

A new species of Genus *Limacella* from Changa Manga Forest, Pakistan

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Abstract

During a study of the genus *Leucoagaricus* in Pakistan, we collected a putatively new species with a distinct morphology, anatomy and ITS nrDNA profile from Changa Manga, Punjab. The new species is characterized by the presence of reddish brown central squamules on a milk-white background, thick-walled and ellipsoid spores, narrowly sub-cylindrical to subclavate cheilocystidia and cylindrical stipe. A full description, color photographs, line drawings and a phylogenetic tree of the new species are provided.

Introduction

The Changa Manga Forest is the largest artificial (manmade) forest and lies in the subtropical area of Pakistan and favors the growth of fungi. Previously 118 species of Basidiomycota belonging to 62 genera and 22 families have been identified from this forest reserve (Niazi et al. 2015). Lepiotaceous fungi occur widely in Punjab at lower elevations. Previously only fifteen *Leucoagaricus* species have been reported from Pakistan (Aman *et al.* 2022), out of which only four species are known from Changa Manga i.e. *Leucoagaricus crystallifer* Vellinga (2000), *Leucoagaricus lahorensis* Qasim, T. Amir & Nawaz (2015), *Leucoagaricus leucothites* (Vittad.) Wasser (1977) and *Leucoagaricus nivalis* (W.F. Chiu) Z.W. Ge & Zhu L. Yang (2017).

Around 135 species are known (He et al. 2019), which are characterized by the white to cream spore print, entire or slightly striate but not plicate pileus margin, free lamellae, metachromatic basidiospores in Cresyl blue, and absence of clamp connections and pseudoparaphyses (Singer 1948 & 1986).

Leucoagaricus is placed in Agaricaceae based on its agaricoid habit and basidiospore morphology (Singer, 1948 & 1986). Molecular studies show that the genus may not be monophyletic (Johnson & Vilgalys 1998, Johnson 1999, Vellinga 2004, Vellinga *et al.* 2011).

This research is an effort to establish the fungal diversity of this forest. During a field survey in 2020–2021 for the collection of macrofungi to explore the diversity of these fungi from Changa Manga, several basidiomata of *Leucoagaricus* were collected.

Material And Methods

Morphological characters

Specimens were obtained from Changa Manga Forest during the monsoon season. Samples were photographed in natural sunlight and vouchered. Specimens examined were deposited in the Herbarium of the University of the Punjab, Lahore, Pakistan (LAH), respectively. Macroscopic characters were recorded based on fresh material given the color codes following the Munsell color chart (1975).

Microscopic characters

These were observed from material rehydrated in 5% aqueous KOH (w/v). Pileal structure, cheilocystidia and basidia were observed and measured in 5% KOH, with 0.5% aqueous Congo Red (w/v). Melzer's reagent was used to test the amyloidity of spores. Spores and basidia were taken from lamella fragments of mature basidiomata. Basidium measurements do not include sterigmata. At least 20 spores were measured in side view for each mature basidioma. The following abbreviations (n/m/p) indicate that measurements were made on n basidiospores in m basidiocarps out of p collections (Liang *et al.* 2011). Dimensions for basidiospores are reported as (a–) b–c (–d). The range b–c contains a minimum of 90% of the measured values. Extreme values (a and d) are in parentheses. The mean value for spore length and width, the Q value (ratio of the mean spore lengths divided by the mean spore widths), and the average Q (avQ) of all basidiospores \pm sample standard deviation were calculated.

DNA extraction, PCR and sequencing

Genomic DNAs were isolated from pieces of lamellae following a modified CTAB method (Doyle and Doyle 1987). PCR was performed using primer sets ITS1F / ITS4 (Gardes & Bruns 1993, Liu *et al.* 1999) to amplify the nuc rDNA ITS1-5.8S-ITS2 region (ITS). PCR reaction parameters, sequencing reactions and methods for editing the sequences are the same as described in Ge *et al.* (2015).

