Changes in fungal diversity and composition along a chronosequence of *Eucalyptus grandis* plantations in Ethiopia

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Abstract

Eucalyptus tree species are widely used in Ethiopian plantations, but the impact of these plantations on the soil fungal communities is still unknown. We assessed the changes in diversity, species composition and ecological guilds of the soil fungal communities across tree ages of *Eucalyptus grandis* plantations by DNA metabarcoding of ITS2 amplicons. Changes in soil fungal species composition, diversity and ecological guilds were related to stand age but also to fertility changes. The relative abundance of saprotrophs and pathogens (i.e. mainly free-living fungi) were negatively correlated with stand age, and positively with soil fertility. In contrast, the relative abundance and diversity of ectomycorrhizal species were higher in older, less fertile stands, including well-known cosmopolitan species but also species associated to *Eucalyptus*, such as *Scleroderma albidum* and *Descomyces albellus*. We show soil fungal community changes linked to a progressive soil colonization by tree roots but also related to soil fertility changes.
Keywords: *Metabarcoding, Fungal community, Mycorrhizal, Stand age, Soil fertility, Fungal diversity*

Introduction

Ethiopian natural forest cover has dramatically decreased during the last decades in an estimated deforestation rate between 150,000 and 200,000 ha. $year^{-1}$ (Zewdie et al., 2010). As a result, natural forests represent today less than 3% of the total country lands (Lemenih and Bekele, 2008; Taddese, 2001). During the last decades, plantations with fast-growing tree species have been established to obtain timber and reduce pressure on natural Ethiopian forests (Bekele, 2011; Zewdie et al., 2010). These plantations are mainly composed of *Eucalyptus*, *Cupressus*, *Pinus* and *Acacia* species (Bekele, 2011; Moges et al., 2010), which represent around 506,000 ha in Ethiopia (FAO, 2011). *Eucalyptus* species are the most widely used species in these plantations, representing more than half of the total national plantation area (Bekele, 2011). *Eucalyptus* species are often chosen for their adaptation to different ecological conditions, management and fast-growing nature, serving as main source of firewood, poles, posts and farm implements in Ethiopia (Kelemu and Tadesse, 2010).

Impacts of *Eucalyptus* on native flora has already been reported in other studies; e.g. *Eucalyptus* is known to compete with native species for nutrients and moisture, inhibits the understorey by exudating phytotoxic chemicals (Jaleta et al., 2016) and may promote nutrient depletion (Temesgen et al., 2016). In contrast to these negative effects, other studies have shown that these plantations host some native herbaceous species and can promote natural regeneration of Ethiopian flora (Lemenih, 2004; Yirdaw, 2002), allowing the regeneration of understorey vegetation under natural successional dynamics (Onaindia et al., 2013). *Eucalyptus* plantations are a source of non-timber forest products, such as edible mushrooms (Dejene et al., 2017a), although information on the impact caused by these plantations on soil fungal communities is still unknown.

Soil microbes are essential components of forest ecosystems. Among soil fungi, mycorrhizal species are especially important because they form a beneficial symbiotic association with plants, providing them nutrients in return for photosynthetically fixed carbon (C) (Smith and Read, 2008). Ectomycorrhizal (ECM) fungi are also key players in the alleviation of drought stress for trees (Mohan et al., 2014), and the role of these organisms is especially relevant for nutrient uptake by plants under nutrient-limited conditions (Read and Perez-Moreno, 2003). Arbuscular mycorrhizal (AM) species are especially efficient to uptake and transfer inorganic nutrient forms such as phosphorus

(Read and Perez-Moreno, 2003). In contrast, other fungal species such as saprotrophs have a paramount role in litter and SOM degradation (Baldrian et al., 2011). Understanding soil fungal communities interactions in forest ecosystems is important, because such interactions determine many important ecosystem processes such as C storage and nutrient cycling (Kyaschenko et al., 2017). Interguild fungal interactions and fungal community shifts may be modulated by changes in the environment such as pH (Rincón et al., 2015), nitrogen (N) (Kjøller et al., 2012), climate (Castaño et al., 2018; Geml et al., 2016), but also human disturbances, such as tree harvesting (Kohout et al., 2018). Thus, such environmental changes potentially cause direct and indirect alterations in ecosystem functioning with respect to SOM decomposition, plant nutrition and C cycling by affecting the soil-associated microbiome (Kohout et al., 2018; Clemmensen et al., 2015; Averill et al., 2014).

Eucalyptus tree species have dual mode of mycorrhizal symbiosis, with both ECM and AM taxa found associated to these tree species (Adjoud-Sadadou and Halli-Hargas, 2017), although association with AM species seems to be restricted in seedlings or younger trees (Adams et al., 2006). Changes in soil chemistry, fertility or changes in host tree status may potentially change the balance of such mycorrhizal associations. For example, depletion of inorganic soil N could negatively affect AM taxa due to their preference for inorganic N forms, although evidences of accession of AM fungi to organic sources has been also shown (Thirkell et al., 2016). However, ECM fungi are especially adapted to mobilize N from organic forms through oxidation chemistry (Tunlid and Lindahl, 2015), which makes these fungi especially adapted to nutrient-stress environments (Read and Perez-Moreno, 2003). Such adaptations could explain why AM species are more abundant on seedlings than in adult trees, when soils seem to be more nutrient stressed (Adams et al., 2006). In addition to this, studies on *Eucalyptus* plantations' impacts on the surrounding environment have neglected the soil microbial communities, especially saprotrophs, despite their importance for understanding plant-soil feedbacks and soil nutrient dynamics.

