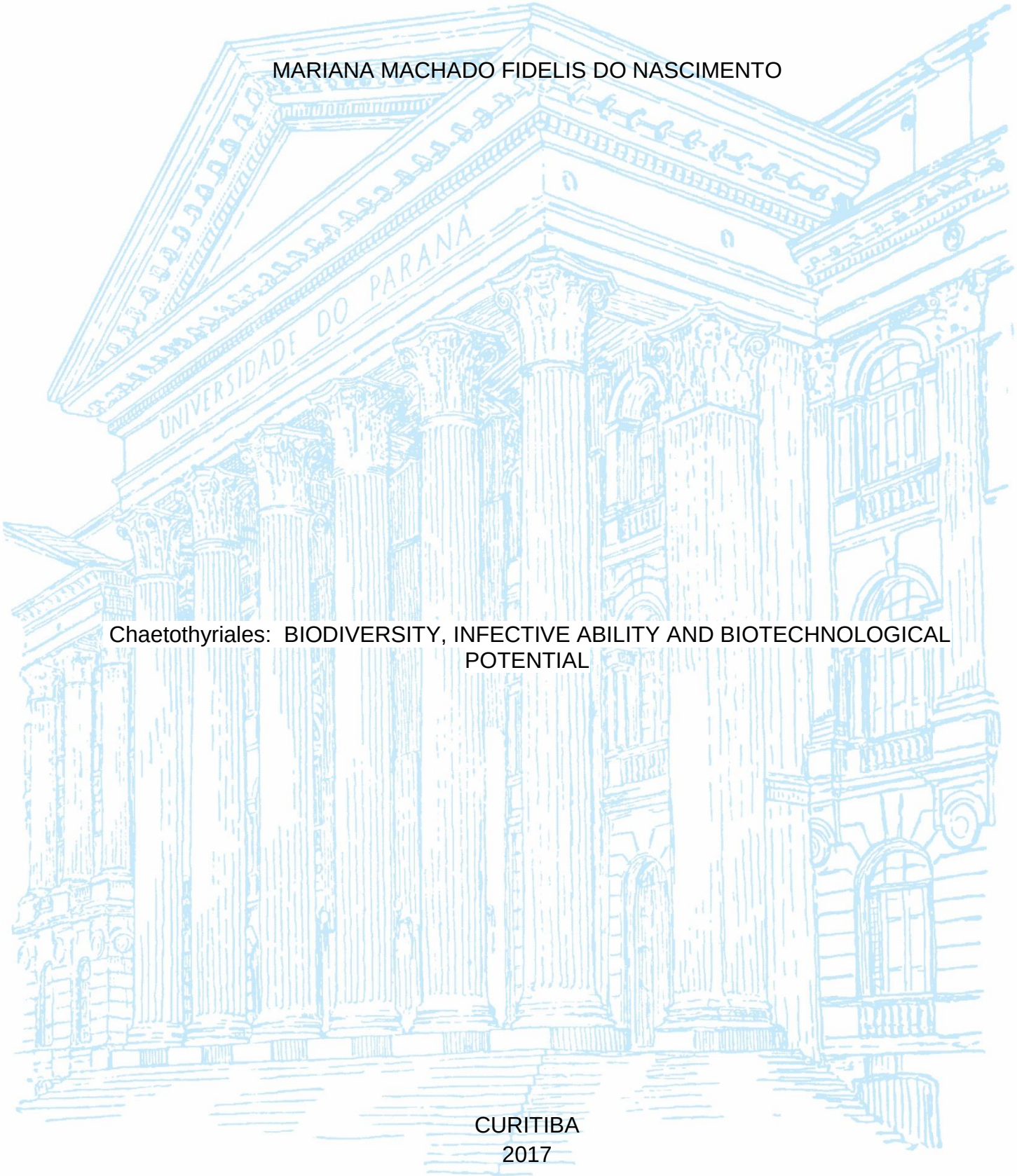
FEDERAL UNIVERSITY OF PARANA

MARIANA MACHADO FIDELIS DO NASCIMENTO



Chaetothyriales: BIODIVERSITY, INFECTIVE ABILITY AND BIOTECHNOLOGICAL POTENTIAL

CURITIBA
2017

MARIANA MACHADO FIDELIS DO NASCIMENTO

Chaetothyriales: BIODIVERSITY, INFECTIVE ABILITY AND BIOTECHNOLOGICAL POTENTIAL

Thesis presented as partial requirement for obtaining the Doctor degree in Microbiology, Parasitology and Pathology, Microbiology Area. Post-graduation Program in Microbiology, Parasitology and Pathology, Federal University of Parana.

Advisor: Gerrit Sybren de Hoog, Ph.D

Co-Advisor:
Vania Aparecida Vicente, Ph.D

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TERMO DE APROVAÇÃO

"*Chaetothyriales*: Biodiversity, Infective ability and Biotechnological potential"

por

Mariana Machado Fidelis do Nascimento

Tese aprovada como requisito parcial para obtenção do grau de Doutor no Curso de Pós-Graduação em Microbiologia, Parasitologia e Patologia, pela Comissão formada pelos professores:

Profa. Dra. Vânia Aparecida Vicente

Dr. Francesc Prenafeta-Boldú

Prof. Dr. Miguel Daniel Noseda

Profa. Dra. Juliana Vitória Messias Bittencourt

Prof. Dr. Diogo Robl

Curitiba, 31 de março de 2017.

ADVISOR COMMENTS

It has been a great pleasure to have Mariana in my lab. She is a competent and motivated worker, understood all the tasks quickly, and soon proved to be able to guide others with the work. Data for essential papers have been produced within a rather short period, which was possible because she was already well-trained in Curitiba when she arrived at CBS. One of the papers was the isolation of black fungi from Babassu coconut shells. Data generation was well organized, and the isolation method efficient, so that she was able to obtain an astonishing number of black yeast-like fungi. The significance of this is that this rather special, ester- and oil-rich microenvironment of gradually decomposing and fermenting shells provides a special environment which proved to be selective for black yeasts of which we did not know the ecological niche before. One of these was *Cladophialophora mycetomatis*, previously only known from a single human infection, and another *Exophiala spinifera*. The latter is a potential agent of human disseminated infections, just as *Exophiala dermatitidis*. For the clinician these two fungi seem similar, but thanks to Mariana's work we now know that they are ecologically very different; this certainly has implications for the route and course of human infection.

A second paper concerns *Arthrocladium*, an enigmatic genus in the Trichomeriaceae family in Chaetothyriales. Mariana described a novel species in this genus, that was the cause of a fatal infection in a human with a rare immune disorder. This is the first black fungus outside the Herpotrichiellaceae to cause such an infection, and will certainly stimulate further work on genomic comparison.

In summary, Mariana did an excellent job with which I would like to congratulate her, and without doubt she deserves the PhD status.

Yours sincerely, Sybren de Hoog

“All we have yet discovered is but a trifle in comparison with what lies hid in the great treasury of nature”.

Antonie van Leewenhoek (1680)

Dedico

Aos meus amados pais, Mariza e José.

Ao meu irmão Francisco.

Muito obrigado por tudo!

I dedicate

To my beloved parentes, Mariza and José.

To my brother Francisco

Thank you so much for everything!

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I am grateful for the support from Federal University of Parana (UFPR) and also to the professors from the Basic Pathology Department.

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I am thankful to my supervisor, Professor Dr. Sybren de Hoog, leader of the group "M*edical Mycology*" at Westerdijk Fungal Biodiversity Institute in Utrecht, The Netherlands, for his excellent scientific supervision, patient guidance, ideas, advice, and critical reading of the manuscripts. I am also thankful for the wonderful experience and opportunity to develop part of my PhD thesis in his research group.

I would like to express my gratitude to my co-supervisor, Professor Dr. Vania Aparecida Vicente for not only their encouragement but for your trust in me, and offer me PDSE fellowship, constant motivation and also for the valuable discussions across the black yeasts world. I am honoured to have her guidance during my PhD.

A part of my PhD research was done in the Institut de Recerca e Tecnologia Agroalimentàries (IRTA), Caldes de Montbui, Spain under the supervision of Dr. Francesc Prenafeta-Boldú. Thank you for receiving me in your lab and assisting me in the Experimental design, data analysis and for contributing to the manuscript.

I am thankful to the Professor Dr. Juliana Vitória Messias Bittencourt from Federal University of Technology – Parana for her encouragement, guidance and confidence.

I would like to thank my fellow co-works at LabMicro, specially Germana, Gheniffer, Juciliane and Renata for the discussions, help, and for all the fun we have had in the last four years. Also I thank and express admiration for Sarah Abdallah Ahmed (Sudan), Leandro Moreno (Brazil), Maycoll Romero (Colombia), Laura Tey (Spain),

Ana Sotrez (Spain), Anne van Diepeningen (The Netherlands), Karolina Dukik (Macedonia), Bert Gerrits van den Ende (The Netherlands), Kittipan Samerpitak (Thailand), Abdullah Mohammed Al-Hatmi (Oman), and Brankovics Balázs (Hungary), Anderson Messias Rodrigues (Brazil) from the following institution Westerdijk Fungal Biodiversity Institute, and Institut de Recerca e Tecnologia Agroalimentàries.

I need to thank all my friends, particularly Carla, Marisol, Lorena, Tamires, and Mary Helen, who always took the time to listen, even when I was just complaining. A special thanks to my dear friend Jason Lee Furuie for all your support, friendship, patience and continuously being so kind and helpful. I also would like for thank his family to makes me feel part of the family.

Finally, I would like to thank all those that contributed to make this effort a success and to God for support me and giving this opportunity. Last but not the least, a special thanks to my parents Mariza and José. Words cannot express my grateful for them. I would also like to thank my beloved boyfriend, Amauri, and my stepson Francisco, for being patience, lovely and so kind with me. I am so grateful and thankful for supporting me for everything.

RESUMO

Todas as leveduras negras membros da ordem Chaetothyriales apresentam melanina na parede celular, um ciclo de vida polimórfico, com uma ecologia significativamente variável, e inclui cinco famílias, Chaetothyriaceae, Cyphellophoraceae, Epibryaceae, Herpotrichiellaceae e Trichomeriaceae. Por um lado, a família Herpotrichiellaceae tem sido a mais estudada, sendo caracterizada por uma ecologia complexa, que compreende espécies consideradas sapróbias, espécies com importância clínica e habilidades assimilativas de hidrocarbonetos monoaromáticos. Por outro lado, a família Trichomeriaceae é uma família recentemente estabelecida e inclui principalmente organismos epífitos e epilíticos. Com base nessa variável e complexa ecologia dos membros da ordem Chaetothyriales, o objetivo da tese foi estudar a diversidade e ecologia da ordem Chaetothyriales com foco em Herpotrichiellaceae e Trichomeriaceae, duas famílias distintas em ecologia e características. Por esta razão, a tese foi dividida em capítulos. A diversidade de leveduras pretas em cascas de coco de babaçu foi investigada aplicando diferentes técnicas de isolamento (Capítulo II). Os isolados de levedura negras obtidos foram identificados pelo sequenciamento da região ITS. Entre os isolados, foram reconhecidas espécies com importância clínica como *Veronaea botryosa*, *Rinhocladiella similis* e *Exophiala spinifera* e duas novas espécies foram descritas na família Herpotrichiellaceae. A capacidade de crescer em tolueno e fenilacetato como única fonte de carbono e energia foi avaliada para oito linhagens. Em relação ao fenilacetato, um composto intermediário da via de degradação do estireno, todas as estirpes testadas foram capazes de assimilar o composto, no entanto, apenas dois isolados assimilam tolueno. A presença de compostos aromáticos voláteis (VOCs) no coco de babaçu foi investigada utilizando GC-ToFMS, e foram identificados um total de 140 compostos, sendo o álcool o composto mais abundante (2023 µg m-3). O gênero Arthrocladium foi restabelecido com base nas análises filogenéticas e atribuído a família Trichomeriaceae (Capítulo IV). A identificação preliminar baseada no sequenciamento da região ITS mostrou que as três espécies não descritas se agrupavam próximas a *Arthrocladium caudatum,* linhagem tipo do gênero. A análise em multilocus confirmou a posição filogenética destas espécies assim como a posição taxonômica do género na família Trichomeriaceae. Com base nesta inferência foram descritas três espécies sem esporulação procedentes de diferentes substratos tais como, o coco da palmeira babaçu, formigas e de amostras de tecido cerebral a partir de uma infecção disseminada fatal em um paciente com distúrbio imunológico genético. Os resultados deste estudo nos levaram a elucidar alguns aspectos da ecologia das leveduras negras, bem como desvendar o potencial biotecnológico dos microrganismos de procedência ambiental.

Palavras-chave: diversidade, Trichomeriaceae, Herpotrichiellaceae, assimilação de tolueno, fungos sem esporulação

ABSTRACT

All black yeasts members of the Chaetothyriales order presents melanin in the cell wall, a polymorphic life cycle, with a significantly variable ecology, which includes five families, Chaetothyriaceae, Cyphellophoraceae, Epibryaceae, Herpotrichiellaceae and Trichomeriaceae. On the one hand, Herpotrichiellaceae has been the most studied family, characterized by a complex ecology, which comprises species considered saprobes, species with clinical importance and assimilative abilities of monoaromatic hydrocarbons. On the other hand, the family Trichomeriaceae is a recently established family and mostly includes epiphytic and epilithic organisms. Based on this variable and complex ecology of the Chaetothyriales members, the purpose of the thesis was to study diversity in Chaetothyriales focusing in Herpotrichiellaceae and Trichomeriaceae, two distinct families in ecology and characteristics. The diversity of black yeast in babassu coconut shells was investigated applying different isolation techniques (Chapter II). One-hundred and sixty isolates of black yeast isolates obtained were identified by the sequencing of ITS region. Among the isolates, species with clinical importance as *Veronaea botryosa*, *Rinhocladiela similis* and *Exophiala spinifera* were recognized and two novel species were described in Herpotrichiellaceae. The ability to grow on toluene and phenyl acetate as the sole carbon and energy source was assessed in eight strains. Regarding to the phenyl acetate, an intermediate compound of styrene degradation route, all strains tested were able to assimilate the compound however only two isolates assimilates toluene. The presence of Volatile aromatic compounds (VOCs) in the babassu coconut were investigated using GC-ToFMS, and a total of 140 compounds were identified, which alcohol was the most abundant compound (2023 µg m^{-3}). The genus *Arthrocladium* were re-established based on the phylogenetic analyses and assigned to Trichomeriaceae family (Chapter IV). Preliminary identification based on the sequencing of ITS region showed three undescribed species clustering near to *Arthrocladium caudatum.* Additional multilocus analysis confirms the phylogenetic position of the undescribed species and the position of the genus in Trichomeriaceae*.* Three non-sporulating species were described, one of the species was isolated from babassu coconut, another associated with ants and one of them from a fatal disseminated infection in a patient with a genetic immune disorder. The findings in this study led us to elucidate some aspects in the ecology of black yeast, as well unravel the biotechnological potential of these microorganisms.

Keys-words: diversity, Trichomeriaceae*,* Herpotrichiellaceae*,* toluene assimilation, non-sporulating fungi

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LIST OF ACRONYMS

ABTS - 2, 2-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid diammonium salt
ALC - Alcohols
ALD - Aldehydes
ALK - Alkanes
AROM - aromatic hydrocarbons
BT2 - β-tubulin gene BT2
BTEX - toluene, ethylbenzene, and xylene
CAPES - Coordination for the Improvement of Post-Graduate Education
CBS - Fungal Biodiversity Centre, Centraalbureau voor Schimmelcultures
CCFEE - Culture Collection of Fungi from Extreme Environments
CMRP - Microbiological Collections of Paraná Network
CNPq - Brazilian Agency for Scientific and Technological Development
CPC - Working collection Pedro Crous
CTAB - Cetyltrimethylammonium Bromide
DAOM - Plant Research Institute, Department of Agriculture (Mycology)
DNA - Deoxyribonucleic Acid
GC-ToFMS - Gas Chromatograph coupled to a time-of-flight mass spectrometer
GE - gas-phase enrichment
HAL - Halocompounds
IFM - Research Center for Pathogenic Fungi and Microbial Toxicoses
IRTA - Institut de Recerca e Tecnologia Agroalimentàries
ITS - Internal Transcribed spacer region
KET - Ketones
LSU - Large ribosomal subunit
MFC - Matsushima Fungus Collection
MFLUCC - Mae Fah Luang University Culture Collection, Thailand
MUCL - Culture Collection Catholic University of Louvain
OAC - Organic acids
OF - Oil flotation technique
RT - Retention time
SULF - Organosulphur compounds

T- Type strain

TEF1 - Translation Elongation Factor 1-alpha

TERP - Terpenes and terpenoids

TOLUENE-D8 - Deuterated Toluene

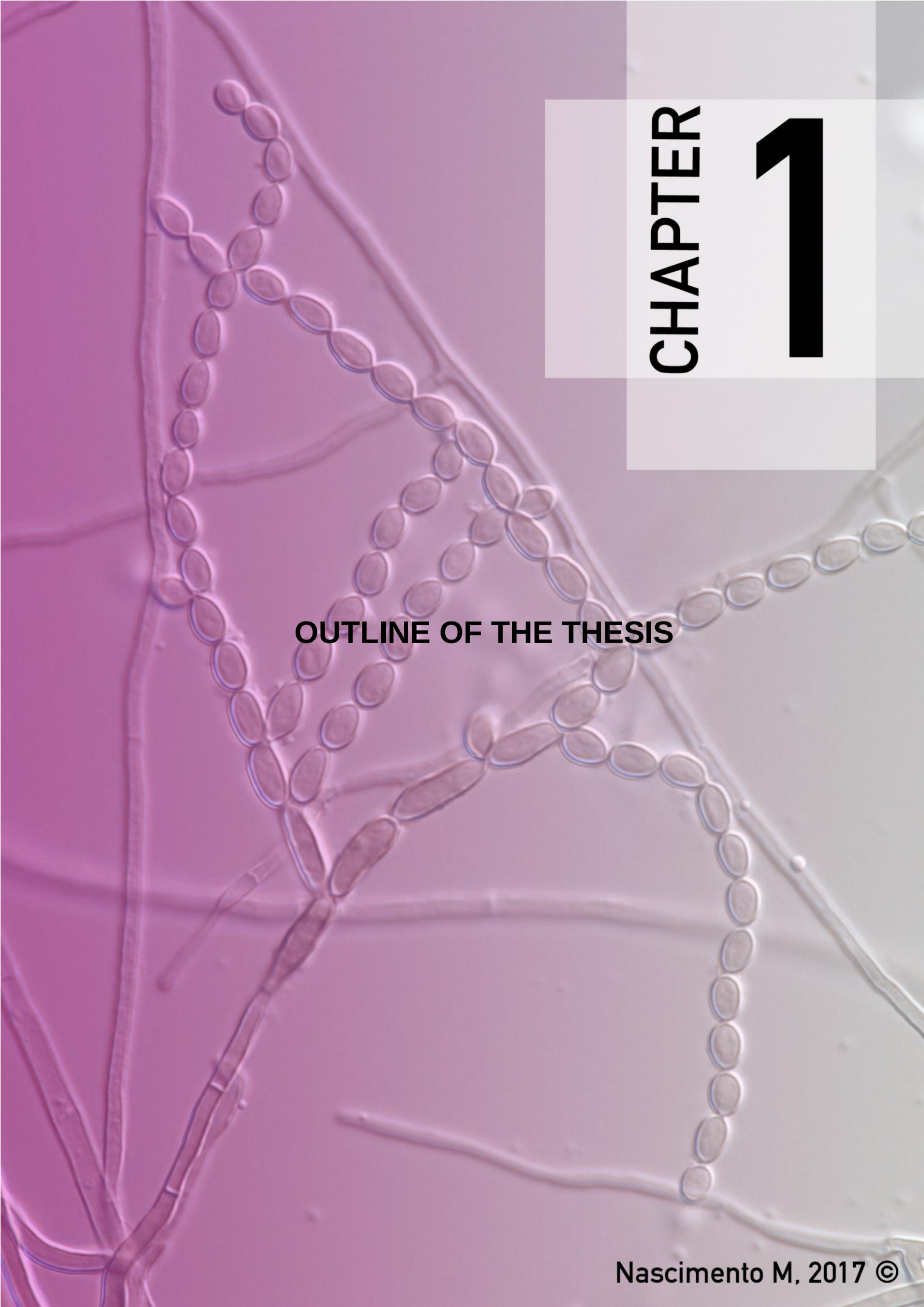
UAMH - University of Alberta Microfungus Collection and Herbarium

UFPR - Federal University of Parana

UTFPR - Federal University of Technology – Parana

VOCs - Volatile Organic Compounds

SUMMARY



CHAPTER 1

OUTLINE OF THE THESIS

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1. GENERAL INTRODUCTION

Pollution of the natural environment with synthetic organic compounds has become an issue in recent decades. Aromatic hydrocarbons and alkanes are toxic pollutants causing serious problems to the soil, water, and air around the world (NETTO *et al*., 2000). In recent years, the use of bioremediation increased as a recovering alternative of impacted environments, since it is a process, which exploits the metabolic properties of microorganisms to degrade pollutants (ILYNA *et al*., 2003). Bioremediation process using bacteria is a cheaper, and sustainable form to remove various types of pollutants, however, in acidic and dry environments these organisms are inhibited and cannot be applicable (SINGH *et al*., 2014). Fungi generally tolerate such conditions and several groups, mainly melanized fungi have been reported as active degraders of monoaromatic hydrocarbon compounds (BADALI *et al.,*2011; BLASI *et al*., 2016).

In recent years, black yeasts, members of Chaetothyriales order have been consistently isolated from environments contaminated with aromatic hydrocarbons (PRENAFETA-BOLDU *et al*., 2001; SEYEDMOUSAVI *et al*., 2011; ISOLA *et al*., 2013), as well as the assimilation of toxic aromatics compounds, such as toluene and styrene as the sole carbon source and energy has been demonstrated by an increasing number of species (PRENAFETA- BOLDÚ *et al*., 2006; ISOLA *et al*., 2013).

Black yeasts-like-fungi is a heterogeneous group of fungi that belongs to Chaetothyriales and Dothideales orders (Ascomycota) (DE HOOG, MCGINNIS, 1987; DE HOOG *et al*., 2000), which the main characteristic is the presence of melanin in the cell wall of the vegetative and reproductive cells (DIXON, POLAK-WISS, 1991). Black yeasts belonging to Dothideales order exhibit adaptive ability for survival in hostile environmental conditions (STERFLINGER *et al*., 1999).

Chaetothyriales order comprises black yeast-like fungi with a vast and diverse ecology (GUEIDAN *et al*., 2014; MADRID *et al*., 2016), and includes species with clinical relevance, pathogens and opportunists of human infection, causing diseases such as Chromoblastomycosis, Mycetoma and Phaeohyphomycosis (VICENTE *et al*.,

2008; BONIFAZ *et al*., 2013), species associated with infection in cold-blooded animals (VICENTE *et al*., 2012, GUERRA *et al*., 2013), ant associated species (VOGLMAYR *et al*., 2011; HUBKA *et al*., 2014), epiphytic and epilithic species (CROUS *et al.,*2006).

In addition, the pathogenicity and virulence along Chaetothyriales fungi differ significantly between the species and many factors are involved (SEYEDMOUSAVI *et al*., 2014). In contrast, some species are found colonizing environments with extreme conditions, such as low nutrient availability, high pH and temperature (GOSTINČAR *et al*., 2011) or polluted with aromatic hydrocarbons (PRENAFETA-BOLDÚ *et al*., 2001). Different works (Marques *et al.*, 2006; Vicente *et al.* 2008, 2014) also reported the presence of black yeast species in babassu coconut shell in Maranhão region and seems to be a possible source of infection for the nut breakers (NASCIMENTO et al., 2017).

Based on the overview presented about black yeast, it is necessary to gather information about the diversity of this group of fungi in different environments, being bioprospecting, an essential tool to verify this diversity and species capable to metabolize aromatic hydrocarbon in order to be applied in bioremediation processes. Bioprospecting is a technique for the exploration of the diversity in the most diverse environments (CARDOSO, 2013, LOOSE, 2011). Several reports have been increased the knowledge about biodiversity in different environments, discover new microorganisms and molecules with biotechnological interest to be applied in various areas of the industry, as well as, in bioremediation processes (SINGH et al 2011; OLIVEIRA; SETTE; FANTINATTI-GARBOGGINI, 2006).

