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A quantitative and molecular examination of *Tuber melanosporum* mycorrhizae in *Quercus ilex* seedlings from different suppliers in Spain

P. Alvarado* and J. L. Manjon

Departamento de Biología Vegetal. Facultad de Biología. Universidad de Alcalá.
28771 Alcalá de Henares (Madrid), Spain

Abstract

Aim of study: The aim of the work was to determine the degree of mycorrhization of *Quercus ilex* L. subsp. *ballota* (Desf.) Samp. by the black truffle fungus *T. melanosporum* Vittad. by quantitative and molecular analyses.

Area of study: seedlings inoculated by different Spanish suppliers.

Material and methods: The internal transcribed spacers (ITS) of mycorrhizae from different plants were amplified by nested PCR involving fluorescently-labelled primers, and the amplicons either directly sized by ARISA or analysed by TRFLP following their digestion with restriction endonucleases. TRFLP analysis distinguished between mycorrhizae of *T. melanosporum*, *T. indicum* Cooke & Massee and *T. borchii* Vittad., as suggested possible by virtual (*in silico*) TRFLP analysis and real TRFLP analysis of the ascomata of these species.

Main results: Significant differences between suppliers were detected in terms of the mean number of mycorrhizae established per plant and percentage mycorrhization. These results allowed the following quality standards for 2 year-old plants to be proposed: a) good quality: > 3,000 mycorrhizae/plant, > 40% mycorrhization, b) medium (acceptable): > 3,000 mycorrhizae/plant, > 30% mycorrhization, c) low quality: < 3,000 mycorrhizae/plant or <30% mycorrhization, always supposing the mycorrhizae counted represent the species of interest as confirmed by the presence of its DNA and the absence of DNA belonging to contaminating species. Finally, a new microsatellite allelic map obtained from the analysis of several *T. melanosporum* populations across Spain was used to provide a tool capable of determining the geographic origin of the fungi used to inoculate plants.

Research highlights: The proposed quality standards can be useful for the evaluation and certification of commercialized *Q. ilex* plants mycorrhized with *T. melanosporum*.

Key words: mycorrhiza; certification; truffle; TRFLP; fungi.

Introduction

Over the last century, ectomycorrhizal plants have been produced in tree nurseries for purposes such as the production of edible fungi and improving the outcome of afforestation programs. Independent evaluation of the mycorrhization methods used by such nurseries, and of the mycorrhizal quality of their products, is in the interests of both producers and customers (Vosátka, 2009). The Spanish authorities have increasingly been asking for the control of the quality of mycorrhized plants before granting funds for new plantations, especially truffle orchards. In fact, this has been of academic concern since the 1st International Truffle Congress (Spoleto, Italy, 1968) through to the recent ICOM6 (Belo Horizonte, Brazil, 2009). Several protocols for evaluating the mycorrhizal quality of plants exist (Bencivenga *et al*., 1987; Abourouh, 1994; Fischer and Colinas, 1996; Miko and Gažo, 2007), but these are not strictly followed. Those charged with quality control largely rely on personal adaptations of these methods (Bach *et al*., 2010) or even completely different methods.

A reproducible and inexpensive method for evaluating the mycorrhizal quality of plants is particularly

* Corresponding author: pablo.alvarado@gmail.com
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Abbreviations used: ARISA: automated ribosomal intergenic spacer analysis; TRFLP: terminal restriction fragment length polymorphisms; RTF: root tip fraction.

necessary when dealing with highly prized edible fungal species, such as those of the genus *Tuber*. The presence of morphologically similar species (*e.g., Tuber melanosporum* and *Tuber indicum*) in nursery seedlings could lead to the involuntary introduction of undesired taxa and even the displacement of local varieties (Murat *et al*., 2008; Bonito *et al*., 2011). Further, the mycorrhizal status of nursery plants directly affects the growth of the fungus after planting, the extent of the symbiotic association achieved (Garbaye and Wilhelm, 1984), and probably the onset of truffle production. Low rates of mycorrhization might also increase the chances of contamination as the local mycobiota take advantage of free niches. Finally, the geographical origin of the fungal strain inoculated into commercial plants is important if the success of plantations is to be assured (Zambonelli *et al*., 2010).

The methods currently used for the evaluation of mycorrhizal quality generally involve three main steps. First, some sort of root system cleaning is performed (Abourouh, 1994). This is followed by the sampling of different mycorrhizal tips, and finally by fungal identification (commonly performed under a stereomicroscope or even using the naked eye). However, the morphological typing of mycorrhizae is time-consuming and requires training and experience (Rinaldi *et al*., 2008; Suz *et al*., 2008a), and even then mistakes can be made, *e.g.,* strains of the same species can appear different and lead to misidentifications (Giomaro *et al*., 2000). Moreover, morphological similarities between structures of closely related species could lead to misidentifications (Manjón *et al*., 2009; Bonuso *et al*., 2010). In contrast, DNA-based methods can be easily standardized, require less training, allow for sample-pooling, and provide more accurate identifications than morphotyping. The presence of unwanted mycorrhizal contaminants, *e.g.,* competitors that might displace a desired fungal species*,* can be assessed by PCR targeting each taxon of interest if specific primers are available, or by following a universal mixed-DNA profiling method such as fragment analysis. The latter is based on the sizing of either undigested or endonuclease-digested amplicons labelled with a fluorescent signal. Theoretically, fragment analysis is more informative than PCR with specific primers since it can offer a holistic view of the fungal community without any prior knowledge of the species present. This is especially true if it is combined with sequencing. However, while sequencing methods (*e.g.,* cloning, massive sequencing) are very informative, they are too expensive for use in routine analysis.

