

Boletus himalayensis (Basidiomycota; Boletales), a new porcini species from Pakistan

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Abstract: Morphology and phylogenetic analyses using the ITS and LSU regions of nrDNA were carried out on bolete collections belonging to *Boletus* s. str., originating from the Himalayan region of Pakistan. The phylogenetic analysis outlined the existence of previously unknown species of this genus. It is described here as *Boletus himalayensis*. Illustrations of the macroscopic characters and the microscopic features of the new species are provided. The distinction of *B. himalayensis* from allied species is discussed.

Key words: *Boletus* s. str., molecular analysis, morphology, phylogeny, taxonomy

1. Introduction

The Himalayan region is considered a biodiversity-rich hotspot and an ecosystem of global importance. Despite its importance, this region is one of the least studied in terms of the diversity of macrofungi (Mirjam, 2010). The boletes are no exception. Bolete diversity from the entire Himalayan region 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; Hernández-Restrepo et al., 2016).

The present work is an attempt to determine the diversity of the genus *Boletus* L. (Boletales, Boletaceae), which is considered polyphyletic (Nuhn et al., 2013). In this genus, all porcini (*B. edulis* and allies) form a group, *Boletus* s. str., and the remaining species have instable state (Dentinger et al., 2010a). Many species have been described within *Boletus* s. str. based on morphological characters, but all these characters are mostly like typical of fleshy pored mushrooms and not unique to this group except a stalk with an enlarged base and a raised netted pattern at least over the uppermost portion, and a layer of tangled white hyphae that covers the immature tubes (Dentinger et al., 2010a); and hence the taxonomic and classification status of species within this group is confusing (Wang and Yao, 2005). The literature showed that species in *Boletus* s. str. 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). Recent research with molecular data has been very useful in understanding phylogenetic relationships and resolving taxonomic issues within this group (Leonardi et al., 2005; Dentinger and McLaughlin, 2006; Beugelsdijk et al., 2008; Dentinger et al., 2010a; Wu et al., 2014; Cui et al., 2015).

The major goals of this study were assessing bolete diversity in Pakistan and contributing to the knowledge on boletes in this part of Asia by description of a new species at molecular level and also highlighting the important hidden diversity found in Pakistan.

2. Materials and methods

2.1. Collection of samples

Specimens were collected during 2011–2013 in early summer (July) and the monsoon season, until the end of September. Field notes were recorded from fresh basidiomes and digital photographs were taken in their habitat. Color codes were designated following Munsell (1975). Basidiomata were then dried with hot air by keeping them near a fan heater. After drying, specimens were kept in paper bags for processing in the lab. Voucher specimens were deposited in the Zahoor Memorial

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Herbarium (LAH), Department of Botany, University of the Punjab, Quaid-e-Azam campus, Lahore, Pakistan.

2.2. 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), and Muñoz (2005). The following morphological characters were recorded from fresh fruiting bodies.

Pileus: Diameter, shape, 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, 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 Q_m is the mean of Q (=length/width ratio of an individual spore). A total of 30 spores from each collection were measured. Voucher specimens were deposited in the Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan (LAH).

2.3. Molecular analyses

DNA was extracted from dried basidiomata by enzymatic digestion and glass-fiber filtration (EDGF) protocol following Dentinger et al. (2010b); for some samples, a modified CTAB method was used (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). For LSU amplification, LR0R as forward and LR5 as reverse primers were used (Ge et al., 2014). After purifying PCR products and sequencing reactions, the sequencing chromatograms obtained were edited by comparing overlapping reads using BioEdit (Hall, 1999) and compared to GenBank records (Table) using BLAST at NCBI (<https://www.ncbi.nlm.nih.gov/>). Multiple sequences were aligned using the Goldman Group Software-PRANK (Probabilistic Alignment Kit) at the European Bioinformatics Institute web (<http://www.ebi.ac.uk/goldman-srv/webprank/>). Phylogenetic analyses were done with the maximum likelihood algorithm and general time-reversible model (Nei and Kumar, 2000) of sequences evolution using the model testing feature of MEGA5 software (Tamura et al., 2011). Bootstrap consensus trees were inferred from 1000

replicates, and corresponding bootstrap values >50% are cited in the tree (Figures 1 and 2).

