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First record of *Boletus himalayensis* (*Basidiomycota, Boletaceae*) from Kalatop Wildlife Sanctuary, Himachal Pradesh, India

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ABSTRACT

A member of porcini mushroom was collected from Western Himalayan region of Himachal Pradesh and critical literature survey, taxonomic investigation along with nrITS based phylogenetic analysis establish that this is the first report of *Boletus himalayensis* from India. A detailed morphological description, illustration, molecular phylogeny along with taxonomic note is given.

Keywords: Macrofungi, nrITS, Phylogeny, Taxonomy

INTRODUCTION

Members of the *Boletaceae* are considered to be one of the largest groups of fleshy poroid mushrooms. They have immense importance in forest ecosystem as they are largely ectomycorrhizal and also economically important due to its edible members which are taken with delicacy across the continent. With the advent of molecular phylogeny and integrated morphology, members of this family are distributed into seven major clades and about 70 genera (Kirk *et al.,* 2008; Feng *et al.,* 2012; Wu *et al.,* 2014; Cui *et al.,* 2016). The genus *Boletus* L. s.s. commonly known as porcini mushrooms, comes under subfamily *Boletoideae* featured by its stuffed pores at young stage, unchanging context color (when exposed), smooth basidiospores and it is considered polyphyletic (Dentinger *et al.,* 2010; Feng *et al.,* 2012; Nuhn *et al.,* 2013; Wu *et al.,* 2014).

The Himalayan region is considered as a biodiversity hotspot and also shows mycofloral diversity. Kalatop Wildlife Sanctuary (KWLS) is a *Cedrus* dominated forest situated at an altitude of 2400 m (a.s.l.) in Chamba district of Himachal Pradesh, India. During a routine macrofungal survey to KWLS in July 2021, an interesting member of genus *Boletus* was collected and after detailed morphological study coupled with nrITS based phylogenetic analysis confirmed its conspecificity and identity with a recently established species namely, *Boletus himalayensis* S. Jabeen, S. Sarwar & A. N. Khalid from Pakistan but hitherto unrecorded (Sarwar *et al.,* 2018). In this communication *B. himalayensis* is being reported for the first time from India with macro- and micromorphological illustrations, phylogenetic analysis and comparisons with allied taxa.

MATERIALS AND METHODS

Macro- and micromorphology observations

Fresh basidiomata (young to mature) were collected during mushroom forays to KWLS of Himachal Pradesh in July, 2021. Macromorphological characters were recorded in the forest and at the basecamp from fresh and dissected fruitbodies. Field photographs of basidiomata were taken with a Sony DSC-RX100 camera. Colors were coded as per the Methuen Handbook of Colour (Kornerup and Wanscher, 1978). Samples were dried with a field drier. Micro-morphological characters were observed with a compound microscope (Olympus CX 41) from free hand sections of dried materials mounted in a solution of 5% KOH, 1% Phloxin and 1% ammoniacal Congo red. Drawings of the anatomical features were made with a drawing tube at 1000× magnification. Microscopic photographs were taken with an Olympus BX 53 camera. The basidiospores were measured in lateral view. Basidiospore measurements and length/width ratios (Q) are recorded as: minimum mean maximum. Basidium length excludes the length of sterigmata.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from 100 mg of dried basidiomes with the InstaGeneTM Matrix Genomic DNA isolation kit (Biorad, USA), following the manufacturer's instructions. The nrITS gene region was amplified with primer pairs ITS1-F and ITS4 (White *et al.,* 1990; Gardes and Bruns, 1993). PCR protocol for the amplification of ITS regions followed after Chakraborty *et al.* (2022). The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany). Both strands of the PCR fragment were sequenced on the 3730xl DNA Analyzer (Applied Biosystems, USA) using the amplifying primers. The sequence quality was checked using Sequence Scanner Software v. 1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious v. 5.1 (Drummond *et al.,* 2010). All sequences newly generated in this study were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

RESULTS

Phylogenetic analysis

The newly generated ITS sequences of *B. himalayensis* from India and its closely related species were retrieved from nBLAST search against GenBank (https://www.ncbi.nlm.nih.gov/genbank) and relevant published literature (Sarwar *et al.,* 2018). The ITS dataset was aligned using the online version of the multiple sequence alignment program MAFFT v. 7, with the L-INS-i strategy (Katoh *et al.,* 2019) with default settings and then trailing ends of the alignment trimmed manually with MEGA v. 7 (Kumar *et al.,* 2016). To eliminate ambiguously aligned positions in the alignment as objectively as possible, Gblocks 0.91b (Talavera and

