True truffles (*Tuber spp.*) are among the most appreciated mushroom in gastronomy. Due to their particular taste and limited natural availability the price of these underground mushrooms is high. In Croatia, the most important presence of *Tuber spp.* was reported in Istria. Since seven to eight decades ago there have been stories of Zadar's truffles which are transmitted orally from generation to generation but, till now, no written document that could confirm it has been found. In this study, truffles were searched for on different locations in Zadar County, the presumed truffles were harvested and molecular analyses were performed to identify them. The results undoubtedly show that the summer truffle, *Tuber aestivum* Vittad. is present in Zadar County.

**Key Words**: truffles, *Tuber aestivum* Vittad., Zadar County
UVOD

Tartuf je uobičajen naziv za hipogene mikorizne gljive. Ovo ime uključuje različite rodove, ali pravi tartuf ima smanjena u odnošenju na Tuber. Procjenjuje se da rod Tuber uključuje više od 180 vrsta, od kojih neke imaju prilično veliku rasprostranjenost, dok ostale rastu u manje ili više ograničenim geografskim područjima (Zambonelli i dr., 2015.). Crni ljetni tartuf (T. aestivum) spada među najrasprostranjenije vrste, a prisutnost mu je zabilježena u gotovo cijeloj Europi, od Španjolske do zemalja Istočne, Sjeverne i Srednje Europe, Sjeverne Afrike i Kine (Weden i dr., 2004.; Gazo i dr., 2005.; Hilszczanska i dr., 2008.; Zambonelli i dr., 2012.; Bungten i dr., 2017.). S druge strane, geografska rasprostranjenost bijelog tartufa (T. magnatum) znatno je manja. Zabilježen je u ograničenim područjima Italije, južne Francuske, hrvatske regije Istre, Slovenije, Srbije, Švicarske i Mađarske (Pomarico i dr., 2007.; Vásquez i dr., 2014.; Riccioni i dr., 2016.). Ekologija i fiziologija roda Tuber nije u potpunosti poznata te se istraživaju i dalje provode. Različiti biotski i abiotski čimbenici mogu utjecati na životni ciklus i geografsku rasprostranjenost tartufa. Razmjerno širok raspon vrste domaćina zajednički je za sve vrste tartufa, te pitanje dostupnosti vrste domaćina ne bi trebalo biti presudno za rasprostranjenost. Naprotiv, vezano za abiotiske čimbenike, vrlo je izvjesno da sastav tla ima vrlo važnu ulogu. Tartuf se obično nalazi u vapnenastim, blago bazičnim tlima (pH između 7 i 8), s izuzetkom T. borchii koji dolazi i na blago kiselim tlima. Klimatski uvjeti važni su za rasprostranjenost nekih vrsta tartufa (Thomas, 2014.; Boddy i dr., 2014.). M. Gryndler i dr. (2017.) naglašavaju utjecaj temperature na prisutnost T. aestivum u Češkoj, gdje se ljetni tartuf pronalazi samo na toplijim lokacijama.

Od davnina su se tartufi cijenili zbog njihova posebnog ukusa i koristili kao hrana (Hall i dr., 2007.; Ross, 2011.). Zapisi o prisutnosti tartufa kao kulinarne delicije prisutne su u knjigama od 17. stoljeća do danas (Rosa-Gruszeczka i dr., 2017.). Zbog ograničene dostupnosti, cijene tartufa su višoke. Bijeli tartuf, T. magnatum, jedna je od najskupljenih namirnica, a ovisno o raspoloživim go- dišnjim količinama, cijene mogu dosegnuti i 4.000 eura za kilogram (Figuolo i dr., 2013.). Cijene truffle is a common name for hypogeous mycorrhizal mushrooms. This name includes different genera, but true truffles are considered only those of the genus Tuber. The genus Tuber is estimated to include over 180 species, some of which have a rather wide distribution while the other grow in more or less restricted geographic areas (Zambonelli et al., 2015). The black summer truffle (T. aestivum) is among the most widespread species. Its presence was reported almost in all Europe from Spain to Eastern European countries, Northern and Central Europe, North Africa and China (Weden et al., 2004; Gazo et al., 2005; Hilszczanska et al., 2008; Zambonelli et al., 2012; Bungten et al., 2017). On the other hand, the geographical distribution of T. magnatum, the white truffle, is much more limited. It was reported to be found in restricted areas of Italy, Southern France, Istria, a Croatian region, Slovenia, Serbia, Switzerland and Hungary (Pomarico et al., 2007; Vásquez et al., 2014; Riccioni et al., 2016). The ecophysiology of the genus Tuber is not completely understood and the research is still ongoing. Different biotic and abiotic factors can influence truffles life cycle and geographic distribution. The relatively wide host species range is common for all truffle species and therefore the host availability should be a minor issue. Among the abiotic factors, the soil composition seems to have an important role. Generally speaking, truffles are found in calcareous, slightly basic soils (pH between 7 and 8), with the exception of T. borchii which can grow in slightly acidic soils. The climatic conditions seem to be important for the distribution of some Tuber species (Boddy et al., 2014; Thomas, 2014). M. Gryndler et al. (2017) underline the influence of temperature on the presence of T. aestivum in the Czech Republic, since the summer truffle was found only on warmer locations.

