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On the presence of truffles in Armenia

Abstract

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This study reports the finding of *Tuber rufum*, *T. rapaeodorum* and *T. scruposum* in Armenia. The morphological characters of the ascomata together with the climate, vegetation and soil features of the areas where the truffles grow are described. The ascomata were also molecularly characterized by sequence analyses from the ITS region of ribosomal DNA.

Introduction

The Republic of Armenia occupies an area of 29800 km² and is situated in the mountainous region of the South Caucasus and it is part of a region conventionally called the Armenian Highland. This is a wedge shaped portion of land between the Iranian and Minor Asiatic mountains which covers more than 300.000 km² and has a mean altitude of 1700 m above sea level. The neighbouring countries of Armenia are Georgia, Azerbaijan, Turkey and Iran.

The climate of Armenia is characterized by the northern humid area and the southern dry area. Six climate zones are recognized: dry subtropical, dry continental, warm dry temperate, cool forest temperate, cold-mountain and sever high-mountain (Baghdasaryan 1962). The highest value of air temperature is from the Arax river valley (+ 42 °C) whereas in the higher mountainous region the temperature does not rise above + 20 °C with a minimum temperature of – 46 °C and up to 2 m of snow.

The main soil types are: mountainous meadows, meadow-steppes, grey sylvan, turf – carbonate sylvan, brown sylvan, black, black soils of meadows, brown, grey soils of semi-deserts, grey soils of irrigated meadows, flood-land - terraces soils, saline-alkaline and palaeohydromorphe cemented alkaline ground soils and ground soils near lake Sevan (Edilyan 1990).

Armenia has a rich and heterogeneous flora. Approximately 3200 species of vascular plants have been described. Vegetation types include: desert, semi-desert, phryganoid (xerophyte), mountain steppes (1500-2200 m asl), subalpine meadow (2200-2800 m asl), alpine meadow (2700-4000 m asl), wetlands and forests (6-9 % of north-eastern 1900-2000 m asl) and south (2200-2400 m asl) parts of the country. The main forest-forming trees are *Quercus* (35 %), *Fagus* (32 %), *Carpinus* (18 %), *Pinus*, as well as *Fraxinus* and *Acer* (15 %) (Takhtajyan 1936).

Studies on truffles have scientific and practical significance because of their role as the fungal partner in ectomycorrhizal associations primarily with species of the genera *Quercus*, *Fagus*, *Corylus*, *Carpinus*, *Populus*, *Salix*, *Tilia* and *Pinus*. Some of them, such as *Tuber melanosporum* Vittad. and *T. magnatum* Pico, are also economically important because of the very high prices paid for their fruiting bodies whilst *T. mesentericum* Vittad., *T. brumale* Vittad., *T. aestivum* Vittad., *Choiromyces meandriformis* Vittad. and *Terfezia arenaria* (Moris) Trappe, are recognized as medicinal mushrooms (Garibova 1976; Denisova 1998; Tardif 2000; Pegler & al. 1993; Pegler 2002).

Up to now the only record of truffles from Armenia is *Tuber aestivum* Vittad. (Taslakhchyan & Nanagulyan 1988) although Armenia's geographic position, eastward the Mediterranean and northward the Middle East, would suggest that other species truffles could be present in this region. The study reported here attempts to increase the knowledge on Armenian truffles. The biotic, climatic and edaphic environmental details where truffles were found were also recorded by several Authors and such data are very important to support field investigations (Lawrynowicz 1988; Tibiletti & Zambonelli 1999; Bencivenga 2001).

Material and Methods

Sample collection

Ascomata of truffles were collected in Armenian forests characterized by more than 10 years old trees and not less than 5 cm of humus layer. The field trips were carried out from April to October in the years 2002-2004 near Dilijan (July, 2003; August, 2004), Vanadzor (June, 2003; July, 2004), Kapan (August, 2003; June, 2004), Ijevan (August, 2004) and Megri (April, 2002) towns (Fig. 1).

