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Morphological characterization of molecular-typed *Tuber magnatum* ectomycorrhizae

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Abstract Recent reports of discrepancies between the morphological and molecular-based characterization of *Tuber magnatum* ectomycorrhizae suggest misleading morphological typing of these structures. This study reports a new morphological and anatomical description of *T. magnatum* ectomycorrhizae at different development stages using nursery-inoculated host plants. Both ascocarps, used as spore donors in the inoculation process, and the resulting ectomycorrhizae were first checked by single-step PCR analysis using a *T. magnatum* species-specific ITS primer pair. Direct sequence analysis of the entire ITS1–5.8S-ITS2 fragment of DNA from mycorrhizal pools demonstrated the purity of the samples processed. Thus, the validity of the criteria reported here for the morphological identification of *T. magnatum* symbiotic structures is supported by molecular analysis. When diagnostic morphological traits appear only occasionally, as a result of ageing or of particular edaphic conditions, only molecular markers such as the species-specific ITS primers designed here ensure reliable typing of *T. magnatum* mycorrhizae for the purpose of controlling and/or certifying mycorrhizal plants present on the market.

Keywords Ectomycorrhizae · ITS · Morphology · Species-specific primers · *Tuber magnatum*

Introduction

The life cycle complexity of the symbiotic *Tuber* species has hindered definition of the different phases of fungus-plant interaction. Description of the biological steps leading from mature spores to their germination, to mycelium growth and to colonization of root tips, or from ectomycorrhizal formation to ascocarp production, are only guesswork. These are derived mostly from studies of other fungal species, since neither molecular nor physiological factors driving these steps have been characterized so far in *Tuber* spp. However, due to the great economic importance of the flourishing truffle market, its production has been encouraged by promoting plantations of truffle-inoculated host plants. Nursery production of mycorrhizal plants is routinely performed by inoculating host seedlings grown in semi-sterile conditions with spores from morphologically determined ascocarps (Grente et al. 1972; Chevalier and Grente 1979; Chevalier 1994).

Identification of the resulting mycorrhizae is also based on morphological criteria. However, such criteria are based on descriptions of the mycorrhizal structures resulting from plant inoculation with spores of a given fungal species. Following this approach, Palenzona and Fontana (1979) gave the first description of *T. magnatum* Pico ectomycorrhizae obtained on *Quercus pubescens* Willd. Later papers reported similar results whilst giving a more detailed morphological description of mycorrhizae obtained with various host plants (Fontana et al. 1990; Zambonelli et al. 1993; Granetti 1995). However, the presence of fungal species actively competing with *Tuber* spp. in colonizing host root tips and the great morphological similarity between mycorrhizae of different *Tuber* spp. may lead to mischaracterization of the mycorrhizal plants produced. To overcome such a problem, molecular markers and methods for reliable identification of these symbiotic fungi throughout their biological cycle have been proposed (Henrion et al. 1994; Paolocci et al. 1995, 1997, 1999, 2000; Mello et al. 1996, 1999a; Gandeboeuf et al. 1997; Amicucci et al. 1997, 1998, 2000; Bertini et al. 1998; Rubini et al. 1998). Biochemical and species-specific molecular markers have shown that mycorrhizae obtained by host plant inoculation with *T. magnatum* can be ascribed instead to other minor white truffle species such as *T. borchii* Vittad. and *T.*

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maculatum Vittad. or to other still undefined species (Bullini et al. 1994; Gandeboeuf et al. 1997; Amicucci et al. 1998). This demonstrates how unreliable morphological analysis alone may be as a diagnostic tool.

Thus a molecular approach is the only way to certain typing of truffle ectomycorrhizae. The reasons why artificial mycorrhization of host plants with *T. magnatum* often fails remain obscure. Since this species produces one of the finest edible fruit bodies and its geographical distribution is quite restricted, large-scale cultivation of plants colonized by *T. magnatum* has been strongly supported, especially in Italy. This paper describes *T. magnatum* ectomycorrhizae on nursery-inoculated *Quercus cerris* L., the identification of which was confirmed by analysis of the nuclear rDNA ITS region.

