

Cyanoboletus macroporus (Boletaceae), a new bolete species from Pakistani forests

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Abstract

Cyanoboletus macroporus belonging to *C. pulverulentus* species complex is designated as a new species from the moist temperate and sub-alpine oak forests of Pakistan after in depth macroscopic, microscopic and phylogenetic analyses using the ITS region of nrDNA as well as comparison with allied taxa. This species belonging to Boletoid group is morphologically distinguished from allied taxa (*Cyanoboletus flavosanguineus*, *C. hymenoglutinosus*, *C. pulverulentus*, *C. rainisii*, and *C. sinopulverulentus*) by wider openings of pores. *C. macroporus* is also phylogenetically distinct from *C. sinopulverulentus* and *C. pulverulentus*, the most closely related spe-

cies. Phylogenetic analysis outlined the existence of previously unknown species of this genus. Field photographs of fresh basidiocarps and line drawings of micro-characters are provided along with a phylogenetic tree as well as a comparison table and a key of distinctive features of all the species in this genus. This is the first authentic species belonging to *Cyanoboletus* from Pakistan. Previously, only *C. pulverulentus* has been mentioned in literature, but no morphological data is available regarding this report. With the addition of this taxon, species number of *Cyanoboletus* will increase to eight.

From Pakistan, despite of the fact that there is great diversity of mushrooms in moist temperate areas (Yousaf *et al.* 2012), this is the first study that describes a species belonging to *Cyanoboletus* genus. Previously only one ambiguous species, *Cyanoboletus pulverulentus*, has been mentioned in literature (Iqbal & Khalid 1996), but with no available materials that could confirm this finding. In this study, *Cyanoboletus macroporus* is described as a new to science and increase the current species number of *Cyanoboletus* to eight.

Introduction

Cyanoboletus is a fungal genus in the family Boletaceae circumscribed in 2014. Currently, the genus accommodates seven species: *Cyanoboletus flavosanguineus* (Lavorato & Simonini) Pierotti, *C. rainisii* (Bessette & O.K. Mill.) Gelardi, Vizzini & Simonini (North America), *C. sinopulverulentus* (Gelardi & Vizzini) Gelardi, Vizzini & Simonini, *C. hymenoglutinosus* D. Chakr., K. Das, A. Baghela, S.K. Singh & Dentinger, *C. brunneoruber* G. Wu & Zhu L. Yang, *C. instabilis* (W.F. Chiu) G. Wu & Zhu L. Yang and the type, *C. pulverulentus* (Opat.) Gelardi, Vizzini & Simonini (Gelardi *et al.* 2013, Vizzini 2014, Pierotti 2015, Li *et al.* 2016, Wu *et al.* 2016 a, b). The generic name is derived from the Ancient Greek cyano («blue»), referring to the deep ultramarine blue bruising oxidation reaction of the fruit bodies (Vizzini 2014). The type species of this genus is *C. pulverulentus*, commonly known as the ink stain bolete. It is an edible bolete mushroom (Smotlacha & Vejrych 1947) growing in ectomycorrhizal association with deciduous and mixed forests, particularly on moist soil on slopes and under beech and oak trees. *C. pulverulentus* is a common species found in northern Asia, Europe, North Africa, Central and northern South America, and eastern North America. All parts of the mushroom will stain dark bluish-black after handling. A recent study has revealed this mushroom hyperaccumulates arsenic (with concentrations exceeding 1000 mg/kg in dry weight) and therefore its consumption should be restricted (Cocchi & Vescovi 1996).

Materials and methods

Sampling and morphological analyses

Sporocarps were collected from different localities of (moist temperate forests dominated by *Quercus incana*) Swat (34.34–35.55°N 72.08–72.50°E), in Toa, (Shangla district), Sultan, Khyber Pakhtunkhwa Province, Pakistan, as well as from Himalayan moist temperate forests of Pakistan dominated by conifers. Basidiocarps were photographed in the field. Fresh morphological characters were recorded in the field and colors were recorded following Munsell (1994) by Color Meter application (Yanmei He, Mac App Store). Field data on basidiocarps (site, habitat, association, etc.) was noted represented in result section below. Specimens were studied plectologically in the laboratory following the methods of Bessette *et al.* (2000). For the spore dimensions, the first values represent the range of lengths and widths, and the values in parentheses present mean spore lengths and widths \pm standard deviations followed $Q_m \pm$ standard deviation, where Q_m is the mean of Q (= length/width ratio of an individual spore). Other measurements are given as a range with exceptional values in parentheses. Voucher specimens were deposited in the Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan (LAH). Voucher numbers are mentioned in holotype material and material examined section.

