

Synthesis of *Tuber mesentericum* ectomycorrhizae with *Quercus pubescens*: a morphological review and DNA characterization

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Tuber mesentericum Vittad., also known as the “black truffle of Bagnoli Irpino”, is a lesser known ectomycorrhizal hypogeous fungus in the Pezizales. Known from the majority of European countries, this species characteristically lives in loose, easily warming up, calcareous soils. The aim of this study was to propose a comprehensive morphological description of *T. mesentericum* ectomycorrhiza (ECM) supported by photographs and molecular data. Previous data suggested that *T. mesentericum* ECM have high similarity to those of *T. aestivum* Vittad. However, the high variability of the ITS region of the nuclear ribosomal DNA in *T. mesentericum* makes it impossible to design primers able to selectively amplify all the genotypes of this species. Mycorrhization percentages on 16 *Quercus pubescens* Willd. seedlings inoculated with *T. mesentericum*, were assessed over a period of 9 (first time-point) and 14 (second time-point) months. Two different quantities of the spore-slurry were used to inoculate two batches of plants (batches A and B). At the first time-point the presence of non-target ECM contaminant *Pulvinula constellatio* (Berk. & Broome) Boud. was detected with an average level of root colonization of 15 %. After 14 months *T. mesentericum* produced well-formed ECM with a rate of 20 % and 40 % in batches A and B, respectively. ITS region from ECM and ascomata used for the inoculum were sequenced and compared with *T. aestivum* and *T. mesentericum* sequences available in GenBank.

Keywords: trufficulture, ectomycorrhiza, truffle, ITS region, specific primers

Tuber mesentericum Vittad. is an edible truffle species belonging to the Pezizales. It is also known as “black truffle of Bagnoli Irpino” because it is historically and traditionally harvested and used in the preparation of local dishes in the city of Bagnoli Irpino (Avellino, Italy) (Garofoli 1906). *Tuber mesentericum* has a developed market in southern Italy as well as in north eastern France, where it is more expensive than *Tuber aestivum* Vittad. (Summer or Burgundy truffle), being able to reach the price of 450 Euro/kg. In Italy, it is sold at a price ranging from 100 to 200 Euro/kg (Benucci et al. 2016; www.laceno.net). However, from the Italian market, *T. mesentericum* is also exported and sold in Germany at higher prices than in Italy (Suriano E., pers. comm.). Its gastronomical use and economical value are strongly related to local traditions and customs, mostly due to its peculiar organoleptic properties. *Tuber mesentericum* has a strong and very distinct phenolic-like aroma that makes it not always appreciated outside its traditional areas of

production (Vittadini 1831, Granetti et al. 2005, Rioussset et al. 2012).

Tuber mesentericum is widespread throughout Europe, spanning from Spain to Turkey and from Sweden to Italy (Pegler et al. 1993, Montecchi & Sarasini 2000, Wedén et al. 2001, Ceruti et al. 2003, Granetti et al. 2005, Sica et al. 2007, Castellano & Türkoğlu 2012). It usually associates with hazelnut (*Corylus avellana* L.), beech (*Fagus sylvatica* L.), oaks (*Quercus robur* L., *Q. cerris* L., *Q. petraea* (Mattuschka) Liebl., and *Q. pubescens* Willd.), pine (*Pinus nigra* Arnold and *P. sylvestris* L.), hornbeams (*Carpinus betulus* L. and *Ostrya carpinifolia* Scop.), and lime (*Tilia* spp.) that grow on hilly and/or mountainous areas up to 1800 m a.s.l. (Ceruti et al. 2003, Granetti et al. 2005, Miko et al. 2006, Marjanović et al. 2010, Bencivenga & Baciarelli Falini 2012). *Tuber mesentericum* often fruits in humid environments with closed vegetation cover. In Italy and Eastern Europe, fruiting bodies are usually found in sloped calcareous soils

oriented to the north (Granetti et al. 2005, Miko et al. 2006, Marjanović et al. 2010). In Central Europe, the most productive habitats of *T. mesentericum* are warm hillsides mostly oriented to the south (Chevalier & Frochot 1997, Stobbe et al. 2012). In Italy, the production of *T. mesentericum* begins in early summer with a peak between September and January. Occasionally, in the mountain belt at high altitudes, (1500–1800 m a.s.l.) snowfalls can delay the harvest to April or May. Specimens collected in spring are often in a perfect state of preservation because of the snow cover and low soil temperature. However, this optimal condition of the ascomata lasts only for a few days (Suriano E., pers. comm.).

