Soil drying procedure affects the DNA quantification of *Lactarius vinosus* but does not change the fungal community composition

<table>
<thead>
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</table>

Abstract

Drying soil samples before DNA extraction is commonly used for specific fungal DNA quantification and metabarcoding studies, but the impact of different drying procedures on both the specific fungal DNA quantity and the fungal community composition has not been analyzed. We tested three different drying procedures (freeze-drying, oven-drying, and room temperature) on 12 different soil samples to determine (a) the soil mycelium biomass of the ectomycorrhizal species *Lactarius vinosus* using qPCR with a specifically designed TaqMan® probe and (b) the fungal community
composition and diversity using the PacBio® RS II sequencing platform. Mycelium biomass of *L. vinosus* was significantly greater in the freeze-dried soil samples than in samples dried at oven and room temperature. However, drying procedures had no effect on fungal community composition or on fungal diversity. In addition, there were no significant differences in the proportions of fungi according to their functional roles (moulds vs. mycorrhizal species) in response to drying procedures. Only six out of 1139 operational taxonomic units (OTUs) had increased their relative proportions after soil drying at room temperature, with five of these OTUs classified as mould or yeast species. However, the magnitude of these changes was small, with an overall increase in relative abundance of these OTUs of approximately 2%. These results suggest that DNA degradation may occur especially after drying soil samples at room temperature, but affecting equally nearly all fungi and therefore causing no significant differences in diversity and community composition. Despite the minimal effects caused by the drying procedures at the fungal community composition, freeze-drying resulted in higher concentrations of *L. vinosus* DNA and prevented potential colonization from opportunistic species.

**Keywords**

Drying treatment  
Ectomycorrhizal biomass  
Fungal community  
qPCR  
Metabarcoding  
*Lactarius*

**Introduction**

The use of high-throughput sequencing platforms such as 454 pyrosequencing, Illumina, PacBio, or Ion Torrent in fungal ecology has increased during the last years (Lindahl and Kuske 2013). The latest reported accuracy values of PacBio data (>99%) together with higher average read lengths (Roberts et al. 2013) make this platform a promising tool for fungal community studies. Thus, data obtained from this platform will allow undertaking deep and comprehensive ecological studies about soil-borne fungal species and communities. Real-time PCR (qPCR) is also a commonly used molecular technique, which has been employed to study the seasonal dynamics and the persistence of extraradical mycelia of the ectomycorrhizal species *Lactarius deliciosus* (Parladé et al. 2007;
Hortal et al. 2008), Boletus edulis (De la Varga et al. 2013), and Tuber melanosporum (Parladé et al. 2013). Due to increasing relevance and application of these molecular techniques, it is important to establish standardized protocols that will enable meaningful comparison of final results generated by different metabarcoding studies. Although there have been important advances in establishing a standard protocol for fungal metabarcoding studies (Lindahl et al. 2013), the impact of different commonly used procedures for drying soil samples have not been quantified and tested yet.

The extraction of DNA is one of the most studied and well-known critical steps for DNA recovery from soil (Plassard et al. 2012), despite the fact that no bias-free DNA extraction method is available (Feinstein et al. 2009). Drying soil samples before DNA extraction has important advantages as it facilitates soil homogenization (Lindahl et al. 2013), reduces colonization by opportunistic microbes, and permits equalization of soil sample size by weight by eliminating the variable water content. However, drying soil samples has some important disadvantages. Storing soil at room temperature (also referred to as “soil incubation”) can alter the DNA yield of certain species (Gryndler et al. 2013; Herdina et al. 2004). There are also evidences that soil incubation in the field may lead to saprotrrophic organisms feeding on ectomycorrhizal mycelia after mycelia death (Lindahl et al. 2010), which could distort the community composition of the soil samples. Community distortion can also occur after long exposure to airborne communities or under the presence of abundantly sporulating fungi (Adams et al. 2013a). All these problems can occur during sample preparation and, consequently, influence the fungal community structure and possibly distort the final ecological interpretation.

Presently, the most common soil drying procedures reported in the literature are (i) drying at room temperature (RT; 20–25 °C), (ii) drying in an oven (O; 40–60 °C), and (iii) freeze-drying (FD). Drying at 40–60 °C (Parladé et al. 2007) allows a rapid soil drying with negligible thermal DNA degradation (Karni et al. 2013). However, potential DNases (enzymes that break down the single- or double-stranded DNA molecule into its component nucleotides) may affect the DNA to a different degree, depending on the amount of DNA bound to humic acids and the amount of free DNA in soil (Crecchio and Stotzky 1998). Drying at room temperature (De la Varga et al. 2013; Parladé et al. 2013; Tedersoo et al. 2014) has insignificant heat impact on soil samples but requires more drying time. This increases the risk of microorganisms feeding on mycorrhizal mycelia and may facilitate DNA degradation caused by DNase activity (Crecchio and Stotzky 1998). Freeze-drying (Clemmensen et al. 2015; Sterkenburg et al. 2015) is a more expensive drying technique but, theoretically, allows for a more efficient drying, preventing the growth of opportunistic fungal species. Despite
the potential effects reported for specific fungal yields and the potential
distortion of the fungal community structure, no comparative studies of these
different drying techniques applied to the same soil samples have been reported
to date.

