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A NEW FUNGISTIC RECORD OF *BOLETUS HIMALAYENSIS* - A MORPHOLOGICALLY COMPLEX PORCINI MUSHROOM FROM PAKISTAN

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Abstract

Porcini mushrooms (Boletus sect. Boletus) have both economic and ecological importance. During this study, a specimen of the phenotypically complex species *Boletus himalayensis* was analyzed morphologically and molecular genetically. This bolete species is characterized by a combination of porcini features: whitish pileus margin and context, pore surface and overall basidiomata having a whitish look before maturity and it has a considerably longer stipe compared to the pileus diameter when immature, with whitish reticulation extending longitudinally towards base. The whitish context and the white to white yellowish pore surface both do not change color upon bruising. Although the specimen exhibited a long stipe and a small non−cracked pileus as compared to other collections of *Boletus himalayensis*, molecular genetic analysis revealed that it belongs to this species.

Introduction

Moist temperate forests of Pakistan are considered as one of the biodiversity hotspots. Despite their importance, these forests are under of the least studied ones in terms of the diversity of macrofungi (Mirjam, 2010). The boletes are no exception, their diversity from the entire Himalayan region of Pakistan is only represented by some sporadic publications (Ahmad *et al*., 1997; Das *et al*., 2012; Das, 2013a, 2013b; Das and Chakraborty, 2014; Sarwar *et al*., 2011, 2012, 2014a, 2014b, 2014c, 2015, 2016, 2018 a, b, 2021 a, b, Hernández−Restrepo *et al*., 2016; Naseer *et al*., 2019). The present work presents a fungistic (=mycofloristic) record of a porcini mushroom from Pakistan. Porcini (*Boletus* section *Boletus*: Boletaceae: Boletineae: Boletales) are a conspicuous group of wild, edible mushrooms characterized by fleshy fruiting bodies with a poroid hymenophore that is "stuffed" with white hyphae when young (Dentinger *et al*., 2010). Together with its ectomycorrhizal plant symbionts they are distributed throughout the Northern Hemisphere (Dentinger *et al*., 2010; Sarwar *et al*., 2018a). Little progress has been made on the systematics of this group using modern molecular phylogenetic tools. Molecular genetic analysis supports the monophyly of the porcini group. Porcini mushrooms have a high diversity and worldwide distribution and are a group of commercially valuable mushrooms that may provide an economic incentive for conservation and support the hypothesis of a tropical origin of the ectomycorrhizal symbiosis (Dentinger *et al*., 2010; Pérez-Moreno, 2021).

A distinguishing feature of porcini boletes is their young mostly ventricose and later cylindrical stipe sometimes with an enlarged base, and with a raised netted pattern at least over the

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uppermost portion, and a layer of tangled white hyphae that covers the immature tubes (Dentinger *et al*., 2010). The taxonomy and classification of these taxa within this group is still confusing (Wang and Yao, 2005). Species in this group have a wide ecological range and a wide distribution pattern including Asia (importantly Pakistan, India, and China) (Thiers, 1975; Bessette *et al*., 2000; Oria de Rueda and Diez, 2002; Leonardi *et al*., 2005; Wang and Yao, 2005; Águeda *et al*., 2006, 2008; Arora, 2008; Beugelsdijk *et al*., 2008; Oria de Rueda *et al*., 2008). Research with molecular genetic data has been very useful in understanding morphological complexity, phylogenetic relationships and taxonomic issues within this group (Leonardi *et al*., 2005; Dentinger and McLaughlin, 2006; Beugelsdijk *et al*., 2008; Dentinger *et al*., 2010; Wu *et al*., 2014; Cui *et al*., 2015).

Materials and Methods

Site description and Collection of samples

During fungal field surveys to the Khyber Pakhtunkhwa (KPK) area, we collected an interesting bolete sample from the forests of the Hindu Kush foothills and Himalayan. Sampling areas included Malam Jabba Valley in Swat District and Ayubia and Nathia Galli in Abbottabad, Khyber Pakhtunkhwa Province. Specimens were collected in early summer (July) and the monsoon season, until the end of September. Field notes were done from fresh basidiomata and photographs were taken in their natural habitat. Colors were designated following Munsell (1975). Basidiomata were dried by keeping them near a fan heater and then kept in paper bags for processing in the laboratory. Specimens are deposited in the LAH Herbarium, Institute of Botany, University of the Punjab, Lahore, Pakistan.

Macromorphological and microscopic studies

Samples were studied macroscopically and microscopically in the laboratory following the methods described by Bessette *et al*. (2000), Ladurner and Simonini (2003), Muñoz (2005) and Dentinger *et al*. (2010). The following morphological characters were recorded from fresh fruiting bodies. Pileus: Diameter, shape, surface color, ornamentation, texture, color and bruising reaction of the context, margin color and shape. Stipe: Length and width, shape, color, ornamentation and texture, color and bruising reaction of the context, attachment of the stipe to the pileus, presence/absence of annulus on stipe. Hymenium: Color and size of pores and tubes, and bruising reactions of the pore surface. For plectological analysis a CXRII, Labomed, Labo America Inc., Fremont, CA, USA microscope was used. Small tissues of each specimen were mounted in lactic acid, KOH, Trypan blue, and Melzer's reagent and the length, width, shape, and contents of cytoplasm of basidiospores, basidia, hymenial cystidia, pileipellis and its terminal cells, and their color reactions were recorded. For the spore dimensions, the first values present the range of lengths and widths and Qm is the mean of Q (=length/width ratio of an individual spore). A total of 20 spores from two collections were measured.

