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Tricholoma punctatum sp. nov. from Serbia

Nebojša Lukić[1], Eleonora Čapelja[2], Maja Karaman[2]

[1] *Mycological Society "Šumadija"*
Daničićeva 120/36,34000 Kragujevac, Serbia
[2] *University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology*
Trg Dositeja Obradovića 2, 21000 Novi Sad, Serbia

*Correspondence to: *eleonora.capelja@dbe.uns.ac.rs*

Abstract–A new species of *Tricholoma* recorded from a temperate forest in central Serbia is described and illustrated. It is characterized by the presence of punctate, often elevated, almost black spots arranged in one to three circular zones on the pileus. The new species showed morphological differences from closely related taxa and molecular phylogenetic analysis of ITS and LSU regions confirm the distinct taxonomic status of the finding.

Key words: *Atrosquamosa*, *Tricholomataceae*, taxonomy, fungal diversity

Introduction

Tricholoma (Fr.) Staude (*Tricholomataceae*, *Agaricales*) is a cosmopolitan genus, mainly distributed in temperate and subtropical zones of both hemispheres (Tedersoo & al. 2010). The majority of *Tricholoma* species are described from North America where more than one hundred species have been reported so far, and this region appears to be the centre of species richness (Bessette & al. 2013). There are about ninety species listed from Europe (Bon 1991, Riva 1988, 1998, 2003; Kibby 2010), but the overall diversity of *Tricholoma* taxa in Europe is still poorly resolved.

Serbia presents an exceptional place for mycological research due to the presence of various habitats: thermophilous oak forest at lower altitudes (usually *Quercus cerris* L.); succeeded by mesophilous beech and coniferous forests (usually *Fagus sylvatica* L.; *Picea abies* (L.) H. Karst.; *Abies alba* Mill.) in higher altitudes. Pine forests can be sporadically found at all altitudes. Although they are some published data about diversity of *Agaricomycetes* in Serbia (Čolić 1967, Karaman & al. 2005, Karadžić & Milijašević 2008, Lukić 2009, 2013; Ivančević & Davidović 2011, Sadiković & al. 2012), a thorough and systematic investigation of this group of fungi is still lacking. *Tricholoma* is quite frequent in Serbia (Uzelac 2009), with basidiomes often appear in late October and November in thermophilous deciduous forests. Since *Tricholoma* species show limited microscopic variation, and are characterized by hyaline, subglobose to oblong spores, simple pileipellis structures, and lack of well-differentiated sterile elements (e.g., cystidia), species identification and partly also the infrageneric classification have mainly been based on macromorphology. In a recent work, the combination of morphological and molecular data showed that characters such as pileus color, pileipellis structure, presence of clamp connections and spore size seem to be rather conserved within accepted sections, while the presence of a distinct ring, and especially host specificity were highly variable (Heilmann-Clausen & al. 2017).

The first comprehensive molecular work on European *Tricholoma* (Heilman-Clausen & al. 2017) revealed ten clades/sections which were not completely in agreement with Singer's classification. Also, they considered European species of sections *Caligata*, *Atrosquamosa*, and *Terrea* to be well evaluated taxonomically, while for all other sections their sampling was limited and are in need for further phylogenetic studies. Heilman-Clausen & al. (2017) discovered several clades with unassigned names, and one of them included two European species *T. josserandii* Bon and *T. fucatum* (Fr.) P. Kumm., taxa that were grouped together with North and Central American species described and mentioned in study by Ovrebo & al. (2019). A phylogenetic analysis by Reschke & al. (2018) of *Tricholoma* collections from Asia, Europe, and North America revealed major clades that were similar to those presented by Christensen & Heilman-Clausen (2013). Also, sequences of *T. imbricatum* (Fr.) P. Kumm. specimens from USA formed a highly supported clade that was separated from the Asian/European *T. imbricatum* clade. Additionally, *T. aurantiipes* Hongo, *T. davisiae* Peck, *T. muscarium* Kawam. ex Hongo, and *T. muscarioides* Reschke & al. comprised a clade that was formally described as a new section *Muscaria*. All of the preceding analyses were based only on ITS sequences, which has proved to be a stable marker for species delimitations; but this region cannot resolve higher taxonomic relationships at a sufficiently detailed level (Frøslev & al. 2005). Infrageneric classification proposed in previous studies should be viewed as preliminary and further studies including multiple molecular markers should be conducted. Here we present Serbian specimens with unique morphological characters differing from previous *Tricholoma* species recorded from Europe (Riva 1988, 1998, 2003); and phylogenetic analyses based on ITS and LSU confirm these specimens as a new species, proposed here as *T. punctatum*.

