SHORT TERM SCIENTIFIC MISSION
STSM-SCIENTIFIC REPORT

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STSM TOPIC: Bioinformatic tools to study soil fungal communities

REPORT SUMMARY

1. Purpose of the STSM

Introduction

The importance of studying the fungal communities

Soils are essential components in terrestrial ecosystems that support living forms. Microbial communities are the main actors living in soils and responsible for the nutrient acquisition of plants. However, the complexity of these communities has made very difficult to understand them and how they influence and interact with the other organisms and ecosystem processes. Only recently, we realised the high complexity and diversity of the fungal communities (Buée et al., 2009). However, since the apparition of the next-generation sequencing (Margulies et al., 2005), we are now able to understand better these communities and start drawing the first ecological interpretations of belowground fungal communities. Just to cite some examples, In boreal ecosystems, Clemmensen et al., (2013) found that these communities, especially the ericoid fungal communities, have the ability to accumulate carbon belowground. At global scale, Tedersoo et al., (2014) revealed insights about the biogeography of fungal communities and started drawing the first ecological drivers that explain fungal community assemblage.

Despite the fact that new molecular technologies have allowed the apparition of several studies concerning fungal communities, we still have many questions to be answered. One of these would be, for instance, how resistant and resilient are these communities in stressed ecosystems, such as Mediterranean forests, with continuous drought periods as a recurrent disturbance. Apart from the ecological applications, we can also use the molecular tools to predict the potential use of ecosystem services such as mushroom productivity. Thus, understanding the relationship between aboveground and belowground communities may help

us in predicting mushroom productivity but also may provide insights about optimum management, since it has been observed that forest management can positively increase mushroom production (De Miguel et al. 2014).

Study design and objectives

During 2009, 28 plots (10x10 m.) were experimentally established to analyse the effect of different environmental parameters on sporocarp production (Bonet et al., 2012). Different thinning intensities were applied with the objective to study the effect of the forest management at the fungal communities. The results showed the importance of climatic parameters but also an effect of the thinning to the sporocarp production. With these results, we had the great opportunity to work on another level; The microbial level. For this, 491 samples were taken during 14 months on all the plots. The **specific objectives** of this study were:

i) To understand the relationship of the above and belowground communities and study the belowground communities as a potential predictors of sporocarp production at plot level,

ii) To understand how forest management affect diversity and richness of fungal communities,

iii) To understand how the environmental parameters assemble fungal communities.

These objectives should provide the basis to accomplish our **general objectives** for the next 3 years:

1) To develop tools to understand, identify and maintain fungal diversity,

2) To develop management models to optimize their production in terms of mushroom productivity but also carbon sequestration.

3) To identify those critical factors that may cause major impacts and shifts on their structure.

Mission and work plan at the Host University

We planned to prepare the extracted DNA for high-throughput sequencing, using PacBio RS II platform. Thus, we planned to extract the DNA from the samples, measure their DNA concentration, amplify using general fungal primers the ITS2 region, obtain an ultrapure sample using magnetic beads, measure the DNA concentration of the amplicons, prepare the equimolar sample and make a quality control. Then, Raw data of PacBio RS II platform was planned to be filtered and clustered to be able to assign the most important OTU (Operational

Taxonomic Unit) to species or genus level. In addition, we planned to extract the total biomass using the ergosterol biomarker. All of these activities were planned to be carried out at the Department of Forest Mycology and Plant Pathology from the Swedish University of Agricultural Sciences (Uppsala):

- Prepare the extracted samples, from the amplicon production to the equimolar mixture of all the extracted samples, in order to sequence the samples using PacBio RS II platform.
- Analyse using bioinformatic tools the raw data, from the data filtering to the OTU assignation.
- Extract the ergosterol from the soil samples, to obtain the total fungal biomass of the samples.
- Make the ecological interpretation of the data obtained after the bioinformatic analysis and discuss the implications of the results to increase the value from the non wood forest products, specially the mushrooms.

2. Description of the work carried out during the STSM

DNA preparation and sequencing

The 491 soil samples from *Pinus pinaster* forests were extracted with NucleoSpin Soil (Macherey-Nagel) and 0.5 g. of freeze-dried soil. We first optimized the DNA extraction method by testing DNA yield and quality using different lysis reagents (Fig. 1).

