PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF CLINICAL ISOLATES OF BIPOLARIS, CURVULARIA, EXSEROHILUM AND PITHOMYCES

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Phenotypic and Molecular Characterization of Clinical Isolates of *Bipolaris*, *Curvularia*, *Exserohilum* and *Pithomyces*

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Doctoral Thesis

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CERTIFY THAT:

The present work entitled "Phenotypic and Molecular Characterization of Clinical Isolates of *Bipolaris*, *Curvularia*, *Exserohilum* and *Pithomyces*" presented by Keith Cássia da Cunha to obtain the degree of doctor by Universitat Rovira i Virgili, has been carried out under their supervision at the *Unitat de Micologia i Microbiologia Ambiental, Departament de Ciències Mèdiques Bàsiques*, and that it fulfills the requirements to obtain the International Doctorate Mention.

Reus, 14 March 2014

Josepa Gené Dìaz José F. Cano Lira Josep Guarro Artigas

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List of frequently used abbreviations

ACT - a fragment of the actin gene

AFG - Anidulafungin

AMB - Amphotericin B

AFLP - Amplified Fragment Length Polymorphism

ATCC - American Type Culture Collection, Bethesda, USA

BAL - Bronchoalveolar lavage

BCC - BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology (BIOTEC), Bangkok, Thailand

BCCM/IHEM - Biomedical Fungi and Yeasts Collection, Brussels, Belgium

BLAST - Basic Local Alignment Search Tool

bp - Base pair(s)

BRIP - Queensland Department of Primary Industries Plant Pathology Herbarium, Brisbane, Australia

bs - Bootstrap support

***BT2* -** a fragment of the β-tubulin gene

CAL - a fragment of the calmodulin gene

CAS - Caspofungin

CBS - Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

ºC - Celsius degrees

CHS - a fragment of the chitin synthase gene

CLSI - Clinical and Laboratory Standards Institute

CMW - culture collection of the Forestry and Agricultural Biotechnology Institute, Pretoria, South Africa

CNS - Central nervous system

CO2 - Carbon dioxide

D1/D2 - variable domain of the 28S rRNA gene

DAOM - National Mycological Herbarium, Ottawa, Canada

DNA - Deoxyribonucleic Acid

dNTPs - Deoxyribonucleotide triphosphate

***EF*-1α -** a fragment of the 1 α elongation factor gene

List of frequently used abbreviations

EMBL - European Molecular Biology Laboratory

et al. - *et alii,* Latin expression meaning "and others"

Fig. - Figure

5FC - Flucytosine

FLC - Fluconazole

FTL - Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio

FMR - Faculty of Medicine culture collection, Universitat Rovira i Virgili, Reus, Spain

g - Gram(s)

G - Gamma distribution

GPDH - a fragment of the glyceraldehyde 3-phosphate dehydrogenase gene

HCL - Hydrochloric acid

HHUF - Herbarium of Hirosaki University, Japan

HKUCC - University of Hong Kong Culture Collection, Department of Ecology and Biodiversity, Hong Kong, China

HTUs - Internal Nodes

i.e. - *id est*, Latin expression meaning "that is" or “namely”

IGS - Intergenic Spacer region

IMI - International Mycological Institute, Kew, United Kingdom

IP - Institut Pasteur, Paris, France

IT - Isotype strain

ITC - Itraconazole

ITS - Internal Transcribed Spacer

KCl - Potassium chloride

L - Liter(s)

LCP - Laboratory of Cryptogamy, National Museum of Natural History, Paris, France.

LSU - Large Subunit

MAFF - Ministry of Agriculture, Forestry and Fisheries, Japan

MCMC - Markov chain Monte Carlo

MEGA - Molecular Evolutionary Genetics Analysis

List of frequently used abbreviations

MFC - Minimum Fungicidal Concentration

MFG - Micafungin

µm - Micrometer

µL - Microliter

MgCl2 - Magnesium chloride

MIC - Minimum Concentration of Antifungal Agent

min - Minutes

mL - Mililiter(s)

ML - Maximum likelihood

mm - Milimeter(s)

mM - Millimolar

MP - Maximum Parsimony

MRC - Medical Research Council, South Africa

MUCL - Mycotheque de L`Universite Catholique de Louvain, Faculté des Sciences Agronomiques, Louvain-la-Neuve, Belgium

Nº - number

NBRC - Biological Resource Center, Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan

ng - Nanogram

NITE - National Institute of Technology and Evaluation, Tsukuba, Japan

NJ - Neighbor-joining

NT - Neotype strain

OA - Oatmeal Agar

OTUs - Operational Taxonomic Units

PCA - Potato Carrot Agar

PCR - Polymerase Chain Reaction

PDA - Potato dextrose agar

PHT - Partition homogeneity test

pp - Posterior probabilities

PSC - Posaconazole

List of frequently used abbreviations

RAPD - Random Amplified Polymorphic DNA

rDNA - Ribosomal DNA

RFLPs - Restriction Fragment Length Polymorphism

RNA - Ribonucleic acid

rRNA - Ribosomal RNA

***RPB2* -** a fragment of the second-largest subunit of polymerase II gene

SEM - Scanning Electron Microscopy

sp. nov. - *species nova*, Latin expression meaning "new species"

sp., spp. - Species

SSU - Small subunit

T - type strain

TN93 - Tamura-Nei

TRB - Terbinafine

TUB - a fragment of the β-tubulin gene

Tx - transplant

U - Unit

UBC - herbarium, *University of British Columbia*, Vancouver, BC

USA - United States of America

UTHSC - Fungus Testing Laboratory, University of Texas Health Science Center, Texas, USA

VRC - Voriconazole

YES - Yeast Extract Sucrose agar

1. INTRODUCTION

1.1. Overview of filamentous fungi

The Kingdom Fungi is characterized by heterotrophic eukaryotic organisms, which obtain their nutrients through absorption and the use of organic compounds as their primary source of energy. Their cell walls are rich in chitin and β-glucan, and they are largely aerobic (with some exceptions). The majority of fungi are saprobes, and thus secrete enzymes to break down dead organic material. Other fungi display parasite behavior and can attack a wide range of eukaryotic organisms plants, animals, algae, and other fungi in recycling to obtain their nutrients from living cells. Some species have come to form mutualistic relationships with plants, animals and other fungi, and can benefit their host through a number of means (Kirk et al. 2008, Webster & Weber 2009).

Based on their cellular organization, fungi can possess two different thalli, yeast or filamentous; however, there are some fungi that can have both filamentous and yeast-like characteristics, depending on environmental conditions (temperature, pH, CO2 concentration). These organisms are known as dimorphic fungi (Ruiz-Herrera 2012). Yeasts are unicellular organisms that usually reproduce asexually by budding. The filamentous fungi are those with multicellular thalli formed by filaments called **hyphae** (Kwon-Chung & Bennett 1992). Most fungi are filamentous form.

From an economic standpoint, fungi can deliver multiple benefits. For example, in the food industry, they are used in the fermentation of wine and beer, in the maturation process of cheeses, or in the biological control of agricultural pests. In medicine and bio-industries, they are used in the production of important substances, such as antibiotics, and are also used to stimulate the development of plants (mycorrhizal fungi). However, fungi can cause serious damage by acting as parasites of plants and animals, or as food contaminants (Singer 1964, Dreyfuss et al. 1976, Alberts 1988, Andersen 1995, Lo et al. 2004, Kirk et al. 2008). In humans, more than 400 species of fungi have been reported as causal agents of infections thus far, and that number increases every year due to the increased opportunities of fungi to produce infections (de Hoog et al. 2000, Guarro et al. 2002, Ferrer et al. 2009).

During the life cycle of the fungi **(Fig. 1)**, two different reproductive modes can be observed: sexual and asexual. In sexual reproduction, the fungus produces spores of meiotic origin (meiospores); and in asexual reproduction, it produces spores of mitotic

origin (mitospores). The sexual and asexual morphs of a fungus are called **teleomorph** and **anamorph**, respectively. When a fungus presents a complete life cycle, the cycle is called a **holomorph** (Kirk et al. 2008). Both teleomorph and anamorph can appear together in nature or in culture (Stalpers et al. 1991); however, depending on environmental conditions, a fungus may develop only one of those reproduction modes (sexual or asexual state). Furthermore, some fungal species are able to produce two or more different types of asexual morphs, which are called "synanamorphs" (de Hoog et al. 1995). Fungi that exhibit different reproductive states are said to be "pleomorphic" (Kirk et al. 2008).

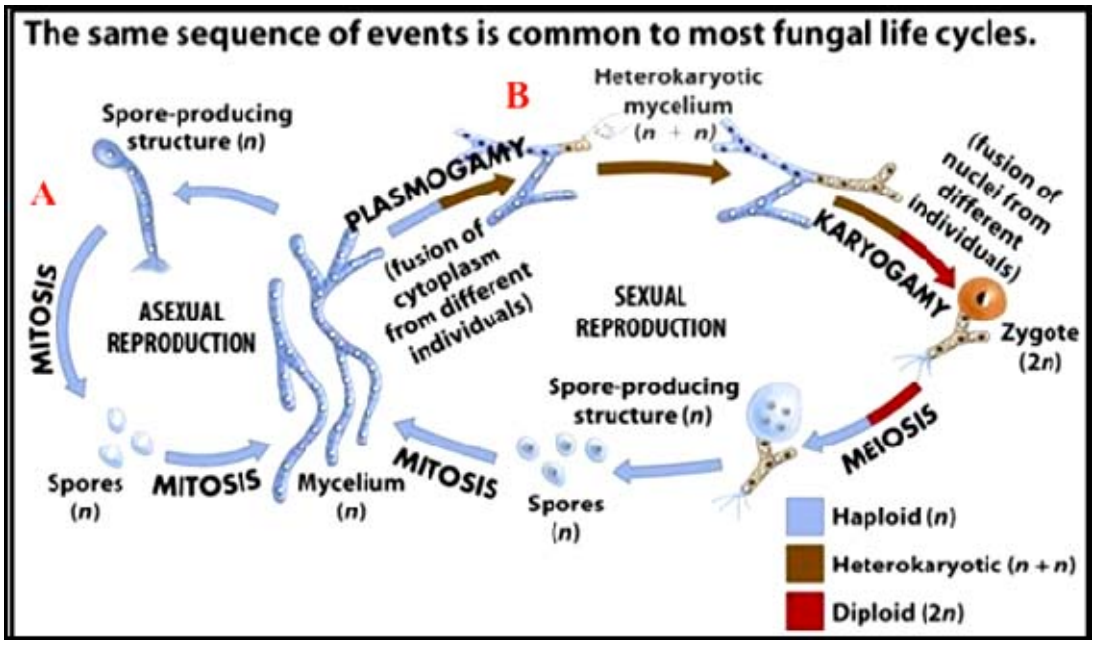

Fig. 1. Fungal life cycle. **A.** Asexual reproduction. **B.** Sexual reproduction (adapted from Freman et al. 2008).

Both the classification and taxonomy of fungi are tradicionally based on the morphology of their reproductive structures, which gives emphasis to teleomorph. However, due to the development and application of molecular biology techniques in recent years, many changes have been observed in fungal taxonomy. For example, the Kingdom Fungi has been divided in two subkingdoms (**Fig. 2**): Basal Fungi and Dikarya (Hibbett et al. 2007). In the former one, the obsolete group named Zygomycotina was spread into the phylum Glomeromycotina and four *incertae sedis*

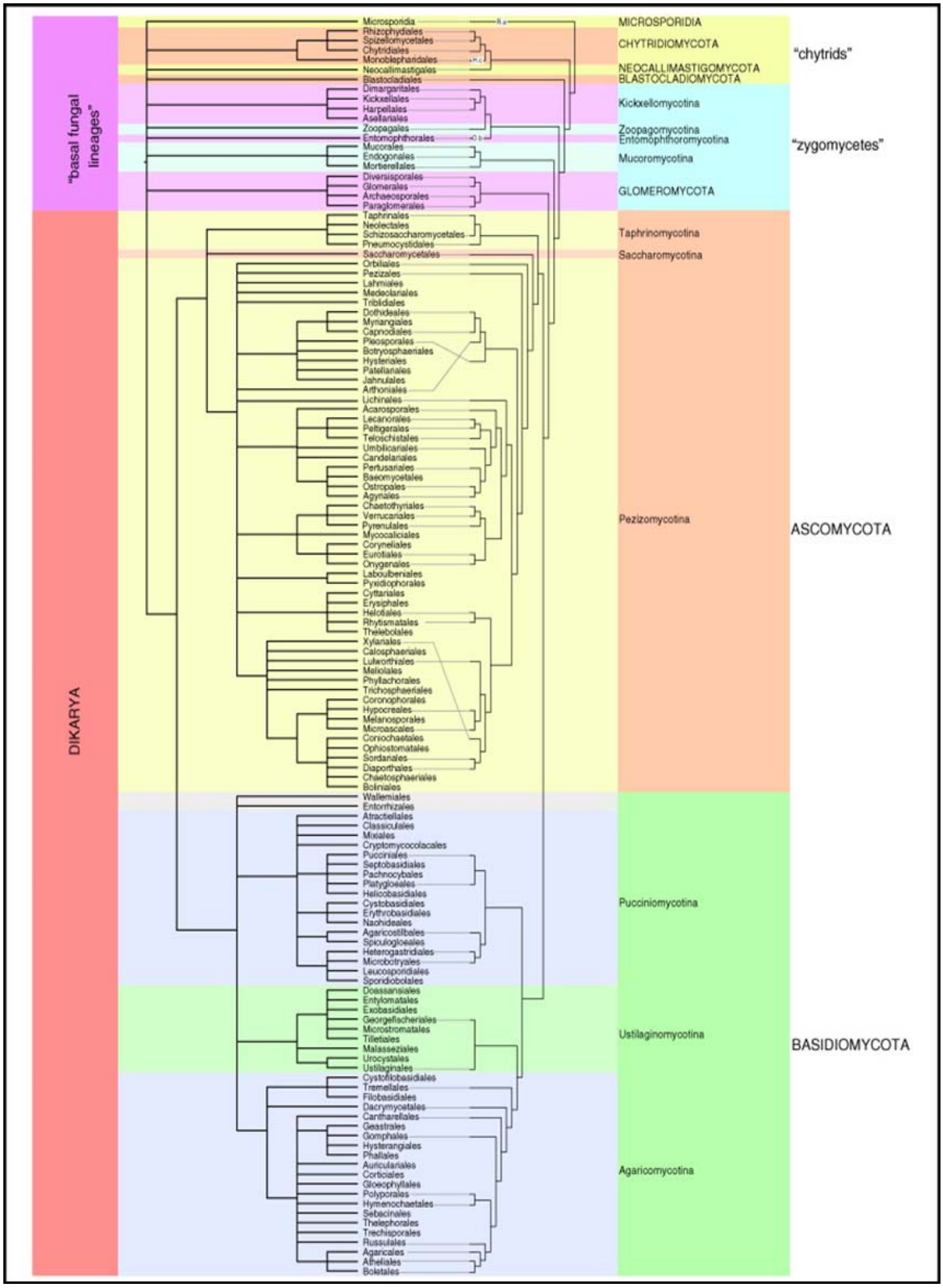

Fig. 2. Phylogeny and classification of the Kingdom Fungi (McLaughlin et al. 2009). Phyla (-mycota), subphyla (-mycotina) and subkingdom-level taxa (Dikarya) are labelled. Names in quotation marks are informal, non-monophyletic groups. The tree on the right reflects taxon sampling and tree topology from James et al. (2006).

subphyla (Entomophthoromycotina, Kickxellomycotina, Mucoromycotina and Zoopagomycota). The subkingdom Dikarya included two phyla: Ascomycota and Basidiomycota. Most filamentous fungi of clinical importance are included in the phylum Ascomycota (Guarro et al. 1999).

During their sexual state, ascomycetes are characterized by the formation of **ascospores** (endogenous spores) **(Fig. 3a),** which are formed inside a sack-like cell **(ascus) (Fig. 3b).** The number of ascospores within an ascus varies and can be from one to thousands. The variability depends on the number of post-meiotic mitoses and the degeneration of nuclei after meiosis. Usually, the ascI are developed into fruiting bodies called **ascocarps** or **ascomata (Fig. 3c).** Most ascomycetes found in clinical practice do not develop the structures of sexual reproduction *in vitro*, and appear only in the asexual state.

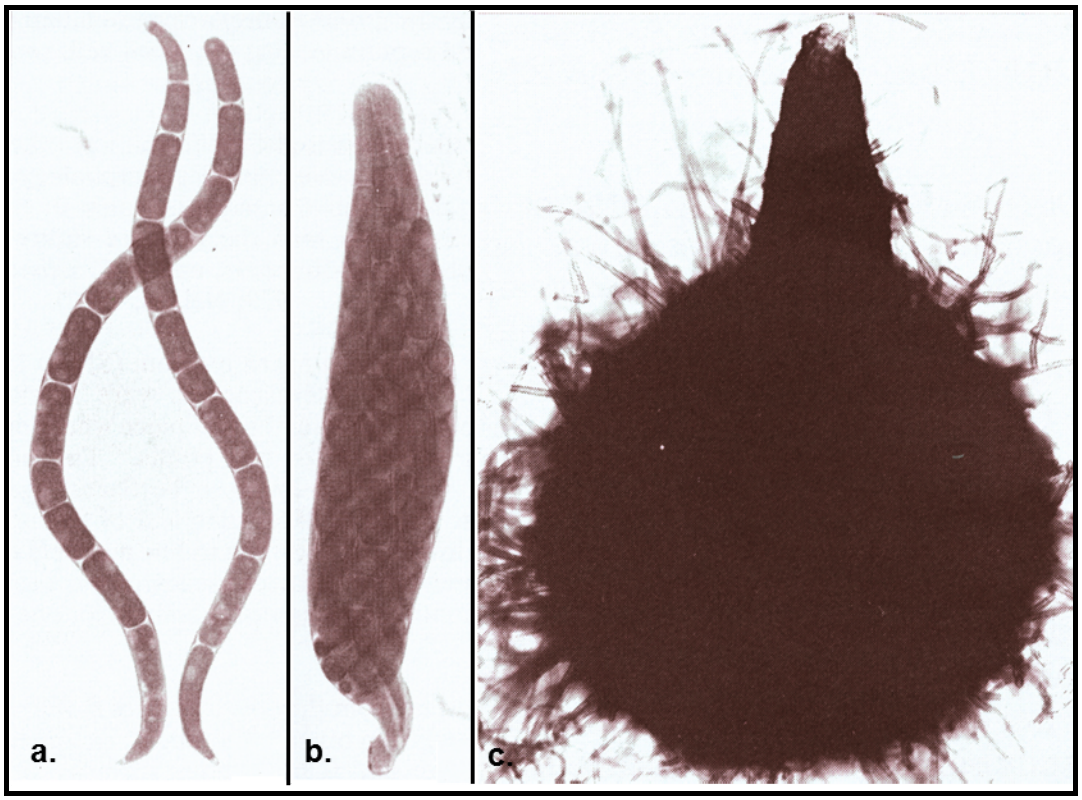

Fig. 3. Strutures of an ascomycete in sexual form. *Cochliobolus microlaenae* a. Ascospores x430. b. Ascus x430. c. Ascoma x105 (adapted from Alcorn 1990).

Filamentous fungi with unknown sexual states have traditionally been included in the division Deuteromycota and, depending on the structures in which

mitospores (**conidia**) were developed, that taxonomic group was subdivided into two classes: Hyphomycetes, in which closed fruiting bodies were lacking (e.g. *Penicillium, Acremonium* or *Curvularia*); and Coelomycetes, which produced conidia into fruiting bodies (e.g. *Phyllosticta*, *Phomopsis* or *Phloeospora*). However, this classification of the filamentous anamorphic fungi was abandoned at the end of the last century (Kirk et al. 2008), but the nomenclature associated with this taxonomycal organization are still in use. It is important to note that most filamentous fungi that are pathogenic for humans are hyphomycetes (de Hoog et al. 2000). Their identification is based on the morphological study of their conidial structures, and they are characterized by having colonies with variable texture and color, septate hyphae, conidiophores, and conidia (Kirk et al. 2008). All the genera included in this thesis are dematiaceous hyphomycetes, which are fungi that have melanin in some of their structures (Kirk et al. 2008, Seifert et al. 2011).

Conidiophores (Fig. 4) are fertile hyphae in which **conidiogenous cells** (cells that produce conidia) are developed. Conidiophores can be classified depending on their degree of differentiation or complexity. **Macronematous** conidiophores **(Fig. 4a)** are those that differ morphologically from vegetative hyphae; **semimacronematous** conidiophores **(Fig. 4b)** differ slightly from the hyphae, and **micronematous** conidiophores **(Fig. 4c)** are morphologically indistinguishable from vegetative hyphae (Ellis 1971). Furthermore, the conidiophore considered **determinate** when the growth of the conidiophore stops with the production of a conidium. If the conidiophore continues to grow after the production of the first conidium, it is considered **percurrent**.

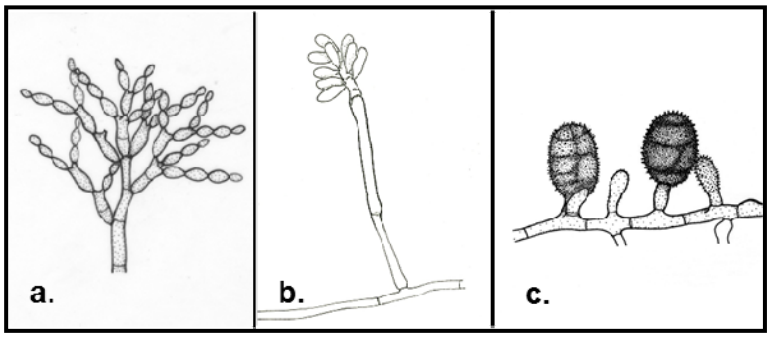

Fig. 4. Conidiophores. a. Macronematous. b. Semimacronematous. c. Micronematous.

In some fungi, the conidiophores can group together and form a compact structure known as a **conidioma**. When a conidioma is comprised of a more or less compact group of erect and sometimes fused conidiophores with conidia only at the apex, it is called a **synnema (Fig. 5a)**. If the conidioma is formed by a cushion-like mass of short conidiophores, it is called a **sporodochium (Fig. 5b)**. Other types of conidioma that are usually associated with coelomycetes are: the **acervulus (Fig. 5c)**, which is characterized by a saucer-shape conidioma (the surface of which is covered by fertile hyphae that bear conidia); and the **pycnidium (Fig. 5d),** which is a flask-shaped fruiting body with an ostiole. In addition, the inner surface of the fungal tissue is lined with conidiogenous cells**.**

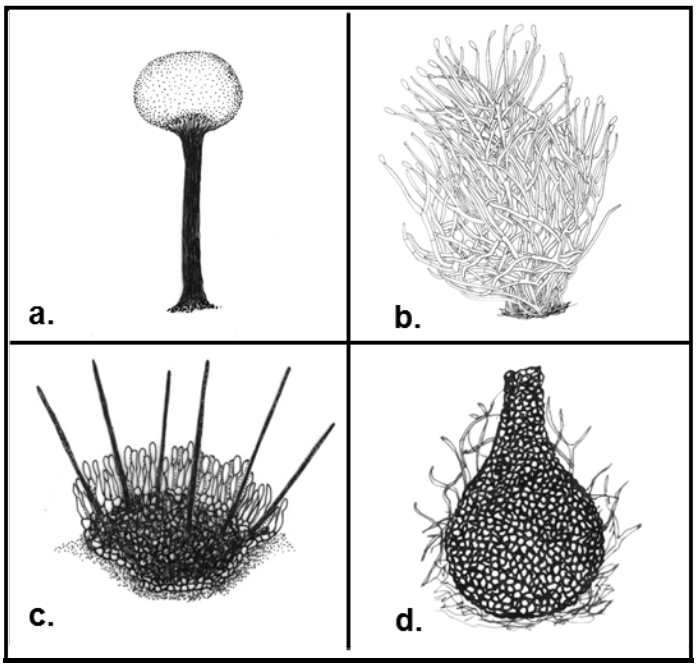

Fig. 5. Conidioma. a. Synnema. b. Sporodochium. c. Acervulum. d. Pycnidium.

Other morphological features that allow us to classify and describe anamorphic filamentous fungi are the different processes involved in the development of conidia from the conidiogenous cells (conidiogenesis). Two main types of **conidiogenesis** are described: **thallic (Fig. 6 a-c),** during which the conidia are formed from the conversion of a portion of pre-existing hyphae; and **blastic (Fig. 6 d-f)**, during which conidia begin to swell or thicken and are then cut off by a septum. Most hyphomycetes undergo blastic conidiogenesis, which itself can be differentiated into **holoblastic (Fig. 6d)**, during which the outer wall of the

conidiogenus cell is continuous with the conidia wall; or **enteroblastic (Fig. 6d)**, in which the inner wall of the conidiogenous cell gives rise to the wall of the conidia. In turn, enteroblastic conidiogenesis can be divided into different processes. The first type is known as **phialidic (Fig. 6e),** during which the conidia are formed from the synthesis of new material from the cell wall, and often after the formation of the first conidium. The conidiogenous cells associated with this type of conidiogenesis are called phialides. Often during this process, cell wall debris remains at the apex of the phialide, giving rise to collarettes. The other type of enteroblastic conidiogenous is described as **annellidic (Fig. 6f).** During this process, the conidiogenous cells (anellides) produce successive enteroblastic proliferations, which give rise to darkened transverse bands called annellations.

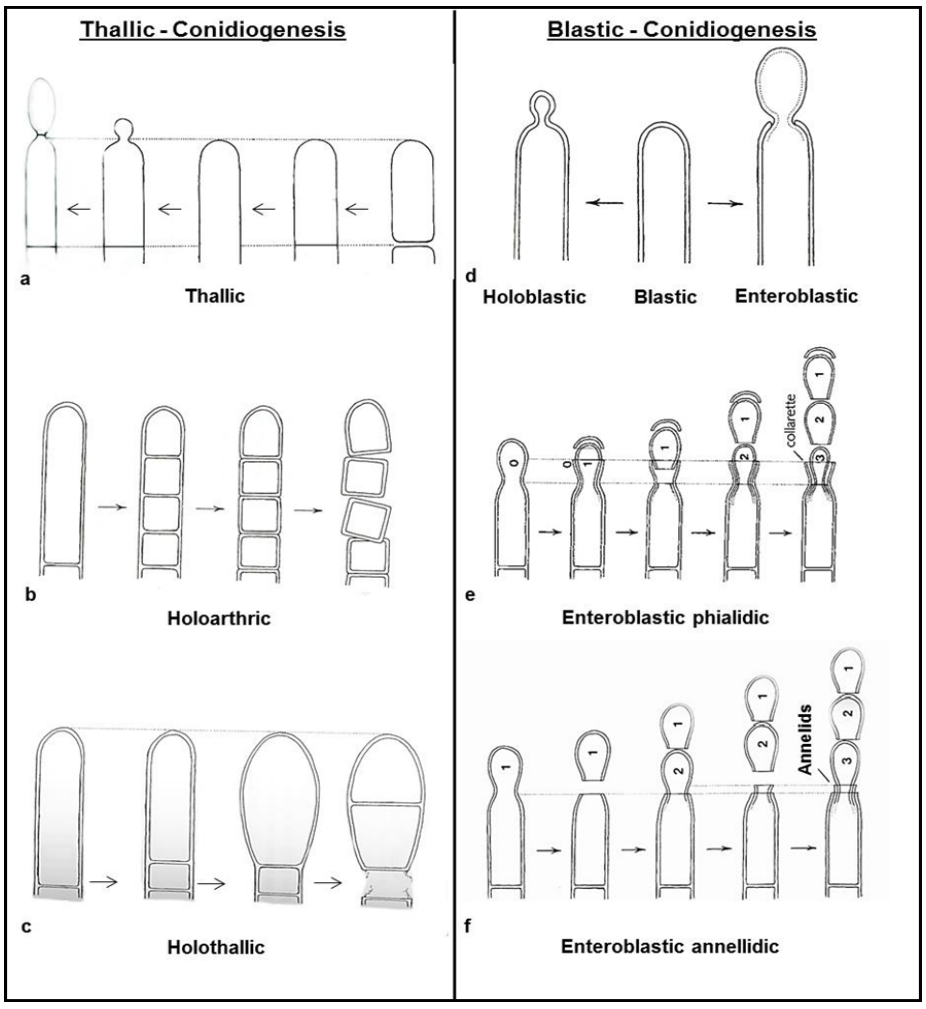

Fig. 6. Basic modes of conidiogenesis. a-c. Thallic. d-f. Blastic. a. Thallic. b. Holoarthric. c. Holothallic. d. Holoblastic, Blastic and Enteroblastic. e. Enteroblastic phiallidic. f. Enteroblastic annellidic (adapted from de Hoog et al. 2000).

In addition to these types of conidiogenesis, some hyphomycetes can present **tretic** conidiogenesis. During this process, the conidia are produced from a pore in the wall of the conidigenous cell. These conidia are referred to as **poroconidia,** and are formed through an extension of the inner wall of the conidigenous cell. Most of the anamorphic genera studied in this thesis show this type of conidiogenesis.

Other conidiogenous events have been described, and usually combine features such as initiation and formation of the cell wall (Kirk et al. 2008).

In addition to the aforementioned structures others such as **sclerotia** (structures comprised of a compacted mass of hyphae on both its surface and its interior, and one that is devoid of spores) **(Fig. 7a)**, and **chlamydospores** (swollen cells with thickened walls that vary in shape, and that can appear at terminal, lateral or intercalary position) **(Fig. 7b)** are very useful in the identification of hyphomycetes (Willetts & Bullock 1992, Stalpers 2000, Kirk et al. 2008).

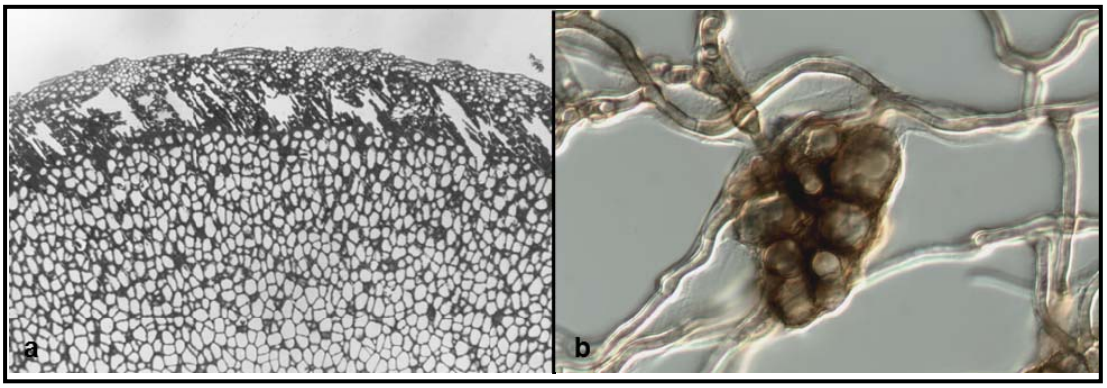

Fig. 7. Fungal structures of resistance. a. Sclerotia of *Cenococcum geophilum* (from http://website.nbm-mnb.ca/mycologywebpages/NaturalHistoryOfFungi/Sclerotia.html). b. A chlamydospore of *Curvularia*.

The study of morphological features for the identification of fungi isolated from clinical specimens can be a problem for several reasons: 1) the slow growth of many fungi, 2) they can loss the ability to produce spores in culture and 3) the fact that sometimes the fungus simply cannot grow *in vitro*. Furthermore, the morphological similarity between species or the lack of experience of the professionals can lead to incorrect identifications or to identifications only at the generic level (Guarro et al. 1999). For these reasons, progressively more molecular techniques have been used in the identification of fungal isolates of clinical origin.

1.2. Molecular biology applied to the taxonomy and the identification of filamentous fungi

A correct morphological fungal identification often requires a lot of time and a wide range of mycological experience, because there are a great number of genera, many of them include numerous species, and often, these species possess a poorly differentiated morphology. Furthermore, in medical mycology, fungal identification is especially problematic because the morphology of the filamentous fungi isolated from clinical specimens can vary significantly *in vitro* compared to the morphology of the same fungus growing on the natural substratum from whence they were originally described. In addition, as mention previously, the fungus may even lose the ability to sporulate, a factor which makes identification impossible.

1.2.1. Molecular techniques

The increase in different molecular diagnostic techniques that can be used in medical mycology has become essential for a fast and correct identification of the agent responsible for an infection. These techniques also allow for the identification of taxa with greater precision and the establishment of phylogenetic relations between the fungi. One of the molecular techniques commonly used for identification is the Restriction Fragment Length Polymorphism (RFLPs), which is based on restriction enzyme digestion of the amplification products of specific genes or polymorphic DNA fragments (Spadaro et al. 2012, Verrier et al. 2012). Another technique is the Random Amplified Polymorphic DNA (RAPD), which is useful for detecting genetic differences within species. Short synthetic oligonucleotides (10 bases long) of random sequences are utilized as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures using PCR. The limitation of this technique is the difficulty in reproducibility (Bardakci 2001). The method known as Amplified Fragment Length Polymorphism (AFLP) is based on selective PCR amplification of restriction fragments from a total digest of genomic DNA. The number of fragments that can be analyzed is 50-100 bands (Vos et al. 1995). This technique has been widely used to establish phylogenetic relations (Kirk et al. 2008, Seifert et al. 2011).

In recent years however, the most widely used tool in the identification of fungi and in phylogenetic studies is DNA sequencing. The most commonly used genes are

those that code for ribosomal RNA (rRNA). These genes **(Fig. 8)** are 18S, 5.8S and 28S. They are separated by two internal transcribed spacers (ITS1 and ITS2) and intergenic spacers (IGS1 and IGS2). The small subunit (SSU) 18S rRNA has approximately 1800 bp, and it is a highly conserved region of fungi. It is often used to determine family and order at the taxonomic level. The large subunit (LSU) 28S rRNA is composed of 3400 bp. It is the largest ribosomal subunit that can be used to identify a fungal species at the genus level. Although it is a conserved gene, there is a variable region known as the D1/D2 domain. The 5.8S gene and the ITS1 and ITS2 regions are often used to distinguish species. The size of this fragment ranges from 450 to 700 bp (Bellemain et al. 2010). In many cases, the ITS region varies slightly among species, such as those in the genera *Aspergillus* and *Penicillium*. In these cases, other genes have been sequenced, mainly those belonging to structural proteins.

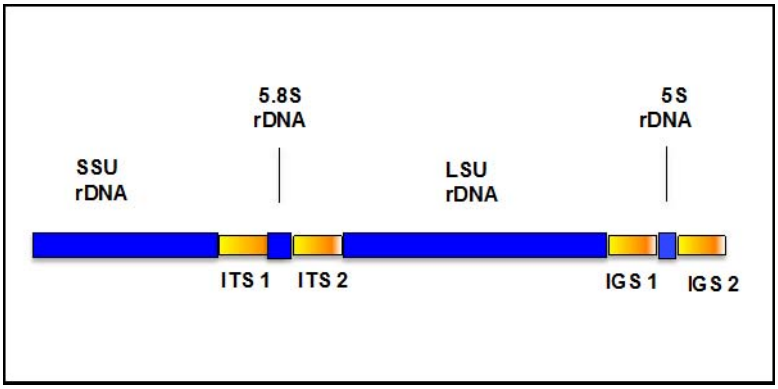

Fig. 8. Structure of the ribosomal RNA gene cluster in fungi.

The combined analysis of protein-coding loci (including elongation factor (*EF-1a*), ß-tubulin (*TUB* and *BT2*), actin (*ACT*), chitin synthase (*CHS*), calmodulin (*CAL*), the second-largest subunit of polymerase II (*RPB2*) and glyceraldehyde 3-phosphate dehydrogenase (*GPDH*)) are used in multilocus studies to elucidate the taxonomy of complex genera, such as *Fusarium, Scedosporium, Sporothrix* and *Cochliobolus* (Gilgado et al. 2005, Marimon et al. 2006, O'Donnell et al. 2009, Manamgoda et al. 2012). The use of DNA sequence databases to facilitate fungal identification gave rise the **barcoding project**. This project seeks to identify species of any organism by sequencing a genome segment (500 – 800 bp) using primers that can be applied to a greater number of different taxonomic groups (Hollingsworth et al. 2009, Maturana et al. 2011, Scarcelli et al. 2011, Schoch et al. 2012). One gene that can be used for all

organisms has not yet been found. In the case of fungi, the ITS region has been used as a universal barcode marker (Schoch et al. 2012). However, because the region may be largely conserved in some fungal groups, there has been debate over which other genes could help in the identification of all fungal species (Seifert & Crous 2008).

When the sequence of a problem fungus is compared to the sequences deposited in international databases such as GenBank (http://www.ncbi.nlm.nih.gov/genbank/), MycoBank (http://www.mycobank.org/BioloMICS.aspx), EMBL (http://www.ebi.ac.uk /embl/), or NBRC (http://www.nbrc.nite.go.jp), the results obtained provide significant taxonomic information about the fungus studied due to the number of sequences of different fungi and different that are deposited in these databases. Nevertheless, these results should be interpreted with caution, especially when the sequences obtained are of fungi identified by people without recognized expertise.

Due to both the lack of standardization among taxonomic studies and the absence of a single universal marker, the sequencing of more than one region of the genome is frequently required to distinguish between fungal genera or species.

1.2.2. Phylogenetic analyses

The methods of phylogenetic reconstruction study the evolution of organisms by analyzing sequences of DNA or amino acids, thus providing more reliable evolutionary hypotheses for the classification of organisms (Holder & Lewis 2003). Phylogenetic analyses are constructed from multiple DNA sequence alignment arrays that are generated with software such as Clustal, Mafft, Muscle (Thompson et al. 1997, Edgar 2004, Katoh et al. 2009). These programs reveal the differences and similarities between the sequences by calculating the number of identical characters divided by the total length of the alignment, so that the percentage of identity or similarity between two sequences can be established (Lemey et al. 2009). The results are a graphical representation known as **phylogenetic trees (Fig. 9)**. These trees are comprised of nodes and branches; the external nodes are also known as **operational taxonomic units** (OTUs), and the internal nodes are called **hypothetical taxonomic units** (HTUs) and represent hypothetical ancestors of the OTUs. The branches represent the relationships in terms of descent and ancestry.

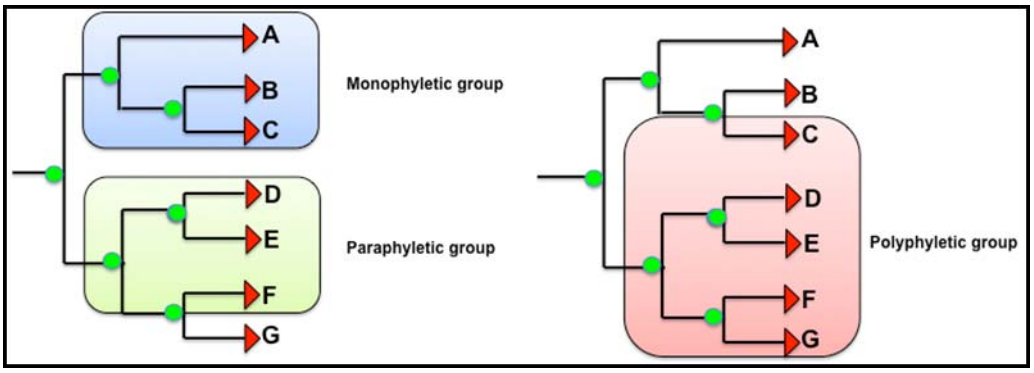

Fig. 9. Schematic representation of a phylogenetic tree ▶ External nodes (OTUs), ● Internal nodes (HTUs) (adapted from Holder & Lewis, 2003).

Two different methods can be used to infer phylogenetic relationships: one is based on **genetic distance,** and the other is based on **preserved character** (Lemey et al. 2009). Genetic distance is calculated using a difference index for each pair of the sequences compared. With all distances calculated, a distance matrix is established, and it serves to build the phylogenetic tree that is deemed most appropriate using different algorithms. A disadvantage of this method is that all of the information sequences are reduced to a single number, and it is assumed that this parameter reflects the existing evolutionary divergence (Bowman et al. 1992). The **neighbor-joining (NJ)** method is an example of a method based on genetic distance— it groups the sequences with lower genetic distance according to the minimum evolution principle, and the tree obtained is one in which the sum of the branches is the smallest (Saitou & Nei 1987).

The methods based on preserved characters consider each nucleotide position as an independent character. Based on the alignment of sequences, this method evaluates the changes that have taken place in each nucleotide position between different sequences and establishes possible phylogenetic relationships (DeSalle et al. 1994). The **Maximum Parsimony (MP)** and the **Maximum Likelihood (ML)** methods utilize preserved characters. The MP method selects trees that require the lowest number of changes from among all possible trees. The ML method requires a base substitution model to establish the probability of each type of mutation. It considers the theory that the greater the number of mutations among the internal nodes of a tree, the less likely it is that the tree represents the true evolutionary history. In order to determine the robustness of the trees obtained from MP and ML statistical tests, bootstrap analysis is used (Felsenstein 1985). The results of the bootstrap analysis are expressed as a

percentage that provides support for the branches of the tree. When the value is greater than or equal to 70%, the branch is statistically supported.

A group that includes an ancestor and all of its descendants is considered **monophyletic.** When a group includes an ancestor but not all of its descendants, it is referred to as **paraphyletic,** and a group that is made up of descendants from different ancestors is considered **polyphyletic (Fig. 9).** The natural taxa represented by genera, families and / or higher taxonomic ranks should be monophyletic. However, numerous fungal groups were established on the basis of morphological features, and they later were proven especially those of the anamorphic fungi, to be polyphyletic (Huhndorf et al. 2004, Miller & Huhndorf 2004, Shenoy et al. 2006, Arzanlou et al. 2007, Crous et al. 2007, Shenoy et al. 2010, Perdomo et al. 2011a, Seifert et al. 2011). This type of study has revealed that, during the course of evolution, anamorphic fungi presented independent morphological characteristics and may therefore be associated with different teleomorphic genera, families, orders or classes (Shenoy et al. 2006, 2010, Perdomo et al. 2011a).

Currently, when one or more species of the same genus are phylogenetically separated from the type species of the genus, these species can be considered to be part of a new genus. Two species can remain in the same genus when they share morphological characteristics and belong to the same family or order based on the analysis of DNA sequences (Arzanlou et al. 2007, Crous et al. 2007).

In conclusion, both species recognition and correct identification are very important, because species sometimes differ in important traits, such as antifungal susceptibility, clinical manifestations, geographical distribution, or host range (de Hoog et al. 2006, Marimon 2007).

1.3. Infections caused by melanized filamentous fungi

Mycosis is the name of the infectious processes caused by fungi. The development of mycosis depends on the ability of the fungus to adapt to host tissues and to resist the defense mechanisms of the host (Casadevall & Pirofski 2001). Both the increased human population with compromised immune systems due underlying or infectious diseases and the use of immunosuppressive drugs in transplant surgeries

(such as those to replace organs and bone marrow) have resulted in increased occurences of opportunistic infections in recent decades.

Though melanized fungi are found in the environment, they are often considered contaminants when isolated in the microbiology laboratory, and typically only 10% of isolates have clinical significance. Melanin is a compound found in many microbes and animals. One of its characteristics is its resistance to many destructive physical-chemical agents. Its role in pathogenic fungi has been studied extensively in recent years (Revankar & Sutton 2010).

The infections caused by melanized fungi are differentiated by histological findings and are divided into categories known as eumycetoma, chromoblastomycosis, and phaeohyphomycosis, but these fungi can cause a variety of other clinical syndromes, including onychomycosis, keratitis, allergic disease, pneumonia, brain abcesses, and disseminated disease (Revankar & Sutton 2010, Guarner & Brandt 2011). Eumycetoma is a chronic suppurative granulomatous infection that usually affects the subcutaneous tissue of the feet. Several genera of melanized fungi, including *Madurella*, *Exophiala* or *Curvularia,* can be the etiologic agents in eumycetoma (Garnica et al. 2009, Revankar & Sutton 2010). Chromoblastomycosis is a chronic infection caused by melanized fungi belonging to different genera, such as *Botryomyces, Cladophialophora, Exophiala, Fonsecaea Phialophora* or *Rhinocladiella.* It is common in tropical and subtropical areas of South America, but it is also regularly described in the Caribbean, Madagascar, Mexico, India, Japan and Australia. Its main etiologic agents are *Cladophialophora carrionii* and *Fonsecaea pedrosoi* (Queiroz-Telles et al. 2009). The most common form of phaeohyphomycosis is described as a chronic infection located in the deep dermis and subcutaneous tissue (Garnica et al. 2009). In immunocompetent patients, the infection is characterized by the presence of an inflammatory cyst located in the extremities, which may progress to diffuse forms and which may possess different degrees of pigmentation. In immunocompromised patients, the characteristics vary and patients may develop lesions in the form of sores, bumps or pustules. In these patients, other organs may be affected due to the hematogenous spread of the infection (Garnica et al. 2009). The agents responsible for phaeohyphomycosis are species of the genera *Alternaria, Aureobasidium, Bipolaris, Cladophialophora, Cladosporium, Curvularia, Exophiala, Exserohilum* and *Phialophora* (Ajello et al. 1997). Infections such as

eumycetoma, chromoblastomycosis and phaeohyphomycosis are preceded by trauma or environmental exposure (Guarner & Brandt 2011).

Melanized fungi rarely cause deep infections; however, when this type of infection occurs, a considerable morbidity rate is observed due to complications and difficulties in treatment (Revankar & Sutton 2010). An example of deep tissue infection is a pulmonary infection, which it is usually acquiered through the inhalation of spores. This infection is typically observed in immunocompromised patients or in those with underlying lung disease, and several genera of fungi are involved, including *Curvularia*, *Cladophialophora* and *Exophiala.* Another type of deep mycoses is a central nervous system (CNS) infection. Frequently, this manifestation is fatal (60 - 100% mortality) and can affect immunocompetent hosts. The most common clinical manifestation is the brain abscess, but other manifestations, such as encephalitis with diffuse brain involvement and meningitis, have also been reported (Revankar & Sutton 2010). Several genera have been associated with CNS infections, including *Cladophialophora, Rhinocladiella, Bipolaris* and *Exserohilum* (Revankar & Sutton 2010).

The melanized fungal species studied in this thesis belong to the genera *Bipolaris, Curvularia, Exserohilium* and *Pithomyces* and have been mainly described as causal agents of infections in the respiratory tract (see section 1.4.2 and 1.5).

1.4. General features of the genera *Bipolaris, Curvularia* and *Exserohilum*

These three anamorphic genera belong to the order Pleosporales and, morphogically, are characterized by having all dark-pigmented structures (i.e. hyphae, conidiophores and conidia). Their conidiophores are percurrent, the conidiogenous cells are tretic and usually placed apically or intercalary in the conidiophore, and their conidia are sympodial, large, more or less elongated and with transverse septa. These genera can be differentiated mainly by the shape and size of the latter structures **(Fig. 10).**

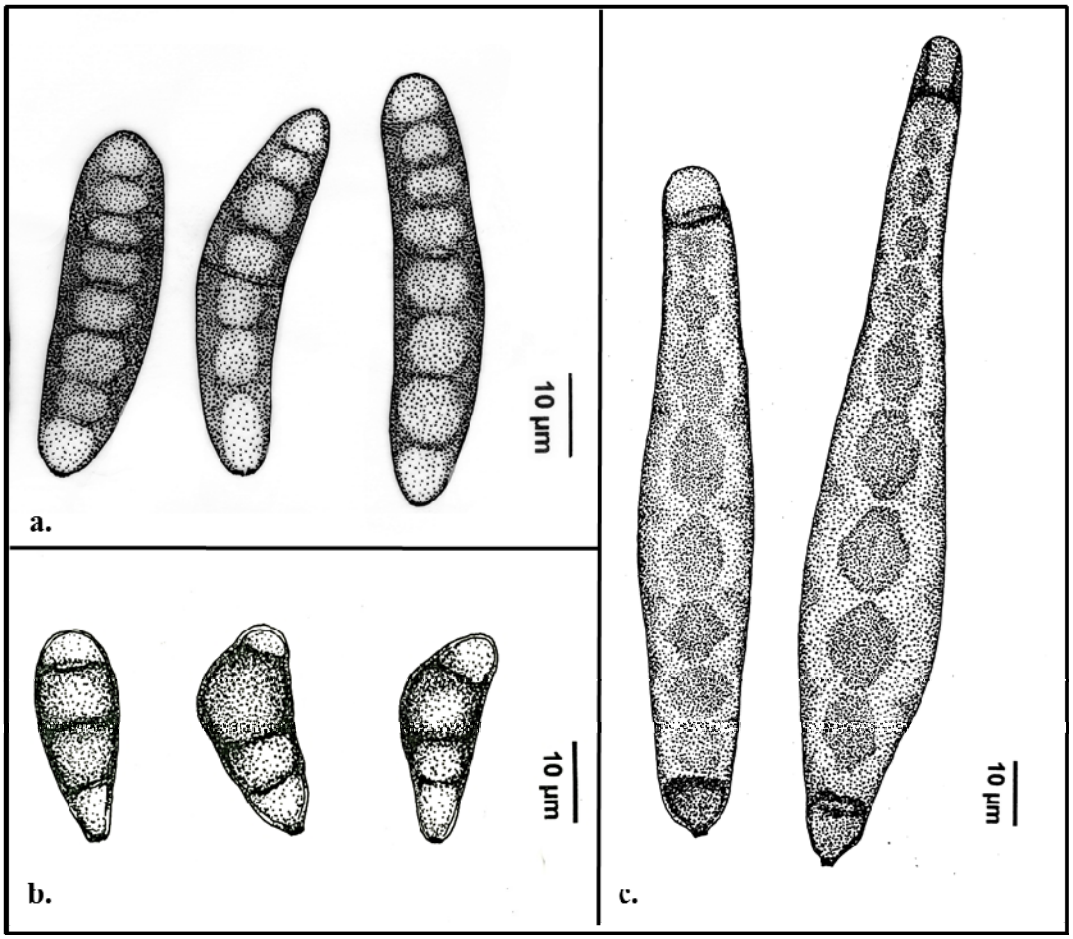

Fig. 10. Conidia of the genera: **a**. *Bipolaris*, **b**. *Curvularia*, **c**. *Exserohilum*.

