Detection of Fungal DNA in Forest Soils:
Optimization of Molecular Techniques for High-throughput Sequencing

Student:

Rana Adamo (MEDFOR Candidate)

Supervisors:

Christine Fischer (Forest Sciences Center of Catalonia - CTFC)

José Antonio Bonet (University of Lleida - UDL)

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Abstract

The applications of molecular methods have expanded our knowledge of fungal ecology and diversity, and are particularly valuable for investigating the microscopic fungal structures that abound in forest soils. Extraction of fungal DNA from soils has several challenges due to the complexity of soil chemical properties. One of the most important steps in using any extraction method is the efficient recovery of DNA that ensures successful downstream applications. In this study we compared the extraction efficiency of two commercial kits designed for extracting DNA from soils. Using eleven different forest soils with a wide range of pH and organic matter, we evaluated the yield and the quality of extracted DNA using NucleoSpin® soil (NSP) kit (Macherey-Nagel, Duren, Germany) and Powersoil® DNA isolation kit (MO Bio, Carlsbad, CA, USA). Extracted DNA was quantified using a NanoDrop spectrophotometer. Yield and quality evaluated by 260:280 nm ratio were higher for DNA extracted from the NSP kit compared to DNA extracted with Powersoil kit. The quality evaluated by 260:230 ratio was lower than the optimal range for extracts from both kits but was higher for NSP compared to Powersoil. Further comparisons of DNA quality and quantity were made by testing the PCR outcomes for each extraction. For our soils the DNA extracted using the NSP kit with its enhancer solution was the most successful in amplifying the ITS2 fungal region for all soils tested using a reduced range of PCR cycles (25-28). Finally we tested these extraction methods using soils collected from both natural and cultivated truffle sites and we observed that DNA extracted with NSP was more efficient than that extracted with Powersoil in detecting one of the Tuber species (*T.brumale*) which was suspected to be present in one of the sites. These sequential tests lead us to the development of an optimized protocol designed to address our future inquiries into fungal diversity in these soils.

Keywords: DNA extraction; Extraction kits, PCR optimization; *Tuber melanosporum* detection.

Introduction

Soil fungal communities play a key role in forests ecosystems. Saprobic species are mainly responsible for organic matter and litter decomposition, which results in nutrient recycling at the ecosystem level. Ectomycorrhizal species form a mutualistic association with living trees by colonizing their fine roots where the absorption of water and nutrients occurs in exchange for assimilated plant carbon (Courty et al. 2010). Recent studies suggest that ectomycorrhizal communities, in addition to supporting forest growth, are a significant source for soil carbon sequestration (Clemmensen et al. 2013), providing more evidence for the important role of ectomycorrhizal fungi. Inventory studies of these diverse communities based on sporocarp or ectomycorrhizal identifications tend to provide incomplete or non-representative results. (Koide et al. 2005; Kjoller. 2006).

Soil fungi have been problematic to study due to various technical limitations associated with identification and quantification of microscopic organisms in highly diverse soil systems. Because the majority of microorganisms are yet to be cultured (less than 1% of microorganisms are cultivable) (Amann et al. 1995; Hugenholtz et al. 1998), studies of fungal community composition must rely on direct analysis of environmental samples (Gillings. 2014). The introduction of culture-independent techniques, based on analyses of microbial DNA have revolutionized environmental microbiology, yielding a wealth of new information on uncultured microbial populations (Maron et al. 2011; Delmont et al. 2011; Pan et al. 2010). Advances in sequencing technologies have made the investigation of fungal ecology and community dynamics more manageable. With the widespread use of next-generation sequencing technologies we may be able to address questions related to ectomycorrhizal community diversity and shifts in response to disturbances, to better understand their role in forests ecosystems.

The first fungal community studies using high-throughput sequencing have highlighted the complexity and the high diversity of fungal species in forests soils (Buée et al. 2009; Jumpponen et al. 2010). However, one of the greatest limitations of using these emerging technologies is the optimization of the molecular procedures for a reliable comparison among the studies. The first critical step in using such

technologies is the efficient recovery of genomic DNA from complex environmental samples such as soil (Plassart et al. 2012).The problem of getting representative extracts of gDNA from complex soil substrates have been widely studied and different methods for extraction including commercial kits have been compared (Töwe et al. 2011; Yankson et al. 2009; Knauth et al. 2012; Yang et al. 2007; Whitehouse et al. 2007). Until present there is no method free from bias (Feinstein et al. 2009; Martin et al. 2001; Frostegard et al. 1999). Also the ability to get standard gDNA in terms of good yield and quality is highly challenging because DNA yields from soils are usually low (McIlroy et al. 2009; Töwe et al. 2011) and often are highly dependent on soil properties such as pH and carbonates (Barton et al. 2006). Another serious issue is the co-extraction of organic substances such as humic and fulvic acids which show anionic characteristics similar to DNA such that they co-precipitate with DNA during the purification step (Tebbe and Vahjen. 1993; Tsai and Oslen. 1992; Técher et al. 2010). As a consequence, low quantities of about 10 ng of those molecules (with molecular weight ranging between 2000-300000 g/mol) may inhibit the subsequent PCR amplification by either inhibiting the enzymatic activity of the DNA polymerase or cause template inhibition by binding to the template preventing it from being amplified (Robe et al. 2003; Rajendhran and Gunasekaran. 2008; Engel et al. 2011; Matheson et al. 2010).

