**Soil analysis reveals the presence of an extended mycelial network in a Tuber magnatum truffle-ground**

# *Soil analysis reveals the presence of an extended mycelial network in a Tuber magnatum truffle-ground*

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## Abstract

Truffles are hypogeous ectomycorrhizal fungi. They belong to the genus *Tuber* and are currently considered a hot spot in fungal biology due to their ecological and economic relevance. Among all the species, *Tuber magnatum* is the most appreciated because of its special taste and aroma. The aim of this work was to set up a protocol to detect *T. magnatum* in soil and to assess its distribution in a natural truffle-ground. We used the β-tubulin gene as a marker to identify *T. magnatum* in the soil. This gene allowed us to trace the distribution of the fungus over the entire truffle-ground. *Tuber magnatum* was found, in one case, 100 m from the productive host plant. This study highlights that *T. magnatum* mycelium is more widespread than can be inferred from the distribution of truffles and ectomycorrhizas. Interestingly, a new haplotype – never described from fruiting body material – was identified. The specific detection of *T. magnatum* in the soil will allow to unravel the ecology of this fungus, following its mycelial network. Moreover, this new tool may have practical importance in projects aimed to increase large-scale truffle production, checking for *T. magnatum* persistence in plantations.


Keywords

- *Tuber magnatum*
- truffle-ground
- β-tubulin
- soil
- nested PCR
- specific primers

## Introduction

Truffles, ectomycorrhizal fungi belonging to the genus *Tuber*, are currently considered a hot spot in fungal biology (Bohannon, 2009). In a series of 'gourmet' genome projects, begun with sequencing of the grape genome, the precious black truffle *Tuber melanosporum* genome has just been sequenced by a French–Italian consortium (Murat & Martin, 2008). Mycologists have described around 200 European species, varieties and forms of *Tuber* over the centuries (Ceruti *et al.*, 2003); however, only the development of molecular tools has allowed construction of reliable phylogenetic trees (Jeandroz *et al.*, 2008) and validation of around 32 species in Europe (Ceruti *et al.*, 2003). Meanwhile, species-specific probes have allowed truffle species to be identified at different phases of their life cycle (Mello *et al.*, 2006), and the same technology can identify truffle fraudsters (Murat *et al.*, 2008). Moreover, it has been possible to widen the range of known truffle hosts: they are traditionally associated with trees and shrubs, but have now also been located inside orchid roots (Selosse *et al.*, 2004), suggesting that truffles possess a more flexible host range. Only recently has the truffle's complex life cycle been elucidated, underlining that they are primarily haploid and reproduce by outcrossing (Paolocci *et al.*, 2006).

Unlike *T. melanosporum*, which can be cultivated in orchards by inoculating oaks and hazelnuts with spore suspensions (Chevalier, 1994), *Tuber magnatum*, the most expensive and appreciated truffle species, has so far resisted the charms of domestication. It is, in fact, found only in a limited number of Italian and eastern European regions, usually associated with lime trees, oaks, willows and poplars. In a long-term investigation in a *T. magnatum* natural truffle-ground in Piedmont, fruiting bodies and mycorrhizas were carefully sampled. During the screening of mycorrhizal tips, *T. magnatum* mycorrhizas were found to be very rare, suggesting that this fungus invests more in forming fruiting bodies than in root colonization and that there is no direct linkage between mycorrhizas and fruiting bodies (Murat *et al.*, 2005). In the same Piedmont truffle-ground, a long-term survey of *T. magnatum* fruiting bodies (1997–2002) allowed us to identify two different sequences (haplotypes) in a sequence-characterized amplified region (SCAR), thanks to the finding of a single-nucleotide polymorphism (SNP), and to follow their spatial and temporal distribution (Mello *et al.*, 2005).

