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Article

Cryptic Risks to Forest Biosecurity Associated with the Global Movement of Commercial Seed

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Abstract: The import and export of tree seed carries with it risks of inadvertent introduction of pests and pathogens to hitherto unaffected regions. Although trade in seed of specified trees is regulated, phytosanitary requirements for most tree species are minimal, even those related to the most important forest tree species in a given region. A better understanding of the microbiome associated with seed intended for commercial production or ornamental use, and their potential risk with the transport from the source origin of distributors, will help regulatory agencies implement measures to safeguard seed health and avoid trade-related spread of potentially harmful pathogens. In this study we used high throughput sequencing to show that highly diverse fungal communities were associated with seed of 14 different *Pinus* species obtained from seed banks (seed orchards) and retail sources (online distributors) in North America and Europe. Fungal diversity differed among the 23 seedlots tested. Community composition did not relate to the species of *Pinus* nor the country of origin. Assigned potential functions based on sequence identity using FUNGuild provided an overall understanding of the likely life strategies of fungal Operational Taxanomic Units (OTUs). Of those sequences classified to a trophic level, 453 were plant pathogens, with the Dothideomycetes having the highest prevalence. The most common plant pathogens included *Sydowia polyspora*, *Lasiodiplodia theobromae*, *Diplodia intermedia* and *Diplodia sapinea* that were detected from the majority of *Pinus* species The evidence presented here illustrates an urgent need for plant protection authorities, practitioners and the general public to recognize the potential risk of introducing harmful pathogens through innocent transport of seed

Keywords: alien invasive forest pathogens; emerging forest diseases; global trade; *Pinus*; *Diplodia sapinea*; *Sydowia polyspora; Lasiodiplodia theobromae*; mycobiome

1. Introduction

Despite advances in vegetative propagation technologies, production of gymnosperm planting stock in tree nurseries is largely through seed and is likely to remain so for the foreseeable future for most species used in forestry. Seed used for restocking large-scale commercial forests are usually locally sourced, ideally from seed orchards or certified stands growing in the region of production. Conversely, for operations requiring smaller numbers of trees or having specific requirements on the

host species-variety, as in the ornamentals market, local seed sources generally are not available and the propagation materials, whether it be seed or young plants, must be sourced elsewhere.

Importing tree seed carries an associated risk of inadvertent introduction of harmful pathogens living on or in the seed themselves. Such introductions could lead to establishment and spread of these organisms, affecting nurseries, the young plants therein, and ecosystems into which the seedlings subsequently are planted. This problem is further complicated by the lack of phytosanitary regulations regarding the trade of tree seed in most countries [1]. The *Eucalyptus* stem canker pathogen *Teratosphaeria zuluensis* is commonly detected in seeds and seed capsules, and has been moved around the world with *Eucalyptus* seed used in plantation establishment [2]. Nuts harvested from planted American chestnut (*Castanea dentata*) were found to be infected by the chestnut blight fungus *Cryphonectria parasitica*, posing a risk to blight-free areas if fruit were imported [3]. Seed was also noted as the source material by which several tree pathogens arrived to, or moved within, Europe during the 19th and 20th century including *Diplodia sapinea* (syn. *Sphaeropsis sapinea*), *Fusarium circinatum*, *Nematospora coryli*, and *Pestalotiopsis maculans* (syn. *Pestalotiopsis guepinii*) [4].

Seed of gymnosperms are known to carry, both externally and internally, a number of pathogenic fungi and fungus-like organisms, that may or may not be transmitted to plants growing from the seeds. Some of these fungi cause primarily diseases of the seed (e.g. *Caloscypha fulgens* causes a seed rot in a number of conifers [5] but has minor effects on other developmental stages of trees). In contrast, seed-borne pathogens of conifers can in some cases result in severe economic and ecological consequences if introduced and become established outside of their natural distribution range. For example, *Lasiodiplodia theobromae* destroys seeds of various *Pinus* species in USA, South Africa and Brazil [6-8]. *Diplodia sapinea* causes tip blight of pines in trees of all ages [9] and has been commonly associated with cones and seeds of various pine species [10,11].

The global and regional transport of seed is also an important pathway for introduction and dissemination of *Fusarium circinatum*, the causal agent of pine pitch canker. The pathogen is a chronic problem in seedling production because it can cause severe pre- and post-emergence damping off, and lead to dieback and mortality of older pine trees in plantations and forests [12,13]. Seed coats and seedling coleoptiles may be colonized by *F. circinatum* [12]. Dwinell [14] found more post-emergence damping-off in *P. radiata* seedlings grown from *F. circinatum*-contaminated seeds than from uncontaminated seeds. Moreover, it is known that internal contamination of seed results in high rates of infection in germinating seedlings compared with superficial contamination [15]. *Fusarium circinatum* may also be transported in seed of Douglas-fir (*Pseudotsuga menziesii*), the only gymnosperm other than members of the genus *Pinus* known to be susceptible to this pathogen [16,17]. *Fusarium circinatum* is regulated in the EU which prohibits the introduction of live plants or plant parts of *Pinus* spp. and *P. menziesii* other than fruit and seed from non-European countries in all member states (Commission Decision 2007/433/EC) [18].

The transport and use of latently infected and/or contaminated seed has been implicated to be a factor in the global movement of several foliar and shoot pathogens. For example, genotypic diversity of *D. sapinea* was consistent with historical records of seed and germplasm importation to and within the southern hemisphere [19]. Seeds of *P. menziesii* have been suggested as a potential source of infection of the needle cast pathogen *Rhabdocline pseudotsugae* in addition to the ascospore-based dissemination [20,21]. Bentele *et al.* [22] also suggested that the needle cast pathogen *Lophodermium seditiosum* on Scots pine (*Pinus sylvestris*) could be seed-borne. In general, there is little information on what diversity of fungi (including contaminants) are found in routinely traded seed materials. Knowledge of these so-called 'plant-associated hitchhikers' may be important to assess the potential risk for their establishment and spread in new locations.