Phylogenetic analyses

The initial BLAST results revealed that the most similar sequences to the novel taxa are sequences of species within *Leucoagaricus* sect. *Rubrotincti*; thus ITS sequences from species within groups were included in the phylogenetic analysis. Sequences were assembled using BioEdit (Hall 1999). BLAST analysis was used to retrieve highly similar sequences of the ITS region and multiple sequence alignment was performed with Mafft 6.8 (Kato & Ton 2008), followed by minor improvements. *Lepiota vellingana* (OM987454, AY176485) was designated as an outgroup for the dataset. On the CIPRES Portal (Miller *et al.* 2010), the HYK + G + I model was selected using jModelTest (Posada 2008). Using RAXML-HPC2 v. 8.1.11 on CIPRES, maximum likelihood analysis was also implemented (Stamatakis 2014), using 1000 bootstraps for rapid bootstrap option performed to assess the bootstrap support (BS) for branches. FigTree v 1.4.3 (Rambaut *et al.* 2014) was used for displaying phylogeny from ML analysis.

Results

Molecular and Phylogenetic Analysis

ITS sequences of the holotype of the new species and the other collection, CHM-14, were identical. The initial BLAST result showed 95.20% similarity with *Leucoagaricus rubrotinctus* (JX827166). The final dataset of ITS consisted of 37 sequences including *Pluteus salicinus* (HM562174, HM562051) as an outgroup (Table 1). The aligned ITS1-5.8S-ITS2 region comprised 776 sites, of which 358 were conserved and 408 variable; 340 sites were parsimony-informative. The phylogenetic analysis showed that *Leucoagaricus changamangaensis* clusters with the sequences of *Leucoagaricus* belonging to section *Rubrotinctii*. In the analysis of the ITS region, the Pakistan sequences clustered with already

reported species from Pakistan (KJ701794, KJ701795), from China (JX827166, JX481050, MW554226), from USA (JN944081, AY176442), and from Russia (JX133167). The phylogenetic tree showed that the two newly generated sequences formed a distinct lineage with high bootstrap support 100. The new species was sister to [GT1] *Leucoagaricus lahorensis* Qasim, T. Amir & Nawaz (KJ701794 and KJ701795), *Leucoagaricus rubrotinctus* (Peck) Singer (JX827166, FJ481050, JN944081, and JX133167), *Leucoagaricus sublittoralis* (Kühner ex Hora) Singer (AY176442) and *Leucoagaricus sp* (MW554226) with a high bootstrap value of 100. All these *leucoagaricus* formed a strongly supported clade with *L. vassiljevae* (JX133170, JX133169, and JX896447).

Taxonomy

Leucoagaricus changamangaensis* Hajra, Thorn & Niazi *sp. nov. (Figures 1, 2).

Mycobank #

Holotype:—PAKISTAN. Punjab: Kasur district, Changa Manga, on humus-rich soil, 192 m a. s. l., gregarious, 02 September 2020, A. R. Niazi & Hajra (CM-14, LAH#02920). GenBank OP905641 (ITS).

Etymology:—The specific epithet '*changamangaensis* refers locality from where the holotype is collected.

Diagnosis:—Differs from *Leucoagaricus rubrotinctus* by having larger, broadly ellipsoid spores with a thin wall; narrowly clavate cheilocystidia and cylindrical stipe.

Pileus 2–3 cm in diameter, plane and flat, with straight margins at maturity; central disc obtuse, red to reddish brown (10 R 4/4) in the center; surface squamulose, with radially arranged reddish brown fibrils, becoming sparse and light brown (7.5R5/16) towards margins over a milk white background; context white, fleshy; margins entire, with cracks developing at maturity. Lamellae free, close, thin, membranous, white to cream (5YR9/2), edges entire; lamellulae in 2–5 tiers. Stipe 3.2–4.1 × 0.3–0.5, central, equal to sub-equal, cylindrical, slightly attenuating towards pileus, with a shiny white surface (5YR9/2), hollow, smooth; base same as stipe. Annulus present, membranous, white to cream, ascending, superior. Volva absent. Taste and odor not recorded.

