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Macrolepiota distribution extends to the montane temperate forests of Pakistan

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Abstract — Basidiomata of *Macrolepiota dolichaula* and *M. excoriata* were collected from mixed coniferous forests in Pakistan. The species were identified using ITS nrDNA sequence analyses and morphological and anatomical characters. Detailed morphological and anatomical descriptions and illustrations are provided along with results of molecular phylogenetic analysis. Both species are regarded as new records for Pakistan, and this report considerably extends their known distribution.

Key words — *Agaricaceae*, basidiomata, *Basidiomycota*, saprotrophic

Introduction

Macrolepiota was established by Singer (1948). Currently, there are about 33 species recognized in the genus worldwide (Kirk et al. 2008; Ge et al. 2010, 2012), of which several are edible (Ding & Huang 2003). Representatives of the genus typically have large fleshy basidiomata, a pileal surface covered by squamules composed of long subcylindric elements, white to cream lamellae, and a prominent annulus. The basidiospores are relatively big and thick-walled with a germ pore covered by a hyaline cap, and stipe squamules are often visible as colored bands in mature specimens (Singer 1948; Vellinga 2003; Vellinga et al. 2003). Some *Macrolepiota* species have been transferred to *Chlorophyllum* Massee, which differs from *Macrolepiota* s. str. by the presence of pileal squamules made up of a hymenodermal layer, spore germ pores that are either absent or truncated, and a smooth stipe (Vellinga & Kok 2002).

Macrolepiota species are saprotrophic and grow in the soil litter layers that contain mostly fresh leaves (Vellinga 2004). Some species, such as *M. excoriata* and *M. phaeodisca* Bellù, have a restricted distribution (Courtecuisse & Duhem 1994; Nauta & Vellinga 1995; Candusso & Lanzoni 1990), although the exact distribution is still unknown for many species. *Macrolepiota procera* (Scop.) Singer is the only species that has been reported from Pakistan (Ahmad et al. 1997).

The present study focuses on *M. dolichaula* and *M. excoriata*, newly recorded from Pakistan from two different areas of Khyber Pakhtunkhawa (KPK) Province. *Macrolepiota dolichaula* has been previously reported from China (Chiu 1948; Shao & Xiang 1981; Zang et al. 1996; Bi et al. 1994, 1997; Ge et al. 2010, 2012), East Africa (Pegler 1977), eastern Australia (Grgurinovic 1997; Vellinga 2003), India (Manjula 1983; Natarajan & Manjuk 1983), Sri Lanka (Pegler & Rayner 1969), Thailand (Ge et al. 2010), and Vietnam (Kiet 1998; Yang 2000). *Macrolepiota excoriata* is native to Europe and has also been reported from India (Kumari 2012), Israel (Barseghyan et al. 2012), South Africa (Doidge 1950), Turkey (Afyon & Yağiz 2004; Doğan & Öztürk 2006), China (Teng 1996; Mao 2000; Ge et al. 2010), and North America and South Africa (http://www.gbif.org/). The correct identity of some of these records, however, requires confirmation.

Materials & methods

Sampling site description

The two sampling sites are situated in the Mansehra and Swat districts of Khyber Pakhtunkhwa.

Mansehra district is located at the eastern border of the KPK. Mountain ranges, plains, valleys, and numerous lakes and rivers are the main features, with the Kunhar and Siran rivers among the largest in the area. The climate is warm in summer and cold in winter, although the northern high mountainous region remains cold in summer due to snow cover. Evergreen and deciduous trees are well represented. *Pinus wallichiana* A.B. Jacks. and *Pinus roxburghii* Sarg. are dominant tree species, while *Juglans regia* L. is the most common broadleaf tree (Mustafa 2003).

The Swat valley, which lies in northern Khyber Pakhtunkhwa, is surrounded by mountains that are offshoots of the Hindu Kush. The Swat River is formed by the joining of the Ushu and Utrot rivers, which descend from the Hindu Kush Mountains. Kalam, a village along the upper reaches of the Swat River, is known for its waterfalls, lakes, and lush green hills dominated by *Cedrus deodara* (Roxb. ex D. Don) G. Don forests, *Pinus* spp., and *Quercus* sp. The climate is moist temperate to dry temperate (Champion et al. 1965). Following the standard classification of Pakistan's forests (Champion et al. 1965), Shah & Khan (2006) classify the Swat forest type as belonging to "montane temperate forest."

