



Tuber fulgens Quél., A New Record for Turkish Truffles

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ABSTRACT

Tuber samples were collected from Kırklareli province on the 10th of August 2020 and they are identified by implementing both traditional methods and molecular phylogenetic analysis using the rDNA sequences including Internal Transcribed Spacer (ITS) and 28S Ribosomal Large Subunit (LSU) regions. By taking into account the high sequence similarity between the collected samples (ANK Akata 7351) and the truffle species *Tuber fulgens* Quél. the collected specimen was regarded as *T. fulgens* and the morphological data also consolidated this finding. As a result, *T. fulgens* was reported for the first time from Turkey. A short description of the newly reported species is given along with its macro and microphotographs, and spore images taken by a scanning electron microscope (SEM). Additionally, ITS and LSU rDNA based evolutionary history of the specimen is provided with phylogenetic trees.

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Introduction

Tuber, also known as truffles, is an ectomycorrhizal hypogean genus of the family *Tuberaceae* belonging to the order *Pezizales* within the division *Ascomycota*. The genus includes over 180 widespread species which have highly valued, gastronomic importance and they are regarded as the diamond of the kitchen. *Tuber* species are known to establish ectomycorrhizal associations with both gymnosperm and angiosperms such as pine, fir, beech, birch, common hazel, poplar, and willow (Patel, 2012; Wan et al., 2017). Members of the genus are mainly characterized by globose to lobed, hypogeous and sessile ascomata sometimes with basal mycelial tuft, solid and firm gleba usually marbled, smooth or warted peridium, globose, sub globose or pear-shaped asci usually with 1-6 spores, ornamented spores with warts, spines or ridges (Hansen and Knudsen, 2000).

T. fulgens grows on calcareous soil from summer to winter and it is associated with various deciduous trees such as oak and hornbeam (Ławrynowicz, 2009). Considering the literature on Turkish *Tuber* (Castellano and Türkoğlu, 2012; Elliot et al., 2016; Gezer et al., 2014; Öztürk et al., 1997; Sesli and Denchev, 2014; Şen et al., 2016; Türkoğlu and Castellano, 2014), up until now, 8 species (*Tuber aestivum* (Wulfen) Spreng., *T. brumale* Vittad., *T. excavatum* Vittad., *T. ferrugineum* Vittad., *T. mesentericum* Vittad., *T. nitidum* Vittad., *T. puberulum* Berk. & Broome and *T. rufum* Picco.) which were identified based on morphological characteristics have been reported from Turkey. However, there was not any report of *Tuber fulgens* Quél. in Turkey. The purpose of this paper is to contribute to Turkish *Tuber*.

Material and Methods

Morphological Study

Tuber specimens were collected from Kırklareli provinces (Turkey) in 2019. In their natural habitat, the macroscopic and ecological characteristics of the specimens were noted. At the fungarium, microscopic features were scrutinized using both binocular light microscope (LM) and scanning electron microscope (SEM). In light microscopy, measurements were repeated for about 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. Fungarium materials were prepared from the identified specimens and deposited into Ankara University Herbarium (ANK).

Determination of the ITS and LSU rDNA Sequences

For the genomic DNA isolation from ANK Akata 7351, CTAB method was utilized as described elsewhere (Rogers and Bendich, 1994). The isolated genomic DNA was spectrophotometrically (Nanodrop Lite Thermo Scientific) analyzed for the quality and quantity measurements and then it was utilized in a polymerase chain reaction as the template to amplify the Internal Transcribed Spacer (ITS) and 28S Ribosomal Large Subunit (LSU) rDNA regions. PCR amplification of the ITS and LSU rDNA regions was conducted using the ITS1/ITS4 and LR5/LROR universal primer couples as previously reported (Stielow et al., 2015). The presence of amplicons was electrophoretically confirmed as single and clear bands on an agarose gel and later they were purified with Expin Gel, PCR, and CleanUp SV Kit (GeneAll) and sequenced with Sanger dideoxy sequencing method. Same ITS1/ITS4 and LR5/LROR primer couples were utilized for the sequencing PCR performed using the BigDye™ Direct Cycle Sequencing Kit (Thermo Fisher Scientific) and the fragment analyses were conducted using ABI Prism 3130 Genetic Analyzer. Agarose gel electrophoresis and the Sanger sequencing were carried out as described elsewhere (Chen et al., 2014).