This work evaluates the effect of three different soil drying procedures described
above (FD; oven at 60 °C, O; room temperature at 20–25 °C, RT (Freeze-
drying, FD; oven at 60 °C, O; room temperature at 20–25 °C, RT) on the
recovery of DNA from the ectomycorrhizal species *Lactarius vinosus* (species-
specific analysis). In addition, we tested the differences in diversity and
community composition of the whole DNA pool (community analysis). To
assess the impact of the drying procedures on the quantity of DNA, we applied
real-time PCR with a TaqMan® probe designed to specifically amplify the ITS
ribosomal DNA (rDNA) region of *L. vinosus*, one of the most important
ectomycorrhizal species collected in this study area with regard to fruit body
yield (Bonet et al. 2012). To examine the differential impact of the drying
procedures on the fungal community, we sequenced multiplexed fungal
amplicons using the PacBio® RS II system based on the real-time (SMRT®)
technology, and analyzed the diversity indices as well as the fungal community
composition. We hypothesized that (1) DNA yield of *L. vinosus* will be lower in
samples dried at O and RT in comparison with FD samples. In addition,
expecting colonization of opportunistic fungal species in the RT samples, both
(2) fungal diversity and (3) fungal community composition are expected to be
different among drying procedures. Finally, colonization or growth of
opportunistic species can be expected, and (4) the number or the relative
proportion of opportunistic species are expected to increase, especially in the
samples dried at room temperature.

**Material and methods**

**Soil sampling**

Soil samples were taken in 12 plots (10 × 10 m) from the long-term experimental
site established in the protected forest (Paratge Natural d’Interès Nacional,
PNIN) of Poblet (Tarragona, Northeastern Spain, 41° 21’ 6.4728” lat, 1° 2’
25.7496’’ long), where fungal fruit body production has been monitored for
7 years with weekly mushroom sampling during the autumn season since 2008
(Bonet et al. 2012). Our soil samples were taken from even-aged *Pinus pinaster*
plots (approx. 50 years old) and were analyzed for organic matter (OM), pH, and
soil humidity. These parameters are shown in Table 1 with plot altitude and
corresponding *L. vinosus* fruit body yields. Organic matter content in soil
 samples was obtained with the Walkley-Black method and expressed as percentage of dry soil sample (Walkley and Black 1934). The pH was measured in a 1:2.5 H₂O solution. Previous studies of *L. viscosus* fruit body productivity in this area have shown an average yield of 35.9 kg fruit bodies ha⁻¹ (Table 1).

**Table 1**

List of sampled plots with corresponding soil organic matter, pH, altitude, humidity of soil samples, and *Lactarius viscosus* fruit body yields averaged for the period 2008–2014. Average values are shown with their standard deviation.

<table>
<thead>
<tr>
<th>Plot no.</th>
<th>Organic matter (%)</th>
<th>pH</th>
<th>Altitude (m.a.s.l.)</th>
<th>Humidity of soil samples (%)</th>
<th>Averaged <em>L. viscosus</em> mushroom yield (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>303c</td>
<td>7.54</td>
<td>6.8</td>
<td>903</td>
<td>23.60</td>
<td>0.64</td>
</tr>
<tr>
<td>304c</td>
<td>7.79</td>
<td>6.9</td>
<td>879</td>
<td>24.87</td>
<td>8.52</td>
</tr>
<tr>
<td>307c</td>
<td>5.79</td>
<td>6.7</td>
<td>796</td>
<td>23.69</td>
<td>115.57</td>
</tr>
<tr>
<td>312</td>
<td>4.08</td>
<td>6.6</td>
<td>633</td>
<td>24.46</td>
<td>9.06</td>
</tr>
<tr>
<td>312c</td>
<td>2.98</td>
<td>6.5</td>
<td>633</td>
<td>22.23</td>
<td>30.77</td>
</tr>
<tr>
<td>316c</td>
<td>2.95</td>
<td>6.8</td>
<td>644</td>
<td>24.08</td>
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</tr>
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<td>6.6</td>
<td>879</td>
<td>12.21</td>
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<tr>
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<td>744</td>
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<tr>
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<td>6.7</td>
<td>759</td>
<td>13.25</td>
<td>27.09</td>
</tr>
<tr>
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<td>6.8</td>
<td>759</td>
<td>8.94</td>
<td>36.53</td>
</tr>
<tr>
<td>309</td>
<td>4.49</td>
<td>7</td>
<td>852</td>
<td>11.32</td>
<td>3.39</td>
</tr>
<tr>
<td>309c</td>
<td>5.31</td>
<td>6.9</td>
<td>852</td>
<td>12.25</td>
<td>53.09</td>
</tr>
<tr>
<td></td>
<td>5.38 ± 2.24</td>
<td></td>
<td>777.15 ± 99.6</td>
<td>17.5 ± 6.71</td>
<td>35.91 ± 46.36</td>
</tr>
</tbody>
</table>

