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# New record of *Melanoleuca cinereifolia* in Himalayan moist temperate forests of Pakistan

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Abstract — Basidiomata of *Melanoleuca cinereifolia* have been collected underneath *Pinus wallichiana* trees in Himalayan moist temperate forests of Pakistan. Its identification was confirmed by nrDNA ITS sequence analysis. A detailed morphological description and illustrations are provided. This is the first report of *M. cinereifolia* from Asia.

Key words —Khyber Pakhtoonkhaw, pine forest, saprobic fungi, *Tricholomataceae*

## Introduction

The Himalayan moist temperate forests of Pakistan, located at elevations of 1373–3050 m, are characterized by vigorous thick vegetation of conifers and some deciduous trees. These mixed forests have an average rainfall of 59.3 cm with humidity up to 57% (Champion et al. 1968). Although the Himalayas are considered one of twenty-five biodiversity hotspots, most mushroom species are yet to be reported (Myers et al. 2000; Razaq et al. 2013). During research on saprobic fungi from pine-dominated forests of Western Himalayas, *Melanoleuca cinereifolia* was identified as a new record for Pakistan. *Melanoleuca* Pat. is a cosmopolitan genus characterized by collybioid to tricholomatoid basidiomata, convex to slightly depressed pilei (often with a shallow umbo), emarginate to adnate to shortly decurrent lamellae, absence of a veil, white to pale-yellowish spore print, cutis to trichoderm pileipellis, hyaline spores with amyloid ornamentations, and absence of clamp connections (Singer 1986, Boekhout 1988, Vizzini et al. 2012).

Singer (1935, 1943, 1986), Kühner (1978), Bon (1978), and Boekhout (1988) gave much importance to morphology (e.g., pileus, stipe and lamellar colour, and stipe ornamentation) and anatomy (cystidial shape and the basidiospore Q value) for infrageneric classification in *Melanoleuca*. Vizzini et al. (2012) used ITS sequence analyses to check the traditional tripartite *Melanoleuca* subgeneric classification (*M.* subg. *Acystis* without cystidia; *M.* subg. *Urticocystis* with

urticiform cystidia; *M.* subg. *Melanoleuca* with macrocystidia). He concluded that *Melanoleuca* is a monophyletic genus in which only two emended subgenera, *Urticocystis* and *Melanoleuca*, are supported; species previously placed in *M.* subg. *Acystis,* shown to be polyphyletic and no longer accepted, are now supported in *M.* subg. *Urticocystis*.

Two other *Melanoleuca* species — *M. exscissa* (Fr.) Singer and *M. angelesiana* A.H. Sm. — have previously been reported from Pakistan (Ahmad 1980, Razaq 2013).

## Materials & methods

### Collection and morphological examination

The basidiome (MSM#005) was collected, photographed, vouchered, dried under fan heater, and characterized morphologically. Specimen sections were mounted in 5% KOH for examination under a MX4300H biological microscope (Meiji Techno Co., Ltd., Japan); phloxine was used to increase contrast, and Melzer's reagent was used to test for amyloidity of the basidiospores.

Measurements of anatomical features (basidiospores, basidia, cystidia, pileus hyphae and stipe hyphae) were calculated from at least 20 measurements made with an ocular micrometer and 100× oil-immersion objective; abbreviations include x = mean spore length and width for all spores measured, Q = spore length / width ratio. Line drawings were made with a Lucida camera. Color designations are from Munsell (1975).

### DNA extraction, PCR amplification, DNA sequencing

Genomic DNA was extracted from a small piece of pileus by a modified CTAB method (Bruns 1995). The internal transcribed spacers (ITS1, ITS2) + 5.8S region of the nuclear ribosomal RNA gene were targeted by the ITS1F/ITS4 primer pair (White et al. 1990; Gardes & Bruns 1993) using an Extract-N-Amp plant DNA extraction Kit (Sigma-Aldrich, St. Louis, MO, USA). The PCR amplification parameters were: 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 72 °C (8 min). Amplified PCR products were sent for purification and bidirectional sequencing to Macrogen (Korea).

