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NEW RECORDS AND DATA ON HYPOGEOUS ECTOMYCORRHIZAL FUNGI IN BULGARIA

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Abstract

In this article, new data on species diversity of hypogeous macrofungi in Bulgaria are reported. Three species: *Tuber rufum*, *Gautieria graveolens* and *Lactarius stephensii* were recorded for first time in the country. New localities of noteworthy fungi *Tuber excavatum* and *Gautieria morchelliformis* are also reported. A DNA sequencing for most studied hypogeous fungi and detailed phylogenetic analysis for *T. excavatum* were performed. Results confirmed that representatives of *T. excavatum* group have a very high intraspecific genetic variability.

Key words: ascomycetes, basidiomycetes, Bulgaria, fungal conservation, hypogeous fungi, phylogenetic analysis.

Introduction

Almost all of hypogeous macrofungi (ascomycetes and basidiomycetes) are ectomycorrhizal species, living in symbiotic relationship with roots of trees. Because of their cryptic, subterranean fruit bodies, they are often overlooked and neglected in different studies until now, when a rapidly development of molecular tools takes main part of scientific research. The design and construction of specific primers can be used as an easier and more sensitive tool for the identification of truffle species in any stage of their life cycle, including the mycorrhizal phase (Bertini et al. 1999).

Many hypogeous fungi, for instance truffles, are pioneer species. They are often reported in present studies as one of the main components of ectomycorrhizal associations in various forest ecosystems under particular ecological conditions (Benucci et al. 2011). Truffles generally grow in calcareous soils, but some of them are well adapted to slightly acidic soils (Jaillard et al. 2014, Pacioni and Comandini 1999). Most of them have a relatively wide plant partners range and also growing conditions and only a few number is restricted to particular ones.

The Balkan Peninsula and the western parts is still insufficiently explored regarding hypogeous fungi, including ’true‘ truffles of the genus *Tuber* (Gyosheva et al. 2012a, Marjanović et al. 2010, Dimitrova and Gyosheva 2008).

Geographic position of Bulgaria and

respectively the great diversity of ecological conditions let to assume that the large number of hypogeous ectomycorrhizal fungi could be found in the country. We can expect slightly genotype difference for some known species because of particular conditions in a process of adaptation to their plant partners.

Eleven hypogeous ascomycetes (truffles and truffles-like fungi) have been published until now from Bulgaria: *Choiromyces meandriformis* Vittad., *Elaphomyces granulatus* Fr., *Hydnotrya cerebriformis* Harkn., *H. tulasnei* (Berk.) Berk. & Broome, *Stephensia bombycina* (Vittad.) Tul., *Tuber aestivum* Vittad., *T. brumale* Vittad., *T. borchii* Vittad., *T. excavatum* Vittad., *T. macrosporum* Vittad., *T. puberulum* Berk. & Broome (Dimitrova and Gyosheva 2008, 2009; Gyosheva et al. 2012a,b; Lacheva 2012; Peev et al. 2015).

There is a great species diversity of larger basidiomycetes in Bulgaria and up-to-date more than 1500 species are recorded (Denchev and Assyov 2010). Some of them from genera *Gautieria* Vittad., *Hymenogaster* Vittad., *Macowanites* Kalchbr., *Melanogaster* Corda, *Octaviania* Vittad. and *Rhizopogon* Fr. are hypogeous and ectomycorrhizal.

Hypogeous macrofungi are with high conservation value. Ten species (5 ascomycetes and 5 basidiomycetes) were included in the Red List of Fungi in Bulgaria (Gyosheva et al. 2006) and also in the Red Data Book of the Republic of Bulgaria (Peev et al. 2015). All species are from categories Critically Endangered (CR) – 1 and Endangered (EN) – 9.

This paper presents three species of hypogeous fungi: *Tuber rufum* Picco, *Gautieria graveolens* Vittad. and *Lactarius stephensii* (Berk.) Verbeken and Walleyn, recorded for first time in Bulgaria. New data for the distribution of *Tuber excavatum* and *Gautieria morchelliformis* Vittad are also presented. Most of fungal specimens are DNA sequenced. Phylogenetic analyses have been done for *T. excavatum* and *T. fulgens* Quél. Recent molecular studies have shed some light on complex diversity among closely related *Tuber taxa.* Molecular and phylogenetic studies on well-known species *T. excavatum* are insufficient until now (Alvarado et al. 2012; Bonito et al. 2010a,b,c; Zampieri et al. 2009; Chen and Liu 2007; Wang et al. 2006, etc.).

Materials and Methods

The investigated specimens were harvested in several localities in Bulgaria in 2014–2015. Some information concerning their habitats was recorded – habitat type, nearby trees, soil structure and type (where data are available). One permanent plot (3×3 m) in the area near Gradets village was chosen on a productive site for periodical observation and ecological studies (at least 1 time per month from September to April).

Morphological observation of main character features of fungal specimens was studied on fresh specimens using Carl Zeiss Jena Citoval stereo microscope. Micromorphological characters were observed under Amplival, Leitz Laborlux Phase-contrast microscope and Zeiss light microscope. As mounting medium deionized water was used. For better observation and for important diagnosing chemical reactions some colorizing reagents were used – KOH, Congo Red in ammonia solution, Cotton Blue in lactophenol, and Melzer’s reagent. The microphotographs were taken by AmScope MA1000, 10 MP× camera. Measurement of asci, spores and peridium were performed by Piximetre 5.8 software (http://ach.log.free.fr/Piximetre/).

