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Morphological and molecular analyses of ectomycorrhizal diversity in a man-made *T. melanosporum* plantation: description of novel truffle-like morphotypes

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Abstract Below-ground ectomycorrhizal communities are often species-rich, and monitoring their dynamics is important to understand the conditions that promote truffle fructification. Characterization of the different ectomycorrhizas (ECM) at the species level can now be achieved by combining detailed morphological and anatomical descriptions with molecular approaches. Following this strategy, we have characterized ectomycorrhizal biodiversity in an artificial *Tuber melanosporum* plantation. Although the plantation was unproductive, *T. melanosporum* mycorrhizas were the most present and two *Tuber*-like mycorrhizal morphotypes, named ECMm1 and ECMm3, showing distinctive features were found. Internal transcribed spacer (ITS) phylogenetic analysis demonstrated that ECMm3 is related to the *Tuber rufum*/*Tuber ferrugineum* species complex, whereas ECMm1 shows the highest ITS similarity with *Tuber scruposum* and fungi-colonizing *Epipactis* roots. The results presented here provide more insights into genetic variability, mycorrhizal morphology, and below-ground distribution of fungi associated with artificial truffle plantations.

Keywords *Tuber* spp. · Ectomycorrhiza · ITS · Morphological characterization

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Introduction

Tuber species form ectomycorrhizas (ECM) on a wide range of tree and shrub species (Harley and Smith 1983) and produce fruiting bodies known as truffles; some of these are edible. Successful nursery inoculation of host plants with some of the most important *Tuber* species has encouraged the cultivation of these fungi as a means of offsetting a rapidly falling spontaneous production and of meeting the increasing demand for truffles worldwide. Changes in land use are believed to be one of the reasons that have, in fact, led to a dramatic reduction in spontaneous truffle production over the last century, so that more than half of the production of the fine black truffle *Tuber melanosporum* Vittad. is now cultivated (Hall et al. 2003).

Thus, in addition to truffle production recording, it has become of crucial importance in both productive and unproductive truffle fields to monitor microflora dynamics next to and on apical root-tips (Donnini et al. 1999). A clear picture of how ectosymbiotic fungal dynamics vary with changing environmental situations and agronomic practices is a prerequisite to gaining insights into the conditions favoring the development and fruiting of a given truffle species. The typing of ectomycorrhizal root-tips generally relies on morphological analysis (Agerer 1986). However, although quite fast and inexpensive, the morphological identification of ECMs produced by some species may be difficult due to the scarcity of diagnostic traits, which depend to a great extent on the host and on environmental conditions (Egger 1995). Thus, diverse *Tuber* species that differ vastly in economic value, ecological requirements, and distribution range can show strikingly similar mycorrhizal structures. The recent devel-

opment of molecular markers provides new tools to type the most important truffle species throughout their life cycle (Paolocci et al. 1995, 1999; Amicucci et al. 1998; Rubini et al. 1998, 2001.

Combined morphological and molecular approaches also offer the opportunity to shed light on microflora interactions and dynamics in truffle grounds. The ongoing acquisition in public databases of sequence information relative to phylogenetically important genomic traits, such as the small subunit (SSU) and the internal transcribed spacers (ITS) of the rDNA region, for an increasing number of ECM fungi greatly improves the possibility of putting species names to mycorrhizal structures (Horton and Bruns 2001). The work present here illustrates the usefulness of combining morphological and molecular approaches to shed light on ECM biodiversity in an unproductive man-made *T. melanosporum* truffle ground. Two novel *Tuber*-like ECM morphotypes have been characterized, and genetic keys are provided in relation to nontruffle morphotypes that may be commonly associated with truffle grounds.

Materials and methods

Collection of samples

All the mycorrhizal samples examined here were collected from a truffle plantation located in Umbria (central Italy) and established in 1988 by planting 110 *Quercus pubescens* Willd., 107 *Quercus ilex* L., and 218 *Corylus avellana* L. plantlets originally inoculated with *T. melanosporum*. A physical, chemical, and pedological description of the site under investigation was reported previously (Baciarelli Falini and Bencivenga 2002). A set of 70 root samples were collected from 17 downy oak, 24 holm oak, and 29 hazel trees randomly distributed over the entire truffle-growing site (Fig. 1). Samples were collected in autumn (October 2000) at 50 cm from the trunk and at a depth of 25 cm. A second sampling was made in late spring in the following year (June 2001) and a third in late spring 2005. All root samples were stored at 4°C before processing. Each sample was individually soaked overnight in tap water and sieved to separate root fragments and ECM from the soil. Analysis of mycorrhizal morphotypes was performed using a stereomicroscope and a light microscope. Mycorrhizas belonging to different morphotypes were selected, collected, and stored in 95% ethanol. Single mycorrhizal root-tips and pools of ECM were also stored at −80°C for subsequent molecular analysis. Vouchers of ectomycorrhizal specimens were deposited in the Perugia University Herbarium (PERU).