Material and methods

Studied material

Specimens studied for this paper were collected by the first author during field trips in central Serbia during October–November 2020. Nearly one hundred basidiomes were recorded during three visits at the defined location (close to the city of Kragujevac, 44.0181°N 20.8839°E, altitude 240 m). The habitat of the newly proposed *Tricholoma* species (widely observed) is an old oak forest (mostly *Quercus robur* L. and *Q. cerris*) with several other interspersed trees (*Pinus nigra* J.F. Arnold, *P. sylvestris* L., *Carpinus betulus* L., *Tilia cordata* Mill., *Populus alba* L., *Betula pendula* Roth) within the Memorial area of Šumarice near the city of Kragujevac with an approximate area of 350 ha. By virtue of the forest age and absence of economic exploitation the defined area represents a very valuable mycological habitat. During twenty years of research in this area twenty-one *Tricholoma* species (and several varieties and forms) have been found mostly within sections *Genuina, Tricholoma, Atrosquamosa,* and *Terrea* (Lukić & al., unpublished results).

Basidiomes were photographed in their natural habitat, at the area of their appearance. Morphological data were recorded from the fresh specimens, which were collected within a period of two months, on three occasions. Color codes follow the Munsell soil color charts (Munsell 1975). Two collections (about ten specimens in each collection) were dried by natural ventilation in the shadow and were preserved in sealed glass containers. Microscopic characters were observed under a Motic SFC 28 microscope (Germany), measured using an ocular micrometer, and recorded by a BCAM3 camera (Germany). The fresh sections of basidiomes were stained by Congo red. Basidiospore dimensions were recorded without 5% of the largest and 5% of the smallest measured values and Q indicates the length/width ratio of the spores. Measurements of the other microscopic characters are given as ranges. The voucher specimens have been deposited in the Fungarium at BUNS Herbaria, Department of Biology and Ecology, University of Novi Sad, Novi Sad, Serbia (BUNS).

Molecular & phylogenetic analysis

Total DNA was extracted from dry specimens employing a modified protocol based on Murray & Thompson (1980). Amplification of ITS and LSU regions was performed under following conditions: 2 min denaturation at 95°C, followed by 35 amplification cycles, each consisting of 30 s denaturation at 95°C, a 30 s annealing step at 54°C, and 1 min extension at 72°C; the final step included 10 min incubation at 72°C. The primers ITS1F and ITS4B (White & al. 1990, Gardes & Bruns 1993) were employed toamplify the ITS rDNA region, while LR0R and LR5 (Gardes & Bruns 1993, Vilgalys

& Hester 1990) were used for the 28S rDNA region. PCR products were checked in 1% agarose gels, and amplicons weresequenced with one or both PCR primers. Sequence chromatograms were inspected and manually edited with FinchTV 1.5.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com). Newly generated ITS and LSU sequences were deposited in GenBank.