In order to obtain a representative sample from the selected plots, 8 cylinders of soil (12 cm deep and 5 cm in diameter) were collected randomly in each of the 12 plots. Soil samples from each plot were then homogenized and pooled in situ to obtain a composite sample. We discarded the litter but included humus and mineral soil, obtaining approximately 500 g composite soil sample from each plot. Soil samples were sieved (3 mm mesh) and stored a maximum of 24 h at 4 °C. Individual soil samples were homogenized, separated in four different subsamples, and kept at −20 °C until use. Three of the four subsamples were subjected separately to each of the three drying procedures, and the fourth
subsample was used to calculate the soil moisture, which was determined by weighting the soil before and after 48 h at 105 °C.

Drying procedures

Subsamples selected for drying procedures were subjected to (a) drying at room temperature (RT procedure, 20–25 °C), (b) drying in the oven (O procedure, 60 °C), or (c) freeze-drying (FD procedure during 48 h). Subsamples dried at room temperature were placed on a table within an isolated room, free from evident fungal contamination during 5 days. Subsamples dried in the oven were subjected to 60 °C during 3 h. Freeze-dried subsamples were dried during 48 h to ensure complete water removal. Once dried to constant weight, subsamples were homogenized using mortar and pestle, resulting in a very fine powder. Each homogenized subsample from each of the three drying procedures was used for DNA extraction.

DNA extraction

Total DNA was extracted from an aliquot (500 mg) of each soil subsample using the NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer’s protocol, with one modification: lysis buffer (SL1) was increased up to 900 μl. Total DNA extracts were quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Design and validation of *L. vinosus* primers and TaqMan® probe

We designed species-specific primers and a TaqMan® probe to amplify an 88-bp fragment of *L. vinosus* DNA using the Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA): LVIN-F 5’(TCGACGAGACAACGTGGG)3’, LVIN-R 5’(GGTAGTCTCAACCGATTTGAG)3’ and the TaqMan® probe LVIN-TQ 5’(6FAM-TCCCTTCTCGGAACACACTCAAC-MGB)3’. The probe was designed based on the variability of the rDNA internal transcribed spacer ITS1 region detected in the alignments among different *L. vinosus* sequences. A search for highly similar sequences (MEGABLAST) was performed in the GenBank database to test the specificity of the designed oligonucleotides. Probe specificity was validated using template DNA obtained from eight fruit bodies of *L. vinosus* collected in the study area and DNA obtained from fruit bodies of different related fungal species: *L. deliciosus*, *Lactarius deliciosus*, *Lactarius sanguifluus*, *Lactarius volemus*, *Lactarius sphagnetti*, *Lactarius bertollini*, *Lactarius chrysoceus*, *Russula aeruginea*, *Russula drimeia*, *Russula aurea*, and *Russula delica*. The DNA obtained from fruit bodies to test the specificity of
probe and primers was extracted from 20 mg of fruit body using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the protocol established by the manufacturer.

Quantitative assays

Standard curves ($R^2 = 0.998$, efficiency = 99.7 %) for mycelium quantification were constructed according the procedure described by Parladé et al. (2007) and Hortal et al. (2008). In short, a mixture of soil and mycelium was prepared using 0.480 g of a soil obtained nearby the sampled forest plots mixed with 0.020 g of fungal tissue obtained from the inner part of a dried *L. vinosus* fruit body. Soil used in these mixtures was previously checked for the absence of *L. vinosus* by real-time PCR using the probe and primers described in the previous section. Total DNA was purified from soil with the added *L. vinosus* using NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany), as previously specified. Serial tenfold dilutions from the purified DNA were prepared to obtain standards of 4, 0.4, 0.04, 0.004, and 0.0004 mg *L. vinosus* g soil$^{-1}$.

Real-time PCR reactions were prepared using 2× Premix Ex Taq™ (Probe qPCR) (Takara Bio Inc. Otsu, Shiga, Japan) according to the manufacturer’s instructions, with 5 µl of DNA template, 800 nM of each oligo, 200 nM of TaqMan probe, and 0.8 µl ROX and HPLC water to adjust a final reaction volume of 20 µl. Real-time cycling conditions for the StepOnePlus instrument (Applied Biosystems) were 30 s at 95 °C, followed by 40 cycles at 95 °C for 5 s and 34 s at 60 °C. Three replicates from each sample and the standards were included in the analysis, as well as a negative control with water instead of template. Data processing and fungal quantification were performed as described by Parladé et al. (2007).

