Towards a Better Understanding of the Epidemiology and Control of Eumycetoma

Inauguraldissertation

Zur

Erlangung der Würde eines Doktors der Philosophie

von

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2022

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

auf Antrag von

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Basel, 19. November 2019

Prof. Dr. Martin Spiess

Dekan

Dedication

I dedicate this work to all mycetoma patients who suffers as we are working in the laboratories, as we are running code and analyzing genomes, as we are wondering which solution could be rapid enough to help alleviate their pain and the horrible consequences of mycetoma.

Acknowledgments

I hereby would like to acknowledge the Swiss Excellence Scholarship board of the State Secretariat for Education, Research and Innovation (SERI) and the coordinators at Universität Basel, Prof. Susanne Bickel and Mrs. Andrea Delpho for the kind support who made the bridge that brought this work to a conclusion.

I want to thank my Swiss supervisors, Prof. Marcel Tanner and Prof. Pascal Mäser along with my Sudanese Supervisor prof. Abdalla Elkhawad for their support and kind supervision. I also would like to thank prof. Sami Khalid for opening his laboratory at which he is developing an *in-vitro* method for screening new molecular entities and extracts on *M. mycetomatis* strains. Dr. Matthias Witschel for his support with the agrochemical fungicide library and Prof. Reto Brun for his support and advice with regards to the diamidine library selection.

I would like to acknowledge the endless support I got from the department of training and education, Swiss Tropical & Public Health Institute and more especially Mrs. Christine Mensch who has really dedicated herself to solving our emergencies and guiding us through the whole study period.

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Abbreviations

Abbreviation	In full script
1 NADH OR	Complex I NADH Oxido-reductase
Acam	Aspergillus compestris
ADA	Adenosine deaminase
Afum	Aspergillus fumigatus
Aneo	Aspergillus neoniger
Anid	Aspergillus nidulans
Anig	Aspergillus niger
Anovo	Aspergillus novofumigatus
Aram	Aspergillus rambellii
Ater	Aspergillus terreus
BLAST	Basic Local Alignment Tool
blastp	Basic Local Alignment Tool for Proteins
CHF	Congective Heart Failue
CHS	Chitin synthase
DNDi	Drugs for Neglected Diseases Initiative
EMA	European Medicines Agency
ESKAS	Swiss Scholarship for Foreign Scholars
FDA	US Food and Drug administration
FNAC	Fine Needle Aspiration Cytology
FRAC	Fungicide Resistance Action Committee
GyrB	DNA topoisomerase type II (gyrase)
HMM	Hidden-Markov Models
HMMer	Program used to search for Hidden-markov model profiles
IV	Intravenous
KDE	Kernel Density Estimation
LFT	Liver Function Test
MRC	Mycetoma Research Center
NTD	Neglected Tropical Disease
PAM	Partitioning Around Medoids analysis
PCA	Principal Component Analysis
PCs	Principal components
Pfam	Protein Family Database
PKS	Polyketide synthase
PmtA	Phospholipid methyltransferase
QGIS	Guantum GIS software
RNApol	RNA polymerase I
SD	Scytalone dehydratase
SDH	Succinate dehydrogenase
SJS	Stevens-Johnson syndrome
Tax ID	Taxonomical identifier
WHO	World Health Organization

Summary

Eumycetoma, a neglected tropical disease with considerable knowledge gaps. Eumycetoma has shown worldwide pattern of incidence, yet most of the patients are living in the tropics and subtropics and are mostly living in poor communities. *Madurella mycetomatis*, a fungus which is considered the first in the broad list of fungal pathogens, recently *M. mycetomatis* was exploited with modern biological techniques which hopefully ended with whole genome sequencing, development of PCR diagnostic tool, and revealing many aspects of the host-parasite interactions.

The aim of this PhD thesis were (i) advance the knowledge on aspects of eumycetoma epidemiology (ii) investigate potential drug targets and active compounds for prospect control and elimination (iii) conduct comparative genomics to answer the questions in (ii) and to search for specific virulence genes that could explain the human invasion by the causative agents.

The list of eumycetoma pathogens featured 130 fully identified species along with 13 partially identified pathogens (*genus* only), these agents have shown differences in their spatial distribution; some species were of limited distribution including *Aspergillus fumigatus* and *Leptospheria senegalensis*, while M. mycetomatis and Madurella grisea have shown worldwide distribution. Comparing the proteomes of eumycetoma fungi to those from famous phytopathogens revealed the potential of adenosine deaminase, chitin synthase, and succinate dehydrogenase to serve as future drug targets against eumycetoma fungal pathogens.

We have also identified myosine related proteins and other set of proteins to have remarkable conservation bias towards eumycetoma fungal pathogens compared to apathogenic species closely related which makes them potential virulence factors.

1. CHAPTER -1-

General Introduction

Eumycetoma is the fungal type of mycetoma which is caused by fungi from varying taxonomical orders, it is an infection of the subcutaneous layers with bone involvements in most of the progressive stages. However, eumycetoma has been reported as disseminating infection at late stages of presentation for a limited number of cases (Zijlstra, van de Sande, Welsh, et al., 2016). Eumycetoma is differentiated from the other subcutaneous fungal infections by the presence of granulation and massive tissue sclerotization with the lesion fistulae discharging purulent and seropurulent fluid. Eumycetoma is a disfiguring, disabling disease, and in some cases accompanied by social stigma, which is aggravated as eumycetoma is common with poor people living in remote areas (Abbas et al., 2018; Emmanuel et al., 2018; Zijlstra, van de Sande and Fahal, 2016; van de Sande, 2013).

The pressing priorities for tackling mycetoma:

Epidemiology of eumycetoma:

The geographical distribution of Eumycetoma was described earlier to extend between the latitudes 15° S and 30° N, representing what is known as “the Mycetoma belt”. The mycetoma-belt includes India, Yemen, Somalia, Sudan, Senegal, Mexico, Venezuela, Colombia, and Argentina (Relhan et al., 2017). The systematic review conducted by van de Sande showed the world map with eumycetoma endemic countries highlighted, one map reflected both the number of mycetoma cases and the prevalence in ratio to the country population. However, this map included both actinomycetoma and eumycetoma statistics (shown below). Countries of high incidence of mycetoma are Sudan, Venezuela, Mexico, and India, Sudan and Mexico are the countries of high number of cases reported. The map showing mycetoma prevalence (Figure 1 A) points at Mexico, Mauritania, Senegal, Sudan, and Somalia as the top endemic countries with prevalence rates of 1.1-1:100,000 (van de Sande, 2013).

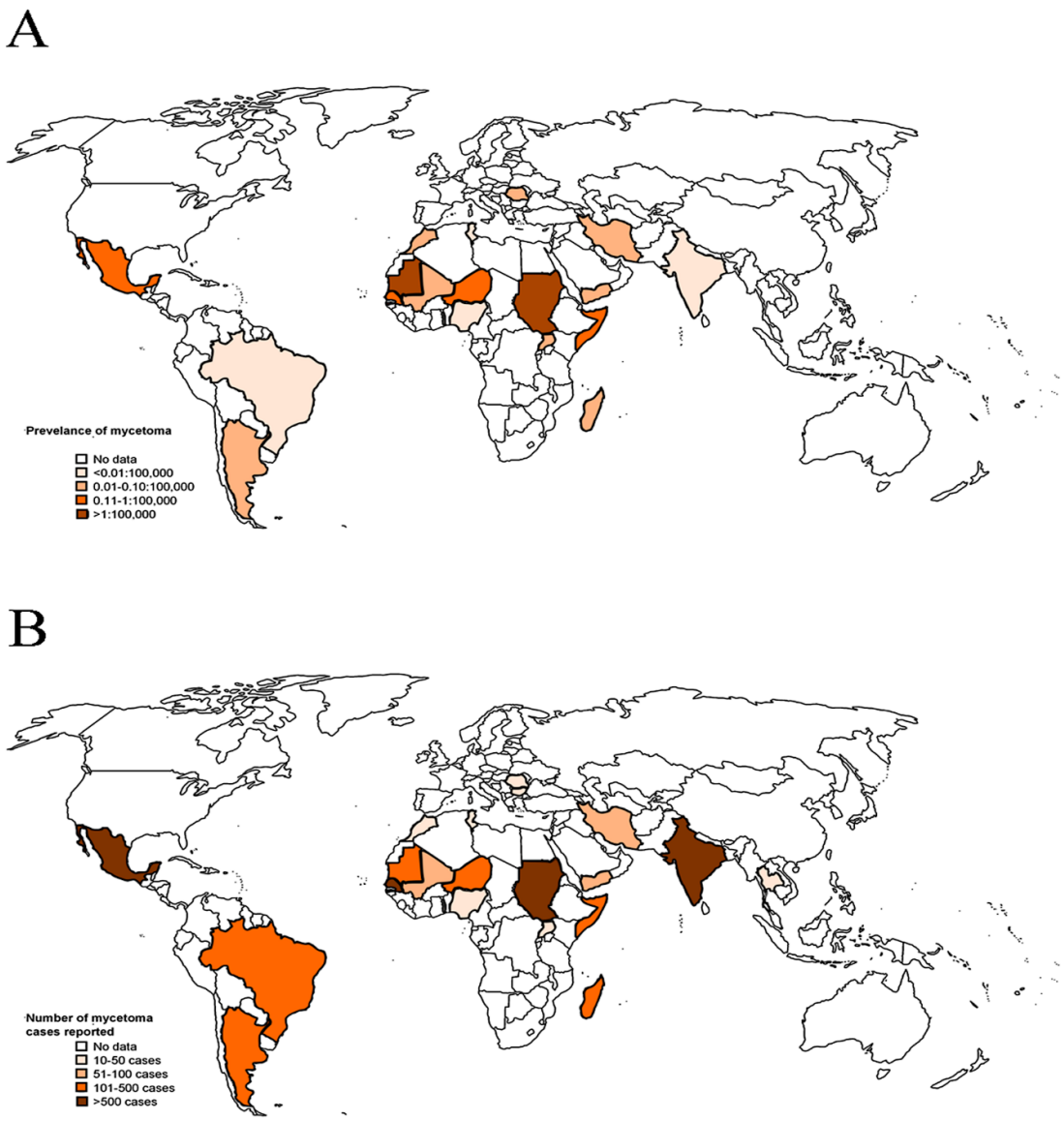

Figure 4: Global burden of mycetoma map

Source: *(van de Sande, 2013)*

Most of the literature about the topic eumycetoma included mentions of the etiologic agents, in particular the review articles and case series. However, the most conclusive lists were provided by Taha H and van de Sande in their review articles mentioning 22 different fungal species as pathogenic agents of eumycetoma. In chapter 2 we shall discuss a systematic

review we conducted, which revealed 143 different species identified as eumycetoma pathogens (Mohamed et al., 2015a; van de Sande, 2013).

Diagnosis of eumycetoma:

The presentation of eumycetoma (or generally mycetoma) varies according to the time lag between the first symptoms and the time to seek healthcare, it ranges from minor lesions to massive lesions as shown in Figure 2 (Fahal et al., 2018)

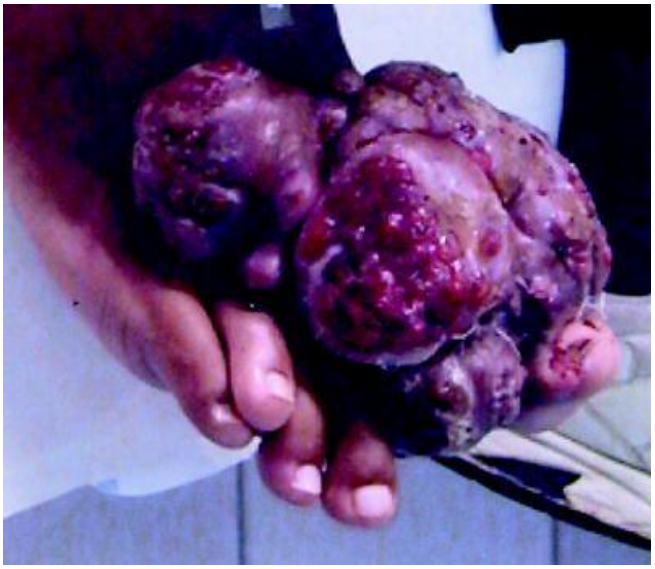

Figure 5: Eumycetoma caused by Aspergillus species presenting at progressive stage

Source: ***(Bustamante and Campos, 2011)***

In contrast to the late presentation as shown in Figure 2, cases with early stage eumycetoma are also clinically encountered (Figure 3). The stage of presentation determines the prognosis and the extent of surgical interventions to be planned.

Eumycetoma caused by *M. mycetomatis* was described as a slow-progressive disease. The progression period was reported to be a year (Maheshwari et al., 2010) or could be longer; ranging from 3 months to 9 years (Maiti et al., 2002)

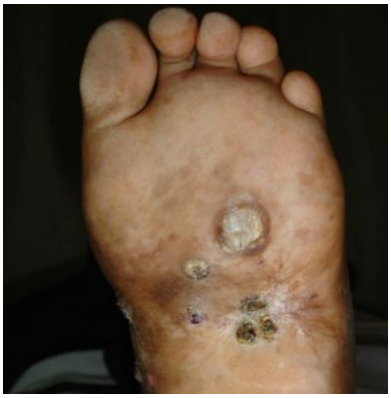

Figure 6: Eumycetoma caused by *Madurella fahalii*, showing small lesions

Source from (de Hoog et al., 2012)

The methods used in the identification of eumycetoma pathogens:

Many techniques have been described aiming at identifying the etiological agents of eumycetoma. These techniques range from simple conventional techniques like culture and phenotypic identification to the most advanced molecular and genomic techniques. Eumycetoma can be successfully diagnosed with Fine Needle Aspiration Cytology and imprint smears which are simple and affordable cytological techniques (Afroz et al., 2010). However, is worth mentioning that the diagnostic techniques described are of varying merits and pitfalls when mycetoma is considered. Furthermore, the diversity of the pathogenic fungi complicates the diagnostic techniques using biological markers and the genomic techniques as well (van de Sande, Fahal, et al., 2014).

Parts of the human body afflicted by eumycetoma:

It is very clear that the body extremities are more susceptible to mycetoma infection. The legs and the hands only represent 75.6% of the reported sites. The deduction has always been made from this perception that mycetoma was attributed to traumatic inoculation in most of the reported cases (Bonifaz et al., 2014; Padhi et al., 2010; Fahal, 2004). Nevertheless, researchers developed predictive models for mycetoma incidence probability by developing geospatial correlation models mapping mycetoma cases versus the spatial

distribution of acacia plant (thorny plant) in Sudan and singling out a potential mycetoma-acacia correlation (Samy et al., 2014). However, mycetoma was extensively reported in deep body parts including the lungs, brain, spinal cord, liver, and oral cavity, which question the aforementioned route of transmission (Beeram et al., 2008; Gumaa et al., 1986).

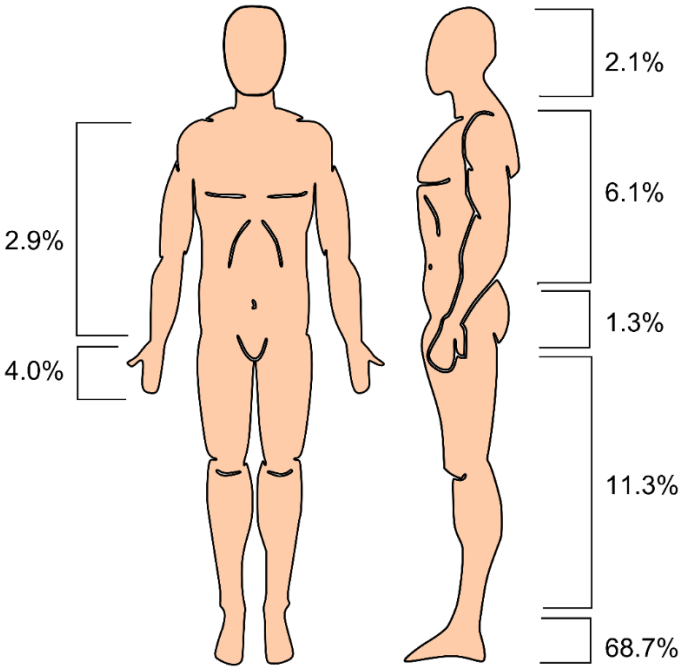

Figure 7: Lesion sites of eumycetoma

Source: *from (van de Sande, 2013)*

The site of infection is crucial at identifying the possible route of transmission. As eumycetoma afflicts the extremities, it was sound to consider the traumatic innoculation of infected soil or plant material to the subcutaneous layers and hence the establishment of eumycetoma. Nevertheless, it is also thoughtful to consider other modes of transmission including air, water, and inhalation as potential routes of transmission. It is also noteworthy there are reports on eumycetoma agents belonging to the normal flora for important hymenoptera insects (besides being virulant entomopathogens in another hand) (Humber, 2000). More investigation is needed to assess the hymenoptera and other insects as potential

vectors that could do the traumatic inoculation via stings and deliver the eumycetoma fungi along with their venomes and fluids.

The multiplicity of eumycetoma pathogens and mutual colonizers:

It is reported in many cases that more than one pathogen is responsible for causing eumycetoma (Pilsczek and Augenbraun, 2007) or that other opportunistic fungi or bacteria contaminating the mycetoma wound

Medical treatment of eumycetoma:

The medical treatment of mycetoma (Table 1) and hence the expected prognosis depends on the right diagnosis at the first place. Eumycetoma and actinomycetoma are presenting with similar clinical pictures which sometimes leads to misdiagnosis. The right procedure before starting medical treatment is to make sure the mycetoma has been appropriately diagnosed; then comes the medical treatment which involves drug treatment and surgical intervention if needed (Welsh et al., 2014a). Early presenting eumycetoma is usually not invasive and treated easily without the need of massive surgical procedure. However, a considerable percentage of eumycetoma patients present for medical care in late stages and therefore require surgical intervention. The more the damage to the tissues and bones, the poorer is the prognosis, and the greater is the likelihood of surgical amputation of the afflicted parts (Suleiman et al., 2016). In general, the current treatment of eumycetoma is not satisfactory and the patients suffer at numerous aspects. The medicines currently used produce a spectrum of harmful adverse effects involving liver injury, hormonal imbalances, and cardiovascular damage (Research, n.d.). Moreover, imidazole antifungal agents which are key component of the current guidelines, have been labelled with a Blackbox warning by both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (Szefler et al., 2006).

In case the eumycetoma patients were correctly diagnosed and offered the best doses of antifungal agents with close monitoring, another uncertainty arises, the drug resistance which is extensively reported and considered the leading challenge with eumycetoma (Welsh et al., 1995; Venugopal and Venugopal, 1993).

It has an equivocal is the main eumycetoma pathogen *Madurella mycetomatis* is having controversial susceptibility to antifungal drugs; it was found susceptible to ravuconazole (Ahmed et al., 2014) and to flucytosine (Elfadil et al., 2015). However, it showed resistance to the echinocandin class of antifungal agents (Sande et al., 2010). These facts prioritize the pursual of susceptibility testing as routine in all clinical settings to shorten the drug refractoriness period for the good of all the eumycetoma patients.

Table 1: Antifungal drugs against eumycetoma

Antifungal	**Class**	**Year**	**Target**	***in-vitro activity***	**human infection**
Amphotericin B	Polyenes	1959	Ergosterol	Moderate	Not effective
Fluconazole	Azole	1990	Lanosterol demethylase (CYP51A1)	Limited	Not effective
Ketoconazole	Azole	1981	CYP51A1	High	Variable efficacy
Itraconazole	Azole	1992	CYP51A1	High	Variable efficacy
Voriconazole	Azole	2002	CYP51A1	High	Effective in a few cases
Posaconazole	Azole	2006	CYP51A1	High	Effective in a few cases
Isavuconazole	Azole	2016	CYP51A1	High	No data
Caspofungin	Echinocandins	2001	Glucan synthase	Low	No data
Terbinafine	Allylamine	1992	Squalene epoxidase	Moderate	No data
Fosravuconazole*	Azole	2017-2018	CYP51A1	Phase II clinical trial	

Adverse effects of fungicide treatment:

Most of the drugs available for eumycetoma patients are contraindicated in case of pregnancy and for children. Furthermore, they showed drastic side effects and are linked to many therapeutic warnings and precautions (Table 2)

Table 2: Therapeutic warnings and precautions of eumycetoma antifungal drugs

Class	Warnings and therapeutic precautions
Azoles Itraconazole Ketoconazole Posaconazole Isavuconazole Fluconazole	• Can cause or exacerbate congestive heart failure (CHF) • When itraconazole IV was administered to healthy human volunteers and dogs, negative inotropic effects were seen • Do not use for the treatment of onychomycosis in patients with ventricular dysfunction (eg, history of CHF) • If signs or symptoms of CHF occur during administration, reassess benefit and risk of continuing treatment
Polyenes Amphotericin-B	• Hypersensitivity • Indicated for patients with progressive and potentially fatal fungal infections • Risk of nephrotoxicity, however, do not withhold if risk of infection outweighs renal risk • Risk of acute infusion reactions, especially with first dose • Caution when coadministration with other drugs that cause hypokalemia (eg, corticosteroids, digoxin) • Cases of new-onset dilated cardiomyopathy with subsequent heart failure have been reported; symptoms normalized within 6 months of discontinuation
Echinocandins Caspofungin	• Anaphylaxis reported, discontinue and administer appropriate treatment; possible histamine-mediated adverse reactions, including rash, facial swelling, angioedema, pruritus, sensation of warmth or bronchospasm • Hepatic effects: Abnormalities in LFTs and isolated cases of clinically significant hepatic dysfunction, hepatitis, or hepatic failure • Cases of Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN
Allylamine Terbinafine	Hypersensitivity

Source: Medscape database (www.medscape.com)

The role of the fungal melanin in drug resistance:

Melanin is associated with the ability of the fungi to penetrate the host tissues at the first inoculation besides being a contributor in hyphal spreading and the establishment of the infection (Langfelder et al., 2003; Bell and Wheeler, 1986). In M. mycetomatis the in vitro inhibition of melanin biosynthesis through tricyclazole, a known melanin biosynthesis inhibitor, increased the efficacy of the imidazole antifungals itraconazole and ketoconazole

by 16- and 32- fold, respectively (van de Sande et al., 2007a). As melanogenic proteins are partially conserved in the mycetoma agents as revealed by our work (see chapter 2), we expect a potential targeting synergy between MBI and fungicides. As no further virulence genes were previously identified to have contribution in eumycetoma invasion, we have also identified candidate virulence genes and elucidated relevant literature (See chapter 4).

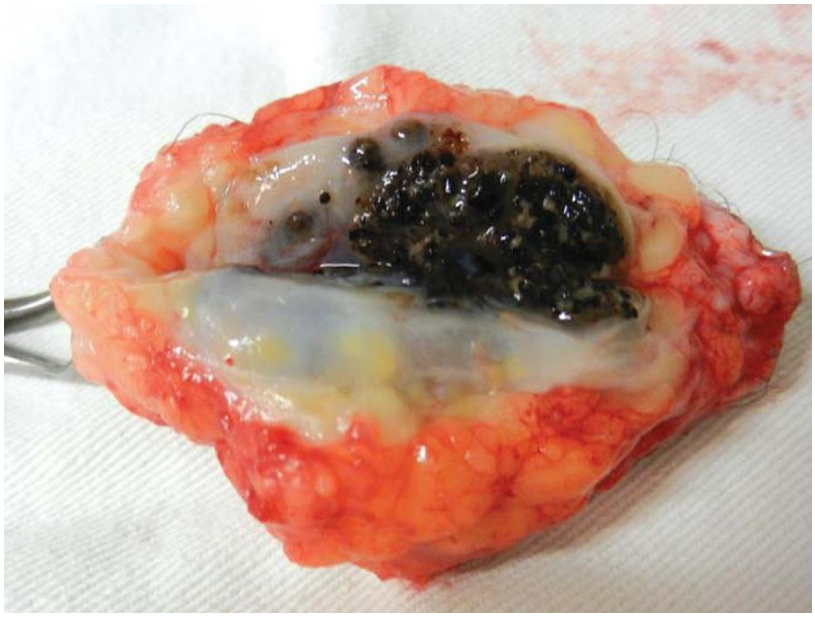

Figure 8: M. mycetomatis granulation after tissue excision

Source: *from (Sampaio et al., 2013)*

Drug discovery for eumycetoma, the pipeline:

The development of antifungal drugs is more challenging than to antibacterial drug development, this because fungal pathogens are closely related to the host. For example, the success of S. cerevisiae as a model eukaryotic organism is because many fundamental biochemical and cell biological processes are conserved from fungi to humans. Consequently, many small molecules that are toxic to yeast are also toxic to humans. As such, it is therefore not surprising that the three major classes of antifungal drugs target structures that are unique to fungi. In addition to the scientific challenges that hinder the identification of new lead compounds, the evaluation of new antifungal agents also presents

a number of challenges with respect to clinical trial design that further complicates development (Ahmed et al., 2003).

Currently, the Drugs for Neglected Diseases Initiative (DNDi) in Geneva has in partnership with the Mycetoma Research Center (MRC), Khartoum, Sudan, launched a phase II, single-center, comparative, randomized, double-blind, parallel-group, active-controlled trial to assess the clinical superiority of Fosravuconazole versus Itraconazole combined with surgery in subjects with eumycetoma in Sudan (https://clinicaltrials.gov/ct2/show/NCT03086226, n.d.).

In February 2018, the University of Sydney, Erasmus MC, and DNDi launched the open source "MycetOS" project which uses the "Open Pharma" approach aiming at discovering new molecules that could bring new treatment options for eumycetoma patients (https://www.dndi.org/2019/media-centre/news-views-stories/news/mycetoma_rnd_status_2019/, 2019). Recently fenarimols were discribed as novel drug candidates for eumycetoma after screening both the "Pathogen" and "Stasis" boxes via an open research model (Lim et al., 2018a).

