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Fungal DNA barcode (ITS nrDNA) reveals more diversity than expected in *Tulostoma* from Macedonia

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Abstract: With the aim of clarifying the number of *Tulostoma* species in Macedonia, and to verify some previous collections, molecular analyses were carried out on four morphologically identified species collected in different habitats (at the edges of oak or juniper forests, pine plantations, meadows, rocky places, roadsides). Sixty-two new sequences were generated and compared with recently published sequences included in public databases. According to the barcoding sequences fourteen *Tulostoma* species are identified in Macedonia and ten of them were assigned to known species: *Tulostoma brumale*, *T. fimbriatum*, *T. lusitanicum*, *T.* cf. *moravecii*, *T. melanocyclum*, *T. punctatum*, *T. simulans*, *T. squamosum*, *T. subsquamosum*, and *T. winterhoffii*. However, four of them remain as *Tulostoma* sp. 1, 2, 3, and 4, since more collections should be found and analyzed before giving them a specific name.

Key words: Morphology, molecular analyses, phylogeny, stalked puffballs, taxonomy

1. Introduction

The genus *Tulostoma* Pers. (Order Agaricales; Basidiomycota) is characterized by a globose spore-sac, where the spores are formed, attached onto a stipe; they are known commonly as stalked puffballs. The spore-sac is formed by the peridium that envelopes the gleba; when spores are mature, they are released via an opening found on the apical part of the sac called a mouth or stoma that can be tubular, circular, or elliptical; fibrillose or fimbriate; or a simple crevice. The current genus was initially divided into two sections: *Eutylostoma* and *Schizostoma* (Fischer, 1900, 1933; Petri, 1909; Fries, 1921), which are differentiated by mouth morphology. Pouzar (1958) offered a more precise classification based on the morphology and mode of opening of the exoperidium, as well as the morphology of the mouth and stipe, proposing four sections: *Brumalia*, with tubular mouth; *Poculata*, with mouth fibrillose; *Fimbriata*, with fimbriate mouth; and *Volvulata*, with irregular mouth.

Wright (1987) supplemented Pouzar's classification with primary and secondary micromorphological characteristics. The primary characteristics are the endoperidium form, size, and color; exoperidium color, persistence, and decay; mouth form; and spore size and ornamentation. Secondary characteristics are stipe size, and color and morphology of stipe surface; thickness of capillitium hyphae; and morphology of capillitium transverse septa. In Wright's classification two subgenera are included: *Tulostoma* Pers. and *Lacerostoma* J. E. Wright, encompassing 137 species worldwide. As pointed out by Cunningham (1925), Moreno et al. (1992) considered that this genus consists of a taxonomic complex, since some characters are not always easy to differentiate.

The genus has been studied in various areas of Europe, Asia Minor, and Africa. In the work of Kreisel (2001), 28 species were cited in Europe, 14 in Asia Minor, and 38 in Africa. In the Mediterranean area, Calonge et al. (2007) described 22 species for Spain, while Tkalčec et al. (2005) and Sesli and Denchev (2008) reported only five species in Croatia and Turkey. In Macedonia, based on morphological identification, Karadelev and Rusevska (2009) confirmed four species: *Tulostoma brumale* Pers., *T. fimbriatum* Fr., *T. melanocyclum* Bres., and *T. squamosum* (J.F. Gmel) Pers. (Table). Wright (1987) reported, for the first and only time, *T. caespitosum* Trab.

Molecular analysis based on the internal transcribed spacers (ITS) of nuclear ribosomal DNA (nrDNA), accepted as the DNA barcode for fungi (Schoch et al., 2012), provides valuable information for species identification. Recently, Jeppson et al. (2017) demonstrated that the ITS region is very useful to discriminate *Tulostoma* species in Europe; in this paper the barcode sequence was obtained

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Table. *Tulostoma* taxa from Macedonia according to morphological features.

Species	Reference	Localities
T. brumale Pers.	Karadelev and Rusevska (2009)	Bistra, Galichica, Gradishtanska Planina, Kichevo Valley, Mangovica, Osogovski Planini, Pogana, Skopska Crna Gora, Skopsko Pole, Taorska Klisura and Badar, Vodno, Zhegligovo
T. caespitosum Trab.	Wright (1987)	Valandovsko Pole
T. fimbriatum Fr.	Lindtner (1939), Pilát and Lindtner (1939), Tortić (1988), Karadelev and Rusevska (2009)	Babuna; Dub – Pogana; Gevgelisko Pole; Kozjak-Kumanovo; Pogana; Prespa; Ruen; Slan Dol; Sredorek; Vodno; Zhegligovo
T. melanocyclum Bres.	Tortić (1988), Karadelev and Rusevska (2009)	Babuna, Mangovica, Prespa, Serta, Vodno, Zhegligovo
T. squamosum J.E. Gmel.: Pers.	Karadelev and Rusevska (2009)	Bistra, Gradishtanska Planina, Mangovica, Taorska Klisura and Badar, Tikvesh, Torbeshija – Karadzhica, Vodno, Zhegligovo

for 26 described species and 19 possibly undescribed species.

The aim of this paper is to review the *Tulostoma* species from Macedonia using a barcoding approach combined with an accurate revision of morphological characters. As a result of our analyses we report some novelties within this genus among specimens from Macedonia.

2. Materials and methods

2.1. Material

The reference material used in the study originates from the Macedonian Collection of Fungi (MCF) and the Macedonian Fungi Database (FUNGI MAK) located at the Mycological Laboratory at the Institute of Biology, Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University, Skopje, with a few duplicates at MA-Fungi (Real Jardín Botánico-CSIC, Madrid). A part of the data comes from amateurs and members of the Macedonian Mycological Society (MMS), the Biology Students' Research Society (BSRS), and the Macedonian Ecological Society (MES). A total of 64 specimens from 34 localities in 16 different habitats were analyzed in this study.

2.2. Morphological analyses

Morphological analyses were performed using light microscopy (LM; LW Scientific, Mycological Laboratory Institute of Biology, Skopje). Slides for taxonomically relevant characters (spores, basidia, capillitium, etc.) were prepared in KOH (5%) or ethanol (70%) and stained or contrasted with Cotton Blue or Melzer's reagent. Scanning electron microscopes (SEMs; Hitachi S-3000N SEM, Real Jardín Botánico, CSIC, Madrid, Spain; and Cambridge S4 Stereoscan, Cambridge Instruments Ltd., Cambridge, UK, and Friedrich Hustedt Study Center for Diatoms (BRM), Bremer, Germany) were also used to examine selected characters from capillitium and spores. SEM stubs were prepared using glebal tissue coated with gold (Balzers SCD 004 sputter coater) or gold-palladium (Polaron SC7640 sputter coater, Quorum Technologies, Sussex, UK). The assessed macroscopic and microscopic features were used for the morphology-based identification of the species following descriptions and identifications given by Calonge (1998) and Knudsen and Vesterholt (2012), as well as literature for individual taxa. The MycoBank website (Stalpers and Cock, 2013; accessed 29.11.2017) was also consulted.