Through the different studies evaluated, the hypothesis of black yeasts present in babassu possibly degrades monoaromatic hydrocarbon is the subject of the present thesis. In Brazil, the babassu palm tree (*Orbignya phalerata* Mart., *Arecaceae*) is native to the humid forests and distributed along Maranhão, Piaui, Tocantins and Goias States (DOS SANTOS et al., 2017; TEIXEIRA, 2008). Particularly in Maranhão state, babassu coconut has been exploited for industrial purpose, being applied in food, soap, and skin products (SOUZA et al., 2011), as well as, has economic importance for local development as a complementary activity to rural workers (SOUZA et al., 2011). On the other hand, Maranhão is an endemic area of chromoblastomycosis (Azevedo and Rodrigues et al. 2015) and the disease in this region has been associated with babassu exploitation (Marques et al. 2006).

Little is known about babassu shell composition, according to Garcia et al. 1995, babassu is rich in lipids and terpenes. Based on it, this study was investigated the coconut shell as an environment possible rich in volatile organic compounds, once the presence of black yeast has been registered by different authors (Marques et al. 2006, Vicente et al. 2014).

Through the previously bioprospecting studies (Blassi et al. 2016, Isola et al. 2013, Badali et al. 2011, Prenafeta-Boldú et al. 2010) had been allowed the selection of black fungi with assimilative abilities for application in toluene degradation (bioremediation), besides enabling the discovery of new black yeast species or species with biotechnological potential and clarify aspects of different ecology observed in this group of microorganisms.

In order to organize the different topics addressed in the thesis are presented in four chapters:

Chapter I, provides a general overview about black yeast-like-fungi, as well presents the hypothesis and the aims that guided the thesis, introduce a brief literature review about the main topics evaluated in the Thesis, focusing on the biodiversity of black yeast-like-fungi belongs to *Chaetothiryales* (Ascomycete) as well in the biotechnological potential of this group of fungi.

Chapter II, the diversity of black yeast-like on babassu coconut shells was assessed in order to investigate the possible relation between babassu coconut and transmission of Chromoblastomycosis in Maranhão region (Brazil). Two novel species of the black yeast assigned to order Chaetothyriales were described, as well as the volatile organic compounds (VOCs) present on babassu.

Chapter III, the strain previously isolated from babassu coconut shell by Vicente et al. in 2008 clustered near to the type strain of *Arthrocladium caudatum* was here described as a new species with two other species of non-sporulating fungi belonging to genus *Arthrocladium*. Pappendorf in 1969 proposed the genus *Arthrocladium* based only on the morphology of a single strain isolated from *Acacia karroo* leaf litter in South Africa. The genus *Arthrocladium* was re-established and assigned belonging to *Thricomeriacea* family. One of the described new species *Arthrocladium fulminans* caused a fatal infection in patient with a rare genetic immune disorder.

A general conclusion and final consideration highlighting the main scientific findings is presented in the **Chapter IV**.

2. OBJECTIVE

2.1. GENERAL

The main objective of this study was bioprospecting the diversity of black yeast-like-fungi in Chaetothyriales focusing in Herpotrichiellaceae and Trichomeriaceae, in order to clarify the different ecological trends among this group of fungi.

2.2. SPECIFIC

- Bioprospect black yeast from babassu coconut;
- Identify by sequencing and phylogenetic analyses the isolates of black yeasts;
- Describe of new species;
- Recognize of potentially human pathogens among the isolated species,
- Identify and select species potentially applicable in bioremediation of aromatic hydrocarbons.
- Verify the laccase activity by black yeasts;
- Characterize of Volatile Organic Compounds from Babassu coconut.
- Elucidate the ecology of black yeast species in Chaetothyriales focusing in Herpotrichiellaceae and Trichomeriaceae.

3. EXPERIMENTAL DESIGN

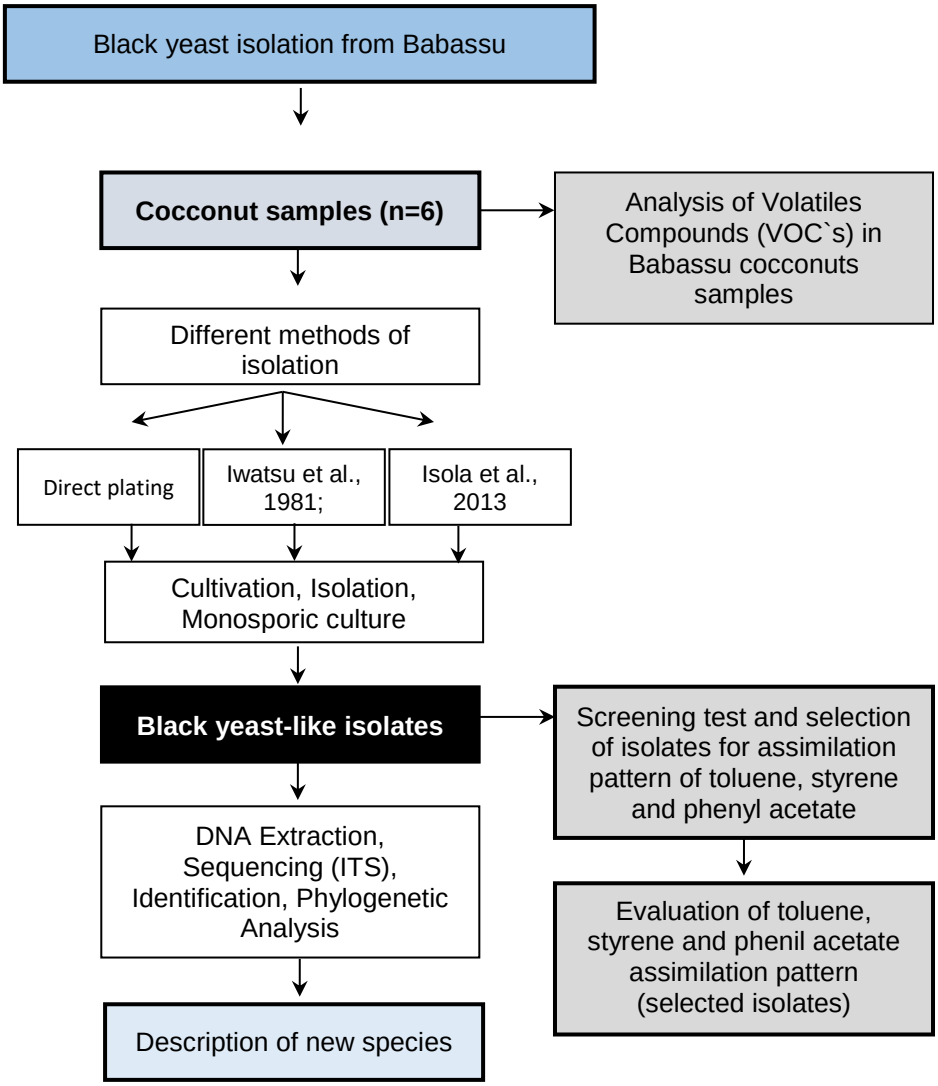

Figure 1 - Thesis Experimental Design

4. LITERATURE REVIEW

4.1. CHARACTERISTICS OF BLACK YEASTS-LIKE-FUNGI

"Black yeasts-like-fungi" comprises a group of melanized fungi (Figure 2) with a polymorphic life cycle, being able to shift from a mycelial to a meristematic growth as well shares some morphological and physiological characters (DE HOOG, MCGINNIS, 1987; STERFLINGER, 2006; ISOLA *et al*., 2016). These group of fungi are phylogenetically heterogeneous, belonging to different orders of Ascomycota Chaetothyriales and Dothideales, with diverse ecologies and morphology (Figure 2) (DE HOOG, MCGINNIS, 1987; VICENTE *et al*., 2012; HUBKA *et al*., 2014).

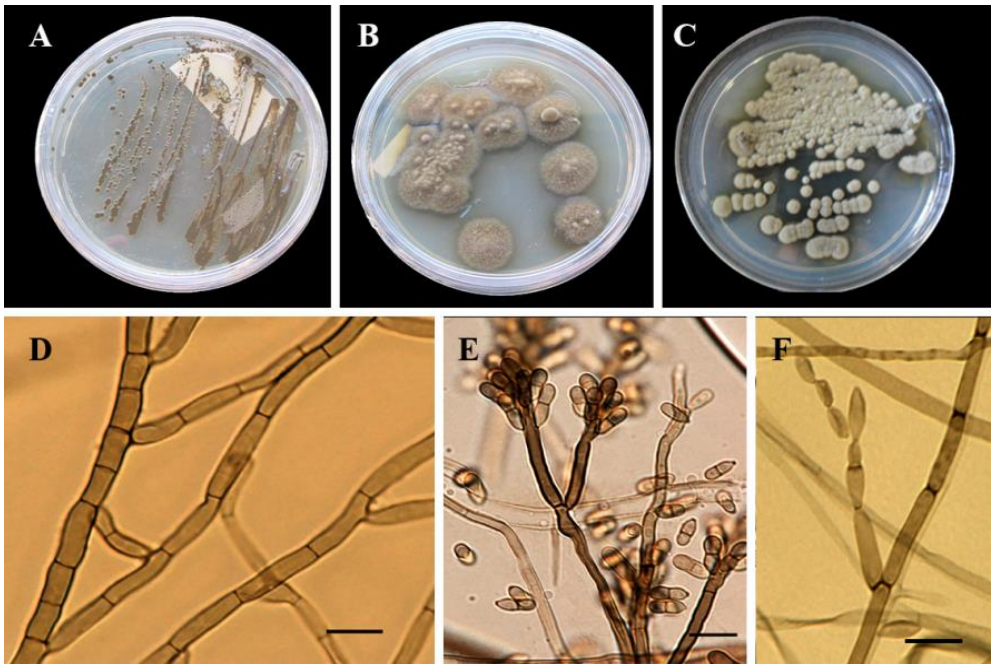

Figure 2 - Black yeast like-fungi macro and micromorphology. A – C: Aspects of melanized colony of different species of black yeast species; D – F: Microscopic appearance of vegetative and reproductive cells of black yeast species D - Hyphae with anastomoses, E - Conidiophores and conidia of *V. botryosa,* F - Cylindrical, septate conidiophores of *C. mycetomatis.* Scale bars=10µm (Adapted from NASCIMENTO, SANTOS, VICENTE, 2017).

The thermophilic species *Exophiala dermatitidis* is an example of black yeasts with a complex life cycle and ecology (SUDHADHAM *et al*., 2011), exhibiting an oligotrophic behavior, once inhabit bath facilities (SUDHADHAM *et al*., 2011),

dishwashers (ZALAR *et al*., 2011), creosoted railway ties, either causing systemic infections in human (SUDHADHAM *et al*., 2008). As observed by Lian and de Hoog (2010), oligotrophic black fungi from humid indoor environments shows a potential to cause human infection.

Many black yeast species are considered extremotolerant or extremophilic, being able to adapt wide stressful environments (ISOLA *et al*., 2016, GOSTINČAR *et al*., 2011) inhabiting strange, extreme, poor, or toxic environments (DE HOOG *et al.,*2014), as well as tolerating high and low temperatures, high and low pH, osmotic stress, high solar and UV radiation or prolonged period of water deficiency (STERFLINGER, 2006; GOSTINČAR *et al*., 2011), also being able to produce extracellular polysaccharides (SELBMANN *et al.,*2005)

Over the last decades, black yeast members of the Chaetothyriales has been consistently isolated from different environments (Figure 1), such as decaying material (VICENTE *et al.,*2008, 2012, GUERRA *et al*., 2013), environments polluted with aromatic hydrocarbons (PRENAFETA-BOLDÚ *et al*., 2001; ZHAO *et al.,*2010; ISOLA *et al*., 2013), human-made environment (ZALAR *et al*., 2011; ISOLA *et al*., 2013), from disseminated infections in mammals and cold-blooded animals (DE HOOG *et al*., 2011; NAJAFZADEH *et al*., 2011; VICENTE *et al*., 2012).

The order Chaetothyriales their teleomorphs (genus *Capronia*) (Figure 3) includes five families, with variable and complex ecology (HUBKA *et al*., 2014), and the Herpotrichiellaceae, the most studied family, presenting the majority of clinical relevant species, which include agents of Chromoblastomycosis, Mycetoma and Phaeohyphomycosis (SEYEDMOUSAVI *et al.*, 2014; AZEVEDO *et al*., 2015) also causing systemic fatal infection (AZEVEDO *et al*., 2015) or disseminated infection (BONIFAZ *et al.,* 2013; AZEVEDO *et al*., 2015) often in apparently healthy individuals (SEYEDMOUSAVI *et al*., 2014).

The Cyphellophoraceae*,* a group of species, which the natural habitat of the members is not well elucidated, however are only known colonizing human skin (SAUNTE *et al*., 2011; FENG *et al*., 2014). The Chaetothyriaceae family is poorly studied and comprehends species considered epiphytes, however most species have not been sequenced or cultured (CHOMNUNTI *et al.,*2012b). The Epibryaceae family was recently proposed by GUEIDAN *et al*., 2014 on the basis of molecular DNA data, which contains genera *Epibryon, Lichenodiplis* and *Muellerella* (MUGGIA *et al.*, 2015).

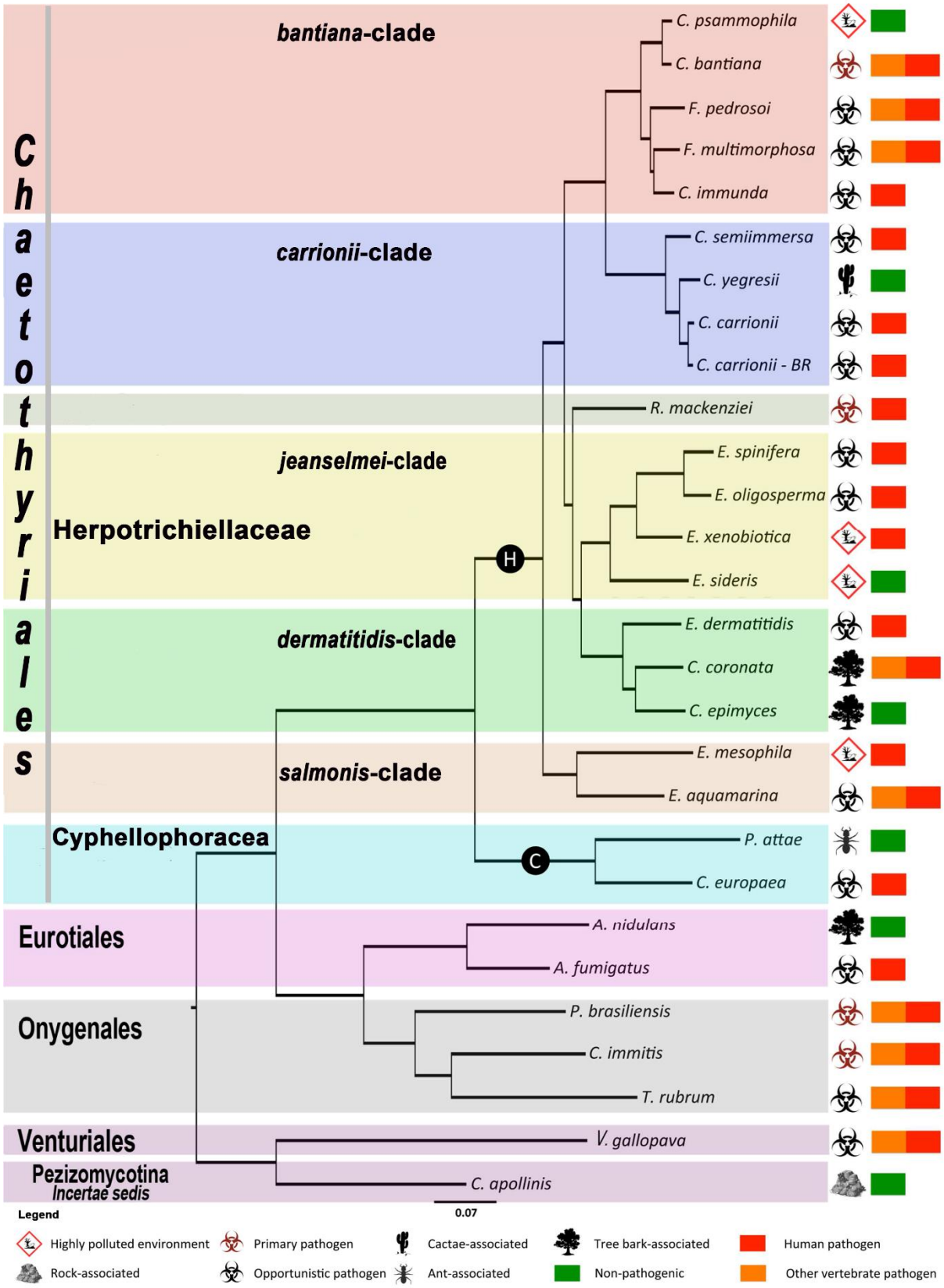

Figure 3 - Phylogenomic distribution of Chaetothyriales representing families Cyphellophoraceae (C) and Herpotrichiellaceae (H) and related ascomycetes. The main characteriscs such as niche, isolation source (red boxes – anthropophilic pathogens; orange boxes - zoophilic pathogens, and, green boxes geophilic) (Adapted from TEIXEIRA et al., 2017).

The family Trichomeriaceae was stablished by Chomnunti *et al.* (2012a) to allocate members of ascosporulating *Trichomerium* genus housed before in Chaetothyriaceae. The family comprises epiphytic species (CHOMNUNTI *et al.,* 2012a), ant associated species (VOGLMAYR *et al*., 2011), and rock inhabiting species (ISOLA *et al*., 2016). Among the latter, Isola and co-workers (2016) widely sampled Italian rock monuments in search of highly destructive and extremotolerant black fungi. As a result of this search one new genus and five new species were proposed in Trichomeriaceae.

4.2. BLACK YEAST INFECTIVE ABILITY

The black yeasts members of Chaetothyriales present an invasive ability and a variable pathogenic potential (DE HOOG *et al.,* 2011). Three ecological groups are apparent in Chaetothyriales, saprobes species without infectious abilities; agents of chromoblastomycosis and, species causing disseminated or systemic infection, as well *Cladophialophora bantiana*, and grows very well at 36-37ºC (SEYEDMOUSAVI *et al*., 2014; DÖĞEN *et al.,* 2013).

The virulence factors present in Chaetothyriales members, such as thermotolerance, presence of melanin, assimilation of aromatic hydrocarbons, osmotolerance, hydrophobicity, and yeast-like phases, plays important role in virulence and pathogenicity (DIXON, POLAK, WISS, 1991; SEYEDMOUSAVI *et al*., 2014). The preference for extreme, poor, or toxic environments, which is considered inappropriate for the most microorganisms also has been supposed a link with virulence in black yeast (SUDHADHAM *et al*., 2011). Nevertheless, Teixeira *et al.* (2017), in a comparative genome analysis with a set of 23 black yeast species find no specific virulence differences between closely related, pathogenic versus environmental sibling species, such as *C. bantiana*, *C. psammophila*, concluding that pathogenicity of black fungi is primarily of opportunistic nature, enhanced by the combination of extremotolerance and assimilative abilities of aromatic compounds.

According Seyedmousavi *et al*. (2014), cellular plasticity is also an important virulence factor. Some species express meristematic growth during the most stressful parts of their life cycle (SUDHADHAM *et al*., 2011). In nature hyphal forms are

produced, however in the infected tissue presents meristematic bodies (muriform cells) (SEYEDMOUSAVI et al*.,* 2014)

Thermotolerance, an important virulence factor, which influences in the host and habitat choice of the species (DE HOOG *et al*., 2011) and viewed as a basic requirement for human infection (NASCIMENTO *et al*., 2016; DE HOOG *et al*., 2011). According to De Hoog *et al*., (2011), the tolerance to human body temperature is an essential requirement for pathogenicity. Chaetothyriales species in bantiana, dermatitidis, and jeanselmei clades (Figure 3) grows at 37°C or higher temperatures (DE HOOG *et al*., 2011), on the other hand, waterborne *Exophiala* species lack this ability and can cause infection in cold-blooded animals (Salmonis clade) (Figure 3) (NASCIMENTO *et al*., 2016).

Melanin pigments, another important virulence factor, are considered a complex polymer secreted or constitutes the structure of fungal cell wall (ZHANG *et al.,*2013), protect the fungi against oxygen radicals (LANGFELDER *et al*., 2003), reduce phagocytosis, promote protection against hydrolytic enzymes produced by macrophages (BOCCA *et al*., 2006) and improve the survival of fungi in hostile environments (DE HOOG *et al*., 2014).

Melanized fungi are more resistant to environmental factors than non-melanized mutants, albinos or normally non-melanized fungi (BUTLER *et al*., 2005). Melanin is a common term used to designate pigments ranging from brown to black, with high molecular weight and formed by the oxidative polymerization of several phenolic compounds (BELL; WHEELER, 1986) by phenoloxidases enzymes, divided into two subgroups, laccases and tyrosinases (LANGFELDER *et al.,*2003). In general, fungi produce several types of melanin using different pathways, however, the predominantly are derived from dihydroxyphenylalanine (DOPA) and dihydroxynaphthalene (DHN) pathways (GÓMEZ, NOSANCHUK, 2003). In addition, recently comparative genomic studies with different Chaetothyriales species suggests that members of Herpotrichiellaceae possess several melanin-associated genes and being able to produce melanin using both pathways (TEXEIRA *et al.,*2017).

Concerning the fungal infection caused by Chaetothyriales members, chromoblastomycosis is a chronic cutaneous and subcutaneous infection characterized by verrucous skin lesions, presenting muriform cells, the invasive form of the fungi, in the tissue (QUEIROZ-TELLES, 2015; QUEIROZ-TELLES *et al*., 2017). Considered a traumatic infection characterized by the implantation of fungal elements

into the skin (BADALI *et al*., 2008) affecting mostly people living in tropical and subtropical areas (QUEIROZ-TELLES *et al*., 2017).

Chromoblastomycosis is worldwide distributed (Figure 4) and prevalent in tropical and subtropical region, such as in Latin America, the Caribbean, Africa, and Asia. Brazil, Mexico, Venezuela, India, Australia, and southern China report the majority of the cases (QUEIROZ-TELLES *et al.,* 2017).

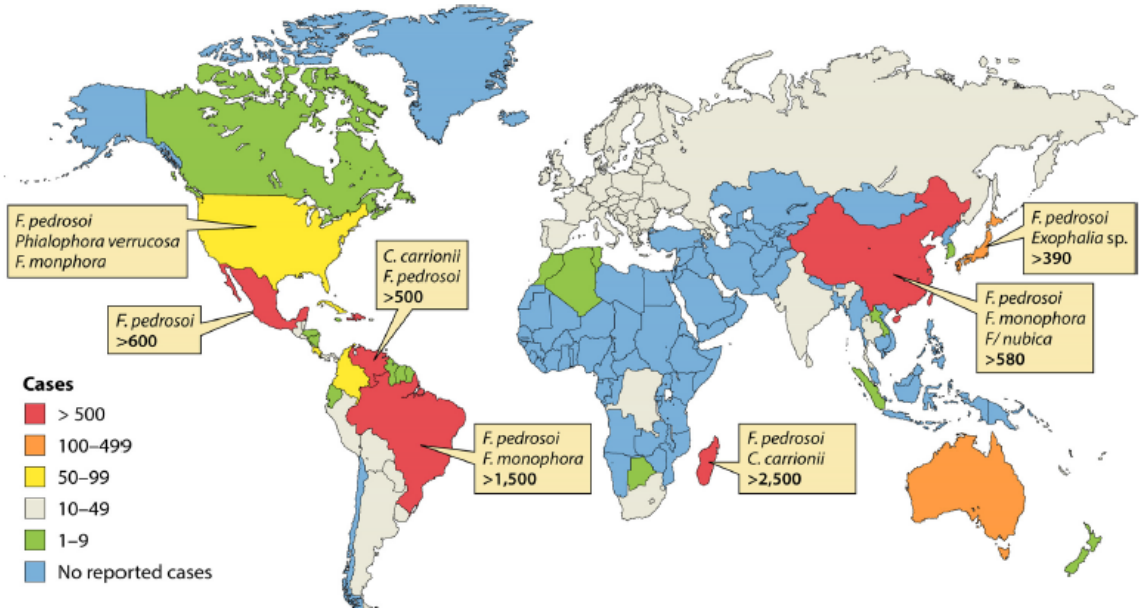

Figure 4 - Global distribution of chromoblastomycosis cases and species prevalence (Adapted from QUEIROZ-TELLES *et al.,*2017).

