environmental microbiology

Environmental Microbiology (2015) doi:10.1111/1462-2920.12741

Whose truffle is this? Distribution patterns of ectomycorrhizal fungal diversity in *Tuber melanosporum* brûlés developed in multi-host Mediterranean plant communities

Elisa Taschen,[1]* Mathieu Sauve,[1] Adrien Taudiere,[1] Javier Parlade,[2] Marc-André Selosse[3] and Franck Richard[1]

[1]*UMR 5175, CEFE, CNRS – Université de Montpellier – Université Paul-Valéry Montpellier – EPHE – 1919 route de Mende, Montpellier 34293, France.*

[2]*Sustainable Plant Protection, IRTA, Centre de Cabrils, Ctra. Cabrils km. 2, Cabrils, Barcelona 08348, Spain.*

[3]*Département Systématique et Evolution (UMR 7205 ISYEB), Muséum National d'Histoire Naturelle, CP 50, 45 rue Buffon, Paris 75005, France.*

Summary

In the Mediterranean region, patches of vegetation recovering from disturbance and transiently dominated by shrubs produce one of the world's most prized fungi, the black truffle (*Tuber melanosporum*). In these successional plant communities, we have fragmentary knowledge of the distribution of *T. melanosporum* in space among ectomycorrhizal (ECM) host species and in time.

Molecular identification of hosts (Restriction Fragment Length Polymorphism) and fungi (Internal Transcribed Spacer sequencing) and quantification of *T. melanosporum* mycelium (quantitative Polymerase Chain Reaction) were employed to evaluate the presence of *T. melanosporum* on four dominant ECM host species (*Quercus ilex*, *Quercus coccifera*, *Arbutus unedo*, *Cistus albidus*) and the extent to which their respective ECM communities shared fungal diversity, over the course of development of truffle grounds, from recent unproductive brûlés to senescent ones where production has stopped.

We found that truffle grounds host rich communities in which multi-host fungal species dominate in frequency. When considering both ECM tips and soil mycelia, we documented a dynamic and spatially heterogeneous pattern of *T. melanosporum* distribution in soils and a presence of ECM tips restricted to *Q. ilex* roots.

This study advances our knowledge of the ecology of *T. melanosporum*, and provides insight into the extent of ECM fungal sharing among plant species that dominate Mediterranean landscapes.

Received 6 August, 2014; revised 1 December, 2014; accepted 2 December, 2014. *For correspondence. E-mail elisa.taschen@cefe.cnrs.fr; Tel. +33 (0) 4 67 61 32 31.

Introduction

Identifying the factors driving the distribution of the astonishing diversity of fungi is a major challenge in microbial ecology. During the last two decades, mycologists have chiefly focused their attention on forest ecosystems (i.e. dominated by trees), largely neglecting the large expanses of shrub-dominated vegetation in Mediterranean regions. During secondary successions, these vegetation types typically occupy the land in most of the period between agricultural abandonment and establishment of forest. This especially applies in Mediterranean shrub-dominated vegetation, which comprises millions of hectares of species-poor plant communities, whose composition, and whose common names (e.g. macchia, garrigues, etc.), vary across the region (Grove and Rackham, 2003; Sirami *et al.*, 2010). These vegetation types are functionally crucial because the establishment of most Mediterranean tree species is often facilitated by shrubs, which buffer abiotic conditions in stress-prone environments (Gómez-Aparicio *et al.*, 2004; Holmgren *et al.*, 2012) and play roles in positive plant–plant interactions mediated by microorganisms (Selosse *et al.*, 2006; Kennedy *et al.*, 2012).

Mediterranean tree species associate with particularly diversified communities of ectomycorrhizal (ECM) fungi that are involved in plant nutrition (Smith and Read, 2008) and tolerance to water stress (Kipfer *et al.*, 2012). Ectomycorrhizal fungal species vary widely in their ability to associate with different plant species, from being highly specific to having multiple hosts (Bruns *et al.*, 2002; Bingham and Simard, 2011). The capacity to have multiple hosts makes it possible for coexisting individuals of different plant species to share ECM fungal mycelia

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd

and indirectly interact (Bingham and Simard, 2011; Simard *et al*., 2012). In the French Mediterranean region, only a few shrub species (e.g. species of *Cistus*, *Helianthemum*, *Arbutus*) have the ability to host ECM fungi on their roots (Richard *et al*., 2009), while others (e.g. species of *Phillyrea*, *Pistacia*) harbour arbuscular mycorrhizal fungi (Maremmani *et al*., 2003). When Mediterranean oaks (*Quercus ilex* and *Q. coccifera*) establish in such vegetation, multiple ectomycorrhizal shrub species transitorily coexist before the complete canopy closure that precedes shrub decline. In Mediterranean ecosystems, the extent to which fungal species are shared among ECM hosts has been documented at the forest stage (Richard *et al*., 2005; Morris *et al*., 2008) but remains largely unknown in shrub-dominated communities.

The highly prized black truffle (*Tuber melanosporum* Vittad.) naturally establishes and is typically collected in these transitory ecosystems, although cultivated truffle grounds with planted host trees are increasingly developed nowadays. This ascomycete is an ECM fungal species that non-specifically associates with various distantly related hosts in the Fagaceae (*Quercus*), Betulaceae (*Corylus*), Malvaceae (*Tilia*) (Callot, 1999) and Cistaceae (*Cistus*) (Wenkart *et al*., 2001; Comandini *et al*., 2006; García-Montero *et al*., 2007) and may even form orchid mycorrhizae (Girlanda *et al*., 2006). In soils of natural and cultivated truffle grounds, studies analyzing ECM fungal diversity, usually considering one single-host tree species, have shown that *T. melanosporum* is a component of diversified ECM fungal communities (Napoli *et al*., 2010) dominated by Tuberaceae and Thelephoraceae (Belfiori *et al*., 2012; De Miguel *et al*., 2014). In natural truffle grounds, *T. melanosporum* fruits in vegetation mosaics that preceed forest stage during secondary succession (Médail and Quézel, 2003). In the French Mediterranean region, natural truffle grounds typically consist of scattered holm oaks (*Quercus ilex* L.) surrounded by a few ECM shrub species (Médail and Quézel, 2003). In these dynamic plant communities, the presence of *T. melanosporum* on roots of the different coexisting ECM hosts, its abundance and/or frequency within the fungal ECM communities on the roots and more broadly the distribution of the different components of this ECM community among co-occurring ECM host species have never been documented.

The vegetative presence of *T. melanosporum* is revealed by the occurrence of patches (called *brûlés*) of poorly productive vegetation neighbouring truffle host trees. The mechanisms underlying the formation of the brûlé are not fully understood (Streiblová *et al*., 2012) and may include a lifestyle in which *T. melanosporum* parasitizes herbaceous plants (Plattner and Hall, 1995) or plant–fungal interactions through volatile organic compounds it emits (Splivallo *et al*., 2009). However, in natural truffle grounds as well as in plantations, the presence of a brûlé does not systematically indicate the production of *T. melanosporum* ascocarps (Suz *et al*., 2008). Indeed, *T. melanosporum* brûlés usually appear first, sometimes becoming productive only years or even decades later (Diette and Lauriac, 2004; Streiblová *et al*., 2012), and eventually become sterile before disappearing, generally as the forest canopy closes. Studies have compared the composition of ECM fungal communities in productive and non-productive *T. melanosporum* brûlés (see De Miguel *et al*., 2014 for a review), but the concomitant changes in soil ECM diversity that accompany the emergence, maturation and decline of brûlés remain unknown. In particular, relationships between *T. melanosporum* ascocarp production, mycorrhizal root tip frequency and mycelium abundance have never been analysed in natural truffle grounds.

Here, we analysed the patterns of ECM fungal communities and *T. melanosporum* distribution in natural brûlés at different stages of ascocarp production. More specifically, we described the ECM communities associated with four co-occurring ECM plant species (*Q. ilex*, *Q. coccifera*, *Arbutus unedo* and *Cistus albidus*) at three different production stages (a–c) defined by the status of the brûlé (immature, productive or post-productive) and in surrounding relict forest (d), using systematic molecular identification of both fungal and plant partners on ECM tips in soil. In addition, we assessed *T. melanosporum* mycelium distribution in the soil of the same sites using quantitative PCR. We addressed the following questions: (i) In these secondary successional landscapes, what part of the ECM fungal diversity is shared among coexisting hosts, and do multi-host fungal species dominate the communities? (ii) In *T. melanosporum* brûlés, do co-occurring ECM plant species host ECM communities that are distinct in composition, and do they differ in their ability to host *T. melanosporum*? (iii) Do different production stages differ in the composition of their ECM fungal communities? (iv) Do the frequency of ECM tips and the distribution patterns of *T. melanosporum* extraradical mycelia fit the fruiting patterns of *T. melanosporum* among brûlés?

Results

General description of the study system

In all, 797 ECM tips were sampled on four host species, *Quercus ilex*, *Q. coccifera*, *Cistus albidus* and *Arbutus unedo* (hereafter abbreviated *Qi, Qc, Ca* and *Au*, respectively) from 192 soil cores on 45 plots (Table 1). Plant and fungal ITS were successfully sequenced for 522 (65.5%) ECM tips, including 25 fungal ITS sequences generated with the basidiomycete-specific primer pair ITS1F-ITS4B.

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*

Table 1. Sampling design and subsequent analyses.