Molecular Phylogeny

For the molecular phylogeny, the sanger reads obtained from ITS1/ITS4 and LR5/LROR primer couples were assembled using DNAMAN Version 10 sequence assembly software (Lynnon Corporation) and BLASTn analyses were performed with the assembled sequences for the identity rate search. Based on these BLAST analyses, the in-group and the out-group members were retrieved from NCBI GenBank for the phylogenetic analyses. The assembled sequences and the nucleotide sequences of the retrieved in-group and out-group members were aligned using the ClustalW algorithm of MEGAX software (Kumar et al., 2018). The phylogenetic trees demonstrating the evolutionary history of ANK Akata 7351 were constructed using the Maximum Likelihood method and K2 nucleotide substitution model with a gamma distribution (Kimura, 1980). The bootstrap method was implemented for the accuracy estimation using 1000 bootstrap replicates (Felsenstein, 1985).

Results

Tuber fulgens Qué. (1880), Figure 1-2)

Macroscopic and Microscopic Features

Ascomata 20-30 mm, hypogeous, subglobose, with deep basal cavities, rugulose, orange to reddish-brown. Gleba solid, firm, pale brown with whitish veins radiating from the cavity, often with orange-red spots. Peridium 250-350 μ m thick, bright orange-brown to reddish-brown. Odor and taste mild. Asci 105-140 \times 95-110 μ m, subglobose to ellipsoid, sessile or short stipitate, and 1-4-spored. Ascospores 45-55 \times 35-45 μ m, globose to subglobose, yellow to yellowish-brown, with alveolate-reticulate ornamentation with high walls.

Material examined: Turkey-Kırklareli, in oak and hornbeam mixed forest, 400 m, 41°44' N, 27°35' E, 10 August, 2020, ANK Akata 7351.

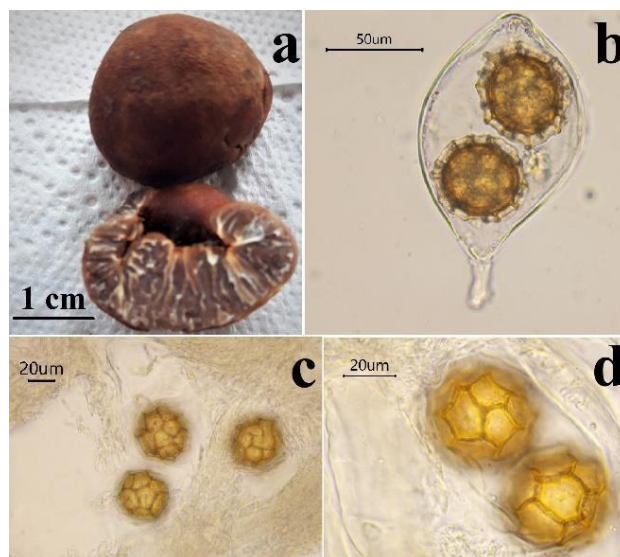


Figure 1. *Tuber fulgens*: a. ascomata, b. spores in an ascus (under LM), c,d. spores (under LM).

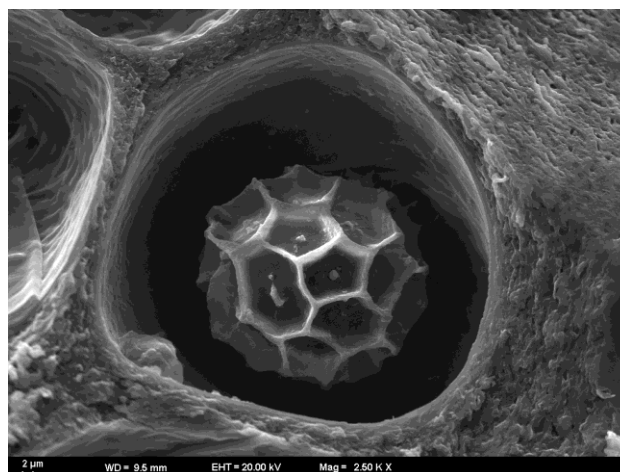


Figure 2. *Tuber fulgens*: a spore in ascus visualized by a scanning electron microscope (SEM).

