CARACTERIZACIÓN FENOTÍPICA Y FILOGENIA MOLECULAR DE HONGOS EXTREMÓFILOS

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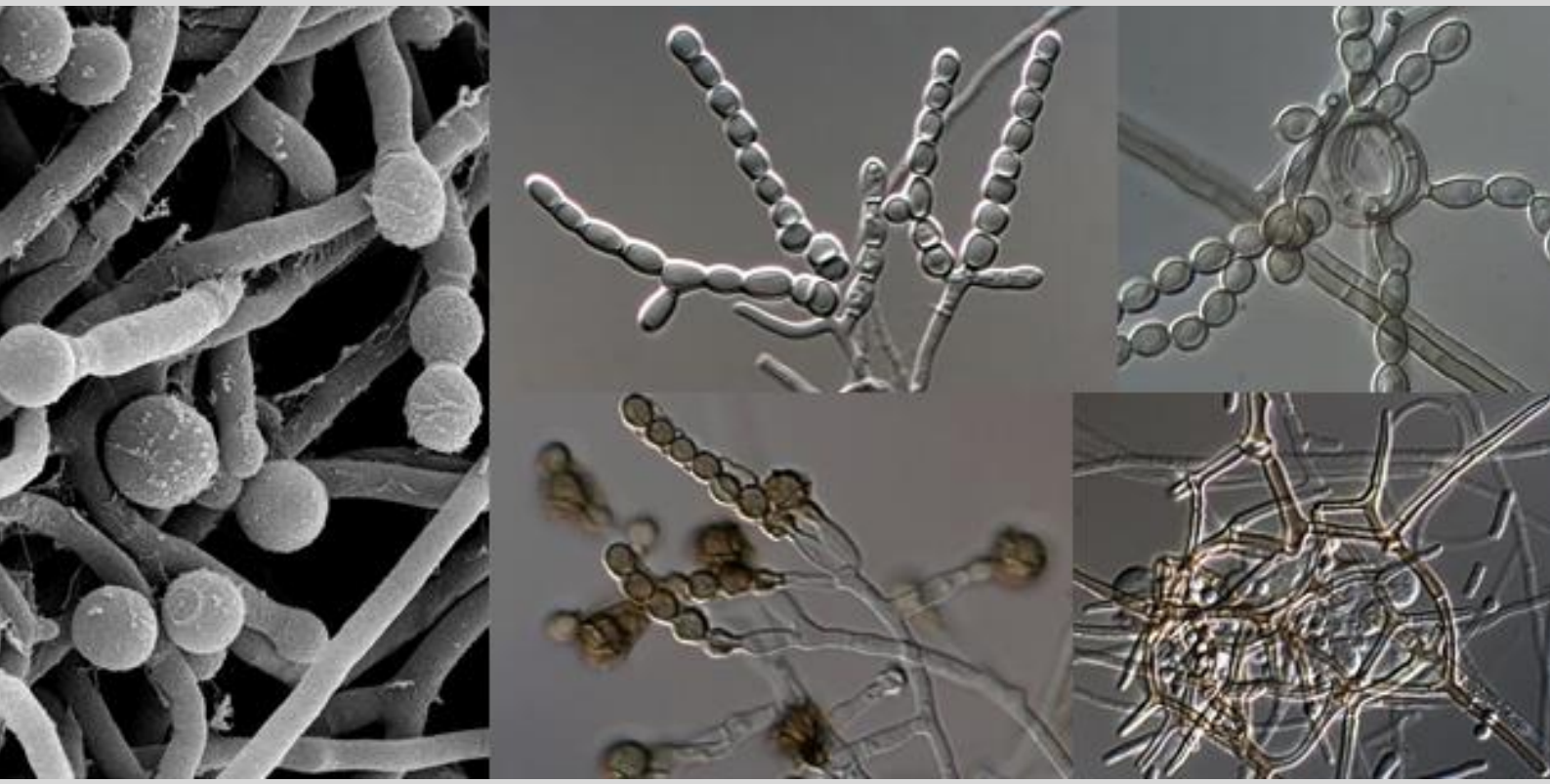

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Caracterización fenotípica y filogenia molecular de hongos extremófilos

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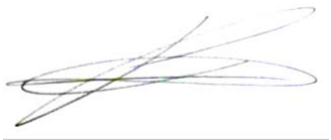
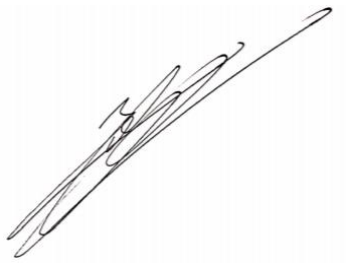
HACEMOS CONSTAR que el presente trabajo, titulado "**Caracterización fenotípica y filogenia molecular de hongos extremófilos**", que presenta D. **Ernesto Rodríguez Andrade** para la obtención del título de Doctor por la URV, ha sido realizado bajo nuestra supervisión en el Departamento **de Ciencias Médicas Básicas**.

Reus, 21/09/2020

Los directores de la tesis doctoral





Alberto M. Stchigel Glikman **José F. Cano Lira** **Josep Guarro Artigas**

ÍNDICE DE ABREVIATURAS

ADN	Ácido desoxirribonucleico
AFG	Anidulafungina
AMB	Anfotericina B
ARNr	Ácido ribonucleico ribosomal
ATCC	*American Type Culture Collection*
a_w	Actividad de agua
BCP-MS-G	Agar glucosado con sólidos de leche y púrpura de bromocresol
BenA	β-tubulina
BI	Inferencia bayesiana
BS	Soporte de bootstrap
CaM	Calmodulina
CBS	*Westerdijk Fungal Biodiversity Institute Culture Collections*
CFG	Caspofungina
CLSI	*Clinical Laboratory Standards Institute*
cm^2	Centímetro cuadrado
CME	Concentración mínima eficaz
CMI	Concentración mínima inhibitoria
CREA	Agar con creatina y sacarosa
DG18	Agar con dicloran y glicerol al 18 %
comb. nov.	Nueva combinación
c.s.p.	Cantidad suficiente para
c/u	Cada una
Cu_2SO_4	Sulfato de cobre
D.O.	Denominación de origen
CYA	Agar de Czapek con extracto de levadura
EDTA	Ácido etilendiaminotetraacético
EMBL-EBI	Instituto Europeo de Bioinformática
Etc.	Etcétera
fam. nov.	Familia nueva
Fig.	Figura
FLC	Fluconazol
FMR	Facultad de Medicina de Reus
g	Gramos
G18	Agar con glicerol 18 %
G25N	Agar con nitrato y 25 % glicerol
H_2SO_4	Ácido sulfúrico
HRA	Humedad relativa ambiental

gen. nov.	Género nuevo
H^+	Protones
ITC	Itraconazol
ITS	Región espaciadora intergénica transcrita del ARNr
keV	Kiloelectronvoltio
L	Litro
MCMC	*Markov chain Monte Carlo*
MEA	Agar con extracto de malta
MEGA	Análisis Genético Molecular Evolutivo
MFG	Micafungina
M	Molar
mg	Miligramos
ML	Análisis de máxima verosimilitud
mL	Mililitros
mm	Milimetros
mM	Milimolar
mm^2	Milimetros cuadrados
mTorr	miliTorricelli
MUSCLE	*Multiple sequence comparison by log-expectation*
MY70FG	Agar con extractos de malta y levadura y 70 % de fructosa/glucosa
Na^+	Ion sodio
NaCl	Cloruro de sodio
OA	Agar con harina de avena
pb	Pares de bases
PCA	Agar con extracto de patata y zanahoria
pCO_2	Presión parcial de dióxido de carbono
PCR	Reacción en cadena de la polimerasa
PDA	Agar con extracto de patata y glucosa
pH	Potencial hidrógeno
pOsm	Presión osmótica
PP	Probabilidad posterior
PSC	Posaconazol
PVA	Alcohol polivinílico
PYE	Agar con fitona y extracto de levadura
p/p	Peso en peso
p/v	Peso en volumen
rpb2	subunidad 2 de la ARN polimerasa
RPM	Revoluciones por minuto
RA	Reproducción asexual
RS	Reproducción sexual

RPMI	Medio Roswell Park Memorial Institute
Sec.	Sección
SDA	Agar de Sabouraud glucosado
SDS	Dodecilsulfato sódico
sp.	Especie
sp. nov.	Especie nueva
spp.	Especies
TOMT	Agar para prueba de opacidad con Tween 80
TRB	Terbinafina
USA	Estados Unidos de América
UTHSC	*Fungus Testing Laboratory of the University of Texas Health Sciences Center*
v.	Versión
var.	Variedad
v/v	Volumen en volumen
VRC	Voriconazol
y col.	Y colaboradores
YES	Agar con extracto de levadura y sacarosa
'	Minutos
''	Segundos
µL	Microlitros
±	Más/menos
>	Mayor que
<	Menor que
≤	Menor o igual a
≥	Mayor o igual a
®	Marca registrada
ºC	Grados centígrados
%	Por ciento
TCA	2,4,6-tricloroanisol
LSU	Gen 28S del ARNr
5FC	5-fluorocitosina
$7H_2O$	Heptahidratada

1. INTRODUCCIÓN

1.1. Biología de los hongos

Los hongos son un grupo monofilético (con un ancestro común) de organismos eucariotas no fotosintéticos (Whittaker 1969). Las células fúngicas están rodeadas de una pared químicamente compleja, que siempre contiene una importante cantidad de polisacáridos. La quitina es uno de ellos, y está presente en la mayoría de los taxones fúngicos (Pontón 2008). Sin embargo, la composición cuali-cuantitativa de los mismos es muy variable, y ha resultado ser una valiosa herramienta taxonómica (Bartnicki-García 1968; Leal y Bernabé 1998). La membrana plasmática no difiere en composición y estructura de la de otros organismos eucariotas, pero al contrario que los protozoos y animales, carece de colesterol y de sus ésteres, siendo estos reemplazados por otros esteroles, como por ejemplo el ergosterol (Pontón 2008; Brandt y Warnock 2015). En cuanto a su organización celular, es de destacar el pequeño tamaño de los núcleos en comparación con otros organismos eucariotas (Griffin 1994; Markham 1995; Madigan y col. 2003), lo poco desarrollado de su aparato de Golgi (Markham 1995; García-Rodríguez y col. 2006), los centriolos difícilmente detectables salvo cuando ocurren eventos ligados a la meiosis (Roberson y col. 2010), y que las mitocondrias contienen membranas internas distribuidas de forma paralela al eje mayor de dicho orgánulo (Alexopoulos y col. 1996; Carlile y Watkinson 2001).

La mayoría de los hongos, a excepción de los grupos taxonómicos menos evolucionados, tales como las divisiones *Cryptomycota* y *Chytridiomycota* (James y col. 2006; 2013; Spatafora y col. 2016), presentan dos tipos de organización somática o vegetativa básica (también denominado talo): unicelular, en las levaduras u hongos levaduriformes; y pluricelular, en los hongos filamentosos o miceliares (Fig. 1). La unidad estructural y funcional básica de los hongos filamentosos son las hifas, una estructura tubular multicelular (Brock 2006; Brandt y Warnock 2015). Las células fúngicas dentro de la hifa pueden estar delimitadas por septos (hifas septadas ó tabicadas) o no (hifas aseptadas ó cenocíticas). Estos septos son invaginaciones de la pared celular que separa la hifa en compartimentos intercomunicados o no entre sí, y pueden ser relativamente simples o más complejos, como en los miembros de la división *Basidiomycota*. La organización estructural del septo ha demostrado tener valor taxonómico a nivel de órdenes y clases fúngicas (Berbee y Taylor 1999).

Figura 1. Tipos de talo fúngico. A, B. Unicelular. C. Pluricelular. Barras de escala = 10 µm.

Los núcleos de los hongos están rodeados de una doble membrana biológica, tal como en otros organismos eucariotas, pudiendo ser haploides o diploides (Carlile 1980).

Los hongos pueden multiplicarse mediante la producción de propágulos de dispersión de origen mitótico (mitosporas) y/o de origen meiótico (meiosporas) (Seifert y Samuels 2000). Los mecanismos implicados en la producción de dichos propágulos de dispersión, así como sus características morfológicas han sido las herramientas más empleadas (y aún siguen siendo empleadas) en su clasificación (Hawksworth y col. 1983; Montes y col. 2003).

Los hongos desempeñan un papel fundamental en la homeostasis de los ecosistemas terrestres y acuáticos, participando activamente como descomponedores de la materia orgánica muerta (necrótrofos o saprobios; van Oorschot 1980), mediante la asociación mutualista con organismos como las algas (Honegger 1991), las plantas (Quilambo 2003) y los animales (Ho y col. 2000; Palma y col. 2005), y regulando la población de otros organismos por parasitismo (Zhang y col. 2004; Silva y col. 2007), depredación (Duddington 1951; Drechsler 1960) o mediante la producción de metabolitos tóxicos (Hoffmeister y Keller 2007). Dicho estilo de vida está estrechamente relacionado con un metabolismo energético que depende de la oxidación parcial o total de moléculas orgánicas (quimioorganotrofos), tales como azúcares, aminoácidos, ácidos orgánicos, alcoholes o alcanos (Cano y col. 1987;

Santamaría 1989, Carroll y Wicklow 1992; Cano y col. 2002; Brock 2006; D´Annibale y col. 2006; Stchigel y col. 2006; Crous y col. 2007; Voglmayr y col. 2011; García-Massini y col. 2012), que obtienen de otros organismos vivos o muertos.

1.2. Sistemática de los hongos

Los hongos durante mucho tiempo se incluyeron dentro del reino *Plantae* (Philippi 1860; Haeckel 1866; Briquet 1912), en el subreino *Talobionta* (talobiontas o plantas con talo), considerándose como descendientes de las algas rojas (Scagel y col. 1980; Carlile y Watkinson 2001), incluso cuando Copeland propuso en 1938 una nueva clasificación basada en cuatro reinos (*Animalia*, *Monera*, *Plantae* y *Protoctista*). No fue hasta 1969 que éstos fueron considerados un reino independiente (*Fungi*) (Whittaker 1969; Kirk y col. 2008), y considerándose a los hongos evolutivamente más cercanos a los animales que a las plantas (Shenoy y col. 2007). En el año de 1998 se sumó el reino *Chromista* (entre los cuales se incluyen las clases *Hyphochytridiomycetes* y *Oomycetes*, antes clasificadas en el reino *Fungi*) a los ya existentes (Cavalier-Smith 1998). Actualmente, basados en estudios de filogenia molecular, el reino *Fungi* es uno de los siete reinos y dos super-reinos (dominios o imperios) del árbol de la vida (Brandt y Warnock 2015; Ruggiero y col. 2015; Fig. 2).

