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Soil Fungal Communities under *Pinus patula* Schiede ex Schltdl. & Cham. Plantation Forests of Different Ages in Ethiopia

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Abstract: The cultivation of plantation forests is likely to change the diversity and composition of soil fungal communities. At present, there is scant information about these communities in Ethiopian plantation forest systems. We assessed the soil fungal communities in *Pinus patula* Schiede ex Schltdl. & Cham. stands aged 5, 11, or 36-years-old using DNA metabarcoding of ITS2 amplicons. The ecological conditions of each plot, such as climate, altitude, and soil, were similar. Stand age and soil fertility influenced soil fungal species diversity and ecological guilds. In total, 2262 fungal operational taxonomic units were identified, of which 2% were ectomycorrhizal (ECM). The diversity of ECM fungi was higher in the 5 and 36-year-old stands than in the 11-year-old *P. patula* stands. Contrary to our expectations, a high level of ECM species diversity was observed in young stands, suggesting that these ECM species could compensate for the effects of nutrient stress in these stands. Our results also suggested that the abundance of plant pathogens and saprotrophs was not affected by stand age. This study provides baseline information about fungal community changes across tree stands of different ages in *P. patula* plantations in Ethiopia that are likely related to ECM fungi in young stands where relatively low soil fertility prevails. However, given that the plots were established in a single stand for each age class for each treatment, this study should be considered as a case study and, therefore, caution should be exercised when applying the conclusions to other stands.

Keywords: ectomycorrhizal fungi; Ion torrent sequencing; metabarcoding; *Pinus patula*; soil fungal diversity; stand age

1. Introduction

A recent review of forestry in Ethiopia revealed that deforestation is a continuous process [1]. When all forest use was included, a deforestation rate of 0.93% per year was calculated in 2010 [2,3]. Despite this, establishing plantations of fast-growing exotic tree species is becoming a major part of forestry practice in Ethiopia [4,5]. Exotic tree species plantations are now estimated to cover 1,000,000 ha of land [5,6]. One of these introduced tree species is *Pinus*, which is mainly being grown to meet the increasing demand for woody raw materials [6–8]. As a consequence, *Pinus patula* Schiede ex Schltdl.

& Cham. has received attention largely as a suitable tree for the production of round wood (timber), poles, and posts [5,9], although it also used as a source of resin and turpentine oil constituents [10].

Soil fungi drive many ecosystem processes and, hence, are a vital part of the soil microbial community [11,12]. *Pinus* trees form obligate symbiotic relations with mycorrhizal fungi, which are vital for host nutrition, survival and productivity in forest systems [13]. Root associations with mycorrhizal fungi are beneficial for most plants because they not only increase their access to nutrients and water in the soil but also enhance their stress tolerance [14]. Similarly, mutualistic fungi use carbohydrates from the host tree for their survival [15]. Mycorrhizal associations also increase host tree resistance to pathogens [16,17]. Previous studies have also reported that there tends to be a greater accumulation of organic matter belowground in forests with ectomycorrhizal (ECM) associations than in other forests [18,19]. Furthermore, decomposition in forests is often limited by competition for nitrogen (N) between ECM and free-living saprotrophic fungi [18,20,21]. Mycorrhizal fungi mediate interactions between plants and other soil microorganisms, such as pathogens and mycorrhizosphere mutualists that produce vitamins, and provide protection against antagonists [14]. Saprotrophic fungi also play a crucial role in the decomposition of organic materials [22]. Thus, knowledge of fungi and their community structure in the soil of plantation forests are important because the trees and fungal synergies could determine many vital ecosystem services such as nutrient cycling [23].

The composition and structure of fungal communities are affected by diverse interacting biotic and abiotic factors [11,23], including host plant succession [24–26]. As a forest develops, the composition of the associated fungal communities also changes [27,28]. For example, early-stage fungi emerge from spores in the spore bank that were already in the soil prior to the establishment of the plantation, whereas late-stage fungi are more prevalent as conditions change as the plantation matures [29]. Both early- and late-stage fungi are involved in the stabilization of the soil and the rehabilitation of soil microflora [30]. Furthermore, changes in edaphic variables due to forest growth affect the abundance and diversity of soil fungi and, thereby, dictate the fungal community composition [31,32]. Previous studies have assessed changes in soil fungal communities across stand ages by performing DNA analyses [33,34] and fruit body surveys [35]. We have also previously shown that the fungal community composition differs at different stages of forest development in Ethiopian forests [8,24,36].

Ethiopian fungal flora remains unexplored, as most regions and habitats in the country have been seldom studied and reports regarding fungi diversity rarely exist. This is due to a lack of research infrastructures as well as to a lack of fungal taxonomists and specialists in fungal ecology. Likely as a result, fungi are not included in the biodiversity database of the country. Previous studies conducted on *Pinus* tree species in Ethiopia have focused on ways to assist the management and development of *Pinus* plantations. However, studies on the soil microbial community, including fungi associated with *Pinus* plantations, are very limited. Sporocarps associated with a *P. patula* plantation during a single rainy season have previously been reported [8]. Although sporocarps represent a unique step in the complex life of fungi [8], they do not reflect the entire soil biota [37,38]. However, the maintenance of a vital soil biota is essential for many woodland ecosystems because energy reaches the forest floor mainly through the degradation of organic matter and the recycling of vital elements found in animal and plant remains [24,39]. Furthermore, a rich soil biodiversity guarantees the presence of vital habitats, the integrity of the soil structure and water-holding capacity, soil fertility, and plant growth [40]. In the present study, we sampled soil from different age groups of *P. patula* plantations. We hypothesized that, as stand age increased, soil fertility would decrease. In addition, we expected that soil would become increasingly colonized by roots and associated symbionts and, hence, that root-associated fungal species would be more dominant in soils in older *P. patula* stands than in younger stands. Thus, in older stands, there is more organic matter and lower N than other edaphic elements. Given the potential changes in edaphic variables with increasing stand age [41], we expected that changes in the soil fungal community would also occur. Thus, the main aim of this study was to provide baseline information about the soil fungal communities that occur along a chronosequence of *P. patula* plantations in Ethiopia. We hypothesized that substantial change in the composition of soil fungal communities would be

