academicJournals

Vol. 8(23), pp. 1096-1099, 18 June, 2013
DOI 10.5897/SRE2013.5529
ISSN 1992-2248 © 2013 Academic Journals
http://www.academicjournals.org/SRE

Scientific Research and Essays

Full Length Research Paper

First report of *Tuber macrosporum* occurrence in Poland

Dorota Hilszczańska[1]*, Aleksandra Rosa-Gruszecka[2], Katarzyna Sikora[2] and Hanna Szmidla[1]

[1]Forest Research Institute, Department of Forest Ecology, Braci Leśnej 3, Sękocin Stary, 05-090 Raszyn, Poland.
[2]Forest Research Institute, Department of Forest Protection, Braci Leśnej 3, Sękocin Stary, 05-090 Raszyn, Poland.

Accepted 31 May, 2013

***Tuber macrosporum*, a large spore species is first time confirmed from Poland. It grows naturally in southern part of Poland, in calcareous soils. The fruit bodies of the species were found in mixed fresh forest with dominant tree species such as: *Pinus sylvestris*, *Carpinus betulus* and *Quercus robur*. The soil chemistry and texture of the site was analysed. Fruit body of the truffle is conserved in the collection of biological material in Ecology Department of Forest Research Institute herbarium.**

Key words: *Tuber macrosporum*, habitat, phylogeny.

INTRODUCTION

Truffles (*Tuber* spp.) are hypogeous fungi growing in symbiosis with a broad diversity of gymnosperm and angiosperm hosts in variety of habitats. The fungi are ascomycetes belonging to Pezizales, a large group of ectomycorrhizal fungi. Ascomycete truffle species are commonly referred to as "true truffles" and basidiomycete truffles as "false truffles'. Several members of the true truffle genus Tuber are highly appreciated as delicacies. One of these species is the smooth black truffle (*Tuber macrosporum* Vittad.), which has an excellent flavor. Despite its good quality and the increased interest, *T. macrosporum* fruiting bodies are still sold mixed with some inferior species of black truffles (Iotti et al., 2002).

T. macrosporum is common in central Italy. So far it has been reported from Czech Republic, France, Hungary, Romania, Serbia, Switzerland, Ukraine, and the United Kingdom where is considered as rather very rare (Hall et al., 2007). Recently, the species was found in Germany where were considered extinct (Stobbe et al., 2012).

T. macrosporum grows in an ectomycorrhizal symbiosis with many different tree species, for example, *Quercus robur*, *Fagus sylvatica*, *Corylus avellana*, *Pinus sylvestris*, *Pinus strobus* (Granetti et al., 2005). According to Bencivenga and Baciarelli Falini (2012), *C. avellana*, *Q. pubescens*, *Carpinus betulus* and *Ostrya carpinifolia* are the usual hosts of *Tuber* species in truffle orchards. The species prefers fresh, wet, occasionally flooded, thick, calcareous soils with variable levels of calcium carbonate, often in lowlands or near rivers (Vezzola, 2005; Marjanović et al., 2009; Benucci et al., 2012). In Italy fruiting bodies of the fungus are found in the same areas as *Tuber magnatum* (white truffle), and have the same host plants (Hall et al., 2007). The time when the species fruits is not clearly defined, yet truffle collectors harvest *T. macrosporum* from August till December (Granetti et al., 2005).

According to phylogenetic studies on the Tuber genus based on the internal transcribed spacer (ITS) region and large subunit of the nuclear ribosomal DNA (nrDNA), have revealed that the Macrosporum clade is one of the ancestral lineages of the genus (Jeandroz et al., 2008; Bonito et al., 2010). The Macrosporum group has also been discovered in Japan and seems to represents a complex of species (Kinoshita et al., 2011). In the study we focus on the habitats of *T. macrosporum* in Poland and determine phylogenetic relationship within the

*Corresponding author. E-mail: d.hilszczanska@ibles.waw.pl. Tel: +48 227150502. Fax: +48 227150504.

