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Morphologic and Molecular Diagnosis of Some *Leucoagaricus* Species and Revealing a New Record from Turkey

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Abstract: Members of the genus *Leucoagaricus* (*Agaricaceae*) grow in the temperate climatic zone of the world. Only 10 species have been identified up to now in Turkey even though 238 *Leucoagaricus* species are listed in Mycobank database. In this study, the taxonomic positions of four species, *Leucoagaricus subvolvatus*, *L. barssii*, *L. subcretaceus* and *L. leucothites*, are discussed by evaluating morphological and molecular evidences and *L. subvolvatus* is approved as a new record for Turkey mycobiota. Internal transcribed spacer region (ITS) of each studied species is sequenced to resolve phylogenetic relationship and microscopic and macroscopic features of new record are described in detail.

Key words: *Leucoagaricus*, Molecular taxonomy, New record.

Türkiye’de Bulunan Bazı *Leucoagaricus* Türlerinin Morfolojik ve Moleküler Teşhisleri ve Yeni Bir Kaydın Ortaya Çıkarılması

Öz: *Leucoagaricus* (*Agaricaceae*) cinsinin üyeleri, dünyanın ılıman iklim bölgesinde yetişirler. Mycobank veri tabanında 238 *Leucoagaricus* türü listelenmesine rağmen, Türkiye’de şu ana kadar sadece 10 tür belirlenmiştir. Bu çalışmada cinse ait dört türün (*Leucoagaricus subvolvatus*, *L. barssii*, *L. subcretaceus* ve *L. leucothites*) taksonomik konumları, morfolojik ve moleküler kanıtlar değerlendirilerek tartışılmış ve *L. subvolvatus* Türkiye mikobiyotası için yeni bir kayıt olarak tanımlanmıştır. Her çalışılan türün iç Internal transcribed spacer bölgesi (ITS) filogenetik ilişkileri çözmek için dizilenmiş ve yeni kayıt türün mikroskopik ve makroskopik özellikleri ayrıntılı olarak açıklanmıştır.

Anahtar kelimeler: *Leucoagaricus*, Moleküler taksonomi, Yeni kayıt.

Introduction

The genus *Leucoagaricus* (Locq. ex) Singer is widely distributed in both Northern and Southern Hemispheres. *Leucoagaricus* species are saprophytic, growing on soil, wood chips, grasslands, road verges, and dune vegetation (Vellinga, 2001; Ortiz et al. 2008). In Mycobank database 238 *Leucoagaricus* species are listed (<http://www.mycobank.org>, accessed; 02.05.2019) and only 10 of them have been identified up to now in Turkey (Sesli and Denchev 2014). Members of the genus

are characterized by pluteoid basidiomata, fibrillose to squamulose or silky pileal surface, free lamellae, presence of annulus, white to cream or pink spore print, dextrinoid spores and absence clamp connections (Vellinga, 2001; 2004).

According to some researchers *Leucoagaricus*, as other lepiotoid genera in *Agaricaceae*, is polyphyletic (Johnson and Vilgalys 1998; Johnson 1999; Vellinga et al. 2003; Vellinga, 2004). Taxonomic and phylogenetic relationships of *Leucoagaricus* and *Leucocoprinus*



species are unresolved because molecular data for previously described species are limited (Hussain et al., 2018). These two genera were accepted as separate by Vellinga and Davis (2006) and Kumar and Manimohan (2009).

In the present study, four *Leucoagaricus* species (*Leucoagaricus subvolvatus* (Malençon & Bertault), *L. barssii* (Zeller) Vellinga, *L. subcretaceus* Bon and *L. leucothites* (Vittad.) Wasser) were identified based on morphologically and molecularly. The ribosomal internal transcribed spacers (ITS) comprising ITS1, 5.8S rDNA, ITS2 sub-regions sequences were used to be sure of species delimitation and to determine the phylogenetic relationships of the species within the genus. Moreover, *Leucoagaricus subvolvatus* are identify as a new record

for Turkey based on microscopic, macroscopic and molecular characters.