The advent of high throughput sequencing (HTS) opened a new era in fungal ecology [23] that allows for holistic insights into the immense taxonomic (relying on DNA metabarcoding) and functional (metatranscriptomics) diversity of fungi in different niches, with ever-increasing resolution [24-26]. Despite the growing body of evidence for its usefulness in the early detection and surveillance of potentially invasive forest pathogens [27-29], and its high sensitivity and capacity to

deal with large numbers of samples in less time compared to traditional methods [30], HTS is generally underutilized as a tool in plant biosecurity [31-35].

From a phytosanitary perspective, tree seeds are generally considered safer for trade than live plants [36] primarily because different plant organs on live plants, and in the case of potted plants, also soil, offer a plethora of niches/microhabitats that can carry diseases caused by all pathogen functional groups [37]. However, fungal contamination can occur at many stages of seed handling and storage, and this fact receives little recognition or consideration in the seed trade business. Globally, the interest in purchasing seed through online distributors has escalated in recent years; more than 22 million 'seed catalogues' can be recovered through a simple online Google search [38], which implies even less control in the global movement of seed. Safeguarding seed health is critically important for avoiding trade-related spread of plant pathogens. Thus, a better understanding the mycobiota associated with seeds, prior to transport, can add relevant and scientifically-evidenced information for pathway risk analyses for seeds of a particular tree species that may be used for commercial purposes. The aim of this study was to determine the fungal communities present in seeds of a range of *Pinus* species obtained from both commercial outlets and national seed banks in order to demonstrate the capacity of this pathway to vector potentially harmful plant pathogens and biosecurity risks associated with trade in seed.

2. Materials and Methods

2.1. Plant material

Seed of a range of *Pinus* species from different commercial outlets, including seed stands, seed orchards and online internet retailers from four countries: Turkey, Sweden, United States of America (USA) and Portugal were tested (Table 1). With the exception of Sweden, all other country sources included seed of both native and non-native *Pinus* species. Four species (*P. sylvestris*, *P. pinaster*, *P. radiata*, and *P. pinea*) were represented in more than one country.

Table 1. *Pinus* species, source origin, and type of commercial outlet that seedlots were sourced from in the four different countries.

Sample code	*Pinus* species	Geographic origin	Native state	Type of commercial outlet	Seedlot source or location
Turkey					
Ps1	*P. pinaster*	Europe	Non-native	Seed stand	Kerpe
Ps2	*P. nigra subsp. pallsiana*	Europe	Native	Seed orchard	Bartın
Ps3	*P. brutia*	Europe	Native	Seed orchard	Döşemealtı, Antalya
Ps5	*P. sylvestris*	Europe	Native	Seed orchard	Sarıkamış
Ps6	*P. radiata*	California USA, Mexico	Non-native	Seed stand	Kerpe
Ps7	*P. halepensis*	Europe	Native	Seed stand	Fethiye
Ps26	*P. pinea*	Europe	Native	Seed stand	Döşemealtı, Antalya
Sweden					
Ps10	*P. sylvestris*	Europe	Native	Seed orchard	Moliden
Ps11	*P. sylvestris*	Europe	Native	Seed orchard	Lycksta
Ps12	*P. sylvestris*	Europe	Native	Seed orchard	Gotthardsberg
USA					
Ps13	*P. sylvestris*	Europe	Non-native	Internet order	n/a*
Ps14	*P. thunbergii*	Asia	Non-native	Internet order	n/a
Ps15	*P. patula*	Mexico	Non-native	Internet order	n/a
Ps16	*P. taeda*	Eastern USA	Native	Internet order	n/a
Ps17	*P. densiflora*	Asia	Non-native	Internet order	n/a
Ps18	*P. radiata*	California USA, Mexico	Native	Internet order	n/a

Ps19	*P. elliottii*	Southeastern USA	Native	Internet order	n/a
Portugal					
Ps20	*P. strobus*	Eastern USA and Canada	Non-native	Seed stand	n/a
Ps21	*P. mugo*	Central Europe	Non-native	Seed stand	n/a
Ps22	*P. pinaster*	Europe	Native	Seed stand	n/a
Ps23	*P. sylvestris*	Europe	Native	Seed stand	n/a
Ps24	*P. radiata*	California USA, Mexico	Non-native	Seed stand	n/a
Ps25	*P. pinea*	Europe	Native	Seed stand	n/a

* n/a; information is not available or unknown

2.2. *DNA extraction and sequencing*

From each seed lot, 100 seeds, randomly chosen, were lyophilized for 48 hours in 50 ml Falcon tubes. Samples were homogenized to powder using a MM200 Retsch ball mill (Retsch GmbH, KG, Haan, Germany) in 25 ml metal grinding vessels with a 6 mm diameter stainless steel ball. No surface sterilization was performed on seeds before homogenization since superficial contamination may also represent part of the introduction pathway for some harmful microorganisms. Metal vessels and ball were sterilized by immersing in 5 % sodium hypochlorite for 5 min, followed by 70 % ethanol for 5 min and then left to dry to avoid any cross-contamination between samples. After homogenization, DNA was extracted from 30 mg of homogenized tissue using the E.Z.N.A. SP Plant DNA Kit (Omega Bio-tek, Doraville, Georgia) following the manufacturer's protocol for dry samples. DNA was quantified using NanoDrop (® ND-2000 UV/vis Spectrophotometer, Thermo Fisher Scientific Inc., Wilmington, Delaware, USA).