Replacing native vegetation or grasslands with exotic trees, such as the case of *Eucalyptus* plantations in Ethiopia, is expected to result in changes in soil physico-chemical properties (Temesgen et al., 2016) as well as in the composition of the associated soil microbial communities and related ecosystem processes. Here, we

investigated the changes in fungal diversity, community composition and ecological guilds along a chronosequence of *E. grandis* plantations. We expected that soil fertility would decrease with the age of *E. grandis* stands, with expected lower N and P contents and higher C/N ratios in older stands. In addition, we expected that soils from older *E. grandis* stands would be more dominated by root-associated fungal species than in younger stands due to an increasing soil colonization of roots and associated symbionts. Thus, we hypothesize that (i) decreasing fertility and increasing dominance of *Eucalyptus* along the chronosequence will result in an increase in the diversity and abundance of ECM species. In contrast to old stands, young stands are expected to be more fertile and trees will be exerting less influence on the fungal microbiome, thus we hypothesise that (ii) at younger stands there will be a higher abundance and diversity of other functional groups such as saprotrophs or AM species.

Material and Methods

Study area

The study was carried out in the Wondo Genet plantation forest area in Southern Ethiopia (coordinates 7º 05' 02'' N 38º 37' 08'' E, altitude between 1 600 and 2 580 m. above sea level), located approximately 265 km from Addis Ababa. The site has a mean annual rainfall of 1 210 mm, with most of the rainfall recorded during summer. The mean annual temperature is 20 °C. Three distinct age classes of *E. grandis* plantations were selected for this study: i.e. 10, 19 and 37 years. All these had historically been natural forests, which were cut and converted to grasslands before the introduction of *E. grandis* trees. Three plots of each stand age class were selected for soil sampling, resulting in a total of nine plots. These plots were separated each other by 120 m. Hence, the plots were similar in terms of their ecological conditions such as climate and altitude.

Soil samplings

All nine plots were sampled in July 2015. In each plot, five soil cores (20 cm deep and 4 cm in diameter) were taken using a cylindrical soil borer extracted along the centreline of each transect at 5 m distance from each other. We sampled well-decomposed organic layers and mineral soil, but we discarded the litter layer (intact and partially decomposed leaves), given that the fungal community composition in the leaves tends to diverge from that in soil (Voříšková et al., 2014). Soil cores were dried, sieved

through a 1 mm mesh and grounded to a fine powder using a mortar and pestle. A subsample was stored at -20ºC for molecular analysis whereas another subsample was taken for determination of soil physico-chemical parameters (Table 1). The soil pH and electrical conductivity were measured using soil:water (1:2.5) suspension and in the supernatant, using pH meter and Electrical Conductivity meter, respectively from the same suspension (Reeuwijk, 2002). Organic carbon content was determined using wet digestion of Walkley and Black (1934). Total N content in soils was determined using the Kjeldahl procedure following Kim (1996). Available P was determined using sodium bicarbonate (0.5M $NaHCO_3$) as extraction solution (Olsen and Sommer, 1982). The color intensity was measured spectrophotometrically at 882 nm. For soil particle size analysis hydrometer method (Bouyoucos, 1951) was employed, using sodium hexametaphosphate (Calgon solution) as the dispersing agent. Once the sand, silt, and clay separates were calculated in percent, the soil was assigned a textural class name based on ASTM Software.

Table 1. Soil physico-chemical characteristics of the *E. grandis* plots from Wondo Genet (Ethiopia). OM= Organic matter, P= Phosphorus, K=Potassium, Ca=Calcium, Mg= Magnesium, Na= Sodium.

Age (years)	Sand (%)	Silt (%)	Clay (%)	pH	OM (%)	N (%)	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Na (mg kg^{-1})
10	59.5±5.8	21.8±11.1	18.8±6.8	6.2±0.1	7.7±1.9	0.5±0.1	60.9±22.3	0.7±0.1	27.2±4.6	9.7±1	0.9±0.1
19	61.5±12.3	22±3.3	13.2±7.3	5.2±0.2	5.5±0.1	0.3±0.1	30.7±8.7	0.4±0.1	14.3±1.8	4.9±0.9	1.6±0.2
37	50.1±1.8	24.3±3	25.7±1.5	6.2±0.3	4.6±0.7	0.3±0.1	26.7±5.7	0.5±0.1	16.4±1.2	5±0.9	1.7±0.1

