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TRICHOLOMA USTALOIDES (AGARICALES, BASIDIOMYCOTA) IN POLAND

Marek Halama[1], Rafał Ważny, Matylda Czosnykowska-Łukacka & Tadeusz Dobosz

Abstract. The current distribution of *Tricholoma ustaloides* Romagn. in Poland is presented, based on literature data and the results of mycological investigations in oak-hornbeam forest associations in the Kotlina Raciborska basin. The paper reports the morphology of the discovered basidiomata, the habitat where they were found, and ITS sequence data for the specimens, and briefly discusses similar species and the ecology of the presented fungus. The location given in the paper is currently the only known station of *T. ustaloides* in Poland. The importance of secondary habitats for survival of this taxon is stressed.

Key words: chorology, ectomycorrhizal fungi, ITS sequence data, micromorphology, Poland

Marek Halama, Museum of Natural History, Wrocław University, H. Sienkiewicza 21, 50-335 Wrocław, Poland; e-mail: marek.halama@uwr.edu.pl
Rafał Ważny, Małopolska Centre of Biotechnology, Jagiellonian University, Gronostajowa 7a, 30-387 Kraków, Poland
Matylda Czosnykowska-Łukacka & Tadeusz Dobosz, Molecular Techniques Unit, Department of Forensic Medicine, Wrocław Medical University, M. Curie-Skłodowskiej 52, 50-369 Wrocław, Poland

Introduction

The ectomycorrhizal genus *Tricholoma* (Fr.: Fr.) Staude comprises *ca* 300 species worldwide (Noordeloos & Christensen 1999; Christensen & Heilmann-Clausen 2013). These agarics are generally symbiotic with forest trees of the families Betulaceae, Fagaceae, Salicaceae and Pinaceae. In addition to the *Tricholoma* species that have a broad spectrum of partners, many apparently are limited to a particular host tree genus (Trappe 1962; Christensen & Heilmann-Clausen 2013). There is only one euagarics clade of Agaricomycotina. The systematic unit Agaricomycotina (Hymenomycetes), is one of three taxa of the fungal division Basidiomycota. The euagarics clade includes forms with lamellate basidiocarps formerly classified in the Agaricales, but also forms with other hymenial configurations. Molecular phylogenetic studies have revealed a monophyly of *Tricholoma* (*s.str*.) within the euagarics clade of Agaricomycotina (Moncalvo *et al.* 2002; Sánchez-García *et al.* 2014). In Europe *ca* 70 *Tricholoma* species are known, and in Poland no fewer than 50 members of this genus have been found (Gminder & Krieglsteiner 2001; Riva 2003; Wojewoda 2003). *Tricholoma ustaloides* Romagn. has not been recorded in Poland previously (Wojewoda 2003). A geographical distribution map of *T. ustaloides* in Europe (Christensen & Heilmann-Clausen 2013) indicated its occurrence in Poland but without giving the source of the record or the exact location. The present paper describes the first collections of the species in Poland, made in the Łężczak Reserve in 2014. The species was identified on the basis of morphological analysis and ITS rDNA sequence data.

Material and methods

morphological study

Macroscopic features were studied from fresh material of four collections comprising 20 basidiomata in different stages of development. All microscopic structures were observed in dried material. Freehand sections of

[1] Corresponding author

rehydrated pieces of basidiomata were examined in 5% $NH_3 \cdot H_2O$ reagent with 1% Phloxine B. Image-grabbing and biometric analyses were done with NIS-Elements D 3.1 imaging software. Dimensions of microcharacters are given as (minimum) average ± standard deviation (maximum), and additionally as main data range (10–90 percentile values). The Q value is the length/width ratio of basidiospores. For basidiospores size measurements, randomly selected mature spores were measured without the hilar appendix. The length of basidia was measured excluding sterigmata. Micrographs were taken using a Nikon digital camera (DS-Fi1) digital camera. Statistical computations employed Statistica (StatSoft). Morphological terminology follows Vellinga (1988) and Vellinga and Noordeloos (1999). The studied collections are deposited in the Museum of Natural History, Wrocław University, Poland (WRSL).

