

ITS-1 versus ITS-2 pyrosequencing: a comparison of fungal populations in truffle grounds

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Abstract: In a recent study pyrosequencing of the ribosomal internal transcribed spacer-1 (ITS-1) has validated the effectiveness of such technology in the survey of soil fungal diversity. Here we compare the two ITS regions, ITS-1 and ITS-2, of the fungal populations occurring in *Tuber melanosporum/Quercus pubescens* truffle grounds and sampled in two areas, one devoid of vegetation (“burned”, brulé in French) where *T. melanosporum* fruiting bodies are usually collected, and outside the brulé. TS1F/ITS2 and ITS3/ITS4 were used respectively for the amplification of the ITS-1 and ITS-2 regions. Two amplicon libraries were built, one for inside and the other for outside. A set of 15,788 reads was obtained. After the removal of low quality sequences, 3568 and 3156 sequences were obtained from inside the brulé with the ITS-1 and ITS-2 primers respectively. The sequences obtained from outside the brulé were 4490 with the ITS-1 primers and 2432 with the ITS-2 primers. Most of the sequences obtained for both ITS fragments could be attributed to fungal organisms.

The pair of primers, ITS1-F/ITS2, was more selective, producing fewer non-fungal sequences (1% inside, 3% outside), in addition to a higher number of sequences, than the pair ITS3/ITS4 (6% inside, 11% outside). Although differences are

present in the taxa percentages between ITS-1 and ITS-2, both reveal that Ascomycota were the dominant fungal phylum and that their number decreased moving from inside the brulé to outside, while the number of Basidiomycota increased. Taken together, both the short ITS-1 and ITS-2 reads obtained by the high throughput 454 sequencing provide adequate information for taxon assignment and are suitable to correlate the dynamics of the fungal populations to specific environments.

Key words: Pezizales, *Tuber melanosporum*

INTRODUCTION

Over the past 20 y the internal transcribed spacer (ITS) of the ribosomal DNA has provided much of what is known about ectomycorrhizal taxonomy, diagnostics and ecology (Horton and Bruns 2001, Mello et al. 2006, Lilleskov and Parrent 2007, Reich et al. 2009). Large scale Sanger sequencing of this region has allowed the characterization of fungal diversity in temperate forest soils and has provided insights into the composition of soil fungal communities (O'Brien et al. 2005). Pyrosequencing of the ITS-1 has validated the effectiveness of such technology, which bypass DNA cloning procedures and produces many more sequences more rapidly for the survey of soil fungal diversity (Buée et al. 2009). This technology, which initially was employed to detect single nucleotide polymorphisms, has been applied successfully in large scale DNA sequencing in multidisciplinary fields with important applications for genome sequencing and metagenomics (Ronaghi et al. 1998, Ronaghi 2001). The first comprehensive examination of soil community diversity was applied to bacteria in four soils across the western hemisphere (Roesch et al. 2007) and then to forest fungi (Buée et al. 2009). Among these fungi an unexpectedly high fungal diversity was found, with the majority of species retrieved only rarely. Because this work relies only on the short ITS-1 reads no information is available on how ITS-2 performs in pyrosequencing.

Therefore the aim of our work was to compare the utility of the two ITS regions, ITS-1 and ITS-2, for assessing diversity of the fungal populations occurring in *Tuber melanosporum/Quercus pubescens* truffle grounds. We sampled two selected environments, inside and outside a “burned” area, between which marked differences in fungal populations were shown

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by Napoli et al. (2010). The “burned” area (commonly referred to by the French word *brulé*) is soil devoid of vegetation near or around the host tree of the ectomycorrhizal fungus *T. melanosporum*, whose fruit bodies are known as black truffle. This is where these fruiting bodies are usually collected in late autumn and winter (Suz et al. 2008). Napoli et al. (2010) showed that the fungal populations present inside and outside nine *brulé* areas at Cahors, France, clustered into two groups based on comparison of their denaturing gradient gel electrophoresis (DGGE) profiles: one from the samples inside the *brulé*, and the second one from samples outside. Because the fungal populations of all *T. melanosporum*/*Q. pubescens* truffle grounds presented similar patterns one of them was selected for a deeper investigation (i.e. molecular cloning of the entire ITS region). From 192 clones, 96 from inside and 96 outside the *brulé*, Napoli et al. (2010) demonstrated a dominance of *T. melanosporum* and a reduction of the Basidiomycota ectomycorrhizal fungi within the *brulé*.

In the present work we wanted to determine whether both the short ITS-1 and ITS-2 reads generated by pyrosequencing could provide adequate information for taxon assignment and produce results similar to those we have obtained for truffle ground fungal populations through the more time-consuming and expensive molecular cloning of the full ITS region. To compare these two methods we took advantage of the extensive sampling undertaken for DGGE to validate pyrosequencing. ITS1F/ITS2 and ITS3/ITS4 were used respectively for the amplification of the ITS-1 and ITS-2 regions. Biases introduced by these ITS primers also are discussed.

