Article Title: Optimizing a MALDI-TOF MS database for detection of xerophilic fungi across environments

Authors: Anne Mette Madsen, Victor Carp Kofoed, Christopher Campion

Contact Email: cca@nfa.dk

DOI: https://doi.org/10.14324/ucloepreprints.280.v1

Preprint first posted online: 2024-08-21 12:02

Keywords: cultural heritage collection., detection methods, MALDI-TOF MS, Xerophilic Aspergillus spp.

Optimizing a MALDI-TOF MS database for detection of xerophilic fungi across environments

Christopher Campion[1], Victor Carp Kofoed[1], Anne Mette Madsen.[1*]

[1]National Research Centre for the Working Environment, Lersø Parkallé 105, 2100 Copenhagen Ø, Denmark.

*corresponding author: amm@nrcwe.dk.

Abstract

Xerophilic fungi can proliferate in dry and water limited conditions and have been detected in museums, libraries, and other cultural heritage sites – some despite stringent climate-controlled indoor environments. In order to protect the staff from fungal exposure and ensure the preservation of heritage collections, further research must be placed in improving rapid detection protocols of xerophilic fungi. MALDI-TOF MS provides a fast and efficient method for detection and identification of fungal isolates, however, species identification is highly dependent on the reference database.

The aim of this study was to develop a supplementary MALDI-TOF MS database of 19 xerophilic *Aspergillus* species and use this to complement the current Bruker database, in screening various environments for xerophilic *Aspergillus* species. These environments included a warehouse, a museum, and 27 domestic homes.

The xerophilic *Aspergillus* species included in the supplementary database, formed species-distinct groups, which also formed clade structures, with similar structures to published phylogeny data based on genomic data. In two environments, xerophilic *Aspergillus* species were identified using the supplementary database - *Aspergillus conicus, Aspergillus dometicus, Aspergillus glabribes,* and *Aspergillus pseudogracilis.* Xerophilic/xerotolerant fungi belonging to the *Cladosporium* and *Penicillium* genus, identified using the Bruker library, dominated all environments. Using low water-activity media had a profound effect on growth of these xerophilic *Aspergillus* species.

This work exemplifies the durability and flexibility of using the MALDI-TOF MS platform for detection of specific fungi of interest and is to our knowledge, the first work using the MALDI-TOF MS platform to identify fungi in museums.

Keywords Xerophilic *Aspergillus* spp.*,* MALDI-TOF MS, detection methods; cultural heritage collection.

Introduction

If not limited, bio-deterioration caused by fungal growth can complicate the preservation of heritage collections (Sterflinger 2010, Sterflinger and Pinar 2013, Romero, Giudicessi et al. 2021, Stratigaki, Armirotti et al. 2024), by causing deterioration of cultural heritage artifacts (Borrego, Guiamet et al. 2018, Sterflinger, Voitl et al. 2018, Savkovic, Stupar et al. 2019, Cappitelli, Catto

et al. 2020, Pyzik, Ciuchcinski et al. 2021, Abdel-Maksoud, Abdel-Nasser et al. 2022, Vacar, Mircea et al. 2022, Kujovic, Gostincar et al. 2024). Furthermore, exposure to airborne fungi may pose a health concern for museum staff and visitors (Wiszniewska, Walusiak-Skorupa et al. 2009, Wiszniewska, Swierczynska-Machura et al. 2010, Gorny, Harkawy et al. 2016).

Methods for limiting fungal growth from museum artifacts, exhibition rooms, and storage repositories include controlling indoor climate parameters such as temperature, humidity, sunlight, complemented by various methods to clear fungal growth (Cappitelli, Catto et al. 2020, Pyzik, Ciuchcinski et al. 2021). However, xerophilic species of the genus *Aspergillus* (Sklenar, Jurjevic et al. 2017), and *Cladosporium* (Segers, Meijer et al. 2015) have the ability to proliferate in extreme dry and water limited (low water-activity (a_w) and low relative humidity (RH)) conditions and many reports have identified xerophilic fungi in museums, libraries, cultural heritage sites and their respective repositories (Montanari, Melloni et al. 2012, Micheluz, Manente et al. 2015, Pinar, Dalnodar et al. 2016, Polo, Cappitelli et al. 2017, Liu, Zhang et al. 2018, Piñar, Poyntner et al. 2020, Bastholm, Richter et al. 2023) - some also being reported in places despite stringent climate-controlled environments (Bastholm, Madsen et al. 2022). This suggest xerophilic fungi growing at low RH/a_w, might be overlooked, when attempting to limit fungal growth in such environments.

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) provides a fast and efficient method for identification of fungal isolates. MALDI-TOF MS relies on measurement of the mass-charge ratio of extracted proteins as mass spectrum. For microbial identification species-specific mass spectrum of unknown isolates are compared to a reference spectrum database. Depending on the similarity, this will result in identification to the genus or species level. The accuracy and depth of identification by MALDI-TOF MS, is therefore dependent on the extent of the reference spectrum database. In order to better ensure a safe work environment for the staff and ensuring the preservation of our heritage collection, further research must be placed in improving rapid detection and identification protocols of xerophilic fungi.

The purpose of this study was to construct a supplementary MALDI-TOF MS database for detection of xerophilic *Aspergillus* spp. For this purpose, a supplementary database was constructed, consisting of 19 xerophilic *Aspergillus* spp. These species were obtained from ITB fungal culture collection (https://dtu.bio-aware.com/page/Fungal%20search). These xerophilic fungi were selected because they are described (Sklenar, Jurjevic et al. 2017, Visagie, Yilmaz et al. 2017).