Castresana, 2007) was used. The program was run with settings allowing for smaller blocks, gaps within these blocks and less strict flanking positions. The dataset was phylogenetically analyzed using the Maximum Likelihood (ML) method. ML analysis was performed using raxmlGUI 2.0 (Edler *et al.,* 2021) with the GTRGAMMA substitution model. The ML analysis was executed applying the rapid bootstrap algorithm with 1000 replicates to obtain nodal support values.

Phylogenetic inferences

The ITS data matrix comprised a total of 56 sequences. The alignment comprised 584 characters including gaps. Our phylogenetic analysis shows that sequences derived from our Indian *Boletus himalayensis* (GenBank accession nos. ON725020 and ON725039) are nested (without a support) within the *B. himalayensis* clade (indicated with a blue arrow in **fig. 1**) consisting of collections from Pakistan (GenBank accession nos. KJ131225, KJ828812, KJ131226, MF288902, MF288903) and an Indian collection (GenBank accession no. MK402159; identified as '*Boletus reticuloceps*') suggesting its strong similarity and conspecificity with the Asian species: *B. himalayensis*.

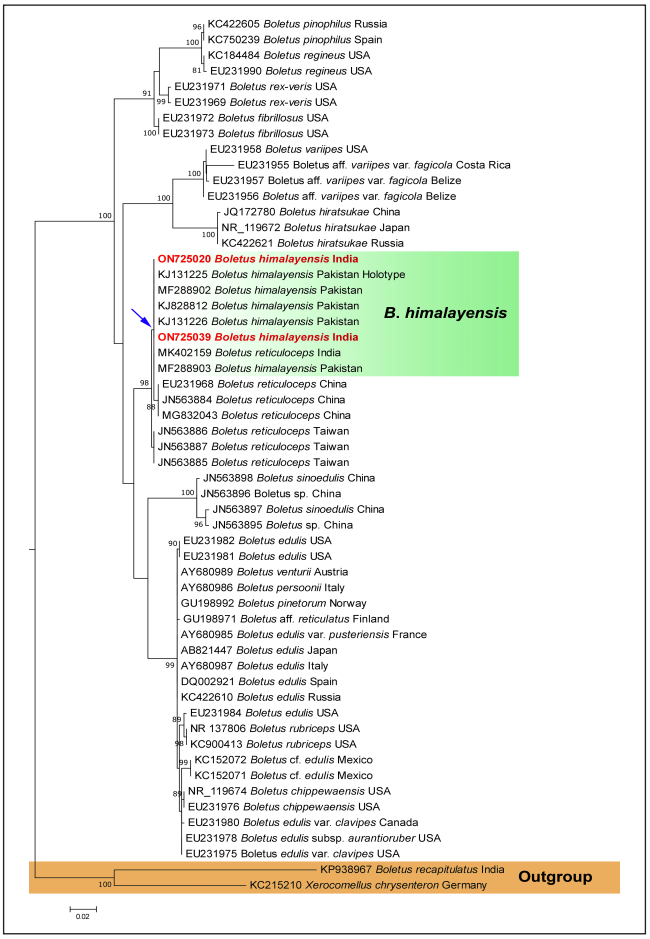

Fig. 1: Phylogeny of *Boletus himalayensis* and their allied species based on a Maximum Likelihood (ML) inference of ITS gene sequences. *Boletus recapitulatus* and *Xerocomellus chrysenteron* are used as outgroup taxa. ML Bootstrap percentage (MLB) ≥70% are shown on the above or below the branches at nodes. Three Indian collections of *B. himalayensis* are highlighted in red and bold font in the phylogram.

