

GENETIC CHARACTERIZATION AND VOLATILE PROFILES OF *TUBER MESENERICUM* FROM BENEVENTO

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Abstract – *Tuber mesentericum* Vitt. is an edible truffle growing in some Mediterranean countries. Its center of production is localized in Italy, in particular in the area surrounding Bagnoli Irpino (Province of Avellino, in land of Naples), where *T. mesentericum* is very abundant.

Considering the variability of its morphological characteristics, we used molecular analysis as a necessary tool for the recognition of the mycorrhizas of *Tuber mesentericum*. The genetic variability of ITS1 and ITS2 nucleotide sequences and volatile organic compounds from *Tuber mesentericum* fruiting bodies were analyzed, focusing our attention on samples collected in the area of Benevento (Italy). Molecular analysis allowed us to identify a genetic difference between the most common *Tuber mesentericum* from Bagnoli and *Tuber mesentericum* from Benevento and. Moreover, the characterization of the profile of volatile organic compounds by Purge and Trap GC-MS analysis let us verify that it diverge across different geographical areas.

Keywords: *Tuber mesentericum*, ITS regions, aroma variability.

1. INTRODUCTION

Distribution of *Tuber mesentericum* Vitt. is spread throughout Europe, with the center of production localized in Italy, in particular in the area surrounding Bagnoli Irpino (Province of Avellino,

inland of Naples), where *T. mesentericum* is very abundant. It is known as the “Bagnoli black truffle” and represents an important economic resource for this rural area [1].

In the past, *T. mesentericum* was sometimes considered a subspecies of *T. aestivum* Vitt. [2] but some morphological studies have defined it as a true species [3] and recent molecular phylogeny analyses have shown that *T. mesentericum* forms a well distinguished species, sister group to *T. aestivum* [4].

The diagnosis of truffles is generally based on biochemical, serological and phenotype identification. However, these methods take time and do not always lead to a precise identification of the microorganism. Since PCR technology promises more rapid and specific identification, we decided to use this molecular biological technique as identification procedure.

In this work, we have evaluated the feasibility of the sequence analysis of the internal transcribed spacer regions (ITS) of the ribosomal DNA for the identification of *Tuber mesentericum* samples founded in the area of Benevento.

Furthermore, we have analyzed the profile of volatile organic compounds of truffle’s fruiting bodies using a Purge and Trap GC-MS analysis.

Since the truffle aroma is typical of each species, the complex composition of its volatile compounds has been the object of several studies over the past years. In fact, the fruiting bodies of truffles release a mixture of volatile compounds which are likely used to communicate with plants, animals and microorganisms [5]. Beyond their biological function, the mixture of volatile compounds

(aroma) emitted from the fruiting bodies determines their economic value [6].

The aim of our work was to characterize *T. mesentericum* samples coming from close areas of Benevento (area of Partenio, side of Benevento, and area of Taburno-Camposauro) and samples from Bagnoli Irpino in order to understand if among them there is a genetic and geographical differentiation which may determine the authenticity linked to territory of these precious mushrooms.

2. MATERIAL AND METHODS

2.1 Genetic and Molecular analysis

Truffle samples were collected from the area of Partenio, side of Benevento, and the area of Taburno-Camposauro (Benevento).

Genomic DNA of the fruiting bodies was extracted with a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

All PCR amplifications were performed using 100 ng of template DNA in a final volume of 50 µL containing 25 µL of Hot Start Taq Master mix (Qiagen, Hilden, Germany), 1µL each of primers, TART F2 (TGCAGAACTCAGTGAATCATC) and TART R2 (CCCTACCTGATCCGAGGTCA) and 18 µL sterile water DNase/RNase (Qiagen, Hilden, Germany) free to adjust to the final volume.

PCRs were carried out in a thermalcycler (Applied Biosystems 2700, USA) after an initial denaturation of 15 min at 95°C, amplified for 30 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and terminated by a final extension step at 72 °C for 7 min.

PCR products were checked on agarose gel through the QiaXcel (Qiagen). Fragment separation on QiaXcel system was performed according to the manufacturer's recommendations .

