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First records of *Bolbitius coprophilus* (Agaricales, Bolbitiaceae) from Pakistan

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Abstract

In efforts to record fungal biodiversity of Pakistan, we collected specimens of the genus *Bolbitius* Fr. from two arid to semi-arid localities in Punjab Province. On the basis of morphological evidence and ITS-nrDNA sequence data, our materials are identified as *B. coprophilus* (Peck) Hongo. These specimens were found to be identical in morphological characteristics to other *B. coprophilus* collections from throughout the world. Based on a phylogenetic analysis, sequences from our material clustered within the *B. coprophilus* clade, which is consistent with our morphological findings. Our three records of *B. coprophilus* from Pakistan are the first from the country. The distribution of *B. coprophilus* is discussed and a morphological comparison with closely related taxa is provided.

Keywords

Khanewal, new report, phylogeny, taxonomy

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Introduction

The genus *Bolbitius* Fr. (Agaricales, Bolbitiaceae) is recognized by the having the pileus with a viscid surface, sulcate-plicate to pectinate pileal margins, free gills, a hymenoderm type of pileipellis, non-capitate cheilocystidia, and thick-walled, brown, smooth spores with a germ pore (Watling 1982; Arnolds 2003; Amandeep et al. 2013; Malysheva et al. 2015). The genus is characterized by elongated having clavate cells packed in gelatinized pileipellis forming a typical hymeniderm. The gelatinous pileus surface of *Bolbitius* taxa distinguishes them from the genus *Conocybe*, which has a dry pileus surface (Tóth et al. 2013). Members of the genus are saprotrophic, preferring a wide range of substrates such as dung, sawdust, humus, sandy or swampy soil and rotten woods (Pegler 1986; Singer 1986; Amandeep et al. 2013). The genus is cosmopolitan (Singer 1986), and it is quite common in some regions of the world such as Great Britain, Ireland, India, and North America (Amandeep et al.

2013). However, there are also some reports of its occurrence from East Africa (Pegler 1977), Japan (Hongo 1958), Malaysia (Watling 1994), Sri Lanka (Pegler 1986), Australia (May and Wood 1997), Czech Republic (Hausknecht et al. 2007), Denmark (Watling 1983), Hungary (Bremer et al. 2007), South America (Singer 1969) and Brazil (Watling 1992). Currently, the genus contains 75 species worldwide (http://www.indexfungorum.org/; accessed on: 2022-5-4). Only *B. vitellinus* (Pers.) Fr. has been reported from Pakistan until now, found on manure heaps (Ahmad 1980; Ahmad et al. 1997). We report *Bolbitius coprophilus* (Peck) Hongo, first described by Hongo (1959), for the first time from Pakistan using morphological data and molecular phylogeny.

Methods

Sampling sites and morphological characterization. We collected *Bolbitius coprophilus* from alluvial plains in three locations in Punjab province, Pakistan: two in Khanewal and one in Nankana Sahib (Fig. 1). These locations were ideal for agricultural activities. The vegetation in these areas is similar to that of a dry desert. Specimens were labelled, photographed, and field notes were prepared. The specimens were dried in a fan heater at >35 °C before preserved in cellophane paper packages. Macromorphological characterization of basidiomata (color, size, and shape), pileus (color, size, and shape), lamellae (type and color), stipe (color, size, and shape), rhizomorphs, and spore print were based on fresh specimens, according to Thomas et al. (2001), Manimohan et al. (2007), and Malysheva et al. (2015).

For microscopic characterization of dried mushroom samples, we prepared fungal slides in 5% aqueous KOH, lactic acid, and trypan blue as the mounting reagent. Details of the basidiospores (shape, size, and position of the germ pore), pileal hyphae, stipe hyphae, cystidia, and basidia were recorded using labomed LX300 binocular microscope at 40× and 100× magnifications. Congo red was used to stain some microscopic characters such as basidiospores, tramal elements, cheilocystidia, caulocystidia, cells/hyphae of pileipellis, and stipitipellis. At least 30 measurements of basidiospores, and 10 measurements of other microscopic characters were recorded per collection. Average and Q values (ratio of length to width) for basidiospores were calculated. Drawings were made with the aid of camera lucida fitted to a light microscope at 40× magnification. The terminology of Pegler (1977), Singer (1986), Atri (2005), and Malysheva et al. (2015) was used to describe the microscopic morphology of our specimens. Specimens are deposited in LAH Herbarium Department of Botany, University of the Punjab, Lahore, Pakistan.