Material and Method

Taxon sampling and morphological studies

The macrofungus samples were collected from Bingöl and Hakkari province of Turkey (Table 1) and deposited in the Fungarium of Van Yuzuncu Yil University (VANF). Macroscopic and microscopic characters were observed in distilled water and 3% KOH solution under a Leica EZ4 stereo microscope while sections were examined under a Leica DM500 research microscope and they were measured with the Leica Application Suite (version 3.2.0) programme and described based on different studies (Singer, 1986; Breitenbach and Kränzlin, 1991; Vellinga, 2001; Kumar and Manimohan, 2009).

Table 1. Location of studied taxa and GenBank accession numbers used in the phylogenetic analysis.

Samples	Location	GenBank number
<i>Leucoagaricus subvolvatus</i>	Bingöl	MN134030
<i>Leucoagaricus barssii</i>	Bingöl	MN134031
<i>Leucoagaricus subcretaceus</i>	Hakkari	MN134032
<i>Leucoagaricus leucothites</i>	Bingöl	MN134033

DNA extraction, PCR amplification and Sequencing

Total DNA was extracted from dried basidiomata using the CTAB method with minor modifications (Doyle and Doyle, 1987). The purity and quantity of extracted DNA were determined by using NanoDrop2000c UV-Vis Spectrophotometer (Thermo Scientific) and 0.8% agarose gel electrophoresis. DNA amplification was performed in a 25 µl volume mixture containing genomic DNA (10 ng/µl), 10X PCR Buffer, MgCl₂ (25 mM), dNTP mixture (10 mM), selected primer pair (10 µM), Taq polymerase (5u/µl) and sterile water. To amplify ITS (ITS1-5.8S-ITS2) region, primer pairs N-nc18S10 5'AGGAGAAGTCGTAACAAG3'/C26A 5'GTTTCTTTTCCTCCGCT3' (Wen and Zimmer, 1996) were used. PCR products were run in a 1.0 % agarose gel and visualized by staining with Gelred dye. Positive reactions were sequenced with forward and reverse PCR primers using ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence alignment and phylogenetic analysis

Forward and reverse sequences were assembled and edited using Alibee Multiple Alignment 3.0 software from the GeneBee website (<http://www.genebee.msu.su/genebee.html>). Ambiguous

sites were checked manually and corrected by comparing the strands. Sequences of studied samples generated from the present study and additional sequences retrieved from NCBI were analyzed together to see phylogenetic relationships among *Leucoagaricus* species in the phylogenetic tree. The sequences downloaded from NCBI were selected considering results of BLAST searches. *Cystolepiota seminuda* (Genbank no: AY176350; Muñoz et al., 2014) was chosen as outgroup taxa. All sequences were aligned with the aid of the program ClustalW (Thompson et al., 1994) and adjusted manually where it was necessary.

Prior to construction of phylogenetic tree, total nucleotide length (bp) and variable sites were calculated using Molecular Evolutionary Genetics Analysis software (MEGA 6.0; Tamura et al., 2013). Phylogenetic tree of ITS region was constructed using two different methods; Maximum Likelihood (ML) and Maximum Parsimony (MP). The sequence data was analyzed by using the Maximum Likelihood (ML) method based on the Tamura-Nei model (Tamura and Nei, 1993). To test branch support, bootstrap analysis was used with 1000 replicates (Felsenstein, 1985). In the ML method, initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the



Maximum Composite Likelihood (MCL) approach, and then the topology with superior log likelihood value was selected. The Tree-Bisection-Reconnection (TBR) search method was employed with 100 random addition replications to construct the MP trees and the consensus tree inferred from 10 most parsimonious trees was used. All positions containing gaps and missing data were eliminated.

Results

Taxonomy

Leucoagaricus subvolvatus (Malençon & Bertault)

Bon (Figure 1-2)

Pileus 50-60 mm, conic when young then flattened with slightly umbo, pale white-cream, surface smooth, silky, robust, radial fibrillose. **Lamellae** crowded, pale white when young, creamy when mature. **Stipe** 40-80 × 5-10 mm, whitish grey, silky, cylindric, expanded towards the base, has universal veil remnants, bulbous base, annulus membranous, persistent, not movable, white. **Spores** 6-8(9) × 4-5.5 μm, hyaline, smooth, amygdaloid. **Basidia** 18-30 × 6-10 μm, clavate to capitate, with 4 spores. **Cheilocystidia** 25-52 × 5.5-12 μm, irregular, lageniform to cylindrical, clavate, usually has crystal shaped at apex. **Pleurocystidia** absent. **Pleipellis cutis** made up of 3-15 μm wide, septate, hyaline in KOH, thin-walled hyphae, clamp absent.