Thus, the recognition of eumycetoma by the WHO as a neglected tropical disease has promoted promising research activities that aim to improve the treatment of patients. To get eumycetoma under control, however, the large knowledge gaps concerning the biology and transmission first need to be closed.

1. Chapter -2-

Etiologic agents of eumycetoma; a systematic review and geographic circumscription mapping

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Data analysis: MH

Interpretation of results: MH, MT

Paper writing: MH, MT, PM

Note: To be submitted in the current format

Abstract:

Eumycetoma is a Neglected Tropical Disease (NTD) recently endorsed by the World Health Organization in 2016. The etiological agents of eumycetoma are fungi of varying taxonomical classification and habitats. The current burden of eumycetoma is found in the tropics and subtropics and has been widely associated with poverty. In this systematic review we attempted to define the currently known fungal pathogens attributed to eumycetoma, and to map their geographical distribution in order to have a conclusive understanding of the geographical circumscription of eumycetoma. We found that 130 species of fungi had been identified as eumycetoma pathogens either from case reports, case series, review articles, or expert communications, plus13 species partially identified with only their genus mentioned. From a taxonomic perspective, eumycetoma is caused mainly by *Ascomycetes*, as this fungal phylum constitutes 94% of the identified species. The methods used in pathogen identification range from conventional culturing and phenotypic identification to DNA-based technologies including PCR, whole genome sequencing and other molecular techniques. The ecological habitats of the identified species included general saprophytic habitats as well as specific habitats, such as animal dung in case of *Madurella* species or industrial waste and urban playgrounds in case of *Scedosporium apiospermum*. Eumycetoma was always referred to as tropical idease barely extending to the subtropics, this would stand true for some species including *S. apiospermum* and *Leptospheria sengalensis* but certainly not for all the fungal species as both *M. mycetomatis* and *M. grisea* shows a worldwide pattern of distribution beyond the predefined circumscription of the tropics.

Keywords: eumycetoma, systematic review, etiologic agent, geographical distribution

Author summary:

Mycetoma was globally neglected by the health organizations for a century and more, since its first description until the recent recognition as a Neglected Tropical Disease (NTD) by the World Health Organization (WHO). The current information about the epidemiology of eumycetoma remains limited, and this knowledge gap is hindering the control of eumycetoma. In this work we have systematically reviewed the literature, aiming to define a complete list of fungal species that were identified as eumycetoma pathogens. This list will not only help to elucidate the epidemiology of eumycetoma, it will also help to understand eumycetoma in terms of burden of disease and risk of exposure. The diversity of identified species allowed to gather information about the natural habitat of each species, which is a crucial to understand the sources of infection and the various exposures in different environments. We have identified 130 pathogenic species that were properly described, in addition to 13 partially identified pathogens. These pathogens had varying habitat and spatial distribution, from narrow geographical circumscription to almost global distribution, as was the case for *Madurella mycetomatis*, the most important eumycetoma pathogen in terms of report per country/territory and in terms of existing literature. We believe this work will help to tailor eumycetoma predictions per country/territory besides providing more conclusive information about the diversity of eumycetoma pathogens, to be considered by clinicians and scientists. Knowing the pathogens will support the choice of treatment options and the prognosis of the disease.

Background:

Mycetoma is a subcutaneous infection with two distinct etiologies: actinomycetoma is caused by higher bacteria and eumycetoma is caused by fungi. The treatment of actinomycetoma with antibiotics has been less problematic than the chemotherapy of eumycetoma, which is jeopardized by drug resistant pathogens, adverse effects of the currently used antifungal medicines, and the high rate of relapses (Reis and Reis-Filho, 2018; Abbas et al., 2018; Welsh et al., 2014a). Fortunately, mycetoma has finally made it to the list of Neglected Tropical Diseases (NTDs), after the 138th Executive Board of the World Health Organization (WHO) recommended the mycetoma resolution to the 69th World Health Assembly for further recognition (WHO, n.d.).

The geographical distribution of eumycetoma was described to extend between the latitudes 15° S and 30° N, representing what is known as "the Mycetoma belt". The mycetoma-belt includes India, Yemen, Somalia, Sudan, Senegal, Mexico, Venezuela, Colombia, and Argentina (Relhan et al., 2017). However, to address the mycetoma knowledge-gaps, experts around the world highlighted as a research priority to establish the incidence and prevalence in order to determine areas/countries where mycetoma is endemic, and to improve the diagnostic tools as well (van de Sande et al., 2018; van de Sande, Maghoub, et al., 2014).

There have been three systematic reviews about mycetoma burden and etiology, which have listed numerous fungal species as eumycetoma pathogens (Al-Hatmi et al., 2017; Bitan et al., 2017; van de Sande, 2013). The development of a complete and up-to-date list of pathogens will help the ongoing clinical studies, the epidemiological work, and certainly the drug discovery efforts, since without a complete list of pathogens neither the control nor the eventual elimination of eumycetoma will be possible. In addition, we summarize the numerous techniques to identify the etiologic agents of eumycetoma.

Methodology:

The study protocol:

This is the first study that is conducted to list all of the fungal species that are reported or known to be causative agents of eumycetoma.) The study was conducted following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement (Moher, 2009). The study was conducted in four steps to develop a database: study design, search strategy, finding relevant original articles, and applying inclusion and exclusion criteria to arrive into the final list of articles to be screened.

Search strategy and database:

The systematic review was conducted via searching the international databases Web of Science (WoS), NCBI PubMed/MEDLINE, and Google scholar. The languages included were English, Arabic, German, French, and Spanish. The search was conducted using a combination of words and Boolean operators (AND & OR), medical subject heading (MeSH) and topic subject (TS) for Google Scholar, PubMed/MEDLINE, and WoS respectively (The word combinations are discussed in detail in the results section). The search was only limited by the current time we conducted this systematic review and included all previous work; the literature was last searched in June 2019.

Inclusion and exclusion criteria:

1.1.1.1. Inclusion criteria:

Case reports and case series were the main target for this systematic review. However, due to the considerable number of review articles and few systematic reviews published, these were also included. The only criteria were the mention of underlying fungal etiological agents that caused eumycetoma.

1.1.1.2. Exclusion criteria:

Articles that were focused on clinical aspects, including surgical procedures, diagnostic imaging, or medical treatment with no mention of etiology were excluded. Articles in the area of veterinary medicine were also excluded. Articles addressing actinomycetoma only were also excluded from the analysis.

1.1.1.3. Selection of the studies:

The final list of captured original articles was exported to EndNote ™ version X9 (Clarivate Analytics Company). The articles were categorized based on the inclusion/exclusion criteria into relevant (included), irrelevant (article topic not eligible), or missing full text when the full text was not retrievable from the databases and other sources; this was the case for publications from 1900 and earlier.

1.1.1.4. Data items and synthesis:

Data captured from the screened articles were presented as tables and data matrix for further clustering and mapping of the components.

1.1.1.5. Data presentation and analysis:

Mapping eumycetoma reporting countries: Mapping was conducted using QGIS version 3.8 (Zanzibar) under the GNU General Public License (http://www.gnu.org/licenses) using data from each location from which eumycetoma etiologic agents had been reported. The analysis involved the generation of Kernel Density (Rosenblatt, 1956) estimated heatmaps for the spatial distribution of locations reporting eumycetoma. The analysis was also carried out using RStudio Version 1.2.1335 (RStudio Team, 2015a) along with Microsoft Excel for development of charts and data management.

Results:

The articles included in this systematic review were published in the period between January 1842 and June 2019, when we conducted the last literature search. The articles were either case reports, case series, or reviews. Searching the databases WoS and PubMed produced 813 hits meeting the defined search strategy. From these records 70 duplicates were removed prior to eligibility screening. Upon eligibility screening, 340 articles were excluded, out of which 168 articles were addressing actinomycetoma and 172 articles were addressing clinical aspects, the diagnostic imaging, the animal models of eumycetoma, or eumycetoma medical treatment with no causative species specified. Furthermore, in some of the excluded studies there were no species identified due to non-sporulating negative cultures despite the authors' attempts of identification (Estrada et al., 2012).

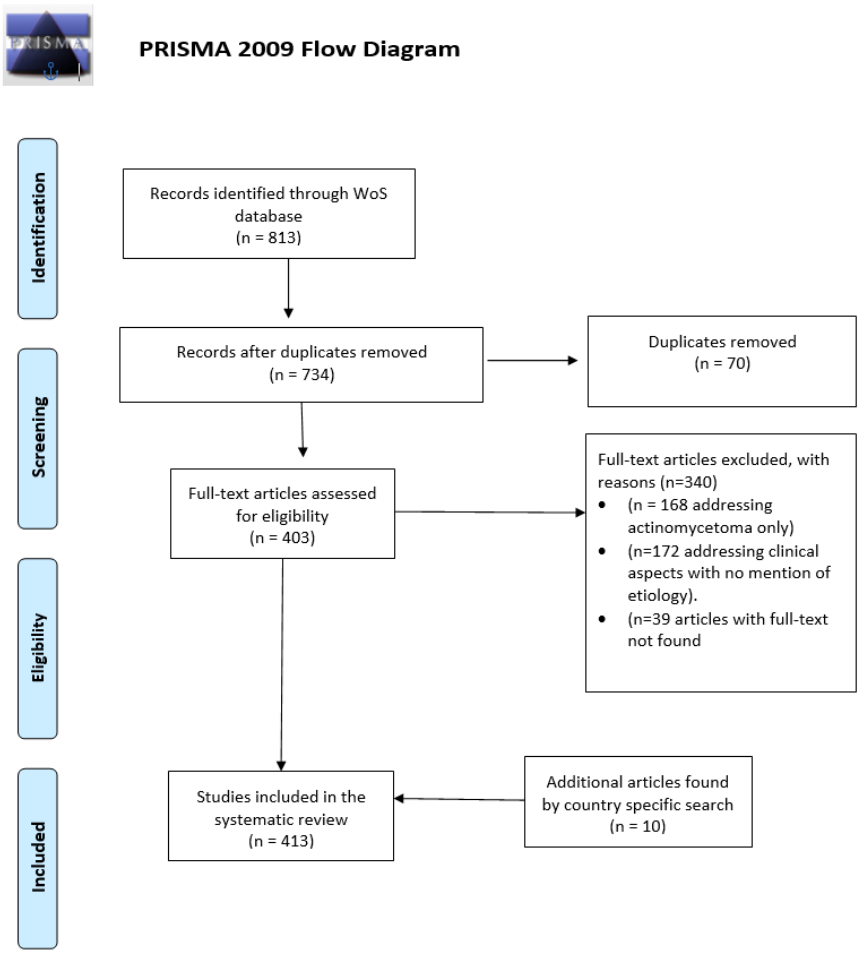

Figure 1: PRISMA flow-chart showing the search steps, eligibility, and the final list of included articles

The most frequently cited technique for identification of the fungal pathogen was culturing followed by phenotypic identification. However, many additional techniques were used including cellular, molecular, and genomic analyses. These techniques are of varying sensitivity, with both merits and pitfalls (van de Sande, Fahal, et al., 2014).

Screening the final list of included articles revealed 130 fungal species reported as eumycetoma pathogens that were fully identified (genus plus epithet) after culture and isolation (Table 1). Furthermore, 13 eumycetoma pathogens had been isolated from eumycetoma patients but their mycological identification was incomplete with only the genus identified (Table 2). The taxonomy

revealed *Ascomycota* as the main fungal phylum to which the majority of eumycetoma causative fungi are classified (94% of the identified species), while the *Basidiomycota* and *Mucormycota* were represented at only 4% and 2%, respectively.

Table 1: Eumycetoma causative pathogens reported worldwide

Species name	Synonym	Author/Year	MycoBank ID	Fungal phylum
Acremoniella lutzi	*None*	*Leão & Lôbo/1939*	292104	*Ascomycota*
Acremoniella rugulosa	*None*	*Leão/1934*	*277097*	*Ascomycota*
Acremonium falciforme	*Cephalosporium falciforme/ Neocosmospora falciformis*	*(Carrion) W. Gams/1971*	*308145*	*Ascomycota*
Acremonium kiliense	*Sarocladium kiliense/Cephalosporium kiliense*	*Grütz/1925*	*252874*	*Ascomycota*
Acremonium potronii	*None*	*Vuillemin/1910*	*248620*	*Ascomycota*
Acremonium recifei	*Xenoacremonium recifei/ Hyalopus recifei*	*(Leão & Lôbo) W. Gams/1971*	*308188*	*Ascomycota*
Acremonium strictum	*Sarocladium strictum*	*W. Gams/1971*	*308201*	*Ascomycota*
Alternaria alternata	*Alternaria tenuis*	*(Fries) Keissler/1912*	*119834*	*Ascomycota*
Arthrographis kalrae	*Oidiodendron kalrai/kalrai*	*(R.P. Tewari & Macpherson) Sigler & J.W. Carmichael/1976*	*309023*	*Ascomycota*
Aspergillus albus	*Aspergillus candidus*	*K. Wilhelm/1913*	*214853*	*Ascomycota*
Aspergillus bouffardii	*Madurella bouffardii*	*Brumpt/1906*	*481743*	*Ascomycota*
Aspergillus flavus	*Monilia flava*	*Link/1809*	*209842*	*Ascomycota*
Aspergillus fumigatus	*Aspergillus cellulosae*	*Fresenius/1863*	*211776*	*Ascomycota*
Aspergillus hollandicus	*Aspergillus vitis*	*Samson & W. Gams/1985*	*114708*	*Ascomycota*
Aspergillus nidulans	*Sterigmatocystis nidulans/Diplostephanus nidulans/Emericella nidulans/Emericella dentata*	*(Eidam) G. Winter/1884*	*182069*	*Ascomycota*
Aspergillus niger	*Sterigmatocystis nigra/Aspergillopsis nigra*	*Tieghem/1867*	*284309*	*Ascomycota*
Aspergillus oryzae	*Eurotium oryzae*	*(Ahlburg) Cohn/1884*	*184394*	*Ascomycota*
Aspergillus terreus	*Aspergillus terrestris*	*Thom/1918*	*191719*	*Ascomycota*
Aspergillus ustus	*Sterigmatocystis ustus/Aspergillus minutus*	*(Bainier) Thom & Church/1926*	*281216*	*Ascomycota*
Aspergillus versicolor	*Sterigmatocystis versicolor*	*(Vuillemin) Tiraboschi/1908*	*172159*	*Ascomycota*
Bipolaris spicifera	*Brachycladium spiciferum/Curvularia spicifera/Drechslera spicifera*	*(Bainier) Subramanian/1971*	*309557*	*Ascomycota*
Blastomyces dermatitidis	*Oidium dermatitidis/Cryptococcus gilchristi/Geotrichum dermatitidis*	*Gilchrist & W.R. Stokes/1898*	*361754*	*Ascomycota*
Candida albicans	*Oidium albicans*	*(C.P. Robin) Berkhout/1923*	*256187*	*Ascomycota*
Candida Guilliermondii	*Blastodendrion*	*(Castellani) Langeron & Guerra/1938*	*252488*	*Ascomycota*

Candida parapsilosis	*Monilia parapsilosis*	*(Ashford) Langeron & Talice/1932*	*253819*	*Ascomycota*
Candida piceae	*None*	*Kurtzman/2000*	*464353*	*Ascomycota*
Candida rugosa	*Mycoderma rugosum*	*(H.W. Anderson) Diddens & Lodder/1942*	*284780*	*Ascomycota*
Candida tropicalis	*Oidium tropicale/Endomyces tropicalis*	*(Castellani) Berkhout/1923*	*280770*	*Ascomycota*
Cephalosporium madurae	*Sarocladium kiliense*	*A.A. Padhye, Sukapure & Thirum./1962*	*327585*	*Ascomycota*
Cephalosporium serrae	*Gibellulopsis serrae/Verticillium serrae*	*Maffei/1929*	*277974*	*Ascomycota*
Chaetomium atrobrunneum	*Amesia atrobrunnea*	*L.M. Ames/1949*	*294685*	*Ascomycota*
Cladophialophora bantiana	*Torula bantiana/Xylohypha bantiana*	*(Sacc.) de Hoog, Kwon-Chung & McGinnis/1995*	*412791*	*Ascomycota*
Cladophialophora mycetomatis	*None*	*Badali, de Hoog & Bonifaz/2008*	*511843*	*Ascomycota*
Coccidioides immitis	*Mycoderma immite*	*C.W. Stiles/1896*	*416228*	*Ascomycota*
Coccidioides posadasii	*None*	*M.C. Fisher, G.L. Koenig, T.J. White & J.W. Taylor/2002*	*484628*	*Ascomycota*
Cochliobolus spicifer	*Pseudocochliobolus spicifer*	*R.R. Nelson/1964*	*328547*	*Ascomycota*
Corynespora cassiicola	*Helminthosporium cassiaecola*	*(Berkeley & M.A. Curtis) C.T. Wie/1950*	*296024*	*Ascomycota*
Cryptococcus neoformans	*Saccharomyces neoformans/Torulopsis neoformans*	*(San Felice) Vuillemin/1901*	*119294*	*Basidiomycota*
Curvularia geniculata	*Helminthosporium geniculatum*	*(Tracy & Earle) Boedijn/1923*	*265873*	*Ascomycota*
Curvularia lunata	*Acrothecium lunatum/Helminthosporium curvulum*	*(Wakker) Boedijn/1933*	*269889*	*Ascomycota*
Curvularia pallescens	*None*	*Boedijn/1933*	*273299*	*Ascomycota*
Cylindrocarpon cyanescens	*Phialophora cyanescens/Neocosmospora cyanescens*	*(G.A. de Vries, de Hoog & Bruyn) Sigler/1991*	*499349*	*Ascomycota*
Cylindrocarpon destructans	*Ilyonectria destructans/Ramularia destructans*	*(Zinssmeister) Scholten*	*329491*	*Ascomycota*
Cyphellophora oxyspora	*Phialophora oxyspora*	*(W. Gams) Réblová & Unter/2013*	*803685*	*Ascomycota*
Delitschia didyma	*Delitschia auerswaldii*	*Auerswald/1966*	*177056*	*Ascomycota*
Diaporthe phaseolorum	*Sphaeria phaseolorum*	*(Cooke & Ellis) Saccardo/1882*	*164797*	*Ascomycota*
Drechslera rostrata	*Exserohilum rostratum*	*(Drechsler) M.J. Richardson & E.M. Fraser/1968*	*330213*	*Ascomycota*
Emarellia grisea	*None*	*Borman AM, Desnos-Ollivier M, Campbell CK, Bridge PD,*	*815959*	*Ascomycota*

		Dannaoui E, and Johnson EM/2016		
Emarellia paragrisea	*None*	Borman AM, Desnos-Ollivier M, Campbell CK, Bridge PD, Dannaoui E, and Johnson EM/2017	*815960*	*Ascomycota*
Epidermophyton floccosum	*Acrothecium floccosum/Blastotrichum floccosum*	(Harz) Langeron & Milochevich/1930	*252200*	*Ascomycota*
Exophiala mansonii	*Exophiala castellanii/Microsporum mansonii*	(Castellani) de Hoog/1977	*314041*	*Ascomycota*
Exophiala spinifera	*Phialophora spinifera/Rhinocladiella spinifera*	(H.S. Nielsen & Conant) McGinnis/1977	*314044*	*Ascomycota*
Exophiala dermatitidis	*Phialophora dermatitidis*	(Kano) de Hoog/1977	*314039*	*Ascomycota*
Exophiala jeanselmei	*Torula jeanselmei/Phialophora jeanselmei/Exophiala castellanii*	(Langeron) McGinnis & A.A. Padhye/1977	*314040*	*Ascomycota*
Exophiala oligosperma	*None*	Calendron ex De Hoog & Tintelnot/2003		*Ascomycota*
Exserohilum rostratum	*Helminthosporium rostratum*	(Drechsler) K.J. Leonard & Suggs/1974	*314059*	*Ascomycota*
Fonsecaea pedrosoi	*Hormodendrum pedrosoi*	(Brumpt) Negroni/1936	*253857*	*Ascomycota*
Fusarium caeruleum	*Selenosporium caeruleum*	Libert ex Saccardo/1886	*522094*	*Ascomycota*
Fusarium Chlamydosporum	*Fusarium sporotrichioide*	Wollenweber & Reinking/1925	*260522*	*Ascomycota*
Fusarium falciforme	*Cephalosporium falciforme/Acremonium falciforme*	(Carrion) Summerbell & Schroers/2002	*483950*	*Ascomycota*
Fusarium fujikuroi	*None*	Nirenberg/1976	*314213*	*Ascomycota*
Fusarium keratoplasticum	*Neocosmospora keratoplastica*	D. Geiser, O'Donnell, Short & Ning Zhang/2013	*802390*	*Ascomycota*
Fusarium pseudensiforme	*None*	Samuels, Nalim & Geiser/2011	*519839*	*Ascomycota*
Fusarium sambucinum	*Fusarium roseum*	Fuckel/1963	*161187*	*Ascomycota*
Fusarium solani	*Neocosmospora solani*	(Martius) Saccardo/1881	*190352*	*Ascomycota*
Fusarium subglutinans	*Fusarium moniliforme*	(Wollenweber & Reinking) P.E. Nelson, Toussoun & Marasas/1983	*115356*	*Ascomycota*
Fusarium thapsinum	*None*	Klittich, J.F. Leslie, P.E. Nelson & Marasas/1997	*437690*	*Ascomycota*
Fusarium verticillioides	*Oospora verticillioides*	(Saccardo) Nirenberg/1976	*314223*	*Ascomycota*
Geotrichum candidum	*Botrytis geotricha*	Link/1809	*182997*	*Ascomycota*
Geotrichum capitatum	*Blastoschizomyces capitatus*	(Diddens & Lodder) Arx/1977	*314418*	*Ascomycota*

Glenospora khartoumense	*Trichosporum khartoumense/Madurella khartoumensis*	*Chalmers & R.G. Archibald/1916*	*481845*	*Basidiomycota*
Glenospora semoni	*Glenosporopsis semoni*	*Chalmers & R.G. Archibald/1917*	*475973*	*Basidiomycota*
Helminthosporium anomalum	*None*	*J.C. Gilman & E.V. Abbott/1927*	*250709*	*Ascomycota*
Histoplasma capsulatum	*Cryptococcus capsulatus*	*Darling/1906*	*102749*	*Ascomycota*
Indiella americana	*Madurella americana*	*Delamare & Gatti/1929*	*250640*	*Ascomycota*
Indiella mansoni	*Madurella mansonii*	*Brumpt/1906*	*431742*	*Ascomycota*
Indiella reynieri	*Madurella reynieri*	*Brumpt/1906*	*492966*	*Ascomycota*
Leptosphaeria thompkinsii	*Falciformispora tompkinsii*	*El-Ani/1966*	*492269*	*Ascomycota*
Leptosphaeria senegalensis	*Falciformispora senegalensis*	*Segretain, Baylet, Darasse & Camain/1959*	*299640*	*Ascomycota*
Madurella bouffardii	*Aspergillus bouffardii*	*(Brumpt) Vuillemin/1931*	*251047*	*Ascomycota*
Madurella brumptii	*Idriella brumptii*	*(Pirajá) Ciferri & Redaelli/1941*	*287883*	*Ascomycota*
Madurella Fahalii	*None*	*G.S. de Hoog, A.D. van Diepeningen, E-S. Mahgoub & W.W.J. van de Sande/2012*	*560128*	*Ascomycota*
Madurella grisea	*Trematosphaeria grisea*	*J.E. Mackinnon, Ferrada & Montemartini/1949*	*287885*	*Ascomycota*
Madurella lackawanna	*None*	*Hanan/1938*	*268785*	*Ascomycota*
Madurella mycetomatis	*Streptothrix mycetomatis/Madurella ikedae/Madurella americana/Madurella mycetomi*			*Ascomycota*
Madurella oswaldoi	*None*	*Horta/1919*	*492022*	*Ascomycota*
Madurella pseudomycetomatis	*None*	*Yan, Deng, Zhou, Zhong & Hao ex de Hoog, van Diepeningen, Mahgoub & van de Sande/2012*	*509682*	*Ascomycota*
Madurella ramiroi	*None*	*Pirajá/1918*	*493016*	*Ascomycota*
Madurella tropicana	*None*	*de Hoog, van Diepeningen, Maghoub & van de Sande/2012*	*800571*	*Ascomycota*
Microascus paisii	*Torula paisii /Scopulariopsis paisii*	*(Pollacci) M. Sandoval-Denis, J. Gené & J. Guarro/2016*	*809213*	*Ascomycota*
Microsporum audouinii	*Sporotrichum audouinii/Trichophyton microsporum/Microsporum langeronii*	*Gruby/1843*	*160505*	*Ascomycota*
Microsporum canis	*Sporotrichum audouinii/Trichophyton microsporum*	*(E. Bodin) E. Bodin/1902*	*160689*	*Ascomycota*
Microsporum ferrugineum	*orosporium bullatum*	*M. Ota/1921*	*361756*	*Ascomycota*