Morphological & anatomical characterization

Fresh basidiomata were collected and catalogued, described morphologically, and photographed using a Nikon D70S digital camera. Color codes follow Munsell Soil Color Charts (1975). The specimens were dried under a fan heater. The specimens were mounted in 5% KOH solution for examination of basidia, cystidia, spores, pileal elements and stipe hyphae under a MX4300H microscope (Meiji Techno Co., Ltd., Japan). Anatomical features were measured with a Carl Zeiss Jena ocular micrometer. Basidiospore dimensions follow the format [*n/m/p* = *n* basidiospores measured from *m* fruit bodies of *p* collections], length × width (l × w), and avQ (= average l/w ratio of all spores). Line drawings were made using Leitz Wetzlar Camera Lucida. Voucher specimens were deposited in either the Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan (LAH) or the Herbarium Hazara University, Dhodial, Pakistan (HUP).

Molecular characterization

A small piece (2 mg) of lamellae was kept in 2% CTAB buffer. DNA was extracted using modified CTAB method following Bruns (1995). The nrDNA ITS regions were amplified using ITS1F (CTTGGTCATTTAGAGGAAGT) and ITS4 (TCCTCCGCTTATTGATATGC) primers under standard conditions (Gardes & Bruns 1993) Agarose Gel Electrophoresis was performed to visualize PCR products in Gel Documentation System (UVtec. Avebury House, Cambridge CB4 1QB UK) at default settings. The products were sent to Macrogen Inc. (Korea) to perform sequencing using the same pair of primers. The consensus sequence was generated from the sequences obtained using both primers. A BLAST search was used to retrieve nucleotide sequences from GenBank (http://www.ncbi.nlm.nih.gov/), with the closest matching sequences selected from the initial blast. The most closely related species were also included in the final data set. ClustalW was used to align sequences in MEGA software. The sequences were trimmed with the conserved motifs 5′-(...GAT)CATTA... and ...GACCT(CAAA...)-3′, and the alignment portion between them was included in phylogenetic analysis. Maximum likelihood (ML) analysis was performed using Jukes-Cantor model and nearest neighbor interchange as ML heuristic search method in MEGA5 software to test the phylogeny at 1000 bootstraps. The rDNA-ITS divergence was reconstructed using MegAlign (DNAStar). Sequences generated in this study were submitted to GenBank (KJ013326; KJ643333; KJ643334).

Taxonomy

Macrolepiota dolichaula (Berk. & Broome) Pegler & R.W. Rayner, Kew Bull. 23: 365 (1969). Fig. 1

Basidiomata medium to large with long stipe. Pileus ≤13.5 cm diam., fleshy, convex to plano-convex, white (5Y9/2), with inwardly projected margins, umbonate, covered with minute, pallid, yellowish brown (10YR5/6) squamules, crowded at centre, become minute and sparse towards margins; disc yellowish brown (10YR5/6); margins rough, fragile at maturity; context thick and white (5Y9/2). Lamellae free, crowded, white (5Y9/2) when young, becoming off-