The PCR products, purified by the QIAquick PCR Purification Kit (Qiagen). Each amplified product was sequenced, using the same primers cited above, with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA). The sequence reaction was performed with a thermal cycler (Applied Biosystems 2700, USA) and provides 25 divided into cycles: 10 sec at 96 °C for DNA denaturation, 5sec at 50°C for the primer

hybridization and 4 min at 60 ° C for the extension of the fragment.

The sequenced products were purified using DyeEX 2.0 spin kit (Qiagen) and analysed on the ABI Prism 3500 automated sequencer (Applied Biosystems, USA).

DNA sequence analyses were performed with BLAST software available through the National Center for Biotechnology Information (NCBI). Tuber magnatum Vittad. was used as outgroup in the phylogenetic tree.

2.2 Purge and Trap GC-MS volatile fingerprinting

For each sample, 100 mg of pulverized truffle were put in 40 ml of high purity water and analyzed.

Analysis of truffle volatile organic compounds was carried out using a suitable technique, that is the Purge and Trap GC-MS analysis. The major component of the Purge and Trap system (TELEDYNE TEKMAR AtomX) is the analytical trap that retains the volatile organic compounds during the purge cycle allows the analytes to be trapped and concentrated before being desorbed and sent to the GC-MS for separation and detection. The GCMS-TQ8030 triple quadrupole gas chromatograph mass spectrometer (SHIMADSU) equipped with a SPBTM-624 Fused Silica Capillary Column (60m x 0.32mm x 1.8 µm film thickness) was used.

The oven was held at 35°C for 5 min, then warmed 4°C/min until 200°C was reached, and held for 15 min. Tentative identification of aroma components was based on mass spectra and NIST 14 library comparison. The peak was considered as identified when experimental spectrum matched that in the library with a score over 90%.

3. RESULTS

The identification of different fungal species can be performed with the direct optical microscope analysis of the sample, by means of the culture plate and on the morphological differentiation of the different structures, with serological tests for detection of antigens or antibodies and biochemical tests.

Today these techniques are no longer satisfactory, mainly for diagnostic level since

techniques are laborious, little standardized and with very long execution times especially with regard to the culture plate on whose times vary from species to species, but require a minimum of 48h incubation [7]. In addition, in most cases, these techniques do not provide accurate results because they have a low specificity in the identification of the species [8].

The use of molecular techniques instead allows rapid and specific identification with a higher sensitivity such as PCR, Real-time PCR, PCR-Elisa, RFLP (Restriction Fragment Polymorphism) and so on [9, 10, 11, 12].

We decided to identify *Tuber mesentericum* species of the Benevento area using PCR of the highly variable region ITS1 and ITS2 present in the ribosomal DNA.

Previous studies propose the use of ribosomal genes as occur in multiple copies within the genome and show conserved regions, as for example the genes 18S, 28S and 5.8S common to all fungi, and highly variable regions between the different species, such as the ITS regions. These variable regions are then exploitable for the phylogenetic classification and therefore also for identification to species level required for diagnostics [11, 13, 14, 15, 16].

The obtained sequences were also compared with other *Tuber mesentericum* sequences in GenBank using the BLAST (Basic Local Alignment Search Tool) of the NCBI (National Center for Biotechnology Information, USA) for the fragment identification. We made a comparison based on the most percentage of homology of the analyzed sequence with respect to the sequences found in the database. In particular, *Tuber mesentericum* from Bagnoli Irpino was compared with our samples in order to define the homology between two samples coming from two close areas.

Analysis of volatiles from *T. mesentericum* fruiting bodies has led to the identification of 11 significant peaks (figure 1).