Molecular Phylogeny of ANK Akata 7351

The ITS and LSU rDNA sequences of ANK Akata 7351 obtained from Sanger sequencing were deposited into NCBI GenBank with the accession numbers of MT984247.1 and MT982616.1 respectively.

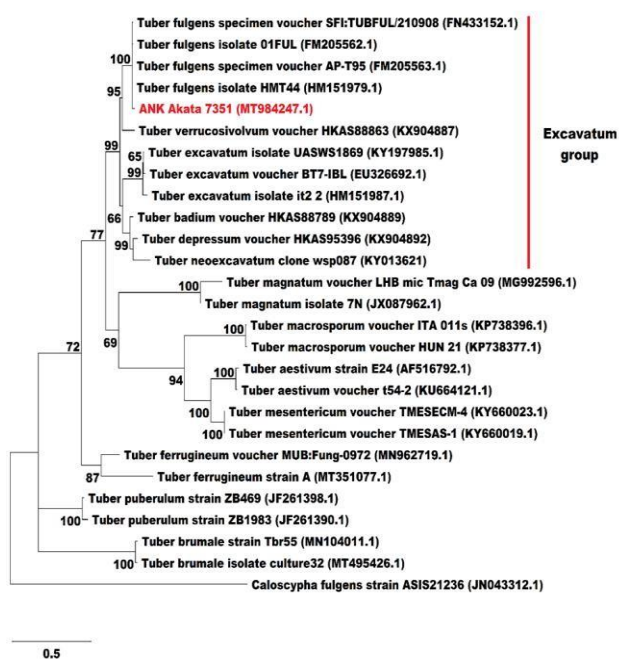


Figure 3. The Maximum Likelihood tree revealing the phylogenetic relationships of 14 fungal species estimated from the nuclear ITS rDNA region. Percentage bootstrap values (≥ 50) were indicated for each branch. All of the sequences included in the phylogenetic analysis were retrieved from GenBank except for ANK Akata 7351. *Caloscypha fulgens* were included as the outgroup sample in the phylogenetic analysis. GenBank accession numbers are also given. The scale bar (lower left) represents a genetic distance of 0.5.

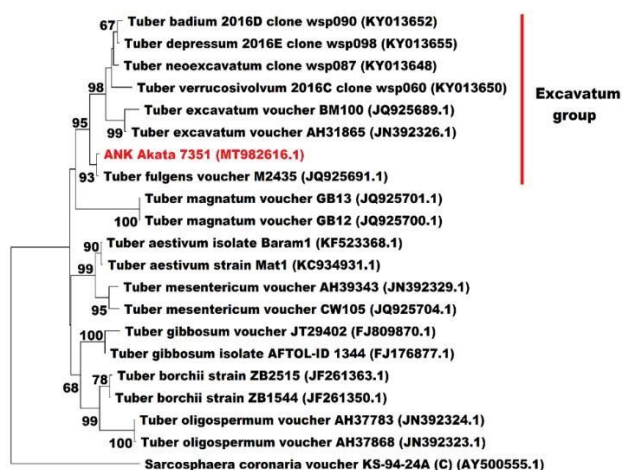


Figure 4. The Maximum Likelihood tree revealing the phylogenetic relationships of 13 fungal species estimated from the nuclear LSU rDNA region. Percentage bootstrap values (≥ 50) were indicated for each branch. All of the sequences included in the phylogenetic analysis were retrieved from GenBank except for ANK Akata 7351. *Sarcosphaera coronaria* was included as the outgroup samples in the phylogenetic analysis. GenBank accession numbers are also given. The scale bar (lower left) represents a genetic distance of 0.05.

