CLINICAL AND ENVIRONMENTAL ASPERGILLUS: MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION, PHYLOGENY, AND ANTIFUNGAL SUSCEPTIBILITY PROFIL·LE

Joao Paulo Zen Siqueire

ADVERTIMENT. L'accés als continguts d'aquesta tesi doctoral i la seva utilització ha de respectar els drets de la persona autora. Pot ser utilitzada per a consulta o estudi personal, així com en activitats o materials d'investigació i docència en els termes establerts a l'art. 32 del Text Refós de la Llei de Propietat Intel·lectual (RDL 1/1996). Per altres utilitzacions es requereix l'autorització prèvia i expressa de la persona autora. En qualsevol cas, en la utilització dels seus continguts caldrà indicar de forma clara el nom i cognoms de la persona autora i el títol de la tesi doctoral. No s'autoritza la seva reproducció o altres formes d'explotació efectuades amb finalitats de lucre ni la seva comunicació pública des d'un lloc aliè al servei TDX. Tampoc s'autoritza la presentació del seu contingut en una finestra o marc aliè a TDX (framing). Aquesta reserva de drets afecta tant als continguts de la tesi com als seus resums i índexs.

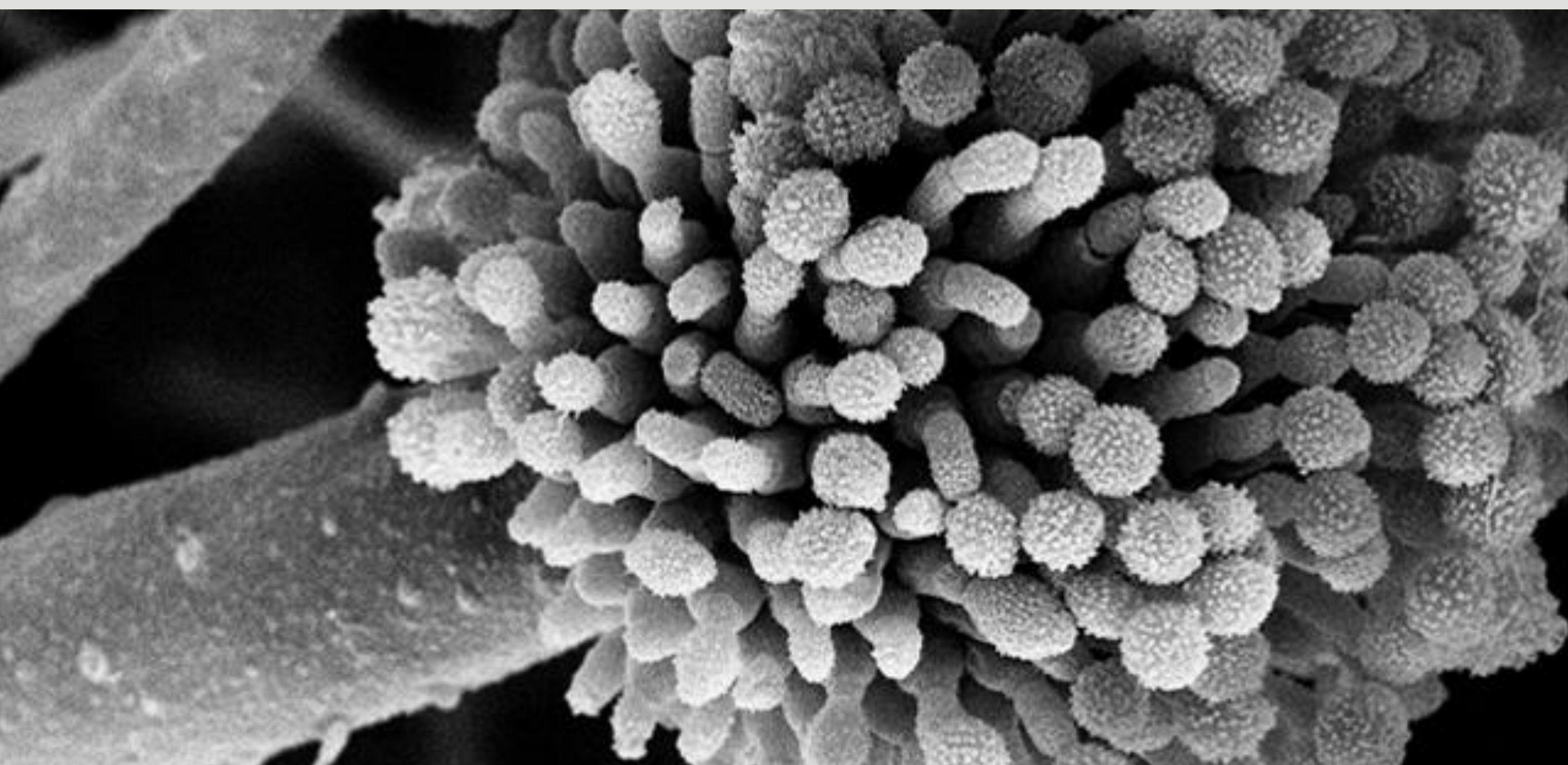
ADVERTENCIA. El acceso a los contenidos de esta tesis doctoral y su utilización debe respetar los derechos de la persona autora. Puede ser utilizada para consulta o estudio personal, así como en actividades o materiales de investigación y docencia en los términos establecidos en el art. 32 del Texto Refundido de la Ley de Propiedad Intelectual (RDL 1/1996). Para otros usos se requiere la autorización previa y expresa de la persona autora. En cualquier caso, en la utilización de sus contenidos se deberá indicar de forma clara el nombre y apellidos de la persona autora y el título de la tesis doctoral. No se autoriza su reproducción u otras formas de explotación efectuadas con fines lucrativos ni su comunicación pública desde un sitio ajeno al servicio TDR. Tampoco se autoriza la presentación de su contenido en una ventana o marco ajeno a TDR (framing). Esta reserva de derechos afecta tanto al contenido de la tesis como a sus resúmenes e índices.

WARNING. Access to the contents of this doctoral thesis and its use must respect the rights of the author. It can be used for reference or private study, as well as research and learning activities or materials in the terms established by the 32nd article of the Spanish Consolidated Copyright Act (RDL 1/1996). Express and previous authorization of the author is required for any other uses. In any case, when using its content, full name of the author and title of the thesis must be clearly indicated. Reproduction or other forms of for profit use or public communication from outside TDX service is not allowed. Presentation of its content in a window or frame external to TDX (framing) is not authorized either. These rights affect both the content of the thesis and its abstracts and indexes.

UNIVERSITAT
ROVIRA i VIRGILI

Clinical and environmental *Aspergillus*: morphological and molecular characterization, phylogeny, and antifungal susceptibility profile

JOÃO PAULO ZEN SIQUEIRA



DOCTORAL THESIS
2017

UNIVERSITAT ROVIRA i VIRGILI

Clinical and environmental *Aspergillus*: morphological and molecular characterization, phylogeny, and antifungal susceptibility profile

João Paulo Zen Siqueira

Doctoral Thesis

Directed by Drs. Josepa Gené Díaz, Josep Guarro Artigas,
and Dania García Sánchez,

Departament de Ciències Mèdiques Bàsiques
Facultat de Medicina i Ciències de la Salut
Universitat Rovira i Virgili

Reus

2017

UNIVERSITAT
ROVIRA i VIRGILI

Facultat de Medicina i Ciències de la Salut
Departament de Ciències Mediques Bàsiques
Unitat de Microbiologia

WE STATE that the present study, entitled **"Clinical and environmental *Aspergillus*: morphological and molecular characterization, phylogeny, and antifungal susceptibility profile"**, presented by **João Paulo Zen Siqueira** for the award of the degree of Doctor, has been carried out under our supervision at the Department Ciències Mèdiques Bàsiques of this university.

Reus, 4 September 2017

Doctoral Thesis Supervisors

Josepa Gené Díaz | Josep Guarro Artigas | Dania García Sánchez

ACKNOWLEDGEMENTS

First I would like to thank my supervisors Dr. Josepa Gené, Dr. Josep Guarro, and Dr. Dania García for accepting me as their student and for the guidance throughout these years.

I would also like to thank for the opportunity to work alongside with the other members of the group, Dr. Alberto Stchigel, Dr. José Cano, Dr. Maria José Figueras, and Dr. Javier Capilla in such a prestigious center.

Thank you to my colleagues and friends at the laboratory, who played a big part in my life during these years: Marcelo, Aleja, Fadua, Adela, Marta, Marcela, Nico, Alba (Pérez Cataluña), Núria (Salas), Ana, Patri, Emmanuel, Damaris, Alba New, Neto, Isabel, Kati, and Loida. Also Kevin, who spent just a few months but was a great friend.

I have to express my gratitude to the laboratory's technicians, who helped me many times, Carolina, Jordi, Núria (Pilas), and Carmen.

A special mention to my mentor in Microbiology, Dr. Margarete Gottardo de Almeida, I am very grateful to all that she has done for me, and Keith Cunha, who also helped me and taught me a lot. They have opened the doors for this PhD and always believed in my work.

I am also thankful to all of the coauthors, who played decisive rolls in the studies included in this thesis and are also a part of it.

I would like to acknowledge CAPES for conceding me the grant which allowed me to stay in Spain.

Finally, I want to thank my family, my friends, and Samia, for the support and patience. The strength to work hard and always do my best I got from you, thank you.

INDEX

LIST OF ABBREVIATIONS i

1. INTRODUCTION 1

1.1. Brief History 3

1.2. General Characteristics and Morphology 4

1.3. Taxonomy and Nomenclature 10

1.4. Species Characterization in *Aspergillus* 11
1.4.1. Morphological Identification 12
1.4.2. Extrolites Profile 14
1.4.3. Molecular Identification 15
1.4.4. Genomics 17

1.5. Importance of *Aspergillus* 17
1.5.1. Biotechnological Aspects of *Aspergillus* 18
1.5.2. *Aspergillus* and Disease 19
1.5.2.1. Laboratory Diagnosis of Aspergillosis 21
1.5.2.2. Antifungal Susceptibility Testing 22

2. INTEREST AND OBJECTIVES 25

3. MATERIAL AND METHODS 29

3.1. Origin of the Isolates 31

3.2. Techniques for the Isolation of Environmental *Aspergillus* 31

3.3. Phenotypic Characterization 32

3.4. Molecular Studies 33
3.4.1. DNA Extraction, Amplification, and Sequencing 33
3.4.2. Molecular Identification and Phylogenetic Analysis 33

3.5. Antifungal Susceptibility Test 34

3.6. Storage and Conservation of the Strains 35
3.6.1. Short Term Conservation 36
3.6.2. Long Term Conservation 36

4. RESULTS 61

4.1. Species diversity of *Aspergillus* section *Versicolores* in clinical samples and antifungal susceptibility 65

4.2. Multilocus phylogeny and antifungal susceptibility of *Aspergillus* section *Circumdati* from clinical samples and description of *A. pseudosclerotiorum* sp. nov. 77

4.3. Species of *Aspergillus* section *Aspergillus* from clinical samples in USA 91

4.4. Cryptic *Aspergillus* species from clinical samples in the USA and description of a new species in section *Flavipedes* 125

4.5. Herbivorous dung, a forgotten source of Eurotialean fungi 161

4.6. New species of *Aspergillus* from soil 293

4.6.1. *Aspergillus bicephalus* sp. nov. 293

4.6.2. *Aspergillus subglobosus* sp. nov. 297

4.6.3. *Aspergillus argentinensis* sp. nov. 303

4.6.4. *Aspergillus tumidus* sp. nov. 309

5. SUMMARIZING DISCUSSION 315

6. CONCLUSIONS 321

7. REFERENCES 325

LIST OF ABBREVIATIONS

1F=1N	One Fungus = One Name
5FC	Flucytosine (5-Fluorocytosine)
AFG	Anidulafungin
AMB	Amphotericin B
Amplific.	Amplification
ATCC	American Type Culture Collection
a_w	Water Activity
BAL	Bronchoalveolar Lavage
BenA	Fragment of the β-tubulin Gene
BI	Bayesian Inference
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
bs	Bootstrap Support
Bx	Biopsy
CaM	Calmodulin
CBS	CBS-KNAW Fungal Biodiversity Centre
Cct8	Chaperonin Containing TCP1 Subunit 8
CFG	Caspofungin
CLSI	Clinical and Laboratory Standards Institute
cm	Centimeter
comb. nov.	*combinatio nova*, Latin expression meaning "new combination"
CREA	Creatine Sucrose Agar
CY20S	Czapek Yeast Autolysate Agar supplemented with 20% Sucrose
CYA	Czapek Yeast Autolysate Agar
d	Days
D1-D2	Domains of the 28S rRNA Gene
DG18	Dichloran 18% Glycerol Agar
diam	Diameter
Dir.	Direction
DNA	Deoxyribonucleic Acid
DRBC	Dichloran Rose-Bengal Chloramphenicol Agar
DTO	Applied and Industrial Mycology Department Collection
e. g.	*exempli gratia*, Latin expression meaning "for example"
EMBL	European Molecular Biology Laboratory

et al.	*et alii,* Latin expression meaning "and others"
etc.	*et cetera*, Latin expression meaning "and the rest (of such things)"
Fig.	Figure
FMR	Faculty of Medicine, Reus
g	Gram
gen. nov.	*genus novus*, Latin expression meaning “new genus”
GM	Geometric Mean
h	Hour
HPLC	High Performance Liquid Chromatography
ICBN	International Code of Botanical Nomenclature
i. e.	*id est*, Latin expression meaning "that is" or “namely”
ILD	Incongruence Length Difference Test
ITC	Itraconazole
ITS	Internal Transcribed Spacer
L	Liter
LSU	Large Subunit of the rRNA
M60Y	Harold’s Agar supplement with 60 % Sucrose
MAFFT	Multiple Alignment using Fast Fourier Transform
MALDI-TOF	Matrix Assisted Laser Desorption Ionization – Time of Flight
MCMC	Markov Chain Monte Carlo Algorithm
MEA	Malt Extract Agar
MEC	Minimal Effective Concentration
MFG	Micafungin
mg	Milligram
MIC	Minimal Inhibitory Concentration
min	Minute
ML	Maximum Likelihood
mL	Milliliter
mm	Millimeter
MS	Mass Spectrometry
MUSCLE	Multiple Sequence Comparison by Log-Expectation
NCBI	National Center for Biotechnology Information
NJ	Neighbour-Joining
No.	Number
NRRL	Agriculture Research Service Culture Collection
OA	Oatmeal Agar
PAUP*	Phylogenetic Analysis Using Parsimony

PCA	Potato Carrot Agar
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
pp	Bayesian Posterior Probabilities
PSC	Posaconazole
rDNA	Ribosomal DNA
RPB1	RNA Polymerase II Largest Subunit
RPB2	RNA Polymerase II Second Largest Subunit
Sect.	Section
SEM	Scanning Electron Microscopy
sp.	species
sp. nov.	*species nova*, Latin expression meaning "new species"
TBF	Terbinafine
Temp.	Temperature
Tsr1	Ribosome Maturation Factor
URV	Universitat Rovira i Virgili
UTHSC	University of Texas Health Science Center
UTHSCSA	University of Texas Health Science Center (San Antonio)
v.	Version
VRC	Voriconazole
YES	Yeast Extract Sucrose Agar
Yr	Year
µg	Microgram
µL	Microliter
µm	Micrometer
°C	Degrees Celsius

1. INTRODUCTION

1.1. Brief History

In 1729, the catholic priest and botanist Pier Antonio Micheli (1679–1737) altered the course of mycology, being considered today one of the founding fathers of this branch of science. This is mainly because of his publication in that year of "*Nova Plantarum Genera: iuxta Tournefortii methodum disposita".* In this work, he described and illustrated around 900 species of fungi, including important genera such as *Botrytis*, *Polyporus*, and *Mucor*. He also described a fungus with a spore-bearing structure that reminded him of a device used by the clergy to sprinkle holy water called *asperges* (in English, aspergillum), and named it *Aspergillus* (Figure 1).

Eighty years after this first description, Link (1809) introduced two species: *Aspergillus glaucus* and *Eurotium herbariorum*, without noticing they represented a unique organism. De Bary (1854) demonstrated their common mycelial origin, showing that *E. herbariorum* was, in fact, the sexual stage of *A. glaucus*.

In 1926, Thom and Church were the first to revise the genus *Aspergillus*, including 69 species divided into 11 groups. Later, Thom and Raper (1945) compiled 77 species with 10 varieties, placing them in 14 groups. Afterwards, the monograph of Raper and Fennell (1965) accepted 132 species subdivided into 18 groups; this is considered one of the most important works regarding the taxonomy of *Aspergillus*. Gams et al. (1985) established a new scheme of classification of the genus in sections and subgenera; they proposed this infrageneric division including six subgenera and 17 sections. It is noteworthy that this classification was, at that time, based almost exclusively on the study of macro and microscopic features of the specimens.

More recently, DNA sequence data have been used in *Aspergillus* systematics to address issues about species boundaries and infrageneric relationships. Relevant works were accomplished by Varga and Samson (2008), with the publication "*Aspergillus* systematics in the genomic era" and by Peterson (2008), who provided the sequences of four different loci of 460 *Aspergillus* isolates. This multilocus analysis has demonstrated to be a very useful approach to identify and infer phylogeny of the members of *Aspergillus* with confidence. These and other molecular studies (Houbraken and Samson 2011) have shown that phenotypic-based groups of Raper and Fennell (1965) largely correspond with the nowadays classification of the genus (Houbraken et al. 2014).

In 2011, in the International Botanical Congress Nomenclature Section at Melbourne, it was accepted the "One Fungus: One Name" (1F=1N) principle, which had a major impact on *Aspergillus* taxonomy (McNeill et al. 2012). Therefore, following

the new nomenclatural rules, several genera that described sexual morphs of *Aspergillus*, such as *Neosartorya*, *Emericella*, *Eurotium*, and *Petromyces*, are now synonymized with *Aspergillus*.

Few years ago, Samson et al. (2014) provided a detailed revision including the latest information about phylogeny, nomenclature, and identification of *Aspergillus*. It must be taken into account that many other publications have contributed substantially to the study of *Aspergillus* throughout the years and shaped the knowledge we have on this genus. Moreover, the number of recent publications shows that the history of the studies in *Aspergillus* is still being written, with new information becoming available every year.

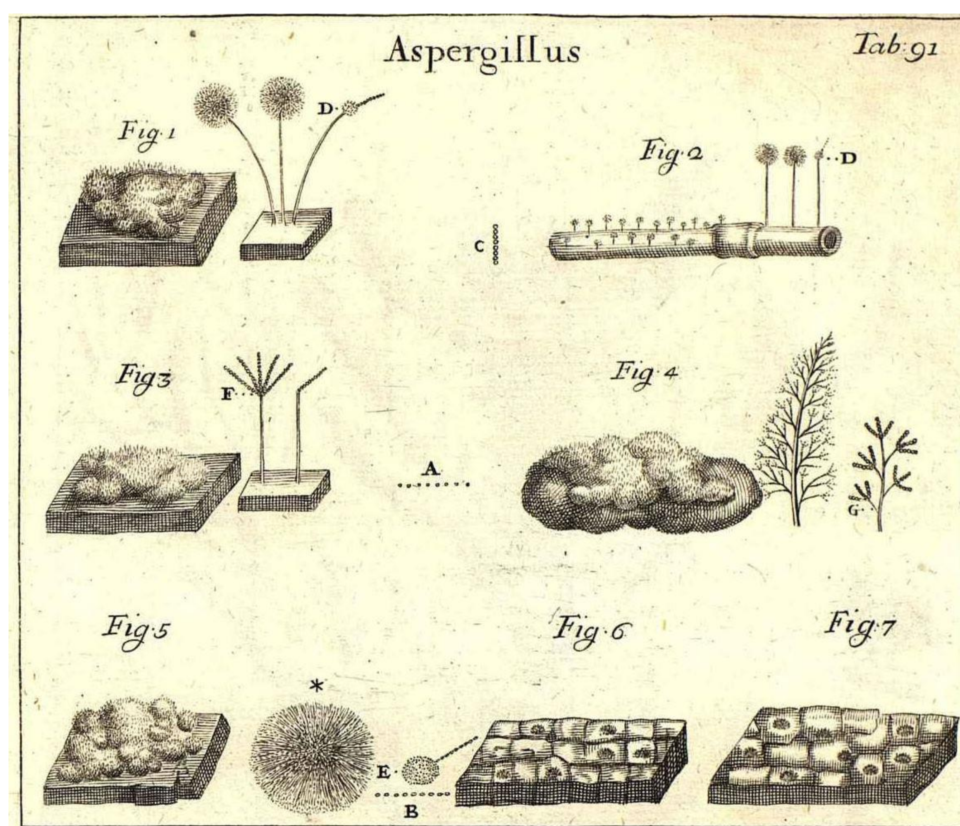


Figure 1. First illustrations of *Aspergillus* made by Pier Antonio Micheli in his publication *Nova Plantarum Genera* (reproduced from Micheli 1729).

1.2. General Characteristics and Morphology

Opposed to yeast, the unicellular life form of fungi, *Aspergillus* is a multicellular organism, known as mold or filamentous fungus. The cells are organized as branched filaments called hyphae, which form a network called mycelium. They are heterotrophic

organisms, able to secrete acids and enzymes into the surrounding environment, breaking down polymeric molecules down into simpler ones that are then absorbed back into the fungal cell (Bennett 2010). In general, genus *Aspergillus* is very well adapted to live in a wide variety of conditions, what makes it one of the most common fungi in the planet. One of the main reasons for such ubiquity is the prolific production of uninucleated, hydrophobic, airborne conidia (Axelrod et al. 1973). The conidia production occurs by the differentiation of vegetative hyphae by asexual development (anamorphic stage). Additionally, the *Aspergillus* life cycle (Figure 2) may also include a sexual stage, (teleomorphic stage), known for many species. It involves the production of closed sexual fruiting bodies containing meiotic spores (ascospores). Where the sexual state is present, aspergilli have either heterothallic or homothallic breeding systems. Homothallic species can enter into the sexual cycle without the need to cross with a compatible partner, but are usually not restricted to self-fertilization. Although *A. fumigatus* and *A. flavus* were reported to present heterothallic systems, the homothallic breeding is far more prevalent among *Aspergillus* species (O'Gorman et al. 2009; Czaja et al. 2011; Dyer and O'Gorman 2012).

Both states, asexual and sexual, can offer adaptive advantages to survive and proliferate in many substrates. *Aspergillus* are commonly saprophyte organisms, many species (e.g., the former *Eurotium* species) are able to grow at low water activity (a_w) (below 0.75); whereas others can survive in a wide range of temperatures (*A. candidus*, from 3 to 44 ºC; *A. fumigatus*, from 12 to 65 ºC) (Lacey and Magan 1991). Regarding pH, some studies report that *A. niger* can grow over a pH range of 1.5–9.8, *A. candidus* over 2.1–7.7, and *A. pseudoglaucus* over 1.8–8.5 at 1.0 a_w (Panasenko 1967). In addition, *Aspergillus* species appear to be tolerant at high CO_2 concentrations (Paster et al. 1983). These physiological characteristics make this genus very common on soil, plant debris, stored feeds and seeds, indoor air environment, among many other substrates.

Most *Aspergillus* species are recognizable by their characteristic conidiogenenous apparatus, i.e. an unbranched conidiophore terminating in a vesicle, on which the conidiogenous cells (phialides) are borne directly or on metulae (defined as "cell proximally adjacent to the phialide"), and the conidia are arranged in dry long chains. The current accepted terminology for the structures present in a typical *Aspergillus*, as well as the classic terminology used by Raper and Fennell (1965), is shown on Figure 3. The term sterigma was traditionally used to design the vesicle surface buds, the primary sterigma called metula and the secondary, phialide (Krijgsheld et al. 2013). However, in general, this terminology has been abandoned for the hyphomycetes. The terms *stipe* and *foot cell* are also being avoided in the

descriptions of aspergilli, and the term conidiophore is considered more appropriate to designate those regions (Minter et al. 1985). Conidial head is generally used to designate the portion that comprehends the vesicle and conidiogenous cells.

It must be considered that, based on phylogenetic analyses, the production of aspergillus-like conidial heads does not guarantee that a given species belongs to *Aspergillus* (Samson et al. 2014). For example, *Penicillium paradoxum* was formerly described in *Aspergillus* because it produces condiophores with a terminal vesicle reminiscent of this genus; however, phylogenetic analyses place it in the genus *Penicillium* (Visagie et al. 2014a). On the other hand, species lacking typical aspergillus-like asexual structures are nested within the *Aspergillus* generic clade (Houbraken and Samson 2011). An example is *Aspergillus inflatus* (Stolk and Malla 1971), which produces penicillium-like conidiophores, although it belongs to *Aspergillus* (Samson et al. 2014) (Figure 4).

The *Aspergillus* species with known sexual stage are able to form a diploid zygote, which undergoes meiosis to produce haploid ascospores. This phase takes place inside an ascoma-type fruiting body called cleistothecium. The cleistothecium is a small spherical body containing thousands of ascoscopores, which may or may not be covered with white mycelium (Raper and Fennell 1965). The bivalve construction of a typical ascospore may have an evident furrow between the two parts or be flanked by crests. They can be smooth, rough, echinulate, or ridged, and all these characteristics are of important taxonomic value for species delimitation (Figure 5).

Also may be present in an *Aspergillus* species Hülle cells and sclerotia (Figure 6a and 6b). Hülle cells are specialized structures with thick walls, usually surrounding the cleistothecia. It may provide protective layer against desiccation and attack by soil dwelling animals, although their true function is uncertain (Johnson and Borman 2010). Sclerotium is a vegetative body consisting of thick-walled parenchyma-like cells shaped as hard masses. The role of sclerotia is to survive environmental extremes, and when conditions are again favorable, the sclerotia can directly produce conidia or form new mycelia (Wicklow 1987; Cary et al. 2007).

Another peculiarity that may be observed in *Aspergillus* is the presence of accessory conidia (Figure 6c). This type of conidia was first observed in *A. terreus*; they are usually globose, produced singly, in clusters, sessile, or in small conidiophores, laterally on the hyphae. These conidia have been observed in vivo during human infection and they have already been reported in other species, such as *A. citrinoterreus* (section *Terrei*) or in many species of the section *Flavipedes* (Balajee 2009; Guinea et al. 2015; Hubka et al. 2015).

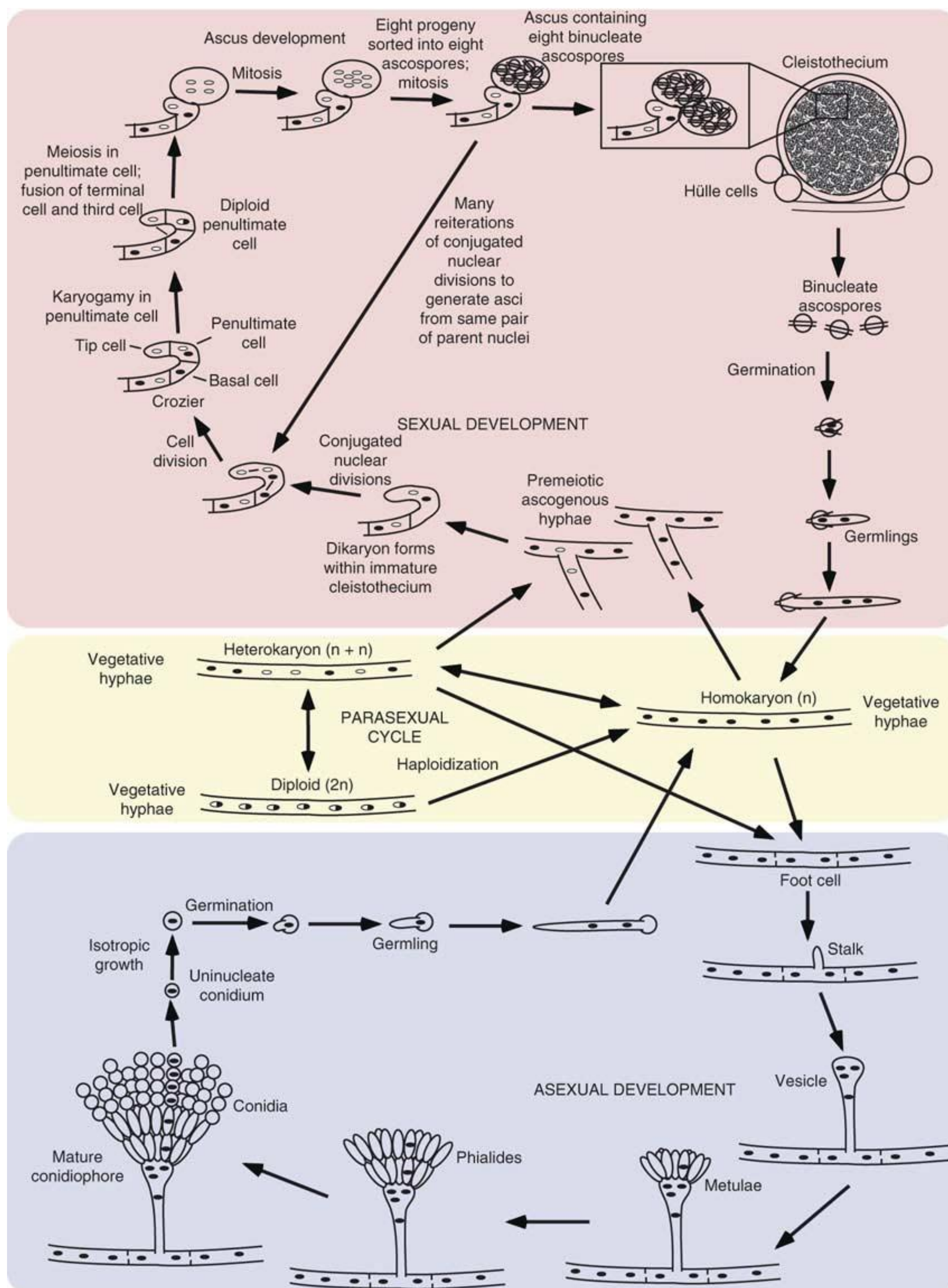


Figure 2. Life cycle of *Aspergillus nidulans*. The asexual development is highlighted in purple, sexual development in pink, and parasexual cycle in yellow. The parasexual cycle consists in vegetative hyphae from two individuals fusing to form a heterokaryon and nuclei in a heterokaryon or a homokaryon fusing to form a diploid (reproduced from Todd et al. 2007).

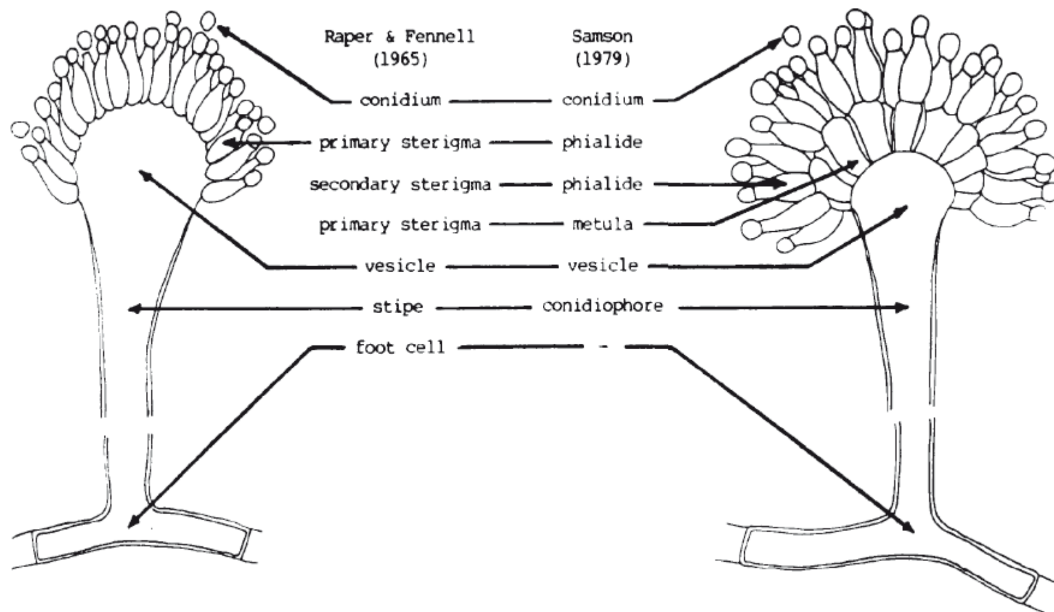


Figure 3. Terms used by some authors to describe the different conidiogenous structures in *Aspergillus* (adapted from Minter et al. 1985).

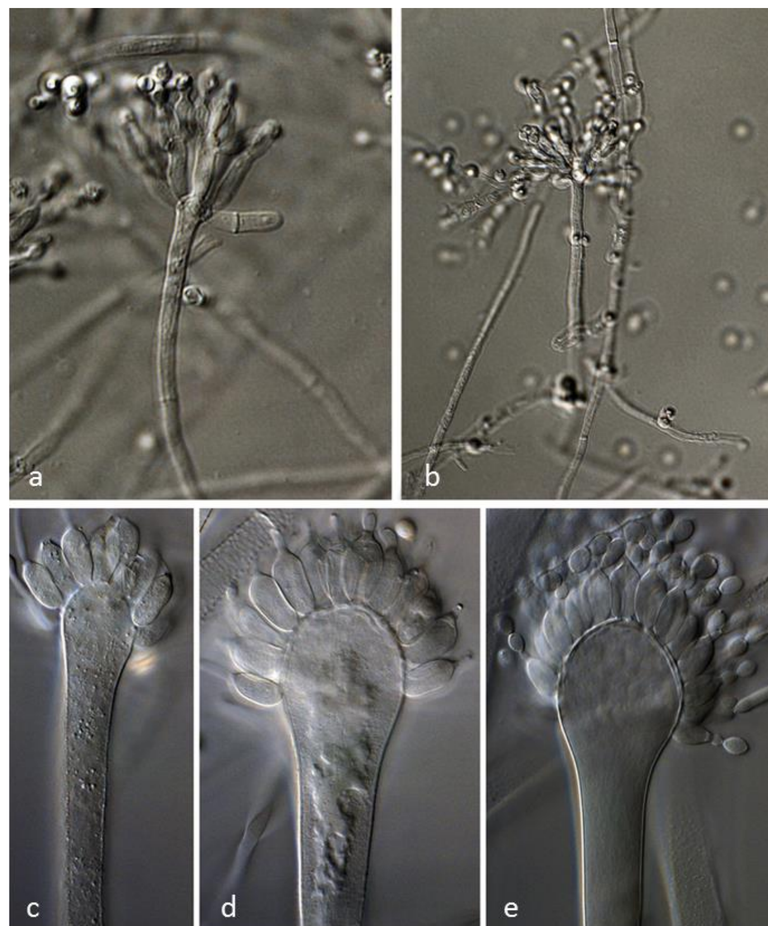


Figure 4. Conidiophores of *Aspergillus inflatus* (a,b) (reproduced from Samson et al. 2014) and *Penicillium paradoxum* (c–e) (reproduced from Visagie et al. 2014a).

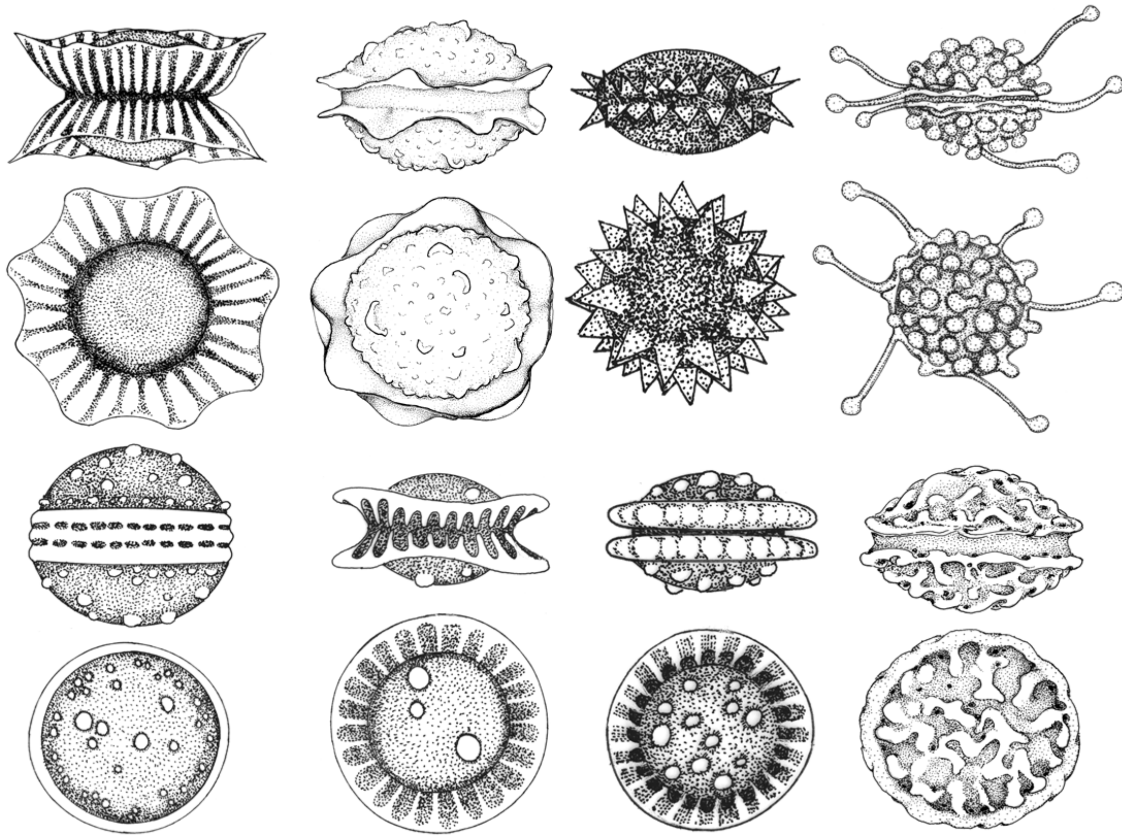


Figure 5. Examples of different morphology and ornamentation of *Aspergillus* ascospores (reproduced from Guarro et al. 2012).

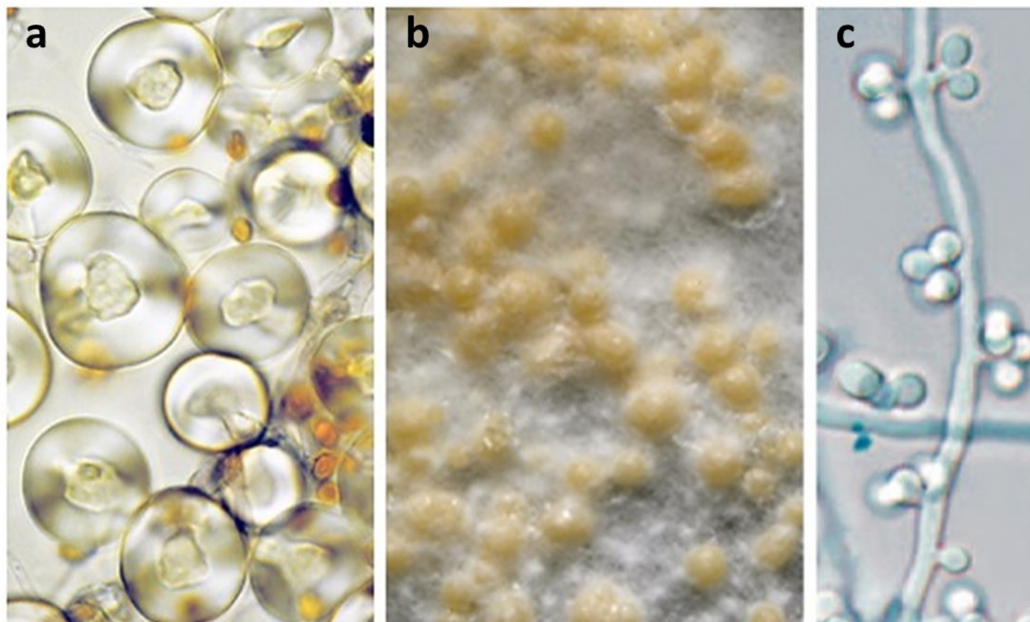


Figure 6. Specialized structures in *Aspergillus*. Hülle cells (a); sclerotia (b); and accessory conidia (c) (reproduced from Hubka et al. 2015).

As mentioned before, after the first description of *Aspergillus* by Micheli in 1729, many reviews and advances were made in the attempt to update the generic concept of the genus. Currently, the *Aspergillus* concept was emended by Samson et al. (2014) and it is as following:

Aspergillus P. Micheli ex Haller, emended description (Samson et al. 2014)

Generic type: *Aspergillus glaucus* (L.) Link

Vegetative mycelium hyaline to brightly pigmented. Conidiophores (aspergillum) consisting of thick-walled basal cells (foot cell) producing stalks, usually aseptate and unbranched, terminating in inflated apex (vesicle) which can be globose, ellipsoidal to clavate; conidiophores in some species may be septate, lack a foot cell, lack a vesicle, or consisting of single conidiogenous cells with one to several loci. Conidiogenous cells phialidic, producing dry conidial chains borne directly on the vesicle (uniseriate) or on metulae (biseriate); in a few species, appearing to be annellidic or polyphialidic. Conidia greatly varying in color, size, shape and ornamentation. Cleistothecia of various structures produced by some species with mostly a thin ascoma wall consisting of a single layer of hyphal networks, sometimes covered by layers of Hülle cells or sclerotium-like. Asci globose usually containing eight ascospores. Ascospores often lenticular, hyaline or coloured, varying in size, shape and ornamentation. Sclerotia or sclerotium-like structures regularly present in some species, varying in colour, size and shape, consisting of thick-walled cells, sometimes containing ascigerous structures. Hülle cells sometimes covering cleistothecia or occurring in compact masses in the mycelium, varying in shape and size, but mostly thick-walled and hyaline.

1.3. Taxonomy and Nomenclature

Taxonomy is a dynamic discipline and inadequate classification or uncertain nomenclature can lead to tremendous confusion (Houbraken et al. 2014). For a long time, *Aspergillus* was considered as an anamorphic genus being placed in the *Deuteromycota* phylum, *Hyphomycetes* class. The problem was that this traditional classification was artificial and usually based on morphology, and not on common phylogenetic background. However, with the progress of DNA sequencing and more precise analytical methods, the classification of anamorphic fungi has been modified

and hence redefined. Consequently, the current taxonomic position of *Aspergillus* is in *Ascomycota* phylum, *Pezizomycotina* subphylum, *Eurotiomycetes* class, *Eurotiales* order, and *Aspergillaceae* family. *Aspergillus* was previously considered as belonging to the *Trichocomaceae* family; however, Houbraken and Samson (2011), based on four genetic markers (Cct8, Tsr1, RPB1, and RPB2), have divided such family into three, *Aspergillaceae*, *Thermoascaceae*, and *Trichocomaceae*.

As previously stated, the taxonomy and nomenclature of the teleomorphs of *Aspergillus* have also suffered important changes. Until recently, the different sexual states of *Aspergillus* were considered different genera. However, in the light of phylogenetic studies and the nomenclature changes proposed by 1F=1N, all teleomorph-based genera were transferred to *Aspergillus*. Therefore, the following 10 genera are considered teleomorphs of this genus and synonymized with *Aspergillus*, i.e., *Cristaspora*, *Emericella*, *Eurotium, Fennellia*, *Hemisartorya*, *Neocarpenteles*, *Neopetromyces*, *Neosartorya*, *Petromyces*, and *Saitoa*. The genera *Sclerocleista* and *Warcupiella*, previously considered *Aspergillus* teleomorphs, do not belong to the *Aspergillus* monophyletic clade, and thus were excluded from this genus (Houbraken et al. 2014; Kocsube et al. 2016).

Considering such a diverse and large genus, the generic classification of *Aspergillus* encompasses a great variability. Consequently, to group species morphologically and genetically related, which form distinct phylogenetic clades, the genus is commonly subdivided in subgenera and sections. The latest review of *Aspergillus* was based on nine loci (18SrDNA, 5.8S rDNA, 28S rDNA (D1-D2), RPB1, RPB2, CaM, BenA,Tsr1, Cct8) and Kocsube et al. (2016) divided it in six subgenera and 22 sections (Figure 7), but they did not included two sections (*Petersonii* and *Tanerii*). More recently, Chen et al. (2016) proposed the adoption of section *Cavernicolus* to group five species previously assigned to section *Usti*. Consequently, up to this date, *Aspergillus* has 25 proposed sections.

1.4. Species Characterization in *Aspergillus*

There are over 350 described species of *Aspergillus* (Samson et al. 2014), many of them based on their morphological and physiological features. However, in the last years, these characters proved to be insufficient to distinguish all the species, especially the called cryptic ones, which are usually phylogenetically very close related and consequently, sharing many phenotypic characteristics.

There are a number of diagnostic features important in the identification of the species of *Aspergillus*. The introduction of molecular technologies has been a great advance in this field, but there may have been a tendency to over-evaluate these techniques in the characterization of species. There is no method, molecular or phenotypic, which works flawlessly in recognizing species (Samson and Varga 2009). To overcome the individual disadvantages of each method, the polyphasic approach proposes the combination of many features to reach species recognition in *Aspergillus* with confidence, such as morphological characters of colony and micromorphology; physiological characters, such as growth on different culture media at different temperatures; extrolite profiles; and multilocus DNA sequence analyses.

1.4.1. Morphological Identification

The classical method for characterizing *Aspergillus* isolates was summarized by Samson et al. (2014). The basic recommended media for species recognition in *Aspergillus* are Czapek Yeast Autolysate agar (CYA) and Malt Extract agar (MEA). However, when describing a new taxon, other media should be included in the characterization; usually, Dichloran 18% Glycerol agar (DG18), Yeast Extract Sucrose agar (YES), Oatmeal agar (OA), and Creatine Sucrose agar (CREA). CREA, first introduced for penicillia characterization, can also provide an important physiological aid in the identification of *Aspergillus* species, being able to detect acid production because of the bromocresol purple pH indicator (Frisvad 1981). Depending on the organism, for example osmophilic *Aspergillus* from section *Aspergillus*, other media can be included, such as CYA supplemented with 20% sucrose and Harrold's agar. These latter media have high content of sugar, a condition required for some species of that section to grow and sporulate (Raper and Fennell 1965; Hubka et al. 2013). The colony morphology, also known as macromorphology, is often recommended to be observed at 7 and 14 days, but it is also important keep the observation of the colony for longer periods of time because some characteristics (e.g. sclerotia, soluble pigments, ascomata) may need more time to appear. Most of the visible features may have taxonomic value, such as colony growth rates, texture, degree of sporulation, production of sclerotia or cleistothecia, mycelium colors, soluble pigments, exudates and colony reverse.

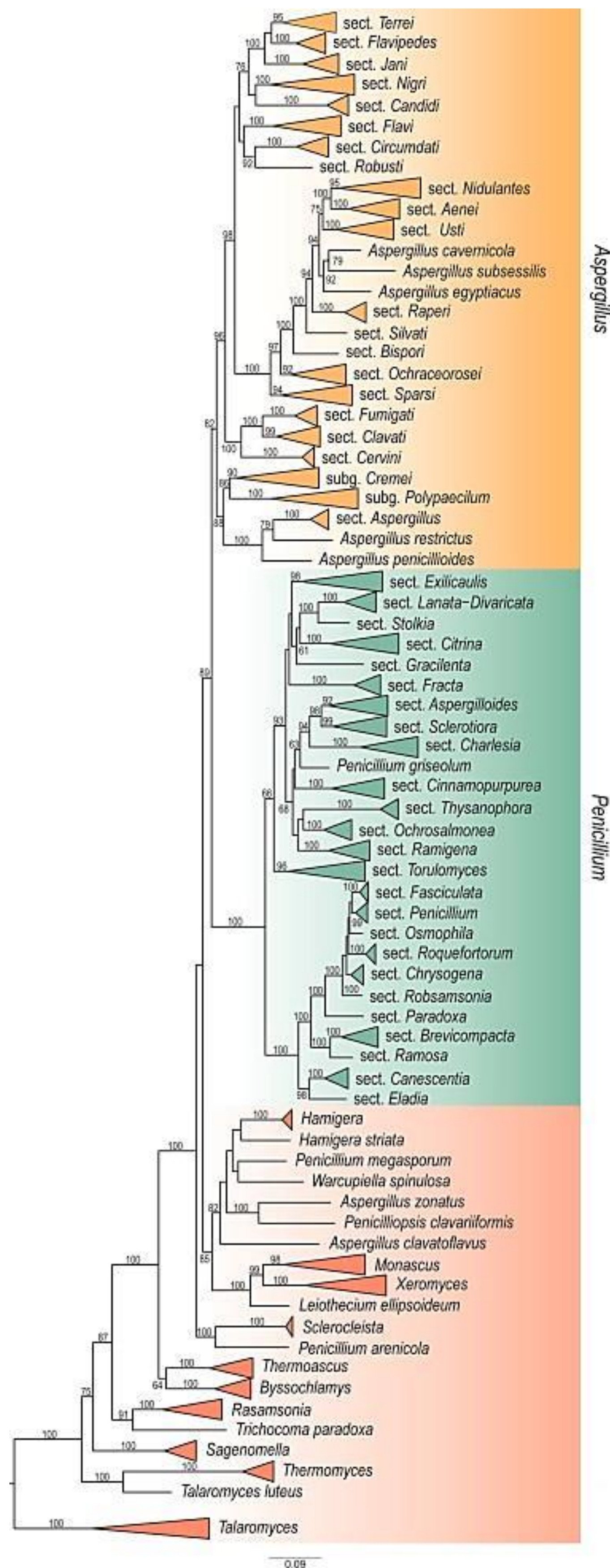


Figure 7. ML tree based on nine loci showing the position of *Aspergillus* and its sections in relation to families *Aspergillaceae*, *Thermoascaceae*, and *Trichocomaceae*. Monophyletic groups are collapsed and shown as triangles (reproduced from Kocsube et al. 2016).

The microscopic features, or micromorphology, are recommended to be observed under light microscopy mainly from MEA cultures after at least 7 days of incubation. The recommended mounting fluid for this purpose is 60% lactic acid or Shear's solution. By contrast, lactophenol is corrosive and it is not recommended. Washing out the excess of conidia with a drop of 70 % ethanol facilitates the visualization and prevents the presence of air bubbles. The useful characters to be examined are: the shape of conidial heads (radiate or columnar), the number of branching points between vesicle and phialides (uniseriate or biseriate), color of conidiophores, and the size, shape and texture of conidiophores, vesicles, metulae (when present), phialides, conidia, Hülle cells (when present), cleistothecia, asci and ascospores. Texture of conidiophores and conidia vary from smooth to rough. The shape of vesicles are usually globose, pyriform, spathulate, or clavate. The use of scanning electron microscopy (SEM) is also a powerful tool for species recognition, especially for visualizing ascospores ornamentation (Samson et al. 2014).

1.4.2. Extrolites Profile

By definition, an extrolite (or secondary metabolite) is an outwardly directed chemical compound produced during differentiation of a living organism. It is usually excreted, but can also be accumulated in the cell wall or membrane. Their regulation is by the genome and influenced by the surrounding biotic and abiotic environment (Samson and Frisvad 2004). Evidences show that some extrolites like xanthocillins, terphenyllins, and emodin are in common within all subgenera of the genus (Frisvad and Larsen 2015) (Table 1). Frequently, within a particular section of *Aspergillus*, a large number of species share the ability to produce a given secondary metabolite, although most metabolites are produced by species in only one or few sections. However, it must be taken into account that some well-known bioactive secondary metabolites, such as penicillin, viridivatin, mevinolin, pseurotin A and cyclopiazonic acid are present in phylogenetically different sections of *Aspergillus* (Frisvad and Larsen 2015).

Production of a particular secondary metabolite can be an efficient identification trait for allocating species to section level and even for species identification (Samson et al. 2014). For this purpose, the best methodology for extracting and separating the extrolites is through High Performance Liquid Chromatography (HPLC) and then, the identification is usually carried out by mass spectroscopy based technology (Frisvad et

al. 2008). Growth media and technical and analytical procedures must be standardized to give reliable profiles. Furthermore, a qualitative database on the verified production of secondary metabolites by different species of *Aspergillus* is required to enable identification (Samson et al. 2014).

Table 1. Extrolites present in *Aspergillus* subgenera based on Frisvad and Samson 2004; Samson et al. 2004; Nielsen et al.2009; Frisvad and Larsen 2015, 2016; Ma et al. 2016 (reproduced from Kocsube et al. 2016).

Extrolites	*Aspergillus and Cremei*	*Fumigati*	*Nidulantes*	*Circumdati*
Pseurotins	−	+	−	+
Kojic acid	−	−	+	+
Terrein	−	−	+	+
Asperphenamate	+	−	−	+
Sterigmatocystin	+	−	+	+
Cyclopiazonic acid	−	+	−	+
Malformins	−	+	−	+
Fumitremorgins	−	+	+	+
Emodin (as precursor)	+	+	+	+
6-Methylsalicylic acid (as precursor)	−	+	−	+
Itaconic acid	+	−	−	+
Viridicatins	−	+	+	+
Penicillins	−	+	+	+
Notoamides	−	−	+	+
Aflavinins	−	+	+	+
Echinulins	+	+	−	+
Diketopiperazines	+	−	−	+
Polythiodiketopiperazines	−	+	+	+
Kotanins/desertorins	+	−	+	+
Falconensin type azaphilones	−	+	+	+
Xanthocillins and terphenyllins	+	+	+	+
Mycophenolic acid	+	+	−	−
Heveadrides	+	+	−	−
Patulin	+	+	−	−

1.4.3. Molecular Identification

DNA sequence data and molecular phylogenetic studies are being applied in *Aspergillus* taxonomy to address issues about species boundaries and infrageneric relationships for nearly thirty years (Geiser et al. 2008). The most frequently sequenced marker, considered the official DNA barcode in fungi, is the internal transcribed spacer rDNA region (ITS), which includes the ITS1, 5.8S gene, and ITS2 regions (Schoch et al. 2012). Although it is broadly used, it sometimes does not contain enough variation for distinguishing among all species of *Aspergillus*, and the rank of discrimination

generally is only to the section level. Based on that, other genetic markers are needed to give more information and to successfully identify close related species. The adoption of other markers should be considered as long as they are easy to amplify and there are sequences available for comparison (Samson et al. 2014). Three additional loci are largely used for identification and taxonomy of *Aspergillus* (Peterson et al. 2008; Samson et al. 2014) (Table 2). A portion of the β-tubulin gene (*BenA*) is easy to amplify and there are universal primers (Glass and Donaldson 1995). However, caution is necessary because the PCR can result in the amplification of paralogous genes, generating confusion and mistakes in phylogenetic inferences (Hubka and Kolarik 2012). Portion of the calmodulin (*CaM*) gene is also widely used, and the sequence database is almost complete for all accepted species of *Aspergillus.* Another useful option is sequencing the RNA polymerase II second largest subunit (*RPB2*) which can provides complementary information, but it is reported not so easy to amplify as the previously mentioned markers. Samson et al. (2014) suggest the use of *CaM* as a provisional secondary identification marker in *Aspergillus*, but the best alternative to the ITS region may vary among the sections. The multilocus study based on these four genetic markers (ITS, *BenA*, *CaM*, and *RPB2)* is currently the most effective way to distinguish all species of *Aspergillus*, including the considered cryptic ones.

Table 2. Primers and annealing temperatures used for amplification and sequencing in *Aspergillus* (adapted from Samson et al. 2014).

Locus	Amplific.	Annealing temp (ºC)	Cycles	Primer	Dir.	Primer sequence (5′–3′)	Reference
ITS	standard	55 (alt. 52)	35	ITS1	F	TCCGTAGGTGAACCTGCGG	White et al. 1990
				ITS4	R	TCCTCCGCTTATTGATATGC	White et al. 1990
				V9G	F	TTACGTCCCTGCCCTTTGTA	De Hoog and Ende 1998
				LS266	R	GCATTCCCAAACAACTCGACTC	Masclaux et al. 1995
BenA	standard	55 (alt. 52)	35	Bt2a	F	GGTAACCAAATCGGTGCTGCTTTC	Glass and Donaldson 1995
				Bt2b	R	ACCCTCAGTGTAGTGACCCTTGGC	Glass and Donaldson 1995
CaM	standard	55 (alt. 52)	35	CMD5	F	CCGAGTACAAGGARGCCTTC	Hong et al. 2005
				CMD6	R	CCGATRGAGGTCATRACGTGG	Hong et al. 2005
				CF1	F	GCCGACTCTTTGACYGARGAR	Peterson et al. 2005
				CF4	R	TTTYTGCATCATRAGYTGGAC	Peterson et al. 2005
RPB2	touch up	50-52-55	5-5-30	5F	F	GAYGAYMGWGATCAYTTYGG	Liu et al. 1999
		(48-50-52)		7CR	R	CCCATRGCTTGYTTRCCCAT	Liu et al. 1999
				5Feur	F	GAYGAYCGKGAYCAYTTCGG	Houbraken et al. 2012
				7CReur	R	CCCATRGCYTGYTTRCCCAT	Houbraken et al. 2012

1.4.4. Genomics

Next-generation sequencing is becoming increasingly affordable, enabling whole-genome sequence-based analyses. Up to date, 44 *Aspergillus* species genomes are available in the fungal genomics portal MycoCosm (http://jgi.doe.gov/fungi), developed by the US Department of Energy Joint Genome Institute, a number more than twice than it was in 2014. *Aspergillus* genomes are similar in size (29 to 36 Mb) and GC content (48 to 53%), slightly larger than in *Onygenales*. Similarly, the number of predicted genes is also a little more elevated in the range of 9113 to 13,553. Larger differences were observed for the repetitive portion of the genome, which is in the range of 100 kb to 1.3 Mb. Genomes of the *Aspergillaceae* have an average repeat content of 2–3% (De Vries et al. 2017).

However, the genome sequence itself does not reveal many secrets and further analyses of functional and comparative genomics are needed to recognize evolutionary differences between strains or species (Knuf and Nielsen 2012). Comparative genome analyses of *Aspergillus* species can help elucidate physiological aspects and characteristics of genome evolution and gene regulation, likely to be common to all eukaryotic organisms (Galagan et al. 2005).

The study of the genome function and evolution of *Aspergillus* has already begun yielding remarkable novel insights regarding genome architecture, sexual reproduction, population biology, secondary metabolism, and virulence mechanisms (Gibbons and Rokas 2013). For instance, in the biotechnological setting, the genomic analyses of *A. niger* strains found characteristic key enzymes and pathways for their industrial fermentation application (Andersen et al. 2011). In the clinical setting, these analyses can help understanding dynamic alterations that occur in a fungal pathogen genome within its host, as well as how the host environment can modify phenotypic properties (Hagiwara et al. 2014). Comparison of the genomes and transcriptomes may help, for example, in predicting which pathogenicity factors are especially important (Cerqueira et al. 2014).

1.5. Importance of *Aspergillus*

Aspergillus is one of the most well-known and studied groups of filamentous fungi, with many important roles in natural ecosystems and human economy. Because it is very diverse, it presents positive and negative aspects in many fields. The ability to

produce a wide range of extracellular enzymes, organic acids, and secondary metabolites are intensely used for different industrial purposes. Nevertheless, numerous species are also able to invade tissue from a host causing infections or to produce harmful metabolites causing plant and/or animal diseases.

1.5.1. Biotechnological Aspects of *Aspergillus*

It is believed that the use of *Aspergillus* species for fermentation purposes goes back many centuries in old Chinese and Japanese recipes that describe the use of fungal cultures in preparation of food stuffs. The physiological properties and metabolic versatility of *Aspergillus* contribute for their use in biotechnological purposes. Like in other filamentous fungi, the abilities of *Aspergillus* in secreting enzymes that hydrolyze starch, cellulose, pectin, proteins, lipids, and other biopolymers are exploited to manufacture enzymes for industrial use (Meyer et al. 2015).

With the development of the recombinant technology, species such as *A. niger*, *A. oryzae*, and *A. terreus* have been developed into important hosts to over produce enzymes and pharmaceutical proteins (Van Dijck 2008). The first cholesterol lowering drug statin approved for human use was isolated from *A. terreus* (Alberts 1998). Other compounds with pharmacological activities produced by *Aspergillus* species include cholecystokinin and neurokinin antagonists, ion channel ligands, and antifungal drugs (An 2005). In the fermentation industry, this genus is frequently used for the production of organic acids, enzymes, vitamins and antibiotics (Kozakiewicz, 1989). Among the acid production, the citric acid is one of the most important. The versatility and non-toxicity of citric acid are its main positive characteristics and it has many applications. In food and beverage industry is used as an acidifier or antioxidant to preserve or enhance the flavors; in the pharmaceutical industry, as antioxidant to preserve vitamins, pH corrector, blood preservative, or in the form of iron citrate; in the chemical industry, is employed as a foaming agent for the softening and treatment of textiles; among other applications (Max et al. 2010). Other acids produced by *Aspergillus* species widely used in the chemical industry are gluconic, itaconic, oxalic, and kojic acid (Ward et al. 2005).

Aspergillus can produce and secrete a variety of industrial enzymes that have been used for commercial purposes for over 120 years, including amylases, pectinases, cellulases, lipases, proteases, and glucose-transforming enzymes (Fogarty 1994). Amylases and proteases are widely used in baking processes, for example to

modify wheat gluten (Souza et al. 2015), and pectinases have, for example, applications in fruit processing and recovery of plant oils (Poletto et al. 2015).

On the other hand, several *Aspergillus* mycotoxins have been identified as contaminants in foods and feeds, the most relevant being the aflatoxins, ochratoxins, fumonisins and patulin. Contamination by mycotoxins can have a huge economic impact on biotechnological industries. The aflatoxins are the most important mycotoxins; they are known to be hepatotoxic, carcinogenic, teratogenic, and mutagenic (Ward et al. 2005). There are six predominant types, the AFB_1 being considered the most potent naturally occurring carcinogen (Gouas et al. 2009). Similarly, the ochratoxin A has also received special attention because of the same effects and its marked nephrotoxic and immunotoxic nature (Scibelli et al. 2003). It is commonly found on staple food crops, and beverages such as beer and wine (Bayman and Baker 2006).

1.5.2. *Aspergillus* and Disease

More than 90% of all reported fungal-related human deaths are consequence of the infections produced by one of the four following genera: *Cryptococcus*, *Candida*, *Pneumocystis*, or *Aspergillus* (Brown et al. 2012). Nevertheless, all *Aspergillus* species have a saprobic life style and infections caused by members of this genus, the aspergillosis, are opportunistic. As opportunistic pathogens, their metabolic apparatus did not evolve in order to take advantage of living hosts. *Aspergillus* diseases are not contagious in the sense that they pass from one infected host to another, instead infections arise from the environment (Bennett 2009). Their conidia are commonly present in the air and, eventually, they can enter our body through lungs and sinuses, mainly, or through traumatized skin, intravascularly (through catheters or drug abuse), gastrointestinal tract, and ear (Stevens 2009). Inhaled conidia of *Aspergillus* spp. are usually effectively cleared by phagocytic cells, such as alveolar macrophages, neutrophils and monocytes, without clinical consequence (Segal 2007). However, in immunocompromised individuals, with prolonged neutropenia, allogeneic hematopoietic stem cell transplant, solid organ transplant, inherited or acquired immunodeficiencies, corticosteroid use, etc.; or who have preexisting diseases, e.g. tuberculosis or diabetes, the risks for establishing an invasive aspergillosis are considerably higher. In individuals with hyperactiveimmune systems, on the other hand, the risk of allergic aspergillosis increases (Figure 8).

No other infectious agent of man produces the diversity of infections as *Aspergillus* species. The aspergillosis can be broadly divided into the following categories (Denning 2010): invasive life-threatening infection in immunocompromised patients; subacute or chronic infection in patients with pre-existing pulmonary or sinus disease and probably some subtle defect in innate immunity; allergic or eosinophilic disease which is manifest in many forms including allergic bronchopulmonary aspergillosis, eosinophilic rhinosinusitis and extrinsic allergic alveolitis; locally invasive infection as a result of trauma or surgery, notably keratitis and post-operative infection. In addition, fungal colonization on the outermost layers of the epithelium may lead to a range of non invasive infections, including onychomycosis, dermatomycosis and dacrocystitis (Van Burik et al. 1998).

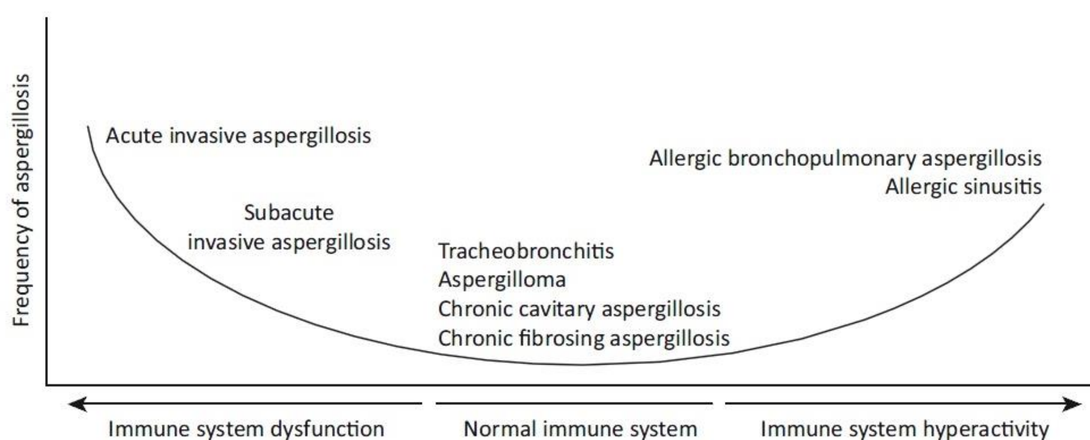


Figure 8. Frequency of aspergillosis in relation to the host immune system (modified and reproduced from a concept developed by Denning; Gibbons and Rokas 2013).

Aspergillus species have a series of virulence factors that allow them to infect a host (Paulussen et al. 2016). These factors may refer to a specific component of the pathogen or relate to fungal structure, capacity for growth, stress adaptation, host damage and mechanisms utilized to evade the immune system (Chotirmall et al. 2014). The cell wall is considered a crucial virulence factor for fungal binding and subsequent invasion of host epithelium (Chotirmall et al. 2014). Others such as adhesion factors, e.g. hydrophobins, which allow the binding of the conidia to host epithelial cells (Tomee and Kauffman 2000) and conidial melanin, that can masks immunostimulatory glucans and protects against immune cells (Valiante et al. 2016) are also important. Molecules such toxins, e.g. gliotoxin, that can suppress the host immune response (Sugui et al. 2007; Sales-Campos et al. 2013) and allergens, e.g. Asp f2, which aids pulmonary colonization by binding to laminin (Rementeria et al. 2005), likewise play significant roles in *Aspergillus* virulence.

The ability of *Aspergillus* to cause diseases is not limited to men. Other animals such as dogs, horses, lizards, and birds, for example, were already reported with aspergillosis (Girling and Fraser 2009; Coyner 2010; Cafarchia et al. 2012; Walker 2012). Plants can also be susceptible to *Aspergillus* diseases, which cause mainly postharvest rots of fruits and vegetables and seeds and grains decay (Agrios 2005). Since many species have the capacity to produce mycotoxins, the ingestion of contaminated foods, especially grains and stored products, can cause mycotoxicosis. The small size of the toxin molecules do not induce response in the human immune system and the most common consequence is deterioration of liver or kidney function, besides, many of them exhibit mutagenic and teratogenic effects (Kamei and Watanabe 2005).

1.5.2.1. Laboratory Diagnosis of Aspergillosis

The diagnosis of a fungal infection should be precise and fast. An accurate diagnosis enables the choice of the proper antifungal therapy and an early diagnosis allows the treatment at a time when it is most likely to be effective (Kozel and Wickes 2014). The gold standard for the diagnosis of an *Aspergillus* infection is isolation and culture of the organism in the microbiology laboratory. However, culturing is often poorly specific and slow. For example, blood cultures are usually negative for invasive aspergillosis (Brown et al. 2012). According to the revised EORTC/MSG consensus, the diagnosis of invasive aspergillosis can only be proved in the presence of a positive histopathology (De Pauw et al. 2008). As confirmatory microbial cultures are not available in all cases, expertise in discerning the specific features of *Aspergillus* infection is critical in determining optimal therapy (De Pauw et al. 2008). Consequently, the diagnosis typically involves not only the isolation of the organism, but also a combination of clinical signs and symptoms, existence of host factors, diagnostic imaging (x-ray, CT scan), among others. The ideal diagnostic test is yet to be discovered and every method has some limitation, but several approaches have shown satisfactory results in many occasions. The detection of fungal components, mostly antigens and DNA, has become the most relevant novelty in the last decade in the field of medical mycology (Bernal-Martinez et al. 2016).

Histopathology can be a rapid and cost-effective direct examination tool that provides a presumptive or definitive diagnosis of invasive fungal infection. In addition, it can differentiate fungal infections caused by hyaline molds from those caused by black

fungi or even mucorales. However, histology does not allow differentiating among species, which is essential for selecting the best therapy (Guarner and Brandt 2011). The hyaline hyphae of *Aspergillus* can be easily seen by Grocott-Gomorri methenamine silver stain or by acid Schiff silver stain.

Detection of fungal polysaccharides in the serum, plasma, or other body fluids can also be of use. The 1,3-β-D-glucan is a carbohydrate present in the majority of fungal species and its detection in the serum is indicative for a presumptive diagnosis of invasive fungal disease. The main advantage of such test is the good negative predictive value (90%) (Odabasi et al. 2004). The galactomannan is a polysaccharide component of the *Aspergillus* cell wall and its detection in the body fluids has demonstrated to be a significant tool in the early diagnosis of invasive aspergillosis, especially in patients with hematological malignancies (Pfeiffer et al. 2006). However, this technique is not useful in all groups of patients and false positives may occur.

Regarding the PCR-based methods, although there have been many studies in the past 20 years; the main disadvantage is still the lack of standardization. The parameters of gene targets, detection system, primers, clinical specimen, volume, DNA extraction procedure and the PCR itself are highly variable, hampering the inter-laboratory comparison. Nevertheless, it may have diagnostic value when results are compatible with the clinical context of the patient (Bernal-Martinez et al. 2016).

It is worth mentioning that in recent years, the MALDI-TOF MS (matrix assisted laser desorption ionization – time of flight mass spectrometry) has been developed for improving the microbial identification in clinical laboratories and it has been reported to yield rapid and accurate identification of filamentous fungi. The method generates a mass spectrum that is compared against a database by specific software, resulting in the identification of the organism. The main limitations of this technique are that databases are not public and low number of entries can result in misidentifications. If the number of strains for each species were expanded, it would improve the usefulness of MALDI-TOF MS (Masih et al. 2016). This technique shows promise, but a lot of work remains to make routine identifications feasible (Visagie et al. 2014a).

1.5.2.2. Antifungal Susceptibility Testing

Antifungal susceptibility testing methods are available to determine the best treatment for an infection by a specific fungus, to detect antifungal resistance, and to know the local and the global epidemiology of antifungal resistance (Alastruey-

Izquierdo et al. 2015). The increased incidence of fungal infections and the emergence of new therapeutic alternatives have led the development and standardization of methods for testing antifungal susceptibility (Colombo et al. 2010). As a result, there are now several methods available for the evaluation of in vitro antifungal activity in filamentous fungi, including broth-based and agar-based methods.

The microdilution is considered the gold standard for antifungal susceptibility testing and two organizations currently have regulated protocols: the Clinical and Laboratory Standards Institute (CLSI), document M38-A2 (2008), and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), document E.DEF 9.3 (2015). Both institutions have developed breakpoints for several antifungals to some *Aspergillus* species, which are currently used to classify resistant strains. Both approaches have proved to yield comparable results (Pfaller et al. 2011).

The agar-based tests are alternatives to the microdilution. They are cheap and easier to perform in a clinical laboratory; however, the results may disagree with the reference microdilution methods (Gupta et al. 2015). The M51 series of the CLSI and the commercial Etest® (bioMérieux, Marcy l'Etoile, France) are the most commonly performed. Other commercial tests are available but not yet fully validated for *Aspergillus*.

Among the clinically available antifungals drugs, the most commonly used and efficient for treating *Aspergillus* infections are the azoles, the echinocandins, amphotericin B, and terbinafine. The azoles act in the fungal cell membrane by blocking the pathway of ergosterol biosynthesis, specifically the enzymes 14-α-sterol demethylases A and B. This mechanism prevents the conversion of lanosterol to ergosterol, resulting in the accumulation of toxic methylsterols and inhibition of fungal cell growth and replication (Mellado et al. 2001; Thompson and Patterson 2010). The first azoles, such as ketoconazole and fluconazole, lacked activity against *Aspergillus* species, but the newer triazoles (e.g. itraconazole, posaconazole, voriconazole, ravuconazole, isavuconazole, and albaconazole) have demonstrated fungicidal activity against this genus. Azoles differ in their affinity for the 14-α-demethylase enzyme and this difference is largely responsible for their varying antifungal potency and spectrum of activity (Thompson and Patterson 2010). Currently, voriconazole is the recommended treatment for aspergillosis by the Infectious Diseases Society of America. Posaconazole is recommended for prophylaxis against invasive aspergillosis and itraconazole for allergic bronchopulmonary aspergillosis (Patterson et al. 2016).

The echinocandin drugs are potent inhibitors of β-1,3-D-glucan synthase, which catalyzes the biosynthesis of the principal glucan component of fungal cell walls, being the first class of antifungal agents to target the fungal cell wall (Morrison 2006).

Caspofungin, micafungin and anidulafungin are the principal drugs of this class and, although their action in *Aspergillus* species is fungistatic, their in vivo effectiveness appears to be derived from drug-induced enhancement of the local immune response (Perlin and Hope 2010).

Amphotericin B is an antifungal agent from the polyene class, commercially available for almost 50 years. The mechanism of action of polyenes remains somewhat controversial, but it is believed that they act by forming pores in the fungal membrane by binding to ergosterol, which leads to membrane leakage and possible fungal cell death (Vanden Bossche et al. 1994; Lewis 2010).

Lastly, terbinafine is a member of allylamine group, which inhibit ergosterol biosynthesis via inhibition of squalene epoxidase. Although it is commercialized to treat dermatophytosis, most of isolates of *Aspergillus* are susceptible in vitro to terbinafine (Cuenca-Estrella and Rodriguez-Tudela, 2010).

2. INTEREST AND OBJECTIVES

Species of *Aspergillus* are ubiquitous and very common in the environment, inhabiting a great diversity of substrates. Some of them are also known as relevant human pathogens, responsible for over 200,000 life-threatening infections per year worldwide (Brown et al. 2012). The advances in modern medicine, in a paradoxical way, contribute to an increasing population with altered immune function. Solid-organ and stem cell transplantation, new chemotherapeutics for cancer and inflammatory conditions, prolonged survival of critically ill patients, among other factors, continually expand the population of immunocompromised patients and, consequently, at risk of fungal infections. The main agent of aspergillosis is *A. fumigatus*, but others such as *A. flavus*, *A. niger*, and *A. terreus* have increased as the cause of severe opportunistic infections, especially in the immunocompromised host (Richardson and Lass-Florl 2008). The change in the epidemiology of fungal infections is noticeable and many authors already reported that this shift occurs towards more resistant and/or cryptic species (Lass-Florl and Perkhofer 2008; Alastruey-Izquierdo et al. 2013; Nedel and Pasqualotto 2014).

The diversity of *Aspergillus* species recovered from clinical samples is also progressively increasing and the difficulty to differentiate among them by classical methods may lead to misdiagnoses. Clinically, the accurate identification of an *Aspergillus* to the species level is important when dealing with infections refractory to antifungal therapy, for investigation of an outbreak, or when performing epidemiologic studies (Balajee et al. 2007).

Improvement of identification methods, especially the sequence-based, may be one of the reasons for the changes in the epidemiology and the greater diversity of *Aspergillus* species found in clinic and environmental samples. Sequencing of many genetic loci and construction of phylogenies helped to understand the relationship among sections and the species delimitation in *Aspergillus*. For example, strains placed in species complexes and/or considered varieties are now better delineated and promoted to the species rank. Although close related species may share morphological features, they may present differences in physiological aspects and susceptibility to antifungal drugs (Varga et al. 2008; Guinea et al. 2015), which should be properly described and reported.

In biotechnology, the production of metabolites of interest and mycotoxins are of highly economic importance. The correct identification is essential, otherwise chemical data could be linked to an incorrect species; incorrect names could be included in quarantine legislation; and food-safety recalls could be done based on scientifically inaccurate information (Samson et al. 2014).

The ubiquity, complexity and diversity of *Aspergillus* are the main reasons why this genus was chosen to be the focus of this thesis. *Aspergillus* has positive and negative impact in many fields, including fungal taxonomy, food and indoor mycology, biotechnology, ecology, medical mycology, and genomics; therefore, the correct identification is a crucial step in many disciplines (Houbraken et al. 2014). Recent reports prove that the diversity of the genus is yet being discovered and there are potential numerous new species to be described in a variety of substrates (Visagie et al. 2014; Hubka et al. 2015; Chen et al. 2016).

Considering that the current studies on *Aspergillus* diversity are focused mainly on sections *Fumigati*, *Flavi* and *Nigri*, the main objective of this thesis was:

To contribute to the better knowledge of genus *Aspergillus,* exploring the species diversity of the less studied *Aspergillus* sections from clinical and environmental sources.

To reach these general purposes, the specific objectives were:

1. To obtain a great number of isolates belonging to poorly studied sections of *Aspergillus* from clinical samples and different environmental sources, such as soil or herbivore dung.
2. To identify the isolates based on morphological criteria and comparison of DNA barcodes.
3. To characterize the detected putative new species by a polyphasic approach, including phenotypic features (i.e. macro and micromorphology, growth rates, acid production) and phylogenetic relationships based on multilocus sequence analyses.
4. To determine the in vitro activity of clinically available antifungals against the most frequent species recovered from clinical samples.

3. MATERIAL AND METHODS

UNIVERSITAT ROVIRA I VIRGILI
CLINICAL AND ENVIRONMENTAL ASPERGILLUS: MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION, PHYLOGENY,
AND ANTIFUNGAL SUSCEPTIBILITY PROFIL·LE
Joao Paulo Zen Siqueire

3.1. Origin of the Isolates

A total of 433 isolates obtained from different sources were included in the studies of this thesis (Table 3). The clinical isolates (n = 248), from animal and human origins, were provided by the Fungus Testing Laboratory of the University of Texas Health Science Center (UTHSCSA, USA), a reference institute that receives clinical isolates collected from different organizations of the country. The great majority of the isolates with known origin were from respiratory tract samples (n = 99; 39.9 % of clinical isolates), mainly from bronchoalveolar lavage (BAL; n = 59; 23.8 %) and sputum (n = 25; 10.1 %) (Figure 9). The environmental isolates (n = 185) were obtained from soil (n = 92; 49.7 %), herbivore dung samples (n = 82; 44.3 %), and plant debris (n = 10; 5.4 %), and one isolate with unknown origin. The environmental samples were collected mainly from different regions from Spain, although soil samples from different countries such as Mexico, Brazil, Argentina, etc., were also studied. The isolation procedure is explained bellow.

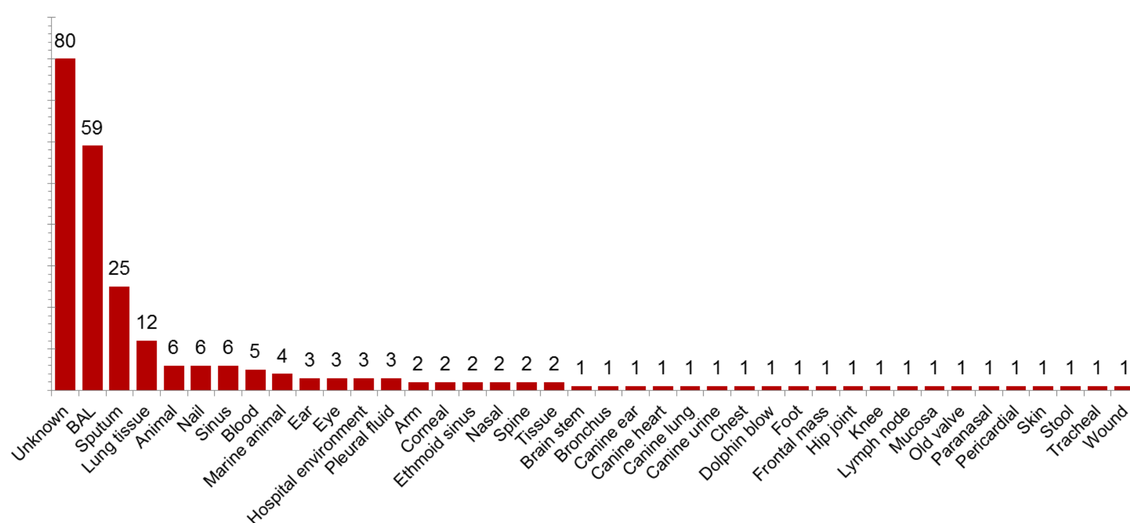


Figure 9. Column graph exhibiting the number of *Aspergillus* isolates included in this thesis according to the clinical origin.

3.2. Techniques for the Isolation of Environmental *Aspergillus*

Soil samples were taken from the superficial layers of soil without the organic material (horizon A-A0 ~1 cm deep), placed in plastic bags and stored at 4 ºC until they

were processed. Dung samples were usually collected in paper bags and processed as soon as possible, without being stored for long periods of time.

Approximately 1 g of each soil or dung sample were put in a tube with 9 mL of sterilized distillate water, homogenized thoroughly, and serially diluted until 10^{-5}. The pour plate method was used with one mL of each dilution pipetted in a new, sterile, plastic Petri dish, and then approximately 20 mL of melted cooled agar medium were poured into the plate and carefully homogenized. Two different media were chosen and prepared at the same time, i.e. Potato Dextrose Agar (PDA, Pronadisa, Madrid, Spain) and Dichloran Rose-Bengal Chloramphenicol Agar (DRBC; 5 g peptone, 10 g glucose, 1 g potassium dihydrogen phosphate [KH_2PO_4], 0.5 g magnesium sulphate [$MgSO_4$], 25 mg rose-bengal, 2 mg dichloran, 200 mg chloramphenicol, 15 g agar, 1000 mL distilled water). Plates were incubated at room temperature and checked continuously for 3 to 4 weeks.

In parallel, small pieces of dung samples were incubated in moist chambers (sterile Petri dishes with filter paper) at room temperature. To maintain wet conditions, sterile water was poured every 3 or 4 days, and Petri dishes were also examined for 3 to 4 weeks. Colonies that exhibited the typical *Aspergillus* morphology were isolated in PDA plates for further investigation.

3.3. Phenotypic Characterization

The *Aspergillus* isolates were characterized following the criteria recommended by Samson et al. (2014). The morphology of the colonies and growth rates were determined after 7 days of incubation on CYA (Becton, Dickinson and Company, Sparks MD, USA) and MEA (Pronadisa, Madrid, Spain) at 25 ºC, and on CYA at 37 ºC. Moreover, the characterization of the isolates belonging to section *Aspergillus* was based on Hubka et al. (2013); i.e., colonies were also studied on CYA supplemented with 20% sucrose (CY20S) at 25 ºC and 37 ºC, and Harrold's Agar containing 60% sucrose (M60Y) (Raper and Fennell 1965). The isolates that represented new species were described on CYA, MEA, DG18 (Hocking and Pitt, 1980), YES (Frisvad 1981), OA (Samson et al. 2010), and CREA (Frisvad 1981) at 25 ºC, and CYA at 30 ºC and 37 ºC. The colony colors in descriptions were based on Kornerup and Wanscher (1967).

Microscopic features were examined and measured on MEA and/or CY20S cultures, after 10 to 14 days of incubation. Wet mounts were prepared with 60% lactic acid and the excess conidia were removed using a drop of ethanol 70%. Photograhs of

the habitat were taken from a Carl Zeiss Stemi Stereo Material Microscope and photomicrographs were made in a Zeiss Axio Imager M1 light microscope, both mounted with a DeltaPix Infinity X digital camera (Zeiss, Oberkochen, Germany). For the photomicrographs, Nomarski differential interference contrast and phase contrast optics were used. Scanning electron microscope (SEM) photographs were obtained with a Jeol JSM- 6400 using techniques described previously by Figueras and Guarro (1988).

3.4. Molecular Studies

3.4.1. DNA Extraction, Amplification, and Sequencing

Total genomic DNA was extracted from MEA cultures after 7 days of incubation at 25 ºC, using one of two methods, i.e. FastDNA® Kit and the FastPrep® Instrument (MP Biomedicals, Irvine CA, USA) according to the manufacturer's specifications, or by a method based on Muller et al. (1998), with modifications. Ammonium acetate and chloroform steps were used to isolate and purify the DNA, removing proteins and other organic compounds, and isopropanol was used to precipitate and concentrate the DNA. Amplification targeted the four most used genetic markers for *Aspergillus* (Peterson 2008; Samson et al. 2014): i.e., ITS regions, including the 5.8S gene, and portions of *BenA*, *CaM*, and *RPB2* genes. The primers used to amplify those regions were: ITS5 and ITS4 (White et al. 1990); Bt2a and Bt2b (Glass and Donaldson 1995); Cmd5 and Cmd6 (Hong et al. 2005); and 5F and 7CR (Liu et al. 1999), respectively.

PCR products were sequenced in both directions, using the same primers, at Macrogen Europe (Macrogen Inc., Amsterdam, Netherlands). Sequences were assembled and edited using SeqMan v.7.0.0 (DNASTAR, Madison, WI, USA).

All sequences newly generated in the studies included here were deposited in GenBank/EMBL databases and included in Table 3.

3.4.2. Molecular Identification and Phylogenetic Analysis

The presumptive molecular identification was provided by pairwise sequence analyses in databases available online (GenBank/EMBL and MycoBank).

The final species identification was obtained through phylogenetic analyses. They were performed individually for each gene and in concatenated studies. Sequences of type and reference strains were obtained from the GenBank and included in the analyses. Outgroups were also included, mostly a member of a different section or, in the case of a general tree, *Penicillium paradoxum* CBS 527.65.

Multiple sequence alignment for each studied section was performed with ClustalW together with MUSCLE in MEGA v.6 software (Tamura et al*.* 2013), with manual adjustments to refine it when necessary. For larger alignments, the MAFFT tool was used in the EMBL-EBI Web Services portal.

MEGA v.6 software was also used to conduct the Maximum Likelihood (ML) analysis, and for the estimation of the best nucleotide substitution method. Support of the internal branches was assessed by the Bootstrap method with 1,000 replications, where values equal or higher than 70 were considered significant. In parallel, Bayesian Inference (BI) method was performed using MrBayes v.3.1.2 software (Ronquist and Huelsenbeck 2003). The evolutionary models that best fit each gene were assessed by the software MrModelTest (Nylander 2004). Markov chain Monte Carlo (MCMC) sampling was performed with two simultaneous runs for 1 million generations, with samples taken every 100 generations. The 50% majority rule consensus trees and posterior probability values (pp) were calculated after removing the first 25% of the resulting trees for burn-in. A pp value of 0.95 or higher was considered significant in the tree.

3.5. Antifungal Susceptibility Test

The isolates that underwent antifungal susceptibility test were confronted against up to nine antifungal drugs following the microdilution broth method, according to the document M38-A2 (CLSI 2008). The inoculum consisted in the preparation of conidial suspensions from pure culture grown on PDA from seven to 10 days. A sterile loop with a drop of Tween 20 was used to scrape the colonies and transfer them to a tube with sterile water. The suspension was then filtered through sterile gauze to remove hyphal fragments and the concentration adjusted according to the protocol by counting on a Neubauer chamber. The antifungal agents tested were amphotericin B (AMB) (Sigma Aldrich Quimica S.A., Madrid, Spain), itraconazole (ITC) (Jansen Pharmaceuticals, Beerse, Belgium), posaconazole (PSC) (Schering-Plough Res., Inst., NJ, USA), voriconazole (VRC) (Pfizer S.A., Madrid, Spain), anidulafungin (AFG) (Pfizer

S.A., Madrid, Spain), caspofungin (CFG) (Merk & Co., Inc., Rahway, EUA), micafungin (MFG) (Astellas Pharma, Madrid, Spain), terbinafine (TBF) (Sigma Aldrich Química S.A., Madrid, Spain), and flucytosine (5FC) (Sigma Aldrich Química S.A., Madrid, Spain). Microplates were prepared for each antifungal drug with concentrations ranging from 0.03 to 16.0 µg/mL. The minimal inhibitory concentration (MIC) was defined as the lowest drug concentration that produced 100% inhibition of visible fungal growth for the AMB and the azoles (ITC, PSC and VRC) or 50% and 80% for 5FC and TBF, respectively. The minimum effective concentration (MEC) was determined for the echinocandins (AFG, CFG and MFG) and was defined microscopically as the lowest concentration of drug that would permit the growth of small, rounded, compact hyphal forms as compared with the long, unbranched hyphal clusters that were seen in the growth control. The period and temperature of incubation, in some cases, were slightly modified to fit the growth requirements of the isolates. For instance, for members of section *Aspergillus*, readings were performed after 72 h of incubation, and, for section *Versicolores* and isolates of *A. pseudoglaucus* and *A. microperforatus*, the microplates were incubated at 30 ºC. *Candida krusei* ATCC 6258, *Aspergillus flavus* ATCC 204304, and *Aspergillus fumigatus* ATCC MYA-3626 were used as quality control strains and their MIC values were within the acceptable MIC range per the CLSI standard. All the tests were carried out in duplicate on different days to assess reproducibility. Results were statistically analyzed using the Prism software for Windows, version 6.0 (GraphPad Software, San Diego, CA).

3.6. Storage and Conservation of the Strains

Pure cultures of all the isolates studied in this thesis were deposited in the *Facultat de Medicina de Reus* (FMR) culture collection. Different methods of conservation were adopted to ensure the survival of the isolates.

Additionally, living cultures of rare and new species, as well as the respective holotypes, were also deposited at the Westerdijk (before CBS-KNAW) Fungal Biodiversity Institute (Utrecht, the Netherlands). Names for the new taxa were registered at the MycoBank database.

3.6.1. Short Term Conservation

The isolates were inoculated in agar slants with PDA and OA, and stored at room temperature in plastic tubes with sealing caps. In addition, spore suspensions were prepared accordingly to Samson et al. (2014). For this, spores from a pure culture were suspended in a solution of 0.2 % agar + 0.05 % Tween 80 and stored at 4 ºC.

3.6.2. Long Term Conservation

To preserve the viability of the isolates for long periods, other methodologies were applied. Pure cultures in agar slant glass tubes with screw caps were covered in mineral oil and stored at room temperature. Additionally, from a pure culture in a plate, blocks of the agar were cut with a sterile scalp and placed in glass flasks with 2–3 ml of sterile water. The flasks were sealed and stored at room temperature. The third method used for long-term conservation was lyophilization. Scrapes of colony were homogenized in flasks with 1–2 ml of skim milk medium (Difco, USA) and lyophilized by the automate system VirTis Advantage 2.0 ES (SP Scientific, USA). Flasks were sealed and stored at room temperature.

Table 3. *Aspergillus* isolates studied in this thesis, final identification, collection numbers, origins, and GenBank/EMBL accession number.