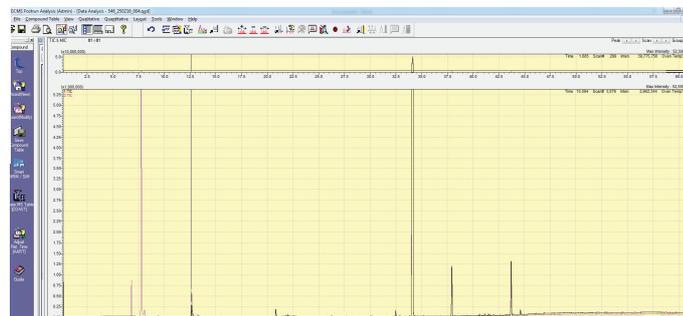


Fig. 1: Profile of volatile organic compounds in the populations of samples coming from Benevento

The compounds identified are listed in Table 1 with their relative quantity.

Table 1: Compounds identified through Purge and Trap GC-MS analysis.

Ret. Time	Name	Area
12.650	2-Propenoic acid	1560207
12.820	2-Propenoic acid	
20.840	2,5-Norbomadiene	738403
32.500	3-Octanone	494144
34.130	1,3,5-Cycloheptatriene, 1-methoxy-	325127738
37.950	2-Ethylphenol, methyl ether	4443045
40.650	3,4-Dimethoxytoluene	155732
41.160	Benzene, 1,2,3-trichloro-	141033
41.520	Phenol, 3-methyl-6-propyl-	110155
41.710	1,3-Butadiene, 1,1,2,3,4,4-hexachloro-	80755
43.720	Benzene, 1,4-dimethoxy-2-methyl-	4724787
44.620	3,5-Dimethoxytoluene	393896

4. DISCUSSION

Although a decline in production has triggered research into truffle cultivation, many basic aspects of truffle biology, such as the reproductive mode and the amount and distribution of population genetic variation, are not well understood.

Genetic analysis of ITS regions of *Tuber mesentericum* shows that the genetic sequences of the four samples from the Benevento area are different from the sequences, relating to ITS regions, present in GenBank from the Bagnoli

Irpino area. For this purpose, the sequences obtained were aligned with the GenBank sequences with accession numbers AB261178.1, AB261180.1, AB261182.1, AB261184.

The alignments, performed with Bioedit, showed that the genetic sequences of *Tuber mesentericum* from Benevento areas are different respect to those from the Bagnoli Irpino area, infact they differ in some nucleotides (figure 2).

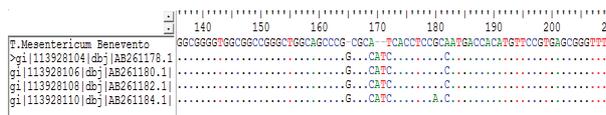


Fig 2: ITS nucleotide sequence alignment of *Tuber mesentericum* from Benevento and the one from Bagnoli Irpino

Numerous factors influence truffle aroma such as the maturity of the fruiting bodies, the presence of the microbial flora that inhabits the fruiting bodies [17, 18].

Gioacchini et al., [19] reported that geographical origin also contributes to aroma variability.

However, although the truffles show significant variability in their aromatic profiles [20], truffles belonging to a given species share a common profile of volatile organic compounds that can serve as fingerprints to identify a species [21].

We reported in the profile of volatiles of *Tuber mesentericum* from Benevento areas compounds typical of *Tuber mesentericum* Vitt. [22] along with other volatiles specific to the only variety from Benevento. Among them a representative compound is anisole or methoxybenzene which have an odor of anise and could give the truffle a unique aroma determining a characteristic flavour linked to the territory and therefore typical of authentic food.

5. CONCLUSIONS

Our results reported a genetic difference between the *Tuber mesentericum* from Benevento areas and the ones from the Bagnoli Irpino area, infact they differ in some nucleotides.

Numerous factors influence truffle aroma including the geographical area.

Despite the significant variability in their aromatic profiles, truffles belonging to a given species share a common profile of compounds that represents a fingerprint of that species.

We reported that the truffles collected from the area of Partenio, side of Benevento, and the area of Taburno-Camposauro (Benevento) show a profile of volatiles typical of *Tuber mesentericum* Vitt. along with other volatiles specific to the only variety from Benevento, in particular the anisole compound with its characteristic anise odor. Therefore it could give the truffle a unique aroma determining a characteristic flavour linked to the territory and therefore typical of authentic food.

5. References

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