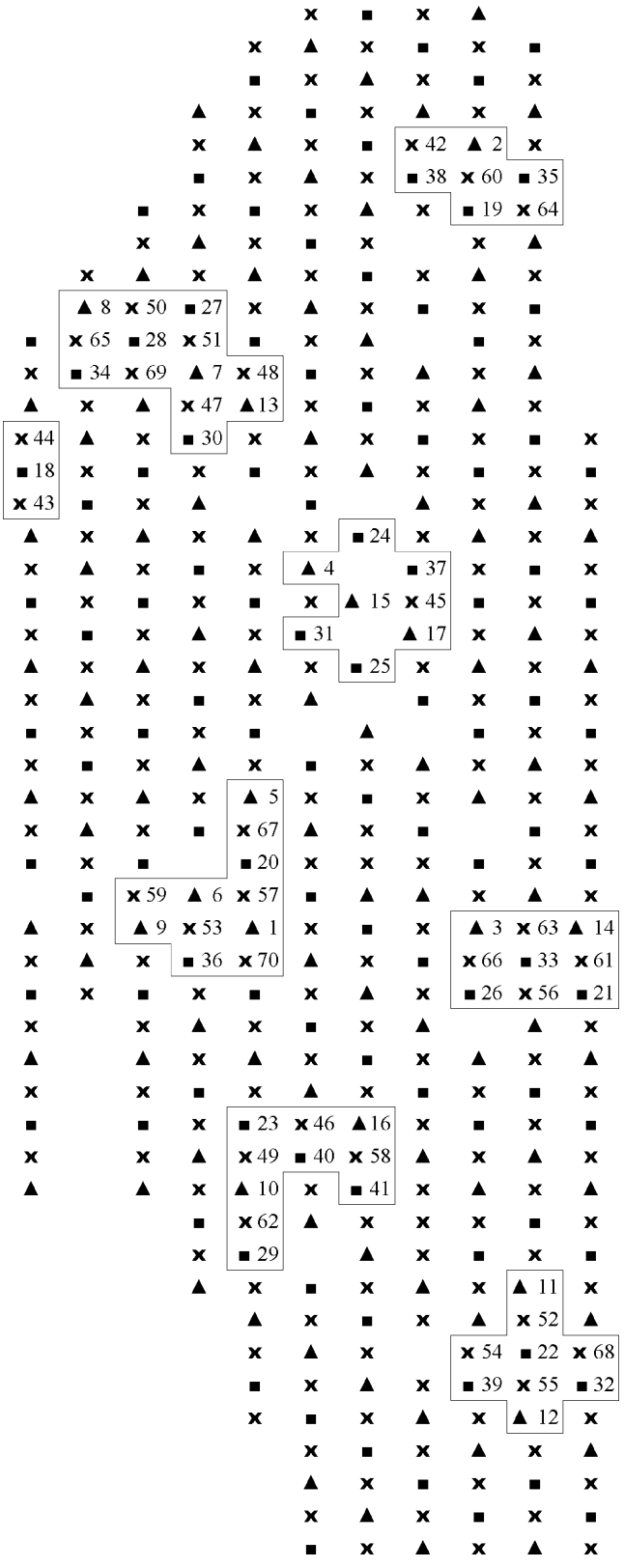

Fig. 1 Map of the artificial truffle plantation. *Triangles* indicate *Q. pubescens*, *x* indicates *C. avellana*, and *squares* indicate *Q. ilex*. *Boxes* indicate the sampled plants

Morphological analysis

Mycorrhizal morphotypes were identified by color and shape of the mycorrhiza, characteristics of the mantle surface, and presence and structure of emanating elements, as previously reported (Fontana et al. 1992; Agerer 1986; Zambonelli et al. 1993). Between 75 and 100 root tips were considered for the morphological description of each mycorrhizal morphotype of uncertain classification (ECMm1, ECMm3, ECMm7–ECMm8, ECMm9, and ECMm10; see "Results"). Photographs were taken using a dissecting microscope on freshly isolated ectomycorrhizal tips. Features of the mantle surface and emanating elements were examined on mycorrhiza mounted in 50% glycerol, using a Normanski interference contrast microscope, according to Agerer (1986).

DNA isolation and PCR amplification

Genomic DNA was isolated from single and pooled mycorrhizal root tips as described by Paolocci et al. (1999). For each mycorrhizal morphotype of uncertain classification (ECMm1, ECMm3, ECMm7–ECMm8, ECMm9, and ECMm10; see "Results"), DNA was isolated and PCR amplified both from two lots of mycorrhizal pools containing up to ten root-tips and from three samples consisting of a single root-tip each. For the morphotypes ECMm2, ECMm4, ECMm5, and ECMm6 (see "Results"), DNA was isolated and PCR amplified from two batches of (up to ten) root-tips.

The ITS1/ITS4 primer pair was used to amplify the ITS region, and the primers NS1/NS8 were used to amplify the SSU rDNA region (White et al. 1990). The ITS1/ITS4 PCR amplification was carried out in a Gene Amp 9700 Thermal Cycler (Applied Biosystems) with the following cycling parameters: an initial denaturation step at 95°C for 3 min, 25 cycles consisting of 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C, and a final extension for 7 min at 72°C. When the NS1/NS8 primer pair was used, the cycling conditions were as above except for the annealing step, which was incremented to 45 s. All PCR amplifications were performed in a 50-µl reaction mixture containing 200 mM of each deoxyribonucleotide triphosphate, 10 pmol of each primer, 4 mM $MgCl_2$, 10 mM Tris–HCL pH 9.0, 50 mM KCl, and 2.5 units of Taq polymerase (Amersham Pharmacia Biotech) supplemented with 7 mg/ml bovine serum albumin (Sigma) to overcome the effect of PCR inhibitors when processing the root samples (Paolocci et al. 1999). All PCR experiments included a negative control (no DNA template).

ITS species-specific primers for *Tuber borchii*, *Tuber maculatum*, *Tuber dryophylum*, and *Tuber puberulum* were derived from Amicucci et al. (1998) and used as suggested by the authors. ITS species-specific primers for *T. melanosporum*, *Tuber brumale*, and *Tuber aestivum* were derived from Rubini et al. (1998) and Mello et al. (2002).

Sequencing and phylogenetic analysis

The PCR products were purified using a Jet-Quick spin column (Genomed) and directly sequenced using a BigDye terminator sequencing kit (Applied Biosystems) according to the supplier's instructions. Sequencing reactions were run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The sequencing primers used were ITS1, ITS4, NS1, NS2, NS3, NS4, NS5, NS8 (White et al. 1990), 5.8sf, and 5.8sb (Rubini et al. 1998). The ITS and SSU sequences were checked for similarity using the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/). The ITS sequences showing the highest score and other ITS sequences representative of the *Tuberaceae* were retrieved from GenBank and aligned with the ECMm1 and ECMm3 ITS sequences using ClustalX software version 1.8 (Thompson et al. 1997). The neighbor-joining tree was obtained using the MEGA version 2.1 (Kumar et al. 2001) based on the two-parameter distance model of Kimura (1980). The ITS sequence of *Terfezia arenaria* (AF276674) was used as an outgroup in the phylogenetic tree.