As the previous screening was focused on the fruiting bodies, which are temporary organs mostly found in autumn, and on mycorrhizas, which mostly depend on the host's physiological activity, we wondered about the distribution of the mycelium, which is expected to be present year-round in the soil. Tracking the dynamics of a given ectomycorrhizal fungus is considered difficult because fruiting bodies do not reflect the distribution of ground networks (Dahlberg, 2001). The extraradical mycelium plays a key role in the uptake of nutrients and water. Because each ectomycorrhizal fungal species is ecologically specialized (Goodman & Trofymow, 1998), contributing to the diversity of soil ecosystems, it is important to understand the structure and the spatial distribution of the extraradical mycelium, which exploits specific resources. *Hebeloma cylindrosporum* was the first ectomycorrhizal fungus to be detected in the soil by DNA extraction (Guidot *et al.*, 2002), and more recently, *Lactarius deliciosus* (Hortal *et al.*, 2008) and the black truffles *T. melanosporum* and *Tuber indicum* have been identified in plantation soils (Suz *et al.*, 2006, 2008; Murat *et al.*, 2008). Use of rDNA was considered reliable for the soil detection of these fungi, whereas internal transcribed spacer (ITS)-specific primers for *T. magnatum* fruiting bodies and mycorrhizas (Mello *et al.*, 1999) were sometimes not sufficiently specific when applied to the soil. The use of SCAR in order to trace *T. magnatum* mycelium was unsuccessful as well (C. Murat, unpublished data). For this reason, we searched for alternative markers. Recently, the β-tubulin gene has been used to develop *Tuber* genus-specific primers (Zampieri *et al.*, 2009); however, it had been used for designing species-specic PCR primers for fungal plant pathogens (McCartney *et al.*, 2003).

In the present paper, we used the β-tubulin gene to: (1) set up a protocol to specifically detect *T. magnatum* in soil and (2) assess the distribution of *T. magnatum* mycelium in a natural truffle-ground, assuming that DNA extracted from soil mirrors the whole extent of an ectomycorrhizal fungus.

# Materials and methods

## Sampling and DNA extraction

A total of 43 samples belonging to 40 species of different geographic origins were used (Table 1). Some fruiting bodies, or their genomic DNAs, were already available in the laboratory, while other samples were kindly sent by colleagues (A. Zambonelli, M. Iotti and G. Bonito). Each fruiting body was washed and the peridium was peeled. The gleba was cut into small pieces and stored at −80 °C. Total DNA was extracted from mycelia and ascocarps using the Dneasy Plant Mini Kit (Qiagen SA, Courtaboeuf, France) following the manufacturer's instructions. To check DNA quality, genomic DNAs, from *Tuber* and non-*Tuber* species, were tested with the β-tubulin universal primers Bt2a/Bt2b (Glass & Donaldson, 1995) using a Gene Amp PCR System 2700 thermocycler (Applied Biosystems), with a denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 90 s, and a final extension step of

5 min. The mix consisted of 10 × buffer (2.5 μL), 2.5 mM dNTPs (2 μL), 10 μM primer f (1 μL), 10 μM primer r (1 μL), water (15.2 μL), Red Taq 1 U $\mu L^{-1}$ (Sigma) (0.7 μL) and 1/10 diluted DNA (2 μL).

Tab. 1 List of the samples (*Tuber* and non-*Tuber* species) and their origin

| Species | Origin |
|---|---|
| *T. melanosporum 080107* | Piedmont |
| *T. borchii F9* | Piedmont |
| *T. rufum 2773* | Bologna |
| *T. oligospermum 2416* | Bologna |
| *T. dryophilum 3006* | Bologna |
| *T. magnatum F8* | Piedmont |
| *T. magnatum F6* | Piedmont |
| *T. magnatum IS1* | Piedmont |
| *T. aestivum* | Veneto |
| *T. excavatum 3438* | Bologna |
| *T. brumale 080130-1* | Piedmont |
| *T. indicum 080110-1* | China |
| *T. oregonense* | USA |
| *T. lyonii* | USA |
| *T. canaliculatum* | USA |
| *T.* cf. *gibbosum* | USA |
| *T. youngii* | USA |
| *T. quercicola* | USA |
| *T. candidum* | USA |
| *T. separans* | USA |
| *T. spinoreticulatum* | USA |
| *T. menseni* | USA |
| *T. mesentericum* | Piedmont |
| *T. foetidum 175* | Piedmont |
| *T. puberulum 174* | Piedmont |
| *T. macrosporum* | Piedmont |
| *T. moschatum 206* | Lombardia |
| *T. uncinatum T2* | Piedmont |
| *T. maculatum crix II* | Liguria |
| *T. maculatum N2* | Liguria |
| *T. panniferum* | Puglia |
| *Tuber* sp. | USA |
| *Tuber* sp. (clade *Rufum*) | USA |
| *Genea* sp. | Puglia |
| *Terfezia 1996* | Bologna |
| *Choiromyces* sp. | Puglia |
| *Humaria hemisphaerica* | Piedmont |

| Species | Origin |
|---|---|
| *Fusarium* sp. | Piedmont |
| *Acremonium* sp. | Piedmont |
| *Penicillium* sp. | Piedmont |
| *Oidiodendron maius* | Poland |
| *Boletus edulis* | Sardinia |
| *Hymenogaster* sp. | Puglia |

