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Analysis of fungal bioaerosols in Athens: a pilot study.

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Abstract

This pilot study is the first attempt to describe the bioaerosol composition found in Athens' urban air by DNA barcoding. There are currently limitations in establishing a direct link between fungal exposure and health effects due to shortcomings of existing sampling and analysis methods, among other reasons. New sampling technologies and molecular techniques can achieve a better understanding of this link.

We collected airborne samples using Rutgers Electrostatic Passive Sampler (REPS). We studied the taxonomy, diversity, and frequency of captured airborne fungal particles by extracting and purifying fungal DNA present in samples and then sequencing it. Four sampling campaigns were conducted in 2019 and one in 2020 at the National Observatory of Athens meteorological and environmental station of Thisseion. Five samples plus two controls were used each time. Sequence analyses are now routine in systematics, taxonomy, and fungi ecology, with the nuclear ribosomal operon being the most frequently targeted genetic region. The variable spacers ITS1 and ITS2, jointly or separately, are often species-specific. Thus, the internal transcribed spacer (ITS) region was our primary choice for molecular identification of fungi. We detected 13 classes of two phyla (Ascomycota and Basidiomycota) and approximately 133.000 OTUs. The dominant classes (>10.000 OTUs) were *Dothideomycetes*, *Malasseziomycetes*, *Leotiomycetes*, and *Microbotryomycetes*.

Keywords: Fungi, bioaerosols, Athens, DNA barcoding, taxonomy

1. Introduction

Fungi constitute a significant fraction of bioaerosols, aerosolized from various terrestrial and aquatic ecosystems (Frohlich-Nowoisky et al., 2009 & 2016). In the broad sense, fungi are ubiquitous in terrestrial, freshwater, marine ecosystems, and the atmosphere (Dix and Webster 1995; Thines 2014; Elbert et al., 2007). Fungi have an economic impact as plant, animal, and human pathogens. Their presence/absence also serves and an indicator of environmental quality, yet they remain relatively understudied (Lücking et al. 2020; Hyde et al. 2019). Due to the immense range of fungal habitats, and the continuous need to compete against a diverse array of other fungi, bacteria, and animals, they have developed numerous survival mechanisms (Hyde et al., 2019). Fungi prevail in the pedosphere (Tedersoo et al., 2014), from which spores, hyphae, fragments, and other propagules are released into the global atmosphere, with an estimated annual emission based on spore counts and molecular tracers of 28–50 Tg y-1 (Elbert et al.,2007). The emitted fungal particles participate in climate systems, health, and ecosystem interactions, and they can serve as ice-forming nuclei (IN) and cloud condensation nuclei (CCN) (Haga et al.,2014; Fröhlich-Nowoisky et al.,2016) and/or by absorbing and reflecting solar and terrestrial radiation (Spänkuch et al., 2000; Woo et al., 2018). Aerosol particles, like fungal aerosols, eventually settle on the Earth's surface due to gravity (dry deposition) and precipitation (wet deposition). Knowledge is scarce regarding the diversity of fungi deposited from the atmosphere (Chen et al.,2018). Most airborne fungi belong to the divisions of Ascomycota and Basidiomycota (Frohlich-Nowoisky et al., 2009). Most Ascomycota and Basidiomycota actively eject their spores with liquid jets or droplets (osmotic pressure and surface tension effects), and they are a major source of bioaerosols. Dry-discharged spore concentrations tend to be enhanced during warm, dry weather conditions, whereas actively wet discharged spores tend to be more present during humid conditions (Elbert et al.,2007; Després et al.,2012). Accurate and precise identification of airborne fungi is challenging. Because of clade-specific evolutionary histories, there is currently no single tool for the identification of fungi, although DNA barcoding using the internal transcribed spacer (ITS) remains the common technique, particularly in metabarcoding studies (Lücking et al., 2020). Sequence analyses are now routine in systematics, taxonomy, and fungi ecology, with the nuclear ribosomal operon being the most frequently targeted genetic region. The variable spacers ITS1 and ITS2, jointly or separately, are often species-specific. Studies based on DNA obtained directly from atmospheric aerosol samples offer new possibilities to identify the origin of fungal aerosols, independent of viability, cultivability, and fragmentation. Amplification of the internal transcribed spacer (ITS) regions of ribosomal ribonucleic acid (rRNA) genes provides good target regions to identify fungi to genus and often to species level (Frohlich-Nowoisky et al., 2009). Regarding the species richness, studies show that airborne fungi consist of 64% Basidiomycota and 34% Ascomycota in a semi-urban area in central Europe; it was found that on the class level within the Ascomycota, *Dothideomycetes, and Eurotiomycetes* seem to be the prevalent groups (Frohlich-Nowoisky et al., 2009)