Molecular characterization and phylogenetical analysis. For molecular studies, genomic DNA was extracted from the dried lamellae using modified CTAB method of Gardes and Bruns (1993). Internal transcribed spacer region (ITS) of nrDNA was amplified using the ITS1F/ITS4 primers (White et al. 1990). PCR conditions for ITS-rDNA included: initial denaturation at 95 °C for 2 min, 95 °C for 1 min, 52 °C for 1 min, 72 °C for 45 s, and 72 °C for 10 min, followed by a 4 °C soak. Amplifications were verified in 1.5% agarose gel prepared with 1× TAE buffer and dyed with ethidium bromide. Bands were visualized in a Gel Documentation System. PCR products were then purified and sequenced at Macrogen in Korea. Consensus sequences

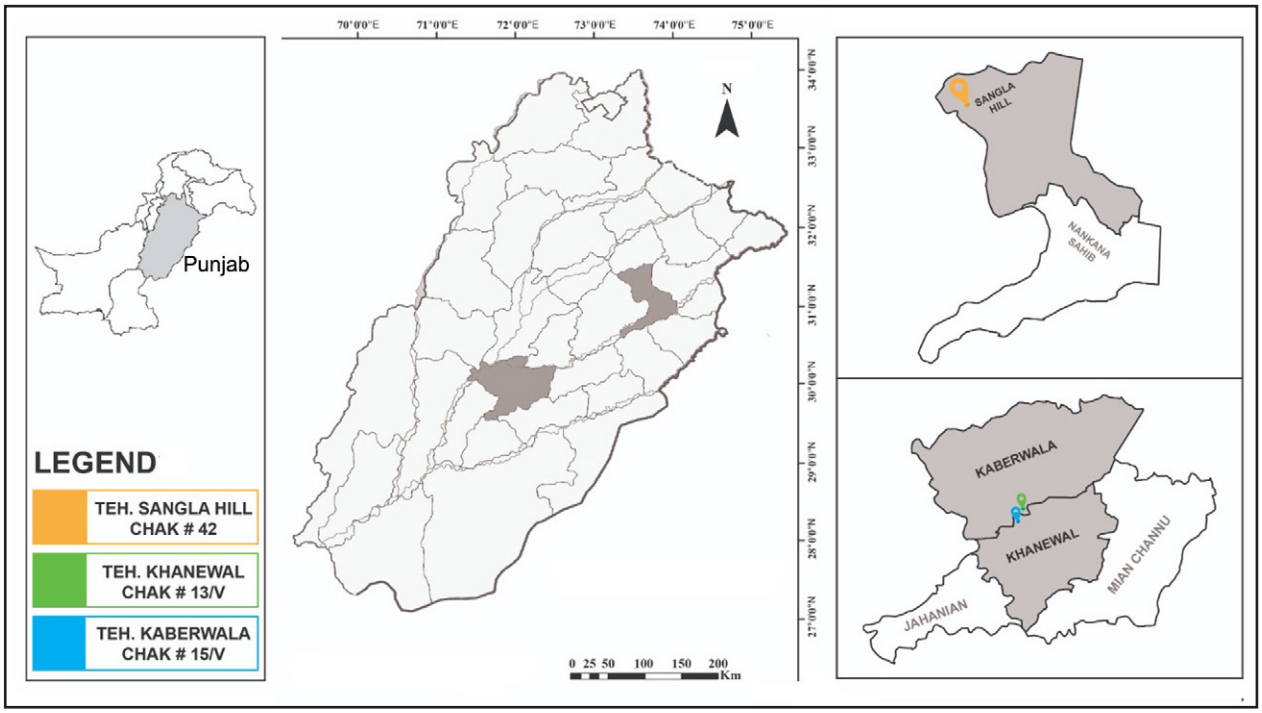

Figure 1. Distribution map of *Bolbitius coprophilus* in Pakistan.

were generated using the BioEdit sequence alignment editor version 7.2.5 using forward and reverse primers (Hall 1999). Initial BLAST search was performed at the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/). Following the results of a BLAST search and published literature (Malysheva et al. 2015), closely related sequences were downloaded from GenBank, and multiple alignment was performed using Clustal W in MUSCLE alignment software (Edgar 2004). Maximum likelihood and the Tamura-Nei model of sequence evolution were used to build a phylogenetic tree using 1000 bootstrap replicates. Phylogenetic analysis was conducted in MEGA X (Kumar et al. 2018). All newly generated ITS sequences were deposited to the GenBank.

