*Full Length Research Paper*

# Morphological and molecular characterization of *Tuber oligospermum* mycorrhizas

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*Tuber oligospermum* (Ascomycota) is an edible truffle growing in some Mediterranean countries. In Morocco, this truffle is regularly harvested and it is exported to Italy. Although *T. oligospermum* produces fruiting bodies of good quality, in Italy, it is fraudulently passed off as the most precious Italian white truffle (*Tuber magnatum*) and "bianchetto" truffle (*Tuber borchii*). In this work, a specific primer able to discriminate *T. oligospermum* from *T. borchii* and *T. magnatum* by multiplex polymerase chain reaction (PCR) assay was designed and tested for selective amplifications. Moreover, *T. oligospermum* ectomycorrhizas were obtained under greenhouse conditions by spore inoculation of *Quercus robur* seedlings and their morpho-anatomical characters were described and compared with those of *T. borchii* and *T. magnatum*. The degree of *T. oligospermum* root colonization was higher than that obtained for the other 2 truffle species but differences in mycorrhizal morphology were only found in terms of cystidia type and dimensions. Our results suggest that, *T. oligospermum* cultivation might represent an interesting agricultural activity for North African countries.

**Key words:** Internal transcribed spacer (ITS), mycorrhizal morphotyping, molecular identification, *Tuber oligospermum*, *Tuber borchii*, *Tuber magnatum*, multiplex polymerase chain reaction (PCR).

## INTRODUCTION

Hypogeous fungi belong to ascomycetes (the true truffle) and basidiomycetes (the false truffles), and some zygomycetes (Trappe and Castellano, 2007). True truffles belong to 37 genera, almost included in the Pezizales order, living in mycorrhizal association with the roots of different species of trees and shrubs (Trappe et al., 2007) and they are worldwide distributed (Trappe and Castellano, 2007; Trappe et al., 2010). However, only a few species of the genera *Tuber, Terfezia,* and *Tirmania* form edible fruiting bodies, which are a priced gourmet food and represent an important economic resource (Hall et al., 2007). *Terfezia* spp. and *Tirmania* spp. (called desert truffles) are found only in the warmest countries of Mediterranean area such as Morocco, Libya, Southern Spain, and Tunisia (Khabar et al., 2001). Within *Tuber* genus, only a few species, such as *T. oligospermum* (Tul. and C. Tul.) Trappe, *Tuber gennadii* (Chatin) Pat. and

*Tuber asa* Tul. And C. Tul., grow in the same areas of the desert truffles, whereas the other economically important members of this genus are widespread in more temperate and cold habitats of Mediterranean and European regions (*T. magnatum* Pico, *Tuber aestivum* Vittad*., Tuber melanosporum* Vittad*., T. borchii* Vittad*.,* etc.).

*T. oligospermum* fructifies abundantly in Northern Africa, establishing ectomycorrhizal symbiosis with coniferous and broad-leaved trees in sandy soils (Khabar et al., 2001). Although, *T. oligospermum* is an edible truffle of good quality, the Italian law forbids its trade. Being morphologically similar to the most precious Italian white truffle (*T. magnatum*) and the "bianchetto" truffle (*T. borchii*), this species is often exported to Italy and is used for commercial frauds (http://www.ilgiornale.it/news/cronache/truffa-

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servitainvasi-tartufo-d-africa.html;
http://bologna.repubblica.it/cronaca/2012/06/19/news/truff
a_tartufo-37491932/).

*T. oligospermum* ascomata are 1 to 4 (6 cm) in diameter, subglobose or irregular in form, solid, firm, of a whitish to yellowish-gray color and with often a lumpy surface finely puberulent or pruinose which becomes smooth with the age (Ceruti et al., 2003). The peridium (140 to 270 μm thick) includes a thin external plectenchimatic layer (40 to 60 μm) formed by hyaline, interwoven and septate hyphae and an internal prosenchimatic layer (Riousset et al., 2001). The gleba, whitish in immature ascomata, tends to grayish-brown color in the mature ones and it is marbled with numerous whitish veins arising from various points of the peridium. The asci contain 1 to 3 (4) globose spores (28 to 50 μm, ornamentations excluded), yellowish-brown at maturity, ornamented with a regular reticulum 5 to 7 μm high, with 4 to 6 meshes along the spores (Ceruti et al., 2003; Montecchi and Sarasini, 2000).