2. Methods

We studied the taxonomy, diversity, and frequency of fungal aerosol in Athens' air particulate matter by DNA extraction, purification, and next-generation sequence analysis. Three sampling campaigns were conducted in June, August, and October 2019, and one in February 2020, near NOA Thisseion meteorological and environmental station in Athens. Airborne particles were collected using Rutgers Electrostatic Passive Sampler (REPS). The collected particles were eluted, and the

DNA was extracted immediately after each sampling campaign. Three samples plus two controls were used each time. After sampling, the REPS were brought back to the laboratory, and we cut the PVDF film of each REPS and put them into kit tubes (2 tubes for each sample) for further analysis. According to the manufacturer's protocol, genetic DNA was extracted from collected air samples using the DNeasy PowerSoil Kit (Qiagen, Valencia, CA). The isolated DNA in ultrapure water was ready for PCR analysis and other downstream applications. Fungal amplicon diversity of samples were characterized by a barcoded amplicon sequencing method under the trademark service (bTEFAP®) at a commercial laboratory MrDNA (Shallowater, TX). Sequence data were processed using the MR DNA microbiota analysis pipeline (MR DNA, Shallowater, TX). In summary, paired sequences were joined and depleted of barcodes; sequences < 150 base pairs or with ambiguous base calls and homopolymer run exceeding 6bp were removed. Sequences were denoised, operational taxonomic units generated, and chimeras removed. Operational taxonomic units were defined by clustering at 3% divergence (97% similarity). Final operational taxonomic units were taxonomically classified using BLASTn against a curated database derived from RDPII and NCBI (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu). Contamination prevention: Owing to the low mass and low biological content, particulate matter samples are sensitive to laboratory and other environmental contaminations, and thus we made extensive efforts to avoid contamination in the sample collection, pretreatment, and DNA extraction steps. The REPS used for sample collection were disinfected thoroughly and cleaned with 75% (vol/vol) ethanol in Rutgers Univ., New Jersey, USA. All the tubes and tools used in sampling or analysis were sterilized (autoclaved if possible) or cleaned with 75% (vol/vol) ethanol before use. To assess the degree of potential contamination, we conducted negative-control experiments. Controls had the same pretreatment and DNA extraction process as samples. If an OTU was detected only in controls or it had counts >10% counts compared to actual samples, the samples were discarded.

Quality control/data filtering. OTUs related to contamination of samples as well as OTUs with low identity (<97%) of sequencing, bitscore <300 and e-value > e-100 (BLASTn) and/or low counts <10 were rejected. The analysis and visualization of data were done by EXPLICET, a Graphical user interface software for metadata-driven management, analysis, and visualization of microbial ecology data (Robertson et al., 2013; website: www.explicet.org)

3. Results and Discussion

Two contaminants were found on the blanks only, e.g., the species *Termitomyces sp* and *Antrodia sinuosa*, and their OTUs were deleted. In Athens from June 2019 to February 2020, we selected 133.666 OTUs of fungal aerosols for further analysis. These OTUs belong to two fungal Phylla (Ascomycota and Basidiomycota), 11 classes, 21 orders, 26 genera, and 45 species, after quality controls and cutoff <10 counts. In Fig.1, the variation of the relative abundance % of fungal Phylla is shown, Ascomycota (AM) is the dominant Phyllum, and its predominance is higher during the hot season.

The dominant order is *Hypocreales* (27%) and major ones are *Pleosporales, Capnodiales and Helotiales* (18% -10%). All the predominant orders belong to Ascomycota except for *Malasseziales*. In the August library, we detected two Orders (*Hypocreales* and *Malasseziales*) in February three (*Helotiales*, *Malasseziales*, and *Pleosporales*). *Malasseziale*s were found in all libraries, while *Pleosporales*, *Helotiales*, and *Hypocreales* in three libraries (Fig.2).