Materials and Methods

Xerophilic Aspergillus species strains

A total of 19 Xerophilic identified *Aspergillus* species were obtained from the ITB fungal culture collection (https://dtu.bio-aware.com/page/Fungal%20search)

(Table 1).

These species were all of the *Aspergillus* sect. *Restricti*, and contained representatives of the *As restrictus/As conicus* clade (*As caesiellus, As pachycaulis*, *As restrictus*, *As domesticus*, *As destruens*, *As gracilis*, *As pseudogracilis*, *As villosus* and *As conicus*), the *As vitricola* clade (*As vitricola*, *As glabripes*), the *As penicillioides* clade (*As penicillioides*, *As hordei*, *As reticulatus*, *As magnivesiculatus*, *As canadensis, As clavatophorus* and *As infrequens*), and the *As halophilicus* clade (*As halophilicus*) (Sklenar, Jurjevic et al. 2017).

The fungi were grown on Dichloran Glycerol agar (DG18, Thermo Fisher Scientific Oxoid, Basingstoke, UK)(Hocking and Pitt 1980) supplemented with 100 mg/l chloramphenicol or Malt-Yeast-50%-glucose agar (MY50) (Pitt and Hocking 2009). All fungi were incubated at 25°C for 14-21 days. Of the 19 *Aspergillus* spp. included in this study, *As. penicillioides* was already present in the BRUKER library BDAL Filamentous Fungi library version 4 (MaldiBiotyperDBUpdate_V4_Fungi-856(RUO)). Therefore, a strain of *As penicillioides* was included in the supplementary library as a positive control.

Sample preparation for MALDI-TOF MS

Protein extraction was performed using a modified version of the manufactures´ recommendations, using an ethanol-formic acid extraction protocol. In brief, the fungal isolates were inoculated in 1.5ml Eppendorf tubes containing either sabouraud broth (SAB), at room temperature for 1-3 days or until sufficient biomass had accumulated. Samples were inactivated by centrifugation, discarding supernatant and resuspending the pellet in 70% ethanol. The ethanol was removed and the sample was dried at room temperature, after which 5-20µl (depending on pellet size) 70% formic acid was added. After 2 min, the same volume (5-20µl, depending on pellet size) of acetonitrile was added. The samples were then centrifuged and 1µl of the supernatant was spotted on a MALDI 96 MSP polished steel target plate (Bruker) along with 1µl HCCA matrix solution (-Cyano-4-hydroxycinnamic acid, #8255344, Merck).

Spectrum acquisition and species identification

Samples were analyzed on a Microflex LT mass spectrometer (Bruker Daltonics Inc.,), using the Bruker Biotyper software (v. 3.1) with the Filamentous Fungi library (library version 4, MaldiBiotyperDBUpdate_V4_Fungi-856(RUO)). Identification of isolates originating from environments were analyzed as technical duplicates, with the cut-offs: scores > 1.70 were unidentified, score 1.70-1.80 were identified at genus level and with scores >1.8 identified to species level.

Main spectrum (MSP) creation for the supplementary MALDI-TOF MS database

Each species in the supplementary database, was represented by a series of MSPs, constructed by combining raw mass spectrums obtained from the species isolates inoculated in four different growth medium (Sabouraud, MY50, Czapek yeast 20% sucrose broth (CY20), or Malt-Yeast-70%-glucose broth (MY70)), at room temperature for 3-5 days or until sufficient biomass had accumulated. For

fungal identification, the standard procedure in this lab, was to inoculate isolates in sabouraud broth, therefore, the majority of the MSPs were created based on raw mass spectrums obtained from isolates inoculated in sabouraud broth. MSPs were later created based on mass spectrums obtained from isolates inoculated on MY50, MY70 and CY20S. Prior to MSP creation, all the raw spectrums were visually examined using the flexAnalysis software (Bruker Daltonics, Inc.), where spectrum with outlier peaks and low m/z (mass to charge ratio) were excluded. Then, using the MALDI Biotyper Compass Explorer module, raw mass spectrum were "preprocessed", by default settings, which includes: Mass Adjustment, Smoothing, Baseline Subtraction, Normalization & Peak picking. This was followed by creating a principal component analysis (PCA) plot of the spectrum, enabling further examination and removal of outlying spectrum. Finally, the MSPs obtained for all species were presented as a MSP dendrogram using the MALDI Biotyper Compass Explorer module. This analysis enables visualization of how close the MSPs representing the different species are related to one another, reflected by an arbitrary distance level, normalized to a max of 1000. In summary, for each species, up to four reference MSPs were created based on the inoculation media (SAB, MY50, CY20 and MY70).

Search for xerophilic Aspergillus species retrospectively

Previously obtained mass spectrum were used to test the supplementary database, using the Bruker Biotyper 3 software and the BDAL Filamentous Fungi library version 4 (MaldiBiotyperDBUpdate_V4_Fungi-856(RUO)). Exposure samples were plated on DG18 and sabouraud agar. For further information on the study, please refer to Daae et al., (Daae, Graff et al. 2024).

Validation of the supplementary MALDI-TOF MS database

Environmental samples from three different environments, were furthermore used to test the supplementary database. The environments included in this study were; a warehouse ($n_{samples}$=9; 6 personal samplers, 3 stationary samplers), a museum ($n_{samples}$=15), and home samples ($n_{samples}$=27). Environmental samples can be collected using different methods, depending on the environment and aim of research. Therefore, for each environment, a different sampling method was utilized. Air samples were taken in the warehouse (Gesamtstaubprobenahme air samplers, GSP). Electrostatic dust collectors (EDC) were used in homes as passive samplers. In an open-air museum environmental surface swabs (eSwab), were taken from surfaces of artefacts.