DNA extraction, amplification, and sequencing

DNA was extracted from dried material of two mature sporocarps (collection numbers are mentioned in material examined and holotype) following the protocol by a modified CTAB extraction method (Gardes & Bruns 1996). The extracted genomic DNA was evaluated using gel electrophoresis (1% agarose gel in Gel documentation system Uvipro Platinum (IM-2000 WL/ LC/26M MANZ) with default settings. After getting gel images, genomic DNA was suspended in nuclease free water and stored at -18°C . PCR conditions (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) were used to amplify the entire rDNA internal transcribed spacer region (ITS = ITS1+5.8S+ITS2) with primer pair ITS1F/ITS4 (White *et al.* 1990). The PCR amplicons were sent to Macrogen (Republic of Korea) for purification and bidirectional sequencing.

Sequence alignment and phylogenetic analysis

Consensus sequences were generated from the obtained sequences with the forward and reverse algorithms in BioEdit (Hall 1999). The data was preliminary identified using BLAST searches at NCBI (<http://www.ncbi.nlm.nih.gov/>). The most similar sequences after BLAST in NCBI for ITS region were retrieved from GenBank. The aim was to retrieve at least 1-2 sequences of each species of *Cyanoboletus* genus that belongs to different countries. No sequence of this genus was present in GenBank database from Pakistan before this study. These sequences were then aligned using MUSCLE (Edgar 2004) alignment tool available online (www.ebi.ac.uk/Tools/msal-muscle). Maximum likelihood tree (mostly used by mycologists for phylogenetic analysis) was inferred for each of the two alignments using RAXML-HPC2 v 8.1.11 (Stamatakis 2014) with a GTR + gamma model of nucleotide substitution. One thousand bootstrap iterations were performed with rapid bootstrapping. Significant support was considered to be $\geq 70\%$. All phylogenetic analyses were performed on the CIPRES Portal v. 3.1. (Miller *et al.* 2010).

Results

Molecular phylogenetic analyses

The extracted DNA from *Cyanoboletus macroporus* was amplified by ITS1F and ITS4 primers for ITS region. Both collections were used for molecular analysis. ITS sequences (as mentioned in Holotype and material examined) were obtained successfully and processed further for phylogenetic analysis. Consensus sequence of 678 base pairs was obtained by trimming the motifs and subjected to BLAST search at NCBI. It showed 89% identity to *Boletus pulverulentus* Opat. (EU819453 & EU819502) from Western Wisconsin and 88% to *Boletus sinopulverulentus* (KC579402) from China with 99% query coverage and 0.0 E value. To analyze phylogeny, ITS sequences of different species of *Cyanoboletus* genus were retrieved from the GenBank and also added from literature. There is scarce data about *Cyanoboletus* in databank. There were 29 sequences in the final dataset (Table 2) and a total of 970 characters in the alignment file after trimming the ends at conserve sites to begin and end with the conserved motifs 5'-(...GAT)CATT— and —GACCT(CAAA...)-3' to facilitate alignment. These motifs correspond to the conserved 3' and 5' termini of the flanking SSU and LSU genes, respectively. Among these 236 characters were constant, 688 variable characters were parsimony-uninformative while 526 characters were parsimony informative. The sequences generated during this study get separated from closest taxa forming its own lineage in same clade with strong bootstrap value (Fig. 3).

