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Expanding the understanding of a forest ectomycorrhizal community by combining root tips and fruiting bodies: a case study of *Tuber magnatum* stands

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Abstract: A survey of both ectomycorrhizal (ECM) root tips and fruiting bodies of ECM fungi was carried out during a four-year period (2009–2012) in two similar forest stands in central Italy, previously investigated in detail being habitats of the Italian white truffle *Tuber magnatum* Pico. This research is one of the few conducted in mixed forest stands and, for the first time, it took into account the cryptic fungi (corticiaceous, hypogeous, and sclerotia). This survey led to an exhaustive description of the ECM community by integrating all the 197 taxa recorded (147 species as fruiting bodies, 65 as ectomycorrhizas, and 15 overlapping taxa recorded by both systems), and it also revealed a discrepancy between the results obtained using these two different approaches. In fact, a prevalence of Russulaceae, Inocybaceae, and Cortinariaceae resulted from fruiting body observations, whereas Thelephoraceae and Sebacinaceae dominated the ECM root tips. This result suggests a probable flaw in the sampling methodology. There may be several causes of this phenomenon, including the different nutritional strategies of ectomycorrhizal fungi and their seasonal turnover. Some species that could be candidates as bioindicators of *T. magnatum* habitat in mixed forest were identified.

Key words: Biodiversity, forest ecology, fungal community, mycorrhizal symbiosis, white truffle

1. Introduction

Plant communities guarantee the functionality of terrestrial ecosystems, but they, in turn, depend on the microorganisms with which they are associated, especially those living in soil and roots. They provide most of the nitrogen and phosphorus taken up by plants, in temperate and boreal forests (Van der Heijden et al., 2008). Conservative estimates indicate that about 20,000 species of plants are completely dependent on soil symbiotic microorganisms for growth and survival, indicating the importance of soil microbes as regulators of plant richness on Earth (Van der Heijden et al., 2008).

Among the various types of symbioses, the ectomycorrhizas (ECMs), which affect mainly the forest formations of temperate areas, are usually produced by fungal species capable of forming fruiting bodies (FBs) (Vogt et al., 1992). In the past, the structure of ectomycorrhizal (ECM) fungal communities was studied by FB surveys; this was then replaced by or coupled with root tip morphological analyses (Pacioni et al., 2001; Ashkannejhad and Horton, 2006). Molecular tools have been introduced and perfected over the past decades (Gardes et al., 1991; Gardes and Bruns, 1993), allowing for the identification, at least as a DNA sequence, of cryptic or nonfruiting ECM taxa. Molecular identification of ECMs is becoming the most common approach to study ECM communities in simplex and complex ecological systems (Mühlmann et al., 2008, Urban et al., 2008). Molecular characterization of ECMs coupled with FB surveying was also applied in several cases to study ECM communities. This double approach, in primary successional settings, makes ECM assemblages simple and gives a good correspondence between above and below ground ECM fungal communities (Nara et al., 2003); the ECM community is, in fact, dominated by a limited set of species that, after initial colonization, readily form sporocarps to disperse their spores to surrounding areas to widen their distribution (Nara, 2008). In contrast, in mature natural stands, there is a pervasive disconnection between above- and below-ground ECM fungal communities (Gardes and Bruns, 1996). Smith et al. (2007) suggest that, in order to reach a complete overlap between FB and ECM fungal

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communities, it is necessary to increase the number of samplings, focused mainly on the so-called "cryptic" FBs, which are corticiaceous, hypogeous, and sclerotia. Indeed, the definition of ECM communities present in complex stands with different species of host plants by means of the double approach has so far been poorly applied.

