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Seasonal and Site-Specific Patterns of Airborne Fungal Diversity Revealed Using Passive Spore Traps and High-Throughput DNA Sequencing

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Abstract: The aim of the present work was to study seasonal and site-specific patterns of airborne fungal diversity, focusing on plant pathogens. The sampling of fungal spores was carried out for twelve months, i.e., between September 2017 and August 2018, using passive spore traps that were placed at three different sites in western (Lenkimai), central (Dubrava), and eastern (Labanoras) Lithuania. Samples were collected every 7–10 days, resulting in 146 samples altogether. Following DNA isolation, samples were individually amplified using ITS2 rRNA as a marker and subjected to high-throughput sequencing. Clustering and taxonomic classification of 283,006 high-quality reads showed the presence of 805 non-singleton fungal taxa. The detected fungi were 53.4% Ascomycota, 46.5% Basidiomycota, and 0.1% Mucoromycota. The most common fungal taxon at Labanoras and Lenkimai was *Hannaella coprosmae* (23.2% and 24.3% of all high-quality fungal sequences, respectively), while at Dubrava it was *Cladosporium macrocarpum* (16.0%). In different sites, plant pathogenic fungi constituted between 1.6% and 14.6% of all fungal taxa and among these the most common were *Protomyces inouyei* (4.6%) and *Sydowia polyspora* (1.9%). The results demonstrated that the diversity of airborne fungi was mainly determined by the surrounding vegetation and climatic factors, while the occurrence of pathogenic fungi was affected by the availability of their hosts.

Keywords: biological invasions; fungal spores; microbial diversity; plant pathogens

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1. Introduction

Biological invasions represent an important component of global change with major ecological impacts on the conservation of native species, and on the integrity of natural and managed ecosystems worldwide [1,2]. Interactions among climate changes, including changes in mean temperatures and precipitation, global trade, and pests and pathogens (indigenous or non-native) can be expected to have a serious impact on health and sustainability of forest ecosystems. Apart from recent biological invasions, such as ash dieback in Europe [3,4], many new, unprecedented forest health problems are likely to occur in the future [5], leading to alterations in forest primary production and ecosystem functioning. For instance, insects and pathogens may modify their ranges of distribution in response to changes in the host tree distribution and changes in climatic conditions, thereby moving into new areas and utilizing existing hosts or shifting to new hosts [6].

Different pathways of introduction of non-native forest pests and pathogens have been suggested [5]. Dispersal by air is one of many mechanisms that allow plant pathogens to spread both locally and globally [7–9]. Indeed, Leyronas et al. [10] demonstrated that many plant pathogenic fungi can be disseminated via the atmosphere from micro- to macro-geographical scales. Fungi are known to disseminate by the release of spores, mycelial fragments, and/or other propagules into the atmosphere and each year they can circulate

across wide geographic areas and over long periods of time [11]. By these means, certain fungal pathogens may travel long distances, while surviving extreme temperatures, UV radiation, and desiccation, remaining viable to cause disease [12]. The airborne fungal structures are eventually deposited on the ground or water surfaces by sedimentation (dry deposition) or precipitation (wet deposition) and contribute to the global cycling of substances [13].

Despite the majority of airborne fungal spores being dispersed locally, they can also travel over long distances [10]. This is determined by many factors including the physical characteristics of the spores (size, shape, degree of surface roughness, density, and electrostatic charges) and environmental factors, including wind (speed, direction, turbulence, gradients near the ground, and pattern of atmospheric circulation), rain, and topography of the area [14]. Indeed, some fungal pathogens can make long distance jumps from one susceptible host to another just in one growing season by following the direction of the prevailing winds. For example, this is the case for oomycetes, responsible for tobacco blue mold and cucurbit downy mildew [10].

Generally, the release of fungal spores is highly dependent on climatic factors [15] which affect the abundance of fungal spores in the atmosphere of different geographical regions [16–18]. For the release of fungal spores, some fungi require rather humid air conditions, whereas others favor dry and windy conditions. For example, the mean air temperature, relative humidity, and wind speed are known to be factors that determine the spore release and distribution of *Alternaria*, *Cladosporium*, *Drechslera*-type, *Epicoccum,* and *Torula* fungi [19–21]. Thus, the dynamics of microbiome in the atmosphere appears to be governed by a combination of air movement, spore production, climatic factors, local vegetation sources, and anthropological activities such as agriculture and large-scale composting [18], all of which affect fungal growth and sporulation. The effect of each factor varies by fungal species, with the complex dynamics not fully understood, even for fungi causing various plant diseases [22]. Seasonal dynamics and life cycles of fungal foliar endophytes, tree pathogens or mycorrhizal fungi can demonstrate their specificity and adaptations for spread as this can be reflected in the aerial spore composition [23]. It was found that the composition of spores in the air may depend on how the different fungal species interact with their hosts, and the release of fungal spores may often coincide with the growing activity of their hosts [24,25].

Despite attempts to study the movement of microorganisms in the atmosphere between different geographical locations [21], seasonal patterns of fungi in the air are still scarce [13,26]. Such studies are often aimed at detecting the presence of fungal spores, which may have an adverse effect on humans or animals [27–29]. However, the atmosphere may also contain various plant pathogenic fungi whose airborne spores may play a crucial role in plant disease dispersal [30–32]. Indeed, many economically important diseases of forest trees and agricultural crops have airborne propagules and may disperse rapidly, and over relatively large distances [33,34]. On the other hand, most fungal spores fall close to their fruitbodies [35] and captured spores largely reflect site-specific and local diversity of fungi [23]. Apart from Glomales, Chytridiomycota, and many Gasteromycetes, most fungi disperse by airborne spores [36], forming a large proportion of the fungal diversity in natural ecosystems [23].

The aim of the present work was to study seasonal and site-specific patterns of airborne fungal diversity, focusing on plant pathogens.

2. Materials and Methods

2.1. Study Sites and Sampling

The sampling of fungal spores was carried out for twelve months, i.e., between September 2017 and August 2018. In order to examine the possible site-specific fungal communities and their variation during the sampling season. Study sites were selected in three different locations situated in western (Lenkimai), central (Dubrava), and eastern (Labanoras) Lithuania (Figure 1). The distance between different sites was at least 150 km.

Study sites (within a radius of 200 m) at Labanoras and Lenkimai were of similar forest type, namely composed of ca. 100-year-old *Pinus sylvestris* trees with *Corylus avellana* and *Juniperus communis* in the understory. The ground vegetation consisted of *Vaccinium myrtillus*, *Vaccinium vitis-idaea*, *Calluna vulgaris,* and *Poaceae* sp. By contrast, the forest at the Dubrava site was composed of ca. 70-year-old *Alnus incana*, *Betula pendula,* and *Alnus glutinosa* trees. The ground vegetation was composed of *Melampyrum nemorosum*, *Hepatica nobilis*, and *Dryopteris* sp.