### Sequence alignment and phylogenetic analysis

The nrITS sequence from MSM#005 comprised 740 base pairs. Initial blast analysis of nucleotide sequences revealed that the specimen showed 99% maximum identity with *Melanoleuca* aff. *cinereifolia*, *M. cinereifolia*, *M. communis* (GenBank JX429210, JX429108, JX429197, JX429207). GenBank sequences were selected based on the phylogenetic studies on *Melanoleuca* by Vizzini et al. (2012) and Sánchez-García et al. (2013).

Sequences were manually edited and assembled using BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html). Following Dentinger et al. (2011) for complete ITS sequences, all sequences were trimmed with the conserved motifs 5′-(…GAT)CATTA– and –GACCT(CAAA…)-3′ and the alignment portion between them were included in the analysis. Gaps were treated as data for construction of phylogeny.

The sequence from MSM#005 was aligned with GenBank sequences of *M. cinereifolia* and other related taxa (Table 1) by Muscle using the default setting in Molecular

Evolutionary Genetics Analysis (MEGA) software (Tamura et al. 2011). A phylogenetic tree was constructed with the Maximum Likelihood (ML) algorithm and Neighbor-Joining method, Jukes & Cantor (1969) model of nrITS sequences, and nearest-neighbor-interchange (NNI) as ML heuristic search method using MEGA5 software (Tamura et al. 2011). Phylogeny was tested by a bootstrap value of 1000 replicates, and bootstrap values >50% are cited in the tree.

Sequence of *M. cinereifolia* generated for this study was submitted to the Genbank and the accession numbers for this, as well as for other closely related taxa used in the phylogenetic analysis are cited in phylogenetic tree. Percent Identities (PID) and DNA divergence were calculated by DNAStar.

Table 1. *Limnoperdon* and *Melanoleuca* ITS-rDNA sequences used in the phylogenetic analysis. New sequence indicated in bold.

| Taxon | Collection/Voucher | Country | Genbank # |
|---|---|---|---|
| *L. incarnatum* | IF030398 | Japan | DQ097363 |
| *M. angelesiana* | ANC M0203 | Italy | JN616420 |
| | ANC M0203 | Italy | JN616421 |
| *M. brevipes* | 4574 | Italy | JF908352 |
| *M.* aff. *cinereifolia* | FCME11225 | Mexico | JX429108 |
| | IBUGJS89 | Mexico | JX429210 |
| *M. cinereifolia* | 319 | Italy | JN052137 |
| | 1471 | Italy | JN052138 |
| | **MSM#005** | **Pakistan** | **KJ182965** |
| *M. cognata* | 13939 | Italy | JF908360 |
| | ANC M0170 | Italy | JN616425 |
| *M. communis* | XAL Murrieta1025 | Mexico | JX429204 |
| | FCME 17116 | Mexico | JX429207 |
| | ENCB Guzman 6329 | Mexico | JX429226 |
| *M. decembris* | ANC M0197 | Italy | JN616426 |
| | ANC M0200 | Italy | JN616428 |
| *M. exscissa* | ANC M0210 | Italy | JN616433 |
| | ANC M0213 | Italy | JN616436 |
| *M. grammopodia* | ANC M0217 | Italy | JN616439 |
| | ANC M0218 | Italy | JN616440 |
| *M. heterocystidiosa* | ANC M0174 | Italy | JN616444 |
| | ANC M0175 | Italy | JN616445 |
| *M. nivea* | MCVE 9578 | Italy | JN392452 |
| | ANC M0177 | Italy | JN616450 |
| *M. paedida* | ANC M0189 | Italy | JN616452 |
| | ANC M0190 | Italy | JN616453 |
| *M. pseudoluscina* | ANC M0194 | Italy | JN616458 |
| | ANC M0195 | Italy | JN616459 |
| *M. strictipes* | ANC M0172 | Italy | JN616465 |
| | ANC M0173 | Italy | JN616466 |
| *M. subpulverulenta* | ANC M0004 | Italy | JN616472 |
| | ANC M0178 | Italy | JN616473 |
| *M. substrictipes* | ANC M0214 | Italy | JN616474 |


Fig. 1. *Melanoleuca cinereifolia* (MSM#005). Basidiomes. Scale bars: 10 mm.