Results

Morphological characterization of ECM

The surveyed ECM were sorted into nine morphotypes (Table 1), four of them, namely, ECMm2, ECMm4, ECMm5, and ECMm6, showed all the diagnostic anatomical and morphological traits specific to *T. borchii*, *T. brumale*, *T. melanosporum*, and *T. aestivum*, respectively. Among the other five morphotypes, ECMm1 and ECMm3 displayed typical *Tuber*-like structures: the mycorrhizas are brown-orange, monopodial-pinnate, simple or scarcely ramified, with straight, club-shaped, unramified ends (Fig. 2a,e,f). The outer surface of the mantle is pseudoparenchymatic with polygonal, ellipsoidal to epidermoid cells (Fig. 2m–o). In particular, the ECMm1 mycorrhizas are 0.4–3.8 mm (mean 2.9±0.5 mm) long and 0.2–0.32 mm (mean 0.24±0.05 mm) in diameter. They have a short-spiny surface because of the presence of abundant yellowish needle-like cystidia (spinulae) (Fig. 2e,n). The spinulae have thick walls and may present septa at their base (Fig. 2k). They have an average basal diameter of 3.5±0.04 µm and are 33.0–75.0 µm (mean 50.1±12.53 µm) long. The cells of the outer surface of the mantle are 12×7 µm and arranged in a complex and irregular pattern (Fig. 2n). At the apex, the

Table 1 ECM morphotypes harvested in the sampled plants

Name	Accession no.		Morphotypes	BLAST analysis of ITS sequences
	ITS	18s		
ECMm1	AY940164	AY940165	*Tuber* sp.	*Tuber* sp.
ECMm2	DQ402505	–	*T. borchii*	*T. borchii*
ECMm3	DQ402504	DQ402510	*Tuber* sp.	*Tuber* sp.
ECMm4	–	–	*T. brumale*	–
ECMm5	–	–	*T. melanosporum*	–
ECMm6	–	–	*T. aestivum*	–
ECMm7/m8	DQ402506/DQ402507	–	AD-type	*Sarcosomataceae* *Pyronemataceae*
ECMm9	DQ402508	–	*Scleroderma* sp.	*Scleroderma* sp.
ECMm10	DQ402509	–	Basidiomycete	*Cortinarius* sp.

cells are mostly epidermoid and arranged in a puzzle-like pattern (Fig. 2o). Conversely, the ECMm3 mycorrhizas are 0.45–4.0 mm (mean 3.1±0.7 mm) long and 0.25–0.4 mm (mean 0.26±0.03 mm) in diameter. These mycorrhizas have a smooth surface without emanating hyphae or cystidia. The outer surface of the mantle shows polygonal to ellipsoidal cells measuring, on average, 16×9 μm (Fig. 2m).

Among the remaining morphotypes, one shows the AD-type morphology described by Giraud (1988), although this morphotype grouped mycorrhizas, named ECMm7 and ECMm8 (see below), with different ITS sequences. These mycorrhizas are brown-orange, monopodial pinnate, straight, simple or scarcely ramified (Fig. 2b), 0.32–4.0 mm (mean 2.95±0.49 mm) long, and 0.14–0.3 mm (mean 0.22± 0.05 mm) in diameter. The emanating hyphae (Fig. 2h) are yellow, slightly bent, thick-walled, ramified with a straight angle, and 2–6 μm (mean 2.98±0.67 μm) in diameter. The hyphae have simple septa without clamps. The outer surface of the mantle is pseudoparenchymatic with polygonal cells measuring 16.7×10.9 μm on average (Fig. 2l).

The ECMm9 mycorrhizas showed structures resembling *Scleroderma* spp. (Waller et al. 1993); they are white-ochre, irregular-pinnate, densely ramified, tortuous (Fig. 2a), 0.35–3.8 mm (mean 1.93±1.1 mm) long, and 0.15–0.31 mm (mean 0.21±0.06 mm) in diameter. The emanating hyphae (Fig. 2g) are white-yellowish, tortuous, ramified, and 1.2–3.5 μm (mean 1.76±0.08 μm) in diameter. They prevalently have simple septa and only occasionally clamp connections. Rhizomorphs are very abundant, moderately ramified, have hairy margins, and are 7.5–15.75 μm (mean 12.75±3.9 μm) in diameter. The outer surface of the mantle is plectenchymatic with loosely woven hyphae. The ECMm10 mycorrhizas are ochre, monopodial pinnate, simply or scarcely ramified (Fig. 2b,c), 0.28–4.8 mm (mean 2.82±0.8 mm) long, and 0.12–0.30 mm (mean 0.17±0.06 mm) in diameter. The emanating hyphae (Fig. 2i) have abundant clamp connections and are white-hyaline, tortuous, ramified, and 2–3.2 μm (mean 1.9±0.06 μm) in diameter. The rhizomorphs are moderately ramified and 6.5–16.75 μm (mean 10.5±2.9 μm) in diameter. The outer surface of the mantle is plectenchymatic with hyphae that are 1.6 μm in diameter on average.

Molecular characterization of *Tuber*-like ECM

A preliminary molecular typing of all truffle-like ECM morphotypes (ECMm1 to ECMm6, Table 1) was performed by PCR analysis in the presence of truffle species-specific ITS primers. Concerning the morphotypes ECMm2, ECMm4, ECMm5, and ECMm6, this analysis confirmed the species attribution inferred by morphological observation: each morphotype only yielded an amplicon when amplified with *T. borchii*, *T. brumale*, *T. melanosporum*, and *T. aestivum* species-specific primers, respectively (data not shown). Because ECMm1 displayed morphological features which differed slightly from the ECM of *T. borchii* and *T. borchii* complex, in addition to the primer pair mentioned above, the ITS species-specific primers available to date for the species of this complex, *T. borchii*, *T. maculatum*, *T. dryophylum*, and *T. pubelurum*, were used. None of these primers produced amplicons in any of the ECMm1 samples processed. Similarly, all the specific primers failed to produce amplicons from the ECMm3 mycorrhizal samples.