The ITS2 region of the rDNA was amplified by PCR using the fungal primers fITS7 and ITS4 [39,40] that were tagged with different 5' identifier sequences. For each sample, three replicate PCRs were conducted. Reactions were carried out in 50 µl volumes each containing: 5 ng/µl template DNA or 5 ng/µl of sterilized water for the blank, 200 µM dNTPs; 750 µM $MgCl_2$; 0.025 µM Phusion High-fidelity DNA Polymerase (Thermo Scientific), and 200 nM of each primer in 1× buffer. All reactions were performed in an Eppendorf® Master Cycler®. The PCR program consisted of denaturation at 98 °C for 3 min, followed by 31 cycles of 98 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 30 s, before a final extension step at 72 °C for 7 min. PCR products were purified using Agencourt AMPure XP (Agencourt Bioscience Corp, Massachusetts USA) and quantified using a Qubit 3.0 Fluorometer with the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA). After quantification, PCR products were pooled in an equimolar mix and sent for sequencing using the Illumina MiSeq platform (Macrogen Inc, Seoul, Korea).

2.3. *Bioinformatics and analysis of sequence data*

Analysis and annotation of output data were carried out using the QIIME data analysis package [41]. Sequences were processed using QIIME version 1.9 [41]. Poor quality reads (<Q20, minimum length = 150 bp, homopolymer = 6 bp) were removed. All sequence files were combined into a single fasta file. Sequences were dereplicated and singletons were removed in USEARCH (http://www.drive5.com/usearch/). Following sequence clustering at 97% similarity using the UPARSE algorithm implemented in USEARCH which included chimera detection and filtering, each sequence was searched by BLAST against NCBI. No fungal sequences were removed. OTUs with identity and coverage ≥ 97% were assigned to a species level and below that threshold, to a genus or family level [35].

The FUNGuild database v1.0 database (https://github.com/UMNFuN/FUNGuild) was used to assess the ecological and functional diversity in samples of OTUs identified to the species level [42]. Trophic levels included: pathogens (in FUNGuild referred to as pathotrophic fungi), saprotrophs, and mutualists (in FUNGuild referred to as symbiotrophic fungi). Fungal taxa categorized into

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combined pathogen-saprotroph or pathogen-mutualist trophic levels included those that may exhibit different life styles depending on life cycle stage, environmental conditions, or both.

All data analyses were conducted in R version 3.5.1 (R Core team, 2018) using the vegan package version 2.4 [43]. The number of sequences per sample was normalized to the smallest sample size in order to remove sample heterogeneity. The relative abundance of a specific fungal OTU was defined as the percentage number of reads where the OTU was detected relative to the total number of reads in a sample. The number of sequences per sample was normalized to the smallest sample size in order to remove heterogeneity. The relative abundance of a specific fungal OTU was defined as the percentage of the number of reads where the OTU was detected relative to the total number of reads in a sample. A rarefaction curve plot was generated showing the number of OTUs versus the number of sequences (reads). Richness was estimated with the Chao1 richness estimator (a nonparametric method for estimating the number of species in the community, considering also rare OTUs) using the formula: Schao1 = S*obs* + {*n1* (*n1*-1)/2 (*n2* + 1)}, where S*obs* is the number of observed species/OTUs, *n1* is the number of OTUs that contained only one sequence, and *n2* is the number of OTUs that contained two sequences. Shannon index, which measures the diversity of communities considering both species richness and evenness, was calculated using the formula H′ = -Σp*i*ln(p*i*), where p is the relative abundance of the phylotypes.

The 'plant pathogen' community composition among samples was visualized using non-metric dimensional scaling (NMDS) on a Bray–Curtis distance matrix. A heatmap of relative abundances of the most dominant plant pathogens was generated and samples were sorted based on Bray–Curtis distance. The sequences of the four most abundant SpOTUs (i.e., plant pathogens identified to species level) were aligned with known sequences in GenBank, using ClustalW [44]. Neighbour joining analysis, based on K2P distances and tested by 1000 bootstrap replicates, was conducted for each alignment to confirm the identification of query sequence to the genus or species level, using PHYLIP 3.6 [45] and trees were drawn using TreeView [46].

3. Results

3.1. *Structure of the fungal communities of Pinus spp.*

After quality filtering and sequence processing, we obtained large data sets, ranging between 52,054 and 230,458 (average =96,330 ± 7,857) fungal sequences per sample. Rarefying at the lowest recovery threshold, a total of 1,997 unique fungal OTUs were recorded from the 23 seedlots. The rarefaction curves based on OTU numbers showed that all the samples reached the saturation plateau, and that the sequencing effort revealed the full diversity of the fungal community (Figure 1).

