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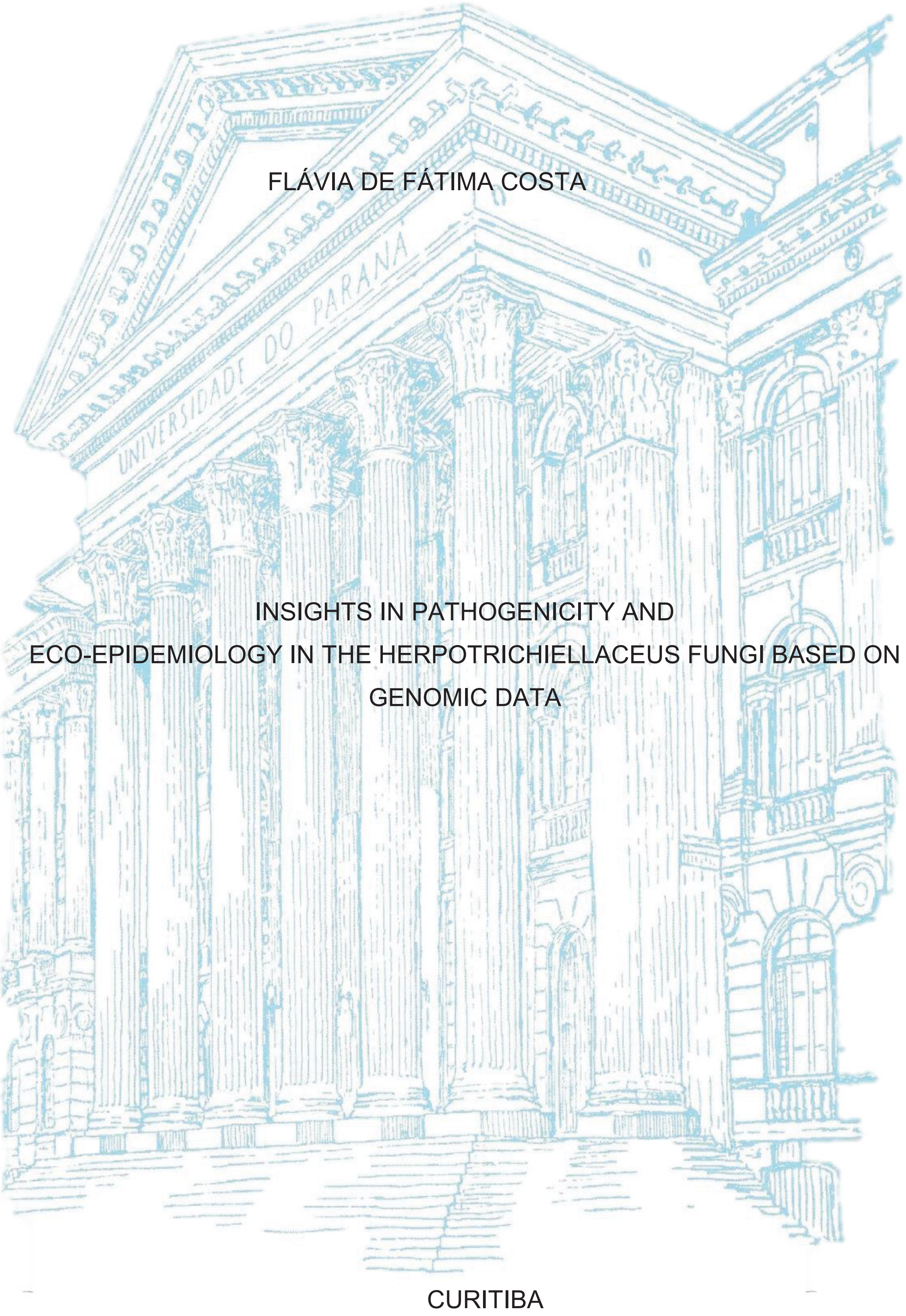

FLÁVIA DE FÁTIMA COSTA

INSIGHTS IN PATHOGENICITY AND ECO-EPIDEMIOLOGY IN THE HERPOTRICHIELLACEUS FUNGI BASED ON GENOMIC DATA

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

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **FLAVIA DE FATIMA COSTA** intitulada: **" Insights in pathogenicity and eco-epidemiology in the Herpotrichiellaceus fungi based on genomic data"**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua aprovação no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 18 de Junho de 2019.

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I dedicate,

To my beloved husband Lucas and to our daughter Laura,

To my parents and brothers.

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RESUMO

As leveduras negras da família Herpotrichiellaceae são extremamente importantes no ponto de vista clínico e ecológico. Estes são oligotróficos e podem estar presentes em uma ampla variedade de nichos ambientais. Além disso, existem algumas espécies associadas a hospedeiros humanos e animais considerados agentes causadores de doenças, como a cromoblastomicose e a feohifomicose. Nesse contexto, o objetivo principal deste trabalho foi utilizar os dados metagenômicos para identificação e detecção desses agentes a partir de amostras ambientais, a fim de elucidar sua eco-epidemiologia. Além disso, a sequenciamento genômico foi utilizado para inferir dados sobre a patogenicidade de espécies associadas à cromoblastomicose. O DNA total de *Fonsecaea nubica* foi seqüenciado nas plataformas Ion e Illumina. Após a análise da qualidade, foi realizada a montagem do genoma. O *draft* compreendeu o tamanho de 33,7 Mb, distribuído em 258 contigs com a anotação de 11.681 genes previstos, disponível em PRJNA312449. Com o objetivo de compreender melhor os aspectos eco-epidemiológicos dos fungos associados à infecção humana, foram analisados os conjuntos de dados da metagenômica do solo do bioma Cerrado brasileiro com foco nas espécies patogênicas. Nesta análise, foram identificadas 41 espécies e dentificou-se a ocorrência ambiental de espécies clínicas da família Herpothrichiellaceae dos gêneros *Exophiala* e *Rhinocladiella*, além de outras espécies causais de infecções humanas de outros gêneros. Além disso, um estudo utilizando os dados metagenômicos disponíveis nas diferentes regiões brasileiras foi realizado utilizando-se os códigos de barras e sondas de cadeados para espécies específicas de Herpothrichiellaceae, a qual revelou a presença destes agentes em diversas fontes ambientais, tais como solos contaminados por hidrocarbonetos, substratos em decomposição, solos, rizosfera e ambientes aquáticos. A identificação *in silico* mostrou a ocorrência ambiental de espécies patogênicas, como a *Cladophialophora bantiana* encontrada em amostras de DNA de cana-de-açúcar e confirmada pelo isolamento usando métodos seletivos. Além disso, neste isolamento foram identificadas novas espécies, descritas como *C. rhizus*, *C. cannae*, *C. molasses* e *E. plantae.*

Palavras-chave: Herpotrichiellaceae. Genômica. Metagenômica.

ABSTRACT

Black yeasts of the Herpotrichiellaceae family are extremely important concerning to the clinical and ecological point of view. They are oligotrophics and can be present in a wide variety of environmental niches. Moreover, there are some species associated to human and animal hosts considered causal agents of diseases, such as chromoblastomycosis and phaeohyphomycosis. In this context, the main objective of this work, it was to use the metagenomic data for identification and detection of these agents from environmental samples, in order to elucitate their eco-epidemiology. In addition, the genomic sequecing was used for inferences regarding the pathogenicity of species associated to chromoblastomycosis. The total DNA of *Fonsecaea nubica* was sequenced in the plataforms Ion and Illumina. After the analysis of the quality, was perfomed genome assembly. The draft comprised genome size 33.7 Mb, distributed in 258 contigs with the annotation of 11,681 predicted genes, and genome is available in PRJNA312449. In order to better understand the eco-epidemiology aspects of fungi associated to human infection, the datasets of soil metagenomics from the Brazilian biome Cerrado were analyzed with focus on the pathogenic species. In this analysis, it was identified 41 species. Among them we identified the environmental occurrence of clinical species from the Herpothrichiellaceae family of the genus *Exophiala* and *Rhinocladiella*, besides the other human infections casual agents species of other genera. In addition, the study using the metagenomic data avaiable from the differents Brazilian regions was provided using the barcodes and padlock probes for Herpothrichiellaceous species-specific. The analysis revealed the presence of these agents in several environmental sources, such as, hydrocarbons contaminated soils, decomposing substrates, soils, rhizosphere and aquatic environments. The *in silico* identification showed the environmental ocuurence of pathogenic species, as the *Cladophialophora bantiana* encountered in DNA samples of sugarcane, which was confirmed by isolation using selective methods. Moreover, in this environmental isolation, it was isolated and identificated new species, described as *C. rhizus, C. cannae, C. molasses* and *E. plantae.*

Keys-words: Herpotrichiellaceae. Genomics. Metagenomics.

FIGURE LIST

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER V

TABLES LIST

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER V

APPENDIX

LIST OF ACRONYMS

ACT1 – actin 1
BT2 – β-tubulin 2
BLASTn – Basic Local Alignment Search Tool Nucleotide
CBM – Chromoblastomycosis
CMRP – Microbiological Collections of Paraná Network
DNA – Deoxyribonucleic acid
EMG – EBI metagenomics
ENA – European Nucleotide Archive
LCD – Lethargic Crab Disease
ITS – Internal transcribed spacer
MEA - Malt Extract Agar
MG-RAST – Rast Server
NCBI – National Center for Biotechnology Information Search database
NGS – Next-Generation Sequencing
RAFTS3 – Rapid Alignment Free Tool for Sequence Similarity Search
tRNA – Transfer ribonucleic acid
SAB - Sabouraud Agar
SRA – Sequence Read Archive
T– Type strain
TEF1 – Translation Elongation Factor 1-alpha

SUMMARY

CHAPTER I: Outline of the thesis

1 INTRODUCTION

The black yeasts fungi have as main characteristic dark pigmentation in vegetative and reprodutive cells walls. This fungal group have clinical and ecological extremely importance (DIXON; POLAK-WISS, 1991; STERFLINGER, 2006). They are oligotrophics and can be present in a wide variety of niches such as soil, tree bark, thorns, fruits, Palmaceae family plants and decomposing organic subtrates (VICENTE, 2000; NASCIMENTO et al., 2017), hydrocarbons contaminated soils (SATOW et al., 2008), among others, including some species associated to host animal infections (DE HOOG et al., 2008).

The majority of pathogenic species are located at Chaetothyriales order in the Herpotrichiellaceae family (REVANKAR; SUTTON, 2010), that harbor the causal agents of human and animal diseases, such as Chromoblastomycosis, Mycetoma and the Phaeohyphomycosis (MCGINNIS, 1992; REVANKAR; SUTTON, 2010). Among the genus the principal agents belong to the *Exophiala, Cladophialophora, Capronia, Fonsecaea, Rinocladiella* and *Veronea* genus (DE HOOG et al., 2000; TEIXEIRA et al., 2017).

Although these agents have a wide distribution their isolation form the environmental samples have been limited and normally done by the specific methods of isolation that explore their oligotrophic nature, which demonstrate their occurrence in niches where common saprobios do not survive (VICENTE et al., 2013).

In this context, culture-independent methods such as metagenomics (HANDELSMAN et al., 1998) has been used as indispensable tool for microbial ecology researchs aims to understanding the metabolism, diversity and ecologic role of an ecosystem specific (TRINGE et al., 2005; SOUZA et al., 2016). Since the first publications referring to the sequencing technique (SANGER; COULSON, 1975) until to the new-founds it was possible a wide range of discoveries. However, new challenges appeared regarding the data organization and analysis generated (CARVALHO; SILVA, 2010). Currently, the new generation sequencing (NGS) techniques have being provided faster sequencings, lowest cost and on a large-scale (KIM et al., 2013).

Therefore, this study aims to infer insights on pathogenicity and eco-epidemiology of black yeasts from genomic and metagenomic data in order to elucidate questions about their environmental occurrence and preference host colonization. This research was divided into six chapters.

Chapter I is an overview of ecology and biodiversity of black yeasts, associated diseases (chromoblastomycosis and phaeohyphomycosis) and NGS applications (genomic and metagenomic).

Chapter II is an article it is presented the genome sequencing data, assembly and annotation of the *Fonsecaea nubica* (CBS 269.64), starting the pathogenicity studies *in silico* of this specie.

In Chapter III, the public metagenomic data generated from Cerrado biome soil were analyzed regarding to presence of pathogenic fungi sequences.

In Chapter IV, it was made an *in silico* identification of black yeasts of many environmental sources applying specific molecular markers in public metagenomes data from Brazil.

In Chapter V, the specific isolation of black yeasts from the sugarcane was carried out. We identified the presence of *Cladophialophora bantiana* and probably four new species belonging to Herpotrichiellaceae family.

Lastly, the Chapter VI contains the conclusions and perspectives.

2 OBJECTIVES

2.1 GENERAL OBJECTIVE

- To infer insights about eco-epidemiology and pathogenicity of Herpotrichiellaceus based on genomic and metagenomic data.

2.2 SPECIFICS OBJECTIVES

- To sequence the genome, assembly and annotation of the fungus of *Fonsecaea nubica* CBS 269.64 by sequencers of NGS and bioinformatics tools.
- To identify pathogenic fungi from metagenomic data of the Cerrado biome by bioinformatics analysis.
- To identify in public data of metagenomics, black yeasts using molecular makers.
- To proceed the environmental isolation of chromoblastomycosis and phaeohyphomycosis agents from sugarcane plants.

3 LITERATURE REVIEW

3.1 BIODIVERSITY AND ECOLOGY OF BLACK YEASTS

The family Herpotrichiellaceae (in Chaetothyriales order of the phylum Ascomycota) includes true pathogens, as well as opportunistic fungal species from human and animal hosts, causing many severe diseases such as chromoblastomycosis (CBM), mycetoma and phaeohyphomycosis (MCGINNIS, 1992; REVANKAR; SUTTON, 2010). Black yeasts are melanized fungi with large melanin deposition in their cell walls, that are macro and microscopically visible by brown, olivaceous, or black tinge (SEYEDMOUSAVI et al., 2014; TEIXEIRA et al., 2017). In addition, other saprobic species commonly isolated from the environmental samples are also included in this family (UNTEREINER; NAVEAU, 1998; VICENTE, 2000; VICENTE et al., 2008).

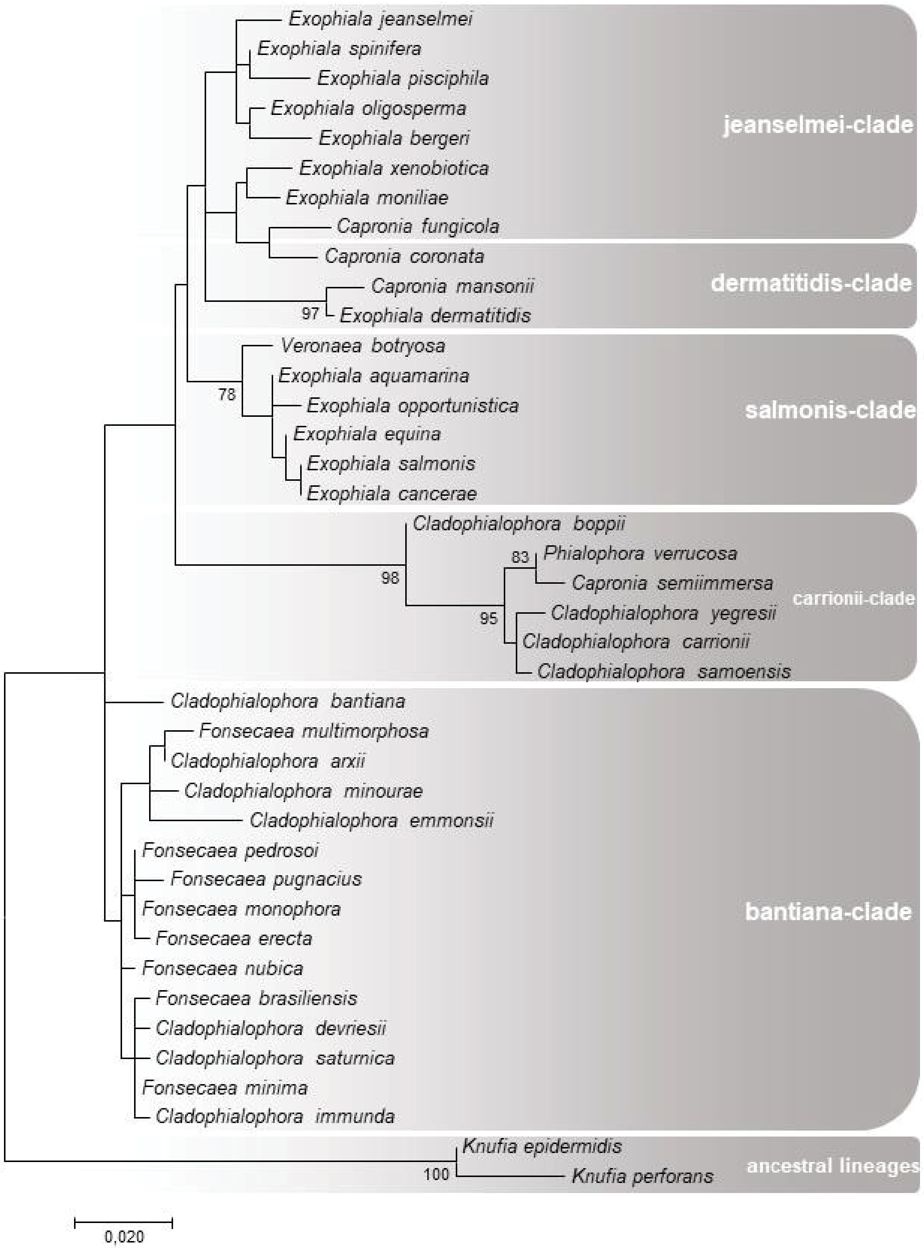
Fungal species of the Chaetothyriales order and their relative species are ecologically as well as clinically very important (DIXON; POLAK-WISS, 1991; STERFLINGER, 2006). Chaetothyriales fungi are generally isolated from a wide variety of niches such as soil, tree bark, thorns, fruits, plants, and decomposing organic matter (VICENTE, 2000; SALGADO et al., 2004; VICENTE et al., 2008; NASCIMENTO et al., 2017); however, due to their oligotrophic metabolism, the black yeasts may also be found in adverse and extreme conditions, such as on rock surfaces in hot and arid climates (DE HOOG, 2014), and in soils contaminated with hydrocarbons (SATOW et al., 2008). In addition, owing to the extensive exploration related to black yeast diversity in the last decades, the number of species within the genera has been increasing continuously (DE HOOG, 2014).

Herpotrichiellaceae family contains species of clinical and environmental importance, which are phylogenetically distributed into five clades (TEIXEIRA et al., 2017). In Herpotrichiellaceae (Figure 1), the bantiana-clade and carrionii-clade include species known as pathogenic agents of chromoblastomycosis (DE HOOG et al., 2007; AZEVEDO et al., 2015) and phaeohyphomycosis (HORRÉ; DE HOOG, 1999), respectively, and the clades jeanselmei, salmonis and *E. dermatitidis* have pathogenic species which occasionally affect animals (DE HOOG et al., 2011). The main asexual genera are represented by *Cladophialophora*, *Exophiala*, *Fonsecaea*, *Phialophora,* and *Rhinocladiella* (DE HOOG et al., 2000; TEIXEIRA et al., 2017).

Cladophialophora is a polyphyletic (BADALI et al., 2009a) genus with 38 species, which have already been described (KIYUNA et al., 2018). The species of this genus are frequently isolated from decomposing organic matter, plants and rocks (BADALI et al., 2008; VICENTE et al., 2012; NASCIMENTO et al., 2017; KIYUNA et al., 2018). Some species may also be found in association with living plants, such *C. yegresii* being endophytic with cactus (DE HOOG et al., 2007) and *C. exuberans* associated with babassu coconut (NASCIMENTO et al., 2017). Furthermore, some species were reported to be of clinical origin (DE HOOG et al., 2000), being *C. bantiana* (Figure 2E and F) the most virulent species described in genus, capable of causing fatal cerebral infections in immunocompromised and immunocompetent patients (HORRÉ; DE HOOG, 1999; KANTARCIOGLU et al., 2017). In addition, *C. carrionii* (Figure 2I and J) has been described as the main causative agent of the Chromoblastomycosis (DE HOOG et al., 2007; BADALI et al., 2008). There are some rare cases of disease caused and disseminated by *C. devriesii* and *C. arxii* (DE HOOG et al., 2000) and *C. boppii*, *C. emmonsii* and *C. saturnica* causing cutaneous infections (DE HOOG et al., 2007; BADALI et al., 2009a). Besides, biotechnological studies introduced two more species of *Cladophialophora* as *C. immunda* and *C. psammophila* (PRENAFETA-BOLDÚ et al., 2001; BADALI et al., 2011).

The genus *Exophiala* comprising of more than 40 species described (BORMAN et al., 2017), and is the genus having the largest number of species among the Herpotrichiellaceae family members. They can be isolated from domestic and or aquatic habitats, environmentally polluted materials with hydrocarbons and also from the clinical patients (LIAN; DE HOOG, 2010; DE HOOG et al., 2011; DÖĞEN et al., 2013; NASCIMENTO et al., 2017; BORMAN et al., 2017; PINHEIRO et al., 2019). The genus includes several pathogens of cold-blooded (DE HOOG et al., 2011) as well as warm-blooded animals (LI et al., 2011). There are several clinical species reported as causal organism of human infection (Figure 2: K,L,O and P), which are *E. jeanselmei*, *E. asiatica*, *E. spinifera* and *E. dermatitidis* (SEYEDMOUSAVI et al., 2013; PINHEIRO et al., 2019; LI et al., 2009); and species related in systemic infections are *E. lecanii-corni, E. oligosperma, E. phaeomuriformis*, and *E. xenobiotica* (ZENG et al., 2007). Furthermore, it is a polyphyletic genus distributed in different clades (TEIXEIRA et al., 2017).

Figure 1 - Phylogenetic tree of Chaetothyriales order

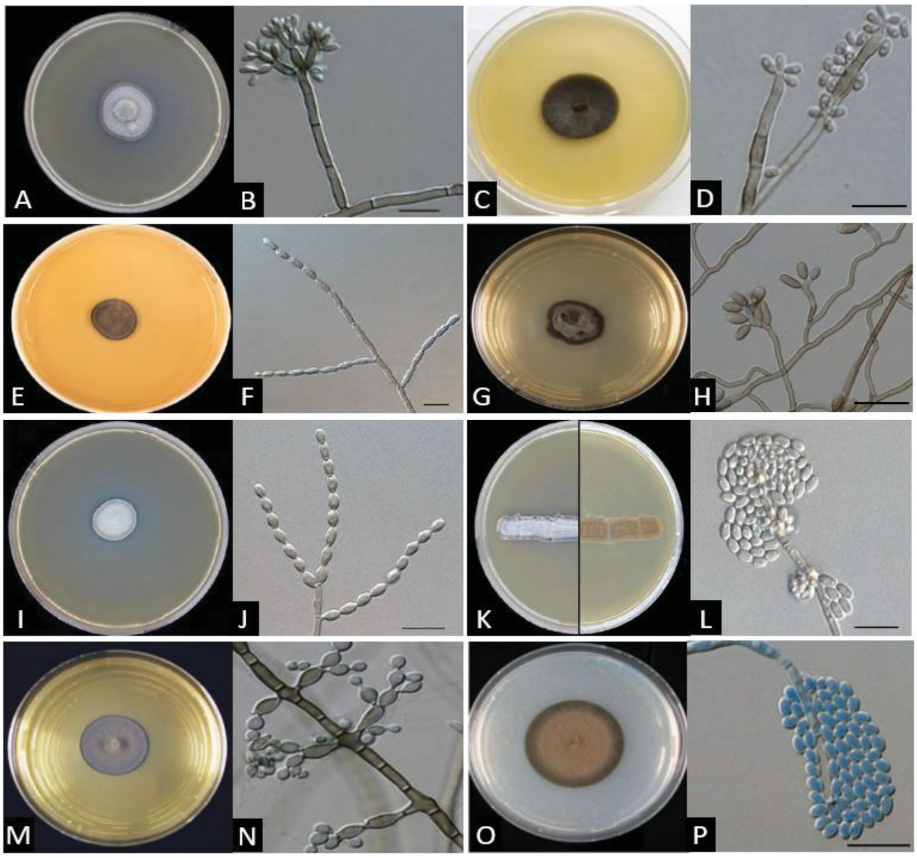


Tree based on LSU sequences constructed with Maximum likelihood implemented in Mega7 with Kimura"s two parameters model. The bootstrap support was calculated using 1000 replicates and values ≥ 75% are shown in the branches.

