Ecological specificities and molecular diversity of truffles (genus *Tuber*) originating from mid-west of the Balkan Peninsula

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Little is known on the diversity and distribution of truffles at the Balkan Peninsula. A first detailed study of hypogeous fungi in Serbia has been started at 1992. To extend the knowledge on European truffles and their natural habitats, data on their diversity in Serbia, and to some extent in Montenegro and FYRO Macedonia are summarised within this paper. Twelve species of the genus *Tuber,* including five varieties of T. *rufum* Pico, are recorded and their habitats briefly described. Four species are reported for the Balkan Peninsula for the first time. In addition, parts of ribosomal DNA (ITS regions) of 46 specimens are sequenced and analysed to confirm the morphological determinations. ITS sequences of specimens morphologically identified as *Tuber fulgens* Quél are reported for the first time. A phylogeny based on ITS sequences of the Balkan truffles and additional 29 records from the GenBank is calculated. The results are discussed towards expanding the information on distribution as well as ecological and molecular diversification of *Tuber spp*. in Europe.

Keywords: hypogeous Ascomycetes, phylogeny, ecology, Europe

Currently, 50 genera of hypogeous fungi are recorded in Europe which implies a lower diversity rate of hypogeous fungi then known in Australia or North America (Simpson 2000; Bougher & Lebel 2001). Recently, Montecchi & Sarasini (2000) described 178 European species, among them 22 species of *Tuber spp*. Based on morphological parameters, Riousset *et al.* (2001) recognize 24 European species of this genus, and divide them in 6 taxonomical groups. Many misidentifications and synonyms based on the morphological descriptions of ascoma created unclear overview of species of genus *Tuber* in Europe (reviewed in Riousset *et al.* 2001), and many authors tried to resolve this by using different methods of comparing DNA sequences originating from European truffles (Henrion *et al.* 1994; Gandeboeuf *et al.* 1997; Roux *et al.* 1999, Mello *et al.* 2000, 2002; Halasz *et al.* 2005; Weden *et al.* 2005; Jeandroz *et al.* 2008). Still, the problem with connecting morphological and the molecular identification of *Tuber spp.* is continuing. Within the most recent comprehensive monographic publication on the *Tuber spp.* in Europe, Ceruti *et al.* (2003) list 32 species and refer to the Mediterranean and sub-Mediterranean zones of Europe (Iberian and Apennine Peninsulas and Southern France) as the regions of greatest diversity of *Tuberaceae*. The Balkan Peninsula, as the largest and the most continental peninsula of the South Europe until now has been almost unexplored for hypogeous fungi.

Due to its geographic position, its geological, topographical and climatic diversity, the biodiversity of Balkan Peninsula is one of the highest in Europe (Stevanović *et al.* 1995; Myers 1999). Moreover, the area served as a refugium site for many European plants in the times of glaciations, and has therefore been the natural centre of plant diversification during times of interglaciations (Bennet *et al.* 1991; Taberlet *et al.* 1998; Petit *et al.* 2002a and 2002b). The western part of the peninsula is dominated by continental climate, with Mediterranean influence that is declining from the Adriatic sea towards central peninsular regions. Calcareous and very high Dinaric Alps start steeply from the Adriatic coast and spread eastward till the Western Serbia and northern Former Yugoslav Republic of Macedonia (FYROM). They form natural barrier for the wet maritime air currents, which causes annual precipitation rates gradient, decreasing from more then 2000ml in Montenegro coast, over app. 1000 ml in Western Serbia, to as low as 500 ml in east Serbia and FYROM (Atlas klime SFRJ 1969). Average annual temperatures vary between 8.8–16.6°C in Montenegro, 9.5–11.7°C in Serbia and 8.8–14.6°C in FYROM (Atlas klime SFRJ 1969). Seasonal variations of precipitation and temperature are pronounced in the northern and central regions of the peninsula. Such climate conditions determined environment supportive for temperate ectomycorrhiza (ECM) forming trees that dominate forest communities in entire investigated area (Stevanović *et al.* 1995, Kojić *et al.* 1997). Only small areas on the predominantly steep Adriatic coast are covered by macchia vegetation on the hillsides, and pines (*Pinus pinea* L., *P. pinaster* Ait.) or Mediterranean oaks (*Quercus pubescens* Wild., *Q. ilex* L.) on the plane areas.

From already known truffle-producing areas, ecological requirements and eco-geographic limits of European truffles are well documented (Szemere 1965, Alvarez *et al.* 1993; Riousset *et al.* 2001; Chevalier & Frochot 2002; Ceruti *et al.* 2003; Jeandroz *et al.* 2008). However, some data on truffle habitats in Serbia are contradictory to these findings (Milenković & Marjanović 2001). Records of *Tuber spp.* from the Balkan Peninsula have been sporadically published (Lindtner 1935;

Frančišković 1950; Hrka 1988; Pázmány 1991; Milenković *et al.* 1992; Glamočlija *et al.* 1997; Marjanović & Milenković 1998; Milenković & Marjanović 2001), but a number of data appeared to be unclear or outdated.

The aim of this report was to provide a comprehensive list of species of *Tuber spp.* found in Serbia during 17 years of investigation, as well as their molecular determination and relation to specimens originating from other European territories. Results from Montenegro and FYROM are included as well. Furthermore, an overview of some differential to previously reported characteristics of the ecosystems that support truffle fructification in the mid-west of Balkan Peninsula are provided. Therefore the distribution, general ecological features and molecular diversity of some European truffles is re-evaluated.

