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Forest Mycology and Plant Pathology

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# Abstract

The fungal kingdom is a large group of eukaryotic organisms consisting of more than 100 000 known and an expected number of more than 1 million species with a wide variety of lifestyles. Among these lifestyles is a symbiotic lifestyle that has been of importance for lichen forming fungi. The lichens are a polyphyletic group consisting of around 18000 different species. These fungi belong to both the Ascomycota and Basidiomycota, with the majority of the lichen forming fungi being Ascomycota. Though lichens have been known of and studied since the 19$^{th}$ century, many of the basic biological features are unknown due to their cryptic nature. With new and modern methods, such as next generation sequencing (NGS) and barcoding, it has become possible and more available to study lichens in different substrates.

Different barcoding regions are used in the three major eukaryotic groups, animals, plants and fungi. The internal transcribed spacer (ITS) region is the adopted default barcoding region in fungi and has proven to be efficient in most taxa. In this study, the fungal ITS2 region is amplified with the primers gITS7, ITS4A and ITS4 from samples collected from naturally decaying Norway spruce logs. The aim of this study is to: (1) provide practical experience, (2) examine where the highest fungal DNA concentration in the logs is and (3) examine the similarity between the technical replicates.

In this experiment, 32 wood discs collected from 8 logs of Norway spruce in two forests in Arvidsjaur, Sweden by my supervisor Veera Tuovinen were examined. From 13 different places on the wood discs, drill-samples were taken and extracted for DNA. Two replicates of each drill-sample were taken. In addition, from each wood disc four biofilm samples were taken and DNA extracted. The samples were run in PCR with tagged primers for preparations to Illumina-sequencing. After the PCR, the samples were cleaned and the DNA-concentrations measured.

The concentration of fungal DNA in the log decreased from the edge to the center of the logs, being twice as high close to the edge compared to the center. The similarity between the two technical replicates was between 39-45% at the different sampling points. The highest similarity was at the outmost and the fourth outmost samples which were 45% similar and the lowest similarity was at the center samples with a similarity of 39%. The PCR-cycles used were also similar for all sampling points with 29 ± 2 cycles.

The distribution of the DNA concentration in the wooden disc could possibly be due to where the fungi get in contact and colonize the log. Mycelial growth into the heartwood will take more time and is likely to explain the lower DNA concentration there. Some fungi may through assistance by bark beetles or by colonizing highly degraded parts enter deeper part of the wood than what they normally tend to colonize.

# 1 Introduction

## 1.1 Background

The fungal kingdom is a large group of eukaryotic organisms. The classic fungi like chanterelle (*Chantharellus cibariua*) and champignons (*Agaricus bisporus*), are species with a large fruiting bodies. However, fungi are normally cryptic and include life forms such as unicellular yeasts or as mycelia with pathogenic saprotrophic and symbiotic lifeforms. The cryptic mycelial stage is impossible to observe directly and therefore, many of them are less studied and less well known. Around 20 % of the yet known fungal species, i.e. about 18000 species, are estimated to form lichens (Nash 2006, Honegger 2012). In lichens a fungus, the mycobiont forms intimate symbioses with a photosynthesizing partner, the photobiont, which can be an algae and/or a cyanobacteria (Honegger 2012). Lichens form a polyphyletic group with mycobionts originating from many different fungal clades. Though much is known about the general structure and functions of the lichens, many basic biological aspects e.g about the life cycle remain enigmatic.