Currently, *Bipolaris* is comprised of more than 100 species, *Curvularia* covers more than 80, and *Exserohilum* includes 35 species. These fungi are widely distributed geographically, although some species may have a limited areal distribution. Most species are saprobes in soil and plants or act as pathogens of wild grasses (Ellis 1971, Sivanesan 1987). Some species are plant pathogens and can cause devastating epidemics in agriculture, such as in plantations of rice, wheat and maize (Sivanesan 1987, Berbee et al. 1999). Furthermore, these genera also include species that can cause several diseases in both humans and animals. In humans, they are frequently reported in cases of keratitis and allergic sinusitis (Revankar & Sutton 2010). In animals, such as cats, dogs, horses and cows, different species have been described as causal agents of systemic mycosis, keratomycosis, mycetomas, or dermal granulomas (Bridges 1957, Whitford et al. 1989, Waurzyniak et al. 1992, Scott & Carter 2014).

Apart from their clinical relevance, these fungi are also important for different industries because of their production of secondary metabolites such as pigments, substances with antimicrobial properties and precursors used for steroid production (Sivanesan 1987, Bashan et al. 1996, Chrysayi-Tokousbalides & Kastanias 2003, Andryushina 2010).

1.4.1. Taxonomy of *Bipolaris*, *Curvularia* and *Exserohilum* complex

Initially, many species of *Bipolaris*, *Curvularia*, *Drechslera* and *Exserohilum* were included in the genus *Helminthosporium* (Sivanesan 1987). The genus *Helminthosporium,* typified by *H. velutinum* (Link 1809), was characterized by solitary, cylindrical, unbranched, brown conidiophores that produced obclavate, distoseptate conidia through small pores in the wall of the apical and intercalary cells of the conidiophores (Goh et al. 1998). The fungi belonging to the aforementioned genera differed from *H. velutinum* in that they produced conidia through sympodial extension from the subepidermal region of the conidiophores (Goh et al. 1998).

Nisikado (1928) divided the genus *Helminthosporium* into two subgenera: *Cylindro-Helminthosporium***,** which included species that possessed straight, cylindrical conidia with germination by one or more germ tubes of any conidial cell; and *Eu-Helminthosporium***,** which included species with conidia that were often curved and fusiform and germinating only by terminal cells. In 1930, Ito raised the subgenus *Cylindro-Helminthosporium* to the rank of genus and referred to it as *Drechslera* (Ito 1930); the sexual state of the latter genus was associated with *Pyrenophora*. To accommodate members of the subgenus *Eu-Helminthosporium* Shoemaker (1959) raised the genus *Bipolaris* with the type species *B. maydis*. In this new genus, the species that had conidia with protuberant hilum were associated with the sexual state *Trichometasphaeria*, while the other species, which had conidia without protuberant hilum, were associated with the teleomorphic genus *Cochliobolus*. Later, Leonard & Suggs (1974) established the genus *Exserohilum* to accommodate species with large conidia and protuberant hilum. The genus was typified by *Exserohilum turcicum* (Pass.) K.J. Leonard & Suggs. The ascomycete genus *Setosphaeria* was established as the teleomorph of *Exserohilum.*

The genus *Curvularia* was established by Boedijn (1933) and typified by *C. lunata*. As in the case of *Bipolaris*, *Curvularia* was also associated with the teleomorphic genus *Cochliobolus.* Another genus of ascomycetes that was associated with species of *Bipolaris* and *Curvularia* was *Pseudocochliobolus* (Tsuda et al. 1977, Tsuda & Ueyama, 1981, 1982, 1983, 1985). This genus was proposed in order to accommodate *Cochliobolus* species that presented ascomata arising from columnar or flattened stroma and ascospores arranged in asci that were parallel or slightly spiraled in shape. Alcorn (1983a) argued that these characters were not sufficiently reliable for the generic distinction and concluded that *Pseudocochliobolus* and *Cochliobolus* were congeneric.

Curvularia and *Bipolaris* have similar characteristics, including the production of stromata, bipolar germination of their conidia, and conidiogenous cells that are either smooth or verrucose (Sivanesan 1987). Because these genera differ only in certain morphological characteristics of the conidia and type of septation, some authors suggested that they were synonymous (Von Arx & Luttrell 1979, Alcorn 1983a, 1988; Sivanesan 1987). However, considering the type of conidial septum, i.e., in *Curvularia* euseptate versus distoseptate in *Bipolaris* spp., both genera were maintained as different taxa, although species with intermediate features of both genera have been reported (Luttrell 1963, Alcorn 1983a, 1988).

With advances in molecular techniques, many uncertainties of conventional taxonomy can be resolved (Valente et al. 1999, Mendoza et al. 2001). Several phylogenetic studies on *Bipolaris* and *Curvularia* have found both genera to be polyphyletic; those species of *Bipolaris* with short and straight conidia were grouped in the clade of with *Curvularia* species (Goh et al. 1998, Berbee et al. 1999, Emami & Hack 2002, Manamgoda et al. 2011, 2012). A recent phylogenetic study confirmed that the complex made up of *Bipolaris, Curvularia* and species identified as *Cochliobolus* should be divided into two monophyletic groups. The species *Bipolaris australiensis*, *B. coicis, B. ellisii, B. graminicola, B. hawaiiensis, B. ovariicola, B. ravenelii, B. spicifera, B. perotidis* and *B. tripogonis* were clustered with the type species of the genus *Curvularia.* Because these *Bipolaris* species did not share morphological characteristics with the type species of *B. maydis* (i.e. large, gently curved conidia) and were

phylogenetically distant, these species were transferred to *Curvularia* (Manamgoda et al. 2012).

The genus *Exserohilum* was characterized by simple conidiophores, geniculate above, by polyblastic and sympodial conidiogeneus cells that have conspicuous conidial scars, and its large conidia. The conidia can be fusiform, cylindrical or obclavate, straight to curved, and have a strongly protruding hilum. This genus was distinguished from *Bipolaris* and *Curvularia* by features associated with the conidia, such as shape, septum ontogeny and hilum morphology. Other criteria used to distinguish this genus included anamorph-teleomorph connections and culture colony morphology (Sivanesan 1987, Alcorn 1988). However, the genus presented a few problems in terms of taxonomy. One of these problems was the morphological similarity among species, which occurs with the three species that are pathogenic for humans (*E. rostratum, E. longirostratum* and *E. mcginnisii*) (Mcginnis et al. 1986). Unfortunately, little is known about the phylogeny of this genus.

1.4.2. Clinical importance of *Bipolaris, Curvularia* and *Exserohilum*

Currently, the genera *Bipolaris Curvularia* and *Exserohilum* are considered emerging pathogens that are responsible for cutaneous and subcutaneous infections **(Fig. 11),** although allergic fungal sinusitis is the most common clinical presentation attributed to those genera (Brandt & Warnock, 2003, Revankar & Sutton 2010, El Khizzi et al. 2010, Derber et al. 2010, Alvarez et al. 2011, Glass & Amedee 2011, Knutsen et al. 2012). Other clinical manifestations that have been described include keratomycosis, endophthalmitis, allergic bronchopulmonary micosis, disseminated infection and cerebral phaeohyphomycosis (Aquino et al. 1995**,** Flanagan & Bryceson 1997, Tessari et al. 2003, Peerapur et al. 2004, Bashir et al. 2009, Chowdhary et al. 2011, Nath et al. 2011, Patel et al. 2012, Gongidi et al. 2013, Chiller et al. 2013). The fungi from these genera can affect immunocompromised patients (Karim et al. 1993, Heinz et al. 1996, Derber et al. 2010, Rosow et al. 2011, Patel et al. 2012) and immunocompetent patients alike (Ashraf et al. 2005, Coop & England 2006, Bashir et al. 2009, Kleinfeld et al. 2013, Skovrlj et al. 2013).

Until recently, the genus *Bipolaris* was composed of four species of clinical importance: *B. spicifera*, *B. australiensis*, *B. hawaiiensis* and *B. papendorfii*. Due to the

phylogenetic results obtained by Manamgnoda et al. (2012), *B. spicifera*, *B. hawaiiensis* and *B. australiensis* were transferred to the genus *Curvularia*. *Bipolaris papendorfii* is currently the only species of the genus involved in clinical reports that has been described as a cause of keratitis; these cases were reported in two patients in the USA (Kumar et al. 2005).

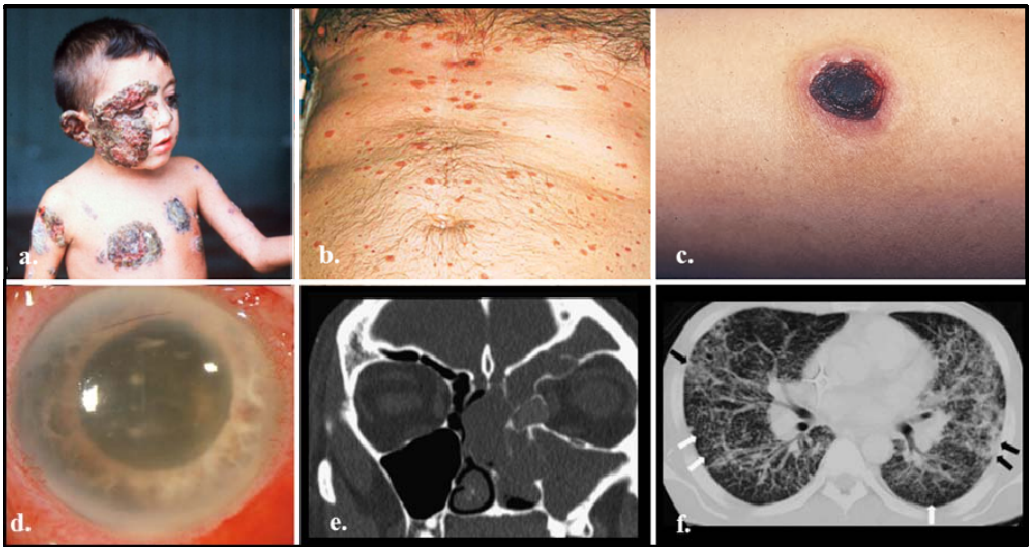

Fig. 11. Infections caused by *Exserohilum* (a, c), *Curvularia* (b, d), *Bipolaris* (e, f). Cutaneous lesions in cases of phaeohyphomycosis (a-c), keratitis (d), sinusitis (e); and pulmonary infection (f). (Figures obtained from Toul et al. 2006, Kobayashi et al. 2008, Levy et al. 2003, Tessari et al. 2003, http://www.doctorfungus.org/mycoses/human/other/phaeohyphomycosis.php).

Several species of the genus *Curvularia* have been reported as human opportunists. These species include *C. lunata*, *C. brachyspora*, *C. clavata*, *C. geniculata*, *C. inaequalis, C. pallescens, C. senegalensis*, and *C. verruculosa* (de Hoog et al. 2000, Wilhelmus & Jones 2001, Revankar & Sutton 2010). The three species of *Bipolaris* that are now included in the genus *Curvularia* (*C. spicifera, C. hawaiiensis* and *C. australiensis*) further increase the number of species of clinical importance.

Although previous studies have reported three species of the genus *Exserohilum* (*E. rostratum*, *E. longirostratum* and *E. mcginnisii*) as being opportunistic pathogens for humans (Padhye et al. 1986, Adler et al. 2006, Al-Attar et al. 2006, Saint-Jean et al. 2007), some molecular studies suggest that they may be the same species (de Hoog et al. 2000, 2011). These fungi have mainly been described as the cause of subcutaneous

phaeohyphomycosis, but also they have been reported as agents of keratitis, as well as the cause of a fatal disseminated infection in a patient with aplastic anemia (Revankar & Sutton 2010, de Hoog et al. 2011).

In October 2012, the outbreak of fungal meningitis, related to methylprednisolone acetate injections that had been contaminated by *E. rostratum,* was a significant public health problem in the United States; it was an important cause of morbidity. Because of this fact, scientists have sought more information regarding the pathophysiology of this fungus in order to optimize the treatment of the infections it causes (Chiller et al. 2013, Kleinfeld et al. 2013).

1.5. The genus *Pithomyces:* taxonomy and clinical importance

The genus *Pithomyces* was proposed by Berkeley & Broome (1873) to accommodate the species *P. flavus,* a fungus found on an unidentified monocotyledon and which caused small yellow patches. Currently, the genus contains more than 40 species that are widely distributed in nature, and which are isolated from dead leaves or stems of many different plants, but also from soil and air (Ellis 1960). These fungi are characterized by either punctiform or effuse colonies that are yellow, olive-green, brown, or black, as well as by subhyaline or brown hyphae, and by short, peg-like conidiophores that arise laterally on the hyphae. Their conidia emerge alone at the apex of conidiophores and are subspherical to clavate, with transverse, oblique or/and longitudinal septa, and are brown and commonly with a verruculose or echinulate wall (Ellis 1960, 1971). The genera *Monodictys*, *Parapithomyces* and *Endosporoideus* are morphologically similar to *Pithomyces* (Seifert et al. 2011). The diagnostic characteristics used to distinguish between *Pithomyces* and similar genera are compared in Table 1.

In 1986, Roux described *Leptosphaerulina chartarum* as the sexual state of *P. chartarum*, the only *Pithomyces* species with teleomorph. *Leptosphaerulina chartarum* was mainly characterized by woolly to appressed and dark olive colonies, producing ascostromata solitary, dark olive brown, globose, large ostiole, with bitunicate 8 - spored asci, and ascospores with usually three transverse and one longitudinal septum, constricted at septa, hyaline to light brown, smooth, broadly ellipsoidal, 23-27 x 7-12 µm (Roux 1986). The members of *Leptosphaerulina* are included in the family

Didymellaceae of the order Pleosporales (Zhang et al. 2009, 2012).

TABLE 1. Diagnostic characteristics of *Pithomyces* and similar genera

	Pithomyces Berk. & Broome (1873)	***Monodictys*** S. Hughes (1958)	***Parapithomyces*** Thaung (1976)	***Endosporoideus*** W.H. Ho, Yanna, K.D. Hyde & Goh (2005)
Conidiophores	Micro- or semi-macronematous Mononematous	Micro- or semi-macronematous, Mononematous	Semi- or macronematous Mononematous	Micronematous Mononematous
Conidiogenous cells	Holoblastic Mono- or polyblastic	Holoblastic Monoblastic	Holoblastic Mono- or Polyblastic	Holoblastic Polyblastic
Conidia	Phragmospores or dictyospores Single	Dictyospores or bulbis with short protuberances Single	Dictyospores Single or in short acropetal chains	Phragmospores (disarticulate at septa) Single
Conidial secession	Rhexolytic	Rhexolytic	Schizolytic	Schizolytic

Pithomyces chartarum is the only species clinically relevant, because of its ability to cause facial eczema in animals such as sheep, cattle, goats and deer. This eczema is a cutaneous response to liver damage caused by a mycotoxin (sporisdesmin) produced by this fungus (Ozmen et al. 2008, Di Menna et al. 2009). This cutaneous lesion has been described in animals from different countries, including New Zealand, Nigeria, the USA and Brazil (Brook 1963, Hansen et al. 1994, Efuntoye & Fashanu 2002, Moreira et al. 2009) **(Fig. 12)**. Althoug *Pithomyces* species are rarely causal agents of human infections, they have recently been reported in cases of peritonitis and onychomycosis (Litz & Cavagnolo 2010, Deplano 2012, Terada et al. 2014). Furthermore, the presence of *Pithomyces* spores in the air has been associated with cases of asthma (Jones et al. 2011, Meng et al. 2012, Premila 2013).

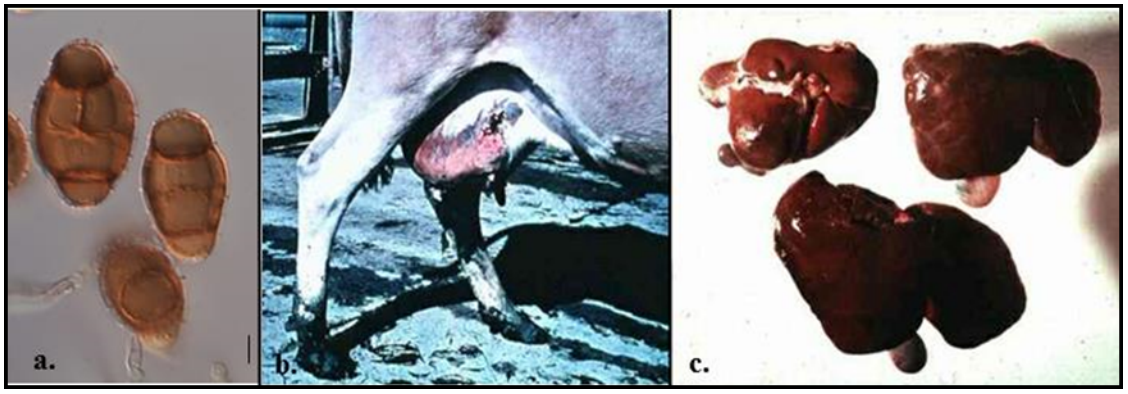

Fig. 12. a. Conidia of *Pithomyces chartarum* (Scale bar = 5 µm). b. Scabbing and sores on the udder of a cow with exposed spores of *P. chartarum.* c. Liver of a healthy sheep (bottom) compared with two livers from affected sheep (top). The affected livers are small and show significant fibrous tissue, which is absent from the healthy liver. Figures b and c were taken from http://archive.bio.ed.ac.uk/ jdeacon/microbes/facial.htm.

1.6. Antifungal Susceptibility

Antifungal susceptibility studies allow for the evaluation of the *in vitro* susceptibility of a fungus to one or more antifungal agents in order to correlate drug activity in *in vitro* versus *in vivo* studies, and also to predict the therapeutic outcome. These studies are also useful for detecting resistant strains and for determining the potential therapeutic value of a newly detected resistant strains, as well as for determining the potential therapeutic value of a new antifungal agent (Espinel-Ingroff 1994). Therefore, it is extremely important to determine the antifungal susceptibility patters not only of the pathogenic fungal species but also of those fungi isolated from clinical specimens, which can prove to be pathogenic species for humans.

1.6.1. Methods of *in vitro* antifungal susceptibility studies

The methods and techniques used to study *in vitro* antifungal susceptibility are similar to the methods used for studies involving bacteria; the design follows the same orientations (Espinel-Ingroff & Shadomy 1989, Shadomy et al. 1991). The most commonly used technique for the study of the *in vitro* susceptibility of filamentous fungi is the liquid medium dilution method (micromethod) following the recommendations from the CLSI (2008). This technique can measure antifungal activity

on fungal growth expressed as **MFC (**minimum fungicidal concentration) or **MIC** (minimum inhibitory concentration).

It is worth mentioning, that most studies carried out recently on the antifungal susceptibility of melanized fungi, the identification of those fungi at the genus or species level have been based only on morphological features. Therefore, considering the results of molecular studies which usually reveal that a morphospecies included different phylogenetic species with different responses to antifungal drugs or even different pathogenic behavior (Lass-Flörl et al. 2008), thus a re-evaluation of the identification of pathogenic melanized fungi is necessary. Once a correct phylogenetic identification has been performed, it is important to determine again the antifungal susceptibility patterns of the fungi molecularly identified. Only the results of the antifungal tests performed with well delimited species will be reliable for choosing an appropriate therapy for patients infected by this type of fungi.

1.6.2. *In vitro* antifungal susceptibility of *Bipolaris*, *Curvularia, Exserohilum* and *Pithomyces*

In recent years, antifungal susceptibility testing has made progress; however, the *in vitro* data available for dematiaceous fungi is relatively scarce, and it often relies on a small number of isolates of each species. Furthermore, most studies were performed before standardized procedures for antifungal susceptibility testing for filamentous fungi were available. Therefore, breakpoints were not available, and the interpretation of the results are not reliable. Until recently, the directives for the interpretation of *in vitro* data were also based on the breakpoints only for *Candida* species (Revankar & Sutton 2010).

With the exception of flucytosine, in the case of most of the drugs used in the treatment of dematiaceous fungal infections, a MIC of ≤1 μg/mL is used to indicate potential susceptibility. In fact, lower MICs generally suggest better activity (Revankar & Sutton 2010). In the case of the genera *Bipolaris* and *Curvularia, in vitro* studies suggest good activity based on consistently low MICs to itraconazole (ITC), voriconazole (VRC) and posaconazole (PSC). *Exserohilum* species present good activity when exposed to amphotericin B (AMB), ITC and VRC. Although *in vitro* antifungal susceptibility studies on *Pithomyces* are even rarer than those available on the

other genera mentioned, the results of these few studies suggest that the genus presents good activity when exposed to VRC and ITC (Espinel-Ingroff et al. 2008, Revankar & Sutton 2010). Therefore, all of these melanized fungi are usually susceptible to azoles, mainly ITC and VRC, such as in the case of other dematiaceous fungi (*Alternaria, Cladophialophora, Exophiala*). However, their response to other antifungal drugs such as terbinafine, flucytosine, anidulafungin are not well understood.

1.6.3. Treatment of infections caused by *Bipolaris*, *Curvularia, Exserohilum* and *Pithomyces*

Clinical experiments involving the treatment of infections caused by *Bipolaris, Curvularia, Exserohilum* and *Pithomyces* are relatively scarce. The treatment choice depends on the site of the infection; sometimes, the treatment consists of both surgery and antifungal therapy (Revankar & Sutton 2010). Different antifungal agents have been used (mainly amphotericin B, natamycin and azoles), but different results have been reported (Revankar & Sutton 2010). Table 2 summarizes the therapy suggested for infections by these fungi when they are associated with different clinical syndromes (Revankar & Sutton 2010, Kainer et al. 2012, Kauffman et al. 2013, Kerkering et al. 2013, Terada et al. 2014).

TABLE 2. Clinical syndromes associated with *Bipolaris, Curvularia, Exserohilum* and *Pithomyces,* and suggested therapies.

Clinical syndrome	**Commonly associated fungal genera or species**	**Therapy**
Allergic fungal sinusitis	*Bipolaris*, *Curvularia*	Surgery + steroids ± ITC
Allergic bronchopulmonary micosis	*Bipolaris*, *Curvularia*	Steroids ± ITC
Keratitis	*Bipolaris*, *Curvularia, Exserohilum*	Topical natamycin ± topical azole
Peritonitis	*Curvularia, Pithomyces*	Catheter removal ± AMB or azole
Meningitis	*Exserohilum*	VRC ± AMB
Disseminated disease	*Bipolaris*	VRC + TRB ± echinocandin, VRC ± echinocandin or L-AMB

AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; TRB, terbinafine (adapted from Revankar & Sutton 2010).

2. INTEREST AND OBJECTIVES

In recent years, a significant increase in infections caused by filamentous fungi has been reported, and these infections sometimes occur with a high morbidity and mortality in spite of the different antifungal treatments and medical techniques available. The high mortality is the result of a lack of knowledge on the epidemiology of infections caused by genera that themselves are understudied; thus little is known about their roles in the diagnosis and treatment of infections.

In this thesis, we have chosen four anamorphic genera of dematiaceous filamentous fungi (*Bipolaris, Curvularia, Exserohilum* and *Pithomyces*) due to the significant number of strains isolated from clinical specimens originating largely from different hospitals in the United States. This study was possible because of a partnership with the Fungus Testing Laboratory at the University of Texas, which is the reference center for fungal identification and antifungal susceptibility testing in the US. The increase in the number of isolates of these fungi isolated in clinical samples represents a real challenge for clinical microbiologists, mainly due to the difficulty in fungal identification. The taxonomy of these fungi is based on the study of the morphological features of their sporulated structures, which are usually only described based on the natural substrate. Clinical isolates of many dematiaceous fungi when grow *in vitro,* i.e. on culture media rich in nutrients, they very often do not sporulate or their morphological features vary respect to those described on the natural substrate. In addition, the genera selected for investigation in this thesis included numerous species (in *Bipolaris* ca 100 spp., in *Curvularia* ca 80 spp., in *Exserohilum* ca 35, *Pithomyces* ca 45 spp.), and distinction among them is largely based on few morphological features (e.g. number of conidial septa or the size of the conidia). Therefore, both the morphological similarity among species and the *in vitro* variation of those morphological features severly hinder the correct identification of the species, particularly those involved in clinical processes. It is worth mentioning that species of these genera are described not only as human pathogens (de Hoog et al. 2000, Revankar & Sutton 2010), but also as causal agents of plant diseases (Ellis 1960, 1971, Sivanesan 1987). Some are also fungi with different applications in the field of mycology (Sivanesan 1987, Andryushina 2010). Thus, the correct identification of the isolates is crucial if we are to understand the real impact of the species on these different fields.

In the recent years, molecular biology has been fundamental in the study of genera with complex taxonomy. DNA sequencing has been the main tool used to identify fungi and also to detect cryptic species, or species that, despite their morphological similarity, are genetically very different. Clear examples are the molecular studies on *Acremonium, Pseudollescheria, Phialemonium* or *Sporothrix* (Gilgado et al. 2005, Marimon 2006, 2007, Perdomo et al. 2011a,b), among other clinically relevant genera. These studies allowed for the characterization of new species of clinical importance, since they were found to have antifungal susceptibility patterns that were different from those of the known species (Tortorano et al. 2008, Lackner et al. 2012). They were also found to have different virulence properties (Revankar & Sutton 2010).

Molecular studies on the genera investigated in the present thesis have thus far been very scarce. Therefore, the real incidence of the species isolated from clinical specimens, as well as their susceptibility to the current antifungal drugs available are unknown. An additional problem is the fact that there are few sequences deposited in databases such as GenBank, which hinders molecular identification through sequence comparison.

For these reasons, the **objectives** of this thesis are:

1. To identify morphologically and molecularly clinical isolates of *Bipolaris, Curvularia, Exserohilum* and *Pithomyces* in order to determine the diversity of species from those genera in clinical samples.
2. To determine the phylogenetic relationships and taxonomic positions of the clinical species identified.
3. To characterize morphologically and molecularly the new species that were not previously recognizable through traditional identification methods.
4. To assess the *in vitro* activity of different antifungals drugs against the species of *Bipolaris, Curvularia, Exserohilum* and *Pithomyces* identified in the present study.

3. MATERIALS AND METHODS

3.1. Origin and storage of the isolates

During the development of this thesis, a total of 281 clinical isolates were studied. The covered four genera: *Bipolaris* (104 isolates), *Curvularia* (101 isolates), *Exserohilum* (34 isolates) and *Pithomyces* (42 isolates). All of the isolates were obtained from the culture collection of the Fungus Testing Laboratory in the Department of Pathology at the University of Texas Health Science Center (UTHSC) (San Antonio, USA). These isolates were recovered over a ten-year period (2003 - 2012). In addition, 123 types or reference strains obtained from different international culture collections and reference centers were studied in the present work, and morphological or genetic comparisons were performed (see Annex I).

All of the strains were conserved as submerged cultures in liquid paraffin and water, and were also lyophilized. In the first procedure, fungi were cultured on potato carrot agar (PCA; 20 g potatoes, 20 g of carrot, 15 g of agar and 1 L distilled water) and oatmeal agar (OA; 30 g oatmeal, 20 g of agar,1 L distilled water) in slant tubes, and the growing part of the agar was covered with the liquid paraffin. The tubes were stored at room temperature.

In the second procedure, small pieces of sporulated cultures on OA and/or PCA were transferred to small dark vials (10 mL) containing 5 mL of sterilized distilled water, and they were then stored at room temperature. To perform lyophilization, the strains were cultured on PCA or OA to obtain abundant sporulation. The colonies were then mixed with 3 mL of a 10% skim milk solution (Difco, USA), a cryoprotectant, which was previously sterilized at 115 ° C for 15 min. Next, 1 mL of this solution was dispensed in 3 mL sterile glass vials and placed in the lyophilizer (Advantage 2.0 Series; Virtis Company Gardiner, USA). Sublimation was achieved when the temperature of the condenser reached 45° C, the vacuum of 200 mTorr was made, then the following lyophilization cycle was programmed: -30° C (240 min), -10ºC (240 min), +10 °C (300 min), and +30°C (300 min). After the process was complete, the vials were sealed under vacuum and stored at room temperature.

3.2. Phenotypic studies

Phenotypic characterization consisted mainly of the evaluation of macroscopic and microscopic features in different culture media. The isolates included in this thesis

were initially identified morphologically following the criteria established for each genus (Ellis 1960, 1971, 1976, Rao & de Hoog 1986, Sivanesan 1987).

All isolates were cultured on PCA and OA media and incubated at 25°C for 7 to 21 days. When necessary cardinal temperatures of the species were obtained on potato dextrose agar (PDA, Pronadisa) at 15, 20, 25, 30, 35, 37 and 40ºC; incubated in darkness. When the fungus grew but did not sporulate, it was subcultured again and incubated under alternating cycles of black light and darkness (12hours/12hours) until its sporulation. For the colony description, a standardized colour nomenclature was used according to Kornerup & Wanscher's manual (1978).

The microscopic features were examined using direct wet mounts in 85% lactic acid from the different culture media. The size of each microscopic structure (i.e. condiophores, conidiogenous cells and conidia) was obtained by measuring a minimum of 15 structures of each type, and through the use of an Olympus CH2 light microscope. Photomicrographs were obtained mostly with a Zeiss Axio Imager M1 light microscope, which had an Olympus DP10 InfinityX digital camera incorporated into it, and images were captured and processed using the DeltaPix software.

In the taxonomic study on *Curvularia,* we examined several species using Scanning Electron Microscopy (SEM), particularly to observe details of the conidial wall ornamentation of the different species investigated. The procedure to prepare fungi for SEM examination was as followed that of Bensch et al. (2012). Sporulated areas of fungal cultures were carefully selected using a stereo microscope (Nikon SMZ1500). Small (approx. 3 x 5 mm) agar blocks were carefully cut out with a surgical blade (no. 11, Swan-Morton, Sheffield, UK) and the disturbance of fungal structures was kept to a minimum during the cutting and transfer of the samples to a copper cup (diam 10 mm, height 8 mm). Agar blocks were glued to the copper cup with a frozen tissue medium (KP-Cryoblock, Klinipath, Duiven, The Netherlands). The copper cup was placed on an agar surface inside a closed Petri dish to prevent sample from drying out. The sample was quickly frozen in nitrogen slush and immediately transferred to a JEOL 5600LV scanning electron microscope equipped with an Oxford CT1500 cryostation. The sample was viewed at 2.5 kV, and ice was removed through the use of sublimation after the SEM-stage was heated to -85 °C. The sample was then sputter-coated in the cryostation by means of a gold target for three periods of 90 s each, with the sample

held at different angles for optimal coating. Electron micrographs were acquired with the F3 or F4 scan at 5 kV, and contrast levels were digitally enhanced in Adobe® Photoshop® Creative Suite v. 6.

3.3. Molecular studies

The internal transcribed spacers (ITS), including the 5.8S rDNA, and the fragment D1/D2 of the large subunit (LSU) were sequenced for isolates studied for identification purposes and also to determine the taxonomic position of the species among Ascomycetes. When the ribosomal sequences were not numerous enough to elucidate the taxonomy of the species investigated, different structural genes were sequenced (see section 3.3.2).

DNA extraction was performed directly from fungal colonies that were on Yeast Extract Sucrose agar (YES medium; 2% yeast extract, 15% sucrose, 2% agar and 1 L of distilled water) after three or four days of incubation at 25°C. The YES medium was used to minimize the presence of pigments, which might inhibit PCR reactions later. We used two extraction protocols: the Fast DNA kit protocol (Bio 101, Inc., Joshua Way, Vista, California, USA) or Prepman Ultra (Applied Biosystems, USA), as described by the manufacturers.

3.3.1. Amplification and sequencing of ribosomal DNA (rDNA)

Amplification and sequencing of the 5.8S RNA gene and the ITS 1 and 2 regions were performed with the ITS5/ITS4 primer pair (Table 3). The PCR reaction was performed in a final volume of 40 µL that contained 20 to 60 ng of the genomic DNA, 10X PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 50 mM of MgCl2, 0.2 mM of dNTPs (for each nucleotide), 10 mM of each primer, and 5 U / µL Taq of DNA polymerase (Invitrogen, The Netherlands). The amplification programs used were described by Álvarez et al. (2010).

For most of the genera included in this thesis, the D1/D2 domains of the large subunit (LSU) were amplified and sequenced with the NL1/NL4b pairs of primers following the protocols published by O'Donnell (1993). In the case of the *Curvularia* species, a larger fragment of the LSU was sequenced, one which includes the D1, D2,

D3, and D4 domains. For this purpose, the LR0R/LR5 pairs of primers were used following the protocols published by Vilgalys & Hester (1990) (Table 3).

In some cases, this protocol was modified in order to amplify the ITS region and D1/D2 rDNA together; in those cases, the primers used were ITS5 and NL4b, and the annealing temperature was 53°C.

3.3.2. Amplification and sequencing of different structural genes

Sequences of genes that differed from those of rDNA analyzed in order to clarify the taxonomy of the different fungi investigated are as follows: Actin 1(*ACT 1*), Elongation factor 1-alpha (*EF-1α*), Glyceraldehyde-3-phosphate dehydrogenase (*GPDH*) and RNA polymerase II second largest subunit (*RPB2*).

The PCR reaction to amplify the fragment of *ACT 1* gene was performed as indicated previously with Act-1/Act4 primer pairs (Table 3)**.** The amplification programs used were described by Voigt & Wöstemeyer (2000).

Amplification and sequencing of a fragment of the *EF-1α* gene was performed with the 983F/ 2218R primer pair (Table 3) according to Sung et al. (2007).

Fragments of the *GPDH* gene were amplified and sequenced with the gpd1/gpd2 primer pairs (Table 3) following the protocol previously described by Berbee et al. (1999).

Finally, fragments of the *RPB2* gene were amplified and sequenced with the 5F2/7cR primer pairs (Table 3) following the protocol previously described by Amaradasa et al. (2014).

The combination of the gene markers used for the different studies was as follows: the sequences of the ITS region, plus fragments of LSU, *GPDH* and *RPB2* were analyzed for the study of *Curvularia* and *Pithomyces* isolates, while only the ITS region was used to elucidate the relationships among *Bipolaris* species. In the cases of *Exserohilum* species*,* the relationships were established using the ITS region and fragments of LSU, *ACT 1* and *EF-1α* genes.

TABLE 3**.** Primers used in PCR and sequencing for different fungi.

Loci	**Product size (bp)**	**Primer *forward* (5' →)**	**Primer *reverse* (5' →)**	**T(ºC)**	**Reference**
LSU	834	LR0 ACCCGCTGAACTTAAGC	LR5 TCCTGAGGGAAACTTCG	53	Vilgalys & Hester 1990
D1/D2 domains LSU	431-464	NL1 GCATATCAATAAGCGGAGGAAAA	NL4 GGTCCGTGTTTCAAGACGG	51	O´Donell et al. 1993
ITS	385-545	ITS 5 GGAAGTAAAAGTCGTAACAAGG	ITS 4 TCCTCCGCTTATTGATATGC	55	White et al. 1990
ACT 1	317-318	Act-1 TGGGACGATATGGAIAAIATCTGGCA	Act-4R TCITCGTATTCTTGCTTIGAIATCCACAT	55	Voigt and & Wöstemeyer 2000
EF - 1α	609	983F GCYCCYGGHCAYCGTGAYTTYAT	2218R ATGACACCRACRGCRACRGTYTG	47	Sung et al. 2007.
GPDH	434	GPD1 CAACGGCTTCCGTCGCATTG	GPD2 GCCAAGCAGTTGGTTGTGC	57	Berbee et al. 1999.
RPB2	793	5F2 GGGGWGAYCAGAAGAAGGC	7cR CCCATRGCTTGYTTRCCCAT	60-54	Amaradasa et al. (2014).

LSU**,** large subunit; ITS, internal transcribed spacers; *ACT 1,* actin 1; *EF - 1α,* elongation factor 1 alpha; *GPDH*, glyceraldehyde -3-phosphate dehydrogenase; *RPB2*, RNA polymerase II second largest subun.

3.3.3. Verification and purification of the amplified products

PCR products were analyzed through the use of agarose gel electrophoresis at 2% (Boehringer-Mannheim), followed by RedSafe DNA Stain (ChemBio) staining. To check the size of amplicons, 100-bp molecular weight markers were added in the first and last well of each gel (Invitrogen, Holland). In the remaining wells, 4 µL of PCR product and 2 µL of Fermentas 6X DNA Loading Dye were added (Thermo Fisher Scientific, France).

PCR products were purified using a GFXTM PCR DNA kit (Pharmacia Biotech, Cerdanyola del Valles, Spain).

3.3.4. Sequencing of the amplified product

For the isolates from the genera *Bipolaris* and *Exserohilum,* the sequencing process was carrie out using the *Taq* DyeDeoxy Terminator cycle sequencing kit protocol (Applied Biosystems, Gouda, The Netherlands). DNA sequencing reaction mixtures were analyzed on a 310 DNA sequencer (Applied Biosystems).

For the *Curvularia* isolates, 10 µL of the ITS PCR products and 10 µL of the primer employed in the PCR-amplification were added to a 1,5 mL microtube. These microtubes were sequenced to Macrogen (Korea) using a 3739 XL DNA analyzer (Applied Biosystems). The *GPDH*, LSU and *RPB2* loci were sequenced at the CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands) using the BigDye terminator sequencing kit v. 3.1 (Applied Biosystems) and an ABI Prism^{TM} 3100 DNA sequencer (Applied Biosystems).

For *Pithomyces* isolates*,* 10 µL of the PCR product of the gene studied and 10 µL of the primer employed in amplification were added to a 1,5 mL microtube. These microtubes were sent to Macrogen (Korea) or to the scientific technical service of our university for sequencing.

3.3.5. Consensus sequences and molecular identification

The SeqMan software (Lasergene, Madison, Wisconsin) was used to obtain consensus sequences from the complementary sequences of each isolate. The sequences obtained for each gene were compared to those deposited mainly in GenBank using BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3.6. Sequence alignment

Nucleotide sequence alignments were performed using MAFFT (Katoh et al. 2009) or ClustalW (Chenna et al. 2003), followed by manual adjustments with MEGA5 (Tamura et al. 2011) or with a text editor.

3.3.7. Phylogenetic analyses

Individual alignments of the loci studied in the present thesis, as well as concatenated dataset were analyzed with maximum likelihood (ML) using MEGA5 (Tamura et al. 2011), with partial deletion of gaps and the substitution models proposed by this program and 1000 bootstrap replicates. Bootstrap support values (bs) ≥ 70% were considered significant.

The partition homogeneity test (PHT) (Farris et al. 2005) as implemented in PAUP v. 4.0b10 (Swofford 2003) was performed ir order to check the extent of incongruence among datasets in the *Curvularia* and *Pithomyces* multilocus analyses. A Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes v. 3.1.1 (Ronquist & Huelsenbeck 2003). The best models of nucleotide substitution for each locus for the Bayesian analysis were determined using MrModeltest v. 2.3 (Nylander 2004). Two analyses of four MCMC chains were run from random trees for 4598100 generations and sampled every 100 generations, resulting in 45981 trees, of which 25% of which were discarded as part of the burn-in phase. Posterior probabilities (pp) were determined from the remaining trees.

Sequences of reference strains extracted from international databases such as GenBank and used in our phylogenetic analyses are in Annex 2.

3.4. Deposit of isolates and sequences

All isolates were deposited into the FMR collections, and were preserved using the methods explained in the section 3.1 (pag. 35). Each isolate was labelled with an accession number, which in the FMR database includes all information on the origin, date of isolation, and collector. If an isolate was considered to be of taxonomic interest, it was also deposited in international public collections, and most frequently in that of

the CBS-KNAW Fungal Biodiversity Centre (Utrecht, the Netherlands), the BCCM/IHEM biomedical fungi and yeasts collection (Belgian Co-ordinated Collections of Micro-organisms / IPH-Mycology) and the MUCL, Mycothèque de L'Université Catholique de Louvain, Faculté des Sciences Agronomiques (Louvain-la-Neuve, Belgium).

Most of the sequences generated in this thesis were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Acession numbers of both culture collection and sequence are indicated in the tables included in the annexes (I, pag. 201; II, pag. 221) and in the different publications included in the Results Section (pag. 45).

3.5. *In vitro* antifungal susceptibility test

In vitro susceptibility studies were performed against different antifungal drugs using a total of 77 *Bipolaris* strains, 99 *Curvularia* strains, 34 *Exserohilum* strains, and 31 *Pithomyces* strains**.**

In the case of *Bipolaris* and *Curvularia,* a total of 9 antifungal drugs were tested. They were anidulafungin (AFG, Pfizer Inc., New Cork, NY, US); caspofungin (CAS, Merck&Co., Inc., Rahway, NJ, US); fluconazole (FLC, Pfizer, Madrid, Spain); voriconazole (VRC, Pfizer Central Research, Sandwich, UK); micafungin (MFG, Astellas Pharma, Osaka, Japan); flucytosine (5-FC, Sigma-Aldrich Corp., St. Louis, EEUU); AMB (Sigma-Aldrich, Saint Quentin Fallavier, France); ITC (Janssen-Cilag, Issy-les-Moulineaux, France); and PSC (Schering-Plough Ltda., Hertfordshire, United Kingdom, Schering-Plough Research Institute, Kenilworth, NJ, US, and Schering-Plough Europa, Brussels, Belgium).

In the case of *Exserohilum* and *Pithomyces*, 8 antifungal drugs were tested. Two drugs that had been used in *Bipolaris* and *Curvularia* testing FLC and 5-FC were not used in *Exserohilum* and *Pithomyces*. Thus, the drugs tested were AFG, AMB, CAS, ITC, MFG, PSC, VRC and TRB (Novartis Pharma AG, Basel, Switzerland).

The isolates were subcultured on OA for 7 to 10 days at 30°C. The colonies were scraped, and the mycelium obtained was suspended in a physiological solution. This suspension was adjusted to 4 $x10^5$- $5x10^6$ conidia/mL after counting in a Neubauer chamber. Antifungal susceptibility testing was performed using a broth microdilution technique according to the guidelines of the National Committee for Clinical Laboratory

Standards M38A-2 (CLSI 2008). The antifungal drugs were dissolved either in dimethyl sulfoxide (DMSO, Panreac Química S.A., Barcelona, Spain), or in distilled water in the case of MFG. Next, the stock solutions of antifungal drugs were serially diluted in DMSO, and each drug concentration was then diluted to 1:50 in RPMI. Wells of 96-well microtiter plates were filled with 100 µL of each drug concentration. A drug-free well containing RPMI + 2% DMSO in the medium served as the growth control. Each well was inoculated with 100µL of conidial suspension diluted to 1:50 in RPMI. In order to evaluate viability, 100 µL of the dilutions were transferred to dichloran rose bengal chloramphenicol agar (DBRC) plates. The microtiter plates were incubated for 24-48 h at 30ºC in the case of *Pithomyces* and at 35ºC when the other genera were tested.

The MIC endpoint was 100% inhibition for AMB, ITC, VRC, and PSC and 50% inhibition for FLU and 5-FC. The minimum effective concentration (MEC) for the echinocandins was the lowest concentration at which a visible change in the growth characteristics compared to the growth control could be observed. Endpoint determinations were read at 48 hours. In the event that sufficient growth was not observed at the prescribed reading time, the test materials were incubated until sufficient growth was observed in order to enable accurate endpoint readings.

4. RESULTS

4.1. Diversity of *Bipolaris* species in clinical samples in the United States and their antifungal susceptibility profiles.

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Diversity of *Bipolaris* Species in Clinical Samples in the United States and Their Antifungal Susceptibility Profiles

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A set of 104 isolates from human clinical samples from the United States, morphologically compatible with *Bipolaris*, were morphologically and molecularly identified through the sequence analysis of the internal transcribed space (ITS) region of the nuclear ribosomal DNA (rDNA). The predominant species was *Bipolaris spicifera* (67.3%), followed by *B. hawaiiensis* (18.2%), *B. cynodontis* (8.6%), *B. micropus* (2.9%), *B. australiensis* (2%), and *B. setariae* (1%). *Bipolaris cynodontis*, *B. micropus*, and *B. setariae* represent new records from clinical samples. The most common anatomical sites where isolates were recovered were the nasal region (30.7%), skin (19.2%), lungs (14.4%), and eyes (12.5%). The antifungal susceptibilities of 5 species of *Bipolaris* to 9 drugs are provided. With the exception of fluconazole and flucytosine, the antifungals tested showed good activity.

Bipolaris is a large genus of dematiaceous hyphomycetes with more than 100 species, most of them being saprobes in soil and pathogens of plants, while some of the saprobic species are potentially able to infect humans and animals (27). They are anamorphs of the ascomycetous genera *Cochliobolus* and *Pseudocochliobolus* (family Pleosporaceae, order Pleosporales) (20).

The typical morphological features of *Bipolaris* species include rapidly growing dark colonies, geniculate conidiophores with sympodial conidiogenesis, and large conidia with transverse distosepta, usually without a protuberant hilum (a basal scar indicating the point of attachment in the conidiogenous cell) and with bipolar germination. Morphologically similar anamorphic genera are *Drechslera*, *Curvularia*, and *Exserohilum* (27).

Clinically relevant *Bipolaris* species are *B. australiensis*, *B. hawaiiensis*, *B. spicifera*, and, to a lesser extent, *B. papendorfii* (8). These fungi are able to infect both immunocompetent and immunosuppressed patients, mainly in tropical and subtropical areas. The most common clinical presentations are allergic sinusitis, keratitis, endophthalmitis, onychomycosis, peritoneal dialysis-associated peritonitis, lung and skin infections, and, less frequently, central nervous system (CNS) infections (2, 4, 5, 10, 16, 26, 28, 31).

The prevalence of the different species of *Bipolaris* in human infections is poorly known since only a few studies involving this genus have been published and the isolates were usually identified only by morphological criteria. Considering the similarity among the species of *Bipolaris* and the fact that the separation of species is based on subtle characters, some published identifications are doubtful or remain unresolved (10, 26). In the present study, we have identified a large number of isolates of clinical origin by molecular methods and by comparison of their sequences with those of type or reference strains in order to assess the spectrum of *Bipolaris* species in clinical samples in the United States.

MATERIALS AND METHODS

Fungal isolates. A total of 104 isolates from human clinical samples, presumably belonging to the genus *Bipolaris*, were morphologically examined and sequenced in the present study (see the supplemental material). They were received at the Fungus Testing Laboratory in the Department of Pathology at the University of Texas Health Science Center at San Antonio, mostly over a period of 5 years (2006 to 2010), for identification or antifungal susceptibility determination. In addition, 14 ex-type or reference strains were also included in the study.

Morphological study. All isolates were cultured on potato carrot agar (PCA; 20 g of potatoes, 20 g of carrots, 20 g of agar, 1 liter of distilled water) and oatmeal agar (OA; 30 g of filtered oat flakes, 20 g of agar, 1 liter of distilled water) media and incubated at 25°C for 10 to 21 days. The identification criteria were after Ellis (11, 12) and Sivanesan (27). Using light microscopy, microscopic features were examined by making direct wet mounts in 85% lactic acid from the different culture media.

Molecular study. Isolates were grown on yeast extract sucrose (YES; yeast extract, 2%; sucrose, 15%; agar, 2%; water, 1 liter) for 3 days at 25°C, and DNA was extracted using a PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Briefly, the fungal mycelium from a colony was gently mixed with 100 μl of the PrepMan Ultra sample reagent (Applied Biosystems, Foster City, CA). The mixture was then incubated at 100°C for 10 min, pelleted by centrifugation for 8 min at 13,000 rpm, and stored at 4°C. The DNA was quantified using GeneQuant pro (Amersham Pharmacia Biotech, Cambridge, England). The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA) was amplified and sequenced following the protocol described by Álvarez et al. (1). The specific primers used for the amplification of the ITS region were ITS 5, 5′ GGAAGTAAA AGTCGTAACAAGG 3′, and ITS 4, 5′ TCCTCCGCTTATTGATATGC 3′, and the expected size of the PCR products was 575 bp.