The genus *Fonsecaea,* monophyletically grouped in the bantiana-clade, was established in 1930 and was reported from the chromoblastomycosis agent *Hormodendrum pedrosoi* (BRUMPT, 1922). Currently, the genus *Fonsecaea* is composed of eight species from pathogenic and environmental sources (AZEVEDO et al., 2015). The clinical species reported as causative agents of chromoblastomycosis are *F. pedrosoi* (Figure 2A and B) (QUEIROZ-TELLES et al., 2009; 2017), *F. monophora* (XI et al., 2009), *F. nubica* (NAJAFZADEH et al., 2010a; CHEN et al., 2016), and *F. pugnacius* (AZEVEDO et al., 2015). Moreover, the genus encompasses two species related to animal infections, which are *F. multimorphosa* isolated from a feline cerebral abscess (NAJAFZADEH et al., 2011) and *F. brasiliensis* (Figure 2M and N) associated with the infections of cold-blooded animals (VICENTE et al., 2012). The two ecological species are *F. erecta* and *F. minima* isolated by specific methods from the living plants (VICENTE et al. 2013).

Likewise, the *Rhinocladiella* genus includes clinical and environmental species such as *R. mackenziei* (BADALI et al., 2009b), obtained from severe infections, *R. aquaspersa* (GONZÁLEZ et al., 2013), *R. tropicalis* and *R. similis* causing chromoblastomycosis (GOMES et al., 2016; HEIDRICH et al., 2017). In the Figure 2 was presented the macro- and micromorphology of mainagents associated to animl and human infection.

Figure 2 – Main Heportrichielaceous species associated to animal and human infections



A, C, E, G, I, K, M and O were presented fungal cultures in the Malt Extract Agar (MEA) and in the B, D F, H, J, L, N and P the conidiophores of the species, as following: A and B *Fonsecaea pedrosoi*; C and D *Rhinocladiella tropicalis*; E and F *Cladophialophora bantiana*; G and H *Rhinocladiella mackenziei*; I and J *Cladophialophora carrionni*; K and L *Exophiala dermatitidis*; M and N *Fonsecaea brasiliensis*; O and P *Exophiala cancerae*. Adapted from: DE HOOG et al. 2014; VICENTE et al., 2011; DE HOOG et al., 2011; GOMES et al., 2016; QUEIROZ-TELLES et al., 2017.

3.2 CHROMOBLASTOMYCOSIS AND PHAEOHYPHOMYCOSIS

Chromoblastomycosis (CBM) occurs worldwide; however, most of the clinical cases and isolates have been reported from tropical and subtropical regions such as Brazil (SILVA et al., 1992, QUEIROZ-TELLES,1997; VICENTE, 2000; SALGADO et al., 2004; GOMES et al., 2016), China (NAJAFZADEH et al., 2010b; CHEN et al., 2016; XI et al., 2009), India (AGARWAL et al., 2017), and other countries. It has also been reported as a rare instance in non-tropical regions (OUÉDRAOGO et al., 2017).

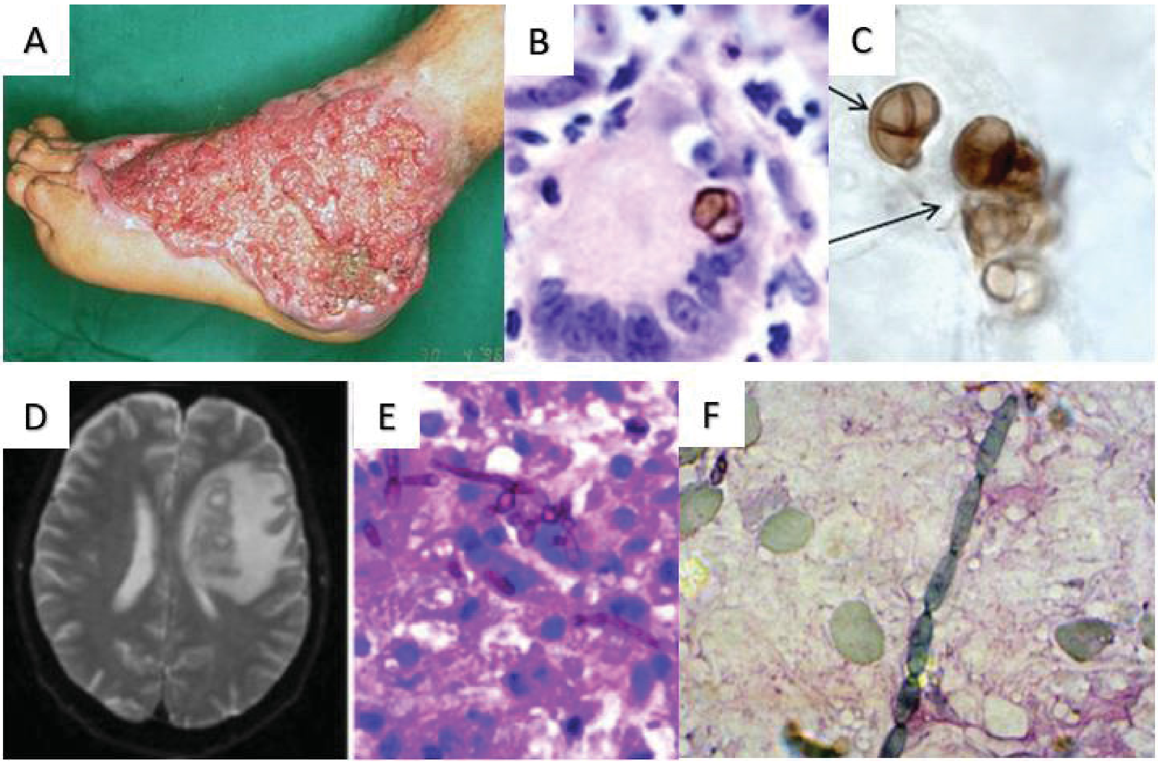
CBM is characterized by the appearance of cutaneous and subcutaneous lesions that may emerge as tumor-like ulcerative eruptions (DE HOOG et al., 2004; NAJAFZADEH et al., 2009, 2010b, 2011; VICENTE et al., 2012) (Figure 2). Melanin is a virulence factor and plays a crucial role in environmental protection mostly against solar radiation (CHOWDHARY et al., 2014). CBM is diagnosed through the presence of muriform cells, a septate brownish structure in the tissues (VICENTE, 2000; BONIFAZ et al., 2001). The muriform cells can invasively be formed within the host tissue (DA SILVA et al., 2002; DA SILVA et al., 2008) or wet preparations of pus or scrapings (CHOWDHARY et al., 2014). Previous studies on melanization reported that *Fonsecaea* pathogens can produce cell-wall associated melanin-like components (CUNHA et al. 2005; FRANZEN et al., 2006; SANTOS et al., 2007). Likewise, there are hypotheses suggesting that melanin is either used immunological response as an activator or may possibly interact with host immune cells (FARBIARZ et al., 1992).

C. carrionii, *F. monophora*, *F. pedrosoi*, *R. aquaspersa* and, occasionally, *P. verrucosa* are the fungal species that are associated with CBM (CHOWDHARY et al., 2014). *Fonsecaea* species are endemic in tropical regions with typical climatic conditions, and four species of this genus are known as CBM agents (QUEIROZ-TELLES et al., 2017), which are *F. pedrosoi* (QUEIROZ-TELLES et al., 2009), *F. nubica* (NAJAFZADEH et al., 2010a; CHEN et al., 2016), *F. monophora* and *F. pugnacius* (AZEVEDO et al., 2015). *F. pedrosoi* is identified as the main etiological agent of cutaneous and subcutaneous lesions (NAJAFZADEH, et al., 2009). In clinical studies, *F. monophora* has been isolated commonly from brain lesions (BONIFAZ et al., 2001), lymphatic system (NAJAFZADEH et al., 2010b), bile (SURASH et al., 2005), and central nervous system in case of phaeohyphomycosis (DOYMAZ et al., 2015). Recently, chromoblastomycosis lesions were found in lung tissues caused by

F. monophora (CLEINMAN et al., 2017) and in-ear injury by *F. nubica* (CHEN et al., 2016).

Phaeohyphomycosis is a disease that is distributed worldwide; however, it occurs with higher prevalence in tropical climatic regions such as Madagascar and northern Venezuela (REVANKAR et al., 2017). In Brazil, it has been described in the Amazon in the northeast (GOMES et al., 2016) and also in other regions (MINOTTO et al., 2014; OLIVEIRA et al., 2016). This is a sporadic infection which apparently affects immunocompetent as well as immunosuppressed individuals (REVANKAR et al., 2017). The phaeohyphomycosis agents are not restricted to a single genus of the Chaetothyriales order (FERNANDEZ-PITTOL et al., 2019), but some other species of this order are also considered as causal agents such as *C. bantiana* (HOWLETT et al., 2019) and *E. dermatitidis* (KIRCHHOFF et al., 2019) (Figure 3).

Figure 3 - Clinical aspect of chromoblastomycosis and phaeohyphomycosis

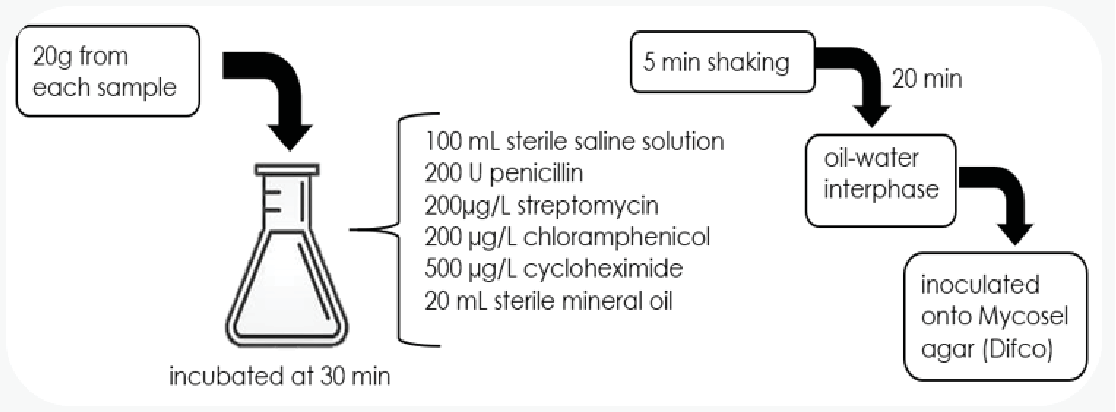


Clinical aspect of chromoblastomycosis (A to C) and phaeohyphomycosis (D to F). A: vegetant and papillomatous lesions; B: Histopathology of cutaneous tissue with cells brownish; C: muriform cells; D: lesions cerebral; E and F: histology section of brain biopsy consistent with cerebral phaeohyphomycosis. Adapted from: KANTARCIOGLU et al., 2017; QUEIROZ-TELLES et al., 2017; HOWLETT et al., 2019.

3.3 ISOLATION METHODS AND MOLECULAR MAKERS

The black yeasts have ability to metabolize aromatics compounds (PRENAFETA-BOLDÚ et al., 2006). Besides, the isolation of the species of Herpotrichiellaceae family is very difficult, and their recovery through the conventional protocols is also very poor; therefore, it becomes important to develop specific methods, like as of flotation in mineral oil (SATOW et al., 2008; VICENTE, 2000; VICENTE et al., 2008). This method (Figure 4), is based the hydrophobicity of the melanized fungal cells, which have affinity with the mineral oil inserted in the solution (IWATSU et al., 1981; VICENTE et al., 2008).

Figure 4 - Mineral oil flotation isolation process



Other method, to recoverd these species and to understand various aspects of pathogenicity, is the use the animais like vectors (DIXON et al., 1980; VICENTE et al., 2017; OLSOWSK et al., 2018; FORNARI et al., 2018; LIU et al., 2019).

After the recovering these black yeasts, your identification is based on micro and macro morphological characteristics, as well as, also on phylogenetic analysis, which are based on molecular biology methods aiming at the efficient identification of fungi (QUEIROZ-TELLES et al., 2017). Thus, molecular biology tools help identify (DE HOOG et al., 2006) species and generally, the Internal Transcribed Spacer (ITS) region is considered the barcode for fungal identification (SCHOCH et al., 2012). However, for the species of the Herpotrichiellaceae family, other genes are needed to identify correctly, such as translation elongation factor (TEF1) and β-tubulin (BT2) and cell division cycle 42 (Cdc42) (QUEIROZ-TELLES et al., 2017; TEIXEIRA et al., 2017).

Other molecular tools can also be used for fungal identification, such as padlock probes and barcodes. The padlock probes, which are specific oligonucleotide with the ability to identify single nucleotide polymorphisms (SNPs), have been proposed for the recognition of several groups of black agents (NAJAFZADEH et al., 2011, 2013, 2018; HAMZEHEI et al., 2013; DENG, et al., 2014; SCHNEIDER et al., 2019). DNA barcoding, based on the ITS region and applying short sequences (25–41 bp) of nucleotides specific for a single taxonomic species (HEINRICHS et al., 2012), can additionally be used to recognize herpotrichiellaceous species by variable regions in the ribosomal operon.

3.4 NEXT GENERATION SEQUENCING AND ITS APPLICATIONS

The process of the sequencing, since your first report (SANGER; COULSON, 1975), even its latest enhancements have been an important tool for determining a sequence of nucleotides contained in a DNA molecule.

In 2004, it started the next generation sequencing (NGS) with techniques are faster, cost-effective, and capable sequence the samples on a large-scale in one shot (KIM et al., 2013). Since then, sequencers have been marketed in the biological field and the first to be launched on the market was 454 Life Sciences technology, based on pyrosequencing (RONAGHI et al., 1998: RONAGHI, 2001; MARGULIES et al., 2005). Nowdays, there are other sequencers available as: Solexa/Illumina sequencer, SOLiD system from Applied Biosystems, Ion Torrent PGM and PacBio technologies; which vary in methodology, sequencing capacity, cost, processing time, etc (MUNROE, HARRIS, 2010; SCHADT et al., 2010).

Widely used for sequencing, the Illumina platform is based on DNA molecule synthesis methodology through cyclic reversible termination using the enzyme DNA polymerase and fluorophore-labeled terminator nucleotides (CARVALHO; SILVA, 2010). Occurring nucleotide attachment, cleavage and fluorescence imaging by high resolution camera (LIU et al., 2012). However, the Ion Proton platform methodology resembles pyrosequencing, but is based on semiconductors that transform chemical information contained in DNA directly into computational information (ROTHBERG et al., 2011).

With the recent advancements in functional genomic research, the NGS technologies have become an essential and powerful tool for understanding various molecular aspects of a biological process. The Fungal Genomics Program (http://jgi.doe.gov/fungi) expedites the key role of this tool which brings together fungal genomes and allows for a number of online bioinformatic analyses (GRIGORIEV et al., 2013). Currently, the project covers more than 800 genomes and other analyses of transcriptome and metagenome, including fungal microbiota that began in 2011 with *E. dermatitidis* genome (CHEN et al., 2014) and as of today, it encompasses about 37 black yeasts genomes and transcripts (MORENO; VICENTE; DE HOOG, 2018).

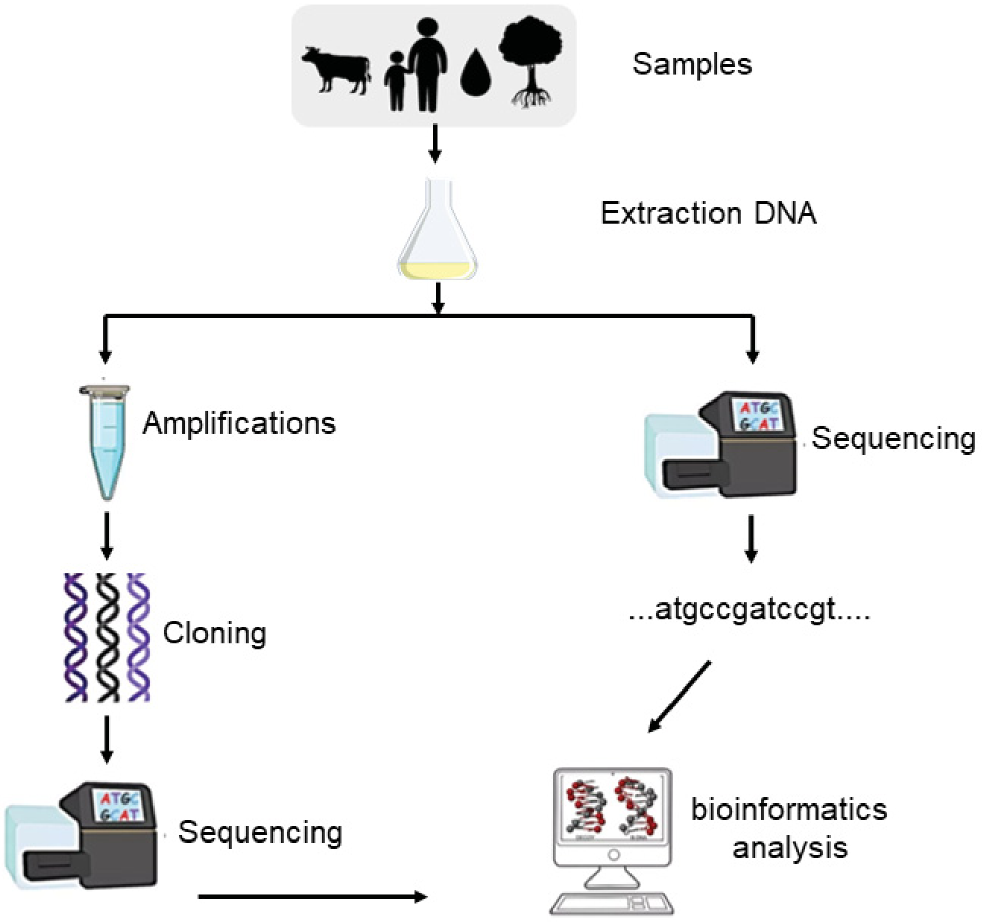
The investigation involved the Herpotrichiellaceae genome analysis and comparative account of different species of black yeasts to ascertain phylogenomic relationships, transposable elements, sex-related genes, protein family evolution, genes related to protein degradation (MEROPS), carbohydrate-active enzymes (CAZymes), melanin synthesis, and secondary metabolism. In addition (TEIXEIRA et al., 2017; VICENTE et al., 2017), the studies also involved the specific gene (laccase) annotation (MORENO et al., 2017) and transcriptome analysis of these black yeasts (LI et al., 2016; POYNTNER et al., 2016; BLASI et al., 2017).

3.5 METAGENOMICS

In the 1970s, DNA identification processes in soil samples were initiated, which made it possible to isolate pure DNA molecules (TORSVIK; GOKSØYR, 1978). In the following decade, from environmental samples, the purification, amplification and cloning processes were used in bacterial communities (PACE et al., 1986).

Currently, direct sequencing of genomic material from any environmental sample is called metagenomics (HANDELSMAN et al., 1998; WOOLEY; GODZIK; FRIEDBERG, 2010). Moreover, after the advent of NGS, the sequencing technologies have evolved unceasingly and rapidly, and as a consequence, the huge data generated necessitates the requisite for databases for storage and ease of access (KIM et al., 2013). In this context, direct sequencing of genomic material from any ecological samples designated as metagenomics has immense potential (HANDELSMAN et al., 1998; WOOLEY; GODZIK; FRIEDBERG, 2010). The samples that generate the metagenomic data are very diverse, and can be environmental metagenomes (water, air or soil), host-associated metagenomes (plants, invertebrates or non-human mammals) and humans (skin, gut, oral and other mucosal organs) (CUADROS-ORELLANA et al., 2013) (Figure 5).

Figure 5 - Summary diagram of the stages of metagenomics



Adapted from: CHIU; MILLER, 2019.

The understanding of metagenomic data has been used as an indispensable tool in microbial ecology research aimed at determining the metabolism, diversity and ecological role in a specific ecosystem (CUADROS-ORELLANA et al., 2013).

3.5.1 Databases

However, organization and analysis of the data generated have emerged as a new challenge (CARVALHO; SILVA, 2010). And the most well-known metagenomic databases are Rast Server (MG-RAST) and EBI metagenomics (EMG); both the databases store (upload) on their servers and make data publically available and offer free services. MG-RAST is an open source web server and has a unique set of tools to analyze data (MEYER et al., 2008), such as the non-redundant M5nr database that has protein sequences and notes from other consolidated consortia (WILKE et al., 2012). EMG is a platform that analyzes data on a large scale and is independent of its data source (metagenomics, metatranscriptome, amplicon or assembly). The data are processed in a standardized flow, capable of producing a rich taxonomic diversity and functional annotations (MITCHELL et al., 2016). EMG in partnership with the European Nucleotide Archive (ENA) (SILVESTER et al., 2015); and Sequence Read Archive (SRA) (ALNASIR; SHANAHAN, 2015), which index and make the metagenomic data as well as raw sequencing data available.

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CHAPTER II: Draft Genome Sequence of *Fonsecaea nubica* strain CBS 269.64, Causative Agent of Human Chromoblastomycosis

Draft Genome Sequence of *Fonsecaea nubica* Strain CBS 269.64, Causative Agent of Human Chromoblastomycosis

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1 ABSTRACT

Fonsecaea species are the etiologic agents chromoblastomycosis disease, being *F. pedrosoi*, *F. monophora*, *F. nubica* and *F. pugnacius* the four species of genus that can cause this implantation disease. Based on multilocus phylogenetic data, *Fonsecaea nubica* was described in 2010 as a molecular sibling of *F. monophora*, an established agent of the human skin disease chomoblastomycosis in tropical zones. The total DNA of *F. nubica* was sequenced in the plataforms Ion and Illumina. After the analysis of the quality, was perfomed genome assembly. The draft comprised genome size 33.7 Mb, distributed in 258 contigs with the annotation of 11,681 predicted genes, and genome is available in PRJNA312449. Genome analysis of these pathogens is mandatory to identify genes involved in the interaction with host and virulence.

2 INTRODUCTION

The genus *Fonsecaea* comprises anamorph members in the Chaetothyriales, an ascomycete order of black yeasts and filamentous relatives covering numerous opportunistic pathogens on humans (XI et al., 2009; DE HOOG et al., 2000a; BADALI et al., 2008). *Fonsecaea* is one of the prevalent genera of etiologic agents of chromoblastomycosis (MARTINEZ; TOVAR, 2007; TANABE et al., 2004), a chronic, cutaneous, and subcutaneous infection characterized by slowly expanding, polymorphic skin lesions with muriform cells in tissue, provoking a granulomatous immune response (DE HOOG et al., 2004; BONIFAZ et al., 2001). The disease occurs preferentially in humans, although some cases have been reported in other mammals (GAMS; MCGINNIS, 1983; RIPPON, 1988; DE HOOG et al., 2000b). Several *Fonsecaea* species are involved as etiologic agents of the disease, i.e., *F. pedrosoi*, *F. monophora*, *F. nubica*, and *F. pugnacius*, each with different virulence potentials (DE HOOG et al., 2004; NAJAFZADEH *et al*., 2011). *F. nubica* was first described in 2010 with type strain CBS 269.64, isolated from a human patient with chromoblastomycosis in west Cameroon (DE HOOG et al., 2004; NAJAFZADEH et al., 2010). Genome analysis of the pathogenic fungus *F. nubica* is needed to identify genes involved in the interaction with host cells and molecular mechanisms in response to cytotoxic agents (NAJAFZADEH et al., 2009).

3 MATERIALS AND METHODS

3.1 STRAIN USED

Strain *F. nubica* CBS 269.64 was first described in 2010 with type strain CBS 269.64, isolated from a human patient with chromoblastomycosis in west Cameroon (DE HOOG et al., 2004; NAJAFZADEH et al., 2010).

3.2 GENOMIC DNA EXTRACTION, SEQUENCING AND ASSEMBLY

Strain *F. nubica* CBS 269.64 was grown in Sabouraud’s broth, with shaking at 150 rpm at 28°C for 7 days and DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method with phenol-chloroform/isoamyl alcohol. Total DNA was purified with the microbial DNA UltraClean kit. DNA of *F. nubica* was used for library construction using the Ion Plus Fragment library kit (Thermo, FisherScientific) and Nextera XT (Illumina) following the manufacturer’s instructions. The libraries were sequenced on an Ion Proton (Thermo, FisherScientific) for singleend reads and in Miseq (Illumina) for paired-end reads. The quality of the reads was assessed by means of FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The reads were assembled *de novo* using SPADES v3.6.2 (BANKEVICH et al., 2012). For the gap closure performed using FGAP (PIRO et al., 2014). To measure the assembly coverage Bowtie2 was used (LANGMEAD, SALZBERG, 2012).

