



Short Communication

Addition to the Boletes: *Boletus pakistanicus* sp. nov. from the Coniferous Forests of Pakistan

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Abstract

Boletus pakistanicus (Fungi, Basidiomycota, *Boletaceae*) characterized by uniformly pale yellowish flesh; is described as a new species from the coniferous forests of Pakistan based on morphological and molecular analysis. A description, illustrations, phylogenetic analysis and comparison with related taxa are presented. © 2014 Friends Science Publishers

Keywords: Boletes; Basidiospore; Ectomycorrhizae; Pileipellis

Introduction

The *Boletaceae* Chevall. is a monophyletic family of mushrooms (Binder and Besl, 2000) having pores on the underside of the pileus. There are 35 genera and approximately 787 species in this family (Kirk *et al.*, 2008). The genus *Boletus sensu auct* has almost 300 species (Kirk *et al.*, 2008). Boletes, like many other macrofungi are an important part of forest ecosystems because they form ectomycorrhizae with a variety of green plant symbionts. In this role, they are an integral part of all forest systems since they are involved with basic processes such as nutrient cycling, nutrient uptake and decomposition of organic matter (Halling *et al.*, 2007). From Pakistan until present, only twenty-one *Boletus* species *i.e.*, *B. aestivalis* (Paulet) Fr., *B. appendiculatus* Schaeff., *B. barrowsii* Thiers and A.H. Sm., *B. calopus* Pers., *B. chrysenteron* Bull., *B. edulis* Bull., *B. elegans* Bull., *B. erythropus sensu* Pers., *B. fraternus* Peck, *B. luridus* Sowerby, *B. parasiticus* Bull., *B. placidus* Bonord., *B. piperatus* Bull., *B. pulverulentus* Opat., *B. queletii* Schulzer, *B. reticulatus* Boud., *B. rubellus* Krombh., *B. spadiceus* Schaeff., *B. subtomentosus sensu* Bolton, *B. subvelutipes* Peck and *B. ustalis* Berk. have been reported (Ahmad *et al.*, 1997; Razaq, 2007; Niazi, 2008).

Recently an additional *Boletus* species resembling *B. dryophilus* Thiers was collected from the moist coniferous forests of Pakistan, associated with *Pinus wallichiana* A.B. Jacks. Based on macro- and micromorphological features in combination with sequence data of rDNA-ITS, this bolete represents a new species. A description, illustrations, phylogenetic analyses and comparison with related taxa are presented herein.

Materials and Methods

Basidiocarps were collected during the rainy season (July-

August, 2010) and macromorphological characteristics were recorded in the field. Specimens were studied macroscopically and microscopically in the laboratory. In the description of spore dimensions, the first values corresponds to the range of the lengths and widths, the values in the parentheses indicate the mean spore length and width \pm their standard deviation followed by Q_m value, which represents the mean Q value \pm the standard deviation. The mean Q was taken as the mean of all E values, where $E = \text{length/width}$ of each spore measured. Similarly measurements of other structures were taken in range with exceptional values in parentheses.

After drying with a fan heater specimens were deposited in the Punjab University herbarium, Botany department (LAH).

Molecular Analysis

DNA was extracted from dried basidiocarps using Extract N. Amp.TM Plant kit (SIGMA), and the nrDNA *i.e.* ITS1-5.8S-ITS2 was amplified using fungal specific primers pairs (ITS1F/ITS4). Amplification parameters were - denaturation at 94°C for 4 min followed by 35 cycles of 45 s at 94°C, 45 s at 54°C and 1 min 30 s at 72°C, and a final extension at 72°C for 2 min. After purification of PCR products, bidirectional sequencing was done by Macrogen (South Korea) and the sequence subjected to BLAST, and searched in GenBank for comparing with available sequences. For calculating percent identity, similarity and divergence, the selected sequences were aligned using Clustal W and corrected manually. All ambiguous insertions and deletions were removed prior to further analyses. Percent Identities (PID) and DNA divergence were calculated by DNASTar. Maximum Likelihood and Maximum Parsimony criteria were used to describe the phylogenetic placement with other species

included in the present study. Phylograms were made using Mega5.