Species	Section	Collection numbers	Origin	Substrate	Locality	GenBank/EMBL accession number			
						ITS	*BenA*	*CaM*	*RPB2*
A. chevalieri	*Aspergillus*	FMR 14116 = UTHSCSA DI16-382	Clin.	BAL	Texas, USA	LT627250	LT627275	LT627300	LT627325
		FMR 14120 = UTHSCSA DI16-414	Clin.	Unknown	Texas, USA	LT627255	LT627280	LT627305	LT627330
		FMR 14171 = UTHSCSA DI16-397	Clin.	Sinus	Texas, USA	LT627253	LT627278	LT627303	LT627328
		FMR 14219 = UTHSCSA DI16-375	Clin.	Sputum	Utah, USA	LT627248	LT627273	LT627298	LT627323
		FMR 14320 = UTHSCSA DI16-413	Clin.	Unknown	Texas, USA	LT627254	LT627279	LT627304	LT627329
		FMR 14401 = UTHSCSA DI16-381	Clin.	BAL	Texas, USA	LT627249	LT627274	LT627299	LT627324
		FMR 14402 = UTHSCSA DI16-394	Clin.	BAL	Florida, USA	LT627251	LT627276	LT627301	LT627326
		FMR 14403 = UTHSCSA DI16-396	Clin.	Corneal	Illinois, USA	LT627252	LT627277	LT627302	LT627327
		FMR 14454 = UTHSCSA DI15-18	Clin.	BAL	Minnesota, USA	LT627247	LT627272	LT627297	LT627322
		FMR 15878	Env.	Dung	Extremadura, Spain	-	LT798954	-	-
A. costiformis	*Aspergillus*	FMR 14452 = UTHSCSA DI15-16	Clin.	Hospital Env.	Tennessee, USA	LT627256	LT627281	LT627306	LT627331
A. microperforatus	*Aspergillus*	FMR 14071 = UTHSCSA DI16-407 = CBS 142376	Clin.	Lymph node	Texas, USA	LT627271	LT627296	LT627321	LT627346
		FMR 14405 = UTHSCSA DI16-400 = CBS 142377	Clin.	Toe nail	Maryland, USA	LT627270	LT627295	LT627320	LT627345
A. montevidensis	*Aspergillus*	FMR 14069 = UTHSCSA DI16-411	Clin.	Unknown	Texas, USA	LT627266	LT627291	LT627316	LT627341
		FMR 14070 = UTHSCSA DI16-406	Clin.	Tissue	Massachusetts, USA	LT627264	LT627289	LT627314	LT627339
		FMR 14172 = UTHSCSA DI16-408	Clin.	Paranasal	Colorado, USA	LT627265	LT627290	LT627315	LT627340

		FMR 14322 = UTHSCSA DI16-403	Clin.	Sputum	Minnesota, USA	LT627262	LT627287	LT627312	LT627337
		FMR 14344 = UTHSCSA DI16-412	Clin.	Unknown	Texas, USA	LT627267	LT627292	LT627317	LT627342
		FMR 14404 = UTHSCSA DI16-401	Clin.	Lung tissue	Montana, USA	LT627261	LT627286	LT627311	LT627336
		FMR 14406 = UTHSCSA DI16-405	Clin.	Sinus	Pennsylvania, USA	LT627263	LT627288	LT627313	LT627338
		FMR 14455 = UTHSCSA DI15-19	Clin.	Ethmoid sinus	California, USA	LT627257	LT627282	LT627307	LT627332
		FMR 14456 = UTHSCSA DI15-20	Clin.	Sputum	California, USA	LT627258	LT627283	LT627308	LT627333
		FMR 14457 = UTHSCSA DI15-21	Clin.	BAL	California, USA	LT627259	LT627284	LT627309	LT627334
		FMR 14458 = UTHSCSA DI15-22	Clin.	Sputum	Minnesota, USA	LT627260	LT627285	LT627310	LT627335
		FMR 15608	Env.	Dung	Castile and Leon, Spain	-	LT798956	-	-
		FMR 15738	Env.	Dung	Extremadura, Spain	-	LT798955	-	-
		FMR 15739	Env.	Soil	Salta, Argentina	-	-	-	-
A. pseudoglaucus	*Aspergillus*	FMR 14169 = UTHSCSA DI16-410	Clin.	Stool	Minnesota, USA	LT627269	LT627294	LT627319	LT627344
		FMR 14453 = UTHSCSA DI15-17	Clin.	Nasal	Minnesota, USA	LT627268	LT627293	LT627318	LT627343
		FMR 15607	Env.	Dung	Castile and Leon, Spain	-	LT798957	-	-
		FMR 15612	Env.	Dung	Castile and Leon, Spain	-	LT798958	-	-
A. canariensis	*Candidi*	FMR 15733 = CBS 142983	Env.	Dung	Canary Islands, Spain	LT798905	LT798924	LT798925	LT798926
		FMR 15736 = CBS 142982	Env.	Dung	Canary Islands, Spain	LT798906	LT798927	LT798928	LT798929
A. candidus	*Candidi*	FMR 15172	Env.	Dung	Catalonia, Spain	-	LT798959	-	-

		FMR 15218	Env.	Dung	Balearic Islands, Spain	-	LT798960	-	-
A. coprophilus	*Candidi*	FMR 15224 = CBS 142984	Env.	Dung	Castile and Leon, Spain	LT798902	LT798915	LT798916	LT798917
		FMR 15226 = CBS 142985	Env.	Dung	Castile and Leon, Spain	LT798903	LT798918	LT798919	LT798920
A. longipes	*Candidi*	FMR 15444 = CBS 142752	Env.	Dung	Galicia, Spain	LT798904	LT798921	LT798922	LT798923
		FMR 15601	Env.	Dung	Galicia, Spain	-	-	-	-
A. subalbidus	*Candidi*	FMR 15730	Env.	Dung	Canary Islands, Spain	-	-	-	-
A. tritici	*Candidi*	FMR 14920	Env.	Soil	Mexico State, Mexico	-	-	-	-
A. verruculosus	*Candidi*	FMR 15877 = CBS 142667	Env.	Dung	Canary Islands, Spain	LT798907	LT798930	LT798931	LT798932
A. affinis	*Circumdati*	FMR 15602	Env.	Dung	Galicia, Spain	-	LT798961	-	-
A. elegans	*Circumdati*	FMR 14927	Env.	Soil	Angkor, Cambodia	-	-	-	-
		FMR 15053	Env.	Soil	Mexico D.F., Mexico	-	-	-	-
A. insulicola	*Circumdati*	FMR 14130 = UTHSCSA DI16-402	Clin.	Marine	California, USA	LT574682	LT574717	LT574752	LT574787
		FMR 14221 = UTHSCSA DI16-374	Clin.	Marine	California, USA	LT574681	LT574716	LT574751	LT574786
A. ochraceopetaliformis	*Circumdati*	FMR 14177 = UTHSCSA DI16-392	Clin.	Marine	California, USA	LT574684	LT574719	LT574754	LT574789
		FMR 14226 = UTHSCSA DI16-387	Clin.	BAL	Texas, USA	LT574683	LT574718	LT574753	LT574788
A. ochraceus	*Circumdati*	FMR 14223 = UTHSCSA DI16-384	Clin.	Ear	Florida, USA	LT574685	LT574720	LT574755	LT574790
		FMR 14446 = UTHSCSA DI15-10	Clin.	BAL	Arizona, USA	LT574686	LT574721	LT574756	LT574791
		FMR 14447 = UTHSCSA DI15-11	Clin.	Old valve	Nevada, USA	LT574687	LT574722	LT574757	LT574792
A. pseudosclerotiorum	*Circumdati*	FMR 14124 = UTHSCSA DI16-373	Clin.	Sputum	Maryland, USA	LT574707	LT574742	LT574777	LT574812

		FMR 14174 = UTHSCSA DI16-380	Clin.	BAL	Florida, USA	LT574708	LT574743	LT574778	LT574813
		FMR 14175 = UTHSCSA DI16-383	Clin.	BAL	South Carolina, USA	LT574709	LT574744	LT574779	LT574814
		FMR 14224 = UTHSCSA DI16-385	Clin.	Sputum	Missouri, USA	LT574710	LT574745	LT574780	LT574815
		FMR 14225 = UTHSCSA DI16-386	Clin.	Lung tissue	Texas, USA	LT574711	LT574746	LT574781	LT574816
		FMR 14449 = UTHSCSA DI15-13 = CBS 141845	Clin.	Lung tissue	Pennsylvania, USA	LT574713	LT574748	LT574783	LT574818
		FMR 14450 = UTHSCSA DI15-14	Clin.	BAL	Texas, USA	LT574714	LT574749	LT574784	LT574819
		FMR 14451 = UTHSCSA DI15-15	Clin.	Lung tissue	Maryland, USA	LT574715	LT574750	LT574785	LT574820
		FMR 14746	Env.	Soil	Mexico D.F., Mexico	-	-	-	-
		FMR 15052	Env.	Soil	Mexico D.F., Mexico	-	-	-	-
A. sclerotiorum	*Circumdati*	FMR 14075 = UTHSCSA DI16-395	Clin.	Sputum	Wisconsin, USA	LT574688	LT574723	LT574758	LT574793
		FMR 14076 = UTHSCSA DI16-399	Clin.	BAL	Florida, USA	LT574691	LT574726	LT574761	LT574796
		FMR 14215 = UTHSCSA DI16-409	Clin.	Eye	Michigan, USA	LT574692	LT574727	LT574762	LT574797
		FMR 14323 = UTHSCSA DI16-398	Clin.	BAL	Florida, USA	LT574689	LT574724	LT574759	LT574794
		FMR 14324 = UTHSCSA DI16-404	Clin.	Sputum	Texas, USA	LT574690	LT574725	LT574760	LT574795
		FMR 14448 = UTHSCSA DI15-12	Clin.	Sputum	North Carolina, USA	LT574693	LT574728	LT574763	LT574798
A. subramanianii	*Circumdati*	FMR 14074 = UTHSCSA DI16-390	Clin.	Foot	Florida, USA	LT574696	LT574731	LT574766	LT574801
		FMR 14173 = UTHSCSA DI16-378	Clin.	Lung tissue	Texas, USA	LT574694	LT574729	LT574764	LT574799
		FMR 14222 = UTHSCSA DI16-389	Clin.	Wound	Florida, USA	LT574695	LT574730	LT574765	LT574800

		FMR 15729	Env.	Dung	Canary Islands, Spain	-	LT798962	-	-
A. westerdijkiae	*Circumdati*	FMR 14072 = UTHSCSA DI16-376	Clin.	Unknown	Maryland, USA	LT574697	LT574732	LT574767	LT574802
		FMR 14073 = UTHSCSA DI16-377	Clin.	Unknown	Maryland, USA	LT574698	LT574733	LT574768	LT574803
		FMR 14127 = UTHSCSA DI16-379	Clin.	BAL	Texas, USA	LT574699	LT574734	LT574769	LT574804
		FMR 14176 = UTHSCSA DI16-388	Clin.	Lung tissue	Texas, USA	LT574700	LT574735	LT574770	LT574805
		FMR 14227 = UTHSCSA DI16-391	Clin.	Lung tissue	Florida, USA	LT574701	LT574736	LT574771	LT574806
		FMR 14363 = UTHSCSA DI16-393	Clin.	Sputum	Wisconsin, USA	LT574702	LT574737	LT574772	LT574807
		FMR 14441 = UTHSCSA DI15-5	Clin.	BAL	California, USA	LT574703	LT574738	LT574773	LT574808
		FMR 14442 = UTHSCSA DI15-6	Clin.	Sputum	California, USA	LT574704	LT574739	LT574774	LT574809
		FMR 14443 = UTHSCSA DI15-7	Clin.	Nail	California, USA	LT574705	LT574740	LT574775	LT574810
		FMR 14444 = UTHSCSA DI15-8	Clin.	Marine	California, USA	LT574706	LT574741	LT574776	LT574811
A. clavatus	*Clavati*	FMR 13543 = UTHSCSA DI14-235	Clin.	Sputum	- , USA	-	-	-	-
		FMR 14925	Env.	Soil	Angkor, Cambodia	-	-	-	-
		FMR 15414	Env.	Soil	Catalonia, Spain	-	-	-	-
		FMR 15610	Env.	Dung	Castile and Leon, Spain	-	LT798963	-	-
		FMR 15611	Env.	Dung	Castile and Leon, Spain	-	LT798964	-	-
A. dimorphicus	*Cremei*	FMR 15938	Env.	Soil	Catalonia, Spain	LT899656	LT899658	LT899659	LT899660
		FMR 15943	Env.	Soil	Catalonia, Spain	LT899657	LT899661	LT899662	LT899663

A. esporlensis	*Cremei*	FMR 14605 = CBS 142750	Env.	Soil	Balearic Islands, Spain	LT798908	LT798933	LT798934	LT798935
A. europaeus	*Cremei*	FMR 14163 = UTHSCSA 13-652	Clin.	Unknown	- , USA	LT899469	LT899523	LT899571	LT899626
		FMR 14589	Env.	Soil	Balearic Islands, Spain	LT899467	LT899521	LT899569	LT899624
		FMR 14591	Env.	Soil	Balearic Islands, Spain	LT899468	LT899522	LT899570	LT899625
		FMR 15216	Env.	Dung	Balearic Islands, Spain	-	LT798965	-	-
		FMR 15626	Env.	Soil	Sao Paulo, Brazil	-	-	-	-
A. albertensis	*Flavi*	FMR 15321	Env.	Dung	Balearic Islands, Spain	-	-	-	-
A. alliaceus	*Flavi*	FMR 13544 = UTHSCSA DI14-236	Clin.	BAL	- , USA	-	-	-	-
		FMR 13545 = UTHSCSA DI14-237	Clin.	Sputum	- , USA	-	-	-	-
		FMR 14123 = UTHSCSA 03-1711	Clin.	Unknown	Texas, USA	-	-	-	-
		FMR 14445 = UTHSCSA DI15-9	Clin.	BAL	Minnesota, USA	-	-	-	-
A. flavus	*Flavi*	FMR 14026 = UTHSCSA 14-66	Clin.	Unknown	Ohio, USA	-	-	-	-
		FMR 14028 = UTHSCSA 14-89	Clin.	Unknown	Massachusetts, USA	-	-	-	-
		FMR 14032 = UTHSCSA 14-432	Clin.	Unknown	Florida, USA	-	-	-	-
		FMR 14034 = UTHSCSA 14-669	Clin.	Unknown	California, USA	-	-	-	-
		FMR 14035 = UTHSCSA 14-788	Clin.	Unknown	Florida, USA	-	-	-	-
		FMR 14037 = UTHSCSA 14-1138	Clin.	Unknown	Pennsylvania, USA	-	-	-	-
		FMR 14042 = UTHSCSA 14-1603	Clin.	Unknown	Ohio, USA	-	-	-	-

FMR 14043 = UTHSCSA 14-1806	Clin.	Unknown	Delaware, USA	-	-	-	-
FMR 14045 = UTHSCSA 14-1875	Clin.	Unknown	Texas, USA	-	-	-	-
FMR 14047 = UTHSCSA 07-2799	Clin.	Unknown	Pennsylvania, USA	-	-	-	-
FMR 14059 = UTHSCSA 14-1033	Clin.	Unknown	Texas, USA	-	-	-	-
FMR 14109 = UTHSCSA 14-176 A	Clin.	Unknown	Alabama, USA	-	-	-	-
FMR 14110 = UTHSCSA 14-176 B	Clin.	Unknown	Alabama, USA	-	-	-	-
FMR 14119 = UTHSCSA 06-2208	Clin.	Unknown	Texas, USA	-	-	-	-
FMR 14128 = UTHSCSA 05-2894	Clin.	Skin	North Carolina, USA	-	-	-	-
FMR 14214 = UTHSCSA 14-725	Clin.	Unknown	Michigan, USA	-	-	-	-
FMR 14321 = UTHSCSA R-4162 C	Clin.	Unknown	Texas, USA	-	-	-	-
FMR 14341 = UTHSCSA 11-2648 A	Clin.	Unknown	New York, USA	-	-	-	-
FMR 14371 = UTHSCSA 11-2648 B	Clin.	Unknown	New York, USA	-	-	-	-
FMR 14400 = UTHSCSA 05-2476	Clin.	Unknown	Arizona, USA	-	-	-	-
FMR 14459 = UTHSCSA DI15-23	Clin.	BAL	Pennsylvania, USA	-	-	-	-
FMR 14460 = UTHSCSA DI15-24	Clin.	Spine	California, USA	-	-	-	-
FMR 14608	Env.	Soil	Catalonia, Spain	-	-	-	-
FMR 14928	Env.	Soil	Angkor, Cambodia	-	-	-	-
FMR 15399	Env.	Soil	Catalonia, Spain	-	-	-	-

		FMR 15400	Env.	Soil	Mexico D.F., Mexico	-	-	-	-
		FMR 15448	Env.	Dung	Galicia, Spain	-	-	-	-
		FMR 15616	Env.	Plant debris	Catalonia, Spain	-	-	-	-
A. hancockii	*Flavi*	FMR 15874	Env.	Dung	Canary Islands, Spain	-	-	-	-
A. nomius	*Flavi*	FMR 14055 = UTHSCSA 14-417	Clin.	Unknown	California, USA	-	-	-	-
		FMR 14183 = UTHSCSA 05-3194	Clin.	Corneal	Texas, USA	-	-	-	-
A. parasiticus	*Flavi*	FMR 14067 = UTHSCSA 04-950	Clin.	Leukemia	Florida, USA	-	-	-	-
		FMR 15384	Env.	Soil	Sao Paulo, Brazil	-	-	-	-
		FMR 15447	Env.	Soil	Sao Paulo, Brazil	-	-	-	-
		FMR 15604	Env.	Dung	Castile and Leon, Spain	-	-	-	-
A. tamarii	*Flavi*	FMR 14622	Env.	Soil	- , Venezuela	-	-	-	-
A. albodeflectus	*Flavipedes*	FMR 15175 = CBS 142665	Env.	Dung	Balearic Islands, Spain	LT798909	LT798936	LT798937	LT798938
A. ardalensis	*Flavipedes*	FMR 14590	Env.	Soil	Balearic Islands, Spain	-	-	-	-
		FMR 15057	Env.	Dung	Catalonia, Spain	-	LT798966	-	-
		FMR 15058	Env.	Dung	Catalonia, Spain	-	LT798967	-	-
A. hemisphaericus	*Flavipedes*	FMR 13523 = UTHSCSA DI14-215	Clin.	Sputum	- , USA	LT899487	LT899536	LT899589	LT899644
A. iizukae	*Flavipedes*	FMR 13527 = UTHSCSA DI14-219	Clin.	BAL	- , USA	LT899477	LT899528	LT899579	LT899634
		FMR 15051	Env.	Dung	Catalonia, Spain	LT899475	LT798968	LT899577	LT899632
		FMR 15606	Env.	Dung	Castile and Leon, Spain	LT899476	LT798969	LT899578	LT899633

A. micronesiensis	*Flavipedes*	FMR 13522 = UTHSCSA DI14-214	Clin.	Canine urine	- , USA	-	-	-	-
		FMR 15214	Env.	Dung	Balearic Islands, Spain	-	LT798970	-	-
		FMR 15323	Env.	Plant debris	Catalonia, Spain	-	-	-	-
		FMR 15734	Env.	Dung	Canary Islands, Spain	-	-	-	-
		FMR 15737	Env.	Dung	Canary Islands, Spain	-	LT798971	-	-
A. movilensis	*Flavipedes*	FMR 14921	Env.	Soil	Quang Ninh, Vietnam	-	-	-	-
A. spelaeus	*Flavipedes*	FMR 14182 = UTHSCSA 04-3307	Clin.	Unknown	Missouri, USA	LT899491	LT899538	LT899593	LT899648
		FMR 14606	Env.	Soil	Balearic Islands, Spain	LT899488	LT899537	LT899590	LT899645
		FMR 14610	Env.	Soil	Balearic Islands, Spain	-	-	-	-
		FMR 15176	Env.	Dung	Balearic Islands, Spain	LT899489	LT798972	LT899591	LT899646
		FMR 15178	Env.	Dung	Balearic Islands, Spain	-	LT798973	-	-
		FMR 15180	Env.	Dung	Balearic Islands, Spain	-	LT798974	-	-
		FMR 15215	Env.	Dung	Balearic Islands, Spain	-	LT798975	-	-
		FMR 15223	Env.	Dung	Balearic Islands, Spain	LT899490	LT798976	LT899592	LT899647
A. templicola	*Flavipedes*	FMR 14628	Env.	Soil	Balearic Islands, Spain	-	-	-	-
		FMR 15055	Env.	Dung	Catalonia, Spain	-	LT798977	-	-
		FMR 15059	Env.	Dung	Catalonia, Spain	-	LT798978	-	-
		FMR 15179	Env.	Dung	Balearic Islands, Spain	-	LT798979	-	-

		FMR 15324	Env.	Plant debris	Catalonia, Spain	-	-	-	-
A. arcoverdensis	*Fumigati*	FMR 14617	Env.	Soil	Ceara, Brazil	-	-	-	-
A. aureolus	*Fumigati*	FMR 14636	Env.	Soil	- , Venezuela	-	-	-	-
		FMR 14637	Env.	Soil	Alajuela, Costa Rica	-	-	-	-
A. fischeri	*Fumigati*	FMR 14594	Env.	Soil	- , Venezuela	-	-	-	-
A. fumigatiaffinis	*Fumigati*	FMR 13524 = UTHSCSA DI14-216	Clin.	Thrombus pulm. artery	- , USA	-	-	-	-
		FMR 13525 = UTHSCSA DI14-217	Clin.	Nasal	- , USA	-	-	-	-
		FMR 14114 = UTHSCSA 14-1113	Clin.	Unknown	Colorado, USA	-	-	-	-
A. fumigatus	*Fumigati*	FMR 14058 = UTHSCSA 14-723	Clin.	Unknown	Wisconsin, USA	-	-	-	-
		FMR 14118 = UTHSCSA 06-244 B	Clin.	Unknown	Texas, USA	-	-	-	-
		FMR 14141 = UTHSCSA 07-1018 A	Clin.	Unknown	California, USA	-	-	-	-
		FMR 14154 = UTHSCSA 10-1394	Clin.	Unknown	California, USA	-	-	-	-
		FMR 14595	Env.	Soil	Catalonia, Spain	-	-	-	-
		FMR 14607	Env.	Soil	Catalonia, Spain	-	-	-	-
		FMR 14612	Env.	Soil	Catalonia, Spain	-	-	-	-
		FMR 14629	Env.	Soil	Galicia, Spain	-	-	-	-
		FMR 14631	Env.	Soil	- , Spain	-	-	-	-
		FMR 14632	Env.	Soil	Morelos, Mexico	-	-	-	-
		FMR 14639	Env.	Soil	Sao Paulo, Brazil	-	-	-	-
		FMR 14745	Env.	Soil	Mexico D.F., Mexico	-	-	-	-

		FMR 14922	Env.	Soil	Quang Ninh, Vietnam	-	-	-	-
		FMR 15390	Env.	Dung	Andalusia, Spain	-	-	-	-
		FMR 15393	Env.	Dung	Balearic Islands, Spain	-	-	-	-
		FMR 15394	Env.	Dung	Balearic Islands, Spain	-	-	-	-
		FMR 15395	Env.	Dung	Balearic Islands, Spain	-	-	-	-
		FMR 15397	Env.	Dung	Balearic Islands, Spain	-	-	-	-
		FMR 15398	Env.	Dung	Castile and Leon, Spain	-	-	-	-
		FMR 15409	Env.	Soil	Sao Paulo, Brazil	-	-	-	-
		FMR 15410	Env.	Soil	Bali, Indonesia	-	-	-	-
		FMR 15614	Env.	Dung	Castile and Leon, Spain	-	-	-	-
		FMR 15620	Env.	Dung	Balearic Islands, Spain	-	-	-	-
A. fumisynnematus	*Fumigati*	FMR 14217 = UTHSCSA 14-1486	Clin.	Unknown	- , USA	-	-	-	-
A. lentulus	*Fumigati*	FMR 13529 = UTHSCSA DI14-221	Clin.	Respiratory	- , USA	-	-	-	-
		FMR 13530 = UTHSCSA DI14-222	Clin.	Canine lung	- , USA	-	-	-	-
		FMR 13531 = UTHSCSA DI14-223	Clin.	Lung tissue	- , USA	-	-	-	-
		FMR 13532 = UTHSCSA DI14-224	Clin.	Unknown	- , USA	-	-	-	-
		FMR 13556	Clin.	Unknown	- , USA	-	-	-	-
		FMR 13557	Clin.	Unknown	- , USA	-	-	-	-
A. udagawae	*Fumigati*	FMR 14626	Env.	Soil	- , Venezuela	-	-	-	-
		FMR 15617	Env.	Dung	Galicia, Spain	-	-	-	-

Aspergillus sp.	*Fumigati*	FMR 14027 = UTHSCSA 14-75	Clin.	Unknown	Minnesota, USA	-	-	-	-
		FMR 14029 = UTHSCSA 14-169	Clin.	Unknown	California, USA	-	-	-	-
		FMR 14030 = UTHSCSA 14-249	Clin.	Unknown	Texas, USA	-	-	-	-
		FMR 14031 = UTHSCSA 14-365	Clin.	Unknown	Texas, USA	-	-	-	-
		FMR 14033 = UTHSCSA 14-513	Clin.	Unknown	North Carolina, USA	-	-	-	-
		FMR 14036 = UTHSCSA 14-1114	Clin.	Unknown	Arizona, USA	-	-	-	-
		FMR 14038 = UTHSCSA 14-1177	Clin.	Unknown	California, USA	-	-	-	-
		FMR 14039 = UTHSCSA 14-1225	Clin.	Unknown	Tennessee, USA	-	-	-	-
		FMR 14040 = UTHSCSA 14-1485	Clin.	Unknown	Maryland, USA	-	-	-	-
		FMR 14041 = UTHSCSA 14-1556	Clin.	Unknown	Utah, USA	-	-	-	-
		FMR 14044 = UTHSCSA 14-1808	Clin.	Unknown	Texas, USA	-	-	-	-
		FMR 14046 = UTHSCSA 03-3384	Clin.	Unknown	Utah, USA	-	-	-	-
		FMR 14053 = UTHSCSA 14-105	Clin.	Unknown	California, USA	-	-	-	-
		FMR 14054 = UTHSCSA 14-195	Clin.	Unknown	Minnesota, USA	-	-	-	-
		FMR 14060 = UTHSCSA 14-1108	Clin.	Unknown	Wisconsin, USA	-	-	-	-
		FMR 14063 = UTHSCSA 14-1442	Clin.	Unknown	Texas, USA	-	-	-	-
		FMR 14064 = UTHSCSA 14-1443	Clin.	Unknown	Texas, USA	-	-	-	-
		FMR 14065 = UTHSCSA 14-1444	Clin.	Unknown	Texas, USA	-	-	-	-

		FMR 14066 = UTHSCSA 14-1555	Clin.	Unknown	Tennessee, USA	-	-	-	-
		FMR 14117 = UTHSCSA 06-244 A	Clin.	Unknown	Texas, USA	-	-	-	-
		FMR 14129 = UTHSCSA 06-1190	Clin.	Unknown	Florida, USA	-	-	-	-
		FMR 14131 = UTHSCSA 03-365	Clin.	Blood	Florida, USA	-	-	-	-
		FMR 14145 = UTHSCSA 08-1003	Clin.	Emphysema	- , USA	-	-	-	-
		FMR 14166 = UTHSCSA 14-846	Clin.	Unknown	California, USA	-	-	-	-
A. janus	*Jani*	FMR 15883	Env.	Soil	Jujuy, Argentina	-	-	-	-
A. amoenus	*Nidulantes*	FMR 14134 = UTHSCSA 05-2980	Clin.	Animal	Nebraska, USA	LN898664	LN898818	LN898741	LN898895
		FMR 14138 = UTHSCSA 06-1721	Clin.	BAL	Florida, USA	LN898665	LN898819	LN898742	LN898896
		FMR 14142 = UTHSCSA 07-1668	Clin.	Sinus	Minnesota, USA	LN898666	LN898820	LN898743	LN898897
		FMR 14143 = UTHSCSA 07-2785	Clin.	Pleural fluid	- , USA	LN898667	LN898821	LN898744	LN898898
		FMR 14144 = UTHSCSA 07-2881	Clin.	Pleural fluid	- , USA	LN898668	LN898822	LN898745	LN898899
		FMR 14146 = UTHSCSA 08-2366	Clin.	Unknown	Maryland, USA	LN898669	LN898823	LN898746	LN898900
		FMR 14157 = UTHSCSA 11-476	Clin.	Sputum	Missouri, USA	LN898670	LN898824	LN898747	LN898901
		FMR 14158 = UTHSCSA 11-1419	Clin.	BAL	South Carolina, USA	LN898671	LN898825	LN898748	LN898902
		FMR 14188 = UTHSCSA 06-4284	Clin.	BAL	South Carolina, USA	LN898672	LN898826	LN898749	LN898903
		FMR 14198 = UTHSCSA 09-125	Clin.	BAL	- , USA	LN898673	LN898827	LN898750	LN898904
		FMR 14209 = UTHSCSA 12-340	Clin.	Animal	California, USA	LN898674	LN898828	LN898751	LN898905

		FMR 14329 = UTHSCSA 07-443	Clin.	BAL	Florida, USA	LN898675	LN898829	LN898752	LN898906
		FMR 14334 = UTHSCSA 07-3621	Clin.	Chest	- , USA	LN898676	LN898830	LN898753	LN898907
		FMR 14368 = UTHSCSA 09-2582	Clin.	Lung tissue	Massachusetts, USA	LN898677	LN898831	LN898754	LN898908
		FMR 14627	Env.	Soil	Alajuela, Costa Rica	-	-	-	-
A. argentinensis	*Nidulantes*	FMR 15740	Env.	Soil	Salta, Argentina	LT903690	LT903681	LT903684	LT903687
A. aureolatus	*Nidulantes*	FMR 15442	Env.	Dung	Galicia, Spain	-	LT798994	-	-
A. austroafricanus	*Nidulantes*	FMR 15174	Env.	Dung	Balearic Islands, Spain	-	LT798995	-	-
A. creber	*Nidulantes*	FMR 13534 = UTHSCSA DI 14-226	Clin.	BAL	- , USA	LN898678	LN898832	LN898755	LN898909
		FMR 13536 = UTHSCSA DI 14-228	Clin.	Nail	- , USA	LN898679	LN898833	LN898756	LN898910
		FMR 14112 = UTHSCSA 14-223	Clin.	Arm	North Carolina, USA	LN898680	LN898834	LN898757	LN898911
		FMR 14132 = UTHSCSA 03-2409	Clin.	Hospital Env.	Texas, USA	LN898681	LN898835	LN898758	LN898912
		FMR 14133 = UTHSCSA 05-2359	Clin.	BAL	Utah, USA	LN898682	LN898836	LN898759	LN898913
		FMR 14149 = UTHSCSA 09-1670	Clin.	BAL	Minnesota, USA	LN898683	LN898837	LN898760	LN898914
		FMR 14151 = UTHSCSA 09-3357	Clin.	BAL	Pennsylvania, USA	LN898684	LN898838	LN898761	LN898915
		FMR 14168 = UTHSCSA 14-188	Clin.	BAL	Delaware, USA	LN898685	LN898839	LN898762	LN898916
		FMR 14186 = UTHSCSA 06-3435	Clin.	BAL	Ohio, USA	LN898686	LN898840	LN898763	LN898917
		FMR 14201 = UTHSCSA 10-1327	Clin.	Nail	Minnesota, USA	LN898687	LN898841	LN898764	LN898918
		FMR 14207 = UTHSCSA 11-2813	Clin.	Mucosa	Minnesota, USA	LN898688	LN898842	LN898765	LN898919
		FMR 14237 = UTHSCSA	Clin.	BAL	Pennsylvania,	LN898689	LN898843	LN898766	LN898920

		09-2679			USA				
		FMR 14238 = UTHSCSA 10-639	Clin.	BAL	North Carolina, USA	LN898690	LN898844	LN898767	LN898921
		FMR 14325 = UTHSCSA 04-799	Clin.	Sputum	Tennessee, USA	LN898691	LN898845	LN898768	LN898922
		FMR 14333 = UTHSCSA 07-2788	Clin.	BAL	- , USA	LN898692	LN898846	LN898769	LN898923
		FMR 14364 = UTHSCSA 04-434	Clin.	Sputum	Minnesota, USA	LN898693	LN898847	LN898770	LN898924
		FMR 14369 = UTHSCSA 10-582	Clin.	BAL	Virginia, USA	LN898694	LN898848	LN898771	LN898925
		FMR 14634	Env.	Soil	- , Uruguay	-	-	-	-
		FMR 15379	Env.	Plant debris	Catalonia, Spain	-	-	-	-
A. cvjetkovicii	*Nidulantes*	FMR 14153 = UTHSCSA 10-479	Clin.	Hospital Env.	Ohio, USA	LN898695	LN898849	LN898772	LN898926
A. fructus	*Nidulantes*	FMR 14162 = UTHSCSA 12-3194	Clin.	Pericardial	California, USA	LN898696	LN898850	LN898773	LN898927
		FMR 15728	Env.	Dung	Canary Islands, Spain	-	LT798996	-	-
A. jensenii	*Nidulantes*	FMR 13528 = UTHSCSA DI 14-220	Clin.	Nail	- , USA	LN898697	LN898851	LN898774	LN898928
		FMR 14136 = UTHSCSA 05-3600	Clin.	Sputum	Minnesota, USA	LN898698	LN898852	LN898775	LN898929
		FMR 14150 = UTHSCSA 09-2299	Clin.	Sputum	Illinois, USA	LN898699	LN898853	LN898776	LN898930
		FMR 14152 = UTHSCSA 10-327	Clin.	Sputum	Pennsylvania, USA	LN898700	LN898854	LN898777	LN898931
		FMR 14161 = UTHSCSA 12-79	Clin.	BAL	Ohio, USA	LN898701	LN898855	LN898778	LN898932
		FMR 14193 = UTHSCSA 07-3790	Clin.	BAL	Pennsylvania, USA	LN898702	LN898856	LN898779	LN898933
		FMR 14200 = UTHSCSA 10-71	Clin.	BAL	Connecticut, USA	LN898703	LN898857	LN898780	LN898934
		FMR 14234 = UTHSCSA 09-425	Clin.	Nail	Utah, USA	LN898704	LN898858	LN898781	LN898935

		FMR 14613	Env.	Soil	Catalonia, Spain	-	-	-	-
		FMR 14615	Env.	Soil	- , Uruguay	-	-	-	-
A. latus	*Nidulantes*	FMR 14593	Env.	Soil	Sao Paulo, Brazil	-	-	-	-
A. nidulans	*Nidulantes*	FMR 14057 = UTHSCSA 14-648	Clin.	Unknown	Maryland, USA	LT899482	LT899531	LT899584	LT899639
		FMR 14135 = UTHSCSA 05-3563	Clin.	Sinus	Texas, USA	LT899481	LT899530	LT899583	LT899638
		FMR 14160 = UTHSCSA 11-3215	Clin.	Unknown	Minnesota, USA	-	-	-	-
		FMR 15219	Env.	Dung	Balearic Islands, Spain	-	LT798980	-	-
		FMR 15229	Env.	Dung	Andalusia, Spain	-	LT798981	-	-
		FMR 15377	Env.	Dung	Balearic Islands, Spain	-	LT798982	-	-
		FMR 15378	Env.	Dung	Balearic Islands, Spain	-	-	-	-
A. pachycristatus	*Nidulantes*	FMR 14113 = UTHSCSA14-805	Clin.	Unknown	Minnesota, USA	LT899484	LT899533	LT899586	LT899641
		FMR 15741	Env.	Soil	Salta, Argentina	LT899483	LT899532	LT899585	LT899640
A. protuberus	*Nidulantes*	FMR 14140 = UTHSCSA 06-4104	Clin.	BAL	Maryland, USA	LN898705	LN898859	LN898782	LN898936
		FMR 14148 = UTHSCSA 09-246	Clin.	Animal	Connecticut, USA	LN898706	LN898860	LN898783	LN898937
		FMR 14156 = UTHSCSA 11-269	Clin.	BAL	Illinois, USA	LN898707	LN898861	LN898784	LN898938
		FMR 14191 = UTHSCSA 07-2433	Clin.	BAL	- , USA	LN898708	LN898862	LN898785	LN898939
		FMR 14195 = UTHSCSA 08-3392	Clin.	BAL	Massachusetts, USA	LN898709	LN898863	LN898786	LN898940
		FMR 14205 = UTHSCSA 11-2175	Clin.	Sputum	Ohio, USA	LN898710	LN898864	LN898787	LN898941
		FMR 14208 = UTHSCSA	Clin.	Animal	California, USA	LN898711	LN898865	LN898788	LN898942

		12-338							
		FMR 14244 = UTHSCSA 12-256	Clin.	BAL	South Carolina, USA	LN898712	LN898866	LN898789	LN898943
		FMR 14328 = UTHSCSA 06-2837	Clin.	BAL	- , USA	LN898713	LN898867	LN898790	LN898944
		FMR 14336 = UTHSCSA 08-1574	Clin.	BAL	Colorado, USA	LN898714	LN898868	LN898791	LN898945
		FMR 14619	Env.	Soil	Andalusia, Spain	-	-	-	-
		FMR 14924	Env.	Soil	Quang Ninh, Vietnam	-	-	-	-
A. puulaauensis	*Nidulantes*	FMR 14159 = UTHSCSA 11-1436	Clin.	BAL	Washington, USA	LN898715	LN898869	LN898792	LN898946
		FMR 15630	Env.	Soil	Bangkok, Thailand	-	-	-	-
A. quadrilineatus	*Nidulantes*	FMR 14068 = UTHSCSA 05-237	Clin.	Blood	- , USA	LT899486	LT899535	LT899588	LT899643
		FMR 14621	Env.	Soil	Sao Paulo, Brazil	LT899485	LT899534	LT899587	LT899642
A. rugulosus	*Nidulantes*	FMR 15173	Env.	Dung	Catalonia, Spain	-	LT798983	-	-
A. spinulosporus	*Nidulantes*	FMR 14242 = UTHSCSA 11-3214	Clin.	Unknown	Minnesota, USA	LT899492	LT899539	LT899594	LT899649
A. sydowii	*Nidulantes*	FMR 14147 = UTHSCSA 09-48	Clin.	Blood	Kentucky, USA	LN898716	LN898870	LN898793	LN898947
		FMR 14155 = UTHSCSA 11-204	Clin.	Eye	Pennsylvania, USA	LN898717	LN898871	LN898794	LN898948
		FMR 14164 = UTHSCSA 13-2518	Clin.	Eye	Utah, USA	LN898718	LN898872	LN898795	LN898949
		FMR 14165 = UTHSCSA 13-2630	Clin.	Sinus	Minnesota, USA	LN898719	LN898873	LN898796	LN898950
		FMR 14184 = UTHSCSA 06-2186	Clin.	BAL	Florida, USA	LN898720	LN898874	LN898797	LN898951
		FMR 14185 = UTHSCSA 06-2780	Clin.	Bronchus cast	Minnesota, USA	LN898721	LN898875	LN898798	LN898952
		FMR 14187 = UTHSCSA	Clin.	Sinus	Minnesota,	LN898722	LN898876	LN898799	LN898953

06-4167			USA				
FMR 14189 = UTHSCSA 07-1018	Clin.	Animal	California, USA	LN898723	LN898877	LN898800	LN898954
FMR 14197 = UTHSCSA 09-97	Clin.	BAL	California, USA	LN898724	LN898878	LN898801	LN898955
FMR 14210 = UTHSCSA 12-934	Clin.	BAL	Minnesota, USA	LN898725	LN898879	LN898802	LN898956
FMR 14212 = UTHSCSA 13-2674	Clin.	BAL	Minnesota, USA	LN898726	LN898880	LN898803	LN898957
FMR 14239 = UTHSCSA 10-1222	Clin.	Unknown	New York, USA	LN898727	LN898881	LN898804	LN898958
FMR 14240 = UTHSCSA 10-3180	Clin.	Sputum	Minnesota, USA	LN898728	LN898882	LN898805	LN898959
FMR 14241 = UTHSCSA 11-2683	Clin.	Spine	Minnesota, USA	LN898729	LN898883	LN898806	LN898960
FMR 14326 = UTHSCSA 06-727	Clin.	BAL	Minnesota, USA	LN898730	LN898884	LN898807	LN898961
FMR 14337 = UTHSCSA 08-3215	Clin.	Animal	Connecticut, USA	LN898731	LN898885	LN898808	LN898962
FMR 14338 = UTHSCSA 09-1708	Clin.	Lung tissue	Utah, USA	LN898732	LN898886	LN898809	LN898963
FMR 14342 = UTHSCSA 12-3109	Clin.	Lung tissue	North Carolina, USA	LN898733	LN898887	LN898810	LN898964
FMR 14366 = UTHSCSA 08-865	Clin.	Hip joint	Minnesota, USA	LN898734	LN898888	LN898811	LN898965
FMR 14440	Clin.	Ear exudate	Catalonia, Spain	LN898735	LN898889	LN898812	LN898966
FMR 14588	Env.	Soil	Balearic Islands, Spain	-	-	-	-
FMR 14623	Env.	Soil	- , Venezuela	-	-	-	-
FMR 15603	Env.	Dung	Castile and Leon, Spain	-	LT798997	-	-
FMR 15618	Env.	Dung	Galicia, Spain	-	LT798998	-	-
FMR 15880	Env.	Dung	Canary Islands, Spain	-	LT798999	-	-

A. tabacinus	*Nidulantes*	FMR 14179 = UTHSCSA 03-1197	Clin.	Sputum	Florida, USA	LN898736	LN898890	LN898813	LN898967
		FMR 14190 = UTHSCSA 07-2427	Clin.	BAL	- , USA	LN898737	LN898891	LN898814	LN898968
		FMR 14202 = UTHSCSA 10-1677	Clin.	Pleural fluid	California, USA	LN898738	LN898892	LN898815	LN898969
		FMR 14232 = UTHSCSA 08-2898	Clin.	BAL	- , USA	LN898739	LN898893	LN898816	LN898970
A. tumidus	*Nidulantes*	FMR 15743	Env.	Soil	- , Chile	LT903691	LT903682	LT903685	LT903688
A. unguis	*Nidulantes*	FMR 14206 = UTHSCSA 11-2524	Clin.	Unknown	Minnesota, USA	LT899498	LT899545	LT899600	LT899655
A. versicolor	*Nidulantes*	FMR 14181 = UTHSCSA 03-3679	Clin.	BAL	Florida, USA	LN898740	LN898894	LN898817	LN898971
A. versicolor	*Nidulantes*	FMR 14614	Env.	Soil	Catalonia, Spain	-	-	-	-
A. viricatenatus	*Nidulantes*	FMR 15446	Env.	Dung	Galicia, Spain	-	LT799000	-	-
A. aculeatus	*Nigri*	FMR 15382	Env.	Soil	Sao Paulo, Brazil	-	-	-	-
		FMR 15406	Env.	Soil	Sao Paulo, Brazil	-	-	-	-
A. awamori	*Nigri*	FMR 13537 = UTHSCSA DI14-229	Clin.	BAL	- , USA	-	-	-	-
		FMR 13538 = UTHSCSA DI14-230	Clin.	Ear	- , USA	-	-	-	-
		FMR 13539 = UTHSCSA DI14-231	Clin.	Canine ear	- , USA	-	-	-	-
		FMR 13540 = UTHSCSA DI14-232	Clin.	Ethmoid sinus	- , USA	-	-	-	-
		FMR 13541 = UTHSCSA DI14-233	Clin.	Dolphin blow	- , USA	-	-	-	-
A. brasiliensis	*Nigri*	FMR 15386	Env.	Plant debris	Catalonia, Spain	-	-	-	-
A. carbonarius	*Nigri*	FMR 14638	Env.	Soil	Sao Paulo, Brazil	-	-	-	-
A. japonicus	*Nigri*	FMR 14592	Env.	Soil	Sao Paulo, Brazil	-	-	-	-

		FMR 14641	Env.	Soil	Sao Paulo, Brazil	-	-	-	-
A. luchuensis	*Nigri*	FMR 15413	Env.	Dung	Galicia, Spain	-	-	-	-
A. niger	*Nigri*	FMR 14640	Env.	Soil	- , Venezuela	-	-	-	-
		FMR 14714	Env.	Soil	Mexico D.F., Mexico	-	-	-	-
		FMR 15385	Env.	Soil	Sao Paulo, Brazil	-	-	-	-
		FMR 15387	Env.	Plant debris	Catalonia, Spain	-	-	-	-
		FMR 15388	Env.	Soil	Andalusia, Spain	-	-	-	-
		FMR 15392	Env.	Dung	Balearic Islands, Spain	-	-	-	-
		FMR 15396	Env.	Dung	Balearic Islands, Spain	-	-	-	-
		FMR 15441	Env.	Dung	Galicia, Spain	-	-	-	-
A. tubingensis	*Nigri*	FMR 14630	Env.	Soil	Asturias, Spain	-	-	-	-
		FMR 14635	Env.	Soil	- , Venezuela	-	-	-	-
		FMR 14712	Env.	Soil	Mexico D.F., Mexico	-	-	-	-
		FMR 15326	Env.	Soil	- , Morocco	-	-	-	-
		FMR 15389	Env.	Soil	Andalusia, Spain	-	-	-	-
A. alabamensis	*Terrei*	FMR 13542 = UTHSCSA DI14-234	Clin.	Tracheal	- , USA	LT899447	LT899501	LT899549	LT899604
		FMR 14616	Env.	Soil	- , Uruguay	LT899444	LT899499	LT899546	LT899601
		FMR 15383	Env.	Soil	Sao Paulo, Brazil	LT899445	LT899500	LT899547	LT899602
		FMR 15407	Env.	Soil	Sao Paulo, Brazil	-	-	-	-
		FMR 15408	Env.	Soil	Sao Paulo, Brazil	-	-	-	-
		FMR 15412	Env.	Dung	Galicia, Spain	LT899446	LT798984	LT899548	LT899603

		FMR 15731	Env.	Dung	Canary Islands, Spain	-	LT798985	-	-
A. allahabadii	*Terrei*	FMR 15221	Env.	Plant debris	Catalonia, Spain	-	-	-	-
		FMR 15879	Env.	Dung	Extremadura, Spain	-	-	-	-
A. aurantiosulcatus	*Terrei*	FMR 15182 = CBS 142981	Env.	Dung	Balearic Islands, Spain	LT798912	LT798945	LT798946	LT798947
A. bicephalus	*Terrei*	FMR 14918	Env.	Soil	Mexico State, Mexico	LT601380	LT601381	LT601382	LT601383
A. carneus	*Terrei*	FMR 13521 = UTHSCSA DI14-213	Clin.	Canine heart	- , USA	LT899466	LT899520	LT899568	LT899623
		FMR 15380	Env.	Plant debris	Catalonia, Spain	LT899465	LT899519	LT899567	LT899622
A. citrinoterreus	*Terrei*	FMR 15876	Env.	Dung	Canary Islands, Spain	-	LT798989	-	-
A. fimeti-brunneus	*Terrei*	FMR 15228 = CBS 142751	Env.	Dung	Andalusia, Spain	LT798913	LT798948	LT798949	LT798950
A. floccosus	*Terrei*	FMR 15061	Env.	Dung	Catalonia, Spain	-	LT798986	-	-
A. hortai	*Terrei*	FMR 13526 = UTHSCSA DI14-218	Clin.	BAL	- , USA	LT899474	LT899527	LT899576	LT899631
		FMR 14597	Env.	Soil	- , Venezuela	LT899471	LT899525	LT899573	LT899628
		FMR 15220	Env.	Plant debris	Catalonia, Spain	LT899472	LT899526	LT899574	LT899629
		FMR 15227	Env.	Dung	Andalusia, Spain	LT899473	LT798987	LT899575	LT899630
A. majoricus	*Terrei*	FMR 15181 = CBS 142986	Env.	Dung	Balearic Islands, Spain	LT798910	LT798939	LT798940	LT798941
		FMR 15217 = CBS 142987	Env.	Dung	Balearic Islands, Spain	LT798911	LT798942	LT798943	LT798944
A. subglobosus	*Terrei*	FMR 15381	Env.	Soil	Sao Paulo, Brazil	LT903689	LT903680	LT903683	LT903686
A. terreus	*Terrei*	FMR 14056 = UTHSCSA 14-555	Clin.	Unknown	California, USA	LT899496	LT899543	LT899598	LT899653
		FMR 14061 = UTHSCSA	Clin.	Unknown	Texas, USA	LT899494	LT899541	LT899596	LT899651

		14-1147							
		FMR 14062 = UTHSCSA 14-1441	Clin.	Unknown	Texas, USA	LT899495	LT899542	LT899597	LT899652
		FMR 14126 = UTHSCSA 05-1115	Clin.	Unknown	Texas, USA	LT899493	LT899540	LT899595	LT899650
		FMR 14213 = UTHSCSA 14-724	Clin.	Unknown	Texas, USA	LT899497	LT899544	LT899599	LT899654
		FMR 14926	Env.	Soil	Angkor, Cambodia	-	-	-	-
		FMR 15054	Env.	Dung	Catalonia, Spain	-	LT798988	-	-
		FMR 15325	Env.	Plant debris	Catalonia, Spain	-	-	-	-
A. calidokeveii	*Usti*	FMR 15225 = CBS 142666	Env.	Dung	Castile and Leon, Spain	LT798914	LT798951	LT798952	LT798953
		FMR 13517 = UTHSCSA DI14-209	Clin.	Knee	- , USA	LT899455	LT899509	LT899557	LT899612
		FMR 13518 = UTHSCSA DI14-210	Clin.	Sputum	- , USA	LT899456	LT899510	LT899558	LT899613
		FMR 13519 = UTHSCSA DI14-211	Clin.	BAL	- , USA	LT899457	LT899511	LT899559	LT899614
		FMR 13520 = UTHSCSA DI14-212	Clin.	BAL	- , USA	LT899458	LT899512	LT899560	LT899615
		FMR 13546 = UTHSCSA DI14-238	Clin.	Arm	- , USA	LT899459	LT899513	LT899561	LT899616
		FMR 13547 = UTHSCSA DI14-239	Clin.	BAL	- , USA	LT899460	LT899514	LT899562	LT899617
		FMR 13548 = UTHSCSA DI14-240	Clin.	Frontal mass	- , USA	LT899461	LT899515	LT899563	LT899618
		FMR 13549 = UTHSCSA DI14-241	Clin.	BAL	- , USA	LT899462	LT899516	LT899564	LT899619
		FMR 13550 = UTHSCSA DI14-242	Clin.	Tissue	- , USA	LT899463	LT899517	LT899565	LT899620
		FMR 13551 = UTHSCSA DI14-243	Clin.	BAL	- , USA	LT899464	LT899518	LT899566	LT899621
		FMR 14115 = UTHSCSA	Clin.	Unknown	- , USA	LT899454	LT899508	LT899556	LT899611

		14-1227							
		FMR 14121 = UTHSCSA 09-518	Clin.	Unknown	- , USA	LT899452	LT899506	LT899554	LT899609
		FMR 14139 = UTHSCSA 06-3415	Clin.	Unknown	- , USA	LT899448	LT899502	LT899550	LT899605
		FMR 14170 = UTHSCSA 14-1036	Clin.	Unknown	- , USA	LT899453	LT899507	LT899555	LT899610
		FMR 14194 = UTHSCSA 08-1449	Clin.	Unknown	- , USA	LT899450	LT899504	LT899552	LT899607
		FMR 14199 = UTHSCSA 09-1887C	Clin.	Unknown	- , USA	LT899451	LT899505	LT899553	LT899608
		FMR 14230 = UTHSCSA 07-2558	Clin.	Unknown	- , USA	LT899449	LT899503	LT899551	LT899606
		FMR 15609	Env.	Dung	Castile and Leon, Spain	-	LT798990	-	-
		FMR 15942	Env.	Soil	Catalonia, Spain	-	-	-	-
A. granulosus	*Usti*	FMR 13533 = UTHSCSA DI14-225	Clin.	Brain stem	- , USA	LT899470	LT899524	LT899572	LT899627
A. insuetus	*Usti*	FMR 15322	Env.	Dung	Andalusia, Spain	-	LT798991	-	-
		FMR 15631	Env.	Soil	Jujuy, Argentina	-	-	-	-
		FMR 15632	Env.	Soil	Salta, Argentina	-	-	-	-
		FMR 15936	Env.	Soil	Catalonia, Spain	-	-	-	-
A. keveii	*Usti*	FMR 14596	Env.	Soil	Colonia, Uruguay	-	-	-	-
		FMR 14711	Env.	Soil	Mexico D.F., Mexico	-	-	-	-
		FMR 15732	Env.	Dung	Canary Islands, Spain	-	-	-	-
A. pseudodeflectus	*Usti*	FMR 14744	Env.	Soil	Canary Islands, Spain	-	-	-	-
		FMR 15376	Env.	Dung	Andalusia,	-	LT798992	-	-

					Spain				
		FMR 15619	Env.	Soil	Andalusia, Spain	-	-	-	-
		FMR 15625	Env.	Unknown	- , Spain	-	-	-	-
		FMR 15628	Env.	Soil	Sao Paulo, Brazil	-	-	-	-
		FMR 15727	Env.	Dung	Canary Islands, Spain	-	LT798993	-	-
		FMR 15939	Env.	Soil	Catalonia, Spain	-	-	-	-
A. pseudoustus	*Usti*	FMR 15937	Env.	Soil	Catalonia, Spain	-	-	-	-
A. puniceus	*Usti*	FMR 14847	Env.	Soil	Catalonia, Spain	-	-	-	-

CBS: Westerdijk Institute (former CBS Fungal Biodiversity Centre, The Netherlands); FMR: Facultat de Medicina de Reus (Spain); UTHSCSA: University of Texas Health Science Center (San Antonio, USA); Clin.: clinical; Env.: environmental; ITS: Internal transcribed spacer regions of the rDNA and 5.8S region; *BenA*: β-tubulin; *CaM*: calmodulin; *RPB2*: partial RNA polymerase II second largest subunit; - : not available.

4. RESULTS

The *Aspergillus* isolates from clinical samples included in this thesis were preliminary identified by morphological examination and analysis of, at minimum, one of the recommended phylogenetic markers, revealing the existence of at least 49 species in 11 sections (Figure 10). Considering we focused on the species diversity of not well-known sections of *Aspergillus* in clinical specimens, 72 isolates that belonged to sections *Fumigati*, *Flavi*, and *Nigri* were not studied further in the thesis. On the other hand, 176 isolates were selected to complete the multilocus sequence analysis for their final identification. Based on that, 40 species were identified from the sections *Aspergillus*, *Circumdati*, *Clavati*, *Cremei*, *Flavipedes*, *Nidulantes*, *Terrei*, and *Usti*. The sections with most isolates were *Versicolores* (currently *Nidulantes*), *Circumdati*, and *Aspergillus*, which resulted in three publications (sections 4.1–4.3 of this thesis). The remaining clinical isolates are part of a study on cryptic species of *Aspergillus* from clinical samples, which is currently being prepared to be submitted (4.4). Among the species identified, the most frequent were *A. sydowii* (n = 20) and *A. creber* (n = 17) in section *Nidulantes*; *A. calidoustus* (n = 17) in section *Usti*; *A. amoenus* (n = 14) in section *Nidulantes*; *A. montevidensis* (n = 11) in section *Aspergillus*; and *A. westerdijkiae* (n = 10) section *Circumdati*. It is also worth mentioning the proposal of three new species. Two of them were already described, *A. pseudosclerotiorum* (n = 8) in section *Circumdati* and *A. microperforatus* (n = 2) in section *Aspergillus*. The third new species belongs to section *Flavipedes* and it is included in the publication of cryptic *Aspergillus* species (4.4).

Regarding the environmental isolates, they were preliminary identified by sequencing of the *BenA* marker, the best informative locus to discriminate among *Aspergillus* species. A total of 79 species (n = 185 isolates) were identified and distributed in 13 sections (Figure 10). The most frequent species were *A. fumigatus* (n = 19) in section *Fumigati*, *A. niger* (n = 8) in section *Nigri*, *A. pseudodeflectus* (n = 7) in section *Usti*, *A. spelaeus* (n = 7) in section *Flavipedes*, and *A. alabamensis* (n = 6) in section *Terrei*. Forty eight species (n = 92) were recovered from soil samples collected in Argentina, Brazil, Cambodia, Chile, Costa Rica, Indonesia, Mexico, Morocco, Spain, Thailand, Uruguay, Venezuela, and Vietnam. Another 48 species (n = 82) were isolated from dung samples collected from different natural areas from Spain, including Balearic and Canary Islands. Isolates from plant debris (n = 10), collected also in Spain, corresponded to 10 species. Among the environmental isolates, 17, nine from herbivore dung and five from soil, could not be identified using only *BenA*. Sequencing of other markers and phenotypic characterization confirmed that they corresponded to 14 new species. The descriptions of these new species are included in two different studies according to their origin, i.e. from herbivore dung (4.5) or from soil (4.6).

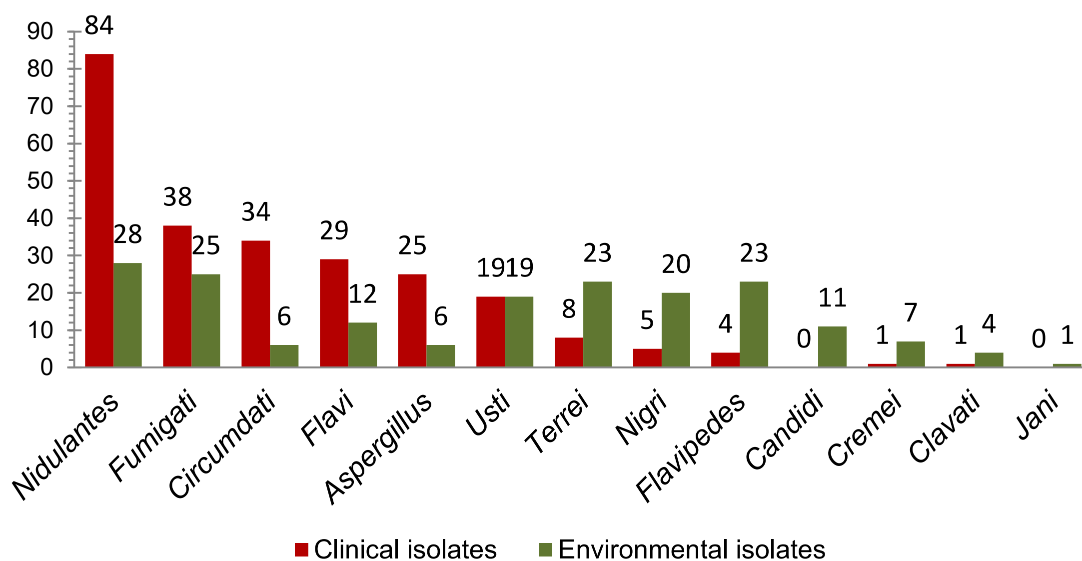


Figure 10. Column graph exhibiting the number of *Aspergillus* isolates included in this thesis divided into sections. Red bars correspond to the clinical and green bars to environmental isolates.

4.1. Species diversity of *Aspergillus* section *Versicolores* in clinical samples and antifungal susceptibility

João Paulo Zen Siqueira, Deanna A. Sutton, Dania García, Josepa Gené, Pamela Thomson, Nathan Wiederhold, Josep Guarro

Published in: Fungal Biology
November 2016, Volume 120, Issue 11, Pages 1458–1467

FUNGAL BIOLOGY 120 (2016) 1458–1467

British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/funbio

Species diversity of *Aspergillus* section *Versicolores* in clinical samples and antifungal susceptibility

João Paulo Zen SIQUEIRA[a,b], *Deanna A. SUTTON*[c], *Dania GARCÍA*[a], *Josepa GENÉ*[a,*], *Pamela THOMSON*[a], *Nathan WIEDERHOLD*[c], *Josep GUARRO*[a]

[a]*Unitat de Micologia, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, 21 Sant Llorenç St., 43201 Reus, Spain*
[b]*Laboratório de Microbiologia, Faculdade de Medicina de São José do Rio Preto, 5416 Brigadeiro Faria Lima Ave., 15090-000 São José do Rio Preto, Brazil*
[c]*Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, TX, USA*

ARTICLE INFO

Article history:
Received 15 December 2015
Received in revised form
9 February 2016
Accepted 16 February 2016

Corresponding Editor:
Pedro W. Crous

Keywords:
Antufungal activity
Ascomycetes
Aspergilli
Multilocus phylogeny
Taxonomy

ABSTRACT

Aspergillus section *Versicolores* includes species of clinical relevance and many others that have been poorly studied but are occasionally found in clinical samples. The aim of this study was to investigate, using a multilocus phylogenetic approach, the spectrum of species of the section *Versicolores* and to determine their *in vitro* antifungal susceptibility. The study was based on a set of 77 clinical isolates from different USA medical centres, which had been previously identified as belonging to this section. The genetic markers used were internal transcribed spacer (ITS), β-tubulin (*BenA*), calmodulin (*CaM*), and RNA polymerase II second largest subunit (*RPB2*), and the drugs tested, following the CLSI guidelines, were amphotericin B (AMB), itraconazole, posaconazole, voriconazole, anidulafungin, caspofungin, micafungin, terbinafine (TBF), and flucytosine (5FC). The most frequent species were *Aspergillus sydowii* (26 %), *Aspergillus creber* (22 %), and *Aspergillus amoenus* (18.2 %), followed by *Aspergillus protuberus* (13 %), *Aspergillus jensenii* (10.4 %), and *Aspergillus tabacinus* (5.2 %); while *Aspergillus cvjetkovicii*, *Aspergillus fructus*, *Aspergillus puulaauensis*, and *Aspergillus versicolor* were represented by only one isolate each (1.3 %). This is the first time that *A. jensenii* and *A. puulaauensis* have been reported from clinical samples. Considering the high number of isolates identified as belonging to this fungal group in this study, its clinical relevance should not be overlooked. *Aspergillus versicolor*, traditionally considered one of the most common species in this section in a clinical setting, was only rarely recovered in our study. The *in vitro* antifungal results showed that echinocandins and TBF were the most potent drugs, the azoles showed variable results, AMB was poorly active, and 5FC was the less active.

© 2016 British Mycological Society. Published by Elsevier Ltd. All rights reserved.

* *Corresponding author*. Tel.: +34 977 759359; fax: +34 977 759322.
E-mail address: josepa.gene@urv.cat (J. Gené).

http://dx.doi.org/10.1016/j.funbio.2016.02.006
1878-6146/© 2016 British Mycological Society. Published by Elsevier Ltd. All rights reserved.

Introduction

Aspergillus is one of the most ubiquitous genera of ascomycetes. It includes many species of biotechnological and industrial relevance (Houbraken *et al.* 2014). Some of them, particularly *Aspergillus fumigatus*, are involved in allergic diseases and severe infections in both animals and humans (de Hoog *et al.* 2011). Therefore, the correct identification of the fungal isolates is crucial for a better knowledge of the actual prevalence of the different species in their habitats and substrates. Traditionally, *Aspergillus* identification is based on macro- and micromorphological characteristics, and the species organized in groups or sections (Raper & Fennell 1976; Gams *et al.* 1985). Recent molecular studies have demonstrated that most of the *Aspergillus* sections are in fact monophyletic groups of closely related species. However, the boundaries of some sections still remain unclear (Houbraken & Samson 2011; Houbraken *et al.* 2014; Samson *et al.* 2014; Hubka *et al.* 2015). The section *Versicolores* is a clear example. It includes a group of relevant species but with a taxonomy not yet resolved. Some authors consider the delimitation of the members of this section from those of the section *Nidulantes* to be unresolved (Peterson 2008; Buzina 2013; Houbraken *et al.* 2014; Negri *et al.* 2014), while others treat *Versicolores* and *Nidulantes* as different sections (Jurjevic *et al.* 2012; Samson *et al.* 2014; Visagie *et al.* 2014; Hubka *et al.* 2015). Despite their being closely related and being two monophyletic clades with low statistical support, both sections show some phenotypic characteristics that allow their distinction. Specifically, the *Versicolores* species are characterized by conidiophores with subglobose to pyriform vesicles, biseriate conidial heads, usually radiated, with greenish rough-walled usually globose to subglobose conidia (Raper & Fennel 1976; Klich 1993; Jurjevic *et al.* 2012). However, they are particularly difficult to distinguish among species because even though their cultural morphology is considerably different, their microscopic structures are very similar (Klich 1993; Jurjevic *et al.* 2012). The taxonomy of *Versicolores* has been investigated molecularly in recent years and 20 species have so far been accepted (Jurjevic *et al.* 2012; Samson *et al.* 2014; Visagie *et al.* 2014), *Aspergillus versicolor* and *Aspergillus sydowii* being the most well-known and studied species. The interest of the species of this section lies in their common occurrence in indoor environments (Zahradnik *et al.* 2013; Sharpe *et al.* 2015), the ability to produce sterigmatocystin, a carcinogenic, and mutagenic precursor to aflatoxin B_1, and in their different biotechnological applications (Schmitt *et al.* 2002; Batista *et al.* 2003; Jurjevic *et al.* 2013; Dou *et al.* 2014; Li *et al.* 2015). Moreover, they have been reported as human and animal opportunistic pathogens (de Hoog *et al.* 2011; Buzina 2013) able to cause a variety of infections, including onychomycosis (Torres-Rodrígues *et al.* 1998; Takahata *et al.* 2008), endophthalmitis (Perri *et al.* 2005), ear infection (Rotoli *et al.* 2001), invasive pulmonary infections (Charles *et al.* 2011), aspergilloma (Kane *et al.* 2014), homograft valve infection (Huh *et al.* 2013), endodontic infection (Gomes *et al.* 2015), and vaginitis (Borsa *et al.* 2015); as well as infections in animals, such as dogs (Zhang *et al.* 2012) and horses (Ludwig *et al.* 2005; Lee *et al.* 2012). However, the spectrum of species of the section *Versicolores* in the clinical setting, considering modern taxonomic criteria proposed for *Aspergillus* (Jurjevic *et al.* 2012; Samson *et al.* 2014; Visagie *et al.* 2014), has not been fully explored. Additionally, the antifungal susceptibility of these species is practically unknown because it has only occasionally been reported (Torres-Rodríguez *et al.* 1998; Chavez *et al.* 2010; Negri *et al.* 2014). The aim of this study, therefore, was to investigate, using a multilocus sequence analysis, the diversity of species of *Aspergillus* section *Versicolores* in clinical samples in the USA and to determine their *in vitro* susceptibility to the currently available antifungal drugs.

Materials and methods

Fungal isolates

A total of 77 isolates of *Aspergillus* section *Versicolores* were investigated (Table 1), 69 from human origin, six from animal specimens and two from an environmental source. These isolates were received at the Fungus Testing Laboratory of the University of Texas Health Science Center (USA) from other centres in the country to identify them and/or to determine their antifungal susceptibility. Most of the isolates had been provisionally morphologically identified as *Aspergillus versicolor* ($n = 74$) and three as *Aspergillus* spp.

Morphological characterization

The fungal isolates were characterized morphologically following the criteria recommended by Samson *et al.* (2014). Briefly, the macromorphology of the colonies and the growth rates were determined on Czapek Yeast Autolysate Agar (CYA, Becton, Dickinson and Company®, Sparks, MD, USA) and Malt Extract Agar (MEA, Pronadisa®, Madrid, Spain) after 7 d of incubation at 25 °C and 37 °C. The microscopic structures were examined and measured on MEA cultures after 10–14 d of incubation at 25 °C, in wet mounts with 60 % lactic acid. Photographs were taken with a Zeiss Axio Imager M1 light microscope (Zeiss, Oberkochen, Germany) with a mounted DeltaPix Infinity X digital camera using Nomarski differential interference contrast and phase contrast optics.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from MEA cultures after 7 d of incubation at 25 °C, using the FastDNA® Kit and the FastPrep® Instrument (MP Biomedicals, Irvine, CA, USA), according to the manufacturer's specifications. Four genetic markers were amplified, i.e. the internal transcribed spacer (ITS) region of the rDNA, which comprises ITS1, the 5.8S gene, and ITS2, and fragments of β-tubulin (*BenA*), calmodulin (*CaM*), and RNA polymerase II second largest subunit (*RPB2*) genes (Peterson 2008; Samson *et al.* 2014). The primers used were ITS5 and ITS4 for the ITS region (White *et al.* 1990), Bt2a and Bt2b for the *BenA* gene (Glass & Donaldson 1995), Cmd5 and Cmd6 for *CaM* gene (Hong *et al.* 2005), and 5F and 7CR

Table 1 – GenBank accession numbers of the sequences of each of the *Aspergillus* strains included in this study.

Species (no. of isolates)	Isolate number	Origin	GenBank accession number			
			ITS	*BenA*	*CaM*	*RPB2*
A. amoenus (14)	UTHSC 05-2980	Animal	LN898664	LN898818	LN898741	LN898895
	UTHSC 06-1721	BAL	LN898665	LN898819	LN898742	LN898896
	UTHSC 07-1668	Sinus	LN898666	LN898820	LN898743	LN898897
	UTHSC 07-2785	Pleural fluid	LN898667	LN898821	LN898744	LN898898
	UTHSC 07-2881	Pleural fluid	LN898668	LN898822	LN898745	LN898899
	UTHSC 08-2366	–	LN898669	LN898823	LN898746	LN898900
	UTHSC 11-476	Sputum	LN898670	LN898824	LN898747	LN898901
	UTHSC 11-1419	BAL	LN898671	LN898825	LN898748	LN898902
	UTHSC 06-4284	BAL	LN898672	LN898826	LN898749	LN898903
	UTHSC 09-125	BAL	LN898673	LN898827	LN898750	LN898904
	UTHSC 12-340	Animal	LN898674	LN898828	LN898751	LN898905
	UTHSC 07-443	BAL	LN898675	LN898829	LN898752	LN898906
	UTHSC 07-3621	Chest	LN898676	LN898830	LN898753	LN898907
	UTHSC 09-2582	Lung biopsy	LN898677	LN898831	LN898754	LN898908
A. creber (17)	UTHSCDI 14-226	BAL	LN898678	LN898832	LN898755	LN898909
	UTHSCDI 14-228	Nail	LN898679	LN898833	LN898756	LN898910
	UTHSC 14-223	Arm	LN898680	LN898834	LN898757	LN898911
	UTHSC 03-2409	Environment	LN898681	LN898835	LN898758	LN898912
	UTHSC 05-2359	BAL	LN898682	LN898836	LN898759	LN898913
	UTHSC 09-1670	BAL	LN898683	LN898837	LN898760	LN898914
	UTHSC 09-3357	BAL	LN898684	LN898838	LN898761	LN898915
	UTHSC 14-188	BAL	LN898685	LN898839	LN898762	LN898916
	UTHSC 06-3435	BAL	LN898686	LN898840	LN898763	LN898917
	UTHSC 10-1327	Nail	LN898687	LN898841	LN898764	LN898918
	UTHSC 11-2813	Skin mucosa	LN898688	LN898842	LN898765	LN898919
	UTHSC 09-2679	BAL	LN898689	LN898843	LN898766	LN898920
	UTHSC 10-639	BAL	LN898690	LN898844	LN898767	LN898921
	UTHSC 04-799	Sputum	LN898691	LN898845	LN898768	LN898922
	UTHSC 07-2788	BAL	LN898692	LN898846	LN898769	LN898923
	UTHSC 04-434	Sputum	LN898693	LN898847	LN898770	LN898924
	UTHSC 10-582	BAL	LN898694	LN898848	LN898771	LN898925
A. cvjetkovicii	UTHSC 10-479	Environment	LN898695	LN898849	LN898772	LN898926
A. fructus	UTHSC 12-3194	Pericardium	LN898696	LN898850	LN898773	LN898927
A. jensenii (8)	UTHSCDI 14-220	Nail	LN898697	LN898851	LN898774	LN898928
	UTHSC 05-3600	Sputum	LN898698	LN898852	LN898775	LN898929
	UTHSC 09-2299	Sputum	LN898699	LN898853	LN898776	LN898930
	UTHSC 10-327	Sputum	LN898700	LN898854	LN898777	LN898931
	UTHSC 12-79	BAL	LN898701	LN898855	LN898778	LN898932
	UTHSC 07-3790	BAL	LN898702	LN898856	LN898779	LN898933
	UTHSC 10-71	BAL	LN898703	LN898857	LN898780	LN898934
	UTHSC 09-425	Nail	LN898704	LN898858	LN898781	LN898935
A. protuberus (10)	UTHSC 06-4104	BAL	LN898705	LN898859	LN898782	LN898936
	UTHSC 09-246	Animal	LN898706	LN898860	LN898783	LN898937
	UTHSC 11-269	BAL	LN898707	LN898861	LN898784	LN898938
	UTHSC 07-2433	BAL	LN898708	LN898862	LN898785	LN898939
	UTHSC 08-3392	BAL	LN898709	LN898863	LN898786	LN898940
	UTHSC 11-2175	Sputum	LN898710	LN898864	LN898787	LN898941
	UTHSC 12-338	Animal	LN898711	LN898865	LN898788	LN898942
	UTHSC 12-256	BAL	LN898712	LN898866	LN898789	LN898943
	UTHSC 06-2837	BAL	LN898713	LN898867	LN898790	LN898944
	UTHSC 08-1574	BAL	LN898714	LN898868	LN898791	LN898945
A. puulaauensis	UTHSC 11-1436	BAL	LN898715	LN898869	LN898792	LN898946
A. sydowii (20)	UTHSC 09-48	Blood	LN898716	LN898870	LN898793	LN898947
	UTHSC 11-204	Eye	LN898717	LN898871	LN898794	LN898948
	UTHSC 13-2518	Eye	LN898718	LN898872	LN898795	LN898949
	UTHSC 13-2630	Sinus	LN898719	LN898873	LN898796	LN898950
	UTHSC 06-2186	BAL	LN898720	LN898874	LN898797	LN898951
	UTHSC 06-2780	Bronchus	LN898721	LN898875	LN898798	LN898952
	UTHSC 06-4167	Sinus	LN898722	LN898876	LN898799	LN898953
	UTHSC 07-1018	Animal	LN898723	LN898877	LN898800	LN898954
	UTHSC 09-97	BAL	LN898724	LN898878	LN898801	LN898955
	UTHSC 12-934	BAL	LN898725	LN898879	LN898802	LN898956
	UTHSC 13-2674	BAL	LN898726	LN898880	LN898803	LN898957

Table 1 – (continued)

Species (no. of isolates)	Isolate number	Origin	GenBank accession number			
			ITS	*BenA*	*CaM*	*RPB2*
	UTHSC 10-1222	–	LN898727	LN898881	LN898804	LN898958
	UTHSC 10-3180	Sputum	LN898728	LN898882	LN898805	LN898959
	UTHSC 11-2683	Spine	LN898729	LN898883	LN898806	LN898960
	UTHSC 06-727	BAL	LN898730	LN898884	LN898807	LN898961
	UTHSC 08-3215	Animal	LN898731	LN898885	LN898808	LN898962
	UTHSC 09-1708	Lung biopsy	LN898732	LN898886	LN898809	LN898963
	UTHSC 12-3109	Lung biopsy	LN898733	LN898887	LN898810	LN898964
	UTHSC 08-865	Hip joint	LN898734	LN898888	LN898811	LN898965
	FMR 14440	Ear exudate	LN898735	LN898889	LN898812	LN898966
A. tabacinus (4)	UTHSC 03-1197	Sputum	LN898736	LN898890	LN898813	LN898967
	UTHSC 07-2427	BAL	LN898737	LN898891	LN898814	LN898968
	UTHSC 10-1677	Pleural fluid	LN898738	LN898892	LN898815	LN898969
	UTHSC 08-2898	BAL	LN898739	LN898893	LN898816	LN898970
A. versicolor	UTHSC 03-3679	BAL	LN898740	LN898894	LN898817	LN898971

BAL, bronchoalveolar lavage fluid specimen; FMR, Facultat de Medicina, Universitat Rovira i Virgili, Reus, Spain; UTHSC, Fungus Testing Laboratory at the University of Texas Health Science Center, San Antonio, USA.

for *RPB2* gene (Liu *et al.* 1999). PCR products were sequenced in both directions, using the same primers, at Macrogen Europe (Macrogen Inc., Amsterdam, the Netherlands). Sequences were assembled and edited using Sequencher v.4.1.4 (Gene Codes Corporation©, Ann Arbor, MI, USA).

Molecular identification and phylogenetic analysis

The phylogenetic analyses were carried out first individually for each gene and after the topologies proved to be congruent, a concatenated study was then carried out. To give support to our analyses, sequences of the type strains of 19 species of the section *Versicolores* and of *Aspergillus multicolor* (outgroup) were obtained from GenBank and added to the analyses. For multiple sequence alignment, the ClustalW tool was used together with the MUSCLE tool inside MEGA v.6 software (Tamura *et al.* 2013), with manual adjustments for refinement. The Maximum Likelihood (ML) phylogenetic method was also run with MEGA v.6 software, as well as the estimation of the best nucleotide substitution method. Support of the internal branches was assessed by the Bootstrap method with 1000 replications, where values ≥70 were considered significant. The Bayesian Inference (BI) method was performed using MrBayes v.3.1.2 software (Ronquist & Huelsenbeck 2003). The evolutionary models that best fit each gene were assessed by MrModelTest software (Nylander 2004). Markov chain Monte Carlo (MCMC) sampling was performed with two simultaneous runs for one million generations, with samples taken every 100 generations. The 50 % majority rule consensus trees and posterior probability values (pp) were calculated after removing the first 25 % of the resulting trees for burn-in. A pp value of ≥0.95 was considered in the tree.

The type strain of *Aspergillus griseoaurantiacus* was not included in the final tree because the sequence for the *RPB2* gene was not available, although sequence comparison for the other three loci was done.

Antifungal susceptibility testing

A total of 73 isolates of the most frequent *Aspergillus* species identified here were tested against nine antifungal drugs following the microdilution broth method, according to the document M38-A2 (Clinical and Laboratory Standards Institute 2008). The antifungal agents, obtained as pure power, were amphotericin B (AMB) (Sigma Aldrich Química S.A., Madrid, Spain), itraconazole (ITC) (Jansen Pharmaceuticals, Beerse, Belgium), posaconazole (PSC) (Schering-Plough Res., Inst., NJ, EUA), voriconazole (VRC) (Pfizer S.A., Madrid, Spain), anidulafungin (AFG) (Pfizer S.A., Madrid, Spain), caspofungin (CFG) (Merk & Co., Inc., Rahway, EUA), micafungin (MFG) (Astellas Pharma, Madrid, Spain), terbinafine (TBF), and flucytosine (5FC) (Sigma Aldrich Química S.A., Madrid, Spain). The minimal inhibitory concentration (MIC) was defined as the lowest drug concentration that produced 100 % inhibition of visible fungal growth for the AMB and the azoles (ITC, PSC, and VRC) or 50 % and 80 % for 5FC and TBF, respectively. The minimum effective concentration (MEC) was determined for the echinocandins (AFG, CFG, and MFG) and was defined microscopically as the lowest concentration of drug that would lead to the growth of small, rounded, compact hyphal forms as compared with the long, unbranched hyphal clusters that were seen in the growth control following 48 h of incubation. The incubation temperature was set to 30 °C given the growth requirements of the most species of *Versicolores* (Jurjevic *et al.* 2012; Visagie *et al.* 2014). *Aspergillus flavus* (ATCC® 204304) and *Aspergillus fumigatus* (ATCC® MYA-3626) strains were used as quality controls. All tests were carried out in duplicate. Results were statistically analysed using the Prism software for Windows v.6.0 (GraphPad Software, San Diego, CA).

Nucleotide sequence accession numbers

Sequences newly generated in this study were deposited in GenBank under accession numbers LN898664–LN898740

(ITS), LN898818–LN898894 (*BenA*), LN898741–LN898817 (*CaM*), and LN898895–LN898971 (*RPB2*) (Table 1).

Results

The single gene phylogenetic analyses proved that ITS, *BenA*, *CaM*, and *RPB2* were consistent for a concatenated study (see Supplementary material). Therefore, a phylogenetic analysis combining the four mentioned markers was done for species recognition. The concatenated sequence alignment consisted of 2392 base pairs (ITS, 508 bp; *BenA*, 413 bp; *CaM*, 520 bp; *RPB2*, 951 bp), from which 486 were parsimony informative sites (ITS, 44; *BenA*, 76; *CaM*, 154; *RPB2*, 212). With only minor differences observed in the value of the supports of the internal nodes, the topologies of the trees obtained with ML and BI analyses were virtually the same. Based on that, our results showed that the 77 isolates included in the study clustered unambiguously with the type strains of ten of the 20 species of the section *Versicolores* (Fig 1). The majority of the strains nested to the *Aspergillus sydowii* (26 %) clade, followed by *Aspergillus creber* (22 %), *Aspergillus amoenus* (18.2 %), *Aspergillus protuberus* (13 %), *Aspergillus jensenii* (10.4 %), *Aspergillus tabacinus* (5.2 %), *Aspergillus cvjetkovicii* (1.3 %), *Aspergillus fructus* (1.3 %), *Aspergillus puulaauensis* (1.3 %), and *Aspergillus versicolor* (1.3 %).

The six isolates from animal specimens were identified as *A. amoenus*, *A. protuberus*, and *A. sydowii*, with two isolates per species. The two environmental isolates belonged to *A. creber* and *A. cvjetkovicii*.

All isolates showed the typical morphological characteristics described for the *Versicolores* section. As expected, morphological identification at the species level was difficult to carry out due to the similarity of the features observed among the different species of this section. Macro- and micromorphological features of the most frequent identified species are depicted in Fig 2.

The majority of human clinical isolates included in the study were from bronchoalveolar lavage fluid (44.2 %), followed by sputum (11.7 %), nail (5.2 %), sinus (3.9 %), lung biopsy (3.9 %), pleural fluid (3.9 %), and eye (2.6 %).

Table 2 shows the antifungal susceptibility results of the isolates tested. In general, all the drugs tested, with the exception of 5FC and AMB in some cases, demonstrated potent activity. The drugs that exhibited the best results were the echinocandins and TBF, with MIC values ranging from 0.03 to 0.125 $\mu g\ ml^{-1}$. The azoles tested also showed potent activity, with MICs ranging from 0.6 to 4.0 $\mu g\ ml^{-1}$, but with geometric means (GM) closer to the lowest MIC value (ITC, 0.283 $\mu g\ ml^{-1}$; PSC, 0.343 $\mu g\ ml^{-1}$; VRC, 0.88 $\mu g\ ml^{-1}$). The highest MICs were those of 5FC, ranging from 1.0 to greater than 16.0 $\mu g\ ml^{-1}$, especially against *A. amoenus*, *A. creber*, and *A. protuberus*, with GM MICs higher than 11.0 $\mu g\ ml^{-1}$. For AMB, more variable results were observed with MIC values ranging from 0.5 to 16.0 $\mu g\ ml^{-1}$. For this drug, the lowest GM MIC values was observed against *A. jensenii* (0.6 $\mu g\ ml^{-1}$), and the highest was against *A. sydowii* (4.7 $\mu g\ ml^{-1}$).

Discussion

Clinical interest in the species of *Aspergillus*, and particularly of those of the section *Versicolores*, is increasing because of the reported number of infections that are affecting not only humans but other mammals too (Arabatzis *et al.* 2011; Zhang *et al.* 2012; Huh *et al.* 2013; Kane *et al.* 2014; Negri *et al.* 2014; Borsa *et al.* 2015; Gomes *et al.* 2015; Heo *et al.* 2015). However, all those reports include a single isolate, or just a few, and, to date, no study has been conducted on a significant number of isolates. Thus, the diversity and the relative frequency of the species of *Versicolores* in the clinical setting are practically unknown. Here, using the molecular criteria proposed by Samson *et al.* (2014), we found that, among the isolates belonging to that section that were received by a reference center in the USA, the most frequent species was *Aspergillus sydowii*, followed by *Aspergillus creber*, *Aspergillus amoenus*, *Aspergillus protuberus*, and *Aspergillus jensenii*. Interestingly, this latter species together with *Aspergillus puulaauensis*, two species recently proposed by Jurjevic *et al.* (2012), have never been identified from clinical samples before. These results show a relative frequency and high diversity of the members of this section in this particular habitat. Although the high number of isolates recovered seems to suggest that these fungi might be opportunistic pathogens, further studies are needed to elucidate this because they might merely be contaminants or colonizers. Although *Aspergillus versicolor* has always been considered to be of some clinical relevance, its pathogenic importance might be overestimated.

The poor knowledge of the distribution and the habitat of the species of the *Versicolores* section are due to the difficulties in their morphological identification. According to Jurjevic *et al.* (2012), some phenotypic characteristics, such as conidial ornamentation, presence of soluble pigments, and the ability to grow at 37 °C can be useful for differentiating some of these species. Although *A. amoenus* and *Aspergillus tabacinus* have been described with smooth conidia (Jurjevic *et al.* 2012), all the isolates in the present study identified molecularly as belonging to those species have finely roughened to rough conidia (Fig 2). Only the stipe ornamentation of the conidiophores in *A. protuberus*, or growth at 37 °C in *A. amoenus*, *Aspergillus fructus*, *Aspergillus griseoaurantiacus*, *A. sydowii*, and *A. versicolor* were useful for differentiating them from the rest. Our study, therefore, seems to confirm that reliable identification of these fungi is dependent on the use of molecular methods. However, in this sense, it is worth of mentioning that the analysis of ITS barcode, which is very useful for many other fungi, does not provide enough resolution for species recognition on this group of aspergilli (Jurjevic *et al.* 2012; Samson *et al.* 2014; Visagie *et al.* 2014). Jurjevic *et al.* (2012) proposed a multilocus phylogenetic scheme to infer the phylogenetic relationship and identification of the members of the section *Versicolores*, which was based on the analysis of the markers *CaM*, *RPB2*, DNA replication licensing factor, and prerRNA processing protein. Samson *et al.* (2014) have since advocated the use of four different markers (ITS, *BenA*, *CaM*, and *RPB2*) for *Aspergillus* identification in general. The combined use of these latter

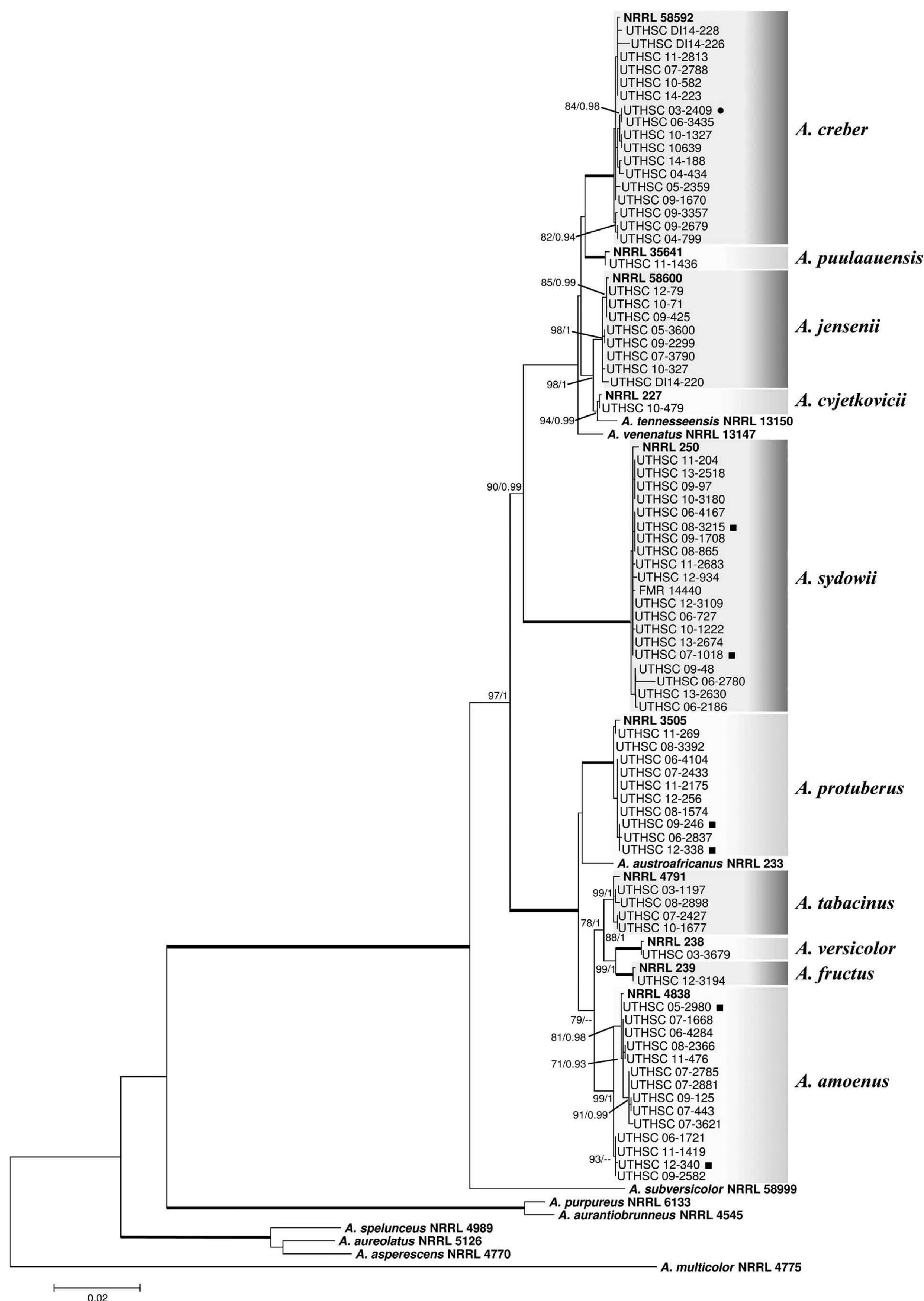


Fig 1 – ML tree obtained from the combined ITS, *BenA*, *CaM*, and *RPB2* sequences of the isolates. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian pp scores over 70/0.95 are indicated on the nodes. The fully supported branches (100/1) and type strains are shown in bold. ■ indicates strain of animal origin, ● indicates the environmental strain. UTHSC, University of Texas Health Science Center (USA); FMR, Facultat de Medicina de Reus (Spain).

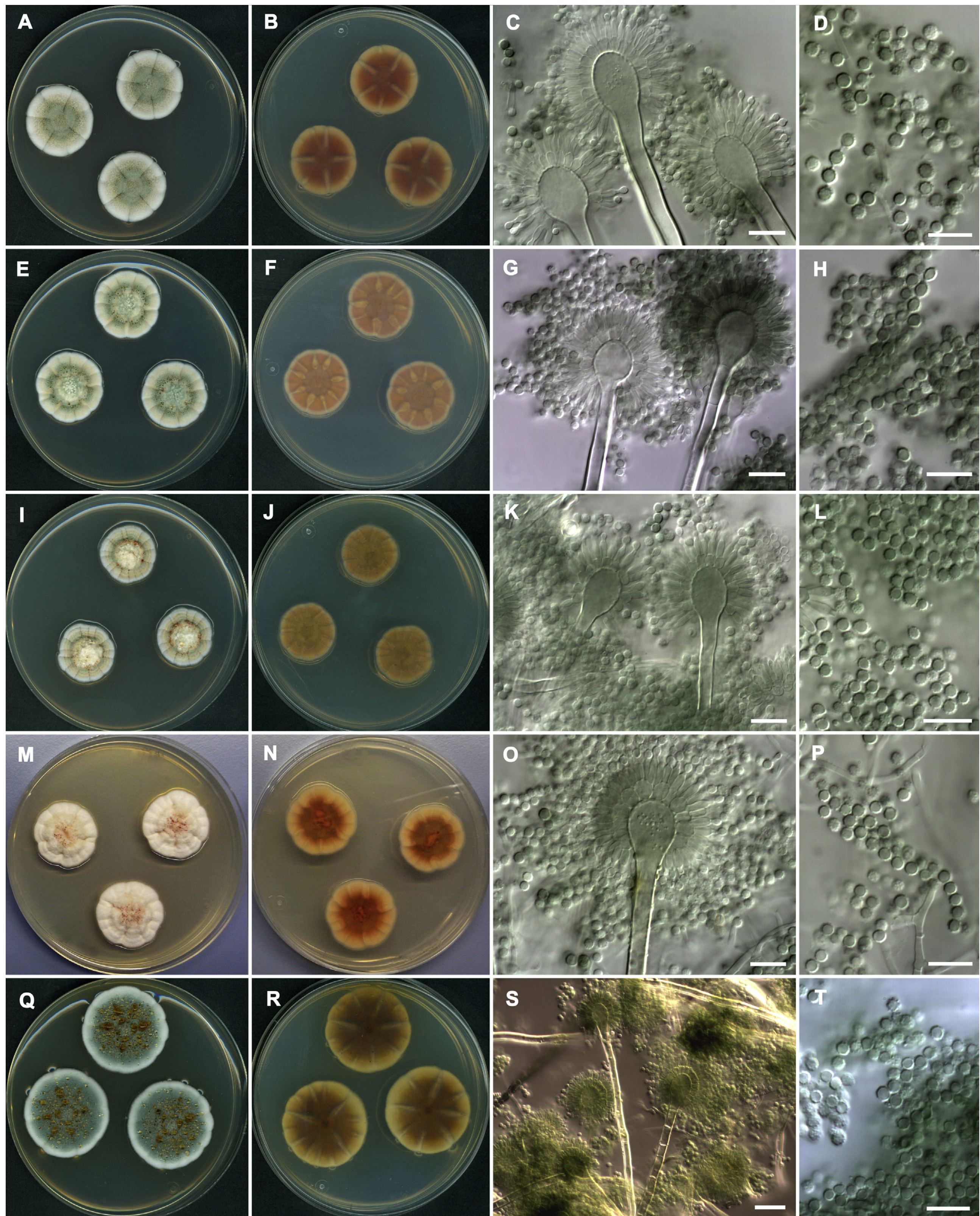


Fig 2 – Morphological features of *A. amoenus* (A–D), *A. creber* (E–H), *A. jensenii* (I–L), *A. protuberus* (M–P), and *A. sydowii* (Q–T). Colonies on CYA at 25 °C after 7 d, front (A, E, I, M, Q), and reverse (B, F, J, N, R). Conidiophores (C, G, K, O, S) and conidia (D, H, L, P, T). Scale bars: C, D, G, H, K, L, O, P, T = 10 μm; S = 20 μm.

Table 2 – Results of *in vitro* antifungal susceptibility test for 73 isolates of *Aspergillus* section *Versicolores*.

Species (no. of isolates)	Parameter	MIC or MEC (μg ml^{-1}) for:								
		5FC	AFG	AMB	CFG	ITC	MFG	PSC	TBF	VRC
A. sydowii (20)	GM	6.616	0.03	4.757	0.03	0.334	0.03	0.595	0.0318	1.498
	MIC range	1.0–16	0.03	1.0–16.0	0.03	0.125–2.0	0.03	0.125–2.0	0.03–0.125	1.0–4.0
	MIC_{90}	8.0	0.03	8.0	0.03	0.5	0.03	2.0	0.125	4.0
A. creber (17)	GM	11.81	0.03	2.378	0.03	0.31	0.03	0.354	0.033	1.091
	MIC range	1.0–>16	0.03	1.0–8.0	0.03	0.125–1.0	0.03	0.125–0.5	0.03–0.125	0.5–2.0
	MIC_{90}	>16.0	0.03	8.0	0.03	0.5	0.03	0.5	0.03	2.0
A. amoenus (14)	GM	16.81	0.03	1.903	0.03	0.086	0.03	0.13	0.03	0.25
	MIC range	8.0–>16	0.03	1.0–4.0	0.03	0.06–0.125	0.03	0.06–0.25	0.03–0.03	0.125–0.5
	MIC_{90}	>16.0	0.03	4.0	0.03	0.125	0.03	0.25	0.03	0.5
A. protuberus (10)	GM	12.13	0.03	0.707	0.03	1.072	0.03	0.466	0.03	1.149
	MIC range	2.0–>16.0	0.03	0.5–1.0	0.03	0.5–4.0	0.03	0.25–0.5	0.03–0.03	1.0–2.0
	MIC_{90}	>16.0	0.03	1.0	0.03	2.0	0.03	0.5	0.03	2.0
A. jensenii (8)	GM	4.416	0.03	0.609	0.03	0.112	0.03	0.136	0.03	0.609
	MIC range	1.0–>16.0	0.03	0.5–1.0	0.03–0.06	0.06–0.25	0.03	0.06–0.25	0.03–0.03	0.25–1.0
A. tabacinus (4)	GM	4.595	0.03	2.297	0.03	0.6	0.03	0.66	0.03	1.149
	MIC range	2.0–8.0	0.03	2.0–4.0	0.03	0.25–1.0	0.03	0.5–1.0	0.03–0.03	1.0–2.0
Total (73)	GM	8.844	0.03	2.132	0.03	0.283	0.03	0.343	0.031	0.88
	MIC range	1.0–>16.0	0.03	0.5–16.0	0.03–0.06	0.06–4.0	0.03	0.06–2.0	0.03–0.125	0.06–2.0
	MIC_{90}	>16.0	0.03	8.0	0.03	1.0	0.03	1.0	0.03	2.0

four genetic markers has allowed the successful identification of all the isolates investigated here.

The prevalence of *A. sydowii* in clinical samples demonstrated here has been reported previously in Czech isolates by Hubka *et al.* (2012). In that study, *A. sydowii* was the second most common species after *Aspergillus fumigatus*, with 17 of the 178 isolates (9.6 %), and was involved mainly in superficial infections, affecting nails and skin, but also in ear and respiratory infections. Other studies have also reported this species to be an opportunistic pathogen (de Hoog *et al.* 2011; Sabino *et al.* 2014; Nouripour-Sisakht *et al.* 2015). In our case, *A. sydowii* was identified from very different human specimens, including superficial and deep tissues (Table 1). Although we are not able to demonstrate the pathogenic role of the isolates investigated, the high number of strains reinforces the importance of *A. sydowii* in the clinical setting.

Aspergillus creber, *A. amoenus*, and *A. protuberus* represented here by 17, 14, and ten of the isolates, respectively, have been recently reported as causal agents of infections in Brazil (*A. creber*, Negri *et al.* 2014) and in Turkey (*A. protuberus*, Borsa *et al.* 2015), while *A. amoenus*, a species previously identified as *A. versicolor*, was isolated from mammary gland in the USA (Jurjevic *et al.* 2012). Other species identified in our study, although with a lower frequency, were *A. tabacinus* with four isolates and *A. fructus* with one isolate. The former was previously isolated in Brazil from respiratory secretions (Negri *et al.* 2014) and the latter in Portugal from a patient suspected to have allergic bronchopulmonary aspergillosis (Sabino *et al.* 2014).

The species *Aspergillus cvjetkovicii*, *A. jensenii*, and *A. puulaauensis*, closely related to *A. creber* and *A. sydowii*, constituted together a well-supported clade that represent the 61 % (47 of 77) of all the isolates identified. Due to the similarity among the species of this clade, some of them might have been misidentified in previous studies as *A. sydowii*, which may have hampered the significance of the other species.

The data available on the antifungal susceptibility of these fungi are very scarce and usually limited to occasional reports and with no confirmation of the correct identification of the species involved. In our study, the echinocandins and TBF showed the lowest MICs, and 5FC and AMB were the least potent. However, the data provided here are to some extent similar to those previously reported (Cuenca-Estrella & Rodriguez-Tudela 2010; Arabatzis *et al.* 2011; Buzina 2013). For instance, in the case of AMB, the MICs of our isolates were similar to those of the study of Heo *et al.* (2015), in which six strains of this section were studied and the range observed was from 1.0 to 2.0 μg ml^{-1}. Against *A. sydowii*, we observed lower potency for AMB than in previous reports, in which the isolates may have been misidentified (García-Martos *et al.* 2005; Buzina 2013; Heo *et al.* 2015). With respect to the azoles, the results were more variable, depending on the species and drugs tested; the less active being VRC against *A. sydowii*. In general, potent activity of these drugs has been reported (Pfaller *et al.* 2002; Arabatzis *et al.* 2011; Buzina 2013). However, triazole resistance and elevated MIC values have also been reported for *A. versicolor* previously (Torres-Rodríguez *et al.* 1998; Baddley *et al.* 2009; Espinel-Ingroff *et al.* 2010).

In conclusion, the clinical relevance of the species of *Aspergillus* section *Versicolores* should not be overlooked, and it seems highly likely that apart from *A. sydowii* other species of the section can also be responsible of human infections. Further studies are needed, at least in animal models, to prove the pathogenic role of these species and to evaluate the most appropriate therapies.

Acknowledgements

This study was supported by the Spanish Ministerio de Economía y Competitividad, grant CGL2013-43789-P, and by

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil, grant BEX 0623/14-8.

Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2016.02.006.

REFERENCES

Arabatzis M, Kambouris M, Kyprianou M, Chrysaki A, Foustoukou M, Kanellopoulou M, Kondyli L, Kouppari G, Koutsia-Karouzou C, Lebessi E, Pangalis A, Petinaki E, Stathi A, Trikka-Graphakos E, Vartzioti E, Vogiatzi A, Vyzantiadis TA, Zerva L, Velegraki A, 2011. Polyphasic identification and susceptibility to seven antifungals of 102 *Aspergillus* isolates recovered from immunocompromised hosts in Greece. *Antimicrobial Agents and Chemotherapy* **55**: 3025–3030.

Baddley JW, Marr KA, Andes DR, Walsh TJ, Kauffman CA, Kontoyiannis DP, Ito JI, Balajee SA, Pappas PG, Moser SA, 2009. Patterns of susceptibility of *Aspergillus* isolates recovered from patients enrolled in the transplant-associated infection surveillance network. *Journal of Clinical Microbiology* **47**: 3271–3275.

Batista LR, Chalfoun SM, Prado G, Schwan RF, Wheals AE, 2003. Toxigenic fungi associated with processed (green) coffee beans (*Coffea arabica* L.). *International Journal of Food Microbiology* **85**: 293–300.

Borsa BA, Ozgun G, Houbraken J, Okmen F, 2015. The first case of persistent vaginitis due to *Aspergillus protuberus* in an immunocompetent patient. *Mikrobiyoloji Bulteni* **49**: 130–134.

Buzina W, 2013. *Aspergillus*: classification and antifungal susceptibilities. *Current Pharmaceutical Design* **19**: 3615–3628.

Charles MVP, Joseph NM, Easow JM, Ravishankar M, 2011. Invasive pulmonary aspergillosis caused by *Aspergillus versicolor* in a patient on mechanical ventilation. *The Australasian Medical Journal* **4**: 632–634.

Chavez J, Rivas P, Cortés J, Cuervo S, Sánchez R, Parra C, 2010. Sensibilidad *in vitro* de hongos miceliales de aislamientos clínicos en pacientes con cáncer en el Instituto Nacional de Cancerología ESE. *Infectio* **14** (Suppl. 2): 116–126.

Clinical and Laboratory Standards Institute, 2008. *Reference Method for Broth Dilution Antifungals Susceptibility Testing of Conidium-forming Filamentous Fungi: approved standard*, 2nd edn. CLSI, Wayne, PA, M38-A2.

Cuenca-Estrella M, Rodriguez-Tudela JL, 2010. Terbinafine. In: Pasqualotto AC (ed.), *Aspergillosis: from diagnosis to prevention*. Springer, Netherlands, pp. 317–323.

de Hoog GS, Guarro J, Gené J, Figueras M, 2011. *Atlas of Clinical Fungi* CD-ROM version 3.1. CBS-KNAW Fungal Biodiversity Centre, Utrecht.

Dou Y, Wang X, Jiang D, Wang H, Jiao Y, Lou H, 2014. Metabolites from *Aspergillus versicolor*, an endolichenic fungus from the lichen *Lobaria retigera*. *Drug Discoveries & Therapeutics* **8**: 84–88.

Espinel-Ingroff A, Diekema DJ, Fothergill A, Johnson E, Pelaez T, Pfaller MA, Rinaldi MG, Canton E, Turnidge J, 2010. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *Journal of Clinical Microbiology* **48**: 3251–3257.

Gams W, Christensen M, Onions AH, 1985. Infrageneric taxa of *Aspergillus*. In: Samson RA, Pitt JI (eds), *Advances in* Penicillium *and* Aspergillus *Systematics*. Plenum Press, New York, pp. 55–62.

García-Martos P, García-Agudo L, Gutiérrez-Calzada J, Ruiz-Aragon J, Saldarreaga A, Marin P, 2005. *In vitro* activity of amphotericin B, itraconazole and voriconazole against 20 species of *Aspergillus* using the Sensititre microdilution method. *Enfermedades Infecciosas y Microbiologia Clinica* **23**: 15–18.

Glass NL, Donaldson GC, 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**: 1323–1330.

Gomes CC, Pinto LC, Victor FL, da Silva EA, Ribeiro AA, Sarquis MI, Camoes IC, 2015. *Aspergillus* in endodontic infection near the maxillary sinus. *Brazilian Journal of Otorhinolaryngology* **8**: 527–532.

Heo MS, Shin JH, Choi MJ, Park YJ, Lee HS, Koo SH, Lee WG, Kim SH, Shin MG, Suh SP, Ryang DW, 2015. Molecular identification and amphotericin B susceptibility testing of clinical isolates of *Aspergillus* from 11 hospitals in Korea. *Annals of Laboratory Medicine* **35**: 602–610.

Hong S-B, Go S-J, Shin H-D, Frisvad JC, Samson RA, 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia* **97**: 1316–1329.

Houbraken J, Samson RA, 2011. Phylogeny of *Penicillium* and the segregation of Trichocomaceae into three families. *Studies in Mycology* **70**: 1–51.

Houbraken J, de Vries RP, Samson RA, 2014. Modern taxonomy of biotechnologically important *Aspergillus* and *Penicillium* species. *Advances in Applied Microbiology* **86**: 199–249.

Hubka V, Kubatova A, Mallatova N, Sedlacek P, Melichar J, Skorepova M, Mencl K, Lyskova P, Sramkova B, Chudickova M, Hamal P, Kolarik M, 2012. Rare and new etiological agents revealed among 178 clinical *Aspergillus* strains obtained from Czech patients and characterized by molecular sequencing. *Medical Mycology* **50**: 601–610.

Hubka V, Novakova A, Kolarik M, Jurjevic Z, Peterson SW, 2015. Revision of *Aspergillus* section *Flavipedes*: seven new species and proposal of section *Jani* sect. nov. *Mycologia* **107**: 169–208.

Huh HJ, Lee JH, Park KS, Jun T-G, Kang IS, Kim Y-J, Ki C-S, Lee NY, 2013. A case of misidentification of *Aspergillus versicolor* complex as *Scopulariopsis* species isolated from a homograft. *Annals of Clinical Microbiology* **16**: 105–109.

Jurjevic Z, Peterson SW, Horn BW, 2012. *Aspergillus* section *Versicolores*: nine new species and multilocus DNA sequence based phylogeny. *IMA Fungus* **3**: 59–79.

Jurjevic Z, Peterson SW, Solfrizzo M, Peraica M, 2013. Sterigmatocystin production by nine newly described *Aspergillus* species in section *Versicolores* grown on two different media. *Mycotoxin Research* **29**: 141–145.

Kane S, Pinto JM, Dadzie CK, Dawis MA, 2014. Aspergilloma caused by *Aspergillus versicolor*. *Pediatric Infectious Disease Journal* **33**: 891.

Klich MA, 1993. Morphological studies of *Aspergillus* section *Versicolores* and related species. *Mycologia* **85**: 100–107.

Lee SK, Kang H-G, Na K-J, Han J-I, 2012. Fungal dermatitis caused by *Aspergillus sydowii* in a thoroughbred horse. *Journal of Equine Veterinary Science* **32**: 835–839.

Li XB, Zhou YH, Zhu RX, Chang WQ, Yuan HQ, Gao W, Zhang LL, Zhao ZT, Lou HX, 2015. Identification and biological evaluation of secondary metabolites from the endolichenic fungus *Aspergillus versicolor*. *Chemistry & Biodiversity* **12**: 575–592.

Liu YJ, Whelen S, Hall BD, 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. *Molecular Biology and Evolution* **16**: 1799–1808.

Ludwig A, Gatineau S, Reynaud MC, Cadoré JL, Bourdoiseau G, 2005. Fungal isolation and identification in 21 cases of guttural pouch mycosis in horses (1998–2002). *The Veterinary Journal* **169**: 457–461.

Negri CE, Goncalves SS, Xafranski H, Bergamasco MD, Aquino VR, Castro PT, Colombo AL, 2014. Cryptic and rare *Aspergillus*

species in Brazil: prevalence in clinical samples and *in vitro* susceptibility to triazoles. *Journal of Clinical Microbiology* **52**: 3633–3640.

Nouripour-Sisakht S, Mirhendi H, Shidfar MR, Ahmadi B, Rezaei-Matehkolaei A, Geramishoar M, Zarei F, Jalalizand N, 2015. *Aspergillus* species as emerging causative agents of onychomycosis. *Journal de Mycologie Médicale/Journal of Medical Mycology* **25**: 101–107.

Nylander JAA, 2004. *MrModeltest v2. Program Distributed by the Author*. Evolutionary Biology Centre, Uppsala University.

Perri P, Campa C, Incorvaia C, Parmeggiani F, Lamberti G, Costagliola C, Sebastiani A, 2005. Endogenous *Aspergillus versicolor* endophthalmitis in an immunocompetent HIV-positive patient. *Mycopathologia* **160**: 259–261.

Peterson SW, 2008. Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. *Mycologia* **100**: 205–226.

Pfaller MA, Messer SA, Hollis RJ, Jones RN, SENTRY Participants Group, 2002. Antifungal activities of posaconazole, ravuconazole, and voriconazole compared to those of itraconazole and amphotericin B against 239 clinical isolates of *Aspergillus* spp. and other filamentous fungi: report from SENTRY antimicrobial surveillance program, 2000. *Antimicrobial Agents and Chemotherapy* **46**: 1032–1037.

Raper KB, Fennel DI, 1976. *The Genus* Aspergillus, 1st edn. Robert E. Krieger Publishing Company, New York.

Ronquist F, Huelsenbeck JP, 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Rotoli M, Sascaro G, Cavalieri S, 2001. *Aspergillus versicolor* infection of the external auditory canal successfully treated with terbinafine. *Dermatology* **202**: 143.

Sabino R, Verissimo C, Parada H, Brandao J, Viegas C, Carolino E, Clemons KV, Stevens DA, 2014. Molecular screening of 246 Portuguese *Aspergillus* isolates among different clinical and environmental sources. *Medical Mycology* **52**: 519–529.

Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CH, Perrone G, Seifert KA, Susca A, Tanney JB, Varga J, Kocsube S, Szigeti G, Yaguchi T, Frisvad JC, 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Studies in Mycology* **78**: 141–173.

Schmitt EK, Eilinghoff B, Olliger R, Decker H, Kuck U, 2002. Development of molecular tools for the mulundocandin producer *Aspergillus sydowii*: DNA-mediated transformation and reporter gene expression. *Applied Microbiology and Biotechnology* **58**: 625–631.

Sharpe RA, Bearman N, Thornton CR, Husk K, Osborne NJ, 2015. Indoor fungal diversity and asthma: a meta-analysis and systematic review of risk factors. *Journal of Allergy and Clinical Immunology* **135**: 110–122.

Takahata Y, Hiruma M, Sugita T, Muto M, 2008. A case of onychomycosis due to *Aspergillus sydowii* diagnosed using DNA sequence analysis. *Mycoses* **51**: 170–173.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S, 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725–2729.

Torres-Rodríguez JM, Madrenys-Brunet N, Siddat M, López-Jodra O, Jiménez T, 1998. *Aspergillus versicolor* as cause of onychomycosis: report of 12 cases and susceptibility testing to antifungal drugs. *Journal of the European Academy of Dermatology and Venereology* **11**: 25–31.

Visagie CM, Hirooka Y, Tanney JB, Whitfield E, Mwange K, Meijer M, Amend AS, Seifert KA, Samson RA, 2014. *Aspergillus*, *Penicillium* and *Talaromyces* isolated from house dust samples collected around the world. *Studies in Mycology* **78**: 63–139.

White T, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Shinsky J, White T (eds), *PCR Protocols: a guide to methods and applications*. Academic Press, San Diego, pp. 315–322.

Zahradnik E, Kespohl S, Sander I, Schies U, Khosravie-Hohn J, Lorenz W, Engelhart S, Kolk A, Schneider G, Bruning T, Raulf-Heimsoth M, 2013. A new immunoassay to quantify fungal antigens from the indoor mould *Aspergillus versicolor*. *Environmental Science: Processes & Impacts* **15**: 1162–1171.

Zhang S, Corapi W, Quist E, Griffin S, Zhang M, 2012. *Aspergillus versicolor*, a new causative agent of canine disseminated aspergillosis. *Journal of Clinical Microbiology* **50**: 187–191.

4.2. Multilocus phylogeny and antifungal susceptibility of *Aspergillus* section *Circumdati* from clinical samples and description of *A. pseudosclerotiorum* sp. nov.

João Paulo Zen Siqueira, Deanna A. Sutton, Josepa Gené, Dania García, Nathan Wiederhold, Stephen W. Peterson, Josep Guarro

Published in: Journal of Clinical Microbiology
March 2017, Volume 55, Issue 3, Pages 947–958

AMERICAN SOCIETY FOR MICROBIOLOGY Journal of Clinical Microbiology®

MYCOLOGY

Multilocus Phylogeny and Antifungal Susceptibility of *Aspergillus* Section *Circumdati* from Clinical Samples and Description of *A. pseudosclerotiorum* sp. nov.

J. P. Z. Siqueira,[a,b] D. A. Sutton,[c] J. Gené,[a] D. García,[a] N. Wiederhold,[c] S. W. Peterson,[d] J. Guarro[a]

Unitat de Micologia, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Reus, Spain[a]; Laboratório de Microbiologia, Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, Brazil[b]; Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, Texas, USA[c]; Mycotoxin Prevention and Applied Microbiology Research Unit, National Center for Agricultural Utilization Research, Peoria, Illinois, USA[d]

ABSTRACT A multilocus phylogenetic study was carried out to assess species identity of a set of 34 clinical isolates from *Aspergillus* section *Circumdati* from the United States and to determine their *in vitro* antifungal susceptibility against eight antifungal drugs. The genetic markers used were the internal transcribed spacer (ITS) region, and fragments of the beta-tubulin (*BenA*), calmodulin (*CaM*), and RNA polymerase II second largest subunit (*RPB2*) genes. The drugs tested were amphotericin B, itraconazole, posaconazole, voriconazole, anidulafungin, caspofungin, micafungin, and terbinafine. The most common species sampled was *A. westerdijkiae* (29.4%), followed by a novel species, which was described here as *A. pseudosclerotiorum* (23.5%). Other species identified were *A. sclerotiorum* (17.6%), *A. ochraceus* (8.8%), *A. subramanianii* (8.8%), and *A. insulicola* and *A. ochraceopetaliformis*, with two isolates (5.9%) of each. The drugs that showed the most potent activity were caspofungin, micafungin, and terbinafine, while amphotericin B showed the least activity.

KEYWORDS *Aspergillus*, *Circumdati* section, clinical isolates, molecular identification, phenotypic identification

Received 30 September 2016 **Returned for modification** 22 November 2016 **Accepted** 30 December 2016

Accepted manuscript posted online 4 January 2017

Citation Siqueira JPZ, Sutton DA, Gené J, García D, Wiederhold N, Peterson SW, Guarro J. 2017. Multilocus phylogeny and antifungal susceptibility of *Aspergillus* section *Circumdati* from clinical samples and description of *A. pseudosclerotiorum* sp. nov. J Clin Microbiol 55:947–958. https://doi.org/10.1128/JCM.02012-16.

Editor David W. Warnock, University of Manchester

Copyright © 2017 American Society for Microbiology. All Rights Reserved.

Address correspondence to J. Gené, josepa.gene@urv.cat.

Section *Circumdati* includes aspergilli with biseriate conidial heads in shades of yellow to ochre, with mostly globose vesicles, and sclerotia variable in shape and color (1–3). It contains 26 species (3), with *A. ochraceus* being the best known and described as an important producer of many extrolites, including the mycotoxin ochratoxin A (3–5). This metabolite has nephrotoxic, immunosuppressive, teratogenic, and carcinogenic properties (6, 7) and is commonly found in coffee, rice, beverages, and other contaminated foodstuffs (3, 8). Several species in this section have been involved in different types of infections, such as onychomycosis, caused by *A. insulicola*, *A. melleus*, *A. ochraceopetaliformis*, *A. persii*, *A. sclerotiorum*, and *A. westerdijkiae* (9–14); otomycosis, caused by *A. sclerotiorum* (15); skin infection, caused by *A. westerdijkiae* (12); and pulmonary aspergillosis and osteomyelitis, caused by *A. ochraceus* (16, 17). Moreover, *A. ochraceus*, *A. sclerotiorum*, and *A. westerdijkiae* have been repetitively isolated from clinical specimens of immunocompromised patients, although in such cases their pathogenic role is uncertain (18–22).

There are few data on the *in vitro* antifungal susceptibility of species within section *Circumdati*. The azoles, especially itraconazole, appear to have good activity against *A.*

ochraceus and *A. sclerotiorum* (18, 23). In contrast, amphotericin B shows limited activity against species in this section (18, 23, 24), particularly against *A. westerdijkiae* (25).

Identification of *Aspergillus* species, traditionally based on morphological and physiological aspects (2), has changed recently with the use of DNA sequencing and multilocus analyses (26). Therefore, to assess the diversity of clinically relevant species within this section, a set of isolates with features characteristic of *Circumdati* section were identified molecularly. These clinical isolates were recovered between 2003 and 2015 in a U.S. reference laboratory. Moreover, the antifungal susceptibility of the most frequent species was determined against eight antifungal drugs.

RESULTS

Single-gene analyses of sequences revealed similar topologies for all of them, especially for the terminal branches. The internal transcribed spacer (ITS) marker was the least informative, being unable to discriminate between closely related species. However, the most basal clades could still be discerned in the analysis of this region, providing useful data in the concatenated tree. A limitation of the concatenated analysis that included all of the species in the *Circumdati* section was the lack of RNA polymerase II second largest subunit (*RPB2*) sequences for the ex-type strains of *A. affinis*, *A. occultus*, *A. pulvericola*, *A. salwaensis*, *A. sesamicola*, and *A. westlandensis*. However, analyses of the other three markers, i.e., ITS, beta-tubulin (*BenA*), and calmodulin (*CaM*), unequivocally demonstrated that none of the strains studied here corresponded to any of the above-mentioned species.

The final concatenated sequence alignment, with 58 strains and the 4 sequenced markers, consisted of 2,451 bp (ITS, 482 bp; *BenA*, 470 bp; *CaM*, 481 bp; *RPB2*, 1,018 bp), of which 941 sites were variable (ITS, 85; *BenA*, 250; *CaM*, 231; *RPB2*, 375) and 686 parsimony informative (ITS, 57; *BenA*, 182; *CaM*, 159; *RPB2*, 288). Topology trees inferred by the two phylogenetic methods were basically the same, with only minor differences in the support values of the internal nodes. The ML phylogenetic tree and the bootstrap and posterior probability values (Fig. 1) show that 26 of the strains included in this study clustered with the ex-type strains of six species from section *Circumdati*, i.e., *A. westerdijkiae* ($n = 10$; 29.4%), *A. sclerotiorum* ($n = 6$; 17.6%), *A. ochraceus* ($n = 3$; 8.8%), *A. subramanianii* ($n = 3$; 8.6%), *A. insulicola* ($n = 2$; 5.7%), and *A. ochraceopetaliformis* ($n = 2$; 5.9%). Interestingly, a group of eight isolates (25.7%) formed a well-supported clade together with sequences of two unidentified *Aspergillus* strains (NRRL 35028 and NRRL 35056). This clade represents an undescribed species, proposed here as *Aspergillus pseudosclerotiorum*.