This species was described from the brothers Tulasne and Tulasne (1851) as *Terfezia oligosperma* because of the globose spores; later, Mattirolo (1904 to 1905) proposed to move it in the new genera *Delastreopsis*, considering the similarity of its spores to those of *Delastria rosea* Tul. and C. Tul. Finally, Trappe (1979) classified it in the genus *Tuber*.

*T. oligospermum*, as other similar whitish smooth truffles (*T. borchii, Tuber dryophilum* Tul. and C. Tul.*, Tuber puberulum* Berk. and Broome, *Tuber maculatum* Vittad.*,* etc.), is often misidentified (Zambonelli et al., 2000) and a number of the rDNA Internal transcribed spacer (ITS) accessions deposited in public sequence databases have inconsistent reliability (Zambonelli et al., 2012). As the ITS regions are widely used as reference for fungal species identification, especially when distinctive morphological characters are lacking (such as in immature fruiting bodies or mycorrhizas), caution is warranted in their use for taxonomic determinations of *T. oligospermum* through basic local alignment search tool (BLAST) in GenBank database (NCBI, http://www.ncbi.nlm.nih.gov).

With the aim of providing reliable molecular markers to identify this species, a specific primer able to discriminate *T. oligospermum* from *T. borchii* and *T. magnatum* by a multiplex PCR assay was designed. Moreover, the morphology of mycorrhizas of these 3 *Tuber* species was compared on *Quercus robur* L., an oak species forming extensive natural forests in Europe, from Norway (64°N) to Sicily (37°N) and from Ireland to the Balkans, Urals and Caucasus (Reyes and Casal, 2006).

## MATERIALS AND METHODS

### Morphological and molecular characterization of ascomata

Three ascomata (52 g in total) analyzed in this study were collected in April 2011 under *Pinus pinaster* Aiton from the Marmora forest (Rabat, Morocco) and it was identified by morphology according to Pegler et al. (1993), Riousset et al. (2001) and Ceruti et al. (2003). Dried specimens were deposited in the herbarium of the "Centro di Micologia" of Bologna University (CMI-UNIBO). ITS1-5.8S-ITS2 region of each ascomata was amplified by the direct PCR approach (Iotti and Zambonelli, 2006; Bonito, 2009), using the primer pair ITS1f-ITS4 (Gardes and Bruns, 1993). Sanger sequencing was performed using the ABI PRISM 3700 DNA Analyzer (Applied Biosystems) and sequences have been deposited in GenBank under the accession numbers KF021622 (CMI-UNIBO 4234), KF021623 (CMI-UNIBO 4231), and KF021624 (CMI-UNIBO 4230). BLASTN searches were done against the non-redundant nucleotide database at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify ITS sequences with the highest similarity to that generated in this work. Sequence analysis was performed by the MEGA5 software (Tamura et al., 2011).

### Preparation of spore inoculum and plant infection

After morphological characterization, the ascomata were surface flame-sterilized and stored at 4°C in sterile sand for 1 month. Spore inoculum was prepared as described by Iotti et al. (2012). Ascomata were crushed by a blender with the addition of sterile sand and water to facilitate tissue grinding. Spore suspension was then absorbed by sterile vermiculite and used to inoculate the seedling root systems.

Seeds of *Q. robur* were collected from a single plant in October 2010, sterilized in a 1% sodium hypochlorite solution for 10 min, rinsed under tap water and kept at 4°C in moist sterile sand until use. They were placed to germinate in a peat sand mixture (1:9) under greenhouse conditions and the developing root systems were washed and lightly pruned before inoculation.

In May 2011, 20 seedlings were inoculated with the spore inoculum, using approximately 2.5 g of ascomata per seedling ($10^5$ to $10^6$ spores per seedling). For comparison, other 20 seedlings were inoculated either with *T. borchii* or *T. magnatum* using the same procedures. 20 seedlings were not inoculated and were used as control. Plantlets were grown in 250 ml pots filled with a calcareous sandy loam soil recovered from a natural forest, close to Bologna (Italy), known for *T. magnatum* and *T. borchii* production. The soil was sterilized twice at 120°C for 1 h. The pots were kept in a greenhouse under daylight conditions and watered weekly with 100 ml of tap water.

