Retention of seed trees fails to lifeboat ectomycorrhizal fungal diversity in harvested Scots pine forests

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Abstract

Fennoscandian forestry has in the past decades changed from natural regeneration of forests towards replantation of clear-cuts, which negatively impacts ectomycorrhizal fungal (EMF) diversity. Retention of trees during harvesting enables EMF survival, and we therefore expected EMF communities to be more similar to those in old natural stands after forest regeneration using seed trees compared to full clear-cutting and replanting. We sequenced fungal ITS2 amplicons to assess EMF communities in 10-60 year-old Scots pine stands regenerated either using seed trees or through replanting of clear-cuts with old natural stands as reference. We also investigated local EMF communities around retained old trees. We found that retention of seed trees failed to mitigate the impact of harvesting on EMF community composition and diversity. With increasing stand age EMF communities became increasingly similar to those in old natural stands and permanently retained trees maintained EMF locally. From our observations we conclude that EMF communities, at least common species, post-harvest are more influenced by environmental filtering, resulting from environmental changes induced by harvest, than by the continuity of trees. These results suggest that retention of intact forest patches is a more efficient way to conserve EMF diversity than retaining dispersed single trees.

Keywords: clear-cutting, seed trees, ectomycorrhizal fungi, high-throughput sequencing, retention trees, *Pinus sylvestris*

Introduction

Disturbances, stress and competition between species are all selective pressures that shape biological communities. Human-induced disturbances often differ from the ones under which the ecosystems evolved (Steffen *et al.* 2015), which is why they frequently have particularly large impacts on biological communities (Shade *et al.* 2012). Boreal forests have a history of frequent fires (c.f. Zackrisson 1977), although in northern Europe, the forest fires have mainly been of low-severity with surviving trees and only rarely resulting in complete stand-replacement (Kuuluvainen & Aakala 2011). Biological communities in these forests may therefore have low resistance to stand-replacing disturbances.

Industrialized clear-cutting was introduced in Fennoscandian forestry in the beginning of the 20th century and became common in the 1950s (Framstad *et al*. 2013; Lundmark, Josefsson & Östlund 2013). In Sweden, clear-cutting and planting has gradually replaced natural regeneration and is today the major forest regeneration method (Skogsstyrelsen 2016). Since stand-replacing disturbances have been historically rare in Fennoscandian forests, clear-cutting only partly resembles natural disturbances. It is one of the main reasons that almost 5000 forest-dwelling species are nationally red-listed in Fennoscandia (Rassi *et al*. 2010; ArtDatabanken 2015; Henriksen and Hilmo 2015).

Ectomycorrhizal fungi (EMF) form symbiotic relationships with trees. When trees are harvested, the host supply of sugars ceases, leading to an immediate strong reduction in EMF species richness and a shift in EMF community composition (Högberg *et al*. 2001; Jones, Durall & Cairney 2003; Twieg *et al.* 2007; Wallander *et al.* 2010; Hartmann *et al*. 2012). However, it remains uncertain whether there are lasting impacts on EMF diversity throughout the length of a forest rotation, or if EMF

communities recover during stand development and become similar to those of an unharvested forest. Previous studies have indicated that relative abundances of EMF species may change during the development of regenerated stands, and that some genera, such as *Tylospora* and *Thelephora*, can be more common in young stands while other genera, like for example *Cortinarius* and *Russula,* increase in abundance with stand age (Twieg *et al.* 2007; Wallander *et al.* 2010; Kyaschenko *et al*. 2017).

It seems that the post-harvest recovery of EMF communities towards a composition similar to unharvested stands takes several decades. Visser (1995) observed community stabilisation 41 years after stand-replacing wildfire. Varenius *et al.* (2016) observed an altered composition of EMF frequencies still 50 years after harvest, and Twieg *et al.* (2007) and Kyaschenko *et al.* (2017) found that communities tended to reach a composition similar to unharvested stands 60 years after clear-cutting. However, even though diversity and relative abundances of species differ, the set of common EMF species seems to be largely the same in harvested and unharvested stands (Byrd *et al*. 2000; Varenius *et al.* 2016). This indicates that the EMF community develops over time primarily through accumulation of species rather than species replacement, as suggested by Bradbury *et al.* (1998) and Kranabetter *et al*. (2005).

Individual EMF genets can extend over several square metres and become several decades or even centuries old (Bonello P., Bruns T. D., Gardes M. 1998; Dahlberg & Mueller 2011; Douhan *et al*. 2011), and sugars from connecting host roots can be distributed throughout large parts of the mycelial individual (Finlay and Read, 1986). Thus, trees retained through the clear-cut phase may act as a ‘lifeboat’ for EMF mycelia (Amaranthus & Perry 1987; Luoma *et al.* 2006; Rosenvald & Lõhmus 2008), enabling long-term local survival of EMF. The Swedish forestry

practice of “seed tree regeneration”, in which 50–150 trees per hectare are temporarily retained for ten years (Karlsson & Örlander 2004), should therefore have a major potential to enable survival of EMF, and maintenance of diversity. Previous observations that EMF communities on seedlings that were seeded or planted close to mature trees largely mirroring those of the mature trees support this idea (Jonsson *et al.* 1999; Cline *et al.* 2005; Heinonsalo *et al.* 2007). Seedlings planted in clear-cut areas originally harbour EMF species from the nursery (Leski *et al.* 2010; Menkis *et al.* 2016), which are later replaced, primarily by spore inflow from close-by surrounding forests (Dahlberg & Stenström 1991; Peay *et al*. 2012) and potentially, by a few species surviving the harvest as a dormant spore bank (Bruns *et al*. 2009; Nguyen, Hynson & Bruns 2012; Glassman *et al*. 2015).

Permanently retained trees may lifeboat EMF through the clear-cut phase, although only within about 10 m from the tree (Cline *et al.* 2005; Luoma *et al.* 2006; Jones *et al.* 2008). In Swedish forestry the practice of permanently retaining some trees at harvest was introduced in the 1990s with the aim to mitigate negative effects of clear-cutting on biodiversity (Fedrowitz *et al*. 2014). Today, single trees or groups of trees corresponding to on average 8% of the basal forest area are permanently retained in Sweden (Skogsstyrelsen 2015). However, assuming a ten-meter radius of life-boated EMF around the few permanently retained trees, it seems unlikely that those trees have an influence on the overall EMF community in the entire stand.