			Sampled plants per species				Analyses	
Sites	Prod. stages	No. of plots	Au	Ca	Qc	Qi	ECM root tip identification	*T. mel.* mycelium in soil
S1	***a***	2	1	1		2	✓	✓
	b	2	2	1		2	✓	✓
	c	2	2	2		2	✓	✓
	d	1	1			1	✓	✓
S2	***a***	3	3	1	3	3	✓	✓
	b	3	3	3	3	3	✓	✓
	c	3	3	1	3	3	✓	✓
	d	1	1		1	1	✓	✓
S3	***a***	3		2	3		✓	✓
	b	3	1		3	3	✓	✓
	c	2	1	1	2	1	✓	✓
	d	4		1	4	1	✓	✓
S4	***a***	2		2	2		✓	
	b	3		3	3		✓	
	c	2		2	2		✓	
S5	***a***	3	3	2		3	✓	
	b	3	3			3	✓	
	c	3	3			3	✓	
5 sites		**45**	**27**	**22**	**29**	**31**	**192 soil cores: 522 ECM tips**	**29 plots**

Fungal ITS revealed 151 OTUs (Table 2) predominantly belonging to Basidiomycetes (70.2% of ECMs and 79.6% of OTUs). Among Basidiomycetes, Thelephoraceae was the most abundant (37.1% of ECM tips), frequent (63.2% of soil cores) and diverse (38.8% of OTUs) family (Table 2). The second most abundant family, Pyrenomataceae, accounted for 13.6% of ECM tips, 28.8% of soil cores and 9.9% of OTUs. Thelephoraceae and Pyrenomataceae dominated consistently across sites (data not shown), host species and production stages (Table 2; Fig. 1A and B). A small number of species accounted for a large proportion of all occurrences. Six OTUs (4%) represented 20% of the total number of ECM types, and these species were also the most frequent both in the soil core (present in nine to 18 soil cores out of the total number of cores) and at the site (data not shown). These few common species were accompanied by a much larger number of infrequent species, with 33.8% of OTUs represented by only one sequenced ECM tip.

Table 2. Relative abundance (number of ECM tips as a percentage of the total number of ECM tips), frequency (number of soil cores as a percentage of the total number of soil cores) and richness (number of OTUs as a percentage of the total number of OTUs) of 12 dominant ECM taxa.

ECM taxa	Abundance (%)	Frequency (%)[a]	Richness (%)[b]
Thelephoraceae	37.1	63.4	38.8 (59)
Pyrenomataceae	13.6	28.8	9.9 (15)
Tuberaceae	8.4	19.4	3.9 (5)
Cortinariaceae	7.3	16.2	8.6 (8)
Inocybaceae	6.7	12.0	9.9 (15)
Sebacinales	6.1	12.6	5.9 (9)
Tricholomataceae	5.5	12.6	5.9 (9)
Helvellaceae	3.3	6.3	3.3 (5)
Russulaceae	3.1	5.8	2.6 (4)
Pezizaceae	1.5	3.7	1.3 (2)
Gloniaceae	1.3	3.7	0.7 (1)
Boletaceae	1.1	2.6	2.0 (3)
Others	5.0	9.9	7.2 (16)
Ascomycetes	**30**	**55**	**20.5 (30)**
Basidiomycetes	**70**	**86**	**79.5 (121)**

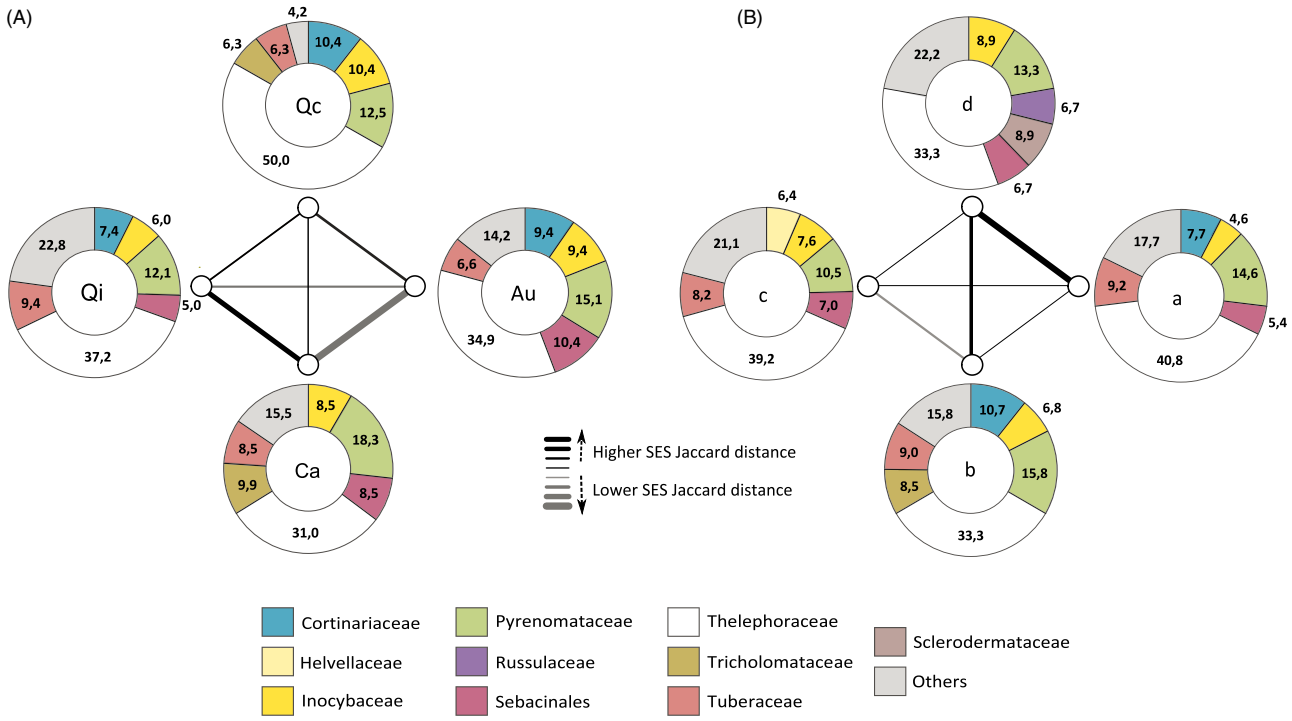
a. Percentages in this column do not add up to 100, as multiple OTUs were often present in a single soil core.
b. Values in brackets are numbers of OTUs.

In 98 soil cores (51%), 282 ECM tips (54% of the total number of ECM tips successfully examined) provided RFLP patterns that did not correspond to that of the above-ground canopy species present in the plot (Table S1). The proportion of ECM tips that did not correspond to the above-ground canopy species ranged from 19.8% under *Au* to 79.5% under *Qc* (Table S1).

Multi-scale patterns of ECM communities

The variance in the composition of ECM communities was poorly explained by among-site variation (PERMANOVA; df = 4; $P = 0.001$; $R^2 = 0.047$) and was more closely related to among-plot variation (PERMANOVA; df = 41; $P = 0.001$; $R^2 = 0.396$, Table 3). A second set of analyses showed weak but significant effects of plant species present either as the ECM tip host (as identified by ITS RFLP; df = 3; $P = 0.018$; $R^2 = 0.029$) or as above-ground canopy species (df = 3; $P = 0.001$; $R^2 = 0.036$), and of

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*



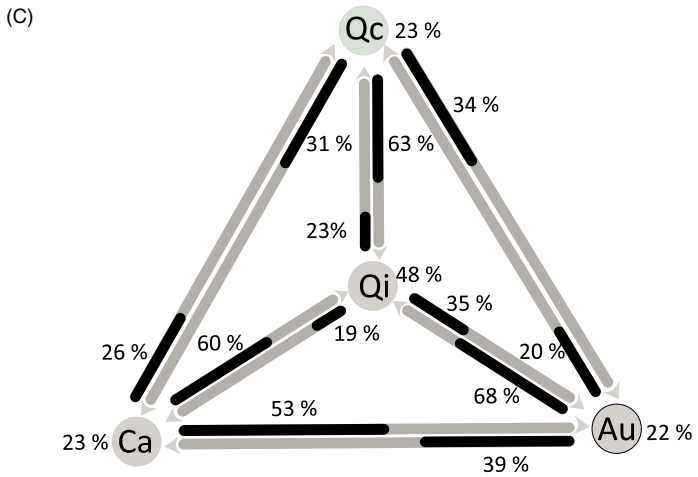


Fig. 1. Composition and similarities of ECM fungal communities among four host plant species (A) and four production stages (B), with detail of reciprocal sharing of OTUs between pairs of host species (C).
Host species are *Arbutus unedo* (*Au*), *Cistus albidus* (*Ca*), *Quercus ilex* (*Qi*) and *Q. coccifera* (*Qc*). Production stages are immature brûlés (*a*), productive brûlés (*b*), post-productive brûlés (*c*) and relict forests (*d*). The width of the links indicates the Standard Effective Size Jaccard distances between host plant species (A) and production stages (B), and the black/gray shade indicates whether they tend to be higher or lower, respectively, than the mean distance under the null model. None of these deviations from null expectations are significant.
(C) Reciprocal sharing of OTUs is expressed as the proportion of the OTUs associated with the focal species that is shared with the opposite host species indicated by the arrow. The proportion of OTUs specific to the focal host is indicated beside its name.

production stage (df = 3; $P = 0.002$; $R^2 = 0.031$/$P = 0.023$; $R^2 = 0.032$; Table 3b and c respectively) on variation in composition of ECM communities, but no significant interaction between plant species and production stage (Table 3).

Patterns of ECM communities across host species

When considering the four production stages together, each of the four host species showed patterns sharing three similarities: (i) unsaturated OTU accumulation

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*

Table 3. Permutational multivariate analyses of variance (PERMANOVA) of ECM community composition according to a) scales of analysis, b) host species (*Arbutus unedo* versus *Cistus albidus* versus *Quercus ilex* versus *Q. coccifera*) and production stage (immature brûlés versus productive brûlés versus post-productive brûlés versus relict forests) and c) above-ground canopy species and the production stage (as above).