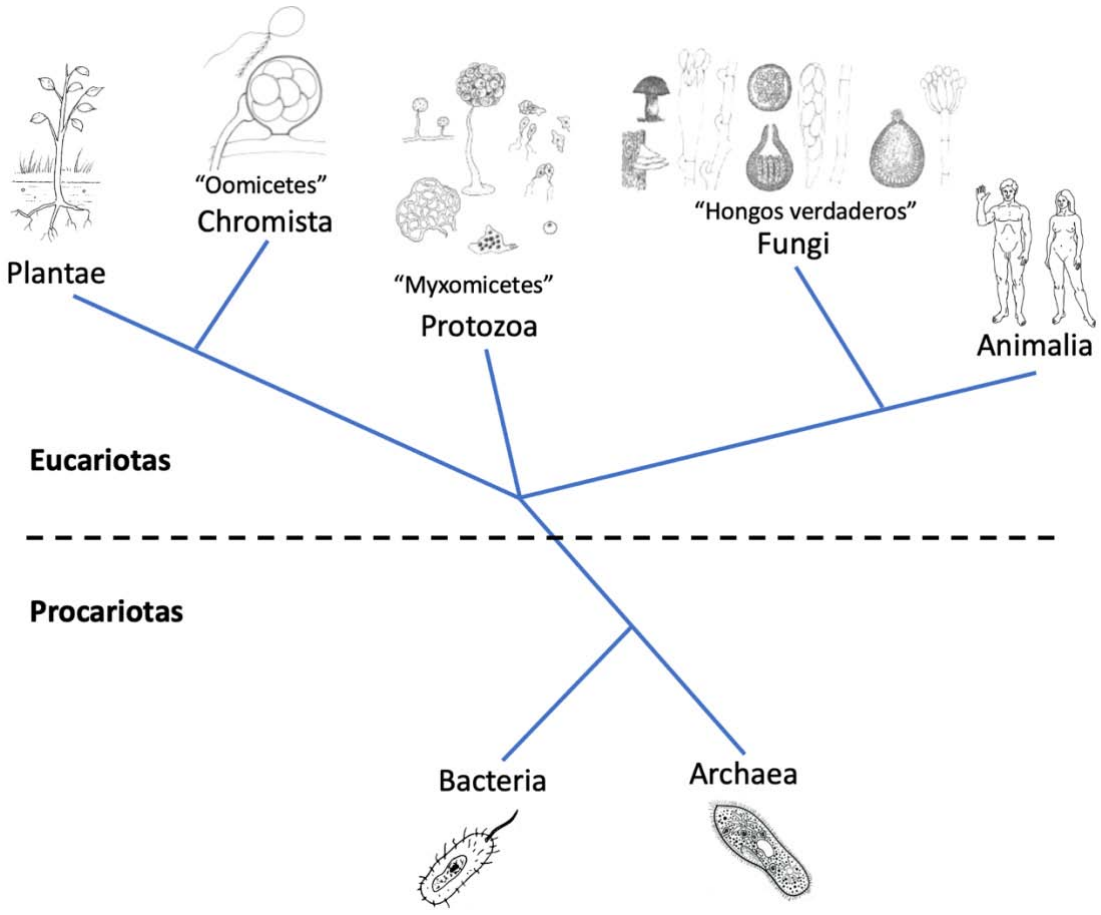


Figura 2. Clasificación jerárquica de los super-reinos y reinos en el árbol de la vida (modificado de Hernandéz-Restrepo 2013; Ruggiero y col. 2015).

La taxonomía clásica de hongos estaba basada en la comparación de sus características morfológicas (y menos frecuentemente en las fisiológicas), dando especial énfasis a la caracterización exhaustiva de las estructuras implicadas en la reproducción sexual (teleomorfo, "sexual morph"). Así, en la séptima edición del "*Ainsworth & Bisby´s Dictionary of the Fungi*" (Hawksworth y col. 1983), los hongos fueron clasificados en dos divisiones: *Myxomycota* (hongos mucilaginosos, que incluyen una fase fagocítica ameboide durante su ciclo de vida), y *Eumycota* (los hongos verdaderos, que son aquellos que carecen de una etapa ameboide y tienen nutrición absortiva). Dentro de *Eumycota* fueron incluídas cinco subdivisiones: I) *Ascomycotina*, caracterizada por la producción de meioesporas dentro de estructuras denominadas ascos; II) *Basidiomycotina*, con meioesporas producidas en la superficie de células llamadas basidios; III) *Mastigomycotina*, caracterizada por la producción de meioesporas flageladas y con motilidad; IV) *Zygomycotina*, un grupo caracterizado por sus hifas aseptadas y cuyas meioesporas, denominadas zigosporas, son estructuras únicas y de

paredes gruesas, oscuras y ornamentadas; y V) *Deuteromycotina*, la cual incluía aquellos hongos sin reproducción sexual conocida (Fig. 3).

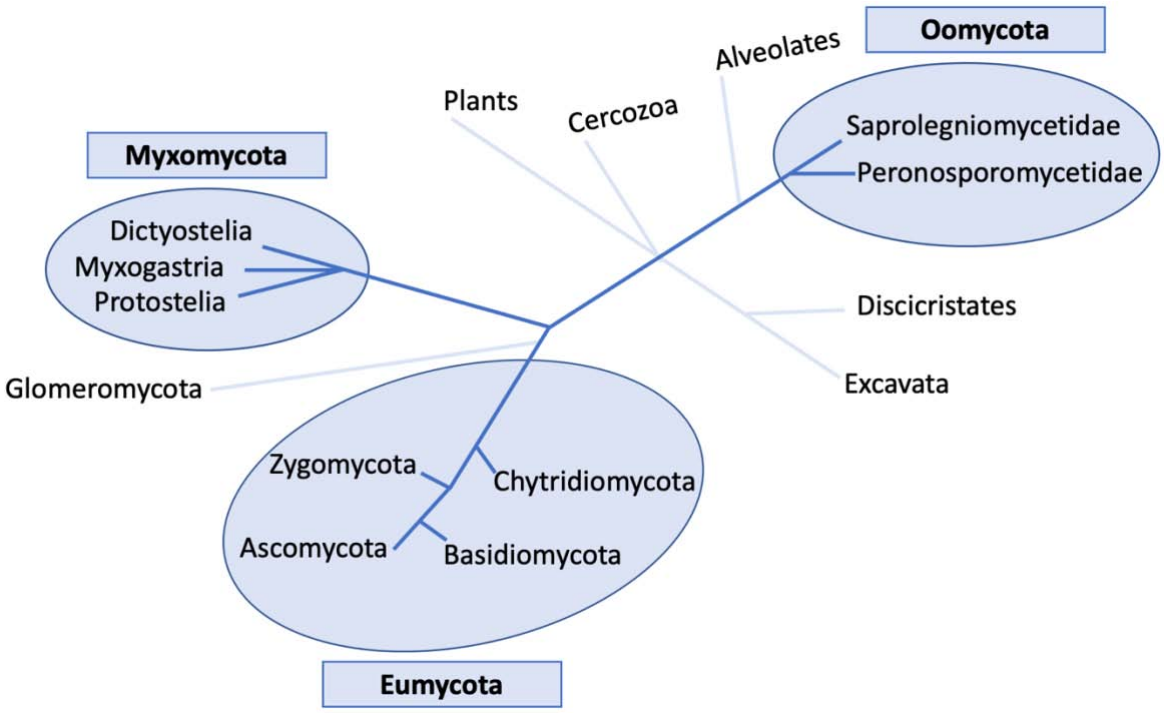


Figura 3. Clasificación de los hongos microscópicos y filamentosos en el árbol filogenético de la vida (modificado de Jobard y col. 2010).

Desde mediados de la década de los ochenta, los análisis filogenéticos basados en el estudio comparativo de las secuencias nucleotídicas de los genes ribosomales y de otros genes estructurales, a los que se han sumado los denominados *housekeeping* (genes que están involucrados en pasos clave del metabolismo celular; Goldman 2001), vienen marcando las pautas para proponer un sistema de clasificación de los organismos fundamentado en criterios más objetivos que los precedentes, que se basaban preferentemente en el empleo de criterios taxonómicos fenotípicos. Consecuentemente, los hongos han sufrido un proceso severo de reclasificación. Mientras que *Myxomycota* fue excluida del reino *Fungi* (Cavalier-Smith 1993), los *Microsporidia*, un grupo de organismos parásitos obligados, fueron incluidos en este reino (Keeling y col. 2000; Hibbett y col. 2007). La subdivisión *Deuteromycotina* fue eliminada como un taxón válido (Kirk y col. 2008), siendo la mayoría de sus miembros transferidos a las divisiones *Ascomycota* (mayoritariamente) y *Basidiomycota* (Saikawa y col. 1994; de Beer y col. 2003; 2006). También *Mastigomycotina* pasó a ser un taxón obsoleto, y sus miembros fueron incluidos en tres divisiones segregadas del reino *Fungi*: *Chytridiomycota*

(James y col. 2006; Hibbertt y col. 2007), *Blastocladiomycota* (Cooke y col. 2000) y *Neocallimastigomycot*a (Cavalier-Smith y Chao 2003). Por otro lado, la subdivisión *Zygomycotina* tampoco fue aceptada como taxón válido, siendo sus miembros repartidos en cuatro subdivisiones (*Entomophthoromycotina*, *Kickxellomycotina*, *Mucoromycotina* y *Zoopagomycotina*; Hibbett y col. 2007). Posteriormente, basados en la filogenia molecular, y en la reconstrucción de las historias evolutivas teniendo en cuenta genomas completos o de fragmentos muy extensos de los mismos (filogenómica; Patané y col. 2017), se reordenó el reino *Fungi* en ocho divisiones, doce subdivisiones y cuarenta y seis clases (Fig. 4; Spatafora y col. 2017). Más recientemente, basados en posteriores estudios moleculares, se aceptan dentro del reino *Fungi* nueve divisiones: *Opisthosporidia*, *Ascomycota*, *Basidiomycota*, *Blastocladiomycota*, *Chrytridiomycota*, *Glomeromycota*, *Mucoromycota*, *Neocallimastigomycota* y *Zoopagomycota* (Naranjo-Ortiz y Gabaldón 2019).

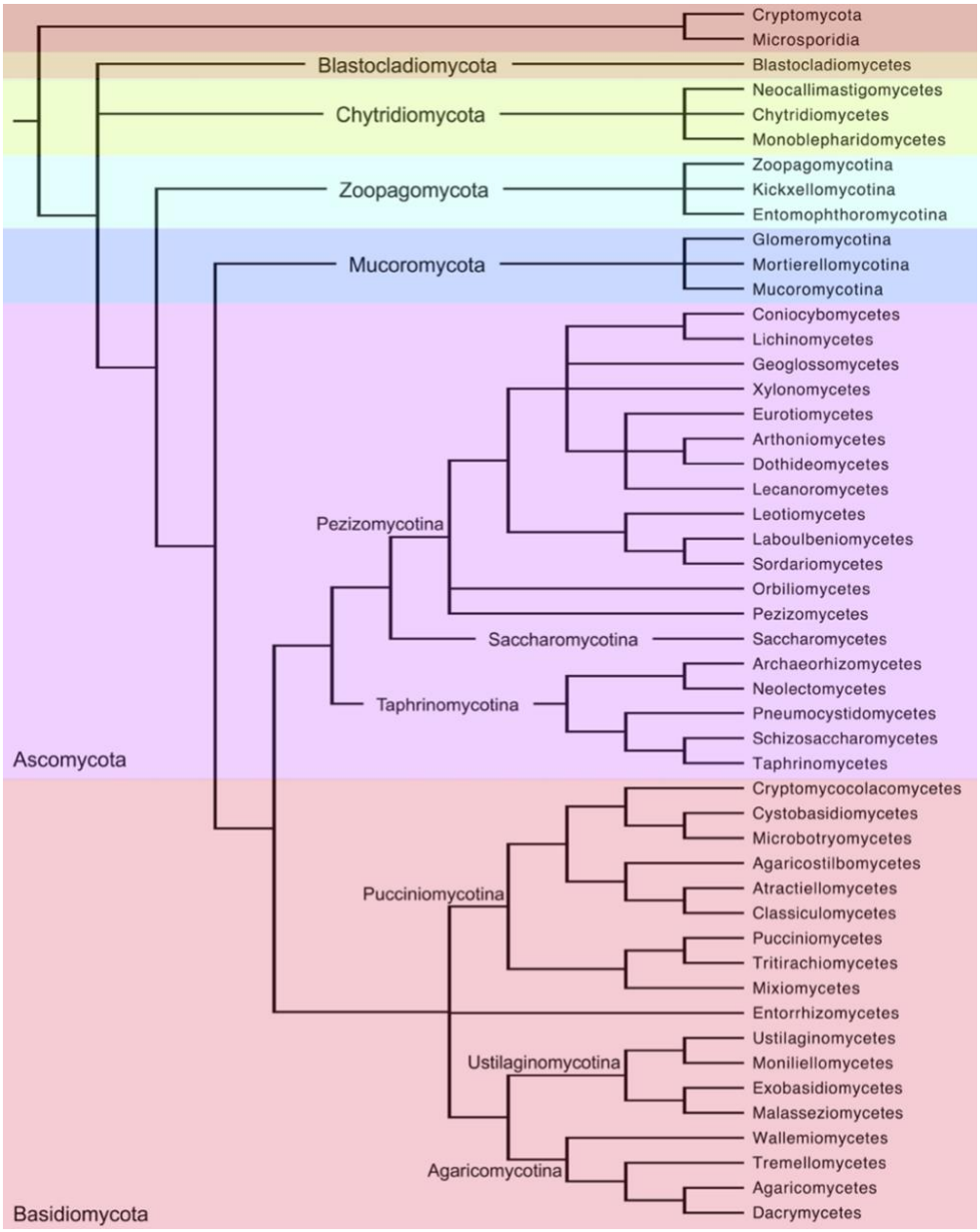


Figura 4. Árbol filogenético teórico del reino *Fungi*. De izquierda a derecha figuran las divisiones, las subdivisiones y las clases (tomado de Spatafora y col. 2017).