In phylogenetic analysis of ANK Akata 7351, considering the BLAST search results of the specimen's nuclear ITS rDNA sequence, the various members of the genus *Tuber* from the family *Tuberaceae* were used as the ingroup sequences and the nuclear ITS rDNA sequences of *Caloscypha fulgens* (Pers.) Boud. from the family *Caloscyphaceae* was selected for the outgroup sequence. On the other hand, for the LSU rDNA based phylogenetic analysis of ANK Akata 7351, LSU sequences of various members of the genus *Tuber* were used as the ingroup sequences, and the LSU sequence of *Sarcosphaera coronaria* (Jacq.) J. Schröt. from the family *Pezizaceae* was selected as the outgroup sequence.

As a result of the phylogenetic analysis conducted with the ITS sequences including from ANK Akata 7351, a highly supported distinct clade came out (Figure 3). This clade, named as Excavatum group, included different isolates of *Tuber fulgens* and the specimen ANK Akata 7351 along with different isolates of *Tuber excavatum* and closely related, recently described *Tuber* species such as *T. verrocusivolum*, *T. badium*, *T. depressum*, and *T. neoexcavatum* (Figure 3). On the other hand, *Caloscypha fulgens* fell into a distinct branch separate from the ingroup species and formed an outgroup as predicted. The BLAST analysis implemented with the nuclear ITS rDNA sequence of ANK Akata 7351 revealed identity rates as high as 99.23% between the specimen and different isolates of *T. fulgens*. The phylogenetic analyses performed herein, further strengthen the close identity relationship of this specimen with *T. fulgens* with a bootstrap value of 100%.

As a result of the phylogenetic analysis performed with the LSU sequences including from ANK Akata 7351, again a highly supported distinct clade (Excavatum group) came out (Figure 4). This clade also included one isolate of *Tuber fulgens* and the specimen ANK Akata 7351 along with different isolates of *Tuber excavatum* and closely related, recently described *Tuber* species such as *T. verrocusivolum*, *T. badium*, *T. depressum*, and *T. neoexcavatum* (Figure 4). On the other hand, *Sarcosphaera coronaria* fell into a distinct branch separate from the ingroup species and formed an outgroup as predicted. The BLAST analysis performed with the nuclear LSU rDNA sequence of ANK Akata 7351 revealed identity rates as high as 99.21% between the specimen and one isolate of *T. fulgens*. The phylogenetic analyses performed herein, further strengthen the close identity relationship of this specimen with *T. fulgens* with a bootstrap value of 93%.

Discussion

Although *T. fulgens* resembles other excavatum group members (*Tuber badium* S.P. Wan, *T. depressum* S.P. Wan, *T. excavatum* Vittad., *T. neoexcavatum* L. Fan & Yu Li and *T. verrucosivolum* S.P. Wan) in terms of its macro and micromorphology, its distinguishable characteristics are orange-brown ascomata and a relatively single type of ascospores (globose to subglobose) (Fan et al., 2013; Lange, 1956; Wan et al., 2017). However, ITS and nrLSU rDNA regions-based molecular phylogeny and SEM images of spores can help distinguish *T. fulgens* from other excavatum group members.

For the precise identification of fungal species, conventional methods relied on morphological data may not always sufficient. For this reason, conserved regions of genomic DNA such as nrITS, and nrLSU, as well as sequences of protein-coding genes, are utilized for molecular taxonomic studies for the last few decades (Raja et al., 2017). Besides, ITS and LSU are two of the most generally used DNA barcoding markers for fungi and thus confers precious information for molecular phylogenetic studies. Therefore, we employed nuclear ITS and LSU rDNA sequences for the molecular identification of ANK Akata 7351. Nuclear ITS and LSU rDNA-based molecular phylogeny exhibited almost 100% identity between *Tuber fulgens* and the specimen (GenBank ID: MT984247.1 and MT982616.1 for ITS and LSU rDNA regions respectively) (Figure 3 and 4).

Conclusion

With this study, *T. fulgens* was reported for the first time from Turkey and it is the 9th reported *Tuber* species for Turkish truffles. The morphological data obtained from the specimen ANK Akata 7351 corresponded to the description of *T. fulgens*. Additionally, the ITS and LSU rDNA based molecular phylogenetic studies of the specimen further consolidated the accuracy of the conventional identification of ANK Akata 7351.

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