PacBio RS II sequencing

Each subsample was subjected to PCR amplification using 25 ng of genomic DNA and gITS7 and ITS4 primers (Ihrmark et al. 2012) to amplify the ITS2 region. A unique tag composed of eight bases was added to both primers. The number of necessary PCR cycles was optimized until a visible band was obtained. PCR amplifications of samples and both negative controls from DNA extraction and PCR were conducted in a 2720 Thermal Cycler (Life Technologies) in solutions of 50 µl. Final concentrations in the PCR reaction mixtures were 25 ng template, 200 µM of each nucleotide, 2.75 mM MgCl$_2$, primers at 200 nM, and 0.025 U µl$^{-1}$ polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA) in 1× buffer PCR. PCR cycling conditions were as follows: 5 min at 95 °C, followed by 24–30 cycles of 30 s at 95 °C, 30 s at 56 °C, 30 s at 72 °C, and a final extension step at 72 °C for 7 min before storage.
at 4 °C. Each sample was amplified by triplicate, purified using AMPure kit (Beckman Coulter Inc. Brea, CA, USA), and quantified using Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Equal amounts of DNA from each sample were pooled before sequencing. The final equimolar mix was purified using EZNA Cycle Pure kit (Omega Bio-Tek). Quality control of purified amplicons was carried out using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA) and a 7500 DNA chip. The samples were included with another set of samples to use with PacBio RS II system based on the real-time (SMRT®) technology. The molecular data are archived at the Sequence Read Archive under accession number PRJNA309233 (www.ncbi.nlm.nih.gov/sra).

Quality control and bioinformatic analysis

Quality control, filtering, and clustering was assessed with the SCATA pipeline (scata.mykopat.slu.se). Sequences were filtered removing data with an average quality score <20 and/or with a minimum sequence length of 100 bp, using the amplicon quality option. Sequences were also screened both for primers (using 0.9 as a minimum proportional primer match for both primers) and sample tags. We used “usearch” as a search engine, with an established minimum match length of 85 %. Homopolymers were collapsed to 3 bp before cluster analysis. Pairwise alignments were conducted using a mismatch penalty assigned at 1, gap open penalty of 0, and a gap extension penalty with value 1. Sequences were clustered in operational taxonomic units (OTUs) with single linkage clustering, using 1.5 % as a threshold distance with the closest neighbor.

Taxonomic and functional identification

We assigned a putative taxonomy to the 559 most abundant OTUs, which represented around 95 % of the global number of reads. We selected the most abundant sequence from each OTU for taxonomical identification, using the option BLASTn massblaster in PlutoF from the UNITE (Abarenkov et al. 2010) and INSD database. Sequences from UNITE database were preferentially selected due to their accurate selection and revision (Köljalg et al. 2005). When there was no consistency, we classified the OTU as unknown until the level in which we observed taxonomical consistency. In the case where two or more species showed the same or similar homology at 1.5 % level, we included all the species names as potentially representing the OTU. We validated the taxonomical classification by constructing phylogenetic trees using the neighbor joining clustering method including reference sequences from the UNITE and INSD databases. Functional roles of the species were assigned as follows: (a) ectomycorrhizal or ericoid fungi, (b) moulds, (c) yeast, (d) saprotrophs or litter-decay fungi, (e) pathogens, (f) moss-associated fungi, (g) root-associated fungi, and (h) unknown function, based on the UNITE database (Abarenkov et al.)
2010) and from DEEMY (www.deemy.de) or other published literature. OTUs classified as mould species belong mainly to the genera *Penicillium* sp., *Talaromyces* sp., *Trichoderma* sp., *Mortierella* sp., *Umbelopsis* sp., and *Mucor* sp. OTUs classified as “Not considered” were those with very low representation in the database (Table 4) or fungal species not relevant for this study such as animal pathogens.

**Statistics**

All statistical analyses were carried out using the R software environment (v3.0.2 version; R Core Team 2015), using the vegan package (Oksanen et al. 2015) for Hill’s numbers and multivariate analysis, and the indicspecies package (De Caceres and Legendre 2009) for species indicator analysis.

The drying procedure effects on DNA yield of *L. vinosus* obtained by qPCR were tested using linear mixed-effects (LME) models. Here, samples nested within plots were included as random factors to deal with the spatial structure of the data. The same test was carried out using the 260/230 DNA quality ratios in order to study whether these values affected the qPCR data from specific plots or drying procedures.

Hill’s series of diversity indices were used to compare differences in diversity values between drying procedures (Hill 1973). Hill’s series of diversity (Hill 1973) consists of three numbers: N0 is the richness (number of OTUs per sample); N1 is the antilogarithm of Shannon’s diversity index; and N2 is the inverse Simpson diversity. We did not rarefy the community due to the potential loss of information or incorrect interpretation of results (McMurdie and Holmes 2014). However, an uneven read distribution across samples is very common due to the sequencing bias, which may affect these indices. Thus, as an alternative, we used the square root transformation of read counts as an explaining variable when testing for the Hill’s numbers (Bálint et al. 2015). LME models were used to test the effect of the drying procedures on Hill’s numbers, with the samples nested within plots treated as random variables.