Source of variation		df	MS	R^2	*P*
a) Sampling scale	Site	4	4.607	0.047	0.001**
	Plot	41	3.765	0.396	0.001**
	Residuals	78	2.783	0.557	
	Total	123	0	1	
b) Host and stage	Host species	3	3.752	0.029	0.018*
	Production stage	3	4.04	0.031	0.002**
	Host sp. × prod. stage	9	2.374	0.055	1
	Residuals	109	3.167	0.885	
	Total	124	0	1	
c) Canopy and stage	Canopy species	3	4.539	0.036	0.001***
	Production stage	3	3.921	0.032	0.023*
	Canopy sp. × prod. stage	9	3.076	0.074	0.788
	Residuals	97	3.301	0.858	
	Total	112	0	1	

*$P \leq 0.05$; **$P \leq 0.005$; ***$P \leq 0.001$.

curves (Fig. S1A); (ii) high OTU richness (32.5 to 35.4 OTUs per 45 ECM root tips; Table S2) and (iii) high values of the three diversity indices: the Shannon–Wiener information index (from 3.41 for *Qc* to 4.42 for *Qi*), Simpson's diversity index (from 0.96 for *Qc* to 0.98 for *Qi*) and Fisher's alpha (from 46.2 for *Ca* to 66.3 for *Qi*; Table S2). On each of the four host species, Thelephoraceae and Pyrenomataceae were the most represented families (Fig. 1A). The third families in terms of abundance depended on the host (Sebacinales on *Au*, 10.4% of identified ECM tips; Tricholomataceae on *Ca*, 9.9%; Cortinariaceae and Russulaceae on *Qc*, 10.4% each; Tuberaceae on *Qi*, 9.4%). According to SESJ-distances, the composition of ECM communities tended to be more similar between *Au* and *Ca*, and to a lesser extent between *Au* and *Qi*, but these distances did not significantly differ from those obtained under the null model (Fig. 1A).

Patterns of ECM communities across production stages

When considering the four host species together, production stages *a* (immature brûlé) and *c* (post-productive brûlé) showed the steepest species accumulation curves, while stage *b* (productive), and even more so stage *d* (relict forest), accumulated species more slowly (Fig. S1B). Similarly, production stage *a* showed the highest rarefied number of OTUs and values of Fisher's alpha, and stage *c* showed the highest Shannon–Wiener information index and Simpson diversity index, while stage *d* showed the lowest values for all indexes (Table S2).

Thelephoraceae and Pyrenomataceae dominated the ECM communities in each production stage, accounting for 55.4, 49.2, 49.7 and 46.7% of the identified ECM root tips in stages *a*, *b*, *c* and *d* respectively (Fig. 2B). The third most represented ECM family on roots was Tuberaceae in stages *a* and *c* (9.2 and 8.2% respectively), Cortinariaceae in stage *b* (10.7%), and Inocybaceae and Sclerodermataceae in stage *d* (8.9% each; Fig. 1B). In the three different stages of brûlés (*a*, *b, c*), the same fungal families dominated the ECM communities (Fig. 2B). In the relict forest soils (stage *d*), the distribution pattern of ECM fungal families slightly differed from that in brûlés (*a*, *b, c*), with Sclerodermataceae and Russulaceae replacing Cortinariaceae and Tuberaceae among the eight most represented families.

According to SESJ-distances, the composition of ECM communities tended to be more similar between stages *b* and *c* than between all other pairs of productive stages, with a marked difference between stages *a* and *d* (Fig. 2A).

Plant host sharing by ECM fungi

Of the 151 ECM fungal OTUs, 84 (55.6%) were found on roots of one single host species (Fig. 2). Multi-host fungal species included 41 (27.2%), 21 (13.9%) and five (3.3%) OTUs shared by two, three and four host plant species respectively (Fig. 2). Fungal OTUs shared by all host species included two species from the Ascomycetes [*Genabea sphaerospora* (OTU 164) and *Tuber oligospermum* (OTU 147)] and three species from the Basidiomycetes [*Tricholoma scalpturatum* (OTU 155), *Inocybe tenebrosa* (OTU 43) and *Thelephoraceae* (OTU 86)].

A significant and positive correlation was found between the frequency of fungal OTUs and the number of their host species ($P < 0.001$ by Pearson's test; Fig. 2B).

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*

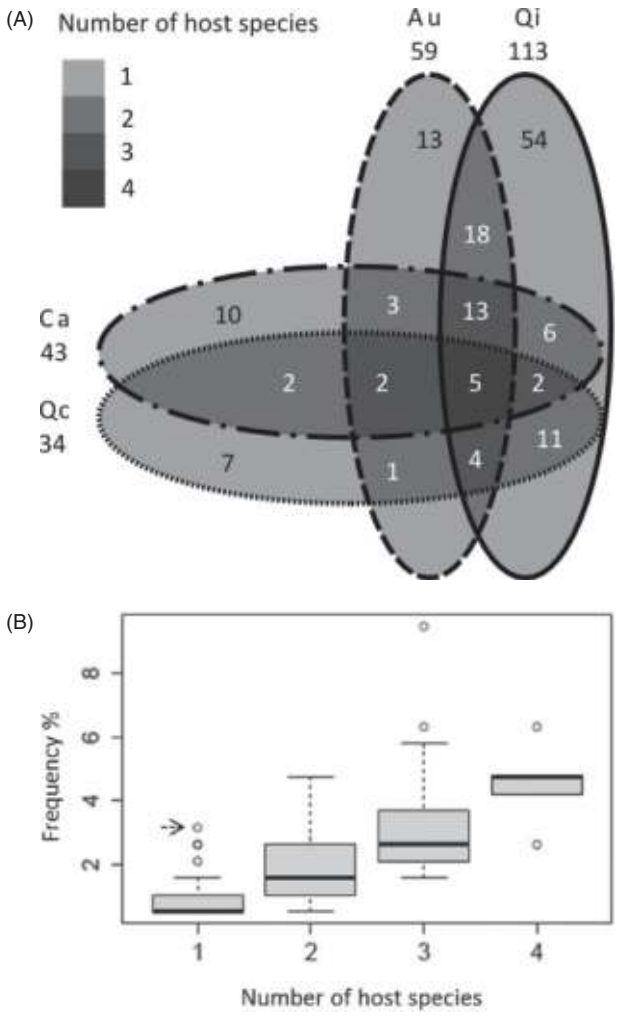


Fig. 2. A. Venn diagram representing the distribution of ECM fungal OTUs on *Arbutus unedo* (*Au*), *Cistus albidus* (*Ca*), *Quercus ilex* (*Qi*) and *Q. coccifera* (*Qc*) root tips. Shaded zones indicate the number of fungal OTUs found on roots of one, two, three and all four host species.
B. Distribution of OTUs according to the number of their associated host species and their frequency in soil cores. The arrow indicates the location of *T. melanosporum*.

Accordingly, the five fungal OTUs present on four host species belonged to the eight most frequent OTUs on the roots of all host species.

Fungal sharing by host plant species

Of the total number of root tips examined, the proportion occupied by fungal OTUs that were shared with at least one other species ranged from 52.2% for Qi to 79.4% for Qc (Fig. 2A). *Ca* and *Au* showed similar patterns to *Qc*, with 76.7% and 78% of fungal OTUs shared with other host species (Fig. 1C). A bidirectional representation of symbiont sharing between host species showed a different pattern on *Qi* than on the three other host species: the proportion of OTUs of the ECM community of Qi also shared with other host species varied from 19% to 35% from the *Qi* side, while it represented 60%, 63% and 68% of OTUs, of the community of *Ca*, *Qc* and *Au* respectively (Fig. 2C).

Distribution patterns of Tuber *species across host species and production stages*

In all, five OTUs were assigned to Tuberaceae as defined by Bonito and colleagues (2010), including *Tuber ferrugineum* (OTU 146) and *Tuber sp.1* (OTU 149) in the rufum clade, *T. oligospermum* (OTU 147) and *T. borchii* (OTU 150) in the *puberulum* clade and *T. melanosporum* (OTU 148; Fig. S2).

The analysis of the distribution patterns of the five species of Tuberaceae across host species and production stages showed that (i) all species but *T. melanosporum* were shared by at least two host species; (ii) *T. melanosporum* was restricted to *Qi*; (iii) the five species of *Tuber* were present on only recently arisen brûlés (stage *a*); (iv) *Tuber ferrugineum* (Table 4) within the rufum clade (Fig. S2) was the only species present across the four production stages and in the relict forest and (v) *T. oligospermum* was the only species present on roots of all four host species (Table 4).

Table 4. Relative abundance of Tuberaceae species across production stages and host species. Abbreviations indicate the identity of species of Tuberaceae assigned as defined by Bonito and colleagues (2010): T. fer for *T. ferrugineum*; T. oli for *T. oligospermum*; T. bor for *T. borchii*; *T. mel* for *T. melanosporum* and T. sp1 for a species within the rufum clade. Shading indicates the cumulative abundance in percent of species of Tuberaceae per host per production stage (0–5%, 5–10%, 10–15%, > 15%).

	Production stages			
Host species	Immature brûlés	Productive brûlés	Post-productive brûlés	Relict forest
A. unedo	T. bor/T. oli	T. oli	T. oli/T. sp1	
C. albidus	T. oli/T. fer	T. oli	T. sp1	
Q. coccifera		T. oli	T. fer	
Q. ilex	T. bor/T. oli/T. mel/T. sp1	T. oli/T. mel/T. fer	T. bor/T. oli/T. fer	T. fer
Number of OTUs	5	3	4	1

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*

Distribution patterns of T. melanosporum *across host species and production stages*

Root tips of *T. melanosporum* were found in one out of 13 recently arisen brûlés (stage *a*) and in five out of 14 productive brûlés (stage *b*), where it was the second most frequent species (4.25%) after OTU 33 (uncultured Pyrenomataceae). Based on comparison of its observed frequency (number of soil cores in which it was present) with that given by the null model, *T. melanosporum* was a significantly over-represented OTU among the single-host fungi ($P = 0.049$): according to its frequency, more than one associated host species would have been expected according to the null model. In other words, *T. melanosporum* is over-represented on *Qi* compared with the general distribution pattern of OTUs among host species according their frequency, which indicates a particular host affinity of *T. melanosporum* for *Qi*.

