Sveriges lantbruksuniversitet
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Sammanfattning

Under det senaste årshundradet har de svenska skogarna genomgått en omfattande förvandling, från mer eller mindre naturligt dynamiska till hårt brukade skogar bestående av monokulturer av tall eller gran. Denna förändring har lett till en minskning av den biologiska mångfalden, att många arter som är beroende av skog har minskat radikalt och hotas av utrotning. Som ett försök att främja variation, biologisk mångfald i allmänhet och förhindra minskningen av rödlistade skogsarter introducerades i början av 90-talet en viss naturvårdshänsyn i brukade skogar. I den här studien utvärderas den påverkan som kalavverkning har och den betydelse hänsynsträd har på artsammansättningen och förekomsten av ektomykorrhizasvampar (EMF) under de första 20 åren efter kalavverkning. Studien utfördes genom extrahering och identifiering av svamp-DNA från jordprover tagna på kalavverkade ytor, nära hänsynsträd och i gammal skog i ett begränsat geografiskt område i norra Sverige. Resultatet visar att kalavverkning har stor påverkan på EMF. Få arter hittades på de kalavverkade ytorna, hänsynsträd verkar ha en viss betydelse där några fler arter hittades, medan flertalet arter hittades enbart i den gamla skogen. Resultaten visar att det inte räcker med hänsynsträd vid avverkning för at klara mångfalden av EMF utan att det behövs flera åtgärder.

Abstract

Swedish forests have gone through large changes during the last century. By the introduction of clear-felling, forests have changed from more or less natural dynamics, through extensive management, to production monoculture forests of Norway spruce (Picea abies) or Scots pine (Pinus sylvestris). This has led to a decrease in biodiversity and many species are red-listed as they are declining and pose a risk of becoming locally and nationally extinct. Retention forestry was introduced in Swedish forestry in the early 90's as a measure to promote variation and biodiversity in general and preventing decline of red-listed forest species. In this study the effect that clear-cutting have on EMF and the importance that retention trees may have on EMF during the first 20 years after clearcutting is evaluated. This was done by extracting DNA from soil samples taken on clear-cut areas, close to retention trees in clear-cuts and in old growth forests, in a restricted area in northern Sweden. The result clearly shows that EMF is affected by clear-cutting. Very few species was found on the youngest clear-cuts. By retention trees some more species were found, so retention trees clearly support mycelia of some species through the clear-cut phase, but several EMF species was only found in the old growth forests. Clearly this measurement needs to be more carefully evaluated before all forests have been managed this way and many species it's meant to support are gone.

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1 Introduction

1.1 Background

The boreal forest is a large terrestrial biome covering more than 1.4 billion hectares globally which is about 14 % of the earth's vegetation covered land, extending over North America and Eurasia, including Sweden (Soja *et al.* 2007). Forests cover more than 28 million hectares of Sweden's total 48 million ha land cover. According to forest statistics, 25 % of the Swedish forest is exempted from forestry, but much of that are unproductive forests close to the mountains and voluntarily set asides. Only 3.6 % of the productive forest has a formal protection, which is about 844 000 hectare (Skogsstyrelsen 2014, b).

To satisfy domestic and international demands for fibre and fuel, the use of Swedish forests has gone through a large change during the last century (Nilsson 1990). Within one century, the northern Swedish forests have largely changed from more or less natural, to regulated production forests (Östlund *et al.* 1997), leaving small remnants of natural forest (Bryant *et al*. 1997). In Sweden the common silviculture method used since the early 50's has been clear felling with replacement to monoculture of Norway spruce (*Picea abies*) or Scots pine (*Pinus sylvestris*). Since the introduction of clear-felling, forest management has changed to be more large-scale and mechanical (Esseen *et al.* 1997). The results of this forest management is that old growth forests with natural dynamic are becoming scares as well as early successional forests arising after natural disturbances (Johansson *et al*. 2013). Disturbance has historically been an important factor shaping ecosystems in boreal forests. Before man started managing the forests, fire was the primary large scale disturbance creating larger gaps in the landscape. Storms and pests then and still create smaller gaps (Olsson 2011). Today's forestry has resulted in more homogenous forests with less structural variation and biodiversity (Johansson *et al*. 2013). Fires have become less common and pests and storms affect larger areas (Olsson 2011).

Forestry affects many groups of forest species, both species that are associated to old growth forests, and those associated with early successional stages following natural disturbances (Kuuluvainen 2009). The population of a relatively large number of forest species has been negatively affected and are therefore red-listed (ArtDatabanken 2015). This list over threatened species is a measurement taken by many countries, to support the work to counteract species decline and ultimately national extinction. The most recent Swedish Red List contains more than 4000 species and of them more than 2000 lives in forests (ArtDatabanken 2015). One group of species on this list is fungi with almost 800 species.

Another conservation measures taken to promote variation and biodiversity in general and preventing decline of red-listed forest species is retention forestry (Gustafsson *et al*. 2010). In retention forestry, different considerations are being taken in account during harvest, all from leaving solitary trees (retention trees, green tree retention) on clear-cuts, creating and leaving dead wood and snags on clear-cuts, leaving small patches of forest on clear-cuts, buffer zones along streams and mires, to leaving patches of intact forest in the size of up to more than one hectare (Gustafsson *et al*. 2012).

Retention forestry was first introduced in North America in the early 90's, but other countries soon followed, among them Sweden, where it has been common practice (Gustafsson *et al*. 2010). From the laws point of view retention are still recommendations (Skogsstyrelsen 2014, a). However Forest certificate organizations demands differen kind of retention measurements (FSC 2010) and in Sweden

about 50 % of the productive forest land are certified by FSC (Johansson *et al*. 2013). The idea of retention forestry is to decrease the harmful effects of intensive forest management, by supporting flora, fauna and funga through retaining a more heterogeneous landscape, both by mimicing natural disturbances, by leaveing elements found after natural disturbances, and by providing refugees for species, source areas for re-colonizing the harvested area, lowering the amount of disturbance directly after the harvest and helping species to recover (Vanha-Majamaa and Jalonen 2001).

Fungi are a large group of organisms with species of different important ecological functions. Some fungi are saprotophs, decomposing dead material, some are symbionts, living in a close symbiotic relationship with plants and some are parasites, attacking and killing other living organisms (Deacon 2006). Some of the symbiotic fungi belong to a group called ectomycorrhizal fungi (EMF).

These EMF's are symbionts with many of the dominant trees in the boreal forest (Hobbie and Agerer 2010) and they play a vital functional role in many of the world's forest ecosystems (Smith and Read 2008, Peay *et al*. 2012). Many EMF form, what is known as mushrooms, but mushrooms are only the fungus fruiting body/sporocarp and consist of a small portion of the fungi, only (Smith and Read 2008). The greater part of the fungus is the mycelium, a network of filaments called hyphae which grows abundantly in the soil where it ties together its hyphae around the tree roots and in between the tree roots cells (Martin 2007). Here an exchange between the fungus and the tree occurs, where the tree get nutrients such as phosphorus and nitrogen and water from the fungi (Smith and Read 2008). The mycelium has a much longer range than the tree roots, extending/ broadening/ the trees absorption area (Agerer 2001, Berner *et al*. 2012). Fungi also have enzymes that can break down minerals and thus releases nutrients otherwise unavailable to trees that on their own only are able to absorb free nutrients. The fungus may also protect the tree against parasites (Smith and Read 2008). In return the EMF gets carbon from the tree. Fungi don't have the ability to photosynthesize like plants, as they are heterotrophic, meaning that they cannot fix carbon and uses organic carbon for growth (More *et al*. 2011). EMF is dependent on carbon from plants, mostly trees for their survival. When the host-tree is cut down, the flow of assimilate from the tree to the fungus is cut of and the fungal mycelium dies if it doesn't have or find another host tree it can connect to.

New establishment of fungi is dependent on dispersal by spores from fruiting fungi. Giving that many fungi only forms sporocarps sporadically and have a limited dispersal capacity they may have difficulties establishing in a new areas (Peay *et al*. 2012). In addition to that EMF's are obligate symbionts and in that way dependent on trees that they can live in symbiosis with to survive, they are also dikariotic, which means that their cells consist of two nuclei from two different individuals. A germinated spore forms a haploid mycelia that needs to find another haploid mycelia (germinating spore) of the same species that it can fuse with to form a long-lived dikaryotic mycelia (More *et al*. 2011).

Until recently, studies on fungi was restricted to survey of fungal sporocarps, but recent years advances in molecular technology has made it possible to identify fungal mycelia in soil samples (e.g. Guidot *et al*. 2003, Lilleskov *et al*. 2011). Many fungal species only fruit occasionally and some may live their entire life only as hyphal network in the ground without producing sporocarps (More *et al*. 2011).

1.2 Aim

This study aimed to evaluate the importance that retention trees may have on EMF during the first 20 years after clearcutting, by comparing clear-cut areas with and without retention trees and with nearby old growth forest.

1.3 Research questions

1. How important are retention trees for the survival of EMF after clearcutting? To what extent will species richness and community composition of EMF in managed forests with retention trees correspond to that of old growth forests?

2. Will clear-cut areas without retention trees and clear-cut areas with retention trees have the same similar EMF species richness and composition?

3. Does the species richness and species composition of EMF change over the first 20 years after clearcutting? Will this potential change differ between areas with and without retention trees?

1.4 Hypothesis

My hypothesis is that some generalist species will be found in all areas, while some late successional species will only be found in the old growth forests. Mycelia of these late successional species may be sensitive to clearcutting. As they may be supported through the clear-cut phase by the retention trees, I expect them to be found by retention trees. I expect some early successional species to be more common in the clear-cuts than in the old growth forests, but fewer in the younger forest than in the forests that was re-established 20 years ago, after clear cutting (e.g. Kranabetter *et al*. 2013, Luoma *et al*. 2004).