Results

B. pakistanicus Sarwar and Khalid nov. sp. (Plate 1)

GenBank Number: JQ178324.

Number of Mycobank: MB 564279.

Pileus usque 7 cm, siccus, tomentosus saepe rimosus, rufus vel rufo-brunneus. Tubuli flavi tactu coerulei; pori 0,5-1 mm lati. Stipes siccus, subtiliter flocculosus-striatus, sursum ochraceous, basim versus rubris floccis ornatus, basi flavidus. Contextus pallide citrinus, in sectione cyanescens. Spores 14-18 x 6-7 μ m, L/l ellipsoidae, laeves. Pileipellis trichodermica, articulis terminalis sub-diametricis lageniformibus, laevibus.

Habitat sub Pinis, in locis montanis, mensibus aestivalis. Holotypus in the University of Punjab Herbarium sub LAH S. B. # 49 conservatus.

Etymology

Based on the country, where the new species was found.

Macrocharacers

PILEUS 6-7 cm wide, depressed to infundibuliform to irregularly shaped at maturity, surface dry, rough, tomentose, patches present on pileus surface, dull red to brownish red, margins uplifted to slightly recurved, of same color as pileus, not entire but cracked. CONTEXT creamish to light yellowish, bluing when exposed. STIPE 4-7 cm long, 1 - 2 cm thick, central, equal, dry, solid, curved, brownish yellow near hymenium, dull red below, becoming yellowish near base, smooth, lacking reticulations, sometimes slightly longitudinally striated, context light yellowish throughout the stipe, slowly bluing when exposed like pileus context. PORE SURFACE yellow to olive yellow, free and approximate to adnexed, readily bluing when bruised, pores circular to elliptical to irregularly shaped, 1-2 per mm, tubes 5-9 mm deep. SMELL AND TASTE not distinctive. EDIBILITY not known.

Microcharacers

BASIDIOSPORES fusiform to sub-fusoid, smooth, olive brown, 14 - 18 x 6 - 8 μ m, ($16.06 \pm 1.4 \times 7.15 \pm 0.5$; $Q_m = 2.25 \pm 0.14$). BASIDIA broadly clavate, long, 2 - 4 sterigmate, sterigmata long and sharp, (34-) 38 - 43 x (7-) 11 - 12 (-15) μ m, yellowish brown contents visible in Meltzer's. CYSTIDIA cylindrical to subclavate, (26-) 33 - 40 x 7 - 8 μ m. PILEIPELLIS trichoderm consisting of slender, cylindrical to subclavate, septate, more or less interwoven hyphae 95 - 110 (-130) x 10 - 11 μ m, terminal elements clavate lageniform, 57 - 80 x 22 - 24 μ m. CLAMP CONNECTIONS not observed. CHEMICAL REACTIONS

