*Article*

# Drivers of Ectomycorrhizal Fungal Community Structure Associated with *Pinus sylvestris* Var. *mongolica* Differ at Regional vs. Local Spatial Scales in Northern China

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**Abstract:** *Pinus sylvestris* var. *mongolica*, a widely planted tree species, is facing long-lasting, unresolved degradation in desertified Northern China. Ectomycorrhizal fungi (EMF) are closely related to the stand status, because they substantially participate in ecological processes of terrestrial forest ecosystems. EMF may be key to solving the introduction recession. Therefore, we performed DNA sequencing of *P. sylvestris* root samples from plantations and natural forests as control to characterize the EMF from semi-arid and dry sub-humid regions, using ITS Illumina sequencing and conventional soil physicochemical index determination. The results indicated that (1) the dominant EMF genera were *Suillus, Rhizopogon,* and *Wilcoxina* in the Hulunbuir, Mu Us, and Horqin Sandy Lands, respectively. Their dominance retained with stand ageing. (2) Plantation EM fungal diversity differs significantly among the three sandy lands and was significantly lower than in natural forest. The diversity varied with stand age, showing distinct trends at the local scale. (3) At the regional scale, the mean annual sunshine times and the soil organic carbon content affect EMF diversity. The community composition and structure were more characterized by temperature and precipitation. At the local scale, besides the soil organic carbon content, the EM fungal community composition and structure were correlated with total nitrogen and phosphorus content (Hulunbuir), the total phosphorus content (Mu Us), and the pH and total soil porosity (Horqin). The EM fungal community composition and structure have the obvious geographical distribution variation; they were strongly correlated with the meteorological elements and soil nutrients at the regional scale. At the local scale, they were jointly driven by stand age and soil properties. This improved information contributes to increasing the understanding of the interaction between EMF and forest ecosystems and guides sustainable forest management of degraded *P. sylvestris* plantations.

**Keywords:** ectomycorrhizal fungi; *Pinus sylvestris* var. *mongolica*; stand age; sandy land; soil properties

## 1. Introduction

Ectomycorrhizal fungi (EMF) are widely found in forest ecosystems [1]. The EMF mycelium infects vegetative roots of trees and forms symbiotic structures [2]. Through this symbiosis, EMF obtain carbon from host plants for growth, and host plants increase the absorption and utilization of

soil moisture and mineral nutrients [3]. Ectomycorrhizae (EM) can improve host plant resistance to environmental changes and stress, thus maintaining forest ecosystem stability [4].

Pineaceae was the oldest known EM plant taxon in the word and is widely distributed in the boreal forests [5]. Many EMF were identified in pine forests [6–8]. In China, more than 30% of the reported EMF were pine-associated [9]. *Pinus sylvestris* var. *mongolica* (*P. sylvestris* for short below) is native to the Hulunbuir Sandy Land. It is the most widely planted evergreen arbor in desertified Northern China [10]. At first, plantations were generally successful, but many introduction areas experienced large-scale decline and death over time. Currently, this is a critically unresolved issue hindering development and ecological restoration in China.

*P. sylvestris* is a typical ectomycorrhizal-dependent species [11]. EMF have a mutually beneficial relationship with *P. sylvestris* [12]. Some scholars believe that EMF loss is a major reason for the decline of *P. sylvestris* plantations as their crucial function throughout the pine life cycle [13,14], including the role of promoting host resistance to drought, diseases, and other poor surroundings [15,16]. Moreover, EMF also play an irreplaceable role in the natural regeneration and seedling establishment and survival [17]. The positive role of EM in the tree physiology and ecosystem function is widely known. Conversely, the EM fungal community variation will also retroact to plant health and ecosystem stability [18]. Consequently, EMF should not be neglected in studies of the sustainable management of *P. sylvestris* plantations, because the disrupted EMF symbiosis may have a significant effect on the *P. sylvestris* growth and metabolism.

Soil EMF have numerous species and geospatial variabilities [19], which are driven by the host, environment, and geographical distance. In Northern China, all *P. sylvestris* were introduced from the Hulunbuir Sandy Land; hence, genetic variation among trees is small [20]. Therefore, environmental elements, rather than the host species, likely influence the EM fungal community. Generally, the geographical climate has directly and indirectly shaped and regulated EMF [19]. Soil physical and chemical factors alter the community composition and EMF structure [21]. At the regional scale, the fungal dispersal process which was limited by geographical distance played an important role in the EM fungal distribution [22]. At the same time, EM fungal community succession occurs with aging of the host plant [23]. In addition, mycorrhizal functional traits themselves modulate and stabilize EMF geographic pattern and ecosystem functions. For example, the role of nutrient capture may regulate the community distribution along the biogeochemical pattern [24]. Consequently, differences in ectomycorrhizal fungal populations between introduced and native areas may be key factors affecting the vigorous growth of *P. sylvestris*. However, the connection between EMF and *P. sylvestris* decline remains largely unknown.

We investigated how *P. sylvestris*-associated EM fungal communities respond to host ageing and bioclimatic zones. We tried to find the key impact factors of EM fungal communities on different scales during the introduction and forest development. It contributes to the further study of identifying relevant functional relationships between EM fungal communities and *P. sylvestris* plantation degradation. We hypothesized that 1) the natural forest has rich EM fungal diversity which should also be higher in the original habitat among plantation, and the EM fungal diversity increased with the plantation stand ageing. 2) Climate factors drive EMF communities at the regional scale, with variable EMF composition driven by soil environmental factors at the local scale.

## 2. Material and Methods

### 2.1. Study Site

This study was conducted in *P. sylvestris* origin and introduction areas (Figure 1). The natural forest plot was located in Honghuaerji Forest Park (Inner Mongolia Autonomous Region). The introduction areas included Hailar Forest Park (Inner Mongolia Autonomous Region), Hongshixia Sandy Botanical Park (Shaanxi Province), and Zhanggutai Sandy Land Forest Park (Liaoning Province). The selected plantations were afforested by the same method, and there was no manual management after planting. Hulunbuir Sandy Land (6400 km$^2$) is the origin area of *P. sylvestris*. Honghuaerji and Hailar Forest Parks are located in the Hulunbuir Sandy Land, which has a semi-