2.3 Molecular analyses

2.3.1. DNA extraction and sequencing

A part of the glebal tissue from specimens was selected for DNA isolation; in a few cases other parts, such as the peridium or volva, were used for DNA isolation. DNA extraction, amplification, and sequencing of the ITS regions including the 5.8S of the ribosomal RNA gene cluster followed the protocols mentioned by Phosri et al. (2009). The ITS region was successfully amplified and sequenced from dried basidiomes using the ITS1F/ITS4 primer pair, except from a few collections. In general, after purification of the amplified product, if the DNA concentration was greater than 20 ng/µL, the purified products were sequenced directly by Macrogen (Korea).

To identify possible DNA contamination a negative control was run at all steps (Sundquist and Bessetti, 2005). BLAST searches with the MEGABLAST option (Altschul et al., 1997) were used to compare the sequences obtained against sequences in the National Center of Biotechnology Information (NCBI) nucleotide databases. The new

consensus sequences have been lodged in the EMBL-EBI and UNITE databases (Kõljalg, 2013) under the accession numbers indicated in the taxonomy section of this paper.

2.3.2. Alignment and phylogenetic analyses

The ITS nrDNA sequences were aligned in Se-Al v2.0a11 Carbon (Rambaut, 2002) using protocols for multiple sequence alignment. Sequences were compared with homologous sequences from GenBank. Where ambiguities in the alignment occurred, the alignments generating the fewest potentially informative characters were chosen. Alignment gaps were marked "–"; unresolved nucleotides and unknown sequences were indicated with "N". The polarity of characters was assessed with outgroup comparison using *Phellorinia* (AF097754, AF097753, DQ311083, DQ311087, GQ249887) and *Dictyocephalos attenuatus* (AF095688). The alignment was analyzed using the programs PAUP*4.0b10 (Swofford, 2003) and MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) as indicated by Phosri et al. (2009). Phylogenetic trees were drawn using TreeView (Page 1996).

2.4. Taxonomy

A comprehensive analysis of the distribution of *Tulostoma* species in Macedonia was performed through revision of published information and tracking the unpublished data relevant for the area of the Republic of Macedonia, the revision of available collections to identify the "expected" species, and molecular analyses of 64 selected specimens from four morphological taxa.

After molecular analyses, a second morphological revision was done. In the taxonomic part ten species are included with the following data: a) short description of the taxon, including photos of macroscopic and microscopic characters; b) descriptions of locality, habitat, altitude, and accession number; and c) remarks.

3. Results

3.1. Molecular analyses

A total of 64 extractions were performed. Genomic DNA concentration ranged from 1.5 to 15 ng/µL. Approximately 70 PCR products were purified and sequenced, which resulted in 62 new sequences.

The new *Tulostoma* sequences were aligned with 16 sequences from GenBank. Of the 818 positions, 473 were constant, 69 were variable but parsimony-uninformative, and 276 were parsimony-informative. In the phylogenetic analysis under heuristic search, 100 most parsimonious trees (MPTs) were obtained (tree length = 776 steps, consistency index CI = 0.6160, homoplasy index HI = 0.3840, retention index RI = 0.8945). The 50% majority-rule tree of the Bayesian analysis (Figure 1) has similar topology to the parsimony strict consensus tree (data not shown).

Generated sequences of *Tulostoma* species comprise a monophyletic group with high support. Within the Bayesian tree two main lineages (I and II) were revealed, where the Macedonian specimens are distributed in nine major terminal clades (a–b, f–g, i–j, l–n) and five singletons (c–d, e, h, k).

Sequences of lineage I split into two clades, IA and IB. In clade IA sequences were distributed in two very strongly supported subclades (a and b), with spores ornamented by rather regular warts. Subclade a (PP = 1.00; BS = 99%) consisted of Macedonian samples identified as *Tulostoma* sp., *T. brumale*, *T. melanocyclum*, *T. squamosum*, and one GenBank sequence under *T. brumale* (EU784434). Sequences of this subclade show 100% similarity through BLAST search with *T. simulans* (KU519050), also proven by spore ornamentation with clearly isolated warts (observed under SEM), compared with those of Jeppson et al. (2017). The Macedonian specimens in this group were collected in different localities, from 100 to 900 m altitude and from various associations, such as oak or willow habitats, park, and yard.

Subclade b (PP = 1.00, BS = 97%) is formed by one GenBank sequence under *T. brumale* (EU784433) and many Macedonian samples previously identified as *T. brumale*, *T. melanocyclum*, and *T. squamosum*; as in the previous group, all samples from this branch are from different localities in the altitude range from 150 to 1400 m, mainly from meadows or oak forests, while one collection is from pine plantations. After the second morphological revision, we can confirm that Macedonian specimens under subclade b belong to *T. brumale*, due to their globose to subglobose and slightly asperulate spores under LM, as well as the presence of a brown zone around the mouth in most of the collections.

Sequences in clade IB are also distributed in two branches: a singleton, indicated with c in the tree, represented by *Tulostoma* sp. (07MCF9359), and clade d (PP = 1.0, BS = 91%), formed by a sequence under *Tulostoma* sp./*T.* cf. *moravecii* (07MCF8292) together with a GenBank sequence under *T. niveum* (EU784437). The collection in branch c has a relatively small basidiome (2.5 cm), with white ocher color and almost smooth to asperulate spores under LM; the species was collected on rocky calcareous ground, among grass and mosses, in open shrubby area, at 1400 m altitude; concerning the spore ornamentation, this collection shows similarity with *T. moravecii*, and according to the sequence it is 100% *Tulostoma* sp. 13 from Jeppson et al. (2017). The collection in clade d (07MCF8292) has asperulate spores under LM and conical warts under SEM (a feature of *T. moravecii*); it is also found among mosses, in open areas in *Juniperus excelsa* forest at 300 m altitude; this specimen is indicated in the tree as *T.* cf. *moravecii*.