detected along the chronosequence of *P. patula* plantations. Specifically, we hypothesized that: (1) there would be changes in the total fungal diversity and community composition, and (2) that the ecological guilds would change along the chronosequence of *P. patula* plantations in the study area.

2. Materials and Methods

2.1. Study Area

The study was conducted in *P. patula* stands in Wondo Genet, Ethiopia. Wondo Genet is approximately 265 km from the Ethiopian capital, Addis Ababa (Figure 1). The study site ranged from 7°06′ North to 7°07′ North and from 38°37′ East to 38°42′ East, and was 1600 to 2580 meter (m) above sea level [42,43]. The climate of the study area is characterized by the Woyna Dega agro-climatic zone, which has a bimodal rainfall pattern: a rainy season in spring followed by the main rainy season in summer, with a mean annual rainfall of 1210 millimeter (mm) [43,44]. The mean annual temperature is 20 °C [43,44]. The study area has a slightly undulating topography and an Andisol soil with a sandy loam texture [45] that has an average pH value of 5.7 [46].

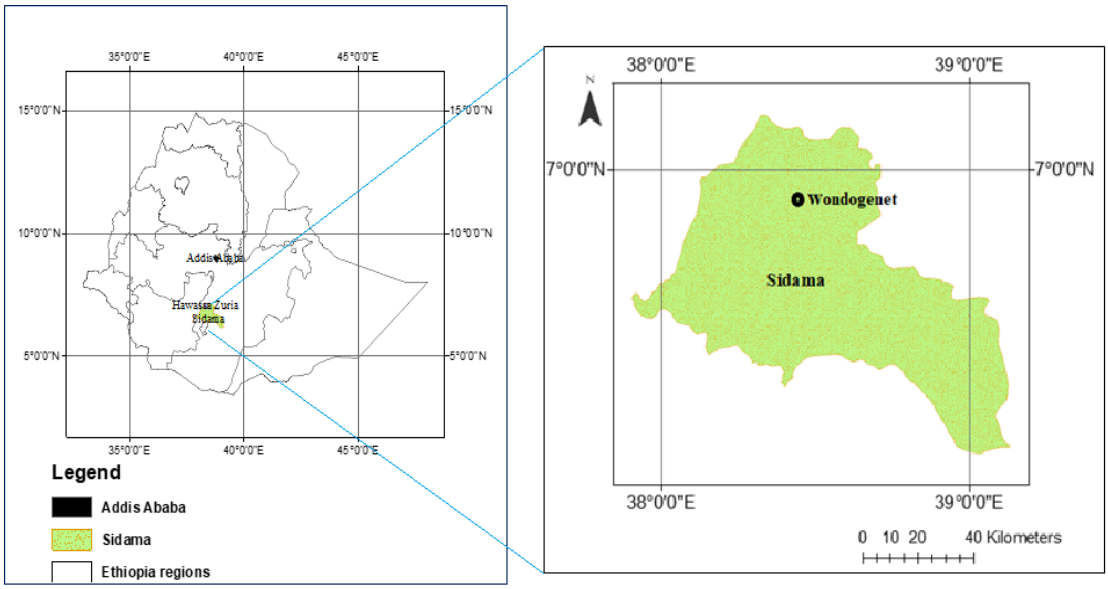

Figure 1. Maps showing the location of the study area, Wondo Genet, in the Sidama region of Ethiopia.

The native vegetation that originally grew in the study area was destroyed before the inception of the nearby college of forestry (1976) by logging and clearance for cultivation [10]. However, in recent decades, a mass planting scheme of exotic tree species has been undertaken, resulting in approximately 100 ha of non-native plantations of *Cupressus lusitanica* Mill., *Grevillea robusta* A. Cunn. ex R.Br., and *P. patula* in the study area [10,47]. In *P. patula* plantations, stands of three different ages (i.e., 5, 11, and 36-years-old) were selected and three 2 × 50 m plots were established in each stand age category [27,29], with a minimum distance of approximately 120 m between plots within a stand [28]. All plots were similar in terms of their ecological conditions such as climate, altitude, and soil.

The three stands were far enough apart (minimum 4 km) that they are not interacting and they could be clearly differentiated with the silvicultural treatments applied to them. For example, weeding and slashing were conducted for the 5 and 11-year-old stands while thinning was applied for the 36-year-old stands in the study area [8].

