UNIVERSIDADE FEDERAL DO PARANÁ

NICKOLAS MENEZES DA SILVA

OMICS ANALYSIS OF CHAETOTHYRIALIAN BLACK YEASTS FOCUSING ON VIRULENCE AND BIOREMEDIATION GENES

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NICKOLAS MENEZES DA SILVA

OMICS ANALYSIS OF CHAETOTHYRIALIAN BLACK YEASTS FOCUSING ON VIRULENCE AND BIOREMEDIATION GENES

Tese apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, da Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Engenharia de Bioprocessos e Biotecnologia.

Orientadora: Profª Drª. Vania A. Vicente

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“To live by grace means to acknowledge my whole life story,
the light side and the dark. In admitting my shadow side
I learn who I am and what God’s grace means"
(Brennan Manning)

“Viver pela graça significa reconhecer toda a minha história de vida,
o lado claro e o escuro. Ao admitir meu lado sombrio,
aprendo quem eu sou e o que a graça de Deus significa"
(Brennan Manning)

RESUMO

As primeiras aparições do termo "levedura negra" ocorreram entre as décadas de 1890 e 1920, usado provisoriamente para descrever fungos polimórficos que não formam esporos ou possuem poder fermentativo, mas foram incluídos entre os fungos secretores de pigmento que ocorrem no leite, ar, sangue de insetos. De 1895 até fevereiro de 2020, envolvendo 92 países, 2.065 instituições e 9.622 autores, a revisão bibliométrica (Capítulo 1) revela que a partir da década de 1970 as publicações aumentaram exponencialmente. Os conceitos agrupados demonstram uma alta relação com o aprimoramento das técnicas moleculares e ciências ômicas por meio da bioinformática. As leveduras negras são definidas como fungos melanizados, encontrados em diferentes nichos e habitats, com ecologia variada e muitas vezes encontradas em ambientes altamente tóxicos e assim, um grupo representativo pertence à ordem Chaetothyriales. Esta ordem é muito diversa, incluindo muitos agentes oportunistas de doenças em humanos e vertebrados de sangue frio. Por outro lado, algumas espécies têm sido descritas com capacidade de degradar compostos aromáticos e xenobióticos voláteis em aplicações de biorremediação como *Cladophialophora exuberans* (Capítulo 2). No total, fornecemos 12 sequenciamentos de genomas e montagens de dois gêneros bem conhecidos, *Cladophialophora* (1), *Exophiala* (11). A comparação dos dados do genoma foi realizada entre *E. dermatitidis* e *E. spinifera*, produtoras de cápsulas, revelando que existiam perfis de virulência nessas duas leveduras negras, incluindo genes que estão possivelmente ligados à invasão cerebral, compartilhados com a espécie neurotrópica *Rhinocladiella mackenziei*. A ausência de perfis associados à virulência apoia a hipótese de que as leveduras negras são oportunistas, e não patógenos primários (Capítulo 3). Também foram avaliados os fatores de virulência, aptidão e as principais diferenças genéticas e de expressão gênica na árvore de *Exophiala* spp. por meio da tecnologia de RNAseq. Um total de 48 genes únicos diferencialmente expressos foram e exclusivamente encontrados para a linhagem clínica, incluindo genes de vários genes processos metabólicos e regulação transcricional, e os genes regulados positivamente estão principalmente envolvidos no transporte transmembrana, processos biossintéticos e metabólico (Capítulo 4). Este estudo forneceu novos insights a respeito da virulência intrínseca das espécies dentro da ordem Chaetothyriales e indicando variabilidade intraespecífica e genes chave associados as interações patógeno hospedeiro. A identificação dos mecanismos de patogenicidade e das estratégias adaptativas aos tecidos de hospedeiros especialmente em imunocompetentes, podem auxiliar na elucidação do potencial destes agentes para as aplicações em processos de biorremediação.

Palavras-chaves: leveduras negras, genômica comparativa, variabilidade intraespecífica, biorremediação, virulência, oportunistas.

ABSTRACT

The first appearances of the term “black yeast” occurred in the between the 1890s and 1920s used provisionally to describe polymorphous fungi which do not form spores and do not possess fermentative power but have been included among the pigment-secreting fungi occurring in milk, air, the blood of insects. Until February of 2020, involving 92 countries, 2065 institutions and 9622 authors. The bibliometric review (Chapter 1) reveals that after 1970s publications increased exponentially. The clustered concepts demonstrate a high relationship with the improvement of molecular techniques and omics sciences through bioinformatics. Nowadays, the black yeasts are defined as melanized fungi, found in different habitats and having different ecologies, often in polluted environments and a representative group belongs to the Chaetothyriales order. This order is much diverse, including many opportunistic agents of diseases in humans and cold-blooded vertebrates. On the other hand, some species has been described with capability to degrate aromatic compounds and xenobiotic volatiles in bioremediation applications as *Cladophialophora exuberans* (Chapter 2). In total, it was provided 12 genomes sequencing and assemblies of some well-known genera as following *Cladophialophora* (1) and *Exophiala* (11). Comparison of genome data were performed between the capsule-producing *E. dermatitidis* and *E. spinifera* revealing that virulence profiles existed in these two black yeasts, including genes genes that are possibly linked with brain invasion, shared with the neurotropic species *Rhinocladiella mackenziei*. The absence of consistent virulence-associated profiles supports the hypothesis that black yeasts are opportunists rather than primary pathogens (Chapter 3). It was also evaluated the virulence factors, fitness, and the major genetic and gene expression differences along the tree *Exophiala* spp. through of RNAseq technology. There were 48 unique genes differentially expressed exclusively to the clinical strain, including genes from various metabolic processes and transcriptional regulation, and the up-regulated genes are mainly involved in transmembrane transport, biosynthetic and metabolic processes (Chapter 4). This study provided new insights into the intrinsic virulence of species within the Chaetothyriales order indicating intraspecific variability and key genes associated with interactions between host and pathogen. The identification of pathogenic mechanisms and adaptive strategies to host tissues, especially in immunocompetent ones, can help in elucidating the potential of these agents and so, discriminate strains for bioremediation applications.

Key-words: black yeasts, comparative genomics, intraspecific variability, virulence mycoremediation, opportunists.

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GENERAL INTRODUCTION

Black yeasts are a heterogeneous group of fungi belonging to the Dothideales and Chaetothyriales order, with the presence of melanin in the cell wall in the reproductive and vegetative structures, which may show dark colorings, ranging from olive green, brownish and black (HOOG; MCGINNIS, 1987; HOOG et al., 2019; TEIXEIRA et al., 2017). Black yeasts of the order Dothideales exhibit the ability to survive in hostile environmental conditions (STERFLINGER; HOOG; HAASE, 1999).

The Chaetothyriales emerged about 387 Mya, during the end of Devonian (416–359 Mya) and with many speciation events occurred in the Jurassic (201–145 Mya) (QUAN et al., 2020). This order comprises a vast group of black yeasts associated with different ecological niches such as soils, fruits, decomposing organic matter (VICENTE et al., 2008; VOIDALESKI et al., 2020; NASCIMENTO et al., 2017; LIMA et al., 2020; COSTA et al., 2020). They have an oligotrophic metabolism, as they can live in an environment that offers very low levels of nutrients and exhibit a competitive ability occupying niches that can not be used by other fungal (NASCIMENTO et al., 2017; TEIXEIRA et al., 2017). In this context, they are often associated with regions of tropical and subtropical climate and terminals of desert soils, rocky surfaces and glacial environments (VICENTE et al., 2014; NASCIMENTO et al., 2017; TEIXEIRA et al., 2017). Moreover, it includes species with important clinical related to humans and associated with invertebrate animals (VICENTE et al., 2008, 2012; BONIFAZ et al., 2013; GUERRA et al., 2013; SEYEDMOUSAVI et al., 2018).

The genus *Cladophialophora*, comprises several species of clinical, environmental and biotechnological interest, changes 38 species, often associated with organic matter, rocks and plants (BADALI et al., 2009; VICENTE et al., 2012; NASCIMENTO et al., 2017; KIYUNA et al., 2018). In the clinical scope, *C. bantiana* is one of the most virulent species applied, capable of causing lesions in immunocompromised and immunocompetent patients, in addition to being a chromoblastomycosis agent (HORRÉ; HOOG, 1999; BADALI et al., 2008; KANTARCIOGLU et al., 2016). Other species may be associated with skin infections, such as *C. boppii*, *C. emmonsii* and *C. saturnica* (HOOG et al., 2007; BADALI et al., 2009). Finally, some species have a biotechnological potential, mainly in hydrocarbon bioremediation, such as species such as *C. immunda*, *C. psammophila* and *C. exuberans* (PRENAFETA-BOLDÚ et al., 2001; BADALI et al., 2011; NASCIMENTO et al., 2017).

Furthermore, the *Exophiala*, is distributed in a polyphyletic clade and comprises more than 40 species, grouped in the Herpotrichiellaceae family (HOOG et al., 2011; BORMAN et al., 2017; TEIXEIRA et al., 2017). The species are common in domestic environments, environments polluted with hydrocarbons and in the clinical scope (DÖĞEN; ILKIT; HOOG, 2013; BORMAN et al., 2017; PINHEIRO et al., 2019). In

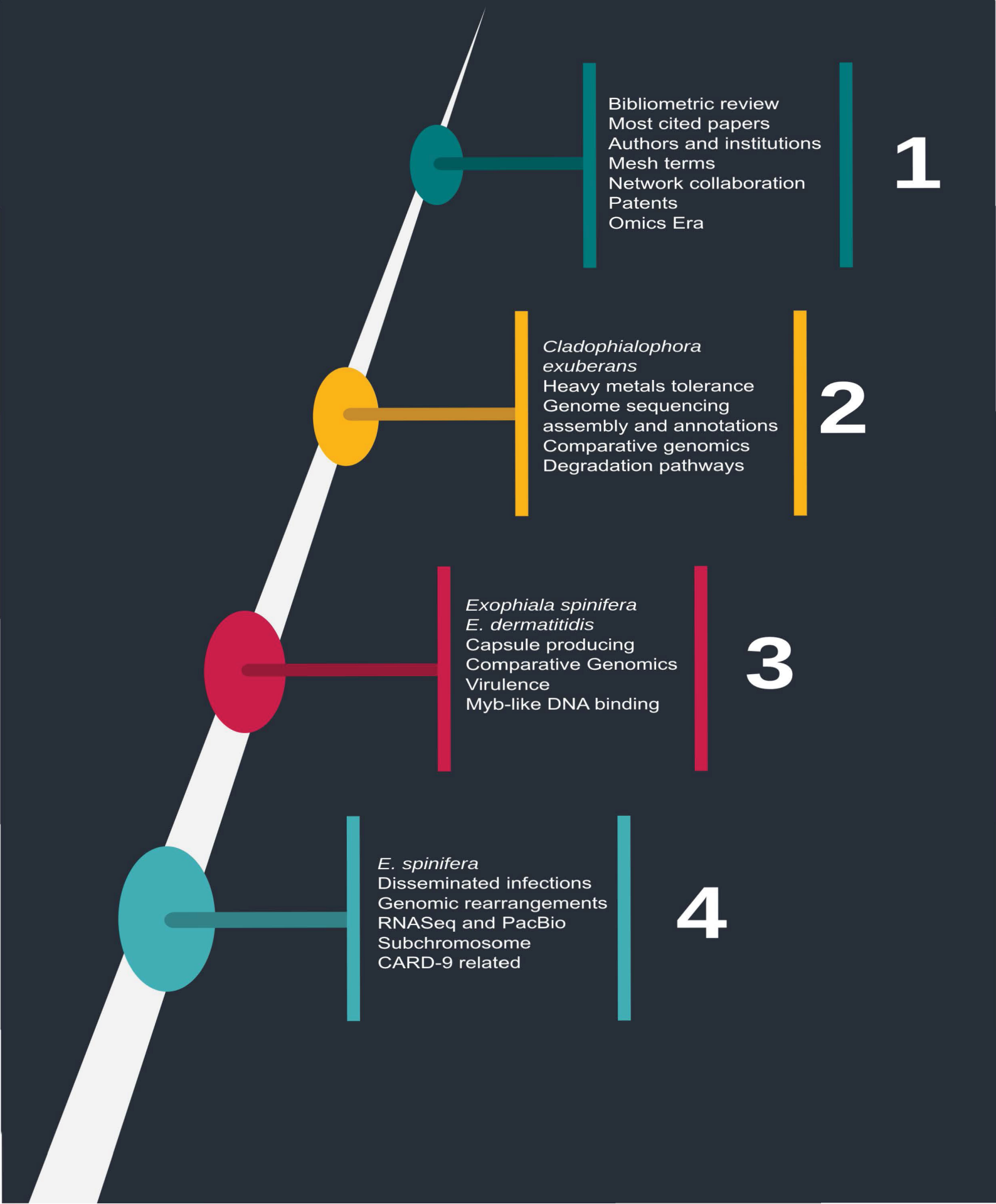
concern to pathogenicity, the genus may be associated with invertebrate animals (crab) and cold-blooded animals (fish and amphibians), in addition to containing numerous opportunistic and human immunocompetent pathogens (SUDHADHAM et al., 2008; LI; DURBIN, 2009; HOOG et al., 2011; PINHEIRO et al., 2019).

Focusing to understanding of the virulence and the mechanisms of adaptation of these fungi, becomes necessary to study the genera as *Cladophialophora* and *Exophiala* because both have members associated with cases of infection in humans, including cases of cerebral phaeohyphomycos for *E. dermatitidis* (WANG et al., 2019) and *C. bantiana* (KHALIQ; IHLE; SCHIRTZINGER, 2019) and on the other hand they have ability to survive in hostile environments (BADALI et al., 2011; TAFER et al., 2015).

In 2020, the black yeasts research completed 125 years of existence. With the advancement of molecular techniques, isolation methods, genomic sequencing, a massive production of data has been generated and through of the combination of technology advances, available supercomputers and databases. It has been possible to develop software with high scalability of processes aimed at transforming of raw data into knowledge glimpsing the understating of ecology, pathogenicity, host relationship, virulence and adaptive mechanisms.

This thesis was organized in chapters. The chapter 1 provided the first bibliometric review focusing on the principle and current situation of research related to black yeasts. In the second chapter, it was presented a genomic study of the *C. exuberans* looking for to explore the biotechnological potential of the specie. The chapter 3 and 4 were realized in cooperation with China and the Netherlands being already published approaching the intraspecific variability of the *Exophiala* species and the relation between environmental and clinical strains, including analyzing a transcriptome data of strain isolated from a CARD9 deficient patient.

Thesis Overview



OBJECTIVES

The main objective of this study was to explore the well-know genders of the Chaetothyriales order as *Cladophialophora*, *Exophiala* based on the generation of "Omics" data comparing the clinical and the environmental strains to elucidate the different virulence profiles and the possible use to bioremediation applications.

SPECIFIC OBJECTIVES

- To produce the first bibliometric review of the black yeasts;
- To execute the DNA extraction, genome sequencing, assembly and annotation of the *Cladophialophora exuberans*;
- To execute the DNA extraction, genome sequencing, assembly and annotation of *Exophiala* species comparing the clinical and environmental strains;
- To execute the PacBio Sequencing and the RNASeq of the *Exophiala spinifera* comparing the different expression levels from two environmental strains and one CARD9-deficient patient.

CHAPTER 1

125 YEARS OF RESEARCH ABOUT BLACK YEASTS SPECIES, A GLOBAL BIBLIOMETRIC REVIEW STUDY

1 125 YEARS OF RESEARCH ABOUT BLACK YEASTS SPECIES, A GLOBAL BIBLIOMETRIC REVIEW STUDY

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ABSTRACT

The application of bibliometrics in black yeast related paper allows to analyze the total available data in different levels. 2020 is the 125th birthday of the first document collected. The objective of this study is to analyze the historical changes, the effect of the methods and techniques and show the world network collaboration. It was used the Dimensions database. In total 6424 documents were collected and analyzed, between 1895 and 2020, it relates with 44.698 different concepts and 2950 Mesh Terms. The number of publications has been increased after 1970, this fact is related with the improvements of the molecular techniques, the large production of genome and transcriptomes data set and the development of the bioinformatics area.

Keywords: bibliometrics, black yeast, publications.

1.1 INTRODUCTION

The first appearances of the term “black yeast” (BY) occurred in the between the 1890s and 1920s used provisionally to describe polymorphous fungi which do not form spores or possess fermentative power but have been included among the fungi occurring in milk, air, the blood of insects (BURGWITZ, 1928).

To the clinical area, in the beginning of 20th century the first cases of a distinct cutaneous mycotic infection were described, caused by black yeasts and which disease are named as Chromoblastomycosis. Looking to the first’s cases in south America, in specific to Brazil, it was in 1911 identified by A. Pedroso, but only being published in 1922, with the causal agent *Fonsecaea pedrosoi*, which in time was described with a different name *Hormodendrum pedrosoi* by E. Brumpt, in honor to A. Pedroso, (MEDLAR, 1915; PEDROSO; GOMES, 1920; BRUMPT, 1922; CARRIÓN, 1942). This specie of BY is a very important in clinical area, being the most cause of the disease in South America.

In 1942 there an impressive article showing the Biotechnology potential of black yeasts (CHRISTENSEN et al., 1942), which in this case showed *Hormodendrum resinae* (current *Cladosporium resinae*, taxon name updated in 1955 by G.A. de Vries) growing in a wood impregnated with coal tar and creosote, demonstrating the capacity of BY to grown and survive in a hostile environment. During the next nine years, cases of chromoblastomycosis was growing in United States, when 13 cases were reported by (BARWASSER, 1953), at the same time other diseases caused by black yeasts was in to evidently, such as Phaeohyphomycosis and Mycetoma (AJELLO, 1978).

In nowadays, the black yeasts are present on everyday life, in places like dishwashers (ZUPANČIČ et al., 2016), sinks (NISHIMURA et al., 1987), baths and saunas facilities (MATOS et al., 2002) and also in extreme conditions like volcanoes (PULSCHEN et al., 2015), glaciers (PERINI; GOSTINČAR; GUNDE-CIMERMAN, 2019), rocks (ZAKHAROVA et al., 2012; AMETRANO; MUGGIA; GRUBE, 2019) and even growing in the Chernobyl’s reactor (ROBERTSON et al., 2012). It’s possible to find them in several environmental sources (VICENTE et al., 2008, 2012; VOIDALESKI et al., 2020) such as coconuts (VICENTE et al., 2012; NASCIMENTO et al., 2017), railways (YAZDANPARAST et al., 2017), polluted soils (ZHAO et al., 2010) and in specific niches as already report in ant nested (LIMA et al., 2020). The black fungi are almost omnipresent fungi, sometimes being dangerous to human health, sometimes useful to biotechnological applications (BLASI et al., 2017; HOOG; VICENTE; GORBUSHINA, 2013), like bioremediation, due to its potential for aromatic compounds’ degradation and ability to survive in extremely conditions; alkaline and toxic conditions, high temperature, low nutrient availability (PRENAFETA-BOLDÚ et al., 2001; PRENAFETA-BOLDÚ; SUMMERBELL; HOOG, 2006).

Nevertheless, the black yeast have been associated with infection in human and animals. Among the most common infections are phaeohyphomycosis, eumycetoma and chromoblastomycosis (ESPINEL-INGROFF; SHADOMY, 1989; VICENTE et al., 2008; ALVIANO et al., 2004). Moreno et al. (2018) reported as casual agents of human cerebral infections, provoking disorders in animals as eastern hellbenders (HOPF et al., 2020), saffron cod (MEYERS et al., 2019), crab (VICENTE et al., 2012) and atlantic halibut (OVERY et al., 2014). One of the main gateway for BY in human hosts occurs through traumatic inoculation of the fungus into the skin (VICENTE et al., 2008). Chromoblastomycosis is a chronic infection, of slow evolution, with nodules and verrucous plaques in the skin and subcutaneous tissue, with possible ulceration, usually located in the lower limbs (CORREIA et al., 2010; PIRES et al., 2012). Eumycetoma is treated by the presence of black yeast in the form of dark granules in the tissues, being more common in the lower extremities of the body (REVANKAR; SUTTON, 2010) and Phaeohyphomycosis are mycoses that can appear in cutaneous, subcutaneous and systemic form, without the formation of muriform bodies, and can be caused by species of the genera *Cladophialophora*, *Phialophora* and more commonly genus *Exophiala*. It is considered a sporadic, cosmopolitan disease that affects both individuals considered healthy and those with compromised immune systems (REVANKAR, 2007; ROSSETTO et al., 2010).

These diseases rarely present in the disseminated or invasive form, but they have an important impact on public health because they are difficult to control and present recurrences, requiring an effective diagnosis for the efficiency of the treatment (QUEIROZ-TELLES et al., 2011, 2017). Recently, the chromoblastomycosis was recognized by World Health organization as a neglected disease (QUEIROZ-TELLES et al., 2017).

Several studies discussed the immunological aspects of the diseases (WANG et al., 2014; GRUMACH et al., 2015). Wang et al. (2019) in 2019, showed CARD-9 mutations related with TH17 cell deficiencies seen to highlight this immunological aspect of fungal infections. A recent report shows a fatal case of neuro phaeohyphomycosis in a patient with CARD-9 homozygous for loss-of-function mutation, demonstrating patients with this deficiency has a predisposition to systemic fungal infection, in special to infection on the central nervous system (DRUMMOND et al., 2015; WANG et al., 2019). CARD-9 mutations seen to be related with absence of neutrophils on the central nervous system and a down regulation of the innate immune system on patients with fungal infection, with increase the risk of that (DRUMMOND et al., 2015).

The correlation studies between the environment where the black yeasts are and diseases caused by them show to the scientific community the importance of this kind of studies. Seyedmousavi et al. in 2014, explain how the melanin, produced by

fungus, provides protection against UV radiation, extreme temperatures and oxidizing agents, and thus provides a high virulence and greater competitive ability against sporulating hyaline fungi (SEYEDMOUSAVI et al., 2014).

The species of the order Chaetothyriales showed a remarkable association with environments contaminated with monochromatic hydrocarbons, showing a competitive advantage when these compounds are present, and the potential role in the virulence of this assimilation capacity has been inferred based on the structural similarity of these compounds and neurotransmitters, explaining the fungal predilection for nervous system tissue (JACOBSON, 2000; ZHAO et al., 2010).

Scientometrics is a branch of informatics, according to the Organisation for Economic Co-operation and Development (OECD) the bibliometric analysis use data on numbers, authors, institutions, fields of science, countries, patents to measure the results of science production, making it possible to analyze the formation of collaboration networks and the evolution of the science subjects.

In this context this study aimed at to show the most cited papers by subject, the current collaboration networks and it's formation, publications per country and the impact of the omics science to the BY research.

1.2 MATERIALS AND METHODS

1.2.1 Database selection and data collection

The searches were performed by the most populars scientometric databases (Table 1) to identify articles published related to black yeasts. Considering the fields available, number of publications, patents, availability and data extraction formats, it was selected the dimensions database. All fields were recovered in JSON format using the Dimensions API (https://www.dimensions.ai/) the following terms "black yeasts" or "black yeast" or "black-yeast" or "black-yeasts" to create the dataset as title, abstract, keywords, citations, authors, grant numbers and the results were not limited.

1.2.2 Data treatment and text mining

The data acquisition was performed by Dimensions API. It was used the REST protocol to recovery the data. The data treatment and statistical analyses were performed in R Language and their packages. The manipulations, filters, groupings were performed using the sqldf package (https://cran.r-project.org/web/packages/sqldf/README.html) and the data visualization were generated by plotly package (https://plotly.com/r/).

1.2.3 Publications classification

To evaluate the publication evolution, the acquired data were distributed in 3 periods of forty-one years. 1895-1936, 1937-1978, 1979-2020. A R script was made to classify the publications according to keywords.

1.2.4 Network analysis

The networks analysis was performed in R language, using the igraph (https://igraph.org/r/) and RederR (https://bioconductor.org/packages/release/bioc/html/RedeR.html) packages.

1.2.5 Fields of Research (FOR) and Mesh Terms

The data was classified by Dimensions API using the Fields of Research system developed by the Australian Bureau of Statistic. The classification is divided into 22 divisions and 157 groups including arts, humanities, science and engineering. It also categorized by the Medical Subject Headings (Mesh Terms, https://www.nlm.nih.gov/mesh/meshhome.html), that represents a vocabulary hierarchically and organized produced by the National Library of Medicine to use to indexing, cataloging, and searching publications.

1.3 RESULTS AND DISCUSSION

The first occurrence of the word "black yeast" emerged in 1895, without concise definition, by (TURCK, 1895), reporting a group of cases of various pathological conditions of stomach carcinoma, followed by other reports (WILEY, 1902) and summaries of current researches, used only to describe and classify types of yeasts. Between the first well-known articles, by (MEDLAR, 1915) and (KANO, 1934), not indexed by Dimensions, the first chromoblastomycosis case reported was founded in Dimension's database, it's a Brazilian medical report made in 1922 and published by The Journal of the American Medical, entitle, "Current Medical Literature". The Brazilian study suggested the term "chromoblastomycosis" to describe ulcerated nodules scattered thickly in one leg, from hip to toes. (JAMA, 1923).

A total of 6424 documents were collected in the Dimensions Database on February 19th, 2020, being 3285 articles, 279 patents, 2623 chapters, 179 books and 42 monographs, 12 preprint and 4 proceeding documents, between 1895 to Feb/2020. The number of journals is 160, the number of authors related is 9622 and the average of citations per document was 29.6. The average of references to the papers was 61 references/document. Based on the search in title, the most cited document is the

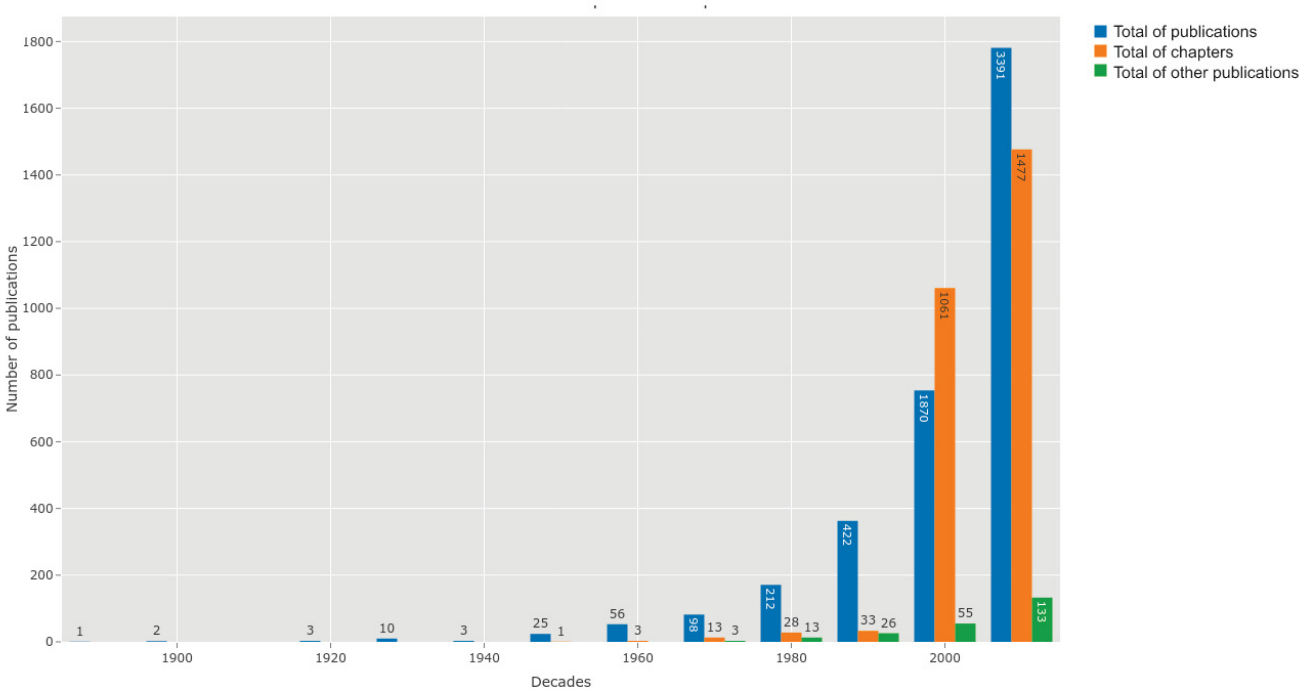
TABLE 1 – Scientometric databases comparisons about the black yeasts related papers.

Database	Total of Publications	Total of Patents	First Document	Chapters	Grant ($)	API/Export
Dimensions	3289	279	1895	2623	Yes	Yes
Scopus	533	336	1951	-	No	Yes
Web of Science	562	-	1969	-	No	Yes
Google Scholar	5480	210	1844	Yes/Na	No	No

clinical guidelines for the diagnosis and management of systemic phaeohyphomycotic: diseases caused by black fungi, cited 155 times (Table 2).

Concerning the articles of Chromoblastomycosis and phaeohyphomycosis cited (Table 3), in the first place, it is the article published by (MCGINNIS, 1983) in the Journal of the American of Dermatology, cited 354 times, revising and explaining the major concept and until then confused terminologies of the mycoses caused by dematiaceous fungi. After the few published papers during 1900s to 1950s, after 1980s, the number of publications increased exponentially, highlighting to the 2000s where the number of chapters were more representative than the articles (Figure 1).