3.3 GENE PREDICTION AND ANNONTATION

Protein-coding genes were predicted with GeneMark-ES (TER-HOVHANNISYAN *et al*., 2008). For annotation genes were assigned based on similarity searches against the *nr* database using RAFTS3 (VIALLE *et al*., 2016) and InterProScan (JONES *et al*., 2014) comparisons. The tRNAs identified using ARAGORN (LASLETT; CANBACK, 2004).

3.4 MITOCHONDRIAL DNA

The mitochondrial genome was assembled by extracting reads using the complete mtDNA of Exophiala dermatitidis as reference. The reads were mapped using Bowtie2 (LANGMEAD, SALZBERG, 2012) and the mapped reads assembled with SPAdes v3.6.2 (BANKEVICH et al., 2012).

4 RESULTS

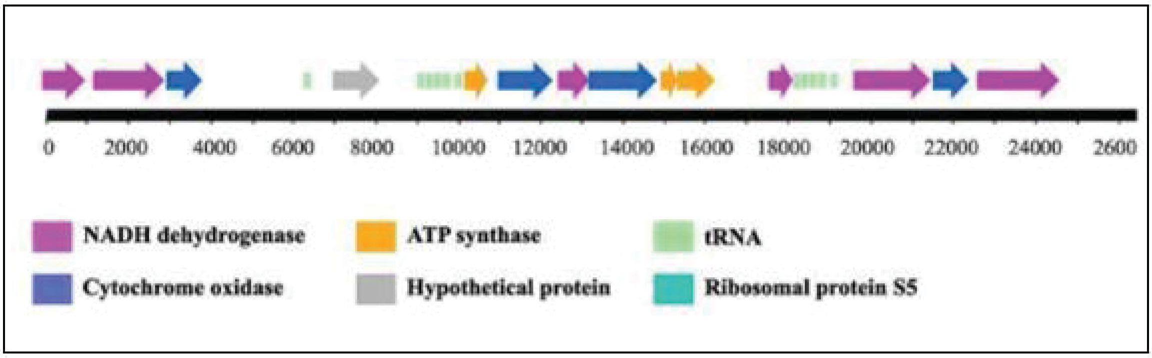
The final high-quality draft genome of *Fonsecaea* nubica comprised 258 contigs and the genome size was 33.7 Mb, with average coverage of 85X and G+C content of 52.46%. Annotation for 11,681 predicted and 36 tRNA genes were predicted (Table 1). Comparing *F. nubica* with species of *Fonsecaea* it was observed that species are similar in size, in conserved hypothetical proteins and GC content (VICENTE et al., 2017).

Table 1 - *Fonsecaea nubica* genome data assembly

Information	**Value**
Genome size	33.79 Mb
DNA G+C contente	52.46%
DNA scafollds	258 contigs
Coverage	85X
Proteins	11,681
tRNAs	36

The mtDNA of *F. nubica* resulted in a single contig with 24.5 Kb. The gene composition of the mitochondrial genome contains 16 protein coding genes involved in the respiratory chain and ATP synthesis (Figure 6).

Figure 6 - Mitochondrial genome of *Fonsecaea nubica*



Adapted from: VICENTE et al., 2017.

4.1 ANNOTATIONS

After genome assembly, it was possible to annotate the genes in several databases. The Gene Ontology (GO) annotation made it possible to statement about the functions of a genes. As shown in Table 2, among the genes identified, we highlight those that decode biological process, cellular component, molecular function and othres functions obsolete. With the annotation of KEGG Metabolic Pathways, it was possible to observe the number of genes involved in certain Metabolic Pathways. With KOG was deduced the gene families deduced by annotations. And in carbohydrate-active enzymes (CAZymes) database and in Peptidase-encoding genes (Merops), observing an enzymatic profile.

Table 2 - Annotation the genes in several databases.

Database	**Term**	Genes
Gene ontology	Biological process	4143
	Cellular component	2218
	Molecular function	6117
	Obsolete biological process	515
	Obsolete cellular component	7
	Obsolete molecular function	80
KEGG Metabolic Pathways	Amino Acid Metabolism	635
	Biosynthesis of Polyketides and Nonribosomal Peptides	42
	Biosynthesis of Secondary Metabolites	467
	Carbohydrate Metabolism	605
	Energy Metabolism	133
	Glycan Biosynthesis and Metabolism	121

	Lipid Metabolism	598
	Metabolism of Cofactors and Vitamins	423
	Metabolism of Other Amino Acids	201
	Nucleotide Metabolism	143
	Overview	500
	Xenobiotics Biodegradation and Metabolism	705
KOG	Cellular Processes and Signaling	1512
	Information Storage and Processing	1104
	Metabolism	2813
	Poorly Characterized	1790
CAZY	AA (Auxiliary Activities family)	44
	CBM (Carbohydrate-Binding Module family)	12
	CE (Carbohydrate Esterase family)	9
	EXPN (Distantly related to plant expansins)	2
	GH (Glycoside Hydrolase family)	139
	GT (Glycosyl Transferase family)	93
	MYOSIN MOTOR (Glycosyltransferase Family 2)	2
	PL (Polysaccharide Lyase family)	0
MEROPS	A (Aspartic)	22
	C (Cysteine)	30
	G (Glutamic)	1
	I (Trypsin)	4
	M (Metallo)	52
	P (Mixed)	6
	S (Serine)	177

Adapted from: VICENTE et al., 2017.

4.2 ACCESS

This whole-genome shotgun project has been deposited in DDBJ/ENA/GenBank under accession number LVCJ00000000 and available in PRJNA312449.

5 DISCUSSION

Morphologically *F. nubica* it is very similar to *F. pedrosoi* e *F. monophora* and has been reported as an etiological agent of infection in several countries such as China (SLESAK et al., 2011), França (CATEAU et al., 2014) and southern African countries (NAJAFZADEH et al., 2011a; SUN et al., 2012). And it has recently been reported in cases of chromoblastomycosis in the Amazon region of Brazil (ANDRADE et al., 2019). This black yeast is also responsible for first auricular case of chromoblastomycosis (CHEN et at., 2016).

Compareted the genome of F. nubica with the other genomes of fonsecaea species are quite similar (VICENTE et al., 2017). The annotation of *F. nubica* genome allowed the understanding of genes involved in carbohydrate metabolism, respiration and virulence and pathogenicity mechanisms. Genome analysis of these pathogens is mandatory to identify genes involved in the interaction with host and virulence.

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CHAPTER III: New insights on environmental occurrence of pathogenic fungi based on metagenomic data of Brazilian Cerrado biome

New insights on environmental occurrence of pathogenic fungi based on metagenomic data of Brazilian Cerrado biome

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Running title: Metagenomic of the cropped soils from Brazil

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1 ABSTRACT

The Cerrado is the second largest biome in Brazil and contributes to most of the country's grain production. Previous studies on soil metagenomics from the Cerrado have revealed an outstanding microbial diversity. In this study, the diversity of pathogenic fungi were analyzed from genomic sequences of the Cerrado soils under native vegetation, or with agriculture in the no-tillage and conventional tillage. Of the 128,627 sequences of fungi identified, 43,439 belonging to pathogenic fungi distributed as follows: native 17,301 (40%), no-tillage 13,780 (32%) and conventional tillage 12,358 (28%). We identified 41 pathogenic fungal species associated with human and animal infections, being agents of primary infections and opportunistic.

2 INTRODUCTION

The Cerrado biome is a savannah-like region, in the central part of Brazil covering about 2 million km^2 area (DE CASTRO et al., 2008). It is the second largest Brazilian biome (SILVA & BATES, 2002) and considered as one of the most biodiverse sites on the planet (MYERS et al., 2000). Nowadays, the greatest production of grains in the country occurs in this biome (JEPSON, 2005), which has consequently led to the changes in the native vegetation due to agricultural activities and deforestation (SANO et al., 2018). Studies reporting the rich biodiversity of the Cerrado encompass the fauna (COSTA, 2017), flora (SANO et al., 2018), and microorganisms (SOUZA et al., 2016; DE ARAUJO et al., 2017; NORILER et al., 2018).

Fungi are considered pathogens when they complete their life cycle in a host (DE HOOG et al., 2018) and are the agents of infections in humans, animals, and plants (GINTER-HANSELMAYER & NENOFF, 2018). Human pathogenic fungi are known as hidden killers and are responsible for approximately 1.5 million deaths per year (BROWN et al., 2012), causing superficial, (sub)cutaneous and systemic infections (GINTER-HANSELMAYER & NENOFF, 2018). Most etiologic agents are found in soil, vegetation, and decaying matter in humid environments and colonize a host by necessity or opportunity. Being present in nature, the fungi can reach their host in a variety of ways. While many routes of infection are still unknown, it is known that fungal spores dispersed in air can cause pulmonary or disseminated infections (SEPHTON-CLARK et al., 2018), propagules present in soil and plant debris may be traumatically inoculated into the cutaneous/subcutaneous tissues of the warm-blooded host (SANCHOTENE et al., 2015), and that the fungi may be transmitted to humans via plants or by animals (QUEIROZ-TELLES et al., 2019). In addition, fungi that have source of nutrients the keratin present in the skin, hair and nails, colonizing them (de Hoog et al., 2017).

Culture-independent methods such as the metagenomics have become a robust technique to understand and compare microbial diversity in the most diverse environments (HANDELSMAN et al., 1998; TRINGE et al., 2005). Therefore, it is a widely used tool to identify microorganisms that are hardly recovered from the environment. In this context, the main objective of this study was to identify pathogenic fungi from the metagenomic data of differently treated Cerrado soils, including non-disturbed soil covered with native vegetation, and soils with agriculture under the no-tillage and conventional tillage systems.

3 MATERIAL AND METHODS

3.1 SAMPLES

The DNA ribosomal sequences were accessed from the previous published study by Souza and collaborators (2016), were it was collected soil samples from the experimental station of Embrapa Cerrados in Planaltina, Federal District, Brazil (15°36′34” S and 47°44′36” W). The samples were classified as native soil (undisturbed Cerrado stricto sensu with original soil condition) and two cultivable soils. The cultivable soils were cropped for 23 years with soybean/maize under conservation tillage “no-till” (NT) and conventional tillage (CT) with breaks in the winter (dry season). The CT area was annually prepared by ploughing and disking the soil before sowing and for incorporation of weeds after harvest, whereas the NT area was managed without ploughing or disking (SOUZA et al., 2016).

3.2 DATASET ACQUISITION

The data analyzed in this study were obtained from the SRA NCBI database under the accession number PRJNA292758. The datasets were named as mgp10523, mgp10541, and mgp10450, and are also available online on Metagenomics RAST server-MG-RAST (GLASS, MEYER, 2011). We analyzed nine samples from Cerrado soil classified as per their source as native soils (NATIVE 1, NATIVE 2 and NATIVE 3) grown under no-tillage (NT 1, NT 2 and NT 3), and conventional tillage (CT 1, CT 2 and CT 3) soil preparation.

3.3 DATA MINING

The metagenome sequencing generated a total of 49,182,419 sequences derived from the total DNA of three biological replicates of each of the three treatments (SOUZA et al., 2016). The MG-RAST annotated data was filtered and only sequences with eukaryotic taxonomy were used in this study. A total of 406,972 eukaryotic sequences were downloaded of which, 128,627 sequences belong to fungi (Table 3). The data mining was performed using in-house scripts in Java programming language (http://www.java.com).

Table 3 - Summary of the data mining of metagenomic surveys

Sample Name	ID in MG-RAST server	Eukaryotic sequences	Fungi sequences
NATIVE1	mgm4577669.3	46,172	18,669
NATIVE2	mgm4578924.3	36,052	15,208
NATIVE3	mgm4578925.3	40,824	17,944
NT1	mgm4577671.3	50,849	15,321
NT2	mgm4578714.3	45,729	13,077
NT3	mgm4577672.3	50,210	12,023
CT1	mgm4577670.3	49,117	12,739
CT2	mgm4578926.3	51,228	13,803
CT3	mgm4578927.3	36,791	9,843
Total	-	406,972	128,627

Native vegetation of Cerrado (Native1, 2 and 3) or cropped with soybean/corn under no-tillage (NT1, 2 and 3) or conventional tillage (CT1, 2 and 3) systems.

The data of all graphs were normalized to balance the sizes of the different datasets using the ggplot package (WICKHAM, 2016) in R software (http://www.r-project.org/).

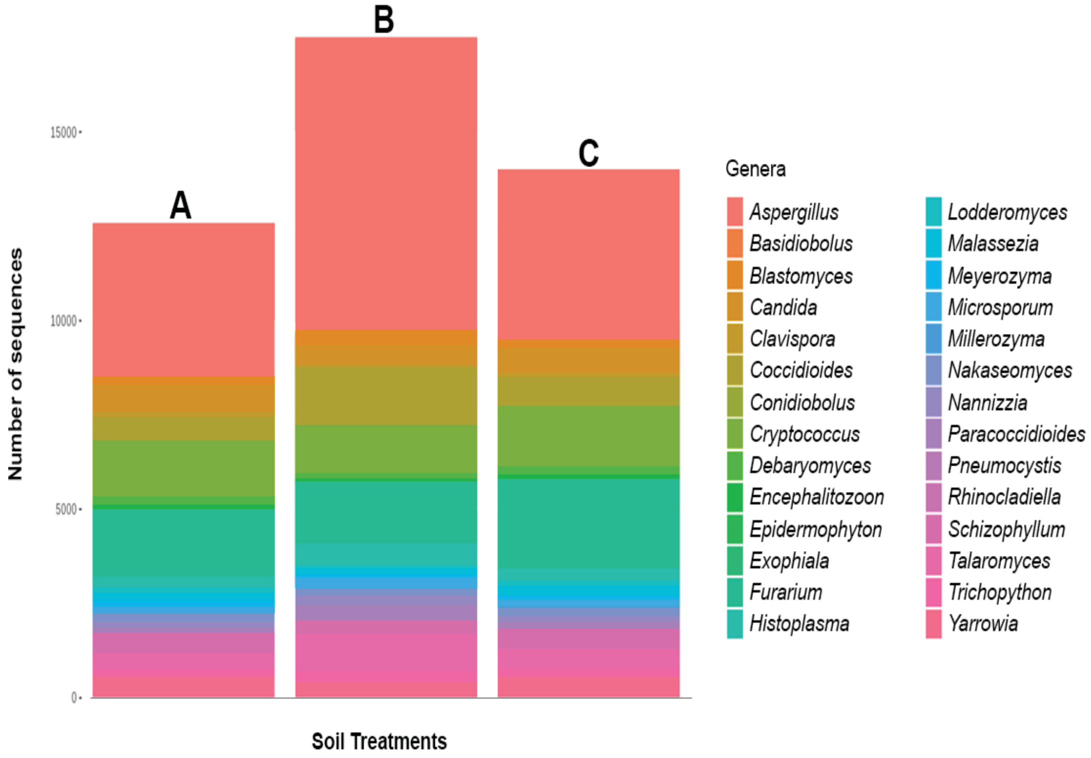
4 RESULTS

Of the 128,627 sequences of fungi identified, 43,439 belonging to pathogenic fungi distributed as follows: native 17,301 (40%), no-tillage 13,780 (32%) and conventional tillage 12,358 (28%) (Figure 7). In terms of taxonomic abundance, considering all the treatment evaluated, it was observed 4 phyla, 9 classes, 11 orders, 18 families, 28 genera, and 41 different species.

Among the 28 genera identified, the most abundant were *Aspergillus* (38%), followed by *Fusarium* (13%), *Cryptococcus* (10%), and *Coccidioides* (7%). Moreover, genera with 4% abundance were *Candida*, *Talaromyces*, and *Yarrowia*, those with 3% abundance included *Histoplasma* and *Schizophyllum* and lastly *Blastomyces*, *Malassezia*, *Nakaseomyces*, *Paracoccidioides*, and *Trichophyton* showed 2% of abundance. Less abundant genera, representing less than 1% included *Clavispora*, *Debaryomyces*, *Encephalitozoon*, *Lodderomyces*, *Meyerozyma*, *Microsporum* and *Nannizzia*. The less abundant genera comprised *Basidiobolus*, *Conidiobolus*, *Epidermophyton*, *Exophiala*, *Millerozyma*, *Pneumocystis*, and *Rhinocladiella* with 0.1% (Figure 7).

In addition, when comparing between the three treatments it was observed that the genera *Aspergillus*, *Coccidioides*, *Talaromyces*, *Histoplasma*, *Blastomyces*, *Paracoccidioides*, *Trichophyton*, *Microsporum*, *Nannizzia*, *Pneumocystis, Basidiobolus* and *Rhinocladiella* showed the highest number of sequences in the native soils. The NT soils showed higher abundance of *Fusarium*, *Cryptococcus*, *Schizophyllum*, *Malassezia*, *Meyerozyma*, *Encephalitozoon*, *Epidermophyton*, *Exophiala* and *Millerozyma* genera while CT soil showed predominance of *Candida*, *Yarrowia*, *Clavispora*, *Lodderomyces, Conidiobolus* and *Debaryomyces* (Figure 7).

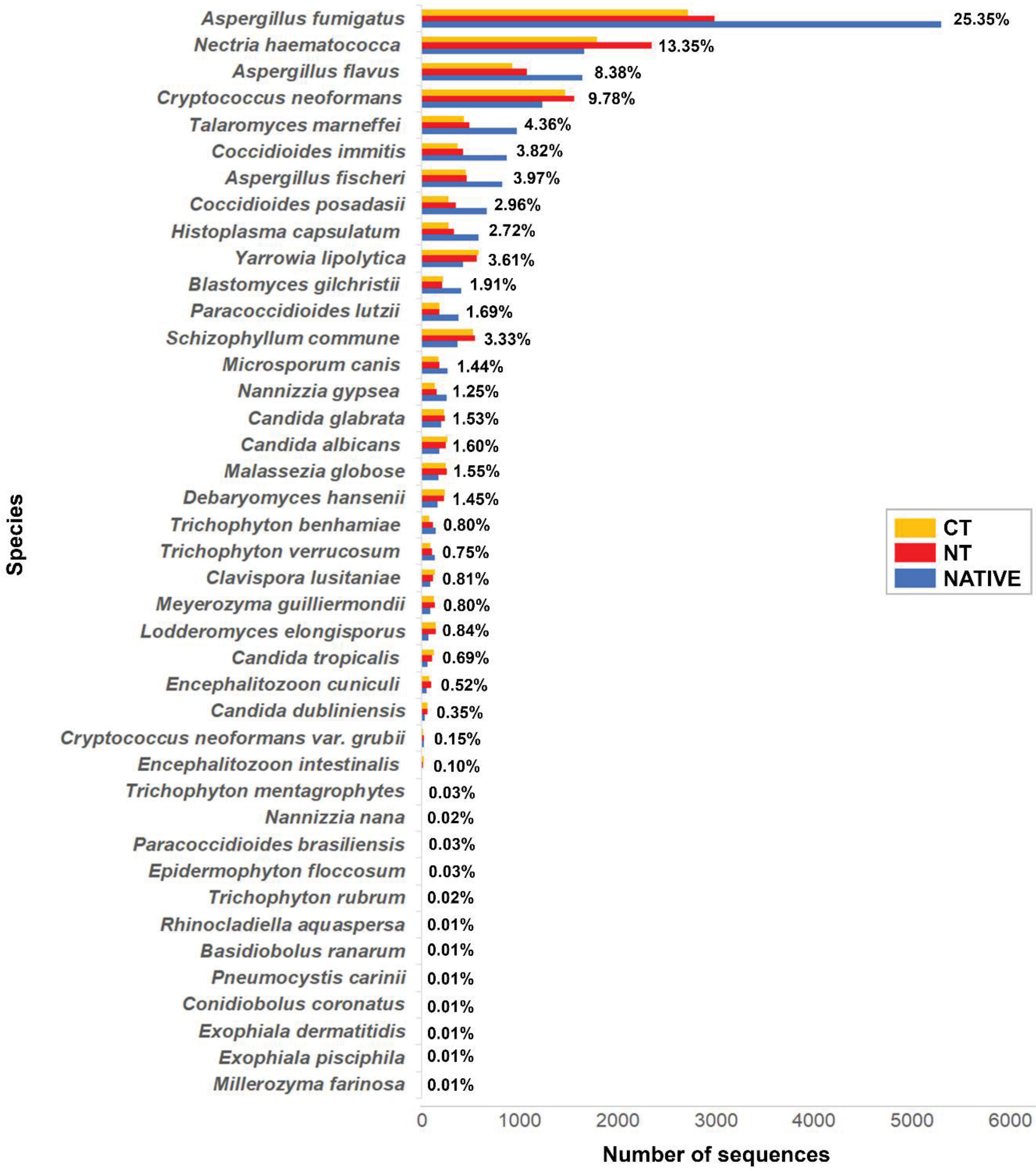
Figure 7 – Relative abundance of the genera associated to human and animal infection



In A: conventional tillage (CT), in B: undisturbed Cerrado soil (Native) and in C: no-tillage (NT).

Among the genus were identified 41 species that have already been reported as casual agents of diseases in humans and/or animals, these agents of primary infections and fungi opportunistic (Figure 8). Another important result is that among the 41 species identified, 31 species were arranged in the three treatments, 6 species in two treatments and 4 species in only one treatment. Considering the sequences total identified, the species with more predominance were the *Aspergillus fumigatus* encountered in the native soils corresponding to 25,35% of the total sequences, following by the *Nectria haematococca* (*Fusarium solani*) with 13,35% and *Cryptococcus neoformans* with 9,78%, both with more predominance in soils cropped (Figure 8).

Figure 8 - The distribution of the species by grouping on the samples



No-tillage (NT), undisturbed Cerrado (Native) and conventional tillage (CT).

5 DISCUSSION

The metagenomic analysis of soils belonging to the Cerrado biome from three different treatments revealed the presence as saprophytic fungi, real opportunistic and pathogens. In this study, were identified 43,439 sequences belonging to pathogenic fungi distributed as follows: native 17,301 (40%), no-tillage 13,780 (32%) and conventional tillage 12,358 (28%) (Table 3). Therefore, the results highlight that undisturbed Cerrado soils covered with native vegetation show higher relative abundance of pathogenic fungi than soils submitted to agricultural practices. This result indicates a reduction in fungal biodiversity associated with anthropogenic activity, also observed in previous studies from Cerrado biome (CASTRO et al., 2008; BRESOLIN et al., 2010; SOUZA et al., 2016).

Epidemiological data evidence the significant increase in the incidence of invasive fungal diseases in humans (DE HOOG et al., 2018; GUINEA et al., 2014; QUEIROZ-TELLES et al., 2019) and in animals (SEYEDMOUSAVI et al., 2014). Among the species identified, the one with highest relative abundance among the species was *Aspergillus fumigatus* (25.35%) (Figure 8), which is important allergen that causes aspergillosis, responsible for the major cause of human morbidity and mortality around the world (LAMOTH, 2016). In Brazil, epidemiological data are quite scarce due to difficulty of correct diagnosis (COSTA et al., 2019) and studies with environmental isolates of *A. fumigatus* and *A. flavus* demonstrated 20–25% rate of the resistance itraconazole (BEDIN DENARDI et al., 2018). Often found in soil and air samples (PRINGLE et al., 2005), here was identified in more abundance in soils natives. It is suggested that soil management in some way seems to be alters the frequency of the environment fungal occurrence.

The sequence data showed *Fusarium solani* as the second major relative abundant species and predominant in cultivated soils, being higher in NT than CT (Figure 8). Recognized as phytopathogen that can be which can cause losses on soils cropped, this fungus is involved in opportunistic fungal infections and keratitis in humans (ZHANG et al., 2006). Studies of invasive fusariosis in Brazil showed that *Fusarium* spp. was associated with agricultural activities in a hematology sector (NUCCI et al., 2013).

In our analyses, the third most abundant fungus was *Cryptococcus neoformans* (9.78%) distributed in the three treatments but more frequently in no-tillage (Figure 8). This fungal is opportunistic due to its ability to grow at body temperature, produce melanin, and polysaccharide capsule and causal agent of cryptococcosis by affecting immunocompetent and immunocompromised individuals (MAY et al., 2016; HUANG et al., 2016). Epidemiological data showed that in Brazil, mortality reached up to 60% in HIV infected patients (COSTA et al., 2019).

