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Tuber melanosporum, when dominant, affects fungal dynamics in truffle grounds

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Summary

- The fruiting bodies of the ectomycorrhizal (ECM) fungus *Tuber melanosporum* are usually collected in an area devoid of vegetation which is defined as a 'burnt area' (*brulé* in French).
- Here, the soil fungal populations of inside and outside *brulé* were compared in order to understand whether the scanty plant cover was related to a change in fungal biodiversity. Both denaturing gradient gel electrophoresis (DGGE) and molecular cloning of the internal transcribed spacer (ITS) marker were employed on soil DNA to obtain profiles from nine truffle grounds and fungal sequences from one selected truffle ground sampled in two years.
- Denaturant gradient gel electrophoresis profiles from the two areas formed two distinct clusters while molecular cloning allowed 417 fungal sequences to be identified. *T. melanosporum* was the dominant fungus within the *brulé*. There were nine new haplotypes, which had never been detected in fruiting bodies. The Basidiomycota ECM fungi decreased within the *brulé*, indicating a competitive effect of *T. melanosporum* on the other ECM fungi.
- Among other factors, the dynamics of fungal populations seems to be correlated to *brulé* formation.

Introduction

Truffles are ectomycorrhizal (ECM) fungi that produce hypogeous fruiting bodies, belonging to the *Tuber* genus (Ascomycota, Pezizales). Truffle species have common ecological features such as a wide range of host species (oak, willow, poplar, hazel and some shrubs) and the requirement of calcareous soil (pH between 7 and 8), except for *Tuber borchii*, which tolerates slightly acidic soils (Mello *et al.*, 2006). Among the *Tuber* species, the black truffle *T. melanosporum* and the white truffle *T. magnatum* are highly appreciated because of their special taste and smell (Mello *et al.*,

2006). *T. melanosporum* grows in symbiosis with several oak species and hazelnut trees in Mediterranean conditions, in France, Italy and the Iberian Peninsula (Ceruti *et al.*, 2003). Truffle plantations have, however, been introduced in New Zealand, Australia, Israel and North America (Bonet *et al.*, 2006).

The development of mycorrhizal symbiosis in some *Tuber* species is associated with the production of a burnt area (commonly referred to by the French word *brulé*) around their symbiotic plants. This *brulé* is usually circular, characterized by scanty vegetation and typical of *T. melanosporum* and *T. aestivum* (Pacioni, 1991). Explanations for the formation of the *brulé* have been proposed. Plattner & Hall (1995) hypothesized parasitism of the *Tuber* spp. on the nonhost herbaceous plants, whereas Delmas (1983) thought that *Tuber* ectomycorrhizas may compete for nutrients or water. A phytotoxic effect by truffle metabolites was highlighted by Pacioni (1991) and Lanza *et al.* (2004). Recently, Splivallo *et al.* (2007) showed that truffle volatiles inhibit growth and induce an oxidative burst in *Arabidopsis thaliana*, indicating that fungal volatiles are molecules that can mediate fungal–plant interactions, at least in *in vitro* conditions. However, the mechanisms are still unknown, as is its ecological meaning. To our knowledge, only two studies, limited to cultivable fungi, have tested the hypothesis that the presence of *Tuber* spp. could affect fungal biodiversity (Luppi-Mosca, 1972; Luppi & Fontana, 1977). On the basis of this knowledge, we have focused attention on the fungal composition in the *brulé* associated with *T. melanosporum*, where the fruiting bodies of this species are usually collected in late autumn and winter.

Suz *et al.* (2008), through relative quantification of DNA from *T. melanosporum* mycelia, suggested that the *brulé* appears only when a certain amount of mycelium has formed; they also demonstrated that the onset of truffle production in orchards is unpredictable (4–12 yr) and variable among trees in the same orchard, and that the *brulé* appears before the onset of truffle production, but it does not guarantee that the tree will produce truffles. It is totally unknown which populations live within a *brulé*. Therefore, our aims were to compare soil microorganisms between the two areas – inside and outside the *brulé*– and to understand whether the scanty plant cover is correlated with a change in fungal biodiversity. Denaturing gradient gel electrophoresis (DGGE) is the main technique used to detect microbial community shifts through the simultaneous comparison of band patterns originated from numerous samples (Anderson & Cairney, 2004). In order to achieve our goal, soil samples were collected in nine truffle grounds located in Cahors, France, and the internal transcribed spacer (ITS) region was processed with both DGGE and molecular cloning. Since the *brulé* is a dynamic area that can move from one year to the next (Sourzat, 1997), one truffle ground was also surveyed 2 yr later in order to compare the temporal dynamics of the fungal communities between the two areas. In this case, only molecular cloning was applied. DGGE profiles from the two areas – inside and outside the *brulé*– formed two distinct clusters. In both years, *T. melanosporum* turned out to be the most dominant ECM fungus within the *brulé*; unexpectedly, it was possible to type more haplotypes than those described in the literature. Finally, the biodiversity of ECM fungi decreased within the *brulé*, which would seem to indicate a competitive effect of *T. melanosporum* on other ECM fungi.

Materials and Methods

Soil Sampling

The sampling sites are *T. melanosporum* truffle grounds in Cahors (France) which belong to La Station de la Trufficulture de Cahors-Le Montat (Station d'expérimentation sur la truffe, Lycée professionnel agricole Lacoste, Le Montat, France). All the truffle grounds proved to be productive, with a yield of between 100 and 1000 g (P. Sourzat, pers. comm.).