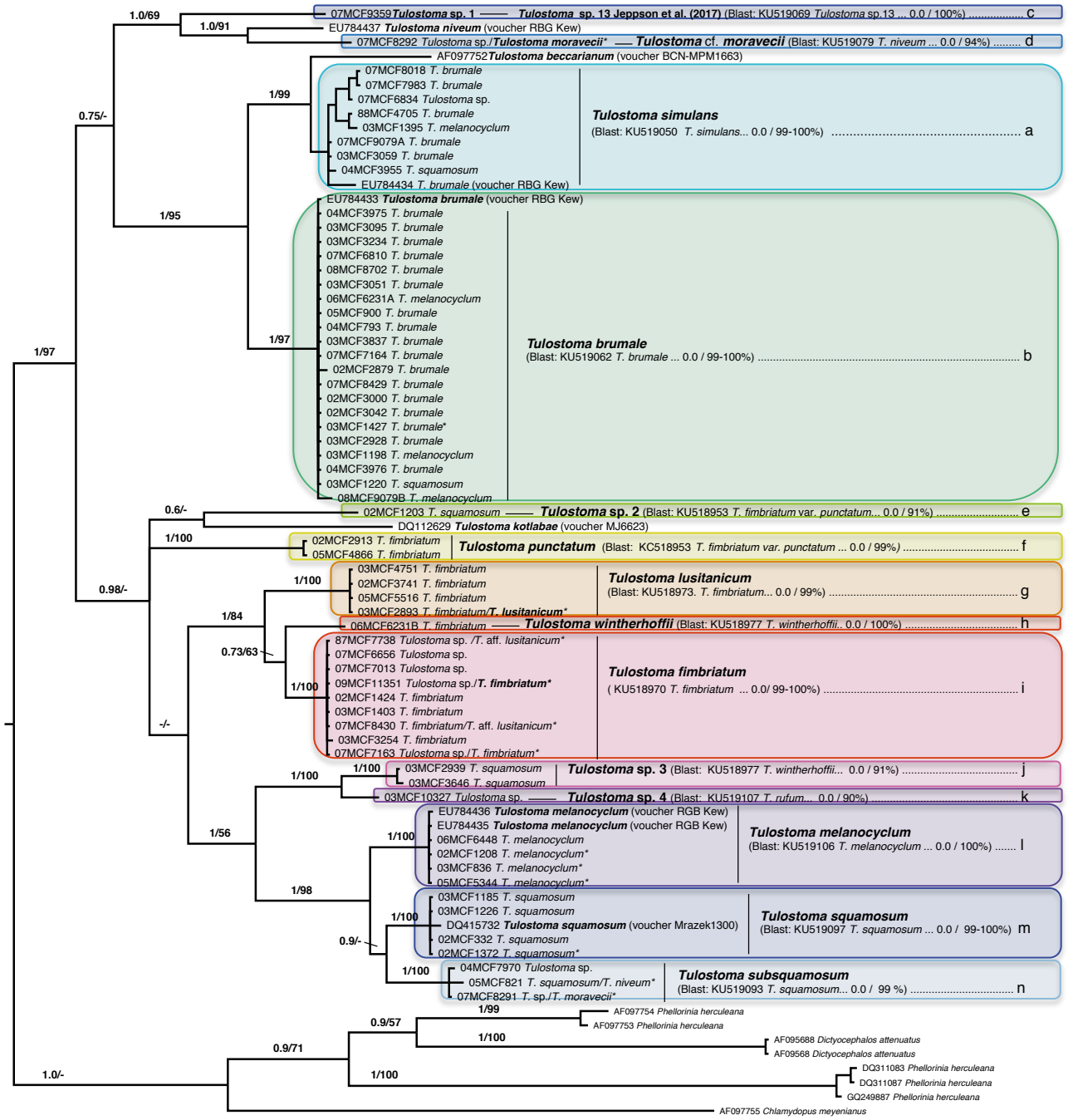

Figure 1. The 50% majority-rule consensus tree of Bayesian analysis inferred from ITS nrDNA sequences of *Tulostoma* specimens from Macedonia (indicated with MCF), as well as sequences retrieved from GenBank. Bootstrap and posterior probabilities values are indicated on the branches. Sequences of *Phellorinia herculeana* (AF097754, AF097753, DQ311083, DQ311087, GQ249887) and *Dictyocephalos attenuatus* (AF095688) as outgroup.

In lineage II, sequences are distributed in at least seven main subclades and three singletons (e, h, k). Singleton e corresponds to Macedonia 02MCF1203, preliminarily described as *T. squamosum*; this sample has a dark brown layer on the stipe, misidentified as squamules, which took us to a preliminary identification as *T. squamosum*), but its sequence under BLAST search is 91% similar to *T. fimbriatum* var. *punctatum* (KU518953), representing a sister branch of *T. kotlabae* (DQ112629), from which it differs by the dark brown stipe. The Macedonian collection

is indicated in the tree as *Tulostoma* sp. 2.

Subclade f is highly supported (PP = 1.00, BS = 100%) and consists of two Macedonian samples (02MCF2913 and 05MCF4866), collected from deciduous forest, preliminarily identified as *T. fimbriatum* due to macromorphology, i.e. fibrillose-fimbriate mouth. BLAST results show 99% similarity with a sequence under *T. fimbriatum* var. *punctatum* (KC518953). Wright (1987) pointed out that a punctuated endoperidium is an easily distinguished feature to identify this variety; however, it was difficult to observe in our collections. Jeppson et al. (2017) mentioned that this character is "of little taxonomic value" and "could not be observed in the European specimens". Instead of its phylogenetic position as *T. fimbriatum* var. *punctatum* according to Jeppson et al. (2017), SEM photos of spores from our collection (coarsely ornamented, but slightly densely anastomosed) are more close to those of *T. punctatum* from Slovakia (MJ 10058, GB) analyzed in the cited publication. Thus, for this species, additional analyses (morphological and phylogenetic) from additional collections are needed.

Clade g, with significant support (PP = 1.00, BS = 100%), contains specimens from oak forests, preliminarily identified as *T. fimbriatum* (three samples) or *T. lusitanicum* (one sample). According to Calonge (1998) the main difference between these two species is the structure of the exoperidium (hyphal or membranous), which is "difficult to observe due to being covered with remains of the substratum" in *T. fimbriatum*, or "with sand grains" in *T. lusitanicum*. However, their distinction by ITS is clear and well supported. Observations of their spores under SEM show differences, i.e. in *T. lusitanicum* spores have well-defined conical warts, at some places grouped in pyramids, and in *T. fimbriatum* spores have verrucae coalescing into ridges. A singleton, indicated with h in Figure 1 and collected in pine plantings, corresponds to *T. wintherhoffii*, which, under SEM, has spores very similar to those of *T. lusitanicum*; however, in the tree it is well supported (100% under BLAST) as a separate branch.

Clade i is also formed by a number of specimens identified as *T. fimbriatum* or *T. lusitanicum*; however, the BLAST search and a careful morphological examination, as well as SEM showing ornamented spores with crests, confirm the species as *T. fimbriatum*.

Clade j is formed by two collections identified as *Tulostoma squamosum* (03MCF2939 and 03MCF3646) with high support (PP = 1.0, BS = 100%), a sister group of sequence k under *Tulostoma* sp. (03MCF10327); this relationship is highly supported (PP = 1.00, BS = 100%). These three collections have a tubular mouth, without any obvious dark zone, and they were found in different habitats: hornbeam-oak, Greek juniper forest, and meadow, respectively. After a second morphological revision, some of the features in samples grouped in the j and k branches do not fit with *T. squamosum*. Spore ornamentation of *Tulostoma* sp. 4 is like that of *T. rufum* of Jeppson et al. (2017), which also fits with the BLAST results in our tree.

Clade l clearly grouped four Macedonian collections (06MCF6448, 02MCF1208, 03MCF836, and 05MCF5344) and two GenBank sequences (EU784436 and EU784435), all identified as *T. melanocyclum* by their morphology, habitat (oak forest), and BLAST results (KU519106); clearly this clade represents the species *T. melanocyclum*.

Clade m grouped four Macedonian collections (03MCF1185, 03MCF1226, 02MCF332, and 02MCF1372) and one GenBank sequence (DQ415732) under *Tulostoma squamosum*. All Macedonian collections were collected in oak forests. Macedonian collections have easily recognizable squamules together with other characters, like relatively long stipe (in most of the collections) and spores with warts united in small pyramidal groups at some parts, as well as 100% confirmation of sequences from Jeppson et al. (2017); there is no doubt about the taxonomy of this species.