3. Results

3.1. Taxonomy

Boletus himalayensis S. Jabeen, S. Sarwar & A. N. Khalid *sp. nov.* Figures 3 and 4

Mycobank Number MB 820806

GenBank number: for ITS KJ131225; for LSU MF288904

Etymology: The specific epithet refers to the Himalayan range, from where the sample was first collected.

Holotype: PAKISTAN. Khyber Pakhtunkhwa Province, Malakand Division, Swat District, Mashkun, 2500 m asl, on soil under *Cedrus deodara*, 5 Sep 2013, Sana Jabeen and Abdul Nasir Khalid SJ6 (LAH35028; GenBank for ITS KJ131225; for LSU MF288904)

Diagnosis: This fungus resembles *B. reticuloceps*, *B. edulis*, and *B. sinoedulis* B. Feng, Yang Y. Cui, J.P. Xu and Zhu L. Yang, but is distinguished from all these species by a narrow stipe base as compared to them and differences in ITS region.

Description: Pileus up to 6.5 cm wide, convex to plano-convex; surface dry, smooth, sometimes cracked, whitish context visible between cracks, yellowish brown (10YR7/8) to dark brown (10R6/16), margin entire, rimose, incurved to straight. Tubes free, 11–18 mm deep, off white to yellowish brown (2.5Y7/6), pores rounded, up to 3 per mm. Stipe up to 4.7 × 2.4 cm, base and apex tapering up to 1 cm, central, stipe covered with well-developed reticulum, reticulum off white on yellowish to brownish background at the upper 1/3 of the stipe, basal part off white, nonreticulated. Context whitish, no color change to brownish upon exposure. Odor and taste not distinctive.

Basidiospores (16.3) 17–19 (20.7) × (6) 7–8 μm , $Q_m = 2.7$, ellipsoid, smooth, apiculus prominent. Basidia 21–32 × 12–14 μm , clavate 4-spored, thin walled, guttulate, hyaline 5% KOH. Cystidia, long, 21.5–31 × 6–8 μm , densely clustered at the edges of tubes, clavate to subclavate or cylindrical, hyaline in 5% KOH. Bilateral tube trama divergent from the central strand, thin walled, clamp connections absent, sterile, very distinct, trama composed of long dense cluster of cheilocystidioid elements that often intermingle with the adjoining tubes to give distinct hairy mat beyond tubes, terminal cells 25–60 μm long, cylindrical to lanceolate or ventricose with rounded, fusoid to subfusoid, or slightly appendiculate apex, stuffed pores, subhymenium pseudoparenchymatous. Pileipellis of interwoven hyphae, protruding out in the form of hyphal clusters, septate, terminal elements of hyphae erect, hyaline in 5% KOH. Stipitipellis hyphae thin, filamentous,

Table. Information of ITS and LSU sequences retrieved for phylogenetic purpose, their sequence accession number, country of origin, host tree/habitat, and reference to published work where those sequences were obtained.

Taxon	Sequence accession number		Location	Host tree	Reference
	LSU	ITS			
<i>Phylloporus pumilus</i>		JQ003627	Indonesia		
<i>Boletus edulis</i> var. <i>pusteriensis</i>		AY680985	France: Montminal		Leonardi et al. (2005)
<i>B. edulis</i>		AY680987	Italy: Pattanella, Grosseto		Leonardi et al. (2005)
<i>B. edulis</i>		EU231981	USA: CA	<i>Pinus muricata</i>	
<i>B. edulis</i>		EU231982	USA: OR	<i>Pinus contorta</i>	
<i>B. venturii</i>		AY680989	Austria		Leonardi et al. (2005)
<i>B. persoonii</i>		AY680986	Italy	<i>Castanea sativa</i>	Leonardi et al. (2005)
<i>B. pinetorum</i>		GU198992	Finland	<i>Pinus sylvestris</i>	Korhonen et al. (2009)
<i>B. aff. reticulatus</i>		GU198971	Finland	<i>Quercus, Tilia, Corylus, Betula</i>	Korhonen et al. (2009)
<i>B. edulis</i>		KC422610	Russia		Fedosova and Kovalenko (2014)
<i>B. edulis</i>		DQ002921	Spain	<i>Cistus ladanifer</i>	A'gueda et al. (2006)
<i>B. edulis</i>		AB821447	Japan		Endo et al. (2014)
<i>B. edulis</i> var. <i>clavipes</i>		EU231980	Canada: ON		
<i>B. edulis</i> var. <i>clavipes</i>		EU231975	USA: NY	<i>Picea abies</i>	
<i>B. edulis</i> sub sp. <i>aurantioruber</i>		EU231978	USA: MI		
<i>B. chippewaensis</i>		EU231976	USA: MI		
<i>B. chippewaensis</i>		NR119674	USA		
<i>B. cf. edulis</i>		KC152072	Mexico		
<i>B. cf. edulis</i>		KC152071	Mexico		
<i>B. edulis</i>		EU231984	USA: CO	<i>Picea</i> sp.	
<i>B. rubriceps</i>		NR137806	USA: NM	<i>Abies, Picea, Pinus</i>	Arora and Frank (2014a)
<i>B. rubriceps</i>		KC900413	USA: NM	<i>Abies, Picea, Pinus</i>	Arora and Frank (2014a)
<i>B. fibrillosus</i>		EU231972	USA: CA	<i>Pseudotsuga menziesii, Tsuga heterophylla, Abies grandis, Notholithocarpus densiflorus</i>	
<i>B. fibrillosus</i>		EU231973	wUSA: CA	<i>Pseudotsuga menziesii, Tsuga heterophylla, Abies grandis, Notholithocarpus densiflorus</i>	
<i>B. rex-veris</i>		EU231971	USA: CA	<i>Pinus ponderosa, Pinus lambertiana, Pinus contorta, Abies concolor</i>	