Additional sequences were retrieved from GenBank or UNITE database: for ITS, 26 *Tricholoma* sequences from 15 taxa, with a *Clitocybe* outgroup sequence; and for LSU, 19 *Tricholoma* sequences from 13 taxa, with a *Ganoderma* outgroup sequence. A separate phylogenetic analysis was performed on each of the gene regions. Alignments for both analyses were carried out with MUSCLE 3.7 (Edgar 2004) using the default settings. Substitution models were selected using the ModelTest-NG v0.1.6 (Darriba & al. 2020). GTR + I + G (General time reversible model + Proportion of invariant + Gamma) model was chosen for ITS datasets while K80+I+G (Kimura 2-parameter model + Proportion of invariant + Gamma) was chosen for LSU dataset. Maximum Likelihood (ML) analyses were performed in MEGA X (Kumar & al. 2018) with the number of bootstraps set to 1000. MUSCLE 3.7andModelTest-NG v0.1.6 were used through CIPRES Science Gateway (Miller & al. 2010). The alignments and phylogenetic trees were deposited in Treebase.

Taxonomy

Tricholoma punctatum Lukić, **sp. nov.** FIGS 1–3

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Basidiomes of *T. punctatum* differ by the presence of dark colored, elevated, circularly arranged spots on the lobed gray pileus.

TYPE: Serbia, Šumadija, Kragujevac, "Memorial complex of Sumarice", 240 m a.s.l., on soil in mixed thermophilous deciduous forest (*Quercus robur*, *Q. cerris*, *Carpinus betulus*), October 2020, Nebojša Lukić (**Holotype,** BUNS 12-00766; GenBank OK631797, OL310859).

ETYMOLOGY: The specific epithet refers to the distinctly spotted surface of pilei.

PILEUS: 50–110 mm, at first convex, becoming flattened with broad, low umbo. Fully developed pileus is often irregularly circular, lobed, with wavy margin (FIG. 1). Surface is densely felt, then breaking up in small fine scales. Pileipellis can be separated easily by half of the pileus radius. Color is gray [N7/0-N4/0], grey-brown [5YR6/4-5YR7/3] with lighter margin zone (FIG. 1 A–C). it is characterized by punctate, almost black spots (more or less pronounced) placed in one, usually two or even three circular zones at half and at 3/4 of the pileus radius (FIG. 1 A, C, D). These punctate black-colored spots are often elevated and can be removed by nail quite easily. LAMELLAE strongly sinuate (clearly expressed, FIG. 1 B, C), rather broad, quite thick and deep, whitish [N9/0], then pale grey [N7/0], not spotted. Intermediate lamellae numerous. The number of