Materials and methods

Sample source and DNA isolation

Tuber magnatum ascocarps collected in Umbria (Italy) were used as spore donors to inoculate a plot of 25 *Quercus cerris* plantlets grown in semi-sterile conditions, basically following the procedure of Palenzona and Fontana (1979) with minor modifications (A. Rubini, unpublished results). The inoculated plants were grown in a nursery under normal environmental conditions. About 6 months after plant inoculation, roots were sampled from five plants, washed in running tap water and analysed under a dissecting microscope for the presence of ectomycorrhizae. Genomic DNA was isolated from ascocarps of *T. magnatum* and from single or pooled mycorrhizal root tips. In order to verify the successful mycorrhization of plants with *T. magnatum*, root tip samples were randomly collected from all five plants at different physiological stages, to include mycorrhizal tips with and without peritrophic elements (cystidia and emanating hyphae). DNA from both mycorrhizae and small portions of the ascocarps used for inoculation (about 10 mg) were collected in a 2-ml microcentrifuge tube, frozen in liquid nitrogen and used immediately for DNA isolation or stored at –70°C. As a control for the specificity of the *T. magnatum*-specific ITS primers, DNA was also isolated from ascocarps of the fungal species listed in Table 1 and from the host species *Quercus cerris* L. and *Populus alba* L. DNA was isolated as previously described by Paolocci et al. (1999). Voucher specimens are listed in Table 1.

Morphological analysis of ectomycorrhizae

Morphological observations and photography were performed on freshly isolated ectomycorrhizal tips using a dissecting microscope, according to Agerer (1986). Features of the mantle surface and emanating elements were examined on ectomycorrhizal tips mounted in 90% lactic acid using a Normanski interference contrast microscope. For anatomical analyses, mycorrhizal tips were fixed in 5% glutaraldehyde in 0.075 M sodium cacodylate buffer pH 7.0, dehydrated in ethanol and embedded in glycol methacrylate (Sigma, Milan) using a standard protocol (Leduc and Bernhard 1967). Cross-sections of about 3 µm were cut with a microtome using a tungsten carbide knife.

Table 1 List of ascocarps of *Tuber* spp. and their collection sites. Voucher specimens (one for each species) have been deposited at the Herbarium of Istituto Sperimentale per la Patologia Vegetale, (ROPV) Via C.G. Bertero, 22 00156 Rome, Italy (*A* the number of ascocarps processed)

Species	Vouchers	Collection sites		A
Tuber magnatum	AR-Ma211	Città di Castello (Umbria)	Italy	1
	–	Pietralunga (Umbria)	Italy	4
	–	Alba (Piemonte)	Italy	4
	–	Isernia (Molise)	Italy	5
Tuber melanosporum	AR-Me369	Norcia (Umbria)	Italy	1
	–	Spoleto (Umbria)	Italy	10
	–	Abruzzo	Italy	10
	–	Lazio	Italy	5
	–	Toscana	Italy	3
	–	Navarra	Spain	4
	–	Provence	France	5
Tuber macrosporum	AR-Mc11	Pietralunga (Umbria)	Italy	1
	–	Pietralunga (Umbria)	Italy	2
	–	Marche	Italy	2
Tuber rufum	AR-R2	Città di Castello (Umbria)	Italy	1
	–	Umbria	Italy	1
Tuber oligospermum	AR-O5	Città di Castello (Umbria)	Italy	1
	–	Umbria	Italy	2
Tuber borchii	AR-Bo6	Città di Castello (Umbria)	Italy	1
	–	Umbria	Italy	5
	–	Basilicata	Italy	6
Tuber brumale	AR-Br1	Monte S.M. Tiberina (Umbria)	Italy	1
	–	Umbria	Italy	3
	–	Abruzzo	Italy	2
	–	Lazio	Italy	3
Tuber indicum	AR-In80	Truffle imported into Italy in 1997	China	1
	–	Truffle imported into Italy in 1997	China	14
Tuber magnatum × *Quercus cerris* (ectomycorrhizae)	AR-MM1			