Results

***Bolbitius coprophilus* (Peck) Hongo**, Memoirs of the Faculty of Education, Shiga University, Natural Science: 82 (1959)
Figures 2–4

New records. PAKISTAN – **Punjab** • Khanewal District, Tehsil Kaberwala, Chak #13/V; 30°24′24.37″N, 071° 52′00.12″E); 138 m alt.; 07.VIII.2019; M. Usman leg.; near *Oryza sativa* L. fields, on ground, in groups; GenBank MT489696, MT489697, MT489698; KU30, LAH36428 • Tehsil Khanewal, Chak #15/V; 30°18′00″N, 071° 56′00″E); 128 m alt.; 15.VIII.2019; M. Usman leg.; in *Gossypium arboretum* L. fields, solitary; KU48; LAH36428 • Nankana Sahib District, Tehsil Sangla Hill, Morar village, Chak #42; 31°42′41.9472″N, 073°23′18.7908″E); 297 m alt.; 21.VII.2019; A.N. Awan leg.; on wheat straw, in groups; SH42, LAH36428.

Taxonomic description. Basidiomata 7.0–20 cm high, bright. **Pileus** 2–7 cm in diameter, 1–3 cm high, convex to campanulate when young, flat at maturity, umbonate; umbo extended, milky white to pale brown, with distinct pinkish tinge when young, light brownish grey with age; surface viscid, fragile; margins wavy, split at maturity, flesh thin, non-deliquescent; pileal veil absent. **Lamellae** 0.2–0.35 cm broad, free, crowded, unequal, somewhat narrow to moderately broad, white when young, pink to brown with age, fragile, deliquescent, gill edges curled with age. **Stipe** 7.0–18.5 cm long, 0.6–0.8 mm broad, tubular, hollow, fragile, pale yellow, unchanging with time, silky, shiny, fibrillose, thin towards the top, broader towards the base, centrally attached with the pileus. **Base** equal. **Taste and odor** not quite distinct. **Spore print** reddish brown. **Rhizomorphs** present.

Basidiospores 11.4–17.0 × 9.4–11.4 µm (avg = 14.50 × 10.16 µm), Q = 1.39–1.49 (Q_{avg} = 1.42), ellipsoid to ovoid, thick walled, smooth, truncated by a broad germ pore, light brown in 5% KOH. **Basidia** 23–26 × 13.5–17.0 µm, turbinate to clavate, thin walled, 2–4 sterigmata, broader at the top; sterigma 2.6–3.0 µm long. **Cheilocystidia** 24.0–37.5 × 8.0–19.0 µm, cylindrical, thin walled, clavate-verriculose, hyaline. **Pleurocystidia** absent. **Hymenophoral trama** 8.5–15.5 µm, thin walled, hyaline. **Pileipellis** hymeniform; hyphae occasionally septate, branched, irregular, thick walled, 5.7–8.6 µm wide, hyaline, clamp connections absent; pileal terminal cell 19.95–91.2 × 8.55–17.1 µm, clavate, thick walled, elongated, tapering towards the base. **Stipitipellis** cutis, unbranched, regular, thick walled 5.7–28.5 µm wide, hyphoid, clavate, spherical and irregular with projection, hyaline, clamp connections absent. **Caulocystidia** 23.0–25.9 × 6.9–12.5 µm, scattered on the entire stipe surface, clavate, cylindrical, thin walled, hyaline.