Taxonomy

Cyanoboletus macroporus Sarwar, Naseer & Khalid, *sp. nov.*

Figures 1, 2

MycoBank:—MB837538

DIAGNOSIS: *Cyanoboletus macroporus* is characterized by dark brown pileus, Instantly basidiomata color change to olivaceous black to dark greenish black, stipe without reticulations, reddish tint near

Table 1: Comparison of *Cyanoboletus* species

Character/ Species name	<i>Cyanoboletus flavosanguineus</i>	<i>C. hymenoglutinosus</i>	<i>C. macroporus sp. nov.</i>	<i>C. pulverulentus</i>	<i>C. rainisii</i>	<i>C. sinopulverulentus</i>	<i>Cyanoboletus brunneoruber</i>	<i>Cyanoboletus instabilis</i>
Color change upon bruising and handling	Turn strongly blue upon bruising	instantaneously changing (to blue-black) pore surface and context	Instantly trun olivaceous black to dark greenish black	instantly bruising deep ultramarine blue on handling	Pore surface turn dark green (not blue-black) when bruised	Basidiomata turns Indigo blue	prompt bluing basidioma when hurt	bluing context when bruised
Pileus	Dry, Primrose yellow color in every part	highly glutinous basidiomata	dark brown pileus	lack the typical glutinous pileipellis	Yellow cap flesh blues instantly, lack the typical glutinous pileipellis	Dark brownish tints on both pileus and stipe	Surface carrot orange to grayish orange	surface yellowish brown to reddish brown
Stipe	Lower part of stem covered with hairs visible with optical microscope	surface scaly pruinose with longitudinal striations on the upper half	Without reticulations, reddish tint near hymenium, black downwards, whitish at base	pruinose and peculiarly coloured stipe which is of a bright yellow in the upper half but more or less sharply reddish to brownish-red from the middle zone downwards	Bright yellow stem has red tints near the base	stipe without reticulum but finely scarbrous-scissurate radially resembling a zebra pattern with whitish basal mycelium	pruinose to furfuraceous, sometimes with faint longitudinal striations on the upper part of stipe,	eccentric to subcentral, subcylindrical; sur- face reddish orange to brownish orange on the apex or upper part, and reddish brown towards the base, nearly glabrous to faintly pruinose
Hymenium	Primrose yellow color, Pores round	yellow- to brown- orange pore surface with stuffed pores, pore2-3 mm	yellowish brown to dark brown, angular to irregular, much wide pores as compared to all related species	yellow when young, darkening to golden yellow to brownish yellow when mature, 1-2 angular pores per mm	Yellow pores, stain green,	deep yellow pore surface and unstuffed pores	brownish red hymenophoral surface	whitish yellow to pale yellow when young, and yellow to deep chrome yellow
Basiospores	Very large spores	slightly longer	slightly longer	slightly longer and narrower spores	spores are much larger	ellipsoid-fusiform, smooth walled, bright yellow spores	Subfusiform, brownish- yellowish, smooth	brownish- yellowish
Reference	Pierotti 2015	Li et al. 2016	Li et al. 2016	Li et al. 2016	Li et al. 2016	Gelardi et al. 2013	Wu et al. 2016a	Wu et al. 2016b