MATERIALS AND METHODS

Soil sampling and DNA extraction.—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 are productive with a yield of 100–1000 g (Sourzat pers comm). One well defined *brulé* was selected for each truffle ground on the basis of its features (e.g. the almost complete absence of vegetation). Soil samples were collected in May 2006 from the nine *T. melanosporum*/*Q. pubescens* truffle grounds already investigated by Napoli et al. (2010). Samples of about 200 g soil were collected inside and outside each *brulé* at a depth of approximately 10–15 cm and stored at -80°C until they were analyzed. The number of samples (generally 2–3) collected for each area was different because of the heterogeneity (i.e. having different shapes and sizes) of the *brulé* and the distance of the *brulé* limit from a tree trunk. After removing any visible

roots or small pebble the soil samples (about 50 g) from each area were mixed to make them homogeneous. Two pools (inside and outside the *brulé*) per truffle ground were created, for a total of 18 pools (nine inside the *brulé* and nine outside).

The total DNA was extracted from 0.5 g soil with 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 to obtain the most representative DNA of the soil sample. In this way 18 DNA samples were obtained. All these DNA samples were quantified with NanoDrop™ and concentrations of 20 ng/μL were used in the subsequent PCR procedures.

PCR with fusion primers and purification.—The two pair of primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3')/ITS2 (5'-GCTGCGTTCCTCATCGATGC-3') and ITS3 (5'-GCATCGATGAAGAACGCAGC-3')/ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were chosen respectively for the amplification of the ITS-1 and the ITS-2 from 18 DNA samples. Because the DNA-sample preparation for amplicon sequencing consists of a PCR amplification reaction with special fusion primers these were generated by addition of primer A (5'-GCCTCCCTCGCGCCATCAG-3') and primer B (5'-GCCTTGCCAGCCCGCTCAG-3') sequences to the 5' ends of the template-specific primers ITS1F/ITS2 and ITS3/ITS4.

The PCR conditions were: denaturation at 94 C for 4 min, followed by two cycles 92 C, 55 C, 68 C; two cycles 92 C, 53 C, 68 C; two cycles 92 C, 51 C, 68 C; two cycles 92 C, 49 C, 68 C; and 29 cycles 92 C, 47 C, 68 C. The reaction was followed by a final extension of 68 C for 10 min. The PCR high fidelity system (Roche) was used. The DNA samples amplified with the fusion primers were purified with an Agencourt AMPure PCR Purification Kit according to the protocol procedure.

Amplicon library construction.—All PCR products from the nine *brulé* obtained with the same pair of primers were pooled to constitute one sample. Another sample was obtained, pooling the PCR products from the corresponding samples outside. Four subsamples were obtained: IN (A-ITS1F/B-ITS2), OUT (A-ITS1F/B-ITS2), IN (A-ITS3/B-ITS4) and OUT (A-ITS3/B-ITS4). The quality of these samples was checked with the ND-1000 Spectrophotometer NanoDrop and the Experion™ System (Biorad), whereas the quantification was obtained with the ND-1000 Spectrophotometer NanoDrop. Two final amplicon libraries were built, one for inside and the other for outside, each containing fragments amplified with the two pairs of fusion primers. A total of 10 μL containing 10^{10} molecules/μL was necessary for each library. The concentration (ng/μL) of the samples to be mixed was converted into molecules/μL with:

$$\text{Molecules}/\mu\text{L} = \frac{\text{Sample concentration}(\text{ng}/\mu\text{L}) \times (6.022 \times 10^{23})}{(656.6 \times 10^9) \times \text{sample size}(\text{bp})}$$

The two libraries were pyrosequenced with Genome Sequencer FLX System (454 Life Science, Roche) by BMR

Genomics s.r.l. (Padoa, Italy). The samples were processed in two lines out of the 16 available in the GS-FLX System.

Sequences analysis.—The four groups of sequences, two from inside the brulé and two from outside, were separately analyzed. To reduce the overall error rate the reads containing one or more ambiguous nucleotides (N) and reads shorter than 60 nucleotides were discarded (Sogin et al. 2006).