In order to assess the distribution of *T. magnatum* mycelium, a 7000-$m^2$ truffle-ground in a valley near Montemagno (Asti, Piedmont), North-western Italy, 8°19′35″4E, 44°59′2″40N (Mello *et al.*, 2005; Murat *et al.*, 2005), was selected. The soil was sampled twice, in January and May 2005, with each soil sample placed in a labelled bag, and caution had to be exercised while digging the soil under owner scrutiny. During the first survey, the collection sites were recorded for the second sampling in which soils were sampled approximately 50–70 cm from the site of the first sampling. Thirteen and 18 samples, in January and May, respectively, of about 100 g were taken at a depth of 10–15 cm and stored at −80° (Fig. 1). Soil DNAs were extracted following the protocol of the Fast DNA Spin Kit for Soil (Qbiogene), adding 10 washes with 5.5 M guanidine thiocyanate (Luis *et al.*, 2004). Two replicate samples of 0.5 g were extracted and pooled for amplification. The soil DNAs were amplified with the fungal ITS universal primers ITS1f/ITS2 (White *et al.*, 1990; Gardes & Bruns, 1993) under the same conditions as those used for Bt2a/Bt2b primers.

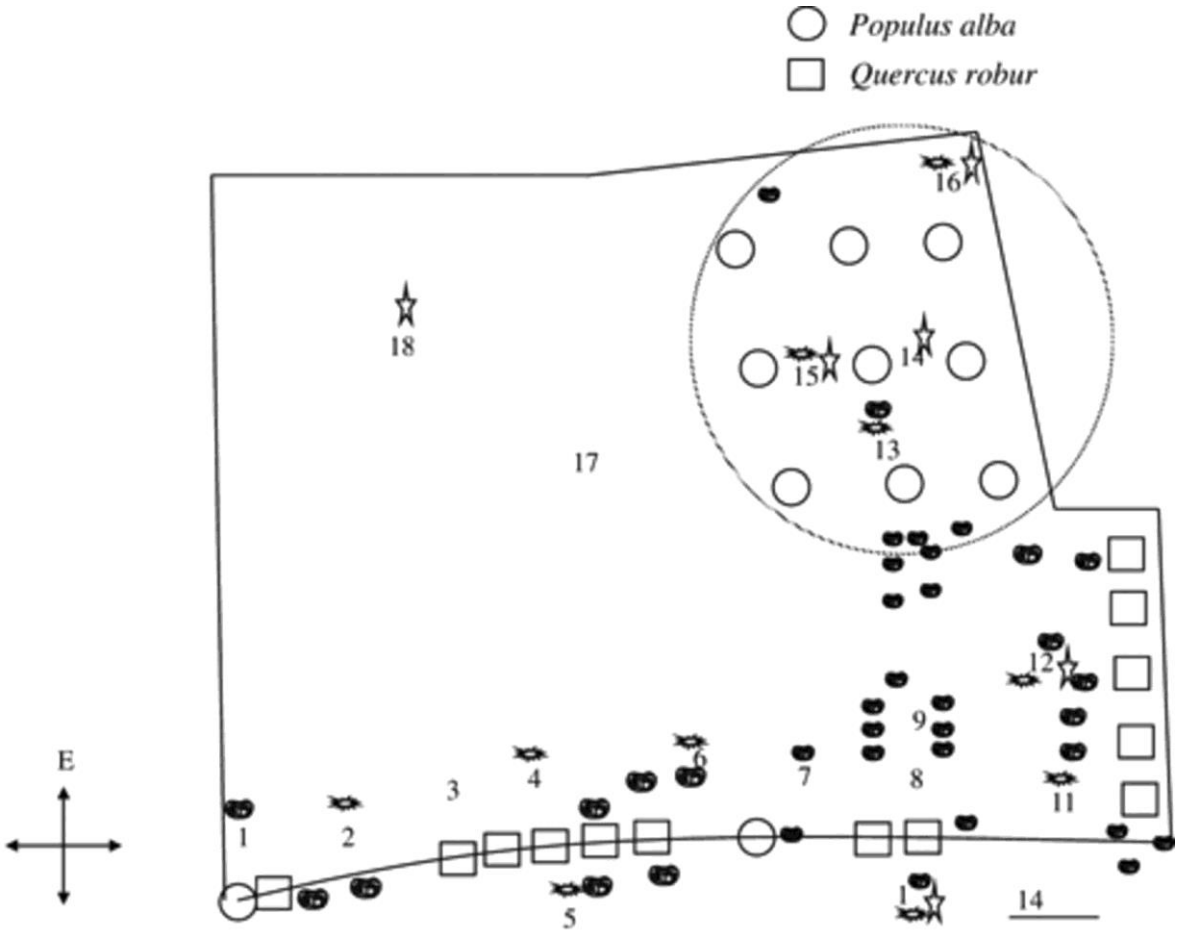


Fig. 1 Map of the truffle-ground in Montemagno (AT). The large circle indicates a nonproductive area. The numbers show the collection sites, which are the same for the first (January) and the second (May) sampling, except for numbers 7, 8, 14, 17 and 18, which were harvested only in May.

