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

Marjanović, Ž.[1*], Grebenc, T.[2], Marković, M.[3], Glišić, A.[4], and Milenković, M.[5]

[1]Institute for Multidisciplinary Research, Kneza Višeslava 1a, 11 030 Beograd, Serbia,

[2]Slovenian Forestry Institute, Večna pot 3, SI-1000 Ljubljana, Slovenia

[3]Hajduk Veljkova 51/9, 14 000 Valjevo, Serbia

[4]Makedonska 34, 26340 Bela Crkva, Serbia

[5]Institute for Biological Research "Siniša Stanković", Bulevar Despota Stefana 142, 11000 Beograd, Serbia

Marjanović, Ž.[1], Grebenc, T.[2], Marković, M.[3], Glišić, A.[4], and Milenković, M.[5] (2009) **Molecular diversity and ecological specificity of truffles (genus *Tuber*) originating from mid-west of the Balkan Peninsula** Sydowia XX (X): pp-pp

*

* corresponding author E-mail: zaklina@ibiss.bg.ac.yu

Balkan Peninsula is the last part of the South Europe to be investigated for truffles (*Tuber spp*.). The first study of hypogeous fungi (truffles and truffle-like fungi) in Serbia has been underway since 1992. In order to upgrade the data on European truffles and their natural habitats, we summarized the up to date results on their diversity in Serbia, and to some extent in Montenegro and FYRO Macedonia, as a part of scarcely explored Balkan Peninsula. Twelve species of the genus, including five varieties of *Tuber rufum* Pico, were recorded and their habitats briefly described. Four species are for the fist time reported for the Balkan Peninsula. In addition, parts of ribosomal DNA (ITS regions) of 46 specimens were analysed to confirm morphological determinations. ITS sequences of specimens morphologically described as *Tuber fulgens* Quél are reported for the first time here. In order to illustrate approximate phylogenetic relations to the specimens originating from the other European areas, additional 29 truffle ITS sequences from the GenBank were used for construction of phylogenetic tree. The results were discussed towards expanding the information on distribution as well as ecological and molecular diversification of *Tuber spp*. in Europe.

Key Words: hypogeous Ascomycetes, phylogeny, ecology, Europe

Currently, 50 genera of hypogeous fungi are recorded for Europe, only two being endemic, implying that diversity of fungi producing hypogeous carpophore in Europe is lower than in Australia or North America (Simpson 2000; Bougher & Lebel 2001). In the most recent publication Montecchi & Sarasini (2000), 178 European species were described, 22 being species of genus *Tuber*. Comprehensive monographic publication on the *Tuber spp*. in Europe by Ceruti *et al*. (2003), lists 32 species, signing Mediterranean and sub-Mediterranean zones of Europe (Iberian and Apennine Peninsulas and Southern France) as the regions of greatest diversity of hypogeous *Ascomycota*, and especially *Tuberaceae*. Still, the largest and the most continental peninsula of the South Europe (Balkan Peninsula) was almost unexplored for hypogeous fungi, including “true” truffles (*Tuber spp*.).

On the other hand, due to its geographic position, geological, topographical and climatic diversity, Balkan Peninsula have produced an environment conducive to highest biodiversity rates in Europe (Stevanović *et al*. 1995; Myers 1999). Through the geological history, area served as a refugium site for many European plants in the times of glaciations, and therefore the natural centre of plant diversification in the times of interglaciations (Bennet *et al*. 1991; Taberlet *et al.* 1998; Petit *et al*. 2002a and 2002b). Nowadays, western part of Peninsula (Fig 1) is dominated by continental climate with more or less pronounced Mediterranean influence coming from the Adriatic sea. Inside the Peninsula, continental climate supports formation of temperate forests dominated by numerous ectomycorrhiza forming species (ECM) of *Fagaceae* (up to 1700m altitude) and *Pinaceae* (presumably higher altitudes, Stevanović *et al*. 1995). Accordingly, ECM tree species dominate in 34 types of forest communities in Serbia recorded by now (Kojić *et al.* 1997), and 14 of them potentially hosts *Tuber* species. On the predominantly steep Adriatic coast, typical Mediterranean climate causes formation of the macchia vegetation on the slopes and pines (*Pinus pinea, P. pinaster*) or Mediterranean oaks (*Quercus pubescens, Q. ilex*) dominating patches on the more plane areas. Continental climate of Former Yugoslavian Republic of Macedonia (FYROM) is characterised by generally lower precipitation rates then other two countries.

Available information from already known truffle-producing areas revealed an impression that eco-geographic limits of European truffles were quite clear (summarised in Cerruti *et al.* 2003, Jeandroz *et al*. 2008). However, some data coming from the research conducted in truffle habitats in Serbia did not match these findings (Milenković & Marjanović 2001). Collections 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.

This report provides a comprehensive list of species of the genus *Tuber* found in Serbia during 17 years of investigation, as well as their molecular determination and approximate correlation to specimens originating from other European territories. Results of less detailed study of selected areas of Montenegro and FYROM were included as well, as they represent first data coming from those regions. Furthermore, an overview of potential ectomycorrhizal truffle hosts and some characteristics of the ecosystems that support truffle fructification in the mid-west of Balkan Peninsula are provided as well. Considering these data, we discuss the new reported areas of distribution of some European truffles, as well as their differentiating ecological features and molecular diversity.

Materials and Methods

Calcareous areas of Serbia and to some extent Montenegro and FYROM have been explored in search for truffles throughout truffle-production seasons since 1992. Ascomata were discovered with a help of trained dogs and identified after the keys for hypogeous fungi of Szemere (1965), Pegler *et al.* (1993), Zambonelli *et al.* (2000), Montecchi & Sarasini (2000), Ceruti *et al*. (2003). Over two hundred collections of the ascomata belonging to genus *Tuber* were supplemented with comprehensive photo documentation and data on microscopic characters, and preserved either by drying, or immersion in a 2:1 ethanol-glycerol mixture. Collections, supplemented with all data, and photographic material were deposited in the Institute for Biological Research „Siniša Stanković“ and presently in Institute for Multidisciplinary Research in Belgrade, Serbia.

