

## Two New Additions to *Turkish Tulostoma*

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### ABSTRACT

The purpose of the present work is to identify *Tulostoma* samples collected from Ankara and Kırıkkale provinces (Turkey). Both traditional methods and ITS rDNA-based molecular phylogeny were implemented to identify the specimens. When the high sequence similarities were taken into account, the collected specimens ANK Akata & Altuntaş 647 and ANK Akata & Altuntaş 675 were identified as *T. simulans* and *T. subsquamosum* respectively; and the morphological data further supported these findings. Short descriptions of the species are given together with their macro- and micromorphology and spore images taken by a scanning electron microscope (SEM).

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## Türkiye *Tulostoma*' larına İki Yeni Katkı

### ÖZET

Bu çalışmanın amacı Ankara ve Kırıkkale illerinden (Türkiye) toplanan *Tulostoma* örneklerini tespit etmektir. Örnekleri tanımlamak için hem geleneksel yöntemler hem de ITS rDNA temelli moleküler filogeni uygulanmıştır. Yüksek dizi benzerlikleri dikkate alındığında, toplanan ANK Akata & Altuntaş 647 ve ANK Akata & Altuntaş 675 örnekleri, sırasıyla *T. simulans* ve *T. subsquamosum* olarak teşhis edilmiş; ve morfolojik veriler de ayrıca bu bulguları desteklemiştir. Türlerin kısa tanımları, makro ve mikromorfolojileri ve taramalı elektron mikroskobu (SEM) ile alınan spor görüntüleri ile birlikte verilmektedir.

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## INTRODUCTION

*Tulostoma* Pers., commonly known as stalked puffballs, is a large gasteroid genus containing approximately one hundred and seventy currently existing species ([www.indexfungorum.org](http://www.indexfungorum.org); accessed 20 November 2020). Members of the genus are characterized by globose and stalked spore-sac opened by an apical mouth, double peridium, pulverulant gleba, globose to ovoid, smooth or ornamented basidiospores, simple or branched capillitium with septa. Members of the genus are cosmopolitan, which prefer primarily sandy and calcareous soil in temperate and tropical regions (Pegler et al., 1995; Calonge, 1998).

The genus was ascertained by Persoon (1794, 1801) to compile puffball species having "peridium pedicellatum", a small hole with an "ore cylindraceo cartilagineo". Two species (*T. squamosum* (J.F. Gmel.) Pers. and *T. brumale* Pers.) were accommodated in the

genus by Persoon. While Tournefort (1700) formerly illustrated *T. brumale* as *Lycoperdon parisiense*, Linnaeus (1753) described *T. squamosum* as *Lycoperdon pedunculatum*. Four species *T. fimbriatum*, *T. laceratum* (syn. *Schizostoma lacerata*), *T. tortuosum*, and viz. *T. mammosum* (including *T. squamosum*) were described by Fries (1829). However, Fries (1829) subsequently renamed *Tulostoma* with *Tulasnodea*, for the honor of the Tulasne brothers, who were his mycologist colleagues from France. However, considering the nomenclature rules, "*Tulostoma*" coined by Persoon should be regarded as the legitimate name for this typical genus (Persoon, 1801). In older literature, sometimes an orthographic variant, *Tylostoma*, is encountered. The knowledge of *Tulostoma* was developed by the early works of some researchers. Schroeter (1876) described the development of basidia and capillitium, parts of basidiomata, in this genus. Later Wright (1955) elucidated the unique morphological characters of the

genus. In his work entitled “The Genus *Tulostoma/Gasteromycetes: A World Monograph*” Wright (1987), included 139 species. Wright’s species concept mostly relied on studies of type specimens and various herbarium samples. With the advancements in scanning electron microscopy (SEM), fine spore morphology could be employed as a guide for the taxonomic revisions and appearance of new species.

