

## MOLECULAR PHYLOGENETIC ANALYSIS OF FLESHY PORED MUSHROOMS: *NEOBOLETUS LURIDIFORMIS* AND *HORTIBOLETUS RUBELLUS* FROM WESTERN HIMALAYAN RANGE OF PAKISTAN

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### Abstract

Fleshy pored mushrooms is the name given to boletes due to their porous hymenium and fleshy nature. These are ectomycorrhizal basidiomycetes found in all continents except Antarctica. These mushrooms are important economically due to their edibility and medicinal value. This research work highlights the diversity of boletes in Pakistan and their correct identification by using molecular phylogenetic techniques. Western Himalayan range (WHR) of Pakistan is considered as diversity rich area. During present investigation regarding diversity of boletes in these areas, two bolete taxa viz. *Hortiboletus rubellus* and *Neoboletus luridiformis* were found under conifers. These mushrooms were collected and analyzed morphologically as well as phylogenetically by using Internal Transcribed Spacer (ITS) region of nrDNA sequences, and compared with their allies. All description and comparison with related taxa is provided in detail. These boletes are first time analyzed using molecular method from Pakistan.

**Key words:** *Boletus*, *Cedrus*, nrDNA, taxon.

### Introduction

Boletaceae (Boletales; Basidiomycota) is a large family characterized by porous hymenium rather than gills in its members mostly found in moist temperate areas all over the world (Corner, 1972). Members of this family form mycorrhizal association with pines but a few exceptions are there. *Hortiboletus rubellus* (Kromb.) Simonini, Vizzini & Gelardi and *Neoboletus luridiformis* (Rostk.) Gelardi, Simonini & Vizzini are two important taxa of this family with porous hymenium and fleshy fruiting bodies (Šutara, 2008).

*Neoboletus luridiformis* is generally called as “dotted stem bolete”. This taxon has been given new name by transferring from *Boletus* to *Neoboletus* Gelardi, Simonini & Vizzini, after analyzing it genetically during 2014. Now it became the type species of *Neoboletus* (Gelardi *et al.*, 2014). This species is edible only after cooking (David, 1986).

*Hortiboletus* Simonini, Vizzini & Gelardi is another newly described genus like *Neoboletus* based upon molecular phylogenetic analysis (Vizzini, 2015). This genus likes to grow in urban areas and gardens as its name indicates. Previously the members of this genus were placed in *Xerocomellus* Šutara. This genus is distinguished from *Xerocomellus* due to smooth spores in all *Hortiboletus* species (Nuhn *et al.*, 2013; Wu *et al.*, 2014; Vizzini, 2015). *Hortiboletus rubellus* is the type species of this genus and commonly known as “ruby bolete”. Previously this species was known as *Xerocomellus rubellus* (Krombh.) Šutara. It is very famous due to its bluing bruising reaction after cutting. *Hortiboletus rubellus* is characterized by velvety, dry pileus surface, slow bruising reaction and smooth spores (Smith & Theirs, 1971; Theirs, 1975).

The climate of Western Himalayan range in Pakistan is moist temperate with maximum biodiversity. Many mushroom species including boletes have been reported from this region. During

present investigation *H. rubellus* and *N. luridiformis* have been identified and reported from Pakistan first time based on molecular phylogenetic analysis.

### Materials and Methods

**Sampling:** Basidiocarps were collected during rainy season (July-September) from selected sampling sites of WHR including, Khaira Gali and Khanspur, Abbottabad district; Kaghan valley, Hazara district; Kalam and Mashkun, Swat district; Murree, Rawalpindi district and Shringal, Upper Dir district in Punjab and Khyber Pakhtunkhwa, Pakistan. Field notes were prepared for each specimen by recording its fresh characters, taking photographs and habitat description. Voucher number was given to each sample. Samples were dried in the laboratory with the help of fan heater. After drying each specimen was kept in separate sealing bag for further processing. After analysis voucher specimens were deposited in the fungal section of the Herbarium (LAH), Department of Botany, University of the Punjab, Lahore, Pakistan.