Moesziomyces bullatus	*Pseudozyma aphidis/Moesziomyces rugulosus*	*(J. Schröter) Vánky/1977*	*317784*	*Basidiomycota*
Neoscytalidium dimidiatum	*Scytalidium dimidiatum*	*(Penzig) Crous & Slippers/2006*	*500869*	*Ascomycota*
Neotestudina rosatii	*Pseudophaeotrichum sudanense*	*Segretain & Destombes/1961*	*335143*	*Ascomycota*
Nigrograna mackinnonii	*Pyrenochaeta mackinnonii*	*(Borelli) Gruyter, Verkley & Crous/2012*	*564795*	*Ascomycota*
Paecilomyces lilacinus	*Purpureocillium lilacinum/Penicillium lilacinum*	*(Thom) Samson/1974*	*319114*	*Ascomycota*
Parathyridaria percutanea	*Roussoella percutanea*	*(S.A. Ahmed, D.A. Stevens, W. van de Sande & S. de Hoog) Jaklitsch & Voglmayr/2016*	*817777*	*Ascomycota*
Penicillium mycetogenum	*Penicillium mycetomagenum*	*G.P. Martelli & C.L. Negri/1915*	*171166*	*Ascomycota*
Petriellidium boydii	*Allescheria boydii/Pseudallescheria/Pseudallescheria shearii*	*(Shear) Malloch/1970*	*319444*	*Ascomycota*
Phaeoacremonium fuscum	*None*	*L. Mostert, Damm & Crous/2008*	*505140*	*Ascomycota*
Phaeoacremonium inflatipes	*None*	*W. Gams, Crous & M.J. Wingfield/1996*	*415957*	*Ascomycota*
Phaeoacremonium krajdenii	*None*	*L. Mostert, R.C. Summerbell & P.W. Crous/2005*	*334052*	*Ascomycota*
Phaeoacremonium parasiticum	*Phialophora parasitica*	*(Ajello, Georg & C.J.K. Wang) W. Gams, P.W. Crous & M.J. Wingfield/1966*	*415651*	*Ascomycota*
Phaeoacremonium sphinctrophorum	*None*	*L. Mostert, Summerbell & P.W. Crous/2006*	*500231*	*Ascomycota*
Phialemoniopsis curvata	*Phialemonium curvatum*	*(W. Gams & W.B. Cooke) Perdomo, Dania García, Gené, Cano & Guarro/2013*	*563876*	*Ascomycota*
Phialemonium obovatum	*None*	*W. Gams & McGinnis/1983*	*108343*	*Ascomycota*
Phialophora richardsiae	*Pleurostomophora richardsiae/Cadophora richardsiae/Phialophora calyciformis*	*(Nannfeldt) Conant/1937*	*276522*	*Ascomycota*
Phialophora verrucosa	*None*	*Medlar/1915*	*214996*	*Ascomycota*
Pleurostomophora ochracea	*Pleurostoma ochraceum*	*N. Mhmoud, S. Ahmed, A. Fahal,S. de Hoog, van de Sande/2012*	*800514*	*Ascomycota*
Pseudochaetosphaeronema larense	*Chaetosphaeronema larense*	*(Borelli & R. Zamora) Punithalingam/1979*	*321757*	*Ascomycota*

Pyrenochaeta unguis-hominis	*Neocucurbitaria unguis-hominis*	*Punithalingam & M.P. English/1975*	*322137*	*Ascomycota*
Pyrenochaeta mackinnonii	*Nigrograna mackinnonii/Biatriospora mackinnonii*	*Borelli/1976*	*322132*	*Ascomycota*
Pyronchaeta remoroi	Medicopsis romeroi	*Borelli/1959*	*338075*	*Ascomycota*
Rhinocladiella atrovirens	*Melanchlenus eumetabolus*	*Nannfeldt/1934*	*257799*	*Ascomycota*
Rhinocladiella mackenziei	*Ramichloridium mackenziei*	*(C.K. Campbell & Al-Hedaithy) Arzanlou & Crous/2007*	*504554*	*Ascomycota*
Rhizopus microsporus var. rhizopodiformis	*Mucor rhizopodiformis/Rhizopus rhizopodiformis/Rhizopus pusillus*	*(Cohn) Schipper & Stalpers/1984*	*116953*	*Mucoromyceta*
Rhytidhysteron rufulum	*Hysterium rufulum/Rhytidhysterium rufulum*	*(Sprengel) Spegazzini/1921*	*121714*	*Ascomycota*
Roussoella percutanea	*Parathyridaria percutanea*	*S.A. Ahmed, D.A. Stevens, W. van de Sande & S. de Hoog/2014*	*804857*	*Ascomycota*
Scedosporium apiospermum	*Glenospora clapieri/Monosporium apiospermum/ Monosporium sclerotiale/Polycytella hominis*	*(Saccardo) Saccardo ex Castellani & Chalmers/1919*	*432048*	*Ascomycota*
Scedosporium boydii	*Cephalosporium boydii/Hyalopus boydii*	*(Shear) Gilgado, Gené, Cano & Guarro/2008*	*538387*	*Ascomycota*
Sporothrix schenckii	*Sporotrichum tropicale/Dolichoascus schenckii*	*Hektoen & C.F. Perkins/1900*	*101184*	*Ascomycota*
Trichophyton mentagrophytes	*Trichophyton sarkisovii/Trichophyton granulosum*	*(C.P. Robin) R. Blanchard/1896*	*185902*	*Ascomycota*
Trichophyton rubrum	*Trichophyton kanei/Epidermophyton rubrum*	*(Castellani) Sabouraud/1911*	*254498*	*Ascomycota*
Trichophyton schoenleinii	*Sporotrichum schoenleinii/Achorion schoenleinii/Oidium schoenleinii*	*(Lebert) Langeron & Milochevich/1934*	*254610*	*Ascomycota*
Trichophyton terrestre	*Trichophyton terrestre-primum*	*Durie & D. Frey/1957*	*307101*	*Ascomycota*
Trichophyton violaceum	*Trichophyton yaoundei/Achorion violaceum*	*Sabouraud/1902*	*355796*	*Ascomycota*
Trichosporon cutaneum	*Trichosporon aneurinolyticum/Monilia cutanea/Geotrichum cutaneum*	*(Beurmann, Gougerot & Vaucher bis) M. Ota/1926*	*122298*	*Basidiomycota*
Xenoacremonium recifei	*Cephalosporium recifei*	*(Leão & Lôbo) L. Lombard & P.W. Crous/2015*	*810272*	*Ascomycota*

Table 2: Species which were partially identified

Genus name	Author/Year	Mycobank ID	Fungal phylum
Aureobasidium	Viala & G. Boyer/1891	7297	*Ascomycota*
Cladosporium	Link/1816	7681	*Ascomycota*
Cunninghamella	Matruchot/1903	20150	*Mucoromyceta*
Glenosporella	Nannizzi/1931	8336	*Ascomycota*
Glomus	Tulasne & C. Tulasne/1845	20244	*Mucoromyceta*
Hormonema	Lagerberg & Melin/1927	8562	*Ascomycota*
Phoma	Saccardo/1880	9358	*Ascomycota*
Phyllosticta	Persoon/1818	9384	*Ascomycota*
Roussoella	Saccardo/1888	4799	*Ascomycota*
Scopulariopsis	Bainier/1907	9854	*Ascomycota*
Septobasidium	Patouillard/1892	16312	*Basidiomycota*
Sterigmatocystis	C.E. Cramer/1815	10091	*Ascomycota*
Syncephalastrum	J. Schröter/1886	20556	*Mucoromyceta*

The species identified (Table 1) were in some cases identified with the countries from which the eumycetoma case was reported; however, 47 species were mentioned in review articles without denoting the respective country of origin. The top reported eumycetoma pathogens (Figure 2) were *M. mycetomatis* (reported in 53% of the countries), *S. apiospermum* (27%), *M. grisea* (26%), *P. boydii* (21%), *A. falciforme* (13%), *F. solani* (13%), *P. remoroi* (12%), *E. jeanselmei* (11%), and *L. senegalensis* (10%).. The complete list of eumycetoma pathogens with their corresponding country or territory is provided as supplementary data (Table S1).

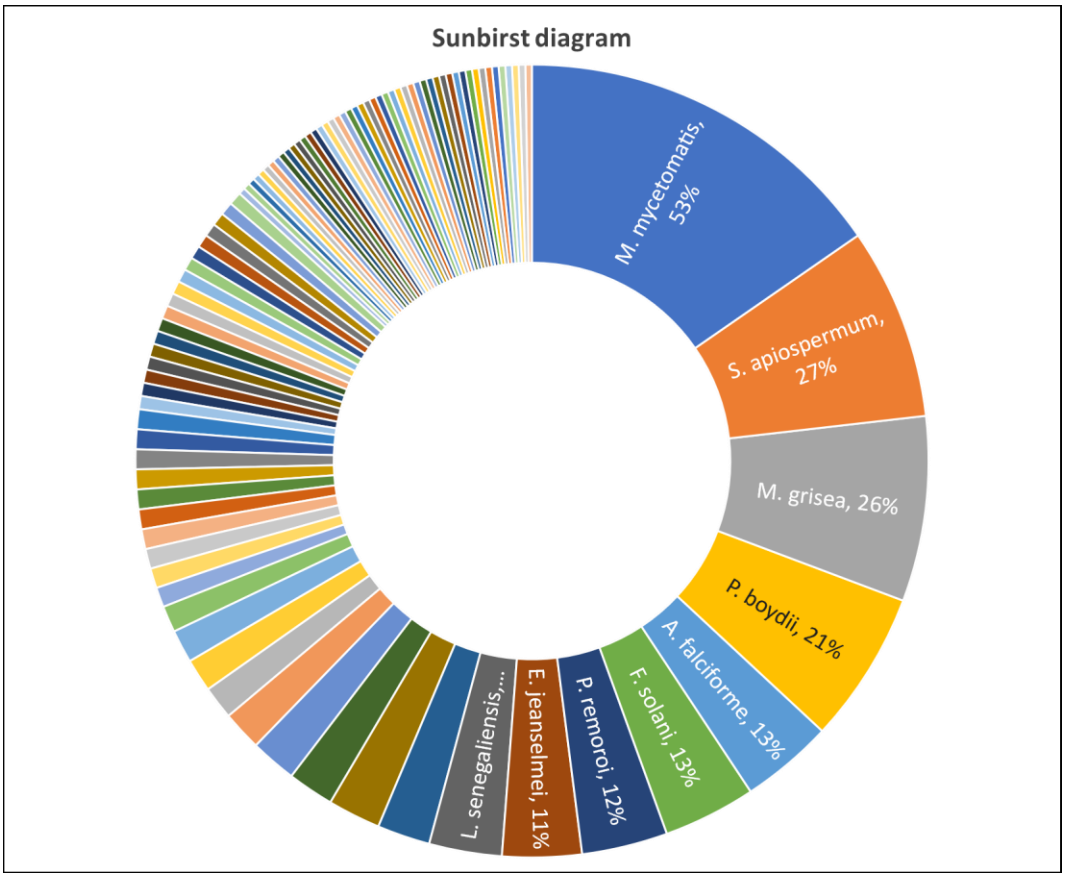

Figure 2: Top reported eumycetoma pathogens world-wide.

Eumycetoma was also reported as a zoonotic disease afflicting a wide range of animals including dogs, goats, horses, and mares (Elad et al., 2010, 2014; Gumaa et al., 1978; Seibold, 1955). Furthermore, some of the pathogens are also well known to be phytopathogens. Phytopathogens linked to eumycetoma include *Aspergillus* and *Fusarium* species (Dean, Van Kan, et al., 2012). Many species have been identified as entomopathogens, and some were also explored as potential biocontrol agents for insects that impact crop production or serve as disease vectors (Zhang et al., 2018; Humber, 2000; Teetor-Barsch and Roberts, 1983; Hasan and Vago, 1972).

The most reported eumycetoma pathogen worldwide, *M. mycetomatis*, belongs to the order *Sordariales* of the phylum *Ascomycota*. Limited data are available about the natural habitat of *M.*

mycetomatis. However, a phylogenetic study suggested possible nesting within *Chaetomiaceae* based on comparison to neighboring taxa (ref). *Chaetomiaceae* are inhabitants of indoor environments, enriched soil, and animal dung (de Hoog et al., 2013). Furthermore, a study suggested a correlation in susceptibility to eumycetoma and schistosomiasis, which might denote that eumycetoma pathogens also present in aquatic environments (van Hellemond et al., 2013). *M. mycetomatis* was found to be reported from 53% of all countries/territories that reported eumycetoma. Furthermore, we found that the countries/territories reporting *M. mycetomatis* tended to report more other fungal species (nearly double) compared to countries/territories not reporting *M. mycetomatis*; the mean number of eumycetoma species was ~5 and ~2 in the two clusters, respectively (See Figure 3). This finding might suggest that many eumycetoma pathogens either share the same ecological habitat as *M. mycetomatis* or have a similar route of infection. We also suggest that *M. mycetomatis* might prefer an environment that is inhabitable for many other eumycetoma fungal pathogens as well, specifically in the tropics and subtropics as shown in the maps. *A. falciforme*, which belongs to the order Sordariales and family *Chaetomiaceae*, which might suggest similarity to *M. mycetomatis* in ecology and natural habitat. However, *A. falciforme*is a plant parasite with more selectivity to grass (Glenn et al., 1996).

S. apiospermum and *P. boydii* belong to the order *Microascaceae*, which is characterized by brown ascospores. Their ecological abundance was found to correlate with the concentration of nitrogen and decreasing pH (ref). They were most abundant in industrial areas, urban playgrounds, and agricultural fields. Their ecology is greatly affected by human activity and recent reports suggested changing patterns in the spectrum of clinical diseases they cause in humans (Luplertlop et al., 2019; Kaltseis et al., 2009; Guarro et al., 2006).

F. solani belongs to the order *Sordariales* of the phylum *Ascomycetes*, which have a worldwide distribution in soil and are important plant pathogens. Approximately 20% of fusariosis infections are caused by *F. solani* (Kosmidis and Denning, 2017). *Pyronchaeta remoroi* or *Medicopsis remoroi*, which belong to the order *Pleosporales* of the phylum *Ascomycota*, along with the *Leptospheria* species *L. senegalensis* and *L. thompkinsii*, are saprophytes which live in soil or associated to plants. The species *E. jeanselmei* and *E. dermatitidis* (*Chaetothyriales* of the phylum *Ascomycota*) grow in a wide range of temperatures and pH and tolerate high concentrations of NaCl. Their environmental recovery rate is high from man-made habitats, in particular when polluted with aromatic hydrocarbons. Furthermore, they are recovered from glacial meltwater, mineral water, and indoor environments (Babič et al., 2018).

The species *A. fumigatus* and *A. flavus* (*Eurotiales* of the phylum *Ascomycota*) are also important eumycetoma pathogens.The availability of dead leaves alongside moist soil represents the ideal habitat for *A. fumigatus* during winter, resulting in a seasonality of airborne spores (Anaissie et al., 2009).

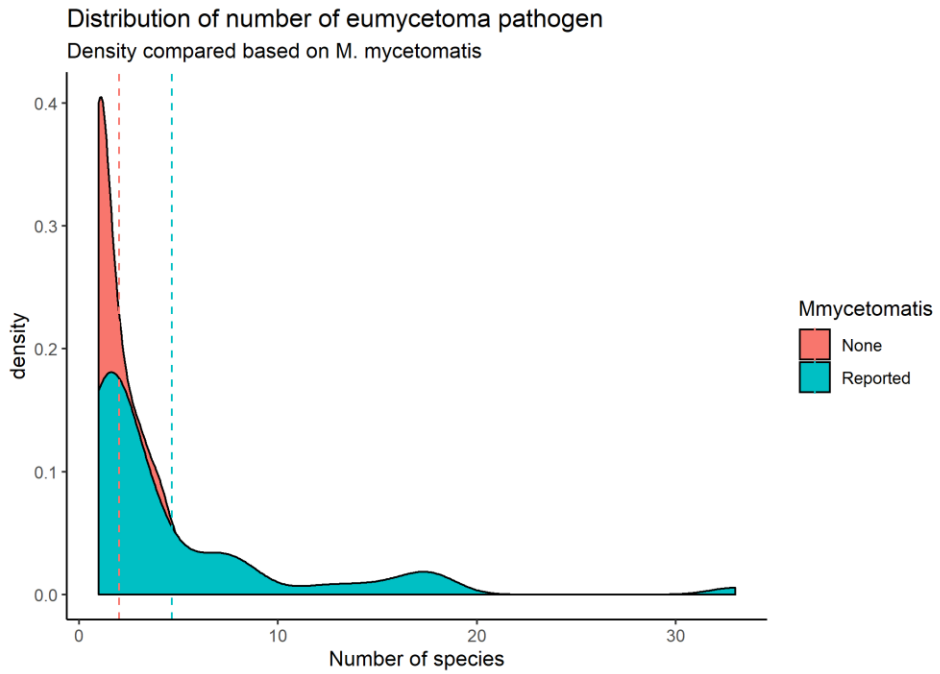

Figure 3: Distribution density of the number of eumycetoma species identified per location compared based on whether M. mycetomatis was reported in the given location or not.

Worldwide patterns of eumycetoma geographical distribution:

The distribution of eumycetoma has been circumscribed to countries between 30°N and 15°S, the so-called mycetoma-belt of the tropics and subtropics (Mohamed et al., 2015a). In this work, we assessed the locations from which eumycetoma was reported aiming at a more conclusive understanding of the geographic distribution of eumycetoma. To pursue this geographic visualization, we have appointed one geographic location to represent either a country or territory from which eumycetoma cases were reported. This approach allowed insights into the spatial patterns of important eumycetoma pathogens and enabled us to draw conclusions about the distribution of species and the distribution density inferred via Kernel density estimation.

We found that in terms of species diversity, eumycetoma has indeed the tropical and subtropical pattern of distribution described by the mycetoma-belt. The countries India (33 different species), Iran (16 species), Sudan (18 species), Senegal (18 species), Brazil (14 species), Venezuela (12 species), and Mexico (17 species) had reported the highest numbers of different eumycetoma fungal pathogens. However, when looking at the overall picture and including countries that had reported only one or two eumycetoma causative species, a worldwide pattern emerged except for the far global North (i.e. Canada, Russia, and Scandinavian countries; Figure 4).

Using Kernel density-based spatial interpolation of the eumycetoma locations, we found discrepancies when the species were mapped individually. *M. mycetomatis* showed a distribution that was close to the overall distribution for all species, and the highest predicted kernel density estimates matched the hypothesized Mycetoma-belt (Figure 5). In contrast, *M. grisea* showed scattered distribution with spots of high density located far to the North or to the South in Europe or Africa/South America, respectively (Figure 6).

Species with narrow spatial circumscription included *L. senegalensis*, which had been reported from the tropics and subtropics of Africa, Asia, and South America but never from the global North. (Figure 7). In contrast, *A. fumigatus* and *S. apiospermum* showed a distribution to the global North with highest densities in Europe following a narrow circumscription pattern (Figures 8 and 9).

Comparing the top 10 geographically reported eumycetoma pathogens worldwide (the species from Figure 3), we plotted the maximum kernel density estimated per species and observed that both *S. apiospermum* and *M. mycetomatis* had estimates above the mean of the maximum kernel density. This might indicate a geographical niche. *S. apiospermum* had its niche located in the global North (Southern Europe), and for *M. mycetomatis* the niche was located within the equator

region of central Africa (see Figure 10). *S. apiospermum* has ecological characteristics that help explain its narrow geographical circumscription, as it spread in the environment via ascospores and is abundant in man-manipulated environment as discussed above (Kaltseis et al., 2009).

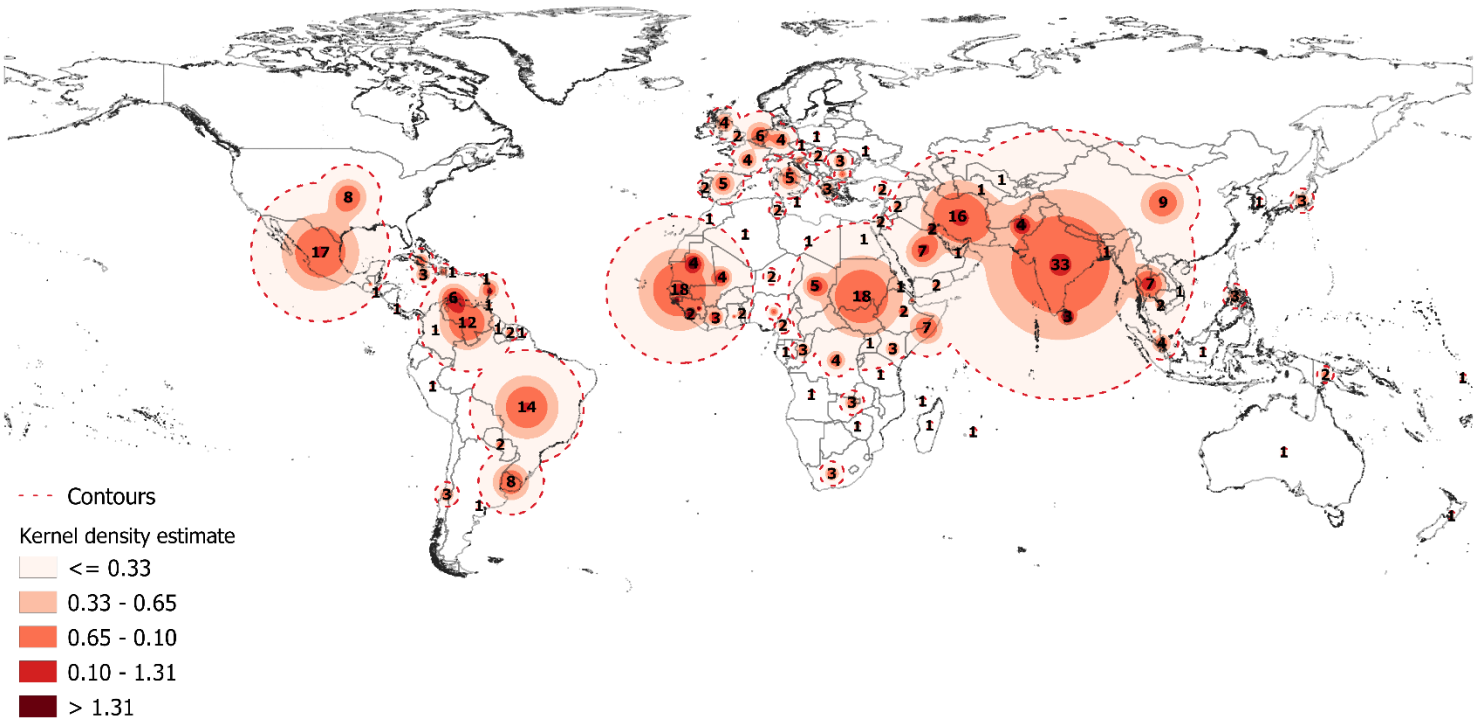

Figure 4: Worldwide distribution of eumycetoma reports per country/territory with the corresponding number of species. The raster layer in red palette range shows the estimated density calculated using Kernel density (Quartic algorithm) weighted by the number of species. The highest density aligned to the defined mycetoma-belt across Africa, South America, Asia continents. Low density is also observed in Europe and Oceania continents.

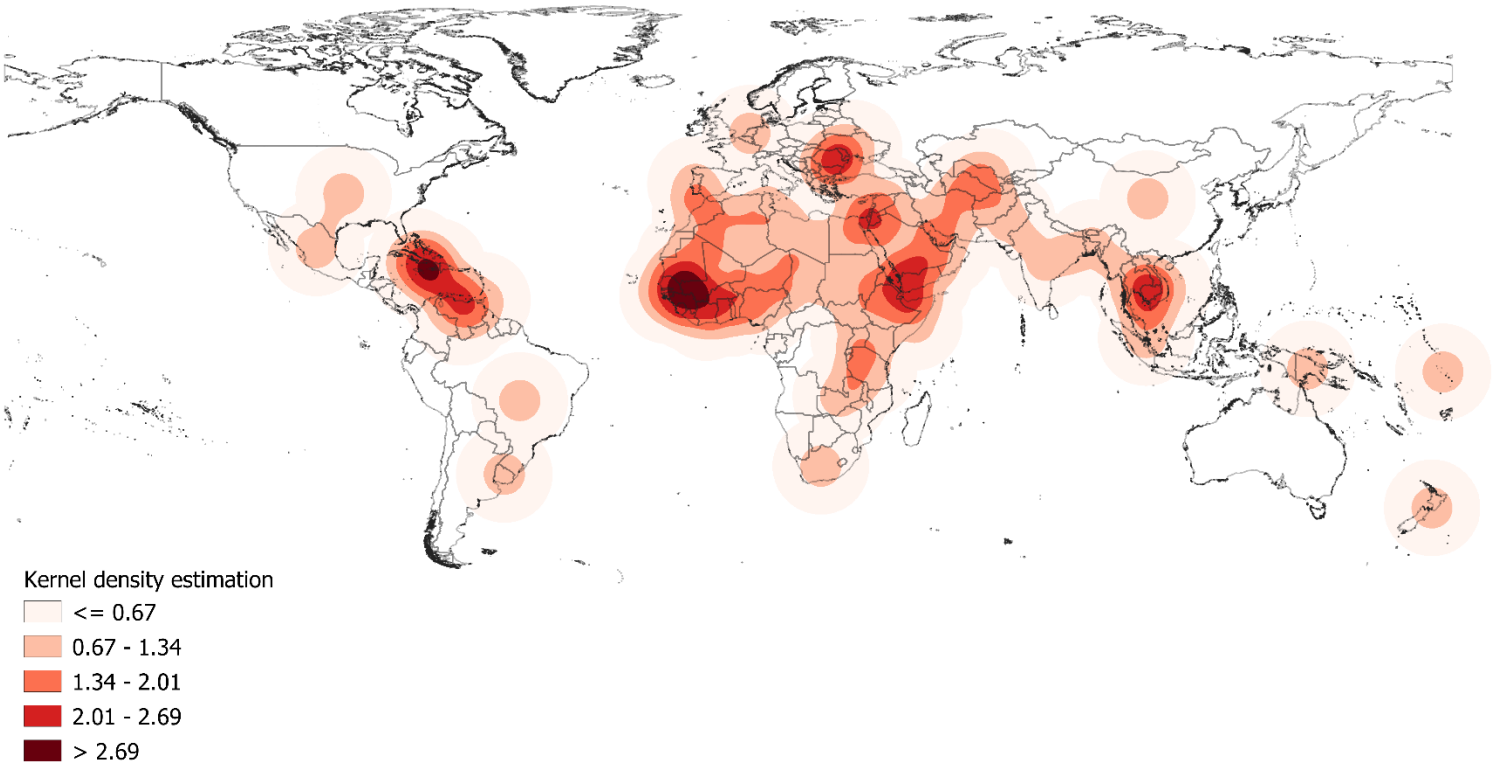

Figure 5: Predicted spatial distribution of *M. mycetomatis* eumycetoma, inferred by kernel density using the reporting countries/territories as mapped locations.