Soil samples were collected in May 2006 and March 2008, the months in which *T. melanosporum* mycelium spreads, colonizing the soil, and sexual reproduction takes place (Sourzat, 1997). For the first soil sampling (May 2006), we chose nine *T. melanosporum/Quercus pubescens* truffle grounds, and one well-defined brulé was analyzed for each truffle ground. Seven truffle grounds are natural; only two are artificial. The mean values of the granulometric fraction contents are: 19.3% clay, 48.6% silt and 32.1% sand. Chemical soil features present 5.8–6.8% organic matter and an approximate pH of 8. The host ages, the date of *T. melanosporum* inoculation in the artificial truffle grounds and the origin of the sites are shown in Table 1. Samples of *c.* 200 g of soil from each area were taken at a depth of *c.* 10–15 cm and stored at –80°C until they were analyzed. A number of samples, ranging from one to five, were collected inside and outside each brulé (Table 1). The numbers of samples collected for each area were different because of the heterogeneity – different shapes and sizes – of the brulé, the distance of the brulé limit from the tree trunk (Supporting Information, Fig. S1) and because some resistance was encountered by the owners to digging the soil of such economically important environments. In total, 45 soil samples were collected from inside and outside nine brulé areas.

Table 1. Details of each considered truffle ground and Shannon index values resulting from denaturing gradient gel electrophoresis (DGGE) profiles of the first sampling

Truffle ground number	Place	Origin	Typology and <i>Tuber melanosporum</i> inoculation age (yr)	Host age (yr)	First year sampling		Second year sampling		Shannon index (<i>H'</i>)	
					No. of samples Inside	No. of samples Outside	No. of samples Inside	No. of samples Outside	Inside	Outside
1	Fontanes, Lot	Fallow land	Natural	40–50	1	2			1.09	1.1
2	La Bigouse, Lalbenque	<i>Quercus pubescens</i> plantation	Natural	60	2	5	12	12	1.12	1.33
3	La Bigouse, Lalbenque	<i>Q. pubescens</i> plantation	Natural	60	1	3			0.79	1.2
4	Le Montat	Fallow land	Natural	20–25	2	1			1.04	1.27
5	Le Montat	Fallow land	Natural	20–25	2	2			1.05	1.31
6	Le Montat	Pasture	Natural	50–60	3	3			1.09	1.24
7	Le Montat	Pasture	Natural	50–60	3	3			1.2	1.29

Truffle ground number	Place	Origin	Typology and <i>Tuber melanosporum</i> inoculation age (yr)	Host age (yr)	First year sampling		Second year sampling		Shannon index (H')	
					No. of samples Inside	No. of samples Outside	No. of samples Inside	No. of samples Outside	Inside	Outside
8	Le Montat	Q. <i>pubescens</i> plantation	17	16	2	1			1.06	1.33
9	Escayrac	Q. <i>pubescens</i> plantation	10	10	2	1			1.18	1.29

Truffle ground 2 was also surveyed 2 yr later (March 2008). In this case, it was possible to obtain a number of more representative soil samples of the checked area. Twelve samples were collected inside and outside the brulé, as shown in Fig. S1. The same sampling and soil storing method as previously used were adopted. After removing any visible roots or small pebbles from the soil collected in both 2006 and 2008, the soil samples (*c.* 50 g) from each area were mixed to make them homogeneous. Two pools (inside and outside the brulé) per site were created. In this way, 18 pools (nine inside the brulé and nine outside) and two pools, respectively, were obtained for the first and second samplings. Those from the soil samples collected in 2008 from truffle ground 2 were air-dried and sieved through a 2 mm sieve for further homogenization.

Soil DNA extraction

The total DNA was extracted from 0.5 g of soil using a Fast DNA Spin Kit for Soil (Qiagen), with modifications according to [Luis *et al.* \(2004\)](#). Two extractions were made for each pool, and mixed in order to obtain the most representative DNA of the soil sample. In this way, 18 DNA samples were obtained for the first sampling and two were obtained for the second sampling. All these DNA samples were quantified with NanoDrop (Thermo Scientific, Wilmington, DE, USA) and concentrations of 20 ng μl^{-1} were used in the subsequent PCR procedures.

Denaturing gradient gel electrophoresis

All the 18 DNA pools from the first sampling were analyzed in the DGGE experiment. The fungal ITS region was amplified with the ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') fungal-specific primers ([White *et al.*, 1990](#); [Gardes & Bruns, 1993](#)) and a GC clamp of 40 bp ([Muyzer *et al.*, 1993](#)) was added to the 5'-end of the ITS1F primer (ITS1F-GC). For the DGGE molecular marker preparation, fungal organisms were isolated directly from a single soil sample and morphologically identified (Table S1). DNA was extracted from the obtained cultures and the ITS region was amplified with the primers. ITS fragments which covered a broad range in DGGE were chosen and pooled to constitute the marker. ITS rDNA of *T. melanosporum* was added to the marker in order to follow its presence in all of the samples studied.

Polymerase chain reaction was performed in a 40 μl reaction volume containing *c.* 5 ng of template DNA; 20 pmol of each primer; 3 mM MgCl_2 ; 0.4 μl BSA (100 \times); 4 μl 10 \times buffer; 4 μl dNTPs (2.5 mM each) and 0.3 μl Expand High Fidelity PCR System (5 U μl^{-1}) (Roche). The PCR

programme was conducted according to [Yergeau *et al.* \(2007\)](#), except for the final extension, which was at 68°C for 10 min.

The DGGE analysis was performed on 8% (w/v) acrylamide/bisacrylamide (37.5 : 1) gels with a denaturing ranging from 15 to 55%. All the gels were run in a Bio-Rad DCode System (Bio-Rad, Hemel Hempstead, United Kingdom) at a constant temperature of 60°C, for 17 h at 60 V in a 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8). The gels were stained using the silver nitrate method ([Bassam *et al.*, 1991](#)) and the image was captured using a digital camera.

DGGE analysis

The digitized DGGE images were analyzed with Quantity One image analysis software (Version 4.5.2, Bio-Rad), which was used to apply lanes and bands to the image with additional manual adjustment of the band designations. A similarity matrix that related the band patterns was automatically calculated with the Dice coefficient. Dendrogram and clustering were done applying UPGMA algorithms.