In this study we investigated the importance of seed tree regeneration for the composition of EMF communities during 60 years following harvest of Scots pine stands. In addition, we evaluated the local effect of permanently retained trees. We focused on the overall EMF community composition and abundances of dominant EMF species, since they are probably most important for major ecosystem functions.

We hypothesized (1) that EMF communities are less impacted by regeneration using seed trees than by clear-cutting and replanting, (2) that EMF communities in stands regenerated using seed trees return to a composition similar to communities in old natural stands faster than communities in stands that were replanted after clear-cutting, and (3) that local EMF communities close to permanent retention trees mirror communities of old natural stands but differ from those in surrounding harvested areas.

2. Materials and Methods

2.1 Experimental design

The study was repeated in three regions within the boreal zone of Sweden: Dalarna, Jämtland and Norrbotten located at increasing latitude, and differing in climate (Fig. 1). In each region we selected five *Pinus sylvestris* (Scots pine) forest stands that had been replanted after clear-cutting, five stands that had been regenerated using seed trees and three old natural stands, totalling 39 stands. The regenerated stands had been harvested between 1960 and 2000 and were evenly distributed across stand ages (Table 1). The old natural stands (with 100–200-year-old trees) had a structure and dynamic that had not been significantly affected by humans, as defined by Rouvinen and Kouki (2008). In ten of the younger stands (4–26-year-old); (two stands in Dalarna, four in Jämtland and four in Norrbotten), occasional retention trees were permanently left during harvest. These trees were used to compare EMF communities close to retention trees with those of the surrounding harvested areas.

2.2 Selection of stands

The stands were selected using information in stand databases provided by the principal forest owner in each region: Bergvik skog AB in Dalarna, SCA in Jämtland and Sveaskog in Norrbotten. Stands were selected based on the following criteria: (i) *P. sylvestris* constituted at least 70% of the basal area of the trees in the stand and (ii) the stand was located in a mesic environment with ground vegetation dominated by *Vaccinium vitis-idaea L.* (Table S1). The selected stands were located from about 1 km apart to up to 20 km apart in Norrbotten and from about 1 km apart to up to 100 km apart in the other regions. Regeneration methods (clear-cut or seed trees) were verified using detailed aerial photographs (scale 1:30.000; provided by Metria, state-owned GIS company) of the stands taken 5–10 years before and after the reported years of harvest. The selected stands ranged from 1.6 to 56.6 ha in size.

2.3 Sample collection

Ten soil cores (3 cm in diameter, 10 cm in depth) were collected from each forest stand during July and August 2014. Sampling was performed at least 25 m from the stand edge along two transects located 25 m from each other. Five samples were collected along each transect, with 10 m between each of the sampling points (Fig. 1). Soil cores were divided into organic and mineral soil: the organic samples were kept separate since organic soil is known to harbour the majority of EMF (Lindahl *et al.* 2007) whereas the mineral samples were pooled within transects. In stands with permanent retention trees (ten stands), three to five trees that were at least 25 m apart from each other were selected, and additional soil samples were collected 1 m north, east, south and west of each retention tree and pooled into one sample per tree. All samples were frozen at –18°C on day of collection.

2.4 Sample preparation

Sample preparation followed the procedure described by Clemmensen *et al.* (2016). Samples were freeze-dried and all material was then homogenized by grinding in a mortar. DNA was extracted from approximately 400 mg of material using the NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany) and DNA concentrations were checked using Nanodrop (Thermo Fisher Scientific Inc., Wilmington, Delaware, USA). Milli-Q water was extracted as a negative control. The internal transcribed spacer 2 (ITS2) region was PCR amplified with samples diluted to 0.25 ng/µl in a 50-µl reaction volume (3 min at 95°C; 25 cycles of 30 s at 95°C, 30 s at 56°C and 30 s at 72°C; 7 min at 72°C) using the fungal-specific primer combination gITS7-ITS4/ITS4arch (Ihrmark *et al*. 2012; Kyaschenko *et* al. 2017) with both primers fitted with sample-specific tags of eight bases (with minimum three bases difference between tags) designed using BARCRAWL (Frank 2009). Three PCR reactions for each sample were pooled. Milli-Q water was used for negative PCR controls. Amplification success was visually checked based on band intensity after gel electrophoresis. Bands assessed as too weak (not visible) or too strong (stronger than the ladder) were subjected to a new round of PCR with increased (at most 35) or decreased (at least 22) numbers of cycles (Lindahl *et al.* 2013). PCR products were cleaned using the AMPure kit (Beckman Coulter Inc., Brea, California, USA), and DNA concentrations were established using a Qubit fluorometer (Life Technologies, Carlsbad, California, USA). Equal amounts of DNA from each sample were pooled, and the pooled sample was purified using the E.Z.N.A.® Omega cycle pure kit (Omega Bio-tek, Norcross, Georgia, USA). Amplicon size distribution was pre-checked using the Agilent 2100 Bioanalyzer system, and the composite sample was sequenced on the PacBio RSII platform by SciLifeLab, Uppsala. The PacBio platform

was chosen to minimize bias due to size variation in the amplicon pool, which is considerable for the fungal ITS2 region.

2.5 Sequence analysis

Sequences were filtered and clustered using the SCATA pipeline (scata.mykopat.slu.se) (Ihrmark *et al.* 2012). Sequences were quality checked to remove reads shorter than 100 bp, with mean quality scores lower than 20, with individual bases with a quality score of less than three, or with a missing 3' or 5' tag. Sequences were screened for the gITS7 and ITS4 primers, requiring a minimum match of 90%, and reverse complemented if necessary. Sequences that passed quality filtering (44% of the total) were clustered into species hypotheses (SHs) (Kõljalg *et al*. 2013) through pair-wise comparison by USEARCH (Edgar 2010) followed by single linkage clustering, with the maximum distance to the closest neighbour allowed to enter a cluster set at 1.5%. After clustering, singletons, samples with non-matching tags (presumably due to tag swapping) and samples with less than 100 reads were excluded from further analysis, leaving 367 individual humus soil samples, 73 pooled mineral soil samples and 44 retention tree humus soil samples for further analysis.