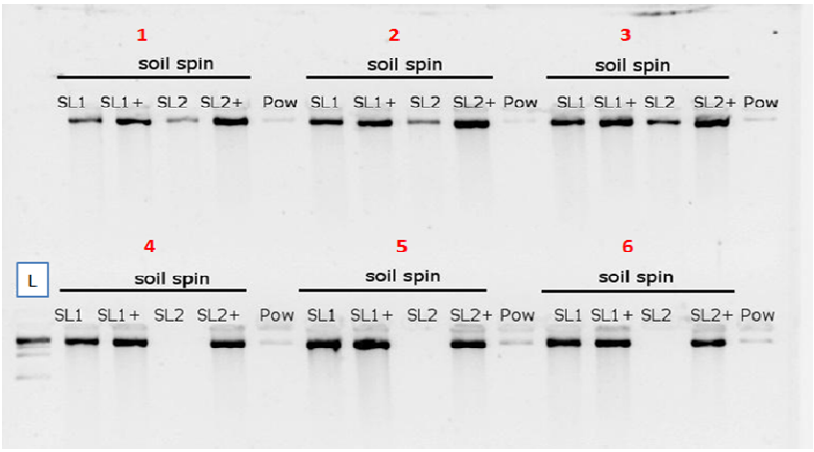


Fig. 1: Agarose gel of genomic DNA to study to determine the best Lysis combination to get the highest yield. Lysis combination are SL1 (Lysis SL1 without enhancer), SL1+ (SL1 with enhancer), SL2 (SL2 without enhancer), SL2+ (SL2 with enhancer), Pow (A different extraction kit).

DNA extracts were quantified using Nanodrop and checked for quality using 280/260 and 280/230 ratios (Figure 2). Negative controls were included every 24 samples. All samples

were diluted using double-distilled water. We calculated the approximated amount of DNA needed to successfully establish a PCR but finally all samples were first diluted 1:10 due to the low variability in total genomic DNA observed in the samples.

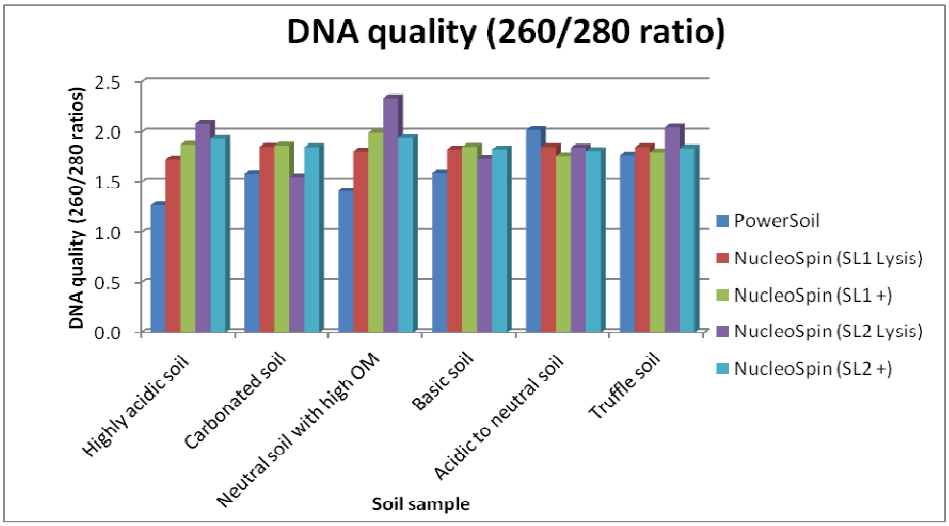


Figure 2: DNA quality ratios 260/280. Despite the small variations in DNA concentrations, the quality ratios 260/280 were good for all the samples and Lysis combinations, specially using Nucleo-Spin Soil kit.

DNA extracts were subjected to PCR-amplification of ITS2 markers using the two primer combinations gITS7 and ITS4A, which allow the amplification of the ITS2 region (Ihrmark et al. 2012). Both primers were degenerated, which made necessary a PCR cycle optimization in order to avoid potential biases in the amplification step. Thus, a first round of PCR test was made in order to estimate the most suitable number of PCR cycles and DNA concentration in order to obtain a medium-weak band intensity (Fig. 3). The number of cycles and DNA concentration was assessed by taking 8 µL. of PCR reaction when the extension temperature was reached (72ºC) in each of the PCR cycles.

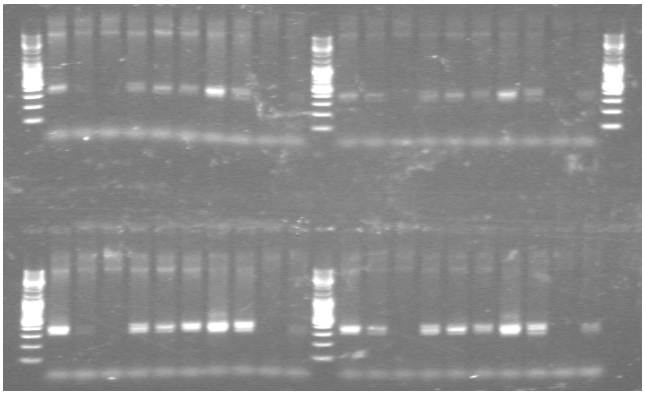


Figure 3: PCR optimization test using 21 cycles (Above) and 24 cycles (below). Results indicated the different amplification potential of the samples and thus every sample was studied for PCR cycle optimization. This test was carried out using 21, 24, 27 and 30 cycles and DNA concentrations ranging from 0.5 ng/uL, 1.0 ng/uL and 10 ng/uL.