PCR amplification

Universal ITS1 and ITS4 primers (White et al. 1990) and the specific ITS primer pairs for *T. magnatum*, ITSMAGN (5′-GTCACTGAAAACCCACTCACG – 3′) and ITSBACK3 (5′-TGAGGTCAACCCAGTTGGACAGT – 3′), were used to check the identity of *T. magnatum* mycorrhizae.

PCR amplification was carried out in a Gene Amp 9700 Thermal Cycler (Applied Biosystems, Foster City) with the following cycling parameters for the specific primers: an initial denaturation step at 95°C for 3 min, 25 cycles consisting of 30 s at 95°C, 30 s at 63°C and 45 s at 72°C and a final extension for 7 min at 72°C. When using the universal primers, the cycling conditions were as above but with a lower annealing temperature of 55°C.

All PCR amplifications were performed in 50-µl reaction mixtures containing 200 µM of each dNTP, 10 pmol of each primer, 4 mM $MgCl_2$, 10 mM Tris-HCL pH 9.0, 50 mM KCl, 2.5 units of Taq polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden) supplemented with 7 mg/ml BSA (Sigma, Milan) to overcome the effect of PCR inhibitors when processing the root samples (Paolocci et al. 1999). All PCR experiments included fungal DNA other than from *T. magnatum*, as well as a negative control (no DNA template).

Sequencing analysis

PCR products were purified using a G50 spin column (Amersham Pharmacia Biotech, Uppsala, Sweden), run on 1% agarose gel electrophoresis and quantified by comparison with DNA Quantitation Standards Phage λ DNA (Life Technologies, Glasgow) after gel staining with ethidium bromide. Double-strand direct sequencing of PCR products from ascocarp and mycorrhizal samples was carried out on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City) using a Big Dye terminator cycle sequencing kit according to the supplier's instructions.

The sequencing primers used were ITS1, ITS4 and 5.8sf /5.8sb (Rubini et al. 1998). The ITS sequence from ectomycorrhizal tips of *T. magnatum* is deposited in GenBank under the accession number AF325174. ITS sequences obtained from mycorrhizal tissues were tested for similarity using a BLAST algorithm at NCBI and were aligned to four ITS sequences of *T. magnatum* ascocarps retrieved from GenBank (accession numbers AJ002509, AF003911, AF003912, AF003913) using ClustalV software (Higgins and Sharp 1988)