Molecular identification of ascomata

About 50ng of fungal material was used for DNA extraction after careful removal of the inner part of ascomata under 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 resuspended in pre-warmed, sterile Milli-Q water to the approximate final concentration 100 ng/μl.

PCR amplification

Fungal specific primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al*. 1990) were used for PCR amplification of the entire ITS regions. 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 after Kraigher *et al*. (1995), using a PE 9700 DNA thermocycler and annealing temperature of 55ºC. For several samples giving only low amplification the annealing temperature was lowered down to 50 ºC to obtain sufficient amount of DNA. Negative controls were run for each experiment to check for the contamination of reagents. Amplified DNA was separated and analysed as described in Grebenc *et a*l. (2000).

Sequencing

Prior to sequencing the amplified DNA was separated on 2% agarose gel, excised and purified using the Promega Wizard SV Gel and PCR Clean-Up System. Fragments were re-amplified in sequencing reaction using the same primers as in an initial amplification, purified, and analysed on 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 searched for similarities to a GenBank nucleotide database using a nucleotide query (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to check for correct amplification. Obtained sequences have been lodged in the EMBL database with the accession numbers indicated in Table1.

Phylogenetic analyses

Sequences were aligned using CLUSTAL W software package (Larkin *et al.* 2007), and used for construction of approximate phylogenetic tree. As the purpose of this analysis was to confirm ascoma determination performed using morphological descriptions of macro and micro features, only few additional sequences from the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) were used for the tree construction. These sequences were chosen to represent diverse geographic origin of ascoma within Europe. The NJ tree based on 393 informative sites was constructed by using *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

At the present, twelve species of the genus *Tuber* were recognised according to morphological criteria after Montecchi & Sarasini (2000), from the collections originating from investigated area. Four species were new recorded from the Balkan Peninsula. The study also revealed first lists of six recorded species from Montenegro, and five from FYROM.

Ecosystems supporting truffle production in the conditions of Balkan Peninsula

Some common features characterise truffle supporting forest ecosystems in the mid-west of Balkan Peninsula: neutral to weakly basic soils and presence of appropriate ECM forming tree host. As the host trees distribution is strongly determined by altitude, exposition and soil water status, ECM forming truffles are consequently following same distribution pattern. While some species can be found in majority of temperate forests dominated by ECM trees and formed on the suitable soils, habitats of other, more demanding species is eco-topologically defined by the exposition. Lowlands, especially the wide river valleys of the Balkan rivers, and hilly regions in the mid-west of Balkan Peninsula generally host qualitatively and quantitatively different truffle communities.

Truffle species common and wide spread in the entire investigated part of mid-west Balkan Peninsula

Tuber rufum Pico

Though never abundant, *Tuber rufum* Pico is probably the most widely distributed *Tuber spp*. in investigated area. In Serbia, all varieties described in Montecchi and Sarasini (2000) were recorded (*var rufum* Pico, var. *apiculatum* E. Fisch.; var. *ferrugineum* (Vittad.) Montecchi & Lazzari; var. *lucidum* (Bonnet) Montecchi & Lazzari; var. *nitidum* (Vittad.) Montecchi & Lazzari), but the most common by far was var. *rufum*, whereas var. *lucidum* was recorded only twice. In Montenegro and FYROM only var. *rufum* Pico was recorded. *T. rufum* in Balkan Peninsula is host non-specific species, as it is commonly found with native *Fagus sylvatica, Corylus avellana, Quercus robur, Quercus cerris, Quercus fraineto, Populus alba, Populus nigra, Carpinus betulus, Betula pendula, Abies alba* and an introduced *Quercus borealis.* Ascomata can be found from the July to November in all studied forests, but also in adverse conditions like polluted city parks, or near high-traffic roads.

Tuber excavatum Vittad.

In the Balkan Peninsula, *Tuber excavatum* Vitt. is another very common and soil non-specific truffle. Common hosts of this species are: *Pinus sp., Quercus cerris, Q. frainetto, Quercus robur, Populus alba, Salix sp, Fagus sylvatica* and *Corylus avellana.* Ascomata can be abundant, ripening in early summer to autumn and occurring both in broadleaved and conifer dominated forests. Particularly preferring moist seasons, this species forms unusually large ascomata (up to 50g) at the upper elevation ranges. On the other side, small (radius less then 1cm) but very abundant ascomata can be found on the soil surface of the lowland forests in late spring, disappearing in early summer from the spot.

Tuber aestivum Vittad.

On neutral to weakly basic soils, *T. aestivum* is widely distributed throughout ECM-supporting forests in the entire investigated area. *Tuber uncinatum* Chatin (Chevalier & Frochot 1997), 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 the slopes exposed to north or west. Ascomata were found under various broad-leaved host trees - *Quercus robur, Fagus sylvatica, Corylus avellana, Carpinus betulus, Populus nigra, Betula pendula, Tilia sp.*, including non-native *Quercus rubra*. In damp places with Mediterranean oaks in Serbia, or in the *Fagus sylvatica* dominating mountain forests of Montenegro, ripe ascomata of *T. aestivum* were collected even in late winter.

Tuber brumale Vittad.

Investigation of habitats in Serbia and Montenegro that could be expected to host *T. melanosporum* (Mediterranean forests of *Quercus pubescens, Q. ilex, Q, coccifera, Ostria carpinifolia* and *Carpinus orientalis,* on the shallow or stony calcareous soils) or in Mediterranean coast forests dominated by *Pinus pinea* and *Pinus halepensis*, revealed always *T. brumale* Vittad. as abundant and dominant species. It is also very common in the lowland forests dominated by *Quercus robur* and *Populus spp*. with the fructification maximum in early winter (January, February), when the dominating in such forests, *Tuber magnatum* Pico, is strongly declining. In late autumn and winter *T. brumale* is the most ubiquitous species in Serbia and Montenegro. A strong fragrance differentiation was observed between specimens found in aerated, limestone-rich soils in hills, and those found in thick clay-rich soils of lowlands.