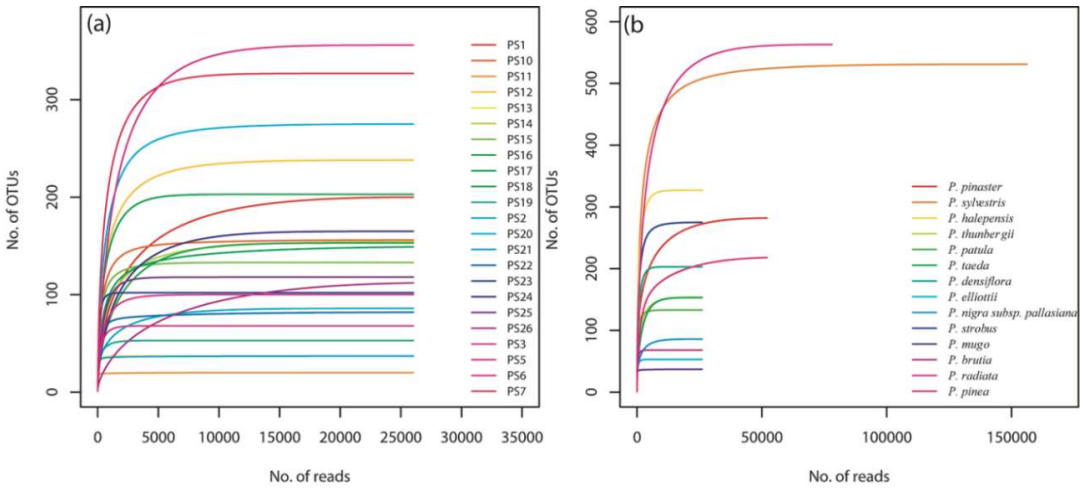

Figure 1. Diversity of fungi in a range of *Pinus* spp. seedlots. **(a)** Rarefaction curve of OTUs in each seed sample plotted against the number of pyrosequencing reads excluding singletons. The numbers

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represent sample ID (Table1); **(b)** Rarefaction curve of OTUs for each *Pinus* species plotted against the number of pyrosequencing reads excluding singletons. OTUs were clustered conservatively at 97% similarity.

A diverse population of fungi was associated with seeds of *Pinus* spp. sourced from the four different countries (Supplementary File 1). Ascomycota dominated the fungal community, representing 53% of the OTUs. The second most abundant phylum was Basidiomycota, representing 21% of the OTUs. The abundance of the OTUs assigned to the Chytridiomycota and Zygomycota phyla were 0.1% and 1.4%, respectively. Approximately 25% of reads could not be assigned to any phylum.

Chao1 richness estimates showed a large disparity among samples. An average of 144.5 taxa per sample were detected. Chao1 values ranged from 20 (P. sylvestris, Sweden, Ps11) to 356 (*P. radiata*, Turkey, Ps6). Sample Ps26 (*P. pinea*, Turkey) had the lowest Shannon index value (0.8114) while Ps7 (*P. halepensis*, Turkey) had the highest (4.222) (Table 2). However, both total fungal community and plant pathogen community were not significantly affected by geographic origin of seeds. In addition, based on Chao and Shannon diversity indices, there were no significant differences among native and non-native *Pinus* spp.

The ecological functions of approximately 47% OTUs (n=945) were not defined. The remaining OTUs comprised saprotrophs, pathogens-saprotrophs-symbionts and pathogens, at 21%, 12% and 10%, respectively. A total of 212 OTUs (10%) had life strategies in five other guilds (Figure 2).

Table 2. Shannon index (H) and Chao1 richness of the total fungal community associated with seed samples of *Pinus* spp. from different countries. .

Sample code	*Pinus* species	Total fungal community		"Plant pathogens"	
		Chao1	Shannon_H	Chao1	Shannon_H
Turkey					
Ps1	*P. pinaster*	200	2.833	26	1.424
Ps2	*P. nigra subsp. pallsiana*	86	2.538	22	1.621
Ps3	*P. brutia*	68	1.899	23	0.8489
Ps5	*P. sylvestris*	100	1.871	24	1,472
Ps6	*P. radiata*	356	2.895	36	1.603
Ps7	*P. halepensis*	327	4.222	65	2.165
Ps26	*P. pinea*	112	0.8114	43	0.661
Sweden					
Ps10	*P. sylvestris*	156	2.562	28	0.6453
Ps11	*P. sylvestris*	20	1.91	10	1.449
Ps12	*P. sylvestris*	238	3.254	36	1.47
USA					
Ps13	*P. sylvestris*	37	2.377	6	0.931
Ps14	*P. thunbergii*	154	2.461	59	1.692
Ps15	*P. patula*	133	3.215	18	2.182
Ps16	*P. taeda*	153	1.78	54	1.181
Ps17	*P. densiflora*	203	3.119	52	1.902
Ps18	*P. radiata*	149	2.439	51	1.405
Ps19	*P. elliottii*	53	2.608	28	2.254
Portugal					
Ps20	*P. strobus*	275	3.898	69	2.541
Ps21	*P. mugo*	37	2.583	8	1.769
Ps22	*P. pinaster*	82	2.323	28	0.9963
Ps23	*P. sylvestris*	102	3.79	18	2.136
Ps24	*P. radiata*	165	1.495	30	0.2639
Ps25	*P. pinea*	118	2.147	86	2.115

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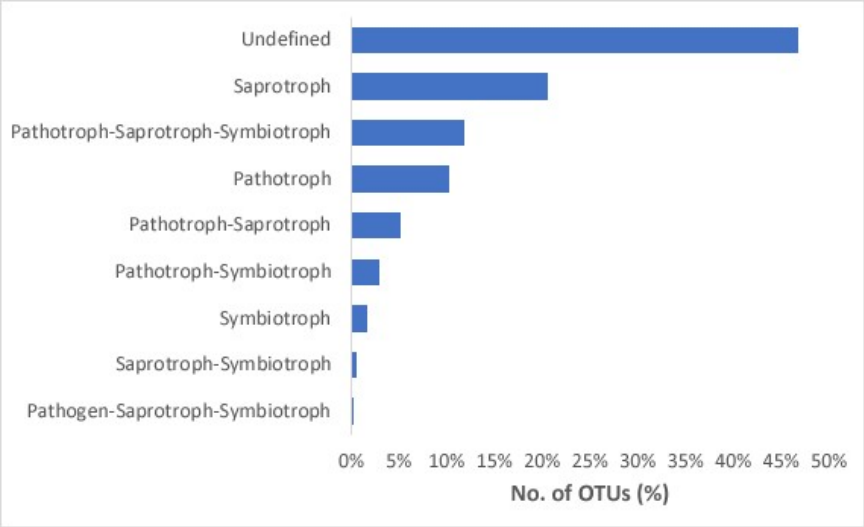

Figure 2. Relative abundance of fungi in seed assign to different trophic levels in FUNGuild. Trophic modes include: pathogen [pathotroph], saprotroph, and mutualist [symbiotroph], and combinations thereof.