Finally, subclade n, the sister group of clade m, is formed by one unidentified Macedonian collection (04MCF7970), one identified as *Tulostoma niveum* (05MCF821), and one as *T. moravecii* (07MCF8291), collections from different localities and habitats (oak, Greek juniper forests, and roadsides). Spores with anastomosed verrucae and high support of the branch with *T. subsquamosum* (KU519093) clearly confirm this taxon.

Thus, based on morphological and molecular data, 15 species are recognized for Macedonia (Figures 2a–2n and 3a–3n).

3.2. Taxonomy

1. *Tulostoma brumale* Pers. (Figures 2b and 3b)

Mature *basidiomata* stipitate. Endoperidial body creamy to pale ochraceous, 7–13 mm in diameter. Mouth conical, with or without brown to dark brown ring zone. Stipe cylindrical, 13–60 × 1–3 mm, creamy-ocher to pale brown, smooth, furrowed or with small light to dark brown scales. The remnants of volva are present in all analyzed samples. Gleba ocher to light brown.

Basidiospores globose to subglobose, 3.8–4.9 µm in diam., with very small verrucae (under LM), which appeared as almost isolated, irregular, ±conical warts under SEM (Figure 3b), 0.4–0.6 µm long. Capillitium branched, with strongly widened septa, without crystals.

Material studied (Figure 1, clade b). **Baba Sach and Ljuben**: St. Bogorodica Prechista monastery (vicinity), along r. Treska, 850 m a.s.l., meadow, 02.05.2007, 07MCF6810 (MG768860); **Bistra** Mt.: Izvor vill., 500 m a.s.l., sandy soil, 23.03.2004, 04MCF793 (MG768865); **Galichica**: Ohrid side, near spring, 1400 m a.s.l.,

Figure 2. Basidiomes. a- *Tulostoma simulans* (03MCF3059); b- *T. brumale* (03MCF1220); c- *Tulostoma* sp. 1 (07MCF9359), as *Tulostoma* sp. 13 in Jeppson et al. (2017); d- *T.* cf. *moravecii* (07MCF8292); e- *Tulostoma* sp. 2 (02MCF1203); f- *T. punctatum* (05MCF4866); g- *T. lusitanicum* (03MCF4751); h- *T. winterhoffii* (06MCF6231B); i- *T. fimbriatum* (07MCF6656); j- *Tulostoma* sp. 3 (03MCF2939); k- *Tulostoma* sp. 4 (03MCF10327); l- *T. melanocycum* (06MCF6448); m- *T squamosum* (03MCF1226); n- *T. subsquamosum* (07MCF8291). Scale bars: 0.5 cm. Letters (a–n) correspond to subclades and branches shown in Figure 1.

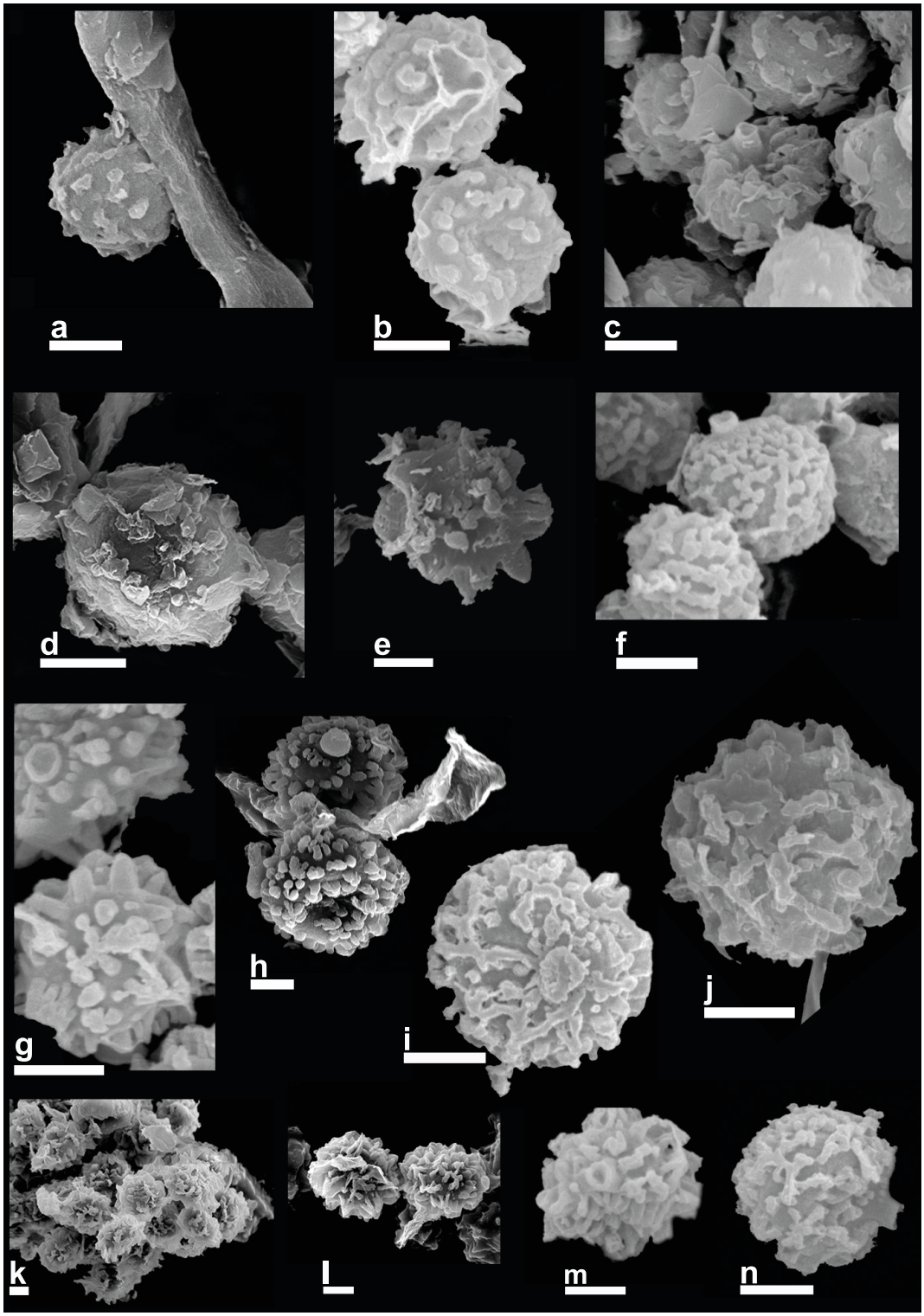

Figure 3. SEM photos of basidiospore ornamentation. a- *Tulostoma simulans* (03MCF3059); b- *T. brumale* (03MCF1220); c- *Tulostoma* sp. 1 (07MCF9359), as *Tulostoma* sp. 13 in Jeppson et al. (2017); d- *T.* cf. *moravecii* (07MCF8292); e- *Tulostoma* sp. 2 (02MCF1203); f- *T. punctatum* (05MCF4866); g- *T. lusitanicum* (03MCF4751); h- *T. winterhoffii* (06MCF6231B); i- *T. fimbriatum* (07MCF6656); j- *Tulostoma* sp. 3 (03MCF2939); k- *Tulostoma* sp. 4 (03MCF10327); l- *T. melanocycum* (06MCF6448); m- *T squamosum* (03MCF1226); n- *T. subsquamosum* (07MCF8291). Scale bars: 2 µm. Letters (a–n) correspond to subclades and branches shown in Figure 1.