FIGURE 1 – Evolution of the black yeast publications per decade.



Source: the author

TABLE 2 – Top 15 most cited documents of black yeast, search based only on the title

year	title	times cited	doi
2014	ESCMID and ECMM joint clinical guidelines for the diagnosis and management of systemic phaeohyphomycosis: diseases caused by black fungi	155	10.1111/1469-0691.12515
1993	Role of black fungi in color change and biodeterioration of antique marbles	145	10.1080/01490459309377952
2000	Hypersaline waters in salterns – natural ecological niches for halophilic black yeasts	138	10.1111/j.1574-6941.2000.tb00716.x
2002	High prevalence of the neurotrope *Exophiala dermatitidis* and related oligotrophic black yeasts in sauna facilities	108	10.1046/j.1439-0507.2002.00779.x
2000	Hypersaline waters in salterns - natural ecological niches for halophilic black yeasts.	102	10.1016/s0168-6496(00)00032-5
2003	Species diversity and polymorphism in the *Exophiala spinifera* clade containing opportunistic black yeast-like fungi	102	10.1128/jcm.41.10.4767-4778.2003
2008	The neurotropic black yeast *Exophiala dermatitidis* has a possible origin in the tropical rain forest	97	10.3114/sim.2008.61.15
2008	Resistance of Antarctic black fungi and cryptoendolithic communities to simulated space and Martian conditions	95	10.3114/sim.2008.61.10
2008	Environmental isolation of black yeast-like fungi involved in human infection	89	10.3114/sim.2008.61.14
2001	Removal of toluene in a vapor-phase bioreactor containing a strain of the dimorphic black yeast *Exophiala lecanii-corni*	84	10.1002/bit.10066
1995	Black fungi in marble and limestones — an aesthetical, chemical and physical problem for the conservation of monuments	82	10.1016/0048-9697(95)04590-w
2006	Black yeasts and meristematic Fungi: Ecology, diversity and identification	78	10.1007/3-540-30985-3_20
2000	Black fungi: clinical and pathogenic approaches.	77	10.1080/714030907
2008	Black yeast symbionts compromise the efficiency of antibiotic defenses in fungus-growing ants	74	10.1890/07-0815.1
2002	Cellular responses to environmental salinity in the halophilic black yeast *Hortaea werneckii*	74	10.1046/j.1365-2958.2002.03021.x

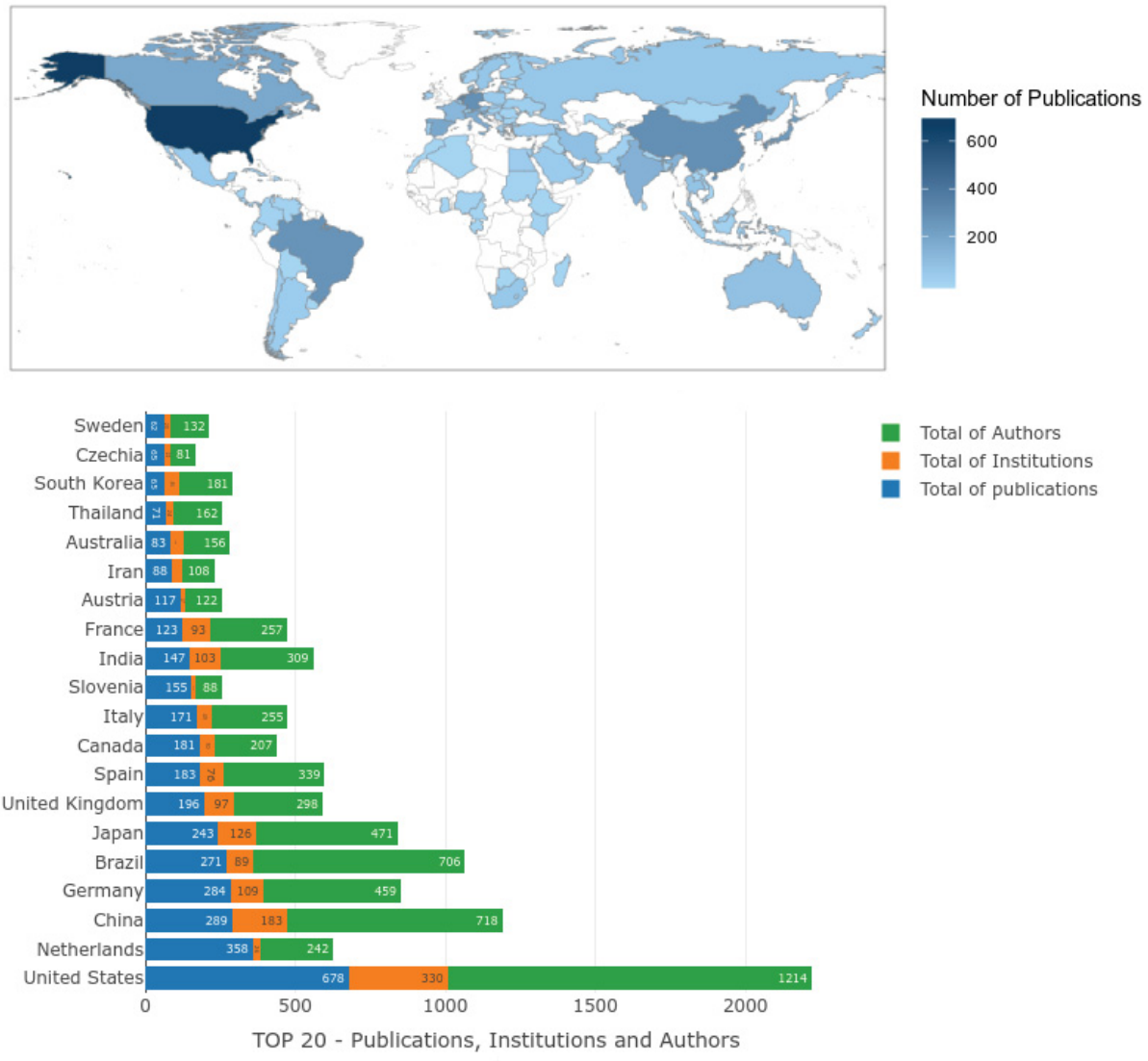
TABLE 3 – Top 15 most cited documents of chromoblastomycosis, search based only the title

Year	Title	Times cited	doi
1983	Chromoblastomycosis and phaeohyphomycosis: New concepts, diagnosis, and mycology	354	10.1016/s0190-9622(83)70001-0
2007	Biology and pathogenesis of *Fonsecaea pedrosoi*, the major etiologic agent of chromoblastomycosis	68	10.1111/j.1574-6976.2007.00077.x
2010	*Fonsecaea nubica* sp. nov, a new agent of human chromoblastomycosis revealed using molecular data	67	10.3109/13693780903503081
2017	chromoblastomycosis	62	10.1128/cmr.00032-16
2010	Rapid detection of pathogenic fungi using loop-mediated isothermal amplification, exemplified by *Fonsecaea* agents of chromoblastomycosis	46	10.1016/j.mimet.2009.10.002
2013	Challenges in the Therapy of chromoblastomycosis	42	10.1007/s11046-013-9648-x
1958	Chromoblastomycosis caused by a rare yeast like dematiaceous fungus	38	10.1007/bf02051408
2014	Chromoblastomycosis	31	10.5114/pdia.2014.40949
2015	*Fonsecaea pugnacius*, a Novel agent of disseminated chromoblastomycosis	30	10.1128/jcm.00637-15
2014	Environmental siblings of black agents of human chromoblastomycosis	29	10.1007/s13225-013-0246-5
2010	*Rhinocladiella aquaspersa*, proven agent of verrucous skin infection and a novel type of chromoblastomycosis	28	10.3109/13693780903471073
2010	Successful treatment of chromoblastomycosis of 36 years duration caused by *Fonsecaea monophora*	27	10.3109/13693780903008813
2016	Molecular epidemiology of agents of human chromoblastomycosis in Brazil with the Description of Two Novel Species	23	10.1371/journal.pntd.0005102
2007	Rapid identification of *Fonsecaea* by duplex polymerase chain reaction in isolates from patients with chromoblastomycosis	23	10.1016/j.diagmicrobio.2006.08.024
2005	Invasive chromoblastomycosis and sinusitis due to *Phialophora verrucosa* in a child from northern Africa	22	10.1111/j.1439-0507.2005.01150.x

1.3.1 Authors and Institutions

The black-yeast related studies are in all continents, involves 92 countries, 2065 institutions and 9622 authors. The USA has more 678 publications (P), 1214 authors (A) and 330 institutions related (I), followed by Netherlands (P: 358, A: 242, I: 26) and China (P: 289, A: 718, I: 183) (Figure 2).

FIGURE 2 – World Map of the Publications. a) the bubble chart represent the number of the publications by country b) The TOP 20 countries and their number of authors, institutions and publications.



Source: the author

Although, analyzing manually the documents it was possible to identify authors and institutions and their countries (Figure 2). The USA was the first country identified publishing, in 1936, about the black-yeast related papers in the Dimension's Institutions Database. Two countries debuted in 2019 being then Mongolia and Armenia.

Even having the largest number publications, authors and institutions the USA shows 0.5583 publications per author (p/a). Mauritius is the TOP 1 comparing this index, 2.33 p/a. Another 4 countries showed more than 1 document per author, highlighting to the Netherlands (1.5 p/a) for having 26 institutions, showing concise distribution and very productive working groups.

The formation of collaboration between the countries can be evaluated since 1970. Two groups were formed before 1980 decade. The first one started by United States with Italy and Brazil and the another one including the Canada (Figure 3). After 1980, fourteen countries joined to the network collaboration. The two groups before separated were found publishing together and several countries like the USA, the South Africa and the Netherlands can be noted centralizing, being responsible to integrate other countries. However, in the 1990 (c), the network lost density and the groups are more defined and the hub effect is very clear for the Netherlands, Germany, USA and Austria.

In the 2000s, the force of attraction of the core of the network becomes greater and more countries join the cooperation network on a peripheral way following to the current global Network noted in (Figure 4). According to the figure the relationships are very density and it's possible to see the green nodes that more and more countries are joining to the black yeast related studies.

In addition the fact that accounting all the publications without any cut off there's no evidence if exists some groups directing the research, moreover if we look only to the countries that has more than 10 publications in the last decade, it's possible to visualize that the network nucleus is composing by USA, Germany, United Kingdom, Italy, China, Brazil and the Netherlands. The USA connects Slovenia, Sweden, Belgian and Japan to the nucleus. The Turkey group collaborate exclusively with the Netherlands and the Russians are connected by Spain.

1.3.2 Mesh terms and concepts

In total, the publications are related with 44698 distinct concepts and 2950 MeSh Terms. The Mesh terms Based on the machine learning approach available offered by the Dimensions API, it was found 30 concepts and 10 Mesh Terms per document, on average. The main Mesh terms are "Humans" present in 625 publications(p), "Ascomycota" (339 p), the phylum that the black-yeast belongs, followed by Animals (313 p) and Fungi (311). Advancing from left to right in the (Figure 5) it's possible to

FIGURE 3 – Evolution of cooperation networks among countries per decade. a) before 1980s; b) 1980 to 1990; c) 1990 to 2000; d) 2000 to 2010.

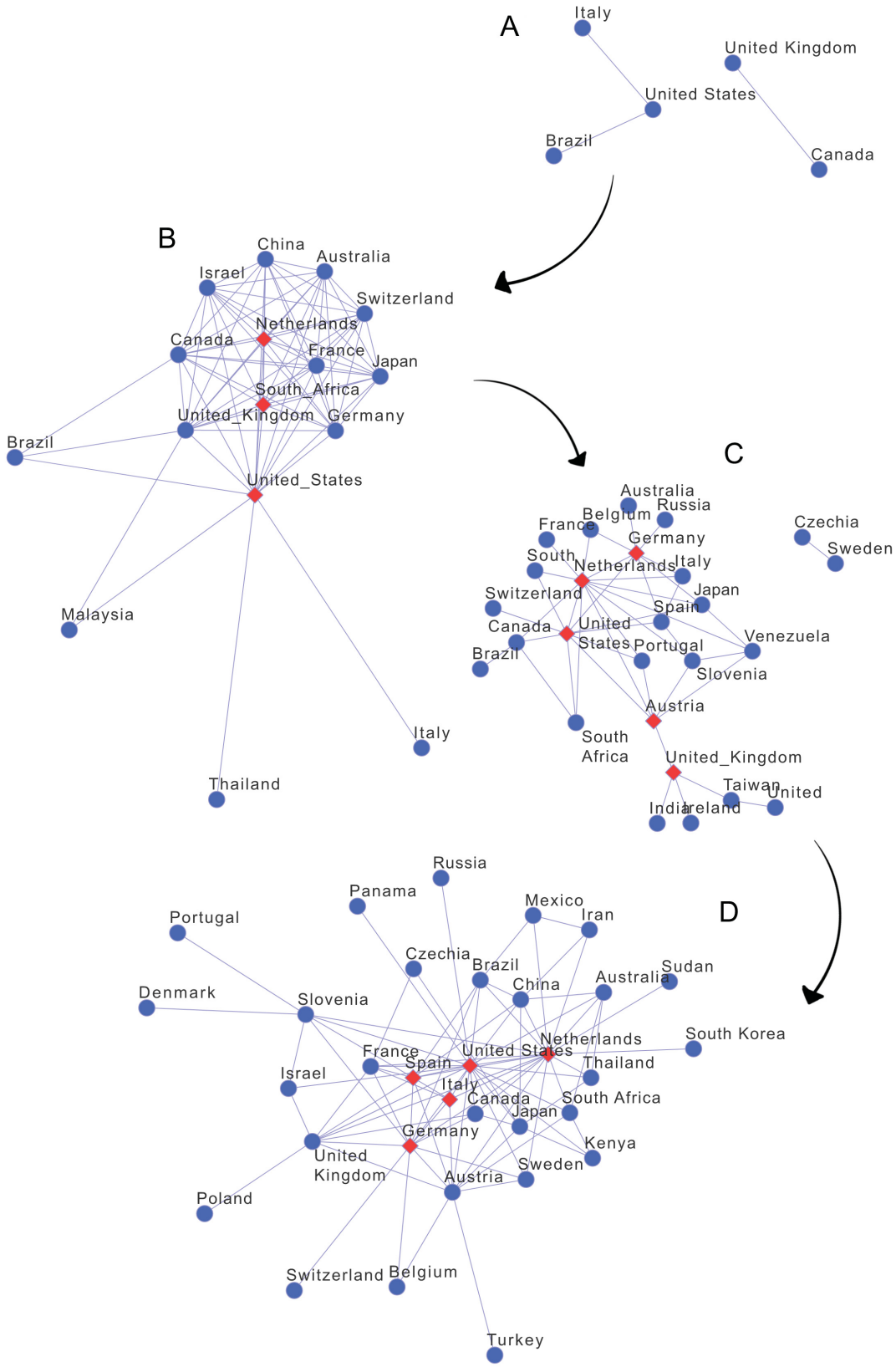


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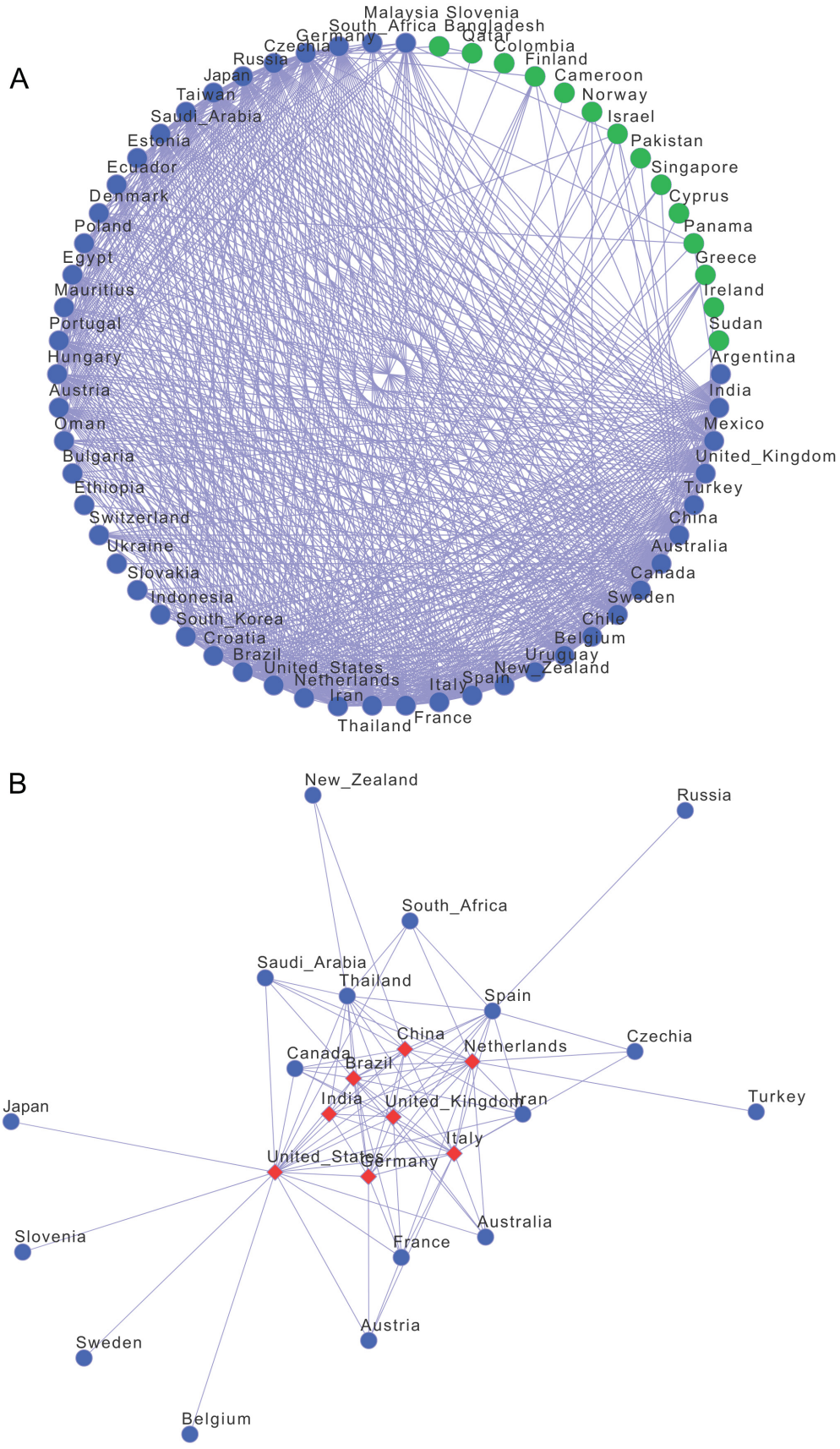
FIGURE 4 – Current global network collaboration in the black yeast research between the countries. a) the "hairball" representation, the green nodes represent the new countries incorporated in the research network. b) Network collaboration with cutoff of 10 publications.



Source: the author

note the diversification and the main topics that the black yeast research is evolved. On the second and third column, "Phylogeny", "DNA, Fungal", "Molecular Sequence Data" and "Sequence Analysis" appears like major techniques and strategies for the investigations. The concepts obtained by the clustered abstracts shows a very representative "vocabulary" associated with the black yeasts papers. The increase in multidisciplinary can be noticed by the number of concepts covered evaluated in the three periods, to 1895-1936, 66 concepts found, 1937-1978 (1750 concepts) and to 1979-2020 (43.907 concepts).

FIGURE 5 – More relevant Mesh Terms. The size of the boxes and the numbers together with the term represents the number of publications.

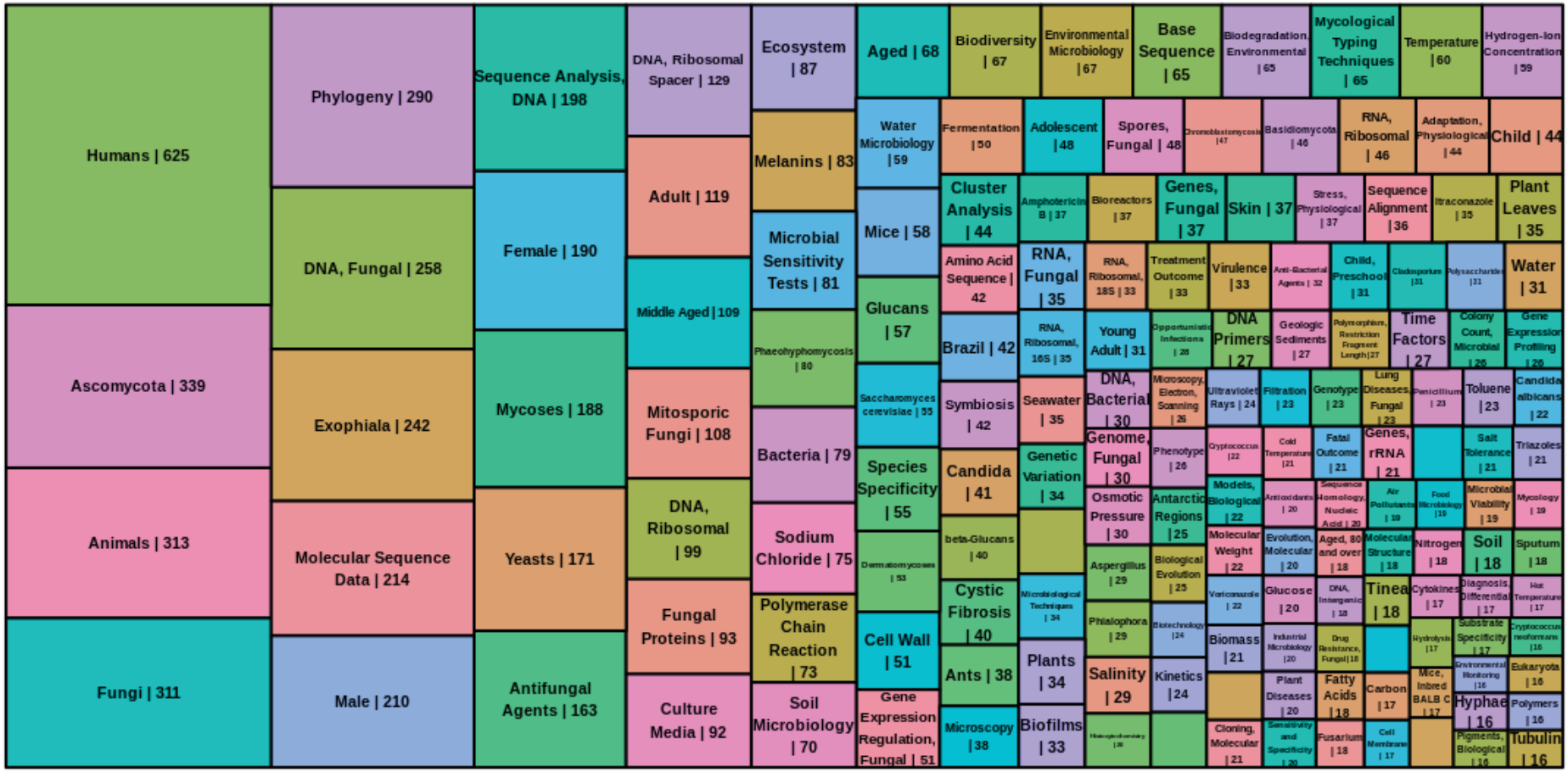


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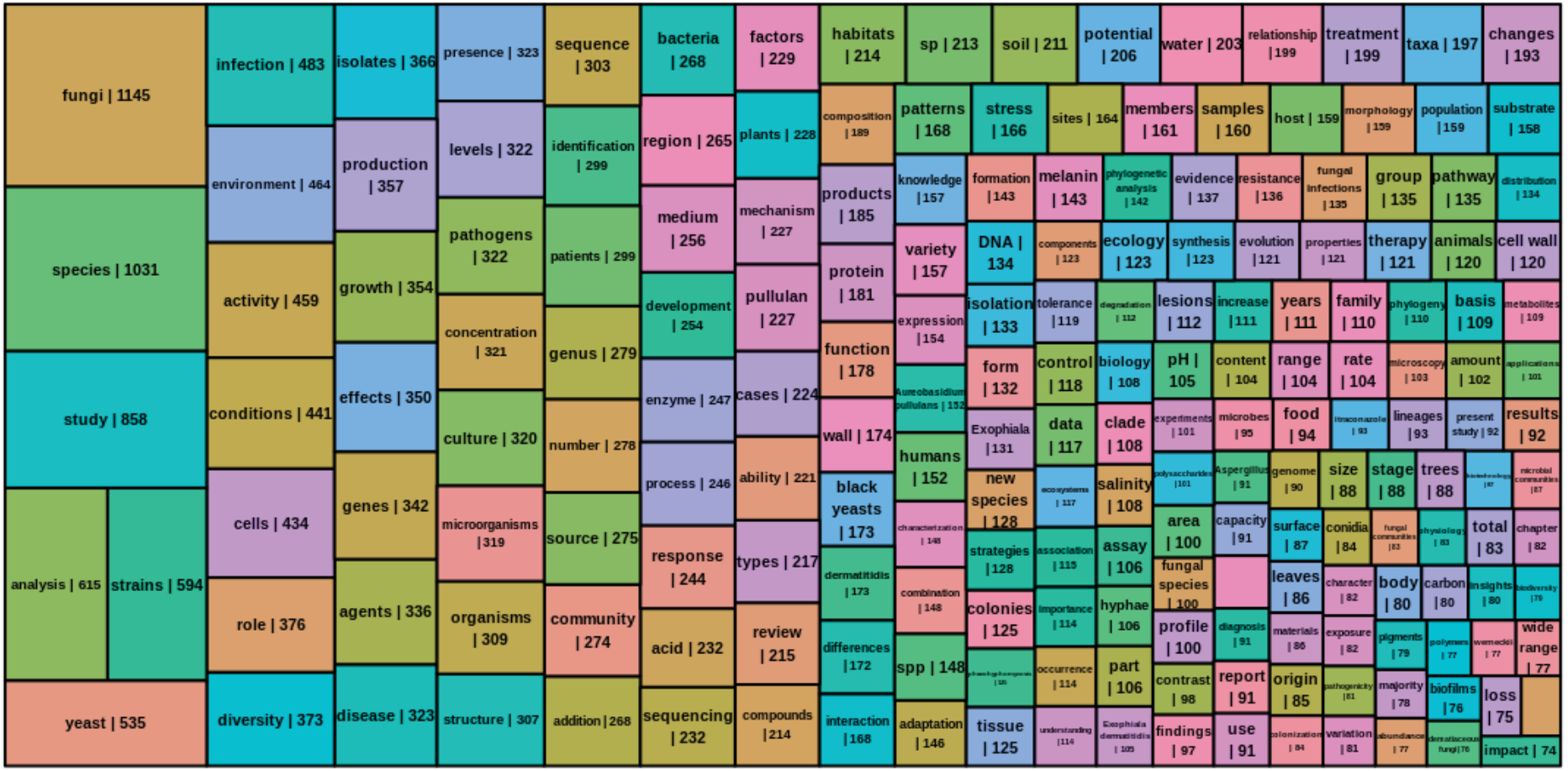
FIGURE 6 – More relevant Concepts. The size of the boxes and the numbers together with the term represents the number of publications.

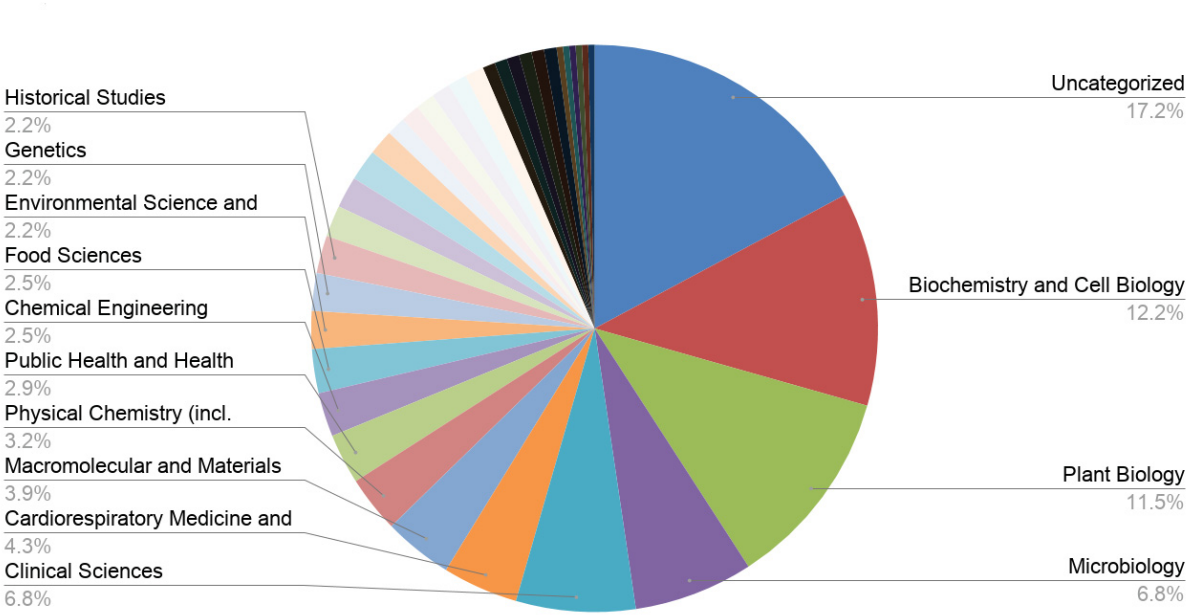


Source: the author

1.3.3 Patents and Financial supports

In total, 279 were collected. Some patents are deposited in more than one Country, so removing the duplicate records, the current number of black yeast related patents is 153. The main patents are classified to the Biochemistry and Cell Biology, Plant Biology, Microbiology and Clinical Sciences categories and 48 were not categorized (Figure 7). Focusing in the biotechnological applications, several patents are noticed using the *Aureobasidium pullulans* from the Dothideales order like to support to produce of xanthan gum (EP0698662A2), use of pullulan as a slow digestive carbohydrate (CN1708236A) and to degrate chemical pollutants (US5399495A). The *Exophiala* genus appears being used to produce melanin-degrading enzyme extracted and applied on the surfaces of the skin, the nails or in the hair to degrade the melanin and to provide whitening effect in the patent nº US7291340. The strain *Exophiala wilhansii* was patented by advantageously capability to be applied in isomerization and oxidation reactions. The *E. spinifera* ATCC 74269 and *Rhinocladiella atrovirens* have potential use in the crop agriculture to degrade the mycotoxin fumonisin (US5792931A). There's no patent related with complete bioremediation process of the BTEX compounds and the major applications are related with the melanin production. Finally to concern the financial supports, it was found 23 grants related with the black yeast papers, totalizing 906.172,00 USD and supporting 838 publications.