Primary fungi infection agents were identified, being these dimorphic fungi such as *Paracoccidioides lutzii,* (1.69%) and *P. brasiliensis* (0.03%), that causes paracoccidioidomycosis, an endemic disease of the Brazilian Cerrado region and restricted to Latin America (TEIXEIRA et al., 2014). It is the chief systemic mycosis affecting the Brazilian population, the eighth largest cause of mortality due to mycoses (COUTINHO et al., 2015; SHIKANAI-YASUDA et al., 2017), and besides humans, it can also infect animals (BAGAGLI et al., 2003; RICHINI-PEREIRA et al., 2008). In this study, *P. lutzii,* and *P. brasiliensis* were identified in all treatments, but in more frequent in native soils (Figure 8). Moreover, the *Histoplasma capsulatum* recognized as endemic agent in Brazil particularly from the Midwest (ALMEIDA et al., 2019) was identified mainly in native soil (Figure 8). This fungal inhabits soils with high nitrogen concentrations (MUNIZ et al., 2001), such as the soils reported in this study because they consist of nitrogen-fixing bacteria (SOUZA et al., 2016).

Although the semi-arid region of the Brazilian Northeast has been classified as an endemic area of systemic coccidioidomycosis and the cases are usually related to armadillo hunting (DE MACÊDO et al., 2011; WANKE et al., 2000), the etiological agents *Coccidioides immitis* (3.82%) and *C. posadasii* (2.96%) were found in the all the soils analyzed, likewise with more predominance in native soil (Figure 8).

Among the opportunistic species, the sequences of *Candida* spp. identified in this study did not show differences in frequencies (Figure 8). The most abundant species was *C. albicans* (1.60%) that is related as a prevalent causal agent of the onychomycoses in northeast of Brazil (SILVA-ROCHA et al., 2017). In addition, it was observed *Yarrowia lipolytica* anamorph of *C. lipolytica* (3.61%) that cause blood infections (TRABELSI et al., 2015) and as well as *Malassezia globosa* (1.55%) considered a relevant agent of superficial mycoses in humans and animal (VELEGRAKI et al., 2015).

Dermatophytes are recognized by infections of skin, nails and hair (DE HOOG et al., 2017). Fungi classified as anthropophilic, zoophilic and geophilic (DE HOOG et al., 2017) were identified in this study, but in a small relative abundance. As well, the fungus *Talaromyces marneffei* (4.36%) and *Blastomyces gilchristii* (1.91%) also were identified in more predominance in native soil, but in low frequency (Figure 8). This fact may justify the rare cases in Brazil and Latin America (CHAN et al., 2016; DALCIN et al., 2016).

Likewise, the Herpotrichiellaceous fungi was identified in low relative abundance (Figure 8), e.i. *Rhinocladiella aquaspersa* a rare agent of chromoblastomycosis, was present in native soils, *Exophiala pisciphila* which is associated with infection in cold blood animals (DE HOOG et al., 2011), although in isolated cases, it can infect humans (KEBBE, MADOR, 2016) and *E. dermatitidis* is an opportunistic pathogen and this genus can cause peritonitis (PINHEIRO et al., 2019), cystic fibrosis, phaeohyphomycosis and chromoblastomycosis in humans (KIRCHHOFF et al., 2019).

Although identified low in relative abundance, the zygomycetes *Conidiobolus coronatus* and *Basidiobolus ranarum* have clinical importance because they cause conidiobolomycosis and basidiobolomycosis, respectively (VILELA, MENDONZA, 2018). Furthermore, a basidiomycete and an occasional human pathogen agent of respiratory infections, *Schizophyllum commune* was also identified (CHOWDHARY et al., 2013). With respect to animal pathogenic fungi, *Pneumocystis carinii*, the species responsible for lung infections in rats (WEISBROTH, 2006) were identified. In addition, to *Encephalitozoon cuniculi* and *E. intestinalis* that causes microsporidiosis in rats, and several other infections in mammals (GOODWIN et al., 2006; LINDSAY et al., 2009; MALčEKOVÁ et al., 2010) were observed (Figure 8).

6 CONCLUSIONS

The fungi are considered ubiquitous organisms found in the environment in association with soil, plants, rocks animals and water sources, where human and animals are in frequent exposure. However, relatively few fungal species are capable of infecting human and animal host and likewise its environmental isolation are not always correlated to epidemiological data, which normally could be attributed to the limitations of isolation methods and / or frequency of the species in highly specific niches. In this scenario the metagenomic assays represent a relevant tool for this inference.

This exploratory metagenome study of soils from the Cerrado region in Brazil identified the presence of forty-one fungal species considered pathogenic to human and animal hosts. The data analysis revealed that the native soils have a higher relative abundance of genera and species, than other conventional and no-tillage treatments, corroborating with previous studies, in which observed a reduction in fungal biodiversity associated with anthropogenic activity. Furthermore, it was inferred about the environmental occurrence of causal agents of primary and opportunistic human infections rarely isolated from the environmental sources.

Therefore, culture-independent methods can identify the presence of microorganisms in several substrates and conditions contributing to the elucidation of niches and disease infection routes.

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CHAPTER IV: Environmental sources of black yeasts in Brazil based on the metagenomic data

Environmental sources of black yeasts in Brazil based on the metagenomic data

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1 ABSTRACT

Melanized fungi known black yeasts from the Herpotrichiellaceae family in the Chaetothyriales order are important agents associated to human and animal infection such as chromoblastomycosis and phaeohyphomycosis. The oligotrophic nature of these fungi enables them to survive in adverse environments which common saprobes are absent. Despite of that, isolation studies demonstrated a low frequency of clinical species in environmental substrates. The metagenomic technique allows the characterization of microorganisms regardless of culture, while padlock probes and barcodes are specific molecular markers for identification of genotype and/or species identification. In this context, this work aimed to identify species of Herpotrichiellaceae family, using molecular markers to screen public environmental metagenomic datasets from Brazil available in Sequence Read Archive (SRA). The species characterization was performed with the BLAST comparison of barcodes and padlock probe sequences already described with metabarcoding. A total of 18,329 sequences comprising the genera *Cladophialophora*, *Exophiala*, *Fonsecaea*, *Rhinocladiella* and *Veronaea* was found, including species related to the chromoblastomycosis disease. The data obtained in this study showed the presence of these pathogens in environmental metagenomics datasets from Brazil. The metagenomic and molecular markers are an additional tool that contribute for acknowledgement about the environmental occurrence, and epidemiology of these pathogens.

2 INTRODUCTION

A large number of species of black yeast-like fungi that belong to ascomycetous order Chaetothyriales, family Herpotrichiellaceae, are renowned as opportunistic pathogens in immunocompetent vertebrate hosts (DENG et al., 2015; QUEIROZ-TELLES et al., 2017). Agents are particularly involved in subcutaneous, systemic and disseminated infections, known as chromoblastomycosis and phaeohyphomycosis (GOMES et al., 2016; QUEIROZ-TELLES et al., 2017; KIRCHHOFF et al., 2019), and are chronic and often severely mutilating or even fatal.

Chromoblastomycosis is a relatively common disease in rural tropical climate zones around the world. This implantation disorder is characterized by the presence of a specialized tissue form of the fungus known as muriform cells (SEYEDMOUSAVI et al., 2014; AZEVEDO et al., 2015; QUEIROZ-TELLES et al., 2017). Infection is hypothesized to take place via traumatic inoculation of environmental material such as plant thorns and/or wood fragments (MARQUES et al., 2006; SALGADO et al., 2004). Epidemiological data confirmed by studies using selective isolation methods (VICENTE et al., 2001; 2008; SATOW et al., 2008; GUERRA et al., 2013) suggest an environmental origin of this disease, presence of these agents is low, only few isolates having been recovered even even after extensive sampling in endemic areas (VICENTE et al., 2001; 2008; 2013; NASCIMENTO et al., 2017); culture usually yields only non-pathogenic relatives. Novel molecular methods are required for understanding the ecology and environmental occurrence of these agents.

Metagenomics are culture-independent methods for the study of microbial diversity, based on next generation sequencing (NGS), allowing characterization of fungi in complex environmental system, using specific molecular markers for identification (CUADROS-ORELLANA et al., 2013). In addition, abundant metagenomic data are available in public databases such as Sequence Read Archive (SRA; LEINONEN et al., 2010), Rast Server (MG-RAST; MEYER et al., 2008), and EBI metagenomics (EMG; MITCHELL et al., 2016). Likewise, sequences of several molecular markers are available that are used for conventional identification of species in Herpotrichiellaceae, i.e. *ITS*, *TEF1*, *BT2*, and *ACT1* (TEIXEIRA et al., 2017).

Alternatively, padlock probes, which are specific oligonucleotide with the ability to identify single nucleotide polymorphisms (SNPs), have been proposed for the recognition of several groups of black agents (NAJAFZADEH et al., 2011, 2013, 2018; HAMZEHEI et al., 2013; DENG, et al., 2014; SCHNEIDER et al., 2019). DNA barcoding, based on the ITS region and applying short sequences (25–41 bp) of nucleotides specific for a single taxonomic species (HEINRICHS et al., 2012), can additionally be used to recognize herpotrichiellaceous species by variable regions in the ribosomal operon.

The present study aims to explore the environmental occurrence of agents of chromoblastomycosis in the family Herpotrichiellaceae in environmental samples in tropical Brazil. We compare metagenomic data present in public databases, using barcodes and padlock probes for species identification. This approach should lead to better understanding of the sources and routes of infection of patients with chromoblastomycosis.

3 MATERIALS AND METHODS

3.1. DATABASE

The metagenomic database was assembled applying the following criteria: (1) samples with Brazilian origin; (2) environmental samples from a wide variety of sources including arthropods and other animals, aquatic bodies, hostile environments including rocks, decomposing materials with plant debris and soil; (3) metagenomics with public data available for download in the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra). A total of 3,786 runs of approximately 2 terabytes of data was analyzed.

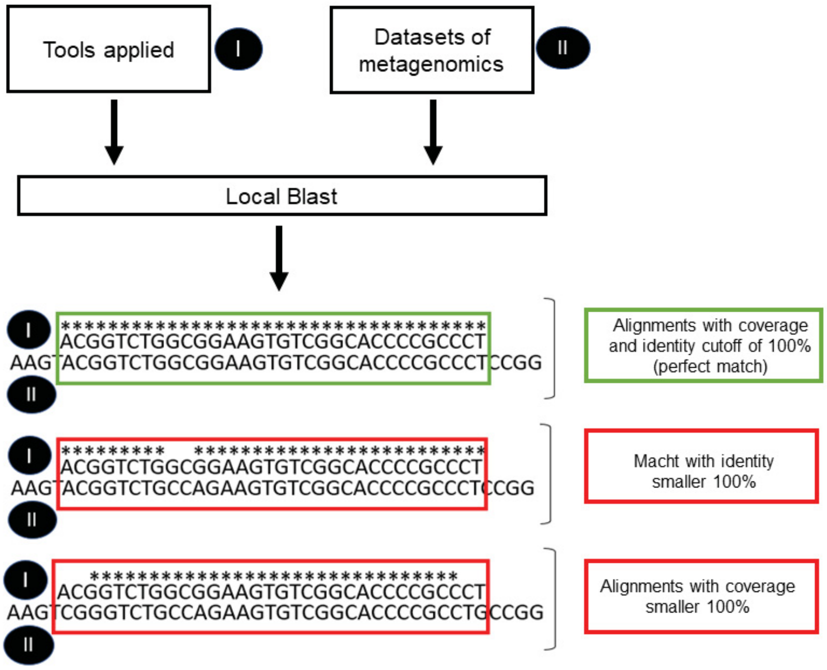
3.2. MOLECULAR MARKERS

Molecular markers for members of the family Herpotrichiellaceae described in the literature (Table S1) were used for species identification in the metagenome datasets. A total of 97 barcode identifiers with 25–41 bp (HEINRICHS et al., 2012) and 25 padlock probes sequences with 28–42 bp with different SNPs, were designed based on from rDNA internal transcribed spacer (ITS2; NAJAFZADEH et al., 2011, 2013, 2018; HAMZEHEI et al., 2013; DENG et al., 2014, SCHNEIDER et al., 2019).

3.3. SAMPLE MINING

Comparison of metagenomes with molecular markers sequences was performed with local BLASTn (v2.6.0. +). For the data mining only alignments with coverage and identity cutoff of 100% (perfect match) were considered (Figure 9). Matches with values below the previously described were excluded. Because padlock and barcode probes are extremely specific for species identification, cases of perfect misalignment and low sequence identity do not characterize the fungus in the analyzes (Figure 9). Metagenome reads from double-strand sequencing where considered once in the final read count.

Figure 9 - Fluxogram of identification *in silico*



In green criteria of selection and in red rejected criteria.

4 RESULTS

4.1. DATASETS CONTAINING HERPOTRICHIELLACEOUS

In total 169 large datasets distributed in 3,786 samples were analyzed from Brazil. Of these, only 11 large datasets arranged in 179 samples have sequences of members of Herpotrichiellaceae, originated from five states, representing environmental samples from different geographic areas in Brazil (Table 4, Figure 10A). The datasets which were rearranged according to eight types of sources, i.e. rhizosphere (PRJNA379918, PRJNA362455, PRJEB24131), ant (PRJNA321130), aquatic (PRJNA237344), biotechnological (PRJNA285006, PRJEB5245), mycorrhizal fungi (PRJNA339563), plant (PRJNA522264), mangrove (PRJNA478407), and soil (PRJNA421085) (Table 4).

Table 4 **-** Summary of selected datasets that contain Herpotrichiellaceae fungi sequences

Accession large datasets	Accession samples	Dataset description
PRJNA339563	SRR4065317, SRR4065319, SRR4065500	Mycorrhizal fungi: interactions of tropical mycoheterotrophic plants and their arbuscular mycorrhizal fungal hosts
PRJNA379918	SRR5399784, SRR5399785, SRR5399787, SRR5399789	Rhizosphere: the maize rhizosphere community under different phosphate source
PRJNA237344	SRR1786616, SRR1790680, SRR4833059	Aquatic: evaluation of the waters of the Amazon River to the Atlantic Ocean due to their immense scale and their apparent sensitivity to climate variability and anthropogenic forces.
PRJEB5245	ERR957350, ERR957352 to ERR957355	Biotechnological: development of a microbial enrichment for sugarcane bagasse breakdown.
PRJNA321130	SRR3493327	Ant: the fungal diversity found on the integument of *Atta capiguara* and *A. laevigata alate* ants.
PRJNA522264	SRR8585376, SRR8585377, SRR8585380, SRR8585381, SRR8585384 to SRR8585386, SRR8585391, SRR8585392, SRR8585395 to SRR8585398, SRR8585411, SRR8585412, SRR8585414, SRR8585416, SRR8585417, SRR8585420, SRR8585425, SRR8585428, SRR8585430, SRR8585434 to SRR8585437, SRR8585453 to SRR8585457, SRR8585459, SRR8585461 to SRR8585465, SRR8585467, SRR8585468, SRR8585471, SRR8585474, SRR8585475, SRR8585489, SRR8585492, SRR8585494 to SRR8585497, SRR8585501 to SRR8585503, SRR8585506, SRR8585507, SRR8585509, SRR8585510, SRR8585513 to SRR8585518, SRR8585520, SRR8585530 to SRR8585533, SRR8585536, SRR8585538	Plant: characterization of the microbiomes associated with two plant species (*Vellozia epidendroides* and *Barbacenia Macrantha*) that thrive in the extremely P-impoverished soils of the Brazilian campos rupestres.
PRJEB24131	ERR2233399 to ERR2233446	Rhizosphere: root-associated microbiome of maize genotypes with contrasting phosphorus use efficiency.
PRJNA285006	SRR2086459, SRR2086461, SRR2086464, SRR2086481	Biotechnological: microbiome sugarcane filter cake compost piles were carried out to analyses the dynamics of fungal and bacterial communities along the process

		and biomass degrading profile for second generation bioethanol.
PRJNA478407	SRR7450155 to SRR7450157, SRR7450161 to SRR7450169, SRR7450174, SRR7450176, SRR7450177, SRR7450179 SRR7450181, SRR7450186	Mangrove: metagenomics and metatranscriptomics of the microbial community involved in the transformation of organic carbon in mangrove sediments at São Paulo state.
PRJNA421085	SRR6354886	Soil: soils contaminated with crude oil.
PRJNA362455	SRR5195137, SRR5195141 SRR8056346, SRR8056347 SRR8056355 to SRR8056358	Rhizosphere: citrus rhizosphere microbiome.

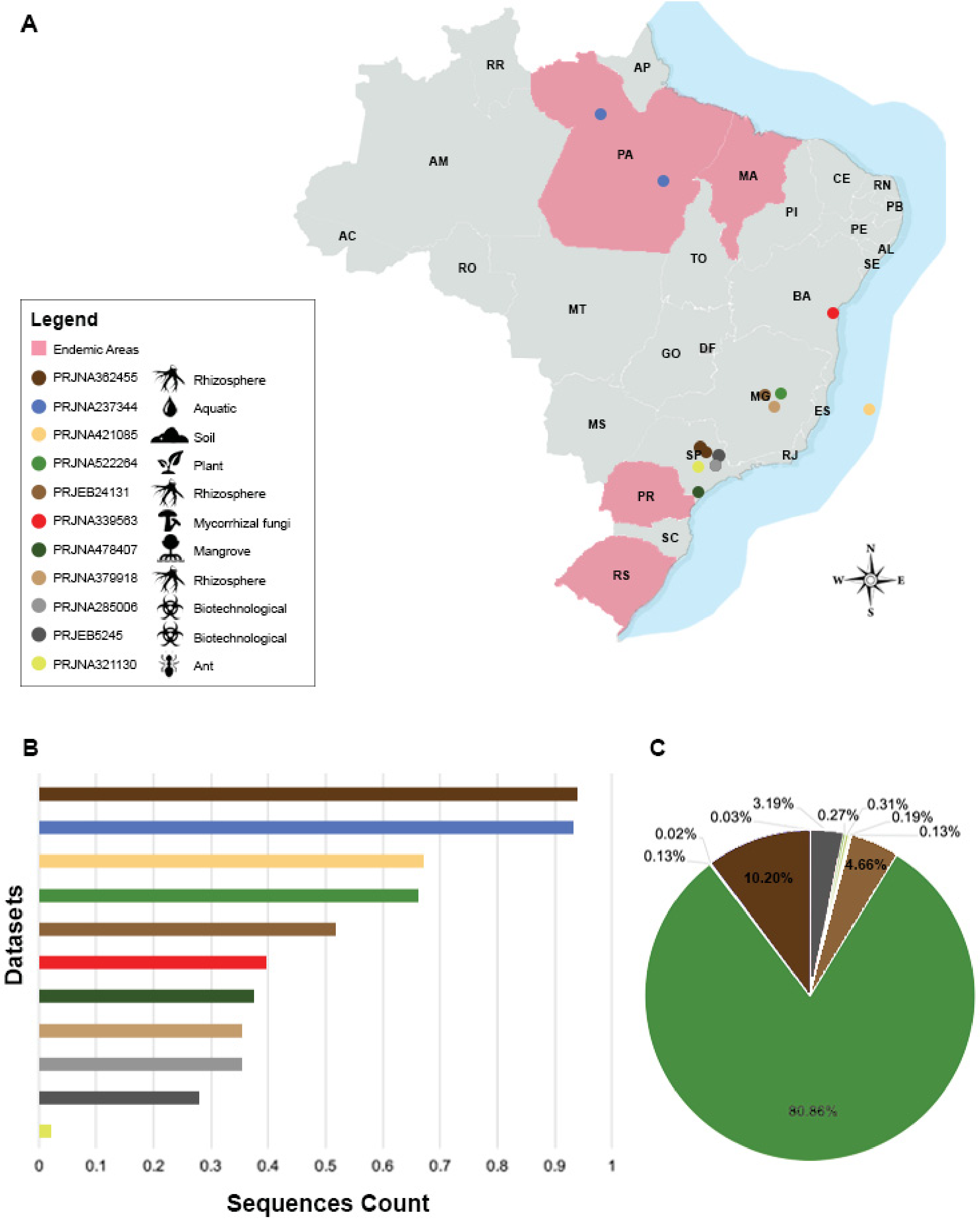
Each metagenome project generated the data according to its scope, which resulted in a high variation in size of the datasets. The read number ranged from 14,293 to 1,394,769,476, with the rhizosphere metadata (PRJNA362455) being the one with the highest number of reads (Table 5, Figure 10B;). Within each read pool, the ones matching Herpotrichiellaceae ranged from 4 reads to 14,821 sequences, with the highest concentration in the plant metadata (PRJNA522264). All counting results considered normalized data (Table 5, Figure 10C).

The total number of reads matching herpotrichiellaceous fungi was 18,329. Of this data pool, 84% (15,526 reads) where identified by barcode markers, and only around 5.6% (1,032 reads) exclusively by padlock probe markers. The number of sequences identified simultaneously by both markers were 1,771 reads (Table 5), which underlined the requirement to use more than a single tool for *in silico* identification.

Table 5 **-** Overview of sequences identified as Herpotrichiellaceae fungi

Accession large datasets	**Number of sequences total**	**Number of sequences of Herpotrichiellaceae identified**	**Criteria of species identification**		
			Barcodes*	**Padlock probes****	**Both*,****
PRJNA339563	4,146,905	24	4	17	3
PRJNA379918	2,895,509	57	0	11	46
PRJNA237344	1,104,240,094	4	2	0	2
PRJEB5245	1,498,794	584	0	565	19
PRJNA321130	142,930	6	6	0	0
PRJNA522264	35,414,582	14,821	13,634	136	1,051
PRJEB24131	11,210,858	855	0	252	603
PRJNA285006	2,889,538	50	6	1	43
PRJNA478407	3,505,958	35	8	27	0
PRJNA421085	38,540,232	23	0	23	0
PRJNA362455	1,394,769,476	1,870	1,866	0	4
Total	**2,599,126,239**	**18,329**	15,526	1,032	1,771

Figure 10 - Herpothrichiellaceus sequences encountered in different dataset investigated



A: Geographic metagenomic data distribution. **B:** Total of reads in the different dataset investigated. **C**: Herpotrichellaceues sequence per dataset.

4.2. SPECIES IDENTIFIED

In the datasets investigated the genera *Cladophialophora*, *Exophiala*, *Fonsecaea*, *Rhinocladiella*, and *Veronaea* were identified. The sequences mainly belonged to the genus *Exophiala* which was identified by barcodes and padlock probes. Among the fungi of the family Herpotrichiellaceae, *Exophiala* species were the most abundantly represented (18,113 reads) corresponding to 98.77% of the total sequences belonging to 16 described *Exophiala* species; *E. bergeri* (46.01%), *E. sideris* (27.86%), and *E. pisciphila* (11.42%) were prevalent. The presence of *Fonsecaea pedrosoi*, the major agent of chromoblastomycosis in Brazil, was detected at low incidence (0.74%). *Cladophialophora* species (0.14%) was represented by *C. chaetospira* (0.12%), and *C. arxii* and *C. immunda*, both with 0.01%. In the genus *Rhinocladiella* (0.30%), two species were identified, i.e. *R. similis* (0.08%) and *R. atrovirens* (0.22%). *Veronaea botryosa* was present in low numbers (0.05%) (Table 6).

The species *E. angulospora* was identified in mangrove (PRJNA478407), *E. castellanni* in mycorrhizal fungi (PRJNA339563), *E. equina* in root-associated of maize (PRJEB24131), *E. heteromorpha* in river (PRJNA237344), *E. jeanselmei* in lignocellulosic biomass (PRJEB5245), *E. sideris* in soil-associated material and in plants (PRJNA522264), *E. mesophila* in sugarcane filter cake (PRJNA285006). Moreover, species of *Exophiala* found in two or more different metagenomic datasets were *E. dermatitidis* in river (PRJNA237344) and in sugarcane filter cake (PRJNA285006); *E. spinifera* in sugarcane filter cake (PRJNA285006) and root-associated debris of maize (PRJEB24131); *E. bergeri* in sugarcane filter cake (PRJNA285006), in soil-associated and plants (PRJNA522264) and in maize root-associated soil (PRJEB24131); *E. exophialae* in river water (PRJNA237344), in maize rhizosphere (PRJNA379918) and in ants (PRJNA321130); *E. oligosperma* in mycorrhizal fungi (PRJNA339563), in soil-associated material and in plants (PRJNA522264) and in lignocellulosic biomass (PRJEB5245); *E. brunnea* in mycorrhizal fungi (PRJNA339563), in citrus rhizosphere (PRJNA362455), in soil-associated and plants (PRJNA522264) and in root-associated soil of maize (PRJEB24131); *E. cancerae* in sugarcane filter cake (PRJNA285006), in soil-associated soil and plants (PRJNA522264), lignocellulosic biomass (PRJEB5245) and in root-associated soil of maize (PRJEB24131); *E. pisciphila* in mangrove (PRJNA478407), in soils contaminated with crude oil (PRJNA421085), in citrus rhizosphere (PRJNA362455), in maize rhizosphere (PRJNA379918) and in root-associated soil of maize (PRJEB24131); *E. xenobiotica* in mycorrhizal fungi (PRJNA339563), in citrus rhizosphere (PRJNA362455), in sugarcane filter cake

(PRJNA285006), in soil-associated soil and plants (PRJNA522264), in maize rhizosphere (PRJNA379918). All the sequences of the genus *Cladophialophora* were identified by padlock probes, being *C. arxii* and *C. chaetospira* in mangrove (PRJNA478407), and *C. immunda* and *C. chaetospira* in soils contaminated with crude oil (PRJNA421085). The *F. pedrosoi* was identified by padlock probe in soil-associated material of plants of the family Velloziaceae (PRJNA522264). In addition, *R. similis* was observed in maize rhizosphere (PRJEB24131), and *R. atrovirens* was identified in soil associated with plants (PRJNA522264). *V. botryosa* was identified in mangrove, root-associated soil of maize and in sugarcane filter cake (PRJNA478407, PRJEB24131 and PRJNA285006) (Table 6).