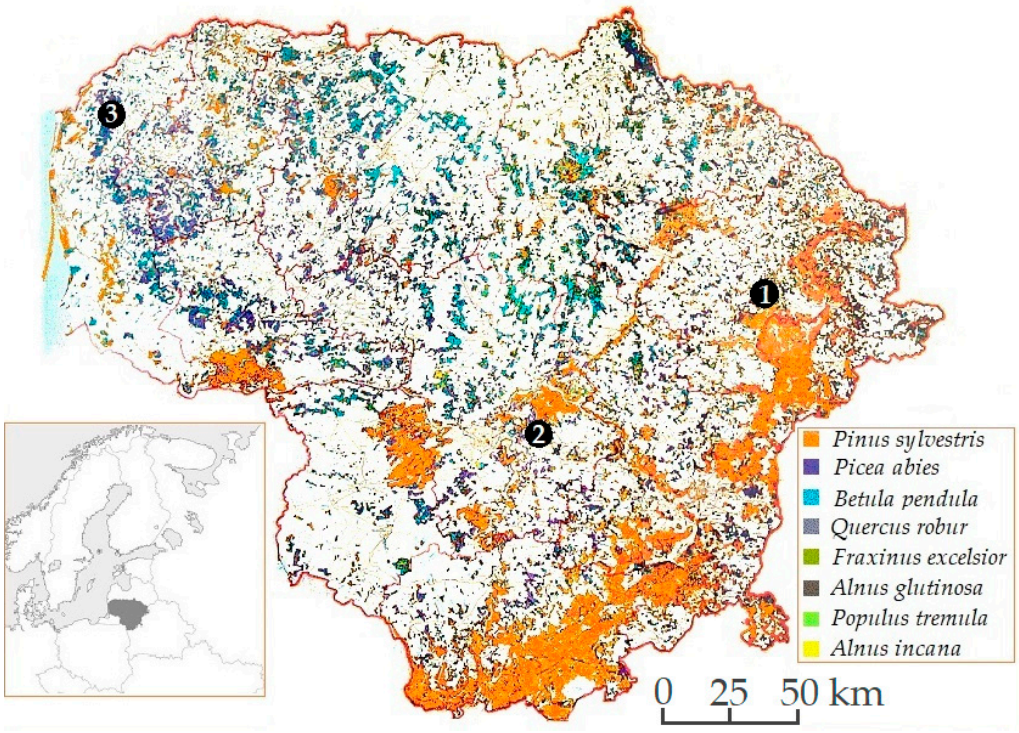

Figure 1. Map of Lithuania (position on the north European map is shown in the lower left corner) showing distribution of forests and tree species (colors of different tree species are shown in the lower right corner). Study sites are indicated by black circles and numbered: 1—Labanoras (N 55°14′3.38″, E 25°33′21.81″), 2—Dubrava (N 54°51′47″, E 24°4′0.75″), and 3—Lenkimai (N 56°11′1.99″, E 21°18′42.23″). The map was reproduced with permission from FORESTGEN, www.forestgen.mi.lt (accessed on 25 January 2023).

At each site, fungal spores were collected using a passive spore trap with a filter paper [37,38]. Specifically, each trap consisted of a 9 cm diameter Munktell filter paper (made of cotton linters, particle retention 5 to 6 μm, grade 1F) (Ahlstrom-Munksjö, Stockholm, Sweden), which was placed in between two 10 × 10 cm stainless steel grills (mesh size 1 × 1 cm), which were horizontally attached to a 1.5 m-long stick to anchor the trap into the ground (Figure 2).

After installing the filter paper, the upper and lower grills were tightened together using clips. At each site, the trap was placed at a height of ca. 1 m above the ground and approx. 100 m from the forest edge. Filters with deposited fungal spores were collected every 7–10 days [39] during a period of 12 months, resulting in a total of 146 samples. Collected samples with fungal spores were labelled, packed into plastic bags, transported to the laboratory, and stored at −20 °C before further processing.

The climate data, i.e., air temperature and precipitation, were obtained from the meteorological stations nearest to each study site.

Figure 2. The passive spore trap with a filter paper at the Dubrava study site.

2.2. DNA Extraction, PCR Amplification and Sequencing

In the laboratory, samples with fungal spores were freeze-dried (Labconco FreeZone Benchtop Freeze Dryer, Cole-Parmer, Vernon Hills, IL, USA) at −60 °C for 24 h. For isolation of DNA, half of each filter paper was taken, cut into smaller pieces, and placed into three 2 mL screw-cap centrifugation tubes together with sterile 3 glass beads, which were 3 mm in diameter. The remaining materials were stored at −20 °C as a backup. A total of 438 sub-samples (146 samples × 3 replicates = 438) were used for isolation of DNA. Prepared samples were homogenized for 2 min at 4500 rpm using Precellys-24 biological sample homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Total DNA was extracted by adding to each centrifugation tube 1000 µL of CTAB buffer (0.5 M EDTA pH 8.0, 1 M Tris-HCL pH 8.0, 5 M NaCl, 3% CTAB) followed by incubation at 65 °C for 1 h. After centrifugation, the supernatant was mixed with an equal volume of chloroform. 2-Propanol was used to precipitate the DNA into a pellet. The pellet was washed in 500 µL 70% ethanol, dried and dissolved in 30 µL of sterile milli-Q water. All sub-samples from the same filter paper were pooled together, resulting in a total of 146 DNA samples. The concentration of DNA was measured using a NanoDrop One spectrophotometer (Thermo Scientific, Rodchester, NY, USA) and adjusted to 10 ng/µL.

Amplification of ITS2 rRNA region was carried out using a fungal specific primer gITS7 [40] and a universal primer ITS4 [41], both containing sample identification barcodes. PCR reactions were performed in 50 µL reactions and included 0.25 ng/µL of template DNA, 200 µM of dNTPs, 750 µM of $MgCl_2$, 0.025 µM polymerase (5 U/µL) (DreamTaq Green, Thermo Scientific, Waltham, MA, USA), and 200 nM of each primer. Amplifications were carried out using the Applied Biosystems 2720 thermal cycler (Waltham, MA, USA). The polymerase chain reaction (PCR) conditions included an initial denaturation step at 95 °C for 5 min, which was followed by 27 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s and 72 °C for 30 s. The final extension step was at 72 °C for 7 min. The PCR products were examined on 1.5% agarose gel stained with Nancy-520 using gel electrophoresis system (Sigma-Aldrich, Stockholm, Sweden). The purification of PCR products was conducted using 3 M sodium acetate (pH 5.2) (Applichem GmbH, Darmstadt, Germany) and 96% ethanol mixture (1:2). The quantification of purified PCR products was carried out using a Qubit fluorometer 4.0 (Life Technologies, Sweden). High-throughput sequencing of amplified samples, which were pooled in an equimolar mix, was achieved using the PacBio RSII platform and two SMRT cells (SciLifeLab, Uppsala, Sweden).

2.3. Bioinformatics

The sequences produced were quality-filtered and subjected to clustering using SCATA NGS sequencing pipeline (http://scata.mykopat.slu.se (accessed on 16 January 2023)). Quality filtering of the sequences was performed by removal of short sequences (<200 bp), sequences with low mean read quality, primer dimers, and homopolymers, which were collapsed to 3 base pairs (bp) before clustering. The sequences that were missing a barcode or primer were removed. Following quality filtering, the sequences were clustered into different taxa using single linkage clustering based on 98% similarity, which was selected following several preliminary clustering runs. For each cluster, the most common genotype (real read) was used to represent each taxon. For clusters containing two sequences, a consensus sequence was produced. The GenBank (NCBI) database and the Blastn algorithm were used for taxonomic identification of different taxa. The following criteria were used for identification: sequence coverage > 80%, similarity to taxon level 98–100%, and similarity to genus level 94–97%. Sequences deviating from these criteria remained unidentified and were given unique names. Representative sequences of fungal non-singletons are available from GenBank under accession numbers MW757346–MW757980.