Often, ascomata of *T. mesentericum* are confused with *T. aestivum* because of their very similar morphology. In particular, they both have transverse streaks on the peridium warts and the surface of their spores is reticulate-alveolate with complete, irregular, polygonal meshes. In the early nineties, some authors proposed the hypothesis of a “*Tuber aestivum-mesentericum* complex” because of the high similarity between the two species (Pacioni & Pomponi 1991). Nevertheless, subsequent morphological studies have defined *T. mesentericum* as a separate species (Rauscher et al. 1995). In a mix of freshly harvested *T. aestivum* and *T. mesentericum* truffles, the last can be recognized because of the characteristic aroma, which is generally strong with frequent unpleasant note of phenol, tar and/or iodine. It has been demonstrated that truffle’s aroma is caused by VOCs (volatile organic compounds) which are influenced by the fungal genotype (Splivallo et al. 2012) and by the microbial communities of gleba and peridium (Buzzini et al. 2005, Splivallo et al. 2014), which can vary according truffles geographical origin. Beyond the aroma, there are other unique features ascribed to *T. mesentericum*, such as the presence of a basal depression or cavity in the ascomata (Ceruti et al. 2003). However, a basal cavity can be present also in *T. aestivum* (especially its commercial type, *T. uncinatum*) which makes this characteristic a poor taxonomic trait. Previous molecular investigations have confirmed that *T. mesentericum* is a well distinguished species, sister to *T. aestivum* (Wedén et al. 2005). To avoid any misidentification between these two species, both morphological and molecular analysis for truffle characterization are strongly recommended (Sica et al. 2007). Although PCR-based methods to detect *T. aestivum* were successfully developed (Mello et al. 2002), species-specific primers able to characterize the whole genotypic

variability of *T. mesentericum* are lacking (Gryndler et al. 2011).

Tuber mesentericum ectomycorrhizae (ECM) were firstly found by Giraud in 1988 in a natural truffle productive site. Afterwards, ECM were obtained, after spore inoculation in laboratory conditions, by Rauscher et al. (1995) on *C. avellana* and by Zambonelli et al. (1993, 1995) on *Pinus pinea*. *Tuber mesentericum* ECM obtained in the above-mentioned studies had similar morphological features with ECM of *T. aestivum*. Both these truffles have ECM with a densely wooly surface, due to the presence of many, long, and curly cystidia. Cystidia are awl shaped, bristle-like (type A), sometimes with proximal (basal) ramification in *T. mesentericum*. Young *T. aestivum* and *T. mesentericum* ECM have a yellowish-brown colour, whereas at the old stages ECM of *T. aestivum* turn to dark-brown and start to lose their cystidia (Molinier et al. 2016, Benucci et al. 2016). Both species have a pseudoparenchymatous mantle with angular pseudo-cells (type L). The main morphological feature that distinguishes *T. mesentericum* from *T. aestivum* ECM is the presence of cystidia with a proximal ramification (Zambonelli et al. 1995). To the best of our knowledge, there is no literature regarding selective isolation and characterization of DNA from *T. mesentericum* ECM.

This study aimed to: i) obtain *T. mesentericum* ectomycorrhizae (ECM), ii) generate a morphological and anatomical characterization of formed ECM, supported by photographs and molecular sequence data, iii) calculate mycorrhization levels of *T. mesentericum* and of other concurrent fungi, and iv) describe specific growing conditions that may benefit the production of seedlings inoculated with *T. mesentericum* for the market.

Materials and methods

Quercus pubescens acorns were collected in Umbria (Italy) in a naturally wooded site. Seeds were surface sterilized with a 5 % sodium hypochlorite solution for 20 min, rinsed, and set in a mix of 50 % sterile perlite and 50 % sterile vermiculite, in a climatic controlled environment to await germination. A single *T. mesentericum* ascoma weighing 36 g was accurately taxonomically identified through morphological and molecular analysis (see below). A spore slurry was prepared by blending it (approximately 31 g of truffle gleba) in 360 ml of sterile water. Sixteen four-months-old seedlings were inoculated either with 15 ml (batch A) or 30 ml (batch B)

spore slurry. Seedlings of batches A and B were placed in two different trays, leaned in separated shelves inside a growth chamber (16 hours light/day, 22 °C), and grown for a total of 14 months.

The level of ectomycorrhizal colonization was assessed after nine months on four seedlings (first time-point) while the other 12 were harvested and analyzed after 14 months (second time-point). The same number of samples was taken from each batch and the negative controls were analyzed at the second time-point. To properly visualize ECM, roots were gently washed in cold tap water and placed in a glass Petri dish containing distilled water as suggested by Avis et al. (2003). Mycorrhization levels were assessed using an official standard method for quality control published by Donnini et al. (2014), which is used to officially certify truffle-inoculated seedlings, produced by commercial nurseries, in Italy.

Mycorrhization data of the two seedlings groups at the two time-points were tested for normality of data distribution using Shapiro-Wilk test. Analysis of Variance (ANOVA, $p \leq 0.05$) and Tukey test were used to test for differences between group means. Morphological and anatomical traits of the ECM were evaluated using a stereomicroscope (Leica Leitz Wild MZ8, Wetzlar, Germany) and a light microscope (Leica Leitz DMRB, Wetzlar, Germany). Digital photographs were taken on a Leica DFC320 digital camera. Description of ECM was performed according to Agerer (2006).