Table 6 – Species identified in selected metagenomics datasets

Sources sampling/ Species identified *in silico*	PRJNA 478407 Mang.	PRJNA 339563 Myco.	PRJNA 237344 Aq.	PRJNA 421085 Soil	PRJNA 362455 Rhizos.	PRJNA 379918 Rhizos.	PRJEB 24131 Rhizos.	PRJNA 285006 Biotec.	PRJEB 5245 Biotec.	PRJNA 522264 Plant	PRJNA 321130 Ant	Total of sequences	%
E. angulospora	4*	0	0	0	0	0	0	0	0	0	0	4	0.02
E. bergeri	0	0	0	0	0	0	1**	1**	0	8,431**	0	8,433	46.01
E. brunnea	0	4**	0	0	10**	0	2**	0	0	1**	0	17	0.09
E. cancerae	0	0	0	0	0	0	7**	1**	562**	35**	0	605	3.30
E. castellanni	0	3***	0	0	0	0	0	0	0	0	0	3	0.02
E. pisciphila	23*	0	0	5*	1,856**	10**	200**	0	0	0	0	2,094	11.42
E. exophialae	0	0	1**	0	0	1**	0	0	0	0	6**	8	0.04
E. equina	0	0	0	0	0	0	20**	0	0	0	0	20	0.11
E. dermatitidis	0	0	2***	0	0	0	0	1***	0	0	0	3	0.02
E. heteromorpha	0	0	1**	0	0	0	0	0	0	0	0	1	0.01
E. jeanselmei	0	0	0	0	0	0	0	0	19***	0	0	19	0.10
E. sideris	0	0	0	0	0	0	0	0	0	5,106**	0	5,106	27.86
E. spinifera	0	0	0	0	0	0	603***	42***	0	0	0	645	3.52
E. xenobiotica	0	16*	0	0	4***	46***	0	1*	0	1,051***	0	1,118	6.10
E. oligosperma	0	1**	0	0	0	0	0	0	3**	20**	0	24	0.13
E. mesophila	0	0	0	0	0	0	0	3**	0	0	0	3	0.02
C. arxii	1*	0	0	0	0	0	0	0	0	0	0	1	0.01
C. chaetospira	6*	0	0	16*	0	0	0	0	0	0	0	22	0.12
C. immunda	0	0	0	2*	0	0	0	0	0	0	0	2	0.01
F. pedrosoi	0	0	0	0	0	0	0	0	0	136*	0	136	0.74
R. atrovirens	0	0	0	0	0	0	0	0	0	41**	0	41	0.22
R. similis	0	0	0	0	0	0	14**	0	0	0	0	14	0.08
V. botryosa	1*	0	0	0	0	0	8**	1**	0	0	0	10	0.05
Total	35	24	4	23	1,870	57	855	50	584	14,821	6	18,329	

The identification by: only padlock probes*, only barcodes **, and padlock probes and barcodes simultaneously***. Legend: Mang. - mangrove place; Myco. - mycorrhizal fungi; Aq. -aquatic; Rhizos. – rhizosphere; Biotec. - sugarcane filter cake and lignocellulosic biomass

5 DISCUSSION

In this study we investigated the presence of sequences of herpotrichiellaceous fungi in metagenomic datasets that were generated after analysis of divergent environmental sources, using molecular markers for *in silico* identification of the causal agents of chromoblastomycosis and phaeohyphomycosis. The tools used as reference were padlock probes developed for the rapid detection of pathogenic *Fonsecaea* species in clinical samples (*F. pedrosoi*, *F. nubica*, *F. monophora* and *F. pugnacius*; NAJAFZADEH et al., 2011; SCHNEIDER et al., 2019), the agent of neurotropic phaeohyphomycosis *Cladophialophora bantiana* (HAMZEHEI et al., 2013), and other opportunistic species with variable pathology (DENG et al., 2014; NAJAFZADEH et al., 2013, 2018). ITS rDNA barcoding sequences had previously been recommended for rapid identification of clinical and environmental sequences (ZENG & DE HOOG, 2008), and were suggested for taxonomic identification in metagenomic data (HEINRICHS et al., 2012).

The results indicated that this methodology represents complementary data to studies on direct isolation via culture (SATOW et al., 2008; GUERRA et al., 2013; VICENTE et al., 2001, 2008, 2013; FENG et al., 2013; NASCIMENTO et al., 2017), which all reported low frequency of these agents in the environment. Judging from the number of sequences present in the evaluated datasets, the low frequency of herpotrichiellaceous fungi, compared to the total number of fungal sequences, was confirmed (Table 5). For example, *Fonsecaea pedrosoi,* a major agent of chromoblastomycosis in Brazil (QUEIROZ-TELLES et al., 2017), was detected in metagenomic data from plant- and soil-associated materials. This habitat is in line with the hypothesis of chromoblastomycosis as an implantation disease from inoculated plant-derived material. This demonstrates that *in silico* identification can be used as a new tool to elucidate the natural habitat of agents of opportunistic diseases, and assist in elucidating the environmental occurrence and the route of infection of causative species. The infection route of agents of chromoblastomycosis nevertheless remains controversial. Their occurrence in living plants has extensively been discussed. DE HOOG et al. (2007) and VICENTE et al. (2013) demonstrated that the main *Fonsecaea* species occurring in living plant belong to other species than those occurring on the human host. FORNARI et al. (2018) presented an *in vitro* plant infection model showing that the agents of chromoblastomycosis have a certain degree of plant-invasive ability, but it remains questionable why in our study the non-pathogenic *Fonsecaea* were not detected.

Species of the genus *Rhinocladiella* have been described as less common agents of chromoblastomycosis (RESENDE et al., 2000; RICHARZ et al., 2018), like *R. aquaspersa, R. similis* and *R. tropicalis*. *Rhinocladiella similis* has also been isolated from dialysis water and from babassu coconuts (FIGEL et al.,2013; NASCIMENTO et al., 2017). In our *in silico* data, *R. similis* was observed in the rhizosphere of maize. The saprobe *R. atrovirens* was identified in plant and soil-associated habitats. In addition, *Veronaea botryosa,* an extremely rare agents of disseminated infections in patients with CARD9 immune disorders (BONIFAZ et al., 2013; WELFRINGER et al., 2017), had previously been isolated from babassu coconuts (NASCIMENTO et al., 2017) and from creosote-treated railway ties (VICENTE et al., 2008). In this study, was identified in mangrove, maize rhizosphere and in sugarcane filter cake, indicating a wider saprobic occurrence.

Presence of herpotrichiellaceous opportunists in the environment has been shown by several authors (SALGADO et al., 2004; SATOW et al., 2008; GUERRA et al., 2013; FENG et al., 2013; VICENTE et al., 2001, 2008, 2013; NASCIMENTO et al., 2017). Our *in silico* data showed that the most common sequences in metagenomics databases belonged to the genus *Exophiala*. This is the largest genus the family Herpotrichiellaceae containing numerous species, many of which are opportunistic pathogens of cold- and warm-blooded animals (DE HOOG et al. 2019; TEIXEIRA et al., 2017). We detected species reported from various types of disease other than chromoblastomycosis, i.e. *E. bergeri*, *E. dermatitidis*, *E. jeanselmei*, *E. heteromorpha*, *E. mesophila*, *E. spinifera*, *E. oligosperma* and *E. xenobiotica* (ZENG et al., 2007). Also *E. angulospora*, *E. pisciphila* and *E. equina,* associated with infections of cold-blooded animal such as frogs, toads and fish (DE HOOG et al., 2011; SARAIVA et al., 2019) were detected. *Exophiala cancerae* was first described from the Lethargic crab disease (LCD) occurring along the Brazilian coast (BOEGER et al., 2007; DE HOOG et al., 2011). This species hitherto had only been found in endemic coastal areas. However, in our study it was identified in soil, plant roots and in a sugar filter cake, indicating a wider environmental occurrence. Other unexpected encounters were *E. castellannii,* previously isolated from water (BIEDUNKIEWICZ, SCHULZ, 2012) but in our data from in mycorrhizal fungi, *E. brunnea,* known from litter (DE HOOG et al., 2011) but here in association with mycorrhizal fungi, rhizosphere and plant, and *E. sideris* from the hydrocarbon-polluted environments (SEYEDMOUSAVI et al., 2011) but here from plant- and soil-associated materials, and finally *E. exophialae* know from straw in burrow of *Dasypus septemcinctus*, but here from river, rhizosphere and associated with ants.

The genus *Cladophialophora* was represented by two opportunistic species, *C. arxii* and *C. immunda*. *Cladophialophora arxii* was originally reported from a disseminated infection (TINTELNOT et al., 1995) and *C. immunda* from patient with a subcutaneous ulcer (BADALI et al., 2008). The latter species was later detected in sites polluted with hydrocarbons (PRENAFETA-BOLDÚ et al., 2001), which matches with its presence in soils contaminated with crude oil analyzed in this study. The environmental saprobe *C. chaetospira* is known to occur in plant litter (VICENTE et al., 2008; BADALI et al., 2008), while in our study it was found in mangroves and in soil contaminated with crude.

6 CONCLUSION

The methodology presented in this study was shown to be a reliable and quick alternative to identify the presence of agents of clinical interest in environmental samples, which is particularly valid for fungi that are difficult to bring in culture, such as black yeasts and other opportunistic agents of human disease. The use of molecular markers as tools for the identification of Herpotrichiellaceae in metagenomics datasets proved to be an effective way to study microhabitats of these fungi, demonstrating the importance of mining databanks for tracking fungal agents. However, data availability is still limited, since the barcode sequences and padlocks described in the literature are restricted to the few species. This may explain why in a number of cases our data are significantly different from existing literature, in that common saprobic relatives were not detected, while species with supposedly limited distribution were found in remote, variable habitats suggesting a low degree of host- or habitat-specificity. Expansion of databases may provide a more balanced picture in the future.

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CHAPTER V: Sugarcane as a source of Chaetothyriales fungi

Sugarcane as a source of Chaetothyriales fungi

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1 ABSTRACT

Sugarcane (*Saccharum officinarum* L., Poaceae) is a perennial grass cultivated on a large scale in (sub) tropical regions, such as Brazil, as well as, the distribuition of the black yeasts frm the Chaetothyriales order. These fungi could be classified as environmental and pathogenic, being recovered from the human and animal tisuues host, indoor environments and environmental samples. The present study assessed the diversity of Chaetothyriales fungi present on sugarcane using two methods, the identification *in silico* and isolation selective method. In the *in silico*, were identificaded 5,834 fungi and in the isolation using two selective methods a total of 639 isolates black yeasts-like were recoverd. In both strategies, it was identified fungi as members of the genera *Cyphelophora*, *Exophiala*, *Cladophialophora*, *Knufia* and *Rhinocladiella*. In this work, we reported endophytic, saprophytic and pathogenic black fungi present in sugarcane and its rhizosphere. Among the isolates should be highlighted the *Cladophialophora bantiana*, a restrict human pathogen, and four novel species of Herpotrichiellaceae family.

2 INTRODUCTION

Black yeasts-like are melanized ascomycetes fungi belonging to the Dothideales and Chaetothyriales orders (DE HOOG et al., 2014). The Herpotrichiellaceae family includes both environmental species (VICENTE et al., 2013; NASCIMENTO et al., 2017) and highly pathogenic species (AZEVEDO et al., 2015), which are related to opportunistic infections such as chromoblastomycosis and phaeohyphomycosis (REVANKAR et al., 2017; QUEIROZ-TELLES et al., 2017). These fungi have been reported in the literature isolates from environmental samples such as soil, decomposing material and plants (SALGADO et al., 2004; VICENTE et al., 2008; 2013), as well as in domestic environments (WANG et al., 2018). Other sources of these fungi are biopsy material from human and animal tissue lesions (VICENTE et al., 2012; AZEVEDO et al., 2015; GOMES et al., 2016).

Chromoblastomycosis and phaeohyphomycosis are worldwide distributed diseases with high prevalence in regions of (sub)tropical climate (REVANKAR et al., 2017; QUEIROZ-TELLES, et al., 2017). Chromoblastomycosis is an occupational disease that affects rural workers that were exposed to contaminated soil and plant material. This disease is endemic in Brazil, especially in Amazon region and Maranhão State. Regard to phaeohyphomycosis, this is a sporadic and cosmopolitan infection which affects immunocompetent and immunosuppressed individuals (REVANKAR et al., 2017).

The epidemiological data of the chromoblastomycosis suggest an environmental route; however, few studies using methods of selective isolation showed the presence of pathogenic agents in the environment (VICENTE et al., 2008). Therefore, culture-independent methods, as metagenomics, are an important tool which allows characterizion of the microbial diversity in complex environmental sources by identification of DNA sequences (CUADROS-ORELLANA et al., 2013).

Sugarcane (*Saccharum officinarum* L., Poaceae) is a perennial grass cultivated on a large scale in tropical and subtropical regions (LIMA et al., 2001). The economic interest in sugar cane is based on its main derivatives, which are: sugar, alcohol and bagasse, beyond other by-products (CHEAVEGATTI-GIANOTTO et al., 2011). In Brazil, sugarcane is an expanding crop covering the Midwest, Southeast (mainly São Paulo), South and Northeast regions (CHEAVEGATTI-GIANOTTO et al., 2011, ARRUDA; GILLER; SLINGERLAND, 2017).

Recent studies have been demonstrated the occurrence of fungi from the Herpotrichiellaceus family in metagenomic data of sugarcane plant in Brazil (SOUZA et al., 2016). Therefore, the present study aimed to identify the Herpotrichiellaceous fungi associated to human and animal infection in the samples of sugarcane plants trought the *in silico* and by the isolation methods.

3 MATERIALS AND METHODS

3.1. METAGENOMICS DATASET

The public metagenomic datasets of the sugarcane used was available in the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra). The Bioproject access number is PRJNA319259. The reads comprise the ITS2 region described by Souza et al. (2016).

3.2. DATA MINING

The *in silico* identification of molecular markers for Herpotrichiellaceae family described in the literature (Table S1) were used for species identification in the metagenome datasets. A total of 97 barcodes with 25-41 bp (HEINRICHS et al., 2012) and 25 padlock probes sequences with 28-42 bp based in SNPs, both designed based on the internal transcribed spacer - ITS2 (NAJAFZADEH et al., 2011, 2013, 2018; HAMZEHEI et al., 2013; DENG et al., 2014, SCHNEIDER et al., 2019). The DNA comparison was performed with BLASTn (v.2.6.0. +) online and it was considered only alignments with coverage and identity cutoff of 100% (perferct match). Metagenome reads from double-strand sequencing where considered once in the final read count.

3.3. SAMPLES

The sugarcane samples were collected from two different locations, denominad as A and B. The samples collected in A are composed of three sugarcane and were collected in Paulinia city, São Paulo State, Brazil (22°46'33.2"S, 47°05'55.7"W), in april of 2018. The samples from B are from a previous study described by Souza et al. (2016). For this study, the samples collected were stored in the greenhouse at the Center for Molecular Biology and Genetic Engineering (CBMEG - http: // www.cbmeg.unicamp.br), State University of Campinas (Unicamp). All the sugarcane samples were partitioned into: leaf, stalk, root and the soil of layer rhizosphere.

3.4. ISOLATION METHODS

3.4.1 Oil flotation

The samples were submitted to oil flotation method (IWATSU et al., 1981; VICENTE et al., 2008) in which approximately 20 g from each sample was incubated at room temperature for 30 min in 100 mL of sterile saline solution containing 200 U penicillin, 200µg/L streptomycin, 200 µg/L chloramphenicol and 500 µg/L cycloheximide. Subsequently 20 mL of sterile mineral oil was added, followed by vigorous shaking for 5 min. The flasks were left to settle for 20 min. Ten aliquots of the oil-water interphase of each sample were carefully collected and inoculated onto plates Mycosel agar (Difco) and incubated for 55 days at 28 °C.

3.4.2. Endophytic isolation

Following the protocols of Petrini (1991) with adaptation of Lima (2008), the sugarcane parts were cut into four fragments (approximately 0.5 cm^2) by a flame-sterilized blade in a laminar flow hood to prevent the air contamination. The surface was sterilized by immersion in 70% ethanol for 1 min, sodium hypochlorite 124 (2-2.5% active chlorine) for 4 min, 70% ethanol for 30 s and washed three times with sterile distilled water. The fragments were transferred aseptically to Petri dishes containing Mycosel agar (Difco) and incubated for 55 days at 28 °C.

3.5. FUNGAL IDENTIFICATION

3.5.1. Morphological identification

The colonies resembling black yeasts characteristics were selected and transferred to Mycosel agar. All isolates were evaluated by slide culture (DE HOOG et al., 2011) with adaptations. The fungi were inoculated on Oatmeal agar blocks, covered with glass slide and incubated at 28°C for 7, 14 and 21 days. The micro morphology was used to preliminary identification and establishment of groups.

3.5.2. Molecular and Phylogenetic identification

3.5.2.1. DNA Extraction

To extract the DNA the protocol described by Vicente et al. (2008) was used. The fungal sample was macerated in a microtube containing silica: celite (2: 1), 200 µL of CTAB and 500 µL of CIA (acidic chloroform isoamyl alcohol solution) with centrifugation for 7 min. to 16000g. The DNA was precipitated with 96% ice-cold alcohol followed by two washes with 500 µl of 70% ethanol, dehydrated and again hydrated with ultrapure water. The DNA was quantified using NanoDrop 2000® spectrophotometer and integrity was checked by 0.8% agarose gel electrophoresis.

3.5.2.2. Amplification and Sequencing

The partial large subunit of the nuclear ribosomal RNA gene (LSU) was amplified using primers NL1 and LR5 (O'DONNELL, 1993) for phylogenetic assessment. Three gene regions were chosen for species delimitation: rDNA Internal Transcribed Spacer (ITS), Elongation Factor (EF1) and β-tubulin (BT2). ITS amplicons were generated with primers V9G and LS266 (DE HOOG; GERRITS VAN DEN ENDE, 1998; MASCLAUX et al., 1995) and were sequenced with primers ITS1 and ITS4. BT2 amplification and sequencing was generated with Bt-2a and Bt-2b (GLASS; DONALDSON, 1995). PCR was performed in a 12.5 µL volume of a reaction mixture containing 1× PCR buffer, 2.0 mM MgCl2, 25 µM dNTPs, 0.5 µM of each forward and reverse primers, 1 U of DNA polymerase (LudwingBiotec) and 20 ng of genomic DNA. Amplification was performed in an ABI Prism 2720 thermocycler (Applied Biosystems, Foster City, USA), as follows: 95°C for 4 min followed by 35 cycles consisting of 95°C for 45 s, 52°C for 30 s and 72°C for 2 min and a delay at 72°C for 7 min. For some samples, annealing temperatures were changed from 50 °C to 55 °C. For BT2 the annealing temperature was changed to 58 °C. Amplicons were cleaned with Exonuclease I and Shrimp Alkaline Phosphatase (SAP) according to manufacturer's instructions. PCR products were used for sequencing using Big Dye terminator cycle sequencing RR mix protocol (ABI PRISM v. 3.1, Applied Biosystems) with the following conditions: 95 °C for 1 min, 30 cycles of 95 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Products were purified using Sephadex G-50 fine (GE Healthcare Bio-Sciences,Uppsala, Sweden) and analysed on an ABI 3730XL automatic sequencer (Applied Biosystems).

3.5.2.3. Phylogenetic analysis

Consensus sequences of the ITS, BT2, EF and the LSU regions were adjusted using the BioEdit Sequence Alignment Editor 7.2.5 (HALL, 1999) and the alignment of obtained sequences was performed using the online MAFFT (KATOH, ROZEWICKI, YAMADA, 2017). The ITS, EF and BT2 genes were first separately analyzed and them multilocus analysed. The LSU region was used to reconstruct the phylogeny of the Herpotrichiellaceae groups previously recognized (DE HOOG et al., 2011; TEIXEIRA et al., 2017). Trees were constructed with 500 bootstrap replicates using the Maximum

Likelihood Implemented in Mega v. 7 software (KUMAR et al., 2016), with the best evolutionary model to this dataset. To explore the species isolated, their sequences were compared to those deposited at GenBank.

3.5.2.4. Physiology

Cardinal growth temperatures were determined on Sabouraud media incubated for 3 weeks at temperatures of 18–42 °C at intervals of 3 °C (VICENTE et al., 2013). All tests were performed in triplicate and the diameters of the colonies were recorded in the last week. Experiments consisted of three simultaneous replicates for each tested strain; averages of three measurements were calculated. Growth velocities per species were obtained by calculation of the average including the respective standard deviations. Results were plotted with temperature (°C) versus colony diameter (mm) as parameters. Optimum range (= average ± standard deviation) and m aximum growth temperatures among species were determined three replicates. The isolates strains were represented in different colors in the graphic.

3.6. NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The DNA sequences of isolated species in this study have been deposited in GenBank and the isolates were deposited in the Microbiological Collections of Paraná Network – CMRP.

4 RESULTS

Black yeasts were identified in *silico* and in the isolation from sugarcane samples. A total of 5,834 sequeces related to the black yeasts were identified from metagenomics data. However, in the selective isolation, a total of 639 black yeasts-like fungi were recovered. All the sugarcane fragments analyzed showed colonies of black yeasts, being 591 isolates obtained from selective isolation by mineral oil flotation and 48 isolates from in the endophytic isolation method (Table 7).

Table 7 - Count of Black yeast isolates from fragmented sugarcane samples by isolation methods

Parts of sugarcane	Identification *in silico*	Isolation methods			% Isolation per sample
		Oil flotation		Endophytic	
		Samples A	Samples B	Samples A	
Rhizosphere	1,014	11	24	-	5.47
Root	-	192	1	32	35.21
Stalk (out)	3,281	17	200	-	33.95
Stalk (inside)	1,280	-	37	16	8.29
Leaf	201	109	0	0	17.05
Total	5,834	591		48	639

The isolates were clustered was performed based on the morphology and 50 isolates were selected and analyzed for taxonomic studies (Table 8). These strains were identified based on of their rDNA ITS sequences compared with reference sequences avaliable in Genbank.

The isolates were clustered was performed based on the morphology and 50 isolates were selected and analyzed for taxonomic studies (Table 8). These strains were identified based on of their rDNA ITS sequences compared with reference sequences avaliable in Genbank. A comparative analysis revealed species belonging to the genera *Cladophialophora*, *Exophiala*, *Rhinocladiella*, *Knufia*, *Cyphellophora*, *Cladopsporium*, *Minimelanolocus* and *Pleosporales*. The sugarcane samples yielded the isolation of a diversity of species of black yeasts, as *Cladophialophora bantiana*, *C. tortuosa*, *Cladosporium sphaerospermum*, *Cyphellophora oxyspora*, *Exophiala cancerae*, *E. lecanii-corni*, *E. spinifera*, *Rhinocladiella similis*, *Knufia* sp., *Minimelanolocus* sp. and *Pleosporales* sp. Some of the isolates belonged to species of clinical interest, being *C. bantiana* and *E. spinifera* were a known agent of human phaeohyphomycosis and *Rhinocladiella similis*, recently described as agent of chromoblastomycosis (Table 8).