Fungal functional groups were assigned using the FUNGuild database (version 1.1) [42], and, if needed, were further refined using information at the MycoBank database. In the case where the fungus had two possible functional groups, it was classified based on the FUNGuild categorization.

2.4. Statistical Analyses

The rarefaction analysis was carried out using Analytical Rarefaction v.1.3 available at http://www.uga.edu/strata/software/index.html (accessed on 16 January 2023). Differences in the richness of fungal taxa at three study sites (Dubrava, Labanoras, and Lenkimai) were compared using the nonparametric chi-square test [43]. The qualitative Sørensen similarity index, the Shannon diversity index (H-index), and principal coordinates analysis (PCA) in Canoco v.5.02 (Microcomputer Power, Ithaca, NY, USA) were used to characterize the diversity and composition of airborne fungal communities [43,44]. Permutational multivariate analysis of variance (PERMANOVA) with the Bray–Curtis distance metric, using adonis2 function from the vegan package [45] in R [46], was used to assess the significance of fungal community similarity among different study sites. Using the nonparametric Mann–Whitney test in SAS v. 9.4 (Cary, NC, USA), we tested if the Shannon diversity index among different study sites was statistically similar or not. Correlation analysis was carried out to reveal the relationship between the obtained high-quality sequences or fungal taxa and climatic factors (temperature and precipitation) using SAS v. 9.4 (Cary, NC, USA).

3. Results

A total of 652,193 sequences (403 bp on average) was generated by PacBio sequencing. Quality filtering showed that 283,006 (43.4%) sequences were of high-quality and were retained, while the remaining 362,399 (56.6%) low quality sequences were removed from further analyses. Clustering of high-quality sequences resulted in 808 non-singletons and 5367 singletons, which were excluded.

Among the non-singletons, 805 (99.9%) represented fungi (Table S1). The climatic data, the number of fungal sequences, the number of fungal taxa, and the H-diversity from each study site are presented in Table 1.

A plot of fungal taxa from three sampling sites vs. the number of fungal sequences resulted in rarefaction curves that approached the asymptote (Figure 3). When the same number of sequences was taken from each site, species richness was significantly higher in Labanoras and Lenkimai than in Dubrava ($p < 0.0001$). In a similar comparison, Labanoras and Lenkimai did not differ significantly from each other ($p > 0.05$). Ascomycota was the most dominant phylum, which accounted for 467 (53.4%) of fungal taxa of the study, followed by 331 (46.5%) taxa of Basidiomycota, and 7 taxa (0.1%) belonging to Mucoromycota and Chytridiomycota.

Table 1. Generated high-quality fungal sequences and detected diversity of fungal taxa in spore trap samples from Labanoras, Dubrava, and Lenkimai sites in Lithuania. The temperature and precipitation data show average values for the sampling period. Shannon diversity index is denoted by H-diversity.

		2017				2018								
		IX	X	XI	XII	I	II	III	IV	V	VI	VII	VIII	Avg./Total
Labanoras	Temperature. °C	13.2	6.9	3.5	0.9	−1.9	−6.9	−2.4	5.2	15.7	16.7	19.7	18.6	6.8
	Precipitation, mm	76	87	54	55	60	19	24	32	38	29	76	60	610
	No. of sequences	13,016	21,939	11,160	10,897	11,757	7123	11,504	13,890	13,219	12,427	4020	12,805	143,757
	No. of fungal taxa	55	106	68	75	143	60	132	69	123	107	96	149	532
	H-diversity	2.39	2.90	2.57	2.49	2.89	2.30	2.63	2.50	2.69	3.23	3.04	3.40	3.64
Dubrava	Temperature. °C	13.4	7.6	3.9	1.1	−1.6	−5.8	−1.8	5.6	17.3	17.5	20.2	19.1	7.4
	Precipitation, mm	87	111	45	74	58	24	23	36	18	58	138	66	737
	No. of sequences	11,139	8976	15,279	7127	7574	6575	3103	8805	8785	7864	8499	4257	97,983
	No. of fungal taxa	38	19	27	28	53	32	16	43	32	34	29	25	183
	H-diversity	2.82	2.21	2.57	2.39	2.85	2.50	1.79	2.73	2.33	2.44	2.32	2.41	3.49
Lenkimai	Temperature. °C	12.7	7.1	4.0	1.6	−1.0	−6.2	−2.1	5.4	16.2	16.3	20.3	18.5	7.1
	Precipitation, mm	145	97	87	71	47	15	16	48	22	24	78	60	710
	No. of sequences	4108	5854	5572	1772	5189	2216	487	3822	2218	-	3339	6689	41,266
	No. of fungal taxa	132	187	49	81	33	5	2	81	32	-	35	116	411
	H-diversity	3.28	3.51	2.47	2.33	2.75	1.16	0.01	2.66	2.20	-	1.22	2.83	3.65

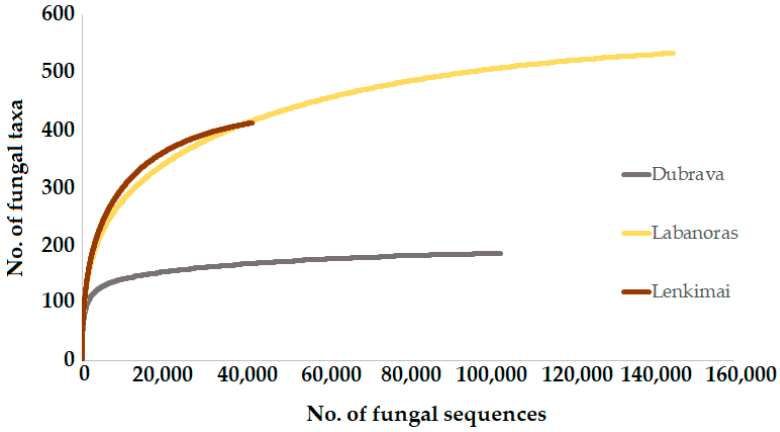

Figure 3. Rarefaction curves showing the relationship between the cumulative number of fungal taxa and the number of ITS2 rRNA sequences from three sampling sites.

Among all fungal taxa, 276 (34.2%) were exclusively found in Labanoras, 89 (11.2%) in Dubrava, 169 (20.1%) in Lenkimai, and only 50 (6.3%) were common to all sites (Figure 4A). There were 177 shared fungal taxa between Lenkimai and Labanoras, 29—between Labanoras and Dubrava, and 15—between Lenkimai and Dubrava (Figure 4A). At all study sites, 45 plant pathogenic fungi were detected and six of these were shared among all study sites (Figure 4B). There were 15 unique plant pathogenic fungi at the Labanoras site, and four unique plant pathogenic fungi at both the Dubrava and Lenkimai sites (Figure 4B).

