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ORIGINAL PAPER

The AD-type ectomycorrhizas, one of the most common morphotypes present in truffle fields, result from fungi belonging to the *Trichophaea woolhopeia* species complex

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Abstract Belowground ectomycorrhizal communities are often species rich. Characterization of the ectomycorrhizas (ECMs) underneath native truffle areas and/or cultivation sites is particularly relevant to identifying fungal species that might interfere with or promote truffle propagation and fruiting. Fungal identification at the genus/species level can now be achieved by combining detailed morphological and anatomical descriptions with molecular approaches. In a survey of the mycorrhizal biodiversity of *Tuber melanosporum* orchards and inoculated host plants in nurseries, we repeatedly sampled ECMs with morphological features resembling those of the ECMs widely known as the AD type. Despite the fact that the AD type is regarded as one of the most competitive fungal species towards *Tuber* spp., its taxonomical rank has yet to be resolved. By analyzing the 28S and internal transcribed spacer (ITS) rDNA regions, here, we show that AD-type ECMs result from host plant colonization by the pyronemataceous species *Trichophaea woolhopeia*. Further to this, the 28S and ITS phylogenetic trees built from the AD-type ECMs analyzed sustain the hypothesis that *T. woolhopeia* is a species complex.

Keywords Ectomycorrhiza · *Tuber* · Molecular identification · LSU · ITS · *Trichophaea woolhopeia*

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Introduction

The production of truffles, the edible ascomata produced by some *Tuber* spp., results from a complex interplay between these symbiotic fungal species and their host plants. However, a largely uncharacterized network of belowground interactions between these fungi and many other microbial species, which may interact antagonistically or synergistically within the same habitats, appears to play a pivotal role in controlling the dynamics of *Tuber* spp. vegetative growth and fructification. Studies on fungal species richness and distribution underneath truffle plantations relied mainly on accurate morphological analyses of ectomycorrhizas (ECMs) and/or on in vitro cultivation of saprotrophic organisms (Luppi 1972; Luppi and Fontana 1977; Donnini and Bencivenga 1995; Granetti and Angelini 1992; Granetti and Baciarelli Falini 1997).

The recent advent of molecular techniques for genotyping soil-living organisms and even single mycorrhizal root tips, combined with improved high-throughput sequencing techniques and the development of DNA databases that store increasing numbers of phylogenetically informative nucleotide sequences, has greatly improved the chances of assigning a species name to unculturable organisms or ectomycorrhizal structures (Horton and Bruns 2001; Buée et al. 2009). The identification of ECMs from Ascomycetes

remains problematic at both the genus and species levels since they exhibit only a few diagnostic morphological traits. Moreover, unlike Basidiomycetes, tracking the hyphal strands that connect each fruiting body to its relative ECM is difficult in Ascomycetes. In addition, many Ascomycetes produce hypogeous and inconspicuous fruit bodies which can be easily overlooked (Agerer 1991, 2001). AD-type ECM, named from the initials of the French words "angles droits," meaning right angle, in reference to the characteristic hyphae having perpendicular ramifications, was originally described by Giraud (1988). The very distinctive morphological characteristics of the emanating hyphae and the cells of the mantle surface, which have a perfect polygonal shape, make the identification of this ectomycorrhizal morphotype fairly simple, and the occurrence of the AD morphotype on different host plants has been reported by many authors. In particular, the AD morphotype has frequently been found in natural and manmade *Tuber melanosporum* truffle grounds (Giraud 1988; Donnini and Bencivenga 1995; Mamoun and Oliver 1997; Sourzat et al. 1999; De Miguel and Sáez 2005; Baciarelli Falini et al. 2006). The AD-type ECM can coexist with *T. melanosporum* without preventing truffle production over a short-term period. However, this species has been described as highly competitive with respect to *T. melanosporum* and able to form a "brulé," an area devoid of vegetation around host plants, such as those colonized by *T. melanosporum* and *Tuber aestivum* (Giraud 1988). Therefore, the presence of this ectomycorrhizal species in natural and manmade truffle grounds could represent a risk for the long-term spreading and survival of *T. melanosporum* mycorrhizas on host trees. However, whether this species can influence the formation of truffle ascocarps is currently unknown. Even though the AD type has been regarded by mycologists as one of the most common ectomycorrhizal morphotype associated with *T. melanosporum* orchards, its taxonomical and phylogenetic rank has remained largely unresolved up to now. Using internal transcribed spacer (ITS) markers, our group has previously genotyped ECMs sampled from a *T. melanosporum* plantation located in central Italy and showed the presence within the ECMs exhibiting the AD morphotype of two molecular types, namely, ECMm7 and ECMm8, whose sequences featured Sarcosomataceae and Pyronemataceae, respectively (Baciarelli Falini et al. 2006). More recently, Agueda et al. (2008a, b) described "*Quercirhiza quadratum*" as an ECM belonging to the AD type and reported that ECMs with this morphotype showed high similarity with either ECMm7 or ECMm8 previously described Baciarelli Falini et al. (2006). It is worth of noting that the "*Quercirhiza quadratum*" ECM that showed, on the 84% of the query coverage, 100% identity with the ITS from ECMm8 also showed similarity, to the same extent, to the ITS from *Trichophaea woolhopeia* (Cooke and W. Phillips) Arnould (Agueda et al. 2008a, b).