Materials and Methods

Sampling, morphological identification, preservation

Calcareous areas of Serbia and to some extent of Montenegro and FYROM (Fig. 1) have been explored for truffles by few collectors since 1992 with a help of trained dogs. More than three hundred collections (362) of *Tuber spp.* have been supplemented with comprehensive photo documentation and data on microscopic characters. Specimens have been preserved either by drying, or immersion in a 2:1 ethanol-glycerol mixture. Collections are presently deposited at the Institute for Biological Research „Siniša Stanković“ and at Institute for Multidisciplinary Research in Belgrade, Serbia. Specimens (great majority of them dry) have been morphologically identified according to Szemere (1965), Pegler *et al.* (1993), Zambonelli *et al.* (2000), Montecchi & Sarasini (2000), Riousset *et al.* (2001) and Ceruti *et al.* (2003). Only ecological data supplied together with specimens by collectors were analyzed: potential plant host as registered on the site, date of collection, approximate elevation and altitude of the site. Soil data were taken from Soil map of Serbia (Tanasijević *et al.* 1965, 1966) or registered by collector.

DNA isolation

About 50ng of dry fungal material of the inner part of ascomata was used for DNA extraction. Samples were taken using a binocular, to avoid contamination with soil particles or parasitic fungi. The DNA was extracted either by using 2% CTAB (Rogers & Bendlich 1985; Doyle & Doyle 1990) or by Plant DNeasy Mini Kit (Promega). After both procedures the DNA was suspended in pre-warmed, sterile Milli-Q water to the approximate final concentration 100 ng/μl.

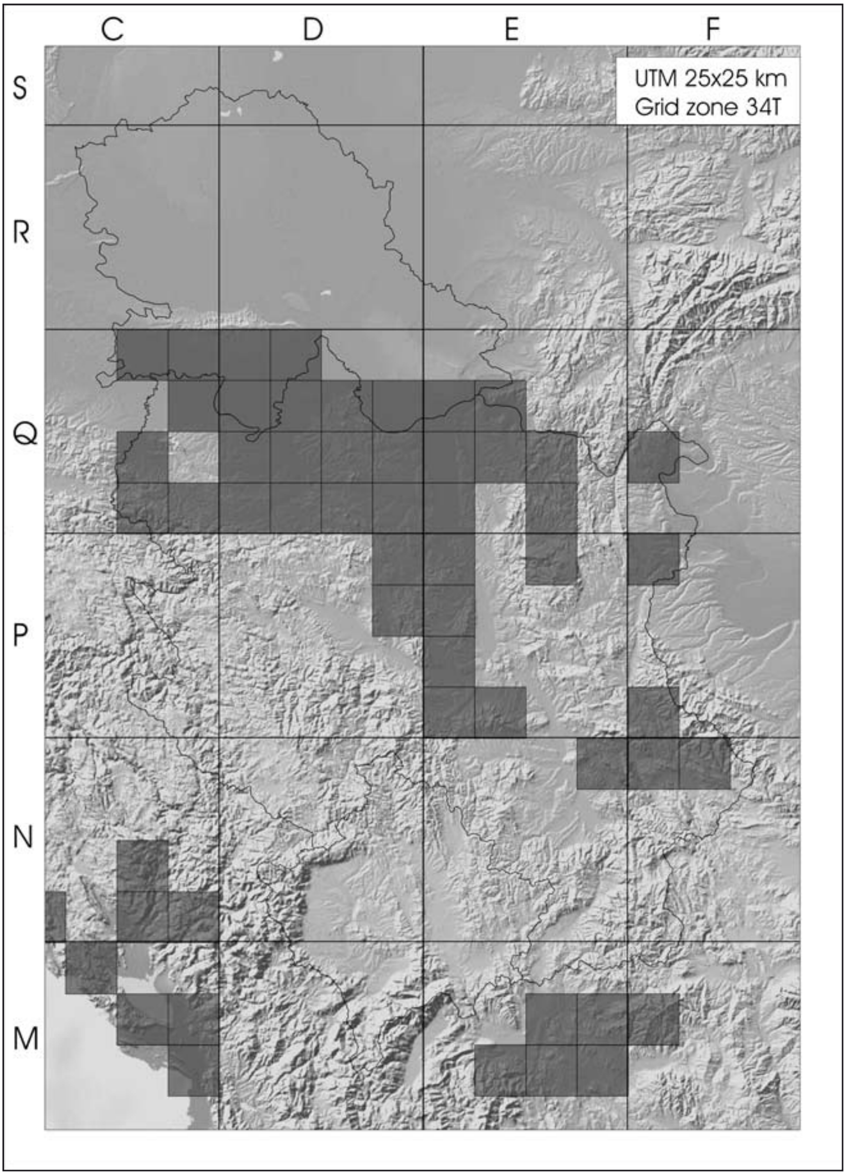

Figure 1. UTM map of the mid-west of Balkan Peninsula (Grid zone 34T). The codes of UTM quadrants are given. Shaded quadrants (size 25 x 25 km) represent area covered by investigation

PCR amplification

The fungal specific primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) were used for PCR amplification of the ITS1-5.8S-ITS2 rDNA region. Amplifications were done using standard procedure described in White *et al.* (1990) in a total reaction volume of 40 µl with AmplyTaq Gold polymerase and modified according to Kraigher *et al.* (1995), using a PE 9700 DNA thermocycler and annealing temperature of 55°C. When no or little PCR product could be obtained the annealing temperature was lowered down to 50 °C to. Negative controls were run for each experiment to check for the contamination of reagents. Amplified DNA was purified and analysed as described in Grebenc *et al.* (2000).

Sequencing

Prior to sequencing the amplified DNA was separated on 2% agarose gel and purified using the Promega Wizard SV Gel and PCR Clean-Up System. For sequencing the primers mentioned above were used, sequencing was conducted using a ABI Prism 310 automatic sequencer (Applied Biosystems). Sequencer 4.8 software (Gene Codes) was used to read, identify and clean the consensus sequence from the two strands of each isolate. All consensus sequences were blasted against the GenBank nucleotide database using a nucleotide query (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Sequences have been lodged in the EMBL database with accession numbers indicated in Tab. 2.

Phylogenetic analyses

Sequences were aligned using CLUSTAL W software package (Larkin *et al.* 2007). The NJ tree based on 393 informative characters was constructed applying *MEGA* version 4 (Tamura *et al.* 2007) using the K2P model presuming uniform substitution rates. Bootstrap values were calculated based on 10000 bootstrap replicates. Gaps were treated as missing data.