Scanning electron microscopy (SEM) was carried out at the Institute of Physical Chemistry, Bulgarian Academy of Sciences. Specimens were examined on JSM-5510 scanning electron microscope at 10 kV.

All studied specimens are kept at the Mycological Collection of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia (SOMF).

The nomenclature of hypogeous fungi follows Kirk et al. (2001). The author's abbreviations of fungi are according to Kirk and Ansell (2004). Hypogeous fungi were identified following Riousset et al. (2012), Dissing (2000), Montecchi and Sarasini (2000), Montecchi and Lazzari (1993), Pegler et al. (1993), Breitenbach and Kränzlin (1984), Trappe (1979), Dennis (1978) and Moser (1963). The threat status follows the Red List of Fungi in Bulgaria (Gyosheva et al. 2006).

For DNA analysis was used only properly dried material (5–6 h, 55–60 °C) stored in freezer at – 20 °C. For DNA extraction, was followed and modified Dellaporta et al. (1983) protocol for plant tissue isolation. PCR amplification was performed with the primers ITS1 and ITS4 (White et al. 1990, Gardes and Bruns 1993) for the ITS regions. After gel-electrophoresis, successful DNA fragments were visualized and cleaned using GeneMATRIX Basic DNA Purification Kit. Samples were sequenced at GATC biotech laboratory in Germany.

The results were compared with their closest relatives obtained with BLAST algorithm. Phylograms are constructed for *T. excavatum* and *T. fulgens*. Sequences were first aligned in CLC Genomics workbench 8.0 and then corrected manually. Dendrogram was constructed and analyzed for best fit model – lowest-scoring maximum likelihood tree. To obtain this, several tests were used: hierarchical likelihood ratio test (hLRT), BIC test (Bayesian information criterion) – $BIC = -2\ln(L) + K\ln(n)$, where $\ln(L)$ = loglikelihood of the best tree found, K = number of parameters and AIC Model test – $AIC = -2\ln(L)+2K$, where $\ln(L)$ = loglikelihood of the best tree found and K = number of parameters.

Results and Discussion

New records of hypogeous macrofungi for Bulgaria

Ascomycota
Pezizales
Tuberaceae

Tuber rufum Picco, Meleth. Bot.: 80 (1788)

Ascomata hypogeous or semihypogeous, irregular, lobed and very rare subglobose (Fig. 1a), 1.5–4 cm in diameter, yellow-brown to reddish-brown, fine warted and areolate, cartilaginous. Warts are fine and almost pyramidal. **Peridium** is very thick (380–480 μm) and two layers can be distinguished – outer, which is composed by 3–5 rows of thick-walled large hyphae and inner layer, which is paler and almost white, forming pseudoparenchymatous tissue (Fig. 1b). **Gleba** of interwoven to parallel hyphae is more loosely structured than the inner layer of peridium, hard and solid, initially whitish, than yellowish, ochre to olive-brown or olive-gray at maturity, with a close network of whitish meandering and translucent veins. **Odor:** relatively strong, not specific and unpleasant. **Taste** is mild. **Asci** 50–70 × 80–100 μm, clavate to almost globose (depending of spore position and view – Fig. 1d), short-stalked