Ecology

Under *Quercus* sp., Tarlabası village, Bingöl province, 37° 21'409"K - 44° 30'002"D, 1481 m, 28.10.2018, Acar 1079.

Molecular phylogeny

Sequences of *Leucoagaricus* generated from the current study and additional sequences retrieved from GenBank were combined and analyzed together to identify our samples correctly and see phylogenetic relationships among species in the phylogenetic tree. The amplified

DNA fragment of the region was approximately 700 bp length encompassing complete ITS1, 5.8S and ITS2 subregions. ITS data matrix comprised a total of 39 sequences including 35 from NCBI. The aligned data included a total of 717 positions, of which 379 were conserved, and 326 were variable (216 variable sites in ITS1 and 110 in ITS2 subregion) nucleotides. *Leucoagaricus leucothites* (JQ683081) was used as reference sequence.

The Maximum Likelihood analysis resulted in similar phylogenetic topologies with Maximum Parsimony, so only one tree (ML) was given to indicate phylogenetic relationships and taxonomic position of studied species. In the ML phylogenetic tree, two *Leucoagaricus* sections, *L. sect. Rubrotincti* Singer and *L. sect. Piloselli* (Kühner ex) Singer, separated from each other and caused two main clades (Figure 3). The taxa that clustered together in a section carried morphological similarities.

L. sect. Rubrotincti is characterized by a reddish-brown pileus, ellipsoid spores, and a negative ammonia reaction (Singer 1986; Vellinga 2001). Section *Piloselli* is distinguished by basidiomes usually staining orange-red when bruised and turning green with ammonia and spores without germ pore (Singer 1986; Vellinga 2010).

Leucoagaricus subvolvatus grouped with its representative (KP300878) and several retrieved samples in section *Rubrotincti* with a bootstrap value of 100% (Figure 3). *Leucoagaricus barssii*, *L. leucothites* and *L. subcretaceus* placed in section *Piloselli*. *Leucoagaricus leucothites* clustered close to *L. subcretaceus*. Although these two species are molecularly similar, they can be distinguished from each other based on macroscopic and microscopic characters (Table 2). *Leucoagaricus barssii* grouped with three representatives from NCBI with 99% bootstrap.