Molecular analysis

DNA extraction was performed from 0.25 g of soil per sample with the PowerSoil™ DNA Isolation Kit (MoBio laboratories Inc., Carlsbad, CA, USA). PCR reactions were performed in triplicate for each sample to minimize PCR biases. PCR reactions were performed in 40 µl reaction volumes containing 24 µl of sterile water, 1.00 µl of DNA template, 4.00 µl of 10x buffer, 5.60 µl of MgCl2 (25 mM), 1.50 µl dNTPs (10 mM), 0.50 µl BSA (2%), 1.50 µl of reverse and forward primers (10 µM) and 0.4 µl Taq polymerase (Invitrogen, Carlsbad, CA). We used the following PCR conditions: an initial denaturation step at 95°C for 5 min; then 35 cycles of 95 ºC for 20 s, 54 ºC for 30 s and 72ºC for 1.5 min; and ending with one cycle of 72ºC for 10 min. The ITS2 rDNA region amplified using the forward primer fITS7 (Ihrmark et al., 2012) and reverse primer ITS4 (White et al., 1990). These primers have been especially designed to detect

a wide-range of fungal species, but they unmatch with several AM families (Lekberg et al. 2018). Similarly, copy gene number per biomass unit in AM species is much lower than in ECM species. Therefore, a reliable description of AM species is not possible, neither a comparison between AM and ECM species, although an evaluation of the changes in relative abundance of AM species across samples is still possible. To be able to identify each sample, the ITS4 primer was labelled with sample-specific Multiplex Identification DNA-tags. A negative control consisting of sterile water instead of DNA was included in each PCR replicate and underwent the PCR under the same experimental conditions and was shown on a gel to be amplicon free. Ion Torrent sequencing was carried out at the Naturalis Biodiversity Center. We used the sequencing Ion 318TMChip to allow for highest possible sequencing coverage.

Quality control and bioinformatics

Raw sequence reads were obtained from the Ion Torrent output that comprise demultiplexed sample reads. Primers and poor-quality ends were trimmed based on 0.02 error probability limit in Geneious Pro 8.1.8 (BioMatters, New Zealand). Subsequently, sequences were filtered using USEARCH v.8.0 (Edgar, 2010) based on the following settings: all sequences were truncated to 200 bp and sequences with expected error >1 were discarded. The remaining sequences were collapsed with USEARCH v.8.0 (Edgar, 2010) into unique sequence types on a per-sample basis while preserving read counts. Singleton sequence types were discarded and the resulting 305,520 high-quality sequences were grouped into 3 286 operational taxonomic units (OTUs) with USEARCH at 97% sequence similarity while simultaneously excluding 181 chimeric sequences. We assigned sequences to taxonomic groups based on pairwise similarity searches against the curated UNITE+INSD fungal ITS sequence database containing identified fungal sequences with assignments to species hypothesis groups (Kõljalg et al., 2013). After excluding OTUs with <70% similarity or <150 bp pairwise alignment length to a fungal sequence, the dataset contained 2 886 fungal OTUs, representing total of 296,384 high quality sequences. Ecological guilds of identified taxons at species or genera level was performed using FUNGuild (Nguyen et al., 2016).

Data analysis

Statistical analyses were implemented in the R software environment (version 2.15.3; R Development Core Team 2013) using the vegan package for multivariate analysis (Oksanen, 2015), "nlme" package for linear mixed models (LME: Pinheiro et al., 2016) and "indicspecies" for indicator species analysis (De Cáceres et al., 2017). "iNEXT" package (Hsieh et al., 2016) was used for diversity analysis and interpolation of fungal diversity data. Ordination of community data (Detrended Correspondence Analysis and Canonical Correspondence Analysis: DCA and CCA, respectively) was carried out using CANOCO version 5.0 (Biometris Plant Research International, Wageningen, The Netherlands).

First, we investigated whether there was a strong correlation between some of the measured environmental variables, especially soil variables (OM, P, Mg, N, K, Ca), with variance inflation factor (VIF) values > 20, except for pH (Fig. S1). Accordingly, the first axis (PCA1) of a PCA considering these variables was used as a soil chemistry index. Soil chemistry index was also representing a fertility gradient (Fig. S1). Similarly, the first axis (PCA1) of a PCA considering soil texture values (sand, silt, clay) was used as a soil texture index.

DCA considering the fungal species composition was used to obtain graphical representations of fungal community similarity across stand ages and the parameters related to soil chemistry and textures. Effects of these environmental parameters were tested by CCA over the Hellinger transformed community data, by testing their simple and conditional effects. Simple term effects are the effects of an individually tested factor, whereas conditional effects are the effects of each factor before removing the effects of other factors. Significance of the environmental variables were tested by Monte Carlo permutations test (999 permutations). Similarly, significance of the environmental variables were also confirmed using Permutational multivariate analysis of variance based on distance matrices (function "adonis" and Bray-Curtis distance). Responses of specific fungal taxa to either stand age or soil fertility were tested by running species response curves using Generalized Linear Models (GLM) and indicator species analysis was used to identify taxa associated to a certain stand age. The same analyses (DCA, CCA) were carried out using the relative proportions of each guild as a response variable, and the environmental variables (stand age, fertility index) as an explanatory variable. Significant effects of these environmental variables to each guild

was specifically tested by ANOVA and confirmed by Linear Mixed Effects Models (LME), in which the effect of stand age was specifically tested by defining this variable as fixed factor and the soil fertility as a random variable. Similarly, the effect of soil fertility over each specific guild was tested by defining this variable as fixed factor and tree age as random variable. Effects of these LME were tested by ANOVA ($P<0.05$ was considered significant effect).

Hill's series of diversity indices (Hill, 1973) were used to compare differences in diversity values across fertility indices and tree ages. Hill's diversity consists of three numbers: N0 is species richness; N1 is the antilogarithm of Shannon's diversity index; and N2 is the inverse of Simpson's diversity index. These tests were performed over the whole fungal diversity values, but also separate diversity analyses were carried out considering only species belonging to specific guilds (e.g. ECM, saprotrophs). N0, N1 and N2 Hill's diversity indices were calculated from the asymptotic estimates implemented in "iNEXT". Diversity comparison between tree ages and soil fertility indices were performed using ANOVA.