The program SeqMan (Lasergene, Madison, Wisconsin) was used to obtain consensus sequences from the complementary sequences of each isolate.

Phylogenetic analysis. Nucleotide sequence alignments were performed with Clustal X version 1.81 (30), followed by manual adjustments with a text editor. Distance trees were constructed with the maximum likelihood (ML) method using the Kimura 2-parameter substitution model with a pairwise deletion of gaps, as implemented in the MEGA 5.0 computer program (29). The robustness of branches was assessed by a

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bootstrap analysis of 1,000 replicates. The sequences of *Corynespora cassiicola* (IMI 056007, U95173.1) and *Alternaria alternata* (CBS 916.96, FJ196306) were used as outgroups in the ITS analysis.

Antifungal susceptibility. A total of 77 isolates with sufficient conidial production to standardize an inoculum were tested. Antifungal susceptibility testing was accomplished via methods outlined in CLSI document M38-A2 (7). This involves standardizing the inoculum by spectrophotometer to 0.4×10^4 to 5×10^4 CFU/ml (0.25 to 0.3 optical density at 530 nm [OD_{530}]), use of RPMI 1640 with L-glutamine but without bicarbonate, and incubation at 35°C. The minimum effective concentration (MEC) was determined at 24 h for the candins, and the MIC was determined at 48 h for the remaining drugs. The MIC was defined as the lowest concentration exhibiting 100% visual inhibition of growth for amphotericin B, itraconazole, posaconazole, and voriconazole while at a 50% reduction in growth for fluconazole and flucytosine. In the event that sufficient growth was not observed at the prescribed reading times, the tests continued to be incubated until sufficient growth was observed, enabling accurate endpoint determination.

RESULTS

Of the 104 clinical isolates studied, 100 were morphologically identified as members of the genus *Bipolaris*. Four isolates did not sporulate (UTHSC 07-3454, UTHSC 08-1495, UTHSC 10-2300, and UTHSC 10-2807), but the analysis of their ITS sequences demonstrated that they were *B. spicifera*.

With the primers used, we were able to amplify and sequence 571 to 575 bp of the ITS region. The predominant species was *Bipolaris spicifera* (70 isolates, 67.3%), followed by *B. hawaiiensis* (19 isolates, 18.2%), *B. cynodontis* (9 isolates, 8.6%), *B. micropus* (3 isolates, 2.9%), *B. australiensis* (2 isolates, 2%), and *B. setariae* (1 isolate, 1%). The correlation between the morphological and molecular identification was 89.7%.

In addition to the 14 reference and ex-type strains, 70 of 104 clinical isolates were included in the phylogenetic analysis. Of the 70 isolates identified as *B. spicifera*, 36 were randomly chosen for the analysis since the sequences of the isolates of the mentioned species were very similar (99.4 to 100% similarity). Figure 1 shows the neighbor-joining (NJ) tree inferred from the analysis of the ITS region of a representative number of isolates treated in this study, including the type and reference strains. The isolates from clinical origin that were included in the analysis clustered in six well-supported clades, each of them representing a different phylogenetic species. The largest clade, comprising the ex-type strain of *B. spicifera* and 36 clinical isolates, received 88% of bootstrap support (BS). The sequences of the isolates of this clade deviated maximally by 0.6% from that of the ex-type strain of *B. spicifera* (MUCL 3017). Isolates were characterized by brown, gray, or black colonies and 3-distoseptate cylindrical conidia with rounded ends, measuring 13 to 41 by 7 to 14 μm, without a protuberant hilum that were medium brown, except for a narrow hyaline to subhyaline area just above the conidial scar (Fig. 2A).

The ex-type strain of *B. australiensis* and two clinical isolates, morphologically identified as this species, constituted another clade (BS, 77%). The similarity of the sequences of such isolates with the ex-type strain was 98.1%. They showed gray to blackish brown, velvety colonies and pale brown to medium reddish brown, 3-distoseptate, ellipsoidal or oblong, straight conidia with rounded ends, measuring 15 to 32 by 7 to 11 μm, with a nonprotuberant hilum and smooth to finely roughened walls (Fig. 2B).

The clade corresponding to *B. hawaiiensis* (BS, 64%) encompassed 19 clinical isolates in addition to the ex-type strain with which it showed 99.1 to 100% similarity. The species was characterized by pale to medium brown, 2- to 6-distoseptate, oblong, cylindrical, or ellipsoidal conidia, measuring 13 to 37 by 4 to 11 μm, with a nonprotuberant hilum (Fig. 2C).

Three reference strains of *B. micropus* were grouped with three clinical isolates that had been morphologically identified as this species (BS, 96%). These isolates produced conidia that were pale brown, 3- to 7-distoseptate, cylindrical, and straight, measuring 19 to 47 by 10 to 15 μm, with smooth walls and a short protuberant hilum (Fig. 2D).

Isolate UTHSC 05-3211 showed 99% similarity with a reference strain of *B. setariae* (CBS 141.31). The clinical isolate showed pale brown, 3- to 9-distoseptate conidia that were cylindrical and straight to slightly curved, measuring 37 to 92 by 11 to 16 μm, with a nonprotuberant hilum (Fig. 2E). These conidial features were similar to those described for *B. sacchari* and *B. setariae* (30). However, no type strains of those species are known to exist and, in addition, our isolate only showed a similarity of 95.5% with a reference strain of *B. sacchari* (CBS 324.64).

Nine clinical isolates were grouped with four reference strains of *B. cynodontis* (BS, 97%). The sequence similarity among the isolates of this clade was 99.4 to 100%. However, important morphological differences were observed among them. Most showed conidial features typical of *B. cynodontis*; i.e., conidia with 3 to 8 distosepta, narrowly ellipsoidal to oval, irregularly curved, without a protuberant hilum, pale brown, and smooth-walled, measuring 19 to 61 by 8 to 15 μm (Fig. 2E). In contrast, two isolates (UTHSC 07-557 and UTHSC 07-3862) showed considerably shorter (up to 41-μm-long) conidia with fewer septa (from 3 to 6).

The majority of the isolates included in this study were from the nasal region (30.7%), followed by various cutaneous presentations (19.2%) and the lungs (14.4%), eyes (12.5%), nails (7.6%), and blood (4.8%). The remaining 10.8% of the isolates were from other sites like the ear, the brain, bile, and the chest, among others.

With the exception of fluconazole and flucytosine, the antifungal drugs tested showed good activity against all the isolates tested (Table 1). The MICs of the three drugs most commonly tested in these types of infections, i.e., amphotericin B, voriconazole, and itraconazole, were <0.03 to 2, 0.05 to 2, and <0.03 to 4 μg/ml, respectively.

DISCUSSION

This is the first study where a large panel of clinical isolates of *Bipolaris* has been identified down to the species level using both phenotypic and molecular methods. Although it seems that *Bipolaris* is an uncommon fungus involved in human infections, the fact that more than 250 clinical isolates belonging to that genus were received at the Fungus Testing Laboratory over a period of 5 years demonstrates that its low incidence is only relative. Close to 100 of such isolates were included in the present study. However, it should be mentioned that the role of these isolates as a cause of infection was not demonstrated.

A recent retrospective study, performed in Saudi Arabia (10), recorded a total of 23 clinical isolates of *Bipolaris* from different anatomical sites, collected over a period of 2 years. Although they were identified only morphologically, the most frequent species were *B. spicifera*, *B. australiensis*, and *B. hawaiiensis*, a finding which, in general, correlates with the prevalent etiologic agents in human infections reported in the literature (5, 15, 17, 19, 21, 23). In our study, *B. spicifera* and *B. hawaiiensis* were also the most

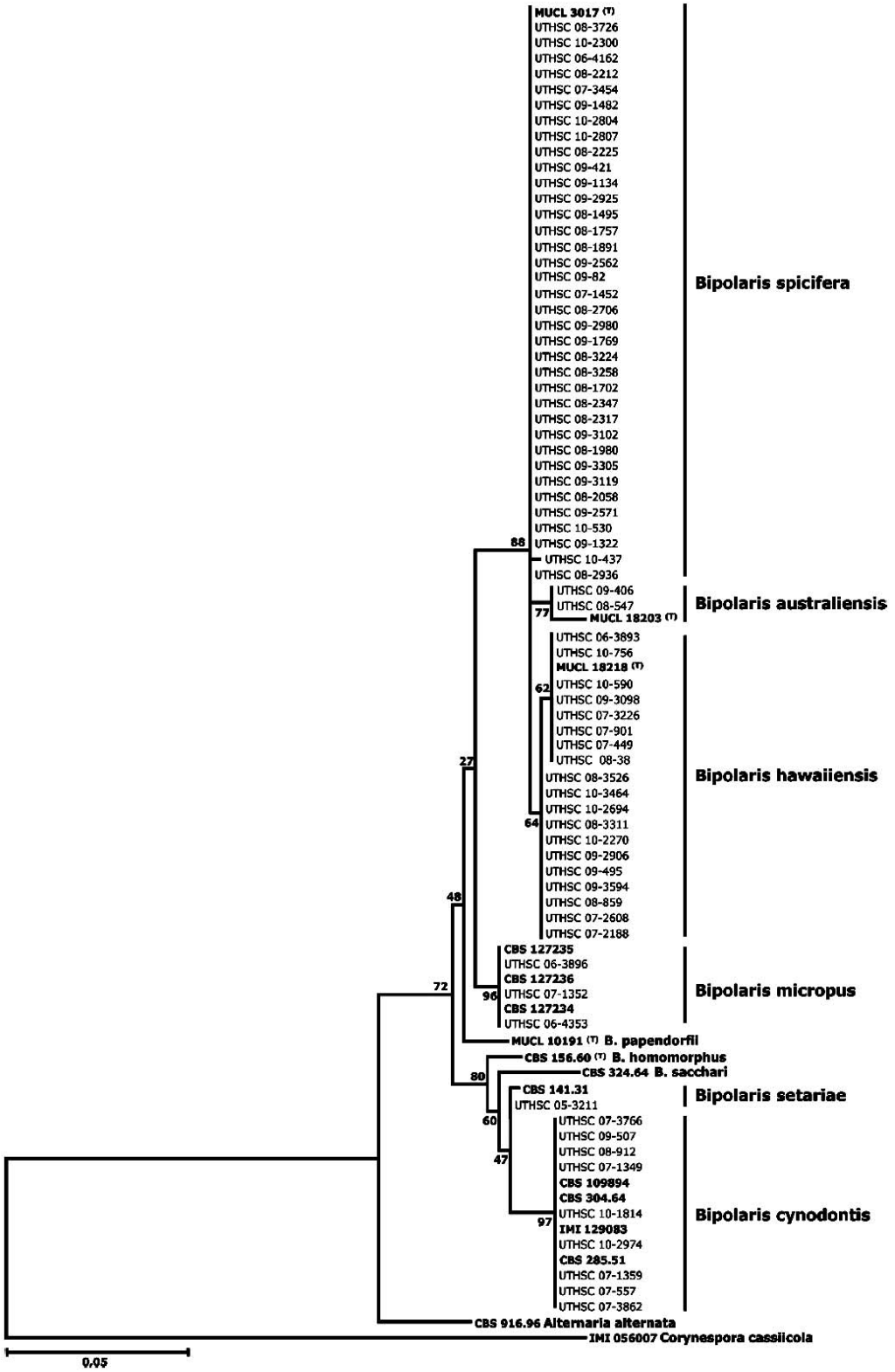

FIG 1 Maximum likelihood tree inferred from ITS sequences of *Bipolaris* listed in Table S1 in the supplemental material. Branch lengths are proportional to the distance. Type or reference strains are shown in bold.

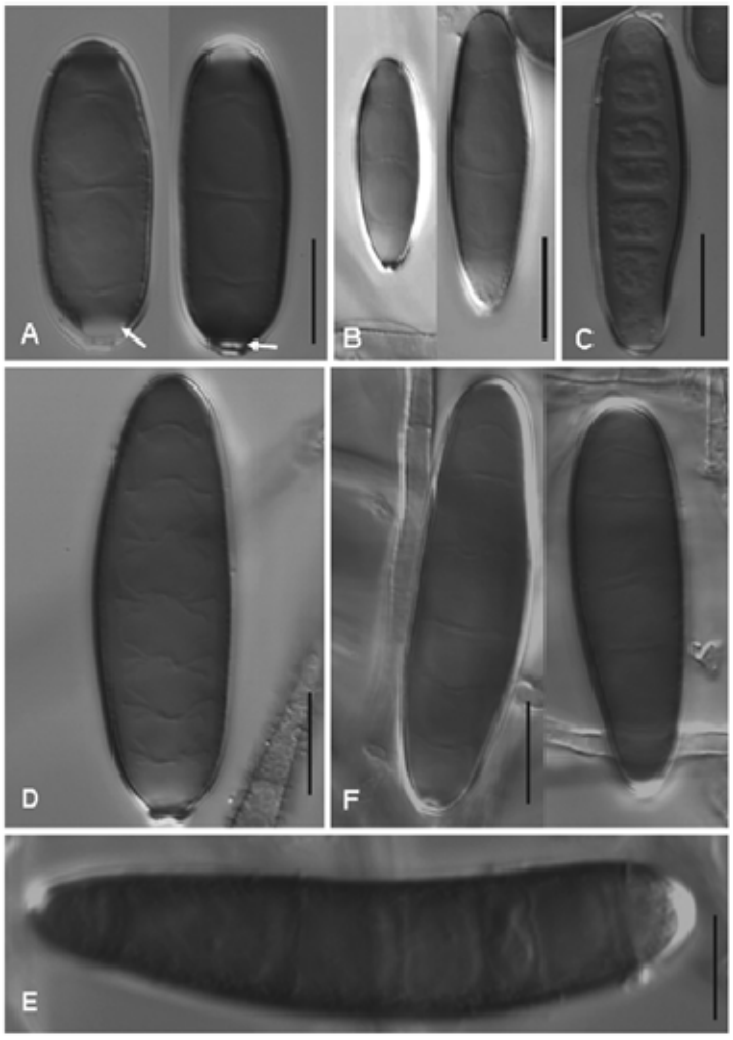

FIG 2 Conidia of *Bipolaris* species on OA at 25°C after 10 days. (A) *B. spicifera*, UTHSC 08-1706; (B) *B. australiensis*, UTHSC 09-406; (C) *B. hawaiiensis*, UTHSC 09-2906; (D) *B. micropus*, UTHSC 06-3896; (E) *B. setariae*, UTHSC05-3211; and (F) *B. cynodontis*, UTHSC 09-507. Scale bars, 10 μm.

commonly identified species, but, surprisingly, *B. australiensis* was represented only by two isolates. The fact that *B. spicifera* and *B. australiensis* are morphologically similar, especially when they grow *in vitro*, may have been a cause of misidentification in many cases. *Bipolaris spicifera* differs from *B. australiensis* by its 3-distoseptate conidia, which show a pale area at the base, just above the scar, while those of *B. australiensis* are usually 3- to 4(−5)-distoseptate, and the pale basal area is absent (27). The ITS sequences of the type strains of both species showed a similarity of 97.8%. *Bipolaris hawaiiensis* is morphologically distinguished from *B. spicifera* and *B. australiensis* by its 5(−7)-distoseptate narrow conidia (up to 7 μm wide), while those of *B. australiensis* and *B. spicifera* are 6 to 11 μm and 9 to 14 μm wide, respectively (27). The ITS sequence of the type strain of *B. hawaiiensis* showed similarities of 93.7 and 94.8% with those of the type strains of *B. australiensis* and *B. spicifera*, respectively. A BLAST search comparing ITS sequences is the procedure usually used in recent years to confirm *Bipolaris* species identification (3, 6, 9, 16, 18). However, this procedure may not be useful when comparisons are made with inaccurate sequences or when sequences of authentic strains of the species to which the problem isolate belongs have not been deposited.

Interestingly, we identified three species, *B. cynodontis*, *B. micropus*, and *B. setariae*, which, up to now, never had been found in clinical samples. In contrast, *Bipolaris papendorfii*, reported in three clinical cases (8), was not found in this study.

In our study, the predominant anatomic sites where strains were isolated were the nasal region, the eye, and the skin, a finding which agrees with the clinical cases of *Bipolaris* infections reported in the literature (5, 10, 15, 17, 19, 21, 23, 26).

The remarkably high *in vitro* activity of most of the antifungal drugs tested against *Bipolaris* species agrees with previous studies

TABLE 1 Results of *in vitro* antifungal susceptibility testing for *Bipolaris* species

Species (no. of isolates)	Antifungal agent	MIC (μg/ml)		
		Range	GM[a]	90%
B. australiensis (2)	Anidulafungin	<0.015–0.06	0.06	
	Amphotericin B	0.06–0.125	0.08	
	Caspofungin	1	1	
	Itraconazole	0.25–0.5	0.35	
	Fluconazole	8–16	11.3	
	Flucytosine	>64	>64	
	Micafungin	<0.015–0.06	0.06	
	Posaconazole	0.06	0.06	
	Voriconazole	0.05–1	0.70	
B. cynodontis (6)	Anidulafungin	0.03–0.06	0.04	
	Amphotericin B	<0.03–1	0.25	
	Caspofungin	<0.015–1	0.21	
	Itraconazole	<0.03–1	0.24	
	Fluconazole	2–16	4	
	Flucytosine	32–>64	>64	
	Micafungin	<0.015–0.03	<0.015	
	Posaconazole	<0.03–0.125	0.08	
	Voriconazole	0.25–1	0.39	
B. hawaiiensis (14)	Anidulafungin	<0.015–>8	0.07	0.125
	Amphotericin B	0.125–0.25	0.18	0.25
	Caspofungin	0.5–1	0.90	1
	Itraconazole	<0.03–0.5	0.32	0.5
	Fluconazole	2–32	8	16
	Flucytosine	>64	>64	>64
	Micafungin	<0.015–0.06	0.04	0.06
	Posaconazole	<0.03–0.5	0.14	0.25
	Voriconazole	0.25–2	0.67	1
B. micropus (3)	Anidulafungin	<0.015–0.125	0.015	
	Amphotericin B	0.06–0.25	0.09	
	Caspofungin	0.25–0.5	0.39	
	Itraconazole	<0.03–0.25	0.25	
	Fluconazole	0.125–1	0.5	
	Flucytosine	>64	>64	
	Micafungin	<0.015–0.06	<0.015	
	Posaconazole	<0.03	<0.03	
	Voriconazole	0.125–0.5	0.25	
B. spicifera (52)	Anidulafungin	<0.015–>8	0.06	0.25
	Amphotericin B	<0.03–2	0.21	1
	Caspofungin	0.25–2	0.89	1
	Itraconazole	<0.03–4	0.63	1
	Fluconazole	4->64	38.7	>64
	Flucytosine	>64	>64	>64
	Micafungin	<0.015–0.125	0.05	0.125
	Posaconazole	<0.03–1	0.26	0.5
	Voriconazole	0.25–4	1.56	2

[a] GM, geometric mean.

(13, 14, 24, 25). Owing to the infrequent occurrence of *Bipolaris* infections, clinical experience on their treatment is very scarce. In the reported clinical case studies, different antifungal drugs, such as amphotericin B, itraconazole, and voriconazole, have been used, with variable results (6, 15, 17, 22, 26, 31).

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Supplemental material

TABLE Clinical isolates, type or reference strains of *Bipolaris* spp. included in the study.

Species	Isolate	Origin	GenBank accession no. ITS
B. australiensis	**MUCL 18203$^{(T)}$**	Seed of *Oryza sativa*, Vietnam	JN601026.1[a]
	UTHSC 08-547	Foot nail, Texas	HE792906
	UTHSC 09-406	Middle ear, Texas	HE792907
B. cynodontis	**CBS 285.51**	*Cynodon transvaalensis*, Kenya	HE792937
	CBS 109894	*Cynodon dactylon*, Hungary	HE792938
	CBS 304.64	*Cynodon dactylon*, USA	HE792939
	IMI 129083	*Cynodon dactylon*, Australia	HE792940
	UTHSC 07-3766	BAL, California	HE792922
	UTHSC 07-3862	Leg wound, Texas	HE792930
	UTHSC 07-557	Nasal sinus, Minnesota	HE792929
	UTHSC 07-1359	Nail, Florida	HE792927
	UTHSC 07-1349	Toe nail, Florida	HE792924
	UTHSC 08-912	Sputum, Minnesota	HE792926
	UTHSC 09-507	Cornea, Utah	HE792928
	UTHSC 10-2974	Nasal sinus, Minnesota	HE792925
	UTHSC 10-1814	Abscess, Colorado	HE792923
B. hawaiiensis	**MUCL 18218 $^{(T)}$**	*Oryza sativa*, Hawaii	JN601029.1 [a]
	UTHSC 06-3893	Blood, Arkansas	HE792915
	UTHSC 07-449	BAL, Florida	
	UTHSC 07-3226	Lung, Hawaii	
	UTHSC 07-2608	Nasal sinus, Utah	
	UTHSC 07-2188	Nail, South Carolina	
	UTHSC 07-901	Blood, Ohio	HE792911
	UTHSC 08-3311	Nasal tissue, Texas	

Species	Isolate	Origin	GenBank accession no. ITS
B. hawaiiensis	UTHSC 08-3526	Peritoneal fluid, Texas	HE792917
	UTHSC 08-859	BAL, Texas	HE792916
	UTHSC 08-38	Nasal sinus, Texas	HE792912
	UTHSC 09-3098	BAL, Arizona	
	UTHSC 09-2906	Burn wound tissue, Texas	
	UTHSC 09-495	BAL, Texas	
	UTHSC 09-3594	Left shoulder, Texas	
	UTHSC 10-756	Nasal sinus, Arizona	HE792914
	UTHSC 10-590	Peritoneal fluid, Minnesota	HE792913
	UTHSC 10-3464	Brain, Florida	HE792909
	UTHSC 10-2694	Nasal sinus, Minnesota	HE792908
	UTHSC 10-2270	Nasal sinus, Texas	HE792910
B. homomorphus	**CBS 156.60 (T)**	Air, Kansas	JN192380.1 [a]
B. micropus	**CBS 127235**		HE792934
	CBS 127234		HE792933
	CBS 127236		HE792935
	UTHSC 06-3896	Toe, Flori	HE792918
	UTHSC 06-4353	Toe nail, South Carolina	HE792919
	UTHSC 07-1352	Toe nail, Florida	HE792920
B. papendorfii	**MUCL 10191 (T)**	*Acacia karroo*, South Africa	HE792931
B. sacchari	**CBS 324.64**	*Saccharum officinarum*	HE792932
B. spicifera	**MUCL 3017 (T)**	Soil, Spain	JN192387.1 [a]
	UTHSC 06-4162	BAL - Lung Tx, Texas	
	UTHSC 06-4551	Sinus aplastic anemia, Ohio	
	UTHSC 06-3737	Nasal sinus, California	
	UTHSC 06-3277	Skin foot, Utah	
	UTHSC 06-3088	Toe nail, Florida	
	UTHSC 06-3056	Maxillary sinus tissue, Missouri	

Species	Isolate	Origin	GenBank accession no. ITS
B. spicifera	UTHSC 06-3229	Sternal (wound), Indiana	
	UTHSC 06-4011	Peritoneal fluid, Texas	
	UTHSC 07-1452	Bile, Tennessee	
	UTHSC 07-3454	Eye, Minnesota	
	UTHSC 07-3282	BAL, Maryland	
	UTHSC 07-3140	BAL, Ohio	
	UTHSC 07-1723	Nasal tissue, Texas	
	UTHSC 07-2844	Ear, Ohio	
	UTHSC 07-2843	Knee, Ohio	
	UTHSC 07-2159	Nasal sinus, Texas	
	UTHSC 07-2048	Cornea, Arizona	
	UTHSC 07-1687	Nasal sinus, Arizona	
	UTHSC 07-1822	Sputum, Utah	
	UTHSC 08-2706	Skin, Colorado	
	UTHSC 08-2347	Foot tissue, Utah	HE792901
	UTHSC 08-2317	Cornea, Pennsylvania	
	UTHSC 08-2225	Maxillary sinus, Tennessee	HE792900
	UTHSC 08-2936	Thigh, Washington DC	
	UTHSC 08-2212	Nasal sinus tissue, Texas	
	UTHSC 08-2058	Nail, South Carolina	
	UTHSC 08-1980	Leg, Texas	
	UTHSC 08-1891	BAL, South Carolina	
	UTHSC 08-1757	Maxillary sinus, North Carolina	HE792905
	UTHSC 08-1702	Eye, Texas	HE792902
	UTHSC 08-1495	Left knee, Arizona	
	UTHSC 08-1479	Maxillary sinus, Utah	
	UTHSC 08-533	Blood, Arizona	HE792904
	UTHSC 08-3726	Tissue - Mediastinal, Texas	

Species	Isolate	Origin	GenBank accession no. ITS
B. spicifera	UTHSC 08-3258	Ankle, Nebraska	
	UTHSC 08-3224	Left thigh hematoma, Colorado	HE792899
	UTHSC 09-1134	Eye, Arizona	
	UTHSC 09-1769	Nasal sinus, Utah	
	UTHSC 09-3305	Eye, Minnesota	
	UTHSC 09-2925	Cornea, Arizona	
	UTHSC 09-2571	Blood, Texas	
	UTHSC 09-3119	Nasal sinus, Utah	
	UTHSC 09-421	Hand wound cellulitis, Texas	
	UTHSC 09-3449	Maxillary sinus, Texas	
	UTHSC 09-3102	Maxillary sinus, Oklahoma	
	UTHSC 09-2980	Sputum, Arizona	HE792903
	UTHSC 09-2562	Blood stem cell Tx, California	
	UTHSC 09-82	Pansinusitis, Texas	
	UTHSC 09-1482	Eye vitreous fluid, Texas	
	UTHSC 09-1322	Brain, Massachusetts	
	UTHSC 10-1784	Hand, Arizona	
	UTHSC 10-1657	Nasal sinus, Arizona	
	UTHSC 10-250	Maxillary sinus, Tennessee	
	UTHSC 10-2008	Sphenoid sinus, Arizona	
	UTHSC 10-908	Leg wound, Florida	
	UTHSC 10-2655	Chest, Texas	
	UTHSC 10-1565	Eye, Texas	
	UTHSC 10-1586	Cornea, Texas	
	UTHSC 10-437	Maxillary sinus, California	
	UTHSC 10-530	Maxillary sinus, Arizona	
	UTHSC 10-640	BAL, Arizona	
	UTHSC 10-3055	Eye, Texas	

Species	Isolate	Origin	GenBank accession no. ITS
B. spicifera	UTHSC 10-3335	BAL, Arizona	
	UTHSC 10-3145	Membrane, Texas	
	UTHSC 10-3143	Nasal sinus, South Carolina	
	UTHSC 10-2804	Nasal sinus, Texas	HE792897
	UTHSC 10-2488	BAL, Arizona	
	UTHSC 10-2807	Mastoid sinus, Oklahoma	HE792896
	UTHSC 10-2300	Nasal sinus, Ohio	HE792898
	UTHSC 11-60	Tissue - Canine, Arizona	
B. setariae	**CBS 141.31**		HE792936
	UTHSC 05-3211	Eye, Michigan	HE792921

[a] Sequences retrieved from Genbank; BAL, Bronchoalveolar lavage; Tx, transplant; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; IMI, International Mycological Institute, Surrey, England; MUCL, Mycothèque de L'Université Catholique de Louvain, Faculté des Sciences Agronomiques, Louvain-la-Neuve, Belgium; UTHSC, Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, TX; [(T),] ex-type strain. Reference and ex-type strains are indicated in boldface.

UNIVERSITAT ROVIRA I VIRGILI
PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF CLINICAL ISOLATES OF BIPOLARIS, CURVULARIA, EXSEROHILUM AND PITHOMYCES
Keith Cassia Da Cunha
DL:T 1479-2014

4.2. *In vitro* antifungal susceptibility and molecular identity of 99 clinical isolates of the opportunistic fungal genus *Curvularia*.

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In vitro antifungal susceptibility and molecular identity of 99 clinical isolates of the opportunistic fungal genus *Curvularia*☆

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ABSTRACT

The in vitro antifungal susceptibility of a set of 99 clinical isolates of *Curvularia* was tested against 9 drugs using a reference microdilution method. The isolates had been identified previously to species level by comparing their ITS rDNA and glyceraldehyde-3-phosphate dehydrogenase gene sequences with those of reference strains. We were able to reliably identify 73.2% of the isolates, the most frequent species being *Curvularia aeria*, *Curvularia geniculata*/*Curvularia senegalensis*, *Curvularia lunata*, *Curvularia inaequalis*, *Curvularia verruculosa*, and *Curvularia borreriae*. Most of these isolates had been recovered from nasal sinus, which is generally considered one of the most frequent sites of infection by these fungi. In addition, at least 3 phylogenetic species that have not yet been formally described were detected. The most active drugs were the echinocandins, amphotericin B, and posaconazole, whereas voriconazole and itraconazole showed poor activity.

1. Introduction

The genus *Curvularia* comprises more than 80 species. Most of them are saprobes in soil or plant pathogens (Manamgoda et al., 2011; Manamgoda et al., 2012; Sivanesan, 1987), but some species have been reported to cause infections in humans and animals (de Hoog et al., 2000).

The identity of most of the clinical isolates of *Curvularia* is confusing. Most literature citations are usually short case reports, in which the *Curvularia* isolates had been identified only by phenotypic criteria. Considering the morphological similarity of *Curvularia* spp. and that differences between them are based on subtle characters that can vary depending on culture conditions, species identification may be incorrect, doubtful, or remain unresolved (Revankar and Sutton, 2010; Vermeire et al., 2010). Recent studies have shown that morphological identification does not correlate with molecular identification (Manamgoda et al., 2012; Yanagihara et al., 2010). In particular, clinical isolates morphologically identified as *Curvularia lunata*, the most commonly reported clinical species (de Hoog et al., 2000; Revankar and Sutton, 2010), were placed in different, unrelated phylogenetic clades (Yanagihara et al., 2010). Other species, such as *Curvularia brachyspora*, *Curvularia clavata*, *Curvularia geniculata*, *Curvularia inaequalis*, *Curvularia pallescens*, *Curvularia senegalensis*, and *Curvularia verruculosa*, have also been reported in clinical cases (de Hoog et al., 2000; Revankar and Sutton, 2010; Wilhelmus and Jones, 2001). These species have been associated with different types of infection, such as keratitis, sinusitis, cutaneous and subcutaneous infections, peritonitis, onychomycosis, endocarditis, endophthalmitis, cerebral phaeohyphomycosis, and allergic bronchopulmonary as well as disseminated disease (Alvarez et al., 2011; Bryan et al., 1993; Ehlers et al., 2011; Forster et al., 1975; Gupta et al., 2007; Moody et al., 2012; Mroueh and Spock, 1992; Sharma et al., 2011; Singh et al., 2008; Tessari et al., 2003; Varughese et al., 2011; Yau et al., 1994). *Curvularia* is able to infect both immunocompetent and immunosuppressed patients, mainly in tropical and subtropical areas. Cases have been reported mainly in India, the United States, Brazil, Japan, and Australia (Agrawal and Singh, 1995; Carter and Boudreaux, 2004; Guarro et al., 1999; Tanabe et al., 2010; Thew and Todd, 2008). However, as mentioned above, the real incidence of the different species of *Curvularia* in human infections is inconclusive. Furthermore, clinical data on the treatment of *Curvularia* infections are scarce, and the most appropriate therapies are unknown.

Species of *Curvularia* are traditionally characterized by dark mycelium, geniculate conidiophores with sympodial, tretic conidiogenous cells and elongated conidia. The conidia are smooth to tuberculate-walled, with several false septa (distosepta) and straight or curved due to an enlarged middle cell that is often more pigmented than the other cells. *Bipolaris*, *Drechslera*, and *Exserohilum* are

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Table 1
Clinical isolates, type, and reference strains of *Curvularia* spp. included in the study.

Species	Strain	Origin	GenBank no.	
			ITS	GPDH
C. aeria	CBS 294.61 (T) (as *C. lunata* var. *aeria*)	Air, Brazil	HE861850	HF565450
	CBS 337.64 (as *C. lunata* var. *lunata*)	*Agropyron repens*, USA		
	CBS 533.70 (as *C. lunata* var. *aeria*)	Seed, *Pennisetum*, Denmark		
	UTHSC 06-3039	Nasal sinus, Arizona		
	UTHSC 07-481	Ethmoid sinus, Arizona		
	UTHSC 07-2859	Leg, Florida	HE861852	HF565451
	UTHSC 07-3183	Nasal sinus, Minnesota		
	UTHSC 08-2	Maxillary sinus, South Carolina		
	UTHSC 08-1226	Maxillary sinus, Vermont		
	UTHSC 08-2586	Nasal sinus, Florida		
	UTHSC 08-2625	Nasal sinus, South Carolina		
	UTHSC 08-2923	Nasal sinus, South Carolina		
	UTHSC 08-3003	Blood, Texas		
	UTHSC 08-3299	Nasal sinus, Washington		
	UTHSC 08-3398	Bronchial wash, Texas		
	UTHSC 08-3490	Frontal sinus, Missouri		
	UTHSC 09-494	Nasal sinus, Texas		
	UTHSC 09-2019	Wound, South Carolina		
	UTHSC 09-2085	Nasal sinus, Minnesota	HE861844	HF565452
	UTHSC 09-2318	Nasal sinus, Texas	HE861851	HF565453
	UTHSC 09-2546	Middle turbinate, Arkansas		
	UTHSC 09-3123	Nasal sinus, South Carolina		
	UTHSC 09-3124	Nasal sinus, South Carolina	HE861843	HF565454
	UTHSC 10-370	Skin, Utah		
	UTHSC 10-543	Peritoneal dialysis fluid, South Carolina		
	UTHSC 10-816	Nasal sinus, Texas		
C. borreriae	CBS 859.73	Volcanic ash soil, Chile	HE861848	HF565455
	UTHSC 08-2957	Corneal ulcer, West Virginia	HE861846	HF565456
	UTHSC 08-3433	Nasal sinus, California	HE861845	HF565457
	UTHSC 09-2408	Sputum, Washington		
	UTHSC 09-3510	Peritoneal dialysis fluid, District of Columbia	HE861847	HF565458
C. cf. *clavata*	UTHSC 10-1041	Bronchial wash, Utah	HE861819	HF565460
C. geniculata/*C. senegalensis*	CBS 220.52	Discolored wood, triplex plank, Suriname		
	CBS 332.64	*Setaria italica*, USA		
	CBS 149.71 (as *C. senegalensis*)	Unknown, Nigeria		
	CBS 431.75 (as *C. senegalensis*)	Sorghum, Fiji		
	UTHSC 07-3111	Eye, Texas	HE861839	HF565461
	UTHSC 07-3044	Nasal sinus, South Carolina		
	UTHSC 07-3620	Skin scraping, Texas		
	UTHSC 07-3740	Toe nail, Minnesota		
	UTHSC 08-2979	Toe nail, Texas		
	UTHSC 08-3314	Bronchial wash, Minnesota	HE861841	HF565462
	UTHSC 08-3531	Eye, Louisiana		
	UTHSC 08-3728	Eye, Missouri		
	UTHSC 09-1824	Knee biopsy, Florida		
	UTHSC 09-2568	Leg, Texas		
	UTHSC 09-2592	Finger wound, Utah		
	UTHSC 09-2753	Corneal ulcer, Tennessee		
	UTHSC 09-3005	Bronchial wash, Tennessee	HE861840	HF565463
	UTHSC 09-3435	Ear, Florida		
C. cf. *inaequalis*	UTHSC 07-3495	Cerebrospinal fluid, Wisconsin		
	UTHSC 08-2346	Foot, Utah		
	UTHSC 08-2860	Ethmoid tissue, Utah	HE861821	HF565464
	UTHSC 08-3472	Nasal sinus, Utah	HE861822	HF565465
	UTHSC 08-3685	Arm, Utah		
	UTHSC 09-1077	Lung biopsy, Utah	HE861820	HF565466
C. intermedia	CBS 334.64	*Avena versicolor*, North Carolina	HE861853	HF565467
	UTHSC 08-1041	Nail, South Carolina	HE861854	HF565468
	UTHSC 09-3240	Tissue, Colorado	HE861855	HF565469
C. lunata	CBS 730.96 (NT)	Lung biopsy, Florida	JX256429[a]	JX256429[a]
	CBS 157.34	Unknown, Indonesia		
	UTHSC 07-3452	Toe nail, Florida		
	UTHSC 08-172	Toe nail, Florida		
	UTHSC 08-959	Nail, South Carolina		
	UTHSC 08-1381	Eye, North Carolina		
	UTHSC 09-31	Wound, Minnesota	HE861816	HF565470
	UTHSC 09-191	Toe nail, South Carolina		
	UTHSC 09-2114	Nail, South Carolina		
	UTHSC 09-2395	Ethmoid sinus, South Carolina	HE861818	HF565471
	UTHSC 09-2719	Wound, South Carolina	HE861817	HF565472
	UTHSC 10-145	Contact lens, Minnesota		
C. protuberata	CBS 376.65 (T)	Leaf, *Deschampsia flexuosa*, Edinburgh	HE861823	HF565473
	UTHSC 08-2588	Left tibia, Minnesota		
	UTHSC 08-2880	Leg, North Carolina	HE861825	HF565474

Table 1 (*continued*)

Species	Strain	Origin	GenBank no.	
			ITS	GPDH
	UTHSC 08-2880	Leg, North Carolina	HE861825	HF565474
	UTHSC 09-1969	Abscess—penis, South Carolina	HE861824	HF565475
C. pseudorobusta	UTHSC 08-3458	Nasal sinus, Texas	HE861838	HF565476
C. cf. sorghina	UTHSC 08-809	Sphenoid sinus, South Carolina	HE861826	HF565477
	UTHSC 08-3445	Wound, Minnesota		
	UTHSC 09-868	Nasal sinus, South Carolina	HE861827	HF565478
	UTHSC 09-3575	Leg wound, Texas	HE861828	HF565479
C. verruculosa	CBS 148.63 $^{(T)}$	*Typha* sp., India	HE861829	HF565480
	CBS 149.63	*Elaeis guineensis*, Nigeria		
	UTHSC 07-3093	Cornea, Texas	HE861830	HF565481
	UTHSC 08-827	Corneal ulcer, Texas	HE861831	HF565482
	UTHSC 09-2658	Inferior turbinate, Texas		
	UTHSC 09-2246	Ethmoid sinus, Texas		
	UTHSC 09-2471	Maxillary sinus, Tennessee		
	UTHSC 10-709	Wound, Minnesota		
Curvularia sp. I	CBS 144.63 (as *C. lunata* var. *lunata*)	Leaf, India		
	UTHSC 07-2791	Cornea, Utah		
	UTHSC 07-3105	Nasal sinus, Texas		
	UTHSC 07-3184	Nasal sinus, Arkansas		
	UTHSC 07-3581	Nail, Minnesota		
	UTHSC 08-849	Eye, Louisiana	HE861837	HF565483
	UTHSC 08-1296	Nail, Texas		
	UTHSC 08-2418	Bronchial wash, Texas		
	UTHSC 08-2517	Foot, Texas		
	UTHSC 08-2905	Chest, Utah	HE861836	HF565484
	UTHSC 08-3737	Bronchial wash, Texas		
	UTHSC 09-464	Cornea, Florida		
	UTHSC 09-1692	Nasal sinus, Ohio		
	UTHSC 09-2197	Nasal sinus, Minnesota	HE861835	HF565485
	UTHSC 09-2532	Nasopharynx, Texas		
	UTHSC 09-3403	Tissue, Texas		
Curvularia sp. II	UTHSC 07-2649	Toe tissue, Texas	HE861834	HF565486
	UTHSC 08-84	Nasal sinus, Utah		
	UTHSC 08-278	Peritoneal dialysis fluid, Ohio	HE861832	HF565487
	UTHSC 08-2697	Leg, Tennessee		
	UTHSC 08-3414	Ankle, Minnesota	HE861833	HF565488
	UTHSC 09-2907	Toe nail, Oklahoma		
	UTHSC 09-2806	Bone marrow, Virginia		
	UTHSC 09-2863	Bronchial wash, Texas		
	UTHSC 10-1276	Maxillary sinus (antrochoanal polyp), California		
Curvularia sp. III	UTHSC 07-2764	Toe nail, Montana		
	UTHSC 08-1283	Nasal sinus, Nevada		
	UTHSC 09-2092	Nasal sinus, California	HE861842	HF565459

UTHSC = Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, TX; CBS = CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands; $^{(T)}$ = type strain; $^{(NT)}$ = neotype strain.
[a] Sequences from Manamgoda et al. (2012).

morphologically similar genera, and they are differentiated mainly by their conidial morphology and teleomorph associations (Sivanesan, 1987). *Curvularia* and *Bipolaris* are associated to the teleomorph genus, *Cochliobolus* and *Exserohilum* to *Setosphaeria* (Ellis, 1971, 1976; Sivanesan, 1987). In a recent taxonomic re-evaluation of these genera based on a multigene analysis, Manamgoda et al. (2012) amended the concept of *Curvularia* and some clinically relevant species of *Bipolaris*, such as *Bipolaris australiensis*, *Bipolaris hawaiiensis*, and *Bipolaris spicifera*, were transferred to *Curvularia*.

In this study, we have reliably identified a large set of clinical isolates of *Curvularia* from the USA, comparing the sequences of 2 DNA regions (internal transcribed spacer [ITS] region of the rDNA and glyceraldehyde-3-phosphate dehydrogenase [GPDH] gene) with those of reference strains in order to assess the real spectrum of *Curvularia* spp. in clinical samples, and determined their susceptibility to the available antifungal drugs.

2. Materials and methods

2.1. Fungal isolates

A total of 101 clinical isolates morphologically identified as *Curvularia* were included in the study. These isolates were randomly chosen from nearly 250 *Curvularia* isolates stored in the repository of the Fungus Testing Laboratory (FTL) at the University of Texas Health Science Center at San Antonio, which had been received over a period of 5 years (2006–2010). In addition, 15 type or reference strains of *Curvularia* spp. were also included in the study (Table 1).

2.2. Morphological study

All isolates were cultured on potato carrot agar (20 g of potatoes, 20 g of carrots, 20 g of agar, 1 L of distilled water) and oatmeal agar (30 g of filtered oat flakes, 20 g of agar, 1 L of distilled water) and incubated at 25 °C for 10–21 days in the dark. The identification criteria were according to Ellis (1971, 1976) and Sivanesan (1987). Microscopic features were examined on direct wet mounts with 85% lactic acid from the different culture media using light microscopy.

2.3. Molecular study

Isolates were grown on yeast extract sucrose (2% of yeast extract, 15% of sucrose, 2% of agar, 1 L of water) for 3 days at 25 °C, and DNA was extracted using a PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol. The DNA was quantified using GeneQuantpro

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(Amersham Pharmacia Biotech, Cambridge, United Kingdom). The 5.8S and flanking ITS regions and a fragment of the GPDH gene were amplified and sequenced with the primer pairs ITS5/ITS4 and gpd1/gpd2, respectively, following previously described protocols (Álvarez et al., 2010; Berbee et al., 1999). The ITS PCR products were purified and sequenced at Macrogen Europe (Amsterdam) with a 3739XL DNA analyzer (Applied Biosystems). The PCR and GPDH gene sequencing was carried out at the CBS-KNAW Fungal Biodiversity Centre (Utrecht, the Netherlands). This locus was sequenced using the BigDye terminator sequencing kit v. 3.1 (Applied Biosystems) and an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems). SeqMan (Lasergene, Madison, WI, USA) was used to obtain consensus from the complementary sequences of each isolate. BLAST sequence identity searches with ITS sequences were carried out to compare data from the isolates studied with those deposited in the GenBank database.

2.4. Phylogenetic analysis

Nucleotide sequences were aligned with Clustal X version 1.81 (Thompson et al., 1997), followed by manual adjustments with a text editor. Phylogenetic analyses were conducted using MEGA v. 5.05 with the maximum likelihood (ML) algorithm, using Kimura 2-parameter model with pairwise deletion of gaps (Tamura et al., 2011). The robustness of branches was assessed by bootstrap analyses of 1000 replicates. Two sequences retrieved from GenBank were used as outgroup in the combined analysis, one of *Exserohilum rostratum* ATCC32197 (ITS, AF071342.1; GPDH, AF081379.1) and another of *Alternaria alternata* EGS 34–016 (ITS, AF347031.1; GPDH, AF081400.1).

2.5. Antifungal susceptibility

Antifungal susceptibility was tested using methods outlined in CLSI document M38-A2 (CLSI, 2008). With the exception of 2 isolates that did not sporulate, all the *Curvularia* isolates were included in the antifungal susceptibility study. The drugs tested included anidulafungin (AFG), amphotericin B (AMB), caspofungin (CAS), itraconazole (ITC), fluconazole (FLC), voriconazole (VRC), micafungin (MFG), posaconzole (PSC), and 5-flucytosine (5-FC).

Testing involved standardizing the inoculum by spectrophotometer to 0.4–5 × 10^4 CFU/mL (0.25–0.3 OD at 530 nm), the use of RPMI 1640 with L-glutamine but without bicarbonate, and incubation at 35 °C. The MIC endpoint was 100% inhibition for AMB, ITC, VRC, and PSC and 80% inhibition for FLU and 5-FC. The minimum effective concentration (MEC) for the echinocandins was the lowest concentration where a visible change in the growth characteristics compared to the growth control was observed. Endpoint determinations were read at 48 hours. In the event that sufficient growth was not observed at the prescribed reading time, the tests continued to be incubated until sufficient growth was observed enabling accurate endpoint determination.

3. Results

With the primers used, we were able to amplify and sequence the ITS region and a fragment of the GPDH gene, and the length of the alignment used in the combined phylogenetic analysis was 886 bp (385 bp for ITS and 501 bp for GPDH). Fig. 1 shows the ML phylogenetic tree inferred from the analysis produced from combined ITS and GPDH sequences. The isolates were placed in 14 well-supported clades, which represented different phylogenetic species of *Curvularia*. Three of them did not include any type or reference species and did not match morphologically with any known species of *Curvularia*; they were therefore considered as putative new species, which we refer to here as *Curvularia* sp. I to III.

We were able to identify 72.3% of the isolates at species level, those being: *Curvularia aeria* (23 isolates), *C. geniculata*/*C. senegalensis* (14 isolates), *C. lunata* (10 isolates), *C. verruculosa* (6 isolates), *Curvularia protuberata* (3 isolates), *Curvularia intermedia* (2 isolates), *Curvularia borreriae* (4 isolates), *Curvularia pseudorobusta* (1 isolate), *C.* cf. *inaequalis* (6 isolates), *Curvularia* cf. *sorghina* (4 isolates) and *C.* cf. *clavata* (1 isolate). *Curvularia inaequalis*, *C. sorghina*, and *C. clavata* were identified only on the basis of morphological criteria and could not be confirmed molecularly because there were no reference strains or sequences available for comparison.

The clade representing *Curvularia* sp. I contained 15 clinical isolates and 1 strain from the CBS culture collection received as *C. lunata*. The members of this clade were morphologically compatible with *C. lunata*, showing conidia usually curved, obovoid, 3-4-distoseptate, with the third cell from the base curved and often larger and darker than the others, smooth to roughly warted, intermediate and end cells pale brown to brown and with smooth walls. They measured 16–30 µm long, 7–14 µm wide in the broadest part. However, the ex-type strain of *C. lunata* nested in a different clade in the present analysis. The similarity of the sequences of this clade was 98.1–100%.

The clade of *Curvularia* sp. II comprised 9 clinical isolates with identical sequences. They were characterized by straight to curved, broadly ellipsoidal conidia, usually with the third cell much larger than the others and unequally sided, predominantly 3-distoseptate, pale brown to brown, and with a smooth to slightly rugose basal cell, and measuring 13–28 µm long by 7–15 µm wide in the broadest part.

The clade representing *Curvularia* sp. III included 3 clinical isolates. They showed conidia curved, obovoid, 3-distoseptate, with one or more septa thicker and darker than the others, with the third cell from the base frequently larger and darker than the others, end cells usually pale brown, intermediate cells brown or dark brown, smooth, 11–25 µm long, 7–12 µm wide in the broadest part. These morphological features were similar to those observed in the isolates of the clade of *C. aeria*; however, the similarity was of only 95%.

The majority of the clinical isolates included in this study were from the nasal region (36.6%), followed by skin and nails (21.8%), eyes (10.8%), bronchial washings (6.9%), and peritoneal dialysis fluid (2.9 %).

The results of the susceptibility tests are shown in Table 2. AMB, MFG, and PSC were the most active drugs. AFG showed generally good activity, although 2 isolates of *C. lunata* showed high MICs (>8 µg/mL). The 3 echinocandins displayed different results, and although AFG and MFG showed very low MICs (geometric mean [GM] total of 0.7 and 0.2 µg/mL, respectively), those of CAS were considerable higher (GM 1 µg/mL). ITC and VRC showed high MICs for some species such as *C. aeria*, *C. borreriae*, *C. protuberata*, and *C. pseudorobusta*. Fluconazole and flucytosine were not active against any of the isolates tested.