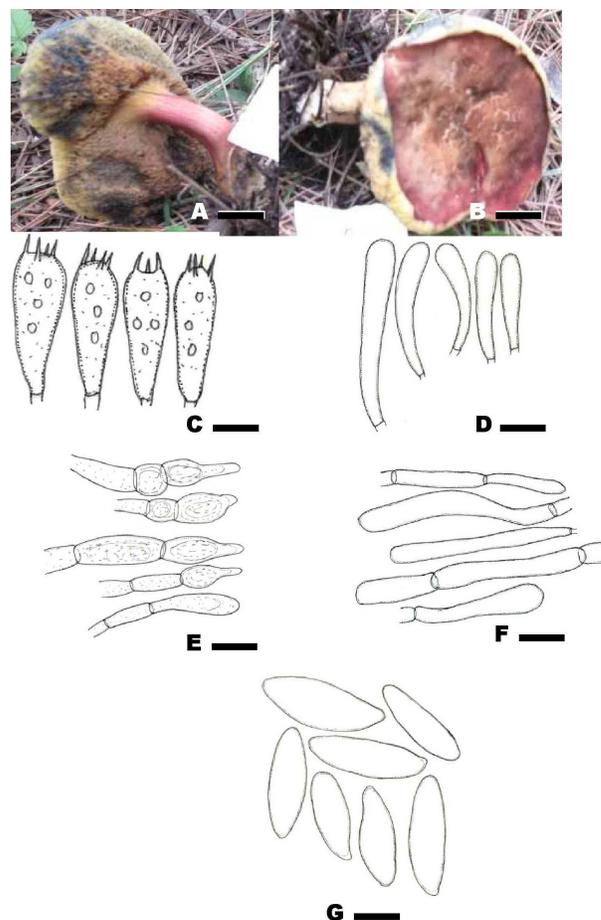


Plate. 1: *Boletus pakistanicus*. A & B, Sporocarps; C, Basidia; D, Cystidia; E, Terminal elements of Pileipellis hyphae; F, Pileipellis hyphae; G, Basidiospores. Scale Bars: for A & B = 1 cm; C = 13 μ m; D = 11 μ m; E = 34 μ m; F = 36 μ m; G = 7 μ m.

pileipellis stains dark blue with the application of FeSO_4 , hyaline to light yellowish in KOH, Meltzer's and Lactic acid, spores olive brown to brown in Meltzer's, light yellow to honey yellow in Lactic acid.

Material Examined Pakistan Khyber Pakhtunkhwa, Khera Gali

2347 m.a.s.l., under *Pinus wallichiana* A.B. Jacks., solitary, on ground, 17th June 2010, LAH S. B.# 49 (holotypus), (GenBank accession. JQ178324); Helipad, 2438 m.a.s.l., under *Pinus wallichiana* A.B. Jacks, solitary, on ground, 6th July 2010 (S. B.# 62); Nathia gali, 2493 m.a.s.l., under *Pinus wallichiana* A.B. Jacks, solitary, on ground, 7th July 2010 (S. B.# 76).

Molecular and Phylogenetic Analyses

When *B. pakistanicus* was submitted for closest matches,

Table 1: rDNA sequences downloaded from GenBank for molecular characterization and phylogenetic analysis

FUNGAL SPECIES	COLLECTION NO.	ORIGIN	ACCESSION NO.
<i>Boletus cf. chrysenteron</i>	JMP0007	USA	EU819456.1
<i>B. chrysenteron</i>	TENN60896	USA	FJ596906.1
<i>B. dryophilus</i>	3035	USA	AY185181.1
<i>B. dryophilus</i>	3049	USA	AY185183.1
<i>B. erythropus</i>	SU46	Italy	DQ131633.1
<i>B. erythropus</i>	SU47	Italy	DQ131634.1
<i>B. subtomentosus</i>	C 1064254	USA	EU526005.1
<i>B. zelleri</i>	src444	USA	DQ974704.1
<i>B. pakistanicus</i>	S.B.49	PAK	JQ178324
<i>Octaviania columellifera</i>	TNS-F-11695	Japan	EF183532.1
<i>O. columellifera</i>	TNS-F-11697	Japan	EF183542.1
<i>X. armeniacus</i>	MA-Fungi 46678	Spain	AJ419221.1
<i>X. chrysenteron</i>	KGP62	USA	DQ822793.1
<i>X. chrysenteron</i>	TDB635	USA	DQ533981.1
<i>X. cisalpinus</i>	BB28_409_Oh_161006 (DNA660)	Germany	HM190085.1
<i>X. cisalpinus</i>	BB28_409_Oh_161006 (DNA479)	Germany	HM190084.1
<i>X. porosporus</i>	BB28_403_Ah_161006 (DNA451)	Germany	HM190086.1
<i>X. pruinatus</i>	BB28_305_Of_Fa_070507 (DNA799)	Germany	HM190106.1
<i>X. pruinatus</i>	BB06_105_Of_Pi_230407 (DNA750)	Germany	HM190107.1
<i>Xerocomus sp. MHM129</i>	MHM129	USA	EU569235.1
<i>X. zelleri</i>	KGP68	USA	DQ822794.1