A general fungal community analysis was carried out using permutational multivariate analysis of variance based on distance matrices (using the function “adonis” and Bray-Curtis distances). Here, plots and drying procedures were included as factors, and count data were Hellinger transformed. Subsequently, nonmetric multidimensional scaling (NMDS) was used to visualize community variation, and the effects of the drying treatments on the fungal community composition. Similarly, the effects of the drying procedures on the low abundant
taxa were tested using adonis on fourth-root transformed data.

Changes in the relative abundance of functional groups were tested using ANOVAs. Also, we checked the possibility for a certain OTU to be identified as an indicator species for a certain type of drying treatment using Species Indicator Index (De Caceres and Legendre 2009). Here, we also considered the relative abundance of each OTU as an approach to take into account the bias caused by the different sequencing depths. We used the function multipatt together with the parameter IndVal.g to manage the unequal group size. In both analyses, relative abundances were arcsine transformed.

Results

Impact of drying procedures on the DNA yield of *L. vinosus* (hypothesis 1)

Specific quantification of *L. vinosus* obtained by qPCR showed a wide range of values among soil samples, which ranged from 0.51 to 0.002 mg of *L. vinosus*·g$^{-1}$ soil. In addition, from one plot, we did not detect DNA of *L. vinosus* and the data from this plot were discarded from further analysis. Mean values obtained for DNA yield were 0.15 ± 0.03, 0.11 ± 0.02, and 0.09 ± 0.02 mg of *L. vinosus*·g$^{-1}$ soil for the FD, O, and RT procedures, respectively, with significant differences according to the drying treatment (df = 10, F value = 3.65, $p < 0.05$). *L. vinosus* detection was greater from soils dried using the FD procedure in comparison with O and RT, with values 26 and 42 % higher in FD. DNA quality ratios 260/230 of soil samples showed no significant differences among the plots ($F$ value = 0.763, $p > 0.05$) nor according to drying procedures ($F$ value = 1.15, $p > 0.05$) or their interaction ($F$ value = 2.14, $p > 0.05$). DNA quality ratios 260/280 ranged from 1.8 to 2.

Impact of the drying procedure on diversity (hypothesis 2)

After quality control and discarding singletons and sequences having two different tags (sequences with tag jumps), we obtained 30,588 reads. None of the tags from the negative controls were detected in the database, but one sample was discarded due to low sequence output. We recovered 1139 OTUs and an average of 987 reads in each sample. Analysis of Hill’s numbers showed that diversity indices were not affected by the drying procedure after accounting for the sequencing bias (df = 17, $p > 0.05$, Table 2, Fig. 1). As expected, the effect of the sequencing bias was significant for richness ($p < 0.001$, Table 2).

Table 2

Variation in Hill’s diversity numbers obtained from the linear mixed-effects (LME) models
and their significance

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>Hill’s N0</th>
<th></th>
<th>Hill’s N1</th>
<th></th>
<th>Hill’s N2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F value</td>
<td>Pr (&gt;F)</td>
<td>F value</td>
<td>Pr (&gt;F)</td>
<td>F value</td>
<td>Pr (&gt;F)</td>
</tr>
<tr>
<td>Intercept</td>
<td>1</td>
<td>621.86</td>
<td>&lt;.001</td>
<td>341.43</td>
<td>&lt;.001</td>
<td>146.79</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Sqrt (read counts)</td>
<td>1</td>
<td>63.19</td>
<td>&lt;.001</td>
<td>0.97</td>
<td>0.340</td>
<td>0.25</td>
<td>0.625</td>
</tr>
<tr>
<td>Drying procedure</td>
<td>2</td>
<td>2.59</td>
<td>0.105</td>
<td>1.69</td>
<td>0.215</td>
<td>0.37</td>
<td>0.702</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

At the LME. plot was considered a random factor and the bias caused by the different number of reads included in the model. I am not sure but, should this part, together with the line below, be moved to the upper part of the table? ...

*Hill’s N0* richness, *Hill’s N1* antilogarithm of Shannon’s diversity, *Hill’s N2* inverse of Simpson’s diversity, *Sqrt* square root

**Fig. 1**

The effect of the drying procedures represented by Hill’s numbers. *Boxplots* of Hill’s diversity numbers show diversity profile of the soil fungal communities in response to the soil drying procedures. No statistical differences in Hill’s numbers were observed between drying procedures (*p > 0.05*). *Values on the Y axis* indicate the Hill’s diversity series numbers in absolute terms. *Codes on the X axis* refer to the drying procedures (*FD* freeze-dried samples, *O* samples dried at the oven, *RT* samples dried at room temperature)

**Impact of the drying procedures on the fungal community (hypothesis 3)**
Permutational multivariate analysis of variance showed that there were no differences in the fungal community composition between the three drying procedures considered ($F = 1.34, \ p > 0.05, R^2 = 0.03$, Table 3). Here, most of the variation was observed among plots ($F = 5.78, \ p < 0.01, R^2 = 0.74$, Table 3), representing 74% of the total variation, whereas the drying procedures represented only 3%. Similarly, the drying procedures had no significant effect on the low-abundance taxa ($F = 1.20, \ p > 0.05, R^2 = 0.03$). The visual display of results is presented in the NMDS plot (Fig. 2), indicating that most of the observed variation in the fungal community was plot-related, whereas the three different drying procedures were mostly clustered together.