Sequencing of genomes was until recently a long and expensive process but has since the introduction of next generation sequencing (NGS) been made easier. NGS methods were developed to make sequencing of large genomes faster and cheaper. They also allow the study of whole organism communities by using genetic markers in a much larger scale than with the traditional Sanger sequencing. The Illumina sequencing is an example of NGS methodology. Illumina sequencing utilizes single strand DNA. The ends of the strands are attached to a glass plate and then sequenced all at the same time. The sequencing is made using nucleotides that give of a fluorescent light when bound. The fluorescence given off by the nucleotides is of different wavelength depending on which base it is comes from. The difference at wavelength is detected by the sequencing machine and processed to give the sequence (reviewed by Mardis 2008). Since NGS techniques were adopted they have been welcomed in the mycological world and used to study the cryptic fungal diversity in different substrates. New previously unknown species are being found and described on a regular basis and the scientific world has come to a new understanding of the complexity of the fungal world. In order to make species identification easier, the animal, plant, fungal and bacterial kingdoms each have different barcoding regions to make species identification easier. An efficient barcoding region for ascomycetes has been sought after. Different regions of fungal DNA have been targeted as candidate barcoding regions with different success and efficiencies (Schoch *et al.* 2012). The *c* oxidase subunit 1 (CO1) and RNA polymerase II (RPB2) have both been tested as barcoding regions and has been somewhat successful, especially in the lichen family *Cladonia* (Pino-Bodas *et al.* 2013). In plants regions in the chloroplast DNA, namely rbcL and matK, have been used as barcoding regions. In other eukaryotes as animals the COI gene in the mitochondrial DNA has been used for barcoding. In fungi however, neither of the barcoding regions in animals or plants have been effective. Instead the Internal Transcribed Spacer (ITS) region has been the default barcode. The ITS barcode is the most sequenced region in the fungal genome, especially in lichens. The reference database is therefore large and provides much data to use in comparative studies. ITS has two regions that are used as individual barcodes, the ITS1 region which is located between the nuclear ribosomal small subunit 18S and 5.8S rRNA genes and the ITS2 region that is located between the 5.8S and the large subunit 28S rRNA genes.

During the summer of 2015 I worked with Veera Tuovinen at the Swedish University of Agricultural Science (SLU). The project aimed to search for and identify potential free-living wood

inhabiting lichen mycobionts living in Norway spruce tree logs. These logs were from Arvidsjaur in northern Sweden. Wood inhabiting lichens, also known as lignicolous lichens, consists of many species that are less studied. The lichen mycobionts may potentially be either obligate or facultative and grow both the outer and inner part of wood. Many of the obligate lignicolous lichens are sexually reproducing and spore-dispersing (Spribille *et al.* 2008).

Many spore-dispersing lichens do not spread along with their photobiont and need to quickly reform the symbiosis after their initial germination. For a short time the mycobiont is free-living. However it needs to encounter a photobiont for reproduction and long-term survival (Ott 1987). The extent to which the free-living stage occurs is however very poorly known and it is speculated that this potentially may occur for a limited time and the growth of the mycobiont will stop if it is not able to establish a symbiosis (Meeßen & Ott 2013). However, mycobionts have been grown in laboratories on different growth media in the absence of their photobiont. These mycobionts have not been able to sexually reproduce without their photobiont and hence their occurrence in nature has been doubted (Nash 2008). Mycobionts have been detected and identified in wood in a study made by *Kubartova et al.* (2012). These same extractions were examined for the presence of the lichen photobionts and as several symbiotic algal strains where encountered. The algal strains where found in wood but it was suggested by *Tuovinen et al.* (2015) that the occurrence of the lichen mycobiont DNA rather was due to cracks and fissures in the logs. However, it is unlikely that the thallus fragments get sufficient light to photosynthesize. It is yet unclear if lichen mycobiont may occur as free-living in wood or not and a more detailed studies for the occurrence of free-living lichens is required (Tuovinen *et al.* 2015).

## 1.2 Aim

The main objective in my work was to learn and practice the methodology behind studies of fungal communities in environmental samples. In addition I explored the following two questions;

- Where in decaying logs is the concentration of fungal DNA the highest?
- How does the amount of fungal DNA differ between the technical replicates taken from the same wood sample?

# 2 Materials and Methods

## 2.1 Material collection

The field work was conducted before I joined the project. My part of the project was to extract DNA from the samples, amplify ITS2 with tagged primers by PCR and prepare the samples for Illumina sequencing by cleaning the products with AMPure and measuring the final concentrations by Qubit.