grassy place, 22.11.2002, 02MCF3042 (MG768871); **Gradishtanska Planina**: D'lga vill., 400–450 m a.s.l., Querco-Carpinetum orientalis, 12.03.2003, 03MCF3837 (MG768866); **Kozjak** (Kumanovo): Zhegljane vill., *Quercus* forest, 09.2005, 05MCF900 (MG768864); **Mangovica**: between Zubovce village and Shuplji Kamen village, 400 m a.s.l., Querco-Carpinetum orientalis, sandy soil, 15.04.2003, 03MCF3051 (MG768862); 03MCF1427 (MG768872); 03MCF1198 (MG768874); Zubovce vill., Bislim, 500 m a.s.l., degraded forest, 07.03.2004, 04MCF3976 (MG768875); **Skopska Crna Gora**: between Gornjane vill. and Blace vill., 700 m a.s.l., oak forest, degraded, 25.01.2003, 03MCF3234 (MG768859); **Skopsko Pole**: Nikishtane vill., 400 m a.s.l., *Pinus nigra* plantings, 22.12.2007, 07MCF8429 (MG768869); Orman vill. (near Volkovo), oak forest, degraded, 16.02.2008, 08MCF8702 (MG768861); *Quercus petraea* forest, 270 m a.s.l., 23.02.2008, 08MCF9079B (MG768877); **Taorska Klisura and Badar**: St. Jovan Veterski monastery, 150–200 m a.s.l., *Juniperus excelsa* forest, 23.10.2002, 02MCF2879 (MG768868); **Vodno**: Sredno Vodno, 500–1000 m a.s.l., Querco-Carpinetum orientalis, 20.10.2002, 02MCF3000 (MG768870); Sredno Vodno, 400 m a.s.l., meadow with *Juniperus*, 01.01.2003, 03MCF2928 (MG768873); between Sredno Vodno and Matka, 900 m a.s.l., 04.2004, 04MCF3975 (MG768857); Vodno peak: 900 m a.s.l., meadow, sandy soil, 04.05.2003, 03MCF3095 (MG768858); Gorno Sonje vill., *Pinus nigra* plantings, 21.10.2006, 06MCF6231A (MG768863); **Zhegligovo**: Querco-Carpinetum orientalis, 20.03.2003, 03MCF1220 (MG768876); Staro Nagorichane vill., meadow in deciduous forest (*Quercus, Crataegus*), 07.2007, 07MCF7164 (MG768867).

Remarks: *Tulostoma brumale* is recognizable by the cylindrical mouth with brown-colored peristome, verrucose spores, and capillitium with broadly swollen septa. Dozens of varieties of this species sometimes makes the determination difficult; therefore, Tiffney and Tiffney (1971) recommended that species description should be based on populations and collections from different periods and ecological conditions, not only from a single specimen. It is rare in some European countries, as in Greece or Hungary, and in Poland where it is included in the national Red Lists (Adamczyk, 2009). According to the NMV Dispersion Atlas of Mushrooms (https://www.verspreidingsatlas.nl/0230010) it is common in the Netherlands, especially in the coastal part of the country. Although it is recorded in 60 localities in southwest Sweden, it is red-listed as a species growing on rare and vulnerable steppe habitats (Gärdenfors, 2010). It is known from a few provinces in Turkey growing in black pine, Greek juniper, or cedar associations (Doğan et al., 2012). In Macedonia it is known from 13 localities (Karadelev and Rusevska, 2009); in this paper it is cited as new to four localities. In Macedonia it is found mainly in oak-hornbeam, as well as in degraded oak forests, pine plantings, and meadows, and one collection is from Greek juniper association, at 100 to 1000 m altitude. It grows on sandy soil or humus, among grass or mosses during the whole year, except the summer period.

2. *Tulostoma fimbriatum* Fr. (Figures 2i and 3i)

Mature *basidiomata* stipitate. Endoperidial body whitish, ochraceous, light gray to brownish, 9–15 mm in diameter, papery, almost without grains on the surface, but always with a layer of soil particles in the lower part, up to 5 mm; with a short collar around the stipe. Mouth fimbriate, plane, concolorous with endoperidium. Stipe cylindrical, 25–40 × 3–6 mm, slightly fimbriate to scaly or woody in some samples, dark brown; slightly enlarged in the basal part. Gleba ochraceous to light brown or ferruginous.

Basidiospores globose to subglobose, 4.5–5.7 µm in diam., with verrucae (0.4 µm long) coalescing into ridges (Figure 3i). Capillitium branched, septate.

Material studied (Figure 1, clade i). **Babuna**: Markovi Kuli, meadow (rocky place), 09.02.2007, 07MCF7165 (MG768896); **Gevgelisko Pole**: Bogdanci, 150 m a.s.l., 27.02.1987, 87MCF7738 (MG768888); **Prespa**: Nakolec vill., 850 m a.s.l., Salicetum, 12.06.2003, 03MCF3254 (MG768895); **Ruen**: r. Pchinja, Alginja vill., deciduous forest (*Quercus, Crataegus*), 04.2007, 07MCF6656 (MG768889); **Skopsko Pole**: Nikishtane vill., 400 m a.s.l., *Pinus nigra* plantings, 22.12.2007, 07MCF8430 (MG768894); **Sredorek**: Kuklica, Stone Dolls, 450 m a.s.l., meadow, 29.11.2009, 09MCF11351 (MG768891); **Zhegligovo** (Kumanovo): Staro Nagorichane vill., meadow, 20.05.2007, 07MCF7013 (MG768890), 600 m a.s.l., degraded habitat (*Paliurus spina-christi*, *Quercus pubescens*), 30.11.2002, 02MCF1424 (MG768892), 400 m a.s.l., Querco-Carpinetum orientalis, 20.03.2003, 03MCF1403 (MG768893).

Remarks: In comparison with other species from the genus, *T. fimbriatum* is characterized by the fimbriate mouth and robust basidiome. According to Wright (1987) it is a typical European species, introduced in other regions, but according to the latest research by Jeppson et al. (2017) this species is synonymous with *T. campestre* and could not be considered as a typical European species. It is a rare or protected species in Greece and on the Red Lists in Poland (Adamczyk, 2009), and Sweden (Gärdenfors, 2010), where a significant decrease of its population is noticed (Hansson and Jeppson, 2005). Jaworska et al. (2012) mentioned this species as an indicator for xerothermic grasslands in Poland. In Turkey it is reported from pine, Greek juniper, and cedar forests (Doğan et al., 2012). It grows predominantly in oak-hornbeam, degraded oak forests, pine plantings, *Salix* associations or meadow, between 150 and 800 m altitude.