Phylogenetic results. ITS dataset contains 34 nucleotide sequences used in molecular phylogenetic analysis, including 32 ingroup sequences of *Bolbitius* and two outgroup sequences (*Conocybe apala* (Fr.) Arnolds JX968209 and *Galeropsis desertorum* Velen. & Dvorák AY194534). Thirty-three sequences, based on the dataset of Malysheva et al. (2015), were retrieved from GenBank, and we added three new generated sequences. In our maximum-likelihood analysis of 779 positions, 393 positions were constant, 169 parsimony-uninformative, and 317 were variable. A phylogenetic tree with a superior log likelihood value (−3716.39) is shown in Figure 5. The genus has been shown to be monophyletic in almost all phylogenetic studies (Moncalvo 2002; Rees et al. 2003; Golden et al. 2005; Matheny et al. 2006). Based on morphological and phylogenetic analyses, our specimens were identified as *B. coprophilus*. In the *B. coprophilus* clade, the sequences from our collections clustered with those from Italy and Russia. This relationship is strongly supported by a significant bootstrap value of 99.

Discussion

Distribution of *Bolbitius coprophilus*. *Bolbitius coprophilus* was originally described from North America by Hongo (1959). Since then, investigators have reported it from various regions of the world as follows: dung heaps in New York (Peck 1893); wheat fields in England (Watling 1982); horse and deer dung mixed with straw in Denmark (Rald 1991), India (Atri et al. 1992, Amandeep et al. 2013), and Italy (Hausknecht and Zuccherelli 1993); scattered on cow dung, compost, and rice straw in Singapore (Watling 1994); compost and wheat straw in Argentina (Alberto et al. 1996); elephant dung in Kerala, India (Thomas et al. 2001; Manimohan et al. 2007). There are only species reports from Europe (Hausknecht et al. 2007), including Poland (Szczepkowski et al. 2009); horse dung in France (Garnier-Delcourt et al. 2015) and Austria (Hausknecht and Krisai-Greilhuber 2003); and straw, dung, and compost in Russia (Malysheva et al. 2015). *Bolbitius coprophilus* has been reported from Indian Punjab by Amandeep et al. (2013).

Morphological comparison with closely related taxa. *Bolbitius coprophilus* is characterized by a broad pileus

Figure 2. *Bolbitius coprophilus*. **A.** Basidiomata (KU30). **B**. Upper side of pileus (KU30). **C.** Lamellae (KU30). **D, E.** Different views of basidiocarp (SH42). **F.** Pileus (SH42). **G.** Underside of pileus (SH42). **H.** Lamellae discoloration (SH42). Scale bars: A–D, F–H = 1.4 cm, E = 3.3 cm.