1.3. Los hongos extremófilos

El término “extremófilo” fue utilizado por primera vez hace casi medio siglo. Dicho término fue empleado para clasificar algunos microorganismos capaces de prosperar en condiciones adversas para la gran mayoría de las formas de vida (MacElroy 1974). Sin embargo, “ambiente extremo” es un término relativo, ya que aquellas condiciones ambientales que para algunos organismos pueden ser extremas, para otros pueden ser esenciales para su supervivencia

(Ramírez y col. 2006). Por dicho motivo es difícil establecer el significado real que tienen los términos "extremo" y "normal" para los microorganismos (Gorbushina y Krumbein 1999). Algunos ambientes sometidos a temperaturas extremas (por debajo de 0 ºC y por encima de 40 ºC), a concentraciones elevadas de metales pesados, moléculas inorgánicas u orgánicas tóxicas, con una muy baja disponibilidad de agua, con elevadísimas concentraciones de solutos, entre otros, son considerados inhabitables para la mayoría de las formas de vida y, a pesar de ello, pueden albergar y dar sustento a ciertas formas de vida (Rampelotto 2013). Recientes estudios han ido estableciendo las condiciones medioambientales límites para los diferentes organismos (Kashefi y Lovley 2003; Cowan y Tow 2004; Hallsworth y col. 2007; Bhaganna y col. 2010; Chin y col. 2010; Golyshina 2011; Cray y col. 2013; Harrison y col. 2013; Krisko y Radman 2013; Yakimov y col. 2015). Sin embargo, escasean las investigaciones centradas en los mecanismos fisiológicos y bioquímicos implicados en la supervivencia de los organismos en ambientes extremos (Stevenson y col. 2015).

Ciertos hongos tienen la habilidad de colonizar ambientes cuyas características físicas y/o químicas son altamente adversas para la supervivencia de la mayoría de otras formas de vida (Ramírez y col. 2006; Onofri y col. 2011), tales como suelos polares sometidos a muy bajas temperaturas y a una intensa desecación de forma constante (Selbmann y col. 2005; Maggi y col. 2013), aguas hipersalinas (Zalar y col. 1999), fuentes hidrotermales (Appoloni y col. 2008; Yamazaki y col. 2010), ecosistemas desérticos (Staley y col. 1982; Selbmann y col. 2005), suelos y aguas alcalinas (Nagai y col. 1998; Kroll 1990) o ácidas (Battley y Bartlett 1966; Baker y col. 2004; Selbmann y col. 2008), o substratos con una actividad de agua (a_w) muy reducida (Pitt y Christian 1968; Hocking 1986; Pérez-Sánchez y col. 1997; Pettersson y col. 2011; Seijo y col. 2011; Terrab y col. 2019). Además, es conocido que ciertos hongos pueden sobrevivir a la exposición de dosis de radiaciones ionizantes letales para la gran mayoría de los seres vivos (Zhdanova y col. 2004; Kimura y col. 2006; Dadachova y Casadevall 2008; Selbmann y col. 2011), siendo además capaces de proliferar en ambientes con elevadas concentraciones de metales pesados (Ceci y col. 2012) o de radionúclidos (Dadachova y col. 2007), o en condiciones similares a las del espacio exterior (Onofri y col. 2012).

1.3.1. *Hongos xerófilos*

La disponibilidad de agua es el contenido efectivo de agua expresado como su fracción molar y expresada como actividad de agua (a_w), teniendo el agua pura un valor de a_w de 1 y

todas las demás soluciones una $a_w < 1$. Así mismo, la disponibilidad de agua determina la vitalidad y funcionalidad de las diferentes formas de vida (Zajc y col. 2014). La mayoría de los microorganismos no pueden multiplicarse una a_w por debajo de 0,9 (Brown 1976; Manzoni y col. 2012; Moyano y col. 2013), pero algunos organismos son capaces de multiplicarse hasta valores de a_w de 0,61 (Pitt 1975; Williams y Hallsworth 2009, Stchigel 2014). Aquellos microorganismos capaces de crecer a una $a_w \leq 0,85$ se les denomina xerotolerantes, y aquellos cuyo desarrollo óptimo ocurre a dichos valores de a_w se les llama xerófilos (Pettersson y Leong 2011). Los hongos xerófilos son mucho más tolerantes al estrés hídrico que otros tipos de organismos, siendo *Xeromyces bisporus* la especie xerófila más extrema conocida hasta el presente, capaz de crecer a a_w menores a 0,70, e inclusive formar colonias visibles a una a_w de 0,61–0,62 (Pitt y Hocking 2009; Stchigel 2014). Un género filogenéticamente cercano a *X. bisporus*, y que también tiene la capacidad de crecer en condiciones de bajas a_w, es *Monascus* (Hawksworth y Pitt 1983; Hocking y Pitt 1988; Snowdon y Cliver 1996; Barbosa y col. 2017). Dentro del género *Aspergillus*, la especie más xerófila es *A. halophilicus*, cuyas ascosporas son capaces de germinar a una a_w de 0,675 (Christensen y col. 1959). La mayoría de las especies xerófilas del género *Aspergillus* se encuentran en la sección *Restricti* (Peterson 2008). Por ejemplo, *A. restrictus* y *A. penicilloides* son capaces de crecer a $a_w < 0,73$ (Andrews y Pitt 1987). Otras especies xerotolerantes/xerófilas del género como *A. appendiculatus*, *A. montevidensis* y *A. pseudoglaucus*, entre otras, habitualmente contaminan alimentos con una a_w relativamente baja, como los cereales (Kozakiewicz 1989), o las especies *A. niger*, *A. sydowii* y *A. tubingensis*, que afectan preferentemente a los frutos secos o chocolates (Pitt 1966; Pitt y Christian 1968; de Clercq y col. 2015). *Basipetospora halophila* (actualmente *Phialosimplex halophilus*) es otro hongo xerófilo que tiene una a_w óptima de crecimiento de 0,73, aunque crece más rápidamente en medios de cultivo con la a_w equivalente de cloruro de sodio que con glucosa (comportamiento halofílico), motivo por el cual se la suele clasificar cómo una especie halófila (Pettersson y Leong 2011). Por otro lado, se sabe de algunas especies del género *Chrysosporium* que son xerófilas extremas, tales como *Chrysosporium farinicola* y *C. inops*, que pueden crecer a $a_w < 0,72$, *Bettsia fastidia* a a_w 0,69 y *Xerochrysium xerophilum* a a_w 0.71 (anteriormente clasificadas como *C. fastidium* y *C. xerophilum*, respectivamente) (Kinderlerer y Kellard 1984; Kinderlerer 1997; Gock y col. 2003; Leong y col. 2011; Pettersson y Leong 2011; Pitt y col. 2013). El género *Wallemia* contiene varias especies xerófilas, tales como *W. itchthyophaga*, *W. muriae* o *W. sebi* con crecimientos en a_w de 0,77 y 0,83 para las

dos últimas, estas especies usualmente contaminan y alteran alimentos ricos en azúcares (frutas, pasteles, azúcar de caña), en sal (pescado, carne, cacahuates) y alimentos deshidratados (Samson y col. 2002). Estos hongos han sido también aislados de sal marina (Høye 1902), suelos (Domsch y col. 1980), y del aire de hogares (Takahashi 1997). Otro de los sustratos de donde se han reportado un número importante de hongos xerófilos y xerotolerantes es la miel, con especies tales como *Ascosphaera apis*, *Aspergillus* spp., *Bettsia alvei*, *Blastobotrys meliponae*, *Metschnikowia reukaufii*, *Monascus* spp., *Schizosaccharomyces octosporus*, *Trichosporon mucoides* y *Zygosaccharomyces* spp., entre otros, algunos de los cuales están estrechamente ligados a la forma de vida de los organismos que la producen: las abejas (Lochhead y Farrel 1931; Ruiz-Argueso y Rodríguez-Navarro 1975; Snowdon y Cliver 1996; Magyar y col. 2005; Kačániová y col. 2009; Carvalho y col. 2010; Pettersson y Leong 2011; Kačániová y col. 2012; Saksinchai y col. 2012a, 2012b; Sinacori y col. 2014; Čadež y col. 2015; Grabowski y Klein 2015; Crous y col. 2016; Barbosa y col. 2017, 2018). Por otro lado, se han aislado varios hongos xerófilos/xerotolerantes de diversos artículos deteriorados pertenecientes al patrimonio histórico-cultural, tales como tallas de madera, pinturas, artículos de cuero y metal de órganos de iglesias, de libros y papel, entre otros (Pinzari y Montanari 2011; Borrego y col. 2012; Montanari y col. 2012; Micheluz y col. 2015; Sterflinger y col. 2018).

Muchos de los microorganismos expuestos a un elevado estrés hídrico han desarrollado métodos para sobrevivir en estas condiciones, pero pocos han desarrollado las adaptaciones fisiológicas que les permiten crecer de forma óptima en entornos con una baja a_w. Los hongos xerófilos son organismos-modelo indispensables para investigar los mecanismos moleculares que determinan la supervivencia de estas y otras formas de vida en condiciones de extrema aridez (Onofri y col. 2004; Beaty y Buxbaum 2006; Tosca y col. 2008). Así, la acumulación citoplasmática de glicerol por parte de hongos en respuesta a una baja a_w medioambiental fue reportada en 1982 para los xerófilos *Chrysosporium fastidium*, *Wallemia sebi* y *Xeromyces bisporus* (Pettersson y Leong 2011), así como en los hongos xerotolerantes *Aspergillus niger* y *Penicillium chrysogenum* (Hocking 1993; de Clercq y col. 2015). También ciertas especies fúngicas tales como *Mucor hiemalis*, *Phytophthora cinnamomi* y *Pythium debaryanum* sintetizan el aminoácido prolina cuando crecen en medios con alta concentración de solutos

iónicos o no-iónicos, actuando en una vía similar a la del glicerol, permitiendo así una función eficiente de sus enzimas (Luard 1982).

1.3.2. *Hongos osmófilos y caofílicos/caotolerantes*

Como se ha mencionado previamente, muchos hongos tienen la capacidad de tolerar y crecer activamente bajo condiciones de estrés hídrico (Magan 2007). Entre estos, los llamados hongos osmófilos y osmotolerantes son capaces de crecer en ambientes con altas concentraciones de solutos (polioles o azúcares, generalmente) (Pettersson y Leong 2011). Los solutos disueltos en el entorno reducen la cantidad de agua disponible (y consecuentemente la a_w), pero también incrementan la presión osmótica y desorganizan la red tridimensional del agua, ejerciendo efectos tóxicos sobre los microorganismos debido a la capacidad de promover la desnaturalización de sus macromoléculas (agentes caotrópicos) (Zajc y col. 2014). En el año 2000, varios hongos osmófilos (*Aureobasidium pullulans*, *Hortaea werneckii*, *Phaeotheca triangularis* y *Trimmatostroma salinum*) fueron reportados cómo habitantes de las denominadas salinas solares (Gunde-Cimerman y col. 2000). A partir de este primer reporte, muchas especies nuevas para la ciencia han sido descubiertas en entornos hipersalinos naturales de todo el mundo (Wasser y col. 2003; Zalar y col. 2005; Zajc y col. 2014). Algunas de las especies aisladas de dicho habitat eran previamente conocidas como contaminantes de alimentos con baja a_w debido a que eran conservados mediante el empleo de altas concentraciones de sales o azúcares (Tresner y Hayes 1971; Munitis y col. 1976; Pitt y Hocking 1977; Samson y col. 2002; Pitt y Hocking 2009; Carvalho y col. 2010; Kunčič y col. 2009; Saksinchai y col. 2012a, b; Čadez y col. 2015; Crous y col. 2016; Barbosa y col. 2017, 2018).

Recientemente ha sido propuesta una nueva categoría eco-fisiológica de microorganismos: los caofílicos/caotolerantes. Estas formas de vida crecen de forma óptima o toleran (respectivamente) ambientes con elevadas concentraciones salinas, cuya naturaleza físico-química determina su condición de agentes caotrópicos. Algunas especies fúngicas, tales como *Aspergillus amstelodami*, *A. chevalieri*, *Hortaea werneckii* y *Wallemia ichthyophaga* son capaces de prosperar en medios acuáticos hipersalinos (Samson y col. 2002; Butinar y col. 2005; Zalar y col. 2005; Cantrell y col. 2006; Hallsworth y col. 2007; Butinar y col. 2011; Oren y Gunde-Cimerman 2012). Con respecto a su fisiología, sabemos que los hongos caotolerantes producen moléculas orgánicas (solutos compatibles) para equilibrar la presión osmótica del

entorno, acumulando glicerol y manteniendo bajas concentraciones intracelulares de sales, tales como las que contienen iones de Na^+, las que pueden mostrar una elevada toxicidad (Gostinčar y col. 2011; Zajc y col. 2012). Esta estrategia les permite una adaptación más flexible a los cambios de salinidad del medioambiente (Zajc y col. 2014).

1.3.3. *Hongos termófilos*

De todos los factores que afectan la estabilidad celular, la temperatura tiene la mayor influencia sobre la homeostasis celular. Así, la mayoría de los organismos solo pueden crecer dentro de un estrecho rango de temperaturas. Sin embargo, la existencia de actividad geotérmica en medioambientes terrestres y marinos ha permitido la selección y persistencia de microorganismos que no solo resisten, sino que requieren de altas temperaturas para crecer de forma óptima (de Oliveira y col. 2015). Los organismos termófilos se encuentran en los tres dominios en que clasificamos todas las formas de vida. Sin embargo, en el dominio *Eukarya* la tolerancia a altas temperaturas es más estrecha que en *Archaea* y *Bacteria*, y especies que crecen de forma óptima a temperaturas por encima de 61 ºC aún no han sido observadas (Tamsey y Brock 1972; de Oliveira y col. 2015). Solo unas pocas especies de hongos tienen la habilidad de prosperar a temperaturas entre 45 y 55 ºC (Maheshwari y col. 2000). Estos hongos pueden clasificarse como termófilos o termotolerantes, y pueden distinguirse basándose en su temperatura mínima y máxima de crecimiento (Cooney y Emerson 1964): los hongos termófilos crecen en un rango de temperatura entre 20 ºC y más de 50 ºC, y los hongos termotolerantes pueden crecer a una temperatura inferior a 20 ºC y hasta los 50 ºC (Cooney y Emerson 1964). Pero esta definición no es aplicable al conjunto de todos los hongos. Por ejemplo, *Aspergillus fumigatus* puede crecer a temperaturas ≥ 50 ºC y ≤ 20 ºC (Mouchacca 2000). Alternativamente, Maheshwari y colaboradores (2000) propusieron una clasificación más simple, la cual es utilizada ampliamente en la industria y la micología aplicada, en la cual los hongos termófilos son definidos como aquellas especies que tienen una temperatura de crecimiento óptima ≥ 45 ºC. Posteriormente, Morgenstern y colaboradores (2012) utilizaron el criterio de que los hongos termófilos son aquellos que crece más rápido a 45 ºC que a 34 ºC. Sin embargo, la definición más reciente (de Oliveira y col. 2015) considera que los hongos termófilos son aquellos que crecen óptimamente en un rango entre 40 - 50 ºC, separándolos de las especies termotolerantes por su incapacidad de crecer por debajo de los 20 ºC.