3.2. *Structure of the plant pathogen community in Pinus spp. seed*

A considerable number of sequences grouped in the fungal guild "Plant Pathogen". Of the total number of OTUs, 453 (23%) were classified as plant pathogens and 60% of those were identified to the species level (SpOTUs = 276 OTUs). Ascomycota was the most prevalent phylum (representing 95% of all SpOTUs). Taxonomic classifications of the SpOTUS from the Ascomycota are shown in Figure 3. The class Dothideomycetes (57%) was the most abundant and consisted of fungal sequences in the orders Botryosphaeriales (36%), Capnodiales (14%), Dothideales (9%), Pleosporales (37%) Venturiales (4%). The phylum Basidiomycota accounted for 5% of the plant pathogens sequences identified at the species level. It included Agaricomycetes (43%), with only Mycenaceae, and Exobasidiomycetes (57%), dominated by Exobasidiaceae (75%), followed by Quambalariaceae (25%).

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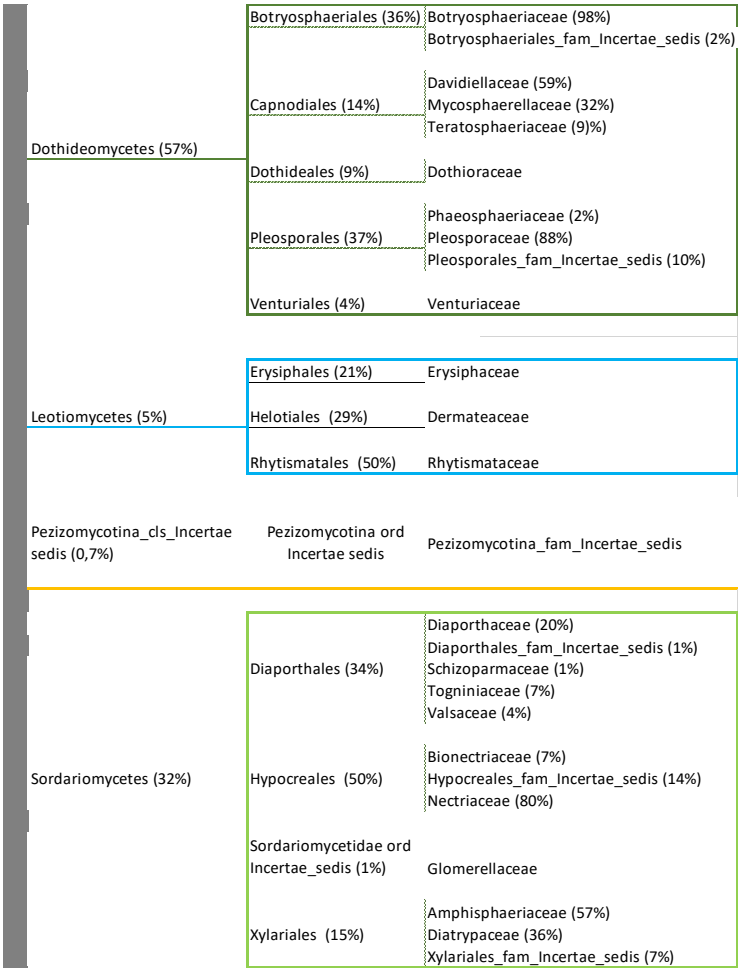

Figure 3. Relative abundance of different taxonomic groups within the Ascomycota of OTUs classified as plant pathogens.

NMDS biplot revealed that the fungal community differed among samples. *Pinus* species and country of origin were not related to the fungal community (Figure 4). For example, fungal communities from *P. sylvestris* (Ps10, Ps11 and Ps12 from Sweden, Ps13 from USA, Ps5 from Turkey and Ps23 from Portugal) did not cluster together.

Plant pathogen SpOTUs grouped as 141 different species, of which 35% were associated to *Pinus* spp. from all over the world. Among those species, *Pestalotiopsis microspora* was never recorded in Europe [47]. However, it was detected on samples of *P. brutia* (P3) and *P. radiata* (P6) from Turkey and *P. radiata* (P24) from Portugal. *Pinus radiata* was associated with more than 55% of pine pathogens. The double hierarchical dendrogram showed the fungal community distribution among samples related to taxa present with more than 10% of taxa in seed samples (Figure 5). Most of the SpOTUs were identified as *Sydowia polyspora* (31%), *Lasiodiplodia theobromae* (13%), *Diplodia intermedia* (12%) and *Diplodia sapinea* (18%), which were the most abundant species found, representing over 10% of all SpOTUs (n=276). These species were present in all samples, except Ps13 and Ps5. Sequence data for the four most dominant fungal species were deposited in GenBank (Accessions MK635056-MK635059).