<i>B. rexveris</i>		EU231969	USA: WA	<i>Pseudotsuga menziesii</i> , <i>Picea engelmannii</i> , <i>Pinus ponderosa</i> , <i>Abies</i> sp.	
<i>B. regineus</i>		KC184484	USA: OR, Williams	<i>Arbutus</i>	Arora and Frank (2014b)
<i>B. regineus</i>		EU231990	USA: CA	<i>Quercus</i> , <i>Willits</i>	
<i>B. variipes</i> var. <i>fagicola</i>		EU231963	USA: PA	<i>Conium maculatum</i> , <i>Quercus rubra</i> , <i>Fagus</i>	
<i>B. variipes</i> var. <i>fagicola</i>		NR119673	USA: Michigan	scattered to <i>Fagus</i> , <i>Acer</i> , <i>Populus</i> sp.	
<i>B. variipes</i>		EU231961	USA: NY	<i>Quercus</i> , <i>Castanea</i>	
<i>Porphyrellus brunneus</i>	JX889646		Australia: Queensland		Halling et al. (2012)
<i>Boletus edulis</i>	KF112455		Russia		
<i>B. edulis</i>	AF462353				Jarosch and Bresinsky (2000)
<i>B. edulis</i>	AF462352				Jarosch and Bresinsky (2000)
<i>B. edulis</i>	DQ071747				Garnica et al. (2007)
<i>B. edulis</i>	AF291300		Germany		Weiß and Oberwinkler (2001)
<i>B. edulis</i>	AF462354				Jarosch and Bresinsky (2000)
<i>B. rubriceps</i>	KC900411		USA: AZ		Arora and Frank (2014a)
<i>B. edulis</i> var. <i>grandedulis</i>	KC184482		USA: CA		Arora and Frank (2014a)
<i>B. edulis</i> var. <i>grandedulis</i>	KC900423		USA: NM		Arora and Frank (2014a)
<i>B. rubriceps</i>	KC900408		USA: CO		Arora and Frank (2014a)
<i>B. rubriceps</i>	KC900407		USA: CO		Arora and Frank (2014a)
<i>B. rubriceps</i>	KC900412		USA: AZ		Arora and Frank (2014a)
<i>B. himalayensis</i>	MF288904		Pakistan	<i>Cedrus deodara</i>	
<i>B. reticuloceps</i>	JN563843				Feng et al. (2012)
<i>B. reticuloceps</i>	KF112454		China		
<i>B. fibrillosus</i>	KF030344		USA: CA		Nuhn et al. (2013)
<i>B. fibrillosus</i>	KF030343		USA: WA		Nuhn et al. (2013)
<i>B. aurantioruber</i>	KF030342		USA: MA		Nuhn et al. (2013)
<i>B. subcaerulescens</i>	KF030341		USA: NCES, NY		Nuhn et al. (2013)
<i>B. pinophilus</i>	AF462358				Jarosch and Bresinsky (2000)
<i>B. subalpinus</i>	KF030340				Nuhn et al. (2013)
<i>B. regineus</i>	KC184485		USA: OR		Arora and Frank (2014b)
<i>B. aereus</i>	KF030339		USA: CA		Nuhn et al. (2013)
<i>B. edulis</i>	AF071457				Grushiba et al. (2001)
<i>B. pinophilus</i>	AF462359				Jarosch and Bresinsky (2000)
<i>B. barrowsii</i>	KC184480		USA: NM		Arora and Frank (2014b)
<i>B. sp.</i>	JN563847				Feng et al. (2012)
<i>B. cf. edulis</i>	AF462355		Turkey		Jarosch and Bresinsky (2000)
<i>B. sp.</i>	JN563852				Feng et al. (2012)
<i>B. sp.</i>	JN563851				Feng et al. (2012)