Among the etiologic agents of chromoblastomycosis, *Fonsecaea* and *Cladophialophora* species are considered the most prevalent agents (Figure 4) (QUEIROZ-TELLES, 2015). In China, *Fonsecaea monophora* is the predominant etiologic agent of chromoblastomycosis (ZHANG *et al.,*2013). Differently, in Brazil, *Fonsecaea pedrosoi* is supposed to be the main etiologic agent and Maranhão region (northern) is considered an endemic area of the disease (VICENTE *et al*., 2014). Recently, Gomes *et al. (*2016), developed an extensively study among the diversity of chromoblastomycosis agents in endemic areas of Brazil. A set of 123 clinical strains were analyzed regarding sequence data, clinical aspects, direct mycological examination, and culture characteristics. The chromoblastomycosis agents clustered in different clades within the Chaetothyriales, belonging to *Fonsecaea*, *Rhinocladiella*, and *Cyphellophora* genera.

Among to the chromoblastomycosis agents in *Fonsecaea* genus, four species are recognized *F. pedrosoi*, *F. nubica*, *F. monophora*, and *F. pugnacious,* and produce muriform cells in tissue (AZEVEDO *et al*., 2015, QUEIROZ-TELLES *et al*., 2017). However, morphological distinction of sibling species is difficult (Figure 5) being distinguished based on molecular data (NAJAFZADEH *et al.,*2010). Once the *Fonsecaea* sibling species presents branched conidiophores, with the formation of primary and secondary conidia with variations (Figure 5) (GOMES *et al*., 2016; VICENTE *et al*., 2012, 2014).

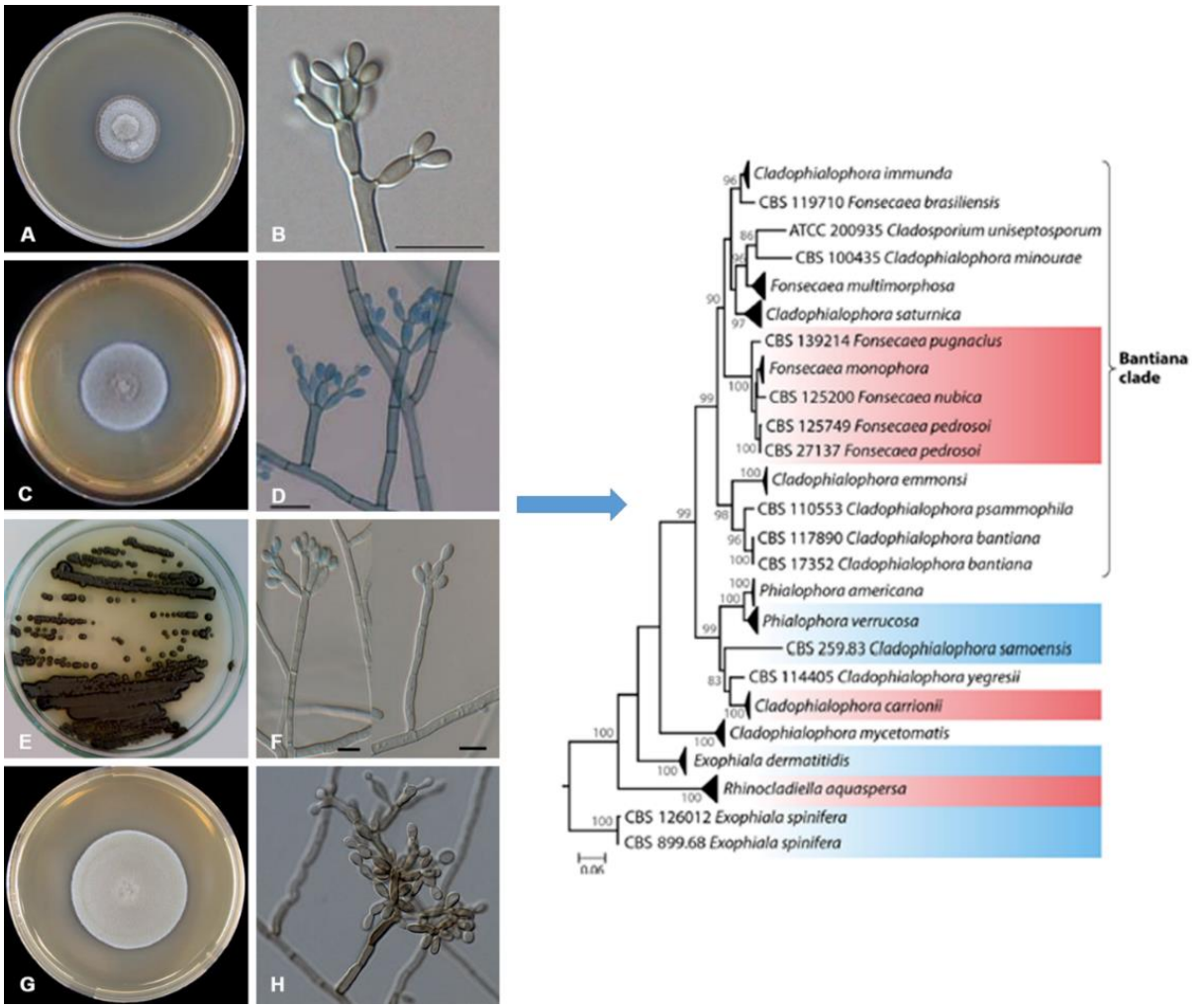

Figure 5 - Macro and micromorfological characteristics of *Fonsecaea* agents of chromoblastomycosis, **A-B**: *F. pedrosoi*, **C-D**: *F. nubica*, **E-F**: *F. monophora*, **G-H**: *F. pugnacius*, and phylogenetic affiliation of *Fonsecaea* sibling species.
Note: A to D and **E to H** from Online Atlas of Clinical Fungi (http://atlas.clinicalfungi.org), **D** from Bombassaro, 2016, and Phylogenetic tree adapted from Queiroz-Telles et al., 2017.

Normally, the clinical presentation of chromoblastomycosis is characterized by primary lesion at the site of inoculation evolving to chronic involvement of cutaneous and subcutaneous tissues (QUEIROZ-TELLES, 2015) with diverse clinical presentation, such as nodular, tumoral (cauliflower-like) or verrucous (QUEIROZ-TELLES, 2015). *Fonsecae pedrosoi* and *F. nubica* causes localized infection and *F. monophora* and *F. pugnacius* causes disseminated infection to the brain (QUEIROZ-TELLES, 2015; AZEVEDO *et al*., 2015).

Fonsecaea sibling species (Herpotrichiellaceae*)* agents of chromoblastomycosis belongs to the bantiana-clade (Figure 4) (QUEIROZ-TELLES *et al*., 2017), which also contains *Fonsecaea* species associated with animal hosts, as *F. multimorphosa* causing infection in cats (NAJAFZADEH *et al*., 2011), *F. brasiliensis* associated with disseminated infection in crabs (VICENTE *et al*., 2012), either plant related species as *F. erecta* and *F. minima* (VICENTE *et al*., 2014), as well as the pathogenic *Cladophialophora* species *C. bantiana*, *C. arxii*, and *C. devriesii* and the environmental species *C. minourae, C. saturnica* (BADALI *et al*., 2008)*,* which only cause opportunistic infections.

Cladophialophora bantiana is consistently associated with brain infections (HORRÉ, DE HOOG, 1999), moreover also being recovered from soil (PRENAFETA-BOLDÚ *et al*., 2006). The invasive potential of black yeast-like fungi varies significantly between species (BADALI *et al*., 2008; SEYEDMOUSAVI *et al*., 2011) even with short phylogenetic distance, such as observed in bantiana-clade. Prenafeta-Boldú *et al.* (2006), proposed that the neurotropism inside Herpotrichiellaceae family could be explained, by the ability of some species metabolize catechol substrates as a carbon source. Isola *et al.* (2013), highlight that the assimilation of phenolic compounds and hydrocarbons by black yeast-like fungi, may represent an additional virulence factor, since it may enable to infect the central nervous system which has a high content of monoaromatic catecholamine neurotransmitters and aliphatic amino alcohols.

4.3. BIOTECHNOLOGICAL POTENTIAL

The ability to metabolize aromatics compounds shows to be restricted to the black yeasts belonging to the family Herpotrichiellaceae (Chaetothyriales), in *Cladophialophora* and *Exophiala* genus (PRENAFETA-BOLDÚ *et al*., 2006; ISOLA *et*

al., 2016). According to Prenafeta-Boldú *et al*., (2006), in general, fungi promotes the degradation of aromatic compounds basically in three modes: I) partial transformations reactions; II) degradation of hydrocarbons in the presence of a second substrate; III) use of the hydrocarbon as the sole carbon source. Regarding black yeasts, however, the assimilatory metabolism of xenobiotics is due to a well-arranged pathway of energy-yielding reactions that leads to the degradation of those substrates. As in all no coincidental metabolisms, the evolution of this capability must be the result of the selective pressure of specific environmental factors which have yet to be fully understood (BLASI *et al*., 2016).

In an extensively review study Prenafeta-Boldú *et al*., (2006), observed that black yeasts species in Chaetothyriales order are potential agents to degrade monoaromatic hydrocarbons. Several species of black yeast-like fungi were recovered from environments contaminated by hydrocarbons using selective techniques, as well assimilative abilities of toxic organic compounds were described (Figure 5) (ISOLA *et al*., 2013; BADALI *et al*., 2011; SEYEDMOUSAVI *et al*., 2011, PRENAFETA-BOLDÚ *et al*., 2001).

The Oil Flotation method (Figure 6.A), developed by Iwatsu *et al.,* (1981), is based the hydrophobicity of the melanized fungal cells, and has been one of the methodologies widely applied to recover several species of black yeasts from the environment (VICENTE *et al*., 2008, 2012, 2014; FENG *et al*., 2014; NAJAFZADEH *et al*., 2011; MARQUES *et al*., 2006). Using this method, Iwatsu *et al*., (1981) isolated strains of *Phialophora verrucosa* and *Fonsecaea pedrosoi* from enviroment in Japan. The oil flotation isolation technique was applied by Satow *et al., (*2008) to recover black yeasts from contaminated soil samples in a landfarming area. Several species of black yeasts-like fungi were recovered, such as *Exophiala xenobiotica* the preponderant isolated species, which is frequently isolated from habitats rich in hydrocarbons and sporadically reported causing infection on human skin (ZHAO *et al*., 2010; ISOLA *et al*., 2013). Feng *et al*., (2014), applying the same method isolated *Cladophialophora abundans* species from plant sources in Brazil, and from the environment of the mangrove land crab (*Ucides cordatus*). The species is morphologically very similar to *Cladophialophora bantiana*, however the species presents maximum growth temperature of 36 °C and seems to be a strict saprobe. According to Satow *et al*., (2008) mineral oil (complex mixture of petrol hydrocarbon) is an enrichment factor in black yeasts isolation, once those fungi are able to assimilate monoaromatic

hydrocarbons (PRENAFETA-BOLDÚ *et al*., 2001, 2004; ZHAO *et al*., 2010; BADALI *et al*., 2011, SEYEDMOUSAVI *et al*., 2011).

Figure 6 - Selective isolation methods for black yeast-like fungi which degrade monoaromatic hydrocarbon. A – Oil flotation method. B – Solid state-like incubations under controlled atmospheres; C – Liquid batch cultures in sealed vials with Teflon, D – Air biofilter method. **Note: A – C:** authors personal collection; **D:** Prenafeta-Boldú, 2013.

Three enrichment techniques were stablished by Prenafetra-Boldú *et al*., (2001) in order to isolate black yeast-like fungi capable of growing on toluene. The solid state-like batches method (Figure 6.B) is based on a controlled atmosphere condition in a desiccator, consisting of gas phase supplied of volatile organic substrates, and water activity control through salt solutions. The batches containing a sterile inert porous support (perlite) are previously prepared with a mineral medium.

The liquid batch cultures (Figure 6.C) method is based on the cultivation of fungi growing in a defined mineral medium in closed vials (sealed with Teflon coated valves Mininert™) with headspace containing volatile monoaromatic compound incubated at 25ºC. The consumption of volatile monoaromatic compound is monitored by gases on head-space.

Concerning to Air biofilters (Figure 6.D) isolation, the system consists in four glass columns (A, B, C and D) packed with perlite granules previously saturated with mineral medium and mixed with the sample to be analyzed (i. e. contaminated soil). The system is feed with humidified air containing toluene. The medium in columns A and C initially present pH at 4 and in columns B and D at 8. All filters operate at 25ºC.

Using the three different techniques previously described, Prenafetra-Boldú *et al*. (2001), isolated black yeast-like fungi, belonging to *Cladophialophora* and *Exophiala* genus, from different samples, which were able to grow on toluene as sole carbon and energy sources. In another study Zhao *et al*. (2010), isolated several black yeasts species, such as *Exophiala xenobiotica*, *Exophiala bergeri*, from natural ecological niches and man-made environments, applying the solid state-like batch culture technique in a hydrocarbon atmosphere.

In addition, the study developed by Isola *et al*. (2013), isolated black fungi reported as degraders of VOC from hydrocarbon-polluted sites and indoor environments, presenting a higher biodiversity of *Exophiala* species, as *E. mesophila, E. oligosperma, E. sideris*. The black yeast species *Cladophialora immunda* have been isolated from a toluene-charged air biofilter, hydrocarbon-polluted soil, from *Syagrum romanzoffianum*, as well as from a patient with a sub-cutaneous ulcer (BADALI et al., 2008), becoming evident an association between metabolism of aromatic hydrocarbons and opportunistic pathogenicity (BADALI *et al*., 2011).

Rustler *et al.* (2008), isolated a specie of black yeast, *Exophiala oligosperma*, which shows the ability to use numerous organic nitriles under acidic conditions as nitrogen sources, as well grow with phenylacetonitrile as sole source of carbon, energy and nitrogen. Badali *et al*. (2011), described a new species *Exophiala psammophila,* isolated from soil in a gasoline station polluted with toluene, ethylbenzene, and xylene (BTEX), using solid state-like cultivation batch system, as a selective method. The species was proven to assimilate toluene.

A screening study developed by Blasi *et al*. (2016) with 163 black yeast species for the ability to grow on hexadecane, toluene and polychlorinated biphenyl 126 (PCB126) as the sole carbon and energy source, revealed, only two fungal strain with positive growth for toluene, *C. immunda* and *E. mesophila*. Previous study (BADALI *et al.,*2011) described the ability of *C. immunda* to grow with toluene as the sole carbon source. Rustler *et al.,* (2008) described the opening of aromatic rings via the homogentisate pathway by *Exophiala oligosperma*. Another example *is Exophiala*

lecanii-corni, capable to degrade VOCs including toluene and ethylbenzene as sole carbon and energy source, which would be viable to use in biofiltration, once was able to degrade a wide range of VOCs, even under harsh environmental conditions (WOERTZ, KINNEY *et al*., 2001). Differently, *E. sideris* was unable to grow in the presence to the aromatic compound (toluene) as the sole carbon and energy source, nevertheless appears to be tolerant to arsenate (SEYEDMOUSAVI *et al*., 2011). According to Seyedmousavi *et al*., (2011), with the recent and consistent finds regarding to *Exophiala* species and hydrocarbons assimilation, another type of ecology becomes apparent in the Chaetothyriales, although the precise nature of this relation needs to be elucidated.

The extremotolerant fungi into black yeasts group are able to metabolize aromatic pollutants and their extremophilic nature (GOSTINČAR *et al*., 2011) makes them ideal candidates for specific bioremediation purposes (ZHAO *et al*., 2010). Nowadays, microbial remediation would be urgent in such sites (SEYEDMOUSAVI *et al*., 2011) and investigations related to the pathogenicity of this agents are important, in order to select safety microorganism to be used in microbial remediation (PRENAFETA-BOLDÚ *et al*., 2006, ZENG *et al*., 2007).

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CHAPTER 2

Diversity of opportunistic black fungi on Babassu coconut shells, a rich source of esters and hydrocarbons

Diversity of opportunistic black fungi on Babassu coconut shells, a rich source of esters and hydrocarbons

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1. ABSTRACT

The present study assessed the diversity of black yeast-like fungi present on babassu coconut shells, substrate rich in lipids, and several volatile organic compounds (VOCs) including aromatic hydrocarbons. Using different isolation methods, one-hundred-six isolates were obtained and were identified by ITS sequencing as members of the genera *Exophiala*, *Cladophialophora*, *Veronaea* and *Rhinocladiella*. Two novel species were discovered. Eight strains were selected for assessing their ability to grow on toluene and phenyl acetate as the sole carbon and energy source. All strains tested were able to assimilate phenyl acetate, while two out of eight were able to use toluene. VOCs profiling in babassu samples was also investigated by GC-ToFMS, revealing that a complex mixture of VOCs was emitted, which included alkylbenzenes such as toluene. Assimilation of alkylbenzenes by the black yeasts might therefore be the result of evolutionary adaptation to symbiotic interactions with higher plants. The potential relationship between lipid/aromatic hydrocarbon metabolism and pathogenicity is also discussed.

Key-words: Black yeasts, Chaetothyriales, Volatile Organic Compounds, toluene

Running title: Black yeast-like fungi from Babassu.

2. INTRODUCTION

Babassu (*Orbignya phalerata* Mart., *Arecaceae*) is a native palm tree in the northern and northwestern regions of Brazil, especially in Maranhão, Piaui, Tocantins and Goias States (TEIXEIRA, 2008). Particularly in Maranhão state, babassu exploration for application in food, soap and skin products has great economic importance for local development and as a complementary activity to rural people's subsistence (SOUZA et al., 2011). On the other hand, the Maranhão province is known as one of the main endemic areas of chromoblastomycosis, a chronic, mutilating skin disease caused by black fungi (QUEIROZ-TELLES et al., 2015, AZEVEDO et al.,

2015). Several authors (MARQUES et al., 2006, VICENTE et al., 2012, VICENTE et al., 2014) reported on a possible connection between the presence of black fungi on babassu shells and the prevalence of chromoblastomycosis in Maranhão, suggesting that chromoblastomycosis in this province might be an occupational disease.

Black yeast-like fungi are phylogenetically diverse, but the recurrent human opportunists nearly all belong to a single order, the Chaetothyriales. Many species display polymorphic life cycles (DE HOOG and MCGINNIS, 1987). The natural habitats of most species are unknown; despite their probably opportunistic nature, many have been reported from human-dominated environments and from humans only. Potential agents of the disease probably inhabit adverse (micro)-niches in decaying plant material (VICENTE *et al*., 2008, VICENTE *et al*., 2012, GUERRA *et al*., 2013), hydrocarbon-polluted sites (PRENAFETA-BOLDÚ *et al*., 2001a, ISOLA *et al*., 2013), railway sleepers (DÖĞEN *et al*., 2013) or bathing facilities (SUDHADHAM *et al*., 2008). Some species, primarily in the genera *Exophiala* and *Cladophialophora*, are able to assimilate toxic volatile aromatics, to an extent that this might be beneficial for bioremediation of industrial pollution (PRENAFETA-BOLDÚ *et al*., 2001b, ZHAO *et al*., 2010). However, the same species have been associated repeatedly with infectious disease (DE HOOG *et al*., 2004, NAJAFZADEH *et al*., 2010, NAJAFZADEH *et al*., 2011, AZEVEDO *et al*., 2015). An ecological connection between aromatic hydrocarbon assimilation and human pathogenicity has been presumed (PRENAFETA-BOLDÚ *et al*., 2006). Elucidation of ecological and evolutionary parameters is necessary to understand those factors of habitat choice that seem to enhance opportunism on the human host (VICENTE et al., 2008, DÖĞEN *et al*., 2013, GÜMRAL *et al*., 2014).

Babassu coconuts are rich in lipids, terpenes and other hydrocarbons (Garcia *et al*., 1995) and are a commodity for industrial purposes (TEIXEIRA, 2008, Santos *et al*., 2013). The shells provide a rather special environment recalcitrant to microbial decomposition. However, abundant presence of black fungi has been observed. The hypothesis of babassu shells possibly transmitting disease by these fungi is the subject of the present paper. We describe the coconut shell as an environment rich in volatile organic compounds (VOCs), possibly enhancing enrichment of agents of chromoblastomycosis.

3. MATERIAL AND METHODS

3.1. SAMPLING AREA AND ISOLATION METHODS

Six samples of babassu coconut were collected in the municipality of São Luis, in Maranhão state, Brazil, on January 2014. The epicarp and mesocarp of the coconut were selected for fungal isolation. Three methods focused on black fungi were applied:

(i) Direct plating. Of each sample, approximately 1 g was added to 5 mL saline solution (8.5 g/L). After homogenization, 100 µL was plated directly onto Mycosel agar (Difco, Detroit, MI, U.S.A.) and Dichloran Rose Bengal Chloramphenicol Agar (DRBC, Himedia, Mumbai, India). Samples were incubated at 28 °C for 2 weeks.

(ii) Selective enrichment on volatile aromatics was adapted from Prenafeta-Boldú *et al.* (2001a) and Isola *et al.* (2013). Czapeck agar without glucose (sodium nitrate 2.0 g/L, dipotassium phosphate 1.0 g/L, magnesium sulphate 0.5 g/L, potassium chloride 0.5 g/L, and ferrous sulphate 0.01 g/L, chloramphenicol 0.1 g/L) was used for enrichment in 6 mL liquid culture medium in 15 mL flasks, inoculated with 100 µL of the suspension solution. Flasks were incubated at 25 °C for three weeks under controlled atmosphere rich in volatile aromatic hydrocarbon (toluene) which was supplied as a sole carbon and energy source. Hundred µL of the enriched culture was plated on Mycosel Agar (Difco) and incubated at 28 °C for two weeks and black colonies were transferred to Malt Extract Agar (MEA, malt extract 20 g/L, glucose 10 g/L, peptone 1 g/L, agar 15 g/L).

(iii) Oil flotation (VICENTE *et al*., 2008). Approximately 20 g from each sample were incubated at room temperature for 30 min in 100 mL sterile saline solution containing 200 U penicillin, 200 µg/L streptomycin, 200 µg/L chloramphenicol and 500 µg/L cycloheximide. Subsequently 20 mL sterile mineral oil were added, followed by vigorous 5 min shaking. The flasks were left to settle for 20 min. The oil-water interphase was carefully collected, inoculated onto Mycosel agar (Difco) and incubated for 4 weeks at 28 ºC.

3.2. PHYSIOLOGY AND MORPHOLOGY

Laccase activity was assayed for nine selected isolates (CMRP1196, CMRP1198, CMRP1216, CMRP1227, CMRP1262, CMRP1256, CMRP1258, CMRP1226, CMRP1259) according to Sun *et al.* (2012) using ABTS-agar medium containing 5 mM 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (Sigma Aldrich). Agar plugs (3 mm diam) obtained from the edge of growing mycelium of tested fungi were inoculated and incubated at 24 °C in the dark for 7 d. Positive reaction was indicated by the formation of a green halo around the colony.

Cardinal growth temperatures were determined on MEA plates incubated in the dark for 3 weeks at temperatures of 18–36 °C at intervals of 3 °C; growth was also recorded at 37 °C and at 40 °C. All tests were performed in triplicate and the diameters of the colonies were recorded weekly. Microscopic morphology was done using slide cultures. Slide cultures were prepared with Sabouraud's Glucose Agar (SGA); 2-week-old slide cultures were made in lactic acid or lactophenol cotton blue and observed with a Nikon Eclipse 80i microscope with a Nikon digital sight DS-Fi1 camera.

3.3. MOLECULAR IDENTIFICATION

Initial identification using molecular techniques was based on sequencing of the ITS rDNA region. DNA extraction, amplification, and sequence reconstruction were performed as described by Isola *et al* (2013). Comparisons of ITS region were performed using the CBS databank (www.cbs.knaw.nl) which searches all major sequence databases including GenBank, and a black yeast research database maintained at CBS.

For phylogeny of new species, two additional genes were amplified: the large ribosomal subunit (LSU) and the partial beta tubulin gene (*BT2*) using primers LROR and LR5 (VILGALYS AND HESTER, 1990) and BT2a and T2 (GLASS AND DONALDSON, 1995; O'DONNELL and CIGELNIK, 1997), respectively. PCR reactions mixtures containing 1 µL template DNA (40 ng), 1.25 µL 10× PCR buffer, 1 µL dNTP mix (2.5 mM), 0.5 µL of each primer (10 pmol), 0.1 µL Taq polymerase (Biotaq, Bioline, Germany) (5 U), DMSO 0.5 µL, and water to complete the final volume of 15 µL. All

PCR reactions were performed in an ABI PRISM 2720 (Applied Biosystems, Foster City, U.S.A.) with amplifications conditions performed as follows: 95 ºC for 5 min, followed by 35 cycles consisting of 94 ºC for 45 s (denaturation), 52 ºC for 45 s (annealing), and 72 ºC for 2 min (extension), with a final delay step at 72 ºC for 7 min. For *BT2* the annealing temperature was changed to 58 ºC. 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).