The inclusion of the molecular analysis resulted in fundamental changes in the fungus systematics and taxonomy. The order *Agaricales* was established to include basidiomycetous gasteroid fungi including the puffball genus *Tulostoma* by Hibbett et al. (1997) Although a considerable amount of morphological data concerning the genus *Tulostoma* have been accumulated, relatively less molecular phylogenetic studies related to this genus have been reported. These published studies mostly focus on a low number of species and they are geographically restricted (Jeppson et al., 2017).

Considering the literature on Turkish *Tulostoma* (Gücin and Öner, 1982; Sesli, 1995; Afyon, 1996; 1997; Solak et al., 1999; Afyon et al., 2000; Kaşık et al., 2000; Sesli et al., 2000; Işiloğlu, 2001; Solak et al., 2002; Aktaş et al., 2003; Türkekul and Sesli, 2003; Kaya, 2005; 2006; Doğan and Öztürk, 2006; Doğan and Türkoğlu, 2006; Allı et al., 2007; Doğan et al., 2007; Türkoğlu et al., 2007; Yağız et al., 2007; Doğan et al., 2011; Kırış et al., 2012; Akata et al., 2014), so far 5 species (*T. brumale*, *T. fimbriatum* Fr., *T. squamosum*, *T. pluriosteum* Long & S. Ahmad and *T. wightii* Berk.) which were identified based on morphological features have been listed for Turkish mycobiota. However, there was not any report of *Tulostoma simulans* Lloyd and *Tulostoma subsquamosum* Long & S. Ahmad in Turkey. The aim of the present paper is to contribute new species for Turkish *Tulostoma*.

## MATERIAL and METHOD

### Morphological study

*Tulostoma* samples were collected from Ankara and Kırıkkale provinces (Turkey) in 2019. At their site of collection, the macroscopic and ecological features of the samples were recorded. At the laboratory, microscopic features were scrutinized using both simple light microscope (LM) and scanning electron microscope (SEM). In light microscopy, measurements were repeated roughly 30 times under a light microscope (Euromex Oxion Trinocular microscope). Each microscopic structure was examined with 100X magnification rates and the compiled data were assessed statistically. For SEM, pieces of mass inside the gleba were fixed on stubs using double-sided sticky tape, coated with gold particles, and visualized using an EVO 40XVP (LEO Ltd., Cambridge, UK) scanning electron microscope with an accelerating voltage of 20

kV. Sample identification was performed in light of the relevant literature (Pegler et al., 1995; Calonge, 1998; Jeppson et al., 2017; Rusevska et al., 2019). Fungarium materials were prepared from the identified specimens and deposited into Fungarium of Ankara University Faculty of Science, Department of Biology.

### Determination of the ITS rDNA Sequences

For the genomic DNA extraction from ANK Akata and Altuntaş 647 and ANK Akata and Altuntaş 675, CTAB method was implemented as described previously (Rogers and Bendich, 1994). For the quality and quantity measurements, the isolated genomic DNA was spectrophotometrically (Nanodrop Lite Thermo Scientific) analyzed, and later it was utilized as the template in order to amplify the Internal Transcribed Spacer (ITS) rDNA regions via the polymerase chain reaction (PCR) method. By using the ITS1 forward and ITS4 reverse universal oligonucleotides, PCR amplification of the ITS rDNA regions was implemented as described elsewhere (Stielow et al., 2015). The presence of the amplification products was electrophoretically verified on an agarose gel and then they were purified with Expin Gel, PCR, and CleanUp SV Kit (GeneAll) and sequenced with Sanger dideoxy sequencing method. For the sequencing PCR conducted using the BigDye™ Direct Cycle Sequencing Kit (Thermo Fisher Scientific), the same ITS1 and ITS4 oligonucleotides were employed and the fragment analyses were carried out by using ABI Prism 3130 Genetic Analyzer. Agarose gel electrophoresis and the Sanger sequencing were conducted as described previously (Chen et al., 2014).