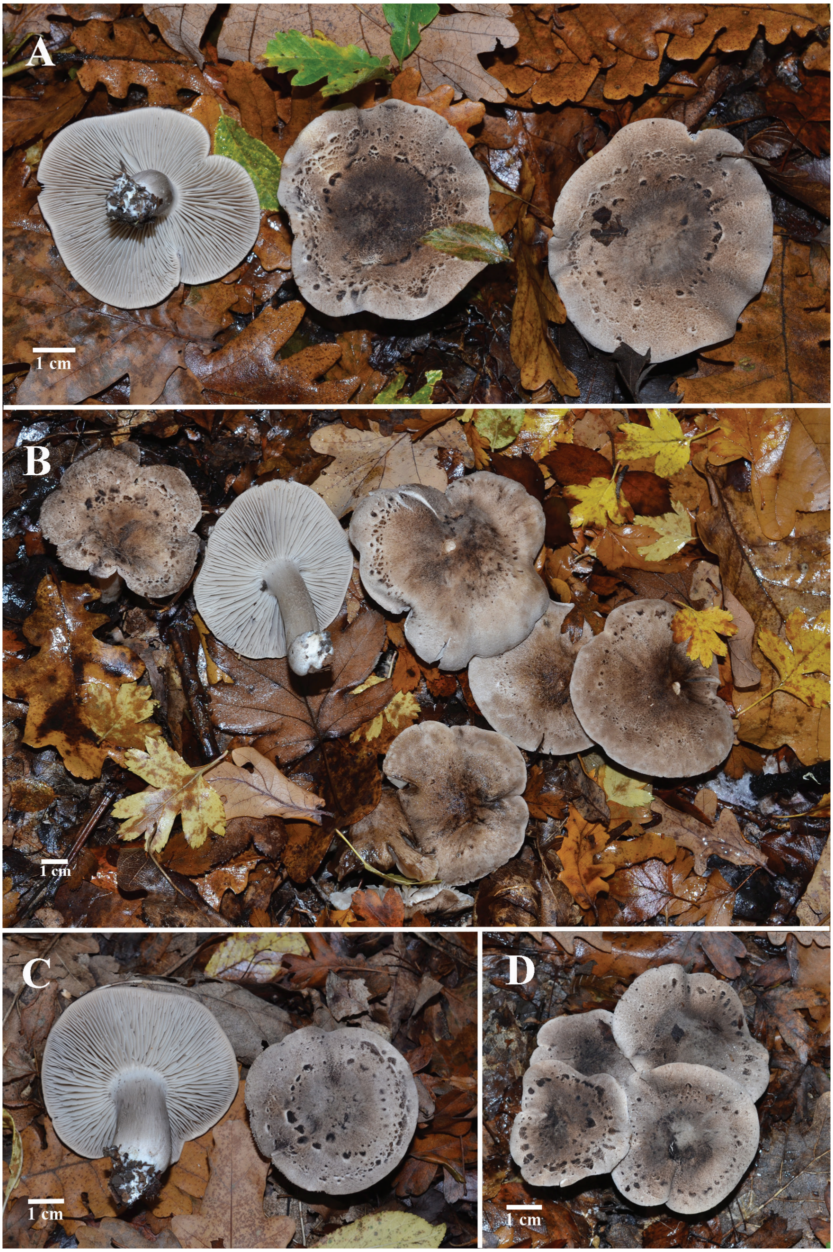

Fig. 1. *Tricholoma punctatum*: basidiocarps in situ.

those half-length lamellae is nearly equal to the number of ordinary lamellae. Edges are entire and wavy. Color changes are not observed. STIPE: 40–90 × 10–20 mm, robust and firm, cylindrical but not so rare flattened or with vertical grooves, widened at apex, tapering at base, white on ends, rough and covered for the most part with gray [N6/0] or gray-brown fibers (FIG. 1, B, C). No cobweb ring zone observed. Basal mycelium whitish. FLESH: white then faintly gray. ODOR: clearly farinaceous, strong after cutting. TASTE: farinaceous without bitter component.

BASIDIOSPORES: 4.6–5.1 × 3.6–4.1 µm (60 spores, from 3 basidiomes), subglobose to ellipsoid, Q = 1.20–1.35 (FIG. 2 A). BASIDIA: 25–35 × 3.5–6µm, mainly 4-spored (FIG. 2 B). PILEIPELLIS: a cutis, breaking upon trichoderm scales (FIG. 2 C); individual hyphal elements generally 33–75 × 7–14µm; subpellis poorly differentiated; mainly globose inflated cells 20-30 µm in diameter (fully developed), present as separated clusters in punctate colored elevated fibers within pileipellis (FIGS 2 and 3). CLAMP-CONNECTIONS: absent.

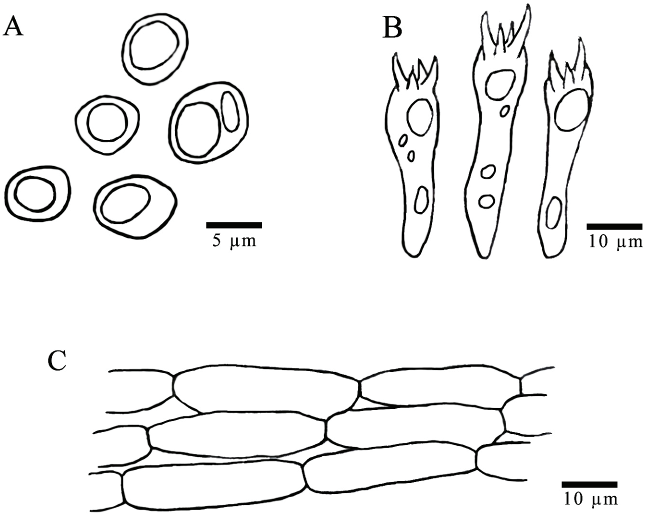

FIG. 2. *Tricholoma punctatum* (holotype BUNS 12-00766): A. basidiospores; B. basidia; C. pileipellis.