Pieces of the *T. mesentericum* ascoma, which was used to prepare the inoculum, were ground in liquid nitrogen and used for DNA extraction with the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, Missouri, USA). One ECM was randomly picked from four different seedlings, belonging to both batches A and B, and used for genetic DNA analysis. DNA was extracted with the Extract-N-Amp kit (Sigma-Aldrich, St. Louis, Missouri, USA), using only 10 µl of extract and dilution solutions. All the DNA extracts were PCR amplified for the Internal Transcribed Spacer (ITS) region using the ITS1F/ITS4 primer pair (Gardes & Bruns 1993). Each 50 µl PCR reaction consisted of 1× DreamTaq Buffer (with MgCl₂ included), 10 micrograms BSA (bovine serum albumin), 0.2 mM dNTPs, 10 ng of ITS1F and ITS4 primers, 2U DreamTaq DNA Polymerase (Thermo Scientific, Waltham, Massachusetts, USA) and ultrapure water to reach the volume mix of 45 µl. A volume of 5 µl of DNA extract was used to get a satisfying DNA amplification. The PCR thermal profile included 5 min at 95 °C as initial denaturation, fol-

lowed by 40 cycles of 30 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C and a final extension of 7 min at 72 °C. Three microliters of PCR products were added of 1× of DNA gel loading dye and run on 1.8 % (w/v) agarose gels stained with ethidium bromide. PCR products were then purified using the EuroGOLD Cycle-Pure kit (EUROCLONE, Pero, Italy) and Sanger sequenced from both sides on an ABI PRISM 3130xl genetic analyzer (Applied Biosystems, Foster City, California, USA). Assembled sequences were used as queries against the Genbank (Benson et al. 2013) database using Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al. 1990) available on the NCBI portal (<http://www.ncbi.nlm.nih.gov>) to confirm their taxonomic match to *T. mesentericum*. Representative assembled sequences were also deposited in NCBI with accession no. KY660019–KY660023. ITS sequences obtained from ascomata of *T. mesentericum*, *T. aestivum* and *Tuber magnatum* Pico were also downloaded from GenBank and used in a phylogenetic reconstruction together with the sequences produced in this study. Sequences produced in this study were labelled as TMESECM (ectomycorrhiza) and TMESAS (ascoma) while downloaded sequences were labeled as *Tuber mesentericum*, *T. aestivum* and *T. magnatum*. This last species was included as outgroup. The complete set of sequences was aligned using ClustalW of MEGA7 to avoid the inclusion of mislabeled sequences or misidentifications. The phylogenetic tree was constructed with MEGA7 (Kumar et al. 2016) using the maximum likelihood method based on the Tamura 3-parameter model (Tamura 1992).

Results and discussion

At the first time-point (9 months after truffle-spore inoculation), no *T. mesentericum* ectomycorrhizae (ECM) were found in the four (2 from batch A and 2 from B) analyzed seedlings. On the contrary, at the second time-point (14 months after the truffle-spore inoculation), *T. mesentericum* ECM were found in 6 of the remaining seedlings, 4 seedlings of batch A (A3, A6, A7, A8) and 2 of batch B (B6, B8). The highest mycorrhization level was found in sample B6 (40 %) and the lowest in samples A3 and A7 (5 %) (Tab.1). ECM (and ascomata, which were growing on the surface of the seedling substrate) of *Pulvinula constellatio* (Berk. & Broome) Boud. were also detected in 12 seedlings collected either at the first or second time-point in rates of 10–80 % (Tab. 1). In particular its highest presence was found in seedling B5 (80 %) and low-

Tab. 1. (A) Description of *Quercus pubescens* root colonization with *Tuber mesentericum* and contaminant fungi at time-point 1 (9 months after inoculation) and time-point 2 (14 months after inoculation). The analysis was carried out evaluating 400 root tips randomly picked from each seedling. Sample name, % of identified *T. mesentericum* and % of *Pulvinula constellatio* are shown. Letters A and B in sample names stay for the different inoculated batch. An average value at each time-point is also reported. **(B)** Overall mean value of mycorrhization level for group A and B.

(A)	Sample	<i>T. mesentericum</i> (mean %)	<i>P. constellatio</i> (mean %)
Time-point 1	A1	0	0
	A2	0	30
	Group mean	0	15
	B1	0	0
	B2	0	10
	Group mean	0	5
	Time point mean	0	10
Time-point 2	A3	5	5
	A4	0	45
	A5	0	55
	A6	20	0
	A7	5	0
	A8	10	10
	Group mean	6.67	19.17
	B3	0	55
	B4	0	65
	B5	0	80
	B6	40	0
	B7	0	20
	B8	15	0
Group mean	9.17	36.67	
Time point mean	7.92	27.92	
Controls	C1	0	10
	C2	0	35
	Group mean	0	22.5
(B)	Group	<i>T. mesentericum</i> (mean %)	<i>P. constellatio</i> (mean %)
	A	5.00	18.13
	B	6.88	28.75

est in seedling A3 (5 %). A co-contamination of *P. constellatio* with *T. mesentericum* was noted in samples A8 and A3. At the second time-point, the two negative controls (no inoculum added) were free of *T. mesentericum* but 10 and 35 % of *P. con-*

stellatio ECM were detected. The mean contamination value of *P. constellatio* in the negative controls is close to the average reported for all the seedlings (23.44 %). The overall *T. mesentericum* average mycorrhization level was significantly higher after 14 months from the inoculation (ANOVA, $p \leq 0.05$) than after only 9 months. *Tuber mesentericum* and *P. constellatio* average mycorrhization levels were not significantly different in the groups A and B (ANOVA, $p \leq 0.05$).