The Shannon index (H') and the Fisher alpha (α), based on the intensity and number of bands, were calculated for each lane, according to [Konstantinov *et al.* \(2003\)](#) and [Scanlan *et al.* \(2006\)](#), respectively. Each band was presumed to correspond to a single fungal microorganism, and the band density to its abundance. A statistical analysis was carried using Student's *t*-test (Microsoft Excel), and a probability level of 0.05 was considered to be statistically significant.

DGGE band recovering

Most bands from two lanes were excised from the gel with a sterile razor blade and DNA extraction was carried out using the QIAquick Gel Extraction Kit (Qiagen) developed for extraction of DNA fragments from polyacrylamide gels. The recovered DNA was eluted in 10 µl of sterile water. A PCR with ITS1F-GC/ITS2 primers (20 pmol of each) and 1 µl of DNA was performed. After fragment purification (QIAquick PCR Purification Kit, Qiagen) and ligation (pGEM-T Easy Vector System, Promega), transformation was carried out with XL1 Blue competent cells (Stratagene, Agilent Technologies, Santa Clara, CA, USA), according to the manufactures' instructions. Six blank colonies were recovered for each cloning and the insert was re-amplified with the same primers. By doing so, we obtained a useful PCR product for a new DGGE. Four PCR products were run for each band in a new gel against the initial sample to ensure that the target band had been amplified. The fragments that ran in the same position of the corresponding band were selected and sequenced. Similarity comparisons of the 25 sequences obtained (accession numbers from [FN377830](#) to [FN377854](#)) were performed using the National Center for Biotechnology Information (NCBI) online standard BLAST (Basic Local Alignment Search Tool) programme.

Molecular cloning

Four cloning experiments were performed for samples collected in both 2006 and 2008 on truffle ground 2, leading to four different clone libraries (inside 2006, outside 2006, inside 2008 and outside 2008). The ITS region of the fungal rDNA was amplified with the common ITS1F and ITS4 fungal primers ([White *et al.*, 1990](#); [Gardes & Bruns, 1993](#)), using the Expand High Fidelity DNA PCR System (Roche). The cycling parameters were as follows: 94°C for 4 min followed by 30 cycles at 94°C for 45 min, 55°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 10 min. The purified PCR products were cloned using a TOPO Cloning Kit (Invitrogen). White colonies were screened for each insert, and random colonies that showed an insert of *c.* 600–800 bp were sequenced. The accession numbers for the four clone library sequences are: [FN391297-](#)

[FN391375](#) (inside 2006), [FN397102-FN397186](#) (outside 2006), [FN397187-FN397322](#) (inside 2008) and [FN397323-FN397439](#) (outside 2008).

Sequences analysis

The sequences from each clone library were analyzed separately. Putative chimeric sequences were identified with a Bellerophon program and removed from the analysis. Multiple alignments were generated for each group of sequences using ClustalW (with default parameters). Alignments were adjusted manually using Gene-Doc to ensure that all the sequences had the same start and end points. A distant matrix was constructed using DNAdist from PHYLIP version 3.68 with default parameters. Each output file served as input for DOTUR 1.53. This programme allows sequences to be assembled in operational taxonomic units (OTUs), rarefaction curves to be generated, and the richness and diversity indices, Ace and Chao1, to be calculated. OTUs were defined for 97% of pairwise similarity. A representative sequence was chosen for each OTU and a search of the GenBank database was conducted using the online BLAST program.

All the sequences grouped in the OTUs corresponding to *T. melanosporum* were brought into alignment separately to highlight any possible single nucleotide polymorphisms (SNPs). The chromatogram of each sequence was carefully analyzed, focusing on the peak for which the polymorphism was detected, in order to ensure that the chromatogram reading was correct.

Results

Denaturing gradient gel electrophoresis was applied to all the samples from the first sampling in order to have a comprehensive picture of the soil fungal biodiversity inside and outside the brulé ([Table 1](#)). The identification of the main taxa occurring in the two different areas was obtained from a single truffle ground with both DGGE and molecular cloning.

Denaturing gradient gel electrophoresis

The DGGE analysis was performed with a fragment of 300 bp, representing the fungal ITS1 region. All the 18 different sample pools, belonging to the nine truffle grounds sampled in 2006, ran in the gel, revealing a general view of the fungal communities. A comprehensive DGGE gel of all the nine sampled truffle grounds was performed in duplicate to verify the repeatability of the fingerprints. As a result, the two gels were overlapped ([Fig. 1](#) and Fig. S2). Since this, the following further analyses were made for one of the two gels, which is the one that presents a clearer and sharper image ([Fig. 1](#)). For an easier comparison of the community profiles inside and outside the brulé, the soil samples collected outside the brulé were run on the gel before the corresponding soil samples collected inside the brulé. The DGGE profiles were compared, using the Quantity-one software, which led to a dendrogram ([Fig. 2](#)). Two clusters were formed: one by the samples inside the brulé, and the second one by the samples outside the brulé. The marker profiles clustered together, as expected. The Shannon index was calculated for all the nine truffle grounds ([Table 1](#)). The inside brulé values were significantly lower than the outside values (P -value = 0.0007). The Fisher α index, which is independent of the sample size, confirmed this result (Table S2).