El primer reporte de un hongo termófilo fue el de *Mucor pusillus* (sinónimo de *Rhizomucor pusillus*), hace ya más de un siglo (Lindt 1886). Unos pocos años después, se describe *Thermomyces lanuginosus*, capaz de crecer a temperaturas por encima de 50 ºC (Tsiklinsky 1899). El primer estudio sistemático de hongos termófilos es atribuido a Miehe (1907), quien describió dos de las especies más importantes para este grupo: *Malbranchea pulchella* var. *sulfurea* (sinonimizada con *Malbranchea cinnamomea*) y *Thermoascus aurantiacus*. Sin embargo, la primera mención sobre hongos "termófilos" se debe a Apinis, en 1953.

Estos hongos tienen una distribución geográfica amplia, que abarca desde zonas tropicales, pasando por regiones templadas y llegando hasta los polos. Algunos de estos hongos se conocen hace más de cien años, tales como *Malbranchea cinnamomea* (Libert 1857), *Thermoascus aurantiacus* (Miehe 1907), *Thermomyces dupontii* (originalmente *Penicillium dupontii*; Griffon y Maublanc 1911) y *Thermomyces lanuginosus* (Tsiklinsky 1899), mientras que otros han sido descritos más recientemente, tales como *Chaetomium thermophilum* (La Touche 1950), *Thermomyces thermophilus* (Stolk 1965), y *Thermothelomyces thermophilus* (originalmente *Sporotrichum thermophile*; Apinis 1963; Marín-Félix y col. 2015.

Este tipo de hongos pueden resistir temperaturas elevadas debido a la modificación de diferentes sistemas metabólicos respecto a los mesófilos, por ejemplo, la solubilidad de lípidos, la síntesis rápida de metabolitos esenciales, producción de moléculas termo-resistentes y la termo-estabilidad estructural de estas mismas (Crisan 1973). Sin embargo, estos sistemas se han enfocado preferentemente a la producción de proteínas resistentes a elevadas temperaturas, denominadas como "*shock proteins*" (Magan 2007). Entre los *Eukaria* termófilos existe un gran abanico de mecanismos involucrados en la estabilización de estas proteínas, por ejemplo, la termo-estabilidad intrínseca, la estabilización por iones, la adhesión de moléculas chaperonas, autoagregación y la posible asociación con los constituyentes poliméricos de la pared celular (Maheshwari y col 2000).

1.3.4. *Hongos psicrófilos*

Los microorganismos que tienen la habilidad de crecer a temperaturas ≤ 0 ºC, que poseen una temperatura óptima de crecimiento ≤ 15 ºC, y una temperatura máxima de crecimiento ≤ 20 ºC se conocen con el nombre de psicrófilos (Morita 1975). Por otro lado, los organismos psicrotróficos son aquellos que tienen los mismos rangos de crecimiento que los psicrófilos,

pero su temperatura óptima de crecimiento está por encima de los 15 ºC (Morita 1975; Gounot 1991; Cavicchioli y col. 2002). En cuanto a los organismos psicrotolerantes, éstos pueden crecer alrededor de los 0 ºC, tienen una temperatura óptima de crecimiento > 15 ºC y una temperatura máxima de crecimiento > 20 ºC (Morita 1975). Los microorganismos psicrófilos, psicrotróficos y psicrotolerantes se desarrollan en regiones de climas fríos distribuidas a lo largo y ancho de todo el planeta (denominadas en su conjunto cómo criosfera), de los cuales podemos destacar aquellas localizaciones que contienen agua en estado sólido −hielo− en glaciares, lagos y ríos congelados (Kudryashova y col. 2013; Musilova y col. 2015; Walsh y col. 2016; Salazar y Sunagawa 2017), en congeladores (Ahmad y col. 2010) y neveras (Flores y col. 2012). El hielo ha sido considerado como un reservorio de microorganismos encerrados aleatoriamente en su matriz debido a la deposición de estos sobre su superficie (Ma y col. 2000). Sin embargo, existen diferentes tipos de hielo (en cuanto a su forma cristalina, composición química, etc.), tales como la nieve, el hielo glaciar o el hielo marino, que proveen diferentes ambientes en los cuales pueden crecer y reproducirse organismos psicrófilos (Christner y col. 2000; Price y Sowers 2004; Rohde y Price 2007; Price 2009).

Se sabe que las bajas temperaturas pueden dañar las células debido a la formación de cristales de hielo tanto en el exterior como el interior de estas, reducir significativamente la actividad enzimática, disminuir la fluidez de la membrana plasmática y del citosol, obstaculizando así el funcionamiento celular normal en entornos de baja temperatura sin las herramientas de adaptación adecuadas (Raymond y col. 2007; Chandler 2018). Sin embargo, los hongos cuyo metabolismo y estructura celular están adaptados a proliferar en condiciones ambientales de bajas temperaturas permanentemente sobreviven en las profundidades marinas y oceánicas, en los glaciares, en el ártico y en el antártico. Dichos hongos han desarrollado ciertas respuestas fisiológicas, tales como la producción de enzimas con propiedades adaptativas al frío, cambios en la fluidez de la membrana citoplasmática y la producción de ciertos metabolitos que les permiten crecer en dichas condiciones a velocidades comparables con las de los mesófilos (que tienen un óptimo de crecimiento a temperaturas moderadas, de entre 20 y 30 ºC) (D´Amico y col. 2006; Ruisi y col. 2007). Además, este tipo de hongos están ampliamente distribuidos en la criosfera (Hoshino y

Matsumoto 2012) y juegan un papel importante en el reciclaje de nutrientes y tienen la capacidad de descomponer compuestos orgánicos a temperaturas bajo cero (Tsuji 2016).

Los microorganismos psicrófilos fueron descritos por primera vez por Forster (1887), demostrando su presencia en regiones polares, tanto en el agua cómo en los suelos, y sobre una gran variedad de sustratos (Stokes 1963). El número de especies de hongos psicrófilos conocidos es relativamente escaso. Sin embargo, en las últimas dos décadas la diversidad de dicho grupo de hongos se ha ido incrementando paulatinamente (Möller y Dreyfuss 1996; Robinson 2001; Stchigel y col. 2001; Blanchette y col. 2004; Arenz y col. 2006; Connell y col. 2006; Held y col. 2006; Malosso y col. 2006; Duncan y col. 2008; Onofri y col. 2008; Selbmann y col. 2008; Arenz y Blanchette 2009; Jurgens y col. 2009; Lopatina y col. 2013; Bakermans y col. 2014; de Menezes 2016). *Thelebolus microsporus* junto con *Pseudogymnoascus pannorum* (previamente *Geomyces pannorum*) son dos de las especies psicrófilas más extendidas a nivel mundial, y dominantes en diferentes ecosistemas antárticos terrestres (Kobayasi y col. 1967; Montemartini y col. 1993; Marshall 1998; Godinho y col. 2013; Minnis y Lindner 2013). *Thelebolus microsporus* coexiste con otras dos especies recientemente descritas, *Thelebolus ellipsoideus* y *Thelebolus globosus*, en lagos antárticos (de Hoog y col. 2005). *Antarctomyces psychrotrophicus*, un hongo tan solo reportado para la Antártida, también ha sido reportado en ecosistemas terrestres (Stchigel y col. 2001). Recientemente, *Antarctomyces pellizariae* (de Menezes 2016) se reporta cómo un nuevo "snow mould" (moho de la nieve) psicrófilo, que viene a unirse a los ya conocidos *Coprinopsis psychromorbida*, *Microdochium nivale* y *Sclerotinia borealis* (Schipper 1967; Dejardin y Ward 1971; Traquair y Smith 1982; Weinstein y col. 1997; Hsiang y col. 1999; Tronsmo y col. 2001; Singh y col. 2006; Gargas y col. 2009; Hoshino y col. 2010; Anupama y col. 2011; Minnis y Lindner 2013). Además, existen reportes de que *Pseudogymnoascus destructans*, una nueva especie fúngica descrita como productora del "*white-nose syndrome*" en murciélagos hibernantes del hemisferio norte (especialmente letal en individuos de América del Norte), puede crecer en un rango de temperaturas de 3 ºC y 20 ºC, pero no a temperaturas superiores (Gargas y col. 2009; Chaturvedi y col. 2010; Verant y col. 2012). Varias levaduras también han sido descritas como psicrófilas: *Mrakia cryoconiti*, *M. frigida* y *M. psychrophila* han sido reportadas para la Antártida; *Mrakiella aquatica* y *M. niccombsii* en los Alpes y el Ártico; y con distribución cosmopolita, diferentes especies de los géneros *Rhodotorula* y *Cryptococcus* (Margaret 1966; Vishniac y Onofri 2002; Abe y col. 2006;

Xin y Zhou 2007; Margesin y Fell 2008; Robin y col. 2010). Recientemente, de un total de 1.428 aislados fúngicos procedentes de la meseta Qinghai (República Popular de China), usualmente conocida como "*the world´s roof*" o "*the third pole*", se obtuvieron nuevos taxones de hongos psicrófilos, tales como el nuevo género *Psychrophila* y sus especies *P. antarctica*, *P. lutea* y *P. olivacea*, y las especies *Tetracladium ellipsoideum*, *T. globosum* y *T. psychrophilum* (Wang y col. 2015).

Estos hongos han cobrado especial importancia como fuente de enzimas de interés biotecnológico y metabolitos secundarios bioactivos de interés farmacéutico (Flam 1994; Pietra 1997; Biabini y Laatch 1998; Gudjarnnson 1999; Höller y col. 2000; Verbist y col. 2000; Bhadury y col. 2006; Ebel 2006; Blunt y col. 2007; Rateb y Ebel 2011; Wang y col. 2017), así como por la producción de proteínas anticongelantes (Duman 2001), tales como la aislada de *Antarctomyces psychrotrophicus* (Xiao y col. 2010).

1.3.5. *Hongos acidófilos*

Los hongos acidófilos son aquellos que pueden prosperar hasta un valor de pH de 1,0 y crecer activamente hasta un pH < 4,0, y los acidotolerantes los que pueden crecer a pH menores a 4,0, pero también lo pueden hacer a pH neutro, o incluso alcalino (Magan 2007). Los ambientes extremadamente ácidos, tales como los manantiales volcánicos, los drenajes de las minas y las aguas residuales de procedencia industrial (Gross y Robbins 2000; Rothschild y Mancinelli 2001; Tiquia-Arashiro y Rodrigues 2016) tienen a menudo una baja concentración de nutrientes (Brake y Hasiotis 2010) además de altas concentraciones de metales pesados tóxicos (Aguilera y col. 2007) y/o temperaturas extremas (González-Toril y col. 2015). Aun así, hay diferentes formas de vida, incluidos los *Eukarya*, capaces de sobrevivir y proliferar en condiciones medioambientales tan adversas.

Los microorganismos acidófilos son mayoritariamente *Bacteria* (Ingledew 1990), aunque también encontramos *Archea* como *Thermoplasma acidophilum* (González-Toril y col. 2003), o *Eukarya* como el alga unicelular *Cyanidium caldarium* (Seckbach 1994), capaz de crecer en un rango de pH de entre 0 a 4,0. Entre los *Eukarya* acidófilos, las algas y los protozoos han recibido mucha más atención que los hongos. Sin embargo, el rol ecológico de los hongos acidófilos es de indiscutible importancia, habiendo sido reconocidos como participantes activos en la acidificación de medioambientes acuáticos ricos en sulfuros (Armstrong 1921;

Gross y Robbins 2000; Oggerin y col. 2016). La mayoría de los hongos que viven en hábitats ácidos son acidotolerantes (Gross y Robbins 2000). El análisis de secuencias ha demostrado que los hongos *Basidiomycota* acidófilos tienen más del 97 % de similitud con las secuencias nucleotídicas de especies fúngicas disponibles en bases de datos (tales como GenBank), siendo por lo tanto asimilables a taxones conocidos, tales como *Cryptococcus humicola* o *Cystofilobasidium bisporidii*; sin embargo, los hongos acidófilos pertenecientes a las divisiones *Zygomycota* y *Chytridiomycota* tienen menos del 93 % de similitud con las secuencias de las especies más próximas (Gadanho y Sampaio 2006), motivo por el cual existe un gran potencial de nuevos taxones por describir.

Muchas especies de *Aspergillus*, *Fusarium* y *Penicillium* son ácido-tolerantes, y crecen en condiciones de pH ≤ 2,0, mostrando un crecimiento óptimo a pH ligeramente ácidos o incluso alcalinos (Magan 2007). La mayoría de las levaduras tienen un pH óptimo de crecimiento entre 5,5 – 6,0; sin embargo, *Candida acidothermophilum* (nombre actual *Issatchenkia orientalis*), *Cyniclomyces guttulatus*, *Kazachstania exigua*, *Rhodotorula mucilaginosa* y *Saccharomyces cerevisiae* pueden crecer a valores de pH entre 1,5 y 2,5 (Recca y Mrak 1952; Battley y Bartlett 1966).

Hasta el momento actual, el hongo más extremadamente acidófilo que se conoce es *Acontium velatum*, capaz de crecer a una concentración 2,5 M en H_2SO_4 (con pH alrededor de 0), pero también en soluciones saturadas de Cu_2SO_4, en las cuales no sobreviven otras formas de vida (Starkey y Waksman 1943). *Acidomyces acidophilus* es capaz de desarrollarse en condiciones semejantes a las anteriores: aguas ricas en sulfatos y pH próximo 1,0 (Selbman y col. 2008) así como *A. richmondensis* presente en drenajes ácidos de minas (Baker y col. 2004). *Teratosphaeria acidotherma* fue aislada de matas microbianas y biopelículas que se desarrollan en arroyos termales extremadamente ácidos (Yamazaki y col. 2010). Más recientemente, en un estudio llevado a cabo en Río Tinto (Huelva, España), considerado como uno de los ecosistemas ácidos más grandes del planeta, se aislaron 350 cepas fúngicas, determinándose que en su gran mayoría pertenecían a la división *Ascomycota* (*Eurotiomycetes* 52%, *Dothideomycetes* 27%, *Sordariomycetes* 17% y *Leotiomycetes* 2%), mientras que los que pertenecían a la división *Basidiomycota* y *Mucoromycota* representaban menos del 2% de del total (Oggerin y col. 2016).