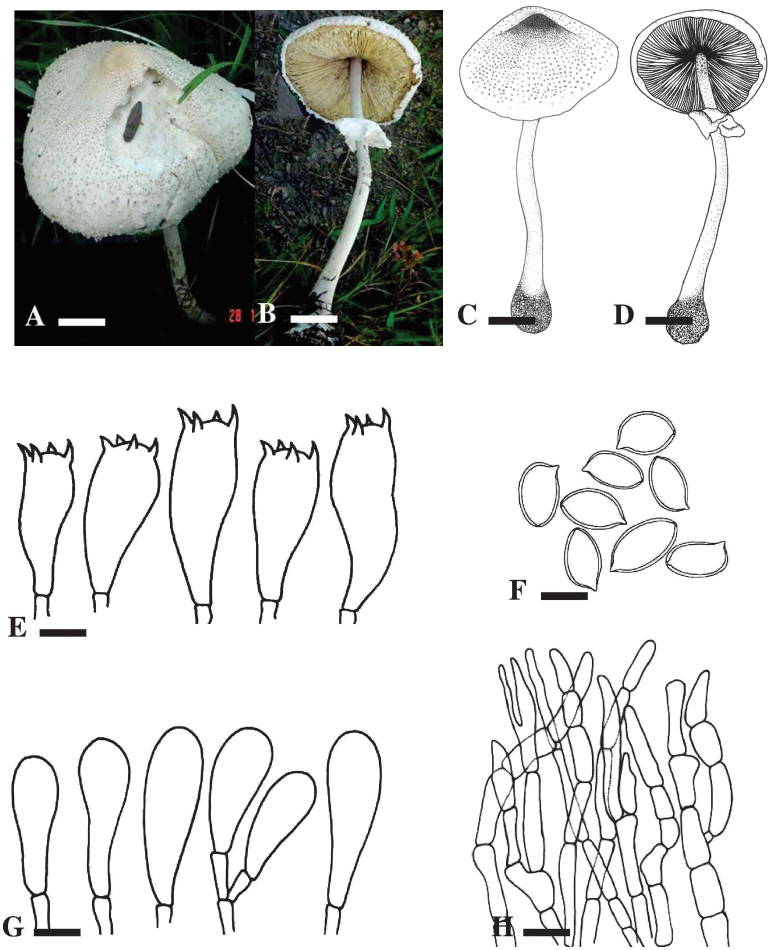

Figure 1: *Macrolepiota dolichaula* (FM-22). A–D. Basidiomata; E. Basidia; F. Basidiospores; G. Cheilocystidia; H. Hyphae from pileus. Scale bars: A = 3 cm; B–D = 4.5 cm; E = 13 µm; F = 9 µm; G = 10 µm; H= 15 µm.

white to cream (2.5Y8/4) at maturity. Lamellulae few in general. Stipe ≤26 cm long, gradually tapering towards the apex, diam. 0.5 cm towards the pileus and ≤1.3 cm towards the base, farinose, mostly glabrous, off-white to cream (2.5Y8/4), scabrous just below the annulus, with brown (10YR7/6) scales; base bulbous, ≤2 cm in diam. Annulus ascending, whitish (2.5Y8/4), superior, membranous, moveable. Odor and taste not recorded.

Basidiospores [50/1/1] 9.4–15.6 × 7.5–11.3 µm (average = 12.5 × 9.4 µm), avQ = 1.33, ovoid to broadly ellipsoid, ellipsoid, hyaline in 5% KOH, reddish brown in Melzer's reagent, with a germ pore on the rounded apex with a hyaline cap; apiculus 1–1.5 µm high. Basidia 29–39.6 × 9.9–14.4 µm, clavate to subclavate, thin-walled, hyaline in 5% KOH, 4-spored. Pleurocystidia absent. Cheilocystidia 18.4–28.4 × 7.1–12.8 µm, clavate, thin walled, hyaline. Squamules on pileus consisting of cylindric to subcylindric, branched, clampless hyphae, 3–10 µm in diam., terminal elements cylindric to slightly attenuating towards the tip, hyaline to yellowish brown vacuolar pigment. Clamp connections occasional at basidial bases.

Material examined—**PAKISTAN**, **Mansehra District**, Khabbal Paien, 1450 m asl, on soil under *Pinus wallichiana*, August 2009, Muhammad Fiaz FM–22 (HUP MFM–331; GenBank KJ643334).

Macrolepiota excoriata (Schaeff.) Wasser, Ukr. Bot. Zh. 35: 516 (1978) Fig. 2

Basidiomata medium to large. Pileus ≤8 cm in diameter, fleshy, convex to plano-convex with smooth margins, slightly umbonate, white to off white (2.5Y8/4), covered with furfuraceous brownish squamules (10YR5/6), which become sparse toward margin; disc smooth, brownish (10YR5/6), margins rough and fragile. Lamellae free, ≤9 mm broad, crowded with 1–2 tiers of short lamellulae, white to off white (2.5Y8/4). Stipe 7.3–14.5 cm long, diam. 0.4 cm towards the point of attachment and 0.7 cm towards the base, with abruptly bulbous base ≤1.7 cm diam., central, hollow, fibrillose, white to off white (2.5Y8/4), cylindrical, white to off-white (2.5Y8/4), covered with minute brownish (10 YR 5/6) squamules above the annulus. Annulus persistent, descending, whitish (2.5Y8/4), membranous, superior, about 3 cm below the stipe apex. Context white, not changing when bruised. Odor and taste not recorded.