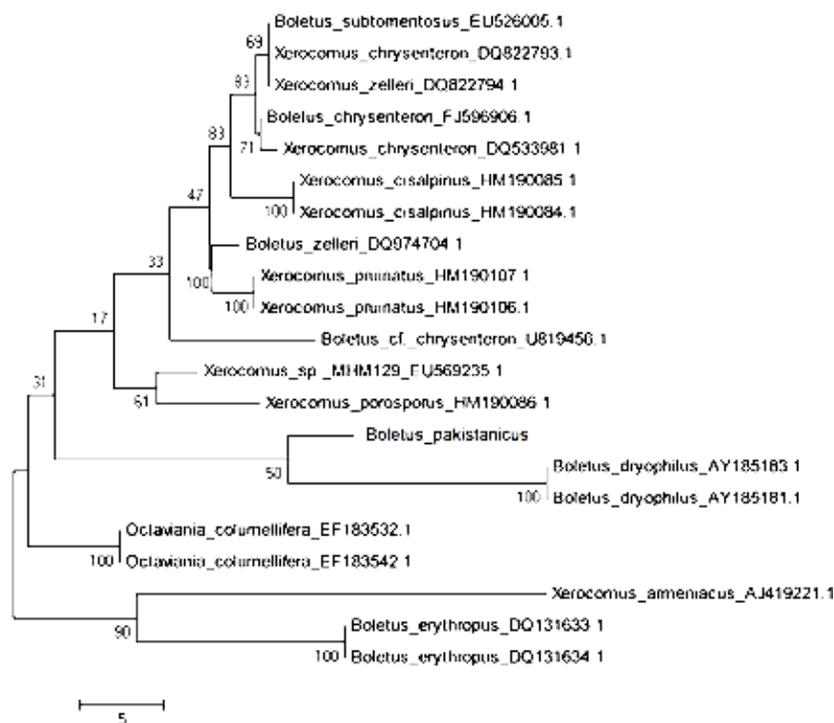


Fig. 1: Bootstrap consensus tree inferred from 100 replicates. Cladogram based on parsimony analysis of rDNA-ITS region of *Boletus pakistanicus* with respect to other species. MP tree generated by parsimony analysis of rDNA-ITS with 5.8s gene. The number across branches refers to Bootstrap values. The accession numbers of analyzed sequences are shown after each taxon name

the top BLAST results were *B. subtomentosus* (Accession No. EU526005.1), *Xerocomus zelleri* (Accession No. DQ822794.1) and *Xerocomus chrysenteron* (Accession No. DQ822793.1) with 89% similarity and *B. dryophilus* (Accession No. AY185183.1) with 88% similarity. These matches are well below the 97% cutoff value for species identification. Phylogenetic placement of *B. pakistanicus*

was confirmed by making phylogenetic trees using maximum parsimony and maximum likelihood criteria.

The sequences included in this analysis had approximately 925 characters, from which 729 characters were used for further analysis after alignment and trimming from both 3' and 5' sites of rDNA-ITS. After that, none of characters were excluded from final analysis.