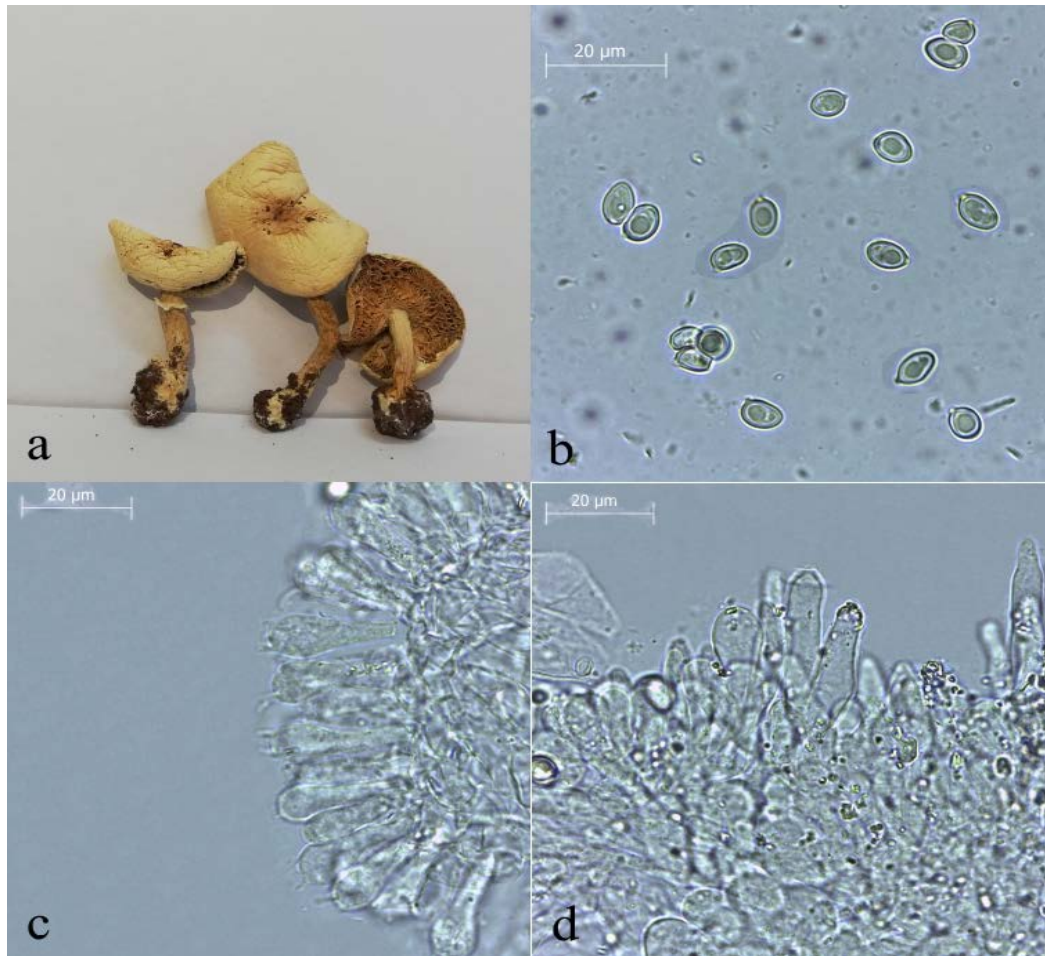


Figure 1. Macroscopic and microscopic characters of *Leucoagaricus subvolvatus*.
a. basidiocarps b. spores c. basidia d. cheilocystidia

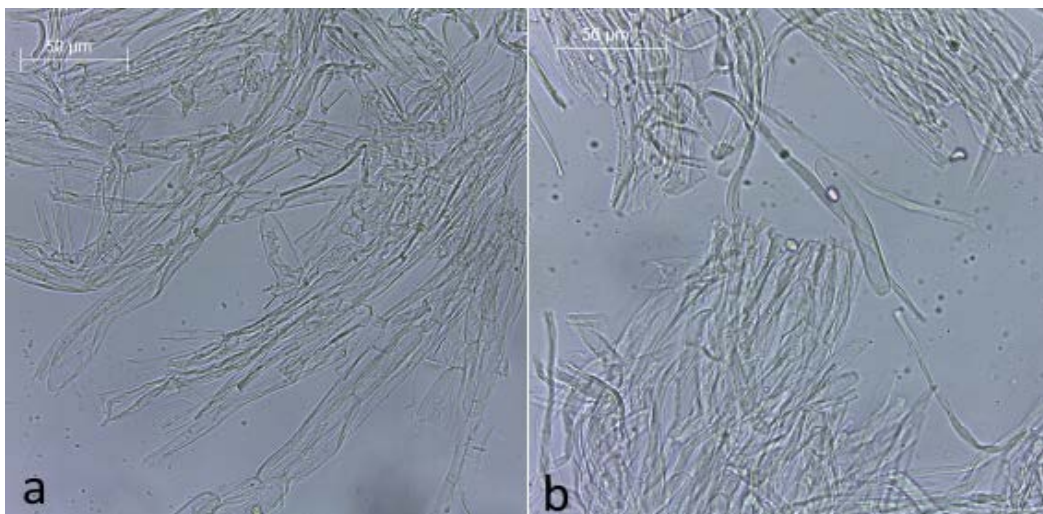


Figure 2. Pleipellis a. in dH₂O b. in KOH

Table 2. Considerable morphologic characters of studied *Leucoagaricus* samples.