3. *Tulostoma lusitanicum* Calonge & M.G. Almeida (Figures 2g and 3g)

Mature *basidiomata* stipitate. Endoperidial body whitish, ochraceous, light gray to brownish, 7–11(–15) mm in diameter, papery, with or without grains on the surface, but always has a layer of soil particles in the lower part, up to 5 mm; with a short collar around the stipe. Mouth fimbriate, plane, concolorous with the endoperidium. Stipe cylindrical, 19–(37)–45(–50) × 2–5 mm, slightly fimbriate to scaly or woody in some samples, dark brown; slightly enlarged in the basal part. Gleba yellowish to light brown.

Basidiospores globose to subglobose, 4.6–5.6 µm in diam., with well-defined conical or cylindrical warts, 0.7–0.9 µm long, grouped in pyramids at some places (Figure 3g). Capillitium branched, septate.

Material studied (Figure 1, clade g). **Belasica**: Koleshino vill., between r. Daravica and r. Baba, 350 m a.s.l., *Quercus* and *Castanea* forest, 01.01.2003, 03MCF2893 (MG768886); **Dub**: Gorni Bolovan, 200 m a.s.l., Coccifero-Carpinetum orientalis, 10.08.2002, 02MCF3741 (MG768884); **Gevgelisko Pole**: Bogdanci, meadow in Coccifero-Carpinetum orientalis, 150 m a.s.l., 12.12.2005, 05MCF5516 (MG768885); **Pogana**: Kuchalat (vicinity), 200–300 m a.s.l., Coccifero-Carpinetum orientalis, 02.01.2003, 03MCF4751 (MG768883).

Remarks: The best match of our BLAST search of Macedonian sequences fits with the sequence KU518973 (voucher MJ8773) under *T. fimbriatum* in GenBank and described as *T. calongei* Jeppson, Altés, G. Moreno & E. Larss., sp. nov. by Jeppson et al. (2017).

4. *Tulostoma melanocyclum* Bres. (Figures 2l and 3l)

Mature *basidiomata* stipitate. Endoperidial body cream-colored, ochraceous to light gray, 8–12(–14) mm in diameter. Mouth tubular, concolorous with the endoperidium or surrounded by ash-colored zone. Stipe cylindrical, 22 × 2–3 mm, ochraceous brownish, furrowed or with small adpressed dark brown scales; with bulb at the base. Gleba yellowish to brown.

Basidiospores globose to subglobose, (5.2–)5.7–6.6 µm in diam., echinulate under LM, with well expressed, relatively dense, conical warts (0.6–1.3 µm long), anastomosed in parts, under SEM (Figure 3i). Capillitium branched, thick-walled, septate.

Material studied (Fig 1, clade j). **Serta** Mt.: St. Gjorgija monastery (vicinity), 200 m a.s.l., Coccifero-Carpinetum orientalis, 16.12.2006, 06MCF6448 (MG768900); **Zhegligovo** (Kumanovo): Staro Nagorichane vill., 600 m a.s.l., degraded habitat (*Paliurus spina-christi*, *Quercus pubescens*), 30.11.2002, 02MCF1208 (MG768901); 400 m a.s.l., Querco-Carpinetum orientalis, 20.03.2003, 03MCF836 (MG768902).

Extralimital collections: **SERBIA, Bosilegrad**: Mlekominci vill., Trlishte, 800 m a.s.l, 30.04.2005, 05MCF5344 (MG768903).

Remarks: *Tulostoma melanocyclum* is represented by small basidiomata with cylindrical ostiolum. Usually grows together with *T. brumale*, a similar species in macroscopic features, from which it differs by exoperidial structure, septa of capillitium, and spore ornamentation (Esqueda et al., 2004), as well as by the color of the peristom and stem, which in *T. melanocyclum* is dark brown, while in *T. brumale* is light brown (Tomaszewska et al., 2011). *Tulostoma melanocyclum* has been found in many European countries (Kreisel, 2001) and is a typical European species rather frequent in South and West Europe (Wright, 1987). In Poland, it is considered an endangered species, and according to Stasińska (2009) it is also found in xerothermic swards. It is included in the Red List of Wales, where it grows in sand dunes (Rotheroe, 2003), and Sweden (Gärdenfors, 2010), where populations in steppe habitats are reported to be in drastic decline (Hansson and Jeppson, 2005). In Macedonia it is found at five localities, in oak forests and riparian willow association, between 400 and 850 m altitude. It grows on humus, during March, April, June, and November.

5. *Tulostoma* cf. *moravecii* Pouzar (Figures 2d and 3d)

Mature *basidiomata* stipitate. Endoperidial body white to cream-colored, 1–2 cm in diameter. Mouth conical concolorous with endoperidium. Stipe cylindrical, 20–30 × 3–4 mm, light to dark brown, longitudinally furrowed. Gleba brown.

Basidiospores globose to subglobose, 4–5.4 µm in diam., verrucose under LM. SEM photos of spores show spherical to conical ornamentations (0.7–1.8 µm long) composed of different patches (like rosette but in opposite way), irregularly dispersed, forming furrowed veil structures on some parts (Figure 3d). Capillitium branched, 1.9–3.2 µm broad, septate.

Material studied (Figure 1, branch d). **Belasica**: Chalakli vill., 200 m a.s.l., *Juniperus excelsa* forest, 26.10.2007, 07MCF8292 (MG768879).

Remarks: *Tulostoma moravecii* is a European species described for the first time from the Czech Republic (Pouzar, 1958), where it is now probably extinct (Holec and Beran, 2006). Based on morphological studies, it is also reported from Austria, Hungary, Slovakia, and Spain (Kreisel, 2001). According to Jeppson et al. (2017), the sequences of isotype material of this species belong to *T. simulans*. There are only two collections from one locality in the southern part of Macedonia, growing in Greek juniper forest. The sequences from Macedonian collections do not fit with *T. simulans*, nor with other available sequences. Therefore, further analyses are needed for this specimen.

6. *Tulostoma punctatum* Peck (Figures 2f and 3f)

T. fimbriatum var. *punctatum* (Peck) J.E. Wright

Mature *basidiome* stipitate. Endoperidial body whitish, creamy to ochraceous, 9–14 mm in diameter, papery, spotted with very small sandy grains, forming a layer in its lower part, up to 4 mm; with short collar around the stipe. Mouth fimbriate, circular, ±plane, without brown ring zone, concolorous with endoperidium. Stipe cylindrical, shiny, creamy, light to dark brown, (30–)40–50 × 2–4 mm, slightly woody, with small adpressed dark brown squamules. Gleba light ocher to light brown.

Basidiospores globose, 3.5–4.8 µm in diam., slightly asperulate under LM, but with clearly expressed verrucae, mostly anastomosed in ridges (0.2–0.3 µm high), rarely single under SEM (Figure 3f). Capillitium branched, thick-walled, septate.

Material studied (Figure 1, branch f). **Kozjak** (Kumanovo): Zhegljane vill., 800 m a.s.l., *Pinus* plantation, 18.11.2002, 02MCF2913 (MG768881); **Zhedligovo** (Kumanovo): Dobroshane vill., 350 m a.s.l., mixed forest, 10.04.2005, 05MCF4866 (MG768882).