2.2. Collection of Soil Samples for Molecular Analysis

In total, nine transects, one in each of the 2 × 50 m plots, running perpendicular to the slope, were established [28,48]. Five cores 5 m apart were extracted along the centerline of each transect using a cylindrical soil borer (2 cm radius, 20 cm deep, and 250 cm^3) [49] to collect samples with spatial variability while minimizing the likelihood of repeatedly sampling the same genet. Before these cores were sampled, the litter layer, which comprised intact and partially decomposed leaves, was removed because the fungal community in leaves tends to differ from that in soil [50]. The five cores from each transect, comprising well-decomposed organic layers and mineral soils, were pooled to produce a composite soil sample of each transect. Next, the cores were dried, sieved through a 1-mm mesh and then ground to a fine powder using a mortar and pestle. A subsample of each composite sample was stored at −20 °C while awaiting molecular analysis.

2.3. Molecular Analysis

A PowerSoil™ DNA Isolation Kit (MoBio laboratories Inc., Carlsbad, CA, USA) was used to extract DNA from 0.25 g of soil per sample. PCR reactions of each sample were carried out in triplicate to minimize PCR biases. PCR reactions were performed in 20 µL reaction volumes containing 11.22 µL of Modified Quantization (MQ) water, 1.60 µL of DNA template, 2.00 µL of 10× buffer, 1.40 µL of $MgCl_2$ (50 mM), 1.60 µL of dNTPs (10 mM), 0.50 µL of Bovine Serum Albumin (BSA) (2%), 0.80 µL of reverse and forward primers (10 µM), and 0.08 µL of Platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA). The following PCR conditions were used: an initial denaturation step at 94 °C for 3 min; then 35 cycles of 94 °C for 45 s, 50 °C for 1 min, and 72 °C for 1.5 min; ending with one cycle of 72 °C for 10 min. To amplify the ITS2 rDNA region, we used the forward primer fITS7 [51] and the barcoded reverse primer ITS4 [52]. Sample-specific Multiplex Identification DNA-tags were used to label the ITS4 primer. Each set of PCR replicates also included a negative control comprising MQ water instead of DNA that underwent PCR under the same experimental conditions and was shown to be amplicon-free on a gel. Ion torrent sequencing was performed at the Naturalis Biodiversity Center using the sequencing Ion 318^{TM} Chip to obtain the greatest possible sequencing coverage.

2.4. Collection of Soil Samples for Chemical Analysis

To examine the relationship between soil fungal composition and edaphic variables, soil samples were extracted from the center and the four corners of every plot after removing plant material and debris from the surface. Soil down to a depth of 20 cm was extracted using an auger and spade. The samples were mixed thoroughly to create a composite sample before placing approximately 500 g of soil in a plastic bag for laboratory analysis. After air-drying the soil in the shade, standard extraction methods (i.e., diethylene triamine pentaacetic acid extraction, KH_2PO_4 extraction, Olsen, Kjeldahl digestion Walkley Black, and ammonium acetate) and instrumental analysis were used to determine the pH, organic matter, cation exchange capacity, sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), N, available phosphorus (P), and the physical properties (% of sand, silt, and clay) of the soil.

Soil analysis was conducted by Water Works Design and Supervision Enterprises, Laboratory Service Subprocess, Soil Fertility Section at Addis Ababa, Ethiopia. Soil pH and electrical conductivity were determined by analyzing a soil:water (1:2.5) suspension and the supernatant from the same suspension with the aid of a pH meter and an electrical conductivity meter, respectively [53]. The organic carbon content of the soil was determined using wet digestion [54]. The Kjeldahl procedure was used to determine the total N content in soils [55]. Sodium bicarbonate (0.5 M $NaHCO_3$) was used as an extraction solution to determine the available P [56]. Available Na, available K, Ca, and Mg were also determined. To assess soil particle size we used a hydrometer [57] and sodium hexametaphosphate (Calgon solution) was used as a dispersing agent. After calculating the proportions of sand, silt, and clay, the soil was assigned a textural class name using ASTM free software, Version 4, Available: http://www.astm.org. The results of the soil analysis are presented in Table 1.

Table 1. Selected chemical properties of soil samples from the study plots in Pinus patula plantations in Wondo Genet area, Ethiopia.

Soil Chemical Properties	Stand Age		
	5 Years	11 Years	36 Years
pH-KCl (1:2.5)	6.04 ± 0.19	5.42 ± 0.20	5.44 ± 0.44
Exch. Na^+ (meq/100 g of soil)	0.85 ± 0.03	0.78 ± 0.05	1.10 ± 0.49
Exch. K^+ (meq/100 g of soil)	1.32 ± 0.25	0.42 ± 0.08	0.77 ± 0.05
Exch. Ca^{2+} (meq/100 g of soil)	22.62 ± 3.82	13.66 ± 2.99	16.01 ± 7.23
Exch. Mg^{2+} (meq/100 g of soil)	7.96 ± 1.16	4.36 ± 0.42	6.66 ± 1.15
Organic matter	5.96 ± 0.68	7.27 ± 0.84	13.33 ± 0.98
Nitrogen (%)	0.83 ± 0.07	0.71 ± 0.06	0.34 ± 0.02
Available P (mg P_2O_5/kg soil)	35.70 ± 5.02	36.99 ± 8.10	38.56 ± 7.46
C/N	3.61 ± 0.28	5.17 ± 0.41	19.56 ± 2.11

Note: Values shown are means ± the standard deviation.