The concentration of *T. melanosporum* mycelium in soil varied significantly across production stages and among plots of the same stage (Fig. 3). A Fligner–Killeen test showed a significantly over-dispersed distribution pattern across plots (df = 3; Chi2 = 10.45; $P = 0.015$). The mycelium biomass was significantly higher in productive brûlés than in post-productive brûlés and relict forests ($P = 0.018$ and 0.028 by Wilcoxon test for *b* versus *c* and *b* versus *d* respectively).

The distribution patterns of *T. melanosporum* mycelia were consistent with patterns of root tip occurrence, the highest values of mycelium concentration corresponding to plots where *T. melanosporum* was also detected as root tips.

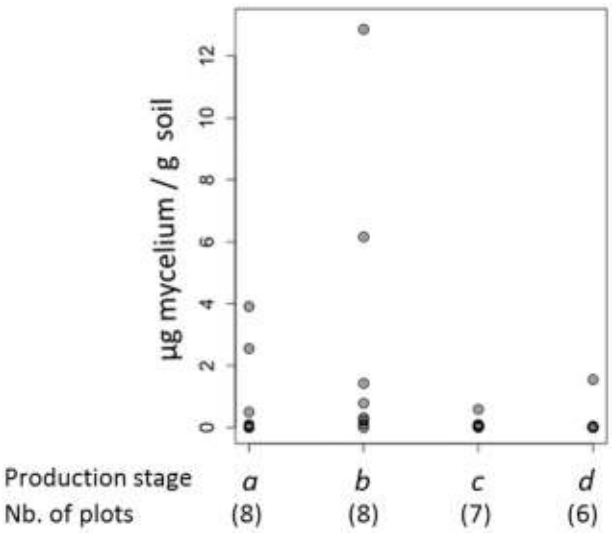


Fig. 3. Concentration of *T. melanosporum* mycelia in soils collected in immature brûlés (*a*), productive brûlés (*b*), post-productive brûlés (*c*) and relict forests (*d*) from sites 1, 2 and 3.

Discussion

Our study provides three main insights into the structure and dynamics of ECM fungal communities during ecological succession in Mediterranean regions. First, before canopy closure, shrubs and trees harbour multi-host fungi within species-rich and highly connected ECM below-ground communities, where multi-host fungal symbionts dominate in frequency (Fig. 2B). Second, in natural *T. melanosporum* truffle grounds, ECM communities are spatially structured at the among-brûlé scale and little influenced by host species and brûlé production status (i.e. presence or absence of truffle ascocarps; Table 4). Third, in this ecological context where *T. melanosporum* establishes spontaneously, both the frequency of ECM tips and the distribution patterns of soil mycelia only weakly mirror fruiting patterns within brûlés.

Thelephoraceae and Pyronemataceae dominate in Q. ilex *forests*

On all host species and in all production stages, half of ECM tips revealed either a Thelephoraceae or a Pyronemataceae (Fig. 1A). The dominance of these two families was expected because a similar pattern has been regularly observed in natural truffle grounds, whatever the focal species of *Tuber*, including *T. melanosporum* (Belfiori *et al*., 2012), *T. macrosporum* (Benucci *et al*., 2014) and *T. magnatum* (e.g. Murat *et al*., 2005; Leonardi *et al*., 2013; Salerni *et al*., 2014), as well as in truffle plantations (see De Miguel *et al*., 2014 for a review).

The observed ratio of Ascomycetes to Basidiomycetes strikingly contrasts with that reported from mature forest ecosystems (see Horton and Bruns, 2001 for a review), and particularly with that from late successional *Q. ilex* forests: in old and mature *Q. ilex* forests (Richard *et al*., 2005; 2011; Shahin *et al*., 2013), Thelephoraceae consistently dominate ECM communities, and to a lesser extent Russulaceae and Cortinariaceae, but very few ECM Ascomycetes colonize roots, except the dominant *Cenococcum geophilum*. Our results suggest that Thelephoraceae may continuously make up the dominant family during aging of *Q. ilex* forests, while the proportion of Ascomycetes (particularly Pyronemataceae) may decline during succession, especially after canopy closure. These ascomycetes encompass species that may preferentially establish in early-successional vegetation. In all, these distribution patterns may reflect the contrasted ecological strategies (pioneer versus late stage) underlying ECM fungal diversity (Dickie *et al*., 2013) and suggest that some temporal partitioning among fungal species may occur during forest development.

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*

T. melanosporum *brûlés reveal hyper-diverse and structured ECM communities*

We showed that ECM communities in the three stages of brûlés, whatever their production status, are highly diverse (Table S2) and display a clustered OTU distribution at the levels of core (43% of all OTUs were found in a single core), plot (49.7% in a single plot) and site (58.3% in a single site). Interestingly, the contribution of each host species to these hyper-diverse and patchy communities was in the same range, as indicated by close rarefied richness values (Table S2) and similar OTU accumulation curves (Fig. S1) for the different hosts. The predominance of infrequent OTUs is in accordance with previously observed patterns in old *Q. ilex* forests (Richard *et al*., 2011) and in naturally established brûlés of other truffle species. In a study comparing natural and cultivated *T. melanosporum* truffle grounds, Belfiori and colleagues (2012) reported high values of diversity indexes at natural sites and lower values in plantations. Conversely, high values of Shannon–Wiener indexes were calculated for natural truffle grounds of *T. magnatum* (Leonardi *et al*., 2013) and *T. macrosporum* (Benucci *et al*., 2014), with no clear difference between productive and non-productive locations for the latter species.

Three successive analyses of variance (PERMANOVA) showed that all the controlled factors included in our sampling scheme (i.e. site, plot, host species, production stage and canopy host species) significantly contributed to the distribution of fungal OTUs among samples, when a sample was defined as all ECM tips from a given host species in a given plot (Table 3). The tested factors explained a significant but moderate part of the variance of species distribution patterns: site, host species, canopy species and production stage accounted for only 2.9% to 4.7% of the variance among samples, whereas plot explained 39.6% of the variance among samples. The plot factor, of course, had a larger number of degrees of freedom than did other factors (Table 3). In detail, four noteworthy trends were revealed. First, two samples from the same site shared more OTUs on average than did two samples randomly taken from the whole dataset. Second, two co-occurring host species within the same plot shared more OTUs on average than did two samples randomly taken from the whole dataset. These two results illustrate the multi-scale patchiness and structuring by distance of ECM diversity (Lilleskov *et al*., 2004; Pickles *et al*., 2010; Anderson *et al*., 2014). Moreover, this suggests that the measured host sharing at the level of the whole dataset (Fig. 2A) is locally effective at the plot scale. Noticeably, fungal sharing was certainly underestimated in our study, because our sampling strategy excluded picking replicates of the same morphotype within a single core. Third, two samples taken under the canopy of the same plant species shared more OTUs on average than did two samples randomly taken from the whole dataset, even when some ECM root tips belonged to a host other than the one forming the canopy (Table S1). Our systematic identification of plant and fungal partner of each ECM tip showed that the spatial distribution of canopies hardly reflects the distribution of roots in these mixed ECM plant communities, and that roots of ECM hosts intermingle in soils (Table S1). Our results indicate that the composition of the forest canopy influences the composition of the ECM fungal communities as much as root host species ($R^2 = 0.036$; $R^2 = 0.029$ respectively; Table 3). This may result from influences of canopy on direct (chemical composition of the resulting litters; e.g. Belsky *et al*., 1993) and/or indirect (resulting micro-environmental conditions at ground level, e.g. humidity, temperature) environmental parameters (Conn and Dighton, 2000; Cullings *et al*., 2003; Prescott and Grayston, 2013), which still need to be deciphered. Fourth, two samples taken in separate plots of the same production stage shared more OTUs on average than did two samples randomly taken from the whole dataset. This influence was as weak as that of other factors (e.g. host species), and we could not find fungal OTUs specific to a given host species or a production stage. However, a global signal emerged at the whole community scale (Table 3).

Yet the relatively high similarity between ECM communities in productive and non-productive brûlés contrasts with results of previous studies comparing ECM communities within and outside fruiting zones of focal species of Ascomycetes or Basidiomycetes. For instance, fungal communities beneath *Tricholoma matsutake* fairy rings are particularly species poor, and significantly differ from those inside and outside the fairy rings (Lian *et al*., 2006). Our results suggest that stochastic changes in composition of ECM fungal communities, rather than directional shifts, accompany the dynamics of natural *T. melanosporum* truffle grounds from immature to post-productive brûlés.