## Taxonomy

***Melanoleuca cinereifolia*** (Bon) Bon, Docum. Mycol. 9(33): 71 (1978). Figs 1–3

Description based on a single basidiome:

Pileus 21 mm diam., plano-convex, flat, thin (collybioid); margin straight or flaring, smooth; surface dull, off-white to grayish near margin (5R9/2), central disc chocolate brown (5YR1/2). Lamellae free or approximate, close, off-white to gray (5YR9/2). Stipe 26 mm, central, subequal, often with subbulbous base, hollow, fibrillose, light brown (5YR7/4). Rhizomorphs white, few. Odor and Taste not recorded. Basidiospores 4.3–6.1 × 7.2–9.5 µm [x = 5.4 × 8.5 µm, Q = 1.57], oblong or ellipsoid, apiculus absent, warty, thin-walled, hyaline in KOH, amyloid. Basidia 8.5–11.8(–13) × 28.7–38.2(–44.8) µm, clavate, mono-, bi-, or tetra-spored, thin-walled, hyaline in KOH; sterigmata 1.8–4.2(–5.1) µm. Trama hyphal, 6.1–8.9 µm, thin-walled, hyaline in KOH. Cheilocystidia 11.8–14(–16.9) × 47–71(–77.4) µm, lageniform with crystals at the apex, sometimes septate, hyaline, slightly thick walled, up to 1 µm. Pleurocystidia similar to cheilocystidia. Pileipellis a cutis, hyphae cylindrical, 9–16 µm, thin-walled, hyaline in KOH. Stipe Hyphae cylindrical, 5–11.8(–14.8) µm, hyaline to pale yellow in KOH. Clamp connections absent.

Material examined: **PAKISTAN**, **Khyber Pakhtoonkhaw**, Shangla, Yakh Tangay, under *Pinus wallichiana* A.B. Jacks., 2 September 2013, coll. M. Saba & A.N. Khalid, MSM#005 (LAH; GenBank KJ182965).