4. Discussion

This study demonstrates that a wide spectrum of *Curvularia* spp. is represented in clinical samples. In addition, the high number of clinical isolates received by the FTL over a 5-year period highlights the possible importance of *Curvularia* in the clinical setting.

Most of the isolates included in the present study were morphologically identified as *C. lunata*; however, the phylogenetic analysis demonstrates that, in many cases, that identification was incorrect. According to Sivanesan (1987) and Ellis (1971), this species comprised 2 varieties: *C. lunata* var. *aeria*, which was characterized by smooth conidia and a presence of stromata in culture; and *C. lunata* var. *lunata* characterized by smooth to roughly warted conidia and an absence of stromata. Nakada *et al.* (1994), based on DNA RFLP, considered them as 2 different species, i.e., *C. lunata* and *C. aeria*; this is confirmed in our study, in which the type strains of both species are placed in very distant clades. There is also confusion in the taxonomy of *C. geniculata* and *C. senegalensis*, which have traditionally been described as 2 different, clinically relevant species (de Hoog et al.,

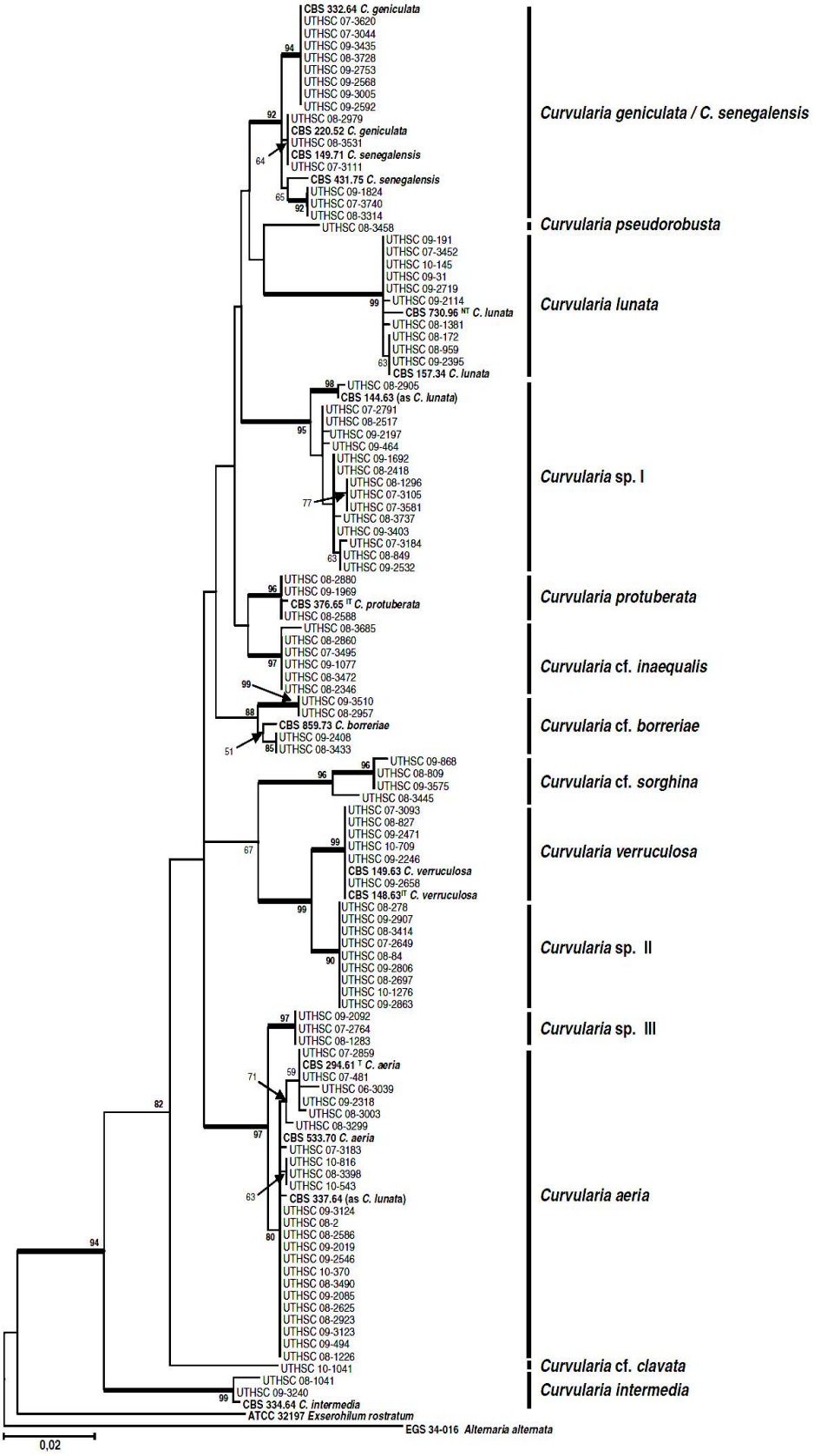

Fig. 1. ML tree inferred from combined ITS and GPDH gene sequences of the isolates listed in Table 1. Branch lengths are proportional to the distance. Bootstrap values of more than 50% are shown in the tree nodes. Branch with bootstrap support higher than 90% are indicated in bold. Ex-type ^{T} and reference strains are in bold.

Table 2
Results of in vitro antifungal susceptibility testing for *Curvularia* spp.

Species (no. of isolates)	MIC or MEC(μg/mL)														
	AFG			AMB			CAS			ITC			FLC		
	Range	GM	90%	Range	GM	90%	Range	GM	90%	Range	GM	90%	Range	GM	90%
C. aeria (23)	0.03–0.25	0.06	0.125	0.06–2	0.125	0.25	0.5–1	1	1	0.25 to >16	>16	>16	4–64	32	64
C. borreriae (3)	<0.015–0.06	0.03		0.06–0.25	0.15		0.5–1	0.79		0.5 to >16	4		8–16	10	
C. cf. clavata (1)	0.06			0.25			1			0.5			8		
C. geniculata/C. senegalensis (14)	<0.015 to >8	0.10	0.125	0.06–0.5	0.20	0.5	0.5–2	0.86	1	0.06–1	0.23	0.25	2–16	5.65	8
C. cf. inaequalis (5)	0.06–0.125	0.06		0.125–0.25	0.14		1	1		0.125–2	0.32		2–4	2.63	
C. intermedia (2)	0.03	0.03		0.5–4	1.41		0.5	0.5		0.125–0.25	0.17		2–8	4	
C. lunata (10)	<0.015 to >8	0.06	>8	0.125 to >16	0.3	0.5	0.5 to >8	1	1	0.125 to >16	0.25	0.25	2–64	3	8
C. protuberata (3)	0.03–0.06	0.03		0.25–0.5	0.31		0.5	0.5		>16	>16		64 to >64	>64	
C. pseudorobusta (1)	0.06			0.06			0.5			>16			64		
C. cf. sorghina (4)	0.03–0.5	0.10		0.125	0.125	0.125	0.5–1	0.84		<0.03–0.5	0.17		1–4	2.37	
C. verruculosa (6)	0.03–0.125	0.05		0.125–0.25	0.19	0.25	0.5–1	0.79		0.5–1	0.7		4–32	11.3	
Curvularia sp. I (15)	0.03 to >8	0.06	0.125	0.25–1	0.5	1	0.5–1	1	1	0.25–1	0.5	1	2–32	8	32
Curvularia sp. II (9)	0.03–0.125	0.05		0.06–0.25	0.19	0.25	0.5–1	0.62		0.125–2	0.42		2–16	5.03	
Curvularia sp. III (3)	0.06	0.06		0.125–0.25	0.25		1	1		0.125–1	0.125		2–16	4	
Total (99)	<0.015 to >8	0.7	0.125	0.06 to >16	0.6	0.5	0.5 to >8	1	1	<0.03 to >16	9	32	1 to >64	17	32

Species (no. of isolates)	MIC or MEC (μg/mL)											
	VRC			MFG			PSC			5-FC		
	Range	GM	90%	Range	GM	90%	Range	GM	90%	Range	GM	90%
C. aeria (23)	0.5–16	8	16	0.03–0.125	0.06	0.125	0.125–2	1	1	>64	>64	>64
C. borreriae (3)	1–4	2.51		<0.015–0.06	0.02		0.125–0.5	0.31		>64	>64	
C. cf. clavata (1)	0.5			0.06			0.25			>64		
C. geniculata/C. senegalensis (14)	0.125–4	0.67	1	<0.015–0.06	0.04	0.06	<0.03–0.5	0.10	0.25	64 to >64	>64	>64
C. cf. inaequalis (5)	0.5–2	0.75		0.03–0.125	0.06		<0.03–1	0.06		>64	>64	
C. intermedia (2)	0.125–0.5	0.25		0.06	0.06		0.06–0.125	0.08		>64	>64	
C. lunata (10)	0.25–1	0.5	1	0.015 to >8	0.03	0.06	<0.03–0.5	0.06	0.25	>64	>64	>64
C. protuberata (3)	8–16	10		<0.015–0.03	<0.015		0.5–1	0.79		>64	>64	
C. pseudorobusta (1)	>16			0.03			4			>64		
C. cf. sorghina (4)	0.25–2	0.70		0.015–0.06	0.04		0.06–0.025	0.12		>64	>64	
C. verruculosa (6)	0.5–2	1.12		0.015–0.125	0.06		0.06–1	0.22		>64	>64	
Curvularia sp. I (15)	0.5–2	1	2	<0.015–0.125	0.03	0.06	0.06–1	0.25	0.25	>64	>64	>64
Curvularia sp. II (9)	0.5–1	0.62		<0.015–0.03	0.02		<0.03–0.5	0.10		> 64	>64	
Curvularia sp. III (3)	0.5–1	0.5		0.06–0.125	0.06		<0.03–0.25	0.125		>64	>64	
Total (99)	0.125 to >16	3	8	<0.015 to >8	0.2	0.125	<0.03–4	0.4	1	64 to >64	>64	>64

GM, geometric mean; AFG, anidulafungin; AMB, amphotericin B; CAS, caspofungin; ITC, itraconazole; FLC, fluconazole; VRC, voriconazole; MFG, micafungin; PSC, posaconazole; 5-FC, flucytosine.

2000; Guarro et al., 1999). However, our study seems to confirm data published previously that indicates they could be conspecific (Hosokawa et al., 2003; Sun et al., 2003). Our study also demonstrated that several species, not previously identified and probably new, are also widely represented in clinical samples.

Although the BLAST search using ITS sequences is a useful tool for the identification of a variety of pathogenic fungi, it is not so for *Curvularia* because there are so few reference sequences deposited in GenBank. Isolate identification carried out in this study was made by comparing ex-type or reference strains from international culture collections and sequenced in our laboratory. However, even using this procedure only 72.3% of them could be confidently identified. This was due mainly to the fact that some isolates belonged to putative new species and that the taxonomy of the genus is not yet resolved. Although *Curvularia* spp. have considerable economic importance being plant pathogens, either in the production of secondary metabolites or as biological control agents, few molecular studies on the taxonomy of the genus have been carried out, and only a few species have been included (Berbee et al., 1999; Hosokawa et al., 2003; Manamgoda et al., 2011; Sun et al., 2003). In a recent phylogenetic study using a multigene analysis, Manamgoda et al. (2012) tried to clarify the taxonomy of *Curvularia*, redefining 20 species and concluding that there are still many species of *Curvularia* that need to be delimited.

As indicated above, *Curvularia* may cause a wide spectrum of human infections that affect various organs. This study agrees with several clinical studies that have reported the eyes, nasal region, and nails as the most common sites of *Curvularia* infections (Alvarez et al., 2011; Berbel et al., 2011; Ehlers et al., 2011; Gupta et al., 2007; Moody et al., 2012; Tessari et al., 2003; Varughese et al., 2011). It is worth mentioning that, in this study, most of the strains identified as *C. aeria* (73.9%) were isolated from the nasal region, whereas only 1 isolate of *C. lunata* and another of *C. geniculata/C. senegalensis* were found from that anatomical area.

In the reported clinical cases, different antifungal drugs such as AMB, natamycin, and azoles have been used, with variable results (Arora et al., 2011; Ehlers et al., 2011; Moody et al., 2012; Posteraro et al., 2010; Singh et al., 2008; Varughese et al., 2011). In allergic fungal sinusitis, treatment usually consists of surgery and administration of steroids and ITC or AMB, with good tolerance and favorable clinical outcomes (Alvarez et al., 2011; Revankar and Sutton, 2010). Allergic bronchopulmonary mycosis by these fungi is often treated with steroids and ITC (Revankar and Sutton, 2010). Cutaneous infections have been treated successfully with ITC, VRC, and ketoconazole (Moody et al., 2012; Vermeire et al., 2010). In ocular infections, the suggested therapy is natamycin and azoles, such as ITC, FLC, PSC, or VRC (Arora et al., 2011; Qiu et al., 2005; Revankar and Sutton, 2010). However, treatment failures of AMB in peritonitis (Pimentel et al., 2005; Varughese et al., 2011) and of AMB, VRC, and 5-FC in CNS and disseminated infections (Carter and Boudreaux, 2004; Singh et al., 2008; Tessari et al., 2003) have also been reported. The in vitro activity of the drugs tested here is similar to that reported in previous in vitro studies that tested a small number of *Curvularia* isolates (González, 2009; Guarro et al., 1999; Qiu et al., 2005; Unal et al., 2011) and also to

the results obtained in recent studies against *Exserohilum* and *Bipolaris*, 2 genera closely related to *Curvularia* (da Cunha et al., 2012a, 2012b). However, the general in vitro activity showed by VRC and ITC, 2 of the drugs most commonly used to treat infections by *Curvularia*, was considerably lower in the present study, with MICs 90 of 8 and 32 μg/mL, respectively.

The fact that several clinically relevant *Curvularia* spp. were unequivocally identified here mainly by comparing them with type strains and their sequences deposited in GenBank, we hope that will be of help in any future identification of clinical isolates. Similarly and for the same reason, the in vitro data provided here could be useful for guiding the therapy against human infections caused by these opportunistic fungi.

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4.3. Novel *Curvularia* species from clinical specimens.

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Novel *Curvularia* Species From Clinical Specimens

Running head: Novel Clinical *Curvularia* spp.

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ABSTRACT:

The fungal genus *Curvularia* includes numerous plant pathogens and some emerging opportunistic pathogens of humans. In a previous study (da Cunha et al. 2013. Diagn Microbiol Infect Dis 76:168–174) we used morphology and sequences of the nuclear ribosomal internal transcribed spacer region (ITS) and the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene to identify species within a set of 99 clinical *Curvularia* isolates from the USA. Seventy-two isolates could be identified while the remaining 27 isolates belonged in three unclassified clades that were tentatively labelled *Curvularia* sp. I, II and III. In the present study, we further assess the taxonomic placement of these isolates using sequences of ITS, *gpd*, the large subunit rDNA, and the second largest subunit of RNA polymerase II. DNA sequence comparisons with a set of 87 isolates representing 33 *Curvularia* spp. and members of the closely-related genera *Bipolaris* and *Exserohilum* revealed that *Curvularia* sp. I, II and III represent novel lineages in *Curvularia.* These lineages are morphologically different from the currently accepted species. In the phylogenetic tree, *Curvularia* sp. I and sp. III were each split into two distinct lineages. Morphology and phylogeny supported the proposal of five new species, to be named *C. americana*, *C. chlamydospora*, *C. hominis*, *C. muehlenbeckiae*, and *C. pseudolunata.* The concatenated 4-locus phylogeny revealed the existence of six clades in *Curvularia* which are associated with particular morphological features. They were named after representative species, namely *americana*, *eragrostidis*, *lunata*, *hominis*, *spicifera*, and *trifolii*.

Key words: *Curvularia*, *Bipolaris*, Pleosporales, phylogeny, taxonomy

INTRODUCTION

Curvularia, typified by *C. lunata*, is a species-rich genus which includes numerous grass pathogens and saprobes occurring on plant material, dung and soil (Faurel & Schotter 1965, Sivanesan 1987, Jiang & Zhang 2007). At least eight species of this genus have been reported from opportunistic diseases in humans ranging from mild skin and nail infections to severe invasive disease, depending on route of infection and immune status of the host (Kamalam et al. 1992, Ismail et al. 1993, Lopes & Jobim 1998, Ebright et al. 1999, de Hoog et al. 2000). Morphologically, *Curvularia* is characterized by the production of sympodial conidiophores with tretic, terminal and intercalary conidiogenous cells and elongate, transversely septate conidia with a dark basal scar. Conidia are often curved at an asymmetrically swollen intermediate cell, but species with straight conidia also have been described (Sivanesan 1987). Authors such as Ellis (1971, 1976), de Hoog et al. (2000) and Revankar & Sutton (2010) have described the conidia as truly septate or "euseptate", i.e. composed of a single wall with septa that are formed as inward extensions of that wall (Luttrell 1963). A similar genus is *Bipolaris*, type species *B. maydis*, which traditionally has been distinguished from *Curvularia* by producing conidia which lack an asymmetrically swollen intermediate cell and are "distoseptate" (Domsch et al. 2007, Revankar & Sutton 2010), i.e., they have a common outer wall enclosing more or less spherical cells, each of which is surrounded by an individual wall (Luttrell 1963). The separation of the two genera has been a matter of controversy and many authors have stated that *Curvularia* species also have distoseptate conidia (Alcorn 1983a, Sivanesan 1987, Seifert et al. 2011).

Sexual stages of *Bipolaris* and *Curvularia* were traditionally placed in *Cochliobolus.* Typically, they feature thick-walled, ostiolate ascomata with pseudoparaphyses, and bitunicate asci that give rise to filiform, multiseptate ascospores (Sivanesan 1987, Zhang et al. 2012). The ascospores often appear more or less helically coiled within the ascus A similar genus, *Pseudocochliobolus*, was segregated from *Cochliobolus* to accommodate species producing ascomata on columnar stromata, with ascospores appearing linearly parallel or loosely coiled within the asci. The asexual stages of *Pseudocochliobolus* species were *Curvularia* and *Bipolaris* species with short, rather straight conidia (Tsuda et al. 1977, Tsuda & Ueyama 1981). Most authors have not accepted *Pseudocochliobolus* as a separate genus because the degree of coiling of

the ascospores can vary greatly within a species. Also, the addition of a second genus with *Curvularia* and *Bipolaris* asexual stages would introduce unnecessary complexity into the taxonomy of this group of fungi instead of clarifying it (Alcorn 1983a, 1988; Sivanesan 1987).

Cochliobolus, Pseudocochliobolus and their *Bipolaris* and *Curvularia* asexual morphs were previously considered to be related either to the Dothideales (Eriksson 1981) or to the Pleosporales (Barr 1979, Sivanesan 1984). Molecular data confirmed their placement in the latter order and more precisely in its largest family, Pleosporaceae, along with other important genera of plant pathogens and clinically-relevant fungi such as *Alternaria* and *Exserohilum* (Olivier et al. 2000; Zhang et al. 2009, 2012). Berbee et al. (1999) performed a phylogenetic study to assess the evolutionary relationships of *Cochliobolus*, *Pseudocochliobolus*, *Curvularia* and *Bipolaris*. Their phylogenetic trees, based on the internal transcribed spacer (ITS) region of the rDNA and the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene, revealed that isolates were distributed mainly in two clades which were named "*Cochliobolus* groups 1 and 2". Group 1 exclusively encompassed species with *Bipolaris* asexual morphs, including the type species, *B. maydis*, agent of southern corn leaf blight, as well as other economically-relevant phytopathogenic species. The sexual morph of *B. maydis* is *Cochliobolus heterostrophus*, type species of *Cochliobolus* (Sivanesan 1987). Group 2 included mostly plant pathogens and saprobes with *Bipolaris* and *Curvularia* asexual morphs, including the type species of the latter genus, *C. lunata* and all species of *Pseudocochliobolus.*

Manamgoda et al. (2012), with a wider sampling of species and based on the analysis of ITS, large subunit (LSU) rDNA, *gpd* and elongation factor 1-α (EF1- α) genes, applied the one fungus = one name concept (Hawksworth et al. 2011) to the *Bipolaris-Curvularia Cochliobolus-Pseudocochliobolus* complex. Their phylogenies confirmed the existence of the same two main groups reported by Berbee et al. (1999). Based on those results, *Cochliobolus* and *Pseudocochliobolus* were synonymized with the more commonly used generic names *Bipolaris* and *Curvularia*, respectively, and the generic concept of the latter genus was expanded to accommodate some species with rather straight conidia formerly placed in *Bipolaris* but grouping in the *Curvularia* clade (Manamgoda et al. 2012). These included important agents of opportunistic infections

in vertebrates, such as *B. australiensis*, *B. hawaiiensis* and *B. spicifera* (de Hoog et al. 2000). The last of these had been previously considered a *Curvularia* species by Boedijn (1933).

Curvularia spp. have been identified mostly based on morphology, but the names applied often do not correlate with DNA sequence-based identifications. Furthermore, the species most commonly reported from humans, *C. lunata*, appeared to be a species complex (Berbee et al. 1999, Yanagihara et al. 2010). da Cunha et al. (2013) recently characterized a set of 99 clinical *Curvularia* strains from the USA using sequences of the ITS region and the *gpd* gene. They could identify 73.2% of the isolates, including *C. aeria,* which was the most common species. The remaining isolates were distributed over three different lineages which did not correlate with any known species. In this study we used DNA sequence data of four nuclear loci to further assess the taxonomic position of these isolates.

MATERIALS AND METHODS

Fungal Isolates

Twenty-seven clinical *Curvularia* isolates from the USA were studied (Table I). These isolates were obtained from the Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, and represent the clades named *Curvularia* sp. I, II and III in the study by da Cunha et al. (2013). These isolates were compared with ex-type or reference strains of different *Curvularia* spp. from the CBS Fungal Biodiversity Centre, Utrecht, The Netherlands.

Phenotypic Study

Colony morphology and growth rates were studied on potato carrot agar (PCA; 20 g of potatoes, 20 g of carrots, 20 g of agar, 1L of distilled water) and oatmeal agar (OA; 30 g of filtered oat flakes, 20 g of agar, 1 L of distilled water) after 7 days of incubation at 25 °C in the dark. Microscopic features were studied in lactic acid from colonies on the same media after 10–21 days of incubation. Size ranges in the species descriptions are derived from at least 30 measurements.

Cryo- Scanning Electron microscopy

Relevant areas of fungal cultures were carefully selected by means of a stereo microscope (Nikon SMZ1500, Nikon, Amsterdam, The Netherlands). Small (approx. 3 × 5 mm) agar blocks were carefully cut out with a surgical blade (no. 11, Swan-Morton, Sheffield, UK), while disturbing of fungal structures was kept to a minimum during cutting and transferring of the samples to a copper cup (diam 10 mm, height 8 mm). Agar blocks were glued to the copper cup with frozen tissue medium (KP-Cryoblock, Klinipath, Duiven, the Netherlands). The copper cup was placed on an agar surface inside a closed Petri-dish to prevent drying of the sample. The sample was quickly frozen in nitrogen slush and immediately transferred to a JEOL 5600LV scanning electron microscope (JEOL, Tokyo, Japan) equipped with an Oxford CT1500 cryostation. The sample was viewed at 2.5 kV and ice was removed by sublimation after heating of the SEM-stage to -85 ºC. Then the sample was sputter-coated in the cryo-station by means of a gold target for three times 90 s holding the sample at different angles for an optimal coating. Electron micrographs were acquired with the F3 or F4 scan at 5 kV and contrast levels digitally enhanced in Adobe® Photoshop® Creative Suite v. 6.

Molecular study

DNA extraction of *Curvularia* spp. I–III was performed with the PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA, USA) as described by da Cunha et al. (2013). DNA extraction of isolates of the other species studied was carried out from colonies growing on malt extract agar (MEA; Oxoid, Basingstoke, England) with the UltraClean® Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA). Amplification and sequencing of the ITS and RNA polymerase II second largest subunit (*RPB*2) were performed with primers ITS5 + ITS4 (White et al. 1990) and 5F2 + 7cR (O'Donnell et al. 2007) following the protocols of Amaradasa et al. (2014). Amplification of the *gpd* and LSU genes were performed with primers gpd1 + gpd2 (Berbee et al. 1999) and LR0R + LR5 (Vilgalys & Hester 1990) as described in Manamgoda et al. (2012). The ITS PCR products were purified and sequenced at Macrogen Europe (Amsterdam) using a 3739 XL DNA analyzer (Applied Biosystems). The *gpd*, LSU and *RPB*2 loci were sequenced at the CBS-KNAW Fungal

Biodiversity Centre (Utrecht, The Netherlands), using the BigDye terminator sequencing kit v. 3.1 (Applied Biosystems) and an ABI Prism™ 3100 DNA sequencer (Applied Biosystems). The program SeqMan Pro (Lasergene, Madison, Wisconsin) was used to obtain consensus sequences from the complementary sequences of each isolate. Sequences of the clinical isolates were aligned with those of a set of 60 isolates representing 33 species of *Curvularia*, and two phylogenetically related genera of Pleosporaceae, i.e. *Bipolaris* (nine spp.) and *Exserohilum* (one sp., used as outgroup) using ClustalX v. 1.81 (Thompson et al. 1997), followed by manual adjustments with a text editor. Individual alignments of ITS, LSU, *gpd* and *RPB2* and a concatenated four-locus dataset were analyzed with maximum likelihood (ML) using MEGA5 (Tamura et al. 2011) with partial deletion of gaps, substitution models proposed by this program and 1000 bootstrap replicates. Bootstrap support values (bs) ≥ 70% were considered significant. Incongruence among datasets was tested by a visual inspection of all groups with ≥ 70 bs in the partial trees to search for potentially conflicting groups. A Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes v. 3.1.1 (Ronquist & Huelsenbeck 2003). The best models of nucleotide substitution for each locus for the Bayesian analysis were determined using MrModeltest v. 2.3 (Nylander 2004). Two analyses of four MCMC chains were run from random trees for 4598100 generations and sampled every 100 generations, resulting in 45981 trees, of which 25% were discarded as the burn-in phase. Posterior probabilities (pp) were determined from the remaining trees. The sequences generated during this study and the alignments used in the phylogenetic analyses were deposited in GenBank (Table I) and TreeBASE (Submission ID http://purl.org/phylo/treebase/phylows/study/TB2:S14881), respectively.

RESULTS

Phylogenetic study

After removing ambiguously aligned regions, we obtained ITS, LSU, *gpd* and *RPB2* alignments of 533, 830, 434, and 793 positions of which 64 (12%), 39 (4.69%), 111 (25.57%), and 259 (32.66%) were variable, respectively. MEGA5 proposed a K2 + G + I model for the ITS and *RPB2* loci, K2 + I for LSU, T92 + G for *gpd* and GTR + G + I for the concatenated four-locus dataset. These models were used in the ML analyses.

Partial trees (not shown) were congruent except for the following clades: *Curvularia gladioli* CBS 210.79 grouped with *C. ischaemi* CBS 630.82 (93% bs) in the ITS tree, but in the *RPB2* tree the former isolate grouped with *Curvularia trifolii* CBS 173.55 (77% bs), while the CBS 630.82 grouped with *Curvularia coicis* CBS 192.29 (100% bs). These incongruencies affected species which are not closely related to *Curvularia* sp. I–III of da Cunha et al. (2013) and therefore the four loci were combined. Partial trees revealed that *RPB2* was the most informative locus with 35 clades with significant bs, followed by *gpd* with 23. ITS and LSU both showed only 10 clades with significant bs. The ITS and LSU ML trees provided good support for a clade representing the genus *Bipolaris*, but *Curvularia* species appeared in several clades, some of which had low bootstrap support. The *gpd* ML tree separated *Bipolaris* and *Curvularia* as two clades with 93% and 70% bs, respectively, whereas these clades showed 99% and 95% bs in the *RPB2* tree. In the concatenated four-locus ML tree (not shown) the *Bipolaris* and *Curvularia* clades had 100% and 97% bs, respectively. For Bayesian analysis, MrModeltest proposed a SYM + I + G model for the ITS locus and GTR + I + G for LSU, *gpd* and *RPB2*. These models were incorporated in the analysis. The consensus tree obtained from the Bayesian analysis (Fig. 1) agreed with the topology of the ML tree (not shown) for the four-locus dataset.

The four-locus tree (Fig. 1) revealed that *C. carica-papayae*, listed as a synonym of *C. aeria* by Sivanesan (1987), is a phylogenetically distinct species. The concatenated tree also corroborated that isolates in *Curvularia* spp. I–III of da Cunha et al. (2013) are different from accepted species of this genus represented in the CBS collection (Fig. 1). However, *Curvularia* spp. I and III were each split into two lineages that are sufficiently distant from each other to represent different species. These lineages were named here Ia, Ib and IIIa, IIIb, accordingly. One of them, Ib, shows considerable genetic variation, but is treated here as a single taxon because its complex topology does not seem to suggest a clear separation of species within it. The ITS and LSU ML trees did not provide enough resolution to separate lineages within *Curvularia* spp. I and III, but showed 87% and 99% bs for *Curvularia* sp. II. The *gpd* ML tree gave 80% bs to *Curvularia* sp. II and separated lineages Ia (98% bs) and Ib (52% bs) of *Curvularia* sp. I, but did not separate the two lineages of *Curvularia* sp. III. The *RPB2* ML tree gave 100% bs to *Curvularia* sp. II and provided enough resolution to separate

lineages Ia, Ib, IIIa and IIIb with bs ≥ 75%. *Curvularia* sp. Ia, Ib, II, IIIa and IIIb are morphologically and phylogenetically different from other members of the genus and therefore are proposed here as new taxa. These species were respectively named *C. muehlenbeckiae*, *C. hominis*, *C. americana*, *C. chlamydospora*, and *C. pseudolunata* and described in alphabetical order in the Taxonomy section.

Within the *Curvularia* clade, six well-supported lineages were associated with certain combinations of morphological features. These lineages were named the *americana*-, *eragrostidis*-, *hominis*-, *lunata*-, *spicifera*- and *trifolii*-clades (Fig. 1). The *eragrostidis*-clade (82% bs, 1 pp) was formed by *C. eragrostidis*, *C. graminicola* and *C. intermedia,* and is characterized by producing inconspicuously distoseptate (i.e., the two cell wall layers within the conidium are difficult to distinguish in mature conidia), straight to somewhat unequal-sided, 4-celled conidia. In mature conidia of *C. graminicola*, all septa are accentuated by dark transverse bands, whereas in *C. eragrostidis* and *C. intermedia*, only the median septum is accentuated (Sivanesan 1987, Alcorn 1998). The *americana*- (99% bs, 1 pp), *hominis*- (99% bs, 1 pp) and *lunata*- (93% bs, 0.99 pp) clades included species with mostly 4-celled, inconspicuously distoseptate conidia with the central cells usually darker than the end cells. In these clades the conidia are often curved at the third cell from base, and this cell is usually larger than the others (the only exceptional case is *C. brachyspora*, in which both central cells are more or less the same size (Sivanesan 1987)). This morphology, however, is also observed in *C. ischaemi*, which falls outside these three clades. The *americana*-clade included *C. americana* and *C. verruculosa*. These species appeared in Fig. 1 as two distinct species separated by relatively long branches. The *hominis*-clade included two new species, *C. hominis* and *C. muehlenbeckiae*. One isolate of the latter taxon, CBS 144.63, had been labelled "*C. lunata*" in the CBS collection, but in this study it proved to be phylogenetically quite distant from the ex-neotype strain of that species, CBS 730.96. The *lunata*-clade was formed by *C. aeria*, *C. brachyspora*, *C. carica-papayae*, *C. chlamydospora*, *C. lunata*, *C. prasadii* and *C. pseudolunata*. Accentuated septa can be observed in all members of this clade and elongate blackish stromata have been reported in *C. carica-papayae* and *C. aeria* (Mathur & Mathur 1959; Ellis 1966, 1971; Sivanesan 1987). This kind of stromata is also produced by old cultures of the ex-neotype strain of *C. lunata*, CBS 730.96 (unpublished data). Isolates

of *C. chlamydospora* and *C. pseudolunata* can produce aggregates of brown chlamydospores in culture (Figs. 3K and 6I). The *spicifera*-clade (98% bs, 1 pp) was formed by *C. australiensis*, *C. ellisii*, *C. hawaiiensis*, *C. perotidis* and *C. spicifera*. Members of this clade produce conspicuously distoseptate conidia which are straight in all species, except in *C. ellisii* which produces both straight and curved conidia (Sivanesan 1987). Three taxa of this clade are agents of opportunistic infections in humans, i.e. *C. australiensis*, *C. hawaiiensis* and *C. spicifera* (McGinnis et al. 1986, de Hoog et al. 2000). The *trifolii*-clade (95% bs, 1 pp) included *C. akaii*, *C. heteropogonis*, *C. gladioli* and *C. trifolii*. These species produce 4-celled, usually curved, inconspicuously distoseptate conidia which, in contrast to those seen in the other clades discussed here, show a strongly protruding hilum (Sivanesan 1987, Boerema & Hamers 1989, Alcorn 1990). Two other species in our study produce conidia with a protruding hilum, i.e. *C. cymbopogonis* and *C. protuberata*. Their conidia, however, are 5-celled (Sivanesan 1987).

Not all *Curvularia* species were included in the six clades previously mentioned, and other well-supported lineages were observed. *Curvularia oryzae* and *Curvularia tuberculata*, for example, appeared as sister taxa with 99% bs and 1 pp. These species are morphologically very different, i.e. the conidia of *C. oryzae* are 3-distoseptate and smooth while those of *C. tuberculata* are 3–8 distoseptate and tuberculate at maturity (Sivanesan 1987). We preferred not to name morphologically heterogeneous lineages because future studies including more taxa might reveal more homogeneous groupings within such lineages.

Taxonomy

Curvularia americana Da Cunha, Madrid, Gené & Cano, sp. nov. — MycoBank MB 806052; Fig. 2 A–K

Etymology: The name refers to the continent where this species was found.

Colonies on OA and PCA attaining 62 and 69 mm diam, respectively, in 7 days at 25°C, funiculose and greenish grey to dark green at the center, effuse and greyish white towards the periphery, with a fimbriate margin; reverse olive to dark green. Vegetative hyphae septate, branched, subhyaline to brown, smooth to asperulate, 1.5–4

wide, anastomosing. Conidiophores semi- to macronematous, mononematous, septate, usually simple, slightly geniculate, subhyaline to dark brown, smooth to asperulate, with cell walls often thicker than those of the vegetative hyphae, 60–299 × 2–5 µm. Conidiogenous cells terminal or intercalary, polytretic, proliferating sympodially, subcylindrical to slightly swollen, 8–22 × 4–8 µm. Conidia 4(–5)-celled, straight to slightly curved, 13–28 × 7–15 µm, usually with the third cell unequally sided and larger than the others, second and third cells pale brown to brown, apical and basal cell subhyaline, apical cell smooth-walled, intermediate smooth (slightly verruculose under SEM), basal cell often verruculose; hilum non-protruding, flat, darkened and thickened, 1.5–3 µm wide. Microconidiation sometimes present, forming one-celled, pale brown, globose conidia 5–6 µm wide. Chlamydospores not observed. Sexual morph not observed.

Specimens examined. USA: Minnesota, culture from ankle (human), *D.A. Sutton* 2008 (**Holotype:** CBS H-21465; cultures ex-type: FMR 11551, UTHSC 08-3414, CBS 136983); California, culture from maxillary sinus (human), *D.A. Sutton* 2010 (FMR 11500, UTHSC 10-1276); Ohio, culture from peritoneal dialysis fluid (human), *D.A. Sutton* 2008 (FMR 11691, UTHSC 08-278); Oklahoma, culture from toe nail (human), *D.A. Sutton* 2009 (FMR 11005, UTHSC 09-2907); Tennessee, culture from leg (human), *D.A. Sutton* 2008 (FMR 11674, UTHSC 08-2697); Texas, culture from toe tissue (human), *D.A. Sutton* 2007 (FMR 11687, UTHSC 07-2649); Texas, culture from bronchial wash (human), *D.A. Sutton* 2009 (FMR 11514, UTHSC 09-2863); Utah, culture from nasal sinus (human), *D.A. Sutton* 2008 (FMR 11693, UTHSC 08-84); Virginia, culture from bone marrow (human), *D.A. Sutton* 2009 (FMR 11515, UTHSC 09-2806).

Notes: *Curvularia americana* is similar to *C. lunata* and *C. prasadii* in conidial morphology. However, the conidia of *C. lunata* are slightly narrower, up to 13 µm wide (Manamgoda et al. 2012) and, in contrast to *C. americana*, all septa in conidia of *C. prasadii* are accentuated and up to 2.4 µm wide (Mathur & Mathur 1959; Ellis 1966, 1971). The phylogenetic study placed *C. lunata* and *C. prasadii* in the *lunata*-clade, a lineage relatively distant from *C. americana*. The four-locus tree indicated that *C. americana* is the sister taxon of *C. verruculosa*, but these species were separated by a considerable genetic distance (Fig. 1). The conidia of *C. verruculosa* are slightly larger

(20–40 × 12–17 µm) than those of *C. americana* and show distinctly verruculose intermediate cells (Tandon & Bilgrami 1962; Ellis 1966, 1971; Sivanesan 1987).

Curvularia chlamydospora Madrid, Da Cunha, Gené & Guarro, sp. nov. — MycoBank MB 806053; Fig. 3 A–K

Etymology: The name refers to the presence of chlamydospores.

Colonies on OA attaining 76 mm diam in 7 days at 25 °C, funiculose, greenish grey or dark green, margin fimbriate; reverse olive grey to dark green. Colonies on PCA attaining 68 mm diam at the same temperature and time of incubation, funiculose at the center, effuse towards the periphery, dark green, with a fimbriate margin; reverse dark green. Vegetative hyphae septate, branched, subhyaline to brown, smooth-walled, 1.5–4 wide, anastomosing. Conidiophores semi- to macronematous, mononematous, septate, usually simple, geniculate or bent at the apex, brown to dark brown, smooth to asperulate, 22–323 × 2–5 µm. Conidiogenous cells terminal or intercalary, polytretic, proliferating sympodially, subcylindrical to irregularly shaped, 7–18 × 5–10 µm. Conidia 4-celled, mostly slightly curved, 16–25 × 7–12 µm wide in the broadest part, smooth-walled (basal cell verruculose under SEM), usually with the central septum appearing slightly accentuated, the third cell from the base slightly larger and unequal sided, second and third cells darker than the others, brown to dark brown, end cells paler; hilum non-protruding, flat, darkened and thickened, 1.5–3 µm wide. Chlamydospores present, initially as intercalary chains but later forming clusters of swollen cells, 13–80 µm, smooth to verruculose and thick-walled. Microconidiation present, forming conidia 1–2-celled, pale brown, globose to subglobose, 4–6 µm diam. Sexual morph not observed.

Specimens examined. USA: Montana, culture from toe nail (human), *D.A. Sutton* 2007 (**Holotype:** CBS H-21466; cultures ex-type: FMR 11709, UTHSC 07-2764, CBS 136984); Nevada, culture from nasal sinus (human), *D.A. Sutton* 2008 (FMR 11040, UTHSC 08-1283).

Notes: *Curvularia chlamydospora* is superficially similar to three species producing 4-celled conidia with an accentuated median septum, namely *C. brachyspora*, *C. eragrostidis* and *C. intermedia*. However, the third cell from base is usually larger and more pigmented than the second one in *C. chlamydospora*, while in the three

similar taxa both intermediate cells are rather equal in size and pigmentation. These species have not been reported to produce chlamydospores in culture and have wider conidia, i.e. 10–14 µm in *C. brachyspora*, 11–20 µm in *C. eragrostidis* and 13–20 in *C. intermedia* (Sivanesan 1987). *Curvularia eragrostidis* and *C. intermedia* reside in the *eragrostidis*-clade, while *C. chlamydospora* belongs to the *lunata*-clade. *Curvularia brachyspora* appeared as the sister taxon of *C. chlamydospora* but this relationship received poor statistical support (Fig. 1).

Curvularia hominis Da Cunha, Madrid, Gené & Cano, sp. nov. — MycoBank MB 806054; Fig. 4 A–I

Etymology: The name refers to the origin of the isolates, all of which were isolated from clinical human specimens.

Colonies on OA and PCA attaining 70–72 mm diam in 7 days at 25 °C, funiculose and dark green at the center, floccose and olive to white towards the periphery, with a fimbriate margin; reverse olive to dark green. Vegetative hyphae septate, branched, subhyaline to brown, smooth to slightly asperulate 1.5–5 µm wide, anastomosing. Conidiophores semi- to macronematous, mononematous, septate, simple or branched, geniculate towards the apex, subhyaline to dark brown, smooth to asperulate, with cell walls often thicker than those of the vegetative hyphae, 55–325 × 2–5 µm wide. Conidiogenous cells terminal or intercalary, polytretic, proliferating sympodially, subcylindrical to irregularly shaped, 6–26 × 4–9 µm; conidiogenous loci usually somewhat thickened and darkened. Conidia 4–5-celled, slightly curved, 18–30 × 7–14 µm wide in the broadest part, with the third cell from the base often larger and unequal sided, intermediate cells usually verruculose and darker than the others, brown, end cells subhyaline to pale brown and smooth-walled; hilum non-protruding, flat, darkened and thickened, 1.5–3 µm wide. Microconidiation and chlamydospores were not observed. Sexual morph not observed.

Specimens examined. USA: Florida, culture from cornea (human), *D.A. Sutton* 2009 (**Holotype:** CBS H-21467; cultures ex-type: FMR 11539, UTHSC 09-464, CBS 136985); Arkansas, culture from nasal sinus (human), *D.A. Sutton* 2007 (FMR 11172, UTHSC 07-3184); Louisiana, culture from eye (human), *D.A. Sutton* 2008 (FMR

11688, UTHSC 08-849); Minnesota, culture from nail (human), *D.A. Sutton* 2007 (FMR 11698, UTHSC 07-3581); Minnesota, culture from nasal sinus (human), *D.A. Sutton* 2009 (FMR 11527, UTHSC 09-2197); Ohio, culture from nasal sinus (human), *D.A. Sutton* 2009 (FMR 11535, UTHSC 09-1692); Texas, culture from nasal sinus (human), *D.A. Sutton* 2007 (FMR 11704, UTHSC 07-3105); Texas, culture from nail (human) *D.A. Sutton* 2008 (FMR 11683, UTHSC 08-1296); Texas, culture from bronchial wash (human) *D.A. Sutton* 2008 (FMR 11680, UTHSC 08-2418); Texas, culture from bronchial wash (human) *D.A. Sutton* 2008 (FMR 11542, UTHSC 08-3737); Texas, culture from foot (human), *D.A. Sutton* 2008 (FMR 11678, UTHSC 08-2517); Texas, culture from nasopharynx (human), *D.A. Sutton* 2009 (FMR 11521, UTHSC 09-2532); Texas, culture from tissue (human), *D.A. Sutton* 2009 (FMR 11509, UTHSC 09-3403); Utah, culture from cornea (human), *D.A. Sutton* 2007 (FMR 11708, UTHSC 07-2791).

Notes: Although all isolates of this fungus are from humans, the species might also be common in the environment. *Curvularia hominis* resembles other species of the genus with 4-celled conidia and an asymmetrically swollen, dark third cell, such as *C. aeria*, *C. carica-papayae*, *C. lunata* and *C. prasadii*, but differs from them in producing conidia with verruculose intermediate cells (Fig. 4E–I). The latter four species are members of the *lunata*-clade, whereas *Curvularia hominis* and *C. muehlenbeckiae* form a distinct lineage, the *hominis*-clade (Fig. 1).

Curvularia muehlenbeckiae Madrid, Da Cunha, Gené Guarro & Crous sp. nov. — MycoBank MB 806055; Fig. 5 A–J

Etymology: The name refers to the substrate from which the ex-type strain was obtained, *Muehlenbeckia* sp.

Colonies on OA attaining 76 mm in 7 days at 24 °C, cottony to funiculose, pale grey at the center, dark olive towards the periphery, with a fimbriate margin; reverse olivaceous black. Colonies on PCA attaining 40 mm diam at the same temperature and period of incubation, radiate, funiculose, dark olive with a slightly fimbriate margin; reverse concolorous with surface. Vegetative hyphae septate, branched, subhyaline to brown, smooth-walled, 1.5–5 µm wide, anastomosing. Conidiophores semi- to macronematous, mononematous, septate, simple to branched, straight or flexuous,

geniculate towards the apex, subhyaline to dark brown, smooth to asperulate with cell walls often thicker than those of the vegetative hyphae, 21.5–398 × 2–5 µm with subnodulose and nodulose intercalary swellings up to 9.5 µm wide, swellings coinciding with conidiogenous loci. Conidiogenous cells integrated, terminal and intercalary, subcylidrical to irregularly shaped, mono- to polytretic, proliferating sympodially; intercalary conidiogenous cells 5–18 µm long, terminal conidiogenous cells 5–25 µm long. Conidia 4-celled, asymmetrical to more or less curved at the third cell from base, 17–26 × 8.5–12 µm, intermediate cells dark brown and usually verruculose, end cells paler and smooth-walled or less ornamented than central cells. Chlamydospores and microconidiation not observed. Sexual morph not observed.

Specimens examined. INDIA, locality unknown, from *Muehlenbeckia* sp. leaf, 1962, *K.S. Bilgrami*, holotype Herb. CBS H-10451, culture ex-type CBS 144.63. USA: Utah, culture from chest (human) *D.A. Sutton* 2008 (UTHSC 08-2905, FMR 11671, CBS 136986).

Notes: This species is the sister taxon of *C. hominis*, which has slightly larger conidia (18-30 x 7-14 µm) with a similar ornamentation consisting of small but conspicuous warts. Some *Curvularia* species outside the *hominis*-clade produce conidia ornamented with warts, e.g. *C. tuberculata*, *C. verruculosa* and *C. verruciformis*. The former two species produce larger conidia, i.e. 23–52 × 13–20 µm and 20–40 × 12–17 µm, respectively and the latter differs from members of the *hominis*-clade by having mostly 5-celled, more strongly ornamented conidia (Jain 1962, Agarwal & Sahni 1963, Ellis 1966, Sivanesan 1987). *Curvularia* species with warted conidia appear in different clades, suggesting that this kind of ornamentation evolved several times in *Curvularia*.

Curvularia pseudolunata Da Cunha, Madrid & Gené, sp. nov. — MycoBank MB 806056; Fig. 6 A–I

Etymology: The name refers to the morphological resemblance and phylogenetic closeness of this species to *Curvularia lunata*.

Colonies on OA attaining 71 mm diam in 7 days at 25 °C cottony to lanose, greenish grey, with a fimbriate margin; reverse dark green. Colonies on PCA attaining 78 mm diam at the same temperature and time of incubation, lanose at the center, floccose towards the periphery, greyish green, with a fimbriate margin; reverse olive

green. Vegetative hyphae septate, branched, subhyaline to brown, smooth-walled, 1.5–5 µm wide. Conidiophores macronematous, mononematous, septate, unbranched, geniculate near the apex, brown, smooth-walled, 100–350 × 2–4.5 µm. Conidiogenous cells mostly terminal, polytretic, proliferating sympodially, subcylindrical, subglobose to irregularly shaped, 4.5–30 × 6–10 µm. Conidia 4-celled, mostly curved, 20–27 × 8–12 µm, with the third cell from base usually unequally sided, larger and darker than the others, brown, second and end cells pale brown to subhyaline, smooth-walled, basal cell often verruculose; hilum non-protruding, flat, darkened and thickened, 1.5–2.5 µm wide. Chlamydospores abundant, initially as intercalary chains, later forming clusters of swollen cells, up to 60 µm diam, smooth- and thick-walled. Microconidiation not observed. Sexual morph not observed.

Specimens examined. USA: California, culture from nasal sinus (human), *D.A. Sutton* 2009 (**Holotype:** CBS H-21468; cultures ex-type: FMR 11529, UTHSC 09-2092, CBS 136987).

Notes: *Curvularia pseudolunata* is morphologically similar to *C. lunata* and these taxa grouped together in the four-locus phylogeny (Fig. 1). However, the conidia of *C. lunata* are slightly larger (21–31 × 9–13 µm) and this species is separated from *C. pseudolunata* by a considerable genetic distance.