Morpho-anatomically *Tuber mesentericum* ECM showed a ramified monopodial-pinnate pattern (Fig. 1a) and were ochre to brown at complete formation. The unramified ends were straight, cylindrical, inflated and club-shaped, densely covered by the emanating hyphae. The outer mantle surface was formed of angular cells (type L) of 3–11 × 6–20 µm, organized in a pseudoparenchymatous tissue (Fig. 1d). Septed, awl-shaped, brownish cystidia were present in the outer mantle; they were bristle-like (type A) with proximal ramification (Fig. 1b,c). Ten *T. mesentericum* ECM were randomly selected to perform anatomic measurements. Ectomycorrhizae length averaged 1.30±0.45 mm, while diameter averaged 0.24±0.02 mm (Tab. 2a). Cystidia length averaged 270.8±128 µm, while diameter averaged 2.25±0.45 µm (Tab. 2b).

The five ITS rDNA sequences (4 sequences obtained from single ECM tips and 1 from the ascoma used to prepare the spore inocula) matched (identity ≥99 %), *T. mesentericum* reference sequences present in GenBank. A maximum likelihood (ML) phylogenetic tree was also produced to support the result of BLAST. The ML tree shows the presence of four main clades belonging to the *Aestivum* Clade (Bonito & Smith 2016), all together separated from the outgroup. Two clades include only sequences of *T. mesentericum*, other two clades include only sequences of *T. aestivum*. This is consistent with Bennucci et al. (2016), who showed the existence of different species within the *T. mesentericum* species complex. All the sequences produced in this study clustered together with the *T. mesentericum* accessions AB261253 and AB261267 (Italy), AB261401 (Hungary), AB261423 and AB261425 (Germany), marked as Clade 2 in Fig. 2. Sequences belonging to *T. mesentericum* from Spain, France and Switzerland (Clade 1), and sequences belonging to *T. aestivum* collected in Italy, Slovakia, Poland (Clade 3) and Sweden (Clade 4) were also included. *Tuber magnatum* sequences were included as outgroup (JQ925645 and AF003913) (Fig. 2).

Tuber mesentericum is an edible and legally commercialized truffle species in Europe. Although,