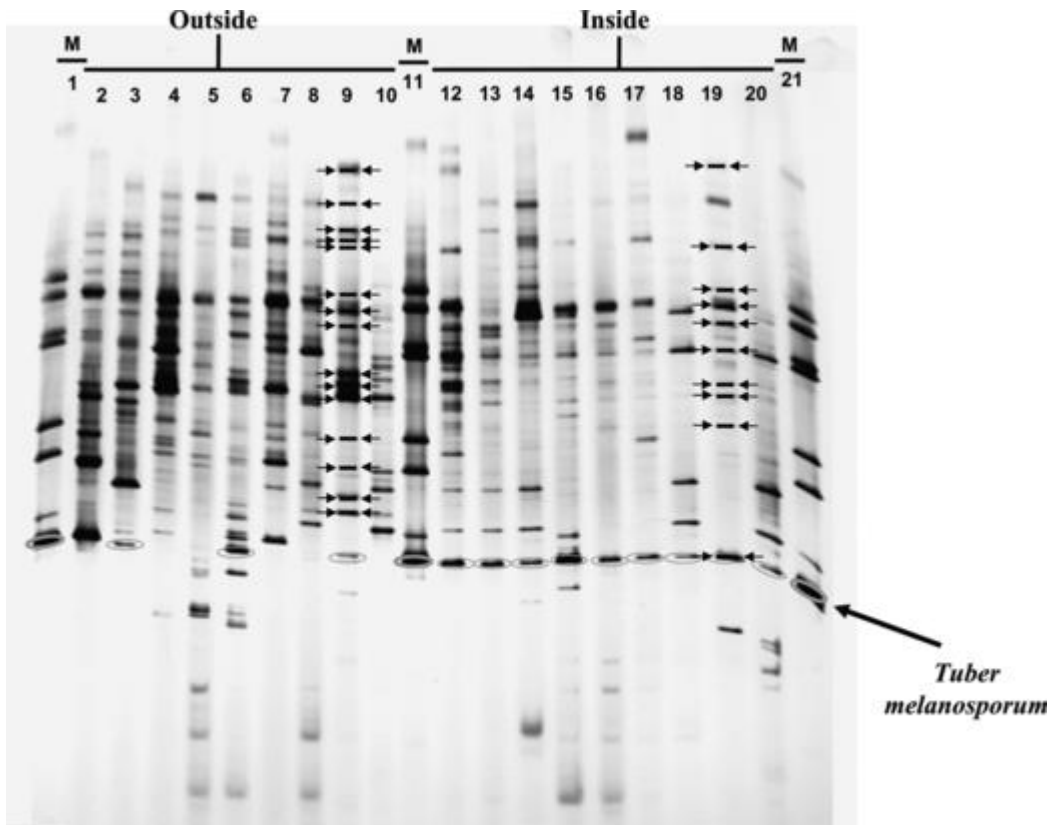


Figure 1. The denaturing gradient gel electrophoresis (DGGE) of 300 bp fungal internal transcribed spacer (ITS) regions from nine *Tuber melanosporum* truffle grounds. Lanes 2–10 represent the soil collected outside the brulé, while lanes 12–20 represent the soil collected inside the brulé. Lanes 1, 11 and 21, indicated by M, represent the markers. The position of the *T. melanosporum* fragment (also present in the marker pattern) is underlined. Lanes 9 and 19 correspond to the 'La Bigouse' truffle ground 2, whose bands (indicated with arrows) were sequenced. (gel 8% acrylamide; gradient from 15 to 55%).

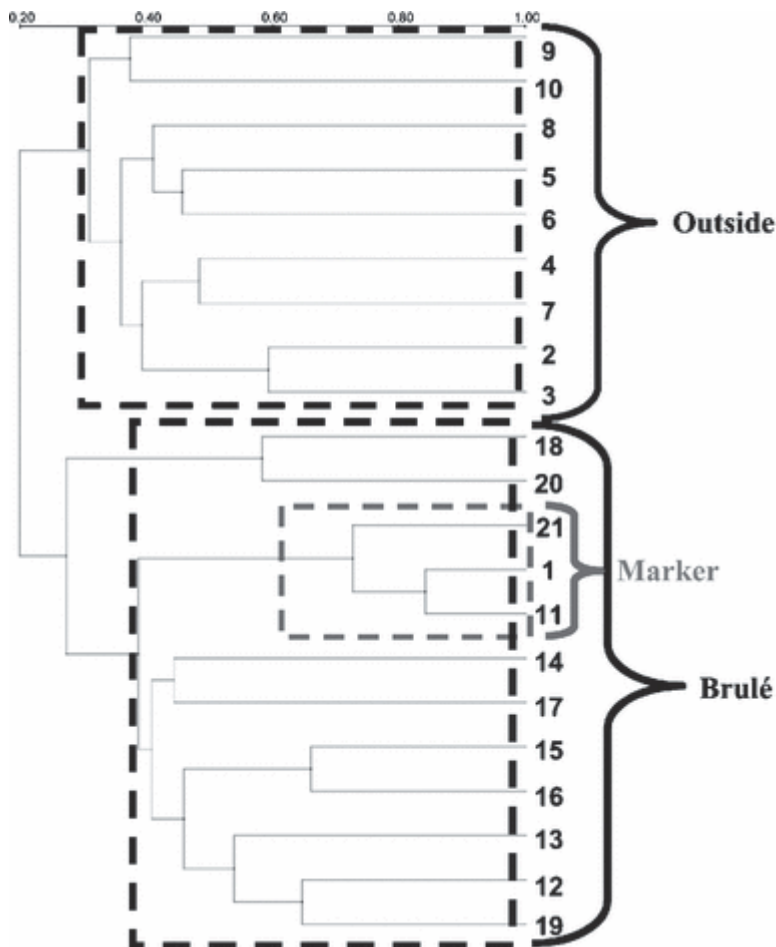


Figure 2. The dendrogram (UPGMA) generated from the denaturing gradient gel electrophoresis (DGGE) profiles. Profiles 2–10 refer to the samples collected outside of the brulé areas, while profiles 12–20 refer to the samples collected in the brulé areas. Numbers 1, 11 and 21 are the marker profiles. Numbers 9 and 19 are the profiles of truffle ground 2.