According to literature data (Garibova 1976; Prokhorov 1976; Pegler & al. 1993) hypogeous fungi grow commonly in sylvan ecosystems. Ascomata were sought in moist forested areas with sylvan soils, close to the possible host plants (species of *Quercus*, *Fagus*, *Carpinus*, *Tilia*, *Populus*, *Salix*, etc.). The ascomata were found by digging in soil 1-1.5 m around the trunks. The soil was dug to 30-50 cm and afterwards replaced and covered with leaf-litter.

Morphological characterization

Fresh samples of *Tuber* ascomata were preliminary described (colour, surface, smell, etc.), numbered using the first letter of location plus an accession number (e.g. "K 20"), dried and stored both in the herbarium of Laboratory of Fungal Biology and Biotechnology (FBBL), Yerevan State University (Armenia) and in the herbarium of Dipartimento di Protezione e Valorizzazione Agroalimentare (CMI-Unibo), University of Bologna (Italy).

The micro-morphological characteristics of both fresh and dry ascomata were described. Part of the asomata were embedded in Tissue Tek OCT (Sakura, Zoeterwoude, Netherlands) compound and then cut with a rotary cryomicrotome (Tissue Tek® II, Miles, Elkhart, IN, USA) (8-10 µm thickness). Serial sections were

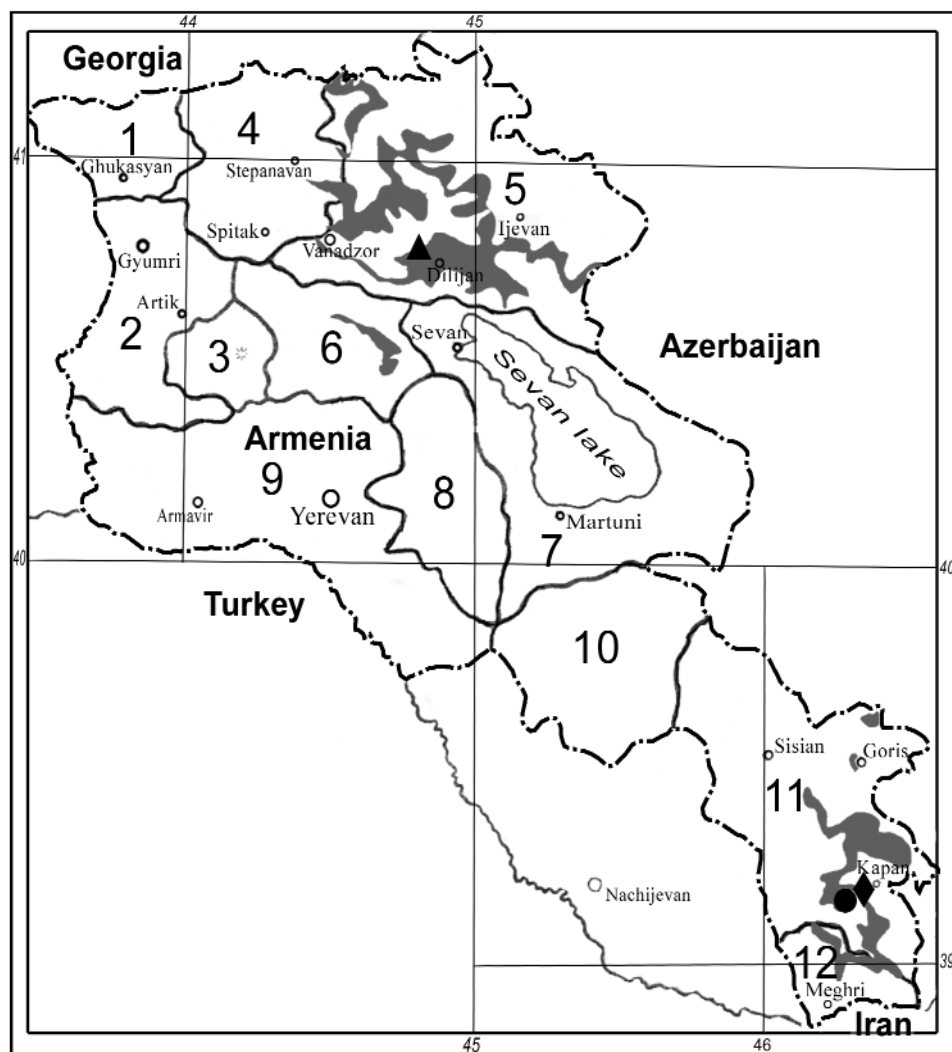


Fig. 1. Map of flora regions of Armenia (Takhtajyan 1978): 1. Upper-Akhuryan; 2. Shirak; 3. Aragats; 4. Lori; 5. Ijevan; 6. Aparan; 7. Sevan; 8. Geghama; 9. Yerevan; 10. Daralagez; 11. Zangezour; 12. Meghri. The favourable regions for growth of truffles in Armenia marked in grey: ▲ – *Tuber scruposum*, ◆ – *Tuber rufum*, ● – *Tuber rapaeodorum*.

mounted in lactic acid and observed under a microscope ECLIPSE TE 2000-E (Nikon, Tokyo, Japan).

Main dimensions of the peridium cells, asci and spores were determined using Axio Vision 2.05 software (Carl Zeiss Vision GmbH, Hallbergmoos, Germany) from images captured with a DXM1200F digital camera (Nikon, Tokyo, Japan).

Molecular analyses