2.6 Species assignment

Representative sequences from SHs were compared against database references in UNITE (Kõljalg *et al*. 2013) using BLAST. Non-fungal SHs were discarded (15% of the high quality reads), and fungal SHs were annotated in order of declining abundance until at least 90% of all reads in each sample were annotated. The requirements for assigning SHs to species were at least 98.5% similarity and a BLAST score of at least 400 to a sequence validated by an expert taxonomist

(exceptions are described in Table S2). Nomenclature follows the Swedish Taxonomic Database (2016), except when Latin names are given with the naming authority. All identified SHs identified to species or genus were classified as "EMF" or "not EMF" and the remaining SHs as "unknown function". The EMF classification was done based on published literature (Hallingbäck and Aronsson 1998; Tedersoo and Smith 2013).

2.7 Data analysis

2.7.1 Community composition

All analyses were performed in R (R Core Team 2016). Relative abundances of all fungal SHs (proportion of SH reads out of the total number of fungal reads, including unknowns) were calculated for each individual sample and the resulting abundances were averaged per stand. EMF SH abundances were divided by the sum of all EMF relative abundances per stand, resulting in EMF SH abundances relative to the total EMF community, which were square root arc sine transformed in order to reduce heteroscedasticity. Differences in EMF community composition between stands were illustrated graphically by DCA (function decorana() in vegan). Potential factors explaining differences in EMF community composition were evaluated for statistical significance using CCA, applying the function anova(cca) in vegan, which implements an ANOVA like permutation test of the joint constraints of the CCA. Using this approach we tested the effect of (1) regenerated vs. old natural stands; (2) regeneration method within regenerated stands; and (3) stand age within regenerated stands (region was partialled out using option "Condition" in the CCA). CCA permutation tests were also performed for pairwise comparisons of EMF communities in stands less than 30 years old, 30–60-year-old stands and old natural stands, in order

to detect stand-age specific effects that may be lost in the overall ordination. Organic and mineral soil samples were analysed separately. In order to capture the spatial structure of the EMF communities, frequencies of the number of samples in which a SH were present out of the ten soil cores collected from each stand were calculated, based on a threshold of >1% of the total fungal reads for a SH being classified as present. Differences between regenerated and old natural stands were determined using a CCA permutation test (with region as the Condition). In order to determine whether EMF communities in regenerated stands become increasingly similar to old natural stands as they age, an "average old natural EMF community" was obtained by averaging the relative abundances of each SH among all old natural stands. Similarity in community composition of each regenerated stand to the "average old natural EMF community" was calculated as the Sørensen index (1-Bray Curtis dissimilarity, vegdist function, method "bray" in the vegan package). In order to determine whether the similarity to the "average old natural EMF community" changed depending on regeneration method, stand age or region, these variables were included as explanatory variables in a linear regression model (function lme in the lme4 package with region as a random variable when not tested specifically). CCA permutation tests were also performed in order to pairwise compare EMF communities close to retention trees with those in surrounding harvested areas, and with those in old natural stands (with region as the Condition).

2.7.2 Individual species test and indicator species

Differences in abundances of individual SHs, present in at least ten soil cores, were tested *post hoc* with generalized linear mixed models of the number of sequence reads in individual organic samples, using the function glmer in package lme4, assuming a

Poisson distribution with log-transformed total fungal reads as offset and the forest stand within a region as a random factor. The models were applied in order to determine: (1) differences between old natural and regenerated stands; (2) differences between regeneration methods; (3) the effect of regenerated stand age; and (4) differences between retention trees, surrounding harvested areas and old natural stands. SHs were also collapsed to the level of genera, and for each genus, generalized linear mixed models were used to test differences in abundance between old natural and regenerated stands. Indicator species analyses were also performed on square root arc sine transformed EMF mean relative abundances per stand (function multipatt in the indicspecies package). Comparisons were made between: (1) old natural and regenerated stands and (2) retention trees, surrounding harvested areas and old natural stands.

2.7.3 Proportion and species richness of EMF

To obtain comparable numbers of species richness per stand, the total fungal sequence data was pooled within stands, rarefied (function rrarefy in the vegan package) to lowest total number of fungal reads (2121), after which EMF SH richness was calculated. Species richness was related to old natural and regenerated stands, regeneration methods and stand age, using linear mixed models (function lme in the nlme package) with region as a random factor. The same tests were also performed for the average proportion of EMF reads per stand (out of the total fungal reads) with the same explanatory variables.

3. Results

3.1 Sequencing output

Sequencing, quality filtering and clustering resulted in 130,572 reads and 1,786 SHs from the 367 organic samples, of which 13,777 reads (11%) and 112 SHs (6%) were assigned to EMF (Table 2, Table S3). In total 17,392 reads and 741 SHs were obtained from the 44 retention tree samples, of which 1,987 reads (11%) and 63 SHs (9%) were assigned to EMF (Table 3, Table S4). The five most common EMF SHs in the organic soil (retention trees included) were *Suillus variegatus* (16% of EMF reads), *Piloderma sphaerosporum* (13%), *Piloderma olivaceum* (9%), *Cenococcum geophilum* (7%) and *Cortinarius semisanguineus* s.l. (7%). In total, 23,835 reads and 815 SHs were obtained from the 73 transect mineral samples, of which 1,855 reads (8%) and 78 SHs (10%) were assigned to EMF. In general, the same EMF SHs were detected in the mineral samples as in the organic samples (Table S5).

3.2 Comparing old and regenerated stands as well as regeneration methods

3.2.1 EMF community composition

Within regenerated stands the EMF community composition differed marginally depending on stand age ($P = 0.05$) but was not significantly influenced by regeneration method (seed trees or clear-cut) ($P = 0.2$) (Fig. 3). The EMF community composition was significantly different depending on region ($P = 0.001$) and differed between old natural and regenerated stands both in terms of relative abundances ($P = 0.001$, Fig. 2) and frequencies ($P = 0.004$, Fig. S1). When grouping the regenerated stands in “0-30-year-old” and “30-60-year-old”, EMF composition differed significantly between the groups ($P = 0.009$) as well as between each group and old natural stands ($P = 0.001$ and $P = 0.01$). EMF communities in regenerated stands became more similar to those in old natural stands (Sørensen index) with increasing stand age ($P = 0.001$, Fig. 3) and more dissimilar to those in old natural stands with

higher latitude (P = 0.001); however, the regeneration method had no detectable effect (P = 0.9). The results were similar for mineral samples (Fig. S2).