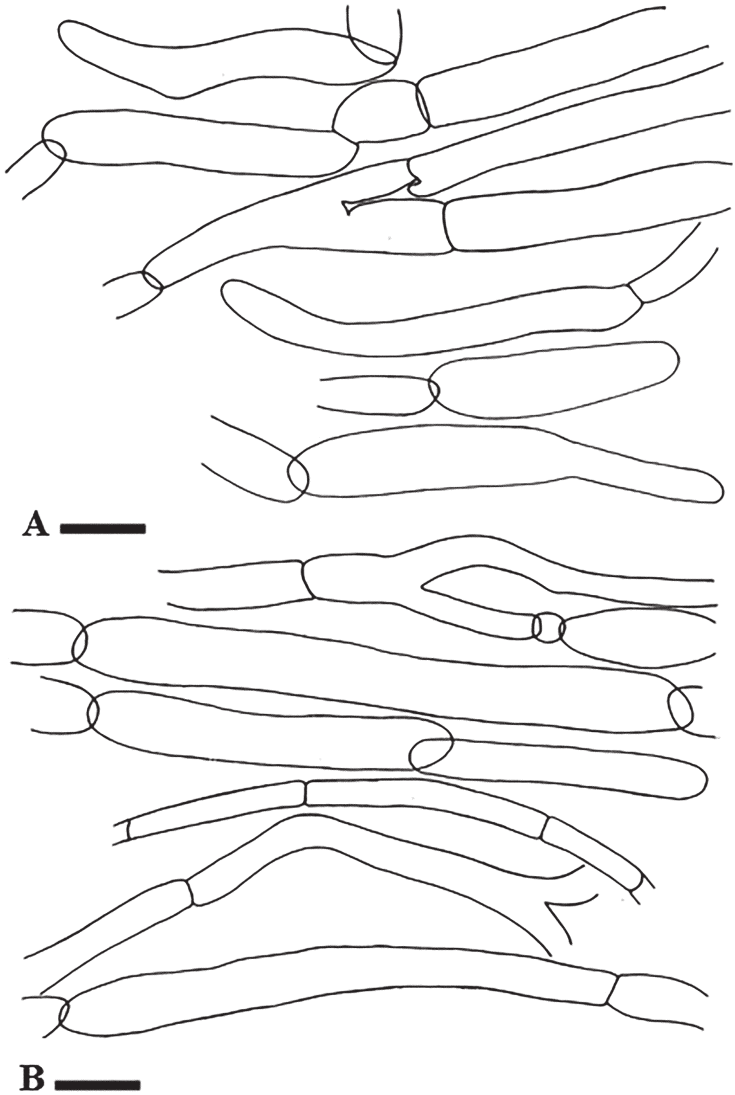


Fig. 2. *Melanoleuca cinereifolia* (MSM#005). A, pileipellis; B, stipitipellis. Scale bars: A = 20 µm, B = 16 µm.

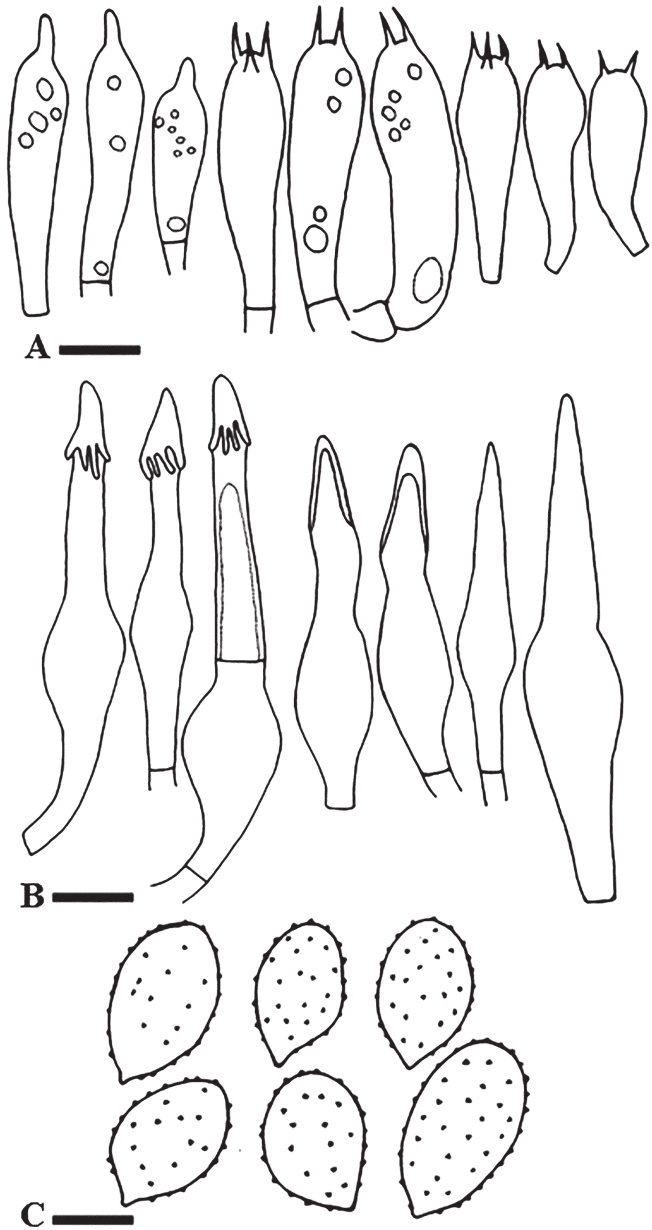


Fig. 3. *Melanoleuca cinereifolia* (MSM#005). A, basidia; B, cheilocystidia; C, basidiospores. Scale bars: A, B = 10 µm; C = 3.6 µm.