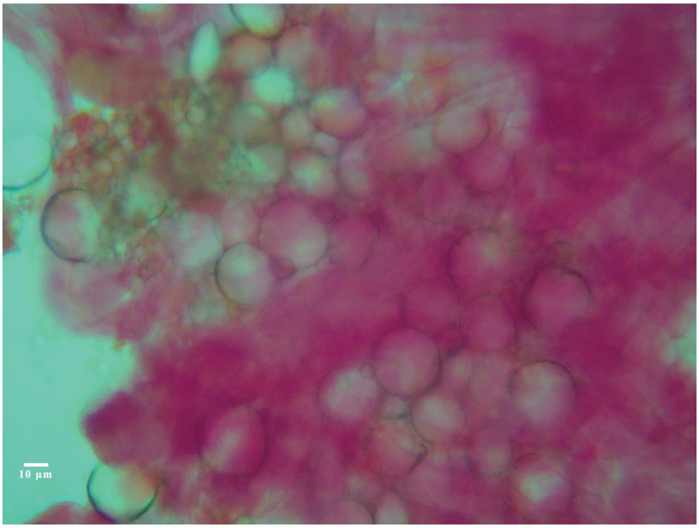

Fig. 3. *Tricholoma punctatum* (holotype BUNS 12-00766): globose inflated cells in punctate colored areas within pileipellis.

Additional specimen examined: **SERBIA, Šumadija, Kragujevac**, "Memorial complex of Sumarice", 240 m a.s.l., on soil in mixed thermophilous deciduous forest (*Quercus robur*, *Q. cerris*, *Carpinus betulus*), October 2020, Nebojša Lukić (BUNS 12-00782, GenBank ON598607)

Habitat & ecology: Basidiomes solitary or often in small groups. Habitat is a mixed thermophilous deciduous forest (*Quercus robur*, *Q. cerris*, *Carpinus betulus*) overgrown with *Crataegus monogyna*, *Prunus spinosa*, and *P. cerasifera*, on neutral or slightly acidic clayey soil.

Key to *Tricholoma* sect. *Atrosquamosa* Kühner & Romagn.

Cap greyish to almost black but also greyish-brown, densely squamulose-fibrillose, stem often squamulose too, smell different but farinaceous after cutting, spores medium sized.

1. Stem white, more or less shiny, often with weakly expressed, scattered blackish scales, smell of uncut fruit bodies sweetish 2

1. Stem white to greyish, with a dense covering of darker fibrils (or scales), smell of uncut fruit bodies spicy or farinaceous 3

2. Basal mycelium yellow, cap mostly fibrillose, especially gills and cap margin reddening when old, in deciduous forests, mostly *Fagus* and *Quercus* .... ***T. orirubens***

2. Basal mycelium white; cap mostly with dark (black) scales, with conifers, but also with *Tilia* and *Corylus* .... ***T. atrosquamosum***

3. In *Picea* forests (Fennoscandia), cap often with an olivaceous tinge, especially at margin .... ***T. olivaceotinctum***
3 In thermophilous deciduous forests on clayey or calcareous soil, cap without olivaceous tinge 4
4. Stem less fibrillose or squamulose, widened stem base strongly reddening .... ***T. basirubens***
4.Stem base not reddening 5
5. Cap margin woolly in young fruit bodies, cap strongly squamulose .... ***T. squarrulosum***
5 Cap with conspicuous darker spots mainly arranged in two circular zones and often lobed, cap margin paler, spores mostly subglobose .... ***T. punctatum***

Phylogenetic results

Newly generated ITS and LSU sequences of *Tricholoma punctatum* were approximately 780 bp and 750 bp long, respectively. BLAST sequence analysis showed maximum similarity with *T. orirubens* AMB 17410 (MT462641) for ITS sequence, while LSU sequence of *T. punctatum* showed maximum sequence match with *T. sulphurescens* G0243 (MK278617) and *T. orirubens* (MK278611) with exactly the same scores. Phylogenetic analysis of ITS (Fig. 4) and LSU (Fig. 5) datasets shows that ITS and LSU sequences of *T. punctatum* generated in this study formed their own lineage within the clade corresponding to sect. *Atrosquamosa*. In the analysis of ITS sequences *T. punctatum* was shown as sister clade of *T. orirubens,* while in the LSU tree *T. punctatum* is placed within the *T. orirubens* clade.

