ISSN (print) 0093-4666 ISSN (online) 2154-8889

MYCOTAXON

http://dx.doi.org/10.5248/126.133

Volume 126, pp. 133–141 *October–December 2013*

Lepiota brunneoincarnata and *L. subincarnata:* distribution and phylogeny

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Abstract — An updated phylogeny of the clade of toxic *Lepiota* species is presented, and new insights in the distribution of *L. brunneoincarnata* and *L. subincarnata* are given. *Lepiota brunneoincarnata* is widespread in Europe and temperate Asia, and *L. subincarnata* is now known from Asia, Europe, and North America. Morphological and anatomical descriptions are provided for these two species based on material from the western Himalayan forests in Pakistan, where they are reported for the first time.

Keywords — amatoxins, lepiotaceous fungi, mushroom diversity, rDNA

Introduction

Among lepiotaceous fungi the genus *Lepiota* (Pers.) Gray (*Agaricales*, *Basidiomycota*) has a worldwide distribution and is highly diversified (Bon 1993; Ahmad et al. 1997; Vellinga 2003; Kirk et al. 2008; Razaq et al. 2012; Nawaz et al. 2013). The genus is characterized by having a scaly (rarely smooth) pileus, free lamellae, partial veil in the form of annulus, a universal veil, smooth white dextrinoid spores (in most species), and clamp connections (present in all but one or two species) (Vellinga 2001, Kumar & Manimohan 2009). The nature of the pileus covering elements and the spore shape are very important characters for infrageneric classification (Candusso & Lanzoni 1990; Bon 1993; Vellinga 2001, 2003). The pileus covering of *Lepiota* is a hymeniderm, a trichoderm, an epithelium made up of long or globose elements, or cutis-like (Vellinga 2001, 2003). Bon (1993) and Vellinga (2001) recognized two subsections in sect. *Ovisporae* (J.E. Lange) Kühner, a section morphologically characterized by ellipsoid spores and a trichodermal pileus covering based on structure and size of the pileus elements: subsect. *Helveolinae* Bon & Boiffard with a trichoderm made up of only longer pileus elements and subsect. *Felininae* Bon with long and basal short elements. Vellinga (2003) discovered that sect.

Ovisporae is not monophyletic: species of subsect. *Felininae* clustered with sect. *Lepiota*, while species of subsect. *Helveolinae* clustered with species with spurred spores in sect. *Stenosporae* (J.E. Lange) Kühner. The clade of subsect. *Helveolinae* is represented by the European species *L. brunneoincarnata*, *L. subincarnata*, and *L. farinolens* Bon & G. Riousset, plus the tropical species *L. elaiophylla* Vellinga & Huijser. Both *L. brunneoincarnata* and *L. subincarnata* are infamous for amanitin poisoning (Gérault & Girre 1975; Bresinsky & Besl 1985). Such poisonings from *Lepiota* species result in liver and kidney failure and, if not treated in time, death in humans (Khelil et al. 2010; Delacour et al. 2009; Donnelly et al. 2000; Haines et al. 1985).

Collected during fieldwork in the moist temperate forests of western Himalaya dominated by coniferous vegetation, *L. brunneoincarnata* and *L. subincarnata* have not previously been documented for Pakistan and are here described morphologically and molecularly. *Lepiota subincarnata* is known from several parts of North America, Europe, and eastern Himalaya, and *L. brunneoincarnata* occurs throughout Europe and in eastern China. ITS-rDNA sequences of these two species are now available from different regions of the world. The objective of this paper is to provide a precise description of the Pakistani material, to evaluate the phylogenetic relationship among all isolates of these species from Asia, Europe, and North America, and to place the species in a phylogenetic context.

Materials & methods

The basidiomata were carefully dug up using a knife and photographed in the field. Material was characterized morpho-anatomically and molecularly. For microscopic observation, sections were stained with Congo Red and Melzer's reagent. Dimensions were determined for 25 basidiospores, 20 basidia, 20 cheilocystidia, and 20 elements of pileus covering from each basidioma. The following abbreviations are used: avl for average length, avw for average width, Q for the basidiospore length-width ratio, and avQ for average of all Q. Drawings were made using a camera lucida attached to a compound microscope. Dried specimens were deposited in the LAH Herbarium, Department of Botany, University of the Punjab, Lahore.