Taxonomy

Boletus himalayensis Jabeen & Sarwar, Khalid, *Turkish Journal of Botany* **42:** 791, 2018 (**Fig. 2 & 3**)

Pileus 20-110 mm diam., convex, surface smooth to subvelvety when young, gradually squamulose or becoming areolate at or surrounding the centre with maturity, light brown (6D7-8) when young, gradually apricot yellow (5B7, 6B6) to yellow brown or darker with maturity, copper red to burnt sienna (7C8-D8) with KOH; margin entire, decurved with narrow sterile flap of tissue. Pore surface yellowish white (2A2), depressed at stipe juncture, becoming golden yellow (5B5-7) at maturity, or when wet; pores rounded, 2-32/mm, covered by a white tomentum when young. Tubes sinuate, 2-11 mm long, light yellow (4A4-4B4), no change on exposure. Stipe 30-70 × 12-25 mm, subclavate to cylindric with tapering base mostly bent, golden yellow (5B7) at the upper one third of the stem, faded towards the base; surface with a shallow or coarse white reticulation that is most pronounced at the apex, basal mycelium white. Context 7-18 mm thick in pileus, solid, yellowish white, no color changing when exposed., apricot (yellow) (5B6) with KOH, no color change with $FeSO_4$. Taste none. Spore print not found.

Basidiospores 13-16.0-18.6 × 5-4.3-7 µm, (n= 30, 2.4-2.61-3.54), elliptic to oblong, thin-walled, smooth under light microscope. Basidia 25-35 × 10-13 µm, 4-spored, clavate; sterigmata 2 µm wide. Pleurocystidia 28-42 × 6-9 µm, cylindrical to ventricose with fusoid, to rounded apices, thin walled. Tube edge fertile with cystidia and cystidoid eliments; cheilocystidia 52-55 × 7-8 µm, subfusoid, to cylindrical, in a cluster with cystidoid eliments when young. Tube trama divergent, gelatinous. Pileipellis 280-300 µm thick, a trichoderm composed of erect hyphae, unbranched, septate; terminal cell cylindrical with rounded to subfusoid apex. Stipitipellis 40-60 µm thick; hyphae erect, in tufts, fertile near apex, composed basidia and cystidia; basidia 4-spored, clavate; caulocystidia 30-43 × 8-17 µm, mostly clavate, cylindrical with rounded apex. Clamp are absent in all parts of basidiomata.

Habit and Habitat: Scattered to subgregarious in coniferous forest dominated by *Cedrus deodara*.

Distribution: This species was originally described from Pakistan (Kiran *et al.,* 2018), and is now known from India.

Specimens examined: India, Himachal Pradesh, Chamba, Kalatop, 32°32.550′N, 76°01.317′E, alt. 2380 m a.s.l., 18 July 2021, Dyutiparna Chakraborty, DC 21-26; Chamba district, Khajjiar, 32°32.514′N, 76°03.455′E, alt. 1930 m a.s.l., 21 July 2021, Dyutiparna Chakraborty, DC 21-42.

Discussion: The combination of characters like tapering stipe base, reticulation over stipe surface, yellowish brown pilus,

Fig. 2: *Boletus himalayensis*. a-c. Fresh basidiomata in the forest and in basecamp; d. Pileipellies; e,f. Pleurocystidia; g. Caulocystidia; h. Basidiospores. Scale bars: d = 50 µm; e-g = 10 µm.