The LSU region was used to reconstruct the phylogeny of the Chaetothyriales. Sequences were adjusted using the SEQMAN package (DNAStar, Madison, U.S.A.) and aligned using the server version of the MAFFT program (http://www.ebiac.uk/Tools/mafft) and manually corrected using MEGA 6. Phylogenetic relationships were estimated using the maximum likelihood method using MEGA 6, Jukes Cantor correction and 500 bootstrap replicates.

3.4. EVALUATION OF VOLATILE ORGANIC COMPOUND (VOC) ASSIMILATION

Assimilation of toluene and phenyl acetate as the sole source of carbon and energy was tested for seven selected isolates (CMRP1196, CMRP1198, CMRP1216, CMRP1227, CMRP1219, CMRP1262, CMRP1256), as previously described (Prenafeta-Boldú *et al*., 2001a, Prenafeta-Boldú *et al*., 2001b). Experiments were performed in 250 mL Boston flasks sealed with Teflon Mininert valves (Phase Separations, Waddinxveen, The Netherlands). Each flask contained 25 mL mineral salts medium (0.5 g/L K_2HPO_4, 4.5 g/L KH_2PO_4, 2 g/L NH_4Cl, 0.1 g/L $MgSO_4.7H_2O$; pH 7) with 0.01% yeast extract. Flasks were supplemented with toluene (7.7 µL) and phenyl acetate (9.3 µL) and, after gas-liquid substrate equilibration, inoculated with a spore suspension of the fungus (10^4 cells/mL). Incubation was at 25 °C under static conditions. Growth was assessed by measuring the substrate consumption and the carbon dioxide accumulation in the headspace by injecting 100 µL gas samples in a Trace Ultra GC (Thermo Finnigan, Langerwehe, Germany) and Varian 3800 GC

(Varian, San Diego, U.S.A.), respectively. For carbon dioxide, the chromatograph was fitted with a Varian Hayasep Q (80e100 mesh) packed column and a Thermal Conductivity Detector (TCD). The temperature of the injector port and the TCD was set at 180 °C, and the temperature of the oven was 90 °C. Helium (He) was used as a carrier gas (45 mL/min). For toluene and phenyl acetate, the stationary phase was a TRB-624 capillary column and a Flame Ionization Detector (FID) was used. The temperatures of the column and the FID were 180 and 250 °C, respectively. The carrier gas was He at a flow rate of 2 mL/min.

3.5. VOC IN BABASSU COCONUT SHELLS

Analysis of VOCs in babassu coconut shells was carried out using a Gas Chromatograph (Agilent 7890, U.S.A.) coupled to a time-of-flight mass spectrometer (GC-ToFMS) (BenchTOF-dx, Almsco, Germany). Dried fragments of a babassu coconut shell were introduced in a micro-chamber and confined in the thermal extraction unit of the GC-ToFMS. Chamber conditions were adjusted to 60 ºC for 15 min in order to equilibrate the emission of VOCs at that temperature. Approximately 400 mL of the headspace were collected and flown through adsorption columns. Once desorbed, compounds were captured in a trap at low temperature (−20 to 10 °C). Subsequently, the cold trap was heated to a maximum of 300−350 °C according to a programmed temperature profile, to release all VOC up to the GC for subsequent chromatographic separation. The emitted VOCs were separated and identified by gas chromatography coupled with time-of-flight mass spectrometry (GC-TOFMS, BenchTOF-dx, ALMSCO International, UK), as described previously (GUTIÉRREZ *et al.,*2015). Mass spectra and the total ion chromatograms were obtained by automatic scanning a mass range (m/z) of 45−400. Volatile components were identified by comparing mass spectra with those available in the spectra library. Chemical composition was reported as the percentage of relative area, after obtaining the sum of all peak areas in the chromatogram. For the data analysis, the threshold of 10 µg m^{-3} was adopted and compounds with a concentration below that were not considered.

4. RESULTS

A total of 106 black yeast-like fungi were isolated from babassu coconut shells by a combination of techniques (Table 1). All six processed samples were positive for black fungi, but significantly higher isolation yields were obtained in samples (3,5 and 6) from rotting babassu coconut (Table 2). While black yeasts were isolated successfully from all analysed samples by means of the oil flotation method (103 isolates in total), the results were rather limited when using the selective enrichment method on toluene (three isolates from three samples/CMRP1219, CMPR1227 and CMRP1247). No black yeast-like isolates were obtained with the direct plating method.

Strains were identified on basis of their rDNA ITS sequences. Reference sequences from the CBS collection (housed at Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands) were included in a comparative analysis. Comparison of sequence data led to assignment to nine phylogenetic groups belonging to the genera *Cladophialophora*, *Exophiala*, *Rhinocladiella,* and *Veronaea*.

The babassu coconut samples yielded the isolation of a diversity of species of black yeast, as *Cladophialophora chaetospira*, *Cladophialophora matsushimae*, *Cladophialophora mycetomatis*, *Exophiala alcalophila*, *Rhinocladiella similis, Exophiala spinifera* and *Veronaea botryosa*.

Several isolates belonged to two species of clinical interest, *E. spinifera* and *V. botryosa,* and one strain fully matched with *C. mycetomatis*, thus far only known from a single subcutaneous infection. No *Fonsecaea* species was isolated. None of the isolated species was a known agent of human chromoblastomycosis.

In order to assess the phylogenetic position of species that did not match with any described taxon, combined partial LSU (Figure 7) and ML trees based on ITS and β-tubulin (Figure 8) were constructed using sequence data of representative species in Herpotrichiellaceae (Chaetothyriales). Both phylogenetic trees showed that isolates of *Cladophialophora* sp. grouped in a distinct cluster, relatively close to *C. mycetomatis* (Figure 7 and 8) and the unknown *Exophiala* sp. grouped close to *Capronia parasitica.* Both taxa are described below as new species.

Table 1 - Isolated strains from Babassu coconut.

SPECIES / POSITIVE SAMPLES[A]	COLLECTION NUMBER OF ISOLATES	GENBANK ACCESSION NUMBER		
		LSU	ITS	BTUB
C. chaetospira sample 4	**CMRP1226**	-	KY680415	-
C. matsushimae sample 2	**CMRP1198**	-	KY680416	-
C. exuberans sample 3	**CMRP1219**	KY570930	KY680430	KY689827
C. exuberans sample 5	CMRP1194, **CMRP1190**, CMRP1409, CMRP1211, **CMRP1227(T)** CMRP1228, CMRP1206	KY570931	KY680431, KY680429,	KY689826
C. exuberans sample 6	CMRP1247, CMRP1205, **CMRP1204**, CMRP1176		KY680432	
C. mycetomatis sample 1	**CMRP1232**	-	KY680417	-
C. mycetomatis sample 3	**CMRP1221**, CMRP1237		KY680418	
C. mycetomatis sample 5	**CMRP1208**, CMRP1215, CMRP1261, CMRP1393, CMRP1192 CMRP1222, CMRP1233, CMRP1217, CMRP1229, CMRP1209	-	KY680419	-
C. mycetomatis sample 6	CMRP1216, CMRP1240, CMRP1269, CMRP1218, CMRP1249 **CMRP1250**, CMRP1252, CMRP1395, CMRP1186, CMRP1396 CMRP1267, CMRP1386, CMRP1177, CMRP1193, CMRP1391	-	KY680420	-
E. alcalophila sample 4	**CMRP1258**	-	KY680421	-
E. palmae sample 3	**CMRP1196 (T)**	KY570929	KY680434	KY689829
E. palmae sample 5	**CMRP1207**, CMRP1255	KY570928	KY680433	KY689828
E. palmae sample 6	CMRP1188, CMRP1189	-	-	-
E. spinifera sample 3	**CMRP1184**, CMRP1197, CMRP1266, CMRP1195, CMRP1183 CMRP1243, CMRP1223, CMRP1412, CMRP1245, CMRP1408 CMRP1257, CMRP1241, CMRP1191, CMRP1235, CMRP1225 CMRP1234, CMRP1411, CMRP1178, CMRP1260, CMRP1231 CMRP1242, CMRP1179, CMRP1187, CMRP1263	-	KY680422	-
E. spinifera sample 5	**CMRP1256**, CMRP1254, CMRP12700, CMRP1253, CMRP1268 CMRP1239	-	KY680423	-

Continued on next page

SPECIES / POSITIVE SAMPLES[A]	COLLECTION NUMBER OF ISOLATES	GENBANK ACCESSION NUMBER		
		LSU	ITS	BTUB
E. spinifera sample 6	**CMRP1230, CMRP1246, CMRP1212, CMRP1182, CMRP1248 CMRP1210, CMRP1244, CMRP1185, CMRP1180, CMRP1213 CMRP1214, CMRP1413, CMRP1220**	-	KY680424	-
R. similis sample 4	**CMRP1259**	-	KY680425	-
V. botryosa sample 2	**CMRP1181**	-	KY680426	-
V. botryosa sample 5	**CMRP1199**, CMRP1200, CMRP1201, CMRP1202, CMRP1203 CMRP1224	**-**	KY680427	-
V. botryosa sample 6	**CMRP1236**, CMRP1238, CMRP1251, CMRP1262, CMRP1264 CMRP1265, CMRP1175	-	KY680428	-

[a]Origin: São Luiz, Maranhão, Brazil; **T-** Type strain; *CMRP – Coleção Microbiológica da Rede Paranaense (Microbiological Collections of Paraná Network); The collection numbers in bold correspond to the isolates which sequences have been deposited in GenBank

Table 2 - Black yeast isolates obtained from different Babassu coconut samples and isolation methods

SAMPLE NUMBER	ISOLATION METHODS			TOTAL ISOLATES	% ISOLATION PER SAMPLE
	Direct plating	Oil Flotation	Gas-phase enrichment		
1[a]	0	1	0	1	0.94
2[a]	0	2	0	2	1.88
3[b]	0	27	1	28	26.42
4[a]	0	3	0	3	2.83
5[b]	0	30	1	31	29.25
6[b]	0	40	1	41	38.68
Total	0	103	3	106	-

[a]Sample in initial stage of decomposition; [b]Sample in advanced stage of decomposition.

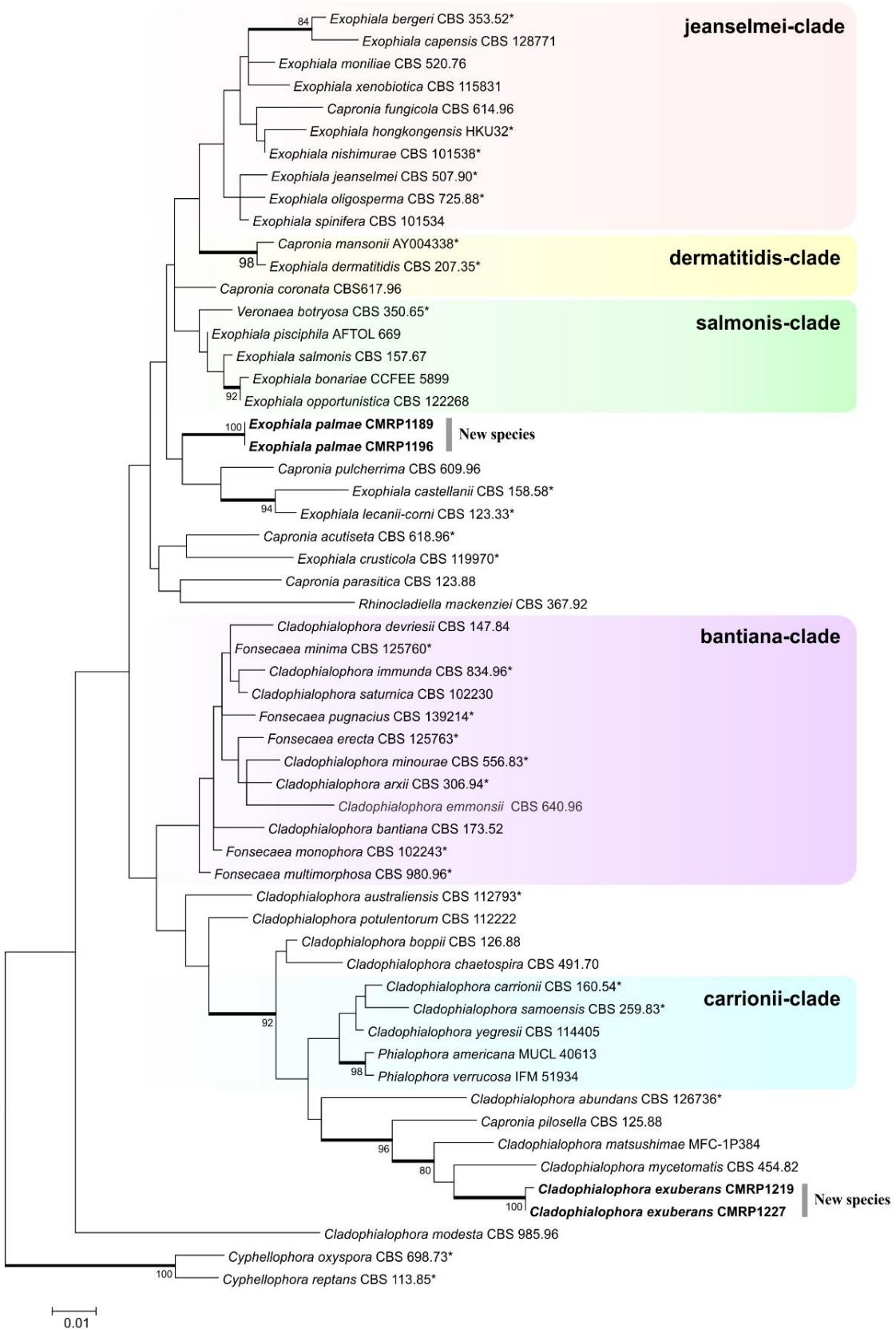

Figure 7 - Phylogenetic tree of Herpotrichiellaceae based on the alignment of D1/D2 domain of LSU sequences constructed with Maximum likelihood implemented in Mega6 with Kimura's two parameters model. *Cyphellophora oxyspora* (CBS 968.73) and *Cyphellophora reptans* (CBS113.85) were used as outgroup.The bootstrap support was calculated using 500 replicates and values ≥ 70% are shown in the branches.

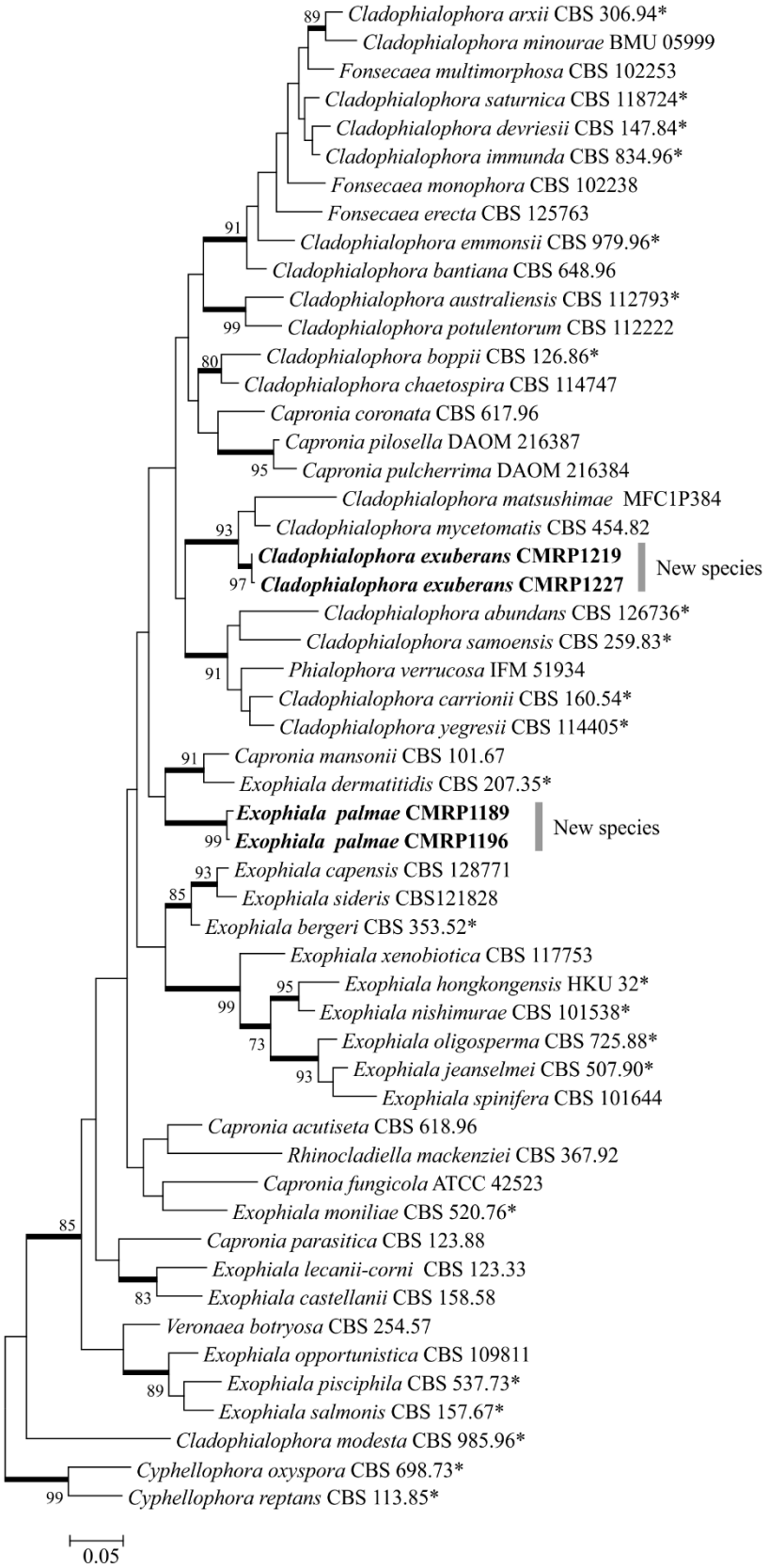

Figure 8 - Phylogenetic tree of Herpotrichiellaceae based on the alignment of B-tubulin and ITS1-5.8S-ITS2 sequences constructed with Maximum likelihood implemented in Mega6 with Jukes-Cantor model. *Cyphellophora oxyspora* (CBS 968.73) and *Cyphellophora reptans* (CBS113.85) were used as outgroup. Bootstrap values (500 replicates) above 70 % are added to supported branches.

Nine isolates (one representative of each identified phylogenetic group) were cultured on ABTS-agar medium to determine extracellular laccase activity. Halos were consistently observed around the colonies, with a colour intensity and halo diameter that was nearly invariable between species.

A subset of seven strains (one representative for each identified phylogenetic group) was tested for their capacity to assimilate phenyl acetate and toluene as the sole source of carbon and energy. Phenyl acetate was readily metabolized by the tested isolates as carbon and energy source, but two distinct groups were apparent. On the one hand, isolates CMRP1196, CMRP1219 and CMRP1262 showed substrate carbon recovery as carbon dioxide higher than 60 % after 32 days of incubation, with a comparatively short lag-phase for growth (<7 days). On the other hand, isolates CMRP1198, CMRP1216 and CMRP1256 displayed lower substrate mineralization levels (<20 %) and longer lag-phases (about 18 days). Strain CMRP1229 occupied an intermediate position with phenyl acetate carbon recovery of 41 % by the end of the incubation period.

Concerning toluene, only two isolates (CMRP1227 and CMRP1219, both belonging to *Cladophialophora exuberans* described below) were capable of growing with toluene as the sole source of carbon and energy. Carbon mass balances demonstrated that more than the 70 % of the carbon contained in the added toluene was recovered as carbon dioxide upon complete substrate degradation (Table 3). A prolonged lag-phase of around 35 days was observed prior to any significant biomass development and carbon dioxide accumulation in the culture headspace, thus indicating difficulty of fungal toluene metabolism (Figure 12).

Table 3 - Assimilation of phenyl acetate and toluene by selected black yeasts isolates.

			PHENYL ACETATE		TOLUENE	
STRAIN NR	**IDENTIFICATION**	**ISOLATION METHOD[a]**	C-recovery (%)	Lag-phase (days)	C-recovery (%)	Lag-phase (days)
CMRP1196	*Exophiala palmae*	OF	60	<7	–[B]	–
CMRP1198	*Cladophialophora matsuhimae*	OF	16	18	–	–
CMRP1216	*Cladophialophora mycetomatis*	OF	10	18	–	–
CMRP1227	*Cladophialophora exuberans*	GE	64	<7	75	35
CMRP1219	*Cladophialophora exuberans*	GE	41	15	91	35
CMRP1262	*Veronaea botryosa*	OF	98	<7	–	–
CMRP1256	*Exophiala spinifera*	OF	19	17	–	–

[a] OF: Oil flotation technique; GE: gas-phase enrichment on volatile substrates.
[b] No growth.

A total of 140 VOCs was identified in babassu coconut samples. Most of these compounds were detected in trace amounts and only 42 were observed at headspace concentrations above 10 µg m^{-3} (Table 4), and might therefore play a more significant ecological role in the babassu-associated indigenous mycobiota. Alcohols (nine compounds, 2023 µg m^{-3}), and particularly ethanol (1051 µg m^{-3}), were the most abundant compounds. Other important detected chemical families included aldehydes (five compounds, 223 µg m^{-3}) and ketones (7 compounds, 801 µg m^{-3}). Terpenoids (7 compounds, 885 µg m^{-3}) were also present in significant amounts. Volatile hydrocarbons were quite common, and encompassed mainly alkanes (seven compounds, 313 µg m^{-3}) and alkylbenzenes (four compounds, 74 µg m^{-3}). The latter group included environmentally relevant contaminants from the chemical industry, like toluene (34 µg m^{-3}), ethylbenzene (15 µg m^{-3}), and xylene isomers (13 µg m^{-3}). Other chemical families represented with less compounds and/or at lower concentrations were organic acids, organosulphur and organohalogen compounds (Table 4).

Table 4 - Head space GC-ToF-MS profile of predominant volatile organic compounds (>10 µg m^{-3}) emitted from babassu coconut upon heating dried samples at 60°C.

RT (min)	COMPOUND	CAS No.	CHEMICAL FAMILY [a]	CONCENTRATION (µg /m^{-3})
7.9	Ethanol	64-17-5	ALC	1051
9.3	2-Propanol	67-63-0	ALC	544
15.0	1-Propanol, 2-methyl-	78-83-1	ALC	26
16.6	1-Butanol	71-36-3	ALC	73
19.7	1-Butanol, 3-methyl-	123-51-3	ALC	50
19.9	1-Butanol, 2-methyl-	137-32-6	ALC	23
21.1	1-Pentanol	71-41-0	ALC	19
26.1	2-Heptanol	543-49-7	ALC	58
31.0	1-Hexanol, 2-ethyl-	104-76-7	ALC	179
11.2	Propanal, 2-methyl-	78-84-2	ALD	70
15.7	Butanal, 3-methyl-	590-86-3	ALD	68
16.0	Butanal, 2-methyl-	96-17-3	ALD	51
22.1	Hexanal	66-25-1	ALD	14
29.4	Benzaldehyde	100-52-7	ALD	20
10.2	Pentane, 2-methyl-	107-83-5	ALK	15
10.8	Pentane, 3-methyl-	96-14-0	ALK	20
35.0	Dodecane	112-40-3	ALK	22
38.0	Tridecane	629-50-5	ALK	24
40.8	Tetradecane	629-59-4	ALK	94
46.3	Hexadecane	544-76-3	ALK	119
4.5	Cyclopropane	75-19-4	AROM	19
20.2	Toluene	108-88-3	AROM	34
24.2	Ethylbenzene	100-41-4	AROM	15
24.5	m,p-xylene	108-38-3/106-42-3	AROM	13
25.6	Benzocyclobutene	694-87-1	AROM	12
30.5	Benzene, 1,4-dichloro-	106-46-7	HAL	13
9.0	Acetone	67-64-1	KET	354
13.2	2-Butanone	78-93-3	KET	23
25.9	2-Heptanone	110-43-0	KET	296
29.5	5-Hepten-2-one, 6-methyl-	110-93-0	KET	13
29.6	2-Octanone	111-13-7	KET	23
33.2	Acetophenone	98-86-2	KET	65
39.3	2-Undecanone	112-12-9	KET	26
15.5	Acetic acid	64-19-7	OAC	31
9.5	Carbon disulfide	75-15-0	SULF	45
30.2	D-Limonene	5989-27-5	TERP	59
32.3	Citronellyl formate	105-85-1	TERP	560
33.3	Linalool, formate	115-99-1	TERP	111
36.0	(-)-Alcanfor	464-48-2	TERP	13
36.3	L-(-)-Menthol	2216-51-5	TERP	80
38.8	(-)-Carvone	6485-40-1	TERP	15
40.5	a-Guaiene	88-84-6	TERP	47

[a] ALD: Aldehydes; ALC: Alcohols; ALK: Alkanes; AROM; aromatic hydrocarbons; HAL: Halocompounds; KET: Ketones; OAC: Organic acids; SULF: Organosulphur compounds; TERP: Terpenes and terpenoids.