The isolates examined here showed typical morphology of section *Circumdati* and matched those of the respective species. We found, however, that identification to the species level based only on phenotypic characteristics is difficult, but combining some of the phenotypic characteristics can make this feasible (Table 1). Among the species identified here, *A. westerdijkiae* and *A. ochraceus* were the only ones with finely roughened conidia; these two species could be distinguished from each other by the lack of or only slight growth at 37°C (0 to 9 mm) for *A. westerdijkiae*, while *A. ochraceus* reached 23 to 26 mm in diameter in 7 days at the same temperature. The other species identified here had smooth-walled conidia. In addition, *A. insulicola* was the only species that did not produce sclerotia but did produce a reddish-brown soluble pigment on Czapek yeast autolysate agar (CYA); *A. subramanianii* showed good growth at 37°C (39 to 46 mm in 7 days); the colonies of *A. ochraceopetaliformis* had dense white mycelial areas and poor sporulation after 7 days; and *A. sclerotiorum* produced yellow (3A7) to brownish-orange (6C3) colonies, which reached 56 to 58 mm in diameter in 7 days on CYA, with white sclerotia, abundant sporulation, and profuse growth at 37°C (32 to 36 mm). *Aspergillus pseudosclerotiorum* shares similar morphological features with *A. sclerotiorum* but with a slightly lower growth rate at 25°C (45 to 55 diameter in 7 days) and at 37°C (22 to 38 mm), smaller metulae (3 to 9 by 2.5 to 6 μm, compared with 7 to 15 by 4 to 7 μm in *A. sclerotiorum*), and its sclerotia become yellow to orange yellow with age.

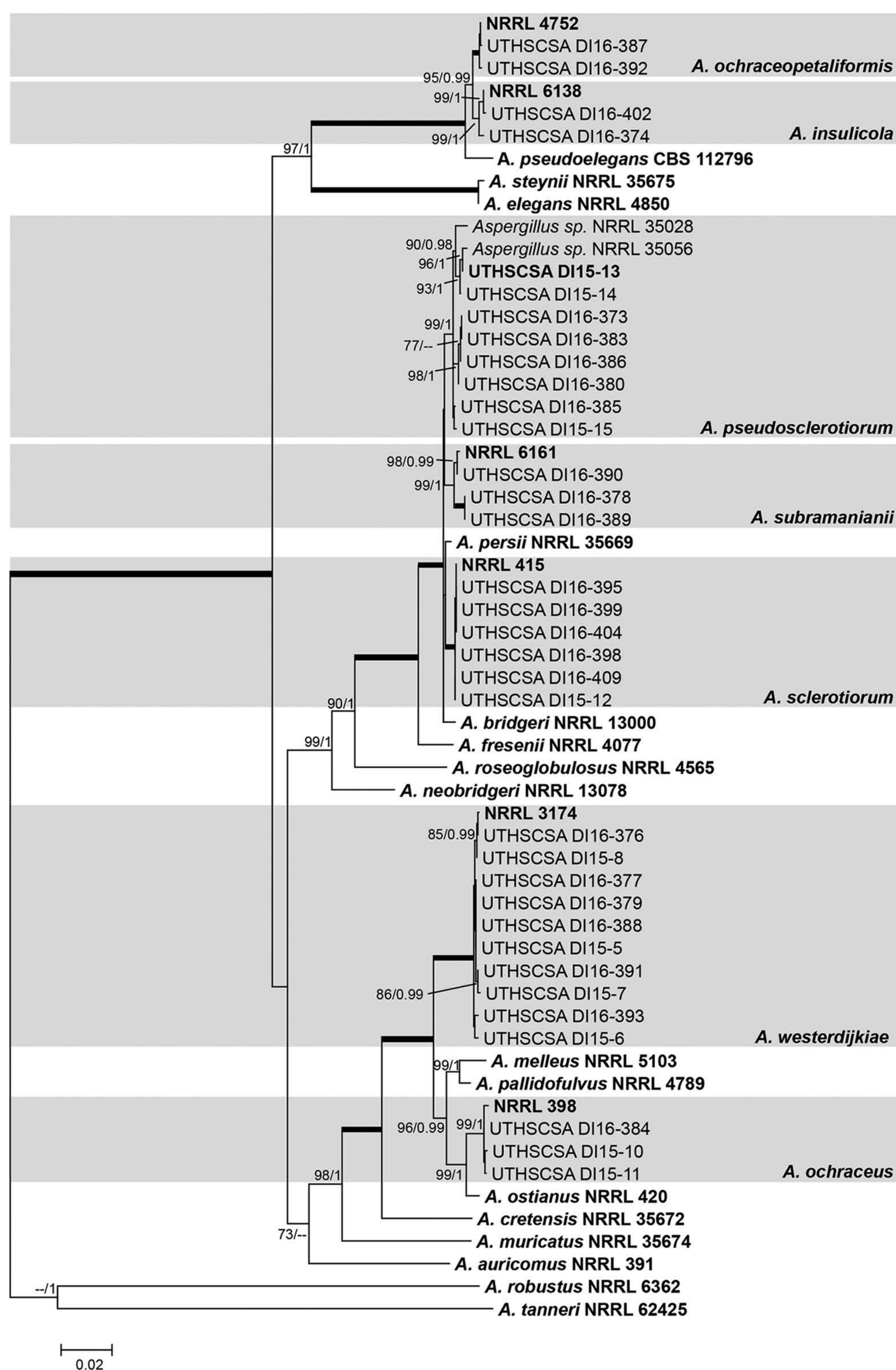


FIG 1 Maximum likelihood tree obtained from analysis of combined ITS, *BenA*, *CaM*, and *RPB2* data set. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. Fully supported branches (100/1) and ex-type strains are shown in boldface. UTHSCSA, University of Texas Health Science Center (San Antonio, Texas, USA).

In vitro susceptibility testing showed that the drugs with the most potent activity against all of the isolates tested were caspofungin (CFG), micafungin (MFG), and terbinafine (TBF), while amphotericin B (AMB) showed the lowest activity. The azoles (itraconazole [ITC], posaconazole [PSC], and voriconazole [VRC]), showed good activity

TABLE 1 Key morphological features of *Aspergillus* section *Circumdati* species identified in this study

Species	Sclerotium	Metula dimensions (μm)	Conidial ornamentation	CYA colony diam (mm) in 7 days at:	
				25°C	37°C
A. insulicola	Absent	6.5–12 by 3–5	Smooth	46–49	14–15
A. ochraceopetaliformis	Present	9–18 by 3.5–6	Smooth	38–46	27–29
A. ochraceus	Present	7–14 by 3–6	Finely roughened	44–49	23–26
A. pseudosclerotiorum	Present	3–9 by 2.5–6	Smooth	45–55	22–38
A. sclerotiorum	Present	7–15 by 4–7	Smooth	56–58	32–36
A. subramanianii	Present	8.5–14 by 3.5–6.5	Smooth	52–53	39–46
A. westerdijkiae	Present	8–18 by 4–7	Finely roughened	41–51	0–9

in general, with the exception of ITC against *A. sclerotiorum*. Interestingly, according to statistical analyses based on the Mann-Whitney test, the ITC MIC values showed significant differences between *A. sclerotiorum*, *A. ochraceus*, and *A. westerdijkiae* (GM of 11.31 μg/ml, 1.0 μg/ml, and 0.46 μg/ml, respectively; $P < 0.05$); however, differences were not significant between *A. sclerotiorum* and *A. pseudosclerotiorum* (0.89 μg/ml; $P = 0.06$) and *A. subramanianii* (4.0 μg/ml; $P = 0.43$). Regarding the new species, in general the drugs tested showed good activity against *A. pseudosclerotiorum*. Higher MIC values were observed only for AMB and VRC. Results of the *in vitro* susceptibility test are summarized in Table 2.

Taxonomy. *Aspergillus pseudosclerotiorum* J. P. Z. Siqueira, Deanna A. Sutton & Gené sp. nov. (MycoBank accession no. MB818572) (Fig. 2). Etymology: the name refers to the morphological similarity to *A. sclerotiorum*. Holotype: USA, Pennsylvania, isolated from

TABLE 2 Results of *in vitro* antifungal susceptibility test for 30 isolates of *Aspergillus* section *Circumdati*

Species (no. of isolates) and parameter[a]	MIC or MEC (μg/ml) for[b]:							
	AMB	AFG	CFG	MFG	ITC	PSC	VRC	TBF
A. ochraceus (3)								
GM	16.0	0.25	0.04	0.03	1.0	0.31	2.0	0.03
MIC range	16.0	0.12–0.5	0.03–0.06	0.03	1.0	0.25–0.5	2.0	0.03
Mode	16.0	0.5	0.03	0.03	1.0	0.25	2.0	0.03
A. subramanianii (3)								
GM	>16.0	0.10	0.03	0.03	4.0	0.79	4.0	0.03
MIC range	16.0–>16.0	0.03–0.25	0.03	0.03	4.0	0.5–1.0	4.0	0.03
Mode	>16.0	0.25	0.03	0.03	4.0	1.0	4.0	0.03
A. sclerotiorum (6)								
GM	4.76	0.03	0.04	0.03	11.31	1.0	3.36	0.03
MIC range	4.0–8.0	0.03	0.03–0.06	0.03	4.0–>16.0	1.0	2.0–4.0	0.03
Mode	4.0	0.03	0.03	0.03	>16.0	1.0	4.0	0.03
A. pseudosclerotiorum (8)								
GM	5.04	0.04	0.03	0.03	0.89	0.25	1.41	0.03
MIC range	2.0–>16	0.03–0.12	0.03–0.06	0.03	0.25–>16.0	0.12–0.5	1.0–2.0	0.03
Mode	4.0	0.03	0.03	0.03	0.5	0.25	2.0	0.03
A. westerdijkiae (10)								
GM	>16.0	0.14	0.03	0.03	0.46	0.29	1.08	0.03
MIC range	>16.0	0.03–1.0	0.03–0.06	0.03–0.06	0.12–1.0	0.12–0.5	1.0–2.0	0.03
Mode	>16.0	0.25	0.03	0.03	0.5	0.25	1.0	0.03
MIC_{90}	>16.0	0.5	0.06	0.06	0.5	0.5	1.0	0.03
Total (30)								
GM	12.82	0.08	0.03	0.03	1.28	0.39	1.74	0.03
MIC range	2.0–>16.0	0.03–1.0	0.03–0.06	0.03–0.06	0.12–>16.0	0.12–1.0	1.0–4.0	0.03
Mode	>16.0	0.03	0.03	0.03	0.5	0.25	1.0	0.03
MIC_{90}	>16.0	0.5	0.06	0.03	4.0	1.0	4.0	0.03

[a]GM, geometric mean.
[b]AMB, amphotericin B; AFG, anidulafungin; CFG, caspofungin; MFG, micafungin; ITC, itraconazole; PSC, posaconazole; VRC, voriconazole; TBF, terbinafine; MEC, minimum effective concentration for AFG, CFG, and MFG.

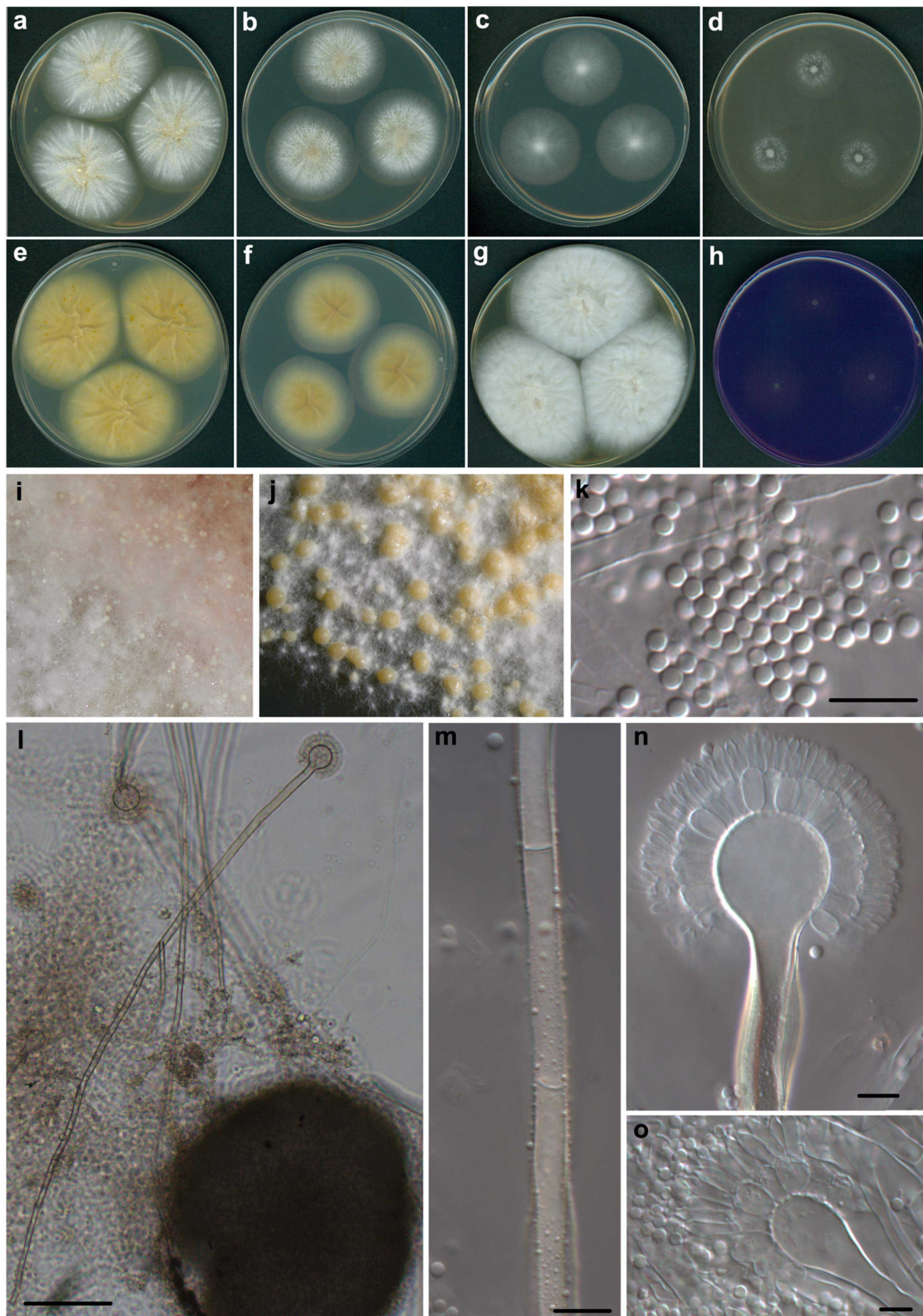


FIG 2 Morphological features of *Aspergillus pseudosclerotiorum* sp. nov. (UTHSCA DI 15-13 [a to n] and UTHSCSA DI16-383 [o]). (a, b, e, and f) Front and reverse of colonies on CYA and MEA, respectively, after 7 days at 25°C. (c, d, g, and h) Front of colonies on DG18, OA, YES, and CREA, respectively, after 7 days at 25°C. (i) Enlarged view of conidial heads on CYA after 7 days at 25°C. (j) Sclerotia on CYA after 14 days at 25°C. (k) Conidia. (l) Conidiophores and a sclerotium. (m) Detail of conidiophore stipe. (n and o) Details of conidial heads. Scale bars: 10 μm (k, m, n, and o) and 100 μm (l).

UNIVERSITAT ROVIRA I VIRGILI
CLINICAL AND ENVIRONMENTAL ASPERGILLUS: MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION, PHYLOGENY,
AND ANTIFUNGAL SUSCEPTIBILITY PROFIL·LE
Joao Paulo Zen Siqueire

TABLE 3 List of *Aspergillus* section *Circumdati* species, their isolate information, sequences generated in this study, and those retrieved from GenBank[d]

Species	Isolate no.[a]	Origin[b]	Yr	GenBank/EMBL accession no.[c]			
				ITS	BenA	CaM	RPB2
A. affinis	ATCC MYA-4773[T]			GU721090	GU721092	GU721091	
A. auricomus	NRRL 391 [T]			EF661411	EF661320	EF661379	EF661301
A. bridgeri	NRRL 13000 [T]			EF661404	EF661335	EF661358	EF661290
A. cretensis	NRRL 35672 [T]			FJ491572	AY819977	FJ491534	EF661311
A. elegans	NRRL 4850 [T]			EF661414	EF661349	EF661390	EF661316
A. fresenii	NRRL 407 [T]			EF661409	EF661341	EF661382	EF661296
A. insulicola	NRRL 6138 [T]			EF661430	EF661353	EF661396	EF661286
	UTHSCSA DI16–374	**Marine**	**2003**	**LT574681**	**LT574716**	**LT574751**	**LT574786**
	UTHSCSA DI16-402	**Marine**	**2009**	**LT574682**	**LT574717**	**LT574752**	**LT574787**
A. melleus	NRRL 5103 [T]			EF661425	EF661326	EF661391	EF661309
A. muricatus	NRRL 35674 [T]			EF661434	EF661356	EF661377	EF661314
A. neobridgeri	NRRL 13078 [T]			EF661410	EF661345	EF661359	EF661298
A. occultus	CBS 137330 [T]			KJ775443	KJ775061	KJ775239	
A. ochraceopetaliformis	NRRL 4752 [T]			EF661429	EF661350	EF661388	EF661283
	UTHSCSA DI16-387	**BAL**	**2006**	**LT574683**	**LT574718**	**LT574753**	**LT574788**
	UTHSCSA DI16-392	**Marine**	**2007**	**LT574684**	**LT574719**	**LT574754**	**LT574789**
A. ochraceus	NRRL 398 [T]			EF661419	EF661322	EF661381	EF661302
	UTHSCSA DI15-10	**BAL**	**2012**	**LT574686**	**LT574721**	**LT574756**	**LT574791**
	UTHSCSA DI15-11	**Heart valve**	**2013**	**LT574687**	**LT574722**	**LT574757**	**LT574792**
	UTHSCSA DI16-384	**Ear**	**2006**	**LT574685**	**LT574720**	**LT574755**	**LT574790**
A. ostianus	NRRL 420 [T]			EF661421	EF661324	EF661385	EF661304
A. pallidofulvus	NRRL 4789 [T]			EF661423	EF661328	EF661389	EF661306
A. persii	NRRL 35669 [T]			FJ491580	AY819988	FJ491559	EF661295
A. pseudoelegans	CBS 112796 [T]			FJ491590	AY819962	FJ491552	EF661282
A. pseudosclerotiorum	NRRL 35028			EF661407	EF661343	EF661362	EF661293
	NRRL 35056			EF661405	EF661344	EF661364	EF661294
	UTHSCSA DI15-13 [T]	**Lung biopsy**	**2014**	**LT574713**	**LT574748**	**LT574783**	**LT574818**
	UTHSCSA DI15-14	**BAL**	**2014**	**LT574714**	**LT574749**	**LT574784**	**LT574819**
	UTHSCSA DI15-15	**Lung tissue**	**2015**	**LT574715**	**LT574750**	**LT574785**	**LT574820**
	UTHSCSA DI16-373	**Sputum**	**2003**	**LT574707**	**LT574742**	**LT574777**	**LT574812**
	UTHSCSA DI16-380	**BAL**	**2006**	**LT574708**	**LT574743**	**LT574778**	**LT574813**
	UTHSCSA DI16-383	**BAL**	**2006**	**LT574709**	**LT574744**	**LT574779**	**LT574814**
	UTHSCSA DI16-385	**Sputum**	**2006**	**LT574710**	**LT574745**	**LT574780**	**LT574815**
	UTHSCSA DI16-386	**Lung mass**	**2006**	**LT574711**	**LT574746**	**LT574781**	**LT574816**
A. pulvericola	CBS 137327 [T]			KJ775440	KJ775055	KJ775236	
A. robustus	NRRL 6362 [T]			EF661176	EU014101	EF661357	EF661033
A. roseoglobulosus	NRRL 4565 [T]			FJ491583	AY819984	FJ491555	EF661299
A. salwaensis	DTO 297B3 [T]			KJ775447	KJ775056	KJ775244	
A. sclerotiorum	NRRL 415 [T]			EF661400	EF661337	EF661384	EF661287
	UTHSCSA DI15-12	**Sputum**	**2014**	**LT574693**	**LT574728**	**LT574763**	**LT574798**
	UTHSCSA DI16-395	**Sputum**	**2007**	**LT574688**	**LT574723**	**LT574758**	**LT574793**
	UTHSCSA DI16-398	**BAL**	**2008**	**LT574689**	**LT574724**	**LT574759**	**LT574794**
	UTHSCSA DI16-404	**Sputum**	**2009**	**LT574690**	**LT574725**	**LT574760**	**LT574795**
	UTHSCSA DI16-399	**BAL**	**2009**	**LT574691**	**LT574726**	**LT574761**	**LT574796**
	UTHSCSA DI16-409	**Eye**	**2014**	**LT574692**	**LT574727**	**LT574762**	**LT574797**
A. sesamicola	CBS 137324 [T]			KJ775437	KJ775063	KJ775233	
A. steynii	NRRL 35675 [T]			EF661416	EF661347	EF661378	JN121428
A. subramanianii	NRRL 6161 [T]			EF661403	EF661339	EF661397	EF661289
	UTHSCSA DI16-378	**Lung tissue**	**2005**	**LT574694**	**LT574729**	**LT574764**	**LT574799**
	UTHSCSA DI16-389	**Wound**	**2006**	**LT574695**	**LT574730**	**LT574765**	**LT574800**
	UTHSCSA DI16-390	**Foot**	**2006**	**LT574696**	**LT574731**	**LT574766**	**LT574801**
A. tanneri	NRRL 62425 [T]			JN853798	JN896582	JN896583	JN896585

(Continued on next page)

TABLE 3 (Continued)

Species	Isolate no.[a]	Origin[b]	Yr	GenBank/EMBL accession no.[c]			
				ITS	BenA	CaM	RPB2
A. westerdijkiae	NRRL 3174 T			EF661427	EF661329	EF661360	EF661307
	UTHSCSA DI15-5	**BAL**	**2014**	**LT574703**	**LT574738**	**LT574773**	**LT574808**
	UTHSCSA DI15-6	**Sputum**	**2014**	**LT574704**	**LT574739**	**LT574774**	**LT574809**
	UTHSCSA DI15-7	**Nail**	**2015**	**LT574705**	**LT574740**	**LT574775**	**LT574810**
	UTHSCSA DI15-8	**Marine**	**2011**	**LT574706**	**LT574741**	**LT574776**	**LT574811**
	UTHSCSA DI16-376	**Unknown**	**2004**	**LT574697**	**LT574732**	**LT574767**	**LT574802**
	UTHSCSA DI16-377	**Unknown**	**2004**	**LT574698**	**LT574733**	**LT574768**	**LT574803**
	UTHSCSA DI16-379	**BAL**	**2005**	**LT574699**	**LT574734**	**LT574769**	**LT574804**
	UTHSCSA DI16-388	**Lung mass**	**2006**	**LT574700**	**LT574735**	**LT574770**	**LT574805**
	UTHSCSA DI16-391	**Lung nodule**	**2007**	**LT574701**	**LT574736**	**LT574771**	**LT574806**
	UTHSCSA DI16-393	**Sputum**	**2007**	**LT574702**	**LT574737**	**LT574772**	**LT574807**
A. westlandensis	CBS 137321 T			KJ775434	KJ775066	KJ775230	

[a]ATCC, American Type Culture Collection; CBS, CBS-KNAW Fungal Biodiversity Centre (Utrecht, the Netherlands); DTO, Applied and Industrial Mycology Department Collection (Utrecht, Netherlands); NRRL, Agriculture Research Service Culture Collection (Peoria, NY); UTHSCSA, University of Texas Health Science Center (San Antonio, TX). A superscript T indicates an ex-type strain.
[b]BAL, bronchoalveolar lavage fluid specimens.
[c]ITS, internal transcribed spacer regions of the rDNA and 5.8S region; *BenA, β-tubulin; CaM*:, calmodulin; *RPB2*, partial RNA polymerase II, second largest subunit.
[d]Sequences generated in this study are in boldface.

lung biopsy specimen (human), D. A. Sutton, 2014 (CBS H-22808; culture ex-types: UTHSCSA DI15-13, FMR 14449, CBS 141845).

Colonies on CYA at 7 days reached 45- to 55-mm diameter at 25°C; at 30°C exhibited optimum growth, reaching 55- to 64-mm diameter; at 37°C reached 22- to 38-mm diameter; and at 40°C showed restricted growth. Colonies on CYA were pale yellow (3A3) to reddish white (7A3) at the center, white toward the periphery, cottony to floccose, and usually granulose due to the presence of abundant sclerotia, margin fimbriate; reverse yellow (3A7) to greyish yellow (3B5); colorless exudates present in most isolates; little soluble pigment produced, yellow (3A6), or absent. On malt extract agar (MEA), colonies similar to CYA but with slower growth, reaching 34 to 42 mm at 7 days. On yeast extract sucrose agar (YES), colonies showed fastest growth, reaching 56 to 66 mm at 7 days, white, cottony to floccose, with abundant sclerotia; reverse yellow (3A6) to greyish yellow (4B5), sulcate; exudates abundant, colorless to yellowish white (3A2). On dichloran 18% glycerol agar (DG18), colonies reaching 28 to 34 mm at 7 days, with white to light orange (5A4) compact center, and white fluffy mycelium toward periphery; reverse yellowish white (3A2) to pale yellow (3A3); sporulation sparsely produced only with age; sclerotia absent. On oatmeal agar (OA), colonies reaching 24 to 27 mm at 7 days, yellowish white (3A2) to greyish yellow (4B4), sandy to dusty, with a more compact center, margin regular; reverse yellowish white (4A2) to greyish yellow (4B6). On creatine-sucrose agar (CREA), colonies reaching 22 to 28 mm at 7 days, white, dense at the center, sparse aerial mycelium toward the periphery; acid production absent. Micromorphology consisting of conidiophores with biseriate and radiating conidial heads; stipes septate with rough walls, subhyaline to pale brown, 120 to 980 μm long by 2.5 to 8 μm wide; vesicles mainly globose, occasionally subglobose, 7- to 31-μm diameter; metulae cylindrical, 3 to 9 by 2.5 to 6 μm, usually covering 100% of vesicle, with exception of the strain UTHSCSA DI16-383, which covered 75% of vesicle; phialides ampulliform, 4.5 to 8 by 1.25 to 3 μm; conidia globose, smooth-walled, 1.5- to 3-μm diameter; sclerotia present (except in UTHSCSA DI16-380), 150- to 507-μm diameter, white to light orange (5A4), becoming yellow (3A6) to orange yellow (4A6) with age.

DISCUSSION

In this study, we identified a total of six species in the section *Circumdati* from clinical samples, some of which contained a relatively large number of isolates. Although their role as etiologic agents in these cases is unknown, detection of 34 isolates

of this section over a period of 12 years in a single reference center, together with some reports on infections produced by members of this section in the same period (15, 17, 18, 22, 27), highlights the importance of these fungi in the clinical setting. The degree of morphological similarity among the species of the *Circumdati* section, as with other groups of *Aspergillus*, requires DNA sequencing analysis for a definitive identification.

As was mentioned above, the most common *Aspergillus* species in the set of isolates studied here was *A. westerdijkiae*, a species described in 2004 and known to produce ochratoxin (28). It is noteworthy that the *A. ochraceus* strain from which ochratoxin A was discovered was later reidentified as *A. westerdijkiae*. This means that some isolates reported as *A. ochraceus*, especially the ones identified before 2004, in fact may be *A. westerdijkiae* (29). Growth rates at 37°C can be a useful feature to differentiate between these species without sequencing (3). *Aspergillus westerdijkiae* is commonly found in environmental samples (30) and as a food (31) and indoor contaminant (31–35). In the clinical setting, *A. westerdijkiae* has been linked to superficial infections (12) and isolated from sputum of immunocompromised patients in Tunisia (19). In our case, this species was mainly identified from respiratory specimens but also from a nail and in a sample from a marine animal (Table 3).

It is worth noting that the second most frequently identified species in the present study was a novel one, *A. pseudosclerotiorum*. This species is closely related to *A. bridgeri*, *A. persii*, *A. salwaensis*, *A. sclerotiorum*, and *A. subramanianii*. While these species could not be discriminated from each other using the ITS-based fungal barcode, *A. pseudosclerotiorum* was noted to have unique sequences for the other three markers (*BenA*, *CaM*, and *RPB2*). Phenotypically, *A. pseudosclerotiorum* generally can be distinguished from the above-mentioned aspergilli by its growth rate on different media and temperatures, colony pigmentation, and degree of sporulation, as well as sclerotia and conidiophore features. *Aspergillus bridgeri* produces brown colonies (3, 36). *A. persii* grows faster on OA (35- to 38-mm diameter in 7 days) and DG18 (45- to 50-mm diameter in 7 days) (3). *Aspergillus salwaensis* produces a characteristic yellowish-orange soluble pigment and usually has conidiophores with vesicles flattened at the apex (3). *Aspergillus subramanianii* grows faster on CYA at 37°C (39- to 46-mm diameter in 7 days). *Aspergillus sclerotiorum* grows faster on CYA at 25°C (54- to 57-mm diameter in 7 days), and at 37°C (32- to 36-mm diameter at 37°C) it shows a higher level of sporulation and its sclerotia are white to cream colored. However, one of the eight isolates of *A. pseudosclerotiorum* (UTHSCSA DI16-380), which showed 99.6% similarity with the other isolates, produced atypical colonies (i.e., brownish and profusely sporulated). The size of metulae is also a diagnostic feature for *A. pseudosclerotiorum*, because they are smaller (3 to 9 by 2.5 to 6 μm) than those of the related species (6.5 to 10 by 3.5 to 5.5 μm in *A. bridgeri*, 9 to 17.5 by 4 to 7.5 μm in *A. persii*, 8 to 21 by 3.5 to 6 μm in *A. salwaensis*, 8 to 16 by 4.5 to 7 μm in *A. sclerotiorum*, and 9 to 14 by 4 to 6.5 μm in *A. subramanianii*) (3). Although all isolates of *A. pseudosclerotiorum* were from the human respiratory tract (i.e., BAL fluid samples, sputum, and lung tissue), further studies are needed to determine the pathogenic role of this new fungus.

The third most common species sampled was *A. sclerotiorum*, which has been reported to cause superficial infections, such as onychomycosis and otomycosis (10, 14, 15). Here, most of the isolates were also from the human respiratory tract. *Aspergillus sclerotiorum* is found worldwide, commonly isolated from soil, and reported as a species of biotechnological importance due to its ability to produce a wide range of compounds (37–39).

The best-known species in the section, *A. ochraceus*, was poorly represented in this study (8.8%). In contrast, it is commonly found on coffee, rice, dried fruits, and nuts (8, 40, 41) and is capable of producing different metabolites (42–44). It was reported previously in pulmonary infections based on morphological identifications (16, 20). More recently, it has been identified in a case of osteomyelitis (17) and has also been isolated from immunocompromised patients (18, 19). Carpagnano et al. often found *A. ochraceus* in exhaled breath condensate of lung cancer patients (27). In other mammals,

it was associated with a case of otomycosis in a dog (45). Here, the three isolates were from different clinical origins (i.e., BAL fluid, ear, and heart valve).

Of the three other species identified, *A. insulicola* and *A. ochraceopetaliformis* have been reported from cases of onychomycoses (9, 12), while *A. subramanianii* was recovered for the first time from clinical specimens. Concerning the latter species, it is noteworthy that two isolates (UTHSCSA DI16-378 and UTHSCSA DI16-389) formed a clade slightly separate from the other *A. subramanianii* isolates (Fig. 1); however, the genetic identity (99.3%) with the ex-type strain and phenotypic similarity confirm their identification as *A. subramanianii*. This species could be considered a potential agent of human infections because of its ability to grow at 37°C and the deep-tissue origin of the isolates (lung tissue and wound).

Data available on the *in vitro* susceptibility of section *Circumdati* aspergilli against antifungal drugs are limited to a few reports with a small number of isolates tested. Here, the three echinocandins and TBF exhibited potent activity against the fungi tested. Similar results were obtained in our previous study on *Aspergillus* section *Versicolores* (46). TBF also has been reported to be highly effective *in vitro* against clinically relevant *Aspergillus* species, such as *A. flavus*, *A. niger*, *A. nidulans*, or *A. terreus*, and even against numerous isolates of *A. fumigatus sensu stricto* (47–49). To our knowledge, however, there is no previous information available on the activity of TBF against section *Circumdati* species. Results observed for echinocandins, especially MFG and anidulafungin (AFG), could be expected since, in general, they have been reported to be effective *in vitro* on *Aspergillus* species (50, 51). With respect to *Circumdati* aspergilli, Arabatzis et al. (18) tested three echinocandins against two isolates of *A. ochraceus* and one of *A. sclerotiorum* and reported high MICs only for CFG. In contrast, Gheith et al. (21) tested CFG against one isolate of *A. ochraceus* and one of *A. westerdijkiae* and reported low MICs, which is similar to our findings. AMB showed the least activity against the isolates tested, especially for *A. ochraceus*, *A. subramanianii*, and *A. westerdijkiae*. High AMB MICs were also observed for species in section *Circumdati* (i.e., *A. melleus*, *A. ochraceous*, and *A. pallidofulvus*), recently identified from human clinical specimens in India, in contrast to the results obtained in the same study for most isolates of *A. fumigatus*, *A. flavus*, and *A. terreus*, which were susceptible to antifungals tested there (51). PSC was the azole with the most potent activity against the strains tested, which agrees with Alastruey-Izquierdo et al. (25), Gheith et al. (21), and Masih et al. (51); however, the study of Arabatzis et al. (18) showed higher MICs for PSC. Recently, Babamahmoodi et al. (17) reported a case of osteomyelitis by *A. ochraceus*, for which the strain showed azole MICs (PSC, 0.032 μg/ml; VRC and ITC, 1.0 μg/ml) similar to ours (Table 2), and the patient improved after 4 months of treatment with VRC.

In conclusion, taxonomic studies are very important to assess the distribution of fungal species and their identity in clinical settings. In our study of clinical isolates within section *Circumdati* from a reference collection in the United States, we not only identified *A. subramanianii* as being associated with human specimens for the first time but also described a new taxon, *A. pseudosclerotiorum*, as one of the most frequent species of the section in this set of isolates. However, data from more isolates are needed to determine more reliable MICs of the different antifungal drugs against the species of this section and to determine the pathogenic role of these fungi in human and animal infections.

MATERIALS AND METHODS

Fungal isolates. A total of 34 *Aspergillus* isolates received from the Fungus Testing Laboratory at the University of Texas Health Science Center (San Antonio, TX, USA) were investigated. Based on morphological features, the isolates were identified as belonging to section *Circumdati*. Most isolates studied were from human clinical specimens, mainly from the respiratory tract ($n = 22$; 64.7%), although other human clinical sources were noted as well ($n = 8$; 23.5%). In addition, four isolates were from marine animals (Table 3).

Morphological characterization. The isolates were characterized morphologically by following the criteria recommended by Samson et al. (1). Briefly, colony morphology and growth rates were determined after 7 days of incubation on CYA (Becton, Dickinson and Company, Sparks, MD, USA) at 25°C and 37°C and on MEA (Pronadisa, Madrid, Spain) at 25°C. After 10 to 14 days of incubation, microscopic

structures were examined and measured from MEA cultures in wet mounts with 60% lactic acid and a drop of 70% ethanol to wash out the excess conidia. A minimum of 20 of each structure was measured in order to cover all of the size ranges. Photographs were made using a Zeiss Axio Imager M1 light microscope (Zeiss, Oberkochen, Germany) with a mounted DeltaPix Infinity X digital camera using Nomarski differential interference contrast and phase-contrast optics.

DNA extraction, amplification, and sequencing. Total genomic DNA was extracted from MEA cultures after 7 days of incubation at 25°C using the FastDNA kit and the FastPrep instrument (MP Biomedicals, Irvine, CA, USA) according to the manufacturer's specifications. Four genetic markers were amplified, i.e., the ITS region of the rRNA, which comprises ITS1, the 5.8S gene, and ITS2 regions, and fragments of the *BenA*, *CaM*, and *RPB2* genes (1, 26). The primers used were ITS5 and ITS4 for the ITS region (52), Bt2a and Bt2b for *BenA* (53), Cmd5 and Cmd6 for *CaM* (54), and 5F and 7CR for *RPB2* (55). PCR products were sequenced in both directions, using the same primers, at Macrogen Europe (Macrogen Inc., Amsterdam, Netherlands). Sequences were assembled and edited using SeqMan v.7.0.0 (DNASTAR, Madison, WI, USA).

Molecular identification and phylogenetic analysis. Phylogenetic analyses were first performed individually for each gene. Since the topologies proved to be congruent with the incongruence length difference test (56), a concatenated analysis was performed. Sequences of the ex-type strains of all the species in section *Circumdati* were obtained from GenBank and added to the analyses. *Aspergillus tanneri* (section *Tanneri*) and *A. robustus* (section *Robusti*) were used as outgroups. In addition, GenBank sequences of two strains identified only as *Aspergillus* spp. (NRRL 35028 and NRRL 35026) were also added to the analyses because they formed a distinct lineage in section *Circumdati* (26). For multiple-sequence alignment, ClustalW was used together with MUSCLE in MEGA v.6 (57), followed by manual adjustments. The maximum likelihood (ML) analysis was conducted with MEGA v.6, as well as to estimate the best nucleotide substitution model. Support of the internal branches was assessed by the bootstrap method with 1,000 replications, where values of ≥70 were considered significant. Bayesian inference (BI) was performed using MrBayes v.3.1.2 (58). The evolutionary model that best fit each gene was assessed by MrModelTest (59). Markov chain Monte Carlo (MCMC) sampling was performed with two simultaneous runs for 1 million generations, with samples taken every 100 generations. The 50% majority rule consensus trees and posterior probability (pp) values were calculated after removing the first 25% of the resulting trees for burn-in. A pp value of ≥0.95 was considered significant.

Antifungal susceptibility testing. Isolates of the most frequent *Aspergillus* species identified here were tested against eight antifungal drugs using the methods in the CLSI M38-A2 reference standard (60). The antifungal agents, obtained as pure powders, were AMB (Sigma-Aldrich Quimica S.A., Madrid, Spain), ITC (Jansen Pharmaceuticals, Beerse, Belgium), PSC (Schering-Plough Research Institut, NJ, USA), VRC (Pfizer S.A., Madrid, Spain), AFG (Pfizer S.A., Madrid, Spain), CFG (Merk & Co., Inc., Rahway, USA), MFG (Astellas Pharma, Madrid, Spain), and TBF. The MIC was defined as the lowest drug concentration that produced 100% inhibition of visible fungal growth for AMB and the azoles (ITC, PSC, and VRC) and 80% for TBF. The minimum effective concentration (MEC) was determined for the echinocandins (AFG, CFG, and MFG) and was defined microscopically as the lowest concentration of drug that permitted growth of small, rounded, compact hyphal forms, as opposed to the long, unbranched hyphal clusters that were seen in the growth control. The quality control strain *Candida krusei* ATCC 6258 was used in each test, and the MIC values were according to CLSI guideline ranges. All tests were carried out in duplicate, on different days, to assess reproducibility. Statistical analyses were performed using Prism software for Windows v.6.0 (GraphPad Software, San Diego, CA).

Accession number(s). Newly generated sequences from this study were deposited in GenBank/EMBL databases under the accession numbers listed in Table 3 and in MycoBank under accession number MB818572.

ACKNOWLEDGMENTS

This study was supported by the Spanish Ministerio de Economía y Competitividad, grant CGL2013-43789-P, and by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil), grant BEX 0623/14-8.

REFERENCES

1. Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CH, Perrone G, Seifert KA, Susca A, Tanney JB, Varga J, Kocsube S, Szigeti G, Yaguchi T, Frisvad JC. 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. Stud Mycol 78:141–173. https://doi.org/10.1016/j.simyco.2014.07.004.
2. Raper KB, Fennell DI. 1965. The genus *Aspergillus*. Robert E. Krieger Publishing Co., Inc., Huntington, NY.
3. Visagie CM, Varga J, Houbraken J, Meijer M, Kocsube S, Yilmaz N, Fotedar R, Seifert KA, Frisvad JC, Samson RA. 2014. Ochratoxin production and taxonomy of the yellow aspergilli (*Aspergillus* section *Circumdati*). Stud Mycol 78:1–61. https://doi.org/10.1016/j.simyco.2014.07.001.
4. Ciegler A. 1972. Bioproduction of ochratoxin A and penicillic acid by members of the *Aspergillus ochraceus* group. Can J Microbiol 18: 631–636. https://doi.org/10.1139/m72-100.
5. Varga J, Kevei E, Rinyu E, Téren J, Kozakiewicz Z. 1996. Ochratoxin production by *Aspergillus* species. Appl Environ Microbiol 62:4461–4464.
6. Lea T, Steien K, Stormer FC. 1989. Mechanism of ochratoxin A-induced immunosuppression. Mycopathologia 107:153–159. https://doi.org/10.1007/BF00707553.
7. Pfohl-Leszkowicz A, Manderville RA. 2007. Ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans. Mol Nutr Food Res 51:61–99. https://doi.org/10.1002/mnfr.200600137.
8. Duarte SC, Pena A, Lino CM. 2010. A review on ochratoxin A occurrence and effects of processing of cereal and cereal derived food products. Food Microbiol 27:187–198. https://doi.org/10.1016/j.fm.2009.11.016.
9. Brasch J, Varga J, Jensen JM, Egberts F, Tintelnot K. 2009. Nail infection by *Aspergillus ochraceopetaliformis*. Med Mycol 47:658–662. https://doi.org/10.1080/13693780902803032.

10. Amri M, Gorcii M, Essabbah N, Belhajali H, Letscher-Bru V, Zili J, Azaiez R, Babba H. 2010. *Aspergillus sclerotiorum*: à propos d'un cas d'onychomycose en Tunisie. J Med Mycol 20:128–132. https://doi.org/10.1016/j.mycmed.2010.03.004.
11. Zotti M, Machetti M, Perotti M, Barabino G, Persi A. 2010. A new species, *Aspergillus persii*, as an agent of onychomycosis. Med Mycol 48:656–660. https://doi.org/10.3109/13693780903420641.
12. Hubka V, Kubatova A, Mallatova N, Sedlacek P, Melichar J, Skorepova M, Mencl K, Lyskova P, Sramkova B, Chudickova M, Hamal P, Kolarik M. 2012. Rare and new etiological agents revealed among 178 clinical *Aspergillus* strains obtained from Czech patients and characterized by molecular sequencing. Med Mycol 50:601–610. https://doi.org/10.3109/13693786.2012.667578.
13. Zotti M, Agnoletti AF, Vizzini A, Cozzani E, Parodi A. 2015. Onychomycosis from *Aspergillus melleus*, a novel pathogen for humans. Fungal identification and *in vitro* drug susceptibility. Exp Dermatol 24:966–968.
14. García-Martos P, Guarro J, Gené J, Mira J, Linares M, Ortoneda M. 2005. Onychomycosis caused by *Aspergillus sclerotiorum*. J Mycol Med 11:222–224.
15. Harima N, Inoue T, Kubota T, Okada O, Ansai S, Manabe M, Ichinoe M, Kasai T. 2004. A case of otomycosis caused by *Aspergillus sclerotiorum*. J Dermatol 31:949–950. https://doi.org/10.1111/j.1346-8138.2004.tb00635.x.
16. Novey HS, Wells ID. 1978. Allergic bronchopulmonary aspergillosis caused by *Aspergillus ochraceus*. Am J Clin Pathol 70:840–843. https://doi.org/10.1093/ajcp/70.5.840.
17. Babamahmoodi F, Shokohi T, Ahangarkani F, Nabili M, Afzalian Ashkezari E, Alinezhad S. 2015. Rare case of *Aspergillus ochraceus* osteomyelitis of calcaneus bone in a patient with diabetic foot ulcers. Case Rep Med 2015:509827.
18. Arabatzis M, Kambouris M, Kyprianou M, Chrysaki A, Foustoukou M, Kanellopoulou M, Kondyli L, Kouppari G, Koutsia-Karouzou C, Lebessi E, Pangalis A, Petinaki E, Stathi A, Trikka-Graphakos E, Vartzioti E, Vogiatzi A, Vyzantiadis TA, Zerva L, Velegraki A. 2011. Polyphasic identification and susceptibility to seven antifungals of 102 *Aspergillus* isolates recovered from immunocompromised hosts in Greece. Antimicrob Agents Chemother 55:3025–3030. https://doi.org/10.1128/AAC.01491-10.
19. Gheith S, Saghrouni F, Bannour W, Ben Youssef Y, Khelif A, Normand A-C, Ben Said M, Piarroux R, Njah M, Ranque S. 2014. Characteristics of invasive aspergillosis in neutropenic haematology patients (Sousse, Tunisia). Mycopathologia 177:281–289. https://doi.org/10.1007/s11046-014-9742-8.
20. Wierzbicka M, Podsiadlo B, Janczarski M. 1997. Inwazyjna aspergiloza pluc wywolana przez *Aspergillus ochraceus*. Pneumonol Alergol Pol 65:254–260.
21. Gheith S, Saghrouni F, Bannour W, Ben Youssef Y, Khelif A, Normand A-C, Piarroux R, Ben Said M, Njah M, Ranque S. 2014. *In vitro* susceptibility to amphotericin B, itraconazole, voriconazole, posaconazole and caspofungin of *Aspergillus* spp. isolated from patients with haematological malignancies in Tunisia. Springerplus 3:19–26. https://doi.org/10.1186/2193-1801-3-19.
22. Negri CE, Goncalves SS, Xafranski H, Bergamasco MD, Aquino VR, Castro PT, Colombo AL. 2014. Cryptic and rare *Aspergillus* species in Brazil: prevalence in clinical samples and *in vitro* susceptibility to triazoles. J Clin Microbiol 52:3633–3640. https://doi.org/10.1128/JCM.01582-14.
23. García-Martos P, García-Agudo L, Gutiérrez-Calzada J, Ruiz-Aragón J, Saldarreaga A, Marín P. 2005. Actividad *in vitro* de anfotericina B, itraconazol y voriconazol frente a 20 especies de *Aspergillus* empleando el método de microdilución Sensititre. Enferm Infecc Microbiol Clin 23:15–18. https://doi.org/10.1157/13070403.
24. Wildfeuer A, Seidl HP, Paule I, Haberreiter A. 1998. *In vitro* evaluation of voriconazole against clinical isolates of yeasts, moulds and dermatophytes in comparison with itraconazole, ketoconazole, amphotericin B and griseofulvin. Mycoses 41:309–319. https://doi.org/10.1111/j.1439-0507.1998.tb00344.x.
25. Alastruey-Izquierdo A, Mellado E, Cuenca-Estrella M. 2012. Current section and species complex concepts in *Aspergillus*: recommendations for routine daily practice. Ann N Y Acad Sci 1273:18–24. https://doi.org/10.1111/j.1749-6632.2012.06822.x.
26. Peterson SW. 2008. Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. Mycologia 100:205–226. https://doi.org/10.3852/mycologia.100.2.205.
27. Carpagnano GE, Lacedonia D, Palladino GP, Logrieco G, Crisetti E, Susca A, Logrieco A, Foschino-Barbaro MP. 2014. *Aspergillus* spp. colonization in exhaled breath condensate of lung cancer patients from Puglia region of Italy. BMC Pulm Med 14:22. https://doi.org/10.1186/1471-2466-14-22.
28. Frisvad JC, Frank JM, Houbraken JAMP, Kuijpers AFA, Samson RA. 2004. New ochratoxin A producing species of *Aspergillus* section *Circumdati*. Stud Mycol 50:23–43.
29. Samson RA, Hong SB, Frisvad JC. 2006. Old and new concepts of species differentiation in *Aspergillus*. Med Mycol 44:S133–S148.
30. Sabino R, Verissimo C, Parada H, Brandao J, Viegas C, Carolino E, Clemons KV, Stevens DA. 2014. Molecular screening of 246 Portuguese *Aspergillus* isolates among different clinical and environmental sources. Med Mycol 52:519–529. https://doi.org/10.1093/mmy/myu006.
31. Gil-Serna J, Patino B, Cortes L, Gonzalez-Jaen MT, Vazquez C, Patiño B, Cortes L, Gonzalez-Jaen MT, Vazquez C. 2015. *Aspergillus steynii* and *Aspergillus westerdijkiae* as potential risk of OTA contamination in food products in warm climates. Food Microbiol 46:168–175. https://doi.org/10.1016/j.fm.2014.07.013.
32. Mikkola R, Andersson MA, Hautaniemi M, Salkinoja-Salonen MS. 2015. Toxic indole alkaloids avrainvillamide and stephacidin B produced by a biocide tolerant indoor mold *Aspergillus westerdijkiae*. Toxicon 99:58–67. https://doi.org/10.1016/j.toxicon.2015.03.011.
33. Bacha N, Atoui A, Mathieu F, Liboz T, Lebrihi A. 2009. *Aspergillus westerdijkiae* polyketide synthase gene "aoks1" is involved in the biosynthesis of ochratoxin A. Fungal Genet Biol 46:77–84. https://doi.org/10.1016/j.fgb.2008.09.015.
34. Marino A, Nostro A, Fiorentino C. 2009. Ochratoxin A production by *Aspergillus westerdijkiae* in orange fruit and juice. Int J Food Microbiol 132:185–189. https://doi.org/10.1016/j.ijfoodmicro.2009.03.026.
35. Gil-Serna J, Patiño B, Cortés L, González-Jaén MT, Vázquez C. 2011. Mechanisms involved in reduction of ochratoxin A produced by *Aspergillus westerdijkiae* using *Debaryomyces hansenii* CYC 1244. Int J Food Microbiol 151:113–118. https://doi.org/10.1016/j.ijfoodmicro.2011.08.012.
36. Christensen M. 1982. The *Aspergillus ochraceus* group: two new species from western soils and a synoptic key. Mycologia 74:210–225. https://doi.org/10.2307/3792887.
37. Whyte A, Gloer J. 1996. Sclerotiamide: a new member of the paraherquamide class with potent antiinsectan activity from the sclerotia of *Aspergillus sclerotiorum*. J Nat Prod 59:1093–1095. https://doi.org/10.1021/np960607m.
38. Whyte A, Joshi B. 2000. New cyclic peptide and bisindolyl benzenoid metabolites from the sclerotia of *Aspergillus sclerotiorum*. J Nat Prod 63:1006–1009. https://doi.org/10.1021/np000103v.
39. Zheng J, Zhu H, Hong K, Wang Y, Liu P. 2009. Novel cyclic hexapeptides from marine-derived fungus, *Aspergillus sclerotiorum* PT06-1. Org Lett 11:5262–5265. https://doi.org/10.1021/ol902197z.
40. Logrieco A, Bottalico A, Mulé G, Moretti A, Perrone G. 2003. Epidemiology of toxigenic fungi and their associated mycotoxins for some Mediterranean crops. Eur J Plant Pathol 109:645–667. https://doi.org/10.1023/A:1026033021542.
41. Taniwaki MH, Teixeira AA, Teixeira ARR, Copetti MV, Iamanaka BT. 2014. Ochratoxigenic fungi and ochratoxin A in defective coffee beans. Food Res Int 61:161–166. https://doi.org/10.1016/j.foodres.2013.12.032.
42. Trenk H, Butz M, Chu F. 1971. Production of ochratoxins in different cereal products by *Aspergillus ochraceus*. Appl Microbiol 21:1032–1035.
43. Steyn P, Holzapfel C. 1967. The synthesis of ochratoxins A and B: metabolites of *Aspergillus ochraceus* Wilh. Tetrahedron 23:4449–4461. https://doi.org/10.1016/S0040-4020(01)88843-8.
44. Dai J, Carté B. 2001. Circumdatin G, a new alkaloid from the fungus *Aspergillus ochraceus*. J Nat Prod 64:125–126. https://doi.org/10.1021/np000381u.
45. Ghibaudo G, Peano A. 2010. Chronic monolateral otomycosis in a dog caused by *Aspergillus ochraceus*. Vet Dermatol 21:522–526. https://doi.org/10.1111/j.1365-3164.2010.00884.x.
46. Siqueira JPZ, Sutton DA, García D, Gené J, Thomson P, Wiederhold N, Guarro J. 2016. Species diversity of *Aspergillus* section *Versicolores* in clinical samples and antifungal susceptibility. Fungal Biol 120:1458–1467. https://doi.org/10.1016/j.funbio.2016.02.006.
47. Jessup CJ, Ryder NS, Ghannoum MA. 2000. An evaluation of the *in vitro* activity of terbinafine. Med Mycol 38:155–159. https://doi.org/10.1080/mmy.38.2.155.159.
48. Moore CB, Walls CM, Denning DW. 2001. *In vitro* activities of terbinafine against *Aspergillus* species in comparison with those of itraconazole and amphotericin B. Antimicrob Agents Chemother 45:1882–1885. https://doi.org/10.1128/AAC.45.6.1882-1885.2001.

UNIVERSITAT ROVIRA I VIRGILI
CLINICAL AND ENVIRONMENTAL ASPERGILLUS: MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION, PHYLOGENY,
AND ANTIFUNGAL SUSCEPTIBILITY PROFIL·LE
Joao Paulo Zen Siqueire

49. Fernández MS, Rojas FD, Cattana ME, Sosa Mde L, Iovannitti CA, Lass-Flörl C, Giusiano GE. 2015. *In vitro* activities of amphotericin B, terbinafine, and azole drugs against clinical and environmental isolates of *Aspergillus terreus sensu stricto*. Antimicrob Agents Chemother 59:3619–3622. https://doi.org/10.1128/AAC.00045-15.
50. Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Segal BH, Steinbach WJ, Stevens DA, van Burik J-A, Wingard JR, Patterson TF. 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis 46:327–360. https://doi.org/10.1086/525258.
51. Masih A, Singh PK, Kathuria S, Agarwal K, Meis JF, Chowdhary A. 2016. Identification by molecular methods and matrix-assisted laser desorption ionization–time of flight mass spectrometry and antifungal susceptibility profiles of clinically significant rare *Aspergillus* species in a referral chest hospital in Delhi, India. J Clin Microbiol 54:2354–2364. https://doi.org/10.1128/JCM.00962-16.
52. White T, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p 315–322. *In* Innis MA, Gelfand DH, Sninsky JJ, White TJ (ed), PCR protocols: a guide to methods and applications. Academic Press Inc., New York, New York.
53. Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol 61:1323–1330.
54. Hong S-B, Go S-J, Shin H-D, Frisvad JC, Samson RA. 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. Mycologia 97:1316–1329. https://doi.org/10.3852/mycologia.97.6.1316.
55. Liu YJ, Whelen S, Hall BD. 1999. Phylogenetic relationships among Ascomycetes: evidence from an RNA Polymerase II Subunit. Mol Biol Evol 16:1799–1808. https://doi.org/10.1093/oxfordjournals.molbev.a026092.
56. Farris J, Kallersjo M, Kluge A, Bult C. 1994. Testing significance of incongruence. Cladistics 10:315–319. https://doi.org/10.1111/j.1096-0031.1994.tb00181.x.
57. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729. https://doi.org/10.1093/molbev/mst197.
58. Ronquist F, Huelsenbeck JP. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574. https://doi.org/10.1093/bioinformatics/btg180.
59. Nylander JAA. 2004. MrModeltest v2. Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden.
60. Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungals susceptibility testing of conidium-forming filamentous fungi: approved standard, 2nd ed. M38-A2. CLSI, Wayne, PA.

4.3. Species of *Aspergillus* section *Aspergillus* from clinical samples in USA

João Paulo Zen Siqueira, Deanna A. Sutton, Josepa Gené, Dania García,
Nathan Wiederhold, Josep Guarro

Accepted in: Medical Mycology, manuscript MM-2017-0048 (July 2017)

Medical Mycology - Decision on Manuscript ID MM-2017-0048.R3

12-Jul-2017

Dear Dr. Josepa Gene:

It is my pleasure to inform you that your manuscript, MM-2017-0048.R3, Species of Aspergillus section Aspergillus from clinical samples in USA, **has been accepted for publication in Medical Mycology**. You will receive proofs from Oxford University Press in approximately 8 weeks. You will have an opportunity to review the PDF proof and make minor changes at that time, should need be.

In order to publish your article, Oxford University Press requires that you complete a licence agreement online. A link to the online licensing system, and instructions on how to select and complete a licence, will be provided to you by the Production Editor at Oxford University Press in due course. You will receive your official acceptance date from Oxford University Press once you have signed your licence to publish. (N.B. If you are a UK-based author and are looking to comply with the HEFCE policy on open access in the Research Excellence Framework, you should use the official acceptance date when depositing in your repository).

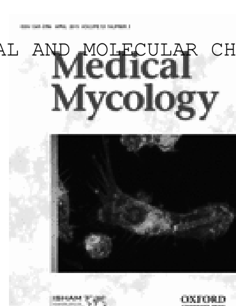
Once you have returned the proofs, with any corrections, the article will be electronically published ahead of print on the publisher's website. While there will be no page numbers associated with the report, it will carry a DOI, making it trackable and citable. Your manuscript will remain published online in this manner until it is published in a future issue of Medical Mycology.

In the event that you are not an ISHAM member, I would like to take this opportunity to invite you to visit the society's Web site at www.ISHAM.org to obtain a membership form and information on the many benefits provided by ISHAM.

Thank you for submitting your manuscript to Medical Mycology. If you have any questions or require further information, please don't hesitate to contact me.

Sincerely,

Dr. Karl V. Clemons
Editor-in-Chief
Medical Mycology



Species of Aspergillus section Aspergillus from clinical samples in USA

Journal:	*Medical Mycology*
Manuscript ID	MM-2017-0048.R3
Manuscript Type:	Original Article
Date Submitted by the Author:	04-Jul-2017
Complete List of Authors:	Zen Siqueira, Joao; Universitat Rovira i Virgili Facultat de Medicina I Ciences de la Salut, Departament de Ciències Mèdiques Bàsiques Sutton, Deanna A.; University of Texas Health Science Center, Fungus Testing Lab/Dept. Path/Mail code 7750; Gene, Josepa; Facultad de Medicina y Ciencias de la Salud c, Unidad de Microbiología Garcia, Dania; Universitat Rovira I Virgili, Mycology Unit Medical School Wiederhold, Nathan; University of Texas at Austin, Pharmacy; University of Texas Health Science Center at San Antonio, PERC Guarro, Josep; Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Unitat de Microbiologia
Keyword:	Aspergillus, phylogenetic analysis, antifungal susceptibility, taxonomy, Eurotium
Abstract:	The diversity of Aspergillus species in clinical samples is continuously increasing. Species under the former name Eurotium, currently accommodated in section Aspergillus of the genus, are xerophilic fungi widely found in the human environment and able to grow on substrates with low water activity. However, their prevalence in the clinical setting is poorly known. We have studied the presence of these species in a set of clinical samples from the USA using a multilocus sequence analysis based on the internal transcribed spacer (ITS) region of the rRNA,and fragments of the genes β-tubulin (BenA), calmodulin (CaM), and polymerase II second largest subunit (RPB2). A total of 25 isolates were studied and identified as follows:A. montevidensis (44%), A. chevalieri (36%), A. pseudoglaucus (8%), and A. costiformis (4%).A new species Aspergillus microperforatus is also proposed, which represented 8% of the isolates studiedand is characterized by uniseriate conidial heads, subglobose to pyriform vesicles, rough conidia, globose to subglose cleistothecia, and lenticular and smooth ascospores.The in vitro antifungal activity of 8 clinically available antifungals was also determined against these isolates, with the echinocandins and posaconazole having the most potent activity.

SCHOLARONE™
Manuscripts

Title: Species of *Aspergillus* section *Aspergillus* from clinical samples in USA

Runing title: *Aspergillus* section *Aspergillus* in clinical setting

João Paulo Zen Siqueira[1,2], Deanna A. Sutton[3], Josepa Gené[1*], Dania García[1], Nathan Wiederhold[3], Josep Guarro[1]

[1]Unitat de Micologia, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Reus, Spain.
[2]Laboratório de Microbiologia, Faculdade de Medicina de São José do Rio Preto, 5416 Brigadeiro Faria Lima Ave., 15090-000, São José do Rio Preto, Brazil.
[3]Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, TX, USA.

Keywords: *Aspergillus*, phylogenetic analysis, antifungal susceptibility, taxonomy, *Eurotium*

Corresponding author:

Josepa Gené

Unitat de Micologia

Facultat de Medicina i Ciències de la Salut

Universitat Rovira i Virgili

C/ Sant Llorenç 21

43201 Reus, Spain

Ph: +34-977-759359; F: +34-977-759322; email: josepa.gene@urv.cat

Abstract

The diversity of *Aspergillus* species in clinical samples is continuously increasing. Species under the former name *Eurotium*, currently accommodated in section *Aspergillus* of the genus, are xerophilic fungi widely found in the human environment and able to grow on substrates with low water activity. However, their prevalence in the clinical setting is poorly known. We have studied the presence of these species in a set of clinical samples from the USA using a multilocus sequence analysis based on the internal transcribed spacer (ITS) region of the rRNA, and fragments of the genes β-tubulin (*BenA*), calmodulin (*CaM*), and polymerase II second largest subunit (*RPB2*). A total of 25 isolates were studied and identified as follows: *A. montevidensis* (44%), *A. chevalieri* (36%), *A. pseudoglaucus* (8%), and *A. costiformis* (4%). A new species *Aspergillus microperforatus* is also proposed, which represented 8% of the isolates studied and is characterized by uniseriate conidial heads, subglobose to pyriform vesicles, rough conidia, globose to subglose cleistothecia, and lenticular and smooth ascospores. The in vitro antifungal activity of 8 clinically available antifungals was also determined against these isolates, with the echinocandins and posaconazole having the most potent activity.

Introduction

The number of species of *Aspergillus* involved in human infections is continuously increasing, and most of these species have nowadays been identified using modern molecular techniques. Until recently, the dual nomenclature system permitted different names for the sexual and asexual forms of *Aspergillus*. One such example is the genus *Eurotium*, the name for the sexual state for species in the former *Aspergillus glaucus* group[1]. However, following the recent changes in fungal nomenclature[2,3] and based on phylogenetic studies[4,5], all generic names for sexual states of *Aspergillus* are now included under the name *Aspergillus,* and former species of *Eurotium* now comprise the aspergilli in the section *Aspergillus*[4]. A new approach to phylogenetic study supports the current wide range of *Aspergillus*[5], with *A. glaucus* (= *E. herbariorum*) being the type species of the genus.

Species in the section *Aspergillus* are usually osmophilic, with optimum growth on substrates with high sugar or salt concentrations. Commonly the asexual morph has smooth conidiophores, with uniseriate, radiate to somewhat columnar conidial heads, and ellipsoidal to globose echinulate conidia[1,6]. The sexual morph is usually characterized by globose to subglobose, thin-walled cleistothecia, eight-spored asci, and lenticular, smooth to rough-walled ascospores, generally showing an equatorial line or furrow[1,7]. These species are found worldwide, and often on organic materials, dust, and stored cereals and other food products[1,7]. Although these aspergilli are of minimal clinical importance, some, such as *A. glaucus*, has been reported in orofacial[8] and brain infections[9]. In addition, *A. montevidensis* has been involved in cases of otitis, mycetoma, cerebral abscess, keratitis, and pulmonary infections[10] and *A. glaucus* and *A. montevidensis* can also cause hypersensitive pneumonitis[11,12]. *Aspergillus chevalieri* and *A. pseudoglaucus* have been linked to cutaneous aspergillosis[13] and maxillary sinusitis[14], respectively. Hubka et al.[15] recovered five species of section *Aspergillus* among isolates from probable cases of superficial infections (e.g., skin and nails), including *A. montevidensis*, *A. costiformis*, *A. pseudoglaucus*, *A. proliferans*, and *A. ruber* in Czech Republic. The antifungal susceptibility patterns of members of section *Aspergillus* are largely unknown[16], with little published data. Masih et al.[17] demonstrated potent activity of posaconazole, anidulafungin, and micafungin

against 3 strains of *A. montevidensis* (GM MICs of 0.04 µg/mL, 0.015 µg/mL, and 0.015 µg/mL, respectively) and one of *A. chevalieri* (MICs of 0.015 µg/mL, 0.03 µg/mL, and 0.015 µg/mL, respectively). García-Martos et al.[18] alsodemonstrated low MIC values for amphotericin B, itraconazole, and voriconazole against 3 strains of *A. glaucus* (MIC ranges of 0.125–0.5 µg/mL, 0.25–0.5 µg/mL, and 0.125–0.25 µg/mL, respectively) and 2 strains of *A. chevalieri* (MIC ranges of 0.125–0.5 µg/mL, 0.125–0.25 µg/mL, and 0.125–0.25 µg/mL, respectively). Wildfeuer et al.[19] tested 8strains of *A. glaucus* against 4 drugs and observed that itraconazole exhibited the most potent activity (GM MIC of 0.39 µg/mL).

In order to assess the diversity of species from *Aspergillus* section *Aspergillus* in the clinical setting and to observe their response to antifungal drugs, the aim of this study was to identify to the species level a set of clinical isolates from the USA using a multilocus phylogenetic study, and to determine the susceptibility pattern of 8 clinically available antifungals against these species.

Materials and Methods

Fungal isolates

A total of 25 isolates of section *Aspergillus* were investigated in this study. Most of them were from human clinical samples, primarily from the respiratory tract (BAL, sputum, and sinus), but also in fewer numbers from corneas, nails, stool, and lymph nodes. One of them was of environmental origin and the origin of four were unknown (Table 1). These isolates were received at the Fungus Testing Laboratory of the University of Texas Health Science Center (San Antonio, TX, USA) from different institutions across the USA over a period of 11 years (2004-2015), for identification and/or antifungal susceptibility testing.

Morphological characterization

The morphology of the fungi was characterized by the traditional criteria[4,20]. Briefly, this is determined after 7 days of incubation on Czapek Yeast Autolysate agar (CYA, Becton, Dickinson and Company®, Sparks MD, USA), CYA supplemented with 20% sucrose (CY20S), and Malt Extract agar (MEA, Pronadisa®, Madrid, Spain) at 25 ºC; and CY20S and Harrold's Agar containing 60% sucrose[1] (M60Y) at 37 ºC. Colors

match Kornerup & Wanscher[21]. Microscopic features were examined and measured on MEA and CY20S cultures, after 10–14 days of incubation, on wet mounts with 60% lactic acid and a drop of ethanol 70% to wash out the excess conidia. Photomicrographs were taken with a DeltaPix Infinity X digital camera mounted on a Zeiss Axio Imager M1 light microscope (Zeiss, Oberkochen, Germany), using a Nomarski differential interference contrast and phase contrast optics. Scanning electron microscope (SEM) photographs were taken with a Jeol JSM- 6400 using techniques described previously[22].

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from MEA cultures after 7 days of incubation at 25 ºC, using the FastDNA® Kit and the FastPrep® Instrument (MP Biomedicals, Irvine CA, USA), according to the manufacturer's specifications. After extraction, four different genetic regions were amplified for each strain[20,23]; i.e., the internal transcribed spacer (ITS) region of the rRNA, comprising ITS1, 5.8S gene, and ITS2 regions, using ITS5 and ITS4 primers[24]; a portion of β-tubulin gene (*BenA*), using Bt2a and Bt2b primers[25]; a portion of calmodulin gene (*CaM*), using Cmd5 and Cmd6 primers[26]; and a portion of RNA polymerase II second largest subunit gene (*RPB2*), using 5F and 7CR primers[27]. PCR products were sequenced in both directions, using the same primers, at Macrogen Europe (Macrogen Inc., Amsterdam, the Netherlands). Sequences were assembled andedited using SeqMan v.7.0.0 (DNASTAR, Madison, WI, USA).

Molecular identification and phylogenetic analysis

The phylogeny was analyzed first individually for each partition and then in a concatenated study, once the topologies proved to be congruent. To give support to our analyses, sequences of the ex-type strains of all species of the section *Aspergillus* obtained from GenBank were also included, and *A. halophilicus* (section *Restricti*) was used as the outgroup. To increase the robustness of the *A. pseudoglaucus* clade, sequences of 15 other strains of this species were additionally retrieved from GenBank and included in the analyses. A multiple sequence alignment was performed using ClustalW inside MEGA v.6 software[28]. When necessary, the MUSCLE tool and manual adjustments were used to refine the alignment. Maximum Likelihood (ML) was conducted with MEGA v.6 software, as well as the estimation of the best nucleotide substitution method. Support values of the

internal branches were assessed using the Bootstrap method with 1,000 replications (values equal or higher than 70% were considered significant). Bayesian Inference (BI) was performed using MrBayes v.3.1.2 software[29]. The evolutionary models that best fit each partition were assessed by MrModel Test software[30]. Markov chain Monte Carlo (MCMC) sampling was performed with two simultaneous runs for 1 million generations, with samples taken every 100 generations. The 50% majority rule consensus trees and posterior probability values (pp) were calculated after removing the first 25% of the resulting trees for burn-in. Values of 0.95 or higher were considered significant.

Antifungal susceptibility testing

The isolates were tested against eight antifungals, following the microdilution broth method[31]. The antifungal agents tested were: amphotericin B (AMB) (Sigma Aldrich Quimica S.A., Madrid, Spain), itraconazole (ITC) (Jansen Pharmaceuticals, Beerse, Belgium), posaconazole (PSC) (Schering-Plough Res., Inst., NJ, USA), voriconazole (VRC) (Pfizer S.A., Madrid, Spain), anidulafungin (AFG) (Pfizer S.A., Madrid, Spain), caspofungin (CFG) (Merk & Co., Inc.,Rahway, USA), micafungin (MFG) (Astellas Pharma, Madrid, Spain), and terbinafine (TBF) (Sigma Aldrich Química S.A., Madrid, Spain). Readings were taken after 72 h to allow the strains to grow properly. Strains of *A. pseudoglaucus* (UTHSCSA DI15-17 and UTHSCSA DI16-410) and *A. microperforatus* (UTHSCSA DI16-400 and UTHSCSA DI16-407) were incubated at 30 ºC, while the others were incubated at 35 ºC to fit the growth requirements of the isolates under the CLSI protocol. Minimal inhibitory concentration (MIC) was defined as the lowest drug concentration that produced 100% inhibition of visible fungal growth for the AMB and azoles (ITC, PSC and VRC) and 80% for TBF. For echinocandins (AFG, CFG, and MFG), the minimum effective concentrations (MEC) were determined microscopically as the lowest concentration of drug that allowed the growth of small, rounded, compact hyphal forms, as opposed to the long, unbranched hyphal clusters that are seen in the growth control. *Candida krusei* ATCC 6258 was used as the quality control strain in each test and the MIC values were within the acceptable MIC range per the CLSI standard. All tests were carried out in duplicate, on different

days, for reproducibility. Statistical analyses of the results were performed using the Prism software for Windows v.6.0 (GraphPad Software, San Diego, CA).

Nucleotide sequence accession numbers and alignments

Newly-generated sequences from this study have been deposited in GenBank/EMBL databases under the accession numbers listed on Table 1. The alignments were deposited in TreeBASE (submission number S20583).

Results

In the present study, as expected, the ITS region was the least informative marker, being unable to discriminate some of the species includedin the analysis (Figure S1 in supplemental material). Two main clades were defined using this genetic marker, one grouping the species *A. chevalieri*, *A. intermedius*, *A. montevidensis*, *A. cristatus*, and *A. costiformis,* and the second one grouping the species *A. pseudoglaucus*, *A. glaucus*, *A. neocarnoyi*, *A. niveoglaucus*, *A. brunneus*, *A. proliferans*, *A. ruber*, *A. appendiculatus*, *A. cibarius*, *A. tonophilus*, and *A. sloanii*. The other markers (*BenA*, *CaM*, and *RPB2*) were more informative, with better delineation in well-supported monophyletic groups (Figures S2–S4 in supplemental material). The single phylogenetic analysis corresponding to the different genes showed very similar tree topologies, and a concatenated study was performed. The final concatenated sequence alignment consisted of 2,653 bases (ITS, 641; *BenA*, 433; *CaM*, 596; *RPB2*, 983), of which 794 were variable sites (ITS, 105; *BenA*, 176; *CaM*, 255; *RPB2*, 258) and 461 parsimony informative (ITS, 31; *BenA*, 104; *CaM*, 160; *RPB2*, 166). The ML tree (Figure 1) shows significant support values for both phylogenetic methods (bootstrap/posterior probabilities).

The clinical isolates grouped together with the following species: *A. montevidensis* (11 isolates, 44%), *A. chevalieri* (9 isolates, 36%), and *A. pseudoglaucus* (2 isolates, 8%). The environmental isolate was identified as *A. costiformis* (4%). The isolates UTHSCSA DI16-400 (= CBS 142377) and UTHSCSA DI16-407 (= CBS 142376), from toenail andlymph node samples, respectively, and sequences of two environmental isolates (CCF 5387 and CCF 5388) retrieved from GenBank, formed a full-supported clade close to the *A. pseudoglaucus* clade, which represents an

undescribed phylogenetic lineage for the section *Aspergillus*. Therefore, we propose the new species *A. microperforatus*.

The morphology of the isolates shows the expected phenotypic characters that agree with the previous species descriptions[1,4,32–34]. *Aspergillus montevidensis* exhibits rough ascospores, with irregular crests; *A. chevalieri* shows smooth ascospores, with prominent crests; *A. costiformis* is the only species with smooth conidia; and *A. pseudoglaucus* and *A. microperforatus* demonstrated smooth ascospores, with no crests, and rough conidia. In fact, these last two species have a similar morphology, being differentiated by the slow growth and restricted sporulation of the novel species on CYA at 25 ºC and on M60Y at 37 ºC and the absence of soluble pigment in any of the culture media tested. *Aspergillus pseudoglaucus* isolates identified here grew and sporulated well on both media and temperatures, and produced a brownish soluble pigment on CYA at 25 ºC in 14 days of incubation. Table 2 shows the key phenotypic features of the species of *Aspergillus* section *Aspergillus* already reported in clinic, including those recovered in this study.

In general, all isolates were inhibited by each of the antifungal drugs tested, with overall geometric mean (GM) values lower than 1.0 µg/ml. The most potent activity was observed with the echinocandins (GM of 0.03 µg/ml), while VRC had the highest MIC values (GM of 1.0 µg/ml for *A. pseudoglaucus*, and 0.77 µg/ml for *A. montevidensis*, with individual values up to 2.0 µg/ml). The results of the *in vitro* susceptibility test are summarized in Table 3.

Taxonomy

Aspergillus microperforatus J.P.Z. Siqueira, Deanna A. Sutton & Gené sp. nov. (MycoBank MB 820080, Fig. 2).

Colonies on CYA 8–15 mm diam in 7 days at 25 ºC, floccose, yellowish white (3A2) at the center, white towards the periphery, sporulation scarce, margin entire; reverse pale (2A2) to olive (3A3); exudate and soluble pigment absent. On CY20S, colonies 30–45 mm diam in 7 days at 25 ºC, granulose due to the presence of ascomata, sporulation abundant, conidial masse greyish green (25E5); reverse brownish orange (7C5) to olive (3A6) at the centre, pale yellow (2A3) to yellow (2A6) towards the periphery; exudate

and soluble pigment absent. On MEA, colonies 11 mm diam in 7days at 25 ºC; sporulation absent in UTHSCSA DI16–407, abundant in UTHSCSA DI16–400, with conidial masse brown (5A5), margin entire; reverse pale (2A2) to brownish orange (5C3); exudate and soluble pigment absent. On YES, colonies 33–40 mm in 7 days at 25 ºC, velutinous to downy, slightly granulose at the centre due to the presence of ascomata, sporulation abundant, with conidial masse dark green (27F5), margin entire; reverse pale yellow (4A3) to light yellow (4A4). On DG18, colonies 30–45 mm in 7 days at 25 ºC, fuzzy, white to light orange (5A5), sporulation abundant, conidial mass honey yellow (4D6); reverse light yellow (1A4) to yellow (3A7). On CREA, colonies up to 5 mm in 7 days at 25 ºC, acid production absent. No growth on OA at 25 ºC, or on CY20S at 37 ºC. Conidiophores up to 550 μm long, with uniseriate and radiating conidial heads; stipes occasionally septate, 260–500 x 6.5–9.5 μm, hyaline to subhyaline, smooth to finely roughened; vesicles subglobose to pyriform, 24–36 μm diam; phialides variable in shape and size, ampulliform to cylindrical, 7–18 (30) x 2–5 μm; conidia globose to elongate, sometimes pyriform, 6–9.5(–11) x 4.5–9 μm, in shades of brown, rough. Cleistothecia globose to subglobose, 90–130 μm diam, light yellow (2A5) to deep yellow (4A8); asci globose, 10–14 μm in diam; ascospores lenticular, 4–5.5 x 2.5–4.5 μm, hyaline, with a slight furrow in the equatorial region, convex surface smooth with very small pits only visible under SEM.

Etymology: Referring to the presence of small pits in the ascospore wall under SEM.

Type: USA, Texas, isolated from human lymph node, D.A. Sutton, 2011 (CBS H-22998 holotype; cultures ex-type: UTHSCSA DI16-407, CBS 142376, FMR 14071).

Discussion

Although the diversity of *Aspergillus* section *Aspergillus* species is well known in osmophilic substrates, house dust, indoor air or stored products, in the clinical setting it is poorly documented. As previously noted, the taxonomy and nomenclature of the species of section *Aspergillus* has recently changed. In addition to that, recent advances in molecular tools have allowed for the description of new cryptic species that are almost impossible to differentiate using classical morphological tools[35]. Clinically, identification of *Aspergillus* isolates at the species level may be important given that

susceptibilities to antifungal drugs vary for different species and that species identity can influence the choice of appropriate antifungal therapy[36]. In the present study, a total of 25 isolates of section *Aspergillus* were used, most of them being recovered from human respiratory specimens, although further studies are needed to elucidate the role of these fungi as pathogens. Five different species of the section have been identified here, including a novel one (i.e., *A. chevalieri*, *A. costiformis, A. microperforatus, A. montevidensis* and *A. pseudoglaucus*). To facilitate comparison, their key morphological features are presented in Table 2.

Aspergillus montevidensis was the most prevalent (44%). Clinically, this species may be the most relevant pathogen of this group because it has been isolated from different bodies sites, from superficial to deep tissue infections[10,15,37]. It was first reported and described from a case of human otomycosis[38]. This species currently includes strains that were formerly accepted as different but now considered conspecific. *Aspergillus hollandicus* (incorrectly associated to the sexual state *Eurotium amstelodami*), *A. heterocaryoticus*, and *A. vitis* are all synonyms of *A. montevidensis* and these names should no longer be used[4]. The nine isolates of *A. montevidensis* in the present study,with the exception of two of unknown origins, were from respiratory specimens (i.e. sputum, sinuses, and lung biopsies).

The second most prevalent species in the present study is *A. chevalieri* (36%), known until recently by its teleomorph name, *Eurotium chevalieri*. This species has been reported from a case of cutaneous aspergillosis[13] and more recently was the cause of fatal cerebral aspergillosis acquired by traumatic inoculation[17]. It is worth noting that *A. chevalieri* and *A. montevidensis* represent 80% of the isolates included in this study, in fact they are also some of the most commonly species found in indoor environments[4]. These two species, and the others reported here, were able to grow well at 37 ºC in vitro (Table 2), the basic pathogenic feature that enables them to invade deep tissue[39].

The isolate of *A. costiformis* identified in this study is the third known strain of this species since its description. It was originally recovered from a moldy paper-box in China[34]. The second strain was isolated from a human nail in the Czech Republic[15], and the present study has recovered a third strain from hospital environment. The difficulty in the phenotypic characterization of this species might explain why it is rarely reported.

The key characteristic that identifies *A. costiformis* is the presence of smooth conidia (Table 2) and the asexual morph is not usually formed in standard culture conditions. To overcomethis problem, the production of conidial heads can be induced on M60Y at 37 ºC, as previously reported[4].

The remaining isolates included in this study were genetically and morphologically similarbut they could be distinguished as two different species (each representing 8% of the isolates). Firstly, isolates UTHSCSA DI15-17 and UTHSCSA DI16-410 were identified as *A. pseudoglaucus*, a species described by Blochwitz in 1929[33]. This species was more recently delineated phylogenetically by Hubka et al.[4] in whose study other species were shown to be conspecific with *A. pseudoglaucus*, i.e. *A. glaucoaffinis*/*E. pseudoglaucum*[40], *A. glaber*[40], *A. fimicola*[41], and *A. reptans*/*E. repens*[40]. The phylogenetic tree constructed in the present study includes sequences of the ex-type strains of synonymous species and of numerous reference strains, giving more support to the *A. pseudoglaucus* clade. Our analysis shows that the genetic similarity among all those strains is 99.9% or higher in the concatenated alignment, in agreement with the proposal mentioned above. *Aspergillus pseudoglaucus* iscommonly found in stored products[7]and produces metabolites that are potentially toxic[42]. There are few clinical reports involving *A. pseudoglacus*; it has been reported from a mixed infection in a case of maxillary sinusitis[14] and occasionally recovered from human skin and nails[15], although its pathogenicity has not been confirmed. The two isolates of *A. pseudoglaucus* identified in the present study were from nasal and stool samples. Secondly, the isolates UTHSCSA DI16-400 and UTHSCSA DI16-407, recovered from toenail and lymph node, respectively, and sequences of two isolates retrieved from GenBank group in a clade close to *A. pseudoglaucus*. Although these latter isolates were labelled as *A. pseudoglaucus*, both phylogenetic methods (ML and BI) and the three most informative markers (*BenA*, *CaM*, and *RPB2*) all show that they represent together with our isolates investigated a distinct lineage in the section[4]. Thus, they have been proposed as the novel species *A. microperforatus*. The possible role of this species in the clinical disease is yet unknown. Although section *Aspergillus* includes some species that are closely related, some characteristics can be useful for discriminating *A. microperforatus* (Table 2). For example, the novel species shows restricted growth on

CYA at 25 ºC (up to 15 mm), in contrast to *A. pseudoglaucus* (up to 24 mm), which also differs by growing better on M60Y at 37 ºC (41 to 46 mm, vs. 28 to 32 mm diam in *A. microperforatus*) and, according to our results, on CYA at 25º C it produces a diffusible brown pigment that is absent in *A. microperforatus*. The novel species can be differentiated from *A. glaucus* and *A. proliferans* by its ability to grow on M60Y at 37 ºC and *A. glaucus* has larger ascospores (6.0 to 7.5 µm, vs. 4.0 to 5.5 µm in *A. microperforatus*). Based on the descriptions and other reports, it might be difficult to differentiate the morphologies of *A. microperforatus* and *A. ruber* although the ascospores of *A. ruber* have an evident furrow and the conidia are usually ellipsoidal[1,4]. Although *A. glaucus* is a known opportunistic pathogen, being reported from many types of infections[8,9,11], it was not recovered in the present study, as was the case in a study of clinical aspergilli in the Czech Republic[15]. Therefore, it is possible that the clinical prevalence of this species has been overestimated, probably due to the limitations of diagnostic tools for *Aspergillus* identification and for filamentous fungi in general.

The CLSI have established epidemiological cut-off values for triazoles (ITC, VRC, and POS) and AMB for only six *Aspergillus* species, i.e., *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *A. nidulans*, and *A. versicolor*[43,44]. However, the limited number of isolates of other species available in different clinical laboratories precludes the determination of epidemiologic cutoff values, and members of section *Aspergillus* have only been rarely tested for antifungal susceptibility[16]. With few exceptions, the eight antifungals used in this study showed good activity against the aspergilli tested, with MIC values equal to or less than 1.0 µg/ml (Table 3). Recently, Masih et al.[17] provided the in vitro antifungal susceptibility profiles of rare *Aspergillus* species in clinical samples from India. Althoughthey did not test TBF, the MIC values for the other seven antifungals against three isolates of *A. montevidensis* and one strain of *A. chevalieri* were similar to the values observed in the current study. Most available in vitro data is with *A. glaucus*. Wildfeuer et al.[21] and García-Martos et al.[22] include eight and three clinical isolates of *A. glaucus*, respectively, and both reported good activity for ITC (MIC range of 0.25–0.5 µg/ml), VRC (0.125–0.78 µg/ml), and AMB (0.125–1.56 µg/ml). Although we did not study any *A. glaucus* strains, these values are similar

to the overall values observed for the strains tested here. However, our MIC values were slightly lower for AMB and higher for VRC. Furthermore, García-Martos et al.[21] also included two isolates of *A. chevalieri* and report the same MIC range (0.125–0.25 µg/ml) for ITC, VRC, and AMB. These results are very similar to those found in our *A. chevalieri* isolates, with the exception of one isolate that had an ITC MIC of 1.0 µg/ml.

In summary, this study has assessed the species diversity of *Aspergillus* section *Aspergillus* from a set of clinical isolates from the USA and demonstrated that *A. montevidensis* and *A. chevalieri* were the most frequently identified species. We also describe *A. microperforatus* as a new species. The antifungals tested showed potent activity against these isolates, especially the echinocandins and PSC.

Acknowledgments: This study was supported by the Spanish Ministerio de Economía y Competitividad, grant CGL2013-43789-P and by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil), grant BEX 0623/14-8.

Disclosure of conflict of interest: No conflict of interest.

References

1. Raper KB, Fennell DI. *The Genus Aspergillus*. Huntington, NY: Robert E. Krieger Publishing Co., Inc.; 1965.
2. McNeill J, Barrie FF, Buck WR, et al. *International Code of Nomenclature for Algae, Fungi and Plants (Melbourne Code)*. Koeltz Scientific Books; 2012.
3. Norvell LL. Melbourne approves a new code. *Mycotaxon*. 2011; 116(1): 481–490.
4. Hubka V, Kolarik M, Kubatova A, Peterson SW. Taxonomic revision of *Eurotium* and transfer of species to *Aspergillus*. *Mycologia*. 2013; 105(4): 912–937.
5. Kocsubé S, Perrone G, Magistà D, et al. *Aspergillus* is monophyletic: evidence from multiple gene phylogenies and extrolites profiles. *Stud Mycol*. 2016; 85:199–213.
6. Thom C, Church MB. *The Aspergilli*. Baltimore, MD: Williams & Wilkins; 1926.
7. Kozakiewicz Z. *Aspergillus Species on Stored Products*. Ferry Lane: C.A.B. International; 1989.
8. Dreizen S, Bodey GP, McCredie KB, Keating MJ. Orofacial aspergillosis in acute leukemia. *Oral Surgery, Oral Med Oral Pathol*. 1985; 59(5): 499–504.
9. Traboulsi RS, Kattar MM, Dbouni O, Araj GF, Kanj SS. Fatal brain infection caused by *Aspergillus glaucus* in an immunocompetent patient identified by sequencing of the ribosomal 18S-28S internal transcribed spacer. *Eur J Clin Microbiol Infect Dis*. 2007; 26(10): 747–750.
10. de Hoog GS, Guarro J, Gené J, Figueras M. *Atlas of Clinical Fungi*. CD-ROM version 3.1, CBS-KNAW fungal biodiversity centre, Utrecht; 2011.
11. Kaukonen K, Savolainen J, Viander M, Kotimaa M, Terho EO. IgG and IgA subclass antibodies against *Aspergillus umbrosus* in farmer's lung disease. *Clin Exp Allergy*. 1993; 23(10): 851–856.
12. Reboux G, Piarroux R, Mauny F, et al. Role of molds in farmer's lung disease in eastern France. *Am J Respir Crit Care Med*. 2001; 163(7): 1534–1539.

13. Naidu J, Singh SM. A*spergillus chevalieri* (Mangin) Thom and Church: a new opportunistic pathogen of human cutaneous aspergillosis. *Mycoses*. 1994; 37(7–8): 271–274.
14. Aznar C, de Bievre C, Guiguen C. Maxillary sinusitis from *Microascus cinereus* and *Aspergillus repens*. *Mycopathologia*. 1989; 105(2): 93-97.
15. Hubka V, Kubatova A, Mallatova N, et al. Rare and new etiological agents revealed among 178 clinical *Aspergillus* strains obtained from Czech patients and characterized by molecular sequencing. *Med Mycol*. 2012; 50(6): 601–610.
16. Buzina W. *Aspergillus* – Classification and Antifungal Susceptibilities. *Curr Pharm Des*. 2013; 19(20): 3615–3628.
17. Masih A, Singh PK, Kathuria S, Agarwal K. Identification by molecular methods and matrix-assisted laser desorption ionization – time of flight mass spectrometry and antifungal susceptibility profiles of clinically significant rare *Aspergillus* species in a referral chest hospital in Delhi, India. *J Clin Microbiol*. 2016; 54(9): 2354–2364.
18. García-Martos P, García-Agudo L, Gutiérrez-Calzada J, Ruiz-Aragón J, Saldarreaga A, Marín P. Actividad in vitro de anfotericina B, itraconazol y voriconazol frente a 20 especies de *Aspergillus*empleando el método de microdilución Sensititre®. *Enferm Infecc Microbiol Clin*. 2005; 23(1): 15–18.
19. Wildfeuer A, Seidl HP, Paule I, Haberreiter A. In vitro evaluation of voriconazole against clinical isolates of yeasts, moulds and dermatophytes in comparison with itraconazole, ketoconazole, amphotericin B and griseofulvin. *Mycoses*. 1998; 41: 309–319.
20. Samson RA, Visagie CM, Houbraken J, et al. Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Stud Mycol*. 2014; 78: 141–173.
21. Kornerup A, Wanscher JH. *Methuen Handbook of Colour*. 3rd edn. Hastings House, New York, NY: Methuen, 1967.
22. Figueras MJ, Guarro J. A scanning electron microscopic study of ascoma development in *Chaetomium malaysiense*. *Mycologia*. 1988; 80(3): 298–306.
23. Peterson SW. Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. *Mycologia*. 2008; 100(2): 205–226.

24. White T, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: A Guide to Methods and Applications*. New York: Academic Press inc.; 1990: 315–322.

25. Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol*. 1995; 61(4): 1323–1330.

26. Hong SB, Go SJ, Shin HD, Frisvad JC, Samson RA. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia*. 2005; 97(6): 1316–1329.

27. Liu YJ, Whelen S, Hall BD. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. *Mol Biol Evol*. 1999; 16(12): 1799–1808.

28. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol*. 2013; 30(12): 2725–2729.

29. Ronquist F, Huelsenbeck JP. MRBAYES 3: bayesian phylogenetic inference under mixed models. *Bioinformatics*. 2003; 19: 1572–1574.

30. Nylander JAA. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University. 2004.

31. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungals susceptibility testing of conidium-forming filamentous fungi: approved standard, 2nd ed. M38-A2. CLSI, Wayne, PA. 2008.

32. Mangin L. Quest-ce que L'*Aspergillus glaucus*? Etude critique et experimentale des formes groupers sous ce nom. *Ann Sci Nat Bot*. 1909; 9: 303–371.

33. Blochwitz VA. Die Gattung *Aspergillus*. Neue Species. Diagnosen. Synonyme. *Ann Mycol*. 1929; 27(3–4): 205–240.

34. Kong H, Qi Z. Two new species of *Eurotium* Link. *Acta Mycol Sin*. 1995; 14(1): 10–16.

35. Alastruey-Izquierdo A, Mellado E, Cuenca-Estrella M. Current section and species complex concepts in *Aspergillus*: recommendations for routine daily practice. *Ann N Y Acad Sci*. 2012; 1273: 18–24.

36. Balajee SA, Houbraken J, Verweij PE, et al. *Aspergillus* species identification in the clinical setting. *Stud Mycol*. 2007; 59: 39–46.

37. Marr KA, Patterson T, Denning D. Aspergillosis. Pathogenesis, clinical manifestations, and therapy. *Infect Dis Clin North Am*. 2002; 16(4): 875–894.

38. Talice R V., MaKinnon JE. *Aspergillus* (*Eurotium*) *montevidensis*, n. sp. isolé d'un cas d'otomycose chez l'homme. *Compt Rend Soc Biol*. 1931; 108: 1007–1009.

39. Kobayashi GS. Disease of mechanisms of fungi. In: Baron S, ed. *Medical Microbiology*. 4th ed. Galveston: University of Texas Medical Branch at Galveston; 1996.

40. Samson RA, Gams W. Typification of the species of *Aspergillus* and associated teleomorphs. In: Samson RA, Pitt JI, eds. *Advances in Penicillium and Aspergillus Systematics*. New York: Plenum Press; 1985: 31–54.

41. Kong H, Qi Z. Two new species of *Eurotium* isolated from Xizang (Tibet), China. *Acta Microbiol Pol*. 1995; 14(2): 86–91.

42. Podojil M, Sedmera P, Vokoun J, et al. *Eurotium* (*Aspergillus*) *repens* metabolites and their biological activity. *Folia Microbiol (Praha)*. 1978; 23(6): 438–443.

43. Espinel-Ingroff A, Diekema DJ, Fothergill A, et al. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *J Clin Microbiol*. 2010; 48(9): 3251–3257.

44. Espinel-Ingroff A, Cuenca-Estrella M, Fothergill A, et al. Wild-Type MIC distributions and epidemiological cutoff values for amphotericin b and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *Antimicrob Agents Chemother*. 2011; 55(11): 5150–5154.

Fig 1 – Maximum likelihood tree obtained from the combined ITS, *BenA*, *CaM* and *RPB2* sequences of the isolates. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The fully supported branches (100/1) and type strains are shown in bold. The new species is shown in the colored box.

Fig 2 – Morphological features of *Aspergillus microperforatus* sp. nov. (UTHSCSA DI16-407). Panels: a, b, c, f, g, h, front and reverse of colonies on CY20S, DG18, and YES, respectively,after 7 days at 25 ºC; d, e, front of colonies on CYA, and MEA, respectively, after 7 days at 25 ºC; i, front of colonies on M60Y after 7 days at 37 ºC; j, front of colonies on CYA after 14 days at 25º; k, l, ascoma; m, n, asci; o, p, ascospores; q, r, s, conidial heads; t, u, conidia. Scale bars: k, 100 µm, l–u, 10 µm.

TABLE 1 – Origins, year of isolation, and GenBank/EMBL accession numbers of the *Aspergillus* strains included in this study

Species	Isolate number	Origin	Year	GenBank/EMBL accession number			
				ITS	*BenA*	*CaM*	*RPB2*
A. chevalieri (9)	UTHSCSA DI15-18	BAL	2014	LT627247	LT627272	LT627297	LT627322
	UTHSCSA DI16-375	Sputum	2004	LT627248	LT627273	LT627298	LT627323
	UTHSCSA DI16-381	BAL	2006	LT627249	LT627274	LT627299	LT627324
	UTHSCSA DI16-382	BAL	2008	LT627250	LT627275	LT627300	LT627325
	UTHSCSA DI16-394	BAL	2007	LT627251	LT627276	LT627301	LT627326
	UTHSCSA DI16-396	Corneal	2008	LT627252	LT627277	LT627302	LT627327
	UTHSCSA DI16-397	Sinus	2008	LT627253	LT627278	LT627303	LT627328
	UTHSCSA DI16-413	Unknown	2008	LT627254	LT627279	LT627304	LT627329
	UTHSCSA DI16-414	Unknown	2008	LT627255	LT627280	LT627305	LT627330
A. costiformis (1)	UTHSCSA DI15-16	Environmental	2014	LT627256	LT627281	LT627306	LT627331
A. microperforatus (2)	UTHSCSA DI16-400	Toe nail	2009	LT627270	LT627295	LT627320	LT627345
	UTHSCSA DI16-407	Lymph node	2011	LT627271	LT627296	LT627321	LT627346
A. montevidensis (11)	UTHSCSA DI15-19	Ethmoid sinus	2014	LT627257	LT627282	LT627307	LT627332
	UTHSCSA DI15-20	Sputum	2014	LT627258	LT627283	LT627308	LT627333

	UTHSCSA DI15-21	BAL	2015	LT627259	LT627284	LT627309	LT627334
	UTHSCSA DI15-22	Sputum	2015	LT627260	LT627285	LT627310	LT627335
	UTHSCSA DI16-401	Lung tissue	2009	LT627261	LT627286	LT627311	LT627336
	UTHSCSA DI16-403	Sputum	2009	LT627262	LT627287	LT627312	LT627337
	UTHSCSA DI16-405	Sinus	2010	LT627263	LT627288	LT627313	LT627338
	UTHSCSA DI16-406	Lung tissue	2010	LT627264	LT627289	LT627314	LT627339
	UTHSCSA DI16-408	Paranasal	2013	LT627265	LT627290	LT627315	LT627340
	UTHSCSA DI16-411	Unknown	2008	LT627266	LT627291	LT627316	LT627341
	UTHSCSA DI16-412	Unknown	2008	LT627267	LT627292	LT627317	LT627342
A. pseudoglaucus (2)	UTHSCSA DI15-17	Nasal	2011	LT627268	LT627293	LT627318	LT627343
	UTHSCSA DI16-410	Stool	2014	LT627269	LT627294	LT627319	LT627344

UTHSCSA: University of Texas Health Science Center (San Antonio, USA); ITS: internal transcribed spacer regions of the rDNA and 5.8S region; *BenA*: β-tubulin; *CaM*: calmodulin; *RPB2*: partial RNA polymerase II second largest subunit.

TABLE 2 – Relevant features of the *Aspergillus* section *Aspergillus* already reported in clinical setting.

	Growth rate (mm) at 7d									
	25ºC		37ºC		Ascospores			Conidia		
Species	CYA	CY20S	CY20S	M60Y	Long axis (µm)	Equatorial region	Ornamentation	Size (µm)	Ornamentation	References
A. chevalieri	16–24	45–65	30–49	65–>70	4.5–6.5	crests prominent	smooth	3.5–5.5	rough	this study
A. costiformis	18–21	33–38	36–39	>70	6–8	crests irregular	rough	4–8 (12)	smooth	this study
A. glaucus	3–20	30–45	0	0	6–7.5	crests absent	smooth	4.5–8.5	rough	[1, 4]
A. microperforatus	8–15	40–46	0	28–32	4–5.5	crests absent	smooth	6–9.5(11)	rough	this study
A. montevidensis	17–21	36–48	39–55	68–>70	3.5–5.5	crests irregular	rough	4.5–5.5	rough	this study
A. proliferans	n.a.	15–22	0	0	4.5–6	crests absent	smooth	5–9	rough	[1, 4]
A. pseudoglaucus	22–24	38–44	0	41–46	3.5–5.5	crests absent	smooth	7–8.5	rough	this study
A. ruber	n.a.	>30	0	n.a.	5–6	crests absent	smooth	5–7.5	rough	[1, 4]

CYA: Czapek yeast autolysate agar; MEA: malt extract agar; CY20S: CYA supplemented with 20% sucrose; M60Y: Harrold's agar; n.a.: not available.

TABLE 3 − Results of in vitro antifungal susceptibility test for 25 isolates of *Aspergillus* section *Aspergillus*

Species (no. of isolates)		MIC or MEC (μg/ml) for:							
		AMB	AFG	CFG	MFG	ITC	PSC	VRC	TBF
A. chevalieri (9)	GM	0.14	0.03	0.03	0.03	0.24	0.03	0.37	0.09
	MIC range	0.06–0,5	0.03	0.03	0.03	0.12–1.0	0.03	0.12–0.5	0.03–0.12
	Mode	0.12	0.03	0.03	0.03	0.5	0.03	0.5	0.12
A. costiformis (1)	Values	0.25	0.03	0.03	0.03	0.25	0.06	0.5	0.12
A. microperforatus (2)	GM	0.03	0.03	0.03	0.03	0.12	0.03	1.0	0.06
	MIC range	0.03	0.03	0.03	0.03	0.12	0.03	1.0	0.06
A. montevidensis (11)	GM	0.25	0.03	0.03	0.03	0.19	0.03	0.77	0.13
	MIC range	0.12–0.5	0.03–0.06	0,03	0.03	0.12–0.5	0.03–0.06	0.5–2.0	0.06–0.25
	Mode	0.25	0.03	0.03	0.03	0.12	0.03	0.5	0.12
	MIC90	0.5	0.03	0.03	0.03	0.25	0.03	1.0	0.25
A. pseudoglaucus (2)	GM	0.03	0.03	0.03	0.03	0.12	0.03	1.0	0.12
	MIC range	0.03	0.03	0.03	0.03	0.12	0.03	1.0	0.12
Total (25)	GM	0.14	0.03	0.03	0.03	0.2	0.03	0.57	0.1
	MIC range	0.03–0.5	0.03–0.06	0.03	0.03	0.12–1.0	0.03–0.06	0.12–2.0	0.03–0.25
	Mode	0.12	0.03	0,03	0.03	0.12	0.03	0.5	0.12
	MIC90	0.5	0.03	0.03	0.03	0,5	0.03	1.0	0.12

AMB, amphotericin B; AFG, anidulafungin; CFG, caspofungin; MFG, micafungin; ITC, itraconazole; PSC, posaconazole; VRC, voriconazole; TBF, terbinafine; MIC, minimum inhibitory concentration; MEC, minimum effective concentration, for AFG, CFG, and MFG; GM, geometric mean.

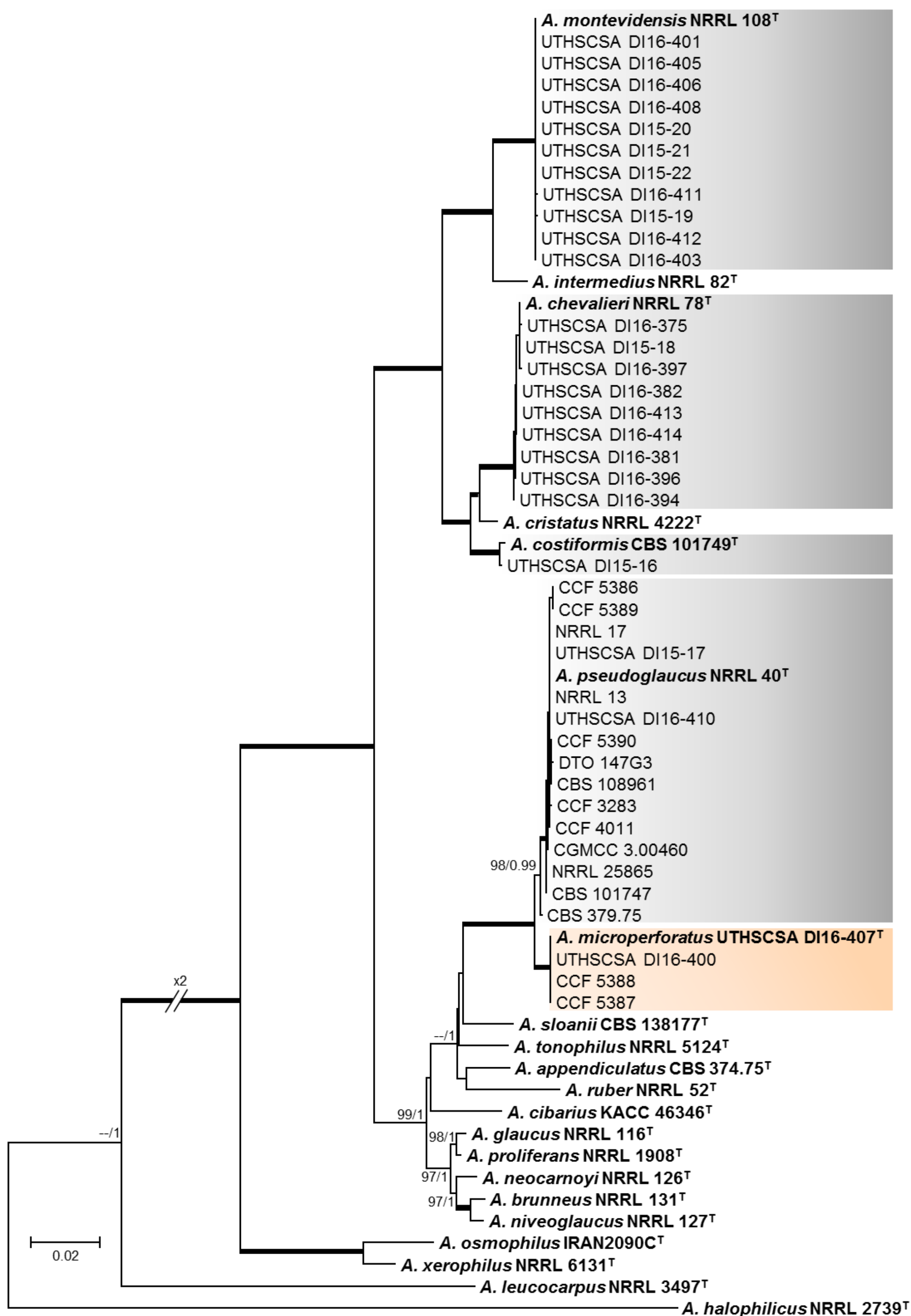


Fig 1

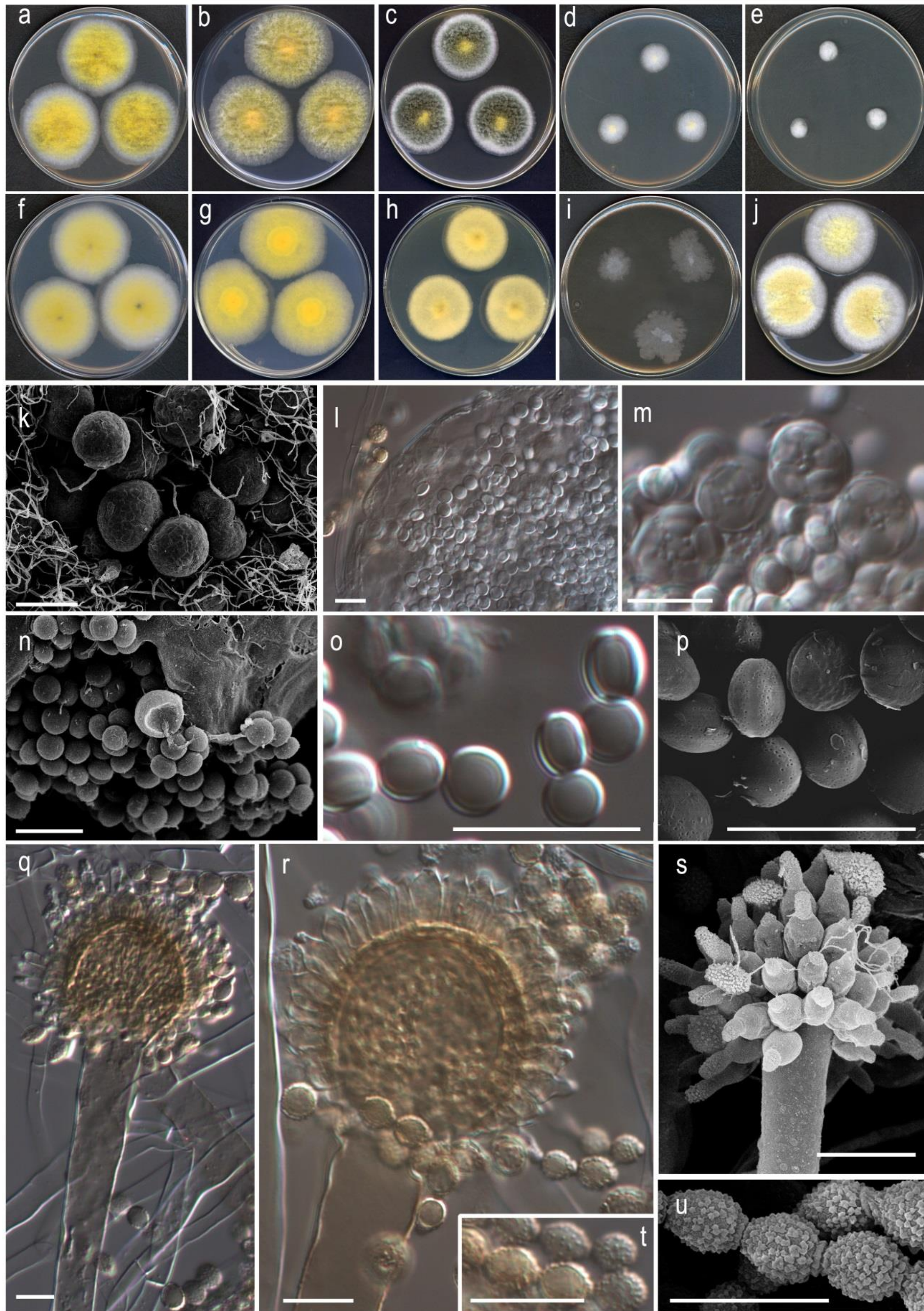


Fig 2

Fig S1 – Maximum likelihood tree obtained from the ITS sequences of the isolates. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. Type strains are shown in bold. The new species is shown in the colored box.