The ‘splash’ symbol shows *Tuber magnatum* identification in January samples, while the ‘star’ symbol shows *T. magnatum* identification in May samples. The symbol shows the fruiting body collection sites, according to Mello *et al.* (2005).

## Development of specific primers for *T. magnatum*β-tubulin

β-Tubulin sequences from the following 11 samples (*Fusarium oxysporum*EF450110, *Acremonium* sp. FJ430785, *Boletus edulis*FN252808, *Choiromyces* sp. FN252809, *T. magnatum*FN252812, *T. melanosporum*FN252815, *Tuber excavatum*FN252816, *Tuber borchii* FN252810, *Tuber rufum*FN252814, *Tuber oligospermum*FN252813 and *Tuber dryophilum*FN252811) were taken from the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and aligned using clustalw (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html) to search the different regions among *T. magnatum* and the other fungi.

A forward primer was designed (tubmagnf: CCTCCCAA TTTGCAATACAC) within the intron region of the β-tubulin gene and was used with the reverse primer (elytubr: AAAGACGAAGTTATCTGGCCTGA) (Zampieri *et al.*, 2009) (Fig. 2). The primers were tested *in silico* using the amplify 3.1 software (http://engels.genetics.wisc.edu/amplify/).

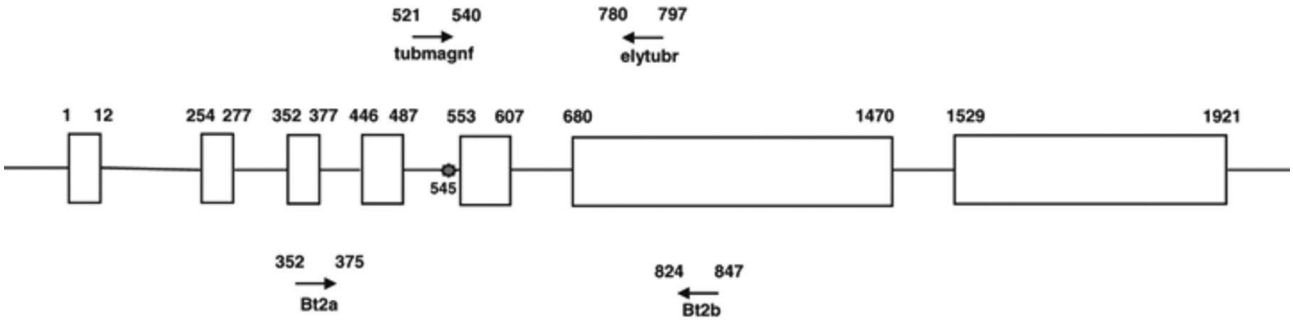


Fig.2 β-Tubulin structure showing the nucleotide positions of introns (lines) and exons (rectangles). The arrows indicate the position of the universal primers Bt2a/Bt2b and that of the *Tuber magnatum*, specific primers tubmagnf/elytubr, on the basis of the *Neurospora crassa*β-tubulin structure (Glass & Donaldson, 1995). The star shows the position of the SNP, found in the soil 10 (May sampling).