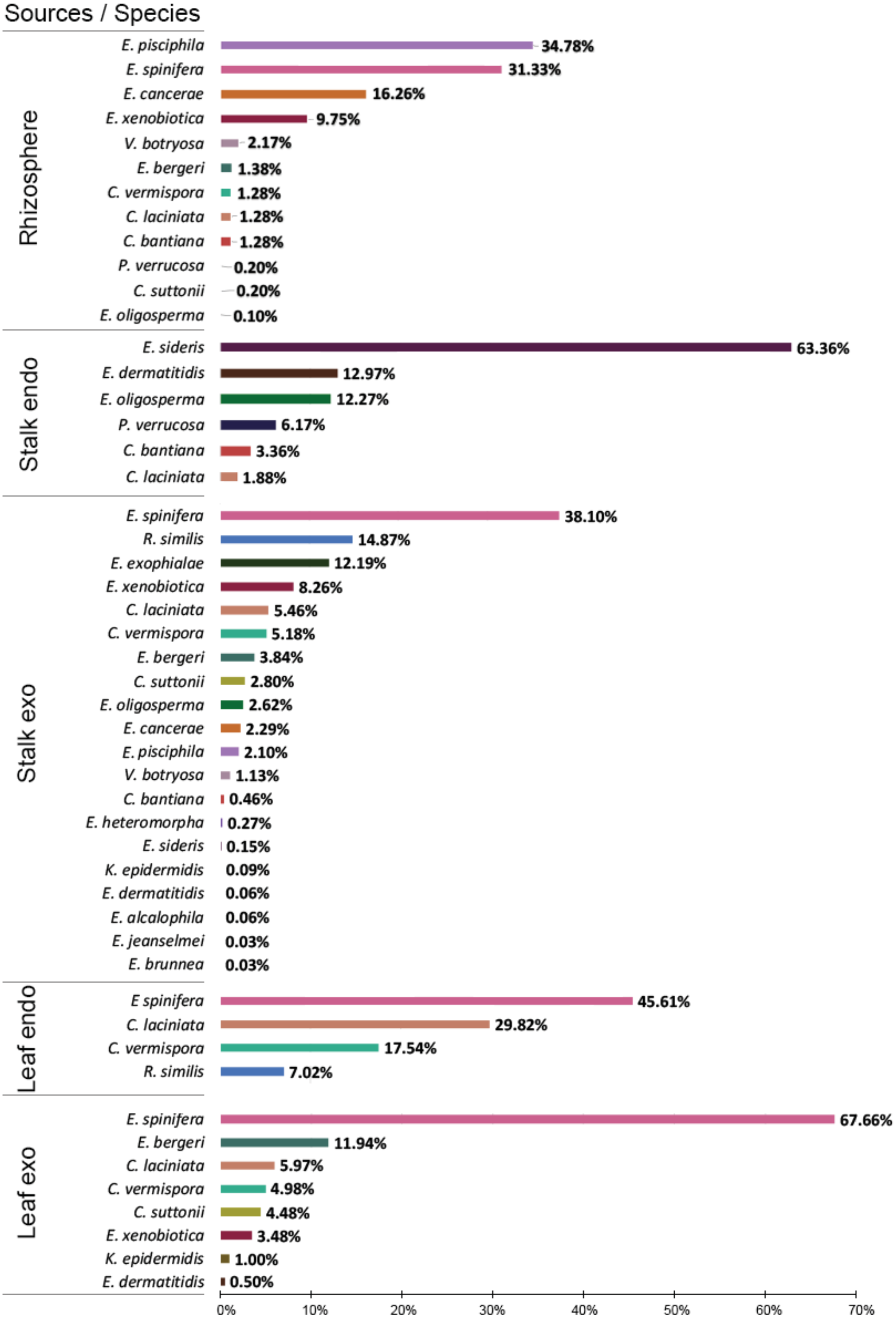
In the analysis *in silico*, a total of the 20 species were identified, being these *Cladophialophora bantiana*, *Cyphellophora laciniata*, *C. suttonii*, *C. vermispora*, *Exophiala alcalophila*, *E. bergeri*, *E. brunnea*, *E. cancerae*, *E. dermatitidis*, *E. exophialae*, *E. heteromorpha*, *E. jeanselmei*, *E. oligosperma*, *E. pisciphila*, *E. sideris*, *E. spinifera*, *E. xenobiotica*, *Knufia epidermidis*, *Phialophora verrucosa*, *Rhinocladiella similis* and *Veronaea botryosa*, distributed throughout the sugarcane compartments (Table S2).

Table 8 - Isolated strains from sugarcane

Species	Collection number of isolates	
Molecular identification		**Source**
Cladophialophora rhizus	**CMRP3553**^{T}, CMRP3556	Rhizosphere
Cladophialophora cannae	**CMRP3446**^{T}, CMRP3441, CMRP3518, CMRP3520	Root
Cladophialophora molasses	CMRP3456	Stalk
	CMRP3525, CMRP3461	Root
	CMRP3536, **CMRP3450**^{T}	Leaf
Cladophialophora bantiana	CMRP3443, CMRP3437, CMRP3438, CMRP3439	Rhizosphere
Cladophialophora tortuosa	CMRP3574, CMRP3579	Root
Cladophialophora chaetospira	CMRP3519	Root
Cladosporium sphaerospermum	CMRP3434, CMRP3435	Rhizosphere
Cyphellophora oxyspora	CMRP3526	Rhizosphere
Exophiala plantae	CMRP3444, **CMRP3436**^{T}	Rhizosphere
Exophiala cancerae	CMRP3522, CMRP3664, CMRP3440, CMRP3452	Root
	CMRP3447, CMRP3460	Endo root
Exophiala lecanii-corni	CMRP3664	Root
Exophiala spinifera	CMRP3442,	Endo root
Rhinocladiella similis	CMRP3524, CMRP3523	Root
	CMRP3448	Endo stalk
	CMRP3457, CMRP3457	Leaf
Knufia tsuneadae	CMRP3451	Endo root
Minimelanolocus sp.	CMRP3488	Root
Pleosporales sp.	CMRP3459	Endo root

The metagenomic samples were divided into rhizosphere, leaf (endo and exophytic) and stalk - bottom, medium and upper (endo and exophytic), the exophytic samples being larger with the number of sequences identified than the endophytic samples (Figure 11).

Figure 11 - Black yeasts distribution on sugarcane metagenomic samples



In order to assess the phylogenetic position of the species isolates that did not match with any described taxon, a combined partial LSU (Figure 12) and multilocus trees based on ITS, β-tubulin and Ef1 were done (Figure 13 and 14).

The trees were constructed using sequences data of representative species in Herpotrichiellaceae (Chaetothyriales). The phylogenetic analyses the isolates of *Cladophialophora* and *Exophiala* grouped separated from the taxon already described (Figure 13 and 14).

According to the LSU analisys the new isolates of *Cladophialophora* species are located amindst carrionii and salmonis clades (Figure 12). Based on the multilocus tree it was observad that the isolates CMRP3553 and CMRP3556 grouped in a distinct cluster, relatively close to *C. boppii* and *C. chaetospira*, while that the isolate strains CMRP3446 and CMRP3441 was related to *C. mycetomatis* and the strains CMRP3461 and CMRP3450 was close to *C. exuberans* (Figure 12).

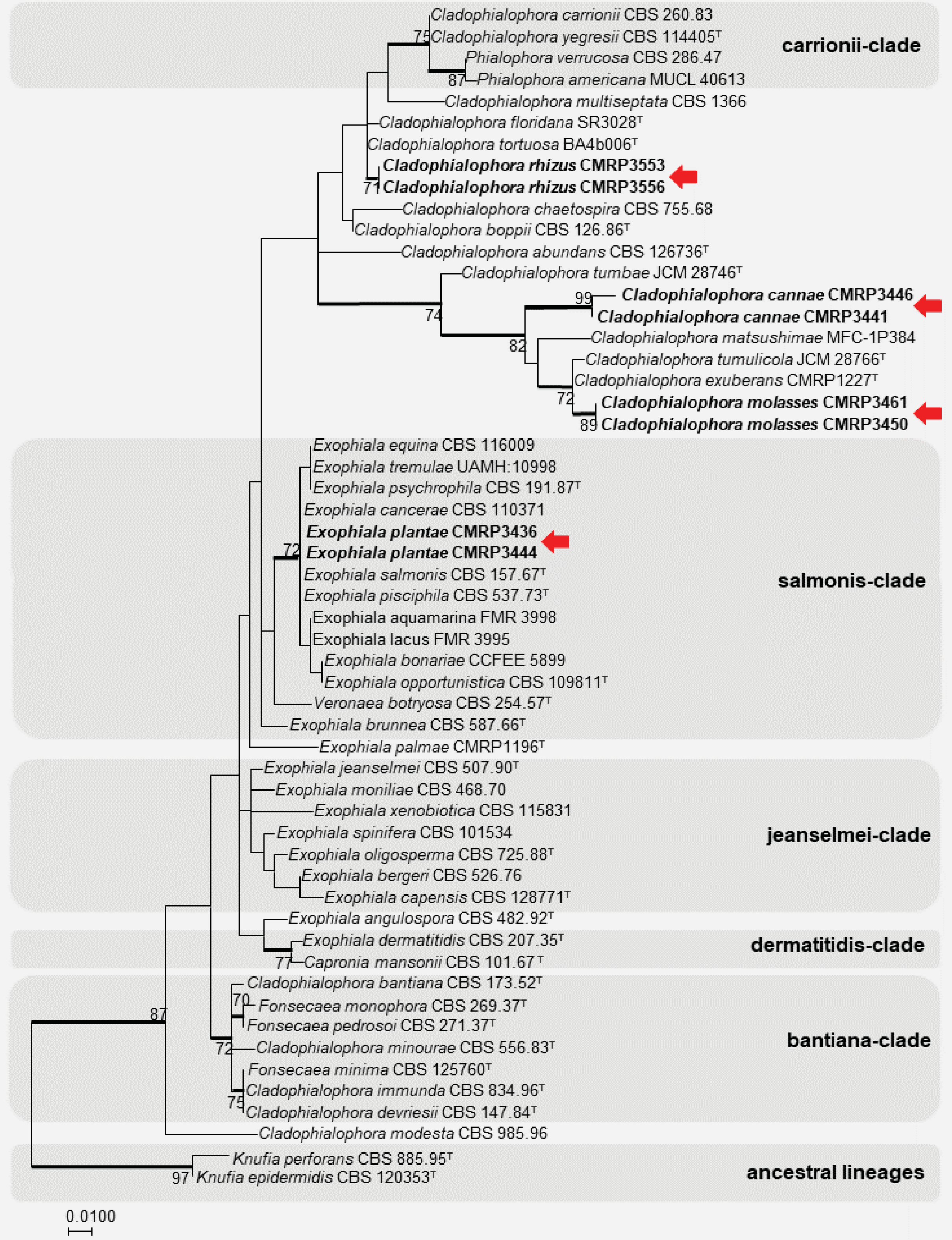
All phylogenetic trees showed the isolates of *Cladophialophora* grouped separated from the taxon already described and we will be introduced here as *Cladophialophora rhizus*, *C. cannae* and *C. molasses.*

Moreover, among the isolates showed in the tree are the four (n=4) environmental isolates of *C. bantiana* obtained from the sugarcane rhizosphere (Figure 13).

In addition, in LSU tree (Figure 12) the isolates of *Exophiala* (strains CMRP3436 and CMRP3444) were located at the salmonis clade amidst *Exophiala* spp.

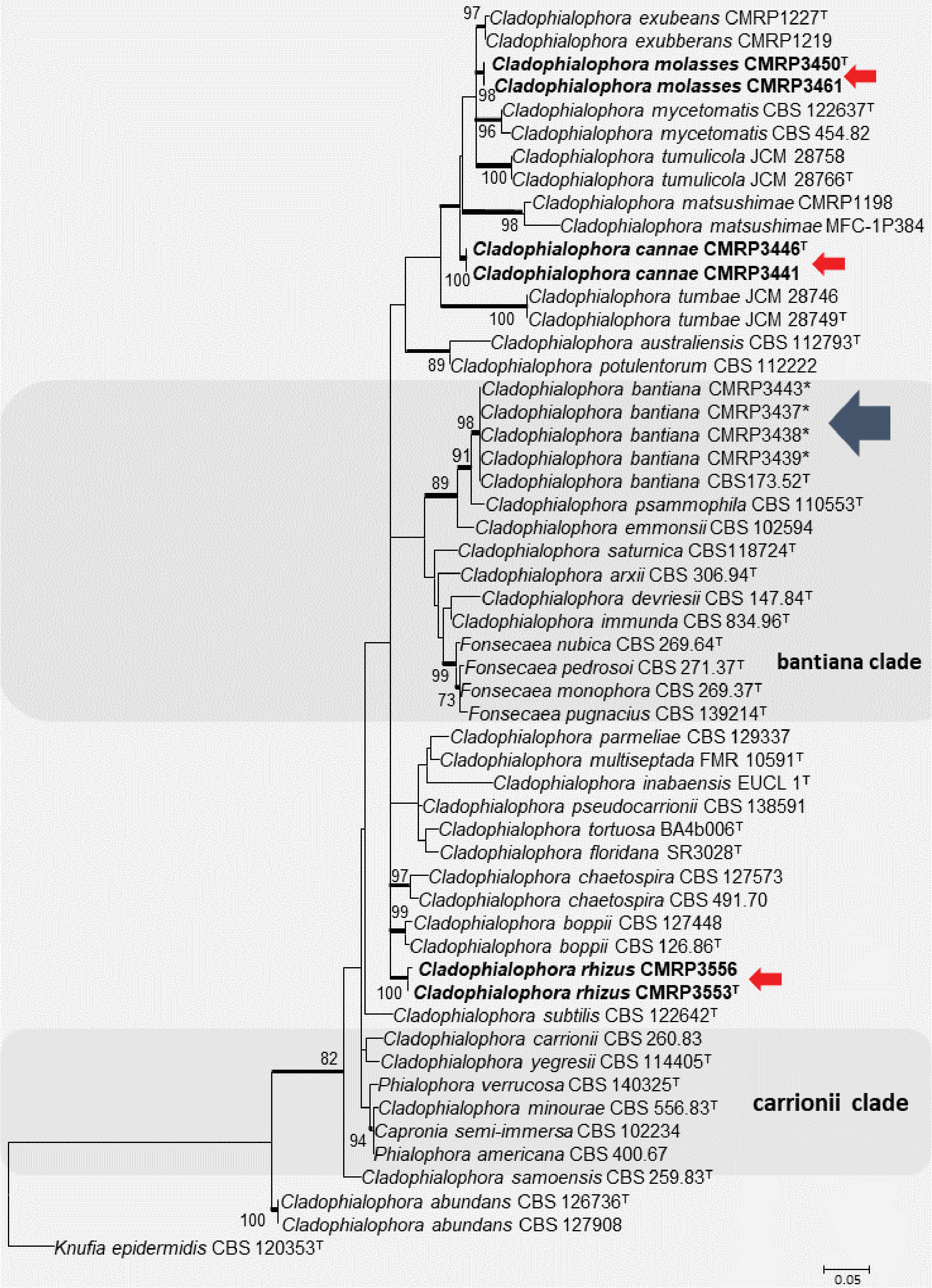
The multilocus analisys demonstrated that the isolates were close related to *E. pisciphila*, located at significantly distance from known *Exophiala* species (Figure 14). Based on it, the two strains isolated were judged to represent a novel taxon in *Exophiala* genus introduced as new species *E. plantae*.

Figure 12 - Phylogeny of a representative selection of species in Chaetothyriales based on confidently aligned LSU sequences



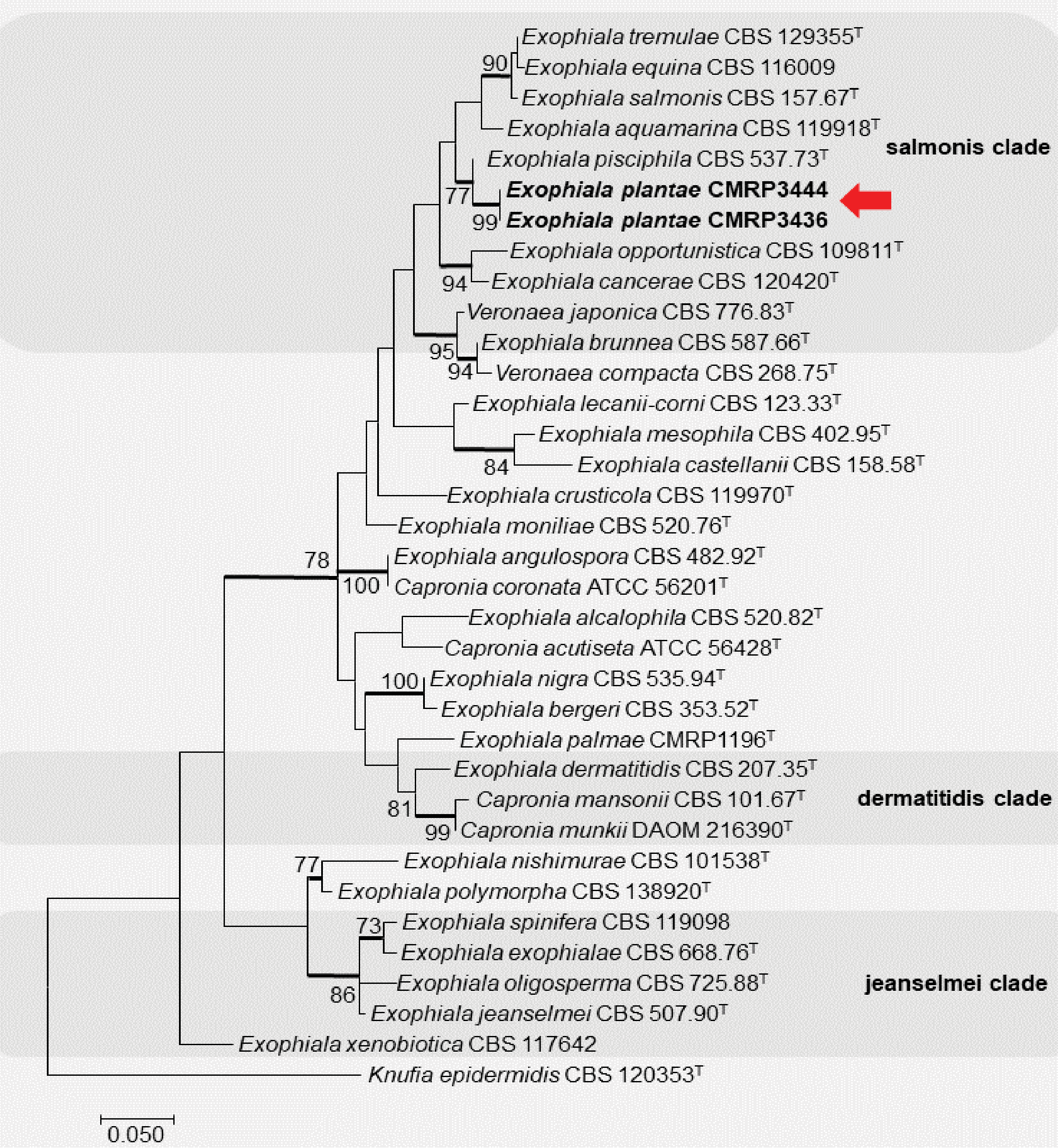
Constructed with Maximum likelihood implemented in MEGA 7. Bootstrap values > 70% from 1000 resampled datasets are shown with branches. Novel species are indicated in bold with arrows. *Knufia perforans* (CBS 885.65) and *Knufia epidermidis* (CBS120353) were used as outgroup. (T) Type strains.

Figure 13 - Multilocus tree of Cladophialophora based on ITS, partial *BT2* and partial *EF1* sequences



Constructed with maximum likelihood implemented in MEGA 7. Bootstrap values of >80% from 1000 resampled data sets are shown with branches. Novel species are indicated in bold with arrows. *Knufia epidermidis* (CBS120353) were used as outgroup. (T) Type strains. (*) Strains of *C. bantiana* isolated in this study.

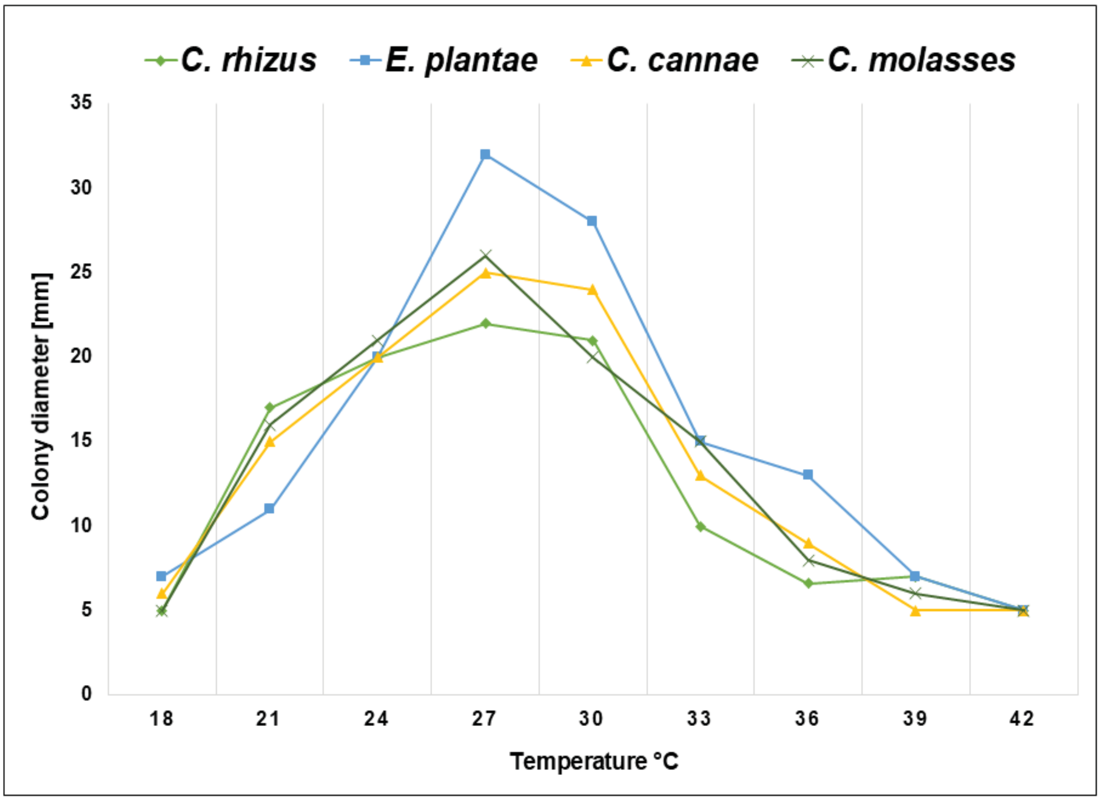
Figure 14 – Multilocus tree of *Exophiala* based on ITS, partial *BT2* and partial *EF1* sequences



Constructed with maximum likelihood implemented in MEGA 7. Bootstrap values of >70% from 500 resampled data sets are shown with branches. Novel specie is indicated in bold with arrows. *Knufia epidermidis* (CBS120353) were used as outgroup. (T) Type strains.

The environmental *Cladophialophora* isolates formed a consistent separation frim the clinical strains of the genera. The *Cladophialophora* like strains showed optimal development at 27 °C while growth was observed in the entire range between 19−37°C. In addiotional, the isolated of *Exophiala* also showed optimal development at 27°C while growth was observed in the entire range between 21−37°C. The maximum growth temperature of all strains analyzed was found to be 37°C and no growth was observed at 40°C (Figura 15).