The internal transcribed spacer region (ITS) of the nuclear ribosomal DNA is the formal DNA barcoding region for molecular identification of fungi (Schoch et al. 2012; Blaalid et al. 2013). The fungal ITS region varies roughly, with some exceptions, between approximately 450 and 750 base pairs (bp) in length and consists of three subregions: the variable spacers ITS1 and ITS2 and the intercalary 5.8S gene (Blaalid et al. 2013). Primer sets have been designed and successfully applied to amplify this fungal region (Schoch et al. 2012). Recently it has been suggested that ITS2 region is better for fungi species determination (Koetschan et al. 2010). New primers have been developed to amplify this region to provide better resolution of fungal species and much more preserved community composition resulting in less bias during the DNA amplification (Ihrmark et al. 2012). Further bias occurs with the increase in the number of cycles during the amplification, because excessive cycles may result in preferential amplification of rare sequences, short sequences and the creation and further propagation of chimeric sequences (Kanagawa. 2003; Huber et al.

2011). Particularly when using degenerate primers with high cycle numbers, depletion of specific primers in the mixture may cause a bias in the amplification, favoring species that match other less depleted primers (Polz and Cavanaugh. 1998). Therefore, the number of PCR cycles should be minimized. Generally, one should aim for weak to medium-strong amplicon band, as visualized on agarose gel (Lindahl et al. 2013). In addition, reactions using relatively high concentrations of DNA template have proven to reduce bias (Polz and Cavanaugh 1998) but also might increase the inhibition during amplification, especially in soils with a high content of humus. One solution is the dilution of templates which may often improve PCR outcome as a result of the dilution of the inhibition component (Wilson. 1997). Considering all the issues addressed above, researchers can follow guidelines from previous comparative studies and perform preliminary tests to identify which protocols are most successful in overcoming problems associated with their particular environmental samples. These studies are essential ground work to create an efficient, reliable and cost effective protocol for further downstream applications.

Our objective in this study was to (i) Test the extraction efficiency of two commercial DNA kits: NucleoSpin® soil (NSP) kit (Macherey-Nagel, Duren, Germany) and Powersoil® DNA isolation kit (MO Bio, Carlsbad, CA, USA) on different soils with a wide range of pH and organic matter collected from Spain (ii) test the inhibition of the forest soils used in the study (iii) test different PCR cycles (22, 25, and 30 cycles) for PCR amplification using the fungal primers fITS7-ITS4 to obtain ready-to-use PCR products with small to medium band size for high-throughput sequencing (iiii) Compare the efficiency of the extraction methods for the detection of *Tuber melanosporum*, an important ectomycorrhizal fungus in the several soils used in this study.

Materials and methods

Soil properties and sampling

Soils from eleven experimental sites have been considered in this study. Five are forest soils sampled from Catalonia (NE, Spain) with predominantly Black pine (*Pinus nigra*) and/or Scots pine (*Pinus sylvestris* L.) trees, and the other six soils are from black truffle sites (both natural and cultivated plantations) sampled from Catalonia and other regions of Spain (La Rioja and Soria). The geographical locations of the different experimental sites are shown in Fig. 1. Soil Chemical properties are shown in Table1.

Table 1. Soil pH and organic matter percentage of the experimental sites considered in the study. NA= data not available

Sample number	Site Ref	Water pH	Organic matter (%)
1	210	5.8	3.48
2	213	>8,2	3.31
3	222	6.7	9.25
4	227	8	11.77
5	311	6.7	3.91
6	TOF2	8.2	11.15
7	TOF10	8.4	10.76
8	TOF28	8.2	4.59
9	LR1	NA	NA
10	LR2	NA	NA
11	RAG18	NA	NA

Fig. 1 Map showing the geographical distribution of the sampling sites. LR refers to sample 9 and sample 10 collected from non producing truffle plantations in La Rioja.

Four individual cores (20 cm depth) were sampled at each site with 10 meters distance between each core. Samples were transported in sealed plastic bags in a portable cooler to the University of Lleida and stored overnight at 4 °C. The soils were sieved the next day (4 mm sieve) to remove stones, roots, and debris then freeze dried for three days. After freeze drying, 10g of each of the four cores which correspond to each experimental site were bulked and homogenized with mortar and pestles and frozen at −20 °C prior to further processing.

DNA extraction

The DNA extraction experiment was conducted on the first six soils listed in Table 1. Soil DNA was extracted using the NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany) and the Powersoil® DNA isolation kit (MO Bio, Carlsbad, CA, US) according to the manufacturer's instructions. The NSP kit included two separate buffers (SL1, SL2) and an enhancer solution (SX) to optimize the extraction efficiency according to the properties of the soil of interest, offering four possible buffer combinations of the NSP kit. These four options and the Powersoil (P) provided five extraction methods tested to extract DNA from each soil and to compare the extraction efficiency in terms of yield and quality (Table 2). For all extraction methods, gDNA was extracted from the same amount of soil (250 mg) and eluted in the same final volume of 100 μL of the elution buffer provided with each commercial kit. DNA extraction for each soil sample was performed in triplicate (6 soils * 5 methods * 3 replicates = 90 samples) and all DNA extracts were stored at −20 °C.

Table 2. The five extraction methods tested in the study.

Lysis buffer combination	Method reference	Method
SL1 without enhancer solution (SX)	SL1	NucleopSpin (NSP)
SL1 with enhancer solution (SX)	SL1+	NucleopSpin (NSP)
SL2 without enhancer solution (SX)	SL2	NucleopSpin (NSP)
SL2 with enhancer solution (SX)	SL2+	NucleopSpin (NSP)
	P	Powersoil (P)

Evaluation of DNA yield and purity

The yield and the purity of the gDNA were evaluated quantitatively using nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE USA). A total of 1 µL was loaded to determine the concentration and the quality of gDNA. DNA concentration was measured as ng/µL and DNA quality was determined by the ratios of 260:280 nm and 260:230 nm. The recommended ideal value for the 260:280 nm ratio is between 1.8 and 2. Values above or below this range indicate protein or other contaminants. The optimal range for 260:230 nm ratio is between 1.7-2, and values below 1 indicate the presence of humic acids. The readings of the nanodrop spectrophotometer for both yield and quality were used to compare the DNA extraction protocols for each soil using two-way ANOVA tests (method, soil type and the possible interaction between the two factors) at $P = 0.05$ level of significance. The *Tukey's HSD* (honest significant difference) test was applied to compare the treatments means. The statistical analysis was conducted using the software JMP V.11 statistical discovery from SAS. To further confirm the nanodrop results, comparison of DNA extractability from the different soils and tested methods was qualitatively assessed by agarose gel electrophoresis. 5 µL of genomic DNA extract was run in 2% agarose gel at 80V for 30 min in 1X TBE buffer and with 1 kb DNA Ladder (Sigma-Aldrich, USA) as the molecular size marker. Gels were prestained with GelRed™ fluorescent nucleic acid dye and visualized under UV light.