The aim of the present work was to determine the degree of mycorrhization of *Quercus ilex* L. subsp. *ballota* (Desf.) Samp. by the black truffle fungus *T. melanosporum* Vittad. by quantitative and molecular analyses in seedlings inoculated by different Spanish suppliers. In addition, a new microsatellite allelic map obtained from the analysis of several *T. melanosporum* populations across Spain was used to characterize the inoculated fungi in terms of geographic origin.

Material and methods

Mycorrhized plants

The experimental plants included 11 *Quercus ilex* subsp. *ballota* seedlings (from a Spanish supplier) inoculated with no mycorrhiza-producing fungus (control, set 1), 103 *Quercus ilex* subsp. *ballota* seedlings inoculated with *T. melanosporum* by different Spanish suppliers (sets 2-11), 10 *Quercus ilex* subsp. *ilex* seedlings inoculated with *T. melanosporum* (set 12), 10 *Quercus ilex* subsp. *ilex* seedlings (from a high quality Italian supplier) inoculated with *T. borchii* (set 13), and 10 *Quercus ilex* subsp. *ballota* seedlings inoculated with *T. indicum* produced *ex professo* at the University of Alcalá (set 14) (Table 1). The different suppliers planted their seedlings in different types of substrate and pot (Table 1) and employed different mycorrhization procedures, although their exact nature was not disclosed to the authors. Note that the plants in some groups were 1 year-old, while those in others were two years old; this allowed the influence of duration of mycorrhization to be examined. The plants were grown in glasshouses under the standard conditions employed by the different suppliers. Note also that the substrate for the plants of set 5 was not sterilized; this allowed the influence of contamination to be more easily seen.

Preparing the root for observation

The entire root system of each plant was cleaned of substrate under gently running tap water, taking care not to break the secondary root system (and recovering the broken parts if this did occur). The root tip fraction (RTF) of each plant was obtained by rubbing the se-

Table 1. Sets of mycorrhized plants studied, and mean (± SD) values reflecting mycorrhizal status

Set number	Supplier	Plants	Age (years)	Substrate	Plant	Fungus	Total number of roots extracted	Mean number of mycorrhizae	% Mycorrhization
1	Supplier 4	11	1	Peat	*Q. ilex* subsp. *ballota*	Uninoculated	14,786.843 ± 9,526.711	575.087 ± 525.561	4.0 ± 3.1
2	Supplier 1	8	2	Melfert	*Q. ilex* subsp. *ballota*	*T. melanosporum*	3,889.201 ± 1,731.758	866.250 ± 447.430	23.8 ± 12.4
3	Supplier 2	17	2	Soil-perlite-vermiculite	*Q. ilex* subsp. *ballota*	*T. melanosporum*	23,100.919 ± 13,164.747	9,913.470 ± 5,198.173	44.5 ± 6.8
4	Supplier 2	16	2	Soil-perlite-vermiculite	*Q. ilex* subsp. *ballota*	*T. melanosporum*	25,876.251 ± 12,886.809	10,959.516 ± 5,635.054	44.2 ± 17.1
5	Supplier 2	14	1	Peat*	*Q. ilex* subsp. *ballota*	*T. melanosporum*	4,312.389 ± 5,201.575	1,344.826 ± 658.871	14.2 ± 17.3
6	Supplier 3	4	2	Peat	*Q. ilex* subsp. *ballota*	*T. melanosporum*	20,601.105 ± 9,412.514	6,261.764 ± 2,363.187	33.3 ± 9.8
7	Supplier 5	10	2	Peat-perlite	*Q. ilex* subsp. *ballota*	*T. melanosporum*	12,224.119 ± 4,079.823	5,222.745 ± 1,865.266	52.1 ± 4.9
8	Supplier 5	10	2	Peat-perlite	*Q. ilex* subsp. *ballota*	*T. melanosporum*	15,092.375 ± 5,197.650	6,803.511 ± 1,801.069	49.7 ± 5.2
9	Supplier 5	10	1	Peat-perlite	*Q. ilex* subsp. *ballota*	*T. melanosporum*	12,542.153 ± 6,869.090	3,913.164 ± 1,801.848	32.6 ± 9.2
10	Supplier 6	10	2	Peat-perlite	*Q. ilex* subsp. *ballota*	*T. melanosporum*	22,732.829 ± 8,589.942	8,152.643 ± 4,303.897	28.5 ± 5.3
11	Supplier 8	4	2	Peat	*Q. ilex* subsp. *ballota*	*T. melanosporum*	16,740.756 ± 10,804.812	3,078.413 ± 1,417.570	22.5 ± 10.3
12	Supplier 7	10	2	Peat-vermiculite	*Q. ilex* subsp. *ilex*	*T. melanosporum*	31,656.644 ± 9,653.25	13,709.523 ± 5,600.469	44.1 ± 12.0
13	Supplier 10	10	2	Soil	*Q. ilex* subsp. *ilex*	*T. borchii*	—	—	—
14	Supplier 9	10	1	Soil-perlite	*Q. ilex* subsp. *ballota*	*T. indicum*	—	—	—

* Substrate not sterilized prior to inoculation. Number of mycorrhizae and number of roots are expressed per plant.

condary root system over a stainless steel sieve of pore size 1 mm. The mycorrhizae were forced through the mesh using a strong tap water stream. The RTF was retained on a 0.2 mm mesh below the 1 mm mesh, and weighed after removing the excess water.