After determination of the most appropriate PCR cycle and DNA concentrations, final amplifications were carried out with tagger primers. Both primers were tagged with eight bases, which allow the future identification of all the samples and allows the detection of the sequences having switched Tags. If a PCR reaction was not successful, cycles were adjusted until a medium-weak band was obtained (Fig. 4).

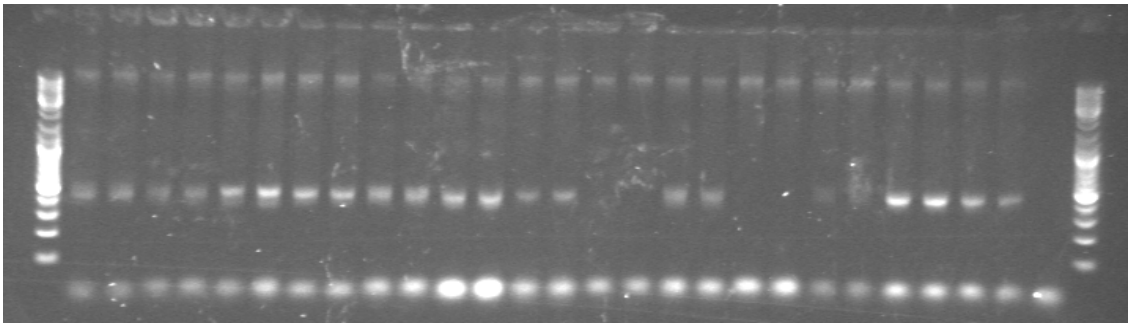


Figure 4: Example of weak-medium bands suitable for sequencing. In this case most of the samples were considered suitable for sequencing

PCR conditions were as follow for all the samples;

gITS7-ITS4A mix	Stock, µM	50 µl reaction
		x1 reaction
ddH_2O		30.75
Buffer	x10	5
dNTPs	2000	5
$MgCl_2$	25000	1.5
DreamTaq polymerase	5u/µl	0.25
Primers		5
SUM		47.5
Template DNA		2.5
Reac vol.		50

Table 1: PCR mixture for 1 reaction considering 50 uL. of total PCR mixture. ddH2O: Double distilled water. Buffer: Green Taq Buffer. dNTPs: Mixture of nucleotide triphosphates containing deoxyribose. MgCl2: Magnesium chloride. DreamTaq Polymerase: Taq Polymerase, Primers: gITS7 and ITS4A.

The PCR cycle conditions using gITS7-ITS4 primers were as follows:

95°C 5 min
95°C 30 sec
56°C 30 sec } 18-30 cycles
72°C 30 sec
72°C 7 min

Figure 5: PCR conditions for all the samples, with PCR cycles ranging from 18 to 30 cycles. Annealing time was reduced to 30 sec since ITS2 region is expected to have between 150 and 600 bp.

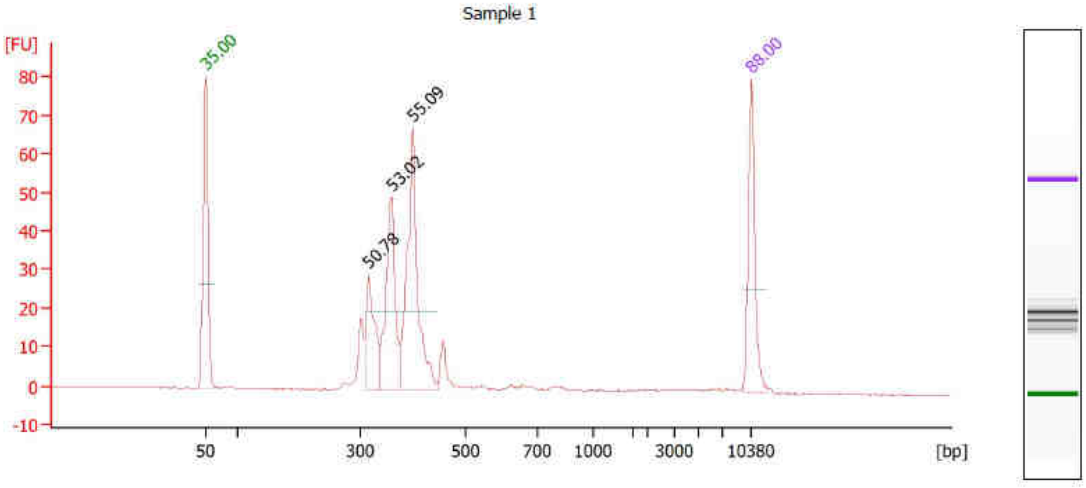
Each sample was amplified by duplicate and purified with AMPure kit (Beckman Coulter Inc., Brea,CA, USA). Concentration of the purified PCR products was calculated with Qubit fluorometer (Life Technologies, Carlsbad,CA, USA). In order to determine the total DNA concentration, we applied the next formula:

Concentration of the sample= QF value * (200/x),

Where: QF Value is the value given by Qubit fluorometer,

X is the number of microliters of sample that we added to the assay tube.