Based on the statistical analysis (Fig. 2) conducted for the first DNA extraction test, the three methods which had significantly different yields among the five tested methods (SL1+, SL2 and P) were used for the second set of soil extractions performed for the truffle soils (number 7 to 11 in Table 1). Together with the first set of soil extracts, they were included in the following amplification tests.

PCR analysis

For the purpose of PCR optimization for future sequencing analysis, inhibition and PCR cycle numbers were tested simultaneously using the DNA extractions from all 11 soils with the following three methods: SL1+, SL2 and P. Preparing different dilutions from the stock DNA to obtain template DNA concentrations of 0.5, 1 and 2

ng/µL was performed for the PCR inhibition test with all soil samples. Amplification of fungal ITS2 region was performed using fungi-specific primer fITS7 (Ihrmark et al. 2012) (5′ -GTGARTCATCGAATCTTTG-3′, Eurofins Genomics, Ebersberg, Germany) and universal primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′, Eurofins Genomics, Ebersberg, Germany). Reactions were carried out using puReTaq Ready- To- Go PCR beads (GE Healthcare, Buckinghamshire, UK) in a final volume of 25 µL, containing BSA, Stabilizers, Reaction Buffer, 200 µM of each dNTP, 1.5 mM MgCl2, 0.2 pM/µL of each primer and 2.5 units of puReTaq DNA polymerase. PCR cycling was done using Biometra thermocycler (Goettingen, Germany). The PCR program included an initial denaturation at 95 °C for 5 min followed by either 22, 25 or 30 cycles of 95 °C for 30 sec, 57 °C for 30 sec and 72 °C for 30 sec, and a final extension at 72 °C for 7 min. Since that different PCR cycle numbers (22, 25, 30) were used for the purpose of the study, to achieve the targeted cycle number, PCR thermocycler was paused when the designated cycle number was completed, 7 µL of each amplicon were transferred to a new tube and incubated in a water bath at 72 for 7 min, and then kept in refrigeration to guarantee the same condition for all cycles tested in this experiment. Negative Controls with no DNA were included in every series of amplifications. PCR products were checked to confirm successful amplification with 5 µL of amplicon run on 2% agarose gel at 80V for 30 min in 1X TBE buffer and with 1 kb DNA Ladder (Sigma-Aldrich, USA) as the molecular size marker. Gels were prestained with GelRed™ fluorescent nucleic acid dye and visualized under UV light.

Detection of *Tuber* species

Based on the conclusions obtained from PCR inhibition and cycles tests, the two methods of extraction (SL1+ and P) were used to compare their success in the detection of *Tuber* species in the six truffle soils included in this study. For this purpose PCR was performed using two different concentrations of gDNA templates for each sample. The first was 2µl of the stock DNA and the second was 5 µl of 1:10 dilution of the same stock DNA. The concentrations and quality of the DNA extracted for both methods are given in Table 3.

Table 3. The concentration and quality of the DNA extracted for SL1+ and P

Soil number	Soil Ref	Conc (ng/ µl)	260:280 nm	260:230 nm
6	SL1+	44.3	1.81	1.37
	P	3	1.89	0.6
7	SL1+	35.8	1.83	1.61
	P	1.7	3.27	0.54
8	SL1+	28.7	1.75	0.6
	P	3.1	1.98	0.72
9	SL1+	35.1	1.7	0.97
	P	1.1	8.73	0.14
10	SL1+	29.5	1.1	0.57
	P	1.3	3.73	0.11
11	SL1+	5.4	1.78	0.4
	P	2	2.31	0.33

The amplification was carried out using multiplex *Tuber* primers (*T. melanosporum*, *T.brumale* and *T.indicum* species-specific forward primers: ITSML, ITSB, and ITSCHCH in combination with the reverse primer ITS4LNG (Paolocci et al., 1999). Reactions were carried out using puReTaq Ready- To- Go PCR beads (GE Healthcare, Buckinghamshire, UK) in a final volume of 25 µL, containing BSA, Stabilizers , Reaction Buffer , 200 µM of each dNTP , 1.5 mM MgCl2, 0.2 pM/µL of each primer and 2.5 units of puReTaq DNA polymerase. PCR cycling was done using Biometra thermocycler (Goettingen, Germany). Positive controls of DNA from previously identified sporocarps (*T. melanosporum*, *T.brumale*, and *T.indicum)* and controls with no DNA were included in every series of amplifications. PCR steps were as follows: initial denaturation at 94 °C for 5 min followed by 5 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min followed by 33 cycles of 94 °C for 30 sec, 48 °C for 30 sec and 72 °C for 1min and a final extension at 72 °C for 10 min. PCR products were checked to confirm successful amplification using 10 µL of amplicon run in 2% agarose gel at 80V for 30 min in 1X TBE buffer and with 100 bp DNA Ladder (Sigma-Aldrich, USA) as the molecular size marker. Gels were prestained with GelRed™ fluorescent nucleic acid dye and visualized under UV light.