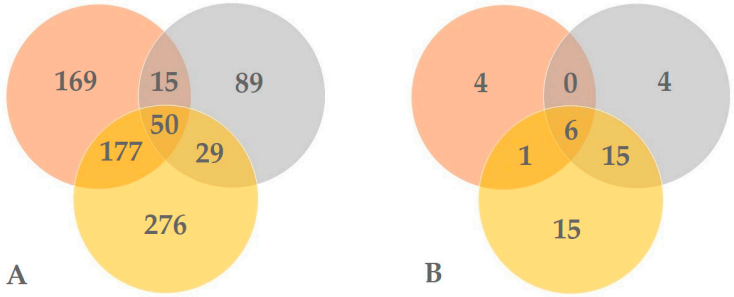

Figure 4. Venn diagram showing the diversity and overlap of fungal taxa in spore trap samples from the three sampling sites: (**A**)—all fungal taxa; (**B**)—plant pathogenic fungal taxa. Different colors represent different sites: Grey—Dubrava, Orange–Labanoras, Pink—Lenkimai (Numbers indicate the amount of unique and overlapping taxa in the study sites).

The distribution and relative abundance of fungal classes varied among differed sampling sites and the four seasons of the year (Figure 5). Over the whole year, the most dominant fungal classes at Lenkimai were Tremellomycetes (33.2%), Dothideomycetes (15.1%), and Leotiomycetes (13.0%), at Labanoras—Tremellomycetes (37.1%) and Dothideomycetes (24.5%), while at Dubrava—Dothideomycetes (23.8%), Malasseziomycetes (15.5%), Eurotyomycetes (14.3%), Tremellomycetes (10.3), and Agaricomycetes (10.3%) (Figure 5).

Identification, at least to genus level, was successful for 495 (60.6%) out of 805 fungal taxa. Information on the 10 most common fungal taxa in each sampling site, representing 59.8–65.5% of all high-quality fungal sequences per site, is presented in Table 2. Among these, two fungal taxa in Labanoras, two in Dubrava, and three in Lenkimai could not be identified at the species or genus level (Table 2).

The most common fungi in Labanoras were *Hannaella coprosmae* (23.2%), *Leucosporidium drummii* (8.5%), and *Epicoccum nigrum* (5.5%); in Dubrava—*Cladosporium macrocarpum* (16.0%), *Penicillium chrysogenum* (10.9%), and Unidentified sp. 4258_1 (10.7%); in Lenkimai—*H. coprosmae* (24.3%), Unidentified sp. 4258_8 (9.2%), and *E. nigrum* (5.7%) (Table 2).

The chi-square test showed that the relative abundance of the 10 most common fungal taxa varied greatly among the three sampling sites (Table 2). For example, the relative abundance of *E. nigrum* and *Protomyces inouyei* was significantly higher in Labanoras and Lenkimai than in Dubrava ($p < 0.05$). The relative abundance of Unidentified sp. 4258_7 was significantly higher in Dubrava than in the other two sites ($p < 0.05$). The relative abundance of Unidentified sp. 4258_8 was significantly higher in Lenkimai than in the other two sites ($p < 0.05$).

Information on the 10 most common plant pathogenic fungal taxa, representing between 1.6% and 14.6% of all high-quality fungal sequences per each site, is presented in Table 3. The most common plant pathogenic fungi at Labanoras and Lenkimai were *P. inouyei* (4.9% and 4.4%, respectively) and *S. polyspora* (2.3% and 1.3% respectively), while at Dubrava they were *Erysiphe heraclei* (0.5%) and *Fusarium sacchari* (0.4%) (Table 3).

The assessment of fungal functional groups showed that the great majority of fungal taxa could not be assigned to any functional group, primarily because they could not be identified. Among the remaining taxa, 10.2–18.6% per each site constituted plant pathogenic fungi, 13.2–24.6%—saprotrophs and 0.0–1.8% mycorrhizal fungi (Figure 6). In terms of sequence reads, the distribution of fungal functional groups differed substantially as compared to fungal taxa (Figure 6). Consequently, fungi of unknown functional group constituted 40.9–73.2% of reads per different sites, pathogens—5.8–28.9%, saprotrophs—19.2–50.7%, and mycorrhizal fungi—0.0–0.6%.

The correlation analysis of sequence reads vs. climate data showed that precipitation had a more profound impact on the number of generated sequences ($R^2 = 0.0206$, $p < 0.01$) than had air temperature ($R^2 = 0.0021$, $p < 0.05$) (Figure 7A,C). The analysis also showed the significantly positive correlation between the richness of fungal taxa and air temperature ($R^2 = 0.0258$, $p < 0.05$) or precipitation ($R^2 = 0.0538$, $p < 0.001$) (Figure 7B,D).

Principal coordinate analysis (PCA) of fungal communities explained 16.4% variation on Axis 1 and 6.8% on Axis 2. The PCA showed that fungal communities from the same sampling site were more or less well clustered together (Figure 8). Although fungal communities at Labanoras and Lenkimai sites were largely overlapping, they differed significantly from each other ($p < 0.03$) (Figure 8). Furthermore, fungal communities at both Labanoras and Lenkimai sites were largely separated (on Axis 1) from those at the Dubrava site ($p < 0.0001$) (Figure 8). The Sørensen similarity index of fungal communities was as follows: Labanoras vs. Dubrava—0.22 (low), Lenkimai vs. Dubrava—0.22 (low), and Labanoras vs. Lenkimai—0.48 (moderate). The Shannon diversity index of fungal communities ranged between 2.3 and 3.4 at Labanoras, between 1.79 and 2.85 at Dubrava, and between 0.01 and 3.51 at Lenkimai (Table 1). Despite this variation, the Shannon diversity index did not differ significantly among different sampling sites ($p > 0.05$).