FIGURE 7 – Patents categorized in the Fields of Research (FOR) categories.



Source: the author

1.3.4 Articles classification and Influence of the Omics Era

The most papers are related with clinical and description of new species, but a significant number of papers using Omics science were found to the black yeasts research (Figure 8). The first full DNA genome sequenced was a bacteriophage ΦX174 by Sanger in 1977, in the time the sequencing was a radioactive carbon DNA marker. This Sanger sequencing was developed and is still used in methods of several works, but not with a radioactive carbon anymore. However, with moderns technologies others methods of sequencing were developed, bring the new generations sequencing (NGS) and consequently, new approaches form the omics era (DIJK et al., 2014; MORENO et al., 2018).

Advances in the era of Omics technologies, sequencing performance of the whole-genome and low-cost availability, have enabled genome assemblies for a wide variety of fungal species (GRIGORIEV et al., 2011). Current mainly used approaches to genomic analysis include methods such as short reading sequence technology (eg Illumina) and long reading technology beneficial to a repetitive genome (e.g Pacific Biosciences and Oxford Nanopore), among others (SOHN; NAM, 2016; TREANGEN; SALZBERG, 2011; BENTLEY, 2006).

Within this framework of work in recent decades, there has been great insight into the composition of the genome, revealing expansions of the gene family and events of gene loss, contractions, expansions, which outlines its functional potential for biotechnology, virulence and host infection of various species of fungi (BALL; LANGILLE; GEDDES-MCALISTER, 2020).

Black yeasts differ from other yeasts in their ecological behavior, the occurrence of melanin deposits, and because many BY are capable of forming a synapomorphy consisting of abundant septate mycelium (HOOG; MCGINNIS, 1987). In contrast, black yeasts have significant similarities between groups of species, causing a difficulty to elucidate their taxonomy, and a need to use molecular methods to help solve clades and new species (HOOG et al., 2007). On the other hand, molecular tools used until then were not meeting the need to elucidate some species or to understand infection routes in animal and human hosts caused by black yeasts. This led to the era of omics, when, in 2014, Chen et al published the first black yeast genome, which was *E. dermatitidis*. After that, several genomes of black yeast species were published, in 2015 three genomes in total, which were *Cladophialophora immunda* (STERFLINGER et al., 2015), *E. mesophila* (TAFER et al., 2015) and *Phialophora attae* (MORENO et al., 2015), in 2016 others three genomes were published, *Fonsecaea nubica* (COSTA et al., 2016), *C. bantiana* (KUAN et al., 2016), *F. monophora* (BOMBASSARO et al., 2016). In 2017, 26 genomes were published, they are *F. multimorphosa* (LEAO et al., 2017), *Rhinocladiella mackenziei* (MORENO et al., 2018), *F. erecta* (VICENTE et al., 2017),

and others 23 of Chaetothyriales order by (TEIXEIRA et al., 2017).

Teixeira et al. (2017) evaluated genomic diversity of black yeasts and relatives in the Chaetothyriales order by the genomic information which a wide range of abilities of melanin biosynthesis was revealed among genes related to metabolically distinct DHN, DOPA and pyomelanin pathways. According to this study all *Capronia* species are homothallic as both MAT1-1 and MAT1-2 genes were found in each single genome. In addition, they noticed the genomic synteny of the MAT-locus flanking genes (SLA2-APN2-COX13) is not conserved in black fungi as is commonly observed in Eurotiomycetes, indicating a unique genomic context for MAT in those species and suggested that a parasexual cycle may play an important role in generating diversity among those fungi. The MAT (MAting Type) locus and other sex-related genes were recognized in all 23 black fungi. Members of the asexual genera *Fonsecaea* and *Cladophialophora* appear to be heterothallic with a single copy of either MAT-1-1 or MAT-1-2 in each individual. Vicente et al. (2017) used a comparative genomic analysis of environmental and pathogenic siblings of *Fonsecaea* and *Cladophialophora*, including *de novo* assembly of *F. erecta* from plant material in order given insights about pathogenic siblings share virulence factors enabling survival in mammal tissue after coincidental inoculation driven by pathogenic adaptation. The study shows the similarity of carbohydrate-active vs. protein-degrading enzymes associated with the occurrence of virulence factors suggested a general tolerance to extreme conditions, which might explain the opportunistic tendency of *Fonsecaea* sibling species. Virulence was tested in the *Galleria mellonella* model and immunological assays were performed in order to support this hypothesis.

In addition, Moreno et al. (2018) published a paper with the history of the omics era of black yeasts, showing very interesting data on comparing ecology, etiology and genomic patterns in BY history. The study also suggests a relationship between ants and black yeast, as the first produce many toxic compounds which can be metabolized by black yeast species. Silva-Bailao et al. (2018) provided data add knowlegde about aspect of black yeasts biology that may be useful in the understanding of their pathogenicity, when they reported an *in silico* analysis of four black yeasts genomes concerning zinc and copper homeostasis, demonstrating a relationship between the niches of black yeasts and pathogenicity in human hosts. In the analyzed genomes by the authors showed these organisms share apparatus of metal uptake, storage and detoxification with other pathogenic and non-pathogenic fungi, such as genes coding plasma membrane and organelle transporters, as well as metal binding proteins.

Bombassaro et al. (2020) in order to understand a neurotropic infection by an agent of Chromoblastomycosis, assumed as neurotropic dissemination from a chronic cutaneous case in an immunocompetent patient. The authors correlated by the genomic analysis the ability of the species to metabolize aromatic hydrocarbons with its

disseminate brain infection. That analysis was performed with *de novo* assembly, gene prediction, annotation and mitochondrial genome assembly, supplemented with animal infection models. The genome draft of 34.8 Mb was assembled with a total of 12,217 protein-coding genes. Several proteins, enzymes and metabolic pathways related to extreme tolerance and virulence were recognized.

Moroever, Moreno et al. (2020) studied several species of melanized fungi in the order Chaetothyriales live in symbiotic association with ants inhabiting plant cavities (domatia) or with ants that use carton-like material for the construction of nests and tunnels. According to the results the genomes of domatia-associated species are relatively small compared to other Chaetothyriales, with low numbers of protein-coding genes and with a high content of repetitive elements. Based on this analysis the authors suggested an ecological classification for Chaetothyriales based on genomic features considering derived species with high abundance of paralogs colonizing habitats rich in polyaromatic and potential producers of secondary metabolites with antimicrobial activities, beneficial for symbiotic interactions, occupying specific micro-habitats such as ant *domatia*.

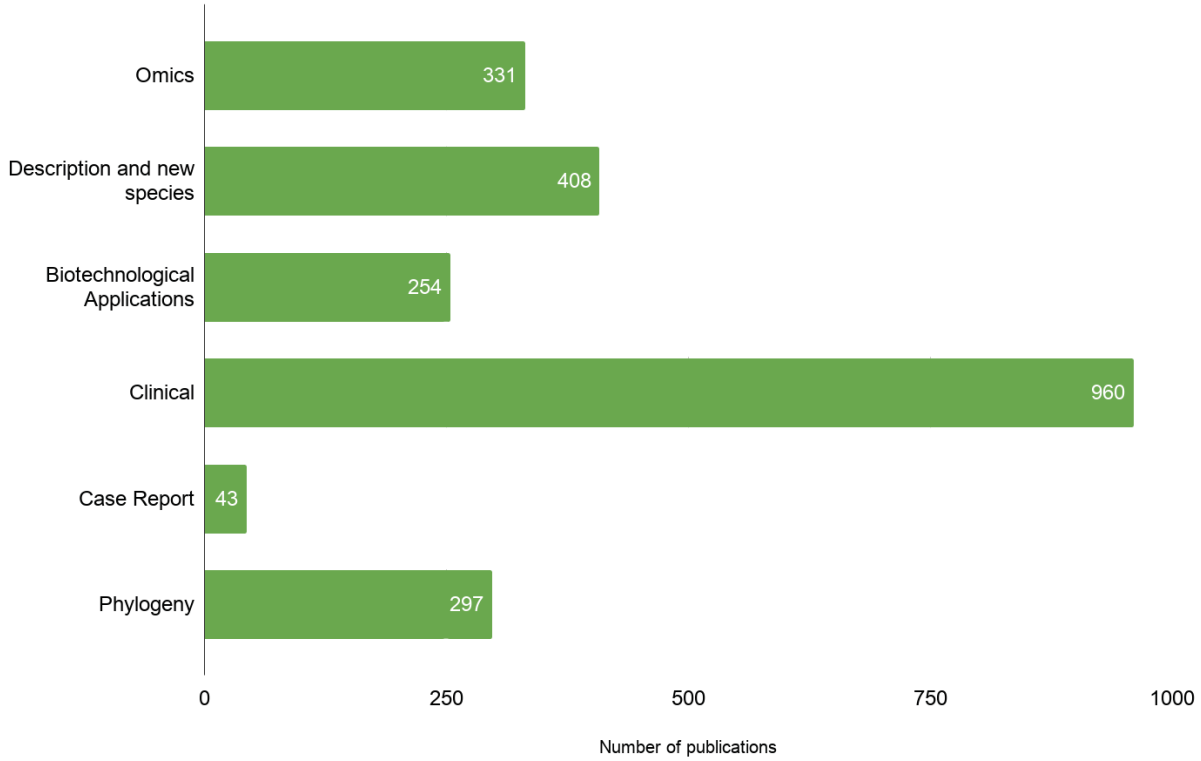
Furthermore, the transcriptomic studies results appear from 2002, which the first one was a transcriptome analysis of *Hortaea werneckii* for understanding the cell response to the environmental salinity (PETROVIC; GUNDE-CIMERMAN; PLEMENITAS, 2002). Looking forward the others results appears from 2010, which are several aims, such as pathogenicity (LIU et al., 2013; SONG et al., 2020), cell regulations and others.

Li et al. (2016) used *de novo* assembly transcriptomics using Illumina paired-end sequencing to investigate the transcriptome and RNASeq expression data, which are valuable resources to better understand the molecular and biological mechanisms regulating melanisation in *F. monophora*. The authors compared by the differential expression gene profiling analyses of parent and albino strains of *F. monophora*, using the Illumina RNA-seq system, role of melanin pathways in extremotolerance and virulence of *Fonsecaea*, demonstrated extensive down-regulation of key genes in the DHN pathway, while up-regulation was noted in the DOPA pathway of the albino mutant. Meanwhile, numbers of genes involved in light sensing, cell wall synthesis, morphology and environmental stress were identified in the transcriptome of *F. monophora*.

In 2016, more transcriptomes studies related to BYs start to appears, which differentiate some aspects related to virulence *Exophiala dermatitidis* (POYNTNER et al., 2016). Later, Blasi et al. (2017) report the genome and transcriptome of the toluene degrading black yeast *C. immunda*, showing that this species is able to mineralized hydrocarbon molecules. Shi et al. (2019) explored the potential mechanism of macrophages against *F. monophora* by the transcriptional profiling. The Functional

analyses suggested the biological functions of differentially expressed genes were closely related to immune response, and the melanin might affect the interactions by regulating the MAPK signalling pathway of macrophages. On the same year, Poyntner et al. (2018) developed an *E. dermatitidis* melanin deficient strain with Crisp-CAS9, and compare its transcriptome with a wild-type from the same species, with results showing differences in the expression of copper homeostasis, proteases and cell wall genes.

FIGURE 8 – Articles classification to the black yeast research lines, according to the keywords search on the title and abstract.



Source: the author

1.4 CONCLUSION

In conclusion, this study provides a overview of the black yeast research, which will help in evidence-based descriptions, comparisons, and visualizations of the publications. A total of 6145 publications were collected and analyzed, being that 86% of the documents were produced in the last 20 years by 1841 different institutions and 92 countries. It was also provided the current collaboration networks between the countries, the patents produced for the industry and the amount invested in the search for the understanding of the ecology, virulence and mechanisms of adaptation of these fungi.

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CHAPTER 2

GENOME SEQUENCING OF THE *Cladophialophora exuberans*, A NEW CANDIDATE FOR BIOREMEDIATION APPLICATIONS OF HYDROCARBON POLLUTED ENVIRONMENTS AND HEAVY METALS

2 GENOME SEQUENCING OF THE *Cladophialophora exuberans*, A NEW CANDIDATE FOR BIOREMEDIATION APPLICATIONS OF HYDROCARBON POLLUTED ENVIRONMENTS AND HEAVY METALS

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ABSTRACT

Cladophialophora exuberans is a black yeast fungi, belonging to the Ascomycota phylum and to the Chaetothyriales order. These melanized fungi are found in many different ecologies and habitats, often in high toxic environments. Even the Herpotrichiellaceae family being frequently involved in subcutaneous human infections, some members, include C. exuberans, *C. immunda*, *C. psammophila* and *Exophiala mesophila* has been described with capability to degrate aromatic compounds and xenobiotic volatiles in bioremediation applications. The objective of this present study is sequencing, assembly and describe the whole-genome of the *C. exuberans* focusing to show genes and pathways related with the ability to grown using BTEX compounds, phenyl acetate and

heavy metals as sole carbon source and energy, through genomics comparisons of siblings species including human pathogen and environmental strains.

Keywords: bioremediation, aromatic compounds, toluene, black yeast.

2.1 INTRODUCTION

Hydrocarbon contamination is a great worldwide worry because of the effect in the ecosystems, affecting environments, agricultural and food safety (SRIVASTAVA et al., 2019). The contamination can occurs in several ways because of the extensive use by humans, as generated during production, operations, refining, transportation, storage, and the treatment in the downstream process (MARIC et al., 2018), generally accidental dis-charge or oil spill that not only destroys the ecosystem but also causes soil and water pollution's (NAEEM; QAZI, 2019). Elimination of pollutants from soil, air, and water is better way to the environmental remediation in natural and human-transformed environments, being a low-cost and a clean technology (SRIVASTAVA et al., 2019).

In the last several decades, different methods have been employed and applied for the cleanup of our environment which includes mechanical, chemical, and biochemical remediation methods (NAEEM; QAZI, 2019) including the search of fungi based on the thermotolerance and ability of adaptation to grown in other stress conditions. The stress tolerance is a key attribute to use microorganisms for industrial applications (NAEEM; QAZI, 2019).

Recently, an increasing number of fungi isolated from polluting hydrocarbon sources ,biofilters, dry and acidic soil shown the capability to assimilate volatile aromatic hydrocarbons compounds as the only source of carbon and energy and notorious levels of adsorption and tolerance of heavy metals (PRENAFETA-BOLDÚ et al., 2001; MOHAMMADIAN et al., 2017), consequently being interesting to bioremediation applications (PRENAFETA-BOLDÚ; SUMMERBELL; HOOG, 2006).

Concern to the biotechnological applications, the comparison of the CytochromeP450 fungi enzyme system is necessary to help elucidated the details of its catalytic mechanism which is partially responsible for the transformation processes of the detoxification of xenobiotics (HUSSAIN et al., 2019; CHEN et al., 2014). In this context it has being highly used to as biobricks in synthetic biology for producing valuable compounds in recombinant hosts and due to their tailoring properties of catalyzing oxidative reactions (HUSSAIN et al., 2019; URLACHER; GIRHARD, 2019). In addition, it's also related with some pathogenicity cases (KARLSSON et al., 2008; TEIXEIRA et al., 2017; SONG et al., 2020).

Some genomics and transcriptomes of some Chaetothyriales order members has been produced, as *C. psammophila*, *C. immunda*, *E. mesophila* (BADALI et al., 2011; BLASI et al., 2017; TAFER et al., 2015), that they demonstrate degradation of aromatic hydrocarbons and ability to degrade BTEX compounds. This family has mainly been scientifically investigated in connection with human pathogenesis associated with certain thermotolerant members of the group (PRENAFETA-BOLDÚ; SUMMERBELL;

HOOG, 2006).

Nascimento et al (2017), showed the ability of the *Cladophialophora exuberans* to grown using toluene as carbon and energy source. Besides that, the soil naturally consists of heavy metals, and due to human action like refining of oil and use of pesticides, their concentration in soil is rising. Several areas have such high heavy metal and metalloid concentration that surrounding natural ecosystem has been severely affected (NAEEM; QAZI, 2019). Many mechanisms in fungi to tolerate and detoxify metals has been studied, like extra and intracellular precipitation, transformation of metals and biosorption to cell wall.

Considering the above mechanisms of metal resistance and aromatic compound assimilation, the objective of the present paper is to describe the genome sequence of the *C. exuberans* and to show the capability to survive in metal toxicity, exploring the genomic comparisions between some clinical and environmental strains using the GO, PFAM and KEGG databases.

2.2 MATERIALS AND METHODS

2.2.1 Broth microdilution assays

The minimum inhibitory concentration (MIC) will be determined in a microdilution test in a 96-well plate broth, according to CLSI document M38-A2 (2008). Different concentrations of CuCl2 and PbCl2 (39 - 20000 ppm) were added to evaluate the development of the *Cladophialophora exuberans* CMPR 1227. 1x104 CFU/mL spore suspension in RPMI 1640 medium, with pH 7.2 to 7.4 was inserted. Aliquots of a volume of 100 µ L of the fungal suspension were added to each well. The plates were incubated at 120h at 28 ºC and a visual reading was performed, where complete growth inhibition was evaluated. The negative control will be the absence of metals in the culture medium.

2.2.2 Determination of minimum fungicidal concentration (MFC)

The minimum fungicidal concentration was determined according (BOCATE et al., 2019). 20 µ L of each well with complete inhibition of fungal growth was withdrawn and cultured in plates with Czapek for 72 h at 30 ℃. The MFC was defined as the lowest drug dilution that yielded fewer than three colonies or complete absence of growth.

2.2.3 Agar diffusion assay

The agar diffusion assay was determined in 20 mL of Czapek culture medium, pH 5.6 were added in a 90 mm Petri dish. CuCl2 and PbCl2 solutions were added in concentrations (156 - 10000 ppm). After solidification of the culture medium, a mycelial point (from a plate with 14 days of growth), of the *C. exuberans*, was inserted into

the center of the plate. The plate was incubated at 28 ℃ until mycelial growth in the positive control reached 21 days. Mycelial growth was measured with the aid of a caliper, for tolerance indexes and mycelial growth speed index from the heavy metals. The tolerance index was determined follow the Oladipo's equation (2018). Tolerance index rating values indicate:

$$\frac{\text{Radial growth(mm) of test fungus in heavy metal incoporated medium}}{\text{Radial growth (mm) of fungus in non-incorporated medium}}$$

- 0.00–0.39 – extremely low metal tolerance.
- 0.40–0.59 – low metal tolerance.
- 0.60–0.79 – moderate metal tolerance.
- 0.80–0.99 – high metal tolerance.
- 1.00–1.00 – exceedingly high metal tolerance.

2.2.4 Statistical analyses

Means and standard deviation of the values obtained were performed and the results are tabulated using the Graphpad Prism 7.0, normality test and Kruskall-Walis has used in BioStatisc 5.0.

2.2.5 Strains, DNA extraction and sequencing

The strain *C. exuberans* (CBS 120420) was first described in 2017 evidencing exuberant conidiophores and to its ability to degrade toluene and phenyl acetate (Nascimento et al., 2017). The strain was cultivated in Sabouraud Glucose Agar at 28℃ for 7 days and DNA extraction was performed by the cetyltrimethylammonium bromide (CTAB) method with Chloroform:Isoamyl Alcohol (CIA) 24:1 v/v. To confirm the strain identification, the internal transcribed spacer region was amplified and sequenced using ITS1 and ITS4 primers. To genome sequencing the strain was grown in Sabouraud's broth, with shaking at 150 rpm at 28 ℃ for 7 days and DNA was extracted by CTAB with CIA method. DNA extraction was quantified by Nanodrop 2000/2000c and Qubit Fluorometer (Invitrogen). The library construction used the DNA of *C. exuberans* with the Nextera XT (Illumina) following the manufacturer's instructions and the sequencing was performed by Miseq (Illumina) for paired-end reads.

2.2.6 *De novo* assembly and annotation

The quality control of the reads was performed using FastQC v0.11.8 (http://www.bioinfor\matics.bbsrc.ac.uk/). The low-quality sequences were removed by BBMap

(https://sourceforge.net/projects/bbmap/).The high-quality reads were assembled *de novo* using SPADES v3.6.2 (BANKEVICH et al., 2012). Genes were predicted by Genemark-ES v4.30 (LOMSADZE, 2005) and to predict functional annotations and proteins it was used InterProScan v5.27-66.0 (QUEVILLON et al., 2005). The completeness of the genome assembly was evaluated by BUSCO v4.0.2 using the chaetothyriales_odb10 dataset (SIMÃO et al., 2015).

2.2.7 Genome Comparison

The orthologous analysis was performed by OrthoVenn2 (XU et al., 2019) with E-value 1e-2 and inflation value 1.5 parameters. The InterPro, PFAM, Gene Ontology databases were used to inference of the functions. The genomic tree was made using the JolyTree (CRISCUOLO, 2019).

2.3 RESULTS AND DISCUSSION

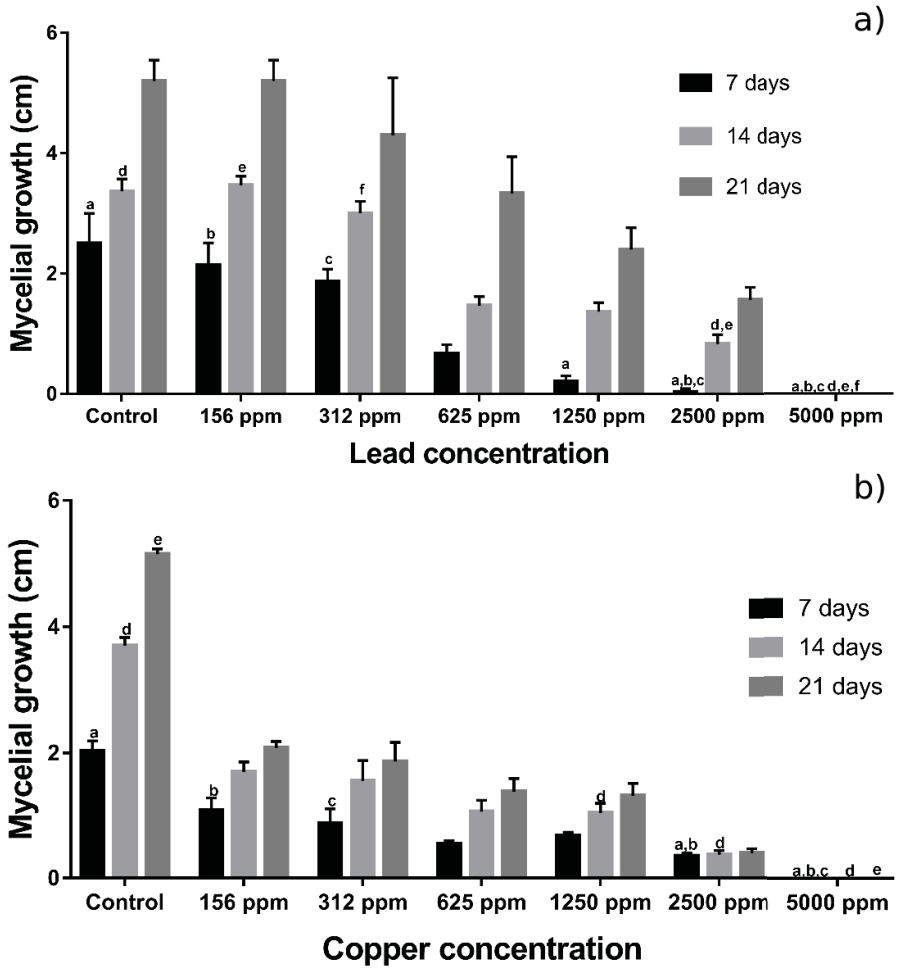
The use of different concentrations of Copper (Cu) and Lead (Pb), demonstrated the sensitivity of the species used. The MIC used for copper was 1250 ppm and for lead 625 ppm. In our studies, it was observed that the spores of the fungi remain viable in concentrations below 5,000 and 10,000 ppm of Cu and Pb, respectively. Although high concentrations inhibit its development, when the external conditions become great for its growth, the reproductive structures of the fungi start the germination process, when compared to the MIC and MFC methods. Regarding to the methodology, Ribeiro and collaborators (2017) performed in vitro susceptibility tests using copper and lead against *Phytium insidiosum*, given the inherent toxicity in heavy metals to fungal growth. Shams et al. (2014) evaluated the toxicological potential of copper against strains of *Aspergillus niger* and *Candida albicans*.

In fungal growth tests on agar, it was observed the strain growth until the threshold of 2500 ppm of lead. Higher concentrations showed no growth at all defined time intervals, as seen in figure 9. In 7 days at 156 ppm of lead, the strain *C. exuberans* CMRP 1227 showed a high tolerance (of 0.84) while at the concentration of 312 ppm there was a moderate tolerance (0,74). In the other concentrations evaluated (of 625, 1250 and 2500 ppm) it was observed that there was an exceptionally low tolerance (0.264, 0.08 and 0.012 respectively). From 14 days, it was evidenced an adaptation to the concentration of 156 ppm, which made possible a high tolerance to lead (1,00), as seen in the concentration of 312 ppm (0,89), 625 ppm (0,43) e 1250 ppm (0,4), in which tolerance rates have increased.

The profile of increase in fungal growth rates is related to the adaptive strategies of the fungal lineage. It was observed that after 21 days of cultivation, the strain showed tolerance of 1.00, 0.82, 0.63, 0.46 and 0.3 in the concentrations of 156, 312, 625,

1250 and 2500 ppm, respectively, not showing statistical differences in the exposures when compared to the control. The strain showed a remarkably high tolerance in the concentration of 156 ppm, however, in the other concentrations (312, 625, 1250 and 2500 ppm) the strain showed high, moderate, low and very low tolerance respectively after 21 days of treatment.

FIGURE 9 – Agar diffusion in Czapek culture medium with the *Cladophialophora exuberans* CMRP 1227 strain in contractions of 5000 - 156 ppm of lead (a) and cooper(b). The same letters represent the statistical differences between the concentrations. The black bars represent the standard deviation.



Related to the copper tests, it was also demonstrated mycelial growth up to the threshold of 2500 ppm with absence in higher concentrations, as seen in the (Figure 9). At 7 days, at concentrations of 156 and 312 ppm, a low tolerance was observed (0.55 and 0.43, respectively). In the other concentrations of 625 ppm, 1250 ppm and 2500 ppm we observed a very low tolerance to the metal that was tested (0,26, 0,33 and 0,175. At 14 days of testing the strain *C. exuberans* CMRP 1227 showed the same

tolerance profile calculated at 7 days, while at 156 ppm and 312 ppm, the strain showed a low tolerance (0,45 and 0,40). Moreover, in the concentration of 625 ppm, 1250 and 2500 was evidenced a very low tolerance (0,27, 0,27 and 0,09, respectively). Ultimately, at 21 days of testing only the 156 ppm concentration showed a low tolerance to copper (0,40), the rest of the concentrations entered in the test (312, 525, 1250 and 2500 ppm) expressed very low tolerances to copper (0,36, 0,26, 0,25 and 0,07).

Different filamentous fungi and yeasts, mainly hyaline, are used to assess their tolerance to the presence of metals, mainly lead and copper. Mao et al. (2019) evaluated the tolerance of a strain of the genus *Mucor* and observed that they showed growth sensitivity at 5 ppm of lead. Bansal and collaborators (2018) observed that *Candida parapsilosis* performed tolerance tests on metals, mostly Cu and Pb. In their results, it was observed that concentrations of 12 mM (24 ppm for Pb e 72 ppm for Cu) lose cell viability by 50%.As mentioned above, many strains of fungi are sensitive to low metal concentrations. The *C. exuberans* CMRP 1227 strain evaluated in this study demonstrated the potential to tolerate high concentrations. According to the literature the genus Cladophialophora has the potential for hydrocarbon bioremediation, mainly low molecular weight like the toluene (BLASI et al., 2017; NASCIMENTO et al., 2017) and the melanin seems to be an essential role (NASCIMENTO et al., 2017).