2 Material and Methods

2.1 Study sites:

The selection of sites for this study was done by Kerstin Varenius and Maria Elene Johansson, researcher at the Department of Forest Mycology and Plant Pathology in SLU, Uppsala who is doing a larger study on EMF in managed pine forests. The project is conducted in in collaboration with the forest company Sveaskog, who provided the information and selection of forests in an area about 30 km north of the town Kalix in the county of Norrbotten (Fig. 1 and 2). Sveaskog provided documentation, ecological, geological information and aerial photographs over the forests. This information was used to select forests that met the required criteria. Information of the chosen study sites is found in table 1.

In total 6 stands were selected for the study, four managed young forests and two old unharvest forest according to the following criteria they should consist of:

1. Two different age intervals of the managed stands (5 to 15 years (site 1 and 2) and of 15 to 25 years (site 3 and 4) after cutting).
2. The managed stands in each age category should consist of one clear-cut with retention-trees and one clear-cut with retention-trees plus seed trees cut.
3. Two older forests acting as controls (Site 5 and 6) should be located nearby site 1-4.

Plant establishment at site 1 and 3 were conducted by planting and at site 2 and 4 from natural regeneration from the seed trees.

The criteria for the selected stands were; a. pine as the dominant tree-species (> 70% of the basal area), b. lingonberry as the main vegetation cover, c. at least five solitary retention-trees with at least 20 meters to the neighbouring retention tree or the edge of the stand, large enough surface without retention-trees to be able to take five samples, preferable on a transect with ten meters between the samples, and at least 20 meters to the closest retention-tree and the edge of the stand. Aerial photographs and the location of the sampling points are shown in Fig 4 – 6.

Retention trees were sampled at site 1-4. At site 1 and 3, sampling were also conducted at areas without any trees left at clearcutting. At site 2 and 4, sampling were also conducted at areas without retention trees but with seed trees, which were cut within 10 years after clear cutting.

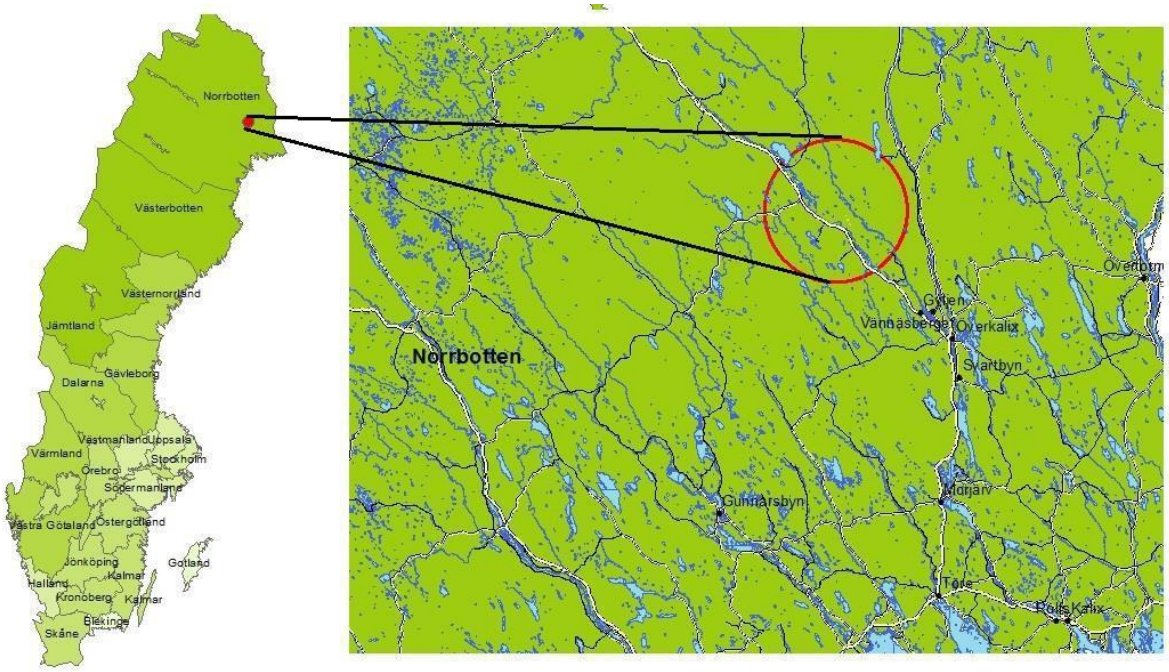
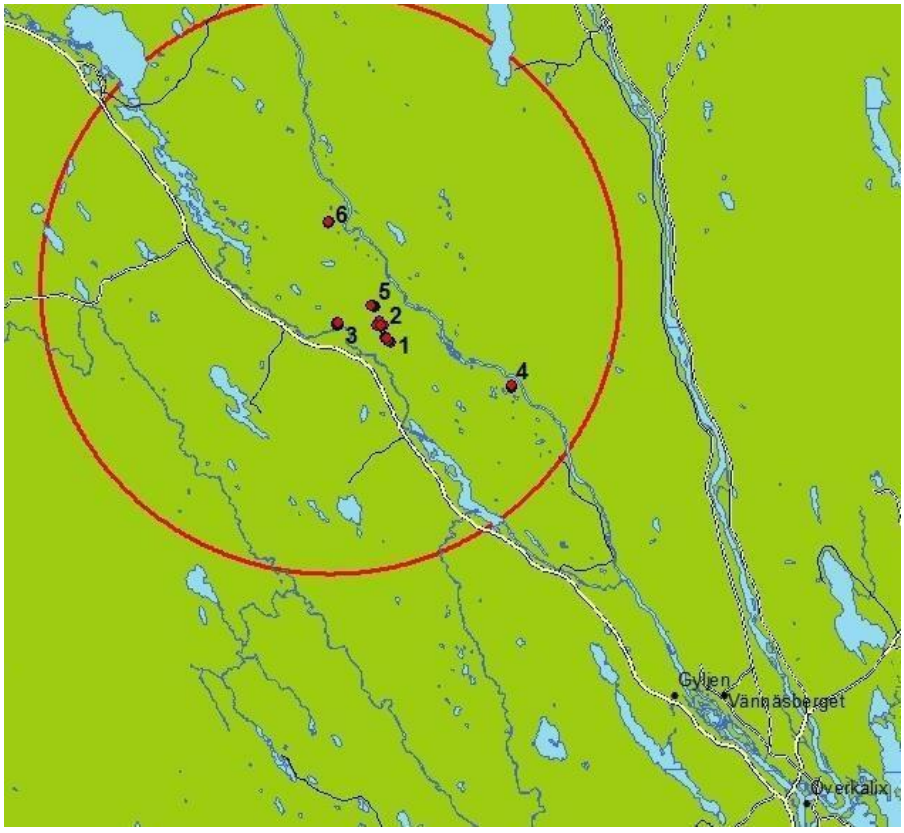


Figure 1. Map of Sweden and the study area.



Figur 2. Map showing the location of the study sites.

Table 1. *Management description of the study sites*
Year of management operations (cut, seed tree cut, thinning and planting). No indicate no such management. Est. age = the estimated age of the forest. Site quality is the expected growth in m^3 per ha and year of the area. E and N is the east and north coordinates of the sites given in RT90.

Site	Cut	Seed tree	Planted	Thinned	Est. Age	Fertilized	Cleared	Scarified	Site qualit	E	N
1	2004/2008	No	2010	No	5	No	No	2009	3.6	7397564	1795381
2	2004	2009	No	No	10	1982/1989	No	No	3.2	7398205	1795142
3	1991	No	1993	2012	22	1972	1991	1992	3.6	7395394	1801036
4	1988	1993/2000	No	1996	23	No	1988	No	3.6	7398264	1793171
5	No	No	No	No	>120	No	No	No		7399017	1794826
6	No	No	No	Yes	>120	No	No	No		7402860	1792762

2.1.1 Soil sampling

All soil samples were taken within two days, in the middle of July and within a range of 10 km from each other. Even if you have a good historical, environmental and geological record when selecting the appropriate stands in the office, the entire site may not in reality meet the requirements altogether. In the field the selected study sites were first visually studied to locate the parts of the Stands that fit the requirements the best. The exact spot for sampling were chosen by locating all possible spots with retention trees and by random choosing five trees and a large enough clear-cut surfaces to fit a transect for five samples of 10 meters apart. In the stands that did not have a larger surface that met the requirements than was needed, samples were taken where possible (more detailed description of each area is found in appendix 1). Samples were taken 1m north of the retention trees using measuring tape and compass and in the clear-cut surfaces, a transect for five samples with a distance of 10 m between was preferred, but when this was not possible due to shortage of space the samples were taken on as close to a transect as possible. In the older forests two times five samples was taken on as straight transects as possible (Fig 3).

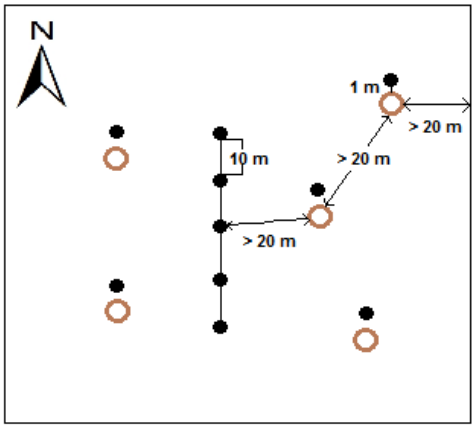


Figure 3. schematic image illustrating ideal soil sampling design. Black dots = sampling points, Brown rings = retention trees.