An interesting band was observed, which in the run showed the same behavior as the *T. melanosporum* present in the marker. It had high intensity (meaning dominance) in all the samples corresponding to the soil collected inside the brulé (mean = 110 ± 24.44), but it was absent or had a low intensity in the profiles outside the brulé (mean = 25 ± 41.11). Analysis of its density showed a significant abundance inside compared with that outside the brulé (P -value = 0.00003). This band was sequenced from lanes 19, 16 and 13 (Fig. 1), corresponding to the inside profiles of truffle grounds 2, 5 and 8. The sequences showed 100% similarity with Genebank *T. melanosporum* sequences.

In order to obtain more information about the fungal taxa distribution, we focused on a single truffle ground and chose truffle ground 2 situated in ‘La Bigouse’ (Table 1). Ten and 15 bands, from inside and outside the brulé, respectively, were excised and sequenced (lanes 19 and 9, Fig. 1). Of all the sequences obtained, five out of 25 were identical in the two profiles, as expected, given that these bands ran in the same position in the gel. BLAST analysis showed a high similarity with sequences from *Cylindrocarpon* sp., *Ceratobasidium* sp., two species of *Mortierella*, and an uncultured member of Pyronemataceae. Another seven bands matched sequences belonging to Basidiomycota. Among these, five were found in the outside profile and corresponded to *Tephrocybe* sp., *Schizophyllum commune*, *Inocybe splendens*, *Tricholoma* sp. and *Ceratobasidium* sp.; only two were found inside the brulé (*Rhizoctonia* sp. and an uncultured Russulaceae). Another five

sequences were found among the Ascomycota outside the brulé, corresponding to uncultured Amphisphaeriaceae, *Stachybotrys* sp., *Parepichloe* sp. and two species of *Peziza*, while sequences corresponding to *T. melanosporum*, *Spizellomyces* sp. and *Fusarium oxysporum* were found inside the brulé. The remaining sequences outside the brulé belonged to Zygomycota and were represented by Mortierellales (data not shown).

From a general comparison of the two areas, the number of Ascomycota remained stable at 40% in both areas, the number of Basidiomycota moved from 30% outside to 20% inside the brulé, while the number of Zygomycota increased from 20 to 30%.

Molecular cloning of fungal rDNA ITS

Molecular cloning was applied to truffle ground 2 in La Bigouse by sequencing the ITS region from samples collected inside and outside the brulé in two years (2006 and 2008).

The number of clones sequenced from each area in both years, and the number of OTUs are shown in Fig. 3. In spite of the different percentages for the two years, the OTU number inside the brulé was consistently lower than that outside. For each year, the rarefaction curves of the inside brulé community show lower plots than the curves of the outside brulé community, indicating a lower richness inside than outside the brulé (Fig. S3). At the minimal number of 79 sequences, we found, respectively, 18 and 11 OTU less inside the brulé, in the first and second years.

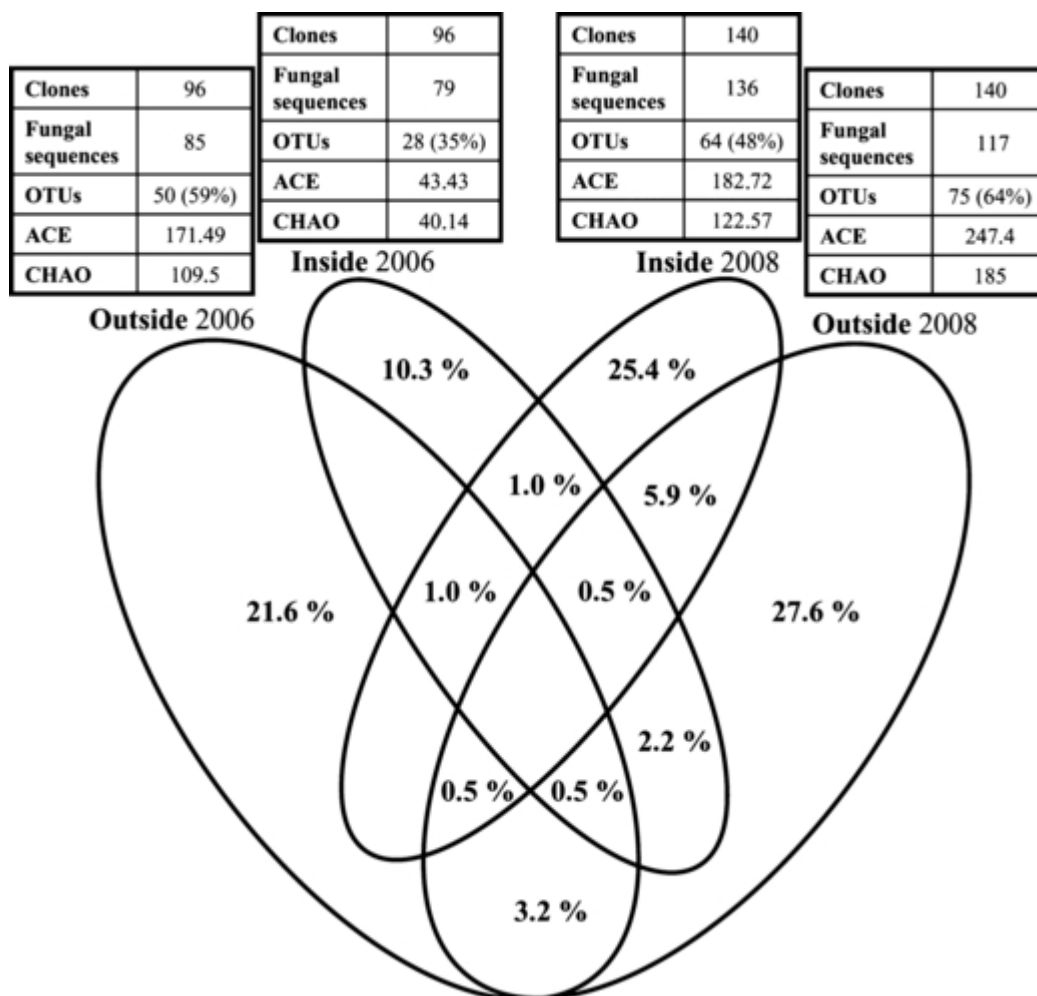


Figure 3. Numbers of clones, fungal sequences, operational taxonomic unit (OTU) and richness estimators Chao1 and ACE from each area (inside and outside the brulé) are given for the two years (2006 and 2008). Nonfungal and chimeric sequences were omitted. The 'Venn' diagram shows the percentages of overlap of the OTUs between the four internal transcribed spacer (ITS) clone libraries.