From all the samples, we obtained 5 different libraries for sequencing (representing 491 soil samples in total). All of the samples in each of the libraries were pooled in equal amounts of DNA, obtaining a total DNA ranging from 2.5 and 3 micrograms in total, measured with Qubit. All of these 5 libraries were purified again using E.Z.N.A. Cycle Pure Kit (Omega Biotek) and eluted in 100 µL. Finally, the 5 libraries were subjected for quality control using BioAnalyzer (Fig. 5), which gives an estimation of the length size and DNA concentration of the final pools. All 5 libraries were delivered for DNA sequencing to PacBio RS II, using 20 SMRT cells, with an expected data output of 500.000 sequences in total.



Overall Results for sample 1 : Sample 1

Number of peaks found: 3

Peak table for sample 1 : Sample 1

Peak	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
1	50	8.30	251.5	Lower Marker
2	315	3.08	14.8	
3	359	5.32	22.4	
4	400	7.66	29.1	
5	10,380	4.20	0.6	Upper Marker

Figure 6: Final concentration and fragment length distribution of the resulting sample (After equimolar mix). As deduced by length distribution, we can observe that basidiomycetes are clearly dominant at the amplicon pool.

Analysis of DNA data

Sequences from all the 5 libraries were filtered and clustered using SCATA pipeline developed at the Swedish University of Agricultural Sciences (SLU-Uppsala), (scata.mykopat.slu.se). All the sequences with quality score less than 20 were removed from the data. Parameters for low single quality bases were left at minimum and sequences were discarded only when they were below 2. We used the "Amplicon quality" option for sequence clustering, this method was proved to successfully recover more good sequences that all the other available methods (Data not shown). Quality filtering also considered the identification of primers and Tags. For identification of primers, we choose the threshold 0.9, which means that it accepts 10% of sequence variation within the primer sequence. Also, sequences with less than 100 bp were discarded. Pairwise alignments were scored using a mismatch penalty of 1, gap open penalty of 0, and gap extension penalty of 1, clustering distance was considered 0.015 since the pipeline is based on single-linkage clustering. Homopolymers were collapsed to 3 bp before clustering. Finally, all the sequences with switched tags were manually discarded and only the sequences with the same tag both at the gITS7 and ITS4A primers were included in the analysis. After quality control and filtering, we obtained i) The cluster list and, ii) The Tag list with the read counts of each OTU (See example in Figure 7).

Tag	scata2671_0	scata2671_3	scata2671_5	scata2671_9	scata2671_2	scata2671_1	scata2671_13	scata2671_10	scata2671_8
test 4 library 1 ITS4a_tag_1_gITS7_tag_1	2	0	18	29	7	19	22	0	0
test 4 library 1 ITS4a_tag_10_gITS7_tag_10	13	32	29	52	1	0	10	28	17
test 4 library 1 ITS4a_tag_100_gITS7_tag_100	3	0	0	19	4	23	10	0	0
test 4 library 1 ITS4a_tag_101_gITS7_tag_101	38	0	10	3	107	14	6	0	3
test 4 library 1 ITS4a_tag_104_gITS7_tag_104	23	0	29	71	13	0	6	3	0
test 4 library 1 ITS4a_tag_105_gITS7_tag_105	15	0	17	1	25	0	11	0	3
test 4 library 1 ITS4a_tag_106_gITS7_tag_106	25	61	21	20	1	0	3	40	7
test 4 library 1 ITS4a_tag_107_gITS7_tag_107	11	8	2	0	2	0	12	1	5
test 4 library 1 ITS4a_tag_108_gITS7_tag_108	30	40	4	2	0	57	10	0	1
test 4 library 1 ITS4a_tag_109_gITS7_tag_109	6	1	4	3	27	44	7	0	0
test 4 library 1 ITS4a_tag_11_gITS7_tag_11	7	64	26	2	0	17	25	40	18
test 4 library 1 ITS4a_tag_112_gITS7_tag_112	19	0	7	6	0	0	4	8	5
test 4 library 1 ITS4a_tag_113_gITS7_tag_113	22	5	6	1	1	0	17	9	35
test 4 library 1 ITS4a_tag_114_gITS7_tag_114	21	18	4	2	9	0	3	0	1
test 4 library 1 ITS4a_tag_116_gITS7_tag_116	3	1	0	19	12	9	5	0	0
test 4 library 1 ITS4a_tag_117_gITS7_tag_117	0	65	17	0	0	0	1	0	4
test 4 library 1 ITS4a_tag_12_gITS7_tag_12	31	12	0	0	0	0	30	9	0
test 4 library 1 ITS4a_tag_13_gITS7_tag_13	0	14	5	0	7	13	6	0	18
test 4 library 1 ITS4a_tag_14_gITS7_tag_14	0	0	3	4	0	0	6	0	0
test 4 library 1 ITS4a_tag_15_gITS7_tag_15	17	0	2	0	0	0	12	3	0
test 4 library 1 ITS4a_tag_16_gITS7_tag_17	11	0	0	1	17	0	5	0	6
test 4 library 1 ITS4a_tag_17_gITS7_tag_17	0	0	0	0	0	0	0	0	0
test 4 library 1 ITS4a_tag_17_gITS7_tag_16	0	0	2	0	7	5	3	0	0
test 4 library 1 ITS4a_tag_18_gITS7_tag_18	2	0	5	1	0	3	16	0	0
test 4 library 1 ITS4a_tag_20_gITS7_tag_20	21	0	31	16	43	0	20	0	2
test 4 library 1 ITS4a_tag_21_gITS7_tag_21	1	12	0	0	1	2	4	8	6