### Molecular Phylogeny Study

DNAMAN Version 10 sequence assembly software (Lynnon Corporation) was used to assemble the sanger reads obtained from ITS1 and ITS4 primers were assembled and BLASTn search was conducted with the assembled sequence for the identity index analysis. Based on the results of the BLAST search, the in-group and the out-group sequences were retrieved from NCBI GenBank and used in the phylogenetic analysis (Table 1) (Pawlik et al., 2015; Jeppson et al., 2017). The ClustalW algorithm of MEGAX software was used to align the assembled sequences and the nucleotide sequences of the retrieved in-group and out-group members (Kumar et al., 2018). The phylogenetic tree exhibiting the evolutionary history of ANK Akata and Altuntaş 647 and ANK Akata and Altuntaş 675 was predicted using the Maximum Likelihood method and GTR+G+I nucleotide substitution model (Nei and Kumar, 2000). The bootstrap method was selected for improving the accuracy of the estimation using 1000 bootstrap replicates (Felsenstein, 1985).

Table 1. GenBank accession numbers of the ITS sequences belonging to the 16 fungi specimens used in this study  
*Tablo 1. Bu çalışmada kullanılan 16 mantar örneğine ait ITS dizilerinin GenBank erişim numaraları*

Tulostoma Species	GenBank number (ITS)	Geographical origin	References
ANK Akata & Altuntaş 647 ( <i>Tulostoma simulans</i> )	MT798590.1	Turkey	Current study
<i>Tulostoma simulans</i>	KU519053.1	Hungary	Jeppson et al., 2017
<i>Tulostoma simulans</i>	KU519046.1	Spain	Jeppson et al., 2017
<i>Tulostoma brumale</i>	KU519061.1	Slovakia	Jeppson et al., 2017
<i>Tulostoma grandisporum</i>	KU519004.1	Hungary	Jeppson et al., 2017
<i>Tulostoma striatum</i>	KU518959.1	Spain	Jeppson et al., 2017
<i>Tulostoma fimbriatum</i>	KU518981.1	Spain	Jeppson et al., 2017
<i>Tulostoma berkeleyi</i>	MK578704.1	USA	Jeppson et al., 2017
<i>Tulostoma winterhoffii</i>	KU518977.1	Sweden	Jeppson et al., 2017
<i>Tulostoma squamosum</i>	KU519097.1	France	Jeppson et al., 2017
<i>Tulostoma rufum</i>	KU519107.1	USA	Jeppson et al., 2017
<i>Tulostoma calcareum</i>	KU519088.1	Hungary	Jeppson et al., 2017
<i>Tulostoma subsquamosum</i>	KU519092.1	Spain	Jeppson et al., 2017
ANK Akata & Altuntaş 675 ( <i>Tulostoma subsquamosum</i> )	MT798591.1	Turkey	Current study
<i>Tulostoma subsquamosum</i>	KU519093.1	Hungary	Jeppson et al., 2017
<i>Coprinus comatus</i>	JQ901445.1	Poland	Pawlik et al., 2015

## RESULTS and DISCUSSION

The systematics of the newly reported *Tulostoma* species was in accordance with Index Fungorum ([www.indexfungorum.org](http://www.indexfungorum.org); accessed 20 November 2020). Short descriptions were provided together with collection dates, localities, notes on habitats, geographical positions, herbarium numbers, and images of their macro- and micromorphology, and spores viewed by a scanning electron microscope (SEM).

### 1. *Tulostoma simulans* Lloyd (1906), (Figure 1).

**Syn.:** *Tulostoma mammosum* var. *simulans* (Lloyd) Sacc. & Trotter (1912).

#### Macroscopic and microscopic features

**Spore-sac** 6-10 mm diam., globose. **Mouth** circular. **Exoperidium** membranous. **Endoperidium** papery, smooth, yellowish to light brown. **Stipe** 15-30 × 2-3 mm, yellow to light brown, cylindrical. **Gleba** light yellow to light brown. **Basidia** not seen. **Basidiospores** 4-5 µm diam, globose, almost smooth to slightly warted under LM, with conical or isolated warts under SEM. **Capillitium** up to 9 µm diam, branched, septate, and thick-walled, sometimes with irregular crystals on the capillitial wall.