**Morphological and anatomical characterization:** 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 anatomical analysis, small sporocarp tissues of each specimen were mounted in Lactic acid, KOH, Trypan blue and Melzer's reagent and length, width, Shape, and contents of cytoplasm of basidiospores, basidia, hymenial cystidia, pileipellis and its terminal cells, and their color reactions were recorded.

**Phylogenetic analysis:** Enzymatic digestion and glass-fibre filtration (EDGF) protocol was used for DNA extraction from dried sporocarps and internal transcribed spacer region (ITS) of nrDNA was amplified following Dentinger *et al.* (2010). ExoSAP-IT® (Affymetrix, High Wycombe, UK) was used for PCR products purification and BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies/ABI, California, USA) was used for dye-terminated unidirectional sequencing in 10 µL reactions by using protocol of Dentinger *et al.* (2010). After cleaning sequencing reactions with ethanol precipitation following the manufacturer's instructions, these were resuspended in 30 µL of distilled water, and run on an ABI 3730 DNA sequencer. Sequence chromatograms were edited by BioEdit software and comparisons to GenBank records using BLAST. All sequences were deposited in GenBank. The sequence alignment was carried out using MUSCLE alignment software. Phylogenetic trees were constructed with the maximum likelihood criterion in MEGA6 software.

## Results

*Neoboletus luridiformis* (Rostk.) Gelardi, Simonini & Vizzini *Index Fungorum*, 192: 1 (2014) **Figs. 1&2**

**Pileus** 7–13 cm wide, plane to convex and hemispheric, dark brown to reddish brown or yellowish brown, smooth, dry, margins incurved, sometimes decurved, entire, smooth. **Context** yellowish, bluing upon bruising. **Stipe** 2–3.5 cm wide, 7–11 cm long, clavate to subclavate to equal, yellowish to off-white background with reddish dots, centric, solid, dry; **Context** yellowish, ring absent, turns blue upon exposure. **Pore surface** adnate and ascending, reddish to brick red to orangish red, bruised blue upon exposure, pores small, frequent, tubes 11–18 mm deep, yellowish. **Basidiospores** 13–16 × 5–7 µm, (14.06 ± 0.7 × 6.15 ± 0.77;  $Q_m = 1.97 ± 0.15$ ), smooth, subfusoid, thick walled. **Basidia** 20–27 × 9–13 µm, clavate, hyaline, four sterigmata, contents visible in KOH.

**Cystidia** 34–48 × 10–13 µm, subclavate to cylindrical, cylindrical-fusoid. **Pileipellis** 68–73 × 14–18 µm, long cylindrical hyphae in a tangled layer, thick walled, yellowish brown contents, most terminal elements of pileipellis 47–52 × 7–8 µm, subclavate to cylindrical. **Taste** mild, **Odour** unpleasant. **Edibility:** edible after cooking. **Chemical reactions:** spores yellowish brown in Melzer's reagent, pileipellis stains light brown in Melzer's reagent, bluish in FeSO<sub>4</sub>, light yellowish in lactic acid and KOH.

**Habit and habitat:** Solitary to gregarious, on ground.

**MATERIAL EXAMINED: PAKISTAN: PUNJAB,** Murree, 2559 m a.s.l., under *Pinus wallichiana* A.B. Jack., 18 June 2010, Sarwar S.B. # (LAH90), (GenBank KJ802930). **KHYBER PAKHTUNKHWA,** Kaghan valley, 2011 m a.s.l., under *Abies pindrow* Royle, 18 June 2011, Tayiba A. # (LAH59), (GenBank KJ802931); Kalam, 2400 m a.s.l. under *Cedrus*

*deodara* (Roxb. ex D. Don) G. Don, 3 September 2013, S. Jabeen K2-3 (LAH35032) and K2-4 (LAH35033); Mashkun, 2500 m a.s.l., under *C. deodara*, 5 September 2013, S. Jabeen SJ4 (LAH35031); (GenBank KM199730); MTT (LAH35034); MT (LAH35035).