Since the ancient times truffles have been appreciated for their particular taste and used as food (Hall et al., 2007; Ross, 2011). The records of the presence of truffles as delicacies are present in books from the 17th century to the present day (Rosa-Gruszeczka et al., 2017). Due to the limited availability, the price of true truffles is high. White truffle, T. magnatum, is among the most expensive food; depending
za *T. aestivum* su znatno niže i to zbog većih dostupnih količina i rasprostranjenosti, duljeg razdoblja branja i mogućnosti uzgoja (Mello i dr., 2006.). Unatoč tome, cijena ljetnih tartufa i dalje je visoka, a ovisno o prinosu može dosegnuti i nekoliko stotina eura. Crni ljetni tartuf stoga je zanimljiv i za uzgoj i zbog branja u prirodi. Tartufi su lako kvarljivi pa se vrše istraživanja da im se produži trajnost (Romanazzi i dr., 2016.). U Hrvatskoj je Istra jedina regija poznata po branju tartufa (Zambonelli i dr., 2015.; Riccioni i dr. 2016.). Jedno od najvažnijih područja rasprostranjenosti bijelog tartufa (*Tuber magnatum*) u Istri je Motovunška šuma u kojoj se tartufi beru od 1930-ih (Bragato i dr., 2004.). Tek je odnedavno uloženo više napora u dokumentiranje i istraživanje i drugih područja rasprostranjenosti i pogodnosti za uzgoj tartufa u Hrvatskoj. I. Tikvić i dr. (2017.) istraživali su prirodnu rasprostranjenost tartufa u Hrvatskoj, ali je njihovo istraživanje obuhvaćalo samo područje Posavine (regija uz rijeku Savu) i SZ Hrvatsku gdje su pronašli primjerke crnih i bijelih tartufa. Prema I. Tikviću i dr., druge poznate lokacije tartufa, osim Istre, postoje na različitim područjima u Slavoniji, a samo je jedno poznato područje u cijeloj južnoj Hrvatskoj i to na poluotoku Pelješcu. Dakle, do sada se njegle nije navodilo zadarsko područje koje ima sve okolišne i prirodne uvjete za rast tartufa, međutim činjenica je da do sada tartufi nisu ni traženi na tom području. Po usmenoj predaji, tartufi su se u prošlosti povremeno nalazili u različitim dijelovima zadarskog područja. Spominje se i da je u novinama tijekom talijanskog upravljanja gradom objavljen članak koji govori o golemom tartufu pronađenom u blizini Zadra. Nažalost, istraživanje ovog članka u arhivima nije dalo rezultata jer nije pronadan. Uzimajući kao polazište u obzir ovu usmenu informaciju te okolišno-prirodne preduvjete za rast tartufa, 2014. su potvrđeni nalazi tartufa na različitim lokacijama Zadarske županije.