**Ecology:** Fall to winter, in sandy soil (Rusevska et al., 2019).

**Distribution:** Asia, Australia, Europe, North, and South America (Jeppson et al., 2017).

**Material examined:** TURKEY—Ankara: Ankara University Beşevler 10.Yıl campus, under *Picea pungens* Engelm. (*Pinaceae*), 867 m, 39°56' N, 32°50' E, 02.09.2019, ANK Akata & Altuntaş 647.

**Remarks:** Although it is difficult to distinguish *T. simulans* and *T. brumale* in detail by conventional methods, the exoperidial characteristic, spore dimensions, and the presence of crystals may help to separate these species (Jeppson et al., 2017; Rusevska et al., 2019).

### 2. *Tulostoma subsquamosum* Long & S. Ahmad (1947), (Figure 2).

#### Macroscopic and microscopic features

**Spore-sac** 10-15 mm diam., globose. **Mouth** tubular or circular. **Exoperidium** membranous. **Endoperidium** papery, smooth, whitish, ochraceous to light brown. **Stipe** 25-40 × 2-3 mm, brownish squamules on a white background, slightly woody. **Gleba** light yellow to brownish. **Basidia** not seen. **Basidiospores** 4-5 µm diam, globose, verrucose-echinate under LM, with conical or cylindrical warts anastomosed in ridges under SEM. **Capillitium** up to 8 µm diam, branched and septate.

**Ecology:** Fall, in sandy soil (Rusevska et al., 2019).

**Distribution:** Asia, Europe, North, and South America (Jeppson et al., 2017).

**Material examined:** TURKEY—Kırıkkale: Bahşili, near road, 710 m, 39°44' N, 33°27' E, 10.10.2019, ANK Akata & Altuntaş 675.

**Remarks:** *T. subsquamosum* can be distinguished from other *Tulostoma* members by its verrucose-echinate spores (under LM) and hyphal exoperidium with scattered sphaerocyst-like cells (Rusevska et al., 2019)

The nuclear ITS rDNA sequences of ANK Akata and Altuntaş 647 and ANK Akata and Altuntaş 675 obtained from Sanger dideoxy sequencing were



deposited into NCBI GenBank with the accession numbers MT798590.1 and MT798591.1 respectively. In phylogenetic analyses of ANK Akata and Altuntaş 647 and ANK Akata and Altuntaş 675, 13 different nuclear ITS rDNA sequences belonging to 11 different

*Tulostoma* species were retrieved from NCBI GenBank database and used as in-group sequences in the phylogenetic analysis. As the out-group sequence, nuclear ITS rDNA sequences of *Coprinus comatus* (O.F. Müll.) Pers. was selected.



Figure 1. *Tulostoma simulans*: a. basidiomata, b. spores (LM), c-d. capillitium (LM), e. spores (SEM).  
Şekil 1. *Tulostoma simulans*: a. bazidiyomata, b. sporlar (LM), c-d. kapilisyum (LM), e. sporlar (SEM).