2.5. Bioinformatic Analysis

Raw sequence reads comprising demultiplexed sample reads were obtained from the Ion Torrent output. Primers and poor-quality ends were removed based on a 0.02 error probability limit in Geneious Pro 8.1.8 (BioMatters, Auckland, New Zealand). Next, all sequences were truncated to 200 bp and then filtered with USEARCH v.8.0 [58] to discard sequences with an expected error of >1. The remaining sequences were collapsed into unique sequence types on a per-sample basis using USEARCH v.8.0 [58] while preserving read counts. First, we discarded singleton sequence types before grouping the remaining 187,332 high-quality sequences into 2262 operational taxonomic units (OTUs) with USEARCH at a 97% sequence similarity level while simultaneously excluding 3161 sequences representing 67 OTUs with <70% similarity or <150 bp pairwise alignment length to a fungal sequence. Sequences were assigned to taxonomic groups based on pairwise similarity searches against the curated UNITE+INSD fungal ITS sequence database, which contains identified fungal sequences with assignments to species hypothesis groups [59]. The FUNGuild database (http://www.funguild.org) was initially used to perform functional classification of OTUs at the genus level and it was manually checked afterwards. OTUs with >90% similarities to a fungal SH with known ecological function were assigned either to plant pathogens, mycoparasites, animal parasite, ECM fungi, arbuscular mycorrhizal, and saprotrophs functional groups. For genera that are known to comprise species from multiple functional guilds, their ecological function was assigned individually based on available ecological information for the matching SH in the UNITE database.

2.6. Statistical Analysis

To normalize the OTU table for subsequent statistical analyses, we rarefied the number of high-quality fungal sequences to the smallest library size (1951 reads). The resulting matrix contained 1216 fungal OTUs representing 17,559 high-quality sequences. Thus, the analyses used sequence read abundance data, which is not necessarily equivalent to true biological abundance. Shannon's diversity index, H = −Σpi(lnpi) [60], was estimated for each stand, where pi indicates the relative abundance of fungal OTUs [61]. We also calculated Simpson's diversity, D = 1 − Σ (pi2), where pi is the importance probability in element i, and the Evenness, J = H′/H′max, where H′ is the number derived from the Shannon diversity index and the H′max is the maximum possible value of H′ [62]. We also estimated the richness values of all fungal OTUs (S) based on stand type. The Biodiversity RGUI package [63] in R was used to calculate all the diversity measures [64], such as diversity indices and richness.

Ordination of community data was carried out by performing detrended correspondence analysis (DCA) and canonical correspondence analysis (CCA) using CANOCO version 5.0 [65] based on a Hellinger-transformed abundance data matrix. The relative sequence read abundance contribution of each ecological group to community composition was assessed using the relative abundance of the fungal OTUs. A Monte Carlo Permutation test was used to test the statistical significance of the

environmental variables (999 permutations). Correlation between the CCA axes scores and edaphic variables were assessed using linear regression. We also tested whether fungal compositions were statistically different across forest stands of different ages using the multiple-response permutation procedure (MRPP) and permutation-based nonparametric MANOVA (PerMANOVA) [66]. Data were subjected to 4999 iterations per run using the Sørensen similarity (Bray–Curtis index) and a random number to start. To identify which fungal species were most responsible for the patterns observed and to calculate the percentage contribution of fungal taxa to significant dissimilarities among the three stands, we undertook an analysis of similarity percentages (SIMPER) [67,68].

3. Results

3.1. Diversity of Fungal OTUs

A total of 187,332 high-quality sequences were grouped into 2262 OTUs. We obtained between 1951 and 37,245 high-quality reads from each sample. The OTUs were assigned to six fungal phyla. The majority of the OTUs belonged to the Ascomycota (58%), followed by the Basidiomycota (26%). The Chytridiomycota, Glomeromycota, Rozellomycota, and Zygomycota made up to 4%, 3%, 1% and 2% of the total OTUs, respectively, and approximately 7% of OTUs were not assigned to a fungal phylum.

Saprotrophs were the most abundant species found in *P. patula* plantations, comprising 41% of the fungal community, followed by plant pathogens (7%) and ECM species (2%). Less abundant groups were categorized as animal parasites, arbuscular mycorrhizal fungi, mycoparasites, and endophytes. These groups were excluded from further analysis. About 48% of the species were not categorized into any of these functional groups.

The highest mean values for Shannon and Simpson diversity indices for the ECM and saprotrophic fungi were found in 5-year-old stands. Contrary trend was observed for plant pathogens which showed higher diversity in 36-year-old stands. On the other hand, the estimated evenness values of the ECM (range 0.24 to 0.30), plant pathogens (range 0.21 to 0.24), and saprotrophic fungi (range 0.12 to 0.25), showed that each functional groups distributed uniformly in each of the three stands.

The relative abundance of ECM fungi was greater in the 11-year-old *P. patula* stand, whereas the plant pathogens obtained higher mean values in the 5-year-old stands. By contrast, the relative abundances of saprotrophic fungi were homogeneous along the chronosequence of *P. patula* stands.

3.2. Fungal Community Composition and Edaphic Variables

PerMANOVA analyses indicated that the composition of fungal OTUs in stands of different ages were significantly different ($F = 2.76$, $p = 0.013$). MRPP tests also confirmed that the plantation age effect was significant, with a distance effect size of $A = 0.017$ and $p = 0.006$. More specifically, the composition of 5 and 36-year-old stands differed from the 11-year-old stand, as indicated by the Sørensen average distance measures (Table 2). In addition, SIMPER analysis indicated that the overall between-group dissimilarity based on Bray-Curtis measures was 76.09% for the 5 and 11-year-old stands, 77.67% for the 5 and 36-year-old stands and 83.58% for the 11 and 36-year-old stands (Table S1).