Basidiospores [50/2/2] 12.7–18.4 × 11.1–12 µm (average = 15.7 × 11.5 µm), avQ = 1.36, ellipsoid to ovoid with one or many guttules, thick-walled (0.8 µm), smooth, off-white or pale yellow in 5% KOH, light brown to reddish brown in Melzer's reagent, with rounded apex; germ pore visible; apiculus prominent. Basidia 40.3–47.4 × 16.0–18.6 µm (average = 43.6 × 17.2 µm), at base 5.7 µm wide on average, clavate, guttulate, thin-walled, commonly 4-spored, hyaline to off-white or pale yellow in KOH; sterigmata 6 µm long. Pleurocystidia absent. Cheilocystidia 29.3–32.1 × 11.1–13.9 µm (average = 31 × 11.9 µm), base 5 µm, thin-walled, hyaline to off-white or pale yellow in 5% KOH, clavate. Squamules made up of filamentous, branched, cylindrical, clampless, hyphae 3.3–16 µm in diam. with yellowish pigment; terminal segments 32.5–153.4 × 6.3–12.5 µm. Stipe hyphae thin walled, hyaline to cream. Clamp connections not observed.

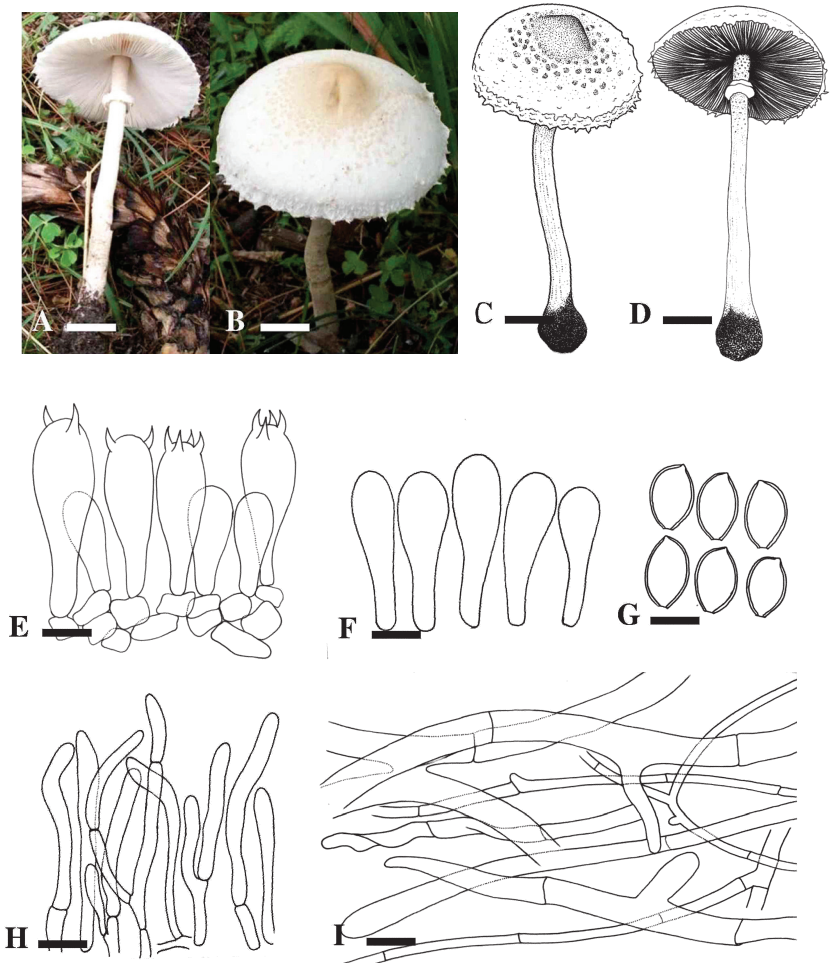

Figure 2: *Macrolepiota excoriata* (FM-305 [A–D]; F2; K3-1 [E–I]). A–D. Basidiomata; E. Basidia; F. Cheilocystidia; G. Basidiospores; H. Hyphal squamules; I. Hyphae from pileipellis. Scale bars: A, C, D = 3 cm; B = 2 cm; E = 10 µm; F = 9 µm; G = 8 µm; H = 18 µm; I = 21 µm.