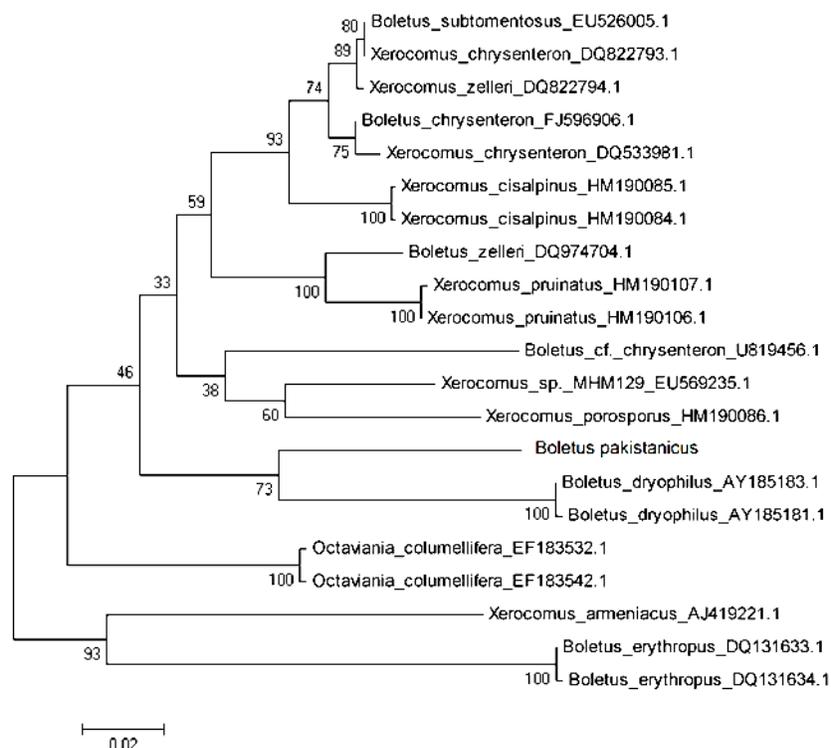


Fig. 2: Phylogenetic position of *Boletus pakistanicus* with respect to other species. Tree inferred by maximum likelihood analysis based on rDNA sequences, including 5.8S and ITS2 region (Ln likelihood = -5878.41). The numbers against branches indicate the percentage at which a given branch was supported in 1000 bootstrap replications. GenBank accession number are given at the end of species names