The novel species of *Cladophialophora* and *Exophiala* are described below.

Cladophialophora exuberans Nascimento, Vicente & de Hoog, **sp. nov.** – Figure 9; MycoBank **MB819715**

Etymology: The name refers to its exuberant conidiophores and to its ability to degrade toluene and phenyl acetate.

Holotype: UPCB86821, Brazil, São Luis, Maranhão state, from decaying shell of babassu coconut (*Orbignya phalerata)*; ex-type culture CMRP1227. Additional material examined listed in Table 1.

Description after 2 wk incubation on SGA, 25 °C.

Colonies growing moderately rapidly, reaching up to 25 mm diam, olivaceous to smoke brown centre with grey margin, velvety, restricted, with cottony aerial mycelium and regular margin. Reverse olivaceous black, becoming darker with age and without diffusible pigment in the medium. Hyphae septate, 1.5–3.5 µm diam, smooth- and thin-walled, with occasional anastomoses and some spirally twisted hyphae. Conidiophores solitary, pale brown, cylindrical without septa, arising terminally on hyphae, 3.0−3.5 µm wide. Conidiogenous cells pale brown, smooth-walled, sympodially proliferating with a few conidiogenous loci. Ramoconidia oblate to ellipsoidal, 2.5−4.0 × 2.5−3.0 µm. Conidia holoblastic, ellipsoidal, produced in long chains; subhyaline to pale brown. Optimal growth at 24−30 °C, no growth above 36 °C (Figure 11).

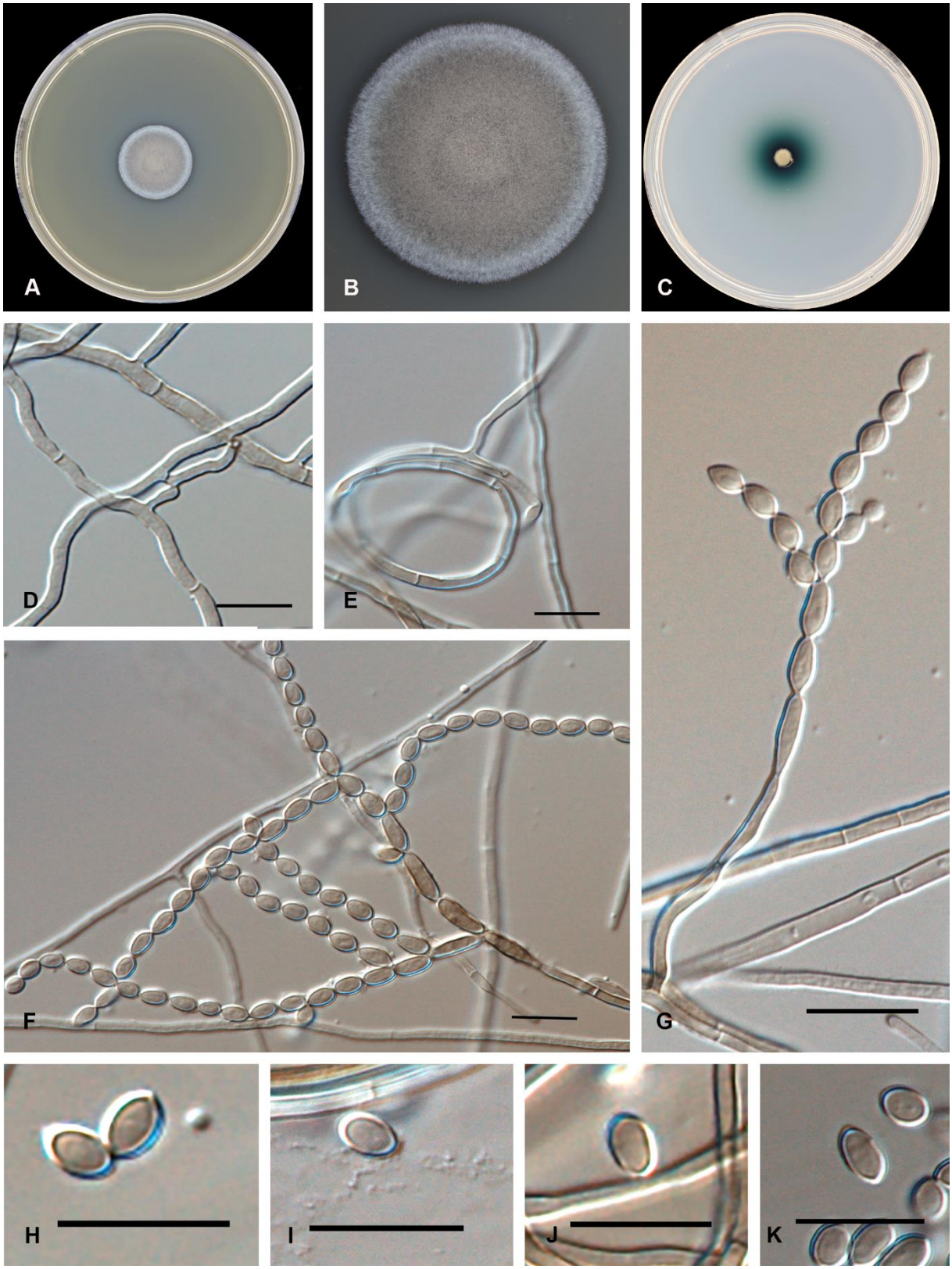

Figure 9 - *Cladophialophora exuberans* (CMRP1227). (A- B) Colony on SAB; (C) Laccase activity on ABTS medium; (D) Hyphae with anastomoses; (E) Spirally twisted hyphae; (F) Conidial chains; (F–K) Conidiophores, conidiogenous cells and conidia. Scale bars = 10 µm

Exophiala palmae Nascimento, Vicente & de Hoog, **sp. nov.** – Figure 10; MycoBank **MB 819714.**

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

Holotype: UPCB86822, Brazil, São Luis, Maranhão state, from decaying shell of babassu coconut (*Orbignya phalerata)*; ex-type culture CMRP1196. Additional material examined listed in Table 1.

Description after 2 wk incubation on SGA, 25 °C.
Colonies growing slowly, reaching up to19 mm diam, greyish olivaceous; reverse olivaceous black, velvety, downy, with cottony aerial mycelium and regular margin. No diffusible pigment produced. Yeast cells (sub)hyaline. Hyphae pale brown, moniliform, with anastomoses, producing conidia apically and laterally, septate 1.5–4.5 µm wide hyphae. Conidiophores (sub)hyaline, inserted laterally or terminally on undifferentiated hyphae. Conidia (sub)hyaline, ellipsoidal to subcylindrical, 1.3–1.9 × 3.0–4.3 µm. Optimal growth at 24–30 °C, no growth at 36, 37 and 40 °C (Figure 11).

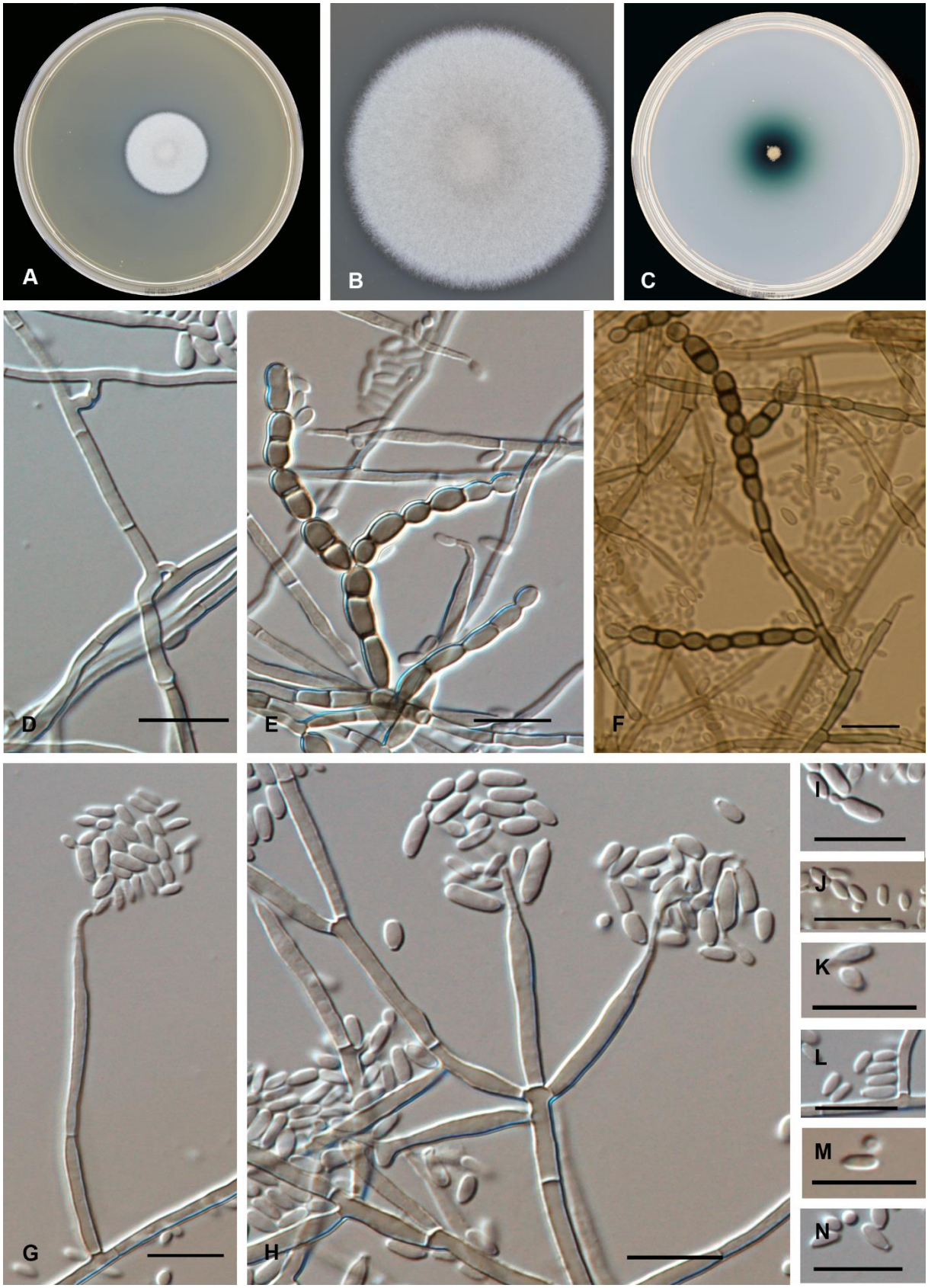

Figure 10 - *Exophiala palmae* (CMRP1196). (A- B) Colony on SAB; (C) Laccase activity on ABTS medium; (D) Hyphae with anastomoses (E-F) Pale brown moniliform hyphae; (G-H) Erect, cylindrical, solitary and multi-celled conidiophore; (I) Budding cell; (J–N) Conidia. Scale bars = 10 µm

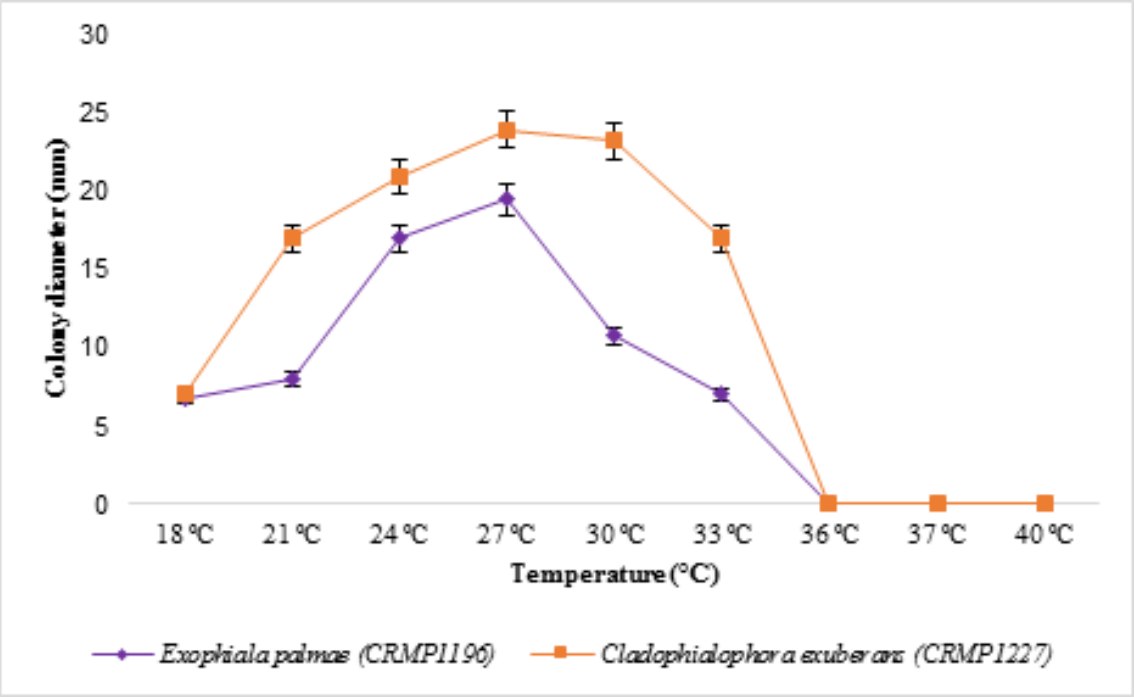

Figure 11 - Colony diameters of novel species at different temperatures ranging from 18 to 40 °C, measured after two weeks on 2 % MEA

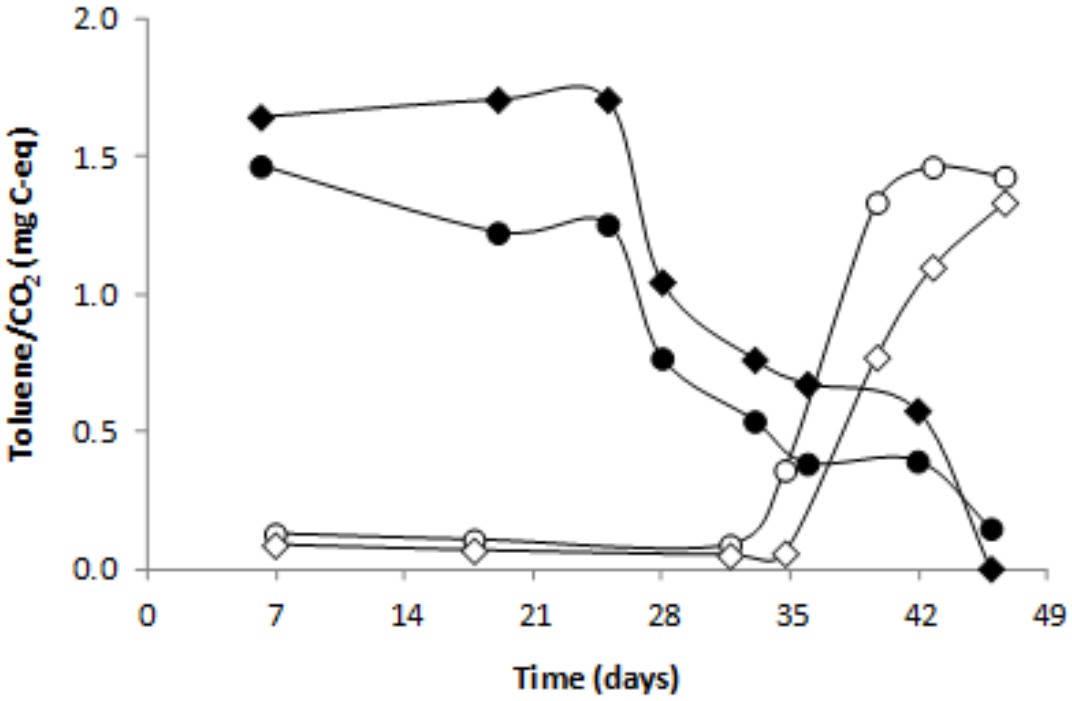

Figure 12 - Biodegradation of toluene (solid signs) linked to the production of carbon dioxide (empty signs) measured in the headspace of sealed axenic liquid cultures of *Cladophialophora exuberans* CMRP1227 (circles) and CMRP1219 (rhombs), both incubated at 25°C.

5. DISCUSSION

In this study we applied different methods to recover black yeast-like fungi from babassu coconut samples (*Attalea speciosa*). The decaying shells provide a rather special environment for selected mycobiota, being rich in fatty acids, terpenes and hydrocarbons. Numerous publications have suggested that chaeotothyrialean black yeasts have a certain association with toxic hydrocarbons, expressed by the fact that these environments tend to be conducive for their prevalence. The oil flotation isolation technique showed the best results in obtaining black yeasts (n=103 isolates). The efficiency of this technique for the recovery of black yeasts from different environmental samples is well-established (IWATSU *et al.,* 1981, MARQUES *et al.*, 2006, VICENTE e*t al.,* 2008, 2012*,* 2014, GUERRA *et al*., 2013). The high success rate and selectivity has been ascribed to the hydrophobicity of the strongly melanized fungal cells and to the capacity of tolerating and even metabolizing toxic hydrocarbons (PRENAFETA-BOLDÚ *et al*., 2006, SATOW *et al*., 2008). Black yeasts in the Chaetothyriales have also consistently been isolated through solid state-like enrichment cultures with vapor-phase substrates. Alkylbenzenes such as toluene appeared to be used as the sole source of carbon and energy by certain species in the genera *Exophiala* and *Cladophialophora* (PRENAFETA-BOLDÚ *et al*., 2001a). The comparatively low number of isolates obtained in our study upon gas-phase enrichment cultures with toluene (only 3 strains) confirms previous claims that a well-developed toluene assimilation pathway might only be present in very few black yeast species (BLASI *et al*., 2016).

Based on ITS sequencing identification, the majority of the obtained isolates were members of the genus *Exophiala*, but representatives of the phylogenetically related genera *Cladophialophora*, *Rhinocladiella* and *Veronaea* were also found. No *Fonsecaea* species was encountered, and none of the isolated *Cladophialophora* or *Rhinocladiella* species was known as an agent of human chromoblastomycosis. However, black yeast-like fungi causing other types of human infection, i.e. *C. mycetomatis, E. spinifera*, and *V. botryosa* were surprisingly frequent. The 28 obtained isolates of *C. mycetomatis* are particularly noteworthy since this species thus far is only known from a single subcutaneous infection in a 49-yr-old male farmer from Jicaltepec, Mexico (BADALI *et al*., 2008). *Veronaea botryosa* has been repeatedly encountered in

human infections worldwide and causes severe disseminated infections in patients without known underlying immune disorders (e.g. BONIFAZ *et al*., 2013), but as yet no cases have been reported from Brazil. *Exophiala spinifera* causes serious, sometimes fatal infections, mostly in adolescents, and has occasionally been diagnosed in Brazil in cases that were unrelated to babassu (CAMPOS-TAKAKI and LOBO JARDIM, 1994, DABOIT *et al*., 2012). *Exophiala alcalophila* was reported from alkaline environments (DE HOOG *et al.,*2011), treated dental unit waterlines (PORTEOUS *et al.,*2009), biofilms on water taps (HEINRICHS *et al.,*2013) and shows a low virulence (DE HOOG *et al.,*2011). *Cladophialophora matsushimae* and *C. chaetospira* were reported occurring in decayed plant material (VICENTE *et al*., 2008, BADALI *et al*., 2008). *Rhinocladiella similis* was first reported from biofilter eliminating gasoline vapors (VIGUERA *et al.,*2009), dialysis water (FIGEL *et al.,*2013) and also causing infection in human skin (RESENDE *et al*., 2000).

In general, our data show that direct inoculation and infection of rural babassu coconut workers with development of chromoblastomycosis is highly unlikely. Judging from these results, chromoblastomycosis of babassu workers is not an occupational disease. It is possible that the used isolation methods were counter-selective for human opportunists, and the application of animal baits might yield yet another set of species, but this seems unlikely since Vicente *et al.* (2014) did isolate agents of chromoblastomycosis from other substrates using the same flotation method.

A hitherto undescribed species of *Cladophialophora* was found. Members of the genus *Cladophialophora* are nearly monomorphic (except for occasional phialides on nutrient-poor media) and our fungus morphologically resembled species such as *C. mycetomatis, C. subtilis*, and *C. samoënsis*. *Cladophialophora exuberans* differs from these species by oblate to ellipsoidal conidia, absence of conidiophores and by physiology, being unable to grow at temperatures above 36 °C. The novel *Exophiala* species, *E. palmae*, showed well-differentiated conidiogenous cells with very long annellated zones.

In addition to potential opportunists, also strict saprobes were recovered, such as *Cladophialophora chaetospira*. Species able to degrade toluene are *Cladophialophora exuberans, C. immunda, C. psammophila*, and *C. saturnica*, as well as *Exophiala oligosperma* and *E. xenobiotica* (BADALI *et al*., 2011). The respective *Cladophialophora* species have never been associated with human disease, while the two *Exophiala* species are occasionally involved in rather mild, superficial infections.

Thus, the relationship between hydrocarbon metabolism and human opportunism is not straightforward, and the exact determinants of pathogenicity remain unknown.

Assimilation of toluene was observed in two strains of the novel species *Cladophialophora exuberans* (CMRP1229 and CMRP1221). These hydrocarbonoclastic isolates were among the three isolates obtained by culture enrichment under toluene-rich atmospheres, thus demonstrating the selectivity of this method towards the isolation of fungi capable of growing on volatile aromatics. In contrast, none of the tested isolates obtained from the oil flotation method were able to assimilate toluene and this method therefore must be considered as a more general technique for isolating black yeasts.

Synthesis of fungal melanin and other pigments is associated with the oxidation of phenolic compounds by phenoloxidases (laccase and tyrosinase) (GRIFFITH, 1994). All tested black yeast isolates had a positive reaction for the laccase using ABTS as secretion indicator for this enzyme. Several studies (FENG *et al*., 2012, SUN *et al*., 2012) used ABTS as an indicator for laccase activity which proved to be very sensitive in the detection of this enzyme in different concentrations. Laccases from fungal origin have been extensively studied because of their ability to oxidize a wide range of important compounds in industry (VISWANATH *et al*., 2014). Many applications have been developed (BOLLAG *et al*., 2003), which a main application concerns bioremediation of toxic recalcitrant compounds. Most strains tested in this study were able to assimilate phenyl acetate. This compound is a relatively common intermediate in the biodegradation of complex aromatic compounds that are oxidized by laccases (e.g. lignin, melanin, aromatic hydrocarbons, etc.), and hence it was expected that this compound will be readily metabolized by fungi producing extracellular laccases.

We interpreted the decaying babassu coconut shell as an ecological niche for black yeasts after comprehensive chemical characterization of the volatile fraction using GC/Time-of-Flight Mass Spectrometry (Table 4). Previous reports on babassu chemistry focused on the lipid fraction of fruits and on its extracted oil (VINHAL *et al*., 2014). Long-chain fatty acids, especially lauric acid, were consistently found to be predominant. In contrast, because of their very low volatility, none of these fatty acids were detected in the present study, where we found only the lighter and more volatile acetic acids in significant amounts in the gas phase. Acetic acid might be the product of ethanol metabolism, which was the most abundant volatile organic compound and

probably arose from natural fruit ripening and fermentation processes. Likewise, other potential by products of fermentation that were measured in comparatively high concentrations included some longer-chain alcohols and their corresponding aldehydes and ketones. The most relevant ones were 2-propanol and acetone, which have previously been reported from the coconut of the phylogenetically related *Cocos nucifera* (JIRAPONG *et al*., 2015). Terpenes and terpenoids were found among the most abundant volatile organic compounds in babassu coconut samples, especially concerning the terpenic acids citronellyl formate and linalool formate (Table 4). The former compound is major constituent of pelargonium oil and its presence has been related to poor storage conditions (KAUL *et al*., 1997), while the latter was detected in trace amounts in essential oils from different plants (WRIGHT *et al*., 2013). Hence, these predominant terpenoids might have been the result of the coconut decay. The main function of terpenoids in plant tissue is to confer protection against microbial attack by fungi and bacteria, as well as from grazing animals (CANNAS *et al*., 2016). Black yeasts are known to tolerate a wide range of toxic chemicals, such as hydrocarbons and heavy metals (SEYEDMOUSAVI *et al*., 2011) and might therefore have an advantage in occupying natural niches rich in such biocidal battery of chemicals, which also include both alkenes and alkylbenzenes like toluene that can be used as growth substrates.