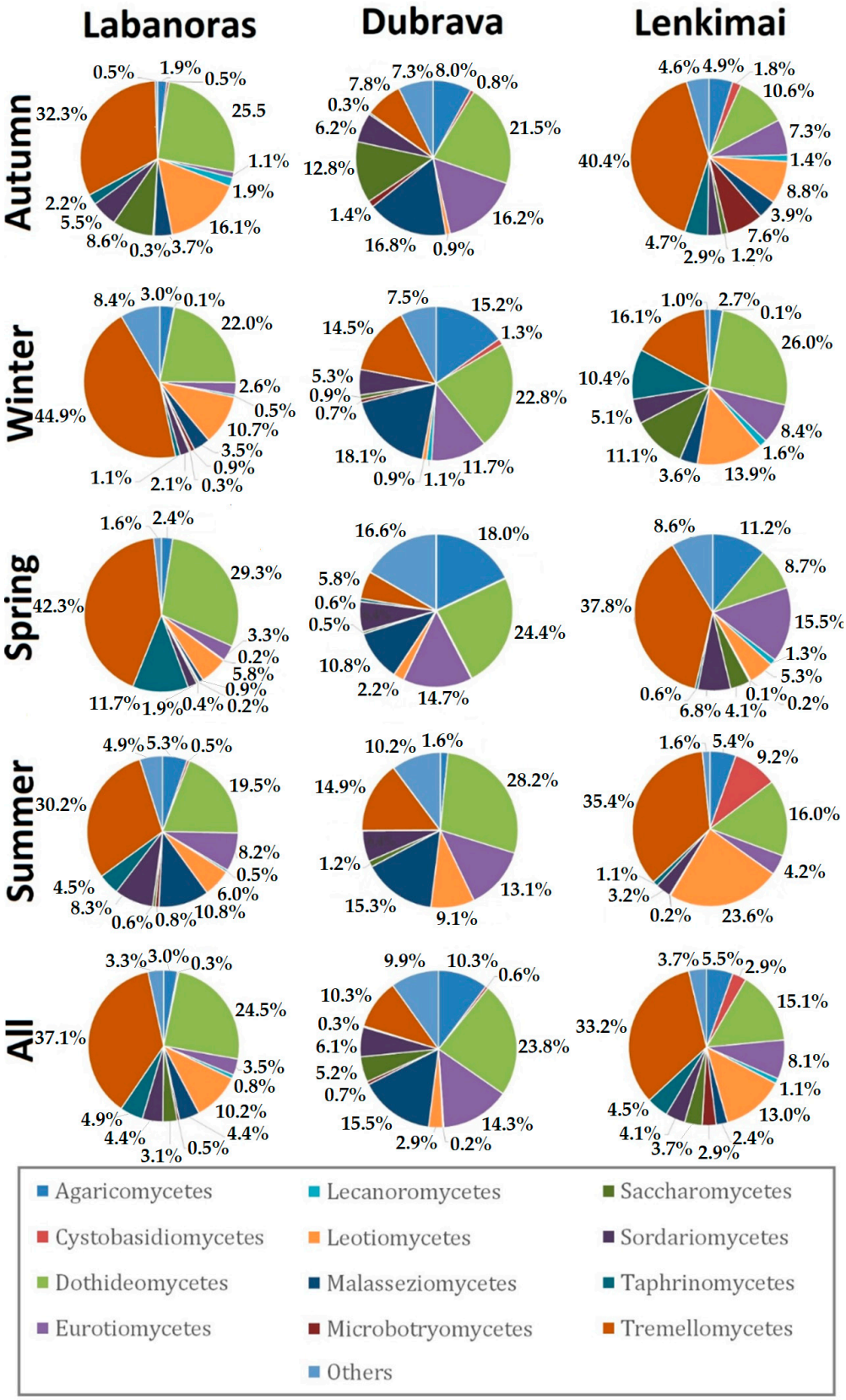

Figure 5. Composition and relative abundance of different fungal classes in trapped fungal spore samples from the three study sites (Labanoras, Dubrava, Lenkimai) and different seasons of the year (Autumn, Winter, Spring, and Summer). *Others* represent fungal classes for which the relative abundance was <1%.

Table 2. Occurrence and relative abundance of the 10 most common fungal taxa (shown as a proportion of all high-quality fungal sequences) from spore trap samples collected between September 2017 and August 2018 in Labanoras, Dubrava, and Lenkimai sites.

					2017				2018								
	Phylum	Fungal Taxa	Genbank Reference	Compared, bp/Similarity, %	IX	X	XI	XII	I	II	III	IV	V	VI	VII	VIII	All
Labanoras	Basidiomycota	*Hannaella coprosmae*	KY460840	217/218 (99)	21.98	17.44	23.19	22.16	35.03	38.76	16.57	24.15	32.35	15.12	11.19	23.09	23.23
	Basidiomycota	*Leucosporidium drummii*	MK679580	234/234 (100)	3.09	10.86	0.58	0.73	6.61	15.09	22.51	11.32	7.94	0.47	25.52	8.62	8.47
	Ascomycota	*Epicoccum nigrum*	MH102081	249/249 (100)	0.65	0.33	0.72	20.26	1.54	5.88	6.48	10.32	7.88	6.22	14.25	2.61	5.53
	Ascomycota	*Protomyces inouyei*	KX067824	261/261 (100)	0.63	0.16	8.06	2.01	0.70	-	1.39	20.03	11.93	9.25	-	1.38	4.98
	Ascomycota	*Cladosporium macrocarpum*	MK690548	243/243 (100)	2.64	5.28	7.10	0.01	3.73	3.24	4.59	3.70	10.38	1.76	5.70	4.80	4.48
	Basidiomycota	*Vishniacozyma dimennae*	KY105820	247/247 (100)	-	3.88	5.55	13.35	3.21	-	0.02	4.59	4.06	2.23	0.17	0.09	3.32
	Ascomycota	*Candida parapsilosis*	MK638869	218/218 (100)	29.47	-	-	-	-	-	-	-	-	-	-	-	2.67
	Ascomycota	*Aureobasidium pullulans*	MK686043	249/249 (100)	0.17	-	0.46	0.37	0.07	1.63	18.28	1.81	0.53	0.13	0.80	6.18	2.44
	Ascomycota	Unidentified sp. 4258_20	MG827923	243/243 (100)	-	11.71	7.61	-	-	-	-	-	-	-	-	-	2.38
	Basidiomycota	Unidentified sp. 4258_1	MH451188	369/369 (100)	5.85	1.40	0.91	1.70	3.11	3.44	0.79	0.75	0.39	3.54	0.97	5.22	2.34
		Total Labanoras			64.47	51.05	54.18	60.59	53.99	68.05	70.63	76.69	75.46	38.73	58.61	52.00	59.83
Dubrava	Ascomycota	*Cladosporium macrocarpum*	MK690548	243/243 (100)	10.63	8.69	11.58	21.24	6.89	28.17	-	11.22	30.86	8.95	30.80	24.83	16.02
	Ascomycota	*Penicillium chrysogenum*	MK696383	258/258 (100)	1.18	31.47	9.50	7.90	17.53	5.63	2.06	4.52	17.19	15.41	7.09	4.77	10.88
	Basidiomycota	Unidentified sp. 4258_1	MH451188	369/369 (100)	12.90	11.82	9.78	13.23	13.97	13.72	22.24	3.98	2.98	9.30	9.70	17.62	10.72
	Basidiomycota	Unidentified sp. 4258_7	KX222221	302/302 (100)	0.66	11.33	5.88	4.42	4.96	2.56	45.38	14.21	3.63	2.81	8.31	4.72	7.10
	Ascomycota	*Debaryomyces hansenii*	MH595408	288/288 (100)	3.01	15.14	16.47	-	-	2.27	-	0.86	0.18	0.78	2.06	-	4.78
	Basidiomycota	*Leucosporidium drummii*	MK679580	234/234 (100)	-	-	14.71	7.82	10.42	-	-	6.73	1.79	-	-	-	4.43
	Basidiomycota	*Hannaella coprosmae*	KY460840	217/218 (99)	-	-	3.73	1.25	-	4.78	-	1.83	-	16.15	20.65	0.23	4.25
	Basidiomycota	*Malassezia sympodialis*	LT671825	327/327 (100)	1.21	3.35	0.19	3.23	4.61	1.02	13.57	2.40	0.57	1.59	4.28	4.70	2.53
	Ascomycota	*Alternaria alternata*	MH892844	253/253 (100)	15.17	-	3.12	-	-	-	-	1.42	0.17	-	0.14	-	2.37
	Ascomycota	*Epicoccum nigrum*	MH102081	249/249 (100)	2.78	-	2.05	1.70	3.54	5.25	-	-	0.24	4.92	-	12.99	2.37
		Total Dubrava			47.54	81.81	77.02	60.78	61.92	63.38	83.24	47.17	57.61	59.91	83.03	69.86	65.45
Lenkimai	Basidiomycota	*Hannaella coprosmae*	KY460840	217/218 (99)	4.58	25.67	40.08	37.02	6.92	15.12	-	32.08	20.83		48.55	21.51	24.29
	Ascomycota	Unidentified sp. 4258_8	MG827641	244/244 (100)	-	0.12	3.54	9.88	4.41	33.75	-	1.65	1.13		38.78	15.94	9.22
	Ascomycota	*Epicoccum nigrum*	MH102081	249/249 (100)	2.22	3.91	1.04	1.19	14.72	46.44	-	-	1.40		0.27	1.91	5.72
	Ascomycota	*Protomyces inouyei*	KX067824	261/261 (100)	-	1.83	9.92	22.74	11.51	-	-	0.86	0.14		0.24	1.49	4.37
	Basidiomycota	*Leucosporidium drummii*	MK679580	234/234 (100)	7.08	8.76	-	1.02	-	-	-	15.67	-		1.26	4.05	4.20
	Ascomycota	*Candida palmioleophila*	KC111442	288/288 (100)	0.07	-	3.30	-	16.34	-	-	6.10	1.22		-	-	3.14
	Ascomycota	*Cladosporium macrocarpum*	MK690548	243/243 (100)	2.68	1.35	1.17	3.50	1.08	-	0.21	0.13	0.99		2.40	11.94	3.10
	Basidiomycota	Unidentified sp. 4258_29	MG827488	289/295 (98)	0.29	0.26	1.44	0.34	-	0.14	-	-	-		6.23	10.72	2.52
	Ascomycota	*Penicillium chrysogenum*	MK696383	258/258 (100)	-	-	0.09	-	7.65	-	-	7.51	2.84		-	2.08	2.16
	Basidiomycota	Unidentified sp. 4258_1	MH451188	369/369 (100)	0.56	5.40	3.50	1.19	4.10	-	-	0.03	0.14		-	0.22	1.91
		Total Lenkimai			17.48	47.30	64.07	76.86	66.74	95.44	0.21	64.02	28.67	-	97.72	69.88	60.63