		Percent identity																						
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
Divergence	1	■	77.8	99.5	100.0	94.4	88.9	88.3	93.7	89.0	80.2	85.0	94.7	94.7	88.9	88.8	84.5	84.6	81.6	81.6	83.8	83.7	1	<i>Boletus subtomentosus_EU526005</i>
	2	9.5	■	77.6	77.6	77.1	74.3	77.2	76.8	77.9	75.2	76.4	75.4	75.4	77.0	77.1	76.0	76.1	71.6	71.6	78.5	78.5	2	<i>Boletus pakistanicus</i>
	3	0.3	9.5	■	99.5	94.5	88.8	88.5	93.8	88.9	80.7	85.2	94.7	94.7	88.8	88.6	84.6	84.8	81.6	81.6	84.0	83.8	3	<i>Xerocomus zelleri_DQ822794</i>
	4	0.0	9.6	0.3	■	94.4	88.9	88.3	93.7	89.0	80.2	85.0	94.7	94.7	88.9	88.8	84.5	84.6	81.6	81.6	83.8	83.7	4	<i>Xerocomus chrysenteron_DQ822793</i>
	5	1.4	9.9	1.3	1.4	■	87.8	87.7	89.0	87.5	78.9	84.2	90.8	90.8	87.5	87.4	82.7	82.9	78.5	78.5	83.1	83.1	5	<i>Boletus chrysenteron_FJ596906</i>
	6	8.5	15.8	8.4	8.5	8.9	■	87.4	86.8	85.6	77.2	83.0	87.4	87.4	85.9	85.7	83.5	83.4	78.3	78.1	81.2	81.1	6	<i>Boletus cf_chrysenteron_U819456</i>
	7	8.4	13.7	8.2	8.4	8.6	10.7	■	86.7	87.0	79.0	86.3	86.4	86.4	86.3	84.8	84.9	77.6	77.6	83.5	83.4	7	<i>Xerocomus sp_MHM129_EU569235</i>	
	8	1.7	10.1	1.6	1.7	0.6	9.6	9.3	■	86.6	78.3	83.5	90.0	90.0	86.8	86.7	82.3	82.4	77.5	77.5	82.2	82.2	8	<i>Xerocomus chrysenteron_DQ533981</i>
	9	6.4	10.8	6.2	6.4	6.6	11.2	9.6	7.3	■	79.3	85.5	85.6	85.6	94.9	95.1	83.1	82.7	78.1	77.8	83.1	83.0	9	<i>Boletus zelleri_DQ974704</i>
	10	15.2	15.5	14.9	15.2	15.7	19.9	17.8	16.0	16.7	■	77.0	79.6	79.6	78.6	78.6	76.8	76.7	77.1	77.1	75.9	75.7	10	<i>Xerocomus armeniacus_AJ419221</i>
	11	8.5	12.4	8.0	8.5	8.2	12.0	7.9	8.9	9.2	15.1	■	83.5	83.5	85.7	85.6	86.0	86.0	75.6	75.6	80.8	80.7	11	<i>Xerocomus porosporus_HM190086</i>
	12	3.5	10.7	3.5	3.5	3.3	7.7	8.1	3.8	6.7	15.4	8.0	■	100.0	87.0	86.8	82.9	83.0	82.3	82.3	81.6	81.5	12	<i>Xerocomus chrysenteron_DQ533981</i>
	13	3.5	10.7	3.5	3.5	3.3	7.7	8.1	3.8	6.7	15.4	8.0	8.0	■	87.0	86.8	82.9	83.0	82.3	82.3	81.6	81.5	13	<i>Xerocomus cisalpinus_HM190084</i>
	14	7.7	12.3	7.5	7.7	8.4	12.3	10.8	9.1	3.4	17.8	11.0	7.4	7.4	■	99.9	84.0	83.5	78.1	78.3	82.7	82.6	14	<i>Xerocomus pruinatus_HM190107</i>
	15	7.7	12.3	7.5	7.7	8.4	12.3	10.8	9.1	3.4	18.0	11.0	7.4	7.4	0.0	■	83.8	83.4	78.2	78.5	82.7	82.6	15	<i>Xerocomus pruinatus_HM190106</i>
	16	10.5	14.4	10.0	10.5	11.5	12.9	10.8	11.9	11.9	15.5	11.6	10.3	10.3	12.6	12.6	■	99.6	76.0	76.0	81.5	81.3	16	<i>Octaviania columellifera_EF183532</i>
	17	10.5	14.4	10.0	10.5	11.5	12.9	10.8	11.9	12.3	15.6	11.8	10.3	10.3	13.0	13.0	0.3	■	75.9	75.9	81.3	81.2	17	<i>Octaviania columellifera_EF183542</i>
	18	17.4	18.7	17.4	17.4	16.5	19.5	19.0	17.3	17.7	19.6	17.8	14.6	14.6	18.1	18.1	19.3	19.4	■	80.7	74.9	74.8	18	<i>Boletus erythropus_DQ131633</i>
	19	17.6	19.0	17.6	17.6	16.7	19.7	19.2	17.5	17.9	19.8	18.0	14.8	14.8	17.9	17.9	19.6	19.6	0.1	■	74.8	74.5	19	<i>Boletus erythropus_DQ131634</i>
	20	12.4	10.2	12.2	12.4	12.3	17.9	14.0	13.1	13.2	19.6	12.6	12.6	12.6	14.1	14.3	13.6	13.6	23.5	23.5	■	89.9	20	<i>Boletus dryophilus_AY185183</i>
	21	12.6	10.4	12.4	12.6	12.3	18.1	14.2	13.1	13.4	19.8	12.8	12.8	12.8	14.3	14.5	13.8	13.8	23.7	23.7	0.1	■	21	<i>Boletus dryophilus_AY185181</i>
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		

Fig. 3: Percent divergence was calculated by comparing sequence pairs in relation to the phylogeny reconstructed by MegAlign (DNASTAR). Percent identities compare sequences directly without accounting for phylogenetic relationships

All characters were of type 'unord'. There were 209 parsimony-informative sites, 438 constant sites, 282 variable characters were parsimony-uninformative. All the gaps were treated as "missing" data. Multistate taxa were interpreted as uncertain.