Figure 15 - Colony diameters of novel species at different temperatures ranging from 18 to 42 °C, measured after two weeks



Based on the described above, the novel species encountered clearly separated from the other taxon already described and proposed as a novel taxon, will be introduced with the following descriptions:

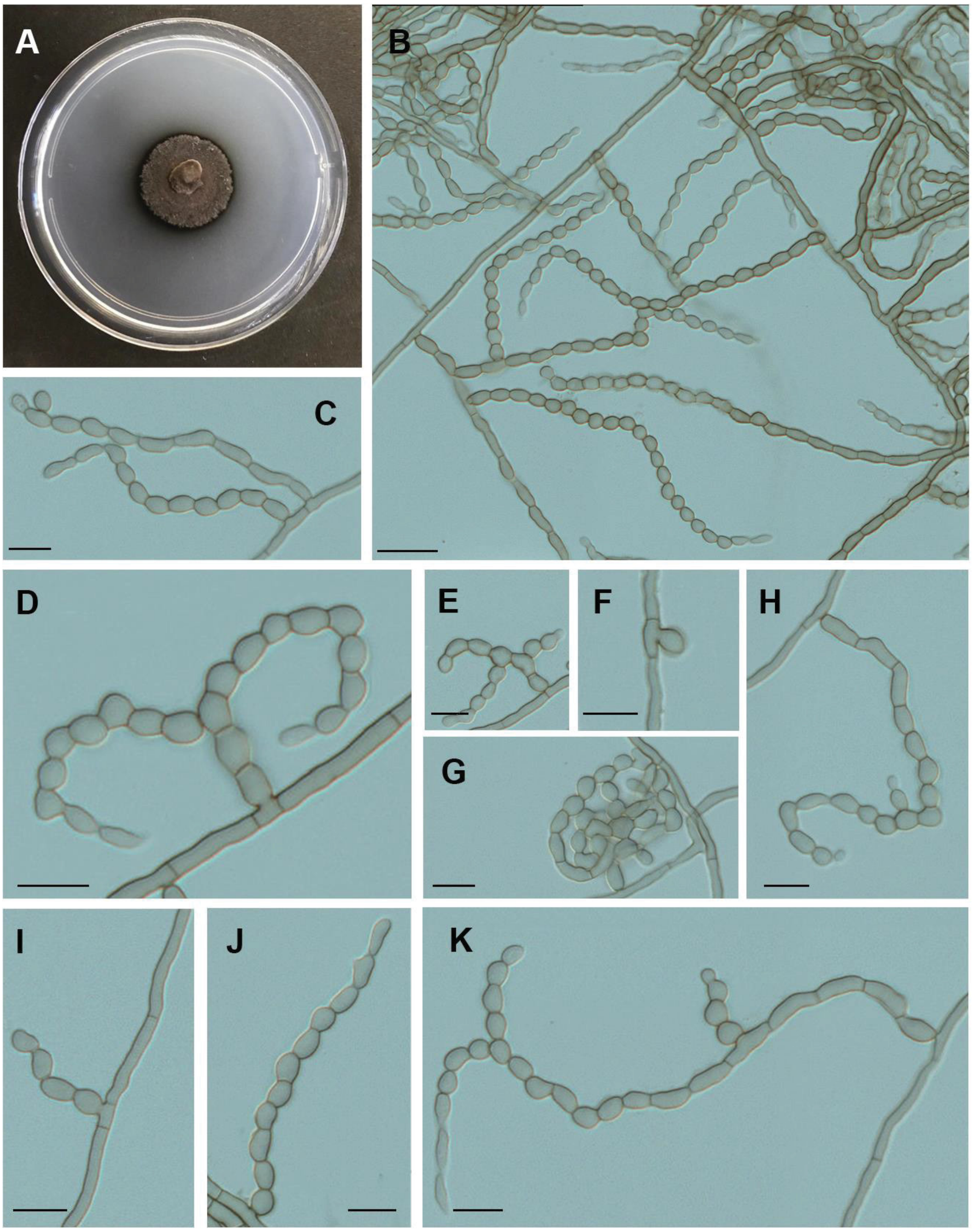
Cladophialophora rhizus Costa, de Hoog & Vicente **sp. nov.** – Figure 16.

Etymology: The name refers to the rhizhosphere substrate from which the isolated were recovered.

Holotype: Brazil, Campinas, São Paulo state, from rhizosphere of sugarcane (*Saccharum officinarum* L., Poaceae); dried holotype will be deposited at Department of Botany Herbarium at Federal University of Paraná (UPCB); Type strain culture CMRP3553. Additional material examined listed in Table 8. Description after 2 weeks incubation on MEA, 28 ºC.

Colonies moderately expanding, with color greyish olive in MEA and dark olive in agar Sabouraud with olivaceus black reverse. Hyphae, mid olivaceus to pale olivaceous brown, 2 µm wide, septate every 7.1-18.9 µm, forming long acroptal conidia chains mostly unbranched terminal or lateral, with lateral sinuously conidia chains, eventually arising in clusters. Conidiogenous cells are undifferentiated with ellipsiodal to lemon-shaped conidia with 4.5-8.2 x 2.5-3.9 µm diameters.

Figura 16 – *Cladophialophora rhizus* microscopic morphology



(A) colony on MEA; (B-C) long conidia chains; (D-E) Conidial chains arranged sinuously; (G) clusters; (H-K) Conidial chains with of ellipsoid to ovoid conidia. Scale bars 10 µm.

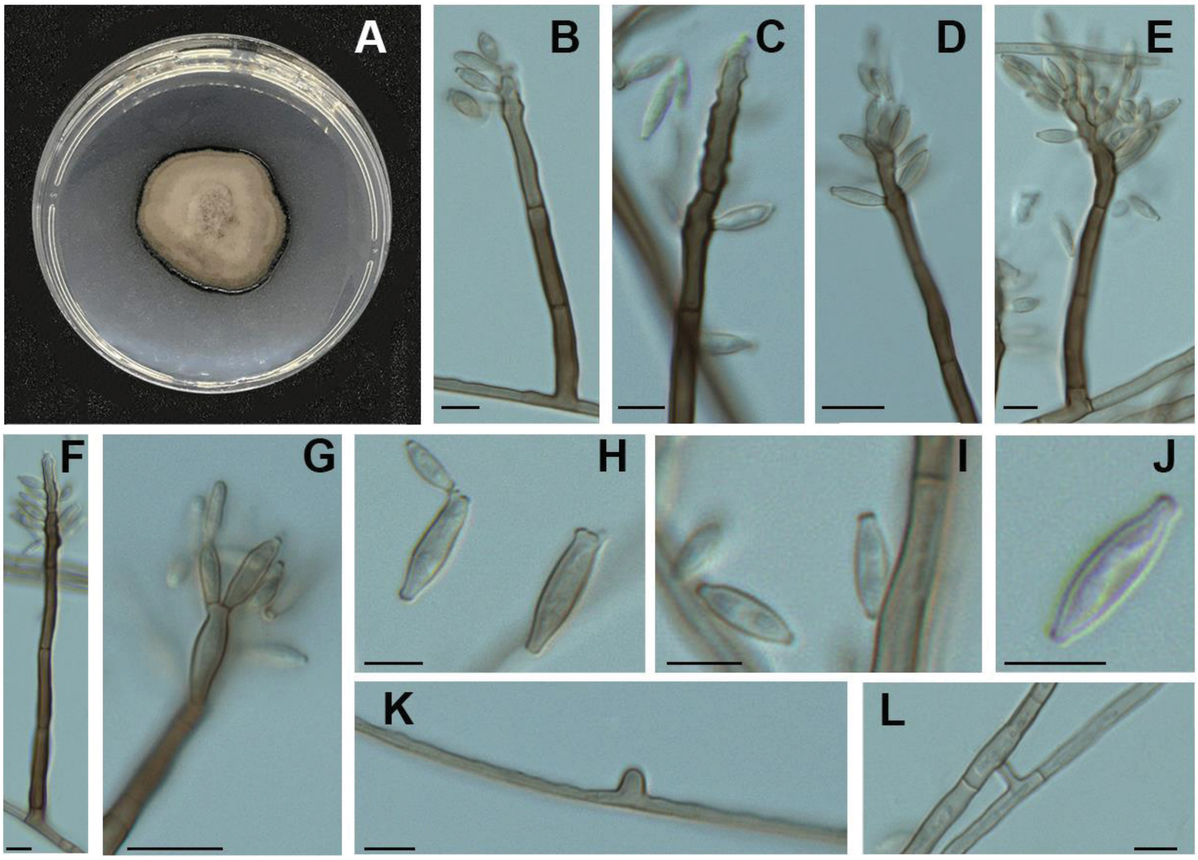
Cladophialophora cannae Costa, de Hoog & Vicente **sp. nov.** – Figure 17.

Etymology: The name refers to the isolation source of this species: the sugarcane.

Holotype: Brazil, Paulinia, São Paulo state, from sugarcane plant (*Saccharum officinarum* L., Poaceae); dried holotype will be deposited at Department of Botany Herbarium at Federal University of Paraná (UPCB); Type strain culture CMRP3446. Additional material examined listed in Table 8. Description after 2 weeks incubation on SAB, 28 ºC.

Colonies moderately expanding, with color greyish olive to and with olivaceus black reverse. Hyphae pale olivaceous to brown,1.5 µm wide, septate every 18.7–26.3 µm, with simpodial conidia in conidiophoros dark brown slightly differentiated thick -walled bears denticles up to 20 µm long. Conidia fusiform pale to olivaceous, with discernible scars 3.8-8.4 x 1.1-2.4 µm.

Figura 17 - *Cladophialophora cannae* microscopic morphology



(A) colony on SAB; (B-G) conidiophores with conidia produced in sympodial order; (H-J) conidia; (K) hyphae with chlamydospores; (L) anastomosis. Scale bars 10 µm.

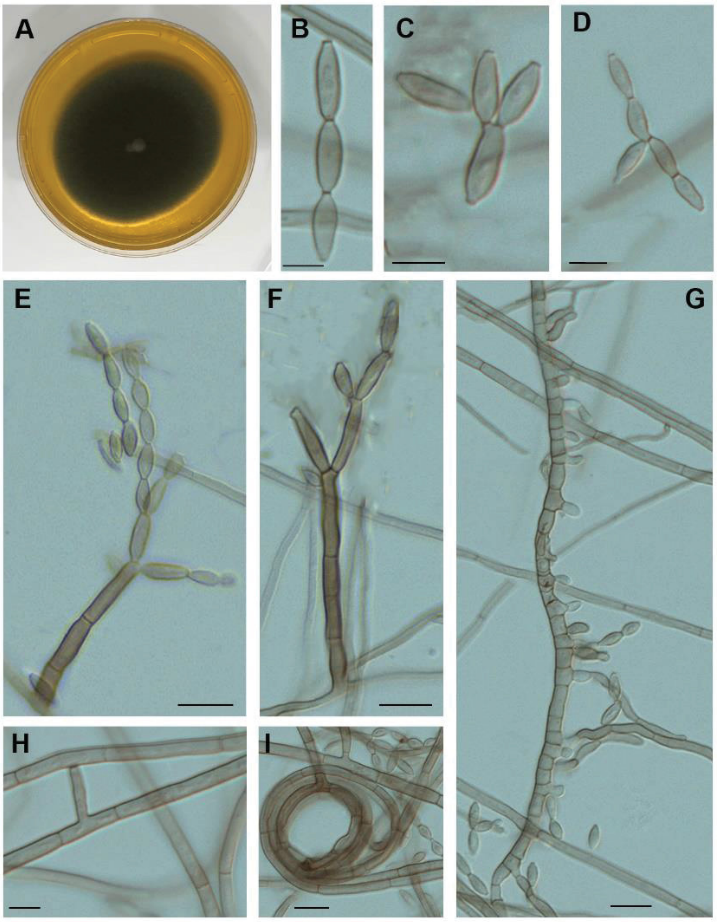
***Cladophialophora* molasses** Costa, de Hoog & Vicente **sp. nov.** – Figure 18.

Etymology: The name refers at subproduct of the sugarcane, the molasses.

Holotype: Brazil, Paulinia, São Paulo state, from sugarcane plant (*Saccharum officinarum* L., Poaceae); dried holotype will be deposited at Department of Botany Herbarium at Federal University of Paraná (UPCB); Type strain culture CMRP3450. Additional material examined listed in Table 8. Description after 2 weeks incubation on SAB, 28 ºC.

Colonies moderately expanding, with color greyish olive to and with olivaceus black reverse. Fertile hyphae pale olivaceous brown, 1.9-3.2 µm wide, septate every 16.6-37.7 µm, forming log acroptal conidia chains, branched and unbranched. Conidiophore erect eventually present. Thickened hyphae with septated every 6.5-11.3 µm presenting lateral extentions. Spirally twisted hyphae eventually present. Conidia pale brouwn lemon-shape to fusiform with dark scars, one celled 3.8-6.9x1.4-2.6 µm.

Figura 18 - *Cladophialophora molasses* microscopic morphology



(A) colony on MEA; (B-D) conidia; (E) Fertile hyphae; (F) Conidiophore; (G) Thickened hyphae; (H) anastomosis; (I) spirally twisted hyphae. Scale bars 10 µm.

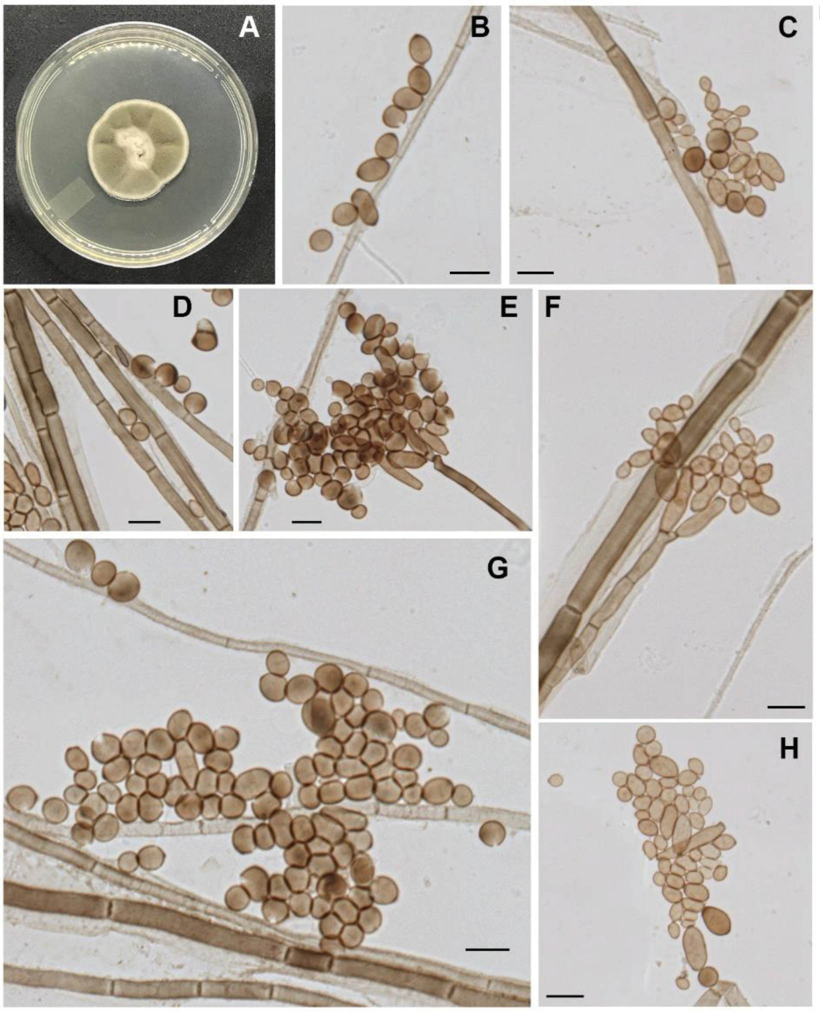
Exophiala plantae Costa, de Hoog & Vicente **sp. nov.** – Figure 19.

Etymology: The name refers to the source ot the isolation of this species, the sugarcane plant.

Holotype: Brazil, Campinas, São Paulo state, from rhizosphere of sugarcane (*Saccharum officinarum* L., Poaceae); dried holotype will be deposited at Department of Botany Herbarium at Federal University of Paraná (UPCB); Type strain culture CMRP3436. Additional material examined listed in Table 8. Description after 2 weeks incubation on Sabouraud, 28 ºC.

Colonies moderately expanding, with color greyish to black in agar Sabouraud with olivaceus black reverse. Hyphae septaded, pale brown to brown, 2.4-3.9 μm wide septate every 14-35 μm. Conidiogenous cells flask-shaped, mostly in loose clusters or branched systems. Conidia 1.6-14.1x2.6-6.6 μm with shape ellipsoidal, cylindrical and lemon-shaped.

Figura 19 – *Exophiala plantae* microscopic morphology



(A) colony on SAB; (B-E, G-H) conidia; (F) Conidiophore. Scale bars 10 μm.

5 DISCUSSION

The black yeasts and their relatives harbour numerous agents of human and animal infection (QUEIROZ-TELLES et al., 2017). In the environment they to occupy specific micro-habitats, with by a low competitive ability towards other microorganisms, and because of that they can be isolated with selective methods only (VICENTE et al., 2013). The oligotrophism enables them to survival and maintain at low density on adverse substrates where common saprobes are absent (VICENTE et al., 2001, 2008; SATOW et al., 2008). Usually, agents of disease are morphologically indistinguishable from their environmental relatives (VICENTE et al., 2013), however, the development of reliable moleculars and genomics tools are necessary for species accurate identification (VICENTE et al., 2017; MORENO; VICENTE; HOOG, 2018).

In this study we investigated the occurrence of black yeast in sugarcane samples trough *in silico* and *in vitro* methods. This study was based in a previously work done by Souza and colaborates (2016), which observed the presence of Chaetothyriales fungi in sugarcane from metagenome data. In this study a re-evaluation was proceeded using molecular tools aimind to distigui spcies in theses sample metagenomic data. According to our resuts a total of 5,834 sequences were associated to genus *Cladophialophora*, *Cyphellophora*, *Exophiala*, *Knufia*, *Phialophora*, *Rhinocladiella* and *Veronaea*. Among the species identified it was observed the presence the neurotropic fungi *C. bantiana* that causing brain infections (BADALI et al., 2008) and affects both immunocompromised and immunocompetent humans and animals (KANTARCIOGLU et al., 2016). The oil flotation method based on Vicente and colaborates (2013) was used in order to confirm the metagenomic identification with a largest number of isolates were obtained confirming a variety of fungi identified computationally.

The *in silico* analisiy reveleted the occurrence of pathogenic black yeasts rarely recovered from the environmental sources. The culture-independent methods in environmental samples may aid in the diseases ecoepidemiology elucidation. In this case, the *in silico* analysis of sugarcane samples revealed a favorable environment for black yeasts that was confirmed by the isolation by mineral oil flotation establishing a targeted isolation previously *in silico* identified.

The oil flotation isolation method recovereded some causal agents of chromoblastomycosis and the phaeohyphomycosis from environmental sources, such as *C. bantiana* (KANTARCIOGLU et al., 2016) from the rhizosphere of sugarcane, *Rinocladiella similis* (RICHARZ et al., 2018) isolated from the endophytic stalk, root and leaf, *Exophiala spinifera* (BOHELAY et al., 2016) from the endophytic root and *E. lecanii-corni* (LEE et al., 2016) from the root. Known isolates of *C. bantiana* are generally recovered from clinical or isolated cases through animal vectors, although some isolates have been recovered from hot water and brick wall scrappings samples (KANTARCIOGLU et al., 2016).

Since *C. bantiana* was described (HORRE and DE HOOG, 1999), it is environmental niche has not yet been fully elucidated (BADALI et al., 2008). There are some studies that have revealed the environmental occurrence of the specie from bark of plant and sawdust (DIXON et al., 1987), scrapings (ESPINEL-INGROFF et al., 1982) and from of hot tub water samples (JURCEVIC, 2013). However, the isolates were never sequenced, and this work was to identify and prove phylogenetically the occurrence of this pathogen in environmental sources.

Moreover, phylogenetically we identified four new species, with one belong to the *Exophaila* genus clearly delimited in salmonis-clade with 72% bootstrap support (Figure 11 and 13). In addition, it was introduced more three recognized *Cladophialophora* species. Being, this genus is polyphylethic, the isolates are located along to the Herpotrichiellaceae family. The species here described present cardinal growth temperatures optimal development at 27 °C, while that growth was observed over the entire range between 19 - 37°C, demonstrating that these fungi are saprobies, since that environmental isolates do not grow at 40 °C (Figure 14). However, the environmental strains of *C. bantiana* isolated from the rhizosphere growth until 42 °C demonstrating your pathogenicity potential.

Therefore, *in silico* methodology associated with specifics molecular tools showed a wide variety of fungus in the sugarcane, for instance the species such *C. bantiana*, *R. similis*, *E. spinifera* and *E. lecanii-corni* was isolated from the isolation methods and it also have been identified *in silico*.

Few isolation studies have proved the recovery of pathogenic black yeasts from environmental sources (MARQUES et al., 2006; VICENTE et al., 2001, 2008, 2013; NASCIMENTO et al., 2017), demonstrating that the recovery of pathogenic environmental strains is limited and rare. Besides, they can be having niches specific, not yet undiscovered (VICENTE et al, 2008). However, culture-independent methods in environmental samples may aid in the ecoepidemiology of diseases. In this case, the *in silico* analysis of sugarcane samples revealed a favorable environment for black yeasts, which was demonstrated by the isolation using mineral oil flotation. Likewise, it was possible to verify without culture independent methods the presence of these agents trought targeted tools in silico investigations draw based on the genomic data of pathogenic and environmental fungal strains.

6 CONCLUSION

The results obtained in this study showed that the combination of in silico methods such as metagenomics allied with barcodes was able of orientate the isolation method to the more efficient recover of pathogenic black yeasts. With this combination of techniques, it was possible the establishment of the *C. bantiana* environmental niche as well of other pathogenic species such as *R. similis*, *E. spinifera* and *E. lecanii-corni* recovered from associated with sugarcane plant.

Future works could be beneficiated from the joint use of metagenomics and barcodes, contributing to the elucidation of niches and to bioprospecting black yeasts in other substracts.

An important agent of the phaeohyphomycosis *Cladophialophora bantiana*, was isolated from an environmental source. As well as, was identified endophytic, saprophytic and pathogen black yeast in addition to description of four new species Herpotrichiellaceae family.

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CHAPTER VI: General Discussion and Future perpectives

GENERAL DISCUSSION AND FUTURE PERPECTIVES

This thesis focused to infer insights on eco-epidemiology and pathogenicity based on genomic and metagenomic data of black yeasts fungi belongs to Chaetothyriales. As observed in the chapter II, the genome of the Fonsecaea nubica was realiazed understand more about your pathogenicity, because with the assembly of its genome and through the annotation, it was possible to identify proteins that have basic functions related to the respiration process, structural proteins and proteins involved in the degradation process and in the metabolisms of carbohydrates, lipids, zinc and vitamins (VICENTE et al., 2017). As well as, protein-related mechanisms of tolerance and resistance and thermal shock. These data are public and available on GenBank (LVCJ00000000.1) for further study (COSTA et al., 2016).

In the chapters III, IV and V, through the analysis *in silico* with metagenomics data was possible infer insights on eco-epidemiology of the black yeast fungi belongs to Chaetothyriales in environmentals samples.

As observed in the chapter III, a variety of pathogenic agents causing different diseases were identified through by metagnomics data from Cerrado biome. The applied method demonstrated the efficiency, because with culture-independent methodologies it is possible to identify DNA fragments in samples (HANDELSMAN et al., 1998), evidencing the presence of these fungi in the samples studied. Were identified 41 pathogenic fungal species associated with human and animal infections. The variety of pathogenic fungi identified in these soils indicates the versatility of these microorganisms, such as dermatophytes, hyphomycetes, dimorphic, yeasts-like, zygomycetes, basidiomycetes, and fungi causing animal infections. Among them we identified the environmental occurrence of clinical species from the Herpothrichiellaceae family of the genus *Exophiala* and *Rhinocladiella*, besides the other human infections casual agents species of other genera.

In chapters IV and V, the *in silico* identification was based on looking for black yeasts through tools, called the molecular markers described in the literature for fungi of the Herpotriclhacae family (NAJAFZADEH et al., 2011; 2013; 2018; SCHNEIDER et al., 2019; HAMZEHEI et al., 2013; DENG et al., 2014; HEINRICHS et al., 2012), in metagenomics data from Brazil. The methodology presented in this study was shown to be a reliable and quick alternative to identify the presence of agents of clinical interest in environmental samples, which is particularly valid for fungi that are difficult to bring in culture, such as black yeasts and other opportunistic agents of human disease. This method proved to be an effective way to study microhabitats of these fungi, demonstrating the importance of mining databanks for tracking fungal agents. The results indicated that this methodology represents complementary data to studies on direct isolation via culture (SATOW et al., 2008; GUERRA et al., 2013; VICENTE et al., 2001, 2008, 2013; FENG et al., 2013; NASCIMENTO et al., 2017), which all reported low frequency of these agents in the environment. In the datasets investigated the genera *Cladophialophora*, *Exophiala*, *Fonsecaea*, *Rhinocladiella* and *Veronaea* were identified. The *Fonsecaea pedrosoi*, a major agent of chromoblastomycosis in Brazil (QUEIROZ-TELLES et al., 2017), was detected in metagenomic data from plant- and soil-associated materials. This habitat is in line with the hypothesis of chromoblastomycosis as an implantation disease from inoculated plant-derived material. The infection route of agents of chromoblastomycosis nevertheless remains controversial. Their occurrence in living plants has extensively been discussed. DE HOOG et al. (2007) and VICENTE et al. (2013) demonstrated that the main Fonsecaea species occurring in living plant belong to other species than those occurring on the human host. FORNARI et al. (2018) presented an in vitro plant infection model showing that the agents of chromoblastomycosis have a certain degree of plant-invasive ability. However, data availability is still limited, since the barcode sequences and padlocks described in the literature are restricted to the few species. But future works could be beneficiated from the joint use of metagenomics and barcodes, contributing to the elucidation of niches and to bioprospecting black yeasts in other subtracts.