**PRIRODNE OSOBITOSTI MJESTA ISTRAŽIVANJA**

Istraživanja mogućih nalaza tartufa provedena su na različitim potencijalnim lokacijama u Zadarskoj županiji, preciznije u okolici Novigrada, on year’s harvest the prices can reach 4,000 euro per kg (Figliuolo et al., 2013). The prices of *T. aestivum* are much lower, due to the major presence and distribution, longer harvesting period and a major possibility of cultivation (Mello et al., 2006.). Nevertheless, the price of summer truffles is still high; depending on yield it can reach a few hundred euro per kg. That makes the black summer truffle interesting both for cultivation and harvesting in the wild. Truffles are highly perishable, and strategies to extend their shelf life are under evaluation (Romanazzi et al., 2016.). In Croatia, the only region known for collecting truffles is Istria (Zambonelli et al., 2015; Riccioni et al. 2016.). One of the most important production areas for *T. magnatum* in Istria is Motovun Forest, where truffles have been collected since the 1930s (Bragato et al., 2004.). Recently more effort has been made in documenting other areas in Croatia suitable for truffles growth. I. Tikvić et al. (2017) researched natural distribution of truffles in Croatia but their research comprised only the area of Posavina (a region along Sava River) and NW Croatia where they found black and white truffle specimens. According to I. Tikvić et al. (2017), other known truffle locations, besides Istria, are in different areas in Slavonia and there is only one known location in all Southern Croatia (on Pelješac peninsula). There is no mention of Zadar region which also has all environmental conditions for truffle growth, but they were never even searched for in that area. According to the tales of older population, in the past the truffles were occasionally found in different parts of the County. Apparently, there was an article in a newspaper during the Italian governance of the city about a huge truffle found near the city of Zadar. Unfortunately, the search for this article in archives gave no results. Considering this story and the environmental conditions suitable for spontaneous truffle growth, in 2014 we verified the presence of truffles in different locations of Zadar County.

**NATURAL FEATURES OF STUDY SITE**

The search for truffles was conducted in different meadows in Zadar County, and to be more precise, in the surroundings of cities of Sv. Rok, Novigrad, Benkovac, Maslenica, Miljevci area, Rovanijska and...
Benkovca, Maslenice and Rovanjske. Lokacije su oda-
brane na temelju prirodnih značajki tih područja s
obzirom na to da su klima i tla slična područjima
rasta tartufa u Istri. Od svih istraživanih područja
tartufi su u Zadarskoj županiji pronadjeni na tri lo-
kacije: Žegar, Rovanjska i Maslenici.

Sve lokacije su u krškom području, uglavnom
vapnenace i dolomitne geološke građe, najčešće
sa sredinim tlima (kalkokambisoil) ili rendzinizama
na vapnencu, te ponešto crnice na vapencu (Bašić,
2012.).

Područja pripadaju mediteranskoj vegetacijskoj
regiji, mediteransko-ovalnom vegetacijskom pojazu
i submediteranskoj vegetacijskoj zoni (Trinajstić,
1998.). Tipična biljna zajednica ovoga područja je
šuma hrasta medunca i bijeloga graba (Querco-
Carpinetum orientalis, Horvatić, 1939.) (Vu
kević i dr., 2008.). Svojstvene biljne vrste su: hrast me-
dunac (Quercus pubescens Willd.), bijeli grab (Carpinus
orientalis Mill.), hrast cer (Quercus cerris L.), crni ja-
sen (Fraxinus ornus L.) i druge (Trinajstić, 1998.).

Zadarsko zaleđe, što je područje istraživanja, ima
umjerenu klimu (klimatska zona C prema Köpp-
enoj klasifikaciji) te pripada klimatskim tipovima
Csa (sredozemna klima s vrućim ljetom) i Csb (sre-
dozemna klima s toplim ljetom) (Filipčić, 2000.)
koji u Sj dijelu županije prelaze u hladniju i vlaž-
niju klimu Cfa (umjereno topla vlažna klima s vru-
ćim ljetom) i Cfb (umjereno topla vlažna klima s
toplim ljetom). Zanimljivo je da se sva tri područja
gdje su pronadjeni tartufi u sjevernoj Dalmaciji na-
laže na granici između klimatskih tipova Cs i Cf. U
Istri su glavna područja tartufa u unutrašnjosti
istarskog poluotoka (poput Motovunskе šume) s
klimatskim tipom Cfb. Istri klimatski tip prisutan
je u SZ i istočnoj Hrvatskoj (Filipčić, 2000.) gdje
su i druge lokacije pronalaska tartufa.