MOLECULAR PROCEDURES

The fungal mycelium was isolated from the basidiocarp into pure cultures. DNA was extracted from mycelium samples with a Genomic Mini AX Plant Kit (A&A Biotechnology) according to the manufacturer's instructions. Prior to sequencing, the ITS rDNA region (ITS1+5.8S+ITS2) was amplified using ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) primers. PCR conditions were optimized for the Maxima Hot Start Green PCR Master Mix (Thermo Scientific). Each reaction contained 10–100 μg/μl DNA, 9.5 μl nuclease-free water, 12.5 μl Master Mix and 1 μg 10 pmol of each primer. The total volume of each reaction was 25 μl. The PCR parameters were as follows: initial denaturation at 95°C for 3 min, then denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. This cycle was repeated 35 times. The final extension was carried out at 72°C for 5 min. The PCR products were visualized by agarose electrophoresis. The ITS rDNA amplicons were sequenced with ITS4 primer by the Laboratory of Molecular Biology of Adam Mickiewicz University in Poznań (Poland). All obtained sequences were analyzed by Chromas (www.technelysium.com.au) and compared with the sequences deposited in the UNITE and NCBI databases using the BLASTn algorithm.

RESULTS

Tricholoma ustaloides Romagn. Figs 1 & 2

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Fig. 1. Known distribution of *Tricholoma ustaloides* Romagn. in Poland.

Basidiomata solitary, gregarious or subfasciculate. Pileus 20–100 mm, initially hemispherical to broadly campanulate, with strongly involute margin, then convex with low umbo, after which plano-convex, smooth or subsquamulose at the center in some basidiomata, margin somewhat lobed, involute, often sulcate in old basidiomata, orange brown to dark brick, sometimes with slightly paler margin, strongly glutinous-viscid when moist, ± sticky when dry. Lamellae, L = 50–90, l = 2–5, moderately crowded, narrowly adnate to emarginate, subventricose to ventricose, white to cream when fresh, often with dark brown stains and spots near edges when old or bruised, with entire, concolorous edge. Stipe 30–100 × 10–25 mm, fusiform, tapering upwards, subclavate, rooting, sometimes ± swollen to bulbous towards base, smooth to fibrillose, rarely with appressed scales, generally dark pinkish buff to dark brick, usually at the top with rather distinctly and sharply delimited, 5–10 mm broad, white and almost smooth zone. Context whitish, often with brownish tinges near surface. Smell after cutting strongly farinaceous. Taste farinaceous and distinctly bitter, particularly the pileipellis. Basidiospores (4.5) 5.5 ± 0.4 (7.5) × (4.0) 4.5 ± 0.3 (5.5) μm, 5.0–6.0 × 4.5–5.0 μm, Q = (1.1) 1.2 ± 0.1 (1.4), Q = 1.1–1.3, n = 188, subglobose to ellipsoid, with distinct hilar appendage (predominantly broadly ellipsoid), smooth, thin-walled. Basidia (26.5) 33.0 ± 3.3 (41.0) × (6.5) 7.5 ± 0.4

Fig. 2. *Tricholoma ustaloides* Romagn. A – basidiospores, B – basidia, D – top, side and bottom views of basidiomata (all photographed from WRSL MH-2014-0284).

(8.5) μm, 29.5–37.5 × 7.0–8.0 μm, n = 86, narrowly clavate, 2–4-spored. No cystidia observed. Pileipellis an ixocutis to an ixotrichoderm, made up of cylindrical hyphae embedded in gelatinous layer, 2.5–5.5 μm wide, with subclavate terminal elements. Pigment membranal and incrusting, mainly in suprapellis, also brown, intracellular in some terminal elements of pileipellis. No clamp-connections seen.