The degree of ectomycorrhizal colonization was checked 6 months after inoculation. The root system of each seedling was washed and examined under a stereomicroscope (12×) to assess the presence of ectomycorrhizas. Ten rootlets (3 to 5 cm long) were randomly excised from each mycorrhized seedlings and the degree of root colonization was measured by counting the whole number of infected and uninfected tips. Results were expressed as a percentage of infected tips with *Tuber* spp. out of the total number of tips examined. Description of ectomycorrhizas was performed using a set of morpho-anatomical characters listed by Agerer (1987 to 1998) such as branching type, shape, color, mantle surface of unramified ends, mantle type and cystidia. Dimensions of the mantle cells in plan view (area, perimeter, maximum and minimum Feret's diameter), cystidia (length, basal diameter) and hyphae (diameter) were also measured by an ECLIPSE TE 2000-E microscope (1000×) (Nikon) using a NIS-elements AR 3.10 software (Nikon) from images captured with a DXM1200F digital camera (Nikon). Data are presented as the mean of 90 measurements obtained from 3 colonized tips excised from 3 different seedlings. Statistical differences between biometric data of *T. oligospermum, T. magnatum,* and *T. borchii* mycorrhizas were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test.

**Primer selection and PCR multiplex**

*T. oligospermum* specific primer for multiplex PCR was selected comparing all its ITS sequences analyzed in this work with some of those of *T. magnatum* (AF106888, FM205627, AF003913, AJ586271, AJ879679) and *T. borchii* (FJ554524, FJ554516, FJ554490, FJ809852, HM485342) deposited in GenBank database. A ClustalW alignment was performed to search species-specific and conserved domains which position, within ITS region, allowed to amplify PCR amplicons significantly different in size from those generated by TmagI (5'-GGATGCGTCTCCGAATCCTGAAT-3')-ITS4 (specific for *T. magnatum*) and rTboII (5'-GAAGTTGACCGTGGTAATAG-3')-ITS4 (specific for *T. borchii*) primer pairs (Amicucci et al., 2000). Primer selection was carried out with Primer3 software (http://frodo.wi.mit.edu/primer3/) and secondary structures and dimer formation were verified using Oligo Analyzer 1.0.3 software (Freeware, Teemu Kuulasmaa, Finland). Specificity was tested *in silico* using BLASTN algorithm and then confirmed by multiplex PCR against genomic DNA of different truffle species (*T. magnatum*, *T. borchii.*, *T. maculatum*, *T. dryophilum* T*, T. aestivum, Tuber rufum* Pico, *Tuber macrosporum* Vittad., *T. brumale* Vittad., *T. melanosporum*, *Choiromyces meandriformis* Vittad.) isolated from the corresponding ascomata, using the Nucleo Spin Plant II kit (Macherey-Nagel), following the manufacturer's protocol. PCRs were performed on 25 µl reaction mixture volumes containing 50 ng of total DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 µM for each dNTP, 400 nM for each primer (3 species-specific primers as forwards and ITS4 as reverse) and 1.5 U of *Taq* DNA polymerase (Fermentas).

Thermocycler conditions were the same used by Amicucci et al. (2000): 30 cycles of 94°C for 20 s, 63°C for 15 s, 72°C for 30 s, with an initial denaturation at 95°C for 5 min and a final extension at 72°C for 10 min. PCR products were electrophoresed in 1.5% agarose gels and visualized by staining with ethidium bromide in an GeneGenius Imaging System (SynGene, Cambridge, UK). Multiplex PCRs of mycorrhizas selected for morphotyping were also carried out using a direct PCR approach (Iotti and Zambonelli, 2006).

# RESULTS

## Phylogenetic analysis

The 3 ascomata used in this study, morphologically identified as *T. oligospermum*, generated 568-bp long ITS1-5.8S-ITS2 sequences each, with 2 variable positions both within ITS1 region. BLASTN searches against GenBank nucleotide database (latest date of accession: April, 2013) resulted in 45 sequences with a similarity >90%, mostly identified as belonging to *T. oligospermum* and *T. sphaerospermum* by Alvarado et al. (2012) and Grebenc et al. (2010). Phylogenetic analysis separated these entries in 3 major clades (Figure 1). Mean divergence among these 3 clades ranged from 7.6 and 4.9%, while intraclade ITS variation was <1.8% (Table 1). The sequences generated in this study were grouped in the 3 clades with ascomata and mycorrhizas collected in the western part of the Mediterranean area (Morocco, Iberian peninsula, and Sardinia), whereas, the members of the other 2 clades resulted to come mainly from European Mediterranean countries, from Spain to Israel.