Figure 7: Read counts associated to each of the Tags in Library 1. This figure only shows the first 9 most representative species in the data set.

The 2000 most frequent OTUs were identified using the workbench PlutoF from UNITE (Kõljalg et al., 2005), using the massBLASTer option (See example in Figure 9).

Query 2 of 500: scata267_3_repseq_0

Reference	Score	E-value	Prcnt	MisM
UDB019725|SH176300.07FU|Inocybe geophylla var. geophylla	545	3e-154	100.00	0000
FJ897193|SH176300.07FU|uncultured mycorrhizal fungus|Inocybe	503	2e-141	99.64	0000
JF899559|SH176304.07FU|Inocybe geophylla	501	7e-141	97.32	0003
KF297017|SH176298.07FU|uncultured fungus|Inocybe	497	9e-140	97.29	0004
UDB022395|SH176304.07FU|Inocybe pudica	496	3e-139	96.98	0003
KF617410|SH176298.07FU|uncultured fungus	494	1e-138	96.95	0005
KF296950|SH176298.07FU|uncultured fungus|Inocybe	492	4e-138	96.95	0005
JX316675||uncultured fungus|Inocybaceae 492 4e-138 96.95 0005 1 295 652 362				
JX316674||uncultured fungus 492 4e-138 96.95 0005 1 295 652 362				
KJ792561||uncultured Inocybe 490 1e-137 96.95 0004 1 295 307 18				
KF618017|SH176304.07FU|uncultured fungus	473	1e-132	95.30	0007
KF296732||uncultured fungus|Inocybe 473 1e-132 95.64 0006 1 295 570 277				
FJ196926|SH176308.07FU|uncultured Inocybe|Inocybe	460	1e-128	94.97	0007
JF908152|SH176300.07FU|Inocybe posterula|Inocybe posterula	455	5e-127	99.60	0001
EF417817||uncultured Inocybe|Inocybe 448 9e-125 94.59 0004 1 295 634 350				
HE820692|SH176305.07FU|uncultured ectomycorrhizal fungus|Inocybe	436	2e-121	93.67	0005
HE820368|SH176305.07FU|uncultured ectomycorrhizal fungus|Inocybe	436	2e-121	93.67	0005
EF619710|SH176305.07FU|uncultured Inocybe|Inocybe	436	2e-121	93.67	0005
DQ985375||uncultured Basidiomycota 422 5e-117 97.22 0002 47 295 316 67				
DQ985376|SH176300.07FU|uncultured Basidiomycota|Inocybe	398	9e-110	98.25	0001
KF041342|SH176308.07FU|uncultured Inocybe	390	2e-107	95.90	0004
KM403046||uncultured Inocybe 361 1e-98 89.60 0012 1 295 680 399				
KJ146718|SH176296.07FU|Inocybe whitei	361	1e-98	89.60	0012
KJ146717|SH176296.07FU|Inocybe whitei	361	1e-98	89.60	0012
HQ604284|SH176296.07FU|Inocybe geophylla	361	1e-98	89.60	0012
HQ604283|SH176296.07FU|Inocybe pudica	361	1e-98	89.60	0012
HQ604282|SH176296.07FU|Inocybe pudica	361	1e-98	89.60	0012
HQ604281|SH176296.07FU|Inocybe cf pudica MLB 2010a	361	1e-98	89.60	0012

Figure 9: Example of massBLASTer output. In the figure it is shown the Query number 2, which is considered the most abundant ectomycorrhizal specie in our dataset. In this case, this OTU was assigned to *Inocybe geophylla* var. geophylla.