Tuber magnatum Pico is the most precious truffle because of a limited growth area and because there is not yet any valid farming alternatives, as there are for the other species of edible truffles (Murat et al., 2005). For this reason, it is important to protect its natural habitat by obtaining information on its autecology and soil-associated microorganisms. Previous studies of this habitat have focused on the ECM fungal communities on roots (Murat et al., 2005; Bertini et al., 2006). Recently, the abundance and frequency of ECM species of four natural truffle grounds distributed along the Italian peninsula have been studied (Leonardi et al., 2013). In this study the authors examined more than 8000 root tips but *T. magnatum* ECM were never found, even in *T. magnatum* productive points. These results emphasize the importance of coupling ECM characterization with FB surveying. However, FB diversity has never been systematically surveyed and the only report about mushrooms in *T. magnatum* habitats in Monferrato (Piedmont region, Italy) dates back to 1983 and reports the presence of numerous species of the genera *Inocybe* and *Tuber* together with some other species of *Russula* (Giovannetti, 1983). The aim of this study is to supplement the information inferred from the survey of root tips with that gathered from both identifiable and cryptic FBs collected in two similar stands in central Italy, with a view to:

- increase the level of knowledge of the community of ECM fungi that structure the habitat of the white truffle (*T. magnatum*);

- verify the effectiveness of the double sampling (root-tips and fruiting bodies) for the ecological characterization of forest environments.

2. Materials and methods

2.1. Study areas

The research was carried out in two *T. magnatum* highly productive areas of central-southern Italy. The two stands are localized in the Abruzzo region (ASD Torre di Feudozzo, Castel di Sangro, AQ, lat 41°45′55″80 N, long 14°11′12″80 W, altitude 950 m ASL, c 5400 m^2) and Molise region (Riserva "Man & Biosphere" Collemeluccio, Pescolanciano, IS, lat 41°42′07″60 N, long 14°20′34″50 W, altitude 810 m ASL, c 4050 m^2). They are characterized by the same type of vegetation syntaxa: the association *Aremonio agrimonioidis-Quercetum cerridis* (order *Fagetalia sylvaticae*, alliance *Erythronio-Carpinion betuli)*, which is the typical mesophilic mixed *Quercus cerris* L. woods of the Apennines. Details of the soil and vegetation characteristics are published on the website http://www.agrsci.unibo.it/magnatum. The ECM host plants found in Abruzzo (Feudozzo) are *Quercus cerris* L., *Fagus sylvatica* L., *Corylus avellana* L., *Carpinus betulus* L., *Ostrya carpinifolia* Scop., *Populus tremula* L., *Salix caprea* L., and *Salix purpurea* L., while in Molise (Collemeluccio) they are *Q. cerris*, *Abies alba* Mill., *Populus canadensis* L., *C. betulus*, *C. avellana*, and *Alnus cordata* (Loisel.) Desf.

2.2. ECM sampling

Soil sampling was done by taking soil cores 30 cm in length and 6 cm in diameter, after removing litter and organic soil horizon in December 2009. In the Feudozzo and Collemeluccio stands, 12 and nine plots of 30 × 15 m were delimited, respectively. In each plot, two random samplings were carried out along diagonals at 1 and 2 m from their crossing point, oriented to the point of greatest presence of *Tuber magnatum* fruiting bodies. These samples were added to those previously collected in September–December 2008 (Leonardi et al., 2013). In total, 32 soil cores from Feudozzo and 20 from Collemeluccio were studied. Cores were disrupted in water and soaked in tap water for 1 h. Visible roots were collected and washed under a gentle stream of tap water. The soil suspension was sieved through a 2-mm sieve to recover the remaining root tips. Colonized root tips were morphologically characterized according to Agerer (1987–2008), and then divided into two lots: one was stored in FAA (formaldehyde, 70% ethanol, acetic acid, 5:90:5) at 5 °C as a reference for morphotyping; the other was deep-frozen at –80 °C for molecular analysis.

2.3. Mushroom fruiting bodies

For four consecutive years (2009–2012), systematic harvests of FBs and microsclerotia (every week in spring and autumn) were carried out. They were then identified based on morphology, considering as ECM the species belonging to the genera listed in the checklist of Rinaldi et al. (2008). Hypogeous fungi were collected with trained dogs, while the microsclerotia were recovered by means of the wet-sieving improved technique according to Pacioni (1991) with soil suspensions originating from root cleaning. At least one sample from each species, from which DNA was extracted, is preserved as a voucher in the University of L'Aquila herbarium (AQUI), with specimen numbers reported in Table S1, published on the website http://dipsa.unibo.it/umiweb/magnatum/Table%20S1.pdf.

The nomenclature follows http://www.indexfungorum.org/names/names.asp.