Despite this, the analysis of the transcriptome of the sister species, *C. immunda* revealed that the genus has a capacity for development in places contaminated, which can serve as possible bioremediation indicators (BLASI et al., 2017). A relevant fact is that the melanin present in the vegetative and reproductive structures of this strain can react with metals due to the different amine, carboxyl and hydroxyl groups present in the pigment. Some studies report that copper can bind to carboxylic groups and also in hydroxyphenolic groups (FRONCISZ; SARNA; HYDE, 1980). The melanin present in demeaceous fungi demonstrated to assist in heavy metal biosorption processes performed experiments with albino and melanized *Cladosporium resine* and *Aureobasium pullans* (GADD; ROME, 1988). In their results had an increase of 2,9x and 2,5x of capitation of copper for melanized lines of *C. resine* and *A. pullans*, respectively, when compared to albino strains. Siegel and collaborators (1986) also performed experiments with *Cladosporium cladosporioides* pigmented with Nickel, Copper, Zinc, Lead. The group's results showed a capitation of 155, 140, 90 e 165 nmol/mg/h of Nickel, Copper, Zinc, Lead, respectively.

The biomass production, based on the results obtained in our study, is closely related to the process of bioremediation by the species *C. exuberans*. According to Bishnoi and Garima (2004), fungal biosorption depends on the concentration of the biomass. Moreover, the removal of metals by microorganisms is considered better than conventional adsorbents.

The study made by Buszman et al. (2006) demonstrated the adsorption of copper and zinc ions by pigmented fungi of the *Cladosporium cladosporioides* species using paramagnetic resonance spectroscopy. The authors verified the alteration of free radicals o-semiquinone in the melanin of *C. cladosporioides* in the presence of zinc and copper. Consequently, the homogeneity of the distribution of these radicals influenced the process of biosorption of metals by the fungus, as well as stimulating growth and pigmentation. Refering to the our results of the radial growth of *C. exuberans* (CMRP 1227) in the presence of copper and lead, the tolerance of the fungus to these metals was observed. The melanin in the cell wall justifies the tolerance and biosorption capacity of metals by fungi, resulting in their growth.

The recent study reported by Ahmad and Ganjo (2020) demonstrated the capacity of fungi for bioremediation of heavy metals, which showed a reduction in the concentration of lead of 26,70 mg/Kg (control) to 10.99 mg/kg (in two weeks) and 3.96 mg/kg (in two months). As well as for the remediation of arsenic, by the species *Aspergillus niger*. According to the results, it is evident the minimum inhibitory concentration (MIC) of 1250 ppm and 625 ppm, respectively, for Cooper and lead metals. Talukdar et al. (2020) observed the removal of chromium and cadmium by fungi in which metal removal rates were 70 to 74%, associated with the tolerance of pollution-resistant microorganisms, therefore, knowledge about their resistance to heavy metals is essential.

Kumar et al. (2014) verified the removal of lead, cadmium and chromium in liquid medium at a concentration of 50 ppm by different species of fungi, of the genus *Aspergillus*, *Rhizopus* and *Trichoderma*. This study corroborates the results obtained for *C. exuberans* demonstrating that capacity of bioaccumulation and biosorption by microorganisms could allow its application in bioremediation techniques.

The studies by Hassan et al. (2020) also demonstrated the capacity of different fungal species for bioremediation of lead, nickel and zinc, in which the reduction of the concentration of metals was observed in the interval of 100 days. Consequently, the bioaugmentation with the use of fungi it improves the process of removing heavy metals in the soil. The studies by Joshi et al. (2011), unlike other studies, observed the tolerance capacity of fungi above 400 ppm for lead, cadmium, chromium and nickel. In addition to highlighting the potential application of fungi as biosorbents for the removal of metals from wastewater and industrial effluents.

Silva-Bailao et al. (2018) demonstrated the mechanisms of copper and zinc homeostasis in pathogenic black fungi. The authors observed in the genome analyzes structural differences in the transcription factors possibly associated with zinc and copper, when comparing with the regulators already defined. Moreover,it was showed transporters from the ZIP and CDF families, associated with the transition of zinc through

cell membranes. Related to the copper, although the mechanisms aren't very clear, the presence of Ctrs transporters is evident, resulting in copper uptake and distribution in cell compartments.

Conforming this study, related to growth in presence of metals conditions, probably, *C. exuberans* (CMRP 1227) also has mechanisms related to the metabolism of copper and lead. Therefore, genomic analyzes of black yeasts are essential to understand their potential for bioremediation.

The genome sequencing of the *C. exuberans* comprises in 38.1 MB with 95.55 % of the assembly completeness. The number of predicted proteins is 14.851 Table 4.

TABLE 4 – Assembly and protein prediction of the *Cladophialophora exuberans*.

Genome Size	38.1MB
Assembly completeness (BUSCO)	95.5%
Reads	14913242
Scaffolds	1205
GC content	50.8%
Coverage	89.9X
Nº of proteins	14851

The largest annotated domains of the *C. exuberans* were compared with six species from the Chaetothyriales order. In general, *C. exuberans* annotation show many genes associated to the metabolic process (630 genes), highlighting catalysis of the redox reaction, with impressive 1419 genes to the oxidation-reduction process (GO:0055114) (Figure 10a) and 1174 oxidoreductase activity (GO:0016491) (Figure 10b), followed by *E. mesophila* and the *C. carrioni*, they showed together high levels to the zinc ion binding and ATP binding domains. Regarding the cellular components, there's no significant difference between the species, except for the integral component of membrane (GO:0016021) and nucleus (GO:0005634) (Figure 10c).

In total, 14.536 genes orthologous clusters were formed, with 9503 clusters at least contained two species and 5033 single-copy gene orthogroups. The number of genes clusters shared between *C. exuberans* and the siblings species variated in 165 genes and sharing more genes with *C. immunda* (12.096 genes), also known for its ability to toluene degrading (BLASI et al., 2017).

FIGURE 10 – Gene Ontology terms comparison. a) Biological Process; b) Molecular Functions; c) Cellular Component. It was selected the top 20 ontologies more representative in the *Cladophialophora exuberans* proteins and compared with the siblings species.

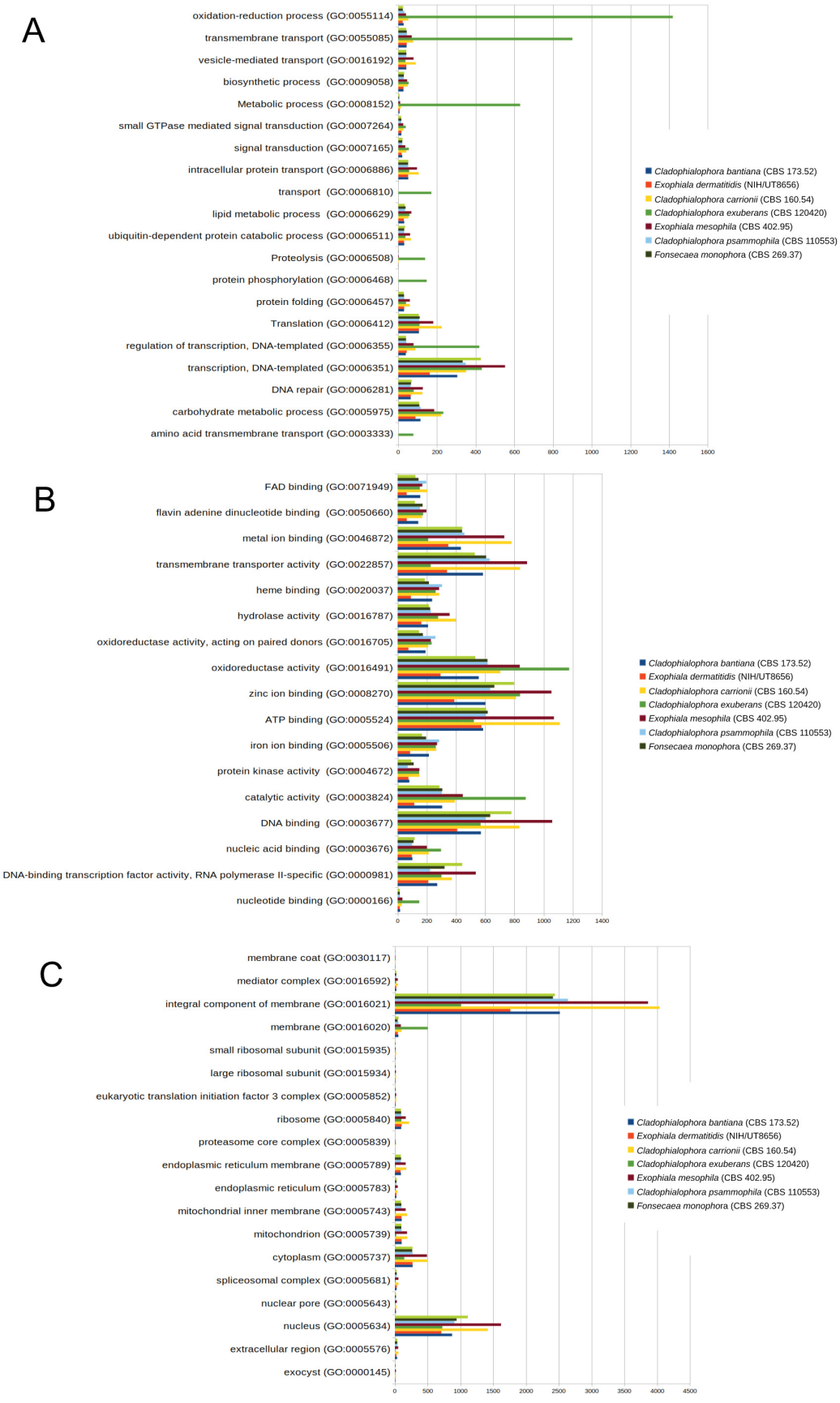
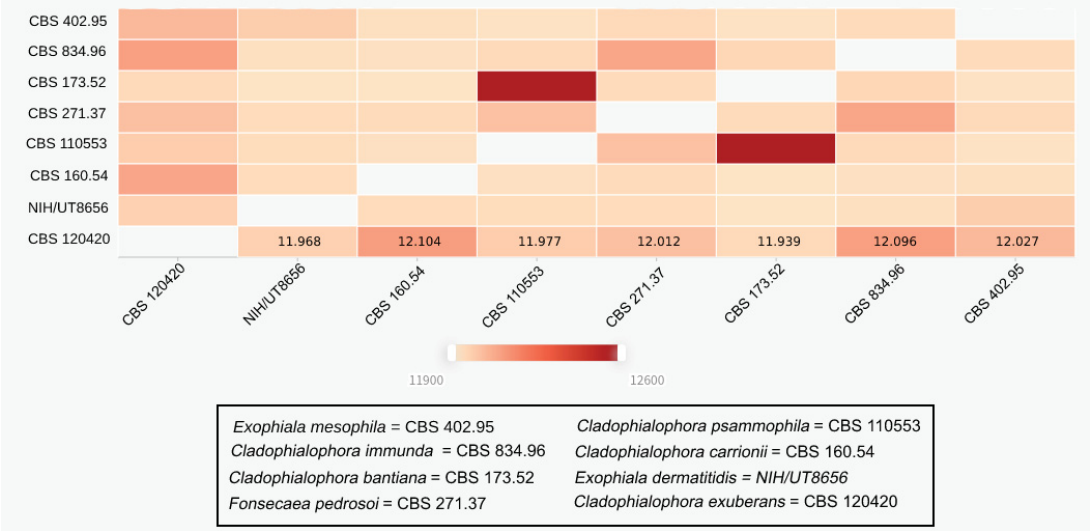
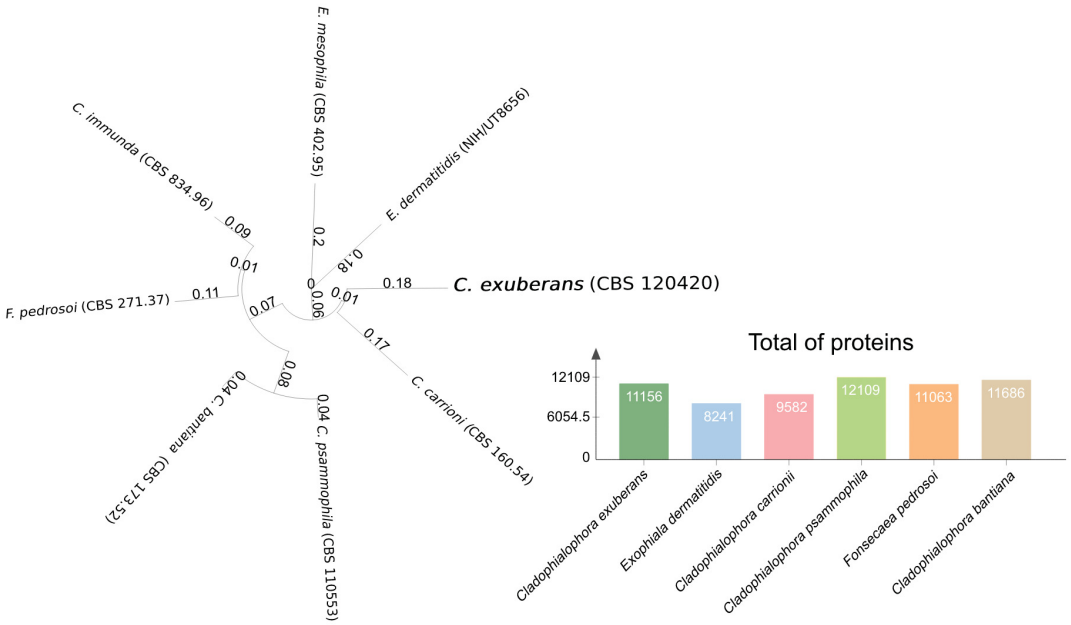


FIGURE 11 – Pairwise HeatMap representation of the orthogroups. The graphs shows a similarity matrix between each pair of genomes.



Two strains had the number of singletons (genes not belonged to any cluster) more than the average (1340 genes), being *C. exuberans* and *C. immunda* with 3109 and 1923 genes, respectively. A total of 68 genes were found related to the protein degrading peptidase enzyme families. It was expected more affinity between the *C. exuberans* and *C. psammophila*, however the genome of the *C. exuberans* is more closer than *C. carrioni* (Figure 12), while *C. psammophila* have more afinity with the neutropic specie *C. bantiana* based on the gene clusters shared (Figure 11).

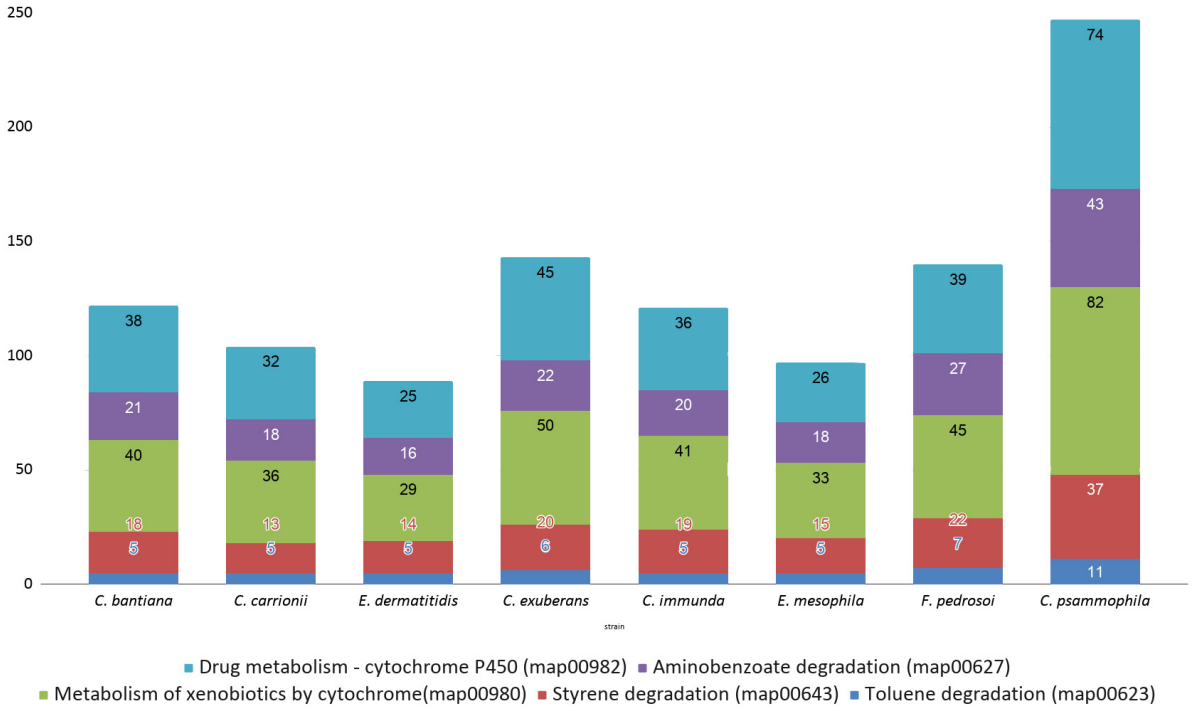
FIGURE 12 – Genomic tree based on the computation of the dissimilarity between the genomes and estimation of the number of substitution events throughout evolution.



Although there is no occurrence of a clinical case for *C. exuberans*, in concern to the virulence genes, it was found 35 genes of the Myb-like DNA-binding domain (SONG et al., 2020) and it was not detected to the *C. psammophila*, also investigated to the *E. dermatidis* and *E. spinifera* by (SONG et al., 2020).

The KEGG pathways analyzed (Figure 13), all strains have genes associated with the degradations pathways, *C. psammophila* had more genes, followed by the *C. exuberans*. However it's was no possible to find consists differences between the clinical and enviromental strains being necessary further studies, preferably transcriptomes in toxic conditions and some animals hosts, should be developed to investigate and understand the relation of the ability to degrade aromatic compounds and the virulence and pathogenicity domains.

FIGURE 13 – Pathways from KEGG database comparision.



2.4 CONCLUSION

In conclusion, thorough genome assembly and annotation of *Cladophialophora exuberans* combined with tolerance test of toxic conditions allows to get a better understanding of the mechanisms used by this fungus to adapt in extreme conditions and to protect itself. Moreover, it was found orthologous virulence genes of *Exophiala dermatitidis* that it can serve as insights into the relationship between aromatic degradation mechanisms and virulence factors. The previously tests performed by Nascimento et al. (2017) to assimilation of toluene in *C. exuberans* was confirmated by the fact that there genes involved in all steps to degrate the toluene. Although there are no described metal degradation pathways available in KEGG db, the resistance observed in *C. exuberans* can be explained by the degradation pathways found in the genomes as justified by the abundance of the annotated genes for the domains as metabolism of xenobiotics by cytochrome, aminobenzoate degradation and oxidation-reduction process. Transcriptome studies, including comparisons with data already available, should be developed in order to find the expression levels of genes related in this domains.

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CHAPTER 3

COMPARATIVE GENOMIC ANALYSIS OF CAPSULE-PRODUCING BLACK YEASTS *Exophiala dermatitidis* AND *E. spinifera*, POTENTIAL AGENTS OF DISSEMINATED MYCOSES

3 COMPARATIVE GENOMIC ANALYSIS OF CAPSULE-PRODUCING BLACK YEASTS *Exophiala dermatitidis* AND *E. spinifera*, POTENTIAL AGENTS OF DISSEMINATED MYCOSES

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ABSTRACT

The two black yeasts *Exophiala dermatitidis* and *Exophiala spinifera* that are clinically considered as the most virulent species potentially causing disseminated infections are both producing extracellular capsule-like material, are compared. In this study, 10 genomes of *E. spinifera* and *E. dermatitidis* strains, including both clinical and environmental isolates, were selected based on phylogenetic analysis, physiology tests and virulence tests, sequenced on the Illumina MiSeq sequencer and annotated. Comparison of genome data were performed between intraspecific and interspecific strains. We found capsule-associated genes were however not consistently present in both species by the comparative genomics. The prevalent clinical species, *E. dermatitidis*, has small genomes containing significantly less virulence-associated genes than *E. spinifera*, and also than saprobic relatives. Gene OG0012246 and Myb-like DNA-binding domain and SANT/Myb domain, restricted to two strains from human brain, was shared with the neurotropic species *Rhinocladiella mackenziei*. This study indicated that different virulence profiles existed in the two capsule-producing black yeasts, and the absence of consistent virulence-associated profiles supports the hypothesis that black yeasts are opportunists rather than primary pathogens. The results also provide the key virulence genes and drive the continuing research forward pathogen–host interactions to explore the pathogenesis.

Keywords: black yeasts, comparative genomics, intraspecific variability, interspecific differences, virulence profiles, opportunists

3.1 INTRODUCTION

Black yeasts in the ascomycete order Chaetothyriales are relatively frequent opportunistic agents of human disease. Species of Chaetothyriales producing budding cells in any stage of their life cycle are classified currently in the genus *Exophiala*. The Atlas of Clinical Fungi (HOOG et al., 2019) lists 19 species that were proven to have been involved in infections of humans or cold-blooded vertebrates. Of these, *Exophiala dermatitidis* and *E. spinifera* are the most common species in clinical settings, and are the only recurrent agents of severe, deep and disseminated infections in humans, then often with fatal outcome. In the past, patients were reportedly without significant underlying disease, but recent research has shown that such infections are mostly associated with an inherited defect in the dectin signaling pathway due to mutations in the CARD9 gene (LANTERNIER et al., 2015).

Virulence of black yeasts has been attributed to the presence of melanin in cell walls (TABORDA et al., 2008), but since all members of the order Chaetothyriales are consistently melanized, this does not explain the difference in infective ability between species. Thermotolerance, for which marked differences are noted between species, is another important factor: eleven out of 19 opportunistic *Exophiala* species are at least weakly tolerant of 37 °C (HOOG et al., 2019), with *E. dermatitidis* and *E. spinifera* having the most pronounced thermotolerance. However, Hoog et al. (2011) described a 'waterborne clade' of mesophilic species in the genus, comprising species commonly infecting fish, frogs, toads or crabs, often at epidemic proportions (VICENTE et al., 2012; SARAIVA et al., 2019).

Consequently, black yeasts lacking thermotolerance are also able to cause vertebrate infection. Gostinčar et al. (2018) suggested polyextremotolerance as a prerequisite for opportunism, highlighting the interplay of several, independent factors enabling growth under non-optimal conditions of animal tissue. The black yeasts and relatives mitigate external stress of e.g. dryness and irradiation by melanin, and detrimental effects of toxin can be compensated by pathways of the cytochrome P450 (MORENO et al., 2018) aiding degradation and co-assimilation of monoaromatic hydrocarbons. This combination of vitality factors provides a wide array of survival strategies, and it may be hypothesized that species with the most pronounced development of such pathways may have a higher ability of tissue invasion. In addition, (YURLOVA; HOOG, 2002) noted that *E. dermatitidis* and *E. spinifera* are unique in producing extracellular capsule-like structures during early exponential growth of budding cells. This may further enhance opportunism. Both species are able to disseminate in susceptible patients with formation of cutaneous acanthosis, but a difference in clinical predilection has been noted: *E. dermatitidis* has a tendency of neurotropism, whereas *E. spinifera* seems to be somewhat osteotropic (SONG et al., 2017). This suggests that opportunism in these

fungi might be fine-tuned.

The aim of the present paper is to compare the genomes of the above opportunists in *Exophiala* with each other and with species with other types or with no opportunistic potentials, in search of genes that might play a role in the above described differences. Several genomes were sequenced of each species, enabling to compare the intra-species variability in the genome in general and in potential virulence genes.

3.2 MATERIALS AND METHODS

3.2.1 Strains and DNA extraction

To extract genomic DNA, fungal mycelia of *E. dermatitidis* strains CBS 109144, CBS 115663, CBS 120473, CBS 132758, CBS 132754, and CBS 578.76, and *E. spinifera* strains CBS 101539, CBS 116557, CBS 123469, CBS 126013, and CBS 131564 were harvested from fresh cultures on Sabouraud's Glucose Agar (GSA), washed using sterile Tris-EDTA buffer (TE), pH 8.0 in 2 mL vol screw-capped tubes, and then resuspended in 500 ml TE buffer. Fungal cell walls were disrupted using 0.5 mm glass beads in a BioSpec Mini-Beadbeater-16 (BioSpec) for 5 min and cooled on ice for an additional 5 min. DNA solutions were separated using two phenol/chloroform (1:24, pH 8.0) extractions. DNA was then precipitated by isopropanol, washed with 70% ethanol, dried at room temperature, and resuspended in 35 mL TE buffer, pH 8.0. DNA quantity and quality were determined using Qubit (Invitrogen, Applied BioSystems), and an Agilent Bio Analyzer 2100 using a 1000 DNA Chip (Agilent). Cardinal growth temperatures were determined on 2% malt extract agar (MEA; Difco). Plates were incubated at 37 ℃, 40 ℃, 42 ℃, 45 ℃ in the dark for 2 weeks; plates contained double quantities of medium and were sealed to prevent drying out. Colony diameters were measured for studied strains.

3.2.2 De novo assembly

Quality control of the reads was performed using FastQC v0.11.8 (http://www.bioinformatics.bbsrc.ac.uk/), and low-quality sequences were removed by Trimmomatic (Leading: 3, Trailing: 3, Slidingwindow: 4:15) (BOLGER; LOHSE; USADEL, 2014). High-quality reads were assembled de novo using SPAdes v3.13.0 (BANKEVICH et al., 2012).

3.2.3 Read mapping and SNP calling

For SNP calling, high-quality sequencing reads of each of the genomes were mapped against the reference genome of the reference strains *E. dermatitidis* CBS 525.76 = NIH/UT8656 and *E. spinifera* CBS 899.68 deposited at GenBank, respectively,

by using Burrows Wheeler Aligner (BWA) v0.7.17-r1188 mem (LI; DURBIN, 2009) and sorted to the bam format using SAMtools v1.7. They were then marked duplicated using Picard v1.8 (https://broadinstitute.github.io/) and indexed using SAMtools. Variants were identified using GATK HaplotypeCaller v3.4.9.0. SNP annotation was performed by VCFannotator (http://vcfannotator.sourceforge.net/) to assess whether the SNP was found within an untranslated region, intron, or coding exon, and mutations were classified into synonymous (SYN), nonsynonymous (NSY), read-through (RTH), and nonsense (STP).

3.2.4 Protein Detection

To look for the protein sequences of the newly sequenced *E. dermatitidis* strains, 9,285 proteins of a reference strain *E. dermatitidis* CBS 525.76 (NIH/UT8656) were aligned to the genomes of the strains CBS 109144, CBS 115663, CBS 120473, CBS 132758, and CBS 578.76 using the homology-based predictor Exonerate v2.4.7 [11] with the parameters-model protein2genome-percentage 90. Similarly, 12,049 proteins of the type strain of *E. spinifera* CBS 899.68 were also aligned to the genomes of the strains CBS 101539, CBS 116557, CBS 123469, CBS 126013, and CBS 131564. Protein sequences of the ten strains were extracted from the aligned exon regions using a custom Python script.

Possible relationships of analyzed gene families with invasion of cerebral versus bone tissue which are critical virulence genes for the understanding of black yeast virulence, were searched in the literature, with functional confirmation in the online InterPro database (http://www.ebi.ac.uk/interpro/). Relevant genes and domains were blasted against the *E. spinifera* and *E. dermatitidis* genomes.

3.2.5 Ortholog detection

The protein sequences of the ten strains of the two species *E. dermatitidis* and *E. spinifera* were clustered using OrthoFinder v2.1.2 (EMMS; KELLY, 2015) to determine which proteins were shared or specific by the two species. In addition, the protein sequences of the five *E. dermatitidis* strains (CBS 109144, CBS 115663, CBS 120473, CBS 132758, and CBS 578.76) were also clustered to see which proteins were shared between isolates from brain (CBS 578.76, CBS 120473) and environmental isolates (CBS 109144, CBS 115663, CBS 132758).