To identify the taxonomic group or species to which the ECMm1 and ECMm3 morphotypes can be ascribed, the 18S and ITS rDNA regions were PCR amplified and sequenced from these samples. Both ECMm1 and ECMm3 in the presence of NS1/NS8 primers produced an amplicon of about 1,700 base pairs. The two amplicons were very similar in sequence (identity of 99.2%) and showed high similarity with SSU sequences from *Tuberaceae* (*Tuber*, *Choiromyces*, *Labyrinthomyces*, *Reddellomyces*, and *Dingleya*). The highest similarity (about 99% of sequence identity) was with *Tuber gibbosum*.

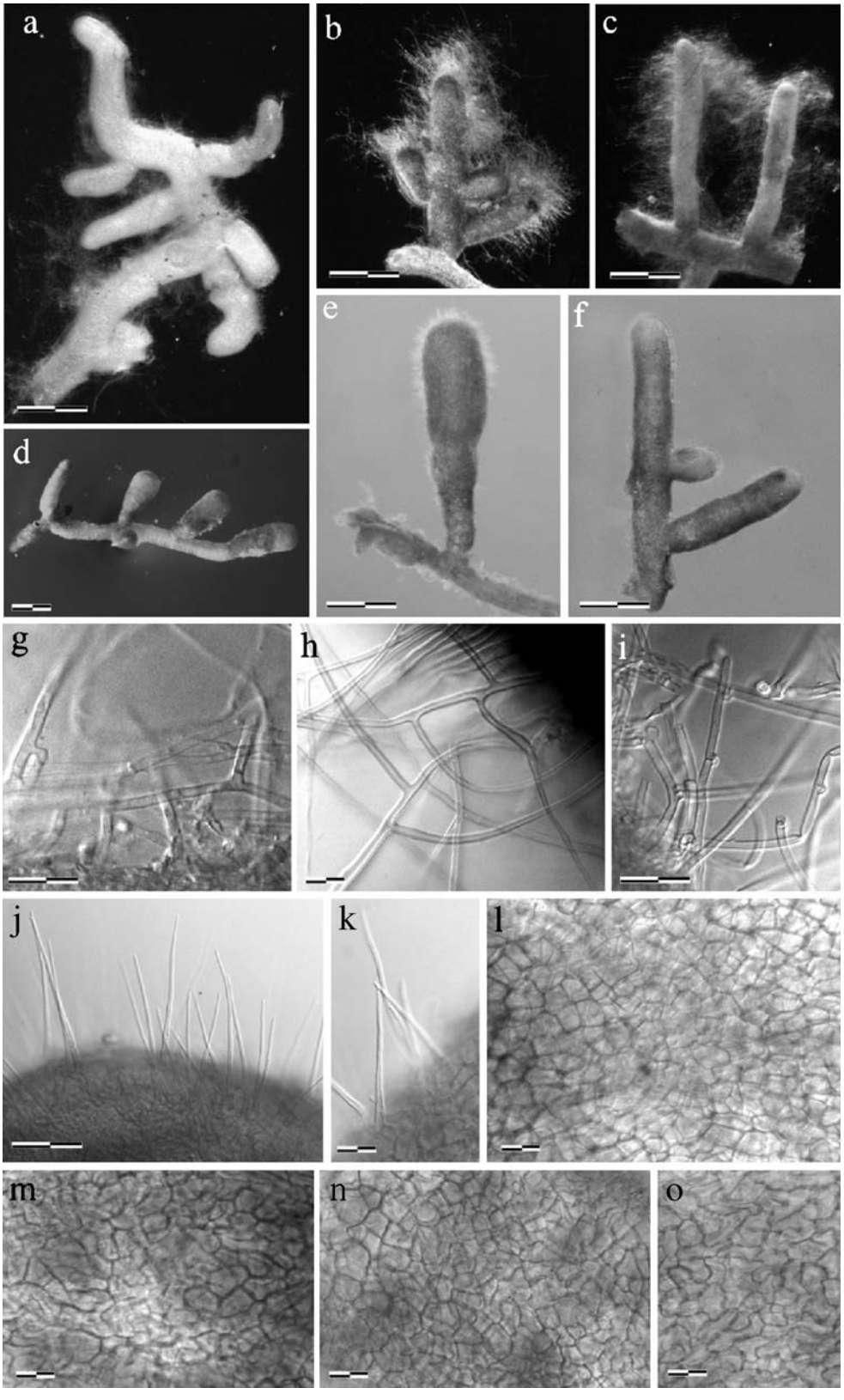

Fig. 2 Morphological characteristics of ECM morphotypes. **a**–**f** simple or ramified mycorrhizal tips; *bar*=340 μm. **a** ECMm9. **b** ECMm7/8. **c** ECMm10. **d** ECMm3. **e** and **f** ECMm1. **g**–**m** Detail of the emanating hyphae. **g** ECMm9, emanating hyphae without clamp connections; *bar*=12 μm. **h** ECMm7/8, ramified cystidia; *bar*=12 μm. **i** ECMm10, emanating hyphae with clamp connections; *bar*=12 μm. **j** ECMm3, plan view of outer mantle layer with cystidia; *bar*=30 μm. **k** ECMm1, detail of needle-like cystidia showing a basal septa; *bar*=12 μm. **l**–**o** plan view of outer mantle layer; *bar*=12 μm. **l** ECMm10, polygonal-shaped hyphal cells. **m** ECMm3, epidermoid hyphal cells. **n** ECMm1, polygonal-shaped hyphal cells (middle part of the mycorrhiza). **o** ECMm1, epidermoid hyphal cells (mycorrhizal tip)

Because of the still limited number of *Tuber* spp. SSU sequences available from public databases and because of their putative scarce interspecific polymorphism, a more finely tuned classification was sought through ITS sequencing. All of the ECMm1 samples analyzed produced an ITS amplicon of identical length (about 540 base pairs), which appeared to be among the shortest among *Tuber* spp. The ECMm3 samples produced an ITS amplicon of about 600 base pairs. Direct sequencing analysis of ECMm1 and ECMm3 ITS products showed them to be absolutely identical across the apical root-tips analyzed, regardless of their being amplified individually or as pools. Additionally, it revealed that the length variation observed in the ECMm1 compared to the other *Tuber* ITS sequences resides on the ITS1 region.