As a general conclusion it may be stated that (1) Toluene-degrading species were isolated only when toluene was offered as enrichment factor, showing that the species spectrum is at least partly dependent on the isolated method. (2) Environments rich in VOCs are selective for black yeast-like fungi including opportunistic pathogens and sibling environmental isolates. Based on the present results the black yeast-colonized babassu coconut is an unlikely source of human infection. (3) To exclude the babassu as potential source of agents of human chromoblastomycosis, this study should be repeated using a warm-blooded animal as bait.

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CHAPTER 3

Arthrocladium, an unexpected human opportunist in Trichomeriaceae (Chaetothyriales)

Arthrocladium, an unexpected human opportunist in Trichomeriaceae (Chaetothyriales)

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1. ABSTRACT

The family Trichomeriaceae (Chaetothyriales) mainly comprises epiphytic and epilithic organisms. In some species elaborate ascomata are formed, but for the great majority of the species no asexual conidium formation is known other than simple fragmentation of the thallus. The present paper re-establishes the genus *Arthrocladium* with three non-sporulating species. One of these is described for a strain causing a fatal infection in a human patient with a rare genetic immune disorder.

Key words: Black yeasts, Disseminated infection, Phylogeny, Sterile fungi

Running title: Opportunistic *Arthrocladium*

2. INTRODUCTION

The Chaetothyriales is an order of ascomycetes comprising black yeasts (anamorph genus *Exophiala*) and its filamentous relatives. At first glance the members of this order show very different ecological traits, regularly occurring in hydrocarbon-containing environments (ZHAO *et al.,*2010), bathing facilities (SUDHADHAM *et al*., 2008), dead plant material (VICENTE *et al.,*2008, 2013), as agents of disease in cold-blooded vertebrates (DE HOOG *et al.,*2011) and in humans (CHOWDHARY *et al.,*2015), and in ant nests (VOGLMAYR *et al.,*2011). However, common factors in these dissimilar habitats are found in several types of extremotolerance (GOSTINCAR *et al.,*2015): oligotrophism, osmo- and thermotolerance, tolerance of irradiation and toxicity, and growth under acidic and alkaline conditions (GÜMRAL *et al.,*2015). The order is unique in the significant role of monoaromatic hydrocarbon assimilation (PRENAFETA-BOLDÚ *et al.,*2006, ISOLA *et al*., 2013).

The best known family is the Herpotrichiellaceae, containing numerous human-pathogenic opportunists: about one-third of members of the family has been described from diseases of the human host, and occasionally infections are disseminated or systemic taking a fatal course (LI *et al.,*2010). Of special significance is that the majority of infected hosts seem to have no underlying metabolic or immune

disorder (BONIFAZ et al., 2013). The Cyphellophoraceae are closely related, and are mainly found as human skin colonizers with enigmatic environmental habitat (FENG *et al.,* 2013). Of the family Chaetothyriaceae only very few members have been sequenced (CHOMNUNTI *et al.,*2012a). The Epibryaceae contain minute intracellular pathogens of liverworths (DÖBBELER 1980) and was recently suggested to belong to the Chaetothyriales on the basis of sequence similarities (GUEIDAN et al., 2014).

The family Trichomeriaceae was recently added to the Chaetothyriales (CHOMNUNTI *et al.,*2012a). It accommodates the ascosporulating genus *Trichomerium*, characterized by small, setose ascocarps with eight transversely septate ascospores which indeed resemble those of *Capronia*, the teleomorph genus in Herpotrichiellaceae. *Trichomerium* species lack anamorph sporulation. In nature they grow together with sooty mold in plant exudates or the sugary honeydew secreted by insects (CHOMNUNTI *et al.,* 2012a). Recently several authors revealed that some genera of extremotolerant fungi growing on exposed surfaces were phylogenetically related to *Trichomerium* (ISOLA *et al.,* 2015). Genera involved were *Bradymyces, Knufia*, and *Lithophila.* Most members of these genera inhabited bare rock and were slow-growing without recognizable sporulation. The taxonomic position of the non-sporulating fungi is ambiguous as they have variously been assigned to Trichomeriaceae and Chaetothyriaceae on the basis of sequence similarity only (TSUNEDA *et al.,* 2012; REBLOVÁ *et al.,* 2013, HUBKA *et al.,* 2014, ISOLA *et al.,*2015).

A genus *Arthrocladium* was introduced by Papendorf (1969) to accommodate a single strain from *Acacia* leaf litter in South Africa. Sequencing of the type strain of *A. caudatum* revealed that the genus is related to *Knufia*; phenotypically it is similar by absence of recognizably differentiated sporulation. The present paper introduces three further species that cluster with *Arthrocladium*. Two of these were isolated as inhabitants of rotten wood, whereas the third caused a fatal disseminated infection in a human with a GATA-2 immune defect (EGENLAUF *et al.,*2015).

3. MATERIAL AND METHODS

3.1. STRAINS ANALYSED

Strains from woody substrates were obtained using the oil flotation method (IWATSU *et al.,*1981, VICENTE *et al.,*2008). A clinical isolate was isolated from a brain biopsy sample. Reference strains were obtained from the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre (CBS) reference collection and are listed in Table 1. Stock cultures were maintained on slants of malt extract agar (MEA; Oxoid, U.K.) and potato dextrose agar (PDA; Oxoid). Taxonomic information for new species was deposited in MycoBank (www.MycoBank.org).

Table 5 - Strain analysed used in multilocus phylogeny.

Species	Strain no.	Source	Locality	Sporulation type	Genbank Accession Number		
					ITS	LSU	BTUB
Arthrocladium caudatum	CBS 457.67 T	Leaf litter *Acacia karroo*	South Africa	None	KT337438	KT337443	KT806374
Arthrocladium fulminans	CBS 136243 T	Disseminated infection human	Africa	None	KT337439	KT337444	KT806375
Arthrocladium tropicale	CBS 134926 T	*Petalomyrmex phylax* ant domatia in *Leonardoxa Africana*	Cameroon	None	KT337440	KT337445	KT806376
Arthrocladium tardum	CBS 127021 T	Shell of coconut Babassu palm	Brazil	None	KT337441	KT337446	KT806377
Arthrocladium tardum	CBS 134919	*Petalomyrmex phylax* ant domatia in *Leonardoxa Africana*	Cameroon	None	KT337442	KT337447	KT806378
Bradymyces alpinus	CBS 138368 T	Rock 3200 m a.s.l.	Italy	Endoconidia	HG793052	GU250396	LN589970
Bradymyces alpinus	CBS 133066	Rock 3200 m a.s.l.	Italy	Endoconidia	HG974249	-	-
Bradymyces oncorhynchi	CBS	Fish (*Oncorhynchus mykiss*)		Endoconidia	HG426062	HG4260693	HG426060
Capronia villosa	MUCL 39951	Decorticated wood	New Zealand	Ascospores	AF050261	-	-
Capronia peltigerae	UAMH 11090 T	Thallus of *Peltigera rufescens*	Luxembourg	Ascospores	HQ709322	HQ613813	-
Cladophialophora abundans	CBS 127907	Soil from mangrove environment	Brazil	Catenate	KC776597	-	-
Cladophialophora abundans	CBS 127890	Leaf of living tree in mangrove environment	Brazil	Catenate	KC776593	-	-
Cladophialophora carrionii	CBS 108.97	Chromoblastomycosis; skin	Venezuela	Catenate	EU137306	-	EU137188
Cladophialophora carrionii	CBS 857.96	Chromoblastomycosis; skin	Venezuela	Catenate	EU137294	-	EU137177
Cladophialophora matsushimae	MFC-1P384	Decaying petiole of palm	Japan	Catenate	FN549916	FN400758	-
Cladophialophora minutissima	UAMH 10710	*Sphagnum fuscum* bog with *Picea mariana*	USA	Catenate	EF016386	-	-
Cladophialophora minutissima	UAMH 10711	*Sphagnum*-dominated wetland	USA	Catenate	EF016385	-	-
Cladophialophora modesta	CBS 985.96 T	Brain, human	USA	Catenate	KF928421	KF928485	KF928549
Cladophialophora mycetomatis	CBS 122637	Mycetoma, human	Mexico	Catenate	NR111364	KC809991	-

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Species	Strain no.	Source	Locality	Sporulation type	Genbank Accession Number		
					ITS	LSU	BTUB
Cladophialophora sylvestris	CBS 350.83 T	Pine needle	Netherlands	Catenate	EU137330	EU035413	-
Cyphellophora olivacea	IFM 53357	-	-	Phialidic	AB190379	-	-
Cyphellophora sessilis	CBS 238.93	Styrene	Netherlands	Phialidic	AY857541	KC455264	-
Cyphellophora suttoni	CBS 449.91 T	Dog´s ear subcutaneous lesion	USA	Phialidic	KC455243	KC455256	-
Cyphellophora vermispora	CBS 228.86 T	Root of *Triticum aestivum*	Germany	Phialidic	KC455244	KC455257	-
Epibryon hepaticola	M10	-	-	-	JX298885	EU940091	-
Epibryon lecanora	ANT050904	Moist coastal rocks and weathered whale bone	King George Island	-	DQ534469	-	-
Exophiala placitae	CPC 13707	Leave of *Eucalyptus placita*	Australia	Annellidic	EU040215	-	-
Knufia cryptophialidica	DAOM 216555 T	*Populus tremuloides*	-	Endoconidia	JN04050	JN040500	-
Knufia endospore	UAMH 10396 T	*Populus tremuloides*	-	Endoconidia	JN040510	-	-
Knufia epidermidis	CCFEE 5814	Gasoline car tank	Italy	Arthroconidia	JX681060	-	
Knufia karalitana	CBS 139720 T, CCFEE 5656	Marble, Cathedral S. Maria, Cagliari	Italy	Arthroconidia	KP791781	-	-
Knufia karalitana	CCFEE 6001	Marble tombstone, CB	Italy	Arthroconidia	KP791785	-	-
Knufia marmoricola	CCFEE 5721	Pietraforte (sandstone), Boyl Palace, Cagliari	Italy	Arthroconidia	KP791787	-	-
Knufia marmoricola	CCFEE 5886	Marble (catalogue n. 22635), CP	Vatican City State	Arthroconidia	KP791779	-	-
Knufia marmoricola	CCFEE 5923	Travertine, Albani family coat of arms, CP	Vatican City State	Arthroconidia	KP791777	-	-
Knufia marmoricola	CCFEE 5726	Pietraforte (sandstone), Boyl Palace, Cagliari	Italy	Arthroconidia	KP791789	-	-
Knufia mediterranea	CCFEE 5768	Marble, G. Todde funerary monument, CB	Italy	Endoconidia	KP791792	-	-
Knufia mediterranea	CBS 139721 T, CCFEE 5738	Marble, F. Warzee funerary monument, CB	Italy	Endoconidia	KP791791	-	-
Knufia mediterranea	CCFEE 6211	Marble, Frau-Carta funerary monument, CB	Italy	Endoconidia	KP791793	-	-

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Species	Strain no.	Source	Locality	Sporulation type	Genbank Accession Number		
					ITS	LSU	BTUB
Knufia mediterranea	CCFEE 5710	Marble, Frau-Carta funerary monument, CB	Italy	Endoconidia		-	-
Knufia perforans	CBS 885.95 T	Marble	Greece	Endoconidia	AJ244230	FJ358237	-
Knufia petricola	CCFEE 5709	Marble monument	Italy	Endoconidia		-	-
Knufia petricola	CCFEE 5777	Marble monument	Italy	Endoconidia		-	-
Knufia petricola	CCFEE 5741	Marble monument	Italy	Endoconidia		-	-
Knufia vaticanii	CCFEE 5939 T	Travertine, St Peter's colonnade	Vatican City State	Arthroconidia	KP791780	-	-
Lithophila guttulata	CCFEE 5910	Marble (catalogue n. 37106), CP	Vatican City State	Endoconidia	KP791772	-	-
Lithophila guttulata	CBS 139723 T, CCFEE 5907	Marble (catalogue n. 37106), CP	Vatican City State	Endoconidia	KP791773	-	-
Lithophila guttulata	CCFEE 5908	Marble (catalogue n. 37106), CP	Vatican City State	Endoconidia	KP791770	-	-
Metulocladosporiella musicola	CBS 113865	*Musa sapientum*	Uganda	Sympodial	DQ008134	DQ008158	-
Metulocladosporiella musicola	CBS 113873	*Musa sapientum*	Mozambique	Sympodial	DQ008135	DQ008159	-
Metulocladosporiella musicola	CBS 110960	*Musa* sp.	South Africa	Sympodial	DQ008127	DQ008153	-
Phaeococcomyces catenatus	CBS 650.76 T	Air	Switzerland	Arthroconidia	JN040512	-	-
Trichomerium deniqulatum	MFLUCC 10-0884 T	Living leaf of *Psidium guajava*	Thailand	Ascospores	JX3136554	JX313660	-
Trichomerium dioscoreae	CBS 138870	Leaf of *Dioscorea* sp.	Japan	Ascospores	KP004468	-	-
Trichomerium foliicola	MFLUCC 10-0054	*Mangifera indica*	Thailand	Ascospores	JX313651	JX313657	-
Trichomerium foliicola	MFLUCC10-0073	*Psidium guajava*	Thailand	Ascospores	JX313652	JX313658	-
Trichomerium foliicola	MFLUCC10-0078	*Murraya paniculata*	Thailand	Ascospores	JX313655	JX313661	-
Trichomerium gloeosporum	MFLUCC10-0087	*Ficus* sp.	Thailand	Ascospores	JX313656	JX313662	-

CBS: CBS Fungal Biodiversity Centre, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CCFEE: Culture Collection of Fungi from Extreme Environments, Dept. of Ecological and Biological Sciences, University of Tuscia, Viterbo, Italy; CPC: Working collection Pedro Crous, housed at CBS; DAOM: Plant Research Institute, Department of Agriculture (Mycology); IFM: Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba, Japan; MFC: Matsushima Fungus Collection, Kobe, Japan; MFLUCC: Mae Fah Luang University Culture Collection, Thailand; MUCL: Culture Collection, Catholic University of Louvain; UAMH: University of Alberta Microfungus Collection and Herbarium, Canada.
T = type culture.

3.2. MORPHOLOGY

Strains were cultured on MEA and PDA and incubated at 24 °C for 3 weeks. Microscopic preparations were performed by slide cultures on MEA and PDA using lactic acid, lactic acid-cotton blue and Shears as mounting fluids. Micrographs were taken using a Nikon Eclipse 80i microscope and DS Camera Head DS-Fi1/DS- 5 m/DS-2Mv/DS-2MBW using NIS-Element freeware package (Nikon Europe, Badhoevedorp, The Netherlands).

3.3. PHYSIOLOGY

Cardinal growth temperatures were determined on MEA plates incubated in the dark for 2 weeks at temperatures of 18–36 °C at intervals of 3 °C; growth was also recorded at 37 °C and at 40 °C with two replicates for each isolate. Laccase enzyme activity was tested according FENG *et al.* (2012), using ABTS agar medium containing 0.03% ABTS (2, 2-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid diammonium salt). ABTS plates were incubated at room temperature for 1 week.

3.4. DNA EXTRACTION

Approximately 1 cm^2 of 14-day-old cultures was transferred to a 2 mL screw-capped tubes filled with 490 µL 2% cetyltrimethylammonium bromide (CTAB) buffer and 6–10 acid-washed glass beads (diam 1.5-2.0 mm, Sigma). 10 µL Proteinase K were added and mixed on a MoBio vortex for 10 min, the mixture was incubated at 60 ºC for 30 min. After incubation, 500 µL Chloroform: isoamylalcohol (24:1) was added and shaken for 2 min. The tubes were centrifuged for 10 min at 20,400g force value, supernatants were collected in new 1.5 mL Eppendorf tubes, ~270 µL of ice-cold iso-propanol was added followed by centrifugation again at 14,000 r.p.m. for 10 min. Pellets were washed with 1 mL ice-cold 70% ethanol, dried using a vacuum dryer and re-suspended in 50 µL TE-buffer. DNA concentrations were measured with NanoDrop 2000

spectrophotometer (Thermo Fisher, Wilmington, U.S.A.). Extracted DNAs were stored at –20 ºC until use.

3.5. MULTILOCUS SEQUENCING

Four gene regions were amplified: rDNA the internal transcribed spacer region (ITS), (partial) large ribosomal subunit (LSU), and the partial β-tubulin gene (*BT2*) and translation elongation factor 1-alpha (*TEF1*) loci. Primers used for amplification and sequencing are listed in Table 2. PCR reactions mixtures containing 1 µL template DNA (50 ng), 1.25 µL 10× PCR buffer, 1 µL dNTP mix (2.5 mM), 0.5 µL of each primer (10 pmol), 0.2 µL Taq polymerase (Biotaq, Bioline, Germany) (5 U/µL), BSA 0.5 µL, and water to complete the final volume of 12.5 µL. PCR was performed in an ABI PRISM 2720 (Applied Biosystems, Foster City, U.S.A.). Amplifications were performed as follows: 95 °C for 5 min, followed by 35 cycles consisting of 94 °C for 45 s, 52 °C for 45 s and 72 °C for 2 min and a post-elongation step at 72 °C for 7 min. Annealing temperature was changed to 58 °C for the *BT2* gene. PCR products were visualized by electrophoresis on a 1% (w/v) agarose gel. Sequencing reaction mixtures contained 1 µL template DNA (0.1 pmol), 1 µL primer (4 mM), 1 µL of BigDye™ terminator (Applied Biosystems), 3 µL buffer and 5.5 µL ultra-pure water to 10 µL final volume. Sequencing PCR was performed as follows: 95 °C for 1 min, followed by 30 cycles consisting of 95 °C for 10 s, 50 °C for 5 s and 60 °C for 2 min. Reactions were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) and sequencing was done on an ABI 3730XL automatic sequencer (Applied Biosystems).

Table 6 - Primer list used for amplification and sequencing in this study.

LOCUS	PRIMER	PRIMER SEQUENCE (5´- 3´)	REFERENCE
ITS	V9G	TTACGTCCCTGCCCTTTGTA	DE HOOG AND GERRITS VAN DEN ENDE (1998)
	LS266	GCATTCCCAAACAACTCGACTC	MASCLAUX *et al.,*1995
	ITS1	TCCGTAGGTGAACCTGCGG	WHITE *et al.,*(1990)
	ITS4	TCCTCCGCTTATTGATATGC	
LSU	LROR	ACCCGCTGAACTTAAGC	VIGALYS AND HESTER, (1990)
	LR5	ATCCTGAGGGAAACTTC	
BT2	BT-2a	GGTAACCAAATCGGTGCTGCTT	GLASS AND DONALDSON, (1995)
	T2	TAGTGACCCTTGGCCCAGTTG	O'DONNELL AND CIGELNIK (1997)
TEF1	EF-983F	GCYCCYGGHCAYCGTGAYTTYAT	REHNER (2001)
	EF-2218R	ATGACACCRACRGCRACRGTYTG	

3.6. ALIGNMENT AND PHYLOGENETIC ANALYSES

Sequences were ajusted using SeqMan from the Lasergene package (DNAStar, Madison, WI, U.S.A.) and pre-aligned in the BioNumerics package v. 4.61 (Applied Maths, Sint-Martens-Latem, Belgium). The ITS region was used to reconstruct the phylogeny of the Chaetothyriales. Sequences were aligned using the server version of the MAFFT program (http://www.ebi.ac.uk/Tools/mafft) and manually corrected in the program MEGA 6. Phylogenetic relationships were estimated using the maximum likelihood method with the server version of RAxML-VI-HPC v.7.0.0 (https://www.phylo.org/), as implemented on the Cipres portal. The robustness of the trees was estimated by a bootstrap analysis with 1,000 replicates. The tree was rooted by the nearest neighbours.

4. RESULTS

Cardinal growth temperature tests showed that all cultures evaluated in this study had their optimal development at 27–33 °C, with growth abilities ranging between 18–40 °C (Figure 13). For strains CBS 457.67, CBS 127021, CBS 134926, CBS 136243, and CBS 134919 the optimum growth temperature on MEA was 30 °C, with a maximum of 36 °C for CBS 134926, 37 °C for CBS 127021 and 40 °C for CBS 457.67, CBS 136243 and CBS 134919 (Figure 13).

Phylogeny of Chaetothyriales using the D1–D2 region of rDNA LSU confirmed that the type strain of *Arthrocladium caudatum*, CBS 457.67 clustered among members of T*richomeriaceae*, with *Trichomerium dioscoreae* in a paraphyletic position (data not shown). An ITS tree was constructed including all currently sequenced members of Trichomeriaceae, together with representatives of remaining families of Chaetothyriales (Figure 13). The family, currently comprising the genera *Bradymyces, Lithomyces, Knufia*, and *Trichomerium* was found clearly separate from remaining genera of Chaetothyriales included for comparison. *Arthrocladium* and *Trichomerium* formed sister clades, while *Knufia* was found in a derived position (Figure 13). *Capronia peltigerae* was found amidst *Knufia* species. *Cladophialophora cladoniae* and *C.*

parmeliae, described by Diederich (2012) from rock and growing association with lichens, proved to be distant, outside the Trichomeriaceae (data not shown).

The type strain of *Arthrocladium caudatum*, CBS 457.67 (Figure 16), the generic type of *Arthrocladium*, clustered in the ITS tree with four additional strains in a separate cluster at 99% bootstrap support (Figure 13). For multilocus sequencing using BT2 and TEF1 data the *Arthrocladium* cluster was too distant from nearest neighbours to produce a meaningful phylogeny. In Figure 14, a selection of strains was included to underline the gap between this cluster and remaining species of Trichomeriaceae. Concordance of trees and high bootstrap values between subcluster or individual strains suggests that three separate species were concerned in addition to *A. caudatum*. No ecological trend is apparent between strains. All nearest neighbours of the *Arthrocladium* clade concern as yet undescribed fungi and were located at large distance from all species reported in this paper. The species that can currently be recognized in *Arthrocladium* are the following:

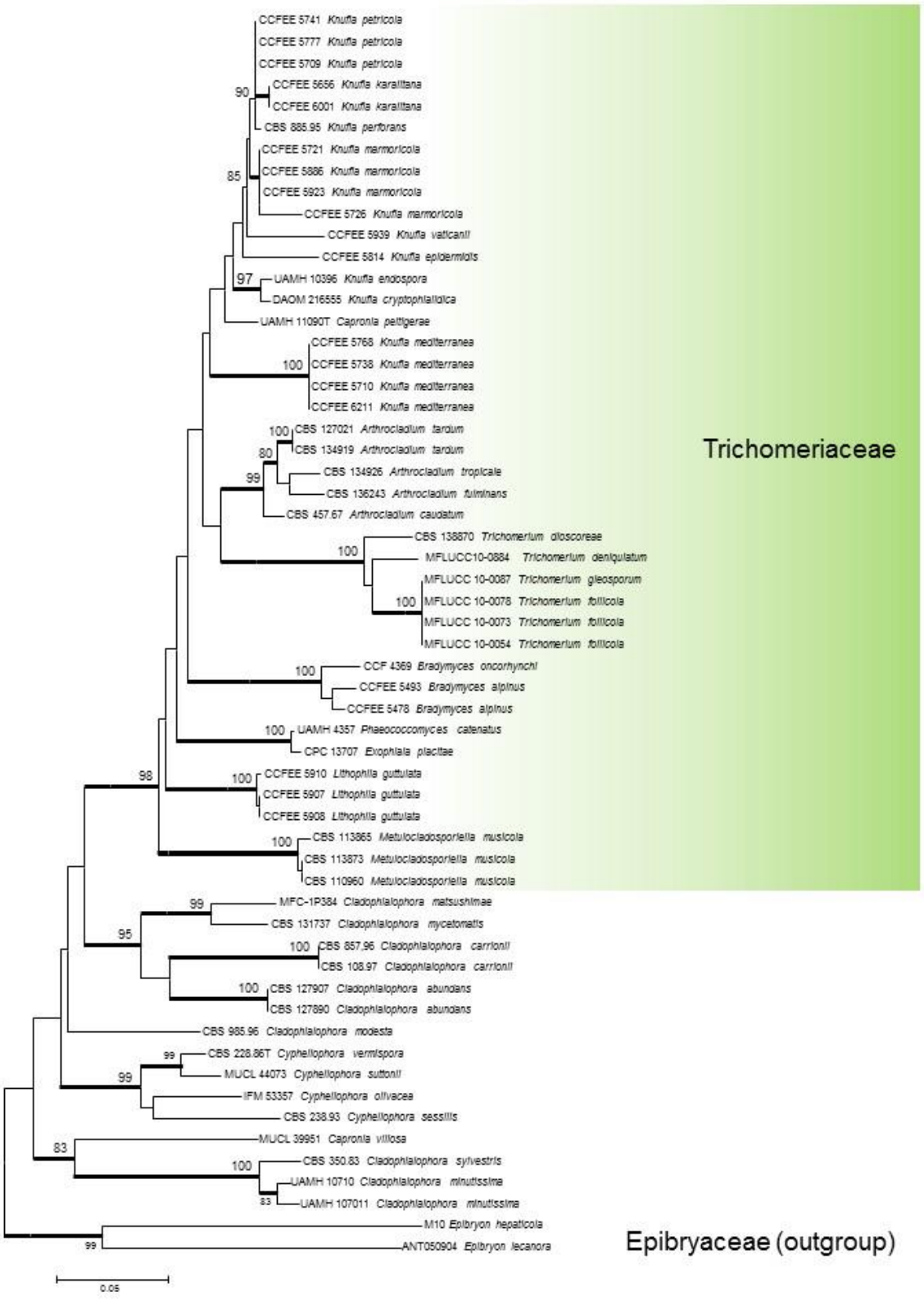

Figure 13 - Phylogenetic tree generated by maximum likelihood analysis using sequences of the rDNA ITS region. Bootstrap values (1,000 replicates) above 80% are added to supported branches (ML). *Epibryon lecanora* (ANT050904) and *E. hepaticola* (M10) are used as outgroup.