The warehouse was used for storage of gardening and roadwork equipment, tools and machinery used by workers for the maintenance of public spaces. The warehouse is made of heat-treated Pine tree wood (Thermowood) and fungal growth had been reported, suggested to be caused by fluctuations in humidity (unpublished data). Samples were collected using GSPs (personal GSP sampled for 11-21min, stationary GSP sampled for 240min), which were carried by workers or placed at specified locations. The GSP samplers used 37 mm polycarbonate filters (pore size of 0.8/1 m; SKC or CIS by BGI, Inc., Cambridge, MA, USA

respectively).

The museum is an open-air museum and consists of historical houses, buildings and indoor exhibitions representing various times throughout history. Here, surface samples were collected using E-swab (Copan's Liquid Amies Elution Swab, eSwab; Copan, Brescia, Italy) from artefacts in various locations. The home environments utilized long-term passive sampling (11-19 days) of dust using an electrostatic dust collector cloth (EDC, ZEEMAN, Alphen, Netherland, surface exposure area 209 cm^2).

Quantification of fungi

Material from these samples were extracted using an extraction solution (MilliQ water, 0.85 % NaCl and 0.05% Tween80), while shaking (GSP and eSwab – 15min at 500rpm; EDC – 60min at 300rpm).

Samples were stored at -80°C in 30% glycerol. The GSP samples were plated on DG18 and MY50. The eSwap samples were plated on sabouraud, DG18 and MY50. The EDC samples were plated on DG18, MY50, and MEA. If required, samples were diluted (MilliQ water, 0.85% NaCl, 0.001% Bac. peptone). All samples were incubated at 25°C; DG18, sabouraud, and MEA for 1 week, MY50 for 3 weeks. Following incubation, fungi colonies were counted. Sample and fungi concentrations were calculated, taking into account how much samples were diluted, sampling time, volume extracted and flow rate, depending on the sample technique in use.

Statistical analysis

Data analysis was conducted using R (v.4.2.3, R Core Team, 2013) in Rstudio (rstudio.com), and following Rpackages "tidyverse" (Wickham et al., 2019), ampvis2 (Andersen, et al., 2018a) and visualized using ggplot2 (Wickham, 2016)).

Results

Creation of supplementary MALDI-TOF MS database

For sixteen of the species, it was possible to create four MSPs, however, for *As clavatophorus, As restrictus, As penicillioides* and *As halophilicus*, it was not possible to obtain raw spectrum from all four media (Table 1). Of note, it was only possible to obtain mass spectrum of *As halophilicus* if the fungus was inoculated in broth with low water activity (MY50 and MY70). It was not possible to obtain satisfactory quality mass spectrum of *As restrictus, As clavatophorus* and *As penicillioides*, when inoculated in MY50, MY70, CY20S (Table 1).

Discrimination between the 19 xerophilic *Aspergillus* species of the supplementary database was evaluated by a MSP dendrogram (Fig.1). The majority of the MSPs formed species specific groups, with the exception of MSPs of *As destruens* and *As penicillium*, which were indistinguishable from each other. Generally, the MSP species groups, formed clades, with similar patterns to published data based on genomic data, albeit with some distinctions (Sklenar, Jurjevic et al.

2017). The ***As restrictus* clade**, consisting of *As pachycaulis*, *As caesiellus* and *As restrictus* were grouped together, forming a distinct cluster. The ***As conicus* clade** (*As villosus, As gracilis, As domesticus, As pseudogracilis, As conicus*, *As destruens*), instead of forming one clade, formed two clades of *As villosus, As gracilis,* and *As domesticus, As conicus, As pseudogracilis*, with *As destruens* being indistinguishable from *As penicillioides* from the *As penicillioides* clade. The two species included from the ***As vitricola* clade** (*As glabripes*, *As vitricola*), were grouped together with species of the *As penicillioides* clade and *As conicus* clade. The species included of the ***As penicillioides* clade** (*As magnivesiculatus, As hordei*, *As penicillioides, As clavatophorus, As infrequens, As canadensis*), were split into two distinct clades.

Only the control, *As penicillioides*, which already is present in the Bruker library, was identified, when the supplementary database was run against the Bruker Fungi library. The remainder MSPs were not identified, as expected. The MSPs of *As destruens*, were indistinguishable from *As penicillioides* and would be identified as *As penicillioides* by the Bruker Fungal library. The MSPs were kept in the supplementary database regardless.

Search for xerophilic Aspergillus species retrospectively in stored mass spectrum

The supplementary database was used to identify mass spectrum previously obtained from a study on drilling waste treatment plants (Daae, Graff et al. 2024). Xerophilic/xerotolerant fungi had been reported in this environment, therefore, a total of 313 mass spectrum originating from isolates grown on DG18 and/or sabouraud agar plates (n_{DG18}=197; n_{SAB}=116), respectively, were attempted re-identified, using the supplementary database. Of these, two previously unidentified isolates (originating from the same DG18 agar plate), were now identified as *As caesiellus*, with ID scores > 2.00 (Fig. S1).

The two media used in this study, appeared to affect the presence and total number of xerophilic/xerotolerant species (Fig. S1). We observed that the samples inoculated on DG18 agar plates, contained a higher amount of xerophilic/xerotolerant species (n=7/21) of the genera *Penicillium*, *Cladosporium* and *Aspergillus*, when compared to the samples inoculated on sabouraud agar plates (n=3/14).