Material examined–**PAKISTAN**, **Mansehra District**, Khabbal Paien, Dub, 1700 m asl, on soil under *Pinus wallichiana*, July 2009, Muhammad Fiaz FM–305 (HUP MFM–332; GenBank KJ643333); **Swat District**, Kalam, 2070 m asl, on soil under *Cedrus deodara*, 4 September 2013, Sana Jabeen F2; K3-1 (LAH-SJ1-2013; GenBank KJ013326).

Phylogeny

The nrITS sequences of *Macrolepiota excoriata* comprised 750–753 (ITS1F primer) and 760–769 (ITS4 primer) base pairs; a consensus sequence of 654 base pairs was obtained by trimming the motif of both sequences. *Macrolepiota dolichaula* sequences comprised 1390 (ITS1F primer) and 768 (ITS4 primer) base pairs. The BLAST of *M. excoriata* (KJ013326) revealed that it was 99% identical to JQ683100, JQ683089, HM246504, AF482840, AY243606, and JQ683118 with 100% query cover and 0.0 E value; *M. dolichaula* was100% identical to AF482839 with 100% query cover and 0.0 E value. Published sequences from different *Macrolepiota* sections were included to reconstruct the phylogeny with *Leucoagaricus barssii* (Zeller) Vellinga as outgroup. The analysis revealed three major clades within *Macrolepiota* (Fig. 3; /macrolepiota, /macrosporae, /volvatae), which correspond to sections *Macrolepiota*, *Macrosporae*, and *Volvatae* in earlier studies (Vellinga et al. 2003; Ge et al. 2010). Within /macrolepiota, *M. dolichaula*, *M. detersa*, *M. procera*, *M. colombiana*, *M. fuliginosa*, *M. subcitrophylla* and *M. clelandii* clustered with 58% bootstrap support; within /macrosporae, *M. phaeodisca*, *M. konradii*, *M. mastoidea*, *M. subsquarrosa*, *M. orientiexcoriata*, and *M. excoriata* clustered with 97% bootstrap support; and within /volvatae, the two volvate species, *M. eucharis* and *M. velosa,* clustered with 100% bootstrap support.

Discussion

Macrolepiota is a monophyletic group in accordance with ITS based findings (Vellinga et al. 2003). In present study, all three clades recovered by the ITS data set received strong bootstrap support. Morphological characters also support separation of these clades.

Macrolepiota dolichaula (KJ643334) from Pakistan clustered with similar specimens in sect. *Macrolepiota,* which is characterized by a complex annulus, relatively big (usually 14–20 µm) ovoid-ellipsoid spores, frequent clamp connections at the bases of the cheilocystidia and basidia, and a long stipe (Bon 1996). Within this section, the stipe surface usually has fine brown squamules, but *Macrolepiota dolichaula* with a farinose stipe surface is an exception. The pileus within this section usually forms plate-like squamules, but again *M. dolichaula* is an exception, with its pileus covered by minute, pallid squamules.

In the phylogenetic analysis, the *M. dolichaula* specimen from Pakistan shared a 100% identity with HM125516 (China) and AF482839 (Australia), 99.7% identity with AY083193 (Australia), and 99.6% identity with HM125517 (China). All analyzed *M. dolichaula* sequences form a sister clade with *M. detersa* (Fig. 3).