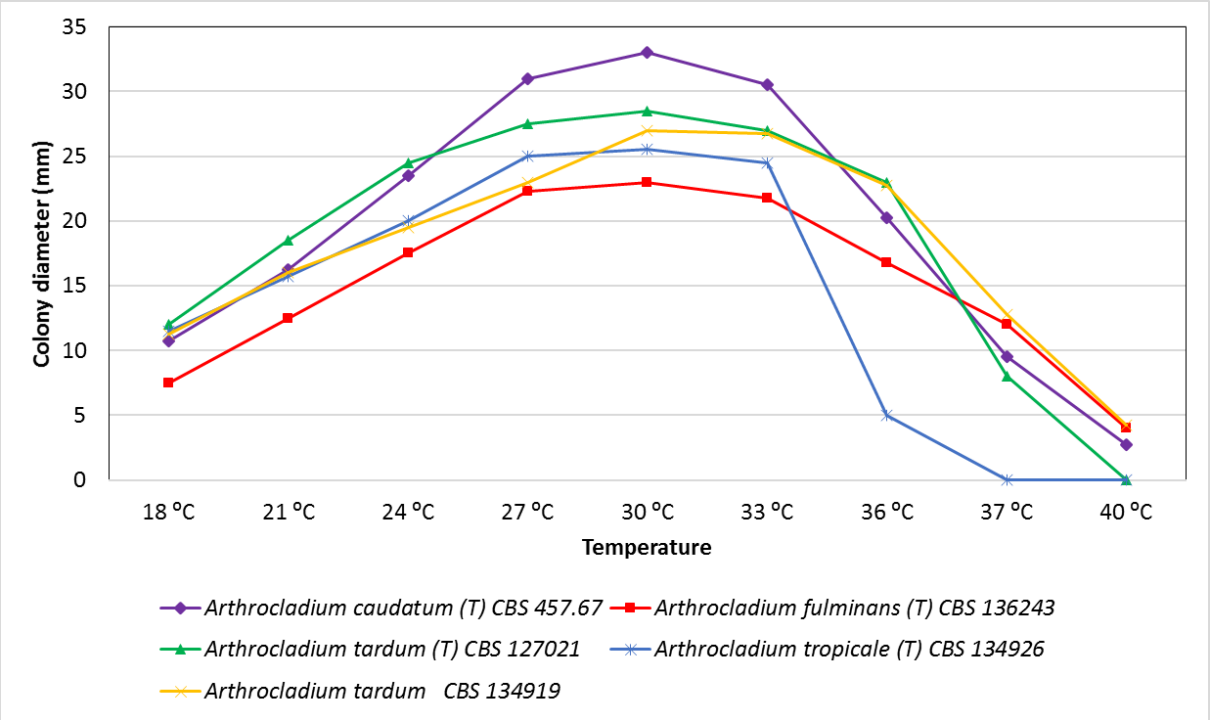

Figure 14 - Colony diameters of strains at various temperatures of *Arthrocladium* species ranging from 18 to 40 °C, measured after 2 weeks on MEA.

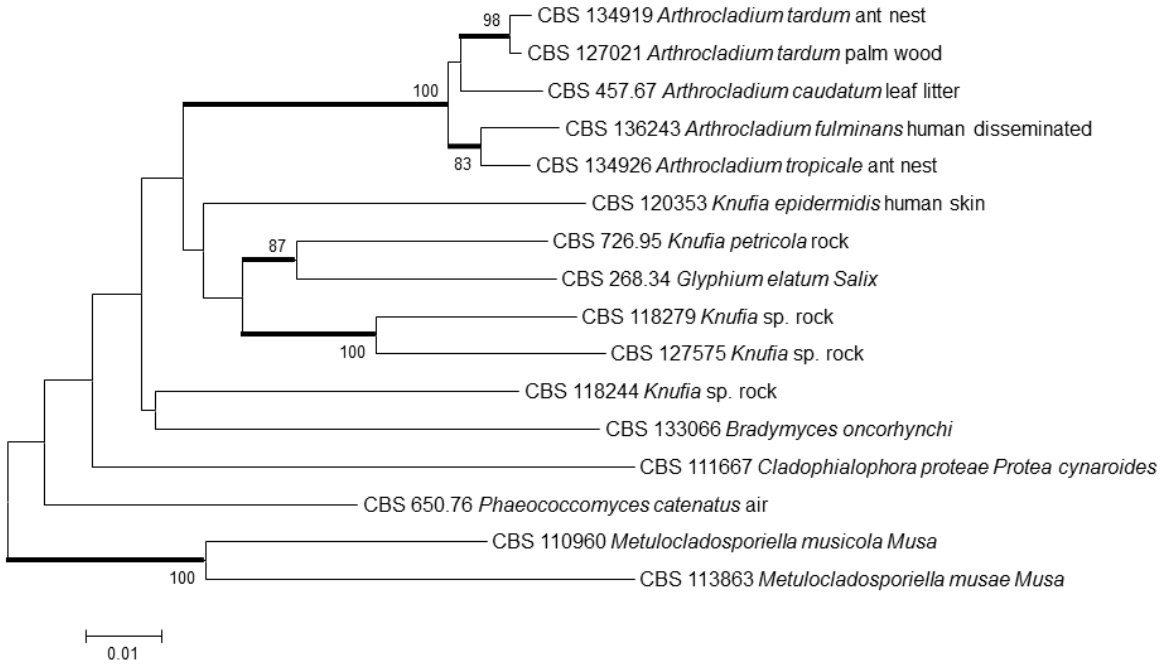

Figure 15 - Phylogenetic tree generated by maximum likelihood analysis using sequences of partial BT2 and TEF1 regions. Bootstrap values (1,000 replicates) above 80 % are added to supported branches (ML). *Metulocladosporiella musicola* (CBS110960) and *Metulocladosporiella musae* (CBS113836) was taken as outgroup.

Arthrocladium caudatum Papendorf, Trans. Br. Mycol. Soc. 52: 483, 1969 – Figure 16. MycoBank MB326486.

Description of CBS 457.67 after 2 week incubation on MEA, 25 °C.

Colonies greyish olivaceous, restricted, circular, with evenly coloured, velvelty aerial mycelium; reverse olivaceous, becoming darker with age. No diffusible pigment produced on any medium. Hyphae septate, 1.5–4.0 µm diam. Conidia not observed; some hyphal cells consisted of swollen and occasionally disarticulated into conidium-like cells. Sexual state unknown. Optimal growth between 27−33 °C, scant growth at 37 and 40 °C.

Type strain CBS 457.67, isolated from *Acacia karroo* leaf litter, Potchefstroom, South Africa.

Notes: The genus *Arthrocladium* was described for a single species isolated from leaf litter. No recognizable sporulation could be noted when CBS 457.67. Papendorf (1969) provided a diagrammatic drawing of short series of swollen cells which occasionally were liberated, which he interpreted as conidia. Similar structures were observed in *Arthrocladium* species described below (Figs 5, 6). Since differentiated conidiation is absent in Trichomeriaceae and asexual propagation takes place by irregular fragmentation of swollen hyphal parts at most, we are reluctant to interpret these structures as conidia that should be dedicated to dispersal. Due to their nondescript morphology, *Arthrocladium* species may have been overlooked in ecological studies.

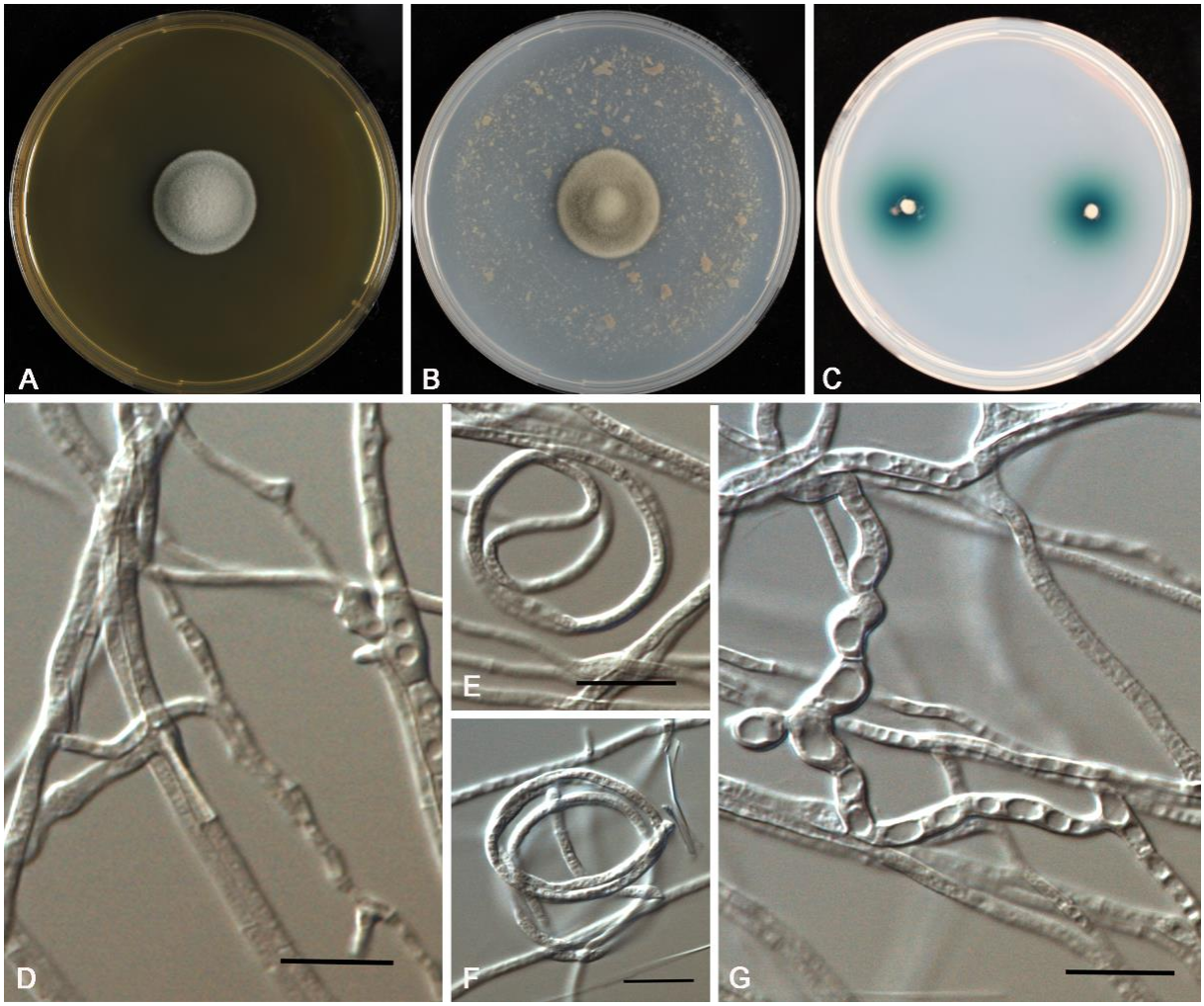

Figure 16 - *Arthrocladium caudatum* CBS 457.67. (A) Colony on MEA; (B) Colony on PDA; (C) Laccase activity on ABTS medium; (D) Hyphae with anastomoses; (E–F) Spirally twisted hyphae; (G) Conidium-like cell — Scale bars = 10 µm.

Arthrocladium fulminans Nascimento, Vicente & de Hoog, **sp. nov.** – Figure 17. MycoBank MB812849.

Etymology: named after the fulminant disease caused in a human patient.

Description of CBS 136243 after 2 week incubation on MEA, 25 °C.

Colonies olivaceous to smoke brown, restricted, circular, with cottony aerial mycelium; reverse olivaceous, becoming darker with age. No diffusible pigment produced on any medium. Colonies slow-growing at 25 °C, but developing slightly more rapidly at higher temperatures. Hyphae septate, 1.5–4.0 µm diam. Conidia not observed; some hyphal cells consisted of swollen and occasionally disarticulated into conidium-like cells.

Sexual state unknown. Optimal growth between 27−33 °C, scant growth at 37 and 40°C.

Holotype CBS H-22287, ex-type culture CBS 136243, isolated from an African immigrant with a fatal disseminated infection, Germany.

Notes: The single strain known of this species was from a patient from Africa with a MonoMAC immunodeficiency syndrome (EGENLAUF *et al.,*2015). This genetic disorder involves mutations in the GATA2 family of transcription factors which contain zinc fingers in their DNA binding domain. The autosomal dominant syndrome leads to severe mono- and lymphocytopenia and B- and NK-cell lymphopenia, which renders the patient susceptible to mycobacterial infections such as tuberculosis, and to viral and fungal infections. The patient acquired a fatal disseminated mycosis by *A. fulminans* following an infection with *Mycobacterium sherrisii*. The origin of the infection is not known.

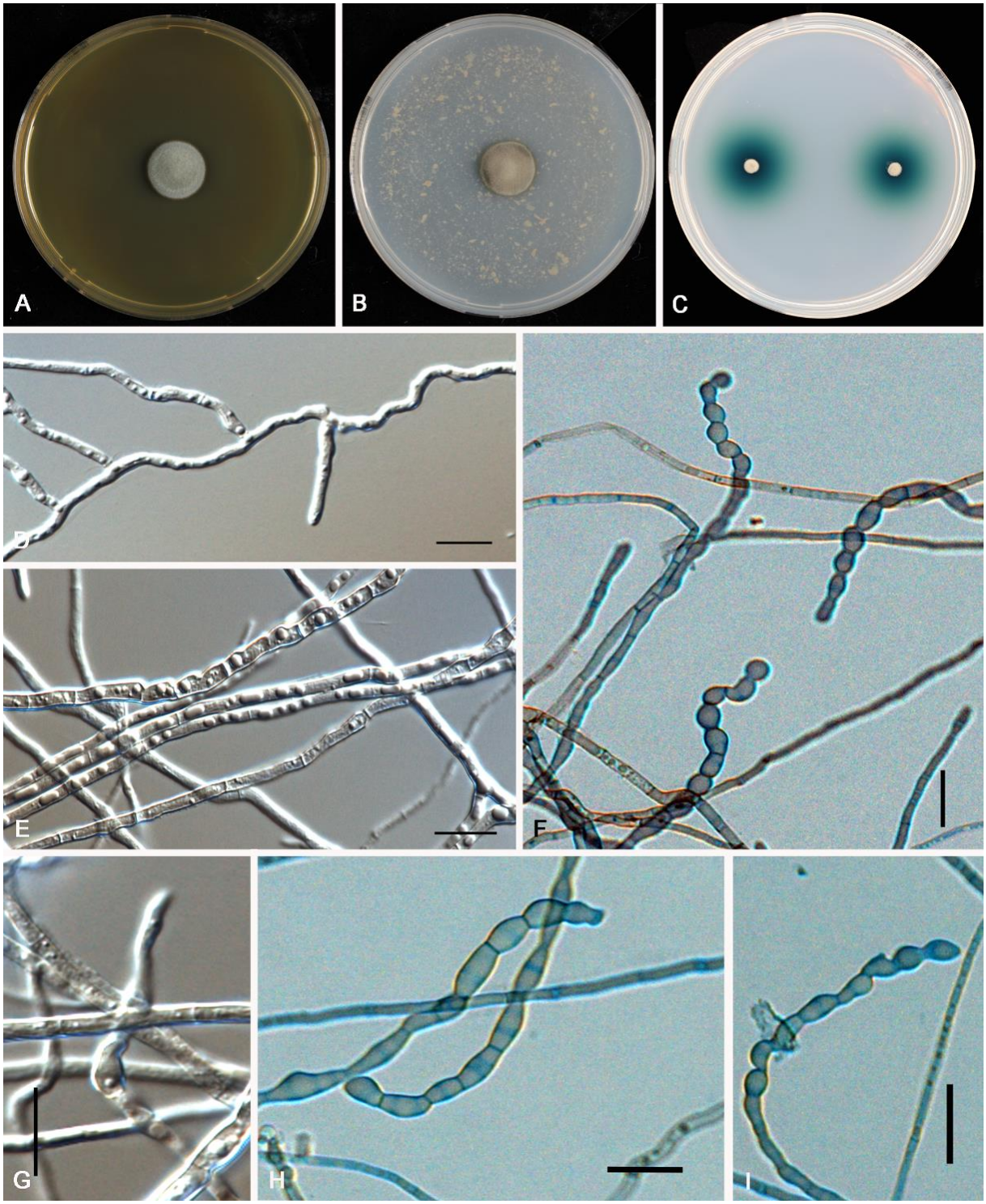

Figure 17 - *Arthrocladium fulminans*, CBS 136243. (A) Colony on MEA; (B) Colony on PDA; (C) Laccase activity on ABTS medium; (D–E) Septate hyphal cells (F–I) Terminally Conidium-like cells — Scale bars = 10 µm.

Arthrocladium tropicale Nascimento, Vicente & de Hoog, **sp. nov.** – Figure 18. MycoBank MB812850.

Etymology: the species was first found in the tropical climate of Cameroon.

Description of CBS 134926 after 2 week incubation on MEA, 25 °C.

Colonies growing slowly, circular, low convex, velvety with entire edge, olivaceous grey, with cottony aerial mycelium. Reverse olivaceous black, becoming dark, without diffusible pigment. Septate hyhae 1.2–4.0 µm diameter, locally consisting of series of inflated cells, with frequent anastomoses. Conidia not observed. Optimal growth at 27−33 °C, scant growth at 36 °C, no growth at 37 and 40 °C. Sexual state unknown.

Holotype CBS H-22288, ex-type culture CBS 134926, isolated from *Petalomyrmex phylax* ant domatia in *Leonardoxa africana*, Cameroon.

Notes: The only known strain was isolated from ant domatia. *Petalomyrmex phylax* ants are regularly associated with chaetothyrialean fungi (VOGLMAYR *et al.,* 2011, BLATRIX *et al.,* 2013), and experimental data indicate a mutualistic relationship involving feeding of the ants on their symbiotic fungi (BLATRIX *et al.,* 2012). *Petalomyrmex phylax*, like many tropical ants inhabiting domatia, cultivate host-specific chaetothyrialean symbionts in distinct patches. Apart from the main symbionts, other chaetothyrialean fungi like *A. tropicale* (and *A. tardum*, see below) are sporadically isolated from these fungal patches (VOGLMAYR *et al.,* 2011). *Arthrocladium tropicale* and *A. tardum* may be considered casual commensals of this ant-plant-fungus association. Recent investigations recorded a high biodiversity of Chaetothyriales associated with ants (VOGLMAYR *et al.,* 2011, NEPEL *et al.,* 2014), which is assumed to be linked to physiology of both ants and fungi. Ants produce a huge diversity of organic, including aromatic, compounds in their glands which have antibacterial and antifungal properties. Due to their abilities to tolerate and metabolize these toxic substances, Chaetothyriales are highly pre-adapted to occupy various niches within ant nests which cannot be used by other fungal competitors.

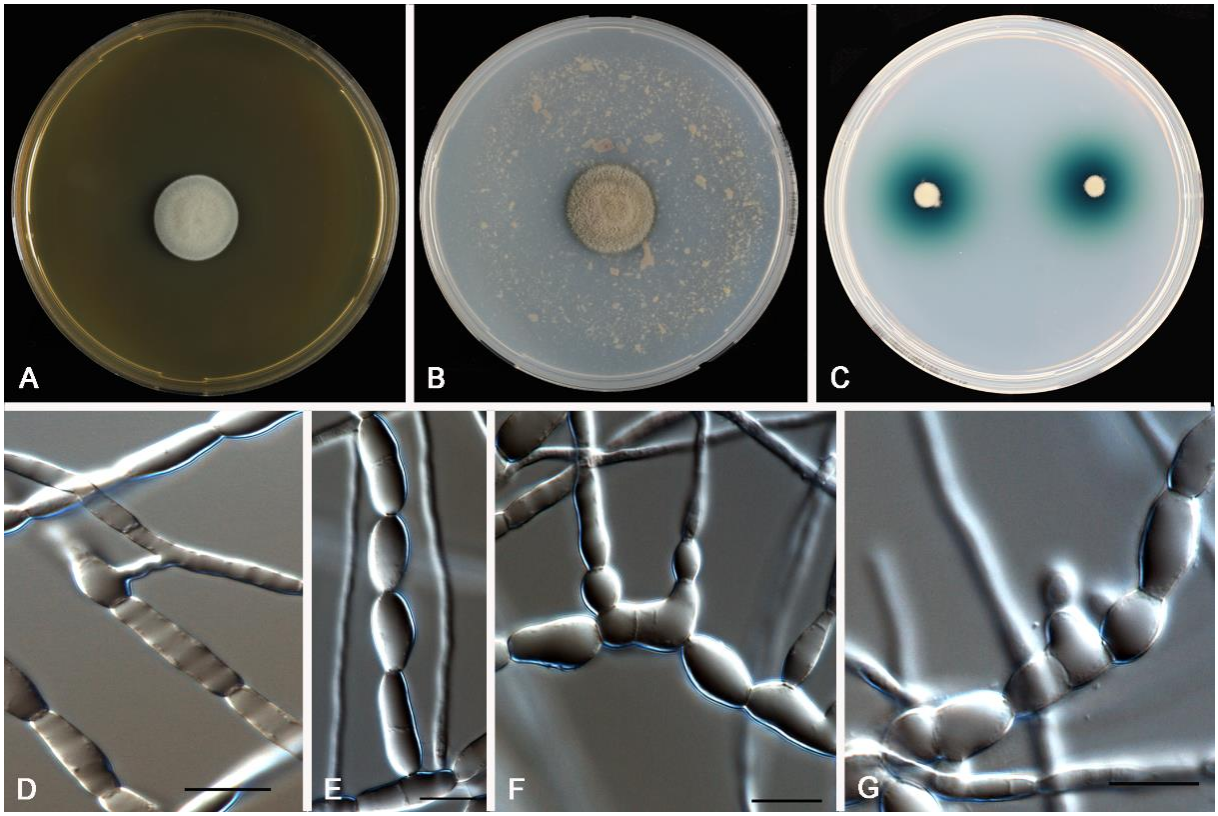

Figure 18 - *Arthrocladium tropicale*, CBS 134926. (A) Colony on MEA; (B) Colony on PDA; (C) Laccase activity on ABTS medium; (D) Hyphae with anastomoses; (E–G) Conidium-like cells — Scale bars = 10 µm.

Arthrocladium tardum Nascimento, Vicente & de Hoog, **sp. nov.** – Figure 19. MycoBank MB812828.

Etymology: named after slow and reluctant growth and sporulation of this fungus.

Description of CBS 127021 after 2-week incubation on MEA, 25 °C.

Colonies slow-growing, olivaceous to smoke brown, velvety, circular, restricted; reverse olivaceous black, without diffusible pigment on the media. Hyphae septate, 1.5–4.0 µm diam, locally consisting of series of inflated cells, with occasional anastomoses. Conidia not observed. Optimal growth at 27−33 °C, scant growth at 37 °C, no growth at 40 °C. Sexual state unknown.

