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Research Article

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## First Record of *Bolbitius reticulatus* (Agaricales: *Bolbitiaceae*) in Turkey, with Nuclear ITS and LSU rDNA Sequences Data

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**Abstract:** *Bolbitius reticulatus*, which is pertained to European region, is reported here for the first time from Black Sea Region, north-eastern Turkey based on both morphological characters and constructed multilocus dataset (nrITS, nrLSU rDNA). A complete macro- and micro-morphological description of the species is provided along with the photographs of fresh basidiomes in natural habitat and the line drawings of its microscopic structures. Comparisons of the presented species with phenotypically and molecularly related taxa are elucidated and discussed in detail.

**Keywords:** Basidiomycota, New record, Phylogeny, Taxonomy, Turkey

### *Bolbitius reticulatus*'un (Agaricales: *Bolbitiaceae*) Nükleer ITS ve LSU rDNA Sekans Verileriyle Türkiye'deki İlk Kaydı

**Öz:** Avrupa bölgesine ait olan *Bolbitius reticulatus*, hem morfolojik karakterlere hem de yapılandırılmış çok lokuslu veri setine (nrITS, nrLSU rDNA) dayalı olarak, Türkiye'nin kuzey doğusundaki Karadeniz Bölgesi'nden ilk kez rapor edilmektedir. Türün makro ve mikro morfolojik deskripsiyonu, doğal ortamdaki taze bazidiomların fotoğrafları ve mikroskopik özelliklerinin çizimleri eşliğinde sağlanmaktadır. Sunulan türün fenotipik ve moleküler olarak ilişkili taksonlarla karşılaştırılması aydınlatılmış ve detaylı olarak tartışılmıştır.

**Anahtar kelimeler:** Bazidiyomikota, Yeni kayıt, Filogeni, Taksonomi, Türkiye

#### Introduction

Currently, *Bolbitiaceae* Singer is composed of six genera, namely *Bolbitius* Fr., *Conocybe* Fayod, *Descolea* Singer, *Galerella* Earle, *Gastrocybe* Watling, and *Pholiotina* Fayod. *Bolbitius* is a small and worldwide distributed genus in the family *Bolbitiaceae*. Above 70 species of *Bolbitius* are known to occur in the world (www.indexfungorum.org). The genus is characterized by mostly fragile basidiomata with a brightly-colored, glutinous and viscid pileus surface, free lamellae with ochraceous to rusty brown, a brown spore-print and a hymeniderm pileipellis (Singer, 1951; Arnolds, 2003, 2005; Malysheva et al., 2015). The members of the genus grow on manure, humus sawdust, rotten wood or soil (Watling, 1982; Enderle et al., 1985; Singer, 1986; Pegler, 1986; Arnolds, 2003; Hausknecht et al., 2007; Malysheva et al., 2015). Recent molecular analysis have verified the monophyletic position of *Bolbitius* and its species which form a well supported sister clade to *Pholiotina* (Moncalvo et al., 2002; Matheny et al., 2006; Tóth et al., 2013).

Recently, numerous studies have been done to contribute to the fungal diversity in Turkey (Bozok et al., 2018; Akata and Erdoğan, 2020; Akata et al., 2020; Çağlı and Öztürk, 2020; Keleş, 2020; Kocakaya et al., 2020; Sesli, 2020; Sesli et al., 2020; Şengül et al., 2020; Acar et al., 2021; Doğan, 2021; Kaplan et al., 2021; Keleş and Kaya, 2021; Sesli, 2021; Şengül et al., 2021; Uzun, 2021). The aim of this study is to determine the taxonomic position of *Bolbitius reticulatus* (Pers.: Fr.) Ricken, the first time reported from Turkey, based on both morphological and multilocus dataset, and also to compare it with phenetically similar and phylogenetically allied species.

#### Material and Methods

##### Morphology

The basidiomata were collected from the Black Sea Region, during a field survey in the Hopa district of Artvin Province, located in northeastern Turkey.