To check the efficiency of RTF extraction, the material that remained on the 1 mm mesh [unsieved fraction (UF)], and that which passed through the 0.2 mm mesh [sedimentary fraction (SF)] was also collected and weighed after removing the excess water from plants in sets 3 and 4. Three samples of random weight (5-100 mg) of the RTF, UF and SF fractions were then taken the number of mycorrhizae counted. The efficiency of RTF extraction was calculated as the relationship between the estimated number of root tips in the RTF over the sum of the root tips in all fractions combined. It proved to be highly efficient, with a mean extraction rate of 70.95 ± 11.13% for the 0.2 mm mesh alone. The UF and SF fractions were therefore not required in ensuing assays.

Number of mycorrhizae established per plant and percentage mycorrhization

Three subsamples of random weight were taken of the RTF of each plant in all sets, the number of mycorrhized and non-mycorrhized roots counted, and the percentage mycorrhization determined (*nº mycorrhized roots/nº roots*). However, since mycorrhiza can differ in size, large mycorrhizae were counted as more than 1, depending on their size compared to the average

mycorrhiza (*i.e.,* a mycorrhiza twice the average size was counted as 2 mycorrhizae). Following the same reasoning, mycorrhizae broken during the sampling process were counted as fractions of a whole mycorrhiza.

Hierarchical cluster analysis (SPSS Statistics v.19, IBM) of the number of mycorrhizae and percentage mycorrhization (standardized in the interval 0-1) was performed in an attempt to classify the mycorrhizal quality of the different sets of plants.

Molecular analysis of mycorrhizae

An *in silico* database was constructed using published sequences of several well-known European species of the genus *Tuber* following the procedure of Alvarado and Manjón (2009). Virtual automated ribosomal intergenic spacer analysis (ARISA) was then performed to estimate the length of the ITS region. In addition, virtual terminal restriction fragment length polymorphism (TRFLP) analysis was performed to predict the fragment size to be produced following digestion of the ITS with the restriction enzymes *Mae*II and *Dde*I.

Real ARISA and TRFLP analyses were performed on the DNA extracted from the RTF fractions of all plants, as well as on that of real ascomata from different *Tuber* species (Table 2), and the results compared to the *in silico* predictions. Total DNA from the ascomata was extracted following the standard CTAB method. Total RFT DNA was extracted from 250 mg RTF samples using the PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad CA), following the manufacturer's protocol. Two microlitres of total DNA were amplified using primers NSI1 and NLB4 (Martin and Rygiewicz, 2005) or ITS1F and ITS4 (Gardes and Bruns, 1993; White *et al*., 1990). Two microlitres of the resulting PCR product were then used in a nested PCR reaction involving the fluorescently labelled primers 6FAM-ITS1 and VIC-ITS4. All PCR reactions were performed in 50 µL and included one unit EcoTaq DNA polymerase (Ecogene), 1X EcoTaq Buffer (Ecogene), $MgCl_2$ 2 mM and DNTPs 0.2 mM each. Amplifications were started using a hot start step at 95°C for 5 min, followed by 30 cycles of three steps at 94°C for 30 s, 54°C for 90 s and 72°C for 120 s, and a final extension step at 72°C for 10 min.

The amplification products were then subjected to ARISA and TRFLP. For ARISA, 1 µL of each PCR product was directly examined in an ABI Prism 3130xl sequencer (Applied Biosystems) using the internal standard LIZ1200 and a POP 7 polymer. For TRFLP analyses, 18 µl of each PCR product were digested with two units of *Hpy*CH4IV (isoschizomer of *Mae*II) or *Dde*I (New England Biolabs) (Alvarado & Manjón 2009) in 2 µL of their respective 10X buffers for 2 h at the temperature recommended by the manufacturer. One microlitre of the digested amplicons was loaded in the same way as the undigested fragments. All results were checked in PeakScanner v1.0 software (Applied Biosystems).

Cloning and sequencing

The ITS amplicons from three randomly selected plants from each set (except for sets 11 and 12) were cloned, and sequenced. The primer pair ITS1-ITS4 was used to amplify the ITS region in a nested PCR reaction following the procedures mentioned above. The PCR products were purified using the UltraClean™ PCR Clean-up kit (MoBio, Carlsbad, US) and eluted in 50 µl of buffer. Four microlitres of each purified PCR product were used in independent cloning reactions involving the plasmid pCR®2.1-TOPO®. The plasmids were then transferred into TOP10 chemically competent cells, using the TOPO TA Cloning® Kit (Invitrogen, Carlsbad, US) according to the manufacturer's instructions. One or two hundred microlitres of transformed cells in SOC medium were spread on LB agar plates supplemented with 50 µg mL^{-1} ampicillin, 40 mg L^{-1} X-gal, and 23.83 mg L^{-1} IPTG. Transformed colonies were verified as such in PCR reactions performed as described above, using the primers M13F (located in the vector), and ITS4 (from the insert). The PCR products were examined in MetaPhor® agarose gel (Lonza, Rockland, US). Clones with different ribotypes were purified and sequenced with ITS4 as above.