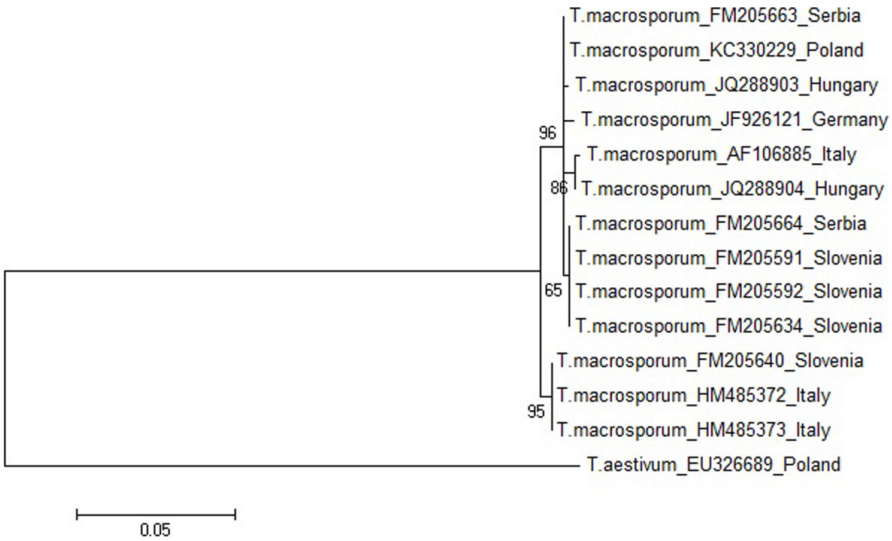

Figure 1. Phylogenetic analysis of ITS sequences obtained from GenBank (NCBI) with the sequence of *T. macrosporum* found in Poland. The tree was constructed using the Maximum Likelihood method. *Bar* represents 0,05 changes. *Tuber aestivum* sequence was used as an out group.

European species.

MATERIALS AND METHODS

The truffles were found accidentally looking for *T. aestivum* with help of the trained truffle dog (Lagotto Romagnolo Breed). Inventory was made in September 2012. The localities were chosen on the basis of pedological, geological and floristic structure (Brożek and Zwydak, 2003). All fruit bodies of truffles were packed into vacuum boxes and gently washed in the laboratory. Small parts of these fungi were taken in order to prepare slides for microscopic observation. Species of truffles were identified on the basis of microscopic features and compared to the criteria by Granetti et al. (2005). Samples of fruiting bodies were also taken for DNA analysis.

DNA extraction

Total DNA was extracted from lyophilised fruit body tissue by using DNeasy Plant Mini Kit (Qiagen, USA), following the manufacturer's protocol. Quality of the DNA was checked with NanoDrop ND-1000 (Thermo Fisher Scientific, USA) and on the basis of electrophoregram in 1% TBE-agarose gel.

Polymerase chain reaction (PCR) amplification and sequencing

Polymerase chain reaction (PCR) amplification of the ITS region of the template DNA was performed using primers ITS1 and ITS4 (White et al., 1990) in a 25 µl reaction containing 50 ng genomic DNA, 250 nM of each primer, 200 µM of each dNTP, 1U RED*Taq*® polymerase, and 1 x PCR buffer (SigmaAldrich, USA). The reaction was performed in a PTC-200™ Programmable Thermal Controller (MJ Research, Inc.) for 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 50 s, with initial denaturation of 3 min at 94°C before cycling and a final extension of 10 min at 72°C after cycling. A portion 2 µl of the amplified product was run on 1% TBE-agarose gel; the presence of a single band (ca. 600 bp) was a check for successful amplification. The PCR product was purified using the A&A Biotechnology (Gdynia). Clean-up kit, following the manufacturer's protocol prior the sequencing. The sequences of *T. macrosporum* are deposited at GenBank (National Center for Biotechnology Information, NCBI). The truffles collected by the inventory are deposited in Ecology Department of Forest Research Institute herbarium.