MATERIJALI I METODE
Uzimanje uzoraka tartufa

Tartufi su traženi na različitim lokacijama ma-
kije i garige u Zadarskoj županiji. Točnije, traženi
su u okolici Svetoga Roka, Novigrada, Benkovca,
Maslenice, Rovanjske i drugdje. Za traženje tartufa
korištena su dva psa trenirana za traženje tartufa
pasmine Lagotto Romagnolo i njihov voditelj s do-

other. Locations were chosen based on natural fea-
tures of the area since climate and soils are similar
to the sites where truffles can be found in Istria. Out
of all research area, truffles were found in three lo-
cations: in Žegar, Rovanjska and Maslenica area,
all situated in Zadar County.

All locations are in karst area, mostly built of
limestone and dolomite and covered with brown
soils (kalkokambi soil) or rendsina soil on lime-
stone and also black soil (Bašić, 2012)

Those areas belong to the Mediterranean vegeta-
tion region, Mediterranean-littoral vegetation belt
and the Submediterranean vegetation zone (Trina-
jstić, 1998). The typical plant community of this
area is forest of pubescent oak and oriental horn-
beam (Querco-Carpinetum orientalis, Horvatić,
1939) (Vu
ekvić et al., 2008). Common species are:
ubescent oak (Quercus pubescens Willd.), oriental
hornbeam (Carpinus orientalis Mill.), turkey oak
(Quercus cerris L.), South European flowering ash
(Fraxinus ornus L.) and others (Trinajstić,
1998.).

Zadar hinterland, including researched area, has a
temperate climate (C climate zone according to Köppen
classification), mostly Csa (hot-summer Medi-
terranean climate) and Csb (warm-summer Medi-
terranean climate) which in NE part of the County
change to cooler and more humid Cfa (humid-subtropical
climate) and Cfb (temperate oceanic climate) climate
types. It is interesting that all three areas where truffles
were found in the North Dalmatia, are situated on the
border between Cs and Cf climate types. In the Istria
the main locations of truffles are in the hinterland of
the peninsula (such as Motovun forest) which is situated
in Cfb climate zone. The same climate type is present
in the NW and East Croatia (Filipčić, 2000) where
others truffle locations were found.

MATERIAL AND METHODS
Truffle hunt

The search for truffles was carried out in differ-
etent meadows in Zadar County, and, to be more
precise in the surroundings of cities of Sv. Rok,
Novigrad, Benkovac, Maslenica, Rovanjska and
other. A truffle hunter and his two Lagotto Romagn-
olo dogs trained for truffle hunt were recruited for
the research. The presumed truffles were collected, put in sterile paper bag, sealed and put in freezer at -20 °C until the analysis.

**Molecular analysis**

The DNA from *T. aestivum* was extracted using the following method. The truffles were first brushed under a stream of tap water and subsequently washed with deionized water and lyophilized. About 30 mg of fruiting bodies was ground in liquid nitrogen and incubated o/n at 55 °C with 1 mL C-TAB extraction buffer (NaCl 2.8 M; Tris-HCl 200 mM pH 8.0; EDTA 40 mM; C-TAB 4 % w/v; proteinase K 100 mg/mL). After incubation, samples were placed at 65 °C for 20 min and then on ice for 10 min. The samples were centrifuged at 12,000 rpm at 4 °C and supernatant was collected. The supernatant was then mixed with equal volumes of phenol-chloroform-isoamyl alcohol (Phe:Chl:IAA 25:24:1 v:v:v) and left at 25 °C for 30 min. After centrifugation, 1/10 volume of C-TAB II buffer (C-TAB 10 % w/v, NaCl 0.7 M) and 1 volume of Chl:IAA 24:1 v:v was added to the upper aqueous layer. The solutions were centrifuged at 13,000 rpm for 5 min and collect upper aqueous layer. The same volume of CTAB III solution (C-TAB 1 % w/v; Tris-HCl 50 mM pH 8.0; EDTA 10 mM) and Chl:IAA 24:1 v:v were added and left at 25 °C for 30 min. After centrifuge at 13,000 rpm for 5 min, the nucleic acids were precipitated by adding 0.6 volume of cold 2-propanol. The pellet was washed with 70 % v/v cold EtOH, dried and resuspended in 30 μL sterile water containing 20 mg/mL RNase.