Common to all chosen stands was a stony ground. To find suitable places to take samples in this stony environment, the ground was examined at the selected sample spot with the foot in a half circle in direction towards the continued orientation of the outsourced transect, to locate the closest possible spot with no stones. When a suitable spot was located, first the vegetation cover was removed and the samples were taken using a soil corer. Earlier studies indicate that EMF's species composition differs somewhat or changes between the humus layer and the mineral soil (Landeweert *et al*. 2003), so the samples were divided between these two layers. Each humus sample was placed in separate bags marked with sample number and coordinates, but to restrict the amount of samples, the mineral soil was pooled together and further analysed only on stand-level. To get a clear separation between the two layers, the layer between the humus and mineral soil containing a mix of both layers were discriminated. If the humus layer were thick (> 2 cm) the sample was vertically divided in half and

half of the sample left. All samples were frozen within eight hours and kept frozen until further analysis in the lab.

2.1.2 Age control of forest stands

In all study sites, branch nodes were counted on trees of the regenerating forest to verify the tree age reported by Sveaskog. Common in all sites was that the regenerating trees were severely browsed by moose. Trees that had managed to escape browsing were chosen for node counts. The age of the retention trees was determined by drilling with an increment borer. Normally trees are drilled at breast height, however in areas with low productivity the age growth of the trees can differ by many years at breast height (Berggren 2004), so the drilling was done as close to the ground as possible. Each core was placed in tubes marked with sample number and coordinates and saved so the age could be determined in the lab by counting the annual rings using a stereo microscope with 60 times magnifications.

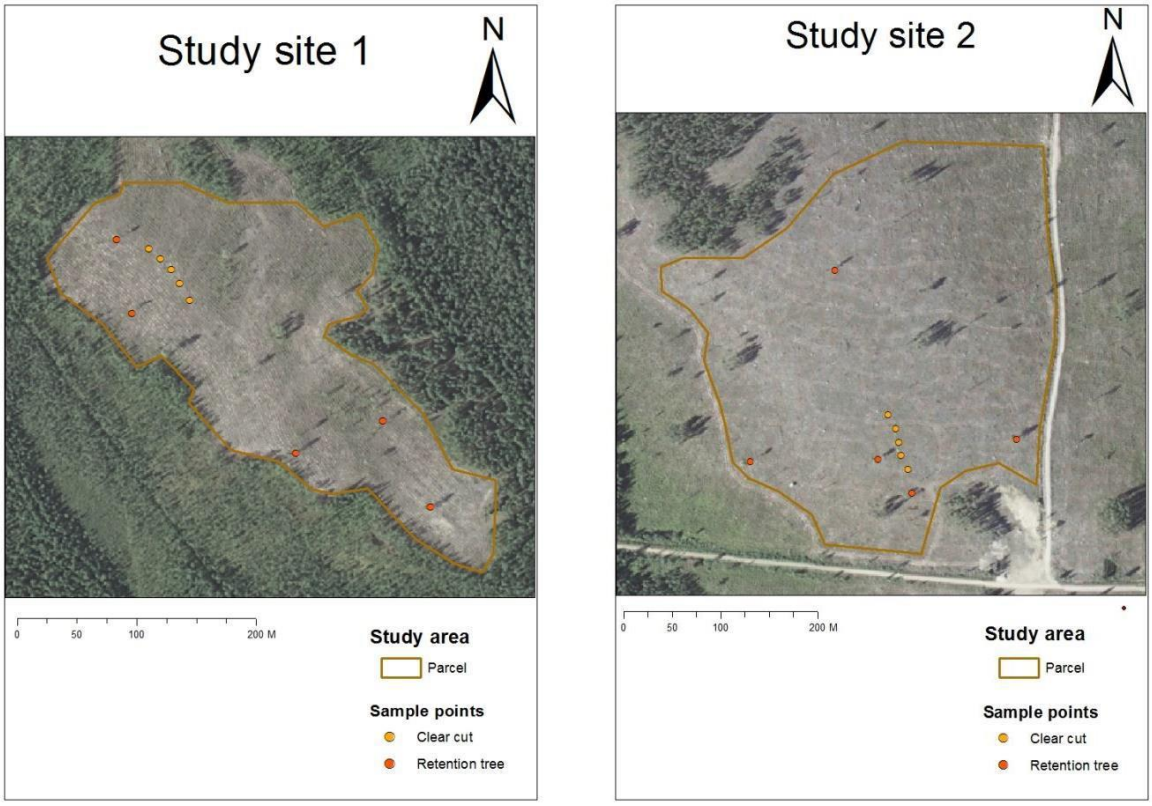


Figure 4. Close up image of study site 1 and 2 age interval 5-15 year. Red and yellow dots show the position for the soil samples.

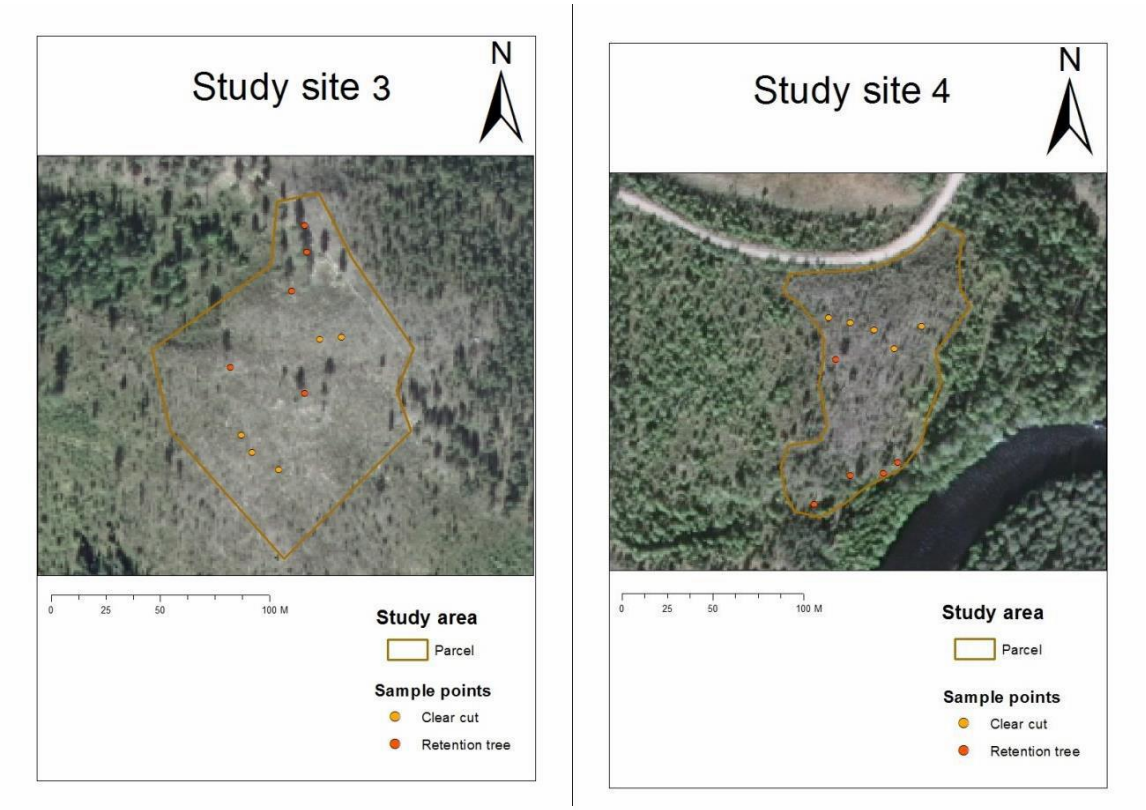


Figure 5 Close up image of study site 3 and 4, age interval 20-30 year. Red and yellow dots show the position for the soil samples.

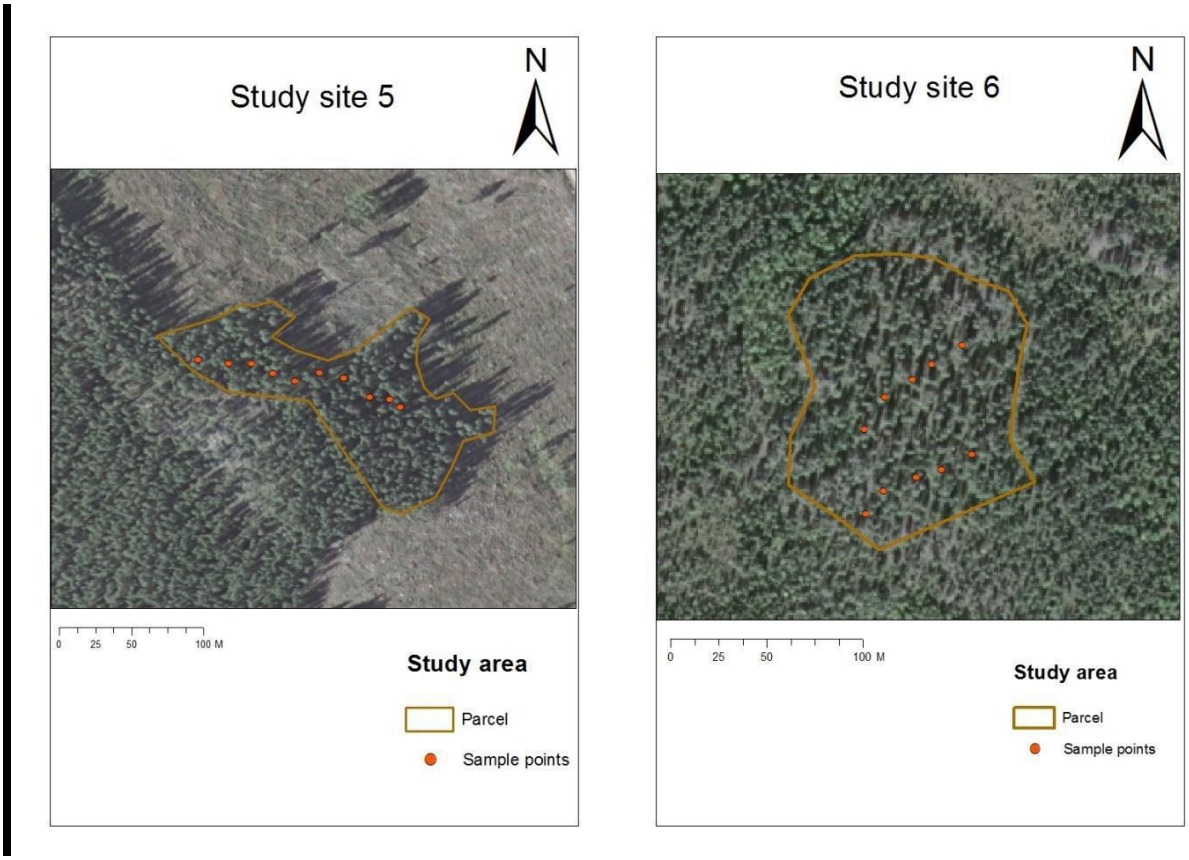


Figure 6. Close up image of study site 5 and 6, old growth forest. Red and yellow dots show the position for the soil samples.