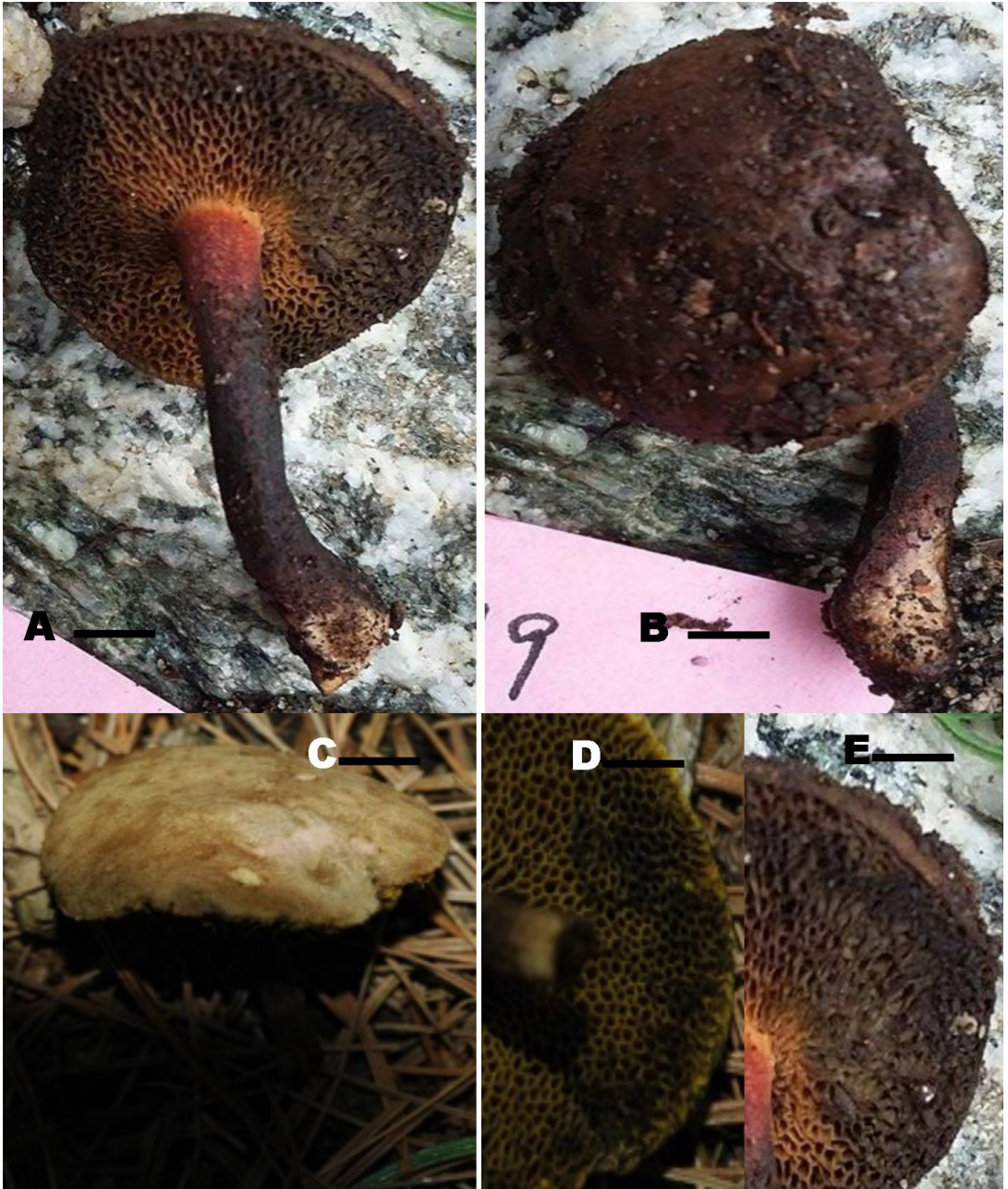


Figure 1. Morphology of *Cynoboletus macroporus*. A–C. Basidioma, D–E. Pore surface. Scale Bars A–C = 0.345 cm.

Table 2. Information of ITS 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	ITS sequence accession number	Country	reference
<i>Cyanoboletus</i> sp.	KT990767	China	Wu et al. 2016b
<i>Cyanoboletus</i> sp.	KT990766	China	Wu et al. 2016b
<i>Xerocomellus rainisiae</i>	KU144790	USA	Frank et al. 2020
<i>Cyanoboletus sinopulverulentus</i>	MH684757	India	Chakraborty 2018
<i>Boletus rainisii</i>	KM213664	USA	Frank et al. 2020
<i>Boletus rainisii</i>	KM213663	USA	Frank et al. 2020
<i>Boletus rainisii</i>	KM213662	USA	Frank et al. 2020
<i>Xerocomellus rainisiae</i>	KU144789	USA	Unpublished
<i>Rubroboletus rhodoxanthus</i>	MH011921	France	Unpublished
<i>Boletus cf subulridellus</i>	MN128246	USA	Unpublished
<i>Lanmaoa pallidorosea</i>	MN906175	USA	Unpublished
<i>Lanmaoa pallidorosea</i>	MN906173	USA	Unpublished
<i>Cyanoboletus hymenoglutinosus</i>	NR164239	India	Li et al. 2016
<i>Cyanoboletus</i> sp.	KT907355	India	Unpublished
<i>Boletus pulverulentus</i>	EU819453	USA	Palmer et al. 2018
<i>Boletus pulverulentus</i>	EU819502	USA	Palmer et al. 2018
<i>Cyanoboletus</i> sp.	LT714710	Czech Republic	Unpublished
<i>Cyanoboletus pulverulentus</i>	MT939495	USA	Unpublished
<i>Cyanoboletus pulverulentus</i>	LT714709	Czech Republic	Unpublished
<i>Cyanoboletus pulverulentus</i>	LT714708	Czech Republic	Unpublished
<i>Cyanoboletus pulverulentus</i>	KT157055	Italy	Gelardi et al. 2015
<i>Cyanoboletus pulverulentus</i>	KT157054	Italy	Gelardi et al. 2015
<i>Boletus sinopulverulentus</i>	KC579402	Italy	Li et al. 2016
<i>Cyanoboletus macroporus</i>	MW045557	Pakistan	Present Study
<i>Cyanoboletus macroporus</i>	MW369503	Pakistan	Present Study