Molecular analysis was performed using sequence data of the ITS regions of the ribosomal DNA. Total genomic DNA was isolated from 100 µg of dehydrated ascomata tissue by DNeasy[®] Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA was then eluted in 50 µl of sterile water. ITS-1, 5.8S and ITS-2 regions were amplified in a 50 µl volume reaction containing 1-10 ng of genomic DNA, using the primers pair ITS1 and ITS4 (White & al. 1990) in a T gradient Thermal Cycler (BIOMETRA, Göttingen, Germany) according to Amicucci & al. (1996). PCRs were performed using 1.5 U of TaKaRa[™] *Taq* DNA polymerase (Takara, Otsu, Japan). The amplified products were first purified by Gene Clean II kit (BIO 101, Vista, CA, USA) and then sequenced using both the primers mentioned above. Sequence reaction was performed using the ABI PRISM 3700 DNA Analyzer (Applied Biosystem, Foster City, CA, USA) with Big Dye terminator v. 3.1 chemistry. The obtained ITS sequences of ascomata were compared to those of GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>) using the BLASTN search (Altschul & al. 1997). Ru1 species-specific primer and ITS4 were used to identify *T. rufum* ascomas (Iotti & al. 2005).

Results

Truffles were found on the northern and north-eastern slopes of mountains covered by woods of *Fagus orientalis* Lipsky, *Carpinus betulus* L., *Tilia cordata* Mill., *Corylus avellana* L., *Pinus* sp. In particular *Fagus* and *Carpinus* woods were investigated near to mountain streams and small rivers (Table 1). Generally the truffles were usually found in the humus-layer of sandy-clayish soils (Hovsepyan 2004).

Currently, the following species of truffles have been firstly identified in Armenia:

***Tuber scruposum* R. Hesse** (Herbarium samples n. [FBBL-YSU / CMI-Unibo]: D 7 / 2192; D 3 / 2197; D 11 / 2201; D 13 / 2194; D 19 / 2207).

The ascomata were found in oak-hornbeam forest (near the town of Dilijan) where the main trees are *C. betulus*, *T. cordata* and *F. orientalis*.

Morphology of ascoma - subglobose or globose, orifice or cavity absent, 0.3-0.6 cm in diameter, yellow or ochre, smooth, peridium slightly warty, finely pubescent, colour of spore-bearing tissue brown, veins wide and sparse (Fig. 2a). Peridium 150-400 µm, pseudoparenchymatous formed of small cells 8-17 × 4-12 µm (Fig. 2b). External cystidia present with septa.