Co-occurring hosts share frequent ECM symbionts on T. melanosporum *brûlés*

We showed that in brûlés and in the *Q. ilex* forest, a majority of ECM tips of all host species were associated with multi-host fungi (Fig. 1C). The steep slope of the interaction accumulation curve compared with that of the OTU accumulation curve summarizes this property of the ECM community (Fig. S1). Thanks to a systematic typing of hosts and symbionts, our study revealed that, in a complex plant community, frequent ECM fungal OTUs tend to be shared by several host species (Fig. 2B; right-hand part). These results confirm and extend previous research that mostly documented pairs of coexisting host

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*

species (e.g. Horton and Bruns, 1998; Kennedy *et al*., 2003; Richard *et al*., 2005; Morris *et al*., 2008) and sometimes more than two coexisting host species (Ishida *et al*., 2007; Lang *et al*., 2011). Our results also provide evidence in favour of a previously hypothesized trade-off between specificity and abundance of ECM fungi (Vazquez *et al*., 2005; Bahram *et al*., 2014). Alternatively, these patterns could arise from under-sampling of ECM communities, as indicated by the unsaturated OTU accumulation curves obtained for all host species (Fig. S1). For this reason, caution is required before concluding about the apparent specificity of infrequent OTUs (Fig. 2B; left-hand part). Assuming under-sampling, the more frequently sampled an ECM OTU, the larger the number of its hosts. However, this scenario cannot readily explain the apparent absence of frequent single-host fungal OTUs (Fig. 2B; right-hand part) in ECM communities, which generally include fungal species that widely vary in their degree of specificity toward host species (Bruns *et al*., 2002). Thus, the observed absence of abundant highly specific OTUs may indicate an ecological pattern of these ECM communities rather than a consequence of under-sampling.

This study provides a detailed view of fungal sharing among four different ECM hosts in Mediterranean landscapes. The picture obtained shows that *Q. ilex* shares a considerably smaller part (*ca.* one-half) of its OTU diversity with coexisting host species than do the three other species (which have *ca.* three-fourths of their OTUs shared with another host Fig. 2A). Our results also reveal a negative correlation between the sampling effort (number of sequenced ECM tips per host species) and the level of fungal sharing (comparing Fig. 2A and Table S1). Both results suggest that fungal sharing among host species must be analysed on samples of similar size for a satisfactory comparison of the relative contribution of each host species to plant-to-plant fungal links. When we used SESJ-distances to minimize sampling effects, we showed that two pairs of host plant species among the six possible pairs maximized their fungal sharing in terms of ECM assemblage resemblances, *A. unedo* and *C. albidus*, and to a lesser extent *A. unedo* and *Q. ilex* (Fig. 1A). Interestingly, the first pair are phylogenetically distant (*A. unedo* is the most distant phylogenetically from the three other species studied) but are the only two shrub species in our study. This result is in line with previous research showing that during ecological succession, co-occurring host species share more ECM fungal symbionts than do plants of different successional stages (e.g. Horton *et al*., 2005; Ishida *et al*., 2007; Teste *et al*., 2009). This trend had more impact here than any phylogenetic conservatism. Fungal sharing between *Q. ilex* and *A. unedo* has been previously documented for acidic soils (Richard *et al*., 2005), where *A. unedo* also appears in early succession (as in the present study), and where shared fungi represent 15% of taxonomic diversity, but 69% of the sampled roots. During secondary successions, various Arbutoideae species are supposed to be involved in facilitation processes that may be mediated by shared ECM fungal symbionts (Richard *et al*., 2009; Kennedy *et al*., 2012). Our results extend our knowledge of the ecology of these mid-successional shrubs that may be involved in the establishment of potential *T. melanosporum* hosts in Mediterranean landscapes.

ECM tips and mycelium partially reflect the production status of T. melanosporum *brûlés*

In our study, *T. melanosporum* ECMs were found only on *Q. ilex*, in spite of their frequency (Table 4). This is not a consistent pattern for the *Tuber* genus, since the four other OTUs were found on at least two host species each. The absence of *T. melanosporum* on *Q. coccifera*, *C. albidus* and *A. unedo* roots was unexpected regarding the evidence of ECM association, respectively, between *T. melanosporum* and diverse oak species (Callot, 1999), Tuberaceae and Cistaceae (Giovannetti and Fontana, 1982; Comandini *et al*., 2006; García-Montero *et al*., 2007) and Tuberaceae and *Arbutus* spp. (Kennedy *et al*., 2012; Lancellotti *et al*., 2014). Concerning *Q. coccifera*, we provided the first insights into its ECM fungal diversity in a season that is classically considered as optimal to describe Mediterranean ECM communities (Richard *et al*., 2005). However, despite similar sampling efforts under the canopy of each host species, the number of ECM tips finally assigned to this species after host molecular identification was modest. This result suggests that the temporal and/or spatial distribution of ECM diversity on roots of *Q. coccifera* may differ from the other studied ECM hosts. Interestingly, the production of *Q. coccifera's* fine root is reduced in winter (Kummerow *et al*., 1990) when conditions are optimal to describe ECM diversity on *Q. ilex* and *A. unedo* roots (Richard *et al*., 2005). At the scale of the whole study system, additional sampling in different seasons would specify the distribution of *T. melanosporum* on its potential Mediterranean hosts, and usefully increase our knowledge of the temporal dynamics of ECM communities (Courty *et al*., 2008) in an ecological context that is still poorly documented.

In *T. melanosporum* brûlés, the black truffle was detected more frequently (as ECM tips) and more abundantly (as mycelia) before or at the production stage (*a* and *b*) than in non-productive stands (*c* and *d*; Fig. 3; Table 4). For each stage, and particularly at the production stage (*b*), the distribution pattern of soil mycelium tended to be heterogeneous (Fig. 3). Heterogeneous distributions of vegetative *T. melanosporum* structures were observed in previous studies comparing productive and

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*

non-productive truffle grounds (Águeda *et al*., 2010; Parladé *et al*., 2013), and the biomass of mycelium in soils may vary significantly between seasons, as reported for some ECM fungal species (De la Varga *et al*., 2013). The significant finding of our study was that some samples from immature and productive brûlés did contain high levels of truffle mycelium. These results support a developmental sequence where vegetative growth occurs before some fruiting eventually starts, and is substantially reduced in brûlés that no longer fruit. Similarly, in *T. melanosporum* orchards, Liu *et al*. (2014) reported significant expansion patterns of the spatial distribution of extra radical mycelium from a 20-year chronosequence design.

In our study, *T. melanosporum* became temporally the second most frequent OTU among ECM tips at the stage of productive brûlés (*b*). However, the frequency of ECM tips only partially reflects the presence of a *T. melanosporum* brûlé: in a majority of productive and all but one newly arisen brûlés (Table 4), we could not detect root tips infected by *T. melanosporum*. Our results again illustrate the well-known discrepancy between the distribution patterns of fruiting bodies and ECM tips (e.g. Gardes and Bruns, 1993; Koide *et al*., 2005). Similarly, the distribution patterns of soil mycelia only partially mirrored the production status of brûlés (Fig. 3). Additional soil sampling would be necessary to compare the respective efficiency of mycelia and root tips in predicting fungal presence in this context (Anderson *et al*., 2014). For the specific case of *T. melanosporum*, a recent study showed that the detection of the mating type in soil extracts can be a promising predictor of *T. melanosporum* production (Zampieri *et al*., 2012).

In published work dealing with the particular case of *T. melanosporum* truffle grounds, all vegetative parts, the ECM tips and the mycelium are generally reported to be more abundant in productive stands than non-productive ones (Águeda *et al*., 2010; Zampieri *et al*., 2012; Parladé *et al*., 2013), with few exceptions (Suz *et al*., 2008). There is no clear threshold indicating the onset of ascocarp production (De la Varga *et al*., 2012; Parladé *et al*., 2013) and the presence of ECM tips can even be maintained over several decades in plantations without production (Águeda *et al*., 2010). Similarly, the distribution of ECM tips poorly reflects the production of *T. magnatum* (Murat *et al*., 2005; Leonardi *et al*., 2013).

Interestingly, mycelium distribution in soils was highly heterogeneous, and mycelium abundance was remarkably low in all brûlés, even in productive ones, as compared with the observed values in plantations, albeit at a different season (Zampieri *et al*., 2012; Parladé *et al*., 2013). For instance, the measured mycelium concentration was more than 10 times lower than the concentrations measured by Parladé and colleagues (2013). As suggested for ECM root tips, potential seasonal mycelium dynamics may occur and should be investigated in diachronic studies.

Conclusion

Beyond a detailed description of the diverse ECM communities in early-successional shrub Mediterranean vegetation, which has been poorly documented by fungal ecologists so far, the present work throws a spotlight on the ecology of *T. melanosporum* in natural ecosystems. Although the sampling of the fungal biodiversity in such ecosystems is still far from complete, and needs to be replicated in other seasons, our results raise questions about the way some ECM ascomycetes achieve an efficient pioneer strategy, i.e. establish early and subsequently dominate in hyper-diverse ECM communities. This work, which has documented for the first time the patterns of distribution of different vegetative parts of *T. melanosporum* (ECM tips versus mycelium), confirms the patchiness of this fungus, in contrasting contexts of natural production. In contrast to most ECM fungal species surrounding it, which are multi-host, *T. melanosporum* shows a marked affinity for *Q. ilex*. This work emphasizes the ecological strategy of *T. melanosporum* and mainly its surprising ability to establish and temporarily dominate hyper-diverse fungal communities.

Experimental Procedures

Study area

The research was performed in a set of private forests located around Pézilla-de-Conflent (southern France; 42°44′20.71′N, 2°29′12.02’E; elevation 240–763 m). The climate is Mediterranean with most rainfall in spring and autumn, reaching 572.4 mm yr^{-1}; monthly average temperatures vary between 4.4°C (mean January minima) and 29.2°C (mean July maxima). Soils are developed on a metamorphic limestone substrate. Physico-chemical properties of the organo-mineral horizon include a slightly basic pH (mean pH = 8.3 and mean pH_{KCl} = 7.6), with a silt loamy texture (11.64% clay, 40.3% silt, 48.1% sand) containing from 2.4% to 4.6% of organic matter (mean C: N ratio = 17.8).