**Table 3**

Permutational multivariate analysis of variance test considering the plot as the main factor and the drying procedure as the second explanatory factor

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>Sums of Sq</th>
<th>Mean Sq</th>
<th>$F$ model</th>
<th>$R^2$</th>
<th>Pr ($&gt;F$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plot</td>
<td>10</td>
<td>4.70</td>
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<td>5.78</td>
<td>0.74</td>
<td>0.001</td>
</tr>
<tr>
<td>Drying procedure</td>
<td>2</td>
<td>0.22</td>
<td>0.11</td>
<td>1.34</td>
<td>0.03</td>
<td>0.060</td>
</tr>
<tr>
<td>Residuals</td>
<td>18</td>
<td>1.47</td>
<td>0.08</td>
<td></td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>6.39</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 2**

Nonmetric multidimensional scaling (NMDS) of the community data showing the effect of the drying procedures on the fungal community composition. Each dot represents a soil sample with a given soil drying treatment for a given plot.

The relative abundance of functional groups was similar among drying procedures analyzed, with no significant differences ($p > 0.05$). Ectomycorrhizal
fungi in the FD procedure represented 44.6 % of the total fungal proportions, and soil from the O and RT treatments showed similar values of 41.2 and 42.0 %, respectively \((F \text{ value} = 0.148, p > 0.05; \text{ Table 4})\). The second most abundant group was composed of OTUs that were not possible to identify or assign to a functional role, representing 25.6 % of the read counts for the FD, 26.4 % for the O, and 24.2 % for the RT procedures. Mould species were the third most abundant functional group, with percentages ranging from 10.3 % for FD samples and 13.4 % for RT samples, with no significant differences \((F \text{ value} = 0.117, p > 0.05; \text{ Table 4})\).

**Table 4**

Average relative abundances of the functional groups obtained for each of the three drying procedures

<table>
<thead>
<tr>
<th>Function</th>
<th>Freeze-dried</th>
<th>Oven</th>
<th>Room temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectomycorrhizal</td>
<td>44.6 ± 0.07</td>
<td>41.2 ± 0.06</td>
<td>42.0 ± 0.07</td>
</tr>
<tr>
<td>Unknown</td>
<td>25.6 ± 0.04</td>
<td>26.5 ± 0.05</td>
<td>24.2 ± 0.04</td>
</tr>
<tr>
<td>Moulds</td>
<td>10.3 ± 0.28</td>
<td>10.4 ± 0.25</td>
<td>13.4 ± 0.37</td>
</tr>
<tr>
<td>Saprotroph</td>
<td>3.6 ± 0.02</td>
<td>3.7 ± 0.02</td>
<td>3.7 ± 0.02</td>
</tr>
<tr>
<td>Root-associated</td>
<td>2.4 ± 0.16</td>
<td>3.1 ± 0.22</td>
<td>3.3 ± 0.29</td>
</tr>
<tr>
<td>Moss-associated</td>
<td>1.7 ± 0.16</td>
<td>1.8 ± 0.14</td>
<td>0.2 ± 0.13</td>
</tr>
<tr>
<td>Yeasts</td>
<td>5.6 ± 0.09</td>
<td>5.8 ± 0.13</td>
<td>6.6 ± 0.13</td>
</tr>
<tr>
<td>Plant pathogen</td>
<td>0.4 ± 0.21</td>
<td>0.5 ± 0.20</td>
<td>0.2 ± 0.13</td>
</tr>
<tr>
<td>Not considered</td>
<td>5.8</td>
<td>7</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Data is given in percentage of total OTUs. Ectomycorrhizal species include those classified as ectomycorrhizal and ericoid mycorrhizal. Unknown species were those that could not be assigned to a functional role. OTUs classified as “Not considered” were those with very low representation in the database or fungal species not relevant for this study.

**Effect of the drying procedures to specific fungal species (hypothesis 4)**

From the 1139 OTUs recovered, there were four and six OTUs that were significantly more frequent after the O and RT procedure, respectively \((\text{Table 5, } p < 0.05)\). The OTUs with increased frequency associated with the RT procedure, mostly belong to moulds or yeasts (Cryptococcus sp., Umbelopsis sp., Trichoderma spirale, Exophiala sp., and Mucor fragilis), opportunistic or R-strategist fungal species \((\text{Table 5})\). In the case of samples from the O procedure, two OTUs were classified as moulds (Mortierella sp., Penicillium sp.) and two
were of uncertain functional classification (unknown Capnodiales, unknown Helotiales) (Table 5). However, the magnitude of these changes was small for both drying procedures, with an overall increase of the indicator OTUs from the O samples at 0.4 %, and the increase of the indicator OTUs from the RT samples at 1.84 % (Table 5).