2.4. Molecular analysis

Molecular identification of ECMs was performed as described by Iotti and Zambonelli (2006). A very small fragment of mantle from three root tips for each

morphotype was used as a DNA amplification template. The nuclear ribosomal internal transcribed spacer (ITS) regions were directly amplified by polymerase chain reaction, using the primer pair ITS1F–ITS4 (White et al., 1990; Gardes and Bruns, 1993) in 50 µL of final volume. Next 2 µL of 20 mg/mL BSA solution (Thermo Scientific) was added to each reaction tube to prevent PCR inhibition. DNA from fresh and dried FBs (25–100 mg) was isolated as described by Paolocci et al. (1999). The amplifications were carried out using the same primer pairs and the following cycling parameters: an initial denaturation at 94 °C for 2 min and 30 s; 25 cycles consisting of 30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C; a final extension at 72 °C for 7 min. The product of each PCR reaction was checked on a 1% agarose gel, purified, and then sequenced by Eurofins MWG Operon (Ebersberg, Germany). The sequences of the ITS1, 5.8S, and ITS2 regions of the nuclear rDNA obtained were compared with those present in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) and with those obtained from the FBs using the BLASTN search tool (Altschul et al., 1997). Sequences of ECM fungi were regarded as belonging to operational taxonomic units (OTUs) following the criteria cited in Landeweert et al. (2003).

Fungal species were identified if their ITS sequences matched a named sporocarp or voucher specimen with at least 97% sequence similarity over at least 500 base pairs with an 80% query coverage according to Smith et al. (2007). ITS sequences obtained in this study have been deposited in the GenBank database; the accession numbers are given in Table S1 (http://dipsa.unibo.it/umiweb/magnatum/Table%20S1.pdf).

2.5. Data analyses

Bray–Curtis (polar) ordination (Bray and Curtis, 1957) was used to investigate the similarity of the FB and ECM communities in the two areas of study (Collemeluccio and Feudozzo). Analyses were performed using PC-ORD (McCune and Grace, 2002).

3. Results

As expected, summing the results from the double sampling approach, a record of ECM taxa was obtained containing more taxa than that obtained from the single sampling of fruiting bodies or ectomycorrhizas. The checklist obtained by the double sampling included 197 taxa, which represents an increase of 34% compared to the result that would be obtained with the sampling of only ECM FBs and a 3-fold improvement compared to the sampling of the ECM root tips only. The results with all details concerning taxa recorded as FBs and/or ECMs in both forestry stands, their herbarium voucher and GenBank accession numbers, are shown in Table S1 available at the link (http://dipsa.unibo.it/umiweb/magnatum/Table%20S1.pdf).