*Hortiboletus rubellus* (Kromb.) Simonini, Vizzini & Gelardi *Index Fungorum* 244: 1 (2015) **Figs. 3&4**

**Pileus** convex to hemispheric to obtuse, 5–11 cm wide, dull red to brownish red to orangish red, surface smooth to areolate to subtomentose, dry, ruptured in distinct patches, margins entire, off-white to reddish white, color different from rest of pileus. **Context** straight to deflexed, off-white to yellowish, becomes blue upon bruising. **Taste and odour:** not distinct. **Stipe** 2–3 cm thick, 5–9 cm long, clavate when young, equal to slightly tapering at ends when mature, central, solid, reddish at apex with off-white striations, yellowish context which turns blue upon bruising. **Pore surface** adnate to sinuate, ascending to free, cream to yellowish, bruise blue, tubes 9–13 mm deep, pores 2–3 per mm. **Basidiospores** 12.5–15.5 × 4.8–7.2 µm, (13.8 ± 0.75 × 6.02 ± 0.69;  $Q_m = 2.3 ± 0.43$ ), smooth, apiculate, fusiform. **Basidia** 47–53 × 13–15 µm, clavate, dark brown granular contents in Melzer's reagent, 1–4 long and sharp sterigmata. **Cystidia** 44–49 × 10–11.5 µm, cylindrical, subclavate, fusoid to ventricose or ampullaceous. **Pileipellis** 47–52 × 7–8 µm, long, thin walled, septate, cylindrical, erect hyphae, terminal elements 41–45 × 19–20 µm, versiform, slightly globose, clavate to cylindrical, ventricose, fusoid, septate, thick walled. **Edibility:** edible. **Chemical reactions:** pileipellis stains yellowish in KOH, hyaline to light yellow in Melzer's reagent; olive green in FeSO<sub>4</sub>, spores yellowish brown to blackish brown in Melzer's reagent.

**Habit and habitat:** Solitary on ground.

**MATERIAL EXAMINED: PAKISTAN: KHYBER PAKHTUNKHWA,** Khanspur, 2350 m a.s.l., under *Quercus incana* Roxb., 7 July 2010, S. Sarwar S.B. # 45 (LAH0710), (GenBank KJ802928); Khaira gali, 2347 m a.s.l., under *Pinus wallichiana* A.B. Jack., 21 August 2010, S. Sarwar S.B. # 45A (LAH0810), (GenBank KJ802929); Shiringal, 2465 m a.s.l., under *Cedrus deodara* (Roxb. ex D. Don) G. Don, 29 August 2014, S. Jabeen & A. N. Khalid SJ113 (LAH35030), (GenBank KX907539).

## Molecular phylogenetic characterization Fig. 5

Internal Transcribed Spacer (ITS) region of nrDNA was successfully amplified from sporocarps of *Neoboletus luridiformis* and *Hortiboletus rubellus*. BLAST showed 99% similarity of Pakistani sequences of *N. luridiformis* with *Boletus erythropus* (DQ131633 & DQ131634); while sequences of *H. rubellus* samples from Pakistan during present study showed 97% similarity with *Xerocomus rubellus* (EF644119) and *B. rubellus* (GQ166883).

Phylogenetic tree was constructed using maximum likelihood criterion (Fig. 5). Closely related sequences retrieved from GenBank. The analysis involved 47 nucleotide sequences. After alignment and trimming from both 3' and 5' sites had 993 genetic characters of rDNA-ITS sequences.



Fig. 1. *Neoboletus luridiformis*: A–D. Fresh basidiomata in the field showing distinct features. E. Stipe surface with distinct blue color change. F. Dotted stipe. G. Orangish red pore surface & hymenium with blue color upon bruising. Bar: A–D = 3 cm.

