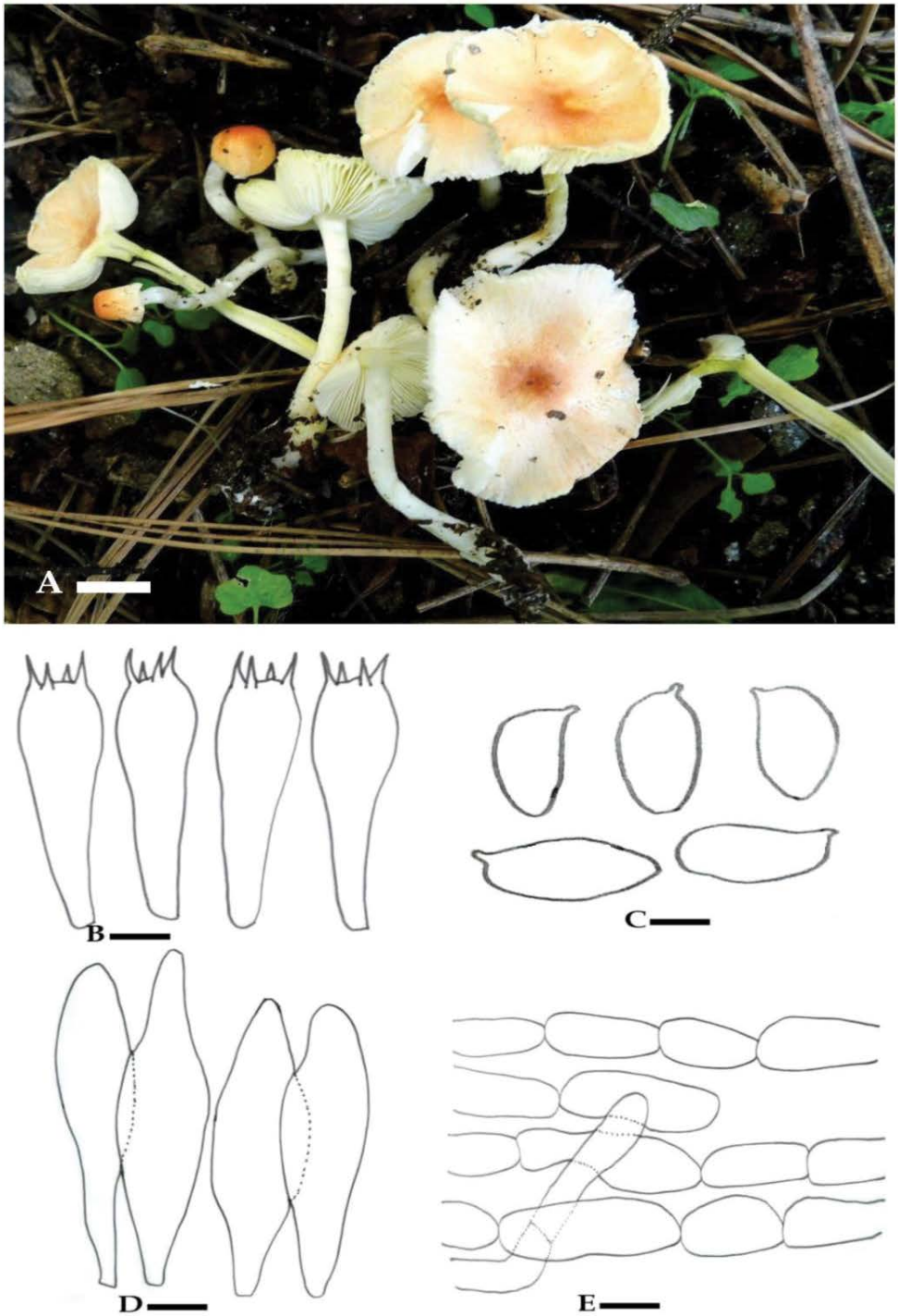

Figure 2: A–E: Morpho-anatomical features of *Leucoagaricus flavoaurantiacus* HUP-11545 (holotype) A Basidiocarps, B. Basidia, C. Basidiospores, D. Cheilocystidia, E. Pileipillis Scale bars: A= 8mm, B = 5µm, C = 4µm, D = 8µm, E = 5µm.