Truffle species with ecological optimum in hills and mountains of mid-west Balkan Peninsula

Tuber borchii Vittad.

After years long search, *Tuber borchii* Vittad. was recorded only once in Serbia, on the Avala mountain (Belgrade county) in the mixed forest of *Quercus cerris, Q. frainetto* with some introduced conifers. This is the first and only confirmed record of the species for Balkan peninsula. Contineous examination of *T. magnatum* habitats for the *T. borchii* revealed no results.

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

Quite a few recent collections of *Tuber oligospermum* (Tul. & C. Tul.) Trappe were heretofore the first records for Balkan Peninsula. Ascomata were collected in late winter, in natural mixed deciduous forests (*Quercus cerris; Q. frainetto; Carpinus betulus,* or near *Populus tremula L.*) and in planted forest of *Pinus halepensis* (not native for the area), on the limestone hills of Western Serbia. The species was also recorded in early spring in *Quercus ilex* dominating communities on coastal sandy soils of Montenegro. Serbian samples were previously misidentified as *T. puberulum* due to untypical habitat, but when a typical example was found in Montenegro, it became clear that all collections were *T. oligospermum*. Molecular analysis confirmed this outcome.

Tuber mesentericum Vittad**.**

In mid-west Balkan Peninsula, *Tuber mesentericum* Vittad**.** occurs in environments similar to those of the sister-species *T. aestivum*, but always in soils with very high $CaCO_3$ content. *Tilia spp., Quercus cerris, Quercus frainetto* and *Carpinus betulus* are potential plant hosts. This truffle is not common but can be abundant and dominant in suitable habitats, for example in *Tilia*-dominated forests on wet calcareous sandy soils. It appears to prefer mesic conditions and fruits in autumn and early winter.

Tuber fulgens Quél**.**

In the similar ecosystems as *T. mesentericum*, *Tuber fulgens* Quél was recently collected in Western Serbia, again the first records for the Balkan Peninsula. It is ubiquitous in mixed forests of *Fagus sylvatica* and *Abies alba* at altitudes more then 1000 m (mountain Tara), but also occurs in *Tilia*-dominated forests near the Deliblato Sands (North-East Serbia), always sharing habitat with *T. mesentericum.* Even though of a very different origin, the soil in both localities was very calcareous (Marjanovic *et al*, unpublished).

Truffle species with ecological optimum in Serbian lowland forests

Tuber macrosporum Vittad. and *Tuber magnatum* Pico

In autumn, *Tuber macrosporum* Vittad. and *Tuber magnatum* Pico are dominant and abundant in the lowland, mixed forests dominated by native poplars (*Populus alba, P. nigra*), pedunculate oak (*Quercus robur, Q. robur ssp. slavonica*) and ash trees (*Fraxinus angustifolia, F. exelsior*). Even though they often share habitats, *T. macrosporum* has been mostly found in the drier micro locations then *T. magnatum*. Sporadically, it was also collected in communities typical for the hilly regions of the continental part of Mesian Balkans (mixed forests of *Quercus cerris*, *Q. frainetto* and other white oaks, and *Carpinus betulus*) 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), 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*) than oaks (except *Q. robur*) 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, we found additional localities of *Tuber foetidum* Vittad, heretofore recorded only once in Serbia (Milenković & Marjanović, 2001). The potential hosts for this truffle were *Salix fragilis, Salix alba and Populus alba.*

Tuber maculatum Vittad.

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, revealed *Tuber maculatum* Vittad., for the first time recorded in Balkan Peninsula. In the same season it was also recorded near the planted trees in parks (*Populus nigra, Picea abies, Tilia sp*., *Pinus sp.)* and quite rarely in natural lowland forests. Heretofore it has never been found again.

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. *Tuber melanosporum Vittad.,* previously reported from Serbia (Milenković *et al.* 1992; Glamočlija *et al.* 1997) was not encountered in the present study. Revision of morphologic and molecular characters of previously published and herbarised collections showed them all to be *Tuber brumale* specimens. Therefore, this species cannot be accepted as present in Serbia. 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 Balkan Peninsula.

A total of 46 new rDNA ITS sequences from the genus *Tuber* spp. were generated (see Table 1 for accession numbers). 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 into the phylogenetic analysis. Sequence similarity to samples available in GenBank confirmed morphological identification. The first molecular identification of specimens morphologically described as *Tuber fulgens* Quél is presented here (Table 1). All newly obtained and the selected public available sequences were used to produce phylogenetic tree (Figure 2). As the tree was primarily constructed to support morphological identifications, distances and relations between taxa do not represent in-depth phylogenetic relations and will not be discussed.

Discussion

Ecological and chorological specificities of truffles in the mid-west of Balkan Peninsula

The number of native species of *Tuber spp.* reported for Europe ranges from 22 to 32, depending on the species interpretations of various authors (Alvarez *et al*. 1992; Alvarez *et al.* 1993; Pegler *et al.* 1993; Montecchi & Sarasini 2000; Ceruti *et al.* 2003). Out of these, *T. maculatum* and possibly *T. rufum* have also been recorded in North America (Gilkey 1939, 1954, Trappe & Cázares 2000). As to species shared by Europe and Asia, *Tuber maculatum* has been reported from Japan under the name *Mukagomyces hiromichii* Umai as well as from Taiwan (Trappe & Cázares 2000), and *T. borchii* was reported from China (Yun 1988). The rest of the species recorded in Balkan Peninsula so far, can be assumed as European.