To gain more insight into the taxonomical rank of AD-type ECM, here, we undertook a detailed morphological and molecular characterization, by means of 28S and ITS rDNA sequence analyses, of AD-type mycorrhizas collected from different environmental conditions, including natural and manmade truffle fields and nursery host plants inoculated with *T. melanosporum.* We show that this ectomycorrhizal morphotype results from the colonization of host plants by the ascomycete *T. woolhopeia.* ITS and 28S rDNA phylogenetic trees converge to support the hypothesis that *T. woolhopeia* is indeed a species complex.

Materials and methods

Sample source

Mycorrhizal samples were collected from natural and manmade *T. melanosporum* truffle grounds located in Umbria and Lazio (central Italy), as well as from nursery plants inoculated with *T. melanosporum.* Root samples were collected from *Quercus pubescens*, *Ostrya carpinifolia*, and *Corylus avellana* plants. Information relative to host plants, ECMs collection sites, and dates are given in Table 1.

All root samples were stored at 4°C before processing. Each root sample was individually soaked in tap water and sieved to separate root fragments and ECMs from the soil. Analysis of mycorrhizal morphotypes was performed using a stereomicroscope and a light microscope. Mycorrhizas belonging to the AD morphotype were collected, stored in 95% ethanol, and preserved at the CNR, Plant Genetics Institute, Perugia Division. Single mycorrhizal root tips were also stored at −80°C and used for molecular analysis.

Morphological analyses

Morphological analysis was performed according to Agerer (1986). Photographs of freshly isolated ECMs were taken using a dissecting microscope. Features of the mantle surface and emanating elements were examined on mycorrhizae mounted in 50% glycerol using a Nomarski interference contrast microscope. Measure of ECM, outer mantle layer cells, and diameter of cystidia were performed on ten ECMs for each sample.

DNA isolation and amplification of rDNA

Genomic DNA was isolated from single mycorrhizal root tips as described by Paolocci et al. (1999). The ITS region

Table 1 List of samples used in this study

Host plants	Collection sites	Collection date	ECM sample	GenBank number	
				ITS	LSU
Q. pubescens	Montemartano, Spoleto (c)	September 2007	AD1	GU174760	GU174771
Q. pubescens	Montemartano, Spoleto (c)	September 2007	AD3	GU174761	GU174772
Q. pubescens	Montemartano, Spoleto (c)	June 2007	AD2	GU174762	GU174773
Q. pubescens	Vallunga, Leonessa, Rieti (c)	November 2007	AD5	GU174764	GU174769
Q. pubescens	Villa Gizzi, Leonessa, Rieti (n)	November 2007	AD4	GU174763	GU174770
Q. pubescens	*T. melanosporum* inoculated plant (g)	October 2007	AD6	GU174765	GU174767
Q. pubescens	*T. melanosporum* inoculated plant (g)	October 2007	AD7	GU174766	GU174768
O. carpinifolia	Fosso, Spoleto (n)	February 2010	245a^{+}	GU811252	GU811243
			245b^{+}	GU811251	GU811242
O. carpinifolia	Borgo Cerreto, Spoleto (n)	February 2010	247b	GU811253	GU811244
Q. pubescens	Borgo Cerreto, Spoleto (n)	February 2010	242b	GU811248	GU811241
C. avellana	Torre Matigge, Trevi (c)	February 2010	G81^{++}	GU811246	GU811236
			G82^{++}	GU811245	GU811237
			G83^{++}	GU811247	GU811238
C. avellana	Torre Matigge, Trevi (c)	February 2010	B111^{+++}	GU811249	GU811239
			B112^{+++}	GU811250	GU811240

c cultivated truffle ground, *n* natural truffle ground, *g* greenhouse

$^{+}$, $^{++}$, and $^{+++}$ indicate samples collected from the same host plant

was amplified with the ITS1/ITS4 primers pair (White et al. 1990). The 5′ end of the large subunit (LSU) rDNA region was amplified using the LR0R/LR7 primers pair (Moncalvo et al. 2000; Tedersoo et al. 2006). Polymerase chain reaction (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 (ITS) or 2 min (LSU) at 72°C, and a final extension for 7 min at 72°C. All PCR amplifications were performed in a 50-μl reaction mixture containing 200 μM 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 (GE Healthcare) supplemented with 0.35 μg of 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).