M. mycetomatis showed the broadest geographical contribution compared to the other species.

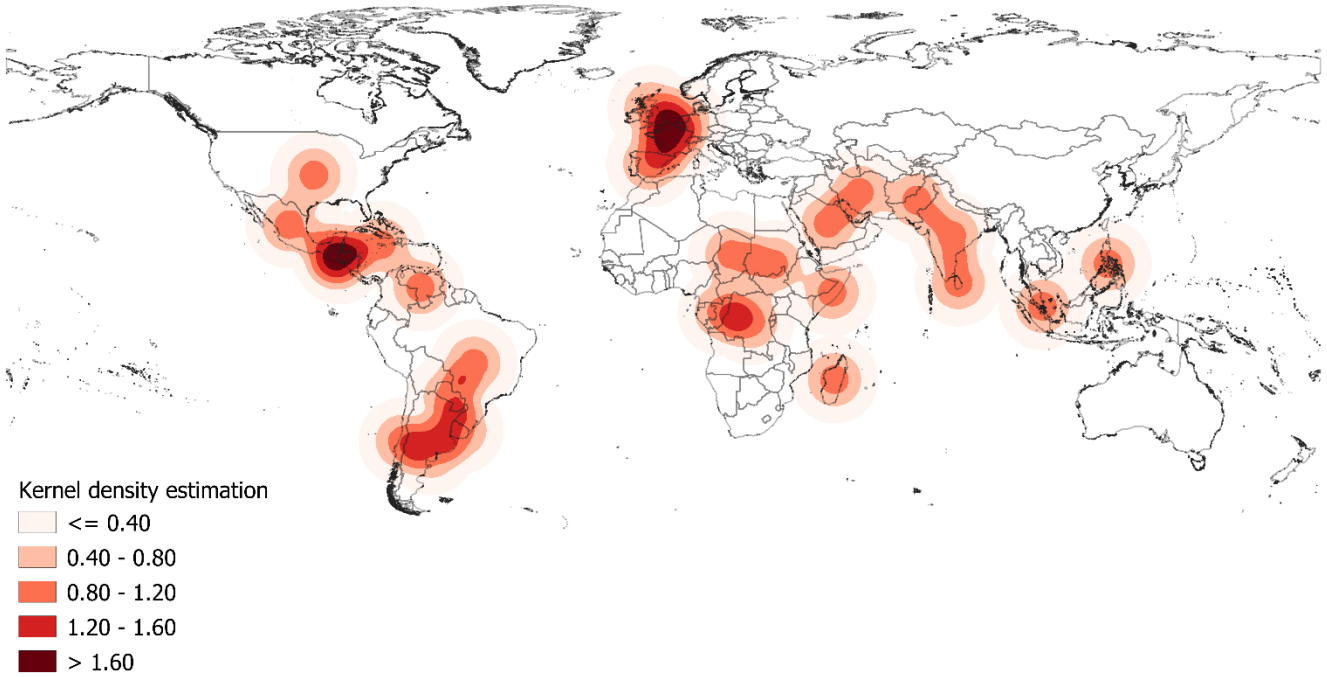

Figure 6: Predicted spatial distribution of *M. grisea* eumycetoma inferred by kernel density using the reporting countries/territories as mapped locations

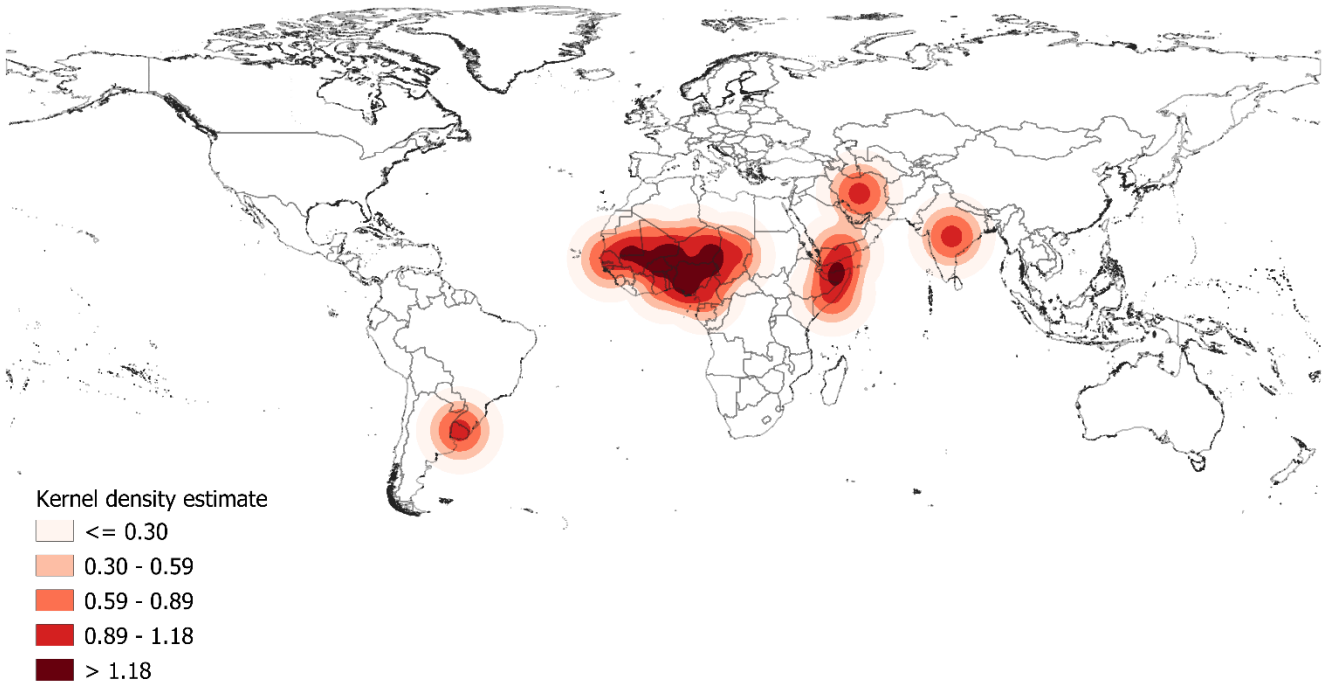

Figure 7: Predicted spatial distribution of *L. senegalensis* eumycetoma inferred by kernel density using the reporting countries/territories as mapped locations

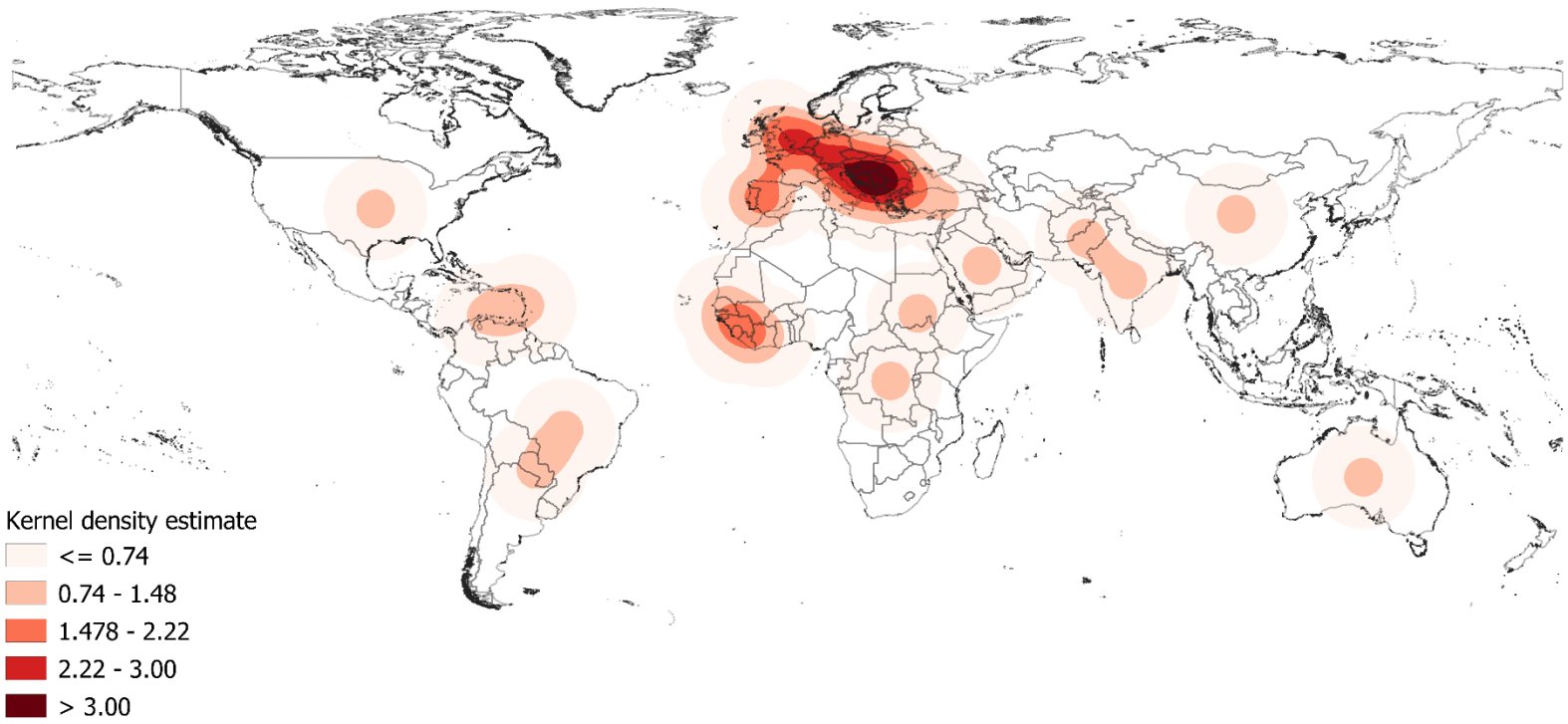

Figure 8: Predicted spatial distribution of *S. apiospermum* eumycetoma inferred by kernel density using the reporting countries/territories as mapped locations

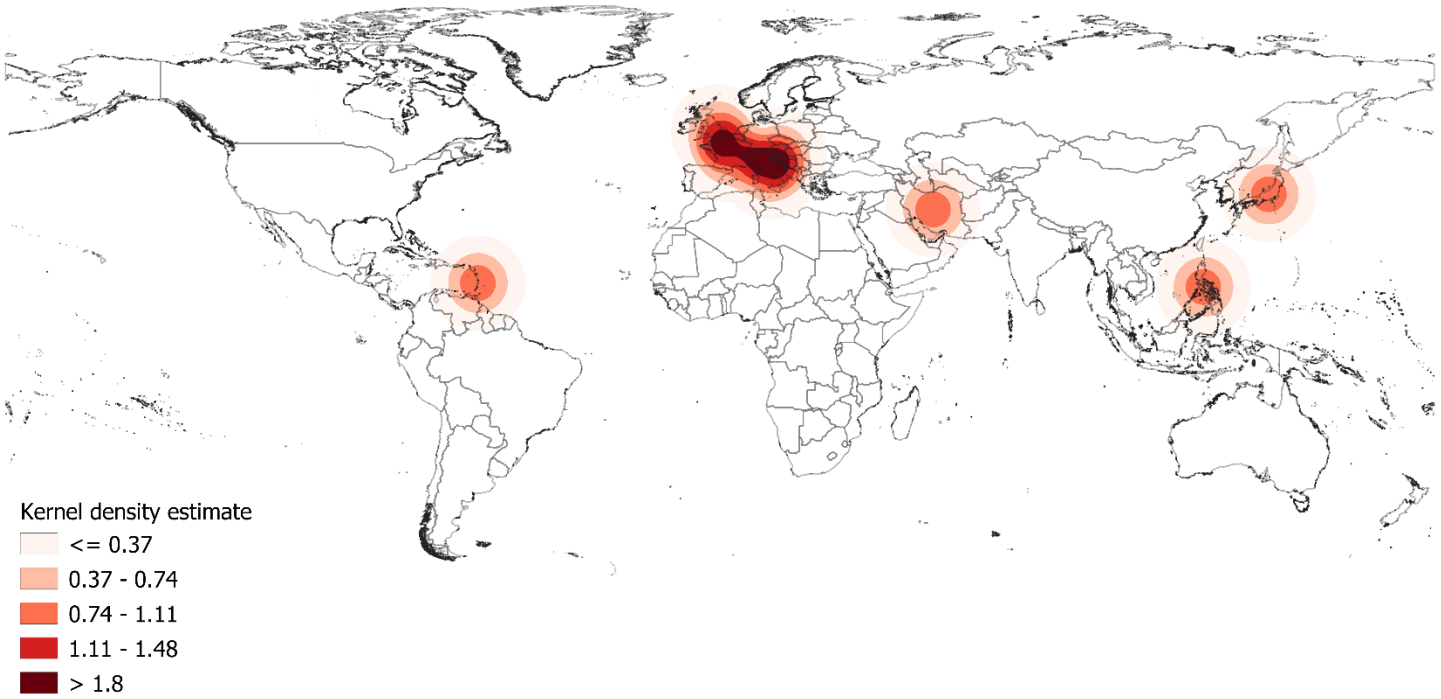

Figure 9: Predicted spatial distribution of *A. fumigatus* eumycetoma inferred by kernel density using the reporting countries/territories as mapped locations

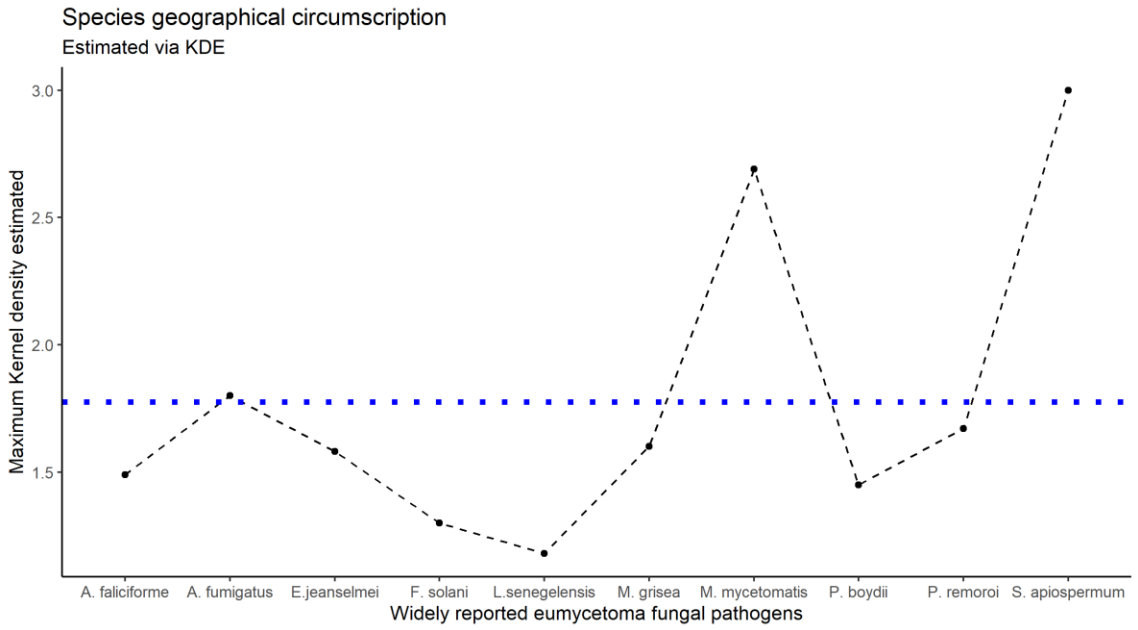

Figure 10: Species circumscription inferred by max kernel density estimated for the eumycetoma pathogens top reported as identified in this work. The species of max kernel density above average are S. apiospermum, M. mycetomatis, and A. fumigatus. M. mycetomatis possess duality of worldwide estimated spatial distribution while maintaining a specific high-density region which could possibly be considered as niches.

In conclusion, eumycetoma appears to be a worldwide disease considering the existing literature. In fact, when eumycetoma is treated as zoonosis the spatial distribution would become even broader, extending to nearly all countries. Host heterogeneity of eumycetoma pathogens, which includes vertebrates, insects and plants, raises a clear ecological threat to the control of eumycetoma, as the transmission dynamics are not predictable. Pathogen heterogeneity is further complicating the situation. Eumycetoma pathogens showed considerable diversity in terms of their taxonomical classification, habitat, and geographic distribution patterns of the reported cases. Some eumycetoma species, including *M. mycetomatis*, *M. grisea*, and *S. apiospermum*, showed considerable geographical distribution; however, these species also tended to have country-

specific clusters of high density representing a region of high endemicity. *L. senegalensis* showed narrow circumscription and exhibited the highest clustering pattern on tropics and subtropics with clear intensity near the equator. Biological characteristics of individual species help to explain their tendency to cause eumycetoma in specific parts of the body, e.g. when considering the sporulation of *S. apiospermum* and the attributed cases of lung eumycetoma. The epidemiologists and the fellow clinicians tackling eumycetoma should be aware of the species diversity we have hereby brought to conclusion.

Limitations:

Accessibility of literature published in country-specific journals was of difficulty more especially for publications from Africa and Asia. The mapping approach tends to be rather broad than specific and only makes sense when overlooked from a worldwide perspective.

Acknowledgements:

I would like to thank Dr. Daniela Rodríguez for her input in the mapping component of this work. Also, I would like to thank Dr. Thomas Früst and Mr. Geovanni Casagrande for their help with data management and the search of international databases.

Funding:

This work was funded by the Swiss government through the Swiss Excellence Scholarship for Foreign Scholars (ESKAS), many thanks for their support and special care for mycetoma patients and the contribution to many aspects from clinical to bench work in Sudan and in Swiss institutions.

Supplementary information:

Supplementary information:

Table S1: The eumycetoma pathogens and the countries from which they were reported

Eumycetoma pathogen	Countries reporting	Percent
M. mycetomatis	57	53%
S. apiospermum	29	27%
M. grisea	28	26%
P. boydii	23	21%
A. falciforme	14	13%
F. solani	14	13%
P. remoroi	13	12%
E. jeanselmei	12	11%
L. senegaliensis	11	10%
A. fumigatus	8	7%
L. thompkinsii	8	7%
M. canis	7	7%
N. rosatii	7	7%
C. albicans	6	6%
A. nidulans	5	5%
M. pseudomycetomatis	5	5%
P. mackinnonii	5	5%
C. lunata	4	4%
A. flavus	3	3%
A. kiliense	3	3%
A. recifei	3	3%
C. bantiana	3	3%
F. pedrosoi	3	3%
F. subglutinans	3	3%
M. Fahalii	3	3%
P. lilacinus	3	3%
R. rufulum	3	3%
T. rubrum	3	3%
A. alternata	2	2%
A. kalrae	2	2%
C. cyanescens	2	2%
C. destructans	2	2%
C. immitis	2	2%
C. serrae	2	2%
C. tropicalis	2	2%
E. grisea	2	2%
F. verticillioides	2	2%
G. candidum	2	2%
M. lackawanna	2	2%
M. audouinii	2	2%
P. parasiticum	2	2%
P. richardsiae	2	2%
P. verrucosa	2	2%
Roussoella	2	2%
S. schenckii	2	2%
T. metagrophytes	2	2%
A. lutzi	1	1%

A. niger	1	1%
A. oryzea	1	1%
A. rugulosa	1	1%
A. strictum	1	1%
A. terreus	1	1%
A. ustus	1	1%
Aureobasidium	1	1%
B. spicifera	1	1%
C. atrobrunneum	1	1%
C. cassiicola	1	1%
C. geniculata	1	1%
C. Guilliermondii	1	1%
C. madurae	1	1%
C. mycetomatis	1	1%
C. oxyspora	1	1%
D. didyma	1	1%
D. phaseolorum	1	1%
E. floccosum	1	1%
E. dermatitidis	1	1%
E. oligosperma	1	1%
E. spinifera	1	1%
F. falciforme	1	1%
F. sambucinum	1	1%
G. khartoumense	1	1%
Glensporella	1	1%
I. americana	1	1%
M. bullatus	1	1%
M. ferrugineum	1	1%
M. tropicana	1	1%
Mucor	1	1%
N. dimidiatum	1	1%
P. curvata	1	1%
P. larense	1	1%
P. ochracea	1	1%
P. percutanea	1	1%
P. sphinctrophorum	1	1%
P. unguis-hominis	1	1%
Phoma	1	1%
Phyllosticta	1	1%
R. atrovirens	1	1%
R. microsporus var. rhizopodiformis	1	1%
R. percutanea	1	1%
S. boydii	1	1%
Scopulariopsis	1	1%
Syncephalastrum	1	1%
T. cutaneum	1	1%
T. schoenleinii	1	1%
X. recifei	1	1%
A. albus	0	0%
A. bouffardii	0	0%
A. hollandicus	0	0%
A. potronii	0	0%
A. versicolor	0	0%
B. dermatitidis	0	0%
C. neoformans	0	0%
C. pallescens	0	0%

C. parapsilosis	0	0%
C. piceae	0	0%
C. posadasii	0	0%
C. rugosa	0	0%
C. spicifer	0	0%
Cladosporium	0	0%
Cunninghamella	0	0%
D. rostrata	0	0%
E. mansonii	0	0%
E. paragrisea	0	0%
E. rostratum	0	0%
F. caeruleum	0	0%
F. fujikuroi	0	0%
F. keratoplasticum	0	0%
F. pseudensiforme	0	0%
F. thapsinum	0	0%
G. capitatum	0	0%
G. semoni	0	0%
Glomus	0	0%
H. anomalum	0	0%
H. capsulatum	0	0%
Hormonema	0	0%
I. mansoni	0	0%
I. reynieri	0	0%
M. bouffardii	0	0%
M. brumptii	0	0%
M. oswaldi	0	0%
M. paisii	0	0%
P. fuscum	0	0%
P. inflatipes	0	0%
P. krajdenii	0	0%
P. mycetogenum	0	0%
P. obovatum	0	0%
R. mackenziei	0	0%
Septobasidium	0	0%
Sterigmatocystis	0	0%
T. terestre	0	0%
T. violaceum	0	0%
Trichosporium	0	0%

Chapter -3-

Comparative genomics of fungicide targets to support drug repurposing for eumycetoma

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Author contributions:

Conceptualization: MH, PM, AE

Data capturing and management: MH

Data analysis: MH, PM

Interpretation of results: MH, PM

Paper writing: MH, MT, PM, AE

Note: Submitted for publication

3.1. Abstract:

Eumycetoma is a neglected tropical disease that contributes to disability, disfiguration, and social stigma of afflicted patients. It is treated by a combination of chemotherapy and surgical intervention. However, the long duration of treatment, drug resistance, and relapses result in poor treatment outcomes and eventual amputation of the affected parts when possible. The current treatment of eumycetoma is based on azoles, allyamines and echinocandins, agents that target fungal cell wall integrity and biosynthesis/function of membrane sterols. Here we perform comparative genomics between plant-pathogenic fungi and the causative agents of eumycetoma based on drug targets. We implement a hidden Markov model-based profiling of fungal species (n=30 genomes) by their scores towards known protein profiles of agrochemical fungicide targets (n=9). The recorded bit scores allowed to cluster the species and to rank the target enzymes. Clustering according to drug target profiles neither followed the phylogeny nor the host specificity of the included fungal species. *Madurella mycetomatis,* the main causative agent of eumycetoma clustered with plant pathogens distant from the rest of eumycetoma fungal pathogens, rationalizing the idea of repurposing agrochemical fungicides for eumycetoma. Of all the analyzed targets, adenosine deaminase, succinate dehydrogenase, and chitin synthase contributed the least to variation of the profile score vectors, indicating a potential for broad-spectrum inhibitors. However, inhibition of adenosine deaminase and succinate dehydrogenase also bears the risk of toxicity, since they are present in the human proteome as well. Chitin synthase was a conserved fungal drug target that was absent in the human host. The melanogenic enzymes polyketide synthase and scytalone dehydratase were specific to the fungi as well, but less conserved considering all species. This work supports the repurposing of agrochemical fungicides for eumycetoma.

3.2. Author summary:

Eumycetoma, a fungal infection that has recently been recognized as a neglected tropical disease, faces big challenges since the currently used drugs lack efficacy, safety, and availability for poor patients. New drugs are needed urgently. Here we try to promote the repurposing of agrochemical fungicides for the treatment of eumycetoma by comparative genomics between phytopathogenic fungi and the fungal agents of eumycetoma. We perform profiling of fungal species based on their scores towards known fungicide targets. The resulting profiles serve to cluster the species and to rank the target enzymes. *Madurella mycetomatis*, the most frequent cause of eumycetoma, clusters together with phytopathogenic fungi based on its agrochemical target profile conservation estimates, which encourages drug repurposing. Of all the analyzed targets, adenosine deaminase, succinate dehydrogenase, and chitin synthase contribute the least to variation, bearing the potential for broad-spectrum inhibitors. However, inhibition of these enzymes also bears the risk of toxicity, since they are present in the human proteome as well. Chitin synthase is a well-conserved fungal drug target that is absent in the human host. Overall, our approach supports the notion of drug repurposing from the agrochemical sector to the treatment of eumycetoma, and it suggests drug targets that could serve for a broad-spectrum targeting scheme for eumycetoma drug discovery to meet the urgent need for efficacious, safe, and also affordable drugs.