arid continental climate (Table 1). The dominant shrub and herb vegetation mainly include *Artemisia desertorum*, *Salix kochiana*, and *Saussurea japonica*. Hongshixia Sandy Botanical Park is located in the Mu Us Sandy Land and has a semi-arid continental monsoon climate (Table 1). The Mu Us Sandy Land is the westernmost sandy land in China (42,200 km²), and it is one of the most important *P. sylvestris* introduction areas. The dominant shrub and herb vegetation types mainly include *A. desertorum*, *S. cheilophila*, and *Agropyron cristatum*. Zhanggutai Sandy Land Forest Park is located in the Horqin Sandy Land, which has a dry sub-humid continental monsoon climate (Table 1). The Horqin Sandy Land is the largest sandy land in China (63,600 km²). It is the first *P. sylvestris* introduction area in China. The *P. sylvestris* recession was first found here. The dominant shrub and herb vegetation mainly includes *A. desertorum*, *Lespedeza bicolor*, and *A. argyi.*
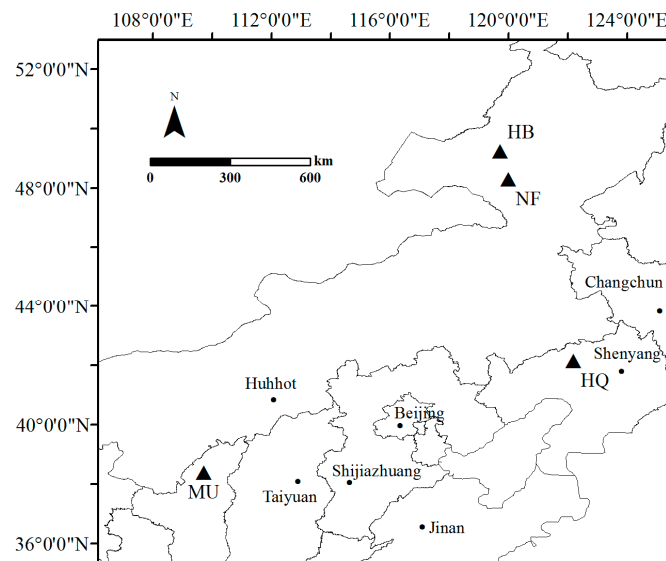


**Figure 1.** The location of study sites. NF: natural forest; HB: the Hulunbuir Sandy Land; MU: the Mu Us Sandy Land; HQ: the Horqin Sandy Land.

**Table 1**. General information of study and sampling sites

| Plots | | Hulunbuir Sandy Land | | | | Mu Us Sandy Land | | | Horqin Sandy Land | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | NF | HBh | HBn | HBm | MUh | MUn | MUm | HQh | HQn | HQm |
| Geographic coordinates | N47°36'-48°35', E118°58'-120°32' | N49°07'-49°13', E119°21'-119°44' | | | N38°16'-38°20', E109°12'-109°43' | | | N42°37'-42°50', E122°11'-122°40' | | |
| The mean annual temperature (°C) | -0.69 | -1.05 | | | 9.42 | | | 8.15 | | |
| The mean maximum temperature (°C) | 26.98 | 27.13 | | | 29.64 | | | 29.37 | | |
| The mean minimum temperature (°C) | -31.94 | -31.10 | | | -13.57 | | | -17.09 | | |
| The mean annual precipitation(mm) | 343.66 | 353.38 | | | 499.91 | | | 517.29 | | |
| The mean annual sunshine times (h) | 239.82 | 218.29 | | | 228.61 | | | 210.27 | | |
| Soil type | | aeolian sandy soil | | | aeolian sandy soil | | | aeolian sandy soil | | |
| Stand age (a) | 6–56 | 24 | 33 | 42 | 26 | 32 | 43 | 26 | 33 | 43 |
| Average tree height (m) | | 11.98 ± 1.62 | 12.63 ± 1.71 | 13.40 ± 2.36 | 12.48 ± 3.69 | 13.95 ± 2.38 | 14.14 ± 1.84 | 10.26 ± 1.47 | 10.61 ± 1.03 | 11.12 ± 1.74 |
| Average DBH (cm) | | 14.66 ± 3.40 | 18.36 ± 2.42 | 22.74 ± 1.98 | 11.76 ± 3.72 | 13.58 ± 3.44 | 19.95 ± 3.03 | 16.93 ± 2.81 | 14.06 ± 2.44 | 21.07 ± 1.02 |
| Canopy density | 0.83 | 0.75 | 0.82 | 0.71 | 0.79 | 0.86 | 0.73 | 0.72 | 0.75 | 0.68 |
| Host status | Stable growth with multiple age class | normal growth, natural regeneration | | | decline with partial deadwood and no-regeneration | | | decline with partial deadwood and no-regeneration | | |

NF: natural forest; HB: the Hulunbuir Sandy Land; MU: the Mu Us Sandy Land; HQ: the Horqin Sandy Land; h: half-mature; n: nearly mature; m: mature.

*2.2. Sample Collection*

Soil-roots samples were collected in July–August 2017 during the peak of plant biomass production and soil microbial activity. Plantations of three age groups (half-mature, nearly mature, and mature forest) without manual management in each sandy land area were selected for study [25]. The natural *P. sylvestris* origin forest was used as a control (Table 1). Fine *P. sylvestris* roots were excavated along the base of the standard tree trunk. Roots and accompanying soil were pooled into a plastic bag, but litter, herbs roots, and undergrowth humus layers were excluded. There was at least 10 m apart between each sampling tree. Five composite samples (consisting of three replicate root samples) were collected from each stand. All the composite samples ((3 sandy lands × 3 stand ages + NF) × 5=50 composite samples) were stored at 4 °C for transport to the lab for analysis. The general soil samples from 0–20 cm were collected to analyze physical and chemical soil properties. The undisturbed soil samples were additionally collected with cutting rings.

*2.3. DNA Extraction and PCR Amplification*

The roots composite samples were thoroughly mixed before DNA extraction. The sampled EM roots were cleaned under running water prior to DNA extraction. 2g roots were used to extract DNA. DNA was extracted with ground tissue homogenated by using a Powersoil DNA Isolation Kit (MoBio, USA). Extracted DNA concentration was quantified on a NanoDrop spectrophotometer (Thermo Scientific, USA). EMF ITS regions were amplified using the common fungal primers ITS1F (5-GGAAGTAAAAGTCGTAACAAGG-3) and ITS2 (5-TCCTCCGCTTATTGATATGC-3). The PCR mixtures were as follows: 4 μL 5× FastPfu Buffer, 1 μL of each primer (5 μM), 2 μL of dNTP mixture (2.5 mM), 2 μL template DNA, and 10 μL $H_2O$. The PCR amplification program consisted of an initial denaturation at 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 secs, 55 °C for 30 secs, 72 °C for 30 secs, and a final extension at 72 °C for 5 min. PCR amplification was performed in triplicate to account for potentially heterogeneous amplification from the environmental template for each sample. PCR products were purified using an AXYGEN Gel Extraction Kit (QIAGEN, Germany). DNA extracted from one sample can be used several times. In order to reduce the error of amplification, one sample was amplified three times (equivalent to three experimental replicates), and then the three amplification products were mixed equimolarly into one amplification product for subsequent library construction. The amplified products of all samples were mixed uniformly according to the pooling ratio. The mixed products were sequenced using NEB Next Ultra II DNA Library Prep Kit for sequencing library construction. Illumina sequencing adapters were completed in the step of library building. An equimolar mix of all three amplicon libraries was used for sequencing at Allwegene Technology Inc. China, using an Illumina MiSeq sequencing system (Illumina, USA).