In case of unknown species, we also developed phylogenetic trees in order to determine the OTU at species or genus level, if possible. To study the phylogenetic relationships within and between OTUs, we used the neighbour-joining clustering using MEGA 5, using the UNITE sequences as reference species.

Ergosterol extraction

We first made an ergosterol test that assessed the reliability of the extraction and the most suitable soil weight to determinate this fungal biomarker. Thus, we used two replicates and two different amount of soil (100 mg. and 1 g.). Finally, the other samples were extracted in 1 g of dried material using 4 ml of 10% KOH in methanol. Samples were sonicated for 15 minutes and incubated 90 minutes. After incubation, we add 1 ml of re-demineralized water and 3 ml of cyclohexane. After centrifugation, upper fase was transferred to a new tube. This last step was repeated until 4 ml. of the upper phase was obtained. Samples were evaporated under air using N2. Finally, we dissolved the evaporated samples in methanol by heating at 40ºC for 15 minutes. Samples were finally filtered through a 0.5 µm of Teflon syringe filter

into autosampler vials and analysed in a reversed-phase column HPLC with a flow rate of 1 ml/min. Ergosterol was detected using a UV lamp at 282 nm and the time for ergosterol detection was 10 minutes, with expected retention time of 6 minutes. Ergosterol was analysed in a reversed-phase column (Chromolith Performance RP-18; Merck Millipore, Darmstadt, Germany) HPLC (auto sampler L2130 with UV-detector L2400; Hitachi, Tokyo, Japan) with a flow rate of 2 ml/min. We included a standard of known amounts of ergosterol in order to estimate the retention time.

3. Description of the main results obtained

DNA sequencing, bioinformatics and species composition

Before preparing the samples for sequences we optimize the extraction method for an efficient DNA amplification. According to the higher DNA yields and DNA quality ratios, we used the Lysis combination SL1 together with Enhancer, since higher yield was obtained under these Lysis combinations. At the amplification step, we also obtained different performance for each sample (Figure 3). Thus, the samples were amplified with a number of PCR cycles ranging from 18 to 30 cycles, which indicate strong differences in DNA concentration among the samples. After completing the equimolar mixing, BioAnalyzer results indicated that the community was dominated mostly by basidiomycetes, since these species have normally longer ITS region (Figure 6). Sequence data confirmed these results, since most of the species identified are basidiomycetes, especially ectomycorrhizal basidiomycetes. In total, we obtained 287.593 sequences after quality control and sequence filtering. Among all the quality filtering methods, the "amplicon quality" was the best option, when up to almost 70% of the sequences passed the quality control. However, library 5 was characterized by especially low sequence output and also showed a low percentage of sequence recovery after quality control and clustering. For this reason, this library will be sequenced again using 2 additional SMRT cells. Not accounting for the poor sequence performance at the Library 5, our results suggest that sequencing with PacBio RS II implies that approximately 60-70% of the sequences have good quality after control quality and sequence filtering (Figure 10), higher than initially expected. The main reason for sequence discarding in all the libraries was Missing 5' and 3' primer and Missing 5' and 3' Tag. One of the advantages of using both tagged primers is the detection of switched primers. Although not very common, this phenomenon was observed, with an average of 20 switched sequences per sample, which may represent 5% of the sequences.

Clustering with SCATA allowed us to detect 2869 global clusters and 6322 singletons (Figure 10). These singletons will be removed for further analysis since they may represent artefacts

or just contaminations. In this sense, from all the negative controls included in the study, only one control gave 2 reads, indicating that no major contaminations happened during the process. However, singletons may not have ecological importance and it is recommendable to remove them before statistical analysis and ecological interpretation.

General statistics for clustering run SCATA_LAST1						
	test_4_librar	test_4_librar	test_4_librar	test_4_librar	test_4_librar	Total
Total number of pyro reads:	93816	102007	108347	105024	74364	483558
Sequences passing QC:	60595	64208	68576	66826	27388	287593
Quality screening method:	Amplicon qu	Amplicon qu	Amplicon qu	Amplicon qu	Amplicon quality	
Reads matching after reverse comp	30027	31740	33437	33123	13338	141665
Number of reads truncated:	0	0	0	0	0	0
Number of reads discarded:	33221	37799	39771	38198	46976	195965
Reads too short:	1	2	3	0	1	7
Reads with too low mean quality:	1915	2112	2011	2300	63	8401
Reads containing bases with too lo	138	174	238	225	74	849
Mean readlength:	294	294	294	298	296	
Maximum read length:	1187	1316	1404	1618	1416	
Minimum read length:	154	154	101	156	156	
Missing 5' primer:	12578	13897	15026	14220	16037	71758
Missing 3' primer:	6785	7566	8344	7605	6615	36915
Missing 5' tag:	6617	8268	8225	8106	15671	46887
Missing 3' tag:	5187	5780	5924	5742	8515	31148
Number of global clusters:	2869					
Number of identified cluster:	1					
Number of global singletons:	6322					
Number of identified singletons:	0					

Figure 10: Summary of the obtained sequences and the result of the quality control after SCATA clustering. For this analysis we included all the 5 different libraries from our study.