Results

Twelve species of the genus *Tuber* were recognised at the investigated area by morphological criteria according to Montecchi & Sarasini (2000). Four species (*T. maculatum*, *T. oligospermum*, *T. borchii* and *T. fulgens*) are new records for the Balkan Peninsula. The study also revealed first records of six species from Montenegro, and five species from FYROM (Tab. 1).

Table 1. – Diversity, distribution and ecological parameters of truffle species recorded in investigated part of Balkan Peninsula. Ascoma maturation periods expressed as Roman numbered months of the year.

Species (according to ascomata morphology)	Potential plant host	Maturation period distribution	Inclination altitude soil type
Tuber aestivum Vittad.	*Quercus cerris* L.	I-XII	0°– 60˚
	Quercus pubescens Wild.		
(exemined 44 specimens)	*Quercus frainetto* Ten.	Serbia	0 – 1000 m a.s.l
	Quercus robur L.	Montenegro	
	Fagus sylvatica L.	FYROM	cambisol
	Corylus avellana L.		rendzina
	Carpinus betulus L.		alluvium
	Betula pendula L.		lithosol
	Tilia sp.		vertisol
Tuber mesentericum Vittad.	*Quercus cerris* L.	I–III, VII–XII	20°– 60˚
	Quercus pubescens Wild	Serbia	
(exemined 13 specimens)	*Ostrya carpinifolia* Scop.	Montenegro	0 – 1000 m a.s.l
	Carpinus betulus L.	FYROM	
	Populus alba L.		rendzina
	Tilia sp.		lithosol
	Pinus pinea L.		cambisol
Tuber brumale Vittad.	*Quercus robur* L.	I–V, IX–XII	0° – 60˚
including *T. brumale var.*	*Quercus cerris* L.		
moschatum;	*Quercus pubescens* Wild	Serbia	0 – 500 m a.s.l
	Fagus silvatica L.	Montenegro	
(exemined 70 specimens)	*Ostrya carpinifolia* Scop.	FYROM	semiglay
	Populus alba L.		rendzina
	Tilia sp.		lithosol
	Pinus pinea L.		cambisol
			alluvium
Tuber macrosporum Vittad.	*Quercus cerris* L.	X – XII(I)	0° – 20˚
	Quercus robur L.		
(exemined 45 specimens)	*Carpinus betulus* L.	Serbia	0 – 500 m a.s.l
	Corylus avellana L.	Montenegro	
	Populus alba L.	FYROM	cambisol
	Salix sp.		aluvium
			rendzina
			vetisol
Tuber magnatum Vittad.	*Quercus robur* L.	IX – XII (I)	0° – 5˚
	Carpinus betulus L		
(exemined 36 specimens)	*Populus nigra* L.	Serbia	0 – 20 m a.s.l
	Populus alba L.		
	Salix sp. L.		semiglay
			humogley
			alluvium
			vertisol
Tuber oligospermum	*Quercus pubescens* Wild	XII – III	30° – 60˚
(Tul. & C. Tul.) Trappe	*Ostrya carpinifolia* Scop.		
	Carpinus orientalis Mill.	Serbia	200 – 500 m a.s.l;
(exemined 8 specimens)	*Pinus halepensis* Mill.	Montengro	
			rendzina

Species (according to ascomata morphology)	Potential plant host	Maturation period distribution	Inclination altitude soil type
Tuber maculatum Vittad. (exemined 15 specimens)	*Carpinus betulus* L. *Populus alba* L. *Populus sp.hybrid*	IV, VI, X – XII Serbia	0°– 5˚ 0 – 20 m a.s.l cambisol alluvium antropogenic soils
Tuber borchii Vittad. (exemined 2 specimens)	*Quercus cerris* L. *Quercus frainetto* Ten. *Carpinus betulus* L. *Populus tremula* L.	II – IV, XI Serbia	app 30˚ app 200 m a.s.l cambisol
Tuber foetidum Vittad. (exemined 3 specimens)	*Salix fragilis* L. *Salix alba* L. *Populus alba* L.	V, VI, XII Serbia	0° – 5˚ 0-20 m a.s.l humogley
Tuber excavatum Vittad. (exemined 54 specimens)	*Quercus robur* L. *Quercus cerris* L. *Quercus pubescens* Wild *Fagus sylvatica* L. *Corylus avellana* L. *Populus alba* L. *Salix* sp. *Pinus* sp.	VI – XII (I) Serbia Montenegro FYROM	0° – 30˚ 0 – 1200m a.s.l cambisol podzol alluvium rendzina
Tuber fulgens Quél. (exemined 16 specimens)	*Fagus sylvatica* L. *Tilia cordata* Mill.	VII, IX – X Serbia	0° – 30˚ 0 – 1200 m a.s.l rendzina cambisol
Tuber rufum Pico var.*rufum* *according to* Montecchi & Sarrasini (2000) (exemined 35 specimens)	*Quercus robur* L. *Quercus cerris* L. *Quercus fraineto* Ten. *Quercus rubra* L. *Fagus sylvatica* L. *Carpinus betulus* L. *Corylus avellana* L. *Betula pendula* Roth. *Populus nigra* L. *Populus alba* L. *Abies alba* L.	I – III, VII – XII; Serbia Montenegro FYROM	0° – 60˚ 0 – 1000 m a.s.l cambisol alluvium rendzina lithosol atropogenic soils
Tuber rufum var. *apiculatum* E. Fisch. *according to* Montecchi & Sarrasini (2000) (exemined 5 specimens)	*Fagus sylvatica* L. *Picea abies* L.	IX – XI Serbia	20° – 60˚ 500 – 1000 ma.s.l. Cambisol