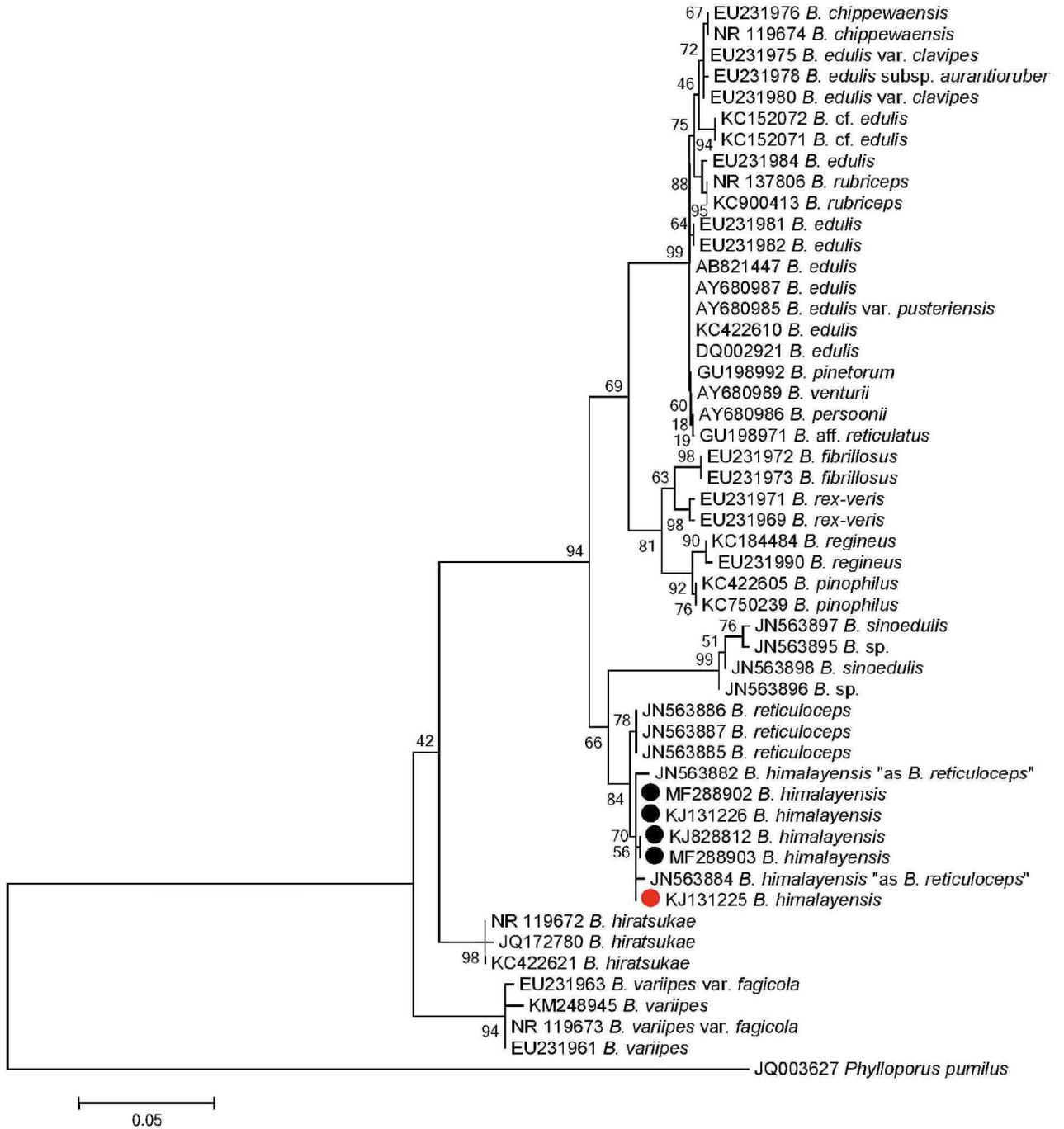


Figure 1. Molecular phylogenetic analyses based on ITS sequences. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Sequences generated during this study are marked with bullets. Red color represents holotype.