Specimens examined: POLAND. Silesian Lowlands, Kotlina Raciborska basin (Łężczak Reserve), on dam (between Babiczak Północny and Salm Duży ponds) – on Oak Alley: 1 – gregarious or subfasciculate on soil (raw humus) under *Carpinus betulus* L., *Crataegus* sp., *Malus sylvestris* Mill., *Quercus* sp. and *Tilia cordata* Mill., alt. 179 m a.s.l., 50°08′27″N, 18°16′57″E, 14 Sept. 2014, *leg. M. Halama* (WRSL MH-2014-0284), 2 – solitary, gregarious or subfasciculate on soil (humus covered by broadleaved litter) under *Crataegus* sp., *Quercus* sp. and *Prunus spinosa* L., alt. 179 m a.s.l., 50°08′27″N, 18°16′56″E, 14 Sept. 2014, *leg. M. Halama* (WRSL MH-2014-0285), 3 – solitary, gregarious or subfasciculate on soil (humus covered by broadleaved litter and humus under herbaceous plants) under *Quercus* sp. and *Tilia cordata*, alt. 179 m a.s.l., 50°08′2.8″N, 18°16′54″E, 14 Sept. 2014, *leg. M. Halama* (WRSL MH-2014-0286), 4 – solitary, gregarious or subfasciculate on soil (humus covered by broadleaved litter and humus under herbaceous plants) under *Cerasus avium* (L.) Moench, *Prunus spinosa*, *Quercus* sp., *Rhamnus cathartica* L. and *Tilia cordata*, alt. 179 m a.s.l., 50°08′30″N, 18°16′48″E, 14 Sept. 2014, *leg. M. Halama* (WRSL MH-2014-0287).

Molecular characterization

Sequencing of the ITS rDNA region of *Tricholoma ustaloides* yielded fragments 680 bp long. The ITS sequence of the tested sample showed 99% similarity with the reference sequences of *T. ustaloides* deposited in the UNITE database as UDB000816, UDB015393 and UDB011564, and with erroneously named sequences of *T. ustale* (UDB000815) and *T. fulvum* (UDB015391), and 99% similarity with the reference sequences of *T. ustaloides* deposited in NCBI GenBank database as LT000126 and LT000094. The obtained ITS sequence was deposited in the NCBI GenBank nucleotide database (Accession number: KX034212).

Discussion

Tricholoma ustaloides is characterized by its orange-brown pileus and lower part of the stipe, rather sharply delimited white zone at the top of the stipe, and distinctly farinaceous and very bitter taste (especially in the cap cuticle). It also seems to be well characterized by having strongly glutinous caps when moist, a white context, rather moderately crowded lammelae, whitish or cream and often brownish stains near the lamella edges, in combination with predominantly broadly ellipsoid basidiospores. The morphology of the *T. ustaloides* specimens collected in Poland agrees with the description and drawings of Christensen and Heilmann-Clausen (2013). The observed occurrence of *T. ustaloides* with *Quercus* in clayey soil is also consistent with the overall ecological pattern of the species. The morphological identification is supported by similarity of the ITS sequence of one Polish specimen with sequences of *T. ustaloides* deposited in UNITE databases.

Tricholoma ustaloides is quite similar to *T. ustale* (Fr.: Fr.) P. Kumm. in having a similar general habit and a yellowish brown to brick pileus. The color of the pileus, however, usually has a rather paler, more yellowish component towards the margin, and the surface of the pileus is less viscid and typically not strongly glutinous. The latter species also differs by the mild taste of the pileipellis and the lack of a sharply delimited white zone at the top of the stipe. Although *T. ustale* is often reported from *Quercus* forests, it is a species particularly accompanying *Fagus* (Christensen & Heilmann-Clausen 2013). *Tricholoma quercetorum* Contu recently described from Southern Europe (Contu 2003) is distinguished from *T. ustaloides* by the same characters as *T. ustale* (apart from the viscidity of the pileus), and additionally differs microscopically by having narrower spores (5.5–7.0 × 3.5–5.0 μm) (Christensen & Heilmann-Clausen 2013). Another similar species, *Tricholoma batschii* Gulden, has, in common with *T. ustaloides*, a well-delimited white zone at the top of the stipe and similar colors of the basidiomata, but differs in having a thin but distinct, membranous and often wavy ring which soon becomes attached to the stipe. Furthermore, *T. batschii* grows with *Pinus* (rarely *Cupressus*), generally in dry, calcareous soil, and may be easily separated microscopically by its smaller basidiospores (3.8–6.4 × 3.1–5.4 μm) (Christensen & Heilmann-Clausen 2013).