Some of the most important 2869 OTUs were *Tricholoma terreum*, several *Inocybe* species, *Russula* species and *Lactarius species* (see list for the 500 most important identified fungal species in Supplementary Table 1), most of them detected in the sporocarp samplings from the last autumn. These results suggest that soil sampling may be a very useful and fast technique to estimates the potential fungal productivity at plot level as well as reliable to understand the ecological status of the forest ecosystems. In addition, the high dominance of ectomycorrhizal species suggests important carbon turnover in soil and high productivity (Clemmensen et al., 2015), although new studies of mycelium turnover and stability should address to what extend this carbon inputs are stable in these Mediterranean soils. No important amplification of non-fungal species have been observed, which make these primers suitable for fungal DNA amplification (Ihrmark et al., 2012). Other possibility that has to be taken in account is the possibility to amplify dying, death or residual DNA from soil. In our dataset we have detected *Cenoccocum geophylum* in relatively high number of samples,

although this should be interpreted with caution, since (Fernandez et al., 2013) observed a very low turnover and decomposition of this specie in the field. Thus, we not neglect the possibility that other species have also low turnover and might be amplified from death tissues.

The dominance of many ectomycorrhizal basidiomycetes in our data suggest that productivity in the study plots is still expected to be high, although it is characterized by recurrent drought periods.

Ergosterol optimization and extraction

Ergosterol extraction gave detectable levels in all the analyzed samples. Also, standards were detected and no variation was observed among repeated measurements. We carried out a preliminary test in order to study the variation between replicates and the effect of soil weight, with consistent results observed both between replicates and soil weights (Table 2)

Sample name	Code	weight	Retention time	area	height
302 11/2014 a	1	1	6.038	631.1	44.4
302 11/2014 b	2	1.0077	6.039	428.1	32.6
302 11/2014 a	3	0.3015	6.04	235.8	13.9
302 11/2014 b	4	0.301	6.041	142.3	11.6
305c 11/2014 a	5	1.0016	6.041	363.3	26.1
305c 11/2014 b	6	1.0015	6.043	288.9	21.2
305c 11/2014 a	7	0.2993	6.033	111	9
305c 11/2014 b	8	0.299	6.035	82.8	6.6
308c 11/2014 a	9	0.995	6.037	617	41.8
308c 11/2014 b	10	1.009	6.036	504.1	35.3
308c 11/2014 a	11	0.3035	6.039	218	13.4
308c 11/2014 b	12	0.3069	6.05	133.8	10.6
neg cont	13	0	0	0	0

Table 2: Preliminary test to study the differences between replicates and the optimum soil weight used to ergosterol extraction

Although some differences have been detected between replicates, we observed much higher variation between plots and between months (ergosterol signal ranged from 12.7 to 158.9 of estimated area). Before calculating the ergosterol in µg./mg soil we will correct the area values since it give better estimation than using the automatic estimation. We have planned to finish the rest of the ergosterol extractions and statistically test the differences between plots, months and thinning intensities, as well as correlate the values with the sporocarp yields in each plot for each of the 4 considered years.

Supplementary Table 1: List of the OTUs identified at species level using UNITE database (Kõljalg et al., 2005), obtained analyzing the 500 most important OTUs, with >97% of similarity at nucleotide level.

1	*Agaricus aff impudicus*
2	*Agaricus hondensis*
3	*Agaricus xanthodermus*
4	*Amanita spissa*
5	*Beauveria bassiana*
6	*Botryobasidium laeve*
7	*Bovista promontorii|Bovista promontorii*
8	*Cadophora finlandica*
9	*Camarophyllus virgineus var. fuscescens*
10	*Cercospora zeae maydis*
11	*Chalara hyalocuspica|Chalara hyalocuspica*
12	*Chalara microchona*
13	*Chroogomphus rutilus*
14	*Cladosporium tenuissimum*
15	*Clavaria asperulospora*
16	*Clavulina rugosa*
17	*Clitocybe aff costata DJL06TN80*
18	*Coniochaeta mutabilis*
19	*Cortinarius belleri*
20	*Cortinarius holophaeus*
21	*Cortinarius obtusus*
22	*Craterellus cornucopioides*
23	*Cryptococcus fuscescens*
24	*Cryptococcus terricola*
25	*Cystolepiota adulterina*
26	*Exophiala salmonis*
27	*Fusarium acuminatum*
28	*Geoglossum barlae*
29	*Geoglossum glabrum*
30	*Geoglossum vleugelianum*
31	*Geopora clausa*
32	*Gorgomyces honrubiae*
33	*Gymnopus aquosus*
34	*Gymnopus erythropus*
35	*Gymnopus ocior*
36	*Hebeloma mesophaeum var crassipes*
37	*Helvellosebacina helvelloides*
38	*Hemimycena pseudolactea*
39	*Humicola nigrescens*
40	*Hydnum ovoideisporum|Hydnum ovoideisporum*
41	*Hygrocybe cf conica*
42	*Hygrocybe chlorophana*
43	*Hygrocybe conica*

