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ORIGINAL PAPER

Assessment of ectomycorrhizal fungal communities in the natural habitats of *Tuber magnatum* (*Ascomycota, Pezizales*)

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Abstract The ectomycorrhizal (ECM) fungal communities of four natural *Tuber magnatum* truffle grounds, located in different Italian regions (Abruzzo, Emilia-Romagna, Molise, and Tuscany), were studied. The main objective of this study was to characterize and compare the ECM fungal communities in the different regions and in productive (where *T. magnatum* ascomata were found) and nonproductive points. More than 8,000 (8,100) colonized root tips were counted in 73 soil cores, and 129 operational taxonomic units were identified using morphological and molecular methods. Although the composition of the ECM fungal communities studied varied, we were able to highlight some common characteristics. The most plentiful ECM fungal taxa belong to the *Thelephoraceae* and *Sebacinaceae* families followed by *Inocybaceae* and *Russulaceae*. Although several ectomycorrhizas belonging to *Tuber* genus were identified, no *T. magnatum* ectomycorrhizas were found. The putative ecological significance of some species is discussed.

Keywords White truffle · Ecology · Biodiversity · Statistics

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Introduction

There are many species of fungi that produce edible fruiting bodies sought after as food delicacies. The most valuable are those produced by ectomycorrhizal (ECM) fungi because they cannot be directly cultivated on organic substrates under controlled conditions. Their production requires the establishment of a symbiotic relationship with specific plants and is also related to seasonal climatic trends and geopedology (Martínez de Aragón et al. 2007).

The finest representatives of edible ECM fungi are some species of the genus *Tuber* (Ascomycota, *Pezizales*), called truffles, which produce underground fruiting bodies. *Tuber magnatum Pico* is undoubtedly the most valuable truffle species. It grows within a very limited distribution area which includes Italy and some parts of the Balkans, reflecting the need for specific environmental conditions (Hall et al. 2007). To date, despite its economic value, scientific knowledge concerning this species has not been sufficient to identify reliable methods for its cultivation. This contrasts with another truffle species, the Périgord black truffle (*Tuber melanosporum* Vittad.), whose first cultivation can be traced back to about 200 years. Some other truffles (*Tuber aestivum* Vittad., *Tuber borchii* Vittad., etc.) have also been successfully cultivated more recently (Hall et al. 2007).

In the absence of guidelines for its cultivation, it is strategic to deepen our knowledge of its ecology and to find methods to preserve and increase the productivity of the

natural productive areas. In particular, the unknown relationship between *T. magnatum* and other ECM fungi in soil could play an important role in the life and fruiting of this precious truffle. Indeed, ECM fungi coexisting in the same soil niche can compete for the main nutritional resources from the roots of host plants or coexist in a dynamic equilibrium (Kennedy 2010).

The use of PCR-based molecular techniques for fungal identification (genotyping) has greatly advanced our understanding of fungal community diversity (Peay et al. 2008). It allows the taxonomic assignment of a greater number of species than the traditional methods based on the detection of fruiting bodies and subsequently integrated with characterization of ECM morphotypes (Dahlberg 2001). Moreover, molecular methods are more precise and reliable than morphotyping because ECM morphology is influenced by age, host plant, and soil conditions. In addition, ectomycorrhizas of different species of the same genus often have overlapping characters (Tedersoo et al. 2006). Thus, although morphotyping is still a useful tool when studying ECM fungal communities, it needs to be complemented by molecular studies to identify previously undetected or misidentified species (Dahlberg 2001).

Recently, this double approach (morphotyping and genotyping) was applied to the study of ECM fungal diversity in *T. melanosporum*, *T. aestivum*, and *T. borchii* truffle grounds (Belfiori et al. 2012; Benucci et al. 2011; Iotti et al. 2010), and in one area producing *T. magnatum* ascomata (Murat et al. 2005). These investigations show that ECM fungal communities in the points where *T. melanosporum*, *T. borchii*, and *T. aestivum* fruiting bodies were collected (productive points) are impoverished and dominated by ectomycorrhizas of these *Tuber* species. In contrast, *T. magnatum* ectomycorrhizas appear rare, even in productive points, with a different growth habit from that of other previously studied *Tuber* species. The objective of this study is to extend our knowledge of the ECM fungal community composition in natural *T. magnatum* grounds and to achieve a better understanding of the ecology of this truffle species. To do this, we studied the abundance and frequency of ECM fungal species in productive and nonproductive points of four natural *T. magnatum* truffle grounds distributed along the Italian peninsula.