Morphology of asci and spores - Asci globose, 50-75 × 45-65 µm, 2-4 irregularly clustered spores per ascus. Spores ellipsoid or subglobose, 25-40 × 20-25 µm, pale brown or medium brown, reticulated (Fig. 2c).

***Tuber rufum* Pico** (Herbarium samples n. [FBBL-YSU / CMI-Unibo]: K 20 / 2203).

Ascomata were found in hornbeam forest, in the environment of the town of Kapan, in hornbeam forest on *C. avellana*.

Morphology of ascoma - globose, orifice or cavity absent, 0.5 cm in diameter, reddish-brown. Peridium 300-450 µm, scabrous; spore-bearing tissue brown, veins wide and

Table 1. Favourable conditions for growth of truffles in Armenia.

Vegetation type	1. Scattered forests — with <i>Juniperus</i> and deciduous trees; 2. Forest vegetation — with <i>Fagus</i> and <i>Quercus</i> complex; dry forests with <i>Quercus araxina</i> and xerophyte scattered forests complex; dry forests with <i>Quercus macranthera</i>; forests of <i>Fagus</i> and <i>Quercus iberica</i>; forests of <i>Fagus</i> and <i>Quercus macranther</i>; 3. Sub-alpine forests — scattered forests of <i>Fagus</i>, <i>Betula</i> and <i>Quercus</i> with high-grass and meadow complex.
Soil type	1. Mountainous-sylvan grey; 2. Mountainous-sylvan brown; 3. Mountainous-sylvan turf-carbonate.
Climate type	Temperate, rather humid during all seasons.

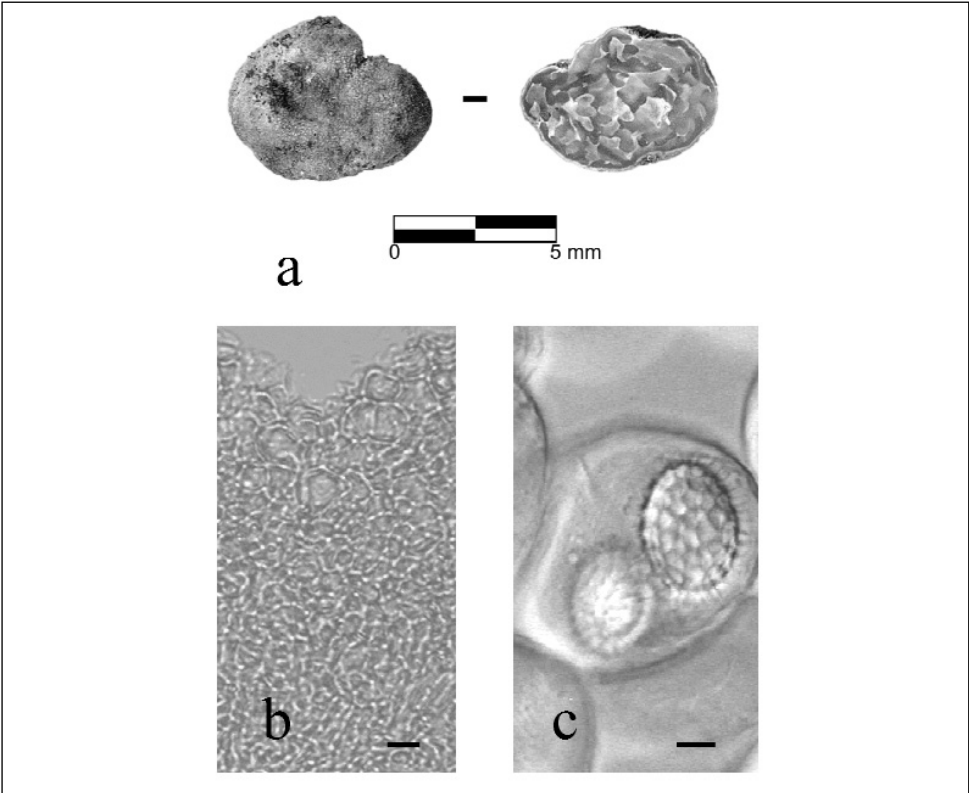


Fig. 2. Ascomata (a), peridium (b) and spores (c) of *Tuber scruposum* (Bars = 10 μm).