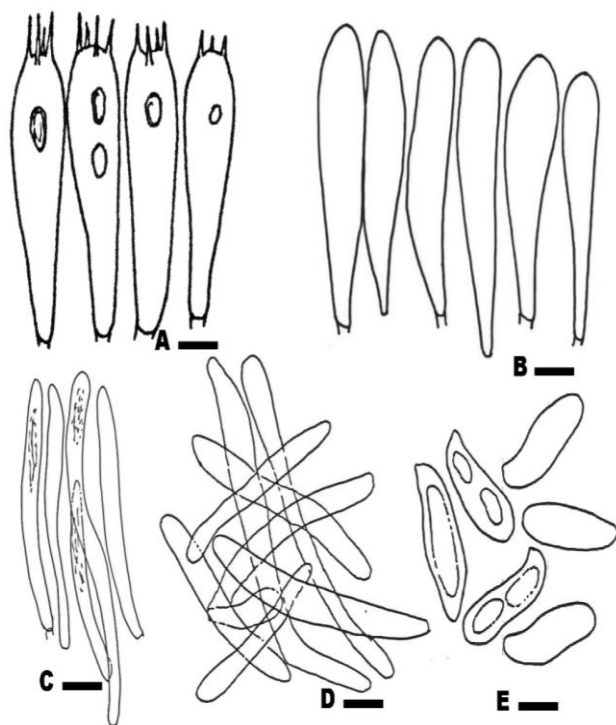


Fig. 2. *Neoboletus luridiformis*: A. Basidia. B. Cystidia. C. Terminal elements of pileipellis hyphae. D. Pileipellis hyphae. E. Basidiospores. Bar: A = 6.5  $\mu\text{m}$ , B = 13.5  $\mu\text{m}$ , C = 10  $\mu\text{m}$ , D = 19  $\mu\text{m}$ , E = 5  $\mu\text{m}$ .

All characters were of type 'unord', gaps were treated as "missing" data while multistate characters were interpreted as uncertain.

### Discussion

Based on available shared genetic and morphological characters analyzed in the present study, *Neoboletus luridiformis* and *Hortiboletus rubellus* were identified and found very close to similar taxa reported from other countries. The phylogram showed two major clades. The sequences of *N. luridiformis* clustered in clade I and found phylogenetically closest to *B. erythropus* (DQ131633 & DQ131634) by sharing more than 99% of genetic characters and only 0.0 to 0.3% genetic divergence. *Hortiboletus rubellus* sequences from Pakistan clustered in clade II with well supported bootstrap value and found phylogenetically closest to *Xerocomus rubellus* (EF644119 and JQ685725) by sharing more than 97% genetic characters with 0.3% genetic divergence. Shared genetic characters and maximum similarity were well above the 97% cutoff value for species delimitation, and genetic divergences were low enough to confirm these species as *H. rubellus* and *N. luridiformis*. *Neoboletus luridiformis* is distinguished by presence of dots on stipe with no reticulations and bright red to orange red pores on the underside of the pileus. Due to presence of dots on stipe it is commonly known as dotted stem bolete. It can be confused morphologically with *Suillellus*