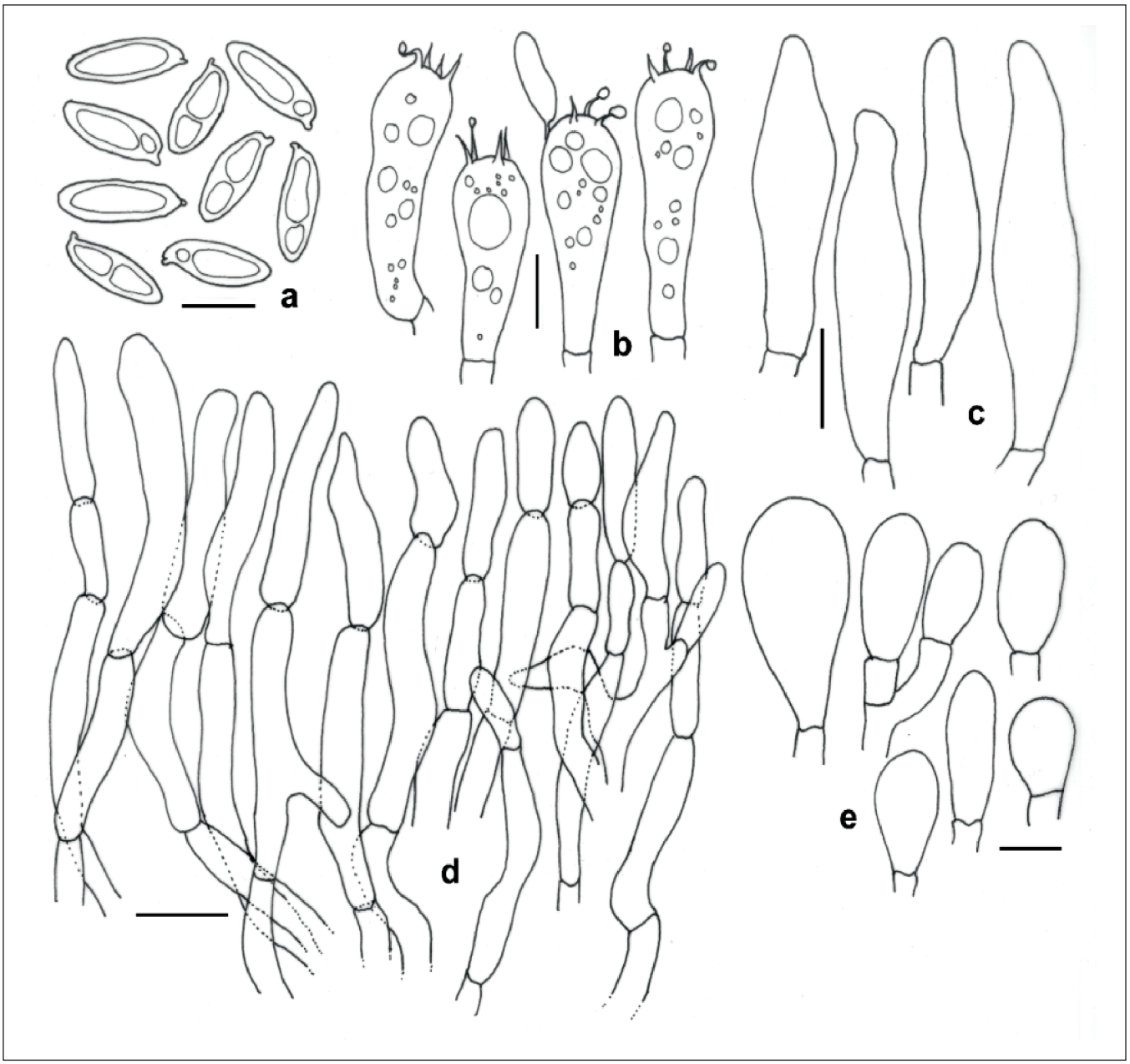

Fig. 3: Line drawings of *Boletus himalayensis*. a. Basidiospores; b. Basidia; c. Pleurocystidia; d. Pileipellis; e. Caulocystidia. Scale bars: a-c, e = 10 µm; d= 25 µm.

white stuffed pore surface at young stage, no color change of context on exposure and ectomycorrhizal association with *Cedrus deodara* completely agrees with the morphological description from the holotype (Sarwar *et al.,* 2018) except the much longer (30-70 mm) stipe than the holotype having shorter stipe (up to 47 mm) and size of pleurocystidia (21.5-31 × 6-8 µm) of the holotype is lesser than the Indian collections. Moreover, nrITS based phylogenetic analysis shows 99.86% similarity with the holotype (GenBank accession no. KJ131225) of *B. himalayensis* from Pakistan. An Indian collection (GenBank accession no. MK402159) was previously submitted to the GenBank as *Boletus reticuloceps* (M. Zang, M.S. Yuan & M.Q. Gong) Q.B. Wang & Y.J. Yao which is considered in this study as *B. himalayensis* because it shows 100% similarity with our collections from Kalatop and nested well in *B. himalayensis* clade (indicated with green highlight in **fig. 1**) in our phylogenetic tree.

A porcini member from India, *Boletus indoedulis* D. Chakr., K. Das, A. Baghela, A. Adhikari & Halling can easily be differentiated from *B. himalayensis* by its bulbous stipe which is wider at base (30-75 mm), ectomycorrhizal association with *Quercus* sp. and smaller, oblong basidiospores (10-14 × 3-4 µm) (Chakraborty *et al.,* 2017). Another closely related species *Boletus sinoedulis* B. Feng, Y.Y. Cui, J.P. Xu & Zhu L.

Yang differs from this newly reported species by its yellowish pore surface with maturity and absence of caulocystidia and caulobasidia (Cui *et al.,* 2016).

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