Sequencing and phylogenetic analyses

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 3130 Genetic Analyzer (Applied Biosystems), and the sequencing primers ITS1, ITS4 (White et al. 1990), LR0R, LR7, LR3R, and LR6 were used (Moncalvo et al. 2000). The sequences are deposited in GenBank, and the accession numbers are given in Table 1.

The ITS and LSU sequences were checked for similarity using the basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/). The sequences showing the highest similarity were retrieved from GenBank and aligned using Muscle V. 3.7 (Edgar 2004).

Phylogenetic trees were inferred with neighbor-joining (NJ), maximum parsimony (MP), and maximum-likelihood (ML) methods. NJ and MP analyses were performed using MEGA v. 4.0 (Kumar et al. 2001). Genetic distances for NJ analysis (Saitou and Nei 1987) were calculated using either the Kimura's two-parameter or *p* distances (Kimura 1980; Nei and Kumar 2000). MP trees were generated using the close neighbor interchange algorithm with search level 1 and the random addition trees option (ten replications). ML analysis was performed with PhyMl software v. 2.4.4 (Guindon and Gascuel 2003). The model of sequence evolution was determined using Modeltest v. 3.7 (Posada and Crandal 1998). The general-time reversible model was used, and transition/transversion substitutions rate, percentage of invariable sites, and Gamma distribution parameter were estimated from the dataset. In all analyses, the gaps were excluded, and bootstrap test was always performed using 1,000 replicates.

Results

Morphological analysis

In a survey of ectomycorrhizal biodiversity underneath natural and artificial *T. melanosporum* truffle plantations located in central Italy, AD-type ECMs were frequently recovered (Baciarelli Falini et al. 2006, Rubini et al., manuscript in preparation). Similarly, AD-type mycorrhizas were observed and collected from host plants artificially inoculated with *T. melanosporum* and grown in a nursery. Detailed morphological analyses were carried out on 16 AD-type samples, reported in Table 1. All the mycorrhizal samples were brown, monopodial pinnate, straight and simple, or scarcely ramified and appeared to be woolly due to the presence of abundant, rather thick, emanating hyphae (Fig. 1a, b). The mycorrhizas were 2–4.5-mm long, with a diameter ranging from 0.15 to 0.4 mm. The outer surface of the mantle showed polygonal cells (Fig. 2c) with an average size ranging from 9.66±0.45×6.28±0.39 to 12.76 ±0.48×9.41±0.67 μm in the apex and from 8.93±0.35× 6.88±0.37 to 12.82±0.61×8.99±0.5 μm in the middle. The mantle showed a pseudoparenchymatic structure consisting of four to five layers of flattened hyphae (Fig. 1d).

The emanating hyphae were pale yellow, slightly bent, thick-walled, and frequently ramified with perpendicularly oriented branches (Fig. 1e). Often, they exhibited a grainy surface for the presence of small crystals in the outer layer of the wall (Fig. 1f). Emanating hyphae with crystals were rarely observed in samples AD6, AD7, 242b, 247b, G83, B111, and B112. The hyphae showed simple septa without clamps (Fig. 1e) and an average diameter ranging from 3.51±0.13 to 4.62±0.36 μm.

Overall, all of the ECMs analyzed showed the diagnostic traits specific to the AD type described by Giraud (1988).

Analysis of LSU and ITS rDNA

LSU analysis

In order to gain insight into the taxonomical rank of ECMs that exhibit the AD morphotype, DNA was isolated from single apical root tips from each of the 16 samples reported in Table 1 and amplified by PCR using the LR0R/LR7 primer pair specific to LSU fungal rDNA gene.

All samples produced an amplicon of about 1,250 bp. Multiple sequence alignment showed that the 16 samples can be sorted in three groups. Among the samples of group 1 (AD1-AD5, G81, G82, and G83), the LSU sequence from AD5 ECM was the most divergent, since it exhibited five single nucleotide polymorphisms (SNPs) with respect to the other six that showed nearly identical sequences. Samples from the second group (AD6, AD7, 245a, 245b, and 247b) had a nearly identical LSU gene with only two SNPs. These sequences differed from those of samples clustered into group 1 for a 3-bp long indel and 12 SNPs. The remaining three samples, 242b, B111, and B112 (group 3), exhibited identical LSU sequences that differed from those of groups 1 and 2, for the presence of 53 SNPs and four short indels (1–5 bp) and for the presence of 34 SNPs and four indels (1–5 bp), respectively.