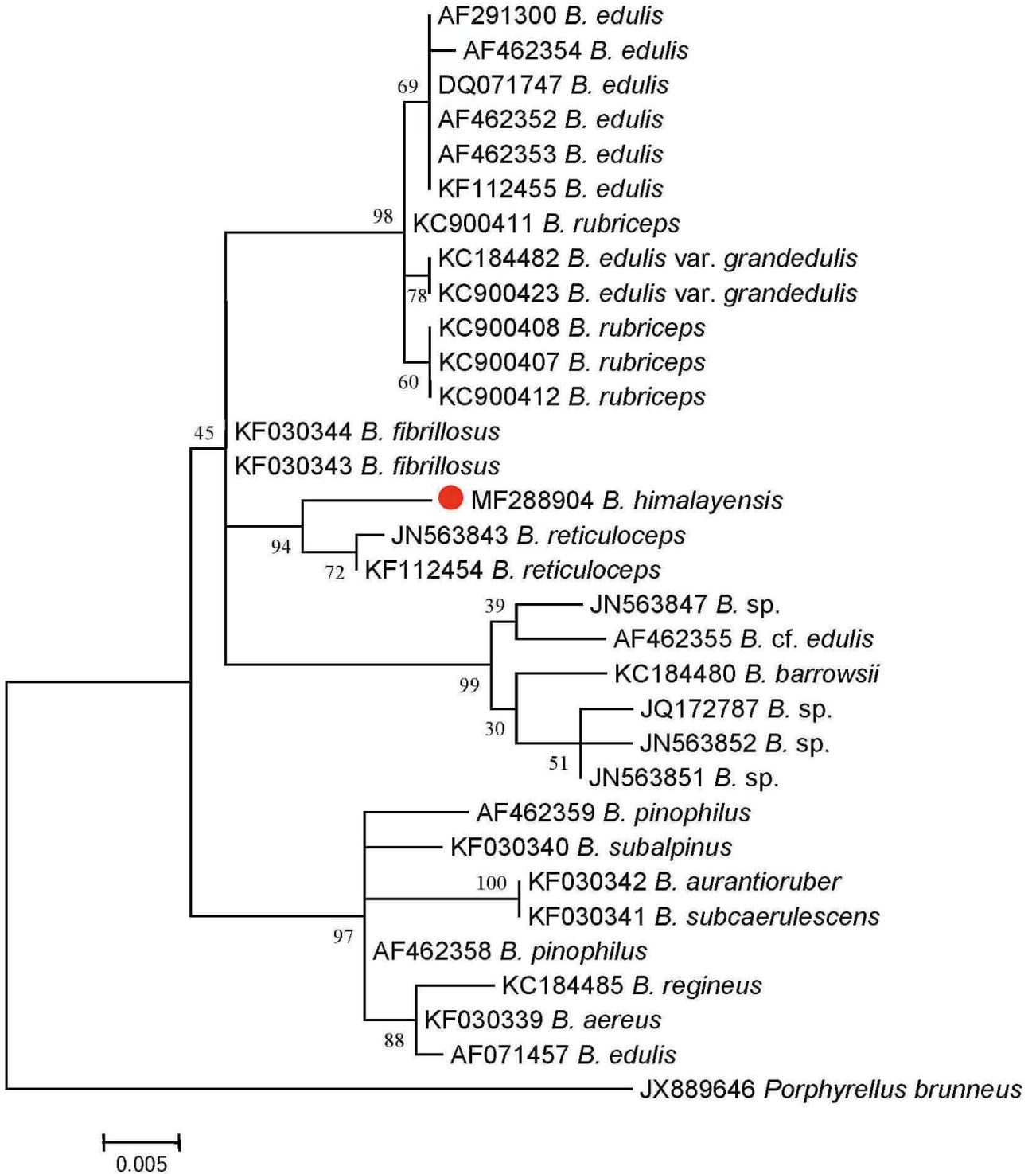


Figure 2. Molecular phylogenetic analysis based on LSU sequences. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The sequence generated during this study is marked with a red bullet indicating holotype.