## Results

The phylogenetic trees were constructed with the Maximum Likelihood algorithm (Fig. 4) and Neighbour-Joining method (Fig. 5), using Jukes & Cantor (1969) model of nrITS sequences and nearest-neighbor-interchange (NNI) as ML heuristic search method using MEGA5 software (Tamura et al. 2011). Codon positions included were 1st+2nd+3rd+Noncoding. The

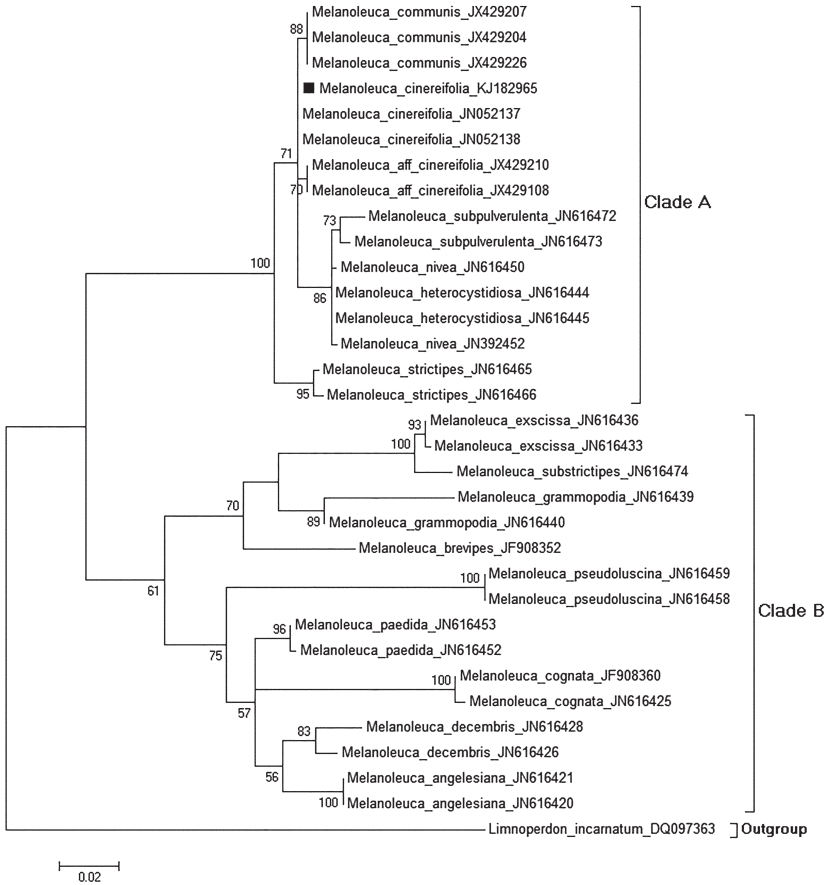


Fig. 4. Phylogenetic relationship of *Melanoleuca cinereifolia* with other *Melanoleuca* spp. based on Maximum Likelihood method inferred from nrITS sequences. The tree with the highest log likelihood (–3381.2856) is shown. Bootstrap values >50 (1000 replicates) are shown below the branches, and the percentage of trees in which the associated taxa cluster together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