In Supplementary Fig. S1, the alignment of LSU sequences from all AD samples investigated is reported.

When BLASTed against the GenBank database, all 28S sequences produced the highest scores with LSU sequences from Pyronemataceae (e.g., *Antrachobia* sp. AY544660; *Aleuria aurantia* AY544654; *Cheilymenia stercorea* AY544661; *Scutellinia scutellata* DQ247806; *T. woolhopeia* DQ220458, DQ220459, and DQ220460; *Trichophaea abundans* DQ220450; *Trichophaea minuta* DQ220452); and many other Pezizales.

ITS analysis

The universal primer pair ITS1/ITS4 was employed to amplify and align the ITS regions of the AD samples. This primer pair produced amplicons of about 580 and 510 bp from samples AD6, AD7, 245a, 245b, and 247b and from samples AD1-AD5, G81, G82, G83, 242b, B111, and B112, respectively (data not shown).

Direct sequence analysis of the ITS amplicons showed that the ECMs could be sorted in the same three groups defined according to the LSU sequences (see above). The sequences within group 1 had nearly identical sequences, with the exception of sample AD5 that slightly differed from the others (97% identity). The sequences from group 2 differed from those of group 1 for the presence of small indels (1–2 bp), SNPs, and a large insertion of 65 bp at the 3′ end of the ITS2 region. Within this group, the sequences showed 99% identity. The identity between group 1 and group 2 ITS sequences was about 82%.

Within group 3, three sequences, B111, B112, and 242b, were present; the first two were identical, whereas the third one showed 98% identity with the others. Group 3 sequences were highly divergent from all the others. The alignment of ITS sequences from all AD-type samples obtained in this study as well as from related sequences retrieved from GenBank is reported in the Supplementary Fig. S2.

BLAST searches on GenBank database showed that group 1 samples produced the best matches (99–100% sequence identity) with the ITS sequence DQ200835 from *T. woolhopeia* fruiting body (Tedersoo et al. 2006) and with ECMm8 (DQ402507) and EU822505, which have been described as AD-type ECMs by Baciarelli Falini et al. (2006) and Agueda et al. (2008a), respectively. BLAST