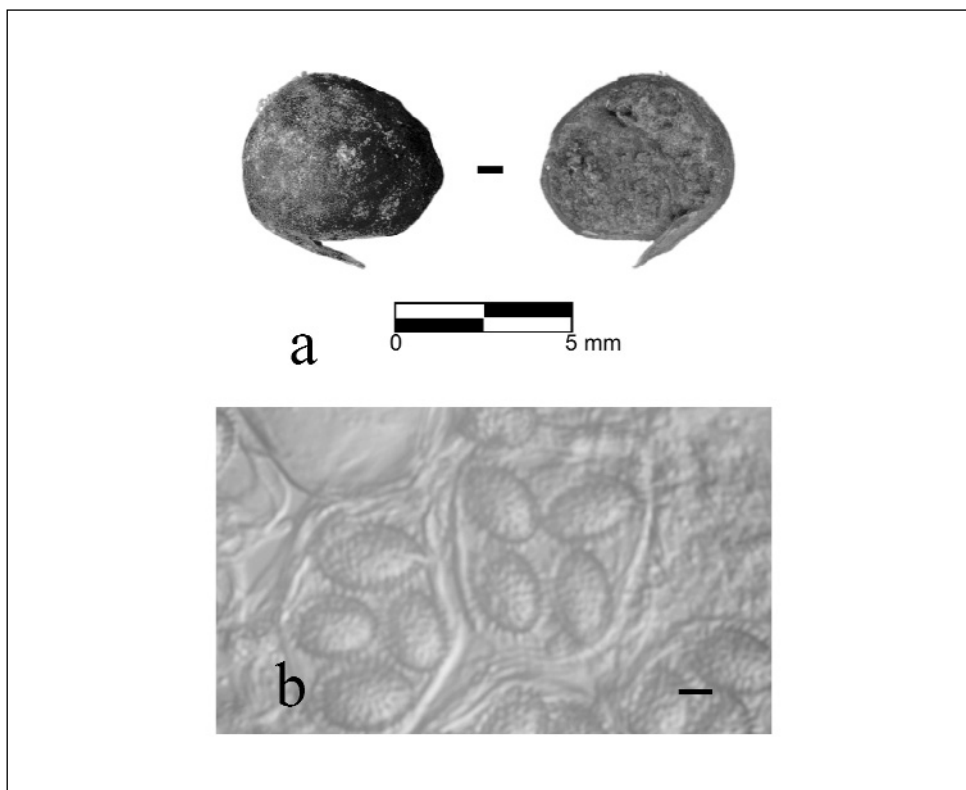


Fig. 3. Ascomata (a), asci and spores (b) of *Tuber rufum* (Bar = 10 µm).

sparse (Fig. 3a). External cystidia absent.

Morphology of asci and spores - Asci saccate or globose with a short or sometimes elonged stalk, $60-70 \times 45-50$ µm. Spores irregularly clusted, 1-5 per ascus, ellipsoid or subglobose, spiny, $25-30 \times 20-25$ µm length, with pale brown or medium brown colour (Fig. 3b).

***Tuber rapaeodorum* Tul.** (Herbarium samples n. [FBBL-YSU / CMI-Unibo]: K 24 / 2483; K 27 / 2481).

Ascomata were collected in the environment of the town of Kapan, under *C. betulus*.

Morphology of ascoma - subglobose or globose, orifice or cavity absent, 0.5-1 cm in diameter, dingy yellow and smooth (Fig. 4a). Peridium approximately 90-230 µm thick, externally pseudoparenchimatous with rounded cells $9-20 \times 7-17$ µm (Fig. 4b). External cystidia absent. Spore-bearing tissue brown, veins numerous and dense.