Basidiospores [20/7/2], (3.7–) 4.4–7.7 (7.1) × (6.5–) 2.1–5.1 (4.6) μm, ellipsoid to somewhat ellipsoid, thin-walled, apex with concentric germ pore, smooth, guttulate. Basidia 19–34 × 6.5–8.5 μm, clavate, thin-walled, hyaline in KOH, guttulate, smooth, 2–4-spored. Cheilocystidia 14–28 × 5.5–7.5 μm, narrowly clavate, thin-walled, hyaline in KOH, smooth. Pileipellis thin-walled, hyaline to light brown in KOH, septate, unbranched; clamp connections absent. Stipitipellis hyphae 1.4–7.6 μm wide, cutis made of hyphae, smooth, thin-walled, hyaline, arranged parallel, with ascending, broadly clavate. Clamp connections are absent in all tissues.

Additional Material Examined:—PAKISTAN. Punjab: Kasur district, Changa Manga, 192 m a. s. l., on the ground, decomposed organic matter, under hardwood trees, 15 September 2021, A. R. Niazi & Hajra (CHM-

14, LAH#15921). GenBank OP905635 (ITS).

[GT1]Not quite correct: sister to L.l. and 3 seqs of L.r.; these and one other seq identified as L.r. a d on as L.sp formed a strongly supported clade with L.v.

Discussion

Basidiomata of *Leucoagaricus changamangaensis* were collected twice from Changa Manga, district Kasur. Preliminary morphological analysis suggested that all collections represented the same species, which was confirmed by molecular analyses. The ITS-nrDNA based phylogenetic tree also supports *L. changamangaensis* as separate taxa from all other *Leucoagaricus* species for which ITS data are available in Genbank. Our ML phylogenetic tree revealed *Leucoagaricus* sect. *Rubrotincti* agrees with those recognized by Singer (1975).

All species in *Leucoagaricus* sect. *Rubrotincti* is characterized by a reddish-brown pileus covering, ellipsoid spores, tetrasporic basidia, no color change upon bruising, and a negative ammonia reaction (Singer 1948 & 1986, Bon 1993, Vellinga 2001). All *Leucoagaricus changamangaensis* sequences clustered together in the *L. sect. Rubrotincti* with high bootstrap support. *Leucoagaricus sp.* forms a sister clade with Chinese collections tentatively called *L. rubrotinctus*. The morphology of these Chinese collections is not known; *L. rubrotinctus* (Peck) Singer is an eastern North American species with an orange-red radially fibrillose pileus, basidiospores are non-amygdaliform (Vellinga 2001). *Leucoagaricus changamangaensis* differs from *L. lahorensis* due to the presence of ellipsoid, thin-walled, apex with germ pore and guttulate spores; while in *L. lahorensis* ellipsoid to broadly ellipsoid and thin-walled spores. *L. changamangaensis* resembles *L. lahorensis* in reddish brown color with squamulose, cracks developing at maturity, 3–4-spored basidia and clamp connections are absent.

Leucoagaricus sublittoralis (Kühner ex Hora) Singer shows similarity with the *L. changamangaensis* in its pinkish brown pileus with white background, 4-spored basidia, and dextrinoid spores, but broadly ellipsoid spores and cheilocystidia without crystals in *L. sublittoralis* renders it a different taxon. Furthermore, *L. sublittoralis* is found in clay, loamy soils (Vellinga 2001), while *Leucoagaricus changamangaensis* is found on humus rich soil.

Leucoagaricus vassiljevae is recognized by the medium to large size of basidiocarps, bright-coloured squamulose pileus with reddish tinges, and microscopically by the relatively large oblong-amygdaliform spores and clavate to subutriform cheilocystidia. Nevertheless *L. changamangaensis* is distinguished from *L. sublittoralis* by the medium-sized and reddish brown basidiome, broad ellipsoid basidiospores and narrowly clavate cheilocystidia.