These taxa belong to 33 genera (21 at Collemeluccio and 27 at Feudozzo), 175 of the Basidiomycota phylum, and 22 of the *Ascomycota* phylum, including microsclerotia of *Cenococcum geophilum* Fr. (Tables 1, S1). In the two forest stands studied, 147 species of ECM mushrooms were identified by means of FBs; of these species 75 and 102 were recorded at Collemeluccio and Feudozzo, respectively, and 30 species of mushrooms were harvested in both stands (Tables 1, S1). The most common mushroom species in these areas belong to Russulaceae: the species of the genera *Russula* and *Lactarius* represent a fifth (20.8%) of the taxa present in both areas. The genera *Cortinarius* (13.2%), *Inocybe* (14.7%), and *Boletus* s.l. (*Aureoboletus*, *Boletus*, *Xerocomellus*, and *Xerocomus*) (5.1%) are well represented, whilst *Tuber* (5.1%) is the most representative among the hypogeous fruiting bodies. Regarding the ECMs, the morphotypes selected following Agerer (1987–2008) on 5252 root tips (3934 at Feudozzo and 1318 at Collemeluccio), totaled 189 at Feudozzo and 42 at Collemeluccio. Among these 231 morphotypes, molecular analysis allowed us to define 64 OTUs at Feudozzo, thereby reducing by almost two-thirds the structure of the hypothetical biodiversity of ECM proposed on the basis of morphology at Feudozzo, while the 42 ECMs of Collemeluccio generated 30 OTUs. The OTU BLASTn analysis within the group further reduced the number of OTUs attributable to different taxa to 65, considering conspecific OTUs with 97% similarity. Four ECM OTUs remained determined at the family or order level (Helotiales, Pyronemataceae, Pezizales, Thelephoraceae). Based on the results of the ECM survey, the communities of the two stands consist of 47 taxa at Feudozzo and 30 at Collemeluccio. Only 12 ECM fungal taxa are in common between the two stands (i.e. *Cenococcum geophilum*, *Inocybe fuscidula* Velen., *Lactarius acerrimus* (Britzelm.) Kuntze, *Sebacina incrustans* (Pers.) Tul. & C. Tul., *Sebacina* sp. 3, *Sebacina* sp. 13, *Tomentella coerulea* (Bres.) Höhn. & Litsch., *T. ferruginea* (Pers.) Pat., *T. viridula* (Bourdot & Galzin) Svrček, *Tomentella* sp. 3, *Tricholoma scalpturatum* (Fr.) Quél., *Tuber rufum* Pico). Only *Cenococcum geophilum* was found both as microsclerotia and ECM in the two stands. In 11 cases, the presence of mycorrhizal species was confirmed by the two sampling systems. In fact, there are seven species (*Boletus subtomentosus* L., *Inocybe quietiodor* Bon, *Lactarius acerrimus*, *Russula foetens* Pers., *Tricholoma scalpturatum*, *Tuber brumale* Vittad., and *Xerocomellus porosporus* (Imler ex Bon & G. Moreno) Šutara) found both as ECMs and as FB at Feudozzo and only four (*Inocybe hirtella* Bres., *I. rimosa* (Bull.) P. Kumm., *Lactarius intermedius* (Krombh.) Berk. and Broome, and *Russula insignis* Quél.) at Collemeluccio. At the Collemeluccio stand, three species, namely *Lactarius intermedius*, *L. salmonicolor* Heim and

Table 1. Data concerning the families and orders recorded using the double-survey. Abbreviations: FB or ECM number of taxa recorded as fruiting bodies or ectomycorrhizas or with both samplings (Double record); % percentage on total of taxa recorded using the double survey; FB % and ECM % percentage of each family or order on the total number of records for FB or ECM respectively; % Tot. percentage of taxa found as FB or ECM on the total number of recorded taxa.

Families/orders	Genus	Taxa	%	FB	% FB	% Tot.	ECM	% ECM	% Tot.	Double record
Amanitaceae	*Amanita*	7	3.6	7	4.8	3.6	0	0	0	0
Boletaceae	*Aureoboletus* *Boletus* *Xerocomellus* *Xerocomus*	10	5.1	10	6.8	5.1	2	3.1	1.0	2
Cantharellales	*Cantharellus* *Clavulina*	2	1.0	2	1.4	1.0	0	0	0	0
Hysteriales	*Cenococcum*	1	0.5	1	0.7	0.5	1	1.5	0.5	1
Cortinariaceae	*Cortinarius*	26	13.2	25	17.0	12.7	1	1.5	0.5	0
Gomphaceae	*Ramaria*	5	2.5	5	3.4	2.5	0	0	0	0
Helotiales		1	0.5	0	0	0	1	1.5	0.5	0
Hygrophoraceae	*Hygrophorus*	7	3.6	6	5.1	3.0	1	1.5	0.5	0
Inocybaceae	*Inocybe*	29	14.7	27	18.4	13.7	6	9.2	3.0	4
Paxillaceae	*Melanogaster* *Paxillus*	2	1.0	2	1.4	1.0	0	0	0	0
Russulaceae	*Gymnomyces* *Lactarius* *Russula*	41	20.8	38	25.9	19.3	8	12.3	4.1	5
Sebacinaceae	*Sebacina*	14	7.1	0	0	0	14	21.5	7.1	0
Strophariaceae	*Hebeloma* *Hymenogaster*	6	3.0	5	3.4	2.5	1	1.5	0.5	0
Telephoraceae	*Telephora* *Tomentella*	21	10.7	1	0.7	0.5	20	30.8	10.2	0
Tricholomataceae	*Tricholoma*	5	2.5	5	3.4	2.5	1	1.5	0.5	1
Tuberaceae	*Tuber*	10	5.1	8	5.4	4.1	4	6.2	2.0	2
Pyronemataceae	*Genea* *Geopora* *Humaria* *Tarzetta*	6	3.0	2	1.4	1.0	4	6.2	2.0	0
Other Pezizales	*Helvella* *Morchella* *Peziza*	4	2.0	3	2.0	1.5	1	1.5	0.5	0
		197		147			65			15

The taxonomy is in agreement with the *Index Fungorum* (http://www.indexfungorum.org/Names/Names.asp).