3.2.2 Individual species tests and indicator species

Two *Cortinarius* SHs were significantly more abundant in old natural stands than in regenerated stands (Table 2, Table S3), and two *Cortinarius* SHs increased in abundance with increasing age of regenerated stands, as did *Suillus variegatus*, *Piloderma sphaerosporum*, and *Rhizopogon rosoleus. Rhizopogon evadens* was significantly more abundant after seed tree regeneration than after clear-cutting and replanting, although this result should be treated with caution since overall community composition was not significantly different. The genera-based analysis revealed that *Cortinarius* was significantly more abundant in old natural stands, while *Lactarius* and *Thelephora* were more abundant in regenerated stands. Six *Cortinarius* SHs, *Hydnellum ferrugineum* and an unidentified *Piloderma* (sp1.) were identified as indicator SHs for old natural stands (Table S3). *Thelephora terrestris* was the only indicator for regenerated stands, was significantly more abundant in regenerated stands than in old natural stands, and also declined significantly in abundance with increasing age of regenerated stands.

3.2.3 Proportion and species richness of EMF

EMF species richness was marginally higher (P = 0.05) in old natural stands (on average 15 SHs per stand) than in regenerated stands (13 SHs). However, EMF species richness in seed-tree-regenerated stands (12 SHs) did not differ significantly (P = 0.1) from that of clear-cut stands (14 SHs) but increased with stand age among the regenerated stands (P = 0.03) (Fig. S3). The proportion of EMF of the total fungal community did not differ significantly between old natural stands (on average 12%)

and regenerated stands (10%) (P = 0.3), nor between seed-tree-regenerated stands (9%) and clear-cut stands (11%) (P = 0.4), but increased with the age of the regenerated stands (P = 0.004) (Fig. S4).

3.3 Comparing EMF communities around retention trees with those in surrounding harvested areas and in old natural stands

3.3.1 Community composition

EMF communities around retention trees did not differ significantly from those in old natural stands (P = 0.5, Fig. 6), but differed from communities in the surrounding harvested areas (P = 0.03). The reported P values were obtained after partialling out the effect of region, which had a significant effect (P = 0.001).

3.3.2 Individual species tests and indicator species

Six SHs (four *Cortinarius* SHs, *S. variegatus* and *C. geophilum*,) were significantly more abundant close to retention trees than in the surrounding harvested areas (Table 3, Table S4). By contrast, *T. terrestris* and *Tylospora fibrillosa* were significantly more abundant in harvested areas than around retention trees. Indicator SHs for retention trees were *Cortinarius obtusus* and *Cortinarius stillatitius s.l.*, and for harvested areas *T. terrestris* (Table S4).

4. Discussion

In this study we investigated whether natural regeneration using seed trees results in an EMF community composition that is more similar to old natural stands than that obtained after replanting clear-cuts. According to our results regeneration method did not influence the EMF community composition. However, permanently retained trees

seemed to maintain EMF communities of old natural stands locally but had no effect in the surrounding harvested areas. EMF communities in regenerated stands became increasingly similar to those of old natural stands with increasing stand age, but significant differences remained still 30–60 years after harvest. Even though relative abundances differed, the pool of common species was largely the same in all stand categories.

4.1. Regeneration method

In contrast to our hypothesis, we found that the ten-year retention of seed trees failed to affect EMF community composition relative to full clear-cutting and replanting. Since EMF survival has been observed within a radius of 10 m from retained trees (Cline *et al*. 2005; Luoma *et al.* 2006; Jones *et al.* 2008), 50-100 temporarily retained seed trees per ha should suffice to ensure that a majority of the area is covered by roots of mature trees, potentially enabling life-boating of EMF. Therefore we expected that seed trees regeneration would result in more diverse and less impacted EMF communities compared to those in clear-cut and replanted stands. Our result shows that the observations of lack of effect of regeneration method on EMF community composition made by Varenius *et al.* (2016), based on a single field experiment of 50-year-old stands, also holds true in a larger geographical context and throughout stand development.

Tree harvesting results in changed soil chemistry, humidity and temperature (Jones, Durall & Cairney 2003). A possible scenario is that EMF species adapted to conditions in old natural stands (e.g. certain *Cortinarius* spp.) could not cope with the overall new environmental conditions induced by the harvest, no matter whether seed trees were retained or not, or were not present in the immediate surroundings to produce sufficient amount of spores that could disperse into and establish in the

logged areas. Thus, these species decreased in abundance and frequency in relation to more generalist species. Our results thus indicate that environmental filtering might have a larger effect on the overall EMF community composition after harvest than regeneration method and tree continuity. It is therefore reasonable to assume that, when aiming to conserve an EMF community similar to an unharvested forest, retention of intact forest patches is probably more efficient than retention of evenly distributed trees. In support of this idea, Jones *et al.* (2008) observed that EMF communities in retention patches as small as 5 m in diameter mirrored those of the unharvested forests, while the effect of the retention patch disappeared 10 m into the harvested area. Kranabetter, De Montigny and Ross (2013) observed reduced abundance of particular dominant EMF species 10 years after harvest along the entire gradient of patch sizes (single trees to 0.12 ha) and suggested a patch size of at least 0.2 ha. Since our results confirm the lack of impact of dispersed retention trees, retaining 0.2 ha forests patches seems like an efficient approach for local preservation of EMF communities that are similar to those of old, natural stands.

4.2. Permanent retention trees

The EMF communities of old natural stands were partly maintained within one meter of permanent retention trees, but not in the harvested areas surrounding the retention trees. This result together with the lack of effect of seed tree retention indicate that retaining single dispersed trees should be viewed upon as a way to life-boat EMF mycelia directly associated with the retained trees but not as a way to maintain EMF diversity throughout an entire stand. This is further supported by the observations by Cline *et al*. (2005), who found that EMF communities of seedlings planted 6 m from mature trees had a composition more similar to the mature trees than seedlings

planted 16 m from the trees, and Luoma *et al.* (2006), who observed a 50% decline in number of EMF taxa 8-25 m from retention trees.