The sequence data were checked between complementary strands and the resulting sequences were aligned with GenBank (NCBI) nucleotide collection.

The multiple sequence alignment was performed by maximum likelihood (ML) algorithm. The optimal tree with the sum of branch length = 0.03062943 is shown (Figure 1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The bootstrap values are given for each node above 50%. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993) and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 566 positions in the final data set. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

Analysis of soils

Soil samples were taken from under of the fruiting bodies of *T. macrosporum*. Analysis of soil texture was done according to PN-ISO 11277(2005). The soil pH in water and essential nutrient contents were measured according to ISO 10390 (1997) and PB-14ed.2 of 1 January, 2010 (using inductively coupled argon-plasma spectrometry following mineralisation in chloric (VII) acid), respectively. The percent of total N (PN-ISO 13878, 2002) and C was analysed according to a method of dry mineralisation (PN-ISO

Table 1. Soil composition at the *T. macrosporum* site.

Soil particle size fractions																
Sand	Silt	Clay	pH_{H2O}	$CaCO_3$ total	P	Ca	K	Mg	Fe	Ca/Mg	K/Mg	C org.	N total	C total	OM	C/N
%				%			g/kg						%			
63.41	22.17	14.42	7.5	4.04	0.23	19.77	2.53	1.51	8.18	13.09	1.68	1.623	0.138	2.108	2.80	15.3

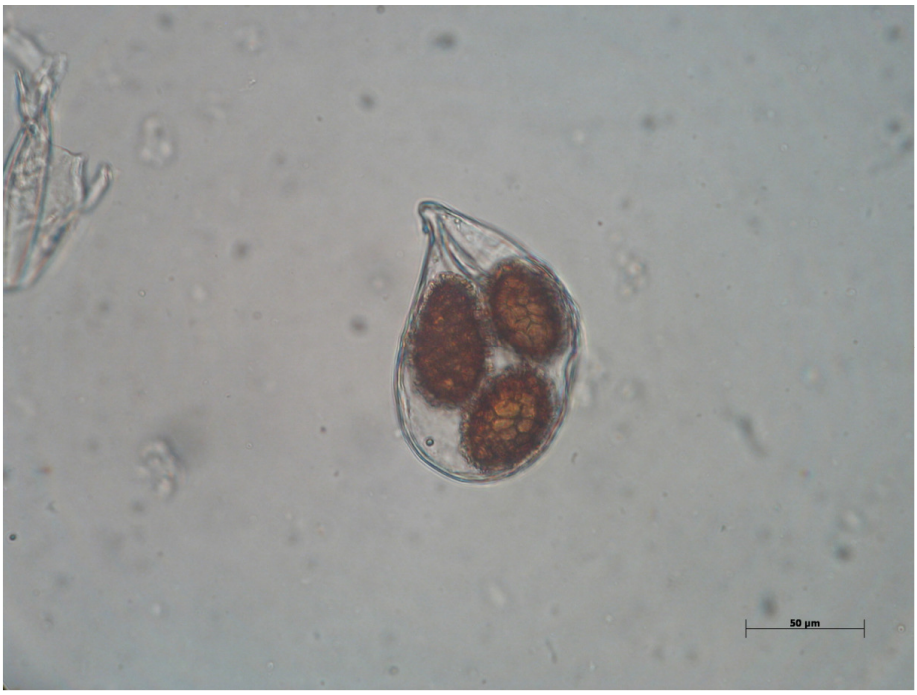

Figure 2. Spores of *Tuber macrosporum* inside the ascus.

10694, 2002). $CaCO_3$ was determined by volumetric method (ISO 10693, 1994). All analyses were carried by the Polish Centre for Accreditation (No. AB740).

RESULTS AND DISCUSSION

Fruiting bodies of *T. macrosporum* were found at the site with latitude 50° 28´N and longitude 20° 45´E. The locality was situated at an elevation of 267 m. The region belongs to one of the warmest Polish zones, the annual mean temperature between 1997 and 2012 was 8.1°C and the annual mean precipitation for the same period was 595 mm.