The materials examined in the study were collected from two different old growth forests in Arvidsjaur in northern Sweden. From each forest four Norwegian spruce logs were sampled. The first logs were taken from a spruce forest with mire and a river close by. The second area samples were collected close to a mire. Four wood discs were taken from each log and transported to the laboratory in Uppsala and stored at - 20°C until sampling of wood took place. First, the cut surface of the discs was burned. Thereafter, thirteen drill-samples were taken from each wooden-disc (Figure 2). The burning was done to destroy any possible DNA contamination on the surface. From each section were also four biofilm samples taken from the surface already at the field. From each drill-sampling point on the section two separate tubes were prepared, these tubes were two separate technical replicates, meaning two replicates from the same sample. The two technical replicates were put in two separate 2 ml screw-cap tubes along with two small metal nuts. The tubes were shaken with a fast-prep machine (Precellys) at 3000 rpm for 30 seconds in order to grind the wood and possible hyphae in the samples for DNA-extraction. Samples were then frozen until DNA-extraction. The biofilms were prepared for the DNA-extraction in a different way since the main interest of the sample was the top most surface of the section. Instead of grinding the whole biofilm it was sprinkled with a little $_{dd}H_2O$ and an approximate surface area of 2*2 cm was scraped off with a scalped and put in a 2 ml tube with screw-cap and ceramic beads included in the kit. The scalpel was sterilized with ethanol and burned between each sample. Any big visible lichens were removed as their presence was inventoried already in the field. The tubes were shaken in a fast-prep machine at 3000 rpm for 30 seconds and frozen until DNA-extraction. A total of 415 different wood samples with two replicates of each and 352 biofilm samples were prepared. All the steps from collection to sequencing are depicted in the flow-chart in figure 1.

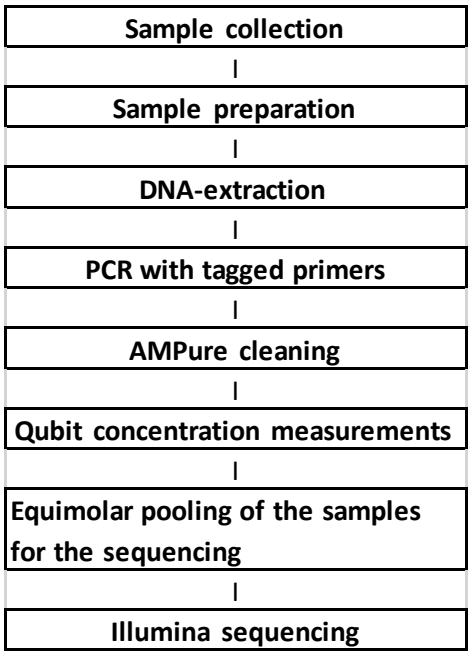


**Figure 1** A flow-chart depicting the different steps of processing before sequencing

## 2.2 Sample preparation

The DNA-extractions were done using the Nucleospin Soil Column kit from Machinary Nagel following the provided protocol (MACHINARY-NAGEL 2014). Based on the previously done testing the SL2 buffer with the addition of the enhancer SX buffer gave the highest DNA yield and

therefore these buffers were chosen for the extractions. The main stages include preparing the sample by adding three different types of buffers: the SL2 buffer, enhancer SX buffer and an SL3 buffer. The purpose of the SL2 and SX buffers is to lyse the cells and free the DNA in the samples. The SL3 buffer creates a precipitation of cell walls and other remaining debris contamination in samples so it isn't pipetted further from the supernatant. The sample supernatant is then added to a filter column in order filter out any leftover precipitants and inhibitors from the supernatant. A binding buffer SB is added to bind the DNA from the supernatant on a new filter column. This column is then washed with buffer SB and washing buffer SW1 and twice with another washing buffer SW2. The samples are eluted into a new collecting tube with lid after the washing steps and then frozen until further processing.


**Figure 2** Sampling locations on the sections. Where the outermost samples being in ring 1, second outermost in ring 2, third outermost in ring 3, fourth outermost in ring 4 and ring 5 being the centermost samples.