The protocol of Extract-N-Amp (XNAP-2) (Sigma, St. Louis, MO, USA) was followed. Dried material of basidioma (approx. 1 mg) was taken in small PCR tubes to which 10 µl of extraction solution was added. These tubes were incubated at 65°C for 10 min then at 94°C for 10 min. After the addition of 10 µl dilution solution (XNAP-2), the tubes remained at room temperature for one hour. ITS regions of rDNA were amplified using the universal primer pair ITS1F and ITS4 (White et al. 1990, Gardes and Bruns 1993). PCR was performed in 25 µL reaction volume following Gardes and Bruns (1993). PCR product of the ITS1+5.8S+ITS2 region was directly sequenced in both directions using the same amplification primers (Macrogen, Korea).

All available ITS sequences of species in subsect. *Helveolinae* plus selected species in subsect. *Felininae* and sect. *Lepiota* were downloaded from GenBank. *Coprinus comatus*

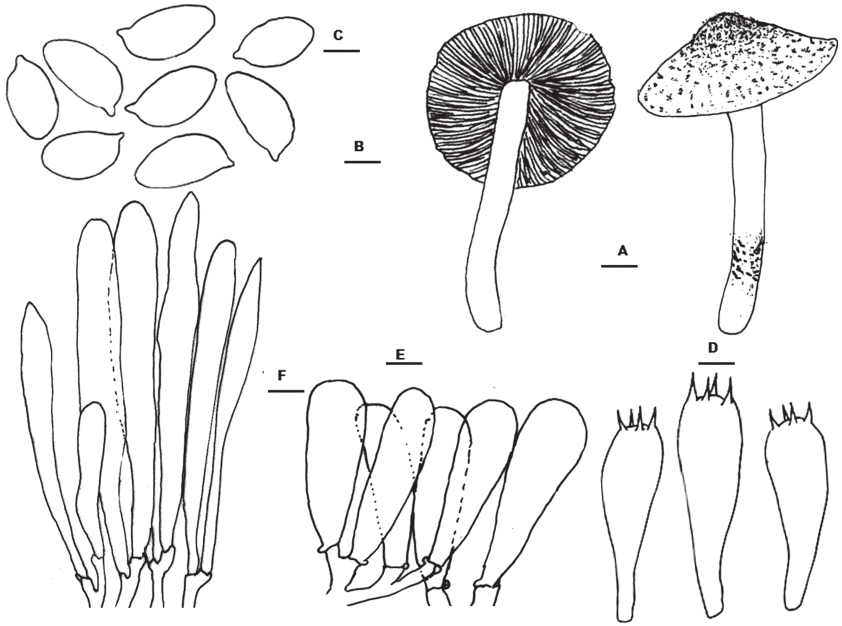

Fig. 1: *Lepiota brunneoincarnata*. A, B. Basidioma. C. Basidiospores. D. Basidia E. Cheilocystidia. F. Pileus covering elements. Scale bars: A, B = 1 cm; C = 3.45 µm; D = 6.25 µm; E = 13 µm; F = 21.25 µm.

was used as outgroup. The sequences were aligned using MAFFT version 6 (Katoh et al. 2002, Katoh & Toh 2008) with default settings. The total 34-sequence dataset was analyzed by a maximum likelihood (ML) method with RAxML 7.2.3 (Stamatakis et al. 2008). All free-model parameters were estimated by RAxML with a general time reversible (GTR) substitution matrix and a proportion of invariable sites estimated. One hundred ML bootstraps were performed.

The newly produced nucleotide sequences were submitted to the European Molecular Biology Laboratory (EMBL) database and are available in GenBank. Numbers are listed with the collections and in Fig. 3.

Results

Lepiota brunneoincarnata Chodat & C. Martin, Bull. Trav. Soc. Bot. Genève 5: 222. 1889. Fig. 1

Pileus 2.6 cm diam., campanulate to plano-convex, with obtuse to slightly umbonate central disc and deflexed margins; central disc with greyish-brown to dark brown scales on white to cream or pinkish buff background, around center concentric crowded and prominent squamules slightly paler or lighter