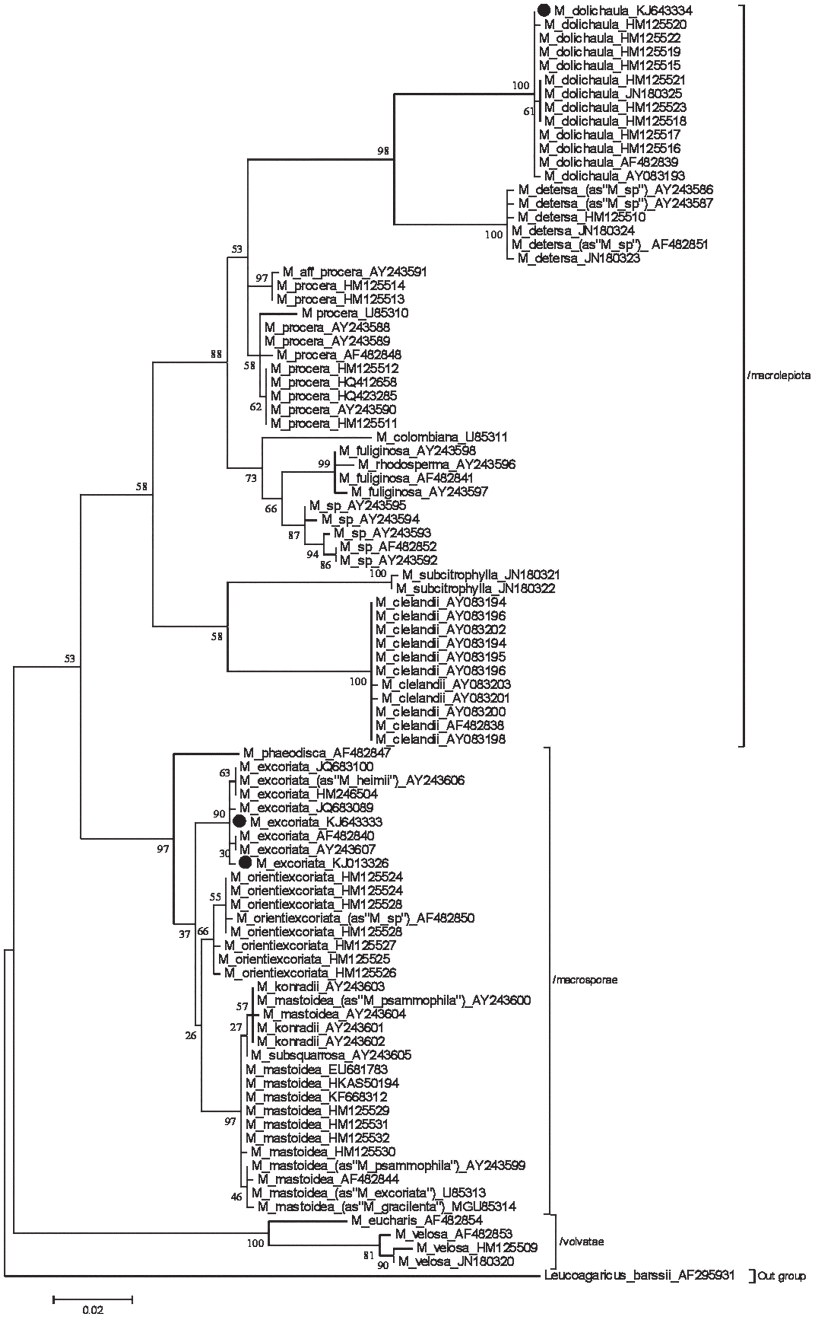
M_dolichaula_KJ643334
M_dolichaula_HM125520
M_dolichaula_HM125522
M_dolichaula_HM125519
M_dolichaula_HM125515
M_dolichaula_HM125521
M_dolichaula_JN180325
M_dolichaula_HM125523
M_dolichaula_HM125518
M_dolichaula_HM125517
M_dolichaula_HM125516
M_dolichaula_AF482839
M_dolichaula_AY083193
M_detersa_(as"M_sp")_AY243586
M_detersa_(as"M_sp")_AY243587
M_detersa_HM125510
M_detersa_JN180324
M_detersa_(as"M_sp")_ AF482851
M_detersa_JN180323
M_aff_procera_AY243591
M_procera_HM125514
M_procera_HM125513
M procera_U85310
M_procera_AY243588
M_procera_AY243589
M_procera_AF482848
M_procera_HM125512
M_procera_HQ412658
M_procera_HQ423285
M_procera_AY243590
M_procera_HM125511
/macrolepiota
M_colombiana_U85311
M_fuliginosa_AY243598
M_rhodosperma_AY243596
M_fuliginosa_AF482841
M_fuliginosa_AY243597
M_sp_AY243595
M_sp_AY243594
M_sp_AY243593
M_sp_AF482852
M_sp_AY243592
M_subcitrophylla_JN180321
M_subcitrophylla_JN180322
M_clelandii_AY083194
M_clelandii_AY083196
M_clelandii_AY083202
M_clelandii_AY083194
M_clelandii_AY083195
M_clelandii_AY083196
M_clelandii_AY083203
M_clelandii_AY083201
M_clelandii_AY083200
M_clelandii_AF482838
M_clelandii_AY083198
M_phaeodisca_AF482847
M_excoriata_JQ683100
M_excoriata_(as"M_heimii")_AY243606
M_excoriata_HM246504
M_excoriata_JQ683089
M_excoriata_KJ643333
M_excoriata_AF482840
M_excoriata_AY243607
M_excoriata_KJ013326
M_orientiexcoriata_HM125524
M_orientiexcoriata_HM125524
M_orientiexcoriata_HM125528
M_orientiexcoriata_(as"M_sp")_AF482850
M_orientiexcoriata_HM125528
M_orientiexcoriata_HM125527
M_orientiexcoriata_HM125525
M_orientiexcoriata_HM125526
M_konradii_AY243603
M_mastoidea_(as"M_psammophila")_AY243600
M_mastoidea_AY243604
M_konradii_AY243601
M_konradii_AY243602
M_subsquamosa_AY243605
M_mastoidea_EU681783
M_mastoidea_HKAS50194
M_mastoidea_KF668312
M_mastoidea_HM125529
M_mastoidea_HM125531
M_mastoidea_HM125532
M_mastoidea_HM125530
M_mastoidea_(as"M_psammophila")_AY243599
M_mastoidea_AF482844
M_mastoidea_(as"M_excoriata")_U85313
M_mastoidea_(as"M_gracilenta")_MGU85314
/macrosporae
M_eucharis_AF482854
M_velosa_AF482853
M_velosa_HM125509
M_velosa_JN180320
/volvatae
Leucoagaricus_barssii_AF295931
Out group
0.02