Species (according to ascomata morphology)	Potential plant host	Maturation period distribution	Inclination altitude soil type
Tuber rufum var. *ferrugineum* (Vittad.) Montecchi & Lazzari *according to* Montecchi & Sarrasini (2000) (exemined 7 specimens)	*Quercus pubescens* Wild *Fagus sylvatica* L. *Tilia sp.* L.	IX, XI; Serbia	20° – 60° 500 – 1000 ma.s.l. cambisol Rendzina
Tuber rufum var. *lucidum* (Bonnet) Montecchi & Lazzari *according to* Montecchi & Sarrasini (2000) (exemined 3 specimens)	*Quercus cerris* L. *Carpinus betulus* L.	VII – X Serbia	20° – 30° 200 – 500 m a.s.l. cambisol
Tuber rufum var. *nitidum* (Vittad.) Montecchi & Lazzari *according to* Montecchi & Sarrasini (2000) (exemined 6 specimens)	*Quercus cerris* L. *Quercus robur* L. *Carpinus betulus* L. *Populus alba* L. *Populus nigra* L.	I, VI, XI – XII; Serbia	0° – 20° 0 – 500 m a.s.l alluvium cambisol

Distribution and ecological preferences of *Tuber* species in mid-west Balkan Peninsula

Neutral to weakly basic soils and presence of appropriate ECM forming tree host encourage the occurrence of *Tuber spp*. As the distribution of the host trees in Balkan Peninsula is strongly determined by altitude, exposition and soil water status, truffles are consequently following same distribution pattern as their host plants. While some species could be found in most forests dominated by ECM trees (*T. rufum, T. excavatum, T. aestivum,* and *T. brumale*), other species are restricted to areas defined by their exposition. Lowlands, especially the wide river valleys, and hilly regions in the mid-west of Balkan Peninsula generally host qualitatively and quantitatively different truffle communities. *Tuber macrosporum, T. magnatum, T. foetidum, and T. maculatum* were found to have their ecological optimum in Serbian lowland forests, while *T. borchii, T. oligospermum, T. fulgens,* and *T. mesentericum* show preferences for hills and mountains of mid-west Balkan Peninsula. Distribution and some general ecological features of truffle species that were registered at Balkan Peninsula are given in Tab. 1. Specific details on every species are described below.

Tuber rufum Pico (species concept according to Montecchi & Sarasini (2000)

Though never abundant, *T. rufum* is probably the most widely distributed species in all studied forests, but also in polluted city parks, or near high-traffic roads (Tab. 1). In Serbia, all varieties described in Montecchi & Sarasini (2000) were recorded, but the most common variety by far was var. *rufum* Pico, whereas var. *lucidum* (Bonnet) Montecchi & Lazzari, was recorded only twice. In Montenegro and FYROM only var. *rufum* Pico was recorded.

***Tuber excavatum* Vittad.**

In the Balkan Peninsula, *T. excavatum* is very common and plant host and soil non-specific truffle (Tab.1). Ascoma can be abundant, ripening in early summer to autumn and occurring in both broadleaved and conifer dominated forests. Particularly preferring moist seasons, this species forms large ascoma (radius up to 6cm) at the upper elevation ranges. On the soil surface of the lowland forests in late spring, small (radius less then 1cm) and very abundant ascoma were registered, but were disappearing in early summer from the spot.

***Tuber aestivum* Vittad.**

T. aestivum is widely distributed throughout ECM supporting forests in the entire investigated area. *T. uncinatum* Chatin (Chevalier & Frochot 2002), lately signed as morphological form of *T. aestivum* (Weden *et al.* 2005; Mello *et al.* 2002), is the most abundant in the lime or oak dominated forests on slopes exposed to north or west. In damp places with Mediterranean oaks in Serbia, or in the European beech dominating mountain forests of Montenegro, mature ascoma of *T. aestivum* were collected even in late winter.

***Tuber brumale* Vittad.**

T. brumale was found to be abundant in all habitats in Serbia and Montenegro that could be expected to host *T. melanosporum* (Mediterranean forests of *Quercus pubescens* Wild., *Q. ilex* L., .*Q. coccifera* L., *Ostrya carpinifolia* Scop. and *Carpinus orientalis* Mill,, on the shallow or stony calcareous soils). It is also very common in the Mediterranean coast forests dominated by *Pinus pinea* L. and *P. halepensis* Ait. or in continental lowland forests dominated by *Quercus robur* L. and *Populus spp*. In early winter, when it reaches fructification maximum, *T. brumale* is the most ubiquitous species in Serbia and Montenegro. A distinct difference in fragrance could be observed between specimens found in aerated, limestone-rich soils in hills, and those found in thick clay-rich soils of lowlands.

***Tuber macrosporum* Vittad. and *Tuber magnatum* Pico**

In autumn, *T. macrosporum* and *T. magnatum* are dominant and abundant in the Serbian lowland, mixed forests (Tab. 1). Sporadically, *T. macrosporum* was also collected in forests typical for the hilly regions of the continental part of Mesian Balkans (Tab.1, Kojić *et al.*1998) where *T. magnatum* has never been recorded. *T. macrosporum* specimens collected in Serbia seem to have significantly larger dimensions then those described in literature (Szemere 1965; Pegler *et al.* 1993; Montecchi & Sarasini 2000; Riuosset *et al.* 2001), obtaining often the weight up to 50g. In conditions of the continental part of Balkan Peninsula, *T. magnatum* is rather associated with the native poplars (predominantly *Populus alba* L.) than oaks (except *Q. robur* L.) or hornbeams, and prefers wet, often occasionally flooded, thick, clay-rich soils in the lowlands. *T. brumale*, *T. macrosporum* and *T. magnatum*, that are common in natural lowland poplar forests, have never been found in introduced tree plantations or parks.

Tuber foetidum **Vittad**

In lowland forests with even higher soil moisture (Tab.1), additional localities of *T. foetidum were* found, heretofore recorded only once in Serbia (Milenković & Marjanović 2001).