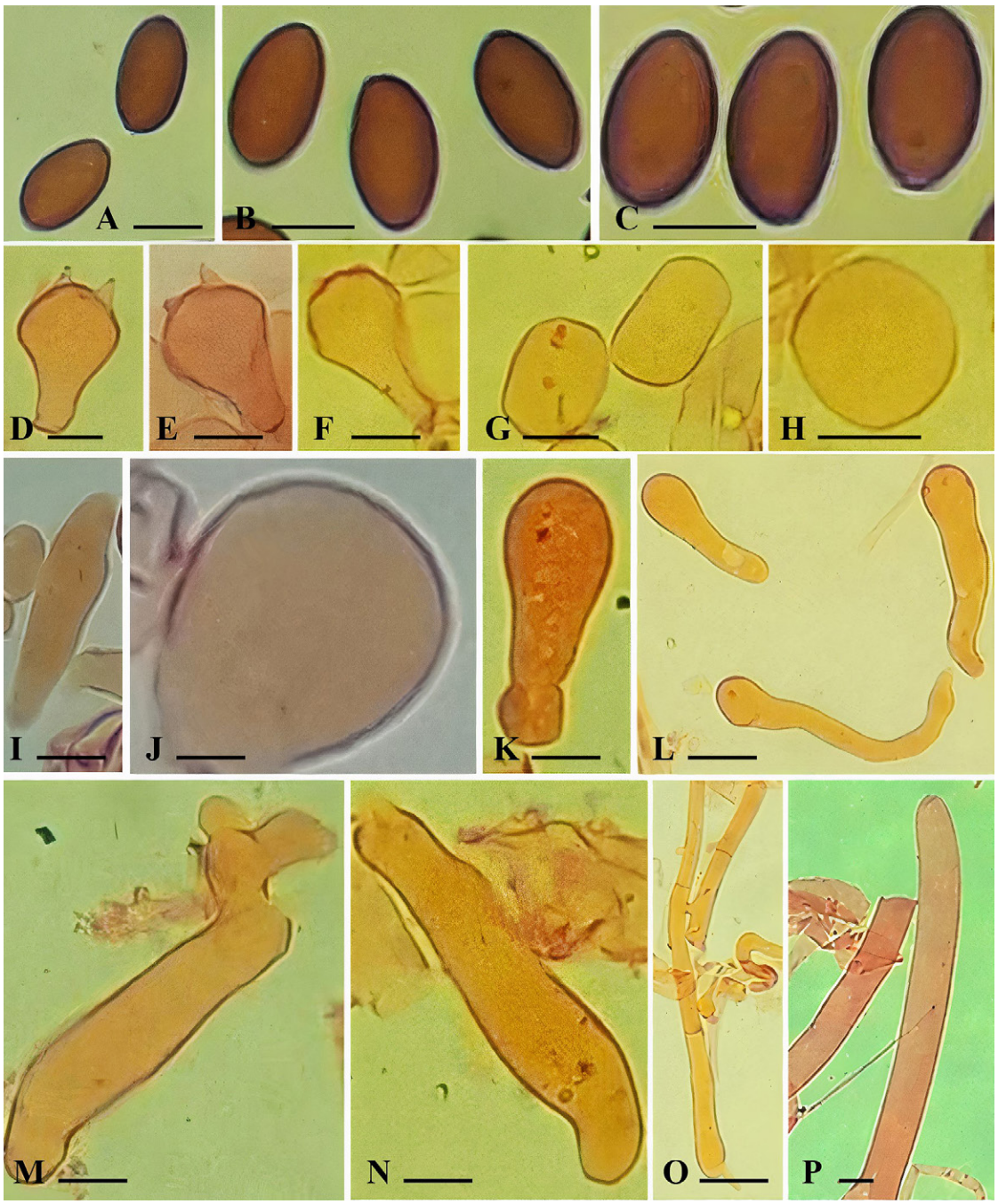

Figure 3. Microscopic features of *Bolbitius coprophilus*. **A–C**. Basidiospores. **D–F.** Basidia. **G, H.** Tramal elements. **I, J**. Cheilocystidia. **K, L**. Elements of pileipellis. **M, N.** Caulocystidia. **O.** Pileipellis hyphae. **P.** Stipe hyphae. Scale bars: 10 µm.

(4.0–7.0 cm), which is pale with a distinct pinkish tinge and a shape that usually varies from convex or campanulate when young and flat at maturity (Watling 1994; Amandeep et al. 2013); the gills are free and non-deliquescent, and the basidiospores are ellipsoid to ovoid (11.4–17.1 × 9.9–11.4 µm) (Szczepkowski et al. 2009; Malysheva et al. 2015). This species prefers to grow on organic substrates that are rich in nutrients, such as dung or compost.

The macroscopic and microscopic characteristics of our specimen are consistent with the taxonomic characteristics of *B. coprophilus* (Singer 1979; Watling 1982; Hausknecht and Krisai-Greilhuber 2003; Amandeep et al. 2013). A morphological comparison of *B. coprophilus* reported from different regions of the world is given in Table 1.

In Ladhar, Pakistan, only *B. titubans* (Bull.) Fr. has been reported (Ahmad 1980), and *B. titubans* appears to be a close relative of *B. coprophilus*. Both species exhibit convex to campanulate pilei, ellipsoidal spores, and clavate basidia. In *B. titubans* (= *B. variicolor* G.F. Atk.), the pileus is yellow to greenish yellow as compared to pink to pale colored pileus in *B. coprophilus*. Additionally, gills are crowded in *B. coprophilus* as compared to close gills in *B. titubans*, and the stipe is also shorter in *B. titubans* (3.0–12 cm) when compared with the longer stipe in *B. coprophilus* (7.0–18.5 cm) (Amandeep et al. 2013).