	<i>Leucoagaricus subvolvatus</i>	<i>Leucoagaricus barssii</i>	<i>Leucoagaricus subcretaceus</i>	<i>Leucoagaricus leucothites</i>
Pileus	expands to 6 cm, conic then flattened with slightly umbo, pale white-cream, surface smooth, silky, robust, radial fibrillose	4-13 cm, hemispherical, margin at first incurved, becoming decurved, surface fibrillose to squamulose	8-12 cm, or more when growing on rich substrates, silky to plushy toward the edge	5-7.5 cm, conico-campanulate with a truncate apex, then flattened, nonstriate margin, cream to dirty white, sometimes grey-brown at the centre, surface granular-punctate
Stipe	4-8 x 0.5-1 cm, whitish grey, silky, cylindric, with universal veil remnants, annulus membranous, white	5-10 x 1.5-2.5 cm, equal, spindle-shaped, white, partial veil fibrillose-membranous, white, forming a medial to superior annulus	4-7 x 0.5-1.5 cm, white, brown at the base, annulus thick, membranous, broad, white, then becoming brown	7-9 x 0.8-1.8 cm, cylindric-clavate, wide at the swollen base, white, fibrillose or with a faint zigzag pattern, and with an apical membranous ring, annulus persistent
Lamellae	crowded, pale white when young, finally cream	free, crowded, cream-colored	dirty white, then brownish toward the edge	free, crowded, pinkish-cream
Spores	(7)8-10 x 2.5-6 µm, amygdaloid	6.5-8.5 x 4-5 µm, smooth, oval to elliptical	(7.5) 8 - 8.5 (10) x 5.5- 6 µm	6-8 x 4-4.75 µm, amygdaliform with a small germ-pore
Cheilocystidia	24-50 x 6.5-13 µm, irregular, lageniform to cylindrical, clavate, cyristal shaped at apex.	23-50 x 7-13 µm, clavate to fusiform	(25) 30-45 (55) x 8 -10 µm, clavate, to lageniform, sometimes capitate	8-10 x 20-40 µm abundant, thin walled, hyaline, very variable in shape