Tuber maculatum **Vittad.**

In the course of investigation of the plantations of different introduced hybrid poplars in the Serbian lowlands in late autumn and early winter of the very moist 2004/2005 season, *T. maculatum* could be recorded, for the first time at Balkan Peninsula. This was the only truffle species that has ever been recorded in the hybrid poplar plantations in investigated area. In the same season it was also found near the planted trees in parks and quite rarely in natural lowland forests (Tab.1).

Tuber borchii **Vittad.**

First time recorded for the Balkan Peninsula, T. borchii could be found only once in Serbia, on the Avala mountain (Belgrade county) in the mixed forest of Quercus cerris L., Q. frainetto Ten. with some introduced conifers. Contineous examination of T. magnatum habitats for the T. borchii revealed no results.

Tuber oligospermum **(Tul. & C. Tul.) Trappe**

T. oligospermum could be found for the first time at Balkan Peninsula, but with only few collections . Ascoma were collected in late winter, in natural mixed deciduous forests (Tab.1) and in planted forest of Pinus halepensis Mill. (not native tree species for the area), on the limestone hills of Western Serbia. The species was also recorded in early spring in Quercus ilex L. dominating communities on coastal sandy soils of Montenegro. Serbian samples were previously misidentified as T. puberulum (Tab. 2) due to untypical habitat, but when a typical example was found in Montenegro, it became clear that all collections were T. oligospermum, which could be confirmed by molecular analysis.

Tuber mesentericum **Vittad.**

In mid-west Balkan Peninsula, *T. mesentericum* occurs in environments similar to those of the sister-species *T. aestivum*, but always in soils with potentially high $CaCO_3$ content (Tanasijević *et al.* 1965; 1966). This truffle is not common but can be abundant and dominant in suitable habitats, for example in *Tilia sp.* dominated forests on wet calcareous sandy soils.

Tuber fulgens **Quél.**

In the similar ecosystems as *T. mesentericum*, *T. fulgens* was collected in Western Serbia, which was the first record for the Balkan Peninsula. It is ubiquitous in mixed forests of *Fagus sylvatica* L. at altitudes more then 1000 m (mountain Tara), but also occurs in *Tilia sp.*-dominated forests in North-East Serbia, always sharing its habitat with *T. mesentericum*. Even though of a very different origin, the soils in both localities were very calcareous (Marjanovic *et al.* unpublished).

T. borchii, T. fulgens, T. macrosporum, T. magnatum, T. foetidum and *T. maculatum* have by now been recorded only in Serbia. *T. oligospermum* was not recorded in FYROM. The rest of the species were present in all investigated countries. *T. melanosporum* Vitt., previously reported from Serbia (Milenković *et al.* 1992; Glamočlija *et al.* 1997) was not encountered in the present study. Revision of morphological and molecular characters of previously published and herbarised collections revealed that all can be assigned to *T. brumale*. Coastal and central areas of Montenegro were frequently searched for truffles but no additional species to the listed ones were discovered.

Molecular characterisation of truffles originating from the mid-west of Balkan Peninsula.

A total of 46 new ITS1-5.8S-ITS2 rDNA sequences of *Tuber* spp. were generated (for accession numbers see Tab. 2). Sequences were aligned with additional 29 truffle ITS sequences retrieved from GenBank for comparison and identification. Amplification of ITS-DNA of *T. mesentericum* was repeatedly unsuccessful, and therefore this species was not included in the phylogenetic analysis A neighbour joining tree is given in Fig. 2. As the tree was primarily constructed to support morphological identifications, distances and relations between taxa do not represent in-depth phylogenetic relations and therefore truffle phylogeny or taxonomy will not be discussed.