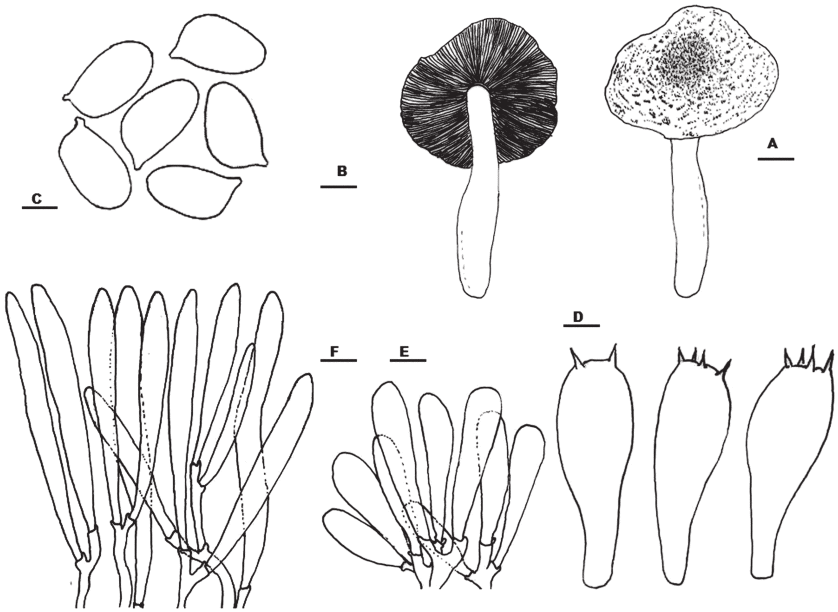

Fig. 2: *Lepiota subincarnata*. A, B. Basidioma. C. Basidiospores. D. Basidia. E. Cheilocystidia. F. Pileus covering elements. Scale bars: A, B = 1 cm; C = 3.45 µm; D = 6.25 µm; E = 13 µm; F = 21.25 µm.

toward margin, made up of small pyramids; margin smooth, at maturity fragile and broken. Lamellae free, moderately crowded, white to cream, with 2–3 tiers of lamellulae alternating with lamellae; edges wavy to denticulate. Stipe 4.2 × 0.3 cm, centrally attached, cylindrical, dark-brown to dull brown, light brown to pinkish brown in apical half, smooth in upper part, scaly in lower part; scales in bands forming incomplete girdles, non-persistent annulus; white basal mycelial strands. Taste not recorded.

Basidiospores 5–8.5 × 3.5–5.5 µm, avl × avw = 6.5 × 4.5 µm, Q = 1.4–1.5, avQ = 1.45, ellipsoid to ovoid, slightly amygdaliform, smooth, moderately thick-walled, colourless or pale yellow in 5% KOH, reddish brown in Melzer's reagent and strongly dextrinoid; wall colouring in Cresyl Blue not observed. Basidia 21–31.5 × 8–9.5 µm, 4-spored, subclavate to clavate, hyaline to pale yellow in 5% KOH, thin-walled, with oil-like contents. Pleurocystidia absent. Cheilocystidia 18.5–31 × 8–10.5 µm, usually clustered, hyaline, thin-walled, narrowly clavate to clavate. Pileus covering a trichoderm with erect or ascending, light brown to hyaline, clamped terminal elements, 90–253 × 7.5–13.5 µm, cylindrical to slightly narrowly fusoid, without any shorter elements at the base. Clamp connections common in all tissues.

Material examined: **PAKISTAN**, **Khyber Pakhtunkhwa**, Khanspur, 2250 m a.s.l., solitary, on moist ground under *Abies pindrow*, 23 August 2010, A. Razaq K-40 (LAH 230840; GenBank HE863668).

Lepiota subincarnata J.E. Lange, Fl. agar. dan. 5 (Taxonomic Conspectus): V, 1940. Fig. 2

Pileus 4.0–4.2 cm diam., hemispherical to plano-convex, with obtuse to slightly umbonate central disc, white, with thick context, with reddish brown to pinkish brown scales on white background on central disc, fading towards margins; margins smooth, sometimes splitting open at maturity. Lamellae free, crowded, white to cream, ventricose, with 3–4 tiers of lamellulae, with wavy to denticulate edge. Stipe 5.5 × 0.8 cm, centrally attached, cylindrical, vinaceous red to pinkish brown, hollow; surface smooth to scaly with fibrillose type scales below annular zone. Odour none. Taste not recorded.