Morphology of asci and spores - Asci globose, sometimes oval, $38-57 \times 37-53$ µm, (1)3(4) irregularly clustered spores (1)3(4). Spores ellipsoidal, $18-47 \times 18-30$ µm, pale brown or medium brown, reticulated (Fig. 4c).

The accession numbers of the obtained ITS1 / ITS4 sequences of the ribosomal DNA

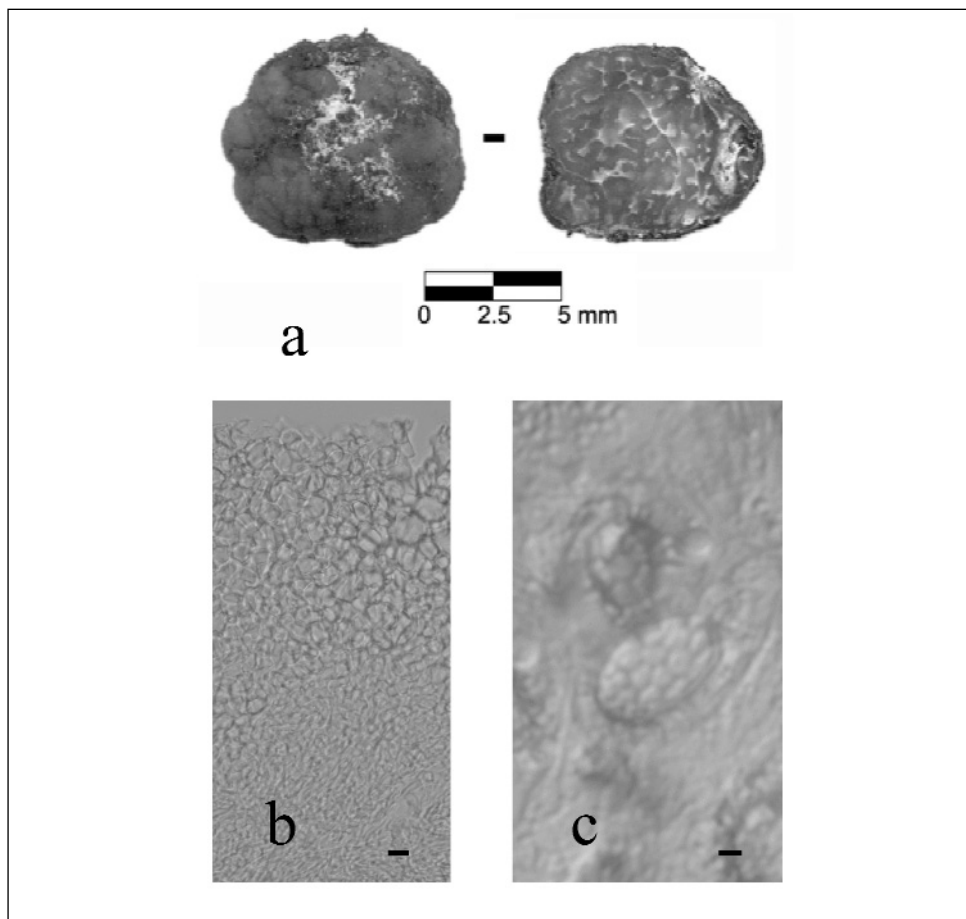


Fig. 4. Ascomata (a), peridium (b) and spores (c) of *Tuber rapaeodorum* (Bars = 7 μ m).

and the results of the Blast searches are listed in Table 2. A specific 568 bp amplicon was obtained by the amplification of the ascoma K 20 / 2203 by Ru1 and ITS4 primers confirming the morphological *T. rufum* identification.

Discussion

Tuber scruposum, *T. rapaeodorum* and *T. rufum* were found associated with *F. orientalis*, *C. betulus*, *T. cordata*, *C. avellana* and *Pinus sp.* in northern region of Ijevan (1400–2000 m asl) and southern region of Zangezur (850–950 m asl) towns on sylvan grey, sylvan brown and sylvan turf-carbonate soils. They were most abundant in moist areas on northern and north-eastern slopes of ravines, gullies, and on the banks of small mountain rivers and streams (Fig. 1, Table 1).