Moreover, the rarefaction curves show no evidence of saturation, indicating that the number of sequences obtained for each cloning experiment did not capture the real richness of our soil samples. The richness of each sample population was estimated using the Chao1 and ACE nonparametric richness estimators. The data confirmed that our results were far from the expected richness of the soil sample and indicated a possible different composition of microbial communities in each sample (Fig. 3).

In order to find OTUs that were common for the two years and the two areas, a set of 417 sequences (164 from the first year and 253 from the second year) was grouped into 185 OTUs. Most of the OTUs (84.9%) were found to be unique to the area and the year they were sampled in. Only a small portion of OTUs were found to be common to more than one library, while no OTU was shared by all four clone libraries ('Venn diagram', Fig. 3).

The taxa composition of the fungal communities was determined (Table S3). The 2006 sampling showed that the few shared sequences between the two areas had high similarity with the fungal genera *Fusarium* and *Mortierella*.

Considering the most represented taxa, the number of Ascomycota was almost unchanged for the two areas (37% in the brulé and 34% outside), the number of Zygomycota increased inside the brulé (from 5 to 32%) and the number of Basidiomycota sequences decreased (from 61 to 23%) (Figs 4a,b). The sequences belonging to Zygomycota consisted of a single genus, *Mortierella*, or were unclassified Zygomycetes. Pezizales and Hypocreales were the most represented orders among the Ascomycota.

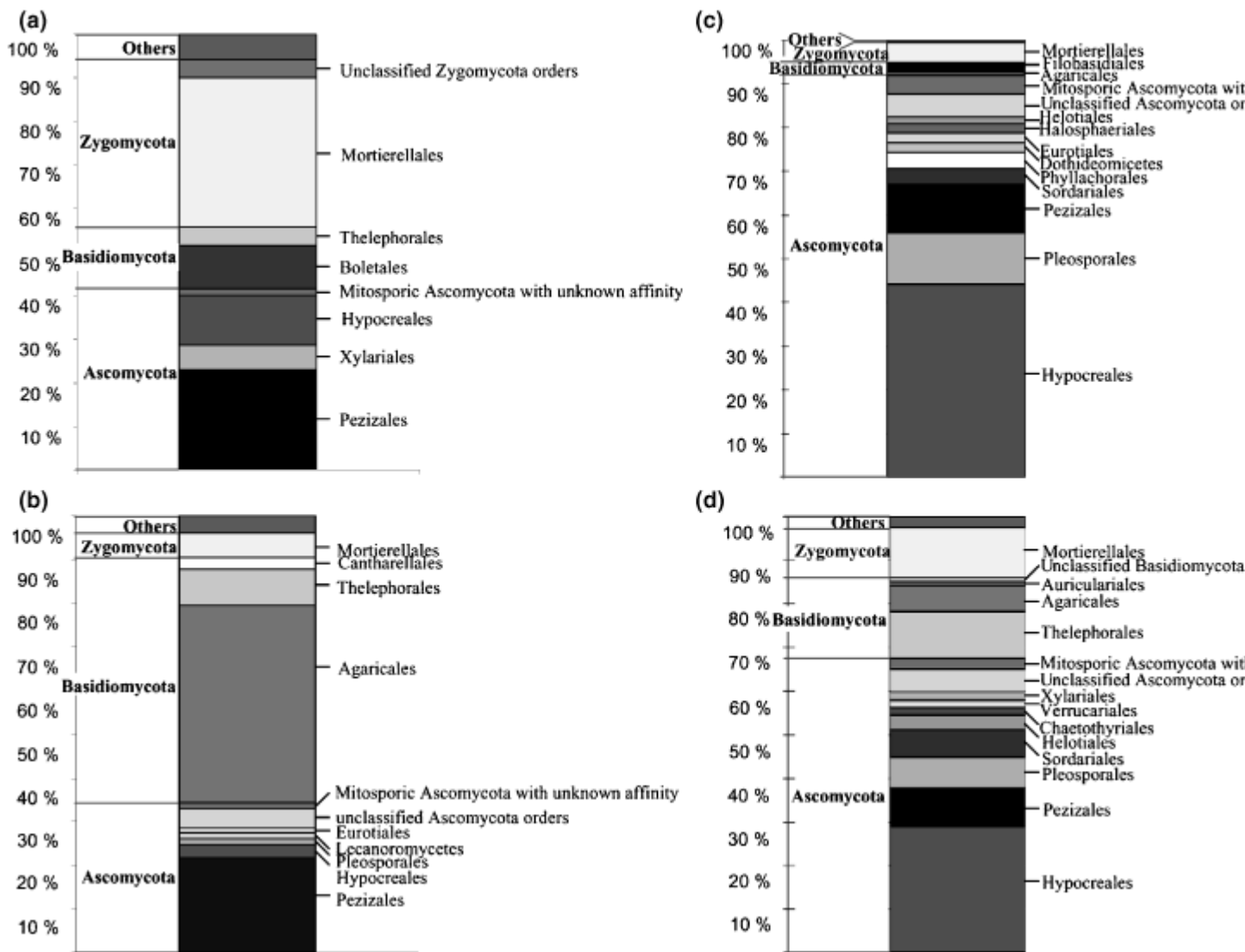


Figure 4. The taxa subdivision generated by the clone library from inside (a, c) and outside (b, d) the brulé sampled in 2006 and 2008, respectively, at La Bigouse.