The primer sequence was trimmed with Sequencher. Each group of sequences (one for ITS-1 from inside the brulé, one for ITS-1 from outside, one for ITS-2 from inside the brulé, one for ITS-2 from outside) was assembled into OTUs, defined for a 97% pairwise similarity, with the RapidOTU program available online (<http://genome.jouy.inra.fr/rapidotu/>). OTU richness was calculated by summing the number of OTUs, including singletons, within ITS-1 and ITS-2, inside and outside the brulé. Rarefaction curves and diversity indices, Ace and Chao1, also were calculated with the RapidOTU program. The non-default parameters used were alignment with Clustal W, distance Kimura 2 parameters and a cut-off of 0.03. One representative sequence for each OTU was used for GenBank similarity queries (BLASTN on NCBI). A species level was assigned only when the e-value was 0.00. When it was not possible to identify a clear similarity with a single-species sequence, the taxonomic rank assigned to each OTU was the genus that was in common with the first results of the top of the BLAST hits with a similar e-value. Results from BLASTN were organized in pie charts illustrating proportions for ITS1 from inside the brulé, for ITS1 from outside, for ITS-2 from inside the brulé, and for ITS2 from outside. Comparisons between ITS-1 and ITS-2 and between inside the brulé and outside were made through percentages. All the sequences grouped in the OTUs corresponding to *T. melanosporum* were brought into alignment separately to highlight any possible single nucleotide polymorphisms (SNPs).

When a polymorphism was identified a quality control for the corresponding base was done on the flow gram recovered for each sequence from the 454 platform to ensure that the polymorphism was correct. The sequences analyzed in this study were deposited in SRA under the accession number SRP006078.

RESULTS

Two pairs of primers, ITS1F/ITS2 and ITS3/ITS4, were used for the amplification of the ITS-1 and the ITS-2 respectively from 18 DNA samples (nine from inside the brulé and nine from outside) and respectively produced fragments of about 350 bp and 400 bp. Two libraries were generated, one for inside and the other for outside, each containing fragments amplified with the two pairs of primers. These libraries produced 15,788 reads; most of them were 200–300 bp long, with a consistent group of almost 300 bp. After the removal of low quality sequences (2142), 3568 and 3156 sequences were

TABLE I. Number of sequences obtained for each amplicon library (one for inside and the other for outside), before and after the removal of sequences of low quality

	Total sequences	Filtered sequences
Inside brulé		
ITS-1 ^a	4101	3568
ITS-2 ^b	3676	3156
Outside brulé		
ITS-1	5160	4490
ITS-2	2851	2432

^aITS-1 reads are obtained with the pair of primers A-ITS1F/B-ITS2.

^bITS-2 reads are obtained with the pair of primers A-ITS3/B-ITS4.

obtained from inside the brulé with the ITS-1 and ITS-2 primers respectively. The sequences obtained from outside were 4490 with the ITS-1 primers and 2432 with the ITS-2 primers (TABLE I).

All 8058 sequences of the ITS-1 region (from both inside and outside the brulé) were assembled to give a total of 900 OTUs; 213 were in common between the two habitats, 301 were exclusive to the inside brulé, while 386 were specific of the outside (FIG. 1A). Eight OTUs surpassed 100 sequences; 18 OTUs grouped from 50 to 100 sequences; 360 OTUs consisted of a single sequence (singletons). The singletons inside and outside were respectively 179 and 182.

The 5588 sequences from the ITS-2 region were assembled into 885 OTUs. In this case 209 OTUs were in common to both inside and outside the brulé; 294 OTUs were exclusive of the outside, while 382 were exclusive of the inside (FIG. 1B). Altogether five OTUs surpassed 100 sequences, 12 OTUs grouped from 50 and 100 sequences and 375 OTUs consisted of a single sequence. The singletons inside and outside were respectively 218 and 161.

BLAST queries indicated that most of the sequences obtained for both ITS fragments could be attributed to fungal organisms (FIG. 2). The non-fungal sequences amplified with the ITS-1 primers were 50 out of the 3568 inside and 151 out of the 4490 outside (1% and 3% respectively) (FIG. 2). Among the remaining ITS-1 sequences only 163 sequences inside (5%) and 175 outside (4%) could not be classified to Phylum because they had low similarity with the specific fungal phyla and sometimes high similarity with sequences classified as uncultured fungi. With the pair of primers for the ITS-2 region, 181 of the 3156 sequences inside and 304 of the 2432 outside sequences (6% and 11% respectively) were not attributable to the fungal kingdom (FIG. 2). One hundred eighty-four sequences could not be assigned