Fig S2 – Maximum likelihood tree obtained from the *BenA* sequences of the isolates. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The fully supported branches (100/1) and type strains are shown in bold. The new species is shown in the colored box.

Fig S3 – Maximum likelihood tree obtained from the *CaM* sequences of the isolates. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The fully supported branches (100/1) and type strains are shown in bold. The new species is shown in the colored box.

Fig S4 – Maximum likelihood tree obtained from the *RPB2* sequences of the isolates. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The fully supported branches (100/1) and type strains are shown in bold. The new species is shown in the colored box.

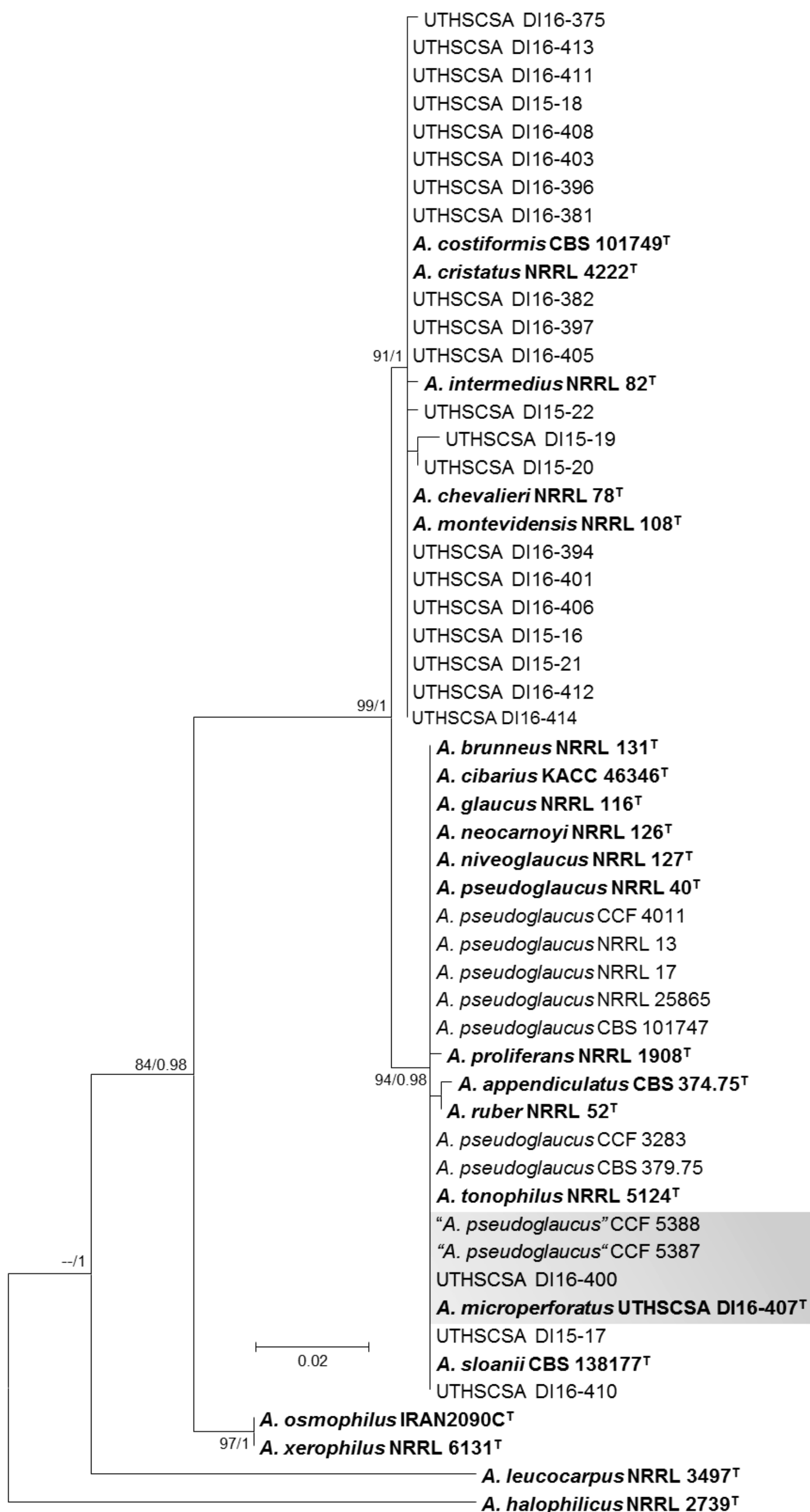


Fig S1

Fig S2

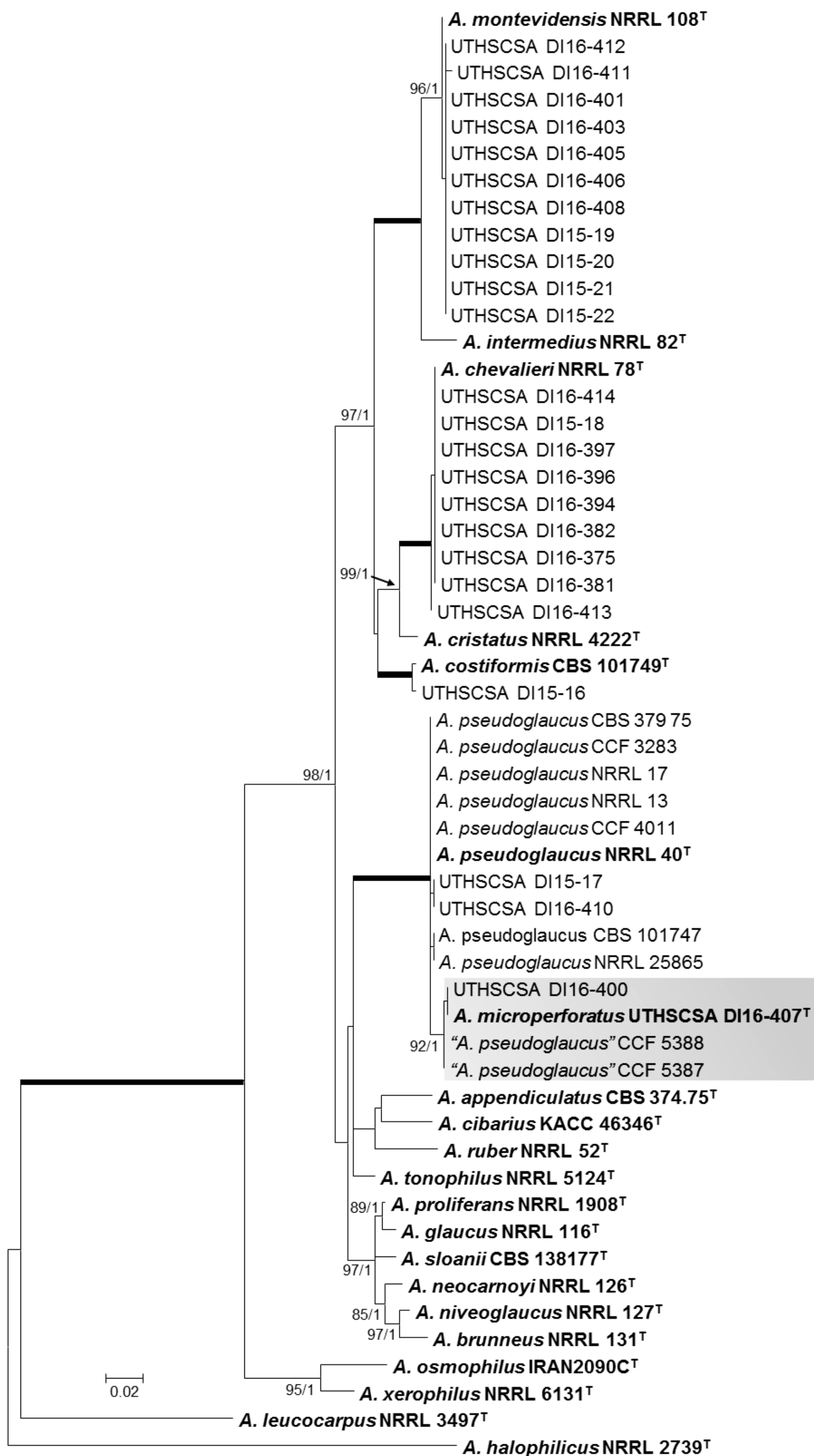


Fig S3

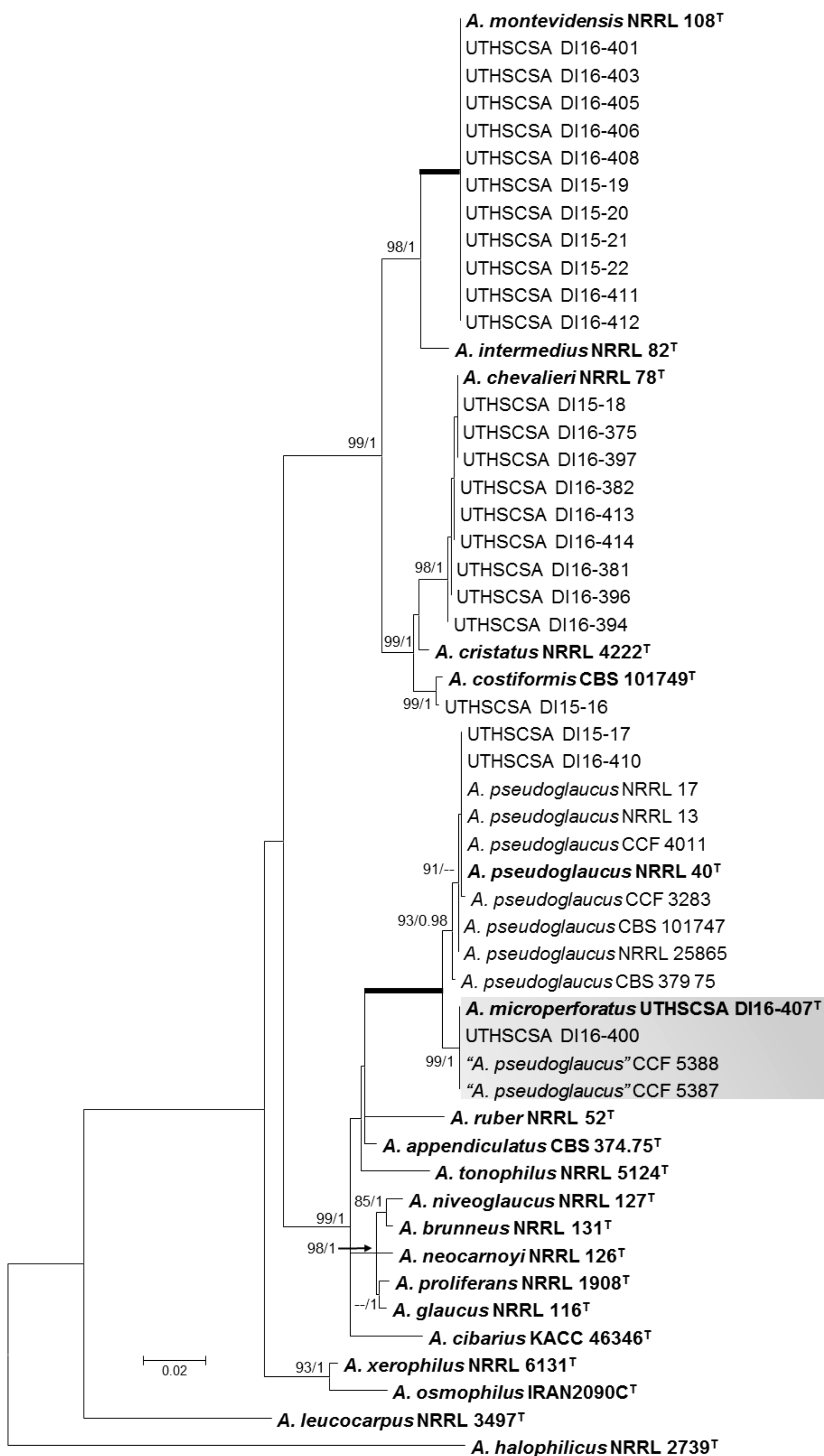


Fig S4

4.4. Cryptic *Aspergillus* species from clinical samples in the USA and description of a new species in section *Flavipedes*

João Paulo Zen Siqueira, Deanna A. Sutton, Josepa Gené, Dania García, Nathan Wiederhold, Margarete T. G. Almeida, Josep Guarro

In preparation to be submitted to: IMA Fungus

1 Cryptic *Aspergillus* species from clinical samples in the USA and description of a new
2 species in section *Flavipedes*
3
4 **Running Head:** Cryptic *Aspergillus* species from the USA
5
6 João Paulo Zen Siqueira[1,2], Deanna A. Sutton[3], Josepa Gené[1], Dania García[1], Nathan
7 Wiederhold[3], Margarete Teresa Gottardo de Almeida[2], Josep Guarro[1]
8
9 [1]Unitat de Micologia, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat
10 Rovira i Virgili, Reus, Spain.
11 [2]Laboratório de Microbiologia, Faculdade de Medicina de São José do Rio Preto, 5416
12 Brigadeiro Faria Lima Ave., 15090-000, São José do Rio Preto, Brazil.
13 [3]Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio,
14 TX, USA.
15
16 **Keywords:** *Aspergillus*, phylogenetic analysis, cryptic species, taxonomy
17
18 **Corresponding author:** Dania García
19 Unitat de Micologia, Facultat de Medicina i Ciències de la Salut
20 Universitat Rovira i Virgili
21 C/ Sant Llorenç 21 – 43201 – Reus, Spain
22 Ph: +34-977-759359; F: +34-977-759322; email: dania.garcias@urv.cat

23 **ABSTRACT**

24 In the last few decades there has been an emergence of cryptic *Aspergillus* species as
25 etiological agents of human infections due to the increase of immunocompromised
26 population, but also to the improvement of identification tools. Continuing our study on
27 *Aspergillus* isolates from clinical origin deposited in a mycological reference center in
28 the USA, we selected 37 isolates belonging to sections *Cremei*, *Flavipedes*, *Nidulantes*,
29 *Terrei* and *Usti*, in order to study their species diversity and detect cryptic species by
30 polyphasic approach. From this set of isolates, a total of 16 species were identified; the
31 most frequent being *A. calidoustus* (48.6%, section *Usti*), *A. terreus* (13.5%, section
32 *Terrei*), and *A. nidulans* (5.7%, section *Nidulantes*). The remaining isolates
33 corresponded to 13 species of rare or cryptic *Aspergillus*, i.e. *A. europaeus* (section
34 *Cremei*); *A. iizukae*, *A. micronesiensis*, *A. spelaeus* (section *Flavipedes*); *A.*
35 *pachycristatus*, *A. quadrilineatus*, *A. spinulosporus*, *A. unguis* (section *Nidulantes*); *A.*
36 *alabamensis*, *A. carneus*, *A. hortai* (section *Terrei*), *A. granulosus* (section *Usti*); and
37 one new species, which is described here as *A. hemisphaericus* (section *Flavipedes*).
38 Correct identification of cryptic species of *Aspergillus* is crucial to reveal new potential
39 pathogens, gather accurate epidemiological data, and to choose an appropriate
40 treatment.

41

42 **INTRODUCTION**

43 Members of *Aspergillus* are important fungal pathogens, mainly due to *A. fumigatus*
44 that is responsible for over 200,000 life-threatening infections per year worldwide
45 (Brown et al. 2012). However, in the last few decades, there has been a clear emergence
46 of new or rare *Aspergillus* as etiological agents of human infections (Lass-Flörl, 2009;
47 Masih et al. 2016). The reasons are primarily attributed to the continuous increasing in
48 the population of immunocompromised patients and, consequently, at risk of
49 opportunistic fungal infections (Kim 2016), but also due to the improvement of the
50 identification tools, such as sequencing and phylogenetic analyses, which enabled the
51 discrimination of close related species, revealing unknown pathogens.

52 Currently, *Aspergillus* has over 350 accepted species and they are organized in
53 subgenera and sections according to their morphological features and phylogenetic
54 relationships (Gams et al. 1985, Samson et al. 2014). Many of them are called cryptic
55 species since they share so many phenotypic features with others that is difficult to
56 differentiate them by classical methods (Howard 2014). Usually, when incapable of
57 being identified, cryptic species are treated as a complex or are presumably identified as
58 the most common pathogen of its section. For this reason, cases of misidentifications in
59 *Aspergillus* are common (Hubka et al. 2014; Khare et al. 2014; Tam et al. 2014), and
60 some species seems to be overestimate as human pathogens, such as *A. versicolor*
61 (Siqueira et al. 2016) or *A. glaucus* (Siqueira et al. 2017b in press). Additionally, two
62 large studies using DNA-based identification, one from transplant recipients in the USA
63 (Balajee et al. 2009a) and the other from a survey study carried out in Spain (Alastruey-
64 Izquierdo et al. 2013), revealed a concerning prevalence of cryptic species of
65 *Aspergillus*, 11 % and 12 %, respectively.

66 Better identification tools allow reliable species delimitation in *Aspergillus* and
67 also the discovery of new species, which is crucial for epidemiological purposes
68 (Balajee et al. 2007). Moreover, correct species identification is fundamental in
69 infections refractory to antifungal therapy, since susceptibility patterns may be variable
70 among phenotypically similar species (Alastruey-Izquierdo et al. 2013).

71 In previous studies we have investigated the occurrence of some less common
72 sections of *Aspergillus* in clinical isolates from the USA identified by multilocus
73 sequence analysis (Siqueira et al. 2016, 2017a, 2017b in press), expanding the diversity
74 of cryptic aspergilli described from human or animal clinical specimens. To complete
75 this search, we have studied by using a polyphasic approach species of *Aspergillus*
76 belonging to sections *Cremei*, *Flavipedes*, *Nidulantes*, *Terrei* and *Usti* isolated from
77 clinical specimens in the USA.

78

79 **MATERIALS AND METHODS**

80 **Fungal isolates**

81 From a set of clinical isolates received from the Fungus Testing Laboratory of the
82 University of Texas Health Science Center (San Antonio, USA), we selected 37 (Table
83 1) belonging to those sections of the genus poorly known in clinical setting (i.e.,
84 *Cremei*, *Flavipedes*, *Nidulantes*, *Terrei* and *Usti*). The isolates were preliminary
85 identified macro- and microscopically to the section level. To give more robustness to
86 the identification other isolates from different sources and locations belonging to those
87 sections were also included to the study (Table 1).

88 **Morphological characterization**

89 The fungal isolates were morphologically characterized following the recommended
90 criteria for *Aspergillus* (Samson et al. 2014). Colony morphology and growth rates were

91 determined after 7 days of incubation on Czapek Yeast Autolysate agar (CYA, Becton,
92 Dickinson and Company®, Sparks MD, USA), at 25 ºC and 37 ºC, and on Malt Extract
93 agar (MEA, Pronadisa®, Madrid, Spain), at 25 ºC. Microscopic structures were
94 examined and measured on slides from MEA cultures after 10–14 days of incubation.
95 Wet mounts were made in 60% lactic acid and the excess conidia were washed using a
96 drop of ethanol 70%. Photographs were made in a Zeiss Axio Imager M1 light
97 microscope (Zeiss, Oberkochen, Germany), using Nomarski differential interference
98 contrast and phase contrast optics, and with a mounted DeltaPix Infinity X digital
99 camera.

100 **DNA extraction, amplification, and sequencing**

101 Total genomic DNA was extracted from MEA cultures after 7 days of incubation at 25
102 ºC, using the FastDNA® Kit and the FastPrep® Instrument (MP Biomedicals, Irvine
103 CA, USA) according to the manufacturer's specifications. The amplification targeted
104 four genetic markers (Peterson 2008; Samson et al. 2014). They are the internal
105 transcribed spacer (ITS) regions of the rRNA, comprising ITS1, 5.8S gene, and ITS2
106 regions, using ITS5/ITS4 primers (White et al. 1990); a portion of the β-tubulin gene
107 (*BenA*), using Bt2a/Bt2b primers (Glass & Donaldson 1995); a fragment of the
108 calmodulin gene (*CaM*), using Cmd5/Cmd6 primers (Hong et al. 2005); and a part of
109 the RNA polymerase II second largest subunit gene (*RPB2*), using 5F/7CR primers (Liu
110 et al. 1999). PCR products were sequenced in both directions with the same primers, at
111 Macrogen Europe (Macrogen Inc., Amsterdam, Netherlands). Sequences were
112 assembled and edited with SeqMan v.7.0.0 (DNASTAR, Madison, WI, USA).

113 **Molecular identification and phylogenetic analysis**

114 Presumptive molecular identification was provided by pairwise sequence comparison in
115 databases available online (GenBank/EMBL and MycoBank). Final identification was

116 based on phylogenetic analyses, which were carried out individually for each gene and
117 also in a concatenated study. Sequences of ex-type strains were obtained from the
118 GenBank and added to the analyses. *Penicillium paradoxum* (CBS 527.65) was used as
119 outgroup.

120 Multiple sequence alignment was performed using ClustalW together with
121 MUSCLE in MEGA v.6 software (Tamura et al. 2013) and manual adjustments to
122 refine it when necessary. MEGA v.6 software was also used to conduct the Maximum
123 Likelihood (ML) analysis, and for the estimation of the best nucleotide substitution
124 method. Support of the internal branches was assessed by the Bootstrap method with
125 1,000 replications, where values equal or higher than 70% were considered significant.
126 In parallel, Bayesian Inference (BI) method was performed using MrBayes v.3.1.2
127 software (Ronquist & Huelsenbeck 2003). The evolutionary models that best fit each
128 gene were assessed by the software MrModelTest (Nylander 2004). Markov chain
129 Monte Carlo (MCMC) sampling was performed with two simultaneous runs for 1
130 million generations, with samples taken every 100 generations. The 50% majority rule
131 consensus trees and posterior probability values (pp) were calculated after removing the
132 first 25% of the resulting trees for burn-in. A pp value of 0.95 or higher was considered
133 significant in the tree.

134 **Nucleotide sequence accession numbers**

135 Sequences newly generated in this study were deposited in GenBank/EMBL databases
136 under accession numbers detailed in Table 1.

137

138 **RESULTS**

139 According to the final identification based on phylogenetic analyses and morphological
140 features, the 37 selected isolates were distributed in these sections as follows: 18 in

141 *Usti*, eight in *Nidulantes*, six in *Terrei*, four in *Flavipedes*, and one in section *Cremei*. A
142 total of 16 species were identified, the most frequent being *A. calidoustus* (n = 17,
143 section *Usti*), *A. terreus* (n = 5, section *Terrei*), and *A. nidulans* (n = 2, section
144 *Nidulantes*). The remaining isolates corresponded to 13 cryptic *Aspergillus* species,
145 each with one isolate. Among them, a new species was found in section *Flavipedes*,
146 which is described further ahead, and other 12 known species: *A. europaeus* (section
147 *Cremei*); *A. iizukae*, *A. micronesiensis*, *A. spelaeus* (section *Flavipedes*); *A.*
148 *pachycristatus*, *A. quadrilineatus*, *A. spinulosporus*, *A. unguis* (section *Nidulantes*); *A.*
149 *alabamensis*, *A. carneus*, *A. hortai* (section *Terrei*); and *A. granulosus* (section *Usti*).
150 At first, identification of these cryptic *Aspergillus* were tentatively carried out by
151 pairwise sequence analyses in the GenBank database using the BLAST tool. However,
152 in many cases, results provided were not satisfactory to identify such species, since
153 some isolates retrieved two or more matches with similarities of 99 % or higher with
154 different species. As expected, ITS sequences showed the least discriminatory power at
155 the species level, being able to identify only two isolates (Table 2). The most reliable
156 marker was RPB2, which allowed identifying unequivocally seven isolates (Table 2).
157 The other two markers (*BenA* and *CaM*) exhibited the same results, being able to
158 identify the same five isolates (Table 2). The BLAST searches could not identify five
159 isolates with any of the markers studied individually, i.e. UTHSCSA DI14-213 (*A.*
160 *carneus*), UTHSCSA DI14-214 (*A. micronesiensis*), UTHSCSA 14-805 (*A.*
161 *pachycristatus*), and UTHSCSA 05-237 (*A. quadrilineatus*). The closest matches with
162 the fifth isolate (UTHSCSA DI14-215) were *A. templicola* (96% for *BenA* and 98% for
163 *RPB2*) and *A. urmiensis* (95% for *CaM*), confirming that it represented an undescribed
164 species.

165 By contrast, the phylogenetic tree reconstructed with the concatenated alignment
166 of the four sequenced loci revealed the identity of all the cryptic species studied (Figure
167 1). The final alignment included sequences of 15 clinical isolates, 18 isolates from
168 different sources and locations, and the ex-type strains of the species closest related to
169 clinical isolates. The ex-type strain of *A. calidoustus* was not included in the
170 concatenate alignment since the *RPB2* sequence was not available for comparison. It
171 consisted of 2,679 nucleotides (ITS, 545; *BenA*, 519; *CaM*, 617; *RPB2*, 998), from
172 which 1332 were variable sites (ITS, 200; *BenA*, 317; *CaM*, 394; *RPB2*, 421) and 1186
173 phylogenetic informative (ITS, 143; *BenA*, 291; *CaM*, 368; *RPB2*, 384). Topologies of
174 the trees by ML and BI methods were very similar, with minor differences on the
175 support values of internal nodes. The ML tree was selected to represent the relationship
176 among the isolates included in the analyses. To confirm that the isolate UTHSCSA
177 DI14-215 constituted a new species, analyses were carried out also considering single
178 gene phylogenies of section *Flavipedes* (supplementary material).

179 The clinical isolates studied here showed the typical morphology of their
180 corresponding sections, except the isolate UTHSCSA DI14-225 (*A. granulosus*) since it
181 did not sporulate in any of media tested. The main characteristics of the cryptic species
182 identified are summarized in Table 3. The most common relevant feature from all these
183 species, except for *A. spelaeus*, is their ability to grow at 37 ºC on CYA, which suggest
184 the potential pathogenicity of these fungi for humans.

185

186 **TAXONOMY**

187 *Aspergillus hemisphaericus* J.P.Z. Siqueira, Deanna A. Sutton, Gené, García & Guarro,
188 sp. nov. MycoBank MBXXXX. Figure 2.

189 In — section *Flavipedes*

190 Etymology — Name refers to the typical shape of the vesicles in the conidiophores of
191 the species.
192 Specimen examined — USA, from human sputum, 2014, D. Sutton (holotype CBS H-
193 XXXX; culture ex-type UTHSCSA DI14-215 = FMR 13523 = CBS XXXX; ITS
194 barcode LT899487, alternative markers: *Ben A*LT899536, *CaM* LT899589, *RPB2*
195 LT899644).
196 Colony diameter in 7d (mm) — On CYA: 25 ºC 24–25, 30 ºC 32–33, 37 ºC 14–16; on
197 MEA: 25 ºC 24–25; on DG18: 25 ºC 16–17; on YES: 25 ºC 16–19; on OA: 25 ºC 20–
198 21; on CREA: 25 ºC 19–20.
199 Colony characters at 25 ºC in 7 d — On CYA, colonies floccose, mycelium white to
200 yellowish white (4A2), margin slightly lobulate and fimbriate; reverse reddish brown
201 (8E5); sporulation dense, with conidial masses white to yellowish white, turning pastel
202 green (10A4) to olive (2E4) in 14 d; soluble pigment light yellow (4A4) to orange
203 yellow (4A7); exudate pale yellow (3A3) to orange yellow (4A6). On MEA, colonies
204 velvety to powdery, furrowed, mycelium white to orange white (5A2), margin
205 fimbriate; reverse pale orange (5A3) to light brown (6D6); sporulation dense, with
206 conidial masses orange white (5A2) to greyish yellow (2B6) towards the center; soluble
207 pigment light yellow (4A5), inconspicuous; exudate colorless. On DG18, colonies
208 floccose to slightly powdery, mycelium white, margin predominantly entire; reverse
209 pale yellow (4A3) to light yellow (4A5); sporulation dense, with conidial masses
210 yellowish white (4A2) to pale orange (5A3); soluble pigment absent; exudate absent.
211 On YES, colonies powdery, slightly furrowed, and center slightly elevated, mycelium
212 white, margin entire; reverse light yellow (4A5); sporulation dense, with conidial
213 masses white; soluble pigment absent; exudate absent. On OA, colonies floccose at the
214 centre, with submerged mycelium towards the periphery, mycelium white, margin

215 entire; reverse yellowish white (4A2) to pale orange (5A3); sporulation dense, with
216 conidial masses pale yellow (4A3); soluble pigment absent; exudate absent. On CREA,
217 colonies floccose, mycelium white, margin predominantly entire; sporulation dense,
218 with conidial masses yellowish white (3A2) to greyish yellow (1A5); acid production
219 absent.
220 Micromorphology — On MEA, conidiophores with conidial heads biseriate and
221 occasionally slightly to strongly bent or bifurcate, mainly columnar, more rarely loosely
222 radiate, orange white (5A2) in 7 d, becoming greyish green (27B5) in 14 d; stipes
223 commonly septate, 180–420 x 4.5–6.5 µm, smooth, hyaline; vesicles mainly
224 hemispherical, occasionally globose, (6)12–17 µm wide, hyaline; metulae cylindrical,
225 covering 75% of the vesicle, 4.5–6.5 x 2.5–4.5 µm, hyaline; phialides, cylindrical with
226 the apex slightly narrower, 6–8 x 2.5–4 µm, hyaline; conidia globose, 2–3.5 µm diam,
227 smooth, hyaline. Hülle cells and ascomata not observed.
228 Distinguish characters — *Aspergillus hemisphaericus* clusters in a terminal clade along
229 with *A. templicola* and *A. urmiensis* in section *Flavipedes*. *Aspergillus templicola* was
230 described from house dust (Visagie et al. 2014) and currently includes the synonym *A.*
231 *mangaliensis* (Hubka et al. 2015). *Aspergillus templicola* differs from our novel species
232 mainly by its consistently elongated vesicles; in *A. hemisphaericus* the vesicles are
233 commonly hemispherical. *Aspergillus urmiensis* was described from hypersaline soil
234 (Arzanlou et al. 2016), and it differs by showing globose vesicles with radiate growth,
235 while in *A. hemisphaericus* has conidial heads mainly columnar.
236

237 **DISCUSSION**

238 The multilocus sequence analysis of a large set of *Aspergillus* isolates from clinical
239 origin carried out in successive studies (Siqueira et al. 2016; 2017a; 2017b in press),

240 including the present one, has been essential to highlight the great species diversity in
241 *Aspergillus* sections poorly studied in clinical setting. In these studies, from a total of
242 173 isolates belonging to the sections *Aspergillus, Circumdati*, *Cremei, Flavipedes,*
243 *Nidulantes, Terrei, Usti* and *Versicolores*, we have identified 38 species, many of them
244 considered cryptic because of their morphological similarity with the most common
245 pathogens of each section studied. It is noteworthy that among these species eight were
246 reported for the first time from animal and human clinical specimens, including three
247 new species, namely *A. pseudosclerotiorum* (Siqueira et al. 2017a), *A. microperforatus*
248 (Siqueira et al. 2017b in press), and *A. hemisphaericus*, proposed here. Despite the role
249 of the species identified in causing infection has not been demonstrated in any case,
250 they should be considered at least as potential opportunistic pathogens.

251 In the present study, we identified *A. europaeus* in section *Cremei*. Members of
252 this section are mainly considered soil-borne fungi and being able to cause spoilage of
253 cereals and nuts (Samson et al. 2010). Although their isolation from clinical samples is
254 rare, Hubka et al. (2016) suggested that *A. europaeus* is a relative common species in
255 clinical setting and it has been overlooked and frequently misidentified, especially as *A.*
256 *wentii*, the most well-known species of this section. The two species can be
257 distinguished by the production of yellow soluble pigment on MEA in *A. europaeus*, not
258 present in *A. wentii* (Hubka et al. 2016). Moreover, they can be differentiated by the size
259 of the conidial heads at the colony center, which usually does not exceed 200 µm diam
260 in *A. europaeus*, and are larger in *A. wentii* (approximately 500 µm diam) (Hubka et al.
261 2016).

262 From section *Flavipedes*, two of the species identified are noteworthy, *A.*
263 *spelaeus*, which has not been previously recovered from clinical samples, and the new
264 species *A. hemisphaericus*. In general, members of this section are common in soil, but

265 can also be isolated from food, indoor environments, and as endophytes (Klich 2002;
266 Hubka et al. 2015). Based on polyphasic taxonomic studies, Hubka et al. (2015)
267 demonstrated that *A. iizukae*, *A. frequens*, and *A. mangaliensis* were the most common
268 and widely distributed species of this section, whereas *A. flavipes* was considered a rare
269 species. It is noteworthy however, that historically *A. flavipes* has been considered the
270 only clinically relevant species in the section (Buzina 2013), being reported in cases of
271 cutaneous aspergillosis (Barson & Ruymann 1986), osteomyelitis (Tack et al. 1982),
272 otomycosis (Stuart & Blank 1955), onychomycosis (Gehlot et al. 2011), and in chronic
273 necrotizing pulmonary aspergillosis (Katou et al. 1999). However, Hubka et al. (2015)
274 re-identified several *A. flavipes* clinical isolates as *A. frequens,* and also suggested that
275 most cases of infection reported up to date could be caused by *A. frequens*. Currently, *A.*
276 *frequens* is considered a synonym of *A. micronesiensis* (Arzanlou et al. 2016); both
277 species were practically described simultaneously (Visagie et al. 2014; Hubka et al.
278 2015), being considered this latter the name valid for the species. We identified *A.*
279 *micronesiensis* from a canine urine sample.

280 In section *Nidulantes*, *A. nidulans* is considered the leading pathogen, being
281 involved in a wide range of infections, such as sinusitis, endophthalmitis, osteomyelitis,
282 catheter-related skin infection, and pulmonary infections (de Hoog et al. 2011). This
283 species is especially relevant in chronic granulomatous disease patients (Henriet et al.
284 2012). Other species identified here, such as *A. quadrilineatus*, *A. spinulosporus* and *A.*
285 *unguis*, have already been reported as cause of human infections. *Aspergillus*
286 *quadrilineatus* has been involved in sinusitis (Polacheck et al. 1992), onychomycosis
287 (Gugnani et al. 2004), and in invasive infections (Verweij et al. 2008). Verweij et al.
288 (2008) did a molecular re-identification of *A. nidulans* isolates, revealing that they were
289 in fact *A. quadrilineatus*. *Aspergillus spinulosporus* has been reported in invasive

290 pulmonary aspergillosis (White et al. 1988; Yu et al. 2013) and in a prosthetic valve
291 endocarditis (Uhrin et al. 2015). *Aspergillus unguis* has been reported as causal agent of
292 several cases of onychomycosis (Grigoriu & Grigoriu 1975; Hubka et al. 2012), and has
293 also been associated with cases of asthma in children (Rabinovitch 2012). This latter
294 species is also morphologically similar to *A. nidulans* (Howard 2003), but they can be
295 differentiated by the ability to grow at 40 ºC; while *A. nidulans* grows profusely,
296 reaching 46 mm in 5 days, *A. unguis* is unable to grow at this temperature (Chen et al.
297 2016). On the other hand, *A. pachycristatus* is the only species of the section *Nidulantes*
298 identified here that has not been reported from clinical samples before. This species was
299 described from Chinese soil (Matsuzawa et al. 2012), and morphologically also
300 resembles *A. nidulans*, but the ascospores of *A. pachycristatus* show thicker crests
301 (Chen et al. 2016). The closest related species to *A. pachycristatus* is *A. rugulosus,*
302 however the latter can be distinguished by the rugulose ornamentation on the convex
303 surface of ascospores; in *A. pachycristatus*, the ascospore surface is smooth (Chen et al.
304 2016).

305 As expected, *A. terreus* has been the most frequent species of the section *Terrei*
306 identified in our study. This species is a well-known pathogen involved in pulmonary,
307 cardiovascular, and disseminated infections among other human and animal diseases (de
308 Hoog et al. 2011). The treatment of this fungus is often complex because it is
309 intrinsically resistant to amphotericin B (Pastor & Guarro 2014), as occurs with other
310 species of the section (Buzina 2013). The species distribution of section *Terrei* in clinic
311 is hard to assess, mainly because *A. terreus* is often considered a species complex
312 (Balajee 2009, Fernandez et al. 2013). Indeed, the species in this section are
313 morphologically very similar and genetically closely related, being susceptible to
314 misidentifications. That is the case of two species reported here, *A. alabamensis* and *A.*

315 *hortai*, which were described after phylogenetic analyses of several clinical isolates
316 previously identified as *A. terreus* (Balajee et al. 2009b, Dodge 1935). Therefore, in
317 agreement with Samson et al. (2011), the frequency of these species in clinic is
318 probably underestimated due to misidentifications. The fourth species in section *Terrei*
319 identified here is *A. carneus*. This species was already associated with pulmonary
320 aspergillosis (Morquer & Enjalbert 1957) and its pathogenicity confirmed in mice (Pore
321 & Larsh 1968). Since *A. carneus* shows white colonies, it has already been
322 misidentified as *A. candidus* (Hubka et al. 2014). However, *A. candidus*, in addition to
323 belong to section *Candidi*, has slow growth rates and it is unable to grow at 37 ºC
324 (Varga et al. 2007). *Aspergillus carneus* is phylogenetically close to *A. niveus*, but they
325 can be differentiated since the while colonies of *A. carneus* acquire pink and purple
326 tones in age, whereas those of *A. niveus* are persistently white (Raper & Fennell 1965).

327 The most frequent species identified in the present study has been *A. calidoustus*
328 in section *Usti*. This species was introduced in 2008 on the basis of clinical strains
329 previously identified as *A. ustus* (Varga et al. 2008). Thenceforth, a rising number of
330 infections attributed to *A. calidoustus* have been reported, which has also acquired
331 significance because of its elevated MICs for triazoles (Varga et al. 2008; Alastruey-
332 Izquierdo et al. 2010; Hubka et al. 2012; Seroy et al. 2017). Another species of the
333 section identified in this study is *A. granulosus*. This species is an uncommon
334 opportunist but clinically relevant since it has been diagnosed as causal agent of a
335 disseminated infection in a cardiac transplant recipient (Fakih et al. 1995) and cerebral
336 aspergillosis (Sutton et al. 2009). In addition, it has been associated to endodontic
337 infections (Gomes et al. 2010), and in our case isolated from a human brainstem sample.

338 The correct identification of cryptic or uncommon species of *Aspergillus* can be
339 a challenging task. DNA-based approaches improved the level of confidence of the

340 identifications but it may not be enough. Pairwise comparison of a sequence in a
341 database, such the BLAST tool in the GenBank, is very useful but not sufficient in
342 many cases. The ITS region, the universal fungal barcode (Schoch et al. 2012), in
343 *Aspergillus* has resolution only to the section level as we could demonstrate here, where
344 only two isolates investigated could be identified with this marker. Although the other
345 markers (*BenA*, *CaM*, and *RPB2*) proved to be more informative, the comparison of a
346 unique marker often cannot distinguish between cryptic species and their closely genetic
347 relatives. Also, it must be taken into account that many isolates in databases may not be
348 correctly identified or their names have not been updated. For example, up to date, the
349 recently described *A. urmiensis* still appears in the GenBank as *Aspergillus* sp., or
350 species now synonymized with *A. quadrilineatus* are shown with their old names such
351 as *Emericella quadrilineata*, *A. nidulans* var. *acristatus*, *A. tetrazonus*, *A. parvathecius*,
352 and *A. miyajii*. Another important limitation is that the *RPB2,* which has demonstrated
353 to be one of the best markers for *Aspergillus*, and that in our study was able to identify
354 seven isolates, is not currently available for all ex-type species.

355 Another difficulty in identifying cryptic species is the fact that the taxonomy is
356 under continuous update, experimenting nomenclatural modifications due to the new
357 criteria on fungal nomenclature (McNeill et al. 2012), synonymizations (Houbraken et
358 al. 2014; Chen et al. 2016) and new species being constantly described in the current
359 literature (Varga et al. 2007, 2008; Balajee et al. 2009b; Samson et al. 2011, 2014;
360 Hubka et al. 2014, 2015, 2016; Arzanlou et al. 2016; Chen et al. 2016; Siqueira et al.
361 2017a,b). Among the 12 known species reported in this study, three of them (*A.*
362 *europaeus*, *A. micronesiensis*, and *A. spelaeus*) had not yet been described at the
363 moment of their isolation.

364 Nowadays, the polyphasic approach, combining multilocus phylogenetic
365 analyses using sequences of ex-type strains and the phenotypic characterization, is the
366 recommended methodology to reach reliable species identification in *Aspergillus*
367 (Samson et al. 2014). In the clinical practice, this methodology may be unfeasible,
368 because it is not cost-effective or in most cases is reserved to basic research. However,
369 it is universally accepted that accurate identification of clinical isolates and better-
370 defined epidemiological data is crucial to understand the true impact of fungal diseases
371 and to determine the outcome of a patient.

372

373 **ACKNOWLEDGMENTS**

374 This study was supported by the Spanish Ministerio de Economía y Competitividad,
375 grant CGL2013-43789-P, and by CAPES (Coordenação de Aperfeiçoamento de Pessoal
376 de Nível Superior, Brasil), grant BEX 0623/14-8.

377

378 **REFERENCES**

379 Alastruey-Izquierdo A, Cuesta I, Houbraken J, Cuenca-Estrella M, Monzón A,
380 Rodriguez-Tudela JL (2010) In vitro activity of nine antifungal agents against clinical
381 isolates of *Aspergillus calidoustus*. Medical Mycology 48: 97–102.
382
383 Alastruey-Izquierdo A, Mellado E, Peláez T, Pemán J, Zapico S, Alvarez M,
384 Rodríguez-Tudela JL, Cuenca-Estrella M, FILPOP Study Group (2013) Population-
385 based survey of filamentous fungi and antifungal resistance in Spain (FILPOP study).
386 *Antimicrobial Agents and Chemotherapy* **57**: 3380–3387.
387
388 Arzanlou M, Samadi R, Frisvad JC, Houbraken J, Ghosta Y (2016) Two novel
389 *Aspergillus* species from hypersaline soils of the National Park of Lake Urmia, Iran.
390 *Mycological Progress* **15**: 1081–1092.
391
392 Balajee SA (2009) *Aspergillus terreus* complex. *Medical Mycology* **47**: S42–S46.
393
394 Balajee SA, Baddley JW, Peterson SW, Nickle D, Varga J, Boey A, Lass-Flörl C,
395 Frisvad JC, Samson RA, ISHAM Working Group on *A. terreus* (2009b) *Aspergillus*
396 *alabamensis*, a new clinically relevant species in the section *Terrei*. *Eukaryotic Cell* **8**:
397 713–722.
398

399 Balajee SA, Borman AM, Brandt ME, Cano J, Cuenca-Estrella M, Dannaoui E, Guarro
400 J, Haase G, Kibbler CC, Meyer W, O'Donnell K, Petti CA, Rodriguez-Tudela JL,
401 Sutton D, Velegraki A, Wickes BL (2009a) Sequence-based identification of
402 *Aspergillus*, *Fusarium*, and Mucorales species in the clinical mycology laboratory:
403 where are we and where should we go from here? *Journal of Clinical Microbiology* **47**:
404 877–884.
405
406 Balajee SA, Houbraken J, Verweij PE, Hong SB, Yaghuchi T, Varga J, Samson RA
407 (2007) *Aspergillus* species identification in the clinical setting. *Studies in Mycology* **59**:
408 39–46.
409
410 Barson WJ, Ruymann FB (1986) Palmar aspergillosis in immunocompromised children.
411 *The Pediatric Infectious Disease Journal* **5**: 264–268.
412
413 Brown GD, Denning DW, Gow NAR, Levitz SM, Netea MG, White TC (2012) Hidden
414 killers: human fungal infections. *Science Translational Medicine* **4**: 1–9.
415
416 Buzina W (2013) *Aspergillus* – classification and antifungal susceptibilities. *Current*
417 *Pharmaceutical Design* **19**: 3615–3628.
418
419 Chen AJ, Frisvad JC, Sun BD, Varga J, Kocsubé S, Dijksterhuis J, Kim DH, Hong SB,
420 Houbraken J, Samson RA (2016) *Aspergillus* section *Nidulantes* (formerly *Emericella*):
421 polyphasic taxonomy, chemistry and biology. *Studies in Mycology* **84**: 1–118.
422
423 De Hoog GS, Guarro J, Gené J, Figueras MJ (2011) Atlas of Clinical Fungi CD-ROM
424 version 3.1. Westerdijk Fungal Biodiversity Centre, Utrecht.
425
426 Dodge CW (1935) Medical mycology; fungous diseases of men and other mammals.
427 The C.V. Mosby Company, USA.
428
429 Fakih MG, Barden GE, Oakes CA, Berenson CS (1995) First reported case of
430 *Aspergillus granulosus* infection in a cardiac transplant patient. *Journal of Clinical*
431 *Microbiology* **33**: 471–473.
432
433 Fernandez MS, Rojas FD, Cattana ME, Sosa ML, Mangiaterra ML, Giusiano GE (2013)
434 *Aspergillus terreus* complex: an emergent opportunistic agent of onychomycosis.
435 *Mycoses* **56**: 477–481.
436
437 Gams W, Christensen M, Onions AH, Pitt JI, Samson RA (1985) Infrageneric taxa of
438 *Aspergillus*. In: Advances in *Penicillium* and *Aspergillus* Systematics. (RA Samson & JI
439 Pitt, eds): 55–62. Plenum Press, USA.
440
441 Glass NL, Donaldson GC (1995) Development of primer sets designed for use with the
442 PCR to amplify conserved genes from filamentous ascomycetes. *Applied and*
443 *Environmental Microbiology* **61**: 1323–1330.
444
445 Gehlot P, Purohit DK, Singh SK (2011) Molecular diagnostics of human pathogenic
446 *Aspergillus* species. *Indian Journal of Biotechnology* **10**: 207–211.
447

448 Gomes C, Fidel S, Fidel R, Sarquis MI (2010) Isolation and Taxonomy of Filamentous
449 Fungi in Endodontic Infections. *Journal of Endodontics* **36**: 626–629.
450
451 Grigoriu D, Grigoriu A (1975) Les onychomycoses. *Revue Medicale Suisse* **95**: 839–
452 849.
453
454 Gugnani HC, Vijayan VK, Tyagi P, Sharma S, Stchigel AM, Guarro J (2004)
455 Onychomycosis due to *Emericella quadrilineata*. *Journal of Clinical Microbiology* **42**:
456 914–916.
457
458 Henriet SSV, Verweij PE, Warris A (2012) *Aspergillus nidulans* and chronic
459 granulomatous disease: a unique host-pathogen interaction. *The Journal of Infectious*
460 *Diseases* **206**: 1128–1137.
461
462 Hong SB, Go SJ, Shin HD, Frisvad JC, Samson RA (2005) Polyphasic taxonomy of
463 *Aspergillus fumigatus* and related species. *Mycologia* **97**: 1316–1329.
464
465 Houbraken J, de Vries RP, Samson RA (2014) Modern taxonomy of biotechnologically
466 important *Aspergillus* and *Penicillium* species. *Advances in Applied Microbiology* **86**:
467 199–249.
468
469 Howard DH (2003) Pathogenic fungi in humans and animals. 2nd edn. Marcel Dekker,
470 USA.
471
472 Howard SJ (2014) Multi-resistant aspergillosis due to cryptic species. *Mycopathologia*
473 **178**: 435–439.
474
475 Hubka V, Kubatova A, Mallatova N, Sedlacek P, Melichar J, Skorepova M, Mencl K,
476 Lyskova P, Sramkova B, Chudickova M, Hamal P, Kolarik M (2012) Rare and new
477 etiological agents revealed among 178 clinical *Aspergillus* strains obtained from Czech
478 patients and characterized by molecular sequencing. *Medical Mycology* **50**: 601–610.
479
480 Hubka V, Lyskova P, Frisvad JC, Peterson SW, Skorepova M, Kolarik M (2014)
481 *Aspergillus pragensis* sp. nov. discovered during molecular reidentification of clinical
482 isolates belonging to *Aspergillus* section *Candidi*. *Medical Mycology* **52**: 565–576.
483
484 Hubka V, Novakova A, Kolarik M, Jurjevic Z, Peterson SW (2015) Revision of
485 *Aspergillus* section *Flavipedes*: seven new species and proposal of section *Jani* sect.
486 nov. *Mycologia* **107**: 169–208.
487
488 Hubka V, Novakova A, Samson RA, Houbraken J, Frisvad JC, Sklenar F, Varga J,
489 Kolarik M (2016) *Aspergillus europaeus* sp. nov., a widely distributed soil-borne
490 species related to *A. wentii* (section *Cremei*). *Plant Systematics and Evolution* **302**: 641–
491 650.
492
493 Katou K, Nanjou K, Kawakami M (1999) A case of chronic necrotizing pulmonary
494 aspergillosis due to *Aspergillus flavipes* associated with pulmonary fibrosis. *The*
495 *Journal of the Japanese Respiratory Society* **37**: 938–42.
496

497 Khare R, Gupta S, Arif S, Jentoft ME, Deziel PJ, Roden AC, Wilhelm MP, Razonable
498 RR, Wengenack NL (2014) Misidentification of *Neosartorya pseudofischeri* as
499 *Aspergillus fumigatus* in a lung transplant patient. *Journal of Clinical Microbiology* **52**:
500 2722–2725.
501
502 Kim JY (2016) Human fungal pathogens: why should we learn? *Journal of*
503 *Microbiology* **54**: 145–148.
504
505 Klich MA (2002) Biogeography of *Aspergillus* species in soil and litter. *Mycologia* **94**:
506 21–27.
507
508 Lass-Flörl C (2009) The changing face of epidemiology of invasive fungal disease in
509 Europe. *Mycoses* **52**: 197–205.
510
511 Liu YJ, Whelen S, Hall BD (1999) Phylogenetic relationships among ascomycetes:
512 evidence from an RNA polymerase II subunit. *Molecular Biology and Evolution* **16**:
513 1799–1808.
514
515 Masih A, Singh PK, Kathuria S, Agarwal K (2016) Identification by molecular methods
516 and matrix-assisted laser desorption ionization – time of flight mass spectrometry and
517 antifungal susceptibility profiles of clinically significant rare *Aspergillus* species in a
518 referral chest hospital in Delhi, India. *Journal of Clinical Microbiology* **54**: 2354–2364.
519
520 Matsuzawa T, Tanaka R, Horie Y, Hui Y, Abliz P, Yaguchi T (2012) The correlation
521 among molecular phylogenetics, morphological data, and growth temperature of the
522 genus *Emericella*, and a new species. *Mycoscience* **53**: 433–445.
523
524 McNeill J, Barrie FF, Buck WR, Demoulin V, Greuter W, Hawksworth DL, Herendeen
525 PS, Knapp S, Marhold K, Prado J, Prud'homme Van Reine WF, Smith GF, Wiersema
526 JH (2012) International code of nomenclature for algae, fungi and plants (Melbourne
527 code). Koeltz Scientific Books, Germany.
528
529 Morquer R, Enjalbert L (1957) Étude morphologique et physiologique d'un *Aspergillus*
530 nouvellement isolé au cours d'une affection pulmonaire de l'homme. *Comptes Rendus*
531 *de l'Académie des Sciences Paris* **244**: 1405–1408.
532
533 Nylander JAA (2004) MrModeltest v2. Program distributed by the author. Evolutionary
534 Biology Centre, Uppsala University.
535
536 Pastor FJ, Guarro J (2014) Treatment of *Aspergillus terreus* infections: a clinical
537 problem not yet resolved. *International Journal of Antimicrobial Agents* **44**: 281–289.
538
539 Peterson SW (2008) Phylogenetic analysis of *Aspergillus* species using DNA sequences
540 from four loci. *Mycologia* **100**: 205–226.
541
542 Polacheck I, Nagler A, Okon E, Drakos P, Plaskowitz J, Kwon-Chung KJ (1992)
543 *Aspergillus quadrilineatus*, a new causative agent of fungal sinusitis. *Journal of Clinical*
544 *Microbiology* **30**: 3290–3293.
545

546 Pore RS, Larsh HW (1968) Experimental pathology of *Aspergillus terreus-flavipes*
547 group species. *Sabouraudia* **6**: 89–93.
548
549 Rabinovitch N (2012) Household mold as a predictor of asthma risk: recent progress,
550 limitations, and future directions. *Journal of Allergy and Clinical Immunology* **130**:
551 645–646.
552
553 Raper KB, Fennell DI (1965) The genus *Aspergillus*. Robert E. Krieger Publishing Co.,
554 USA.
555
556 Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference
557 under mixed models. *Bioinformatics* **19**: 1572–1574.
558
559 Samson RA, Houbraken J, Thrane U, Frisvad JC, Andersen B (2010) Food and indoor
560 fungi. Westerdijk Fungal Biodiversity Centre, The Netherlands.
561
562 Samson RA, Peterson SW, Frisvad JC, Varga J (2011) New species in *Aspergillus*
563 section *Terrei*. *Studies in Mycology* **69**: 39–55.
564
565 Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CH, Perrone G,
566 Seifert KA, Susca A, Tanney JB, Varga J, Kocsube S, Szigeti G, Yaguchi T, Frisvad JC
567 (2014) Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Studies in*
568 *Mycology* **78**: 141–173.
569
570 Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, et al. (2012) Nuclear
571 ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker
572 for fungi. *Proceedings of the National Academy of Sciences of the United States of*
573 *America* **109**: 6241–6246.
574
575 Siqueira JPZ, Sutton DA, García D, Gené J, Thomson P, Wiederhold N, Guarro J
576 (2016) Species diversity of *Aspergillus* section *Versicolores* in clinical samples and
577 antifungal susceptibility. *Fungal Biology* **120**: 1458–1467.
578
579 Siqueira JPZ, Sutton DA, Gené J, García D, Wiederhold N, Guarro J (2017b in press)
580 Species of *Aspergillus* section *Aspergillus* from clinical samples in USA. *Medical*
581 *Mycology*.
582
583 Siqueira JPZ, Sutton DA, Gené J, García D, Wiederhold N, Peterson SW, Guarro J
584 (2017a) Multilocus phylogeny and antifungal susceptibility of *Aspergillus* section
585 *Circumdati* from clinical samples and description of *A. pseudosclerotiorum* sp. nov.
586 *Journal of Clinical Microbiology* **55**: 947–958.
587
588 Stuart EA, Blank F (1955) Aspergillosis of the ear; a report of twenty-nine cases.
589 *Canadian Medical Association Journal* **72**: 334–337.
590
591 Sutton DA, Wickes BL, Romanelli AM, Rinaldi MG, Thompson EH, Fothergill AW,
592 Dishop MK, Elidemir O, Mallory GB, Moonnamakal SP, Adesina AM, Schecter MG
593 (2009) Cerebral aspergillosis caused by *Aspergillus granulosus*. *Journal of Clinical*
594 *Microbiology* **47**: 3386–3390.
595

596 Tack KJ, Rhame FS, Brown B, Thompson RC (1982) *Aspergillus* osteomyelitis. Report
597 of four cases and review of the literature. *The American Journal of Medicine* **73**: 295–
598 300.
599
600 Tam EW, Chen JH, Lau EC, Ngan AH, Fung KS, Lee KC, Lam CW, Yuen KY, Lau
601 SK, Woo PC (2014) Misidentification of *Aspergillus nomius* and *Aspergillus tamarii* as
602 *Aspergillus flavus*: characterization by internal transcribed spacer, beta-Tubulin, and
603 calmodulin gene sequencing, metabolic fingerprinting, and matrix-assisted laser
604 desorption ionization. *Journal of Clinical Microbiology* **52**: 1153–1160.
605
606 Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular
607 evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725–
608 2729.
609
610 Uhrin GB, Jensen RH, Korup E, Grønlund J, Hjort U, Moser C, Arendrup MC,
611 Schønheyder HC (2015) Recurrent prosthetic valve endocarditis caused by *Aspergillus*
612 *delacroxii* (formerly *Aspergillus nidulans* var. *echinulatus*). *Medical Mycology Case*
613 *Reports* **10**: 21–23.
614
615 Varga J, Frisvad JC, Samson RA (2007) Polyphasic taxonomy of *Aspergillus* section
616 *Candidi* based on molecular, morphological and physiological data. *Studies in*
617 *Mycology* **59**: 75–88.
618
619 Varga J, Houbraken J, Van Der Lee HAL, Verweij PE, Samson RA (2008) *Aspergillus*
620 *calidoustus* sp. nov., causative agent of human infections previously assigned to
621 *Aspergillus ustus*. *Eukaryotic Cell* **7**: 630–638.
622
623 Verweij PE, Varga J, Houbraken J, Rijs AJMM, Verduynlunel FM, Blijlevens NMA,
624 Shea YR, Holland SM, Warris A, Melchers WJG, Samson RA (2008) *Emericella*
625 *quadrilineata* as cause of invasive aspergillosis. *Emerging Infectious Diseases* **14**: 566–
626 572.
627
628 Visagie CM, Hirooka Y, Tanney JB, Whitfield E, Mwange K, Meijer M, Amend AS,
629 Seifert KA, Samson RA (2014) *Aspergillus*, *Penicillium* and *Talaromyces* isolated from
630 house dust samples collected around the world. *Studies in Mycology* **78**: 63–139.
631
632 White T, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal
633 ribosomal RNA genes for phylogenetics. In: PCR protocols: a guide to methods and
634 applications. (MA Innis, DH Gelfand, JJ Sninsky & TJ White, eds): 315–322. Academic
635 Press, USA.
636
637 White CJ, Kwon-Chung KJ, Gallin JI (1988) Chronic granulomatous disease of
638 childhood. An unusual case of infection with *Aspergillus nidulans* var. *echinulatus*.
639 *American Journal of Clinical Pathology* **90**: 312–316.
640
641 Yu J, Mu X, Li R. 2013. Invasive pulmonary aspergillosis due to *Emericella nidulans*
642 var. *echinulata*, successfully cured by voriconazole and micafungin. *Journal of Clinical*
643 *Microbiology* **51**: 1327–1329.
644

TABLE 1 − Origins and GenBank/EMBL accession numbers of the *Aspergillus* strains included in this study.

Section	Species	Isolate number	Origin	GenBank/EMBL accession number			
				ITS	*BenA*	*CaM*	*RPB2*
Cremei	*A. europaeus*	UTHSCSA 13-652	--- (USA)	LT899469	LT899523	LT899571	LT899626
	A. europaeus	FMR 14589	Soil (Spain)	LT899467	LT899521	LT899569	LT899624
	A. europaeus	FMR 14591	Soil (Spain)	LT899468	LT899522	LT899570	LT899625
Flavipedes	*A. hemisphaericus*	UTHSCSA DI14-215	Human, sputum (USA)	LT899487	LT899536	LT899589	LT899644
	A. iizukae	UTHSCSA DI14-219	Human, BAL (USA)	LT899477	LT899528	LT899579	LT899634
	A. iizukae	FMR 15051	Dung (Spain)	LT899475	LT798968	LT899577	LT899632
	A. iizukae	FMR 15606	Dung (Spain)	LT899476	LT798969	LT899578	LT899633
	A. micronesiensis	UTHSCSA DI14-214	Canine, urine (USA)	LT899480	LT899529	LT899582	LT899637
	A. micronesiensis	FMR 15214	Dung (Spain)	LT899478	LT798970	LT899580	LT899635
	A. micronesiensis	FMR 15737	Dung (Spain)	LT899479	LT798971	LT899581	LT899636
	A. spelaeus	UTHSCSA 04-3307	--- (USA)	LT899491	LT899538	LT899593	LT899648
	A. spelaeus	FMR 14606	Soil (Spain)	LT899488	LT899537	LT899590	LT899645
	A. spelaeus	FMR 15176	Dung (Spain)	LT899489	LT798972	LT899591	LT899646
	A. spelaeus	FMR 15223	Dung (Spain)	LT899490	LT798976	LT899592	LT899647
Nidulantes	*A. nidulans*	UTHSCSA 05-3563	Human, sinus (USA)	LT899481	LT899530	LT899583	LT899638
	A. nidulans	UTHSCSA 14-648	--- (USA)	LT899482	LT899531	LT899584	LT899639
	A. pachycristatus	UTHSCSA 14-805	--- (USA)	LT899484	LT899533	LT899586	LT899641
	A. pachycristatus	FMR 15741	Soil (Argentina)	LT899483	LT899532	LT899585	LT899640
	A. quadrilineatus	UTHSCSA 05-237	Human, blood (USA)	LT899486	LT899535	LT899588	LT899643
	A. quadrilineatus	FMR 14621	Soil (Brazil)	LT899485	LT899534	LT899587	LT899642
	A. spinulosporus	UTHSCSA 11-3214	--- (USA)	LT899492	LT899539	LT899594	LT899649
	A. unguis	UTHSCSA 11-2524	--- (USA)	LT899498	LT899545	LT899600	LT899655

Terrei	*A. alabamensis*	UTHSCSA DI14-234	Human, tracheal (USA)	LT899447	LT899501	LT899549	LT899604
	A. alabamensis	FMR 14616	Soil (Uruguay)	LT899444	LT899499	LT899546	LT899601
	A. alabamensis	FMR 15383	Soil (Brazil)	LT899445	LT899500	LT899547	LT899602
	A. alabamensis	FMR 15412	Dung (Spain)	LT899446	LT798984	LT899548	LT899603
	A. carneus	UTHSCSA DI14-213	Canine, heart (USA)	LT899466	LT899520	LT899568	LT899623
	A. carneus	FMR 15380	Soil (Spain)	LT899465	LT899519	LT899567	LT899622
	A. hortai	UTHSCSA DI14-218	Human, BAL (USA)	LT899474	LT899527	LT899576	LT899631
	A. hortai	FMR 14597	Soil (Venezuela)	LT899471	LT899525	LT899573	LT899628
	A. hortai	FMR 15220	Leaves (Spain)	LT899472	LT899526	LT899574	LT899629
	A. hortai	FMR 15227	Dung (Spain)	LT899473	LT798987	LT899575	LT899630
	A. terreus	UTHSCSA 05-1115	--- (USA)	LT899493	LT899540	LT899595	LT899650
	A. terreus	UTHSCSA 14-1147	--- (USA)	LT899494	LT899541	LT899596	LT899651
	A. terreus	UTHSCSA 14-1441	--- (USA)	LT899495	LT899542	LT899597	LT899652
	A. terreus	UTHSCSA 14-555	--- (USA)	LT899496	LT899543	LT899598	LT899653
	A. terreus	UTHSCSA 14-724	--- (USA)	LT899497	LT899544	LT899599	LT899654
Usti	*A. calidoustus*	UTHSCSA 06-3415	--- (USA)	LT899448	LT899502	LT899550	LT899605
	A. calidoustus	UTHSCSA 07-2558	--- (USA)	LT899449	LT899503	LT899551	LT899606
	A. calidoustus	UTHSCSA 08-1449	--- (USA)	LT899450	LT899504	LT899552	LT899607
	A. calidoustus	UTHSCSA 09-1887	--- (USA)	LT899451	LT899505	LT899553	LT899608
	A. calidoustus	UTHSCSA 09-518	--- (USA)	LT899452	LT899506	LT899554	LT899609
	A. calidoustus	UTHSCSA 14-1036	--- (USA)	LT899453	LT899507	LT899555	LT899610
	A. calidoustus	UTHSCSA 14-1227	--- (USA)	LT899454	LT899508	LT899556	LT899611
	A. calidoustus	UTHSCSA DI14-209	Human, knee (USA)	LT899455	LT899509	LT899557	LT899612
	A. calidoustus	UTHSCSA DI14-210	Human, sputum (USA)	LT899456	LT899510	LT899558	LT899613
	A. calidoustus	UTHSCSA DI14-211	Human, BAL (USA)	LT899457	LT899511	LT899559	LT899614

A. calidoustus	UTHSCSA DI14-212	Human, BAL (USA)	LT899458	LT899512	LT899560	LT899615
A. calidoustus	UTHSCSA DI14-238	Human, arm (USA)	LT899459	LT899513	LT899561	LT899616
A. calidoustus	UTHSCSA DI14-239	Human, BAL (USA)	LT899460	LT899514	LT899562	LT899617
A. calidoustus	UTHSCSA DI14-240	Human, frontal mass (USA)	LT899461	LT899515	LT899563	LT899618
A. calidoustus	UTHSCSA DI14-241	Human, BAL (USA)	LT899462	LT899516	LT899564	LT899619
A. calidoustus	UTHSCSA DI14-242	Human, tissue (USA)	LT899463	LT899517	LT899565	LT899620
A. calidoustus	UTHSCSA DI14-243	Human, BAL (USA)	LT899464	LT899518	LT899566	LT899621
A. granulosus	UTHSCSA DI14-225	Human, brainstem (USA)	LT899470	LT899524	LT899572	LT899627

645 ---, Unknown clinical specimen; BAL, Bronchoalveolar lavage; FMR, Faculty of Medicine, Reus (Sapin); UTHSCSA, University of Texas
646 Health Science Center in San Antonio (USA)

647

648 TABLE 2 – Identification by BLAST searches of the cryptic *Aspergillus* species found in this studied.

Section	Isolate number	Final identification	BLAST searches			
			ITS	*BenA*	*CaM*	*RPB2*
Cremei	UTHSCSA 13-652	*A. europaeus*	No[1]	Yes	Yes	Yes
Flavipedes	UTHSCSA DI14-219	*A. iizukae*	No	Yes	Yes	No
	UTHSCSA DI14-214	*A. micronesiensis*	No	No	No	No
	UTHSCSA 04-3307	*A. spelaeus*	No	Yes	Yes	Yes
Nidulantes	UTHSCSA 14-805	*A. pachycristatus*	No	No	No	No
	UTHSCSA 05-237	*A. quadrilineatus*	No	No	No	No
	UTHSCSA 11-2524	*A. unguis*	Yes	Yes	Yes	Yes
	UTHSCSA DI14-234	*A. alabamensis*	No	No	No	Yes
	UTHSCSA 11-3214	*A. spinulosporus*	No	No	No	Yes
Terrei	UTHSCSA DI14-213	*A. carneus*	No	No	No	No
	UTHSCSA DI14-218	*A. hortai*	No	No	No	Yes
Usti	UTHSCSA DI14-225	*A. granulosus*	Yes	Yes	Yes	Yes

649 [1] negative cases correspond to matches with two or more species similarities of 99 % or higher

TABLE 3 − Phenotypic features of the cryptic *Aspergillus* species identified in this study from human clinical specimens.

Section	Species	Colony diam (mm) in 7 days			Conidiophore growth	Vesicle		Conidia		Reference
		CYA (25ºC)	MEA (25ºC)	CYA (37ºC)		Shape	Size (µm)	Ornamentation	Size (µm)	
Cremei	*A. europaeus*	35–36	24–25	8–10	Biseriate, radiate	mainly globose	(12)19–38	roughened	2.5–5	This study
Flavipedes	*A. hemisphaericus*	25–26	24–25	15–16	Biseriate, columnar	subglobose	(6)12–17	smooth	2–3.5	This study
	A. iizukae	29–30	23–25	24–26	Biseriate, columnar	mainly subglobose	8.5–21	smooth	2–3	
	A. micronesiensis	21–22	18–19	16–18	Biseriate, columnar	subglobose	7–12	smooth	1.5–3.5	This study
	A. spelaeus	12–13	21–22	0	Biseriate, radiate	mainly subglobose	(7.5)18–23	smooth	2–3	
Nidulantes	*A. pachycristatus*	17–18	14–15	61	Biseriate, columnar	subglobose	9–13	finely roughened	2.5–4	This study
	A. quadrilineatus	59–61	36–37	67–70	Biseriate, columnar	globose to subglobose	7–13	finely roughened	2.5–3.5	This study
	A. spinulosporus	51–53	59–60	>70	Biseriate, columnar	subglobose	9–15	finely roughened	2.5–4	This study
	A. unguis	28–31	33–34	18–19	Biseriate, loosely columnar	subglobose	7.5–14	finely roughened	2.5–3.5	This study
Terrei	*A. alabamensis*	47–51	25–26	65	Biseriate, columnar	subglobose	10–18.5	smooth	2–3	This study
	A. carneus	32–33	30–31	50–52	Biseriate, columnar	subglobose	8.5–18	smooth	2–3	This study
	A. hortai	49–51	33–34	64–68	Biseriate, columnar	subglobose	11–16	smooth	1.5–3	This study
Usti	*A. granulosus*	46–49	46–48	37–40	Biseriate, radiate	subglobose to elliptical	12–18	finely roughened	3.5–5.5	Raper & Fennell 1965

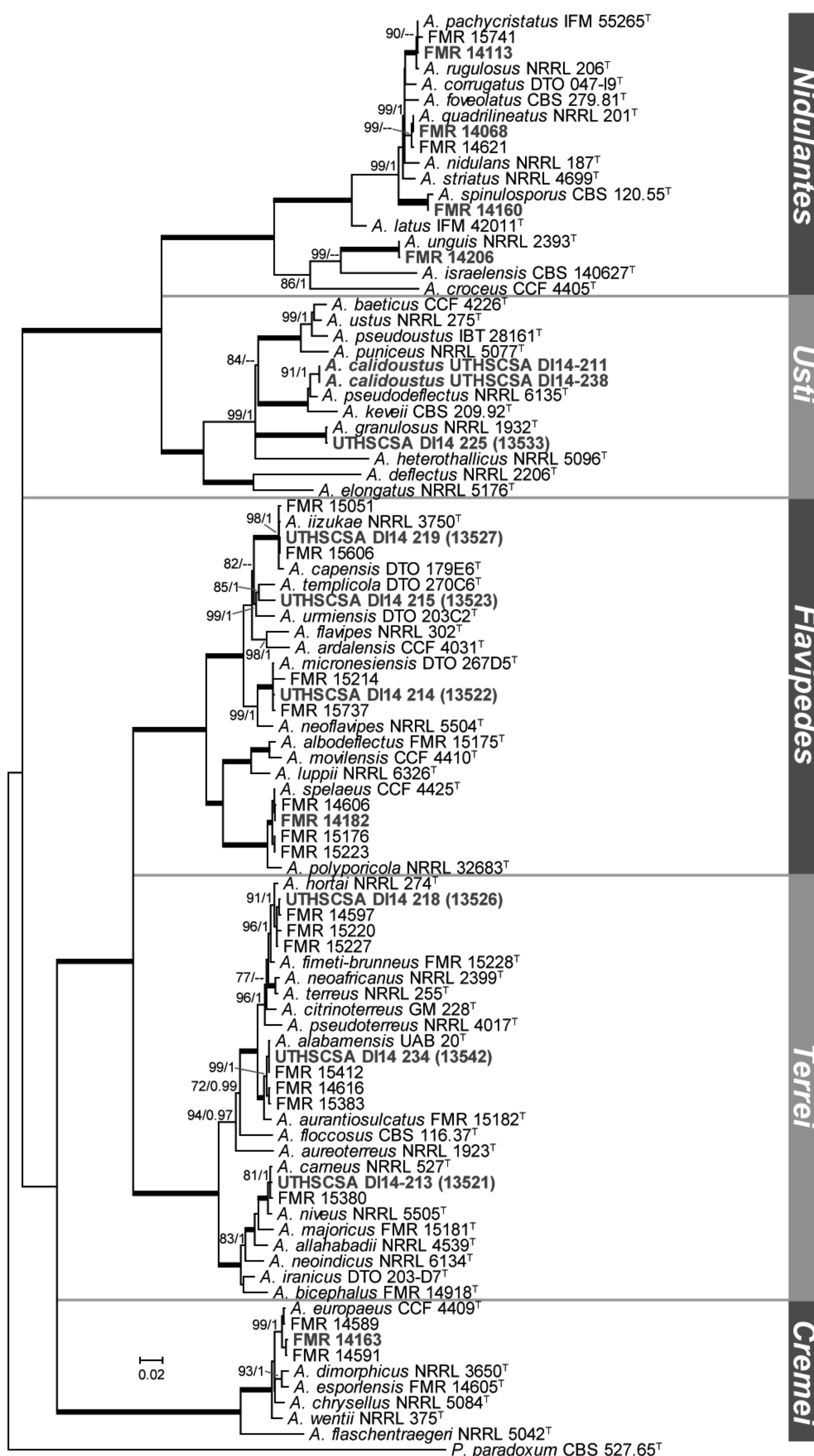
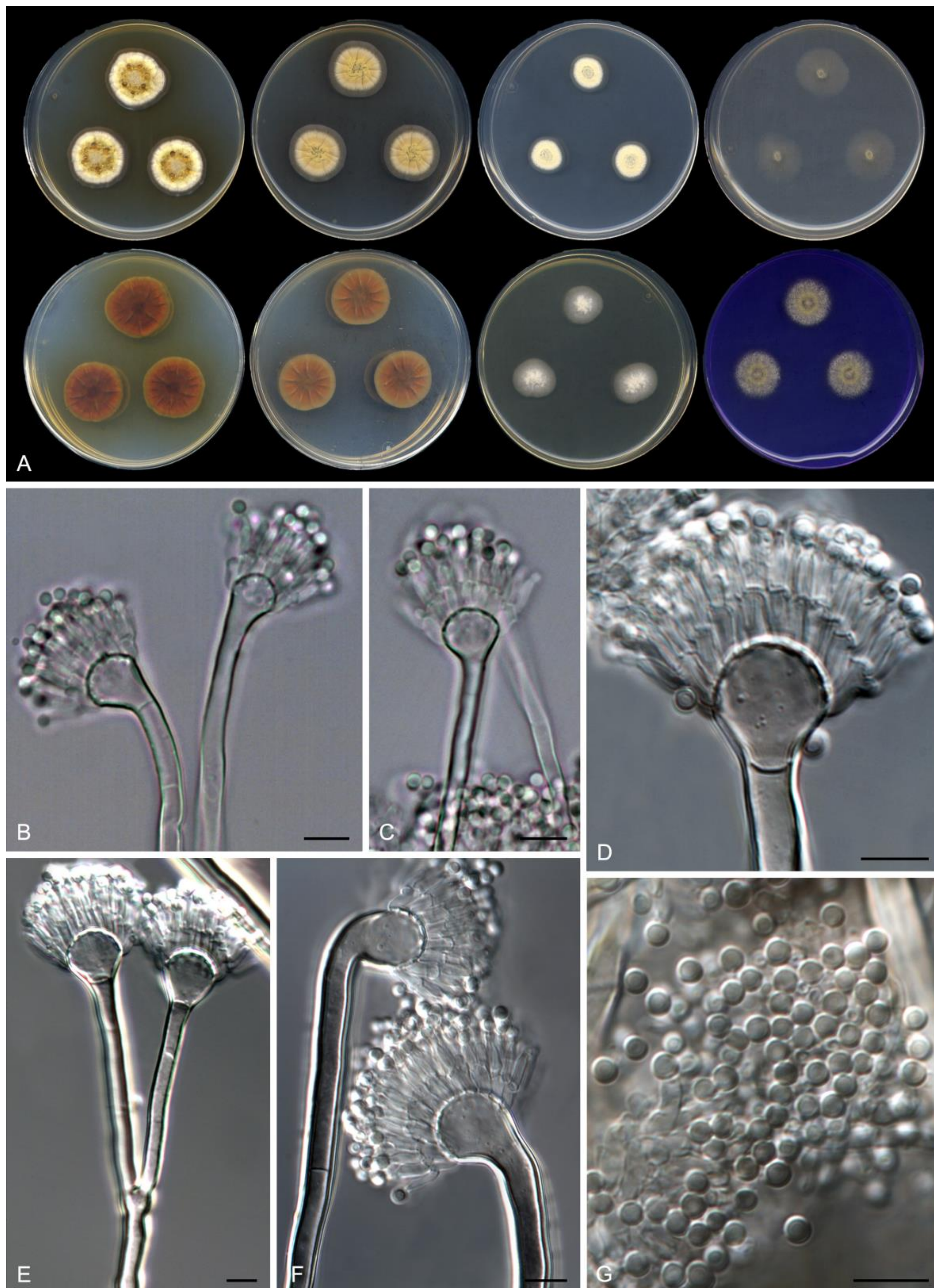
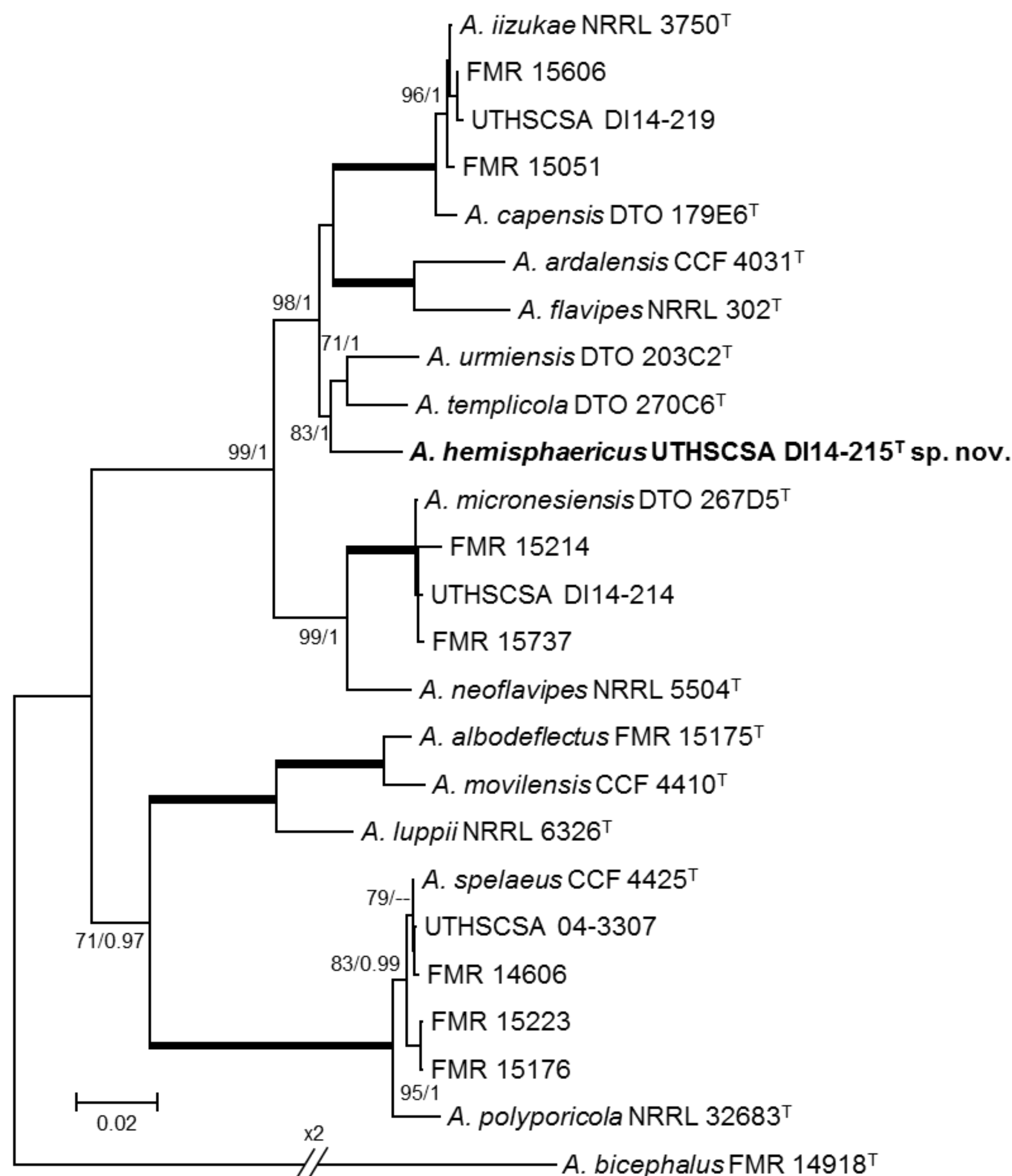


Figure 1 – ML tree from the combined ITS, *Ben*A, *CaM*, and *RPB*2 loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. Thickened branches correspond to full supported clades (100/1). Tree is rooted to *Penicillium paradoxum* CBS 527.75. Names in bold correspond to the clinical isolates. ^{T}: type strain.



657

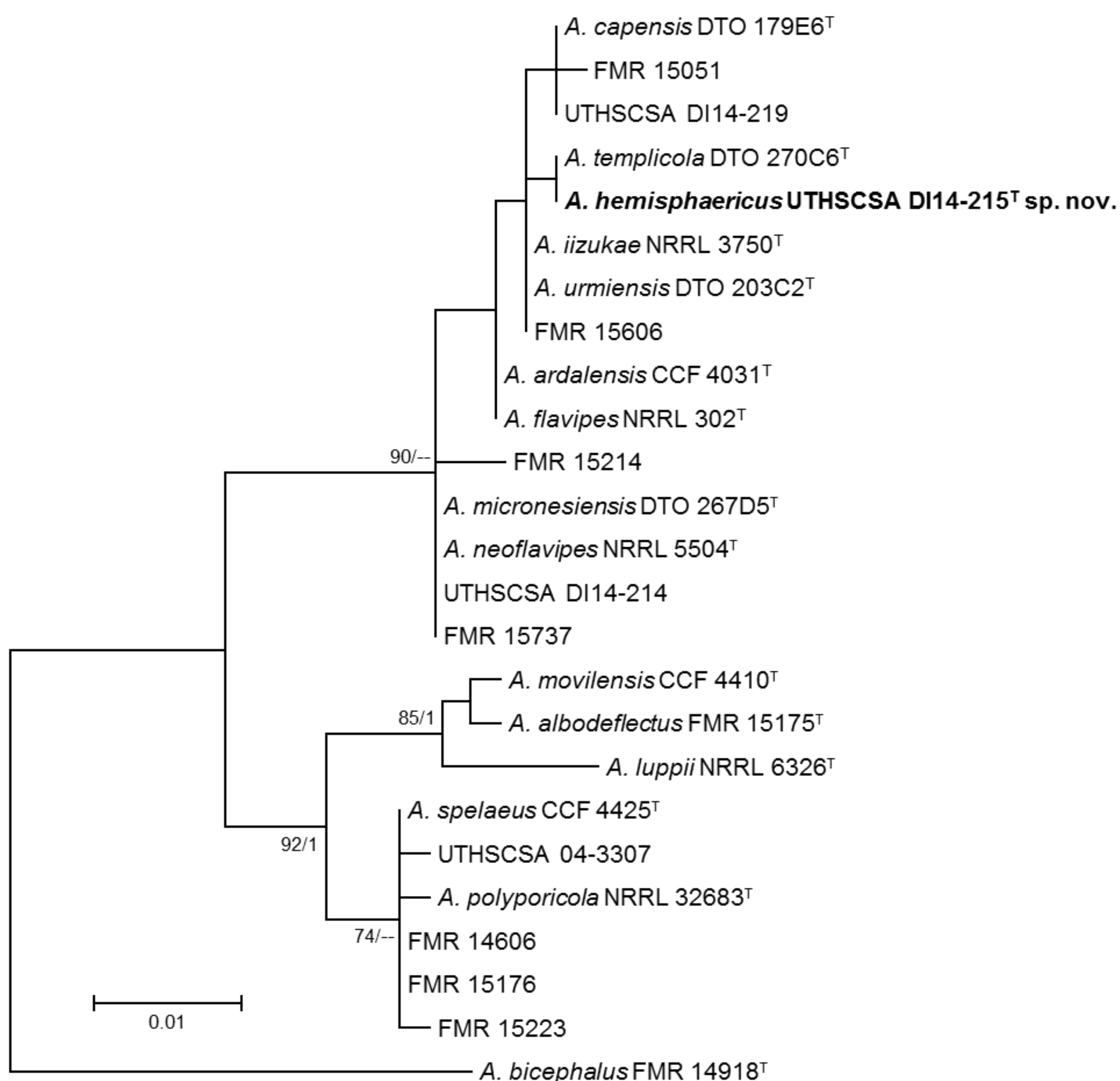
658 Figure 2 – Morphological characters of *Aspergillus hemisphaericus* (UTHSCSA DI14-
659 215^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom
660 row) CYA reverse, MEA reverse, YES, and CREA. B–F. Conidiophores. G. Conidia.
661 Scale bars = 10 µm.



662

663 Figure S1 – ML tree of *Aspergillus* section *Flavipedes* from the combined ITS, *Ben*A,
664 *CaM*, and *RPB*2 loci. Branch lengths are proportional to phylogenetic distance.
665 Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are
666 indicated on the nodes. Thickened branches correspond to full supported clades (100/1).
667 Tree is rooted to *A. bicephalus* FMR 14918. The name in bold corresponds to the new
668 species proposed. ^{T}: type strain.

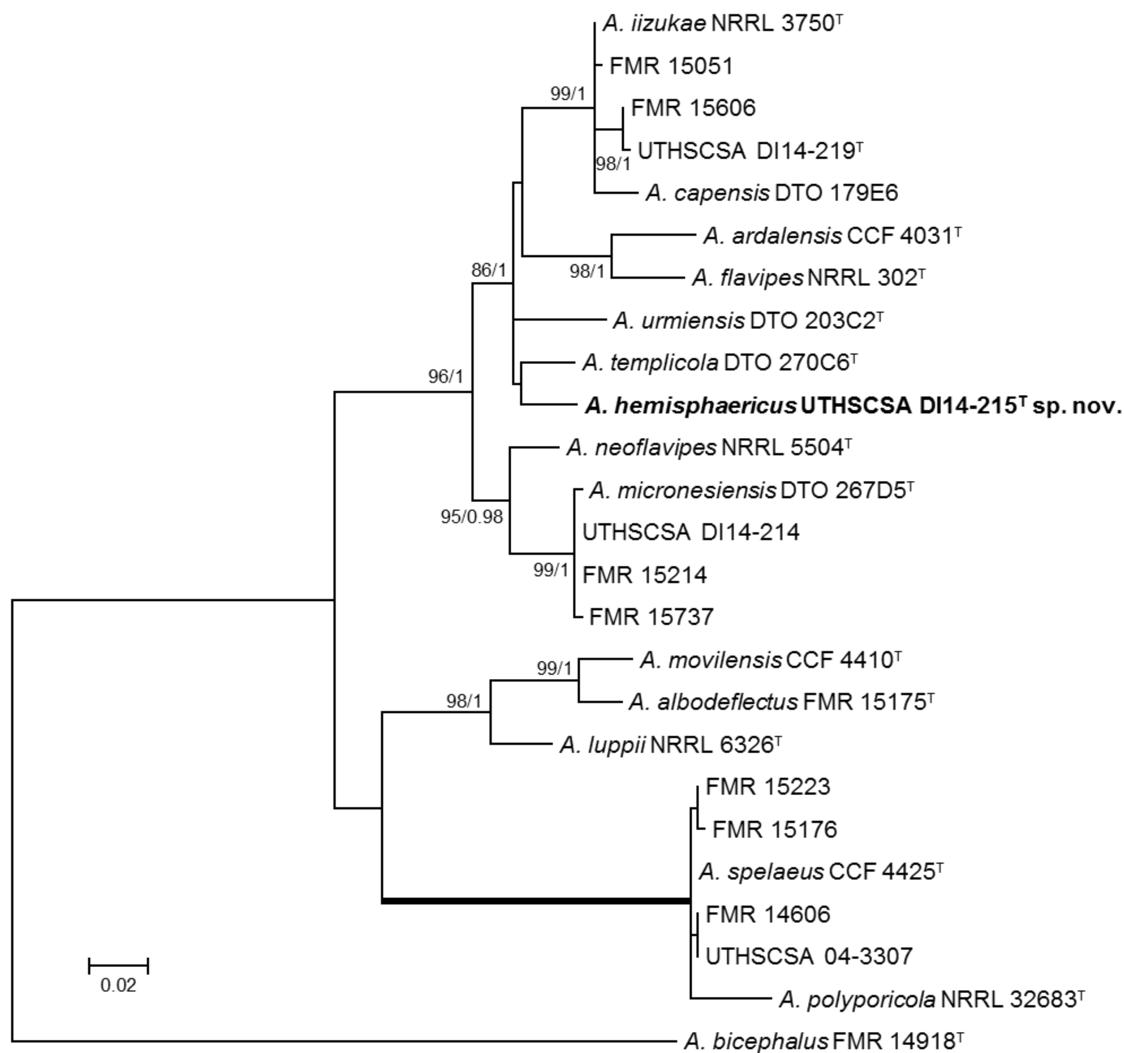
669



670

671 Figure S2 – ML tree of *Aspergillus* section *Flavipedes* from the ITS locus. Branch
672 lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian
673 posterior probability scores over 70/0.95 are indicated on the nodes. Thickened
674 branches correspond to full supported clades (100/1). Tree is rooted to *A. bicephalus*
675 FMR 14918. The name in bold corresponds to the new species proposed. ^{T}: type strain.

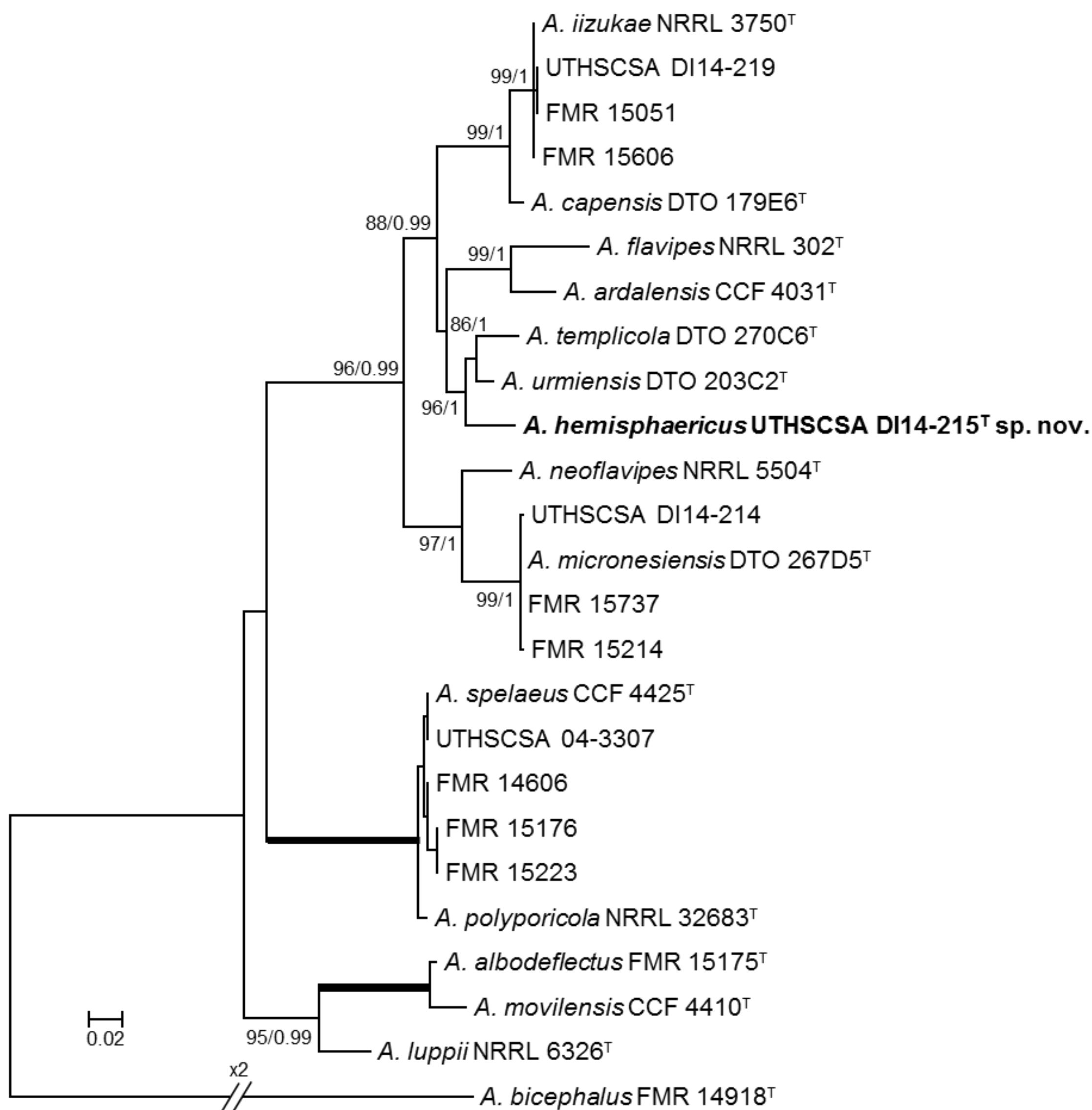
676



677

678 Figure S3 – ML tree of *Aspergillus* section *Flavipedes* from the *BenA* locus. Branch
679 lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian
680 posterior probability scores over 70/0.95 are indicated on the nodes. Thickened
681 branches correspond to full supported clades (100/1). Tree is rooted to *A. bicephalus*
682 FMR 14918. The name in bold corresponds to the new species proposed. ^{T}: type strain.

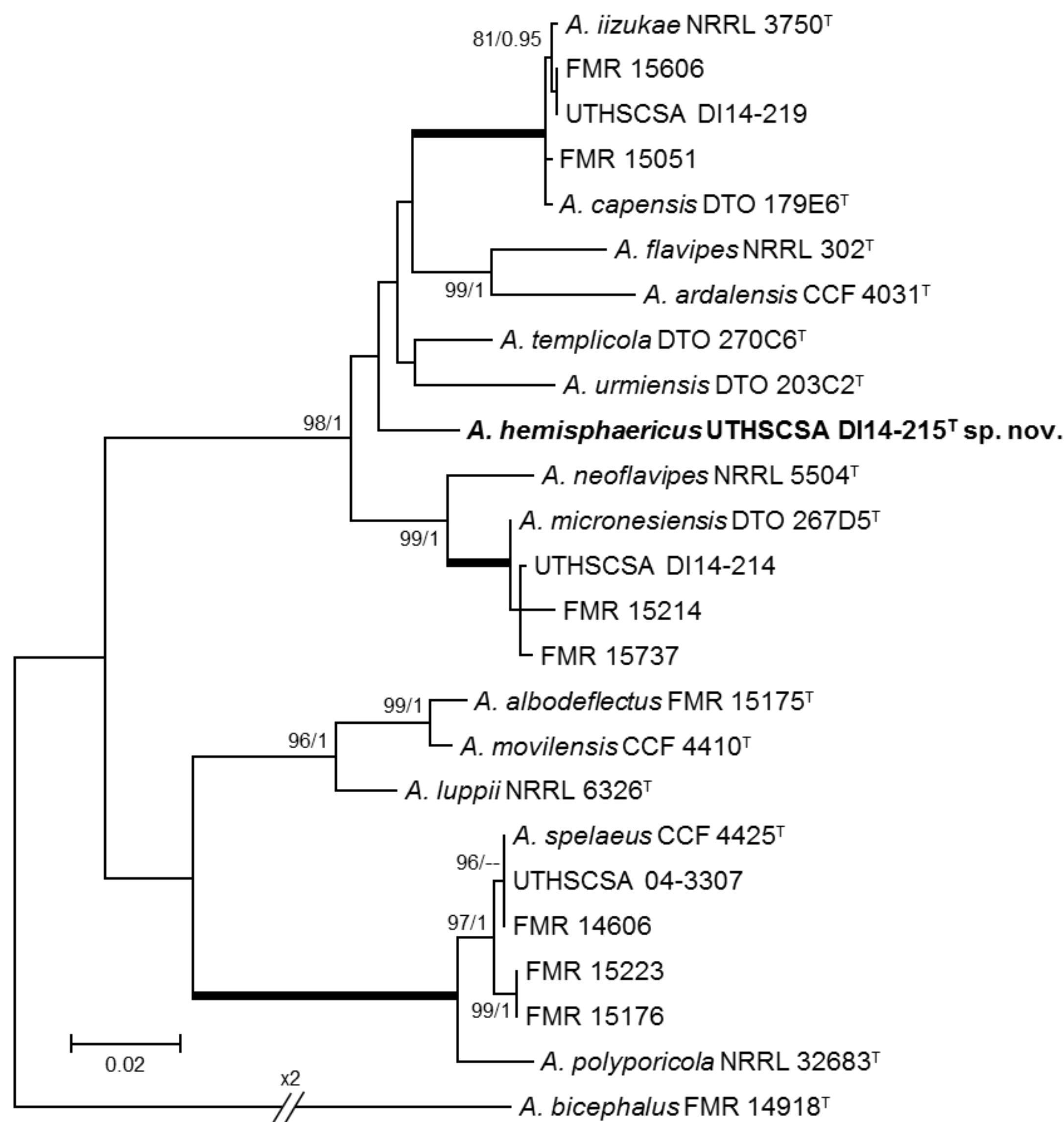
683



684

685 Figure S4 – ML tree of *Aspergillus* section *Flavipedes* from the *CaM* locus. Branch
686 lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian
687 posterior probability scores over 70/0.95 are indicated on the nodes. Thickened
688 branches correspond to full supported clades (100/1). Tree is rooted to *A. bicephalus*
689 FMR 14918. The name in bold corresponds to the new species proposed. ^{T}: type strain.

690



691

692 Figure S5 – ML tree of *Aspergillus* section *Flavipedes* from the *RPB2* locus. Branch
693 lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian
694 posterior probability scores over 70/0.95 are indicated on the nodes. Thickened
695 branches correspond to full supported clades (100/1). Tree is rooted to *A. bicephalus*
696 FMR 14918. The name in bold corresponds to the new species proposed. ^{T}: type strain.

4.5. Herbivore dung, a forgotten source of Eurotialean fungi

Marcela Guevara-Suarez[$], João Paulo Zen Siqueira[$], Dania García, José Francisco Cano-Lira, Josep Guarro, Josepa Gené

[$]These authors contributed equally to the manuscript

Submitted to: Studies in Mycology (July 2017)

Herbivore dung, a forgotten source of Eurotialean fungi

Marcela Guevara-Suarez[1,$], João Paulo Zen Siqueira[1,2,$], Dania García[1], José Francisco Cano-Lira[1], Josep Guarro[1], Josepa Gené[1#].

[1]Unitat de Micologia, Facultat de Medicina i Ciències de la Salut and IISPV, Universitat Rovira i Virgili, Reus, España; [2]Laboratório de Microbiologia, Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, Brazil.

[$]These authors contributed equally to the manuscript

[#]Corresponding author. E-mail: josepa.gene@urv.cat. Unitat de Micologia, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, 21 Sant Llorenç St., 43201, Reus, Spain.

Running head: *Aspergillus*, *Penicillium* and *Talaromyces* from dung.

Key words: coprophilous; *Eurotiales*; *Trichocomaceae; Aspergillaceae*

No conflict of interest declared.

ABSTRACT

Coprophilous fungi are saprotrophic organisms that show a great variability of habitats, herbivore dung being one of the most diverse. The physico-chemical characteristics of this peculiar substrate combined with the high level of fungal adaptation to different environmental conditions offer the perfect setting for discovering new taxa. This study focused on the identification of interesting ascomycetes isolated from 130 dung samples collected in different Spanish localities. A total of 165 isolates of Eurotialean fungi (i.e. 60 *Aspergillus* and 105 penicillium-like) were characterized. The fungi were preliminary identified morphologically and by sequencing of the β-tubulin (*Ben*A) gene. The new taxa were characterized by a multi-gene sequencing analysis testing the *Ben*A, the internal transcribed spacer (ITS), calmodulin (*CaM*), and RNA polymerase II second largest subunit (*RPB*2) genes, and a detailed phenotypic study. Using this polyphasic approach and following the genealogical concordance phylogenetic species recognition concept (GCPSR), we proposed the two new genera *Pseudopenicillium* and *Penicillago* in the family *Aspergillaceae,* and 23 new species, including ten *Aspergillus*, seven *Penicillium* and four *Talaromyces*.

Taxonomic novelties: New genera: *Penicillago* Guevara-Suarez, Gené & D. García, *Pseudopenicillium* Guevara-Suarez, Cano & Guarro. **New species:** *Aspergillus albodeflectus* J.P.Z. Siqueira, D. García & Gené, *Aspergillus aurantiosulcatus* J.P.Z. Siqueira, Guarro & D. García, *Aspergillus calidokeveii* J.P.Z. Siqueira, D. García & Gené, *Aspergillus canariensis* J.P.Z. Siqueira, Gené & Guarro, *Aspergillus coprophilus* J.P.Z. Siqueira, D. García & Gené, *Aspergillus esporlensis* J.P.Z. Siqueira, Gené & Guarro, *Aspergillus fimeti-brunneus* J.P.Z. Siqueira, Gené & D. García, *Aspergillus longipes* J.P.Z. Siqueira, Guarro & D. García, *Aspergillus majoricus* J.P.Z. Siqueira, Gené & Guarro, *Aspergillus verruculosus* J.P.Z. Siqueira, Gené & D. García, *Penicillium balearicum* Guevara-Suarez, Cano & Gené, *Penicillium beceitense* Guevara-Suarez, Gené & Guarro, *Penicillium caprifimosum* Guevara-Suarez, D.

García & Cano, *Penicillium fimosum* Guevara-Suarez, Guarro & D. García, *Penicillium ibericum* Guevara-Suarez, Cano & D. García, *Penicillium mediterraneum* Guevara-Suarez, Gené & Cano, *Penicillium synnematicola* Guevara-Suarez, D. García & Guarro, *Penicillago flava* Guevara-Suarez, D. García & Cano, *Pseudopenicillium coprobium* Guevara-Suarez, D. García & Gené, *Talaromyces catalonicus* Guevara-Suarez, Gené & Guarro, *Talaromyces coprophilus* Guevara-Suarez, Cano & D. García, *Talaromyces gamsii* Guevara-Suarez, Cano & Guarro, *Talaromyces pseudofuniculosus* Guevara-Suarez, D. García & Gené. **New combinations:** *Penicillago nodositata* (Valla) Guevara-Suarez, Gené & D. García, *Pseudopenicillium giganteum* (R.Y. Roy & G. N. Singh) Guevara-Suarez, Gené & Cano, *Pseudopenicillium megasporum* (P. A. Orpurt & D. I. Fennell) Guevara-Suarez, Cano & Guarro.