Holotype CBS H-22286, ex-type culture CBS 127021, isolated from decaying shell of coconut of Babassu palm tree (*Orbignya phalerata*), Maranhao, Brazil.

Notes: Babassu coconuts provide a rather special environment which is rich in lipids, terpenes and aromatic hydrocarbons, and recalcitrant to microbial decomposition. Nascimento *et al.,* (unpublished data) noted significant colonization of coconut shells by a wide diversity of herpotrichiellaceous black yeasts, which are known to be enriched by monoaromatic hydrocarbons (Prenafeta-Boldú *et al.,*2006). A second strain was isolated from *Petalomyrmex phylax* ant domatia in *Leonardoxa africana*, from which also *A. tropicale* has been isolated; hydrocarbons probably play a role in this habitat as well (see above).

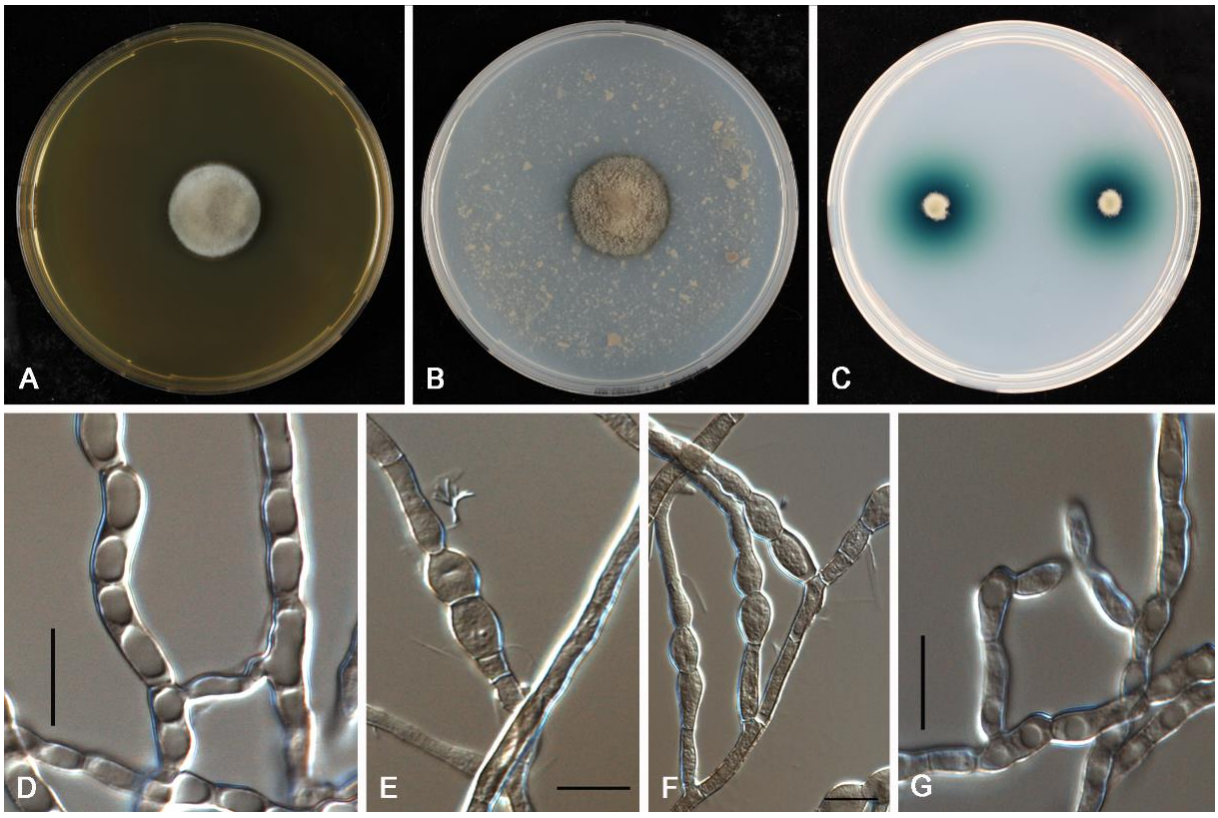

Figure 19 - *Arthrocladium tardum*, CBS 127021. (A) Colony on MEA; (B) Colony on PDA; (C) Laccase activity om ABTS medium; (D) Hyphae with anastomoses; (E–G) Conidium-like cells. — Scale bars = 10 µm.

5. DISCUSSION

The five families of Chaetothyriales, i.e. Chaetothyriaceae, Cyphellophoraceae, Epibryaceae, Herpotrichiellaceae and Trichomeriaceae on average show different phylogenetic and ecological trends. The best-known family, Herpotrichiellaceae, is highly speciose with about 80 described species in six anamorph genera, and a teleomorph genus *Capronia* with a further 80 species, most of which are not known in culture. Numerous species in this family are found causing diseases of human hosts, some of which are systemic (GARZONI *et al.,* 2008) or disseminated (BONIFAZ *et al.,* 2013), or are involved in chromoblastomycosis, a skin disease which is not found outside the family (GONZÁLEZ *et al.,* 2013). The Cyphellophoraceae are a small group of species that are found as colonizers of human skin and nails (SAUNTE *et al.,*2011). The remaining families of Chaetothyriales have very rarely been reported from human hosts, with the exception of *Knufia epidermidis*, causing mild cutaneous infections. *Knufia* and related fungi have earlier been referred to as an 'ancestral clade' to the Herpotrichiellaceae. Isola *et al.* (2015) expanded this clade significantly with rock-inhabiting *Knufia* species and with the novel genus *Lithophila*. Most of the species involved have a natural habitat on bare rock, and seems to propagate by fragmentation of mycelium rather than by specialized propagules.

The 'ancestral clade' and was given the status of family, Trichomeriaceae, by Chomnunti and Hyde (2013), focusing around the ascosporulating genus *Trichomerium.* Members of *Trichomerium* are sooty moulds growing epiphytically on plants in association with honeydew. Species of this genus often lack anamorph sporulation, or the anamorph is a *Tripospermum* species with multiseptate, branched conidia. Also many *Knufia* species combine absence of recognizable sporulation with a life style on exposed surfaces; at most, simple conidiation by disarticulating hyphae takes place. In contrast, absence of anamorph sporulation is a feature that is rarely observed in Cyphellophoraceae and Herpotrichiellaceae, where nearly all species produce abundant conidia from specialized cells.

Trichomerium is characterized by bitunicate asci with multiseptate ascospores produced in small, setose ascocarps. This morphology is rather consistent in the order Chaetothyriales and is also prevalent in *Capronia*, the teleomorph of members of Herpotrichiellaceae. *Capronia peltigerae*, however, clusters in the Trichomeriaceae (Figure 14), parasitizes on *Peltigera* lichens on rock, and lacks anamorph sporulation

(UNTEREINER *et al.,* 2011). This suggests that classification in *Trichomerium* would have been an option. If, we would move towards a purely phylogenetic system, *C. peltigerae* would have to be classified in *Knufia*, as proposed by Réblová and Untereiner (2013). It is likely that other *Knufia* species have hitherto unrevealed sexual stages resembling *Capronia* / *Trichomerium*.

Arthrocladium caudatum was isolated from leaf litter and clustered in the Trichomeriaceae, together with some strains here described as novel species. The *Arthrocladium* type strain was originally described to have septate conidia which resembled swollen hyphal fragments somewhat reminiscent of a *Tripospermum* conidium with a single branch. Despite several attempts, no recognizable sporulation was obtained in the original strain, CBS 457.67. Two of the members of the current genus *Arthrocladium* originated from plant debris, i.e. *A. caudatum* on leaf litter and *A. tardum* on decaying Babassu coconuts. *Arthrocladium tropicale* was isolated from an ant domatium. *Arthrocladium fulminans* caused a fatal disseminated infection in a patient with a genetic GATA-2 immune defect (EGENLAUF *et al.,*2015). With this behaviour *A. fulminans* is unique in the Trichomeriaceae. While *Knufia* is a genus of rock-inhabiting species (Isola *et al.,*2015) and *Trichomerium* comprises epiphytes (CHOMNUNTI *et al.,* 2012a), *Arthrocladium* and related fungi are ecologically undefined (Figure 13).

Knowledge on the family Trichomeriaceae has expanded considerably over the last decade. *Trichomerium* has long been regarded as belonging to the *Capnodiales* in the *Dothideomycetes*, and in numerous databases it is still listed as such. Using molecular phylogeny, Chomnunti *et al.* (2012) recognized its affiliation to Chaetothyriales. Sexual states in Trichomeriaceae and Herpotrichiellaceae are relatively invariant; no significant morphological difference separating *Trichomerium* and *Capronia* is apparent. Obviously the taxonomy of Trichomeriaceae is still in its early stage: recent studies (CHOMNUNTI *et al.,* 2012a, HUBKA *et al.,* 2014, ISOLA *et al.,* 2015) underlined the large changes of generic relationships as a result of better taxon sampling. The great majority of members of Trichomeriaceae are strictly environmental; just *Knufia epidermidis* has been described as a mild colonizer of human skin (LI *et al.,* 2008). With the description of *Arthrocladium fulminans* causing a fatal human infection, the medical significance of the family has changed. Thus far *A. fulminans* takes an isolated position among chaetothyrialean black fungi, and

therefore the species can easily be recognized by the standard rDNA ITS barcoding gene.

6. ACKNOWLEDGEMENTS

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CHAPTER 4

CONCLUSIONS AND FINAL CONSIDERATIONS

CONCLUSIONS AND FINAL CONSIDERATIONS

As general conclusion of the thesis:

- Toluene-degrading species were isolated only when toluene was offered as enrichment factor, showing that the species spectrum is at least partly dependent on the isolated method.

- Environments rich in VOCs are selective for black yeast-like fungi including opportunistic pathogens and sibling environmental isolates. Based on the present results the black yeast-colonized babassu coconut is an unlikely source of human infection.

- To exclude the babassu as potential source of agents of human chromoblastomycosis, this study should be repeated using a warm-blooded animal as bait.

- Re-establish the current *Arthrocladium* genus in Trichomeriaceae family based on molecular data.

- The medical significance of the Trichomeriaceae family has changed with the description of *Arthrocladium fulminans* causing a fatal human infection,

- The species belonging to *Arthrocladium* genus can easily be recognized by the standard rDNA ITS barcoding gene.

As final consideration of the thesis:

This thesis focused on bioprospecting the diversity of black yeast-like-fungi in Chaetothyriales*,* concentrating effort in clarify the different ecological trends among Herpotrichiellaceae and Trichomeriaceae. As observed in the chapter II, a variety of species with different ecologies was isolated through the distinct methods applied demonstrating the efficiency of the methodologies applied to study the black yeast diversity on babassu samples. The oil flotation method showed high success in bioprospecting black yeast, which is attributed to the hydrophobicity of fungi melanized cells.

The consistent finds in the present thesis confirm the hypothesis of black yeasts possibly degrades monoaromatic hydrocarbon. Once the enrichment technique was selective for species with potential to metabolize monoaromatic hydrocarbon, and the new species described, *Cladophialphora exuberans,* and obtained through this method

were able to degrade toluene as sole carbon source and energy. Bioprospecting studies combining both techniques can provide insights into the diversity of black yeast in the evaluated samples, and help to determine the species-specific niche and ecology.

In relation to the potentially human pathogens among the isolated species, most of the black yeast isolates are considered opportunistic or saprobes. Species causing another type of fungal infection, such as *C. mycetomatis*, *E. spinifera*, and *V. botryosa* were consistently isolated. Concerning the agent of chromoblastomycosis, no *Fonsecaea* species were recovered. Although, more studies should be developed in order to exclude the babassu as a potential source of agents of human chromoblastomycosis. Bioprospecting methods cultivation-dependent may not exploit the real diversity in the environments. Previous studies observed the low frequency in the isolation of *Fonsecaea* agents from environmental samples, once a small part of the microorganisms can be cultured under laboratory conditions, however molecular bioprospecting methods, as well as Metagenomics or molecular probes, could be applied in order explore fungal diversity.

Babassu coconut shell was previous characterized containing terpenes and lipids, nowadays based on the range of VOCs identified by GC-ToF MS, is a source of esters and hydrocarbons and enriches for black yeast-like fungi confirming to be an ecological niche for this group of fungi. The compounds found led to elucidate aspects in the ecology of black yeast and unravel a possible biotechnological potential.

Regarding the finds in Chapter III, *Arthrocladium* genus was affiliated to family Trichomeriaceae based on the molecular data. Two of the members were recovered from plant debris as *A. caudatum* and from decaying babassu coconuts demonstrating the species are saprobes with the new ant associated species *Arthrocladium tropicale* isolated from domatium ant while differently, *A. fulminans* caused a fatal disseminated infection in a patient clustered separately from the in the molecular analysis and it was here introduced as a new species.

The present work contributed to re-establish the current *Arthrocladium* genus, which was based before only in morphology of *A. caudatatum*, and elucidate better the ecological trend in Trichomeriaceae, once most of members are strictly environmental and only *K. epidermidis* was described being able to colonize the human skin. The description of *A. fulminans* provides a medical importance for the family.

In summary, continuity of this study is crucial to develop safe bioremediation process applying black yeasts for this purposes. In-depth studies on the pathogenicity

of these organisms should be developed to avoid biohazard problems and establish the relation between monoaromatic hydrocarbon assimilation and pathogenicity. An alternative to exploits the biotechnological potential of pathogenic species is the enzyme immobilization, which allows the use enzymes from the fungi, without any biohazard problems.

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ATTACHMENTS

1. METHODOLOGY DETALAIS

1.1. VOC IN BABASSU COCONUT - QUANTIFICATION

The large amount of digital data was analyzed part automatically, using advanced software and composite libraries. The calibration of the system is performed in-house using a wide range of compounds, such as libraries specific to the instrument of relevant compounds. The analyzes of this study were performed semi-quantitatively, using deuterated Toluene (Toluene-d8) as a reference solution, to prioritize a full scan that quantified all VOCs present in the samples. Previously, two targets (adsorption tubes without sample) were analyzed and the instrumental response of the target average was subtracted in the samples. The quantification of the signal was possible by injecting 50 ng of deuterated Toluene into a tube without sample, which allowed to reference the chromatographic signal obtained from Toluene-d8 with the signals obtained in the real sample and thus obtain the quantification of each compound.

APPENDIX

LIST OF PUBLICATIONS (2013-2017)

1. **NASCIMENTO, M. M. F.;** VICENTE, V. A.; BITTENCOURT, J. V.M.; GELINSKI, J. M. L.; PRENAFETA-BOLDÚ, F. X.; ROMERO, M.; FORNARI, G GOMES, R. R.; SANTOS, G.D.; GERRITS VAN DEN ENDE, A. H. G.; AZEVEDO, C. D. M. P., DE HOOG, G. S. Diversity of opportunistic black fungi on Babassu coconut shells, a rich source of esters and hydrocarbons. **Fungal Biology,** in press, 2017

2. **NASCIMENTO, MARIANA M.F.**; SELBMANN, L.; SHARIFYNIA, SOMAYEH; AL-HATMI, ABDULLAH M.S.; VOGLMAYR, H.; VICENTE, V. A.; DENG, SHUWEN, S.; KARGL, A.; MOUSSA, T. A. A.; AL-ZAHRANI, HASSAN S.; ALMAGHRABI, OMAR A.; DE HOOG, G. S. *Arthrocladium*, an unexpected human opportunist in Trichomeriaceae (Chaetothyriales). **Fungal Biology**, v.120, p. 207-218, 2016.

3. **NASCIMENTO, M. M. F.;** DE HOOG, G. S.; GOMES, R. R.; FURUIE, J. L.; GELINSKI, J. M. L.; NAJAFZADEH, M. J.; BOEGER, W. A. P.; VICENTE, V. A. Shared Physiological Traits of *Exophiala* Species in Cold-Blooded Vertebrates, as Opportunistic Black Yeasts. **Mycopathologia**, v. 181, p. 353-362, 2016.

4. AZEVEDO, C. M. P. S.; GOMES, R. R.; VICENTE, V. A.; CASTRO, D. W.; MARQUES, S. G.; **NASCIMENTO, M. M. F.;** ANDRADE, C. E. W.; SILVA, R. R.; QUEIROZ TELLES, F.; HOOG, S. *Fonsecaea pugnacius*, a Novel Agent of Disseminated Chromoblastomycosis. **Journal of Clinical Microbiology**, v. 53, p. 2674-2685, 2016.

5. FURUIE, J. L.; SUN, J.; **NASCIMENTO, M. M. F.;** GOMES, R. R.; WACULICZ-ANDRADE, C. E.; SESSEGOLO, G. C.; RODRIGUES, A. M.; GALVÃO-DIAS, M. A.; DE CAMARGO, Z. P.; QUEIROZ-TELLES, F.; NAJAFZADEH, M.J.; DE HOOG, S. G.; VICENTE, V. A. Molecular identification of *Histoplasma capsulatum* using rolling circle amplification. **Mycoses**, v. 59, p. 12-19, 2015.

6. MOREIRA, MÔNICA; ADAMOSKI, DOUGLAS; SUN, JIUFENG; NAJAFZADEH, MOHAMMAD J.; **NASCIMENTO, MARIANA M. F.;** GOMES, RENATA R.; BARBIERI, DICLER DE S.; GLIENKE, CHIRLEI; KLISIOWICZ, DÉBORA DO R.; VICENTE, VANIA A. Detection of *Streptococcus mutans* using padlock probe based on Rolling Circle Amplification (RCA). **Brazilian Archives of Biology and Technology**, v. 58, p. 54-60, 2015.

7. GUERRA, R. S.; **NASCIMENTO, M. M. F**.; RIBEIRO, R. O.; OSTRENSKY, A.; MIESCH, S.; NAJAFZADEH, M. J.; HOOG, S.; VICENTE, V. A.; BOEGER, W. A. P. Black Yeast Biota in the Mangrove, in Search of the Origin of the Lethargic Crab Disease (LCD). **Mycopathologia**, v. 175, p. 421-430, 2013.

8. FIGUEL, I. C.; MARANGONI, P. R.; TRALAMAZZA, S. M.; VICENTE, VANIA V. A.; DALZOTO, P. R.; **NASCIMENTO, M. M. F.**; HOOG, S.; PIMENTEL, I. C. Black Yeasts-Like Fungi Isolated from Dialysis Water in Hemodialysis Units. **Mycopathologia**, v. 175, p. 413-420, 2013.

BOOK CHAPTER

1. **NASCIMENTO, M. M. F.**; SANTOS, G. D.; VANIA V. A. Bioprospecção de micro-organismos aplicada à biorremediação. In: ARAÚJO, A. L., MARINHO, R. L. S.; BITENCOURT, J. V. M. (Org.). Gestão da Inovação Agroindustrial. In press, Curitiba: UTFPR, 2017.

2. SANTOS, G. D.; **NASCIMENTO, M. M. F.**; VANIA V. A. Sequenciamento de ácido desoxirribonucleico e taxonomia de procariotos. In: ARAÚJO, A. L., MARINHO, R. L. S.; BITENCOURT, J. V. M. (Org.). Gestão da Inovação Agroindustrial. In press, Curitiba: UTFPR, 2017.

ORAL AND POSTER PRESENTATIONS (2013-2017)

1. **NASCIMENTO, M. M. F.;** VICENTE, V. A.; BITTENCOURT, J. V.M.; PRENAFETA-BOLDÚ, F. X.; ROMERO, M.; FORNARI, G GOMES, R. R.; SANTOS, G.D.; GELINSKI, J. M. L.; GERRITS VAN DEN ENDE, A. H. G.; AZEVEDO, C. D. M. P., DE HOOG, G. S. Enviromental isolation of Black yeasts with assimilative abilities of Volatile Organic Compounds from babassu coconut. Oral Presentation in 6t^{h} meeting of the **ISHAM working groups on Black Yeasts and chromoblastomycosis**, Viterbo, Italy, 2016.

2. SOUZA, B. J. F.; **NASCIMENTO, M. M. F.;** VICENTE, V. A.; BITTENCOURT, J. V. M.; PRENAFETA-BOLDU, F. X.; FORNARI, G.; SANTOS, G.D.; GOMES, RENATA R.; AZEVEDO, C. M. P. S.; HOOG, G.S. Bioprospecting and diversity of black yeasts of babassu coconut with biotechnological potential. Poster Presentation in **8th Brazilian Congress of Mycology**, Florianópolis, Brazil, 2016.

3. 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.

4. **NASCIMENTO, M.M.F.;** VICENTE, V.A.; FURUIE, J.L.; GOMES, R.R.; GELINSKI, J. L.M. N.; BITTENCOURT, J.V.M.; DE HOOG, G.S. Babassu Black yeast flora and Chromoblastomycosis. Poster Presentation **28th Internacional Congress in Microbiology,** Florianópolis, Brazil, 2015

5. FURUIE, JASON L.; ALMEIDA, A. B.; ANDRADE, C. E. W.; **NASCIMENTO, M. M. F;** GOMES, R. R.; GLIENKE, C.; KAVA-CORDEIRO, V.; MARINONI, L.; VICENTE, V. A. Conservation and fungal strains taxonomy of 'Rede paranaense de coleções microbiológicas - TAXon line'. Poster Presentation **28th Internacional Congress in Microbiology**, Florianópolis, Brazil, 2015

6. **NASCIMENTO, M.M. F.;** FURUIE, J. L.; GOMES, R. R.; MORENO, L. F.; GELINSKI, J. M. L. N.; BITTENCOURT, J. V. M.; MARQUES, S G.; AZEVEDO, C.D. M. P. D. S; VICENTE, V. A.; DE HOOG, S. Black Yeast Bioprospecting In Babassu Coconut In Maranhão State, Brazil. Poster Presentation in **Chromoblastomycosis International Symposium**, Maranhão, Brazil, 2014.

7. **NASCIMENTO, M. M. F.;** FURUIE, J. L.; FORNARI, G.; GOMES, R. R.; MORENO, L. F.; GELINSKI, J. M. L.; BITTENCOURT, J. V. M.; VICENTE, V. A.; HOOG, S.G. Bioprospecting black yeasts of clinical and environmental interest. Poster Presentation in **12nd International Meeting on Paracoccidioidomycosis**, Brasília, Brazil, 2014.

8. FORNARI, G.; GOMES, R. R.; MURO, M. D.; PINHEIRO, R. L.; **NASCIMENTO, M. M. F.;** HERKERT, P. F.; FERRARI, C.; TAKIMURA, M.; CARVALHO, V.; VICENTE, V. A.; QUEIROZ TELLES, F. Molecular characterization and susceptibility of *Candida* species from patients with vulvovaginites. Poster Presentation in **12nd International Meeting on Paracoccidioidomycosis**, Brasília, Brazil, 2014.

9. FURUIE, J. L.; GOMES, R. R.; ANDRADE, C. E. W.; SANTOS, G.; GELINSKI, J. M. L.; **NASCIMENTO, M. M. F.;** SUN, J.; VICENTE, V. A. A new probe for *Histoplasma capsulatum* based on RCA method. Poster Presentation in **XII Forum on Fungal Infection in the Clinical Practice**, Curitiba, Brazil, 2014.

10. **NASCIMENTO, M. M. F.;** FURUIE, J. L.; GOMES, R. R.; BOEGER, W. A. P.; HOOG, S.; VICENTE. Assimilation of mineral oil and industrial dye by black yeasts. Poster Presentation in **7th Brazilian Congress of Mycology**, Belém, Brazil, 2013.

11. FORNARI, G.; GOMES, R. R.; **NASCIMENTO, M. M. F.**; VICENTE, V. A.; TAKIMURA, M.; CARVALHO; QUEIROZ TELLES, F. Phylogenetic analysis of the Candida yeasts Isolated of Urogenital Tract. In: **7th Brazilian Congress of Mycology,** Belém, Brazil, 2013.

COURSES AND WORKSHOPS

1. **28th International Congress in Microbiology,** Florianópolis, Brazil, 18-21 October, 2015
2. **Genomics of Neglected Pathogens**, Utrecht, The Netherlands, 20–21 April, 2015.
3. **Course on Medical Mycology**, Utrecht, The Netherlands, November 2014.
4. **29th Meeting of the *Fusarium* working group of Koninklijke Nederlandse planteziektenkundige vereniging**, Utrecht, The Netherlands, 29 October, 2014.
5. **Symposium of the Belgian Society of Medical Mycology**. Fungal Infection in Pediatrics, Antwerp, Belgium, 24 October, 2014.
6. **7th Brazilian Congress of Mycology,** Belém, Brazil, 25-29 November, 2013.