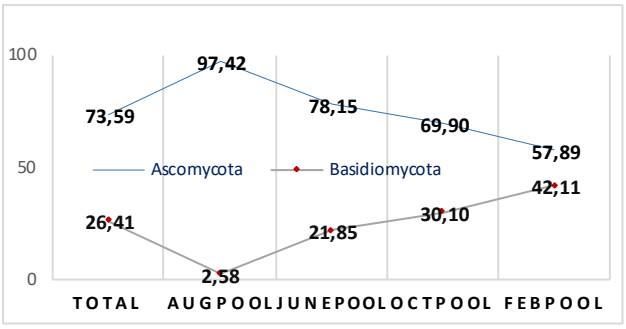

Figure 1. Relative abundance % of fungal Phylla per library

The orders *Gloeophyllales* and *Saccharomycetales* are rare with counts <10. It is noticeable that many Classes were detected in one sample only, e.g., in June, three Classes: the *Agaricomycetes*, *Cystobasidiomycetes*, and l*ecanoromycetes* were detected; in October, two classes were detected: *Saccharomycetes* and *Wallemiomycetes*. The proportion of Classes that were found only in one sample was 42%. In Table 1, the variation of relative abundance % of the main fungal Classes is shown. The dominant Class is *Dothideomycetes* (34,3%), the major Classes are *Sordariomycetes* (27,1%), *Malasseziomycetes* (18%), abundant (10%-8 %) ones are *Leotiomycetes and Microbotryomycetes*, less abundant (3%) is *Eurotiomycetes* and minors (>1%) are *Tremellomycetes*, *Wallemiomycetes*, *Lecanoromycetes*, *Cystobasidiomycetes*, and *Agaricomycetes*. A seasonality in Class abundance is revealed, *Dothideomycetes* is predominant in October, *Sordariomycetes* in August, *Malasseziomycetes*, and *Leotiomycetes* in February.

In bioaerosols of Athens, the dominant species (>14%) were *Engyodontium album*, and *Cladosporium cladosporioides*, the major species (>5%), were *Hyalodendriella betulae, Malassezia restricta, Alternaria alternata, Rhodotorula sp, Nectria mauritiicola, and Malassezia sp*.

The species richness of Basidiomycota (BMC) vs. Ascomycota (AMC) exhibits distinct biogeographic patterns with higher BMC/AMC ratios in continental air compared to marine air (Fröhlich-Nowoisky et al., 2012). We found a high abundance of AMC in Athens' bioaerosols, which can be attributed to Thisseion station's vicinity with Saronicos bay.

In the past, the abundance of the airborne fungi in the city of Athens has been studied by volumetric non-culture-based and culture-based methods from January 1998 until December 2001, and a total of 259,851 fungal spores were recovered (Pyrri & Kapsanaki-Gotsi, 2014). The samplings were performed all year round, and they found that the annual mean concentration of fungi was 865 - 1,206 spores/m3. The airborne fungi recovered were mostly anamorphic with a mean percentage of 72.4 %, followed by the Ascomycota 8.89 %, Basidiomycota 7.03 %, Myxomycota 5.24 %, hyphal fragments 3.58 %, and *Ustilaginale*s 2.85 %. The prevalent genus in Athens was Cladosporium, accounting from 55.2 to 60.1 %, and the second genus Alternaria constituted 3.4–3.8 % of the annual total count, depending on the year. Twenty-four genera and eight groups were registered by the non-culture-based method versus 54 fungal genera, and four groups recovered by the culture-based method (Pyrri& Kapsanaki-Gotsi, 2014). In a 15-year study using a Burkard volumetric trap in Thessaloniki, the taxa of *Cladosporium spp*. (72.2% of total

fungi), and *Alternaria spp*. were the most abundant in the air of Thessaloniki and considered as the most commonly implicated in respiratory allergy symptoms (Gioulekas et al., 2004).

The fungal community of the air in Madrid (Núnez et al., 2021) was examined using volumetric spore traps and DNA sequencing for two years. The fungal community was dominated by Ascomycota, and to a much less extent by Basidiomycota, and it showed a significant increase in richness during Fall (Núnez et al., 2021), as we also recorded (Fig.2) in October sampling. They identified only four core genera (*Cladosporium*, *Alternaria*, *Epicoccum*, and *Eurotium*, all assigned to Ascomycota). Ascomycota belongs to taxa commonly found in soil that dominate across different ecosystems and geographies (Tedersoo et al., 2014). Regarding fungal taxa identified in Madrid, around a third of the total sequences were associated with plant or animal pathogens, with the core genera *Cladosporium*, *Alternaria*, and *Epicoccum* relevant for human health. According to Núnez et al. (2021), the urban air microbiome is dominated by a few cosmopolitan taxa frequently found in soil, with a more homogenous composition than the rural airborne microbiome or pristine environments, or at high altitudes.