3.3 RESULTS

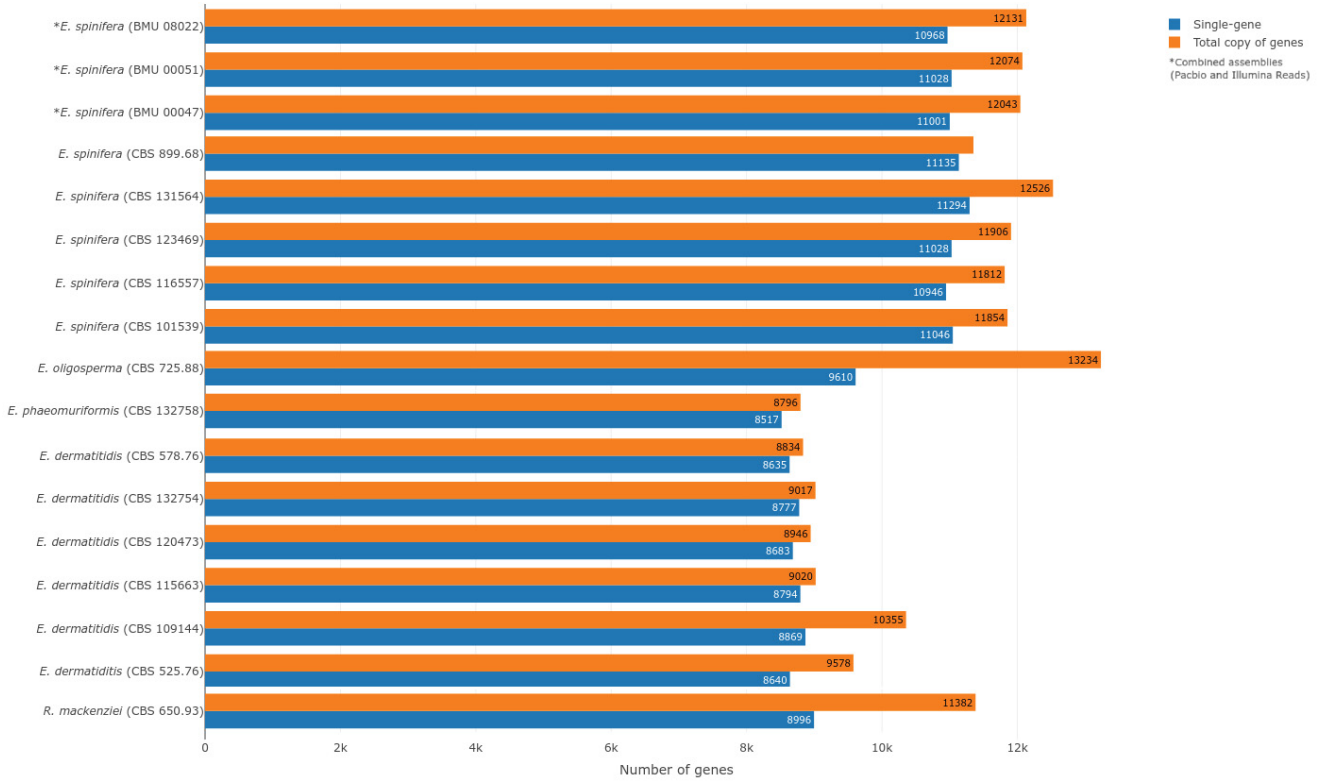
3.3.1 Data quality and identification

Genomes of five strains of each species were sequenced (Table 5). Different techniques were applied, i.e. Pacbio and Illumina. Comparison of genome data of the same strain of *Exophiala spinifera*, BMU00047 (SONG et al., 2020a) with the reference genome (CBS 899.68, JYBY00000000.1) provided perfect match with Illumina, but some translocations were noted compared to PacBio data (Figure 14). The genomes assembled with PacBio had less gaps and had more copies of the same gene. However, when single-genes were counted without duplication, the results of genome assembly are very similar to those assembled with Illumina reads (Figure 14). The reads of the newly sequenced strains of the two black yeasts *Exophiala dermatitidis* and *E. spinifera* were quality-controlled. Low-quality reads were removed. The size of the forward/reverse sequencing reads of these strains ranged from 400 M to 700 M. The number of scaffolds obtained for each genome is provided in Table 6. The high-quality forward/reverse sequencing reads were assembled using *de novo* assembly. The genomes had sizes ranging between 26.2 and 34.3 M. The genomes of *E. dermatitidis* on average were smaller than those of *E. spinifera*. Intraspecific variability was evaluated using Orthofinder, for strains of *E. dermatitidis* and of *E. spinifera* separately. In total, 55,750 clustered genes were found. Percentages of genomic variation ranged from 0.2 % to 8.6 % (1,626 genes) in *E. dermatitidis* genomes, while for *E. spinifera*, with a total of 108,201 genes, variation ranged from 0.5 % to 3.2 % (2,052 genes). In the genomic dendrogram (Figure 15), *E. spinifera* strains demonstrated a higher degree of branching, which is explained by a lower percentage (71.6 %) of single-copy orthogroups (n = 8,979), while for *E. dermatitidis* this was 85.7 % (n = 7,640). Estimation of gene gains and losses shows that *E. spinifera* has higher divergence than *E. dermatitidis*, with averages of 268.13 gains and 74.75 losses, versus 21 gains and 31 losses per speciation event, respectively. Compared to sister species *E. phaeomuriformis* of *E. dermatitidis* and *E. oligosperma* of *E. spinifera*, considerable gene gains and expansions were noted (Figure 15), which deviated significantly (3109.8 genes on average) from intra-specific changes. Gene dynamics also differed between the species of study, i.e. on average 2111 genes (σ314.3 in shared genes and 608.1 in specific genes) in *E. dermatitidis*, and on average 4107.8 (σ121.18 in shared genes and 55.9 in specific genes) in *E. spinifera*.

TABLE 5 – Metadata of strains sequenced in this study, and reference isolates. T = ex-type strain; R = published genomes included for comparison.

Strain ID	Species	Country	Source
CBS 101539	*E. spinifera*	Colombia	Soil
CBS 116557	*E. spinifera*	Thailand	Pine apple
CBS 126013	*E. spinifera*	Brazil	Shell of babasu coconut
CBS 131564	*E. spinifera*	Thailand	Human toenail
CBS 123469	*E. spinifera*	China	Human skin
BMU 08022R	*E. spinifera*	China	Human skin (CARD9 deficient)
BMU 00051R	*E. spinifera*	China	Bark
BMU 00047R	*E. spinifera*	Colombia	Soil
CBS 899.68TR	*E. spinifera*	USA	Human nasal granuloma
CBS 725.88TR	*E. oligosperma*	Germany	Human sphenoid abscess
CBS 132758R	*E. phaeomuriformis*	Turkey	Dishwasher
CBS 109144	*E. dermatitidis*	Netherlands	Turkish bath
CBS 132754	*E. dermatitidis*	Turkey	Bathtub
CBS 578.76	*E. dermatitidis*	Japan	Human brain
CBS 115663	*E. dermatitidis*	Qatar	Endotracheal aspirate
CBS 120473	*E. dermatitidis*	USA	Human brain
CBS 525.76TR	*E. dermatitidis*	Japan	Human sputum
CBS 650.93R	*Rhinocladiella mackenziei*	Saudi Arabia	Human brain

FIGURE 14 – Numbers of genes in analyzed strains of *Exophiala spinifera* and *E. dermatitidis*, compared with reference genomes. *Strains analyzed with combined Illumina and PacBio approach; remaining strains analyzed with Illumina.



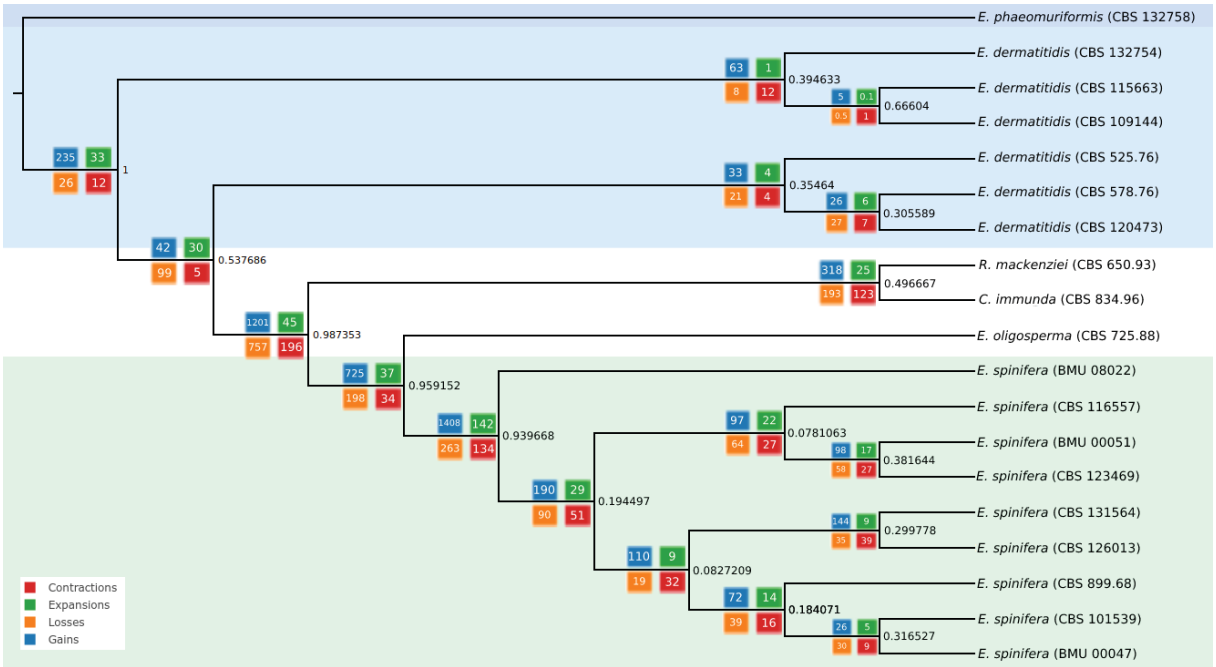
Source: (SONG et al., 2020b)

TABLE 6 – Genome size and scaffold numbers of *Exophiala dermatitidis* and *E. spinifera*.

	Genome size	Number of scaffolds	Number of reads	GC content (%)	Number of genes	Assembly completeness (BUSCO)	Repeated Sequences (Simple Repeats)	Repeated Sequences (Low complexity)
E. dermatitidis								
CBS 109144	29.6 M	2094	21,487,952	51.18%	10.355	99.0%	0.92%	0.07%
CBS 115663	26.8 M	68	20,912,714	51.40%	9.02	99.3%	0.89%	0.07%
CBS 120473	26.4 M	245	19,003,776	51.61%	8.946	97.7%	0.88%	0.07%
CBS 132754	26.8 M	326	11,165,600	51.41%	9.017	99.3%	0.89	0.07%
CBS 578.76	26.2 M	219	20,397,964	51.60%	8.834	99.00%	0.90	0.07%
E. spinifera								
CBS 101539	32.9 M	133	19,532,882	51.56%	11.854	97.7%	0.83%	0.08%
CBS 116557	32.5 M	109	22,950,634	51.81%	11.812	97.3%	0.82%	0.08%
CBS 123469	32.4 M	243	14,594,162	51.85%	11.906	98.0%	0.81%	0.08%
CBS 126013	32.6 M	117	12,808,484	51.81%	11.806	98.0%	0.81%	0.08%
CBS 131564	34.3 M	906	17,069,282	51.60%	12.526	98.3%	0.90%	0.07%
E. phaeomuriformis								
CBS 132758	25.86 M	97	85,740,696	51.60%	8796	99.3%	0.88%	0.07%

The core genomes of 6,812 genes were determined by comparing the shared genes in all *Exophiala* species sequenced to date (Figure 16). The accessory genomes of *E. spinifera* were considerably larger than those of *E. dermatitidis*, but CBS 115663 and particularly CBS 109144 in the latter species deviated considerably from remaining strains by having 580 and 1,586 unique genes, respectively. The use of long reads provides better resolution, which is particularly significant for repetitive regions of the genome. Whole-genome comparison of all genomes of the two species with reference genomes of *Exophiala phaeomuriformis* (nearest neighbor of *E. dermatitidis*), *E. oligosperma* (nearest neighbor of *E. spinifera*), *Cladophialophora immunda* (saprobic hydrocarbon-assimilating species) and *Rhinocladiella mackenziei* (opportunistic neurotropic species) was based on detection of 5,374 single-copy orthogroups representing 41.86 % of orthologous groups. The genomic dendrogram (Figure 15) showed correct clustering of all genomes, but with significant intra-specific variability, which in *E. spinifera* was twice that of *E. dermatitidis*.

FIGURE 15 – Phylogenomic tree based on whole genomic analysis using OrthoFinder; 5,374 single-copy orthogroups were detected, representing 41.86% of orthologous groups.

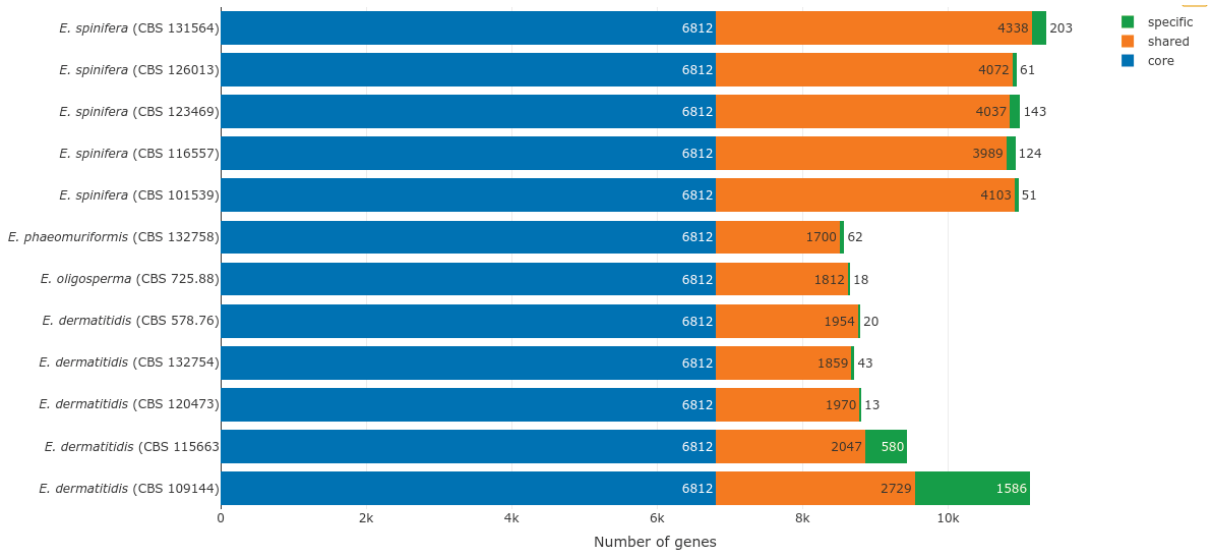


Source: (SONG et al., 2020b)

3.3.2 Protein prediction and SNP calling

Protein sequences of the new sequenced strains were predicted using the homology-based predictor InterProScan v5.27-66.0 (JONES et al., 2014). The numbers of the obtained genes for each of the strain are given in Table 6. Protein sequences of the

FIGURE 16 – Core and accessory genomes of *Exophiala spinifera* and *E. dermatitidis*, showing the number of unique genes in each strain.



Source: (SONG et al., 2020b)

ten genomes of the two species were clustered to detect orthologs using OrthoFinder v2.1.2 (EMMS; KELLY, 2015). A total of 12,690 clusters was detected. Among these, 7,291 clusters had protein sequences shared by *E. dermatitidis* and *E. spinifera*; 1,456 clusters were represented in the *E. dermatitidis* proteome only, and 3,253 clusters were exclusively present in *E. spinifera*.

In order to detect genomic variants, the high-quality sequencing reads of all genomes of the two species were aligned with the respective reference genomes, *E. dermatitidis*
CBS 525.76 = NIH/UT8656 and *E. spinifera* CBS 899.68. The total numbers of genomic variants and non-synonymous SNPs of the *E. dermatitidis* and *E. spinifera* genomes are given in Table 7. The numbers of non-synonymous SNPs in *E. dermatitidis* varied from 8,887 to 30,126, and in *E. spinifera* from 17,978 to 43,282. The two brain-associated isolates of *E. dermatitidis*, CBS 528.76 and CBS 120473, shared two clusters containing genes with unique SNPs. The first cluster contained the genes HMPREF1120_03262 of *E. dermatitidis* and Z518_05210 of *R. mackenziei* that was linked to benzaldenhyde dehydrogenase. The second cluster contained genes HMPREF1120_08762 of *E. dermatitidis* and Z518_06196 of *R. mackenziei*.

3.3.3 Metabolism of aromatic compounds

To identify the presence of genes involved in pathways for the degradation of monoaromatic hydrocarbons (e.g. benzene, toluene, ethylbenzene, xylene, styrene and other volatile pollutants), the genomes of reference strains of *Exophiala der-*

TABLE 7 – Synonymous and non-synonymous SNP numbers of *Exophiala dermatitidis* (CBS 525.76) and E. spinifera (CBS 899.68), compared with reference genomes.

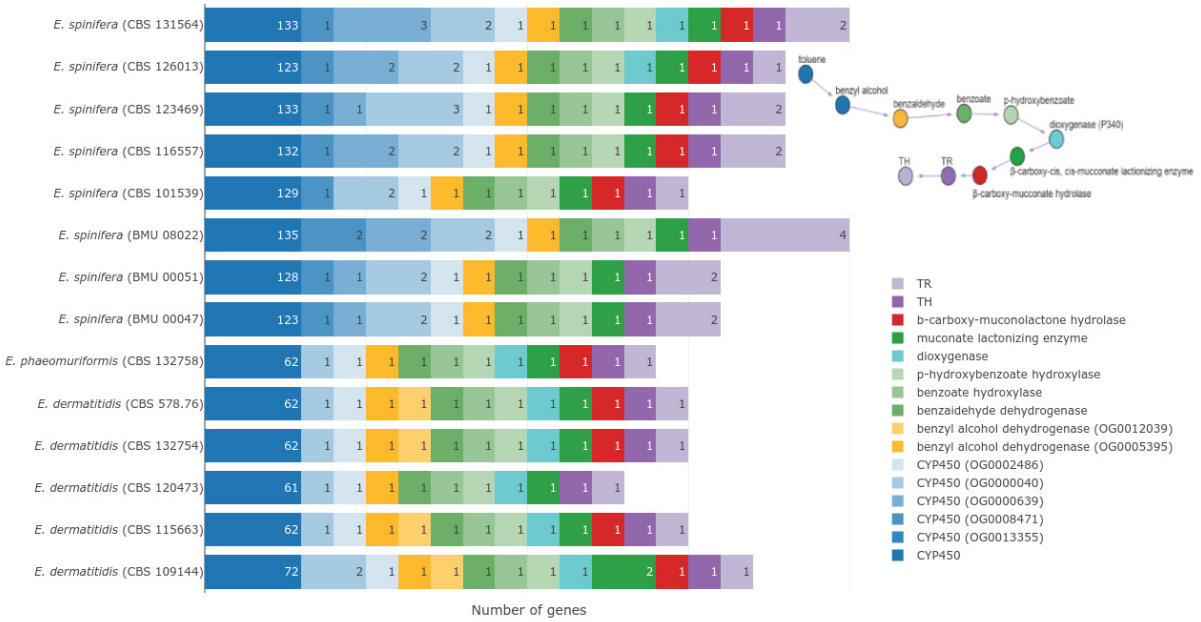
E. dermatitidis	**CBS 109144**	**CBS 115663**	**CBS 120473**	**CBS 132754**	**CBS 578.76**
Number of SNPs	163,572	160,112	94,043	151,141	47,898
Number of non-syn SNPs	30,126	29,542	17,643	11,602	8,887
Number of unique non-syn SNPs	599	807	8618	19	3435
Number of transversions	64,693	65,859	35,208	49,645	18,794
Number of transitions	124,089	126,22	68,215	96,706	36,049
E. spinifera	**CBS 101539**	**CBS 116557**	**CBS 123469**	**CBS 126013**	**CBS 131564**
Number of SNPs	94,675	212,255	242,38	196,728	174,304
Number of non-syn SNPs	17,978	37,075	43,282	35,904	31,316
Number of unique non-syn SNPs	6863	15009	3487	1323	8
Number of transversions	32,874	73,413	83,343	67,764	58,902
Number of transitions	60,654	132,29	150,71	123,03	108,083

matitidis (CBS 525.76 = NIH/UT8656, AFPA00000000.1), *E. spinifera* (CBS 899.68, JYBY00000000.1), *Rhinocladiella mackenziei* (CBS 650.93, JYBU00000000.1), and *Cladophialophora immunda* (CBS 834.96, JYBZ00000000.1) were assessed for the presence of genes required for this assimilation. The protein sequences of these strains clustered to 16,822 groups. No genes of the styrene degradation pathway were detected, but 16 clusters contained protein sequences required in the toluene pathway. *Exophiala spinifera* and *E. dermatitidis* differed significantly in the number of CYP450 genes, which were 123–133 and 61–72, respectively (Figure 17). In *E. spinifera*, dioxygenase (P340), which catalizes the opening of the benzene ring, was missing in most of the strains. Several strains of both species lack β-carboxy-muconolactone hydrolase which is involved in degradation of the product of dioxygenase activity. Some variation was noted in the number of genes between strains of the same species, particularly in *E. spinifera*.

3.3.4 Virulence genes

A total of 813 genes associated with virulence and 18 virulence domains were detected in *E. dermatitidis* and *E. spinifera* (Table 8). The highest number (n = 458)

FIGURE 17 – Genes involved in pathways for the metabolism of aromatic compounds



Source: (SONG et al., 2020b)

was found in *C. immunda*, CBS 834.96, the lowest (n = 267) in *E. dermatitidis*, CBS 578.76. The number of virulence genes in *E. spinifera* ranged between 389 and 432, and in *E. dermatitidis* between 267 and 303. Of the genes that are possibly linked with brain invasion (n = 192), OG0012246 was consistently detected in *E. dermatitidis* and *R. mackenziei*. Hypothetical proteins OG0012600 and OG0012602 were lacking in some *E. dermatitidis*. None of these proteins was present in *E. spinifera*. Thirty-four genes have been suggested to play a role in bone invasion. Of these, 3 were detected in *E. dermatitidis* and *E. spinifera*, of which linker histone H1/H4 and proline racemase were present in both species. In three *E. spinifera* strains, proline racemase was replaced by proline oxidase.

TABLE 8 – Virulence domains detected in strains of *Exophiala spinifera* and *E. dermatitidis* (CBS accession numbers).

IPR/PFAM Accession	Gene	*E. spinifera*					*E. dermatitidis*					
		101539	116557	123469	126013	131564	120473	109144	115663	132754	132758	578.76
IPR013912	Adenylate cyclase associated (CAP) C terminal	1	1	1	1	1	0	0	0	1	1	1
IPR013992	Adenylate cyclase associated (CAP) N terminal	0	0	0	0	0	1	1	1	0	0	0
IPR008441	Capsular polysaccharide synthesis protein	0	0	0	0	0	1	1	1	1	1	1
PF01302	CAP-Gly domain	4	4	3	4	4	3	4	4	3	3	3
PF00188	Cysteine-rich secretory protein family (CRISP)	5	5	5	5	5	5	6	5	5	5	5
PF06058	Dcp1-like decapping family	1	1	1	1	1	1	1	1	1	1	1
IPR001698	F-actin capping protein beta subunit	1	1	1	1	1	1	1	1	0	0	1
IPR018814	Family of unknown function (DUF5427)	1	1	1	1	1	1	1	1	0	0	1
PF00012	Hsp70 protein	12	11	12	11	11	10	10	10	10	10	10
PF03291	mRNA capping enzyme	3	3	3	3	3	3	3	3	3	3	3
IPR019416	Nuclear cap-binding protein subunit 3	1	1	1	1	1	1	1	1	1	1	1
PF01255	Putative undecaprenyl diphosphate synthase	2	2	1	2	3	1	1	1	1	1	1
IPR019012	RNA cap guanine-N2 methyltransferase	1	1	1	1	1	1	1	1	1	1	1
PF11969	Scavenger mRNA decapping enzyme C-term binding	0	0	0	1	0	2	2	2	0	0	2
PF05652	Scavenger mRNA decapping enzyme (DcpS) N-terminal	1	1	1	0	1	0	0	0	1	1	0
PF12658	Telomere capping CST complex subunit	1	1	1	1	1	1	1	1	0	0	1
PF00240	Ubiquitin family	12	11	12	11	12	9	10	9	10	9	9
IPR018814	Maintenance of telomere capping protein 1	0	0	0	0	0	0	0	0	1	1	0

Gene families PFam and IPR in the literature have been associated with virulence; strains were found to differ rather considerably in the presence of these genes. CAP Gly domain, cysteine-rich secretory protein family, Hsp70 protein, mRNA capping enzyme and ubiquitin family were consistently present with higher copy numbers. From the virulence gene prediction, the adenylate cyclase associated (CAP) Gly-domain was present in both species, while capsular polysaccharide synthesis protein and CAP N-terminal were present only in some of the *E. dermatitidis* strains. CAP C-terminal was consistently present in *E. spinifera* but lacking in three *E. dermatitidis* strains.

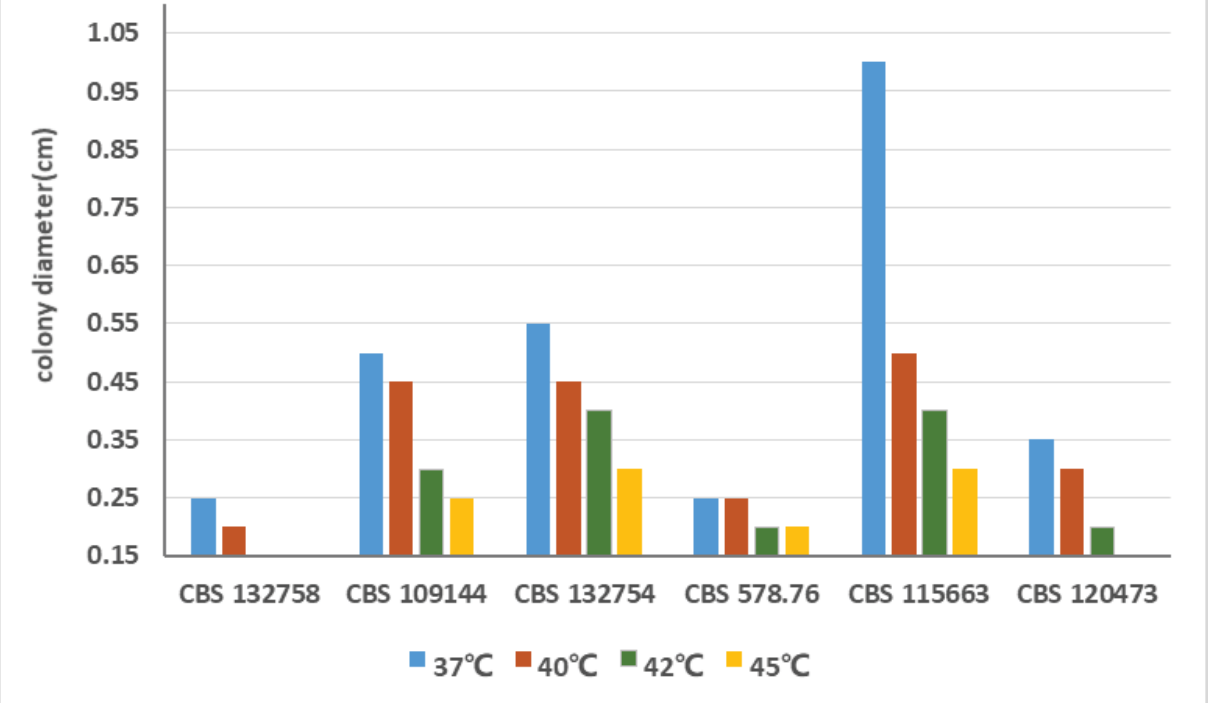
Two *Exophiala dermatitidis* strains, CBS 578.76 and CBS 120473, were isolated from human brain. The species is known to be neurotropic upon dissemination (SUDHADHAM et al., 2008). The strains were found to be close to each other, clustering with strain CBS 132758 collected from a dishwasher (Figure 14). There were 18,283 unique SNPs present in the strains from brain, associated with 1,144 genes. To reveal possible differences between *E. dermatitidis* isolates from human brain and from the environment, the protein sequences of the five *E. dermatitidis* genomes were clustered; 9,151 clusters were obtained. Among these, 876 clusters contained protein sequences that were only found in the two brain isolates CBS 578.76 and CBS 120473. The proteins in CBS 578.76 and CBS 120473 shared two domains, i.e. Myb-like DNA-binding domain and SANT/Myb domain (OG0012246). The former protein has morphogenetic roles (WIESER; ADAMS, 1995), while the latter enhances development and pathogenicity (KIM et al., 2014),. Myb-like DNA-binding domains of RNA polymerase III transcription factor IIIB (TFIIIB) have a function in the assembly of DNA complexes and recruitment of RNA polymerase to the promoter (KASSAVETIS et al., 1997).

Exophiala phaeomuriformis differs phenotypically from *E. dermatitidis* by its lower thermotolerance (HOOG et al., 2019). This was confirmed in the analyzed set of strains: despite significant variation in growth velocity, *E. dermatitidis* strains all were able to grow at 45 ℃, while the maximum of *E. phaeomuriformis* was at 40 ºC (Figure 18).

3.4 DISCUSSION

From clinical data, i.e. the number of published cases and their severity (SUDHADHAM et al., 2008; HOOG et al., 2019) it has been concluded that *Exophiala dermatitidis* and *E. spinifera* are the most virulent species in the genus *Exophiala*. In contrast, *Cladophialophora immunda* has never been observed in clinical settings, which may either be explained by low virulence or its rarity. Remarkably, this fungus was found to have the highest number (n = 458) of genes that have been associated in the literature with virulence. *Exophiala dermatitidis* is a common environmental species occurring in the domesticated environment (GÜMRAL et al., 2014) and thus has a higher chance to

FIGURE 18 – Thermotolerance of *E. phaeomuriformis* CBS 132758 and five *E. dermatitidis* strains measured after 2 weeks incubation on MEA medium.



Source: (SONG et al., 2020b)

of infection; it has a significantly lower number (n = 267–403) of virulence-associated genes than all species compared, including the equally common environmental species *E. oligosperma* (n = 424).