Microsatellite analysis

To determine whether microsatellite analysis of *T. melanosporum* intraspecific variability can be used to determine the geographical origin of the fungal strains inoculated into seedlings, several primers developed by Bertault *et al*. (2001) and Riccioni *et al*. (2008) were used to amplify different microsatellite loci in the mycorrhizae of the different plants, and in fruiting bodies of *T. melanosporum* collected in natural truffle habitats in central and southern Spain (77 samples from three localities in Guadalajara (central Spain), 19

Table 2. ARISA and TRFLP results for several species of the genus *Tuber*. Values predicted i*n silico* are shown in bold text. The upper section of the table represents the predicted fragment sizes after virtual (*in silico*) ARISA and TRFLP, plus the sizes recorded in analyses of the actual fruiting bodies and mycorrhized roots. The lower section contains the fragment sizes predicted *in silico* using GenBank data of other species of the genus *Tuber*

Tuber genotypes	GenBank	Pred. ARISA	Obs. ARISA	Pred. MaeII 1-4	Obs. MaeII 1-4	Pred. MaeII 4-1	Obs. MaeII 4-1	Pred. DdeI 1-4	Obs. DdeI 1-4	Pred. DdeI 4-1	Obs. DdeI 4-1
In silico-Ascomata-Roots											
T. borchii 1	FJ554466	**568**	564	**568**	564	**568**	564	**119**	118	**94**	93
T. brumale 1	FM205692	**914**	911	**379**	379	**533**	531	**99**	95	**285**	288
T. indicum 1	DQ375511	**620**	616	**141**	138	**477**	475	**169**	167	**285**	285
T. indicum 1	DQ375512	**620**	616	**140**	139	**155**	154	**168**	168	**286**	286
T. indicum 2	FJ455097	**624**	620	**180**	178	**442**	475	**169**	167	**94**	92
T. magnatum 1	EU807975	**644**	637	**393**	389	**42**	—	**175**	173	**49**	49
T. malençonii 1	FM205596	**704**	692	**530**	523	**108**	106	**62**	59	**302**	300
T. melanosporum 1	EU753270	**620**	616	**620**	616	**620**	616	**169**	167	**285**	285
In silico											
T. aestivum 1	AF132509	**703**		**203**		**258**		**346**		**305**	
T. aestivum 2	AY226042	**701**		**204**		**257**		**346**		**303**	
T. aestivum 3	AJ492203	**701**		**203**		**256**		**198**		**302**	
T. borchii 2	FJ554495	**568**		**568**		**568**		**60**		**94**	
T. brumale 2	FM205660	**917**		**380**		**535**		**99**		**289**	
T. brumale 3	FJ748900	**886**		**348**		**536**		**99**		**288**	
T. excavatum 1	FN433155	**659**		**659**		**659**		**78**		**141**	
T. excavatum 1	FN433150	**661**		**535**		**124**		**78**		**142**	
T. excavatum 2	FN433145	**659**		**530**		**127**		**202**		**145**	
T. excavatum 3	FN433144	**663**		**533**		**128**		**76**		**146**	
T. excavatum 4	FM205559	**661**		**661**		**661**		**205**		**143**	
T. excavatum 5	FN433141	**700**		**566**		**132**		**77**		**150**	
T. indicum 1	DQ375498	**621**		**142**		**477**		**170**		**86**	
T. indicum 1	U89362	**620**		**463**		**155**		**169**		**285**	
T. indicum 2	DQ375493	**628**		**184**		**442**		**173**		**94**	
T. puberulum 1	AJ557540	**651**		**651**		**651**		**202**		**93**	
T. pseudohimalayense 1	DQ329371	**891**		**781**		**108**		**105**		**282**	
T. pseudohimalayense 1	AY514310	**891**		**891**		**891**		**105**		**282**	
T. rufum 1	EF362473	**660**		**660**		**660**		**660**		**660**	
T. rufum 2	EF362475	**659**		**659**		**659**		**209**		**141**	
T. rufum 3	FM205603	**658**		**658**		**658**		**141**		**141**	
T. rufum 4	FN433161	**663**		**43**		**618**		**189**		**141**	
T. rufum 5	FM205668	**662**		**662**		**662**		**188**		**141**	

samples from 10 localities in Soria (central Spain), 12 samples from 6 localities in Albacete (southern Spain), and 7 samples from 5 localities in Andalusia (southern Spain). The fluorescently-labelled primer pairs FAM-Cro74F/Cro74B, VIC-Cro71F/Cro71B, NED-07ISSR9f/07ISSR9b, PET-07ISSR10f/07ISSR10b, FAM-07ISSR14f/07ISSR14b, NED-F12If/F12Ib and VIC-H1bf/H1bb, were used to amplify the loci ME2, ME4, 07ISSR9, 07ISSR10, 07ISSR14, F12I and H1b respectively. Two microlitres of template DNA were used in two independent multiplex PCR reactions (volume 50 μL), amplifying 3 and 4 of the mentioned loci respectively. The PCR conditions were the same as those stated above.

In addition, the mycorrhizae of plants inoculated with truffle strains coming from different geographical locations were subjected to microsatellite amplification, to test the performance of this kind of analysis on DNA extracted from mycorrhized roots. To this end, 2 μL of template DNA of sets 7 (from Soria) and 12 (from Andalusia) was used to amplify the same microsatellite loci. Fragment analysis was performed in the same conditions as for TRFLP.

Results

Extraction of mycorrhizae

All the inoculated plants showed mycorrhizae; in fact, even the non-inoculated controls had become associated with fungi of some kind. Among the Spanish plants inoculated with *T. melanosporum* (sets 2-11), the number of root tips with mycorrhizae and the percentage mycorrhization varied significantly depending on the supplier (Fig. 1). Cluster analysis of the variables "mean number of mycorrhizae" and "percentage mycorrhization" revealed the existence of different groups of sets: Group I (sets 1, 2, 5, and 11) was formed of plants for which the mean number of mycorrhizae was < 3,000 —these were considered of poor mycorrhizal quality since the non-inoculated set was included in this group; Group II (sets 3, 4, 7, 8 and 12), in which the plants had > 3,000 mycorrhizae and the percentage mycorrhization exceeded 40%— these were considered optimally mycorrhized; and Group III (sets 6, 9 and 10) in which the plants showed intermediate mycorrhizal quality (> 3,000 mycorrhizae/plant, < 40% mycorrhization).