44	*Hygrocybe insipida*
45	*Hygrocybe pratensis*
46	*Hygrocybe spadicea*
47	*Hygrocybe virginea var. fuscescens*
48	*Hygrophorus persoonii*
49	*Hygrophorus quercetorum*
50	*Hygrophorus sp LM2763*
51	*Hymenogaster griseus*
52	*Hypholoma fasciculare*
53	*Inocybe amblyspora*
54	*Inocybe cf reisneri*
55	*Inocybe cf rimosa*
56	*Inocybe cincinnata*
57	*Inocybe geophylla*
58	*Inocybe geophylla var. geophylla*
59	*Inocybe glabripes|Inocybe glabripes*
60	*Inocybe griseolilacina|Inocybe griseolilacina*
61	*Inocybe lilacina|Inocybe lilacina*
62	*Inocybe maculata f fulva*
63	*Inocybe obsoleta*
64	*Inocybe rimosa*
65	*Inocybe subnudipes|Inocybe subnudipes*
66	*Inocybe umbrinella*
67	*Lactarius deliciosus*
68	*Lactarius vellereus*
69	*Lactarius vinosus*
70	*Lepiota echinella*
71	*Lepiota forquignonii*
72	*Leptosporomyces fuscostratus*
73	*Leucopaxillus laterarius*
74	*Lophiostoma cf cynaroidis*
75	*Lycoperdon niveum|Lycoperdon lividum*
76	*Macrolepiota puellaris*
77	*Mycena amicta*
78	*Mycena citrinomarginata*
79	*Mycena clavicularis*
80	*Mycena pura*
81	*Mycena rosella*
82	*Mycena strobilinoidea*
83	*Naemacyclus niveus*
84	*Oidiodendron griseum*
85	*Penicillium arenicola*
86	*Penicillium nodositatum*
87	*Penicillium pancosmium*

88	*Phaeoclavulina curta*
89	*Phialocephala*
90	*Phoma herbarum*
91	*Phomopsis columnaris*
92	*Pseudogymnoascus pannorum*
93	*Pseudoomphalina kalchbrenneri*
94	*Ramaria abietina*
95	*Ramaria quercus ilicis*
96	*Ramariopsis kunzei*
97	*Rasamsonia brevistipitata*
98	*Rhizopogon bacillisporus|Rhizopogon evadens*
99	*Rhizopogon roseolus|Rhizopogon rubescens*
100	*Rhodocollybia butyracea f. asema*
101	*Rhodotorula cresolica*
102	*Russula albonigra*
103	*Russula decipiens*
104	*Russula delica*
105	*Russula littoralis*
106	*Russula livescens (Batsch)*
107	*Russula maculata*
108	*Russula olivacea*
109	*Russula postiana*
110	*Russula praetervisa*
111	*Russula queletii*
112	*Russula risigallina*
113	*Russula subfoetens*
114	*Russula vinosobrunnea*
115	*Sagenomella striatispora*
116	*Suillus bellinii|Suillus bellinii*
117	*Thelonectria veuillotiana*
118	*Thuemenidium atropurpureum*
119	*Trichoderma spirale*
120	*Trichoglossum hirsutum*
121	*Tricholoma batschii*
122	*Tricholoma equestre*
123	*Tricholoma saponaceum*
124	*Tricholoma squarrulosum*
125	*Tricholoma sulphureum*
126	*Tricholoma viridilutescens*
127	*Tuber aestivum*
128	*Tuber rufum*
129	*Xenochalara juniperi*
130	*Xenopolyscytalum pinea*

4. Future collaboration with host institution (if applicable)

We have planned to submit the Library 5 for sequencing again, since it gave us too little sequence output, and start analyzing the data using multivariate statistics. In addition, we have planned to finish all the ergosterol extractions and test for significance with all the environmental parameters, but especially with the sporocarp yields, in order to test if it is possible to predict mushroom productivity at plot level. We have planned to analyse also the sequence data with the environmental parameters. We expect future and continuous collaboration with the host institution and also we aim in publishing our results in international journals.

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