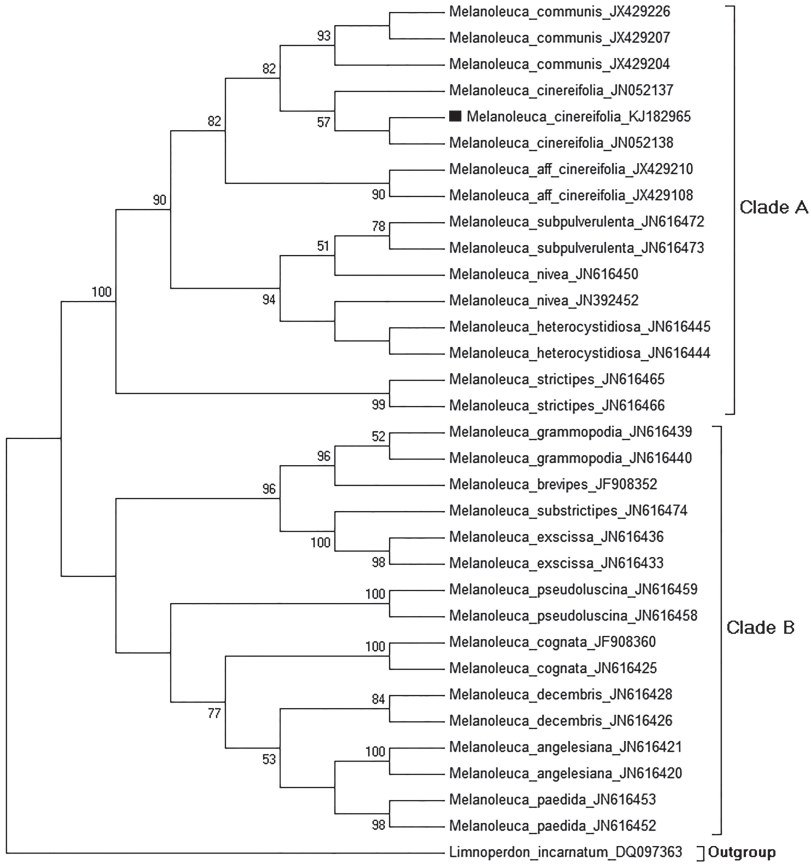


Fig. 5. Phylogenetic relationship of *Melanoleuca cinereifolia* with other *Melanoleuca* spp. based on Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site.

final dataset contains 33 nucleotide sequences. *Limnoperdon incarnatum* (DQ097363) was used as outgroup based on results from Vizzini et al. (2012).

After removing and editing the ambiguous letters from aligned datasheet, a total of 711 characters were subjected for phylogenetic analysis, of which 435 characters were conserved, 274 were variable, 186 were parsimony informative, and 87 were singletons.

The percentage similarity was calculated by MegAlign (DNA Star Inc.). *Melanoleuca cinereifolia* showed 99.4% identity and 0% genetic divergence with sequences retrieved from GenBank (JN052137, JN052138), 99.4% identity and 0.5% divergence with *M.* aff. *cinereifolia* sequences (JX429108, JX429210), and 99.3% identity and 0.5% divergence with *M. communis* (JX429226, JX429204, JX429207).

## Discussion

*Melanoleuca cinereifolia* and *M. communis* M. Sánchez-García & J. Cifuentes closely resemble each other. Both species show 99% maximum similarity to the present species in the Blast search and probably represent the same taxon. In the phylogeny MSM#005 clusters with *M. cinereifolia* and morphologically, it differs from *M. communis* in basidiome and pileus size and pileus and lamellar colour. *Melanoleuca cinereifolia* is usually characterized as a basidiome with a short stipe, grey lamellae, lageniform cystidia that grows in sand dunes (Vizzini et al. 2012). Lantieri et al. (2009), describe it from Italy as frequent on sand dunes characterized by *Cypero capitati-Agropyretum juncei* and as well as at higher elevations characterized by *Medicagini marinae-Ammophiletum australis*. In Pakistan, our *M. cinereifolia* specimen was collected growing on forest floor rich in organic matter in a pure *Pinus wallichiana* forest. Sánchez-García et al. (2013) collected *M.* cf. *cinereifolia* in mountainous mesophilic forests in Mexico.

Other *Melanoleuca* species known from Pakistan are *M. exscissa* and *M. angelesiana* (Ahmad 1980; Razaq 2013). Unlike *M. cinereifolia, M. exscissa* has a urticiform *exscissa* cystidial type while *M. angelesiana* lacks cystidia.

Identification of *Melanoleuca* species is difficult because most morphological characters are homoplastic and influenced by environmental factors (Vizzini et al. 2012), making molecular analysis necessary for species identification.

Our phylogenetic trees (Figs 4, 5) divide *Melanoleuca* into two major clades, supporting recognition of two subgenera (*Melanoleuca* and *Urticocystis*). Clade A in which the Pakistani specimen is nested is supported by a robust (100) bootstrap and includes taxa mainly with lageniform or fusiform macrocystidia. Clade B, which is supported by only 56 bootstrap, includes all taxa with urticoid cystidia, some species lacking cystidia, and the macrocystidiate *M. cognata* complex. *Melanoleuca exscissa* and *M. angelesiana* reported from Pakistan cluster in clade B. Our phylogeny agrees closely with the results reported by Vizzini et al. (2012). Moreover, percent identity and genetic divergence support the identity of MSM#005 as *M. cinereifolia*, as inferred from the phylogenetic tree.