Aerosol samples were collected on glass fiber filters by an active sampler (flow rate ~300 L/ min) over 1 year in Germany (Frohlich-Nowoisky et al., 2009). They also detected Cladosporium sp. as the most frequent species, nearly all detected fungal species belonged to Basidiomycota (64%) or Ascomycota (34%). This result is consistent with the predominance of AMC and BMC in the biosphere. The AMC species found in the air samples were distributed over 4 major classes *(Dothideomycetes, Eurotiomycetes, Leotiomycetes, and Sordariomycetes*), and about one-third could not be attributed to any class. In contrast, most of the detected BMC species belonged to a single class, the Agaricomycetes, a rare Class in our samples. The high proportion of species found only in one sample (70%) and the limited number of investigated samples might imply that the real abundance of the taxa in the air is higher than we recorded (Frohlich-Nowoisky et al.,2009).
Therefore, our short-term study is the first study in Athens for fungal aerosols by NGS and DNA barcoding. We detected many OTUS (>133.000) classified in 45 species with counts >10 OTUs, and the allergenic species *Cladosporium* and *Altenaria* were found as major species with 15%, 9%, respectively. Besides the limited number of sampling campaigns and the use of a passive sampler (the sampled air volume cannot be accurately determined), we recovered a great taxa richness (26 genera and 45 species) in comparison to long-term studies (Núnez et al., 2021; Frohlich-Nowoisky et al., 2009; Pyrri& Kapsanaki-Gotsi, 2014). However, the proportion of Classes found only in one sample is probably high (42%), and this should be analyzed further
This was a successful pilot study using a new technique of passive sampling in combination with DNA barcoding. Future studies will be conducted over a longer period using active and passive samplers in parallel to achieve an accurate determination of air volume and greater taxa richness and diversity.

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References

Chen W., Hambleton S., Seifert K.A., Cariose O., et al. (2018) Assessing performance of spore samplers in monitoring aeromycobiota and fungal plant pathogen diversity in Canada. Appl Environ Microbiol., https://doi.org/ 10.1128/aem.02601-02617

Després V.R., Huffman J.A., Burrows S.M., Hoose C., Safatov A.S., Buryak G., et al, (2012), Primary biological aerosol particles in the atmosphere: a review. Tellus Ser B-Chem Phys Meteorol. **64**, 15598. DOI: 10.3402/tellusb.v64i0. 15598

Dix N.J., Webster J. (1995), Fungal Ecology. Chapman & Hall, London. https://doi.org/10.1007/978-94-011-0693-1

Elbert W., Taylor P.E., Andreae M.O. and Pöschl U. (2007), Contribution of fungi to primary biogenic aerosols in the atmosphere: wet and dry discharged spores, carbohydrates, and inorganic ions. Atmos Chem Phys. **7**, 4569–88.

Fröhlich-Nowoisky, J., Pickersgill, D.A., Despres, V.R., Pöschl, U. (2009) High diversity of fungi in air particulate matter. Proc. Natl. Acad. Sci. 106, 12814–12819. http://dx.doi.org/ 10.1073 /pnas.0811003106.

Fröhlich-Nowoisky J., Burrows S.M., Xie Z., Engling G., et al. (2012). Biogeography in the air: fungal diversity over land and oceans, Biogeosciences **9**, 1125–1136. http://dx.doi.org/10.5194/bg-9-1125-20

Fröhlich-Nowoisky, J., Kampf, C.J., Weber, B., Huffman, J.A., Pöhlker, C., Andreae, M.O., Lang-Yona, N., et al. (2016), Bioaerosols in the Earth system: Climate, health, and ecosystem interactions. Atmos. Res., **182**, 346–376.

Haga D.I., Burrows S.M., Iannone R., Wheeler M.J., Mason R.H., Chen J., et al. (2014), Ice nucleation by fungal spores from the classes Agaricomycetes, Ustilaginomycetes, and Eurotiomycetes, and the effect on the atmospheric transport of these spores, Atmos Chem Phys, **14**, 8611–30.

Hyde K.D., Xu J.C., Lumyong S., Rapior S., Jeewon R.,..et al. (2019), The amazing potential of fungi, 50 ways we can exploit fungi industrially. Fungal Diversity **97**,1–136. https://doi.org/10.1007/s13225-019-00430-9

Lücking R., Aime M.C., Robbertse B., Miller A., Ariyawansa H. et al. (2020), Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding? IMA Fungus 11- 14, DOI: 10.1186/s43008-020-00033-z).