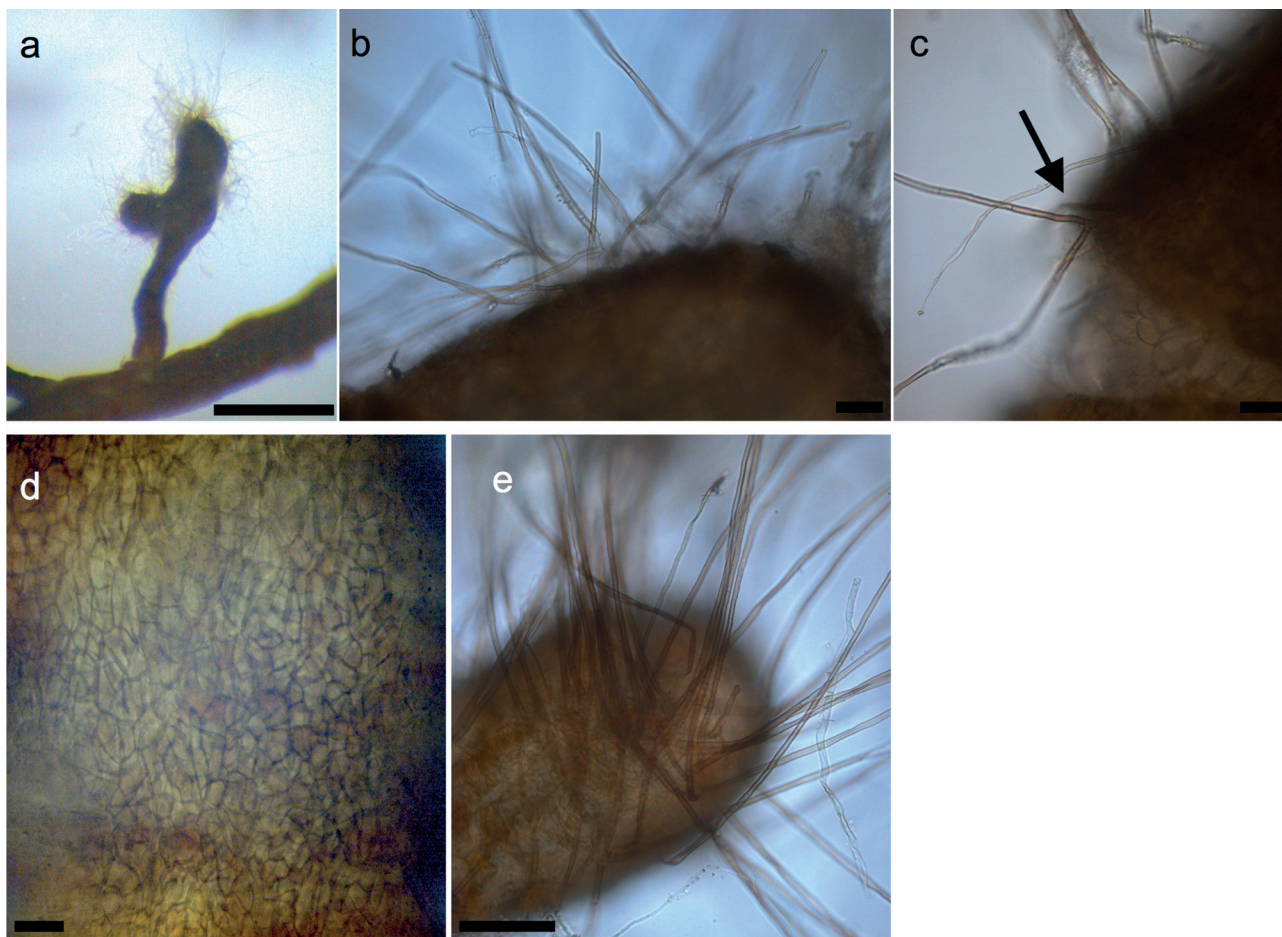


Fig. 1. Anatomical and morphological features of *Tuber mesentericum* ectomycorrhizae (ECM): **a.** ECM with abundant presence of hyphae; **b.** Septed, awl-shaped brownish cystidia; **c.** Single bristle-like (Type A) cystidium with a proximal ramification; **d.** Surface of the outer mantle layer with angular (Type L) pseudo-cells; **e.** Mantle with cystidia. Bars: a 500 μm , b, c 65 μm , d 10 μm , e 100 μm .

it does not have a market as important as that of the most known black truffle species, *T. melanosporum* Vittad. and *T. aestivum*. The low request locates this species on a low range price category and does not stimulate the nurseries to produce seedlings mycorrhized with it. The reason why this truffle is not appreciated like other truffles is its particular aroma. The strong scent, with notes resembling phenol, is unique and different from the more common *Tuber* species used for cooking and appreciated by chefs and consumers.

This study showed that, after 14 months from the truffle spore-slurry inoculation, *T. mesentericum* can successfully form ectomycorrhizae with *Quercus pubescens* in climate-controlled conditions. An average rate of 7.92 % for *T. mesentericum* and 27.92 % of *P. constellatio* was obtained. Only three samples, with 15, 20 and 40 % of *T. mesenteri-*

cum ECM, were free of the other concurrent fungus. The lack of evenness in *T. mesentericum* mycorrhization was probably due to the presence of *P. constellatio*. This concurrent fungus grew fast, arrived first on the roots (less than 9 months), and seemed to negatively impact *T. mesentericum* which arrived later, grew slow, and had less space and resources left. This, so called, “priority effect” (Kennedy 2010) shown by *P. constellatio* is supported by the fact that in four out of six seedlings that presented *T. mesentericum* ECM, this fungus was fully absent.

The two different inoculation treatments (15 and 30 ml) were, in average, not statistically different (ANOVA, $p \geq 0.05$) suggesting that the amount of truffle inoculum does not directly correlate with the obtained mycorrhization percentage (Tab. 1b). We showed that even in a small and controlled environment contaminant fungi can be widely present. This

Tab. 2. Morphological characteristics of ectomycorrhizae (ECM) formed by *Tuber mesentericum* and *Quercus pubescens*. Minimum, mean, maximum, standard deviation, and maximum values are given. **(A)** ECM length and diameter. **(B)** Cystidia length and diameter.

(A)	Sample	Length (mm)	Diameter (mm)	(B)	Sample	Length (μm)	Diameter (μm)
	1	0.97	0.20		1	187.5	2.6
	2	1.91	0.25		2	265	1.87
	3	1.28	0.24		3	368.75	2.87
	4	0.68	0.24		4	255	1.88
	5	1.52	0.28		5	245	1.5
	6	1.65	0.26		6	250	2.25
	7	1.08	0.24		7	262.5	2.5
	8	1.33	0.26		8	125	2.5
	9	1.90	0.23		9	122.5	2.5
	10	0.70	0.22		10	222.5	1.63
	–	–	–		11	327.5	2.5
	–	–	–		12	631.25	1.87
	–	–	–		13	256.25	2.75
	Mean (mm)	1.30	0.24		Mean (μm)	270.67	2.25
	St. dev.	0.45	0.02		St. dev.	128.10	0.45
	Min	0.68	0.20		Min	122.5	1.5
	Max	1.91	0.28		Max	631.25	2.87