BLASTn searches indicated high similarity of the ECMm1 sequence with the ITS of *Tuber scruposum* (92–98% of sequence identity) and with other species of the whitish truffles group (*T. maculatum*, *Tuber foetidum*, *Tuber rapaedorum*, *T. borchii*, *T. dryophylum*, and *Tuber oligospermum*). In addition, high similarity was found with fungal ITS sequences from the roots of *Epipactis* (*Orchidaceae*) (92–97% of sequence identity) (Bidartondo et al. 2004) and with ITS sequences from the ECM community of *Populus* (Kaldorf et al. 2004). The sequence obtained from ECMm3 showed the highest similarity (about 91% of sequence identity) with the ITS of the *Tuber rufum* species complex (*T. rufum*, *Tuber ferrugineum*). The phylogenetic analysis confirmed the BLAST results (Fig. 3). The ECMm1 sample appeared closely related to *T. sruposum* and to fungi colonizing *Epipactis* roots. All these sequences, in fact, were grouped in a distinctive cluster supported by a high-bootstrap-value (100%) group that was closely related to the cluster of *T. borchii* and the complex of whitish truffle species. The ECMm3 sample formed a highly supported cluster (100% of bootstrap replicates) with *T. rufum* and *T. ferrugineum*.

Molecular characterization of non-*Tuber* like ECMs

Following the approach described above, the samples showing non-*Tuber*-like morphotypes were PCR analyzed to gain insights into their taxonomical ranking. The direct sequencing of ITS amplicons from pools of mycorrhizas ascribed to the AD-type showed overlapping peaks, making any nucleotide similarity search impossible. When single mycorrhizas were processed, direct sequencing showed that the AD-like morphotype includes samples exhibiting two ITS sequences (ECMm7 and ECMm8) with only 74.7% of nucleotide identity. According to the BLAST search, however, both ECMm7 and ECMm8 showed the highest similarity with ITS sequences from *Sarcosomataceae* and *Pyronemataceae*, although this similarity was confined only to the conserved 18S, 26S, and 5.8S regions. The ECMm9 displayed the highest ITS similarity to *Scleroderma* sp., confirming what was inferred from morphological observation. The ECMm10 showed 98% of sequence identity with *Cortinarius* sp. (Table 1).

Distribution of mycorrhizas

The presence and distribution of mycorrhizas were monitored on 70 host plants across the entire truffle ground (Fig. 1) and sampled in three different years (see "Materials and methods"). The fungal species and the number of root tips colonized by each species did not vary consistently between samples from each host plant (Fig. 4). *Tuber melanosporum* mycorrhizas were found on 44.3% of the plants (31 out of the 70) with a high variability in mycorrhiza frequency (from 5 up to 80%). In particular, *T. melanosporum* mycorrhizas were found in 9 *Q. pubescens*, 12 *Q. ilex*, and 10 *C. avellana* plants with percentages of mycorrhizal root tips ranging from 5 to 55%, from 5 to 50%, and from 40 to 80%, respectively (Fig. 4). Among the 31 plants colonized by *T. melanosporum*, ten showed only *T. melanosporum* ECM, while mycorrhizas of *Scleroderma* sp. and *Cortinarius* sp. and, less frequently, of other *Tuber* spp. were found in the other 21 plants.

The plants not colonized by *T. melanosporum* showed high percentages (up to 90%) of other ECM fungi reported in Table 1 (*Scleroderma* spp., AD-type, *Cortinarius* spp., *T. borchii*, *T. aestivum*, *T. brumale*, ECMm1, and ECMm3). In particular, five plants were colonized by *T. aestivum* (from 5 to 80%), seven plants by *T. brumale* (from 5% to 50%), two plants by *T. borchii* (from 10% to 25%), and two plants showed the presence of ECMm3. In addition, the ECMm1 morphotype was found in a relatively large number of plants (16) belonging to all three host species and distributed all over the truffle ground (Figs. 1 and 4). In these plants the ECMm1 morphotype was often very abundant, with a percentage of mycorrhization up to 80% (range 5–80%). Interestingly, the ECMm1 mycorrhizas were never found in plants colonized by *T. melanosporum*, although the two species colonized host plants growing near each other. All the plants consistently showed the presence of dried root tips (up to 95%), and this is in agreement with previous observations (Bencivenga et al. 1995; Granetti and Baciarelli Falini 1997).

Discussion

To gain insight into ECM fungal species in an unproductive man-made truffle plantation, we made an extensive morphological and molecular characterization of ECMs present