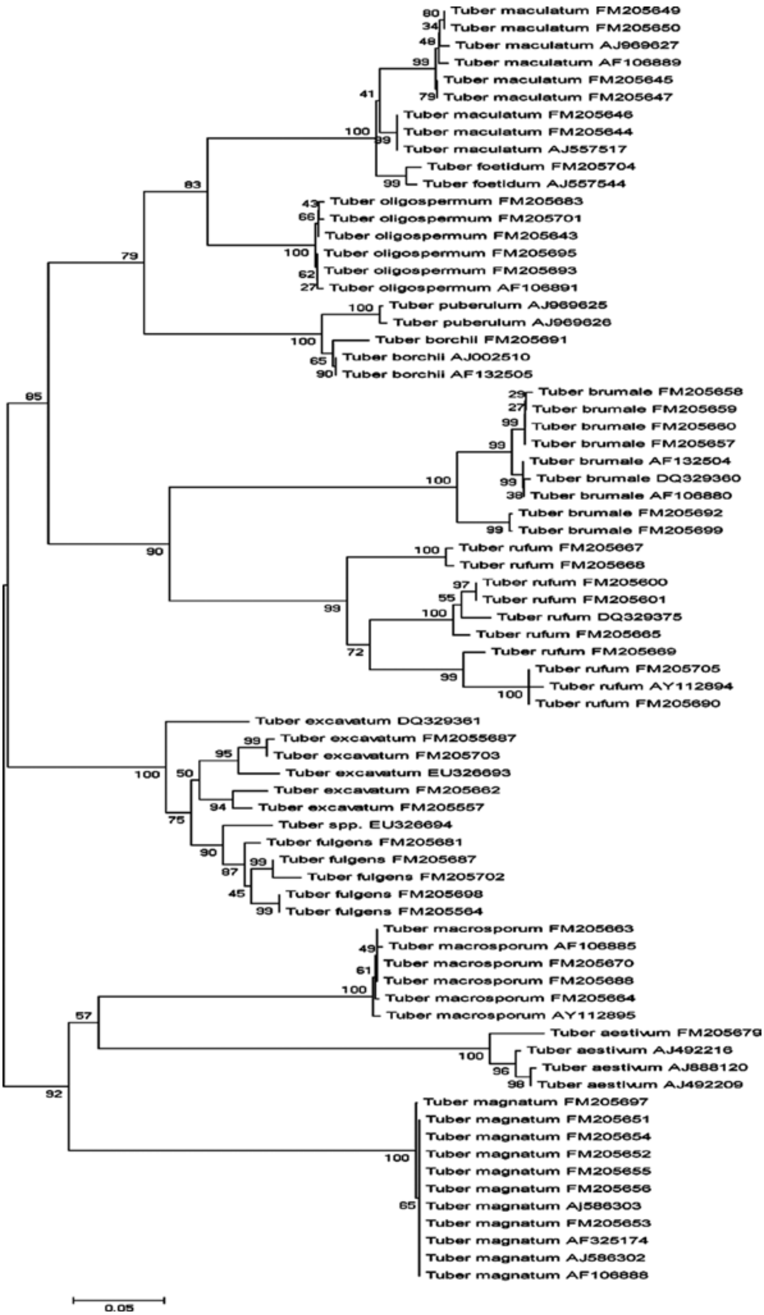

Figure 2. Unrooted NJ phylogenetic tree based on the alignment of ITS regions of truffles originating from Balkan Peninsula and an additional representative samples from other areas of Europe. Taxonomic names, GenBank accession numbers, and origin of the samples are listed in Tab. 2

Table 2. Morphological and molecular identifications of truffles recorded from Balkan Peninsula. GenBank accession numbers for rDNA ITS sequences included in the phylogenetic analysis are given.

Morphological determination	Identities according to BLAST search	GenBank	Geographic origin
Tuber puberulum Berk. & Broome	Tuber oligospermum (Tul. & C. Tul.) Trappe	FM205642	Dinaric Alps, West Serbia
Tuber puberulum Berk. & Broome	Tuber oligospermum (Tul. & C. Tul.) Trappe	FM205643	Dinaric Alps, West Serbia
Tuber maculatum Vittad.	Tuber maculatum Vittad.	FM205644	West Serbia
Tuber maculatum Vittad.	Tuber maculatum Vittad.	FM205645	West Serbia
Tuber maculatum Vittad.	Tuber maculatum Vittad.	FM205646	North Serbia
Tuber maculatum Vittad.	Tuber maculatum Vittad.	FM205647	Centa, Serbia
Tuber maculatum Vittad.	Tuber maculatum Vittad.	FM205649	Obrenovac, Serbia
Tuber maculatum Vittad.	Tuber maculatum Vittad.	FM205650	Maljen, Serbia
Tuber magnatum Pico	Tuber magnatum Pico	FM205651	West Serbia
Tuber magnatum Pico	Tuber magnatum Pico	FM205652	West Serbia
Tuber magnatum Pico	Tuber magnatum Pico	FM205653	Mid Serbia
Tuber magnatum Pico	Tuber magnatum Pico	FM205654	Sumadija, Serbia
Tuber magnatum Pico	Tuber magnatum Pico	FM205655	Sumadija, Serbia
Tuber magnatum Pico	Tuber magnatum Pico	FM205656	Sumadija, Serbia
Tuber magnatum Pico	Tuber magnatum Pico	FM205697	West Serbia
Tuber brumale Vittad	Tuber brumale Vittad	FM205657	Montenegro
Tuber brumale Vittad	Tuber brumale Vittad	FM205658	Montenegro
Tuber brumale Vittad	Tuber brumale Vittad	FM205659	Montenegro
Tuber brumale Vittad	Tuber brumale Vittad	FM205660	Montenegro
Tuber brumale Vittad	Tuber brumale Vittad	FM205692	West Serbia
Tuber brumale Vittad	Tuber brumale Vittad	FM205699	West Serbia
Tuber macrosporum Vittad.	Tuber macrosporum Vittad.	FM205663	Belgrade, Serbia
Tuber macrosporum Vittad.	Tuber macrosporum Vittad.	FM205664	Central Serbia
Tuber macrosporum Vittad.	Tuber macrosporum Vittad.	FM205670	North Serbia
Tuber macrosporum Vittad.	Tuber macrosporum Vittad.	FM205688	West Serbia
Tuber rufum f.lucidum (Bonnet) Montecchi & Lazzari	Tuber rufum Pico	FM205665	Montenegro coast
Tuber rufum Pico	Tuber rufum Pico	FM205667	Tara, Serbia
Tuber rufum Pico	Tuber rufum Pico	FM205668	Tara, Serbia
Tuber rufum var. apiculatum E. Fisch	Tuber rufum Pico	FM205669	Tara, Serbia
Tuber rufum var.nitidum (Vittad.) Montecchi & Lazzari)	Tuber rufum Pico	FM205677	Tara, Serbia
Tuber rufum var. rufum Pico	Tuber rufum Pico	FM205690	City park, Belgrade, Serbia
Tuber rufum var. rufum Pico	Tuber rufum Pico	FM205705	North Serbia
Tuber aestivum Vittad.	Tuber aestivum Vittad.	FM205679	West Serbia
Tuber fulgens Quél	no significant match	FM205681	Tara, Serbia
Tuber fulgens Quél	no significant match	FM205702	Tara, Serbia
Tuber fulgens Quél	no significant match	FM205698	West Serbia
Tuber fulgens Quél	no significant match	FM205685	West Serbia
Tuber excavatum Vittad.	Tuber excavatum Vittad.	FM205662	Tara, Serbia