INTRODUCTION

Fungi are able to grow and colonize the most diverse substrates, especially those rich in nutrients. Therefore, a huge number of taxa developing on herbivore droppings have been described in the most diverse parts of the world (Sarroco 2016), which constitutes one of the most exciting exercises of fungal diversity. Coprophilous fungi are a large group of saprotrophic organisms which represent an important component of the biosphere with a crucial role in recycling the nutrients involved in animal feces (Cooke & Rayner 1984). Animal dung is a complex substrate with a high amount of readily available carbohydrates with high nitrogen content, water-soluble vitamins, impoerant growth factors and mineral ions (Bell 1983). In addition, the physical structure, pH, and varying moisture content of the dung make it a rich medium for fungal growth (Richardson 2001).

Some genera of *Ascomycota* are considered practically exclusive of dung (e.g. *Ascobolus*, *Podospora*), while others are restricted to a particular herbivore animal, such as for instance *Lasiobolus cainii,* which is only found on porcupine dung (Webster 1970). However, the majority of coprophilous fungi can grow on any herbivore dung

due to their ability to survive on any decaying organic matter (Sarroco 2016). A high diversity of fungal taxa, including a large number of species of *Ascomycota*, *Basidiomycota* and *Mucorales*, considered the primary saprotrophs, can be found on this type of dropings (Bell 1983; Richardson 2001). It has been demonstrated that a large and complex succession of fungi appears on dung which makes analogies with the plant sequence in a disturbed area, *Mucorales* being usually the pioneering fungi which are displaced by *Ascomycota* or *Basidiomycota* when fresh propagules arrive or buried spores germinate (Bell 1975; Richardson 2001; Sarroco 2016).

Most of the ascomycetes, and particularly the *Eurotiales,* are not considered predominantly coprophilous (Krug *et al.* 2004), but they are frequently found when the soluble materials are exhausted and a low water availability is present. More particularly, some reports indicate that species of *Aspergillus* and *Penicillium* are more common in feces when the relative humidity decreases below 85 % (Kuthubutheen & Webster 1986). Although the literature on the presence of these groups of fungi on herbivore dung are scarce, some reports show that members of at least nine sections of *Aspergillus* can be present on this substrate. Species of sections *Nidulantes* and *Clavati* are occasionally found on dung samples (Varga *et al.* 2007a; Chen *et al.* 2016a). In addition, several *Aspergillus* species have been discovered from this substrate, such as *A. viridinutans* (section *Fumigati*), *A. monodii* (section *Usti*) (Samson *et al.* 2011a), *A. recurvatus* (section *Nidulantes*) (Raper & Fennell 1965), among others.

In the past, some coprophilous penicillia, such as *P. brevistipitatum*, *P. clavigerum*, *P. concentricum*, *P. coprobium*, *P. coprophilum*, *P. formosanum*, *P. glandicola* and *P. vulpinum*, had been classified in the series *Claviformia* (Frisvad & Samson 2004; Wang & Zhuang 2005). However, the most recent taxonomic classification based on sections, most of the coprophilous species have been reclassified in the section *Robsamsonia* (Houbraken *et al.* 2016), which includes, apart from the mentioned species previously assigned to series *Claviformia* others from

Urticicolae (Frisvad & Samson 2004). Only a few studies on coprophilous organisms have identified penicillium-like fungi at the species level, and many of the fungi identified as *Penicillium* correspond in fact to other morphologically similar genera (Houbraken *et al.* 2016). It is generally known that some species of *Penicillium* sensu stricto and some members of *Talaromyces* share several morphological features; however, Houbraken & Samson (2011) demonstrated that *Penicillium* is phylogenetically more related to *Aspergillus* (family *Aspergillaceae*) than to *Talaromyces* (family *Trichochomaceae*). Concerning *Talaromyces*, some coprophilous species such as *T. atroroseus, T. dupontii, T. emersonii, T. helicus, T. flavus, T. muroii* and *T. trachyspermus* have been reported, although currently some of them (e.g. *T. dupontii* and *T. emersonii*) have been transferred to the genera *Thermomyces* and *Rasamsonia*, respectively (Masunga *et al.* 2006; Frisvad *et al.* 2013; Yilmaz *et al.* 2014).

The high level of adaptation to different substrates of members of *Aspergillaceae* and *Trichocomaceae*, combined with the particular physical and chemical characteristics of the diversity of dung samples, offers the perfect setting for detection of potential new species; however, this constitutes a generally overlooked substrate for these fungal group, due to the difficulties in detecting in most cases the presence of these microorganisms. Currently, the identification of Eurotialean fungi, such as those belonging to the genera *Aspergillus*, *Penicillium* and *Talaromyces*, is achieved using a polyphasic approach that includes the evaluation of morphological and physiological characters (i.e., growth on different culture media at different temperatures and detection and identification of extrolite profiles), and multilocus sequence analyses testing with the internal transcribed spacer region (ITS), and the β-tubulin (*Ben*A), calmodulin (*CaM*), and/or the DNA-dependent RNA polymerase II largest subunit (*RPB*2) genes (Peterson 2008; Houbraken *et al.* 2014a; Samson *et al.* 2014; Visagie *et al.* 2014a; Yilmaz *et al.* 2014). The use of this, already generalized approach has demonstrated to be able to discriminate very close species and to

discover and characterize new taxa (Visagie *et al.* 2014a; Guevara-Suarez *et al.* 2017; Siqueira *et al.* 2016; 2017).

The present study focuses on the detection and identification of species of *Aspergillus*, *Penicillium* and *Talaromyces* isolated from herbivore dung samples collected from different Spanish regions. Their Identification has been carried through by the conjunction of the evaluation of their most remarkable phenotypic features and the analysis of multilocus sequences in comparison with those of ex-type and reference strains. The Genealogical Phylogenetic Species Recognition (GCPSR) criterion has been used to suporty the taxonomic position of numerous putative new species (Taylor *et al*. 2000).

MATERIALS AND METHODS

Sampling and fungal isolation

Dung samples were collected mainly during 2016 in different geographic regions from Spain, with different climates and very diverse fauna and flora, representing very diverse habitats of Andalusia, Balearic and Canary Islands, Cantabria, Castile-Leon, Catalonia, Extremadura and Galicia. Most of the samples were from rabbit, fox, sheep, deer, and goat, although occasionally soil mixed with some of these substrates or even dung from other animals, as cattle, wild pig and horse were also studied. The samples were placed in individual paper or plastic bags and processed not later than three days after collection. Individual samples were divided into two parts; one processed using moist chambers (Richardson 2001) and the other by the dilution method, mainly based on Waksman (1922) with some modifications (see below). In the first procedure, few pieces of the sample were placed on moist filter paper with sterile distilled water in individual Petri dishes, and incubated at room temperature for up to 30 days. For the dilution method, approximately one gram of dung or soil was 1:10 (w/v) diluted in sterile water, and handle shaken for approximately 10 min. Aliquots of the suspensions were pipetted into Petri dishes and mixed with 20 mL of melted cooled agar medium. Culture

media used for isolation were potato dextrose agar (PDA; Pronadisa, Madrid, Spain) and/or potato carrot agar (PCA; 20 g potatoes, 20 g carrot, 20 g agar, 1000 mL distilled water), both supplemented with chloramphenicol (200 mg/L), and dichloran rose-bengal chloramphenicol agar (DRBC; 5 g peptone, 10 g glucose, 1 g potassium dihydrogen phosphate [KH_2PO_4], 0.5 g magnesium sulphate [$MgSO_4$], 25 mg rose-bengal, 2 mg dichloran, 200 mg chloramphenicol, 20 g agar, 1000 mL distilled water). All media were supplemented with dieldrin in dimethyl-ketone (1%). Petri dishes were incubated at room temperature for up to 30 days. The moist chambers and Petri dishes were examined at regular intervals with the aid of a stereo microscope and conidia from sporulated colonies were transferred to PDA supplemented with chloramphenicol.

Molecular identification and phylogenetic analysis

The selected isolates were cultured on PDA or malt extract agar (MEA; Difco, Detroit, USA) for 7-14 days at 25ºC. DNA was extracted using the FastDNA® kit protocol (MP Biomedicals, Solon, OH) and for the homogenization step a FastPrep® FP120 cell disrupter (Thermo Savant, Holbrook, NY).

Preliminary identification of the isolates was carried out by the analysis of a sequence fragment of the *BenA* gene. In the case of putative new species, the ITS region, including the 5.8S rRNA gene, and fragments of *CaM* and/or *RPB*2 genes were also amplified. The primer pairs used were: ITS5/ITS4 for the ITS (White *et al.* 1990), Bt2a/Bt2b for *Ben*A (Glass & Donaldson 1995), CMD5/CMD6 for *CaM* (Hong *et al.* 2006), and RPB2-5F/RPB2-7Cr for *RPB*2 (Liu *et al.* 1999). The amplification protocol and PCR conditions were performed using methods and primers previously described (Peterson 2008; Houbraken & Samson 2011; Yilmaz *et al.* 2014). The amplified products were purified and sequenced at Macrogen Corp. Europe (Amsterdam, the Netherlands) with a 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). Consensus sequences were obtained using SeqMan v. 7.0.0 (DNASTAR, Madison,

WI). The newly generated sequences obtained in this study and their GenBank/EMBL accession numbers are summarized in Table 1.

Phylogenetic analysis using the *Ben*A locus included sequences of ex-type strains of species more closely related to the isolates recovered in this study, based on the results of BLAST searches in the GenBank database. Sequences were retrieved from GenBank taking into account the last update of the database of the International Commission of *Penicillium* and *Aspergillus* (http://www.aspergilluspenicillium.org), which includes all the species accepted in those genera. Single (data not shown) and concatenated phylogenetic analyses were performed to delineate putative new species and the phylogenies corresponding to each section of those genera were properly reconstructed.

Data sets for each locus were aligned individually using ClustalW (Thompson *et al.* 1994), in MEGA v 6.0 software (Tamura *et al.* 2013), refined with MUSCLE (Edgar 2004) under the same platform, and manually adjusted if needed. Larger alignments including different sections of a genus were performed by the MAFFT tool in the EMBL-EBI Web Services portal and manually adjusted in MEGA v 6.0. Phylogenetic reconstructions by maximum likelihood (ML) and Bayesian inference (BI) were carried out using MEGA v. 6.0 and MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001), respectively. For ML analyses, the trees were inferred using Nearest-Neighbour-Interchange as a heuristic method and gaps were treated as partial deletion with a 95 % site coverage cut-off. The estimation of the best nucleotide substitution method was performed in MEGA v 6.0 and phylogeny support for internal branches was assessed by 1,000 ML bootstrapped pseudoreplicates of data. Bootstrap support (bs) ≥ 70 was considered significant. BI analyses were performed using five million Markov chain Monte Carlo (MCMC) generations, with two runs (one cold and three heated chains) and samples were stored every 1,000 generations. The 50% majority-rule consensus tree and posterior probability values (pp) were calculated after discarding the first 25% of the samples. A pp value ≥0.95 was considered significant. The best substitution

models for all gene matrices were estimated using jModelTest v.2.1.3 (Darriba *et al.* 2012; Guindon & Gascuel 2003). The resulting trees were plotted using FigTree v.1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). The alignments and trees were deposited in the TreeBASE (www.treebase.org) under the submission number 21345.

Phenotypic characterization

Phenotypic characterization was carried out using standard growth conditions described previously (Samson *et al.* 2014; Visagie *et al.* 2014a; Yilmaz *et al.* 2014). Briefly, the isolates were cultured onto MEA (Samson *et al.* 2010), oatmeal agar (OA; Samson *et al.* 2010), Czapek yeast autolysate agar (CYA; Pitt 1979), yeast extract sucrose agar (YES; Frisvad 1981), creatine sucrose agar (CREA; Frisvad 1981) and dichloran 18 % glycerol agar (DG18; Hocking and Pitt 1980), incubated at 25 °C for 7 d in darkness. Colony growth rates were also measured after 7 d at 30 and 37 °C on the same agar media above mentioned. Color notations in colony descriptions were from Kornerup & Wanscher (1978). Microscopic features were examined on colonies grown on MEA after 1 to 2 weeks, mounted on slides with Shear's solution or 60% lactic acid, and the excess of conidia were removed with 70 % ethanol, and their photographs were made using a Zeiss Axio-Imager M1 light microscope with Nomarski differential interference contrast and phase-contrast optics (Zeiss, Oberkochen, Germany) with a DeltaPix Infinity X digital camera.

Those isolates identified morphologically as belonging to *Aspergillus*, *Penicillium* or *Talaromyces* were recovered and deposited in the culture collection of the Medicine Faculty of Reus (FMR). Cultures of interesting species, as well as type material and cultures of the new species were deposited at the Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands). Nomenclatural novelties and descriptions were deposited in MycoBank (Crous *et al.* 2004).

RESULTS AND DISCUSSION

In this study a total of 130 dung samples were processed. Our preliminary results (data not shown) on *Aspergillus* identification showed that members of sections *Fumigati*, *Flavi*, and *Nigri* were very common on that substrate, but their species diversity was relatively poor. Therefore, we did not treate these groups of *Aspergillius*. Considering that it is relatively easy to recognize the different types of aspergillli directly on the substrate, we tried to select only isolates belonging to sections different to those above mentioned. On the other hand, a preliminary selection of penicillia was rather harder than in the case of aspergilli, and therefore practically all penicillium-like isolates observed on the samples were recovered. A total of 165 isolates, including five from soil, were studied (Table 1). Preliminary identification using morphological features and *Ben*A phylogeny confirmed that the isolates recovered were *Aspergillus* (n = 60), *Penicillium* (n = 91) and *Talaromyces* (n = 10), while four of them could not be assigned to any of these genera in spite of exhibiting a penicillium-like morphology. To maximize the quality of the alignment, four separate *Ben*A alignments were done corresponding to the different genera studied (Figures 1, 7, 12, 15). We also carried out additional alignments of the single (data not shown) and combined datasets of the different genes corresponding to those genera or sections where putative new species resulted included (Figures 2–6, 8–11, 13, 14, 16). The length, number of variable and phylogenetic informative sites, and substitution models (for BI) for each dataset are summarized in Table 2. All those analyses were performed using ML and BI methods. The topologies of the trees obtained by both methods were similar, the ML ones being used to represent the results. Bootstrap values and BI posterior probability values were marked on relevant branches.

Final identification of the isolates that resulted from the phylogenetic analysis of the different genes and from the exam of their morphological features is shown in Table 1.

Aspergillus phylogeny

The phylogenetic analysis inferred from the 60 *Aspergillus* isolates with the *Ben*A sequences is shown in Figure 1. The alignment was 615 bp long, 408 from which resulted variable and 381 phylogenetic informative. ML substitution model was Kimura 2-parameter (K2) and for BI was General Time Reversible (GTR). Rates among sites were Gamma distributed (G) with invariant sites (I), used for both methods. The isolates were distributed in 38 different species belonging to the following nine sections *Nidulantes*, *Usti*, *Flavipedes*, *Terreus*, *Aspergillus*, *Candidi*, *Circumdati*, *Clavati*, *Cremei* and forming seven well-supported main clades (I – VII). Although some of these clades (i.e. II, III, IV, and V) do not agree with the subgenera phylogeny of *Aspergillus* recently proposed by Kocsube *et al.* (2016), the sections in which our isolates are distributed remain well delineated and presenting high support values. The species delimitation with the *BenA* ML shows, in general, high support values; nonetheless, the identification of some isolates of the sections *Candidi*, *Flavipedes, Terrei* and *Usti* are doubtful. However, the genetic distance of such isolates with ex-type strains of the nearest species, the BI analysis, and the ML analysis with restricted alignments for each section showed that there is not enough variation to consider these isolates different from those species already described. Probably, long alignments including many sections can cause distortions in the tree topology, especially in the terminal branches. This phenomenon makes difficult to reveal phylogenetically relationships between close species, however single trees from each section give more robust identification.

Clade I grouped the sections *Nidulantes* and *Usti* of the subgenus *Nidulantes*. In the first one (90% bs/0.99 pp), eleven isolates were identified as *A. aureolatus* (n=1), *A. austroafricanus* (n=1), *A. fructus* (n=1), *A. nidulans* (n=3), *A. rugulosus* (n=1), *A. sydowii* (n=3), and *A. viridicatenatus* (n=1). The section *Nidulantes* included the majority of species formerly known as *Emericella*, which were transferred to *Aspergillus* by Samson *et al.* (2014) following the new critera for fungal nomenclature. Note that

the current concept of this section by Chen *et al.* (2016a) also includes the members of the former section *Versicolores* (Jurjevic *et al.* 2012; Siqueira *et al.* 2016). We agree with Chen et al. (2016a) that *Nidulantes* is one of the sections with a higher species diversity on dung.

The section *Usti* (93% bs/0.99 pp) comprised four isolates belonging to three known species, i.e. *A. calidoustus* (n=1), *A. insuetus* (n=1) and *A. pseudodeflectus* (n=2), and one isolate (FMR 15225) that could represent an undescribed species. Single (data not shown) and concatenate (Figure 2) analyses of most of the species currently accepted in this section (Samson *et al.* 2014) show that FMR 15225 is closely related to *A. keveii*. However, some phenotypic features such as the ability of this isolate to grow at 37 ºC, and the genetic distance (98 % similar with *Ben*A; 98.9% % in the concatenate dataset) versus the ex-type strain of *A. keveii*, allow to consider them distinct taxa being proposed below as *A. calidokeveii*. Species of the *Usti* section are relatively common in soil and indoor air, being *A. ustus* and *A. monodii*, which were not identified in our study, the only species reported on dung (Samson *et al*. 2011a).

Clade II comprised the sections *Flavipedes* (91% bs/1 pp) and *Terrei* (95% bs/1 pp), which in our study corresponded to the two sections with the largest number of isolates, i.e. 15 (25%) and 10 (16.7%) isolates, respectively. Although members of the sections *Flavipedes* and *Terrei* are very common in soil and dust (Samson *et al.* 2011b; Hubka *et al.* 2015), they have not ever been reported from dung. In the section *Flavipedes,* the species identified were *A. ardalensis* (n=2), *A. iizukae* (n=2), *A. micronesiensis* (n=2), *A. spelaeus* (n=5) and *A. templicola* (n=3); however, although FMR 15175 clearly belongs to this section, it did not match with any of the species of this group. Both *Ben*A analysis (Figure 1) and the concatenated phylogeny (Figure 3) with the currently accepted species of the section (Arzanlou *et al.* 2016) showed that this isolate formed a fully supported clade with the ex-type strain of *A. movilensis*. However, both shows a genetic difference (97.1 % similar with *Ben*A; 98.5 % with the concatenate alignment) enough to be considered distinct species. Thus, FMR 15175 is

described below as *A. albodeflectus* sp. nov. It is noteworthy that the most frequent *Aspergillus* species isolated in this study is *A. spelaeus* (8.33%), a species recently described by Hubka *et al.* (2015) from cave sediment in Spain.

The section *Terrei* currently comprises 17 species (Samson *et al*. 2014) with four of them identified in the present study, i.e. *A. albamenesis* (n=2), *A. citrinoterreus* (n=1), *A. floccosus* (n=1), *A. hortai* (n=1), and *A. terreus* (n=1). However, four isolates (FMR 15182, FMR 15228, FMR 15181 and FMR 15217) did not fit morphologically either genetically with any species of the section. It is noteworthy that the ML general tree based only on *BenA* presented some doubtful results in the identification of *A. terreus* and *A. citrinoterreus* isolates (Figure 1). However, when we carried out a restricted alignment with only members of the section *Terrei* and reconstructing the ML tree from this alignment, both species could be satisfactory identified (see Figure S1 in supplemental material). The concatenated sequence alignment of section *Terrei*, including the four unidentified isolates mentioned above, confirmed that them represent three undescribed phylogenetic species (Figure 4). Three species of this secton, i.e. *A. ambiguous, A. microcysticus* and *A. neoniveus,* were not included in the general alignment because of the long genetic distance versus the other species of this group, and the first two acted as outgroups in the concatenate phylogeny. Two major clades were observed, whereas the first one (98% bs/1 pp) included the isolates FMR 15228 and FMR 15182, which were phylogenetically distant from *A. hortai* and *A. alabamensis*, respectively; the second clade (100% bs/1 pp) included the isolates FMR 15181 and FMR 15217, which formed an independent fully supported lineage clearly distant from the other species of the group. These aspergilli are described below as *A. aurantiosulcatus* (FMR 15182), *A. fimeti-brunneus* (FMR 15228), and *A. majoricus* (FMR 15181 and FMR 15217).

Clade III corresponded to section *Aspergillus* (95% bs/1 pp), which included five isolates identified as *A. chevalieri* (n=1), *A. montevidensis* (n=2), and *A. pseudoglaucus* (n=2). This section included osmophilic organisms, commonly found on organic

materials, dust, and cereals (Kozakiewicz 1989). Some members of this group had already been isolated from dung (Hubka *et al.* 2013), showing that it may be a good reservoir for species of this section. Particular conditions of the samples, as low water activity, may enhance the growth of members of this section in such substrate.

Clade IV, representing the section *Candidi* (94% bs/1 pp), included eight of our isolates, from which two were identified as *A. candidus*. Interestingly, the other six isolates represented at least four undescribed phylogenetic species. Currently, this section comprises six species, two of them described recently, *A. pragensis* from toe nail (Hubka *et al.* 2014) and *A. subalbidus* from house dust (Visagie *et al.* 2014b). The concatenated sequence alignment of the six species of the section, using the four markers, showed that the unidentified isolates were distributed in three moderate to well-supported clades (Figure 5). The first one (99% bs/1 pp) encompassed *A. subalbidus* and *A. taichungensis*, which were basal to the two new species proposed here, *A. canariensis* and *A. verruculosus*. Although these two species were very closely related, with a similarity between each other of 98.2% in the concatenated alignment, their phenotypic differences support their novelty. The second clade (78% bs/0.99 pp) included the ex-type strain of *A. candidus* and, in a separate lineage with a similarity of 98.5 %, the new species *A. longipes*. The third clade (96% bs/1 pp) corresponded to *A. campestris* and two isolates recovered from deer dung which showed a genetic similarity of 98.3 % respect to the ex-type strain of the former species, and thus considered distinct taxa. These two isolates are described below as *A. coprophilus*. The species diversity of the section on herbivore dung seems to be poorly studied and, according to our data, this substrate could be a clear reservoir of new species in *Candidi*. Interestingly, the isolates of our new species show a wide geographic distribution in Spain, being isolated from Balearic and Canary Islands, Castile and Leon, Catalonia, and Galicia.

Clades V and VI corresponded to the sections *Circumdati* (96% bs/1 pp) and *Clavati* (99% bs/1 pp), respectively. In the former, the two isolates identified belonged

to *A. affinis* and *A. subramanianii*, respectively, whereas in the latter the two dung isolates were identified as *A. clavatus*. *Aspergillus affinis* is a rare species only known from submerged leaf litter and soil in Italy and Macedonia, respectively (Davolos *et al.* 2012). *Aspergillus subramanianii* is a widely-distributed species recovered from different substrates and countries (Visagie *et al.* 2014b; c; Siqueira *et al.* 2017), but it has never been reported from animal dung. On the contrary, *A. clavatus* is frequently isolated from dung, soil and even from other types of substrates (Varga *et al.* 2007a).

Two isolates were included in the section *Cremei* (clade VII, 94% bs/1 pp). Whereas FMR 14605 could represent a putative new species, the isolate FMR 15216 was identified as *A. europaeus.* Despite the recent proposal of this new latter species, it is reported as a common fungus on soil (Hubka *et al.* 2016). The section *Cremei* currently comprises 17 species (Samson *et al.* 2104), although only the ex-type strains of the ten species more closely related to the unidentified isolate have been included in the final concatenated phylogeny of the section presented here (Figure 6). The concatenated analysis showed that FMR 14605 was closely related to *A. dimorphicus*, forming both a well-supported terminal clade distant from the other species compared. Although the isolate FMR 14605 and the ex-type strain of *A. dimorphicus* showed identical ITS, they could be distinguished by *BenA* (98 % similar) and *CaM* (98.6 % similar) sequences. Additional phylogenies including more sequences of *A. dimorphicus* available in GenBank and of other closely related species, i.e. *A. chrysellus*, *A. europaeus* and *A. wentii* (see Figures S2 to S6 in supplemental material) supports the novelty of our isolate, which is described below as *A. esporlensis*.

***Penicillium* phylogeny**

The phylogenetic tree based on the *Ben*A locus with the 91 isolates of *Penicillium* is shown in Figure 7. The aligned dataset was 404 bp long, with 257 variable sites and 237 phylogenetic informative. The best substitution model for ML was K2+G, and for BI

it was GTR+G+I. In general, the topology of the phylogenetic tree showed well-delimitated sections.

The analysis distributed the 91 isolates in at least 38 species belonging to 16 sections represented by 14 clades (I-XIV). The two major clades coincided with the two subgenus currently accepted in *Penicillium*, i.e. *Penicillium* and *Aspergilloides* (Kocsubé *et al.* 2016). The former (84% bs/-- pp) includes the following sections: *Fasciculata, Roquefortorum, Chrysogena, Penicillium, Robsamsonia, Turbata, Paradoxa, Ramosa, Brevicompacta,* and *Canescentia;* and the latter (72% bs/0.99 pp) the sections *Exilicaulis, Lanata-Divaricata, Stolkia, Citrina, Sclerotiora, Cinnamopurpurea* and *Aspergilloides*.

Clade I (--% bs/0.99 pp), representing the section *Fasciculata*, included 18 isolates identified as *P. biforme* (n=2), *P. crustosum* (n=15) and *P. polonicum* (n=1). *Penicillium crustosum* was the most frequent species in this study (14.28%). It is a relatively common species, frequently isolated from nuts, meat, cheese, feeds, vegetables, and pomaceous and stone fruits (Sonjak *et al.* 2005). In our study, *P. crustosum* was mainly recovered from Mediterranean areas (Catalonia and Balearic Islands), with the only exception of one isolate that was from Galicia.

Clade II included members of the section *Roquefortorum* (92% bs/1 pp), a section that comprises relevant species used in the cheese industry (Houbraken *et al.* 2016). Within the clade clustered three dung isolates (FMR 15031, FMR 15032, and FMR 15188), that were related to *P. roqueforti* but forming an independent and distant branch that could represent an undescribed species. *Roquefortorum* is a small section of closely related species (Houbraken *et al.* 2010). To evaluate possible intra- and inter-specific variability within the species currently accepted, i.e. *P. carneum, P. paneum, P. psychrosexualis* and *P. roqueforti,* and the phylogenetic position of our putative new species, we performed an additional analysis with *Ben*A gene (see Figure S7 in supplemental material) with more sequences of the species available in GenBank. This demonstrated that *P. roqueforti* is divided in two clades with an intra-

specific variability of around 0.08%, with our isolates forming a separate branch from *P. roqueforti* complex. The concatenated analysis using ITS, *Ben*A, *CaM* and *RPB*2 (Figure 8) supported the novelty of these three isolates, being therefore proposed as *P. mediteraneum*. Although, *P. mediterraneum* and *P. roqueforti* show identical ITS barcode, both species have unique *Ben*A, *CaM* and *RPB*2 sequences.

Clades III and IV were formed by mambers of the the sections *Chrysogena* (74% bs/-- pp) and *Penicillium* (--% bs/1 pp), respectively. In the former, two dung isolates were identified as the species *P. chrysogenum* and *P. flavigenum*; whereas in the latter, other two were identified as *P. expansum*. Until 2016, the species of the section *Penicillium* had been isolated from very different substrates, including dung; however, this section was reevaluated recently, and now mainly contains plant pathogenic species (Houbraken *et al.* 2016). By contrast, the species of the section *Chrysogena* are well-known and usually found on soil, with the exception of *P. chrysogenum*, *P. nalgiovense* and *P. rubens* that commonly occur in indoor environments (Houbraken *et al.* 2012).

Clade V represented the section *Robsamsonia*, a section recently introduced by Houbraken *et al.* (2016) and that includes the majority of coprophilous species described in *Penicillium*. A total of 18 isolates (19.78%) were included here; thirteen of them identified as belonging to the species *P. brevistipitatum* (n=1), *P. concentricum* (n=2), *P. coprobium* (n=2), *P. coprophilum* (n=1), and *P. griseofulvum* (n=7); the five remaining isolates (FMR 15192, FMR 15210, FMR 15211, FMR 16481 and FMR 16491) could not be assigned to any known species. The preliminary *Ben*A analysis, but also the concatenate phylogeny (Figure 9) including the currently accepted species in the section, showed that the five isolates grouped together in a very supported and undescribed lineage closely related to the ex-type strain of *P. glandicola.* An additional *Ben*A analysis (see Figure S8 in supplemental material) with more sequences of *P. glandicola* showed that our isolates and *P. glandicola* were 97.4% similar, confirming that they were new taxa. The genetic differences and morphological peculiarities

observed in such group of isolates allowed to describe the novel species *P. synnematicola.* Interestingly, this species seems to be a common coprophilous fungus in the Mediterranean area, since most isolates have been recovered from goat dung collected in Catalonia and in the Balearic Islands.

Clade VI comprised the sections *Turbata* (95% bs/1 pp) and *Paradoxa* (94% bs/-- pp). In the former, the isolate FMR 15041 closely related to *P. bovifimosum*, did not fit with any species of the section. In *Paradoxa*, which was the third section with most isolates in this study (n=11), two species were identified, *P. atramentosum* (n=4) and *P. magnielliptisporum* (n=1), and five isolates (FMR 15040, FMR 15104, FMR 15107, FMR 15191, and FMR 15196) could no be identified at the species level. These latter were allocated in three single branches, which could represent three putative new species for the genus. The concatenated phylogeny of sections *Paradoxa* and *Turbata* with the six unidentified isolates is shown in Figure 10. The section *Paradoxa* was divided in two fully supported clades, one with *P. crystallinum*, *P. malodoratum* and *P. paradoxum*, and the other one that included the unidentified isolates of the section *Paradoxa*, in addition to the ex-type strains of *P. atramentosum*, *P. magnielliptisporum* and *P. mexicanum*. The isolates FMR 15107 and FMR 15404 clustered together, as well as FMR 15191 and 15196, and their respective single branches were separated from other two terminal branches with FMR 15104 and the ex-type strain of *P. atramentosum.* This latter species has been reported in fact as a species complex (Visagie et al. 2014b), from which *P. mexicanum* and *P. magnielliptisporum* have recently been described as new. The genetic differences showed between the lineage of FMR 15107 and FMR 15040 (97.07% similar with *Ben*A; 98.23% similar with concatenate dataset), that of FMR 15104 (94.68% similar with *Ben*A; 95% concatenate dataset), and that of FMR 15191 and 15196 (96.2% similar with *Ben*A; 96.2% concatenate dataset) respect to *P. atramentosum*, the closest species, allow to consider them distinct taxa. These isolates are described below as *P. ibericum, P. fimosum* and *P. balearicum*, respectively. An additional phylogenetic analysis with

*Ben*A including more sequences of the most closely related species confirms our proposal (see Figure S9 in supplemental material). *Paradoxa* is the section of *Penicillium* with the highest number of new species found in this study. In section *Turbata* the unidentified isolate (FMR 15041) was placed in the same clade than *P. bovifimosum*, but forming a long terminal branch which proved to be a distinc species. Thus, it is described as a new species, *P. caprifimosum. Penicillium bovifimosum* is a monotypic species described from dry cow manure by Tuthill & Frisvad (2002).

Section *Ramosa* was represented by the clade VII (97% bs/0.98 pp), which included only the isolate FMR 15038. Currently, this section counts with 13 accepted species (Visagie *et al.* 2016a), being *P. chroogomphum* the most recent species described in the group (Rong *et al.* 2016). *Penicillium lanosum* and *P. kojigenum* are listed as different species of this section (www.aspergilluspenicillium.org) although Samson & Pitt (2000) had considered them conspecific. In the concatenate analysis performed with three markers (ITS, *Ben*A, and *CaM*), since *RPB*2 sequences were not available for all species in the section (Figure 11), the isolate FMR 15038 was placed in an independent branch between two clades, each one with two species, *P. kojigenum* and *P. lanosum* in the first one, and *P. jamesonlandense* and *P. swiecickii* in the second. The similarity among our isolate and its phylogenetic sisters was 98.3%, proving that our isolate is a distinguishing species, proposed here as *P. beceitense*.

Clade VIII, the last of the subgenus *Penicillium*, comprised the sections *Brevicompacta* (99% bs/1 pp) and *Canescentia* (98% bs/1 pp). In the former, only one isolate was identified as *P. brevicompactum*, a species commonly inhabiting in soil and decaying vegetation, but previously described also from food, cereals, textiles, clinical specimens and feces of snake (Pitt 1979, Guevara-Suarez *et al*. 2016). In the section *Canescentia,* seven isolates were identified as *P. canescens* (n=1), *P. murcianum* (n=3) and *P. radiatolobatum* (n=3). The use of *BenA* for species identification within this section is difficult, especially to distinguish *P. radiatolobatum* and *P. murcianum*.

Therefore, the analysis of *CaM* is recommended to solve this problem (Visagie *et al.* 2016a).

Clade IX corresponded to section *Exilicaulis* (99% bs/1 pp), in which eight of our isolates were included. However, as observed in *Apergillus* section *Terrei*, the general tree based only on *Ben*A was not useful to identify some isolates of the section, especially those belonging to the *P. restrictum*-clade. However, we confirmed the identification of *P. arabicum* (n=2), *P. burgense* (n=1), *P. cinereoatrum* (n=1), *P. momoii* (n=1), and *P. rubefaciens* (n=2) based on a single *Ben*A analysis of the section (see Figure S10 in supplemental material). The only isolate located in *P. restrictum*-clade that could not be identified at the species level was FMR 15841. In the most recent review of the section, Visagie *et al.* (2016b) indicated that such clade needs further revision since it could include some additional cryptic species. Thus, the identification of our isolate remains uncertain until further studies clarifiying the taxonomic structure about the *P. restrictum*-clade.

In section *Lanata-Divaricata* (clade X, 92% bs/1 pp), three isolates were included. One of them was identified as *P. brasilianum*, a widespread species commonly found on soil and recently also reported from human clinical specimens (Pitt 1979, Guevara-Suarez *et al.* 2016); and the other two as *P. cremeogriseum,* a species previously found on forest soil from Ukraine (Houbraken & Samson 2011). To our knowledge, this is the first report of *P. brasilianum* and *P. cremeogriseum* associated to herbivore dung. The section has been recently revised by Visagie *et al.* (2016a), who described 7 new species, mostly from soil.

The only species identified in our study belonging to section *Stolkia* (clade XI, 99% bs/1 pp) was *P. canariense.* This species was described from soil in Canary Islands (Peterson & Sigler 2002), the same geographical origin as our isolate although from different substrates. This is the second isolate of this species obtained so far.

In section *Citrina* (clade XII, 98% bs/1 pp), five isolates were identified as *P. citrinum* and two as *P. sizovae*. Although we have not found previous records of these

species from dung, they have a worldwide distribution, being isolated from soil, foodstuff, and many other types of substrates (Houbraken *et al.* 2011). This section comprises nearly 40 species, but only those identified here were included in the analysis to simplify the *Ben*A phylogenetic tree.

Clade XIII grouped the sections *Sclerotiora* (77% bs/-- pp) and *Cinnamopurpurea* (96% bs/-- pp). In the former, one isolate was identified as *P. lilacinoechinulatum* and in the latter another one as *P. cvjetkovicii.* Houbraken and Samson (2011) revised the taxonomic position of *P. lilacinoechinulatum* and *P. nodositatum* and considered both synonyms of *P. bilaiae.* However, they are currently recognized as three distinc species (Visagie *et al.* 2013). *Penicillium cvjetkovicii* has been recently described from indoor air samples in the USA by Peterson *et al.* (2015).

The last clade XIV (99% bs/1 pp) emcompased *Penicillium* species belongind to the section *Aspergilloides*. The species identified were *P. glabrum* (n=2), *P. frequentans* (n=2), *P. roseoviride* (n=2), and *P. rudallense* (n=1). *Penicillium frequentans* together with *P. spinulosum* and *P. glabrum* are the most common species in the section, being isolated from a wide range of substrates, including soil, food, bark, and indoor environments (Houbraken *et al.* 2014b). Although currently *P. glabrum* and *P. frequentans* are accepted as distinc species, they were considered synonyms for a long time (Houbraken *et al.* 2014b).

Talaromyces phylogeny

The phylogenetic tree based on the *Ben*A locus (Figure 12) shows the relationships of the 10 *Talaromyces* isolates included in the study. The aligned dataset was 384 bp long, from which 193 were variable sites and 156 phylogenetic informative. The ML substitution model was K2 + G +I, while to BI it was GTR + G +I. Two main clades were formed, representing sections *Talaromyces* (97% bs/1 pp) and *Trachyspermi* (99% bs/1 pp). Six out of nine isolates included in the first section (FMR 15489, FMR15490, FMR 15199, FMR 15035, FMR 15307 and FMR 15303) could not be assigned to any

known species of that group, whereas the other three were identified as *T. muroii* (FMR 15496), *T. ruber* (FMR 15839) and *T. sayulitensi* (FMR 15842). *Talaromyces muroii* and *T. sayulitensis* are considered uncommon species mainly associated to soil and indoor environments (Visagie *et al.* 2014b; Chen *et al.* 2016b). On the other hand, only one isolate (FMR 16441) was included in the section *Trachyspermi*, which could not be identified.

To resolve the taxonomy of the unidentified isolates in both sections, we performed the respective phylogeny using sequences of the ITS, *BenA* and *CaM* genes and including all accepted species in each section. Although *RPB*2 was sequenced for all isolates, this marker was not incorporated in the concatenated analysis since there were not sequences available for all the type strains of this section. The combined phylogeny of the members of the section *Talaromyces* is shown in Figure 13. *Talaromyces* is the largest section in the genus and includes nearly 50 species, 15 of them described in the last year from environmental or clinical samples (Cheng *et al.* 2016b; Guevara-Suarez *et al.* 2017; Visagie *et al.* 2015; Wang *et al.* 2016, Yilmaz *et al.* 2016a;b). Our concatenate dataset showed that the unidentified isolates FMR 15489 and FMR 15490 exhibited both a similarity of 99.42% respect to the ex-type strain of *T. angelicus*, a monotypic species described by Sang *et al.* (2013) from roots of *Angelica gigas* in Korea. Although most of their morphological features matched with those of the protologue of *T. angelicus*, we observed some variation respect to the description of this species by Yilmaz *et al.* (2014); i.e, production of diffusible red pigment in one of our isolates (FMR 15490), the colonies of our isolates were deep turquoise and pastel red rather than yellow to white on MEA, they grew faster at 37 ºC (on CYA 34–37 mm diam in 7d vs 25–27 mm in Yilmaz *et al.* 2014), and exhibited stipes up to 100 µm long (up to 120 µm in Yilmaz *et al.* 2014). On the other hand, the isolate FMR 15199 was located in an independent branch clearly distinct from the other species of the section; the isolates FMR 15035 and FMR 15307 clustered together and were closely related to *T. funiculosus* but with a genetic difference enoght to be considered distinct species

(see also Figure S11 in supplemental material); and the isolate FMR 15303 formed an independent branch close to the clade containing *T. francoae, T. kendrickii, T. mangshanicus, T. qii* and *T. thailandensis*. Based on concatenated phylogenetic analysis and supported by phenotypic differences (see taxonomy section), we propose the following three new species, *T. coprophilus, T. pseudofuniculosus*, and *T. gamsii*, respectively. These species can be easily identified with the *Ben*A marker.

The combined phylogenetic analysis of the section *Trachyspermi* (Figure 14) showed that the isolate FMR 16441 was located in a single branch within the same clade that *T. albobiverticillius*, *T. erythromellis, T. heiheensis*, and *T. solicola* (88% bs/0.99 pp). Considering the unique phylogenetic position and the morphological differences observed, we propose the new species *T. catalonicus*. With the inclusion of these new taxa, the section *Trachyspermi* currently comprises 18 species, the most recently described being *T. aerius* from indoor air, *T. heiheensi* from rotten wood, and *T. minnesotensis* from human clinical specimens (Yaguchi *et al*. 1996; Yilmaz *et al*. 2014; Chen *et al*. 2016b; Wang *et al.* 2016; Guevara-Suarez *et al.* 2017). To date, there are no reports of the presence of species of this section on dung samples.

Phylogeny of miscelaneous *Aspergillaceae*

According to the *Ben*A phylogeny, four of our isolates (FMR 15296, FMR 16442, FMR 14718, FMR 15299) were related to some penicillia currenly excluded from the genus *Penicillium* (i.e. *P. giganteum, P. megasporum* and *P. nodositaum*) but belonging to the family *Aspergillaceae* (Figure 15). The aligned dataset of this analysis consisted in 393 bp long, from which 242 were variable sites and 216 phylogenetic informative. The best substitution model for ML was K2 +G+I and for BI it was GTR +G+I. Whereas FMR 15296 and FMR 16442 were located in a well-supported clade along with the ex-type strain of *P. nodositatum*, FMR 14718 and FMR 15299 were allocated in a fully supported distant clade together with the ex-type strains of *P. giganteum* and *P. megasporum.* Despite the morphology of these taxa resambles *Penicillium*, Peterson *et*

al. (2010) based on a multigene phylogeny demonstrated that the two latter species did not belong to *Penicillium* s.str.; and later, Visagie *et al.* (2013) excluded *P. nodositatum* due to its genetic differences respect to the true penicillia. Phylogenetically, these two clades where the mentioned species are allocated could clearly represent two undescribed genera in *Aspergillaceae*. Our phylogeny inferred with sequences of the ITS, *BenA* and *RPB*2 genes, including the isolates under study and members of different genera of *Aspergillaceae* confirms the proposal of two novel genera (Figure 16), which are described below as *Penicillago* (*Pgo.*), typified by *Penicillium nodositatum*, and *Pseudopenicillium* (*Pse.*) with *Penicillium giganteum* and *Penicillium megasporum* as the type. The former genus being more phylogenetically related with *Penicillium,* whereas the latter resulted a sibling genus of *Hamigera* such as it was previously suggested by Peterson *et al.* (2010).

Although in the analysis of *Ben*A sequences (Figure 15), the ex-type strain of *P. nodositatum* and the isolates FMR 15296 and FMR 16442 seemed to be fungi genetically very similar, in the concatenated analysis both isolates resulted to be 85% similar respect to *P. nodositaum* (Figure 16). This difference allows us to propose a new species for the genus *Penicillago*, named *Pgo. flava*, and the new combination for *P. nodositatum* (*Pgo. nodositata*). Morphologically, *Pgo. flava* differs from *Pgo. nodositata* mainly by showing better growth on CYA and MEA at 25°C (40–44 mm and 34–36 mm in 7 days, respectively, vs 13–19 mm and 16–30 mm in *Pgo. nodositata*). According to our results, the proper molecular marker for the identification *Penicilago* species is *RPB*2.

The isolate FMR 14718 matched morphologically and molecularly with *P. giganteum*, whereas FMR 15299 was placed in a new lineage into the *Pseudopenicillium* clade, being more closely related to *P. megarporum* (Figures 15, 16). The morphological differences observed, such as having smaller conidia (up to 6 µm) than those of the other two species in the genus (conidia up to 12 µm in *P. giganteum*, and up to 10 µm in *P. megarporum*), and the genetic differences obtained

(98% similar to *P. giganteum,* and 98.16% similar to *P. megasporum*) allow us to propose it as a new species named *Pseudopenicillium coprobium.*

TAXONOMY

Aspergillus albodeflectus J.P.Z. Siqueira, D. García & Gené, **sp. nov.** MycoBank MB 821808. Figure 17.

Etymology — Name refers to the white color of the colonies and the commonly observed bent conidial heads.

In — Section *Flavipedes*

Specimen examined — Spain, Balearic Islands, Mallorca, Pollença, from herbivore dung, February 2016, J. Guarro, M. Guevara-Suarez, J.P.Z. Siqueira (**holotype** CBS H-23128; culture ex-type FMR 15175 = CBS 142665; ITS barcode LT798909, alternative markers: *Ben*A LT798936, *CaM* LT798937, *RPB*2 LT798938).

Colony diameter in 7d (mm) — On CYA: 25 ºC 19–21, 30 ºC 20–21, 37 ºC 7–8; on MEA: 25 ºC 22–23; on DG18: 25 ºC 17–20; on YES: 25 ºC 20–23; on OA: 25 ºC 8–10; on CREA: 25 ºC 9–11.

Colony characters at 25 ºC in 7 d — On CYA, colonies powdery, forming concentric circles, mycelium white, margin entire; reverse pale yellow (3A3); sporulation dense, with conidial masses white; soluble pigment absent; exudate absent. On MEA, colonies powdery, radially sulcate, mycelium white, margin slightly lobulate; reverse light yellow (4A5); sporulation dense, with conidial masses white; soluble pigment absent; exudate absent. On DG18, colonies velvety, mycelium white, margin entire; reverse pale (2A2); sporulation moderately dense, with conidial masses white; soluble pigment absent; exudate absent. On YES, colonies powdery, radially sulcate, with a slightly elevated center, mycelium white, margin entire; reverse light yellow (4A5); sporulation dense, with conidial masses white; soluble pigment absent; exudate absent. On OA, colonies with floccose center, powdery towards the periphery, mycelium white, margin entire; reverse brownish yellow (5C7); sporulation dense, with

conidial masses white; soluble pigment absent; exudate absent. On CREA, acid production absent.

Micromorphology — On MEA, conidiophores biseriate and often with conidial heads slightly to strongly bent, loosely columnar, white; stipes commonly septate, 150–550 x 5–8 μm, smooth, hyaline; vesicles globose to subglobose, 12–20 μm wide, hyaline; metulae cylindrical, with the apex slightly wider than the bottom, covering 50% to 75% of the vesicle, 6–9 x 3–6 μm, hyaline; phialides flask-shaped, 6–8.5 x 3–5 μm, hyaline; conidia globose, 2–4 μm diam, smooth, hyaline. Hülle cells and ascomata not observed.

Differential diagnosis — The species more closely related to *A. albodeflectus* are *A. lupii* and *A. movilensis*. However, *A. lupii* produces bright yellow colonies and *A. movilensis* produces slightly larger colonies on MEA (25– 30 mm) and on CYA at 37 ºC (10–17 mm). Moreover, in the latter species, colonies become light brown in age (Hubka *et al.* 2015) and both species present Hülle cells, never observed in *A. albodefectus*. However, the production of Hülle cells could be influenced by the composition of the culture media (Hubka *et al.* 2015).

Aspergillus aurantiosulcatus J.P.Z. Siqueira, Guarro & D. García, **sp. nov.** MycoBank MB 821811. Figure 18.

Etymology — Name refers to the orange color, characteristic of the colonies and also to the furrows commonly observed, especially on CYA.

In — Section *Terrei*

Specimen examined — Spain, Balearic Islands, Mallorca, road near Orient**,** from herbivore dung, February 2016, J. Guarro, M. Guevara-Suarez, J.P.Z. Siqueira (**holotype** CBS H-23187; cultures ex-type FMR 15182 = CBS 142981; ITS barcode LT798912, alternative markers: *Ben*A LT798945, *CaM* LT798946, *RPB*2 LT798947).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 37–39, 30 ºC 56–57, 37 ºC 62–65; on MEA: 25 ºC 27–28; on DG18: 25 ºC 34–38; on OA: 25 ºC 20–21; on YES: 25 ºC 55–61; on CREA: 25 ºC 24–26.

Colony characters at 25 ºC in 7 d — On CYA, colonies floccose, radially and concentrically sulcate, mycelium white to pale yellow (3A3), margin entire; reverse brownish orange (6C6); sporulation moderately dense, with conidial masses light orange (6A4); soluble pigment yellowish brown (5D4); exudate colorless. On MEA, colonies floccose, mycelium white to greyish orange (5B3), margin entire; reverse brownish orange (6C6); sporulation moderately dense, with conidial masses white to yellowish brown (5F5); soluble pigment amber yellow (4B6); exudate yellowish white (4A2). On DG18, colonies floccose, mycelium white, margin entire; reverse light yellow (4A4) to orange (5A7); sporulation absent; soluble pigment absent; exudate absent. On YES, colonies cottony, irregularly sulcate, mycelium white, margin entire; reverse orange (6B7); sporulation moderately dense; with conidial masses white to light orange (6A5); soluble pigment absent; exudate absent. On OA, colonies powdery, dense at center, mycelium greyish orange (5B5), margin entire; reverse pale yellow (4A3) to orange (5A6); sporulation dense, with conidial masses light orange (6A5); soluble pigment absent; exudate absent. On CREA, acid production absent.

Micromorphology — On MEA, conidiophores with conidial heads biseriate, loosely radiate to columnar, white to brownish orange (5C6); stipes commonly septate, 110–350 x 2–8 µm, smooth, hyaline; vesicles globose to subglobose, 13–27 µm wide, hyaline; metulae cylindrical, with the apex slightly wider than the bottom, 4.5–6.5 x 2–4 µm, hyaline; phialides flask-shaped, 4–6 x 1.5–3 µm, hyaline; conidia globose to subglobose, 1.5–3 µm diam, smooth, hyaline to shades of yellow. Accessory conidia not observed. Ascomata not observed.

Differential diagnosis — *Aspergillus aurantiosulcatus* is phylogenetically related to *A. alabamensis.* This latter species produces colonies yellowish-brown to cinnamon-

brown, conidial heads are reported as densely columnar and the vesicles subglobose (Balajee *et al.* 2009).

Aspergillus calidokeveii J.P.Z. Siqueira, D. García & Gené, **sp. nov.** MycoBank MB 821814. Figure 19.

Etymology — Name refers to the genetic similarity with *A. keveii* and the ability to grow at 37°C.

In — Section *Usti*

Specimen examined — Spain, Castile and Leon, Palencia, San Juan Valley, from herbivore dung, March 2016, J. Guarro, M. Guevara-Suarez (**holotype** CBS H-23129; cultures ex-type FMR 15225 = CBS 142666; ITS barcode LT798914, alternative markers: *Ben*A LT798951, *CaM* LT798952, *RPB*2 LT798953).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 36–40, 30 ºC 49–50, 37 ºC 23–25; on MEA: 25 ºC 32–34; on DG18: 25 ºC 24–25; on YES: 25 ºC 39–41; on OA: 25 ºC 27–29; on CREA: 25 ºC 22–24.

Colony characters at 25 ºC in 7 d — On CYA, colonies floccose, slightly sulcate, mycelium white to brownish grey (6D2), margin predominantly entire; reverse light yellow (4A5) to yellow (3A7); sporulation dense, with conidial masses olive brown (4E3); soluble pigment yellow (3A7); exudate colorless to light yellow (4A5). On MEA, colonies floccose, mycelium white to brownish grey (6D2), margin entire; reverse light yellow (4A5) to yellow (4A8); sporulation dense, with conidial masses yellowish grey (3D2); soluble pigment yellow (3A7); exudate absent. On DG18, colonies floccose, mycelium white, margin entire; reverse yellowish white (3A2) to greyish yellow (3B5); sporulation sparse; soluble pigment yellow (3A7) in 14 days; exudate absent. On YES, colonies floccose, with raised center, mycelium white to brownish grey (5C2), margin predominantly entire; reverse light yellow (4A4) to greyish orange (6B5); sporulation dense, with conidial masses pale grey (1B1) to medium grey (1E1); soluble pigment yellow (3A7) weakly produced; exudate absent. On OA, colonies floccose to cottony,

mycelium white, margin entire; reverse light yellow (2A4 to 2A5); sporulation dense, with conidial masses olive brown (4A6); soluble pigment absent; exudate absent. On CREA, acid production absent.

Micromorphology — On MEA, conidiophores with conidial heads biseriate, radiate, pale grey (1B1) to olive brown (4E3); stipes commonly septate, (70)110–300 x 3–7.5 μm, smooth to verruculose, in shades of brown; vesicles globose, subglobose to spatulate, (8.5)11–19 μm wide, in shades of brown; metulae cylindrical, covering 50% to 100% of the vesicle, 4.5–7 x 3–5 μm, hyaline to orange brown; phialides flask-shaped, 7–9 x 2–4 μm, hyaline to orange brown; conidia globose to subglobose, 2.5–5 μm diam, rough, in shades of brown. Hülle cells abundant, variably shaped, mostly elongated, 32–120 x 10–28 μm. Ascomata not observed.

Differential diagnosis — The present species is phylogenetically related to *A. keveii*, but they can be phenotipically differentiated mainly by the absence of growth at 37 ºC in the latter (Houbraken *et al.* 2007). In addition, *A. keveii* has conidial heads loosely columnar with pyriform and smaller (9–13 μm) vesicles (Houbraken *et al.* 2007), while *A. calidokeveii* exhibits radiate conidial heads with subglobose to spatulate vesicles, measuring 11–19 μm wide.

Aspergillus canariensis J.P.Z. Siqueira, Gené & Guarro, **sp. nov.** MycoBank MB 821815. Figure 20.

Etymology — Name refers to the Canary Islands, where the fungus was found.

In — Section *Candidi*

Specimens examined — Spain, Canary Islands, Gran Canaria, Santa Brígida, from herbivore dung, September 2016, J. Cano-Lira , D. García, J. Guarro, M. Guevara-Suarez (**holotype** CBS H-23188; cultures ex-type FMR 15736 = CBS 142982; ITS barcode LT798906, alternative markers: *Ben*A LT798927, *CaM* LT798928, *RPB*2 LT798929); Teror, from herbivore dung, June 2016, J. Cano-Lira , D. García, J. Guarro, M. Guevara-Suarez (FMR 15733 = CBS 142983).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 17–20, 30 ºC 19–21, 37 ºC no growth; on MEA: 25 ºC 10–13; on DG18: 25 ºC 15–16; on YES: 25 ºC 22–23; on OA: 25 ºC 13–14; on CREA: 25 ºC 12–13.

Colony characters at 25 ºC in 7 d — On CYA, colonies floccose, with a slightly granulose center, mycelium white, margin entire; reverse pale (5A3) to light orange (5C4); sporulation dense, with conidial masses white to reddish white (7A2); sclerotia absent; soluble pigment brown (6F7) in 14 d; exudate absent. On MEA, colonies floccose, mycelium white, margin slightly lobulate; reverse pale orange (5A3) to brownish orange (5C4); sporulation dense, with conidial masses white to yellowish white (3A2); sclerotia absent; soluble pigment absent; exudate colorless to yellowish white (2A2). On DG18, colonies floccose to velutinous, with submerged mycelium, mycelium white to greenish grey (2B2), margin lobulate; reverse yellowish white (3A2); sporulation moderately dense, especially at borders, with conidial masses white; sclerotia absent; soluble pigment absent; exudate absent. On YES, colonies floccose, slightly sulcate, with elevated center, mycelium white, margin slightly lobulate; reverse pale yellow (4A3) to brownish orange (5C6); sporulation dense, with conidial masses white to greyish white (1B1); soluble pigment absent; exudate absent. On OA, colonies slightly granulose to powdery, with submerged mycelium, mycelium white, margin irregular; reverse yellowish white (3A2); sporulation moderately dense, with conidial masses white; sclerotia absent; soluble pigment absent; exudate colorless. On CREA, acid production absent.

Micromorphology — On MEA, conidiophores with conidial heads biseriate, radiate, white to reddish white (7A2); stipes commonly septate, 200–500 x 4–7.5 µm, smooth, hyaline; vesicles usually globose, (9)12–27 µm wide, hyaline; metulae cylindrical, with the apex slightly wider than the bottom, 6–15(21.5) x 5–8.5 µm, hyaline; phialides flask-shaped, 4–8.5 x 2–3 µm, hyaline; conidia globose, 2–4 µm, smooth, hyaline. Ascomata not observed.

Differential diagnosis — *Aspergillus canariensis* is similar to *A. candidus*, *A. subalbidus* and *A. verruculosus* in its white colonies, smooth conidia and the inability to grow at 37 °C, but differs in the absence of penicillium-like structures typically produced by *A. subalbidus* and *A. candidus* (Visagie *et al.* 2014b), and also by the absence of sclerotia, which are abundantly produced by *A. verruculosus* on CYA.

Aspergillus coprophilus J.P.Z. Siqueira, D. García & Gené, **sp. nov.** MycoBank MB 821816. Figure 21.

Etymology — Name refers to the substrate where the species was found.

In — Section *Candidi*

Specimens examined — Spain, Castile and Leon, Palencia, Monte el Viejo, Deer Reserve Park**,** from deer dung, March 2016, J. Guarro, M. Guevara-Suarez (**holotype** CBS H-23189; cultures ex-type FMR 15224, CBS 142984; ITSbarcode: LT798902, alternative identification markers: *Ben*A LT798915, *CaM* LT798916, *RPB*2 LT798917); Palencia, San Juan Valley, from herbivore dung, March 2016, J. Guarro, M. Guevara-Suarez (FMR 15226 = CBS 142985).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 18–21, 30 ºC 16–18, 37 ºC no growth; on MEA: 25 ºC 13–15; on DG18: 25 ºC 12–14; on YES: 25 ºC 26–28; on OA: 25 ºC 8–12; on CREA: 25 ºC 5–7.

Colony characters at 25 ºC in 7 d — On CYA, colonies floccose at the center, granulose towards the periphery due to the production of scletoria, mycelium white, margin lobulated; reverse light yellow (4A4) to becoming dark brown (9F5) after 14 d; sporulation dense, with conidial masses yellowish white (4A2); sclerotia abundant, dark brown (9F5 to 7F4); soluble pigment dark purple (14F3) present after 14 d; exudate clear to yellowish white (4A2). On MEA, colonies similar to those on CYA; sclerotia absent; soluble pigment dark purple (14F3) in 14d; exudate absent. On DG18, colonies floccose, mycelium white to orange white (5A2), margin entire; reverse colorless; sporulation absent; sclerotia absent; soluble pigment absent; exudate absent. On YES,

colonies floccose, slightly sulcate, mycelium white, margin slightly lobulate; reverse pale yellow (4E3) to orange brown (4B7); sporulation dense, with conidial masses white; sclerotia absent; soluble pigment absent; exudate absent. On OA, colonies with aerial mycelium scarce, white, margin entire and with submerged mycelium; reverse white; sporulation sparse, only at the center and the periphery, with conidial masses white; sclerotia absent; soluble pigment absent; exudate absent. On CREA, poor growth, acid production absent.

Micromorphology — On MEA, conidiophores with conidial heads biseriate, radiate, white; stipes commonly septate, 300–650 x 5–11 µm, smooth, hyaline; vesicles globose to subglobose, occasionally diminutive, (5)13–27 µm wide, hyaline; metulae cylindrical, with the apex slightly wider than the bottom, 7–13 x 5–6 µm, hyaline; phialides flask-shaped, 5–8 x 2–3 µm, hyaline; conidia subglobose to ellipsoidal, 3–6 x 2–4 µm, smooth, hyaline; sclerotia usually globose to subglobose, 300–1100 µm, dark brown (9F5 to 7F4). Ascomata not observed.

Differential diagnosis — *Aspergillus coprophilus* is closely related to *A. campestris*, *A. candidus* and to the new species described here *A. longipes*. *Aspergillus campestris* and *A. longipes* can be distinguished by the absence of sclerotia and the former also by the production of sulphur yellow colonies (Varga *et al.* 2007b). *Aspergillus candidus* differs by its whitish or yellowish brown colony reverse on CYA after 14 d (Hubka *et al.* 2014), while in *A. coprophilus* this is dark brown.

Aspergillus esporlensis J.P.Z. Siqueira, Gené & Guarro, **sp. nov.** MycoBank MB 821817. Figure 22.

Etymology — Name refers to the town where the species was found.

In — Section *Cremei*

Specimen examined — Spain, Balearic Islands, Mallorca, Esporles, from soil mixed with dung, Juny 2012, J. Gené (**holotype** CBS H-23139; cultures ex-type: FMR

14605 = CBS 142750; ITS barcode LT798908, alternative markers: *Ben*A LT798933, *CaM* LT798934, *RPB*2 LT798935).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 25–27, 30 ºC 5–6; 37 ºC no growth; on MEA: 25 ºC 19–20; on DG18: 25 ºC 33–35; on YES: 25 ºC 44–48; on OA: 25 ºC 12–13; on CREA: 25 ºC 14–16.

Colony characters at 25 ºC in 7 d — On CYA, colonies cottony to floccose, mycelium white to greyish yellow (4B4), margin slightly lobulate; reverse pale yellow (4A3); sporulation dense, with conidial masses light yellow (4A4) to golden (4C6), soluble pigment absent; exudate clear to yellowish white (3A3). On MEA, colonies cottony, mycelium white to light brown (5D5), margin predominantly entire; reverse greyish yellow (3B6); sporulation dense, with conidial masses light yellow (3A5) to golden brown (5D7); soluble pigment absent; exudate clear to yellowish white (3A3). On DG18, colonies cottony to lanose, mycelium white, margin entire; reverse white; sporulation sparse, with conidial masses white to yellow (3A6), soluble pigment absent; exudate absent. On YES, colonies floccose, cottony towards periphery, mycelium white to light brown (5D5), margin slightly lobulate; reverse pale yellow (3E3); sporulation dense, with conidial masses light yellow (3A5) to brown (5E5); soluble pigment absent; exudate absent. On OA, colonies floccose, mycelium white to light brown (5D5), margin lobulate; reverse greyish orange (5B5); sporulation dense, with conidial masse light orange (5A5) to brown (5E5); soluble pigment absent; exudate absent. On CREA, acid production weak.

Micromorphology — On MEA, conidiophores with conidial heads biseriate, radiate, white to golden brown (5D7); stipes commonly septate, 200–600 x 4.5–10 µm, smooth, often verruculose towards the apical part , subhyaline; vesicles globose to spatulate, 11.5–50 µm wide, hyaline; metulae cylindrical, 7–15.5 x 5–12 µm, hyaline; phialides flask-shaped, 8–11 x 3–5 µm, hyaline; conidia globose to subglobose, sometimes ellipsoidal, 4–5.5 x 3–5.5 µm, rough, in shades of brown. Ascomata not observed.

Differential diagnosis — The closest related species to *A. esporlensis* is *A. dimorphicus*. Although the two species are genetically distinct, morphologicaly they are rather similar. However, *A. dimorphicus* was described with swollen metulae and branched conidiophores (Mehrotra and Prasad 1969), features not observed in A. *esporlensis*. Moreover, *A. dimorphicus* has very delicately roughened conidia, while in *A. esporlensis* they are clearly roughened. Other closely related species are *A. wentii* and *A. europaeus*. Colonies of *A. wentii* on MEA tend to grow slowly and often with white masses of sterile hyphae on colony surface (Raper and Fennell 1965); *A. europaeus* has mostly pyriform vesicles and produces yellow soluble pigment on MEA (Hubka *et al.* 2016). Other phylogenetically related species is *A. chrysellus* which by contrast produces the sexual morph.

Aspergillus fimeti-brunneus J.P.Z. Siqueira, Gené & D. García, **sp. nov.** MycoBank MB 821818. Figure 23.

Etymology — Name refers to the substrate where it was isolated (dung) and the brown color of the colonies.

In — Section *Terrei*

Specimen examined — Spain, Andalusia, Huelva, Doñana National Park, near Rocina stream, from herbivore dung, March 2016, D. García (**holotype** CBS H-23140; cultures ex-type FMR 15228 = CBS 142751; ITS barcode LT798913, alternative markers: *Ben*A LT798948, *CaM* LT798949, *RPB*2 LT798950).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 37–40, 30 ºC 62–65, 37 ºC 67–70; on MEA: 25 ºC 28–30; on DG18: 25 ºC 42–44; on YES: 25 ºC 65–67; on OA 29–32: 25 ºC; on CREA: 25 ºC 32–34.

Colony characters at 25 ºC in 7 d — On CYA, colonies floccose, cottony towards the periphery, radially sulcate, mycelium white to pale yellow (4A3), margin slightly lobulate; reverse brownish orange (6C6); sporulation dense, with conidial masses brown (6E6); soluble pigment greyish orange (5B6) weakly produced; exudate

yellowish white (3A2). On MEA, colonies floccose, mycelium white to pale grey (1B1), margin entire; reverse golden (4C6); sporulation dense, with conidial masses white to dark brown (6F6); soluble pigment greyish orange (5B6) weakly produced; exudate colorless. On DG18, colonies floccose, dense at center, mycelium white, margin entire; reverse light yellow (3A5); sporulation moderately dense, with conidial masses white; soluble pigment absent; exudate absent. On YES, colonies floccose to cottony, irregularly sulcate, mycelium white to yellowish white (3A2), margin entire; reverse orange (5A7) to deep orange (5A8); sporulation moderately dense, with conidial masses white to pale white (1A3); soluble pigment absent; exudate absent. On OA, colonies powdery, dense at center, mycelium white to greyish orange (5B5), margin entire; reverse light orange (5A4); sporulation dense, with conidial masses brown (6D7); soluble pigment absent; exudate absent. On CREA, colonies floccose to powdery, mycelium white to yellowish white (3A2), margin entire; sporulation moderately dense; with conidial masses yellowish white (3A2) to light orange (5A4); acid production weak.

Micromorphology — On MEA, conidiophores with conidial heads biseriate, columnar, white to dark brown (6F6); stipes commonly septate, 120–320 x 5–9 µm, smooth, hyaline; vesicles globose to subglobose, (9)16–24 µm wide, hyaline; metulae cylindrical, with the apex slightly wider than the bottom, covering 50% to 75% of the vesicle, 5–7 x 2–3.5 µm, hyaline; phialides cylindrical to flask-shaped, 6–9.5 x 1.5–2.5 µm, hyaline; conidia globose to ellipsoidal, sometimes tear-shaped, 1.5–3 x 1.5–3 µm, smooth, hyaline. Accessory conidia commonly present, borne sessile or on short stalks, globose, 3–5.5 µm. Ascomata not observed.

Differential diagnosis — *Aspergillus fimeti-brunneus* forms a clade in the section *Terrei* together with *A. terreus*, *A. citrinoterreus*, *A. hortai* and *A. neoafricanus.* The novel species and *A. citrinoterreus* can be distinguished from the others by the acid production on CREA (Samson *et al.* 2011b). *Aspergillus citrinoterreus* differs in

producing a conspicuous yellow soluble pigment (Guinea *et al.* 2015), while in *A. fimeti-brunneus* it is greyish orange or absent.

Aspergillus longipes J.P.Z. Siqueira, Guarro & D. García, **sp. nov.** MycoBank MB 821819. Figure 24.

Etymology — Name refers to the long conidiophores commonly observed in the species.

In — Section *Candidi*

Specimen examined — Spain, Galicia, Ribeira Sacra**,** Sil Canyon, from herbivore dung, June 2016, J. Cano-Lira, J. Guarro, M. Guevara-Suarez (**holotype** CBS H-23141; cultures ex-type FMR 15444 = CBS 142752; ITS barcodeLT798904, alternative markers: *Ben*A LT798921, *CaM* LT798922, *RPB*2 LT798923).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 20–23, 30 ºC 11–12, 37 ºC no growth; on MEA: 25 ºC 14–17; on DG18: 25ºC 17–18; on YES: 25ºC 28–30; on OA: 25ºC 12–13; on CREA: 25 ºC 10–11.

Colony characters at 25 ºC in 7 d — On CYA, colonies cottony to granulose, with raised center, mycelium white, margin predominantly entire; reverse pale yellow (4A3) to light yellow (4A4); sporulation dense, with conidial masses white to yellowish white (4A2); sclerotia absent; soluble pigment absent; exudate colorless. On MEA, colonies very similar to CYA but with texture rather cottony than granulose. On DG18, colonies powdery to somewhat granulose, mycelium white, margin entire; reverse yellowish white (3A2); sporulation dense, with conidial masses white; sclerotia absent; soluble pigment absent; exudate absent. On YES, colonies cottony to floccose, with raised cottony center, radially sulcate, mycelium white, margin slightly lobulate; reverse light yellow (5A4); sporulation dense, with conidial masses white; sclerotia absent; soluble pigment absent; exudate absent. On OA, colonies similar to DG18. On CREA, colonies powdery, mycelium white; sporulation sparse, acid production absent.

Micromorphology — On MEA, conidiophores with conidial heads biseriate, radiate, white; stipes commonly septate, 250–820 x 4.5–8 µm, smooth, hyaline; vesicles globose to subglobose, (8)12–25 µm wide, hyaline; metulae cylindrical, 5–10(15.5) x 2–5(8) µm, hyaline; phialides flask-shaped 6–14 x 2–3.5 µm, hyaline; conidia subglobose to ellipsoidal, 2–4 x 2–5 µm, smooth, hyaline. Ascomata not observed.

Differential diagnosis — *Aspergillus longipes* is phylogenetically related to *A. campestris*, *A. candidus*, and *A. coprophilus*. However, as mentioned before, *A. campestris* differs by its sulphur yellow colonies (Varga *et al.* 2007b), and *A. candidus* by the whitish or yellowish brown colony reverse on CYA after 14 days (Hubka *et al.* 2014), while *A. coprophilus* can be maliny differentiated by the production of sclerotia.

Aspergillus majoricus J.P.Z. Siqueira, Gené & Guarro, **sp. nov.** MycoBank MB 821820. Figure 25.

Etymology — Name refers to the Mallorca Island (Spain) where the species was found.

In — Section *Terrei*

Specimens examined — Spain, Balearic Islands, Mallorca, Pollença, from herbivore dung, February 2016, J. Guarro, M. Guevara-Suarez, J.P.Z. Siqueira (**holotype** CBS H-23190; cultures ex-type FMR 15181 = CBS 142986; ITS barcode LT798910, alternative markers: *Ben*A LT798939, *CaM* LT798940, *RPB*2 LT798941); Mallorca, Pollença, from herbivore dung, February 2016, J. Guarro, M. Guevara-Suarez, J.P.Z. Siqueira (FMR 15217 = CBS 142987).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 31–36, 30 ºC 40–44, 37 ºC 42–50; on MEA: 25 ºC 24–27; on DG18: 25 ºC 23–27; on YES: 25 ºC 33–37; on OA: 25 ºC 22–25; on CREA: 25 ºC 22–26.

Colony characters at 25 ºC in 7 d — On CYA, colonies floccose, radially sulcate, mycelium white, margin slightly lobulate; reverse light yellow (4A4) to orange

yellow (4A6); sporulation dense, with conidial masses white to pale yellow (3A3); soluble pigment yellowish brown (5D5) weakly produced; exudate colorless to pale yellow (2A3). On MEA, colonies floccose to cottony, mycelium white, margin entire; reverse light yellow (4A4) to yellowish brown (5D5); sporulation moderately dense, with conidial masses white to light yellow (4A4); soluble pigment absent; exudate colorless to orange white (5A2). On DG18, colonies floccose to velvety, with submerged mycelium towards the periphery, mycelium white, margin predominantly entire; reverse white to light yellow (3A5); sporulation dense, with conidial masses white; soluble pigment absent; exudate absent. On YES, colonies floccose, irregularly sulcate, with elevated center, mycelium white, margin lobulate; reverse light yellow (4A4), with brown (6E6) areas; sporulation dense, with conidial masses white; soluble pigment light yellow (4A4 to 4A5) weakly produced; exudate absent. On OA, colonies powdery, more dense at the center, with submerged mycelium towards the periphery, mycelium white, margin predominantly entire; reverse yellowish white (3A2), deep green (28D8) areas may be observed; sporulation dense, with conidial masses white; soluble pigment absent; exudate absent. On CREA, acid production absent.

Micromorphology — On MEA, conidiophores with conidial heads biseriate, columnar, occasionally loosely radiate, white to light yellow (4A4); stipes commonly septate, 130–650 x 2.5–6.5 µm, smooth, hyaline; vesicles subglobose to spatulate, (4.5)10–18 µm wide, hyaline; metulae cylindrical, 4.5–6.5 x 2–3.5 µm, hyaline; phialides flask-shaped, 3.5–5 x 1.5–2.5 µm, hyaline; conidia globose to subglobose, 2–3 µm diam, smooth, hyaline. Accessory conidia absent. Penicillium-like conidiophores often present. Ascomata not observed.

Differential diagnosis — *Aspergillus majoricus* is phylogenetically related to *A. niveus*, *A. carneus* and *A. allahabadii*; but clearly constituting a distinct species. *Aspergillus carneus* differs in its vinaceous fawn colonies (Raper and Fennell 1965), in contrast to those of *A. majoricus* which remain light. *Aspergillus allahabadii* were reported with greenish glaucus-blue conidial heads (Mehrotra and Agnihotri 1962),

wheares those of *A. majoricus* are white to light yellow. *Aspergillus majoricus* and *A. niveus* are phenotypically very similar; however, *A. niveus* shows colony reverse on Czapek agar dark yellow through brown to greenish black, its conidia are rarely globose, and the conidiophores can be up to 1000 µm long (Raper and Fennell 1965).

Aspergillus verruculosus J.P.Z. Siqueira, Gené & D. García, **sp. nov.** MycoBank MB 821821. Figure 26.

Etymology — Name refers to the verruculose conidiophores commonly observed.

In — Section *Candidi*

Specimen examined — Spain, Canary Islands, Gran Canaria, North Coast, from herbivore dung, September 2016, J. Cano-Lira , D. García, J. Guarro, M. Guevara-Suarez (**holotype** CBS H-23130; cultures ex-type: FMR 15877 = CBS 142667; ITS barcode LT798907, alternative markers: *Ben*A LT798930, *CaM* LT798931, *RPB*2 LT798932).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 18–19, 30 ºC 16–17, 37 ºC no growth; on MEA: 25 ºC 10–12; on DG18: 25 ºC 12–13; on YES: 25 ºC 25–27; on OA: 25 ºC 12–13; on CREA: 25 ºC 8–9.

Colony characters at 25 ºC in 7 d — On CYA, colonies granulose, with raised center, mycelium white, margin lobulate; reverse brownish orange (5C5); sporulation dense, with conidial masses white to orange white (5A2); sclerotia usually formed around the border of the colony, greyish yellow (4B3) to greyish orange (5B5), darker with age; soluble pigment dark brown (9F5) in 14 d; exudate colorless. On MEA, colonies floccose to cottony, mycelium white, margin lobulated; reverse light yellow (3A5); sporulation dense, with conidial masses white; sclerotia absent; soluble pigment absent; exudate clear to yellowish white (3A2). On DG18, colonies floccose to loosely cottony, mycelium white, margin predominantly entire; reverse yellowish white (2A3); sporulation moderately dense, with conidial masses white; sclerotia absent; soluble

pigment absent; exudate absent. On YES, colonies floccose, with raised cottony center, slightly sulcate, mycelium white, margin slightly lobulate; reverse light yellow (4A4) to brownish orange (6C6), darker towards the center; sporulation dense, with conidial masses white; sclerotia reddish grey (7B2) to brownish orange (7C4); soluble pigment absent; exudate absent. On OA, colonies powdery to granulose, otherwise very similar to the colonies in DG18. On CREA, poor growth, acid production absent.

Micromorphology — On MEA, conidiophores with conidial heads biseriate, radiate, white; stipes commonly septate, (60)190–500 x 3–6.5 µm, smooth to often verruculose, hyaline; vesicles globose to subglobose, (5)9–18 µm wide, hyaline; metulae cylindrical, with the apex slightly wider than the bottom, covering 75% to 100% of the vesicle, 4.5–10 x 3.5–5.5(7) µm, hyaline; phialides flask-shaped, 4.5–8 x 1.5–3 µm, hyaline; conidia globose, 2.5–3.5 µm, smooth, hyaline; sclerotia globose to elongate, 200–800 x 160–500 µm. Ascomata not observed.

Differential diagnosis — This species is genetically related to *A. subalbidus* and *A. canariensis*. *Aspergillus verruculosus* is the only species with ornamented stipes, but also can be distinguished by the sclerotia, which are purplish black in *A. subalbidus* (Visagie *et al.* 2014b), greyish yellow (4B3) to greyish orange (5B5) in *A. verruculosus*, and absent in *A. canariensis*.

Penicillium balearicum Guevara-Suarez, Cano & Gené, **sp. nov.** MycoBank MB 822061. Figure 27.

Etymology — Name referred to the geographic area, Balearic Islands, where the fungus was isolated.

In — Section *Paradoxa*

Specimens examined — Spain, Balearic Islands, Mallorca, Pollença, from herbivore dung, February 2016, J. Guarro, M. Guevara-Suarez, J.P.Z. Siqueira (**holotype** CBS H-23215; culture ex-type FMR 15191 = CBS 143044; ITS barcode

LT899762, alternative markers: *Ben*A LT898227, *CaM* LT899758, *RPB*2 LT899760); Mallorca, Pollença, from herbivore dung, February 2016, Guarro, M. Guevara-Suarez, J.P.Z. Siqueira (FMR 15196).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 25–36, 30 °C 5–6, 37 °C no growth; on MEA: 25 ºC 14–17, 30 °C 4–6; on YES: 25 ºC 36–45, 30 ºC 4–6; on OA: 25 ºC 26–30, 30 ºC 2–6; on DG18: 25 ºC 14–27, on CREA: 25 ºC 14–20.

Colony characters at 25º C in 7 d — On CYA, colonies velvety, flat, mycelium white, sporulation dense, conidial masses dark dull green (28E3), margin dentate; reverse pale orange (5A3); exudate present, pale droplets; soluble pigment absent. On MEA, colonies velvety, elevated, sporulation dense, conidial masses dark dull green (28E3), margin lobate; reverse light orange (5A4); exudate and soluble pigment absent. On DG18, colonies flat and velvety, mycelium white, sporulation dense, conidial masses dull green (27D4); reverse greenish white (27B2). On YES, colonies cerebriform, raised at the center, concentrically sulcate, mycelium white, sporulation dense at the center, with conidial masses greenish white (27C2), margin lobate; reverse bright orange red (8A8); exudate present, small brown (7E8/7E6) droplets; soluble pigment absent. On OA, colonies fasciculate, mycelium white, sporulation dense, with conidial masses greenish grey (28B2); exudate and soluble pigment absent. On CREA, acid production strong.

Micromorphology — On MEA, conidiophores mostly terverticillate; stipes 250–500 x 2–2.5 µm, smooth; metulae three to four per branch, divergent, 10–14 x 2–3 µm; phialides three to four per metulae, ampulliform, 7–10 x 2–2.5 µm; conidia globose to subglobose, 2.2–2.5 x 2–3 µm, smooth-walled, brownish yellow.

Differential diagnosis — *Penicillium balearicum* is phylogenetically related to species of the *P. atramentosum*-clade, which included six species i.e *P. atramentosum*, *P. balearicum*, *P. fimosum*, *P. ibericum* (the latter three described here), *P. magnielliptisporum* and *P. mexicanum*. *Penicillium atramentosum, P. balearicum, P. magnielliptisporum* and *P. mexicanum* have stipe and conidia smooth, but the latter two

species have larger conidia (5 x 4 µm in *P. magnielliptisporum*, 4 x 3.5 µm in *P. mexicanum*), and *P. atramentosum* can be differentiated by the production of red soluble pigment on CYA and by the absence of growth at 30 ºC (Pitt 1979; Frisvad & Samson 2004). *Penicillium balearicum* differs from *P. fimosum* and *P. ibericum* mainly by having longer stipes (up to 500 µm long), by the production of abundant brown exudate droplets on YES, and by the strong acid production on CREA.

Penicillium beceitense Guevara-Suarez, Gené & Guarro, **sp. nov.** MycoBank MB 822063. Figure 28.

Etymology — Name referred to Beceite, the town where the fungus was found.

In — Section *Ramosa*

Specimens examined — Spain, Aragon, Beceite, from herbivore dung, February 2016, J. Guarro, M. Guevara-Suarez, E. Rosas, (**holotype** CBS H-23183; cultures ex-type FMR 15038 = CBS 142989; ITS barcode LT899780, alternative markers: *Ben*A LT898229, *CaM* LT899764, *RPB*2 LT899798).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 25–27, 30 °C no growth, 37 °C no growth; on MEA: 25 ºC 23–25; on YES: 25 ºC 26–28; on OA: 25 ºC 26–30; on DG18: 25 ºC 19–20, on CREA: 25 ºC 3–5.

Colony characters at 25º C in 7 d — On CYA, colonies sunken at the center, slightly radially sulcate, velvety, mycelium white, sporulation dense, with conidial masses dull green (25E4), margin entire; reverse greyish green (28C5); exudate and soluble pigment absent. On MEA, colonies flat, velvety, mycelium white, sporulation dense, with conidial masses conidial masses dull green (25E4), margin entire; reverse orange (5A6) to greenish grey (30C2); exudate and soluble pigment absent. On DG18, colonies flat, velvety, mycelium white, sporulation dense, conidial masses dull green (26E4), margin entire; reverse greyish green (30B5). On YES, colonies radially sulcate, velvety, mycelium white, sporulation dense, with conidial masses dark green (27F14), margin lobate; reverse greyish yellow (4B5); exudate and soluble pigment absent. On

OA, colonies flat, velvety, sporulation dense, conidial masses dark dull green (28D4), margin entire; reverse greenish grey (30C2); exudate and soluble pigment absent. On CREA, acid production absent.

Micromorphology — On MEA, conidiophores terverticillate, a minor proportion monoverticillate; stipes 190–250 x 3.5–4 µm, smooth; branches 30–40 µm; metulae three to five per branch, divergent, cylindrical, 10–12 (15) x 2.5–3 µm; phialides four to six per metulae, ampulliform, 8–10 x 2.5–3 µm; conidia mostly globose, 2.5–3 x 2.5–3 µm, smooth-walled, dull-green.

Differential diagnosis — *Penicillium beceitense* is closely related to *P. lanosum* and *P. kojigenum,* two species that are indistinguishable according to our phylogeny of the section *Ramosa* (Figure 11). To date, there is no updated description for either *P. lanosum* or *P. kojigenum.* The protologue of *P. kojigenum* described conidia rough-walled, measuring 2.2–2.6 µm (Smith 1961). A relevant feature that distinguishes *P. beceitense* from other species of the section, such as *P. chroogomphum, P. jamesonlandense, P. lanosum, P. ribium,* and *P. soppii*, is the absence of growth at 30 ºC.

Penicillium caprifimosum Guevara-Suarez, D. García & Cano, **sp. nov.** MycoBank MB 822064. Figure 29.

Etymology — From the Latin *capra*= 'goat', and *fimosum* = 'dung-dwelling', describing the subtrate from where the species was isolated.

In — Section *Turbata*

Specimens examined — Spain, Catalonia, Els Ports Natural Park, from goat dung, February 2016, J. Guarro, M. Guevara-Suarez, E. Rosas (**holotype** CBS H-23184; culture ex-type FMR 15041 = CBS 142990; ITS barcode: LT899781, alternative markers: *Ben*A LT898238, *CaM* LT899765, *RPB*2 LT899799)

Colony diameter in 7 d (mm) — On CYA: 25 ºC 30–35, 30 °C 35–38, 37 °C 18–20; on MEA: 25 ºC 24–26, 30 °C 19–21, 37 °C 8–10; on YES: 25 ºC 25–24, 30 ºC 30–

31; on OA: 25 ºC 23–25, 30 ºC 28–30, 37 °C 9–11; on DG18: 25 ºC 16–21, on CREA: 25 ºC 3–6.

Colony characters at 25º C in 7 d — On CYA, colonies flat, velvety, mycelium white, sporulation dense, with conidial masses dull green (28E5), margin entire; reverse purplish grey (14B2); exudate absent; soluble pigment pinkish (12A2). On MEA, colonies flat, velvety, sporulation dense, conidial masses dark dull green (28E3), margin lobate; reverse pale yellow (4A3) to greenish grey (26B3); exudate and soluble pigment absent. On DG18, colonies velvety, flat, mycelium white, sporulation dense, conidial masses dull green (27D4); reverse greyish green (27B5). On YES, colonies velvety, raised at the center, cerebriform, mycelium greenish white (27A2), sporulation sparse, margin lobate; reverse light brown (7D5); exudate and soluble pigment absent. On OA, colonies granulose and flat, radially sulcate towards the periphery, mycelium white, sporulation dense, conidial masses dull green (28E2), margin entire; reverse colorless; exudate and soluble pigment absent. On CREA, acid not produced.

Micromorphology — On MEA, conidiophores mostly biverticillate sometimes with subterminal branches; stipes smooth-walled, 100–250 x 3–3.5 µm; metulae three to four per branch, appressed, 10–12 x 2–3 µm; phialides three to four per metulae, ampulliform, 8–10 x 2–2.5 µm; conidia mostly globose, 2–2.5 x 2.5–3 µm, smooth-walled, brownish yellow.

Differential diagnosis — *Penicillium caprifimosum* can be differentiated from the closest related species, *P. bovifimosum*, by its better growth at 25 ºC and 37º C on CYA. The maximum colony diameter reported for *P. bovifimosum* is 16–21 mm on MEA and 25–29 mm on CYA in 7 days at 25 ºC and 6 mm on CYA at 37 ºC. Furthermore, *P. bovifimosum* is characterized by producing cleistothecia (Tuthill & Frisvad 2002), which are absent in *P. caprifimosum*.

Penicillium fimosum Guevara-Suarez, Guarro & D. García, **sp. nov.** MycoBank MB 822069. Figure 30.

Etymology — Name referred to the substrate from where the species was isolated.

In — Section *Paradoxa*

Specimens examined — Spain, Catalonia, Pratdip, from hervibore dung, February 2016, D. García (**holotype** CBS H-23185; culture ex-type FMR 15104 = CBS 142991; ITS barcode: XXXXXX, alternative markers: *Ben*A LT898273, *CaM* XXXXXX, *RPB*2 XXXXX).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 31–34, 30 °C 3–4, 37 °C no growth; on MEA: 25 ºC 25–30, 30 °C no growth; on YES: 25 ºC 50–55, 30 ºC 5–7; on OA: 25 ºC 25–27, 30 ºC no growth; on DG18: 25 ºC 16–20, on CREA: 25 ºC 20–23.

Colony characters at 25º C in 7 d — On CYA, colonies velvety, slightly radially sulcate at the center, mycelium white, sporulation dense, conidial masses dark dull green (26E4), margin lobate; reverse greyish orange (6B4) at the center, greyish green (28C3) towards the periphery; exudate and soluble pigment absent. On MEA, colonies irregular, flat, velvety, sporulation dense, conidial masses dark dull green (28E3), margin crenate; reverse light orange (5A4) to dull green (28D4) at the center, colorless towards the periphery; exudate and soluble pigment absent. On DG18, colonies velvety, flat, mycelium white, sporulation dense, conidial masses dull green (27D4); reverse greenish white (27B2). On YES, colonies irregularly sulcate, slightly raised at the center, mycelium white, sporulation dense, with conidial masses dull green (26E5), margin almost entire and fimbriate; reverse bright orange red (8A8) at the center; exudate and soluble pigment absent. On OA, colonies slightly granulose, mycelium white, sporulation dense, conidial masses greyish green (28E7); exudate and soluble pigment absent. On CREA, moderate acid production.

Micromorphology — On MEA, conidiophores mostly biverticillate, some irregularly branched; stipes 70–130 x 2.5–3(4) µm, smooth; metulae three to four per branch, rather divergent, 8–14 x 2.5–3(4) µm; phialides three to four per metulae,

ampulliform, (6)8–10 x 2.5–3 µm; conidia globose to subglobose, 2.5–3 x 2.5–3(4) µm, smooth, brownish yellow.

Differential diagnosis — *Penicillium fimosum* is closely related to *P. atramentosum* and to the other novel species *P. ibericum* described here within the *P. atramentosum*-clade. The two former species differ from *P. ibericum* by the absence or by having a very restrictive growth at 30ºC. *Penicilium atramentosum* can be distinguished by the production of exudate, soluble pigment and colony reverse on CYA of reddish brown color*,* features absent in *P. fimosum*. Moreover, this latter has shorter stipes (70–130 µm) than *P. atramentosum* (300–500 µm) (Pitt 1979).

Penicillium ibericum Guevara-Suarez, Cano & D. García, **sp. nov.** MycoBank MB 822070. Figure 31.

Etymology — Name referred to the occurrence of the species in the Iberian Peninsula.

In — Section *Paradoxa*

Specimens examined — Spain, Catalonia, Els Ports Natural Park, from herbivore dung, February 2016, J. Guarro, M. Guevara-Suarez, E. Rosas (**holotype** CBS H-23186; culture ex-type FMR 15040 = CBS 142992; ITS barcode: LT899782, alternative markers: *Ben*A LT898285, *CaM* LT899766, *RPB*2 LT899800); Galicia, Las Dunas de Corrubedo Natural Park, from soil, February 2016, D. García (FMR 15107).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 31–35, 30 °C 18–21, 37 °C no growth; on MEA: 25 ºC 18–20, 30 °C 8–9; on YES: 25 ºC 33–35, 30 ºC 18–20; on OA: 25 ºC 21–23, 30 ºC no growth; on DG18: 25 ºC 5–6; on CREA: 25 ºC 21–22.

Colony characters at 25º C in 7 d — On CYA, colonies flat, velvety, sulcate, mycelium white, sporulation dense, conidial masses dark dull green (26E4), margin slightly lobate; reverse reddish brown (9E5) to reddish (9A2); exudate present, with pale red (10A3) droplets; soluble pigment absent. On MEA, colonies, velvety and flat, sporulation dense, conidial masses dark dull green (28E3); reverse light orange (5A4),

margin slightly irregular; exudate and soluble pigment absent. On DG18, colonies sunken in the middle, velvety, mycelium white, sporulation moderate, conidial masses dull green (27D4); reverse greenish white (27B2). On YES, velvety, sulcate, mycelium white, sporulation dense, conidial masses dull green (26E5), margin crenate; reverse orange grey (5B3); exudate and soluble pigment absent. On OA, colonies irregular, granulose, mycelium white, sporulation dense, conidial masses greyish green (28E7); reverse colorless; exudate and soluble pigment absent. On CREA, moderate acid production.

Micromorphology — On MEA, conidiophores ter- to quarterverticillate; stipes 70–150 x 2–3 (4) µm, smooth; metulae two to four per branch, divergent, 8–12 x 2–2.5 µm; phialides three to four per metulae, ampulliform, 8–10 x 2.5–3 µm; conidia globose, 2.5–3 x 2.5–3 µm, smooth, dull green.

Differential diagnosis — *Penicillium ibericum* is closely related to *P. atramentosum* and *P. fimosum.* It can be distinguished by having shorter stipes (70–150 µm) than *P. atramentosum* (300–500 µm) (Pitt 1979), and for growing more restricted on YES (33–35 mm diam) and DG18 (5–6 mm) at 25º C than *P. fimosum* (YES 50–55 mm, DG18 16–20 mm).

Penicillium mediterraneum Guevara-Suarez, Gené & Cano, **sp. nov.** MycoBank MB 822071. Figure 32.

Etymology — Name referred to the geographical area where the species was found.

In — Section *Roquefortorum*

Specimens examined: Spain, Balearic Islands, Mallorca, Puigpuñent, from herbivore dung, February 2016, J. Guarro, M. Guevara-Suarez, J.P.Z. Siqueira (**holotype** CBS H-23143; culture ex-type FMR 15188 = CBS 142754; ITS barcode: LT899784, alternative markers: *Ben*A LT898291, *CaM* LT899768, *RPB*2 LT899802); Catalonia, Els Ports Natural Park, herbivore dung, February 2016, J. Guarro, M.

Guevara-Suarez, E. Rosas, (FMR 15031 = CBS 142755); Catalonia, Els Ports Natural Park, herbivore dung, February 2016, J. Guarro, M. Guevara-Suarez, E. Rosas (FMR 15032).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 45–60, 30 °C 24–26, 37 °C no growth; on MEA: 25 ºC 45–60, 30 °C 23–26; on YES: 25 ºC >60, 30 ºC 35–40; on OA: 25 ºC 31–38, 30 ºC 8–15; on DG18: 25 ºC 43–45; on CREA: 25 ºC 22–25.

Colony characters at 25º C in 7 d — On CYA, colonies flat, velvety, mycelium white, sporulation dense, conidial masses dull green (25E4), margin entire; reverse greyish green (28C5); exudate and soluble pigment absent. On MEA, colonies flat, velvety, mycelium white, margin crenate, sporulation dense, with conidial masses conidial masses dull green (25E4), margin irregular, slightly lobulate, reverse greyish green (28E5); exudate and soluble pigment absent. On DG18, colonies flat, velvety, mycelium white, sporulation dense, conidial masses dull green (26E4), margin entire; reverse greyish green (30C6). On YES, colonies velvety, raised at the center, concentrically sulcate, mycelium white, sporulation dense, conidial masses dull green (26E4), margin entire and fimbriate; reverse greyish green (28C6); exudate and soluble pigment absent. On OA, colony cottony, mycelium white, sporulation dense, conidial masses dark dull green (28D4), margin entire; reverse colorless; exudate and soluble pigment absent. On CREA, moderate acid production.

Micromorphology — On MEA, conidiophores bi- to terverticillate; stipes 50–100 x 2.5–3 µm, rough-walled; metulae two to three per branch, appressed, cylindrical, 10–14 x 2–3 µm; phialides two to four per metulae, ampulliform, 10–13 x 2–3 µm; conidia mostly globose, 2.5–4 (–5) x 2–4 µm, smooth, dull-green.

Differential diagnosis — All species of the section *Roquefortorum,* including *P. mediterraneum,* show a good growth on CYA and MEA and present rough-walled conidiophores. *Penicillium roquefortii* is the closest related species to *P. mediterraneum,* and it can be distinguished by a more restricted growth on CYA at

30ºC (5–15 mm diam.) and by its blackish green colony reverse on YES (Frisvad & Samsom 2004; Houbraken *et al.* 2010).

Penicillium synnematicola Guevara-Suarez, D. García & Guarro, **sp. nov.** MycoBank MB 822072. Figure 33.

Etymology — Name referred to the formation of synnemata.

In — Section *Robsamsonia*

Specimens examined — Spain, Balearic Islands, Mallorca, Camí Vell d'Orient, from goat dung, February 2016, J. Guarro, M. Guevara-Suarez, J.P.Z. Siqueira (**holotype** CBS H-23132, culture ex-type FMR 15192 = CBS 142669; ITS barcode: LT898167, alternative markers: *BenA* LT898172, *CaM* LT898137, RPB2 LT898142); Mallorca, Escorca, from goat dung, February 2016, J. Guarro, M. Guevara-Suarez, J.P.Z. Siqueira (FMR 15210); Mallorca, Camí Vell d'Orient, from goat dung, February 2016, J. Guarro, M. Guevara-Suarez, J.P.Z. Siqueira (FMR 15211); Catalonia, Barcelona, Montseny Natural Park, from herbivore dung, April 2017, J. Gené, M. Guevara-Suarez, I. Iturrieta-González (FMR 16481 = CBS 143045); Catalonia, Poblet, from herbivore dung, March 2017, J. Guarro, Guevara-Suarez, I. Iturrieta-González (FMR 16491 = CBS 143046).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 33–37, 30 °C 9–10, 37 °C no growth; on MEA: 25 ºC 11–13, 30 °C 5–7; on YES: 25 ºC 30–34, 30 ºC 9–17; on OA: 25 ºC 31–38, 30 ºC 8–15; on DG18: 25 ºC 17–19; on CREA: 25 ºC 6–9.

Colony characters at 25º C in 7 d — On CYA, colonies granular, raised at the center, mycelium white, sporulation dense, conidial masses dark dull green (25E4), margin dentate; reverse light orange (5A5); exudate present, consisting of small hyaline droplets; soluble pigment absent. On MEA, colonies fasciculate due to the presence of small feathery synnemata, mycelium white, sporulation dense, with conidial masses dark green (28F5), margin crenate; reverse orange (6B8); exudate absent and soluble pigment deep orange (5A8). On DG18, colony velvety, mycelium

white, sporulation dense, conidial masses dull green (27E4); reverse greyish orange (5B3). On YES, colonies granular, raised at the center, radially sulcate, mycelium white, sporulation dense, conidial masses dull green (27E4), margin dentate; reverse brownish orange (6C8); exudate and soluble pigment absent. On OA, colony strongly fasciculate, mycelium white, sporulation dense, conidial masses dark dull green (27E4), margin crenate; reverse yellowish white (4A); exudate and soluble pigment absent. On CREA, weak acid production.

Micromorphology — On MEA, synnemata present, up to 1 mm long; conidiophores ter- to quaterverticillate; stipes 100–218 x 3–4 µm, coarsely roughened; metulae three to four per branch, rather appressed, cylindrical, smooth to conspicuously roughened, 10–14 x 2–3 µm; phialides two to four per metulae, ampulliform, 10–13 x 2–3 µm; conidia subglobose to broadly ellipsoidal, 2.5–3 x 2–2.5 µm, smooth, hyaline.

Differential diagnosis — Section *Robsamsonia* include some species producing synnemata, such as *P. coprophilum, P. glandicola* and *P. vulpinum* (Houbraken *et al.* 2016). *Penicillium synnematicola* can be easily differentiated from *P. coprophilum* and from *P. vulpinum* since both have smooth-walled conidiophore stipes, as well as branches and metulae. The closest phylogenetic species is *P. glandicola,* which exhibits like *P. synnematicola* coarsely roughened stipes and branches. However, the former can be distinguished by its orange brown to brown and bright orange-red colony reverse on CYA and YES, respectively, it tends to have a more restricted growth on CYA (17–30 mm) and YES (19–36 mm), its phialides are shorter (7.5–10.5 µm long) and the conidia are yellow green (Houbraken *et al.* 2016).

Penicillago Guevara-Suarez, Gené & D. García, **gen. nov.** MycoBank MB 822073.

Etymology — Name referred to the morphological similarity to *Penicillium*

In — Family *Aspergillaceae*

Mycelium partly superficial and partly immersed, composed of septate, branched, canary yellow to chrome yellow hyphae. *Conidiophores* composed of long, septate, hyaline stipes, often terminating in a small vesicle from which born a verticil of metulae, giving symmetrically biverticillate penicilli, occasionally irregulary branched; metulae appressed to divergent, cylindrical to somewhat obpyriform, bearing in a compact verticil of conidiogenous cells. *Conidiogenous cells* phialidic, ampulliform to acerose, with a fine and long neck. *Conidia* dry, catenate, with conspicuous disjunctors, subglobose to ellipsoidal, coarsely equinulate, subhyaline to brown; conidial chains short to moderately long. *Sexual morph* unknown.

Type species — *Penicillago nodositata* (Valla) Guevara-Suarez, Gené & D. García.

Notes — The genus *Penicillago* is introduced to accommodate *Penicillium nodositatum* and a new species collected from herbivore dung. It is noteworthy that the taxonomy of the former species has been controversial. Initially, Valla *et al.* (1989) classified *P. nodositatum* in the Subgenus *Biverticillium* since its morphological affinity with members of the series *Islandica* (Pitt 1979). Later, based on molecular data, it was considered as member of the *Penicillium* section *Sclerotiora* and tentatively placed in synonymy with *P. bilaiae* by Houbraken and Samson (2011), despite the morphological differences in the conidiophore structure (biverticillate in *P. nodositatum* vs monoverticillate in *P. bilaiae*). However, with the revaluation of both species and new sequence data, Visage *et al.* (2013) showed they were distinct species. These authors also noticed that *P. nodositaum* was related to *P. kabunicum*, however sequences of this latter species are not currently available for comparison. The possibility to examine other two isolates morphologically similar but with different DNA sequences from the ex-type strain of *P. nodositatum* allows us to delineate a new genus of penicillium-like fungi in the family *Aspergillaceae.*

Penicillago flava Guevara-Suarez, D. García & Cano, **sp. nov.** MycoBank MB 822075. Figure 34.

Etymology— From Latin *flavus* = yellow, name referred to the characteristic color of the colonies.

Specimen examined — Spain, Balearic Islands, Mallorca, from wild pig dung, February 2016, J. Guarro, M. Guevara-Suarez, J.P.Z. Siqueira (**holotype** CBS H-23182; culture ex-type FMR 15296 = CBS 142988; ITS barcode: LT899787, alternative markers: *BenA* LT898312, *CaM* LT899771, *RPB*2 LT899805). Extremadura, Badajoz, Granja de Torrehermosa, from herbivore dung, December 2016, J. Cano-Lira (FMR 16442).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 40–44, 30 °C 34–36, 37 °C 7–8; on MEA: 25 ºC 34–36, 30 °C 24–27, 37 °C 0–3; on YES: 25 ºC 50–55, 30 ºC 45–55, 37 ºC 11–10; on OA: 25 ºC 24–26, 30 ºC 22–24, 37 ºC 4–6; on DG18: 25 ºC 2–3; on CREA: 25 ºC 22–30.

Colony characters at 25º C in 7 d — On CYA, colonies cottony, slightly sulcate, mycelium light yellow (3A5) to white, sporulation sparse in 7d, conidial masses yellowish brown (5E8) after 14 d, margin fimbriate; reverse greyish orange (5B4) at the centre, colorless towards the periphery; exudate and soluble pigment absent. On MEA, colonies slightly cottony, flat, mycelium light yellow (3A5) to white, sporulation dense after the 14 d, with conidial masses dark green (28F5), margin slightly fimbriate; reverse light yellow (4A4); exudate and soluble pigment absent. On DG18, colonies restricted, flat, mycelium white, sporulation absent, margin entire; reverse colorless; exudate and soluble pigment absent. On YES, colonies velvety, raised at the center, irregularly sulcate, mycelium yellow (3A7) to white, sporulation sparse, margin fimbriate; reverse greyish orange (5B5); exudate and soluble pigment absent. On OA, colony slightly cottony, mycelium white, sporulation sparse, margin entire; reverse yellowish white (4A2); exudate and soluble pigment absent. On CREA, acid production absent.

Micromorphology — On MEA, conidiophores usually symmetrically biverticillate, occasionally irregularly branched; stipes 200–300 x 2.5–3 µm, often with a small vesicle in the apice, 3.5-5.5 µm wide, smooth, hyaline; metulae divergent, cylindrical to somewhat obpyriform, 9–10 (15) x 3–4(5) µm; phialides four to seven per metulae, ampulliform, tapering abruptly to a long, slender neck, 6–8(9) x 2.5–3.5 µm; conidia subglobose to broadly ellipsoidal, coarsely echinulate, 2.5–4 x 3–4(5) µm, pale brown.

Differential diagnosis — *Penicillago flava* can be easily differentiated from its sister species, *Pgo. nodositata*, by its better growth on CYA (40–44 mm diam) and MEA (34–36 mm) at 25°C in 7d, it is able to growth at 37º C and has pale brown conidia. *Penicillago nodositata* grows at least more restricted on CYA at 25ºC (13–19 mm in 7d), it is unable to grow at 37°C and has brown conidia (Valla *et al.* 1989).

Penicillago nodositata (Valla) Guevara-Suarez, Gené & D. García, **comb. nov.** MycoBank MB 822074.

Basionym: Penicillium nodositatum Valla, Pl. Soil 114: 142–146 1989.

Description and illustration: See Valla *et al.* (1989).

Pseudopenicillium Guevara-Suarez, Cano & Guarro, **gen. nov.** MycoBank MB 822076.

Etymology — *Pseudo*- meaning "false"-, name referred to the morphological similarity but not belonging to *Penicillium*.

In — Family *Aspergillaceae*

Mycelium partly superficial and partly immersed, composed of septate, branched, hyaline to subhyaline hyphae. *Conidiophores* undifferentiated, reduced to conidiogenous cells arising directly from the main hyphae, or differentiated composed of short to moderately long, aseptate or few septate, hyaline to brown stipes, terminating in an irregular penicilli; penicilli varying from monoverticillate to partly biverticillate, with few metulae bearing each a verticil of conidiogenous cells; metulae

terminal or subterminal, generally divergent, cylindrical or apically inflated. *Conidiogenous cells* phialidic, ampulliform, with swollen base and tapering abruptly to a slender neck of varialble length. *Conidia* dry, catenate, with conspicuous disjunctors, globose to subglobose, with spinulose walls, brown, dark in masses; conidial chains short. *Sexual morph* unknown.

Type species — *Pseudopenicillium megasporum* (P. A. Orpurt & D. I. Fennell) Guevara-Suarez, Cano & Guarro.

Notes — The genus *Pseudopenicillium* is proposed to accommodate a new species and two species previously classified in the genus *Penicillium*, *P. giganteum* and *P. megasporum*, being this latter selected as the type since it was the firstly described. These two latter species were recovered from soil samples in UK and India, respectively, and despite their different geographical origin, *P. giganteum* and *P. megasporum* were considered conspecific by Pitt (1979) because their strong morphological similarity. In addition, this author created the series *Megasporus* to include both *P. megasporum* and *P. asperosporium*, since the little morphological affinity with other groups of *Penicillium* (Pitt 1979). However later on, whereas *P. asperosporium* was phylogentically proved to be member of *Penicillium* section *Aspergilloides* (Visage et al. 2014), other multigene studies (Peterson et al. 2010; Houbraken and Samson 2011) demonstrated that *P. megasporum* and *P. giganteum* were distinct species and that they were phylogenetically related to members of the genus *Hamigera*, but without statistical support to be included in this latter genus. Our phylogeny confirms this relationships, showing that *Hamigera* and the mentioned *Penicillium* species comprises two sister clades with enough genetic difference to be considered distinct genus (Figures 15, 16). Morphologically, the novel genus *Pseudopenicillium* differs from *Hamigera* in the lack of sexual morph and by its brown spinulose conidia, and from *Penicillium* and *Penicillago* mainly by its short and often irregularly branched conidiophores producing conidia in short chains.