2.2 Lab work

The total number of soil samples was 72. The suitable amount of samples that could be processed at one time was 24 so the following steps until the evaluation after electrophoresis were conducted four times processing 23 samples and one blank at a time in the first three runs. After the third run samples that was unsatisfying in any way after the evaluation of the electrophoresis, was processed one more time in the fourth run, together with the remaining samples. Any unsatisfied samples after the fourth process was excluded.

2.2.1 Freeze-drying the samples

The frozen samples were transferred from the sample bags into marked 50 ml tubes leaving only a small amount for measuring soil pH later. To reduce the risk that improvements in working methods would affect the results of the samples from the same study site, all samples got new random numbers that they were treated on the basis of during the rest of the lab work (see appendix 2). The tubes were put into a freeze-drying machine for 24 hours. The freeze dried samples were kept at room temperature until further processing.

2.2.2 Homogenizing the samples

The freeze dried samples were grinded into a fine powder using a mortar and returned to the same tubes. The mortar was cleaned with running hot water and sterilised using burning ethanol between each sample. The freeze dried homogenised samples were kept at room temperature until further processing.

2.2.3 Determining age of retention trees

The drill cores where magnified using a stereo microscope with 60 times magnification. Du to that the cores was drilled as close to the ground as possible no age was added to the annual rings counted, so the given age is a bit underestimated.

2.2.4 Measuring pH

The remaining amount of the humus samples from each study site was mixed together and of that 10 g of the humus samples and 20 g of each mineral sample was used to measure the pH of the soil. The samples were mixed in falcon tubes together with 20 ml of deionized water and measured using a pH meter. The pH did not differ much between the sites so this parameter was not further included in the study.

2.2.5 DNA extraction

Prior to the DNA extraction all tubes needed for the whole DNA extraction process was prepared and marked. DNA was extracted using Power Max soil DNA kit, following the manufacturer's instructions with some small adjustments; in step one, the ceramic beads in the bead tubes following the extraction kit was halved, and Buffer SL2 was chosen. In step three, samples were run in a fast-prep machine programmed on 5000x2x30. In the final step, eluting DNA, 50 µl Buffer SE was used to get a medium DNA concentration product. The DNA samples were stored in a freezer at -20°C until further processing.

2.2.6 Concentration measurements

The concentrations of the DNA samples were measured using a NanoDrop machine, following the instructions of the NanoDrop machine. The samples were thawed just prior to the measurement and put directly back in the freezer after the measurement, until further processing.

2.2.7 Sample dilution and PCR

Using the results from the NanoDrop concentration measurements, calculations were done to get 150 µl DNA solutions with a concentration of 0.5 ng/µl.
Equation used for the calculation: C1*V1=C2*V2
C1=Measured DNA concentration, V1= volume of sample needed, C2= required sample concentration (0.5 ng/µl), V2= finish volume (150 µl). The calculated volume of sample needed was rounded up to 0.5 µl

All equipment needed, premarket tubes for the dilutions, tube for mastermix, premarket PCR-stripes and ionized water was put in a PCR preparation hood and exposed to UV light for 20 min before continued work, and further process prior to PCR was performed under the hood. The calculated volume of ionized water needed for each sample was added to the premarket tubes while the samples were allowed to thaw on ice. The calculated amount of sample was added to each tube. The remaining of the samples was returned to the freezer and the diluted samples were kept on ice while the mastermix was prepared.

Prior to the sequencing, all samples were going to be poled in one sample so in this step, identification tags were too be added to all samples, to be able to separate them after sequencing. This was done by using primers pre-marked with tags. Preparation of premarket primers was done by my assistant by measuring up the amount of primers needed in marked tubes. As primers, ITS4: TCCTCCGCTTATTGATATGC and gITS7:GTGARTCATCGARTCTTTG were used. The prepared primers were stored at -20°C until processing.

The total volume required for the PCR was 50 µl; 25 µl sample, 20 µl mastermix and 5 µl tagged primers. A calculation of mastermix volume required for the amount of samples processed was done and the mastermix was mixed following the mastermix recipe. Three replicates of each sample were run in the PCR, so mastermix was prepared for 25*3 samples giving a total volume of 1 500 µl mastermix. During the mixing of the mastermix the tagged primers were allowed to thaw on ice.

20 µl mastermix, 5 µl tagged primer and 25 µl samples was added to the three sets of pre-labelled PCR strips with 25 samples (23 DNA-samples, the blank and one negative). To facilitate the transferring of the solutions a pisteur pipette was used to aliquot the mastermix and a multichannel-pipette to aliquot tagged primer and samples.

The PCR machine was programmed to run 50 µl samples, first at 95°C for 3 minutes then 25 cycles at 95°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec. After the last run, keeping 72°C for 7 min and cool down to 8°C. PCR products were stored in the freezer until further processing.

2.2.8 Gel electrophoresis

A gel tray with room for three combs with 30 pegs was chosen and the open ends were taped thoroughly so no gel could leak out. Combes were placed in the tray at appropriate places. To get the right volume of agarose gel needed for the chosen tray with a concentration of 1.2 %, 2.64 g of

agarose was mixed with 220 ml of SB buffer in a flask. The mixture was heated in a microwave oven at 700 W for 1 minute, gently shaken and run for another minute at 600 w. 4.4 µl Nancy dye was added to the hot liquid gel and the flask was carefully shaken to mix the dye. After allowing the liquid gel to cool for about 5 minutes, it was poured in the gel tray and left to solidify.

While the gel solidified the samples were taken out of the freezer and allowed to thaw. When the gel had solidified, the combs and the tape were removed. 3µl GR ladder was added to both ends of each well row. 5 µl of samples were added to the wells and the samples were put back in the freezer. The tray with the loaded gel was put in an electrophoresis box filled with SB buffer and run for 40 minutes at 230 V.

The gel was scanned using a UV light scanner with a printer. One picture of the whole gel was printed and the gel was cut between each row and a closer-up picture was printed of each row. Each picture was marked to make sure no mix-up would be done.

2.2.9 Evaluation of the electrophoresis

When the three first sets of samples had been run to this point, sample 1-23, sample 24-46 and sample 47-69, the samples were evaluated for further actions before the last set with sample 70-72 was run through the processes.

2.2.10 Evaluation of the first 69 samples

The bands created by the DNA on the gel were compared with the bands from the ladder. With three PCR products of each sample, it was considered ok if two bands from each sample indicated an acceptable DNA concentration. The empty bands or very weak band that had a low concentration of DNA after the extraction was chosen for a new DNA extraction. The empty bands that had a high concentration after the DNA extraction and the weak bands was chosen for a new PCR, running more cycles and the strong band was chosen for a new PCR with fewer cycles. Some of the weak bands were also chosen for a new PCR run with a higher DNA concentration, 2ng/µl.

2.2.11 Evaluation remaining samples and samples chosen for a second run.

The samples from the fourth run that was empty did not get a second chance and was excluded from further analysis. The samples that was run for the second time was evaluated and if they were better than the first run they were used, if not the samples from the first run was used. If both were empty, the sample was excluded. The samples that had been run with a higher DNA concentration did not respond better in the PCR so they were excluded from further analysis.

2.2.12 Final evaluation of all samples prior to AMPure purification

From the evaluation of all samples a plate plan was constructed for the samples chosen for further processing. Samples chosen are shown in the plate plan in appendix 3.

2.2.13 Sample preparation prior to purification

The samples were transferred from the PCR stipes to PCR plates according to the constructed plate plan. The plates were covered with plastic and stored in the freezer until further process. From this part of the process and until the identification of sequenced data, my assistant and a lab assistant did all work.

2.2.14 Purification and measurement

Purification was done with AMPure purification according to manual and concentration measurement was done with Qubit Concentration measurement according to manual.

2.2.15 Evaluation of purified and measured samples

After the last purification the concentration measurement showed that in addition to the samples that were excluded after PCR evaluation, one more sample was empty so the number of samples was now 69. The goal was to get a total of 6000ng DNA in the final sample sent for sequencing, giving 87 ng DNA from each sample would be sent. The calculated amount of each sample was pooled together into one sample, a final purification was done and the sample was sent to IonTorrent sequensing on SciLifeLab in Uppsala for sequencing.

2.3 Processing sequenced data

2.3.1 aning and clustering sequences

The enormous data set on more than 8 Gb, with all sequenced DNA was first processed using Scata (Scata 2014). The file with the sequences was run through a quality filter to erase sequences that were too small, of bad quality or missing primers or tags. Of the 3307678 unique sequences, 513349 passed the quality filter. Within the fungi kingdom, it is recognized that sequences within this part of the DNA which have a similarity of at least 98.5% originates from the same species (Lindahl et al 2013). Therefore, the sequences was first grouped into clusters were all sequences with a similarity of 98,5 % was grouped together. From this an excel-file was created that consisted of:

a) Cluster ID
b) cluster size
c) Reference (a suggested reference from the database UNITE (Unite 2015, a) that end up in the same cluster),
d) Sequence 1 (one representative sequence for each cluster).