3.3. Introduction:

Eumycetoma is a subcutaneous fungal infection characterized by discharge and granulation. Eumycetoma is caused by a wide range of fungi, with *M. mycetomatis* being the top reported pathogen (Mohamed et al., 2015b; Sande, 2013). The current treatment for eumycetoma a is unsatisfactory with a high probability of recurrence (Table 1), leading to end-surgical interventions which include amputations when peripheries are involved, this is in addition to the reported drug resistance (Welsh et al., 2007). Recently the USA Food and Drug Administration (FDA) required a black-box warning for itraconazole (ITZ) of the risk of myocardial toxicity. The FDA had also limited the use of ketoconazole due to the potential of fatal liver injury, the risk of drug interactions, and adrenal gland problems (https://www.aspergillus.org.uk/content/cardiac-toxicity-azole-antifungals, n.d.; Research, n.d.). These drugs are the cornerstone for eumycetoma treatment in third-world countries, where the majority of patients are located. However, drug discovery is a time-consuming process with tremendous costs (Kraljevic et al., 2004). Under the auspices of the Drugs for Neglected Diseases *initiative* (DNDi), a clinical Proof-of-Concept Superiority Trial of fosravuconazole versus itraconazole for eumycetoma is currently carried out in Sudan (https://adisinsight.springer.com/drugs/800018102, n.d.; DNDi – Best science for the most neglected, n.d.; DNDi – Best science for the most neglected, 2015). Recently, diverse molecular libraries have been screened against *M. mycetomatis*, including the Pathogen and Stasis Boxes of the Medicines for Malaria Venture (MMV), open-access tools to boost research and development on neglected diseases (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4965013/, n.d.). Using the MMV libraries, fenarimol analogs, which had been successfully tested against *Trypanosoma cruzi*, were recently found to be active against *M. mycetomatis* (Lim et al., 2018b). Moreover, natural products were tested against *M. mycetomatis* (Lim et al., 2018b; Elfadil et al., 2015).

Recent approaches of drug repurposing suggested using agrochemicals for pharmaceutical drug discovery since agrochemicals are designed following similar approaches of drug-likeliness and optimization of physicochemical properties, although agrochemicals tend to be more hydrophobic. (Jampilek, 2016). Screening of agrochemicals against *Plasmodium falciparum*, *Trypanosoma brucei*, *T. cruzi* and *Leishmania donovani* have provided potential leads for malaria and some neglected tropical diseases (Witschel et al., 2012). Comparative genomics and pharmacogenomics can support drug repurposing by predicting the conservation of known targets in new species (Murphy, 2010). Here we perform comparative genomics of eumycetoma pathogens and phytopathogenic fungi based on predicted targets of antimicrobial agents and agrochemical fungicides.

3.4. Methodology:

3.4.1. Proteomes:

All predicted proteomes were downloaded from the Ensembl fungi database or the NCBI genome database (Agarwala et al., 2018; Hubbard et al., 2002) and tabulated in Table 3.

3.4.2. HMM profile searches:

All HMM profiles were retrieved from the Pfam protein family database (Bateman et al., 2004) (Table 2). HMMer searches (https://www.ncbi.nlm.nih.gov/pubmed/20180275, n.d.) were carried out with the Ugene Unipro bioinformatics toolkit v1.30 (Okonechnikov et al., 2012). HMMer searches against the human proteome were carried out with the hmmsearch web-based search tool (Finn et al., 2011).

For proteomes returning no hits, the value -300 was considered a bit score replacement during data analysis (most negative bit score returned was -200). The data was developed into a data matrix which was centered and scaled for further analysis (van den Berg et al., 2006; Breiger et al., 1975).

3.4.3. Data analysis:

Different methods for clustering were applied including Hierarchical, K-Means, Partitioning Around Medoids (PAM) to elucidate the distances between the study species. Principal Component Analysis (PCA) was used to dissect variation among the developed bit score matrix (Corpet, 1988; Breiger et al., 1975; Hartigan, 1975). Data analyzed was conducted using R-v3.53 and RStudio Version 1.1.453 (KASSAMBARA, 2017; RStudio Team, 2015b; Abdi and Williams, 2010; https://www.jstatsoft.org/article/view/v025i01, n.d.).

3.5. Results:

The current antifungal drugs used against eumycetoma target sterol function (amphotericin B) its biosynthesis (azoles, terbinafine) or fungal cell wall integrity (caspofungin; Table 1). Some of these fungicidal agents were active *in-vitro* but lacked effectiveness *in-vivo* when tested against *M. mycetomatis* in patients (Welsh et al., 2014b; Maertens, 2004; https://adisinsight.springer.com/drugs/800018102, n.d.). Here we aim to find further potential targets that are conserved across multiple pathogenic species. Inhibitors of such targets, if well tolerated by the human host, have the potential for broad-spectrum therapeutics of eumycetoma.

Table 1: Antifungal drugs against eumycetoma

Antifungal	**Class**	**Year**	**Target**	***in-vitro activity***	**human infection**
Amphotericin B	Polyenes	1959	Ergosterol	Moderate	Not effective
Fluconazole	Azole	1990	Lanosterol demethylase (CYP51A1)	Limited	Not effective
Ketoconazole	Azole	1981	CYP51A1	High	Variable efficacy
Itraconazole	Azole	1992	CYP51A1	High	Variable efficacy
Voriconazole	Azole	2002	CYP51A1	High	Effective in a few cases
Posaconazole	Azole	2006	CYP51A1	High	Effective in a few cases
Isavuconazole	Azole	2016	CYP51A1	High	No data
Caspofungin	Echinocandins	2001	Glucan synthase	Low	No data
Terbinafine	Allylamine	1992	Squalene epoxidase	Moderate	No data
Fosravuconazole*	Azole	2017-2018	CYP51A1	Phase II clinical trial	

A panel of fungicide targets was extracted from the website of the Fungicide Resistance Action Committee FRAC (Russell, 2009; http://www.frac.info/home, n.d.; https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2338.1985.tb00271.x, n.d.) and supplemented with antibacterial drug target enzymes to serve as controls in the analysis. The selected targets were RNA polymerase I, adenosine deaminase, NADH reductase (complex I), succinate dehydrogenase (complex II), scytalone dehydratase, polyketide synthase A (Noranthrone Synthase), gyrase (DNA topoisomerase type II), phospholipid N-methyltransferase (PmtA), and chitin synthase (Table 2). For each of these proteins, the respective profile was downloaded from the Protein Family Database Pfam (www.pfam.org) (Bateman et al., 2004). These profiles are position-dependent, hidden Markov-model-based scoring matrices that can be used for searches with the program HMMer (https://www.ncbi.nlm.nih.gov/pubmed/20180275, n.d.). The HMMer bit scores against the human Ensembl protein database (*Homo sapiens* taxid: 9606) (Staff, 2018) are included in Table 2 to predict their conservation in the human host.

Since the goal of this study was to investigate the repurposing potential of agrochemical fungicides against eumycetoma, we have compiled a list of fungal species comprising known plant pathogens (n=7), eumycetoma pathogens (n=20), and shared pathogens (causative agents of both eumycetoma and plant fungal diseases n=3) from the literature (Hashimoto et al., 2017; Mohamed et al., 2015b; Sande, 2013; Dean, Kan, et al., 2012; Zaman and Sarma, 2006) (see Table 3). The Pfam profiles for fungicide targets were run over the predicted proteomes of these 30 species, and the maximum of the HMMer bit scores was recorded for each target and each species. The obtained values showed a large variance, as displayed in Figure 1.

Table 2: Protein domains used in the study, adjusted from RFAC publications (Russell, 2009; https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2338.1985.tb00271.x, n.d.)

FRAC Code: Mechanism of action (MOA)	Target enzyme	Abbrev.	E.C Num	Pfam accession	Bit score of the best hit in the human proteome	Resistance Risk by FRAC
A: Nucleic Acids Metabolism	**A1:** RNA polymerase I	RNApol	2.7.7.6	PF00940	410.71	High
	A2: Adenosin-deaminase	ADA	3.5.4.4	PF00962	52.32	Medium
	A4: DNA topoisomerase type II (gyrase)	GyrB*	5.6.2.2	PF00204	329.50	Not known
C: Respiration	**C1**: Complex I NADH Oxido-reductase	I NADH OR*	7.1.1.2	PF10785	148.52	Not known
	C2: Complex II: succinate-dehydrogenase	SDH	1.2.1.24	PF00171	441.96	Medium to high
F: Lipid Synthesis or Transport / Membrane Integrity or Function	**F2:** Phospholipid biosynthesis, methyltransferase	PmtA*	2.1.1.71	PF04191	114.84	Low to medium
I: Melanin Synthesis	**I2:** Scytalone dehydratase (SD)	SD	4.2.1.94	PF02982	None	Medium
	I3: Polyketide synthase A	PKS	2.3.1.221	PF14765	None	Not known
H: Cell Wall Biosynthesis	**H4:** Chitin synthase	CHS	2.4.1.16	PF03142	None	Medium

*Bacterial enzymes introduced as control domains.

Table 3: Fungal species included in the study:

Num	Figure	Species names	Tax. ID	Family	Etiological class	Assembly ID - Ensembl Fungi	Genome size (Mb)	Protein coding genes
1	2,4,5,8	*Aspergillus flavus*	332952	*Aspergillaceae*	Eumycetoma Pathogen	JCVI-afl1-v2.0	36.80	13'485
2	2,4,5,8,9	*Aspergillus fumigatus*	330879	*Aspergillaceae*	Eumycetoma Pathogen	ASM265v1	28.80	9'623
3	2,4,5,8	*Aspergillus niger*	5061	*Aspergillaceae*	Eumycetoma Pathogen	ASM285v2	33.90	13'942
4	2,4,5,8,9	*Aspergilus Nidulans*	162425	*Aspergillaceae*	Eumycetoma Pathogen	ASM14920v2	30.24	9'536
5	2,4,5,8,9	*Exophiala aquamarina*	1182545	*Herpotrichiellaceae*	Eumycetoma Pathogen	Exop_aqua_CBS_119918_V1	41.50	13'118
6	2,4,5,8	*Exophiala mesophila*	212818	*Herpotrichiellaceae*	Eumycetoma Pathogen	Exop_meso_CBS40295_V1	29.25	9'181
7	2,4,5,8	*Exophiala oligosperma*	215243	*Herpotrichiellaceae*	Eumycetoma Pathogen	Exop_olig_CBS72588_V1	37.90	11'893
8	2,4,5,8,9	*Exophiala sideris*	1016849	*Herpotrichiellaceae*	Eumycetoma Pathogen	Exop_side_CBS121828_V1	29.48	10'191
9	2,4,5,8	*Exophiala spinifera*	91928	*Herpotrichiellaceae*	Eumycetoma Pathogen	Exop_spin_CBS89968_V1	32.87	12'049
10	2,4,5,8	*Exophiala xenobiotica*	348802	*Herpotrichiellaceae*	Eumycetoma Pathogen	Exop_xeno_CBS118157_V1	31.39	12'019
11	2,4,5,8	*Fusarium graminearum*	5518	*Nectriaceae*	Shared Pathogen	RR 1	38.04	14'145
12	2,4,5,8	*Fusarium langsethiae*	179993	*Nectriaceae*	Eumycetoma Pathogen	ASM129263v1	37.54	11'940
13	2,4,5,8	*Fusarium mangiferae*	192010	*Nectriaceae*	Eumycetoma Pathogen	Genome assembly version 1	46.29	15'804
14	2,4,5,8	*Fusarium oxysporum*	426428	*Nectriaceae*	Shared Pathogen	FO2	60.01	17'708
15	2,4,5,8	*Fusarium poae*	36050	*Nectriaceae*	Eumycetoma Pathogen	FPOA1.0	46.48	14'740
16	2,4,5,8	*Fusarium pseudograminearum*	1028729	*Nectriaceae*	Eumycetoma Pathogen	FP7	39.93	12'448
17	2,4,5,8,9	*Fusarium solani*	660122	*Nectriaceae*	Eumycetoma Pathogen	v2.0	51.29	15'705
18	2,4,5,8,9	*Fusarium verticillioides*	334819	*Nectriaceae*	Eumycetoma Pathogen	ASM14955v1	41.79	14'169
19	2,4,5,8	*Geotrichum candidum*	1173061	*Dipodascaceae*	Eumycetoma Pathogen	New2.3_08062011*	24.84	6'799
20	2,4,5,8	*Madurella mycetomatis*	100816	*Sordariales incertae sedis*	Eumycetoma Pathogen	ASM127576v2	36.70	10'707
21	2,4,5,8,9	*Pyrenochaeta*	765867	*Cucurbitariaceae*	Eumycetoma Pathogen	Pyrsp1	38.30	14'990
22	2,4,5,8	*Scedosporium apiospermum*	563466	*Microascaceae*	Eumycetoma Pathogen	ScApio1.0	43.44	8'376
23	2,4,5,8	*Magnaporthe oryzae*	242507	*Magnaporthe*	Plant Pathogen	MG8	40.95	12'593
24	2,4,5,8	*Botrytis cinerea T4*	999810	*Botrytis*	Plant Pathogen	BotFuc_Mar2011	39.51	16'353
25	2,4,5,8	*Colletotrichum chlorophyti*	708187	*Glomerellaceae*	Plant Pathogen	ASM193710v1	52.38	10'310
26	2,4,5,8	*Colletotrichum gloeosporioides*	1213859	*Glomerellaceae*	Plant Pathogen	Colletotrichum gloeosporioides Nara gc-5	55.39	15'381
27	2,4,5,8,9	*Colletotrichum higginsianum*	80884	*Glomerellaceae*	Plant Pathogen	ASM31379v2	49.08	16'141
28	2,4,5,8	*Colletotrichum simmondsii*	703756	*Glomerellaceae*	Plant Pathogen	CSIM01	50.47	13'884
29	2,4,5,8	*Colletotrichum sublineola*	1173701	*Glomerellaceae*	Plant Pathogen	ASM69613v1	46.76	12'699
30	2,4,5,8	*Puccinia triticina*	630390	*Pucciniaceae*	Shared pathogen	ASM15152v1	126.64	11'638

* Genome and proteome data downloaded from NCBI genome database.

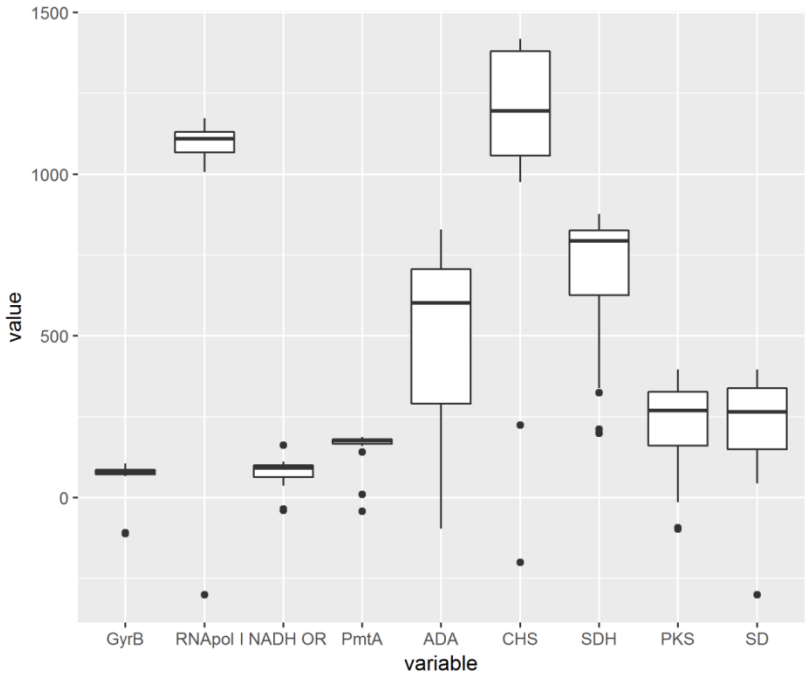

Figure 1: Boxplot showing statistic summary for the spreading of HMM bit scores. The known bacterial profiles GyrB, NADH OR, and PmtA showed low bit scores compared to the known fungal profiles CHS, SD, PKS, SDH, and ADA with the species *A. fumigatus, A. nidulans, M. oryzea, A. niger, and G. candidum* showing low-outlier estimates for the screened profiles.

3.5.1. Patterns of HMMER bit scores across species and clustering

The profile search used each of the studied fungal species as a vector consisting of the obtained best scores for each fungicide target. For a graphical overview of the results, the scores were first scaled then, converted to a heatmap (Figure 2). The heatmap was further clustered using Hierarchical clustering of these vectors based on their pairwise Euclidean distances using complete linkage (Figure 2). The topology of the resulting tree presented no simple pattern: it reflected neither the phylogeny of the 30 species, nor did it follow the host preferences of plant vs. human pathogens.

K-means clustering, and PAM clustering estimated two clusters to be the optimal number of clusters, with silhouette widths s_i of 0.71 indicating the acceptable shape of the clusters (Figure 3).

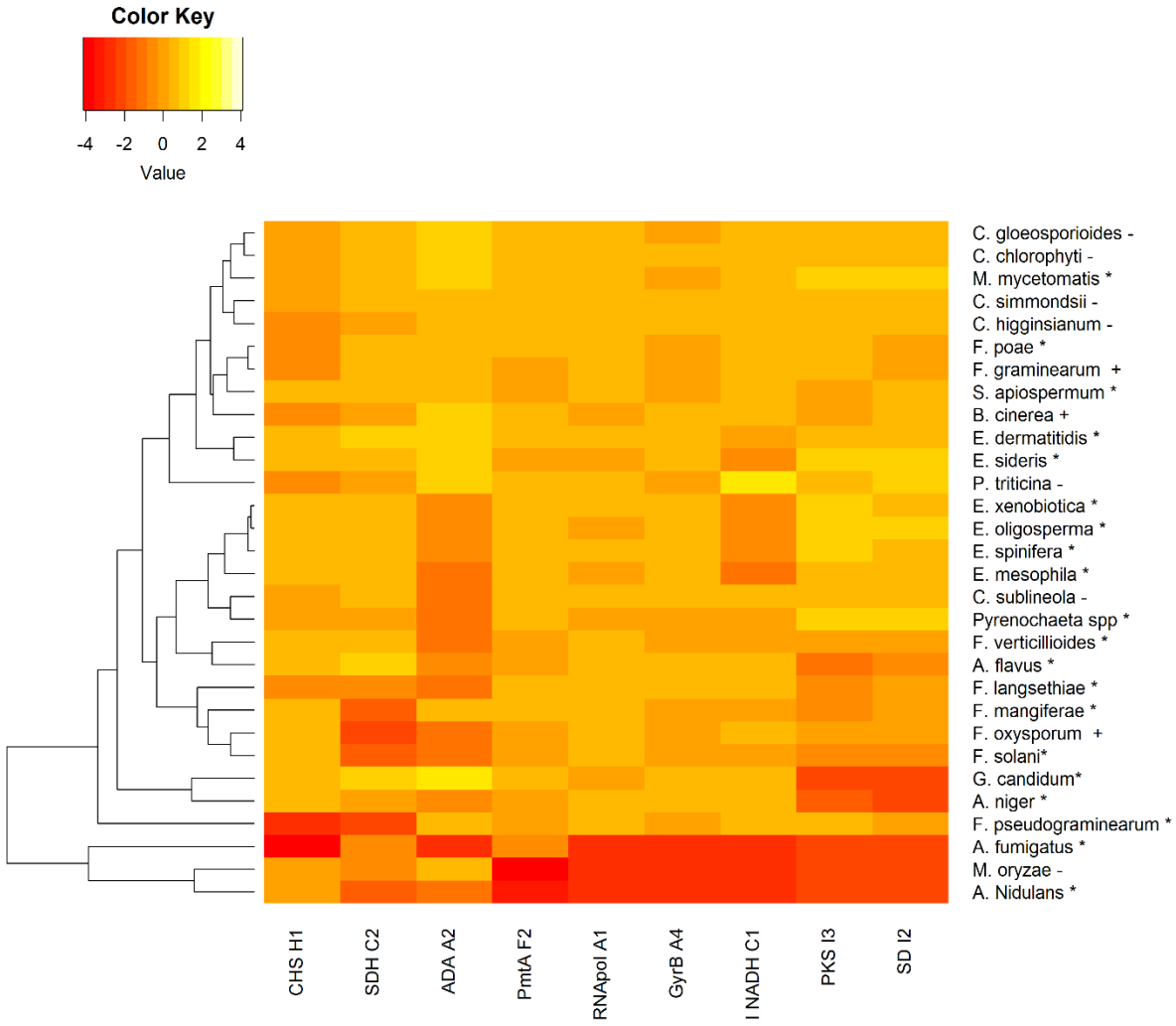

Figure 2: Heatmap using hierarchical clustering of the calculated Euclidean distance, with row clustering. The heatmap has shown eumycetoma agents*, plant pathogens-, and shared pathogens+. *M. mycetomatis* the main eumycetoma pathogen clustered closely to the sole plant pathogens.

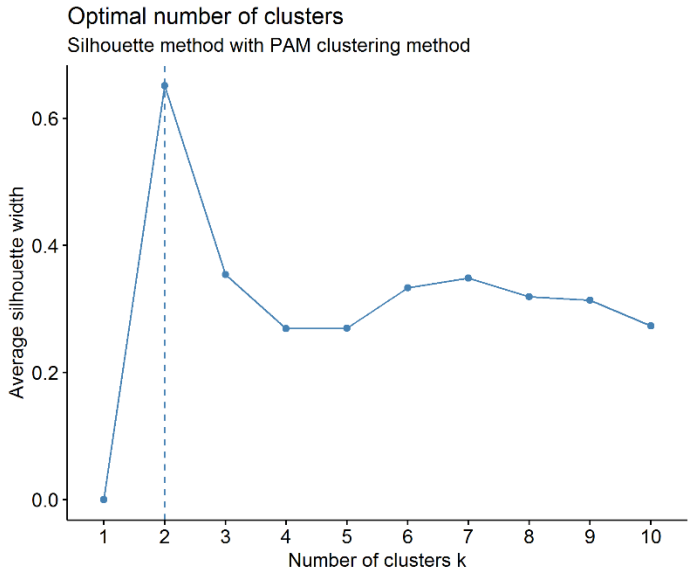

Figure 3: The estimated number of clusters using PAM clustering and average Silhouette width s_i metric. included all species (n=30) revealing 2 clusters as the optimal number to describe the study the species (s_i = 0.65).

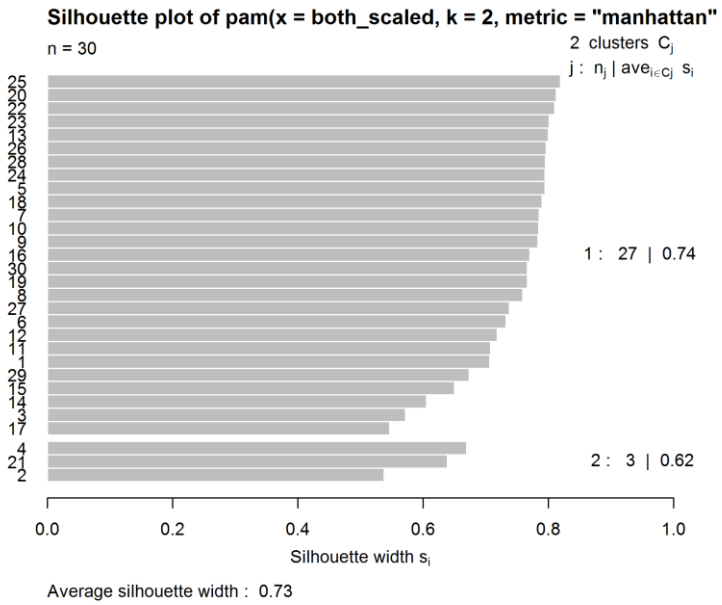

Figure 4: The two clusters suggested when analyzing all species (n=30) using Silhouette width metric, cluster 1 (n=27, s_i=0.67) and cluster 2 (n=3, s_i=0.44). Cluster 2 which included *A. nidulans, A. fumigatus,* and *M. oryzea* showed higher inter-cluster variability compared to cluster 1.

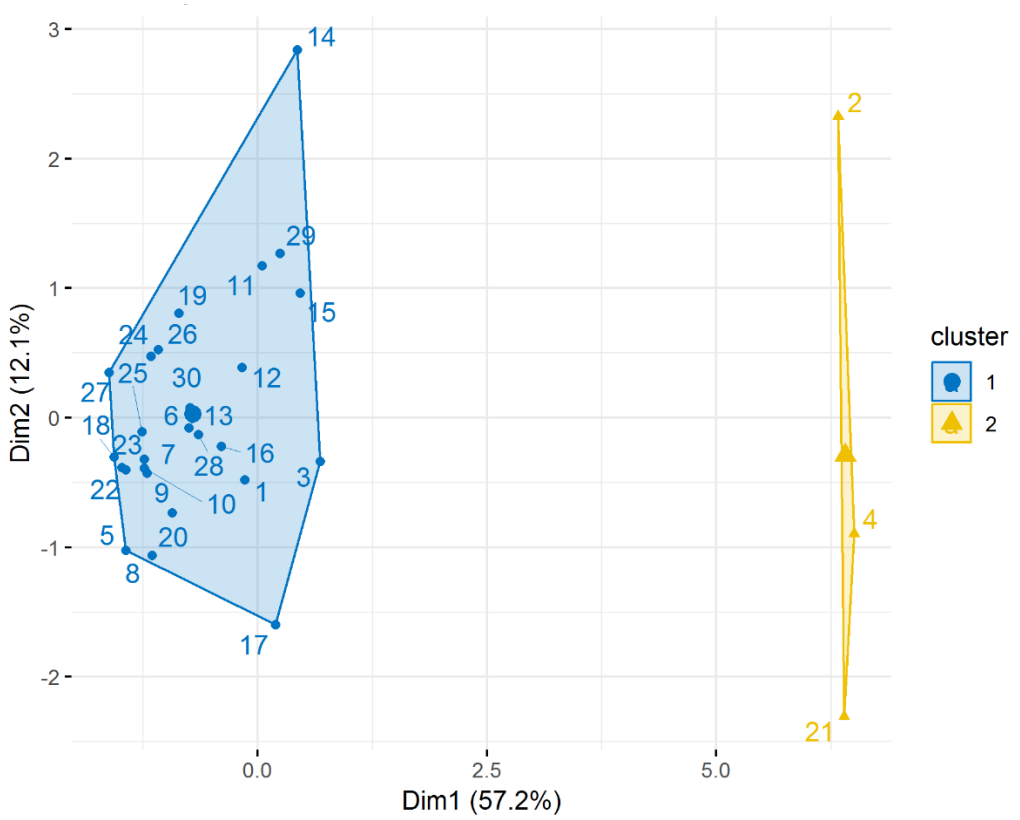

Figure 5: Kmeans **c**luster plot using HMM bit scores showing the studied species forming two distinct clusters when overlaid onto two principal components explaining the variation among these species.