Xerophilic Aspergillus species in environmental samples

The supplementary database was further validated using a collection of samples, originating from three environments and sampled using different techniques (GSP, EDC, and eSwab). These three environments, included a warehouse (n=9, GSP), museum (n=15, eSwab) and home environments (n=27, EDC).

The fungal concentration of the air exposure samplers (GSP) were in the $2.68*10^2$ – $1.15*10^4$ CFU/m^3 range. The fungal concentration of the sedimented dust (EDC) were in the $2.05*10^2$ – $4.45*10^4$ CFU/cm^2/day. The fungal surface concentrations (eSwab) were in the $5*10^1$ – $9.2*10^6$ CFU/location (Fig. 2).

Xerophilic *Aspergillus* species were detected in two environments (warehouse and homes), when using the supplementary library. These specific fungal isolates were not identified using the Bruker library alone, albeit other xerophilic/xerotolerant fungi, were identified from these environments using the Bruker library (Fig. 3). The xerophilic *Aspergillus* species identified using the supplementary library were¸ *As pseudogracilis* (total isolates, n=9), *As glabripes* (total isolates, n=1), *As domesticus* (total isolates, n=3) and *As conicus* (total isolates, n=1). *As domesticus* and *As pseudogracilis* were identified in two of the environments tested.

Several xerophilic/xerotolerant species such as, *As glaucus* (total isolates, n=6), *Penicillium brevicompactum/olsonii* (total isolates, n=64), *Penicillium chrysogenum* (total isolates, n=15), *Penicillium corylophilium* (total isolates, n=14) and *Cladosporium herbarum* (total isolates, n=27), were identified using the Bruker Fungal library and were amongst the most abundant fungi in tested environments.

The indoor homes environment was dominated by the genus *Cladosporium*, with *Cl cladosporioides, Cl herbarum* and isolates identified as *Cladosporium sp.* being present in high concentrations, $7.0*10^3$ CFU/cm^2/day, $4.4*10^3$ CFU/cm^2/day and $9.2*10^3$ CFU/cm^2/day, respectively. Species of *Penicillium* were also present in high concentrations, such as *P italicum*, *P brevicompactum/olsonii, P digitatum* and *P glabrum,* estimated in the 10^3 CFU/cm^2/day range. The three xerophilic *Aspergillus species,* identified from these environments, *As domesticus, As glabripes* and *As pseudogracilis*, using the supplementary database were reported in concentrations of $5.2*10^2$ CFU/day, $4.1*10^2$ CFU/day and $2.1*10^2$ CFU/day, respectively (Fig. 3).

The warehouse samples were dominated by *Penicillium CCC* ($5.5*10^3$ CFU/m^3), *Cladosporium langeronii* ($2.2*10^3$ CFU/m^3) and *filibasidium magnum* ($2.8*10^3$ CFU/m^3). In this environment *As. pseudogracillis, As. domesticus* and *As. conicus* were not as abundant, estimated at $2.7*10^2$ CFU/m^3, $3.9*10^1$ CFU/m^3, $1.0*10^2$ CFU/m^3, respectively.

The most abundant fungi detected in the museum were *Penicillium aurantigriseum* ($6.5*10^4$ CFU/location), *Penicillium chrysogenum* ($1.1*10^4$ CFU/location), and isolates belonging to the *Cladosporum* genus ($2.2*10^4$ CFU/location). No additional xerophilic *Aspergillus* species, were identified from the museum samples.

Effect of media on detection of xerophilic Aspergillus species in environmental samples.

The various agar media used for fungal growth, did appear to affect the presence of xerophilic fungi (Fig. 4). For the domestic home environment, more xerophilic fungi were detected on low water-activity media, MY50 (n=10/21 species, 48% of species), and DG18 (n=8/29 species, 27% of species), contra MEA (n=5/26 species, 19% of species). None of the xerophilic *Aspergillus* species included in the supplementary database were detected on DG18 or MEA.

A similar trend was observed in the warehouse environment (Fig. 4). Albeit, with fewer species detected in the air samples, in general, the media has an observable effect on prevalence of xerophilic/xerotolerant fungi, with the majority of xerophilic/xerotolerant species being detected on low water-activity media (MY50, n=7/10 species, 70% of species), compared to media with higher water-activity (DG18, n=1/5 species, 20% of species).

This trend, observed in the home and warehouse environments, however, was not observed in the museum environment for the media (MY50, 6/22 species, 27% of species; DG18, 5/25 species, 20% of species; SAB, 7/25 species, 28% of species) (Fig. 4).

Discussion

In this study, a MALDI-TOF MS supplementary database consisting of 19 xerophilic *Aspergillus* species, was constructed for the purpose of complementing the current Bruker fungal library, when studying the presence of xerophilic *Aspergillus* species.

Creation of a supplementary MALDI-TOF MS database of selected xerophilic Aspergillus species

Culture condition and sample preparation methods can influence the mass spectrum obtained (Honsig, Selitsch et al. 2022, Topic Popovic, Kazazic et al. 2023). Therefore, the library was constructed using MSPs based on raw mass spectrum originating from isolates inoculated in four different media. Interestingly, raw mass spectrum of the highly xerophilic fungus, *As halophilicus*, was only obtained, when the fungus was inoculated in broth of low water-activity. The inability to obtain raw mass spectrum from *As halophilicus*, might be partly explained by it being obligately xerophilic, and not readily cultured in high water-activity media (Andrews and Pitt 1987, Hocking and Pitt 1988, Stevenson, Hamill et al. 2017, Micheluz, Pinzari et al. 2022). This suggests that, when working with environments where extreme/obligate xerophilic fungi thrive, it might be relevant to re-evaluate the protocols (i.e. inoculation media and inoculation time) used to inoculate unknown fungi isolates, in order to obtain mass spectrum, and hence ensure species identification.