EMF communities are, like most biological communities, composed of relatively few dominant species and many rare ones (Horton and Bruns 2001). The majority of the EMF species occurring in the studied stands, e.g. red-listed species, are too infrequent to be captured by our study design, simply because the probability of finding rare species at levels suitable for statistical analysis is low when sampling soil randomly, as discussed by Varenius *et al.* (2016). These EMF are preferably instead monitored using sporocarp surveys or through analyses of soil from known fruiting areas (van der Linde *et al.* 2012; Gordon and van Norman 2014). However, since our results indicate that permanent retention trees enable local survival of some, relatively common EMF, it is reasonable to conclude that also rare species may survive in such refuges. Although unfeasible in ordinary management, at sites of particular interest, pre-harvest sporocarp inventories could be to carried out, followed by targeted retention of their host trees as a way to maintain EMF species of specific conservation interest. However, certain EMF species require conditions that can only be found in old natural stands, and even if their host trees are retained they may not cope with the environmental changes induced by harvest.

4.3. Regenerated stands vs. old natural stands

We found that regenerated stands differed in EMF community composition from old natural stands still several decades after harvest. Furthermore, as expected, EMF communities in regenerated stands became increasingly similar to communities in old natural stands with increasing stand age, and EMF species richness and the proportion of EMF out of the total fungal community also increased. These results

corroborate several previous reports (Twieg *et al.* 2007; Wallander *et al*. 2010; Varenius *et al*. 2016; Kyaschenko *et al*. 2017).

Since regeneration method did not matter, it seems like the long-term shift in EMF community composition after clear-cutting is mainly induced by changes in soil chemistry, e.g. elevated pH and increased levels of inorganic nitrogen (Jones, Durall & Cairney 2003; Kyaschenko et al. 2017). Priority effects could enable generalist species, established after the disturbance, to persist in high abundance for several decades and delay establishment of more diverse communities (Kennedy, Peay & Bruns 2009; Peay *et al*. 2012). Also, the importance of a few species potentially surviving harvest as a dormant spore bank in the soil is uncertain (Bruns *et al*. 2009; Nguyen, Hynson & Bruns 2012; Glassman *et al*. 2015).

Six *Cortinarius* SHs were indicator species for old natural stands, and the *Cortinarius* genus was significantly more abundant in old natural stands, similar to previous observations (Twieg *et al*. 2007; Kyaschenko *et al*. 2017). The *Cortinarius* genus can access complex nutrient pools and is increasingly being appreciated to take part in the turn-over of organic carbon and nitrogen pools in nitrogen-limited boreal forest soils (Lindahl & Tunlid 2015; Clemmensen *et al*. 2015; Kyaschenko *et al*. 2017). Many *Cortinarius* species prefer low levels of inorganic nitrogen (Lilleskov *et al.* 2002). Thus, the low abundance of *Cortinarius* species in younger forests could be a result of increased nitrogen mineralization by saprotrophic fungi, induced by the tree harvest (Kyaschenko *et al.* 2017). Our results from a large-scale survey highlight the threat that large-scale clear-cutting forestry involve to many *Cortinarius* species and their potentially unique function in boreal forest ecosystems.

Thelephora terrestris, which is known to be a pioneer species (e.g. Colpaert 1999), was, not surprisingly, more abundant in regenerated stands compared with old

natural stands and was the only indicator SH for those stands. It was similarly observed in young Douglas fir stands (Twieg *et al.* 2007) and is common on nursery-grown seedlings (Menkis *et al.* 2016).

Even though abundances of species differed significantly between different types of forests, the ten most common species were present in all stand categories. Together with the observed increase in EMF species richness with age of regenerated stands, this indicates that the EMF community after harvest develops through species accumulation and altered competitive balances rather than by full successional replacement of species (Bradbury *et al.* 1998; Kranabetter *et al*. 2005).

The overall low proportion and richness of EMF observed in this study is expected given the location of the stands in the northern part of Sweden and the relatively low fertility of the stands supported by the results of Sterkenburg *et al.* 2015 observing increasing EMF relative abundance with increasing soil fertility.

As in most fungal community studies, the proportion of explained variation in EMF community composition was generally low, approximately 5% for forest management or stand age and 10% when including the effect of region, indicating large spatial variation.

Author contributions

KV designed the study, performed the sampling, analysed the data and wrote the manuscript. AD and BL contributed to the planning, analysis and writing process.

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Raw data access

Raw sequence data has been submitted to NCBI, accession number SRP109164.

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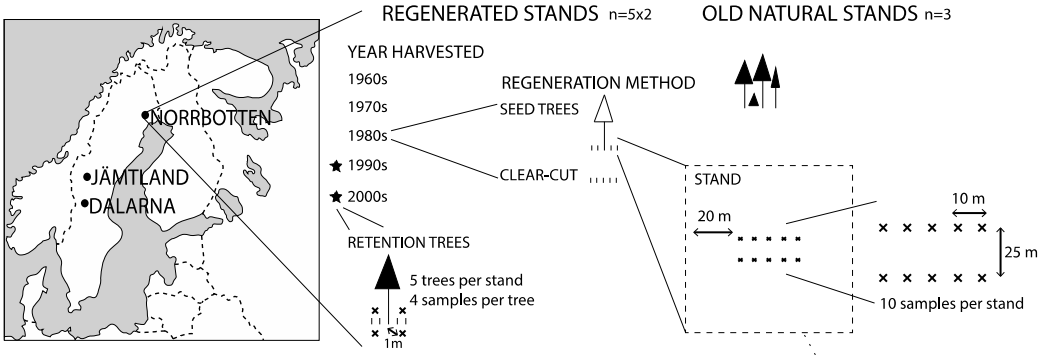

Figure 1. Study design, location of stands and sampling strategy. The study was replicated in three regions (Norrbotten, Jämtland and Dalarna). In each region, 13 forest stands were sampled, of which three were old natural stands (filled group of trees) and 10 were stands that had been regenerated between 1960 and 2000, either by seed-tree regeneration (unfilled trees, five stands) or by replanting after clear-cutting (short vertical lines symbolising stumps, five stands). Ten soil samples (crosses) were collected from each stand along two transects at least 20 m from the stand edge (dashed line) and 25m apart. Samples (crosses) were also collected from ten of the (10–30 year-old) stands (stars) (four in Norrbotten, four in Jämtland and two in Dalarna) at a distance of 1 m from three to five permanently retained trees (filled trees).