*2.4. Sequencing Data Analysis*

The original sequences were processed using the Mothur (v1.30.1, https://www.mothur.org) Sequences with quality scores > 30 and longer than 200 bp were used for analysis. Chimeric sequences were removed using the software package Usearch (Version 8.1.1861, http://www.drive5.com/usearch/). The remaining high-quality sequences were classified as an operational classification unit (OTU) with more than 97% similarity using Uclust [26]. In order to ensure that the coverage of all samples was as high as possible and to reduce the error caused by the different data size, the data size of all samples was homogenized to 120,204 sequences. Singleton tags were removed before data processing. Taxonomic assignment was performed using the Ribosomal Database Project (RDP) classifier. Assigned taxa were verified by NCBI BLAST (https://www.ncbi.nlm.nih.gov/). The non-EM fungal sequences were removed based on the guilds output using FUNGuild v1.0 (http://www.stbates.org/guilds/app.php) [27], the EM fungal OTU sequences were submitted to the NCBI GenBank (Accession number in Table S1). EM fungal OTU richness (Chao1), Shannon, Simpson, and Pielou indices were computed using the vegan package in R (version 3.4.3).

## 2.5. Soil Properties and Climate Data Analysis

The general soil samples were air-dried for 2 days. Roots and stone debris were removed, and soil pH was measured using 1:1 soil and distilled water mixture by a PHS-3E pH meter (INESA, China) [28]. The total soil porosity (TSP) was gravimetrically determined after the undisturbed soil samples soaking in water for 8 h [29].

The total soil organic carbon (SOC) was determined using the dichromate oxidation method [30]. Total nitrogen (TN) and total phosphorus (TP) content were analyzed with a Smartchem Discrete Auto Analyzer (AMS, Italy) with the indophenol-blue spectrophotometric method and Mo-Sh anti-colorimetric analysis methods [31,32], respectively.

Climate data from 2007 to 2016 for each site were obtained from the China Meteorological Data Service Center (CMDC, http://data.cma.cn/en). The mean annual temperature (Ta), mean annual precipitation (Pa), and the mean annual sunshine time (St) were calculated by averaging the annual value (the sum of average monthly value) over ten years. The average maximum and minimum temperature (T+ and T-) was calculated using monthly average maximum/minimum temperature over ten years.

## 2.6. Statistical Analysis

The relative abundance was calculated by the number of OTU divided by the total number of OTUs in this sample. The relative frequency was calculated by the times of OTU occurrences divided by the total times of all OTUs occurrences in this sample.

In order to compare the soil properties (pH, SOC, TN, and TP), fungal richness, and diversity indices among the three age groups within the same sandy land, one-way analysis of variance (ANOVA) and post-hoc Tukey tests after the normality test and the homogeneity test of variance (data conforms to a normal distribution with uniform variance) were conducted. The effects of different sandy lands and stand age on EM fungal diversity indices were tested by two-way ANOVA with post-hoc Tukey tests. EM fungal diversity and soil properties/meteorological factor correlations were evaluated using the Pearson method. All statistical analyses were performed using IBM SPSS 20.0 (IBM, Armonk, NY, USA), with $p < 0.05$ considered significant. The statistical analyses were performed within sandy land (local scale) and over all sandy lands (regional scale).

RDA, NMDS, ANOSIM, and Mantel test were used to visualize the fungal community intergroup similarity and explain the correlation between the soil properties/climate factors and fungal community composition. Redundancy analysis (RDA) of EM fungal communities and environmental factors (meteorological and edaphic) was performed using Canoco for Windows 5.0 [33]. EM fungal composition similarity between different samples was analyzed using non-metric multidimensional scaling (NMDS) and one-way analysis of similarity (ANOSIM). Bray–Curtis distances of EM fungal communities and Euclidean distances of soil variables and geographical distance were used to construct dissimilarity matrices for Mantel tests. NMDS, ANOSIM, and Mantel tests were performed with the vegan package in R (version 3.4.3).

Cluster heatmaps and NMDS figures were generated with R (version 3.4.3, https://www.r-project.org/). Boxplots and histograms were generated with Origin 2016 (OriginLab Corporation, Northampton, MA, USA). Network maps were drawn using Cytoscape v3.6.0.

## 3. Results

### 3.1. Diversity of EMF Communities

A total of 645,186 high-quality sequences were obtained from the samples. The sequences were grouped into 2024 OTUs. We identified 104 OTUs by filtering for ECM fungal taxa using the FUNGuild v1.0.

The diversity indices based on identified OTUs (Figure 2) showed the EMF of natural forests (NF) were more diverse than plantations. Diversity measures differed significantly among the plantations in different sandy lands ($p < 0.05$), with the highest values in the Mu Us Sandy Land and

the lowest in the Hulunbuir Sandy Land. The changing laws of the EM fungal diversity variation with stand age were different among the sandy lands. In the Hulunbir Sandy Land, EM fungal alpha diversity first increased, and then decreased with stand age. However, alpha diversity decreased in the Mu Us Sandy Land and increased continuously in the Horqin Sandy Land. The EM fungal richness in the natural forest (Chao1 = 46.70 ± 4.66) was higher than those in plantations. The EM fungal richness in plantations were HB (44.32 ± 3.39) > MU (41.67 ± 7.11) > HQ (30.64 ± 3.49) at a regional scale.
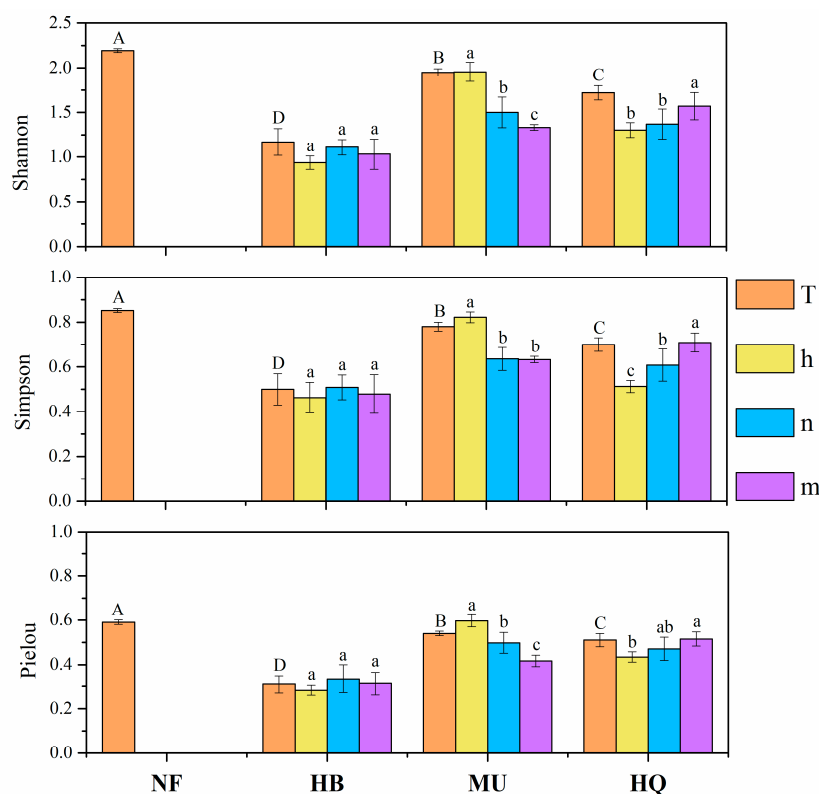