Among the Basidiomycota, the most represented groups outside the brulé were ECM fungi belonging to the Thelephorales and Agaricales orders. Specifically, Thelephorales were represented by Thelephoraceae (mainly *Tomentella*), whereas Agaricales were represented by Tricholomataceae (*Tricholoma*), Hymenogasteraceae (*Hymenogaster*), Inocybaceae (*Inocybe*), Strophariaceae (*Naucoria*) and Amanitaceae (*Amanita*) (Table S3). Among the Ascomycota, the ECM fungi outside the brulé belong exclusively to the Pyronemataceae (*Pulvinula* and unidentified taxa) and Tuberaceae (*Tuber*) of the Pezizales. Moving from outside to inside the brulé, the ECM fungi shift from 73 to 30%, whereas nonECM fungi (saprotrophic, parasitic, ericoid and arbuscular mycorrhizal fungi) move from 27 to 70%. The ratios of ECM : nonECM fungi are 2.7 outside and 0.4 inside.

Despite the different percentages in taxa composition obtained in the second year, the Basidiomycota sequences decreased inside the brulé, as shown in 2006 (Figs 4c,d). This taxon increased in sequences moving outside the brulé (from 2 to 18%) where, as in the first sampling, the most represented groups were ECM fungi belonging to the Thelephorales and Agaricales orders. Table S3 shows that Thelephorales are represented by tomentelloid fungi, whereas Agaricales are represented by Inocybaceae (*Inocybe*). Pyronemataceae (*Pulvinula*) and Tuberaceae (*Tuber*) are the

Ascomycota ECM fungi, which are present outside the brulé. As well as in 2006, the ECM fungi decrease, moving from outside to inside the brulé. They shift from 19 to 9%, whereas nonECM fungi shift from 81 to 91%. In this last group, 4% of ericoid (Helotiales) and 3% of arbuscular mycorrhizal fungi (*Glomus* and *Scutellospora*) are present outside, compared with 0.7% of arbuscular mycorrhizal fungi (*Glomus*) present inside the brulé. The ratios of ECM : nonECM fungi are 0.3 outside and 0.1 inside the brulé. The decrease of ECM fungi inside the brulé is in contrast to the expected dominance of the ECM Basidiomycota associated with *Quercus* in natural conditions ([Richard et al., 2005](#); [Ortega & Lorite, 2007](#)).

Another significant result of this investigation is that the sequences representing *T. melanosporum* were present in high numbers (16% in 2006 and 7% in 2008) inside the brulé, while only one sequence was found outside in the first cloning experiment, and none in 2008.

Tuber melanosporum polymorphism

All of the 24 *T. melanosporum* sequences found in the two years produced unambiguous chromatograms, which showed some variations in the single nucleotides. When these sequences were compared with the 13 haplotypes of *T. melanosporum* described in previous papers ([Murat et al., 2004](#); [Riccioni et al., 2008](#)), 13 sequences obtained in the first and second years were referable to a single ubiquitous haplotype (haplotype I); nine potential new haplotypes were observed; none of the other 12 haplotypes described in previous papers was detected.

Discussion

Two molecular techniques – DGGE and molecular cloning – were used. The first one, which generates community profiles, was applied to all sites; the second, which must be analyzed exclusively by sequencing, was applied to one site only. While DGGE offered a general scenario of the two areas, as well as the possibility of sequencing a few bands, molecular cloning led to the identification of the fungal taxa. Both these techniques demonstrate that *T. melanosporum* is the dominant fungus inside the brulé and its dominance, directly or indirectly, has a great impact on the fungal populations; moreover, they show a lower richness inside the brulé. This data was confirmed 2 yr later by molecular cloning, suggesting that, notwithstanding that the brulé is a dynamic area, its lower fungal richness is a trademark of this area.

DGGE profiles provide a general view of fungal biodiversity in truffle grounds

Denaturing gradient gel electrophoresis enabled us to obtain a comprehensive overview of the fungal communities thriving in nine *T. melanosporum* truffle grounds and to reveal important differences between the fungal communities living inside and outside the brulé. The lower number of bands observed in the profiles of soil collected inside the brulé allowed us to hypothesize a lower degree of biodiversity in this area. Two biodiversity indices confirmed this hypothesis, suggesting that fungal communities are affected in the brulé in which they show decreased biodiversity.

The DGGE band profiles unambiguously identified bands corresponding to *T. melanosporum*, and allowed us to monitor its presence/absence in all the soil samples. The abundance of *T. melanosporum* inside the brulé could be linked to the brulé formation and, however, bears witness to its relationship with the brulé itself. This result is not surprising since *T. melanosporum* fruiting bodies are usually collected in the brulé ([Sourzat, 1997](#); [Suz et al., 2005](#)).

Since the number of sequenced bands from La Bigouse truffle ground 2 was low (25) in comparison to the number of fungal species potentially present in the soil, the DGGE technique only indicated the most represented group of sequences, suggesting some dynamics: moving from outside to inside the brulé, the number of Ascomycota did not change, while the number of Basidiomycota decreased and the Zygomycota increased. Since the DGGE profiles from truffle ground 2 grouped in the dendrogram together with those generated from the other sites, we suggest that the community shown by this truffle ground could be representative of all the investigated truffle grounds. The result from the DGGE sequencing was confirmed, however, when the molecular cloning was applied in 2006 to the same soil of La Bigouse, as far as the taxa composition is concerned. However, most of the sequences turned out to be analysis-specific. This can be explained by the use of two different couples of primers and by the absence of saturation.