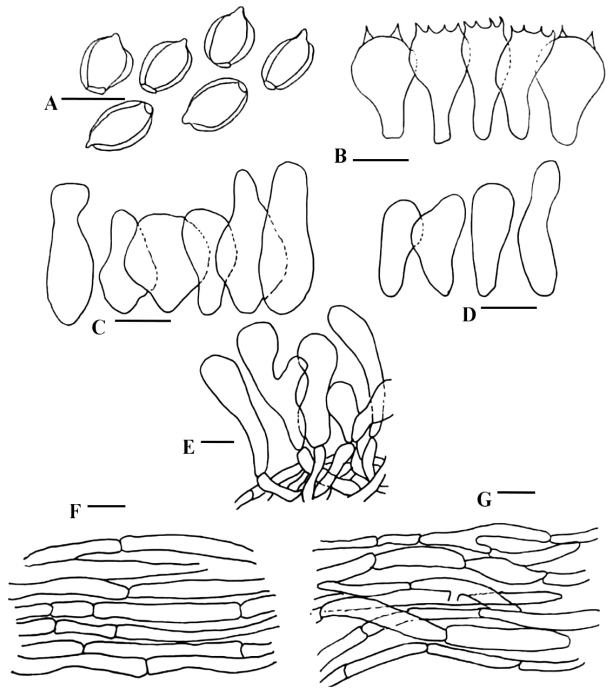

Figure 4. Microscopic features of *Bolbitius coprophilus*. **A.** Basidiospores. **B.** Basidia. **C, D.** Cheilocystidia. **E.** Terminal cells of pileipellis. **F.** Stipitipellis. **G.** Pileipellis. Scale bars: 10 µm.

Bolbitius coprophilus is also morphologically similar to *B.demangei* (Quél) Sacc. & D. Sacc., as both have delicate, campanulate, and umbonate pilei. According to Malysheva et al. (2015), *B. demangei* is very similar to *B. coprophilus*; however, *B. demangei* has smaller basidiomata, a darker pileus with a violaceous tint, and darker lamellae (larger basidiomata and light pinkish pileus and lamellae in *B. coprophilus*) (Amandeep et al. 2013; Melo et al. 2016). *Bolbitius demangei* can also be distinguished microscopically from *B. coprophilus* by its smaller, more slender spores (8.5–14.0 × 6.0–8.0 µm vs. 11.4–17.1 × 9.4–11.4 µm) (Hausknecht and Contu 2006; Malysheva et al. 2015). Although the morphological characters of the two species differ slightly, nrITS sequence data of *B. demangei* (JF907771) is similar (99% similarity) to our specimens of *B. coprophilus*. We consider our specimens to be *B. coprophilus* because they are same clade as in the phylogram by Malysheva et al (2015).

Table 1. Morphological comparison of Pakistani collection of *Bolbitius coprophilus* with specimens of *B. coprophilus* from other countries.

Reference	Pakistan (this study)	Argentina Alberto et al. (1996)	Denmark Ekelund (1991)	India Amandeep et al. (2013)	Malaysia Watling (1994)	Poland Szczepkowski et al. (2009)	Russia Malysheva et al. (2015)
Pileus (cm)	2.0–7.0	1.5–3.0	-	2.3–7.0 × 2–4.2	3.0–7.0	3.0–8.0	1.5–5.0
Stipe, length (cm)	7.0–18.5	(2.4) 5–10	5.0–18	2.4–20.4	5.0–10	5.0–7.0	6.0–13
Gills type	Non-deliquescent	-	Non-deliquescent	-	Non-deliquescent	Non-deliquescent	Deliquescent
Gills attachment	Free	Free, some adnate	Free	Free	Free	Free	Free
Basidia size (µm)	23–26 × 13.5–17	22–25 x 10–15	-	14–34 × 7.0–13.6	20–35 × 13.5–16	17.0–25 × 12–15.3	24–27 × 13–17.5
Basidiospore size (µm)	11.4–17.1 × 9.4–11.4	12–15 × 7–9	12.5–13.75 × 7.5–8.75	10–15.3 × 6.0–9.3	11.5-14.5 × 7.5-9.5	11.5–16.5 × 6.0–10.8	13–16.5 × 8–11
Cheilocystidia (µm)	24–37.5 × 8–19	25–31 × 12–16	-	22.5–39 × 7–20	20–55 × 8.0–20	24–55 × 5.4–19	35–80 × 20–40
Pleurocystidia	Absent	Absent	Absent	Absent	Absent	Present	Absent