The macro-morphological characters were described based on the fresh basidiomata. Micro-characters were studied from fresh and dried material



stained in 1% Congo red or mounted in 3% potassium hydroxide (KOH). Microscopical observations were made on a Leica DM500 light microscope (Leica Microsystems, Wetzlar, Germany), after that the line drawings were made. Among the following abbreviations used:  $L_m$  and  $W_m$  indicate the average length and width of basidiospores,  $Q$  shows the ratios of length/width and  $Q_m$  presents the average quotient of the measured basidiospores or pileipellis elements (Kaygusuz et al., 2021a).

### Reconstruction of the phylogeny

DNA isolation, polymerase chain reaction (PCR) amplifications and sequencing methods were carried out according to the Ref. (Kaygusuz et al., 2021b). The following primer pairs ITS1F and ITS4 were used for the amplification of nrDNA ITS region (White et al., 1990; Gardes and Bruns, 1993), while LR0R and LR5 were used to amplify the nrLSU region (Vilgalys and Hester, 1990). PCR products were purified and sequenced by Sanger DNA sequencing service (Source Bioscience, Berlin, Germany). The newly generated ITS/LSU sequences were deposited in GenBank. Multiple sequence alignment was aligned using MAFFT v7.110 (Kato and Standley, 2013) and manually edited using BioEdit v7.0.5 (Hall, 1999). *Pholiota baeosperma* Singer was used as the outgroup taxa.

For phylogenetic analyses, Maximum Likelihood (ML) and Bayesian Inference (BI) analyses were used. The ML analysis was executed using RAxML v8.2.10 (Stamatakis, 2014) under the GTRGAMMA model of the nucleotide substitution and bootstrap test was done with 1.000 replicates. The BI analysis was conducted with MrBayes 3.2.2 (Ronquist et al., 2012) using Markov chain Monte Carlo (MCMC) method. Four Markov chains were burn-in each for 1.000.000 generations, with sampling every 100 generations. Phylogenetic trees were exhibited in FigTree v1.4.3 (Rambaut, 2016).

## Results

### Molecular studies

For the *Bolbitius reticulatus* study, two nrITS and two nrLSU sequences were generated. Another 57 related sequences (30 nrITS and 27 nrLSU) were taken from GenBank and UNITE databases. The combined data matrix (nrITS and nrLSU) contained 61 sequences with 1045 nucleotide sites. The ML and BI phylogenetic results showed similar topologies and only ML phylogenetic tree with both MLB and BPP values was chosen as the backbone phylogeny (Figure 1). A phylogenetic analysis of a combined data set demonstrated that all sequences of *Bolbitius reticulatus*

formed a monophyletic lineage with strong support (MLB = 80%, BPP = 0.90, Figure 1).