**Figure 2.** Diversity indices (±SE) for Ectomycorrhizae (EM) fungal communities in *P. sylvestris* natural forest and plantations in three sandy lands (*n*=5). **T** for the composed value (included five mixed replicates by adding one of five samples in each age group in the same sandy land together) of each sandy land. **h** for half-mature, **n** for nearly mature, **m** for mature. The capital letters indicate significant differences among sandy lands (columns in orange), the lowercase letters indicate significant differences among age groups within the same sandy land, $p < 0.05$.

Stand age and sandy land have a significant interaction effect on EM fungal diversity indices ($p < 0.05$, Table 2). There were significant differences in EMF alpha diversity indices associated with *P. sylvestris* plantations among different sandy lands ($p < 0.05$). The age main effect on EM fungal diversity was not significant ($p > 0.05$) at the regional scale. While in nested analysis, stand age had a significant effect on diversity indices ($p < 0.05$). This represented that stand age's alpha diversity difference was depending on sandy lands.

**Table 2.** Two-factor variance analysis of effect of stand age and sandy land on EM fungal diversity.

| Source | Dependent variable | Sum of the squares | Mean square | *F* | Significance |
|---|---|---|---|---|---|
| Sandy lands*Age groups | Shannon | 1.233 | 0.308 | 19.551 | **0.000** |
|  | Simpson | 0.218 | 0.054 | 18.429 | **0.000** |
|  | Pielou | 0.101 | 0.025 | 15.189 | **0.000** |
| Sandy lands | Shannon | 2.552 | 1.276 | 80.959 | **0.000** |
|  | Simpson | 0.341 | 0.170 | 57.679 | **0.000** |

| | | | | | |
|---|---|---|---|---|---|
| | Pielou | 0.331 | 0.166 | 99.782 | **0.000** |
| Age groups | Shannon | 0.065 | 0.032 | 2.060 | 0.142 |
| | Simpson | 0.003 | 0.002 | 0.551 | 0.581 |
| | Pielou | 0.004 | 0.002 | 1.242 | 0.301 |
| Age groups (Sandy lands) | Shannon | 1.298 | 0.216 | 13.721 | **0.000** |
| | Simpson | 0.221 | 0.037 | 12.470 | **0.000** |
| | Pielou | 0.105 | 0.017 | 10.540 | **0.000** |
| Error | Shannon | 0.567 | 0.016 | | |
| | Simpson | 0.106 | 0.003 | | |
| | Pielou | 0.060 | 0.002 | | |
| Total | Shannon | 85.890 | | | |
| | Simpson | 16.677 | | | |
| | Pielou | 8.790 | | | |

Values in bold are statistically significant ($p < 0.05$). Sandy lands*Age groups represents the interaction effect of Sandy lands and Age groups

### 3.2. EM Fungal Community Composition and Structure

In total, 104 EM fungal OTUs were successfully identified (Table S1). Of these, 88 belonged to *Basidiomycota* and 16 to *Ascomycota*, representing 30 EMF genera. Natural forest samples contained 50 OTUs, while the plantation samples from three sandy lands included 64 (HB), 55 (MU), and 38 (HQ) OTUs. Only 8 OTUs were shared among all groups (Figure 3). Among them, the Hulunbuir Sandy Land plantations had the most OTUs similar to natural forest. Relative frequency analysis revealed *Basidiomycota* was the most abundant phylum (84%), followed by *Ascomycota* (16%) (Figure 4). *P. sylvestris*-associated EMF in the Hulunbuir (both natural forest and plantations) and Mu Us Sandy land were mainly *Basidiomycota*. The relative abundance of *Basidiomycota* initially decreased and then increased with increasing stand age in these two sandy lands. But in the Horqin Sandy Land, EMF was mainly *Ascomycota*, with significantly decreasing relative abundance ($p < 0.05$, Figure 4).
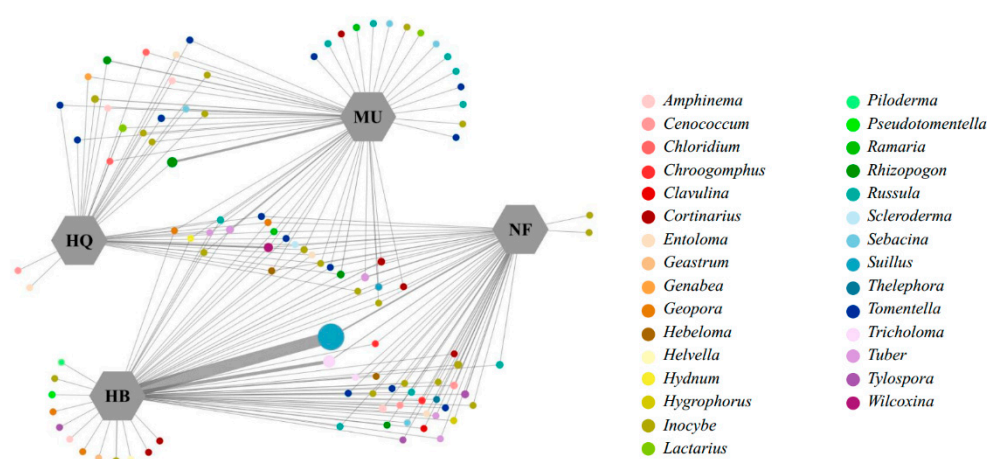