Discussion

Morphologically, the main distinguishing feature of *Tricholoma punctatum* is the pileus appearance and the presence of the globose inflated cells in punctate black-colored are as in pileipellis (Fig. 1). These characters are similar to those of *Tricholoma rufenum* P. Donati which possesses drop-like spots on the pileus. However, the pileus of *T. rufenum* is smooth, shiny and slightly greasy (similar to *T. portentosum* (Fr.) Quél.) (Riva 1998). In addition, *T. rufenum* has bigger spores (5.5–7.2 × 4.6–5.2 µm; Riva 2003) than *T. punctatum* and a white stipe. The drop-like spots are as smooth as the whole pileus surface, in contrast to the elevated spots of *T. punctatum*. Similar smooth drop-like spots appear on the pileus of *T. viridilutescens* M.M. Moser native to Europe. Similar pileus appearance can be also found in the *T. scalpturatum* var. *atrocinctum* Romagn. but the aforementioned feature is not constant (Heap 2020).

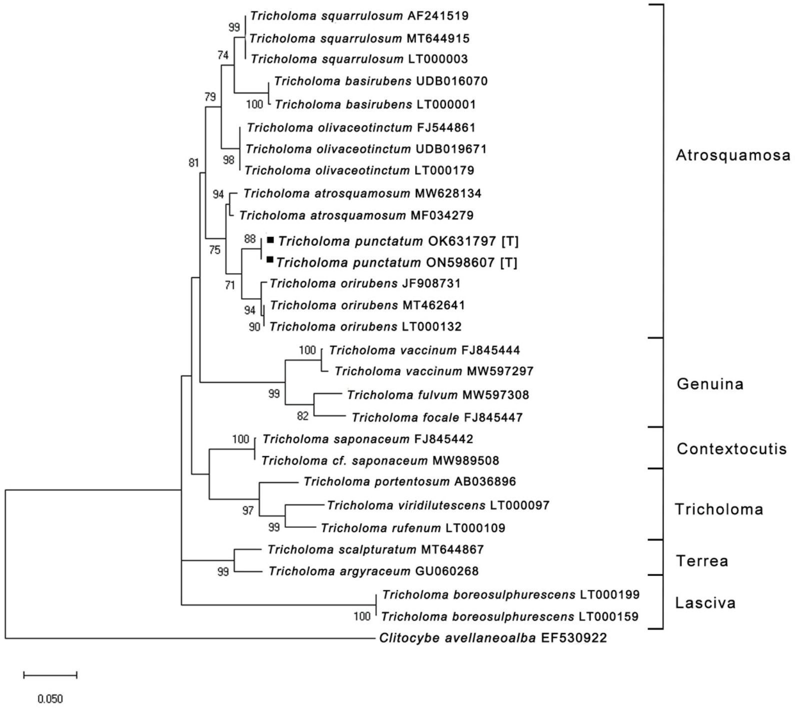

FIG. 4. Maximum likelihood tree inferred from the ITS sequences of *Tricholoma* species, with a *Clitocybe avellaneoalba* outgroup. Bootstrap values ≥50%, based on 1000 replicates are shown at the branch nodes. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 28 *Tricholoma* nucleotide sequences. There were 914 positions in the final dataset.

Besides the particular pileus appearance, *T. punctatum* shows morphologically similar features with *T. orirubens* Quél., *T. atrosquamosum* Sacc., *T. squarrulosum* Bres. and *T. scalpturatum* (Fr.) Quél.