Results

Sequencing output and fungal community composition

Clustering resulted in 2 886 OTUs, of which 350 were singletons. We obtained an average of 24,711±6 325 reads in each site. Overall, saprotrophs (Dung saprotrophs, other saprotrophs) were the most abundant across the whole dataset, representing 47% of the community. The dominance of these groups was followed by ECM species (15%), plant pathogens (12%) and endophytes (12%). Less dominant groups were represented by animal pathogens, AM, wood saprotrophs and soil saprotrophs.

Environmental drivers affecting the fungal species composition

Simple effects of explanatory variables revealed that soil fertility, pH and tree age, but not texture, significantly affected the fungal species composition (Fig. 1a, 1b; Table 2). Conditional effects also identified soil chemistry and tree age significantly affecting the soil fungal species composition (Table 2). Significance of soil chemistry and stand age were also confirmed by PERMANOVA analyses, with both stand age ($F=2.93$, $P=0.007$, $R^2=0.23$) and soil chemistry ($F=3.52$, $P=0.002$, $R^2=0.28$) affecting the fungal

species composition. Indicator species analyses and GLMs showed that several ECM taxa (e.g. *Laccaria* sp., Telephoraceae, *Descomyces*, *Scleroderma*; Fig. 1a, 1b) were associated with older, less fertile stands. In contrast, specific AM species (e.g. Glomeraceae) were more associated with younger, more fertile stands, together with many saprotrophic species such as *Mortierella, Tetracladium,* or fungi belonging to Pleosporales (Fig. 1a, 1b), but also plant pathogens (*Neonectria*, Fig. 1a, 1b). When considering only the ECM species community, there were significant compositional changes across stands of different ages (P=0.05), with specific taxa such as *Laccaria* sp. and *Telephora* sp. being more prevalent in older stands.

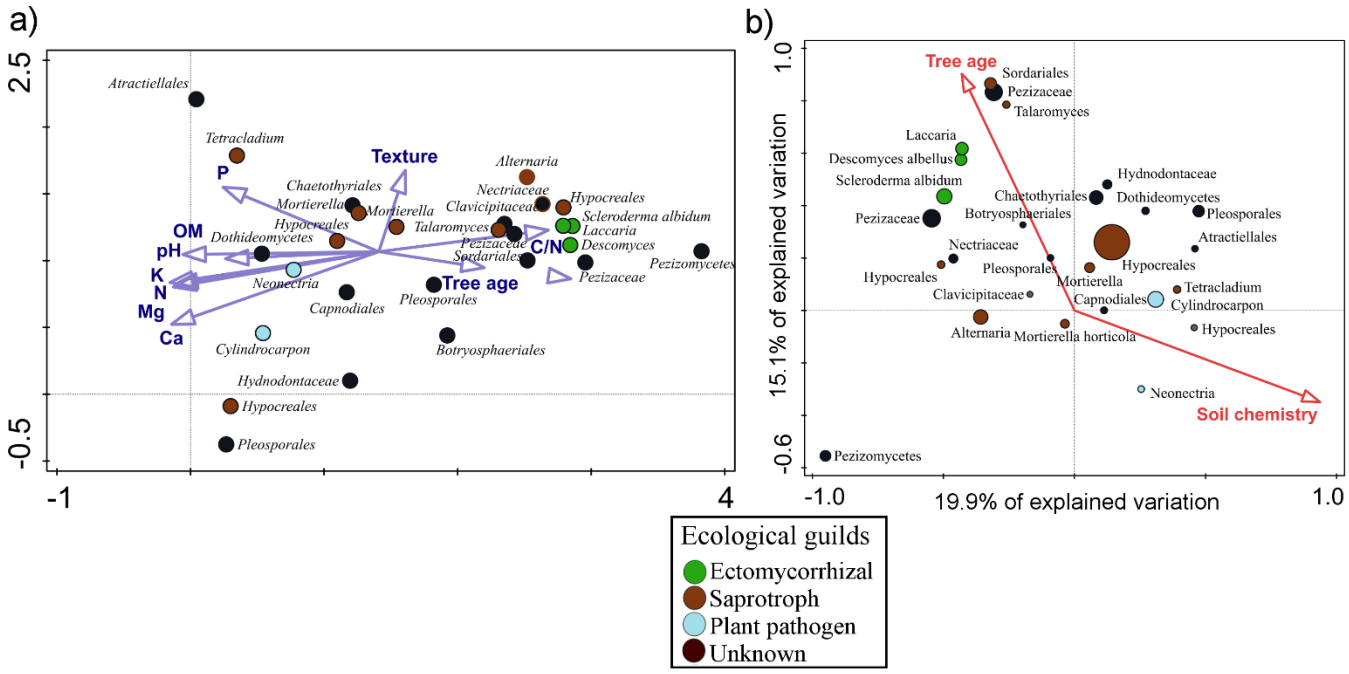

Fig. 1. (a) Detrended correspondence analyses (DCA) and (b) Canonical Correspondence Analysis (CCA) of the species level community composition of soil fungi in Ethiopian forests, as analysed by sequencing internal transcribed spacer 2 amplicons. Environmental parameters are shown as supplementary variables in (a), whereas soil chemistry and age are constrained parameters in (b). In (b), species symbol sizes are proportional to the average relative abundance. The figure shows the 26 most abundant species hypotheses coloured according to their ecological guild. Here, only ectomycorrhizal, saprotrophs and plant pathogens are shown because other fungal guilds were less abundant and therefore not shown in this figure.