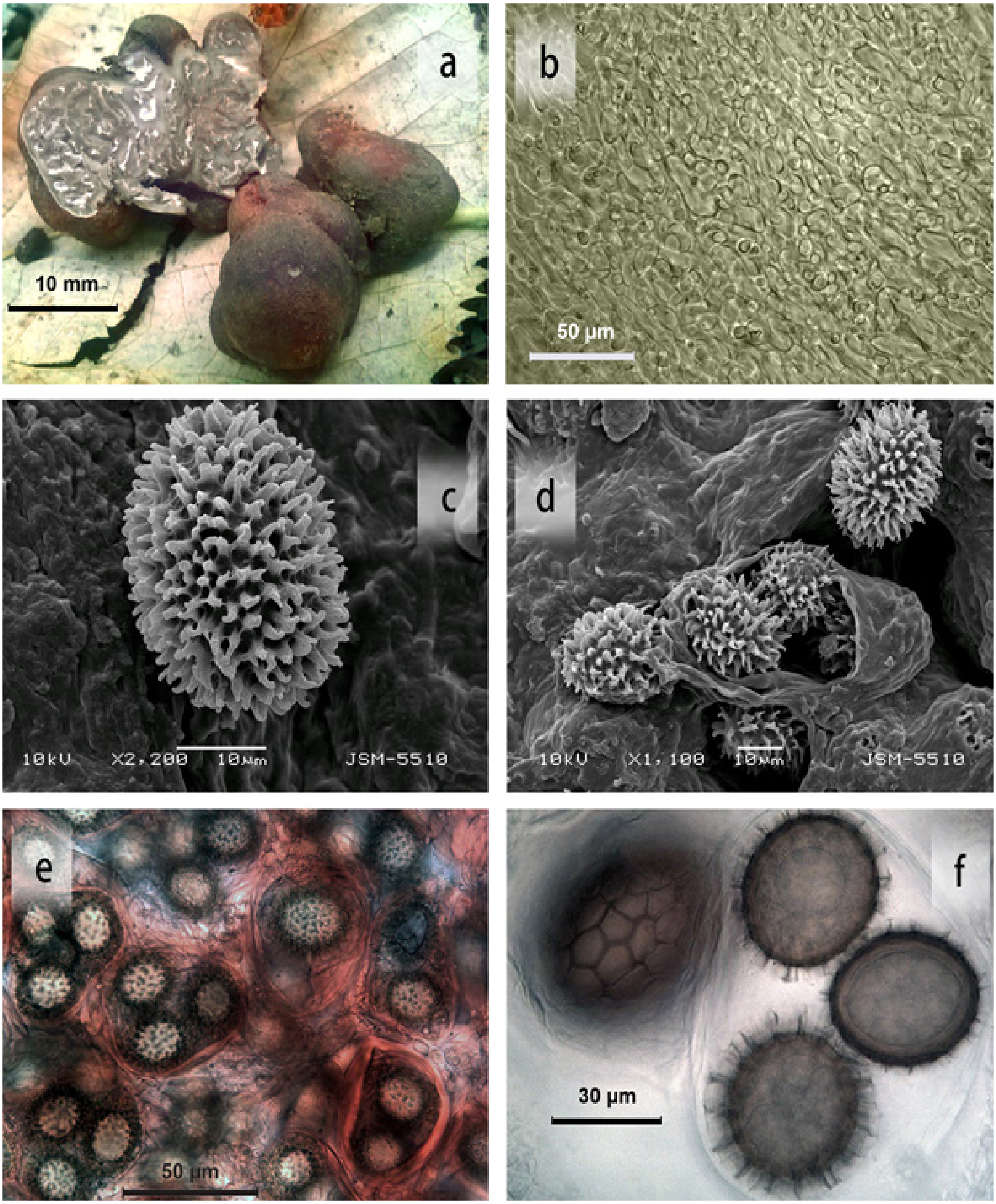

Fig. 1. ***Tuber rufum*** **(BG13AG10): a – general view; b – inner layer of pseudoparenchymatous tissue of peridium in Melzer's reagent; c– SEM detail view of spore; d – SEM general view; e – asci with ascospores in Congo red; f –** ***Tuber excavatum*** **(BG13AG06) – ascospores.**

(10–25 µm) (Fig. 1e), usually 3–4-spored. **Ascospores** 27.1 [32.9; 34.8] 40.7 × 20.2 [24.6; 26.1] 30.5 µm; Q = 1.0 [1.3; 1.4] 1.6; N = 50; C = 95 % M_e = 33.9 × 25.4 µm; Q_e = 1.3, broadly ellipsoid, ornamented with conical spines (Fig. 1c), 2.5–3.5 µm high, initially translucent, yellow – ochre tobrownish at maturity (Fig. 1e).

Habitat. This species is relatively widespread associated with deciduous trees and conifers, ascomata maturing during late summer and autumn (Pegler et al. 1993).

Specimens examined. Western Forebalkan, near Belotintsi village, buried in humus layer of soil, partially on the surface, near *Tilia tomentosa* Moench artificial plantation, 14.09.2014, leg. T. Nedelin, det. M. Gyosheva (SOMF 29671; BG13AG10).

Comments. Although there are several species with similar features, according to Pegler et al. (1993) main differences in close related ones can be observed in type and colour of peridium, its cell organization and structure, also in consistency of gleba. *T. rufum* was also reported recently from Turkey (Türkoğlu and Castellano 2014). Due to its high intraspecific variability a set of specific primers were developed – Ru1f/Ru2f-ITS4 (Iotti et al. 2007). Our result of DNA sequencing matches 100 % for this species in GenBank, with standard fungal primers – ITS1/ITS4, but using specific primers is recommended, because the detection can be more sensitive and it is possible for only 0.2 mg of this truffle to be identified successfully. It is especially important when mycorrhizal tips are used, instead of fungal tissue from ascoma. According to the same authors *Tuber ferrugineum* Vittad. is a morphological form of *T. rufum* characterized by a rusty coloured peridium and spores with long spiny ornamentations.

Basidiomycota
Gomphales
Gomphaceae

Gautieria graveolens Vittad., Monographia Tuberacearum (Milano), p. 27, 1831.

Basidiomata hypogeous or semi-hypogeous, irregularly subglobose, often wrinkled, with small depressions, 2–4 cm in diameter, ochre to reddish-brown. At the base with whitish mycelial tuft of strands (hyphae). **Peridium** is thin (220–330 μm – Fig. 2a) and can be seen only in immature stage and through the development disappear. **Gleba** with a labyrinthine-like elongate or near roundish cavities, situated near ochre, reddish – brown or grayish branches which often enlarge toward the base and forming white or whitish **columella**. **Odor** very strong and unpleasant. **Taste** undefined. **Basidia** 13–20 × 8–10 μm, broadly clavate, usually 2-spored (rare 4-spored). **Cystidia** clavate and hyaline. **Basidiospores** 16–19 × 11.5–13 μm, Q range = 1.3–1.6, broadly ellipsoid, brownish at maturity. **Spore** ribs are smooth and apiculus is rounded (Fig. 2c).

Habitat. In soil, in coniferous and deciduous forests (mainly under oak), from late summer to late autumn.

Specimens examined. Tracian Lowland, near Haskovo town, in the Park ’Kenana‘, buried in organic layer of soil, under *Quercus* sp., 14.09.2014, leg. T. Chokova and E. Nankova, det. M. Gyosheva (SOMF 29672; BG13AG07).