### Taxonomy

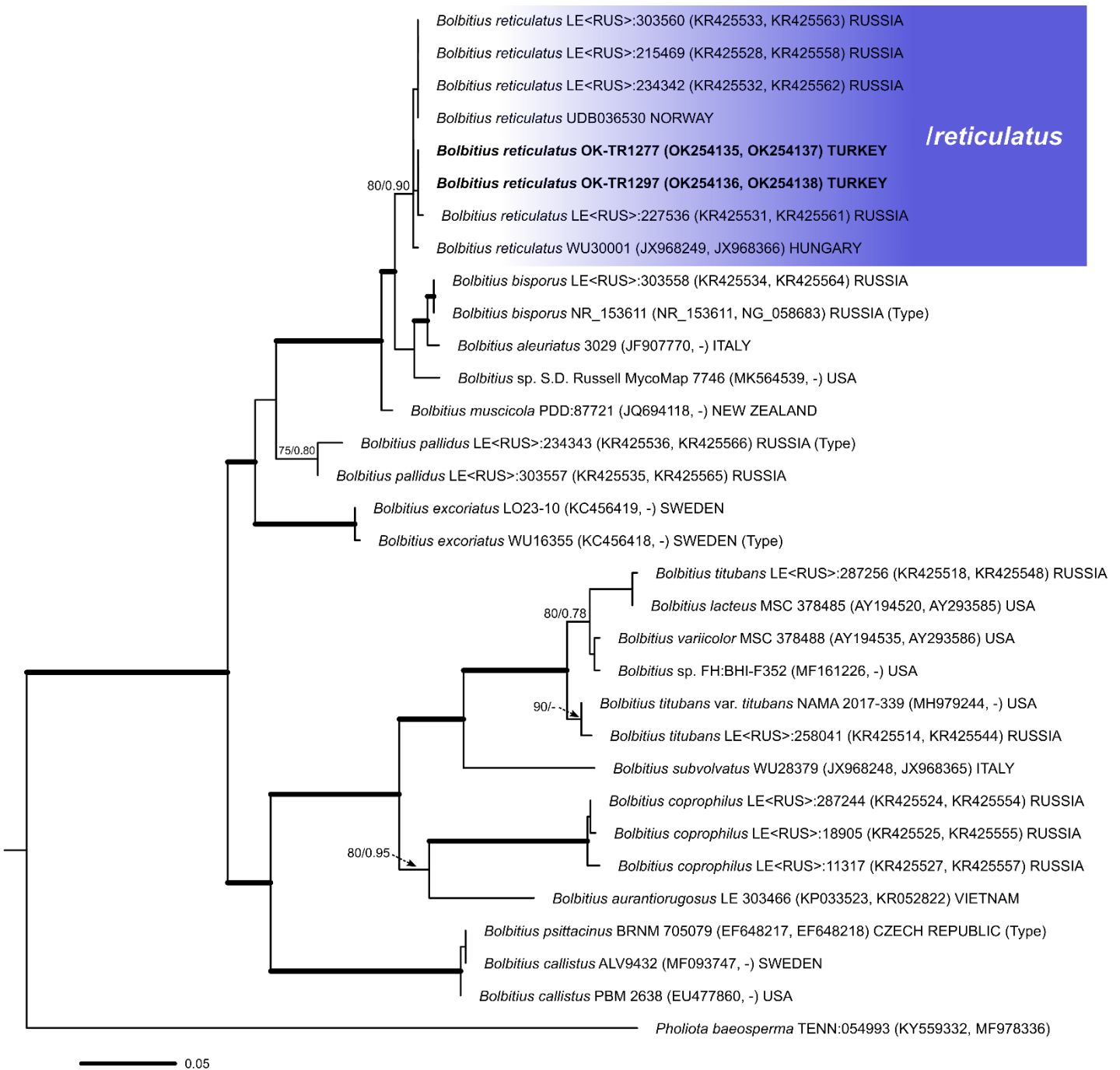
#### *Bolbitiaceae* Singer

*Bolbitius reticulatus* (Pers.: Fr.) Ricken, Die Blätterpilze 1: 68 (1915) (Figure 2 and 3)

Synonyms: *Bolbitius aleuriatus* (Fr.) Singer, Lilloa 22: 490 (1951), *Bolbitius reticulatus* f. *aleuriatus* (Fr.) Enderle, Ulmer Pilzflora 4: 50 (1996), *Bolbitius reticulatus* var. *aleuriatus* (Fr.) Bon, Docums Mycol. 20(no. 78): 39 (1990), *Bolbitius reticulatus* var. *australis* (E. Horak) Garrido, Bibliotheca Mycol. 99: 27 (1985), *Bolbitius reticulatus* var. *reticulatus* (Pers.) Ricken, Die Blätterpilze 1: 68 (1915), *Galera reticulata* (Pers.) P. Kumm., Der Führer in die Pilzkunde: 76 (1871), *Pluteolus aleuriatus* var. *reticulatus* (Pers.) J.E. Lange, Dansk botanisk Arkiv 9 (6): 49 (1938), *Pluteolus reticulatus* (Pers.) Gillet, Hyménomycètes (Alençon): 373 (1876), *Pluteus phlebophorus* var. *reticulatus* (Pers.) Cooke: tab. 422 (1886).