Table 3. Occurrence and relative abundance of the 10 most common plant pathogenic fungal taxa (shown as a proportion of all high-quality fungal sequences) from fungal spore trap samples collected between September 2017 and August 2018 in Labanoras, Dubrava, and Lenkimai sites.

					2017				2018								
	Phylum	Fungal Taxa	Genbank Reference	Compared, bp/Similarity, %	IX	X	XI	XII	I	II	III	IV	V	VI	VII	VIII	All
Labanoras	Ascomycota	*Protomyces inouyei*	KX067824	261/261 (100)	0.63	0.16	8.06	2.01	0.70	-	1.39	20.03	11.93	9.25	-	1.38	4.98
	Ascomycota	*Sydowia polyspora*	MG888613	256/256 (100)	4.74	0.01	0.24	0.24	0.04	12.72	7.86	1.32	2.94	-	0.97	1.36	2.28
	Ascomycota	*Ramularia coleosporii*	MH974744	237/237 (100)	1.10	1.22	20.56	0.34	1.91	0.11	0.12	0.71	0.10	0.39	-	0.41	2.23
	Ascomycota	*Oculimacula acuformis*	MH861289	241/242 (99)	-	13.70	-	-	0.01	-	0.01	-	-	0.01	-	-	2.09
	Ascomycota	*Fusarium lateritium*	MK633904	258/258 (100)	1.82	0.62	2.15	1.62	0.10	-	2.67	1.01	0.04	5.65	0.70	0.57	1.43
	Ascomycota	*Fusarium graminearum*	MK212898	245/245 (100)	-	-	0.12	0.67	-	-	-	0.60	-	3.15	1.17	0.15	0.44
	Basidiomycota	*Fomes fomentarius*	MF563980	286/286 (100)	-	1.73	-	-	-	-	-	-	-	-	-	-	0.26
	Ascomycota	*Gremmeniella abietina*	MH857809	237/237 (100)	-	-	-	-	-	-	0.36	2.15	0.14	-	0.02	-	0.25
	Basidiomycota	*Resinicium bicolor*	MF511087	291/291 (100)	0.05	0.57	-	-	1.90	-	-	-	-	-	-	-	0.25
	Basidiomycota	*Piptoporus betulinus*	MH856908	296/296 (100)	2.35	-	-	-	-	-	-	-	-	-	-	-	0.21
	Ascomycota	*Lophodermium pinastri*	MH856647	239/239 (100)	-	0.77	0.01	0.15	0.03	0.29	0.01	-	0.24	-	0.07	0.21	0.19
		Total Labanoras			10.69	18.78	31.14	5.03	4.68	13.13	12.41	25.82	15.39	18.45	2.94	4.08	14.61
Dubrava	Ascomycota	*Erysiphe heraclei*	MK571420	274/274 (100)	-	-	2.26	-	-	-	-	-	-	-	-	4.30	0.54
	Ascomycota	*Fusarium sacchari*	MK713417	246/246 (100)	-	-	-	5.81	-	-	-	-	-	-	-	-	0.42
	Basidiomycota	*Stereum sanguinolentum*	MH071730	294/294 (100)	-	-	-	-	-	5.02	-	-	-	-	-	-	0.34
	Ascomycota	*Protomyces inouyei*	KX067824	261/261 (100)	-	-	-	-	-	-	-	1.53	-	-	-	-	0.14
	Ascomycota	*Fusarium lateritium*	MK633904	258/258 (100)	-	-	-	-	0.83	-	-	-	-	-	-	-	0.06
	Basidiomycota	*Chondrostereum purpureum*	MK788300	308/308 (100)	-	-	-	-	-	-	-	-	0.67	-	-	-	0.06
	Ascomycota	*Ramularia coleosporii*	MH974744	237/237 (100)	0.01	-	-	-	0.11	-	-	-	-	-	-	-	0.01
	Ascomycota	*Plectosphaerella cucumerina*	MK079567	263/263 (100)	-	-	-	-	0.09	-	-	-	-	-	-	-	0.01
	Ascomycota	*Botrytis cinerea*	MH346332	240/240 (100)	-	-	-	-	-	-	-	-	0.05	-	-	-	0.004
	Basidiomycota	*Ganoderma adspersum*	MN945139	293/293 (100)	-	-	-	-	-	-	-	0.05	-	-	-	-	0.004
		Total Dubrava			0.01	-	2.26	5.81	1.03	5.02	-	1.58	0.72	-	-	4.30	1.58
Lenkimai	Ascomycota	*Protomyces inouyei*	KX067824	261/261 (100)	-	1.83	9.92	22.74	11.51	-	-	0.86	0.14	-	0.24	1.49	4.37
	Ascomycota	*Sydowia polyspora*	MG888613	256/256 (100)	4.92	0.43	-	-	1.77	-	-	0.16	6.18	-	0.06	1.05	1.29
	Ascomycota	*Fusarium lateritium*	MK633904	258/258 (100)	0.44	2.60	-	0.11	-	-	-	-	-	-	0.09	4.16	1.10
	Ascomycota	*Ramularia coleosporii*	MH974744	237/237 (100)	4.80	3.14	-	0.06	-	-	-	-	-	-	0.24	0.09	0.96
	Ascomycota	*Lophodermium pinastri*	MH856647	239/239 (100)	0.19	0.03	3.46	1.81	0.17	-	-	0.03	-	-	-	-	0.59
	Ascomycota	*Pyrenophora tritici-repentis*	AM887511	253/254 (99)	-	-	-	-	-	-	-	1.94	-	-	-	-	0.18
	Ascomycota	*Botrytis cinerea*	MH346332	240/240 (100)	-	0.29	-	-	-	-	-	1.28	-	-	-	-	0.16
	Ascomycota	*Taphrina nana*	MH857501	293/293 (100)	-	0.84	-	-	-	-	-	-	-	-	-	-	0.12
	Ascomycota	*Heterotruncatella spartii*	MK012418	245/245 (100)	0.66	0.26	-	0.11	-	-	-	-	-	-	-	-	0.11
	Basidiomycota	*Exobasidium maculosum*	KR262418	288/288 (100)	-	0.68	-	0.06	-	-	-	-	-	-	-	-	0.10
		Total Lenkimai			11.00	10.10	13.39	24.89	13.45	-	-	4.26	6.31	-	0.63	6.79	8.98