Morphological determination	Identities according to BLAST search	GenBank	Geographic origin
Tuber excavatum Vittad.	Tuber excavatum Vittad.	FM205687	Ub, Serbia
Tuber excavatum	Tuber excavatum	FM205703	Tara, Serbia
Tuber oligospermum (Tul. & C. Tul.) Trappe	Tuber oligospermum (Tul. & C. Tul.) Trappe	FM205683	Montenegro
Tuber foetidum Vittad.	Tuber foetidum Vittad.	FM205704	West Serbia
Tuber borchii Vittad.	Tuber borchii Vittad.	FM205691	Avala, Serbia
Tuber puberulum Berk. & Broome	Tuber oligospermum (Tul. & C. Tul.) Trappe	FM205693	Dinaric Alps, West Serbia
Tuber puberulum Berk. & Broome	Tuber oligospermum (Tul. & C. Tul.) Trappe	FM205695	Dinaric Alps, West Serbia
Tuber sp.	Tuber oligospermum (Tul. & C. Tul.) Trappe	FM205701	West Serbia
Tuber fulgens Quél	no significant match	FM205564	Slovenia
Tuber sp.	no significant match	EU326694	Poland
Tuber excavatum Vittad.	Tuber excavatum Vittad.	FM205557	Slovenia
Tuber excavatum Vittad.	Tuber excavatum Vittad.	EU326693	Poland
Tuber excavatum Vittad.	Tuber excavatum Vittad.	DQ329361	Vaucluse, France
Tuber rufum Pico	Tuber rufum Pico	FM205600	Slovenia
Tuber rufum Pico	Tuber rufum Pico	FM205601	Slovenia
Tuber rufum Pico	Tuber rufum Pico	AY112894	Italy
Tuber maculatum Vittad	Tuber maculatum Vittad	AJ969627	Denmark
Tuber maculatum Vittad	Tuber maculatum Vittad	AF106889	Umbria, Italy
Tuber maculatum Vittad	Tuber maculatum Vittad	AJ557517	Hungary
Tuber foetidum Vittad	Tuber foetidum Vittad	AJ557544	Hungary
Tuber puberulum Berk. & Broome	Tuber puberulum Berk. & Broome	AJ969625	Suserup, South Zealand, Denmark:
Tuber puberulum Berk. & Broome	Tuber puberulum Berk. & Broome	AJ969626	Bromme Plantage, SouthZealand, Denmark:
Tuber borchii Vittad.	Tuber borchii Vittad.	AJ002510	Italy
Tuber borchii Vittad.	Tuber borchii Vittad.	AF132505	France
Tuber oligospermum (Tul. & C. Tul.) Trappe	Tuber oligospermum (Tul. & C. Tul.) Trappe	AF106891	Italy
Tuber brumale Vittad.	Tuber brumale Vittad.	AF106880	Marche, Italy
Tuber brumale Vittad.	Tuber brumale Vittad.	DQ329360	Vaucluse, France
Tuber brumale Vittad.	Tuber brumale Vittad.	AF132504	France
Tuber macrosporum Vittad.	Tuber macrosporum	AF106885	Umbria, Italy
Tuber macrosporum Vittad.	Tuber macrosporum	AY112895	Italy
Tuber magnatum Pico	Tuber magnatum Pico	AJ586303	Piedmont, Italy
Tuber magnatum Pico	Tuber magnatum Pico	AF325174	Italy
Tuber magnatum Pico	Tuber magnatum Pico	AJ586302	Piedmont, Italy
Tuber magnatum Pico	Tuber magnatum Pico	AF106888	Umbria, Italy
Tuber aestivum Vittad.	Tuber aestivum Vittad.	AJ888120	United Kingdom
Tuber aestivum Vittad.	Tuber aestivum Vittad.	AJ492209	Campobasso, Italy
Tuber aestivum Vittad.	Tuber aestivum Vittad.	AJ492216	Teruel, Spain

Discussion

Distribution and ecological preferences of *Tuber* species in mid-west Balkan Peninsula

The number of native species of *Tuber* reported for Europe ranges from 22 to 32, depending on the species interpretations of various authors (Montecchi & Sarasini 2000; Riuosett *et al*. 2001; Ceruti *et al*. 2003). The list of twelve *Tuber* species recorded for the investigated part of Balkan Peninsula is notable, when the relatively small area of investigation is taken into consideration. Among these, few species could be regarded as widespread throughout Europe: *T. rufum*, *T. aestivum, T. excavatum, T. borchii and T. maculatum* (Szemere 1965; Chevalier *et al*. 1986; Wojewoda & Lawrinowicz 1986; Pázmány 1991; Pegler *et al*. 1993; Montecchi & Sarasini 2000; Weden *et al*. 2000; Riuosett *et al*. 2001; Ceruti *et al*. 2003; Kers 2003; Jeandroz *et al*. 2008). The ecological features of these wide spread species in the investigated area, resembled those reported from other European areas (Szemere 1965; Wojewoda & Lawrinowicz 1986; Pázmány 1991; Pegler *et al*. 1993; Montecchi & Sarasini 2000; Riuosett *et al*. 2001; Chevalier & Frochot 2002; Ceruti *et al*. 2003). *Tuber mesentericum* and *Tuber fulgens* are restricted to South and Central Europe (Pegler *et al*. 1983; Ceruti *et al*. 2003; Jeandroz *et al*. 2008). Our results suggest that sister-species of *T. aestivum* and *T. excavatum*, represented by *T. mesentericum* and *T. fulgens* respectively, are adapted to the similar environments, but grow in soils of high $CaCO_3$ content.

In comparison to the rest of the Europe, the peculiarity of the mid-west Balkans in respect to the occurrence of *Tuber* species, is rarity of *T. borchii*, *T. maculatum* and *T. foetidum,* and the absence of *T. puberulum* and *T. dryophylum*, which have been reported as common all over continental Europe and British Isles (Szemere, 1965; Pegler *et al*. 1993; Ceruti *et al*. 2003; Jeandroz *et al*. 2008). The Balkan Peninsula seems not to be a suitable environment for small white truffles. In the ecological constraints of the Balkan Peninsula, *T. maculatum* is often a pioneer species that may help ECM forming trees to establish in adverse environments (plantations, parks). As this species is assumed to be very tolerant to unfavourable environmental conditions (Montecchi & Sarasini 2000; Ceruti *et al*. 2003), it is not clear why is it found so rarely at Balkan Peninsula. Indeed, *T. oligospermum*, that has been assumed to be connected to Mediterranean ecosystems and climate (Montecchi & Sarasini; 2000; Riuosett *et al*. 2001; Ceruti *et al*. 2003; Jeandroz *et al*. 2008), is common in the continental conditions of Serbia.. Even if its habitats in Serbia resemble those from Mediterranean zones in matters of vegetation, soils and climate are quite different, particularly the winter season. It can be postulated that this truffle is more dependent on specific host-plant species then on abiotic factors, as thought previously (Montecchi & Sarasini 2000, Ceruti *et al.* 2003). *T. foetidum*, reported to be distributed in Western Europe (Jeandroz *et al.* 2008) was collected few times at Balkan Peninsula.