Apart from widely known data on commercial species (Chevalier & Frochot 2002; Murat *et al.* 2004; Mello *et al.* 2005), distribution data on the truffles in Europe are rather scarce. The list of twelve *Tuber spp*. species recorded for studied part of Balkan Peninsula is notable, considering the relatively small area of investigation. Among these, few could be regarded as widespread throughout Europe: *T. rufum* and *T. aestivum* probably occur all over Europe (Montecchi & Sarasini 2000; Pegler *et al*. 1993; Chevalier *et al.* 1986; Wojewoda & Lawrinowicz 1986; Pázmány 1991; Weden *et al*. 2000; Ceruti *et al.* 2003, Kers 2003). *Tuber mesentericum* has much the same distribution as *T. aestivum* but appears to be far less frequent (Wojewoda & Lawrinowicz 1986; Pegler *et al.* 1983; Ceruti *et al.* 2003; Kers 2003). Also common all over Europe, *T. excavatum* does not extend north of Denmark, and in Britain it is restricted to southern England beech forests (http://www.netbiologen.dk/rodliste/rodsvampe.htm, Pegler *et al.* 1993). *Tuber fulgens* has been seldom mentioned in the literature and is probably rare, but widely distributed as *T. excavatum* (Ceruti *et al.* 2003). It seems that sister-species of *T. aestivum* and *T. excavatum*, represented by *T. mesentericum* and *T. fulgens* respectively, are adapted to the similar environments, but inhabit soils of high $CaCO_3$ content. Ecological features of these wide spread species (*T. rufum, T. excavatum, T. fulgens, T. aestivum, T. mesentericum*) were resembling those reported from other European areas (Montecchi & Sarasini 2000; Pegler *et al*. 1993; Chevalier *et al.* 1986; Wojewoda & Lawrinowicz 1986; Pázmány 1991; Weden *et al*. 2000; Ceruti *et al.* 2003, Kers 2003). .

The peculiarity of the mid-west Balkans in comparison to the rest of the Europe, is rarity of *T. borchii*, *T. maculatum* and *T. foetidum,* and the absence of *T. puberulum* and *T. dryophyllum*, which have been reported as common all over Europe and British Isles (Wojewoda & Lawrinowicz, 1986; Pegler *et al.* 1993; Ceruti *et al.* 2003, Jeandroz et al, 2008). At this stage, it appears that, to the opposite of the rest of Europe, Balkan Peninsula is not suitable environment for small white truffles. Accordingly, we infer that in the ecological conditions of Balkan Peninsula, *T. maculatum* is often a pioneer species that helps ECM forming trees to establish in adverse environments (plantations, parks). As it is assumed to be very tolerant species in reported European habitats (Montecchi & Sarasini 2000, Ceruti *et al*. 2003, Halász *et al*, 2005, Jeandroz et al, 2008), it is not clear why is it forming ascomata so rarely in Balkan Peninsula. On the other hand, *T. oligospermum*, that was widely accepted as connected to Mediterranean ecosystems and climate (Montecchi & Sarasini, 2000, Ceruti *et al.* 2003, Jeandroz et al, 2008), is more common in the continental conditions of Serbia then *T. borchii*. Even if its habitats in Serbia resemble those from Mediterranean zones in the sense of vegetation, the soils and climate are quite different, particularly the winter season. It can be postulated that this truffle is more dependant on specific host-plant species then on abiotic factors, as previously thought (Montecchi & Sarasini 2000, Ceruti *et al*. 2003). *T. foetidum*, reported to be distributed in Western Europe (Jeandroz et al, 2008) was collected few times in Balkan Peninsula.

All European commercially important truffles, except *T. melanosporum* and *T. borchii*, are widespread in Serbia. Except in Southern Europe and bordering regions, *T. brumale* and *T. macrosporum* were only recorded as rare up to southern England (Pegler *et al*. 1993, Ceruti et al. 2003). These species probably have their distribution optimum in the warmer climates and forest ecosystems of Southern Europe, on the sites where the soil water is not strongly limited. *T. magnatum* had been widely regarded as primarily distributed in Central and Northern Italy, and Istria in Croatia (Hall *et al.* 1998, Ceruti *et al.* 2003), with some additional records from southeast of France and Ticino Canton in Switzerland (Lawrynovicz 1993; Zambonelli & Di Munno, 1991). Its discovery in Serbia (Milenković & Marjanović 2000), Hungary (Bratek *et al.* 2003) and Slovenia (Piltaver & Ratoša 2006) comes as a surprise. Lowland ecosystems where *T. magnatum* in Serbia is usually found are clearly different from descriptions reported from Italy (Lulli & Primavera 2000). It appears that, the so called "Piedmont" white truffle has much wider area of distribution as well as ecological optimum, then previously claimed (Hall *et al*. 1998, Ceruti *et al*. 2003).

In comparison to the other European regions of high truffle diversity (Ceruti *et al*. 2003), Balkan Peninsula is specific in truffle chorology in few points: *T. magnatum* is widespread all over river valleys with the suitable edaphic conditions, but was never recorded on the hills; *T. borchii*, *T. maculatum and T. foetidum* are very rare, while *T. puberulum* and *T. dryophyllum* have not been recorded by now; *T. oligospermum* is not specific for Mediterranean coast-lines only – it is common inside the continental part of 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 and phylogenetic analysis of truffles recorded in the mid-west Balkan Peninsula

This is the first molecular verification of the *Tuber spp*. samples originating from Balkan Peninsula. Analyzing phylogenetic tree, it is obvious that all taxa originating from Serbia fit well into molecular pattern of the other European samples, and the tree clade distribution resembles previous analysis (Jeandroz et al, 2008). However, macroscopic determination of some small white truffles did not appear to be in accordance with molecular data. Two samples initially determined as *Tuber puberulum* Berk. & Broome due to the continental habitat, appeared to be morphologically very similar *Tuber oligospermum* (Tul. & C. Tul.) Trappe, which has previously been detected only in Mediterranean regions (Cerruti *et al*. 2003). Two very small herbarium samples determined as *Tuber borchii* Vittad (according to Montecchi & Sarasini 2000), appeared in the phylogenetic tree to belong to the *T. maculatum* clade. Problems in connecting morphological and molecular determination of small white truffles have been reported before (Halász *et al*, 2005, Kovács & Jakucs, 2006, Jeandroz et al, 2008), and more detailed analysis of small white truffles is necessary for clarifying species delimitation.

Tuber rufum appeared to be molecularly quite diverse species, but morphological forms described by Montecchi & Sarasini (2000) were not supported by present molecular analysis (Fig 2). However, two samples of *T. rufum* originating from the Tara Mountain in Western Serbia did form separate sub-clade within the *T. rufum* clade, but this cannot be signed as significant due to the low number of analysed sequences.