**Figure 3.** Network of EM fungal operational classification units (OTUs) in each *P. sylvestris* stand across three sandy lands. Circle size represents absolute OTU abundance. Line thickness represents relative OTU abundance in sandy land.
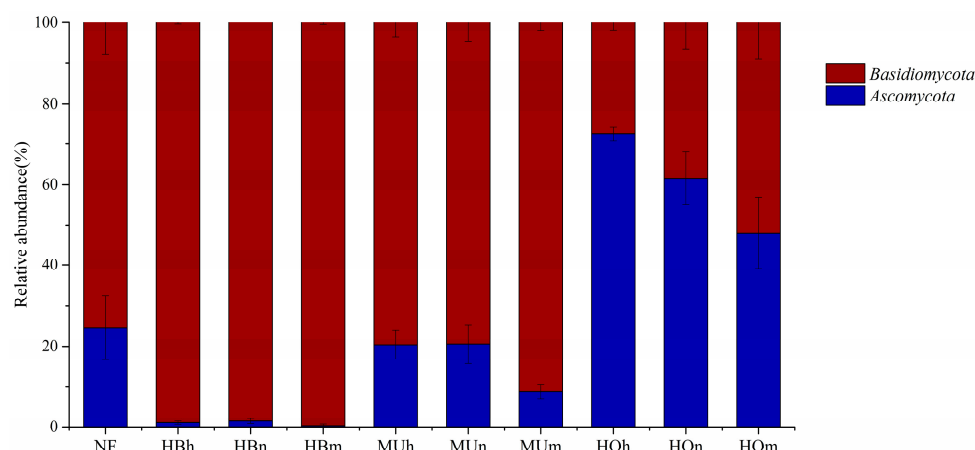
**Figure 4.** Relative abundance of EM fungal phyla in each *P. sylvestris* stand across three sandy lands

The relative abundances were calculated on the OTUs numbers. Of these, among *Basidiomycota*, *Suillus* was the OTU-richest genus, followed by *Tricholoma*. Among *Ascomycota*, *Wilcoxina* was the most abundant genus. A detailed analysis of the EM fungal genera in each *P. sylvestris* stand (Figure 5) revealed that the relative fungal abundance was more balanced in natural forest and was predominantly *Suillus* and *Inocybe*. The dominant genera of the Hulunbuir Sandy Land plantations were *Suillus* and *Tricholoma*. *Suillus* accumulated as stand age increased, but *Tricholoma* did the opposite. In the Mu Us Sandy Land, *Rhizopogon* and *Tuber* were highly abundant. Relative *Rhizopogon* abundance rose with stand age, while *Tuber* decreased. In the Horqin Sandy Land, *Wilcoxina* was the major genus, and abundance decreased with stand age. Finally, *Lactarius* enrichment initially decreased and then increased as stand age increased.
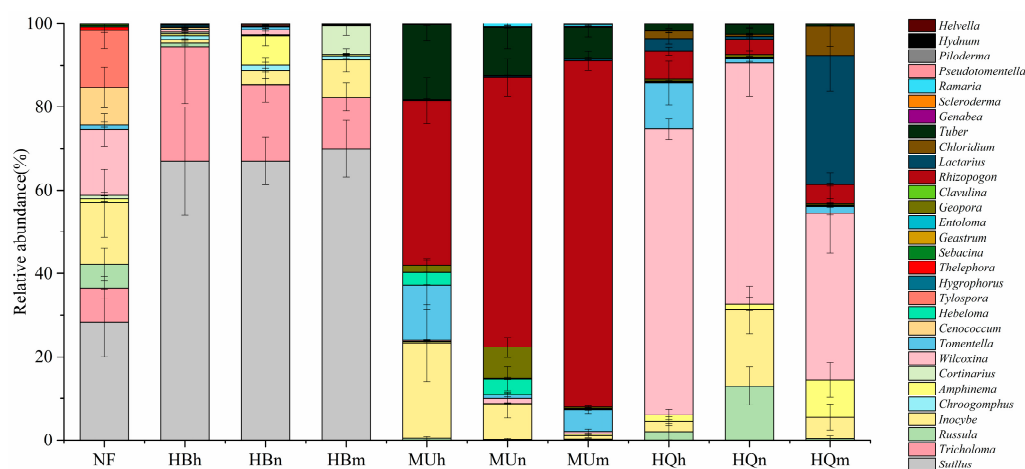


**Figure 5.** Relative abundances of EM fungal genera in each *P. sylvestris* stand across three sandy lands. The relative abundances were calculated as an average of 5 replicates in each stand.

The EMF community composition differed among sampling locations. *Inocybe*, *Russula*, *Amphinema*, *Wilcoxina*, *Lactarius*, *Tomentella*, *Cenococcum*, *Hebeloma*, *Sebacina*, *Geopora*, *Rhizopogon*, and *Ramaria* were detected in samples from all three sandy land areas. *Tricholoma*, *Chroogomphus*, *Hygrophorus*, *Tylospora*, *Thelephora*, *Helvella*, *Clavulina*, *Geastrum*, *Piloderma*, and *Pseudotomentella* were absent in the Mu Us and Horqin Sandy Lands, while *Chloridium* and *Genabea* were not found in the Hulunbuir Sandy Land.

The EM fungal communities of different age groups in the same sandy land clustered well (Figure 6), as did natural forest samples and samples from the Hulunbuir Sandy Land plantations. The EM fungal communities from the Hulunbuir Sandy Land were the most different from the other two. The nearly mature and mature forests showed greater similarity in the Hulunbuir and Mu Us Sandy Land. However, half mature and nearly mature forests were more similar in the Horqin Sandy

Land. NMDS analysis showed clear area separation (Figure 7). The ANOSIM results indicated that location has significant effects on the EM fungal community structure ($r$ = 0.977, $P$ = 0.001).
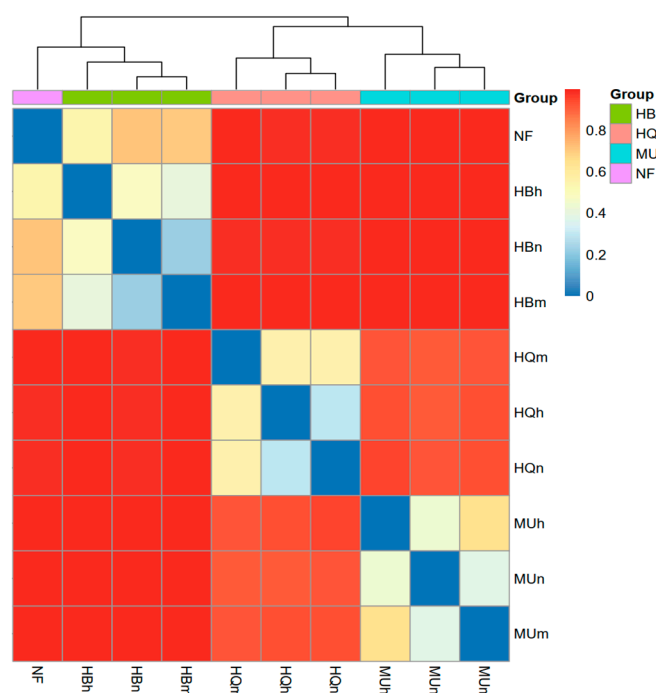