All the plants inoculated with *T. melanosporum* showed the honey-coloured mycorrhizae typical of this fungus, but also brownish or even dark brown mycorrhizae. Some extra-large and pear-shaped mycorrhizal apices were also seen (Fig. 2). The plants inoculated with *T. indicum* (set 14) had mycorrhizae morphologically indistinguishable from those of *T. melanosporum*. The plants of set 13, inoculated with *T. borchii*, showed typical mycorrhizae of this species with cystidia (Kovács and Jakucs 2006). However, the latter were hard to find, perhaps due to the sampling procedure or the fungal strain (Giomaro *et al.*, 2000).

ARISA and TRFLP analysis

The *in silico* ARISA and TRFLP predictions (Table 2) matched closely the results obtained for the real ascomata; the mean difference between them was 3 bp.

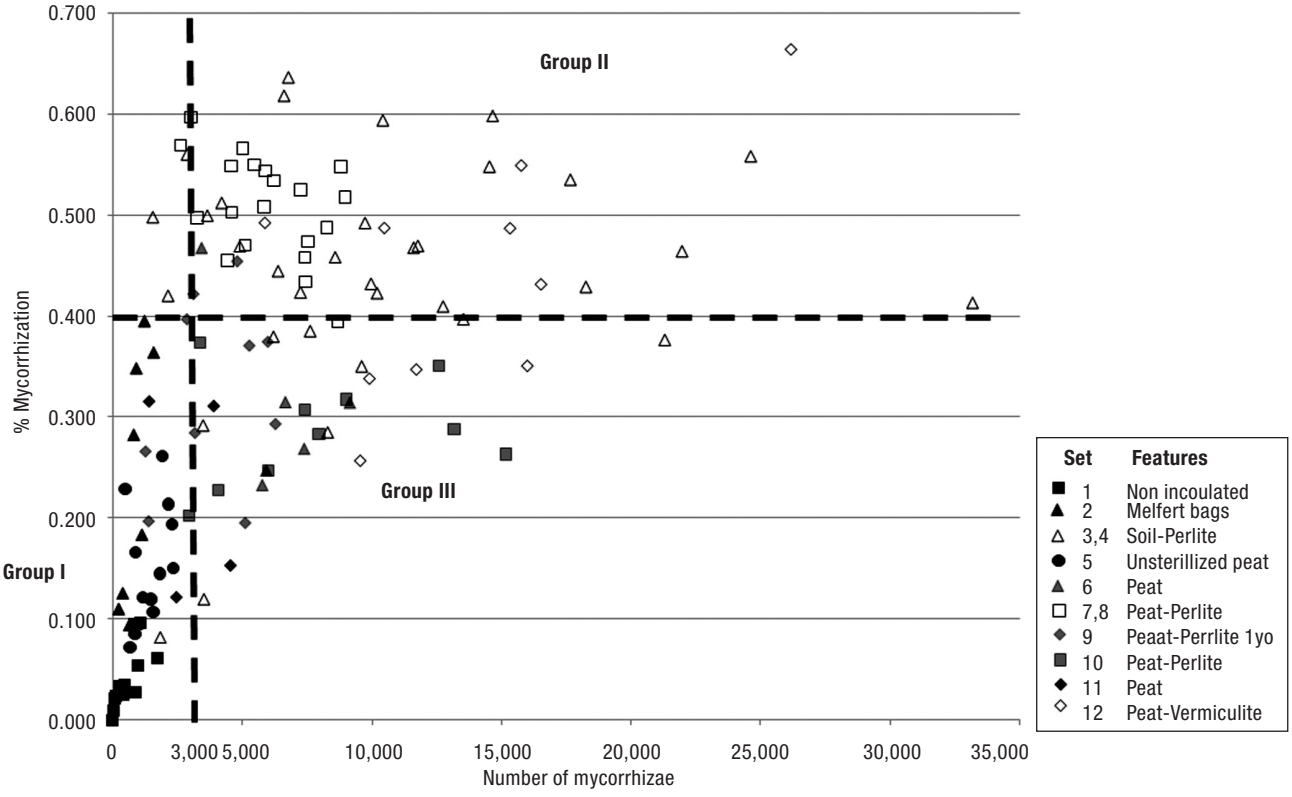

Figure 1. Cluster analysis results showing the proposed mycorrhizal quality groups. Group I includes the "low quality" plants, Group II includes the "optimal quality" plants and Group III the "intermediate quality" plants (*i.e.,* that met the required mycorrhizal number criterion, but not the percentage mycorrhization value).