## Amplification of *T. magnatum*β-tubulin

Nested PCR was applied to the 43 DNA samples listed in Table 1 and 31 DNA from soil samples. The first PCR round was performed on a Gene Amp PCR System 2700 thermocycler (Applied Biosystems) with the universal primers Bt2a/Bt2b. In order to avoid any contamination, the mix composed of 10 × buffer (2 μL), 2.5 mM dNTPs (1.5 μL), 10 μM primer f (0.5 μL), 10 μM primer r (0.5 μL), water (11.1 μL) and Red Taq (Sigma) (1.4 μL) was first decontaminated using 3 U per reaction of MboI enzyme (Carrol *et al.*, 1999) for 30 min at 37 °C. The restriction enzyme was denatured at 95 °C for 2 min. The 1/10 diluted DNA (3 μL) was then added and the PCR was run with an initial denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 90 s, with a final extension at 72 °C for 5 min.

The second round of PCR was performed with the *T. magnatum*-specific primers tubmagnf/elytubr. The mix contained 10 × buffer (2.5 μL), 2.5 mM dNTPs (2 μL), 10 μM primer f (0.8 μL), 10 μM primer r (0.8 μL), water (16.7 μL), Red Taq 1 U $\mu L^{-1}$ (Sigma) (1.2 μL) and template (1 μL of the first PCR product). For the samples listed in Table 1, the PCR product from the first round was diluted

500-fold. The PCR cycles were composed of an initial denaturation at 94 °C for 3 s, followed by 25 cycles of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 5 min.

### Sequence analysis

All soil amplified products, obtained by the second round PCR with tubmagnf/elytubr, were checked on a 1.2% agarose gel in 0.5 × TAE buffer (20 mM Tris-acetate, 0.5 mM EDTA, 8.0 pH) after adding 0.5 μg $mL^{-1}$ ethidium bromide, and compared with the marker (100 bp, Invitrogen). These PCR products were purified following the protocol of the Qiaquick PCR purification (Qiagen SA) kit and sequenced by DiNAMYCODE s.r.l. (Turin, Italy), with both forward and reverse primers, using the CEQ 8000 method (Beckman-Coulter).

The sequences were analysed using sequencer software 4.2.2 (Gene codes corporation Ann Arbor Mi), where chromatograms of each sequence were carefully checked to search for SNPs. The similarity of these sequences was compared using the NCBI online standard basic local alignment search tool (blastn) algorithm (Altschul *et al.*, 1997). Accession numbers of sequences obtained from soil samples are as follows: FN252791–FN252806 for the β-tubulin gene.

# Results

All the DNAs corresponding to samples listed in Table 1 were successfully extracted and yielded an expected band from 300 to around 500 bp, when amplified by PCR with the universal Bt2a/Bt2b primers (data not shown). Similarly, a range of bands from 250 to around 350 bp was obtained, when amplifications with fungal ITS universal primers ITS1f/ITS2 were performed on the extracted soil DNA (Fig. 3a).