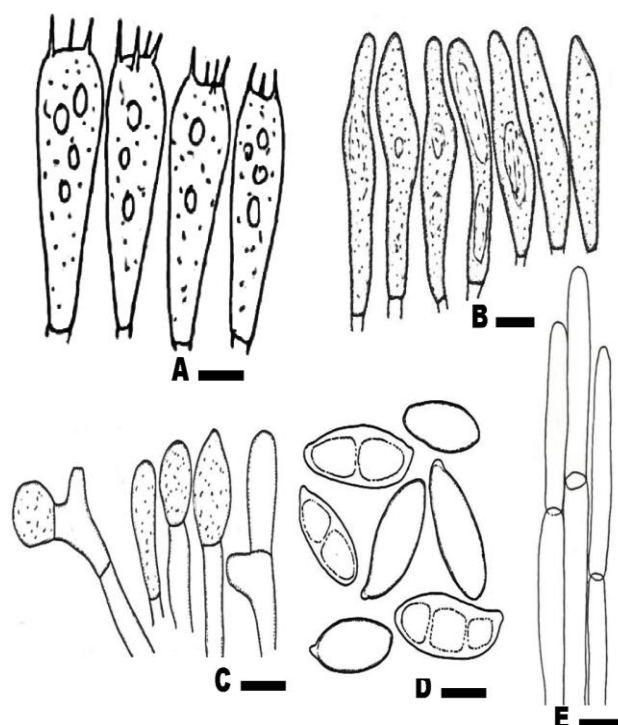


Fig. 4. *Hortiboletus rubellus*: A. Basidia. B. Cystidia. C. Terminal elements of pileipellis hyphae. D. Basidiospores. E. Pileipellis hyphae. Bar: A = 12.5  $\mu\text{m}$ , B = 14.5  $\mu\text{m}$ , C = 18  $\mu\text{m}$ , D = 6  $\mu\text{m}$ , E = 14  $\mu\text{m}$ .

*luridus* (Schaeff.) Murrill (synonym: *Boletus luridus*) and *Rubroboletus satanas* (Lenz) Kuan Zhao & Zhu L. Yang (synonym: *Boletus satanas*); but in all these taxa, stipe characters vary from one another (Haas, 1969; Bessette *et al.*, 2000). Another closely related species is *B. subvelutipes* Peck which has reddish hair on the stipe base and a brighter, more orange colored pileus (Bessette *et al.*, 2000). Phylogenetically it clustered with other *B. erythropus* sequences reported from different countries with significant bootstrap support (Fig. 5). It is an ectomycorrhizal taxon found growing in deciduous and coniferous woodlands. It is widely distributed in Europe and North America (Thiers, 1975; David, 1986).

*Hortiboletus rubellus* was shifted to *Hortiboletus* in 2015 (Vizzini, 2015). This species has punctuate stipe with dull reddish orange dots from the base up to a yellow apex. In comparison with closely related *Boletus campestris* A.H. Sm. & Thiers, the pores present at the underside of the pileus are relatively smaller in diameter (Bessette *et al.*, 2000; Šutara, 2008). Phylogenetically it clustered with *B. rubellus* reported from other countries with highest bootstrap value (Fig. 5). This is an ectomycorrhizal taxon growing mostly in gardens, parks as its name indicates. Molecular analysis during present study indicates that Pakistani sample shared maximum genetic characters with *B. rubellus* sequences retrieved from GenBank. During this study, both taxa analyzed in detail and given name after observing morphological and phylogenetic data.



Fig. 3. *Hortiboletus rubellus*: A – E. Fresh basidiomata in the field showing distinct features. F. Hymenium with blue color upon injury. G – H. Smooth to ruptured pileus surface. F. Areolate to squomulose pileus surface. I – J. Longitudinally striated stipe. Bar: A–E = 1.5 cm.