DISCUSSION:

Traditionally, *Curvularia* and *Bipolaris* have been distinguished by conidial features, i.e. euseptate and typically curved at a swollen intermediate cell in *Curvularia*, but straight to slightly curved and distinctly distoseptate in *Bipolaris* (Kwon-Chung & Bennett 1992, de Hoog et al. 2000, Revankar & Sutton 2010). We agree with the view of authors like Alcorn (1983b), Sivanesan (1987) and Seifert et al. (2011) that both genera have distoseptate conidia. Phylogenetic studies (Berbee et al. 1999, Manamgoda et al. 2012) have demonstrated that species with conspicuously distoseptate conidia previously placed in *Bipolaris* actually belong in *Curvularia*, e.g. members of the *spicifera*-clade (Fig. 1). Furthermore, in the new species described herein, two wall layers were often evident in young conidia (Figs. 2F, 4D, 5F) and septa are already visible at this stage; however, in mature conidia the layers may appear so close to one another that the conidia may look euseptate under the light microscope (Figs. 2G, 4I,

5E). A recently described pleosporalean genus, *Porocercospora*, the causal agent of buffalograss false-smut disease, also shows two-layered conidial cell walls, but mature conidia often seem to have both eu- and distosepta, depending on how closely together the two cell wall layers cohere near the septa. This genus is phylogenetically closely related to *Bipolaris* and *Curvularia* and is similar to them in having tretic conidiogenesis and darkly pigmented mycelium. However, *Porocercospora* has conidiophores without a geniculate rachis, conidiogenous cells with inconspicuous, non-darkened conidiogenous loci and long, obclavate to cylindro-obclavate conidia (Amaradasa et al. 2014).

Although *Bipolaris* and *Curvularia* cannot be distinguished based on the morphology of their conidial septa, other morphological features seem to be of diagnostic value. None of the species of *Bipolaris* s. str. included in this study (Fig. 1) and in previous works (Berbee et al. 1999, Manamgoda et al. 2012) has conidia curved at an intermediate swollen cell. As described by Berbee et al. (1999) for "*Cochliobolus* group 1", the conidia of *Bipolaris* s. str. can show a gentle curve that continues along the whole length of the conidium. Conidia ornamented with small to coarse warts are produced by some *Curvularia* species e.g. *C. verruculosa*, *C. verruciformis* and *C. tuberculata*, but this kind of ornamentation has not been reported in *Bipolaris* s. str. (Jain 1962, Tandon & Bilgrami 1962, Agarwal & Sahni 1963, Ellis 1966, Sivanesan 1987). Another helpful character is the morphology of the hilum. None of the species in *Bipolaris* s. str. has conidia with a strongly protruding hilum, but it is observed in several members of *Curvularia* s. str., such as *C. cymbopogonis* and *C. protuberata*, as well as all species in the *trifolii*-clade (Sivanesan 1987). A protruding hilum is also observed in a closely related genus, *Exserohilum*, which also includes clinically relevant and plant-pathogenic species (McGinnis et al. 1986, de Hoog et al. 2000). Members of this genus sometimes form curved conidia, but the hilum is different from those seen in *Curvularia* spp. In *Exserohilum* the hilum appears as a protrusion of the cell wall that is not delimited by a septum and that often appears double-walled, with the outer wall forming an enveloping collar or "hilar bubble" around it (Alcorn 1983b, 1988). In *Curvularia*, by contrast, when the hilum protrudes, it appears single-walled in light microscopy and is delimited by a septum (Nelson & Hodges 1965, Sivanesan 1987, Zhang et al. 2004). Conidial size might also be helpful to distinguish *Bipolaris* s. str.

from *Curvularia* s. str. Among the species falling in the *Bipolaris* clade in Berbee et al. (1999) and Manamgoda et al. (2012), the longest conidia are those of *B. zeae*, up to 225 µm long (Sivanesan 1987). Conidia of *Curvularia* s. str. tend to be shorter. Among species in the *Curvularia* clade (Berbee et al. 1999, Manamgoda et al. 2012), the longest conidia are produced by *C. tripogonis*, and are up to 130 µm long (Sivanesan 1987).

Boedijn (1933) divided *Curvularia* into three groups of species, i.e. groups Maculans, Lunata and Geniculata. The Maculans group was characterized by producing 4-celled, straight or somewhat asymmetrical conidia with the central cells darker and larger than the end cells. This group included *C. maculans* (currently considered a synonym of *C. eragrostidis*), *C. cesatii* (this species was transferred to the genus *Endophragmiella* as *E. cesatii* by Hughes in 1979), *C. intermedia* and *C. spicifera*, all of which were unable to produce stromata in culture. The Lunata group included species with 4-celled, more or less curved conidia in which one of the intermediate cells is enlarged and darker than the others. Some of its members were *C. lunata*, *C. ramosa* and *C. trifolii.* This group was reported to produce subcylindrical stromata in culture. The Geniculata group was proposed for species with 5-celled conidia which often produced stromata, such as *C. geniculata*, *C. affinis*, *C. fallax* and *C. falcata* (this species was synonymized with *C. senegalensis* by Sivanesan in 1987). In our phylogenetic study, three species of Boedijn's Maculans group were included, i.e. *C. eragrostidis*, *C. intermedia* and *C. spicifera.* The group is polyphyletic since only the former two species grouped together in the *eragrostidis*-clade, a lineage characterized by rather straight, inconspicuously distoseptate, 4-celled conidia. This lineage also included *C. graminicola* (Fig. 1). *Curvularia spicifera* clustered in a different clade with other species whose conidia show evident distosepta. No DNA sequences have as yet been analyzed for *Endophragmiella cesatii*, which, as indicated previously, originally was considered a *Curvularia* species and a member of the Maculans group (Boedijn 1933). Its morphology clearly suggests a phylogenetically distant fungus (Hughes 1979, 1980). The genus *Endophragmiella* is considered a member of the Lasiosphaeriaceae, Sordariales by Seifert et al. (2011). Boedijn's Lunata group also proved to be polyphyletic since two of its members, *C. trifolii* and *C. lunata*, clustered in separate, relatively distant clades in Fig. 1. Two members of Boedijn's Geniculata group included in this study, *C. affinis* and *C. senegalensis*, formed a well-supported clade. CBS

isolates of *C. geniculata* could not be clearly distinguished molecularly from isolates labelled *C. senegalensis* in a study by da Cunha et al. (2013); other authors also suggested that these taxa might be conspecific (Hosokawa et al. 2003, Sun et al. 2003). Unfortunately no ex-type strains of these species are available and epitypification is necessary to clarify their taxonomy. Isolate CBS 155.34, labelled "Syntype" of *C. fallax* has 4-celled conidia and clustered with morphologically similar isolates of *Curvularia* spp. in a preliminary phylogeny (not shown). CBS 155.34 is possibly mislabeled and therefore was excluded from the analysis.

The well-documented *Bipolaris* s.l. opportunists, i.e. *B. australiensis*, *B. hawaiiensis* and *B. spicifera* (McGinnis et al. 1986) were transferred to *Curvularia* (Manamgoda et al. 2012), suggesting that pathogenicity to vertebrates in this group of fungi might be restricted to the latter genus (Manamgoda et al. 2012). Interestingly, however, a recent study by da Cunha et al. (2012) reported that two species of *Bipolaris* s. str., *B. cynodontis* and *B. setariae*, represented 8.6% and 1%, respectively, of a total of 104 clinical *Bipolaris* s.l. isolates from the USA. These species were isolated from various anatomical sites, including the eyes, legs, nasal sinuses, nails and lower respiratory tract. Their pathogenicity still needs to be demonstrated and no cases of human disease by these fungi have been published yet.

The ITS locus has been widely used in the identification of plant pathogenic and clinically relevant fungi and recently has been proposed as a universal barcode marker for these organisms (Iwen et al. 2002, Schoch et al. 2012). It has been used by some authors to identify isolates of *Curvularia* from clinical samples and plants (Fryen et al. 1999, Bagyalakshmi et al. 2008, Dyer et al. 2008, Chowdhary et al. 2011, Funnell-Harris et al. 2013). This marker, however, is not optimal for species identification since it provided little resolution for closely related *Curvularia* species in our study. Similar results were published by da Cunha et al. (2012), in which an ITS tree gave < 70% bs for clades representing *C. spicifera* and *C. hawaiiensis*, two of the main clinically relevant members of the genus. Other authors have found limited species resolution in ITS phylogenies of other members of the Pleosporales (Pryor & Gilbertson 2000, de Hoog & Horré 2002, Pryor & Bigelow 2003, Park et al. 2008, Brun et al. 2013), indicating that additional genes need to be used for reliable species identification in this group of fungi. Protein-coding loci have been reported to be phylogenetically more

informative than rDNA in *Ascomycota* (Schoch et al. 2009) and this is confirmed here in *Curvularia.* In our work, species discrimination improved with the *gpd* and *RPB2* loci, which revealed more than double the percentage of variable sites seen in ITS. These protein-coding loci are promising markers for future phylogenetic studies in *Curvularia* and related genera.

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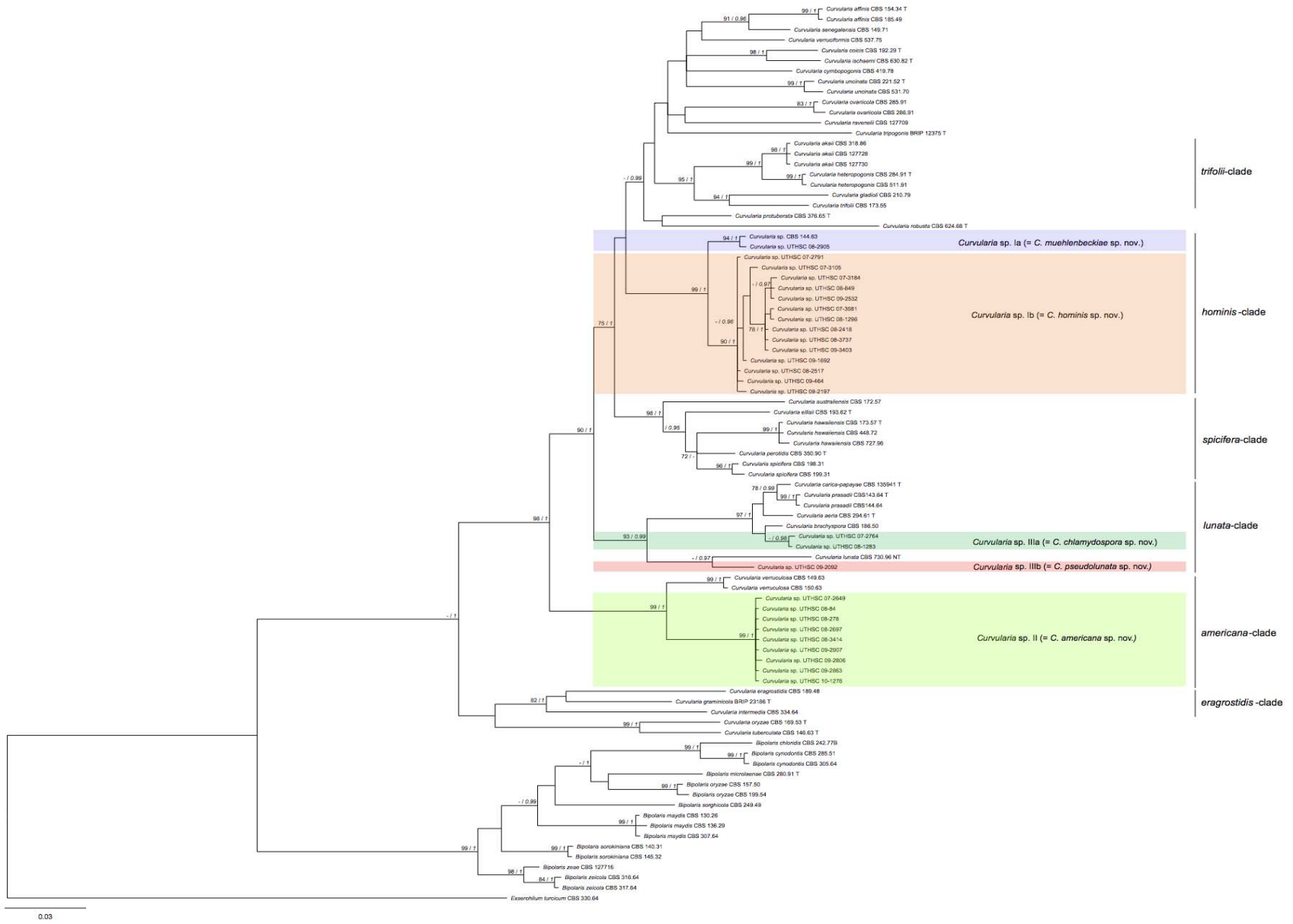
Fig. 1. Bayesian consensus tree obtained from the combined ITS, LSU, *gpd* and *RPB2* alignment of *Curvularia* and related genera. The scale bar represents the average number of substitutions per site. Bootstrap values ≥ 70% and posterior probabilities ≥ 0.95 (in italics) are given near the internodes. The new species proposed in this study are shown in the coloured boxes.



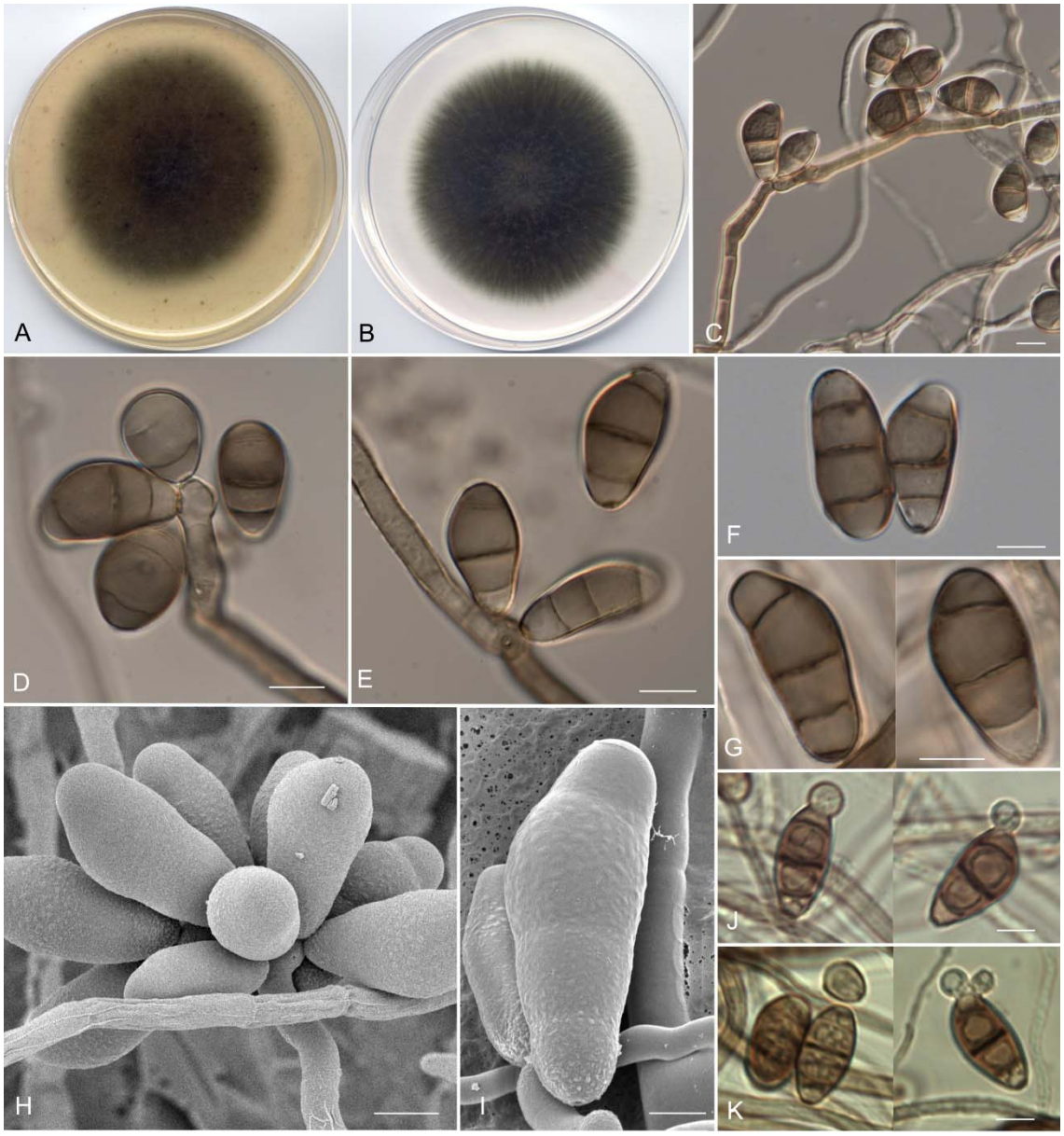


Fig. 2. *Curvularia americana* (A, B, D–I, UTHSC 08-3414; C, K, FMR 11674). A, B. Colonies of OA and PCA, respectively, at 25°C after 7d. C–I. Conidiophores and conidia. J, K. Microconidiation. Scale bar C–I = 10 µm.

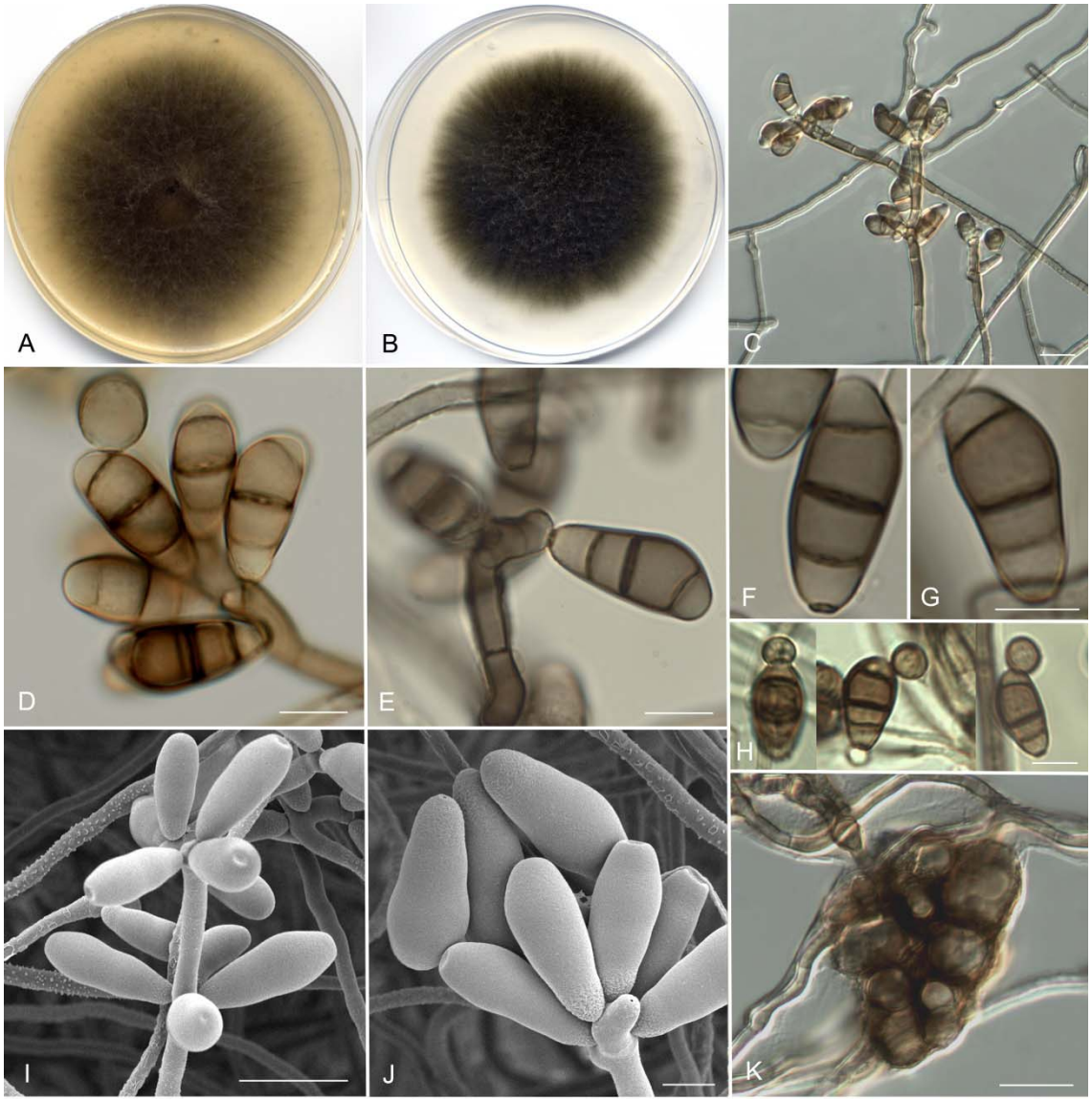


Fig. 3. *Curvularia chlamydospora* (A-D, H–K, UTHSC 07-2764; E–G, FMR 11040). A, B. Colonies of OA and PCA, respectively, at 25°C after 7d. C–G, I, J. Conidiophores and conidia. H. Microconidiation. K. Chlamydospore. Scale bar C, I, K = 20 µm, D–H = 10 µm, J= 5 µm.

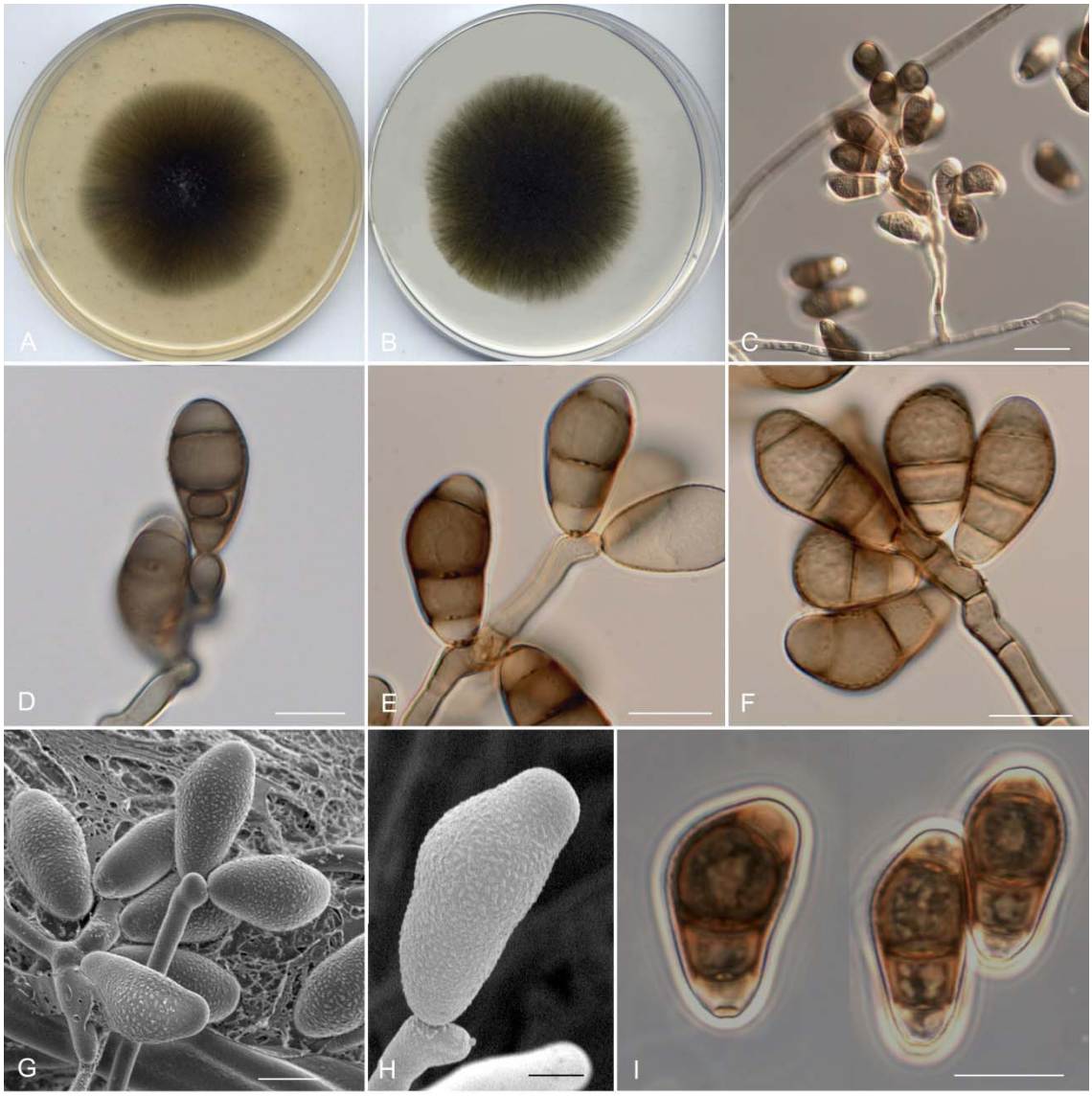


Fig. 4. *Curvularia hominis* (UTHSC 09-464). A, B. Colonies of OA and PCA, respectively, at 25°C after 7d. C, E–I. Conidiophores and conidia. D. Conidium showing two wall layers. Scale bars C = 20 µm, D –G, I = 10 µm, H = 5 µm.

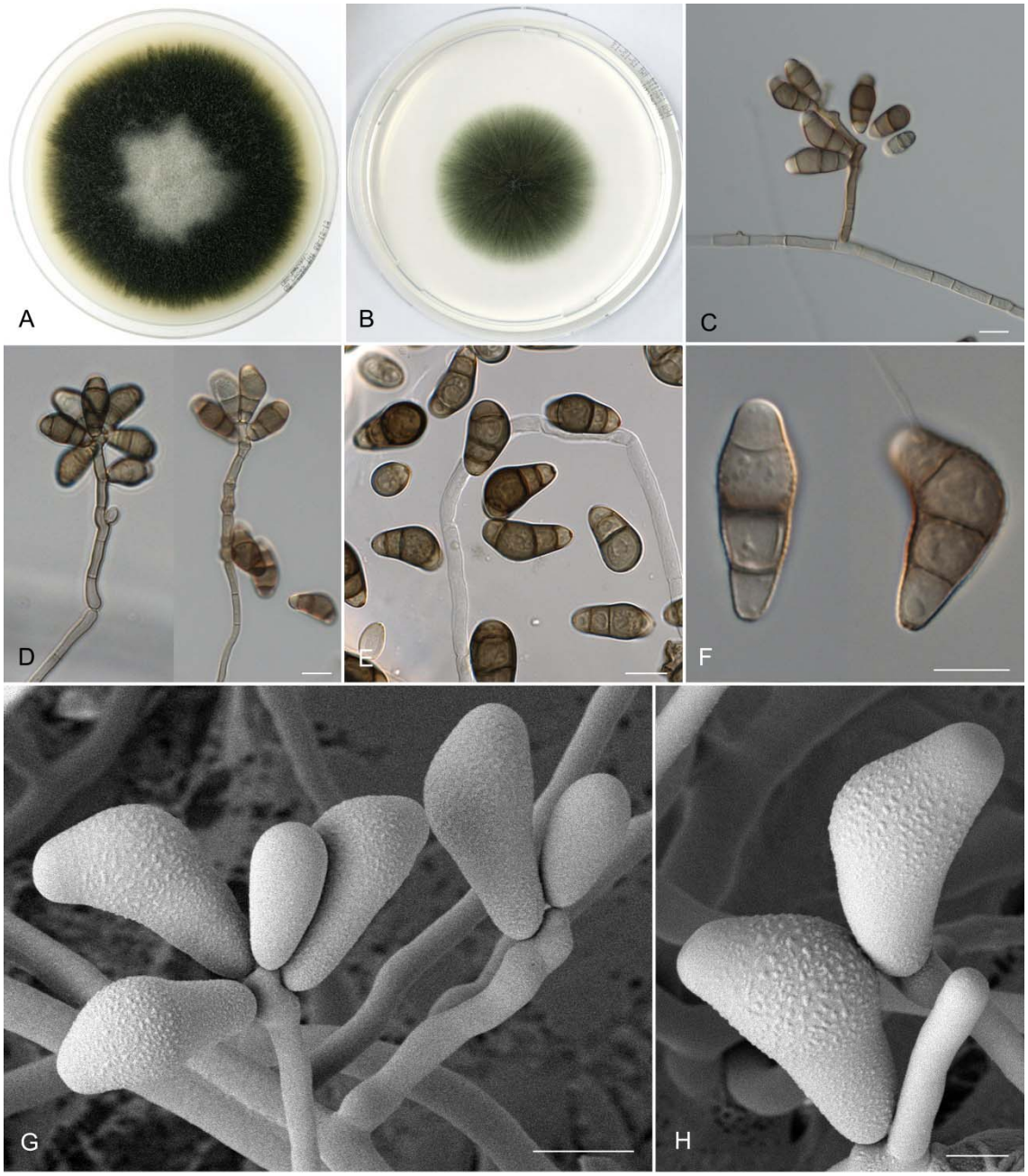


Fig. 5. *Curvularia muehlenbeckiae* (CBS 144.63). A, B. Colonies on OA after 7 days and on PCA after 5 days, respectively, at 25°C. C–H. Conidiophores and conidia. Scale bar C–G = 10 µm, H = 5 µm.

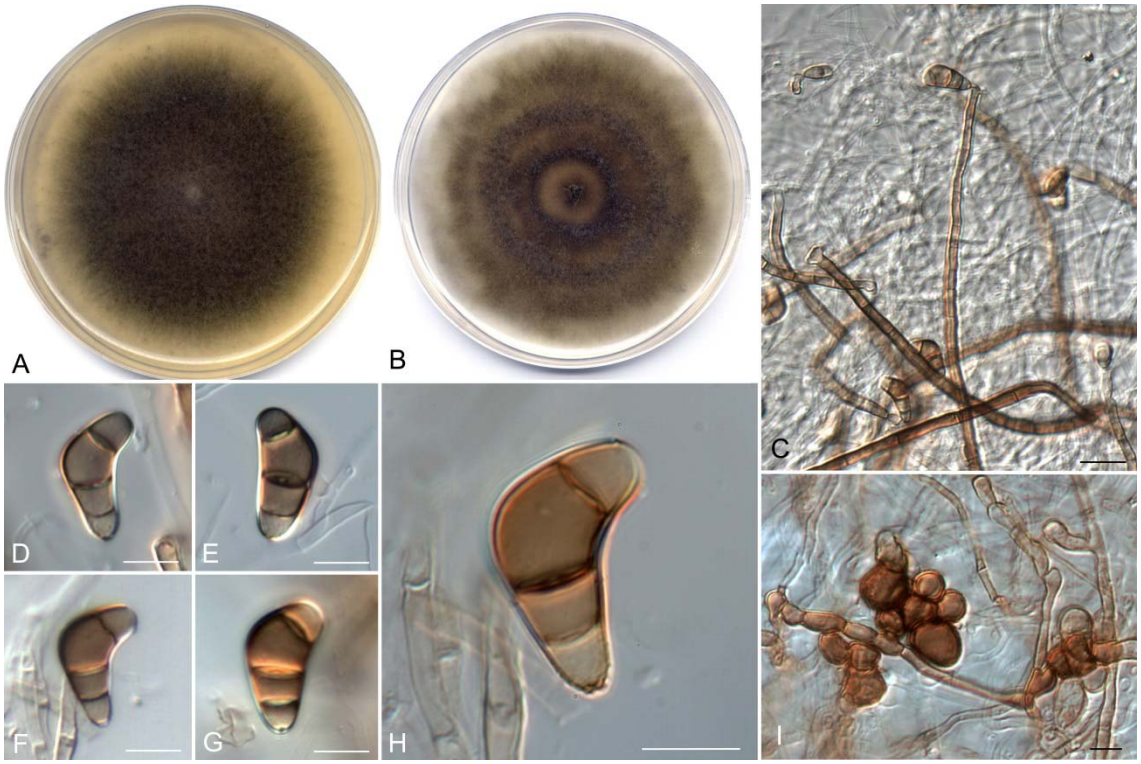


Fig. 6. *Curvularia pseudolunata* (UTHSC 09-2092). A, B. Colonies of OA and PCA, respectively, at 25°C after 7d. C–H. Conidiophores and conidia. I. Chlamydospores. Scale bar C = 20 µm, D–I = 10 µm.

TABLE I. Isolates included in the phylogenetic study, their origins, and GenBank accession no.

Taxon	Isolate no.	Source	GenBank accession no.			
			ITS	LSU	*gpd*	*RPB2*
Bipolaris chloridis	CBS 242.77B	*Chloris gayana*, Australia	HF934928	HF934869	HG779083	HF934830
Bipolaris cynodontis	CBS 285.51	*Cynodon transvaalensis*, Kenya	HF934929	HF934874	HG779081	HF934831
	CBS 305.64	*Cynodon dactylon*, USA	HF934930	HF934883	HG779082	HF934832
Bipolaris maydis	CBS 130.26	Unknown	HF934923	HF934873	HG779084	HF934825
	CBS 136.29	*Zea mays*, Japan	HF934926	HF934879	HG779086	HF934828
	CBS 307.64	*Zea mays*, USA	HF934925	HF934875	HG779085	HF93482
Bipolaris microlaenae	CBS 280.91^{T}	*Microlaena stipoides* leaf, Australia	HF934933	HF934877	HG779092	HF934835
Bipolaris oryzae	CBS 157.50	*Oryza sativa* grain, Indonesia	HF934931	HF934870	HG779090	HF934833
	CBS 199.54	*Oryza sativa* grain, New Guinea	HF934932	HF934884	HG779091	HF934834
Bipolaris sorghicola	CBS 249.49	*Sorghum vulgare* var. *sudanense*, Locality unknown	HF934927	HF934868	HG779087	HF934829
Bipolaris sorokiniana	CBS 140.31	Substrate unknown, Japan	HF934935	HF934876	HG779088	HF934837
	CBS 145.32	*Triticum durum*, Locality unknown	HF934934	HF934885	HG779089	HF934836
Bipolaris zeae	CBS 127716	Unknown	HG778980	HG779027	HG779095	HG779158
Bipolaris zeicola	CBS 316.64	*Zea mays*, USA	HF934938	HF934871	HG779093	HF934840
Bipolaris zeicola	CBS 317.64	*Zea mays*, USA	HF934939	HF934878	HG779094	HF934841
Curvularia aeria	CBS 294.61^{T}	Air, Brazil	HF934910	HF934902	HF565450	HF934812
Curvularia affinis	CBS 154.34^{T}	*Manihot utilissima*, Java	HG778981	HG779028	HG779126	HG779159
	CBS 185.49	*Manihot utilissima*, Java	HG778982	HG779029	HG779127	HG779160
Curvularia akaii	CBS 318.86	Substrate unknown, Japan	HF934921	HF934897	HG779118	HF934823
	CBS 127728	Substrate unknown, Japan	HF934920	HF934898	HG779119	HF934822
	CBS 127730	Substrate unknown, Japan	HF934922	HF934899	HG779120	HF934824
Curvularia australiensis	CBS 172.57	*Oryza sativa* seed, Vietnam	HF934912	HF934901	HG779139	HF934814
Curvularia brachyspora	CBS 186.50	Soil, Java	HG778983	HG779030	HG779150	HG779161

TABLE I (cont.)

Curvularia carica-papayae	CBS 135941[T]	*Carica papaya* leaf, India	HG778984	HG779031	HG779146	HG779162
Curvularia coicis	CBS 192.29[T]	*Coix lacrima-jobi* var. *typica*, Japan	HF934917	HF934895	HG779130	HF934819
Curvularia cymbopogonis	CBS 419.78	*Yucca* sp. leaf, Netherlands	HG778985	HG779032	HG779129	HG779163
Curvularia ellisii	CBS 193.62	Air, Pakistan	HF934913	HF934896	HG779143	HF934815
Curvularia eragrostidis	CBS 189.48	Sorghum seed, Java	HG778986	HG779033	HG779154	HG779164
Curvularia gladioli	CBS 210.79	*Gladiolus* sp. leaf, Romania	HG778987	HG779034	HG779123	HG779165
Curvularia graminicola	BRIP 23186	*Aristida ingrata*, Australia	JN192376	JN600986	JN600964	———
Curvularia hawaiiensis	CBS 173.57[T]	*Oryza sativa*, Hawaii	HG778988	HG779035	HG779140	HG779166
	CBS 448.72	Salt-marsh soil, Kuwait	HG778989	HG779036	HG779142	HG779167
	CBS 727.96	Substrate unknown, USA	HG778990	HG779037	HG779141	HG779168
Curvularia heteropogonis	CBS 284.91[T]	*Heteropogon contortus* leaf, Australia	HF934919	HF934893	HF934919	HF934821
	CBS 511.91	*Heteropogon contortus* leaf, Australia	HF934918	HF934894	HF934918	HF934820
Curvularia intermedia	CBS 334.64	*Avena versicolor*, USA	HG778991	HG779038	HG779155	HG779169
Curvularia ischaemi	CBS 630.82[T]	*Ischaemum indicum* leaf, Solomon Islands	HG778992	HG779039	HG779131	HG779170
Curvularia lunata	CBS 730.96[NT]	Lung biopsy, USA	HF934911	HF934900	JX256429	HF934813
Curvularia oryzae	CBS 169.53[T]	*Oryza sativa* seed, Vietnam	HF934906	HF934867	HF934808	HF934808
Curvularia ovariicola	CBS 285.91	*Eragrostis parviflora*, Australia	HG778993	HG779040	HG779144	HG779171
	CBS 286.91	*Eragrostis parviflora*, Australia	HG778994	HG779041	HG779145	HG779172
Curvularia perotidis	CBS 350.90[T]	*Perotis rara*, Australia	HG778995	HG779042	HG779138	HG779173
Curvularia prasadii	CBS 143.64[T]	*Jasminum sambac*, India	HG778996	HG779043	HG779147	HG779174
Curvularia prasadii	CBS 144.64	Substrate unknown, England	HG778997	HG779044	HG779149	HG779175
Curvularia protuberata	CBS 376.65[T]	*Deschampsia flexuosa* leaf, Scotland	HG778998	HG779045	HG779135	HG779176
Curvularia ravenelii	CBS 127709	Unknown	HG778999	HG779046	HG779109	HG779177

TABLE I (cont.)

Curvularia robusta	CBS 624.68 T	*Dichanthium annulatum* leaf, USA	HG779000	HG779047	HG779125	HG779178
Curvularia senegalensis	CBS 149.71	Substrate unknown, Nigeria	HG779001	HG779048	HG779128	HG779179
Curvularia spicifera	CBS 198.31	*Capsicum anuum*, Cyprus	HF934916	HF934905	HG779136	HF934818
	CBS 199.31	*Cucurbita maxima*, Cyprus	HF934915	HF934903	HG779137	HF934817
Curvularia trifolii	CBS 173.55	*Trifolium repens*, USA	HG779023	HG779077	HG779124	HG779208
Curvularia tripogonis	BRIP 12375 T	*Tripogon jacquemonti,* India	JN192388	JN601002	JN600980	———
Curvularia tuberculata	CBS 146.63T	*Zea mays* leaf, India	HF934907	HF934866	HG779157	HF934809
Curvularia uncinata	CBS 221.52 T	*Oryza sativa* leaf, Vietnam	HG779024	HG779078	HG779134	HG779209
	CBS 531.70	*Oryza sativa* seeds, Denmark	HG779025	HG779079	HG779132	HG779210
Curvularia verruciformis	CBS 537.75	*Lobibyx* sp. feather, New Zealand	HG779026	HG779080	HG779133	HG779211
Curvularia verruculosa	CBS 149.63	*Elaeis guineensis*, Nigeria	HF934909	HF934891	HG779110	HF934811
	CBS 150.63	*Punica granatum* leaf, India	HF934908	HF934892	HG779111	HF934810
Curvularia sp. I Lineage A (= *C. muehlenbeckiae* sp. nov.)	CBS 144.63 T	*Muehlenbeckia* sp. leaf, India	HG779002	HG779049	HG779108	HG779180
Curvularia sp. I Lineage B (= *C. hominis* sp. nov.)	UTHSC 08-2905	Chest, USA	HE861836	HG779050	HF565484	HG779189
	UTHSC 07-2791	Cornea, USA	HG779003	HG779057	HG779105	HG779181
	UTHSC 07–3105	Nasal sinus, USA	HG779004	HG779058	HG779104	HG779182
	UTHSC 07-3184	Nasal sinus, USA	HG779005	HG779059	HG779099	HG779183
	UTHSC 07-3581	Nail, USA	HG779006	HG779060	HG779102	HG779184
	UTHSC 08-849	Eye, USA	HE861837	HG779051	HF565483	HG779185
	UTHSC 08-1296	Nail, USA	HG779007	HG779061	HG779103	HG779186

TABLE I (cont.)

Curvularia sp. I Lineage B (= *C. hominis* sp. nov.)	UTHSC 08-2418	Bronchial wash, USA	HG779008	HG779062	HG779096	HG779187
	UTHSC 09-464 T	Cornea, USA	HG779011	HG779065	HG779106	HG779191
	UTHSC 08-2517	Foot, USA	HG779009	HG779063	HG779107	HG779188
	UTHSC 08-3737	Bronchial wash, USA	HG779010	HG779064	HG779101	HG779190
	UTHSC 09-1692	Nasal sinus, USA	HG779012	HG779066	HG779097	HG779192
	UTHSC 09-2197	Nasal sinus, USA	HE861835	HG779052	HF565485	HG779193
	UTHSC 09-2532	Nasopharynx, USA	HG779013	HG779067	HG779100	HG779194
	UTHSC 09-3403	Unknown tissue, USA	HG779014	HG779068	HG779098	HG779195
Curvularia sp. II (= *C. americana* sp. nov.)	UTHSC 08-3414 T	Ankle, USA	HE861833	HG779056	HF565488	HG779200
	UTHSC 07-2649	Toe tissue, USA	HE861834	HG779054	HF565486	HG779196
	UTHSC 08-84	Nasal sinus, USA	HG779015	HG779069	HG779115	HG779197
	UTHSC 08-278	Peritoneal dialysis fluid, USA	HE861832	HG779055	HF565487	HG779198
	UTHSC 08-2697	Leg, USA	HG779016	HG779070	HG779117	HG779199
	UTHSC 09-2907	Toe nail, USA	HG779017	HG779071	HG779114	HG779201
	UTHSC 09-2806	Bone marrow, USA	HG779018	HG779072	HG779112	HG779202
	UTHSC 09-2863	Bronchial wash, USA	HG779019	HG779073	HG779113	HG779203
	UTHSC 10-1276	Maxillary sinus, USA	HG779020	HG779074	HG779116	HG779204

TABLE I (cont.)

Curvularia sp. III Lineage A (= *C. chlamydospora* sp. nov.)	UTHSC 07-2764 [T]	Toe nail, USA	HG779021	HG779075	HG779151	HG779205
	UTHSC 08-1283	Nasal sinus, USA	HG779022	HG779076	HG779152	HG779206
Curvularia sp. III Lineage B (= *C. pseudolunata* sp. nov.)	UTHSC 09-2092 [T]	Nasal sinus, USA	HE861842	HG779053	HF565459	HG779207
Exserohilum turcicum	CBS 330.64	*Zea mays*, USA	HF934950	HF934887	HG779153	HF934852

Sequences generated during this study appear in bold; other sequences originate from Manamgoda et al. (2012), da Cunha et al. (2013) and Amaradasa et al. (in press). [T] ex-type strain; [NT] ex-neotype strain (Manamgoda et al. 2012); BRIP: Queensland Plant Pathology Herbarium, Queensland, Australia; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; UTHSC: Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, Texas.

UNIVERSITAT ROVIRA I VIRGILI
PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF CLINICAL ISOLATES OF BIPOLARIS, CURVULARIA, EXSEROHILUM AND PITHOMYCES
Keith Cassia Da Cunha
DL:T 1479-2014

4.4. Molecular identification and *in vitro* response to antifungal drugs of clinical isolates of *Exserohilum*.

da Cunha KC, Sutton DA, Gené J, Capilla J, Cano J, Guarro J.

Antimicrobial Agents and Chemotherapy 2012, 56:4951-4954.

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Molecular Identification and *In Vitro* Response to Antifungal Drugs of Clinical Isolates of *Exserohilum*

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***Exserohilum* is an agent of human and animal mycoses. Although classification has been based on a few subtle morphological differences, three species of clinical interest have been traditionally accepted. In this study, by using a multigene sequence analysis, we have demonstrated that *Exserohilum longirostratum* and *E. mcginnisii* are probable synonyms of *E. rostratum*. The isolates tested were mainly from the nasal region. Antifungal susceptibility testing demonstrated high activity of the eight agents tested against this fungus.**

The anamorphic genus *Exserohilum* (teleomorph *Setosphaeria*, family *Pleosporaceae*, order *Pleosporales*) is comprised of approximately 35 species, which are common saprobic fungi on plant debris, although some cause infections in plants, animals, and humans (1, 9, 10, 23, 24). Although these fungi have a broad geographical distribution, human infections occur predominantly in tropical and subtropical regions, affecting mainly immunocompetent patients (1). *Exserohilum* causes mainly allergic sinusitis, keratitis, and, less frequently, endocarditis, endophthalmitis, peritonitis, and invasive infections affecting brain, bones, lungs, and urinary tract (7, 21, 22). Three species, *E. rostratum*, *E. longirostratum*, and *E. mcginnisii*, have been reported as opportunistic pathogens for humans (1, 3, 19, 22). These species are characterized by quick growth, forming dark colonies, geniculate conidiophores, and ellipsoid to fusiform, straight to curved, multidistoseptate conidia with a protruding hilum. Such species can be mainly differentiated by the conidial morphology (7, 17).

To assess the incidence of *Exserohilum* species in clinical samples, we have identified morphologically and molecularly a set of isolates from different clinical specimens, which were sent to a reference laboratory during a period of 7 years (2003 to 2009) from different regions of the United States for identification and/or antifungal susceptibility testing.

A total of 34 clinical isolates, presumably belonging to *Exserohilum* spp., was received in the Fungus Testing Laboratory of the University of Texas Health Science Center at San Antonio, and some type or reference strains were included in the present study. The majority of the isolates were from the nasal region (47%), followed by cutaneous and subcutaneous infections (20.5%) and ocular infections (14.7%). The remaining 11.6% of the isolates were from abscesses, blood, bronchoalveolar lavage fluid, and lumbar disc, while 6.2% were of unknown origin (Table 1). The fungi were cultured on potato carrot agar (PCA) and oatmeal agar (OA).

The internal transcribed spacer (ITS) region and the 28S ribosomal DNA (rDNA) (D1/D2), actin (ACT), and elongation factor 1-alpha (*EF*-1α) genes were amplified and sequenced following previously described protocols (4, 18, 25).

The *in vitro* activity of eight antifungal agents (amphotericin B, itraconazole, posaconazole, voriconazole, anidulafungin, caspofungin, micafungin, and terbinafine) against the isolates tested was evaluated according to reference guidelines (6). *Paecilomyces variotii* ATCC MYA-3630 was used as a quality control strain.

All the isolates examined displayed the typical features of the genus *Exserohilum* (24). Thirty-two isolates showed straight or slightly curved conidia, which were ellipsoidal to fusiform or rostrate, with smooth to finely roughened walls and brown to olivaceous brown coloring, measuring 25 to 91 by 9 to 22 μm, with 4 to 9 distosepta and dark bands at both ends, and were identified as *E. rostratum*. Two isolates were identified as *E. longirostratum*, due to the presence of markedly larger conidia (up to 228 by 12 to 19 μm), which had 6 to 16 distosepta and were centrally curved (7, 24). The type strain of *E. mcginnisii* was examined and showed straight, cylindrical, or slightly clavate conidia, which were smooth-walled and brown, measuring 44 to 76 by 11 to 18 μm, with 4 to 8 distosepta and without dark bands at both ends (7, 17).

With the primers used, we were able to amplify and sequence 392 to 411, 317 to 318, 464, and 609 bp of the ITS, ACT, D1/D2, and *EF*-1α loci, respectively. Sequences of the four regions obtained from the 37 isolates (34 clinical isolates and 3 type or reference strains) included in the study were analyzed phylogenetically. Comparison of such sequences unequivocally proved that all the isolates tested belonged to a single species (Table 2), which demonstrated that *E. longirostratum* and *E. mcginnissii* are synonyms of *E. rostratum*.

In general, all the antifungal drugs tested showed relatively low MICs against *Exserohilum* isolates, with only a few exceptions for echinocandins. Caspofungin and micafungin showed relatively high MICs against seven isolates and anidulafungin against one (Table 3).