Interestingly, *Tuber excavatum* and *Tuber fulgens* were not clearly separated as different species by the method and parameters applied. We propose two explanations for this: either *T. fulgens* molecularly cannot be considered as species, but as variety of *T. excavatum*, or there were not enough sequences available in the GenBank – only three very variable samples of *T. excavatum* and none of *T. fulgens*. From our analysis it appeared that the species published as new by Hilszczanska *et al*. 2008 (Ass No. EU326694 Table1, Fig 2), was actually *T. fulgens*, and was probably signed as new due to the lack of *T. fulgens* ITS sequences in the public data basis. Therefore, the first registered sequence of *T. fulgens* was presented here, but detailed and much more specific investigation is necessary for obtaining real phylogenic relations between species belonging to *Tuber spp*.

We have observed minor, two base pairs variation in the ITS regions of *T. magnatum* sample (Ass. No. FM205697, Table 2) originating from Serbia (not shown). This may come as a surprise since in the available literature almost no ITS diversity was detected in samples from Italy and Istria (Mello *et al*. 2005; Rubini *et al*. 2005). However, this two base pairs difference could hardly be significant for intra-species differentiation comparing to other truffle species (Fig 2). Further investigation should be providing better insight in possible ITS diversity of this species.

To conclude, the truffle species originating from mid-west Balkan Peninsula showed interesting ecological and chorological differences comparing to the specimens found in other parts of Europe, but only slight differences of the ITS sequences. To meet ecological optimum, they have probably been adapting to the specific climatic, edaphic and vegetation conditions of the Balkan Peninsula, as well as climatic changes throughout European bio-geographic history. It is possible that they have been surviving periods of glaciations in the Balkan refugial sites, together with their plant hosts as well (Petit *et al*. 2002a,b, Murat *et al*. 2004). Therefore, the list of truffles originating from the mid-west of Balkan Peninsula could grow with additional collecting, as the so far investigated area is relatively small. Certainly, more species can be expected from regions with strong Mediterranean influences, as well as mountains and canyons, which could be potential glacial refugia.

Acknowledgements

The present work was done as a part of an EU supported Market oriented R&D project EUREKA E!3835. Authors are grateful to all truffle hunters in Serbia that contributed to the collection of specimens, especially to Mr. Momčilo Vićentijević† and Mr. Ljubiša Milojević. We are especially thankful to Dr James Trappe for data supply, support and critical reading of the manuscript, and to Dr Gerard Chevalier and Dr. Zoltan Bratek for organizing several discussions on the subject. For logistic support we are thankful to Prof. Dr. Željko Vučinić and Prof. Dr Hojka Kraigher.

References

1. Alvarez, I.F., Parlade, J., Trappe, J.M. (1992) *Loculotuber gennadii gen. et Comb. Nov* and *Tuber multimaculatum sp.nov*. *Mycologia*, **84** (6): 926-929.
2. Alvarez, I.F., Parlade, J., Trappe, J.M., and Castellano, M.A. (1993) Hypogeous mycorrhizal fungi of Spain. *Mycotaxon* **67**:201-217.
3. Bennet K.D., Tzedakis P.C. and Willis K.J. (1991) Quaternary refugia of north European trees. *Journal of Biogeog*raphy, **18**: 103-115
4. Bougher N.L. & Lebel T. (2001) Sequestrate (truffle-like) fungi of Australia and New Zealand. *Australian Systematic Botany* **14**, 439–484.
5. Bratek, Z., Gogan, A., Halász, K., Bagi, I., Erdei, V., and Bujaku, G. (2004) The Northeast habitats of *Tuber magnatum* known from Hungary. Le premier symposium sur les champignons hypogés du basin méditerranéen, 6–8 Avril, Rabat.
6. Ceruti, A., Fontana, A., and Nosenzo, C. (2003) *Le Specie Europee del genere Tuber—Una Revisione Storica.* Museo Regionale de Scienze Naturali, Torino.

7. Chevalier, G.; Riousset, L.; Dupre, C. (1986) Taxonomie des truffes Européennes. In V. Gianinazzi-Pearson and S. Gianinazzi (eds.), *Proceedings of the 1st European Symposium on Mycorrhizae, Dijon, 1-5 July 1985* pp. 631-635. Paris; INRA
8. Chevalier, G., & Frochot, H. (1997) La Truffe de Bourgogne (*Tuber uncinatum* Chatin). Petrarque, Levallois - Perret Cedex
9. Frančišković, S. (1950) Naši tartufi. *Šumarski List* **74**: 23-38.
10. Gardes, M. and Bruns, T.D. (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology*, **2**:113-118.
11. Gilkey, H. M. (1939) Tuberales of North America. *Oregon State Monographs* **1**: 1-63.
12. Gilkey, H. M. (1954) Tuberales. *North American Flora Series II* **1**: 1-36.
13. Glamočlija, J., Vujičić, R. and Vukojević, J. (1997) Evidence of truffles in Serbia**.** *Mycotaxon*, **LXV**: 211-22.
14. Grebenc, T., Piltaver, A. and Kraigher, H. (2000**).** Establishment of the PCR-RFLP library for *Basidiomycetes, Ascomycetes* and their ectomycorrhizae on *Picea abies* (L.) Karst. *Phyton, Annales rei Botanicae* **40**(4): 79-82.
15. Grebenc, T. & Kraigher, H. (2007). Changes in the community of ectomycorrhizal fungi and increased fine root number under adult beech trees chronically fumigated with double ambient ozone concentration. *Plant Biology*, **9**(2): 279-287. http://dx.doi.org/10.1055/S-2006-924489.
16. Grebenc, T., Kraigher, H., Martin M.P, Piltaver, A., Ratoša, I. (2008), *Research and cultivation of truffes in Slovenia : current status*. In: Chevalier G. (Ed.). *La culture de la truffe : dans la monde*. Brive-la-Gaillarde, INRA, 2008. pp. 183-191.