Table 2. Significance of the CCA analysis based on simple term effects and conditional effects, considering the Hellinger transformed fungal community data at species level. Numbers in bold indicate significant effects (P<0.05).

	Simple Term Effects			Conditional effects		
Variable	**Explains %**	**pseudo-F**	**P**	**Explains %**	**pseudo-F**	**P**
Soil chemistry	**19.3**	**1.7**	**0.004**	**19.3**	**1.7**	**0.002**
Age	**16**	**1.3**	**0.037**	**15.7**	**1.4**	**0.017**

Texture	13.2	1.1	0.273	11.9	1.1	0.411

Fungal functional changes across tree ages and soil fertility

When considering the composition of ecological guilds, simple effects of explanatory variables revealed again that soil chemistry (~ fertility) was responsible for up to 41% of the total fungal functional variation (F=4.9, *P*=0.005). Tree age was responsible for up to 33.4% of the total variation (F= 2.7, *P*=0.01), sharing 15.3% of the variance with soil chemistry. However, there were specific guild responses to either tree age and soil fertility, with some of these functional groups responding differently to each these parameters (Fig. 2). For example, undefined saprotrophs were only significantly increased according to a fertility index, whereas plant pathogens decreased across tree ages and increased with more fertility (Table 3; Fig. 2, Fig. 3). Finally, ECM species decreased with soil fertility but increased with tree age, with a higher ECM abundance in older stands (Table 3; Fig. 2, Fig. 3).

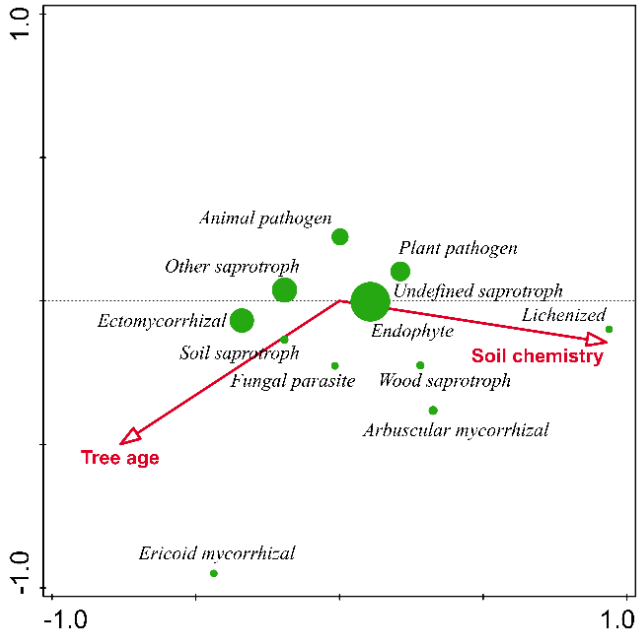

Fig. 2 DCA plots considering the relative proportions of fungal guilds in relation to tree age and soil chemistry in Ethiopian forests, as analysed by sequencing internal transcribed spacer 2 amplicons. Symbol sizes are proportional to average relative abundances of each guild.

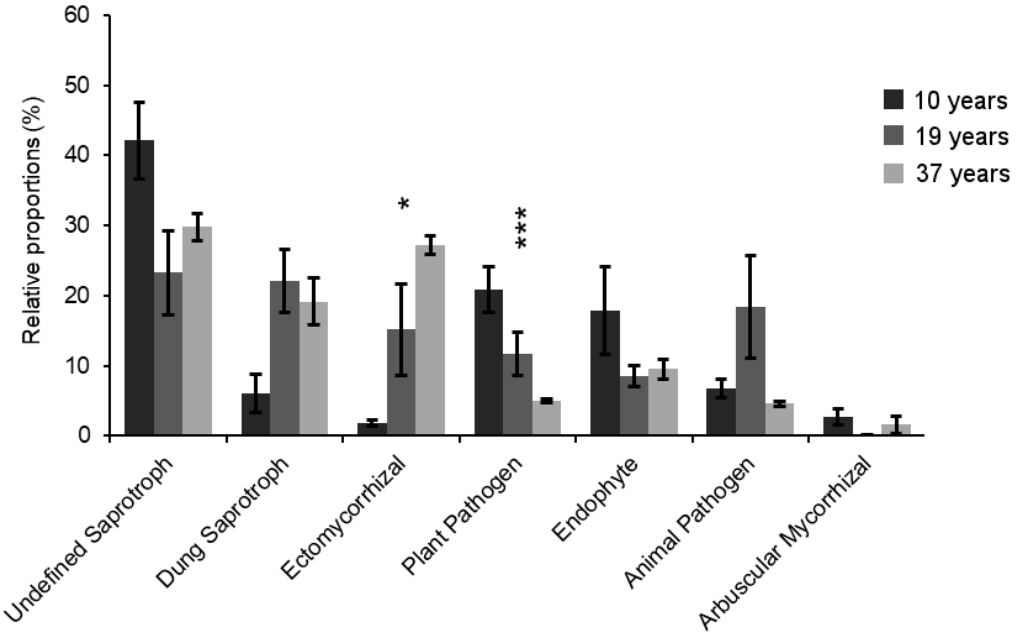

Fig. 3 Fungal functional changes showing the most abundant guilds in 10, 19 and 37 years stands. Here, the seven most abundant guilds are shown, as classified by Nguyen et al., (2016). Significance levels: *** P<0.001, ** P<0.01, * P<0.05.