Most of the samples were diluted 1:10, 20 µl DNA and 180 µl dd$H_2O$ prior to PCR. This was done because the DNA was measured with NanoDrop and gave values under 10 ng/µl. At DNA concentrations that low NanoDrop is not a reliable measuring method, see Machinary Nagels protocol, (MACINARY-NAGEL 2014). The samples that had DNA concentrations over 10 ng/µl in NanoDrop measurements were instead diluted 1:100, 2 µl DNA and 198 µl dd$H_2O$. The biofilm samples were diluted 1:100 due to the significantly higher DNA-concentrations in the samples. After dilution the samples were prepared for PCR. The locus used for the PCR was ITS2, with general primers, gITS7, ITS4A and the ITS4. The PCR was run with the following temperature cycles: 94ºC, 56ºC and 72ºC, in that order, all steps lasted for 30 seconds, with the exception of the initial heating step which had a duration of 5 minutes and the final extension at 72 ºC for 10 minutes. Since the primers used are very general to the fungal genomes, the number of cycles was aimed to get a PCR-product which gives a weak or medium-strong band on an agarose gel. This was done to avoid getting an overabundance of very common fungi. The most common templates will be exponentially amplified during the PCR and many PCR cycles would lead to skewed abundance estimates, as the rare templates will get less readings during the sequencing (Ihrmark *et al.* 2012). The number of PCR cycles used range from 25 cycles to 33 cycles, with the majority of the samples ranging between 28 and 31 samples.

## 2.3 Cleaning and measuring

The PCR-products were cleaned with AMPure (Agencourt). The AMPure procedure is to bind the DNA, both single and double stranded, to magnetic beads. The volume of the AMPure added is 1.8 times the concentration of the PCR-products. The mixture was then vortexed and incubated to further increase binding of DNA and then washed with 70% ethanol. The ethanol was then completely removed by being left to evaporate in 37ºC. The samples were then eluted out with a 10 mM TRIS-HCl buffer. After elution, the eluted PCR-products were put on the magnetic plate and

the buffer was transferred to a new 96 PCR-plate and stored at -20ºC.

The concentration of the cleaned PCR-products was then measured with Qubit (Invitrogen). 5 µl of the PCR-product was diluted in 195 µl Quant-IT active solution using HSbuffer. The samples were then measured in a Quant-IT version two reader. The measurement in Qubit were done on the current dilution concentration, in order to get the actual concentration it had to be calculated using the following formula; Concentration=QF*(200/5). Where QF (Qubit Flourometer value) is the measured concentration.

## 2.4 Data analysis

When analyzing the data I compared each sample to examine how similar in percent the two replicates were in DNA concentrations. I then took the mean value of the similarities for the five sampling locations and their standard deviations (Figure 2). I also examined which were the lowest similarities and the highest similarities at each sampling point. Finally I took the mean value of the concentrations for each sampling point as well. A similar analysis was made for the amount of PCR cycles used for each sampling point in the sections. The mean value, standard deviation and lowest and highest number of cycles were calculated.

# 3 Results

Throughout the experiment a total of 829 drill-samples were DNA-extracted. After PCR, 11 of these samples contained too low concentration of DNA to be measurable by Qubit. For the biofilms a total of 352 samples were DNA-extracted. Due to the lack of time however, the samples were not processed further.

## 3.1 DNA-distribution in the logs

The concentrations varied in the log and were lowest in the center (mean concentration 707ng/mL). The concentrations raised steadily to surface were it was 1537ng/mL (Table 1). No differences in DNA-concentration between the logs from the two different sampling areas, forest and close to mire, was found (results not shown).

| Sample location | Mean concentration ng/μl (n=192) | Concentration similarities % (Mean ± SE, n=96) | Concentration similarity % (min-max) | Mean nr of cycles (mean ± SE,, n=192) | Mean nr of cycles (min-max) |
|---|---|---|---|---|---|
| Outer | 1537 | 45,7% ± 27,8% | 0%-98,2% | 29 ± 3 | 25-33 |
| Second outmost | 1272 | 40,7% ± 28,1% | 0%-96,1% | 29 ± 2 | 25-33 |
| Third outmost | 1198 | 43,9% ± 26,9% | 0%-99,2% | 29 ± 2 | 25-33 |
| Fourth outmost | 981 | 45,8% ± 28,1% | 0%-97,1% | 29 ± 2 | 25-33 |
| Center | 707 | 39,1% ± 27,9% | 0%-95,6% | 29 ± 2 | 25-33 |

**Table 1.** The different sampling locations in the logs from out most to the center taken as a mean value. The difference in concentrations between the two replicate at each sampling point, the mean for the concentration similarity was taken for each sampling location along with a standard deviation. The mean number of cycles used and the standard deviation is also included.