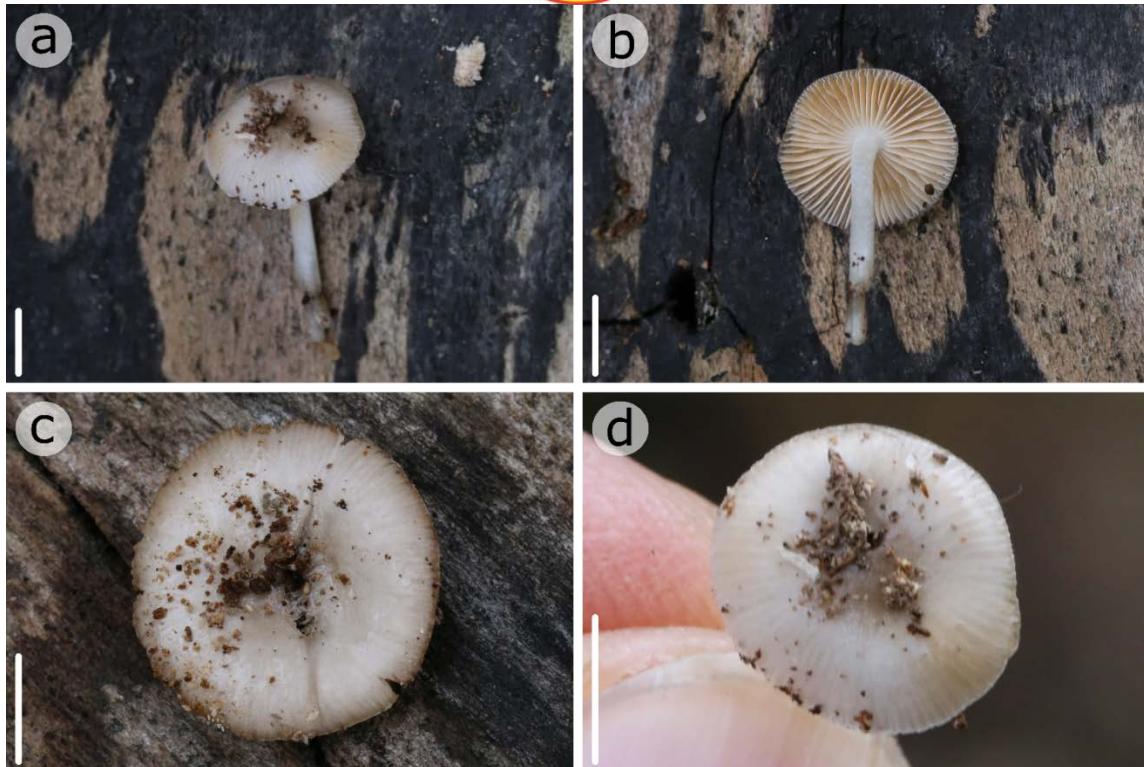
Pileus 8–15 mm diam., subglobose to convex while young, then convex and finally flattened, slightly depressed at center, surface viscid to glutinous, glabrous, not hygrophanous, white pink or greyish beige to white grey, striate margin over one half of the radius in mature. Lamellae moderately crowded, free, thin, pale yellow pink or yellow brown, with flocculose whitish margin. Stipe 10–25 × 1–2 mm, delicate and very fragile, cylindrical or slightly tapering upwards, without bulb, white to pale yellowish, minutely flocculose to pruinose. Context fragile, whitish at the pileus and stipe. Neither the odor nor the taste are distinguishable.

Basidiospores (8.5–)9.6–11.8(–12.7) × (5.0–)5.5–6.5(–7.2) μm,  $L_m \times W_m = 10.7 \times 6.1$  μm,  $Q = 1.5–2.2$ ,  $Q_m = 1.8$ , ellipsoid to oblong, golden brown or light honey brown in KOH, slightly thick-walled. Basidia (14.5–)16.0–20.5(–23.5) × (8.0–)8.5–9.0(–9.5) μm, clavate to broadly clavate, 4-spored, hyaline, thin-walled. Pleurocystidia absent. Cheilocystidia (25.0–)25.5–38.5(–47.5) × (6.5–)10.5–16.5(–17.0) μm, numerous, polymorphic, shape ranging between narrowly utriform to broadly utriform or lageniform with rounded apex, fusiform or rarely clavate, thin-walled, hyaline in KOH. Pleurocystidia absent. Pileipellis hymeniform, made up of clavate, subglobose or globose elements, 15.0–50.0 × 10.0–30.0 μm, often in chains, thin-walled, hyaline in KOH. Stipitipellis a cutis, consisting of 5.0–20.0 μm wide hyphae, smooth and thin-walled, hyaline in KOH.