Importantly, none of the MSPs of the supplementary library, were identified by Brukers fungal library except for the species *As penicillioides* and *As destruens*, with the positive control *As penicillioides* already being present in Brukers database. In the case of *As destruens*, the Bruker database would identify this species as *As penicillioides*.

The MSP dendrogram, showed that the MSPs formed species-specific clusters of the various xerophilic *Aspergillus* species. An exception of the species-specific clusters, was *As penicillioides* and *As destruens*, for which MSPs were indistinguishable. By the time of submission, we observed that the species designation was updated for *As destruens* (strain ITB no. 34818), which is now designated *Aspergillus salinarum* (synonymy – *Phialosimplex salinarum*). This species is a

halophilic fungus isolated from a hypersaline environment (Greiner, Persoh et al. 2014) (Table 1). However, this does not explain why MSPs of these two species are indistinguishable.

Regarding the overall clade structure of the MSP dendrogram, some disagreements in the phylogeny between proteomic based data and published phylogenetic data based on genomic data, were observed (Sklenar, Jurjevic et al. 2017). It should be noted that the supplementary database, was based on a single strain per fungal species. Future studies, improving the supplementary library, by including biological replicates, might be required to clarify this discrepancy.

Testing the supplementary MALDI-TOF MS database

The supplementary database was tested, first by running it against stored mass spectrum from a previous project, leading to the identification of two previously un-identified isolates, now identified as *As caesiellus*. This provides an example of how this group of fungi might have been overlooked previously and shows how already performed studies can easily be revised, with supplementary fungal databases.

Testing the supplementary MALDI-TOF MS database on environmental samples

The supplementary database was used to complement the Bruker database in three different environments. Isolates of xerophilic *Aspergillus* species were identified in two of these environments (Homes and Warehouse).

The museum samples, did not reveal any xerophilic *Aspergillus* species, using the supplementary database, despite other xerophilic/xerotolerant species were identified by the Bruker database, from this environment. It should be mentioned, that the museum environment, from which these samples originate, were not closed, confined or climate-controlled sites and were therefore, not necessary environments that favored growth of the xerophilic *Aspergillus* species included in this study.

Overall, a low number of fungal species were detected from the warehouse, however, the supplementary database detected two species, *Aspergillus glabribes* and *Aspergillus pseudogracilis*. Other xerophilic/xerotolerant were also identified from this environment, by the Bruker database, including *Aspergillus glaucus, Aspergillus pseudoglaucus*, *P brevicompactum,* and *P corylophilum*. The low number of fungal species detected could explained by a relatively short sampling time, for the GSP samplers (11-21 min for the personal samplers (n=6) and 240 min for the stationary samplers (n=3)).

We analyzed samples from 27 randomly selected homes. The most common species observed are in accordance with what has previously been found in homes, such as certain *Cladosporium* (*Cl sphaerospermum, Cl herbarum, Cl cladosporides), Penicillium* (*P brevicompactum, P camemberti, P chrysogenum, P citrinum, P commume, P glabrum, P. olsoni*), and *Aspergillus* (*As glaucus*) species (Segers, Meijer et al. 2015, Knudsen, Gunnarsen et al. 2017, Nastasi,

Haines et al. 2020). The xerophilic *Aspergillus* species added to the database only constituted a small part of the potential exposure.

Effect of media on identification of xerophilic fungi

The media appeared to have an effect on species composition, selection of xerophilic/xerotolerant species and total number of xerophilic/xerotolerant species. With exception of *As caesiellus* (Fig. S1), all xerophilic *Aspergillus* species (from the supplementary database), were only identified on MY50 (Fig. 4). Some species, such as *P brevicompactum/olsonii*, *P chrysogenum, Cl cladosporioides, Cl herbarum, As pseudoglaucus,* and *As glaucus,* were detected regardless on media used. It should be noted that the MY50 plates were incubated longer than DG18, MEA, and sabouraud agar plates, since it takes longer for fungi to appear on this water-restricted media. One explanation as to why the xerophilic *Aspergillus* species in the supplementary database were not detected on DG18 (with exception of *As caesiellis,* Fig. S1), and that fewer xerophilic/xerotolerant fungi were observed on other media, might be that they were out-competed by faster growing strains on less restrictive media.

Another, explanation could also be regarding the inoculation medium used and incubation time, when preparing fungal isolates for MALDI-TOF MS identification. When identifying isolates from the three environments, this study followed Brukers recommendations for sample preparation (liquid cultivation), which included 1-3 days incubation in sabouraud medium, followed by an ethanol extraction with formic acid and acetonitrile. Of note, when creating the supplementary database, raw mass spectrum could not be obtained from *As halophilicus* unless it was inoculated in MY50 or MY70. Therefore, for this fungus, its strict water availability requirements could be another limiting factor, when preparing samples for MALDI-TOF MS through liquid cultivation.

These results, along with other studies (Visagie, Boekhout et al. 2023), underline the importance of appropriate selective media (i.e. low a_w media) and inoculation time, both for database construction and when identifying unknown isolates, when studying the presence of xerophilic species.

Conclusion

In conclusion, the supplementary MALDI-TOF MS database successfully complemented the Bruker database, with the combined databases, identifying additional xerophilic *Aspergillus* species in different environmental samples, previously going undetected, and therefore provided a more in-depth characterization of various environments. Despite some disagreements in phylogeny between proteomic based data (MALDI-TOF MS) and with published phylogeny data (genomic data), regarding overall clade structures, the application of MALDI-TOF MS for identification of fungal species of interest could be used as a less time-consuming method for species identification. This study exemplifies the flexibility and versatility of the MALDI-TOF MS platform for identification of specific filamentous fungi of interest from various environments.