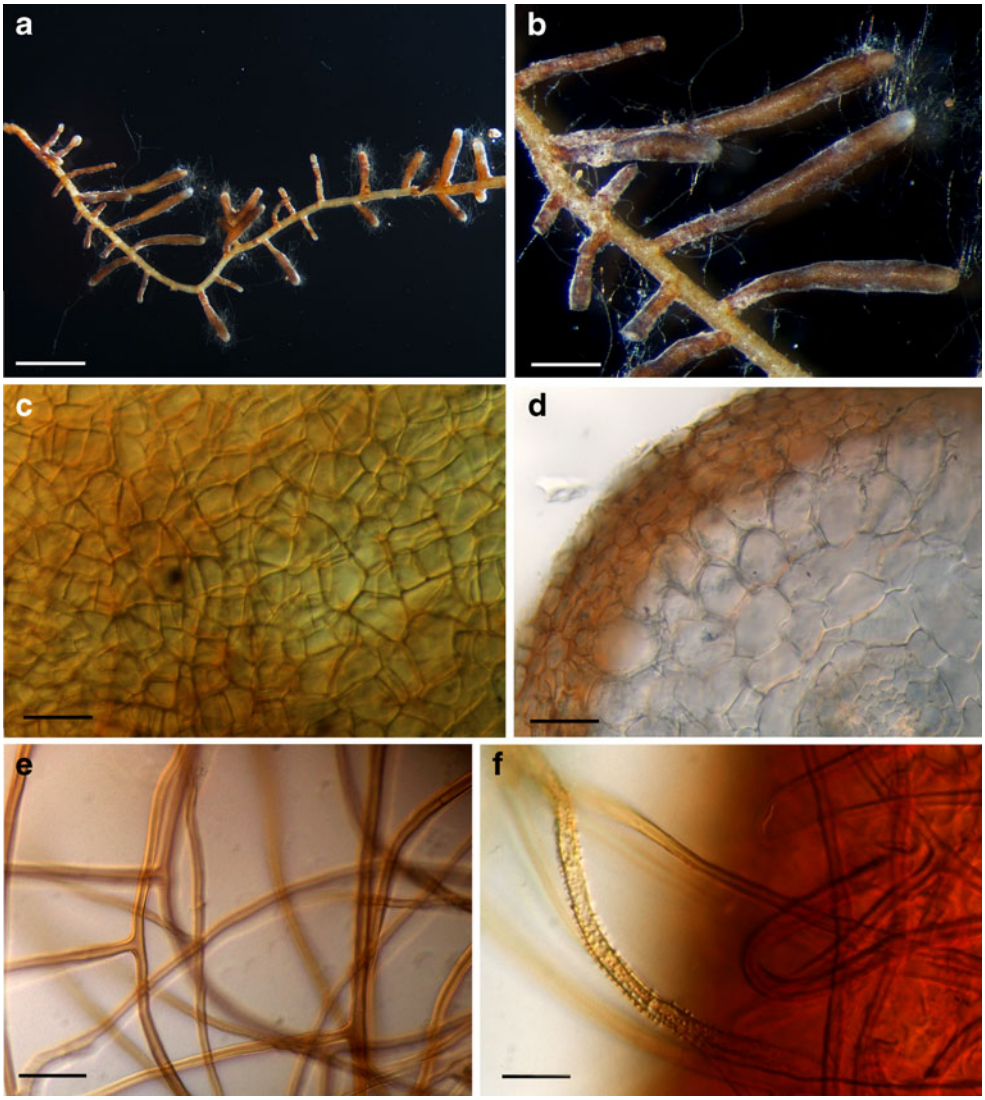

Fig. 1 Morphological characteristics of *Trichophaea woolhopeia* ectomycorrhizas. **a**, **b** Morphology of mycorrhizal tips with emanating hyphae (**a**: bar=2.3 mm; **b**: bar=720 μm). **c** Details of the outer mantle layer with polygonal-shaped cells (bar=20 μm). **d** Cross-section showing pseudoparenchymatous mantle and Hartig net (bar=30 μm). **e** Emanating hyphae with septa and ramifications (bar=35 μm). **f** Details of granulations in the emanating hyphae (bar=25 μm)

searches using the ITS sequences from samples of group 2 as query produced similar results: six of the top 12 scoring BLAST matches were Pyronemataceae and among these, *T. woolhopeia* (DQ200835) showed 94% sequence identity with the query sequence. The group 3 samples showed high similarity (98–100% sequence identity) with ECMm7 (DQ402506) described by Baciarelli Falini et al. (2006) and many other Pyronemataceae, Sarcosomataceae, and Pezizales. Notably, the majority of the ectomycorrhizal sequences deposited in GenBank that showed similarity with the query sequences were labeled as "uncultured ectomycorrhiza" or "uncultured fungi."

Phylogenetic analysis

Both ITS and LSU sequence analyses suggested that ECMs showing the AD morphotype were formed by species belonging to Pyronemataceae. To better understand the taxonomic position of our samples within this ascomycete family, a phylogenetic analysis based on LSU rDNA sequences was then performed. The LSU sequences of Pyronemataceae were retrieved from GenBank according to the BLAST analysis results. The LSU sequences of *Helvella leucomelaena* (AJ972414; Helvellaceae) and *Galiella rufa* (AY789368; Sarcosomataceae) were also included and considered as outgroups.

A phylogenetic analysis based on LSU region was performed following three different methods: MP, NJ, and ML (see "Materials and methods"). Parsimony analysis (Fig. 2) produced seven equally most parsimonious trees (tree length=993, CI=0.469, RI=0.737) with a well-resolved bootstrap consensus tree. The ML (−lnL=6,523.32248) and NJ trees are shown in Supplementary Figs. S3 and S4, respectively.