The DNA (10ng) was used as template to proceed with the PCR analysis to check if the samples belong to the *T. aestivum* species. The PCR was performed as reported by Gryndler et al. (2011) using the following species-specific primers: Tu2sekvF 5'-AGAGCACCACAAACCACAG-3'; Tu2sekvR 5'-ACCACAGCGTCTACCACAA-3'. PCR was performed with the REDTaq® ReadyMix™ PCR Reaction Mix (Merck, US) in a total volume of 25 μL. The program used to perform the PCR was: 4' at 95 °C and steps of 94° for 30”, 56 °C 30” and 72 °C 5’ were repeated for 35 cycles, and the results were visualized on agarose gel (1% w/v) electrophoresis.
RESULTS

Truffles were found in different locations. Three samples collected in different locations and associated with different host plants were chosen for molecular analysis. Sample A was found in the surroundings of Maslenica associated with the roots of oriental hornbeam (*Carpinus orientalis* Mill.) and weighed 71 g. Sample B was found on a location Žegar associated to the roots of a pubescent oak (*Quercus pubescens* Willd.) and it weighed 43 g. Sample C was found on location Rovanjska, it was also associated with pubescent oak (*Quercus pubescens* Willd.) and weighed 69 g (Fig. 1).

In Figure 2 the agarose gel with the amplification results of the DNA of our samples is shown. The amplicon size (800 bp) obtained with the species-specific primers of *T. aestivum* (*Gryndler* et al., 2014) demonstrating that three collected fruiting bodies belong to that species.

**Slika 1.** Crni ljetni tartufi pronađeni na lokacijama Maslenica (A), Žegar (B) i Rovanjska (C)

**Figure 1.** The black summer truffle fruiting bodies found in Maslenica (A), Žegar (B) and Rovanjska (C)

**Slika 2.** Rezultati sekvenciranja uzorka (Linije 1, 2, 3) s početnicama Tu1-sekvF/Tu2-sekvR

**Figure 2.** Products of sample amplification (Lane 1, 2, 3) with primers Tu1-sekvF/Tu2-sekvR

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**REZULTATI**

Tartufi su pronađeni na različitim lokacijama. Za molekularnu analizu odabrana su tri pronađena uzorka prikupljena na različitim lokacijama i povezana uz biljke domaćine. Uzorak A pronađen je u okolici Maslenice, uz korijenje bijelog graba (*Carpinus orientalis* Mill.), a težio je 71 gram. Uzorak B pronađen je na lokaciji Žegar uz korijenje hrasta medunca (*Quercus pubescens* Willd.), a težio je 43 grama. Uzorak C pronađen je na lokaciji Rovanjska uz korijenje hrasta medunca, a težio je 69 grama (Sl. 1.).

Slika 2. prikazuje akrilamidni gel s rezultatima sekvenciranja DNK pronađenih primjeraka. Veličina sekvence (800 bp) dobivene kod vršno specifične početnice *Tuber aestivum* (*Gryndler i dr.*, 2014.) pokazala je da tri prikupljena uzorka pripadaju toj vrsti.
RASPRAVA I ZAKLJUČAK