highlights that truffle selection and handling before preparing the inoculum is a critical step because contaminants' spores persist inside the truffle or on the peridium. Additional source of contamination can come from people who take care of the plants and the growth environment, and/or from inaccurate sterilization (i.e., pasteurization) of cultivation potting-mixes. The average contamination level detected in the negative controls (23.44 %) proves how difficult it is to avoid the presence of external propagules. One reason why commercial nurseries do not usually produce seedlings with *T. mesentericum* is because it is very difficult to obtain a sufficient mycorrhization in the same greenhouse setting they are used to use for other *Tuber* spp. (Baciarelli Falini, pers. comm.).

Well-formed and developed ECM were found, presenting the same anatomy and morphology characteristics as reported by Zambonelli et al. (1993). The length of ECM ranged from 0.7 to 3.6 mm with a maximum value lower than that reported for the same plant-fungal association by Zambonelli et al. (1993). On the other hand, the diameter values varied from 0.20 to 0.28 mm with a mean of 0.24 mm perfectly matching with that proposed by Zambonelli et al. (1993) (mean=0.24 mm). The measures of cystidia were slightly lower than the one reported by Zambonelli et al. (1993, 1995). In these studies authors found an average length of 383 μm and an

average diameter of 2.31 μm ; differently in our study average values of 270.67 μm and 2.25 μm were found. The external layer of the mycorrhiza was fully covered by cystidia as described in the ECM of *T. aestivum* (Müller et al. 1996).

This work indirectly aimed to stimulate the cultivation of this truffle species, providing suggestions to improve the production of inoculated seedlings with *T. mesentericum* under controlled conditions. First, assure to use good quality (e.g., intact, round shaped, not overripe, well cleaned from soil) truffle for the preparation of the spore-inocula, which reduces their contamination with other *Tuber* spp. and ectomycorrhizal fungi. Second, sanitizing periodically and reducing traffic in the growing environment. Third, often check the efficiency of the machine used to sterilize the potting-mixes. Fourth, always include negative controls, seedlings which received no addition of truffle-spore inoculum. Additionally, a routinely check of truffle mycorrhization levels, at different time points, is encouraged before selling the seedlings to the market (Donnini et al. 2014).

For future research in this topic, to more precisely evaluate *T. mesentericum* development and mycorrhization trends over other contaminant fungi, different growth conditions should be tested. Moreover, inoculation of beech (*Fagus sylvatica*) and Turkey oak (*Quercus cerris*), which are other com-