Results

Comparisons of extraction efficiency between Soil extraction kits

DNA Yield

Significant differences for DNA yield among extraction methods were found (Fig. 2). DNA yield also varied with respect to the particular soil samples used in this study but there was no clear trend between yield and the differences in pH and organic matter present in our soils. Regardless of the soil source, extraction using NSP soil kit showed higher DNA concentrations (~35.7 ng/ µL) compared to Powesoil kit (~3.4 ng/ µL), DNA concentrations were superior from tests done with NSP Soil kit using SL1+ and SL2+ to that of SL2 (~18.1 ng/ µL). For each soil, the addition of the enhancer solution SX improved the extraction efficiency when SL2 was used (Fig. 3).
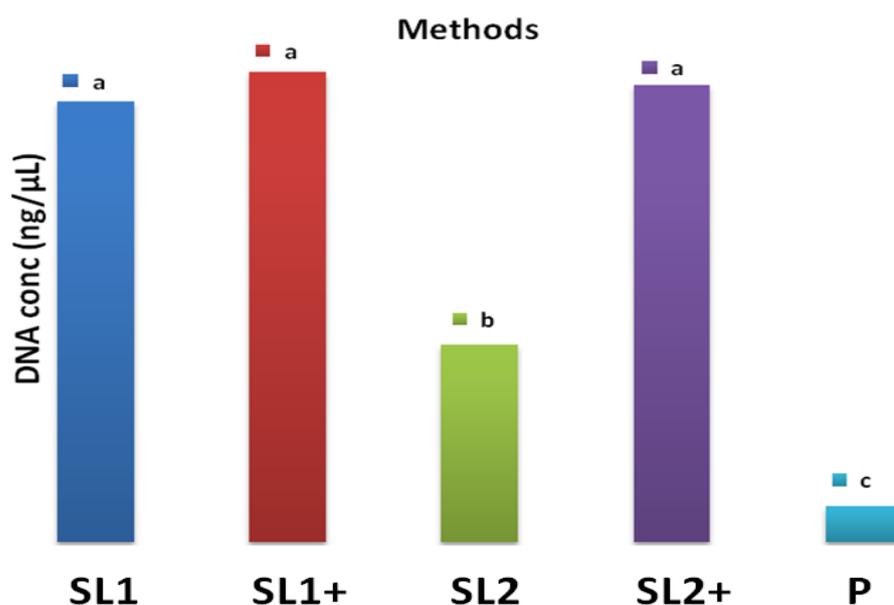
Fig. 2 Average total DNA concentrations obtained from five methods tested: SL1, SL1+, SL2, SL2+ and P. The values used were means of three replicates for each sample for each extraction method. Bars with the same letter are not significantly different at α=0.05.
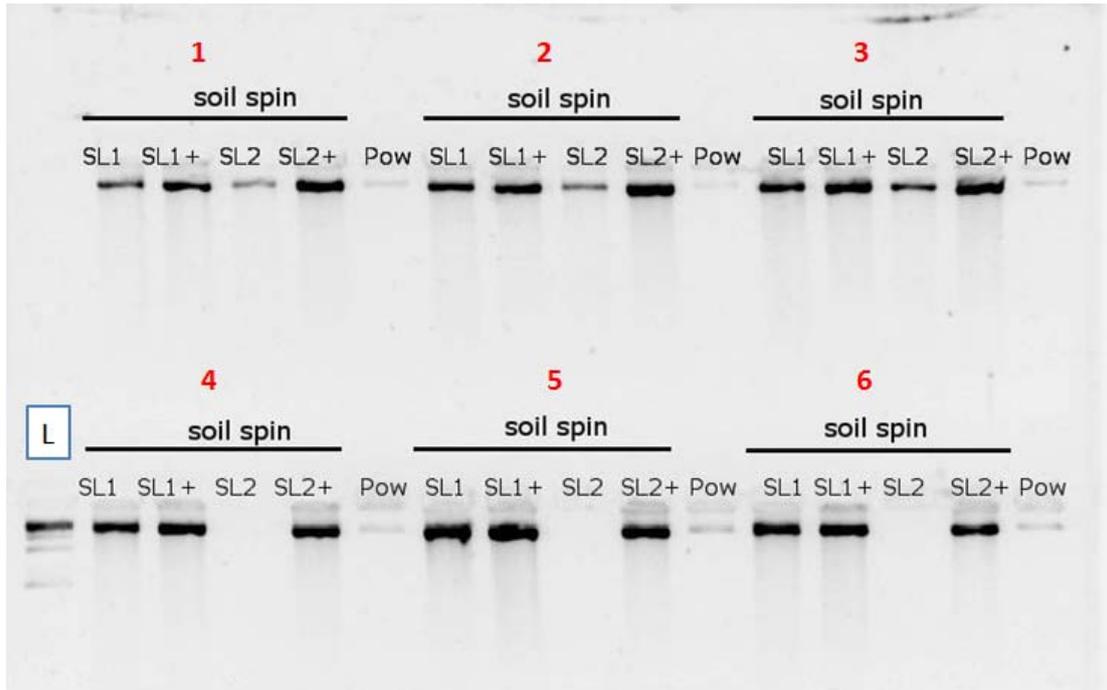
Fig. 3 Agarose 2% gel with bands corresponding to DNA extractions obtained with different extraction methods , soil spin: NSP kit (SL1, SL1+, SL2, SL2+) and Pow: powersoil, L: 1 Kb ladder. The SL1, SL1+ and SL2+ showed brighter bands compared to SL2 and P where weak bands are present. In the second row the lack of bands present for the extractions conducted with SL2 suggest DNA amounts below detection level by agarose gel electrophoresis. Red number refers to soils samples (1 to 6) used in this test.