Materials and methods

Study areas

The research was conducted in four different natural *T. magnatum* production areas, located from north to south in the following Italian regions: Emilia-Romagna [Parco del Museo della Bonifica, Argenta, FE, lat 44°37′10″ N, long 11°48′ 55″ E, altitude 5 m above sea level (ASL)]; Tuscany (Barbialla Nuova, Montaione, FI, lat 43°35′30″ N, long 10° 50′55″ E, altitude 135 m ASL); Abruzzo (FDR Torre di Feudozzo, Castel di Sangro, AQ, lat 41°45′55″ N, long 14°11′12″ E, altitude 950 m ASL); and Molise (Riserva M&B Collemeluccio, Pescolanciano, IS, latitude 41°42′ 07″ N, longitude 14°20′34″ E, altitude 810 m ASL) (Fig. S1). The natural truffle areas, covering between 2 and 5 ha, are characterized by different habitats: in Abruzzo and Molise, the truffle grounds studied are in mixed *Quercus cerris* L. woods, as in Tuscany, while in Emilia-Romagna, the area is a man-made park surrounding some buildings. In Table 1, the soil types and ECM plants of the four experimental areas are reported. More detailed information can be found on the web site http://dipsa.unibo.it/umiweb/magnatum/home.htm. The whole *T. magnatum* ascoma production of the Abruzzo, Molise, Emilia-Romagna, and Tuscany truffle grounds was 327.6, 272.7,

Table 1 Soil and vegetation characteristics of the four truffle grounds

Truffle ground	Soil type[a]	pH	ECM plants
Abruzzo (Feudozzo)	Typic Eutrudepts, fine-loamy, mixed, mesic	6.8–7.8	*Quercus cerris* L., *Fagus sylvatica* L. *Corylus avellana* L., *Carpinus betulus* L. *Ostrya carpinifolia* Scop., *Salix caprea* L. *Populus tremula* L., *Salix purpurea* L.
Molise (Collemeluccio)	Typic Eutrudepts, mixed, mesic	6.8–7.4	*Q. cerris*, *C. betulus*, *C. avellana* *Abies alba* Mill., *Populus canadensis* L. *Alnus cordata* (Loisel.) Desf.
Tuscany (Barbialla)	Typic Ustorthents, coarse loamy, mixed, thermic	7.8–8.5	*O. carpinifolia*, *Q. cerris* *Populus alba* L.
Emilia-Romagna (Argenta)	Aquic Ustochrepts, coarse loamy, mixed, thermic	8.0–8.4	*Populus nigra* L., *Tilia x vulgaris* Hayne *Pinus nigra* J.F. Arnold

[a] USDA (2003) classification

262, and 322 g, respectively, in the years 2008–2010 (Iotti et al. 2012).

Sampling

Soil cores of 30 cm in length and 6 cm in diameter were collected between September and December 2008 in all experimental sites. Sampling was carried out exactly in the points where the trained dog found *T. magnatum* ascomata [productive (P) points] and in nonproductive surroundings areas [nonproductive (NP) points], at distances of at least 20 m to prevent autocorrelation between samples due to ECM patchiness (Lilleskov et al. 2004). After removing litter and organic soil horizon, the soil cores were placed in polypropylene bags, transported in a refrigerator, stored at 4 °C, and then processed within the following 10 days. The numbers of soil samples collected in P points reflected the different numbers of *T. magnatum* ascomata found in the sampling sites during autumn 2008 (Iotti et al. 2012). Indeed, the number of soil cores collected in NP areas was 13 for Emilia-Romagna and 10 for each of the other regions.

Soil cores were disrupted in water. Visible rootlets were collected and the remainder of the sample was soaked in tap water for 1 h before washing under a gentle stream of tap water over a 2-mm sieve. Root samples were then placed in a Petri dish with tap water and observed under a dissecting microscope. ECM tips from each soil sample were sorted in morphotypes, counted, and placed in 1.5-ml tubes containing distilled water. Then, after a further morphological screening, each morphotype was described, photographed, and divided into two lots: the first one was stored in formaldehyde, 70 % ethanol, and acetic acid (5:90:5) at 5 °C as a reference for morphotyping, and the second, deep frozen at −80 °C for molecular analysis.

Morphotyping

Anatomical structures of the mantles, external elements (hyphae, rhizomorphs, and cystidia), and longitudinal and cross-sections of each morphotype were examined under a dissecting microscope Zeiss Imager Z1 Apotome (×630) with differential interference contrast and described after Agerer (1987–2008). Digital photos of ectomycorrhizas were taken using an AxioCam MRm digital camera (Zeiss) and processed with the software Axio Vision (Zeiss). Images and morphological characteristics of ectomycorrhizas are available at eMyCo database (http://emyco.uniss.it; Lancellotti et al. 2012).