Fungal diversity changes across tree ages and soil fertility

Stand age significantly affected the total fungal alpha diversity N0, which represents the fungal richness (Table 3, Fig. S2). N0 decreased with tree age, with a 35% decrease in fungal richness from 10 years to 39 years (Fig. 4). Despite the decreasing tendency in N1 and N2 values across tree ages, these differences were not significant (Table 3). Both N0, N1 and marginally N2 values increased with increasing soil fertility (Table 3), especially in the very fertile sites. However, diversity changes across tree ages and soil fertility were distinct across functional groups. Thus, for ECM species, there was an increase in alpha diversity across years (Fig. 4), concurrent with an increase of their relative abundance (Fig. 3). Although the relative abundance of AM fungi was not affected by tree age neither soil fertility (Fig. 3), this guild was much more diverse at the 10-years old sites (Fig. 4). For undefined saprotrophs, there were no significant changes in alpha diversity neither in abundance across years, although they significantly increased in the most fertile plots (Fig. 4; Table 3). Finally, despite plant pathogens increased their relative abundance in younger stands, the diversity of this group was not affected by tree age neither soil fertility (Fig. 4; Table 3).

Table 3. Significance of the effects between the relative proportions of specific fungal guilds and diversity indices as measured with Hill's numbers, and the tree age and soil chemistry. Hill's diversity consists of three numbers: N0 is species richness; N1 is the antilogarithm of Shannon's diversity index; and N2 is the inverse of Simpson's diversity index. Here, Numbers in bold indicate significant effects (P<0.05).

	Tree age		Soil chemistry	
Guilds	**F**	**P-value**	**F**	**P-value**
Ectomycorrhizal	**11.16**	**0.012**	**7.88**	**0.038**
Other saprotrophs	2.13	0.187	6.05	0.057
Plant pathogen	**50.24**	**<0.001**	**7.83**	**0.038**
Undef. saprotrophs	0.35	0.575	**7.83**	**0.037**
Endophytes	0.73	0.42	1.34	0.3
Animal pathogen	0.69	0.432	0.41	0.549
Arbuscular	0.05	0.833	2.41	0.181
Hill's				
N0	**11.57**	**0.011**	**7.02**	**0.045**
N1	2.40	0.165	**9.96**	**0.025**
N2	0.30	0.599	4.34	0.091

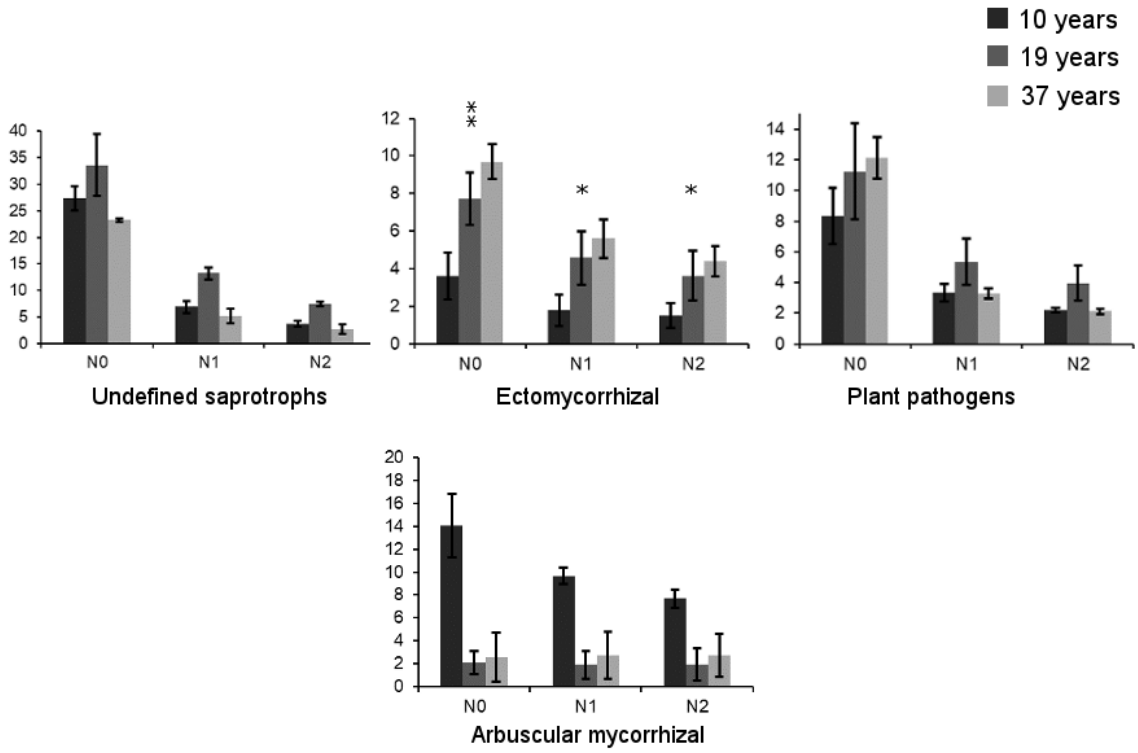

Fig. 4 Changes in interpolated diversity values for each guild, as measured with Hill's numbers, across the three age classes (10, 19 and 37 years old stands) and considering root-associated species and saprotrophs. Hill's diversity consists of three numbers: N0 is species richness; N1 is the antilogarithm of Shannon's diversity index; and N2 is the inverse of Simpson's diversity index. Significance levels: *** P<0.001, ** P<0.01, * P<0.05.