Figure 3. Basidiomata of *Boletus himalayensis*. A–C. Basidiomata. A. LAH35028 (holotype); B & C. LAH35029. Bars: A = 1.5 cm; B & C = 1.2 cm.

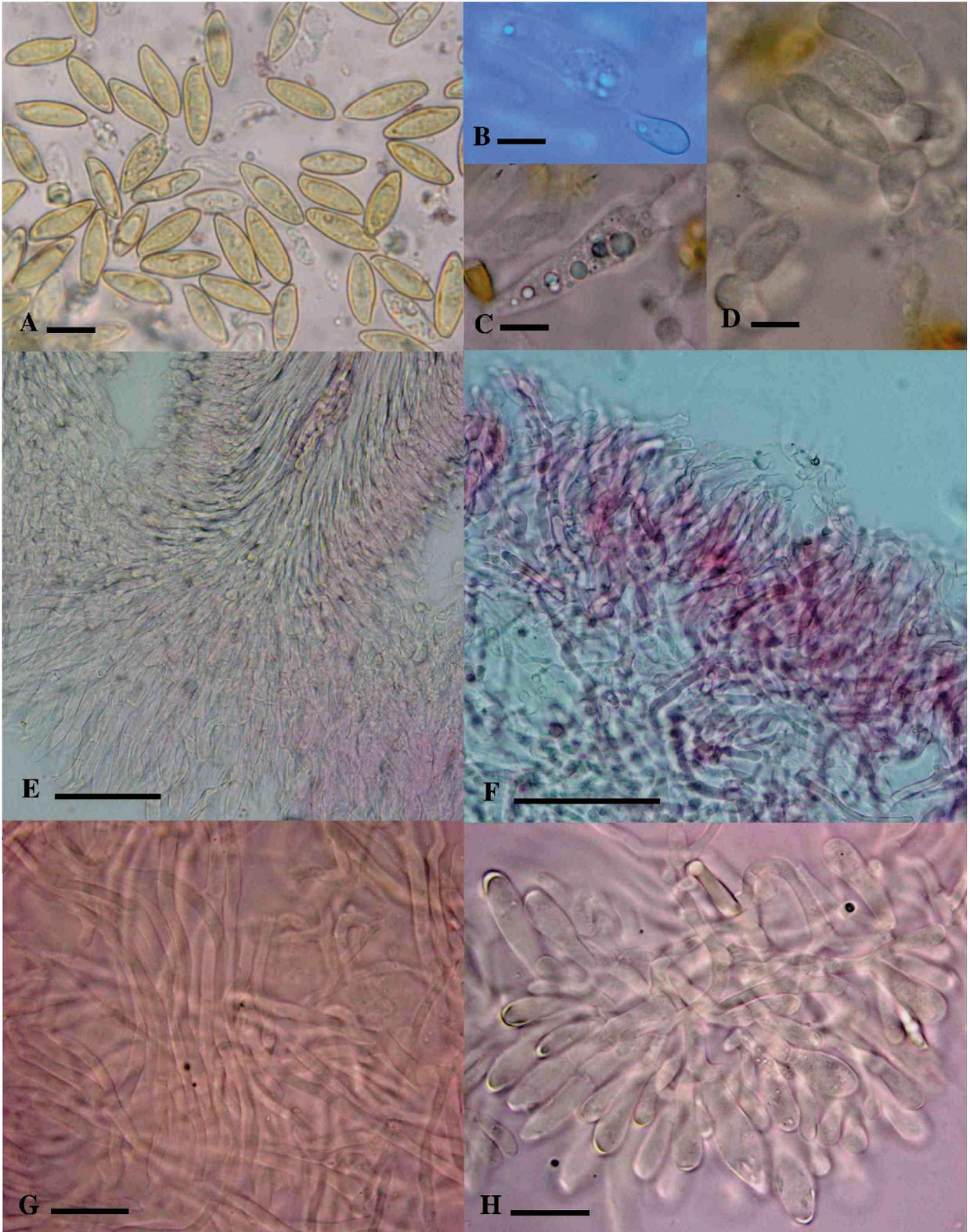


Figure 4. Microscopic characters of *Boletus himalayensis*. A: Basidiospores, B & C: Basidia, D: Hymenal cystidia, E: Tube edges, F: Pileipellis elements, G: Stipitipellis, H: Caulocystidia, A-D: 10 μ m, E: 50 μ m, F: 100 μ m, G & H: 20 μ m.

branched, hyaline in 5% KOH, hyphal tips in stipitipellis erect. Pileus context hyaline to yellowish in Melzer's reagent yellowish red in KOH.