Tricholoma ustaloides has a wide geographical distribution in Europe, where it occurs from the Mediterranean up to southern Scandinavia. It has been reported from Southern Europe (Bulgaria, Corsica, Greece, Italy, Montenegro, Portugal, Sicily, Spain, Turkey), Western Europe (Belgium, France, the Netherlands), Central (Austria, Czech Republic, Germany, Hungary, Lichtenstein, Slovenia, Switzerland) and Northern Europe (Denmark, England, Estonia, Ireland, Norway, Scotland, Sweden) (Riva 1988; Zervakis *et al.* 1998; Christensen 1999; Tóth 1999; Gminder & Krieglsteiner 2001; Denchev & Assyov 2004; Jurc *et al.* 2004; Legon *et al.* 2005; Roux 2006; Walleyn *et al.* 2006; Baptista *et al.* 2010; Kalamees 2010; Běťák 2011; Lazarević *et al.* 2011; Christensen & Heilmann-Clausen 2012, 2013). It is generally rare in Northern Europe and much more common in countries with mild climates in Central and Southern Europe (Riva 1988; Christensen 1999). The northernmost records of the species do not exceed 59°N, and the northern limit of *T. ustaloides* seems to follow the natural distribution of its main hosts (*Quercus*) (Gminder & Krieglsteiner 2001; Kalamees 2010; Christensen & Heilmann-Clausen 2013). *Tricholoma ustaloides* is also known to occur in North Africa (Morocco) and Asia (e.g., Turkey) (Malençon & Bertault 1975; Gezer 2000; Courtecuisse & Duhem 2007; Sesli & Denchev 2008; Riva 2009). Villeneuve *et al.* (1989) indicated a similar species (as *Tricholoma* cf. *ustaloides* Romagn.) from North America (Canada, Quebec) but without any supplementary data.