2.3.2 eparating sequences

To separate all sequences into the samples they belong to and to create a file showing how many sequences of each cluster there are in each sample the program R was used (R 2015). By using R, the sequences from the humus samples and the mineral samples were also separated into different excel-files with the help of their identification tags. During this process some of the sequences had to be discriminated because some of the tags had managed to change place during the sequencing, so they had tags from one sample in one of the ends and tags from another sample in the other end. The two files, one for humus samples and one for mineral samples created here consisted of:

a) Cluster ID
b) Sample number
c) Number of sequences of each cluster in each sample.

2.3.3 eparating between phyla

Now using the file with the 1.5 clusters again, the clusters were grouped into larger clusters using Scata (Scata 2014), where all clusters with a similarity at 92 % were grouped together. This was done to separate between fungi from different phyla. From this, new excel-files were created, one with basidiomycota, one with Ascomycota and one with other fungi containing:

a) Cluster ID

b) cluster size
c) Reference
d) Sequence 1

2.3.4 dentification of clusters

The file containing fungi from the basidiomycota phyla was chosen for further processing. The clusters were now identified by running the representative sequences from each 1.5 cluster against a database of known sequences (Unite 2015, b). The criterion set for approving that a sequence belongs to a species was a score of at least 500 and an identity of more than 98.5 %. Due to that many species still are missing in the databases, some sequences couldn't be determined closer than that they belong to fungi and was excluded. Only the sequences that could be determined at least to family were included in further analysis (fungi within the same family usually have the same ecological function (symbiotic, saprotrophic, parasitic)). When all sequences that were possible to identify was identified, determining which ones belong to ectomycorrhizal fungi (EMF) was done by using the ecological catalogue of macro-fungi (Hällingbäck and Aronsson 1998).

2.3.5 emoving unrepresentative sequences

When all EMF were identified, a new excel-file from the file with humus samples was created and the cluster ID was changed to species/genera name and all clusters not belonging to EMF species were removed. All samples with a sequences size of less than five sequences were also removed. This to minimise the risk of including sequences of spores from fungi not established in the area. The total number of sequences now remaining, with only sequences belonging to EMF, sized at 31818 unique sequences. Now having a file containing:

a) species/genera
b) Sample number
c) Number of sequences (≥ 5) of each species/genera in each sample.

2.3.6 culation

From the latter file, the number of EMF species, the species composition, frequency of EMF species, EMF Sequences and EMF species abundance for each stand were calculated. To determine if there was any significant difference between the different forest structures, a two-sided t-test were done in Excel. Tests were run between the total number of species and the number of species per sample from the different forest structures. From the results, different tables and graphs showing the result was constructed.

3 Results

The total number of samples was 73; 60 humus samples and 12 mineral samples. Only humus samples are included in the following analysis of this study. One sample was lost, hence the analysis is based on 59 samples. Two samples were after two runs of PCR still empty and therefore not Sequenced, but classified as empty of fungal DNA and included in the analysis. After identification of all fungal sequences, six samples did not contain EMF sequences and were treated as lacking EMF. The clear-cut sites in both of the youngest forests only had 2 samples each with DNA from EMF (Table 2).

3.1 Number of EMF species

There was a clear difference in the number of species detected in the old growth forests (site 5 and 6) and the clear-cut areas in the managed forests (site 1-4). The old growth forests also had more species than the retention trees at the clear-cut areas, except one transect in the old growth forest. The retention trees have more species than the clear-cuts except at site 2 were its equal (Table 3, Fig. 7).

Table 2. *Number of samples with EMF, and number of EMF species found in each study site (n=5). Cut= samples taken in clear-cut areas without retention trees. Ret, Samples taken close to retention trees in the clear-cuts. Mean age of retention trees in brackets. *=n= 4.*

Study site	1	1	2	2	3	3	4*	4	5	5	6	6
Structure	Cut	Ret	Cut	Ret	Cut	Ret	Cut	Ret	Old	Old	Old	Old
Forest age	5	(98) 5	10	(160) 10	22	(148) 22	23	(135) 23	> 120	>120	>120	>120
Samples with EMF	2	5	2	4	5	5	4	4	5	5	5	5
No of EMF species	3	9	5	5	6	7	8	11	9	15	14	16

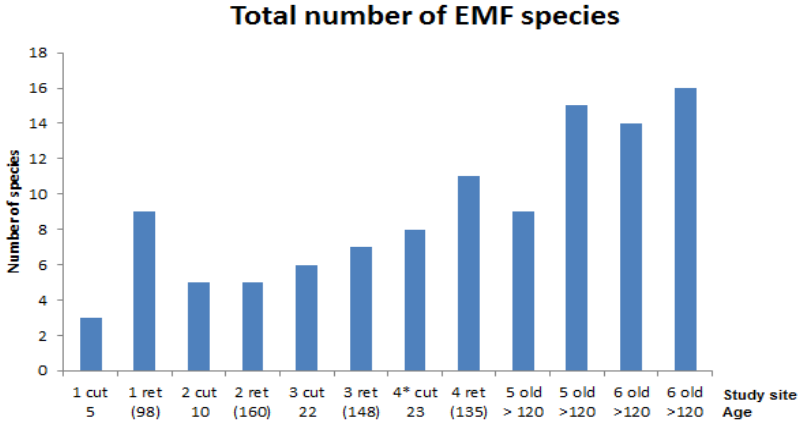


Figure 7. Total number of species found in each study site (n=5). Cut, samples taken in clear-cut areas without retention trees. Ret, Samples taken close to retention trees at clear-cuts. Mean age of retention trees in brackets. *= 4 samples (one sample lost due to error (n=4)).

3.2 Statistical analysis

T-tests shows that there is a significant difference between old growth forests and both retention trees and clear-cuts, when looking at both the total number of species per site and between the number of species per sample. Between retention trees and clear-cuts, the total number of species per site, shows no significant difference, but between the numbers of species per sample there is a significant difference, even between retention trees and clear-cuts (Table 3).

Table 3. P-values from T-tests between the different forest structures. Total number of EMF species per site and Total number of EMF species per sample. > 20 forests older than 20 years, < 10 forests younger than 10 years. < 0.05=significant difference. (per site; 4 values, per sample; 10 values).

Forest structures	Per site	Per sample
Old growth forests versus retention trees	0,025302	0,04055716
Old growth forests versus clear-cuts	0,022006	5,9857E-06
Retention trees versus clear-cuts all	0,155182	0,04460295
Retention trees versus clear-cuts < 10	—	0,05100326
Retention trees versus clear-cuts > 20	—	0,26976816
Clear-cut > 20 versus Clear-cut < 10	—	0,03344784

3.3 EMF species composition

The most common species and also found on most study sites were *Piloderma sphaerosporum*, *P. olivaceum, Suillus variegatus* and *Cortinarius semisanguineus* (Table 4). *Piloderma croceum*, was also common, but was absent in the youngest sites. *Piloderma fallax, Sarcodon glaucopus*, *Tomentellopsis submollis*, unidentified species of *Chroogomphus*, *Phellodon* and *Lactarius*, and eight *Cortinarius* species, of which four are unidentified, were only found in the old growth forests. Seven other species were found both in the old growth forest, and near retention trees, including four other *Cortinarius* species and one species belonging to the *Bankeraceae* family (Table 4).

Table 4. *EMF species found in each study site (n=5). Cut= samples taken in clear-cut areas without retention trees. Ret= samples taken close to retention trees at clear-cuts. Mean age of retention trees in brackets. *=(n=4).*

Study site	6 old	6 old	5 old	5 old	4 ret	4* cut	3 ret	3 cut	2 ret	2 cut	1 ret	1 cut
Species | Forest age	> 120	> 120	> 120	> 120	(135) 23	23	(148) 22	22	(160) 10	10	(98) 5	5
Piloderma sphaerosporum	x	x	x	x	x	x	x	x	x	x	x	
Piloderma olivaceum	x	x	x	x	x	x	x	x		x		
Piloderma croceum	x	x		x	x	x	x	x				
Suillus variegatus	x	x	x		x	x			x	x	x	
Cortinarius semisanguineus			x	x	x	x	x	x	x	x	x	x
Cortinarius obtusus	x	x	x		x							
Cortinarius acutus	x	x	x	x								
Piloderma fallax	x	x	x	x								
Cortinarius causticus/vibratilis	x	x		x	x							
Bankeraceae sp		x		x	x							
Lactarius rufus			x	x					x	x		
Rhizopogon evadens		x		x					x		x	
Cortinarius testaceofolius	x			x		x						
Cortinarius sp				x			x					
Cortinarius biformis		x			x							
Cortinarius neofurvolaesus		x			x							
Cortinarius armillatus											x	x
Cortinarius sp	x											
Sarcodon glaucopus	x											
Chroogomphus sp	x	x										
Cortinarius sp		x		x								
Phellodon sp	x											
Tomentellopsis submollis		x										
Cortinarius sp	x	x										
Lactarius sp			x									
Cortinarius ochrophyllus				x								
Cortinarius vibratilis				x								
Piloderma sp					x							
Cortinarius umbrinolens						x						
Piloderma lanatum						x						
Suillus luteus							x					
Tricholoma stans							x					
Cortinarius sp								x				
Laccaria (laccata/bicolor)								x				
Piloderma byssinum											x	
Suillus bovinus											x	
Cortinarius suberi												x
Tylospora sp											x	
Tylospora fibrillosa											x	
Tot no of species	14	16	9	15	11	8	7	6	5	5	9	3

3.4 Frequency of EMF species

The most frequent species was, *P. sphaerosporum*, detected in 75 % of the samples. The second most common species, *P. olivaceum* occured in 29 % of the samples, while the majority of species was only found in a few samples (Fig 8. Table 5).