All virulence genes in this study can be summarized into the following four major classifications, the capsular polysaccharide synthesis, the CAP associated proteins (including decapping enzyme and capping protein), the Hsp70 protein, and ubiquitin family. Notably, the capsular polysaccharide synthesis protein and CAP N-terminal is present in *E. dermatitidis* strains only. Both species share the same cysteine-rich secretory proteins (CRISP), which can induce an increase of leukocytes in vivo, stimulating the production of cytokines and eicosanoids (XU et al., 2012). Expression of a human CAP superfamily member, the cysteine-rich secretory protein 2 (CRISP2), rescues the phenotype of yeast mutants lacking Pry function and purified CRISP2 binds cholesterol in vitro, indicating that lipid binding is a conserved function of the CAP superfamily proteins playing an important role in lipid and sterol metabolism. These genes are also significant in *Exophiala*.

Exophiala dermatitidis and *E. spinifera* are the only chaetothyrialean black yeasts that possess capsule-like EPS around juvenile cells, while these reportedly

are absent from sister species *E. phaeomuriformis* and *E. oligosperma*, respectively. These genes are known in *Cryptococcus*, where the microtubule-associated CAP-glycine protein (Cgp1) governs growth, differentiation, and virulence of *Cryptococcus neoformans* (GELLI, 2018). Phenotypically, differences are known in the shape of black yeast capsules, which are regular in *E. spinifera* and irregular EPS in *E. dermatitidis* (SONG et al., 2017). In addition,*E. spinifera* loses capsule formation at 37℃, while most E. dermatitidis strains maintain this capacity (SONG et al., 2017). Different from *Cryptococcus*, *Exophiala* species may lose this purported virulence when it is needed at elevated temperature during host invasion.

One of the predicted genes found in both *E. dermatitidis* and *E. spinifera*, Cysteine-rich secretory protein (CRISP), has an effect on potassium channels and inflammatory processes (BERNARDES et al., 2019). Pathogen-Related Yeast (PRY) genes belong to a large CAP protein superfamily (i.e., CRISP, antigen 5, and pathogenesis related 1 proteins). The conserved CAP domain of Pry1 is necessary and sufficient for lipid export and sterol binding (CHOUDHARY; SCHNEITER, 2012).

There are two clusters belonging to the Myb-like DNA-binding domain and SANT/Myb domain only present in the neurotropic species *E. dermatitidis* and *R. mackenziei*, and absent from *E. spinifera* which was suggested to be somewhat osteotropic (SUDHADHAM et al., 2008). SANT/Myb-type genes are involved in conidiation of Cochliobolus carbonum (ZHANG et al., 2014), while Myb-like DNA-binding protein that coordinates initiation of *Aspergillus nidulans* conidiophore development (WIESER; ADAMS, 1995). The virulence gene MYT3 is required for pathogenesis and sexual development in *Fusarium graminearum*. The Multiprotein Transcription Factor TFIIIB is linked to RNA polymerase III-transcribed genes indirectly through interaction with DNA-bound TFIIIC or directly through DNA recognition by the TATA-binding protein, in turn recruits RNA polymerase III to the promoter. It is a key transcription factor in *Saccharomyces cerevisiae* (KASSAVETIS et al., 1997). The genes possibly associated to osteotropy were shared by *E. spinifera* and *E. dermatitidis*, suggesting that the different predilection of disseminated strains might rather be linked to absence of neurotropism in *E. spinifera* rather than presence of bone-related genes. Linker histone H1 is an essential component of chromatin structure, linking nucleosomes into higher order structures and is eventually replaced by H5. Histone proteins have central roles in both chromatin organization as structural units of the nucleosome and gene regulation. Proline racemase catalyses the interconversion of L- and D-proline (FISHER; A.; KNOWLES, 1986; CHAMOND et al., 2003). Three strains deviated by having proline oxidase instead of racemase. The family also contains several proteins that remain hypothetical. FAD-linked oxidoreductase was detected in a clinical *E. spinifera* strain. This concerns a family of bacterial oxidoreductases with covalently linked FAD (BOGACHEV; BAYKOV; BERTSOVA, 2018; MCNEIL; FINERAN, 2013).

Hsp70 proteins is a class of molecular chaperones that are shared by both species. Studies in *Fusarium* showed that knockout of an ER lumenal Hsp70 homolog *FpLhs1* gene reduced growth, conidiation, and pathogenicity. FpLhs1 is likely to act on the development and virulence by regulating protein secretion (CHEN et al., 2019). The ubiquitin family is also essential in both species. The ubiquitin-proteasome system plays an essential role in the regulation of intercellular protein degradation, and the biosynthetic gene cluster for himeic acid A has been proven to be a ubiquitin-activating enzyme (E1) inhibitor in *Aspergillus japonicus* (HASHIMOTO et al., 2018).

Black yeasts in general display remarkably diverse lifestyles, with a predilection for extreme and toxic environments such as those rich in aromatic compounds or heavy metals, or with high temperatures, increased salinity, and scarcity of nutrients (PAOLO et al., 2006; PRENAFETA-BOLDÚ; SUMMERBELL; HOOG, 2006; ZHAO et al., 2010). Moreno et al. (2018) noted that chaetothyrialean black fungi are exceptionally rich in cytochrome P450 genes enhancing toxin management. No genes involved in the styrene pathway, but comparative analysis of *Rhinocladiella mackenziei* against the aromatic hydrocarbon-degrading fungus *Cladophialophora immunda* (MORENO et al., 2018) revealed the presence of orthologs that resemble the published fungal toluene degradation pathway (Figure 17) via protocatechuate (PARALES et al., 2008; BLASI et al., 2017). Toluene was proven to be initially oxidized to benzyl alcohol by a membrane-bound CYP in toluene-growing cells of the closely related black fungus *Cladophialophora saturnica* CBS 114326 (previously confused with *Cladosporium sphaerospermum*) (LUYKX; PRENAFETA-BOLDÚ; BONT, 2003). *Exophiala spinifera* has twice as many CYP as *E. dermatitidis*, a species having the smallest genomes of all members of the family Herpotrichiellaceae sequenced thus far (TEIXEIRA et al., 2017). On the other hand, in most *E. spinifera* strains, dioxygenases needed for the opening of benzene rings via protocatechuate are missing, while they are consistently present in *E. dermatitidis*. Notably, p-hydroxybenzoate hydroxylase is present in both species, while it was absent from the toluene-degrading black fungus *Cladophialophora immunda* (BLASI et al., 2017). It may be surmised that in *E. spinifera* the CYP P450 genes have other functions than toluene degradation. (NASCIMENTO et al., 2017) found rich populations of E. spinifera in degrading coconut shells rich in esters and hydrocarbons, while *E. dermatitidis* is unambiguously associated with monoaromate pollutants, alkanes and creosotes (ISOLA et al., 2013; GRUMACH et al., 2015). Although this ability may enhance neurotropism in *E. dermatitidis*, the human brain is unlikely as a natural habitat for the species, which should be considered as an opportunist rather than a pathogen. A possible additional gene promoting neurotropism is OG0012246 which was consistently detected in *E. dermatitidis* and *R. mackenziei* but was absent from *E. spinifera*.

3.5 DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GenBank Accessions: JAAAJH000000000, JAAAJG000000000, JAAAJF000000000, JAAAJE000000000, JAAAJD000000000, JAAABF000000000, JAAABG000000000, JAAABH000000000, JYBY00000000, JYCA01000000, JAAAJI000000000, WXYG00000000, JAAAJK00-0000000, JAAAJJ000000000, JAAAJM000000000, JAAAJL000000000, AFPA00000000.1 and JYBU00000000.1.

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CHAPTER 4

COMPARATIVE ANALYSIS OF CLINICAL AND ENVIRONMENTAL STRAINS OF *Exophiala spinifera* BY LONG-READS SEQUENCING AND RNASeq REVEAL ADAPTIVE STRATEGIES

4 COMPARATIVE ANALYSIS OF CLINICAL AND ENVIRONMENTAL STRAINS OF *Exophiala spinifera* BY LONG-READS SEQUENCING AND RNASEQ REVEAL ADAPTIVE STRATEGIES

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ABSTRACT

Exophiala spinifera, a capsule-producing black yeast, is over represented as agent of disseminated infection in humans with inherited dysfunction of the CARD9 gene. In a review of published CARD9 cases, black fungi were linked to other mutations than prevalent in yeast and dermatophyte cases, and were found to respond to a larger panel of cytokines. Here, we sequenced and annotated the genomes of BMU 08022 from a patient with CARD9 immunodeficiency and two environmental strains BMU 00051, BMU 00047, and performed genomic and transcriptomic analysis for these different hosts isolates including published black yeasts genomes, using a combination of long-read (PacBio) and short-read (Illumina) sequencing technologies with a hybrid assembly strategy. We identified the virulence factors, fitness, and the major genetic and gene expression differences between the strains with RNAseq technology. Genome assembly reached sub-chromosome level with between 12,043 and 12,130 predicted genes. The number of indels identified in the clinical strain was higher than compared with environmental strains. Moreover, substantial syntenic rearrangements of scaffolds I and III in the CARD9-related isolate were detected. Seventeen gene clusters were involved in the production of secondary metabolites. PKS-cluster 17 was recurrently found to be absent in the clinical strain. Comparative transcriptome analysis demonstrated 16 genes were differentially expressed upon incubation in brain-heart infusion broth vs. Sabouraud's glucose broth. Most of the single-copy genes upregulated with BHI were transporters in KOG classification. This study has provided novel insights into understanding of eventual differences in intrinsic virulence of the species and indicated that intraspecific variability were closely related to host difference, which may provide a warning sign that *E. spinifera* possibly is associated with a differential chance to cause infection in susceptible patient population, and provided important clues for future studies exploring the mechanisms of pathogenic and adaptive strategies of CARD9 immunodeficiency *Exophiala spinifera* isolates.

Keywords: Black yeasts, comparative genomics, transcriptome analysis, intraspecific variability, gene rearrangement, CARD9 deficiency, pathogenicity, virulence.

4.1 CONTRIBUTION TO THE FIELD

Black yeast species *Exophiala spinifera* is most often involved in disseminated infections in humans. Previous research has reported significant differences in the *Exophiala spinifera* ecologically relevant factors between clinical and environmental preferences, but virulence factors, i.e., melanin, capsule and muriform cells were unknown. We first thought that these fungi are extremely virulent, after their clinical profiles were compared and found to be CARD9-associated infection. They are opportunists. To gain understanding of eventual differences in intrinsic virulence of the species, a comprehensive, multi-omic survey was performed and disclosed significant genomic rearrangements between an etiologic agent of CARD9-associated infection in comparison with other two environmental strains. We found environmental strains from different continents were largely identical, whereas the clinical strain was different and deviated in the number of genes. PKS-cluster 17 was recurrently found to be absent in the clinical strain. Single-copy genes upregulated with BHI were key transporters in KOG classification. While we now realize that they do have a special ability of infection. This work has provided novel insights into understanding of eventual differences in intrinsic virulence of the species and indicated that intraspecific variability were closely related to host difference, which may provide a warning sign that *E. spinifera* possibly is associated with a differential chance to cause infection in susceptible patient population.

4.2 INTRODUCTION

Melanized fungi of the order Chaetothyriales are renowned as agents of human infection. The number of cases is low when compared to dermatophytes, *Aspergillus*, *Candida*, but with 86 species in 7 genera that have been proven as potential agents of vertebrate disease order ranges fourth in clinical biodiversity, after Onygenales, Eurotiales and Saccharomycetales. In 19 chaetothyrialean species, systemic or disseminated infection has been observed, which frequently led to death of the patient. The neurotropic species *Cladophialophora bantiana* is one of the most feared fungi, because it causes brain abscesses in healthy-appearing patients, with a case fatality rate of 65% despite antifungal therapy (KANTARCIOGLU et al., 2016). Numerous investigations nevertheless suggest that most members of the order – with a possible exception of agents of chromoblastomycosis – are opportunists rather than pathogens. Opportunists are defined as fungi that complete their natural life cycle without involvement of an animal host, being able only of unintentional infection because of coincidental similarities between conditions of host tissue and natural habitat. Gostinčar et al. (GOSTINČAR et al., 2018) ascribed their infectious ability to polyextremotolerance, which is tolerance to environmental stress supplemented with efficient nutrient scavenging and toxin management. Properties that in pathogenic fungi may have a role in virulence may have

very different functionalities in the fungus' natural habitat. (SONG et al., 2017) noted that hypothesized virulence factors, which enhance infection in truly host-associated fungi like *Candida* or *Histoplasma*, in black yeasts may respond adversely to proxies of host resistance, and thus comparable genes can be functionally different between fungal groups.

An important consequence of opportunism is that all strains taken to have an equal chance to be inoculated into the host, and thus clinical strains are not necessarily different from environmental ones; pathogenic adaptation is less likely to occur because of absence of host-related transmission. However, after coincidental inoculation, some strains may be accidentally more prone to cause symptoms than others, and thus there may be a difference between clinical and environmental strains. Black yeast-like fungi mostly cause local, (sub) cutaneous infections. The host conditions of patients with brain abscesses acquired via the inhalative route are not well understood. For disseminated cases, recent data have shown (LANTERNIER et al., 2014) that these are often associated with host-inherited mutations in caspase recruitment domain-containing protein 9 (CARD9). This protein plays a role in the dectin pathway regulating innate immunity activating pro-inflammatory cytokines. Since most cases of disseminated disease in black yeast-like fungi were described prior to 2013, it is possible that the majority of these cases occurred in patients with CARD9 dysfunctional innate immunity. Several of the infection types of chaetothyrialean fungi show a certain degree of unexplained endemism, such as the prevalence of *Cladophialophora* brain abscess in the Indian subcontinent (CHAKRABARTI et al., 2015). Similarly, the majority of CARD9-associated black fungal cases is found in East Asia: China, Korea, Japan, although the etiologic agents have a global distribution. For an explanation of virulence of members of Chaetothyriales, we thus might expect answers from the interaction of the fungus with windows of opportunity associated with CARD9 regulation and human race.

To study the fungal side of this tripartite relationship, we sequenced the genomes and transcriptomes with different culture condition of *Exophiala spinifera* strain isolated from a patient with a CARD9 related disorder, and compared this with two environmental strains of the same species, and with other published genomes (Table S1). We applied state-of-the-art technology to quantify eventual differences between clinical and environmental strains with maximum precision, and to describe the species' intraspecific variability. A number of genomes of Chaetothyriales are already available, but these were sequenced with somewhat older techniques. One of the aims is therefore to describe the technical and biological variation in these datasets, and determine whether the new techniques provide a better answer to questions as formulated above.

TABLE 9 – Isolation data of strains sequenced in this study, and isolates included for comparison.

Strain ID	Species	Country	Source	GenBank Accession
BMU 08022	*E. spinifera*	China	Human skin (CARD9 deficient)	JAAABF000000000
BMU 00051	*E. spinifera*	China	Bark	JAAABG000000000
BMU 00047	*E. spinifera*	Colombia	Soil	JAAABH000000000
CBS 899.68T	*E. spinifera*	United States	Human nasal granuloma	JYBY00000000
CBS 725.88T	*Exophiala oligosperma*	Germany	Human sphenoid abscess	JYCA01000000

4.3 MATERIALS AND METHODS

4.3.1 Strains and culture conditions

Three strains of *E. spinifera* were analyzed (Table 9): BMU 08022 (ESC1, from a CARD9-deficient patient, Jiangsu, China), BMU00051 (ESE1, from bark, Shenzen, China), and BMU00047 (ESE2, from soil, Colombia). To prepare DNA for genome sequencing, mycelia were harvested from fresh cultures on Sabouraud's Glucose Agar (SGA), frozen in liquid nitrogen, and stored at –80 ℃ until further processing. For RNA extraction, strains were inoculated in Sabouraud's Glucose Broth (SDB) and in Brain Heart Infusion Broth (BHI) and harvested after 20 h, centrifuged and frozen in liquid nitrogen. Liquid nitrogen was used to grind the samples for DNA/RNA extraction.

4.3.2 DNA and RNA extraction

Genomic DNA was extracted using the MOBIO Power Microbial Maxi DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, U.S.A.). DNA was concentrated by QUBIT (Invitrogen, Carlsbad, CA, U.S.A.), and an Agilent Bio Analyzer 2100 using a 1000 DNA Chip (Agilent Technologies, Palo Alto, CA, U.S.A.), and its quality was determined by electrophoresis on agarose gels. Total RNA was extracted from cultures ground in liquid nitrogen using TRIZOL reagent (Thermo Fisher Scientific, Waltham, MA, U.S.A.) according to the manufacturer's instructions, followed by two phenol (pH 4.6)-chloroform-isoamyl alcohol (25:24:1) extraction steps and two chloroform extraction steps after the initial TRIZOL - chloroform phase separation. RNA pellets were dissolved in 88 µL of nuclease-free water and subjected to genomic DNA digestion with DNase (Qiagen, Hilden, Germany). RNA samples were then concentrated using RNA Clean & Concentrator (Zymo Research, Irvine, CA, U.S.A.). RNA quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent). Two micrograms of total RNA per sample was used for cDNA library construction.

4.3.3 cDNA synthesis

The cDNA synthesis was performed using IMPRON II reverse transcriptase kit (Promega, Madison, WI, U.S.A.) with 1 µg of total RNA added according to manufacturer's instructions. The efficiency of the cDNA synthesis process was assessed by PCR using the following reference genes: β-tubulin and ITS. The integrity of PCR amplification products was verified by electrophoresis on 1.2 % agarose gels.

4.3.4 PACBIO sequencing and raw assembly

The extracted DNA of three *E. spinifera* strains were sequenced on the Pacific Biosciences (PacificBiosciences, Menlo Park, CA, U.S.A.) Single Molecule, Real-Time (SMRT) DNA sequencing technology (platform: RS II; chemistry: P6-C4). The raw reads were processed using the standard SMRT analysis pipeline v5.0.1 (CHIN et al., 2013). The de novo assembly was carried out following CANU v1.4 assembly protocol (KOREN et al., 2017) with QUIVER polishing (CHIN et al., 2013). Assemblies were refined by manual curation. Pairwise alignments were carried out using BLASTN v2.6.0+ (ALTSCHUL et al., 1990). The draft assemblies were further improved with FINISHER SC (LAM et al., 2015) and polished with ARROW (CHIN et al., 2013). For each genome assembly, contigs were examined and removed if redundant (i.e. aligning to any other contig in the same assembly with >90% identity). All contigs containing rDNA repeats were excluded from the above step.

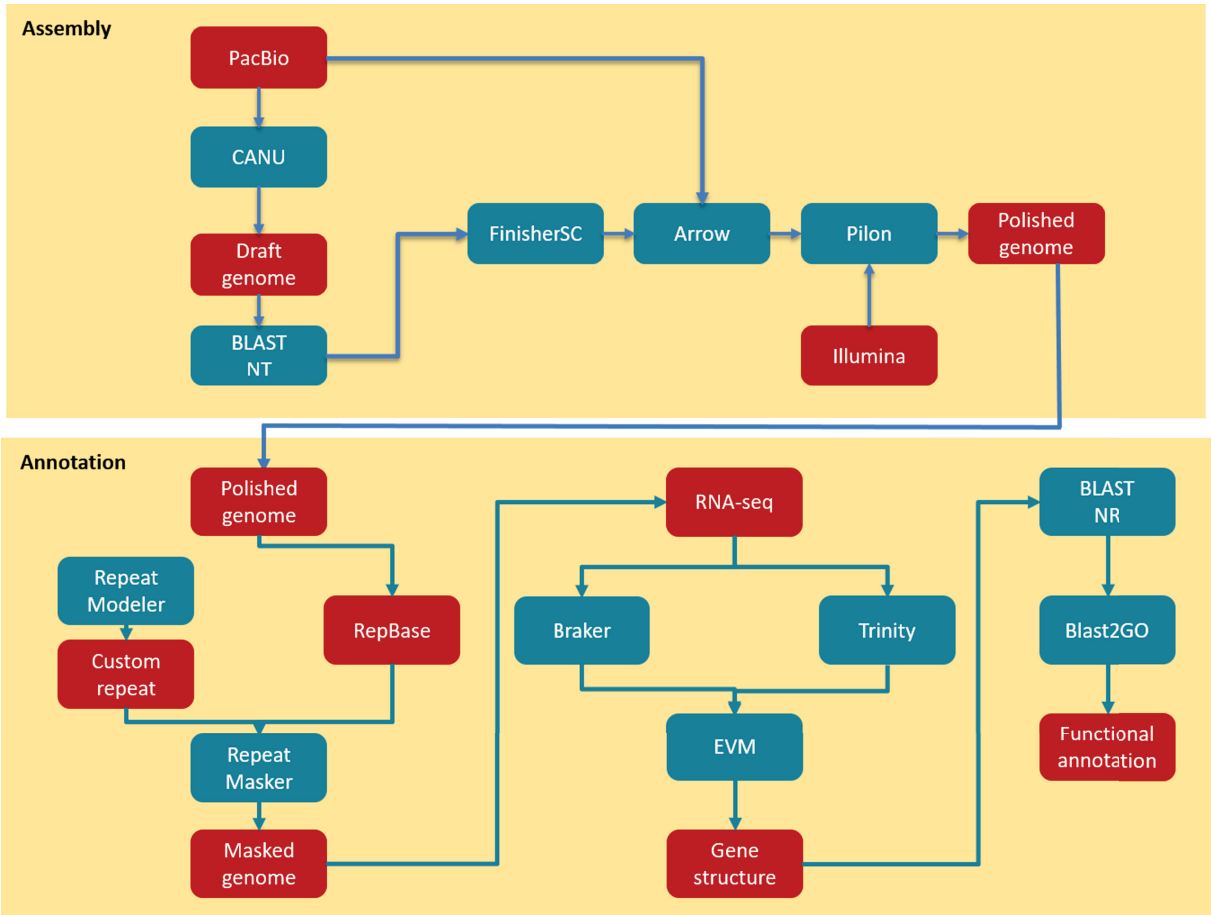
4.3.5 Illumina sequencing, read mapping and error correction

Our genome assembly strategy is summarized in (Figure 19). In addition to the PACBIO sequencing, we also performed Illumina 150-bp paired-end sequencing for each strain. We examined the raw Illumina reads via FASTQC v0.11.5 (http://www.bioinformatics.babraham.ac.uk/) and performed adaptor-removal and quality-based trimming by TRIMMOMATIC v0.36 (BOLGER; LOHSE; USADEL, 2014). For each strain, the trimmed reads were mapped to the corresponding PACBIO assemblies by BWA 0.7.12 and using BWAMEM v0.7.16 (LI; DURBIN, 2009). The resulting read alignments were subsequently processed by SAMTOOLS v1.6 (LI; DURBIN, 2009). On the basis of Illumina read alignments, we further performed error correction with PILON v1.22 (WALKER et al., 2014) to generate final assemblies for downstream analysis. BUSCO v3 (SIMÃO et al., 2015) was used to assess the completeness of the final assembled genomes.

4.3.6 Repetitive sequence prediction and masking

Repetitive elements were predicted using a *de novo* approach by applying REPEAT MODELER v1.0.11 (http://www.repeatmasker.org), which includes RECON

FIGURE 19 – Genome assembly and annotation strategy used in this study. Assembly: The raw reads were processed using the standard SMRT analysis pipeline v5.0. The de novo assembly was carried out following CANU v1.4 assembly protocol with QUIVER polishing. Assemblies were refined by manual curation. Pairwise alignments were carried out using BLASTN v2.6.0+. The draft assemblies were further improved with FINISHER SC and polished with ARROW. Annotation: Ahybrid strategy combining *ab initio* predictions and transcriptomic support (RNA-seq) was applied in gene prediction. Three *ab initio* gene finders, GENEMARK.HMM, FGENESH and AUGUSTUS were used.



Source: (SONG et al., 2020)

v1.08 (BAO, 2002) and REPEATSCOUT v1.0.5 (PRICE; JONES; PEVZNER, 2005) to construct a strain-specific repeat library. To further classify repetitive sequences, BLAST v2.2.28 (CAMACHO et al., 2009) was used to search the repeat library against SWISS-PROT protein database. Sequences with similarities to known proteins were discarded from the repeat library. Repetitive sequences were masked with REPEATMASKER v4.0.7 (http://www.repeatmasker.org using the *de novo* constructed library.

4.3.7 Annotation of rRNA, tRNA and repeat elements

The rRNA loci were predicted by using RNAMMER v1.2(LAGESEN et al., 2007) and tRNA by TRNASCAN-SE v2.0 (LOWE; EDDY, 1997). Repeat elements were identified using REPEATMASKER v4.0.6 (http://www.repeatmasker.org) based on REPBASE LIBRARY 20160829. REPEAT MASKER was run with options that skipped the low-complexity DNAs masking for the purpose of gene prediction.

4.3.8 Gene prediction and functional annotation

A hybrid strategy combining *ab initio* predictions and transcript alignments was applied in annotation of protein coding genes. Firstly, protein coding genes were predicted using BRAKER (HOFF et al., 2015), which was an *ab initio* approach combined with RNA-seq data. Then, RNA-seq was *de novo* assembled by using TRINITY (GRABHERR et al., 2011). The assembled transcripts were aligned to the reference genome of corresponding strains by PASA pipeline (HAAS, 2003). Finally, EVIDENCE MODELER (HAAS et al., 2008) was used to combine *ab initio* predictions and transcript alignments into consensus gene structure. Functional annotation of predicted proteins was done by BLAST (CAMACHO et al., 2009) similarity search of the predicted proteins against NR database with an e-value of 1e–05. Gene Ontology (GO) terms were retrieved by using Blast2go (GOTZ et al., 2008) with default annotation parameters. To further improve the annotations, INTERPROSCAN v5.33-72.0 (QUEVILLON et al., 2005) was used to identify known protein domains. BLAST (CAMACHO et al., 2009) results of NR database and results of the INTERPROSCAN analysis were combined to gain a complete functional annotation of all predicted genes. Genes predicted to encode carbohydrate-active enzymes (CAZYMES) were identified with DBCAN 2 (ZHANG et al., 2018). Also the software SMURF (KHALDI et al., 2010) was used to predict secondary metabolite gene clusters in three genomes of *E. spinifera*.

4.3.9 Comparison of genome assemblies

The synteny among three strains of *E. spinifera* was determined using MUMMER v3.23 (DELCHER; SALZBERG; PHILLIPPY, 2003). We used NUCMER implemented in MUMMER v3.23 to align the genomes of the three strains of *E. spinifera*. In

addition, the DNA DIFF package in MUMMER v3.23 was used to generate coordinates for the differences among genomes of the three strains analyzed. The alignment statistics, SNPs, indels, inversions, etc. were also calculated from the results of MUMMER. We plotted these features circularly with CIRCOS v0.69.6 (KRZYWINSKI et al., 2009).

4.3.10 Identification of homology groups

For nuclear protein-coding genes, we used INPARANOID v4.1 3 to identify gene homology across the three strains of *E. spinifera*. We calculated protein length distributions for each homologous group (1:1:1 association among strains) of the strains. The correlation of exon length and intron length of homologous groups (1:1 association between strains) were calculated by pairwise comparisons among three strains.

4.3.11 Identification of orthologous genes

Orthologous groups were delimited using ORTHOFINDER v2.2.6 (EMMS; KELLY, 2015), in which all predicted protein sequences were compared using a BLAST (CAMACHO et al., 2009) all-against- all search. The single-copy genes, duplicated genes, and strain-specific genes were extracted from the ORTHOFINDER output. To detect the core genome, specific and shared genes, the data was filtered based on the ORTHOFINDER output. The R package UPSETR (Conway et al., 2017) was used to construct statistics of orthologous groups among the three strains under study.

4.3.12 Identification of genes under positive selection

Single-copy orthologous proteins were aligned by using CLUSTAL v1.2.4 (http://www.clustal.org/). To obtain the alignments of codons, the corresponding nucleotide-sequence alignments were derived by substituting the respective coding sequences from the protein sequences. For each single-copy orthologous group, genes from the two environmental strains and their orthologous genes in the clinical strain formed sequence triplets. The test for the asymmetric evolution was constituted as a relative rate test between clinical strain and environmental strains on an unrooted tree. The statistical tests were conducted with a codon-based branch-site model using the CODEML program of PAMLv4.9(YANG, 2007). We compared T0 and T1 between single-copy orthologous genes to detect differences in proportion of selected sites in the two clades. Likelihood ratio (LR) was used to test for significance. To do this, two models were applied to the data: model 1 (T0 = T1) constrains the two Tvalues to be equal for the three sequences, and model 2 (T0 != T1) estimates the two Tvalues as free parameters. Collected maximum likelihood values ML1 and ML2 from the two models were used to calculate the likelihood ratio, LR = 2 (lnML2 – lnML1). LR is then compared against the $\chi 2$ distribution with one degree of freedom.

4.3.13 Transcriptome analysis

The raw reads were assessed for quality by FASTQC v0.11.5 and filtered to remove low-quality reads with TRIMMOMATIC v0.36 (BOLGER; LOHSE; USADEL, 2014). Filtered reads were mapped to the corresponding reference genome of three *E. spinifera* strains using STAR v2.5.3a (DOBIN et al., 2012). The gene expression levels were calculated using FEATURE COUNTS v1.5.2 (LIAO; SMYTH; SHI, 2013) and normalized based on the FPKM method. Differentially expressed genes were detected by R package DESEQ 2 (LOVE; HUBER; ANDERS, 2014). Differentially expressed genes were filtered by adjusted p-value < 0.05 and |log2 fold change| > 1. Gene ontology enrichment analysis was performed by R package TOP GO (https://bioconductor.org/). Only 1-1-1 orthologous genes were considered when detecting differentially expressed genes among different strains.