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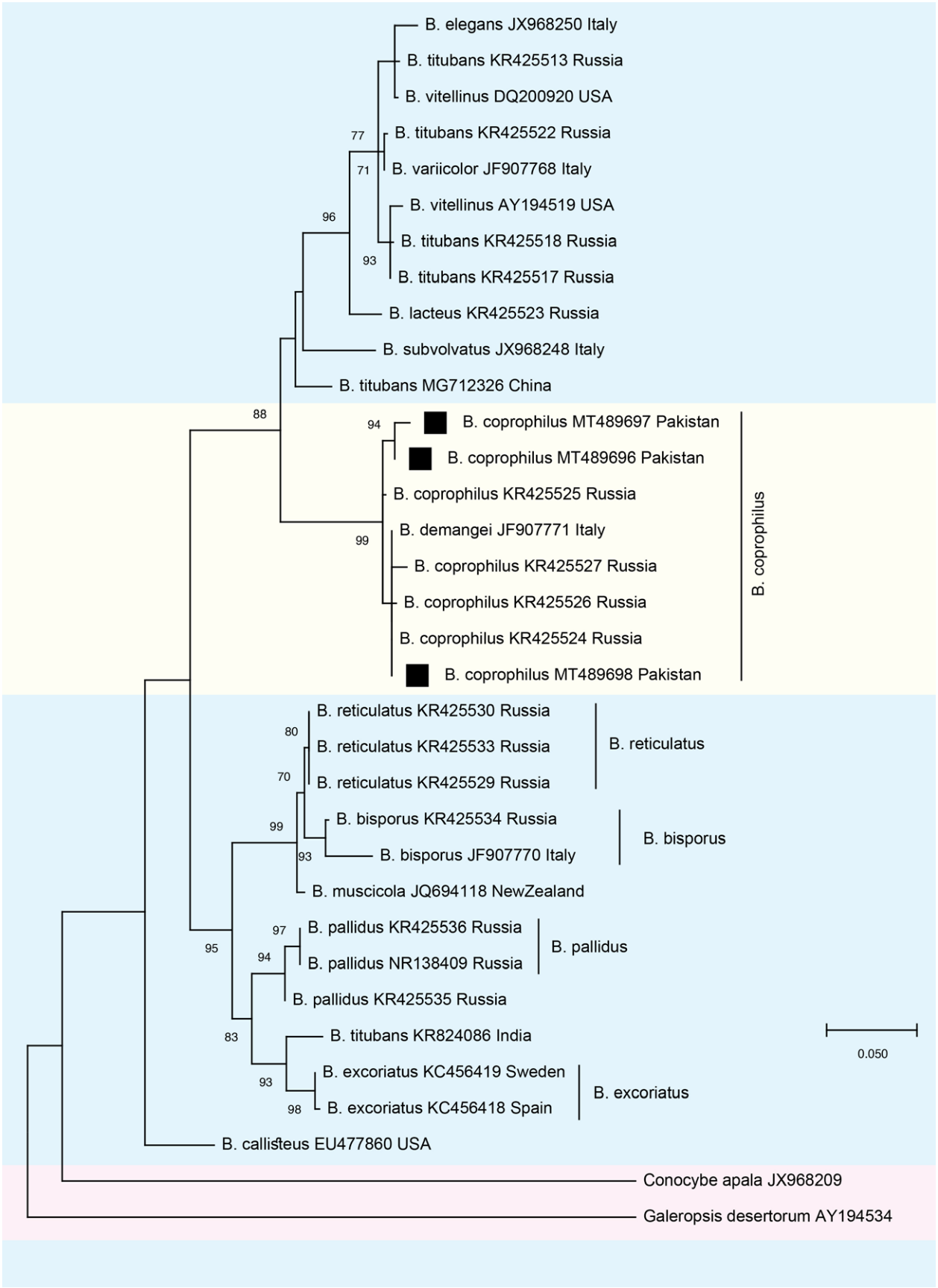

Figure 5. Phylogenetic tree of ITS nrDNA sequences of genus *Bolbitius* (Log-likelihood: –3716.39) based on maximum-likelihood Method. Newly generated sequences are indicated with bullets.

Authors' Contributions

Conceptualization: NY. Data curation: MU, ANA. Formal analysis: NY, MU, ANA. Methodology: MH, ANK, MU. Resources: MU, ANK, ANA, GM, MH. Software: GM. Supervision: NY. Validation: NY. Visualization: MH, ANK. Writing – original draft: ANA, MU. Writing – review and editing: NY.

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