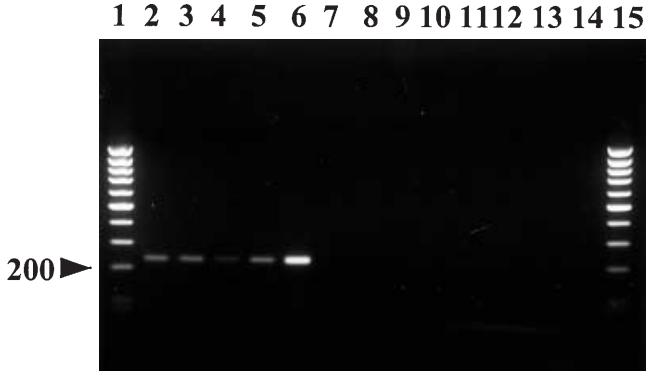


Fig. 1 PCR amplification of DNA isolated from *Tuber* spp. ascocarps and *T. magnatum* mycorrhizae using ITSMAGN and ITSBACK3 specific primers. *Lanes 1 and 15* GeneRuler 100-bp DNA ladder (MBI Fermentas, Vilnius, Lithuania); *lanes 2 and 3* pool of five *T. magnatum* ectomycorrhizae; *lanes 4 and 5* single *T. magnatum* mycorrhizal root-tip; *lane 6 T. magnatum* ascocarp; *lane 7 T. borchii* ascocarp; *lane 8 T. oligospermum* (Tul and Tul.) Trappe ascocarp; *lane 9 T. rufum* Pico ascocarp; *lane 10 T. macrosporum* Vittad ascocarp; *lane 11 T. brumale* Vittad. ascocarp; *lane 12 T. melanosporum* Vittad. ascocarp; *lane 13 T. indicum* Cooke and Massee ascocarp; *lane 14* negative control (no DNA template)

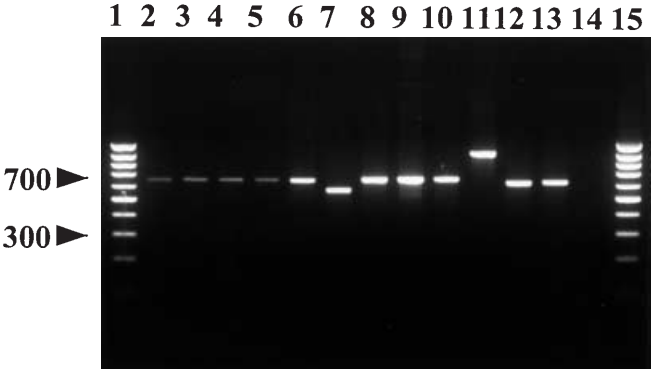


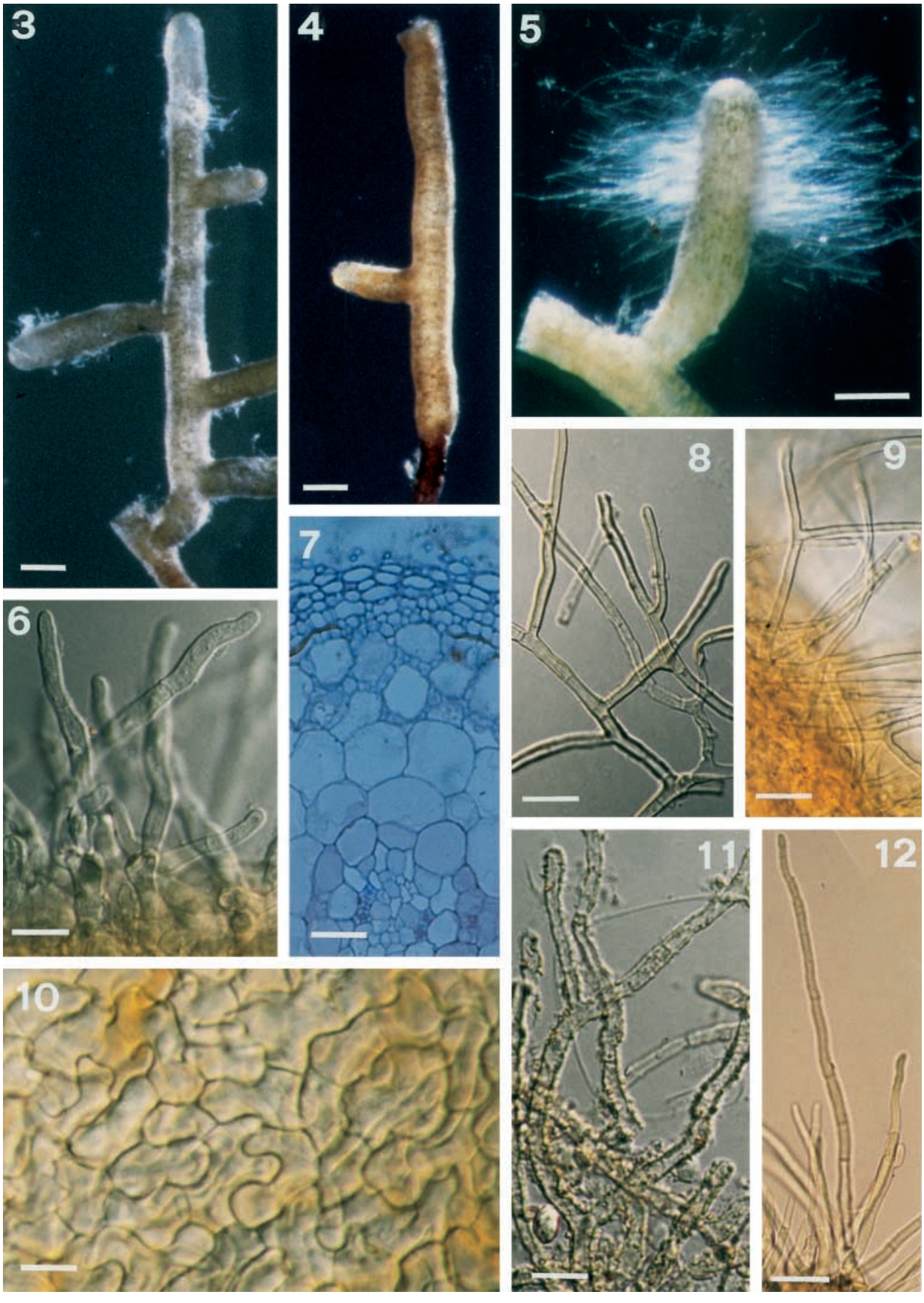
Fig. 2 PCR amplification of DNA isolated from *Tuber* spp. ascocarps and *T. magnatum* mycorrhizae using ITS1 and ITS4 primers. Lanes as in Fig. 1.