Molecular analysis

A direct PCR approach was applied to identify all ECM morphotypes isolated from soil samples. One to three representative ECM tips per morphotype were selected as PCR target. A little fragment of ECM mantle was excised from each selected tip as described by Iotti and Zambonelli (2006) and directly amplified in 50 μl PCR reaction using the primer pair ITS1F/ITS4 (White et al. 1990; Gardes and Bruns 1996). Two microliters of 20 mg/ml BSA solution (Fermentas) were added to each reaction tube to prevent PCR inhibition. The amplification conditions were 6 min of the initial denaturation at 95 °C, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension step of 72 °C for 10 min. PCR products were visualized through 1 % agarose gel electrophoresis, stained with ethidium bromide. The amplified products were purified using the NucleoSpin® Extract II (Macherey-Nagel) and then sequenced using both the primers ITS1F and ITS4. The sequences of the ITS1, 5.8S, and ITS2 regions of the nuclear rDNA obtained were compared with those present in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and UNITE (http://unite.ut.ee/analysis.php) databases using the BLASTN search. Sequences were regarded as belonging to operational taxonomic units (OTUs) on the basis of criteria after Landeweert et al. (2003) except the species with a demonstrated high ITS sequence variability. Sequences were deposited in GenBank database with the accession numbers JX625255–JX625383 (Table S1).

Statistical analysis

Three diversity measures were used to describe ECM fungal communities (general, regional, sampling point): richness (total number of distinct OTUs detected), Pielou's index (describe how evenly the individuals are distributed among the OTUs) ($E=0 \rightarrow 1$, where $E=1$ if all species occur at the same proportion), and Shannon–Wiener index (take into account the taxa richness and their relative abundance) ($H'=0 \rightarrow \infty$, increase of such value is due to additional unique species and/or a greater species evenness) (Magurran 2004). These indices were calculated using the software package Vegan version 1.17-9 (Oksanen et al. 2011), within the R system for statistical computing (version 2.12.2) (R Development Core Team 2011).

Dominance–diversity curves for the four areas were constructed by ranking the abundance values of the OTUs from the highest to the lowest (Magurran 2004). To investigate the effects of the region (fixed at four levels: Abruzzo, Emilia-Romagna, Molise, and Tuscany) and sampling point (fixed at two levels: P and NP) on ECM fungal community structure, we used permutational analysis of variance (PERMANOVA). The advantage of the permutation approach is that the resulting test is "distribution free" and not constrained by many of the typical assumptions of parametric statistics. Type III SS was used as this was appropriate for an unbalanced design.

All tests were performed with 9,999 permutations of residuals under a reduced model. Factors or interactions were considered statistically significant if $P<0.05$. Significant terms were then investigated using a posteriori pair-wise comparisons with the PERMANOVA t statistic and 9,999 permutations.

Similarity percentage analysis (SIMPER) was used to determine the percentage contribution of each OTU to the observed dissimilarity between P and NP soil samplings. All the multivariate analyses were performed on the basis of the Bray–Curtis dissimilarities matrix. Data (number of colonized root tips) were log (x+1) transformed to reduce the weight of dominant and rare species. Analyses were performed using the PERMANOVA routine in the PRIMER v6 computer program, including the add-on package PERMANOVA+ (Anderson et al. 2008).

Results

General characteristics of the ECM fungal communities

Soil samples collected in P points were 11, 7, 10, and 2 in Abruzzo, Emilia-Romagna, Tuscany, and Molise, respectively. More than 8,000 (8,100) colonized root tips were counted in 30 soil cores taken from P points and 43 from NP points and assigned to about 200 morphotypes on the basis of morphological and anatomical features. The diversity found within each sample was very low; nearly half (33 out of 73) of the soil samples showed root tips belonging to one to three morphotypes, and only two soil samples had a higher diversity with nine morphotypes. Moreover, eight soil samples collected from NP points of Argenta truffle ground had no ectomycorrhizas, but only uninfected roots from the numerous non-ECM plant species growing in this park.

Molecular analyses made it possible to identify 129 OTUs (Tables 2 and S1), 37 of which were identified at a species level (six belonging to the *Tuber* genus), 77 at a genus level, and 10 as a family, and 5 could be assigned at an order (Table S1). Intraspecific variability of ITS sequences grouped into each OTU was lower than 1 % except for those identified as *Cenococcum geophilum* and *Tuber rufum*. In particular, the mean genetic variability was 1.7 % for *C. geophilum* and 15.2 % for *T. rufum*. These data are consistent with the hypothesis that these two ECM fungi form species complexes (Douhan et al. 2007; Iotti et al. 2007).

Most of the OTUs (83) were sporadic taxa which are only found in a single sample, highlighting a great heterogeneity in natural truffle areas. Among these, *Inocybe umbrinella*, *Hymenogaster olivaceus*, *Sebacina* sp. 18, *Tomentella galzinii*, and *Tomentella* sp. 9, sp. 11, sp. 12, and sp. 14 showed a very low average abundance (less than 0.10) (Table S1). On the other hand, *C. geophilum*, present in 16 samples, was the most abundant and frequently followed by *T. rufum* (Table S1).