**Primer selection**

Sequence alignment was allowed to identify a number of polymorphic domains, spread in both ITS 1 and ITS 2 regions, potentially suitable to design a specific primer targeted only for the genotypes from Morocco (Clade 3). However, only a domain placed at 3'-end of ITS1 region allowed to select the primer Tolf (5'-CTCCTGAGCTGAGGTGTC-3') which produces specific sized amplicons easy to discriminate, on agarose gel, from those generated from *T. magnatum* and *T. borchii* (Figures 2 and 3). In fact, Tolf, TmagI, and rTboII gave specific amplicons of 513, 596, and 168 bp in size, respectively, when used in conjunction with the reverse primer ITS 4 under multiplex PCR conditions. No amplifications were obtained using the DNA of the other truffle species analyzed and listed in material and methods.

## Morphological and molecular analyses of mycorrhizas

Six months after inoculation, root systems infected with the 3 truffle species were analysed. As shown in Table 2, all the seedlings inoculated with *T. oligospermum* and *T. borchii* were colonized by these species whereas *T. magnatum* ectomycorrhizas were found only in 7 on 20 inoculated seedlings. The highest mean percentage of root tip colonization was obtained with *T. oligospermum* (76.8%). Multiplex PCRs were successfully carried out for each infected tip selected for morphotyping to confirm their identity.

*T. magnatum* and *T. borchii* mycorrhizas obtained on *Q. robur* showed morphological features similar to those reported by Giomaro et al. (2000), Rubini et al. (2001) and Mello et al. (2001). *T. oligospermum* mycorrhizas were simple or monopodial-pinnate with straight, slightly club-shaped and smooth to short-spiny unramified ends; color of infected tips was ochre with a whitish distal end when young and brown at maturity. Both outer and inner mantle layers were pseudoparenchymatous with epidermoid cells; cystidia were hyaline, awl-shaped, uniseptate or biseptate, often provided with a basal inflation (Figure 4).

