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FIRST RECORD OF *LEUCOAGARICUS NIVALIS* FROM PAKISTAN

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Leucoagaricus Locq. ex Singer is represented by more than 150 species of agaricoid, saprotrophic fungi distributed all over the world (Kirk *et al.*, 2008; Kumari and Atri, 2013; Yuan and Liang, 2014; Nabe *et al.*, 2014; Ge *et al.*, 2017; Justo *et al.*, 2015; Qasim *et al.*, 2015; Yu *et al.*, 2016; Hussain *et al.*, 2018; Usman and Khalid 2018; Verma and Vimal, 2018; Sysouphanthong *et al.*, 2018; Yang *et al.*, 2019; Ullah *et al.*, 2020). Only 11 *Leucoagaricus* species have been reported from Pakistan so far (Ahmad *et al.*, 1997; Qasim *et al.*, 2015; Ge *et al.*, 2017; Hussain *et al.*, 2018; Usman and Khalid, 2018; Ullah *et al.*, 2020). *Leucoagaricus* is characterized by small to medium, fleshy or thin basidiomata, ranging in stature from slender to sturdy; a pileus surface that is radially fibrillose, floccose, squamulose to fibrillose-scaly or granulose (rarely); entire or very short striate margins; a central, equal to bulbous stipe with a membranous, sometimes moveable annulus; metachromatic basidiospores generally lack a well-defined germ pore 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 absent in most species. Clamp connections are absent (Singer 1986; Vellinga 2001).

The present study focuses on morphological and molecular characterization of a *Leucoagaricus* species collected in the Changa Manga forest, Kasur district, Punjab, Pakistan. This research is an effort to establish the fungal diversity of this forest. During field survey in 2019 for the collection of macrofungi to explore the diversity of these fungi from Changa Manga. A number of basidiomata of *Leucoagaricus* were collected. Field notes were recorded and the samples were air dried and preserved for future analysis. Macroscopic descriptions were based on the fresh material. Significant characters involve size, shape and color of the pileus; attachment and color of lamellae; presence of annulus on stipe. Color codes were given using Munsell (1975) color system. For micro-morphology, dried samples were examined using standard microscopic techniques. Different chemicals were used as mounting media according to requirements. For rehydration, 5% KOH was used, and for staining the walls of hyaline hyphae, Congo red was used. The anatomical features were observed under microscope Xsz 107BN adjusting at 100× objective lens. Measurements were noted using calibrated Motic Images Plus 2.0 software. For basidiospores, [n/m/p] represents n number of spores, measured from m basidiomata and p collections, l × w represents spore dimensions, extreme values are given in parenthesis. Q values are given as l × w ratio while definitions of the Q values for spores are given following Bas (1969). Drawings were made from the laptop screen. The examined specimens are deposited in the herbarium (LAH), Department of Botany, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan. For DNA extraction, the Extract-N-Amp™ kit (Sigma- Aldrich, St Louis, MO, USA) was used following the manufacturer's protocol. PCR amplification and sequencing was carried

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out from the sequence service using ITS1F and ITS4 primers. Sequences obtained were analyzed in BioEdit sequence alignment editor version 7.2.5 (Hall, 1999). Consensus sequences were generated and BLAST searched at NCBI (http://www.ncbi.nlm.nih.gov/). Sequences with closest match were selected from GenBank to reconstruct phylogeny. The sequences with incomplete ITS region were left aside. Published sequences of the closest relatives of the species were included to reconstruct phylogeny (Hussain *et al.*, 2018; Ullah *et al.*, 2020). *Agaricus bisporus* (J.E. Lange) Imbach (AF432886) and *Agaricus campestris* L. (U85307) were chosen as outgroup to root the phylogenetic tree. The sequences were aligned using an online MUSCLE tool at EMBL-EBI (http://www.ebi.ac.uk/). A maximum likelihood tree was inferred using Tamura 3-parameter model (Tamura, 1992) by best DNA model selection in MEGA 6 (Tamura *et al.*, 2013). 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 a selection of 117 nucleotide sequences. There were a total of 866 positions in the final dataset. The phylogeny was tasted with 1000 bootstrap replicates.