El estudio de la homeostasis ácido-base en algunos hongos acidotolerantes, se ha estudiado en hongos como *Aspergillus niger*, ayudando a desentrañar los mecanismos fisiológicos que les permiten a estos tolerar y mantenerse viables bajo condiciones de extrema acidez (Hesse y col. 2002). Así, el influjo vacuolar de iones hidrógeno (H^+) junto con una elevada capacidad de secreción de los mismos hacia el exterior celular serían los mecanismos fisiológicos principales que le permitiría a dicho hongo mantener un pH citosólico de alrededor de 7.5 (Magan 2007).

1.3.6. *Hongos alcalófilos*

Se definen como alcalófilos a aquellos organismos incapaces de crecer a un pH < 8,5, o a los que tienen un crecimiento óptimo a pH cercano a 9,0, mientras que los llamados alcalotolerantes se definen como aquellos que tienen un crecimiento óptimo a un pH aproximado de 7,0, pero que pueden crecer activamente a valores de entre 9,0 – 9,5 (Kroll 1990). Algunos ambientes alcalinos, tales como ciertos lagos, suelos, manantiales y drenajes de minas, tienen un pH superior a 10,0, muchas veces debido a la presencia de carbonato de amonio, carbonato de potasio, borato de sodio u ortofosfatos de sodio (Magan 2007). Existen bacterias y arqueas alcalófilas que pueden colorear las aguas próximas a suelos alcalinos, como es el caso del lago Magadi (Kenya), el cual tiene una coloración rosa debido a la expansión masiva de *Archaea* alcalófilas rojas (Jones y col. 1998).

Muchas especies de los géneros *Acremonium*, *Botrytis*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Penicillium*, *Purpureocillium* y *Scopulariopsis* son alcalotolerantes (Okada y col. 1993; Nagai y col. 1995; Nagai y col. 1998; Steiman y col. 2004; Magan 2007; Grum-Grzhimaylo y col. 2016). Por ejemplo, *Purpureocillium lilacinum*, el cual fue descrito originalmente como alcalófilo, puede crecer bien a valores de pH entre 7,5 y 9,0, pero también lo hace a pH ligeramente ácidos (Magan 2007). Otros ejemplos de hongos alcalotolerantes son la levadura "negra" *Exophiala alcalophila* y la levadura *Kluyveromyces marxianus* (Basgal 1931; Kursanov y Turkina 1954; Goto y col. 1981).

En 2005, se describe el hongo alcalófilo *Heleococcum alkalinum*, aislado a partir de suelos hipersalinos con pH alrededor de 10,0 (Bilanenko y col. 2005), siendo posteriormente transferido al nuevo género *Sodiomyces*, en base a estudios de filogenia molecular (Grum-Grzhimaylo y col. 2013). *Sodiomyces alkalophilus* crece de forma óptima en un rango de pH

de 8,7 a 10,5, y es incapaz de crecer a pH < 5,2. En 2016, Grum-Grzhimaylo y colaboradores demostraron la existencia de una gran diversidad fúngica en lagos alcalinos de África y Asia. Algunos de dichos hongos se comportan como alcalófilos, tales como *Acrostalagmus luteoalbus*, especies de *Alternaria* sec. *Soda*, *Emericellopsis alkalina*, *Sodiomyces* spp., y *Thielavia* spp.

Los organismos alcalófilos tienen varias vías metabólicas que se han modificado con respecto a las de los neutrófilos. Por ejemplo, las enzimas que secretan en el medio ambiente funcionan de manera óptima en pH superiores a 9,0 para poder proporcionarles las cantidades suficientes de nutrientes (Kladwang y col. 2003). Además, estos organismos han modificado los transportadores de membrana involucrados en la exportación de metabolitos para regular la homeostasis, y modificado la expresión de ciertos genes involucrados en la tolerancia a pH altos (Grum-Grzhimaylo y col. 2013). Queda por descubrir si los diferentes linajes de hongos muestran una evolución convergente en cuanto a estos mecanismos adaptativos, o bien estos se han diversificado para permitirles sobrevivir en dichos ambientes (Grum-Grzhimaylo y col. 2016).

1.4. Taxonomía de los hongos extremófilos

Dentro la división *Ascomycota* encontramos la mayor diversidad de los llamados hongos extremófilos. De este modo, dentro de los hongos tolerantes al estrés hídrico (caofílicos/caotolerantes, osmófilos/osmotolerantes, xerófilos/xerotolerantes) encontramos miembros del orden *Capnodiales,* tales como *Cladosporium* spp. (Montanari y col. 2012; Sinacori y col. 2014) y *Hortaea werneckii* (Kogej y col. 2007; Zajc y col. 2014); del orden *Eurotiales* (uno de los que presenta mayor biodiversidad) *Aspergillus* spp. (Andrews y Pitt 1987; Kozakiewicz 1989; Samson y col. 2002; Peterson 2008; Pinzari y Montanari 2011; Montanari y col. 2012; Grabowski y Klein 2015), *Phialosimplex halophilus* (Pettersson y Leong 2011), *Eurotium* spp. (de Clercq y col. 2015), *Monascus* spp. (Hawksworth y Pitt 1983; Hocking y Pitt 1988; Snowdon y Cliver 1996; Barbosa y col. 2017), *Penicillium* spp. (Thom 1906; Pitt y Christian 1968; http://www.cabri.org/collections.html; http://gcm.wfcc.info/), *Talaromyces* spp. (Barbosa y col. 2018), *Xerochrysium xerophilum* (Pitt y col. 2013) y *Xeromyces bisporus* (Pitt y Hocking 2009; Stchigel 2014); del orden *Hypocreales*, *Acremonium* spp. (Sterflinger y col. 2018); del orden *Onygenales*, *Ascosphaera apis* (Sabatè y col. 2009) y especies de los géneros *Chrysosporium* (Pitt 1966; Kinderlerer y Kellard 1984; Gock y col. 2003; Leong y col.

2011; Pettersson y Leong 2011); del orden *Pleosporales*, *Alternaria* spp. (Kačániová y col. 2009; Kačániová y col. 2012; Sinacori y col. 2014), *Epicoccum nigrum* (Micheluz y col. 2015) y *Phaeosphaeria typharum* (Micheluz y col. 2015); del orden *Saccharomycetales*, las levaduras *Blastobotrys meliponae* (Crous y col. 2016), *Candida* spp. (Gilliam y col. 1974b; Saksinchai y col. 2012a, 2012b), *Metschnikowia reukaufii* (Magyar y col. 2005) y *Zygosaccharomyces* spp. (Lochhead y Farrel 1931); del orden *Schizosaccharomycetales*, la levadura *Schizosaccharomyces octosporus* (Gilliam y col. 1974b); y algunos hongos de posición taxonómica incierta (*incertae sedis)*, tales como *Bettsia alvei* (Burnside 1929; Skou 1972, 1975) y *Lecanicillium kalimantanense* (Micheluz y col. 2015).

Por otro lado, aquellos hongos que pueden crecer a temperaturas extremas (psicrófilos y termófilos) también están principalmente representados dentro de la división *Ascomycota*, con taxones dentro de los órdenes *Amphisphaeriales* (*Microdochium nivale*; Tronsmo y col. 2001), *Capnodiales* (*Cladosporium* spp.; Kostadinova y col. 2009), *Eurotiales* (*Aspergillus* spp. [Ellis 1980; Mouchacca 2000; Kostadinova y col. 2009]; *Penicillium* spp. [Kostadinova y col. 2009], *Rasamsonia* spp. [Su y Cai 2013], *Thermoascus aurantiacus* [Miehe 1907; Apinis 1953] y *Thermomyces* spp. [Tsiklinsky 1899; Griffon y Maublanc 1911]), *Helotiales* (*Botrytis* spp. [Kostadinova y col. 2009], *Sclerotinia borealis* [Hoshino y col. 2010] y *Scytalidium* spp. [de Oliveira y col. 2015]), *Hypocreales* (*Acremonium* spp. [de Oliveira y col. 2015], *Lecanicillium* spp. [Kostadinova y col. 2009] y *Psychrophila* spp. [Wang y col. 2015]), *Onygenales* (*Malbranchea* spp.; Apinis 1953; Sigler y Carmichael 1976), *Pleosporales* (*Alternaria* spp.; Kostadinova y col. 2009), *Rhytismatales* (*Tetracladium* spp.; Wang y col. 2015), *Sordariales* (*Chaetomium thermophilum* [La Touche 1950], *Collariella gracilis* [Maheshwari y col. 1987], *Crassicarpon thermophilum* [Marín-Félix y col. 2015], *Canariomyces* spp., *Humicola* spp., *Melanocarpus* spp., *Myceliophthora* spp., *Thielavia* spp., *Remersonia thermophila* [de Oliveira y col. 2015], *Sordaria* spp. y *Thermothelomyces thermophilus* [Apinis 1963, Marín-Félix y col. 2015]), *Thelebolales* (*Antarctomyces* spp. [Stchigel y col. 2001; Xiao y col. 2010; de Menezes 2016], *Geomyces* spp. [Kostadinova y col. 2009], *Pseudogymnoascus* spp. [Gargas y col. 2009; Chaturvedi y col. 2010; Verant y col. 2012] y *Thelebolus* spp. [de Hoog y col. 2005]), y *Xylariales* (*Arthrinium* spp.; de Oliveira y col. 2015); y la especie *Monodictys arctica* (*insertae sedis*; Kostadinova y col. 2009).

Respecto a los hongos que pueden crecer a pH extremos (acidófilos y alcalófilos, y los respectivos tolerantes), la división *Ascomycota* tiene representantes en los órdenes *Capnodiales* (*Cladosporium* spp.; Magan 2007; Grum-Grzhimaylo y col. 2016), *Chaetothyriales* (*Exophiala alcalophila*; Basgal 1931, Kursanov y col. 1954), *Eurotiales* (*Aspergillus* spp. y *Penicillium* spp.; Magan 2007, Grum-Grzhimaylo y col. 2016), *Glomerellales* (*Colletotrichum* spp. [Magan 2007] y *Sodiomyces* spp. [Grum-Grzhimaylo y col. 2016]; *Helotiales* (*Botrytis* spp.; Magan 2007), *Hypocreales* (*Acremonium alcalophilum* [Grum-Grzhimaylo y col. 2016], *Emericellopsis alkalina* [Grum-Grzhimaylo y col. 2016], *Fusarium* spp. y *Purpureocillium lilacinum* [Magan 2007; Grum-Grzhimaylo y col. 2016]), *Microascales* (*Scopulariopsis* spp. [Grum-Grzhimaylo y col. 2016]), *Pleosporales* (*Alternaria* spp. sect. *Soda* [Grum-Grzhimaylo y col. 2016]), *Saccharomycetales* (las levaduras *Candida acidothermophilum*, *Kazachstania exigua* [Recca y Mrak 1952], *Cyniclomyces guttulatus*, *Saccharomyces cerevisiae* [Pfatt y col. 1978] y *Kluyveromyces marxianus* [Goto y col. 1981]) y *Sordariales* (*Thielavia* sp. [Grum-Grzhimaylo y col. 2016], y especies del género *Acontium* (*incertae sedis*; Starkey y Waksman 1943; Belly y Brock 1974).

Existe un número menor de hongos extremófilos en la división *Basidiomycota*. Dentro de los hongos xerófilos hay representantes de los órdenes *Gloeophyllales* (*Gloeophyllum abietinum*; Micheluz y col. 2015), *Trichosporonales* (*Trichosporon mucoides*; Carvalho y col. 2010) y *Wallemiales* (*Wallemia* spp.; Høye 1902; Domsch y col. 1980; Takahashi 1997; Samson y col. 2002; Zalar y col. 2005; Zajc y col. 2014). Los hongos psicrófilos y termófilos se ubican dentro de los órdenes *Agaricales* (*Coprinopsis psychromorbida*; Traquair y Smith 1982), *Cystophilobasidiales* (las levaduras de los géneros *Mrakia* y *Mrakiella*; Margaret 1966; Xin y Zhou 2007; Margesin y Fell 2008; Robin y col. 2010), *Polyporales* (*Thermophymatospora fibuligera*; de Oliveira y col. 2015), y especies de *Myriococcum* (*incertae sedis*; de Oliveira y col. 2015). Finalmente, *Rhodotorula mucilaginosa* (dentro del orden *Sporidiobolales*) es un representante de levadura acidófila pero también psicrotrófica (Battley y Bartlett 1966; Chaud y col. 2016).

Además de los anteriormente mencionados, existen ejemplos de hongos extremófilos dentro de la división *Mucoromycota*. Los hongos xerófilos se ubican dentro del orden *Mucorales*, con especies de los géneros *Mucor* (Betts 1912; Luard 1982; Grabowski y Klein 2015) y *Rhizopus* (Eltz y col. 2002). Dentro de este mismo orden existen especies

termófilas/termotolerantes en los géneros *Apophysomyces* (Kontoyiannis 2012), *Lichtheimia* (Alvarez-Zúñiga y col. 2017), *Mucor* spp. (Schipper 1967; Ogundero 1981; Kostadinova y col. 2009), *Rhizomucor* spp. (Mouchacca 1997; Zhou y col. 2014), *Rhizopus* spp. (Schipper 1984; Kostadinova y col. 2009), *Saksenaea* (Kontoyiannis 2012) y *Thermomucor* spp. (de Oliveira y col. 2015), y psicrófilas como *Mucor strictus* (Schipper 1967; Kostadinova y col. 2009).

Queda claro pues que los hongos extremófilos no pertenecen a ningún grupo monofilético en concreto, estando distribuidos en la mayoría de las divisiones. Sin embargo, la gran mayoría de ellos pertenecen a la división *Ascomycota*, la que, por otro lado, es la de mayor biodiversidad dentro del reino *Fungi*.

2. INTERÉS Y OBJETIVOS

Los hongos extremófilos son capaces de sobrevivir bajo condiciones ambientales que impiden la proliferación de la gran mayoría de organismos. Estos hongos prosperan en hábitats altamente "estresados", sometidos a altas o bajas temperaturas, en condiciones de elevada acidez o alcalinidad, altas concentraciones de sales inorgánicas y de otras sustancias osmóticamente activas, de baja disponibilidad de agua, contaminados con solventes orgánicos, petróleo y sus derivados, metales pesados o radionúclidos, entre otros.