Leclair., and *Russula cavipes* Britzelm., typical of *Abies alba* were recorded. The last two species were found only as FBs. A summary of the different evaluations of the ECM community from the point of view of the FBs and of the ECMs is shown in Figure 1. A graphical representation of the data by means of Bray–Curtis polar ordination is shown in Figure 2. Axis 1 explains 39.9% of the variance and Axis 2 explains 56.7% of the variance. The two axes together explain 96.7% of the variance, showing a low similarity between the ECM and FB communities between the two stands and within the same stand.

Among the many cases that can be taken into account among the results, we think it is noteworthy that ECMs of *T. magnatum* were not found, which is the most frequent and important species in the two stands. In order to verify if the *T. magnatum* mycorrhizas are camouflaged by other ECM morphotypes as found by Murat et al. (2005), four ECMs molecularly identified as *Sebacina* sp. were amplified

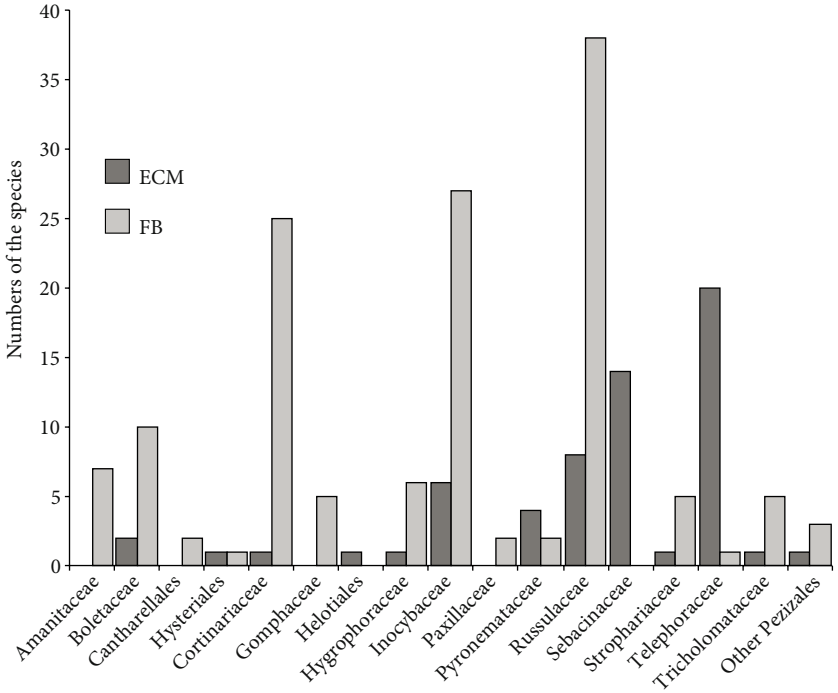

Figure 1. Main taxa recorded in the two stands by means of fruiting bodies (FB) and ectomycorrhizas (ECM) surveys.