**Figure 6.** Bray–Curtis distance heatmap of each *P. sylvestris* stand across three sandy lands. Color varies from blue to red, representing dissimilarities in EM fungal community composition from low (blue) to high (red).
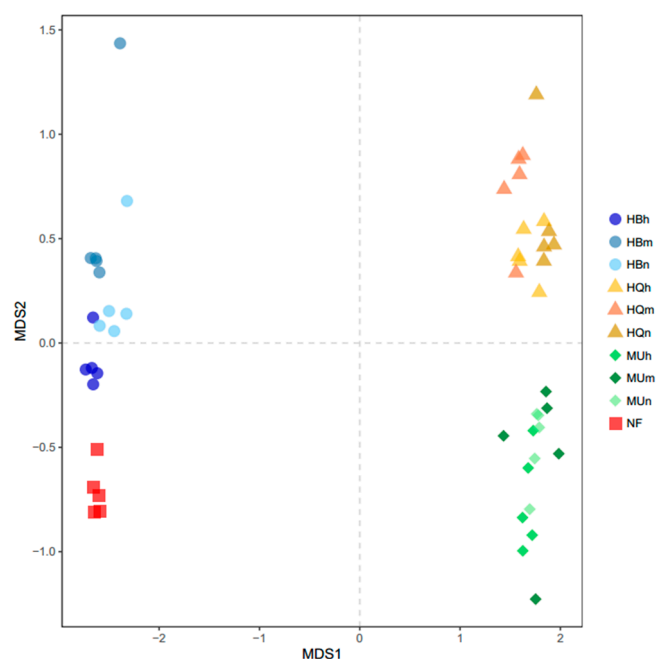


**Figure 7.** EM fungal community structure based on operational taxonomic units (OTUs), as determined by non-metric multidimensional scaling (NMDS), stress= 0.078.

*3.3. The Response of EM Fungal Communities to Environmental Variation*

From Table 3, the Shannon and Simpson diversity and Pielou evenness indices had the strongest positive correlation with the mean annual sunshine times (0.592 and 0.573) and the mean annual temperature (0.506). They also had the strongest negative correlation with the total soil organic

carbon content (−0.601, −0.578, and −0.742, respectively). The Shannon index was significantly correlated with the mean annual sunshine times, soil organic carbon, and total nitrogen content ($p < 0.05$). The Simpson index was significantly correlated with the mean annual temperature, sunshine, minimum temperature, soil organic carbon, and total nitrogen content ($p < 0.05$). The factors that were most significantly associated with the evenness index included meteorological factors and soil organic carbon content ($p < 0.05$).

**Table 3.** Pearson correlation coefficients for EM fungal diversity and climate factor/soil properties.

|  | Ta | Pa | St | T+ | T− | TSP | pH | SOC | TN | TP |
|---|---|---|---|---|---|---|---|---|---|---|
| Shannon | 0.270 | 0.192 | 0.592** | 0.223 | 0.240 | −0.018 | 0.032 | −0.601** | −0.338* | 0.133 |
| Simpson | 0.311* | 0.226 | 0.573** | 0.268 | 0.290* | 0.001 | 0.032 | −0.578** | −0.335* | 0.232 |
| Pielou | 0.506** | 0.448** | 0.410** | 0.466** | 0.474** | −0.079 | 0.014 | −0.742** | −0.212 | 0.217 |

Abbreviations: mean annual temperature (Ta), mean annual precipitation (Pa), mean annual sunshine times (St), mean maximum temperature (T+), mean minimum temperature (T-), total soil porosity (TSP), soil pH, total soil organic carbon (SOC) content, total nitrogen (TN), and total phosphorus (TP). * and ** indicate significant correlation at 0.05 and 0.01, respectively.

Based on the Mantel test (Table 4), the geographical distance, the selected climate factors, and soil properties (Table S2) were significantly correlated with EM fungal community composition (p = 0.001). On the regional scale, the EM fungal communities are strongly significantly affected by geographical distance (r = 0.777, $p$ = 0.001). The effect of the mean minimum temperature (T-) and the mean annual temperature on EM fungal community composition was the most obvious (r = 0.763 and 0.733, respectively) among the climate factors. Soil nutrients had a more pronounced effect than other soil factors. The correlation with stand age was significant (r = 0.103, $p$ = 0.010), though relatively weak at the regional scale. At the local scale, increasing stand age had a significant impact on EMF community structure ($p < 0.01$, Table S3), as did SOC. Moreover, the EMF community structures were strongly significantly associated with TP and TN content in different age groups in the Hulunbuir Sandy Land ($p < 0.01$). The EMF community structures were extremely significantly associated with TP content in the Mu Us Sandy Land, and they were extremely significantly associated with TSP and pH in the Horqin Sandy Land ($p < 0.01$).

**Table 4.** Correlations between EM fungal community composition and climate factors/soil properties/stand age/geographical distance assessed by Mantel test

|  | Ta | Pa | St | T+ | T- | TSP | pH | SOC | TN | TP | Age | GD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| r | 0.733 | 0.725 | 0.592 | 0.726 | 0.763 | 0.312 | 0.266 | 0.440 | 0.529 | 0.500 | 0.103 | 0.777 |
| P | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.010 | 0.001 |

Abbreviations: mean annual temperature (Ta), mean annual precipitation (Pa), mean annual sunshine times (St), mean maximum temperature (T+), mean minimum temperature (T-), total soil porosity (TSP), soil pH, total soil organic carbon (SOC) content, total nitrogen (TN), total phosphorus (TP), stand age (Age), and geographical distance (GD).

From the redundancy analysis (Figure 8), climatic variables and SOC content were the main factors in explaining the differences in community composition between NF+HB and MU+HQ. The differences between NF and HB as well as MU and HQ were mainly caused by differences in TN, TP content, TSP, and soil pH. The difference in EMF community composition between HB and the other types (especially MU and HQ) was caused by the much higher SOC in HB. The difference in EMF community composition between MU and the other types was caused by the much higher TP in MU. Similarly, the difference between HQ and others was caused by TN.
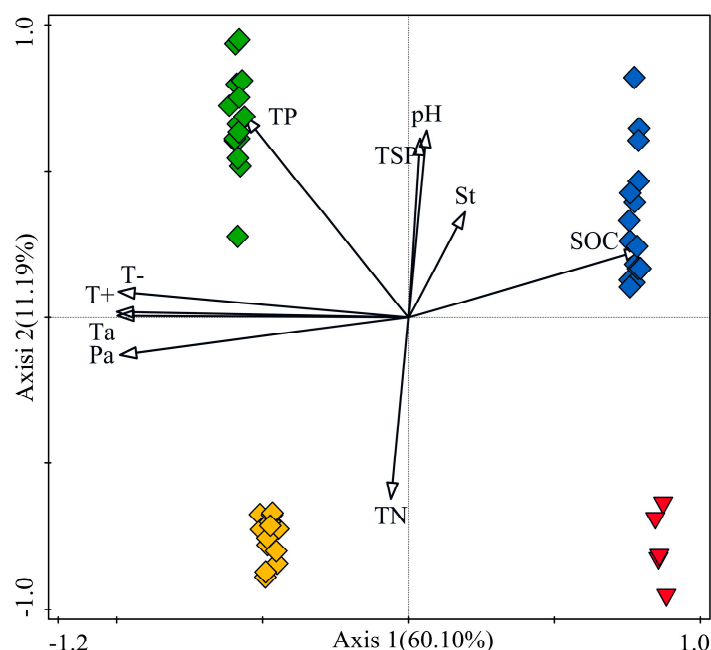
**Figure 8.** EM fungal community dissimilarity in each *P. sylvestris* stand across three sandy lands was tested by redundancy analysis (RDA). Red triangles represent NF (natural forest), blue diamonds represent HB (the Hulunbuir Sandy Land), green diamonds represent MU (the Mu Us Sandy Land), and yellow diamonds represent HQ (the Horqin Sandy Land). Arrows represent environment variables.