Table 5
Significant OTUs and their assigned identification for each of the drying procedures. To test the significance, freeze-drying procedure was defined as a control

<table>
<thead>
<tr>
<th>Method</th>
<th>Ecology</th>
<th>A</th>
<th>B</th>
<th>( P ) value</th>
<th>% total</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oven</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Unknown Capnodiales</strong></td>
<td>Unknown</td>
<td>0.58</td>
<td>0.8</td>
<td>0.03</td>
<td>0.190 (52)</td>
<td>0.099</td>
</tr>
<tr>
<td><strong>Mortierella sp.</strong></td>
<td>Moulds</td>
<td>0.752</td>
<td>0.6</td>
<td>0.02</td>
<td>0.131 (81)</td>
<td>0.106</td>
</tr>
<tr>
<td><strong>Unknown Helotiales</strong></td>
<td>Unknown</td>
<td>0.718</td>
<td>0.6</td>
<td>0.01</td>
<td>0.183 (82)</td>
<td>0.151</td>
</tr>
<tr>
<td><strong>Penicillium sp.</strong></td>
<td>Moulds</td>
<td>0.692</td>
<td>0.6</td>
<td>0.04</td>
<td>0.102 (84)</td>
<td>0.086</td>
</tr>
<tr>
<td>Room temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cryptococcus sp.</strong></td>
<td>Yeast</td>
<td>0.566</td>
<td>1</td>
<td>0.025</td>
<td>0.767 (65)</td>
<td>0.499</td>
</tr>
<tr>
<td><strong>Umbelepopsis sp.</strong></td>
<td>Moulds</td>
<td>0.921</td>
<td>0.6</td>
<td>0.005</td>
<td>0.185 (91)</td>
<td>0.169</td>
</tr>
<tr>
<td><strong>Trichoderma spirale</strong></td>
<td>Moulds</td>
<td>0.511</td>
<td>1</td>
<td>0.02</td>
<td>0.750 (39)</td>
<td>0.292</td>
</tr>
<tr>
<td><strong>Exophiala sp.</strong></td>
<td>Yeast</td>
<td>0.459</td>
<td>1</td>
<td>0.02</td>
<td>1.642 (45)</td>
<td>0.736</td>
</tr>
<tr>
<td><strong>Odontia sp.</strong></td>
<td>Unknown</td>
<td>0.892</td>
<td>0.4</td>
<td>0.045</td>
<td>0.073 (88)</td>
<td>0.064</td>
</tr>
<tr>
<td><strong>Mucor fragilis</strong></td>
<td>Moulds</td>
<td>0.831</td>
<td>0.4</td>
<td>0.02</td>
<td>0.097 (80)</td>
<td>0.077</td>
</tr>
</tbody>
</table>

Component A shows the degree of specificity or the probable predictive value, and component B shows the degree of fidelity or sensitivity, meaning the probability of finding the species in the treatment belonging to the treatment group. \( P \) value is the statistical significance of each OTU. % total represents the total percentage of each significant OTU for a given drying procedure. The increase in percentage with respect to freeze-drying procedure is shown in parenthesis. % increase represents the overall
Discussion

Impact of drying procedures on the total DNA yield and the DNA quantification of *L. vinosus* (hypothesis 1)

Our results have demonstrated that drying procedures had an effect on the DNA yield of the ectomycorrhizal fungus *L. vinosus*, with the greater yield resulting from the freeze-drying (FD) procedure. Similar to our findings, previous studies showed that drying at RT (or soil incubation) results in a decrease of specific DNA of known fungal species measured by real-time PCR such as the ectomycorrhizal *Tuber aestivum* (Gryndler et al. 2013) or the pathogenic *Gaemumannomyces graminis* (Herdina et al. 2004). Gryndler et al. (2013) suggested that DNA was degraded and only the DNA protected by humic acids was remaining after soil incubation. Similarly, Herdina et al. (2004) argued that dead mycelium was degraded after soil incubation, whereas live mycelium was much more stable. Our results, in line with the existing literature, suggest important effects of the drying procedure on the yield of specific fungal DNA, which will affect the estimated ectomycorrhizal fungal biomass. In the case of studies quantifying fungi by real-time PCR, the use of a standard based on known concentrations of genomic DNA (Filion et al. 2003), hyphal biomass (Raidl et al. 2005), or mycelia biomass (Parladé et al. 2007) allows reliable comparison between runs. In addition, the use of this standard could also allow a comparison of absolute abundances of fungal biomass between studies if the same standard and sample handling are used. Finally, the drying procedure will also influence detection of the limit. Thus, when targeting especially low-abundance fungal taxa in soils, freeze-drying would be the most appropriate drying approach.