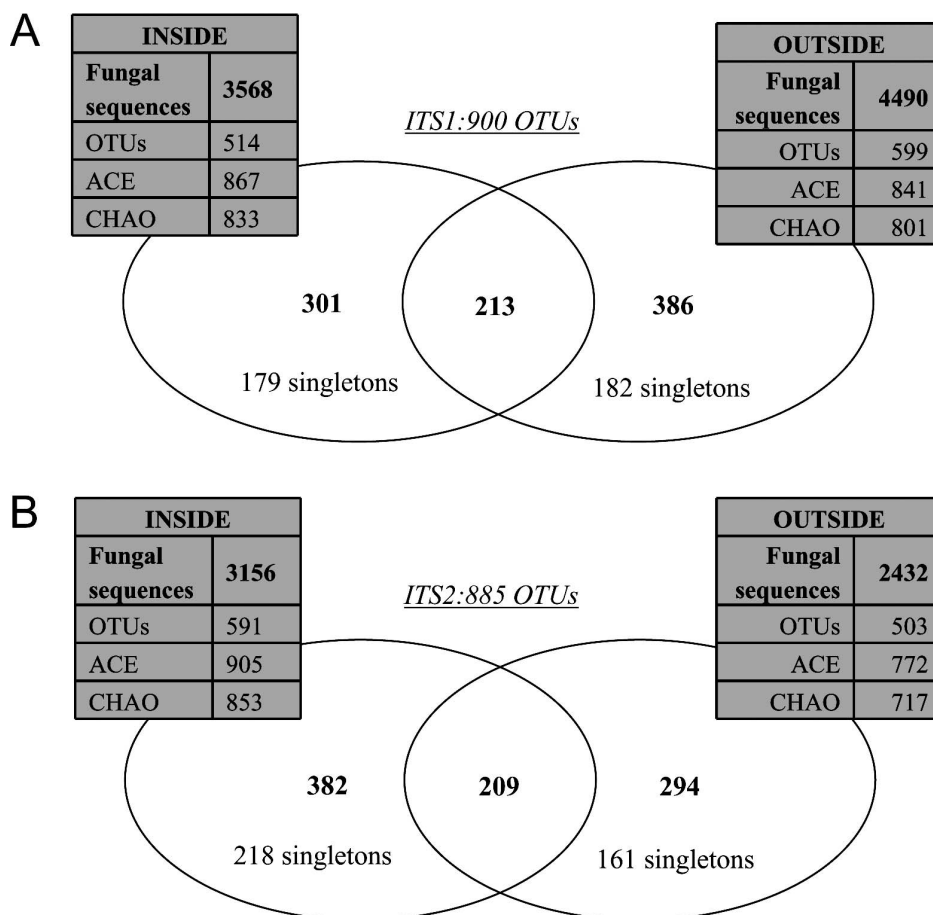


FIG. 1. Number of fungal sequences, operational taxonomic units (OTUs) and richness estimators Chao1 and Ace from ITS-1 (A) and ITS-2 (B) are given for inside and outside the brûlé. Venn diagram shows the overlap of the OTUs (defined at a similarity of 97%).

to any fungal phylum, 95 of which came from the inside library (3%) and 79 from the outside (3%).

Ascomycota and Basidiomycota were the dominant fungal phyla with a clear dominance of Ascomycota, which surpassed 50% of the sequences in all the four groups of sequences. Their number decreased, moving from inside to outside the brûlé with values of 72–57% (ITS-1) and 79–65% (ITS-2). The number of basidiomycetes increased from 11% to 28% (ITS-1) and from 6% to 18% (ITS-2). Other phyla (Zygomycota, Chytridiomycota and Glomeromycota) were under 5%, with the exception of Zygomycota which reached 8% inside the brûlé with the ITS-1 primers. Even though the number of sequences belonging to these phyla was low, the same trend was observed for the two pairs of primers; the number of Zygomycota and Chytridiomycota increased inside the brûlé, while the number of Glomeromycota decreased (FIG. 2).

The Sordariomycetes and Pezizomycetes were well represented in all four groups of sequences. The ITS-

1 reads revealed that 69% of the sequences outside and 78% inside belonged to the Sordariomycetes and Pezizomycetes. The greater number of sequences found inside was due mostly to an increase in Pezizomycetes, which rose from 31% to 45% (SUPPLEMENTAL FIG. 1).

The class composition changed with the ITS-2 reads. A greater percentage of Dothideomycetes and Eurotiomycetes was found in comparison with the ITS-1 reads. In other words, the number of Sordariomycetes increased from 18% to 40% while the number of Eurotiomycetes decreased from 26% to 4%, moving from outside to inside the brûlé (SUPPLEMENTAL FIG. 2).

The most representative orders in three out of the four groups of sequences were Hypocreales and Pezizales. The fourth group from outside the brûlé ITS-2 also presented in addition to a low percentage of Hypocreales (14%) a high percentage of the Onygenales (21%) and was not represented in the ITS-1 reads (SUPPLEMENTAL FIG. 3).