Rezultati nedvojbeno upućuju na to da je *T. aestivum*, ljetni crni tartuf, prisutan na području Zadarske županije. Pronađeno je nekoliko primjeraka na različitim lokacijama što upućuje na to da postoji mogućnost da je rasprostranjen i na širem području. Budući da nisu postojale spoznaje o njegovoj prisutnosti te se nije ni prikupljalo, tartuf je vjerojatno kolonizirao široko područje i ima ga u većim količinama. Dobiveni rezultati su važni sa znanstvenog aspekta. Bilo bi zanimljivo vidjeti kako su zadarski tartufi povezani s ostalim *T. aestivum* kolonijama rasprostranjеним diljem Europe. Jedina hrvatska renomirana regija što se tiče tartufova je Istra (Gryndler et al., 2017.), pa se nameće pitanje o kakvoj je vezi s vezi zadarski i istarski tartuf. Uzeli u obzir razmjerno malu udaljenost između tih dviju regija (oko 250 km) postoji vjerojatnost da vjerojatnost da te dvije populacije imaju zajedničke pretekove. To je bitno s aspekta budućih genetičkih studija koje bi se trebale provesti tako da se prikupe uzorci tartufa u oba područja i analiziraju njihovi genomi. Drugo pitanje koje se postavlja jest je li bilo ili još postoji kontinuirani rast tartufova na području od Istre, duž jadranske obale do zadarskog područja te eventualno i južnije. Provest će se i istraživanje prisutnosti *T. aestivum* između zadarskog područja i Istre. Istraživanje genetičke populacije trebalo bi se provesti kako bi se potvrdila povezanost između dviju populacija te da bi se utvrdilo imaju li isto podrijetlo. Druga mogućnost, koju se ne može isključiti, je da su zadarski tartufi više povezani s talijanskim nego s istarskim. Tijekom dugogodišnje povijesti ovijestio zajedničke uprave, primjerice za vrijeme Mletačke Republike i Austro-Ugarskog Carstva, bila je omogućena lakša trgovina između Zadra i nekih talijanskih regija. Mnoge talijanske regije bogate su tartufima, a među njima je i nekoliko apeninskih regija s druga strane Jadranskog mora poput regija Marche, Umbrija i Abruzzo (Pomarico i dr., 2007.; Vasquez et al., 2014.; Zambonelli i dr., 2015.). Moguće je da su tartufi slučajno uvezeni iz Italije (ili i iz Istre) s nekim drvećem te su se kasnije spontano proširili regijom. Odgovor na ova pitanja može dati samo dublja analiza genetičke populacije što se planira provesti u nastavku istraživanja.

Rezultati također mogu imati utjecaj na gospo-

DISCUSSION AND CONCLUSION

The results show that *T. aestivum*, the black summer truffle, is present in Zadar County. It was found on different locations indicating that its presence could be widespread. The ignorance of its presence, and therefore non-harvesting, has probably allowed the mushroom to colonize a large area and to be present in abundance. From the scientific point of view these results are important. It would be interesting to understand how Zadar’s truffles are related to other *T. aestivum* colonies present in Europe. In Croatia the only renowned region for truffles is Istria (Gryndler et al., 2017), and the question ‘What is the relation between Zadar and Istrian truffles?’ arises spontaneously. Considering a relatively short distance between these two regions (around 250 km) it is rather probable that the two populations have the same ancestors. It is important for further population genetic studies by collecting truffles in both area and analysing their genomes. Another question is if there was or still is a continuous growth of truffles from Istria along the Adriatic coast to Zadar County and, possibly further south. The research of *T. aestivum* presence in the area between Zadar and Istria will be undertaken. The study of population genetics should be performed to verify the links between the two populations and establish whether they have a common origin. The other possibility, that cannot be excluded, is that Zadar truffle population is more linked to the Italian population than to the Istrian one. A long history of common governance, like the Republic of Venice and the Austro-Hungarian Empire, has facilitated the trade between Zadar and some Italian regions. Many Italian regions are reported to be rich with truffles, among whom are also Appennine regions across the Adriatic Sea such as Marche, Umbria and Abruzzo (Pomarico et al., 2007; Vasquez et al., 2014; Zambonelli et al., 2015). The truffles might have been were accidentally imported from Italy (as well as from Istria) with some trees and afterwards spontaneously spread over the region. The answer to these questions could be given only by an in-depth population genetics analysis which is in future plans.

The results could also have an impact on the economy in the region. The truffles are among
the most expensive food, as already told. In Zadar County, they were found in hinterland and some of the areas rich with truffles are depopulated. The truffles could enhance the local economy and give an opportunity for younger population to remain in their villages. The developed tourism in the region could both benefit from the presence of truffles and help the local population to place their truffle products on the market. The spontaneous presence of truffles indicates that this territory is suitable for their growth and therefore in these areas some truffle cultivation could be undertaken. Maybe the best way could be through the reforestation using trees mycorrhized with autochthon strains.

ACKNOWLEDGMENTS

The authors would like to express their gratitude to Mr Ivan Matak who, with his trained dogs, performed the search for truffles in Zadar County.

This research was partially granted by the Agency for Rural Development of Zadar County-AGRRA.
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