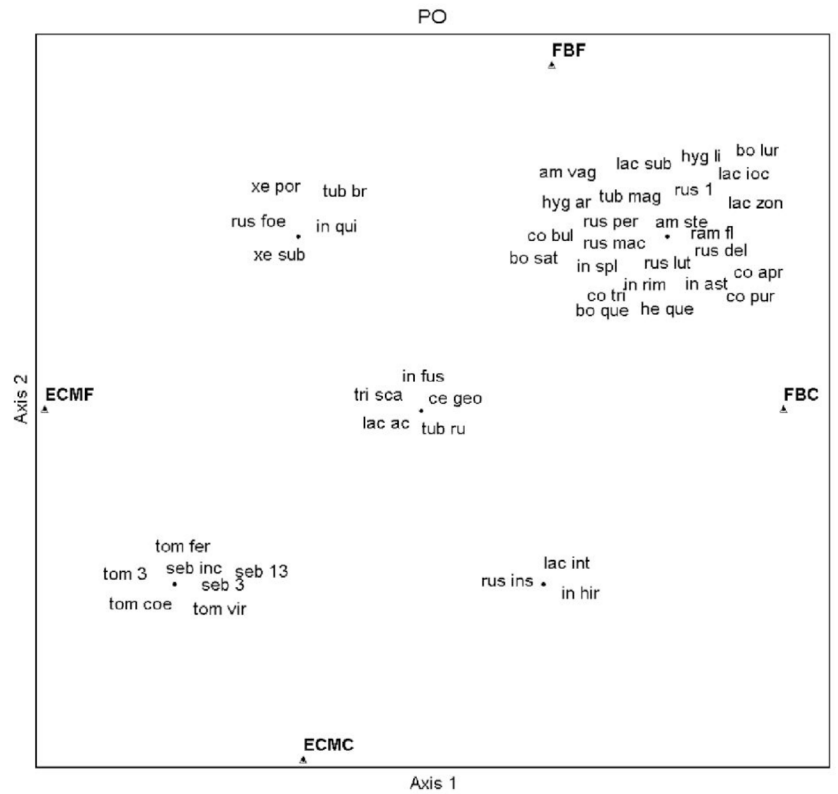

Figure 2. Bray–Curtis polar ordination showing a low similarity between the ECM and FB communities between the two stands and within the same stand. (FBF: Feudozzo fruit bodies species; FBC: Collemeluccio fruit bodies species; ECMF: Feudozzo ectomycorrhizal species; ECMC: Collemeluccio ectomycorrhizal species. Names of the species are reported "in extenso" in Table S1 published on the website http://dipsa.unibo.it/umiweb/magnatum/Table%20S1.pdf.

also with the *T. magnatum* ITS–specific primers (TmagI–TmagII according to Amicucci et al., 1998). *T. magnatum* specific amplicons were not produced (data not shown).

4. Discussion

In this work, two ECM fungal communities in natural *T. magnatum* habitats were studied and compared, analyzing both FBs and ECMs by morphological and molecular methods. The aim was to increase the information about the ECM community that characterizes the natural habitat of *T. magnatum*, verifying, at the same time, the effectiveness of the double sampling of fruiting bodies and root tips to characterize a forest environment.

4.1. *Tuber magnatum* ECM community structure

Even if the FB survey gave only a partial view of the ECM fungal communities, in that they were easily visible and determinable, they could simply be used as a bioindicator of habitats suitable for white truffle development. There are several species with a broad ecological range, such as *Amanita vaginata* (Bull.) Lam., *Cortinarius bulliardii* (Pers) Fr., *C. trivialis* J. E. Lange, *Inocybe rimosa*, *Lactarius volemus* (Fr.) Fr.; most of them (*Boletus luridus* Schaeff., *B. satanas* Lenz, *Hygrophorus lindtneri* M. M. Moser, *Inocybe flocculosa* Sacc., *I. rimosa*, *Lactarius zonarius* (Bull.) Fr., *Russula maculata* Quél., *R. persicina* Krombh., etc.), however, are characteristic of calcareous hardwood forests (Laganà et al., 1999). Some species could be considered as bioindicators of habitats typical of *Tuber magnatum*. They are uncommon species of fresh and deep calcareous soil symbionts of plants of these environments, frequently and consistently collected/recorded (data not shown), such as *Amanita stenospora* Contu, *Cortinarius aprinus* Melot, *Hebeloma quercetorum* Quadr., and *Hygrophorus arbustivus* Fr. var. *quercetorum* Bon & Chevassut.