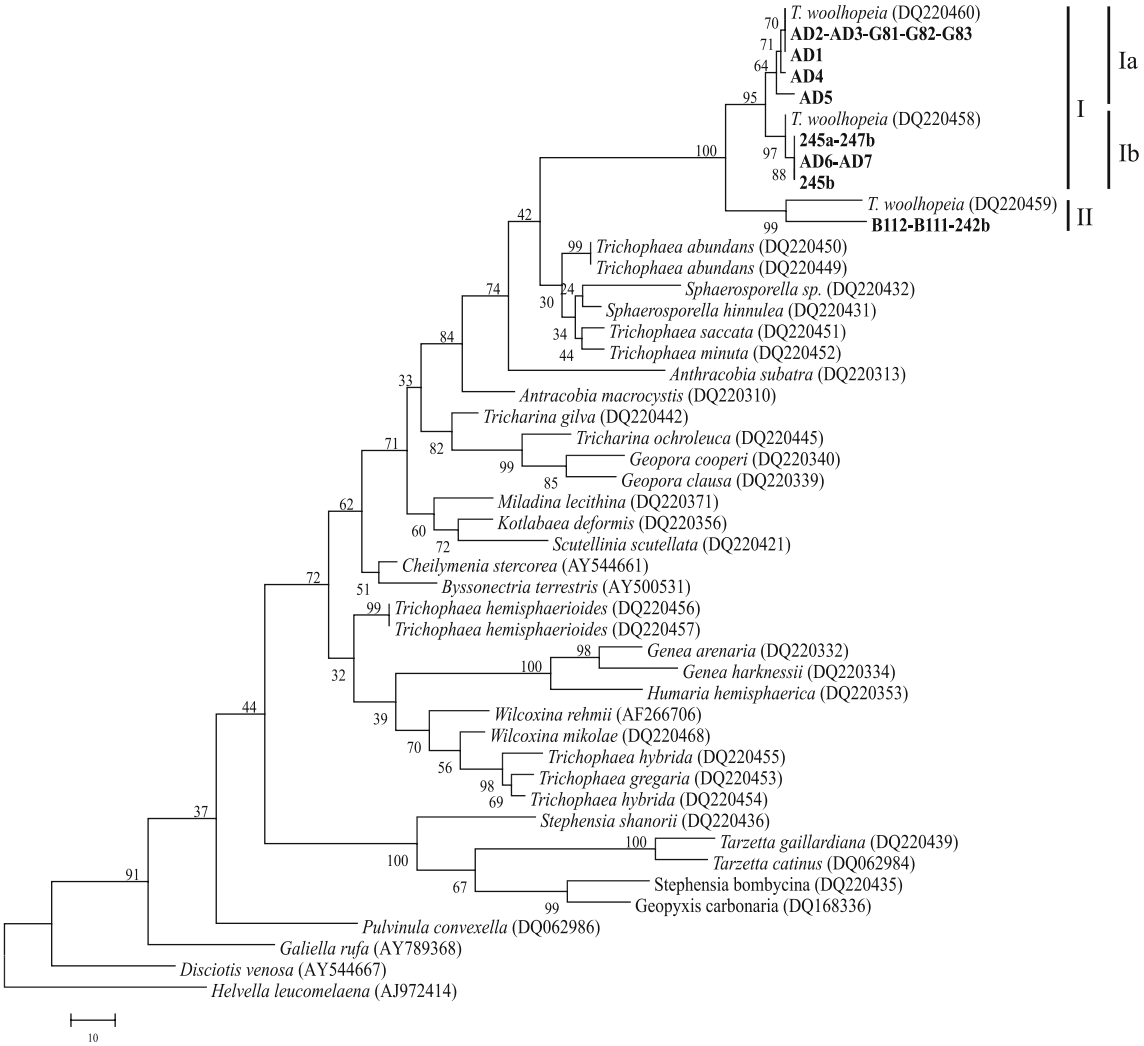

Fig. 2 One of the seven most parsimonious trees based on large subunit (LSU) sequences showing the relatedness of the AD-type ectomycorrhizas with *Trichophaea woolhopeia* and other Pyronemataceae retrieved from the GenBank database. *T. woolhopeia* LSU sequences DQ2204658, DQ2204659, and DQ2204660 were derived from Perry et al. (2007). Numbers near the branches indicate the bootstrap values (percentage of 1,000 replicates). Scale bar indicates the number of substitutions/site

The LSU-based phylogenetic trees obtained with different methods showed very similar topologies. More specifically, the trees showed that all AD-type samples form a highly supported cluster (100% of bootstrap replicates) with the sequences DQ220458, DQ220459, and DQ220460 from *T. woolhopeia* ascocarps (Perry et al. 2007). In addition to this, the phylogenetic trees brought to light that the all the LSU sequences from the AD samples as well as those from *T. woolhopeia* ascocarps present in public databases could be sorted into two main clades: I and II (Fig. 2). Within clade I, emerged the presence of two subclades (LSU Ia and LSU Ib) made up of samples AD1-AD5, G81-G83, and of samples AD6, AD7, 245a, 245b, and 247b, respectively.

NJ and MP trees based on all the ITS sequences from AD-type ECMs from this and previous studies (Baciarelli Falini et al. 2006; Agueda et al. 2008a) as well as from the single *T. woolhopeia* ITS sequence present in public databases were then constructed.