Si bien los hongos extremófilos vienen siendo estudiados hace ya décadas desde diversos enfoques científicos (fisiología y metabolismo, adaptación estructural y funcional a los condicionantes ambientales, complejidad genética, taxonomía y relaciones evolutivas, etc.), todavía queda un gran potencial de taxones por descubrir, debido a que existen nichos ecológicos y sustratos cuya biota fúngica extremófila es poco conocida porque han sido poco estudiados, o lo han sido con herramientas poco apropiadas, o porque no han suscitado el adecuado interés.

Entre los sustratos menos estudiados, y a pesar de su importancia económica, encontramos un edulcorante natural por excelencia: la miel. Este alimento está compuesto mayoritariamente por monosacáridos (dextrosa y fructosa) en una concentración no menor a 60 %. Debido a su naturaleza fisicoquímica, así como a su origen biológico (producida por las abejas –*Apis mellifera*– a partir del néctar o de las secreciones de ciertas plantas), la miel puede ser un sustrato ideal para el estudio de hongos xerófilos y xerotolerantes.

El vino, con una concentración de etanol entre 7 y 14 % v/v y una marcada acidez por su contenido en ácidos orgánicos, es una bebida de gran interés económico, tanto por el volumen de producción como por su elevado valor agregado. Las características físico-químicas determinan que el vino sea una bebida difícilmente deteriorable por la mayoría de los microorganismos. A pesar de ello, existen unas pocas bacterias (productoras de los picados acético y láctico) y levaduras capaces (incluida el *Saccharomyces cerevisiae*, la levadura de cerveza) de crecer a sus expensas y favorecer su alteración, en especial cuando la concentración alcohólica es relativamente baja. A pesar de que existe un limitado número de reportes sobre la potencial implicación de hongos filamentosos en la alteración organoléptica de vinos por la producción metabolitos volátiles con gustos/olores desagradables tales como el 2,4,6-tricloroanisol (TCA), no se sabe si lo hacen a expensas de la alteración del vino o tan solo a expensas del tapón de corcho que sella las botellas.

A pesar de que los ecosistemas antárticos han sido intensamente estudiados a lo largo de más de un siglo, el conocimiento sobre la diversidad microbiana sigue incrementándose año tras año con la incorporación de nuevos taxones. En las últimas dos décadas ha resurgido el interés por el estudio de bacterias y hongos psicrófilos, debido al potencial uso biotecnológico de sus enzimas.

Por otro lado, a pesar de que los hongos que producen infecciones severas en los seres humanos y animales ascienden a poco más de 50 especies, son cientos las especies fúngicas aislados de especímenes clínicos, algunas de los cuales han sido reportados como nuevos taxones para la ciencia. Muchos de estos hongos muestran como característica común su termotolerancia, pudiendo desarrollar a temperaturas de entre 35 y 37 ºC. Entre dichos taxones, las especies del género *Malbranchea* han sido escasamente estudiadas como patógenos oportunistas en humanos y animales.

Finalmente, el suelo es uno de los reservorios más importantes en cuanto a biodiversidad fúngica. Además de ser un sustrato heterogéneo y extremadamente diverso en cuanto a sus características físicas y composición química y biológica, las fuentes de materia inorgánica y orgánica son transformadas y movilizadas continuamente por la actividad metabólica de los micro- y macro-organismos que lo habitan. También es importante la influencia que ejerce la climatología sobre sus poblaciones microbianas. Todos estos factores condicionan la consolidación de unas determinadas poblaciones fúngicas, mientras que otras son suprimidas. A pesar de lo extensivamente estudiado a lo largo de más de un siglo, el suelo continúa siendo una fuente de nuevos hongos para la ciencia.

Por lo anteriormente expuesto, el objetivo general de la presente tesis doctoral es la de estudiar ciertos aspectos de la biología (mediante la caracterización de sus estructuras vegetativas y reproductivas, y de sus peculiaridades fisiológicas) y la evolución (mediante la reconstrucción de su filogenia basada en el análisis de las secuencias nucleotídicas de ciertos genes estructurales) de los hongos aislados de ambientes extremos y, a consecuencia de su estudio, esclarecer la relación evolutiva con otros organismos y su posición taxonómica.

Para su consecución, en la misma se desarrollarán los siguientes objetivos específicos:

1. Contribuir al conocimiento de la diversidad de hongos xerotolerantes y xerófilos en muestras de mieles de España mediante su aislamiento en cultivo puro empleando técnicas

selectivas, su identificación morfológica presuntiva, y su identificación taxonómica final mediante la reconstrucción filogenética teórica gracias al análisis de las secuencias nucleotídicas de diferentes marcadores genéticos.

2. Contribuir al conocimiento de la diversidad de hongos tolerantes al etanol presentes en muestras de cava (vino espumoso mayoritariamente producido en la Comunidad Autónoma de Cataluña) y en los tapones de corcho que sellaban las botellas que lo contenían, mediante su aislamiento en cultivo puro y su posterior identificación mediante su caracterización fenotípica y la reconstrucción filogenética basada en métodos moleculares.

3. Estudiar la biodiversidad de los hongos del suelo de diferentes regiones de la Antártida, Armenia, Chile, Ecuador, España, Irán, México y Vietnam, mediante el empleo de técnicas de aislamiento selectivo, y su identificación basada en el uso de criterios fenotípicos y filogenéticos.

4. Mediante la caracterización fenotípica, secuenciación y análisis de ciertos marcadores genéticos, ubicar taxonómicamente veintidós cepas fúngicas de origen clínico de los Estados Unidos de Norteamérica, identificadas presuntivamente como pertenecientes al género-forma *Malbranchea*, así como determinar sus perfiles de sensibilidad *in vitro* frente a los antifúngicos.

3. MATERIALES Y MÉTODOS

3.1. Origen de las muestras

Se analizaron en total 83 muestras de miel, 72 de las cuales correspondieron a mieles de mielada (producida a partir de las azucaradas de ciertas plantas e insectos áfidos), 45 fueron adquiridas de empresas comerciales y 27 recolectadas y comercializadas por los propios apicultores. Todas las muestras provistas por las compañías comerciales recibieron tratamiento térmico (siendo sometidas por varias horas a una temperatura de 45–55 ºC, incluso algunas a 80 ºC durante 2 minutos). Las restantes 11 fueron mieles multiflorales no sometidas ningún proceso térmico. Todas las muestras fueron colectadas entre 2009 y 2014 en diferentes zonas de España (Asturias, Ávila, Badajoz, Burgos, Cáceres, Castellón, Ciudad Real, Granada, León, Ourense, Salamanca, Tarragona, Toledo, Zamora y Zaragoza). Solo se analizó una muestra proveniente de Argentina, la que fue remitida desde el Instituto Nacional de Tecnología Industrial (INTI, San Martín, provincia de Buenos Aires).

Unas 500 botellas conteniendo vino espumoso (D. O. Cava) y selladas con un tapón de corcho fueron "degolladas" asépticamente y, tras un posterior examen organoléptico por parte de cuatro expertos catadores, un total de 54 muestras de cava y de sus respectivos tapones de corcho fueron seleccionadas para su estudio debido a que eran sospechosas de presentar la alteración organoléptica denominada "*cork taint*". Todas las botellas procedían de una única bodega, localizada en la población de Sant Sadurní d`Anoia, provincia de Barcelona (España), y fueron colectadas y procesadas en abril del 2011.

Se analizaron 104 muestras de suelos procedentes de la Antártida (12 muestras), Armenia (15), Chile (3), Ecuador (1), España (12), Irán (41), México (5) y Vietnam (15), así como 10 muestras de superficies (paredes con alteraciones cromáticas debidas, muy probablemente a la formación de biopelículas), las que fueron colectadas en España.

3.2. Procesado de las muestras

3.2.1. *Muestras de miel*

Se introdujeron 10 g de miel en un recipiente estéril de plástico para la recolección de especímenes biológicos, y se disolvieron en 90 mL de agua estéril. Posteriormente, 1 mL de dilución (1:10) fue vertida asépticamente dentro de una placa Petri de 90 mm de diámetro, se agregaron 15 mL de agar con glicerol 18 % (G18; modificación del medio descrito por Hocking

y Pitt 1980; véase Anexo 1) fundido a 50-55 ºC, procediendo a su homogenización manual. Una vez gelificado el medio, las placas fueron incubadas a 15 ºC y a 25 ºC en oscuridad por un periodo entre uno o dos meses, siendo examinadas bajo el microscopio estereoscópico cada 5-7 días en busca de colonias y estructuras reproductivas.

3.2.2. *Muestras de vino espumoso y de tapones de corcho*

Se filtraron 100 mL de cava a través de una membrana filtrante con un diámetro de poro de 0,45 micrómetros. Seguidamente, las membranas fueron colocadas de forma aséptica sobre la superficie de agar con extracto de patata y glucosa (PDA; Hawksworth y col. 1995; véase Anexo 1) contenido en placas de Petri de 90 mm de diámetro. Las placas fueron incubadas a 25 ºC en oscuridad por un periodo entre uno o dos meses, siendo examinadas bajo el microscopio estereoscópico cada 5-7 días en busca de colonias y estructuras reproductivas. La cara de los tapones de corcho expuesta al vino fue cortada en pequeñas piezas con ayuda de un bisturí desechable estéril, y estas fueron depositadas dentro de placas Petri de 90 mm de diámetro que contenían PDA, y fueron incubadas en las mismas condiciones anteriormente descritas.

3.2.3. *Muestras de suelos*

Aproximadamente 1 g de suelo fue introducido en un tubo de ensayos estéril y se mezcló con 5 mL de una solución de fenol al 2 % (p/v) (Panreac, Barcelona, España), resuspendiendo el sedimento a los 5' mediante agitación manual o con *vortex*, dejando sedimentar espontáneamente durante otros 5'. Posteriormente, el sobrenadante fue desechado y el sedimento resuspendido en 10 mL de agua destilada estéril. Un volumen aproximado de 1,6 mL de dicha suspensión fue mezclado dentro de una placa de Petri de 90 mm de diámetro con 15 mL de agar con extracto de patata y zanahoria suplementado con cloranfenicol (PCA; Stchigel y col. 1998; véase Anexo 1). Una vez gelificado el medio, las placas fueron incubadas a 15 y 25 ºC en oscuridad durante 2-4 semanas hasta la aparición de colonias, las que fueron examinadas bajo el microscopio estereoscópico en busca de estructuras reproductivas.

3.2.4. *Muestras de biopelículas en paredes*

Por medio de un hisopo humectado con solución fisiológica estéril (1 mL), se frotó la superficie de interés en zigzag durante unos 5''-10''. El hisopo impregnado con material

procedente de la alteración cromática fue introducido en un tubo de ensayos estéril al que posteriormente se le agregó 3-5 mL de solución fisiológica estéril, agitando el hisopo hasta resuspender las partículas. A partir de dicha suspensión de material en solución fisiológica, se realizaron diluciones seriadas 1:10 en solución fisiológica, hasta una dilución 10^{-3}. Un volumen de 1 mL de c/u de las diluciones fue introducido en placas de Petri de 90 mm de diámetro, y se mezclaron con 15 mL de PDA fundido a 50-55 ºC. Una vez gelificado el medio, las placas fueron incubadas a 25 ºC en oscuridad durante 2-3 semanas hasta la aparición de colonias, las que fueron examinadas bajo el microscopio estereoscópico en busca de estructuras reproductivas.

3.3. Aislamiento y obtención de cultivos puros

Una cierta cantidad de masa fúngica procedente de las colonias (que presentaban estructuras reproductivas al ser observadas con el microscopio estereoscópico) fue transferida mediante una aguja desechable tipo “tuberculina” o “insulina” a una placa de Petri de 50 mm de diámetro conteniendo G18, PDA ó PCA (dependiendo del medio de cultivo empleado en el aislamiento primario). Una vez inoculadas, las placas fueron incubadas a 25 ºC en oscuridad durante 2-3 semanas hasta el desarrollo de colonias. Una vez verificada su pureza, mediante el estudio microscópico de sus estructuras reproductivas (véase 3.6.3), material procedente de dichas colonias fue inoculado en los respectivos medios de cultivo inclinados en “pico de flauta” (“slant”) dentro de tubos de ensayos estériles que se cerraban mediante una tapa a rosca o a presión.

3.4. Conservación de las cepas fúngicas

Los hongos de interés fueron conservados en la colección de la Facultad de Medicina y Ciencias de la Salud de Reus (FMR), siendo depositados tanto preparaciones microscópicas como material herborizado (para que sirva como tipo fúngico) y cepas viables. El material destinado a herborización fue desecado a 45 ºC en estufa durante un tiempo conveniente, y almacenado posteriormente en las mismas placas Petri que los contenían, selladas con parafilm® y dentro de sobres rotulados convenientemente. Los tipos, al igual que subcultivos de cada una de las respectivas cepas viables (contenidas dentro de tubos de ensayo con un medio de cultivo inclinado en “pico de flauta”) fueron remitidos al “*Westerdijk Fungal Biodiversity Institute*” (CBS) en Ultrecht (Países Bajos) para su depósito. Las cepas viables

fueron conservadas en nuestra colección mediante el empleo de diferentes métodos (véase Anexo 2).

3.5. Cepas de origen clínico

Las cepas de origen clínico (N=22), identificadas presuntivamente como pertenecientes al género-forma *Malbranchea*, fueron aisladas durante el período 2002-2017 en los Estados Unidos de Norteamérica, siendo facilitadas por el "*Fungus Testing Laboratory University of Texas Health Science Center*" (UTHSC, San Antonio, Texas).

3.6. Caracterización fenotípica e identificación presuntiva de las cepas de interés

Las cepas fueron identificadas presuntivamente según criterios establecidos en la literatura especializada para cada género, familia u orden, mediante el estudio pormenorizado de las características de las colonias desarrolladas sobre diferentes medios de cultivo y a diferentes temperaturas, y mediante la caracterización morfológica de las estructuras vegetativas y reproductivas sexuales y/o asexuales. Adicionalmente, se realizaron estudios fisiológicos.