Pseudopenicillium coprobium Guevara-Suarez, D. García & Gené, **sp. nov.** MycoBank MB 822079. Figure 35.

Etymology— Name referred to the source of isolation of the fungus.

Specimen examined — Spain, Castile and Leon, Palencia, from herbivore dung, March 2016, J. Guarro, M. Guevara-Suarez (**holotype** CBS H-23133; culture ex-type FMR 15299 = CBS 142670; ITS barcode: LT899789, alternative markers: *BenA* LT898315, *RPB*2 LT899807).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 26–30, 30 °C 30–32, 37 °C 6–9; on MEA: 25 ºC 13–17, 30 °C 22–24, 37 °C 3–5; on YES: 25 ºC 24–26, 30 ºC 35–38, 37 ºC 23–25; on OA: 24–26, 30 ºC –34, 36 ºC 14–16; on DG18: 25 ºC 16–17; on CREA: 25 ºC 18–20.

Colony characters at 25º C in 7 d — On CYA, colonies flat, velvety, mycelium white, sporulation dense, central area with conidial masses dark green (29F3), margin entire; reverse pale yellow (4A3); exudate and soluble pigment absent. On MEA, colonies growing moderately, velvety, slightly umbonate, mycelium white, sporulation dense, with conidial masses olive-yellow (3C8) to olive (3F8), margin lobate; exudate and soluble pigment absent; reverse greyish yellow (2B4). On YES, colonies dome-shaped, velvety, mycelium white, sporulation absent, margin entire; reverse pale yellow (4A3); exudate and soluble pigment absent. DG18, colones velvety, slightly sulcate, mycelium white, sporulation dense, with conidial masses dull green (28E3), margin entire; reverse yellowish white (4A3). On OA, colonies cottony, mycelium white, sporulation dense, with conidial masses dark green (29F3), margin fimbriate; exudate and soluble pigment absent. On CREA, strong acid production.

Micromorphology — On MEA, conidiophores short, arising as lateral branches from the hyphae, monoverticillate, sometimes biverticillate; stipes 10–35 x 2.5–3 µm, cylindrical, without vesicle, smooth to fine verruculose, hyaline; metulae divergent, cylindrical, 9.5–10 x 2.5–3 µm; phialides in verticils of two to four on the stipe or per

metulae, ampulliform with swollen base, tapering abruptly to a slender neck, (6)7.5–9 x 2.5–3 µm; conidia globose, 5–5.5 x 6 µm, spinulose, thick-walled, pale brown to brown.

Differential diagnosis — *Pseudopenicillium coprobium* is closely related to *Pse. giganteum* and *Pse. megasporum,* and all them have a very similar conidiogenous apparatus and produce globose spinulose conidia. However, they can be distinguished by the conidial size, being up to 6 µm in *Pse. coprobium* and larger in other two species (up to 12 µm in *Pse. giganteum*, and up to 10 µm in *Pse. megasporum*). In addition, *Pse. megasporum* has a more poor growth on CYA at 25 ºC (15–25 mm) and shows vesiculate stipes (Pitt 1979; Peterson *et al.* 2010).

Pseudopenicillium giganteum (R.Y. Roy & G. N. Singh) Guevara-Suarez, Gené & Cano, **comb. nov.** MycoBank MB 822077.

Basionym: Penicillium giganteum R.Y. Roy & G.N. Singh, Trans Br Mycol Soc 51:805. 1968.

Specimen examined — Spain, unknown geographic region, from soil (FMR 14718).

Descriptions and illustrations: See Roy and Singh (1968) and Peterson et al. (2010).

Notes — The isolate identified in the current study represents the second specimen of this taxa found so far. In general, its morphological features are consistent with those of the species giving by Roy and Singh (1968).

Pseudopenicillium megasporum (P. A. Orpurt & D. I. Fennell) Guevara-Suarez, Cano & Guarro, **comb. nov.** MycoBank MB 822078.

Basionym: Penicillium megasporum P. A. Orpurt & D. I. Fennell. Mycologia 47: 233, 1955.

Descriptions and illustrations: See Orpurt & Fennell (1955), Pitt (1979) and Peterson *et al*. (2010).

Talaromyces catalonicus Guevara-Suarez, Gené & Guarro, **sp. nov.** MycoBank MB 822080. Figure 36.

Etymology — Name referred to the region, Catalonia, from where the fungus was isolated.

In — Section *Trachyspermi*

Specimens examined — Spain, Catalonia, Poblet, from herbivore dung, February 2017, J. Guarro, M. Guevara-Suarez, I. Iturrieta-González (**holotype** CBS H-23212; culture ex-type FMR 16441 = CBS 143039; ITS barcode: LT899793, alternative identification markers: *Ben*A LT898318, *CaM* LT899775, *RPB*2 LT899811).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 35–40, 30 °C 38–40, 37 °C 15–18; on MEA: 25 ºC 17–19, 30 °C 19–20, 37 °C 16–18; on YES: 25 ºC 45–50, 30 ºC 50–55, 37 °C 25–30; on OA: 25 ºC 40–45, 30 ºC 40–42, 37 °C 12–14; on DG18: 25 ºC 20–22, on CREA: 25 ºC 17–19.

Colony characters at 25º in 7 d — On CYA, colonies velvety, radially sulcate, mycelium white, sporulation dense, conidial masses dull green (27E4), margin lobate; reverse greyish orange (5B4); exudate absent, soluble pigment only at 30 and 37°C, light yellow (3A5) to light orange (5A6). On MEA, colonies velvety, flat, sporulation dense, with conidial masses greyish green (27E5), margin entire; reverse yellow (2B8); exudate and soluble pigment absent. On DG18, colonies flat, slightly cotton at the center, velvety in the periphery, mycelium white, sporulation dense, conidial masses dull green (26E5), margin entire; reverse greyish green (28B4). On YES, colonies raised at the center, irregularly sulcate, mycelium white, sporulation dense, with conidial masses dark green (30F3), margin entire; reverse orange yellow (4B8); exudate and soluble pigment absent. On OA, colonies velvety, flat, mycelium white,

sporulation dense, conidial masses dark green (27F3), margin entire; reverse greenish grey (29C2); exudate and soluble pigment absent. On CREA, weak acid production.

Micromorphology — On MEA, conidiophores mono- to biverticillate, sometimes irregularly branched, stipes (40) 100–130 x 2–2.5 µm, smooth-walled, hyaline; metulae divergent, 10–11 x 2–2.5 µm; phialides four to six per metulae, acerose, (7) 8–10 x 2–2.5 µm; conidia globose to subglobose, 2–2.5 x 1.8–2 µm, smooth, brownish yellow. Ascomata not observed.

Differential diagnosis — The most closely related species to *T. catalonicus* are *T. albobiverticillius, T. erythromellis, T. heiheensis* and *T. solicola. Talaromyces catalonicus* is characterised by its ability to grow at 37 °C; in contrast, the above-mentioned species do not grow or grow very restricted at this temperature.

Talaromyces coprophilus Guevara-Suarez, Cano & D. García, **sp. nov.** MycoBank MB 822088. Figure 37.

Etymology — Name referred to the source of isolation of the fungus.

In — Section *Talaromyces*

Specimens examined — Spain, Balearic Islands, Mallorca, Escorca, from herbivore dung, February 2016, J. Guarro, M. Guevara-Suarez, J.P.Z. Siqueira (**holotype** CBS H-23144; culture ex-type FMR 15199 = CBS 142756; ITS barcode: LT899794, alternative identification markers: *Ben*A LT898319, *CaM* LT899776, *RPB*2 LT899812).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 38–40, 30 °C 40–45, 37 °C 30–35; on MEA: 25 ºC 33–35, 30 °C 30–35, 37 °C 21–24; on YES: 25 ºC 45–50, 30 ºC 50–55, 37 °C 38–42; on OA: 25 ºC 35–40, 30 ºC 40–45, 37 °C 25–26; on DG18: 25 ºC no growth, on CREA: 25 ºC 8–10.

Colony characters at 25º C in 7 d — On CYA, colonies slightly raised at the center, velvety, mycelium light yellow (4A5) to white, sporulation absent to sparse, with margin entire and fimbriate; reverse light orange (5A4); soluble pigment and exudate

absent. On MEA, colonies slightly raised at center, velvety, flat, mycelium light yellow (4A5) to white, conidial sporulation absent to sparse, young ascomata visible, margin fimbriate; reverse light orange (5A4); soluble pigment and exudate absent. On YES, colonies raised at center, velvety, mycelium pinkish (7A3) to white, sporulation absent, with margin entire; reverse brownish orange (7C6); exudate absent and soluble pigment brownish red (10C7). On OA, colonies granular, flat, with young ascomata visible, conidial sporulation sparse, margin fimbriate; reverse greenish grey (28C3); exudate and soluble pigment absent. On CREA, strong acid production.

Micromorphology — On MEA, conidiophores mono- to biverticillate, stipes 40–70 x 2–2.5 µm, smooth, hyaline; metulae rather appressed, 10–12 x 2.5–3 µm; phialides three to four per metulae, acerose, 9–12 (14) x 2–2.5 µm; conidia ellipsoidal, , 2–3 x 2.5–3 µm, smooth -walled, brownish yellow. Ascomata after 1–2 wk of incubation on OA and MEA at 25 °C, bright orange to orange-red, globose, 200–310 µm diam, with peridal hyphae branched and verruculose; asci globose to subglobose, 6–7 x 7–9 (10) µm; ascospores ellipsoidal, 3.5–4 × 3–4 µm, spiny, thick-walled, golden yellow.

Differential diagnosis — *Talaromyces coprophilus* is characterised by the production of orange-red ascomata with spiny ellipsoidal ascospores, by the lack of growth on DG18, and by having a rapid growth on the other culture media and temperatures tested. Phylogenetically, it forms an independent and distant branch in an unsupported clade along with the ex-type strains of *T. cnidii, T. flavovirens*, *T. funiculosus, T. macrosporus, T. pseudofuniculosus* (described here) *T. rapidus,* and *T. siamensis* are found (Figure 13). Morphologically, *T. coprophilus* resembles to *T. flavovirens* and *T. macrosporus* in the production of sexual morph*;* however, these two latter species mainly differs by having a slower growth on CYA at 25 ºC (19–20 mm in *T. flavovirens*, 22–28 mm in *T. macrosporus*), and larger ascospores (4–7 × 3–4 µm in *T. flavovirens*, 5.5–6.5 × 4.5–5.5 µm in *T. macrosporus*) (Yilmaz *et al.* 2014).

Talaromyces gamsii Guevara-Suarez, Cano & Guarro, **sp. nov.** MycoBank MB 822089. Figure 38.

Etymology — Named as a tribute to the excellent mycologist Walter Gams.

In — Section *Talaromyces*

Specimens examined — Spain, Andalusia, La Rocina, Doñana National Park, from soil, March 2016, D. García (**holotype** CBS H-23213; culture ex-type FMR 15303 = CBS 143040; ITS barcode: LT899795, alternative markers: *Ben*A LT898320, *CaM* LT899777, *RPB*2 LT899813).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 13–15, 30 °C 11–15, 37 °C no growth; on MEA: 25 ºC 25–29, 30 °C 26–30; on YES: 25 ºC 20–23, 30 ºC 10–15; on OA: 25 ºC 12–15, 30 ºC 10–14; on DG18: 25 ºC no growth, on CREA: 25 ºC 8–10.

Colony characters at 25º C in 7 d — On CYA, colonies slightly raised at the center, flat towards the periphery, velvety, mycelium white, sporulation dense, with conidial masses olive (1E8) to greenish yellow (1A7), margin entire; reverse dark orange (5D8) to yellowish orange (4A7); exudate absent; soluble pigment yellowish orange (4A8). On MEA, colonies velvety, flat, sporulation dense, conidial masses greyish green (30C7), margin slightly fimbriate and with submerged mycelium; reverse vivid red (9A8) to pale red (9C3); exudate and soluble pigment absent. On YES, colonies raised at the center, velvety, with mycelium pinkish (11A2) to white; sporulation sparse, margin entire; reverse brownish red (10D6) to pastel red (10B4); exudate absent; soluble pigment brownish red (10C7). On OA, colonies granular, mycelium white, sporulation dense, with conidial masses deep green (28D8), margin regular and slightly fimbriate; reverse greenish grey (28C3); exudate and soluble pigment absent. On CREA, acid production absent.

Micromorphology — On MEA, conidiophores symmetrically biverticillate; stipes 150–220 x 2.5–3 µm, smooth-walled; metulae three to four, divergent, 8–11 x 2–3.5 µm; phialides acerose, four to six per metulae, 9–11 x 2–3 µm; conidia globose to

subglobose, 2.5–3 x 2.5–3(4) µm, finely rough-walled, brownish yellow. Ascomata not observed.

Differential diagnosis — *Talaromyces gamsii* is related to *T. francoae*, *T. kendrickii, T. qii, T. mangshanicus* and *T. thailandensis*. All these species were characterized by the lack of growing at 37 ºC and by having ampulliform phialides. However, *T. gamsii* can be distinguished easily by the absence of growth on DG18 and by its restricted growth on CYA (13–15 mm). In addition to *T. gamsii* and *T. coprophilus* (also describe here), there are other *Talaromyces* species reported to be unable to grow on DG18, such as *T. subinflatus* and *T. udagawae* but they belong to other sections (Yilmaz *et al*. 2014).

Talaromyces pseudofuniculosus Guevara-Suarez, D. García & Gené, **sp. nov.** MycoBank MB 822090. Figure 39.

Etymology — *–pseudo*, meaning "false"-, name referred to its phylogenetic relationship and morphological resemblance to *T. funiculosus*.

In — Section *Talaromyces*

Specimens examined: Spain, Andalusia, La Rocina, Doñana National Park, from herbivore dung, March 2016, D. García (**holotype** CBS H-23214; culture ex-type FMR 15307 = CBS 143041; ITS barcode: LT899796, alternative markers: *Ben*A LT898323, *CaM* LT899778, *RPB*2 LT899814). Catalonia, Els Ports Natural Park, from herbivore dung, February 2016, J. Guarro, M. Guevara-Suarez & E. Rosas (FMR 15035).

Colony diameter in 7 d (mm) — On CYA: 25 ºC 36–43, 30 °C 45–40, 37 °C 31–35; on MEA: 25 ºC 24–29, 30 °C 43–45, 37 °C 23–26; on YES: 25 ºC 34–35, 30 ºC 40–44, 37 °C 32–34; on OA: 25 ºC 33–38, 30 ºC 42–45, 37 °C 27–30; on DG18: 25 ºC 12–15, on CREA: 25 ºC 2–4.

Colony characters at 25º C in 7 d — On CYA, colonies slightly raised at center, radially sulcate, cottony, mycelium white, sporulation dense, conidial masses greyish

green (29D5), with margin entire; reverse dark orange (5D8); soluble pigments and exudate absent. On MEA, colonies velvety to cottony, flat, sporulation dense, conidial masses greyish green (28D5), margin entire to slightly crenate; reverse deep yellow (5A4) to greyish green (30C5); exudate and soluble pigment absent. On YES, colonies slightly raised at the center, sulcate, cottony, mycelium white, sporulation dense in the center, conidial masses greyish green (27D4), margin entire; reverse olive yellow (3C7); exudate and soluble pigments absent. On DG18, colonies flat and velvety, mycelium white; moderate sporulation, conidial masses dull green (27D4); reverse greenish white (27B2). On OA, colonies slightly granular, sporulation dense, conidial masses greenish grey (28B2), margin entre; reverse greyish green (30C5); exudate and soluble pigment absent. On CREA, acid production strong.

Micromorphology — On MEA, conidiophores mostly biverticillate; stipes 40–70 x 2.5–3 µm, smooth, brownish yellow; metulae three to four, mostly appressed, 8–10 x 2–3 µm; phialides three to four per metulae, acerose, 8–10 (11) x 2–3 µm; conidia mostly ellipsoidal, 2–3 x 1.5–2(2.5) µm, smooth. Ascomata not observed.

Differential diagnosis — *Talaromyces pseudofuniculosus* can be distinguished from its closely related species, *T. funiculosus,* by showing appressed metulae (divergent in *T. funiculosus*) and by the pigmentation of the conidiophores, which is brownish yellow in *T. pseudofuniculosus,* and olivaceous in *T. funiculosus.* Moreover, *T. pseudofuniculosus* has a restricted growth on CREA (2–4 mm diam; 20–30 mm diam in *T. funiculosus*) and does not produce funiculous colonies, a typical feature of *T. funiculosus* mainly on MEA and OA (Yilmaz *et al.* 2014).

CONCLUDING REMARKS

This is the first study focused on the diversity of *Aspergillus* and penicillium-like fungi isolated from dung samples, previous findings were limited only to scarce reports. We identified 38 species of *Aspergillus*, including 10 new taxa (*A. albodeflectus*, *A. aurantiosulcatus*, *A. calidokeveii*, *A. canariensis*, *A. coprophilus*, *A. esporlensis*, *A.*

fimeti-brunneus, *A. longipes*, *A. majoricus*, and *A. verruculosus*), 41 of *Penicillium* with seven novel species (*P. balearicum*, *P. beceitense*, *P. caprifimosum*, *P. fimosum*, *P. ibericum*, *P. mediterraneum*, and *P. synnematicola*), eigth *Talaromyces* species, including four new species (*T. catalonicus*, *T. coprophilus*, *T. gamsii*, and *T. pseudofuniculosus*), and the new genera *Penicillago* and *Pseudopenicillium* with one new species each, totalizing 90 species of Eurotialean fungi isolated from dung samples in Spain.

It is remarkable the isolation of species rarely identified before and, never recovered from dung, i.e. *A. austroafricanus*, *A. fructus*, and *A. viridicatenatus* (section *Nidulantes*), *A. ardalensis* (section *Flavipedes*), and *A. floccosus* (section *Terrei*), in *Aspergillus*; and *P. canariense* (section *Stolkia*), *P. cremeogriseum* (section *Lanata-Divaricata), P. momoii* (section *Exilicaulis*), and *P. roseoviride* (section *Aspergilloides*), in *Penicillium*.

The Balearic Islands (n = 45, 27.27 %) and Catalonia (n = 42, 25.45%) were the regions that resulted with the highest number of isolates. Both were also the regions where more new species were found (i.e., four *Aspergillus*, three *Penicillium* and one *Talaromyces* in Balearic Islands, and two new *Penicillium* and two *Talaromyces* in Catalonia), followed by Castile and Leon with three novel *Aspergillus*. The new genera *Penicillago* and *Pseudopenicillium* were recovered in Balearic Islands and Castile and Leon, associated with dung of deer and wild pig, respectively.

In general, our results showed that *BenA* is a good molecular marker to identify Eurotialean fungi, easy to amplify and sequencing, useful for the delimitation of sections, as well as for identification and detection of new species of *Aspergillus*, *Penicillium* and *Talaromyces*. Our results with *Ben*A agree with other studies where large sets of isolates of these genera have been identified (Guevara-Suarez *et al.* 2016; Visagie *et al.* 2014b; Chen *et al.* 2016b).

Our study highlights that herbivore dung is a substrate with a great fungal diversity and that deserves more attention in future taxonomic studies.

ACKNOWLEDGMENT

This study was supported by the Spanish Ministerio de Economía y Competitividad, grant CGL2013-43789-P and by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil), grant BEX 0623/14-8.

REFERENCES

Arzanlou M, Samadi R, Frisvad JC, *et al.* (2016). Two novel *Aspergillus* species from hypersaline soils of The National Park of Lake Urmia, Iran. *Mycological Progress* **15:** 1081–1092.

Balajee SA, Baddley JW, Peterson SW *et al.* (2009). *Aspergillus alabamensis*, a new clinically relevant species in the section *Terrei*. *Eukaryotic Cell* **8**: 713–722.

Bell A (1975). Fungal succession on dung of the brush-tailed opossum in New Zealand. *New Zealand Journal of Botany* **13**: 437–462.

Bell A (1983). *Dung fungi: an illustrated guide to coprophilous fungi in New Zealand.* Victoria University Press, New Zealand.

Cooke RC, Rayner AD (1984). *Ecology of saprotrophic fungi*. Longman Press, USA.

Chen AJ, Frisvad JC, Sun BD, *et al.* (2016a). *Aspergillus* section *Nidulantes* (formerly *Emericella*): Polyphasic taxonomy, chemistry and biology. *Studies in Mycology* **84:** 1–118.

Chen AJ, Sun BD, Houbraken J, *et al.* (2016b). New *Talaromyces* species from indoor environments in China. *Studies in Mycology* **84:** 119–144.

Crous PW, Gams W, Stalpers JA, *et al.* (2004). MycoBank: an online initiative to launch mycology into the 21st century. *Studies in Mycology* **50:** 19–22.

Crous PW, Wingfield MJ, Burgess TI, *et al.* (2016). Fungal Planet description sheets: 469—557. *Persoonia* **37:** 218–403.

Darriba D, Taboada GL, Doallo R, *et al.* (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9:**772.

Davolos D, Persiani AM, Pietrangeli B, *et al.* (2012). *Aspergillus affinis* sp. nov., a novel ochratoxin A-producing *Aspergillus* species (section *Circumdati*) isolated from decomposing leaves. *International Journal of Systematic and Evolutionary Microbiology* **62:** 1007–1015.

Dijksterhuis J, Samson RA (2007). *Food mycology. A multifaceted approach to fungi and food.* CRC Press, The Netherlands: 101–117.

Edgar RC (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32:** 1792–1797.

Guindon S, Gascuel O (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52:** 696–704.

Guinea J, Sandoval-Denis M, Escribano P, *et al*. (2015). *Aspergillus citrinoterreus*, a new species of section *Terrei* isolated from samples of patients with nonhematological predisposing conditions. *Journal of Clinical Microbiology* **53**: 611–617.

Frisvad JC (1981). Physiological criteria and mycotoxin production as aids in identification of common asymmetric penicillia. *Applied and Environmental Microbiology* **41:** 568–579.

Frisvad JC, Samson RA (2004). Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of the food and air-borne terverticillate Penicillia and their mycotoxins. *Studies in Mycology* **49:** 1–173.

Frisvad JC, Yilmaz N, Thrane U, *et al*. (2013). *Talaromyces atroroseus*, a new species efficiently producing industrially relevant red pigments. *PloS One* **8:** e84102.

Glass NL, Donaldson GC (1995). Development of premier sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* **61:** 1323–1330.

Guevara-Suarez M, Sutton DA, Cano-Lira JF, *et al*. (2016). Identification and antifungal susceptibility of penicillium-like fungi from clinical samples in the United States. *Journal of Clinical Microbiology* **54:** 2155–2161.

Guevara-Suarez M, Sutton DA, Gené J, *et al*. (2017). Four new species of *Talaromyces* from clinical sources. *Mycoses:* 1–12. DOI: 10.1111/myc.12640

Guindon S, Gascuel O (2003). A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. *Systematic Biology* **52:** 696–704.

Hocking AD, Pitt JI (1980). Dichloran-glycerol medium for enumeration of xerophilic fungi from low-moisture foods. *Applied and Environmental Microbiology* **39:** 488–492.

Hong SB, Cho HS, Shin HD (2006). Novel *Neosartorya* species isolated from soil in Korea. *International Journal of Systematic and Evolutionary Microbiology* **56:** 477–486.

Houbraken J, de Vries RP, Samson RA (2014a). Modern taxonomy of biotechnologically important *Aspergillus* and *Penicillium* species. In: Advances in applied microbiology. (Sariaslani S, Gadd GM, eds). Academic Press, USA: 199–249.

Houbraken J, Due M, Varga J, *et al*. (2007). Polyphasic taxonomy of *Aspergillus* section *Usti*. *Studies in Mycology* **59**: 107–128.

Houbraken J, Frisvad JC, Samson RA (2010). Sex in *Penicillium* series Roqueforti. *IMA Fungus* **1:** 171–180.

Houbraken J, Frisvad JC, Samson RA (2011). Taxonomy of *Penicillium* section *Citrina*. *Studies in Mycology* **70:** 53–138.

Houbraken J, Frisvad JC, Seifert KA, *et al*. (2012). New penicillin-producing *Penicillium* species and an overview of section *Chrysogena*. *Persoonia:* **29:** 78–100.

Houbraken J, Samson RA (2011). Phylogeny of *Penicillium* and the segregation of *Trichocomaceae* into three families. *Studies in Mycology* **70:**1–51.

Houbraken J, Visagie CM, Meijer M, *et al*. (2014b). A taxonomic and phylogenetic revision of *Penicillium* section *Aspergilloides*. *Studies in Mycology* **78:** 373–451.

Houbraken J, Wang L, Lee HB, *et al*. (2016). New sections in *Penicillium* containing novel species producing patulin, pyripyropens or other bioactive compounds. *Persoonia* **36**:299–314.

Hubka V, Kolarik M, Kubatova A, *et al*. (2013). Taxonomic revision of *Eurotium* and transfer of species to *Aspergillus*. *Mycologia* **105:** 912–937.

Hubka V, Lyskova P, Frisvad JC, *et al*. (2014). *Aspergillus pragensis* sp. nov. discovered during molecular reidentification of clinical isolates belonging to *Aspergillus* section *Candidi*. *Medical Mycology* **52:** 565–576.

Hubka V, Nováková A, Kolarik M, *et al*. (2015). Revision of *Aspergillus* section *Flavipedes*: seven new species and proposal of section Jani sect. nov. *Mycologia* **107**: 169–208.

Hubka V, Nováková A, Samson RA, *et al*. (2016). *Aspergillus europaeus* sp. nov., a widely distributed soil-borne species related to A. wentii (section *Cremei*). *Plant Systematics and Evolution* **302:** 641–650.

Huelsenbeck JP, Ronquist F (2001). MRBAYES: bayesian inference of phylogenetic trees. *Bioinformatics* **17:** 754–755.

Jurjevic Z, Peterson SW, Horn BW (2012). *Aspergillus* section *Versicolores*: nine new species and multilocus DNA sequence based phylogeny. *IMA Fungus* **3:** 59–79.

Kocsubé S, Perrone G, Magistà D, *et al*. (2016). *Aspergillus* is monophyletic: evidence from multiple gene phylogenies and extrolites profiles. *Studies in Mycology* **85:** 199–213.

Kornerup A, Wanscher JH (1978). *Methuen handbook of colour*, 3rd edition. Eyre Methuen Ltd Press, London.

Kozakiewicz Z (1989). *Aspergillus species on stored products*. C.A.B. International, Ferry Lane, UK.

Krug JC, Benny GL, Keller HW (2004). Coprophilous fungi. In: *Biodiversity of fungi: inventory and monitoring methods* (Mueller G, Bills M, Foster G, eds). Elsevier Academic Press: 467–499.

Kuthubutheen AJ, Webster J (1986). Water availability and the coprophilous fungus succession. *Transactions of the British Mycological Society* **86:** 63–76.

Liu YJ, Whelen S, Hall BD (1999). Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. *Molecular Biology and Evolution* **16:**1799–1808.

Masunga GS, Andresen Ø, Taylor JE, *et al.* (2006). Elephant dung decomposition and coprophilous fungi in two habitats of semi-arid Botswana. *Mycological Research* **110:** 1214–1226.

McNeill J, Barrie FR, Buck WR, *et al.* (2012). International code of nomenclature for algae, fungi, and plants (Melbourne Code). Regnum Veg, 154. Koelz Scientific Books, Koenigstein, Germany.

Mehrotra BS, Agnihotri VP (1962). Two new species of *Aspergillus* from India. *Mycologia* **54**: 400–406.

Mehrotra BS, Prasad R (1969). *Aspergillus dimorphicus* and *Emericella cleisto-minuta* spp. nov. from Indian soils. *Transactions of the British Mycological Society* **52**: 331–336.

Müller FMC, Werner KE, Kasai M, *et al.* (1998). Rapid extraction of genomic DNA from medically important yeasts and filamentous fungi by high-speed cell disruption. *Journal of Clinical Microbiology* **36**: 1625–1629.

Orpurt PA, Fennell DI (1955). A new species of *Penicillium* from soil. *Mycologia* **47:** 233–237.

Peterson SW (2008). Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. *Mycologia* **100**: 205–226.

Peterson SW, Jurjević Ž (2013). *Talaromyces columbinus* sp. nov., and genealogical concordance analysis in *Talaromyces* clade 2a. *PloS One* **8:** e78084.

Peterson SW, Jurjevic Ž, Bills GF, *et al.* (2010). Genus *Hamigera*, six new species and multilocus DNA sequence based phylogeny. *Mycologia* **102:** 847–864.

Peterson SW, Jurjević Ž, Frisvad JC (2015). Expanding the species and chemical diversity of *Penicillium* section *Cinnamopurpurea*. *PloS One*, **10:** e0121987.

Peterson SW, Sigler L (2002). Four new *Penicillium* species having *Thysanophora*-like melanized conidiophores. *Mycological Research* **106:** 1109–1118.

Pitt JI (1979). *The genus Penicillium and its teleomorphic states Eupenicillium and Talaromyces.* Academic Press, UK.

Raper KB, Fennell DI (1965). *The genus Aspergillus*. Robert E. Krieger Publishing Co., Inc. USA.

Richardson MJ (2001). Diversity and occurrence of coprophilous fungi. *Mycological Research*, **105:** 387–402.

Rong C, Ma Y, Wang S, *et al.* (2016). *Penicillium chroogomphum*, a new species in *Penicillium* section *Ramosa* isolated from fruiting bodies of *Chroogomphus rutilus* in China. *Mycoscience* **57:** 79–84.

Ronquist F, Huelsenbeck JP (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574.

Samson RA, Houbraken J, Thrane U, *et al.* (2010). *Food and indoor fungi*. CBS laboratory manual series 2. CBS-KNAW Fungal Biodiversity Centre, The Netherlands.

Samson RA, Peterson SW, Frisvad JC, Varga J (2011b). New species in *Aspergillus* section *Terrei*. *Studies in Mycology* **69**: 39–55.

Samson RA, Pitt JI (2000). *Integration of modern taxonomic methods for Penicillium and Aspergillus classification*. Harwood Academic Publishers Press, The Netherlands.

Samson RA, Varga J, Meijer M, *et al.* (2011a). New taxa in *Aspergillus* section *Usti*. *Studies in Mycology* **69:** 81–97.

Samson RA, Visagie CM, Houbraken J *et al.* (2014). Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Studies in Mycology*, **78:** 141–173.

Sang H, An TJ, Kim CS, *et al.* (2013). Two novel *Talaromyces* species isolated from medicinal crops in Korea. *The Journal of Microbiology* **51:** 704–708.

Sarrocco S (2016). Dung-inhabiting fungi: a potential reservoir of novel secondary metabolites for the control of plant pathogens. *Pest Management Science,* **72:** 643–652.

Siqueira JPZ, Sutton DA, García D *et al.* (2016). Species diversity of *Aspergillus* section *Versicolores* in clinical samples and antifungal susceptibility. *Fungal Biology* **120:** 1458–1467.

Siqueira JPZ, Sutton DA, Gené J *et al.* (2017). Multilocus phylogeny and antifungal susceptibility of *Aspergillus* section *Circumdati* from clinical samples and description of *A. pseudosclerotiorum* sp. nov. *Journal of Clinical Microbiology* **55:** 947–958.

Smith G (1961). Some new and interesting species of micro-fungi. II. *Transactions of the British Mycological Society* **44:** 42–50.

Sonjak S, Frisvad JC, Gunde-Cimerman N (2005). Comparison of secondary metabolite production by *Penicillium crustosum* strains, isolated from Arctic and other various ecological niches. *FEMS Microbiology Ecology* **53:** 51–60.

Tamura K, Stecher G, Peterson D, *et al.* (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30:** 2725–2729.

Taylor JW, Jacobson DJ, Kroken S, *et al.* (2000). Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* **31:** 21–32.

Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4673–4680.

Tuthill DE, Frisvad JC (2002). *Eupenicillium bovifimosum*, a new species from dry cow manure in Wyoming. *Mycologia* **94:** 240–246.

Valla G, Capellano A, Hugueney R, *et al.* (1989). *Penicillium nodositatum* Valla, a new species inducing myconodules on Alnus roots. *Plant and Soil* **114:** 142–146.

Varga J, Due M, Frisvad JC, *et al.* (2007a). Taxonomic revision of *Aspergillus* section *Clavati* based on molecular, morphological and physiological data. *Studies in Mycology* **59**: 89–106.

Varga J, Frisvad JC, Samson RA (2007b). Polyphasic taxonomy of *Aspergillus* section *Candidi* based on molecular, morphological and physiological data. *Studies in Mycology* **59**: 75–88.

Visagie CM, Hirooka Y, Tanney JB, *et al.* (2014b). *Aspergillus*, *Penicillium* and *Talaromyces* isolated from house dust samples collected around the world. *Studies in Mycology*, **78**: 63–139.

Visagie CM, Houbraken J, Frisvad JC, *et al.* (2014a). Identification and nomenclature of the genus *Penicillium*. *Studies in Mycology* **78**: 343–371.

Visagie CM, Houbraken J, Rodriques C, *et al.* (2013). Five new *Penicillium* species in section Sclerotiora: a tribute to the Dutch Royal family. *Persoonia* **31:** 42–62.

Visagie CM, Renaud JB, Burgess KMN, *et al.* (2016a). Fifteen new species of *Penicillium. Persoonia* **36:** 247–280.

Visagie CM, Seifert KA, Houbraken J, *et al.* (2016b). A phylogenetic revision of *Penicillium* sect. *Exilicaulis*, including nine new species from fynbos in South Africa. *IMA Fungus* **7:** 75-117.

Visagie CM, Varga J, Houbraken J, *et al.* (2014c). Ochratoxin production and taxonomy of the yellow aspergilli (*Aspergillus* section *Circumdati*). *Studies in Mycology* **78**: 1–61.

Visagie CM, Yilmaz N, Frisvad JC, *et al.* (2015). Five new *Talaromyces* species with ampulliform-like phialides and globose rough walled conidia resembling *T. verruculosus*. *Mycoscience* **56:** 486–502.

Waksman SA (1922). A method for counting the number of fungi in the soil. *Journal of Bacteriology* **7**: 339–341.

Wang L, Zhuang WY (2005). *Penicillium brevistipitatum*, a new species isolated from Jilin Province, China. *Mycotaxon* **93:** 233–240.

Wang QM, Zhang YH, Wang B, *et al.* (2016). *Talaromyces neofusisporus* and *T. qii*, two new species of section *Talaromyces* isolated from plant leaves in Tibet, China. *Scientific Reports* **6:** 18622.

Wang XC, Chen K, Qin WT, *et al.* (2017). *Talaromyces heiheensis* and *T. mangshanicus*, two new species from China. *Mycological Progress* **16:** 73–81.

Webster J (1970). Presidential address: coprophilous fungi. *Transactions of the British Mycological Society* **54:** 161–180.

White TJ, Bruns T, Lee S, *et al.* (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a guide to methods and applications* (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds). Academic Press, USA: 315–322.

Yaguchi T, Someya A, Udagawa SI (1996). A reappraisal of intrageneric classification of *Talaromyces* based on the ubiquinone systems. *Mycoscience* **37:** 55–60.

Yilmaz N, López-Quintero CA, Vasco-Palacios AM, *et al.* (2016b). Four novel *Talaromyces* species isolated from leaf litter from Colombian Amazon rain forests. *Mycological Progress* **15:** 1041–1056.

Yilmaz N, Visagie CM, Frisvad JC, *et al.* (2016a). Taxonomic re-evaluation of species in *Talaromyces* section *Islandici*, using a polyphasic approach. *Persoonia* **36:** 637–656.

Yilmaz N, Visagie CM, Houbraken J, *et al.* (2014). Polyphasic taxonomy of the genus *Talaromyces*. *Studies in Mycology* **78:** 175–341.

Figure 1. ML tree of *Aspergillus* inferred from *Ben*A including the sections recovered from dung in this work. Branch lengths are proportional to phylogenetic distance. Some of the larger branches were condensed, with the proportions showed above the paralell diagonal lines. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. Isolates corresponding to potencially new species are shown in bold. Between paranthesis, GenBank accession numbers of *Ben*A sequences. The tree is rooted to *Penicillium paradoxum* CBS 527.65. ^{T} = type strain.

Figure 2. ML tree of selected *Aspergillus* section *Usti* species inferred from the combined ITS, *Ben*A, *CaM*, and *RPB*2 loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *A. elongatus* NRRL 5176. The name in bold is the new species described in this study. ^{T} = type strain.

Figure 3. ML tree of *Aspergillus* section *Flavipedes* inferred from the combined ITS, *Ben*A, *CaM*, and *RPB*2 loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *A. janus* NRRL 1787. The name in bold is the new species described in this study. ^{T} = type strain.

Figure 4. ML tree of selected *Aspergillus* section *Terrei* species inferred from the combined ITS, *BenA*, *CaM*, and *RPB*2 loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *A. microcysticus* NRRL 4749 and *A. ambiguus* NRRL 4737. Names in bold are the new species described in this study. ^{T} = type strain.

Figure 5. ML tree of *Aspergillus* section *Candidi* inferred from the combined ITS, *Ben*A, *CaM*, and *RPB*2 loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *A. niger* NRRL 326. Names in bold are the new species described in this study. ^{T} = type strain.

Figure 6. ML tree of selected *Aspergillus* section *Cremei* species, inferred from the combined ITS, *Ben*A, *CaM*, and *RPB2* loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *A. inflatus* CBS 682.79. The name in bold is the new species described in this study. ^{T} = type strain.

Figure 7. ML tree of *Penicillium* inferred from *BenA* including the sections recovered from dung in this work. Branch lengths are proportional to phylogenetic distance. Some of the larger branches were condensed, with the proportions showed above the paralell diagonal lines. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. Isolates corresponding to new species are shown in bold. Between paranthesis, GenBank accession numbers of *Ben*A sequences. The tree is rooted to *Talaromyces flavus* CBS 310.38 and *Talaromyces duclauxii* CBS 322.48. ^{T} = type strain.

Figure 8. ML tree of *Penicillium* section *Roquefortorum* inferred from the combined ITS, *Ben*A, *CaM*, and *RPB*2 loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *P. samsonianum* AS 3.15403 and *P. osmophilum* CBS 462.72. Names in bold are the new species described in this study. ^{T} = type strain.

Figure 9. ML tree of *Penicillium* section *Robsamsonia* inferred from the combined ITS, *Ben*A, *CaM*, and *RPB*2 loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *P. brevicompactum* CBS 257.29. Names in bold are the new species described in this study. ^{T} = type strain.

Figure 10. ML tree of *Penicillium* sections *Turbata* and *Paradoxa* inferred from the combined ITS, *Ben*A and *CaM* loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. Names in bold are the new species described in this study. ^{T} = type strain

Figure 11. ML tree of *Penicillium* section *Ramosa* inferred from the combined ITS, *BenA* and *CaM* loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *P. brevicompactum* CBS 257.29. The name in bold is the new species described in this study. ^{T} = type strain.

Figure 12. ML tree of *Talaromyces* inferred from *BenA* including the sections recovered from dung in this work. Branch lengths are proportional to phylogenetic distance. Some of the larger branches were condensed, with the proportions showed above the paralell diagonal lines. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. Isolates corresponding to new species are shown in bold. Between paranthesis, GenBank accession numbers of *Ben*A sequences. The tree is rooted to *T. dendriticus* CBS 660.80 and *T. purpureus* CBS 475.71 (Section *Purpurei*). ^{T} = type strain.

Figure 13. ML tree of *Talaromyces* section *Talaromyces* inferred from the combined ITS, *BenA*, *CaM*, and *RPB*2 loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *T. dendriticus* CBS 660.80 and *T. purpureus* CBS 475.71 (Section *Purpurei*). Names in bold are the new species described in this study. ^{T} = type strain.

Figure 14. ML tree of selected of *Talaromyces* section *Trachyspermi* inferred from the combined ITS, *BenA* and *CaM* loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *T. purpureogenus* CBS 286.36 (Section *Talaromyces*). The name in red bold the new species described in this study. ^{T} = type strain.

Figure 15. ML tree of selected members of *Aspergillaceae* family inferred from *BenA*. Branch lengths are proportional to phylogenetic distance. Some of the larger branches were condensed, with the proportions showed above the paralell diagonal lines. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. Isolates corresponding to new species are shown in bold. Between paranthesis, GenBank accession numbers of *Ben*A sequences. The tree is rooted to *T. flavus* CBS 310.38 and *T. purpureogenus* CBS 286.36. ^{T} = type strain.

Figure 16. ML tree of members of *Aspergillaceae* family inferred from the combined ITS, *BenA*, and *RPB*2 loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *Talromyces flavus* CBS 310.38 and *Talaromyces purpureogenus* CBS 286.36. Names in bold are the new species described in this study. ^{T} = type strain.

Figure 17. Morphological characters of *Aspergillus albodeflectus* (FMR 15175^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B–F. Conidiophores. G. Conidia. Scale bars = 10 µm.

Figure 18. Morphological characters of *Aspergillus aurantiosulcatus* (FMR 15182^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B. Conidial heads in detail. C–F. Conidiophores. G, H. Conidia. Scale bars = 10 µm.

Figure 19. Morphological characters of *Aspergillus calidokeveii* (FMR 15225^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B–E. Conidiophores. F. Conidia. G. Hülle cells. Scale bars: B = 50 µm, C–G = 10 µm.

Figure 20. Morphological characters of *Aspergillus canariensis* (FMR 15736^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B–F. Conidiophores. G. Conidia. Scale bars = 10 µm.

Figure 21. Morphological characters of *Aspergillus coprophilus* (FMR 15224^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B. Sclerotia on CYA at 25 ºC after 7 d. C–E. Conidiophores. F. Conidia. G. Diminutive vesicle. Scale bars = 10 µm.

Figure 22. Morphological characters of *Aspergillus esporlensis* (FMR 14605^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B–E. Conidiophores. F, G. Conidia. Scale bars = 10 µm.

Figure 23. Morphological characters of *Aspergillus fimeti-brunneus* (FMR 15228^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B–D. Conidiophores. E, F. Accessory conidia. G. Conidia. Scale bars = 10 µm.

Figure 24. Morphological characters of *Aspergillus longipes* (FMR 15444^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B, C, E, F. Conidiophores. D. Detailed metula and phialides. G. Conidia. Scale bars = 10 µm.

Figure 25. Morphological characters of *Aspergillus majoricus* (FMR 15181^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B–E. Conidiophores. F. Conidia. Scale bars = 10 µm.

Figure 26. Morphological characters of *Aspergillus verruculosus* (FMR 15877^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B. Sclerotia on CYA at 25 ºC after 7 d. C, E–G. Conidiophores. D. Conidia. Scale bars = 10 µm.

Figure 27. Morphological characters of *Penicillium balearicum* (FMR 15191^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B. Colony texture on YES at 25 ºC after 1-week incubation. C–E. Conidiophores. F. Conidia. Scale bars = 10 µm.

Figure 28. Morphological characters of *Penicillium beceitense* (FMR 15038^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B–F. Conidiophores. G. Conidia. Scale bars = 10 µm.

Figure 29. Morphological characters of *Penicillium caprifimosum* (FMR 15041^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B–F. Conidiophores. G. Conidia. Scale bars = 10 µm.

Figure 30. Morphological characters of *Penicillium fimosum* (FMR 15104^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B. Conidiophores. C. Conidia. D-F. Conidiophores. Scale bars = 10 µm.

Figure 31. Morphological characters of *Penicillium ibericum* (FMR 15040^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B. Colony texture on CYA at 25 ºC after 1-week incubation. C. Conidia. D–G. Conidiophores. H. Scale bars = 10 µm.

Figure 32. Morphological characters of *Penicillium mediterraneum* (FMR 15188^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B–G. Conidiophores. H. Conidia. Scale bars = 10 µm.

Figure 33. Morphological characters of *Penicillium synnematicola* (FMR 15192^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B. Colony texture on CYA at 25 ºC after 1-week incubation. C. Colony texture on OA at 25 ºC after 1-week incubation. D–G. Conidiophores. H. Conidia. Scale bars = 10 µm.

Figure 34. Morphological characters of *Penicillago flava* (FMR 15296^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B–F. Conidiophores. G. Conidia. Scale bars = 10 µm.

Figure 35. Morphological characters of *Pseudopenicillium coprobium* (FMR 15299^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA reverse; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B. Colony texture on OA at 25 ºC after 1-week incubation. C–H. Conidiophores. I. Conidia. Scale bars = 10 µm.

Figure 36. Morphological characters of *Talaromyces catalonicus* (FMR 16441^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA reverse; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B–D. Conidiophores. E. Conidia. Scales bars C–D= 100 µm, E–I = 10 µm.

Figure 37. Morphological characters of *Talaromyces coprohilus* (FMR 15199^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA reverse; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B. Detail of the colony texture with ascomata on MEA after 2 wk incubation. C. Ascoma. D, E Part of a ascoma and peridial hyphae. F. Asci. G. Ascospores. H, I. Conidiophores. J. Conidia. Scales bars C–D= 100 µm, E–I = 10 µm.

Figure 38. Morphological characters of *Talaromyces gamsii* (FMR 15303^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA reverse; (bottom row)

CYA reverse, MEA reverse, YES, and CREA. B–E. Conidiophores. F. Conidia. Scale bars = 10 μm.

Figure 39. Morphological characters of *Talaromyces pseudofuniculosus* (FMR 15307^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA reverse; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B–E. Conidiophores. F. Conidia. Scale bars = 10 μm.

Table 1. Isolates of *Aspergillus*, *Penicillium*, *Talaromyces*, and related genera included in the study and their GenBank/EMBL accession numbers.

Genus/Species*	*Section*	Collection number	Substrate and Origin	GenBank/EMBL accession number			
				ITS	*Ben*A	*CaM*	*RPB*2
A. affinis	*Circumdati*	FMR 15602	Dung, Galicia		LT798961		
A. alabamensis	*Terrei*	FMR 15731	Dung, Canary Islands		LT798985		
A. alabamensis	*Terrei*	FMR 15412	Dung, Galicia		LT798984		
A. albodeflectus	*Flavipedes*	FMR 15175 = CBS 142665	Dung, Balearic Islands	LT798909	LT798936	LT798937	LT798938
A. ardalensis	*Flavipedes*	FMR 15057	Dung, Catalonia		LT798966		
A. ardalensis	*Flavipedes*	FMR 15058	Dung, Catalonia		LT798967		
A. aurantiosulcatus	*Terrei*	FMR 15182 = CBS 142981	Dung, Balearic Islands	LT798912	LT798945	LT798946	LT798947
A. aureolatus	*Nidulantes*	FMR 15442	Dung, Galicia		LT798994		
A. austroafricanus	*Nidulantes*	FMR 15174 = CBS 142994	Dung, Balearic Islands		LT798995		
A. calidokeveii	*Usti*	FMR 15225 = CBS 142666	Dung, Castile and Leon	LT798914	LT798951	LT798952	LT798953
A. calidoustus	*Usti*	FMR 15609	Dung, Castile and Leon		LT798990		
A. canariensis	*Candidi*	FMR 15733 = CBS 142983	Dung, Canary Islands	LT798905	LT798924	LT798925	LT798926
A. canariensis	*Candidi*	FMR 15736 = CBS 142982	Dung, Canary Islands	LT798906	LT798927	LT798928	LT798929
A. candidus	*Candidi*	FMR 15218	Dung, Balearic Islands		LT798960		
A. candidus	*Candidi*	FMR 15172	Dung, Catalonia		LT798959		
A. chevalieri	*Aspergillus*	FMR 15878	Dung, Extremadura		LT798954		
A. citrinoterreus	*Terrei*	FMR 15876	Dung, Canary Islands		LT798989		
A. clavatus	*Clavati*	FMR 15610	Dung, Castile and Leon		LT798963		
A. clavatus	*Clavati*	FMR 15611	Dung, Castile and Leon		LT798964		
A. coprophilus	*Candidi*	FMR 15224 = CBS 142984	Dung, Castile and Leon	LT798902	LT798915	LT798916	LT798917
A. coprophilus	*Candidi*	FMR 15226 = CBS 142985	Dung, Castile and Leon	LT798903	LT798918	LT798919	LT798920
A. esporlensis	*Cremei*	FMR 14605 = CBS 142750	Soil, Balearic Islands	LT798908	LT798933	LT798934	LT798935
A. europaeus	*Cremei*	FMR 15216	Dung, Balearic Islands		LT798965		
A. fimeti-brunneus	*Terrei*	FMR 15228 = CBS 142751	Dung, Andalusia	LT798913	LT798948	LT798949	LT798950
A. floccosus	*Terrei*	FMR 15061	Dung, Catalonia		LT798986		
A. fructus	*Nidulantes*	FMR 15728	Dung, Canary Islands		LT798996		
A. hortai	*Terrei*	FMR 15227	Dung, Andalusia		LT798987		
A. iizukae	*Flavipedes*	FMR 15606	Dung, Castile and Leon		LT798969		
A. iizukae	*Flavipedes*	FMR 15051	Dung, Catalonia		LT798968		
A. insuetus	*Usti*	FMR 15322	Dung, Andalusia		LT798991		
A. longipes	*Candidi*	FMR 15444 = CBS 142752	Dung, Galicia	LT798904	LT798921	LT798922	LT798923

A. majoricus	*Terrei*	FMR 15181 = CBS 142986	Dung, Balearic Islands	LT798910	LT798939	LT798940	LT798941
A. majoricus	*Terrei*	FMR 15217 = CBS 142987	Dung, Balearic Islands	LT798911	LT798942	LT798943	LT798944
A. micronesiensis	*Flavipedes*	FMR 15214	Dung, Balearic Islands		LT798970		
A. micronesiensis	*Flavipedes*	FMR 15737	Dung, Canary Islands		LT798971		
A. montevidensis	*Aspergillus*	FMR 15608	Dung, Castile and Leon		LT798956		
A. montevidensis	*Aspergillus*	FMR 15738	Dung, Extremadura		LT798955		
A. nidulans	*Nidulantes*	FMR 15229	Dung, Andalusia		LT798981		
A. nidulans	*Nidulantes*	FMR 15219	Dung, Balearic Islands		LT798980		
A. nidulans	*Nidulantes*	FMR 15377	Dung, Balearic Islands		LT798982		
A. pseudodeflectus	*Usti*	FMR 15376	Dung, Andalusia		LT798992		
A. pseudodeflectus	*Usti*	FMR 15727	Dung, Canary Islands		LT798993		
A. pseudoglaucus	*Aspergillus*	FMR 15607	Dung, Castile and Leon		LT798957		
A. pseudoglaucus	*Aspergillus*	FMR 15612	Dung, Castile and Leon		LT798958		
A. rugulosus	*Nidulantes*	FMR 15173	Dung, Catalonia		LT798983		
A. spelaeus	*Flavipedes*	FMR 15176	Dung, Balearic Islands		LT798972		
A. spelaeus	*Flavipedes*	FMR 15178	Dung, Balearic Islands		LT798973		
A. spelaeus	*Flavipedes*	FMR 15180	Dung, Balearic Islands		LT798974		
A. spelaeus	*Flavipedes*	FMR 15215	Dung, Balearic Islands		LT798975		
A. spelaeus	*Flavipedes*	FMR 15223	Dung, Balearic Islands		LT798976		
A. subramanianii	*Circumdati*	FMR 15729	Dung, Canary Islands		LT798962		
A. sydowii	*Nidulantes*	FMR 15880	Dung, Canary Islands		LT798999		
A. sydowii	*Nidulantes*	FMR 15603	Dung, Castile and Leon		LT798997		
A. sydowii	*Nidulantes*	FMR 15618	Dung, Galicia		LT798998		
A. templicola	*Flavipedes*	FMR 15179	Dung, Balearic Islands		LT798979		
A. templicola	*Flavipedes*	FMR 15055	Dung, Catalonia		LT798977		
A. templicola	*Flavipedes*	FMR 15059	Dung, Catalonia		LT798978		
A. terreus	*Terrei*	FMR 15054	Dung, Catalonia		LT798988		
A. verruculosus	*Candidi*	FMR 15877 = CBS 142667	Dung, Canary Islands	LT798907	LT798930	LT798931	LT798932
A. viridicatenatus	*Nidulantes*	FMR 15446	Dung, Galicia		LT799000		
P. arabicum	*Exilicaulis*	FMR 15298	Dung, Castile and Leon		LT898226		
P. arabicum	*Exilicaulis*	FMR 15095	Dung, Catalonia		LT898225		
P. atramentosum	*Paradoxa*	FMR 15309	Dung, Castile and Leon		LT898224		
P. atramentosum	*Paradoxa*	FMR 15046	Dung, Catalonia		LT898221		
P. atramentosum	*Paradoxa*	FMR 15092	Dung, Catalonia		LT898222		
P. atramentosum	*Paradoxa*	FMR 15102	Dung, Catalonia		LT898223		
P. balearicum	*Paradoxa*	FMR 15191 = CBS 143044	Dung, Balearic Islands	LT899762	LT898227	LT899758	LT899760

P. balearicum	*Paradoxa*	FMR 15196	Dung, Balearic Islands	LT899763	LT898228	LT899759	LT899761
P. beceitense	*Ramosa*	FMR 15038 = CBS 142989	Dung, Aragon	LT899780	LT898229	LT899764	LT899798
P. biforme	*Fasciculata*	FMR 15312	Dung, Castile and Leon		LT898230		
P. biforme	*Fasciculata*	FMR 15313	Dung, Castile and Leon		LT898231		
P. brasilianum	*Lanata-Diavricata*	FMR 15483	Dung, Galicia		LT898232		
P. brevicompactum	*Brevicompacta*	FMR 15105	Dung, Catalonia		LT898233		
P. brevistipitatum	*Robsamsonia*	FMR 15103	Dung, Catalonia		LT898234		
P. burguense	*Exilicaulis*	FMR 15493	Dung, Galicia		LT898235		
P. canariense	*Stolkia*	FMR 15838	Dung, Canary Islands		LT898236		
P. canescens	*Canescentia*	FMR 15028	Dung, Catalonia		LT898237		
P. caprifimosum	*Turbata*	FMR 15041 = CBS 142990	Dung, Catalonia	LT899781	LT898238	LT899765	LT899799
P. chrysogenum	*Chrysogena*	FMR 15100	Dung, Catalonia		LT898244		
P. cinereoatrum	*Exilicaulis*	FMR 15033	Dung, Catalonia		LT898284		
P. citrinum	*Citrina*	FMR 15646	Dung, Castile and Leon		LT898242		
P. citrinum	*Citrina*	FMR 15647	Dung, Castile and Leon		LT898243		
P. citrinum	*Citrina*	FMR 15094	Dung, Catalonia		LT898239		
P. citrinum	*Citrina*	FMR 15486	Dung, Galicia		LT898240		
P. citrinum	*Citrina*	FMR 15520	Dung, Galicia		LT898241		
P. concentricum	*Robsamsonia*	FMR 15195	Dung, Balearic Islands		LT898245		
P. concentricum	*Robsamsonia*	FMR 15840	Dung, Castile and Leon		LT898246		
P. coprobium	*Robsamsonia*	FMR 15201	Dung, Balearic Islands		LT898247		
P. coprobium	*Robsamsonia*	FMR 15311	Dung, Castile and Leon		LT898248		
P. coprophilum	*Robsamsonia*	FMR 15187	Dung, Balearic Islands		LT898249		
P. cremeogriseum	*Lanata-Diavricata*	FMR 15487	Dung, Galicia		LT898250		
P. cremeogriseum	*Lanata-Diavricata*	FMR 15488	Dung, Galicia		LT898251		
P. crustosum	*Fasciculata*	FMR 15185	Dung, Balearic Islands		LT898259		
P. crustosum	*Fasciculata*	FMR 15186	Dung, Balearic Islands		LT898260		
P. crustosum	*Fasciculata*	FMR 15189	Dung, Balearic Islands		LT898261		
P. crustosum	*Fasciculata*	FMR 15194	Dung, Balearic Islands		LT898262		
P. crustosum	*Fasciculata*	FMR 15197	Dung, Balearic Islands		LT898263		
P. crustosum	*Fasciculata*	FMR 15200	Dung, Balearic Islands		LT898264		
P. crustosum	*Fasciculata*	FMR 15213	Dung, Balearic Islands		LT898265		
P. crustosum	*Fasciculata*	FMR 15034	Dung, Catalonia		LT898252		
P. crustosum	*Fasciculata*	FMR 15036	Dung, Catalonia		LT898253		
P. crustosum	*Fasciculata*	FMR 15037	Dung, Catalonia		LT898254		

P. crustosum	*Fasciculata*	FMR 15042	Dung, Catalonia		LT898255		
P. crustosum	*Fasciculata*	FMR 15043	Dung, Catalonia		LT898256		
P. crustosum	*Fasciculata*	FMR 15045	Dung, Catalonia		LT898257		
P. crustosum	*Fasciculata*	FMR 15098	Dung, Catalonia		LT898258		
P. crustosum	*Fasciculata*	FMR 15494	Dung, Galicia		LT898266		
P. cvjetkovicii	*Cinnamopurpurea*	FMR 15310	Dung, Castile and Leon		LT898267		
P. expansum	*Penicillium*	FMR 15097	Dung, Catalonia		LT898268		
P. expansum	*Penicillium*	FMR 15484	Dung, Galicia		LT898269		
P. fimosum	*Paradoxa*	FMR 15104 = CBS 142991	Dung, Catalonia	XXXXXXX	LT898273	XXXXXX	XXXXXX
P. flavigenum	*Chrysogena*	FMR 15096	Dung, Catalonia		LT898270		
P. frequentans	*Aspergilloides*	FMR 15193	Dung, Balearic Islands		LT898271		
P. frequentans	*Aspergilloides*	FMR 15212	Dung, Balearic Islands		LT898272		
P. glabrum	*Aspergilloides*	FMR 15184	Dung, Balearic Islands		LT898274		
P. glabrum	*Aspergilloides*	FMR 15190	Dung, Balearic Islands		LT898275		
P. glabrum	*Aspergilloides*	FMR 15209	Dung, Balearic Islands		LT898276		
P. griseofulvum	*Robsamsonia*	FMR 15203	Dung, Balearic Islands		LT898280		
P. griseofulvum	*Robsamsonia*	FMR 15204	Dung, Balearic Islands		LT898281		
P. griseofulvum	*Robsamsonia*	FMR 15207	Dung, Balearic Islands		LT898282		
P. griseofulvum	*Robsamsonia*	FMR 15314	Dung, Castile and Leon		LT898283		
P. griseofulvum	*Robsamsonia*	FMR 15029	Dung, Catalonia		LT898277		
P. griseofulvum	*Robsamsonia*	FMR 15030	Dung, Catalonia		LT898278		
P. griseofulvum	*Robsamsonia*	FMR 15093	Dung, Catalonia		LT898279		
P. ibericum	*Paradoxa*	FMR 15040 = CBS142992	Dung, Catalonia	LT899782	LT898285	LT899766	LT899800
P. ibericum	*Paradoxa*	FMR 15107	Soil, Galicia	LT899783	LT898286	LT899767	LT899801
P. lilacinoechinulatum	*Sclerotiora*	FMR 15492	Dung, Galicia		LT898287		
P. magnielliptisporum	*Paradoxa*	FMR 15044	Dung, Catalonia		LT898288		
P. mediterraneum	*Roquefortorum*	FMR 15188 = CBS 142754	Dung, Balearic Islands	LT899784	LT898291	LT899768	LT899802
P. mediterraneum	*Roquefortorum*	FMR 15031 = CBS 142755	Dung, Catalonia	LT899785	LT898289	LT899769	LT899803
P. mediterraneum	*Roquefortorum*	FMR 15032	Dung, Catalonia	LT899786	LT898290	LT899770	LT899804
P. momoii	*Exilicaulis*	FMR 15208	Dung, Balearic Islands		LT898292		
P. murcianum	*Canescentia*	FMR 15304	Dung, Andalusia		LT898293		
P. murcianum	*Canescentia*	FMR 15305	Dung, Andalusia		LT898294		
P. murcianum	*Canescentia*	FMR 15308	Dung, Andalusia		LT898295		
P. murcianum	*Canescentia*	FMR 15491	Dung, Galicia		LT898296		
P. murcianum	*Canescentia*	FMR 15845	Dung, Galicia		LT898297		

P. polonicum	*Fasciculata*	FMR 15099	Dung, Catalonia		LT898298		
P. radiolubatum	*Canescentia*	FMR 15485	Dung, Canary Islands		LT898299		
P. roseoviride	*Aspergilloides*	FMR 15645	Dung, Castile and Leon		LT898300		
P. rubefaciens	*Exilicaulis*	FMR 15202	Dung, Balearic Islands		LT898301		
P. rubefaciens	*Exilicaulis*	FMR 15297	Dung, Castile and Leon		LT898302		
P. rudallense	*Aspergilloides*	FMR 15843	Dung, Canary Islands		LT898303		
P. sizovae	*Citrina*	FMR 15300	Dung, Castile and Leon		LT898304		
P. sizovae	*Citrina*	FMR 15521	Dung, Galicia		LT898305		
P. synnematicola	*Robsamsonia*	FMR 15192 = CBS 142669	Dung, Balearic Islands	LT898167	LT898172	LT898137	LT898142
P. synnematicola	*Robsamsonia*	FMR 15210	Dung, Balearic Islands	LT898168	LT898173	LT898138	LT898143
P. synnematicola	*Robsamsonia*	FMR 15211	Dung, Balearic Islands	LT898169	LT898174	LT898139	LT898144
P. synnematicola	*Robsamsonia*	FMR 16481 = CBS 143045	Soil, Catalonia	LT898170	LT898175	LT898140	LT898145
P. synnematicola	*Robsamsonia*	FMR 16491 = CBS 143046	Dung, Catalonia	LT898171	LT898176	LT898141	LT898146
Penicillium sp.	*Exilicaulis*	FMR 15841	Dung, Castile and Leon		LT898311		
Pgo. flava	-	FMR 15296 = CBS 142988	Dung, Balearic Islands	LT899787	LT898312	LT899771	LT899805
Pgo. flava	-	FMR 16442	Dung, Extremadura	LT899788	LT898313	LT899772	LT899806
Pse. coprobium	-	FMR 15299 = CBS 142670	Dung, Castile and Leon	LT899789	LT898315		LT899807
Pse. giganteum	-	FMR 14718	Soil, unknown	LT899790	LT898314		LT899808
T. angelicus	*Talaromyces*	FMR 15489= CBS 143042	Dung, Galicia	LT899791	LT898316	LT899773	LT899809
T. angelicus	*Talaromyces*	FMR 15490= CBS 143043	Dung, Galicia	LT899792	LT898317	LT899774	LT899810
T. catalonicus	*Trachyspermi*	FMR 16441 = CBS 143039	Dung, Catalonia	LT899793	LT898318	LT899775	LT899811
T. coprophilus	*Talaromyces*	FMR 15199 = CBS 142756	Dung, Balearic Islands	LT899794	LT898319	LT899776	LT899812
T. gamsii	*Talaromyces*	FMR 15303 = CBS 143040	Soil, Andalusia	LT899795	LT898320	LT899777	LT899813
T. muroii	*Talaromyces*	FMR 15496	Dung, Galicia		LT898321		
T. pseudofuniculosus	*Talaromyces*	FMR 15035	Dung, Catalonia	LT899797	LT898322	LT899779	LT899815
T. pseudofuniculosus	*Talaromyces*	FMR 15307 = CBS 143041	Dung, Andalusia	LT899796	LT898323	LT899778	LT899814
T. ruber	*Talaromyces*	FMR 15839	Dung, Castile and Leon		LT898324		
T. sayulitensis	*Talaromyces*	FMR 15842	Dung, Canary Islands		LT898325		

* *A. = Aspergillus; P. = Penicillium; Pgo. = Penicillago; Pse. = Pseudopenicillium; T. = Talaromyces*

Table 2. Overview and details used for phylogenetic analyses of *Aspergilllus*, *Penicillium*, *Talaromyces*, and the related genera.

		Aspergillus					***Penicillium***				***Talaromyces***		**Miscellaneous *Aspergillaceae***
	Section*	***Candidi***	***Cremei***	***Flavipedes***	***Terrei***	***Usti***	***Turbata* and *Paradoxa***	***Ramosa***	***Robsamsonia***	***Roquefortorum***	***Talaromyces***	***Trachyspermi***	
ITS dataset	Length (bp)	490	532	556	564	481	504	485	428	506	361	473	440
	Pvar	49	58	67	54	50	29	47	207	24	73	110	124
	Pi	4	27	32	43	19	18	16	18	13	42	80	104
	Model*	GTR+I	GTR+I	GTR+I	GTR+I+G	GTR+I	K80+G	GTR+I	K80+G	TPM2uf+I	TrN+I+G	TIM2+I+G	TrN+I+G
*Ben*A dataset	Length (bp)	475	474	560	531	440	412	387	352	409	402	381	401
	Pvar	181	195	241	216	151	151	112	122	68	212	160	223
	Pi	54	106	140	159	75	100	64	67	29	166	98	187
	Model*	GTR+G	SYM+G	HKY+I+G	HKY+I	K80+I	TIM2ef+G	TIM2ef+G	GTR+G	TIM1ef	HKY+I+G	TPM3uf+G	HKY+I+G
CaM dataset	Length (bp)	554	500	560	560	465	492	544	486	501	480	474	-
	Pvar	134	211	257	223	214	195	223	164	50	246	233	-
	Pi	32	121	164	144	122	88	92	96	8	212	185	-
	Model*	SYM+G	SYM+I	SYM+G	GTR+I	GTR+I	TIM2+G	TIM2+G	GTR+I	TPM1+G	HKY+I+G	TPM1+I+G	-
*RPB*2 dataset	Length (bp)	900	1014	965	1013	904	915	-	804	915	-	-	956
	Pvar	209	290	265	226	244	239	-	243	106	-	-	463
	Pi	80	173	165	175	142	159	-	127	55	-	-	375
	Model*	GTR+I	SYM+G	SYM+I+G	SYM+G	SYM+I+G	TrN+I+G	-	GTR+I	TIM3ef+I	-	-	TrN+I+G
Concatenated dataset	Length (bp)	2419	2520	2641	2668	2290	2323	1416	2068	2331	1243	1329	1797
	Pvar	573	754	830	719	659	614	382	574	248	531	503	810
	Pi	170	427	501	521	358	365	172	303	105	420	336	666

*Pvar = variable sites; Pi = phylogenetic informative sites; * = substitution model for Bayesian inference.

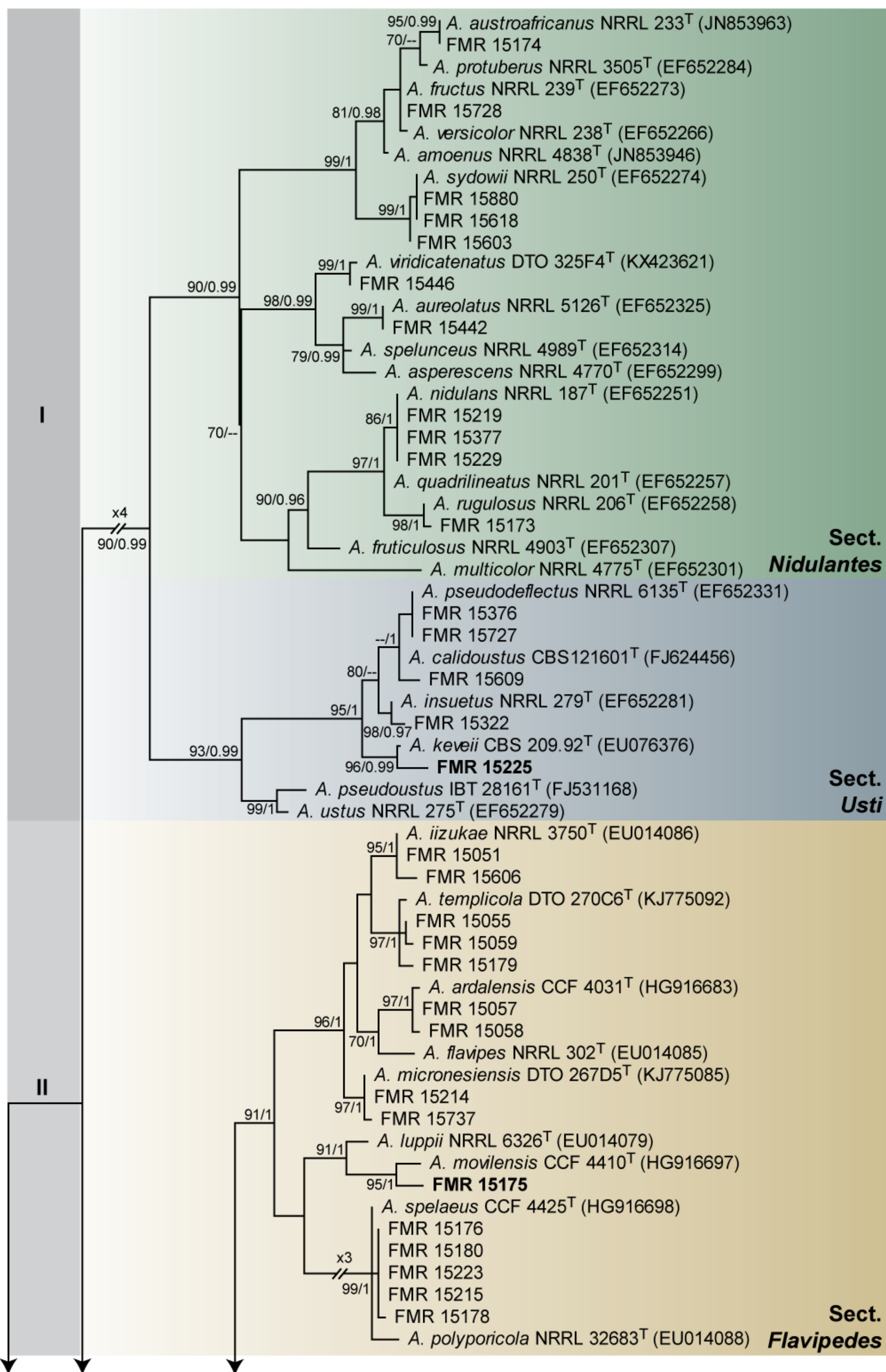


Figure 1.

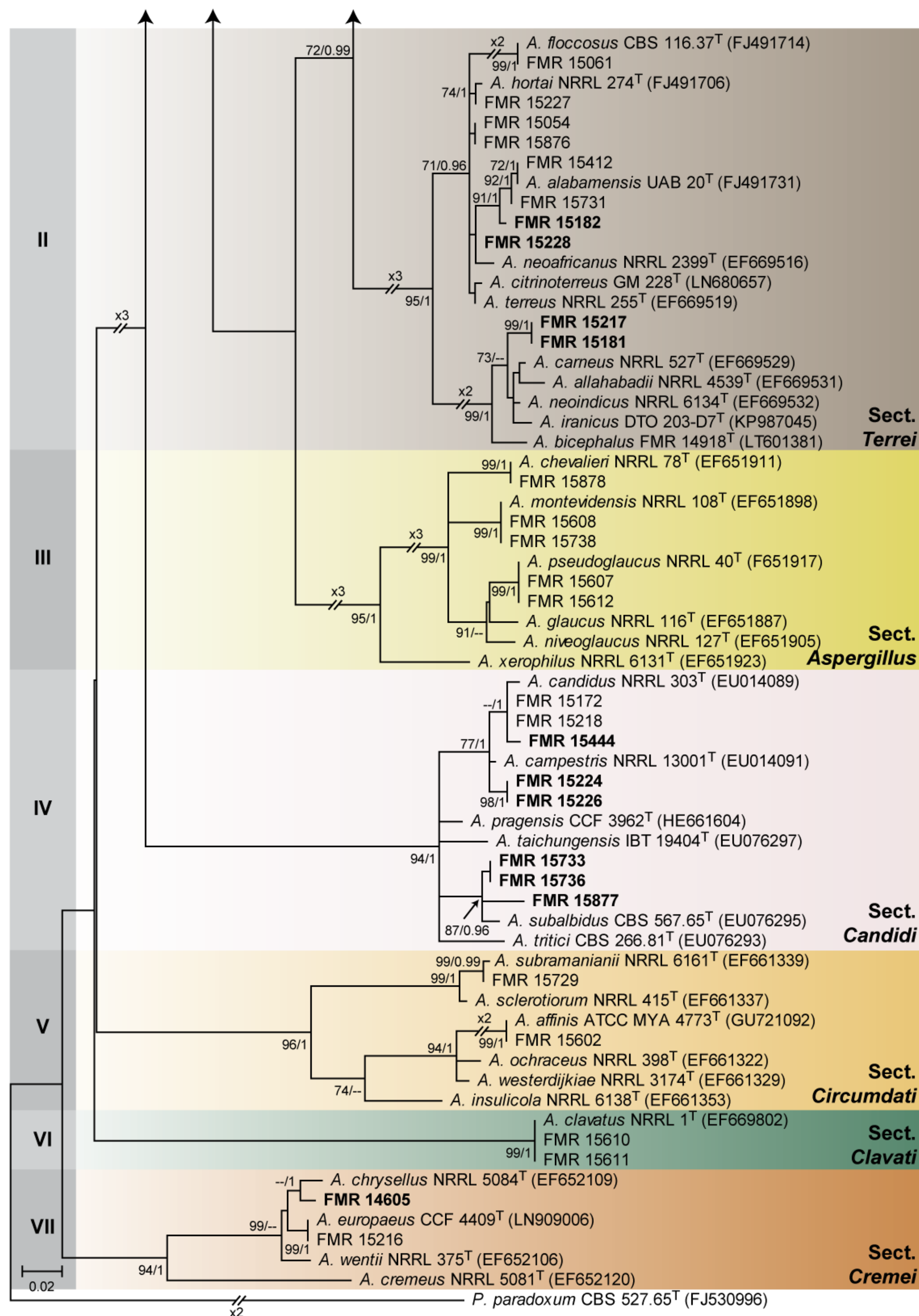


Figure 1. (Continued).

100/1
A. germanicus DTO 27D9T
93/1
A. thesauricus CCF 4166T
100/1
***A. calidokeveii* FMR 15225T sp. nov.**
A. keveii CBS 209.92T
100/1
89/1
A. insuetus NRRL 279T
100/1
A. asper NRRL 35910T
A. pseudodeflectus NRRL 6135T
A. ustus NRRL 275T
--/1
99/1
A. baeticus CCF 4226T
100/1
A. pseudoustus IBT 28161T
A. puniceus NRRL 5077T
88/1
A. granulosus NRRL 1932T
0.02
A. heterothallicus NRRL 5096T
A. elongatus NRRL 5176T

Figure 2.

100/1
A. iizukae NRRL 3750T
A. capensis DTO 179E6T
88/1
A. templicola DTO 270C6T
--/0.99
98/1
A. urmiensis DTO 203C2T
A. ardalensis CCF 4031T
98/1
100/1
A. flavipes NRRL 302T
A. neoflavipes NRRL 5504T
100/1
A. micronesiensis DTO 267D5T
x2
--/1
***A. albodeflectus* FMR 15175T sp. nov.**
100/1
A. movilensis CCF 4410T
100/1
A. luppii NRRL 6326T
94/0.99
A. spelaeus CCF 4425T
100/1
0.02
A. polyporicola NRRL 32683T
x2
A. janus NRRL 1787T

Figure 3.

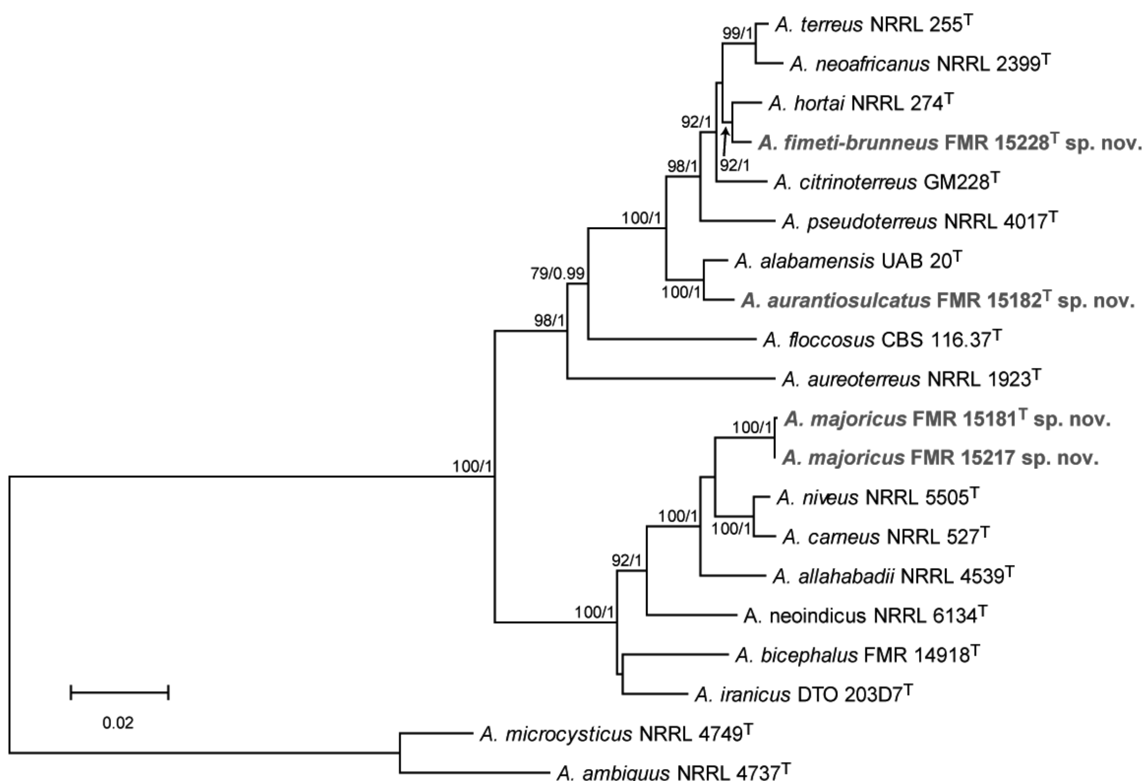


Figure 4.

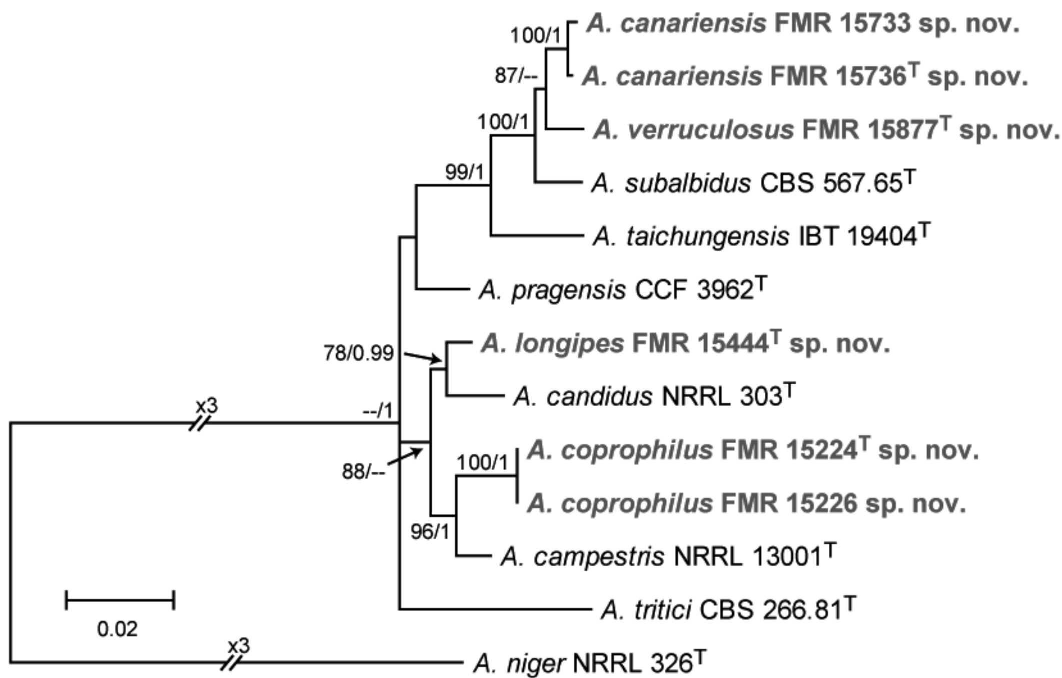


Figure 5.

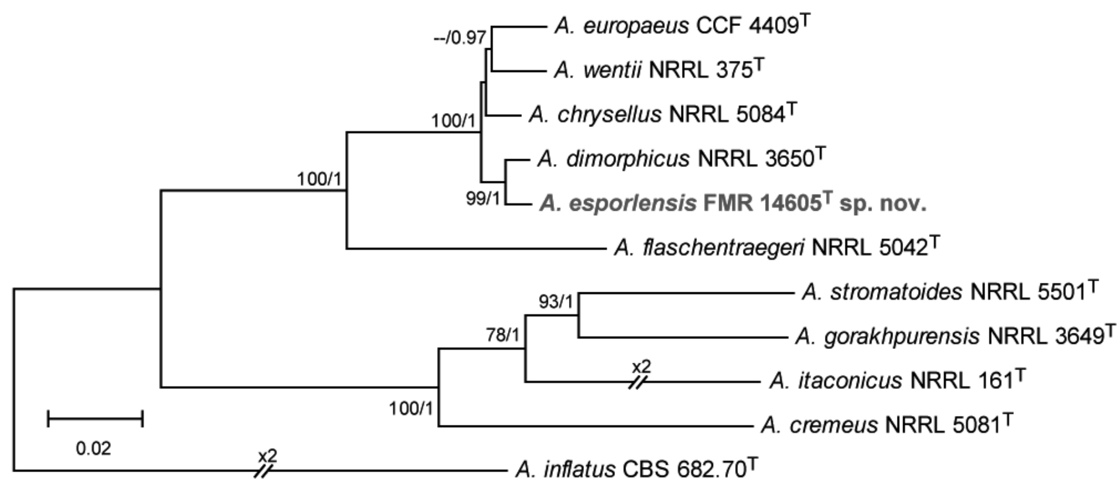


Figure 6.

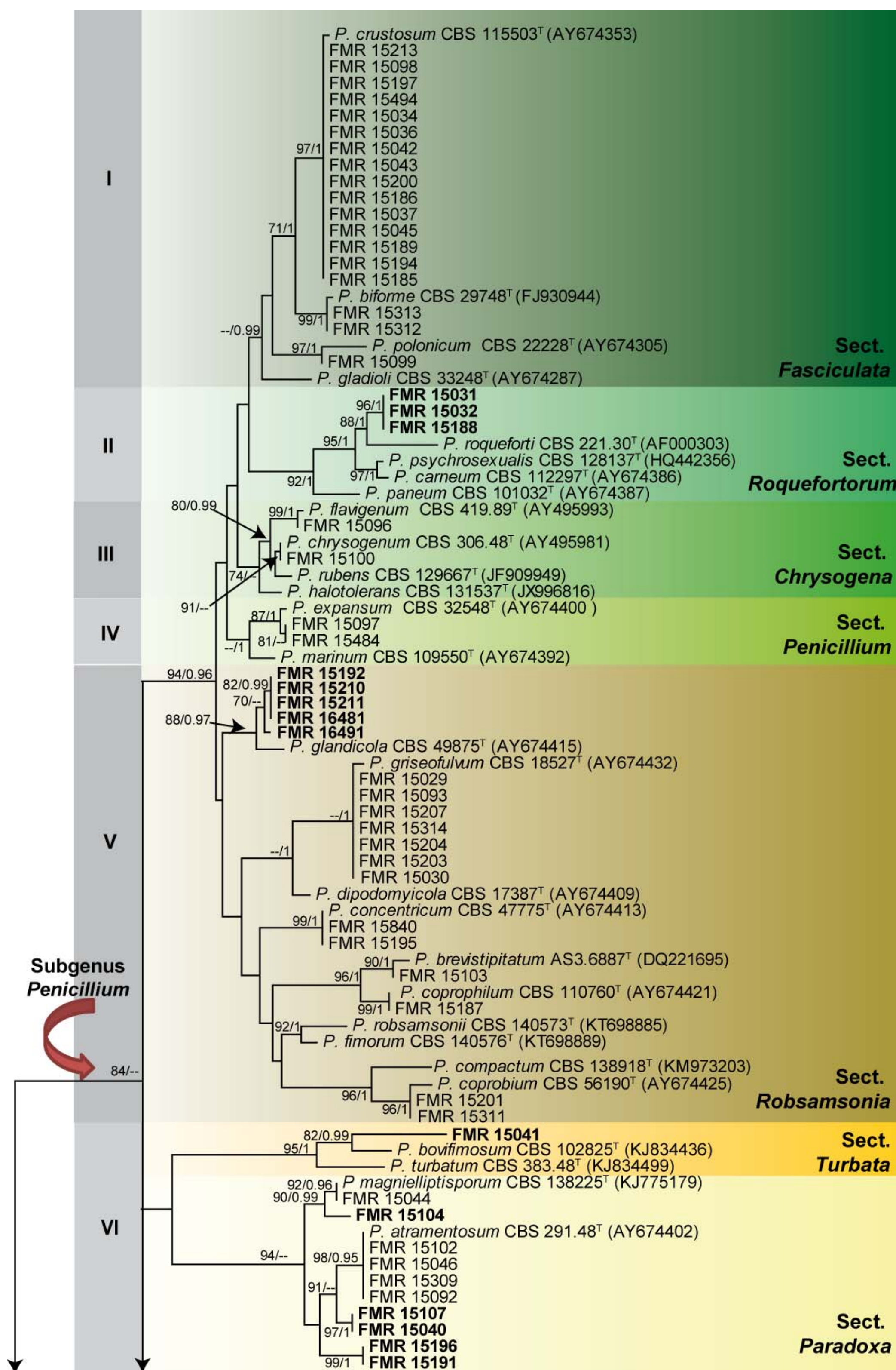


Figure 7.

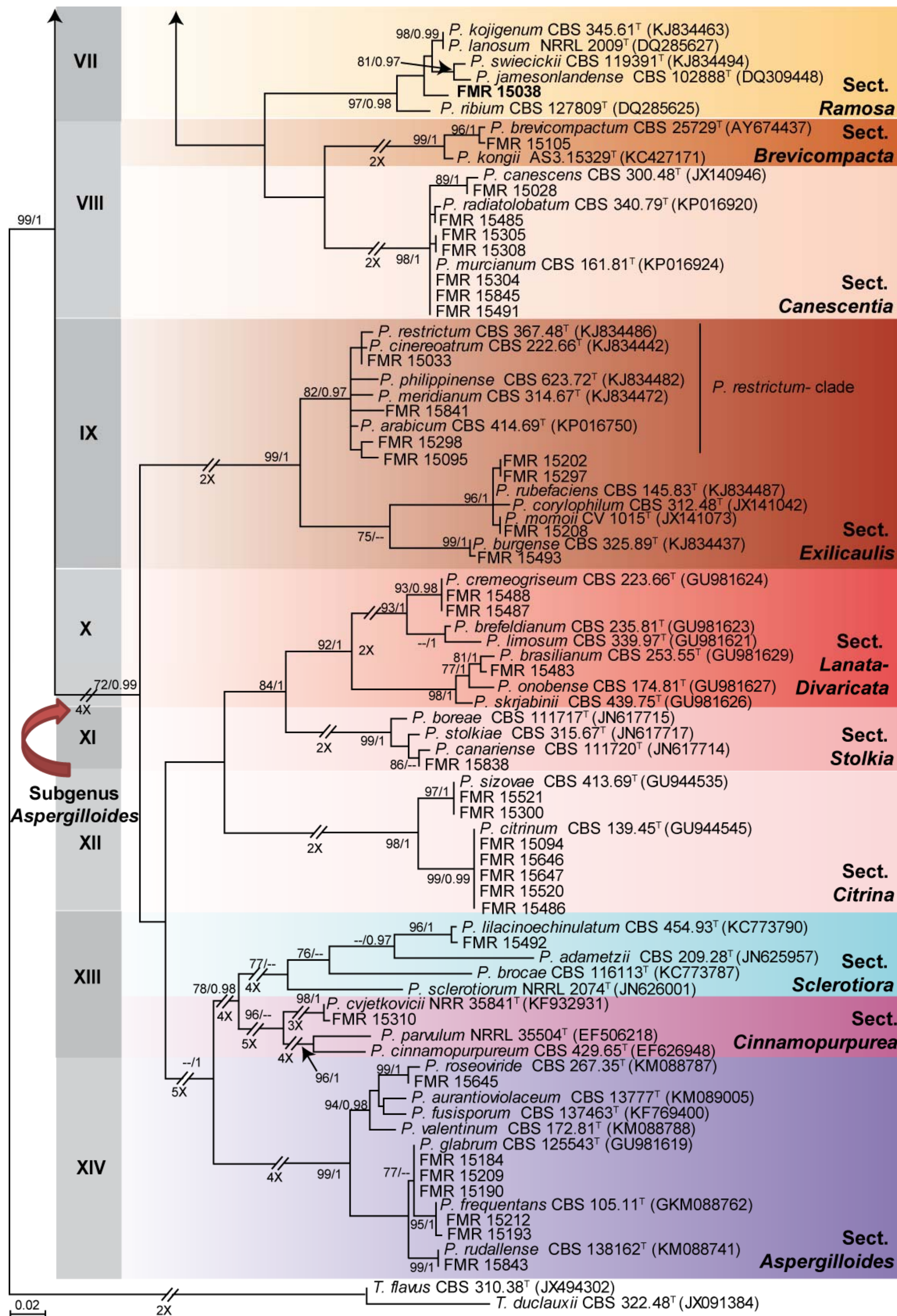


Figure 7. (Continued).

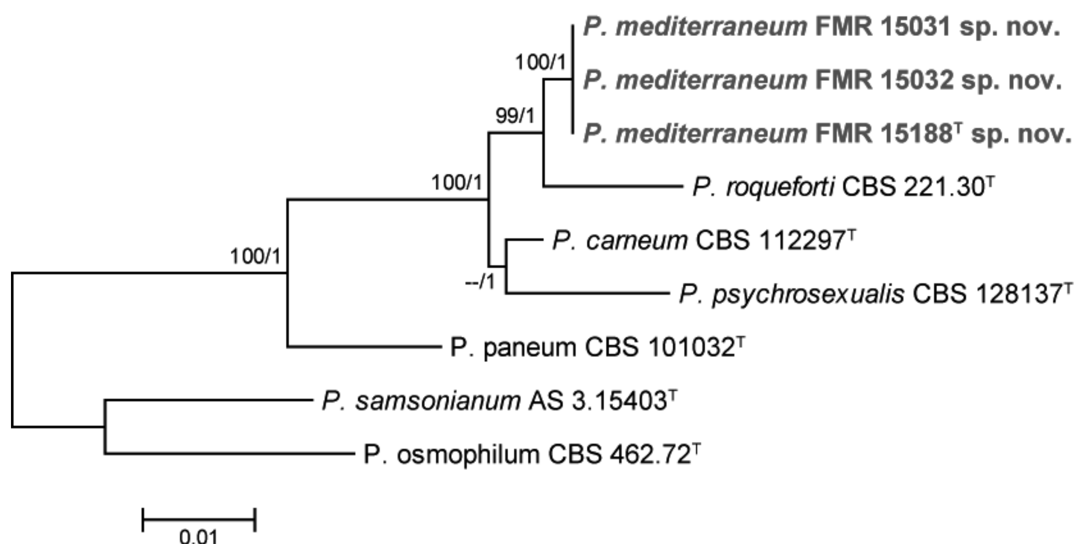


Figure 8.

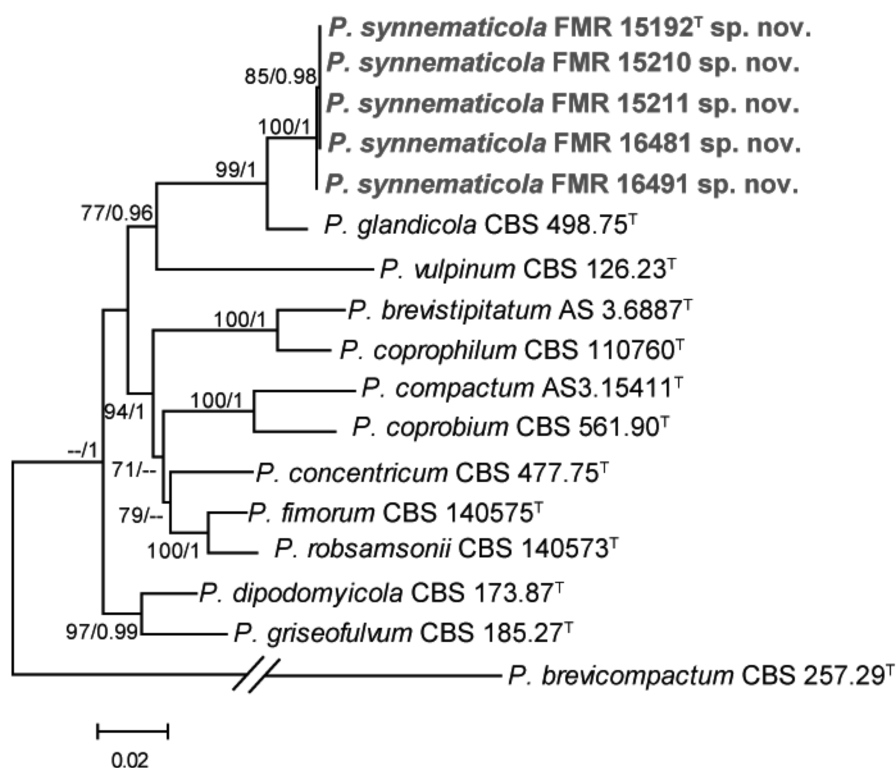


Figure 9.

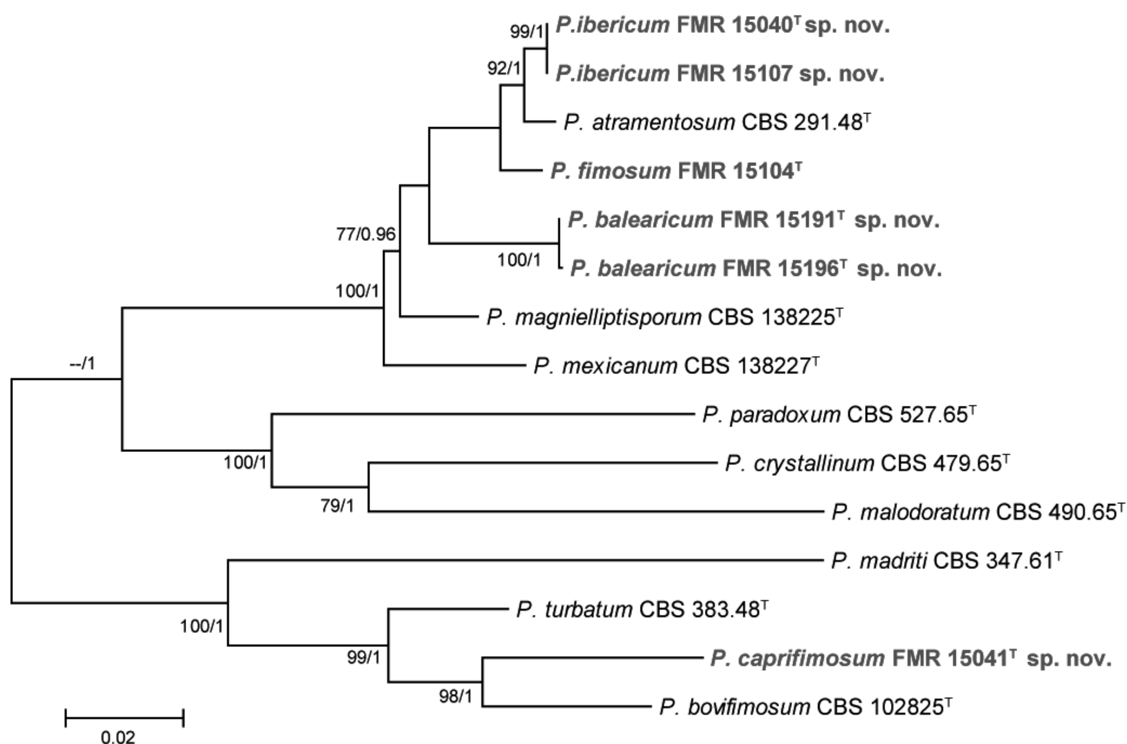


Figure 10.

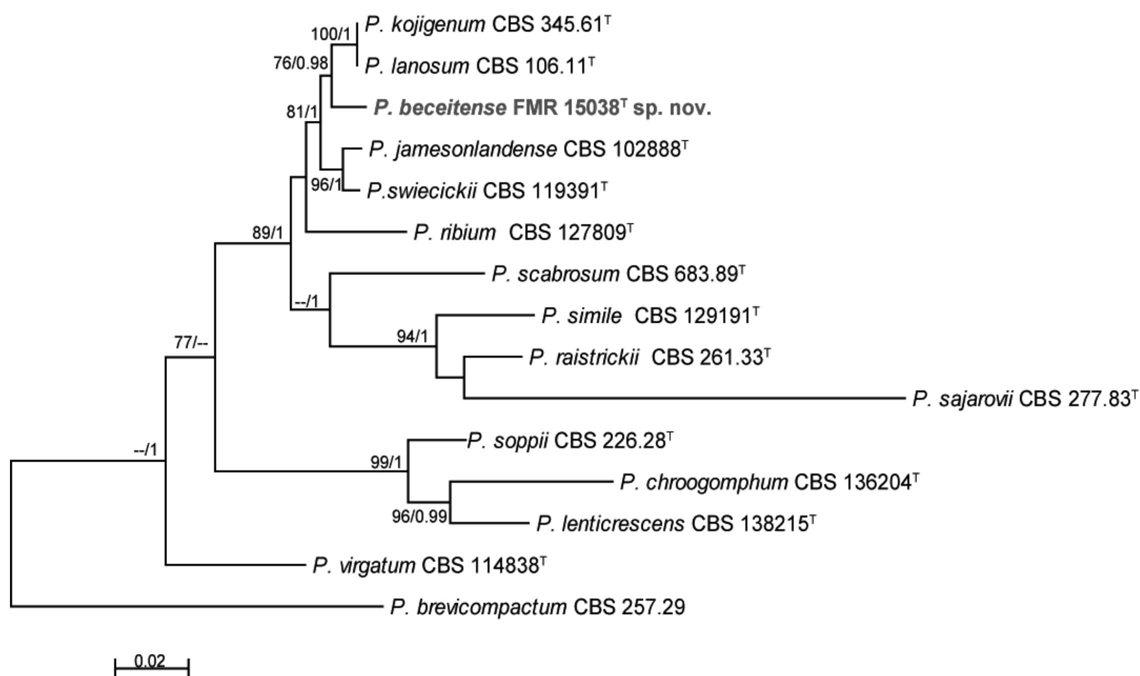


Figure 11.

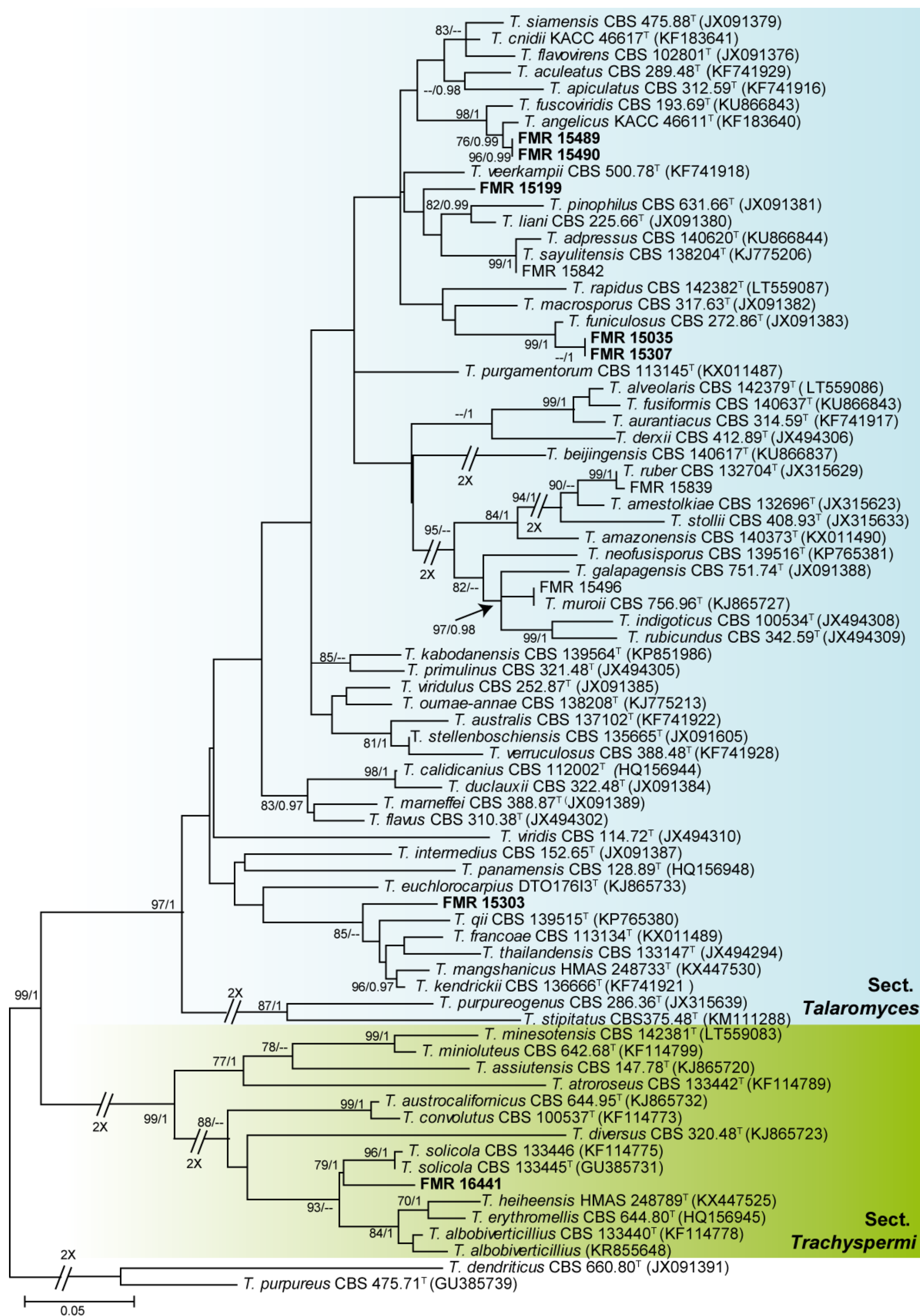


Figure 12.

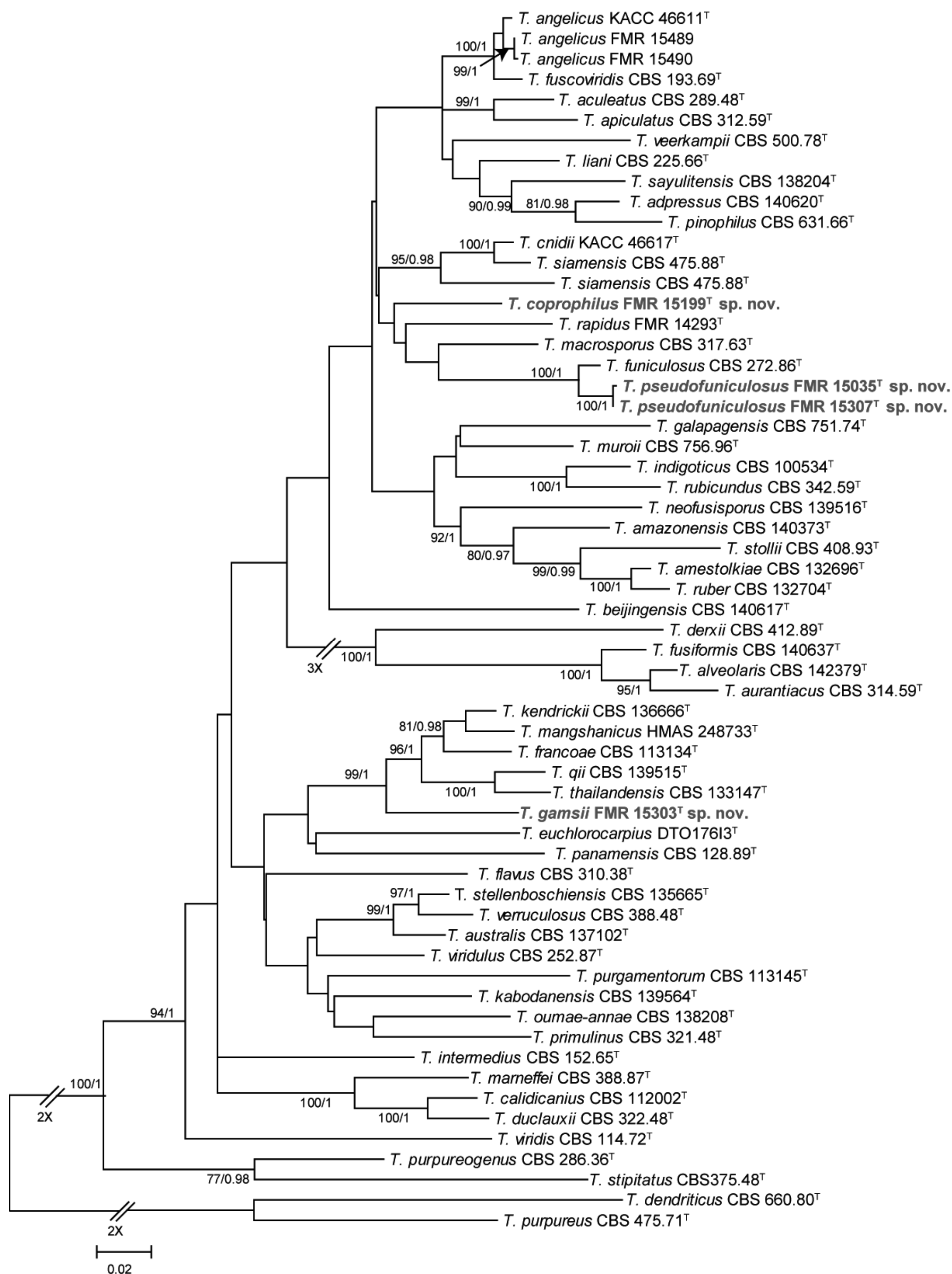


Figure 13.