Phylogenetic trees were constructed for *B.*

pakistanicus, along with species from geographically different localities (Table 1). The phylograms represent a major polytymous Clade I formed by *B. subtomentosus* (EU526005.1), *Xerocomus zelleri* (DQ822794.1), *B. zelleri* (DQ974704.1), *Xerocomus chrysenteron* (DQ533981.1, DQ822793.1), *B. chrysenteron* (FJ596906.1), *Boletus cf.*

chrysenteron (U819456.1), *Xerocomus* sp. MHM129 (EU569235.1), *Xerocomus porosporus* (HM190086.1), *Xerocomus cisalpinus* (HM190084.1, HM190085.1), *Xerocomus pruinatus* (HM190106.1, HM190107.1) and *B. dryophilus* (AY185181.1, AY185183.1).

B. pakistanicus shares maximum of 88.87 of genetic characters and has 78.6% identity compared with *B. dryophilus* (AY185181.1). *B. pakistanicus* shares 88.84% genetic characters and has 77.8% identity compared with *B. subtomentosus* (EU526005.1). *B. pakistanicus* shares minimum genetic characters of 82.44% and has 71.6% identity compared with *B. erythropus* (DQ131633.1). There is significant genetic divergence of rDNA-ITS among *B. pakistanicus* and other rDNA sequences included in the present study (Fig. 3). The nearest rDNA sequence is of *B. dryophilus* (AY185183.1). Both phylogenetic analyses placed *B. pakistanicus* with *B. dryophilus* (AY185181.1, AY185183.1) with comparatively short branches and supportive bootstrapping (Fig. 1 and 2) as sub-clade II of major clade I. This polytomous clade formed by 16 species was included in both analyses. The rDNA-ITS of *B. pakistanicus* was found distinct when the rDNA sequence divergence was compared with other species included in the analyses. Minimum divergence (9.5) was found among the rDNA of *B. pakistanicus* and *X. zelleri* (DQ822794.1) and maximum (19.0) with *B. erythropus* (DQ131634). There was great variability in the ITS1-5.8S-ITS2 region among all the species included in the present investigation.

Comments

Based on available shared genetic characters analyzed in the present study, *B. pakistanicus* was closest to *B. subtomentosus*, *B. dryophilus* and *X. chrysenteron*. None of these species were found to be identical to *B. pakistanicus*. In the phylogenetic analyses, *B. pakistanicus* appeared as a sister clade with *B. armeniacus*, *B. erythropus* and *Octaviania columellifera* (Fig. 2). From the calculated PIDs for the present study, the cutoff value (97%) for species delimitation did not match with *B. pakistanicus* (Fig. 3) and thus designated as a new species.

Morphologically *B. pakistanicus* is characterized by dull red to brownish red, depressed to irregular shaped pileus, yellow to olive-yellow pores, which turns blue upon bruising, with central, equal, solid curved stipe which is smooth with light longitudinal striations. Within the boletes it appears morphologically closest to *B. subtomentosus*, *Xerocomus zelleri*, *X. chrysenteron* and *B. dryophilus* with which it shares many common characters. But this species is clearly distinct from other similar taxa of *Boletus* for its uniformly pale yellow flesh, before the blue color, and terminal elements of pileipellis lageniform.

Morpho-anatomic characterization as well as molecular analysis strongly suggested that this species is distinct from closely related species and is a previously undescribed taxon.

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