BLAST at NCBI revealed that the complete ITS sequences from Pakistani collections of *Leucoagaricus* showed 99–100% similarity with the sequences from China (KY039573) and Pakistan (MK106150–MK106153). These sequences along with other sequences from closely related taxa constituted a final dataset of 117 nucleotide sequences with 866 positions. Among these positions, 296 were conserved, 540 were variable, 436 were parsimony informative and 90 were represented as singletons. The phylogenetic tree recovered from this dataset is shown in Fig. 1. The sequences from our collections were clustered within a clade including sequences of *L. nivalis* (W.F. Chiu) Z.W. Ge & Zhu L. Yang, *L. purpureolilacinus* Huijsman, *L. umbonatus* Hussain *et al.*, and some unidentified taxa. Our sequences clustered within the *L. nivalis* lineage including sequences from Changa Manga forest Pakistan and China with 100% bootstrap value Fig. 1.

Taxonomy:

Leucoagaricus nivalis (W.F. Chiu) Z.W. Ge & Zhu L. Yang Mycosystema **36**(5): 548 (2017)

(**Fig. 2 & 3**)

Pileus 2.3–7.5 cm, broadly convex to flat becoming uplifted when mature, surface smooth to slightly fibrillose, white, slightly umbonate; umbo light yellow (2.5Y9/4), margin striate, undulating to dentate or eroded. Lamellae free, crowded, narrow to ventrocose, entire, white. Lamellulae frequent, of variable lengths. Stipe (1.1–)2–4(–11) × (0.2–)0.3–0.5(–0.6) cm, with upto 0.8 cm wide base, slightly narrow towards the pileus, central, cylindrical, sometimes curved, smooth, white. Annulus present, inferior, white (2.5Y8/4). smell and taste not observed. Basidiospores [60/3/3] (7.8–)8.3–12(–12.6) × (5.7–)6.3–7.7(–8) µm, Q = (1.3–)1.5–1.7(–2), avQ = 1.5, ellipsoid, amygdaliform in side view, ovoid in front view; dextrinoid; apiculus prominent; germ pore absent. Basidia (13.8–)13.9–15.5(–17.2) × (5.4–)5.5–6.4(–7.2) µm, clavate, with 2–4 sterigmata. Lamellae edge sterile Cheilocystidia (11–)14–14.9(–16.1) × (4.2–)4.8–4.9(–5) µm, clavate. Pleurocystidia absent. Pileipellis hyphae (5–)5.4–8.1(–8.8) µm wide, septate; septa frequent; hyphal terminals at the center of the pileus cylindrical (5.2–)5.4–6(–6.6) µm wide. Stipitipellis hyphae (6.2–)9.4–10.8(–12.4) µm wide, septate; septa frequent. Clamp connections absent in all tissues. All hyphal walls are transparent in H_2O and pink in Congo red.

Specimen examined: PAKISTAN. Punjab: Lahore division, Kasur district, Changa Manga, 192 m a.s.l., on soil 6 October 2019, Sana Jabeen SJ51CM4 (LAH36651; GenBank: MT573439); SJ53CM5 (LAH36652; GenBank: MT573440); Tuba SJ58CM12 (LAH36653; GenBank: MT573441).

Fig. 1. Molecular phylogenetic analysis of *Leucoagaricus* spp. based on ITS sequences. Sequence generated during this study are marked by ●. Scale bar = nucleotide substitutions per site.

Fig. 2. *Leucoagaricus nivalis* basidiomata. A. LAH36651; B & C. LAH36652. Scale bars = 2 cm. Photos by Sana Jabeen.