Table 2. Average forest group distances calculated using Sørensen distance matrices to determine whether soil fungal compositions were statistically different across *Pinus patula* stands of three different ages in Wondo Genet, Ethiopia. The average value of all three stand ages ± the standard deviation is also shown.

Group	Forest Stand Type	Sørensen Average Group Distance
1	5-year-old stand	0.60
2	11-year-old stand	0.39
3	36-year-old stand	0.63
Average		0.54 ± 0.13

From the canonical correspondence analysis (CCA) (Figure 2), the eigenvalues indicated that the variability in terms of fungal guild composition, explained by the gradients associated with the first two axes, is high. Together they explained 29.47% of the accumulative variance of fungal guild data. With respect to the edaphic variables, potassium (K) and magnesium (Mg) correlated the most strongly with fungal community composition (Figure 2). The correlation of these edaphic variables with CCAs was evident based on fungal guild Hellinger-transformed abundance data (Table 3).

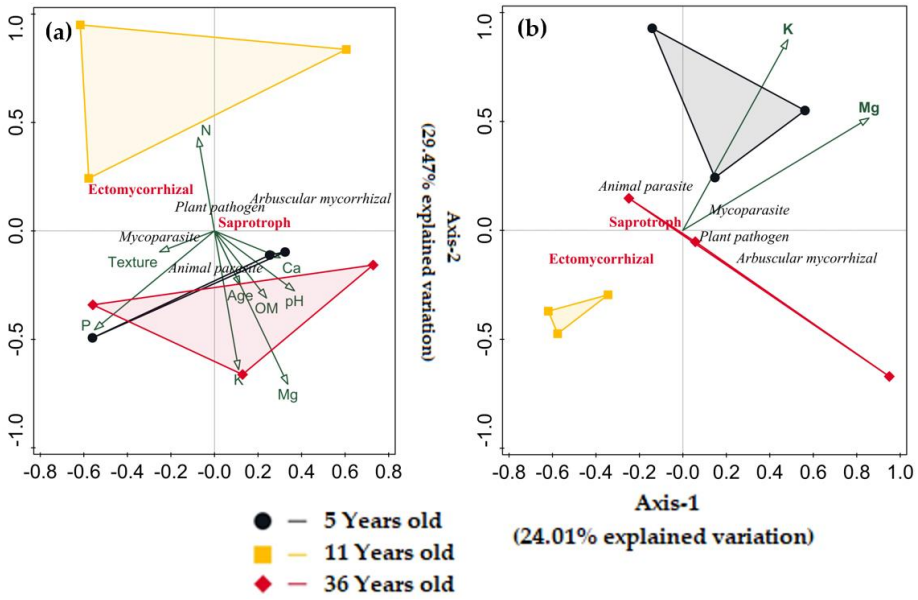

Figure 2. (**a**) Detrended correspondence analyses and (**b**) canonical correspondence analysis (CCA) ordination plots based on Hellinger-transformed abundance data of fungal guilds detected in the soil of three *Pinus patula* stands of different ages. Plots shown in the same color are in the same stand (black, plots in the 5-year-old stand; yellow, plots in the 11-year-old stand; red, plots in the 36-year-old stand). Edaphic variables are shown in green. The percentages of explained variation by each axis are shown in (**b**).

Table 3. Canonical correspondence analysis based on simple term effects showing the significance ($p < 0.05$) of edaphic variables when considering the Hellinger transformed data of the entire fungal guilds at the level of fungal community in the study area.

Edaphic Variables	Df	Sum of Squares	R^2	F	p
K	1	0.018	0.365	26.825	0.010
Mg	1	0.021	0.425	7.518	0.008

Compared with the 5 and 36-year-old stands, we found that a relatively greater proportion of the ECM guild was associated with the 11-year-old stand. By contrast, the saprophytes, mycoparasites, and animal parasites were mainly found associated with the 5- and 36-year-old stands. Based on the cumulative contribution to the dissimilarity of stands, the SIMPER analysis also identified fungal species that typified and distinguished between the soil fungal communities associated with the three *P. patula* plantation age groups (Table S1). *Wilcoxina* sp., *Schizothecium* sp., and *Pustularia* sp. were among the most influential species detected in the soil analyses and cumulatively made the greatest contribution to the differences observed between the three stands, accounting for more than 38.63% of the observed dissimilarity value (Table S1).

When ECM fungi were analyzed separately, the simple term effects in CCA (Figure 3b) revealed that plantation age and the soil fertility variable P significantly influenced the composition of the ECM fungal community in the forest study area (Table 4). The CCA revealed that both axes explained about 45.82% of the cumulative variation in interactions between ECM fungal composition, soil fertility

variables and plantation age. The PerMANOVA analysis also indicated that there were species compositional differences between stands in terms of ECM fungi (F = 3.21, p = 0.003).