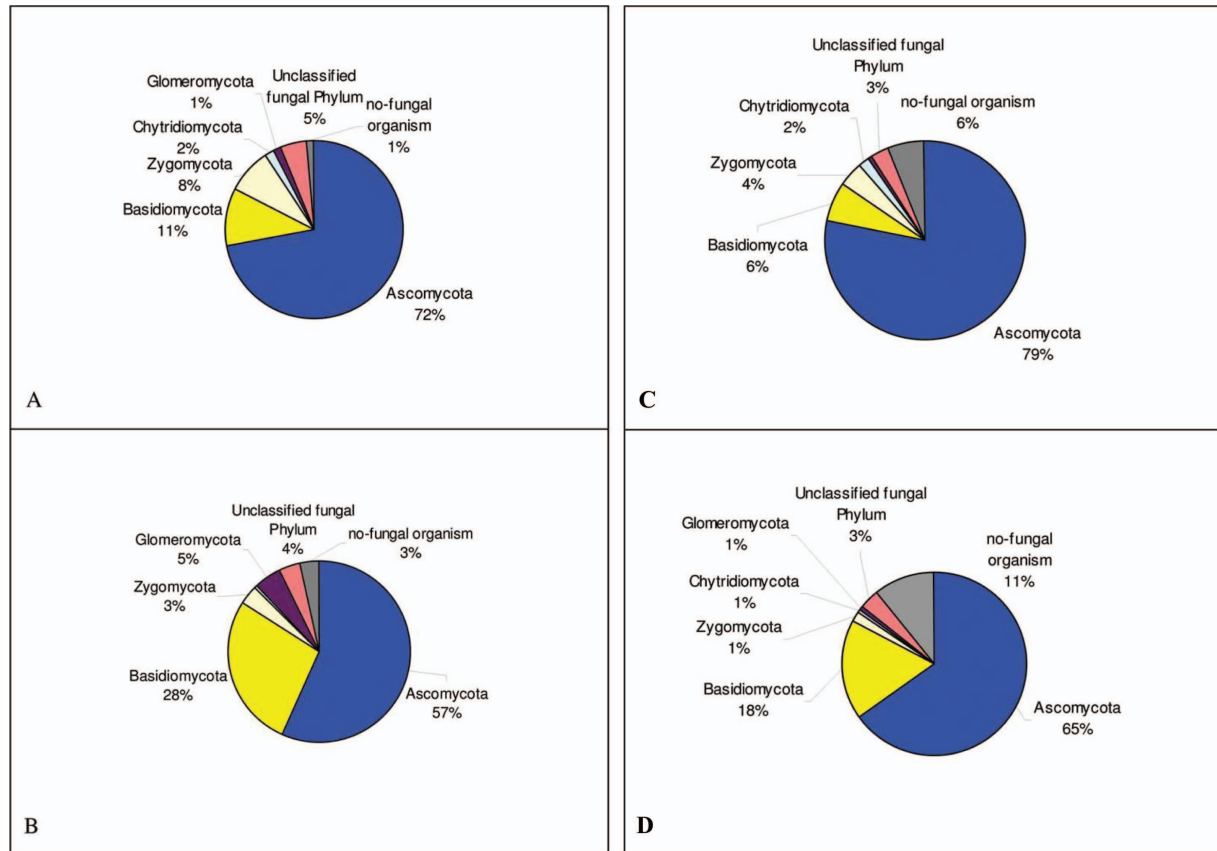


FIG. 2. Phyla subdivision from: ITS-1 reads for inside (A) and outside (B) the brûlé; ITS-2 reads for inside (C) and outside (D) the brûlé.

Among the Basidiomycota more than 80% of the sequences from ITS-1 and ITS-2 represented two classes, Agaricomycetes and Tremellomycetes. Moving from inside the brûlé to outside, the Agaricomycetes increased from 43% to 80% (ITS-1) and from 55% to 82% (ITS-2), while the Tremellomycetes decreased from 43% to 8% (ITS-1) and from 33% to 13% (ITS-2) (SUPPLEMENTAL FIGS. 4, 5). A higher taxa subdivision proved to be difficult because of the abundance of unclassified sequences. In all four groups of sequences more than 10% of those belonging to Basidiomycota were not classified in any order. For the outside ITS-1 sequences the percentage of unclassified sequences reached 39%. Moreover the results showed that the most highly represented orders among the Agaricomycetes were Agaricales and Thelephorales, while among the Tremellomycetes it was the Filobasidiales (SUPPLEMENTAL FIG. 6). Comparisons of relative abundances of classes and orders are illustrated (SUPPLEMENTAL FIGS. 1–6) and summarized (TABLE II).

Eight out of 900 ITS-1 OTUs from both inside and outside contained more than 100 sequences. Among these a single OTU comprised a total of 1305 sequences, 848 from inside and 457 from outside the

brûlé. BLAST queries returned a 100% similarity with GenBank *T. melanosporum* sequences for this OTU. The sequences grouped into this OTU were searched for the presence of SNPs and carefully divided into subgroups. With the exception of one subgroup that contained reads that occurred only once in the entire dataset, which therefore was excluded from this analysis because of possible sequencing mistakes, 16 subgroups differing for 1, 2 or more SNPs were identified. Three subgroups were the most abundant.

Another three abundant OTUs, assigned to Ascomycota, were classified into the *Cordyceps*, *Genea* and *Bionectria* genera. Sequences belonging to *Genea*, which is an ectomycorrhizal genus, were not found inside the brûlé but were found outside (198 sequences). The OTUs assigned to Basidiomycota belonged to the genera *Hymenogaster* and *Cryptococcus*. While the *Hymenogaster* sequences were numerous outside the brûlé, *Cryptococcus* was found mainly inside (TABLE III).