Truffle grounds at the site established naturally on abandoned agricultural terraces that were previously cultivated as vineyards until the vines were uprooted in the early 1970s. The re-colonizing vegetation is made of shrub vegetation (the so-called ‘garrigue’ in French) dominated by *Phyllirea latifolia*, *A. unedo, C. albidus, Cistus ladanifera*, *Fumana ericoides* and *Juniperus oxycedrus* surrounding a discontinuous herbaceous layer. This vegetation is progressively colonized by *Q. ilex* and *Q. coccifera*, which form diffuse patches (< 25% cover) of various sizes. The four most abundant ECM host species, *Q. ilex, Q. coccifera*, *C. albidus* and *A. unedo*, hereafter abbreviated *Qi*, *Qc*, *Ca* and *Au*, respectively, were all sampled in the study.

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*

Sampling design

Five sampling sites located at least 1 km apart were selected according to the simultaneous presence of relict forest patches and truffle grounds naturally established after agricultural activity had stopped. On these sites, plots with four different production stages were sampled, including: immature brûlés of recent origin, where visible impact of *T. melanosporum* on vegetation had been observed for less than 2 years and where truffles were not yet producing (stage *a*); productive brûlés, which had produced at least one truffle per season for at least 6 years (stage *b*); post-productive brûlés, which had ceased to produce truffles at least 6 years previously (stage *c*); and relict forests, i.e. patches of managed forests preserved from agricultural disturbance over at least the last 100 years, dominated by trees at canopy closure (stage *d*). On each site, replicate plots of the four different production stages were selected, thanks to the accurate knowledge of P. Bernadach, the owner of truffle grounds at all investigated sites. Because not all sites contained all production stages, from seven to 12 sampling plots were designated per site (Table 1).

Soil and ECM root sampling

Soil cores (diameter: 10 cm; depth: 15 cm) were collected in February 2011. On each plot, soil cores were drilled under the canopies of the four target host species (*Au*, *Ca*, *Qc*, *Qi*) at a distance of 20 cm from the base of the trunk. For each plant host species in each plot, two to three replications were made. For *Ca* and *Au*, these corresponded to different randomly selected individuals. Because *Quercus* species were often represented by a single individual on each plot, for *Qi* and *Qc*, three replications were made around the focal tree individual (approximately 1 m apart from each other). Because not all plots contained all four host species, from three to eight cores were collected per plot (Table 1). They were stored at 4°C after sampling and treated in the laboratory as follows. In each core, ECM roots were manually separated from the soil matrix. The resulting soil volumes were sieved and stored at –20°C, while sorted roots were washed gently and examined for assessment of ECM diversity. In each soil core, all ECM root tips were classified into morphotypes under a dissecting microscope according to Richard and colleagues (2005). Each core was treated independently, i.e. no attempt was made to assign ECM root tips of similar morphology to a given morphotype when they came from different cores. In each core, from one to five ECM tips of each morphotype were hand-picked, rinsed twice in distilled water and stored dry at –20°C.

Molecular identifications

In each soil core, molecular analysis of one randomly selected ECM root tip per morphotype was conducted in order to identify both plant and fungal partners.

Identification of ECM fungi. Total deoxyribonucleic acid (DNA) was extracted from ECM tips with the REDExtract-N-Amp Plant polymerase chain reaction (PCR) kit (Sigma-Aldrich, St Louis, USA) according to the manufacturer's recommendations. The internal transcribed spacer (ITS1-5.8S-ITS2) region of the fungal nuclear ribosomal DNA was amplified by PCR using the ITS1F – ITS4 primer pair (Gardes and Bruns, 1993). The PCR reaction was performed in a 20 μl reaction volume containing 10 μl of REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich, St Louis, USA), 1 μl of each primer at 10 μM, 6 μl of distilled water and 2 μl of extracted DNA. Amplified DNA was checked on 1.5% agarose gels and (whenever a single clear fragment was observed) sequenced using the primers used in PCR. Amplicons revealing more than one ITS fragment were re-amplified using ITS4B instead of ITS4 to target ECM Basidiomycetes and exclude endophytic or contaminant Ascomycetes (Rodriguez *et al.*, 2009), which are often responsible for multiple ITS amplification. Raw sequence data were edited using CodonCode Aligner (CodonCode, Centerville, MA, USA) and operational taxonomic units (OTUs) were delineated at the 97% similarity threshold (Hughes *et al.*, 2009). For each OTU, consensus sequences were identified using the massBLAST algorithm (http://unite.ut.ee/) and the UNITE, GenBank, EMBL, DDBJ databases. Sequences were taxonomically assigned to the nearest blast name at the species, genus or family level when their similarity was more than 97%, between 95% and 97%, and less than 95% respectively. One sequence per OTU was deposited in GenBank under accessions no. KM247623 – KM247779 (Table S3).

Identification of host species. In order to identify the species hosting, all successfully sequenced ECM tip DNA, extracted DNA was amplified by PCR using the ITS1P – ITS4 primer pair as in Selosse and colleagues (2002). After verification on a 1.5% agarose gel, 15 μl aliquots of amplified DNA were digested by BsaWI endonuclease, which was selected for its ability to provide unambiguously different RFLP patterns for *Au* (163/122/489 bp), *Ca* (707/74 bp), *Qi* (478/301 bp) and *Qc* (779 bp). Polymerase chain reaction products were size fractionated on 2% agarose gels. For each RFLP pattern, five randomly selected samples were sequenced to confirm host identity.

Assessment of T. melanosporum *mycelium distribution in soil.* Total DNA was extracted from sieved soils belonging to stages *a* (eight plots), *b* (eight plots), *c* (seven plots) and *d* (six plots) (Table 1) with the kit Power Soil (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. In order to provide a representative view of each sampled plot, soil aliquots (2 g) from four independent soil cores per plot were pooled and analysed as follows. To quantify the extra radical mycelium of *T. melanosporum*, we used quantitative Taqman PCR (qPCR) with the primers and probe described in Parladé and colleagues (2013). Triplicate real-time PCR was performed on each sample using the 2X Takara Premix Ex Taq (Perfect Real Time, Takara Bio Europe, SAS, France), the specific oligos at a concentration of 800 nM for each primer and 200 nM for the probe, 5 μl of the template DNA and high-performance liquid chromatography (HPLC) water to adjust to a final reaction volume of 20 μl. The thermocycling program was 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 34 s, and was performed in a StepOne Plus Real-Time PCR System machine provided with the StepOne software v. 2.3 (Life

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*

Technologies, Carlsbad, CA, USA). For each site, a standard was prepared by adding 0.01 g of fresh immature *T. melanosporum* ascocarp to 0.24 g of soil collected in an adjacent field without ECM hosts. Deoxyribonucleic acid extraction was performed on each standard as above. A triplicate of four serial 10-fold dilutions of the standard extraction was used to plot a standard curve per site. The number of cycles (Ct values, i.e. number of cycles at which the fluorescent signal exceeds the background level in the exponential phase of the amplification) obtained for each dilution was plotted against the corresponding initial amount of ascocarp to generate the standard curve. Thus, a direct correspondence was obtained between the DNA extracted from known amounts of fresh ascocarp and the Ct values. Absolute quantification of mycelium biomass of *T. melanosporum* was expressed in μg of mycelium per g of soil for each soil sample.

Statistical analyses

Species diversity was estimated using the R package Vegan (Oksanen, 2011) to calculate four diversity estimators classically used in ECM community analyses: (i) the rarefied number of OTUs as a size-independent richness proxy of samples; (ii) Simpson's diversity index (1-D); (iii) the Shannon–Wiener information index (H′) and (iv) Fisher's alpha (α). The influence of sampling scales (site and plot; Table 1) on ECM community composition was tested using permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) in R package Vegan.

The influence of plant species (*Au, Qi, Qc, Ca*; identified on root tips, or as a component of above-ground canopy species) and production stage (*a*, *b*, *c*, *d*) on ECM community composition was analysed as follows. First, the overall effect of the two factors was tested using PERMANOVA as above. Second, paired comparisons were performed to compare the ECM fungal communities of each host and each production stage with those of all the others. These pair-wise comparisons were achieved by calculating Jaccard distances [J(*A*, *B*)] using the R package Vegan (Oksanen, 2011). In order to assess if the observed Jaccard distances differed significantly from dissimilarities that would be expected by chance alone, we used a null model approach (Gotelli and Entsminger, 2001; function *permatswap* in R library Vegan with the argument fixedmar = 'both'). The swap null model randomized the identity of OTUs (composition) among ECM assemblages of a given modality (i.e. either one of the four host species or one of the four production stages) but conserved the number of OTUs (richness) and the relative abundances of each OTU (structure) within each assemblage. The same pair-wise J-distances were calculated on 9999 randomized datasets and were used to calculate a standard effective size Jaccard distance (SESJ-distance), which minimizes the effect of unequal sizes of samples based on the Eq. (1):

$$\text{SESJ-distance}\,(\mathrm{A},\mathrm{B}) = \frac{\mathrm{J}(A,B) - \overline{[\mathrm{J}(A,B)]nm}}{\sigma[\mathrm{J}(A,B)]nm} \qquad (1)$$

where $\overline{[\mathrm{J}(A,B)]nm}$ and $\sigma[\mathrm{J}(A,B)]nm$ are the mean and the standard deviation, respectively, of Jaccard distances given by the null model.

To analyse the relationships between ECM fungal frequency and number of associated host species, OTUs were classified into four classes depending on the number of their associated host species, from one to four. The correlation between the frequency of OTUs and the number of their hosts was estimated using Pearson's correlation coefficient. The influence of host and production stage on *T. melanosporum* abundance in soil was first assessed by examining abundance on ECM tips, by comparing the distribution patterns of *T. melanosporum* ECM tips on the four host species with a random distribution (previous described null model). If there was no significant difference from the distribution under the null model (9999 randomizations), it was considered that the pattern of *T. melanosporum* distribution could not be distinguished from that which would occur at random. The *P*-value was the proportion of the total number of host species in the null model that differed from the observed one.