*T. oligospermum* mycorrhizas were morphologically similar to those of *T. borchii* but slightly different from those of *T. magnatum*, which appeared paler in color (whitish grey) with long-spiny to cottony unramified ends. *T. oligospermum* cystidia were significantly shorter than those of *T. magnatum* and morphologically similar but smaller than those of *T. borchii*, which were also lacking in basal inflation (Table 2). Mantles of these 3 truffle species were similar and difficult to differentiate by their anatomy. Significant differences were found only between cell dimensions of *T. oligospermum* and *T. borchii* (Table 2). Hyphal diameter was significantly
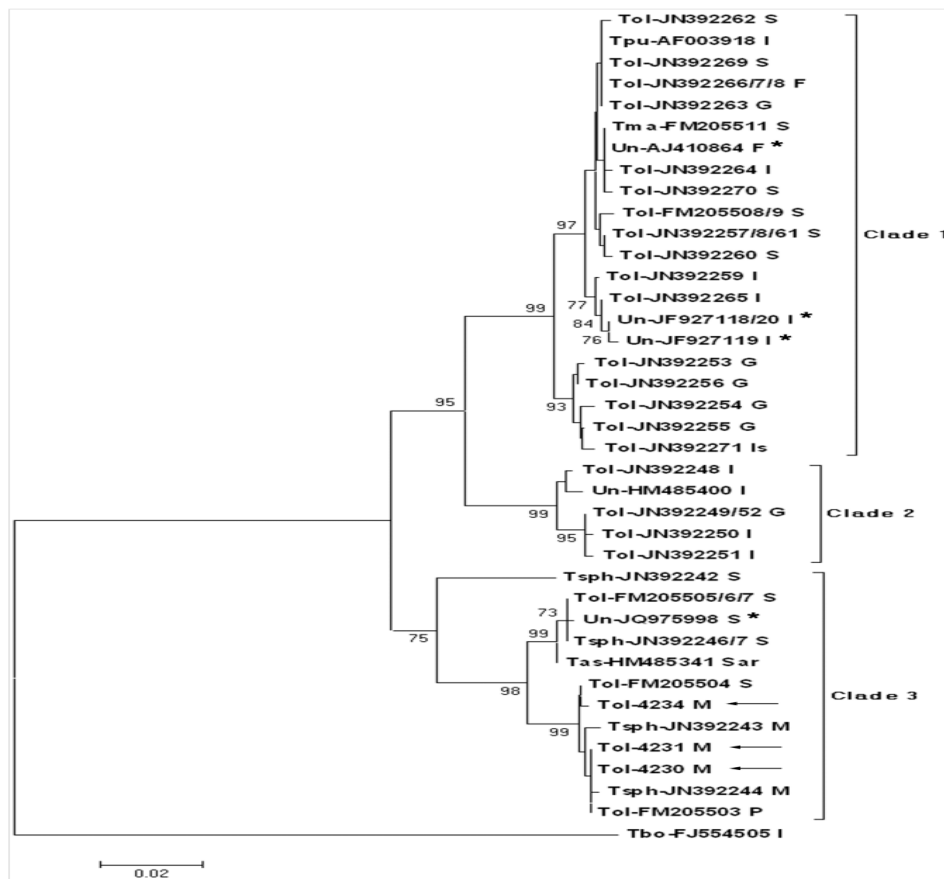
**Figure 1.** Phylogeny of *Tuber oligospermum* based on ITS rDNA sequences. After ClustalW alignment, the phylogenetic tree was generated using the neighbor-joining method with pairwise deletion of gaps and 1000 bootstrap replicates. Redundant entries (identical ITS sequences, species and country of origin) have been removed from the alignment. Taxa are labelled by their organism name (Tol, *T. oligospermum*; Tsph, *T. sphaerospermum*, Tma, *T. maculatum*; Tpu, *T. puberulum*; Tas, *T. asa*; Un, unidentified ascomata or mycorrhizas), GenBank accession number(s) and geographic origin (S, Spain; M, Morocco; I, Italy, Sar, Sardinia; P, Portugal; F, France; G, Greece, Is, Israel). *T. borchii* (Tbo) was used as outgroup. Arrows indicate the sequences generated in this work and asterisks the sequences from ectomycorrhizas or soil clones. Bootstrap values > 75% are reported.
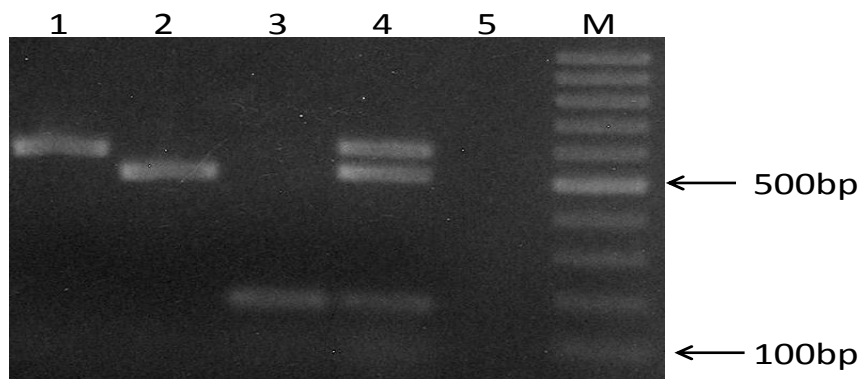


**Figure 2.** Multiplex PCR amplifications from ascomata of *T. magnatum*, *T. oligospermum*, *T. borchii*. For each reaction all species-specific forward primers (TmagI, TolF, and rTboII) were used with ITS 4 as common backward primer. M: GeneRuler 100 bp DNA ladder (Fermentas). Lane 1: *T. magnatum*; Lane 2: *T. oligospermum*; Lane 3: *T. borchii*; Lane 4: mixture of total DNA of the abovementioned *T.* species; Lane 5: negative control.