In all the truffle areas, the most common ECM fungal species belonged to the *Thelephoraceae* and *Sebacinaceae* families. Although several ectomycorrhizas belonging to *Tuberaceae* species were observed, no *T. magnatum* ectomycorrhizas were found (Tables 3 and S1).

The values of the Shannon and Pielou indices (4.348 and 0.894, respectively) calculated for all the points in the study confirm that the community is characterized by a high diversity and an even OTU spread (Table 2).

ECM fungal communities in the different regions

The number of OTUs classified in the different regions was as follows: 60 in Abruzzo, 22 in Emilia-Romagna, 30 in Molise, and 45 in Tuscany (Table 2). The dominance–frequency curves (Fig. 1) show different OTU combinations in the four regions. In Abruzzo and in Molise, the most abundant and frequent is *C. geophilum*; then in Abruzzo, *Inocybe* sp. 11 shows a great abundance and frequency, while in Molise, *Tomentella* sp. 22 and sp. 32 were the only observed twice, and *Sebacina* sp. 2 is relatively abundant, but only found once. In Emilia-Romagna, *T. rufum* s.l. is the most abundant, closely followed by *Tomentella* sp. 8, both present in only one soil sample. As regards frequency, the most common OTU is another truffle species, *Tuber maculatum*. In Tuscany, it should be mentioned that three *Pezizales* species, *Tarzetta* sp., *Genea* sp. 1, and also *T. rufum* were

Table 2 ECM diversity in the *T. magnatum* truffle grounds, in the four areas (Abruzzo, Emilia-Romagna, Molise, and Tuscany), in productive and nonproductive points

	Total	A	E	M	T	P	NP
No. of root tips	8,100	3,634	420	1,208	2,838	3,735	4,365
Species richness	129	60	22	30	45	76	80
Shannon index	4.348	3.684	2.720	3.171	3.390	3.756	4.009
Pielou index	0.894	0.900	0.880	0.932	0.890	0.867	0.915

A Abruzzo, *E* Emilia-Romagna, *M* Molise, *T* Tuscany, *P* productive, *NP* nonproductive

Table 3 Percentage presence of ECM families and higher levels for *Pezizales* and *Helotiales* in the *T. magnatum* truffle grounds, in the four areas (Abruzzo, Emilia-Romagna, Molise, and Tuscany), in productive and nonproductive points

Family/order	Total	A	E	M	T	P	NP
Thelephoraceae	32	33	36	30	29	34	31
Sebacinaceae	18	20	9	30	18	17	16
Inocybaceae	13	7	9	10	20	17	10
Russulaceae	8	8		13	2	3	11
Pyronemataceae	7	7	9	7	4	5	9
Tuberaceae	5	7	23		7	5	8
Pezizales	3	5			2	3	4
Strophariaceae	2	2	9			1	3
Boletaceae	2	3				1	1
Clavulinaceae	2				4	3	1
Pezizaceae	2			3	4	3	1
Tricholomataceae	2	2			2	1	1
Cortinariaceae	1	2		3			1
Helotiales	1	2				1	
Herpotrichiellaceae	1				2	1	
Hydnangiaceae	1		5				1
Hygrophoraceae	1	1				1	
Incertae sedis (*Cenococcum* sp.)	1	1		3	2	1	1
Trichocomaceae	1				2	1	

Orders contain only the OTUs not identified as family

A Abruzzo, *E* Emilia-Romagna, *M* Molise, *T* Tuscany, *P* productive, *NP* nonproductive

observed on many root tips and in different soil samples. The species that most contributed to the exclusive regional compositions are *Inocybe* sp. 11, *Russula vesca* and *Tomentella* sp. 30 for Abruzzo; *Tomentella* sp. 22 for Molise; *Tomentella* sp. 3 and sp. 4 and *Geopora cervina* for Emilia-Romagna; *Genea* sp. 1, *Clavulina* sp. and *Tomentella* sp. 6 for Tuscany (Fig. 1). On the other hand, *Tomentella* sp. 2 is present in all areas even if only in one or two samples, whereas among the most frequent OTUs, *C. geophilum* is absent in Emilia-Romagna, *T. rufum* s.l. in Molise and *Sebacina* sp. 3, in Tuscany.

The family composition of the ECM fungal communities in the four regions varies slightly, even if, in general, the most representative are the *Thelephoraceae* and *Sebacinaceae* families in all the natural truffle areas studied here. In Molise, these two families are equally well represented, while in the northern region of Emilia-Romagna, *Tuberaceae* exceed *Sebacinaceae*. Indeed, *Tuberaceae* are found in three out of four regions, absent only in Molise. It can also be noted that few families were observed in Emilia-Romagna and in Molise. Among the exclusive regional families worth noting there are *Boletaceae* and *Hygrophoraceae* in Abruzzo, *Hydnangiaceae* in Emilia-Romagna, and *Clavulinaceae* in Tuscany (Table 3).