Unlike *T. punctatum*, *T. orirubens* has greenish yellow basal mycelium (Christensen & Heilmann-Clausen 2013), reddening lamellae (mainly in mature stage), white stipe and slightly bigger (and more elongated) spores. When compared with *T. punctatum*, *T. atrosquamosum* has dark gray to black pileus (no brown shade; Riva 1988), blackish spotted gills, white, shiny stem with blackish floccules or green (or red) spots at base, different smell of pepper (Baccardo & al. 2008), as well as distinct habitat and mycorrhizal partners and bigger and more elongated spores (Courtecuisse 1999). *Tricholoma atrosquamosum* is found in North America and Europe, but is generally

Fig. 5. Maximum likelihood tree inferred from the LSU sequences of *Tricholoma* taxa, with a *Ganoderma adspersum* outgroup. Bootstrap values ≥50%, based on 1000 replicates are shown at the branch nodes. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 20 *Tricholoma* nucleotide sequences. There were 762 positions in the final dataset.

recognized as rare in Europe and in danger of extinction in the Netherlands (Noordeloos & Christensen 1999). Basidiomes appear under deciduous and coniferous trees, particularly beech and spruce on calcareous soils.

Tricholoma squarrulosum is smaller (Moser 1983, Phillips 2006), with dense black squamules on pileus with whitish woolly margin and strongly blackish squamulose stipe (Kibby 2010) while *T. punctatum* has grey fibrillose stem. *Tricholoma squarrulosum* also has different smell and bigger and more elongated spores (Q =1.35–1.72) (Christensen & Heilmann-Clausen 2013). *Tricholoma squarrulosum* is also rare (though more widely distributed in southern Europe), and associated with oak, pine, and spruce on chalk soils, with its basidiomes appearing from September to November.

Furthermore, comparing to *T. punctatum,* the species *T. scalpturatum* is smaller in size (Courtecuisse & Duhem 1995), with yellowing lamellae (Galli 2003), white or grey smooth stipe (sometimes with cobweb ring zone) and more elongated spores (Q = 1.4–1.54) (Christensen & Heilmann-Clausen 2013). Likewise, it should be emphasized that new species *T. punctatum* usually have lobed pileus and deep, strongly sinuate lamellae.

Beside the morphological differences between *T. punctatum* and related species, the former species showed differences on molecular level as well, which can be clearly seen from the phylogenetic tree obtained in analysis of ITS sequences (Fig. 4). *T. punctatum* sequence was grouped within the *Atrosquamosa* clade and placed as sister clade of *T. orirubens*. Contrary to this, in the LSU tree (Fig. 5) *T. punctatum* was placed within the *T. orirubens* clade. These results may be due to the fact that LSU is more conserved among species and have less clearly defined barcode gap than ITS (Schoch & al. 2012). Grouping of species in clades/sections in both analyses were in accordance with data of Heilmann-Clausen & al. (2017).

Conclusion

According to the data presented, *Tricholoma punctatum* belongs to section *Atrosquamosa*, which is one of ten proposed *Tricholoma* sections based on molecular and morphological data (Christensen & Heilmann-Clausen 2013). It is characterized by constant morphological features such are pileus color, pileipellis structure, absence of clamp connections and spores size and shape. This section is amongst those (sections *Tricholoma*, *Atrosquamosa*, *Pardinicutis*, and *Terrea*) which include species with a greyish, radially fibrillose, squamulose to felty pileus (Christensen & Heilmann-Clausen 2013). The main distinguishing characteristic of our new species is the presence of globose inflated cells in punctate areas of the pileus, and of the dark colored, elevated, circularly placed (in one to three tiers) spots on the cap. In addition, other characteristics that separate *T. punctatum* from similar species, *T. orirubens*, *T. atrosquamosum, T. squarrulosum,* and *T. scalpturatum*, are the size and shape of spores, fibrillose stipe, lobed pileus, strongly sinuate and deep gills. Furthermore, molecular data based on newly generated ITS sequences provided evidence supporting the distinct taxonomic status of *T. punctatum*.

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