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TABLE 1 Clinical isolates and type and reference strains of *Exserohilum* spp. included in the study[a]

Species	Isolate	Origin	GenBank accession no.			
			ITS	D1/D2	ACT	EF-1α
E. longirostratum	UTHSC 03-3090	Skin BX, Maryland	HE664036	HE664028	HE664076	HE664085
E. longirostratum	UTHSC 05-424	Intervertebral disc, Louisiana	HE664039	HE664030	HE664078	HE664083
E. longirostratum	IP 1229.80	Heart valve prosthesis, Martinique	HE664033	HE664025	HE664072	HE664080
E. mcginnisii	CBS 325.87(T)	Nasal polyp, Arizona	HE664035	HE664027	HE664074	HE664082
E. rostratum	UTHSC 03-1932	Maxillary sinus, Texas	HE664063			
E. rostratum	UTHSC 03-3639	Arm tissue, Colorado	HE664046			
E. rostratum	UTHSC 04-1248	Nasal wash, Texas	HE664038			
E. rostratum	UTHSC 04-2744	Sphenoid sinus, Texas	HE664058			
E. rostratum	UTHSC 04-2416	Sinus, Texas	HE664068	HE664029	HE664077	HE664086
E. rostratum	UTHSC 04-2629	Sinus, Texas	HE664066			
E. rostratum	UTHSC 04-3327	Middle turbinate tissue, Texas	HE664047			
E. rostratum	UTHSC 05-3456	Sinus, Texas	HE664052			
E. rostratum	UTHSC 06-2113	Cornea, Texas	HE664040			
E. rostratum	UTHSC 06-1857	Eye, California	HE664042			
E. rostratum	UTHSC 06-2618	Nasal (frog), Massachusetts	HE664043			
E. rostratum	UTHSC 06-3237	Great toe, Texas	HE664045			
E.rostratum	UTHSC 06-3226	Eye, Texas	HE664060			
E. rostratum	UTHSC 06-530	Ethmoid sinus, South Carolina	HE664064			
E. rostratum	UTHSC 07-263	Sinus, Minnesota	HE664048			
E. rostratum	UTHSC 07-1310	Unknown, Texas	HE664057			
E. rostratum	UTHSC 07-1292	Shin skin, Texas	HE664037			
E. rostratum	UTHSC 07-3092	Nose wound, Arkansas	HE664059			
E. rostratum	UTHSC 07-1498	Unknown, Texas	HE664041			
E. rostratum	UTHSC 07-622	Blood, Texas	HE664049			
E. rostratum	UTHSC 08-3261	Wound, Utah	HE664053			
E. rostratum	UTHSC 08-655	Elbow, Texas	HE664054			
E. rostratum	UTHSC 08-2771	Cornea, Utah	HE664050			
E. rostratum	UTHSC 08-922	Nasal BX, Florida	HE664065	HE664032	HE664075	
E. rostratum	UTHSC 08-3638	Sinus, Utah	HE664061			
E. rostratum	UTHSC 08-2940	Inferior turbinate tissue, Texas	HE664055			
E. rostratum	UTHSC 09-2018	Abscess, South Carolina	HE664069	HE664031	HE664079	HE661084
E. rostratum	UTHSC 09-131	Maxillary sinus, Montana	HE664062			
E. rostratum	UTHSC 09-718	Maxillary sinus, Texas	HE664056			
E. rostratum	UTHSC 09-1259	Eye, Georgia	HE664051			
E. rostratum	UTHSC 09-1211	Inferior turbinate tissue, Texas	HE664067			
E. rostratum	UTHSC 09-109	Bronchial wash, Minnesota	HE664044			
E. rostratum	CBS 467.75(T)	Soil, India	HE664034	HE664026	HE664073	HE664081

[a] UTHSC, Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, TX; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; IP, Institut Pasteur, Paris, France; (T), type strain; BX, biopsy.

Some studies have clearly demonstrated the ability of *Exserohilum* to cause humans infections, although they are mainly single case reports where the fungal identification was based exclusively on morphological criteria (7). This is the first study in which a large panel of *Exserohilum* clinical isolates from different anatomical sites has been identified using phenotypic and genetic criteria. The anatomical sites from where the fungi were isolated agree with those reported in previous studies (8, 15, 20, 21).

Exserohilum species are mainly identified by the conidial morphology seen when growing in the natural substratum (24). *In vitro* identification is more difficult, the conidia tending to be smaller and the isolates losing the ability to sporulate (17, 24). In this study, all the isolates grew well in the media used, although they sporulated better on PCA than on OA. However, the conidia never reached the maximum length described for any of those species in the natural substrate.

Previous taxonomic and molecular studies suggested that *E. rostratum* and *E. longirostratum* could be the same species (7, 13, 14). The present study proved through a multilocus analysis that all isolates, including the type strain of *E. mcginnisii* and reference strains of *E. longirostratum*, showed a high homology with the type strain of *E. rostratum*, indicating that all are probable conspecific species. However, further studies sequencing additional genes are required to demonstrate such synonymy. *In vitro* antifungal susceptibility data for *Exserohilum* spp. are very variable; however, they are scarce and, in general, are from studies performed before the standardization of the procedures for antifungal susceptibility testing for molds (2, 5, 11, 12, 16). The high *in vitro* activity of all the antifungals tested here against *Exserohilum* is remarkable, although it is unknown how this can be translated to clinical practice. Data on the clinical treatment of infections by *Exserohilum* are also very scarce, but recent reviews of cases of sinusitis and cutaneous infections by these fungi report successful outcomes with amphotericin B and more recently with itraconazole and voriconazole (8, 15).

TABLE 3 Results of *in vitro* antifungal susceptibility testing for 34 clinical isolates of *Exserohilum rostratum*[a]

Antifungal agent	MIC or MEC (μg/ml) at 48 h GM range		MIC_{90} (μg/ml) at 48 h	MIC or MEC (μg/ml) at 72 h GM range		MIC_{90} (μg/ml) at 72 h
Amphotericin B	0.02	<0.03 to 0.125	0.03	0.02	<0.03 to 0.125	0.03
Anidulafungin	0.06	<0.03 to 1	0.125	0.10	<0.03 to >16	0.25
Caspofungin	0.06	<0.03 to >16	0.125	0.21	<0.03 to >16	2
Itraconazole	0.02	<0.03 to 0.125	0.03	0.02	<0.03 to 0.125	0.03
Micafungin	0.27	<0.03 to >16	0.05	0.41	0.03 to >16	0.5
Posaconazole	0.03	<0.03 to 0.125	0.03	0.03	<0.03 to 0.5	0.06
Terbinafine	0.02	<0.03 to 0.03	0.03	0.03	<0.03 to 0.25	0.06
Voriconazole	0.10	<0.03 to 1	0.25	0.14	0.03 to 1	0.25

[a] GM, geometric mean.

TABLE 2 Percentages of similarity among type and reference strains of the three clinically relevant *Exserohilum* species[a]

Strain	% identity to indicated strain by: ITS			D1/D2 (28′S)			ACT			EF-1α		
	IP 1229.80 *E. longirostratum*	CBS 325.87(T) *E. mcginnisii*	CBS 467.75(T) *E. rostratum*	IP 1229.80 *E. longirostratum*	CBS 325.87(T) *E. mcginnisii*	CBS 467.75(T) *E. rostratum*	IP 1229.80 *E. longirostratum*	CBS 325.87(T) *E. mcginnisii*	CBS 467.75(T) *E. rostratum*	IP 1229.80 *E. longirostratum*	CBS 325.87(T) *E. mcginnisii*	CBS 467.75(T) *E. rostratum*
UTHSC 04-2416 *E. rostratum*	99.7	99.7	99.5	100	100	100	98.8	98.8	99	99.1	99.2	99.2
UTHSC 09-2018 *E. rostratum*	99.7	99.7	99.5	100	100	100	99.8	99.8	99.2	98.9	99.1	99.1
UTHSC 05-424 *E. longirostratum*	99.7	99.7	99.5	100	100	100	99	99	98.4	98.9	98.7	98.7
IP 1229.80 *E. longirostratum*		100	99.8		100	100		99.6	99		99.9	99.9
CBS 325.87(T) *E. mcginnisii*			99.8			100			99			99.7
CBS 467.75(T) *E. rostratum*												

[a] (T), type strain.

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We alone are responsible for the content and writing of this paper.

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4.5. *Pithomyces* species (Montagnulaceae) from clinical specimens: identification and antifungal susceptibility profiles.

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Pithomyces species (Montagnulaceae) from clinical specimens: identification and antifungal susceptibility profiles

Short title: *Pithomyces* in clinical specimens and antifungal profiles

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Key words: *Pithomyces,* DNA analysis, Clinical specimens, Morphological identification, Antifungal susceptibility.

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ABSTRACT

The fungal genus *Pithomyces* comprises numerous dematiaceous saprobic speciescommonly found on dead leaves or stems of a great variety of plants. Occasionally, they have been recovered also from clinical specimens. We have morphologically and molecularly (rDNA sequences) investigated a set of 42 isolates tentatively identified as *Pithomyces* from clinical specimens in the United States. The predominant species were *Pithomyces chartarum* and *Pithomyces sacchari* (33.3% each), followed by *Pithomyces* sp. I (28.6%) and *Pithomyces maydicus* (4.8%). The isolates were recovered mainly from superficial tissue (50%), the respiratory tract (21.4%), and the nasal region (19%). In general these fungi were highly susceptible *in vitro* to the eight antifungal agents tested.

INTRODUCTION

Pithomyces is a large ascomycetous genus of Pleosporales with species commonly colonizing dead leaves or stems of many different plants [1]. Nevertheless, some species have also been isolated from mammals with various disease symptoms. *Pithomyces chartarum*, the most widespread species of the genus, has been reported to cause facial eczema in some animals (i.e., sheep, cattle, goats or deer), which has been associated with a cutaneous response due to a liver damage caused by a mycotoxin (sporidesmin) produced by this fungus [2-6]. This species has been also reported as an agent of human onychomycosis [7, 8]. In addition, an unidentified *Pithomyces* isolate has been involved in a case of peritionitis in a patient with vulvar cancer [9]. Several studies have reported also the presence of *Pithomyces* conidia in the indoor air of asthma patients [10-12]. However, usually an accurate identification of the *Pithomyces* isolates at species level has not been done.

The genus *Pithomyces* is above all characterized by subspherical to clavate, brown, commonly verruculose or echinulate, and variably septate (i.e. transverse, oblique or/and longitudinal septa) conidia, borne at the apices of short peg-like conidiophores arising laterally on the hyphae [1, 13]. The genus comprises near 50 species. *Pithomyces chartarum* is the only species associated with a sexual stage named *Leptosphaerulina chartarum* [14]. Since *Leptosphaerulina* belongs to the family Didymellaceae (Pleosporales), *Pithomyces* has been taxonomically placed in that family [15].

Considering the few studies on *Pithomyces* from clinical origin and the availability of numerous isolates of that genus obtained from human specimens in a mycology reference laboratory in the United States, the aim of the present work was to identify a set of those isolates by both morphological and molecular methods, and to determine the *in vitro* susceptibility to different antifungal agents.

MATERIALS AND METHODS

Fungal isolates: A total of 42 isolates obtained from clinical specimens, received at the Fungus Testing Laboratory of the University of Texas Health Science Center at San Antonio from the different regions of the United States, were investigated (Table 1). In addition, the ex-type strain of *Leptosphaerulina chartarum* and reference strains of

different *Pithomyces* species, obtained from the Mycothèque de L'Université Catholique de Louvain (MUCL, Belgium) and from the CBS-KNAW Fungal Biodiversity Centre (CBS, The Netherlands), were also included in the study for morphological and molecular comparison (Table 1).

Morphological study: The isolates were cultured on potato carrot agar (PCA; 20 g of potatoes, 20 g of carrots, 20 g of agar, 1 liter of distilled water) and oatmeal agar (OA; 30 g of filtered oat flakes, 20 g of agar, 1 liter of distilled water), incubated at 25°C for 7-21 days. Microscopic features were examined by making direct wet mounts in 85% lactic acid from the different culture media. The isolates were morphologically identified following the criteria of Ellis [1, 13, 16] and Rao and de Hoog [17].

Molecular study: Isolates were grown on yeast extract sucrose (YES; 2% yeast extract, 15% sucrose, 2% agar, 1 L of water) for 3 days at 25°C, and DNA was extracted and purified directly from fungal colonies following the FastDNA kit protocol (Bio101,Vista, CA), with a minor modification, i.e. a homogenization step was repeated three times with a FastPrep FP120 instrument (Thermo Savant, Holbrook, NY). The ITS region and D1/D2 domains of the 28S rDNA were amplified and sequenced with the primer pairs ITS5 and NL4b, following previously described protocols [18, 19]. The ITS PCR products were purified and sequenced at Macrogen Europe (Amsterdam) using a 3739XL DNA analyzer (Applied Biosystems). SeqMan (Lasergene, Madison, WI, USA) was used to obtain consensus from the complementary sequences of each isolate. BLAST sequence identity searches with ITS and D1/D2 regions sequences were carried out to compare data from the isolates studied with those deposited in the GenBank database.

Two alignments were performed in the present study using Clustal X version 1.81 [20]. The first included ITS and D1/D2 sequences of the clinical isolates and a set of seven sequences representing five species of *Pithomyces* (one of these retrieved from GenBank) (Table 1). The second included D1/D2 representative sequences of our clinical isolates, the sequence of the ex-type strain of *L. chartarum* and species representative of different families within the order Pleosporales [15, 21]. Phylogenetic analyses were conducted using MEGA v. 5.05 with the Maximum likelihood (ML) method. A search for the best model of nucleotide substitution was performed for both alignments. In the first analysis the best model was Kimura 2-parameter, and in the

second was Tamura-Nei. The analyses were performed with pairwise deletion of gaps [22]. The robustness of branches was assessed through the use of bootstrap analyses of 1000 replicates. Sequences of *Massarina phragmiticola* (NBRC 105268: 12117501 and 12117502 from NITE) and *Bipolaris maydis* (CBS 136.29: HG326306 and HG326307 from GenBank) were used as out groups in the combined analysis.

Antifungal susceptibility: A total of 31 isolates, those that showed enough sporulation to prepare a standardized conidial inoculum, were evaluated for antifungal susceptibility according to reference guidelines [23], with a minor modification (i.e, incubation at 30ºC). The drugs tested were anidulafungin, amphotericin B (AMB), caspofungin (CAS), itraconazole (ITC), voriconazole (VRC), micafungin (MFG), posaconzole (PSC) and terbinafine (TBF). *Paecilomyces variotii* ATCC MYA-3630 was used as a quality control strain. The MIC endpoint was 100% inhibition for AMB, ITC, VRC, and PSC. The minimum effective concentration (MEC) for the echinocandins was the lowest concentration where a visible change in the growth characteristics compared to the growth control was observed. Endpoint determinations were read at 48 hours.

RESULTS

Of the 42 clinical isolates studied, 35 were morphologically identified as members of the genus *Pithomyces*. Seven isolates did not sporulate, but their ITS and D1/D2 sequences clustered with the rest of *Pithomyces* clinical isolates investigated.

Figure 1 shows the ML phylogenetic tree inferred from the combined analysis of ITS and D1/D2 sequences. The length of the alignment used in the analysis was 976 bp (545 bp corresponding to ITS and 431 bp to D1/D2 regions). In this analysis, all clinical isolates were distributed into 4 clades taxa, of which three were found to correspond to known species, i.e. *Pithomyces chartarum* (14 isolates, 33%), *Pithomyces sacchari* (14 isolates, 33.3%) and *Pithomyces maydicus* (2 isolates, 4.8%). 12 isolates (28.6%) formed an isolated clade. Morphologically, they did not much with any known species and were referred to as *Pithomyces* sp. I.

The *P. sacchari* clade (96% bootstrap support (BS)) included three reference strains and fourteen clinical isolates. The similarity among these seventeen isolates ranged from 99.2-100%. The isolates were characterized by having beige, grey or olivaceous brown colonies. The majority of these isolates (4 did not sporulate) produced

smooth to verruculose, brown, ellipsoidal or slightly clavate conidia, with 2–5 transverse septa and 1 oblique or longitudinal septum, and measuring 12–31 x 5–10 µm in the broadest part (Fig. 2A,B).

The *Phytomyces* sp. I clade comprised twelve clinical isolates and two reference strains, previously identified as *P.atro-olivaceous* (CBS244.96) and *P. cynodontis* (CBS 925.87) respectively. The similarity among the isolates of this clade ranged from 99.6–100%. They showed yellowish white to brownish grey colonies and produced conidia of variable shape, i.e. clavate, navicular, pyriform or turbinate with 1–6 transverse septa and, occasionally, 1–2 oblique or longitudinal septa, verruculose to verrucose, measuring 11–36 x 5–15 µm (Fig. 2C).

The *P. maydicus* clade included two clinical isolates and one reference strain of this species with 100% similarity. The species was characterized by white to greyish yellow colonies and conidia with 2–3 transverse septa, rarely with 1 oblique or longitudinal septum, cylindrical or slightly clavate, pale brown, verrucose and measuring 12–19 x 4–8 µm (Fig. 2D,E).

Finally, the *P. chartarum* clade (90% BS) included nine clinical isolates and three more sequences of that species, two of which corresponding to reference strains and one sequence retrieved from GenBank (UBC F15184). They showed a similarity of 99.5–100%. These isolates had white to dark grey colonies and produced conidia usually broadly ellipsoidal, with 3 transverse septa often constricted, and 1–2 longitudinal septa at the middle cells, brown to mid brown, verruculose to echinulate, 12–33 x 9–19 µm (Fig. 2F-H).

Leptosphaerulina chartarum was described as the teleomorph of *P. chartarum* [14]. However, in our phylogenetic analysis, the ex-type strain of *L. chartarum* was shown to belong neither to the *P. chartarum* clade nor to be related to any of the *Pithomyces* lineages evidenced (data not shown). An additional phylogenetic analysis of the D1/D2 domain sequences was carried out to determine affinities of this ex-type strain (Fig.3). The length of the alignment used in the analysis was of 433 bp and it showed that this strain of *L. chartarum* was related with members of the family Didymellaceae, as well as other species of the genus *Leptosphaerulina*. However, the *Pithomyces* species investigated were close to species of the genus *Letendraea*, that belong to the Montagnulaceae.

The antifungal susceptibility test showed, in general, a high activity for the 8 drugs tested against the isolates tested, with posaconazole, caspofungin and terbinafine being the most potent agents (Table 2).

DISCUSSION

Species of *Pithomyces* have been rarely investigated as far as human health is concerned. Although some species were isolated from mammals, including humans, showing lesions, it is not known whether they are real pathogens. Their relevance as possible human pathogens has been rarely raised and never tested. However, as an increasing number of clinical isolates of *Pithomyces* were received at the Fungus Testing Laboratory in recent years, it seems relevant to explore more accurately their possible pathogenic role. This is the largest study involving of *Pithomyces* isolates from clinical samples.

In the majority of previous studies, *Pithomyces* identifications were based solely on morphological features, and only rarely were they identified to species level [7, 24-26]. BLAST search based on ITS sequences is currently a limited tool for *Pithomyces* identification because there are only a few sequences deposited in GenBank and most of them belong to *P. chartarum* labelled under the name *Leptosphaerulina chartarum*.

In our study, 4 species were recognized, the most commonly identified being, in decreasing order, *P. chartarum, P. sacchari* and *Pithomyces sp.* I. *Pithomyces maydicus* was represented only by two clinical isolates. In the literature, *P. chartarum* is the only species associated with clinical cases [2,3,6,27]. *Pithomyces maydicus* and *P. sacchari* have only been reported as saprobic on plants [1,16]. Nevertheless, twelve clinical isolates, labelled as *Pithomyces* sp. I, could not be identified to any known species. These isolates were highly variable as far as the shape and number of septa of their conidia is concerned. None of the known *Pithomyces* species shows such morphological variation. Therefore, our results suggest that *Pithomyces* sp. I could represent a new species. To ascertain this, an extensive taxonomical study should be carried out with more reference and ex-type strains of the different species described. However, as mentioned before, *Pithomyces* comprises near of 50 species and unfortunately practically no ex-type or reference strains exist. In addition, the type of *P. flavus*, the

type species of the genus, is not available. This is an important handicap to clarifying the taxonomy of this genus.

Pithomyces belongs to the Pleosporales, a large order which includes numerous plant and animal pathogens. Species of Pleosporales are often characterized by dark brown often septate (phragmo- or dictyoseptate) conidia. Well-known genera includes *Alternaria, Curvularia, Exserohilum* or *Stemphylium* [28]. Morphologically they differ from *Pithomyces* in the type of conidiogenesis, which is mono- or polytretic, and in the conidial secession, which is schizolytic in the above-mentioned genera. *Pithomyces* has monoblastic conidia with rhexolytic secession. As mentioned before, since the teleomorph of *P. chartarum* was identified as a member of *Leptosphaerulina* [14], *Pithomyces* was considered an anamorphic genus of the family Didymellaceae [15, 21]. However, in our study none of the *Pithomyces* isolates, including those received from different culture collections and the sequences of *L. chartarum* retrieved from GenBank, were grouped with *Leptosphaerulina* species or other members of the Didymellaceae. According to our D1/D2 analysis, all the *Pithomyces* isolates studied, even those identified as *P. chartarum,* grouped with members of the family Montagnulaceae (Fig 3). To confirm that *L. chartarum* was related to that latter family, we sequenced the ex-type of *L. chartarum* (CBS 329.86), but surprisingly its sequence fell with other *Leptosphaerulina* species into the Didymellaceae clade (Fig 3). We examined morphologically the ex-type strain of *L. chartarum* and found that developed ascomata and ascospores, which matched with the protologue description (Roux 1986), but did not produce the anamorph in any of the culture media and temperatures tested. Considering that the morphology of ascospores of *L. chartarum* and conidia *P. chartarum* are very similar, some confusion in the interpretation of such structures could occur during the study of the former fungus. What is clear is that our data suggest that *L. chartarum* is not the sexual stage of *P. chartarum*, but a more extensive study with more strains of both taxa should be done to clarify this issue.

In our study, the *Pithomyces* isolates originated mainly from superficial tissue, the respiratory tract and nasal region, anatomic sites where other melanized anamorphic fungi such as *Curvularia* or *Exserohilum* are also commonly found [29]. The potent *in vitro* activity of most of the antifungal drugs tested against *Pithomyces* species was

remarkable, which could offer different therapeutic options for the treatment of infections caused by these fungi.

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FIG 1. Maximum Likelihood tree inferred from combined ITS and D1-D2 region of the 28S gene sequences of the isolates listed in Table 1. Branch lengths are proportional to the distance. Reference strains are in bold.


UTHSC 06-3688
MUCL 15288 P. sacchari
UTHSC 04-2746
UTHSC 10-1977
UTHSC 06-3844
CBS 803.72 P. sacchari
UTHSC 10-670
CBS 120504 P. sacchari
UTHSC 03-3221
UTHSC 09-1473
UTHSC 08-426
UTHSC 12-56
UTHSC 04-2483
UTHSC 05-3251
UTHSC 03-1337
UTHSC 07-1285
UTHSC 10-2143
Pithomyces sacchari
71
96
UTHSC 05-3161
UTHSC 06-4528
UTHSC 06-3492
UTHSC 10-2776
UTHSC 07-578
UTHSC 11-3528
CBS 224.96 P. atro-olivaceous
UTHSC 06-3706
UTHSC 07-995
UTHSC 05-3373
UTHSC 08-535
UTHSC 07-1535
UTHSC 09-3190
CBS 925.87 P. cynodontis
Pithomyces sp. I
65
76
58
99
UTHSC 06-3954
CBS 400.73 P. maydicus
UTHSC 06-1549
Pithomyces maydicus
100
UTHSC 04-2495
MUCL 15905 P. chartarum
UTHSC 06-214
UBC F 15184 P. chartarum (DQ384571.1)
UTHSC 08-331
MUCL 9393 P. chartarum
UTHSC 04-678
UTHSC 08-2855
UTHSC 07-3634
UTHSC 10-3088
UTHSC 07-2045
UTHSC 11-2179
UTHSC 07-692
UTHSC 07-2802
UTHSC 03-2472
UTHSC 05-2460
UTHSC 10-2361
Pithomyces chartarum
96
96
90
93
NBRC105268 Massarina phragmiticola
CBS 136.29 Bipolaris maydis
0.05

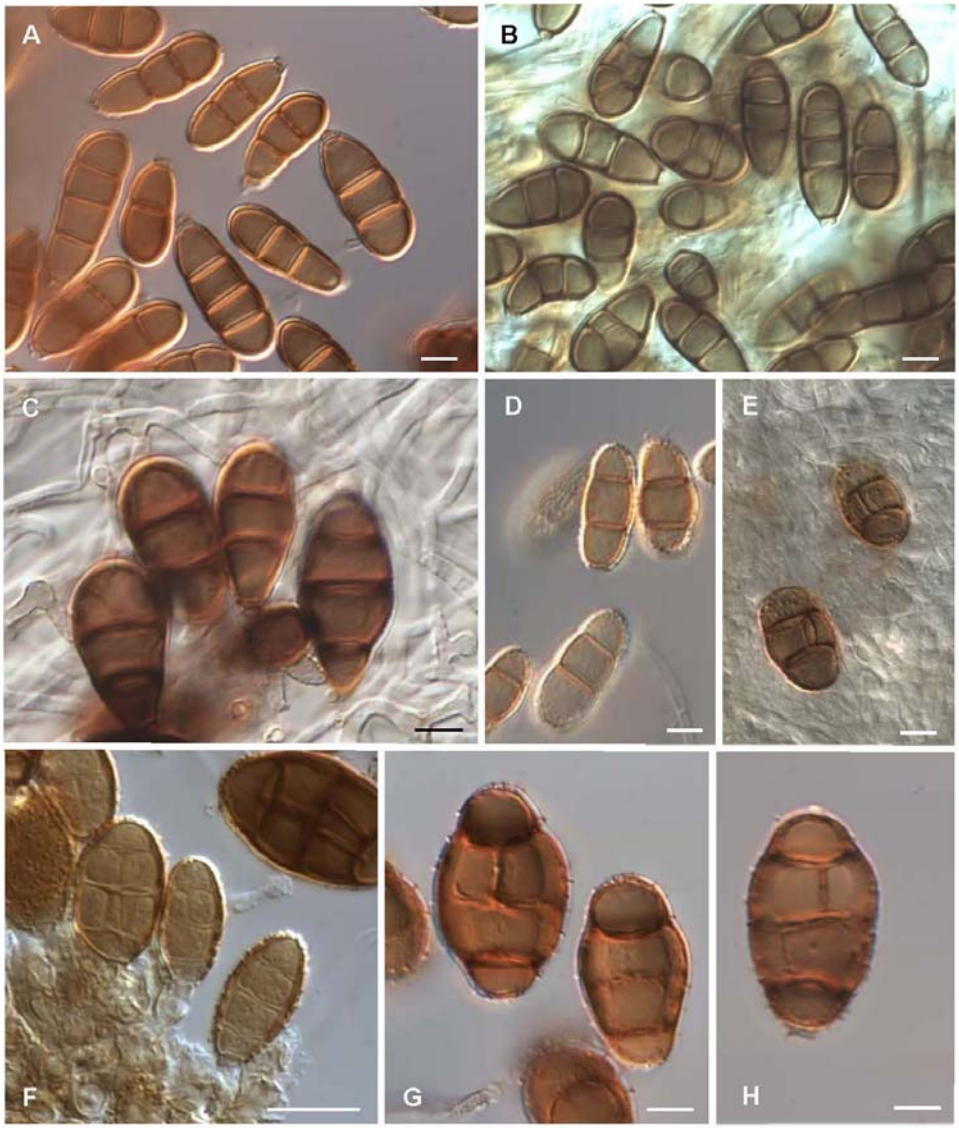


FIG. 2. Conidia of *Pithomyces* spp. on OA at 25ºC after 14 days. A, B. *P. sacchari* (A. UTHSC 07-1285, B. UTHSC 10-2143). C. *Pithomyces* sp. I (UTHSC 06-3492). D, E. *P. maydicus* (UTHSC 06-3954). F-H. *P. chartarum* (F. UTHSC 05-2460; G, H. UTHSC 03-2472). Scale bar: A-E, G, H = 5 µm, F= 10 µm.

FIG 3. Maximum Likelihood tree inferred from D1/D2 region of the 28S gene sequences of species of different pleosporalean families and representatives of our clinical isolates listed in Table 1. Branch lengths are proportional to the distance.

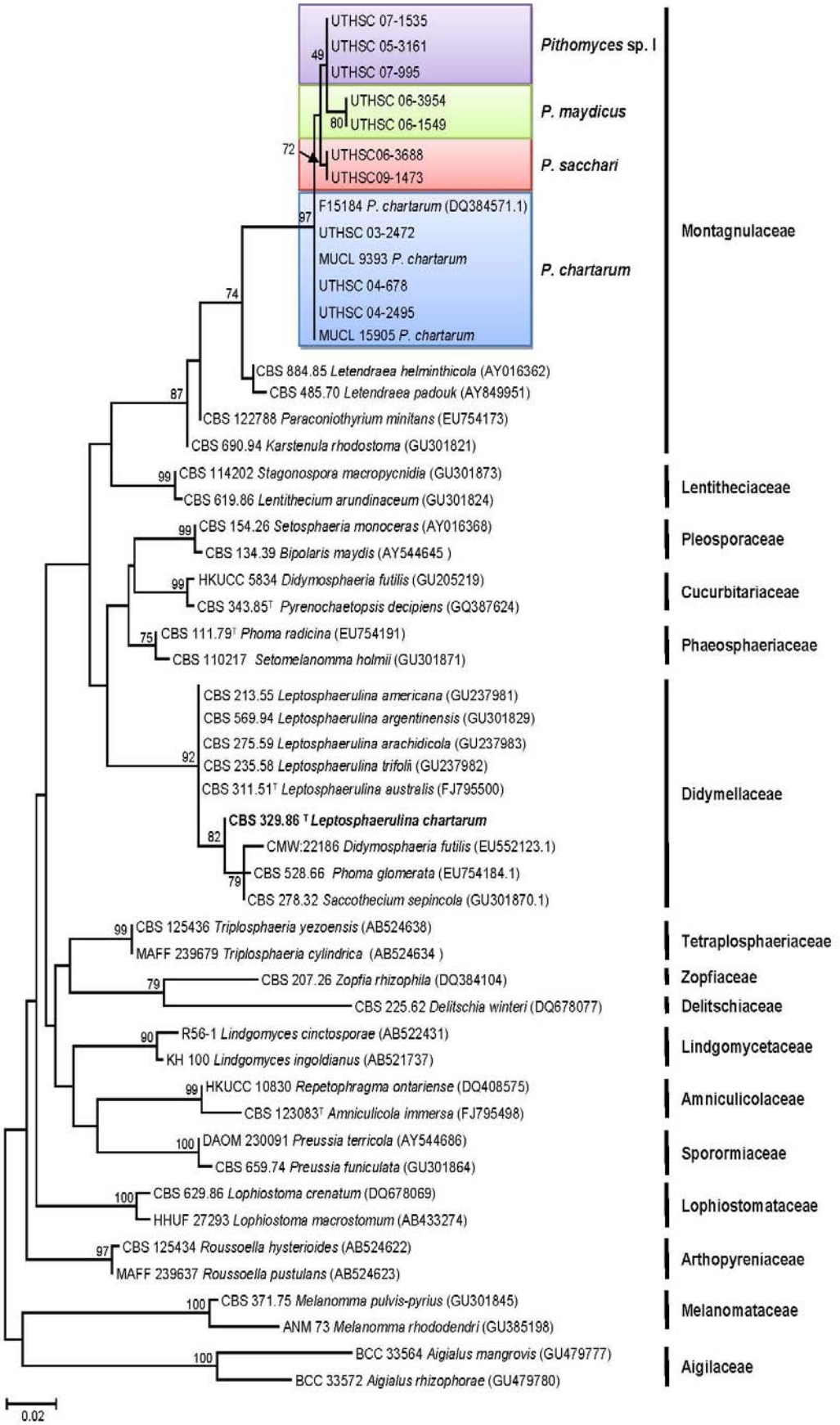

UTHSC 07-1535
UTHSC 05-3161
UTHSC 07-995
Pithomyces sp. I
UTHSC 06-3954
UTHSC 06-1549
P. maydicus
UTHSC06-3688
UTHSC09-1473
P. sacchari
F15184 P. chartarum (DQ384571.1)
UTHSC 03-2472
MUCL 9393 P. chartarum
UTHSC 04-678
UTHSC 04-2495
MUCL 15905 P. chartarum
P. chartarum
Montagnulaceae
CBS 884.85 Letendraea helminthicola (AY016362)
CBS 485.70 Letendraea padouk (AY849951)
CBS 122788 Paraconiothyrium minitans (EU754173)
CBS 690.94 Karstenula rhodostoma (GU301821)
CBS 114202 Stagonospora macropycnidia (GU301873)
CBS 619.86 Lentithecium arundinaceum (GU301824)
Lentitheciaceae
CBS 154.26 Setosphaeria monoceras (AY016368)
CBS 134.39 Bipolaris maydis (AY544645)
Pleosporaceae
HKUCC 5834 Didymosphaeria futilis (GU205219)
CBS 343.85T Pyrenochaetopsis decipiens (GQ387624)
Cucurbitariaceae
CBS 111.79T Phoma radicina (EU754191)
CBS 110217 Setomelanomma holmii (GU301871)
Phaeosphaeriaceae
CBS 213.55 Leptosphaerulina americana (GU237981)
CBS 569.94 Leptosphaerulina argentinensis (GU301829)
CBS 275.59 Leptosphaerulina arachidicola (GU237983)
CBS 235.58 Leptosphaerulina trifolii (GU237982)
CBS 311.51T Leptosphaerulina australis (FJ795500)
CBS 329.86 T Leptosphaerulina chartarum
CMW:22186 Didymosphaeria futilis (EU552123.1)
CBS 528.66 Phoma glomerata (EU754184.1)
CBS 278.32 Saccothecium sepincola (GU301870.1)
Didymellaceae
CBS 125436 Triplosphaeria yezoensis (AB524638)
MAFF 239679 Triplosphaeria cylindrica (AB524634)
Tetraplosphaeriaceae
CBS 207.26 Zopfia rhizophila (DQ384104)
Zopfiaceae
CBS 225.62 Delitschia winteri (DQ678077)
Delitschiaceae
R56-1 Lindgomyces cinctosporae (AB522431)
KH 100 Lindgomyces ingoldianus (AB521737)
Lindgomycetaceae
HKUCC 10830 Repetophragma ontariense (DQ408575)
CBS 123083T Amniculicola immersa (FJ795498)
Amniculicolaceae
DAOM 230091 Preussia terricola (AY544686)
CBS 659.74 Preussia funiculata (GU301864)
Sporormiaceae
CBS 629.86 Lophiostoma crenatum (DQ678069)
HHUF 27293 Lophiostoma macrostomum (AB433274)
Lophiostomataceae
CBS 125434 Roussoella hysterioides (AB524622)
MAFF 239637 Roussoella pustulans (AB524623)
Arthopyreniaceae
CBS 371.75 Melanomma pulvis-pyrius (GU301845)
ANM 73 Melanomma rhododendri (GU385198)
Melanomataceae
BCC 33564 Aigialus mangrovis (GU479777)
BCC 33572 Aigialus rhizophorae (GU479780)
Aigilaceae
0.02

TABLE 1. Clinical isolates, reference strains and sequences of *Pithomyces* spp. included in the study.

Species	**Strain**	**Origin**	**GenBank (or NITE) accession nº**	
			ITS	**D1/D2**
Pithomyces chartarum	MUCL 15905	Unknown substrate, Belgium	pending	pending
	MUCL 9393	Unknown substrate, France	pending	pending
	UBC F15184	Conidial drop from *Rubus spectabilis*, Canada	DQ384571.1	DQ384571.1
	UTHSC 03-2472	Skin, Minnesota	HG518059	HG518064
	UTHSC 04-678	Lt. maxillary sinus, Tennessee	HG518060	HG518065
	UTHSC 04-2495	Foot, Montana	HG518061	HG518066
	UTHSC 05-2460	Maxilary sinus, Tennessee	HG518062	HG518067
	UTHSC 06-214	Toe, Colorado	HG518063	HG518068
	UTHSC 07-692	Scalp, Texas	pending	pending
	UTHSC 07-2045	Skin, Utah	pending	pending
	UTHSC 07-2802	Toe nail, Massachusetts	pending	pending
	UTHSC 07-3664	Unknown, Illinois	pending	pending
	UTHSC 08-331	BAL, Pennsylvania	pending	pending
	UTHSC 08-2855	BAL, Pennsylvania	pending	pending
	UTHSC 10-2361	Sinus, Minnesota	pending	pending
	UTHSC 10-3088	BAL, Washington	pending	pending
	UTHSC 11-2179	Lt. pectoralis muscle, Michigan	pending	pending
Pithomyces maydicus	CBS 400.73	Dead leaf in *Cocos nucifera, Sri Lanka*	pending	pending
	UTHSC 06-1549	Toe nail, South Carolina	pending	pending
	UTHSC 06-3954	Maxillary sinus, Texas	pending	pending
Pithomyces sacchari	CBS120504	Millet, Namibia	pending	pending

Species	Strain	Origin	GenBank (or NITE) accession nº	
			ITS	D1/D2
Pithomyces sacchari	CBS 803.72	Seed, South Africa	pending	pending
	MUCL 15288	Air, South Africa	pending	pending
	UTHSC 03-1337	BAL, Florida	pending	pending
	UTHSC 03-3221	Nasal, Tennessee	pending	pending
	UTHSC 04-2483	BAL, Texas	pending	pending
	UTHSC 04-2746	Maxillary sinus, Texas	pending	pending
	UTHSC 05-3251	BAL, South Carolina	pending	pending
	UTHSC 06-3688	Leg, Arizona	pending	pending
	UTHSC 06-3844	Scalp, Texas	pending	pending
	UTHSC 07-1285	Nail, South Carolina	pending	pending
	UTHSC 08-426	Maxillary sinus, Tennessee	pending	pending
	UTHSC 09-1473	Leg, Texas	pending	pending
	UTHSC 10-670	Lung, Florida	pending	pending
	UTHSC 10-1977	Sputum, North Carolina	pending	pending
	UTHSC 10-2143	Sinus, Tennessee	pending	pending
	UTHSC 12-56	Cornea, Louisiana	pending	pending
Pithomyces sp. I	CBS 244.96 (received as *P. atro-olivaceous*)	Skin scrapings of female diabetic, USA	pending	pending
	CBS 925.87 (received as *P. cynodontis*)	Stem of *Spartocytisus supranubius*, Spain	pending	pending
	UTHSC 05-3161	Nail, South Carolina	pending	pending

Species	Strain	Origin	GenBank (or NITE) accession nº	
			ITS	D1/D2
Pithomyces sp. I	UTHSC 05-3373	BAL, Florida	pending	pending
	UTHSC 06-3492	Unknown, Florida	pending	pending
	UTHSC 06-3706	Cornea, Florida	pending	pending
	UTHSC 06-4528	Foot, Florida	pending	pending
	UTHSC 07-578	Toe nail, Florida	pending	pending
	UTHSC 07-995	Toe nail, Florida	pending	pending
	UTHSC 07-1535	Scalp, Florida	pending	pending
	UTHSC 08-535	Skin feet, Texas	pending	pending
	UTHSC 09-3190	Asian elephant eye, Massachusetts	pending	pending
	UTHSC 10-2776	BAL, South Carolina	pending	pending
	UTHSC 11-3528	Leg tissue, Maryland	pending	pending
Leptosphaerulina chartarum	CBS 329.86 [T]	*Galenia procumbens,* South Africa	pending	pending
Massarina phragmiticola	NBRC105268	Decayed drift wood in the intertidal zone , Japan	(12117501)	(12117502)
Bipolaris maydis	CBS 136.29	Unknown, Japan	HG326306	HG326307

UTHSC, Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, TX; NRBC, NITE Biological Resource Center, Japan; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; MUCL, Mycothèque de L'Université Catholique de Louvain, Faculté des Sciences Agronomiques, Louvain-la-Neuve; UBC Herbarium - Beaty Biodiversity Museum University of British Columbia. [T] Type.

Table 2. Results of *in vitro* antifungal susceptibility testing for *Pithomyces* spp.

Species (nº of isolates)		MIC/MEC (µg/ml) 48 h							
		AMB	**VRC**	**PSC**	**ITC**	**CAS**	**AFG**	**MFG**	**TRB**
Pithomyces chartarum	**GM**	0.06	0.12	0.06	0.06	0.04	0.1	0.1	0.06
(14)	**MIC range**	< 0.03 - 0.12	0.03 - 0.5	< 0.03 - 0.12	< 0.03 - 0.25	0.03 - 0.06	0.03 - 0.25	0.03 - 0.25	0.03 - 0.12
	MIC_{90}	0.05	0.12	0.12	0.12	0.06	0.12	0.12	0.06
***Pithomyces* sp. I (8)**	**GM**	0.02	0.03	0.01	0.02	0.04	0.04	0.04	0.02
	MIC range	<0.03-0.03	<0.03-0.06	< 0.03 - 0.03	<0.03-0.03	<0.03-0.03	0.03-0.12	0.03-0.06	<0.03-0.06
	MIC_{90}	-	-	-	-	-	-	-	-
Pithomyces sacchari	**GM**	0.39	0.66	0.04	0.04	0.05	0.03	0.06	0.03
(7)	**MIC range**	<0.03 - 2	<0.03 - 4	< 0.03 - 0.12	< 0.03 - 0.12	< 0.03 - 0.12	0.03	0.03-0.12	0.03 - 0.06
	MIC_{90}	-	-	-	-	-	-	-	-
Pithomyces maydicus	**GM**	0.04	0.04	< 0.03	<0.03	0.03	0.03	0.03	< 0.03
(2)	**MIC range**	0.03 - 0.06	0.03 - 0.06	< 0.03	<0.03	< 0.03	0.03	0.03	< 0.03
	MIC_{90}	-	-	-	-	-	-	-	-
Total (31)	**GM**	0.12	0.21	0.04	0.06	0.04	0.06	0.07	0.04
	MIC range	< 0.03 - 2	<0.03 - 4	< 0.03 - 0.12	<0.03-0.25	0.03-0.12	0.03 - 0.25	0.03-0.025	<0.03-0.12
	MIC_{90}	0.12	0.25	0.06	0.12	0.06	0.12	0.12	0.06

GM, geometric mean; AFG, anidulafungin; AMB, amphotericin B; CAS, caspofungin; ITC, itraconazole; VRC, voriconazole; MFG, micafungin; PSC, posaconazole; TRB, terbinafine.

4.6. Preliminary results on the taxonomy of *Pithomyces* species

In our previous study on clinical isolates of *Pithomyces*, it became evident that the taxonomy of the genus was problematic and that species delineation was not clear. In this sense, the sequences of several reference strains belonging to the same *Pithomyces* species, i.e. *P. atro-olivaceous*, *P. sacchari* and *P. cynodontis*, were included in different phyloclades. It was also surprising that the isolates that had been morphologically identified as *P. chartarum* were divided in two subgroups, a separation which suggests the possible existence of cryptic species in that morphospecies. In addition, in that first work the taxonomy of the phyloclade labelled as *Pithomyces* sp. I remained unresolved in the original study, mainly because the statistical support that received the clade in the combined analysis with the ITS and the D1/D2 region of the rDNA was rather low, a result which indicates that more than one species is likely to be included there. The ITS-5.8S rDNA operon has previously been selected as the barcoding region to distinguish between fungal species (Schoch et al. 2012), but several molecular phylogenetic studies have demonstrated that rDNA regions have a very low taxonomical resolution in Pleosporales, especially when they are used to distinguish between closely related species (Mugambi & Huhndorf 2009, Zhang et al. 2009, 2012). However, good results have been obtained in the establishment of phylogenetic relationships among pleosporalean fungi with the use of the protein-conding genes *GPDH* and *RPB2* (Manamgoda et al. 2012, Woudenberg et al. 2013). Thus, the objective of this study was to reconstruct phylogenetic relationships among species in the genus *Pithomyces* through the use of increased taxon sampling and more phylogenetically informative loci.

To do so, the clinical isolates of *Pithomyces* species studied previously (see Section 4.5, pag. 119) were morphologically examined and sequenced, along with a total of 42 reference strains of the all *Pithomyces* species that were available the MUCL and in the CBS culture collections (see Annex I). This investigation was carried out in part in the former institution, supervised by the Dr. Cony Decock scientific responsible for the MUCL, and in collaboration with Dr. Hugo Madrid from the CBS.

4.6.1. Phylogenetic study

To perform the molecular phylogenetic study of the genus *Pithomyces,* we used sequences of the ITS region and D1/D2 domains of the 28S rRNA gene and fragments

of the genes *GPDH* and *RPB2* (553, 433, 521 and 810 bp length, respectively). ML analysis was employed in the phylogenetic reconstruction for each locus. The best evolutionary models proposed by the MEGA v.5.0 software in these analysis were: Kimura 2-parameter + discrete Gamma distribution (G) (ITS and D1/D2), Tamura-Nei (TN93) + certain fraction of sites are evolutionarily invariable for *GPDH,* and TN93 + G for *RPB2* as well as for the concatenated four-loci dataset. ML phylogenetic trees of the four loci were congruent (data not shown), and they were therefore combined. Partial trees revealed that *RPB2* and *GPDH* were the most informative loci when compared to ITS and D1/D2 domains, which were found to have 4 and 2 clusters, respectively, with significant bs. The combined analysis of ITS and D1/D2 loci presented in the preliminary publication (see Section 4.5) proved to be insufficient to molecularly differentiate between the species of *Pithomyces* investigated.

In the concatenated four-locus tree (Fig. 13), almost all of the *Pithomyces* strains were included in a strongly supported clade (100% bs), which contained different, well supported subclades that clearly represent different species. However, the reference strains of *P. pulvinatus* and *P. valparadisiacum* were placed very distant from this main clade, what suggests that these species belong to other genera (see below). The multilocus analysis corroborated two of the four *Pithomyces* species identified in our previous study: *P. maydicus* and *P. sacchari.* The other two, represented by the *P. chartarum* and *Pithomyces* sp. I clades, were split into different lineages, each of which had sufficient distance from each other to probably represent five different species, including one strain which resulted the only different from those previously identified as *P. sacchari*.

The clade (79% bs) labelled as *Pithomyces* sp. I was composed of ten of the twelve clinical isolates of our previous study, as well as reference strains of *P. atro-olivaceous*, *P. cynodontis, P. maydicus* and *P. sacchari.* There was a high similarity (98.8 - 100%) between them. However, the isolates included in this sister clade still present morphological variability that should be studied in more detail.

The clade named *Pithomyces* sp. II is composed of two isolates (UTHSC 06-4528 and UTHSC 07-578) with 100% similarity between them and 95.1 – 96% similarity to the isolates of the clade *Pithomyces* sp. I. These two isolates may represent a new species for the genus.

The clade of *P. karoo* (100 bs) did not include any clinical isolates, and was comprised of only a reference strain of *P. quadratus* (MUCL 9365) and the isotype strain of *P. karoo* (CBS 804.72). A similarity of 99.9% was observed between them.

Pithomyces sp. III is represented by only one clinical isolate (UTHSC 03-3221), which is closely related to the *P. sacchari* clade. The well-supported clade of *P. sacchari* (98 bs), which was comprised of three reference strains of the species and 13 clinical isolates, was found to have an intraspecific variability of 98.5 - 99.8%. The similarity between *Pithomyces* sp. III and the *P. sacchari* clade ranged between 93.5 - 95%.

The *P. maydicus* clade (99 bs) included two clinical isolates, seven environmental reference strains of *P. maydicus*, and one strain received as *P. graminicola.* The similarity among all these isolates was 97.8 - 99.8%.

The clinical isolates identified in our previous study as *P. chartarum* were divided into two sister clades through this multilocus analysis, clades which were sufficiently distant enough from each other to represent two different species. The similarity between these sister clades was 94.6%. One clinical isolate (UTHSC 08-2855) of the first study could not be included in this phylogenetic analysis because the amplification of the *RPB2* gene was impossible. The first sister clade (96 bs), labelled *Pithomyces* sp. IV, was composed of two clinical isolates and two MUCL strains identified as *P. chartarum*. The clade (99% bs) that was recognized as a representative of *P. chartarum* was composed of 11 clinical isolates, nine reference strains of this species and one strain that had probably been erroneously identified as *P. cynodontis*.

A reference strain of *P. pulvinatus* (CBS 662.79) was genetically very different (71.9 –73.7% similar) and placed far from the other species of *Pithomyces* that were investigated (Fig. 13). The BLAST search of the ITS region was inefficient for confirming the real identification of this strain; the closest sequences were *Pithomyces valparadisiacus* (EU552152.1) and *Periconia* sp. (HQ607981.1). The result of the ITS BLAST search showed an identity of 86% and query cover of 69% with the sequence of *P. valparadisiacus* (EU552152.1), and of 87% and 54%, respectively, with that of *Periconia* sp. (HQ607981.1).

Two available reference strains of *P. valparadisiacus* were also included in the present analysis. These two strains and other received as *P. atro-olivaceous* comprised a

well-supported clade (100 % bs), but were placed phylogenetically far away from the clade of *Pithomyces* spp. It is worth of mentioning that *P. valparadisiacus* was initially described as the type species of the genus *Stemphyliomma* and, on the basis of morphological features, transferred to the genus *Pithomyces* by Kirk (1983). Our molecular results demonstrated that *P. valparadisiacus* does not belong to the latter genus, and therefore, we preferred to maintain its original identification as *Stemphyliomma valparadisiacum.*

4.6.2. Taxonomy

Based on the results obtained in the multilocus phylogenetic analysis, and also after a preliminary morphological characterization process of the *Pithomyces* strains, we have been able to identify the following known species of *Pithomyces*: *P. chartarum*, *P. karoo*, *P. maydicus* and *P. sacchari*. However, despite the fact that *Pithomyces* sp. I to IV correspond to different phylospecies, we still need to examine herbarium material of holotypes of other known *Pithomyces* species, which are available in different collections, before concluding with certainty that they are new species. Therefore, the following is a detailed description of each *Pithomyces* species detected in the present investigation, organized in alphabetical order in the case of known species and in numerical order in the case of the unnamed *Pithomyces* phyloclades.