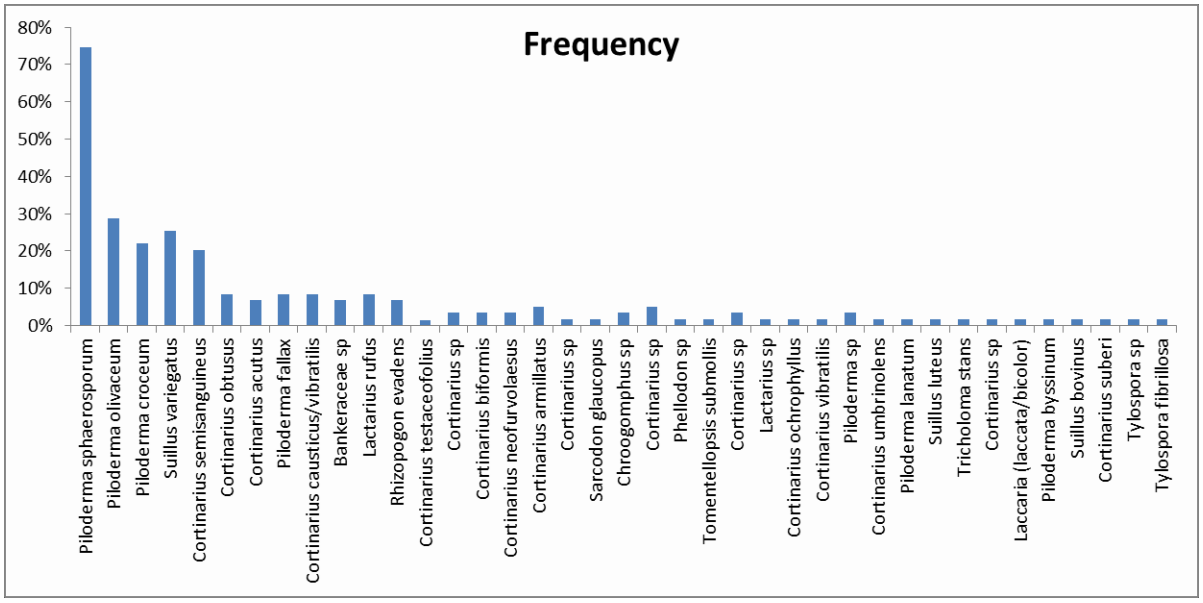


Figure 8. The combined frequency of all detected EMF species in all stands and samples (n=59).

Table 5. *The frequency of species found in each study site (n=5). Cut= samples taken in clear-cut areas without retention trees. Ret= samples taken close to retention trees at clear-cuts. Mean age of retention trees in brackets. *= n=4.*

Study site	6 old	6 old	5 old	5 old	4 ret	4* cut	3 ret	3 cut	2 ret	2 cut	1 ret	1 cut
Species | Forest age	> 120	> 120	> 120	> 120	(135) 23	23	(148) 22	22	(160) 10	10	(98) 5	5
Piloderma sphaerosporum	0,8	0,8	1	1	0,8	1	0,8	0,8	0,8	0,2	0,8	
Piloderma olivaceum	0,8	0,2	0,4	0,6	0,6	0,25	0,2	0,2		0,2		
Piloderma croceum	0,2	0,2		0,2	0,6	0,75	0,6	0,2				
Suillus variegatus	0,6	0,6	0,6		0,2	0,25			0,2	0,4	0,2	
Cortinarius semisanguineus			0,4	0,2	0,2	0,25	0,2	0,4	0,2	0,2	0,2	0,2
Cortinarius obtusus	0,4	0,2	0,2	0	0,2							
Cortinarius acutus	0,2	0,2	0,2	0,2								
Piloderma fallax	0,2	0,2	0,4	0,2								
Cortinarius causticus/vibratilis	0,2	0,2		0,2	0,4							
Bankeraceae sp		0,2		0,2	0,4							
Lactarius rufus			0,2	0,2					0,4	0,2		
Rhizopogon evadens		0,2		0,2					0,2		0,2	
Cortinarius testaceofolius	1			0,4		0,25	0	0				
Cortinarius sp				0,2		0	0,2	0				
Cortinarius biformis		0,2			0,2							
Cortinarius neofurvolaesus		0,2			0,2							
Cortinarius armillatus											0,4	0,2
Cortinarius sp	0,2											
Sarcodon glaucopus	0,2											
Chroogomphus sp	0,2	0,2										
Cortinarius sp		0,4		0,2								
Phellodon sp	0,2											
Tomentellopsis submollis		0,2										
Cortinarius sp	0,2	0,2										
Lactarius sp			0,2									
Cortinarius ochrophyllus				0,2								
Cortinarius vibratilis				0,2								
Piloderma sp					0,4							
Cortinarius umbrinolens						0,25						
Piloderma lanatum						0,25						
Suillus luteus							0,2					
Tricholoma stans							0,2					
Cortinarius sp								0,2				
Laccaria (laccata/bicolor)								0,2				
Piloderma byssinum											0,2	
Suillus bovinus											0,2	
Cortinarius suberi												0,2
Tylospora sp											0,2	
Tylospora fibrillosa											0,2	

3.5 EMF Sequences

The total number of EMF sequences recorded at the different study sites did not mirror the species number. The clear-cut parts in the youngest forests (site 1 and 2) had 122 and 414 sequences respectively, while the number of detected sequences with retention trees in the youngest forest (site 1) had the largest number with 5414. The second largest number was recorded in the clear-cut area in the 22 year old forest (site 3). The clear-cut area in the 23 year old forest (site 4) had a lower total amount, but as this site only had four samples, a likely comparable estimate would probably be about 600 higher, i.e. about 4000 (Table 6 and fig. 9).

Table 6. *Total number of sequences in each study site (n=5). Cut= samples taken in clear-cut areas without retention trees. Ret= Samples taken close to retention trees at clear-cuts. Mean age of retention trees in brackets. *= n=4.*

Study site	1	1	2	2	3	3	4*	4	5	5	6	6
Structure	Cut	Ret	Cut	Ret	Cut	Ret	Cut	Ret	Old	Old	Old	Old
Forest age	5	(98) 5	10	(160) 10	22	(148) 22	23	(135) 23	>120	>120	>120	>120
Tot no of sequences	122	5414	414	2828	4349	1750	3422	2144	1562	3599	3335	2879

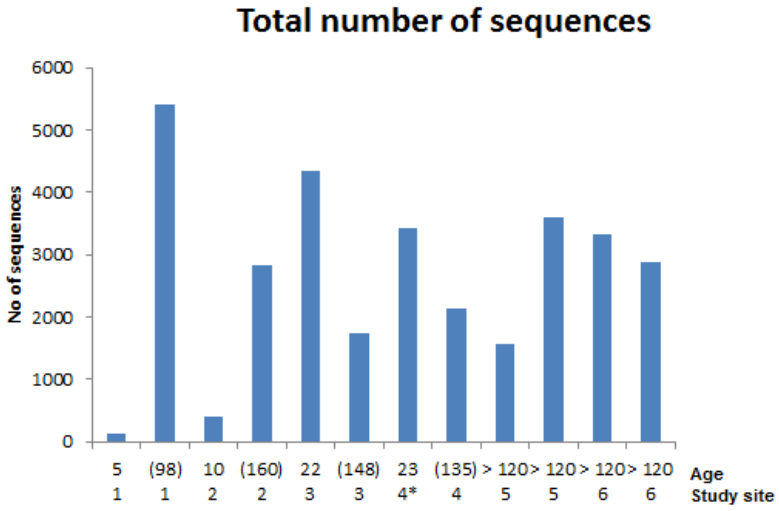


Figure 9. Total number of sequences at each study site (n=5). Age = mean age of the stand. Age of retention trees in brackets. *=n=4.

Piloderma sphaerosporum accounted for most of the sequences in the managed forests; near the retention trees in the youngest forest with 4135 sequences (site1), in the clear-cut surface in the 22 year old forest (site 3) with 1417 sequences together with *Piloderma oliviacus* with 2205 sequences (Table7). The 5 most common species accounted for the largest number of sequences in all sites, except in old-growth forests (site 5 and 6) where the sequences are more spread out between more species.

Table 7. *The specific number of sequences of each species found in each study site (n=5). Cut; samples taken in clear-cut areas without retention trees. Ret; Samples taken close to retention trees at clear-cuts. Forest age = mean age of stand. Age of retention trees in brackets. *= n=4.*

Study site	6 old	6 old	5 old	5 old	4 ret	4* cut	3 ret	3 cut	2 ret	2 cut	1 ret	1 cut
Species | Forest age	> 120	> 120	> 120	> 120	(135) 23	23	(148) 22	22	(160) 10	10	(98) 5	5
Pilodermasphaerosporum	181	332	533	990	1087	1003	1228	1417	2543	312	4135	
Pilodermaolivaceum	813	1465	50	604	511	122	17	2205		34		
Pilodermacroceum	8	13		29	251	1884	354	84				
Suillusvariegatus	735	786	195		10	14			6	53	364	
Cortinariussemisanguineus			173	681	50	40	28	596	73	10	5	17
Cortinariusobtusus	804	6	16		10							
Cortinariusacutus	19	5	84	570								
Pilodermafallax	159	63	497	21								
Cortinariuscausticus/vibratilis	10	7		397	29							
Bankeraceaesp		13		12	12							
Lactariusrufus			5	56					200	5		
Rhizopogonevadens		36		7					6		35	
Cortinariustestaceofolius	6			30		5						
Cortinariussp				161			12					
Cortinariusbiformis		12			43							
Cortinariusneofurvolaesus		26			25							
Cortinariusarmillatus											62	5
Cortinariussp	111											
Sarcodonglaucopus	5											
Chroogomphussp	48	14										
Cortinariussp		79		14								
Phellodonsp	429											
Tomentellopsissubmollis		15										
Cortinariussp	7	7										
Lactariussp			9									
Cortinariusochrophyllus				17								
Cortinariusvibratilis				10								
Pilodermasp					116							
Cortinariusumbrinolens						348						
Pilodermalanatum						6						
Suillusluteus							106					
Tricholomastans							5					
Cortinariussp								28				
Laccaria(laccata/bicolor)								19				
Pilodermabyssinum											419	
Suillusbovinus											317	
Cortinariussuberi												100
Tylosporasp											56	
Tylosporafibrillosa											21	
Tot no of sequences	3335	2879	1562	3599	2144	3422	1750	4349	2828	414	5414	122

3.6 EMF species abundance

Piloderma sphaerosporum had the highest relative abundance, in total representing more than 40 % of the number of EMF sequences. The second most abundant was *S. variegatus*, representing 11 % of the total number of EMF sequences. Most species, however, consisted of a very small proportion of the sequences in only a few samples and has thus a very low abundance (Fig. 10).