3.6.1. *Estudio de las cepas en distintos medios y condiciones de cultivo*

Para la caracterización cultural de los hongos de interés, las cepas se inocularon e incubaron a 15 ºC en el caso de los psicrófilos, 25 ºC para los mesófilos y 35 ºC para los termófilos, durante periodos comprendidos entre los 14 y 21 días, en diferentes medios de cultivo dependiendo de la naturaleza de los taxones: agar de Czapek con extracto de levadura (CYA; Pitt 1979), agar con extracto de malta (MEA; Samson y col. 2010), agar con extracto de levadura y sacarosa (YES; Frisvad 1981), agar con harina de avena (OA; Samson y col. 2010) y agar con dicloran y glicerol 18 % (DG18; Hocking y Pitt 1980) para miembros del orden *Eurotiales*; agar con fitona y extracto de levadura (PYE; Carmichael y Kraus 1959), OA y PDA para miembros del orden *Onygenales*; y agar con nitrato y 25 % glicerol (G25N; Pitt 1979), G18 y PCA para otros hongos. La composición de todos estos medios se describe pormenorizadamente en el Anexo 1.

El color de las colonias fue asignado por comparación con el "*Methuen Handbook of Colours*" (Kornerup y Wanscher 1984). Las características culturales se describieron utilizando

la ayuda de un microscopio estereoscópico. Las características de interés documentadas fueron (Fig. 5):

- Tamaño de la colonia (diámetro en milímetros).
- Color de la superficie y del reverso.
- Forma (puntiforme, circular, irregular, filamentosa, rizoide).
- Superficie (lisa, rugosa, cerebriforme, radialmente plegada, opaca, brillante).
- Textura (mucoide, butirosa, quebradiza, seca, coriácea, afieltrada, aterciopelada, fasciculada, flocosa, algodonosa, lanosa, pulverulenta, granulosa).
- Topografía (plana, elevada, convexa, pulvinada, umbonada, crateriforme).
- Margen (entero, irregular, fimbriado, ondulado, lobulado, filamentoso, ciliado, rizado, filiforme).
- Zonación (bandas, en círculos concéntricos, sectores).
- Presencia de exudados.
- Presencia de pigmentos difusibles.
- Presencia de estructuras reproductivas asexuales y sexuales.

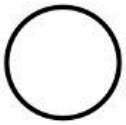
Margen	Topografía	Superficie	Textura
Rizado 	Elevada 	Lisa 	Aterciopelada, algodonosa
Entero (liso) 	Umbonada 	Rugosa 	Brillante, viscosa
Filamentoso 	Plana 	cerebriforme 	Lanosa
Ondulado 	Convexa 	Radialmente plegada 	Afieltrada
Lobulado 	Pulvinada (forma de cojin) 	Opaca 	Granulosa
Serrado 	Crecimiento dentro del medio de cultivo	Brillante 	Pulverulenta

Figura 5. Caracteres descriptivos de las colonias fúngicas.

3.6.2. *Caracterización morfológica e identificación presuntiva de las cepas de interés*

3.6.2.1. *Naturaleza de las estructuras vegetativas y reproductivas a documentar*

Las características documentadas para los hongos pertenecientes a la división *Ascomycota* (correspondientes a la mayoría de las cepas aisladas, independientemente del tipo de substrato; Figs. 6 y 7) fueron:

- **Hifas**: color, forma, ornamentación, diámetro, presencia de septos y anastomosis.
- **Ascomas**: formas iniciales, tipo, color, forma, tamaño; presencia, tipo, color, forma, ornamentación y tamaño de los pelos peridiales; espesor, número de capas y textura del peridio; color, forma, ornamentación y tamaño de las células peridiales; presencia de paráfisis y perífisis y su color, forma, patrón de ramificación y disposición de los septos.
- **Ascos**: número de ascosporas por asco, forma y tamaño, longevidad (evanescente o persistente), presencia de estructuras apicales y reacciones de tinción (amiloides o no amiloides, dextrinoides), presencia y tamaño del estipe.
- **Ascosporas**: color, forma y tamaño, ornamentación de la pared celular, disposición dentro de los ascos, presencia y número de septos y su disposición; presencia, número y posición de poros o surcos de germinación; presencia de una capa mucilaginosa o cápsula; presencia, disposición, forma y tamaño de los apéndices mucilaginosos.
- **Anamorfo (morfo asexual)**: tipo, color, forma, tamaño y ornamentación de los conidióforos y células conidiógenas; tipo de conidiogénesis; color, forma, tamaño, ornamentación, disposición y número de células de los conidios.
- **Clamidosporas**: presencia, color, forma, tamaño, ornamentación y disposición.

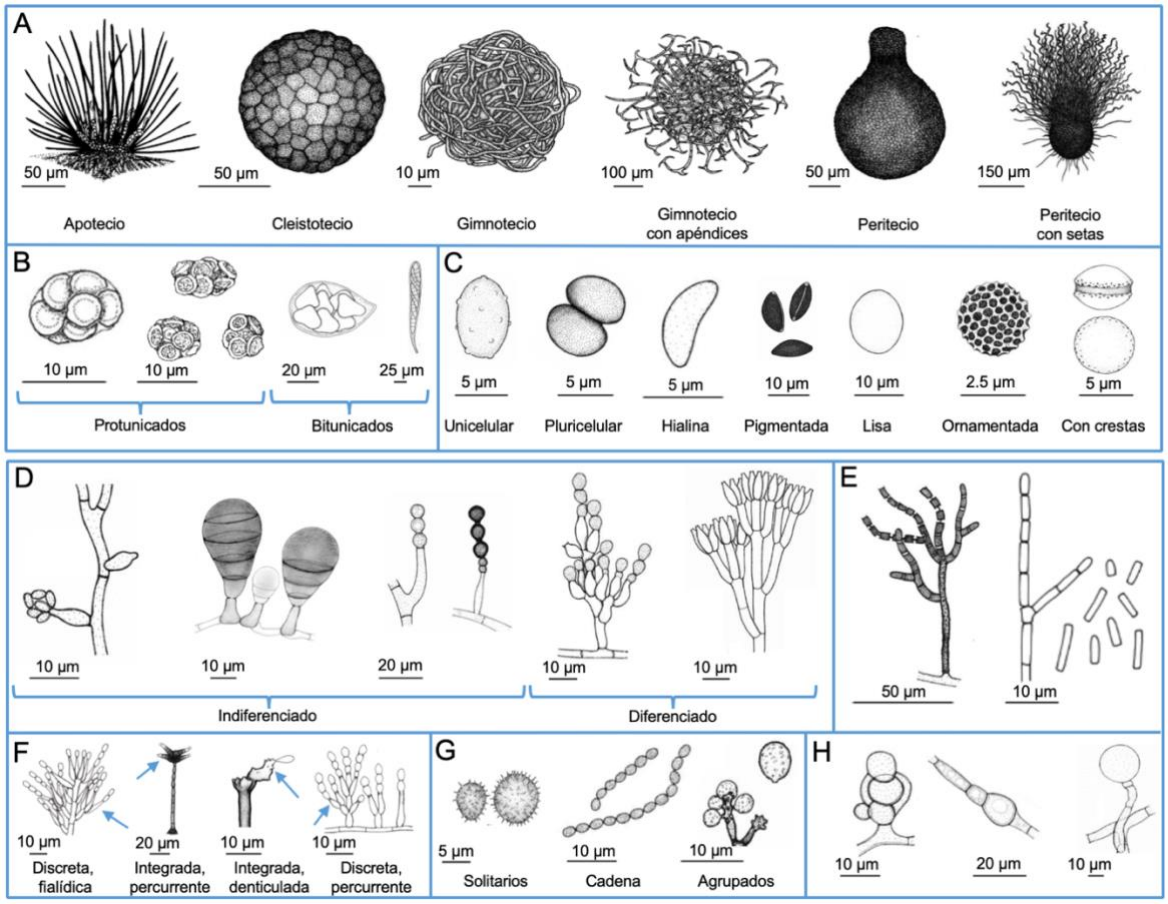


Figura 6. Diversidad de estructuras fúngicas reproductivas (asexual y sexual) de los *Ascomycota*. A. Ascomas. B. Ascos. C. Ascosporas. D. Conidióforos. E. Artroconidios. F. Células conidiógenas (mostradas con flecha). G. Conidios. H. Clamidosporas. Adaptado de Guarro y colaboradores 2012.

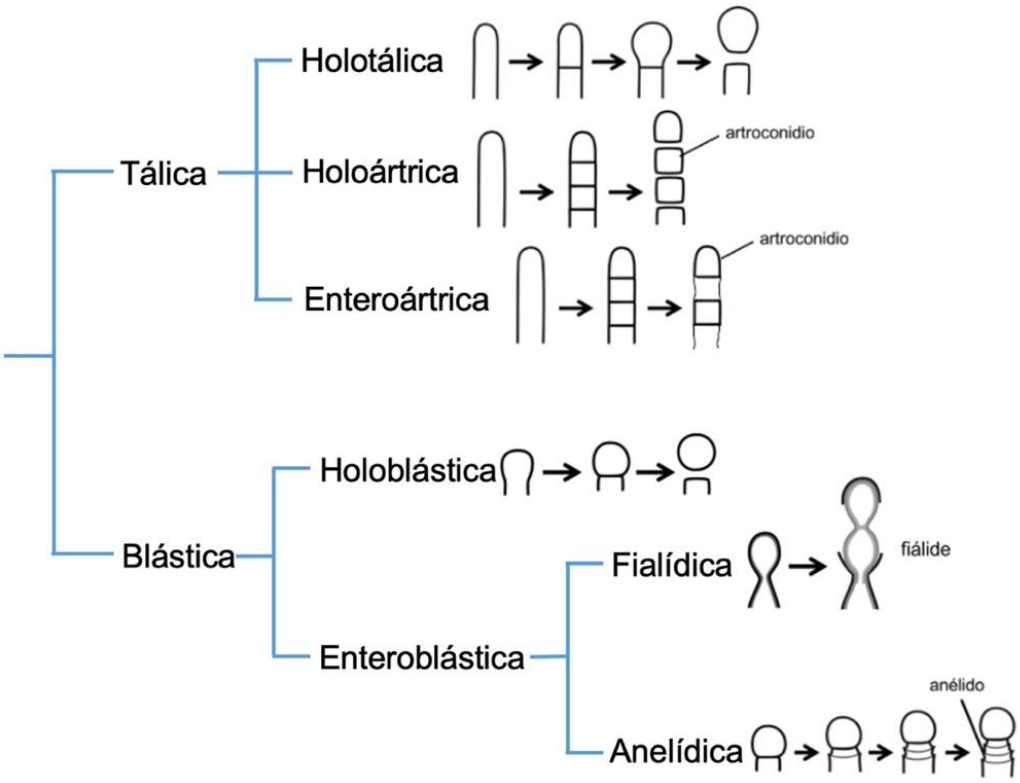


Figura 7. Principales tipos de ontogenia conidial de los *Ascomycota*. Adaptado de M. Piepenbring (https://species-id.net/openmedia/Mycological_teaching_diagrams_by_Meike_Piepenbring).

Las características documentadas para los hongos pertenecientes a la división *Mucoromycota* (el grupo minoritario de hongos aislados; Fig. 8) fueron:

- **Hifas**: color, forma, ornamentación, diámetro, presencia de septos, anastomosis e de inclusiones lipídicas.
- **Zigosporas**: color, ornamentación, forma, tamaño; presencia, ornamentación, forma, tamaño de suspensores y apéndices.
- **Esporangióforos**: color, ornamentación, septación, patrón de ramificación y tamaño; presencia de vesículas terminales.
- **Esporangios**: tipo (esporangíolos, merosporangios o esporangios), color, ornamentación, tamaño, número de esporas que contiene, disposición de las esporas.
- **Esporangiosporas**: color, ornamentación, forma, tamaño.
- **Clamidosporas**: presencia, tipo, color, ornamentación, forma, tamaño y disposición.

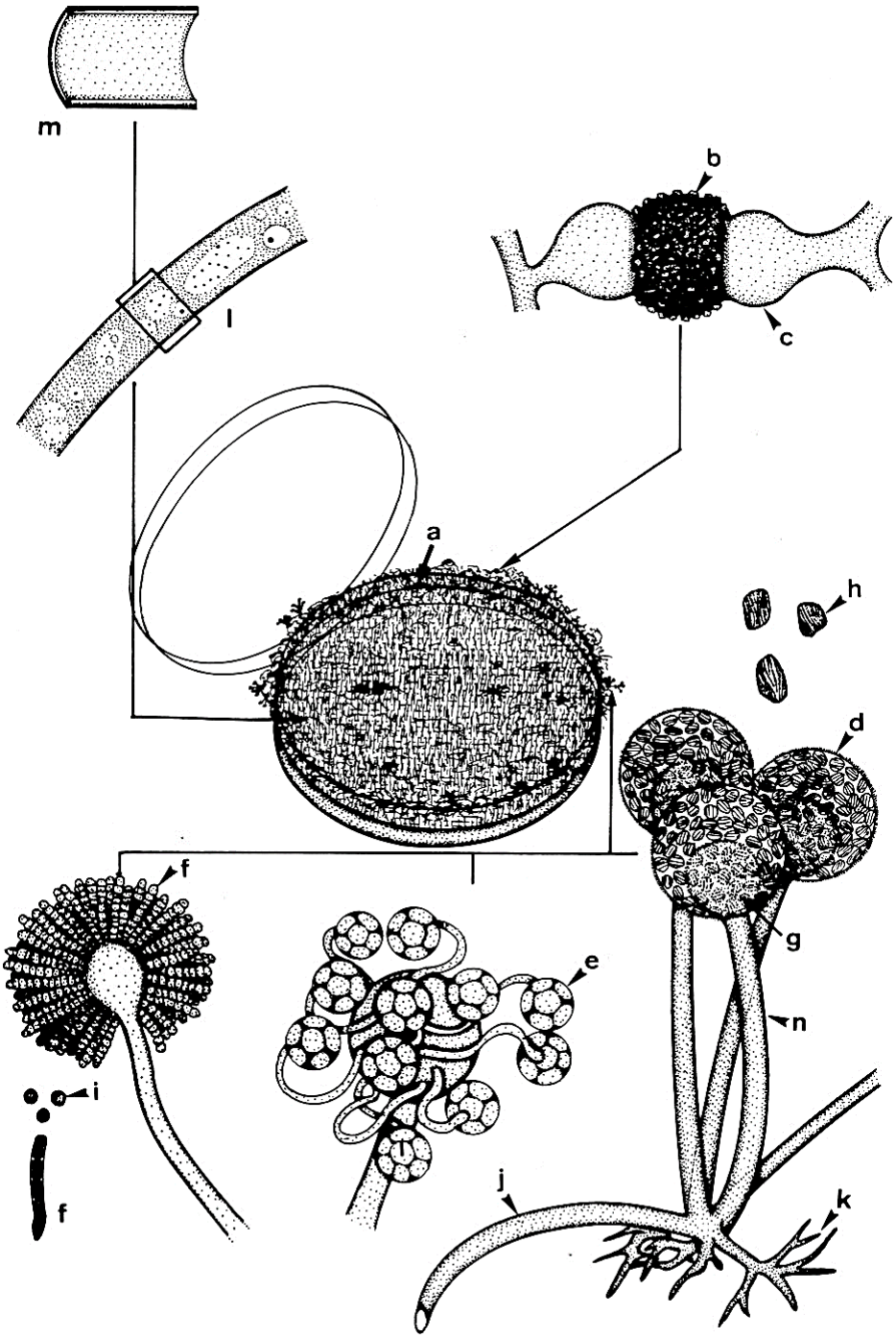


Figura 8. Diversidad de estructuras fúngicas reproductivas (asexual y sexual) de los *Mucoromycota*. a. Micelio expansivo. b. Zigospora (reproducción sexual; RS). c. Suspensorios (RS). d. Esporangios (reproducción asexual; RA). e. Esporangíolos (RA). f. Merosporangios (RA). g. Columenlla. h, i. Esporangiosporas (RA). J. Estolón. K. Rizoides. l. Hifa cenocítica. m. Sección de una hifa. n. Esporangióforo. Adaptado de Guarro y colaboradores 2012.