**Figure 3.** Schematic representation of position of the primers selected for the multiplex PCR in the rDNA ITS region. The black arrowheads represent the 3′ end of the primers.
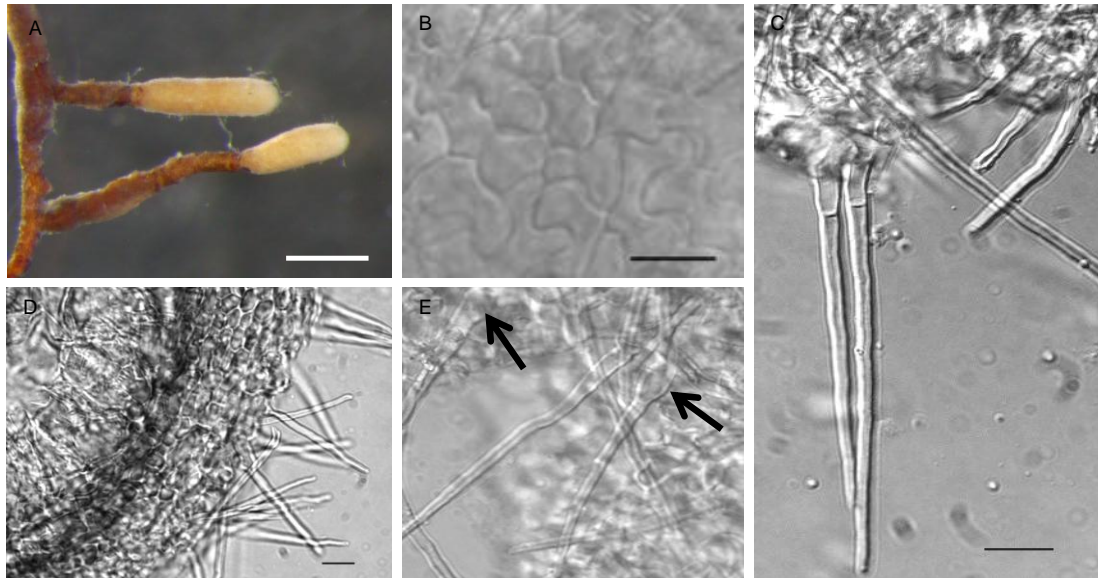


**Figure 4.** *T. oligospermum* mycorrhizas: colonized tips (bar = 300 µm) (A); mantle in plan view (bar = 10 µm) (B); cystidia with the typical basal inflation (bar = 10 µm) (C and E); cross-section (bar = 10 µm) (D).

**Table 1.** Mean distances within and between (in bold) clades of *T. oligospermum* ITS sequences analyzed in this work ($p$ distance ± SE).

| Clade | 1 | 2 | 3 |
|---|---|---|---|
| 1 | 0.010 ± 0.002 | | |
| 2 | 0.049 ± 0.008 | 0.008 ± 0.002 | |
| 3 | 0.073 ± 0.008 | 0.076 ± 0.009 | 0.018 ± 0.003 |

different in the 3 analyzed *Tuber* species, with *T. oligospermum* having intermediate dimensions.

## DISCUSSION

In this study, *T. oligospermum* mycorrhizas were successfully obtained on *Q. robur*, an oak species able to form mycorrhizas with numerous *Tuber* species and thus, largely used as host plant in truffle cultivation (Hall et al., 2007).

*T. magnatum* and *T. borchii* are commonly found associated to *Q. robur* in natural areas, unlike to *T. oligospermum* which does not share the same natural growth environments. Despite this, we obtained a very high percentage of root tip colonization with *T. oligospermum*, greater than that obtained with the other 2 truffle species under the same conditions. In previous works, *T. oligospermum* mycorrhizas were also abundantly obtained on *Quercus cerris* L. (Bencivenga et al., 1997) and *Pinus pinea* L. (Zambonelli unpublished data), which are considered the best host plants for *T. magnatum* and *T. borchii*, respectively (Tocci, 1985; Zambonelli et al., 2012). Being illegally introduced in Italy

**Table 2.** Dimensions of cystidia and mantle cells of *T. oligospermum, T. borchii* and *T. magnatum* mycorrhizas (means ± standard deviation).

| parameter | *T. oligospermum* | *T. magnatum* | *T. borchii* |
|---|---|---|---|
| Mantle cell, area (µm) | 70.72 ± 28.46[a] | 70.38 ± 29.99[a] | 87.62 ± 41.76[b] |
| Mantle cell, major axis (µm) | 14.34 ± 3.15[a] | 13.39 ± 3.22[a] | 14.99 ± 3.56a[b] |
| Mantle cell, minor axis (µm) | 8.36 ± 2.15[a] | 8.33 ± 2.04[a] | 9.15 ± 2.34[b] |
| Mantle cell, perimeter (µm) | 39.07 ± 9.72[a] | 38.33 ± 11.05[a] | 40.79 ± 10.17[a] |
| Length of cystidia (µm) | 46.39 ± 13.58[a] | 109.58 ± 41.49[b] | 64.98 ± 14.81[c] |
| Diameter of cystidia (µm) | 2.09 ± 0.53[a] | 2.08 ± 0.42[a] | 3.34 ± 0.55[b] |
| Hyphal diameter (µm) | 4.17 ± 0.95[a] | 2.49 ± 0.43[b] | 5.82 ± 0.48[c] |
| Number of colonized plants | 20 | 7 | 20 |
| Degree of colonization (%)[†] | 76.8 | 48.2 | 72.2 |