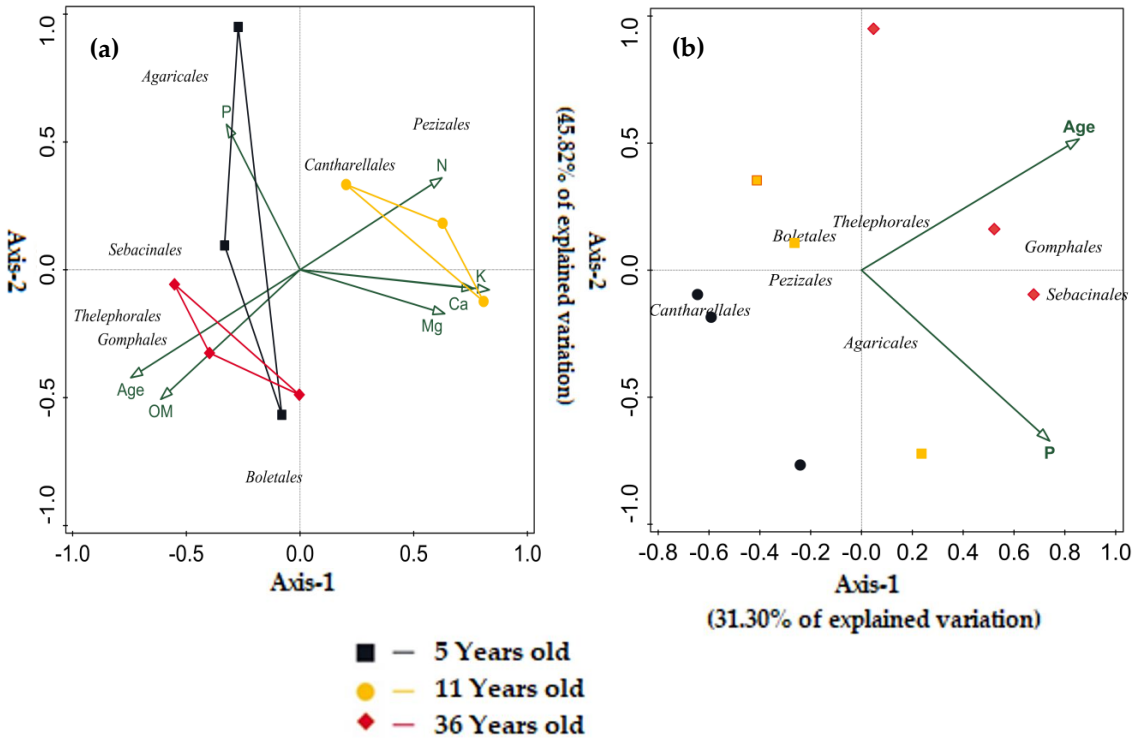

Figure 3. (**a**) Detrended correspondence analyses and (**b**) canonical correspondence analysis (CCA) ordination plots based on Hellinger-transformed abundance data of mycorrhizal fungal species at the order level of fungal communities associated with three age groups of *Pinus patula* stands in the study area in Ethiopia. Plots shown in the same color are in the same stand (yellow, plots in the 5-year-old stand; black, plots in the 11-year-old stand; red, plots in the 36-year-old stand). Edaphic variables are shown in green. The percentages of cumulative explained variation by each axis are shown in (**b**).

Table 4. Canonical correspondence analysis based on simple term effects showing the significance ($p < 0.05$) of edaphic variables when considering the Hellinger transformed data of ectomycorrhizal species at the order level of fungal community in the study area.

Variable	Simple Term Effects		
	Explains %	Pseudo-F	p
Age	26.8	2.60	0.010
Phosphorus	19.0	2.10	0.052

Several species belonging to the orders Gomphales, Sebacinales, and Thelephorales were associated with plots in the oldest stand, where the soil is characterized by relatively high fertility (based on the organic matter content and the C/N ratio). In 5 and 11-year-old stands, which are characterized by low soil fertility (based on organic matter content and the C/N ratio), the species belonging to the order Cantharellales formed an association with other fungal species (Figure 3). Species in the orders Boletales, Pezizales and Agaricales were associated with all age groups of *P. patula* plantations (Figure 3).

4. Discussion

4.1. Diversity of Fungal OTUs

Fungi have been described as the most interesting, enigmatic and species-rich organisms on Earth [69]. The use of molecular methods in recent years has dramatically increased the number of fungal OTUs detected worldwide [70]. In this study, we detected a total of 2262 high-quality OTUs, of which 1303 OTUs (58%) belonged to the Ascomycota, the largest phylum of fungi [71], indicating the dominance of Ascomycota in the *P. patula* forests investigated in this study. Fungi often interact with other organisms, forming beneficial or mutualistic associations. Conifers in the Pinaceae usually form symbiotic relationships with ECM fungi. Thus, ECM fungi were likely to play an important role in the *P. patula* plantations in our study area. However, of the total OTUs identified at genus level and classified by ecological function, 41% were saprotrophs, 7% were plant pathogens, and only 2% were ECM fungi. An explanation for the small proportion of ECM fungi detected might be that the plantation area was located in a non-ECM biome area. The native vegetation that originally grew in the study area was destroyed many years ago by logging and clearance for crop cultivation [10]. Therefore, fungal symbionts compatible with *P. patula* may be absent in the native fungal community in the soil, enabling ECM fungi introduced to the plantation area along with *P. patula* to co-invade the soil habitat [72]. However, the ecological function of 48% of the fungi identified in this study is unknown, indicating that we have hardly scratched the surface in terms of understanding the role played by fungi in these plantation forest systems. It might also an indication for the lack of scientific studies on the local fungal flora in the country. Thus, they are highlighting the need for further studies in the study area.

4.2. Diversity of Functional OTUs along the Chronosequence of Stands

The Shannon and Simpson diversity indices were determined to explain variations in the soil fungal functional groups at different stages of *P. patula* stand development. Only ECM fungi showed differences in their diversity among the stand age groups. Initially, we expected that as stands developed, soil fertility would decrease over time and that these conditions would lead to higher ECM fungal diversity along the chronosequence. By contrast, the soil fertility of young *P. patula* stands was expected to be higher than that of old stands and, therefore, the trees were expected to exert less influence on the fungal microbiome. However, our investigations revealed that the 5 and 36-year-old stands had more diverse ECM fungi in their soils than the 11-year-old stand and were more fertile. Despite this, the relative proportion of ECM abundance was higher in the 11-year-old stand than in the 5 and 36-year-old stands due to the dominance of some ECM species in the 11-year-old stand.