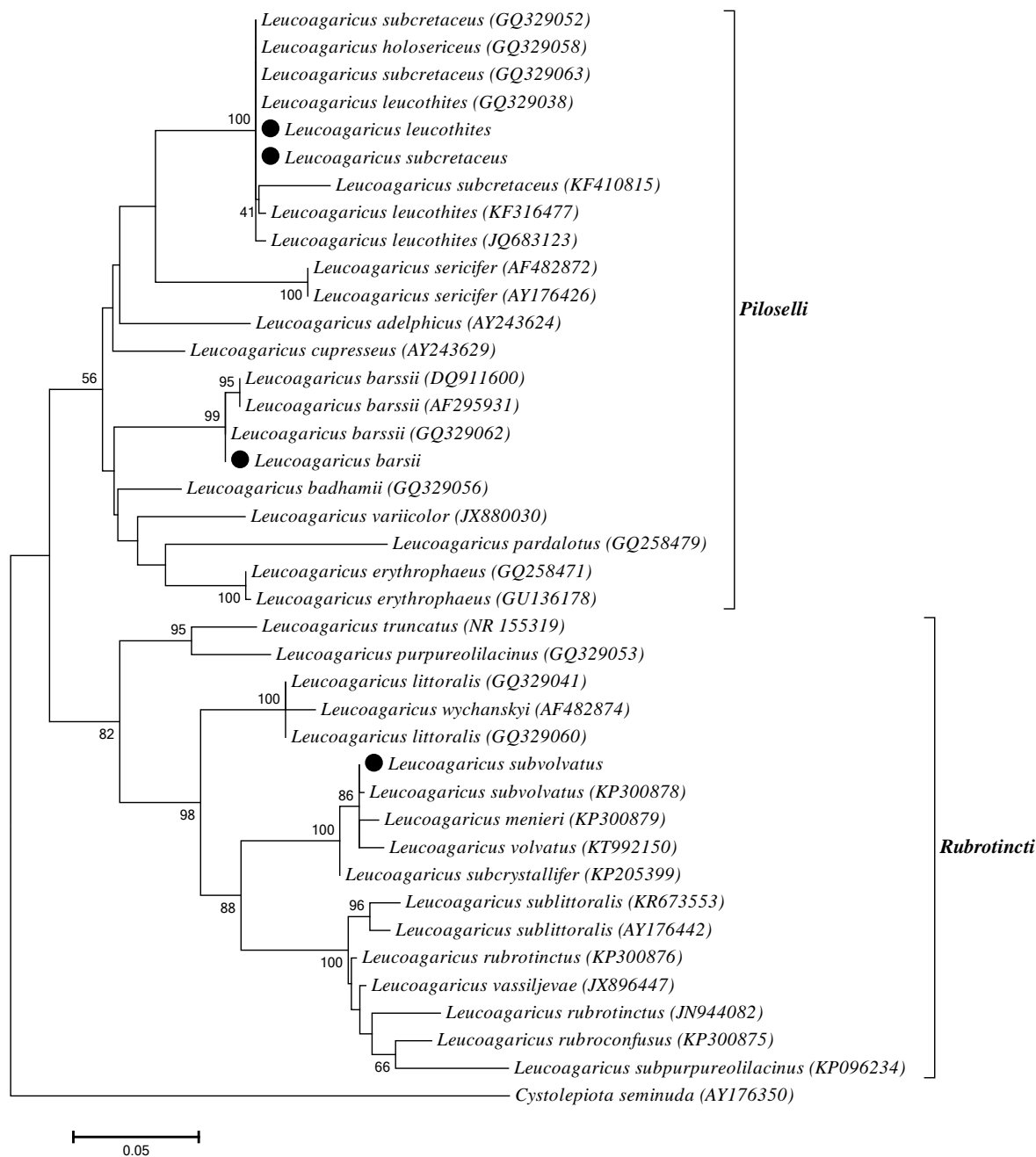


Figure 3. Phylogenetic tree of *Leucoagaricus* species based on ML analysis of the ITS region. Black circle indicates studied specimen. *Cystolepiota seminuda* was used as outgroup. Bootstrap analysis of ML was based on 1000 replicates and values higher than 40% were indicated on branches.

Discussion

Section *Rubrotincti* is characterized a moderately fleshy basidiomata that does not change color when damaged, a red brown, pinkish ochre, olive, or orange pileus with radially arranged hyphae, ellipsoid spores, and a negative ammonia reaction (Singer 1986; Vellinga 2001). Section *Piloselli* is traditionally distinguished by

basidiomes usually staining orange-red when bruised and turning green with ammonia and spores without germ pore (Singer 1986; Vellinga 2010). These characters were also observed for studied samples and used to identify correctly in section and species levels.

The ITS phylogeny showed that *Leucoagaricus subvolvatus* is closely related to *L. menieri* (Sacc.) Singer,



L. volvatus Bon & A. Caball. and *L. subcrystallifer* Ge & Yang. Morphologically these species have white basiomata, similar basidiospores and cheilocystidia with crystals at the apex. However, *L. menieri* has broad, fragile, shining basidiomata and smaller basidiospores (6–7 × 4.7–5 µm; Candusso and Lanzoni 1990; Bas et al., 1999). *Leucoagaricus subcrystallifer* has a greenish gray pileus with a dark gray umbo, amygdaliform basidiospores, narrowly fusiform cheilocystidia with crystals at the apex, and filamentous hyphae (Ge et al. 2015). *Leucoagaricus subvolvatus* and *L. volvatus* are two white volvate species. *L. subvolvatus* has fleshy basidiomata with a white to cream pileus, roundish stipe base that is somewhat volvate and narrowly lageniform to cylindrical cheilocystidia which bear crystal shaped at apex (Candusso and Lanzoni 1990; Bas et al., 1999). *Leucoagaricus volvatus* has some olivaceous tinges in the pileus, but also has crystals on the cystidia and a slightly gelatinized pileus. The current name of *L. subvolvatus* is same name in Mycobank database but *Lepiota subvolvata* in the Index Fungorum.

Leucoagaricus leucothites, *L. subcretaceus* and *L. barssii* clustered in section *Piloselli*. *Leucoagaricus leucothites* is a widespread species and grows grasslands, lawns, meadows and road verges. *Leucoagaricus subcretaceus* and *L. leucothites* are

similar but *L. subcretaceus* is larger and more coloured (Bon, 1981; Reid and Eicker, 1993). In the constructed tree, these three species were located in the *Piloselli* clade. *Leucoagaricus barssii* is one of the few species within the genus to possess pleurocystidia. It has a radially fibrillose and white to greyish pileus (Desjardin et al., 2015; Vellinga 2000; 2001). The ellipsoid spores do not have germ pore, and pleurocystidia are observed close to the edge of lamella (Vellinga 2000; 2001).

Identification of *Leucoagaricus* samples is difficult because of close relationships with some Lepioid genera such as *Leucocoprinus*. Therefore, molecular data is needed to support the diagnosis that is completed based on only morphological characters. In this study, four *Leucoagaricus* species were identified by using both morphological and molecular data and *Leucoagaricus subvolvatus* was determined as a new record. The number of *Leucoagaricus* species in Turkey was increased from 10 to 11 with the results of the current study.

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