Comments. *G. graveolens* was recorded in Greece in forests of *Quercus frainetto* Ten. (Konstantinides 2006). It is also reported from some other European countries and also from North America (Benedek and Pál-Fám 2001, Zeller and Dodge 1918, etc.). There is insufficient data of exact host range of tree species forming ectomycorrhiza with this hypogeous fungus. It can be easily recognized from *Gautieria morchelliformis* (Fig. 2b) by its smaller spores and clear visible peridium in early stages of development.

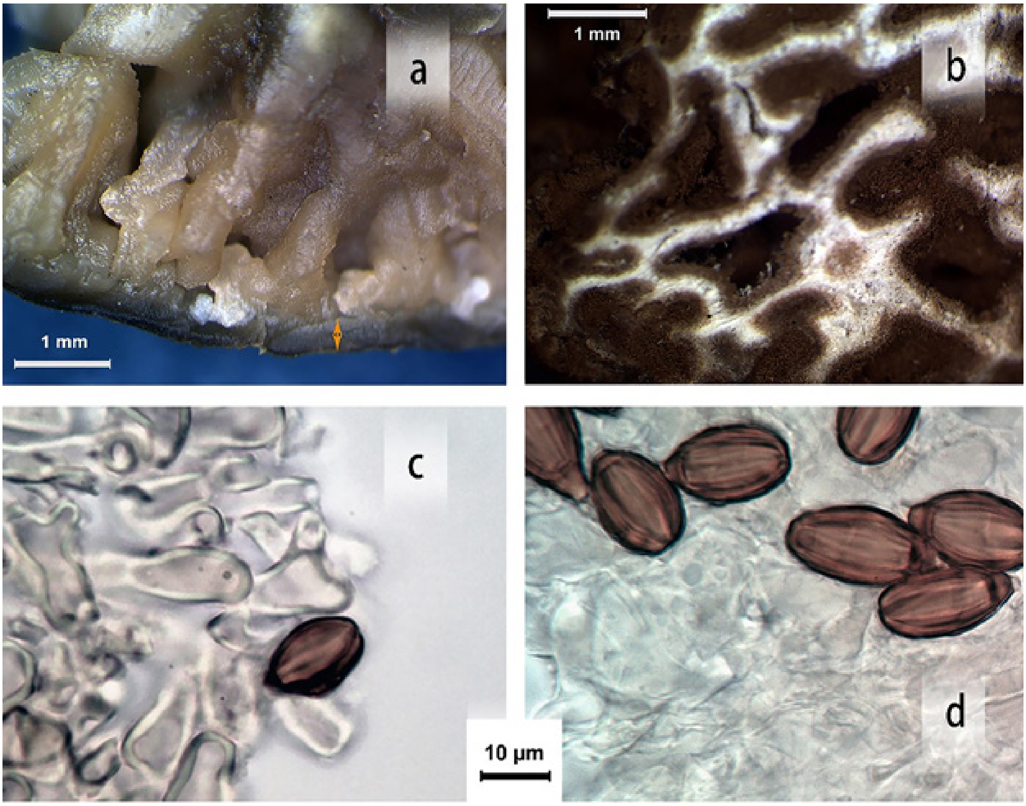

Fig. 2. ***Gautieria graveolens*****: a – immature fruitbody with clear visible peridium (orange arrows); b – section view of** ***Gautieria morchelliformis*****; c –** ***Gautieria graveolens*** **– spore and paraphyses; d –** ***Gautieria morchelliformis*** **– spores.**

Russulales

Russulaceae

Lactarius stephensii (Berk.) Verbeken & Walleyn, in Nuytinck, Verbaken, Delarue & Walleyn, Belg. Jl Bot. 136 (2): 151 (2004) [2003]

Basidiomata hypogeous or semihypogeous 1.0–2.5 cm in diameter (Fig. 3a), mostly irregular or subglobose, lobbed with distinctive radial ridges at the base, together with thin, mycelial cords arising from a minutely projecting sterile base. **Peridium** at first pale ochraceous to orange-red, finally darkreddish-brown, dull, smooth, viscid, easily detached – 150–200 µm total, two layers – 50–80 µm of peridiopellis (Fig. 3b). **Gleba** whitish to pale orange or pinkish, discoloring reddish on exposure after cutting, labyrinthic, with irregularly elongated chambers (Fig. 3c). **Columella** cannot be seen. **Odour** very strong, specific, fruity. **Taste** very strong, fruity, and specific, even in dry specimens. **Basidia** broadly clavate to almost globose, short-stalked, with 3–4-spores. **Basidiospores** 10.91 [12.20; 12.56] 13.84 × 10.37 [11.36; 11.64] 12.62 µm; Q = 0.97 [1.06; 1.09] 1.18; N = 64; C = 95 %; M_e = 2.38 × 11.50 µm; Q_e = 1.08 (excluding ornaments) (Figures 3d, e), globular, ornamented with conical spines rounded apically with length 1.2–1.8–(2.2)