As compared to the other regions, Abruzzo shows a high species diversity with Shannon values of 3.684, while Emilia-Romagna has lower evenness values according to Pielou index (0.880), indicating that species are less equally distributed than in the other regions (Table 2).

Multivariate permutational analysis of variance revealed that region and productivity factors significantly affected community composition as well as their interaction (Table 4). The pair-wise tests showed significant differences between all pairs of regions with the exception of Molise vs Abruzzo and Tuscany (Table 5).

ECM fungal communities in productive and nonproductive sampling points

A similar number of OTUs were found in P and NP points (76 and 80, respectively) (Table 2). However, there is also a high heterogeneity with only one fifth of the OTUs (27) in common; 49 are exclusively found in P points, with *Sebacina* sp. 2 and sp. 11 as the most frequent (10 %), and 53 OTUs are found in NP points, with *Russula vesca* and *Tomentella* sp. 18 and sp. 30 as the most frequent (7–9 %) (Table S1).

Among the ECM fungal community family compositions, *Boletaceae*, *Tricholomataceae*, and *Incertae sedis* (*C. geophilum*) are equally present in both, P and NP points, and *Thelephoraceae* and *Sebacinaceae* are also well represented in both. On the other hand, there are families exclusively present in P or NP points (*Herpotrichiellaceae*, *Hygrophoraceae*, *Trichocomaceae*, and *Cortinariaceae*, and *Hydnangiaceae*, respectively). *Inocybaceae* seem to prefer P points, while *Russulaceae* prefer NP points with the exception of *Lactarius* sp. 4 found only in P points and *Russula* sp. 2 present in both P and NP points. As regards *Tuberaceae*, *T. brumale*, *Tuber dryophilum*, and *T.*