## 3.2 Similarity between the two technical replicates

The similarity in concentration between the replicate is around 40%-45%. The highest similarity is at the fourth outmost and the outmost sampling location which are 45,8% and 45,7% respectively. The standard deviation is similar between all sampling locations, being ± 28% (Table 1).

The mean number of cycles used for the sampling points were at all cases around 29 cycles, with a standard deviation of 2 cycles in all cases. Except the outmost sample which had a standard deviation of 3 cycles.

# 4 Discussion

## 4.1 Methods

### 4.1.1 Extraction methods

The DNA-extraction in the experiment was made using an extraction kit usually used for extraction of fungal DNA in soil samples (MACHINARY-NAGEL 2014). The main issue by utilizing a soil extraction kit is to destroy the wood and the fungal cells within it. Instead of utilizing the ceramic beads, metal nuts were used. They were more efficient to grind up the harder wood than the small ceramic beads. Apart from the grinding, the main procedure was the same. The ceramic beads were efficient enough when grinding the biofilm samples due to their softer tissues. The biofilms were scraped of the surface layer of the bark rather than being a drill dust sample. The looser consistence and the more available fungal cells made the ceramic beads more efficient. Due to the possibility to crack and destroy the tubes containing the samples when using metal nuts, it was preferably to use the ceramic beads when possible.

Of the 829 drill samples, 818 samples had sufficient high DNA-concentration to be measurable by Qubit after the PCR and cleaning steps. In11 samples, I could not detect any DNA. Although lichenized fungi may be present in many samples, the DNA-concentration of the samples does not reflect or correlate the amount of lichen mycobionts as it is the total fungal DNA concentration that is measured. Similarly will the sequencing show the presence of fungi in general but not the concentration. Since the wood in the logs was dead it could be expected that logs contain a high species richness of fungi (Kubartová *et al.* 2012, Ottosson *et al.* 2015). Although there is expected to be lichen mycobionts among the wood-inhabiting fungi, they are in a minority of the total amount of fungi if present. Especially as lichen mycobionts mostly are ascomycetes do not have as effective degrading enzymes as wood-decaying basidiomycetes have. The study by *Kubartová et al.* (2012) reported potentially 100 lichen mycobionts in wood samples. This was however followed up by *Tuovinen et al.* (2015) who also could record lichen photobionts in the same wood samples. Yet there is no evidence for free-living lichen mycobionts in wood being a common and wide-spread phenomenon.

### 4.1.2 Primer choice

The choice of barcoding primers affect which species or groups of species (or taxa) that are amplified and hence which taxa that are identified in the logs. The ITS2 region used is a commonly utilized barcode region in mycology. It is effective to many different fungal taxa but lacks accuracy for some of the earlier diverging fungal lineages (Schoch *et al.* 2012). Neither of the earlier fungal lineages forms a lichen symbiosis. There are several different primers used for the ITS2, e.g. among those are fITS9, ITS1f, fITS7 and the primers used in this lab, ITS4, gITS7 and ITS4A (Ihrmark *et al.* 2012). The primers can be used for different purposes with different efficiencies. The primers used in this work ITS4, ITS4A and gITS7, should be good for the aim of this study. The fITS7 and gITS7 primers amplify a larger number of fungal taxa than the traditionally used ITS1F (Ihrmark *et al.* 2012). Previous barcoding studies show that in the lichen genera *Cladonia*, there is no barcoding gap, which makes it difficult to identify species using the ITS2 region (Pino-Boda *et al.* 2013). Using the gITS7 and the fITS7 primers could lead to some mismatches in some lichen taxa, as the success of these primers for several lichen species has not been tested. These mismatches are however not found in the orders *Penicillium*, *Orbiliales, Mucorales* and *Saccharomycetales* (Ihrmark *et al.* 2012). None of the fungal groups are lichen forming however. As I base the

discussion of the methods on the DNA-concentrations measured after the PCR, it needs to be kept in mind that the concentrations do not mirror the actual amount of lichen fungi but rather all the fungi extracted.