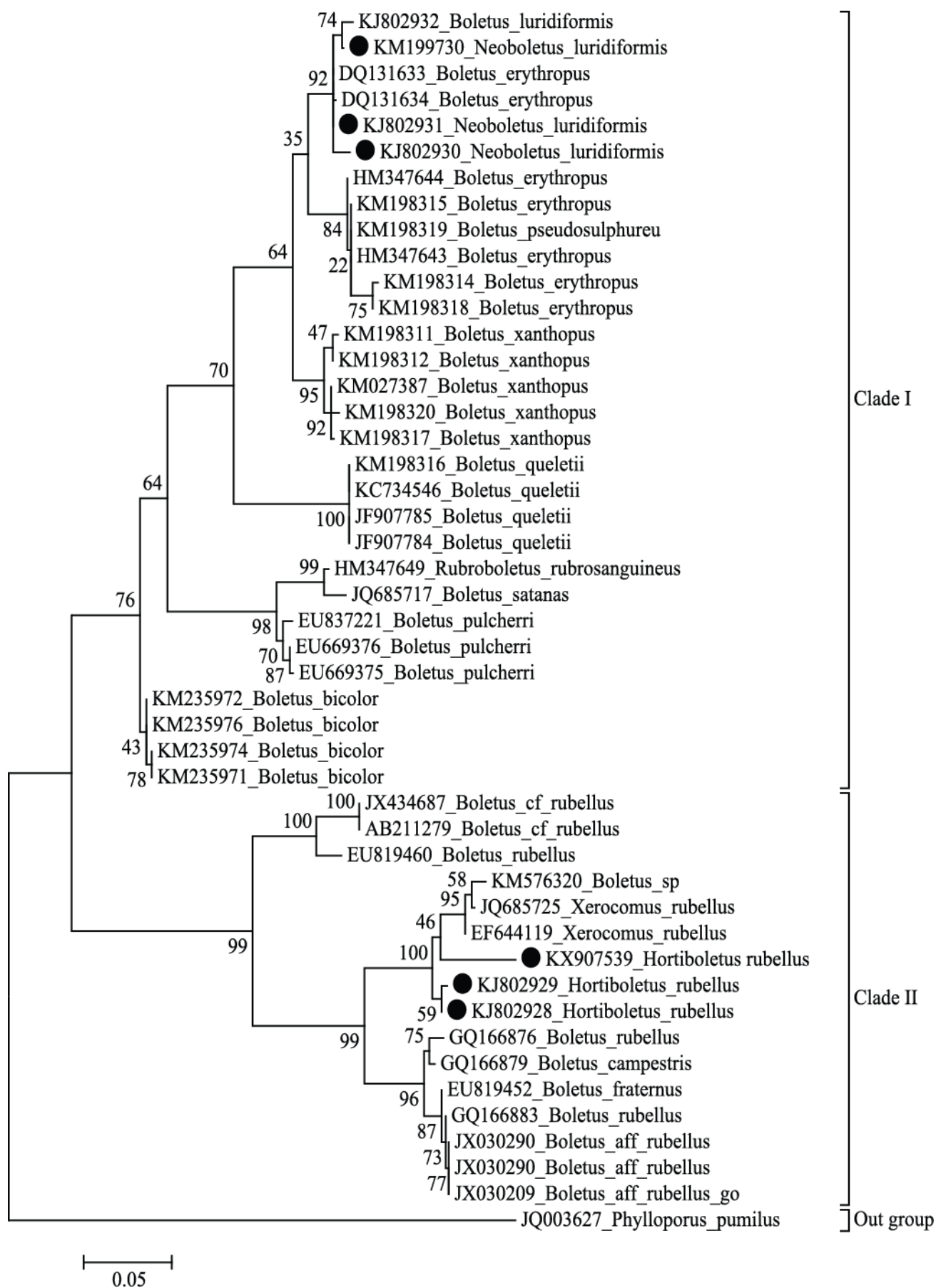


Fig. 5. Phylogenetic position of *Neoboletus luridiformis* and *Hortiboletus rubellus* with respect to other related species. Tree inferred by maximum likelihood analysis based on ITS- nrDNA sequences. *Phylloporus pumilus* M.A. Neves & Halling was included as out group. The numbers against branches indicate the percentage (>50%) at which a given branch was supported in 1000 bootstrap replications.

## Conclusion

From Pakistan, previously boletales were identified based on morphological data only and some species were misidentified. The corresponding author used molecular methods first time in Pakistan to identify this group of mushrooms. During this study two species of boletes were identified based on molecular phylogenetic analysis combining with detailed morphological and anatomical data and given the names according to recent nomenclature

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