hymenium, black downwards, whitish at base, pore surface yellowish brown to dark brown, angular to irregular, much wider pores as compared to all related species and slightly longer ellipsoid basidiospores. Phylogenetically nested in the *C. pulverulentus* clade.

HOLOTYPE: PAKISTAN. Khyber Pakhtunkhwa Province: Sulatanr, Swat Valley, 3400 m a.s.l., on soil, under *Quercus semicarpifolia*, 14 August 2015, Arooj Naseer and Abdul Nasir Khalid, ASM9 (LAH35252, GenBank MW045557).

ETYMOLOGY: The epithet 'macroporus' refers to the large size of pores in hymenium.

DESCRIPTION: *Basidiomata* Large, dry, change instantly olivaceous black to dark greenish black on touch. *Pileus* 2.5 × 2.0 cm diam., broadly convex, dark brown in color (1YR 1.4/1.6), margins slightly incurved, smooth, entire. *Hymenophore* yellowish brown to dark brown (1YR 1.4/1.6) towards margins, angular to irregular, much wider pores. *Stipe* without reticulations, reddish tint near hymenium, black downwards, whitish at base, 2.8 cm long, 0.4 cm at apex, 0.2 cm at centre, 0.6 cm at base in width, dark chocolate brown (9.3RP 1.4/1.6) with brownish red (6.6R 3.5/6.6) top and grayish white (4.8YR 5.5/3.6) base, cylindrical, solid, tough, not ornamented.

Basidiospores slightly longer, 12–14 × 4–7 µm, olive green in 5% KOH, ellipsoid, smooth, non-reticulate, guttulate sometimes bi guttulate. *Basidia* 23–38 × 6–12 µm, olive green in 5% KOH, guttulate, 4 sterigmata, mostly 2, blunt ended. *Pileocystidia* 39–68 × 10–12 µm in diameter, light brown in 5% KOH, fusiform to fusi-ventricose, normal wall thickness, oil content. *Caulocystidia* 2–5.0 × 6–12 µm diameter, fusiform, light brown in 5% KOH. *Pileipellis* 4.0 × 8.9 µm in diameter, hyphal arrangement cutis, light brown in color, septate, cylindrical cell, thin walled, ends are round, yellowish brown pigments. *Stipitipellis* 3 × 7 µm in diameter, hyphal arrangement cutis but with broader cell, septate.

HABIT, HABITAT AND DISTRIBUTION: Swat, Sulatanr, solitary, under the canopy of *Quercus semicarpifolia*; Khanspur, Ayubia, Solitary, under mixed conifers.

ADDITIONAL MATERIAL EXAMINED: Khanspur, Ayubia, mixed Coniferous forests, 15th July, 2011, S. Sarwar & A. N. Khalid, SSB1 (LCWH12011, GenBank for ITS: MW369503).

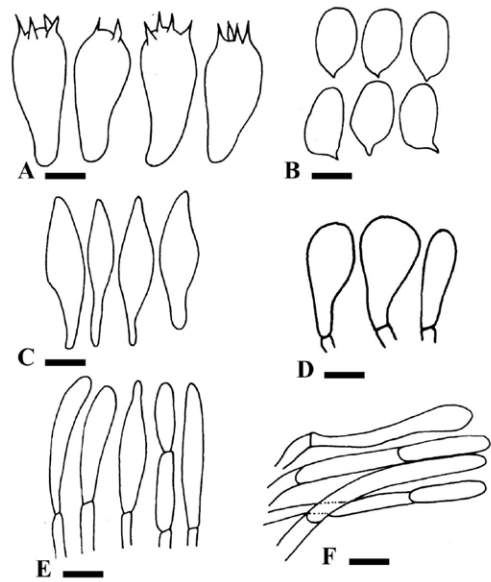


Figure 2. Anatomy of *Cynoboletus macroporus*.