La Bigouse: a blow-up of fungi living in a productive truffle ground

The cloning experiments applied to truffle ground 2 showed that only a few sequences were present in both areas and in both sampling years. The fact that the number of sequences obtained for each cloning experiment did not mirror the real richness of the soil samples could be a further source of the heterogeneity found. Nevertheless, the rarefaction curves consistently showed a lower richness inside the brulé than outside. These data demonstrate that fungal communities are affected in the brulé, which leads to a decreased biodiversity. This scenario comes from an investigation in a single truffle ground at two different times. Interestingly, the same scenario was revealed by DGGE in nine truffle grounds, proving that sample size did not affect this result. One hypothesis is that the lower diversity may be related to the dominance of *Tuber*, which could have affected the fungal community richness, but an inverse casual link cannot be excluded.

Considering the fungal composition, some important traits were also confirmed in the second experiment. First, *T. melanosporum* sequences were almost exclusively present inside the brulé and most Basidiomycota were exclusively present outside the brulé. Interestingly, the Basidiomycota detected outside the brulé in both years were mainly ECM fungi.

On this basis, *T. melanosporum* presence inside the brulé was correlated to a reduction in ECM fungi, suggesting that its mycelium may have an inhibitory effect on ECM biodiversity. A comparable situation has been described by [Lian et al. \(2006\)](#): they revealed that beneath the fairy rings of *Tricholoma matsutake* shiro – a solid and tight white aggregate of mycelia and mycorrhizas linked to *Tricholoma* basidiomas below the litter layer – the ECM community was dominated by this fungus. Only a few other ECM fungal species with low abundance have been detected on root tips of pine trees.

With the exception of *T. melanosporum*, only a few Ascomycota sequences belonging to ECM fungi were found, most of them outside the brulé. Among these, other *Tuber* species, for instance *T. rufum* which was identified in both years, were exclusively found outside the brulé. This molecular result confirms the finding of other *Tuber* species rather than *T. melanosporum* outside the brulé, and that there is a dynamic and temporal evolution of different species of truffles along the development of the brulé ([Serra et al., 2007](#)). Some other sequences, corresponding to *Pulvinula constellatio*, were identified among the Ascomycota ECM fungi. This fungus was found to be a colonizer of *Tuber*-contaminated plants in *in vitro* conditions ([Amicucci et al., 2001](#)). Interestingly, *P. constellatio* was not detected inside the investigated brulé, suggesting that, in natural conditions, *T. melanosporum* is able to exclude competing fungi and to establish plant colonization without disturbance.

Only a few studies have investigated fungal microbial communities that thrive in *T. melanosporum* truffle grounds ([Luppi-Mosca, 1972](#); [Luppi & Fontana, 1977](#)). As these studies were based on traditional cultural-based methods, they only identified cultivable microorganisms, which are often saprotrophic, such as *Penicillium* spp., *Aspergillus* spp., *Fusarium* spp. and *Mortierella* spp. These genera, which are very frequent in traditional isolation practice, were also detected in our libraries, but without any predominance. Our study has therefore overcome the limits of cultural-based methods and, for the first time, has provided an overview of the fungal communities connected to the brulé.

Tuber melanosporum haplotypes in soil reveal its real genetic variability

Our investigation has also produced a second unexpected result. *T. melanosporum* ITS sequences, which turned out to be extensively represented in samples collected inside the brulé, presented a high SNP biodiversity. Among the 23 sequences obtained in our study, we observed nine different haplotypes, instead of the 13 haplotypes found by the previous authors from a total of 353 samples. In order to try to explain the unexpected ITS SNP biodiversity observed here, we should consider that all the sequences obtained here come directly from soil, where the most represented form is mycelium or spores, whereas the regions analyzed by the previous authors were from fruiting bodies. Truffle fruiting bodies are made up of gleba, which has a maternal origin, and asci including spores. As spores in the gleba are not disrupted by the usual nucleic acid extraction protocol applied to fruiting bodies ([Paolocci et al., 2006](#)), the haplotypes found in fruiting bodies are predominantly limited to those of maternal origin. We suggest that the free spores present in soil samples are more easily destroyed during the DNA extraction from soil, and thus our access to haplotypes not yet encountered, including potential male haplotypes, is increased. On this basis, it can be stated that soil is indeed an unexplored source of variability. Unfortunately, unlikely the hypogeous *Rhizopogon* ([Bruns et al., 2009](#)), data on the spore bank origin and dispersion are not available for the genus *Tuber*, preventing us from explaining the additional diversity encountered in the soil.

Although we cannot exclude the possibility of PCR/cloning artifacts, the high diversity observed in our study appears even more surprising if we consider that all the sequences in our research come from a single, well-producing brulé. Furthermore, it is interesting to note that different haplotypes were found in the first- and the second-year experiments. These differences suggest an important dynamism in the *T. melanosporum* population, which is probably linked to the different stages of the *Tuber* life cycle. Even though it cannot be excluded that these haplotypes were present inside fruiting bodies that had not been collected, the minor biodiversity observed in sequences from fruiting body collections confirms that *T. melanosporum* might use sexual reproduction as a common strategy and suggests that only some haplotypes probably reach fruit-body formation. The capacity to form an ascocarp might depend on the mating types encountered. This finding offers us an explanation of unproductive artificial truffle grounds. The *T. melanosporum* genome sequencing that is currently in progress (*Tuber* Genome Consortium) has recently revealed mating-type genes ([Murat & Martin, 2008](#)). This discovery, in the near future, will be a valid tool that could support our hypothesis.

Conclusion

A differential abundance of *T. melanosporum* has been demonstrated through the use of DGGE of two well-defined areas in nine truffle grounds. *Tuber melanosporum* has proved to be the dominant ECM fungus in the brulé, where the truffle is generally collected and nine haplotypes have been identified. As a consequence, it can be stated that the development of the brulé is correlated with *T. melanosporum*. ECM fungi, other than *T. melanosporum*, decreased in the brulé where nonECM fungi increased; as a result, the ratios of ECM : nonECM fungi were different in the two areas.

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