As was evidenced in Chapter V, in which from the computational analysis was performed the direct isolation and recovered important fungi of clinical origin in samples of sugar cane, being the oil flotation method showed high success in recovering black yeasts. The consistent finds in the present thesis (chapetrs IV and V) validate that the of black yeasts can be inside of plants and in your compartments, as root, rhizosfere, leaf and stalk. The results obtained in this study showed that the combination of *in silico* methods such as metagenomics allied with barcodes was able of orientate the isolation method to the more efficient recover of pathogenic black yeasts. With this combination of techniques, it was possible the establishment of the *C. bantiana* environmental niche as well of other pathogenic species such as *R. similis*, *E. spinifera* and *E. lecanii-corni* recovered from associated with sugarcane plant.

An important agent of the phaeohyphomycosis *Cladophialophora bantiana* (KANTARCIOGLU et al., 2016), was isolated from an environmental source. As well as, was identified endophytic, saprophytic and pathogen black yeast in addition to description of four new species Herpotrichiellaceae family.

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APPENDIX

MEDIUM CULTURE AND SOLUTIONS USED

Sabouraud medium

Glucose 40 g
Peptone 10 g
Ágar 15 g
Destilled water 1000 mL
Autoclave 20 min at 121°C.
Final pH, 5.6 ± 0.2

Mycosel medium

Dextrose 40g
Peptone 10g
Agar 15g
Cycloheximide 0,5g
Chloramphenicol 0,05g
Autoclave 20 min at 121°C.
Final pH, 5.6 ± 0.2.

CTAB

CTAB 2 g
NaCl 8,12 g
EDTA 4 mL
Tris 10 mL
PVP 10g
Destilled water 100 mL
CIA - Chloroform Isoamylic alcohol
Chloroform 96 mL
Isoamylic alcohol 4 Ml

TBE 5X

Tris 54g
Boric acid,27,5g
EDTA 0,5M 20 mL

TBE 1X

TBE 5X 100 mL

Ultrapure water 400 mL

PBS

NaCl - 137 mM;

KCl - 2,7 mM;

Na_2HPO_4 -10 mM;

KH_2PO_4 - 2 mM

Destilled water - 1000mL

LIST OF PUBLICATIONS (2015 – 2019)

1. SCHNEIDER, G. X.; GOMES, R. R.; BOMBASSARO, A.; ZAMARCHI, K.; VOIDALESKI, M. F.; **COSTA, F. F**.; LEÃO, A. C. R.; LIMA, B.J. F. S.; SOLEY, B. S.; COLOMBO, I. R. ; CÂNDIDO, G. Z. ; NAJAFZADEH, M. J. ; SUN, J.; DE AZEVEDO, C. M. P. S.; MARQUES, S. G.; DE HOOG, G. S.; VICENTE, V. A. New Molecular Markers Distinguishing *Fonsecaea* Agents of Chromoblastomycosis. Mycopathology, v. 184, p. 493-504, 2019.

2. VICENTE, V. A., WEISS, V. A., BOMBASSARO, A., MORENO, L.F., **COSTA, F. F**., RAITTZ, R. T., LEAO, A.C., GOMES, R.R., BOCCA, A. L., FORNARI, G., CASTRO, R. J. A., SUN, J., FAORO, H., TADRA-SFEIR, M. Z., BAURA, V., BALSANELLI, E., ALMEIDA, S. R., DOS SANTOS, S. S., TEIXEIRA, M. M. S., FELIPE, M.S., NASCIMENTO, M. M. F., PEDROSA, F.O., STEFFENS, M.B., ATTILI-ANGELIS, D., NAJAFZADEH, M.J., QUEIROZ-TELLES, F., SOUZA, E. M., DE HOOG, S. Comparative Genomics of Sibling Species of *Fonsecaea* Associated with Human Chromoblastomycosis. Frontiers in Microbiology, 8, 2017, p.1924. DOI:10.3389/fmicb.2017.01924

3. LEAO, A. C. R.; WEISS, V. A.; VICENTE, V. A.; **COSTA, F**.; BOMBASSARO, A.; RAITTZ, R. T.; STEFFENS, M.B.R.; PEDROSA, F. O.; GOMES, R.R.; BAURA, V.; FAORO, H.; SFEIR, M. Z. T.; BALSANELLI, E.; MORENO, L. F.; NAJAFZADEH, M. J.; DE HOOG, S.; SOUZA, E. M. Genome Sequence of Type Strain *Fonsecaea multimorphosa* CBS 980.96, a Causal Agent of Feline Cerebral Phaeohyphomycosis. GENOME ANNOUNCEMENTS., v.5, p. e 01666-16, 2017.

4. BOMBASSARO, A.; DE HOOG, S.; WEISS, V. A.; SOUZA, E. M.; LEÃO, A. C. R.; **COSTA, F.F.**; BAURA, V.; TADRA-SFEIR, M. Z.; BALSANELLI, E.; MORENO, L. F.; RAITTZ, R.T.; STEFFENS, M.B.R.; PEDROSA, F.O.; SUN, J.; XI, L.; BOCCA, A.L.; FELIPE, M.S.; TEIXEIRA, M.; SANTOS, G. D.; TELLES FILHO, F.Q.; AZEVEDO, C. M. P. S.; GOMES, R.R.; VICENTE, V. A. Draft Genome Sequence of *Fonsecaea monophora* Strain CBS 269.37, an Agent of

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5. **COSTA, F. F**.; DE HOOG, S. RAITTZ, R.T.; WEISS, V.A.; LEÃO, A. C. R.; BOMBASSARO, A.; SUN, J.; MORENO, L.F.; SOUZA, E. M.; PEDROSA, F. O.; STEFFENS, M.B.R.; BAURA, V. TADRA-SFEIR, M.Z.; BALSANELLI, E.; NAJAFZADEH, M. J.; GOMES, R.R.; FELIPE, M.S.; TEIXEIRA, M.; SANTOS, G.D.; XI, L.; ALVES; M.A.C.; VICENTE, V.A. Draft Genome Sequence of *Fonsecaea nubica* Strain CBS 269.64, Causative Agent of Human Chromoblastomycosis. Genome Announcements, v.4, p. e00735-16, 2016.

POSTER PRESENTATIONS (2015-2019)

1. SOUZA, B. J. F.; SANTOS, G. D.; GOMES, R. R.; FORNARI, G.; NASCIMENTO, M. M.F.; **COSTA, F. F**.; BOMBASSARO, A.; HERMAN, T.; VICENTE, V. A. Diversity and antimicrobial potential of fungi belonging to the genus *Diaporthe*. Poster Presentation in 8th Brazilian Congress of Mycology, Florianópolis, Brazil, 2016.

2. **COSTA, F. F**.; BOMBASSARO, A.; WEISS, V. A.; LEAO, A. C. R.; GOMES, R. R.; RAITTZ, R. T.; CASTRO, M. A. A.; SOUZA, E. M.; DE HOOG, S. G.; VICENTE, V. A. ÁNALISE COMPARATIVA DOS GENOMAS MITOCONDRIAIS DE ESPÉCIES DO GÊNERO *Fonsecaea* DE ORIGEM CLÍNICA E AMBIENTAL. Poster Presentation in 8th Brazilian Congress of Mycology, Florianópolis, Brazil, 2016.

3. **COSTA, F. F**.; OLIVEIRA, L. L; SANTOS, G. D.; GOMES, R. R.; GLIENKE, C.; KAVA-CORDEIRO, V.; SVIDZINSKI, T. I. E.; BITTENCOURT, J. V. M.; VICENTE, VÂNIA A. Coleções de Culturas Microbiológicas da Rede Paranaense (CMRP): Rede TAXonline. Poster Presentation in 8th Brazilian Congress of Mycology, Florianópolis, Brazil, 2016.

4. BOMBASSARO, A.; WEISS, V. A.; **COSTA, F**.; LEÃO, A. C. R.; GOMES, R.; RAITTZ, R. T.; TELLES FILHO, F. Q.; SOUZA, E. M.; HOOG, S.; VICENTE, V.

A. DETECÇÃO E ANÁLISE DE GENES ASSOCIADOS À VIRULÊNCIA EM *Fonsecaea monophora*. Poster Presentation in 14th INFOCUS, Curitiba, Brazil, 2016.

5. **COSTA, F. F**.; BOMBASSARO, A.; LEAO, A. C. R.; WEISS, V. A.; RAITTZ, R. T.; VICENTE, V. A. The draft genome of *Fonsecaea nubica*. In: 28º Congresso Brasileiro de Microbiologia, 2015, Florianópolis. Anais: 28º Congresso Brasileiro de Microbiologia, v. 1, p. 1-2, 2015.

6. BOMBASSARO, A.; GOMES, R. R.; WEISS, V. A.; LEÃO, A. C.; **COSTA, F. F.**; VICENTE, V. A. The draft genome of *Fonsecaea monophora*. In: 28º Congresso Brasileiro de Microbiologia, 2015, Florianópolis. Anais: 28º Congresso Brasileiro de Microbiologia, v. 1, p. 1-2, 2015.

CONGRESS AND EVENT ORGANIZATION (2015-2019)

1. **Participant:** 28th Internacional Congress in Microbiology, Florianópolis, Brazil, 18-21 October 2015.
2. **Participant:** 8th Brazilian Congress of Mycology, Florianópolis, Brazil, 3-6 October 2016.
3. **Participant:** 4th Brazilian Congress of Genetic Resources, Curitiba, Brazil, 8-11 November 2016.
4. **Organization:** Workshop das Coleções Microbiológicas Rede *TAXonline*, Curitiba, Brazil, 11-12 August 2016.

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Draft Genome Sequence of *Fonsecaea nubica* Strain CBS 269.64, Causative Agent of Human Chromoblastomycosis

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On the basis of multilocus phylogenetic data, *Fonsecaea nubica* was described in 2010 as a molecular sibling of *F. monophora*, an established agent of the human skin disease chomoblastomycosis in tropical zones. Genome analysis of these pathogens is mandatory to identify genes involved in the interaction with host and virulence.

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The genus *Fonsecaea* comprises anamorph members in the *Chaetothyriales*, an ascomycete order of black yeasts and filamentous relatives covering numerous opportunistic pathogens on humans (1–3). *Fonsecaea* is one of the prevalent genera of etiologic agents of chromoblastomycosis (4, 5), a chronic, cutaneous, and subcutaneous infection characterized by slowly expanding, polymorphic skin lesions with muriform cells in tissue, provoking a granulomatous immune response (6, 7). The disease occurs preferentially in humans, although some cases have been reported in other mammals (8–10). Several *Fonsecaea* species are involved as etiologic agents of the disease, i.e., *F. pedrosoi*, *F. monophora*, *F. nubica*, and *F. pugnacius*, each with different virulence potentials (6, 11). *F. nubica* was first described in 2010 with type strain CBS 269.64, isolated from a human patient with chromoblastomycosis in west Cameroon (6, 12). Genome analysis of the pathogenic fungus *F. nubica* is needed to identify genes involved in the interaction with host cells and molecular mechanisms in response to cytotoxic agents (13).

Strain *F. nubica* CBS 269.64 was grown in Sabouraud's broth, with shaking at 150 rpm at 28°C for 7 days and DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method with phenol-chloroform/isoamyl alcohol. Total DNA was purified with the microbial DNA UltraClean kit. DNA of *F. nubica* was used for library construction using the Ion Plus Fragment library kit (Thermo, FisherScientific) and Nextera XT (Illumina) following the manufacturer's instructions. The libraries were sequenced on an Ion Proton (Thermo, FisherScientific) for single-end reads and in Miseq (Illumina) for paired-end reads. The quality of the reads was assessed by means of FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The reads were assembled *de novo* using SPADES v3.6.2 (14). The draft genome comprised 258 contigs and the genome size was 33.7 Mb, with a G+C content of 52.46%. Protein-coding genes were predicted with GeneMark-ES (15). Gap closure was performed with FGAP software (16). Annotation for 11,681 predicted genes was assigned based on similarity searches against the nr database using RAFTS3 (17) and InterProScan (18) comparisons. The genome contained 36 tRNAs identified using ARAGORN (19).

Information about the genome sequence of this black yeast might provide a better understanding of the basic mechanisms of adaptation to requirements of the environmental habitat, and of pathogenicity and virulence.

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited in DDBJ/ENA/GenBank under accession number LVCJ00000000. The version described in this paper is version LVCJ01000000.

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SUPLEMENTARY TABLES

Table S1 - Molecular markers described in the literature of Herpotrichiellaceae family.

Species	Strains	Number pb		References
		Barcodes	Probes padlock	
Cladophialophora arxii	CBS 306.94 T	35	42	Heinrichs, Gerold & Hoog, 2012; Hamzehei et al., 2013
	CBS 409.96	37	-	Heinrichs, Gerold & Hoog, 2012
Cladophialophora bantiana	CBS 173.52 T	34	41	Heinrichs, Gerold & Hoog, 2012; Hamzehei et al., 2013
Cladophialophora chaetospira	CBS 115468	-	39	Hamzehei et al., 2013
Cladophialophora carrionii	CBS 160.54 LT	29	42 /39	Heinrichs, Gerold & Hoog, 2012; Hamzehei et al., 2013; Deng et al., 2014
	CBS 163.54	29	-	Heinrichs, Gerold & Hoog, 2012
	CBS 260.83	29	-	Heinrichs, Gerold & Hoog, 2012
	FMC.248	29	-	Heinrichs, Gerold & Hoog, 2012
Cladophialophora devriesii	CBS 147.84 T	25	42	Heinrichs, Gerold & Hoog, 2012; Hamzehei et al., 2013
	IFM51369	25	-	Heinrichs, Gerold & Hoog, 2012
Cladophialophora emmonsii	CBS 979.96 T	33	-	Heinrichs, Gerold & Hoog, 2012
	CBS 640.96	33	-	Heinrichs, Gerold & Hoog, 2012
Cladophialophora immunda	CBS 834.96 T	25	41 / 42	Heinrichs, Gerold & Hoog, 2012; Hamzehei et al., 2013
	CBS 102227	25	-	Heinrichs, Gerold & Hoog, 2012
Cladophialophora minourae	CBS 987.96	-	42	Hamzehei et al., 2013
Cladophialophora modesta	CBS 985.96 T	26	-	Heinrichs, Gerold & Hoog, 2012
C.mycetomatis	CBS 122637 T	28	-	Heinrichs, Gerold & Hoog, 2012
	CBS 454.82	28	-	Heinrichs, Gerold & Hoog, 2012
C. psammophila	CBS 110553 T	34	41	Heinrichs, Gerold & Hoog, 2012; Hamzehei et al., 2013
Cladophialophora samoensis	CBS 259.83 T	31	-	Heinrichs, Gerold & Hoog, 2012
Cladophialophora saturnica	CBS 118724 T	27	42	Heinrichs, Gerold & Hoog, 2012; Hamzehei et al., 2013
Cladophialophora subtilis	CBS 122642 T	30	-	Heinrichs, Gerold & Hoog, 2012
Cladophialophora yegresii	CBS 114405 T	30	42	Heinrichs, Gerold & Hoog, 2012; Hamzehei et al., 2013
Exophiala alcalophila	CBS 520.82 T	31	-	Heinrichs, Gerold & Hoog, 2012
Exophiala angulospora	CBS 482.92 T	28	36	Heinrichs, Gerold & Hoog, 2012; Najafzadeh et al., 2018
	CBS 122264	28	-	Heinrichs, Gerold & Hoog, 2012
	CBS 109906	28	-	Heinrichs, Gerold & Hoog, 2012
Exophiala aquamarina	CBS 119918 T	38	37	Heinrichs, Gerold & Hoog, 2012; Najafzadeh et al., 2018
Exophiala asiatica	BMU00015 T	36	-	Heinrichs, Gerold & Hoog, 2012
Exophiala attenuata	CBS110026	29	-	Heinrichs, Gerold & Hoog, 2012
Exophiala bergeri	CBS 353.52 T	31	-	Heinrichs, Gerold & Hoog, 2012
Exophiala brunnea	CBS 587.66 T	38	-	Heinrichs, Gerold & Hoog, 2012
Exophiala cancerae	CBS 120532 T	34	-	Heinrichs, Gerold & Hoog, 2012
Exophiala castellanii	CBS 158.58 T	28	37	Heinrichs, Gerold & Hoog, 2012; Najafzadeh et al., 2018
Exophiala capensis	CBS 128771 T	31	-	Heinrichs, Gerold & Hoog, 2012
Exophiala dermatitidis	CBS 207.35 T	32	36	Heinrichs, Gerold & Hoog, 2012; Najafzadeh et al., 2013
	CBS 100338	32	-	Heinrichs, Gerold & Hoog, 2012
	BMU00035	32	-	Heinrichs, Gerold & Hoog, 2012
Exophiala equina	CBS 119.23 T	38	-	Heinrichs, Gerold & Hoog, 2012
	CBS 122263	38	-	Heinrichs, Gerold & Hoog, 2012
	CBS 122270	38	-	Heinrichs, Gerold & Hoog, 2012
Exophiala exophialae	CBS 668.76 T	32	-	Heinrichs, Gerold & Hoog, 2012
Exophiala halophila	CBS 121512 T	34	-	Heinrichs, Gerold & Hoog, 2012
Exophiala heteromorpha	CBS 232.33 T	29	-	Heinrichs, Gerold & Hoog, 2012
Exophiala jeanselmei	CBS 507.90 T	36	37	Heinrichs, Gerold & Hoog, 2012; Najafzadeh et al., 2013
	CBS 677.76	37	-	Heinrichs, Gerold & Hoog, 2012
Exophiala lacus	CBS 117497 T	37	-	Heinrichs, Gerold & Hoog, 2012
Exophiala lecanii-corni	CBS 123.33 T	29	-	Heinrichs, Gerold & Hoog, 2012
Exophiala mesophila	CBS 402.95 T	28	-	Heinrichs, Gerold & Hoog, 2012
	CBS 121511	28	-	Heinrichs, Gerold & Hoog, 2012
Exophiala moniliae	CBS 520.76 T	26	-	Heinrichs, Gerold & Hoog, 2012
Exophiala nishimurae	CBS 101538 T	32	-	Heinrichs, Gerold & Hoog, 2012

To be continued.

Table S1 - Molecular markers described in the literature of Herpotrichiellaceae family.

Exophiala oligosperma	CBS 725.88 T	38	37	Heinrichs, Gerold & Hoog, 2012; Najafzadeh et al., 2013
	UTHSC95-2041	38	-	Heinrichs, Gerold & Hoog, 2012
	UTHSC91###870	39	-	Heinrichs, Gerold & Hoog, 2012
	IFM41701	40	-	Heinrichs, Gerold & Hoog, 2012
Exophiala opportunistica	CBS109811	39	36	Heinrichs, Gerold & Hoog, 2012; Najafzadeh et al., 2018
Exophiala pisciphila	CBS 537.73 T	39	37	Heinrichs, Gerold & Hoog, 2012; Najafzadeh et al., 2018
Exophiala salmonis	CBS 157.67 T	37	-	Heinrichs, Gerold & Hoog, 2012
Exophiala sideris	CBS 121818 T	29	-	Heinrichs, Gerold & Hoog, 2012
Exophiala spinifera	CBS 899.68 T	32	34	Heinrichs, Gerold & Hoog, 2012; Najafzadeh et al., 2013
Exophiala siphonis	UTHSC88-471	27	-	Heinrichs, Gerold & Hoog, 2012
Exophiala xenobiotica	CBS 118157 T	30	37	Heinrichs, Gerold & Hoog, 2012; Najafzadeh et al., 2013
	CBS 119306	30	-	Heinrichs, Gerold & Hoog, 2012
	CBS 117665	30	-	Heinrichs, Gerold & Hoog, 2012
	CBS 117676	33	-	Heinrichs, Gerold & Hoog, 2012
	CBS 117641	32	-	Heinrichs, Gerold & Hoog, 2012
Fonsecaea erecta	dH20513	33	-	Heinrichs, Gerold & Hoog, 2012
	dH20502	33	-	Heinrichs, Gerold & Hoog, 2012
Fonsecaea minima	dH20511	33	-	Heinrichs, Gerold & Hoog, 2012
Fonsecaea monophora	CBS 269.37 T	36	28	Heinrichs, Gerold & Hoog, 2012; Najafzadeh et al., 2011
	CBS 121732	36	-	Heinrichs, Gerold & Hoog, 2012
	IFM4889	36	-	Heinrichs, Gerold & Hoog, 2012
	IFM54446	36	-	Heinrichs, Gerold & Hoog, 2012
Fonsecaea multimorphosa	CBS 980.96 T	27	-	Heinrichs, Gerold & Hoog, 2012
Fonsecaea nubica	CBS 269.64 T	36	36	Heinrichs, Gerold & Hoog, 2012; Najafzadeh et al., 2011
	CBS 121733	36	-	Heinrichs, Gerold & Hoog, 2012
	CBS 557.76	36	-	Heinrichs, Gerold & Hoog, 2012
Fonsecaea pedrosoi	CBS 271.37 T	36	36	Heinrichs, Gerold & Hoog, 2012; Najafzadeh et al., 2011
	CBS 122741	37	-	Heinrichs, Gerold & Hoog, 2012
Fonsecaea pugnacius	CBS 139214	-	31	Schneider et al., 2019
Phialophora americana	CBS 840.69	30	-	Heinrichs, Gerold & Hoog, 2012
Phialophora verrucosa	CBS 273.37	30	-	Heinrichs, Gerold & Hoog, 2012
	IMTSP.800	30	-	Heinrichs, Gerold & Hoog, 2012
	CBS 286.47	30	-	Heinrichs, Gerold & Hoog, 2012
	CBS 839.68	30	-	Heinrichs, Gerold & Hoog, 2012
Rhinocladiella anceps	CBS 181.65	30	-	Heinrichs, Gerold & Hoog, 2012
	CBS 157.54	32	-	Heinrichs, Gerold & Hoog, 2012
Rhinocladiella aquaspersa	CBS 313.73 T	27	-	Heinrichs, Gerold & Hoog, 2012
Rhinocladiella atrovirens	CBS 264.49	28	-	Heinrichs, Gerold & Hoog, 2012
Rhinocladiella basitona	CBS 101460 T	31	-	Heinrichs, Gerold & Hoog, 2012
Rhinocladiella mackenziei	CBS 650.93 T	37	-	Heinrichs, Gerold & Hoog, 2012
	CBS 367.92	35	-	Heinrichs, Gerold & Hoog, 2012
	CBS 102590	35	-	Heinrichs, Gerold & Hoog, 2012
Rhinocladiella similis	CBS 111763 T	32	-	Heinrichs, Gerold & Hoog, 2012
	dH13054	32	-	Heinrichs, Gerold & Hoog, 2012
Veronaea botryosa	CBS 254.57 T	40	35	Heinrichs, Gerold & Hoog, 2012; Najafzadeh et al., 2018
	CBS 350.65	41	-	Heinrichs, Gerold & Hoog, 2012
Veronaea compacta	CBS 268.75 T	38	-	Heinrichs, Gerold & Hoog, 2012
Veronaea japonica	CBS 776.83 T	38	-	Heinrichs, Gerold & Hoog, 2012

Table S2 - Species and sequence quantities found in the *in silico* identification of sugarcane.

Species	Compartments of sugar cane									
	Rhizosphere	Leaf		Bottom stalk		Medium Stalk		Upper stalk		
		Endo	Exo	Endo	Exo	Endo	Exo	Endo	Exo	**Total**
C. bantiana	13	0	0	43	14	0	1	0	0	71
C. laciniata	13	17	12	0	60	0	83	0	36	221
C. suttonii	2	0	9	0	74	24	7	0	11	127
C. vermispora	13	10	10	0	58	0	80	0	32	203
E. alcalophila	0	0	0	0	0	0	0	0	2	2
E. bergeri	14	0	24	0	38	0	86	0	2	164
E. brunnea	0	0	0	0	0	0	0	0	1	1
E. cancerae	165	0	0	0	13	0	62	0	0	240
E. dermatitidis	0	0	1	0	0	0	2	0	0	3
E. exophialae	0	0	0	58	118	108	208	0	74	566
E. heteromorpha	0	0	0	0	0	0	9	0	0	9
E. jeanselmei	0	0	0	0	1	0	0	0	0	1
E. oligosperma	0	0	0	0	9	0	77	0	0	87
E. pisciphila	353	0	0	10	57	46	12	101	0	579
E. sideris	0	0	0	0	3	0	2	0	0	5
E. spinifera	318	26	136	304	350	202	398	305	502	2541
E. xenobiotica	99	0	7	0	73	0	196	0	2	377
K. epidermidis	0		2	0	0	0	3	0	0	5
P. verrucosa	2		0	0	0	0	0	0	0	2
R. similis	0	4	0	52	131	23	101	4	256	571
V. botryosa	22		0	0	20	0	14	0	3	59
Total	1,014	57	201	467	1,019	403	1,341	410	921	5,834