Declarations

Acknowledgments

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Figures

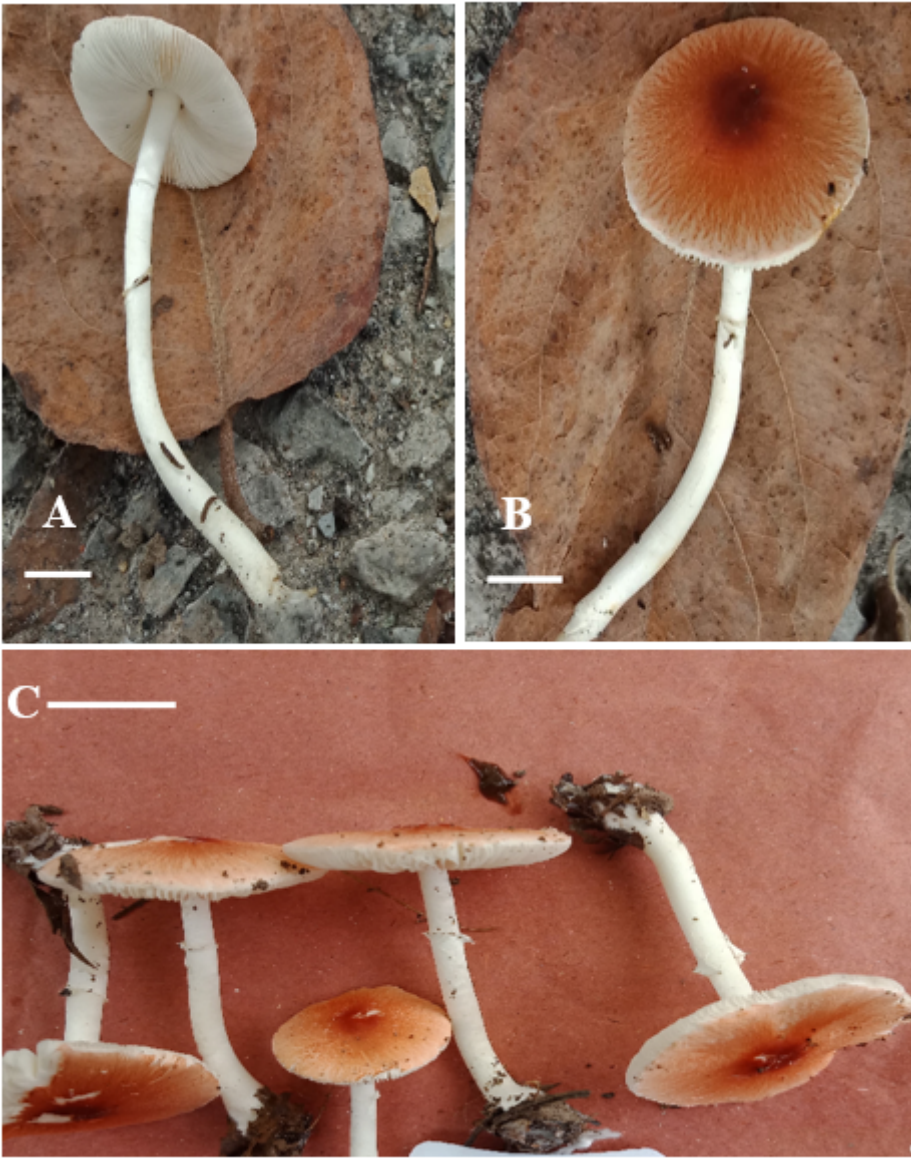


Figure 1

A-C Basidiomata of *Leucoagaricus changamangaensis* from Changa manga (Scale bar A = 0.6 cm, B = 0.8 cm & C = 1.5 cm)