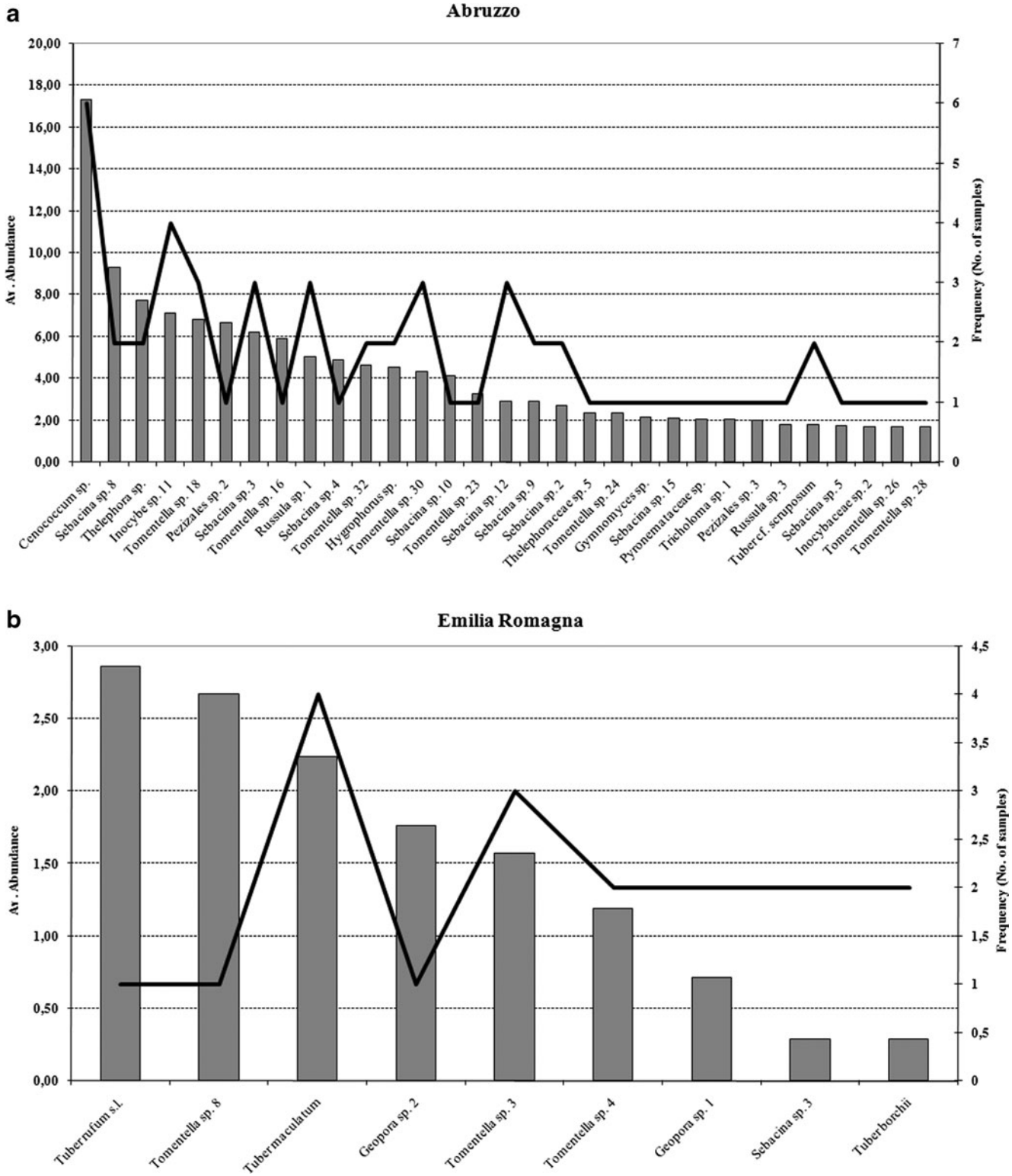

Fig. 1 Average abundance (*column*) and frequency (*line*) for each OTU found in natural truffle grounds in the four areas (Abruzzo, Emilia-Romagna, Molise, and Tuscany). Sporadic (frequency=1) and rare (abundance<1.50) species are not shown

melanosporum, ectomycorrhizas are exclusively linked to points where *T. magnatum* ascomata are absent (NP) (Table 3). Among these latter truffle species, only *T. brumale* was found fruiting in Emilia-Romagna and Abruzzo close to the area where its ectomycorrhizas were found but far from *T. magnatum* P points.

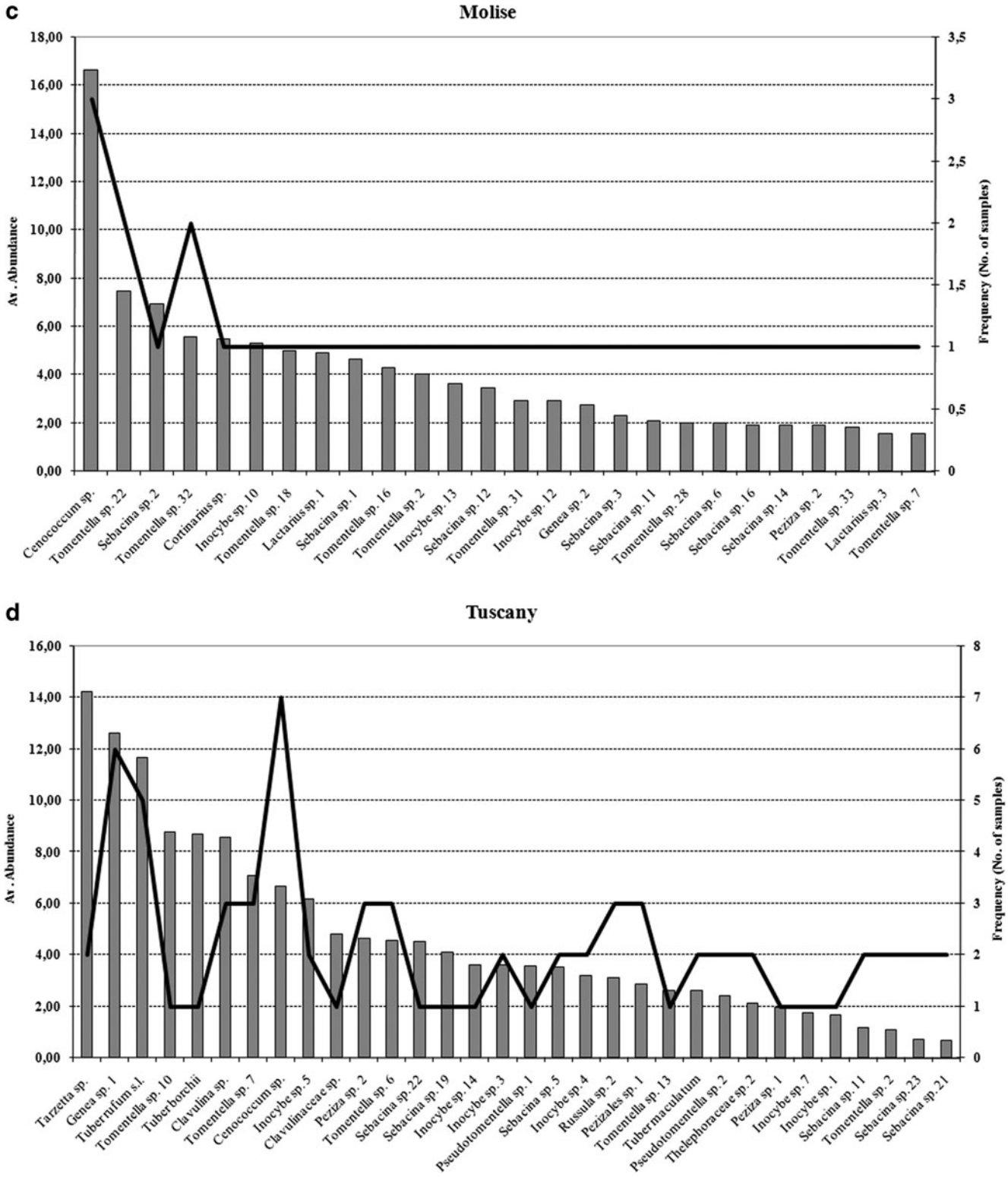

Fig. 1 (continued)