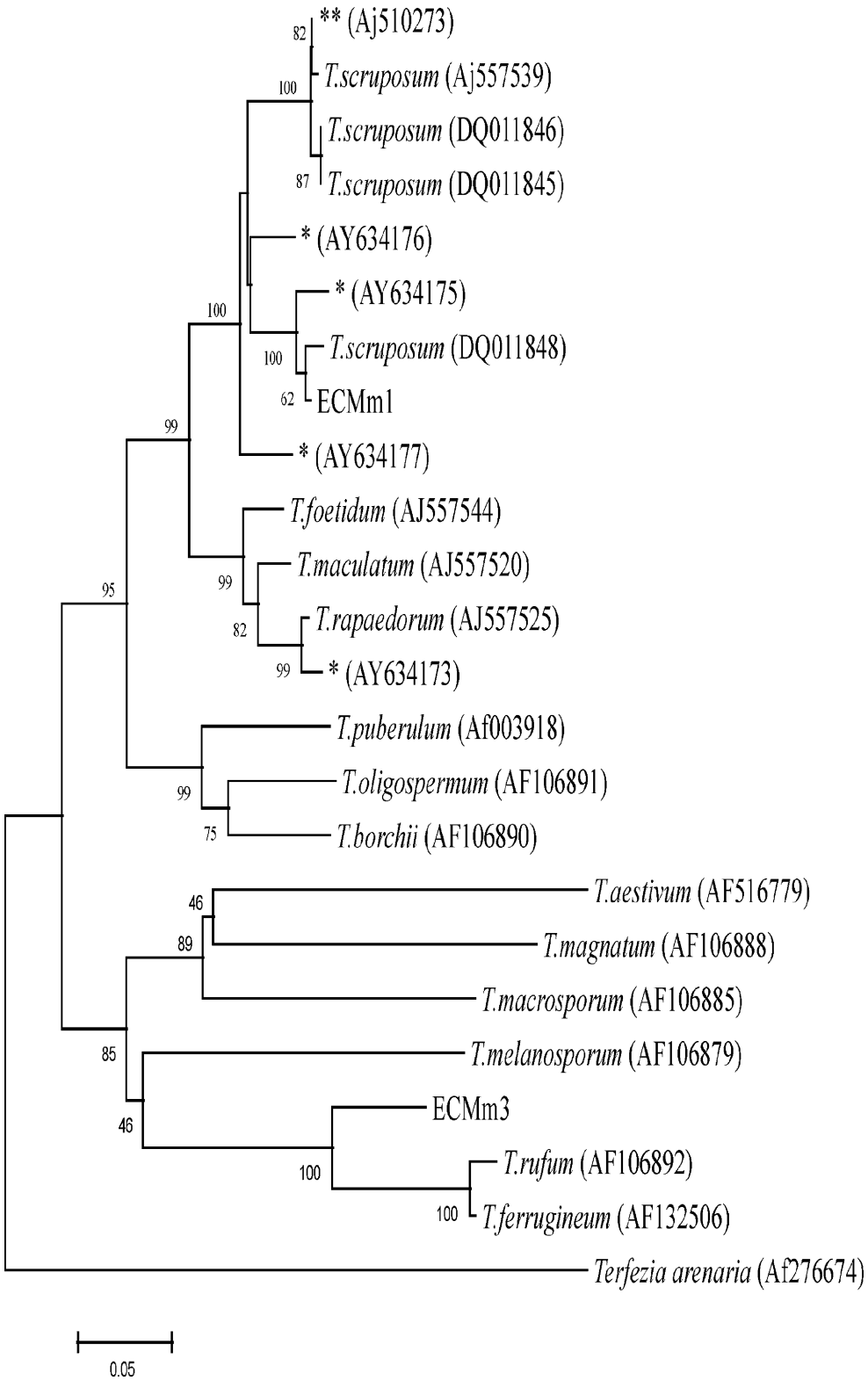

Fig. 3 ITS neighbor-joining phylogenetic tree showing the relatedness of ECMm1 and ECMm3 with other *Tuber* spp. *Single asterisk* indicates sequences from fungal samples associated with orchids; *double asterisk* indicates sequences from unclassified *Tuber* ECM. *Numbers* near the branches indicate bootstrap values (percentage over 1,000 replicates)

in 14-year-old oak and hazel trees originally inoculated with the fine black truffle *T. melanosporum* and out-planted in a site located in Umbria (central Italy). Although no fine black truffles have ever been harvested in the truffle ground examined and in the adjacent area (Mantucci, personal communication), *T. melanosporum* mycorrhizas were present in 44.5% of the sampled plants. To a much lesser extent, mycorrhiza of *T. aestivum*, *T. brumale*, and *T. borchii* were recorded from morphological and ITS PCR analyses. Two morphotypes (ECMm1 and ECMm3) showing morpholog-

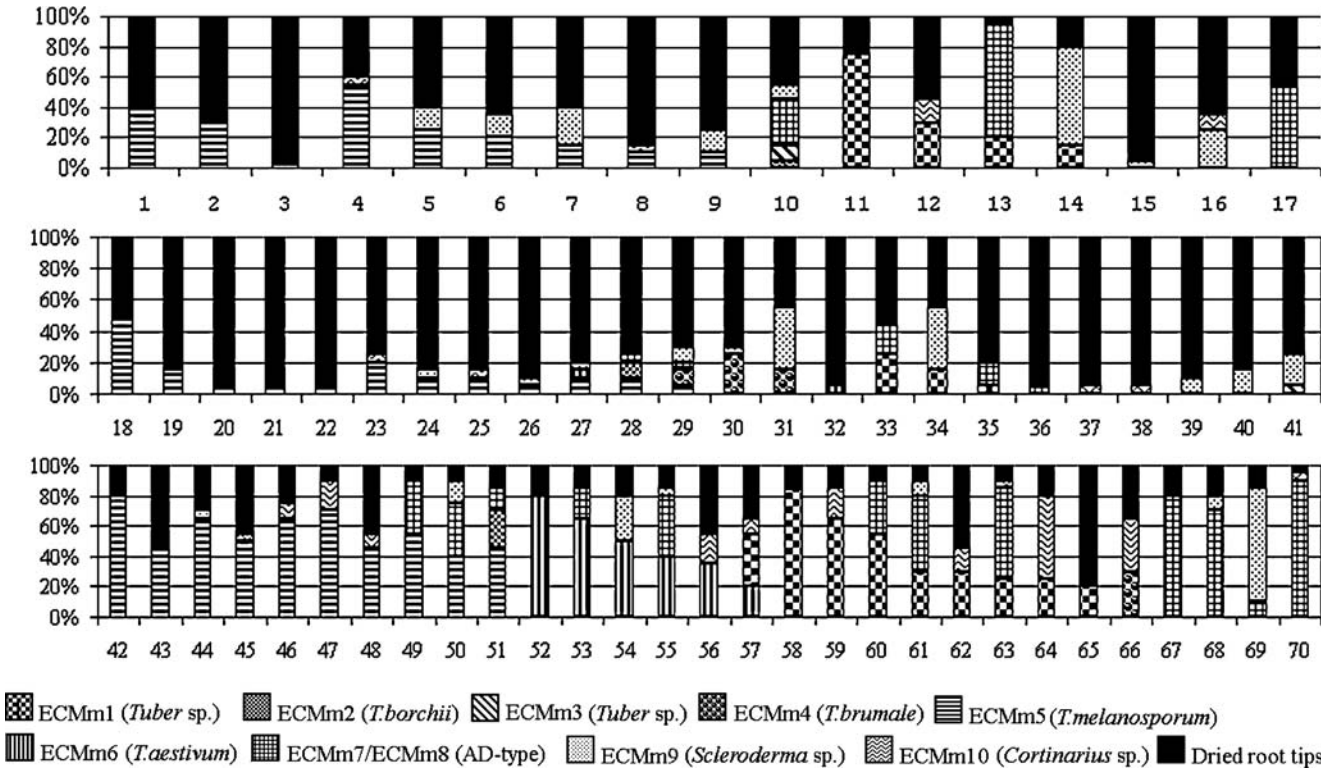

Fig. 4 Distribution of ECM morphotypes on *Q. pubescens* (**a**), *Q. ilex* (**b**), and *C. avellana* (**c**) host plants. Plant numbers are as in Fig. 1. For each plant the data are expressed as percentage of each morphotype in the total number of root tips analyzed in the three samplings