Results

Molecular analysis of ectomycorrhizae

Samples were first checked by PCR to verify the identity of the symbiotic fungus. For this purpose, mycorrhizae with or without peritrophic elements, which are the best diagnostic tool for a morphological analysis, were collected from inoculated plants at different stages of development. All samples tested produced an amplicon of about 230 bp, perfectly matching that produced by DNA from *T. magnatum* ascocarps when amplified with the ITSMAGN/ITSBACK3 primer pair. The two primers were based on the ITS sequence alignment of the most important and widespread species belonging to the *Tuberaceae* in order to specifically amplify the *T. magnatum* rDNA fragment. Amplification experiments confirmed primer specificity, since the expected amplicon was clearly produced only on the prestigious white truffle; no PCR products were obtained using DNA from any other species as target (Fig. 1). Thus, the ITSMAGN/ITSBACK3 primer pair is an effective single-step PCR-based marker for reliable and direct *T. magnatum* identification. Conversely, all target DNAs produced the expected amplicon in the presence of the ribosomal primers ITS1/ITS4 (Fig. 2).

Direct sequencing analysis was also carried out of ITS1/ITS4 PCR products from the amplification of DNA isolated from both a single mycorrhiza and from a pool of mycorrhizal root tips. The absolute identity between the sequences obtained indicated that all the root tips processed in the pools belonged to the same species. Interestingly, a BLAST similarity search confirmed that the sequence obtained was identical to the *T. magnatum* ITS sequences in GenBank.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Morphological characters of *T. magnatum* × *Quercus cerris* mycorrhizae

Morphological characters of *T. magnatum* × *Quercus cerris* mycorrhizae are illustrated in Figs. 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. The mycorrhizae are straight, frail, cylindrical or sometimes club-shaped and always with rounded ends. They can be simple or ramified, following a monopodial-pinnate pattern (Figs. 3, 4). Their colour varies greatly with the different developmental stages. During the active growth phase the new tissues are whitish, diaphanous with pale grey shades (Fig. 3), turning dark grey, greyish-amber or even dark amber (Fig. 4) at the quiescent phase. At any given time, the mycorrhizae are of a uniform colour from base to tip and only rarely is the apex more whitish. Mycorrhizal pigmentation is due to the presence of small, dark grey, variably distributed speckles, probably caused by polyphenol accumulation, which turn amber in old mycorrhizae.