Extraction purity by 260: 280 nm and 260:230 ratio

The purity of gDNA with respect to protein and other contaminants was estimated using the 260: 280 nm ratio. The DNA extracted using methods SL1, SL1+, SL2 and SL2+ showed higher purity (~1.9) compared to that of DNA extracted using the PoweSoil kit (~1.6). The addition of the enhancer solution SX did not improve the extraction purity regardless of the use of SL1 or SL2 (Fig. 4).
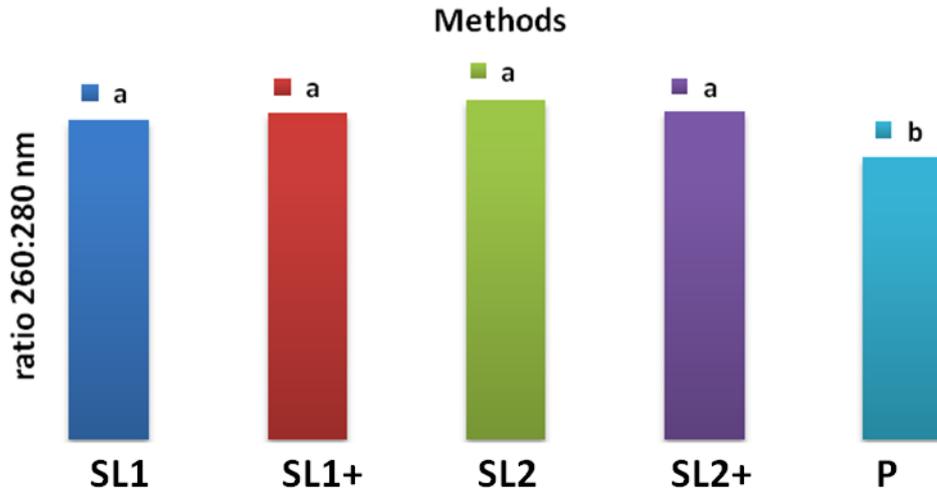
Fig. 4 Average absorbance ratio 260:280 nm of the DNA extracts calculated for five tested method: SL1, SL1+, SL2, SL2+ and P. The values used were means of three replicates for each soil sample for each extraction method. Bars with the same letter are not significantly different at α=0.05.

In contrast to the 260:280 nm ratio, the ratio of 260:230 nm was lower than the ideal range (1.7-2) with values less than 1.2 for all extraction methods regardless of the soil source. However, it was higher with SL1, SL1+ and SL2+ (~1.1) compared with SL2 and PS (~ 0.6) (Fig. 5). The absorption ratio (260:230 nm) obtained from both kits indicated co-extraction of organic molecules such as humic acids or fulvic acids.
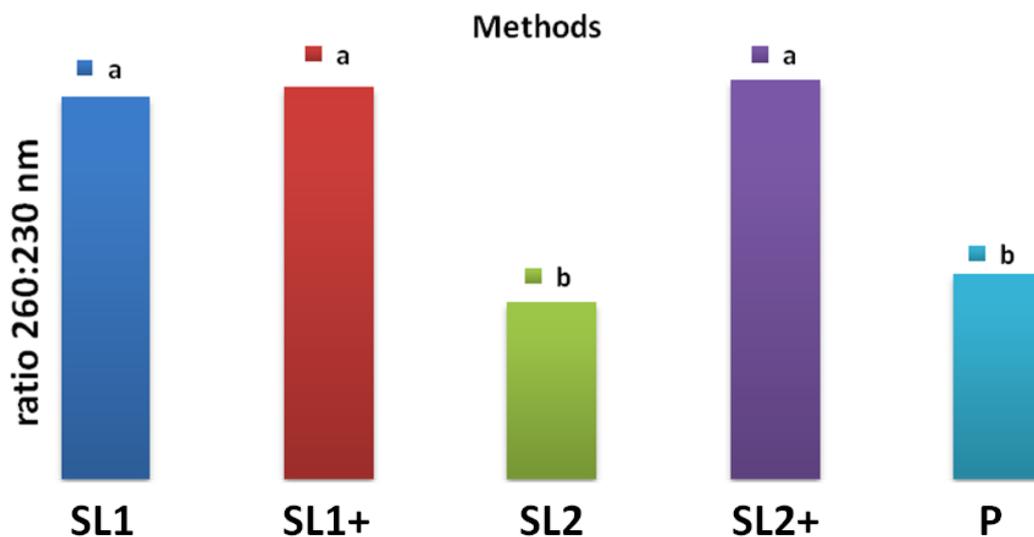

Fig. 5 Average absorbance ratio 260:230 nm of the DNA extracts calculated for five tested method: SL1, SL1+, SL2, SL2+ and P. The values used were means of three replicates for each soil sample for each extraction method. Bars with the same letter are not significantly different at α=0.05

PCR amplification using fungal primers fITS7-ITS4

Despite the low 260:230 nm ratio indicating the presence of organic molecules in all soil samples, results obtained from the amplification of the ITS2 region showed no effect of inhibition for the DNA extracted with the three methods included in the PCR tests (SL1+, SL2 and P). We obtained bands corresponding to expected base pair range below 500bp for fungi amplified in the ITS2 region of 122-245 bp (Ihramark et al. 2012) except for the SL2 where possible inhibition occurred when the gDNA template concentration of both 1 and 2 ng/ μL were tested for 25 PCR cycles (Fig. 6a and Fig. 6b).