**Figure 1.** Phylogenetic tree of *Bolbitius* species inferred from the combined dataset (nrITS and nrLSU) using Maximum-likelihood. Maximum-likelihood bootstrap (MLB) values  $\geq 75\%$  and Bayesian posterior probabilities (BPP)  $\geq 0.78$  are shown on the branches. Bold branches represent MLB  $\geq 90\%$  and BPP  $\geq 0.95$ . The generated sequences are shown in bold. Bar indicates 0.05 anticipated changes per site per branch.





**Figure 2.** *Bolbitius reticulatus*: a-d carpophore growing in natural habitat. Scale bars = 5 mm.

Caulocystidia 35.0–46.5 × 10.5–25.5 µm, as variable as cheilocystidia, clavate to broadly clavate, narrowly utriform or narrowly lageniform, thin-walled, hyaline in KOH. Clamp connections absent in all parts examined.

Habit, habitat and distribution: Saprotrophic, solitary or sometimes in small groups, present at elev. 1500 m, on rotten wood of fallen branch of *Fagus orientalis* L., on mountain slope, in humid, shady and cool places, on moist, weakly acid soils, which are rich in humus.

Specimens examined: TURKEY, Artvin Province, around Borçka town, on decayed wood of *Fagus orientalis*, alt. 1500 m, 11 October 2015, O. Kaygusuz, OKA-TR1277; GenBank: OK254135 for nrITS, OK254137 for nrLSU; *ibid.*, on wood and fallen branch of *F. orientalis*, alt. 1510 m, 15 October 2015, O. Kaygusuz, OKA-TR1297; GenBank: OK254136 for nrITS, OK254138 for nrLSU.

### Discussion

*Bolbitius reticulatus* is a widely distributed but inconspicuous woodland species. It is characterized by delicate and sticky when moist basidiocarps with greyish, greyish-purple or brownish pileus surface, free and ochre-brown lamellae, pruinose to furfuraceous stipe, and habitat on woody substrates (Arnolds, 2003, 2005; Malysheva et al., 2015).

Morphologically, *B. reticulatus* is closely related to *B. coprophilus* (Peck) Hongo, *B. demangei* (Quél.) Sacc. & D. Sacc., *B. excoriatus* Dähncke, Hauskn., Krisai, Contu & Vizzini, *B. ferrugineus* Arnolds, and *B. incarnatus* Hongo. *Bolbitius coprophilus* can be distinguished from *B. reticulatus* by a pale pink or pale orange pileus and slightly larger basidiospores size (11.5–16.5 × 8.0–11.0 µm) (Arnolds, 2003). *B. demangei* is separated by a larger basidiocarps (70 mm broad) and pale to dark violaceous grey pileus (Arnolds, 2005; Hausknecht and Contu, 2006; Malysheva et al., 2015). *B. excoriatus*, originally described from Spain, has a smaller basidiospores size (8.5–10.0 × 6.0–7.0 µm) and mostly clavate pileipellis (Hausknecht et al., 2010). *B. ferrugineus* differs from the alike *B. reticulatus* by orange-brown pileus colour, slightly larger basidiospores size (7.5–10.5 × 4.5–6.0 µm), clavate pileipellis elements, present clamp-connections and habitat on soil (Arnolds, 2003). *B. incarnatus*, initially presented from Japan, has a pink-coloured pileus, a slightly pinkish stipe and larger basidiospores size (up to 16.0 µm long) (Arnolds, 2003; Hausknecht et al., 2007; Malysheva et al., 2015).

Finally, *Bolbitius reticulatus* also can be morphologically confused with *Pluteus longistriatus* (Peck) Peck because of its small wood-rotter with a striate margin and completely free lamellae. However, viscid pileus, lamellae which are hazel, orange-brown or rusty



brown at maturity in addition to some microscopic features distinguish *B. reticulatus* from *P. longistriatus*.