Four OTUs from ITS-2 belonged to Ascomycota and contained more than 100 sequences. Again in this case we found a close correspondence with the genera *Tuber*, *Cordyceps* and *Bionectria*, already found with the ITS-1 reads. The number of sequences for

TABLE II. BLAST results of OTUs from ITS-1 and ITS-2 both inside and outside the brulé

Ascomycota ITS1		Ascomycota ITS2		Ascomycota	Basidiomycota	Basidiomycota ITS1		Basidiomycota ITS2	
Inside	Outside	Inside	Outside	Class	Class	Inside	Outside	Inside	Outside
33% ^a	38%	40%	18%	Sordariomycetes	Agaricomycetes	43%	80%	55%	82%
45%	31%	20%	16%	Pezizomycetes	Tremellomycetes	43%	8%	33%	13%
1%	1%	4%	26%	Eurotiomycetes	Pucciniomycetes	—	2%	6%	2%
8%	8%	14%	18%	Dothideomycetes	Microbotryomycetes	3%	1%	3%	—
3%	4%	5%	10%	Leotiomycetes	Cystobasidiomycetes	1%	—	—	—
—	1%	—	1%	Orbiliomycetes	Unclassified	10%	9%	3%	3%
—	1%	1%	1%	Lecanoromycetes					
—	2%	—	—	Saccaromycetes					
10%	14%	16%	10%	Unclassified					

	Inside	Outside	Order	Order	Inside	Outside
	20%	16%	Pezizales	Agaricales	7%	37%
	2%	21%	Onygenales	Polyporales	1%	—
	1%	5%	Chaetothyriales	Filobasidiales	33%	3%
	8%	9%	Pleosporales	Sporidiobolales	3%	—
	2%	3%	Capnodiales	Cantharellales	—	2%
	3%	2%	Sordariales	Pucciniales	—	1%
	4%	5%	Helotiales	Boletales	13%	2%
	—	1%	Myriangiales	Tremellales	8%	3%
	2%	1%	Xylariales	Russulales	—	1%
	1%	1%	Lecanorales	Telephorales	16%	12%
	—	1%	Botryosphaeriales	Unclassified	19%	39%
	—	1%	Orbiliales			
	1%	—	Verrucariales			
	34%	14%	Hypocreales			
	22%	21%	Unclassified			

^a Percentages refer to the number of sequences after selection obtained from ITS-1, 3568 inside and 4490 outside.

inside and outside the brulé changed for each OTU (TABLE IV).

Rarefaction curves generated within ITS-1 and ITS-2, inside as well as outside the brulé, did not reach saturation, indicating that the number of sequences obtained for each habitat did not capture the real richness of soil samples in ITS-1 or in ITS-2 (FIG. 3). The Chao 1 and Ace non-parametric richness estimators revealed that our results were far from the expected richness of the soil sample (FIG. 1).

DISCUSSION

Napoli et al. (2010) used DGGE to examine differences between fungal populations sampled inside and outside brulé areas of nine *T. melanosporum*/*Q. pubescens* truffle grounds. Because the aim of the present investigation was to see how the short reads from ITS-1 and ITS-2 differed in the sequence and OTU numbers and in the taxa assignment we took the advantage of the extensive sampling previously carried out for DGGE and of our knowledge of truffle ground fungal populations to validate pyrosequencing.

This new technique enables extraordinary progress in assessing biodiversity and contributes to our understanding of the microbial diversity in ecosystems.

Most of the sequences obtained by pyrosequencing were grouped in a few principal OTUs. Although an easier amplification of particular groups could be due to a better DNA accessibility or to primer bias, the results make it possible to hypothesize that these particular groups could be the main fungal taxa present in the sampled soil. Members of the Ascomycota were recovered as the dominant group in our investigation with both ITS regions. Some principal OTUs were assigned for both regions to identical taxa, in particular to *Cordyceps*, *Tuber* and *Bionectria*. Other sequences were not common to the two regions, at least in the principal OTUs, but a clear tendency was identified. Fewer ascomycete sequences were recovered from outside the brulé, while the number of Basidiomycota sequences increased. Within subdivisions of the Basidiomycota and Ascomycota we noted that this trend was due mainly to changes in abundance of members of some principal orders.