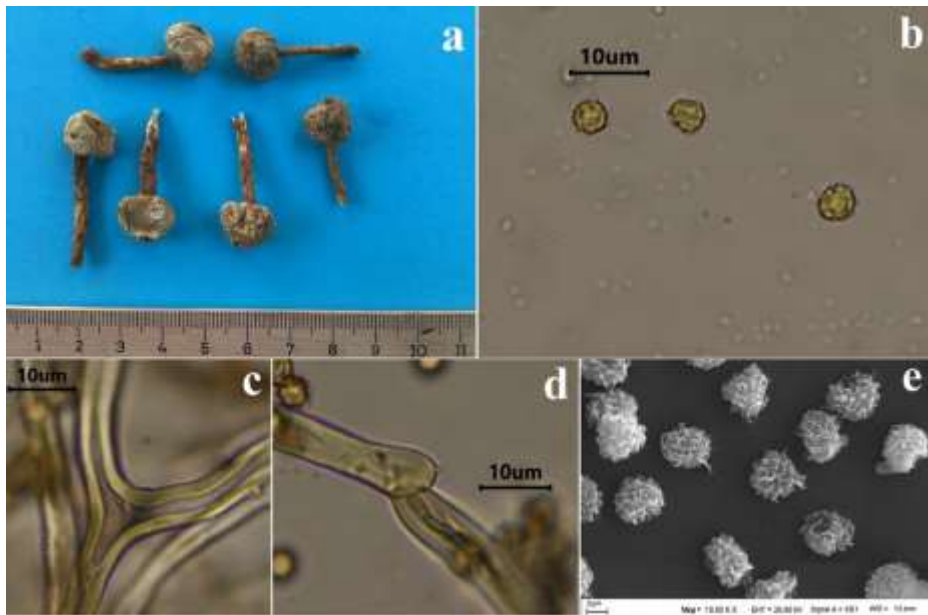


Figure 2. *Tulostoma subsquamosum*: a. basidiomata, b. spores (LM), c-d. capillitium (LM) e. spores (SEM).  
Şekil 1. *Tulostoma simulans*: a. bazidiyomata, b. sporlar (LM), c-d. kapilisyum (LM), e. sporlar (SEM).

As a result of the phylogenetic analysis, the specimens ANK Akata and Altuntaş 647 and ANK Akata and Altuntaş 675 were clustered with species of *T. simulans* and *T. subsquamosum* respectively. On the other side, *Coprinus comatus* fell into a distinct branch separate from the *Tulostoma* species and formed an out-group as expected. The BLAST analysis

implemented with the nuclear ITS rDNA sequence of ANK Akata and Altuntaş 647 and ANK Akata and Altuntaş 675 revealed as high as 100% similarity rates between these specimens and different isolates of *T. simulans* and *T. subsquamosum*. The phylogenetic analyses conducted herein further solidified the close identity relationship of the specimens ANK Akata and

Altuntaş 647 and ANK Akata and Altuntaş 675 with *T. simulans* and *T. subsquamosum* respectively (Figure 3).

For the reliable identification of fungal taxa, conventional methods employing morphological data may not always sufficient per se. Hence, conserved regions of genomic DNA including nrITS, nrSSU, and nrLSU as well as sequences of protein-coding genes are

benefited for molecular taxonomic studies for decades (Raja et al., 2017). Furthermore, ITS is the most generally used DNA barcoding marker for fungi and this reason endows valuable information for molecular phylogenetic studies. Thus, we benefited from nuclear ITS rDNA sequences for the molecular identification of the specimens ANK Akata and Altuntaş 647 and ANK Akata and Altuntaş 675.