A–F LAH35252, A. Basidia; B. Basidiospores; C. Cheilocystidia; D. Pleurocystidia; E. Pileipellis; F. Stipitipellis. Scale Bars: A = 8.28 µm; B = 5.5 µm; C & D = 18 µm; E & F = 14.25 µm. Illustrator & Photographer: Dr. Arooj Naseer & Dr. Samina Sarwar

Discussion

The genus *Cyanoboletus* was erected in 2014 to accommodate three existing species that were phylogenetically shown as a clade distinct from *Boletus* (Wu *et al.* 2014, Vizzini 2014). It is typified by the European *Cyanoboletus pulverulentus*. All three species exhibit an intense bluing colour reaction in the flesh when exposed to air, which, although not unique to the group, is a distinctive field character uniting them. They associate with both coniferous and broadleaf trees worldwide. Eight species are currently accepted in the genus ([www. speciesfungorum. org](http://www.speciesfungorum.org)).

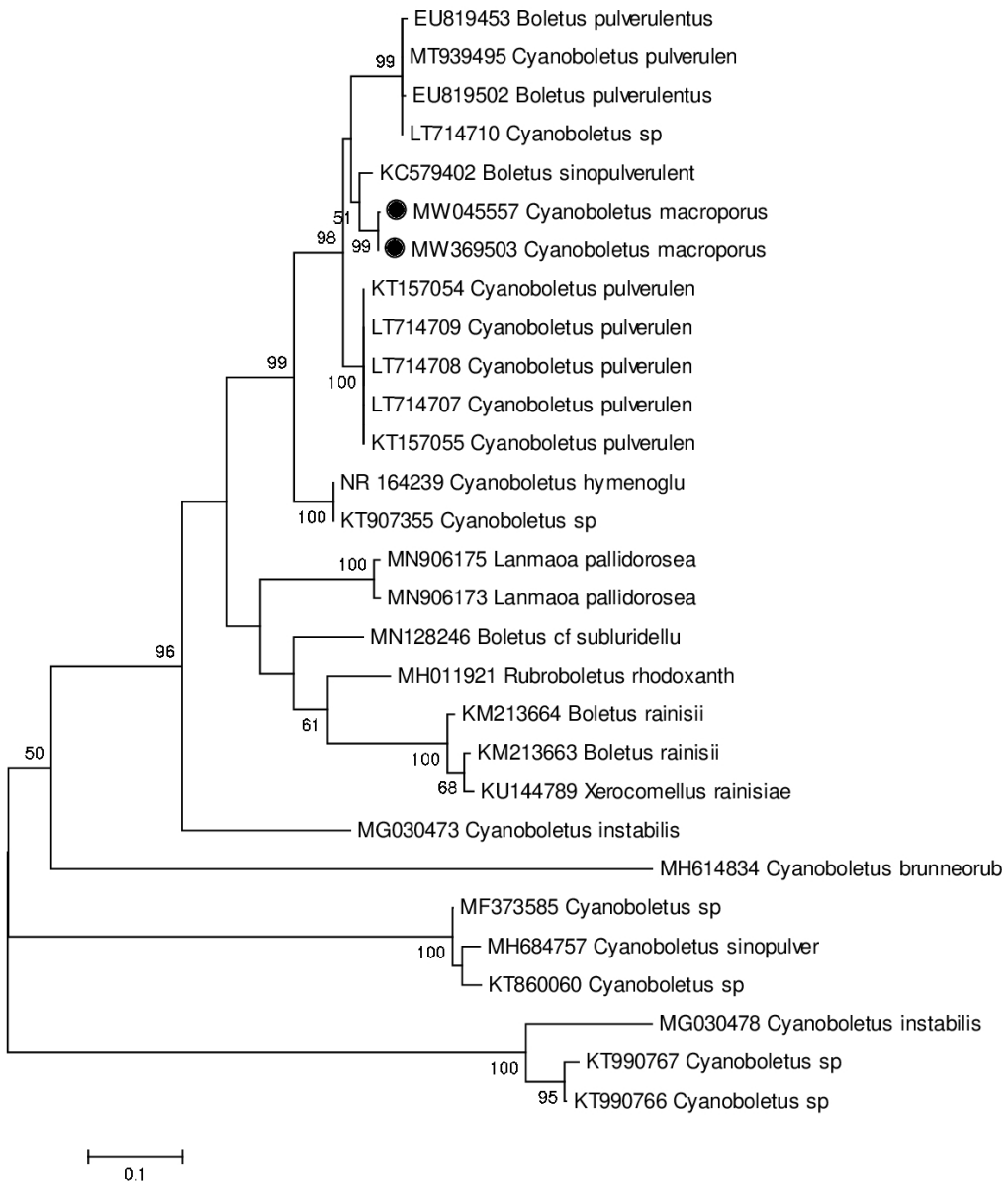


Figure 3. Phylogenetic position of *Cynoboletus macroporus* from Pakistan with respect to other related spp. Tree inferred by maximum likelihood analysis based on rDNA ITS region. The numbers in branches indicate the percentage (>50%) at which a given branch was supported in 1000 bootstrap replications. GenBank accession numbers are given at the end of species names. Highlighted taxa indicate species reported from Pakistan. Illustrator: Dr. Samina Sarwar (Corresponding Author)

Cyanoboletus macroporus described as a novel in this study is characterized by dark brown pileus, instantly basidiomata color change to olivaceous black to dark greenish black, stipe without reticulations, pore surface with much wide pores as compared to all related species and slightly longer basidiospores. Macromorphologically, *Cyanoboletus pulverulentus*, *C. sinopulverulentus*, *C. hymenoglutinosus* and *C. rainisii* are similar to the present species.

C. macroporus differ from *C. sinopulverulentus* due to instantly bruising reaction that is olivaceous black to dark greenish black of basidiomata, pore surface yellowish brown to dark brown, with much wider pores in former while Indigo blue color reaction of basidiomata and deep yellow pore surface and finely scabrous-scissurate radially resembling a zebra