Second, we investigated the influence of production stage on *T. melanosporum* abundance as measured by soil mycelium concentration. As the distribution of *T. melanosporum* concentration in soil at the plot level was non-normal (Shapiro test, $P < 0.05$), the homogeneity of variances among the four different production stages was tested by the non-parametric Fligner–Killeen test, which is robust against deviations from normality. Differences of mycelium abundances among production stages were tested by a Wilcoxon test. All these tests were performed using the R software (R_Development_Core_Team, 2014).

Acknowledgements

E. Taschen's PhD thesis work is funded by the Fondation de France (agroforestry research grants) and the Agence Nationale de la Recherche (grant 'Bases for a sustainable ecological monitoring of ecosystems producing truffles' (SYSTRUF), to M.-A. Selosse and F. Richard). Sequencing was financed by the grant 'Diversité des champignons mycorhiziens des plantes' (DivMyc, to M.-A. Selosse) from the network Bibliothèque Du Vivant funded by CNRS, Museum National d'Histoire Naturelle and INRA. Financial support for mycelium analysis was provided by the Spanish Ministry of Economy (MINECO), project AGL2012-40035-C03 to J. Parladé. We warmly thank Pierre Bernadach for providing access and communicating his knowledge of the study system. We are grateful to D. McKey for precious comments and for correcting the English of the manuscript, to G. Bonito for providing Tuber sequences, to E. Baudouin, M. Ducousso, O. Domergues, A. Galiana, D. Mousain, C. Roux and Y. Prin for help with soil sampling, to J.-M. Bellanger, L. Benoit and M.-P. Dubois for advice on the molecular work and to two anonymous referees for their advice.

References

Águeda, B., Fernández-Toirán, L., Miguel, A.M., and Martinez-Peña, F. (2010) Ectomycorrhizal status of a mature productive black truffle plantation. *For Syst* **19:** 89–97.

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*

Anderson, I.C., Genney, D.R., and Alexander, I.J. (2014) Fine-scale diversity and distribution of ectomycorrhizal fungal mycelium in a Scots pine forest. *New Phytol* **201:** 1423–1430.

Anderson, M.J. (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecol* **26:** 32–46.

Bahram, M., Harend, H., and Tedersoo, L. (2014) Network perspectives of ectomycorrhizal associations. *Fungal Ecol* **7:** 70–77.

Belfiori, B., Riccioni, C., Tempesta, S., Pasqualetti, M., Paolocci, F., and Rubini, A. (2012) Comparison of ectomycorrhizal communities in natural and cultivated *Tuber melanosporum* truffle grounds. *FEMS Microbiol Ecol* **81:** 547–561.

Belsky, A.J., Mwonga, S.M., Amundson, R.G., Duxbury, J.M., and Ali, A.R. (1993) Comparative effects of isolated trees on their undercanopy environments in high- and low-rainfall savannas. *J Appl Ecol* **30:** 143–155.

Benucci, G.M.N., Raggi, L., Albertini, E., Gógán Csorbai, A., and Donnini, D. (2014) Assessment of ectomycorrhizal biodiversity in *Tuber macrosporum* productive sites. *Mycorrhiza* **24:** 281–292.

Bingham, M.A., and Simard, S.W. (2011) Do mycorrhizal network benefits to survival and growth of interior Douglas-fir seedlings increase with soil moisture stress? *Ecol Evol* **1:** 306–316.

Bonito, G.M., Gryganskyi, A.P., Trappe, J.M., and Vilgalys, R. (2010) A global meta-analysis of *Tuber* ITS rDNA sequences: species diversity, host associations and long-distance dispersal: global meta-analysis of *Tuber* ITS rDNA. *Mol Ecol* **19:** 4994–5008.

Bruns, T.D., Bidartondo, M.I., and Taylor, D.L. (2002) Host specificity in ectomycorrhizal communities: what do the exceptions tell us? *Integr Comp Biol* **42:** 352–359.

Callot, G. (1999) La truffe, la terre, la vie. Paris, France: Editions INRA.

Comandini, O., Contu, M., and Rinaldi, A.C. (2006) An overview of *Cistus* ectomycorrhizal fungi. *Mycorrhiza* **16:** 381–395.

Conn, C., and Dighton, J. (2000) Litter quality influences on decomposition, ectomycorrhizal community structure and mycorrhizal root surface acid phosphatase activity. *Soil Biol Biochem* **32:** 489–496.

Courty, P.-E., Franc, A., Pierrat, J.-C., and Garbaye, J. (2008) Temporal changes in the ectomycorrhizal community in two soil horizons of a temperate oak forest. *Appl Environ Microbiol* **74:** 5792–5801.

Cullings, K.W., New, M.H., Makhija, S., and Parker, V.T. (2003) Effects of litter addition on ectomycorrhizal associates of a Lodgepole Pine (*Pinus contorta*) stand in Yellowstone National Park. *Appl Environ Microbiol* **69:** 3772–3776.

De la Varga, H., Águeda, B., Martínez-Peña, F., Parladé, J., and Pera, J. (2012) Quantification of extraradical soil mycelium and ectomycorrhizas of *Boletus edulis* in a Scots pine forest with variable sporocarp productivity. *Mycorrhiza* **22:** 59–68.

De la Varga, H., Águeda, B., Ágreda, T., Martínez-Peña, F., Parladé, J., and Pera, J. (2013) Seasonal dynamics of *Boletus edulis* and *Lactarius deliciosus* extraradical mycelium in pine forests of central Spain. *Mycorrhiza* **23:** 391–402.

De Miguel, A.M., Águeda, B., Sánchez, S., and Parladé, J. (2014) Ectomycorrhizal fungus diversity and community structure with natural and cultivated truffle hosts: applying lessons learned to future truffle culture. *Mycorrhiza* **24:** 5–18.

Dickie, I.A., Martínez-García, L.B., Koele, N., Grelet, G.-A., Tylianakis, J.M., Peltzer, D.A., and Richardson, S.J. (2013) Mycorrhizas and mycorrhizal fungal communities throughout ecosystem development. *Plant Soil* **367:** 11–39.

Diette, S., and Lauriac, A. (2004) La sylviculture truffière: Aperçus historiques, apports techniques et enjeux pour la région méditerranéenne. *Rev For Fr* **56:** 219–230.

García-Montero, L.G., Casermeiro, M.Á., Manjón, J.L., and Hernando, I. (2007) Impact of active soil carbonate and burn size on the capacity of the rockrose *Cistus laurifolius* to produce *Tuber melanosporum* carpophores in truffle culture. *Mycol Res* **111:** 734–739.

Gardes, M., and Bruns, T.D. (1993) ITS primers with enhanced specificity for Basidiomycetes – application to the identification of mycorrhizae and rusts. *Mol Ecol* **2:** 113–118.

Giovannetti, G., and Fontana, A. (1982) Mycorrhizal synthesis between Cistaceae and Tuberaceae. *New Phytol* **92:** 533–537.

Girlanda, M., Selosse, M.A., Cafasso, D., Brilli, F., Delfine, S., Fabbian, R., *et al*. (2006) Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal Russulaceae. *Mol Ecol* **15:** 491–504.

Gómez-Aparicio, L., Zamora, R., Gómez, J.M., Hódar, J.A., Castro, J., and Baraza, E. (2004) Applying plant facilitation to forest restoration: a meta-analysis of the use of shrubs as nurse plants. *Ecol Appl* **14:** 1128–1138.

Gotelli, N.J., and Entsminger, G.L. (2001) Swap and fill algorithms in null model analysis: rethinking the knight's tour. *Oecologia* **129:** 281–291.

Grove, A.T., and Rackham, O. (2003) *The Nature of Mediterranean Europe: An Ecological History*. New Haven, CT, USA: Yale University Press.

Holmgren, M., Gómez-Aparicio, L., Quero, J.L., and Valladares, F. (2012) Non-linear effects of drought under shade: reconciling physiological and ecological models in plant communities. *Oecologia* **169:** 293–305.

Horton, T.R., and Bruns, T.D. (1998) Multiple-host fungi are the most frequent and abundant ectomycorrhizal types in a mixed stand of Douglas fir (*Pseudotsuga menziesii*) and bishop pine (*Pinus muricata*). *New Phytol* **139:** 331–339.

Horton, T.R., and Bruns, T.D. (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Mol Ecol* **10:** 1855–1871.

Horton, T.R., Molina, R., and Hood, K. (2005) Douglas-fir ectomycorrhizae in 40- and 400-year-old stands: mycobiont availability to late successional western hemlock. *Mycorrhiza* **15:** 393–403.

Hughes, K.W., Petersen, R.H., and Lickey, E.B. (2009) Using heterozygosity to estimate a percentage DNA sequence similarity for environmental species' delimitation across basidiomycete fungi. *New Phytol* **182:** 795–798.

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*

Ishida, T.A., Nara, K., and Hogetsu, T. (2007) Host effects on ectomycorrhizal fungal communities: insight from eight host species in mixed conifer–broadleaf forests. *New Phytol* **174:** 430–440.

Kennedy, P.G., Izzo, A.D., and Bruns, T.D. (2003) There is high potential for the formation of common mycorrhizal networks between understorey and canopy trees in a mixed evergreen forest. *J Ecol* **91:** 1071–1080.

Kennedy, P.G., Smith, D.P., Horton, T.R., and Molina, R.J. (2012) *Arbutus menziesii* (Ericaceae) facilitates regeneration dynamics in mixed evergreen forests by promoting mycorrhizal fungal diversity and host connectivity. *Am J Bot* **99:** 1691–1701.

Kipfer, T., Wohlgemuth, T., van der Heijden, M.G.A., Ghazoul, J., and Egli, S. (2012) Growth response of drought-stressed *Pinus sylvestris* seedlings to single- and multi-species inoculation with ectomycorrhizal fungi. *PLoS ONE* **7:** e35275.