Pöschl U., Martin S.T., Sinha B., Chen Q., Gunthe S.S., Huffman J.A., et al., (2010), Rainforest aerosols as biogenic nuclei of clouds and precipitation in the Amazon, Science, **329**,1513–6.

Robertson C., Kirk Harris J., Wagner B.D., Browne K., et al. (2013) Explicet: graphical user interface software for metadata-driven management, analysis and visualization of microbiome data, Bioinformatics Applic. Notes **29.23**,3100–3101 DOI:10.1093/bioinformatics/btt526

Spänkuch D., Döhler W. and Güldner J., (2000), Effect of coarse biogenic aerosol on downwelling infrared flux at the surface, J Geophys Res-Atmos. **105**, 17341–50.

Tedersoo L., Bahram M., Põlme S., Kõljalg U., Yorou N.S., et al. (2014), Global diversity and geography of soil fungi, Science, 346, https://doi.org/10.1126/science.1256688

Thines M. (2014), Phylogeny and evolution of plant pathogenic oomycetes –a global overview, European Journal of Plant Pathology **138**,431–447.DOI:10.1007/s10658-013-0366-5

Woo C., An C., Siyu Xu S., Seung-Muk Yi S.M. and Yamamoto N. (2018), Taxonomic diversity of fungi deposited from the atmosphere. The ISME Journal **12**,2051–2060. https://doi.org/10.1038/s41396-018-0160-7

Figure 2. Actual abundance of fungi at Order level per library

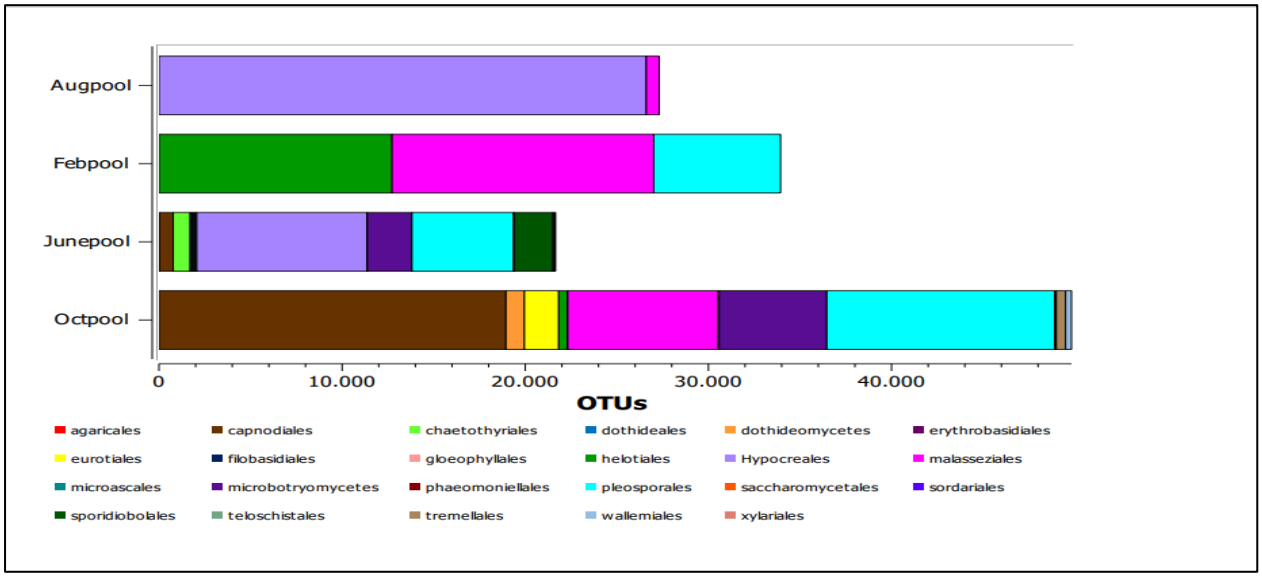

Table 1. Relative abundance % of fungal Classes in libraries and in total

Class/ Rel. Abundance %	Dothideomycetes	Sordariomycetes	Malasseziomycetes	Leotiomycetes	Microbotryomycetes	Eurotiomycetes
Total	*34,33*	*27,08*	*17,77*	*10,00*	*7,86*	*2,12*
June	4,79	7,06	0,00	0,07	3,39	0,71
August	0,00	19,98	0,53	0,00	0,00	0,00
October	24,34	0,03	6,18	0,37	4,46	1,41
February	5,20	0,00	10,73	9,56	0,00	0,00