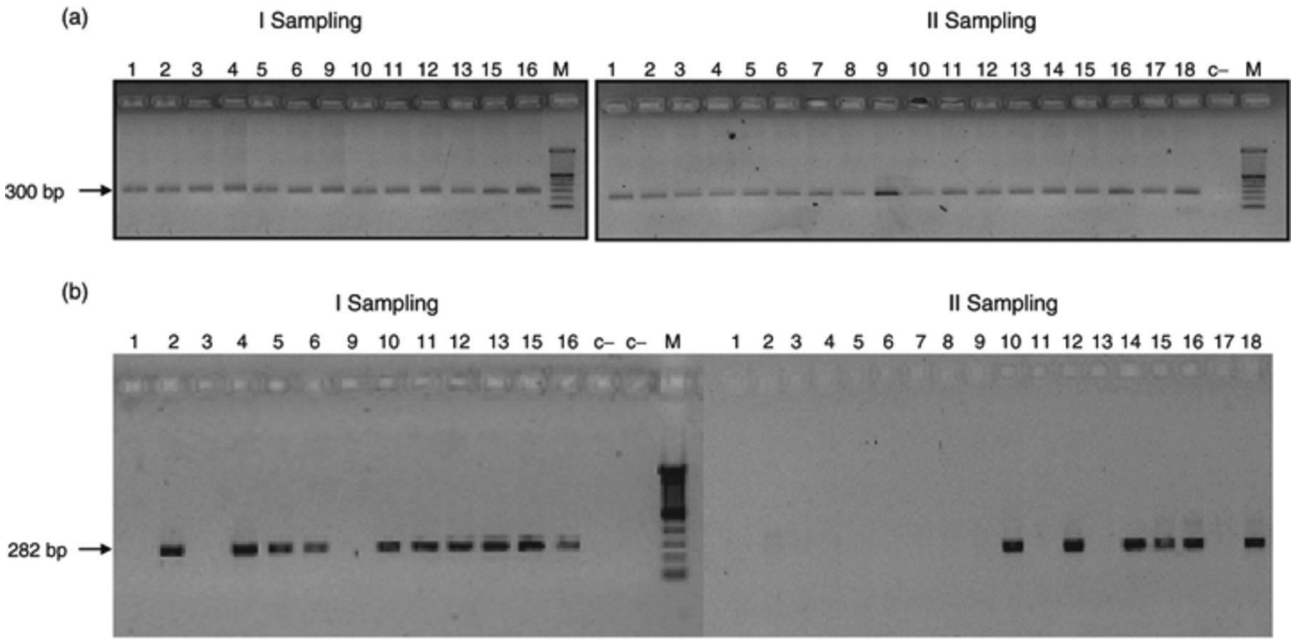


Fig. 3 (a) Bands from PCR with ITS1f/ITS2. (b) Bands from the second-round PCR with tubmagnf/elytubr. The numbers indicate the collection sites in the first (January) and the second (May) sampling; c−, negative control; M, 100-bp marker (Invitrogen).

In order to unambiguously detect *T. magnatum* mycelium, we designed specific primers in the functional β-tubulin gene. A new forward primer was designed on the basis of an alignment of 11 β-

tubulin sequences, arising from *F. oxysporum*, *Acremonium* sp., *B. edulis*, *Choiromyces* sp., *T. magnatum*, *T. melanosporum*, *T. excavatum*, *T. borchii*, *T. rufum*, *T. oligospermum* and *T. dryophilum* (data not shown).

The primers tubmagnf and elytubr were tested for their specificity on three *T. magnatum* fruiting bodies and 40 samples belonging to *Tuber* and non-*Tuber* species (Table 1). A nested PCR on DNAs was set up in order to enhance the possibility to detect *T. magnatum* in the soil. A 282-bp band was obtained, as expected, only for the *T. magnatum* DNA.

Thirteen soil DNAs of the first sampling were processed, as well as 18 from the second sampling. In particular, 10 out of 13 and six out of 18 soil samples gave the expected signal (Fig. 3b). The sequence of the 16 bands obtained fully matched with the β-tubulin *T. magnatum* sequences in the NCBI database. Among all the sequences, one, corresponding to May soil sample number 10 (Fig. 1), showed an SNP at position 545, when compared with the *Neurospora crassa* structure (Glass & Donaldson, 1995) used in Fig. 2, where a *G* was present instead of an *A*.

# Discussion

A truffle-ground is a special environment where plants and microorganisms form a rhizosphere niche within which truffles develop. During a long-term investigation of the natural *T. magnatum* truffle-ground, fruiting bodies and mycorrhizas were sampled (Mello *et al.*, 2006) and productive and nonproductive areas were identified, these last occurring mostly at the right side of the map, where the majority of the *Populus alba* trees are present (Fig. 1). All the data available so far on the ecology of *T. magnatum* have emerged from fruiting body and mycorrhiza studies.

In this study, the main question addressed concerns the distribution of *T. magnatum* in the soil. We were only allowed to collect a few soil samples, given that this truffle is one of the most expensive delicacies (€300–400 per 100 g) and the ground's owner was reluctant to risk his income; despite this, it was still possible to trace the distribution of *T. magnatum* over the entire truffle-ground.