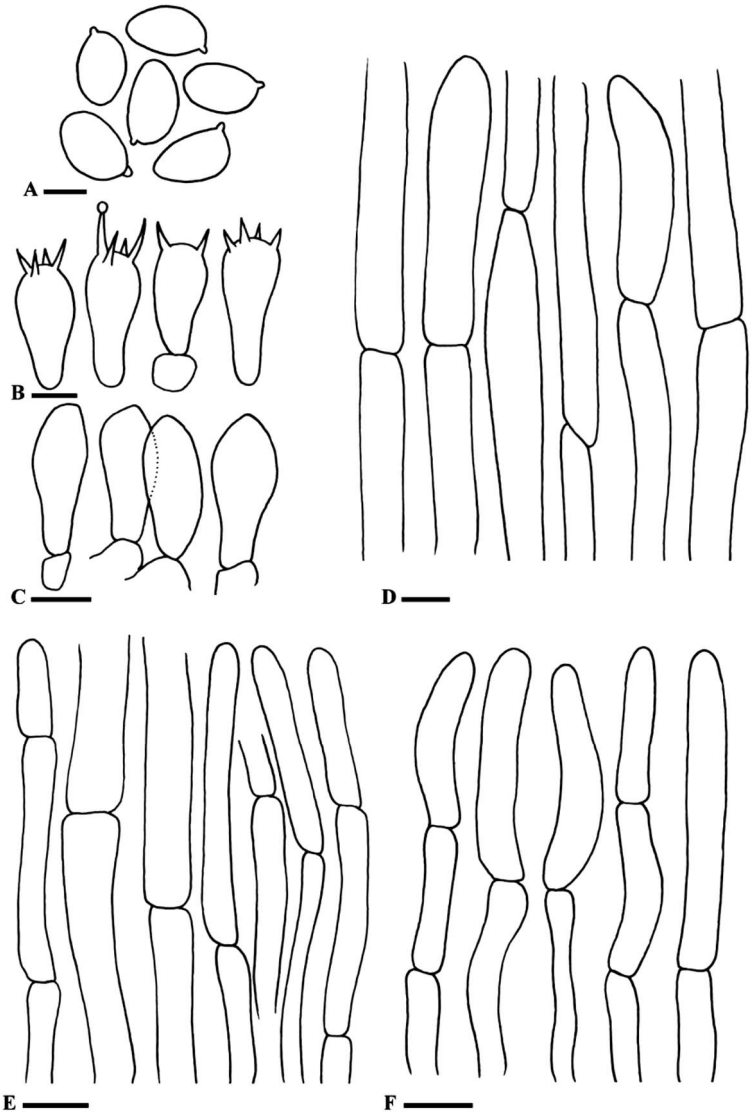

Fig. 3. *Leucoagaricus nivalis* (LAH36652). A. Basidiospores; B. Basidia; C. Cheilocystidia; D. Stipitipellis; E. Pileipellis; F. Pileipellis terminal hyphae from umbo. Scale bars: A–C = 5 µm, D–F = 10 µm. Drawings by Sana Jabeen.

Comments: *Leucoagaricus nivalis* was described in 1948 as *Lepiota nivalis* W.F. Chiu from Kunming, Yunnan province, China (Chiu, 1948). Recent studies by Yang and Ge (2017) revealed that this species belongs to *Leucoagaricus* based on its features of basidiospores and cheilocystidia. *Leucoagaricus nivalis* was morphologically identified based on the type collection and two modern collections from the same area. Our collections showed more or less similar features to the type collection of *L. nivalis* HMAS 4237 (Yang and Ge, 2017). Though there are some minor differences that were observed in comparative study. These features include the size of the basidiomata which is smaller (6–7.5 × 3.5–4.5) and the size of the basidiospores in terms of

Q value that is slightly larger (1.7) in Chinese collections. *Leucoagaricus nivalis* has only been reported from China (Chiu, 1948; Yang and Ge, 2017). Already available sequences in GenBank belong to collections from Changa Manga, Pakistan (MK106150–MK106153) and modern Chinese collection from Yunnan province (KY039573). In phylogenetic tree, the sequences generated during this study clustered with these sequences in the same clade supports its taxonomy as *L. nivalis*. *Leucoagaricus nivalis* has only been validly published from China (Chiu, 1948; Yang and Ge, 2017), occurrence of *L. nivalis* in Pakistan is an addition to the funga of Pakistan.

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