17. Hall, I.R., Zambonelli, A. and Primavera, F. (1998) Ectomycorrhizal Fungi with Edible fruiting Bodies 3. *Tuber magnatum, Tuberaceae*. *Economic Botany*, **52**, No.2: 192-200

18. Halász, K., Bratek, Z. Szegoe, D., Rudnóy, S, Rácz, I., Lásztity, D., and Trappe, J.M. (2005) Tests of species concepts of the small, white, European group of *Tuber* spp. based on morphology and rDNA ITS sequences with special reference to *Tuber rapaeodorum*. *Mycological Progress*, **4**(4): 281–290

19. Hilszczanska, D., Sierota, Z and Palenzona, M. (2008) New Tuber species found in Poland. *Mycorrhiza*, **18**:223–226

20. Hrka, J. (1988) *Tartufi – poznavanje, branje, uzgoj, pripremanje*. Mladost, Zagreb

21. Jeandroz , S., Murat , C., Wang, Y., Bonfante., P. and Le Tacon, F. (2008) Molecular phylogeny and historical biogeography of the genus Tuber, the 'true truffles'. Journal of Biogeography, **35** (5):815 - 829

22. Kers, L. E. (2003) Tryfflarna *Tuber aestivum* och *T. mesentericum* i Sverige. [*Tuber aestivum* Vittad. and *T. mesentericum* Vittad. (Ascomycetes) in Sweden.]. *Svensk Botanisk Tidskr*, **97**: 157–175.

23. Kojić, M., Popović, R., and Karadžić, B. (1997) *Vaskularne biljke Srbije kao indikatori staništa*. Institut za istraživanja u poljoprivredi „Srbija“ i Institut za biološka istraživanja „Siniša Stanković“, Beograd, Serbia

24. Kovács, G.M. and Jakucs, E.(2006) Morphological and molecular comparison of white truffle ectomycorrhizae, Mycorrhiza, 16:567–574

25. Kraigher, H., Javornik, B. and Agerer, R. 1995. Ectomycorrhizae of *Lactarius lignyotus* on Norway spruce, characterized by anatomical and molecular tools. *Mycorrhiza* **5** (3): 175-180.

26. Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A., Lopez R., Thompson J.D., Gibson T.J. and Higgins D.G. (2007). ClustalW and ClustalX version 2**.** *Bioinformatics* **23**(21): 2947-2948.

27. Lulli, L. & Primavera, F. (2000) *Tuber magnatum* Pico: environment of growth. *The Vth International Congress Science and Cultivation of Tuber and other edible hypogeous mushrooms Proceedings, Section Ecology*: 5.269-5.272

28. Lindnter, V. (1935) Podzemne gljive u Srbiji. *Šumarski List* **59**: 15-18.

29. Marjanović,Ž. & Milenković, M. (1998) *Tuber magnatum* Pico and some similar species in Yugoslavia, *Abstracts of Second International Conference of Mycorrhiza*, Uppsala, Sweden: 114.

30. Mello, A., Cantisani, A., Vizzini, A. and Bonfante, P. (2002) Genetic variability of *Tuber uncinatum* and its relatedness to other black truffles. Environmental Microbiology **4**(10): 584-594

31. Mello, A. Murat, C., Vizzini, A., Gavazza, V. and Bonfante, P. (2005) *Tuber magnatum* Pico, a species of limited geographical distribution: its diversity inside and outside a truffle ground. *Environmental Microbiology*, **7** (1): 55-65.

32. Milenković, M., Glamočlija, J., Veljković, V. & Vukojević, J. (1992) Record of two *Tuber* (*T. aestivum* and *T. melanosporum*) species in Serbia. *Archives of Biological Sciences*, **44**: 223-28.

20. Milenković, M. & Marjanović, Ž. (2000) Current results on *Tuber spp.* research in Yugoslavia. *The Vth International Congress Science and Cultivation of Tuber and other edible hypogeous mushrooms Proceedings, Section Ecology*: 4.218 –4.225.

33. Montecchi, A. and Sarasini, M. (2000) *Funghi ipogei d'Europa*. Associaazione Micologica Bresadola – Fondacione centro Studi Micologici, Trento-Vicenza

34. Myers, N. and R.M. Cowling. (1999) *Mediterranean Basin*. In: *Hotspots: Earth's Biologically Richest and Most Endangered Terrestrial Ecoregions* (R.A. Mittermeier, N. Meyers, P. Robles Gil, and C.G. Mittermeier *eds*). CEMEX, Mexico: 254-267.

35. Murat, C., Díez, J., Luis, P., Delaruelle, C., Dupré, C., Chevalier, G., Bonfante, P. and Martin, F. (2004) Polymorphism at the ribosomal DNA ITS and its relation to postglacial re-colonization routes of the Perigord truffle *Tuber melanosporum* New Phytologist, **164** (2): 401–411

36. Pázmány, D. (1990/1991) Conspectus fungorum hypogaeorum Transsilvaniae. *Notes on Botany, Horticulture and Agrobotany Cluj:* XX-XXI.

37. Pegler, D.N., Spooner, B.M. and Young, T.W.K. (1993) *British Truffles*. Royal Botanic Garden, Kew, London

38. Petit, R.J., Csaikl, U. M., Bordács, S., Burg, K., Coart, E., Cottrell, J., van Dam, B., Deans, J. D., Dumolin-Lapègue, S., Fineschi S. *et al.* (2002a) Chloroplast DNA variation in European white oaks: Phylogeography and patterns of diversity based on data from over 2600 populations. *Forest ecology and Management,* **156**: 5-26.

39. Petit, R.J., Brewer, S.,, Bordács, S., Burg, Cheddadi, R., K., Coart, E., Cottrell, J., Csaikl U. M., van Dam, B., Deans, J. D., *et al.* (2002b) Identification of refugia and post-glacial colonisation routes of European white oaks based on chloroplast DNA and fossil pollen evidence. *Forest ecology and Management* **156**: 49-74.

40. Piltaver A., and Ratoša I. 2006. A contribution to better knowledge of hypogeous fungi in Slovenia**.** *Gozdarski vestnik*, **64** (7/8): 303-312, 329-330.