Authorship contributions

CC: Conceptualization, Writing – draft, review and editing, analysis, investigation. AMM: Conceptualization, project administration, resources, supervision, writing – review and editing. VCK: review and editing.

Funding

This research was funded by the Augustinus Foundation (22-1543) and the Aase and Ejnar Danielsens Foundation (22-30-0478).

Competing interest

The authors declare that this research was conducted in absence of any commercial or financial relationships, which could be considered a potential conflict of interest.

Tables

Species	**Section**	**Clade**	**IBT no.***	**MSPs**	**Peak list. Comprising a MSP.**			
				Tot.	**SAB**	**CY20S**	**MY50**	**MY70**
As. destruens#	*Restricti*	*As. conicus*	34818	4	44	9	11	2
As. reticulatus	*Restricti*	*As. penicillioides*	34819	4	22	4	7	2
As. canadensis	*Restricti*	*As. penicillioides*	34642	4	30	6	13	4
As. clavatophorus	*Restricti*	*As. penicillioides*	34632	1	32	-	-	-
*As. halophilicus***	*Restricti*	*As. halophilicus*	34881	2	-	-	7	12
As. vitricola	*Restricti*	*As. vitricola*	34272	4	11	5	4	2
As. villosus	*Restricti*	*As. conicus*	34822	4	40	6	9	4
As. hordei	*Restricti*	*As. penicillioides*	34631	4	56	7	11	4
As. restrictus	*Restricti*	*As. restrictus*	33630	1	6	-	-	-
As. infrequens	*Restricti*	*As. penicillioides*	34524	4	34	4	4	2
As. caesiellus	*Restricti*	*As. restrictus*	34621	4	14	4	1	2
As. gracilis	*Restricti*	*As. conicus*	34817	4	21	5	4	1

Species	Section	Clade	IBT no.*	MSPs	Peak list. MSP.		Comprising a	
As. penicillioides[+]	*Restricti*	*As. penicillioides*	34815	3	21	-	5	4
As. magnivesiculatus	*Restricti*	*As. penicillioides*	34816	4	52	12	14	12
As. pseudogracilis	*Restricti*	*As. conicus*	34813	4	23	11	9	2
As. glabripes	*Restricit*	*As. vitricola*	34820	4	18	5	5	6
As. pachycaulis	*Restricti*	*As. restrictus*	34812	4	17	4	5	3
As. domesticus	*Restricti*	*As. conicus*	34814	4	28	7	4	4
As. conicus	*Restricti*	*As. conicus*	34288	4	30	5	3	2

**https://www.bioengineering.dtu.dk/research/strain-collections/ibt-culture-collection-of-fungi.*
***anamorph – As halophilicus, teleomorph - Eurotium halophilicum.*
[+] *As penicillioides* is present in Brukers BDAL Filamentous Fungi library version 4 (MaldiBiotyperDBUpdate_V4_Fungi-856(RUO)).
[≠] current name -*Aspergillus salinarum* (ex type of *Aspergillus destruens*, synonymy – Phialosimplex salinarum(Greiner, Persoh et al. 2014)).

Table 1. Overview of xerophilic fungi included in this study and Number of main spectrum projections (MSP) for the xerophilic *Aspergillus* species, included in the supplementary library.

Figure legend

Figure 1: A MSP dendrogram based on MSPs of the 19 species of *xerophilic Aspergillus,* using MALDI Biotyper Compass Explorer module. The distance level (arbitrary unit, a.u, normalized to a max of 1000), reflects the differences between species and how related the MSP are to one another. Clade designation based on genomic data is indicated by clade-specific colors and marked on the right-hand side of the image (Sklenar, Jurjevic et al. 2017).

Figure 2: Concentrations of fungi across three environments, each using different sampling techniques (EDC, Homes – A; surface eSwab, Museum – B; GSP air samplers, Warehouse - C). The X-axis shows log10-transformed sample CFU concentrations (CFU/cm^2/day, CFU/location and CFU/m^3), across different media. The black dots represents geometric mean (GM) values of the sample, while the smaller dots represents outliers.

Figure 3: Heatmap of fungal species identified across three environments.

Numbers represent to no. of detections. The color gradient represents the log10-transfor geometric mean of the CFU concentration (CFU/cm^2/day, CFU/location and CFU/m^3). *Cladosporium sp.*, represents isolates identified to genus level as *Cladosporium sp. Penicillium CCC* is an abbreviation for *Penicillium camemberti commune cyclopium*, which designation represents isolates identified as either *Penicillium camemberti, Penicillium commune* or *Penicillium cyclopium*, which cannot be distinguished by MALDI-TOF MS.

Figure 4: Fungal species across environments in various media. Numbers represent the no. of isolates in samples, the species represent. The concentration is represented as GM of samples (EDC, Homes – A; surface eSwab, Museum – B; GSP air sampler, Warehouse – C). Newly added xerophilic fungi (supplementary library) are highlighted in red, xerophilic species present in the Bruker library are highlighted in green, other fungal species are highlighted in blue. *Cladosporium sp.*, represents isolates identified to genus level as *Cladosporium sp. Penicillium CCC* is an abbreviation for *Penicillium camemberti commune cyclopium*, which designation represents isolates identified as either *Penicillium camemberti, Penicillium commune* or *Penicillium cyclopium*, which cannot be distinguished by MALDI-TOF MS.