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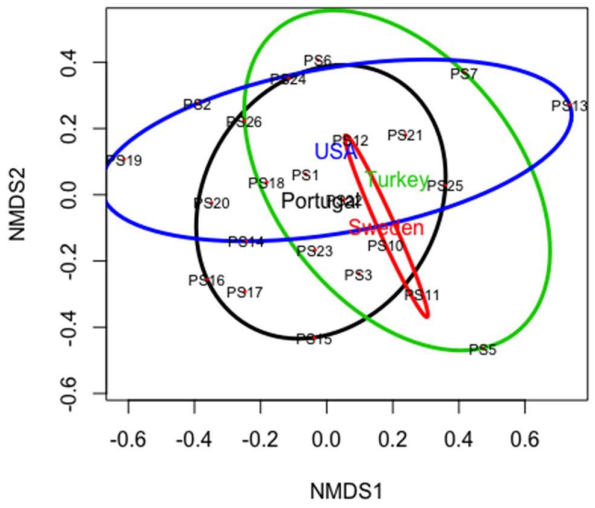

Figure 4. Two-dimensional non-metric multidimensional scaling (NMDS) biplots based on Bray-Curtis distances displaying differences among the fungal community structures identified to the species level. The points represent *Pinus* samples (see sample ID, Table 1). The coloured ellipses represent the country of the samples' origin. R2=0.95.

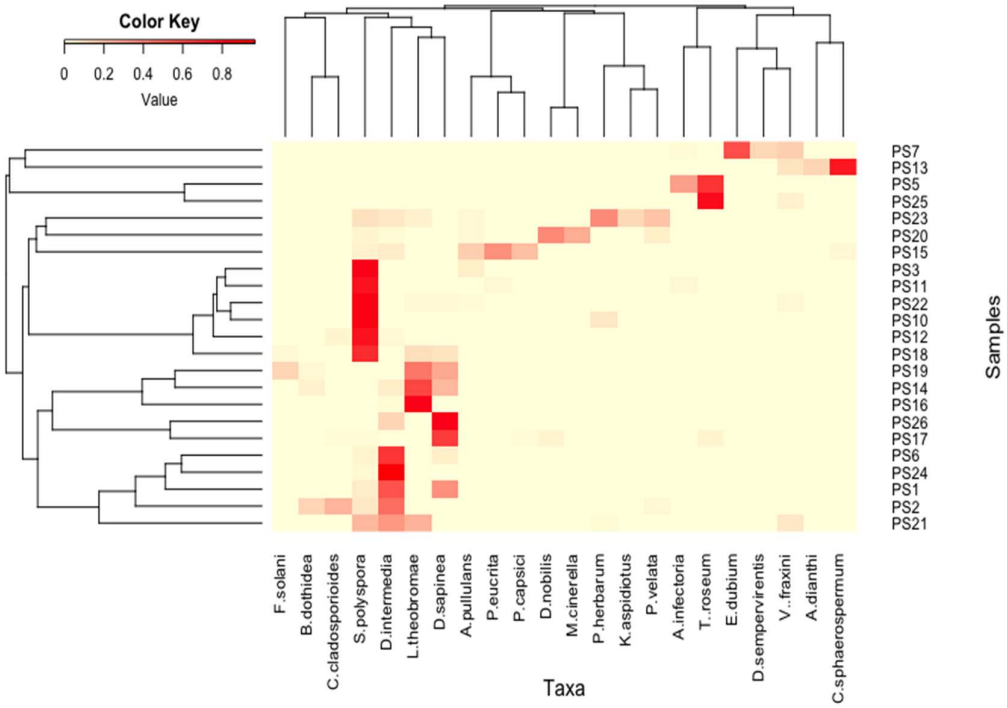

Figure 5. Heatmap analysis of species present with more than 10% of taxa, in seed samples. Numbers indicate the samples of *Pinus* spp. seed (Table 1).

A phylogenetic tree was built to confirm the identity of four SpOTUs (C202, C607, C333 and C625) namely *S. polyspora*, *L. theobromae*, *D. intermedia* and *D. sapinea*, in relation to other taxa (Figure 7). *Diplodia intermedia* and *D. sapinea* clustered together. *Lasiodiplodia* species could not be resolved based on analysis of a portion of the ITS region. Sequences of *S. polyspora* were placed in a distinct clade separate from all other species and supported by a high bootstrap value (Figure 6).

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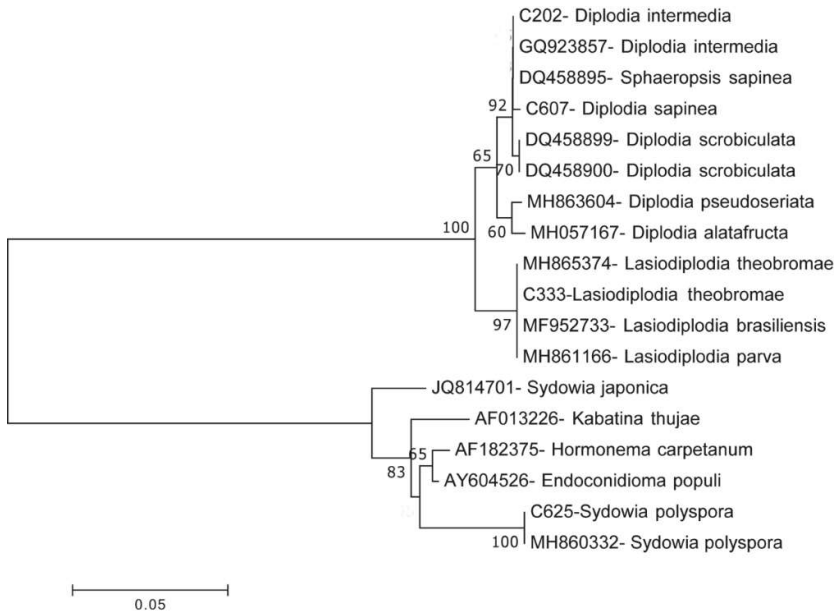

Figure 6. Neighbour joining phylogenetic tree based on the ITS region showing the position of SpOTUs C202, C607, C333 and C625 in relation to other taxa. Bootstrap support values (percentages of 1000 replicates) are shown on branches (values,50 % are not shown).