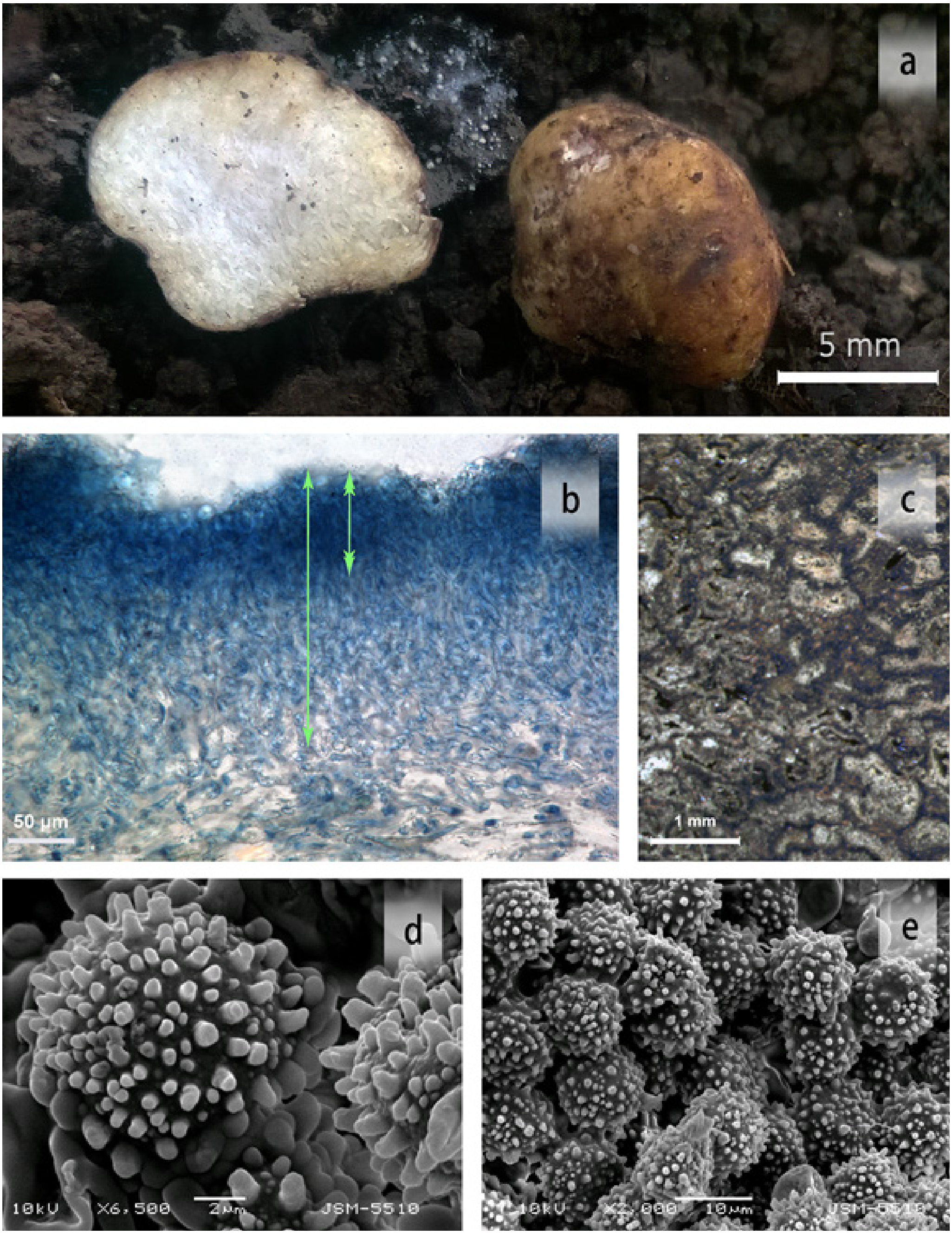

Fig. 3. ***Lactarius stephensii*** **(BG13AG08): a – general view; b – two layers of peridium (green arrows) – outer is with doubled green arrows (≈80 µm); c – detail section view of mature fruit body; d, e – SEM of spores at different magnifications.**

μm, initially translucent, yellow-ochre to brownish at maturity.

Habitat. Usually gregarious, under humus, with ectomycorrhizal hosts from genera *Corylus* L., *Carpinus* L., *Fagus* L., *Populus* L., *Quercus* L., *Tília* L. (Vidal 2004).

Specimens examined. Western Balkan Mts., near Gradets village, buried in organic layer of soil, near *Carpinus betulus* L., 03.09.2014, leg. & det. T. Nedelin (SOMF 29673; BG13AG08).

Comments. Two genera of sequestrate fungi related with genus *Lactarius* Pers. are well recognized presently: *Arcangeliella* Cavara and *Zelleromyces* Singer et A. H. Sm. First of both is secotioid and contrarily, *Zelleromyces* is non-secotioid and sessile. In Europe only 6 species from second are known (Vidal 2004).

New localities of hypogeous fungi in Bulgaria

Ascomycota

Tuber excavatum Vittad.

Specimens examined: BG13AG05 – Northeast Bulgaria, near Razgrad town, buried in organic layer of soil (1–5 cm under litter), near *T. tomentosa* artificial plantation, 13.11. 2013, leg. K. Kyose, det. T. Nedelin (SOMF 29674); BG13AG06 – Western Stara Planina Mts., near Gradets village, buried in organic layer of soil (1–5 cm under litter), near *Carpinus orientalis* Mill., 30.10.2014, leg. &. det. T. Nedelin (SOMF 29675).