Supplementary figure 1: Retrospective search in previous obtained mass spectrum for xerophilic fungi in a drilling waste environment. The figure shows the total species identifications across two media. Newly added xerophilic fungi (supplementary database) are highlighted in red, xerophilic/xerotolerant species present in the Bruker library are highlighted in green, non-xerophilic/xerotolerant fungi species are highlighted in blue. A) shows the total species identification. B) shows species identification based on media (n_{DG18}=197; n_{SAB}=116). *Cladosporium sp.*, represents isolates identified to genus level as *Cladosporium sp. Penicillium CCC* is an abbreviation for *Penicillium camemberti commune cyclopium*, which designation represents isolates identified as either *Penicillium camemberti, Penicillium commune* or *Penicillium cyclopium*, which cannot be distinguished by MALDI-TOF MS.

References

Abdel-Maksoud, G., M. Abdel-Nasser, M. H. Sultan, A. M. Eid, S. H. Alotaibi, S. E. Hassan and A. Fouda (2022). "Fungal Biodeterioration of a Historical Manuscript Dating Back to the 14th Century: An Insight into Various Fungal Strains and Their Enzymatic Activities." Life (Basel) **12**(11).

Andrews, S. and J. I. Pitt (1987). "Further Studies on the Water Relations of Xerophilic Fungi, Including Some Halophiles." **133**(2): 233-238.

Bastholm, C., J. Richter, A. Aalling, A. Bjerre and A. Madsen (2023). Xerophilic fungi have a taste for Danish art and cultural heritage.

Bastholm, C. J., A. M. Madsen, B. Andersen, J. C. Frisvad and J. Richter (2022). "The mysterious mould outbreak - A comprehensive fungal colonisation in a climate-controlled museum repository challenges the environmental guidelines

for heritage collections." Journal of Cultural Heritage **55**: 78-87.

Borrego, S., P. S. Guiamet, I. Vivar and P. A. Battistoni (2018). FUNGI INVOLVED IN BIODETERIORATION OF DOCUMENTS IN PAPER AND EFFECT ON SUBSTRATE.

Cappitelli, F., C. Catto and F. Villa (2020). "The Control of Cultural Heritage Microbial Deterioration." Microorganisms **8**(10).

Daae, H. L., P. Graff, O. A. H. Foss, V. C. Kofoed, A. K. Afanou, M. W. Frederiksen and A. M. Madsen (2024). "A cross-sectional study on occupational exposure to microorganisms, endotoxin, hydrogen sulfide, and dust during work at drilling waste treatment plants." Ann Work Expo Health **68**(1): 58-77.

Gorny, R. L., A. S. Harkawy, A. Lawniczek-Walczyk, J. Karbowska-Berent, A. Wlazlo, A. Niesler, M. Golofit-Szymczak and M. Cyprowski (2016). "Exposure to culturable and total microbiota in cultural heritage conservation laboratories." Int J Occup Med Environ Health **29**(2): 255-275.

Greiner, K., D. Persoh, A. Weig and G. Rambold (2014). "Phialosimplex salinarum, a new species of Eurotiomycetes from a hypersaline habitat." IMA Fungus **5**(2): 161-172.

Hocking, A. D. and J. I. Pitt (1980). "Dichloran-glycerol medium for enumeration of xerophilic fungi from low-moisture foods." **39**(3): 488-492.

Hocking, A. D. and J. I. Pitt (1988). "Two New Species of Xerophilic Fungi and a Further Record of Eurotium Halophilicum." Mycologia **80**(1): 82-88.

Honsig, C., B. Selitsch, M. Hollenstein, M. G. Vossen, K. Spettel and B. Willinger (2022). "Identification of Filamentous Fungi by MALDI-TOF Mass Spectrometry: Evaluation of Three Different Sample Preparation Methods and Validation of an In-House Species Cutoff." J Fungi (Basel) **8**(4).

Knudsen, S. M., L. Gunnarsen and A. M. Madsen (2017). "Airborne fungal species associated with mouldy and non-mouldy buildings – effects of air change rates, humidity, and air velocity." Building and Environment **122**: 161-170.

Kujovic, A., C. Gostincar, K. Kavkler, N. Govedic, N. Gunde-Cimerman and P. Zalar (2024). "Degradation Potential of Xerophilic and Xerotolerant Fungi Contaminating Historic Canvas Paintings." J Fungi (Basel) **10**(1).

Liu, Z., Y. Zhang, F. Zhang, C. Hu, G. Liu and J. Pan (2018). "Microbial Community Analyses of the Deteriorated Storeroom Objects in the Tianjin Museum Using Culture-Independent and Culture-Dependent Approaches." Front Microbiol **9**: 802.

Micheluz, A., S. Manente, V. Tigini, V. Prigione, F. Pinzari, G. Ravagnan and G. C. Varese (2015). "The extreme environment of a library: Xerophilic fungi inhabiting indoor niches." International Biodeterioration & Biodegradation **99**: 1-7.

Micheluz, A., F. Pinzari, E. G. Rivera-Valentin, S. Manente and J. E. Hallsworth (2022). "Biophysical Manipulation of the Extracellular Environment by Eurotium halophilicum." Pathogens **11**(12).

Montanari, M., V. Melloni, F. Pinzari and G. Innocenti (2012). "Fungal biodeterioration of historical library materials stored in Compactus movable shelves." International Biodeterioration & Biodegradation **75**: 83-88.