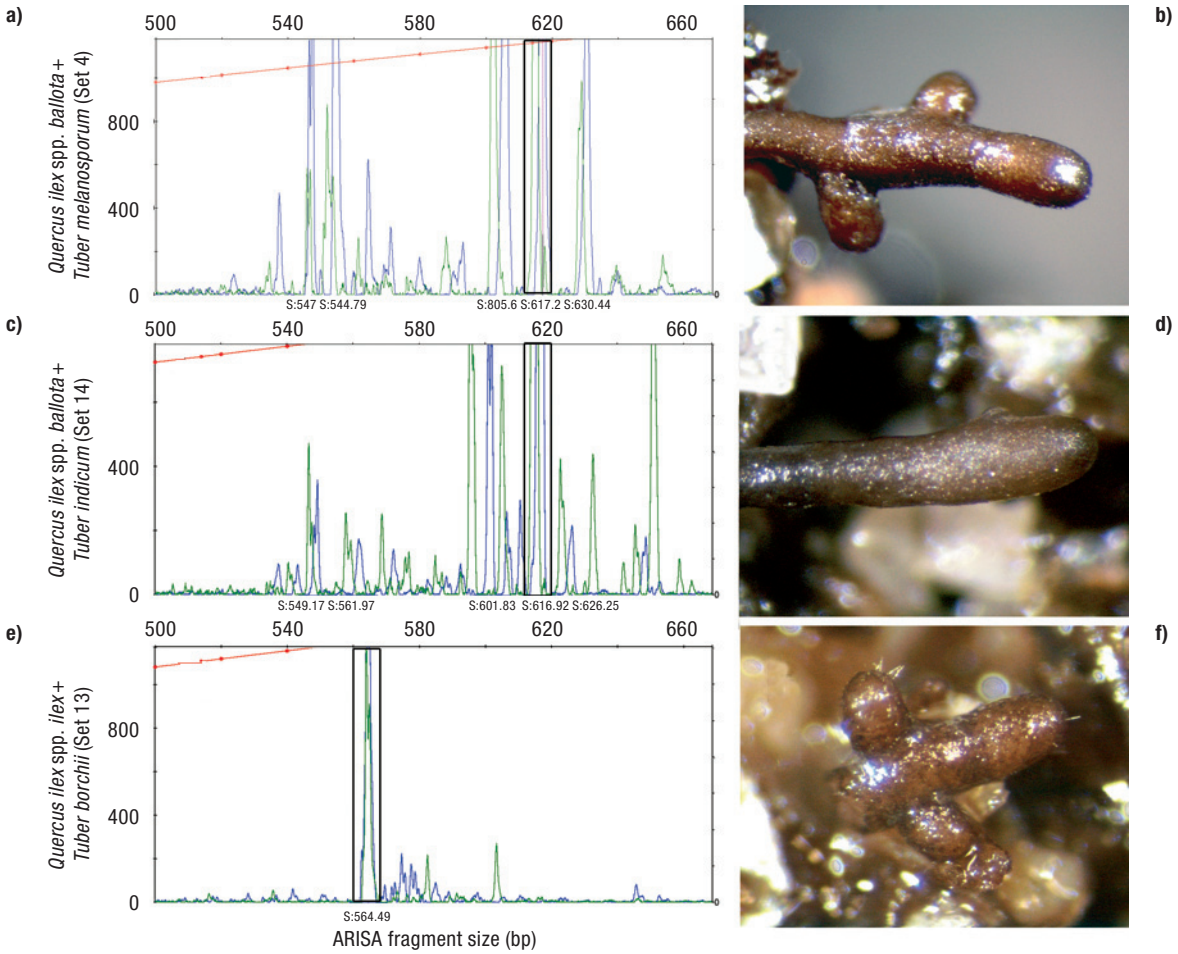

Figure 2. ARISA profiles of the mycorrhized plants. Blue represents the ITS1-FAM fluorescence, while green represents that of ITS4-VIC. The boxes highlight the fluorescence peaks of the inoculated fungi. a-b) *T. melanosporum* mycorrhiza from set 3. c-d) *T. indicum* Clade IV mycorrhiza from set 14. e-f) *T. borchii* Clade I mycorrhiza from set 13.

The error, though variable (2-4 bp) was always the same in comparisons between the *in silico* predictions and the ARISA/TRFLP results for samples of any given species.

ARISA analyses of the mycorrhized plants returned characteristic profiles depending on the supplier. The profiles differed mainly in terms of the most abundant ribotypes, as well as ribotype richness and evenness. The plants of sets 3, 4, 6, 7, 8, 9, 12 and 14 usually showed a dominant peak of 616-617 bp, similar in size to that obtained for the ascomata of *T. melanosporum* and *T. indicum* clade IV (Chen *et al*., 2011). Sometimes other, more or less co-dominant peaks, were seen (Fig. 2). In a few cases, other ribotypes were dominant over that of *T. melanosporum*; in fact, the latter was absent from some supposedly-inoculated samples in sets 2, 5 and 10. As expected, it was also absent from set 1 (the non- inoculated control), and set 13 (inoculated with *T. borchii*). The latter showed a 564 bp peak matching that obtained from an ascoma of this species; this closely approached the *in silico*-predicted fragment size of 568 bp.

The TRFLP profiles of the mycorrhized plants matched those obtained for the ascomata, and allowed discrimination between plants inoculated with *T. melanosporum*, *T. indicum* Clade IV or *T. borchii* Clade I (Bonuso *et al*., 2010).

Cloning and sequencing

ITS sequences matching those of GenBank accessions for *T. melanosporum* (JN392089- JN392094), *T. indicum* Clade IV (JN392096) and *T. borchii* Clade I (JN392095) were recovered from the plants inoculated with these respective fungi. None were recovered from

set 1 (Control) or set 2 (grown in Melfert bags). Set 5 contained sequences of the contaminating mycorrhizal fungus *Thelephora terrestris* (GenBank JN392097), an unidentified fungus of the order Sebacinales (GenBank JN392098), and a sequence from *Hebeloma* sp. (GenBank JN392099), while sets 8 and 12 were contaminated with an unidentified species of *Tomentella* sp. (GenBank JN392100). Most of the remaining sequences obtained through cloning were those of saprophytic or endophytic fungal genera, or failed to match any known genus, as reported by other authors (Lindner and Banik, 2009). *Alternaria* (JN392101), *Blastobotrys* (JN392102), *Cladosporium* (JN392103), *Clitopilus* (JN392104), *Coprinopsis* (JN392105), *Coprinus* (JN392106), *Geomyces* (JN392107), *Neonectria* (JN392108, JN392109), *Mycena* (JN392110), *Penicillium* (JN392111), *Peziza* (JN392112) and *Sphaeropsis* (JN392113) were among the main genera identified using BLAST software. Several other sequences could not be related to any existing genus; these will not be uploaded into public databases until they have been further investigated.