Pithomyces chartarum (Berk. & M.A. Curtis) M.B. Ellis, Mycological Papers 76: 13 (1960).

≡ *Sporidesmium chartarum* Berk. & M.A. Curtis, Grevillea 3 (26): 50 (1874).

≡ *Piricauda chartarum* (Berk. & M.A. Curtis) R.T. Moore, Rhodora 61: 96 (1959).

= *Sporidesmium bakeri* Syd. & P. Syd., Annales Mycologici 12 (2): 204 (1914).

Colonies on OA and PCA reached 70 and 75 mm in diameter, respectively, after 7 days at 25ºC; with dark brown to black cushion-like structures on the agar surface, covered by a white aerial mycelium composed of a network of branched and anastomosing hyphae. Vegetative hyphae, septate, subhyaline to pale olive, smooth-walled, occasionally verruculose, 2-5 µm wide. Conidiophores arising laterally on the hyphae, straight or slightly curved, cylindrical, hyaline or subhyaline, 2.5-10 x 2-3.5 µm. Conidia arising singly as blown-out ends at the apex of each conidiophore, broadly ellipsoidal, (2-) 3 (-5) transverse septa and middle cells usually with 1 or 2 longitudinal

septa, often constricted at the septa, brown to dark brown when mature, verruculose to echinulate, 12-30 x 5-18 μm. Sexual morph not observed.

Specimens examined. THE NETHERLANDS: Wageningen, culture from agricultural soil, *J.H. van Emden* 1971 (CBS 679.71 = IHEM 3811); Wageningen, culture from of root tip on *Solanum tuberosum*, *J.H. van Emden* 1970 (CBS 807.70). FRANCE: unknown location, unknown origin, *W. Marcasas* (CBS 120293 = MRC 6036). SOUTH AFRICA: Deenegeur, Kareedouw, culture from pasture material from outbreak of facial eczema, *M. Schlechter* 1990 (CBS 120283 = MRC 5988); Oudtshoorn, Le Roux, culture from *Lucerne stubble*, *C. Griesel* 1980 (CBS120281 = MRC2090). NEW ZEALAND: unknown location, culture from Ryegrass pasture, *W. Marasas* (CBS 120282 = MRC 4393). BRAZIL: Pernambuco, Recife, culture from soil, *H.P. Upadhyay* 1966 (MUCL 15433 = CBS 486.66 = ATCC 32853 = FMR 13107). USA: Ithaca, New York, culture from soil, *G.L. Hennebert* 1962 (MUCL 4262 = FMR 13115); Sacramento, California, culture from chronic nasal polyposis (human), *M. R. Rinaldi* 1996 (CBS 243.96 = UTHSC 92-1518); Texas, culture from human scalp (human), *D. A. Sutton* 2007 (UTHSC 07-692 = FMR 12461); Pennsylvania, culture from BAL (human), *D. A. Sutton* 2008 (UTHSC 08-331 = FMR 12469); Tennessee, culture from maxillary sinus (human), *D. A. Sutton* 2005 (UTHSC 05-2460 = FMR 12448); Tennessee, culture from human Lt. maxillary sinus (human), *D. A. Sutton* 2004 (UTHSC 04-678 = FMR 12444); Illinois, culture of unknown origin, *D. A. Sutton* 2007 (UTHSC 07-3664 = FMR 12468); Utah, culture from skin (human), *D. A. Sutton* 2007 (UTHSC 07-2045 = FMR 12466); Massachusetts, culture from toenail (human), *D. A. Sutton* 2007 (UTHSC 07-2802 = FMR 12467); Minnesota, culture from skin (human), *D. A. Sutton* 2003 (UTHSC 03-2472 = FMR 12442); Minnesota, culture from sinus (human), *D. A. Sutton* 2010 (UTHSC 10-2361 = FMR 12479); Washington, culture from BAL (human), *D. A. Sutton* 2010 (UTHSC 10-3088 = FMR 12481); Michigan, culture from Lt. pectoralis muscle (human), *D. A. Sutton* 2011 (UTHSC 11-2179 = FMR 12482).

Note. This is an ubiquitous species, which has been found on very different substrates (i.e a wide range of plant species, air, soil, hay, sawn timber and ceiling plaster) and geographical locations (Europe, Africa, Asia, Australasia, North America, Central America and South America) (Ellis 1960, Watanabe 2002, Domsch et al. 2007).

Pithomyces karoo Marasas and I.H. Schum., Bothalia 10: 511 (1972).

Colonies on OA and PCA reached 60 and 47 mm diameter, respectively, after 7 days at 25ºC, effuse, with velvety or powdery mycelium, and black. Vegetative hyphae, septate, pale brown to mid-brown in color, smooth or verruculose, and 2-6 µm wide. Conidia arinsing singly as blown-out ends at the apex of each conidiophore, very variable in shape, ellipisoidal, pyriform, clavate, subglobose or cruciform, commonly with 1-3 transverse and longitudinal septa, straw-colored to dark brown, verrucose, 12-27 x 7-20 µm. Sexual morph not observed.

Specimens examined. SOUTH AFRICA: Colesberg, Cape Province, culture from dead stem on *Gnidia polycephala, W.F.O. Marasas* 1972 (Isotype: CBS H-7655; ex-type cultures: MUCL 54970 = CBS 804.72 = ATCC 24322 = IMI 155881 = PRE 44605 = FMR 13088); Potchefstroom, Transvaal, culture from litter on *Acacia karrroo, M. Papendorf* 1966 (MUCL 9365 = FMR 13112).

Note. It is a species that has been described as saprophytic on different plants (i.e. *Acacia karrroo, Castanopsis diversifolia, Euterpe oleracea, Avena*, *Gnidia, Rhigozum* spp. and wheat rhizosphere) in South Africa, Australia, Brazil, and Thailand (Ellis 1976, Jeamjitt et al. 2006, Castro et al. 2011).

Despite the fact that MUCL 9365 strain was previously identified as *P. quadratus,* it shows morphological features more similar to those of *P. karoo*. The molecular analysis of the isolate confirms that it is a misidentified strain of *P. quadratus*.

Pithomyces maydicus (Sacc.) M.B. Ellis, Mycological Papers 76: 15 (1960).

≡ *Clasterosporium maydicum* Sacc., Nuovo Giornale Botanico Italiano 23 (2): 213 (1916).

≡ *Sporidesmium bakeri* var. *maydicum* (Sacc.) S. Hughes, Mycological Papers 50: 68 (1953).

≡ *Scheleobrachea maydica* (Sacc.) S. Hughes, Canadian Journal of Botany 36 (6): 802 (1958).

Colonies on OA and PCA reached 60 and 22 mm in diameter, respectively, after 7 days at 25ºC, cottony, olive to olivaceous brown, Vegetative hyphae, septate, subhyaline to olive, smooth-walled, and 2-4 µm wide. Conidiophores arising laterally on the hyphae, straight or slightly curved, cylindrical, hyaline or subhyaline, 2-6 x 1.5-2.5 µm. Conidia arising singly as blown-out ends at the apex of each conidiophore, broadly oval, with 2 (or very rarely 3) transverse septa, middle cells occasionally

divided by 1 longitudinal septum, often constricted at the septa, straw-colored or golden brown, verruculose or finely echinulate, 12-24 x 4-11 µm. Sexual morph not observed.

Specimens examined. MARTINIQUE: Fort-de-France, culture from necrotic leaf spot in *Lycopersicon esculentum, B. Houstachy* 1988 (CBS 491.88 = MUCL 54992 = FMR 13087); isolated from an unknown origin and substrate (CBS 134.29 = MUCL 54994 = FMR 13091). SINGAPORE: culture from dead twigs and wood samples, *C. Decock* 2002 (MUCL 48406 = FMR 13102). SRI LANKA: Anuradhapura, culture from dead leaf on *Cocos nucifera,* W. Gams 1973 (CBS 400.73 = MUCL 54971 = FMR 13090). ZIMBABWE: culture from dead wood, *C. Decock* 1996 (MUCL 50413 = FMR 13100). MALAWI: culture from dead wood, *C. Decock* 1991 (MUCL 33070 = FMR 13103); isolated from an unknown origin and substrate; *C. Decock* 1995 (MUCL 39247 = FMR 13110). PAPUA NEW GUINEA, Madang Province, culture from soil in tropical forest, *A. Aptroot* 1995 (CBS 977.95 = MUCL 54993 = FMR 13089); USA: South Carolina, culture from toe nail (human), *D. A. Sutton* 2006 (UTHSC 06-1549 = FMR 12453); Texas, culture from maxillary sinus (human), *D. A. Sutton* 2006 (UTHSC 06-3954 = FMR 13058).

Notes. It is a common species usually found on plant debris from different geographic regions, including Africa, Asia, South America, Central America, North America, and Europe (Ellis 1960, Watanabe 2002, Domsch et al. 2007). Most of clinical isolates of this species examined only rarely had conidia with longitudinal septa and that fact may hinder the correct identification of *P. maydicus*.

Pithomyces sacchari (Speg.) M.B. Ellis, Mycological Papers 76: 17 (1960).

≡ *Sporidesmium sacchari* Speg., Anales del Museo Nacional de Historia Natural Buenos Aires 20 (13): 443 (1910).

≡ *Scheleobrachea sacchari* (Speg.) S. Hughes, Canadian Journal of Botany 36 (6): 802 (1958).

= *Stigmella sacchari* Speg., Revista de la Facultad de Agronomía Universidade Nacional de La Plata 2 (19): 251 (1896).

Colonies on OA and PCA reached 67 and 58 mm in diameter, respectively, after 7 days at 25ºC, firstly punctiform, and often later effused, black. Vegetative hyphae septate, olive to brown, smooth-walled or occasionally verruculose, and 2 to 5 µm wide. Conidiophores arising laterally on the hyphae, straight or curved, cylindrical, hyaline or subhyaline, 2-7 x 1.5-3 µm wide. Conidia arising singly at the apex of each

conidiophore, obovoid, clavate or pyriform, sometimes ellipsoidal, with 2-5 transverse septa and occasionally 1 longitudinal or oblique septa, sometimes slightly constricted at the septa, pale brown when young, becoming dark brown to nearly black when mature, smooth-walled to verruculose, 12-31 x 5-11 μm. Sexual morph not observed.

Specimens examined. NAMIBIA: Okasha Kingondjo, Ovamboland, culture from millet, *A. Lubben* 1975 (CBS 120504 = MRC 0682 = MUCL 54996 = FMR 13095). SOUTH AFRICA: Johannesburg, culture from air, *D. Ordman* 1956 (MUCL 15288 = CBS 306.56 = FMR 13109); Oudshoorn, Cape Province, culture from *Medicago sativa* seed, *W.F.O. Marasas* 1972 (CBS 803.72 = ATCC 24323 = PRE 44585 = MUCL 54974 = FMR 13096). USA: Florida, culture from BAL (human), *D. A. Sutton* 2003 (UTHSC 03-1337 = FMR 12441); Texas, culture from BAL (human), *D. A. Sutton* 2004 (UTHSC 04-2483 = FMR 12445); Texas, culture from maxillary sinus (human), *D. A. Sutton* 2004 (UTHSC 04-2746 = FMR 12447); South Carolina, culture from BAL (human), *D. A. Sutton* 2005 (UTHSC 05-3251 = FMR 12450); Arizona, culture from leg (human), *D. A. Sutton* 2006 (UTHSC 06-3688 = FMR 12455); Texas, culture from scalp (human), *D. A. Sutton* 2006 (UTHSC 06-3844 = FMR 12457); South Carolina, culture from nail (human), *D. A. Sutton* 2007 (UTHSC 07-1285 = FMR 12464); Tennessee, culture from human maxillary sinus (human), *D. A. Sutton* 2008 (UTHSC 08-426 = FMR 12470); Texas, culture from leg (human), *D. A. Sutton* 2009 (UTHSC 09-1473 = FMR 12473); Florida, culture from leg (human), *D. A. Sutton* 2010 (UTHSC 10-670 = FMR 12476); North Carolina, culture from sputum (human), *D. A. Sutton* 2010 (UTHSC 10-1977 = FMR 12477); Tennessee, culture from sinus (human), *D. A. Sutton* 2010 (UTHSC 10-2143 = FMR 12478); Louisiana, culture from cornea (human), *D. A. Sutton* 2012 (UTHSC 12-56 = FMR 12484).

Notes. It is a worldwide species found on soil, air, on millet and rice seeds, on leaves and other parts of various plants (Ellis 1960, Watanabe 2002, Domsch et al. 2007). It is the most identified species among our clinical isolates. However, the morphological features observed were slightly different to those of the description of the species provided by Ellis (1960); i.e., usually ellipsoidal conidia, with 1-3 transverse septa and 1 or 2 longitudinal or oblique septa, and smooth to verruculose walls. Probably it is due to that Ellis' descriptions of *Pithomyces* species are based from specimens growing on the natural substratum and it is well-known that the morphology

of these fungi, especially that of the conidia, can vary considerably *in vitro* (Domsch et al. 2007), fact that makes very difficult the identification of these fungi from cultures.

***Pithomyces* sp. I**

Colonies on OA and PCA reached 70 and 64 mm in diameter, respectively, after 7 days at 25ºC, pulverulent or woolly, yellowish-white to brownish-grey. Vegetative hyphae septate, subhyaline to brown, smooth to asperulate. Conidiophores arising laterally on the hyphae, straight or slightly curved, cylindrical, hyaline or subhyaline. Conidia variable in shape, clavate, navicular, pyriform or turbinate, with 1-6 transverse septa and occasionally with 1 to 2 oblique or longitudinal septa, golden brown to brown, smooth-walled when young, becoming verruculose to verrucose when mature, 11-36 x 5-16 µm, basal cell usually longer than the rest of cells, 6-15 µm long. Sexual morph not observed.

Specimens examined. MALAWI: culture from dead twig, *C. Decock* 1991 (MUCL 33109 = FMR 13104, MUCL 33112 = FMR 13106, MUCL 33110 = FMR 13105*,* MUCL 33111 = FMR 13117). ZIMBABWE: culture from dead twig, *C. Decock* 1996 (MUCL 50391 = FMR 13113, MUCL 50400 = FMR 13101). FRANCE: Nogent-sur-Marne, culture from wood, *G.L. Hennebert* 1966 (as *P. sacchari*, MUCL 8515 = FMR 13111). SPAIN: Tenerife, culture from stem of *Spartocytisus supranubius, J.A. von Arx* 1987 (as *P. cynodontis*, CBS 925.87 = MUCL 54969 = FMR 13085). KUWAIT: culture from salt-marsh soil, *A.F. Moustafa* 1972 (as *P. atro-olivaceous,* CBS 621.72 = ATCC 32852 = MUCL 54967 = 13082); culture from soil, *A.F. Moustafa* 1972 (as *P. maydicus*, CBS 521.72 = MUCL 54972 = FMR 13092). USA: Florida, culture from skin scrapings (human) *M. R. Rinaldi* 1996 (as *P. atro-olivaceous*, CBS 244.96 = MUCL 54968 = FMR13083); Florida, culture from soil *A. Al-Musallam* 1977 (as *P. cynodontis,* CBS 500.77 = MUCL 54991 = FMR 13086); Maryland, culture from leg tissue (human) *D. A. Sutton* 2011 (UTHSC 11-3528 = FMR 12483); Massachusetts, culture from Asian elephant eye, *D. A. Sutton* 2009 (UTHSC 09-3190 = FMR 12475); Texas, culture from foot skin (human), *D. A. Sutton* 2008 (UTHSC 08-535 = FMR 12471); Florida, culture from scalp (human), *D. A. Sutton* 2007 (UTHSC 07-1535 = FMR 12465); Florida, culture from toenail (human), *D. A. Sutton* 2007 (UTHSC 07-995 = FMR 12463); Florida, culture from cornea (human), *D. A. Sutton* 2006 (UTHSC

06-3706 = FMR 12456); Florida, culture from BAL (human), *D. A. Sutton* 2005 (UTHSC 05-3373 = FMR 12451); Florida, culture of unknown origin, *D. A. Sutton* 2006 (UTHSC 06-3492 = FMR 12454); South Carolina, culture from nail (human) *D. A. Sutton* 2005 (UTHSC 05-3161 = FMR 12449); South Carolina, culture from BAL (human), *D. A. Sutton* 2010 (UTHSC 10-2776 = FMR 12480).

Notes. Pithomyces sp. I is a heterogeneous clade in terms of the shape of the conidia. As evidence of this morphological heterogeneity is the fact that it includes strains previously identified as belonging to other species (i.e. *P. atro-olivaceous*, *P. cynodontis* and *P. maydicus).* Despite of that, we can observe a conidial pattern different to that from other species studied, that is elongated conidia with a basal cell usually longer and narrower. This morphology better fits with the conidial morphology of *P. cynodontis* rather than that of *P. atro-olivaceous*. However, we cannot to confirm the identification of *Pithomyces* sp. I until doing a more extensive morphological study, comparing holotypes of the above-mentioned species and reexamining all those isolates growing on agar culture medium containing plant debris. With that we hope to get conidia with a morphology more similar to that described in the literature for the species.

***Pithomyces* sp. II**

Colonies on OA and PCA reached 41 and 35 mm in diameter, respectively, after 7 days at 25ºC, pulverulent, initially yellowish white becoming greyish beige due to the abundant sporulation. Vegetative hyphae, septate, subhyaline to brown, smooth-walled to asperulate. Conidiophores arising laterally on the hyphae, straight or slightly curved, cylindrical, hyaline or subhyaline. Conidia ellipsoidal, obovoid, sometimes clavate, with only 3 (-5) transverse septa, brown to dark brown, verruculose to tuberculate, 23-36 x 7-10 µm, basal cell usually longer than the other cells, up to 15 µm. Sexual morph not observed.

Specimens examined. USA: Florida, culture from foot (human), *D. A. Sutton*, 2006 (UTHSC 06-4528 = FMR 12459); Florida, culture from toenail (human), *D. A. Sutton* 2007 (UTHSC 07-578 = FMR 12460).

Notes. *Pithomyces* sp. II is similar to *P. atro-olivaceous*. However, the conidia of the former are verruculose to tuberculate, concolorous, lack a longitudinal septum, and

possess an elongated basal cell, while *P. atro-olivaceous* has conidia with end cells often paler than those in the middle, they can present 1 oblique or longitudinal septum, and the cell wall is verruculose to echinulate.

***Pithomyces* sp. III**

Colonies on OA and PCA reached 61 and 42 mm in diameter, respectively, after 7 days at 25ºC, floccose, olive brown at the centre, yellowish white towards the periphery. Vegetative hyphae, septate, subhyaline to brown, smooth-walled to asperulate. Conidiophores arising laterally on the hyphae, straight or slightly curved, cylindrical, hyaline or subhyaline. Conidia cylindrical, obvoid or slightly clavate, with only 2 (-3) transverse septa, often constricted at the septa, golden brown, smooth-walled to verruculose, 19-27 x 6-8 µm. Sexual morph not observed.

Specimen examined. USA: Tennessee, culture from Rt. Nasal (human), *D. A. Sutton* 2003 (UTHSC 03-3221 = FMR 12443).

Notes. In the phylogenetic tree (Fig. 13), *Pithomyces* sp. III is a sister taxon of *P. sacchari.* Unfortunately, with the culture media used thus far (PCA and OA), it was impossible to find any morphological differences between *Pithomyces* sp. III and *P. sacchari.*

***Pithomyces* sp. IV**

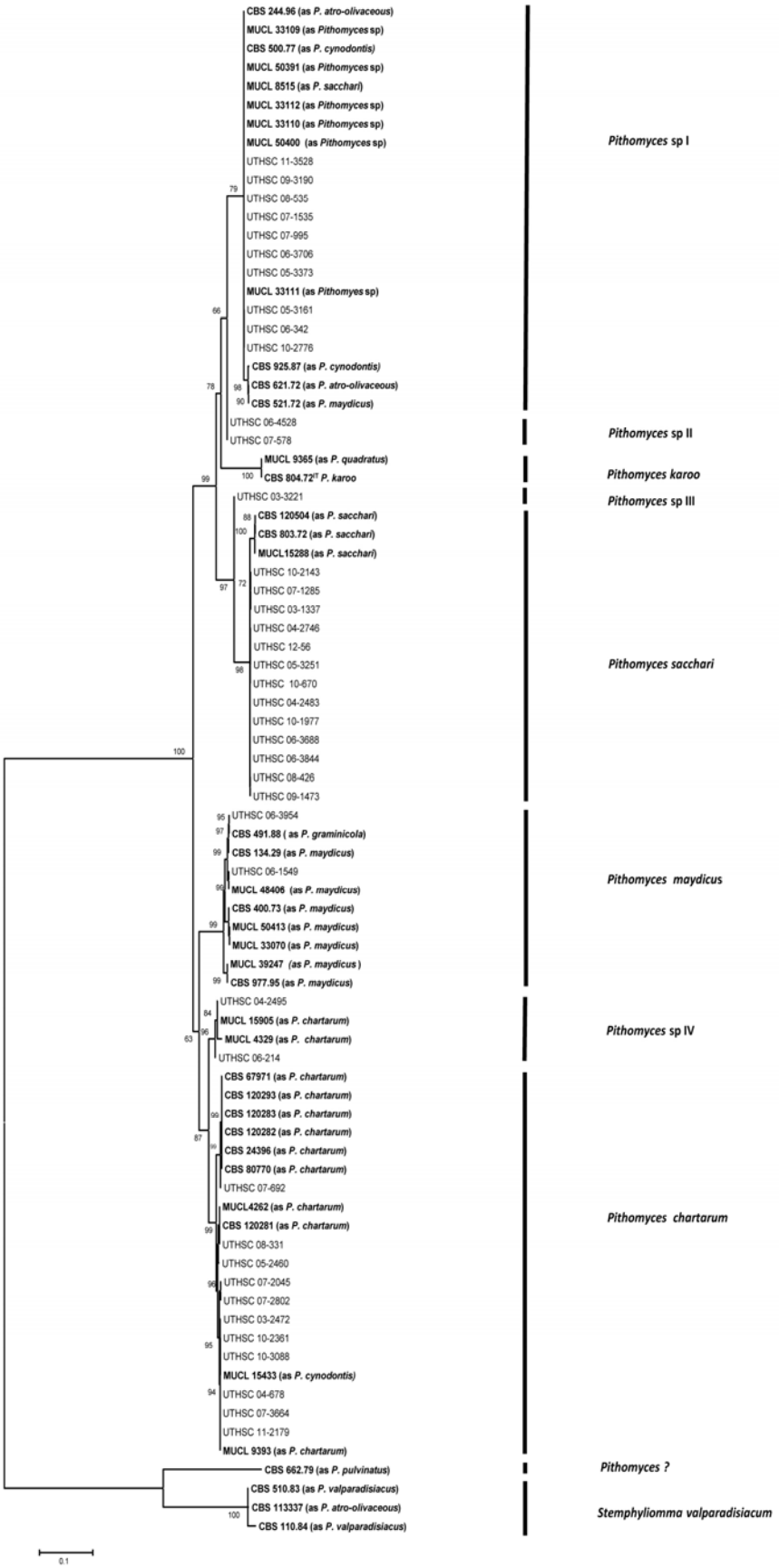
Colonies on OA and PCA reached 65 and 47 mm in diameter, respectively, after 7 days at 25ºC, effuse, granular due to the presence of cushion- like structures, white to olive brown. Vegetative hyphae, septate, subhyaline to brown, smooth to asperulate. Conidiophores arising laterally on the hyphae, straight or slightly curved, cylindrical, hyaline or subhyaline. Conidia cylindrical, broadly ellipsoidal, occasionally slightly clavate, with 3 (-4) transverse septa, sometimes with 1 or 2 longitudinal septa, often constricted at the septa, brown to dark brown when mature, verruculose to tuberculate, 15-33 x 5-15 µm. Sexual morph not observed.

Specimens examined. BELGIUM: Diest, Molenstede, culture from a dust, *G.L. Hennebert* 1970 (MUCL 15905 = FMR 13114). FRANCE: Saône et Loire, culture from calcareous scree, *J. Nicot* 1954 (MUCL 4329 = DAOM 45409 = LCP 1885 = FMR 13116); USA: Montana, culture from foot (human), *D.A. Sutton* 2004 (UTHSC 04-2495

= FMR 12446); Colorado, culture from toe (human), *D.A. Sutton* 2006 (UTHSC 06-214 = FMR 12452).

Notes. Pithomyces sp. IV is similar to *P. chartarum* in conidial morphology. However, the conidia of *P. chartarum* are slightly smaller (up to 29 x 17 μm) and usually the middle cells possess longitudinal septa, while those of *Pithomyces* sp. IV are more variable in the shape and the cell wall can become tuberculate. Our phylogenetic study indicates that *Pithomyces* sp. IV and *P. chartarum* are sister taxa, but with a considerable genetic distance (Fig. 13).

Fig. 13. Maximum likelihood tree obtained from the combined ITS, LSU, *GPDH* and *RPB2* alignment of *Pithomyces*. Branch lengths are proportional to the distance. Reference strains are in bold.


CBS 244.96 (as P. atro-olivaceous)
MUCL 33109 (as Pithomyces sp)
CBS 500.77 (as P. cynodontis)
MUCL 50391 (as Pithomyces sp)
MUCL 8515 (as P. sacchari)
MUCL 33112 (as Pithomyces sp)
MUCL 33110 (as Pithomyces sp)
MUCL 50400 (as Pithomyces sp)
UTHSC 11-3528
UTHSC 09-3190
UTHSC 08-535
UTHSC 07-1535
UTHSC 07-995
UTHSC 06-3706
UTHSC 05-3373
MUCL 33111 (as Pithomyes sp)
UTHSC 05-3161
UTHSC 06-342
UTHSC 10-2776
CBS 925.87 (as P. cynodontis)
CBS 621.72 (as P. atro-olivaceous)
CBS 521.72 (as P. maydicus)
UTHSC 06-4528
UTHSC 07-578
MUCL 9365 (as P. quadratus)
CBS 804.72ET P. karoo
UTHSC 03-3221
CBS 120504 (as P. sacchari)
CBS 803.72 (as P. sacchari)
MUCL15288 (as P. sacchari)
UTHSC 10-2143
UTHSC 07-1285
UTHSC 03-1337
UTHSC 04-2746
UTHSC 12-56
UTHSC 05-3251
UTHSC 10-670
UTHSC 04-2483
UTHSC 10-1977
UTHSC 06-3688
UTHSC 06-3844
UTHSC 08-426
UTHSC 09-1473
UTHSC 06-3954
CBS 491.88 (as P. graminicola)
CBS 134.29 (as P. maydicus)
UTHSC 06-1549
MUCL 48406 (as P. maydicus)
CBS 400.73 (as P. maydicus)
MUCL 50413 (as P. maydicus)
MUCL 33070 (as P. maydicus)
MUCL 39247 (as P. maydicus)
CBS 977.95 (as P. maydicus)
UTHSC 04-2495
MUCL 15905 (as P. chartarum)
MUCL 4329 (as P. chartarum)
UTHSC 06-214
CBS 67971 (as P. chartarum)
CBS 120293 (as P. chartarum)
CBS 120283 (as P. chartarum)
CBS 120282 (as P. chartarum)
CBS 24396 (as P. chartarum)
CBS 80770 (as P. chartarum)
UTHSC 07-692
MUCL4262 (as P. chartarum)
CBS 120281 (as P. chartarum)
UTHSC 08-331
UTHSC 05-2460
UTHSC 07-2045
UTHSC 07-2802
UTHSC 03-2472
UTHSC 10-2361
UTHSC 10-3088
MUCL 15433 (as P. cynodontis)
UTHSC 04-678
UTHSC 07-3664
UTHSC 11-2179
MUCL 9393 (as P. chartarum)
CBS 662.79 (as P. pulvinatus)
CBS 510.83 (as P. valparadisiacus)
CBS 113337 (as P. atro-olivaceous)
CBS 110.84 (as P. valparadisiacus)
0.1
Pithomyces sp I
Pithomyces sp II
Pithomyces karoo
Pithomyces sp III
Pithomyces sacchari
Pithomyces maydicus
Pithomyces sp IV
Pithomyces chartarum
Pithomyces ?
Stemphyliomma valparadisiacum

5. *SUMMARIZING DISCUSSION*

In recent years, there has been a considerable increase in the number of human infections caused by opportunistic fungal species (de Hoog et al. 2011). Most of these fungi are usually identified by the clinical microbiologists through the examination of their microscopic fertile structures obtained *in vitro*. However, due to the great diversity of the species and often to their taxonomic complexity, the mycological diagnosis of the clinical isolates is commonly limited to the genus level. Identification of opportunistic fungi, especially the filamentous ones, is also problematic because they cannot sporulate *in vitro* or if they can produce spores (ascospores, conidia, etc), their morphology can vary considerably respect to that described for the same fungus when it has grown on the natural substratum (Domsch et al. 2007, Seifert et al. 2011). Moreover, it is well known nowadays that the morphological identification of clinical fungi is not enough for a correct diagnosis, because fungi with a great resemblance can be organisms genetically very different; they can be species with a very different virulence or different response to antifungal drugs. Examples of this fact include the relatively recent studies on *Aspergillus*, *Apophysomyces*, *Fusarium*, *Scedosporium* or *Sporothrix* (Lewis et al. 2005, Marimon et al. 2008, Álvarez et al. 2010). A precise diagnosis of fungal opportunists is therefore crucial, not only for establishing a correct therapy for the patients, but also for the advance in the knowledge of clinical fungi in terms of epidemiology, etc. Therefore, the main goal of our study has been to characterize some clinically relevant opportunistic fungi that are poorly understood by clinicians and clinical microbiologists.

Although there are many opportunistic fungi that are understudied, we have focused our study on four melanized asexual genera, i.e. *Bipolaris*, *Curvularia*, *Exserohilum* and *Pithomyces*, for several reasons: 1) most of these genera include species that have been associated with human diseases; 2) they have a complex and confusing taxonomy due to the great number of species that each genus has; and 3) most of their species have been studied only through traditional methodology, which means morphological criteria. In fact, one of the main problems found in the development of the different studies of the thesis has been the existence of very few previous studies on the modern taxonomy of the fungi involved, and, as a consequence, the absence or existence of few reliable DNA sequences deposited in databases that could be compared with the sequences of the fungi investigated. Another serious problem has been the lack

of cultures of type strains of the different species, which were necessary for comparison. Together, these limitations often hindered in taking decisions on the taxonomy of the fungi studied.

Using morphological criteria and DNA sequencing analysis, we investigated a total of 281 clinical isolates. Most of these isolates were obtained from the Fungus Testing Laboratory (FTL) of the University of Texas Health Science Center at San Antonio, Texas, which regularly receives clinical isolates from different hospitals around the US for their identification and/or for antifungal susceptibility testing. Concretely, we studied 104 isolates of *Bipolaris*, 101 of *Curvularia*, 34 of *Exserohilum* and 42 belonging to the genus *Pithomyces*. All of the isolates were received at the FTL between 2003 and 2012, where they had been tentatively identified at the genus or species level considering only morphological features. With the study of those isolates, we intended to determine the real spectrum of the species in clinical samples from the genera selected and to find out whether it truly differed from the information known up to date.

All of these genera are well-known fungi that can be morphologically distinguished by the type of conidiogenesis and of conidial secession; while *Bipolaris, Curvularia* and *Exserohilum* have mono- or polytretic conidiogenesis and schizolytic conidial secession, *Pithomyces* is characterized by monoblastic conidia with rhexolytic secession (Seifert et al. 2011). All four genera belong to the order Pleosporales, but the first three are closely related genera of the family Pleosporaceae and, therefore, are discussed separately from the genus *Pithomyces*, which, according to our phylogenetic analysis, belongs to the family Montagnulaceae.

5.1 Morphological and molecular identification of *Bipolaris*, *Curvularia* and *Exserohilum*

As mention previously, some species of these three genera are relevant fungi, not only because they are causal agents of human diseases, but also because they are important plant pathogens or can be used in the industry to obtain some secondary metabolites of pharmaceutical interest (Ellis 1960, Sivanesan 1987, de Hoog et al. 2011, Manamgoda et al. 2011). Therefore, advances in the taxonomy of all these fungi, at least

in terms of the delineation of their species, are crucial for a wide range of important economic and health-related reasons.

Until relatively recently in the clinical setting, the relevant species of the genus *Bipolaris* were *B. australiensis*, *B. hawaiiensis*, *B. spicifera*, and, to a lesser extent, *B. papendorfii* (Flanagan & Bryceson 1997, Kobayashi et al. 2008, El Khizzi et al. 2010, Revankar & Sutton 2010, de Hoog et al. 2011, Sheyman et al. 2013). In the genus *Curvularia* several species had been associated with human infection, i.e. *C. brachyspora*, *C. clavata*, *C. geniculata*, *C. inaequalis*, *C. lunata*, *C. pallescens*, *C. senegalensis* and *C. verruculosa* (Wilhelmus & Jones 2001, Revankar & Sutton 2010, de Hoog et al. 2011); while in the genus *Exserohilum*, *E. rostratum* was the most clinically reported species, but two other taxa, *E. longirostratum* and *E. mcginnisii,* had also been described as human pathogens (Padhye et al. 1986, Adler et al. 2006, Al-Attar et al. 2006, Saint-Jean et al. 2007). However, according to our results, the spectrum of clinical species of these three genera is susceptible to change. The use of molecular techniques, not only provokes taxonomical changes of the fungi studied, but also allows us to describe new species as potential pathogenic fungi. In the molecular phylogenetic evaluation of *Bipolaris* and *Curvularia*, Manamgoda et al. (2012) transferred the three clinically relevant species of the former genus to *Curvularia* (i.e. *C. australiensis*, *C. hawaiiensis* and C. *spicifera*); and, according to the results of our studies on clinical isolates of both genera (see Sections 4.1-3 of the thesis), the list of the possible opportunistic species of *Bipolaris* and *Curvularia* can be extended to include *Bipolaris cynodontis*, *B. micropus, B. setariae, C. aeria, C. intermedia, C. protuberata* and *C. pseudorobusta,* and the new proposed *Curvularia* species described from clinical isolates *C. americana*, *C. chlamydospora*, *C. hominis*, *C. muehlenbeckiae* and *C. pseudolunata*. Considering the fact, however, that the pathogenicity of all above-mentioned species still needs to be demonstrated and no cases of human disease by these fungi have been published yet, we agree with Manamgoda et al. (2012) in that, thus far, the pathogenic species of this group of fungi might be restricted to the genus *Curvularia* (see further explanations in Section 5.3). In the case of *Exserohilum*, in contrast, the list of clinical species has been reduced to only one, which is *E. rostratum* (see Section 4. 4 −da Cunha et al. 2012).

It is important to note however, that the identification of all of these species has not been an easy task. We have had to examine phenotypically, sequence and compare all of the data obtained from all clinical isolates with that obtained from the ex-type or reference strains available in the different international collections. This was one of the main reasons that we carried out these studies in collaboration with different mycologists from the CBS-KNAW Fungal Biodiversity Centre from Holland and the MUCL from Belgium. Without such collaborations, it would have been impossible to get the results presented, at least in the period of time that the experimental part of this thesis has been performed.

One of the problems involved in the correct morphological identification of the species of these genera is that the original descriptions of the species investigated were based on the fungi growing directly on the natural substratum (Ellis 1960, 1971, 1976, Sivanesan 1987), which do not always coincide with the morphology showed in axenic cultures. Unfortunately, the best culture conditions for recovering these genera from clinical samples are not known. According to our experience, the isolates of the three genera grew well on PCA and OA media, incubated at 25ºC in darkness. However, *Bipolaris* and *Curvularia* sporulated better on OA, while *Exserohilum* on PCA. On those culture media, most of isolates from the different species identified presented morphological characteristics similar to those of the fungi described in the literature; in general, however, they tended to produce shorter and, in the case of *Exserohilum,* even less septate conidia. Therefore, considering the facts that the morphological differences among species of these genera are mainly based on the conidial morphology and that the interspecific differences are very subtle, *in vitro* identification was rather difficult, especially in the case of some *Bipolaris* or *Curvularia* species. For example, some clinical isolates of *Bipolaris* presented features that were compatible with those described for *B. setariae* and *B. sacchari*. Also, in the case of *Curvularia* isolates, it was difficult to distinguish between those of *C. aeria* and those of *C. lunata* (Sivanesan 1987). Only the DNA sequence analysis of the isolates investigated and of respective ex-type or reference strains allowed for a reliable identification of these species. However, in some cases, the lack of reference material impeded the confirmation of the identification of the isolates at the species level, such as occurred with the clinical

isolates identified as *Curvularia* cf *borreriae*, *C.* cf *clavata*, *C.* cf *inaequalis* and *C.* cf *sorghina*.

This taxonomical problem is not only at the species level, but also the separation between *Bipolaris* and *Curvularia* has been a matter of controversy. Traditionally, the members of both genera were mainly differentiated on the basis of conidial septation; while the conidia of *Bipolaris* were described as distoseptate (i.e. conidia with several cells each delimited by an own inner wall and surrounded by a common outer wall), those of *Curvularia* were euseptate (conidia composed of a single wall with septa that are formed as inward extensions of that wall) (Ellis 1971, 1976, de Hoog et al. 2000, Domsch et al. 2007, Revankar & Sutton 2010). However, many authors have stated that *Curvularia* species also had distoseptate conidia (Alcorn 1983b, 1988, Sivanesan 1987). Therefore, based on the morphological similarities of both genera and also because they share the same teleomorphic genus *Cochliobolus*, several authors have suggested that *Bipolaris* and *Curvularia* are congenerics (von Arx & Luttrell 1979, Seifert et al. 2011). In contrast, *Exserohilum* seems to be a well-delineated genus that can be morphologically distinguished by its large distoseptate conidia, with terminal cells that are usually darker than the rest and by the presence of a protuberant basal hilum, and by having *Setosphaeria* as teleomorph. Taxonomic conflicts between the first two genera have been resolved in part with the results obtained from different molecular studies (Berbee et al. 1999, Manamgoda et al. 2011, 2012), some of which were published almost simultaneously with our first two studies on *Bipolaris* and *Curvularia.*

So far, the ITS region and intervening 5.8S nrDNA has been proposed as a universal barcoding marker to differentiate genera and species of fungi (Iwen et al. 2002, Schoch et al. 2012); however, phylogenetic studies based on the ITS region have demonstrated limited species resolution in members of the order Pleosporales (Pryor & Gilbertson 2000, de Hoog & Horré 2002, Pryor & Bigelow 2003, Park et al. 2008, Brunet et al. 2013). As an alternative, protein-coding loci can be used for identification purposes or to clarify the taxonomy of the species included in this order; different genes, such as the reductase melanin biosynthesis gene (*Brn1*), *GPDH*, and *EF1-α*, have all been used for this purpose (Shimizu et al. 1998, Berbee et al. 1999, Zhang et al. 2012).

To clarify the taxonomy of *Bipolaris* and *Curvularia* and the relationships with

Cochliobolus species, several phylogenetic studies have been performed (Shimizu et al. 1998, Berbee et al. 1999, Manamgoda et al. 2011, 2012). In the most recent publication (Manamgoda et al. 2012), using a multilocus analysis with the ITS region, *GPDH*, LSU and *EF1-α*, conclusive phylogenetic results were obtained. The species of the *Bipolaris, Cochliobolus* and *Curvularia* complex were divided into two monophyletic groups: one group included the type species of the genus *Cochliobolus* (*C. heterostrophus*) and its anamorph *B. maydis* (the type species of the former anamorphic genus), and the other group included species of *Bipolaris*, *Cochliobolus,* and *Curvularia* (among them was the type species of the genus *Curvularia*, *C. lunata*). Morphologically, members of the first group could be distinguished by the production of large, canoe-shaped conidia (usually greater than 100 μm) and the lack of stroma associated with ascomata; while the species included in the second monophyletic group have smaller (usually less than 100 μm) and straight to curved conidia, and usually stromata below the ascomata (Berbee et al. 1999, Manamgoda et al. 2012). Therefore, on the basis of these results and following the new criteria on fungal nomenclature (Hawkswork et al. 2011), Manamgoda et al. (2012) proposed that the first group was representative of the genus *Bipolaris* and the second one was representative of the genus *Curvularia*. Both genera were thus redescribed on the basis on the holomorphic concept of these fungi. Subsequently, several *Bipolaris* species (*B. australiensis*, *B. coicis, B. ellisii*, *B. graminicola, B. hawaiiensis, B. ovariicola, B. pterotidis, B. ravenelii, B.spicifera,* and *B. tripogonis*) were transferred to the genus *Curvularia.*

Based on these previous phylogenetic studies, we used the following loci to confidently identify and to establish the phylogenetic relationships among clinical isolates of the respective three genera: for *Bipolaris* the ITS region was used; for *Curvularia,* in addition to the ITS, the loci LSU, *GPDH* and *RPB*2 were also sequenced; and for *Exserohilum,* we sequenced the ITS and the D1/D2 regions of the rDNA and the genes *ACT1* and *EF-1α*.

In the case of *Bipolaris*, with a combination of morphological features and sequence analysis, we were able to identify all of the clinical isolates as, in decreasing order of frequency: *B. spicifera* (now *C. spicifera*), followed by *B. hawaiiensis* (now *C. hawaiiensis*), *B. cynodontis*, *B. micropus*, *B. australiensis* (now *C. australiensis*) and *B. setariae*.

In the case of *Exserohilum*, morphological differences among isolates were observed and although two of them were morphologically identified as *E. longirostratum,* all isolates showed a high percentage of genetic similarity (98.4-100 %) with the four loci analyzed. Therefore, all them were identified as *E. rostratum*. Although our results suggested that the three clinically relevant species of this genus (*E. rostratum, E. longirostratum* and *E. mcginnisii*) were conspecific, the taxonomy of these species and, in general, of the entire genus, is still understudied. The phylogenetic studies on *Exserohilum* are scarce, usually involve a small number of isolates, and are each based on an analysis of a single gene (ShanHai et al. 2011, Sakoda & Tsukiboshi 2012). Thus, extensive studies involving the type or reference strains of most species of the genus would be required to better understand the taxonomy of *Exserohilum*.

The case of the *Curvularia* isolates was a bit complex. Although the first study (in Section 4. 2 −da Cunha et al. 2013), which used the sequence analyses of the ITS region and *GPDH* and morphological features, allowed us to identify a relatively high percentage of isolates at the species level (72.3 %), several phyloclades remained taxonomically unresolved. For example, clinical isolates morphologically identified as *C. geniculata* and *C. senegalensis* were included in the same clade, suggesting that they could be conspecific and that agrees with results obtained previously by other authors (Hosokawa et al. 2003, Sun et al. 2003). On the other hand, twenty-seven isolates could not be identified because, despite their resemblance to *C. aeria* or *C. lunata*, they were grouped into three *Curvularia* phyloclades (I-III) genetically distant from the clades formed by isolates identified as the two species mentioned. To resolve the taxonomy of those unnamed phyloclades, we carried out a second study in which we widened the number of loci analyzed and the number of ex-type or reference strains for comparison (in Section 4. 3 −da Madrid et al. in press). This study allowed us to characterize and propose five new *Curvularia* species; among them *C. americana* and *C. hominis* were the novel species that included the largest number of unidentified clinical isolates in the first study. However, we agree with Manamgoda et al. (2012) that there are still numerous species belonging to those Pleosporalean genera which have not been sequenced and thus not assigned to their current taxonomy, and that is also reflected in the clinical isolates of these three genera studied in the present thesis.

5.2 Morphological and molecular identification of *Pithomyces* species

Human infections by *Pithomyces* are rare, although recently some cases of onychomycosis and peritonitis caused by this fungus have been reported (Litz & Cavagnolo 2010, Deplano et al. 2012, Terada et al. 2014). In addition, several studies have reported the presence of *Pithomyces* spores in the air around asthma patients (Jones et al. 2011, Meng et al. 2012, Premila 2013). In animals, *P. chartarum*, the most ubiquitous species of the genus, has also been relatively recently associated with facial eczemas on several occasions (van Wuijckhuise et al. 2006, Ozmen et al. 2008, Di Menna et al. 2009). Therefore, the apparent incidence of this genus in clinical settings seems to be increasing, and proof of that fact is the increased number of clinical isolates of *Pithomyces* received at the FTL in recent years from various US hospitals for identification purposes. In fact, *Pithomyces* is really an unknown fungal genus among clinical microbiologists, and the only species identified to date in the above-mentioned publications has been *P. chartarum*. Thus, we found interesting to explore the diversity of the species isolated from human specimens, to our knowledge, a study that has never been done before.

The identification of *Pithomyces* is difficult for several reasons. The existing literature on the approximately 50 species of *Pithomyces* is spread among numerous publications, and is often difficult to locate, even for the specialists; in addition, those publications include only descriptions of the fungus growing on the natural substratum. The dichotomous keys to species that are available include very few taxa, and the diagnostic characters used are those from fungi on the nature (Ellis 1960, 1971, 1976, Rao & de Hoog 1986). Furthermore, differences among species can be subtle, and many species exhibit significant intraspecific morphological variability (Ellis 1960, Morgan-Jones 1987). If only *in vitro* cultures of *Pithomyces* isolates are available, the identification is still more difficult because, as in the case of the previously discussed genera, the morphological features, especially those of the conidia, tend to vary from those observed in the specimen on the natural substratum. For this reason, it is very important to examine the isolates of this genus in appropriate *in vitro* conditions. According to our study, the best conditions for growth and for achieving good sporulation for *Pithomyces* strain identification is the OA medium, incubated at room temperature in cycles of 12h of darkness/black light. However, even under these

conditions, the isolates of the different *Pithomyces* species identified in this study tended to produce smaller and less longitudinal septate conidia, features that are relevant for distinguishing species. While we have found relatively easy to identify morphologically isolates of *P. chartarum*, that has not been the case with the isolates of the other species, a finding which agrees with the situation explained previously. For the true identification of the isolates investigated, again the combination of sequence analysis and morphological data of the sporulated cultures has been crucial.

After a preliminary morphological species identification of all clinical isolates, we sequenced the ITS region and the D1/D2 domain of the 28S rRNA gene of all them, as it is generally recommended for molecular fungal identification (Iwen et al. 2002, Schoch et al. 2012). We first used the BLAST search to compare our nucleotide sequences with those of other fungi deposited in GenBank, which is currently considered an easy tool for the identification of fungal species. However, in the case of *Pithomyces* isolates, this tool proved to be very limited. Again, only *P. chartarum* isolates were molecularly confirmed, but the rest of isolates, although related among them, were not possible confirm our morphological identification. Therefore, several reference strains of different species, those morphologically similar to those identified previously, were obtained from culture collections and incorporated into the study. With the combination of the phylogenetic analyses of the ITS and the D1/D2 region and the morphological features, we identified the 42 clinical isolates studied as *P. chartarum, P. maydicus, P. sacchari* and *Pithomyces* sp. I. These latter represented a group of isolates with morphological features that did not match any known *Pithomyces* species, and which formed a phyloclade with a weak statistical support to be considered representative of only one species. To elucidate the taxonomy of this unidentified phyloclade, we expanded our search, including all reference strains of the different *Pithomyces* species deposited in the CBS and MUCL collections, even those from the latter collection identified only at the genus level. In addition to the first two loci (ITS and D1/D2), we sequenced two other genes (*GPDH* and *RPB2*), concretely those which were considered good molecular markers to discriminate among closely related species of the Pleosporales (Berbee et al. 1999, Manamgoda et al. 2012, Zhang et al. 2012, Brun et al. 2013). As a result of this second study, we were able to confirm the previous identification of the clinical isolates as *P. maydicus* and *P. sacchari*, with the latter

being one of the most frequent species identified among the clinical isolates studied. However, the taxonomy of *Pithomyces* sp. I from the first study remained unclear, and now *P. chartarum* seems to be a complex of species, with at least one cryptic species that is practically morphologically indistinguishable from *P. chartarum* s. stric. In fact, in this second study, we have recognized four phyloclades (*Pithomyces* sp. I-IV), all with clinical isolates from the FTL, that represent different species of unclear taxonomy. To resolve the taxonomy of these fungi, we still have to study herbarium material of numerous known species deposited in different personal and public collections, and if the original material (i.e. holotypes, isotypes, etc) is not located, we will either propose new species or to epitypify some of the known species. This is the case of the phyloclade labelled as *Pithomyces* sp. I, which may correspond to *P. cynodontis*, or that of *Pithomyces* sp. II which may correspond to *P. atro-olivaceous*.

Therefore, our study demonstrate that there is a great diversity of *Pithomyces* species associated with clinical samples; even so, the presence of *Pithomyces* species in human specimens should be interpreted with caution because we do not yet have enough clinical data to confirm infections by these fungi.