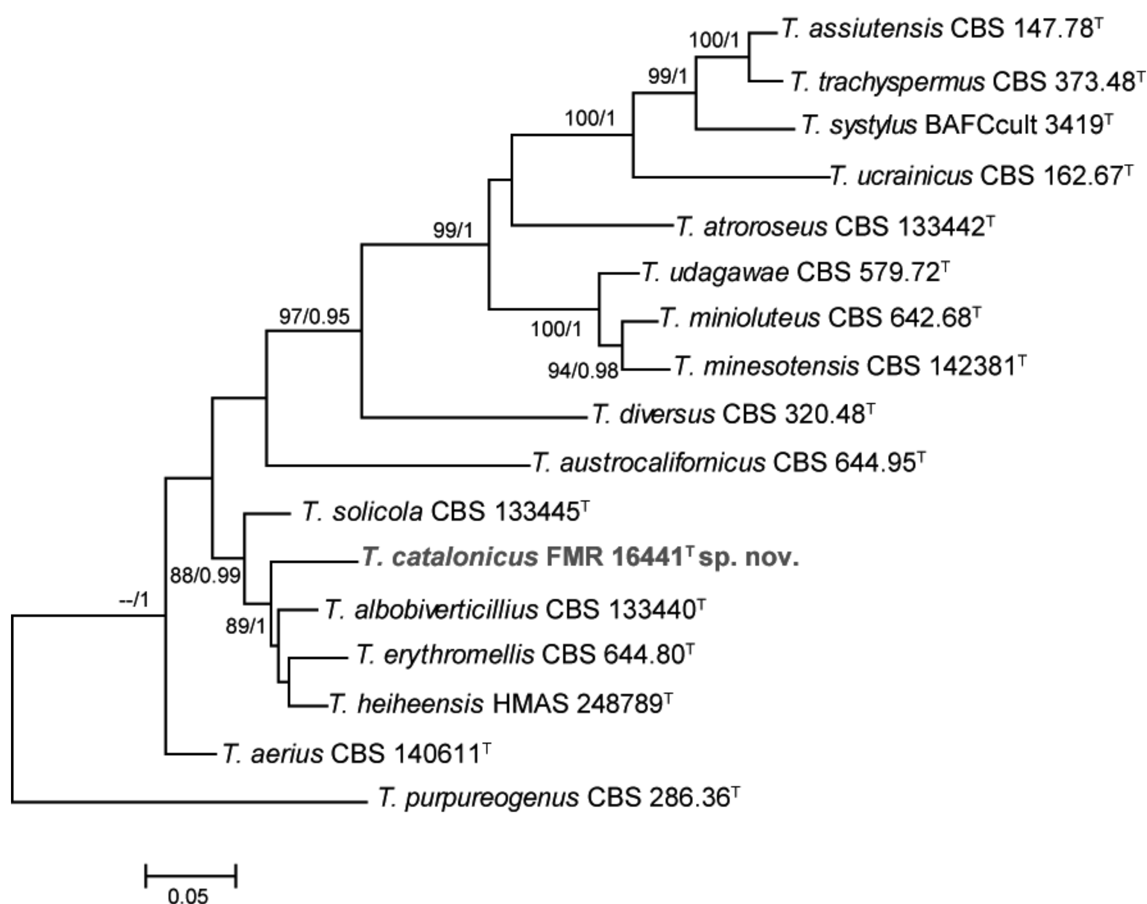


Figure 14.

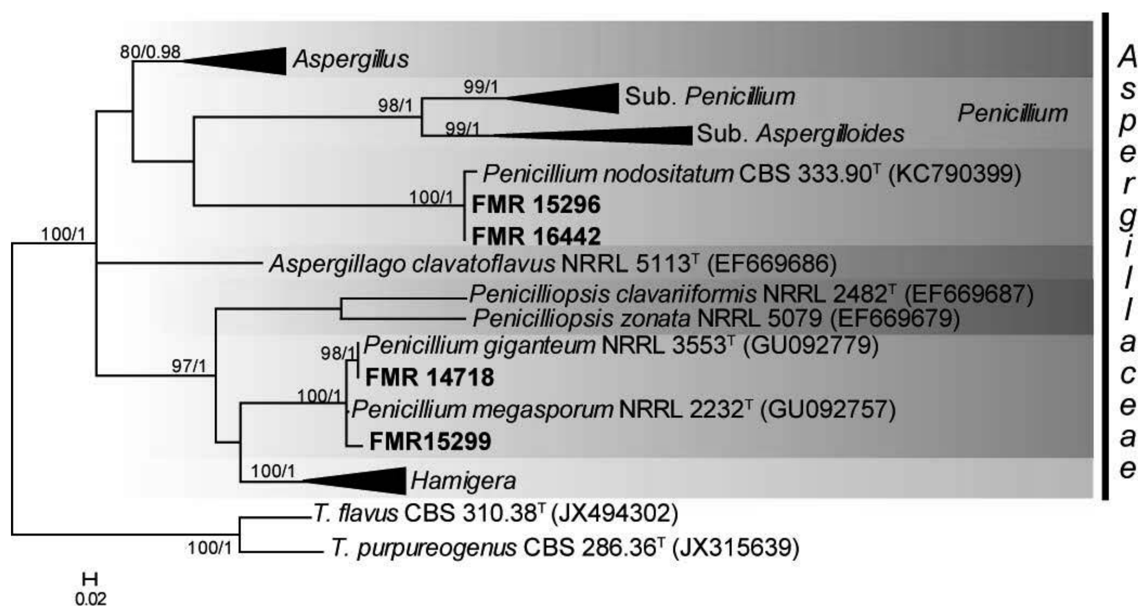


Figure 15.

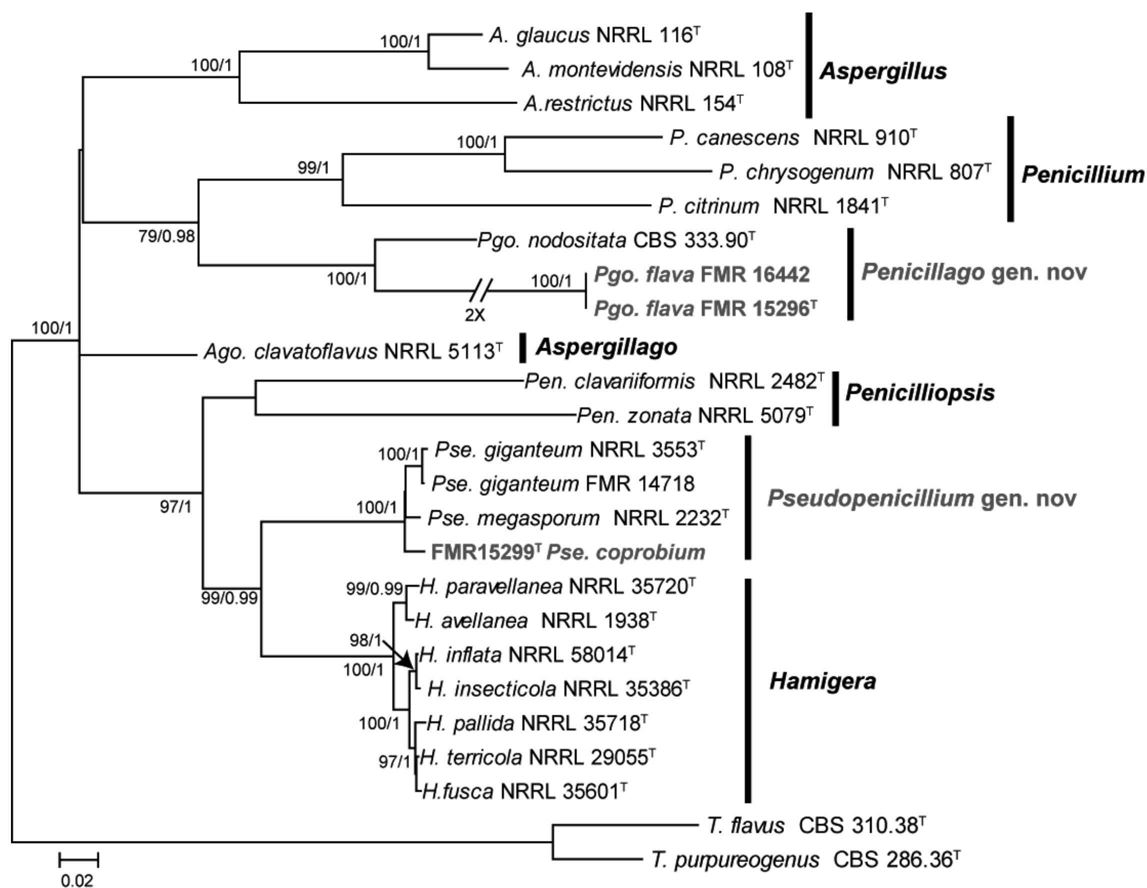


Figure 16.

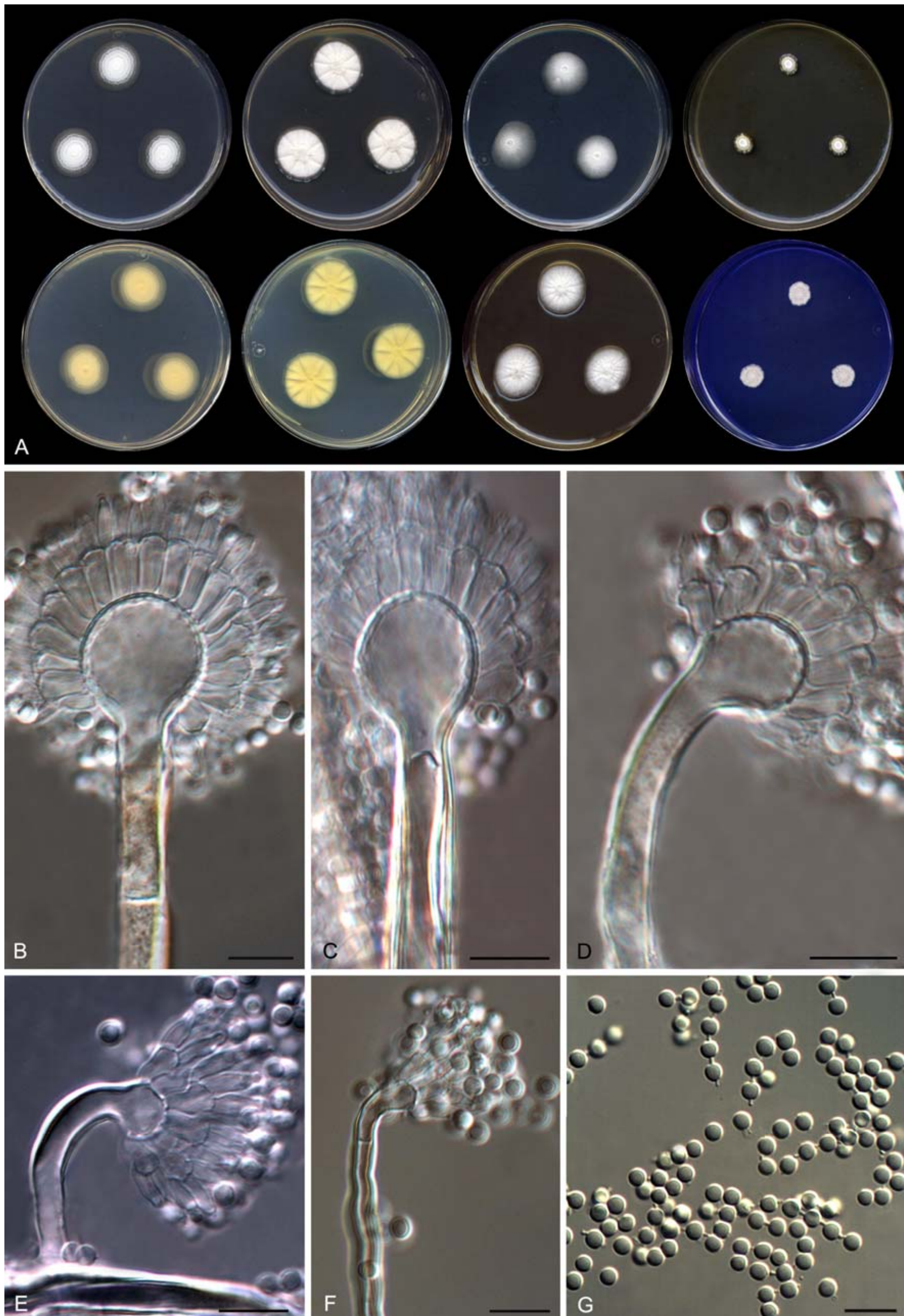


Figure 17.

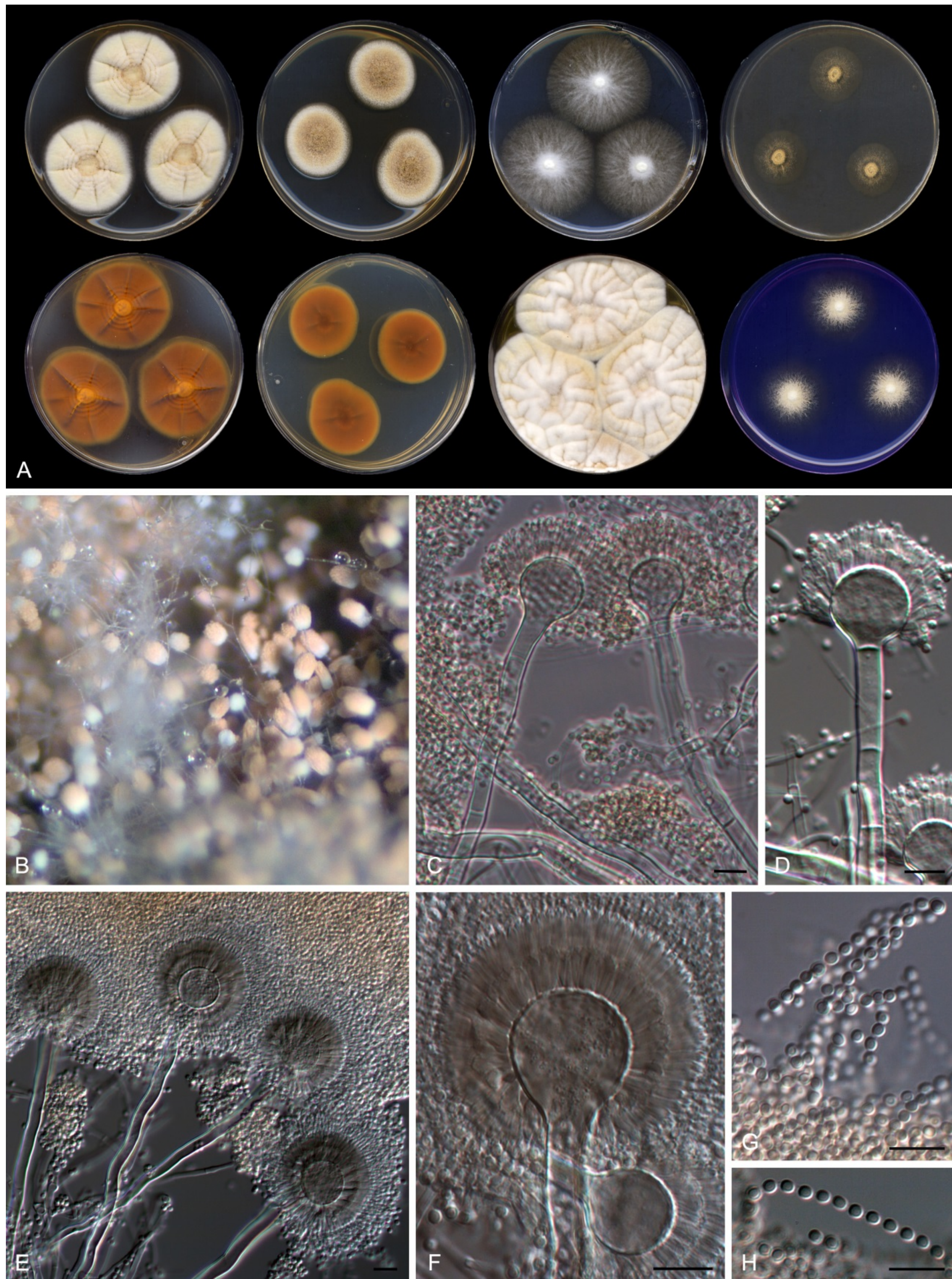


Figure 18.

Figure 19.

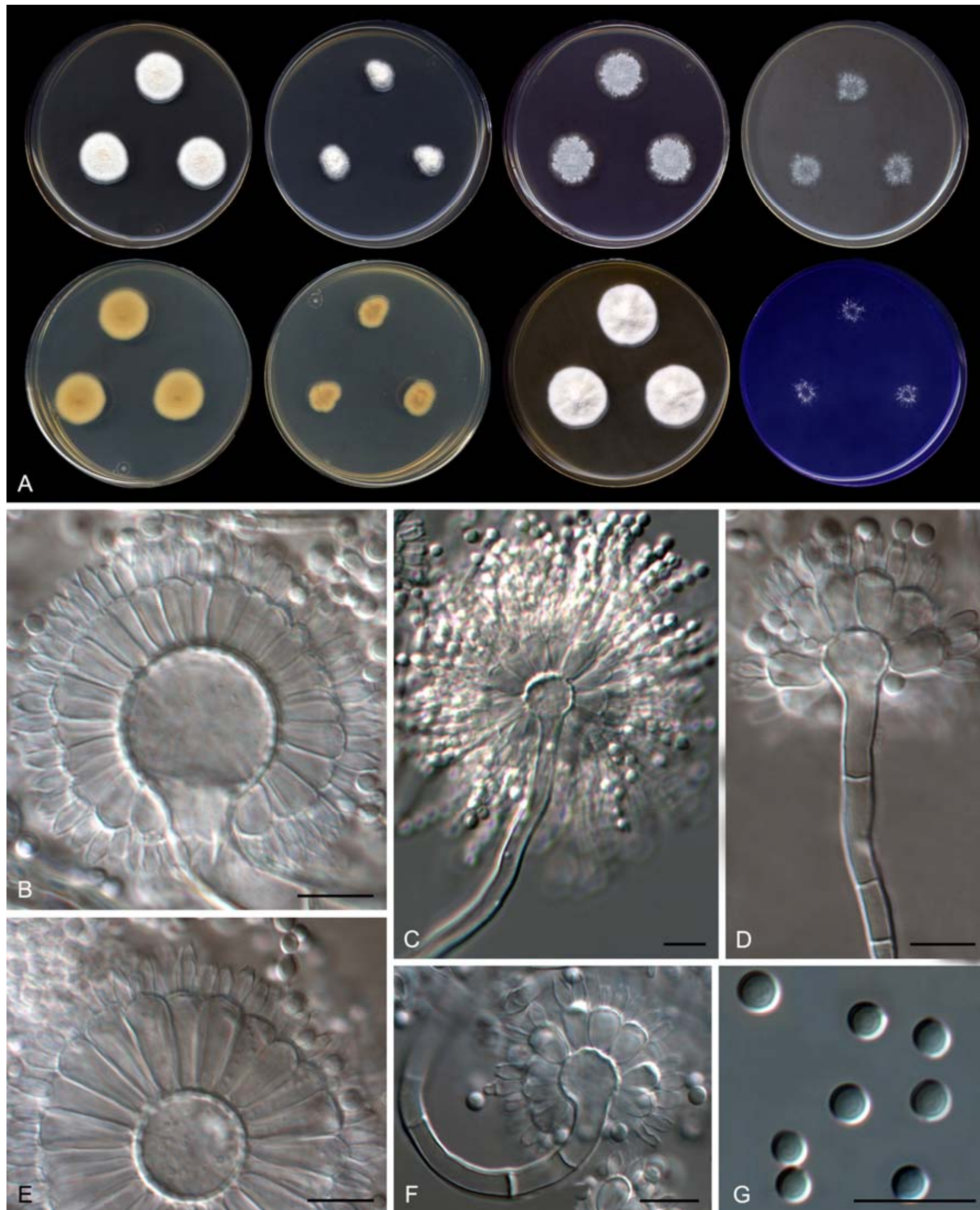


Figure 20.

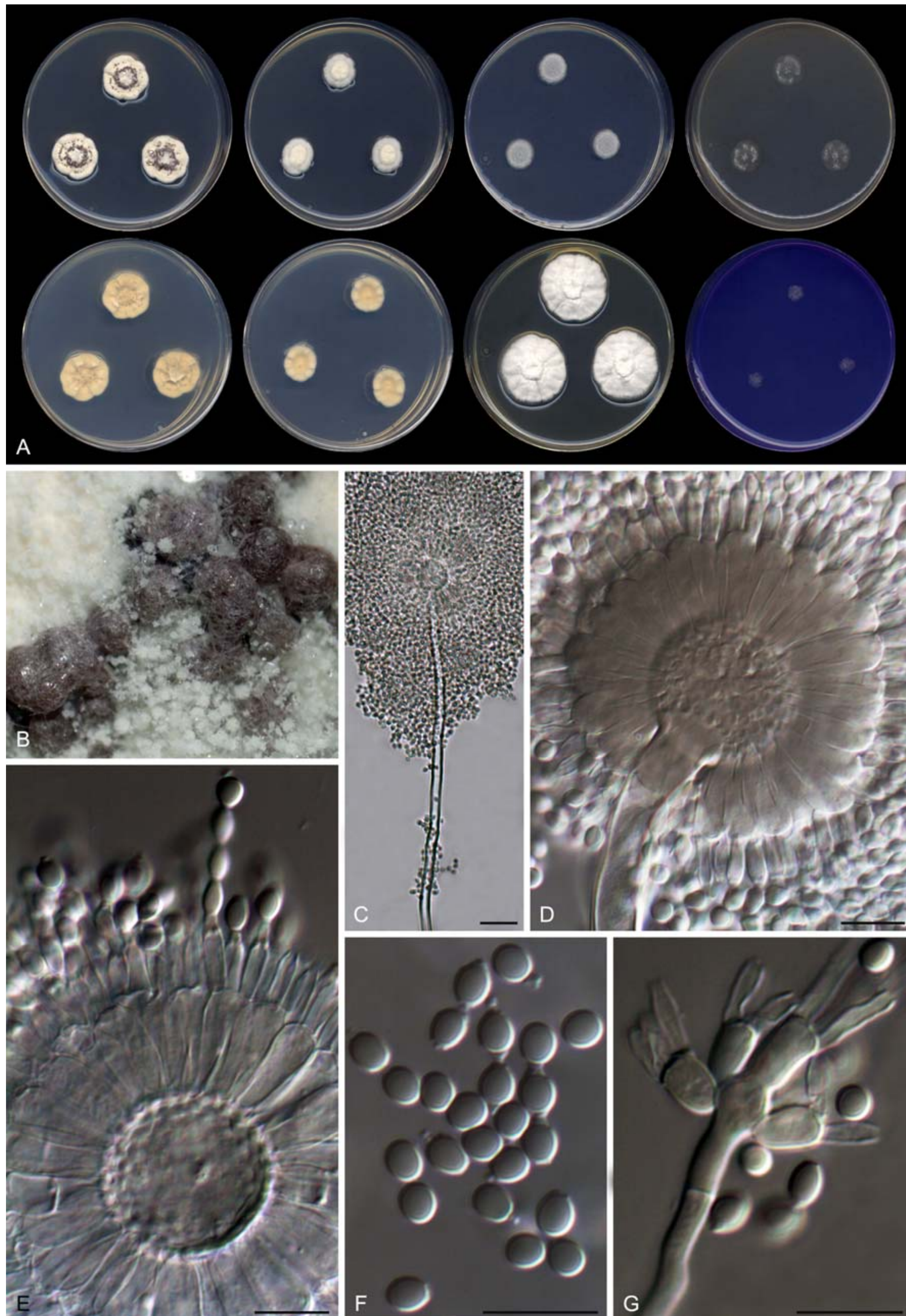


Figure 21.

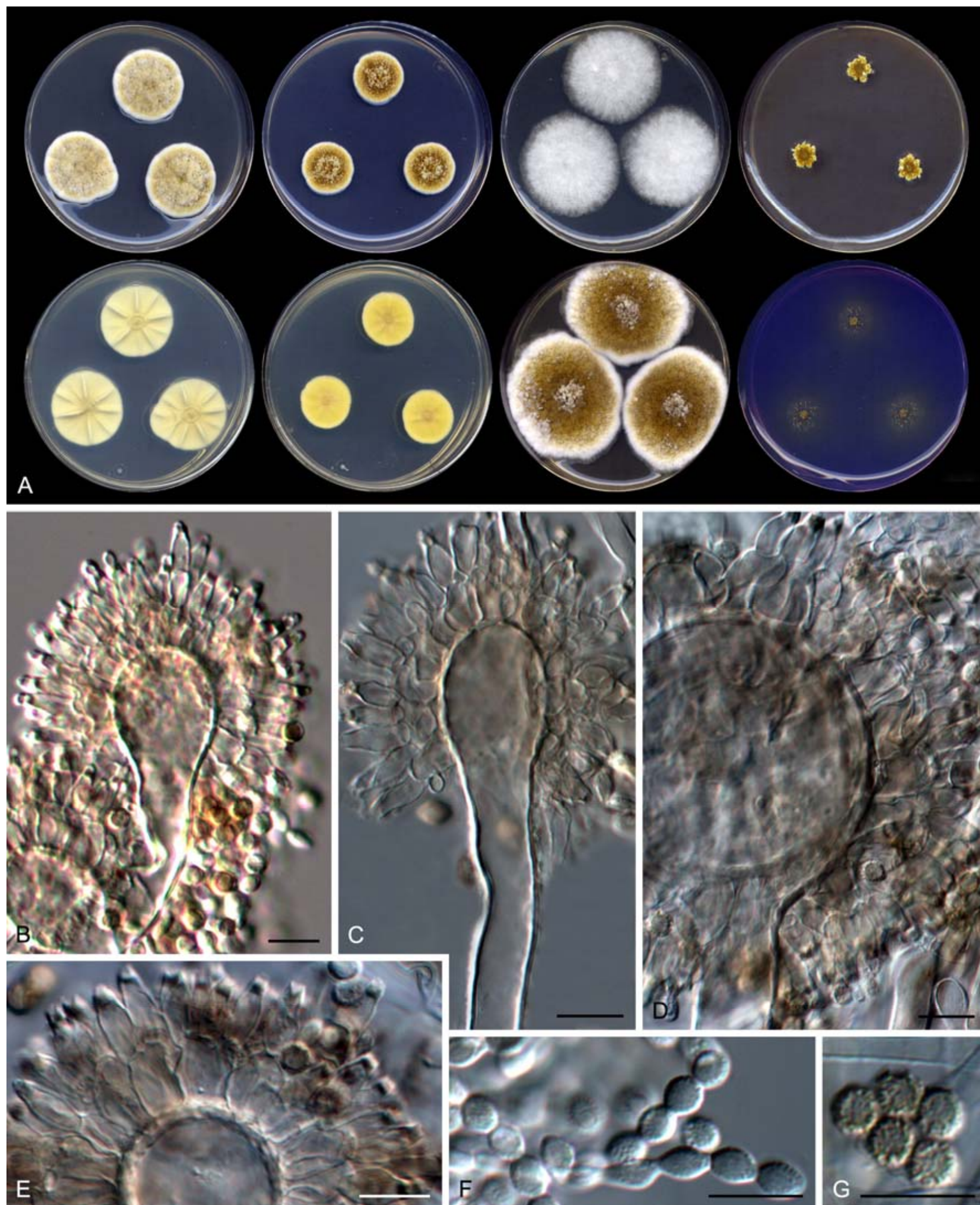


Figure 22.

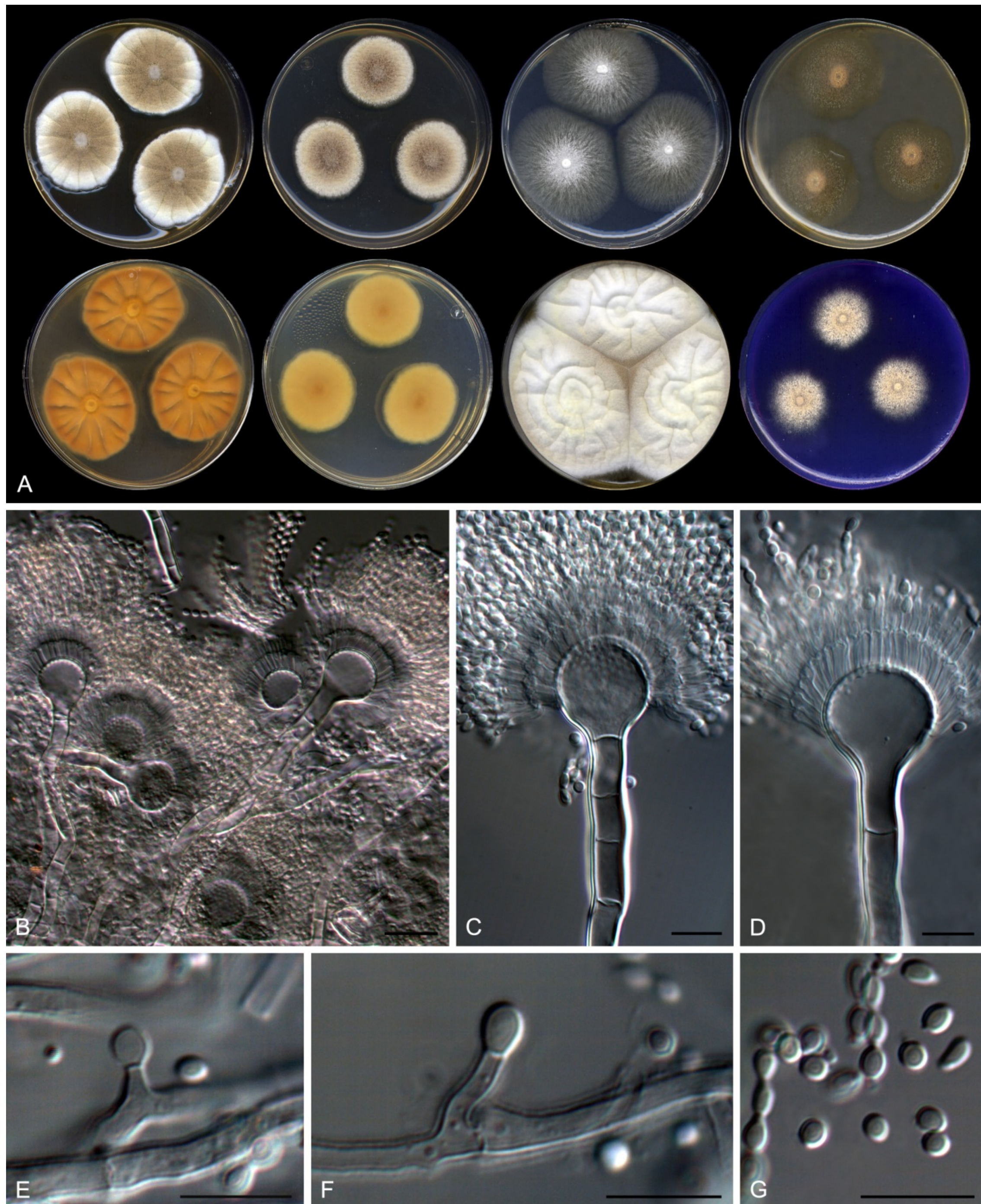


Figure 23.

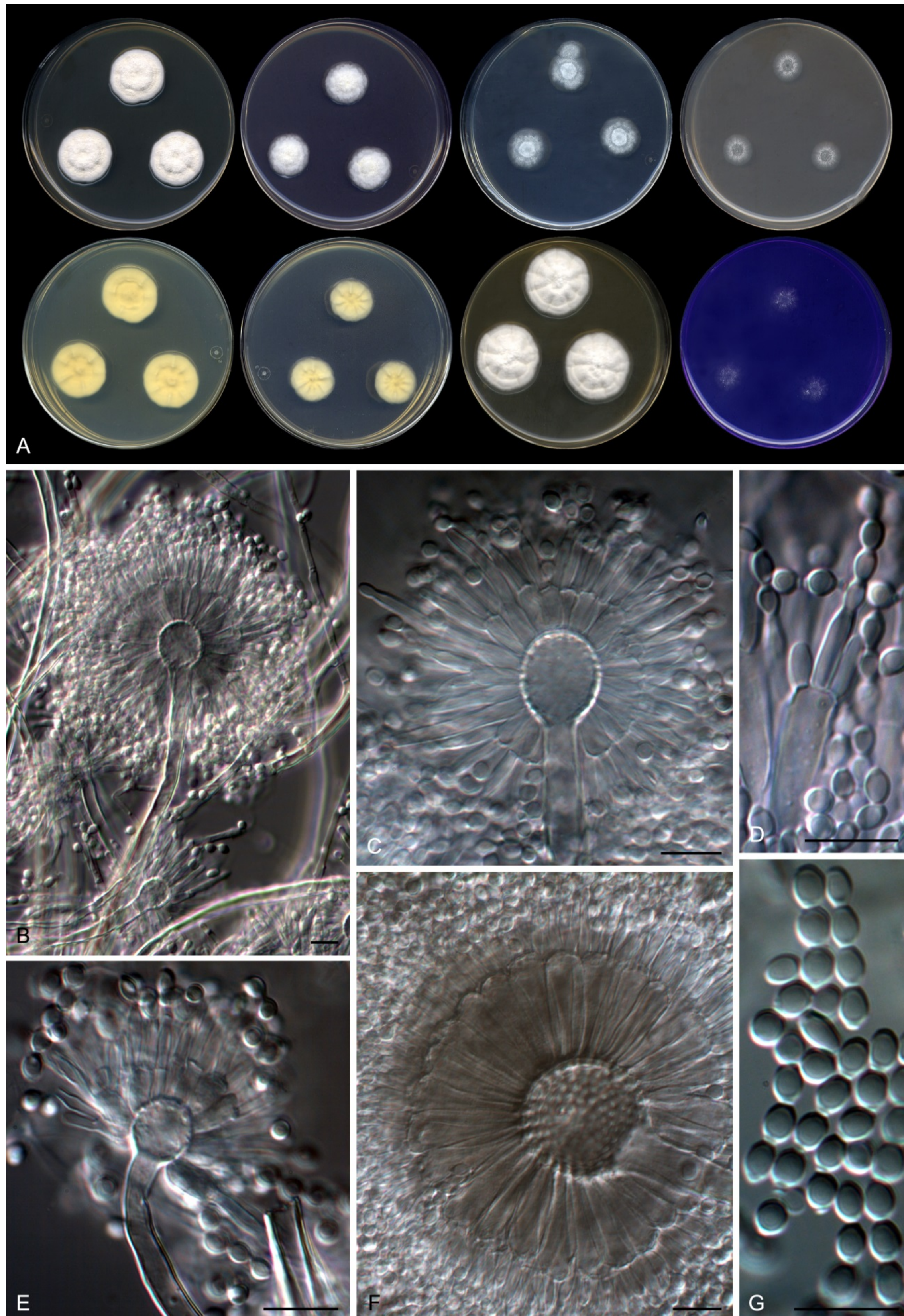


Figure 24.

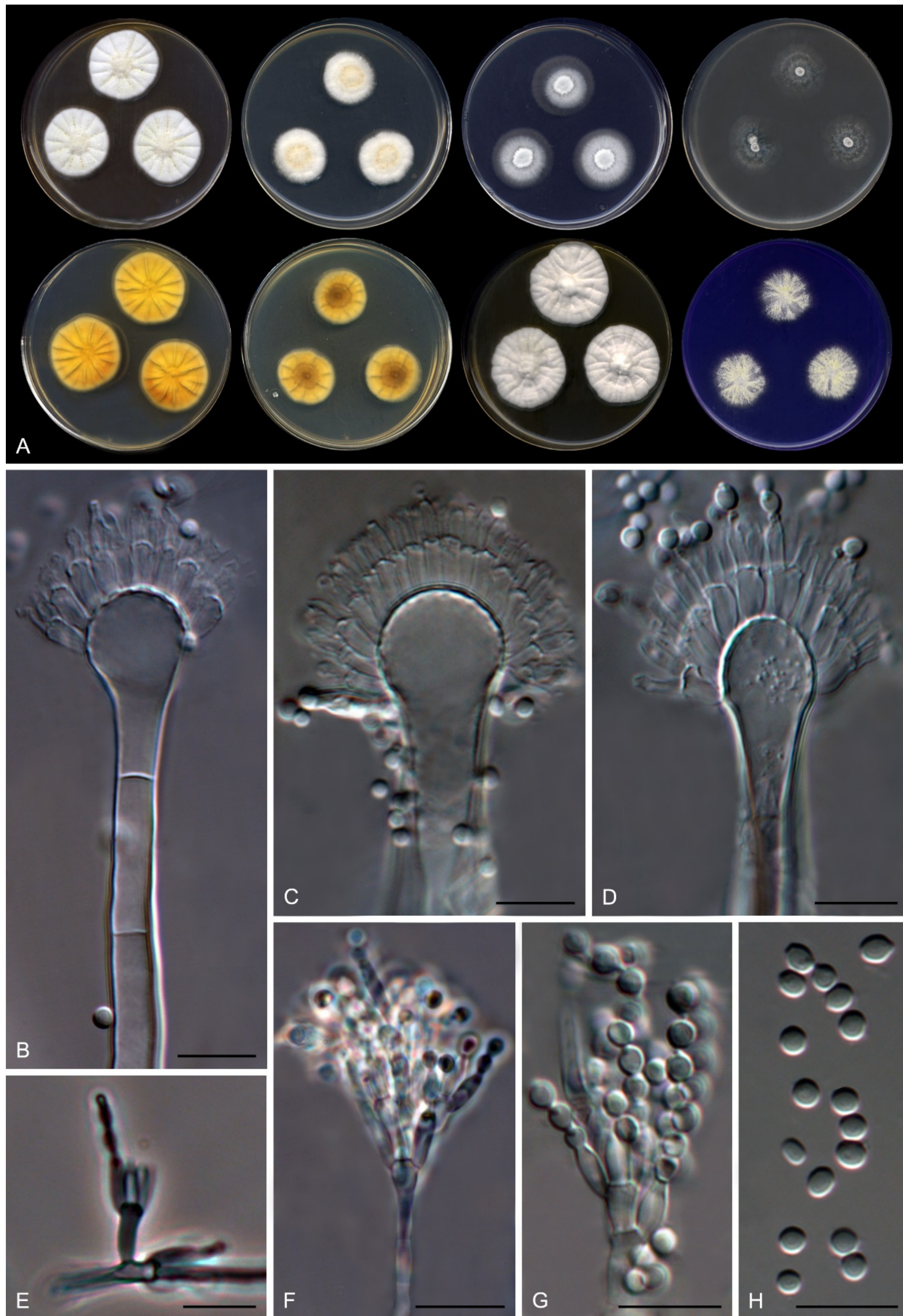


Figure 25.

Figure 26.

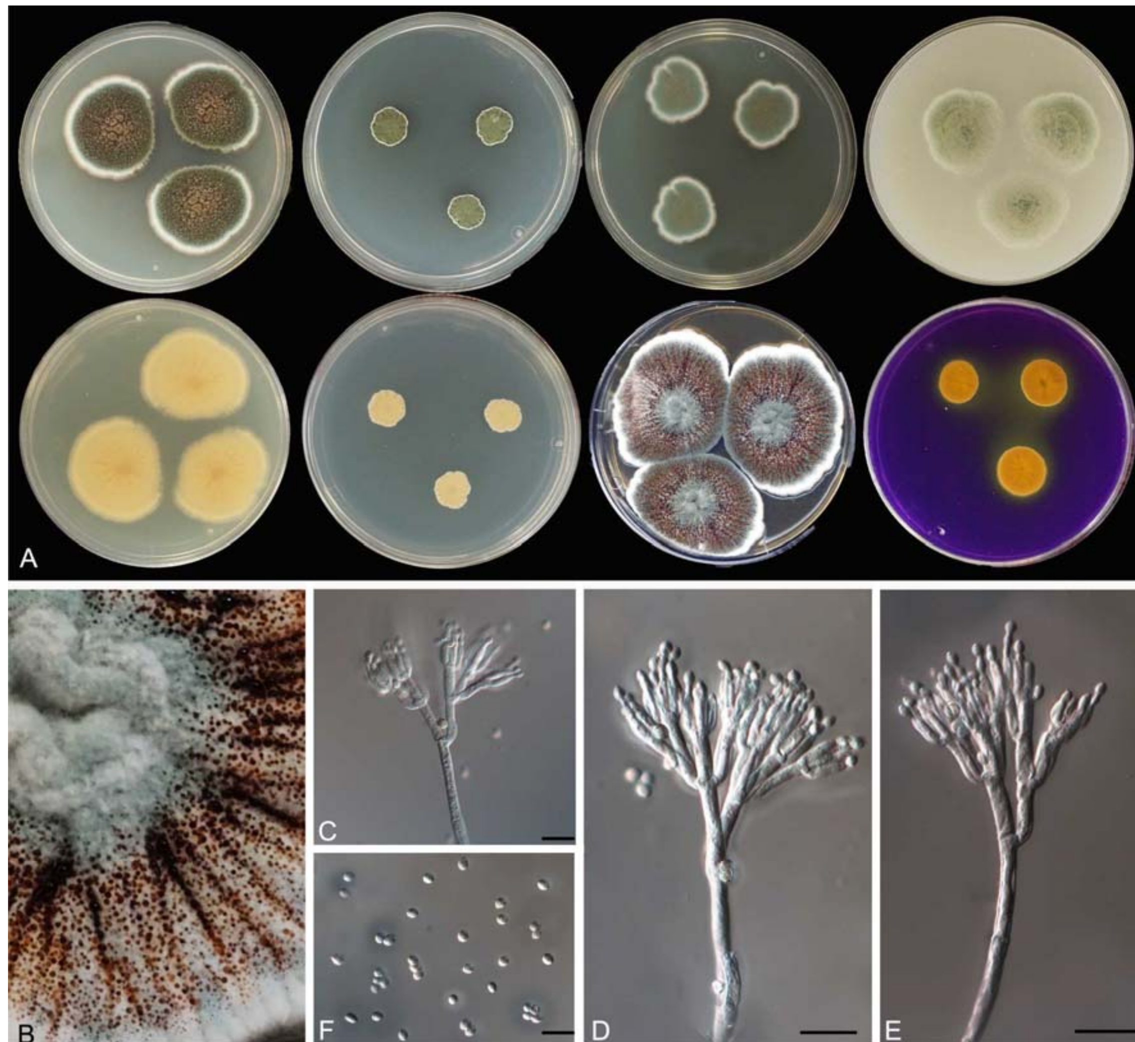


Figure 27.

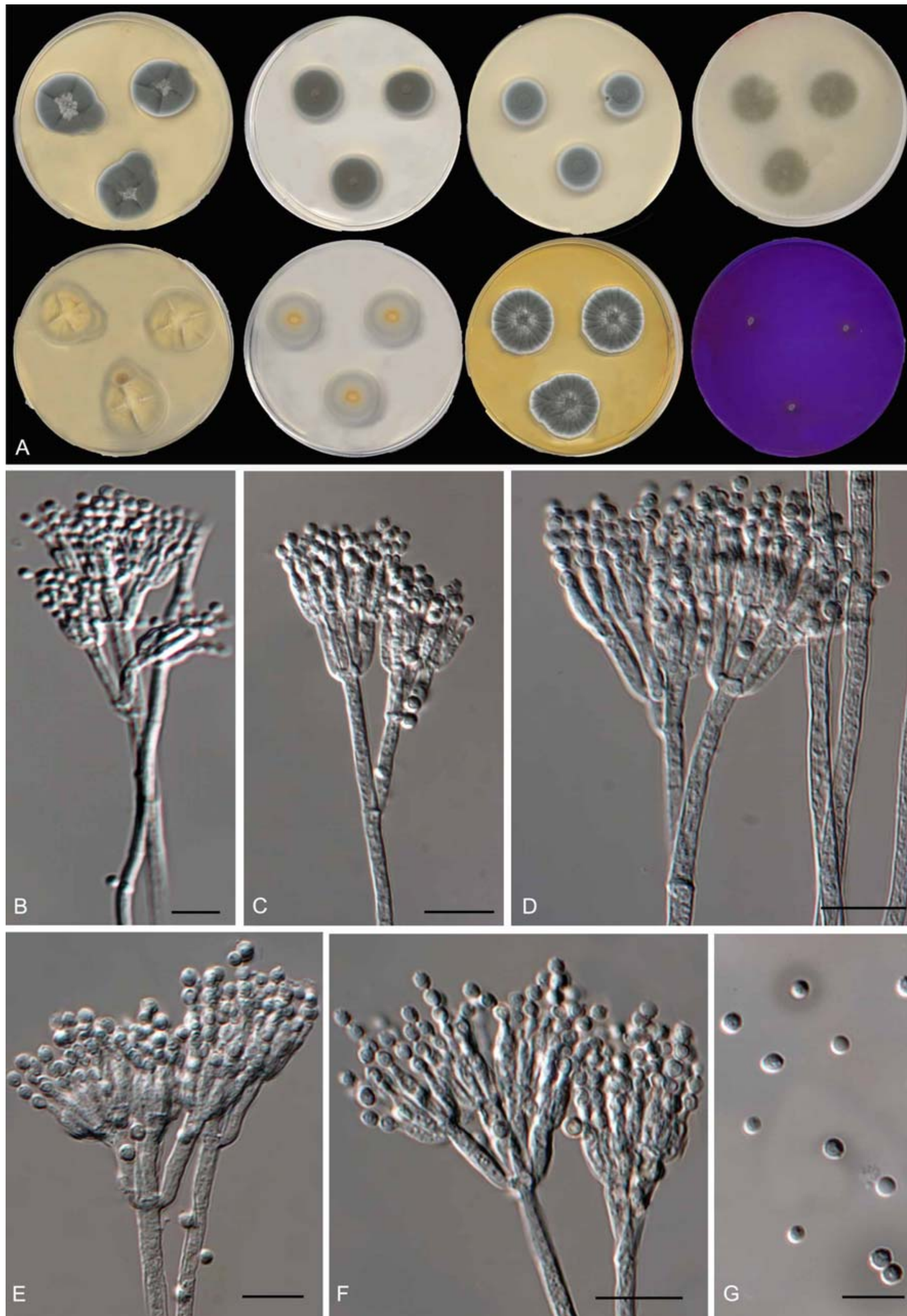


Figure 28.

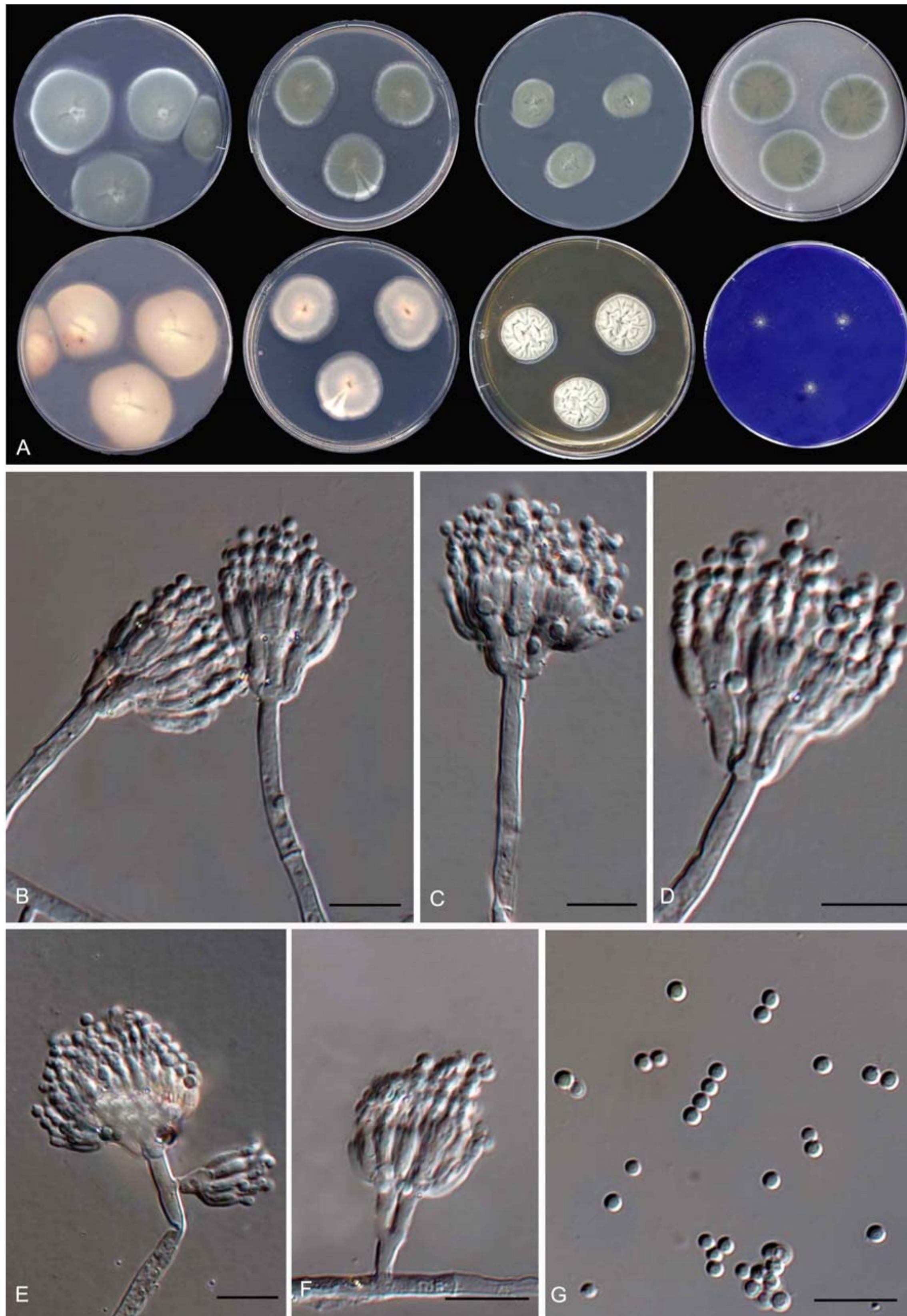


Figure 29.

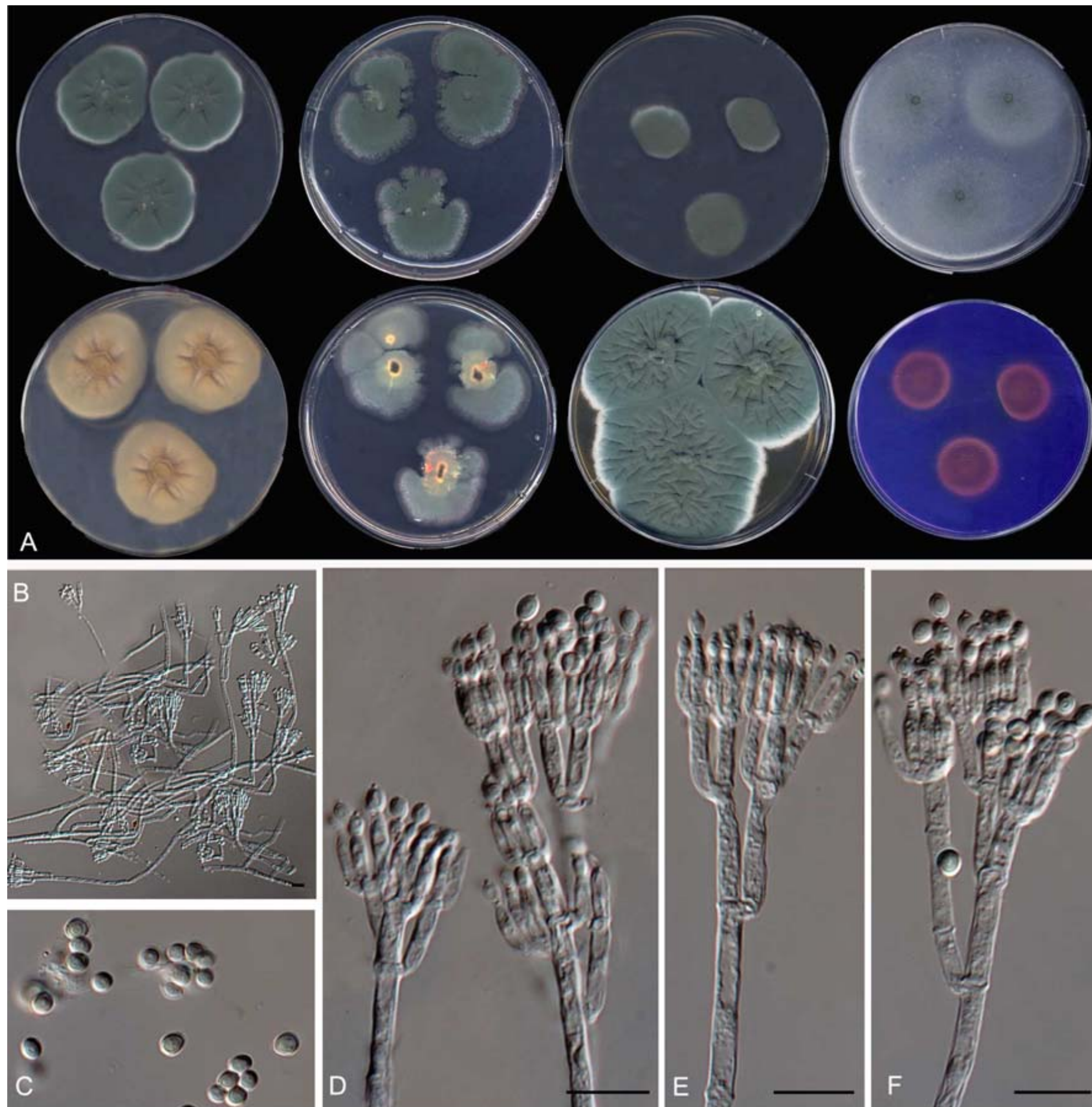


Figure 30.

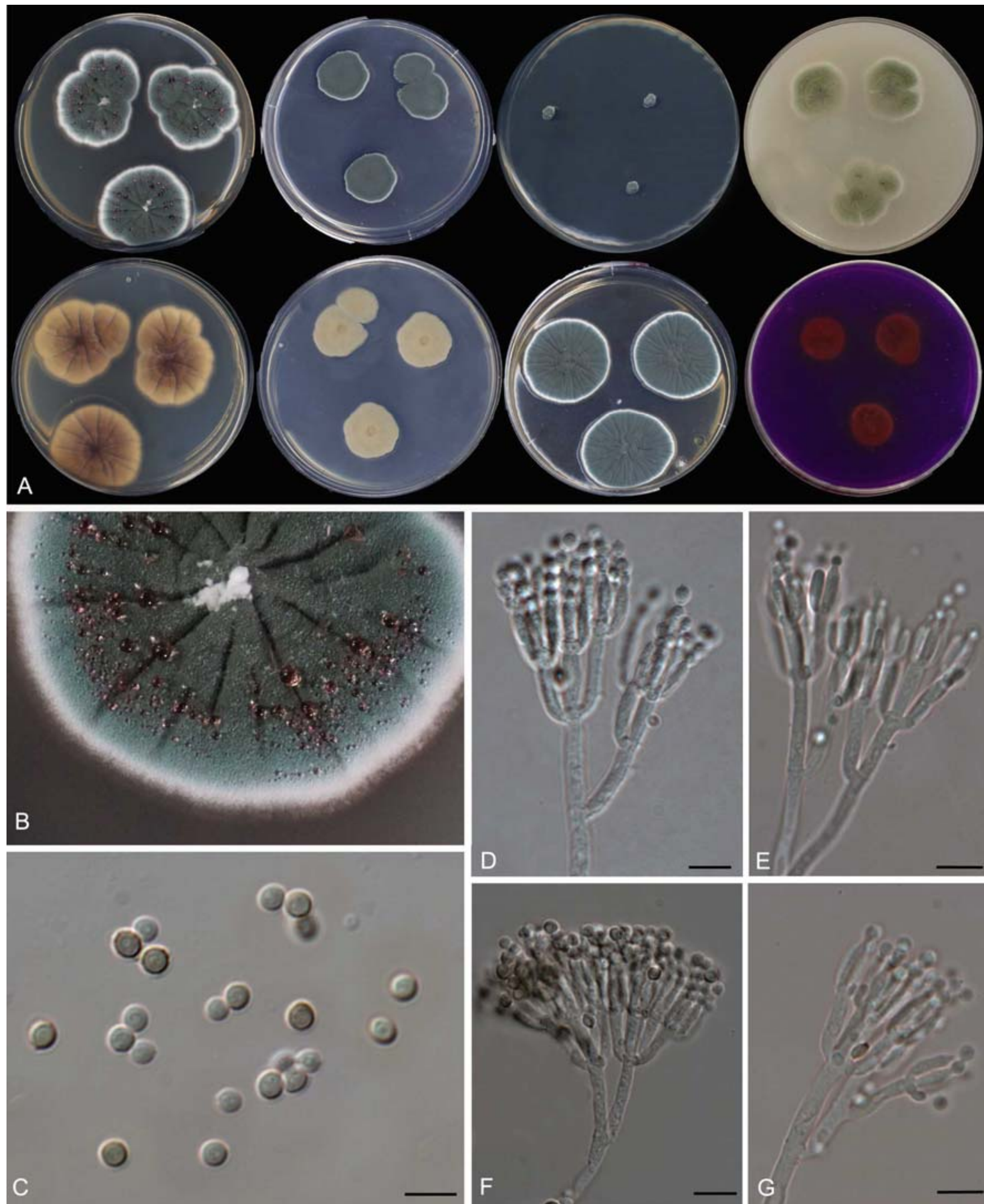


Figure 31.

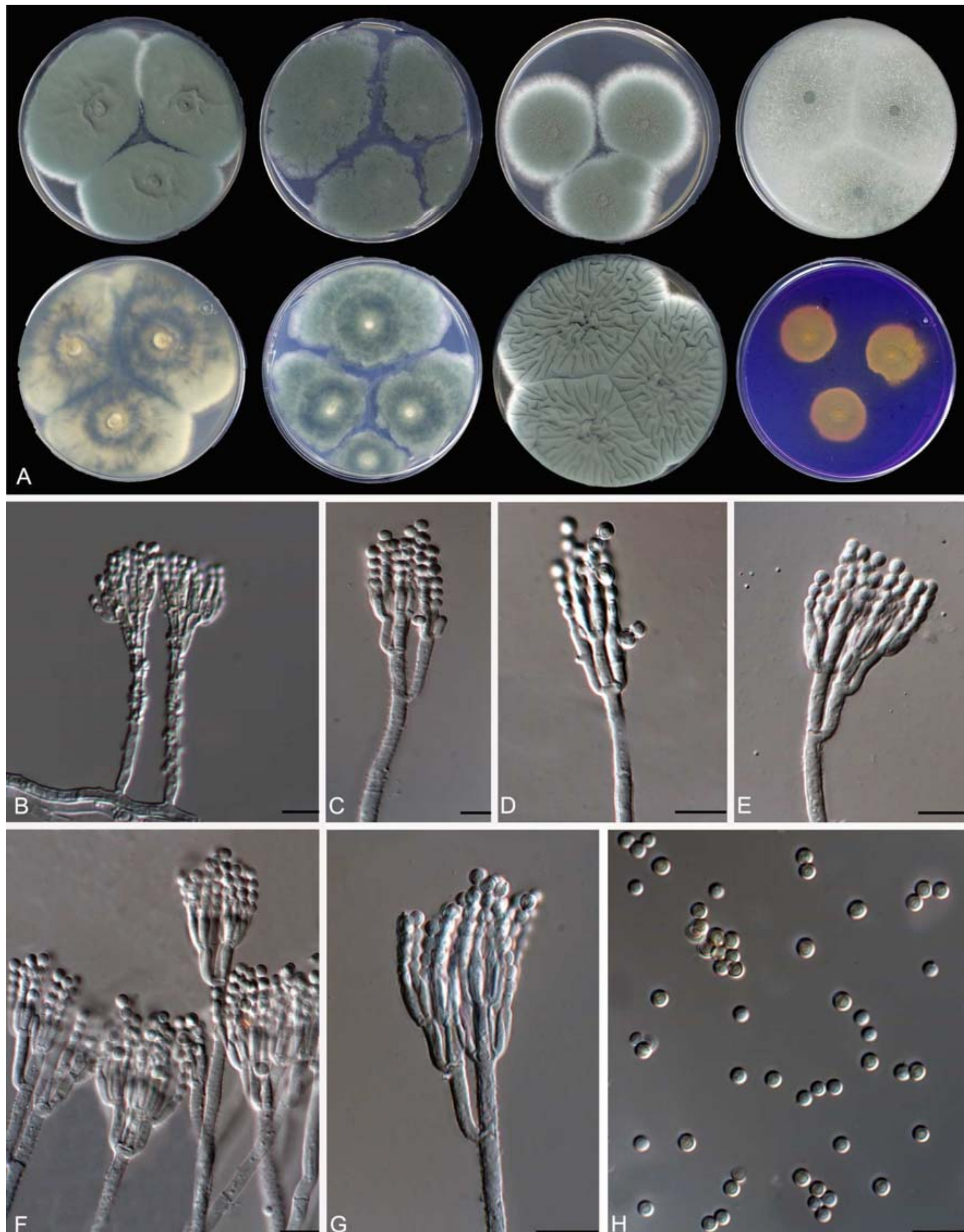


Figure 32.

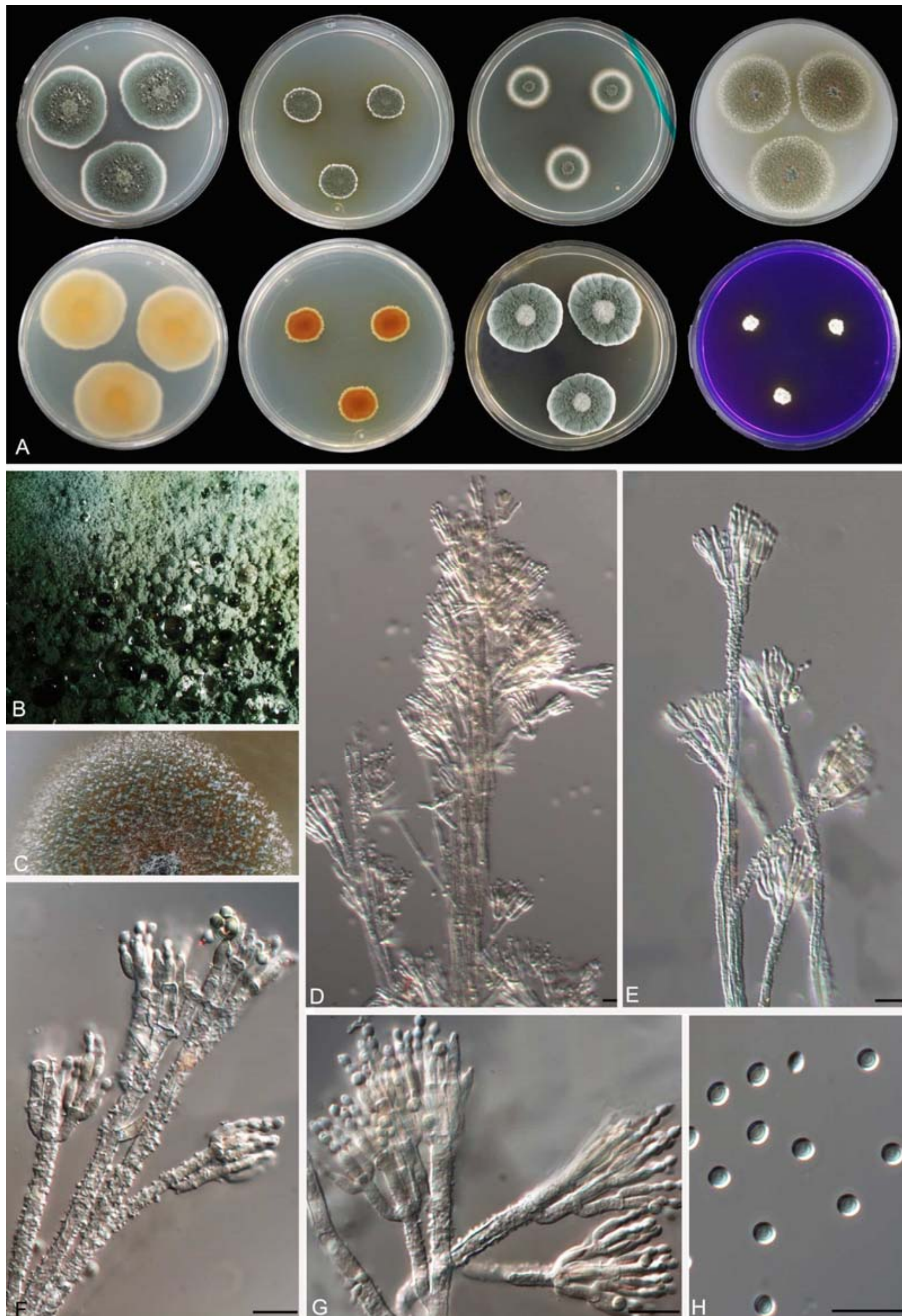


Figure 33.

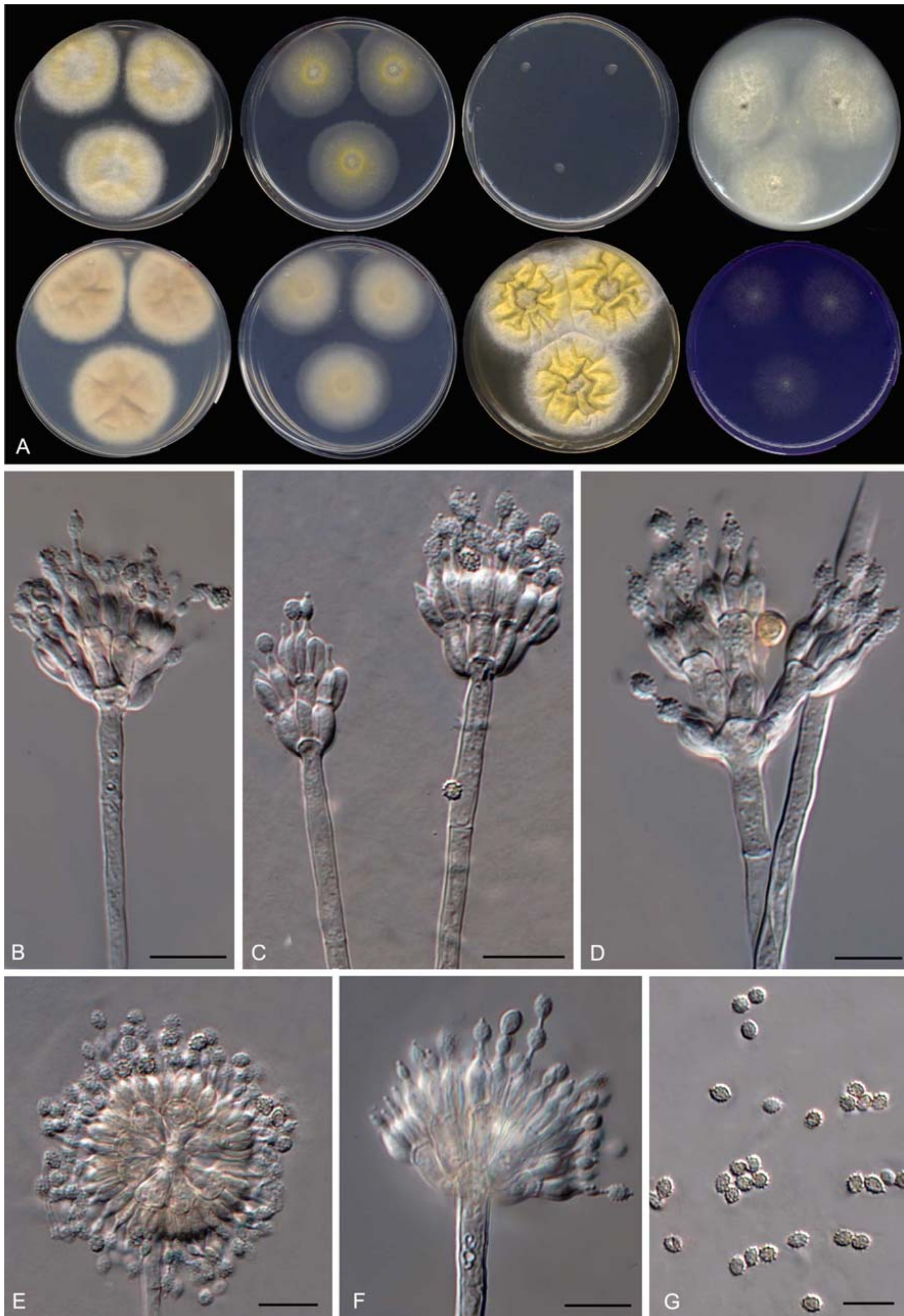


Figure 34.

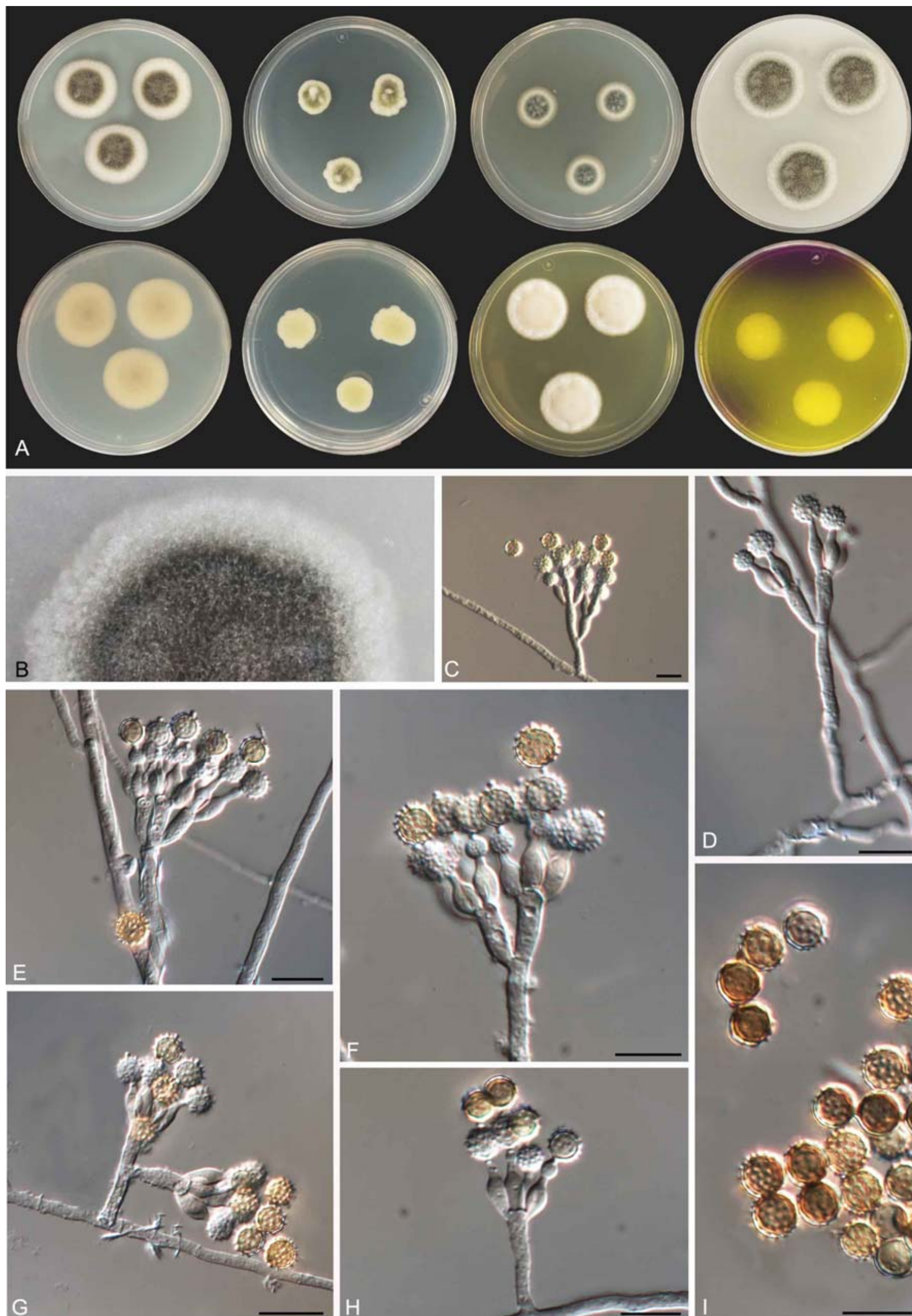


Figure 35.

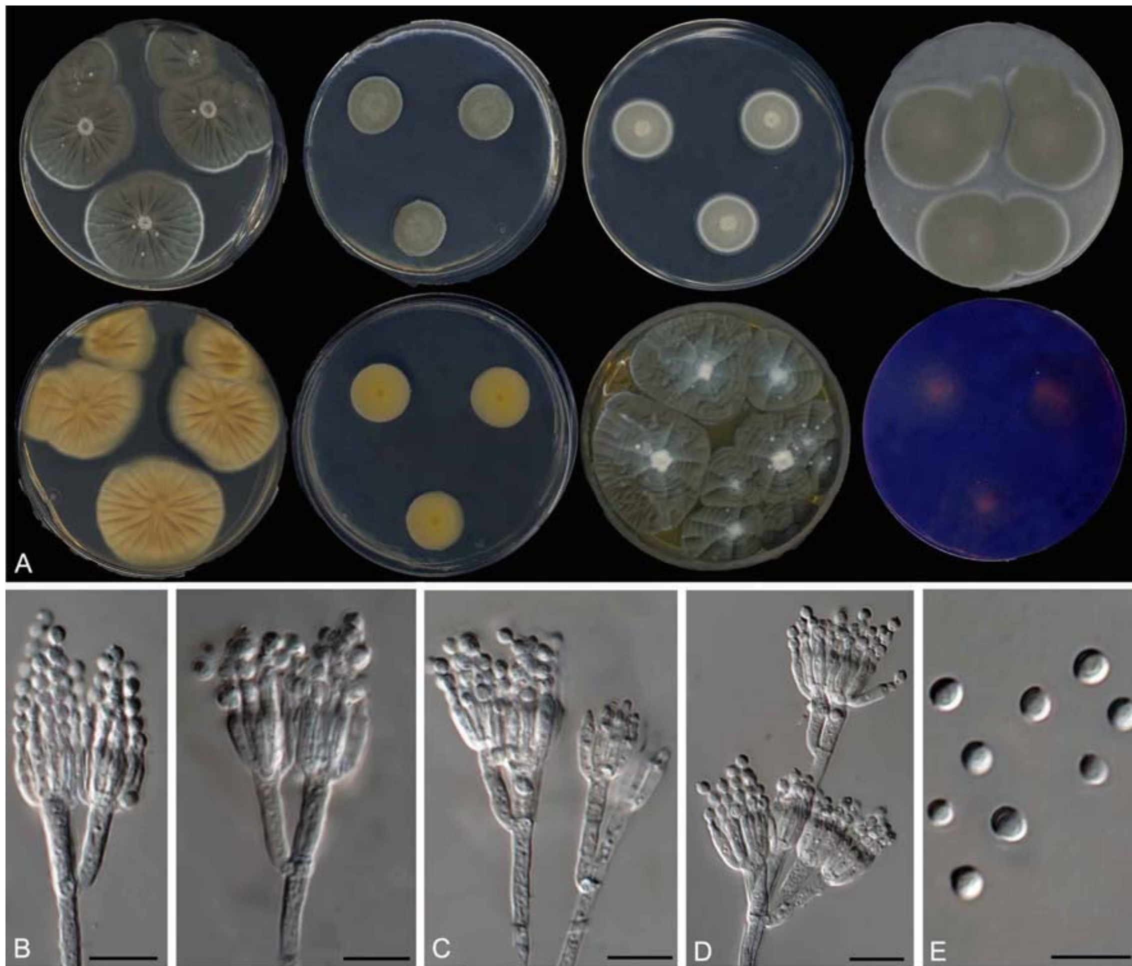


Figure 36.

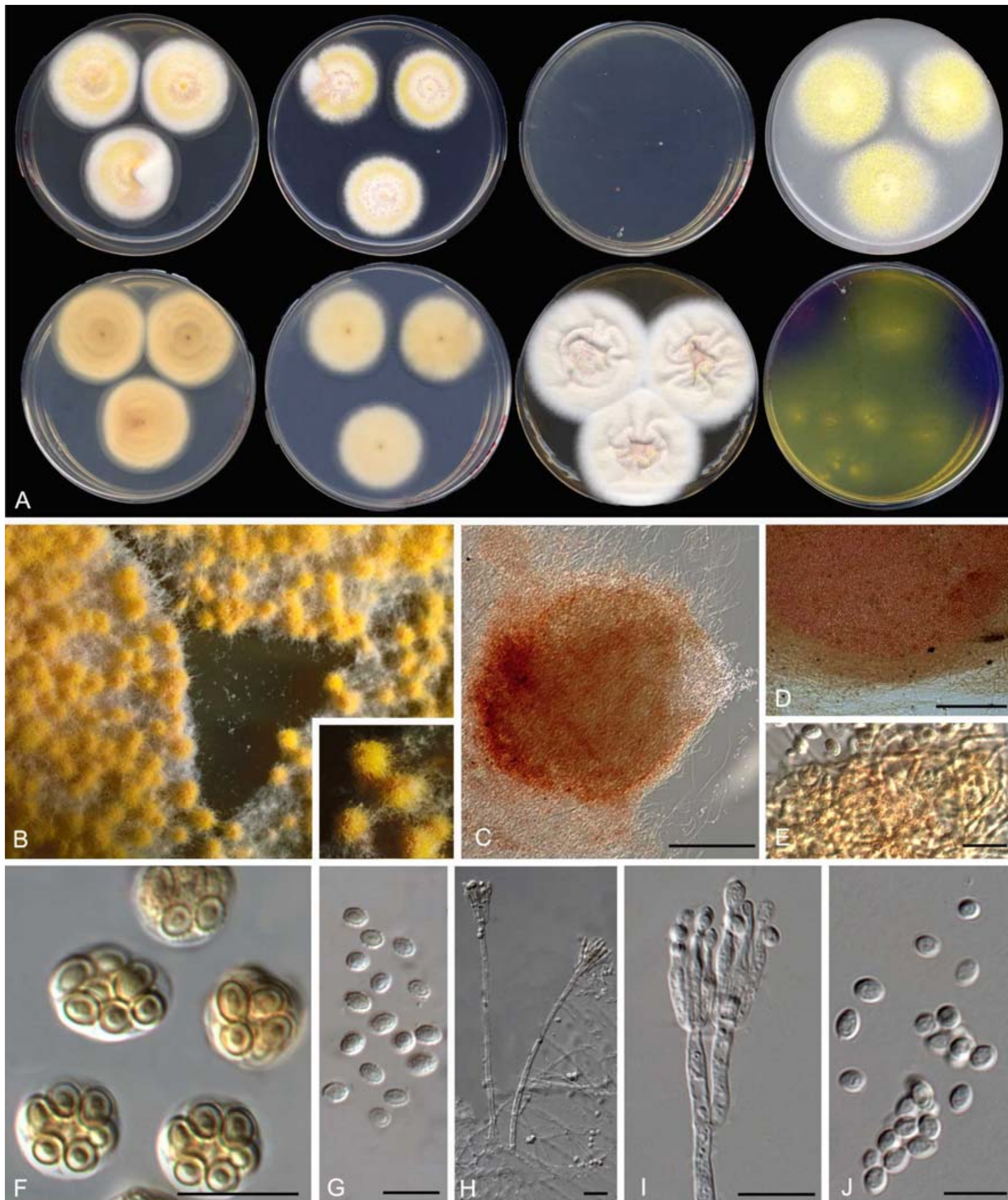


Figure 37.

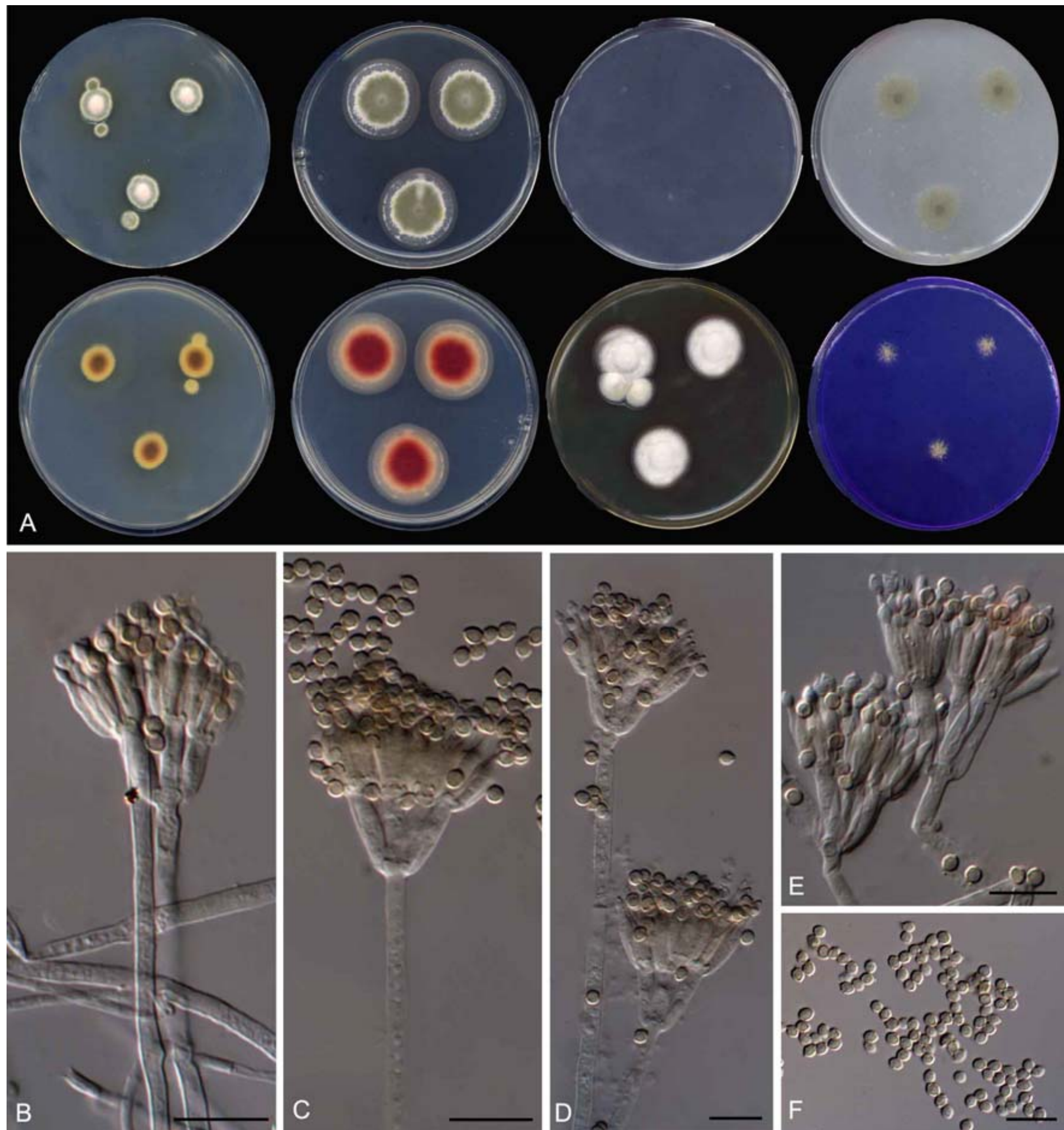


Figure 38.

Figure 39.

SUPPLEMENTARY MATERIAL

Figure S1. ML tree of selected *Aspergillus* section *Terrei* species inferred from *Ben*A, including the isolates belonging to this section recovered in this work. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *A. microcysticus* NRRL 4749 and *A. ambiguus* NRRL 4737. Names in bold are the new species described in this study. ^{T} = type strain.

Figure S2. ML tree of selected *Aspergillus* section *Cremei* species, inferred from ITS. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *A. inflatus* CBS 682.79. The name in bold is the new species described in this study. ^{T} = type strain.

Figure S3. ML tree of selected *Aspergillus* section *Cremei* species, inferred from *Ben*A. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *A. inflatus* CBS 682.79. The name in bold is the new species described in this study. ^{T} = type strain.

Figure S4. ML tree of selected *Aspergillus* section *Cremei* species, inferred from *CaM*. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *A. inflatus* CBS 682.79. The name in bold is the new species described in this study. ^{T} = type strain.

Figure S5. ML tree of selected *Aspergillus* section *Cremei* species, inferred from *RPB*2. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *A. inflatus* CBS 682.79. The name in bold is the new species described in this study. ^{T} = type strain.

Figure S6. ML tree of selected *Aspergillus* section *Cremei* species, inferred from the combined ITS, *Ben*A, *CaM*, and *RPB2* loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *A. inflatus* CBS 682.79. The name in bold is the new species described in this study. ^{T} = type strain.

Figure S7. ML tree of selected *Penicillium* section *Roquefortorum* species, inferred from *Ben*A. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *P. sansonianum* AS 3.5403 and *P. osmophilum* CBS 462.72. Names in bold are the new species described in this study. ^{T} = type strain.

Figure S8. ML tree of selected *Penicillium* section *Robsamsonia* species, inferred from *Ben*A. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *P. brevicompactum* CBS 257.29. Names in bold are the new species described in this study. ^{T} = type strain.

Figure S9. ML tree of selected *Penicillium* section *Paradoxa* species, inferred from *Ben*A. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes.

The tree is rooted to species of section *Tubata*. Names in bold are the new species described in this study. ^{T} = type strain.

Figure S10. ML tree of selected *Penicillium* section *Exilicaulis* species, inferred from *Ben*A, including the isolates belonging to this section recovered in this work. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *P. trzebinskii* CBS 382.48. The isolates identified in the current study are in bold. ^{T} = type strain.

Figure S11. ML tree of selected *Talaromyces* section *Talaromyces* species, inferred from *Ben*A, including the isolates belonging to this section recovered in this work. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The tree is rooted to *T. dendriticus* CBS 660.80 and *T. purpureus* CBS 475.71. Names in bold are the new species described in this study. ^{T} = type strain.

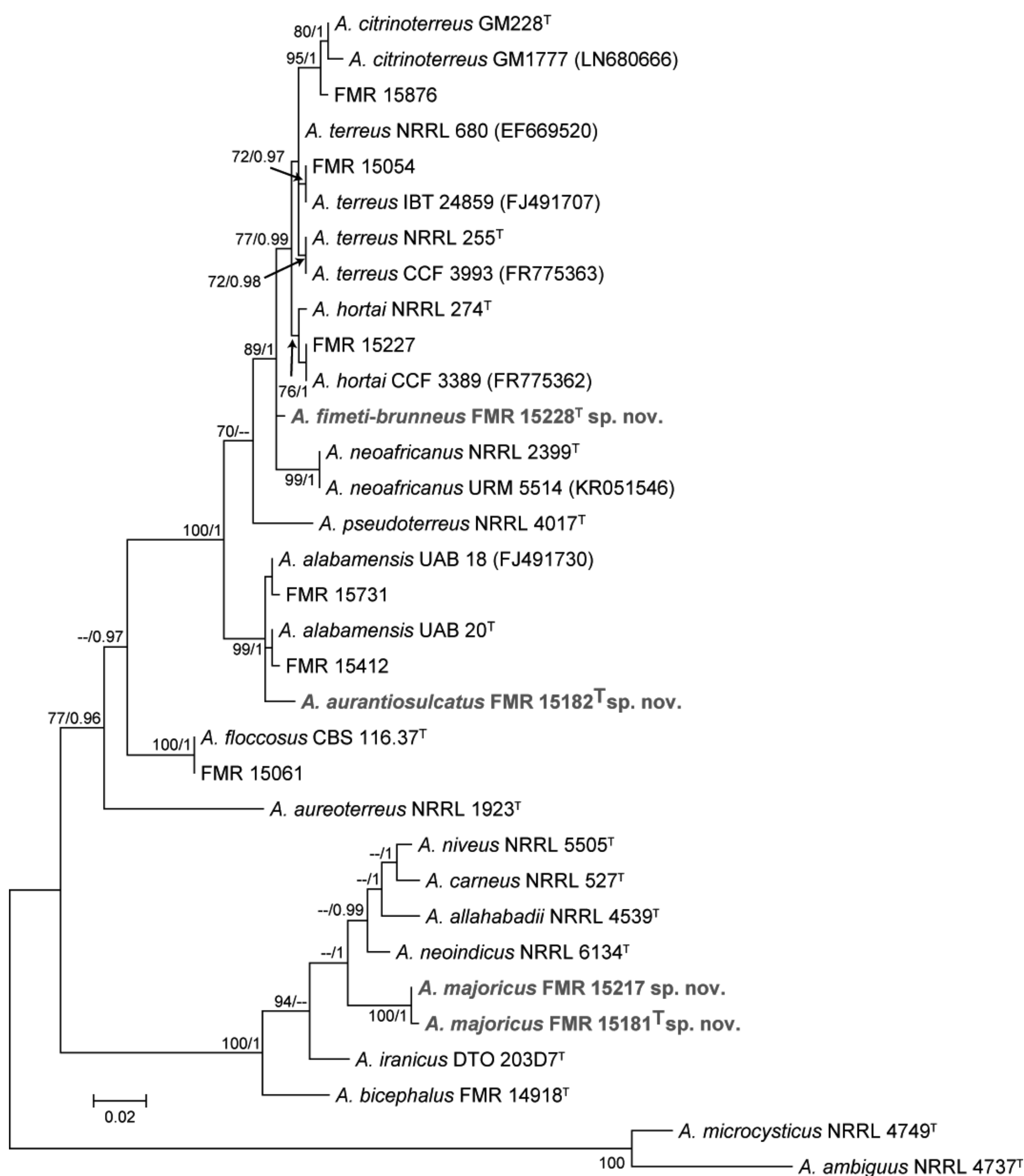


Figure S1.

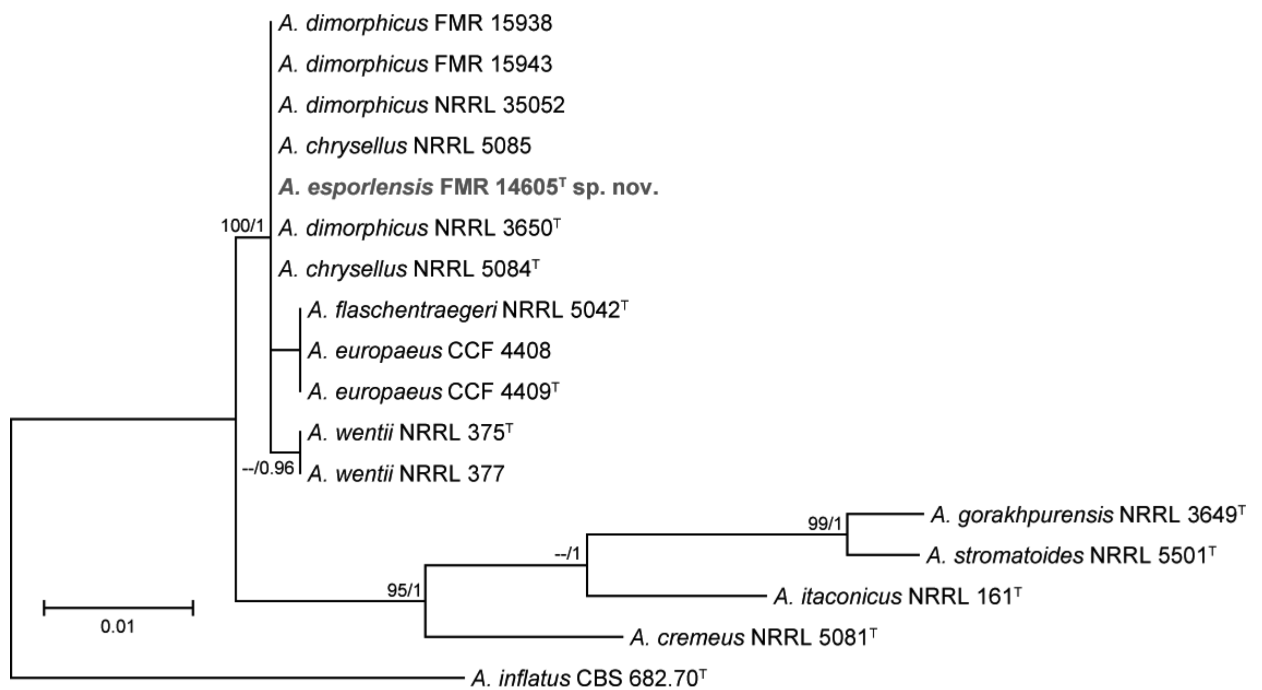


Figure S2.

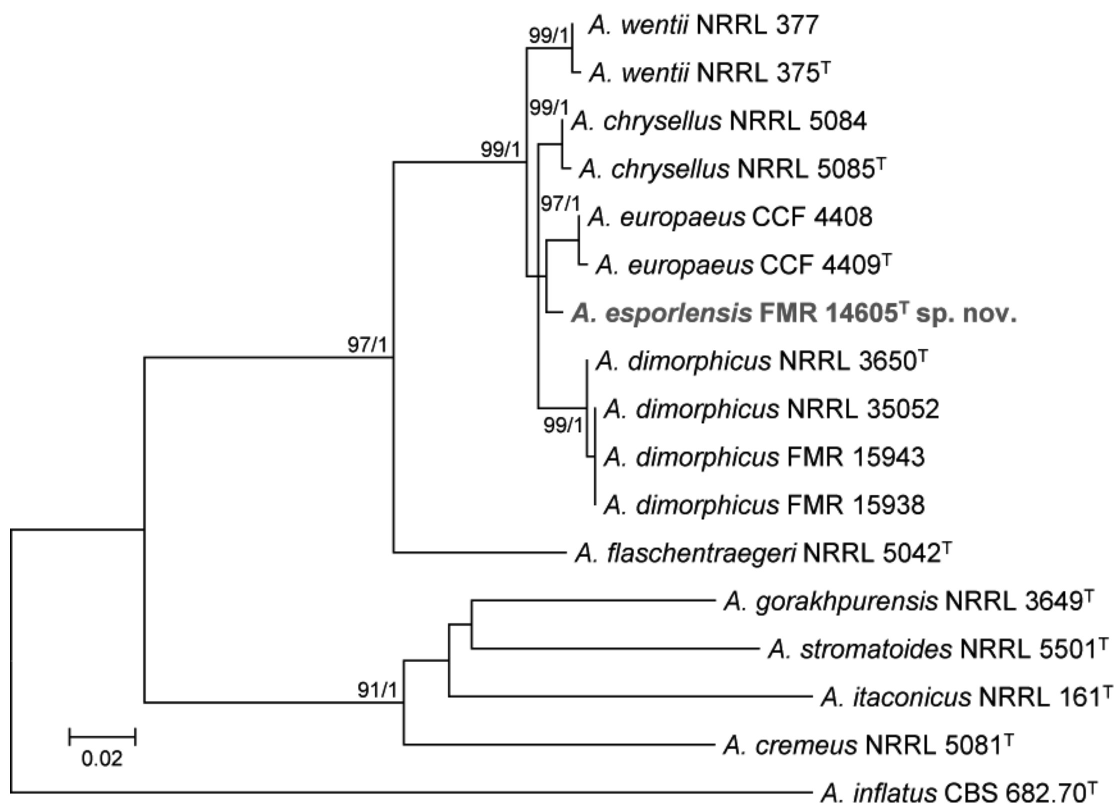


Figure S3.

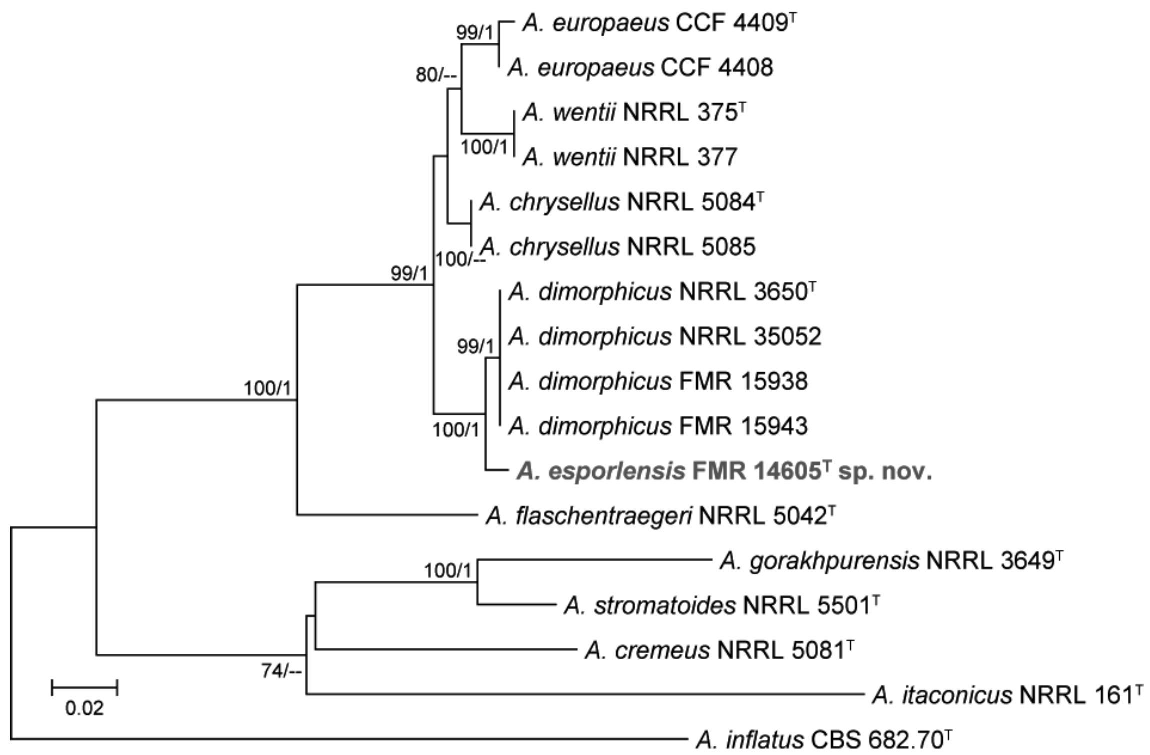


Figure S4.

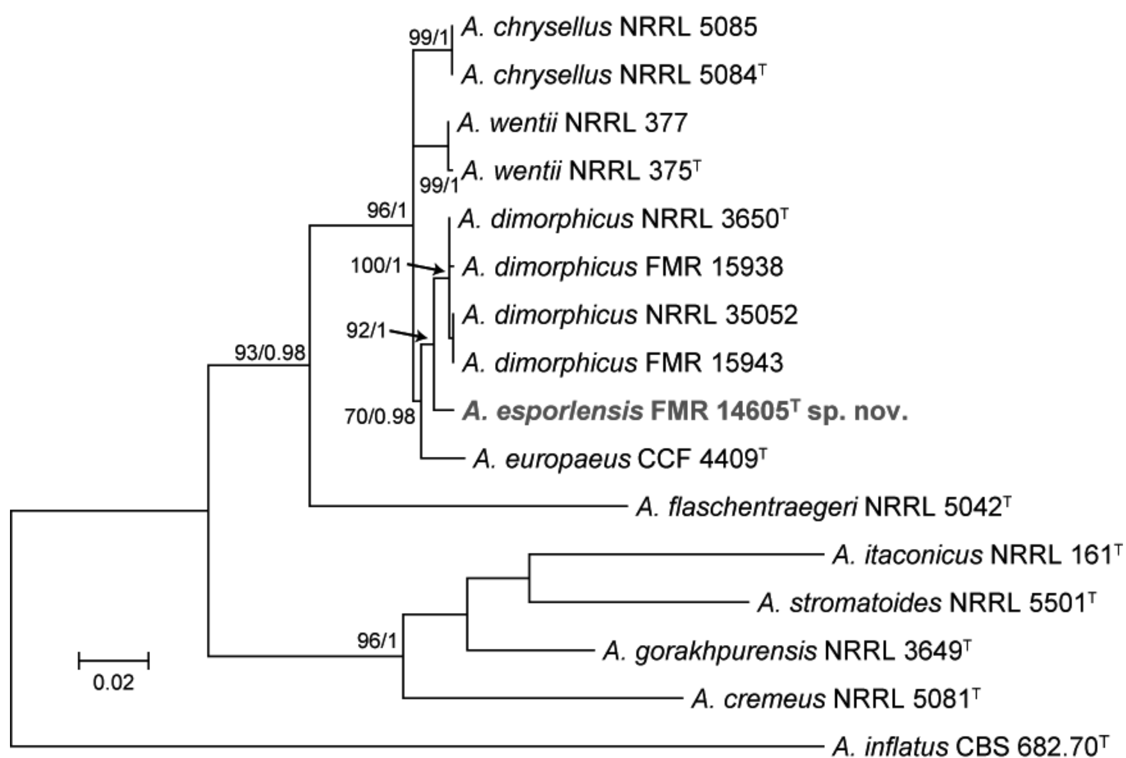


Figure S5.

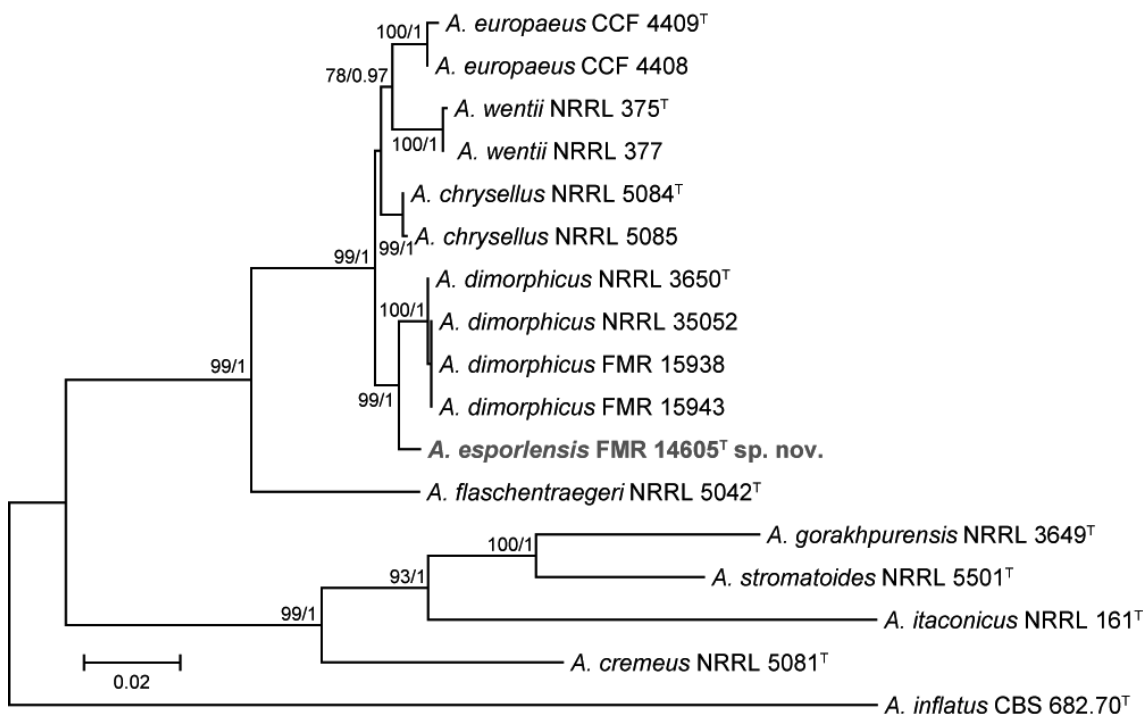


Figure S6.