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**Literature cited**

Ahmad S. 1980. A contribution to the *Agaricales* of Pakistan. Bulletin of Mycology 1: 35–89.

Boekhout T. 1988. Notulae ad floram agaricinam neerlandicam, XVI – new taxa, new combinations in *Melanoleuca* Pat. and notes on rare species in the Netherlands. Persoonia 13(4): 397–431.

Bon M. 1978. *Tricholomataceae* de France et d'Europe occidentale (*Leucopaxilloideae*). Documents Mycologiques 9(33): 1–79.

Bruns TD. 1995. Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. Plant and Soil 170: 63–73. http://dx.doi.org/10.1007/BF02183055

Champion HG, Sethi SK, Khattak GM. 1968. Forests types of Pakistan. Pakistan forest institute, Peshawar.

Dentinger BTM, Didukh MY, Moncalvo JM. 2011. Comparing COI and ITS barcode markers for mushrooms and allies (*Agaricomycotina*). PLoS One 6(9): e25081. http://dx.doi.org/10.1371/journal.pone.0025081

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for *Basidiomycetes*: application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113–118. http://dx.doi.org/10.1111/j.1365-294X.1993.tb00005.x

Jukes TH, Cantor CR. 1969. Evolution of protein molecules. 21-132, in: HN Munro (ed.). Mammalian Protein Metabolism, vol. 3. Academic Press, New York. http://dx.doi.org/10.1016/B978-1-4832-3211-9.50009-7

Kühner R. 1978. Agaricales de la zone alpine. Genre *Melanoleuca*. Bulletin de la Société Linnéenne de Lyon 47: 12–52.

Lantieri A, Gargano ML, Venturella G. 2009. The sabulicolous fungi from Sicily (southern Italy): additions and critical review. Mycotaxon 110: 151–154. http://dx.doi.org/10.5248/110.151

Munsell ™. 1975. Soil color charts. Baltimore.

Myers N, Mittermeier RA, Mittermeier GAB, Fonseca D, Kent J. 2000. Biodiversity hotspots for conservation priorities. Nature 403: 853–858. http://dx.doi.org/10.1038/35002501

Razaq A. 2013. Molecular characterization and identification of gilled fungi from Himalayan moist temperate forests of Pakistan using internal transcribed spacers (ITS) of rDNA. PhD thesis, Department of Botany, University of the Punjab, Lahore.

Razaq A, Khalid AN, Ilyas S. 2013. Molecular identification of *Lepiota acutesquamosa* and *L. cristata* (Basidiomycota, Agaricales) based on ITS-rDNA barcoding from Himalayan Moist Temperate forests of Pakistan. International Journal of Agriculture and Biology 15: 313–318.

Sánchez-García M, Cifuentes-Blanco J, Matheny PB. 2013. Revisión taxonómica del género *Melanoleuca* en México y descripción de especies nuevas. Revista Mexicana de Biodiversidad 84: 111–117. http://dx.doi.org/10.7550/rmb.31569

Singer R. 1935. Étude systématique sur les *Melanoleuca* d'Europe et clé des espèces observées en Catalogne. Cavanillesia 7: 122–132.

Singer R. 1943. Das system der *Agaricales* III. Annales Mycologici 41: 1–189.

Singer R. 1986. The *Agaricales* in modern taxonomy, 4th edn. Koeltz Scientific Books, Koenigstein.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28(10): 2731–2739. http://dx.doi.org/10.1093/molbev/msr121

Vizzini A, Para R, Fontenla R, Ghignone S. Ercole E. 2012 ["2011"]. A preliminary ITS phylogeny of *Melanoleuca* (*Agaricales*), with special reference to European taxa. Mycotaxon 118: 361–381. http://dx.doi.org/10.5248/118.361

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. 315–322, in: MA Innis et al. (eds). PCR Protocols: a guide to methods and applications. Academic, New York.