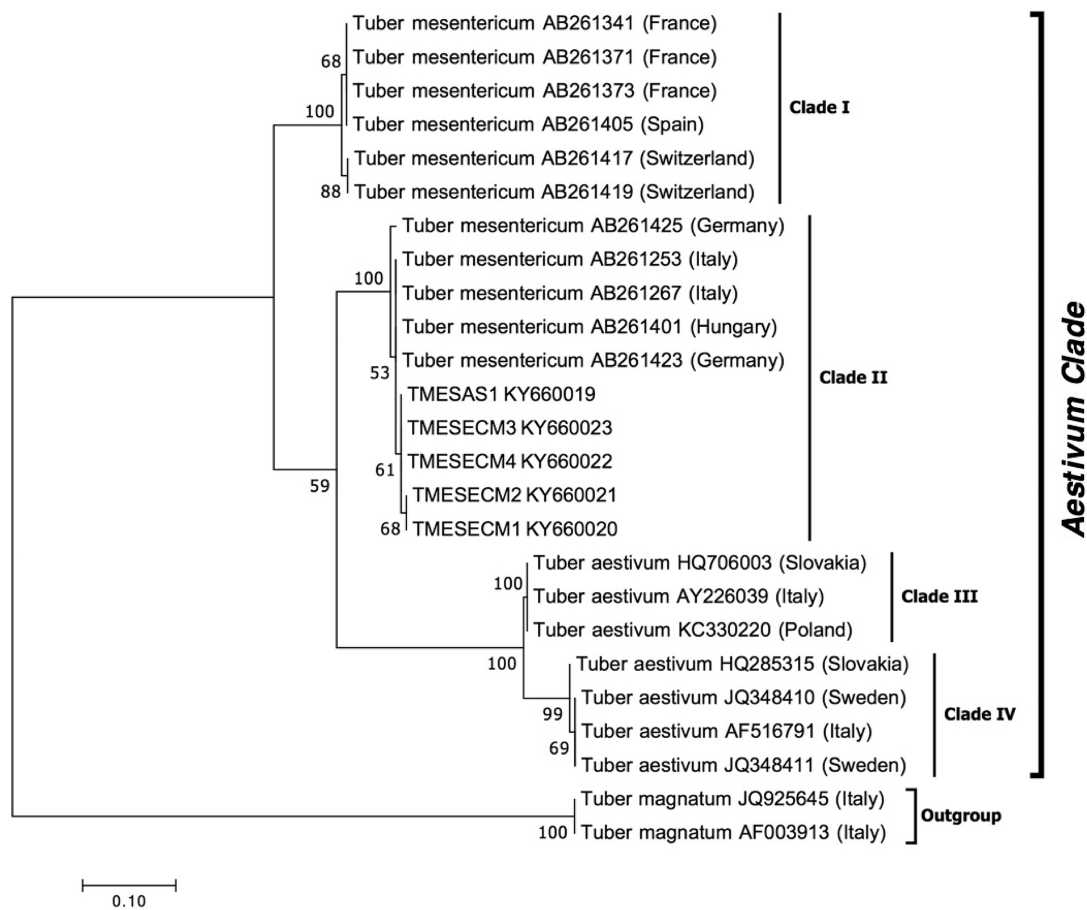


Fig. 2. *Tuber mesentericum/aestivum* maximum likelihood (ML) phylogenetic tree based on the Tamura 3-parameter model (Tamura 1992). A discrete gamma distribution [+I] was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in number of substitutions per site. The analysis involved 25 nucleotide sequences. All positions containing gaps and missing data were eliminated. A total of 179 positions were present in the final dataset.

mon host trees in natural *T. mesentericum* habitats, can improve our understanding of *T. mesentericum* symbiosis and represent an opportunity for commercial nurseries to extend their business in the traditional areas where this truffle is appreciated and consumed. Ultimately, a wider collection of samples would help to deeper investigate the *T. mesentericum* species complex, for example, using multiple-gene phylogenies and genetic population studies.

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References

- Agerer R. (2006) Fungal relationships and structural identity of their ectomycorrhizae. *Mycological Progress* 5(2): 67–107.
- Altschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology* 215: 403–410.
- Avis P.G., McLaughlin D.J., Dentinger B.C., Reich P.B. (2003) Long-term increase in nitrogen supply alters above- and below-ground ectomycorrhizal communities and increases the dominance of *Russula* spp. in a temperate oak savanna. *New Phytologist* 160: 239–253.
- Bencivenga M., Baciarelli Falini L. (2012) *Manuale di Tartuficoltura. Esperienze di coltivazione dei tartufi in Umbria*. Regione Umbria, Assessorato Regionale Agricoltura e Foreste, Artergraf - Città di Castello.
- Benson D.A., Cavanaugh M., Clark K., Karsch-Mizrachi I., Lipman D.J., Ostell J., Sayers E.W. (2013) GenBank. *Nucleic Acids Research* 41(D1): D36–D42.
- Benucci G.M.N., Csorbai A.G., Baciarelli Falini L., Marozzi G., Suriano E., Sitta N., Donnini D. (2016) Taxonomy, biology and ecology of *Tuber macrosporum* Vittad. and *Tuber mes-*