All European commercially important truffles, except *T. melanosporum* and *T. borchii*, are widespread in Serbia. While in Southern Europe and bordering regions *T. brumale* and *T. macrosporum* are rather common, there are only few records in other parts of Europe (Pegler *et al.* 1993, Riousset *et al*, 2001, Ceruti et al. 2003). As unusually big *T. macrosporum* ascoma were recorded often in Serbian lowland forests, this species probably has its distribution optimum in the warmer climates, on the sites where soil water is not strongly limited. Even if the climate, soils and vegetation in Montenegro and Western Serbia resembles conditions optimal for *T. melanosporum*, this species has never been recorded in investigated area. Instead, *T. brumale* ascoma of a very good quality and unusual smell was recorded on hills with calcareous stony soils and Mediterranean vegetation. The historical reasons could be postulated as explanation – when following the routes of oaks recolonization after the last ice age, no oak haplotype that was carrying *T. melanosporum* was registered to originate from the Balkan Peninsula (Murat *et al.* 2004).

T. magnatum was assumed to be primarily distributed in Central and Northern Italy, and in Istria, Croatia (Hall *et al.* 1998, Ceruti *et al.* 2003; Mello *et al.* 2005), with some additional records from southeast of France and Ticino Canton in Switzerland (Lawrynovicz 1993; Zambonelli & Di Munno, 1991), but there are also records published from Serbia (Marjanović & Milenković 1998), Hungary (Bratek *et al.* 2003) and Slovenia (Piltaver & Ratoša 2006). Lowland ecosystems where *T. magnatum* is usually found in Serbia (clay rich, often flooded soils, Tab.1) are clearly different from hilly sites reported from Italy (silty, well aerated soils, Lulli & Primavera 2000). It appears that, the so called "Piemont" white truffle has much wider area of distribution and less restricted ecological requirements then previously claimed (Hall *et al.* 1998, Ceruti *et al.* 2003). However, much more detailed study is required for final conclusions on the ecological limits of this species.

In comparison to other European regions, with high truffle diversity (Ceruti *et al.* 2003), the western Balkan Peninsula is specific for truffle chorology and ecology in a few points: *T. magnatum* is widespread all over river valleys in clay-rich soils, but has never been recorded on hills and well aerated soils; *T. borchii*, *T. maculatum and T. foetidum* are very rare, while *T. puberulum* and *T. dryophylum* by now have not been recorded; *T. oligospermum* is not specific for Mediterranean coast-lines only – it is common at the interior continental parts of the peninsula as well; some true Mediterranean species (*T. asa*, *T. belonae, T. panniferum, T. malenconii*), as well as *T. melanosporum* are by now missing.

Molecular diversity of truffles in the mid-west of Balkan Peninsula

Phylogenetic analysis of our sequence data together with data from GenBank revealed a neighbour joining tree with an over all similar topology of trees already published (Jeandroz *et al.* 2008). Morphological determination of some small white truffles did not appear to be in accordance with molecular data. Two samples initially determined as *T. puberulum* Berk. & Broome due to their continental habitat, appeared to be morphologically very similar *T. oligospermum* (Tul. & C. Tul.) Trappe, the species that had been previously collected only in Mediterranean regions (Cerruti *et al.* 2003). Two very small herbarium samples determined as *T. borchii* (according to Montecchi & Sarasini 2000) belonged to the *T. maculatum* clade in the NJ-tree. Halász *et al.* (2005) reported almost clear correlation between morphological and molecular determination of small white truffles in Hungary. However, other authors that compared sequences from the other European regions, could not observe this correlation (Wang *et al.* 2007, Jeandroz *et al.* 2008). As all these analyses (including our) were performed on quite a small number of sequences, we suggest that more detailed analysis of small white truffles on the global level is necessary for clarifying species delimitation, and realistic phylogenetic relationships.

As reported before (Iotti *et al.* 2007;Wang *et al.* 2007) , *T. rufum* appeared to be genetically very diverse species, but morphological forms (varieties) described by Montecchi & Sarasini (2000) were not supported by the molecular analysis (Fig. 2). *T. excavatum* and *T. fulgens* were not clearly separated in the NJ-tree. More specimens of both taxa must be investigated by morphological and molecular methods for a clear decision if *T. fulgens* is a distinct species, or just a variety of *T. excavatum*. From our analysis it seems likely that *Tuber sp.* published from Poland by Hilszczanska *et al.* 2008 (Acc. No. EU326694 Tab.1, Fig. 2) is *T. fulgens*.

We have observed minor, two base pairs variation within the ITS regions of *T. magnatum* (Acc. No. FM205697, Tab. 2) originating from Serbia (data not shown). This could be important as no ITS diversity was detected in samples from Italy and Istria by Mello *et al.* (2005) or Rubini *et al.* (2005). Still, much more sequences should be investigated in order to confirm possible diversity in ITS region of *T. magnatum*.

Comparing truffels originating from mid-west Balkan Peninsula with those from other parts of Europe, it seems likely that some species differ in their favoured ecological conditions, but only slight differences in the ITS sequences could be detected. An adaptation to the specific climatic, edaphic and vegetational conditions of the Balkan Peninsula can be speculated, due to climatic changes throughout European bio-geographic history. During periods of glaciation, there might have been refugial sites of truffles and their host plant hosts at the Balkan Peninsula (Petit *et al.* 2002a,b; Murat *et al.* 2004). The list of truffles from the mid-west of Balkan Peninsula will be expanded with additional collectings in an extanded area. More species can be expected from regions with strong Mediterranean influences, together with mountains and canyons, which could have been potential glacial refugia.

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