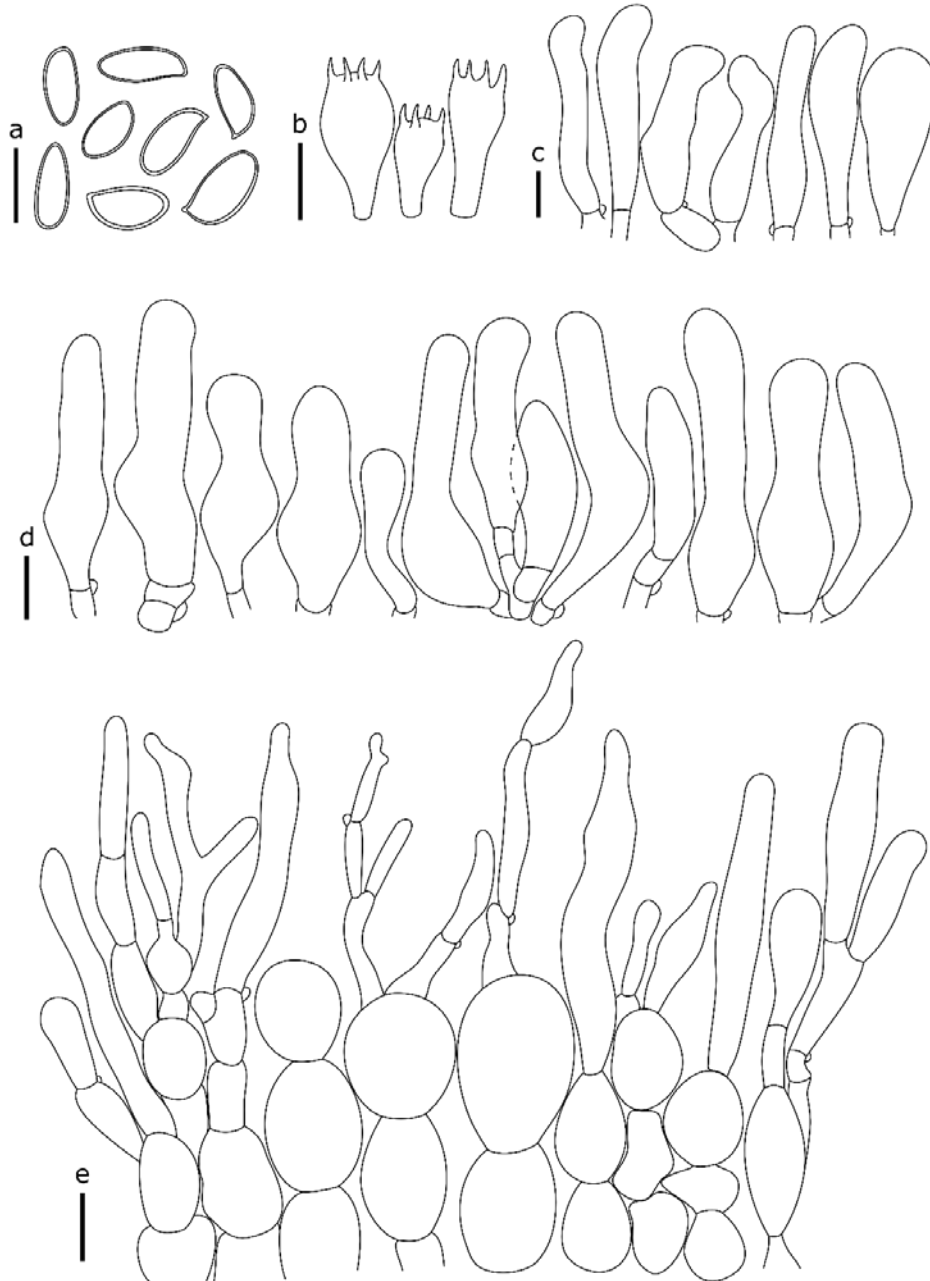
As could be seen from the phylogenetic tree based on the combined nrITS/nrLSU dataset, two collections of *B. reticulatus* from Turkey and six samples from Hungary, Norway and Russia grouped together in a distinct lineage (Figure 1).

Furthermore, *B. reticulatus* formed a sister relationship with *B. bisporus* E.F. Malysheva and *B.*

*aleuriatus* (Fr.) Singer from the European region. However, although *Bolbitius reticulatus* is closely related to *B. bisporus* and *B. aleuriatus*, it is apparently clustered on the separated branch on the phylogenetic tree.

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**Figure 3.** Microcharacters of *Bolbitius reticulatus*: a- basidiospores, b- basidia, c- caulocystidia, d- cheilocystidia, e- pileipellis elements. Scale bars = 10  $\mu$ m.



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