4.3.14 Statistical analyses

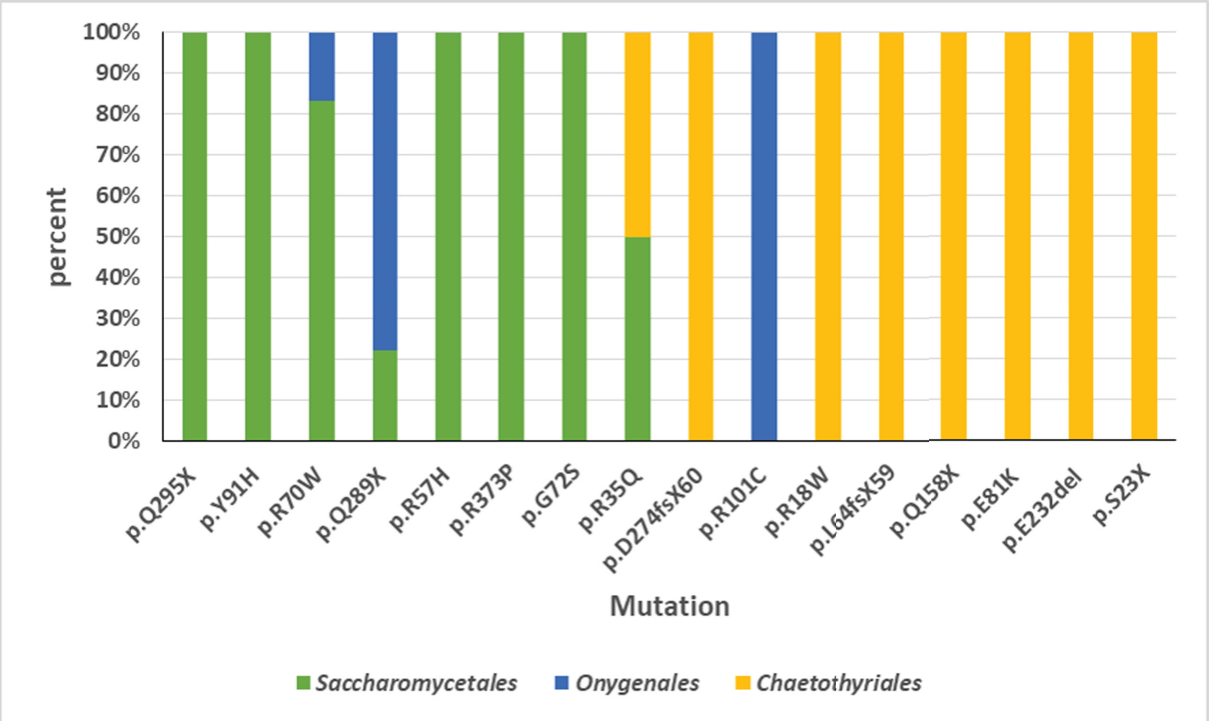
For analysis of RNA-Seq data, differentially expressed genes were detected by R Bioconductor package DESeq2 with the Benjamini-Hochberg adjusted p-value < 0.05 and fold change > 2. Student's t tests were performed for the comparisons, and a p-value <0.05 was considered to represent significance.

4.4 RESULTS

4.4.1 Literature data on black fungi in CARD9 patients

Literature data on black fungi in CARD9 patients An overview was made of published cases of fungal infections in patients with CARD9 deficiencies (Table 10) (LANTERNIER et al., 2013; MEDEIROS et al., 2016; STAMBOULI et al., 2017; CELMELI et al., 2016; CETINKAYA et al., 2018; BRUYNE et al., 2018; CETINKAYA et al., 2018; BRUYNE et al., 2018; DRUMMOND et al., 2015; DREWNIAK et al., 2013; GAVINO et al., 2014, 2016; GLOCKER et al., 2009; GRUMACH et al., 2015; LANTERNIER et al., 2015b,a; HERBST et al., 2015; HUANG et al., 2019; JACHIET et al., 2015; YAN et al., 2016; ARANGO-FRANCO et al., 2018; ZHONG et al., 2018; ZHANG et al., 2018; ZHANG et al., 2017). Sixteen different mutations have been observed. When cases are arranged according to phylogeny of the fungus, three main groups can be recognized: ascomycetous yeasts (*Candida*: order Saccharomycetales), dermatophytes (order Onygenales), and black fungi (mainly order Chaetothyriales, single representatives of orders Pleosporales and Venturiales). A single proven case with a member of Mucorales has been published. Mutations are not randomly distributed in the three fungal groups (Figure 20); p =0.001). For example, p.Q295X is found in 13 cases of *Candida* infection, but was not found among cases by other fungi.

FIGURE 20 – Overview of CARD9 mutations in published cases of fungal infections in patients with CARD9 deficiencies. Sixteen different mutations have been observed. Three main groups can be recognized: ascomycetous yeasts (*Candida*: order Saccharomycetales), dermatophytes (order Onygenales), and black fungi (mainly order Chaetothyriales, single representatives of orders Pleosporales and Venturiales). A single proven case with a member of Mucorales has been published. Mutations are not randomly distributed in the three fungal groups.



Source: (SONG et al., 2020)

TABLE 10 – Overview of published cases of fungal infection in patients with CARD9 deficiency.

Origin	Outcome	Clinical	*Species*	Mutation type	Cytokine deficiency	Reference
Saccharomycetales:						
Iran	*Died*	*CNS*	C. albicans	*NR*	*NR*	Glocker, 2009
Iran	Died	CNS, skin	*C. albicans*	NR	NR	Glocker, 2009
Iran	Died	CNS, mucosa	*C. albicans*	NR	NR	Glocker, 2009
Iran	Chronic	Mucosa	*C. albicans*	p.Q295X	TNFα, IL-17A	Glocker, 2009
Iran	Chronic	Skin	*C. albicans*	p.Q295X	TNFα, IL-17A	Glocker, 2009
Iran	Chronic	Oral, vagina, skin	*C. albicans*	p.Q295X	TNFα, IL-17A	Glocker, 2009
Iran	Chronic	Vagina, skin	*C. albicans,* dermatophyte	p.Q295X	TNFα, IL-17A	Glocker, 2009
Asia	Chronic	CNS	*C. dubliniensis*	p.R373P/p.G72S	IL-6, IL-1β, IL-8, IL-17A	Drewniak, 2013
France	Chronic	CNS	*C. albicans*	p.Y91H	GM-CSF	Gavino, 2014
Turkey	Chronic	CNS	*C. albicans*	p.Q295X	IL-6, TNFα, IL-1β	Herbst, 2015
Turkey	Chronic	CNS	*C. albicans*	p.Q295X	NR	Celmeli, 2016
Turkey	Chronic	CNS	*C. albicans*	p.R70W	IL-6, TNFα	Lanternier, 2015
Turkey	Chronic	CNS	*C. albicans*	p.R70W	IL-6, TNFα	Lanternier, 2015
Iran	Chronic	CNS, sinus, digestive system	*C. glabrata*	p.R35Q	IL-6, TNFα	Lanternier, 2015
Morocco	Chronic	CNS	*C. albicans*	p.Q289X	NR	Lanternier, 2015
Pakistan	Chronic	Digestive system	*C. albicans*	p.Q295X	IL-6, TNFα	Lanternier, 2015
Canada	Chronic	CNS	*C. albicans*	p.Y91H	GM-CSF	Gavino, 2016
Canada	Chronic	CNS, spine	*C. albicans*	p.Y91H	GM-CSF	Gavino, 2016
USA	Chronic	CNS, bone	*C. albicans*	p.R57H	GM-CSF	Drummond, 2015
Turkey	Chronic	Mucosa	*C. albicans*	p.R70W	IL-6, TNFα, IFNγ, IL-1β, IL12p70	Alves de Medeiros, 2016]
Turkey	Chronic	CNS, mucosa	*C. albicans*	NR	IL-6, GM-CSF, IL-22, IL-17A	Alves de Medeiros, 2016]
Asia	Chronic	CNS	*C. dubliniensis*	G72S/R373P	NR	De Bruyne, 2018
El Salvador	Chronic	CNS, osteomyelitis	*C. albicans*	R57H/R57H	IL-6, IL-17A	De Bruyne, 2018
Canada	Chronic	CNS	*C. albicans*	Y91H/Y91H	IL-6, IL-17A	De Bruyne, 2018
Canada	Chronic	CNS	*C. albicans*	Y91H/c.-529TC	IL-6, IL-17A	De Bruyne, 2018
Canada	Chronic	CNS, vertebral osteomyelitis	*C. albicans*	Y91H/c.-529TC	IL-6, IL-17A	De Bruyne, 2018
Canada	Chronic	CNS	*NA*	Y91H/c.-529TC	IL-6, IL-17A	De Bruyne, 2018
Iran	Chronic	CNS	*C. sp.*	Q295X/Q295X	IL-6, IL-17A	De Bruyne, 2018
Iran	Chronic	CNS	*C. sp.*	NA	NA	De Bruyne, 2018

Table 10 continued

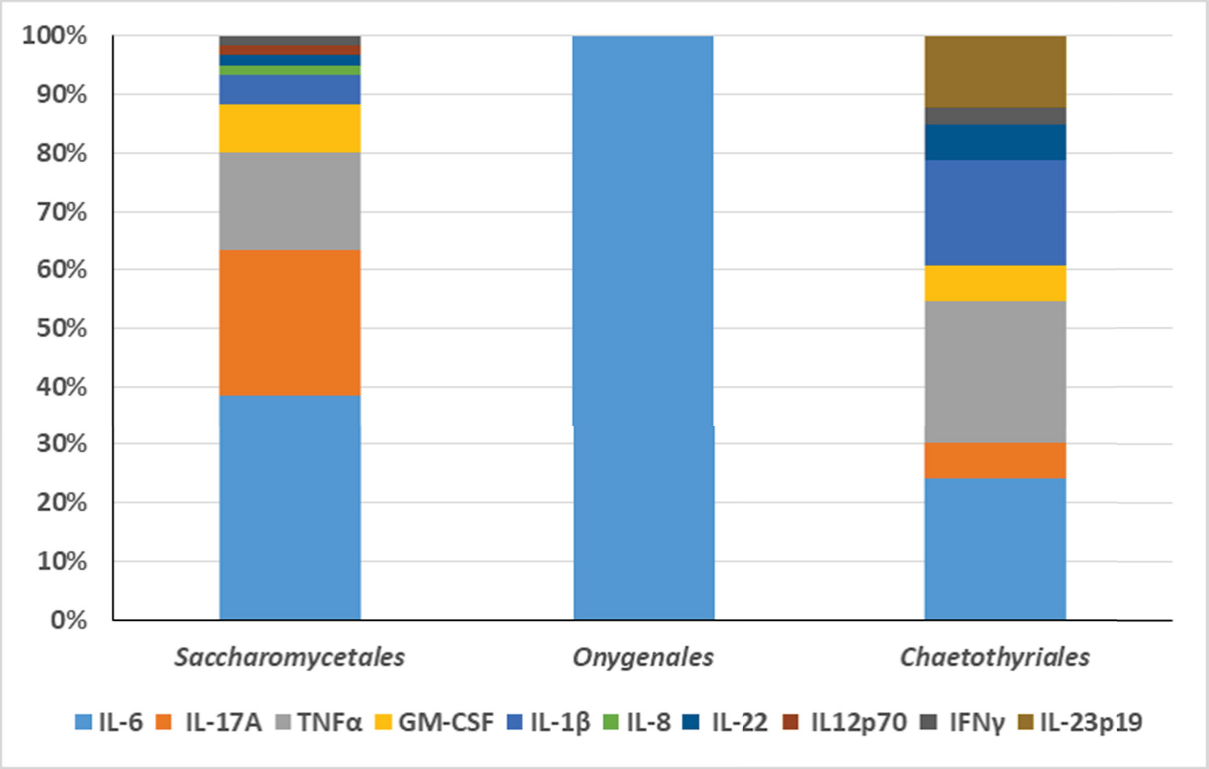
Origin	Outcome	Clinical	Species	Mutation type	Cytokine deficiency	Reference
Saccharomycetales:						
Iran	Chronic	CNS	*C. sp.*	NA	NA	De Bruyne, 2018
Morocco	Chronic	CNS, papillary edema	*C. albicans*	Q289X/Q289X	IL-6, IL-17A	De Bruyne, 2018
Iran	Chronic	CNS	*C. glabrata*	R35Q/R35Q	IL-6, IL-17A	De Bruyne, 2018
Turkey	Chronic	CNS	*C. albicans*	R70W/R70W	IL-6, IL-17A	De Bruyne, 2018
Turkey	Chronic	CNS	*C. albicans*	R70W/R70W	IL-6, IL-17A	De Bruyne, 2018
Turkey	Chronic	CNS	*C. albicans*	NA	NA	De Bruyne, 2018
Turkey	Chronic	CNS	*C. albicans*	Q295X/Q295X	IL-6, IL-17A	De Bruyne, 2018
Turkey	Chronic	CNS	*C. albicans*	Q295X/Q295X	IL-6, IL-17A	De Bruyne, 2018
Turkey	Chronic	CNS	*C. albicans*	Q295X/Q295X	IL-6, IL-17A	De Bruyne, 2018
Turkey	Chronic	CNS	*C. albicans* (*Aspergillus* unconfirmed)	Q295X/Q295X	IL-6	De Bruyne, 2018
Europe	Chronic	CNS	*C. albicans, A. fumigatus*	Q295X/Q295X	IL-6	De Bruyne, 2018
Onygenales:						
Algeria	Chronic	Nail	*T. sp.*	p.Q289X	IL-6, IL-17A	Lanternier, 2013
Morocco	Chronic	Skin, nail, bone, lymph nodes	*T. rubrum*	p.R101C	NR	Lanternier, 2013
Morocco	Chronic	Head, nail	*T. sp.*	p.R101C	IL-6, IL-17A	Lanternier, 2013
Tunesia	Died	Skin, head, nail	*T. sp.*	p.Q289X	IL-6, IL-17A	Lanternier, 2013
Tunesia	Chronic	Head, nail	*T. rubrum*	p.Q289X	NR	Lanternier, 2013
Tunesia	Chronic	Skin, head, nail, lymph nodes	*T. rubrum, T. violaceum*	p.Q289X	IL-6, IL-17A	Lanternier, 2013
Tunesia	Chronic	Skin, head, nail, lymph nodes	*T. rubrum, T. violaceum*	p.Q289X	IL-6, IL-17A	Lanternier, 2013
Egypt	Chronic	Skin, nail	*T. rubrum*	p.Q289X	IL-6, IL-17A	Jachiet, 2015
Brazil	Chronic	Skin	*T. mentagrophytes*	p.R101L	NR	Grumach, 2015
Algeria	Chronic	Skin, nail, head, lymph nodes, CNS	*T. rubrum*	p.Q289X	IL-6, IL-17A	Boudghene, 2017
Turkey	Chronic	Skin, nail, oral cavity, lymph nodes	*T. rubrum, T. violaceum, T. verrucosum, Malassezia*	p.R70W	IL-6, IL-17A	Alves de Medeiros, 2016
Algeria	Died	CNS	*T. violaceum*	NA (family member with Q289X)	IL-6, IL-17A	De Bruyne, 2018

Table 10 continued

Origin	Outcome	Clinical	*Species*	Mutation type	Cytokine deficiency	Reference
Venturiales:						
China	NR	Skin	*Ochroconis musae*	p.D274fsX60	IL-6, TNFα, IL-1β, IL-17A, IL-22, GM-CSF	Wang, 2018
Pleosporales:						
China	NR	Skin, mucosa	*Corynespora cassiicola*	p.L64fsX59/p.D274fsX60	IL-6, TNFα, IL-1β, IL-17A, IL-22, GM-CSF	Wang, 2018
China		Skin	*Corynespora cassiicola*	c.191–192InsTGCT, .L64fsX59		Yan, 2016
Colombia		face	*Corynespora cassiicola*	c.23_29del; p.Asp8Alafs10 c.865C T, p.Q289		Arango-Franco,2018]
Mucorales:						
China	Chronic	Skin	*Mucor irregularis*	c.692CT/c.905_907delTCT	NR	Wang, 2019
Chaetothyriales:						
China	Chronic	Skin	*P. verrucosa*	p.L64fsX59/p.Q158X	IL-6, TNFα, IL-1β, IL-23p19	Wang, 2014
China	Chronic	Skin	*P. verrucosa*	p.D274fsX60	IL-6, TNFα, IL-1β, IL-23p19	Wang, 2014
China	Chronic	Skin	*P. verrucosa*	p.D274fsX60	IL-6, TNFα, IL-1β, IL-23p19	Wang, 2014
China	Chronic	Skin	*P. verrucosa*	p.D274fsX60	IL-6, TNFα, IL-1β, IL-23p19	Wang, 2014
China	Chronic	Skin, mucosa, sinus, CNS	*P. verrucosa*	p.R35Q/p.E81K	IL-6, TNFα	Zhang, 2017
Angola	Chronic	Liver, CNS	*E. dermatitidis*	p.R18W	NR	Lanternier, 2015
Iran	Chronic	Skin, bone, lung	*E. spinifera*	p.E232del	IL-6, TNFα, IL-1β, IFNγ, IL17, IL-22, GM-CSF	Lanternier, 2015
China	Chronic	Skin	*E. spinifera*	p.S23X/p.D274fsX60	IL-6, TNFα, IL-1β, IL-17A, IL-22, GM-CSF	Wang, 2018
Angola	Chronic	CNS	*E. dermatitidis*	R18W/R18W		De Bruyne, 2018
China	Chronic	Skin	*P. americana*	p.D274fsX60	IL-6, TNFα, IL-1β	Huang, 2019

The main cytokine impaired was IL-6 (43/50 cases). In Onygenales, this was the only deficient cytokine reported, while, in contrast, in seven cases of Candida infection GM-CSF or TNFαwere absent instead. Black fungi were associated with a relatively large panel of missing cytokines, particularly TNFαand IL-1β. The relative absence of cytokines in the three fungal main groups is illustrated in (Figure 21).

FIGURE 21 – The relative absence of cytokines in the three fungal main groups is illustrated. The main cytokine impaired in Candida infection was IL-6. GM-CSF or TNFαwere absent in seven cases of *Candida* infection. In Onygenales, IL-6 was the only deficient cytokine reported. Black fungi were associated with a relatively large panel of missing cytokines, particularly TNFαand IL-1β.



Source: (SONG et al., 2020)

4.4.2 Genome sequencing, assembly, and annotation

We sequenced the genomes of three strains: BMU 08022 (clinical strain from human skin, ESC1), BMU 00051 (environmental strain from bark, ESE1), and BMU 00047 (environmental strain from soil, ESE2) (Table 9) using a combination of long-read (PACBIO) and short-read (Illumina) sequencing technologies. The third generation sequencing is supplemented by the second generation sequencing, and combined with a variety of assembly software and parameter optimizations (Figure 19). For each genome, PACBIO sequencing provided more than 100× coverage and Illumina reached 170× coverage. For strain ESC1, we obtained a draft genome assembly of 33,559,924

TABLE 11 – Genome assembly of analyzed strains of *Exophiala spinifera*, compared with deposited genomes of *E. spinifera* (CBS 899.68) and *E. oligosperma* (CBS 725.88).

	ESC1	**ESE1**	**ESE2**	**CBS 899.68**	**CBS 725.88**
Genome size (bp)	33,559,924	32,380,025	32,696,644	32,912,300	38,224,500
Number of contigs	9	7	9		
Number of scaffolds	8	7	7	28	143
L90	7	7	7		
N50 (bp)	4,048,587	4,872,376	4,635,272		
GC content	51.8 %	51.9 %	51.7 %	51.7 %	50.4 %
CEGMA	236 (95.2%)	236 (95.2%)	237 (95.6%)		
BUSCOs	1,282 (97.5%)	1,276 (97.1%)	1,274 (96.9%)		
Number of genes	12.13	12.072	12.043	12.11	11.938
Number of proteins	12,131	12.074	12.043	12.049	13.234
Mean gene length (bp)	2.006	2.012	1.979		
Mean number of exons	2.84	2.92	2.87		
Genome coding	72.4%	75.0%	72.9%		
Coverage of InterPro	78.8%	77.6%	77.9%		
Coverage of GO	70.8%	70.0%	70.5%		
rRNA	18	28	21	16	4
tRNA	42	44	44	45	41
Genome coverage	103×	122×	110×	272×	240×
Sequencing technology	PacBio	PacBio	PacBio	Illumina	Illumina

bp organized into nine contigs with an L90 of 7, and an N50 of 4.0 Mb. The genome of ESE1 was 32,380,025 bp in size and comprised seven contigs with an L90 of 7, and N50 of 4.9 Mb. In ESE2, the genome assembly of 32,696,644 bp contained nine contigs with an L90 of 7, and an N50 4.6 Mb. For all three strains, more than 94% of the Illumina reads were aligned to the draft genome assemblies in post hoc validation (Table 11). The results of genome assembly of the three strains reached sub-chromosome level.

TABLE 12 – Comparison of single nucleotide polymorphisms of clinical vs. environmental strains.

	ESC1 *vs.* ESE1	**ESC1 *vs.* ESE2**
Synonymous SNP	105.095	104,446
Non-synonymous SNP	67.174	66,361
Frameshift deletion	1,231	1,152
Frameshift insertion	1,124	1,133
Non-frameshift insertion	965	962
Non-frameshift deletion	939	909
Stopgain	701	849
Stoploss	153	150

4.4.3 Colinear analysis

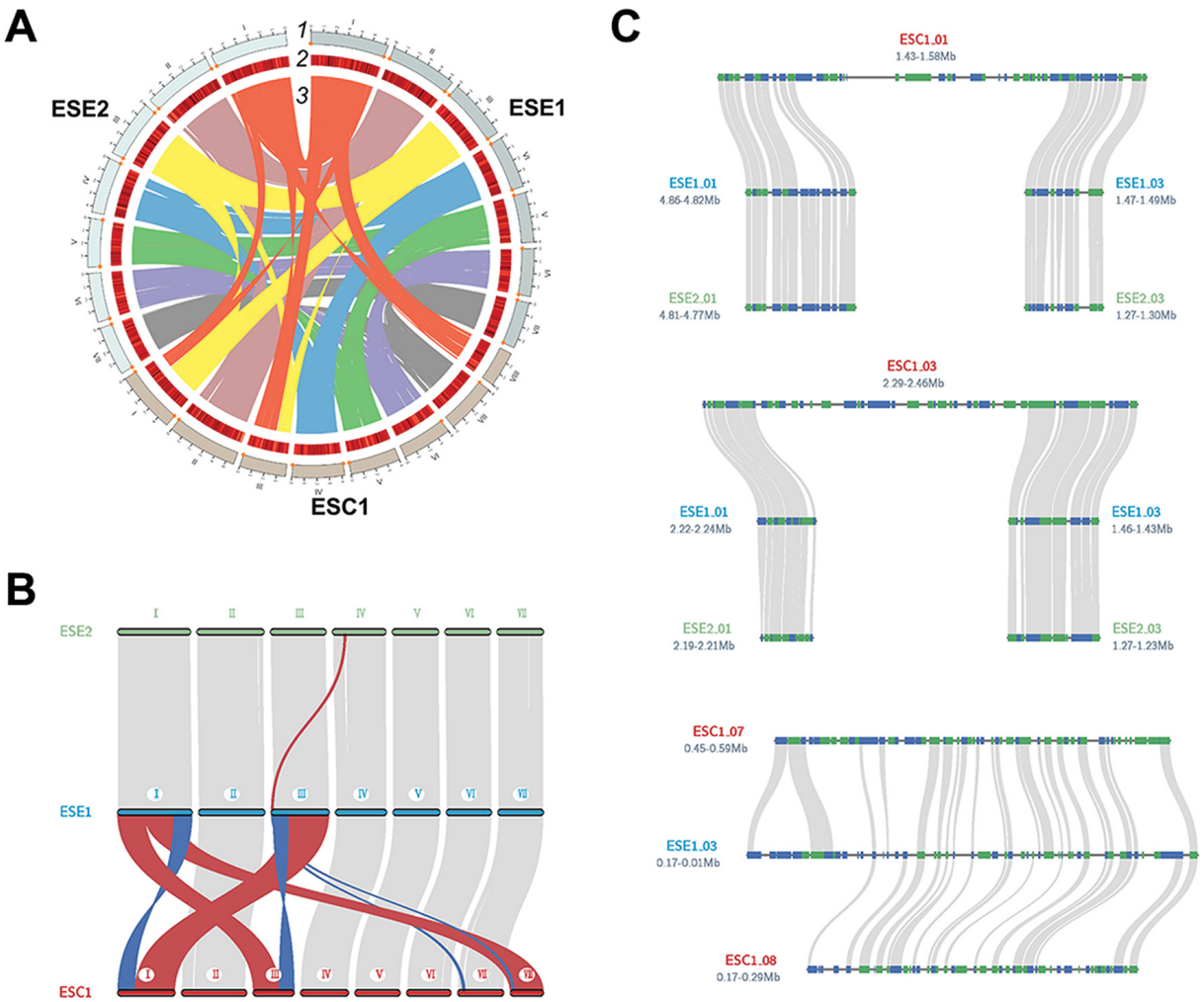
We used whole-genome alignment to identify homologous and strain-specific segments among three *E. spinifera* strains. We identified a total of 172,269 and 170,807 SNPs for the clinical strain compared to the environmental strains BMU 00051 and BMU 00047, respectively (Table 12). However, the number of SNPs between the environmental strains was 362,264, which indicated that the genome of the clinical strain was more remote from the two environmental strains from different continents. Likewise, the number of indels identified in the clinical strain was higher than compared with environmental strains (Table 12). Moreover, we found substantial syntenic rearrangements in scaffolds I and III of ESC1 (Figure 22 a).

Comparing the genomes of ESC1, ESE1 and ESE2, recombination was observed in scaffolds I and III between the clinical and environmental strains (Figure 22b). Scaffold I of ESC1 is equal to part of scaffolds I and III of the environmental strains, and scaffold III of ESC1 is equal to the remainders of scaffolds I and III of the environmental strains. This is shown in more detail in (Figure 23), where scaffold VIII of ESC1 is shown to be comparable to part of scaffold I of the environmental strains, and two areas proved to be inverted between clinical and environmental strains.

4.4.4 Functional annotation

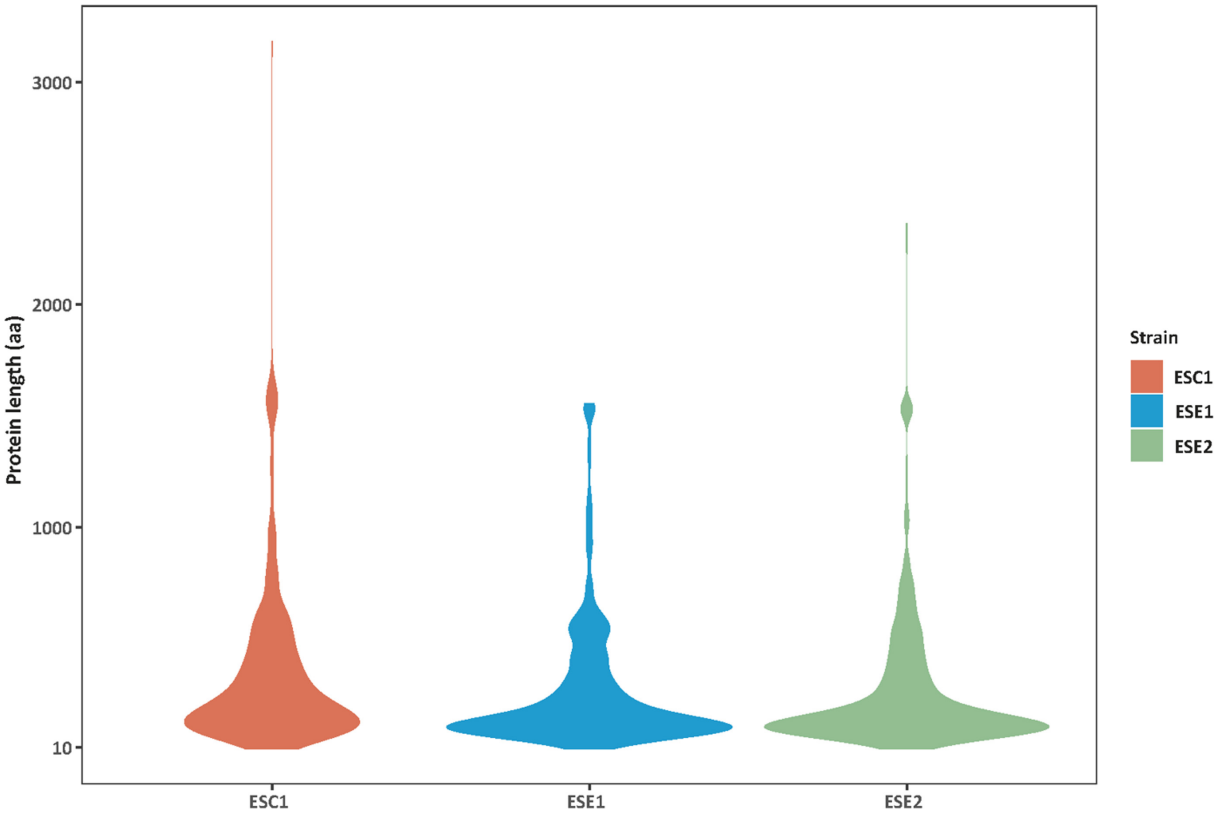
Genome functional annotation was performed with INTERPROSCAN, resulting in over 59% of genes being protein-coding genes annotated with GO term. A hybrid strategy combining ab initio predictions and transcriptomic support (RNA-seq) was applied in gene prediction. Annotation of the strains yielded 12,131 protein-coding genes for *E. spinifera* ESC1, 12,074 protein-coding genes for ESE1 and 12,043 for ESE2 (Table 11). There are rearrangements in scaffolds I and III between ESC1 vs. ESE1 and ESC1 vs. ESE2 (Figure 22), 23), and in scaffolds VII and VIII of ESC1 syntenic rearrangements exist with scaffold III of ESE1 (Figure 23). Plotting the protein lengths of the three strains,

FIGURE 22 – Genome colinear analysis for the three *E. spinifera* genomes. (A) Recombination is observed between clinical strain ESC1 and environmental strains ESE1 and ESE2 in scaffolds I and III. Track 1: genome position; track 2: gene density; track 3: sequence synteny. Orange diamonds: telomere sequences. (B) Location of sequence rearrangements in scaffolds I and III of ESC1. Red: Stands for positive collinearity; Blue: reverse complementary. (C) Scaffold I of ESC1 is equal to part of scaffolds I and III of the environmental strains, and scaffold III of ESC1 is equal to the remainders of scaffolds I and III of the environmental strains. Scaffolds VII and VIII of ESC1 syntenic rearrangements exist with scaffold III of ESE1. Scaffold VIII of ESC1 is shown to be comparable to part of scaffold I of the environmental strains, and two areas proved to be inverted between clinical and environmental strains.