Microsatellite analysis

The analysis of microsatellite variability within *T. melanosporum* populations from central Spain (Guadalajara, Soria) revealed a single dominant haplotype with scattered low-frequency allelic variations (Fig. 3). The southern populations (Andalusia, Albacete), in contrast, showed a greater evenness of alleles at loci F12I, Cro71 and 07ISSR9. Additionally, these alleles were found combined, forming unique haplotypes that were absent from the populations of central and northern Spain (Riccioni *et al*., 2008). Locus H1b showed no apparent variability in the samples analysed, while locus 07ISSR10 (the different alleles of which differed by only 1 bp) had an ambiguous fragment size of 264.5 bp; it was therefore excluded from the analysis. Some degree of stuttering was detected (see Fig. 3f), but a dominant peak was always observed. Direct sequencing revealed the observed allele size to differ slightly (but constantly) from the real size, in agreement with that reported by Riccioni *et al*. (2008). Fig. 3 shows the corrected fragment sizes. Microsatellite analysis of DNA extracted from the mycorrhized roots successfully discriminated between strains with different genotypes from central Spain and Andalusia.

Discussion

All the nursery-produced plants tested showed mycorrhizae of some kind, including the control plants (set 1), although the percentage mycorrhization, and the species involved, depended greatly on the supplier. Further, broad differences in mycorrhization status and molecular traits were detected between the *T. melanosporum* inoculated by the different Spanish suppliers. This seems to be in keeping with the idea that spontaneous mycorrhization occurs in tree nurseries, and that standardised substrate preparation, environmental monitoring and fungal inoculation techniques must be developed and followed if high-quality mycorrhization is to be achieved (Pruett *et al*. 2009).

The set 2 plants, which were grown in Melfert bags, had very few mycorrhizae and returned low percentage mycorrhization values. *T. melanosporum* could not be detected by TRFLP or cloning and sequencing. The sets in which the plants were grown on substrates based on a mixture of peat or soil plus perlite or vermiculite provided better results. However, this could be due to other, unstudied influences on the production process, or even undeclared additions to the substrate mixture that could not be detected visually.

A mycorrhization value of at least 40% and > 3,000 mycorrhizae is here proposed as representing good mycorrhizal quality for 2 year-old *Q. ilex* subsp. *ballota* plants inoculated with *T. melanosporum*. Plants with > 3,000 mycorrhizae and 30% mycorrhization can be considered of acceptable quality, while those with lower values should be considered suboptimal. The literature contains no reports on the relationship between mycorrhizal status and truffle production onset or productivity. However, mycelium abundance in the soil seem to be directly involved (Suz *et al.,* 2008b).

One set of 1 year-old plants (set 9) had a number of mycorrhizae and a percentage mycorrhization similar to that of two 2 year-old sets (sets 7 and 8). It is therefore difficult to draw any conclusions regarding the influence of mycorrhization time on the actual mycorrhization achieved. However, set 9 fell into Group III (acceptable plants) in the cluster analysis, suggesting that the quality standards proposed might also be applied to 1 year-old plants.

This is the first paper to report the use of TRFLP to detect inoculated fungi in commercial mycorrhized plants; the technique would appear to provide an alternative method of fungal community profiling. The fragment sizes showed a small bias when compared to