In the investigated site no other accompanying truffle species were found. The geographical names of the site where the species was found will be available for further research, but not for publication. Publishing site names could lead to reckless prospecting for truffles, resulting in damage to the surrounding flora, as well as the affecting foresters negatively. The texture of soil and chemical properties from the locality are given in Table 1.

According to the Atlas of forest soils of Poland (Brożek and Zwydak, 2003), the region is characterised by bedrock that consists of Cretaceous marlstone, limestone, gypsum, as well as Miocene Period clays and sands. Various Quaternary deposits cover more than three quarters of the region. The soil cover consists primarily of cambisols and chernozems.

The fruiting bodies of *T. macrosporum* occurred in forest where ectomycorrhizal host species *Q. robur*, *C. betulus* and *P. sylvestris* grew together. As non-host trees and shrubs *Acer campestre*, *Betula pendula*, *Lonicera xylosteum*, *Cornus* sp., *Malus* sp., *Pyrus* sp. and *Sambucus nigra* were present. Only five species of ground-layer vegetation were associated with the presence of *T. macrosporum,* viz.: *Asarum europaeum*, *Athyrium filix-femina*, *Neottia nidus-avis* (the host-species), *Polygonatum odoratum* and *Viola reichenbachiana*.

Microscopic identification based on spore examination showed the spore length of *T. macrosporum* up to 55 µm (Figure 2). In order to confirm the proper identification of

the species the molecular techniques were successfully used. Cluster analysis of 14 *T. macrosporum* ITS sequences did not show a clear differentiation of investigated isolates by place of origin (Figure 1).

The findings of *T. macrosporum* widen the list of the truffle species that occur in Poland. This is the first report about presence of new truffle species after five years' time when *T. aestivum*, *T. excavatum* and *T. rufum* were found (Hilszczańska et al., 2008). The fruiting bodies were present in the soil with rather low phosphorus concentration (0.23 $g \times kg^{-1}$) and content of organic matter, what is in agreement with findings by (Morcillo et al., 2007; Ricard, 2003). C/N ratio of soil was 15.3 indicating a preference of *T. macrosporum* for soils that are poor in readily degradable nitrogen and high water pH (7.55). Similar results were found by Miko et al. (2006) who compared the biotopes of *T. macrosporum* in Slovakia. The authors found 17 host and non-host plant species at the one out of two investigated localities of *T. macrosporum.* Yet, at second locality in Slovakia grew together only nine plants. Although some authors (Stobbe et al., 2012; Bencivenga and Baciarelli Falini, 2012) showed that *C. avellana* is commonly associated with *T. macrosporum* we did not found the host-species at our site. It seems the other host species including *Q. robur*, *C. betulus* and *P. sylvestris* are of great importance for *T. macrosporum*. These findings are in agreement with findings of Granetti et al. (2005). Our inventory also showed, that only five plant species of ground vegetation layer were present at the site of *T. macrosporum.* Such a result differs from those of Benucci et al. (2012) who claim that in cooler habitats *T. macrosporum* sites are covered by lush vegetation. Due to the lack of historical Polish records of *T. macrosporum* it is very difficult to estimate to such extent the species occurrence is present in our country. However, every year new findings of truffle species in Poland suggest the diversity of truffle fungi is higher than it was previously estimated.

REFERENCES

Bencivenga M, Baciarelli Falini L (2012). Manuale di Tartuficoltura. Esperienze di coltivazionedei tartufi in Umbria. Regione Umbria, p. 130.

Benucci GMN, Gogan Csorbai A, Di Massimo G, Baciarelli Falini L, Bencivenga M, Donnini D (2012). Mycorrhization of *Quercus robur* L. and *Corylus avellana* L. seedlings with *Tuber macrosporum* Vittad. Mycorrhiza 22(8):639-646. DOI: 10.1007/s00572-012-0441-3.