Discussion

Our study showed profound soil fungal community and changes in ecological fungal guilds along a chronosequence of *E. grandis* stands. Development over time of *E. grandis* plantations was concurrent with a decrease in soil fertility, especially N and P. Thus, fungal compositional and fungal functional changes observed in this study across tree ages parallel with observed changes in soil fertility, suggesting that both drivers (tree aging and soil fertility) may potentially be interrelated. It seems that such changes had distinct effects on the functional community depending on the guild, with both diversity and abundance of ECM species increasing over time, and either abundance and/or diversity of pathogens, saprotrophs or AM fungi decreasing with stand age but increasing with more soil fertility. A reliable description of the AM taxa found in our study is not possible, neither a comparison between AM and ECM species. However, we believe a comparison between samples of the total relative abundance of AM species is still possible.

We observed profound changes in soil fungal communities across the *E. grandis* chronosequence. Changes in fungal communities across stand ages has been reported for soil fungal communities (Blaalid et al., 2012; Clemmensen et al., 2015) but also for fungal fruiting body communities (Bonet et al., 2004; Dejene et al., 2017a, 2017b). Fungal community shifts along chronosequences have been related to several factors, such as changes in soil chemistry or fertility (Blaalid et al., 2012; Clemmensen et al., 2015), changes in root density (Peay et al., 2010), specific life-history events since tree stablishment (Blaalid et al., 2012), or changes in microclimate conditions (Castaño et al., 2018). Our results suggest that observed changes and shifts in community composition of soil fungi may be both related to changes in soil chemistry or fertility, but also related to stand age (i.e. increasing tree root cover in soil). The decreasing soil fertility and increasing plant nutrient stress over time could be compensated by an incresing tree dependency on their fungal symbionts (Read and Perez-Moreno, 2003).

Fungal community changes over time in exotic plantations such as the ones considered in this study may also be related to the distinct colonization strategies of fungi (i.e. first colonization by pioneer species and then replaced by other new species). Pioneer species may be taxa that was already present in the soil in form of spores or resistant structures (Bruns et al., 2009) or taxa whose spores are efficiently dispersed across long

distances by means of wind (Peay and Bruns, 2014). In this sense, we found that some of the fungal symbionts to which were associated to *E. grandis* trees were well-known cosmopolitan fungal species. For example, well-known ECM genera such as *Tomentella* sp., *Inocybe sp.* and *Laccaria* sp. where highly represented even in the oldest stands, and they are among the first species found after disturbances. Some of these genera such as *Laccaria* sp. are also considered or considered pioneer, opportunistic species (Collier and Bidartondo, 2009; Ishida et al., 2008) and are known to develop over several hosts (Roy et al., 2008). Other taxa such as *Scleroderma albidum* and *Descomyces albellus* are well-known fungal species associated to *Eucalyptus*, and probably these species were dispersed from nearby plantations or introduced via seedlings. Since studies reporting and describing the local fungal community inhabiting Ethiopian forests are scarce, we cannot discard the possibility that novel and not yet described ECM taxa was not detected in our study.

As already reported in other systems, ECM richness and diversity increased with stand age (Wallander et al., 2010). However, in our study the abundance and diversity of ECM species was surprisingly low in 10-year old stands, and dominance of mycorrhizal species was not obvious until trees were already 19-years old. Thus, despite that the number of symbionts was significantly increasing over time in our study, the overall low diversity of ECM species observed both at young and older stands suggest the low presence of suitable symbionts at the study site. Similar findings were reported by Dejene et al (2017a), who observed that their fruiting body diversity under *E. grandis* stands was lower when comparing with other countries. Surprisingly, lack of symbionts seemed not to limit the development of *E. grandis*, supporting the hypothesis that a low number of available symbionts is already sufficient for the growth of *Eucalyptus* plantations (Urcelay et al., 2017). It is also possible that the relatively high N and other soil chemistry values measured at the 10-years old stands allowed trees to develop with reduced symbiont dependence. In any case, the survival of *E. grandis* species under very low abundance and diversity of ECM species together with a lack of low competition support the good adaptation of this species to the environment.

As expected, stand age effects on the soil fungal community contrasted with soil fertility effects. N addition in soils has been observed to negatively affect both mycorrhizal diversity and biomass (Ekblad et al., 2013; Kjøller et al., 2012; Lilleskov et