Impact of the drying procedure on the richness and diversity indices (hypothesis 2)

One of the hypothesized effects of drying procedures at community level was the alteration of diversity indices as a consequence of the colonization of soil samples by specific fungal species. Previous works have reported that a very abundant single OTU may distort the community composition and richness (Adams et al. 2013a). This very abundant single OTU could belong to abundantly sporulating fungi (Adams et al. 2013a), or could be a fast-growing opportunistic species (Lindahl et al. 2010). However, our study did not reflect any drying procedure effect on diversity indices, which suggests that no colonization from other organisms occurred or that colonization occurred equally
to all the drying procedures. Although it is not possible to determine the exact cause, we suggest that most likely there was no colonization in any of the drying procedures or this colonization only occurred to a limited extent by a few fungal species.

Impact of the drying procedure on the fungal community (hypothesis 3)

There were no appreciable effects of drying procedures on the fungal community composition, where the plot factor was the main source of variation (74 %). This variation among plots is probably produced by niche effects, which has been commonly reported for soil fungal communities (O’Hanlon and Harrington 2012). This variability was also observed for fruit body production in the studied plots since 2008 (Table 1), but also at a regional level (De Miguel et al. 2014). In any case, differences in fungal mycelia observed between plots (small geographical scales) can be attributed to biotic and abiotic factors (i.e., soil nutrients, aboveground vegetation) or stochastic processes such as dispersal limitation (Bahram et al. 2014). Also, discrete patches of organic matter or mineral nutrients in soil microcosms can also influence mycelia growth (Cairney 2005).

Certain structures from cell walls such as melanin can make the DNA much more resistant to degradation, especially in the short drying period used in this study. Fernandez et al. (2013) reported high stability and low degradation of *Cenococcum geophilum* dead mycelia, most likely due to the presence of melanin, which prevents degradation of the cell walls. Similarly, Clemmensen et al. (2015) report impaired fungal degradation across fungal groups probably due to the presence of the same cell wall component. Although it seems that impaired degradation of fungal DNA may occur, we suggest that the drying procedures applied in this study may not be enough to induce any impaired fungal degradation with time. For instance, Bååth et al. (2004) observed decay in fungal biomarkers after incubation at 25 °C, but the effects after 1 month were very limited. Furthermore, the authors used the 18:2ω6.9 marker, which most likely is more sensitive to degradation than DNA (Wallander et al. 2013). Disconnection from host roots may cause the death of ectomycorrhizal mycelia (Bååth et al. 2004; Wallander et al. 2001), which may become available for other saprotrophs (Lindahl et al. 2010). However, according to our results, no important colonization at expenses of the dead mycelia occurred in the RT samples, most likely due to a fast decrease in moisture content during the drying treatments.
Effect of the drying procedures on specific fungal species (hypothesis 4)

Finally, we observed four and six OTUs increasing in O and RT procedures, respectively. These increases were related to slight increases in read count proportions occurring for specific OTUs, mainly classified as moulds or yeasts and considered to be opportunistic fungal species. Thus, five out of the six OTUs that increased in RT samples were classified as moulds or yeasts, including M. fragilis or T. spirale, and genera represented by Cryptococcus sp., Umbelopsis sp., and Exophiala sp. However, the magnitude of the increase in their frequency was small for both drying procedures, with an overall increase in relative abundance of 0.4 and 1.8 % for O and RT drying procedures, respectively. This small increase in their relative proportions suggests that the drying procedures had a limited impact on these taxa. Some of these species belong to the order Mucorales (i.e., Umbelopsis sp. M. fragilis), which has also been observed to increase in relative abundance after root severing in boreal ecosystems (Lindahl et al. 2010). Mould species, together with yeasts, contribute to increase the turnover of dead mycelium and other forest residues (Clemmensen et al. 2015) and might be considered fungal opportunists. However, these species are also an important component of the airborne fungal community (Adams et al. 2013b) and could alter the samples dried at room temperature.

Conclusions

This study highlights the importance of choosing the appropriate drying method depending on the aims of the study. For real-time PCR studies, we suggest using the same drying technique for all the samples but also for the standard preparation to avoid DNA degradation. When aiming for the detection of low abundant species, we suggest freeze-drying soil samples before DNA extraction. For fungal community analysis, and contrary to our initial hypothesis, we would not expect a significant increase in relative abundance of opportunistic fungal species at the expenses of the ectomycorrhizal mycelia if the drying conditions are optimal, i.e., under low air moisture and temperatures around 20–25 °C. Since we have observed that sample preparation has an effect on the DNA yield of specific fungal species, an open question is whether DNA from dried samples is stable over time. Thus, new studies should address the effect of the long-term storage of soil samples both at the individual species and at the community level.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interests.

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