The species was reported previously in Bulgaria also from Northeast Bulgaria – Razgrad Hills (Dimitrova and Gyosheva 2008) and from Western Rhodopes Mts., near Ravnogor village (Lacheva 2012).

Comments. *T. excavatum* group is well recognized from other truffles because of their usually well-developed basal cavities. This group seems to have a great intraspecific diversity and a dozen of varieties, subspecies and forms were described (Riousset et al. 2012, Gross 1991). For instance the colour of peridium in *monticellianum* form is olive brown. Fischer (1923) distinguishes varieties with small spores (*brevisporum*) and other with larger spores (*longisporum*). Most species have been shown a very close intraspecific ITS to interspecific ITS sequence variation and sometimes overlapped gaps can be recognized (Bonito et al. 2010c). Thus requires comprehensively morphological and phylogenetic studies, especially in case, where degree of variability between examined specimens is higher and there are significant differences in ecological aspects (host plant and period of maturing in respect to geographical latitude). Several samples from each *T. excavatum* locality were compared and macro- and micro-morphologically studied, and were found some slightly distinct features. BG13AG05 has more wrinkled surface (Fig. 4a) and paler colours in the peridium, also its basal cavities are narrowed (Fig. 4c). In BG13AG06 the basal cavities are enlarged, especially in young stage of development (figures 4b and 4d). It has finely papillose peridium, contrary to BG13AG05 which peridium is generally smooth. The colour of its gleba is dark purple to pinkish purple and dark purple to purple brown in BG13AG06 (Fig. 4d), without pink tints in the last. Asci have no significant differences in their morphology or size, but spore ratio (Q) for Razgrad samples is 1.0 [1.2; 1.3] 1.4 (Fig. 4e) and respectively for Gradets village samples – 1.2 [1.4] 1.6 (Figures 1f and 4f). Both specimens (BG13AG05, BG13AG06)

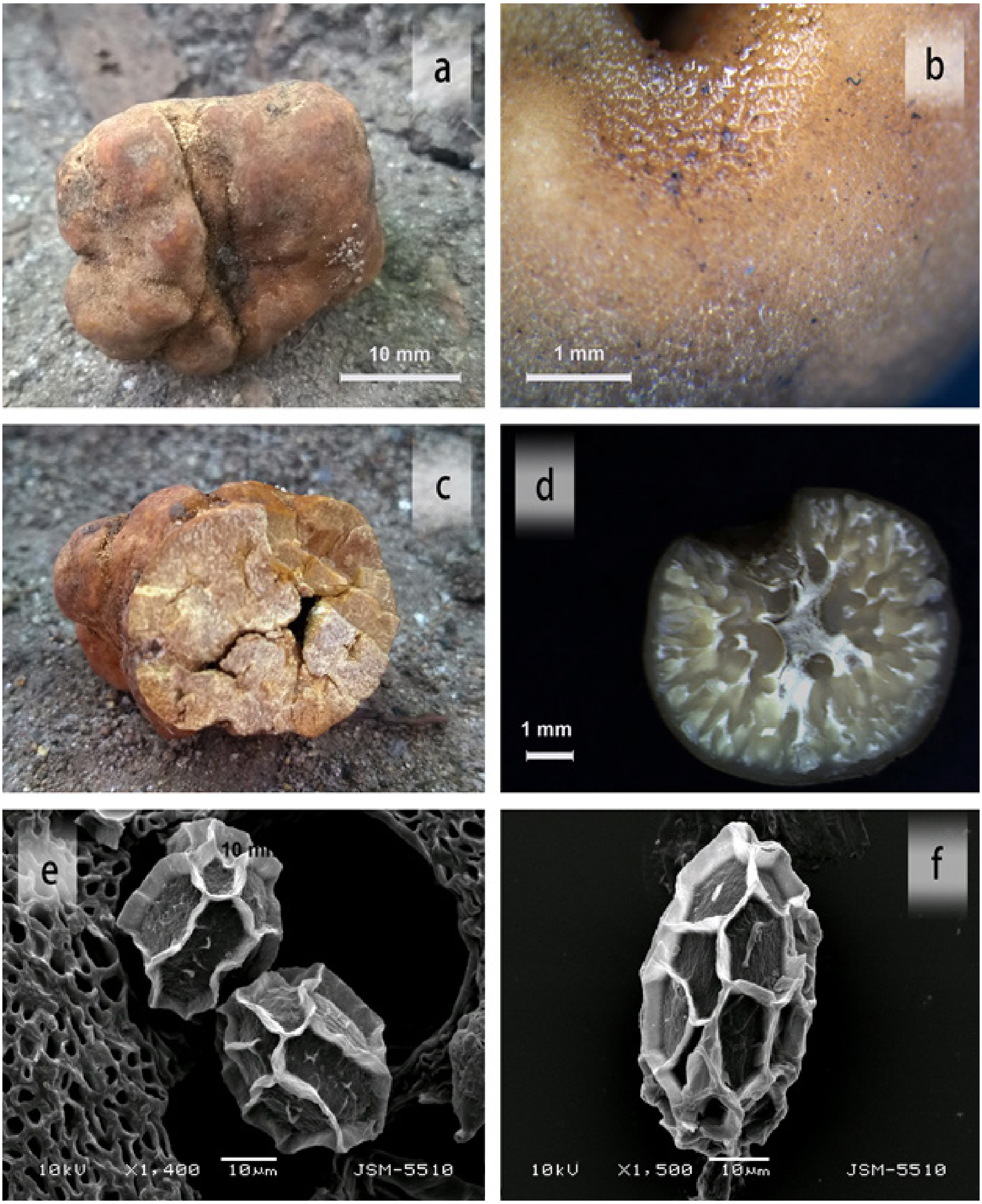

Fig. 4. ***Tuber excavatum*****: a – general view (BG13AG05); b – detailed view of peridium BG13AG06); c – section (BG13AG05), d – section (BG13AG06); e – SEM, BG13AG05; f – SEM, BG13AG06.**

have different tree hosts and there is a great possibility that this might be the most important factor in addition to soil structures, type and climate condition for the ecological plasticity of *T. excavatum* group.