4. Discussion

The fungal communities present in seeds of a variety of *Pinus* species obtained from commercial outlets were examined using HTS in order to assess the presence of known plant pathogens and the potential risk of introduction through the trade of seeds. Identification of nearly 2000 OTUs confirmed diverse fungal communities in or on seed of different *Pinus* species. Fungi that were commonly detected and identified were polyphagous, often occurring in several *Pinus* species, however communities were not structured by host or source origin (country). Replicates of the same *Pinus* species in this study (*P. sylvestris*, *P. pinaster*, *P. radiata*, and *P. pinea*), harbored distinguishable fungal communities, perhaps with the exception of the Swedish seedlots. Species accumulation curves showed that sampling effort in individual seedlots adequately represented the diversity present in these fungal communities.

Many of the fungi associated with the seed tend to have saprotrophic life styles and are unlikely to have a significant impact on seed germination and seedling growth [48-50]. However, a significant proportion of the OTUs detected were potential pathogens that could cause seed damage, seedling diseases (damping-off), and shoot and tip dieback diseases of older pine trees. However, none of the species identified are included in the EPPO A1 and A2 quarantine lists.

In this study we used the ITS region which is generally accepted as the official barcode for fungi by several mycologists due to its ease in amplification, widespread use, and appropriately large barcode gap [51]. However, there are limitations to the use of ITS as a standard for molecular identification because intraspecific variation does not permit sufficient resolution for the identification of some fungal species. In addition, the 97% sequence similarity threshold for the ITS marker gene can be too conservative for species-level identification of some fungal taxa [52]. Specifically, the genera *Fusarium* and *Diaporthe* are known to contain species complexes [53,54]. At least ten putative *Fusarium* taxa were detected in this study using ITS sequencing (Supplementary File 1), though further work would be needed using additional and specific markers [55] to resolve the *Fusarium* species identification. A phylogenetic approach helped resolve some limitations to the barcoding for the identification of dominant plant pathogens in this study, namely, *S. polyspora*, *L. theobromae*, *Diplodia intermedia* and *D. sapinea* which were identified in multiple *Pinus* species.

Sydowia polyspora is commonly known as a foliar endophyte and a weak, opportunistic pathogen that under certain conditions can cause small foliar lesions [56]. Current year needle necrosis caused

by *S. polyspora* has been associated with true fir (*Abies* spp.) across Europe and North America [57-60]. The recent finding of *S. polyspora* behaving as a cryptic pathogen strongly delaying seed emergence in *Pinus ponderosa* [61] supports the idea that fungi may have a broad repertoire of ecological interactions with their hosts that may include the ability to infect and colonize them with and without causing visible damage. Although common foliar endophytes such as *S. polyspora* can become opportunistic pathogens, attacking hosts under conditions conducive to fungal growth, this problem may become more relevant with climate change as additional stresses are added to host growing conditions. A better understanding of the dynamics of fungal life strategies working either positively or negatively on the host, and in some cases cryptically in plant community processes, is needed [61].

Lasiodiplodia theobromae is a fungus that causes black seed rot, resulting in the destruction of slash pine (*Pinus elliottii* var. *elliottii*) seeds in southern US and shoot dieback of slash and loblolly (*P. taeda*) pine seedlings in nurseries [62,63]. *Lasiodiplodia theobromae* is a cosmopolitan, polyphagous, and opportunistic fungal pathogen on a variety of woody hosts across the globe [64]. The pathogen has been detected in seed of *Pinus caribaea, Pinus oocarpa* in Central America [65,66], *Pinus elliottii* in South Africa and southeastern USA [6,62,68] and recently in *P. elliottii* and *P. taeda* in Brazil [7]. Its presence inside the seed impairs germination and causes seedling death. A diverse mycoflora including *L. theobromae*, *Fusarium* spp. and *D. sapinea* was associated with slash pine (*Pinus elliottii* var. *elliottii*) seeds obtained from a seed orchard and cone processing facility in southeastern USA [68]. Cone collection practices can influence the incidence of disease since Fraedrich *et al.* [69] found that seeds from cones removed prematurely from trees and left on the ground for short periods had higher infection levels and contamination by *L. theobromae* than seeds from cones that were close to maturation at the time of collection.

Diplodia sapinea is one of the more economically important damaging agents of *Pinus* species worldwide, occasionally affecting other important conifers such as *Pseudotsuga* sp. *Abies* sp., *Picea* sp. and *Larix* sp. [70]. Severe epidemics have been reported from pine nurseries and plantations in North America [71,72] and more recently in the Baltic and Scandinavian region [9,73]. *Diplodia sapinea* has been detected in seed from several *Pinus* species all around the globe [10,66,68,69,74-82], and can remain alive in seed for several years [10]. Decourcelle *et al.* [10] showed a high percentage of infections in the seeds of Corsican pine trees having no apparent symptoms of *D. sapinea*. It is well known that latent infections exist in symptomless tissue on both diseased and non-diseased trees, and have the potential to become pathogenic [77,83,84]. Though Decourcelle *et al.* [10] found the risk of disease transmission was low in Corsican pine seeds, the frequent importation and use of latently infected pine seed has been implicated as a factor promoting the global movement of *D. sapinea*. A higher genetic diversity in South African populations of *D. sapinea* compared to other southern hemisphere countries was linked to historical records of the frequency and quantity of seed and germplasm importation [85,86]. An added risk with multiple introductions is the possibility of new introduced genotypes that may cross with existing genotypes, allowing for more gene diversity and possibly greater pathogen virulence, leading to more severe disease outbreaks [19]. Thus, the possibility cannot be excluded that seed contamination originating from seed orchards – often the main suppliers of large seed quantities to forest tree nurseries for reforestation efforts - accidentally plays a role in the dissemination of this pathogen to unaffected regions.