Clear differences in band intensity with different PCR cycles and different template DNA concentrations were observed among the three tested methods. Using 22 cycles to amplify the ITS2 region failed to give successful amplification for all soils with different DNA template concentration among all extraction methods. SL1+ showed thick bands in all samples when 30 cycles were used. However, for the same extraction method, using 25 cycles showed clear band when 2 ng/ μL DNA template concentration was used and faint band with 1 ng/ μL. The SL2 method failed to give consistent amplifications for all soil samples, the amplification was successful only when the highest PCR cycle (30) was used with 1 and 2 ng/ μL DNA template concentration (Fig. 6a). Amplification using Powersoil was mostly successful with truffle soils (soil sample 6 to 11) when 30 cycles were used with both 1 and 2 ng/ μL DNA template concentration (Fig. 6b).
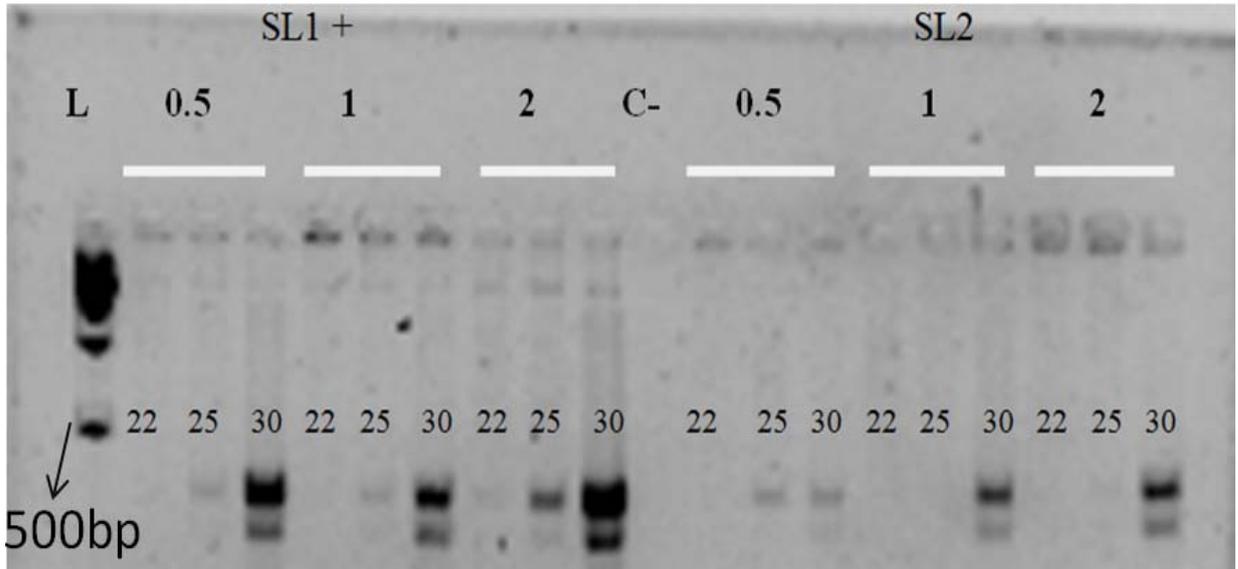
Fig. 6a: Agarose 2% gel displaying bands from PCR amplification using fITS7-ITS4 primers. L:1 KB Ladder, SL1+, SL2: extraction methods C-: Negative control. 22, 25, and 30: different cycle numbers tested. 0.5,1, 2: different DNA template concentrations (ng/ µL). This gel displays the results from our soil sample 3, which is representative of results obtained with all 11 soils.
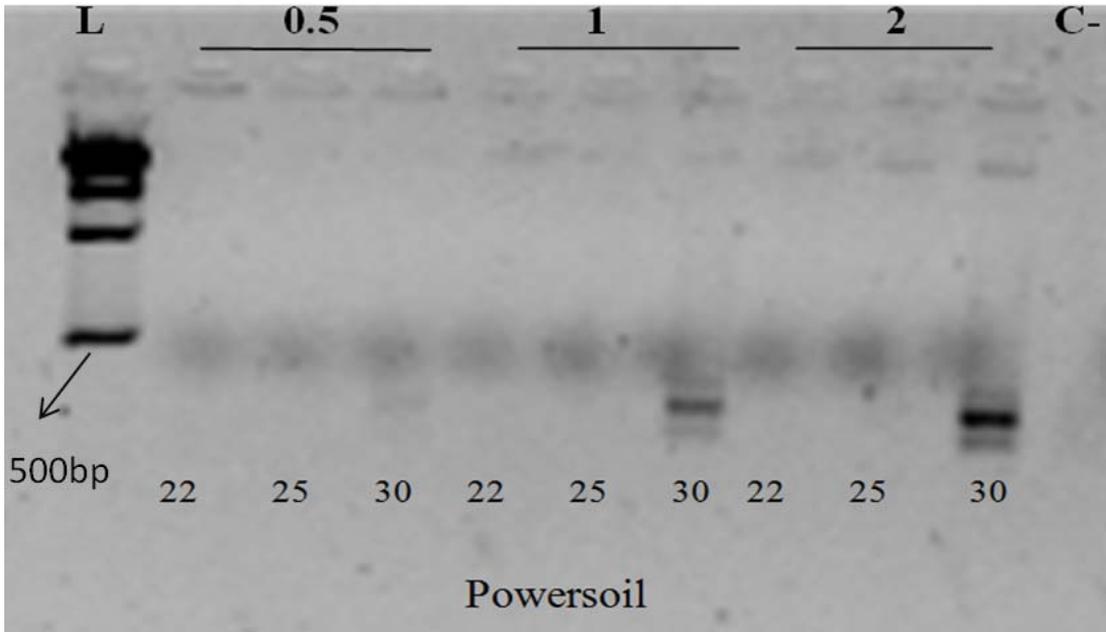

Fig. 6b: Agarose 2% gel displaying bands from PCR amplification using fITS7-ITS4 primers with DNA extracted from Powersoil e kit. L: 1KB Ladder, C -: Negative control. 22, 25, and 30: different cycle numbers tested. 0.5, 1, 2: different DNA template concentrations (ng/ µL). This gel displays the results from our soil sample 6, which is representative of results obtained with all 11 soils.