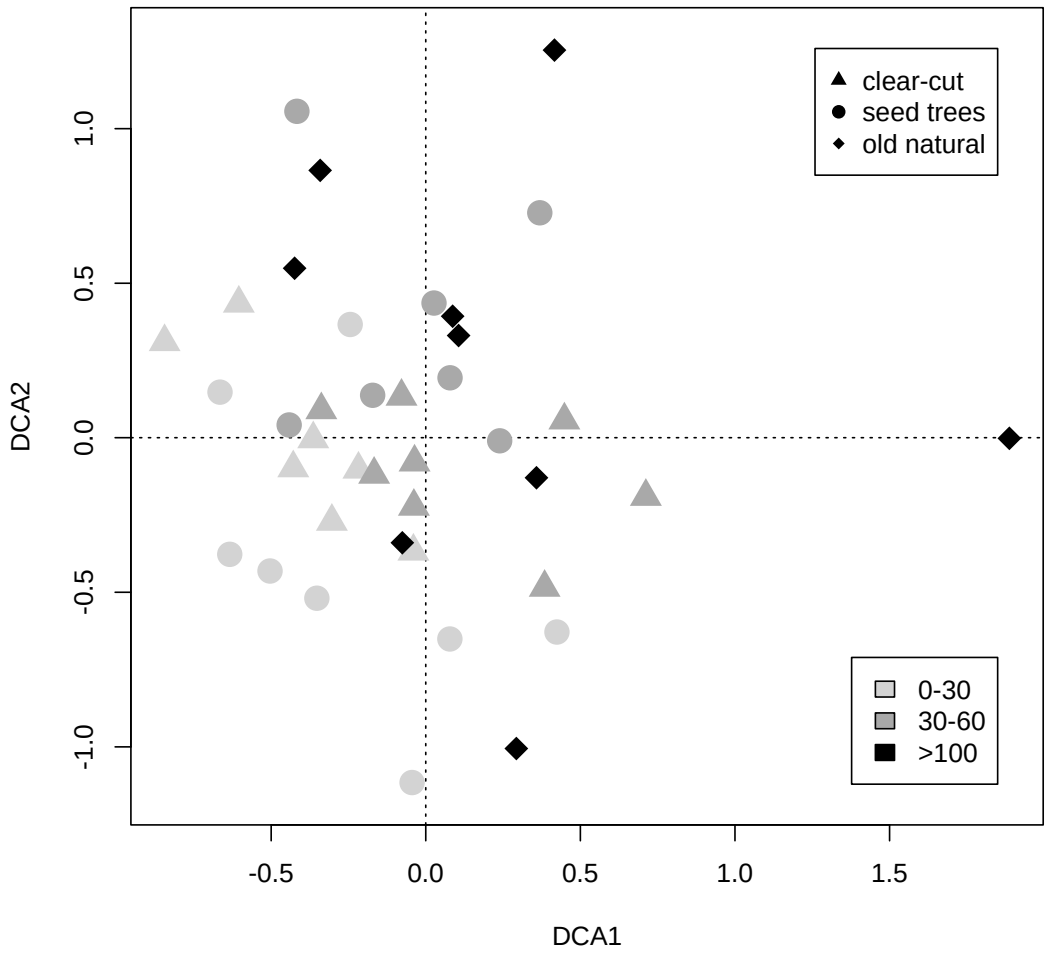

Figure 2. Detrended correspondence analysis (DCA) of ectomycorrhizal fungal (EMF) community compositions in the organic layer of thirty 10-60-year old stands regenerated either by replanting after clear-cutting or naturally using seed trees in comparison with those in nine old natural stands. Each symbol represents the composition of 112 EMF species hypotheses average relative abundances (in 8–10 humus samples) in a stand. Light grey = 0-30 year-old stands, dark grey = 30-60 year-old stands, black = >100 year-old stands, triangle = stands regenerated by replanting clear-cuts, circle = seed tree regenerated stands, and rhomb = old natural stands.