3.5.2. Principal component analysis (PCA) to dissect the sources of variation and agreement

The principal component analysis was conducted to identify the main sources of variation in the matrix of HMM bit scores, and to quantify the contribution to variation of each of the protein domains and the individual species. While conducting PCA, the contribution of the variables (protein profiles) was the end goal since the domains with the least contrition to variation in the data would be our target domains and the probable prospect drug targets. A PCA scree plot of the eigenvalues showed the first 2 principal components (PCs) to possess eigenvalues >1 and describe 57.24% of the variability, indicating their importance in summarizing the variation within the HMMer bit score data matrix (Figure 6). Of all the analyzed target protein profiles, adenosine deaminase (ADA), succinate dehydrogenase (SDH), and chitin synthase showed contribution

beyond the average to both first two important principal components (Figure 7A) and to the first 3 PCs collectively (Figure 7B).

The PCA biplot using the first two principal components shows the relationship between the target enzymes' eigenvectors and the different study species. The target protein profiles adenosine deaminase, succinate dehydrogenase, and chitin synthase showed similar contribution and correlation as seen in the PCA biplot (Figure 8). Interestingly, polyketide synthase and scytalone dehydratase were found correlated, possibly reflecting the functional link as they are both melanogenic enzymes (see the correlation matrix plot, Suppl: Figure S1). PCA analyses also spotted the species with highest contribution to the variation in HMM bit scores of the target protein domains with A. fumigatus as the top contributor (see figure 9)

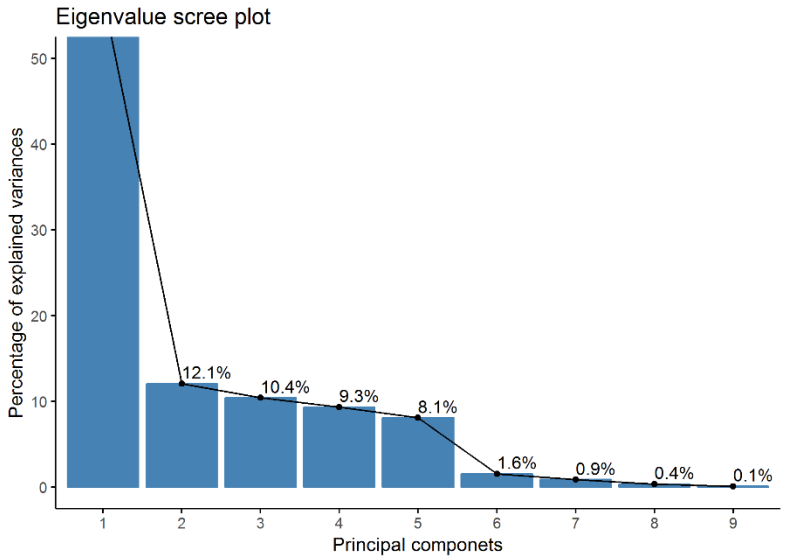

Figure 6: PCA eigenvalues scree plot showing 3 important principal components of eigenvalue>1

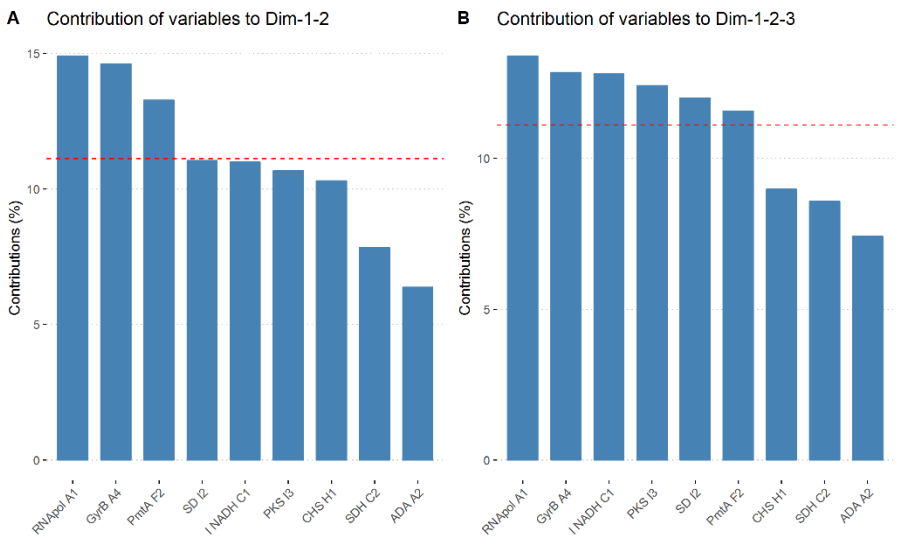

Figure 7: Figure 7A shows the target protein profile contribution to PC1 and 2. Figure 7B shows their contribution to PC1 to 5. The red dotted line resembles the average contribution of all protein profiles. ADA, SDH, and CHS contributed below the average contribution even when 5 PCs are considered.

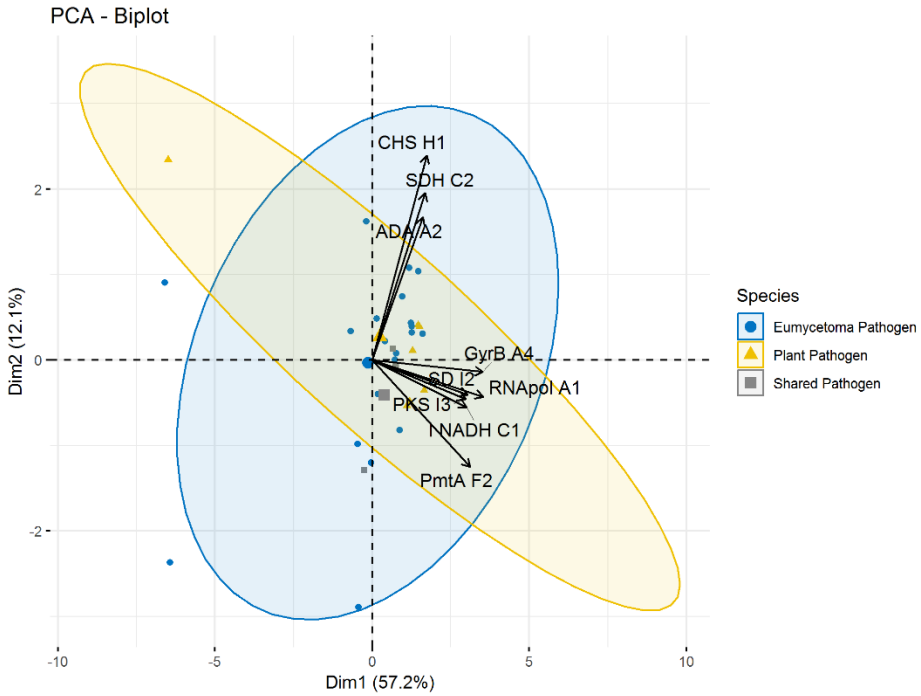

Figure 8: PCA biplot showing the relationship between target protein profiles in the plane of PC1 and PC2, the different groups are shown with an Ellipse indicating their spreading and location to other groups. With few of the studied species spreading away, the majority of the eumycetoma causative fungi and phytopathogenic fungi remain closely related.

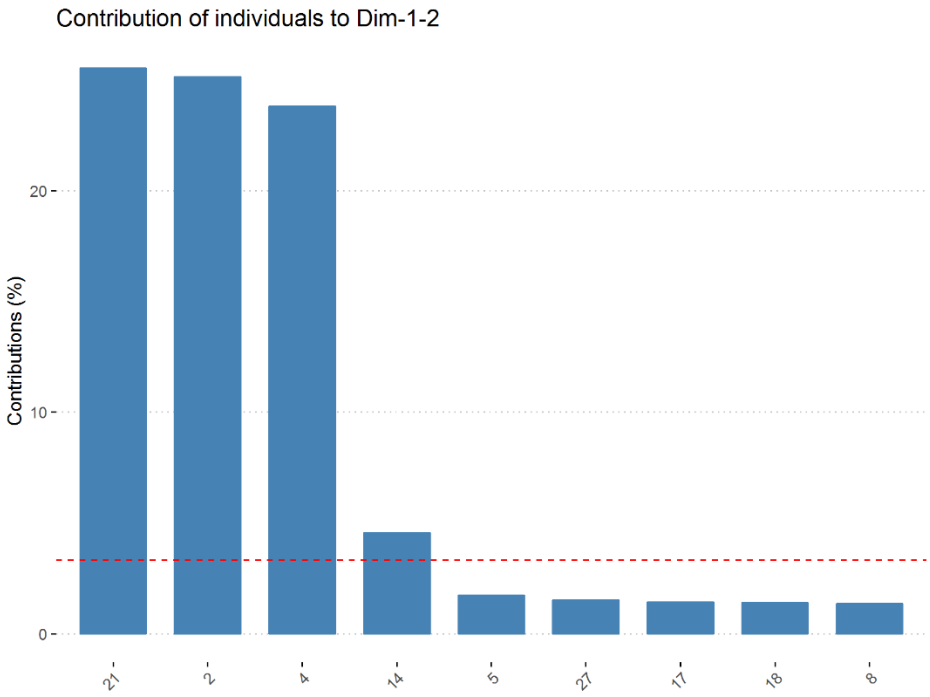

Figure 9: The species with a contribution higher than the average contribution (indicated by the red dotted line)to the variation among data estimating for PC1 and PC2 showing the species *M. oryzea* (21), *A. fumigatus* (2), *A. nidulans*, and *Fusarium pseudograminearum* (14), the contribution of all species tabulated in (Suppl: Table S1).

3.5.3. Conclusions for the repurposing potential by target enzymes

For each of the investigated fungicide target enzymes, inhibitor molecules were obtained from FRAC (Russell, 2009). Drug-likeliness, gastrointestinal (GI) absorption, blood-brain permeability, and skin penetration were estimated using the SwissADME online tool (Daina et al., 2017; Lipinski, 2004; Potts and Guy, 1992; https://onlinelibrary.wiley.com/doi/abs/10.1002/%28SICI%291521-3773%2819991216%2938:24%3C3743::AID-ANIE3743%3E3.0.CO;2-U, n.d.) (see Table 4). Adenosine deaminase (ADA) was the best-suited target enzyme for drug repurposing as estimated based on its low contribution estimated by PCA analyses. Fungicides inhibiting this enzyme are bupiramate, dimethrimol, and ethrimol; these compounds are all druglike based on Lipiniski's rule

of five (Lipinski, 2004). Adenosine deaminase inhibitors other than agrochemical fungicides are the adenosine derivatives cardoceptin and pentostatin, which also satisfy druglikeliness conditions. Bupiramate was tested positive against *Botrytis cinerea* which is both eumycetoma pathogen and reported phytopathogen (Carlile and Coules, 2012). These agents have been tested against *Trypanosoma cruzi* and viruses besides numerous fungi, with positive results (Cristalli et al., 2001; Sugar and McCaffrey, 1998). Also, naringin, a natural flavonoid from grapefruit, targets ADA and possesses antifungal activity against *A. fumigatus* and *B. cinerea* (Li et al., 2015) (Table 4).

Succinate dehydrogenase inhibitors (SDHI) fall into 7 chemical classes and were used against a wide range of fungal species including B. cinerea and showed reliable fungicide activity (https://www.researchgate.net/publication/287690456_Succinate_dehydrogenase_inhibitor_SDHI_fungicide_resistance_prevention_strategy, n.d.).

Chitin synthase inhibitor polyoxins B also resemble an opportunity for repurposing although they violate the druglikeliness roles and have low GI absorption estimates.

Table 4: Currently used agrochemical fungicides that inhibit the investigated targets

Target	Chemical group	Common name	Drug likeliness/Lipiniski Ro5	GI absorption	BBB permeant	skin permeation (Log Kp cm/s)
Adenosine deaminase (ADA)	Hydroxy-(2-amino-) pyrimidines	Bupirimate	Yes;0 violation	High	No	-6.31
		Dimethirimol	Yes;0 violation	High	Yes	-6.23
		Ethirimol	Yes;0 violation	High	Yes	-6.1
	Adenosine derivatives *	Cordycepin	Yes;0 violation	High	No	-8.27
		Pentostatin	Yes;0 violation	Low	No	-9.42
	Flavonoid *	Naringin	Yes;0 violation	Low	No	-10.15
RNA polymerase 1 (RNApol)	Acylalanines	Benalaxyl	Yes;0 violation	High	Yes	-5.87
		Furalaxyl	Yes;0 violation	High	Yes	-6.29
		Metalaxyl	Yes;0 violation	High	Yes	-6.83
	Oxazolidinones	Oxadixyl	Yes;0 violation	High	Yes	-7.43
	Butyrolactones	Ofurace	Yes;0 violation	High	Yes	-7.03
Chitin synthase (CHS)	Polyoxins	Polyoxin B	No; 3 violations: MW>500, NorO>10, NHorOH>5	Low	No	-11.77
Succinate dehydrogenase (SDH)	Phenyl-benzamides	Benodanil	Yes;0 violation	High	Yes	-5.84
		Flutolanil	Yes;0 violation	High	Yes	-4.17
		Mepronil	Yes;0 violation	High	Yes	-5.87
	Phenyl-oxo-ethyl thiophene amide	Isofetamid	Yes;0 violation	High	No	-5.15
	Pyridinyl-ethyl-benzamides	Fluopyram	Yes;0 violation	Low	No	-5.51
	Furan- carboxamides	Fenfuram	Yes;0 violation	High	Yes	-5.95
	Oxathiin- carboxamides	Carboxin	Yes;0 violation	High	Yes	-6.22
		Oxycarboxin	Yes;0 violation	High	No	-7.38
	Thiazole- carboxamides	Thifluzamide	Yes; 1 violation: MW>500	Low	No	-5.25
	Pyrazole-4- carboxamides	Benzovindiflupyr	Yes;0 violation	High	Yes	-5.9
		Bixafen	Yes; 1 violation: MLOGP>4.15	High	No	-5.48
		Fluindapyr	Yes;0 violation	High	Yes	-5.7
		Fluxapyroxad	Yes; 1 violation: MLOGP>4.15	High	No	-6.03
		Furametpyr	Yes;0 violation	High	Yes	-6.46
		Inpyrfluxam	Yes;0 violation	High	Yes	-5.66
		Isopyrazam	Yes;0 violation	High	Yes	-5.62
		Penflufen	Yes;0 violation	High	Yes	-5.14
		Penthiopyrad	Yes;0 violation	High	No	-5.67

		Sedaxane	Yes;0 violation	High	Yes	-5.86
	N-cyclopropyl-N-benzyl-pyrazole-carboxamides	Isoflucypram	Yes; 1 violation: MLOGP>4.15	High	Yes	-5.49
	N-methoxy-(phenyl-ethyl)-pyrazole-carboxamides	Pydiflumetofen	Yes;0 violation	High	No	-5.51
	Pyridine- carboxamides	Boscalid	Yes;0 violation	High	Yes	-4.91
	Pyrazine-carboxamides	Pyraziflumid	Yes;0 violation	High	No	-5.99
Polyketide synthase (PKS)	Trifluoroethyl-carbamate	Tolprocarb	Yes;0 violation	High	Yes	-5.72
Scytalone dehydratase (SD)	Cyclopropane-carboxamide	Carpropamid	Yes;0 violation	High	Yes	-5.3
	Carboxamide	Diclocymet	Yes;0 violation	High	Yes	-5.13
	Propionamide	Fenoxanil	Yes;0 violation	High	Yes	-5.1

* Molecules which are not agrochemical fungicides, they are published inhibitors of ADA. The agrochemical fungicides were retrieved from frac-code-list-2018-final-v2 published by FRAC, Lipinski score and pharmacokinetic properties were calculated using

3.6. Discussion:

The current chemotherapy of eumycetoma is not satisfactory at all (Table 1). Initiatives towards more efficacious and safer drugs are underway, most notably the fosravuconazole trial of DNDi in Sudan, the first randomized, double-blind clinical trial for eumycetoma. Drug repurposing is an attractive option, in particular for a neglected disease like mycetoma. Here we aim to support the repurposing of agrochemical fungicides for eumycetoma by comparative genomics of known drug targets. We have included phytopathogenic fungal species and eumycetoma pathogens; the study focused on finding pathways that are conserved across most of the studies species because such pathways have the potential for broad-spectrum activity.

Profiling of the fungal proteomes according to fungicide targets allowed us to represent each species as a numeric vector (Fügi et al., 2014; Greganova et al., 2013), which was the basis for clustering and further numerical analyses of the fungal species. If the tree built on the target profiles (Figure 2) had simply reproduced the phylogeny of the included species, it would not have been informative. However, there were some interesting deviations from the known phylogeny. *A. fumigatus*, *A. nidulans* and *M. oryzae* consistently formed a separate cluster, indicating that these three species share common features that distinguish them from the rest, i.e. the absence or low conservation of drug targets (Figure 9).

It was interesting to note that the phytopathogenic species and the mycetoma pathogens did not separate into distinct clusters. In particular, *M. mycetomatis*, the most frequently reported eumycetoma pathogen (van Belkum et al., 2013; Ahmed AO et al., 2004), co-clustered with phytopathogens both in hierarchical clustering (Figure 2) and the PAM cluster plot (Figure 5), supporting the idea of repurposing agrochemical fungicides against eumycetoma. Even though the plant-pathogenic species, in general, possess larger genomes and predicted proteomes that the

mycetoma pathogens (Supplementary: Figure S2), *M. mycetomatis* returned positive HMMer bit scores for all the tested profiles of agrochemical fungicide target enzymes.

The most conserved drug targets, as indicated by their low contribution to variability, were adenosine deaminase, succinate dehydrogenase, and chitin synthase (Figure 7). There are several inhibitors for these enzymes among the agrochemical fungicides (Table 4). However, adenosine deaminase and RNA polymerase I are also conserved in the human proteome (Table 2), bearing the risk of toxicity to the human host. Chitin synthase was conserved among the pathogens (Figure 7) but absent in the human proteome (Table 2). Chitin synthase, a glycosyl transferase also known as chitin-UDP N-acetylglucosaminyl transferase, occurs not only in fungi but also in many invertebrates (including parasitic nematodes) and in protists, archaea, bacteria, and even viruses (Gonçalves et al., 2016). Thus, inhibitors of chitin synthase have the potential for broad-spectrum anti-infective activity. Targeting the synthesis of the fungal cell wall, chitin synthase inhibitors could be expected to act synergistically with current fungicides used for eumycetoma, which mostly target the cell membrane or cell wall as well (Table 1). Unfortunately, chitin synthase had only one inhibitor listed, polyoxin B, which violates druglikeliness criteria besides being estimated to have low GI absorption.

Succinate dehydrogenase inhibitor Thifluzamide, had displayed broad-spectrum activity against Basidiomycetes, particularly in plant diseases caused by Alternaria atternata, A. Solani, and B. cinerea which are all known eumycetoma pathogen (Maienfisch and Stevenson, 2016). From 23 molecules drug-likeliness is satisfied with only 4 molecules with one violation to Lipinski role of 5 (Table 4).

Completely different classes of targets that are not conserved in the human host are the fungal melanogenic enzymes scytalone dehydratase and polyketide synthase. Scytalone dehydratase (also

known as scytalone 7,8-hydro-lyase) is involved jointly with 1,3,6,8-tetrahydroxynaphthalene reductase in the biosynthesis of fungal melanin, and polyketide synthase (also known as norsolorinic acid anthrone synthase) is involved in aflatoxin biosynthesis, antibiotic synthesis, and regulates polyketide synthesis and pentaketide cyclization in fungal melanin biosynthesis (Van Lanen and Shen, 2008; Chen et al., 2006). Melanin was identified as an underlying cause of both virulence and drug resistance in pathogenic fungi (Esbelin et al., 2013; Nosanchuk and Casadevall, 2006; Polak, 1990). Molecules targeting melanogenic enzymes could serve as either single treatment, or combined with other fungicides; the combination would attenuate melanogenesis and enhance treatment efficacy. *M. mycetomatis*, which is a black melanized fungal species, was only inhibited by several folds of MICs for azole antifungals when melanin was added to the fungal suspension during *in-vitro* screening (Welsh et al., 2014b; van de Sande et al., 2007b). The species *F. pseudograменiarum* and *G. candidum* returned negative HMMer bit scores for the domains PKS and SD, which indicate that they may lack this pathway for melanin biosynthesis (Figure 9). Several articles and expert communications have suggested agrochemical fungicides as a potential hub for new drugs to target human fungal infections (Lamberth, 2018; Jampilek, 2016; Delaney et al., 2006). The methods adopted in the discovery of agrochemical fungicides are closely linked to pharmaceutical R&D. The main differences concern the safety profile and solubility. Fungicides targeting adenosine deaminase and succinate dehydrogenase possess the desired features of drug-likeliness to a large extent (Table 4), and could, therefore, be starting points for repurposing attempts. Most of the molecules tabulated are orally bioavailable with larger differences in permeation of BBB and skin partitioning (Table 4). The crucial optimization to adapt agrochemical fungicides for future eumycetoma treatment would involve oral bioavailability, pharmacokinetics, and safety. In conclusion, our approach supports the idea of drug repurposing from the

agrochemical sector for the treatment of eumycetoma. It suggests several target enzymes, with a larger diversity of biochemical pathways than targeted by the current eumycetoma treatment. The predicted conservation of known fungicide target enzymes bears the potential for broad-spectrum fungicides to treat eumycetoma, which is necessary given the diversity of fungi that can cause eumycetoma. Effort and time can be spared when starting with molecular structures that possess both fungicide activity and drug-likeliness. Which also represents an opportunity, for the pharmaceutical and agrochemical industries to bridge their efforts in combating fungal pathogens.