Soil microorganisms, including fungi, are influenced by stand development in several ways [73,74]. As the stand develops, the amount of tree cover directly modifies the amount of light available, which affects the composition of the understory, which regulates carbon allocation, nutrient cycling and soil water content [75]. The relatively higher diversity indices of ECM fungi in the 5-year-old stand than in the 11-year-old stand may possibly be explained by the less developed tree canopy at the earlier stage of stand development, which may have allowed diverse ECM fungi to interact extensively with the root systems of understory plants [75]. Another explanation for the relatively higher diversity indices of ECM fungi in the 5-year-old stand may be the previous land use of the plantation area. Deacon and Fleming [76] demonstrated that when afforestation takes place on land initially used for other purposes, the ECM fungal spores are the fundamental inoculum during the early stage of ECM succession. In this case, prior to the establishment of *P. patula* plantations, the study area was previously used for agricultural purposes, which might have contributed to the diversity of ECM fungi in young stands of *P. patula* trees due to the primary succession of ECM fungi through inocula in the spore bank derived from other nearby mycorrhizal-associated plantations such as *Eucalyptus* sp. L'Hér. plantations [24,36].

The relatively higher diversity indices of ECM species in the 36-year-old stand than in the 11-year-old stand may reflect a difference in the management of the 36-year-old stand. Chen et al. [77] indicated that thinning could increase the relative abundance of mycorrhizal fungi because thinning opens up the forest canopy, which increases soil temperature and moisture [78]. Thinning may therefore have a positive effect on microbial activity [79] because soil temperature and moisture influence the reaction of microbial enzymes and, thus, shift the microbial community composition by altering substrates and extracellular enzyme activity [80]. Dove and Keeton [81] and Tomao et al. [82] have suggested that fungal diversity can be conserved or even increased using forest management practices that enhance the structural complexity of stands and the late-successional characteristics of the forest and by carrying out low-impact logging operations. In this study, the 36-year-old stand had undergone thinning as part of a management operation in the study area [8], which could have enhanced the root growth of the remaining trees [83,84], providing new environments for soil microbes, which could lead to an improvement in fungal diversity through root attachments. Castaño et al. [12] observed that the species diversity of soil fungi remained stable after thinning, regardless of its intensity, when sufficient host trees and functional roots from thinned trees were retained. This finding has been supported by a number of different reports. For example, according to Mölder et al. [85], thinning could maintain a high level of ECM fungal diversity in mature stands. Chen et al. [86] and Dang et al. [75] also hypothesized that when thinning operations are performed, light availability, water, and nutrients increase, which improves the forest microclimatic conditions and, hence, the diversity of ECM fungi could be improved by this type of management practice. Furthermore, Goldmann et al. [87] reported that ECM fungi were less diverse in unmanaged forests than in highly managed stands. Thus, we suggest that forest management practices such as thinning could be one of the factors that impact ECM fungal diversity along the chronosequence of *P. patula* stands, although this should be further studied.

4.3. Fungal Composition and Edaphic Variables

Forest soils contain a diverse range of fungal species. The composition of soil fungal communities is influenced by different factors, such as dispersal, plant diversity, soil properties, land use, and climate, which are key components of forest systems [88,89]. Specifically, different fungal species are likely to respond to environmental drivers in different ways, depending on their characteristic traits [90,91], and, thus, in turn, the composition of soil fungal communities is directly correlated with soil fertility and plant growth status [92]. Mycorrhizal species are a particularly important part of the soil fungal community because they form a beneficial symbiotic association with plants, providing them with nutrients in return for photosynthetically fixed carbon [93], which is especially relevant under nutrient-limited conditions [94]. ECM fungi also play a key role in alleviating the drought stress of the host tree [95]. The composition of ECM fungi in the soil is also correlated with soil fertility and the growth status of the host trees [92,96]. In this study, we found that both stand age and soil fertility were factors that affected the fungal community composition in our study area. Our ordination analysis indicated that the 36-year-old stand and the 5-year-old stand had distinctive soil fungal communities, characterized by a relatively high number of ECM species. Previous studies have related similar findings regarding fungal community composition to several factors, such as changes in soil fertility [24], changes in root density [97], specific life-history events that have occurred since the stand was established or changes in microclimate conditions [12]. For example, less litter accumulation in young *P. patula* stands resulted in less organic matter and a lower C/N ratio in the 5 and 11-year-old stands in this study compared with the 36-year-old stand. This situation leads to trees having a greater dependence on mycorrhizal fungal associations for enhanced nutrient and water uptake and availability [24]. Similarly, the greater diversity of ECM species in young stands may indicate that suitable symbionts are present. However, older stands have a greater capacity to reduce fluctuations in temperature and to maintain adequate moisture levels [98–100], which is particularly important for the occurrence of ECM fungi. The abundance of ECM fungi in older stands is generally greater than in younger stands, which could be facilitated by the root systems of old trees, which could increase the

chances of ECM associations forming [85], thereby facilitating the easy uptake of nutrients by trees [94]. Thus, the distinct composition of ECM fungi in young and old stands might not only be related to site quality factors, such as soil fertility, but also to stand age factors (e.g., the increasing area of tree root exploration in the soil with stand age); however, this needs to be investigated further. Given that the amount of organic matter, available P, and the C/N ratio of 5 and 11-year-old stands showed no greater difference in their values, this may have enabled 11-year-old stands to develop an association with only a limited number of ECM fungi, but a higher relative abundance of these ECM fungi, which could indicate increased dependence of *P. patula* trees on a limited number of dominant symbiont species. In any case, the survival of *P. patula* in soils in which ECM species comprise only a small proportion of the microbial community, together with other factors, supports the view that *P. patula* is well adapted to the conditions in this study area; however, this also needs further study.