Irrespective of the distance model used, the topology of the NJ ITS tree showed the presence of two mains clades (ITS I and ITS II) with two subclades each (ITS Ia and ITS Ib; ITS IIa and ITS IIb). The LSU and ITS trees for all the samples from this study nicely overlapped, as such samples AD1-AD5 and G81-G83, samples AD6, AD7, 245a, 245b, and 247b, and samples 242b, B111, and B112 were grouped in subclades Ia, Ib, and clades II, respectively, in both LSU and ITS-based trees (Figs. 2 and 3).

As far as it concerns AD samples for which only the ITS sequence information is provided, Fig. 3 shows that the ITS from ECMm8 (DQ402507, Baciarelli Falini et al. 2006) and from a "*Quercirhiza quadratum*" sample (EU822505,

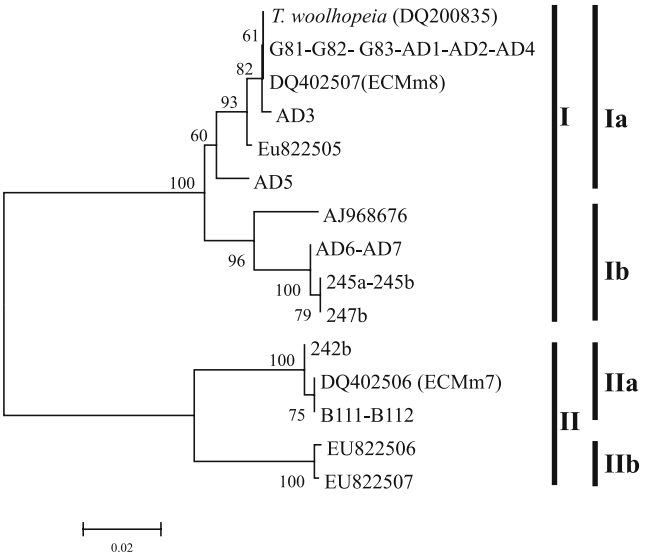

Fig. 3 Midpoint rooted neighbor-joining tree based on internal transcribed spacer sequences showing the clustering of AD-type ectomycorrhizas and their relatedness with similar sequences retrieved from GenBank. Numbers near the branches indicate the bootstrap values (percentage of 1,000 replicates). Scale bar indicates the number of substitutions/site

Agueda et al. 2008a) clustered with group Ia, whereas ECMm7 (DQ402506, Baciarelli Falini et al. 2006) with group II. Additionally, this analysis showed that the "*Quercirhiza quadratum*" samples recorded under the accession numbers EU822506 and EU852507 (Agueda et al. 2008a) clustered apart from all the others within clade II, to form the subclade ITS IIb (Fig. 3). The same sample clustering was obtained with MP analysis (data not shown).

Altogether, the parallel sequencing analysis of the LSU and ITS genomic regions from all the ECM samples considered in this study converged to show that AD-type ECM results from host plant colonization by fungi belonging to the *T. woolhopeia* species complex.

Discussion

The AD-type ECM is one of the most common ECMs associated with truffle plantations. The AD-type species identity however has not yet been clarified. As such, it has been previously reported that AD-type ECMs recovered both in Spanish and Italian truffle plantations were ascribed to Pyronemataceae but also to Sarcosomataceae according to BLAST analysis of ITS sequences (Baciarelli Falini et al. 2006; Agueda et al. 2008a).

To get insight into the taxonomical position of AD-type ECMs, a combined approach based on morphological and molecular typing of ECMs collected from various environmental sites was undertaken. Molecular characterization of AD-type ECMs was initially based on LSU rDNA region, as this is the most widely used marker in field studies of ectomycorrhizal ascomycetes, at least for the Pyronemataceae, and the most complete phylogenies in these fungi are based on this gene (Tedersoo et al. 2006; Perry et al. 2007). Given the higher rate of inter- and intraspecific variability of the ITS region and the lower number of ITS sequences from fungi belonging to the Pyronemataceae and Sarcosomataceae deposited in public databases with respect to the LSU rDNA, the ITS region has been considered in this study as an additional marker to provide evidence for species identification. Following this approach, we show here that AD-type ECMs result from the colonization of host root plants by fungal *taxa* belonging to the *T. woolhopeia* species complex.