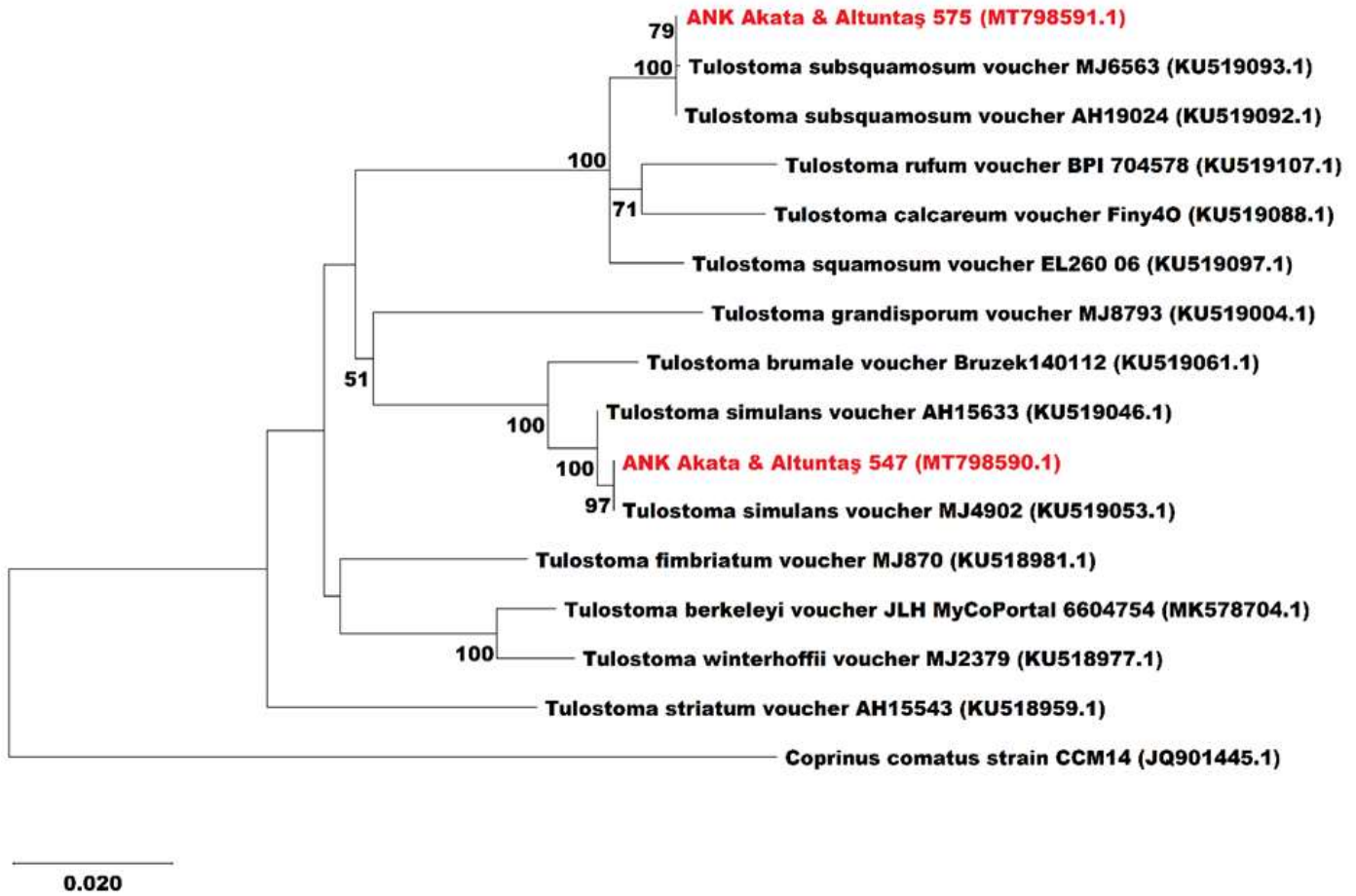


Figure 3. The Maximum Likelihood tree demonstrating the phylogenetic relationships of 39 fungi predicted from the nuclear ITS rDNA region. Percentage bootstrap values that are more than 50 were stated for each branch. All of the sequences included in the phylogenetic analysis were retrieved from GenBank except for specimens ANK Akata and Altuntaş 647 and ANK Akata and Altuntaş 675. Nuclear ITS rDNA sequences of *Coprinus comatus* was included as the outgroup sequence in the phylogenetic analysis. GenBank accession numbers are also stated. The scale bar at the lower left shows a genetic distance of 0.02.

Şekil 3. Çekirdek ITS rDNA bölgelerine göre hesaplanan, 39 fungusun filogenetik ilişkisini gösteren maksimum olasılık ağacı. Her bir dal için yüzde önyükleme değeri 50'den fazla olanlar belirtilmiştir. ANK Akata ve Altuntaş 647 ve ANK Akata ve Altuntaş 675 örnekleri hariç filogenetik analize dahil edilen tüm diziler GenBank'tan elde edilmiştir. Coprinus comatus'un Çekirdek ITS rDNA dizisi, filogenetik analize dış grup dizisi olarak dahil edilmiştir. GenBank erişim numaraları da belirtilmiştir. Aşağı sol tarafta yer alan ölçek çizgisi 0.02 genetik mesafeyi göstermektedir.

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#### Author's Contributions

The contribution of the authors is equal.

#### Statement of Conflict of Interest

Authors have declared no conflict of interest.

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