4.2. The effectiveness of the double sampling

The ECM community characterized through FB sampling conflicts strongly with that emerging from the ECM survey. The most significant components that make up the population represented by the FBs did not find a significant match in the ECMs. Neither ECMs of *Amanita* nor of *Boletus* commonly found as FB were found, and against 25 specific taxa belonging to *Cortinarius*, found as FB, only one ECM was found (*Cortinarius magicus* Eichhorn). On the other hand, we found many ECMs attributable to genera of which we did not find FBs. Even in the case that is well known from numerous studies on the ECM communities defined by molecular tools of the extensive presence of ECMs belonging to *Sebacina* and *Tomentella*, the only species found as FB (*Tomentella lateritia* Pat.) were not found as ECMs. In this survey, an ECM of *Tuber scruposum* R. Hesse was found. This taxon was already found as an ECM in Italy (Baciarelli-Falini et al., 2012) and in the USA (Bonito et al., 2011), but its FBs have never been harvested. The only ITS sequence obtained from FBs of this species originated from Armenia (Badalyan et al., 2005). After a heavy sampling in a small area, Smith et al. (2007) supported the conclusion that with a very high number of soil samples and looking for "cryptic" FBs, mainly hypogeous ones, an almost complete agreement between FB and ECM could be obtained. In order to also detect hypogeous and cryptic species, we used trained dogs and the sieving technique of Pacioni (1991). However, we were able to find only *Cenococcum* microsclerotia and few hypogeous FBs, covering just a fraction of the diversity of this group of fungi found as ECMs. In a natural complex environment such as the forest stands studied, the soil is evolving and subject to water runoff and leaching, and becomes an extremely complex mosaic of microhabitats due to the different soil depth, structure, biological consortium, and even, in some cases, pH and content of solutes or organic matter. In these situations, one may find soil micro-niches suitable for the growth of just some species, but not all. On the other hand, the soil conditions may affect the fungal life cycle, favoring the growth of free-living mycelia and the formation of ECMs (Koide et al., 2005) or the development of fruiting bodies. In such a fragmented habitat, with a clear "patchy structure" of ground cover and plants, a clumped distribution of ECM fungi (Taylor, 2002) is a not completely unexpected consequence. Ideally, in order to find a correspondence between FBs and underground ECMs, soil cores should be taken at the points where the FBs were found. However, the procedure often gave unexpected results. Examples are the lack of ECMs of *Boletus edulis* Bull. s.l. detected by Peintner et al. (2007) and those of *T. magnatum* by Leonardi et al. (2013) detected with this sampling method. A vision closer to reality of the below-ground ECM fungal diversity may be achieved by extending the analyses to the types of root symbioses. In fact, it was shown that some ECM fungal species in nature may give rise to several types of symbiosis different from the canonical ECMs as reviewed by Brundett (2004) and Imhof (2009). On the other hand, Krpata et al. (2007) found many ECM fungi as arbutoid mycorrhizal symbionts of *Arctostaphylos uva-ursi* (L.) Spreng. Lancellotti et al. (2014) also found that a truffle (*Tuber borchii* Vittad.) that is a typical ECM fungus can form arbutoid mycorrhizas on *Arbutus unedo* L. Selosse et al. (2004) first identified as "orchid mycorrhizal fungi" different species of fungi hitherto regarded as "ECM fungi" including *Tuber*, and these results suggest looking for ECM fungi in the root apparatus of nonectomycorrhizal host plants. This suggestion is also supported by a recent study that measured the abundance. By using a quantitative PCR approach, Gryndler et al. (2014) measured the abundance of the *Tuber aestivum* Vittad. mycelium in the roots of host and nonhost (nonectomycorrhizal) plants in a natural

site, showing an important biotic interaction with the nonectomycorrhizal, mostly herbaceous, plants.

In the study of ECM communities research must take into account the fact that the mycelium of these fungi can be saprotroph decaying litter and wood debris (Tedersoo et al., 2003) and that the ECM communities may be subject to changes in their composition in relation to time and seasonal trends (Courty et al., 2008).

Metagenomic approaches may yield a more exhaustive view of the root symbiotic fungal diversity, which represents a very small part of the galaxy of the organisms living and interacting in the rhizosphere (Buée et al., 2009). With the simultaneous use of the two monitoring systems, this study provided for the first time a more comprehensive record of the ectomycorrhizal fungal species sharing the same environment as *Tuber magnatum* and constitutes a reference for further investigation. In particular, the presence of the FB of some fungal species, such as *Amanita stenospora*, *Cortinarius aprinus*, *Hebeloma quercetorum*, and *Hygrophorus arbustivus* var. *quercetorum*, could be a simple tool to reveal a suitable habitat for *T. magnatum* development.

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