41. Rubini, A., Paolocci, F., Riccioni, C., Vendramin, G.G. and Arcioni , S. (2005) Genetic and Phylogeographic Structures of the Symbiotic Fungus *Tuber magnatum, Applied and Environmental Microbiology*, **71 (**11): 6584-6589

42. Simpson, J.A. (2000) More on mycophagous birds. *Australasian Mycologist* **19**

43. Stevanović, V., Jovanović, S., Lakušić, D. & Niketić, M. (1995) *Diverzitet vaskularne flore Jugoslavije sa pregledom vrsta od medjunarodnog znacaja.* In *Biodiverzitet Jugoslavije* (Stevanović, V I V. Vasić *eds*). Biološki Fakultet Univerziteta u Beogradu i Ecolibri, Beograd

44. Szemere, L. (1965) *Die untererdishen Pilze des Karpatenbeckens*. Akademiai Kiado. Budapest

45. Taberlet P., Fumagalli L., Wust-Saucy A-G and Cossons J-F. (1998) Comparative phytogeography and postglacial colonization routes in Europe. *Molecular Ecology* **7**: 453-464.

46. Tamura K, Dudley J, Nei M and Kumar S (*2007*) *MEGA4*: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0**.** *Molecular Biology and Evolution* **24**:1596-1599.

47. Trappe, J. M. and E. Cazares (2000) *Tuber maculatum* around the world. *Bulletin Semestriel de la Fédération des Associations Mycologiques Méditerranénnes n.s.* **18**: 107-112.

48. Weden, C., Danell, E., Camacho, F.J., Backlund, A. (2004) The population of the hypogeous fungus Tuber aestivum syn. T. uncinatum on the island of Gotland. *Mycorrhiza* **14**(1):19-23

49. White, T.J., Bruns, T., Lee, S. and Taylor, J. 1990. *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics.* In: *PCR Protocols. A guide to methods and applications*. Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J. (Eds.). San Diego, Academic Press, p. 315-322.

50. Wojewoda, W. & Lawrinowicz, M. (1986) *Red list of the threatened macrofungi in Poland.* In: *List of threatened plants in Poland* (Zarzyckiego K and W. Wojewoda *eds*) Polaska Akademia Nauk, Komitet Ochrony Przyrody & Institut Botaniki, PWN Warszawa

51. Wedén, C., Danell, E. & Tibell, L. (2005) Species recognition in the truffle genus *Tuber*- the synonyms *Tuber aestivum* and *Tuber uncinatum* Environmental Microbiology **7**(10): 1535-1546

52. Yun, W. (1988) First report of a study on *Tuber* species from China**.** Atti del secondo congresso internazionale sul tartufo, Comunita montana dei Monti Martani e del Serano, Sessione Tassonomia, pp.45-50

53. Zambonelli, A., Riveti, C., Percudani, R. and Otonello, S. (2000) TUBERKEY, A computer program for the description and recognition of truffles. Mycotaxon **74**: 57–76 (http://www.truffle.org/tuberkey/html)

Table 1. Morphological and molecular identifications of truffles recorded from Balkan Peninsula with GenBank accession numbers for rDNA ITS sequences included in the phylogenetic analysis

Name after morphological determination	Name after BLAST search	Accession number in GenBank	Geographical 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*	FM205697	West Serbia

	Pico		
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	Mediterranean coast, Montenegro
Tuber rufum Pico	*Tuber rufum* Pico	FM205667	Tara Mountain, Serbia
Tuber rufum Pico	*Tuber rufum* Pico	FM205668	Tara Mountain, Serbia
Tuber rufum f. apiculatum E. Fisch	*Tuber rufum* Pico	FM205669	Tara Mountain, Serbia
Tuber rufum f nitidum (Vittad.) Montecchi & Lazzari)	*Tuber rufum* Pico	FM205677	Tara Mountain, Serbia
Tuber rufum f. rufum Pico	*Tuber rufum* Pico	FM205690	City park, Belgrade, Serbia
Tuber rufum f. 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 mountain, Serbia
Tuber fulgens Quél	*no significant match*	FM205702	Tara mountain, Serbia
Tuber fulgens Quél	*no significant match*	FM205698	West Serbia
Tuber fulgens Quél	*no significant match*	FM205685	West Serbia
Tuber excavatum Vittad.	*Tuber excavatum*	FM205662	Tara Mountain, Serbia

	Vittad.		
Tuber excavatum Vittad.	*Tuber excavatum* Vittad.	FM205687	Ub, Serbia
Tuber excavatum	*Tuber excavatum*	FM205703	Tara Mountain, 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 Mountain, 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*	AJ557544	Hungary

	Vittad		
Tuber puberulum Berk. & Broome	*Tuber puberulum* Berk. & Broome	AJ969625	Suserup, South Zealand, Denmark:
Tuber puberulum Berk. & Broome	*Tuber puberulum* Berk. & Broome	AJ969626	Bromme Plantage, South Zealand, 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

Legends to figures

Figure 1. Map of the western part of Balkan Peninsula. Investigated countries are shaded.

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. Names, accession numbers and origin of the samples are listed in Table 1.