Pestalotiopsis microspora was the only species associated with *Pinus* spp. not yet present in Europe. The fungus has been detected as endophyte on *Pinus radiata* in Kenya and as pathogen on several fruits, e.g. kiwi, outside Europe [47]. The introduction of *P. microspora* could pose a risk for European cultivations. An earlier introduction of a closely related species, *P. maculans*, now established in Spain, was also attributed to seed [4].

A large majority (approximately 65%) of the plant pathogens identified were not associated to *Pinus* spp. but rather to other plant hosts. Among these included cosmopolitan, polyhagous plant pathogens such as *Botryosphaeria stevensii*, *B. dothidea, Neofusicoccum parvum*, and several species of *Fusarium* (teleomorph *Gibberella*) and *Diaporthe* that are yet unresolved. Pathogens having relatively restricted host range such as *Valsa sordida* – commonly associated to poplar, *Eutypa lata* –the causal

agent of Dead arm of grapevine, and *Ramularia eucalypti* – a common leaf spot disease of *Eucalyptus* hosts, were also detected in *Pinus* spp. seeds. The results suggest that seed transported for commercial use can be subjected to contamination by propagules of a large diversity of fungi (even those that occur outside of its geographic host range), obviously influenced by the external environment to which that commercial seed is cultivated, collected or processed. This raises further questions as to whether there would be a potential for introducing crop pathogens via seed of highly unrelated ornamental species, or vice versa.

Increased knowledge of the mycobiome associated with seeds, including the identification of potentially harmful pathogens, is needed to make informed decisions prior to transport or regarding phytosanitary measures to prevent the introduction and establishment of invasive pathogens. For example, previous detection of the alien invasive fungus *Hymenoscyphus fraxineus* on seeds of *Fraxinus excelsior* [87] supported phytosanitary measures in North America and U.K. to prohibit the import, and in the case of the UK, the internal movement of plant material including seeds. Even though *H. fraxineus* does not appear to be a seed-borne disease [88], infested seed still serve as a potential source of inoculum capable of forming ascomata, and subsequent sporulation under optimal temperature and moisture conditions. For most seed-borne fungi, populations can increase during phases of seed development, seed harvesting and processing, especially under high moisture and temperature conditions which are conducive to their development [89].

Seed fungi are found on and in the seedcoat and in the gametophyte and embryo, and with the exception of molds, are not easily detected through visual observation [89]. Even seedlots infested with known pathogens appear normal and healthy-looking [87]. As in this study, the *Pinus* species seedlots utilized contained multiple plant pathogens but appeared healthy and viable, and undoubtedly would pass a visual phytosanitary inspection. However, the symptomless persistence of the identified pathogens such as *D. sapinea* and *S. polyspora* could have important implications for dissemination and emergence of new disease outbreaks. Even superficial contamination can affect seed germination and subsequent seedling damage at a later stage [14], though the thresholds are not well known. Moreover, only a fraction of the total taxa detected were identified, thus it is reasonable that there may be other pathogens not yet discovered.

Anonymity and transporting seed from 'unknown' locations such as those acquired from online distributors provides an added element of risk for introducing invasive species since it is not known what potential pathogens are carried in or on the seed. We strongly encourage seed production and retail distributors to adopt better biosecurity practices: the mycobiota associated with the commercial seedlots they are selling should be known, and the shipment of seeds containing potentially pathogenic fungi should be avoided. At the least, measures must be taken to reduce or eliminate the risk by appropriate seed treatment (e.g. thermotherapy). Consumers need to adopt socially and environmentally responsible consumer behavior when purchasing commercial seed, requesting basic information on source location of the seed (as opposed to geographic origin of the species), and purchase locally to deter possible entry of new invasive threats. In the EU, the European directive for the marketing of forest reproductive material only mentions that imports of tree seed consignments from suppliers should comply with species purity and germination capacity [90]. Moreover, the European directive on protective measures against the introduction of organisms harmful to plant or plants products and against their spread within, with the exception of *Pinus* spp. and *P. menziesii* [18], mainly exclude seeds of woody plants from controls during import from non-European countries [91]. Plant protection authorities should however be alerted to the potential risk associated with import and domestic movement of seeds and consider phytosanitary measures to minimize the risk of introducing not just quarantine, but other potentially harmful and emerging pathogens.

The study highlights several known pathogenic fungi, and possible cryptic risks associated with the intra- and intercontinental movement of tree seed for commercial, including ornamental, purposes. The use of HTS technologies for screening biosecurity risks remains underutilized in practice. For *Pinus* – which has such a large economic importance globally - the risk of disseminating pathogens (including those causing serious foliar and shoot diseases) through the transport of seeds, is certain. This work, together with evidence from numerous studies [e.g. 2,4,15,19] and cases added

to the curated database for Europe [4] since 2013 such as *Neonectria neomacrospora* and *Sirococcus tsugae* (Alberto Santini, personal communication, May 13, 2019) lend strong support that seed distribution for commercial purposes can contribute to the etiology of several emerging forest diseases.

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