Two kinds of peritrophic elements were observed on *T. magnatum* mycorrhizae: emanating hyphae and cystidia. The emanating hyphae are hyaline, thin-walled, septate and tortuous. They can be simple or ramified, but always have rounded tips (Fig. 11). They bear abundant crystals and generally grow to form a network on the surface of the mycorrhizae. The cystidia are colourless, pluri-septate, with marked walls and rounded tips (Figs. 9, 12). Unlike the emanating hyphae, they are not tortuous and generally show a smooth surface, although they may occasionally bear crystals or warts. The cystidia may be simple or ramified, with perpendicularly or almost perpendicularly oriented branches (Fig. 9). They reach a maximal length of 390 µm, with a diameter of 3–5 µm at their insertion point on the mantle surface. The distal ends of some cystidia can dedifferentiate to form an emanating hypha-like structure. The cystidia grow out radially from the surface of the mantle and are generally arranged in a restricted area, often at the apex of the mycorrhiza (Fig. 5). Only rarely are they distributed over almost the entire surface of the mycorrhiza. Mycorrhizae with cystidia are very rare and the presence of cystidia apparently is not influenced by the age of the mycorrhizae, since young and older mycorrhizae may lack these structures.

Hyphae and cystidia generally show distinctive features. However, peritrophic hyphae resembling cystidia but displaying anastomoses have also been observed (Fig. 8). Moreover, in very young mycorrhizae, simple emanating hyphae with no crystals are found at the initial stage of development (Fig. 6).

The outer surface of the mantle (Fig. 10) has a pseudoparenchymatic structure with epidermoid cells arranged in a puzzle-like pattern. However, both the proximal and the distal ends of mycorrhiza may display cells of variable size and shape, ranging from ellipsoidal, isodiametric and rectangular to square. These cells are generally arranged in complex and irregular patterns.

A cross-section of the mycorrhiza (Fig. 7) shows a 30-µm-deep pseudoparenchymatic mantle arranged in 4–5 layers of cells flattened on their tangential plane. The Hartig net only reaches the epidermal layer and apparently only the first row of the cortex cells.

Discussion

Growing truffles by planting *T. magnatum*-inoculated host plants has not produced the expected results. These prestigious white fruit bodies are de facto only collected in natural truffle areas. Not infrequently, nursery *T. magnatum* mycorrhizal plants unexpectedly produce *T. borchii* or *T. maculatum* ascocarps. Conversely, host plants nursery-inoculated with other species, such as *T. borchii* or *T. melanosporum*, in general have a high yield when planted in open field conditions. It is conceivable that factors other than the inoculation process itself disrupt cultivation of *T. magnatum*, e.g. particular, as yet undefined, pedological and ecological requirements of this fungus. However, Gandeboeuf et al. (1997) questioned the reliability of Italian nursery inoculated seedlings marketed in Europe as *T. magnatum* mycorrhizal plants, since the morphological similarities between *T. magnatum* mycorrhizae and those of other white truffle species do not allow precise species identification. Indeed, previous studies of *T. magnatum* features have all stressed the morphological similarities between *T. magnatum* and *T. albidum* Pico = *T. borchii* Vittad. mycorrhizae. Although, colour, shape and size of the mantle cells, dimensions of the spinule-like cystidia and thickness of the mantle and of the Hartig net have been proposed as discriminating characters of these two species on the basis of accurate comparative studies (Fontana et al. 1992; Zambonelli et al. 1993; Granetti 1995), differences are indeed hardly detectable. Given the unexpected production of white truffles of questionable value by growing nursery-inoculated *T. magnatum* plants, and the marked morphological similarities among mycorrhizae of different truffle spe-