## 4.2 The amount of technical replicates and their biological relevance

The replicates of fungal DNA concentration were in general 40-45% similar with a standard deviation around 28%. The outmost sampling points had the highest similarities at 45,7%-45,8%. The outmost sampling points had a lower standard deviation and should then be more similar throughout all included samples. Each sample was run on a number of cycles to yield an appropriate concentration for Illumina sequencing. Even if the cycles are optimized and the bands on the gels were weak or medium-strong (Ihrmark *et al.* 2012), there are still variations. The mean of the cycles used at each sampling points were similar, with a cycle span of 29 cycles ± 2 cycles, except for the outmost samples which had a deviation of ± 3 cycles. The concentration difference between the two samples is large between some samples and small between others. There is a great deal of variation and it could possibly be due to the difference in the number of PCR-cycles used. If all samples were run on the same amount of PCR-cycles the difference could potentially be smaller. Although many replicates had the same amount of PCR-cycles, the difference is probably more likely to be due to differences in the initial DNA contents. The replicates could contain very different amounts of DNA and could be unevenly distributed. Another probability for the differences is likely to be due to the human factor. Differences in extraction methods and technical variations could cause different amounts of DNA to be extracted from the replicates even if the material contains similar initial DNA-concentrations. Good technical replicates should contain similar amounts of DNA and the differences between these replicates indicate that there is a problem somewhere. As the variation between the technical replicates was similar throughout all samples, I suggest that the difference is due to a real biological difference between the replicates. This suggests that taking more technical replicates will increase the possibility to track all the fungal species in the samples.

Since many fungi thrive in more humid environments, it would be expected that the logs collected from the mire would contain a higher concentration of fungal DNA. The fungal DNA concentration does not, however, mirror the humidity near or in the log. It may also reflect the degree of decomposition and be affected by ground contact and wounds. A wound makes it easier for a fungus to colonize inner wood. Even though the defenses for fungal colonization in a tree have stopped functioning in a dead tree, the bark is still challenge for fungal colonization. Bark burrowing beetles or other insects, however, provide such assistance (Persson *et al.* 2009). A log that had had a high activity of bark beetles may therefore contain more fungi and hence a higher concentration of fungal DNA. The activities of wood inhabiting beetle may also enable lichen fragments to enter into the wood. The fungal DNA would then also include species that are physically present in the wood but not active there. This needs to be taken into account in investigations addressing the questions if lichen mycobiont potentially may be free-living in wood. This question about the function of detected fungi is however impossible to answer based on the amplicon sequencing only.

As seen in Table 1, the highest concentrations are in the outermost samples while the smallest concentrations being in the center. Through-out all sampling points there is a steady decrease in concentration from outermost to centermost samples. Due to the methods of colonization of logs by

fungi, this is to be expected. Fungi either colonize by hyphae from contact other pieces of dead wood or from the ground or from spores dispersed by wind or vectored by insects. Many different fungi are present in the outer areas of wood (Kubartová *et al.* 2012). Some section however had a rather equal concentration throughout the section or even a higher concentration in the center. This could be explained by fungi colonizing the tree before its death and decaying the heartwood. Localized differences in the level of decay in the wood led to some samples containing very decayed sample material while others were hardly visibly decayed. It was impossible to sample one position in one wood disc, as the wood was too decayed. Effects of early fungal colonization causing decay and potentially having caused the death of the tree could be significant for the detected fungal community composition.

A possible approach that was not used is to calculate the expected concentration of DNA in the samples and then to compare the actual, achieved DNA concentration with the expected one. A calculation of expected concentration would normalize the concentration at the same cycle amount. However, as the initial concentrations in the samples were too low to measure with Nanodrop, this could not be done. The concentration of DNA would likely still vary as a result of differences in the initial fungal DNA concentrations between the technical replicates.

## 4.3 Conclusions

The highest DNA concentration in the logs was found close to the log surface. There was then a steady decrease in the concentration into the log center. Since the surface of the log is in contact with the surroundings, the fungal concentration is higher on the surface and lower in the center, which takes longer time to colonize. Between the replicates there was a similarity around 40-45% in the different sampling locations and they had a standard deviation of around 20% . The low similarity could be due to four reasons, (1) the differences in PCR-cycles used, (2) uneven distribution in initial DNA content, (3) different success at extraction (the human factor) and (4) that the variation is due to real biological differences in the samples.

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