## 4. Discussion

### 4.1. Variation in Ectomycorrhizal Fungal Community Composition with Introduction

EM fungal diversity can affect the hosts growth and nitrogen uptake [34]. The highest EM fungal diversity in the natural forest partly supported our first hypothesis. Earlier observations showed that EM fungi were more adapted to the prevailing conditions in the natural forests, with higher richness and more even distribution [35]. The natural forest was highly ecologically stable, with a diverse age group and rich understory vegetation [36]. The average diversity indices of plantations among the three sandy lands were significantly different ($p < 0.05$) as MU>HQ>HB. Prior studies noted EMF biogeographic and ecological patterns were affected by host plant identity [37], while our study objects were the same species without notable genetic variation, but under various growth status in different sandy lands. Contrary to our expectations, the EM fungal diversity of plantations in the Hulunbuir Sandy Land was not higher than that in the Mu Us and Horqin Sandy Land, suggesting that EM fungal diversity is not positively correlated with host status (as mentioned in Table 1, normal growth or decline). No clear relationship between EM fungal diversity and host status has been reported to date. Variation in the diversity indices according to stand age was inconsistent across the tested sandy lands. Results from previous studies on the relationship between EMF diversity and stand age varied widely, with clear differences even within one species across different regions [23,38,39]. However, whether stand age influences fungal diversity remains controversial, this could be known through analyses of long-term soil chronosequences.

Among the 50 samples we obtained, the relative frequency of *Basidiomyceta* was higher than *Ascomycota*, which is consistent with general EM fungal distribution [40]. The relative abundance of *Basidiomyceta* was also higher except in the Horqin Sandy Land, where the dominant genus belonged to *Ascomycota*.

The present genera have a wide geographical distribution, and they are commonly associated with pine [41]. During the stand ageing, some fungi appeared in the early stages, and some occur in late stages [39,42]. In our study, the most dominant genera were consistent in all age groups within

one sandy land area. This result is in consistent with previous data that Atheliaceae was always dominant in the EM fungal community of *P. sylvestris* stands at the 10- to 80-year-old stage [43].

In native areas, both natural and planted forests, the dominant taxon is *Suillus*. Suilloid EMF (specifically, the genera *Suillus* and *Rhizopogon*) are considered to be "pioneers" in human-introduced pine stands and are key to understanding plantation success or failure [44]. These EMF facilitate pine seedling establishment and are important for later stage growth [45]. *Suillus* was predominant in the three age groups in the Hulunbuir Sandy Land, with no significant fluctuation with age. In the Mu Us and Horqin Sandy Lands, the dominant genera changed with stand development, which corresponded to decreased and increased diversity. The dominant genus of the Mu Us Sand Land was *Rhizopogon*, which is a common EMF genus often observed at great distance from the host source area [46]. *Wilcoxina* was dominant in the Horqin Sandy Land. In China, *Wilcoxina* species was first discovered in the northeastern region [47], which is also the location of the Horqin Sandy Land. With the stand aging, its relative abundance was gradually reduced.

### 4.2. Interactions between Environmental Change, Ectomycorrhizal Fungal Community Composition, and Stand Ageing

*P. sylvestris* from one source was introduced into different regions, producing different stand structures under the differing hydrothermal conditions, including the EM fungal communities. At the regional scale, significant differences in geographical distribution of EM fungal community structure were detected. Among them, environmental heterogeneity mainly included climate and soil composition. Climate factors markedly impact the EM fungal diversity and community structure [48]. The biological distribution of terrestrial ecosystems is primary and mostly regulated by temperature and moisture [49]. Mantel tests showed that temperature had the strongest influence on EM fungal communities. Increased EMF diversity occurs with elevated temperature based on the promoted plant productivity [50,51], as the EM fungal alpha diversity indices of plantations are correlated with the mean annual or extreme temperatures. Long-term warming studies in the Artic found an increased abundance and richness of EMF with increasing temperature [50,52]. In a boreal forest, however, warming in combination with drying was found to negatively affect fungal abundance [53]. The response of the below-ground ecosystem to temperature changes requires more in-depth and comprehensive research. Solar radiation is an important heat source, so the mean annual sunshine time was used as a characterization index. Energy from the sun directly affects soil temperature and plant metabolic processes [54]. The mean annual sunshine time had significant impacts on all three indices. EMF obtain carbon from the host plant for growth. We posit that sunlight indirectly influences EMF by driving photosynthesis within the host plant. Fungal species react differently to changes in temperature and moisture. Therefore, large regional climate changes substantially affect the composition and regulation of EM fungal communities [55]. Furthermore, precipitation can also influence EM fungal communities, particularly in desert regions [56]. Generally speaking, drought decreases soil microbial diversity at a global scale [57]. However, the study on the EM colonization of pinyon pine in Northern Arizona found that EM colonization was significantly higher at the much drier cinder site for 5 of 12 months than that in moist sandy-loam soils sites [58]. Drought influenced the EMF community composition in a water-exclusion experiment on beech, and the EMF responded to drought differently in terms of their abundance [59]. In our study, although the precipitation was HQ>MU>HB, evaporation within HQ and MU was double the evaporation of HB. We found that MU and HQ experienced greater drought stress than HB base on the aridity index (potential evaporation/precipitation). This could probably explain why fungi with large fruiting bodies (such as *Suillus*) did not appear in the Horqin and Mu Us Sandy Land. Hypogeous fungi, like *Wilcoxina* and *Rhizopogon*, were thought to be adapted to water scarcity [6] and are dominant in those sandy lands.

Soil conditions are external abiotic factors directly affecting EM fungal communities. In our study, the total soil porosity, pH, and total phosphorus content drove EM fungal community composition, but they had no significant correlation with diversity. This was also reported in previous studies [60]. Most EM fungi were more suitable for slightly acidic or neutral pH, and soil pH exhibited a significant effect on both EM diversity and community structures in Beech, Pine, and

Spruce Forests at both a continental and global scale [61,62]. At the same time, EMF had a large pH adaptation range, EMF could grow in the pH range of 3–8 in both culture media and peat associated with *P. sylvestris* seedlings [63]. The contribution of pH to the variation of EM fungal communities was not highlighted due to the minor differences among our samples.