Basidiomycota

Gautieria morchelliformis Vittad.
(Fig. 2d).

Specimens examined. Western Stara Planina Mts., above the village of Buhovo, semihypogeous in soil, near *Quercus* sp., 25.11.2014, leg. A. Goranov, det. M. Gyosheva (SOMF 29677).

This species was reported only once for Bulgaria from Southern Black Sea coast – Ropotamo Reserve (Kuthan and Kotlaba 1981). It is also found in Greece, under *Quercus ilex* L. (Konstantinides 2006) and in forests of *Pinus nigra* Arn. (Diamandis and Perlerou 2008).

Results from DNA analysis

According to various authors (Rioussett et al. 2012, Montecchi and Sarasini 2000, Pengler et al. 1993) the closest taxonomically species to *T. excavatum* is *T. fulgens*, which differs from it for its brighter, orange-brown colour and its subglobous spores. The phylogram on Fig. 5 is constructed for most *T. excavatum* and *T. fulgens* samples in the GenBank, compared with their identical ITS regions. It was obtained in CLC Genomic Benchmark 8.0 after (computer and then manual) alignments analysis of parallel ITS sequences after several tests for construction of best tree model.

Two major clades clearly emerge with several subgroups amongst them, which can be distinguished also without molecular studies. It was found that in the first group intraspecific variation is slightly below 6 % and in the second slightly above 12 %. This corresponds within the borders accepted by Bonito et al. (2010a) – for intraspecific variation <14.7 % and interspecific variation >9.2 %. *T. excavatum* is the only species from their study, which shows lower interspecific to intraspecific variation and overlaps and we confirm this.

T. excavatum samples from Gradets village are genetically closer to *T. fulgens* than to *T. excavatum* group I. The comparison of BG13AG05 and BG13AG06 is shown in Fig. 6. More symmetric lines correspond to more similarity in their nucleotide basis.

Conclusion

The data of carried out morphological and molecular studies of *Tuber excavatum* samples confirmed the great genetic variability amongst this group. Using specific primers, especially for the group of white truffles, would bring further clarity in their diversity. The dramatic and constant decrease of whole genome sequencing cost price since 2008 (from 100 million USD in 2001 to 1000 USD in 2015 at Illumina HiSeq X10) and establishment of the Next-Gen Sequencers (Bahassi and Stambrook 2014) will make direct sequencing of the entire genome in near future more accessible – up-to-date the most reliable method to determine species. All this, however, must be accompanied by the most possible detailed morphological, anatomical, physiological and other studies of hypogeous fungi from different habitats to determine borders of their ecological plasticity.

All reported hypogeous fungi in the paper are uncommon or threatened in Europe and should be estimated according to IUCN criteria at the next update of Red List of Fungi in Bulgaria.