ical traits specific to *Tuber* spp. mycorrhizas were also sampled. Many of the host plants (about 20%) showed the ECMm1 morphotype which closely resembles mycorrhizas produced by species of the whitish truffle complex. This complex contains truffle species of modest commercial value, and an accurate morphological characterization of the mycorrhizal structures has been provided only for some of these species (*T. puberulum,T. maculatum* and *T. borchii*) (Blasche 1988; Zambonelli et al. 1999; Rauscher et al. 1996). However, ECMm1 differs slightly from both *T. borchii* and other whitish truffle species ECMs for the darker color of the mantle and the shortness of cystidia (spinulae). ECMm1 cystidia are 33–75.0 μm long, while they have been reported to be 80–120, 26–109, and 65–105 μm long in *T. puberulum* (Blasche 1988), *T. maculatum* (Zambonelli et al. 1999), and *T. borchii* (Rauscher et al. 1996), respectively. Repeated samplings in autumn and spring confirmed the persistence of the ECMm1 morphotype, irrespective of host tree species and environmental conditions, lending strong support to the concept that the distinctive morphological features of ECMm1 are not primarily shaped by ecological/environmental factors. The morphological characteristics of ECMm3, which shows some traits (e.g., the absence of cystidia) previously reported for *T. rufum* mycorrhizas (Rauscher et al. 1995), were also stable.

ITS phylogenetic analysis not only confirmed that ECMm1 is related to the whitish truffle species but also revealed that it falls into a cluster grouping ITS sequences derived from *T. scruposum* ascocarps and mycorrhizas of fungi colonizing poplar and orchids (Kaldorf et al. 2004; Bidartondo et al. 2004). However, apart from ITS sequences and host species, no other morphological information is yet available about these latter fungal species. The presence of quite divergent ITS sequences ascribed to *T. scruposum* poses the question of whether all these sequences belong to a unique species or to a *T. scruposum* species complex grouping morphologically similar truffles. Overall, from the ITS phylogenetic tree we can argue that: (1) ECMm1 is the candidate ECM of *T. scruposum* or of a species closely related to *T. scruposum*, and (2) this species or species complex probably colonizes orchids, although these plants may not represent the main niche of these fungi. Molecular ecological analyses have already shown that orchids can form mycorrhizas with fungi, and among them *Tuber* spp., that simultaneously form ECM with the roots of neighboring trees (Taylor and Bruns 1997; Selosse et al. 2002, 2004). Capillary sampling and ITS sequence analysis of *T. scruposum* ascocarps will help to assess the level of genetic polymorphism within this species and verify the hypothesis that ECMm1 is the symbiotic structure of this truffle species. It is worthy to note that neither ascocarps carrying the ECMm1-specific ITS

sequences nor orchids within or surrounding the truffle ground under investigation have been recorded so far. Whether ECMm1 and the other truffle mycorrhizas (*T. aestivum*, *T. brumale*, *T. borchii*) occur spontaneously on the site here sampled or have been introduced with *T. melanosporum* nursery-inoculated mycorrhizal plants remains therefore unknown. Interestingly, although ECMm1 and *T. melanosporum* are the most abundant mycorrhizas and some plants harbored both *T. melanosporum* and *T. borchii* or *T. melanosporum* and *T. brumale* ECMs, ECMm1 and *T. melanosporum* have never been detected on the same root apparatus.

Differently from ECMm1, the species attribution of ECMm3 seems to be a more difficult task. Despite the fact that both morphological and molecular analyses indicate that ECMm3 is closely related to *T. rufum*, the ITS phylogenetic tree clearly shows that ECMm3 clusters apart from *T. rufum* and *T. ferrugineum*. Overall, our data prove once again the high, yet still largely unexplored, level of inter and/or intraspecific genetic polymorphism in truffle species of minor, if any, economic importance. The description of two novel *Tuber* mycorrhizal morphotypes and the report of their ITS fingerprinting help in shedding light on both the morphological and the molecular variability of these *Tuber* species.

Among the non-*Tuber* mycorrhizas, we detected *Cortinarius* and *Scleroderma* spp. as per ITS sequencing. This is not surprising because these morphotypes were not infrequently monitored in both native or cultivated truffle grounds (Baciarelli Falini, unpublished). It should be noted that among the non-*Tuber* mycorrhizas, two divergent ITS sequences were obtained from mycorrhizal samples displaying the same morphotype (AD-type). This suggests that AD-type mycorrhizas, known as a morphotype commonly associated with truffle plantations (Giraud 1988), result indeed from at least two different fungal species.

To the best of our knowledge, this is the first report aimed at assessing, by combining morphological and molecular approaches, the fungal biodiversity underlying an unproductive man-made truffle ground. Chemical and physical soil analyses suggested that the plantation site under investigation does not match the optimal conditions for inducing the fruiting of *T. melanosporum* (Baciarelli Falini and Bencivenga 2002). Hence, besides the description of novel *Tuber* mycorrhizal morphotypes, the present work adds evidence to the thesis that the fructification of ectomycorrhizal fungi might not correlate with their distribution and abundance on host root tips (Horton and Bruns 2001). In this contest, the screening of two natural and productive *T. magnatum* truffle grounds, based upon morphotyping and molecular analysis of ECMs, revealed that *T. magnatum* mycorrhizas were rare and that the production of fruit bodies was not correlated with mycorrhizal abundance (Murat et al. 2005; Bertini et al. 2006). Finally, our finding that *T. melanosporum* mycorrhizas are still present on host roots more than a decade after the onset of the tree plantation might promote attempts to rescue this truffle cultivation by means of appropriate agronomical practices.