Remarks: Very similar to *Tulostoma fimbriatum*, from which it differs by spores that have densely anastomosed ridges seen by SEM.

7. *Tulostoma simulans* Lloyd (Figures 2a and 3a)

Mature basidiomata stipitate. Endoperidial body cream-colored, ochraceous to light brown, 9–20(–22) mm in diameter, smooth, papery, sometimes with very small agglutinated soil or sandy grains, especially in its lower part; with short collar projecting up to 2 mm around the stipe. Mouth circular, more or less conical, without brown ring zone, but sometimes with light orange brown apical region and in two samples (88MCF4705; 03MCF1395) with dark brown zone. Stipe cylindrical, concolorous with endoperidial body, (10–)20–30(–60) × (1–)2–3(–4) mm, slightly woody, with cream to light brown squamules. Gleba light ocher to light brown.

Basidiospores globose (3.5–)3.9–5.1 µm in diam., almost smooth to slightly asperulate under LM, but with clearly expressed conical and isolated warts under SEM, 0.3–0.6 µm long (Figure 3a). Capillitium branched, thick-walled, septate, 3.2–6 µm broad, (6–)7–9(–10) µm at septa.

Material studied (Figure 1, clade a). **Bistra**: Izvor vill., 500 m a.s.l., 23.03.2004, 04MCF3955 (MG768856); **Prespa**: Nakolec vill., 850 m a.s.l. Salicetum, 12.06.2003, 03MCF1395 (MG768853); Ezerani strict nature reserve, 900 m a.s.l. Salicetum, 01.05.2007, 07MCF6834 (MG768851); **Pogana**: Gjavato village, Gjurov Dol, 100 m a.s.l. Coccifero-Carpinetum orientalis, 17.12.1988, 88MCF4705 (MG768852); **Skopsko pole**: Gazi Baba, Botanical Garden, 250 m a.s.l., park, 18.10.2007, 07MCF8018 (MG768849); Hrom, 250 m a.s.l. yard, under planted *Picea* and *Cupressus*, 02.02.2007, 07MCF7983 (MG768850); Orman vill., *Quercus petraea* forest, 270 m a.s.l., 23.02.2008, 08MCF9079A (MG768854); **Zhedligovo**: Querco-Carpinetum orientalis, 20.03.2003, 03MCF3059 (MG768855).

Remarks: Collections under numbers 07MCF6834 and 03MCF1395, both from the vicinity of Prespa Lake, but from different locations, show more slender basidiomata, endoperidial body up to 6 mm in diameter, darker stem (without squamules), and endoperidium with net-forming soil incrustations.

As shown in Figure 1, our material was previously morphologically identified as *T. brumale*, *T. melanocyclum*, or *T. squamosum*; it was particularly impossible to identify *T. simulans* by morphology. As given in its first description by Lloyd (1906), and confirmed also by Jeppson et al. (2017) with sequence data, its name, *simulans*, points to its similarity to other species. The SEM photos of spores in Macedonian collections of *T. simulans* show the presence of rather dense warts, as indicated by Jeppson et al. (2017); the dimensions of the spores help to separate this species from *T. brumale*.

8. *Tulostoma squamosum* (J.F. Gmel.) Pers. (Figures 2m and 3m)

Mature *basidiomata* stipitate. Endoperidial body creamy, ochraceous, light orange to light brown, 10–16 mm in diameter. Mouth tubular with concolorous ring zone. Stipe cylindrical, 45–100 × 2–4 mm, brown, with dark brown scales. Gleba light ocher to light brown.

Basidiospores globose to subglobose, 5.3–6.2 µm in diam., echinulate under LM, with well-developed conical warts (0.6–1.1 µm high), anastomosed in parts, resembling small conical structures under SEM (Figure 3m). Capillitium branched, septate.

Material studied (Figure 1, clade m). **Gradishtanska Planina**: D'lga vill., 650–700 m a.s.l., Querco-Carpinetum orientalis, 12.03.2002, 03MCF1226 (MG768905); **Mangovica**: between Zubovce village and Shuplji Kamen village, 400 m a.s.l., Querco-Carpinetum orientalis, sandy soil, 15.04.2003, 03MCF1185 (MG768904); **Vodno**: 500–1000 m a.s.l., Querco-Carpinetum orientalis, 20.10.2002, 02MCF1372 (MG768907); **Zhegligovo** (Kumanovo): Staro Nagorichane vill., 600 m a.s.l., degraded habitat (*Paliurus spina christi*, *Quercus pubescens*), 30.11.2002, 02MCF332 (MG768906).

Remarks: *Tulostoma squamosum* is distinguished from other species in the genus by its robust stem with dark brown squamules, and cylindrical mouth. Sometimes, the name could be misapplied to *T. brumale* specimens; however, these two species differ in spore and stem morphology. *Tulostoma squamosum* is widely distributed, but uncommon in Europe, principally found in the south and west (Wright 1987). It is red-listed in Hungary and Sweden (Gärdenfors, 2010), but the Scandinavian material

formerly attributed to *T. squamosum* has been shown to be a distinct species that was described as a new species, *T. calcareum*, by Jeppson et al. (2017). In Poland it is a rare and protected species and represents an indicator for extremely thermophilous and dry grassland habitats (Tomaszewska et al., 2011). In Turkey it is known from Greek juniper and pine forests, where it grows on chalky soil (Sesli et al., 2000). In the European part of Russia it is noted as a Mediterranean species that is migrating to the northeast as a result of global warming (Shiryayev, 2009). In Macedonia it is known from several localities (Karadelev and Rusevska, 2009), mainly from the northern part of the country, where it grows on sandy soil, in oak and Greek juniper forests, or in meadows, during the winter, spring, or autumn. The morphology of our collections and their phylogenetic position confirm this species.

9. *Tulostoma subsquamosum* Long & S. Ahmad. (Figures 2n and 3n)

Mature *basidiomata* stipitate. Endoperidial body cinereous white to ochraceous, 9–11 mm in diameter, with soil encrustations on the whole surface, forming a layer in the lower part. Collar slightly developed. Mouth tubular or circular. Stipe cylindrical, 32–40 × 2–3 μm, with small light to dark brown squamules on a white background, slightly woody. Gleba light ocher to light brown.

Basidiospores usually globose, 4.1–5.4 μm in diam., verrucose to echinulate under LM, with conical, more rarely cylindrical, warts (0.5–0.8 μm long), anastomosed in ridges under SEM (Figure 3k). Capillitium branched, septate.

Material studied (Figure 1, clade n). **Belasica**: Chalakli vill., 200 m a.s.l., *Juniperus excelsa* forest, 26.10.2007, 07MCF8291 (MG768910); **Gradishtanska Planina**: Pavleshenci vill., 500–550 m a.s.l., oak forest, 11.2004, 04MCF7970 (MG768908); **Zhegligovo** (Kumanovo): Staro Nagorichane vill., 600 m a.s.l., at roadsides near arable land, 25.10.2005, 05MCF821 (MG768909).