Molecular genetic analyses

DNA was extracted from dried basidiomata by a modified CTAB method (Bruns, 1995). The nuclear ribosomal internal transcribed spacer (ITS) region was amplified using the primers pairs ITS1F/ITS4 (White *et al*., 1990; Gardes and Bruns, 1993). PCR conditions were 5 min denaturation at 95 °C followed by 35 cycles of annealing at 94 °C (1 min), 1.5 min at 55 °C, 1.5 min at 72 °C and a final extension at 72 °C for 5 min. After purifying PCR products and sequencing reactions, the sequencing reaction products were sent to TsingKE, China services. The sequencing chromatograms obtained were edited by comparing overlapping reads using BioEdit

(Hall, 1999) and compared to GenBank records using BLAST at NCBI (https://www.ncbi.nlm.nih.gov/). Sequences were aligned using the MUSCLE Alignment tool. Phylogenetic analyses were done with the maximum likelihood algorithm (Nei and Kumar, 2000) of sequences evolution using the model testing feature of MEGA6 software (Tamura *et al*., 2011). Bootstrap consensus tree was inferred from 1000 replicates, and corresponding bootstrap values >50% are shown in the tree (Fig. 3). *Boletus edulis* was used as outgroup.

Results and Discussion

Morphological analysis

Boletus himalayensis S. Jabeen, S. Sarwar & A. N. Khalid (**Figs 1-2)**

GenBank numbers: OP817154, OP817155

Macroscopic character description: Pileus 2–9 cm in diameter, pulvinate, convex to plano−convex, brownish red to orangish red, surface dry to slightly viscid, smooth, tomentose, sometimes cracked, whitish towards margin. Pileus margin entire, whitish, smooth, rimose, incurved to straight. Context whitish, no color change upon exposure or when bruised. Stipe considerably longer than pileus diameter, about 15 cm long, 2–3 cm thick, central, cylindrical or gradually becoming thicker towards base, straight or slightly curved near base, base itself tapering, whitish towards base, brownish to brownish red towards apex, whitish reticulated allover and mostly very prominent and composed of isodiametric meshes towards apex becoming longitudinally elongated towards base, solid, context whitish, no color change upon exposure or when bruised. Pore surface white, pores 2–3 per mm, circular, adnate and ascending, tubes 9–13 mm long, white to off–white, no color change upon bruising.

Microscopic character description: Basidiospores subfusiform to ellipsoid−elongate, smooth, thick−walled, light brown in KOH, inamyloid, with less prominent apiculus, (13.7–) 14.1–16.0 (–16.7) × (–4.2) 4.8–5.5 (–5.9) µm, [avX= 14.7 ± 0.8 × 5.1 ± 0.4 µm, Qm = 6.8, n = 2×20]. Basidia clavate, 2–4 sterigmate, sterigma long, thick walled, brown contents visible, 17.9–43.2 × 11.0–16.3 µm. Cheilocystidia 21.8–42.7 × 5.3–10.9 µm, clavate, a few spheropendeculate. Pleurocystidia absent. Pileipellis 3.4–6.1 µm in diam., consisting of cylindrical generative hyphae, cylindrical elongated cells observed, and, frequently septate and branched, very few subglobose cells also observed, constricted at the septa. Stipitipellis hyphae 1.4–8 µm in diam., cylindrical elongated cells observed, parallel and branched hyphae, septate and constricted at septa.

Material examined: Pakistan, Khyber Pakhtunkhwa province, Malakand division, Swat district, Malam Jabba, on soil near broadleaf trees, notably oaks, July 2021, Hira Bashir, MJ–02. Genbank OP817154. Pakistan: Khyber Pakhtunkhwa, Ayubia, 2350 m a.s.l., near *Abies pindrow* Royle, solitary, on soil, 19 June 2010, Sarwar S.B. # 76(LCWU0710) Genbank OP817155.

Molecular phylogenetic analysis **(Fig. 3)**

The consensus sequences of the ITS region obtained during this study and used in phylogenetic analysis were about 700 base pairs long, after trimming. We used mostly published species sequences in the final dataset of 29 samples including our consensus sequences. Sequences were BLAST searched at NCBI and showed maximum similarity (100% or almost 100%) with *Boletus himalayensis* (MF288902) meaning that our samples belong to this species. Phylogenetic evaluation the nrITS gene shows that the newly generated sequences OP817154 and OP817155 are nested within a clade containing both *B. reticuloceps* and *B. himalayensis* with strong bootstrap values.