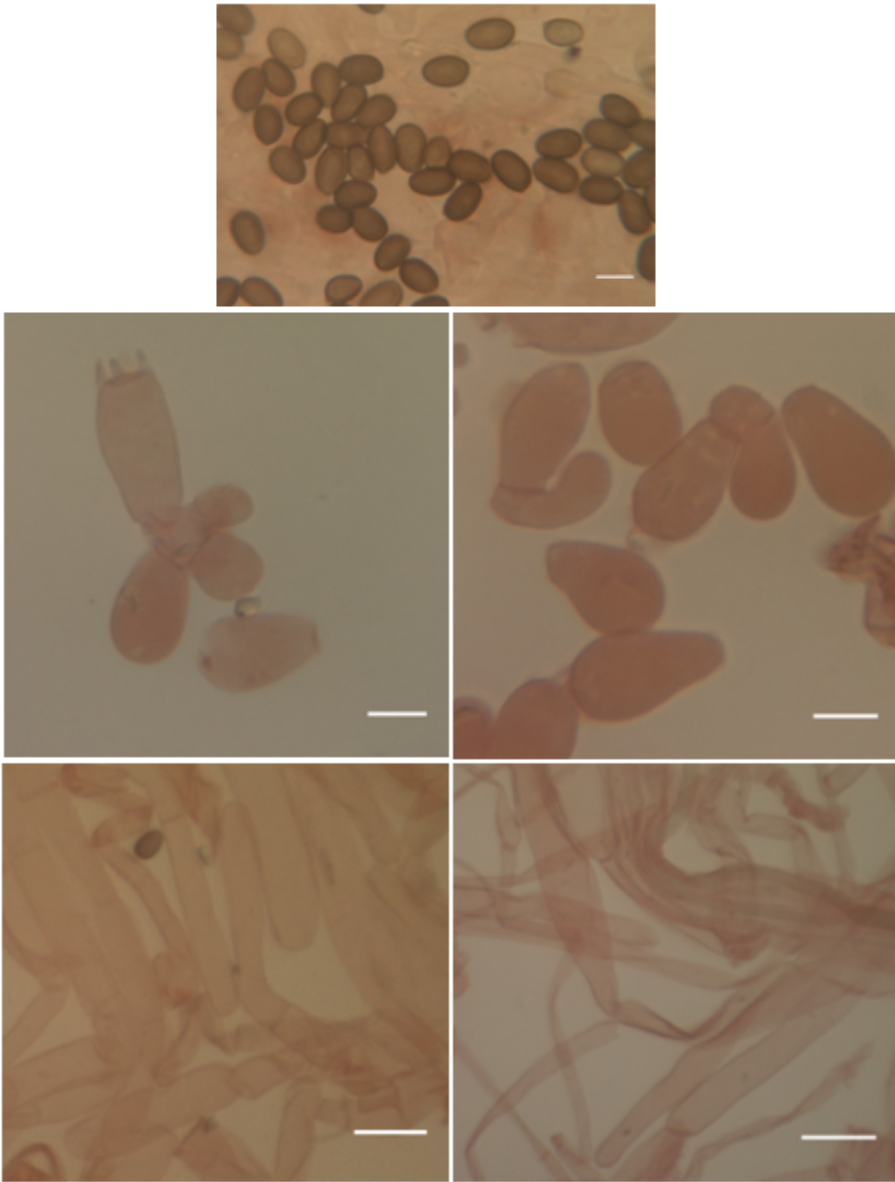


Figure 2

Microscopic characteristics of *Leucoagaricus changamangaensis*. A. Basidiospores in Gills, B-E, features in Basidiomata. B. Basidia C. Cystidia, D. Pileipellis, E. Stiptipellis (Scale bars A= 8 μm , B= 9 μm , C = 10 μm , D= 8 μm , E= 12 μm).