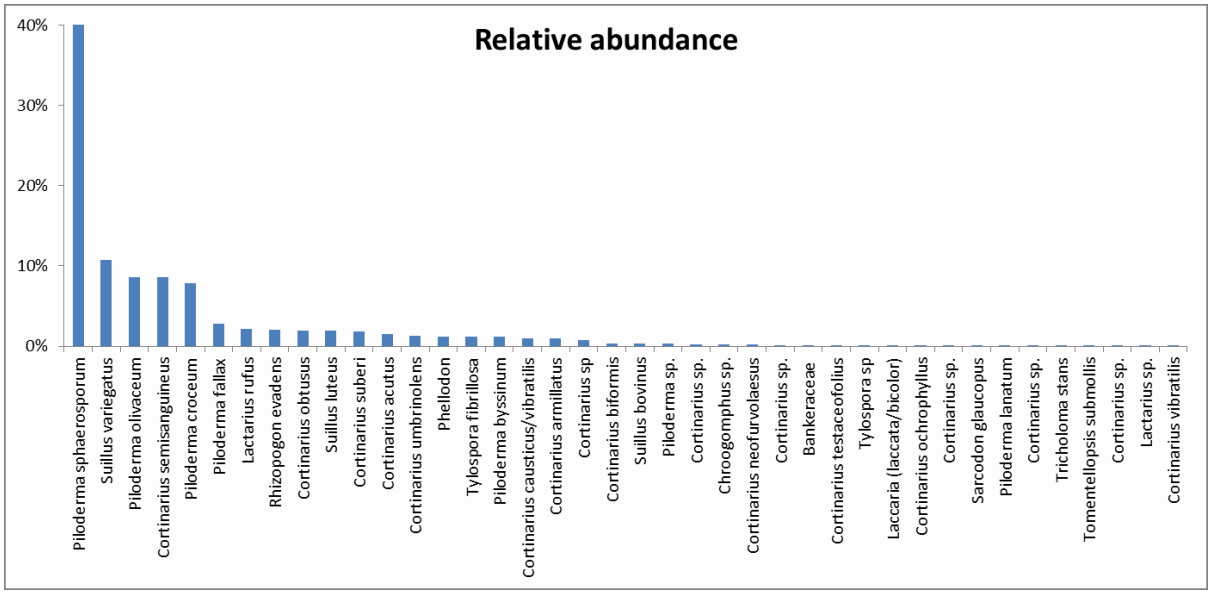


Figure 10. Average relative abundance of each species in all samples combined (n=59).

4 Discussions

This study shows that forest harvesting has significant effects on the species richness and composition of EMF. In the old growth forests, significantly more EMF species were detected than both in the clear-cuts and close to retention trees. The retention trees supported mycelia of some species through the clear-cut phase, and shows an intermediate result. Retention trees have a significant higher number of EMF species per sample number compared to clear-cuts while the total number of detected species did not differ significantly. In the youngest forests, only few EMF species were detected at the clear-cut areas. The clear-cut areas in the forests older than 20 years had, as expected significantly higher number if EMF species established.

Even though there is a significant difference between the number of species found in the old growth forest and by retention trees, one site with retention trees had more species than one transect from the old growth forest with least number of species. In this retention site on the other hand, some of the samples were taken in a slope that had a different vegetation cover than the rest of the sites, with more herbs, and a soil that was undisturbed by forest machines (see appendix 1), which could explain the higher number of species. The retention site in the youngest forest, surprisingly also reached the same number of species as this transect, but four species was only detected in this site; *Suillus bovinus, C. suberi, Tylospora fibrillosa* and one unidentified *Tylospora,* all common species (*S. bovinus;* Dahlberg and Stenlid 1994, *C. suberi;* Soop 2002, *T. fibrillose;* Erland 1995). Due to the dry summer and that this site lay in a wetter area close to a mire (see appendix 1), may explain that species that were not detected at the other sites, occurred in this site. It is also the case that there is a spatial variation in all forests in terms of occurrence of species (which species, species numbers and number of sequences). With few samples - 5 in this case, the chance may also play a role.

The same trend is shown in the clear-cut areas in the youngest forests with few sequences, while in fact the highest number of sequences was recorded near the retention trees in the youngest forest. Interestingly, almost all sequences belong to *P. sphaerosporum,* the most abundant species in this study. The second highest number of sequences was recorded in the clear-cut in one of the forests older than 20 year. Generally, the number of sequences appears to be as high or higher in these forests than in the old growth forest. However, the number of sequences may not well reflect the biomass or the activity of the fungi. On the other hand, as shown in a study done by Wallander et al. (2010) where they both report the number of species and the biomass in forests of different ages, they, showed the highest species number in the older forests but the highest biomass in 10-30 years old forests.

Several species were only found in the old growth forests. According to the Swedish Red List, many EMF species are negatively affected by forestry (Sandström *et al*. 2015). It is important to recognise these species and figure out how to support their existence in the managed forests. Unique for the old growth forests in this study was the red-listed species *Sarcodon glaucopus* (Vulnerable, VU) and one unidentified species of *Phellodon,* which belongs to a genera with several species on the red-list. *Piloderma fallax,* which also occurred only in the old-growth forest, is a species that in earlier studies have shown to be more common in old growth forests (Smith *et al*. 2000). Many species of *Cortinarius,* a genus with more than 100 species on the Swedish Red List, was also only detected in the old growth forests. It is however difficult to make any statistical conclusions with so few observations, but the repeated finding of unique species with few observations in older forest may be indicative.

The most abundant species was detected in all sites. The less frequent species are difficult to say much about due to the few findings, other than that more species were detected in older forests and that the different sites didn't quite mirror each other, neither when it comes to what kind of species, nor the number of EMF species or the number of EMF sequences found. The most common and most abundant EMF in this study, *P. sphaerosporum* is the most frequent and abundant species found when doing DNA analysis on samples from root tips and mycelia in pine forests in the middle and northern parts of Sweden (Personal comment, Dahlberg 2015). This species however had a much higher number of sequences in the younger forests than in the old growth forests, so it seems to be favoured by disturbance, but knowledge about its ecology appear to be inadequate. According to my literature searches, no scientific studies of its biology and ecology are yet published about this species.

As can be seen in the results of this study, some species appeared to survive associated to retention trees, although I cannot exclude the possibility of re-establishment from spores. This indicates that the retention trees can be an important measure for supporting the survival of mycelia of some species. This is also consistent with previous studies on retention trees importance for EMF (e.g. Kranabetter *et al*. 2013, Luoma *et al*. 2004). It was however only a few EMF species in this study that was unique for both old growth forests and retention trees and few EMF species was common within the sites between the retention trees and the clear-cuts, that otherwise would have indicated that retention trees functioned as a source for EMF to establish in the new generated forest. Most of the EMF in the clear-cuts was only found on the clear-cut, indicating that they may have established by spores.

This is a small study, based on what is possible to perform within the framework of a master's thesis. It requires a larger and more extensive study to obtain more data that would enable statistical conclusions about the abundances of the species. In particular, a large number of samples are needed to make statement of rare species. However, these results indicate the impact logging with and without retention trees have on EMF. Considering that only five samples were taken at each site one cannot expect to get a picture, more than of the most common species. Undoubtedly there are many more species in all the forests studied.

A common mushroom collector would probably wonder why many of the more commonly seen/recorded mushrooms in the boreal forest, including *Boletus pinophilus, Amanita muscaria, Leccinum vulpinum, Cortinarius mucosus* were not detected in this study. One reason may be that it's probably the same with the mycelium as with the fungal sporocarps, that they occur more widespread at different time of the year and maybe even between years and sometime with many years between. In this study all samples were taken in the middle of July and it had been a very dry and hot summer so only the species thriving in this condition would be expected to be found.

Several species was not possible to identify down to species level with the sequences and the sequence reference databases available. Even though there sometimes were numerous of sequences in the data bases that correspond to 100 %, indicating that it is not uncommon species, it was unidentified sequences with the origin from soil samples. Several of the identified species were *Piloderma,* that all are EMF fungi forming their thin and often overlooked sporocarps appearing as crust underneath decomposing logs, stumps, or buried wood. For several of these, identification could only be done to the level of the genera. Also several *Cortinarius* species were not possible to identify. *Cortinarius* is a genus generally producing large fruiting bodies ´why it is obviously not only "hard to detect" fungi that is lacking in the databases. Considering that most of the *Cortinarius* species found in this study only was detected in the old growth forests and that more than 100 species of *Cortinarius* is listed on the Swedish red-list, it wold be of great importance to identify them.

Many species may have a long resilience time, meaning that although they still are alive, they have gone under the threshold for what they need to be able to reproduce and survive and they may go locally extinct, eventually regionally extinct and ultimately nationally extinct (extinction debt). Even though a species still is thriving in one part of the country or in other countries, the genetic composition of the species, unique for the particular geographical range may be lost. Nature conservation does not only consider conservation at the species level, but also the genetic variation within a species. Between different populations of the same species it may also be important difference in the genetic composition. This genetic diversity may be critical for species to be able to handle the ongoing and expected continued climate change. When it comes to EMF there is not only the genetic diversity within one species that has adapted to the environment, but through the symbiosis, the trees and the EMF has adapted together to both the environment and each other.