Two other interesting findings derived from our studies on *Pithomyces* are: 1) to demonstrate that *Leptosphaerulina chartarum* described as teleomorph of *P. chartarum* by Roux (1986) is not only a different fungus, but also a different species belonging to a different Pleosporalean family; while the ex-type strain of *L. chartarum* fell into the Didymellaceae, *P. chartarum* and the other closely related *Pithomyces* species fell into the family Montagnulaceae. 2) According to our phylogenetic study *P. valparadisiacum* should be excluded from the genus.

In summary, these results show that there is still a lot of work to be done with isolates that have been morphologically identified as *Bipolaris*, *Curvularia*, *Exserohilum* and *Pithomyces*. Many of these species have still an unclear taxonomy, and only their study with modern methods will allow us to advance in the knowledge of this large group of Pleosporalean fungi of clinical interest.

5.3 Clinical importance and *in vitro* antifungal susceptibility of *Bipolaris*, *Curvularia, Exserohilum* and *Pithomyces*

Most of the isolates investigated in this thesis were from human clinical specimens, and were most commonly isolated from the nasal region, followed by various cutaneous presentations and nails, some from respiratory tract, and eyes (Table 4). These origins agree with the previously reported origins of the *Bipolaris*, *Curvularia* and *Exserohilum* in clinical samples (Padhye et al. 1986, Adler et al. 2006, Al-Attar et al. 2006, Gupta et al. 2007, Saint-Jean et al. 2007, Revankar & Sutton 2010, Alvarez et al. 2011, Berbel et al. 2011, Ehlers et al. 2011, Patil et al. 2011, Chowdhary et al. 2011, Moody et al. 2012). It is important to note that no isolate belonging to *Biporaris* or *Pithomyces* was recovered from deep tissues, while a certain percentage of isolates of the other two genera were isolated from blood, brain, and cerebrospinal fluid, among other clinical specimens, a finding which could suggest a higher pathogenic potential of the *Curvularia* and *Exserohilum* species respect to the *Bipolaris* and *Pithomyces* species. In fact, according to our results, all species identified from the first two genera were able to grow at 37-40ºC, and that could favour their pathogenic power to invade deep tissue. Clinical evidences of this hypothesis could be the recent meningitis outbreak caused mainly by *E. rostratum*, which affected a great number of people from the US who were injected with a steroid solution contaminated by this fungus (Kainer et al. 2012, Bell et al. 2013, Kerkering et al. 2013) or the numerous cases of human deep tissue infections by *Curvularia* spp (Revankar & Sutton 2010, Lee et al. 2011, de Hoog et al. 2011). In our case, although none of these clinical isolates have been proven to be the causal agent of an infection, their repetitive isolation from clinical specimens could be indicative of a pathogenic role that the species identified in the present thesis may play in human or animal diseases.

Studies on *in vitro* antifungal susceptibility testing for dematiaceous fungi are relatively scarce, and they often involve a small number of isolates per species. Furthermore, these studies were performed not only before antifungal susceptibility testing methods were standardized for filamentous fungi, but also when the fungi considered in the present thesis had been identified based only on morphological criteria. Only the knowledge of the antifungal susceptibility data of well-identified fungi can provide reliable options for the treatment of infections caused by these fungi

(Revankar & Sutton 2010). Therefore, this thesis include the first studies where *in vitro* antifungal susceptibility testing were carried out after a reliable identification of the different species of *Bipolaris*, *Curvularia*, *Exserohilum* and *Pithomyces* obtained from clinical samples. In addition, the antifungal activity tests were always evaluated on a relevant number of isolates of the different species identified.

TABLE 4**.** Anatomical origin of clinical isolates studied

Origin	*Bipolaris*	*Curvularia*	*Exserohilum*	*Pithomyces*
	Nº isolates (%)	Nº isolates (%)	Nº isolates (%)	Nº isolates (%)
Nasal region	32 (30.7%)	37 (36.6%)	16 (47%)	8 (19%)
Skin + nails	32 (30.7%)	36 (35.6%)	8 (23.5%)	19 (45.2%)
Respiratory tract	16 (15.8%)	9 (8.9%)	1 (2.3%)	10 (23.8%)
Eyes	13 (12.5%)	11 (10.8%)	5 (14.7%)	2 (4.7%)
Deep tissue	11 (10.89%)*	6 (5.9%)	2 (5.8%)	-
Unknown	-	1 (0.99%)	2 (5.8%)	2 (4.7%)

*Clinical specimens from which *B. hawaiiensis* and *B. spicifera* were isolated, now two species of *Curvularia*

The *in vitro* antifungal activity tests for the *Bipolaris* and *Curvularia* species were performed in the FTL, and the drugs tested were AFG, AMB, CAS, ITC, FLC, VRC, MFG, PSC and 5-FC. The drugs FLC and 5-FC were found to have negligible activity against all species of both genera. The other drugs tested presented good *in vitro* antifungal activity against the isolates of *Bipolaris* and *Curvularia* tested. However, it is worth mentioning that two *Curvularia* species showed higher MICs than the other *Bipolaris* and *Curvularia* species tested; they were *C. lunata* and *C. spicifera* (results on the latter species are in the Section 4.1 under the name *Bipolaris spicifera*). An interesting finding was that several species of *Curvularia*, i.e. *C. aeria*, *C. borreriae*, *C. protuberata* and *C. pseudorobusta*, were found to be resistance to ITC and VRC. Therefore, considering the fact that the azoles are commonly used in the treatment of melanized fungi (Erwin & Fitzgerald 2007, Revankar & Sutton 2010, Vásquez-del-Mercado et al. 2013), only PSC would be the choice therapy in infections caused by these species. This latter drug has been successfully used to treat infections caused by

other dematiaceous fungi such as *Alternaria* or *Cladophialophora* (Dupont et al. 2010, Rammaert et al. 2012).

Based on the lower activity of the FLC and 5-FC against melanized fungi, these drugs were not evaluated in the *in vitro* antifungal activity test carried out in our laboratory against *Exserohilum* and *Pithomyces* species. In the case of *Exserohilum*, with the exception of CAS and MFG, all the drugs tested presented good activity against *E. rostratum*, the only species identified among the number of clinical isolates investigated. In the case of *Pithomyces*, the majority of antifungal drugs tested presented good activity against all of the species identified. It is worth mentioning that no data on antifungal activity against *Pithomyces* species exists in the literature.

The resulting high antifungal activity against the genera studied agrees with previous results from other studies (Jessup et al. 2000, Revankar 2005, Arredondo-Garcia et al. 2009, González 2009, Unal et al. 2011). Although *Bipolaris, Curvularia, Exserohilum* and *Pithomyces* present favourable antifungal results *in vitro*, it is not known how this result can be translated into clinical practice.

Although our studies have increased the data available on the antifungal activity *in vitro* for the genera analyzed based on a considerable number of isolates and using a standardized methodology, other studies must be performed. Some possible tests include an *in vitro* study of possible synergistic combination therapy and the use of animal models to predict results in humans for the development of effective antifungal strategies.

6. CONCLUSIONS

Our study has established the spectra of the species of the genera *Bipolaris*, *Curvularia, Exserohilum* and *Pithomyces* that were present in clinical samples from different hospitals in the United States. In addition, most of the species were confidently identified through the use of morphological and molecular techniques. Based on these studies, we can conclude the following:

1. The most relevant *Bipolaris* species identified from clinical specimens were *B. spicifera* (67.3%), followed by *B. hawaiiensis* (18.2%) and *B. cynodontis* (8.6%), although the former two species were recently transferred to the genus *Curvularia*.
2. The most frequent species of *Curvularia* identified from clinical specimens were *C. aeria* (22.7%), followed by *C. geniculata* / *C. senegalensis* complex (13.86%) and *C. lunata* (9.9%). However, using only the ITS rDNA and the *GPDH* gene as molecular markers nearly 30 % of the isolates studied could not be identified at the species level.
3. The multilocus sequence analysis of four loci combined with an exhaustive morphological study of the unidentified clinical isolates of *Curvularia* allowed us to characterize and propose the following new species: *C. americana, C. chlamydospora*, *C. hominis*, *C. muehlenbeckiae*, and *C. pseudolunata.*
4. In addition to the new taxa, species of *Bipolaris* and *Curvularia* identified in our study but not previously found in clinical settings were *B. cynodontis*, *B. micropus, B. setariae, C. aeria, C. intermedia, C. protuberata* and *C. pseudorobusta,* all of which should be considered potential opportunistic fungi in human infections.
5. Most isolates of *Bipolaris* and *Curvularia* were recovered from nasal sinuses, which is generally considered one of the most frequent sites of infection by these fungi.
6. Our multilocus analysis of clinical isolates and reference strains of *Exserohilum* suggests that *E. longirostratum, E. mcginnisii* and *E. rostratum* were conspecific. Therefore, the only species of this genus identified from clinical specimens was *E. rostratum*.
7 Most of the clinical isolates of *Pithomyces* belonged to the species *P. chartarum* (33.3%), *P. sacchari* (33.3%) and *P. maydicus* (4.8%), the latter two being identified for the first time from human specimens. However, a total of 28.6%

could not be identified at the species level through morphological features or by rDNA sequence analysis.

8. Our phylogenetic analysis of the D1/D2 rDNA sequences revealed that all the species of *Pithomyces* investigated belonged to the family Montagnulaceae while *Leptosphaerulina chartarum,* the supposed teleomorph of *Pithomyces chartarum,* belonged to the family Didymellaceae.
9. The phylogenetic study based on the analysis of four loci revealed 4 cryptic species in *Pithomyces*, which are still being studied.
10. With the exception of Fluconazole and Flucytosine, in general, the antifungal agents tested showed good activity against the identified species of *Bipolaris, Curvularia*, *Exserohilum* and *Pithomyces*. Voriconazole and itraconazole, however, showed high MICs for some *Curvularia* species, particularly for *C. aeria*, *C.* cf *borreriae*, *C. protuberata, C. pseudorobusta* and *C. spicifera.*

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ANNEX -I

Clinical isolates and type or reference strains included in this thesis.

Species	**FMR Nº**	**Other collection**	**Source**
Bipoalris australiensis	11312	CBS 172.57^{T}	*Oryza sativa* seed, Vietnam
(currently *Curvularia australiensis*)		= MUCL 18203^{T}	
	11032	UTHSC 08-547	Foot nail, Texas, USA
	10987	UTHSC 09-406	Middle ear, Texas, USA
Bipolaris chloridis	-	CBS 242.77B	*Chloris gayana*, Australia
Bipolaris cynodontis	11964	CBS 285.51	*Cynodon transvaalensis*, Kenya
	-	CBS 305.64	*Cynodon dactylon*, USA
	11984	CBS 109894	*Cynodon dactylon*, Hungary
	11987	CBS 304.64	*Cynodon dactylon*, USA
	12081	IMI 129083	*Cynodon dactylon*, Australia
	11166	UTHSC 07-3766	BAL, California, USA
	11165	UTHSC 07-3862	Leg wound, Texas, USA
	11283	UTHSC 07-557	Nasal sinus, Minnesota, USA
	11183	UTHSC 07-1359	Nail, Florida, USA
	11185	UTHSC 07-1349	Toe nail, Florida, USA
	11036	UTHSC 08-912	Sputum, Minnesota, USA
	10988	UTHSC 09-507	Cornea, Utah, USA
	11716	UTHSC 10-2974	Nasal sinus, Minnesota, USA
	11725	UTHSC 10-1814	Abscess, Colorado, USA
Bipolaris hawaiiensis	11313	CBS 173.57 ^{T}	*Oryza sativa*, Hawaii
(currently *Curvularia hawaiiensis)*		= MUCL 18218^{T}	
	-	CBS 448.72	Salt-marsh soil, Kuwait
	-	CBS 727.96	Substrate unknown, USA
	11197	UTHSC 06-3893	Blood, Arkansas, USA
	11189	UTHSC 07-449	BAL, Florida, USA
	11171	UTHSC 07-3226	Lung, Hawaii, USA

Species	FMR Nº	Other collection	Source
Bipolaris hawaiiensis (currently *Curvularia hawaiiensis*)	11176	UTHSC 07-2608	Nasal sinus, Utah, USA
	11177	UTHSC 07-2188	Nail, South Carolina, USA
	11187	UTHSC 07-901	Blood, Ohio, USA
	10979	UTHSC 08-3526	Peritoneal fluid, Texas, USA
	10982	UTHSC 08-3311	Nasal tissue, Texas, USA
	11029	UTHSC 08-859	BAL, Texas, USA
	11164	UTHSC 08-38	Nasal sinus, Texas, USA
	10998	UTHSC 09-3098	BAL, Arizona, USA
	11003	UTHSC 09-2906	Burn wound tissue, Texas, USA
	10983	UTHSC 09-495	BAL, Texas, USA
	10993	UTHSC 09-3594	Left shoulder, Texas, USA
	11206	UTHSC 10-756	Nasal sinus, Arizona, USA
	11408	UTHSC 10-590	Peritoneal fluid, Minnesota, USA
	11710	UTHSC 10-3464	Brain, Florida, USA
	11719	UTHSC 10-2694	Nasal sinus, Minnesota, USA
	11724	UTHSC 10-2270	Nasal sinus, Texas, USA
Bipolaris homomorphus	11953	CBS 156.60^{T}	Air, Kansas
Bipolaris maydis	-	CBS 130.26	Substrate and locality unknown
	11960	CBS 136.29	*Zea mays*, Japan
	-	CBS 307.64	*Zea mays*, USA
Bipolaris microlaenae	-	CBS 280.91^{T}	*Microlaena stipoides* leaf, Australia
Bipolaris micropus	-	CBS 127235	Substrate and locality unknown
		CBS 127234	Substrate and locality unknown
	-	CBS 127236	Substrate and locality unknown

Species	FMR Nº	Other collection	Source
Bipolaris micropus	11196	UTHSC 06-3896	Toe, Florida, USA
	11193	UTHSC 06-4353	Toe nail, South Carolina, USA
	11184	UTHSC 07-1352	Toe nail, Florida, USA
Bipolaris oryzae	-	CBS 157.50	*Oryza sativa* grain, Indonesia
	-	CBS 199.54	*Oryza sativa* grain, New Guinea
Bipolaris papendorfii	11917	MUCL 10191^{T}	*Acacia karroo*, South Africa
Bipolaris sacchari	11986	CBS 324.64	*Saccharum officinarum*
Bipolaris setariae	11962	CBS 141.31	Substrate and locality unknown
	11270	UTHSC 05-3211	Eye, Michigan, USA
Bipolaris sorghicola	-	CBS 249.49	*Sorghum vulgare* var. *sudanense*, Locality unknown
Bipolaris sorokiniana	-	CBS 140.31	Substrate unknown, Japan
	-	CBS 145.32	*Triticum durum*, Locality unknown
Bipolaris spicifera (currently *Curvularia spicifera*)	11314	MUCL 3017^{T}	Soil, Spain
	-	CBS 198.31	*Capsicum anuum*, Cyprus
	-	CBS 199.31	*Cucurbita maxima*, Cyprus
	11194	UTHSC 06-4162	BAL - Lung Tx, Texas, USA
	11192	UTHSC 06-4551	Sinus aplastic anemia, Ohio, USA
	11198	UTHSC 06-3737	Nasal sinus, California, USA
	11199	UTHSC 06-3277	Skin foot, Utah, USA
	11202	UTHSC 06-3088	Toe nail, Florida, USA
	11200	UTHSC 06-3229	Sternal (wound), Indiana, USA
	11203	UTHSC 06-3056	Maxillary sinus tissue, Missouri, USA
	11195	UTHSC 06-4011	Peritoneal fluid, Texas, USA
	10986	UTHSC 07-1452	Bile, Tennessee, USA

Species	FMR Nº	Other collection	Source
Bipolaris spicifera (currently *Curvularia spicifera*)	11170	UTHSC 07-3282	BAL, Maryland, USA
	11169	UTHSC 07-3454	Eye, Minnesota, USA
	11173	UTHSC 07-3140	BAL, Ohio, USA
	11181	UTHSC 07-1723	Nasal tissue, Texas, USA
	11174	UTHSC 07-2844	Ear, Ohio, USA
	11175	UTHSC 07-2843	Knee, Ohio, USA
	11178	UTHSC 07-2159	Nasal sinus, Texas, USA
	11179	UTHSC 07-2048	Cornea, Arizona, USA
	11182	UTHSC 07-1687	Nasal sinus, Arizona, USA
	11180	UTHSC 07-1822	Sputum, Utah, USA
	11030	UTHSC 08-2706	Skin, Colorado, USA
	11033	UTHSC 08-2347	Foot tissue, Utah, USA
	11031	UTHSC 08-2317	Cornea, Pennsylvania, USA
	11039	UTHSC 08-2225	Maxillary sinus, Tennessee, USA
	10968	UTHSC 08-2936	Thigh, Washington DC, USA
	11037	UTHSC 08-2212	Nasal sinus tissue, Texas, USA
	11035	UTHSC 08-2058	Nail, South Carolina, USA
	11038	UTHSC 08-1980	Leg, Texas, USA
	11025	UTHSC 08-1891	BAL, South Carolina, USA
	11027	UTHSC 08-1757	Maxillary sinus, North Carolina, USA
	11042	UTHSC 08-1702	Eye, Texas, USA
	11026	UTHSC 08-1495	Left knee, Arizona, USA
	11162	UTHSC 08-533	Blood, Arizona, USA

Species	FMR Nº	Other collection	Source
Bipolaris spicifera (currently *Curvularia spicifera*)	10981	UTHSC 08-3726	Tissue - Mediastinal, Texas, USA
	11024	UTHSC 08-1479	Maxillary sinus, Utah, USA
	11967	UTHSC 08-3258	Ankle, Nebraska, USA
	10970	UTHSC 08-3224	Left thigh hematoma, Colorado, USA
	10997	UTHSC 09-3305	Eye, Minnesota, USA
	10971	UTHSC 09-1134	Eye, Arizona, USA
	10999	UTHSC 09-1769	Nasal sinus, Utah, USA
	11004	UTHSC 09-2925	Cornea, Arizona, USA
	11006	UTHSC 09-2571	Blood, Texas, USA
	10996	UTHSC 09-3119	Nasal sinus, Utah, USA
	10978	UTHSC 09-421	Hand wound cellulitis, Texas, USA
	11001	UTHSC 09-3449	Maxillary sinus, Texas, USA
	11002	UTHSC 09-3102	Maxillary sinus, Oklahoma, USA
	11000	UTHSC 09-2980	Sputum, Arizona, USA
	11009	UTHSC 09-2562	Blood stem cell Tx, California, USA
	10980	UTHSC 09-82	Pansinusitis, Texas, USA
	11008	UTHSC 09-1482	Eye vitreous fluid, Texas, USA
	10984	UTHSC 09-1322	Brain, Massachusetts, USA
	11726	UTHSC 10-1784	Hand, Arizona, USA
	11727	UTHSC 10-1657	Nasal sinus, Arizona, USA
	10995	UTHSC 10-250	Maxillary sinus, Tennessee, USA
	11721	UTHSC 10-2008	Sphenoid sinus, Arizona, USA
	11730	UTHSC 10-908	Leg wound, Florida, USA

Species	FMR Nº	Other collection	Source
Bipolaris spicifera (currently *Curvularia spicifera*)	11720	UTHSC 10-2655	Chest, Texas, USA
	11729	UTHSC 10-1565	Eye, Texas, USA
	11728	UTHSC 10-1586	Cornea, Texas, USA
	10994	UTHSC 10-437	Maxillary sinus, California, USA
	10991	UTHSC 10-530	Maxillary sinus, Arizona, USA
	11205	UTHSC 10-640	BAL, Arizona, USA
	11715	UTHSC 10-3055	Eye, Texas, USA
	11712	UTHSC 10-3335	BAL, Arizona, USA
	11713	UTHSC 10-3145	Membrane, Texas, USA
	11714	UTHSC 10-3143	Nasal sinus, South Carolina, USA
	11723	UTHSC 10-2300	Nasal sinus, Ohio, USA
	11718	UTHSC 10-2804	Nasal sinus, Texas, USA
	11722	UTHSC 10-2488	BAL, Arizona, USA
	11717	UTHSC 10-2807	Mastoid sinus, Oklahoma, USA
	11711	UTHSC 11-60	Tissue - Canine, Arizona, USA
Bipolaris zeae	-	CBS 127716	Substrate and locality unknown
Bipolaris zeicola	-	CBS 316.64	*Zea mays*, USA
	-	CBS 317.64	*Zea mays*, USA
Curvularia aeria	11973	CBS 294.61^{T}	Air, Brazil
	11975	CBS 337.64	*Agropyron repens,* USA
	11974	CBS 533.70	Seed, Pennisetum,Denmark
	11204	UTHSC 06-3039	Nasal sinus, Arizona, USA
	11188	UTHSC 07-481	Ethmoid sinus, Arizona, USA
	11706	UTHSC 07-2859	Leg, Florida, USA
	11702	UTHSC 07-3183	Nasal sinus, Minnesota, USA

Species	FMR Nº	Other collection	Source
Curvularia aeria	11684	UTHSC 08-1226	Maxillary sinus, Vermont, USA
	11694	UTHSC 08-2	Maxillary sinus, South Carolina, USA
	11677	UTHSC 08-2586	Nasal sinus, Florida, USA
	11675	UTHSC 08-2625	Nasal sinus, South Carolina, USA
	11670	UTHSC 08-2923	Nasal sinus, South Carolina, USA
	11667	UTHSC 08-3003	Blood, Texas, USA
	11554	UTHSC 08-3299	Nasal sinus, Washington, USA
	11552	UTHSC 08-3398	Bronchial wash, Texas, USA
	11546	UTHSC 08-3490	Frontal sinus, Missouri, USA
	11538	UTHSC 09-494	Nasal sinus, Texas, USA
	11531	UTHSC 09-2019	Wound, South Carolina, USA
	11530	UTHSC 09-2085	Nasal sinus, Minnesota, USA
	11525	UTHSC 09-2318	Nasal sinus, Texas, USA
	11520	UTHSC 09-2546	Middle turbinate, Arkansas, USA
	11512	UTHSC 09-3123	Nasal sinus, South Carolina, USA
	11511	UTHSC 09-3124	Nasal sinus, South Carolina, USA
	11505	UTHSC 10-370	Skin, Utah, USA
	11504	UTHSC 10-543	Peritoneal dialysis fluid, South Carolina, USA
	11502	UTHSC 10-816	Nasal sinus,Texas, USA
Curvularia affinis	11954	CBS 154.34^{T}	*Manihot utilissima*, Java
	-	CBS 185.49	*Manihot utilissima*, Java
Curvularia akaii	-	CBS 318.86	Substrate unknown, Japan
	-	CBS 127728	Substrate unknown, Japan
	-	CBS 127730	Substrate unknown, Japan
Curvularia americana	11551	UTHSC 08-3414^{T}	Ankle, Minnesota, USA
	11687	UTHSC 07-2649	Toe tissue, Texas, USA
	11693	UTHSC 08-84	Nasal sinus, Utah, USA
	11691	UTHSC 08-278	Peritoneal dialysis fluid, Ohio, USA

Species	FMR Nº	Other collection	Source
Curvularia americana	11674	UTHSC 08-2697	Leg, Tennessee, USA
	11005	UTHSC 09-2907	Toe nail, Oklahoma, USA
	11515	UTHSC 09-2806	Bone marrow, Virginia, USA
	11514	UTHSC 09-2863	Bronchial wash, Texas, USA
	11500	UTHSC 10-1276	Maxillary sinus, California, USA
Curvularia borreriae	11967	CBS 859.73	Volcanic ash soil, Chile
	11669	UTHSC 08-2957	Corneal ulcer, West Virginia, USA
	11550	UTHSC 08-3433	Nasal sinus, California, USA
	11523	UTHSC 09-2408	Sputum, Washington, USA
	11507	UTHSC 09-3510	Peritoneal dialysis fluid, District of Columbia, USA
Curvularia brachyspora	11955	CBS 186.50	Soil, Java
Curvularia carica-papayae	-	CBS 135941^{T}	*Carica papaya* leaf, India
Curvularia chlamydospora	11709	UTHSC 07-2764^{T}	Toe nail, Montana, USA
	11040	UTHSC 08-1283	Nasal sinus, Nevada, USA
Curvularia cf *clavata*	11501	UTHSC 10-1041	Bronchial wash, Utah, USA
Curvularia coicis	-	CBS 192.29^{T}	*Coix lacrima-jobi* var. *typica*, Japan
Curvularia cymbopogonis	-	CBS 419.78	*Yucca* sp. leaf, Netherlands
Curvularia ellisii	-	CBS 193.62	Air, Pakistan
Curvularia eragrostidis	-	CBS 189.48	Sorghum seed, Java
Curvularia geniculata /C. senegalensis	11971	CBS 220.52	Discolored wood, triplex plank, Suriname
	11970	CBS 332.64	*Setaria Italica*, USA
	11978	CBS 149.71 (as *C. senegalensis*)	Substrate unknown, Nigeria
	11979	CBS 431.75 (as *C. senegalensis*)	*Sorghum*, Fiji

Species	FMR Nº	Other collection	Source
Curvularia geniculata /C. senegalensis	11705	UTHSC 07-3044	Nasal sinus, South Carolina, USA
	11703	UTHSC 07-3111	Eye, Texas, USA
	11696	UTHSC 07-3620	Skin scraping, Texas, USA
	11695	UTHSC 07-3740	Toe nail, Minnesota, USA
	11668	UTHSC 08-2979	Toe nail, Texas, USA
	11553	UTHSC 08-3314	Bronchial wash, Minnesota, USA
	11545	UTHSC 08-3531	Eye, Louisiana, USA
	11543	UTHSC 08-3728	Eye, Missouri, USA
	11533	UTHSC 09-1824	Knee biopsy, Florida, USA
	11010	UTHSC 09-2568	Leg, Texas, USA
	11519	UTHSC 09-2592	Finger wound, Utah, USA
	11516	UTHSC 09-2753	Corneal ulcer, Tennessee, USA
	11513	UTHSC 09-3005	Bronchial wash, Tennessee, USA
	11508	UTHSC 09-3435	Ear, Florida, USA
Curvularia gladioli	-	CBS 210.79	*Gladiolus* sp. leaf, Romania
Curvularia graminicola	-	BRIP 23186	*Aristida ingrata*, Australia
Curvularia heteropogonis	-	CBS 284.91^{T}	*Heteropogon contortus* leaf, Australia
	-	CBS 511.91	*Heteropogon contortus* leaf, Australia
Curvularia hominis	11539	UTHSC 09-464^{T}	Cornea, Florida, USA
	11708	UTHSC 07-2791	Cornea, Utah, USA
	11704	UTHSC 07-3105	Nasal sinus, Texas, USA
	11172	UTHSC 07-3184	Nasal sinus, Arkansas, USA
	11698	UTHSC 07-3581	Nail, Minnesota, USA
	11688	UTHSC 08-849	Eye, Louisiana, USA
	11683	UTHSC 08-1296	Nail, Texas, USA

Species	FMR Nº	Other collection	Source
Curvularia hominis	11678	UTHSC 08-2517	Foot, Texas, USA
	11542	UTHSC 08-3737	Bronchial wash, Texas, USA
	11680	UTHSC 08-2418	Bronchial wash, Texas, USA
	11535	UTHSC 09-1692	Nasal sinus, Ohio, USA
	11527	UTHSC 09-2197	Nasal sinus, Minnesota, USA
	11521	UTHSC 09-2532	Nasopharynx, Texas, USA
	11509	UTHSC 09-3403	Unknown tissue, Texas, USA
Curvularia cf *inaequalis*	11168	UTHSC 07-3495	Cerebrospinal fluid, Wisconsin, USA
	11681	UTHSC 08-2346	Foot, Utah, USA
	11673	UTHSC 08-2860	Ethmoid tissue, Utah, USA
	11547	UTHSC 08-3472	Nasal sinus, Utah, USA
	11544	UTHSC 08-3685	Arm, Utah, USA
	11536	UTHSC 09-1077	Lung biopsy, Utah, USA
Curvularia intermedia	11972	CBS 334.64	*Avena versicolor*, USA
	11685	UTHSC 08-1041	Nail, South Carolina, USA
	11510	UTHSC 09-3240	Tissue, Colorado, USA
Curvularia ischaemi	-	CBS 630.82 [T]	*Ischaemum indicum* leaf, Solomon Islands
Curvularia lunata	3394	CBS 730.96 [NT]	Lung biopsy, USA
	11957	CBS 157.34	Substrate unknown, Indonesia
	11700	UTHSC 07-3452	Toe nail, Florida, USA
	11692	UTHSC 08-172	Toe nail, Florida, USA
	11682	UTHSC 08-1381	Eye, North Carolina, USA
	11686	UTHSC 08-959	Nail, South Carolina, USA
	11541	UTHSC 09-31	Wound, Minnesota, USA

Species	FMR Nº	Other collection	Source
Curvularia lunata	11540	UTHSC 09-191	Toe nail, South Carolina, USA
	11528	UTHSC 09-2114	Nail, South Carolina, USA
	11524	UTHSC 09-2395	Ethmoid sinus, South Carolina, USA
	11517	UTHSC 09-2719	Wound, South Carolina, USA
	11506	UTHSC 10-145	Contact lens, Minnesota, USA
Curvularia muehlenbeckiae	11976	CBS 144.63^{T}	*Muehlenbeckia* sp. leaf, India
	11671	UTHSC 08-2905	Chest, Utah, USA
Curvularia oryzae	-	CBS 169.53^{T}	*Oryza sativa* seed, Vietnam
Curvularia ovariicola	-	CBS 285.91	*Eragrostis parviflora*, Australia
	-	CBS 286.91	*Eragrostis parviflora*, Australia
Curvularia perotidis	-	CBS 350.90 ^{T}	*Perotis rara*, Australia
Curvularia prasadii	-	CBS 143.64 ^{T}	*Jasminum sambac*, India
	-	CBS 144.64	Substrate unknown, England
Curvularia protuberata	11977	CBS 376.65 ^{T}	*Deschampsia flexuosa* leaf, Scotland
	11676	UTHSC 08-2588	Left tibia, Minnesota, USA
	11672	UTHSC 08-2880	Leg, North Carolina, USA
	11532	UTHSC 09-1969	Abscess - penis, South Carolina, USA
Curvularia pseudolunata	11529	UTHSC 09-2092^{T}	Nasal sinus, California, USA
Curvularia pseudorobusta	11548	UTHSC 08-3458	Nasal sinus, Texas, USA
Curvularia ravenelii	-	CBS 127709	Substrate and locality unknown
Curvularia robusta	-	CBS 624.68 ^{T}	*Dichanthium annulatum* leaf, USA
Curvularia cf *sorghina*	11690	UTHSC 08-809	Sphenoid sinus, South Carolina, USA
	11549	UTHSC 08-3445	Wound, Minnesota, USA
	11537	UTHSC 09-868	Nasal sinus, South Carolina, USA

Species	FMR Nº	Other collection	Source
Curvularia cf *sorghina*	10992	UTHSC 09-3575	Leg wound, Texas, USA
Curvularia trifolii	-	CBS 173.55	*Trifolium repens*, USA
Curvularia tripogonis	-	BRIP 12375 ^{T}	*Tripogon jacquemonti,* India
Curvularia tuberculata	-	CBS 146.63^{T}	*Zea mays* leaf, India
Curvularia uncinata	-	CBS 221.52 ^{T}	*Oryza sativa* leaf, Vietnam
	-	CBS 531.70	*Oryza sativa* seeds, Denmark
Curvularia verruciformis	-	CBS 537.75	*Lobibyx* sp. feather, New Zealand
Curvularia verruculosa	11982	CBS 148.63^{T}	*Typha* sp., India
	11983	CBS 149.63	*Elaeis guineensis*, Nigeria
	-	CBS 150.63	*Punica granatum* leaf, India
	11701	UTHSC 07-3093	Cornea, Texas, USA
	11689	UTHSC 08-827	Corneal ulcer, Texas, USA
	11518	UTHSC 09-2658	Inferior turbinate, Texas, USA
	11526	UTHSC 09-2246	Ethmoid sinus, Texas, USA
	11522	UTHSC 09-2471	Maxilary sinus, Tennessee, USA
	11503	UTHSC 10-709	Wound, Minnesota, USA
Exserohilum longirostratum	11773	IP 1229.80	Heart valve prosthesis, Martinique
	11259	UTHSC 03-3090	Skin BX, Maryland, USA
	11268	UTHSC 05-424	Intervertebral disc, Louisiana, USA
Exserohilum mcginnisii	11951	CBS 325.87^{T}	Nasal polyp, Arizona, USA
Exserohilum rostratum	11952	CBS 467.75^{T}	Soil, India
	11258	UTHSC 03-1932	Maxillary sinus, Texas, USA
	11260	UTHSC 03-3639	Arm tissue, Colorado, USA
	11266	UTHSC 04-2744	Sphenoid sinus tissue, Texas, USA
	11261	UTHSC 04-1248	Nasal wash, Texas, USA
	11264	UTHSC 04-2416	Sinus tissue, Texas, USA
	11265	UTHSC 04-2629	Sinus, Texas, USA

Species	FMR Nº	Other collection	Source
Exserohilum rostratum	11267	UTHSC 04-3327	Mid. turbinate tissue, Texas, USA
	11271	UTHSC 05-3456	Sinus, Texas, USA
	11278	UTHSC 06-2113	Cornea, Texas, USA
	11277	UTHSC 06-1857	Eye, California, USA
	11279	UTHSC 06-2618	Nasal – FROG, Massachusetts, USA
	11280	UTHSC 06-3237	Great toe, Texas, USA
	11201	UTHSC 06-3226	Eye, Texas, USA
	11275	UTHSC 06-530	Ethmoid sinus, South Carolina, USA
	11190	UTHSC 07-263	Sinus, Minnesota, USA
	11372	UTHSC 07-1310	Unknown tissue, Texas, USA
	11286	UTHSC 07-1292	Shin skin, Texas, USA
	11380	UTHSC 07-3092	Nose wound, Arkansas, USA
	11373	UTHSC 07-1498	Unknown tissue, Texas, USA
	11284	UTHSC 07-622	Blood, Texas, USA
	10969	UTHSC 08-3261	Wound, Utah, USA
	11028	UTHSC 08-655	Elbow (CLL), Texas, USA
	11034	UTHSC 08-2771	Cornea, Utah, USA
	11041	UTHSC 08-922	Nasal sinus, Florida, USA
	11392	UTHSC 08-3638	Nasal sinus, Utah, USA
	11390	UTHSC 08-2940	Nasal inferior turbinate, Texas, USA
	11401	UTHSC 09-2018	Abscess, South Carolina, USA
	11395	UTHSC 09-131	Maxillary sinus tissue, Montana, USA
	11396	UTHSC 09-718	Maxillary sinus, Texas, USA
	10985	UTHSC 09-1211	Rt. Inferior turbinate, Texas, USA
	11394	UTHSC 09-109	Bronch wash, Minnesota, USA
	11399	UTHSC 09-1259	Eye (wood splinter), Georgia, USA
Exserohilum turcicum	-	CBS 330.64	*Zea mays*, USA
Pithomyces chartarum	13108	MUCL 9393	Substrate unknown, France
	13115	MUCL 4262	Soil, Ithaca, USA

Species	FMR Nº	Other collection	Source
Pithomyces chartarum	13107	MUCL 15433 (as *P. cynodontis*)	Soil, Pernambuco, Brazil
	-	CBS 679.71	Agricultiral soil, Wageningen, Netherlands
	-	CBS 120293	Substrate unknown, France
	-	CBS 120283	Pasture material from outbreak of facial eczema, Dennegeur, Kareedouw, South Africa
	-	CBS 120282	Ryegrass pasture, New Zealand
	-	CBS 243.93	White fly, India
	-	CBS 807.70	Toot tip, Wageningem, Netherlands
	-	CBS 1202.81	Lucerne stubble, Oudtshoorn, Le Roux, South Africa
	12442	UTHSC 03-2472	Skin, Minnesota, USA
	12444	UTHSC 04-678	Lt. maxillary sinus, Tennessee, USA
	12448	UTHSC 05-2460	Maxilary sinus, Tennessee, USA
	12461	UTHSC 07-692	Scalp, Texas, USA
	12466	UTHSC 07-2045	Skin, Utah , USA
	12467	UTHSC 07-2802	Toe nail, Massachusetts, USA
	12468	UTHSC 07-3664	Unknown tissue, Illinois, USA
	12469	UTHSC 08-331	BAL, Pennsylvania, USA
	12472	UTHSC 08-2855	BAL, Pennsylvania, USA
	12479	UTHSC 10-2361	Nasal sinus, Minnesota, USA
	12481	UTHSC 10-3088	BAL, Washington, USA
	12482	UTHSC 11-2179	Lt. pectoralis muscle, Michigan, USA
Pithomyces maydicus	13087	CBS 491.88 (as *P. graminícola*)	Necrotic leaf spot in Lycopersicon esculentum, Martinique
	13091	CBS 134.29	Substrate and origin unknown
	13090	CBS 400.73	Dead leaf on *Cocos nucifera,* Anuradhapura, Sri Lanka

Species	FMR Nº	Other collection	Source
Pithomyces maydicus	13089	CBS 977.95	Soil in tropical forest, Madang Prov., Brahman, Papua New Guinea
	13100	MUCL 50413	Dead wood, Zimbabwe, Africa
	13103	MUCL 33070	Dead wood, Malawi, Africa
	13110	MUCL 39247	Substrate and origin unknown
	13102	MUCL 48406	Dead twig and wood samples, Singapore, Asia
	12453	UTHSC 06-1549	Toe nail, South Carolina, USA
	12458	UTHSC 06-3954	Maxillary sinus, Texas, USA
Pithomyces karoo	13088	CBS 804.72 [IT]	Dead stem on *Gnidia polycephala*, Cape Province, Colesberg, South Africa
	13112	MUCL 9365 (as *P. quadratus*)	Litter on *Acacia karroo*, Transvaal, Potchefstroom, South Africa
Pithomyces sacchari	13095	CBS 120504	Millet, Ovamboland, Okasha Kingondjo, Namibia
	13096	CBS 803.72	Seed on *Medicago sativa,* Cape Province, Oudshoorn, South Africa
	13109	MUCL 15288	Air, Johannesburg, South Africa
	12477	UTHSC 10-1977	Sputum, North Carolina, USA
	12478	UTHSC 10-2143	Nasal sinus, Tennessee, USA
	12484	UTHSC 12-56	Cornea, Louisiana, USA
	12441	UTHSC 03-1337	BAL, Florida, USA
	12447	UTHSC 04-2746	Maxillary sinus, Texas, USA
	12445	UTHSC 04-2483	BAL, Texas, USA
	12450	UTHSC 05-3251	BAL, South Carolina, USA
	12455	UTHSC 06-3688	Leg, Arizona, USA
	12457	UTHSC 06-3844	Scalp, Texas, USA
	12464	UTHSC 07-1285	Nail, South Carolina, USA
Pithomyces sp. I	12470	UTHSC 08-426	Maxillary sinus, Tennessee, USA
	12473	UTHSC 09-1473	Leg, Texas, USA

Species	FMR Nº	Other collection	Source
Pithomyces sp. I	12476	UTHSC 10-670	Lung, Florida, USA
	13083	CBS 244.96 (as *P. atro-olivaceous*)	Skin scrapings of female diabetic, Florida, USA
	13086	CBS 500.77 (as *P. cynodontis*)	Soil, Florida, USA
	13111	MUCL 8515 (as *P. sacchari*)	Wood, Nogent-sur-Marne, France
	13113	MUCL 50391	Dead twig, Zimbabwe. Africa
	13106	MUCL 33112	Dead twig, Malawi, Africa
	13105	MUCL 33110	Dead twig, Malawi, Africa
	13104	MUCL 33109	Dead twig, Malawi, Africa
	13101	MUCL 50400	Dead twig, Zimbabwe. Africa
	13117	MUCL 33111	Dead twig, Malawi, Africa
	13085	CBS 925.87 (as *P. cynodontis*)	Stem of *Spartocytisus supranubius*
	13082	CBS 621.72 (as *P. atro-olivaceous*)	Salt-marsh soil, Kuwait
	13092	CBS 521.72 (as *P. maydicus*)	Soil, Kuwait
	12449	UTHSC 05-3161	Nail, South Carolina, USA
	12451	UTHSC 05-3373	BAL, Florida, USA
	12456	UTHSC 06-3706	Cornea, Florida, USA
	12454	UTHSC 06-3492	Unknown tissue, Florida
	12463	UTHSC 07-995	Toe nail, Florida, USA
	12465	UTHSC 07-1535	Scalp, Florida, USA
	12471	UTHSC 08-535	Skin feet, Texas, USA
	12475	UTHSC 09-3190	Asian elephant eye, Massachusetts, USA
	12480	UTHSC 10-2776	BAL, South Carolina, USA
	12483	UTHSC 11-3528	Leg tissue, Maryland, USA

Species	FMR Nº	Other collection	Source
Pithomyces sp. II	12459	UTHSC 06-4528	Foot, Florida, USA
	12460	UTHSC 07-578	Toe nail, Florida, USA
Pithomyces sp. III	12443	UTHSC 03-3221	Nasal sinus, Tennessee, USA
Pithomyces sp. IV	13116	MUCL 4329 (as *P. chartarum*)	Calcareous scree, Saône et Loire, France
	13114	MUCL 15905 (as *P. chartarum*)	Dust, Belgium
Pithomyces sp. IV	12452	UTHSC 06-214	Toe, Colorado, USA
	12446	UTHSC 04-2495	Foot, Montana, USA
Pithomyces ?	13093	CBS 662.79 (as *P. pulvinatus*)	Decaying wood in compost heap, Germany
Stemphyliomma valparadisiacum	13099	CBS 510.83 (as *P. valparadisiacus*)	Appel juis, Germany
	13084	CBS 113337 (as *P. atro-olivaceous*)	Dead culms, on restionaceae, Western Cape province, Kogelberg, South Africa
	13098	CBS 110.84 (as *P. valparadisiacus*)	Soil, Spain
Leptosphaerulina chartarum	-	CBS 329.86 [T]	On *Galenia procumbens,* Cape Province, Middelburg, South Africa

[T] ex-type strain; [NT] ex-neotype strain; [IT] isotype strain; BAL, Bronchoalveolar lavage; Tx, transplant; CBS, *Centraalbureau voor Schimmelcultures*, Holanda; FMR, Colección de la *Facultat de Medicina* de Reus; MUCL, *Mycotheque de l'Universite Catholique de* Louvain, Bélgica; UTHSC, *Fungus Testing Laboratory, University of Texas Health Science Center*, USA; IP, *Institut Pasteur*, Paris; BRIP, *Queensland Plant Pathology Herbarium*, Queensland, Australia; IMI, *International Mycological Institute,* Egham ,Surrey, United Kingdom.

UNIVERSITAT ROVIRA I VIRGILI
PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF CLINICAL ISOLATES OF BIPOLARIS, CURVULARIA, EXSEROHILUM AND PITHOMYCES
Keith Cassia Da Cunha
DL:T 1479-2014

ANNEX -II

Relation of sequences strains extracted from public databases used in different phylogenetic analyzes included in the present thesis.

Taxon	Strain number	Origin	Included in
Aigialus mangrovis	BCC 33564	Substrate and origin unknown	Section: 4.5
Aigialus rhizophorae	BCC 33572	Substrate and origin unknown	Section: 4.5
Alternaria alternata	CBS 916.96 ^{T}	On Arachis hypogaea, India	Section: 4.1, 4.2
Amniculicola immersa	CBS 123083^{T}	Submerged wood on *Salix* sp., Denmark	Section: 4.5
Corynespora cassiicola	IMI 056007	Leaf, *Lycopersicon esculentum*	Section: 4.1
Cochliobolus heterostrophus	CBS 134.39	Leaf on *Zea mays*, origin unknown	Section: 4.5
Delitschia winteri	CBS 225.62	Dung of rabbit, Netherlands	Section: 4.5
Didymosphaeria futilis	HKUCC 5834	Substrate and origin unknown	Section: 4.5
Didymosphaeria futilis	CMW 22186	Substrate and origin unknown	Section: 4.5
Exserohilum rostratum	ATCC 32197	*Zea mays* stalk, Florida, USA	Section: 4.2
Karstenula rhodostoma	CBS 690.94	*Frangula alnus*, Uppland, Sweden	Section: 4.5
Letendraea helminthicola	CBS 884.85	Yerba mate, Montecarlo, Argentina	Section: 4.5
Letendraea padouk	CBS 485.70	*Lactarius gymnocarpus,* Gabon	Section: 4.5
Lentithecium arundinaceum	CBS 619.86	*Phragmites australis,* Zürich, Switzerland	Section: 4.5
Leptosphaerulina americana	CBS 213.55	On *Trifolium pretense,* Georgia	Section: 4.5
Leptosphaerulina argentinensis	CBS 569.94	Leaf spot on *Lonicera periclymenum,* Netherlands	Section: 4.5
Leptosphaerulina arachidicola	CBS 275.59	On *Arachis hypogaea,* unknown origin	Section: 4.5
Leptosphaerulina trifolii	CBS 235.58	On *Trifolium,* Netherlands	Section: 4.5
Leptosphaerulina australis	CBS 311.51^{T}	Lawn, Zürich, Switzerland	Section: 4.5
Lindgomyces cinctosporae	R56-1	Substrate and origin unknown	Section: 4.5
Lindgomyces ingoldianus	KH 100 = NBRC 106126	Twigs of woody plant, Oomijya River, Iriomote, Okinawa, Japan	Section: 4.5
Lophiostoma crenatum	CBS 629.86	On *Prunus spinosa,* Zürich, Switzerland	Section: 4.5
Lophiostoma macrostomum	HHUF 27293	Substrate and origin unknown	Section: 4.5
Massarina phragmiticola	NBRC105268	Decayed drift wood, Kanagawa, Japan	Section: 4.5
Melanomma pulvis-pyrius	CBS 371.75	Decaying wood, France	Section: 4.5

Taxon	Strain number	Origin	Included in
Melanomma rhododendri	ANM 73	Substrate and origin unknown	Section: 4.5
Paraconiothyrium minitans	CBS 122788	Unknown substrate, England	Section: 4.5
Pithomyces chartarum	UBC F15184	Conidial drop from *Rubus spectabilis* (Salmonberry) leaf, Canada	Section: 4.5
Phoma glomerata	CBS 528.66	On *Chrysanthemum,* Netherlands	Section: 4.5
Phoma radicina	CBS 111.79[T]	Grafting base on *Malus sylvestris,* Wanssum, Netherlands	Section: 4.5
Preussia terrícola	DAOM 230091	Substrate and origin unknown	Section: 4.5
Preussia funiculata	CBS 659.74	Soil under *Adansonia* sp., Nianing, Senegal	Section: 4.5
Pyrenochaetopsis decipiens	CBS 343.85[T]	Cyst on *Globodera pallida,* Hoofddorp, Netherlands	Section: 4.5
Repetophragma ontariense	HKUCC 10830	Substrate and origin unknown	Section: 4.5
Roussoella hysterioides	CBS 125434	On culms of *Sasa kurilensis,* Aomori, Shimokita, Yagen, Japan	Section: 4.5
Roussoella pustulans	MAFF 239637	Culm, stalk on *Sasa kurilensis,* Hokkaidou, Japan	Section: 4.5
Saccothecium sepincola	CBS 278.32	On *Ribes nigrum,* Bevarin, North Carolina	Section: 4.5
Stagonospora macropycnidia	CBS 114202	*Phragmites australis,* Södermanland, Sweden	Section: 4.5
Setomelanomma holmii	CBS 110217	Ascospores in fruiting body on *Picea pungens,* Wisconsin, USA	
Setosphaeria monoceras	CBS 154.26	Substrate and origin unknown	Section: 4.5
Triplosphaeria yezoensis	CBS 125436	On culms of *Sasa kurilensis,* Japan	Section: 4.5
Triplosphaeria cylindrica	MAFF 239679	Bamboo, *Sasa kurilensis,* Aomori, Japan	Section: 4.5
Zopfia rhizophila	CBS 207.26	Unknown substrate, United Kingdom	Section: 4.5

[T] ex-type strain; CBS, *Centraalbureau voor Schimmelcultures*, Holanda; IMI, *International Mycological Institute,* Egham, Surrey, United Kingdom; ATCC, *American Type Culture Collection,* Rockville, MD, USA; UBC herbarium, *University of British Columbia*, Vancouver, BC; NBRC, *NITE Biological Resource Centre*, Japan; HKUCC, *University of Hong Kong Culture Collection*, Department of Ecology and Biodiversity, Hong Kong, China; CMW, *Collection of the Forestry and Agricultural Biotechnology Institute* (FABI), University of Pretoria, South Africa; MAFF, *Ministry of Agriculture, Forestry and Fisheries*, Japan; BCC, *BIOTEC Culture Collection*, National Center for Genetic Engineering and Biotechnology (BIOTEC), Bangkok, Thailand; DAOM, *Plant Research Institute*, Department of Agriculture (Mycology), Ottawa, Canada; HHUF, Herbarium of Hirosaki University, Japan; Culture and specimen abbreviations: *ANM* A.N. Miller.