Detection of *Tuber* species

When we compared two extraction methods tested at this phase (SL1+ and P) and two concentrations of DNA (non-diluted and 1:10 dilutions) for the six soil samples from truffles sites using the ITS multiplex primers specific to *T mel, T bru* and *T ind*, we found differences between the 2 methods and between the 2 quantities of gDNA used as template. Amplicons from the PCR carried out with 2 µL of the stock DNA gave sharp and clear bands for both extraction methods. However, amplicons obtained using 5 µL of 1:10 dilution (ranging in concentration between 1.1 and 44.3ng/µl) gave faint bands for nearly all six soils. For Soil 7 no band was observed for the Powersoil method regardless of the DNA template concentration, and only a faint band was observed with SL1+ using non-diluted DNA. (Fig. 7) and (Fig. 8). Results from the multiplex PCR yielded expected bands for the three known sporocarps of *T. melanosporum* at 440 bp, *T. brumale* near 700 bp, and *T. indicum* near 140 bp. For 5 of the 6 soil samples, we observed bands corresponding to *T. melanosporum* with extractions from both methods and at both DNA template concentrations and with no detection for the presence of *T. indicum* or *T. brumale*. In the soil sample 7 we observed bands corresponding to both *T. melanosporum* and *T.brumale*. (Fig. 7). The same results for this test were obtained using a different set of *T.melanosporum* specific primers (Bonito 2009) (Data not shown).
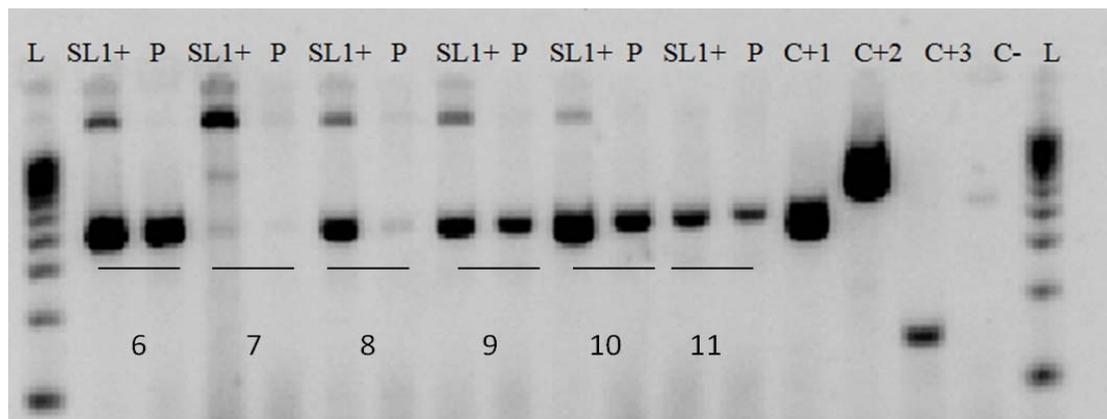

Fig. 7 Amplification with multiplex *Tuber* primers using undiluted DNA template. L: 100bp Ladder, SL1+ and P represents the extraction methods, C+1: positive control (*T.melanosporum*), C+2: positive control (*T.brumale*), C+3: positive control (*T.indicum*), C-: negative control. 6, 7, 8, 9, 10, 11 represents the soil sample according to Table 1.
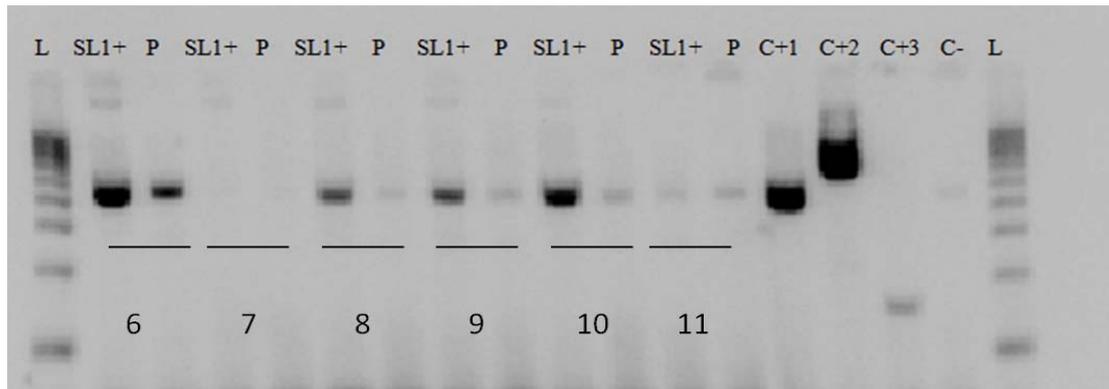
Fig. 8 Amplification with multiplex *Tuber* primers using 1:10 dilution of DNA template. L: 100bp Ladder, SL1+ and P represents the extraction methods, C+1: positive control (*T.melanosporum*), C+2: positive control (*T.brumale*), C+3: positive control (*T.indicum*), C-: negative control. 6, 7, 8, 9, 10, 11 represents the soil sample according to Table 1.