Basidiospores 6–7.5 × 3.5–5 µm, avl × avw = 6.6 × 4.3 µm, Q= 1.5–1.7; avQ = 1.55, oblong ellipsoid to ellipsoid to ovoid, colourless or pale yellow in 5% KOH, reddish brown in Melzer's reagent and strongly dextrinoid, smooth, thin-walled. Basidia 19.5–28.5 × 7–8.5 µm, 4-spored, occasionally 2-spored, subclavate to cylindrical, hyaline, thin-walled with oil contents. Pleurocystidia absent. Cheilocystidia 18–35.5 × 7–11.5 µm, hyaline, thin walled, subclavate to clavate. Pileus covering a trichoderm with erect or ascending, hyaline to light brown, clamped terminal elements, 134–318 × 10.5–12.0 µm, cylindrical to slightly widened, with thickened apices, without any shorter elements at the base. Clamp connections present in all tissues.

Material examined: **PAKISTAN**, **Khyber Pakhtunkhwa**, Khanspur, 2250 m a.s.l., solitary, on moist ground under *Abies pindrow*, 23 August 2010, A. Razaq KP-09 (LAH 230809; GenBank HE863669).

Discussion

Bon (1993), Vellinga (2001), and the authors before them attached much importance to basidiospore shape for infrageneric classification in *Lepiota*. Sect. *Lepiota*, sect. *Ovisporae,* and species in sect. *Stenosporae* share a trichodermal pileus covering but differ in spore shape: fusiform-amygdaliform in sect. *Lepiota*, ellipsoid to oblong in sect. *Ovisporae,* and with a spur in sect. *Stenosporae*. Vellinga (2003) discovered that sect. *Ovisporae* is polyphyletic and that in general the pileus covering is a more significant character for infrageneric delimitation than the spore shape. Bon (1993) and Vellinga (2003) dealt mostly with European species.

In our present analysis, we included all available ITS sequences from different parts of the world and recovered the same results (Fig. 3). Species with a trichodermal pileus covering with long and short elements cluster together in Clade 2 (Fig. 3). This clade corresponds to sect. *Lepiota* and subsect. *Felininae*.