The ascomata of truffles recorded were always very small even when ripe. Such char-

Table 2. Results of molecular identification of Armenian truffles.

Identified species	Herbarium number		Region, town	Collection data	Sequence accession number	Best Blast identities
	FBBL-YSU	CMI-Unibo				
<i>Tuber scruposum</i>	D11	2201	Dilijan	9.07.2003	DQ011845	<i>Tuber sp.</i> , B-1667. AJ557539, identity 99.1%
<i>Tuber scruposum</i>	D7	2192	Dilijan	9.07.2003	DQ011846	<i>Tuber sp.</i> , B-1667. AJ557539, identity 99.1%
<i>Tuber scruposum</i>	D19	2207	Dilijan	10.07.2003	DQ011847	<i>Tuber sp.</i> , B-1667. AJ557539, identity 99.1%
<i>Tuber scruposum</i>	D13	2194	Dilijan	9.07.2003	DQ011848	Uncultured ectomycorrhiza of <i>Tuber sp.</i> AY634175, identity 97.4%
<i>Tuber rapaeodorum</i>	K24	2483	Kapan	13.06.2004	DQ011849	<i>Tuber rapaeodorum</i> , AJ557521, identity 99.6%
<i>Tuber rapaeodorum</i>	K27	2481	Kapan	13.06.2004	DQ011850	<i>Tuber rapaeodorum</i> , AJ557521, identity 99.6%

Notes: FBBL - YSU: Fungal Biology and Biotechnology Lab, Yerevan State University; CMI-Unibo: Herbarium of the Mycological Centre of the Bologna University, Dipartimento di Protezione e Valorizzazione Agroalimentare.

acter is probably due to which to the relatively dry conditions in the areas where they were collected. Besides the tendency to be immature made difficult the morphological characterization of some ascomata (D 7 / 2192, D 19 / 2207 and K27 / 2481). For the reasons stated above the use of molecular techniques is essential and the identification of these *Tuber* species was possible comparing their ITS sequences with those of morphologically identified ripe ascomata (D 11 / 2201, K27 / 2483).

The morphological characters of the specimens D 3 / 2197, D 11 / 2201, D 13 / 2194 correspond to Hesse (1894), Ceruti & al.'s (2003) and Gross's (1987) descriptions of *T. scruposum*. The pseudoparenchimatous peridium formed by small roundish cells is also different from Pegler & al.'s (1993) description of *T. rapaeodorum*. The specimens identified as *T. rapaeodorum* and *T. scruposum* are morphologically different from other species of the *T. borchii* group (*T. puberulum* Berk. & Broome, *T. maculatum* Vittad., *T. foetidum* Vittad. and *T. borchii* Vittad.) held in the CMI-Unibo herbarium and described by

Zambonelli & al. (2000). *T. scruposum* has an almost pseudoparenchymatic peridium with small cells which is different from those of *T. borchii*, *T. dryophilum*, *T. foetidum*, *T. puberulum* and *T. maculatum*. The ellipsoid or subglobose spores also differs from those of *T. puberulum* which are typically spherical. *T. rapaeodorum* has a very thin peridium, thinner than *T. borchii*, *T. foetidum*, *T. puberulum* and *T. maculatum* and similar to *T. dryophilum*. However, the external pseudoparenchymatic and internal plectenchymatic appearance does not resemble *T. dryophilum* which is predominantly pseudoparenchymatous. Identification of *T. rapaeodorum* was confirmed by comparing the ITS sequences of ribosomal DNA with those in the GenBank database (Tab. 2). Molecular identification of *T. scruposum* was not possible because its ITS sequence is lacking in GenBank database. ITS sequences of *T. scruposum* showed a high level of similarity (99.1 % of identity) only with a *Tuber* unidentified species.

The discovery of *T. scruposum* in Armenia confirms Ceruti & al.'s (2003) conclusions that the distribution area of this species is limited to north and central Europe.

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