TABLE III. BLAST results of eight out of 900 OTUs from ITS-1 for both inside and outside the brûlé

bp	Number of sequences		Phylum	Class	Order	Family	Genus	Species
	Total	Inside						
236	1305	848	Ascomycota	Peizomycetes	Pezizales	Tuberaceae	<i>Tuber</i>	<i>Tuber melanosporum</i>
		24% ^a						
253	644	255	Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	<i>Cordyceps</i>	<i>Cordyceps bassiana</i>
		7%						
203	198	0	Ascomycota	Peizomycetes	Pezizales	Pyronemataceae	<i>Genea</i>	
		0						
243	183	4	Basidiomycota	Agaricomycetes	Agaricales	Hymenogasteraceae	<i>Hymenogaster</i>	
		< 1%						
250	154	84	Zygomycota			Mortierellaceae	<i>Mortierella</i>	<i>Mortierella alpina</i>
		2%						
245	153	135	Ascomycota	Sordariomycetes	Hypocreales	Bionectriaceae	<i>Bionectria</i>	
		4%						
103	140	113	Basidiomycota	Tremellomycetes	Filobasidiales		<i>Cryptococcus</i>	
		3%						
266	137	28	Ascomycota					
		< 1%						
		2%						

^a Percentages refer to the number of sequences after selection obtained from ITS-1, 3568 inside and 4490 outside.

TABLE IV. BLAST results of four out of 885 OTUs from ITS-2 both inside and outside the brulé

bp	Number of sequences			Phylum	Class	Order	Family	Genus	Species
	Total	Inside	Outside						
197	569	433 14% ^a	136 6%	Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	<i>Cordyceps</i>	<i>Cordyceps bassiana</i>
89	345	222 7%	123 5%	Ascomycota	Pezizomycetes	Pezizales	Tuberaceae	<i>Tuber</i>	
225	173	160 5%	13 < 1%	Ascomycota	Sordariomycetes	Hypocreales	Bionectriaceae	<i>Bionectria</i>	
60	104	86 3%	18 < 1%	Ascomycota					

^aPercentages refer to the number of sequences after selection obtained from ITS-2, 3156 inside and 2432 outside.

Among the Ascomycota the number of Hypocreales increased inside the brulé. Among the Basidiomycota the number of Agaricales increased while the number of Filobasidiales decreased outside the brulé. It is known that the Hypocreales and Filobasidiales mainly include saprotrophic fungi, while many of the common ectomycorrhizal fungi belong to the Agaricales. On the basis of these data, we suggest a reduction in the number of ectomycorrhizal species within the brulé.

The OTU assigned to the genus *Tuber* was an exception. Although this genus contains ectomycorrhizal fungi, it was found mainly inside the brulé. Species determination for this principal OTU was possible only with one of the two regions. In fact with the ITS-1 region it was possible to assign this principal OTU to *T. melanosporum*. This result agrees with data by Napoli et al. (2010) who suggested a competitive effect of *T. melanosporum* with the other ectomycorrhizal fungi. With the ITS-2 region it was not possible to clearly identify the *Tuber* species, principally because the length of the sequences was less than 90 bp. In an attempt to remove all sequences shorter than 200 bp to improve the match in the BLAST analysis, this OTU was dropped. This prevented us from discarding reads shorter than 200 bp at the beginning of our analysis.

Searching for SNPs, the three most highly represented subgroups of *T. melanosporum* likely belong to the ubiquitous haplotypes 1, 2 or 3 described in fruiting bodies by Murat et al. (2004) and Riccioni et al. (2008); not one subgroup matched the potentially new haplotypes described by Napoli et al. (2010) in soil, whereas five potentially new haplotypes have been identified. This suggests the existence of a greater variability of *T. melanosporum* in the soil than has been recognized in the fruiting bodies, as reported by Zampieri et al. (2010) who found a new haplotype in the β -tubulin gene of *T. magnatum* mycelium in soil. Because a higher variability usually

increases the chance of adaptability and fitness of the mycelia this could ensure mycelia suitable for fruiting body production and must be considered in management of plantations (Zampieri et al. 2010).

PCR biases in ITS-1 and ITS-2.—It is known that various commonly used ITS primers might introduce biases during the amplification of different parts of the ITS region. The pair of primers ITS1-F/ITS2 here used for the amplification of the ITS-1 region produced fewer non-fungal sequences in addition to a higher number of sequences than the pair ITS3/ITS4 amplifying the ITS-2 region. In fact our work produced 15 788 reads among which 12 960 were fungal sequences; 99% (inside) and 97% (outside) originated from ITS-1, whereas 94% (inside) and 88% (outside) came from ITS-2. Given that ITS1-F is a fungal specific primer, it is not surprising to find more fungal sequences in the ITS-1 region data. As a consequence, the pair of primers ITS1-F/ITS2 was more selective. The long fragments inhibit the emulsion-PCR step at the expense of the short amplicons, according with Margulies et al. (2005). In this view the longer fragment of the ITS-2 probably inhibited the emulsion-PCR step at the expense of the shorter fragment of the ITS-1, and this could be the reason for the lower number of sequences obtained from the ITS-2.