Means bearing different letters in the same row are significantly different (ANOVA, Tukey's test, p ≤ 0.05). [†]calculated only for colonized seedlings.

and fraudulently commercialized as *T. magnatum* and *T. borchii*, *T. oligospermum* could be unintentionally included in spore inocula used to produce mycorrhizal plants for truffle cultivation in Italy and in other European regions. In fact, the ascomata of these species are morphologically similar and the presence of some *T. oligospermum* fruiting bodies in a batch of *T. magnatum* and *T. borchii* may escape detection. The high infectiveness of *T. oligospermum* increases the risk of its introduction in non native European habitats and the consequent replacement of the native truffle species on host plant roots may occur at least in certain combinations of soil and climate. Indeed, the introduction of another alien truffle species, the Chinese black truffle *Tuber indicum* Cooke and Massee, has already occurred and demonstrated in Italy and researchers are concerned by the ecological consequences (Murat et al., 2008).

Analysis on *T. oligospermum*, *T. borchii* and *T. magnatum* mycorrhizas synthesized in this work demonstrated that, their morpho-anatomical characters are quite similar, except for cystidia. In fact, *T. oligospermum* cystidia are clearly different from those of *T. magnatum* which are much longer and ramified, as also reported by Rubini et al. (2001). Conversely, they look like those of *T. borchii* but with an additional characteristic basal inflation previously also described by Bencivenga et al. (1997). Significant differences have been also found in mantle cell dimensions, which are however variable, depending from the host plant and fungal strain (Giomaro et al., 2000; 2002). Moreover, considering that the cystidia in some stages of the mycorrhizal development can be absent, their mycorrhizas are difficult to distinguish morphologically and molecular tools are necessary for their identification (Zambonelli et al., 2012). In this context, the *T. oligospermum* specific primer designed in this study for a multiplex PCR approach may represent a reliable and simple tool to discriminate mycorrhizas or fruiting bodies of this truffle species.

On the other hand, *T. oligospermum* may represent an important economic resource for North African countries. In fact, it is an edible truffle with good organoleptic proprieties and infected plants can be easily obtained by spore inoculation. However, in the Northern part of Africa, likewise in other non European countries with no truffle tradition, truffles are harvested by raking the soil (Lefevre, 2012; Xianghua, 2012) and not by trained dogs or pigs. These animal species are able to find only mature truffles because of their typical aroma. Consequently, the sale of immature truffles adversely impacts culinary reputation of *T. oligospermum*. As suggested by Trappe (1989) and successively by Lefevre (2012), to protect local truffle production and to valorize the product, it is necessary to replace raking with trained animals. In this way it would be possible to harvest mature fruiting bodies and also to minimize forest floor disturbance. Another problem that could affect negatively the commercial valorization of *T. oligospermum* is the lack of a reliable phylogenetic definition for this truffle species. In fact, the analysis of sequences obtained in this study and of those retrieved from Genebank, grouped *T. oligospermum* specimens into well separate clades, which provide evidence for a substantial geographic separation of their members. The 3 sequences obtained in this work together with all the sequences from the African countries belong to the Clade 3, where five entries labelled as *T. spaherospermum* are present (Alvarado et al., 2012). This clade should be considered *T. oligospermum sensu stricto* as the morphological features of the analyzed fruiting bodies and, in particular, the anatomy of the peridium (data not shown) are those previously described by Ceruti et al. (2003) who examined a syntype specimen from Mattirolo herbarium.

**Conclusion**

In this work, firstly, we have provided the molecular

methods for the correct identification of *T. oligospermum*, providing the basis for the introduction of this truffle in local and international markets. Moreover, we have shown that, *T. oligospermum* infected plants can be easily obtained by spore inoculation, thus giving the basis for its cultivation. Indeed, *T. oligospermum* cultivation may become a new alternative agricultural activity in North African countries, contributing to their economic development.

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