Bonito GM, Gryganskyi AP, Trappe JM., Vilgalys R (2010). A global meta-analysis of Tuber ITS rDNA sequences: Species diversity, host associations and long-distance dispersal. Mol. Ecol. 19:4994–5008.

Brożek S, Zwydak M (2003). Atlas gleb leśnych Polski. CILP, Warszawa.

Granetti B, De Angelis A, Materozzi G (2005). Umbria, terra di tartufi. Regione Umbria, Terni.

Hall I, Brown G, Zambonelli A (2007). Taming the truffle. The history, lore, and science of the ultimate mushroom. Timber press, Oregon, USA.

Hilszczańska D, Sierota Z, Palenzona M (2008). New Tuber species found in Poland. Mycorrhiza 18(4):223-226.

Iotti M, Amicucci A, Stocchi V, Zambonelli A (2002). Morphological and molecular characterization of mycelia of some Tuber species in pure culture. New Phytol. 155:499–505.

ISO 10693 (1994). Soil Quality. Determination of carbonate content. Volumetric method. International Organization for Standardization. Geneva, Switzerland p. 7.

Jeandroz S, Murat C, Wang YJ, Bonfante P, Le Tacon F (2008). Molecular phylogeny and historical biogeography of the genus tuber the 'true truffles'. J. Biogeogr. 35:815–829.

Kinoshita A, Sasaki H, Kazuhide N (2011). Phylogeny and diversity of Japanese truffles (*Tuber* spp.) inferred from sequences of four nuclear loci. Mycologia 103(4):779-794

Marjanović Z, Grebenc T, Markovic M, Glisic A, Milenkovic M (2009) Ecological specificities and molecular diversity of truffles (genus *Tuber*) originating from mid-west of the Balkan Peninsula. Sydowia 62:67–87.

Miko M, Gazo J, Bratek Z (2006). *Tuber macrosporum* Vitt. and *Tuber mesentericum* Vitt. - One hundred years neglected hypogeus fungi species in the Slovak Republic. Acta fytotechnica et zootechnica 9(4):85-90.

Morcillo M, Moreno B, Pulido E, Sanchez M (2007). Manual de truficultura Andaluza. Ed. Gypaetus y Consejería de MedioAmbiente. Junta de Andalucía. p. 176.

PB-14 ed. 2 of 1 January (2010). Extraction of trace elements using inductively coupled argon-plasma spectrometry following mineralisation in chloric (VII) acid. p. 8.

PN-ISO 10390 (1997). Soil Quality. determination of pH. International Organization for Standardization. Geneva, Switzerland p. 15.

PN-ISO 10694 (2002). Soil Quality. determination of organic and total carbon after dry combustion ("elementary analysis"). International Organization for Standardization. Geneva, Switzerland p. 12.

PN-ISO 11277 (2005). Soil quality. determination of particle size distribution in mineral soil material: Method by sieving and sedimentation. International Organization for Standardization. Geneva, Switzerland p. 46.

PN-ISO 13878 (2002). Soil Quality. Determination of total nitrogen content by dry combustion ("elemental analysis"). International Organization for Standardization. Geneva, Switzerland p. 10.

Ricard JM (2003). La truffe, Guide technique de trufficulture. CTIFL, Párizs p. 268.

Stobbe U, Büntgen U, Sproll, L, Tegel W, Egli S (2012). Spatial distribution and ecological variation of re-discovered German truffle habitats. Fungal Ecol. 5(5):591–599.

Tamura K, Nei M (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10:512-526.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. 28(10):2731-2739.

Vezzola V (2005). Primi risultati produttivi con piante micorrizate da *T. macrosporum* Vittad. Atti Seminario sullo stato attuale della Tartuficoltura Italiana. Grafiche Millefiorini, Spoleto, Italy pp. 51–55.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gefland DH, Sninsky J, White TJ (eds) PCR Protocols. A Guide to Methods and Amplifications, Academic Press pp. 315–322.