Both the Shannon and Pielou indices are higher for the NP points than the P points, indicating a higher diversity and evenness where the fruiting body of *T. magnatum* was not found. The SIMPER indicated that *C. geophilum*, *T. rufum* s.l., *T. maculatum*, *Sebacina* sp. 3, and *Tomentella* sp. 32 made a higher contribution to average dissimilarity between P and NP assemblages, even if the high value of SD and thus a low ratio average dissimilarity/SD revealed

Table 4 PERMANOVA results on OTU abundances in 73 soil samples

Source of variation	*df*	MS	*F*
Region	3	9,613.8	2.92*
Sampling point	1	5,258.9	1.6**
Region × sampling point	3	5,771.8	1.75*
Residual	65	3,290.5	
Total	72		

*$P<0.001$; **$P<0.01$

the high variability of abundance of these species within the two community types (Table 6).

Discussion

This study compares *T. magnatum* ECM fungal communities in different environmental conditions for the first time. The data obtained in the four investigated areas show a high heterogeneity at the ECM fungal species level. This is most likely because these areas—although suitable for *T. magnatum* development—differ as regards soil and vegetation composition. The greatest dissimilarity is between Tuscany and Abruzzo, even if these two areas are localized both in the Apennines of central Italy. The greatest similarity was found between the areas of Feudozzo (Abruzzo) and Collemeluccio (Molise) which are the most similar growth habits with the greatest number of ECM plant species. The area of Argenta (Emilia-Romagna) showed the lowest biodiversity in ECM fungal composition because it is a man-made park where different species of non-ECM plants are also present.

Although this extended monitoring did not provide conclusive information, it broadened our knowledge about the characteristics of ECM ecosystems of white truffle production areas. In this context, the presence of some companion taxa, in particular *Sebacinaceae*, has to be pointed out. Some of these, such as *Sebacina* sp. 2 and sp. 11, were frequently and exclusively recorded within the *T. magnatum* ascomata production points. The behavior and the role of mycorrhizal *Sebacinaceae* within such a microhabitat deserves attention because they have been found associated with ECM ascomycetes in hypothetic tripartite symbiotic situations, such as the already known associations between the Basidiomycetes *Suillus bovinus–Gomphidius roseus* and *Boletus edulis–Amanita excelsa* (Hall et al. 2003).

Coexistence between *Sebacinaceae* and *T. magnatum* was reported in a previous study (Murat et al. 2005) where only one of the two *T. magnatum* ectomycorrhizas, found in the soil sample analyzed, was identified by molecular cloning of a root tip colonized also by *Sebacina* sp.

Whereas *T. magnatum* ectomycorrhizas seem to be absent or very rare adjacent to where its ascomata were found, the ectomycorrhizas of other truffle species such as *T. rufum* and *T. maculatum* were frequent, which are consistent with observation of previous studies (Murat et al. 2005; Bertini et

Table 5 Results of PERMANOVA pairwise test between regions

Region	*t*
A, E	2.15*
A, M	0.79
A, T	1.55*
E, M	1.76*
E, T	2.28*
M, T	1.17

A Abruzzo, *E* Emilia-Romagna, *M* Molise, *T* Tuscany

*$P<0.001$; **$P<0.05$

Table 6 Average density of several prominent taxa in nonproductive and productive sampling points including SIMPER results for contributions from the most important taxa

Taxon	Av density NP	Av density P	Av diss	Diss/SD	Contrib%
Cenococcum geophilum	3.65	18.13	9.12	0.51	9.25
Tuber rufum s.l.	3.42	4.7	3.73	0.36	3.78
Tuber maculatum	1.09	1.57	3.44	0.25	3.49
Sebacina sp. 3	0.14	5.6	3.11	0.27	3.16
Tomentella sp. 32	1.42	3.53	2.42	0.31	2.45
Genea sp. 1	2.4	4.13	2.28	0.4	2.31
Inocybe sp. 11	0.42	4.87	2.17	0.32	2.21
Clavulina sp.	0.21	4.83	2.17	0.25	2.2
Thelephora sp.	0.88	4.67	1.98	0.21	2.01
Sebacina sp. 8	4.98	0	1.89	0.2	1.91
Tomentella sp. 16	1.09	4.5	1.81	0.23	1.83
Tomentella sp. 18	4.91	0	1.74	0.21	1.76
Sebacina sp. 2	0	4.6	1.7	0.27	1.72
Tarzetta sp.	5.95	0	1.65	0.2	1.67
Tomentella sp. 2	1.47	0.93	1.45	0.25	1.47
Sebacina sp. 12	1.98	1.27	1.41	0.26	1.42
Clavulinaceae sp.	0	2.87	1.39	0.17	1.41
Tomentella sp. 8	0	1.87	1.35	0.17	1.36
Tomentella sp. 7	3.12	1	1.33	0.29	1.34
Sebacina sp. 5	1.35	1.5	1.29	0.25	1.31
Sebacina sp. 22	0	2.7	1.27	0.18	1.29
Peziza sp. 2	0.74	2.4	1.23	0.29	1.25