◀ **Fig. 3** Greyish ramified mycorrhiza in active growth phase; *bar* 300 µm

Fig. 4 Old amber mycorrhiza lacking emanating elements; *bar* 300 µm

Fig. 5 Young greyish mycorrhiza with colourless cystidia; *bar* 300 µm

Fig. 6 Emanating hyphae at the initial stage of development; *bar* 13 µm

Fig. 7 Cross-section showing pseudoparenchymatous mantle and Hartig net; *bar* 25 µm

Fig. 8 Emanating hyphae with anastomoses; *bar* 16 µm

Fig. 9 Ramified cystidia; *bar* 16 µm

Fig. 10 Epidermoid cells of mantle outer layer; *bar* 9 µm

Fig. 11 Colourless emanating hyphae with crystals; *bar* 13 µm

Fig. 12 Colourless and septate simple cystidia; *bar* 20 µm

cies, the aim of the present work was to determine the accuracy of the morphological descriptions so far reported for *T. magnatum* mycorrhizae.

We report for the first time a morphological description of *T. magnatum* mycorrhizae supported by molecular marker characterization. The molecular approach provides a safe basis for assessing morphological traits specific to *T. magnatum* symbiotic structures. The greyish colour described here for young actively growing mycorrhizae has not been reported before either for *T. magnatum* or for any other truffle-forming mycorrhizal species. More important, the emanating elements observed on our symbiotic root tips showed features completely different to those reported in other studies. None of our mycorrhizal samples displayed the spinule-like cystidia previously described in *T. magnatum* mycorrhizae (Fontana et al. 1990; Zambonelli et al. 1993; Granetti 1995). Interestingly, the cystidia observed in this study showed a relatively uniform diameter along their entire length, were pluriseptate with rounded ends (Fig. 12) and reached up to 390 µm in length. In contrast, the spinulae described by Fontana, Zambonelli and Granetti were needle-like, not septate, or had exceptionally 1–2 septa, and did not exceed 120 µm in length.

Cystidia are frequently ramified (Fig. 9), which presents a new morphological marker to clearly differentiate *T. magnatum* from *T. borchii* mycorrhizae. *T. borchii* always displays simple cystidia with generally very thick walls (Granetti 1995; Rauscher et al. 1996). Like *T. magnatum*, *T. melanosporum* mycorrhizae show ramified cystidia (Giraud 1979; Zambonelli et al. 1993, 1995; Rauscher and Chevalier 1995; Rauscher et al. 1995; Granetti 1995), but the cystidia of the two species differ in colour, being colourless and yellow-amber, respectively. In addition to differing in colour for both cystidia and the entire mycorrhiza, these two species may display quite a different pattern of surface cell mantle, which is arranged as a puzzle with more sinuous cells in *T. melanosporum* than in *T. magnatum.*

It has emerged from the present study that only the simultaneous presence of simple and/or ramified cystidia and of a puzzle-like pattern of the mycoclena, in addition to the grey colour of the actively growing mycorrhizae, allows the reliable typing of *T. magnatum* symbiotic structures. Conversely, and in contrast to many other reports, not all of the morphological structures so far proposed to be specific to *T. magnatum* were observed in our samples. This is further evidence for inappropriate or misleading morphological descriptions of these structures (Mello et al. 1999b). As a consequence, it is more than likely that mycorrhizae ascribed to *T. magnatum* based on the previously described parameters (Fontana et al. 1990; Zambonelli et al. 1993; Granetti et al. 1995) belong instead to the less valuable *T. borchii* or to other similar white truffle species such as *T. maculatum* (Zambonelli et al. 1999).

The paucity of morphological traits and the influence on them of ageing of host plant species or of particular edaphic conditions make them particularly unreliable for differentiating truffle mycorrhizae throughout the life cycle. Irrespective of the truffle species under investigation, only molecular analysis can ensure reliable identification of the fungal species responsible for host root tip colonization and it is highly recommended for monitoring nursery-inoculated host plants in order to improve truffle cultivation.

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