Nastasi, N., S. R. Haines, L. Xu, H. da Silva, A. Divjan, M. A. Barnes, C. A. Rappleye, M. S. Perzanowski, B. J. Green and K. C. Dannemiller (2020). "Morphology and quantification of fungal growth in residential dust and carpets." Build Environ **174**.

Pinar, G., D. Dalnodar, C. Voitl, H. Reschreiter and K. Sterflinger (2016). "Biodeterioration Risk Threatens the 3100 Year Old Staircase of Hallstatt (Austria): Possible Involvement of Halophilic Microorganisms." PLoS One **11**(2): e0148279.

Piñar, G., C. Poyntner, K. Lopandic, H. Tafer and K. Sterflinger (2020). "Rapid diagnosis of biological colonization in cultural artefacts using the MinION nanopore sequencing technology." International Biodeterioration & Biodegradation **148**: 104908.

Pitt, J. I. and A. D. Hocking (2009). Introduction. Fungi and Food Spoilage. J. I. Pitt and A. D. Hocking. Boston, MA, Springer US**:** 1-2.

Polo, A., F. Cappitelli, F. Villa and F. Pinzari (2017). "Biological invasion in the indoor environment: the spread of Eurotium halophilicum on library materials." International Biodeterioration & Biodegradation **118**: 34-44.

Pyzik, A., K. Ciuchcinski, M. Dziurzynski and L. Dziewit (2021). "The Bad and the Good-Microorganisms in Cultural Heritage Environments-An Update on Biodeterioration and Biotreatment Approaches." Materials (Basel) **14**(1).

Romero, S. M., S. L. Giudicessi and R. G. Vitale (2021). "Is the fungus Aspergillus a threat to cultural heritage?" Journal of Cultural Heritage **51**: 107-124.

Savkovic, Z., M. Stupar, N. Unkovic, Z. Ivanovic, J. Blagojevic, J. Vukojevic and M. Ljaljevic Grbic (2019). "In vitro biodegradation potential of airborne Aspergilli and Penicillia." Naturwissenschaften **106**(3-4): 8.

Segers, F. J., M. Meijer, J. Houbraken, R. A. Samson, H. A. Wosten and J. Dijksterhuis (2015). "Xerotolerant Cladosporium sphaerospermum Are Predominant on Indoor Surfaces Compared to Other Cladosporium Species." PLoS One **10**(12): e0145415.

Sklenar, F., Z. Jurjevic, P. Zalar, J. C. Frisvad, C. M. Visagie, M. Kolarik, J. Houbraken, A. J. Chen, N. Yilmaz, K. A. Seifert, M. Coton, F. Deniel, N. Gunde-Cimerman, R. A. Samson, S. W. Peterson and V. Hubka (2017). "Phylogeny

of xerophilic aspergilli (subgenus Aspergillus) and taxonomic revision of section Restricti." Stud Mycol **88**: 161-236.

Sterflinger, K. (2010). "Fungi: Their role in deterioration of cultural heritage." Fungal Biology Reviews **24**(1): 47-55.

Sterflinger, K. and G. Pinar (2013). "Microbial deterioration of cultural heritage and works of art--tilting at windmills?" Appl Microbiol Biotechnol **97**(22): 9637-9646.

Sterflinger, K., C. Voitl, K. Lopandic, G. Pinar and H. Tafer (2018). "Big Sound and Extreme Fungi-Xerophilic, Halotolerant Aspergilli and Penicillia with Low Optimal Temperature as Invaders of Historic Pipe Organs." Life (Basel) **8**(2).

Stevenson, A., P. G. Hamill, A. Medina, G. Kminek, J. D. Rummel, J. Dijksterhuis, D. J. Timson, N. Magan, S. L. Leong and J. E. Hallsworth (2017). "Glycerol enhances fungal germination at the water-activity limit for life." Environ Microbiol **19**(3): 947-967.

Stratigaki, M., A. Armirotti, G. Ottonello, S. Manente and A. Traviglia (2024). "Fungal and bacterial species richness in biodeteriorated seventeenth century Venetian manuscripts." Sci Rep **14**(1): 7003.

Topic Popovic, N., S. P. Kazazic, K. Bojanic, I. Strunjak-Perovic and R. Coz-Rakovac (2023). "Sample preparation and culture condition effects on MALDI-TOF MS identification of bacteria: A review." Mass Spectrom Rev **42**(5): 1589-1603.

Vacar, C. L., C. Mircea, M. Parvu and D. Podar (2022). "Diversity and Metabolic Activity of Fungi Causing Biodeterioration of Canvas Paintings." J Fungi (Basel) **8**(6).

Visagie, C. M., T. Boekhout, B. Theelen, J. Dijksterhuis, N. Yilmaz and K. A. Seifert (2023). "Da Vinci's yeast: Blastobotrys davincii f.a., sp. nov." Yeast **40**(1): 7-31.

Visagie, C. M., N. Yilmaz, J. B. Renaud, M. W. Sumarah, V. Hubka, J. C. Frisvad, A. J. Chen, M. Meijer and K. A. Seifert (2017). "A survey of xerophilic Aspergillus from indoor environment, including descriptions of two new section Aspergillus species producing eurotium-like sexual states." **19**.

Wiszniewska, M., D. Swierczynska-Machura, P. Cezary and J. Walusiak-Skorupa (2010). "[Fungal allergy among art conservators: prevalence, risk factors and clinical symptoms]." Med Pr **61**(2): 133-141.

Wiszniewska, M., J. Walusiak-Skorupa, I. Pannenko, M. Draniak and C. Palczynski (2009). "Occupational exposure and sensitization to fungi among museum workers." Occup Med (Lond) **59**(4): 237-242.