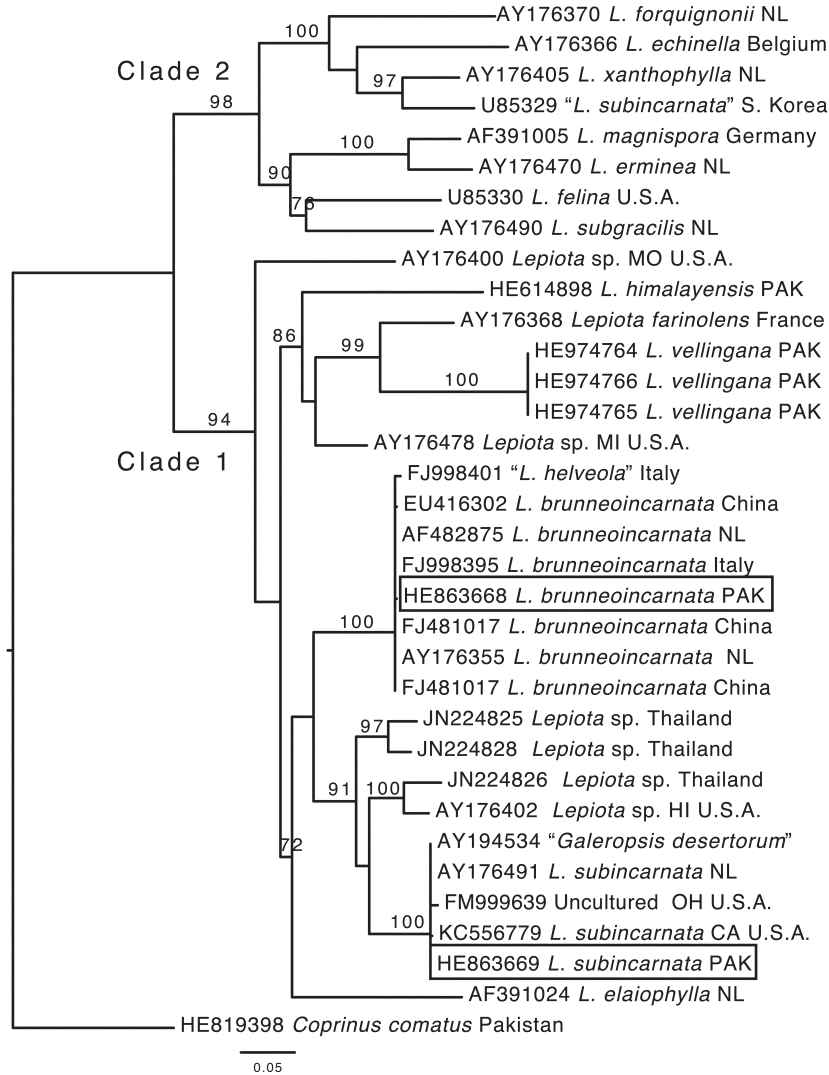

Fig. 3. Phylogenetic relationships of Pakistani collections of *L. brunneoincarnata* and *L. subincarnata* with other *Lepiota* species based on Maximum Likelihood analysis of nrITS sequences. Bootstrap values >70, based on 100 replicates shown at each node. The analysis involved 33 sequences with *Coprinus comatus* as outgroup. Names in quotation marks are discussed in the text. The two new Pakistani collections are outlined.

Species without these short pileus covering elements cluster in Clade 1, which corresponds to subsect. *Helveolinae*. The taxa in this clade are infamous for amatoxins (Vellinga 2003). Two recently described species from Pakistan — *L. himalayensis* Razaq & Khalid and *L. vellingana* Nawaz & Khalid — also cluster in Clade 1.

Both *L. brunneoincarnata* and *L. subincarnata* form well supported clades (both with 100% bootstrap support) and do not show any geographic structure, despite the fact that the sequences originate from very widely separated areas of Asia, Europe, and North America. It might be that ITS sequences alone are not sufficient, and data from other gene regions are needed to differentiate populations within the species.

Lepiota brunneoincarnata and *L. subincarnata* differ mainly in basidiocarp colours: *L. brunneoincarnata* has reddish brown or dark brown scales while *L. subincarnata* has nearly pinkish or vinaceous brown fine scales.

Lepiota brunneoincarnata from Pakistan, which clusters with isolates from Europe and China, is morphologically similar to European collections as described by Vellinga (2001). One GenBank sequence (FJ998401, labelled as *L. helveola* Bres.) falls within the *L. brunneoincarnata* clade, but the sequence is very short and it is likely that the specimen was misidentified.

The occurrence of *L. brunneoincarnata* in Pakistan suggests that it could be expected in other Asian countries; it is already known from China, based on sequence data, and from Israel (Kosakyan et al. 2008) and Turkey (Afyon & Yağiz 2004), based on morphological descriptions. This species is not yet known from North America, although a morphologically similar species was found in Ann Arbor (Michigan; GenBank AY176478).

The Pakistani collection of *L. subincarnata* is in close agreement with Vellinga's (2001) morphological description of Dutch collections. However, our collection is distinguished by longer pileal elements with thickened apices (11–20 μm), a difference that may be due to regional climate and geographical isolation.

In Asia, *Lepiota subincarnata* was previously known from Israel (Kosakyan et al. 2008) and from southwestern China (Yang 1990); the description of the Chinese material is similar to that of our Pakistani collection.

Kumar & Manimohan (2009) list *L. subincarnata* from Kerala, India, but their short description suggests a different species based on the dark brown colours and the pileus covering structure with short basal elements that are up to 400 μm long. A collection from South Korea identified as *L. subincarnata* (U85329; Johnson 1999) does not belong to *L. subincarnata* but falls among the Clade 2 species having a pileus trichoderm composed of longer and shorter cells (Fig. 1).

A sequence in GenBank (AY194534, labelled as *Galeropsis desertorum* Velen. & Dvořák), falls within the *L. subincarnata* clade. According to Hallen et al. (2003), this sequence is from a specimen collected in 1930 in Moravia (Czech Republic). It is possible that contamination or a laboratory mix-up might have caused this unexpected placement.

Acknowledgements

Authors are thankful to Dr. Junfeng Liang (Research Institute of Tropical Forestry, China) and Dr. Zai-Wei Ge (Chinese Academy of Science, China) for acting as expert reviewers to improve the manuscript. We are really thankful to Prof. Tom Bruns (Department of Plant and Microbial Biology, University of California, Berkeley) for providing space to complete this article. This work was financially supported by Higher Education Commission (HEC) of Pakistan under the "Indigenous Ph.D. Fellowship Scheme 5000 Phase IV" and International Research Initiative Support program (IRISP).

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