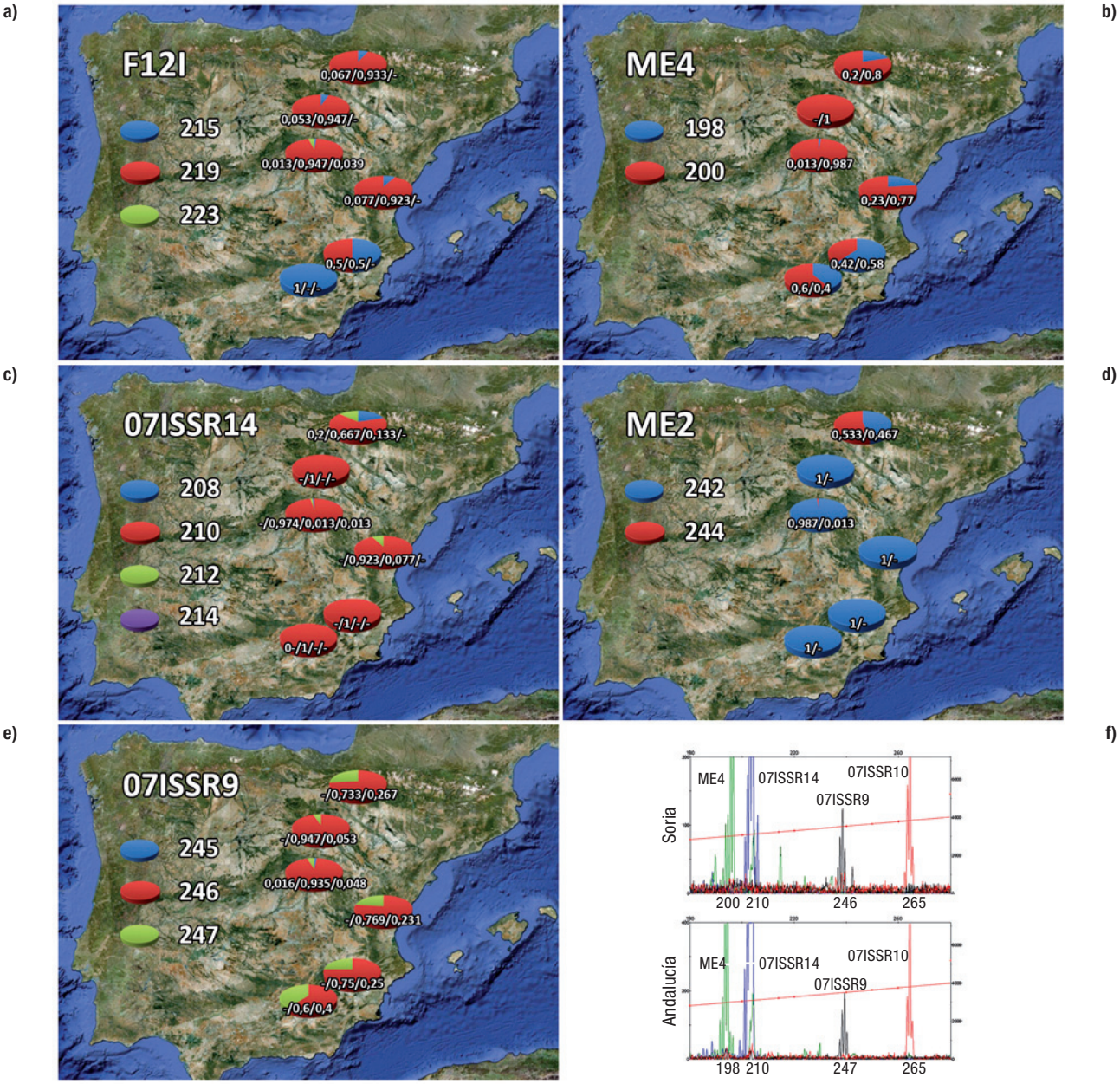

Figure 3. Microsatellite variability of *T. melanosporum* in Spain. a-e) Allelic frequencies of five microsatellite loci taken from the present work and from Riccioni *et al.* (2008). f) Comparison of the microsatellite profiles of plants inoculated with *T. melanosporum* from central Spain (Soria), and from southern Spain (Andalusia).

in silico predictions (although the size of the bias was not related to the fragment size) (Table 2); this was more or less constant for different assays of single samples and for different samples of the same species. The same has been reported by other authors employing techniques based on fragment analysis (Dickie and FitzJhon, 2007) or the use of fluorescently-labelled microsatellites (Riccioni *et al*., 2008). TRFLPs can be used to characterize mycorrhized root tips (Burke *et al*., 2005) cheaply compared to cloning and sequencing. If available, specific primers can be used to detect several taxa in multiplex reactions, but these could return amplicons of equal or similar length. TRFLP-identification requires looking for known peaks, either manually or using an automated system such as TRAMPR (TRFLP analysis and matching package for

R) (FitzJohn and Dickie, 2007). It also offers a semi-quantitative estimation of specific DNA abundance, at least for small quantities of DNA (Manter and Vivanco, 2007), which could be used to assess the dominance of the inoculated fungus.

ARISA successfully discriminated between morphologically similar species such as *T. melanosporum* and *T. indicum* Clade III (Chen *et al*., 2011). However, *T. indicum* Clade IV, which differs from *T. melanosporum* in 6.129% of its sequence (38 variable sites in 620 bp), could only be separated by its TRFLP profile. Even the two cryptic species of *T. borchii* (*sensu* Bonuso *et al*., 2010, or Alvarado *et al*., 2012; but not Halász *et al*., 2005, or Kovács and Jakucs, 2006), which differ in as little as 1.937% of its ITS sequence (11 variable sites in 568 bp) were successfully discriminated by their TRFLP profile.

The microsatellite primers designed to characterize the *T. melanosporum* ascomata were here used for the first time to examine mycorrhized plant roots. Most of the alleles recorded were present — at least at a very low frequency— in all areas, although a single haplotype was the most common in central Spain. This agrees with previous reconstructions of the evolutionary history of *T. melanosporum* (Murat *et al*., 2004; Riccioni *et al*., 2008). The populations of Andalusia and Albacete to the south, and Navarre in northern Spain, showed characteristic alleles or haplotypes that allows fungal strains from these areas to be recognised. Further research into microsatellite profiles should be conducted to determine the reliability of geographical identifications.

Conclusions

This work reports an inexpensive and reproducible method for quantitatively and molecularly assessing the mycorrhiza in the roots of *Quercus ilex* saplings.

The mycorrhizal quality standards proposed for certifying 2 years-old plants mycorrhized with *T. melanosporum* are: a) good quality: > 3,000 mycorrhizae/plant, > 40% mycorrhization, b) medium (acceptable): > 3,000 mycorrhizae/plant, > 30% mycorrhization, c) low quality: < 3,000 mycorrhizae/plant or < 30% mycorrhization. The DNA of the fungus of interest must be present in the absence of DNA from contaminant species dangerous to truffle orchards.

Allelic differences between *T. melanosporum* strains from root samples can be used to determine the geographical origin of some strains.

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