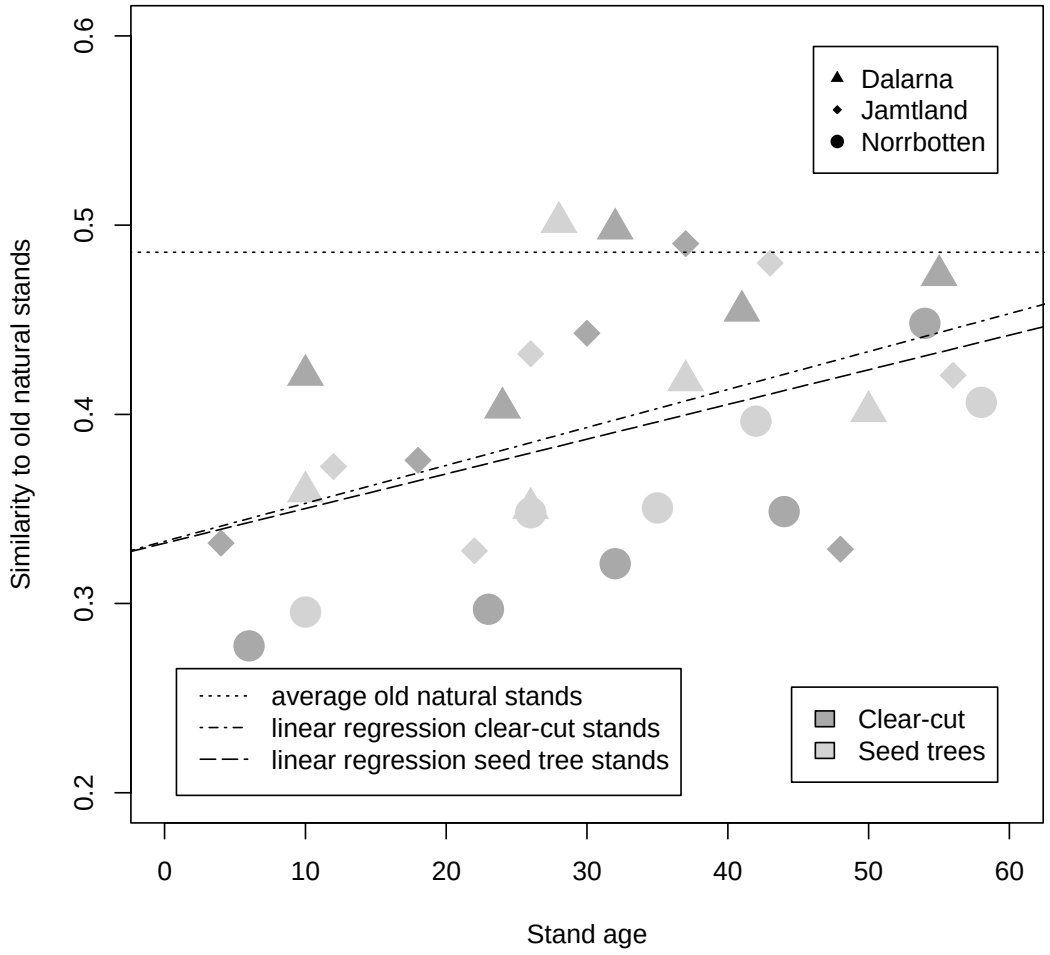

Figure 3. Similarity (Sørensen index) in ectomycorrhizal fungal (EMF) community composition of 112 species hypotheses between stands regenerated either by replanting after clear-cutting or by seed trees and the average EMF community in nine old natural stands, plotted against stand age. Dark grey = seed-tree-regenerated stands, light grey = stands regenerated from clear-cuts, triangle = Dalarna, square = Jämtland, and circle = Norrbotten. The average similarity between communities from individual old natural stands and the average old natural stand community is marked with a dotted line and the linear regressions of the similarity of replanted clear-cut stands ($P = 0.02$) and seed tree regenerated stands ($P = 0.02$) when plotted against stand age are shown as dashed-dotted and dashed lines respectively.

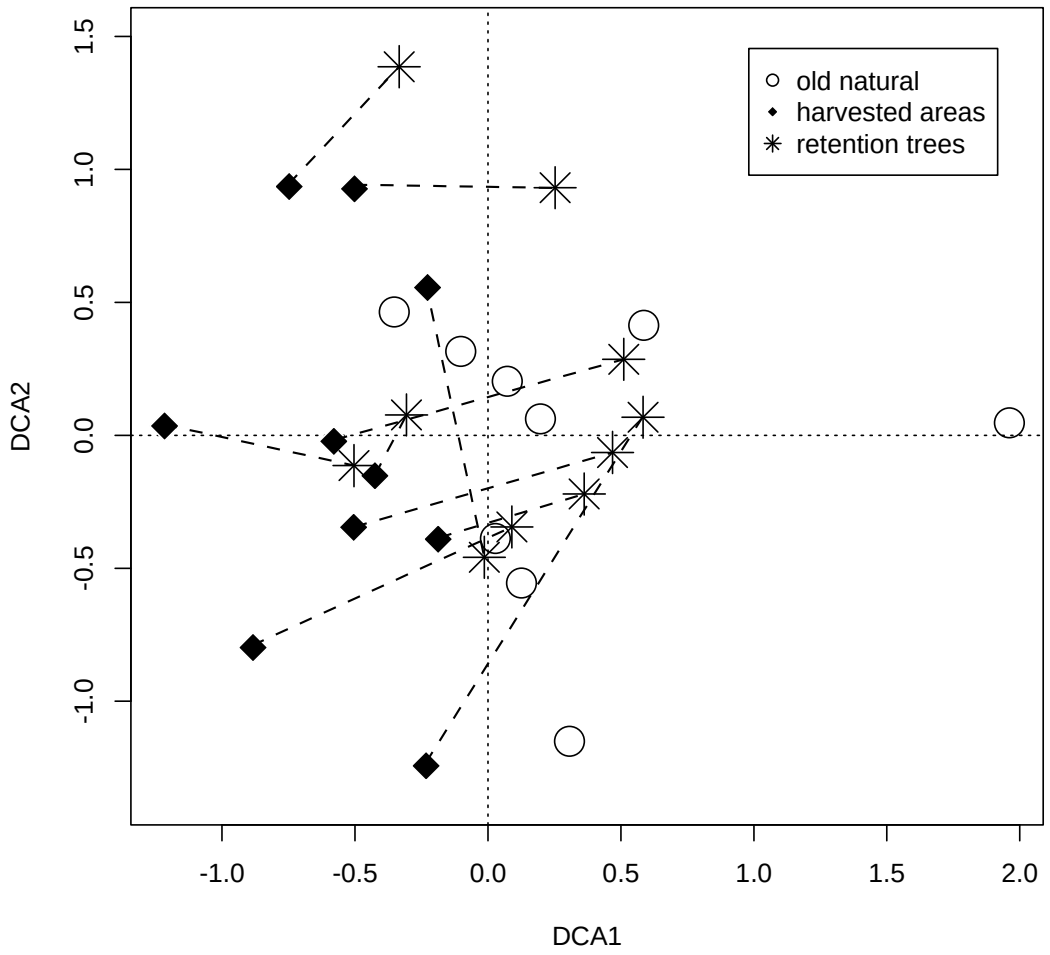

Figure 4. Detrended correspondence analysis (DCA) of ectomycorrhizal fungal community composition in the organic layer close to permanently retained trees (stars) compared with surrounding harvested areas (rhombs) in ten 10-30 year-old stands in comparison with those in nine old natural stands (circles). Each symbol represents the composition of 103 EMF species hypotheses average relative abundances. Dashed lines connect samples from the same stand.

Table 1. Stand age of the studied *Pinus sylvestris* (Scots pine) stands in the three regions, Norrbotten, Jämtland and Dalarna. "r" denotes stands in which permanent retention trees were sampled.