Fig. 1

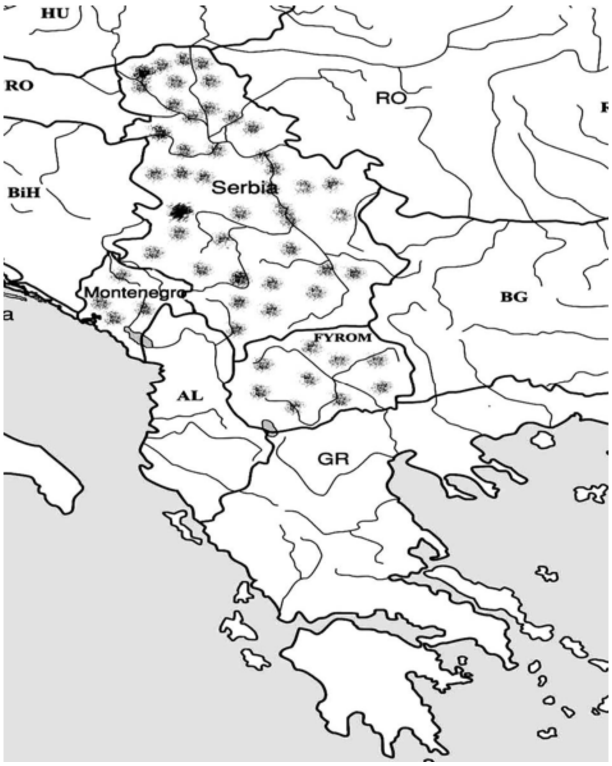
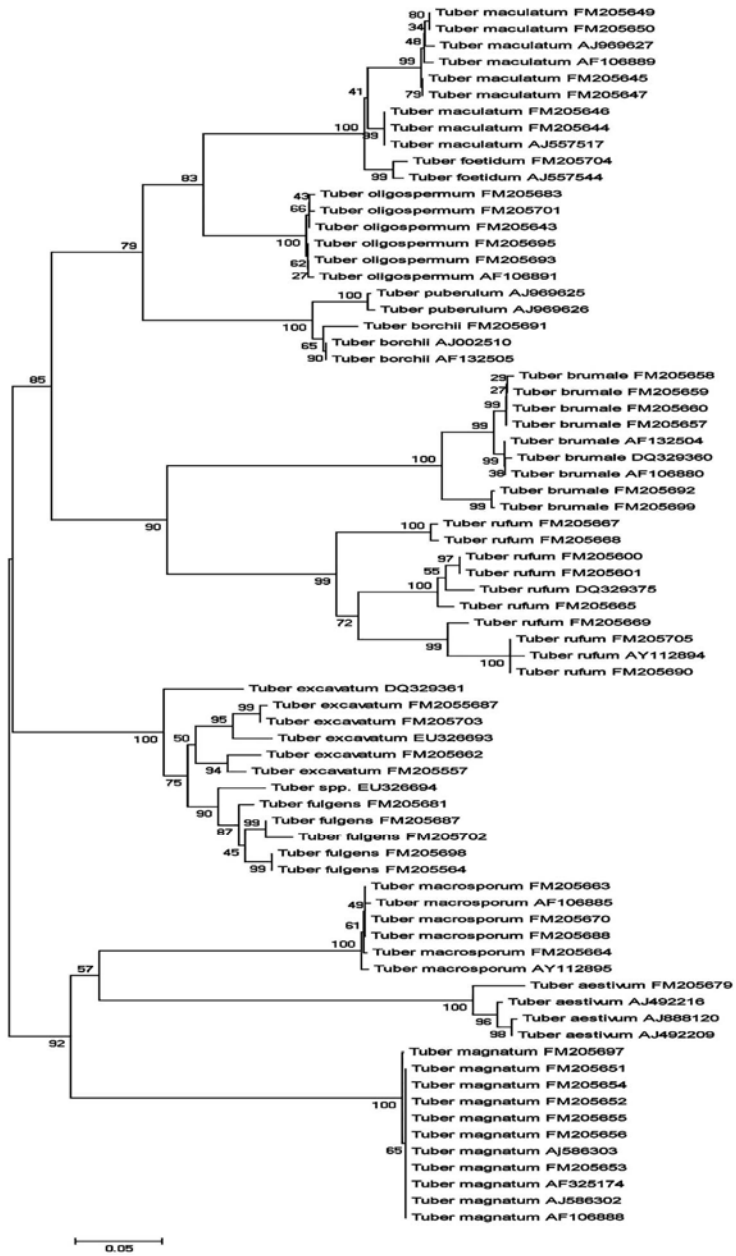


Figure 2.


Tuber maculatum FM205649
Tuber maculatum FM205650
Tuber maculatum AJ969627
Tuber maculatum AF106889
Tuber maculatum FM205645
Tuber maculatum FM205647
Tuber maculatum FM205646
Tuber maculatum FM205644
Tuber maculatum AJ557517
Tuber foetidum FM205704
Tuber foetidum AJ557544
Tuber oligospermum FM205683
Tuber oligospermum FM205701
Tuber oligospermum FM205643
Tuber oligospermum FM205695
Tuber oligospermum FM205693
Tuber oligospermum AF106891
Tuber puberulum AJ969625
Tuber puberulum AJ969626
Tuber borchii FM205691
Tuber borchii AJ002510
Tuber borchii AF132505
Tuber brumale FM205658
Tuber brumale FM205659
Tuber brumale FM205660
Tuber brumale FM205657
Tuber brumale AF132504
Tuber brumale DQ329360
Tuber brumale AF106880
Tuber brumale FM205692
Tuber brumale FM205699
Tuber rufum FM205667
Tuber rufum FM205668
Tuber rufum FM205600
Tuber rufum FM205601
Tuber rufum DQ329375
Tuber rufum FM205665
Tuber rufum FM205669
Tuber rufum FM205705
Tuber rufum AY112894
Tuber rufum FM205690
Tuber excavatum DQ329361
Tuber excavatum FM2055687
Tuber excavatum FM205703
Tuber excavatum EU326693
Tuber excavatum FM205662
Tuber excavatum FM205557
Tuber spp. EU326694
Tuber fulgens FM205681
Tuber fulgens FM205687
Tuber fulgens FM205702
Tuber fulgens FM205698
Tuber fulgens FM205564
Tuber macrosporum FM205663
Tuber macrosporum AF106885
Tuber macrosporum FM205670
Tuber macrosporum FM205688
Tuber macrosporum FM205664
Tuber macrosporum AY112895
Tuber aestivum FM205679
Tuber aestivum AJ492216
Tuber aestivum AJ888120
Tuber aestivum AJ492209
Tuber magnatum FM205697
Tuber magnatum FM205651
Tuber magnatum FM205654
Tuber magnatum FM205652
Tuber magnatum FM205655
Tuber magnatum FM205656
Tuber magnatum Aj586303
Tuber magnatum FM205653
Tuber magnatum AF325174
Tuber magnatum AJ586302
Tuber magnatum AF106888
0.05