Description. Basidiomata medium size. Pileus 6-25 mm diam, parabolic initially becoming plano-convex to saucer to revolute at maturity, pilose-fibrillose squamules, yellowish orange (7.5YR 7/8,8/8) disc, becoming light yelloworange (7.5YR 8/4, 8/6) to yellow-white (5Y 9/4) toward margin, radially arranged and dense at center, white (2.5YR 9/2) background, surface dry, matt, margin entire, incurved at young stage become uplifted upon maturity. Lamellae white (2.5YR 9/2) to light cream (5Y 9/6), close to crowded, free, ventricose, unequal, margin entire. Stipe 25−45 × 1−3 mm, central, flexuous, wide at the base becoming slightly tapered toward apex, with bulbous base 8 mm wide, hollow, slightly longitudinally fibrillose, white. Annulus present, membranous, middle or near to apex, membranous, narrow, white. Context becoming pale yellow when cut.

Basidiospores [40/2/1] (4.6−) 5.6−7.6 (−7.8) × (3.5−) 3.7−4.2 (−4.3) µm, L × W = 6.8 × 4µm, Q =(1.2−) 1.3−1.9(−2), Qe = 1.7, broadly ellipsoid to amygdaliform, hyaline, smooth, attenuate wall, dextrinoid, apiculated, oily content present. Basidia 4-spored, 9.5−11.3 × 5.2−6.5 µm, broadly clavate to clavate. Cheilocystidia 27.5−32 × 11−13.5 µm, lagniform with short nick, obtuse apex, hyaline, clavate to fusiform, attenuate walled. Pleurocystidia absent. Pileipellis 5−7 µm diam, cylindrical to sub-cylindrical. Clamp connection absent.

Habitat: Saprotrophic

Additional material examined.: Pakistan, Province Khyber Pakhtunkhwa, Malakand, Buner, Chagharzai, 1430 m asl, under *Pinus roxburghii* Sarg., August 7, 2018, M. Ishaq (IB-07).

DISCUSSION

Phylogenetic analysis, based on the ITS dataset, revealed that sequences of Pakistani species *Leucocoprinus flavoaurantiacus* sister to *Lc. rubrobrunneus* and *Lc. bulbiger* (Figure 1 and 2). The novel species *Leucocoprinus flavoaurantiacus* is significantly different from the closely related taxa based on micro-morphological characters and molecular analysis. Pileal squamules of *Lc. flavoaurantiacus* is yellow-orange at disc, becoming light yellow-orange to yellow-white toward edges with uplifted margin, whereas the closely related species *Leucocoprinus rubrobrunneus* the squamules is red-brown or brick red with incurved margin. It has lamellae pale cream to yellowish but white to pale cream in *La. flavoaurantiacus*. *Leucocoprinus rubrobrunneus* has smaller basidiospores 5−5.7 × 3.2−4.3 µm, large basidia 18−30 × 6.5−8 µm and cheilocystidia 30−40 × 9.5−13 µm [17]. Another taxon *Leucocoprinus bulbiger* has pileus 23−35 mm yellow-orange or apricot- orange scales, paler and sparser toward the margin, having distant, moderately crowded lamellae and 25−45 × 1−3 mm stipe, which differs from *Lc. flavoaurantiacus* having pileus 6-25 mm diam, yellowish orange disc, becoming light yellow-orange to yellow-white toward margin and white to light creamy close to crowded lamellae. Furthermore it has larger basidiospores 7.5−11 × 4−5.5 and basidia 14.5−22 × 7−9.5 µm. [17]. *Leucocoprinus aurantioruber* pileus nearly hemispherical, umbonate, orange–reddish to orange–brown, covered with minute brown to light orange–reddish radially fibrillose squamules and white context and lamlae. Furthermore it has large basidia, 16.5–22.0 × 8.0–11.0 µm and Cheilocystidia, 34.5–41.0 × 10.0–14.5 µm, and basidiospores 6.0–7.5(8.0) × (3.5) 4.0–5.0µm, ellipsoid to ovoid. In contrast, *Leucocoprinus flavoaurantiacus* has pileus parabolic initially becoming plano-convex to saucer to revolute, pilose-fibrillose squamules have yellowish orange at the disc becoming light yellow–orange to yellow-white. Furthermore it has small basidia 9.5−11.3 × 5.2−6.5 µm, Cheilocystidia 27.5−32 × 11−13.5 µm and basidiospores (4.6−)5.6−7.6(−7.8) × (3.5−)3.7−4.2(−4.3) µm which is broadly ellipsoid to amygdaliform. [19-45].

CONCLUSION

Hence, from all the discussion and comparisons with the closely allied species, it is concluded that our proposed new species, *Lc. flavoaurantiacus* has unique morpho-anatomical characteristics along with DNA profile which make its different from all the previously known species of this genus.

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