Figure 6. Relative abundance (%) of fungal functional groups (shown as fungal taxa and as sequence reads) detected in fungal spore traps from the three sampling sites in Lithuania.

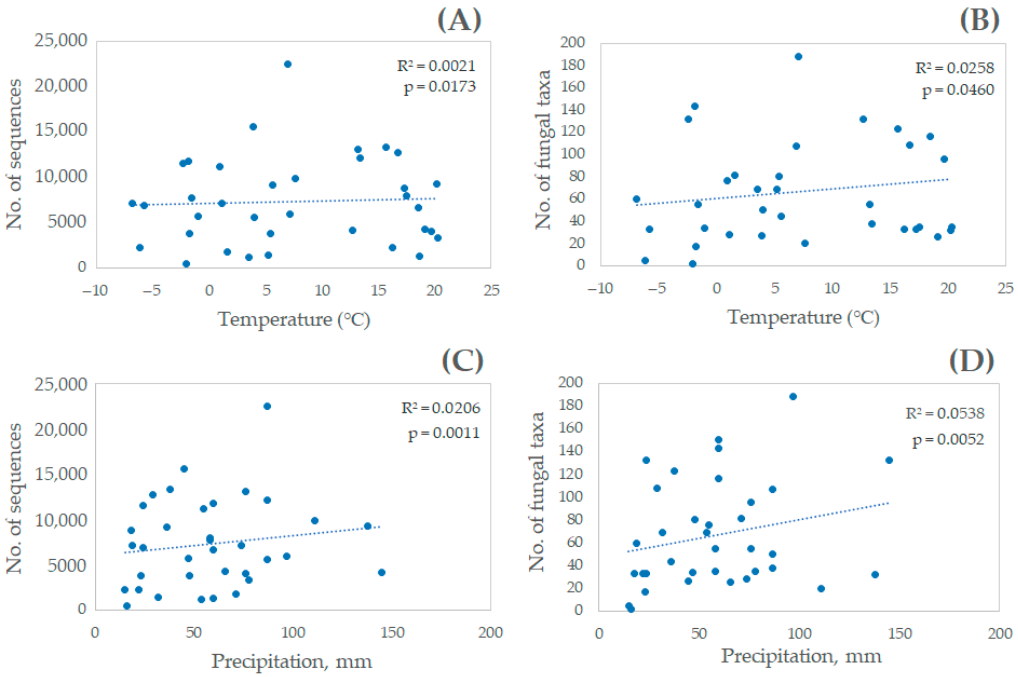

Figure 7. Plots of correlation analysis: (**A**) sequence reads vs. temperature; (**B**) fungal taxa vs. temperature; (**C**) sequence reads vs. precipitation; (**D**) fungal taxa vs. precipitation. The data from all sites are combined.

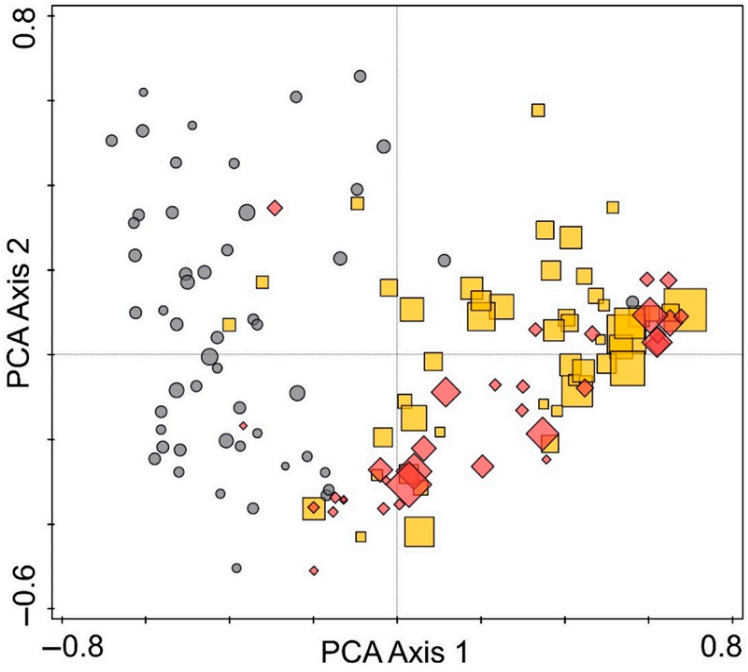

Figure 8. Ordination diagram based on principal coordinate analysis (PCA) of fungal communities from fungal spore traps collected between September 2017 and August 2018 at Labanoras (Orange), Dubrava (Grey), and Lenkimai (Pink) sites. Each point in the diagram represents a single sample and the size of each point reflects the relative richness of fungal taxa.

4. Discussion

Early detection of native and invasive pathogens and identification of changes in their abundance is an important component of forest biosecurity. Since dispersal by air is one of the main mechanisms for many fungal species to spread and reach new susceptible hosts, spore monitoring enables effective tracking of airborne fungal communities and prediction of the risk of new disease outbreaks in agriculture and forestry. In the present study, we characterized the diversity, composition, and specificity of fungal communities deposited in spore traps from the three forest sites using high-throughput DNA sequencing. Our study demonstrated that airborne fungal communities are influenced by a complex of abiotic and biotic factors.

Results showed that the diversity and composition of fungal communities were largely driven by the site conditions (Figures 3–5 and 8), thereby corroborating Redondo et al. [47] that vegetation type determines the local spore deposition. Indeed, fungal communities at the Lenkimai and Labanoras sites, which were dominated by *P. sylvestris* trees, were similar in fungal species composition and richness (Figures 2–5 and 8). By contrast, the fungal community at the Dubrava site, which was dominated by deciduous trees, showed generally different fungal species composition and lower species richness (Figures 5 and 8). There was also a seasonal variation in the abundance and composition of trapped fungi (Table 1, Figure 5), which can probably be explained by seasonal changes in ground vegetation cover and local climate conditions [48].