Most European localities of *T. ustaloides* are from 150–850 m a.s.l. and are confined to colline-subalpine vegetation zones (Pegler 1966; Breitenbach & Kränzlin 1991; Noordeloos & Christensen 1999; Gminder & Krieglsteiner 2001; Laganà *et al.* 2002; Kalamees 2010; Kibby 2010; Ludwig 2012; Hernández-Rodríguez *et al.* 2013; this study). The highest elevation given in available data (1200–1300 m a.s.l.) is from Spain (Llorens van Waveren & Llistosella 2005). Descriptions of the habitat of *T. ustaloides* vary widely from one locality to another and from region to region. Bearing in mind that the climate and the bedrock differ between various sites, the ectomycorrhizal plant hosts should also differ. The most common habitats in Northwestern and Central Europe are described by different authors as (thermophilic) deciduous forest, parks and pond dams, typically on (nutrient-rich) clayey, calcareous or loess soils, mainly under *Quercus robur* L., *Q. petraea* (Matt.) Liebl. and *Fagus sylvatica* L., rarely *Carpinus betulus* (Noordeloos & Christensen 1999; Holec & Beran 2006; Kibby 2010; Ludwig 2012; Christensen & Heilmann-Clausen 2013). In Southern Europe the habitat of the species is generally described as thermophilic (silicolous, calcicolous) deciduous woodland, rarely orchards, with a preference for associations with oak, beech, chestnut and hornbeam (Bon 1984; Riva 1988; Bon 1991; Gminder & Krieglsteiner 2001; Galli 2005; Roux 2006). Edaphic conditions range from acid to neutral and alkaline. *Tricholoma ustaloides* has frequently been characterized in southern regions as a calciphilous species, but there are a number of reports on neutral and acid ground (Bon 1984, 1991; Laganà *et al.* 2002; Galli 2005; Roux 2006; Ortega *et al.* 2010). There are several records on limestone, and its lithological substrates have been described variously as verrucano, sandstone, polychrome sericitic schist, rhyolitic lava flows, clastic quartzitic and phyllitic rocks, gabbro, polygenic conglomerates, periostite and serpentinite (Laganà *et al.* 2001; Laganà *et al.* 2002; Ortega *et al.* 2010). A broad spectrum of accompanying plants have been given, possible mycorrhiza symbionts for *T. ustaloides* in the Mediterranean region: *Castanea sativa* Mill., *Corylus avellana* L., *Quercus canariensis* Willd., *Q. cerris* L., *Q. conferta* Kit., *Q. faginea* Lam., *Q. frainetto* Ten., *Q. humilis* Lam., *Q. ilex* L., *Q. pubescens* Willd., *Q. pyrenaica* Willd. and *Q. suber* L. (Malençon & Bertault 1975; Bon 1984; Riva 1988; Zervakis *et al.* 1998; Gminder & Krieglsteiner 2001; Laganà *et al.* 2001; Laganà *et al.* 2002; Galli 2005; Llorens van Waveren & Llistosella 2005; Comandini *et al.* 2006; Dimou *et al.* 2008; Baptista *et al.* 2010; Ortega *et al.* 2010). Also mentioned are associations of *T. ustaloides* with *Cistus* sp., *Cistus ladanifer* L., *Juniperus oxycedrus* L., *Picea abies* (L.) H. Karst. and *Pinus sylvestris* L. (Gminder & Krieglsteiner 2001; Llorens van Waveren & Llistosella 2005; Comandini *et al.* 2006; Dimou *et al.* 2008; Ludwig 2012; Hernández-Rodríguez *et al.* 2013), but these records may represent other taxa (e.g., *T. ustale*, *T. batschii*) and should be treated with caution.

Tricholoma ustaloides is considered threatened in several countries. It is on the red list of fungi in the Czech Republic in category DD (data deficient), in Denmark, the Netherlands, Norway and Switzerland it is classed as vulnerable (VU), and in Sweden as near threatened (NT) (Benkert *et al.* 1992; Holec & Beran 2006; Senn-Irlet *et al.* 2007; Arnolds & Veerkamp 2008; Dahlberg *et al.* 2010; Anonymous 2012). The only known locality of *T. ustaloides* in Poland is shown in Figure 1. In the neighboring Czech Republic the species is known from only a few localities (Holec & Beran 2006). Its overall distribution in Poland is not known but most likely *T. ustaloides* is very rare here. This species is a possible candidate for the prospective red list of fungi in Poland; the DD (data deficient) threat category is tentatively proposed.

Particularly favorable geographic and climatic conditions in the Łężczak Reserve, combined with past economic activity which led to the development of tree-lined lanes along dams, have made this an important biodiversity area (Duda & Wika 1994). The tree-lined lanes are the only Polish localities of several fungal species (Trząski 1994). At the locality of *T. ustaloides* along the Oak Alley, several other interesting ectomycorrhizal fungi were found, including *Tricholoma sejunctum* (Sowerby: Fr.) Quél., *Aureoboletus gentilis* (Quél.) Pouzar, *Rubinoboletus rubinus* (W. G. Sm.) Pilát

& Dermek, *Caloboletus radicans* (Pers.: Fr.) Vizzini, *Boletus reticulatus* Schaeff., *B. aereus* Bull.: Fr. and *Leccinellum crocipodium* (Letell.) Della Maggiora & Trassinelli.

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