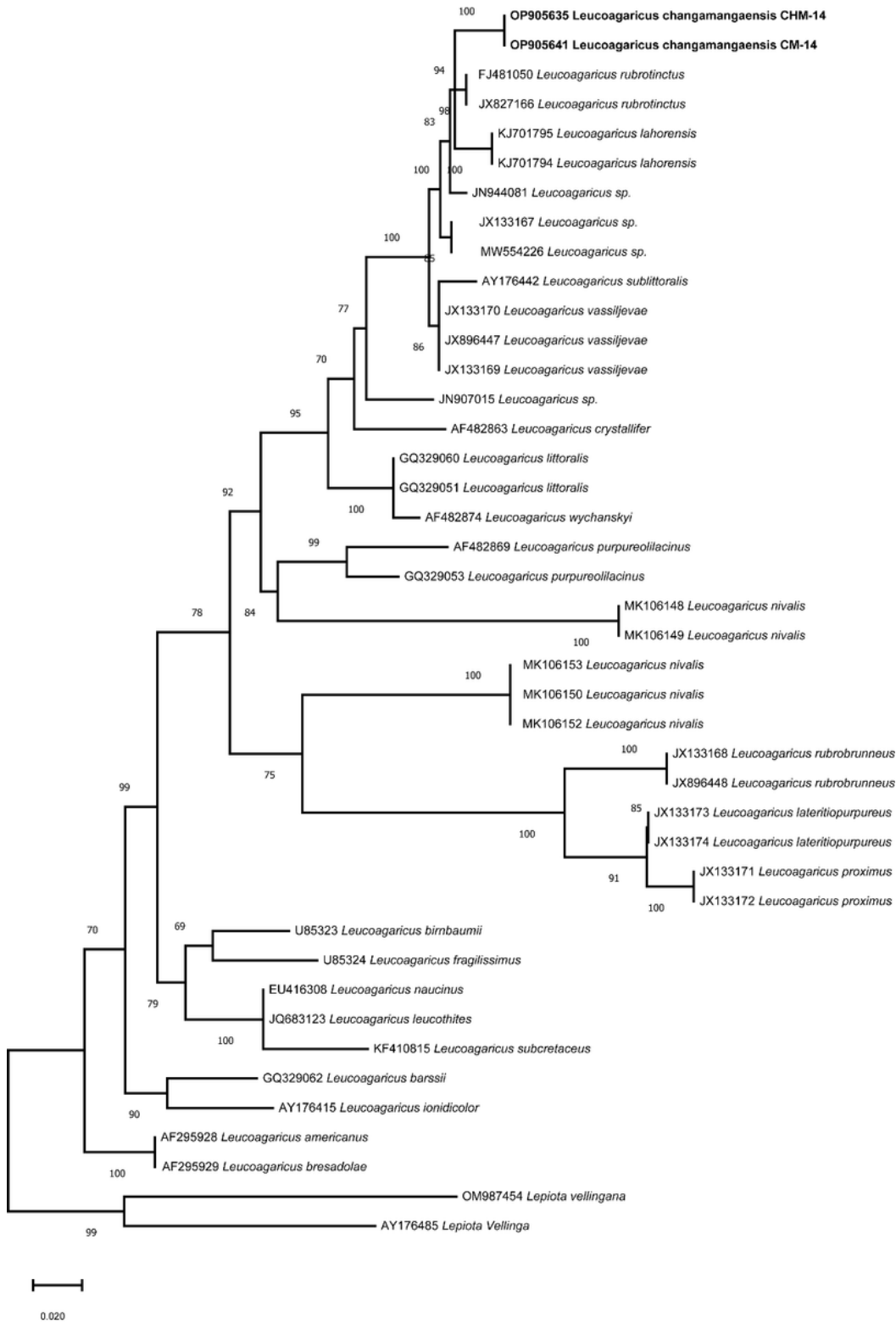


Figure 3

FIGURE 1. Maximum Likelihood phylogram representing the relationships between the new species *Leucoagaricus changamangaensis* and related *Leucoagaricus*. The tree was inferred using ITS sequence data. Bootstrap support values are shown adjacent to each node. Sequences representing the new Pakistani species are in bold.