At the regional scale, soil organic carbon was significantly correlated with EM fungal community composition among the soil nutrient elements. Soil organic carbon in forests is mainly derived from litter, root, and dead microbial cells in the soil decomposition and is an important part of the carbon cycle [64]. EMF mediate carbon cycling, while carbon productivity is also a driver of ectomycorrhizal abundance and diversity [65]. The Mantel test revealed that the total nitrogen content was a major factor influencing the EM fungal community structure in our study. EM fungal communities are affected by available nitrogen and form large-scale patterns by nitrogen variation based on a European biomonitoring network of pine forest plots [66]. Fungal diversity and total soil nitrogen content were negatively correlated in our results. Diverse fungal communities are observed by field experiment in a mixed boreal *P. sylvestris* and *Picea abies* forests in Sweden, where the organic layer is relatively nitrogen poor [67]. Soil phosphorus content has selective influence on EM fungal community composition and ectomycorrhizal role of increasing water absorption [68]. The relationship between nutrient content and EMF diversity are contrary to some studies. This may be due to low soil nutrients in sandy lands (Table S2). The nutrient content was less variable, and our forest stand conditions were distinct from other terrestrial forest ecosystems.

At the local scale, EM fungal richness in the three sandy lands was very different according to stand age. Previous studies showed that EM fungal richness increased, decreased, or did not significantly change with the increased stand age [23,69,70]. There was no clear pattern for the change of EM fungal diversity with stand aging, either among the different tree species or the same tree species in different regions. At the local scale, stand age was the major factor driving the composition and structure of the EMF community (Table S3). It was demonstrated that aging of forest soil determines the EM fungal composition in secondary stands of *P. sylvestris* in the Netherlands [71]. The change in soil properties resulting from stand ageing were considerable factors in EM fungal community composition. The EMF communities were mainly regulated by SOC, TN, and TP content in the Hulunbuir Sandy Land. The SOC and TN content increased with stand ageing, showing consistent homogeneity. Filtration and influence of soil nutrient and organic matter on EMF communities have also been reported in pine forest of California [72]. The EM fungal community composition in Mu Us Sandy Land plantations was also affected by SOC and TP content. In this group, the TP content had a larger effect. The increase in soil phosphorus content promotes the growth of most EMF species based on the field investigation on the production of EM mycelium in the Norway spruce forests in Southern Sweden [73], particularly *Rhizopogon*, which was consistent with our data. However, soil phosphorus has no effect on *Suillus* [74]. Moreover, there is no strong evidence for the impact of phosphorus on EM fungal diversity. In the Horqin Sandy Land, the total soil porosity, pH, and SOC regulated EM fungal community composition. Soil acidity is a crucial factor affecting soil fungi [5]. Our data indicate that the EMF community in the Horqin Sandy Land may be sensitive to soil pH. Soil porosity controls root and fungal mycelial growth [75]. Several hypogeous fungi (*Wilcoxina*) which sporulated mitotically within the soil [6], exist in the Horqin Sandy Land, making TSP a vital factor.

### 4.3. Ectomycorrhizal Fungi and P. sylvestris Plantation Degradation: a Supposition

The EM fungal composition and community structure are filtered and shaped by climate and soil conditions in different sandy lands. Changes in the community structure under introduction of host plant can have consequences for ecosystem function. Community composition and structure of ectomycorrhizal fungi in declined and non-declined forests were significantly different [76]. Considering the benefits from EMF to tree health and ecosystem service, the EM association feedback may be a causal factor of trees degradation [77]. In the declining *Phytophthora cinnamomic*-infected *Quercus ilex* forest, the changes of the EM fungal diversity and abundance caused by human impact

were involved in the *Q. ilex* decline, this counteracted the positive symbiosis effect and might lead to further tree death [78].

Variation in EM fungal community composition can result from nutrient cycle efficiency [79]. At the same time, because of their extremely important role in the nutrient cycle, they would affect the host tree productivity and health in turn [80]. In addition, the loss of fungi which have strong pathogen antagonism and drought tolerance may lead to stand degradation. Host plants suffer from withering dead leaves and low pathogen resistance, likely due to the lack of essential trace elements such as manganese, zinc, and copper [81,82]. The introduction of EMF which have these elements' transporters can help the host plant absorb and transport the elements [83,84]. This could alter trace element shortage and the low absorption rate in desert ecosystems. Simultaneously, the local reduction in the relative abundance of dominant genera may be an overlooked cause of stand decline. The dominant genus in the Hulunbuir Sandy Land was in a relatively stable state during stand evolution, while the other two had larger fluctuations. A population competition mechanism could have a non-negligible influence on EM fungal ecosystem function [85]. The regeneration barriers—"able to germinate, unable to survive"—are important phenomena in plantation degradation. EM may be a key point to alleviate this problem because they have critical benefits to seedling growth and survival [86], especially considering that Suilloid fungi play an essential role in *P. sylvestris* invasion and seedling establishment [44].

In the strong mutual feedback relationship between EMF, host plant, and the environment, the composition and structure of ectomycorrhizal fungi community could be used as a crucial indicator of plant health and ecosystem function. Further, this research could inform an answer to plantation degradation.

## 5. Conclusions

EMF associated with *P. sylvestris* are diverse with various genera and have clear regional differences. The environmental changes caused by the large-scale introduction of *P. sylvestris* strongly affected the diversity and composition of the EM fungal community. We propose that this introduction reduced the EM fungal diversity and richness of *P. sylvestris*. The original plantations were not superior to other introduced areas regarding EM fungal diversity. The variety of EM fungal diversity and richness with the age gradient had no consensus across three sandy lands.

At the regional scale, the mean annual sunshine times and the soil organic carbon content affect EMF species diversity. The EM fungal community composition and structure were more characterized by temperature and precipitation. EM fungal changes were expected to differ among the sandy lands. At the local scale, the factors which drive and modulate the EM fungal communities during stand development were significantly different among three sandy lands. In addition to the soil organic carbon content, the EM fungal community structures were closely correlated with total nitrogen and phosphorus content (Hulunbuir), the total phosphorus content (Mu Us), and the pH and total soil porosity (Horqin).

The primary relationship between EM fungal community and plantation introduction is supported by our results. The EM fungal community changes and related nutrient cycles could reflect stand status. Therefore, we highly considered that study on fungal dynamics of multi-spatial EMF could alleviate the *P. sylvestris* decline and solve the regeneration problem. Our results have the potential to guide sustainable forest management, such as cultivating specific functional mycorrhizal seedlings and mycorrhizal fungal inocula configuration.

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