pattern on stipe in latter. Similarly, from *C. macroporus*, another closely related taxa *C. pulverulentus* can be distinguished by instantly bruising deep ultramarine blue on handling and narrow pores. From *C. hymenoglutinosus* it can be differentiated due to highly glutinous basidiomata and its narrow pores. *C. macroporus* lack the typical glutinous pileipellis and has wider pores. All other species (Table 1) can be separated from this species due to their narrow pores that is remarkable feature of *C. macroporus*. *C. flavosanguineus* can also be differentiated due to its lower part of stem covered with hairs visible with optical microscope. *C. rainisii* has bright yellow glabrous stipe with reddish hues at the extreme base (Smith & Thiers 1971, Bessette *et al.* 2010, Gelardi *et al.* 2013, Vizzini 2014).

Molecular and phylogenetic analysis also supports the morphological comparisons. *C. macroporus* clusters as an independent species, as sister to *C. sinopulverulentus*, they are in turn sister to *C. pulverulentus* sequences. Sequences of *C. macroporus* falls in *C. pulverulentus* clade but showed its distinct identity from *C. sinopulverulentus* and *C. pulverulentus* (Fig. 3). Both morphological and molecular analysis showed that *C. macroporus* is a new species.

Key to *Cyanoboletus* species

Key distinguishing feature of genus

Deep ultramarine blue bruising reaction of the fruit bodies.....*Cyanoboletus*

Lower part of stem covered with hairs visible with optical microscope.....*C. flavosanguineus*

highly glutinous basidiomata, instantaneously changing (to blue-black) pore surface and context.....*C. hymenoglutinosus*

wider pores....*C. macroporus* sp. nov.

young cap and stipe covered with powder....

C. pulverulentus.

bright yellow glabrous stipe with reddish hues at the extreme base, instantly stain greenish-black,*C. rainisii*

stipe without reticulum but finely scabrous-scissurate radially resembling a zebra pattern and yellow pore surface.....*C. sinopulverulentus*

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Conflict of Interest

This work is original and there is no conflict of interest.

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