al., 2002). N addition also changed the composition of mycorrhizal species (Kjøller et al., 2012; Lilleskov et al., 2002). In our study, both the diversity and the relative abundance of ECM species increased with tree age, concomitant with a decrease in soil fertility, decrease in N, P values and increase in soil C / N ratios. In contrast, some saprotrophs and pathogens increased with increasing fertility. These results support recent findings from similar forest systems, in which increasing abundances of saprotrophic and/or pathogenic fungi were observed under fertilization treatments in E*ucalyptus saligna* plantations (Zheng et al., 2017). Thus, in our study saprotrophs responded mostly to changes in fertility rather to changes in tree age. Such positive response of saprotrophs to higher soil N was also reported for the fruiting body community found in *Pinus patula* (Dejene et al., 2017b). These results also support the recent findings from Kyaschenko et al., (2017) and Zheng et al., (2017), who showed that soil fertility was positively related to the abundance of fungal saprotrophs, which may growth at the expense of ECM fungi. These interguild relationships are very relevant, because they potentially affect important ecosystem processes such as C storage and nutrient cycling (Averill and Hawkes, 2016; Kyaschenko et al., 2017). Increases in C/N ratios also correlated with increasing ECM abundance, which may also be indicative of ECM species oxiding organic matter to mine for N (Clemmensen et al., 2013). Phosphorus levels also dramatically decreased with stand age, which could be attributed to the increase of specific ECM taxa. Thus, despite AM species are especially efficient in uptake P, seedlings associated to ECM taxa such as *Scleroderma* or *Laccaria* were observed to retain more phosphorus in their roots than plants inoculated with the other fungal isolates (Burgess et al., 1993). However, whether these ECM species were responsible for the dramatic decrease of P and the mechanisms behind should be further studied.

Conclusions

This study is among the first to characterize the soil fungal communities of introduced *E. grandis* plantations in Ethiopia. Our results suggest that aging of *E. grandis* stands cause profound soil fungal community changes, concurrent with changes in soil chemistry. Such changes may potentially slow soil C cycle, promoting soil C storage, but also may potentially deplete soil nutrients. We also show fungal guild-dependant changes that will potentially affect related several ecosystem processes such as nutrient

cycling. These findings are important because *E. grandis* plantations represent around half a million of hectares only in Ethiopia, but up to date no similar studies on the soil functional fungal community has been carried out. New studies should relate potential changes in fungal traits and guilds with changes in soil C budgets, which should be quantified in order to predict how much C is lost or gained under these plantations.

Data Accessibility

Sequence data are archived at NCBI's Sequence Read Archive under accession number **PRJNA503133** (www.ncbi.nlm.nih.gov/sra).

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Supplementary material

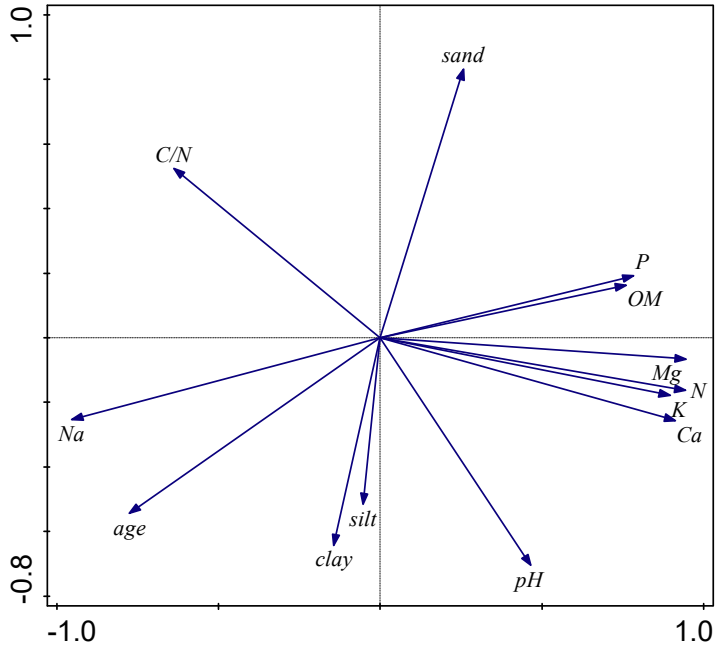

Fig. S1 Principal Component Analysis (PCA) of the soil and stand characteristics of the selected plots.

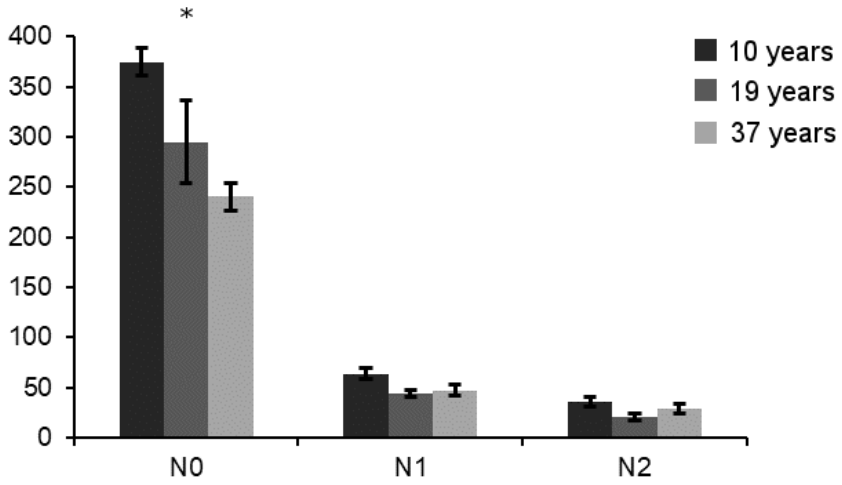

Fig. S2 Changes in intrapolated diversity values, as measured with Hill's numbers, across the three age classes (10, 19 and 37 years old stands). Significance levels: *** P<0.001, ** P<0.01, * P<0.05.