Habitat and distribution: Pakistan, Khyber Pakhtunkhwa Province, moist and dry temperate coniferous forests.

Additional specimens examined: Pakistan, Khyber Pakhtunkhwa Province, Malakand Division, Swat District, Kalam, 2400 m asl, on soil under *Cedrus deodara*, 3 Sep 2013, Sana Jabeen SJ55 (LAH35286; GenBank for ITS: MF288903); Mashkun, 2500 m asl, on soil under *Cedrus deodara*, 5 Sep 2013, Sana Jabeen & Abdul Nasir Khalid SJ7 (LAH35029; GenBank for ITS: KJ131226); Sana Jabeen SJ58 (LAH35287; GenBank for ITS: MF288902), Ishtiaq Ahmad (LAH-ISP080-2013; GenBank for ITS: KJ828812); Hazara Division, Mansehra, Sharan (Kaghan Valley), 2011 m asl, under *Pinus wallichiana* A.B. Jack., 15 Aug 2011, Samina Sarwar and Abdul Nasir Khalid (LAH-TA63-0811; GenBank for ITS: KJ131224).

3.2. Molecular phylogenetic analyses Figures 1 and 2 Sequencing of the PCR products of the ITS region of *Boletus himalayensis* consisted of 790–811 base pairs by using ITS1F and ITS4 primers. The consensus sequence of 698 and 701 base pairs was obtained by trimming the motifs at conserve sites. Sequences obtained were BLAST searched at NCBI and showed maximum similarity with *Boletus reticuloceps* (M. Zang, M. S. Yuan & M. Q. Gong) Q. B. Wang and Y. J. Yao sequences from China (JN563882, JN563882 and JN563885–JN563887) with 89%–99% query cover and 0.0 E-value. Sequencing of the PCR products of LSU yielded 936–948 base pairs by using LR0R and LR5 primers. A sequence of 948 base pairs from LR0R primer was BLAST searched at NCBI. It showed maximum similarity with *Boletus fibrillosus* Thiers (KF030343 and KF030344) from USA and *Boletus reticuloceps* (JN563843 and KF112454) from China. *Porphyrellus* E.-J. Gilbert was chosen as outgroup. Phylogenetic estimations were done by employing the maximum composite likelihood (MCL) criteria. Subsequently the topology with the uppermost log likelihood value was selected. Number of substitutions per site was used for scaling the tree. Evolutionary affiliations of the taxa analyzed were represented by selecting the bootstrap consensus tree inferred from 1000 replicates. Both coding and noncoding positions were included in the analysis. Those positions in which data were missing were not taken into account. The sequences generated from ITS

clustered with similar taxa from China forming a clade of their own with strong bootstrap value. Sequences from the LSU region clustered in a same clade with Chinese taxa but form their own lineage with a valuable bootstrap.

4. Discussion

In the present account, *Boletus himalayensis* has been reported as a new taxon, in *Boletus* s. str. This species is characterized by tapering stipe base, cracked pileus, reticulated stipe, at least in apical part, whitish context with no color change upon bruising, and whitish pore surface in young stages. The most closely related species, both morphologically and phylogenetically, are *B. edulis*, *B. reticuloceps*, and *B. sinoedulis* (Thiers, 1975; Wang and Yao, 2005; Cui et al., 2015). Among these taxa, the closest one is *B. reticuloceps*, but the characters that differentiate *B. himalayensis* from the former is rugulose pileus, gradually broader stipe base, and smaller basidiospores in the former as compared to narrow base stipe and larger sized basidiospores in the latter (Wang and Yao, 2005). *Boletus himalayensis* looks somewhat like *B. sinoedulis*. However, the former differs from the latter by its yellowish to brownish stipe with reticulations often only on the upper half (Cui et al., 2015). This species also resembles *B. edulis*, but a difference is the whitish pileus margin in the latter. Plectologically, like other *Boletus* s. str. species, *B. himalayensis* has sterile tube edges having a long, dense cluster of cheilocystidioid elements and trichoderm, pileipellis composed of a layer of long, erect cylindrical branched hyphae. Molecular phylogenetic analyses based on ITS and LSU also provide strong support for the distinction of *B. himalayensis* from closely related species and confirm this entity as a new taxon.

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