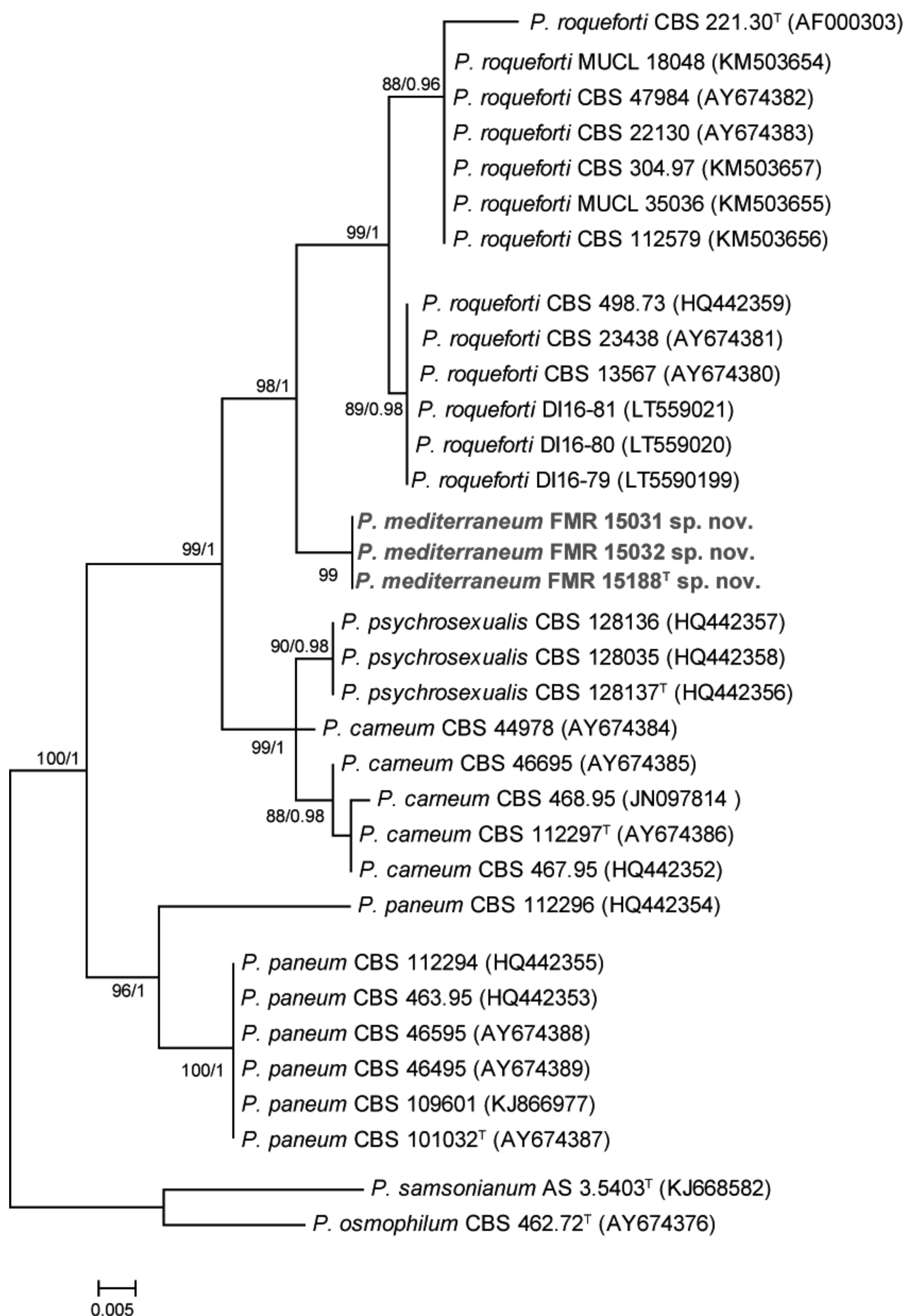


Figure S7.

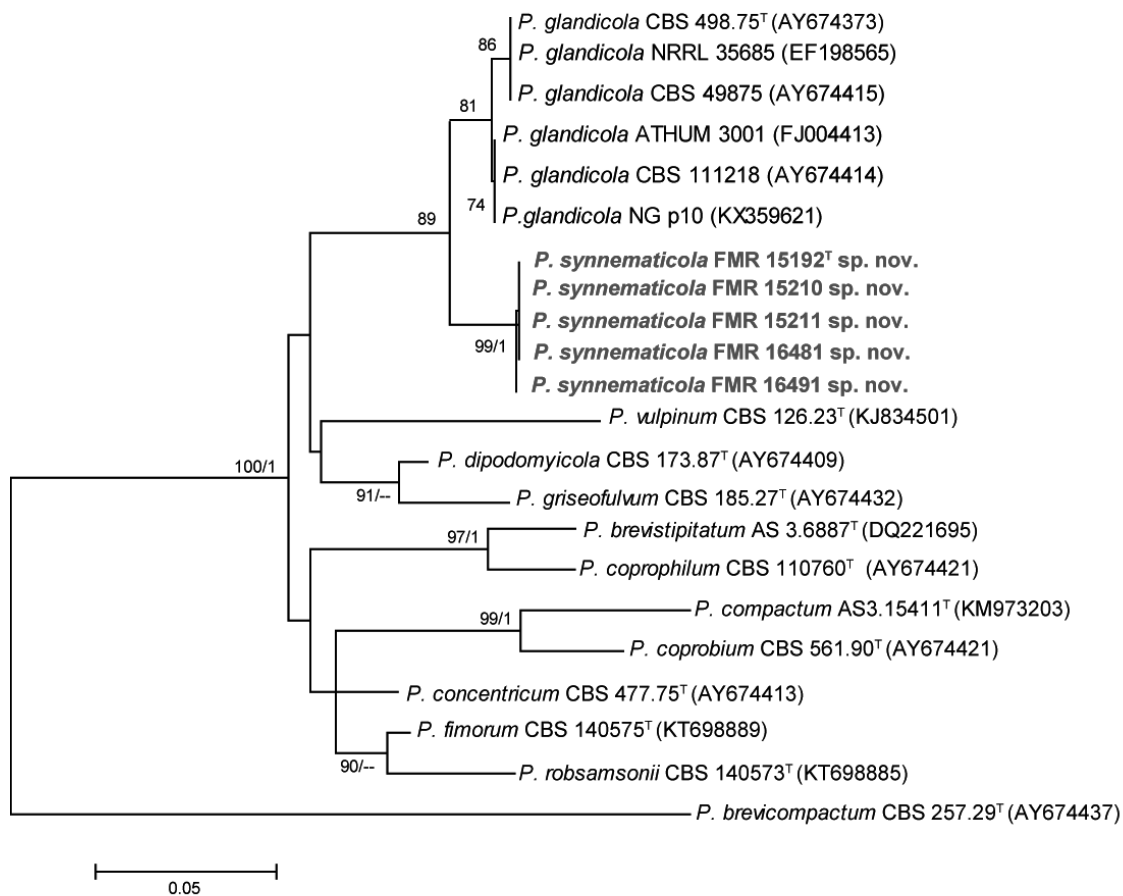


Figure S8.

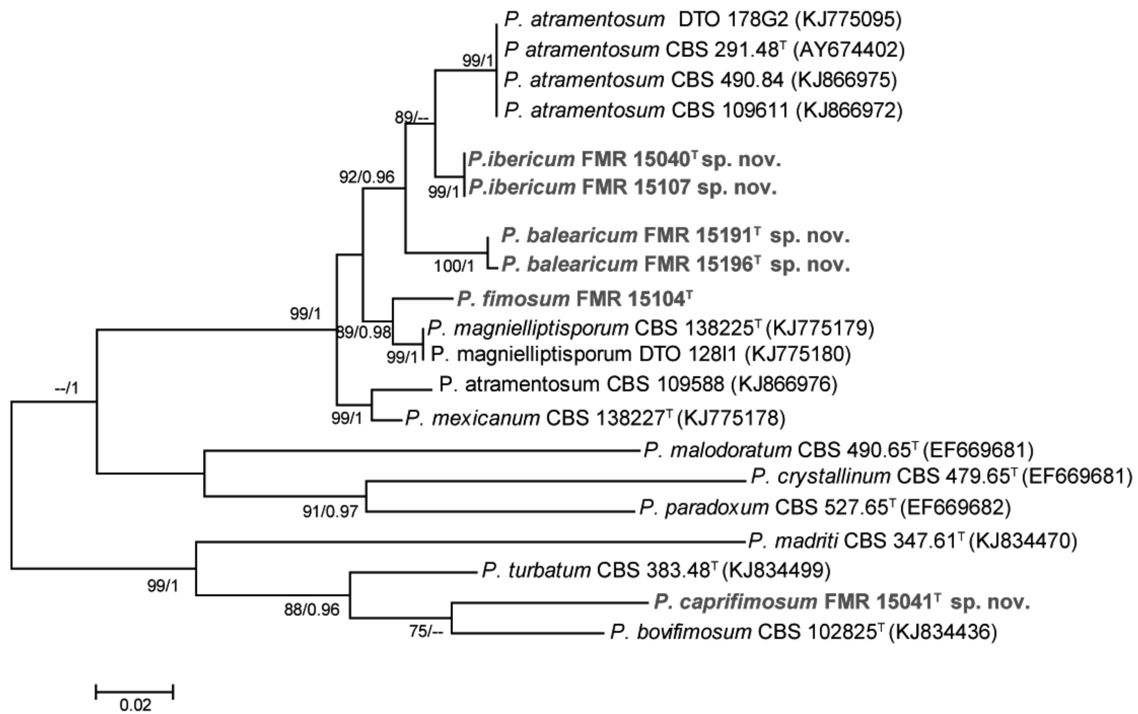


Figure S9.

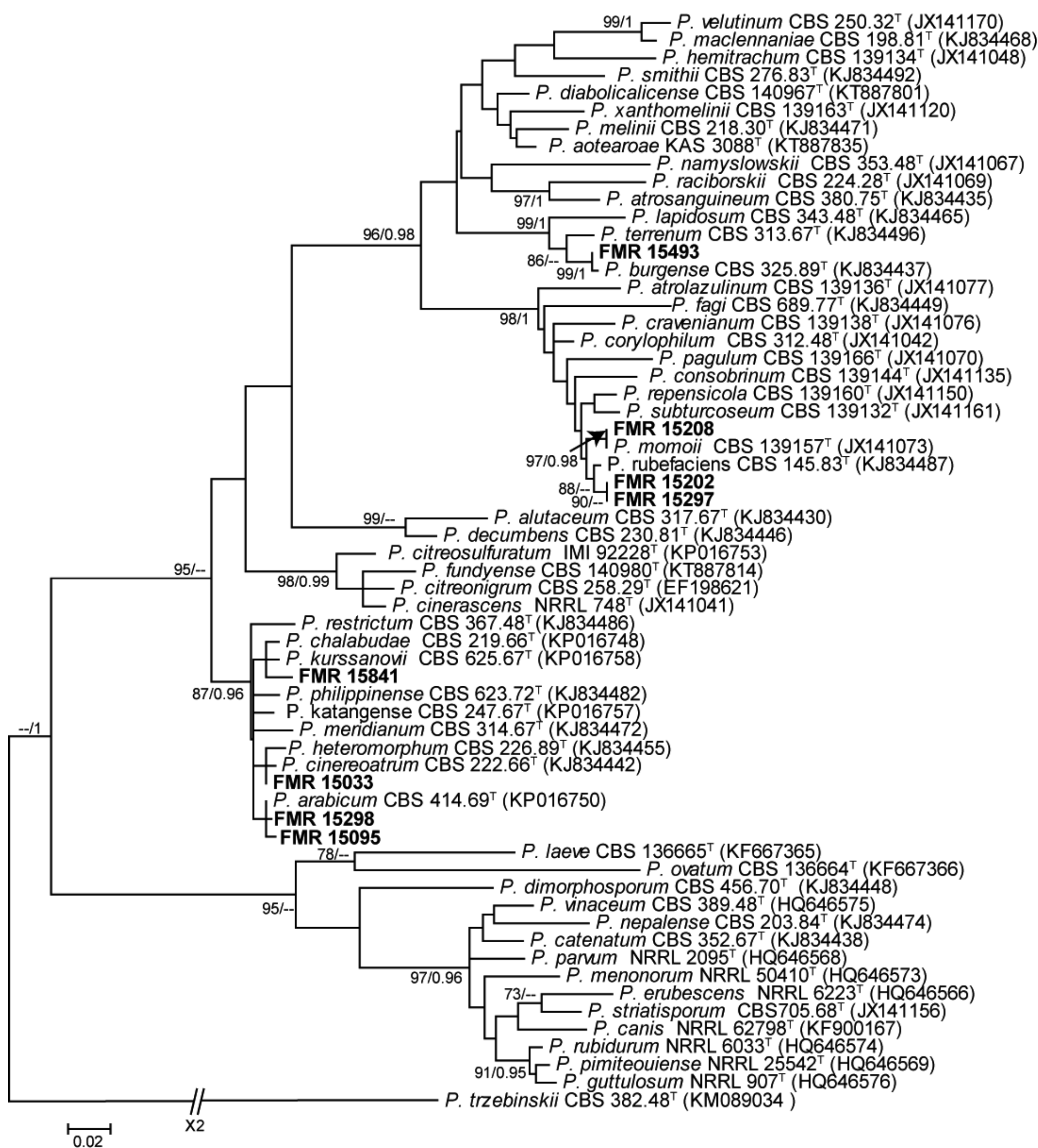


Figure S10.

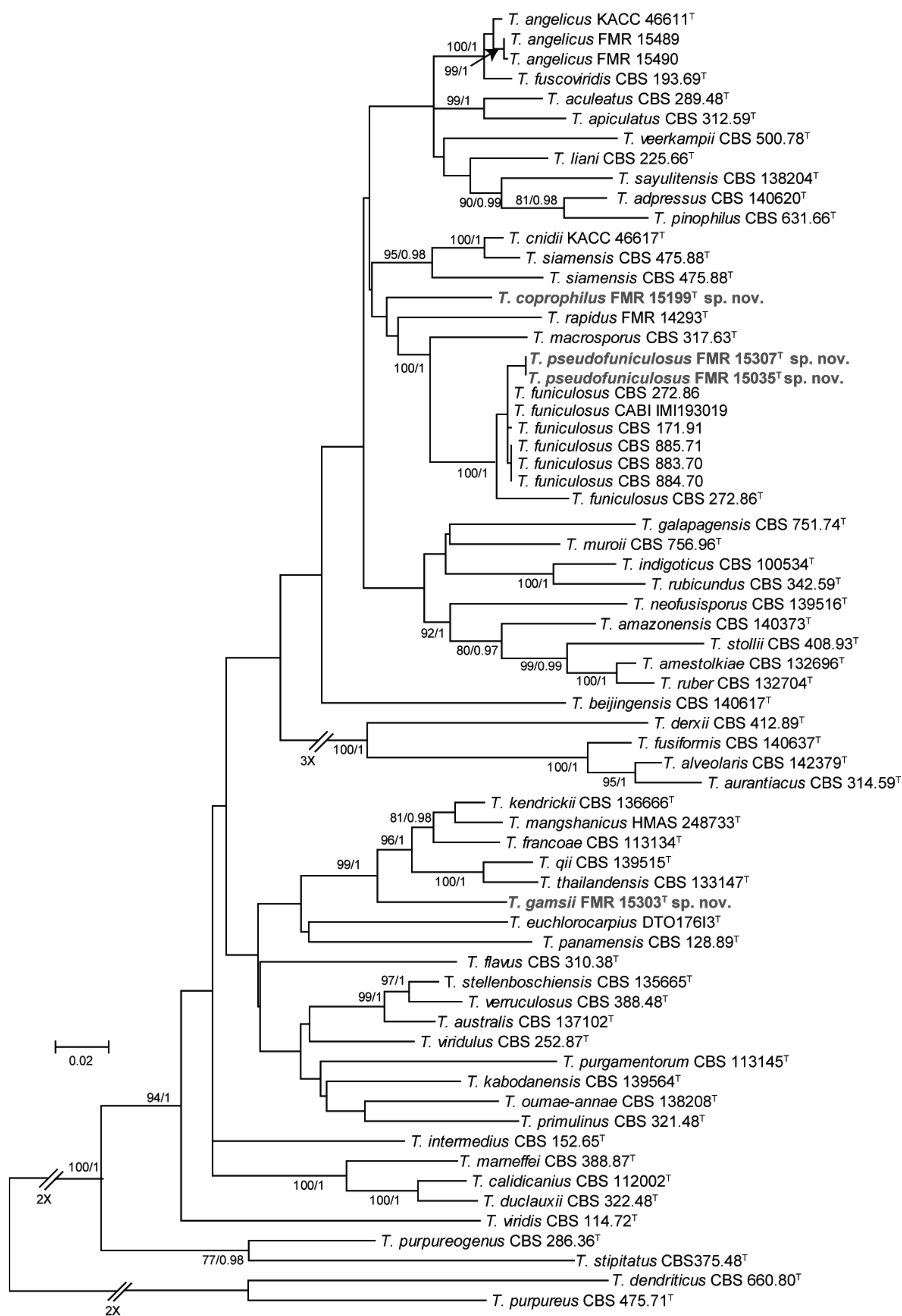


Figure S11.

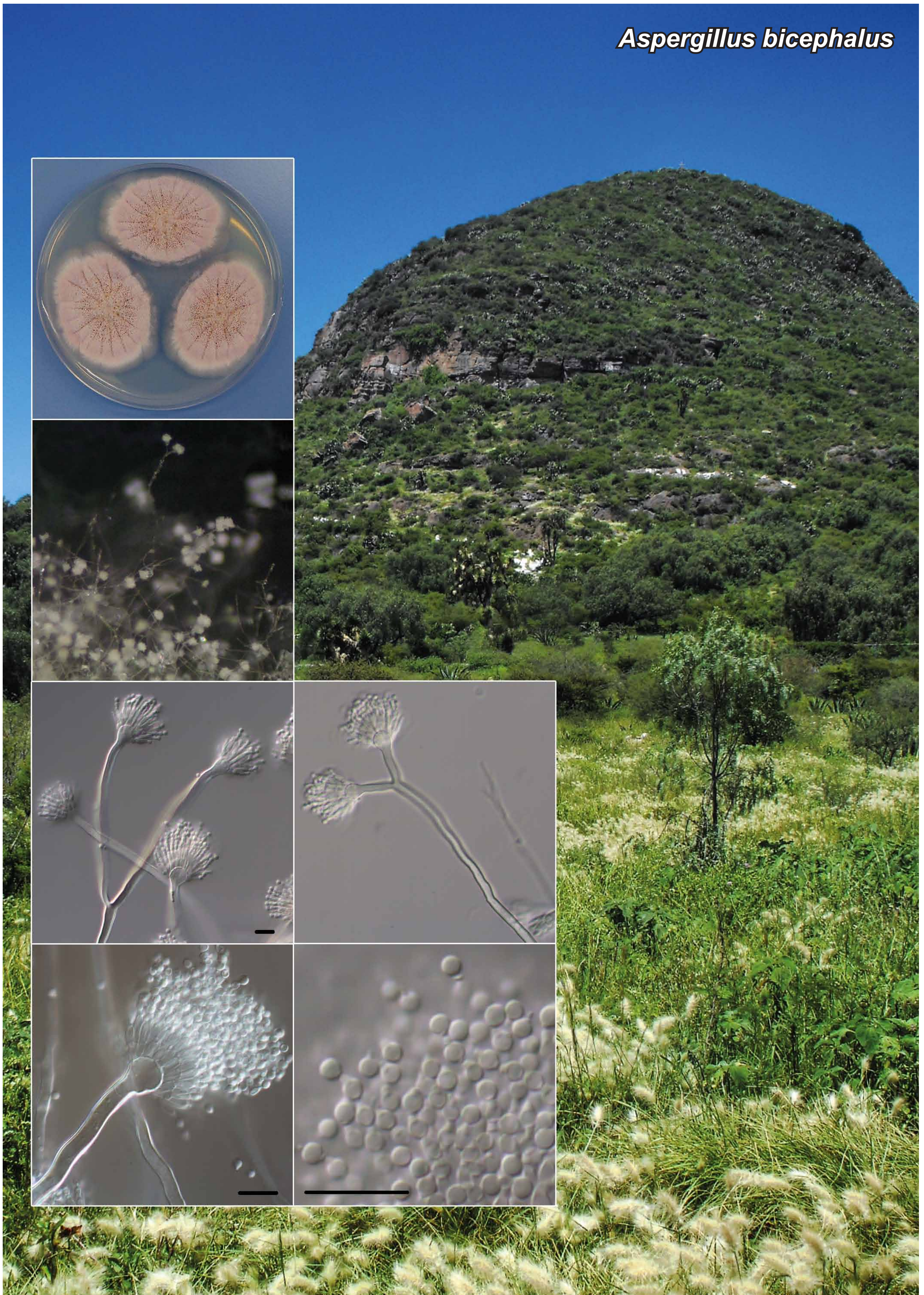
4.6. New species of *Aspergillus* from soil

4.6.1. Aspergillus bicephalus sp. nov.

João Paulo Zen Siqueira, Josepa Gené, Dania García, Josep Guarro

Published in: Fungal Planet description sheets, Journal Persoonia
December 2016, Volume 37, Pages 282–283

UNIVERSITAT ROVIRA I VIRGILI
CLINICAL AND ENVIRONMENTAL ASPERGILLUS: MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION, PHYLOGENY,
AND ANTIFUNGAL SUSCEPTIBILITY PROFIL·LE
Joao Paulo Zen Siqueire


Aspergillus bicephalus

Fungal Planet 497 – 21 December 2016

Aspergillus bicephalus J.P.Z. Siqueira, Gené & Guarro, *sp. nov.*

Etymology. Name refers to the production of conidiophores with two conidial heads.

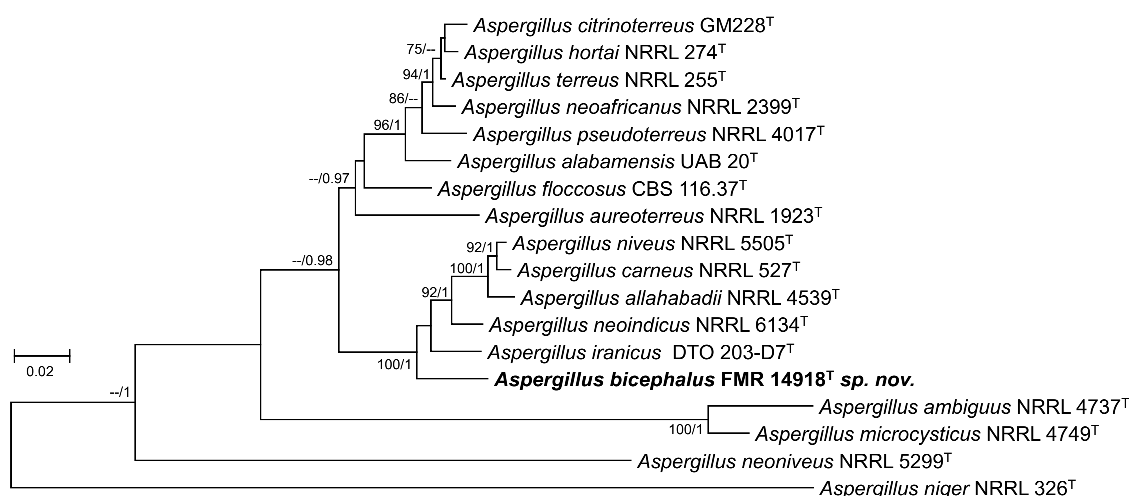
Classification — *Aspergillaceae*, *Eurotiales*, *Eurotiomycetes*.

Conidiophores on MEA hyaline, septate, smooth, thick-walled, often bifurcately branched, producing a terminal conidial head on each branch, 300–950 × 3–9.5 µm. *Conidial heads* columnar, biseriate. *Vesicles* subglobose or pyriform, 8–12 µm wide. *Metulae* cylindrical, covering about 2/3 of the vesicle, 3.5–6.5 × 2–3 µm. *Phialides* ampulliform, 5–8.5 × 2–3 µm. *Conidia* globose to ellipsoidal, hyaline, smooth-walled, 2–2.5 × 1.5–2.5 µm.

Culture characteristics — (in the dark, at 25 °C after 7 d): Colonies on CYA attaining 28–31 mm diam, colony texture velvety to powdery with a floccose centre, sulcate, sporulation strong with conidial mass reddish white (8A2) (Kornerup & Wanscher 1978); reverse light yellow (4A4); exudate colourless; soluble pigments absent. On MEA reaching 20–24 mm diam, velvety to densely floccose, with an elevated centre, sporulation strong with conidial mass reddish white (8A2) to pale red (7A3), mycelium white to yellowish white (4A2) towards the periphery; reverse yellowish white (4A2) to light yellow (4A4); exudate colourless; soluble pigments absent. On YES reaching 32–34 mm diam, velvety to floccose with elevated centre, sulcate, conidial mass white to reddish white (8A2); reverse light yellow (4A4) to greyish yellow (4B4); exudate absent; soluble pigments absent. On OA reaching 19–20 mm diam, colony texture powdery to slightly granular, strong sporulation in centre, conidial mass white; reverse colourless; exudate absent; soluble pigments absent. On CREA, 18–19 mm diam, velvety to powdery, strong sporulation in the centre with conidial mass pale red (9A3), white towards the periphery; acid production absent, even after 14 d. Colonies, mainly on MEA, CYA and YES showing darker shades of rose (11A3 to 11A5) after 14 d. On CYA after 7 d, the colonies reached up to 7.5 mm diam at 15 °C, 44–45 mm diam at 30 °C, 44–47 mm diam at 37 °C, and 32–37 mm diam at 40 °C; growth absent at 45 °C.

Typus. Mexico, Ecatepec de Morelos, from soil, June 2015, coll. *E. Rosas*, isol. *J. Siqueira* (holotype CBS H-22807, culture ex-type FMR 14918; DNA barcode: ITS LT601380 (other barcodes: LSU LT630488; β-tubulin (*BenA*) LT601381; calmodulin (*CaM*) LT601382; RNA polymerase II second largest subunit gene (*RPB2*) sequence LT601383, MycoBank MB818290).

Notes — A BLAST search of the GenBank nucleotide database shows that the sequences of *A. bicephalus* are unique for all the tested markers and confirms that it belongs to *Aspergillus* sect. *Terrei*. This species is clearly differentiated from the others of the section by its bifurcate conidiophores with a terminal conidial head on each branch and by the reddish colour of the conidial mass. *Aspergillus carneus*, a species of the section with similar macroscopic features, has paler colonies, and its vesicles and conidia are slightly larger (9–15 µm wide and 2.5–3 µm diam, respectively) (Klich 2002). The closest phylogenetically related species are *A. iranicus* and *A. neoindicus*. However, the former has white colonies which change to peach after 3 wk and produces accessory conidia (Arzanlou et al. 2016), and the latter species has colonies with yellow-green mycelial tufts and conidiophores with spatulate vesicles (Samson et al. 2011).



Maximum Likelihood tree inferred from the combined ITS, *BenA* and *CaM* regions from all the type strains (^{T}) of the species currently accepted in *Aspergillus* sect. *Terrei*, rooted to *Aspergillus niger* NRRL 326 (section *Nigri*). Maximum likelihood bootstrap support values ≥ 70 % (MEGA v. 6) and Bayesian posterior probabilities ≥ 0.95 (MrBayes v. 3.1.2) are displayed at the nodes. The novel species is indicated in **bold** face.

Colour illustrations. Hill of Ecatepec de Morelos, Mexico State (available at https://www.flickr.com/photos/13383617@N05); 14-d-old colony on CYA showing the characteristic colours of the mature conidial heads, closer look of the conidial heads, detailed conidiophores showing the bifurcation to form two conidial heads, conidia. Scale bars = 10 µm.

João Paulo Zen Siqueira, Josepa Gené, Dania García & Josep Guarro, Mycology Unit, Medical School and IISPV, Universitat Rovira i Virgili (URV), Sant Llorenç 21, 43201 Reus, Tarragona, Spain;
e-mail: jpzensiqueira@yahoo.com.br; josepa.gene@urv.cat; dania.garcias@urv.cat & josep.guarro@urv.cat

© 2016 Naturalis Biodiversity Center & Centraalbureau voor Schimmelcultures

4.6.2. *Aspergillus subglobosus* sp. nov.

In preparation to be submitted as part of the article: “New species of *Aspergillus* from South American soils”

Aspergillus subglobosus J.P.Z. Siqueira, Gené, García & Guarro **sp. nov.** MycoBank MBXXXX. Figure 11.

Etymology — Name refers to the typical shape of the vesicles.

In — Section *Terrei*

Specimen examined — Brazil, Sao Paulo, Sao Jose do Rio Preto, from soil, 2014, J.P.Z. Siqueira (**holotype** CBS H-XXXX; culture ex-type FMR 15381 = CBS XXXX; ITS barcode LT903689, alternative markers: *BenA* LT903680, *CaM* LT903683, *RPB2* LT903686).

Colony diameter in 7d (mm) — On CYA: 25 ºC 42–46, 30 ºC 54–60, 37 ºC 60–61; on MEA: 25 ºC 23–24; on DG18: 25 ºC 46–48; on YES: 25 ºC 68–>70; on OA: 25 ºC 19–21; on CREA: 25 ºC 18–21.

Colony characters at 25 ºC in 7 d — On CYA, colonies floccose to powdery, slightly radially sulcate, mycelium white, margin entire and slightly fimbriate; reverse pale yellow (4A3) to greyish orange (5B5); sporulation dense; with conidial masses white to brownish orange (5C5); soluble pigment absent; exudate absent. On MEA, colonies floccose, mycelium white to yellowish grey (4B2), margin predominately entire; reverse greyish orange (6B6) to brown (6D6); sporulation dense, with conidial masses white to brown (5E4); soluble pigment light orange (5A5) weakly produced; exudate colorless. On DG18, colonies loosely floccose, mycelium white, margin predominately entire and with submerged mycelium; reverse yellowish white (3A2) to light yellow (4A4); sporulation moderately dense, with conidial masses pale (2A2) to light yellow (3A4); soluble pigment absent; exudate absent. On YES, colonies floccose, irregularly sulcate, mycelium white, margin entire; reverse orange (5A7); sporulation dense; with conidial masses white to light brown (5D4); soluble pigment absent; exudate absent. On OA, colonies powdery, mycelium white, margin entire and with submerged mycelium; reverse light yellow (3A4 to 4A5); sporulation dense, with conidial masses pale yellow (3A3) to dull yellow (3B3); soluble pigment absent; exudate absent. On CREA, powdery, with slightly elevated center, mycelium white, margin irregular;

sporulation moderately dense, with conidial masses yellowish white (4A2); acid production absent.

Micromorphology — On MEA, conidiophores with conidial heads biseriate, columnar, in shades of brown; stipes commonly aseptate, 180–400 x 4.5–7 µm, smooth, hyaline; vesicles mostly subglobose, 7–17 µm wide, hyaline; metulae cylindrical, covering 50% to 75% of the vesicle, 5–8 x 1.5–3 µm, hyaline; phialides cylindrical, with the apex slightly narrower, 4.5–8 x 1.5–3 µm, hyaline; conidia globose to subglobose, 1.5–3.5 µm, smooth, hyaline. Accessory conidia not observed. Ascomata not observed.

Differential diagnosis — The species more closely related to *A. subglobosus* are *A. alabamensis* and *A. aurantiosulcatus,* this latter recently described by Guevara-Suarez et al. (in press) (Figure 12). The three species are morphologically very similar and an outstanding feature in all of them is the profuse growth at 37 ºC. The main phenotypic characteristic that allows to distinguish these species is the production of soluble pigment on CYA at 25 ºC in *A. alabamensis* and *A. aurantiosulcatus* (Balajee et al. 2009b; Guevara-Suarez et al. in press), which is absent in *A. subglobosus*.

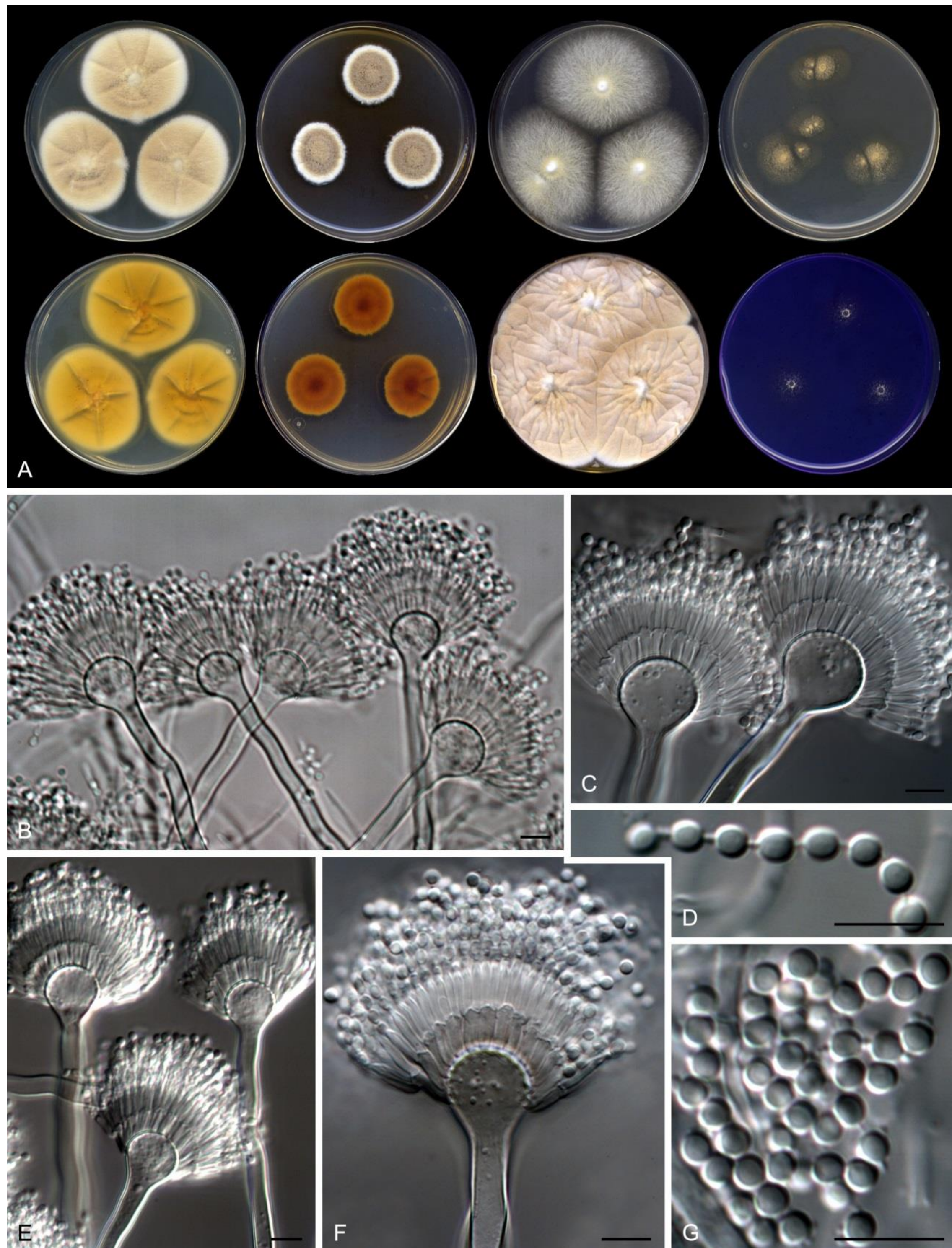


Figure 11. Morphological characters of *Aspergillus subglobosus* (FMR 15381^{T}). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B, C, E, F. Conidiophores. D, G. Conidia. Scale bars = 10 µm.

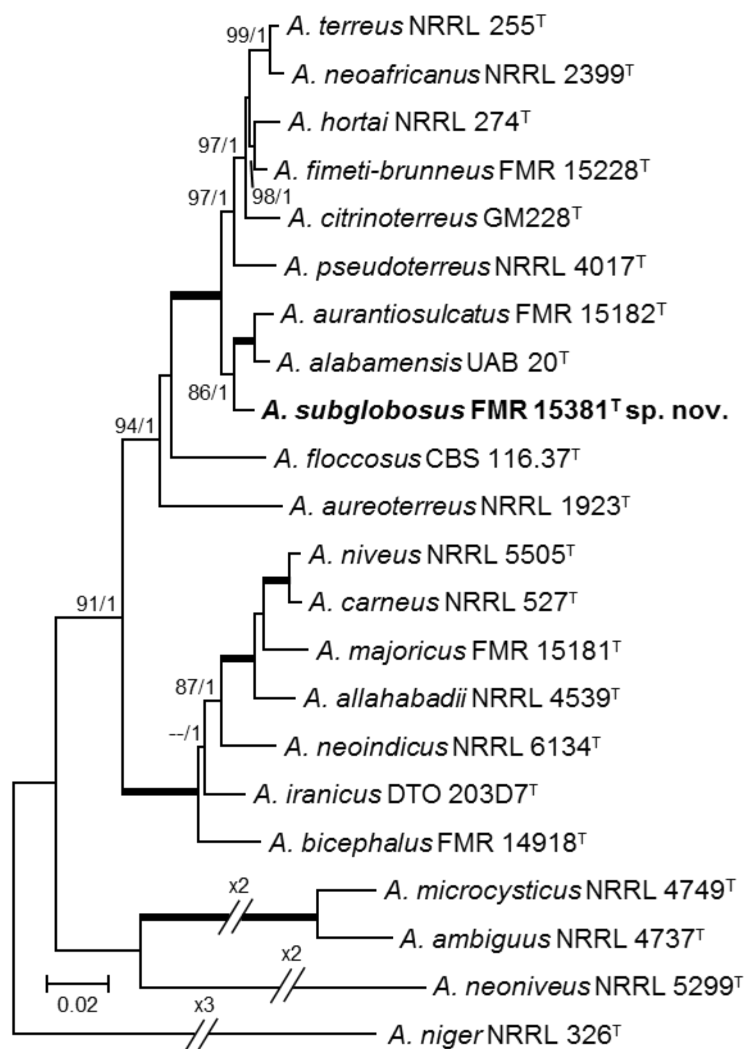


Figure 12. ML tree of *Aspergillus* section *Terrei* inferred from the combined ITS, *Ben*A, *CaM*, and *RPB*2 loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. Thickened branches correspond to full supported clades (100/1). The tree is rooted to *A. niger* NRRL 326. The name in bold represent the new species. ^{T} = type strain.

4.6.3. *Aspergillus argentinensis* sp. nov.

In preparation to be submitted as part of the article: “New species of *Aspergillus* from South American soils”

Aspergillus argentinensis J.P.Z. Siqueira, Gené, García & Guarro, **sp. nov.** MycoBank MBXXXX. Figure 13.

Etymology — Name refers to the country of origin of the isolate.

In — Section *Nidulantes*

Specimen examined — Argentina, from soil, 2016, A. M. Stchigel (**holotype** CBS H-XXXX; culture ex-type FMR 15740 = CBS XXXX; ITS barcode LT903690, alternative markers: *BenA* LT903681, *CaM* LT903684, *RPB2* LT903687).

Colony diameter in 7d (mm) — On CYA: 25 ºC 27–29, 30 ºC 26–28, 37 ºC no growth; on MEA: 25 ºC 21–22; on DG18: 25 ºC 16–18; on YES: 25 ºC 35–37; on OA: 25ºC 10–15; on CREA: 25 ºC 18–19.

Colony characters at 25 ºC in 7 d — On CYA, colonies floccose, slightly elevated, irregularly sulcate, mycelium white to brownish orange (5C6), margin slightly lobulate; reverse light yellow (4A4), with irregular dark brown (6F7) areas; sporulation sparse; soluble pigment absent; exudate absent. On MEA, colonies floccose, slightly radially sulcate, mycelium white to orange white (5A2), margin slightly lobulate; reverse pale orange (5A3) to brownish orange (5C6); sporulation moderately dense, with conidial masses orange white (5A2) to greyish green (29C2); soluble pigment absent; exudate absent. On DG18, colonies floccose to slightly powdery, mycelium white to yellowish white (4A2), margin irregular; reverse light yellow (3A4); sporulation dense, with conidial masses yellow (3A6) at the centre, white at the periphery, with greyish green (28B6) areas; soluble pigment absent; exudate absent. On YES, colonies floccose to slightly cottony, irregularly sulcate, mycelium white to pale orange (5A3), with dull green (29D3) areas, margin slightly lobulate; reverse pale orange (5A3) to orange (6B7); sporulation sparse; soluble pigment absent; exudate absent. On OA, colonies powdery to slightly granulose, with submerged mycelium, mycelium white, margin irregular; reverse yellowish white (3A2) to dull green (29D3); sporulation dense, with conidial masses dark green (27F7); soluble pigment absent; exudate absent. On

CREA, colonies loosely cottony, mycelium white, margin slightly lobulate; sporulation sparse; acid production absent.

Micromorphology — On MEA, conidiophores with conidial heads biseriate, radiate, in shades of green; stipes commonly septate, (400)550–1100 x 5.5–8.5 µm, smooth, hyaline; vesicles mostly spatulate, 11–17 µm wide, hyaline; metulae cylindrical to barrel-shaped, covering 100% of the vesicle, 3.5–6 x 3–4.5 µm, hyaline; phialides cylindrical to flask-shaped, 3.5–7 x 2.5–4 µm, hyaline; conidia globose, subglobose, to ellipsoidal, 2.5–3.5 x 2–3 µm, smooth to finely roughened, hyaline. Ascomata not observed.

Differential diagnosis — Phylogenetically, *A. argentinensis* belongs to the *A. versicolor* clade of section *Nidulantes* (Chen et al. 2016). This new species is closely related to *A. griseoaurantiacus*, *A. pepii*, *A. versicolor*, *A. fructus*, *A. tabacinus*, and *A. protuberus* (Figure 14). *Aspergillus griseoaurantiacus*, *A. versicolor* and *A. fructus* differ from *A. argentinensis* because they are able to grow at 37 ºC (Jurjevic et al. 2012; Visagie et al. 2014b). In addition, whereas *A. argentinensis* does not produce soluble pigment in any of the culture media tested, *A. versicolor* and *A. fructus* produce a reddish pigment on CYA at 25 ºC, (Jurjevic et al. 2012), in the same medium, *A. peppi* produces a brownish pigment (Despot et al. 2017), and in *A. protuberus* it is vinaceous-fawn to pale yellow. In comparison to *A. tabacinus* (Jurjevic et al. 2012), *A. argentinensis* shows a faster growth on MEA at 25 ºC in 7 days (12–16 mm diam vs 21–22 mm) and it has finely roughened conidia (smooth in *A. tabacinus*).

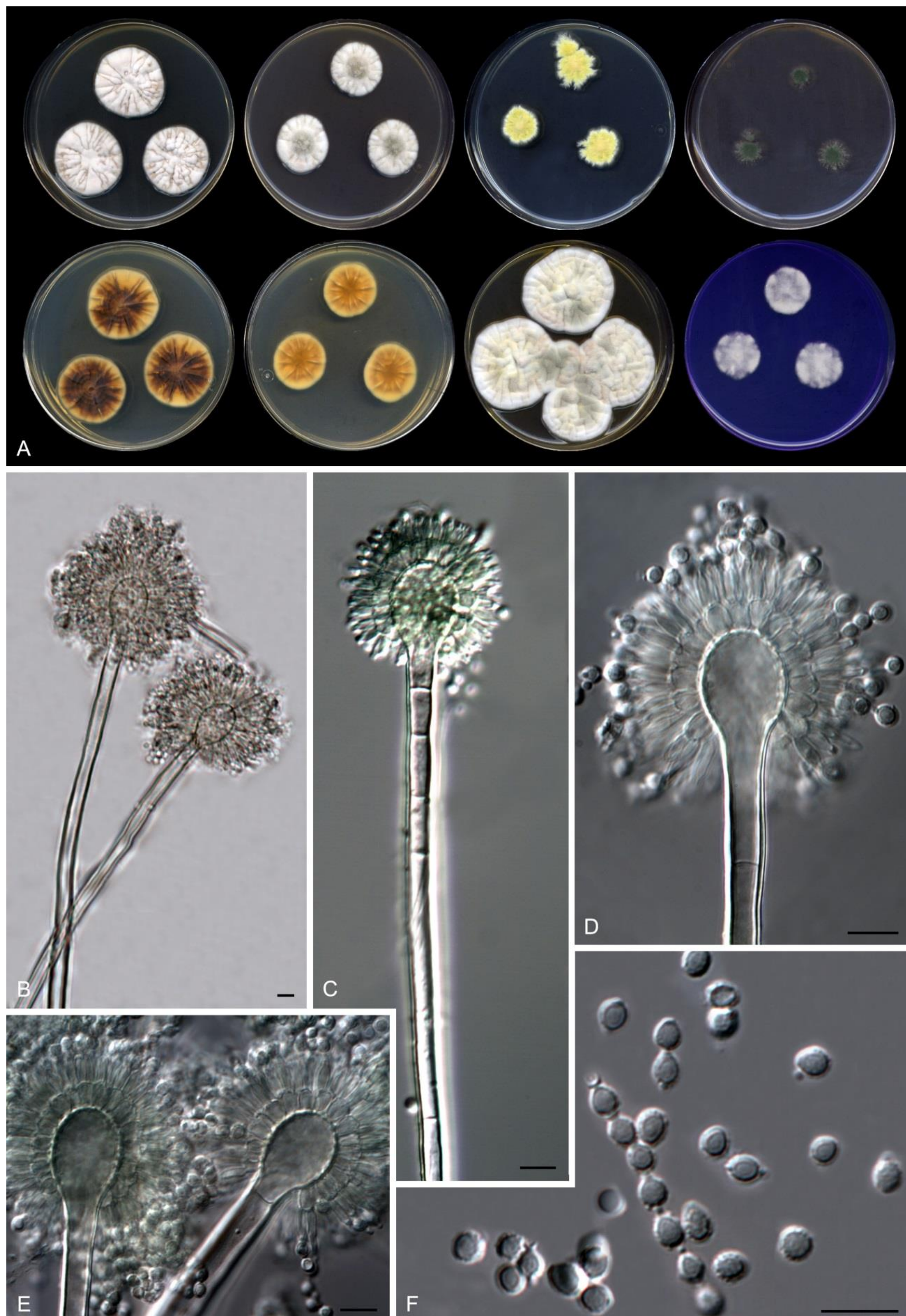


Figure 13. Morphological characters of *Aspergillus argentinensis* (FMR 15740^T). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B–E . Conidiophores. F. Conidia. Scale bars = 10 μm.

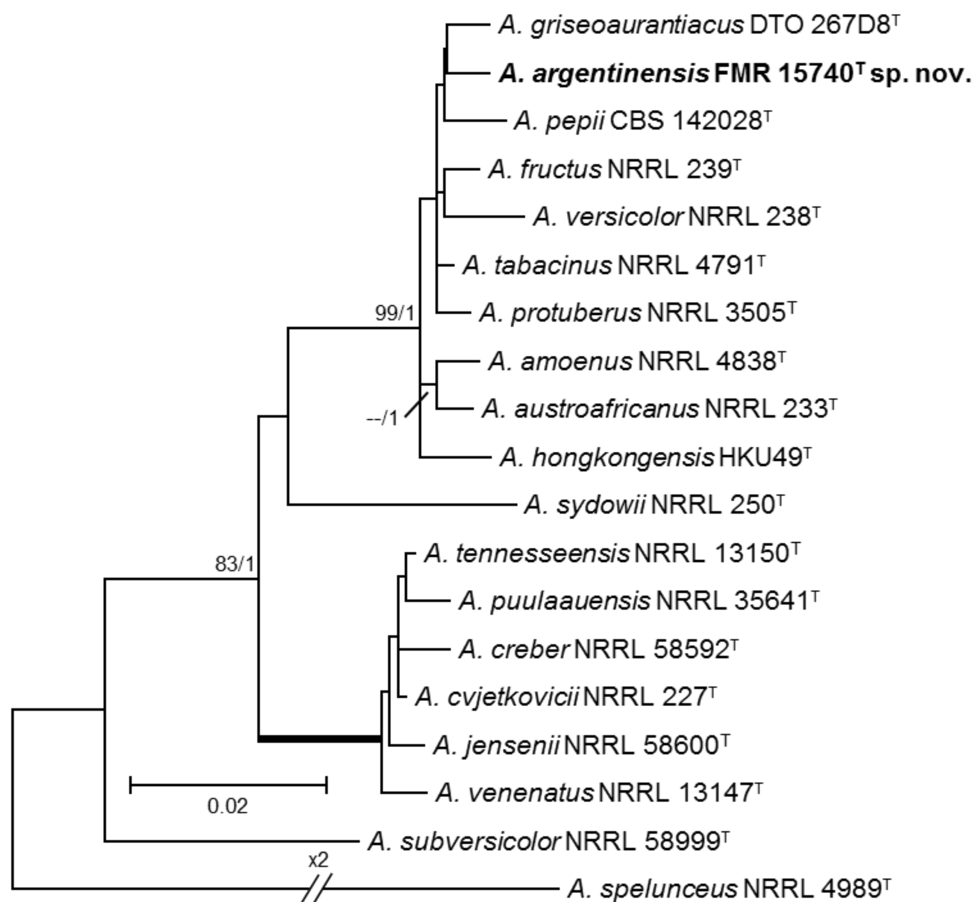


Figure 14. ML tree of selected *Aspergillus* section *Nidulantes* species inferred from the combined ITS, *Ben*A, *CaM*, and *RPB*2 loci. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. Thickened branches correspond to full supported clades (100/1). The tree is rooted to *A. spelunceus* NRRL 4989. The name in bold represent the new species. ^{T} = type strain.

4.6.4. *Aspergillus tumidus* sp. nov.

In preparation to be submitted as part of the article: “New species of *Aspergillus* from South American soils”

Aspergillus tumidus J.P.Z. Siqueira, Gené, García & Guarro, **sp. nov.** MycoBank MBXXXX. Figure 15.

Etymology — Name refers to the swollen characteristic of some vegetative and metulae cells.

In — Section *Nidulantes*

Specimen examined — Chile, Atacama desert, from soil, 2016, A. M. Stchigel (**holotype** CBS H-XXXX; culture ex-type FMR 15743 = CBS XXXX; ITS barcode LT903691, alternative markers: *BenA* LT903682, *CaM* LT903685, *RPB2* LT903688).

Colony diameter in 7d (mm) — On CYA: 25 ºC 34–37, 30 ºC 32–34, 37 ºC no growth; on MEA: 25 ºC 22–23; on DG18: 25 ºC 22–23; on YES: 25 ºC 33–35; on OA: 25 ºC 29–31; on CREA: 25 ºC 20–22.

Colony characters at 25 ºC in 7 d — On CYA, colonies velvety to floccose, slightly radially sulcate, with elevated center, mycelium white, margin entire to slightly lobulate; reverse light green (28A4) to dark brown (6F6); sporulation dense; with conidial masses dark green (29F7); soluble pigment absent; exudate absent. On MEA, colonies floccose to loosely cottony, mycelium white to greenish white (28A2), margin slightly lobulate; reverse light orange (5A4); sporulation dense, with conidial masses pale green (28A3) to dark green (28F8); soluble pigment absent; exudate light green (28A4). On DG18, colonies floccose to powdery, mycelium white to greenish white (28A2), margin slightly lobulate; reverse light orange (5A4); sporulation dense, with conidial masses pale green (28A3) to dark green (28E8); soluble pigment absent; exudate light green (28A4). On YES, colonies floccose to slightly cottony, radially sulcate, mycelium white to greyish green (29B3), margin lobulate; reverse light yellow (4A4) to dark brown(6F6); sporulation dense, with conidial masses greyish green (27C3 to 27E7); soluble pigment absent; exudate yellowish white (3A2) to light yellow (3A4). On OA, colonies cottony at center, powdery towards the periphery, mycelium white, margin slightly lobulate and with submerged mycelium,; reverse white to dull green (28D4); sporulation moderately dense, with conidial masses deep green (29E8);

soluble pigment absent; exudate absent. On CREA, colonies loosely cottony, dense at the centre, mycelium white, margin irregular; sporulation moderately dense, with conidial masses greyish green (28B4); acid production absent.

Micromorphology — On MEA, conidiophores with conidial heads biseriate, radiate, in shades of green; stipes commonly septate, 80–400 x 3–5.5 µm, smooth, hyaline; vesicles subglobose, 5.5–15 µm wide, hyaline; metulae usually inflated, 5.5–9.5 x 2.5–8 µm, covering 75% to 100% of the vesicle, hyaline; phialides flask-shaped, 6.5–10 x 2.5–5 µm, hyaline; conidia globose to subglobose, 3–8 µm, smooth to finely roughened, in shades of green. Hülle cells frequent, mostly globose, sometimes irregular shaped, 12–28 µm. Swollen cells frequently observed along or laterally to the hyphae, mostly globose to ovoid, 8–15 x 10–20 µm. Ascomata not observed.

Differential diagnosis — *A. tumidus* belongs to the *A. multicolor* clade in section *Nidulantes* (Chen et al. 2016), together with *A. multicolor*, *A. mulundensis*, and *A. pluriseminatus* (Figure 16). *Aspergillus multicolor* has pink to purple drab mycelium and pink Hülle cells; *A. mulundensis* presents conidial masses pale green to blue green (Chen et al. 2016); and *A. pluriseminatus* only produces the sexual morph (Stchigel and Guarro 1997).

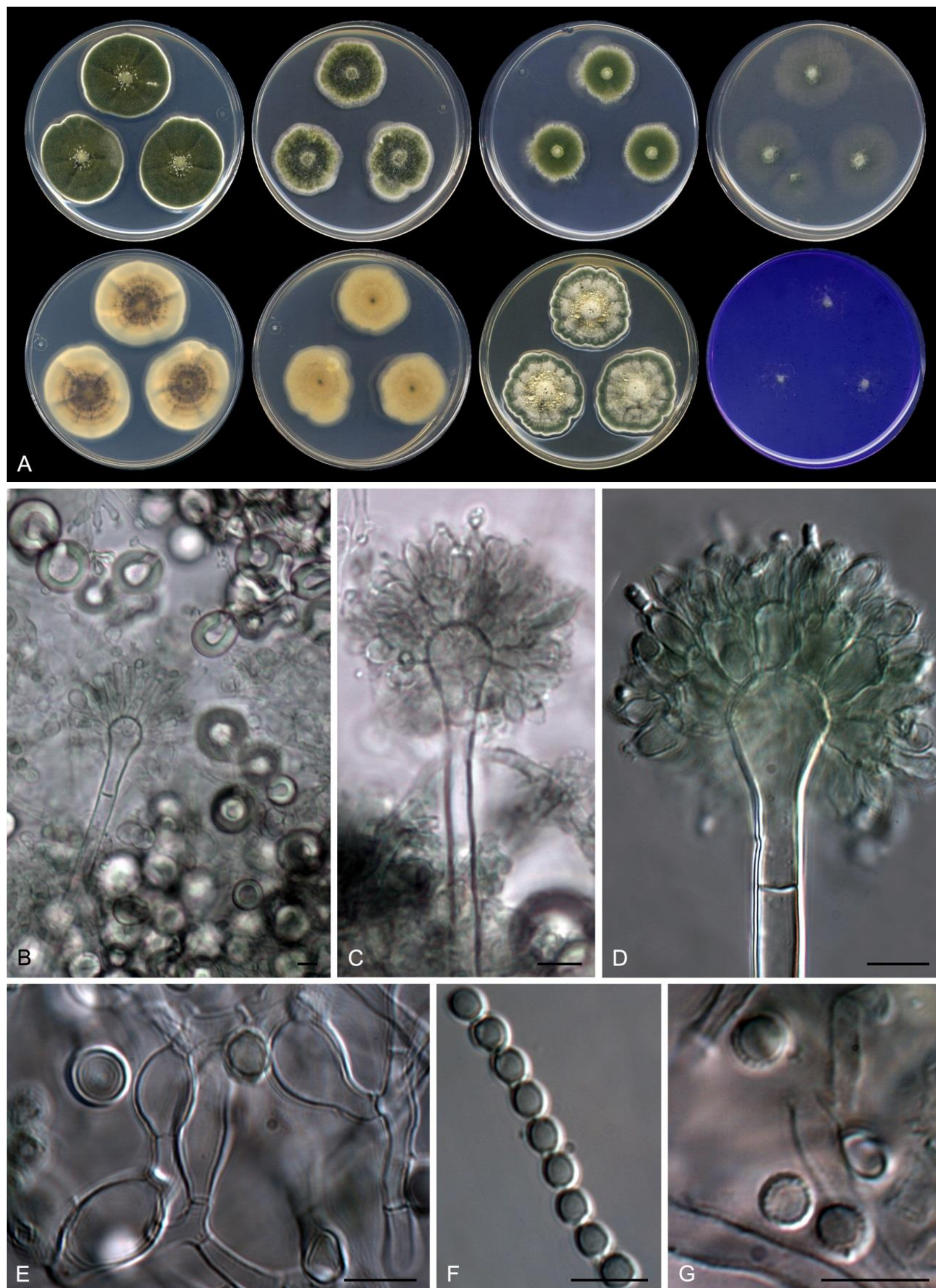


Figure 15. Morphological characters of *Aspergillus tumidus* (FMR 15743^T). A. Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES, and CREA. B. Conidiophore and Hülle cells. C, D. Conidiophores. E. Swollen cells. F, G. Conidia. Scale bars = 10 μm.

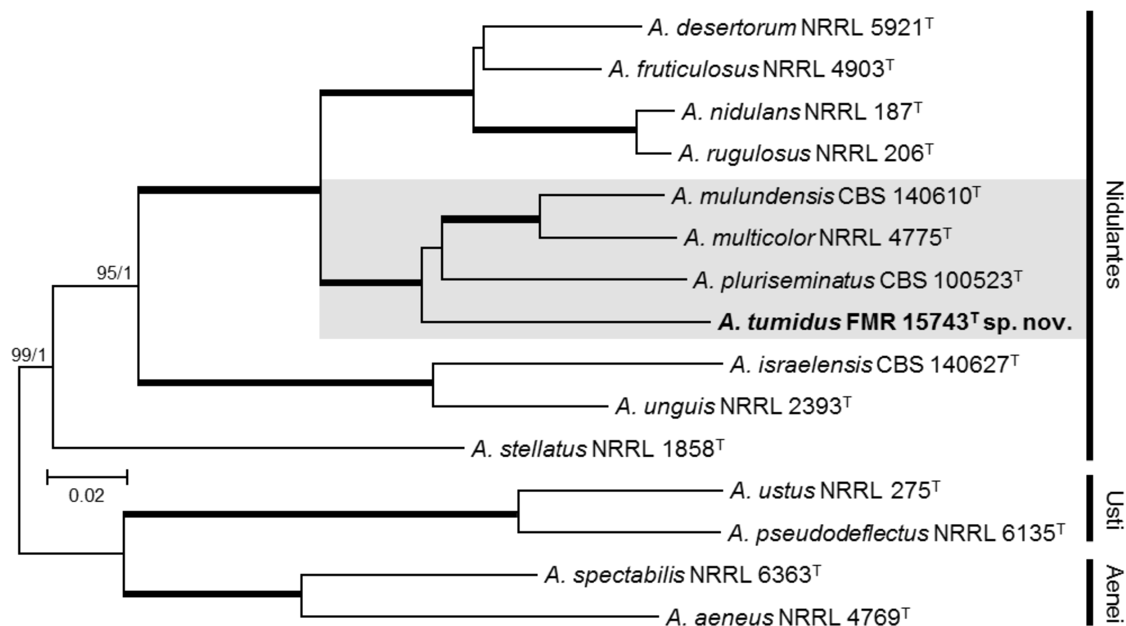


Figure 16. ML tree of selected *Aspergillus* sections *Nidulantes*, *Usti*, and *Aenei* species inferred from the combined ITS, *Ben*A, *CaM*, and *RPB*2 loci. The grey box highlights the *A. multicolor* clade (Chen et al. 2016). Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. Thickened branches correspond to full supported clades (100/1). The name in bold represent the new species. ^{T} = type strain.

5. SUMMARIZING DISCUSSION

The evolutionary history and relationship among the organisms can be estimated by the study of phylogenetic inference (Nei and Kumar 2000). Comparing genetic sequences and reconstructing phylogenetic trees can help us explain similarities and differences among the organisms, including fungi, and better delimitate our currently used classification system, from the higher ranks to the species level. Based on that, using type and reference species, the comparison of sequences cannot only infer relationships, but also identify morphologically similar species. In *Aspergillus*, the sections are in general morphologically recognizable; however, in many cases, the members within the sections may share similar morphological characteristics, being difficult to identify based only on phenotypic features. In the clinical diagnosis, these cases are referred as species complexes (e.g., *A. fumigatus* complex, *A. terreus* complex, *A. nigri* complex) (Balajee 2009; Vermeulen et al. 2014). The species hard to be morphologically distinguished from the leading pathogen or the most common species of the section are usually named cryptic species (Geiser et al. 2007; Balasundaran et al. 2015). Recently, the study of cryptic species of *Aspergillus* has received greater attention owing to advances in molecular identification, which enabled better differentiation. Two large studies, based on DNA-based identification, one from transplant recipients in the USA (Balajee et al. 2009a) and the other on the epidemiology of fungi infections on deep tissue samples, blood cultures, and respiratory samples in Spain (Alastruey-Izquierdo et al. 2013), showed that the prevalence of cryptic species in *Aspergillus* were 11 % and 12 %, respectively. Using phylogenetic analyses, we could observe in the studies enclosed in this thesis the high incidence of cryptic species. For example, in our study of section *Versicolores*, of the 77 clinical isolates tested, 10 species were recovered and only one isolated was identified as *A. versicolor* (1.3 %), which is considered the main pathogenic agent of this group (Buzina 2013). Similarly, among 34 clinical isolates (distributed in seven species) belonging to the section *Circumdati* only three were identified as *A. ochraceus* (8.8 %), and among 25 isolates (five species) of the section *Circumdati* none was identified as *A. glaucus*, which are, respectively, considered the most commonly reported pathogens of these two sections (De Hoog et al. 2011; Buzina 2013).

Consequently, using molecular tools, the diversity of species associated to clinical samples is considerable higher than expected and many *Aspergillus* species are being isolated for the first time from human clinical specimens (Hubka et al. 2012; Negri et al. 2014; Masih et al. 2016). Our studies also confirm this tendency, since we reported eight species recovered for the first time from human samples, including three new species, i.e. *A. pseudosclerotiorum* (Siqueira et al. 2017), *A. microperforatus*

(Siqueira et al. in press), and *A. hemisphaericus*, this latter not published yet (article in preparation for submission).

Many authors have described in recent studies a change in the epidemiology of fungal infections (Richardson and Lass-Florl 2008; Enoch et al. 2017). However, it is unclear whether it is because the improved capability of molecular identification tools and databases, or a genuine trend of increasing frequency of rare species (Howard 2014). It is possible that the considered rare or cryptic species were already causing infections in the past, but that they could not be distinguished or were not even described, overestimating the frequency of the most common species. For example, in our work with cryptic and rare *Aspergillus* (see section 4.4), three of the species were not correctly identified at the moment of their isolation, being assigned to other close related species or considered as part of a complex. Moreover, reports of misidentifications in *Aspergillus* are been increasingly found in the literature (Hubka et al. 2014; Khare et al. 2014; Tam et al. 2014). Consequently, new information provided by taxonomic studies may be altering the epidemiology of *Aspergillus* infections.

Opportunistic infections have gradually increased in recent decades, showing high morbidity and mortality rates, especially in immunocompromised patients. The optimum management and treatment of these infections are often difficult to establish because of the limited understanding of the impact of fungal diseases and the low investment in medical mycology, which cannot be compared with the ones in other areas, such as bacteriology and virology. To understand the true scale of the problem, it is needed to gather better-defined and accurate epidemiological data (Brown et al. 2012). This is only possible through correct identification of the agents and reliable antifungal susceptibility data, allowing appropriate therapeutics.

Regarding antifungal susceptibility testing, we encountered difficulties to compare our results with other works due to the lack of information in the literature respect to the species identified in our studies. Although reports on susceptibility of less common species have increased in the last few years (Alastruey-Izquierdo et al. 2014; Nedel and Pasqualotto 2014), reliable information on the cryptic and rare species is still scarce. The CLSI has established epidemiologic cut-off values for triazoles (ITC, PSC, VRC, and isavuconazole) and AMB for only six *Aspergillus* species, i.e., *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *A. nidulans*, and *A. versicolor* (Espinel-Ingroff et al. 2010; Espinel-Ingroff et al. 2011; Espinel-Ingroff et al. 2013). The limited number of available isolates still precludes the determination of epidemiologic values for other species and antifungal drugs. It has been reported that some species can be intrinsically resistant to some antifungals; for instance, *A. terreus* to AMB; *A. calidoustus* and *A. flavus* to triazoles; and *A. lentulus* to azoles and AMB (Arendrup 2014). However, acquired

resistance became an emerging problem in recent years (Howard et al. 2009). It is now accepted that resistance can develop upon prolonged exposure to antifungal drugs at a sub-lethal concentration (Perlin and Hope 2010; Hagiwara et al. 2016). In addition, environmentally derived resistance mutations have emerged as a major cause of resistance among *Aspergillus* strains over the last decade (Chowdhary et al. 2013). Moreover, susceptibility patterns may be variable among phenotypically similar species (Alastruey-Izquierdo et al. 2014). In our studies, we tried to expand the information regarding antifungal susceptibility, especially from the least studied species, providing in vitro data of 128 isolates from 16 species against at least eight antifungal drugs. Although our results demonstrated that, in general, the most common antifungals available exhibit in vitro activity against the *Aspergillus* species tested, poor activity of AMB could be observed, especially for some members in section *Circumdati*. These results highlight the importance of antifungal susceptibility testing and correct identification.

It is worth mentioning that the lack of information in relation to the origin of the clinical isolates included in this thesis has hampered the analyses of their real role as causal agents of infection. However, they should be considered at least as potential opportunistic pathogens. These findings can promote future studies on epidemiology, pathogenicity and susceptibility, taking into account the novel species described here and also the species reported in clinic for the first time.

Clearly, not only the clinical setting but also the environment is a great source of fungal biodiversity. In fact, the environment is the primary origin of *Aspergillus* diversity, since infections caused by this fungus are commonly opportunistic. As mentioned before, the biology of *Aspergillus* enables it to survive in a wide variety of conditions and substrates, such as stored products and indoor environments. However, since the spores of *Aspergillus* are easily carried by the wind, soil is one of main reservoirs of *Aspergillus* species. Adametz (1886) was one of the pioneers of soil fungus study, reporting *Aspergillus* in this substrate over 130 years ago. *Aspergillus* species seem to favor temperate and tropical soils (Dix and Webster 1995), but they can be found in virtually every type of soil. This was demonstrated in this thesis, where soils from 13 countries were analyzed, including different climates and vegetation, from deserts to Antarctica soil, and 48 species, including four new ones, *A. argentinensis*, *A. bicephalus*, *A. subglobosus*, and *A. tumidus*.

Since we considered droppings as a high source of fungal diversity, we investigated the incidence of *Aspergillus* species on herbivore dung samples collected in Spain. Dung is considered a complex substratum with high amounts of readily available carbohydrates and nitrogen content. Furthermore, the presence of

micronutrients, the physical structure, pH, and varying moisture content make it a rich medium for fungal growth (Richardson 2001). Although some species of *Aspergillus* have been reported from dung, it lacks in the literature studies focused on *Aspergillus* diversity in this substrate. The analyses of 130 samples from different Spanish regions showed great *Aspergillus* diversity, with over 48 species identified distributed in 12 sections. As a result, ten new species of *Aspergillus* were found and described here, highlighting that this substrate can offer a great opportunity for studies of fungal diversity.

The focus of this thesis was on reliable identification of *Aspergillus* isolates and species diversity in different substrates. From the first description by Micheli in 1729, more than 350 species have been described. With this thesis, we have contributed to expand the knowledge on the fungal biodiversity, increasing the number of new species of *Aspergillus*, and the spectrum of species found in the clinical setting.

6. CONCLUSIONS

This thesis assessed the species diversity of the *Aspergillus* sections considered poorly studied in clinical and environmental sources. A polyphasic approach based on a multilocus phylogenetic analyses and phenotypic characterization of the fungi isolated was carried out for their identification to species level with confidence. In addition, the antifungal susceptibility profiles of most of the species isolated from clinical samples were determined. In summary, we identified 303 *Aspergillus* isolates that did not belong to sections *Flavi*, *Fumigati*, and *Nigri*. These corresponded to 76 species, distributed in the sections: *Aspergillus*, *Candidi*, *Circumdati*, *Clavati*, *Cremei*, *Flavipedes*, *Jani*, *Nidulantes*, *Terrei*, and *Usti*.

From *Aspergillus* associated to clinical specimens, we concluded that:

1. The most frequent sections in our set of clinical isolates were *Nidulantes* (including members of the former *Versicolores* section), *Circumdati*, *Aspergillus*, and *Usti*.

2. A total of 49 species were identified, and the most frequently isolated corresponded to: *A. sydowii*, *A. creber*, *A. amoenus*, and *A. protuberus* in section *Nidulantes*; *A. westerdijkiae*, *A. pseudosclerotiorum*, and *A. sclerotiorum* in section *Circumdati*; *A. montevidensis* and *A. chevalieri* in section *Aspergillus*; and *A. calidoustus* in section *Usti*.

3. The species identified for the first time from clinical sources were: *A. jensenii*, *A. pachycristatus*, *A. puulaauensis*, *A. subramanianii*, and *A. spelaeus*. In addition, among the clinical isolates, three new species were characterized, i.e. *A. microperforatus, A. pseudosclerotiorum* and *A. hemisphaericus*.

4. In general, the antifungal drugs tested in vitro showed good activity against the species of the section *Aspergillus*. The species belonging to the other sections studied, i.e. *Circumdati* and *Versicolores*, showed similar susceptibility patterns, with the three echinocandins (AFG, CFG, and MFG) and TBF being the most potent drugs, whereas AMB had less activity. Especially high MICs were observed for AMB against *A. ochraceus*, *A. subramanianii*, and *A. westerdijkiae* in the *Circumdati* section.

From *Aspergillus* isolated from environmental samples, i.e. herbivore dung, soil, and plant material, we found that:

5. Herbivore dung, although poorly studied, was a substrate with a great diversity in *Aspergillus* species. The most frequent sections identified were *Flavipedes*, *Nidulantes*, *Terrei*, and *Candidi*. In addition, ten new species were described from this substrate, i.e. *A. albodeflectus*, *A. aurantiosulcatus*, *A. calidokeveii*, *A. canariensis*, *A. coprophilus*, *A. esporlensis*, *A. fimeti-brunneus*, *A. longipes*, *A. majoricus*, and *A. verruculosus*.

6. Soil also proved to be a great reservoir of rare and new *Aspergillus* species. In this sense, the most frequent sections in our isolates were, *Nidulantes*, *Usti*, and *Terrei*. Furthermore, four new species were characterized, i.e. *A. argentinensis*, *A. bicephalus*, *A. subglobosus*, and *A. tumidus*.

7. Although plant debris was not extensively studied in this thesis, a total of 10 species were identified, i.e. *A. allahabadii*, *A. brasiliensis*, *A. carneus*, *A. creber*, *A. flavus*, *A. hortai*, *A. micronesiensis*, *A. niger*, *A. templicola*, and *A. terreus*. These results show that this substrate should also be considered a source of diversity of *Aspergillus* species.

7. REFERENCES

Adametz L. 1886. Untersuchungen über die niederen Pilze der Ackerkrume. Bär and Hermann, Leipzig, Germany.

Agrios GN. 2005. Plant Pathology. 5th Ed. Elsevier Academic Press, Burlington, USA.

Alastruey-Izquierdo A, Mellado E, Pelaez T, Peman J, Zapico S, Alvarez M, Rodriguez-Tudela JL, Cuenca-Estrella M, FILPOP Study Group. 2013. Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP study). Antimicrobial Agents and Chemotherapy 57: 3380–3387.

Alastruey-Izquierdo A, Alcazar-Fuoli L, Cuenca-Estrella M. 2014. Antifungal susceptibility profile of cryptic species of *Aspergillus*. Mycopathologia 178: 427–433.

Alastruey-Izquierdo A, Melhem MSC, Bonfietti LX, Rodriguez-Tudela JL. 2015. Susceptibility test for fungi: clinical and laboratorial correlations in medical mycology. Revista do Instituto de Medicina Tropical de Sao Paulo 57: 57–64.

Andersen MR, Salazar MP, Schaap PJ, Van de Vondervoort PJ, Culley D, Thykaer J, Frisvad JC, Nielsen KF, Albang R, Albermann K, Berka RM, Braus GH, Braus-Stromeyer SA, Corrochano LM, Dai Z, Van Dijck PWM, Hofmann G, Lasure LL, Magnuson JK, Menke H, Meijer M, Meijer SL, Nielsen JB, Nielsen ML, Van Ooyen AJ, Pel HJ, Poulsen L, Samson RA, Stam H, Tsang A, Van den Brink JM, Atkins A, Aerts A, Shapiro H, Pangilinan J, Salamov A, Lou Y, Lindquist E, Lucas S, Grimwood J, Grigoriev IV, Kubicek CP, Martinez D, Van Peij NN, Roubos JA, Nielsen J, Baker SE. 2011. Comparative genomics of citric-acid-producing *Aspergillus niger* ATCC 1015 versus enzyme-producing CBS 513.88. Genome Research 21: 885–897.

An Z. 2005. Handbook of Industrial Mycology. Marcel Dekker, New York, USA.

Arendrup MC. 2014. Update on antifungal resistance in *Aspergillus* and *Candida*. Clinical Microbiology and Infection 20: 42–48.

Axelrod DE, Gealt M, Pastushok M. 1973. Gene control of developmental competence in *Aspergillus nidulans*. Developmental biology 34: 9–15.

Balajee SA, Houbraken J, Verweij PE, Hong SB, Yaghuchi T, Varga J, Samson RA. 2007. *Aspergillus* species identification in the clinical setting. Studies in Mycology 59: 39–46.

Balajee SA. 2009. *Aspergillus terreus* complex. Medical Mycology 47: S42–S46.

Balajee SA, Kano R, Baddley JW, Moser SA, Marr KA, Alexander BD, Andes D, Kontoyiannis DP, Perrone G, Peterson S, Brandt ME, Pappas PG, Chiller T. 2009a. Molecular identification of *Aspergillus* species collected for the Transplant-Associated Infection Surveillance Network. Journal of Clinical Microbiology 47: 3138–3141.

Balajee SA, Baddley JW, Peterson SW, Nickle D, Varga J, Boey A, Lass-Florl C, Frisvad JC, Samson RA, ISHAM Working Group on *A. terreus*. 2009b. *Aspergillus alabamensis*, a new clinically relevant species in the section Terrei. Eukaryotic Cell 8: 713–722.

Balasundaram SV, Engh IB, Skrede I, Kauserud H. 2015. How many DNA markers are needed to reveal cryptic fungal species? Fungal Biology 119: 940–945.

Bayman P, Baker JL. 2006. Ochratoxins: a global perspective. Mycopathologia 162: 215–223.

Bennett JW. 2009. *Aspergillus*: a primer for the novice. Medical Mycology 47: S5–S12.

Bennett JW. 2010. An overview of the genus *Aspergillus*. In: *Aspergillus* Molecular Biology and Genomics. Eds. M Machida, K Gomi. Caister Academic Press, Norfolk, 1–17.

Bernal-Martinez L, Alastruey-Izquierdo A, Cuenca-Estrella M. 2016. Diagnostics and susceptibility testing in *Aspergillus*. Future Microbiology 11: 315–328.

Brown GD, Denning DW, Gow NAR, Levitz SM, Netea MG, White TC. 2012. Hidden killers: human fungal infections. Science Translational Medicine 4: 1–9.

Buzina W. 2013. *Aspergillus* - classification and antifungal susceptibilities. Current Pharmaceutical Design 19: 3615–3628.

Cafarchia C, Paradies R, Figueredo LA, Padalino B, Greco MF, Greco G, Otranto D. 2012. A case of equine aspergillosis: a novel sampling procedure for diagnosis. Journal of Equine Veterinary Science 32: 634–637.

Cary JW, O'Brian GR, Nielsen DM, Nierman W, Harris-Coward P, Yu J, Bhatnagar D, Cleveland TE, Payne GA, Calvo AM. 2007. Elucidation of veA-dependent genes associated with aflatoxin and sclerotial production in *Aspergillus flavus* by functional genomics. Applied Microbiology and Biotechnology 76: 1107–1118.

Cerqueira GC, Arnaud MB, Inglis DO, Skrzypek MS, Binkley G, Simison M, Miyasato SR, Binkley J, Orvis J, Shah P, Wymore F, Sherlock G, Wortman JR. 2014. The *Aspergillus* genome database: multispecies curation and incorporation of RNA-Seq

data to improve structural gene annotations. Nucleic Acids Research 42: D705–D710.

Chen AJ, Frisvad JC, Sun BD, Varga J, Kocsube S, Dijksterhuis J, Kim DH, Hong SB, Houbraken J, Samson RA. 2016. *Aspergillus* section *Nidulantes* (formerly *Emericella*): polyphasic taxonomy, chemistry and biology. Studies in Mycology 84: 1–118.

Chotirmall SH, Mirkovic B, Lavelle GM, McElvaney NG. 2014. Immunoevasive *Aspergillus* virulence factors. Mycopathologia 178: 363–370.

Chowdhary A, Kathuria S, Xu J, Meis JF. 2013. Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health. PLoS Pathogens 9: e1003633.

Clinical and Laboratory Standards Institute (CLSI). 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard. 2ª Ed. Document M38-A2. Clinical and Laboratory Standards Institute, Wayne, USA.

Colombo AL, Reis V, Godoy P. 2010. Antifungal susceptibility tests of *Aspergillus* species. In Aspergillosis: From Diagnosis to Prevention. Ed. AC Pasqualotto. Springer, Dordrecht, 193–215.

Coyner K. 2010. Otomycosis due to *Aspergillus* spp. in a dog: case report and literature review. Veterinary Dermatology 21: 613–618.

Cuenca-Estrella M, Rodriguez-Tudela JL. 2010. Terbinafine. In: Aspergillosis: From Diagnosis to Prevention. Ed. AC Pasqualotto. Springer, Dordrecht, 317–326.

Czaja W, Miller KY, Miller BL. 2011. Complex mechanisms regulate developmental expression of the matA (HMG) mating type gene in homothallic *Aspergillus nidulans*. Genetics 189: 795–808.

De Barry A. 1854. Ueber die Entwickelung und den Zusammenhang von *Aspergillus glaucus* und *Eurotium*. Botanische Zeitung 12: 425–471.

De Hoog GS, Gerrits van den Ende AH. 1998. Molecular diagnostics of clinical strains of filamentous basidiomycetes. Mycoses 41: 183–189.

De Hoog GS, Guarro J, Gene J, Figueras MJ. 2011. Atlas of Clinical Fungi. CD-ROM version 3.1. CBS-KNAW Fungal Biodiversity Centre, Utrecht.

De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, Pappas PG, Maertens J, Lortholary O, Kauffman CA, Denning DW, Patterson TF, Maschmeyer G, Bille J, Dismukes WE, Herbrecht R, Hope WW, Kibbler CC,

Kullberg BJ, Marr KA, Muñoz P, Odds FC, Perfect JR, Restrepo A, Ruhnke M, Segal BH, Sobel JD, Sorrell TC, Viscoli C, Wingard JR, Zaoutis T, Bennett JE. 2008. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clinical infectious diseases 46: 1813–1821.

De Vries RP, Riley R, Wiebenga A, Aguilar-Osorio G, Amillis S, Uchima CA, Anderluh G, Asadollahi M, Askin M, Barry K, Battaglia E, Bayram O, Benocci T, Braus-Stromeyer SA, Caldana C, Canovas D, Cerqueira GC, Chen F, Chen W, Choi C, Clum A, Santos RA, Damasio AR, Diallinas G, Emri T, Fekete E, Flipphi M, Freyberg S, Gallo A, Gournas C, Habgood R, Hainaut M, Harispe ML, Henrissat B, Hilden KS, Hope R, Hossain A, Karabika E, Karaffa L, Karanyi Z, Krasevec N, Kuo A, Kusch H, LaButti K, Lagendijk EL, Lapidus A, Levasseur A, Lindquist E, Lipzen A, Logrieco AF, MacCabe A, Makela MR, Malavazi I, Melin P, Meyer V, Mielnichuk N, Miskei M, Molnar AP, Mule G, Ngan CY, Orejas M, Orosz E, Ouedraogo JP, Overkamp KM, Park HS, Perrone G, Piumi F, Punt PJ, Ram AF, Ramon A, Rauscher S, Record E, Riano-Pachon DM, Robert V, Rohrig J, Ruller R, Salamov A, Salih NS, Samson RA, Sandor E, Sanguinetti M, Schutze T, Sepcic K, Shelest E, Sherlock G, Sophianopoulou V, Squina FM, Sun H, Susca A, Todd RB, Tsang A, Unkles SE, Van de Wiele N, Van Rossen-Uffink D, Oliveira JV, Vesth TC, Visser J, Yu JH, Zhou M, Andersen MR, Archer DB, Baker SE, Benoit I, Brakhage AA, Braus GH, Fischer R, Frisvad JC, Goldman GH, Houbraken J, Oakley B, Pocsi I, Scazzocchio C, Seiboth B, VanKuyk PA, Wortman J, Dyer PS, Grigoriev IV. 2017. Comparative genomics reveals high biological diversity and specific adaptations in the industrially and medically important fungal genus *Aspergillus*. Genome Biology 18: 28.

Denning DW. 2010. Introduction. In Aspergillosis: From Diagnosis to Prevention. Ed. AC Pasqualotto. Springer, Dordrecht, 3–5.

Despot DJ, Kocsube S, Bencsik O, Kecskemeti A, Szekeres A, Vagvolgyi C, Varga J, Klaric SM. 2017. New sterigmatocystin-producing species of *Aspergillus* section *Versicolores* from indoor air in Croatia. Mycological Progress 16: 63–72.

Dix NJ, Webster J. 1995. Coprophilous fungi. In Fungal Ecology. Springer, Dordrecht, 203–224.

Dyer PS, O'Gorman CM. 2012. Sexual development and cryptic sexuality in fungi: insights from *Aspergillus* species. FEMS Microbiology Reviews 36: 165–192.

Enoch DA, Yang H, Aliyu SH, Micallef C. 2017. The changing epidemiology of invasive fungal infections. In Human Fungal Pathogen Identification: Methods and Protocols. Ed. T Lion. New York, 17–65.

Espinel-Ingroff A, Diekema DJ, Fothergill A, Johnson E, Pelaez T, Pfaller MA, Rinaldi MG, Canton E, Turnidge J. 2010. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). Journal of Clinical Microbiology 48: 3251–3257.

Espinel-Ingroff A, Cuenca-Estrella M, Fothergill A, Fuller J, Ghannoum M, Johnson E, Pelaez T, Pfaller MA, Turnidge J. 2011. Wild-Type MIC distributions and epidemiological cutoff values for amphotericin B and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). Antimicrobial Agents and Chemotherapy 55: 5150–5154.

Espinel-Ingroff A, Chowdhary A, Gonzalez GM, Lass-Florl C, Martin-Mazuelos E, Meis J, Pelaez T, Pfaller MA, Turnidge J. 2013. Multicenter study of isavuconazole MIC distributions and epidemiological cutoff values for *Aspergillus* spp. for the CLSI M38-A2 broth microdilution method. Antimicrobial Agents and Chemotherapy 57: 3823–3828.

Figueras MJ, Guarro J. 1988. A scanning electron microscopic study of ascoma development in *Chaetomium malaysiense*. Mycologia 80: 298–306.

Fogarty WM. 1994. Enzymes of the genus *Aspergillus*. In *Aspergillus*. Ed. JE Smith. Plenum Press, New York, 177–218.

Frisvad JC. 1981. Physiological criteria and mycotoxin production as aids in identification of common asymmetric penicillia. Applied and Environmental Microbiology 41: 568–579.

Frisvad JC, Andersen B, Thrane U. 2008. The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. Mycological Research 112: 231–240.

Frisvad JC, Larsen TO. 2015. Chemodiversity in the genus *Aspergillus*. Applied Microbiology and Biotechnology 99: 7859–7877.

Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, Batzoglou S, Lee SI, Baturkmen M, Spevak CC, Clutterbuck J, Kapitonov V, Jurka J, Scazzocchio C, Farman M, Butler J, Purcell S, Harris S, Braus GH, Draht O, Busch S, D'Enfert C,

Bouchier C, Goldman GH, Bell-Pedersen D, Griffiths-Jones S, Doonan JH, Yu J, Vienken K, Pain A, Freitag M, Selker EU, Archer DB, Peñalva MA, Oakley BR, Momany M, Tanaka T, Kumagai T, Asai K, Machida M, Nierman WC, Denning DW, Caddick M, Hynes M, Paoletti M, Fischer R, Miller B, Dyer P, Sachs MS, Osmani SA, Birren BW. 2005. Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. Nature 438: 1105–1115.

Gams W, Christensen M, Onions AHS, Pitt JI, Samson RA. 1985. Typification of the species of *Aspergillus* and associated teleomorphs. In Advances in *Penicillium* and *Aspergillus* Systematics. Eds. RA Samson, JI Pitt. Plenum Press, New York, 31–54.

Geiser DM, Klich MA, Frisvad JC, Peterson SW, Varga J, Samson RA. 2007. The current status of species recognition and identification in *Aspergillus*. Studies in Mycology 59: 1–10.

Geiser DM, Samson RA, Varga J, Rokas A, Witiak SM. 2008. A review of molecular phylogenetics in *Aspergillus*, and prospects for a robust genus-wide phylogeny. In: *Aspergillus* in the Genomic Era. Eds. J Varga, RA Samson. Wageningen Academic, Utrecht, 17–32.

Gibbons JG, Rokas A. 2013. The function and evolution of the *Aspergillus* genome. Trends in Microbiology 21: 14–22.

Girling SJ, Fraser MA. 2009. Treatment of *Aspergillus* species infection in reptiles with itraconazole at metabolically scaled doses. The Veterinary Record 165: 52–54.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology 61: 1323–1330.

Gouas D, Shi H. 2009. The aflatoxin-induced TP53 mutation at codon 249 (R249S): biomarker of exposure, early detection and target for therapy. Cancer Letters 286: 29–37.

Guarner J, Brandt ME. 2011. Histopathologic diagnosis of fungal infections in the 21st century. Clinical Microbiology Reviews 24: 247–280.

Guarro J, Gene J, Stchigel AM, Figueras MJ. 2012. Atlas of Soil Ascomycetes. CBS-KNAW Fungal Biodiversity Centre. Utrecht, The Netherlands.

Guevara-Suarez M, Siqueira JPZ, Garcia D, Cano-Lira J, Guarro J, Gene J. In press. Herbivore dung, a forgotten source of Eurotialean fungi. Studies in Mycology.

Guinea J, Sandoval-Denis M, Escribano P, Pelaez T, Guarro J, Bouza E. 2015. *Aspergillus citrinoterreus*, a new species of section *Terrei* isolated from samples of patients with nonhematological predisposing conditions. Journal of Clinical Microbiology 53: 611–617.

Gupta P, Khare V, Kumar D, Ahmad A, Banerjee G, Singh M. 2015. Comparative evaluation of disc diffusion and E-test with broth micro-dilution in susceptibility testing of amphotericin B, voriconazole and caspofungin against clinical *Aspergillus* isolates. Journal of Clinical and Diagnostic Research 9: 4–7.

Hagiwara D, Takahashi H, Watanabe A, Takahashi-Nakaguchi A, Kawamoto S, Kamei K, Gonoi T. 2014. Whole-genome comparison of *Aspergillus fumigatus* strains serially isolated from patients with aspergillosis. Journal of Clinical Microbiology 52: 4202–4209.

Hagiwara D, Watanabe A, Kamei K, Goldman GH. 2016. Epidemiological and genomic landscape of azole resistance mechanisms in *Aspergillus* fungi. Frontiers in Microbiology 7: 1382.

Hocking AD, Pitt JI. 1980. Dichloran-glycerol medium for enumeration of xerophilic fungi from low-moisture foods. Applied and Environmental Microbiology 39: 488–492.

Hong SB, Go SJ, Shin HD, Frisvad JC, Samson RA. 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. Mycologia 97: 1316–1329.

Houbraken J, Samson RA. 2011. Phylogeny of *Penicillium* and the segregation of *Trichocomaceae* into three families. Studies in Mycology 70: 1–51.

Houbraken J, Spierenburg H, Frisvad JC. 2012. *Rasamsonia*, a new genus comprising thermotolerant and thermophilic *Talaromyces* and *Geosmithia* species. Antonie Van Leeuwenhoek 101: 403–421.

Houbraken J, De Vries RP, Samson RA. 2014. Modern taxonomy of biotechnologically important *Aspergillus* and *Penicillium* species. Advances in Applied Microbiology 86: 199–249.

Howard SJ, Cerar D, Anderson MJ, Albarrag A, Fisher MC, Pasqualotto AC, Laverdiere M, Arendrup MC, Perlin DS, Denning DW. 2009. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. Emerging Infectious Diseases 15: 1068–1076.

Howard SJ. 2014. Multi-resistant aspergillosis due to cryptic species. Mycopathologia 178: 435–439.

Hubka V, Kolarik M. 2012. β-tubulin paralogue *tubC* is frequently misidentified as the *benA* gene in *Aspergillus* section *Nigri* taxonomy: primer specificity testing and taxonomic consequences. Persoonia 29: 1–10.

Hubka V, Kolarik M, Kubatova A, Peterson SW. 2013. Taxonomic revision of *Eurotium* and transfer of species to *Aspergillus*. Mycologia 105: 912–937.

Hubka V, Lyskova P, Frisvad JC, Peterson SW, Skorepova M, Kolarik M. 2014. *Aspergillus pragensis* sp. nov. discovered during molecular reidentification of clinical isolates belonging to *Aspergillus* section *Candidi*. Medical Mycology 52: 565–576.

Hubka V, Novakova A, Kolarik M, Jurjevic Z, Peterson SW. 2015. Revision of *Aspergillus* section *Flavipedes*: seven new species and proposal of section *Jani* sect. nov. Mycologia 107: 169–208.

Johnson EM, Borman AM. 2010. The importance of conventional methods: microscopy and culture. In Aspergillosis: From Diagnosis to Prevention. Ed. AC Pasqualotto. Springer, Dordrecht, 55–73.

Jurjevic Z, Peterson SW, Horn BW. 2012. *Aspergillus* section *Versicolores*: nine new species and multilocus DNA sequence based phylogeny. IMA Fungus 3: 59–79.

Kamei K, Watanabe A. 2005. *Aspergillus* mycotoxins and their effect on the host. Medical Mycology 43: S95–S99.

Khare R, Gupta S, Arif S, Jentoft ME, Deziel PJ, Roden AC, Wilhelm MP, Razonable RR, Wengenack NL. 2014. Misidentification of *Neosartorya pseudofischeri* as *Aspergillus fumigatus* in a lung transplant patient. Journal of Clinical Microbiology 52: 2722–2725.

Knuf C, Nielsen J. 2012. Aspergilli: systems biology and industrial applications. Biotechnology Journal 7: 1147–1155.

Kocsube S, Perrone G, Magista D, Houbraken J, Varga J, Szigeti G, Hubka V, Hong SB, Frisvad JC, Samson RA. 2016. *Aspergillus* is monophyletic: evidence from multiple gene phylogenies and extrolites profiles. Studies in Mycology 85: 199–213.

Kornerup A, Wanscher JH. 1967. Methuen Handbook of Colour. Methuen, London, England.

Kozakiewicz Z. 1989. *Aspergillus* Species on Stored Products. CAB International, Ferry Lane, UK.

Kozel TR, Wickes B. 2014. Fungal diagnostics. Cold Spring Harbor Perspectives in Medicine 4: 1–14.

Krijgsheld P, Bleichrodt R, Van Veluw GJ, Wang F, Muller WH, Dijksterhuis J, Wosten HAB. 2013. Development in *Aspergillus*. Studies in Mycology 74: 1–29.

Lacey J, Magan N. 1991. Fungi colonising cereal grain: their occurrence and water and temperature relations. In Cereal Grain Mycotoxins, Fungi and Quality in Storage. Ed. J Chelkowski. Elsevier, Amsterdam, 77–118.

Lass-Florl C, Perkhofer S. 2008. In vitro susceptibility-testing in *Aspergillus* species. Mycoses 51: 437–446.

Lewis RE. 2010. Polyene antifungal agents. In Aspergillosis: From Diagnosis to Prevention. Ed. AC Pasqualotto. Springer, Dordrecht, 281–306.

Link HF. 1809. Observationes in ordines plantarum naturales. Mag Gesell Naturf Freunde 3: 1–42.

Liu YJ, Whelen S, Hall BD. 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. Molecular Biology and Evolution 16: 1799–1808.

Masclaux F, Gueho E, De Hoog GS, Christen R. 1995. Phylogenetic relationships of human-pathogenic *Cladosporium* (*Xylohypha*) species inferred from partial LS rRNA sequences. Medical Mycology 33: 327–338.

Masih A, Singh PK, Kathuria S, Agarwal K. 2016. Identification by molecular methods and matrix-assisted laser desorption ionization – time of flight mass spectrometry and antifungal susceptibility profiles of clinically significant rare *Aspergillus* species in a referral chest hospital in Delhi, India. Journal of Clinical Microbiology 54: 2354–2364.

Max B, Salgado JM, Rodriguez N, Cortes S, Converti A, Dominguez JM. 2010. Biotechnological production of citric acid. Brazilian Journal of Microbiology 41: 862–875.

McNeill J, Barrie FF, Buck WR, Demoulin V, Greuter W, Hawksworth DL, Herendeen PS, Knapp S, Marhold K, Prado J, Prud'homme Van Reine WF, Smith GF, Wiersema JH. 2012. International code of nomenclature for algae, fungi and plants (Melbourne Code). Koeltz Scientific Books, Königstein, Germany.

Mellado E, Diaz-Guerra TM, Cuenca-Estrella M, Rodriguez-Tudela JL. 2001. Identification of two different 14-alpha-sterol demethylase-related genes (cyp51A

and cyp51B) in *Aspergillus fumigatus* and other *Aspergillus* species. Journal of Clinical Microbiology 39: 2431–2438.

Meyer V, Fiedler M, Nitsche B, King R. 2015. The cell factory *Aspergillus* enters the big data era: opportunities and challenges for optimising product formation. Advances in Biochemical Engineering/ Biotechnology 149: 91–132.

Micheli PA. 1729. *Nova Plantarum Genera: iuxta Tournefortii methodum disposita.* B. Paperninii, Florence, Italy.

Minter DW, Hawksworth DL, Onions AHS, Kozakiewicz Z. 1985. Descriptive terminology of the conidiogenous structures in *Aspergillus*. In Advances in *Penicillium* and *Aspergillus* Systematics. Eds. RA Samson, JI Pitt. Plenum Press, New York, 71–88.

Morrison VA. 2006. Echinocandin antifungals: review and update. Expert Review of Anti-infective Therapy 4: 325–342.

Muller FM, Werner KE, Kasai M, Francesconi A, Chanock SJ, Walsh TJ. 1998. Rapid extraction of genomic DNA from medically important yeasts and filamentous fungi by high-speed cell disruption. Journal of Clinical Microbiology 36: 1625–1629.

Nedel WL, Pasqualotto AC. 2014. Treatment of infections by cryptic *Aspergillus* species. Mycopathologia 178: 441–445.

Negri CE, Goncalves SS, Xafranski H, Bergamasco MD, Aquino VR, Castro PT, Colombo AL. 2014. Cryptic and rare *Aspergillus* species in Brazil: prevalence in clinical samples and in vitro susceptibility to triazoles. Journal of Clinical Microbiology 52: 3633–3640.

Nei M, Kumar S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York, USA.

Nylander JAA. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

O'Gorman CM, Fuller HT, Dyer PS. 2009. Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. Nature 457: 471–474.

Odabasi Z, Mattiuzzi G, Estey E, Kantarjian H, Saeki F, Ridge RJ, Ketchum PA, Finkelman MA, Rex JH, Ostrosky-Zeichner L. 2004. Beta-D-glucan as a diagnostic adjunct for invasive fungal infections: validation, cutoff development, and performance in patients with acute myelogenous leukemia and myelodysplastic syndrome. Clinical Infectious Diseases 39: 199–205.

Panasenko V. 1967. Ecology of microfungi. The Botanical Review 33: 189–215.

Paster N, Lisker N, Chet I. 1983. Ochratoxin A production by *Aspergillus ochraceus* Wilhelm grown under controlled atmospheres. Applied and Environmental Microbiology 45: 1136–1139.

Patterson TF, Thompson GR, Denning DW, Fishman JA, Hadley S, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Nguyen MH, Segal BH, Steinbach WJ, Stevens DA, Walsh TJ, Wingard JR, Young JAH, Bennett JE. 2016. Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the Infectious Diseases Society of America. Clinical Infectious Diseases 63: e1–e60.

Paulussen C, Hallsworth JE, Alvarez-Perez S, Nierman WC, Hamill PG, Blain D, Rediers H, Lievens B. 2016. Ecology of aspergillosis: insights into the pathogenic potency of *Aspergillus fumigatus* and some other *Aspergillus* species. Microbial Biotechnology 10: 296–322.

Perlin DS, Hope WW. 2010. Echinocandins. In Aspergillosis: From Diagnosis to Prevention. Ed. AC Pasqualotto. Springer, Dordrecht, 263–280.

Peterson SW, Vega FE, Posada F, Nagai C. 2005. *Penicillium coffeae*, a new endophytic species isolated from a coffee plant and its phylogenetic relationship to *P. fellutanum*, *P. thiersii* and *P. brocae* based on parsimony analysis of multilocus DNA sequences. Mycologia 97: 659–666.

Peterson SW. 2008. Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. Mycologia 100: 205–226.

Pfaller M, Boyken L, Hollis R, Kroeger J, Messer S, Tendolkar S, Diekema D. 2011. Use of epidemiological cutoff values to examine 9-year trends in susceptibility of *Aspergillus* species to the triazoles. Journal of Clinical Microbiology 49: 586–590.

Pfeiffer CD, Fine JP, Safdar N. 2006. Diagnosis of invasive aspergillosis using a galactomannan assay: a meta-analysis. Clinical Infectious Diseases 42: 1417–1427.

Poletto P, Borsoi C, Zeni M, Silveira MM. 2015. Downstream processing of pectinase produced by *Aspergillus niger* in solid state cultivation and its application to fruit juices clarification. Food Science and Technology 35: 391–397.

Raper KB, Fennell DI. 1965. The Genus *Aspergillus*. Robert E. Krieger Publishing Co. Inc., Huntington, USA.

Rementeria A, Lopez-Molina N, Ludwig A, Vivanco AB, Bikandi J, Ponton J, Garaizar J. 2005. Genes and molecules involved in *Aspergillus fumigatus* virulence. Revista Iberoamericana de Micologia 22: 1–23.

Richardson MJ. 2001. Diversity and occurrence of coprophilous fungi. Mycological Research 105: 387–402.

Richardson M, Lass-Florl C. 2008. Changing epidemiology of systemic fungal infections. Clinical Microbiology and Infection 14: 5–24.

Ronquist F, Huelsenbeck JP. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Sales-Campos H, Tonani L, Cardoso CR, Kress MR. 2013. The immune interplay between the host and the pathogen in *Aspergillus fumigatus* lung infection. BioMed Research International 2013: 693023.

Samson RA, Frisvad JC. 2004. *Penicillium* subgenus *Penicillium*: new taxonomic schemes, mycotoxins and other extrolites. Studies in Mycology 49: 1–157.

Samson RA, Varga J. 2009. What is a species in *Aspergillus*? Medical Mycology 47: S13–S20.

Samson RA, Houbraken J, Thrane U, Frisvad JC, Andersen B. 2010. Food and Indoor Fungi. CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands.

Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CH, Perrone G, Seifert KA, Susca A, Tanney JB, Varga J, Kocsube S, Szigeti G, Yaguchi T, Frisvad JC. 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. Studies in Mycology 78: 141–173.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Fungal Barcoding Consortium. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proceedings of the National Academy of Sciences of the United States of America 109: 6241–6246.

Scibelli A, Tafuri S, Ferrante MC, Alimenti E, Naso B, Lucisano A, Staiano N, Della Morte R. 2003. Ochratoxin A affects COS cell adhesion and signaling. Toxicology and Applied Pharmacology 192: 222–230.

Segal BH. 2007. Role of macrophages in host defense against aspergillosis and strategies for immune augmentation. The Oncologist 12 Suppl 2: S7–S13.

Siqueira JPZ, Sutton DA, Gene J, Garcia D, Wiederhold N, Peterson SW, Guarro J. 2017. Multilocus phylogeny and antifungal susceptibility of *Aspergillus* section

Circumdati from clinical samples and description of *A. pseudosclerotiorum* sp. nov. Journal of Clinical Microbiology 55: 947–958.

Siqueira JPZ, Sutton DA, Gene J, Garcia D, Wiederhold N, Guarro J. In press. Species of *Aspergillus* section *Aspergillus* from clinical samples in USA. Medical Mycology.

Souza PM, Bittencourt MLA, Caprara CC, Freitas M, Almeida RPC, Silveira D, Fonseca YM, Ferreira-Filho EX, Pessoa-Junior A, Magalhaes PO. 2015. A biotechnology perspective of fungal proteases. Brazilian Journal of Microbiology 46: 337–346.

Stevens DA. 2009. Clinical aspergillosis for basic scientists. Medical Mycology 47: S1–S4.

Stchigel AM, Guarro J. 1997. A new species of *Emericella* from Indian soil. Mycologia 89: 937–941.

Stolk AC, Malla DS. 1971. *Penicillium inflatum* sp. nov. Persoonia 6: 197–200.

Sugui JA, Pardo J, Chang YC, Zarember KA, Nardone G, Galvez EM, Mullbacher A, Gallin JI, Simon MM, Kwon-Chung KJ. 2007. Gliotoxin is a virulence factor of *Aspergillus fumigatus*: gliP deletion attenuates virulence in mice immunosuppressed with hydrocortisone. Eukaryotic Cell 6: 1562–1569.

Tam EW, Chen JH, Lau EC, Ngan AH, Fung KS, Lee KC, Lam CW, Yuen KY, Lau SK, Woo PC. 2014. Misidentification of *Aspergillus nomius* and *Aspergillus tamarii* as *Aspergillus flavus*: characterization by internal transcribed spacer, beta-tubulin, and calmodulin gene sequencing, metabolic fingerprinting, and matrix-assisted laser desorption ionization. Journal of Clinical Microbiology 52: 1153–1160.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30: 2725–2729.

Thom C, Church MB. 1926. The Aspergilli. Williams & Wilkins Comp., Baltimore, USA.

Thom C, Raper KB. 1945. A Manual of the Aspergilli. Williams & Wilkins Comp., Baltimore, USA.

Thompson GR, Patterson TF. 2010. Azoles. In Aspergillosis: From Diagnosis to Prevention. Ed. AC Pasqualotto. Springer, Dordrecht, 231–262.

Todd RB, Davis MA, Hynes MJ. 2007. Genetic manipulation of *Aspergillus nidulans*: meiotic progeny for genetic analysis and strain construction. Nature Protocols 2: 811–821.

Tomee JF, Kauffman HF. 2000. Putative virulence factors of *Aspergillus fumigatus*. Clinical and Experimental Allergy 30: 476–484.

Valiante V, Baldin C, Hortschansky P, Jain R, Thywißen A, Straßburger M, Shelest E, Heinekamp T, Brakhage AA. 2016. The *Aspergillus fumigatus* conidial melanin production is regulated by the bifunctional bHLH DevR and MADS-box RlmA transcription factors. Molecular Microbiology 102: 321–335.

Van Burik JA, Colven R, Spach DH. 1998. Cutaneous aspergillosis. Journal of Clinical Microbiology 36: 3115–3121.

Van Dijck PWM. 2008. The importance of aspergilli and regulatory aspects of *Aspergillus* nomenclature in biotechnology. In: *Aspergillus* in the Genomic Era. Eds. J Varga, RA Samson. Wageningen Academic, Utrecht, 249–257.

Vanden Bossche H, Warnock DW, Dupont B, Kerridge D, Sen Gupta S, Improvisi L, Marichal P, Odds FC, Provost F, Ronin O. 1994. Mechanisms and clinical impact of antifungal drug resistance. Journal of Medical and Veterinary Mycology 32: S189–S202.

Varga J, Samson RA. 2008. *Aspergillus* in the Genomic Era. Wageningen Academic Publishers, Utrecht, The Netherlands.

Vermeulen E, Maertens J, Meersseman P, Saegeman V, Dupont L, Lagrou K. 2014. Invasive *Aspergillus niger* complex infections in a Belgian tertiary care hospital. Clinical Microbiology and Infection 20: O333-O335.

Visagie CM, Houbraken J, Frisvad JC, Hong SB, Klaassen CH, Perrone G, Seifert KA, Varga J, Yaguchi T, Samson RA. 2014a. Identification and nomenclature of the genus *Penicillium*. Studies in Mycology 78: 343–371.

Visagie CM, Hirooka Y, Tanney JB, Whitfield E, Mwange K, Meijer M, Amend AS, Seifert KA, Samson RA. 2014b. *Aspergillus*, *Penicillium* and *Talaromyces* isolated from house dust samples collected around the world. Studies in Mycology 78: 63–139.

Walker JT, Frazho JK, Randell SC. 2012. A novel case of canine disseminated aspergillosis following mating. The Canadian Veterinary Journal 53: 190–192.

Ward OPP, Qin WMM, Dhanjoon J, Ye J, Singh A. 2005. Physiology and biotechnology of *Aspergillus*. Advances in Applied Microbiology 58: 1–75.

White T, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: a Guide to Methods

and Applications. Eds. MA Innis, DH Gelfand, JJ Sninsky, TJ White. Academic Press, New York, 315–322.

Wicklow DT. 1987. Survival of *Aspergillus flavus* sclerotia in soil. Transactions of the British Mycological Society 89: 131–134.

UNIVERSITAT
ROVIRA i VIRGILI