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References

Agerer R (1986) Colour Atlas of ectomycorrhizae. Einhorn, Schwäbisch-Gmünd

Amicucci A, Zambonelli A, Giomaro G, Potenza L, Stocchi V (1998) Identification of ectomycorrhizal fungi of the genus *Tuber* by species-specific ITS primers. Mol Ecol 7:273–277

Baciarelli Falini L, Bencivenga M (2002) Valutazione e tecniche di miglioramento di alcune tartufaie coltivate nello spoletino. Micol Ital 3:29–43

Bencivenga M, Di Massimo G, Donnini D, Tanfulli M (1995) Analisi della micorrizazione e sviluppo delle piante tartufigene messe a dimora da 13 anni a diversa distanza da alcune roverelle adulte. Micol Ital 3:110–116

Bertini L, Rossi I, Zambonelli A, Amicucci A, Sacchi A, Cecchini M, Gregori G, Stocchi V (2006) Molecular identification of *Tuber magnatum* ectomycorrhizae in the field. Microbiol Res 161:59–64

Bidartondo M, Burghardt B, Gebauer G, Bruns TD, Read DJ (2004) Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. Proc R Soc Lond B 271:1799–1806

Blasche H (1988) *Tuber* puberulum. In: Agerer R (ed) Colour atlas of ectomycorrhizae, plate 22. Einhorn, Schwäbish-Gmünd

Donnini D, Baciarelli Falini L, Bencivenga M (1999) Analisi della micorrizazione in tartufaie coltivate di *Tuber melanosporum* Vitt. impiantate da oltre 12 anni in ambienti pedoclimatici diversi. In: Proceedings of the 5th international congress on the science and cultivation of truffle and other edible hypogeous mushrooms, Aix-en-Provence, France, 4–6 March 1999

Egger KN (1995) Molecular analysis of ectomycorrhizal fungal communities. Can J Bot 73:S1415–S1422

Fontana A, Ceruti A, Meotto F (1992) Criteri istologici per il riconoscimento delle micorrize di *Tuber albidum*. Micol Veg Mediterr 7:121–136

Giraud M (1988) Prélévement et analyse de mycorhizes. Bull. FNTP, 10, 49–63. In: La Truffe. CTIFL, Paris

Granetti B, Baciarelli Falini L (1997) Competizione tra le micorrize di *Tuber melanosporum* Vitt. e quelle di altri funghi in una tartufaia coltivata a Quercus ilex L. Micol Ital 3:45–59

Hall IR, Yun W, Amicucci A (2003) Cultivation of edible ectomycorrhizal mushrooms. Trends Biotechnol 21:433–438

Harley JL, Smith SE (1983) Mycorrhizal symbiosis. Academic, London

Horton TR, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. Mol Ecol 10:1855–1871

Kaldorf M, Renker C, Fladung M, Buscot F (2004) Characterization and spatial distribution of ectomycorrhizas colonizing aspen clones released in an experimental field. Mycorrhiza 14:295–306

Kimura M (1980) A simple method for estimating evolutionary rates of nucleotide substitution through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA 2: molecular evolutionary genetics analysis software. Arizona State University, Tempe, AZ

Mello A, Cantisani A, Vizzini A, Bonfante P (2002) Genetic variability of *Tuber uncinatum* and its relatedness to other black truffles. Environ Microbiol 4:584–594

Murat C, Vizzini A, Bonfante P, Mello A (2005) Morphological and molecular typing of the below ground fungal community in a natural *Tuber magnatum* truffle-ground. FEMS Microbiol Lett 245:307–313

Paolocci F, Angelini P, Cristofari E, Granetti B, Arcioni S (1995) Identification of *Tuber* spp. and corresponding ectomycorrhizae through molecular markers. J Sci Food Agric 69:511–517

Paolocci F, Rubini A, Granetti B, Arcioni S (1999) Rapid molecular approach for a reliable identification of *Tuber* spp. ectomycorrhizae. FEMS Microbiol Ecol 28:23–30

Rauscher T, Agerer R, Chevalier G (1995) Ektomykorrhizen von *Tuber melanosporum*, *Tuber mesentericum*und *Tuber rufum* (Tuberales) an Corylus avellana. Nova Hedwig 61:281–322

Rauscher T, Müller WR, Agerer R, Chevalier G (1996) *T. borchii* Vitt. + *Corylus avellana* L. In: Agerer R, Danielson RM, Egli S, Ingleby K, Luoma D, Treu R (eds) Description of ectomycorrhizae, vol 1. Einhorn, Schwäbisch-Gmünd, pp 173–178

Rubini A, Paolocci F, Granetti B, Arcioni S (1998) Single step molecular characterization of morphologically similar black truffle species. FEMS Microbiol Lett 164:7–12

Rubini A, Paolocci F, Granetti B, Arcioni S (2001) Morphological characterization of molecular-typed *Tuber magnatum* ectomycorrhizae. Mycorrhiza 11:179–185

Selosse MA, Weiss M, Jany JL, Tillier A (2002) Communities and populations of sebacinoids basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* and neighboring tree ectomycorrhizae. Mol Ecol 1:1831–1844

Selosse MA, Faccio A, Scappatici G, Bonfante P (2004) Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. Microb Ecol 47:416–426

Taylor DL, Bruns TD (1997) Independent, specialized invasion of ectomycorrhizal mutualism by two non photosynthetic orchids. Proc Natl Acad Sci USA 94:4510–4515

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882

Waller K, Raidl S, Agerer R (1993) Die Ektomykorrhizen von *Scleroderma citrinum*. Z Mykol 59:141–153

White TJ, Bruns TD, Lee S, Taylor J (1990) Analysis of phylogenetic relationship by amplification and direct sequencing of ribosomal RNA genes. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, New York, pp 315–322

Zambonelli A, Salomoni S, Pisi A (1993) Caratterizzazione anatomomorfologica delle micorrize di *Tuber* spp. su *Quercus pubescens* Willd. Micol Ital 3:73–90

Zambonelli A, Iotti M, Amicucci A, Pisi A (1999) Caratterizzazione anatomo-morfologica delle micorrize di *Tuber maculatum* Vittad. su *Ostrya carpinifolia* Scop. Micol Ital 28:29–35