5 Conclusions

Clearcutting both with and without retention trees has significant effects on the species richness and species composition of EMF. Retention trees appear to support some EMF species, but there is still a significant difference between the number of EMF species detected in the old growth forest and by retention trees. The red-listed species found in this study were only found in the old growth forests. This may indicate that retention trees may not be able to support all EMF species. As there were no significant difference between the total number of EMF species between the retention trees and the clear-cuts, it is not possible based on this study to conclude that that retention trees has a significant influence.

A reflection from my side is that today's forestry will affect the community of EMF fungi with large/significant ecological importance so that some species may decline and some also may become locally and potentially also nationally extinct. Different EMF has different ecological functions and probably benefit trees in different ways. As this study shows, EMF is affected by forestry and it is important to evaluate both how different species are affected and their different functions so that forest can be managed to provide the diversity and functions of EMF the society wants to have. Management can mean anything from area protection to forest managed for pulp and timber. This may be a pressing issue as almost all Swedish forests are strongly affected by forestry. More than 60 % of all forest area in Sweden has been clear-cut and most of the remaining subjected to selective cutting and extensively thinned. Not only for the preservation of biodiversity for preservation's sake, but also to ensure that forestry, is conducted in a sustainable way so that future generations can continue to conduct this economically-important industry (Thoren 2013).

6 Acknowledgments

First of all I would like to express my sincere thanks to Anders Dahlberg and Kerstin Varenius. Anders for, in the first place taking me on board and letting me conduct this study and for all the help and support he has been giving me during my work. Kerstin for all help she's been giving me all the way from the start until the end, first picking out the study sites, help in the field, help in the lab, doing the final lab work, processing data, guiding me with the calculations and supporting me in my writing. I would also like to thank all the other people in the lab of the Department of Forest Mycology and Plant Pathology who helped and supported me in the lab.

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Appendix 1

Study site 1

Here the stand was located close to a mire and in parts of the stand the ground was quite peaty. The vegetation differed a bit from the other stands with some ericaceous shrubs and sphagnum mosses in patches. The clear-cut samples was taken in the part of the stand were the vegetation was more similar to the other stands, with lingonberrys (*Vaccinium vitis-idaea*), heather (*Calluna vulgaris*), lichen and mosses. Unfortunately the retention trees were sparse and scattered throughout the stand so here there was not much else to choose from than the few trees that were available, meaning that the soil and the vegetation looked a bit different where these samples were taken.

Study site 2

This stand was large. It had quite a lot of big boulders and the rest of the ground was stony and sandy with almost no vegetation at all, only sparse with lingonberrys (*Vaccinium vitis-idaea*), heather (*Calluna vulgaris*), lichen and mosses sparsely scattered. The retention measurements taken when harvested are some small patches of trees and few solitary trees. The closest retention trees that met the requirements were chosen and the to find a place suitable for clear-cut samples the stand was surveyed and the the less stony part was chosen for sampling (see fig. 5)

Study site 3.

In this stand the area that filled the requirements was small and only five retention trees fulfilled the criteria so no randomisation was needed. A large enough space to fit one transect for five samples was not possible so three samples were taken on one transect and two on another (See fig. 3). The ground was stony and the vegetation was sparse with only lingonberrys (Vaccinium vitis-idaea), heather (*Calluna vulgaris*), some lichen and mosses. The regenerating forest was severely browsed, but the trees that had managed to escape browsing had the approximately age determined by Sveaskog.

Study site 4.

The stand is lying on a hill with a steep slope in the southeast towards a stream. In the slope the vegetation cover was more heterogeneous with more herbs and shrubs, and soil undisturbed by forest machines. In this stand the part that best met the requirements lied on top of the hill, but here the retention trees were few so it was impossible to find five retention trees fulfilling the requirements. The best trees possible was chosen meaning that some of the trees chosen stood in the slope where the vegetation differed some from the rest of the stand where the clear-cut samples was taken (see fig 3), that had a sparse vegetation cover with only lingonberrys (*Vaccinium vitis-idaea*), heather (*Calluna vulgaris*), some lichen and mosses.

Study site 5.

This old growth forest is situated on top of a ridge. No traces of forestry were noted. Most of the stand included rocky ground so the only part where sampling was possible was on a narrow part stretching in north-south direction on the slope on the east part of the ridge bordering a clear-cut. The ground was very stony making it difficult to take samples on a straight transect (See fig. 5). The vegetation

cover consisted of mosses, bilberries (*Vaccinium myrtillus*) lingonberries (*Vaccinium vitis-idaea*) and mosses.

Study site 6.

This stand was also classified as an old growth forest, but here traces after thinning was noted. The ground was quite stony in the entire stand and the vegetation consisted mostly of lingonberries (*Vaccinium vitis-idaea*) but also some bilberries (*Vaccinium myrtillus*), mosses and lichens. The placement of the first transect with five sample points was chosen by a random starting-point in the south west corner and laid in a north-east direction. The second transect for the remaining five samples was randomly chosen with a starting point about 30 meters north of the first transects endpoint and stretched in a south-west direction (See fig. 5).

Appendix 2.

Sample number, randomized lab number and identification tag number of each sample.

Sample no	Lab no	Tag	Sample no	Lab no	Tag
1	22	25	42	26	30
2	53	58	43	41	46
3	15	17	44	59	67
4	12	14	45	33	37
5	61	69	46	56	63
6	16	18	47	49	54
7	55	61	48	35	39
8	14	16	49	42	47
9	66	74	50	45	50
10	8	10	51	71	79
11	13	15	52	31	35
12	10	12	53	51	56
13	4	6	54	32	36
14	9	11	55	50	55
15	1	1	56	29	33
16	6	8	57	72	80
17	21	24	58	37	41
18	2	4	59	69	77
19	20	23	60	47	52
20	24	28	Mineral samples		
21	23	26	1.5	11	13
22	64	72	11.15	60	68
23	62	70	16.20	19	22
24	17	20	21.25	43	48
25	5	7	26.30	58	66
26	18	21	31.35	65	73
27	3	5	36.40	63	71
28	7	9	41.45	67	75
29	52	57	46.50	57	65
30	30	34	51.55	36	40
31	28	32	56.60	54	60
32	44	49	6.10	70	78
33	48	53	Negatives and blanks		
34	38	42	V1		100
35	25	29	V2		101
36	40	45	V3		104
37	68	76	V4		105
38	27	31	N1		106
39	39	44	N2		107
40	46	51	N3		108
41	34	38	N4		109

Appendix 3

Plate 1: Samples 3-31 first extraction, first PCR, 25 cycles. Plate 2: Samples 32-62, first extraction, first PCR, 25 cycles. Plate 3: Samples 63-69 first extraction, first PCR, 25 cycles, PCR-negative (N) and extraction-negative (V) Plate 4: Samples 1-53 new PCR running 25 cycles, 36-65 new extraction, 70-72 first extraction, 22-50 new PCR running 22 cycles. Plate 5: Backup samples: 16 new PCR running 22 cykler, 22-50 first PCR running 25 cycles.

Plate 1	H	G	F	E	D	C	B	A
1	x	x	3	4	5	6	7	8
2	x	x	3	4	5	6	7	8
3	x	x	3	4	5	6	7	8
4	9	10	11	12	13	14	15	16
5	9	10	11	12	13	14	15	16
6	9	10	11	12	13	14	15	16
7	17	18	x	20	x	x	23	x
8	17	18	x	20	x	x	23	x
9	x	x	x	x	x	x	x	x
10	x	x	26	x	x	x	x	31
11	x	x	26	27	x	x	x	31
12	x	x	26	27	x	x	x	31

Plate 2	H	G	F	E	D	C	B	A
1	32	33	34	35	x	37	38	x
2	32	33	34	35	x	37	38	x
3	32	33	34	35	x	37	38	x
4	40	x	42	x	x	45	x	x
5	40	x	42	x	x	45	x	x
6	40	x	42	x	x	45	x	x
7	x	x	x	x	51	52	x	x
8	x	x	x	x	51	52	x	x
9	x	x	x	x	51	52	x	x
10	55	56	x	58	59	60	61	62
11	55	56	x	58	59	60	61	62
12	55	56	x	58	59	60	61	62

Plate 3	H	G	F	E	D	C	B	A
1	63	64	x	66	67	x	69	x
2	63	64	x	66	67	x	69	x
3	63	64	x	66	67	x	x	x
4	N1	N2	N3	N4	N5	x	x	x
5	N1	N2	N3	N4	N5	x	x	x
6	N1	N2	N3	x	N5	x	x	x
7	v1	v2	v3	v4	x	x	x	x
8	v1	v2	v3	v4	x	x	x	x
9	x	v2	v3	x	x	x	x	x
10								
11								
12								

Plate 4	H	G	F	E	D	C	B	A
	1	2	19	21	24	25	28	29
	1	2	19	21	24	25	28	29
	1	2	19	21	24	25	28	29
	30	43	44	53	x	36	39	54
	30	43	44	53	x	36	39	54
	30	43	44	53	x	36	39	54
	57	65	x	71	72	x	x	x
	57	65	70	71	72	x	x	x
	57	65	70	71	x	x	x	x
10	x	22	41	47	48	49	50	x
11	x	22	41	47	48	49	50	x
12	x	22	41	47	48	49	50	x

Plate 5	H	G	F	E	D	C	B	A
1								
2								
3								
4	16	22	41	47	48	49	50	x
5	16	22	41	47	48	49	50	x
6	16	x	41	47	48	49	50	x
7								
8								
9								
10								
11								
12								