Discussion

In this study the extraction efficiency of two commercial kits, well known for their use with environmental samples, were tested on different soil samples collected from different parts of Spain. Both kits applied mechanical disruption of microbial cells with beads of different material and size as well as lysis buffers of unpublished composition. Due to the highly varying composition of different soils (organic matter, inorganic matter, humic substances, metal ions, polysaccahrids, pH), it is difficult to obtain consistent results in DNA yield and purity with one lysis buffer applicable for all sample types. For this reason the NSP kit supplies two separate buffers (SL1, SL2) and an enhancer solution (SX) to assist users in finding the ideal lysis condition for multiple soil characteristics. Higher DNA yield was obtained using the NSP soil kit compared to Powersoil kit. For the SL2 method of the NSP soil kit the yield and the purity from organic contaminants were significantly improved when the enhancer SX was added although no differences were detected when this enhancer was added to SL1 lysis buffer. This result emphasizes the distinct ability of the chemicals contained in this solution to adjust some lysis conditions prior to the mechanical homogenization. Similarly low yield extracts using this same lysis buffer (SL2) without adding enhancer was also encountered by Knauth et al (2012), where gDNA was extracted from three

field soils cultivated with wetland rice. The NSP extraction methods were successful for obtaining a ratio of 260:280 nm within the standard accepted range (1.8-2) indicating DNA extracts free of protein and other contaminants. For our soils the Powersoil method did not produce DNA within this range of purity, which may indicate an incomplete protein precipitation step. The absorption ratio of 260:230 nm was on a low range for both kits. Our results are in agreement with Engel et al (2011) who reported low 260:230 nm ratio after purifying DNA extracts collected from composite soil samples. Suz et al (2006) also reported 260:230 nm values ranging from 0.4 to 1 when DNA was extracted from nursery potting soils inoculated with truffle and yet they achieved PCR amplification despite these values. Overall, the ratios obtained with the NSP kit showed higher 260:230 nm values, indicating a higher purity of the extracted DNA compared to Powersoil with more residual organic co-extracts present. This could be caused by either humic and/or fulvic acids. Adding the enhancer to the lysis buffer SL2 significantly increased the purity of gDNA for this ratio. The positive impact of adjusting lysis conditions was also reported by He et al (2005) who showed that including prelysis washing step using 20 mmol/L of EDTA could significantly increase the gDNA quality.

In our study we were not able to predict a trend to correlate between extraction efficiency and soil properties (pH and organic matter percentage,). We obtained high yield and purity from samples ranging from low (3.31%) to high organic matter content (11.77%) and from acidic soil with PH of 5.8 to basic soil with PH > 8.2. Our results contrast with previous studies (Sheu et al. 2008; Knauth et al., 2012) who reported negative correlation between organic matter content and DNA yield.

There are several possible explanations why the extraction efficiency of NSP in general was superior to that of Powersoil. One reason could be that the composition of lysis buffers is the decisive factor for the varying efficiency between NSP and Powersoil. Another reason could be that NSP kit includes an extra step after precipitating the contaminants by placing the clear supernatant onto an inhibitor removal column before binding the DNA to the silica membrane, while in the Powersoil method the clear supernatant is not treated with a further filtering step. This extra step in the NSP method may filter-out material that remains in solution after the major precipitation step and thus remove additional fine contaminants from the

supernatant to provide more ideal conditions for DNA binding. The third possible reason is that NSP soil kit involves four washing steps to clean the DNA after binding, while Powersoil applies only one washing step before the final elution of the DNA. The number of times this wash is repeated could also affect the final quality of the DNA extracts. Antony-Babu et al (2013) also reported that adding 5 washing steps remarkably improved the purity of the final DNA extracts.

Besides the extraction efficiency, a crucial point in this study was to test the results of these extraction methods for the amplification of the ITS2 region despite the presence of organic co-extracts. And in the case of successful amplification what is the range of PCR cycles that can give us weak to medium-strong amplicons as visualized on agarose gel to ensure an accurate representation of the fungal community composition. SL1+ was the only method which succeeded in fulfilling the quantity and quality criteria of genomic DNA, and this method was sufficient for the amplification of fungal community without adding purifying reagents. Using this method with further PCR programs tested for 26-27-28 cycles and 5 and 10 ng/ µL to ensure the ideal band intensity (data not shown), helped us to specify a range of PCR cycles between 25-28 and DNA template concentration between 2-5 ng/ µL. This range is suitable to obtain an ideal band intensity to avoid further community biases with further downstream applications for our particular soils.

One of the most important ectomycorrhizal fungi currently under study is *Tuber melanosporum,* the black truffle. Due to its economic and scientific significance (Martin et al. 2010), researchers are engaged in studies to better understand the truffle life cycle, evolutionary significance and methods for cultivation. With the development of molecular techniques, researchers have been able to design *Tuber* species-specific primers to detect DNA from truffle sporocarps, ectomycorrhizae and soil mycelia. (Paolocci et al. 1999**;** Suz et al. 2006**;** Bonito. 2009). In the present study we wanted to test the results of DNA extraction methods for a simple and practical downstream application: the detection level of three *Tuber* species in the truffle soils, using species-specific primers.

Both extraction methods (SL1+ and P) successfully detected *Tuber melanosporum*, However, only SL1+ was able to detect the presence of *T.brumale* in one of the soils,

where we had received reports from the owner that fruitbodies of *T. brumale* had been collected, but previous testing had not detected its presence in the soil. It is likely that this truffle species, often considered a problematic or invasive species in black truffle plantations (Fischer et al. 2004), was present with sporadic distribution and low concentrations. However, the implications of these results provide an improved detection capability in service to truffle growers seeking information on the presence of *Tuber* mycelia in their soils. The choice of commercial kits as well as using recommended range of DNA template concentration and cycle numbers during the PCR reaction have been of great importance in this study. The results clearly demonstrate that the commercial DNA extraction kit (NSP) can be used on a wide variety of soils because it provided gDNA consistently amplifiable using eukaryotic primers. In conclusion, the results of this work lead to the development of gDNA extraction and PCR protocols optimized for soil samples with different range of chemical properties including samples collected from sites containing *Tuber* species, Such protocol will contribute to our future studies such as quantitative PCR and next generation sequencing which can be applied to understand the ecology of fungal communities.

Acknowledgments

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