Source: (SONG et al., 2020)

FIGURE 23 – Distribution variability of specific protein length among three strains. The protein lengths (Y-axis) in strains analyzed (n = X-axis) showing an intraspecific variability. Greater differences were found between clinical strain ESC1 and environmental strains ESE1 and ESE2, while the protein length distribution of the two environmental strains was more consistent.



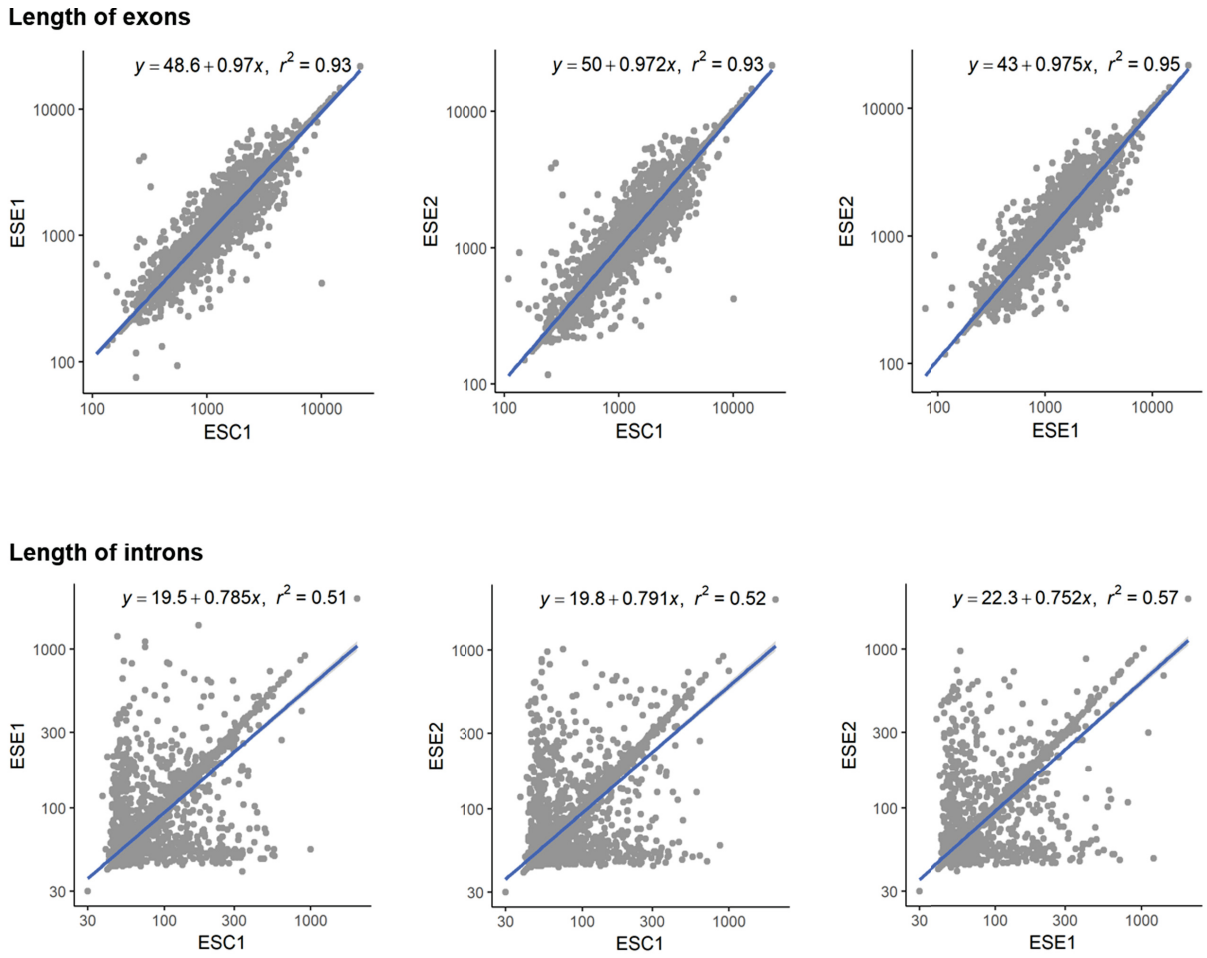
Source: (SONG et al., 2020)

considerable differences were noted (Figure 24): the clinical strain ESC1 deviated by having more genes with very long dimensions. Both environmental strains being very similar, there were 105,095 (ESE1) / 104,446 (ESE2) synonymous versus 67,174 (ESE1) / 66,361 (ESE2) non- synonymous SNPs compared to clinical strain ESC1 (Table 12). Single-copy orthologous genes were compared to detect differences in proportion of selected sites, with likelihood ratio as test of significance. Models 1 (T0 = T1) and 2 (T0 != T1) were used to estimate the Tvalues as free parameters, with likelihood ratios as parameters of significance. A total of 29 genes yielded significant values indicating positive selection (P<=0.0001) (Table S2). In contrast, homologous introns were weakly correlated, with significant length variations (Figure 24).

4.4.5 Secondary metabolite clusters

We identified a total of 9,329 homologous groups at the gene level (1:1:1 association among three strains). In the assumption that the core genomes with basic metabolic functions have similar biological functions, we focused on secondary metabo-

FIGURE 24 – Length variations of introns and exons. The blue lines show trends in exon length distribution, the slopes being different between exons and intron. Positive and negative correlations are shown. Homologous introns were weakly correlated, with significant length variations.



Source: (SONG et al., 2020)

lites. Seventeen gene clusters were involved in the production of secondary metabolites (Figure 26). Clusters 1, 2, 4, 14 and 16 are PKS-related clusters. Cluster 16 is present in environmental strain ESE2 only. The cluster contains the single copy gene benzoate 4-monooxygenase cytochrome P450, which catalyzes the benzoate degradation step in the toluene catabolic pathway (Table 13).

4.4.6 RNA-seq

Sequence clustering at the mRNA level was used to compare the three strains to identify homologous groups among the three strains. For all three strains, poly (A)-enriched, strandspecific RNA-seq from two cultivation conditions (SDB and BHI broth) with different culture degrees was performed. Comparison of differentially expressed genes under different culture conditions demonstrated that ESC1 revealed significant

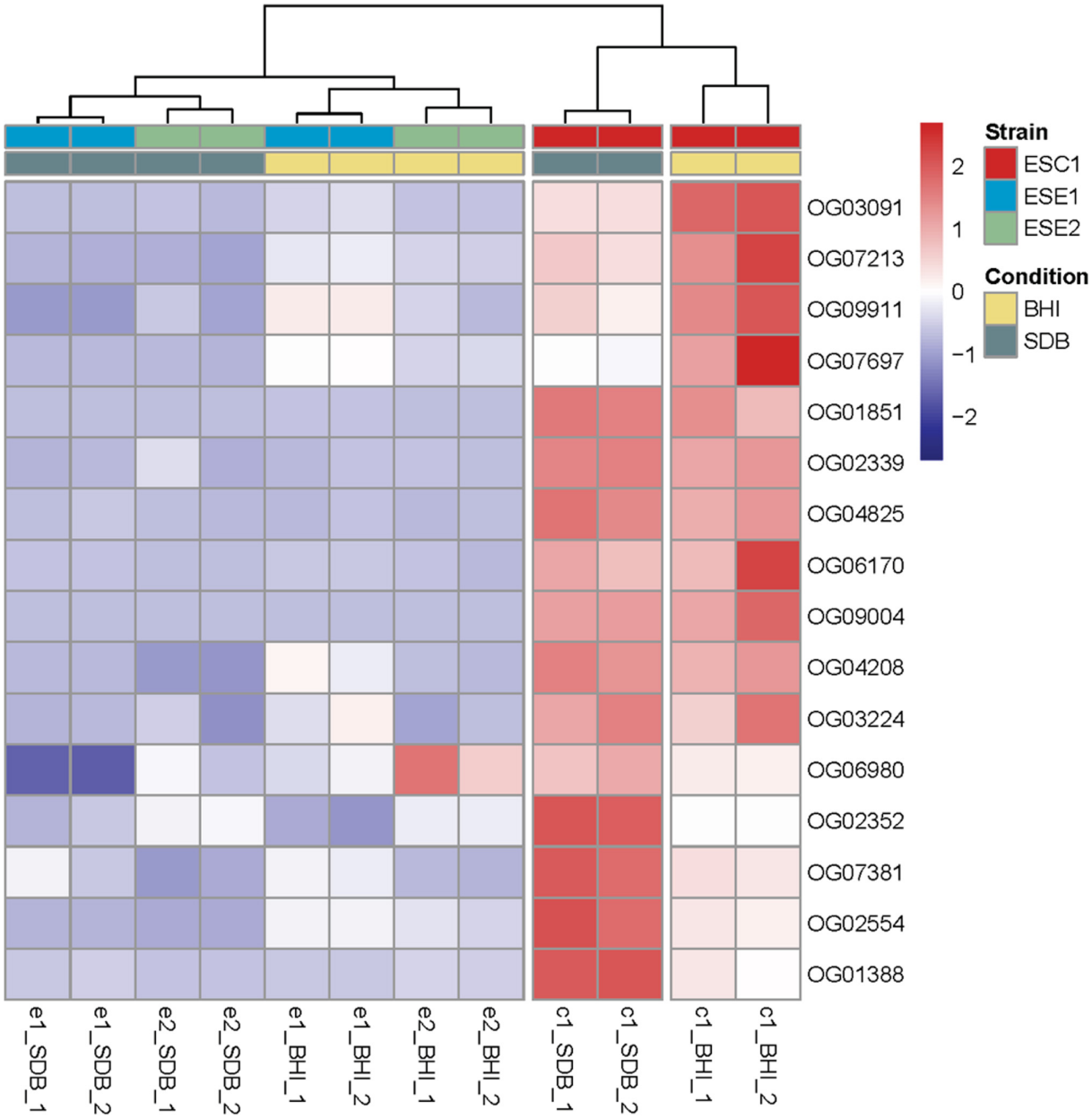
FIGURE 25 – Genome comparison of secondary metabolite clusters of three strains. Seventeen gene clusters were involved in the production of secondary metabolites. Environmental strains ESE1 and ESE2 are predicted to have more secondary metabolite clusters than the clinical strain ESC1. Clusters 1, 2, 4, 14 and 16 are PKS-related clusters. Cluster 16 is present in environmental strain ESE2 only.

Source: (SONG et al., 2020)

TABLE 13 – Function of specific secondary metabolic gene clusters.

Cluster ID	Strain	Backbone
Cluster 3:	ESE1 ESE2	L-aminoadipate-semialdehyde dehydrogenase
Cluster 10:	ESE2	Putative acyl-coenzyme A synthetase
Cluster 11:	ESE1	Nonribosomal peptide synthase Pes1
Cluster 12:	ESE1 ESE2	Beta-ketoacyl-acyl-carrier-protein synthase II
Cluster 15:	ESE1	Nonribosomal peptide synthase GliP
Cluster 16:	ESE2	Polyketide synthase, putative
Cluster 17:	ESE2	NRPS-like enzyme

FIGURE 26 – Comparison of differentially expressed genes upon incubation in SGB and BHI broth. Clustering of deviations from zero and shown in a dendrogram by complete linkage hierarchical clustering using Euclidean distance. All tests are shown in duplicate. The results demonstrated that ESC1 revealed significant differences in response to the two media in 16 genes, while the environmental strains ESE1 and ESE2 remained almost indifferent.



Source: (SONG et al., 2020)

differences in response to the two media in 16 single-copy genes, while the environmental strains ESE1 and ESE2 remained almost indifferent (Figure 26 and Supplementary Table S3). There were 48 unique genes (22 in BHI media and 26 genes in SDB media; Table 24) involved in metabolism and transcriptional regulation differentially expressed exclusively in the clinical strain compared to the two environmental strains, including 12 genes without functional annotation. Supplementary Table S4 displays the functional gene content (name, IPR families, KOG, and GO) and respective expression levels in each scenery/media, to enable the visualization/filters of this data. The table includes the gene ID, description, IPR numbers and the GO information. Differentially expressed genes between clinical isolate and environmental isolates under BHI culture are listed in Supplementary Table S5. There were 637 upregulated genes between ESC1 and ESE1, and 659 up-regulated genes between ESC1 and ESE2. These specific upregulated genes in the clinical strain mainly act in transmembrane transport, translational elongation, ribosome biogenesis, and some other biosynthetic processes (GO enrichment results are summarized in Supplementary Table S6). GO analysis showed that the gene annotations were mainly in three categories: biological processes (BP), molecular function (MF), and cell component (CC). GO categories among three strains showed a similar enrichment distribution (Supplementary Figure S2). However, GO annotations of genes unique to each strain were not identical in BP and MF ontologies. In the biological process, the differentially expressed genes were mainly enriched in response to cellular process and metabolic process. In the MF, differentially expressed genes were mainly related to binding and catalytic activity (Supplementary Figure S3). The correlation between differential expression genes and positive selection genes are listed in Supplementary Table S7. There were 53 positively selected genes that were also upregulated between ESC1 and ESE1 in BHI culture. Comparing ESC1 and ESE2 under BHI conditions, 53 positively selected genes were also up-regulated in ESC1. A total of 29 positively selected genes were up-regulated in ESC1 when compared with both ESE1 and ESE2 in BHI broth. These genes were involved in positive regulation of translation, regulation of translational termination, and ATP synthesis coupled proton transport.

4.4.7 Discussion

Black yeasts are generally considered to be opportunists; consequently, they should lack any specialized adaptation to the vertebrate host (SEYEDMOUSAVI et al., 2018). Infection is coincidentally promoted by factors that enhance survival in the environmental niche of the fungus. Such factors must be present in chaetothyrialean black fungi, given the relatively large number of species reported from infections in humans and in cold-blooded animals. The Chaetothyriales are particularly over-represented in patients with inherited deficiencies in caspase recruitment domain-containing signaling

TABLE 14 – Unique genes differentially expressed exclusively in the clinical strain.

Gene Ontology	Total genes	Media
NA (NA)	10	SDB
integral component of membrane (GO:0016021)	6	SDB
NA (NA)	2	BHI
nucleus (GO:0005634)	2	BHI
oxidation-reduction process (GO:0055114)	2	BHI
oxidoreductase activity (GO:0016491)	2	BHI
zinc ion binding (GO:0008270)	2	BHI
carbon-sulfur lyase activity (GO:0016846)	2	SDB
DNA-binding transcription factor activity (GO:0003700)	2	SDB
GTP binding (GO:0005525)	2	SDB
ligase activity (GO:0016874)	2	SDB
methylation (GO:0032259)	2	SDB
methyltransferase activity (GO:0008168)	2	SDB
phosphopantetheine binding (GO:0031177)	2	SDB
regulation of transcription, DNA-templated (GO:0006355)	2	SDB
transcription factor complex (GO:0005667)	2	SDB
transmembrane transport (GO:0055085)	2	SDB
catalytic activity (GO:0003824)	1	BHI
integral component of membrane (GO:0016021)	1	BHI
protein transport (GO:0015031)	1	BHI
ATP binding (GO:0005524)	1	SDB
cytoplasm (GO:0005737)	1	SDB
DNA-binding transcription factor activity, RNA polymerase II-specific (GO:0000981)	1	SDB
nucleus (GO:0005634)	1	SDB
regulation of transcription by RNA polymerase II (GO:0006357)	1	SDB
ribosome (GO:0005840)	1	SDB
RNA binding (GO:0003723)	1	SDB
transferase activity (GO:0016740)	1	SDB
zinc ion binding (GO:0008270)	1	SDB

protein 9 (CARD9), a disorder impairing the dectin pathway of innate immunity. This protein enhances pattern recognition receptors to induce NF-κB and MAPK activation, leading to a cytokine cascade. Malfunctioning of the protein decreases protection against infection by larger microbes such as parasites and especially fungi. It has been puzzling why the patients, theoretically being susceptible to any fungal infection, usually carry only a single species. Reviewing the 77 fungal cases published to date (Table 9), a possible explanation might be found in the fact that this protection deficiency appears to be highly specific. Only three groups of fungi are prevalent: *Candida spp.* (order Saccharomycetales), dermatophytes (order Onygenales) and black fungi of the order Chaetothyriales. A single case of Mucor irregularis has been published. This is the only species of Mucor that causes chronic infections in apparently healthy individuals (LU et al., 2013). However, most cases were published before CARD9 deficiency was discovered, thus the possibility is not excluded that more patients had a hidden inherited immune disorder. Remarkably, common opportunists such as *Aspergillus* or *Fusarium* are nearly or completely lacking. The over-representation of chaetothyrialean black fungi in this susceptible patient population is also striking because such infections are rare in other patient cohorts, in healthy as well as in otherwise immunocompromised hosts.

As noted by (VAEZI et al., 2018), there is a regional bias in CARD9 cases in that the great majority of cases by *Candida* and dermatophytes are from northern Africa, Turkey and Iran, while chaetothyrialean cases are particularly encountered in East Asia. It may be noted, that prevalence of severe and chronic black fungal infections is well-known in China (de Hoog et al., 2019), but most cases, as in Mucor irregularis, date back from before the discovery of CARD9 deficiencies and consequently at that time these patients were thought to be otherwise healthy. The most common species in CARD9 patients is *Candida albicans*. However, mucocutaneous cases are rare, but CNS-involvement, which in other patient cohorts is generally observed only shortly before death, is preponderant. Some of the patients carried different mutations in the two alleles. With dermatophytes, mutations in all patients had homozygous mutations. In black fungi, i.e. members of Chaetothyriales, Pleosporales and Venturiales, 40% mutations were heterozygous. Mutation types tend to be different between the groups of yeasts, dermatophytes, and black fungi (Figure 20): black fungi responded to other mutations than yeasts and dermatophytes. Also the affected cytokines differed between groups. Dermatophytes consistently responded to IL-6 deficiency alone, while black fungi needed the largest cytokine deficiency to cause infection (Figure 21).

From these data it is obvious that black fungi, and particularly those belonging to the order Chaetothyriales, respond in a rather specific manner to impairment of the human immune system. Comparing the three main groups (yeasts, dermatophytes, black fungi) with respect to their response to cytokines, quite remarkably, the patients with chronic dermatophyte infections all were reported to have IL-6 deficiency alone,

while in black fungi TNFαand often also IL-17A were impaired. In *Candida*, a more variable picture was observed, but IL-6 deficiency was observed in 23 out of 31 cases. IL-6 is a proinflammatory cytokine controlling T-cell differentiation, particularly Th17 and regulatory T cells (ZHAO et al., 2011). These conclusions should however be taken with some care, as cytokine measurements were not consistent between publications.

The data are nevertheless sufficient to surmise that black fungal infections, particularly of members of Chaetothyriales, are not randomly occurring in susceptible patients with impaired immunity in general, but respond specifically to the conditions provided by CARD9 impairment. Because of their pronounced ability to decompose monoaromatic toxins, black yeasts of this order are enriched in the domestic environment (MORENO et al., 2018), and therefore close vicinity to humans is probable. Many species are oligotrophic and have efficient nutrient scavenging systems. Toxin management has been hypothesized to enhance survival strategies in the environment (GOSTINČAR et al., 2018), and industrial pollution by monoaromates might promote growth of these fungi near humans. In this scenario, take-up by susceptible individuals followed by successful infection, may explain their high frequency in CARD9 patients. The cytochome P-450 gene family has been suggested as a possible factor explaining fungal neurotropism, but our clinical strain lacked PKS cluster 16 which is present in one of the environmental strains (Figure 23, Table 12).

Over the past decade, advances in Next Generation Sequencing (NGS) technologies and decreasing sequencing costs allowed an increase in the number of sequenced genomes, with better quality. Genome data of our strains are listed in Table 2, showing a range of intraspecific variability of 863,280 bp and 87 genes. These genomes were sequenced by a combined Illumina and P AC B IO strategy. Comparing the genomes of *E. spinifera* sequenced to date, the older Illumina genomes were within the range of the above established species variance. Similarly, comparable numbers of genes were found, suggesting that published genomes are reliable, despite the fact that the number of scaffolds in the Illumina-only genomes was considerably higher. Despite surmised opportunism in black yeasts, our clinical strain of *E. spinifera*, derived from a CARD9 patient, differed significantly from two environmental strains, even though these originated from different continents. In the environmental / clinical comparison, rearrangements were observed in scaffolds I and III (Figure 22), including two inversions, and ESC1 having an extra scaffold VIII (Figure 22). The rearrangements in this genome area are significant (Figure 23).

E. spinifera shows variable responses with hemolysis (SONG et al., 2017). Although these authors noted that this ability did not match with the division environmental / clinical, the possibility is not excluded that hemolytic strains have an enhanced invasive potency for vertebrate hosts. We registered up- or downregulation of single-copy

proteins in response to SGB versus BHI, and noted that ESC1 showed significant differences with the two media in 16 genes, while the environmental strains remained almost indifferent; in vitro, ESC1 and ESE1 showed hemolysis, while ESE2 was negative. Most of the single-copy genes upregulated with BHI were transporters in KOG classification.

In this study, significant genomic rearrangements between strains of the same species were demonstrated. Two environmental strains from different continents were largely identical, whereas a clinical strain from a CARD9-deficient patient was different and deviated in the number of genes, even though all strains were phenotypically similar (SONG et al., 2017). In general, clinical and environmental strains of a single opportunistic fungus are taken to have comparable infectious abilities, because neither of them is equipped with specialized virulence factors. However, the observed quantitative genetic variability of strains of the single species *E. spinifera* possibly is associated with a differential chance to cause infection in susceptible patient populations; differences may be small but clinically relevant. We compared our NGS approach with older Illumina techniques, which showed some minor flaws e.g. in the number of scaffolds, but for quantitative description of the genome these data proved to be sufficient. Main improvement of PacBio data are expected in qualitative studies of specific gene functions.

4.5 DATA AVAILABILITY STATEMENT

Generated Statement: The datasets generated for this study can be found in the NCBI. The RNA-Seq data can be accessed by PRJNA600825, the PacBio assemblies (PRJNA600825), Illumina assemblies (PRJNA600036). The RNA-Seq is available already deposited at https://www.ncbi.nlm.nih.gov/sra/PRJNA600825).

4.6 AUTHOR CONTRIBUTIONS

We acknowledge Prof. Mihai Netea (Radboud University Medical Center) for advice concerning CARD9 prevalence.

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4.9 SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01880/full#supplementary-material

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FINAL DISCUSSION AND CONCLUSIONS

This study has provided novel insights in the Chaetothyrialian species focusing intrinsic differences in the species virulence indicating intraspecific variability aiming to elucidate key virulence genes and drive the continuing research forward pathogen host interactions. Among the chapters was explored the mechanisms of pathogenic and adaptive strategies of black yeasts including genes envolved on oportunistc animal infecctions and a new possible species and /or strains to the bioremediation applications.

The first Chapter focused to show the historical and current "state of art" of the black-yeasts research using the data visualization applications, text mining, cluster methods and scientometrics analyses. Amidst few bibliometric works in the biological sciences, because they are most common in the areas of statistics, information management and data science, the data represents the first bibliometric review of about black yeasts, the "big bang" of the publications occurred after the 1970s, guided by the USA, Netherlands and Brazil, the globalized science now, allows to have 92 countries involved to work together.

The advancement of omics sciences and the search of the bioinformatics tools has been increased, being noted that in the last decade, the number of publications found in the Dimensions database increased more than 4 times from 3.715 publications in 2010 to 14.630 publications in 2020. In nowadays, it’s possible to analyse biological data by smartphones (PALATNICK et al., 2020), although, many software used are not user-friendly and requires a Information Technology (IT) knowledge and high power computers.

From a context of uncertainty even to the terminology on black yeasts in the 1990s, the lack of techniques that emerged only in the molecular age for identification and isolation, 125 years later it is possible to characterize, identify, sequence genomes and see the profiles of gene expression of these highly complex fungi, providing data focusing to medical treatments and biotechnological applications, even one of the factors related to dissemination to the central nervous system, which maybe it’s related with the ability to assimilate aromatic compounds (MORENO et al., 2018). In fact, the big advances occured after the developed of new molecular techniques and bioinformatics. In the last 20 years based on the data available on the NCBI, 154 species had been described, 41 species of the genus *Cladophialophora*, 47 *Exophiala*, 17 *Phialophora*, 23 *Cadophora*, 8 *Fonsecaea* and 13 species of the *Rhinocladiella*, while before 1996, basically two species were known in the genus *Fonsecaea*and until 2015, the genus comprised in 8 species.

In general, Black yeasts display remarkably diverse lifestyles, with a predilection for extreme and toxic environments such as those rich in aromatic compounds or heavy

metals, or with high temperatures, increased salinity, and scarcity of nutrients. In this context the comparative genome was used in order to elucidate the potential ecology of some species in *Exophila* species and to deepen the knowledge of the mechanisms and of extremetolerance in the recently described species *C. exuberans*, which can survive to high heavy metals conditions as lead and copper and it was evidenced that in the genomic point of view, *C. exuberans* has complete pathways of degradation aromatic compounds as toluene and benzene, using as sole carbon source and energy. According to the genome comparative analysis, it was observed that *C. exuberans* has genes associated with all steps of toluene assimilation confirms the physiological tests by Nascimento et al. (2017).

However, this characteristic is not exclusive to a genus or specie, represented in the Chapter 3, and other black yeasts have the same behavior, even some pathogenic as *E. mesophila* and *E. dermatitidis*. It was expected that *C. exuberans* and *C. psammophila* shared more genes, however, *C. exuberans*, in the genomic organization and the genes clusters shared is more closer of *C. carrionni*, followed of *C. immunda*. *C. psammophila* has more representative genes in the KEGG pathways comparison. Moroever, it is more closer to the neutropic species *C. bantiana* and both did not have the same profile to the degradation process.

The variation of toluene pathways can be noted also to the most virulent species in the genus *Exophiala*, *E. dermatitidis* and *E. spinifera*, not all strains have at least one gene to all steps of the toluene degradation. High numbers of virulence, including cluster of the Myb-like DNA-binding domain and SANT/Myb domain (OG0012246), involved in conidiation of *Cochliobolus carbonum* (ZHANG et al., 2014) and pathogenicity of *Fusarium graminearum* (KIM et al., 2014), has found to these species and some genes can be associated with the brain invasion, because it was also detected shared genes with *R. mackenziei* by Moreno et al. (2018). The *E. dermatitidis* demostrated less expansions and gains than *E. spinifera* throughout evolution.

More studies should be developed to identify the consist relation that such infections are mostly associated with mutations in the CARD9 gene (LANTERNIER et al., 2015b). Furthermore, significant genomic rearrangements between *E. spinifera* were demonstrated (Chapter 4), including 48 genes differentially expressed only in the clinical strain, isolated from the patient with CARD9 deficiency.

In conclusion this study provided relevante genomic and transcriptomic data of *Cladophialophora*, *Exophiala*, in the *Chaetothyriales* order and to future studies. These data comparing the clinical and the environmental strains show different virulence profiles and the possible use to bioremediation applications. According the comparative genomic analyses observed that *C. exuberans* has high number of genes related with the degradation pathways, otherwise has virulence orthologous clusters shared with the

E. dermatitidis.

Therefore, in the current context of researchers on ecology, genera, species, pathogenicity, virulence and biotechnological applications for yeasts, it is necessary to generate more data on genomes, transcriptomes, proteomes linked to the development of software and pipelines. This has to be done respecting the biological individuality of each group in study using the artificial intelligence, data science and big data techniques combined to detect patterns, generate indicators and decision-making for conducting research.

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