3.7. Supplementary information:

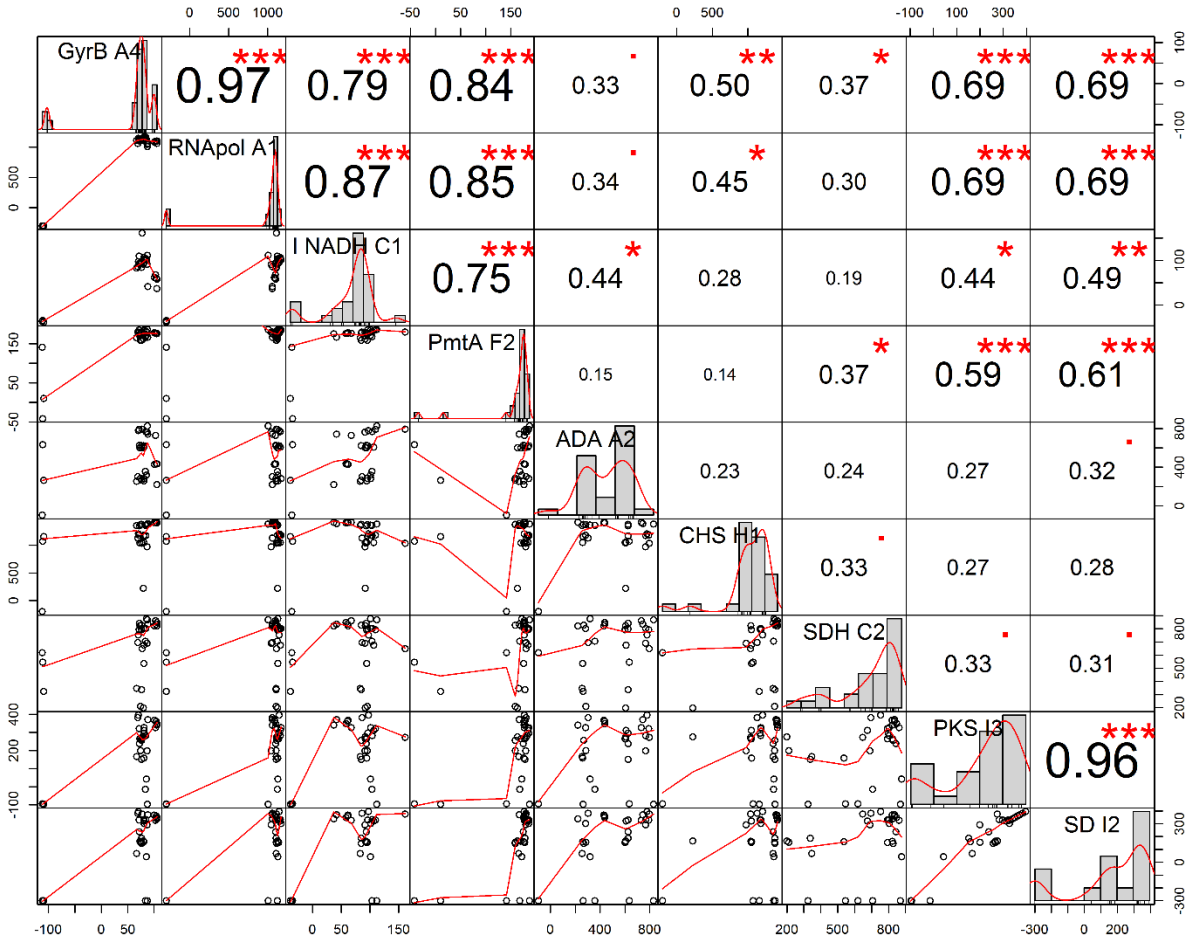

Figure S1: The correlation in bit scores between study protein domains

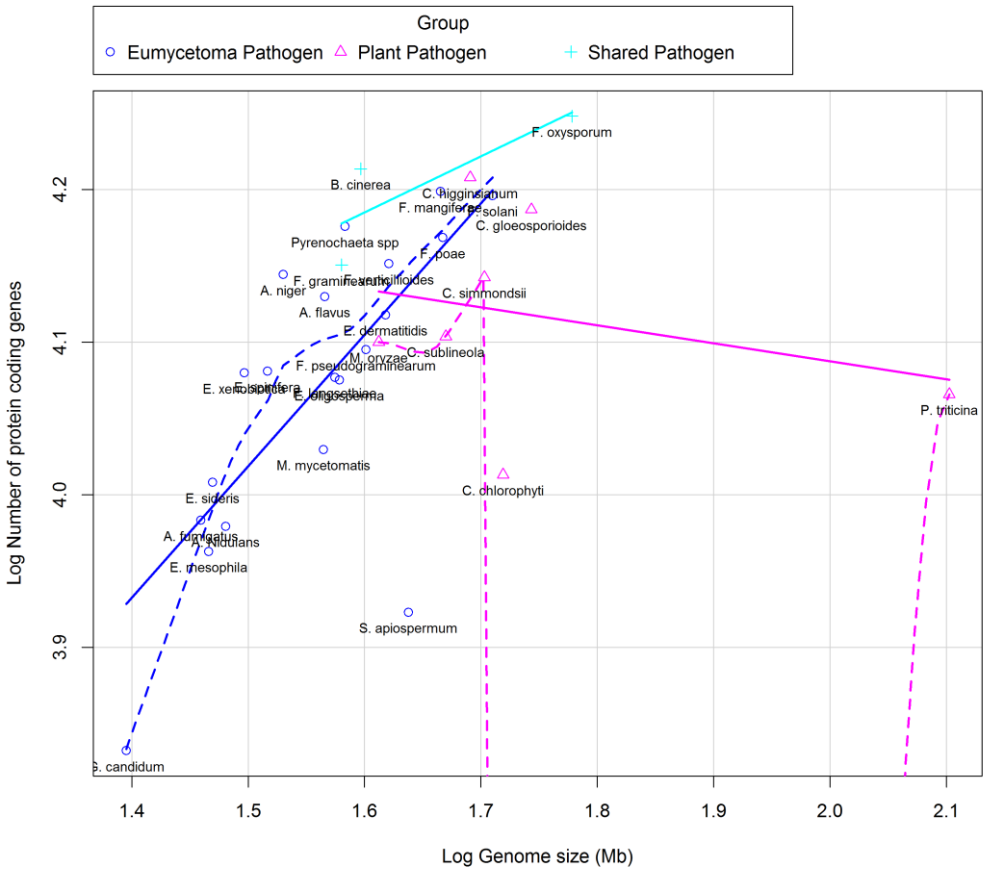

Figure S2: Genomes versus number of protein coding genes for all species; species in blue are eumycetoma pathogens, species in cyan are shared species, and species in magenta are plant pathogens

Table S1: Individual species contribution to variation estimating for PC1 and PC2

Species	PC1	PC2
A. flavus *	0.01	0.73
A. fumigatus *	26.80	17.21
A. niger *	0.31	0.36
A. Nidulans *	28.28	2.54
E. dermatitidis *	1.39	3.33
E. mesophila *	0.38	0.02
E. oligosperma *	1.03	0.33
E. sideris *	0.89	3.59
E. spinifera *	0.97	0.58
E. xenobiotica *	1.03	0.48
F. langsethiae *	0.00	4.39
F. mangiferae *	0.02	0.48
F. poae *	0.34	0.00
F. pseudograminearum *	0.12	25.62
F. solani*	0.14	2.96
F. verticillioides *	0.10	0.16
G. candidum*	0.02	8.10
M. mycetomatis *	1.65	0.29
Pyrenochaeta spp *	0.50	2.06
S. apiospermum *	0.59	1.71
M. oryzae -	27.34	16.89
C. chlorophyti -	1.39	0.51
C. gloeosporioides -	1.47	0.47
C. higginsianum -	0.91	0.72
C. simmondsii -	1.07	0.04
C. sublineola -	0.79	0.89
P. triticina -	1.76	0.39
F. graminearum +	0.28	0.05
F. oxysporum +	0.04	5.11
B. cinerea +	0.37	0.02

3.8. Acknowledgments

- We would like to thank Dr. Matthias Witschel of BASF agrochemicals for opening the collaboration with our institute in agrochemicals repurposing attempts against NTDs and for supplying a library of agrochemical fungicides for future *in-vitro* screening work against mycetoma causative fungi.
- Also, we would like to thank Dr. Jan Hattendorf for his help with adjusting the graphs using R-code.
- Major analysis tool in this work was Ugene Unipro bioinformatics kit, we had great support from the Ugene Unipro support team, especially Mrs. Olga Golosova.

3.9. Funding:

This work was funded by the Swiss government through the Swiss Excellence Scholarship for Foreign Scholars (ESKAS), many thanks for their support and special care for mycetoma patients and the contribution to many aspects from clinical to bench work in Sudan and in Swiss institutions.

Chapter -4-

Potential virulence factors of *Madurella mycetomatis* identified by comparative genomics with *Aspergillus* spp.

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Conceptualization: PM, MH

Data capturing and management: PM, MH

Data analysis: PM, MH

Interpretation of results: PM, MH

Paper writing: PM, MH

Note: To be submitted in the current format

4.1. Abstract:

Madurella mycetomatis is the main causative agent of fungal eumycetoma, a devastating and disfiguring infection of the skin and deeper tissues. eumycetoma is a highly neglected disease: there is no vaccine, no satisfactory therapy, and a lack of diagnostics. Fungal mycetoma also is an enigmatic disease, as there are large knowledge gaps concerning its epidemiology and the basic biology of host-pathogen interaction. Here we aimed to identify virulence factors of *M. mycetomatis* by comparative genomics to selected species of the genus *Aspergillus*. Every predicted protein of *M. mycetomatis* was used as blastp query against four noninvasive, apathogenic species (*A. rambellii*, *A. campestris*, *A. neoniger*, *A. novofumigatus*) and against four invasive pathogens (*A. nidulans*, *A. niger*, *A. fumigatus*, *A. terreus*). This identified all *M. mycetomatis* proteins with higher-scoring orthologues in the pathogens than in the apathogenic controls. The same bioinformatic pipeline was also run with *A. parasiticus* as input proteome. Both approaches, using *M. mycetomatis* as an out-group query or *A. parasiticus* as an in-group query, returned myosin-related proteins as the hits with the highest bias towards the pathogenic *Aspergillus* spp. This suggests an important role for motor proteins as virulence factors of saprophytic fungi not only in plants but also in the human host.

4.3. Introduction:

It is in the very nature of the saprophytic fungi that they possess some of the characteristics required to infect mammals. They make spores and have a protective cell wall; they also have transporters to import organic nutrients such as sugars or amino acids, and the ability to grow hyphae enables them to infiltrate foreign tissue (Brunke et al., 2016; Brand, 2012). Saprophytes that dwell in decomposing organic matter withstand heat stress better than other fungi, and they can survive at our body temperature (Köhler et al., 2017). While such features are necessary for the successful invasion of a mammalian host, they are not sufficient. Pathogenicity usually also requires a certain degree of immunosuppression on the side of the host. This was observed with dramatic clarity in AIDS patients before the introduction of today's highly effective antiretroviral combination therapies (Ramos-e-Silva et al., 2012). Yet host immunosuppression is not sufficient for infection either, since not all saprophytic fungi are opportunistic pathogens. So, what else is needed for saprophytes to become pathogens?

Virulence factors are best known for the plant-pathogenic fungi. They comprise extracellular matrix components that render the spores adhesive to leaves and allow to penetrate into the plant after germination, often supported by mechanical force generated through turgor pressure (Doehlemann et al., 2017). Once inside the host, the fungus will be confronted with defense responses such as oxidative stress, which it overcomes by producing catalase and superoxide dismutase (Mayer et al., 2001). In addition, phytopathogenic fungi transcriptionally silence plant defense responses by secreting small inhibitory RNAs (Huang et al., 2019). Several plant-pathogenic fungi can also cause disease in humans. In particular, *Aspergillus* spp. can cause aspergillosis after inhalation of spores or traumatic inoculation through the skin. The main causative agents of aspergillosis are *A. fumigatus* and *A. flavus*, but many other species of the genus are opportunistic pathogens as well (Sugui et al., 2014). *Aspergillus* virulence factors specific to the mammalian host comprise apoptosis-inducing toxins, immunosuppressive

peptides (Paulussen et al., 2017; Lee et al., 2015), and surface carbohydrates for cytoadherence (Wasylnka et al., 2001).

Much less is known about the virulence factors of *Madurella mycetomatis*, the main causative agent of fungal mycetoma (also called eumycetoma). Eumycetoma is a devastating and disfiguring neglected tropical disease. While the initial lesions caused by *M. mycetomatis* affect subcutaneous tissues, they will invasively progress to deeper tissues and bone (Zijlstra, van de Sande, Welsh, et al., 2016). There is no vaccine for eumycetoma, no satisfactory chemotherapy, and no adequate diagnostics. Fungal mycetoma is not only neglected, it is also an enigmatic disease. There are large knowledge gaps concerning its epidemiology and the basic biology of host-pathogen interaction, rendering *M. mycetomatis* an elusive pathogen (Zijlstra, van de Sande, Welsh, et al., 2016). Here we aim to make use of the published *M. mycetomatis* genome sequence (Smit et al., 2016) to discover potential virulence factors. Our working hypothesis is that convergent evolution of opportunistic parasitism in saprophytic fungi has generated similar virulence factors in different species, and that such virulence factors can be discovered by comparative genomics. For this purpose, we are screening all *M. mycetomatis* proteins against the proteomes of invasive, pathogenic *Aspergillus* species and noninvasive, apathogenic *Aspergillus* species as controls.

4.5. Materials and Methods:

4.5.1. Proteomes

The predicted proteome of *M. mycetomatis* strain m55 was downloaded in August 2019 from Ensembl (www.fungi.ensembl.org/). The predicted proteomes of the *Aspergillus* species were downloaded in September 2019 from the Genome resource of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/genome/). See Table 1 for the sequence identification numbers.

4.5.2. Blast searches:

The proteome-wide blastp (Altschul et al., 1990) surveys were performed with default gap penalties, the Blosum62 scoring matrix (Henikoff and Henikoff, 1992), and an expectation-value (E) threshold of 1. Self-made Perl scripts were used to automate the searches and parse the results; the Perl scripts are available on request. All computation was performed on a BioLinux system (Field et al., 2006).

4.5.3. Phylogenetic trees:

Multiple sequence alignments were performed with the MUSCLE algorithm (Edgar, 2004) using default parameters. Neighbor-Joining trees were constructed based on evolutionary distance measured with the JTT scoring matrix (Jones et al., 1992). Bootstrapping tests were performed on 1000 resampled trees. All evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

4.6. Results:

4.6.1. Selection of the fungal pathogens and control species

Our approach was based on the recently established phylogenomic tree of the genus *Aspergillus* (Steenwyk et al., 2019). As a basis for comparative genomics, we wanted to select noninvasive control species that are pathogenic neither to animals nor to plants. However, of the completely sequenced and annotated genomes of *Aspergillus* spp. available at NCBI Genomes, only five were from species that had been classified as apathogenic; these were *A. rambellii*, *A. campestris*, *A. neoniger*, *A. novofumigatus*, and *A. turcosus* (Steenwyk et al., 2019). The latter had to be eliminated from the control set after the report of several cases of invasive aspergillosis due to *A. turcosus* (Parent-Michaud et al., 2019). The remaining four control species were supplemented with four known pathogens of matching phylogenetic diversity: *A. nidulans*, *A. niger*, *A. fumigatus*, and *A. terreus*. Figure 1A depicts the phylogenetic relationship of the eight included species. They possess similar numbers of protein-coding genes and the

proteins are of similar length (Table 1). The mean proteome size was 10,049 for the pathogenic and 10,248 for the control species; the mean protein length was 508 for the pathogens and 483 for the controls.

4.6.2. Proteome-wide comparison with *Madurella mycetomatis*

The genome of *Madurella mycetomatis* encodes 10,707 predicted proteins (Smit et al., 2016, p. 55). For each, a heuristic local alignment was made to all the proteins of *A. rambellii*, *A. campestris*, *A. neoniger*, *A. novofumigatus*, *A. nidulans*, *A. niger*, *A. fumigatus* and *A. terreus* using blastp (Altschul et al., 1990). This allowed to identify for every *Madurella* protein the best-scoring hit – and thus presumably the most closely related protein – in the different species of the genus *Aspergillus*. The complete blastp output is available as Supplementary Tables S1 to S8, and the compilation of the highest alignment scores as Supplementary Table S9 in MS Excel format.

Forty-seven proteins turned out to be specific to *M. mycetomatis* as they did not return a single hit below the threshold expectancy (E) value of 1 from any of the *Aspergillus* spp. For subsequent analyses, we only included those *M. mycetomatis* proteins (n=9,722) that returned hits from at least three of the four *Aspergillus* species in at least one of the two subsets, i.e. pathogens or controls. Eukaryotic housekeeping proteins that are encoded by single-copy genes (Ren et al., 2016) and that occurred in all eight *Aspergillus* spp. were used as benchmarks to construct phylogenetic trees. Neighbor-joining trees of the eukaryotic translation initiation factor 1A (eIF1A) orthologues and RNA polymerase II subunit A (RPB1) orthologues are shown in Figures 1B and 1C, respectively. While these trees did not perfectly reproduce the phylogeny (Figure 1A) as it had been established based on a sophisticated multigene matrix (Steenwyk et al., 2019), they confirmed that the four selected invasive *Aspergillus* spp. are not

more closely related to each other than to the four control species. The trees also depicted *M. mycetomatis* as a clear outgroup (Figures 1B and 1C).

Table 1. Fungal species used in this study, their total number of proteins, and average length of the proteins. The genome IDs refer to NCBI genomes for *Aspergillus* spp. and to Ensembl for *Madurella*.

Species	Genome ID	Pathogen?	No. proteins	Avg. length
M. mycetomatis	asm127576v2	Y	10707	495
A. rambellii	37156	N	7761	526
A. campestris	66460	N	9756	479
A. neoniger	70036	N	11939	476
A. novofumigatus	66459	N	11534	451
A. nidulans	17	Y	9556	531
A. niger	429	Y	10609	508
A. fumigatus	18	Y	9630	492
A. terreus	53	Y	10401	499
A. parasiticus	12976	Y	8645	512

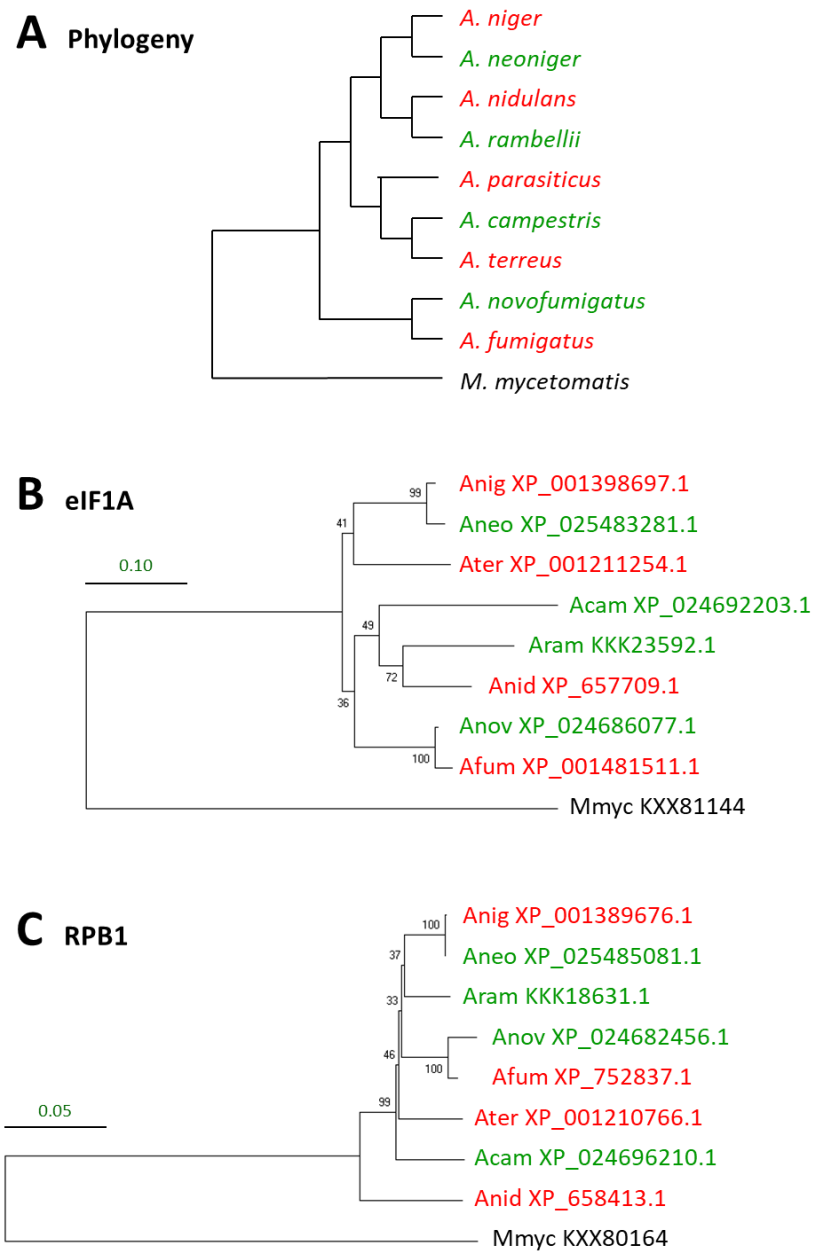

Figure 1. Phylogenetic relationship between *M. mycetomatis*, the four included pathogenic *Aspergillus* species (red), and the four apathogenic control species (green). A) Cladogram representing the established phylogeny (Steenwyk et al., 2019). B) Neighbor-joining tree of eukaryotic translation initiation factor 1A (eIF1A) orthologues. C) Neighbor-joining tree of RNA polymerase II subunit A (RPB1) orthologues. The scale bar indicates amino acid changes per site; the small numbers indicate percent positives of 1000 rounds of bootstrap resampling.

4.6.3. Identification of potential virulence factors in *Madurella mycetomatis*

The average local alignment score of the 9,722 *M. mycetomatis* proteins was 315 against the invasive, pathogenic *Aspergillus* species and 314 against the noninvasive, apathogenic *Aspergillus* species. To identify potential virulence factors of *M. mycetomatis*, we were looking for proteins that had distinctly higher local alignment scores against the pathogens than against the controls. Figure 2 shows for every *Madurella* protein the average alignment score against the pathogens plotted vs. the average alignment score against the apathogenic *Aspergillus* spp. This graphical representation singled out myosin type-2 heavy chain 1 (gene ID KXX80068) as the *M. mycetomatis* protein with the highest difference in score between the pathogenic *Aspergilli* and the controls (Figure 2).

To systematically identify potential virulence factors based on the proteome-wide blast surveys, we proceeded as follows: all the *M. mycetomatis* proteins were considered that had returned hits from all four pathogens (*A. nidulans, A. niger, A. fumigatus, A. terreus*) with an average local alignment score of at least 50 (n=8,009; Supplementary Table S9). *M. mycetomatis* proteins were considered potential virulence factors if they had a relative difference of at least 0.6 in the average alignment scores of pathogens vs. controls. The relative difference was calculated as ($Score^{patho}$ - $Score^{ctr}$) / $Score^{patho}$. This returned 44 different proteins, of which 31 were functionally annotated (Table 2). Besides the myosin identified in Figure 2, this list included orthologues of known virulence factors from other species, as well as a surprising number of enzymes involved in nucleic acid synthesis or degradation (Table 2).

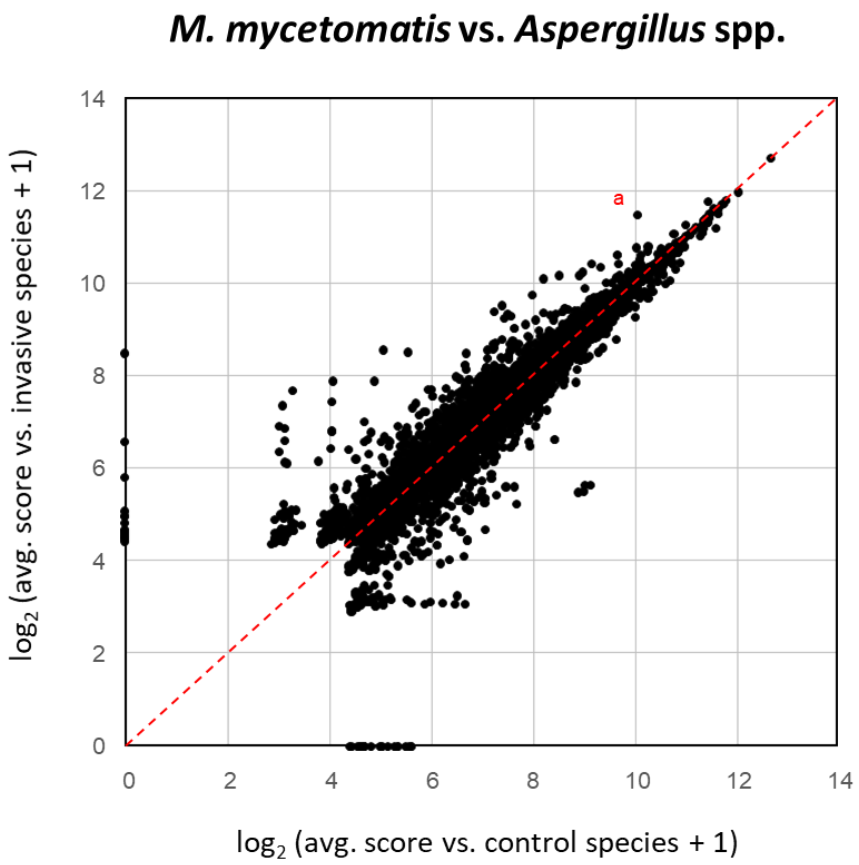

Figure 2. Graphical representation of the blastp local alignment scores obtained for every *M. mycetomatis* protein against the pathogenic (y-axis) and apathogenic (x-axes) species of *Aspergillus*. The addition of 1 to all scores was done to allow plotting on a logarithmic scale also for *Madurella* proteins that returned 0 hits. The protein marked ***a*** is *M. mycetomatis* myosin type-2 heavy chain 1.

Table 2. All *M. mycetomatis* proteins that are more conserved in invasive than in non-invasive *Aspergillus* spp. by a relative difference of at least 0.60 and that could be functionally annotated. There were an additional 13 proteins annotated as hypothetical (Supplementary table S9). Acam, *A. campestris*; Aneo, *A. neoniger*; Anov, *A. novofumigatus*; Aram, *A. rambellii*; Afum, *A. fumigatus*; Anid, *A. nidulans*; Anig, *A. niger*; Ater, *A. terreus*.

Protein ID	Predicted function	Acam	Aneo	Anov	Aram	Afum	Anid	Anig	Ater
Motor function									
KXX80068	Myosin type-2 heavy chain	504	508	509	2828	2856	2887	2864	2887
KXX78552	Myosin-7	43	54	53	446	478	284	462	464
KXX80207	Kinesin light chain	50	62	70	33	82	240	61	199
Nucleic acids									
KXX82433	Copia protein	0	0	31	0	32	112	137	112
KXX81367	Exonuclease	0	0	0	686	748	751	733	745
KXX74984	DNA helicase srs2	33	141	34	514	666	596	690	694
KXX73120	DNA helicase PIF1	0	32	33	645	649	495	647	655
KXX81366	Exonuclease	32	0	145	842	868	858	867	875
KXX79611	DNA repair protein rhp51	45	46	45	635	639	636	636	637
KXX82566	Ribonucleoside-PP reductase	32	0	328	0	331	371	30	295
KXX80270	Poly(A) polymerase 1	115	88	114	100	115	89	114	819
KXX81399	5'-nucleotidase	80	83	80	73	99	599	87	73
KXX77353	DNA damage-inducible protein	142	32	31	392	404	389	382	390
Metabolism									
KXX79334	Tyrosinase	71	42	47	45	64	103	239	286
KXX79244	Imidazolonepropionase	36	90	38	34	35	93	433	76
KXX82805	Tryptophan 2-halogenase	35	523	35	32	64	609	521	526
KXX77307	Esterase TesA	46	45	51	0	34	40	60	256
KXX75950	Alkaline phosphatase D	62	54	616	69	618	697	54	763
KXX76187	Methylglucuronoyl methylesterase	0	0	204	32	284	30	31	266
KXX76086	N-acetyltransferase	40	36	51	52	52	202	35	160
Signaling									
KXX78409	3-phytase	32	33	33	34	32	764	33	718
KXX72837	Heterokaryon incompat. protein	52	61	40	32	45	632	52	749
KXX81888	Platelet-activating factor	0	0	52	0	46	146	43	49
KXX82422	Platelet-activating factor 2	0	33	36	32	36	405	35	39
Glycosylation									
KXX74124	Ceramide galactosyltransferase	0	31	0	33	207	33	32	177
KXX74760	Beta-galactosidase BoGH2A	51	76	67	64	69	615	84	83
KXX79581	Beta-galactosidase BoGH2A	91	113	100	108	96	914	108	112
Protein-protein interaction									
KXX77273	Nucleoprotein TPR	44	41	36	1068	1060	1176	1120	1065
KXX79910	Ankyrin-2	85	75	87	171	246	466	361	393
KXX79186	Ankyrin repeat protein	326	112	122	110	552	220	621	395
Other									
KXX81388	Pea pathogenicity protein 2	0	196	33	33	162	162	267	167

4.6.4. Including *Aspergillus parasiticus* as the query

We performed the same proteome-wide blastp exercise also with *A. parasiticus*, an aflatoxin-producing, invasive species. *A. parasiticus* is an in-group query (Figure 1A); it is closely related to *A. flavus* but has a smaller predicted proteome of only 8,645 proteins (Table 1). We repeated the bioinformatic pipeline using the same inclusion criteria as for the *M. mycetomatis* proteins. This yielded 8,421 *A. parasiticus* proteins that had blastp hits from at least three *Aspergillus* spp. in either subset, pathogens or controls. As expected, the local alignment scores to the best-scoring hits were higher for the *A. parasiticus* proteins than for the *M. mycetomatis* proteins: on average it was 555 against the invasive, pathogenic *Aspergillus* species and 546 against the noninvasive, apathogenic *Aspergillus* species. All the data are in Supplementary Table S10 as a MS Excel file. Plotting the average alignment scores of the *A. parasiticus* proteins to the best-scoring hits in the pathogenic *Aspergilli* vs. the apathogenic *Aspergilli* again singled out two myosin-related proteins as the hits with the strongest bias towards the pathogens (Figure 3). These were a myosin head motor domain protein (gene ID KJK67962) and a myosin-like protein 1/2 (MLP1/2) orthologue (gene ID KJK64803) of *A. parasiticus*.