Indeed, temperature and precipitation were found to have a significant impact on the detected fungal diversity (Figure 6). As climatic conditions change during the year, in addition to other factors, climatic conditions likely affect the seasonal dynamics of the airborne fungi. Other studies have also identified seasonal changes in the composition of fungal communities [47,49,50]. Although previous studies have shown that temperature is an important factor determining fungal spore production, release, and abundance in the air [51,52], in the present study, precipitation was found to be an important factor that can have a direct effect on spore deposition. In addition, precipitation may trigger a

rapid discharge of fungal spores for some fungal species or may provide the humidity [52] required for spore maturation [53].

Nicolaisen et al. [49] found similar fungal communities in the air across northwestern Europe, indicating that the location explained only 8% of the variation between airborne fungal communities. This was even though some fungi (polypores or corticoid fungi) were highly specific for some locations [49]. Interestingly, it was shown that clear differences and separation of fungal communities can be found in the decomposing wood of deciduous and coniferous trees [54,55]. As there was a higher relative abundance of saprotrophic fungi in the deciduous forest of Dubrava site vs. *P. sylvestris* forests at Labanoras and Lenkimai sites (Figure 6, $p < 0.05$), this may have contributed to the observed differences in fungal communities. Furthermore, the relative abundance of dominant fungal pathogens was lower at the deciduous forest site (Dubrava) than at the coniferous forest sites (Lenkimai and Labanoras) (Table 3), thereby repeatedly highlighting the specificity of these sites and the potential impact on the composition of fungal communities.

The most common pathogenic fungus was *P. inouyei* (Table 3), which is ascomycetous yeast causing disease on host plants. Kurtzman [56] stated that all known *Protomyces* spp. have hosts in Apiaceae, Compositae, Umbelliferae, and other plants. *Protomyces inouyei* induces gall symptoms on stems of *Youngia japonica* [57,58]. There are several related species in Lithuania, namely., *Hieracium vulgatum*, *H. murorum*, *H. umbellatum*, that can be potential hosts of this plant pathogen. These plant species grow in sparse, light, and dry forests characterized by infertile soils such as present at the Labanoras and Lenkimai sites.

Another pathogenic fungus that was commonly detected at the Labanoras and Lenkimai sites, was *S. polyspora*. *S. polyspora* is known as an endophyte and/or foliar or seed pathogen [59,60]. *S. polyspora* was shown to be responsible for seasonal needle necrosis, a disease that affects *Abies* spp. trees in Europe [61] and the USA [62]. In agreement with the results of the present study, this pathogenic fungus constituted a high proportion (3.5%) of all sequence reads derived from coniferous forests in Sweden [59]. It appears to have a broad ecological niche (classified as an endophyte, saprotroph, or even pathogen) and can also often be transmitted by insects. Due to a broad ecological niche and efficient spread, it can also be a primary colonizer of woodland litter [63–65].

Among other pathogens, there were three *Fusarium* species detected that were among the ten most abundant plant pathogens in all sampling sites (Table 3). *Fusarium* is a large cosmopolitan genus of imperfect fungi that includes a number of important plant pathogens [66]. In the present study, *Fusarium lateritium* was among the dominant fungal taxa in all sampling sites (Table 3). It was earlier reported as an agent of nut gray necrosis on *Corylus avellana* [67,68] and as a new pathogen damaging fruits of *Prunus persica* [69]. *Fusarium graminearum*, detected only at the Labanoras site, is known as a pathogen of maize, wheat, rice, and barley and is responsible for the disease known as *Fusarium* head blight [70]. *F. sacchari*, that was only found at the Dubrava site, was shown to be associated especially with maize diseases [71].

The pathogenic fungus *E. heraclei*, which was also detected in the present study, causes powdery mildew in several plant species including dill, carrot, and parsley [72]. A higher abundance of this fungus at the Dubrava site can probably be explained by more intense agriculture in this geographical area. The disease cycle starts in the spring when ascospores are dispersed by the wind or water, followed by spore germination on the leaf tissue of relevant hosts while the disease symptoms appear gradually after that [73].

Although a number of other plant pathogens were also detected, their relative abundance was relatively low (Table 3). The study revealed the presence of *Chondrostereum purpureum* and *Ganoderma adspersum* at the Dubrava site, *Piptoporus betulinus* at the Labanoras site, and *Exobasidium maculosum* at the Lenkimai site. Plant pathogen *C. purpureum* is a causative agent of silver leaf disease in fruit trees such as plum, apple, apricot, and cherry and can also infect many species of the Rosaceae family. *E. maculosum* is a causal agent of the leaf and fruit spot disease of blueberry [74]. Furthermore, *Gremmeniella abietina*, which was exclusively detected at the Labanoras site, is known as one of the most

serious coniferous tree pathogens in the Northern hemisphere [75]. *Gremmeniella abietina* attacks different coniferous tree species, including *Picea abies*, *Pinus contorta*, and *P. sylvestris*, causing dieback of shoots and buds, and forming cankers on stems and branches [76,77]. In the present study, *G. abietina* was most abundant in the spring, especially in April, but apart from July, it was undetected for the rest of the year. However, Laflamme and Archambault [78] showed that the spore dispersal of *G. abietina* started in the middle of July and ended in October, with a peak of spore release during the first three weeks of August. The temperature was shown to have no direct effect on the release of *G. abietina* spores, but there was a strong correlation between the relative humidity, rain, and spore dispersal [78,79]. In addition, among 45 plant pathogenic fugal taxa detected in the present study, there were also several important tree pathogens including *Fomes fomentarius*, which causes white rot to forest trees [80], *Heterobasidion annosum*, which causes root rot mainly to *Pinus* spp. and *Picea* spp. trees [81], *Hymenoscyphus fraxineus*, which causes ash dieback [82], and *Dothistroma septosporum*, which causes needle blight of *Pinus* spp. trees [83]. Similar composition of fungal pathogens in forest ecosystems were reported by Candelier et al. [84]. However, in the present study the relative abundance of these fungal pathogens was very low (Table S1), thereby making analyses on their seasonal occurrences, site specificity, and dependence on climatic factors hardly possible. Although the study revealed that time of the year (season) influences airborne fungal communities, the results should be interpreted with caution as long-term observations are needed to infer possible tendences. Nevertheless, certain seasonal regularities in airborne fungal communities may persist in different years, while observed variations may depend on the environmental conditions of the area [85].

5. Conclusions

The diversity and composition of airborne fungal communities was found to largely depend on the vegetation type of each study site. Although the temperature and precipitation were found to have a positive effect on the richness of fungal taxa, the seasonal effect on the composition of fungal communities was less expressed. The majority of the identified fungi were saprotrophs, but the presence at each site of different plant pathogenic fungi demonstrated the relative importance of host plants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15040539/s1. Table S1. The occurrence and relative abundance of fungal taxa sequenced (shown as a proportion of all high-quality fungal sequences) from spore trap samples collected between September 2017 and August 2018 in Labanoras, Dubrava, and Lenkimai sites.

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