Koide, R.T., Xu, B., and Sharda, J. (2005) Contrasting below-ground views of an ectomycorrhizal fungal community. *New Phytol* **166:** 251–262.

Kummerow, J., Kummerow, M., and Trabaud, L. (1990) Root biomass, root distribution and the fine-root growth dynamics of *Quercus coccifera* L. in the garrigue of southern France. *Vegetatio* **87:** 37–44.

Lancellotti, E., Iotti, M., Zambonelli, A., and Franceschini, A. (2014) Characterization of Tuber borchii and Arbutus unedo mycorrhizas. *Mycorrhiza* **24:** 481–486.

Lang, C., Seven, J., and Polle, A. (2011) Host preferences and differential contributions of deciduous tree species shape mycorrhizal species richness in a mixed Central European forest. *Mycorrhiza* **21:** 297–308.

Leonardi, M., Iotti, M., Oddis, M., Lalli, G., Pacioni, G., Leonardi, P., *et al*. (2013) Assessment of ectomycorrhizal fungal communities in the natural habitats of *Tuber magnatum* (Ascomycota, Pezizales). *Mycorrhiza* **23:** 349–358.

Lian, C., Narimatsu, M., Nara, K., and Hogetsu, T. (2006) *Tricholoma matsutake* in a natural *Pinus densiflora* forest: correspondence between above- and below-ground genets, association with multiple host trees and alteration of existing ectomycorrhizal communities. *New Phytol* **171:** 825–836.

Lilleskov, E.A., Bruns, T.D., Horton, T.R., Taylor, D., and Grogan, P. (2004) Detection of forest stand-level spatial structure in ectomycorrhizal fungal communities. *FEMS Microbiol Ecol* **49:** 319–332.

Liu, B., Fischer, C., Bonet, J.A., Olivera, A., Inchusta, A., and Colinas, C. (2014) Pattern of *Tuber melanosporum* extramatrical mycelium expansion over a 20-year chronosequence in Quercus ilex-truffle orchards. *Mycorrhiza* **24** (Suppl. 1): S47–S54.

Maremmani, A., Bedini, S., Matoševic, I., Tomei, P.E., and Giovannetti, M. (2003) Type of mycorrhizal associations in two coastal nature reserves of the Mediterranean basin. *Mycorrhiza* **13:** 33–40.

Médail, F., and Quézel, P. (eds) (2003) *Ecologie et biogéographie des forêts du bassin méditerranéen*. Cachan, France: Editions Lavoisier.

Morris, M.H., Smith, M.E., Rizzo, D.M., Rejmánek, M., and Bledsoe, C.S. (2008) Contrasting ectomycorrhizal fungal communities on the roots of co-occurring oaks (*Quercus* spp.) in a California woodland. *New Phytol* **178:** 167–176.

Murat, C., Vizzini, A., Bonfante, P., and 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.

Napoli, C., Mello, A., Borra, A., Vizzini, A., Sourzat, P., and Bonfante, P. (2010) *Tuber melanosporum*, when dominant, affects fungal dynamics in truffle grounds. *New Phytol* **185:** 237–247.

Oksanen, J. (2011) Multivariate analysis of ecological communities in R: vegan tutorial. R Package Version 1.

Parladé, J., Varga, H., Miguel, A.M., Sáez, R., and Pera, J. (2013) Quantification of extraradical mycelium of *Tuber melanosporum* in soils from truffle orchards in northern Spain. *Mycorrhiza* **23:** 99–106.

Pickles, B.J., Genney, D.R., Potts, J.M., Lennon, J.J., Anderson, I.C., and Alexander, I.J. (2010) Spatial and temporal ecology of Scots pine ectomycorrhizas. *New Phytol* **186:** 755–768.

Plattner, I., and Hall, I.R. (1995) Parasitism of non-host plants by the mycorrhizal fungus *Tuber melanosporum*. *Mycol Res* **99:** 1367–1370.

Prescott, C.E., and Grayston, S.J. (2013) Tree species influence on microbial communities in litter and soil: Current knowledge and research needs. *For Ecol Manage* **309:** 19–27.

R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Richard, F., Millot, S., Gardes, M., and Selosse, M.-A. (2005) Diversity and specificity of ectomycorrhizal fungi retrieved from an old-growth Mediterranean forest dominated by *Quercus* ilex. *New Phytol* **166:** 1011–1023.

Richard, F., Selosse, M.-A., and Gardes, M. (2009) Facilitated establishment of *Quercus ilex* in shrub-dominated communities within a Mediterranean ecosystem: do mycorrhizal partners matter? *FEMS Microbiol Ecol* **68:** 14–24.

Richard, F., Roy, M., Shahin, O., Sthultz, C., Duchemin, M., Joffre, R., and Selosse, M.-A. (2011) Ectomycorrhizal communities in a Mediterranean forest ecosystem dominated by *Quercus ilex*: seasonal dynamics and response to drought in the surface organic horizon. *Ann For Sci* **68:** 57–68.

Rodriguez, R.J., White, J.F., Jr, Arnold, A.E., and Redman, R.S. (2009) Fungal endophytes: diversity and functional roles. *New Phytol* **182:** 314–330.

Salerni, E., Iotti, M., Leonardi, P., Gardin, L., D'Aguanno, M., Perini, C., *et al*. (2014) Effects of soil tillage on *Tuber magnatum* development in natural truffières. *Mycorrhiza* **24:** 79–87.

Selosse, M.-A., Bauer, R., and Moyersoen, B. (2002) Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees. *New Phytol* **155:** 183–195.

Selosse, M.-A., Richard, F., He, X., and Simard, S.W. (2006) Mycorrhizal networks: des liaisons dangereuses? *Trends Ecol Evol* **21:** 621–628.

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*

Shahin, O., Paul, N.M.-S., Rambal, S., Joffre, R., and Richard, F. (2013) Ectomycorrhizal fungal diversity in *Quercus ilex* Mediterranean woodlands: variation among sites and over soil depth profiles in hyphal exploration types, species richness and community composition. *Symbiosis* **61:** 1–12.

Simard, S.W., Beiler, K.J., Bingham, M.A., Deslippe, J.R., Philip, L.J., and Teste, F.P. (2012) Mycorrhizal networks: mechanisms, ecology and modelling. *Fungal Biol Rev* **26:** 39–60.

Sirami, C., Nespoulous, A., Cheylan, J.-P., Marty, P., Hvenegaard, G.T., Geniez, P., *et al*. (2010) Long-term anthropogenic and ecological dynamics of a Mediterranean landscape: impacts on multiple taxa. *Landsc Urban Plan* **96:** 214–223.

Smith, S.E., and Read, D.J. (2008) *Mycorrhizal Symbiosis*, Third edn. Amsterdam, the Netherlands: Academic Press. 3rd edition.

Splivallo, R., Fischer, U., Gobel, C., Feussner, I., and Karlovsky, P. (2009) Truffles regulate plant root morphogenesis via the production of auxin and ethylene. *Plant Physiol* **150:** 2018–2029.

Streiblová, E., Gryndlerová, H., and Gryndler, M. (2012) Truffle brûlé: an efficient fungal life strategy. *FEMS Microbiol Ecol* **80:** 1–8.

Suz, L.M., Martín, M.P., Oliach, D., Fischer, C.R., and Colinas, C. (2008) Mycelial abundance and other factors related to truffle productivity in *Tuber melanosporum* – *Quercus ilex* orchards. *FEMS Microbiol Lett* **285:** 72–78.

Teste, F.P., Simard, S.W., Durall, D.M., Guy, R.D., Jones, M.D., and Schoonmaker, A.L. (2009) Access to mycorrhizal networks and roots of trees: importance for seedling survival and resource transfer. *Ecology* **90:** 2808–2822.

Vazquez, D.P., Poulin, R., Krasnov, B.R., and Shenbrot, G.I. (2005) Species abundance and the distribution of specialization in host-parasite interaction networks. *J Anim Ecol* **74:** 946–955.

Wenkart, S., Roth-Bejerano, N., Mills, D., and Kagan-Zur, V. (2001) Mycorrhizal associations between *Tuber melanosporum* mycelia and transformed roots of *Cistus incanus*. *Plant Cell Rep* **20:** 369–373.

Zampieri, E., Rizzello, R., Bonfante, P., and Mello, A. (2012) The detection of mating type genes of *Tuber melanosporum* in productive and non productive soils. *Appl Soil Ecol* **57:** 9–15.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Richness accumulation curves of ECM communities according to (A) host species, (B) production stages, (C) total community and host species–OTU interactions accumulation curves.

Fig. S2. Position of the five *Tuber* OTUs (annotated by a star symbol) among the nine major *Tuber* clades as defined by Bonito and colleagues (2010). Phylogeny is based on ITS nuclear rDNA (293 included positions) by an HKY85 model of nucleotide substitution. Maximum likelihood bootstrap values are shown above branches. Sequences are labeled with Latin binomials, GenBank accession or collection number and geographical origin (Bonito *et al*., 2010).

Table S1. Sampling design, sequencing efficiency and proportion of reassigned root tips after RFLP analysis on the four sampled plant species. As roots were intermingled in soil, molecular identification led to re-attributions of ECM tips to the different host species.

Table S2. Ectomycorrhizal fungal diversity on four sampled host species and in four different production stages at the Pézilla-de-Conflent study sites. Host species are *Arbutus unedo* (*Au*), *Cistus albidus* (*Ca*), *Quercus ilex* (*Qi*) and *Q. coccifera* (*Qc*). Production stages are immature brûlés (*a*), productive brûlés (*b*), post-productive brûlés (*c*) and relict forests (*d*). Values in brackets indicate the standard error.

Table S3. Operational taxonomic units and accession numbers of the sequences submitted to GenBank.

© 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*