Cut-off for low contributions is 50 %

Av average, *NP* nonproductive, *P* productive, *Av diss* average dissimilarity contribution of the most important taxa, *Diss/SD* dissimilarity divided by the standard deviation of the contribution of each taxa across all pairs of samples, *Contrib%* percentage of contribution of each taxa to the total of 98.62

al. 2006; Iotti and Zambonelli 2006). The widespread presence of *T. magnatum* mycelium in the productive zones, following a patchy distribution model (Zampieri et al. 2010), would favor the hypothesis that the production of *T. magnatum* ascomata is supported by an abundant presence of its mycelium and the absence of its ectomycorrhizas. Thus, this anomaly is dramatically confirmed in this study, given the high number of root tips examined from the four experimental truffle orchards, where in a previous experience, a positive correlation between ascoma production and *T. magnatum* mycelium in soil using a quantitative "real-time PCR" assay was found (Iotti et al. 2012).

The ecological strategy of *T. magnatum* appears different from those of the other edible truffles, which are dominant in both natural or man-made truffle orchards. For example, *T. melanosporum* (Napoli et al. 2010; Belfiori et al. 2012) produces a spectacular conditioning of the vegetation and microbial communities around the host plant (within the area called "brûlé" or "pianello") where its ectomycorrhizas and mycelium are prevalent. A situation almost similar to that found with the ectomycorrhizas of *T. aestivum* in its production areas (Zambonelli et al. 2005; Benucci et al. 2011). Iotti et al. (2010) showed that *T. borchii* ectomycorrhizas accounted for 20 % of the ECM fungal community, and Bonito et al. (2011) found that natural colonization of *Tuber lyonii* F.K. Butters accounted for 17 % of ectomycorrhizas in pecan (*Carya illinoinensis*) orchards in the USA.

The phenomenon of non-correspondence between the production of fruiting bodies and presence of ectomycorrhizas in the soil is interesting due to the hitherto lack of understanding on the reasons which lead to these apparent discrepancies. On the one hand, the ectomycorrhizas of *B. edulis*, *Boletus pinophilus*, and *Boletus aereus* are scarcely present adjacent to fruiting bodies, while on the other, *Boletus aestivalis* and *Tricholoma matsutake* ectomycorrhizas are concentrated just below their fruiting bodies (Lian et al. 2006; Peintner et al. 2007).

Gardes and Bruns (1996) found that *Suillus pungens* ECM root tips were rare in a *Pinus muricata* forest, whereas *S. pungens* fruiting bodies were abundant, while *Russula amoenolens* ectomycorrhizas were abundantly represented, but its fruiting bodies were rare. Again in a *Pinus sylvestris* stand, the genus *Cortinarius* produced 42.3 % of fruiting bodies; however, below ground, only 1.6 % of the ectomycorrhizas could be attributed to this genus (Taylor 2002).

Saprobic ability of *T. magnatum* mycelium was hypothesized but not demonstrated until recently (Barbieri et al. 2010). However, the possibility that this truffle may live as a saprotroph in soil without establishing symbiotic relationships is in contrast to the difficulties of growing it in pure culture on synthetic or semisynthetic media (Iotti et al. 2013). Murat et al. (2005) suggested that the association between plant and *T. magnatum* may not require a well-differentiated mycorrhiza. In greenhouses, this truffle is able to establish well-formed ectomycorrhizas (Mello et al. 2001; Rubini et al. 2001), even if they rapidly disappear and are replaced by other ECM fungal species (Iotti, personal observation). Consequently, we suppose that under natural conditions, *T. magnatum* ectomycorrhizas are camouflaged by the co-presence of other ECM mycelia on the same root tips in a tripartite symbiosis. It cannot be excluded that in soil, *T. magnatum* may form other types of symbiosis, such as orchid-like mycorrhizas, revealed for other truffle species (Selosse et al. 2004).

To test, this hypothesis will still need to take a survey approach different from morphotyping and the direct molecular amplification of ECM mantles. Further studies also need to be carried out to study whether the ECM fungal species found to be strongly linked to productive points have a beneficial effect on *T. magnatum* ascomata, creating favorable truffle growth conditions or establishing a nutritional exchange with *T. magnatum* mycelium.

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