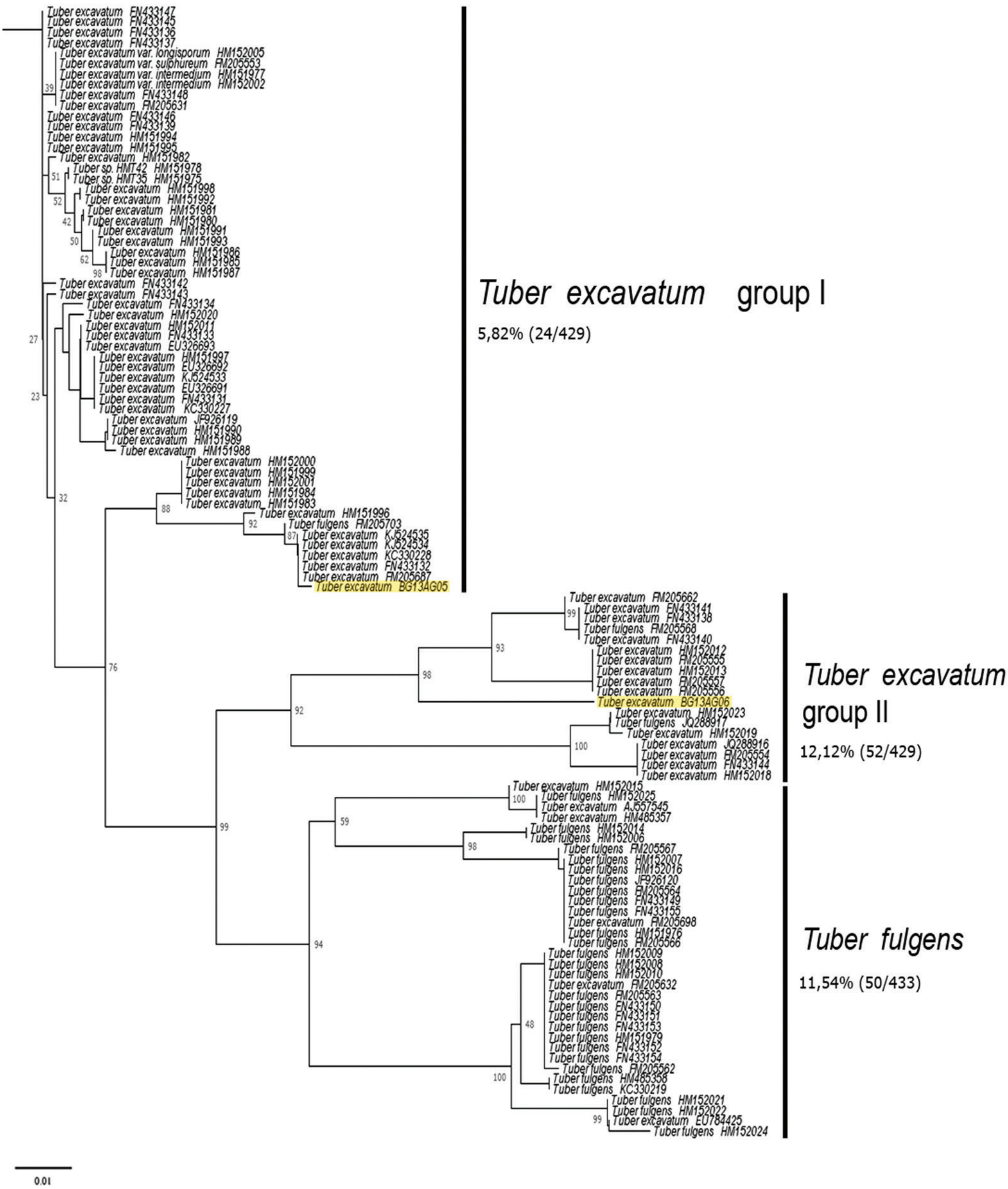

Fig. 5. Phylogenetic tree of *Tuber excavatum* and *Tuber fulgens* specimens from public database.

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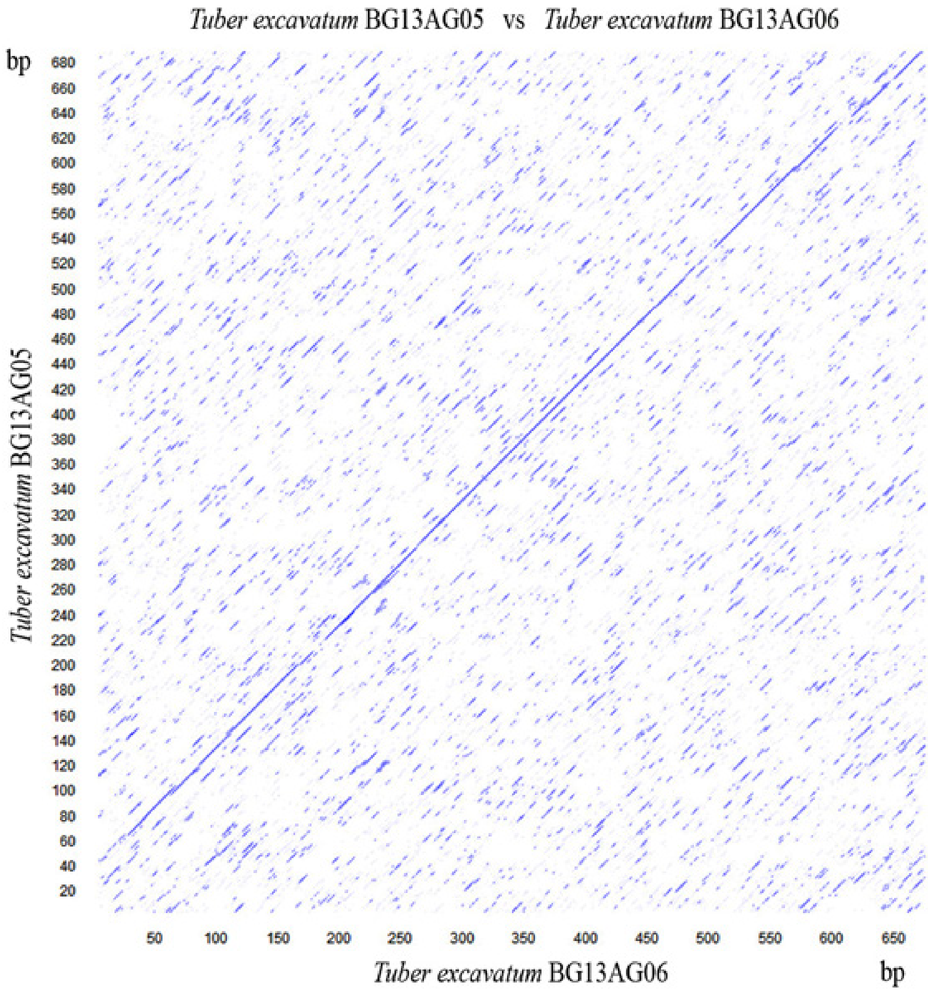

Fig. 6. Dot-plot diagram of both Bulgarian *Tuber excavatum* specimens (BG13AG05, BG13AG06).

'Taxonomy, conservation and sustainable use of fungi'.

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