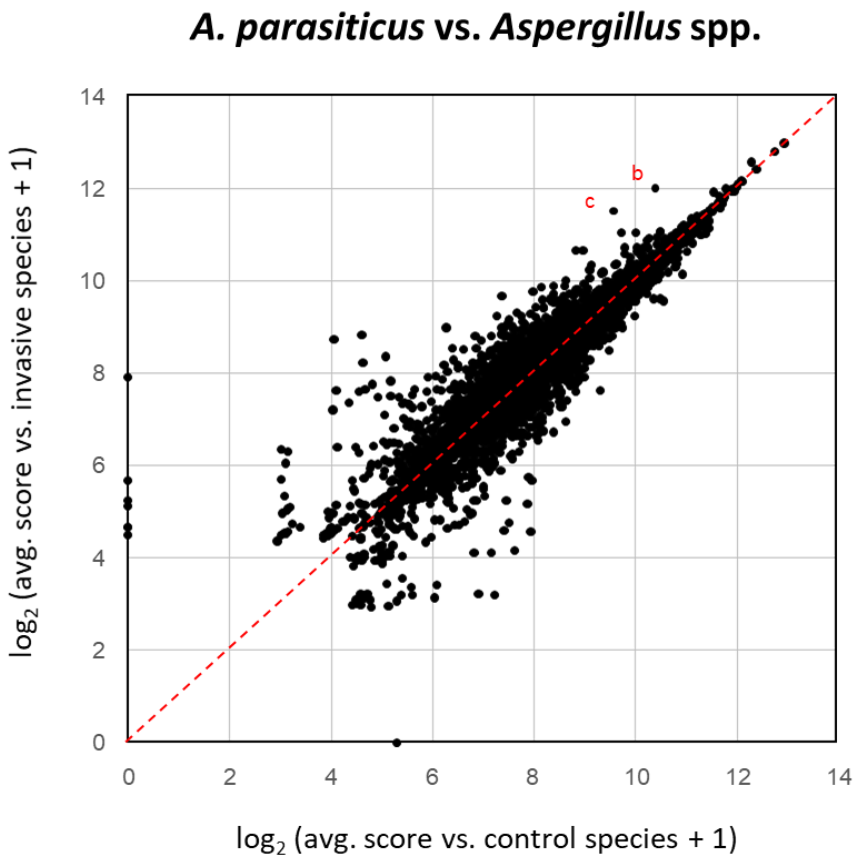

Figure 3. Graphical representation of the blastp local alignment scores obtained for every *A. parasiticus* protein against the pathogenic (y-axis) and apathogenic (x-axes) species of *Aspergillus.* The addition of 1 to all scores was done to allow plotting on a logarithmic scale also for *A. parasiticus* proteins that returned 0 hits. The proteins marked ***b*** and ***c*** are a myosin head motor domain protein and myosin-like protein 1/2-like protein, respectively.

4.6.5. Clustering analysis and the Principal components.

The spreading of the blastp scores of *M. mycetomatis proteins* mapped against the proteomes from both pathogenic and apathogenic *Aspergillus spp.* showed 5 distinct clusters based on the Elbow graph computed using k-means clustering (Lloyd, 1982) (Figure 4). The cluster plot (Figure 5) provided a visualization for the location of each gene in the cluster hyperspace. These findings suggest differences in gene specific blastp scores across the query species.

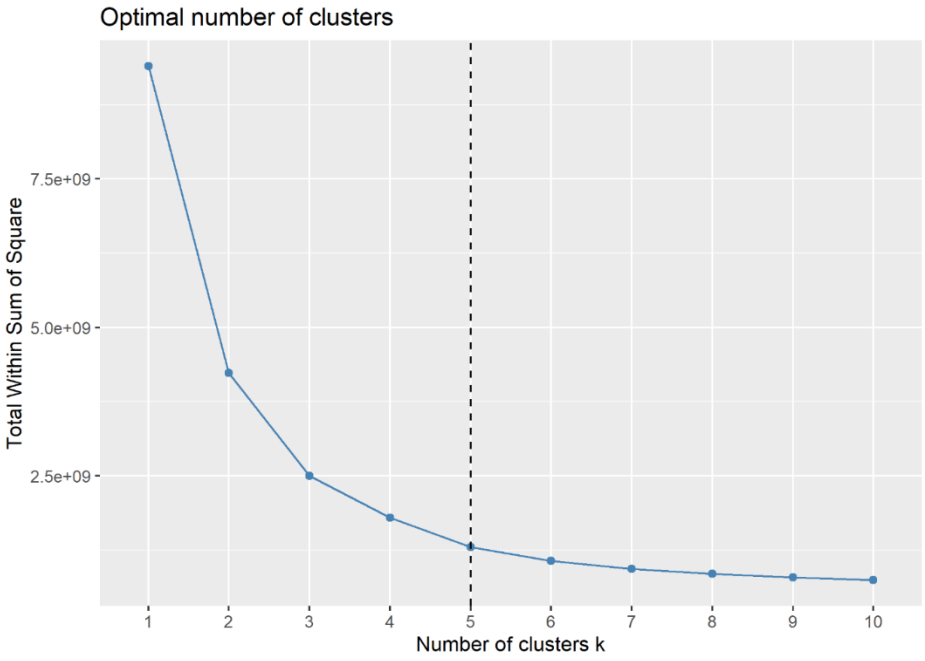

Figure 4. Optimum number of protein clusters computed using Elbow method with k-means clustering showing that at 5 clusters the total within-cluster sum of square (WSS) is at the minimum

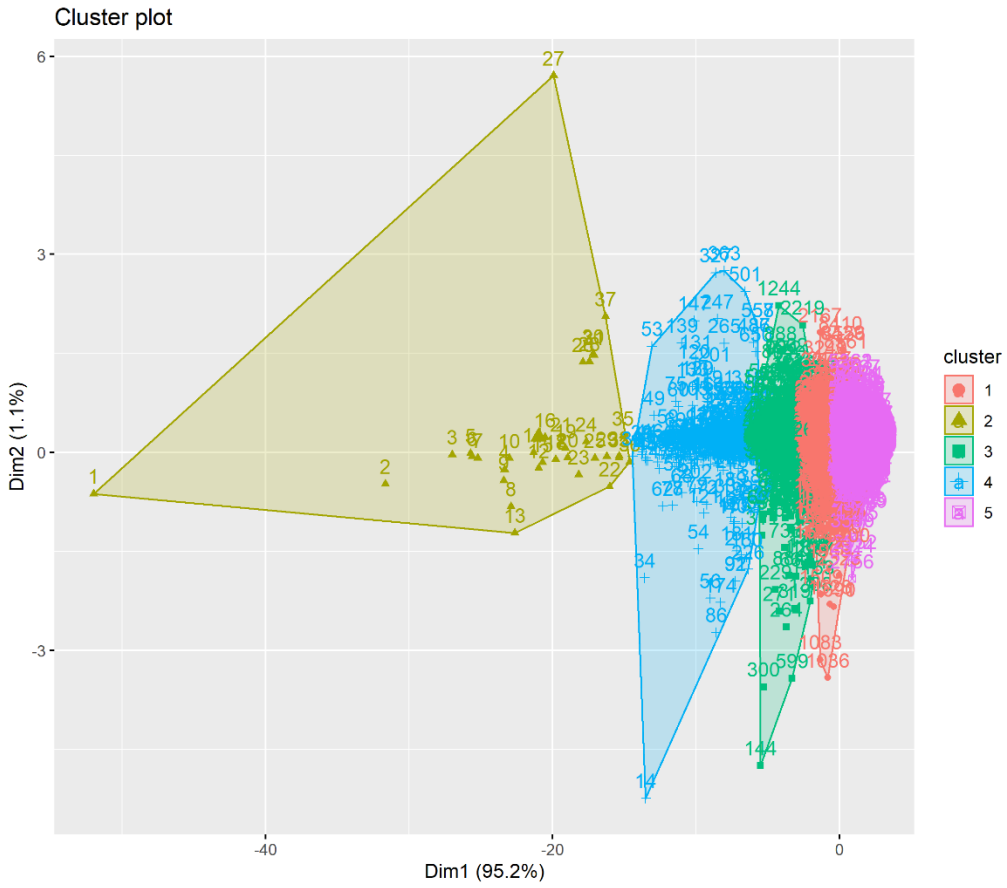

Figure 5. Cluster plot showing the location of each protein in the hyperspace showing the two main components of variation.

4.7. Discussion:

The phylogenetic tree of *Aspergillus* spp. clearly indicates multiple origins of parasitism, for pathogens of plants as well as for pathogens of animals (Steenwyk et al., 2019). This renders the *Aspergilli* excellent subjects to study the convergent evolution of virulence factors that are required for a saprophytic fungus to become an opportunistic pathogen. Here, we use *Aspergillus* spp. as a sieve to identify potential virulence factors of *M. mycetomatis*. We have implemented proteome-wide blastp surveys to identify *Madurella* proteins that return a higher average alignment score to their best-scoring hits in pathogenic, invasive *Aspergilli* than in apathogenic, noninvasive *Aspergilli*. This straightforward approach readily produced a list of candidate virulence factors from *M. mycetomatis*. However, there are two main caveats for this approach (besides the obvious drawback that it is very difficult to experimentally validate the identified candidates (Trieu et al., 2017)). First, some proteins might be missing from the predicted proteome files of the selected *Aspergillus* species due to gaps in sequencing coverage or sequence assembly. This would lead to artefacts in our pipeline. Second, our approach critically relies on a set of apathogenic *Aspergillus* species that serve as controls. However, absence of pathogenicity cannot be proven. For instance, *A. turcosus* was classified as a harmless species (Steenwyk et al., 2019), but only until the genotyping of suspected *A. fumigatus* clinical isolates identified four to be *A. turcosus* (Parent-Michaud et al., 2019). Clearly, our established analysis pipeline will gain in predictive power as the genomes of further species and isolates of the genus *Aspergillus* will be sequenced at high coverage and high quality of gene prediction.

Still, we think that the present proteome-wide blastp survey produced informative results. A stringent (but arbitrary) cut-off of 60% relative difference in the average scores against pathogenic and control *Aspergillus* spp. proteomes returned a manageable list of candidates that contained several M. *mycetomatis* orthologues to known virulence factors (Table 2).

Phytase is a virulence factor in *Candida albicans*, required for tissue adhesion penetration (Tsang et al., 2017). Tyrosinase is the rate-limiting enzyme for the synthesis of melanin, a protective agent and ubiquitous virulence factor in pathogenic fungi (Revankar and Sutton, 2010). The pea pathogenicity proteins are small effector proteins that are secreted by several plant-pathogenic fungi (Rep, 2005). Other hits, such as the identified nucleic acid synthesis or degradation enzymes, are less intuitive to explain and might not be related to pathogenicity.

The most convincing hits in our opinion were the myosins and related proteins. Myosins are known virulence factors of the crop pathogens *Ustilago maydis* (Schuchardt et al., 2005; Weber et al., 2003) and *Magnaporthe oryzae* (W. Tang et al., 2018; Guo et al., 2017). Myosin-based motors are important for plant pathogenic fungi not only for hyphal growth and the associated cytoplasmic streaming and nuclear movement (Xiang, 2018; Renshaw et al., 2016), but also for the secretion of toxins (G. Tang et al., 2018; Song et al., 2013). The finding that the out-group query *M. mycetomatis* (Figure 2) and the in-group query *A. parasiticus* (Figure 3) both returned myosin-related proteins as the hits with the highest difference between harmless and pathogenic *Aspergilli*, indicates the importance of motor proteins as virulence factors of saprophytic fungi not only in plants but also for pathogenesis in the human host.

3.9. Funding:

This work was funded by the Swiss government through the Swiss Excellence Scholarship for Foreign Scholars (ESKAS), many thanks for their support and special care for mycetoma patients and the contribution to many aspects from clinical to bench work in Sudan and in Swiss institutions.

Chapter -5-

General Discussion and Conclusions

From the wide spectrum of the thesis topics, which eventually demonstrate how neglected is eumycetoma? We have collectively answered some questions hoping we offer a common ground for epidemiologists and drug discovery experts to arrive into better options for eumycetoma patients, these questions were as follows:

What are the current therapeutic challenges with eumycetoma? The drug discovery for eumycetoma is a pressing priority since the current medical treatment is unsatisfactory, the development of adverse events and drug resistance are major challenges (Welsh et al., 2014b). Furthermore, both the Food and Drug Administration (FDA) of the United States and the European Medical Agency (EMA) urged the limited use of Ketoconazole and Itraconazole which are major imidazole medicines in use in South American, Asian, and African countries (Szefler et al., 2006). The current therapeutic gaps urge the science community to work together and with all available measures and resources to bring safe, efficacious, and affordable new drugs. Many challenges are expected in the way to achieve this target, more especially with the problems encountered with the currently used antifungal drugs. The problems are of two natures, the first aspect is the subclinical effectiveness of frequently used itraconazole and ketoconazole imidazole antifungals drugs, and secondly the high cost of the new generation which includes posaconazole, voriconazole, and isavuconazole.

What are the etiologic agents of eumycetoma? While working on the proposal of this thesis, we have found many fungal species attributed to eumycetoma, however, some other species were only found in case reports from backdated literature. Accordingly, we have conducted numerous 130 species of fungi had been identified as eumycetoma pathogens either from case reports, case series, review articles, or expert communications, plus13 species partially identified with only their genus mentioned. From a taxonomic perspective, eumycetoma is caused mainly by Ascomycetes, as this fungal phylum constitutes 94% of the identified species.

What are the potential sources of eumycetoma infection? From the systematic review we performed we always examined the sources of infection; however, the data wasn't that informative. We found eumycetoma couples to traumatic inoculation in most of the cases with the exposure to soil, water, plants (more specially the thorny ones), and via inhalation in case of S. apiospermum pulmonary eumycetoma in patients with cystic fibrosis. Moreover, eumycetoma was also reported as iatrogenic disease establishes after septic surgical procedure.

Is eumycetoma a geo-restricted infection? Eumycetoma was always referred to as tropical disease that only extend to the subtropics, this would stand true for some species including S. apiospermum and *L. sengalensis* but certainly not for all the fungal species as both *M. mycetomatis* and *M. grisea* shows a worldwide pattern of distribution beyond the predefined circumscription of the tropics.

Are there any validated drug targets that could be targeted in the future to develop fungicides for eumycetoma? To answer this question, we planned to examine the potential of agrochemical fungicides. Agrochemical fungicides provided the existing activity against fungi which were have compared to eumycetoma fungi using comparative genomics and we concluded that adenosine deaminase, succinate dehydrogenase, and chitin synthase contributed the least to variation of the profile score vectors, indicating a reliable conservation and hence a potential for broad-spectrum inhibitors. However, both adenosine deaminase and succinate dehydrogenase were found to be conserved in human proteome which necessitates further evaluation of cross-toxicity when repurposed for be administered to human.

Regarding *M. mycetomatis*, can we find virulence genes that explain its worldwide reports? We performed a comparative genomic study using the proteome from *M. mycetomatis* and from *A. parasiticus* as known eumycetoma pathogens and we performed a genome-wide blastp search against the genomes of both pathogenic and apathogenic fungi from the genus

Aspergillus. We were able to identify myosin-related proteins as the hits with the highest difference between harmless and pathogenic *Aspergilli*, indicates the importance of motor proteins as virulence factors of saprophytic fungi not only in plants but also for pathogenesis in the human host

In conclusion, eumycetoma is a caused by numerous fungal species of diverse taxonomical and geographical patterns of distribution. Considering the reporposing potential of agrochemical fungicides against eumycetoma pathogenic fungi we found hope with potential drug targets to be considered in the upstream drug discovery efforts. Finally, we have also found myosine related proteins to have clear bias to the eumycetoma pathogens when compared to apathogenic fungi within the same phyla of taxonomy. We hope that our work would provide a helpful insights that collectively could help eumycetoma control and raise key questions withregards to the epidemiology of eumycetoma.

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Annexes:

Report on the screening of agrochemical fungicide library and the library of selected diamidine:

The thesis course included an obkective that was not fulfilled despite the allocation of 1 years and a half to it, the objective was to screen a agrochemical fungicide library and the library of selected diamidine (Table 1 and 2). The screening was preceeded with optimization of the screening protocol which was accompanied with many difficulties which is to be herein summarized.

Table 1: The library of agrochamical fungicides:

Table 2: The library of selected diamidine molecules

Technical report from the laboratory work in Sudan

Mycetoma drug discovery:

Technical report: in-vitro screening in Sudan:

The in-vitro screening was conducted using a protocol used in UST. It is developed by the mycetoma drug discovery group headed by prof. Sami Khalid. They are still working on the validation as they revealed in the very final steps in my work in their laboratory (the protocol is attached!)

Profits from the laboratory work:

In this trip to Sudan we were able to secure a reliable starting laboratory infrastructure for pursue of all experiments intended during the course of my Ph.D.

Secondly, I had the hands-on exercise regarding experiments. I had the chance to carry-out in-vitro assays and interpret the results and understand all potential sources of inaccuracy.

The initial results:

The experiments were adjusted after several attempts due to many reasons.

- First, the strain used was resistant to voriconazole which we used first as standard drug. This finding led us to re-plan the test after looking into the susceptibility.
- Secondly, I used RPMI medium with L-glutamate and $NaHCO_3$ buffer. This wasn't stated in UST protocol. This why I used the RPMI with L-glutamate only (pH adjusted to ~7 using NaOH.
- Then, the reference strain was used again, and the plates were seeded with 7 antifungals only to reveal the resistance issue. The result showed resistance to all drugs except for caspofungin with MIC of 64 µg/ml.
- The susceptibility patterns recorded from prior experiments done by UST team showed no similar susceptibility pattern.
- Strains resistant to voriconazole and posaconazole were found. Itraconazole and ketoconazole remained active in all the set of 12 strains in the laboratory.
- The final experiment was conducted using both Media (buffered and regular). The results showed huge variation between the two media and quite similar agreement in the replicates.

Missing tools to be considered before leaving for field work:

1. Incubator with control for both temperature and CO_2.
2. Inverted light microscope.
3. Microtitre plate shaker.
4. Sterile eppindorf tubes.
5. Sterile falcon tubes of different volumes.
6. Micropipette tips (size 1,2)

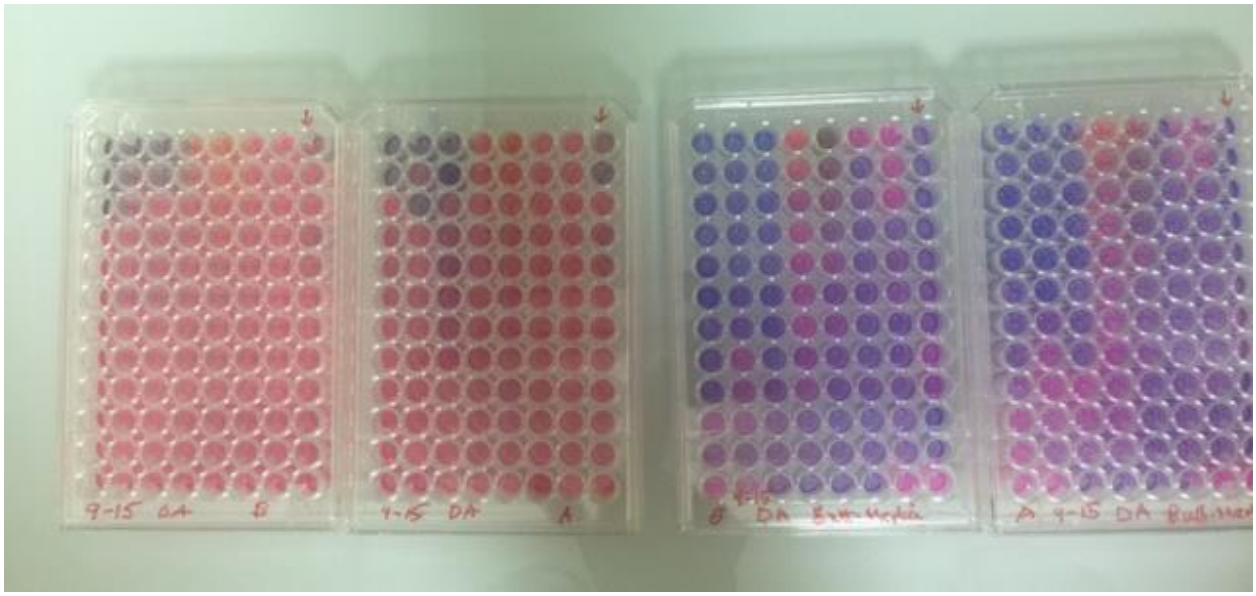

Figure 1: The test outcome using regular RPMI in the left and buffered RPMI in the right for the same set of compounds. Reference antifungals used were Itraconazole, ketoconazole and caspofungin in the wells F, G, and H respectively from left to right.

Summary of the challenges:

- Standard protocol needs to be validated at large scale and compared to other reproducible protocols.
- Diamidine solubility was a challenge, 4 of them were not fully soluble even in the starting concentration of 500 µg/mL.
- The protocol used in UST uses small volumes in seeding microplates. Final volume is 100 µL. For example, fugal cell inoculum is added in 10 µL RPMI. This was a challenge when pipetting.
- Missing equipment should be secured.

Curriculum Vitae

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Summary

I am a pharmacologist with training in the area of infection biology & epidemiology as tools for drug discovery and disease burden alleviation. I aspire to engage in a related field to keep developing myself and find room to serve with creativity and excellence. I also had experience in the field of teaching. I think in terms of objectives, I am enthusiastic, and I work with decisiveness and independence. I have a proactive attitude and find positive ways to engage with people. I believe in teamwork and devotion.

Experience:

Doctoral candidate

Swiss Tropical & Public Health Institute, Basel, Switzerland

Department of Parasite chemotherapy

September 2016 till of today

During this period, I worked to fulfill my doctoral project in the area of Infection Biology & Epidemiology, my project involved comparative genomics, *in-vitro* screening of diverse chemical libraries using fungal-cell culture and performing systematic reviews.

Lecturer, Pharmacology

University of Medical Sciences & Technology, Khartoum, Sudan

October 2015 till August 2016

Duties:

- Teaching pharmacology.
- Teaching drug discovery module.
- Supervising undergraduate research.
- Board member of curricula committee.

Lecturer, Pharmacology

University of Misurata, Misurata, Libya

November 2013 till September 2015

Duties:

- Teaching pharmacology and therapeutics.
- Teaching applied pharmacology using *in-silico* animal models.
- Supervising research involving animal models for graduate students.
- Running animal house, Wister albino and Hamsters.
- Establishment of virtual screening computer laboratory.

Clinical Pharmacist

Khartoun North Teaching Hospital

November 2010 till January 2012

Duties:

- Monitoring of adverse drug reaction in internal medicine wards.
- Provide consultation on treatment options and medication utility.
- Provide assistance in adherence appraisal and outcome follow-up.

Hospital intern pharmacist, National selection service

Khartoum Teaching Hospital

August 2009 till October 2010

Education:

PhD thesis, Epidemiology

University of Basel, and Swiss Tropical & Public Health Institute, Basel, Switzerland

Expected by Nov 2019

Higher Diploma, Research Methodology & Biostatistics

University of Medical Sciences & Technology, Khartou, Sudan

Date completed: February 2nd 2017

Masters, Basic Pharmacology

Higher Diploma, Research Methodology & Biostatistics

University of Medical Sciences & Technology, Khartoum, Sudan

Date completed: March 17th 2015

Bachelor, Clinical Pharmacy

Khartoum College of Medical Sciences, Khartoum Sudan

Currently (Ibn Sina University, Khartoum, Sudan)

Date completed: June 25th 2008

Skills:

- Sound computer skills (MS Office/Unix/Mac OS).
- Performance of numerous statistical analysis including GIS related topics using R-Language, STATA, and SPSS.
- Experience in databases and AI.

Languages:

- Arabic/Nubian as mother tongue.
- English: Fluent
- German: Average

Volunteering tasks:

Goodwill Foundation, Khartoum, Sudan

Juba University Student Association

Specially-ables rehabilitation center

Pharmacists Union, Sudan

Conferences:

- EMBO conference, 2018, Basel CH.
- Bac2 conference of structural biology, 2018, Basel CH.
- We Scientists Shape Science, 2016, Bern CH

Workshops:

- Fosravuconazole Clinical Study preparation workshop, January 2016, Khartoum, Sudan.
- Quality in Academic Institutions, May 2015, Misurata University, Misurata, Libya.

Publications:

1. Elhag DE, Abdallah BS, Hassan M, Suliman A (2018) ESI-LC/MS Method Development and Validation for the Determination of Some Selected Antibiotics in Hospital Wastewater. Pharm Anal Acta 9: 578

2. Eltayeb E. M. Eid, Faizul Azam, Mahmoud Hassan, Ismail M.Taban & Mohammad A. Halim (2018) Zerumbone binding to estrogen receptors: an in-silico investigation, Journal of Receptors and Signal Transduction, 38:4, 342-351

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