- entericum* Vittad. In: True Truffle (*Tuber* spp.) in the World (ed. Zambonelli A. et al.) Springer International Publishing: 69–86.
- Bonito G.M., Smith M.E. (2016) General systematic position of the truffles: evolutionary theories. In: True Truffle (*Tuber* spp.) in the World (ed. Zambonelli A. et al.) Springer International Publishing: 3–18.
- Buzzini P., Gasparetti C., Turchetti B., Cramarossa M.R., Vaughan-Martini A., Martini A., Pagnoni U.M., Forti L. (2005) Production of volatile organic compounds (VOCs) by yeasts isolated from the ascomata of black (*Tuber melanosporum* Vitt.) and white (*Tuber magnatum* Pico) truffles. *Archives of Microbiology* **184**(3): 187–93.
- Castellano M.A., Türkoğlu A. (2012) New records of truffle taxa in *Tuber* and *Terfezia* from Turkey. *Turkish Journal of Botany* **36**: 295–298.
- Ceruti A., Fontana A., Nosenzo C. (2003) *Le specie del genere Tuber. Una revisione storica*. Regione Piemonte, Museo Regionale di Scienze Naturali di Torino.
- Chevalier G., Frochot H. (1997) *La truffe de Bourgogne (Tuber uncinatum Chatin)*. Pétrarque, Levallois-Perret.
- Donnini D., Benucci G.M.N., Bencivenga M., Baciarelli Falini L. (2014) Quality assessment of truffle-inoculated seedlings in Italy: proposing revised parameters for certification. *Forest Systems* **23**(2): 385–393.
- Gardes M., Bruns T.D. (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113–118.
- Garofoli A. (1906) *Funghi e tartufi*. Cassone, Casale Monferato.
- Giraud M. (1988) Prélèvement et analyse de mycorhizes. Congrès de la trufficulture. Saintes, 27–28 novembre 1987. In: La truffe (ed. CTIFL, Parra, C.) Bull FNPT 10: 49–63.
- Granetti B., De Angelis A., Materozzi G. (2005) *Umbria terra di tartufi*. Regione Umbria, Terni.
- Gryndler M., Hršelová H., Soukupová L., Streiblová E., Valda S., Borovička J., Gryndlerová H., Gažo J., Miko M. (2011) Detection of summer truffle (*Tuber aestivum* Vittad.) in ectomycorrhizae and in soil using specific primers. *FEMS Microbiology Letters* **318**(1): 84–91.
- Kennedy P. (2010) Ectomycorrhizal fungi and interspecific competition: species interactions, community structure, coexistence mechanisms, and future research directions. *New Phytologist* **187**(4): 895–910.
- Kumar S., Stecher G., Tamura K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**: 1870–1874.
- Marjanović Ž., Grebenc T., Marković M., Glisic A., Milenković M. (2010) Ecological specificity and molecular diversity of truffles (genus *Tuber*) originating from mid-west of the Balkan Peninsula. *Sydowia* **62**: 67–87.
- Mello A., Cantisani A., Vizzini A., Bonfante P. (2002) Genetic variability of *Tuber uncinatum* and its relatedness to other black truffles. *Environmental Microbiology* **4**(10): 584–594.
- Miko M., Gazo J., Bratek Z. (2006) *Tuber macrosporum* Vitt. and *Tuber mesentericum* Vitt. – One hundred years neglected hypogeous fungi species in Slovak Republic. *Acta Fytotechnica et Zootechnica* **4**: 58–90.
- Molinier V., Peter M., Stobbe U., Egli S. (2016) The Burgundy Truffle (*Tuber aestivum* syn. *uncinatum*): A Truffle species with a wide habitat range over Europe. In: True Truffle (*Tuber* spp.) in the World (ed. Zambonelli A. et al.) Springer International Publishing: 33–47.
- Montecchi M., Sarasini A. (2000) *Funghi Ipogei d'Europa*. Associazione Micologica Bresadola, Trento.
- Müller W.R., Rauscher T., Agerer R., Chevalier G. (1996) *Tuber aestivum* Vitt.+ *Corylus avellana* L. *Descriptions of Ectomycorrhizae* **1**: 167–172.
- Pacioni G., Pomponi G. (1991) Genotypic patterns of some Italian populations of the *Tuber aestivum*-*mesentericum* complex. *Mycotaxon* **42**: 171–179.
- Pegler D.N., Spooner B.M., Young T.W.K. (1993) *British Truffles: a revision of British hypogeous fungi*. Royal Botanic Gardens, Kew.
- Rauscher T., Agerer R., Chevalier G. (1995) Ektomykorrhizen von *Tuber melanosporum*, *Tuber mesentericum* und *Tuber rufum* (Tuberales) and *Corylus avellana*. *Nova Hedwigia* **61**: 281–322.
- Riousset L., Riousset G., Chevalier G., Bardet M.C. (2012) *Truffles d'Europe et de Chine*. Editions Quae. Paris.
- Sica M., Gaudio L., Aceto S. (2007) Genetic structure of *Tuber mesentericum* Vitt. based on polymorphisms at the ribosomal DNA ITS. *Mycorrhiza* **17**: 405–14.
- Splivallo R., Valdez N., Kirchhoff N., Ona M.C., Schmidt J.P., Feussner I., Karlovsky P. (2012) Intraspecific genotypic variability determines concentrations of key truffle volatiles. *New Phytologist* **194**(3): 823–835.
- Splivallo R., Deveau A., Valdez N., Kirchhoff N., Frey-Klett P., Karlovsky P. (2014) Bacteria associated with truffle-fruiting bodies contribute to truffle aroma. *Environmental Microbiology* **17**(8): 2647–2660.
- Stobbe U., Büntgen U., Sproll L., Tegel W., Egli S., Fink S. (2012) Spatial distribution and ecological variation of rediscovered German truffle habitats. *Fungal Ecology* **5**(5): 591–599.
- Tamura K. (1992) Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Molecular Biology and Evolution* **9**: 678–687.
- Vittadini C. (1831) *Monographia Tuberaearum*. Rusconi, Milano.
- Wedén C., Ericsson L., Danell E. (2001) Research on *Tuber aestivum* syn. *T. uncinatum*, and *T. mesentericum* reported from Sweden for the first time. *Svensk Botanisk Tidskrift* **95**: 205–211.
- Wedén C., Danell E., Tibell L. (2005) Species recognition in the truffle genus *Tuber*—the synonyms *Tuber aestivum* and *Tuber uncinatum*. *Environmental Microbiology* **7**: 1535–1546.
- Zambonelli A., Salomoni S., Pisi A. (1993) Caratterizzazione anatomo-morfologica delle micorrize di *Tuber* spp. su *Quercus pubescens* Willd. *Micologia Italiana* **22**(3): 73–90.
- Zambonelli A., Salomoni S., Pisi A. (1995) Caratterizzazione anatomo-morfologica delle micorrize di *Tuber borchii*, *Tuber aestivum*, *Tuber mesentericum*, *Tuber brumale*, *Tuber melanosporum* su *Pinus pinea*. *Micologia Italiana* **24**(2): 119–137.

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