## Mycelial network in a *T. magnatum* truffle-ground

The primers designed in the β-tubulin region were specific for *T. magnatum* fruiting bodies and allowed a reliable and sensitive identification of this species in soil. *Tuber magnatum* was detected in most of the soil samples collected in winter (10 out of 13 soils), while in spring the positive soils were fewer (six out of 18). Considering that all the DNA soil samples had been extracted using the same procedure and that they showed the expected band with fungal ITS universal primers, this result is in line with the biological cycle of the fungus. January is still the time of *T. magnatum* fruiting and spore dispersal whereas in May fruiting bodies are no longer present. The amplification of *T. magnatum* DNA in soil concerns mycelium; although spores and conidial forms are not excluded, the latter have never been described for *T. magnatum*, but have been shown for *T. borchii* (Urban *et al.*, 2004).

In a parallel investigation, all the soil DNAs corresponding to the two samplings have been amplified with *Tuber* genus-specific primers (Zampieri *et al.*, 2009). Among five *Tuber* species, belonging to the *Aestivum*, *Puberulum* and *Rufum* clades (Jeandroz *et al.*, 2008), *T. magnatum* was detected in 10 January samples and two May samples. The specific primers, applied here, confirmed the presence of *T. magnatum* in those samples, and revealed *T. magnatum* in four additional soils in the second sampling. This result demonstrates the higher sensitivity of the *T. magnatum*-specific primers vs. the *Tuber* genus-specific primers.

In the present paper, among the positive soils of the second sampling, one revealed an unexpected polymorphism, because none had been detected in the β-tubulin gene, from 30 *T. magnatum* truffles (Mello *et al.*, 2005). The missed detection of this polymorphism in the fruiting bodies could be explained in two ways: either the DNA extraction method left the spores unbroken (Rubini *et al.*, 2005) or the mycelium carrying that SNP was unable to form fruiting bodies. By contrast, free spores in the soil are probably disrupted, because they are subjected to direct friction during DNA extraction. Recently, the *Tuber* life cycle has been revaluated, showing that truffles are primarily haploid and reproduce by extensive outcrossing rather than from the simultaneous presence of different self-perpetuating clones (Riccioni *et al.*, 2008). On the basis of this reinterpretation, soil is an unknown source of genetic variability. Its potential richness could guarantee fruiting body production and must be considered in the management of plantations.

When we mapped the locations of the fruiting bodies collected in the past survey and of the soil samples, some observations emerged. The belowground propagules were much more widespread than could be inferred from the distribution of truffles: *T. magnatum* was also present in a nonproductive area, clearly suggesting that the presence of the mycelium is independent of fruiting body presence. *Tuber magnatum* DNA (18 in Fig. 1) was detected as far as 100 m away from the nearest productive tree and therefore from the truffle collection sites. Clearly, fruiting bodies are not produced over the whole extent of the belowground mycelium. This result resembles the distribution patterns of *Suillus grevillei* (Zhou *et al.*, 2001) and *Tricholoma matsutake* (Lian *et al.*, 2006) mycelia, whose extent belowground is not always centred around sporocarps. However, our finding is in contrast to the distribution of *H. cylindrosporum* mycelium that was not found >50 cm away from a fruiting body (Guidot *et al.*, 2002). Our survey in the truffle-ground shows that the number of *T. magnatum*-positive soils and that of fruiting bodies harvested (Mello *et al.*, 2005) are much greater than that of mycorrhizas (Murat *et al.*, 2005; Bertini *et al.*, 2006) and that there is no direct linkage among belowground mycelium, mycorrhizas and fruiting bodies. On the basis of the current literature, the analyses that consider all the steps of ectomycorrhizal fungi life cycle as the parameter for their distribution are still limited. Therefore, our study is a step further not only in the context of truffle ecology, but more generally in that of ectomycorrhizal fungi.

The availability of the new tool based on the β-tubulin gene is key in current projects aimed at establishing *T. magnatum* plantations to increase large-scale truffle production. Such a production, under controlled conditions, has rarely been reported, and it is not certain whether failure has resulted from unsuccessful inoculation in the nursery or establishment after planting (Mello *et al.*, 2006). Checking for the presence and persistence of the introduced *Tuber* species will now be possible during common plantation surveys, and for the first time we can obtain an overview of the *T. magnatum* network in the soil.

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