In silico PCR analyses conducted by Bellemain et al. (2010) indicated that ITS3 and ITS4 primers preferentially amplified ascomycetes whereas ITS1-F preferentially amplified basidiomycetes. In agreement with these authors, we have found, using the ITS-2 region, a slighter higher number of the ascomycetes compared to basidiomycetes in both environments.

In term of species richness the ITS-1 and ITS-2 pyrosequencing produced a high richness, 900 OTUs and 885 OTUs respectively. The OTU number inside the brulé was 514 (14%) and 591 (19%) on the basis of ITS-1 and ITS-2 respectively, against 599 (13%) and 503 (21%) outside. Nevertheless, if we consider the high number of singletons found in ITS-1 and in ITS-

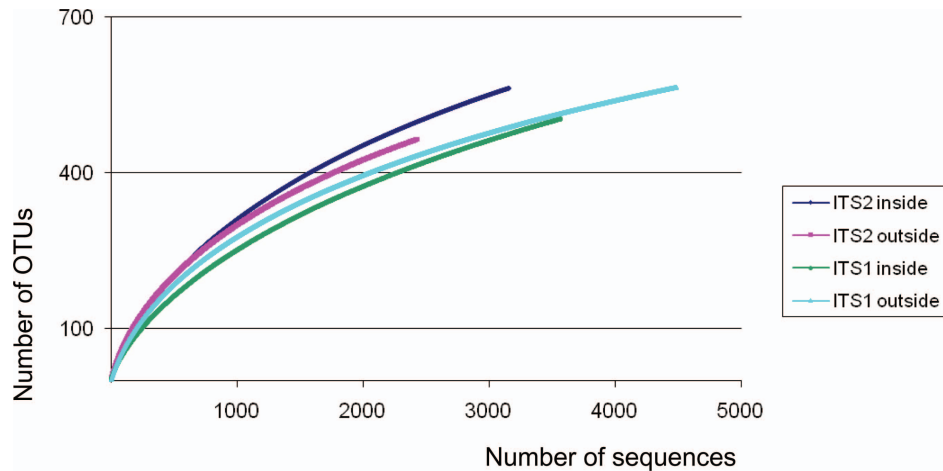


FIG. 3. Rarefaction curves generated by the DOTUR 1.53 software for the fungal operational taxonomic units (OTUs) obtained from ITS-1 and ITS-2 for inside as well as outside the brulé.

2, the richness estimation might not be reliable. This conclusion is supported by Jumpponen and Jones (2009) who found one-half of singletons in the hyperdiverse phyllosphere communities. Regardless of the high richness, the rarefaction curves did not reach saturation in either ITS-1 or ITS-2, suggesting that the real fungal richness could be greater. Furthermore the numerous non-fungal sequences revealed by the ITS-2 region could overwhelm the number of obtained fungal OTUs and consequently affect the slope of the rarefaction curve for this region. In agreement with the analysis by Nilsson et al. (2009), who examined how well the short pyrosequenced reads perform in silico in taxa assignment, we have demonstrated that both ITS-1 and ITS-2 provide adequate information for taxon assignment. Both regions led to the knowledge we gained on fungal populations in one of the previously investigated truffle grounds (i.e. ectomycorrhizal species decreased inside the brulé, but only ITS-1 revealed that the ectomycorrhizal fungus *T. melanosporum* was mainly inside the brulé).

To the authors' knowledge this is the first report on the comparison of the two ITS regions in pyrosequencing. Buée et al. (2009), Jumpponen and Jones (2009) and Jumpponen et al. (2010) used ITS-1 to study soil forest fungi, fungal communities in the *Quercus macrocarpa* phyllosphere and fungal communities in the *Quercus* spp. respectively. Tedersoo et al. (2010) showed on the basis of the ITS-1 sequence data that pyrosequencing and Sanger sequencing of tropical fungi provide similar results but reveal substantial methodological biases. Apart from the ITS region, Lumini et al. (2010) and Opik et al. (2009) used the small subunit ribosomal RNA gene to address arbuscular mycorrhizal fungi biodiversity. On the basis of our

experience, ITS-1 is probably the best choice to study soil forest fungi. Because our pilot experiment of comparison of the short ITS-1 and ITS-2 took advantage of our previous knowledge on truffle ground fungal populations we did not include repetitions and consequently statistical analysis. Nevertheless we suggest considering repetitions when taxa have to be inferred, once the molecular marker has been chosen.

Taken together, we have provided an experimental validation of the efficacy of the short ITS-1 and ITS-2 when applied to 454 pyrosequencing. In times in which high throughput techniques are becoming widely used, bypassing DNA cloning, validation experiments represent an important point from which to build next steps. Thanks to this technology the scientific challenge is a sequencing project to uncover the soil metagenome (Vogel et al. 2009). This surely will unravel the biodiversity associated with soil microorganisms, in addition to providing insights into the ecology of those that are beneficial to crop production.

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