Fig. 1. *Boletus himalayensis* (macroscopic features). A–C, Basidiomata (Swat and Ayubia collection) showing pileus, stipe and hymenium features. Scale Bars: for A–C = 1 cm.

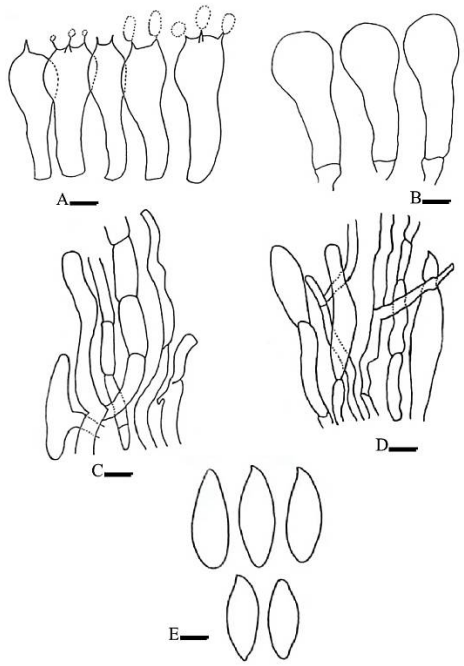

Fig. 2. *Boletus himalayensis* (microscopic features*)*. (Swat collection) A. Basidia; B. Cystidia; C. Pileipellis; D. Stipitipellis; E. Basidiospores. Scale Bar: A–E = 10 µm.

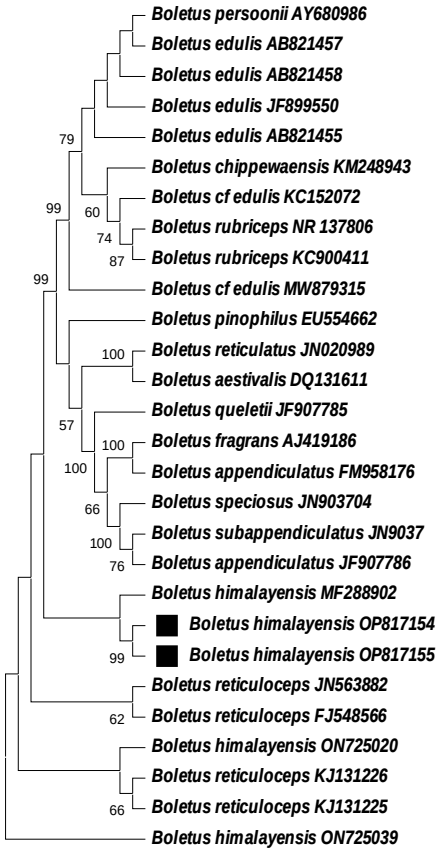

Fig. 3. Phylogenetic position of *Boletus himalayensis* with related species. Tree inferred by maximum likelihood analysis based on nrDNA ITS sequences. The numbers against branches indicate the percentage (>50%) at which a given branch was supported in 1000 bootstrap replications. GenBank accession number are given at the end of species names. ■ indicate newly generated sequences.

In the present study, *Boletus himalayensis*, which was described in 2018 from the Himalayas, could be found again and the analysis shows that it is morphologically quite variable by sometimes having a long stipe and a smooth pileus as compared to the cracked pileus and short stipe in previously reported *B. himalayensis* specimens, but genetically these collections all belong to this species (Sarwar *et al*., 2018a). Other closely related species, both morphologically and phylogenetically, are *B. pinophilus* and *B. reticulatus* (Sarwar *et al*., 2018a; Thiers 1975, Wang and Yao, 2005). A special note has to be made about *B. reticuloceps*. Among these taxa, the closest one is *B. reticuloceps*, but the characters that differentiate *B. himalayensis* from the former are a rugulose pileus and a gradually broader stipe base in the former as compared to long stipe with narrow base in the latter (Wang and Yao, 2005). *Boletus reticuloceps*, which appears to be seeminlgy intermixed with *B. himalayensis* in our phylogenetic analysis, was originally described in the genus *Aureoboletus*, as having a reddish yellow pore surface changing to brownish upon bruising, which is in contradiction to *B. himalayensis*. One explanation for the observed mix could be that samples of *B. himalayensis* have been misidentified. The second possibility would be that *B. reticuloceps* is actually correctly placed in the genus *Boletus* s. str. and thus *B. himalayensis*

would become a later synonym. However, the morphological discrepancy of the different pore color and discoloration would then remain. This taxonomic question can only be resolved by studying the type of *B. reticuloceps* in the future.

Anatomically, like other *Boletus* s. str. species, the samples analyzed during this study had sterile tube edges having long, dense clusters of cheilocystidioid elements and a trichoderm, a pileipellis composed of a layer of long, erect cylindrical or few-branched hyphae. Molecular phylogenetic analyses based on ITS provide strong support that our two samples investigated belong to *B. himalayensis* with some morphological variations. This variability very likely is due to the weather conditions at site with a cracking pileus and short stipe in dry weather conditions and a longer stipe and smooth pileus when moist, mainly due to monsuun season.

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