Figure 3 (left): Molecular phylogenetic analysis of *Macrolepiota* spp. inferred by using the Maximum Likelihood method. Sequences generated from Pakistan are marked with ●. Genbank accession numbers of all the taxa are given. The percentage of trees in which the associated taxa clustered together at 1000 bootstraps 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 93 nucleotide sequences. There were a total of 712 positions in the final dataset.

Macrolepiota excoriata is closely related to *M. orientiexcoriata* Z.W. Ge et al. but forms a separate subclade within /macrosporae. *Macrolepiota* sect. *Macrosporae* is characterized by a long stipe, a simple annulus, and rare clamp connections. The species within this clade have furfuraceous fine squamules with rarely branched, light brown cylindrical hyphae. *Macrolepiota excoriata* (KJ013326) and *M. excoriata* (KJ643333) from Pakistan shared a 99.9% identity and clustered with *M. excoriata* from Israel (JQ683089, JQ683100, JQ683089), Italy (HM246504), the Netherlands (AF482840), the United Kingdom

Figure 4: Map showing distribution of *Macrolepiota dolichaula* (★) and *M. excoriata* (●). Open dots (⭘) represent reports of *M. excoriata* without voucher specimens.

(AY243606), and France (AY243607) with 99.7–99.9% identity and 0.2–0.3% genetic divergence.

Many *Macrolepiota* species have relatively small distribution areas (Courtecuisse & Duhem 1994; Nauta & Vellinga 1995; Candusso & Lanzoni 1990). Occurrence of *M. dolichaula* and *M. excoriata* in KPK, Pakistan extends the known distributions of these species considerably (Fig. 4). These two species were found in two different habitats dominated by obligatory mycorrhizal tree species. Mansehra district, KPK, lies in the moist temperate region dominated by *Pinus wallichiana* where the forest floor is dominated by different grass species. These coniferous forests are very similar to those in other parts of the northern temperate zone of Europe and America (Champion et al. 1965). Swat district, KPK, falls in the dry temperate region dominated by *Cedrus deodara* forests, in which there is no understory vegetation.

Although *Macrolepiota* species are generally very regional and are not widely distributed, *M. dolichaula* is an exception. We still lack enough data on the full distribution of these fungi to be able to predict their occurrence (or non-occurrence) in a certain area.

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