	Stand age (years)		
Region	Old natural	Seed trees	Clear-cut
Norrbotten	123, 124, 136	10r, 26r, 35, 42, 58	6r, 23r, 32, 44, 54
Jämtland	103, 111, 132	12r, 22r, 26, 43, 56	4r, 18r, 30, 37, 48
Dalarna	136, 138, 184	10, 26r, 28, 37, 50	10r, 24, 32, 41, 55

Table 2. Individual species abundance tests and average relative abundances (‰ of all fungal reads) for ectomycorrhizal fungal (EMF) species hypotheses (SHs) occurring among the 20 most common EMF SHs in either old natural stands (O), stands regenerated using seed trees (S) or by replanting after clear-cutting (C)). The *post hoc* tests of individual SH abundances were comparing the number of reads of each SH in all individual soil cores and analysing whether a certain SH were more present in old vs. regenerated stands (O vs. S&C) and if the abundance of the SH increased or decreased with age of regenerated stands (Age +/- 10-60). The two regeneration methods only differed significantly for one SH (*). Letters denoting test results are explained below.

SH	SH name	O vs. S&C	Age +/- 10-60	‰ fungal reads		
				O	S	C
10	*Suillus variegatus*		+	23	21	12
18	*Piloderma sphaerosporum*		+	14	12	18
30	*Russula decolorans*			8	4	4
26	*Cortinarius semisanguineus s.l.*		+	7	4	9
34	*Cortinarius biformis*			5	6	8
60	*Cortinarius cf obtusi s.l.* sp. 1	o	+	7	3	<1
21	*Piloderma olivaceum*			5	8	14
40	*Cenococcum geophilum*			5	4	9
25	*Lactarius rufus*			5	3	15
160	*Cortinarius cf obtusi s.l.* sp. 2			3	<0.1	<1
130	*Cortinarius caperatus*			3	1	
65	*Cortinarius obtusus*			3	<1	
93	*Piloderma byssinum*			5	<0.1	<1
301	*Cortinarius causticus*			1	<1	
126	*Russula vinosa*			1	<0.1	<0.1
64	*Russula paludosa*			3	5	<0.1
193	*Cortinarius testaceofolius*	o		3	<0.1	<1
171	*Tylospora* sp. 1			2	<1	<1
98	*Cortinarius mucosus*			3	2	2
330	*Cortinarius obtusus s.l.*			1		
92	*Tylospora fibrillosa*			1	2	2
134	*Tylospora asterophora*			1	1	1
85	*Cortinarius brunneus*			<1	2	2
198	*Rhizopogon mohelnensis* Velen.			<1	<0.1	1
188	*Cortinarius armillatus*			<1	<1	1
255	*Hebeloma* sp. 1			<1	<1	1
132	*Rhizopogon evadens* A.H. Sm. (*)			<0.1	2	<1
146	*Thelephora terrestris*	s&c	–	<0.1	<1	1
312	*Cortinarius pholideus*			<0.1	<1	<1
162	*Suillus bovinus*				2	<1
189	*Cortinarius clarobrunneus*				1	<0.1
1116	*Phellodon tomentosus*					<0.1
358	*Cortinarius croceocristallinus s.l.*					1

o SH significantly more abundant in old natural (100+ years) than in regenerated stands (10-60 years).

s&c SH significantly more abundant in regenerated stands (10-60 years) than in old natural (100+ years).

+/– SH significantly increasing (+) or decreasing (–) with the age of the regenerated stands (10-60 years).

* SH with significantly more abundant in stands regenerated using seed trees compared to clear-cuts.

Table 3. Individual species abundance tests and average relative abundances (‰ of all fungal reads) for ectomycorrhizal fungal (EMF) species hypotheses (SHs) occurring among the 20 most common SHs in either old natural stands (O), around permanent retention trees (R), or in the surrounding harvested areas (H). The *post hoc* tests of individual SH abundances were comparing the number of reads of each SH in all individual soil cores and anlysing whether a certain SH differed in abundance when comparing retention trees with old natural stands and with harvested areas. Letters denoting test results are explained below.

SH	SH name	Pairwise test	‰ fungal reads		
			O	R	H
10	*Suillus variegatus*	rh oh	23	20	4
21	*Piloderma olivaceum*		5	12	12
40	*Cenococcum geophilum*	rh	5	9	5
26	*Cortinarius semisanguineus s.l.*	rh	7	10	3
65	*Cortinarius obtusus*		3	11	<0.1
18	*Piloderma sphaerosporum*	or	14	6	8
60	*Cortinarius cf obtusi s.l.* sp. 1	rh oh	7	5	<1
25	*Lactarius rufus*		5	3	4
30	*Russula decolorans*		8	4	3
34	*Cortinarius biformis*		5	2	2
90	*Cortinarius stillatitius s.l.*		<1	4	
92	*Tylospora fibrillosa*	rh	1	1	1
286	*Cortinarius traganus*		<1	2	<0.1
238	*Cortinarius lux nymphae*			2	<1
184	*Pseudotomentella tristis*		<1	1	
130	*Cortinarius caperatus*		3	3	
126	*Russula vinosa*		1	1	<0.1
146	*Thelephora terrestris*	rh oh	<0.1	1	2
64	*Russula paludosa*		3	1	<1
188	*Cortinarius armillatus*		<1	<1	<1
85	*Cortinarius brunneus*	rh	<1	1	<1
171	*Tylospora* sp. 1		2	1	1
160	*Cortinarius cf obtusi s.l.* sp. 2		3	<1	<0.1
162	*Suillus bovinus*			<1	1
132	*Rhizopogon evadens* A.H. Sm.		<0.1	<1	1
98	*Cortinarius mucosus*		3	<1	
93	*Piloderma byssinum*		5	<0.1	
301	*Cortinarius causticus*		1	<0.1	
193	*Cortinarius testaceofolius*	oh or	3	<0.1	<1
330	*Cortinarius obtusus s.l.*		1		
494	*Tomentella badia*		<1		<1
1116	*Phellodon tomentosus*				<0.1
284	*Lactarius mammosus*				<1

rh SH with significantly different abundance around retention trees and in harvested areas.
oh SH with significantly different abundance in old natural stands and in harvested areas.
or SH with significantly different abundance in old natural stands and around retention trees.