Fertile soil contains nutrients that enable the growth and development of a soil fungal community [101]. Thus, in turn, the fungi are directly influenced by edaphic parameters [102–104]. In this study, edaphic cation elements, such as Mg and K, were also correlated with the overall fungal community from the whole data set, which indicates that soil cation concentrations could influence the composition of the fungal community [105]. Cations in general play an important part in a number of physicochemical processes, such as photosynthesis [106] and, thus, can affect plant photosynthesis and, hence, the amount of carbon that is available to soil fungi and bacteria [107]. Of the various cations, Ca is one of the main edaphic factors that influence the structure of soil fungal communities worldwide [108]. Other edaphic elements have also been reported to influence the composition of fungal communities in forest systems. For example, in this study, available P and tree age influenced the composition of mycorrhizal fungi (Table 4), which was similar to the findings reported by Rosenstock et al. [109]. The composition of fungi in the soil particularly that of mycorrhizal fungi, can also be influenced by N availability [110]. High levels of available N could decrease the dependency of the host plant on mycorrhizal fungi, which could reduce the amount of carbon allocated to fungi [111], which eventually could cause competition among the fungal species and could lead to changes in their composition [96,110]. In this study, the C/N ratio of soil in 5 and 11-year-old stands was relatively low compared with that of 36-year-old *P. patula* stands (Table 1). This result is inconsistent with Wang and Wang [96] who reported that a high C/N ratio negatively influenced fungal community structure, probably because a high concentration of N restrains the expansion of fungi. Our results also confirmed this, in the sense that the fungal diversity was low in stands where the soil C/N ratio was high, indicating that a high C/N ratio might not favor the fungal community in the forests in the study area. Soil organic matter could also impact the composition of soil fungal communities because fungi generally extend their mycelia at the soil–litter interface [112]. The amount of organic matter affects the water holding capacity of soil and nutrient availability, which could affect mycelial outgrowth and network formation [113,114]. However, soil acidity can also influence the composition of soil fungal communities [115,116] through its influence on spore germination and mycelial development [117].

Fungi have different life-history strategies and, in plantations, early colonizer fungal species that relish disturbance colonize first, followed by superior competitors that can outcompete the early colonizer species in older stands where resources are getting scarcer. In this study, we found that some fungal species associated with *P. patula* trees were detected at all age stages of tree development, such as those belonging to the genera *Tomentella*, *Ramaria*, and *Inocybe*. These genera have several hundreds of species, many still undescribed, and their strategies do not seem to be conserved at the genus level. Of these, *Tomentella* and *Inocybe* are cosmopolitan species that inhabit *Eucalyptus* plantations in Ethiopia [24]. Species of *Rhizopogon* were also associated more with younger stands (5- and 11-year-old stands) in this study, which supports previous findings that they are early colonizer fungal species [118]. The *Rhizopogon* species are known as spore bank species that facilitate the establishment of trees in formerly non-forest habitats. Other taxa belonging to the genera *Amanita* have also been reported in our *P. patula* plantation forests. These fungi species are well-known for their association with conifer forests as the genera is characteristic of late-stage pine stands that are 30–40 years old [119,120].

5. Conclusions

Our study explained the soil fungal community composition associated with a *P. patula* plantation in Ethiopia. The diversity value of vital fungi such as ECM was relatively higher in the youngest stand than in the two older stands. Stand age and soil fertility were also found to affect fungal community composition. Some ECM fungi were found as early colonizer species in the young stand and were replaced by superior competitor species as the *P. patula* stands developed.

The overall low level of ECM species richness detected in the *P. patula* stands is probably because this is a plantation of an ECM tree in a non-ECM biome. Due to the importance of fungi in plantation forest system, the results of this study could be relevant for the promotion and conservation of forests in Ethiopia through the promotion of non-wood forest products such as fungi, which could also provide food resources for poor populations during times of food scarcity. Thus, it is imperative to investigate how soil fungal communities respond to management regimes such as thinning and clear-cutting. The high diversity and relative abundance of plant pathogenic fungi detected in this study also highlights the need to protect Ethiopian plantations from plant diseases and pests.

Although the ecological conditions of all studied plots were similar in terms of climate, altitude, and soil, the results of this study should be considered as a case study, given that the plots were established in a single stand for each age class for each treatment and, therefore, the applicability of any conclusions to other stands should be treated with caution. Furthermore, additional scientific investigations of the plantation forest ecosystem are needed in order to consolidate the Ethiopian fungal biodiversity database.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/10/1109/s1, Table S1: Summary of the Similarity Percentage (SIMPER) analysis showing contrasts between the cumulative total contribution (50% cut-off) and the contribution (%) of the most influential fungal operational taxonomic units to the dissimilarity of the soil fungi detected in three *Pinus patula* stands of different age groups in a plantation in Wondo Genet, Ethiopia.

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