Monitoring the microbial biodiversity underneath truffle orchards helps mycologists understand the determinants of the ability of *Tuber* spp. to colonize a given habitat, establish a mycelial network on the root apparatus of host plants as well as in the soil, and eventually fruit. In a survey aimed at comparing the fungal species composition and richness between natural and manmade truffle fields as well as between productive and unproductive truffle plantations, we repeatedly sampled ECMs of the AD type originally described by Giraud (1988). The presence of diagnostic elements such as emanating hyphae with perpendicular ramifications and the polygonal shape of the outer layer mantle cells indicated that the samples were in fact the AD morphotype. Overall, our morphological and molecular analyses were performed on 16 AD-like ectomycorrhizal samples recovered either from natural or manmade truffle orchards or from host plants artificially inoculated with *T. melanosporum* and grown in a nursery. Some samples (G81, G82, G83, B111, and B112) were recovered from the same *T. melanosporum* orchard (Torre Matigge di Trevi) previously investigated by our group (Baciarelli Falini et al. 2006) in order to monitor the distribution of this ECM over the time and deepen the information on the biodiversity of these fungal species by sequencing the LSU locus in addition to the ITS region analyzed initially (Baciarelli Falini et al. 2006).

Only a subtle morphological difference emerged when all the ECMs were compared, with samples AD6, AD7, 247b, 242b, G83, B111, and B112 showing less abundance of cystidia with accumulation of crystals.

When the LSU region was analyzed, all samples showed a high level of sequence similarity and the highest BLAST scores with Pyronemataceae. The phylogenetic tree resulting from the alignment of LSU sequences relative to Pyronemataceae unequivocally places all of the AD-type samples within a highly supported (100% bootstrap value) cluster with sequences from *T. woolhopeia* fruiting bodies reported by Perry et al. (2007). BLAST and phylogenetic

analyses based on ITS region also prove a close relatedness of these sequences with two *T. woolhopeia* sequences, one from fruit body, the other from ECM (Tedersoo et al. 2006).

In addition to this, Tedersoo et al. (2006) provided a brief description of *T. woolhopeia* ECMs that overlaps well with that reported by Giraud for the AD type and with the morphotype exhibited by the AD-like ECMs sampled in this study.

It is worth of mentioning that LSU sequences from samples B111, B112, and 242b that share nearly identical ITS sequence with ECMm7 also clustered with *T. woolhopeia*. Thus, the original attribution of ECMm7 to Sarcosomataceae has to be interpreted as a misleading result due to the scarce representation, in public sequence databases at the time that the BLAST analysis was performed (Baciarelli Falini et al. 2006), of ITS sequences from phylogenetically close species. In the present study by amplifying from the same ectomycorrhizal samples both the LSU and the ITS regions, we have overtaken such a limitation to eventually attach the species name to the AD morphotype. To rule out the hypothesis that the assignment of our samples to *T. woolhopeia* was biased by the small number of informative polymorphic sites in a generally highly conserved genomic region such as the LSU, we have analyzed the ITS region from all the AD-type ECMs sampled. In agreement with the LSU analysis, all AD-type samples show a remarkably high level of similarity to the ITS from *T. woolhopeia* fruiting body. In addition to this, it has to be pointed out that both LSU and ITS-based phylograms depict the presence of at least two main clades (I and II) within the AD-type ECMs and, in turn, within the *T. woolhopeia* species. On the basis of LSU and ITS loci, clade I can be also subdivided into two highly supported subclades (Ia and Ib). The lack of LSU information relative to AD samples under the accession numbers EU822506 and EU822507 that form the ITS subclade IIb prevents us to test the hypothesis that two subclades can also be distinguished within clade II of the 28S gene.

The high rate of ITS polymorphisms is an expected result for a species collected all over Europe (Baciarelli Falini et al. 2006; Tedersoo et al. 2006; Perry et al. 2007; Agueda et al. 2008a). Further to this, in several fungal molecular ecology studies, ITS sequences have been considered to belong to different species if they differed in 3% or more of the base pairs across the ITS1/5.8S/ITS2 region (Tedersoo et al. 2003; Izzo et al. 2005; Smith et al. 2007; Peay et al. 2008). By using this cut off level, it can be concluded that AD-type and "*Quercirhiza quadratum*" ECMs should be assigned to different species within the *T. woolhopeia* species complex. Better still, here, we show the presence of at least four well-resolved lineages within this species complex that likely represent cryptic species. Further work will be needed to elucidate the taxonomy within this complex.

Notably, the multiple sampling within a given truffle field let us to conclude that both within single manmade (Torre Matigge di Trevi) and natural truffle (Borgo Cerreto) fields, different AD phylotypes can coexist.

In conclusion, based on parallel morphological and molecular screening of ECMs, we have resolved the identity of one of the potentially most aggressive fungal competitors of *Tuber* spp. for host plant colonization.

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