Remarks: Samples in collection 05MCF821 differ by having a distinctly whitish endoperidial body (which refers it to *T. niveum*), almost without soil grains on the surface, but found only on its lower part; with circular mouth and well developed collar up to 1 mm; stipe shorter, 15–26 mm.

According to Wright (1987), *Tulostoma subsquamosum* is a critical species known from Asia and America, later also reported from Spain (Calonge and Wright, 1989). However, sequence data originating from collections from Spain, Hungary, and Slovakia (Jeppson et al., 2017) indicate that it is a distinct and well-defined species. The verrucae of the spores observed under SEM for one of the three collections (05MCF821) show typical "tendency to form a subreticulum", a microscopic feature reported by Jeppson et al. (2017). These authors placed it in the phylogenetic tree as a sister group of *T. squamosum*, which was confirmed by molecular analyses of Macedonian specimens (Figure 1).

10. *Tulostoma winterhoffii* H. Schub. & P. Specht (Figures 2h and 3h)

Mature basidiomata stipitate. Endoperidial body whitish, ochraceous, light gray to brownish, 11 mm in diameter, papery, without grains on the surface; with short dark brown collar around the stipe. Mouth fimbriate, plane, concolorous with endoperidium. Stipe cylindrical, 20 × 4 mm, scaly or woody, dark brown; slightly enlarged in the basal part. Gleba yellowish to light brown.

Basidiospores globose, 6–6.8 μm in diam., with isolated conical or cylindrical warts, 0.6–0.8 μm long, with tendency to form pyramids (Figure 3h). Capillitium branched, septate.

Material studied (Figure 1, branch h). **Vodno**: Gorno Sonje vill., *Pinus nigra* plantation, 21.10.2006, 06MCF6231B (MG768887).

Remarks: According to the phylogenetic position of the single sequence from our material, as well as the ornamentation of spores on SEM, this sample belongs to the new species, *T. winterhoffii*, proposed by Schubert and Specht (2015).

11. *Tulostoma* sp. 1, as *Tulostoma* sp. 13 in Jeppson et al. (2017) (Figures 2c and 3c)

Material studied (Figure 1, branch c). **Bistra**: Lazaropole vill., Cucul, 1300 m a.s.l., rocky place, soil, among grass, 07.04.2007, 07MCF9359 (MG768878).

Remarks: The sequence obtained from specimen 07MCF9359 fit 100% with the sequence under *Tulostoma* sp. 13 of Jeppson et al. (2017), collected from New Mexico, that morphologically fits with *T. excentricum*. More specimens should be located and described to identify these specimens to the species level.

12. *Tulostoma* sp. 2 (Figures 2e and 3e)

Material studied (Figure 1, branch e). **Taorska Klisura and Badar**: St. Jovan Veterski monastery, 150–200 m a.s.l., meadow in *Juniperus excelsa* forest, 23.10.2002, 02MCF1203 (MG768880).

Remarks: In our tree, the sequence obtained from specimen 02MCF1203 is close to *T. kotlabae* (DQ112629), and in the BLAST search it has 91% similarity with sequence KU18973 under *Tulostoma fimbriatum* var. *punctatum*. The dark brown stipe in specimen 02MCF1203 is a feature that does not agree with the description of *T. kotlabae* in the literature (e.g., whitish, creamy, or grayish, according to Wright, 1987; Knudsen and Vesterholt, 2012), and irregular warts on spores do not fit with almost clear verrucae in *T. punctatum*. More specimens should be located to describe and to identify to the species level.

13. *Tulostoma* sp. 3 (Figures 2j and 3j)

Material studied (Figure 1, clade j). **Tikvesh**: Stobi, 150-200 m a.s.l., meadow, sandy soil, 30.12.2003,

03MCF3646 (MG768898); **Zhegligovo** (Kumanovo): beside r. Pchinja, Staro Nagorichane vill., 550 m a.s.l., Querco-Carpinetum orientalis, sandy soil, 18.11.2003, 03MCF2939 (MG768897).

Remarks: According to our molecular analyses (Figure 1, clade j and k), it is close to *Tulostoma* sp. 4. Both species, *Tulostoma* sp. 3 and 4, form highly supported sister branches (PP = 1.00; BS = 100%), but according to BLAST results, *Tulostoma* sp. 3 is closer to T. *winterhoffii* (91%) and *Tulostoma* sp. 4 to *T. rufum* (90%). More specimens should be located to describe in order to identify to the species level.

14. *Tulostoma* sp. 4 (Figures 2k and 3k)

Material studied (Figure 1, branch k). **Belasica**: Chalakli vill., 200 m a.s.l., *Juniperus excelsa* forest, 18.11.2003, 03MCF10327 (MG768899).

Habitat: Oak-hornbeam; Greek juniper forest, and meadow.

Remarks: See remarks under *Tulostoma* sp. 3.

Excluded species

Tulostoma caespitosum Trab. apud Sacc.

There is only one collection for *T. caespitosum* in Macedonia from Wright (1987). Research conducted to date has not yet confirmed the existence of this species.

4. Discussion

In the genus *Tulostoma*, as in other gasteroid fungi (Kruger et al., 2001; Martín et al., 2013; Phosri et al., 2013; Rusevska et al., 2015), the barcoding region is very useful to discriminate species, mainly when the morphological features are very similar, such as a not clearly defined mouth, difficulty in defining the structure of the exoperidium, or indistinguishable spore ornamentation even under SEM. In general, such as in Jeppson et al. (2017), terminal clades are highly supported for *T. fimbriatum*, *T. wintherhoffii*, or the clade of *T. melanocyclum*, *T. squamosum*, and *T. subsquamosum*. Barcoding sequences also help in identification of the species *Tulostoma brumale* in some collections, when a brown ring zone around the mouth is absent or not clearly defined, or *T. simulans*, which also is described as having a brown mouth zone (Jeppson et al., 2017). However, as indicated by many authors (Schoch et al., 2012; Jeppson et al., 2013; Martín et al., 2015; Sousa et al., 2017), the ITS marker alone is not enough to infer the phylogenetic relationship among species, and it is necessary to study at least two or three markers to clearly delimitate the taxa (such as Tef 1a used in the analyses by Jeppson et al. (2017), *RPB*2, or translation elongation factor 1-alpha). Moreover, in our study more collections should be found and studied to define *Tulostoma* sp. 1 (*Tulostoma* sp. 13 in Jeppson et al., 2017), *Tulostoma* sp. 2, *Tulostoma* sp. 3, and *Tulostoma* sp. 4, with additional analyses, both at morphological and molecular levels. *T. caespitosum* is a species that has been cited for Macedonia, but we have not found it again.

Our work shows that the ITS can be used in combination with morphological characteristics to resolve taxonomic uncertainties among the genus *Tulostoma*. The analyses from our research, based on morphological, ecological, and phylogenetic data of 62 sequences belonging to five previously identified *Tulostoma* species, show the presence of at least 14 different taxa. Thus, we can confirm a higher than expected diversity of this genus in Macedonia, as also pointed out recently for some other European countries (Jeppson et al., 2017).

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