3.6.2.2. *Preparaciones microscópicas*

Para el examen microscópico y la caracterización de las estructuras fúngicas vegetativas y reproductivas, se emplearon las siguientes técnicas:

- Montaje directo: las estructuras fúngicas del hongo de interés fueron extraidas mediante la aguja de una jeringuilla hipodérmica desechable tipo “tuberculina” o “insulina” y depositadas en una gota de medio de montaje (lactofenol o ácido láctico; véase Anexo 2) sobre la superficie de un portaobjetos. Luego fue depositado sobre esta un cubreobjetos, y la preparación fue sellada con esmalte para uñas, para preservar su integridad.
- Microcultivo: a partir de una placa de Petri con un medio de cultivo adecuado para el crecimiento del hongo de interés, fueron cortados con la hoja de un bisturí bloques de 1 cm^2 de dicho medio. Tres o cuatro de estos bloques fueron transferidos asépticamente a la superficie del mismo medio de cultivo contenido en una placa de Petri de 90 mm de diámetro. Posteriormente, las cuatro esquinas de los bloques se inocularon con el hongo de interés, y cada bloque se cubrió con un cubreobjetos estéril. Los microcultivos fueron incubados en las condiciones óptimas para cada hongo y fueron periódicamente examinadas bajo el microscopio estereoscópico, hasta la aparición de estructuras reproductivas, momento en que el cubreobjetos fue retirado cuidadosamente del bloque de agar y fue colocado sobre una gota de medio de montaje (lactofenol o ácido láctico; véase Anexo 2) previamente depositado en un portaobjetos. Luego fue depositado sobre este un cubreobjetos, y la preparación fue sellada con esmalte para uñas para preservar su integridad.

3.6.2.3. *Examen y documentación de las estructuras fúngicas mediante el microscopio de campo claro*

Para estudiar las características morfológicas de las estructuras vegetativas y reproductivas de las cepas, se emplearon los siguientes microscopios de campo claro:

- Olympus BH2
- Olympus CH2
- Zeiss Axio Imager M1

Para documentar dichas estructuras, se procedió a la captura de las imágenes con una cámara digital DeltaPix Infinity X acoplada al microscopio Zeiss Axio Imager M1 empleando los condensadores de Nomarski (contraste de interferencia) y de contrastes de fases. La edición de dichas imágenes se realizó con el programa Adobe Photoshop CS6 v. 13.0.

3.6.2.4. *Microscopía electrónica de barrido (SEM)*

Para estudiar en detalle la ornamentación de varias estructuras fúngicas, especialmente de esporas, las muestras fueron procesadas y observadas mediante un microscopio electrónico de barrido siguiendo el protocolo descrito en Figueras y Guarro (1988) con algunas modificaciones (Stchigel 2000). Se empleó un microscopio electrónico de barrido Jeol JSM-6400 de los Servicios Científico-Técnicos de la Universitat Rovira i Virgili (Tarragona, España). La distancia de trabajo a la muestra varió de 8 a 39 mm, y el voltaje aplicado varió entre 10 y 20 keV.

3.6.3. *Estudio fisiológico de las cepas de interés*

3.6.3.1. *Pruebas bioquímicas*

Las siguientes pruebas bioquímicas se llevaron a cabo para algunos taxones pertenecientes al orden *Onygenales* sometidos a estudio durante el desarrollo de esta tesis:

- Resistencia a la cicloheximida®: se evaluó haciendo crecer las cepas en agar de Sabouraud glucosado (SDA; Pronadisa, España) suplementado con 0,2 % de cicloheximida® (Sigma, USA).
- Tolerancia al NaCl: evaluada en agar de Sabouraud glucosado (SDA; Pronadisa; España) suplementado con 3, 10 y 20 % de NaCl p/p.
- Crecimiento y la alcalinización/acidificación del medio: se ensayaron sobre agar glucosado con sólidos de leche y púrpura de bromocresol (BCP-MS-G; Kane y Smitka 1978; véase Anexo1). La acidificación del medio se observó mediante un cambio de color, de azulado a amarillo, y la alcalinización, de azulado a morado intenso.
- Producción de ureasa: se utilizó el agar urea de Christensen (Merck, Darmstadt, Alemania; Christensen 1946). La hidrólisis de la urea se evidencia por un cambio de color del medio, de amarillo o naranja-amarillento a fucsia intenso.
- Actividad de lipasa: mediante prueba de opacidad con Tween 80 (TOTM; Slifkin 2000; véase Anexo 1). La actividad de la lipasa se observa por la opacidad desarrollada alrededor de la colonia.

En el caso de los taxones pertenecientes al orden de los *Eurotiales* se realizaron las siguientes pruebas bioquímicas:

- Producción de ácido: en el medio agar con creatina y sacarosa (CREA; Frisvad 1981; véase Anexo 1). La producción de ácido se observa por el cambio de coloración del violeta al amarillo.
- Crecimiento a baja a_w: fue evaluada mediante agar con extractos de malta y de levadura y 70 % de fructosa/glucosa (MY70FG; Beuchat y Hocking 1990; véase Anexo 1).

3.6.3.2. *Estudios del crecimiento a diferentes a_w*

Para poner a prueba la capacidad de crecimiento de los hongos aislados de muestras de miel en diferentes a_w, medios de cultivo con extracto de malta (1 % p/p), extracto de levadura (0,25 % p/p) y agar-agar (1 % p/p) fueron preparados con seis diferentes a_w (0,97, 0,95, 0,93, 0,92, 0,88 y 0,82), mediante la adición a partes iguales de fructosa y glucosa (correspondiendo a un 22 %, 30 %, 40 %, 44 %, 48 % y 55 % p/p de azúcares, respectivamente) y a un pH de 5,3 (Pitt y Hocking 1977). El ensayo se realizó por triplicado, inoculando una suspensión de propágulos de los hongos de interés en el centro de la placa Petri, e incubándolas a 25 ºC (a 15 ºC en el caso de las cepas pertenecientes al género *Oidiodendron*) en oscuridad. El diámetro de las colonias fue medido después de 21 días de incubación.

3.6.3.3. *Estudios del crecimiento a diferentes concentraciones de etanol*

La tolerancia de las cepas aisladas de los corchos (expuestos al cava) y del vino frente al alcohol se probó en caldo con extracto de malta al 2 % suplementado con diferentes cantidades de etanol (5, 10, 15 y 20 % v/v) en tubos de ensayo de plástico cerrados herméticamente por un tapón del mismo material. Luego de inoculados con la cepa de interés, los tubos de ensayo fueron sellados con parafilm® para asegurar su estanqueidad, e incubados a 15 ºC durante 13 meses en oscuridad y sin agitación, intentando simular los métodos empleados en la elaboración del vino. Estos fueron revisados cada mes para detectar crecimiento fúngico. Ante la ausencia de crecimiento evidente, 0,1 mL del caldo se inoculaba en PDA dentro de placas de Petri de 90 mm, y eran incubadas durante 2 semanas en oscuridad a 25 ºC.

3.6.3.4. *Estudios de tolerancia a diferentes temperaturas*

Todos los nuevos taxones registrados en esta tesis, independiente de su origen, fueron inoculados en placas de Petri de 90 mm de diámetro con el medio de cultivo más apropiado

(el G18 para los aislados de miel; el CYA para los *Eurotiales*; y el PDA para el resto de aislados), siendo posteriormente incubados a 5, 10, 15, 25, 30, 35, 37, 40 y 45 ºC. Las placas fueron examinadas a los 7, 14 y 21 días de incubación, midiendo el crecimiento de acuerdo con el diámetro de las colonias.

3.6.3.5. *Estudios de sensibilidad antifúngica*

Se determinó el patrón de sensibilidad antifúngica para los 22 aislados clínicos con el método de caldo de diluciones del protocolo M38 "*Clinical and Laboratory Standards Institute* (*CLSI*)" (CLSI 2017), con algunas modificaciones. Los antifúngicos analizados fueron: amfotericina B (AMB), fluconazol (FLC), voriconazol (VRC), itraconazol (ITC), posaconazol (PSC), anidulafungina (AFG), caspofungina (CFG), micafungina (MFG), terbinafina (TRB) y 5-fluorocitosina (5FC). Las cepas fueron sembradas en placas Petri de 90 mm de diámetro con medio OA suplementado con cloranfenicol, incubándolas a 25-35 ºC en oscuridad durante 2-4 semanas, hasta conseguir una abundante esporulación. Una vez esporuladas, la superficie de las colonias fue raspada con la ayuda de un asa estéril, resuspendiendo la masa fúngica obtenida en solución fisiológica estéril, la cual fue posteriormente filtrada a través de un algodón estéril para eliminar los restos de micelio. Las suspenciones de conidios fueron cuantificadas mediante lectura en una cámara de Neubauer, ajustadas a una concentración de $4 \times 10^5 - 5 \times 10^6$ conidios/mL y luego diluidas 1:50 en medio de *Roswell Park Memorial Institute* (RPMI-1640, Gibco, Reino Unido). En una microplaca de 96 pocillos, se inocularon 100 µL del inóculo para cada dilución del antifúngico a ensayar. Las microplacas fueron incubadas en la oscuridad, sin agitación, durante 2 a 7 días a 30 ºC. La cepa de *Candida parapsilosis* ATCC 22019 fue usada como control de calidad en todos los experimentos. La lectura de la sensibilidad frente a equinocandinas se realizó a partir de las 48 horas de incubación, determinando la concentración mínima eficaz (CME), definida como la mínima concentración del antifúngico en la cual se observa un crecimiento aberrante de las hifas del hongo, caracterizado por masas compactas, formadas por elementos miceliares estrellados. Para la lectura de la sensibilidad frente al resto de antifúngicos, se determinó la concentración mínima inhibitoria (CMI) a partir de las 48 horas de incubación. Se definió como CMI a la mínima concentración del antifúngico capaz de conseguir el 100 % de inhibición del crecimiento de hongo. Todas las pruebas se realizaron por duplicado y la lectura se llevó a cabo de forma visual, con ayuda de un espejo invertido.

3.7. Estudios moleculares

Para realizar la identificación molecular de aquellos hongos de especial interés taxonómico, se amplificaron y secuenciaron mediante la reacción en cadena de la polimerasa (PCR): la región espaciadora intergénica transcrita del ARNr (ITS), un fragmento del gen 28S del ARNr (LSU) conteniendo los dominios D1 y D2, y un fragmento del gen de la β-tubulina (*BenA*), dependiendo del género, familia u orden a la que el hongo fuera asignado mediante su identificación fenotípica presuntiva. Así, para la mayoría de los hongos, y principalmente aquellos ubicados en el orden *Onygenales*, se secuenció el LSU; para los miembros de la familia *Myxotrichaceae* y las especies del género *Monascus* se secuenció el ITS; y para los pertenecientes al orden *Eurotiales*, utilizamos el *BenA*. Una vez que las secuencias obtenidas fueron comparadas con las existentes en las bases de datos, aquellas cepas que pertenecían a los géneros *Penicillium*, *Rasamsonia* y T*alaromyces*, y que significaban un posible taxón nuevo basados en su secuencia de *BenA*, se les tuvo que amplificar y secuenciar otros marcadores, como un fragmento del gen de la calmodulina (*CaM*) y/o un fragmento del gen de la subunidad 2 de la ARN polimerasa (*rpb*2), para poder completar el análisis molecular.

3.7.1. *Extracción del material genético*

El ADN total fue extraído usando un protocolo de extracción por medio de perlas de cristal, en el cual, a un tubo estéril con dichas perlas, se le agregó una porción de micelio aéreo junto con las estructuras de fructificación procedente de la colonia del hongo y un buffer de lisis (100 mM Tris pH 8,0; 50 mM EDTA; 1 % SDS), posteriormente se trituró el micelio, usando el *FastPrep FP120 cell disrupter* (Thermo Savant, Holbrook, Nueva York) durante 45'', a continuación, se centrifugaron las muestras por 10' a 13000 revoluciones por minuto (RPM), después se les agregó 275 µL de acetato de amonio pH 7,0, incubando las muestras a 65 ºC durante 5' y después a 4 ºC por otros 5'. Posteriormente se les agregó 500 µL de cloroformo y las muestras una vez más se centrifugaron durante 5' a 13000 RPM. El sobrenadante fue recuperado en un tubo Eppendorf estéril, se les agregó 800 µL de isopropanol y se incubaron a 4 ºC durante 15'. El ADN fue precipitado centrifugando los tubos una vez más durante 5' a 13000 RPM, y lavando con etanol al 70 %. Finalmente, el ADN se dejó secar durante una hora y se resuspendió en agua miliQ, agregándole 2 µL de RNAsa (10 mg/mL) e incubando durante 30' a 37 ºC.

3.7.2. *Amplificación y secuenciación*

Para la amplificación de los diferentes loci mediante PCR se utilizarón los siguientes cebadores:LR0R (Rehner y Samuels 1994) y LR5 (Vilgalys y Hester 1990) para LSU; ITS5 e ITS4 (White y col. 1990) para la región de ITS; T10 y Bt2b (Glass y Donaldson 1995) para *BenA*; Cmd5 y Cmd6 (Hong y col. 2005) para *CaM*; y RPB2-5F and RPB2-7cR (Liu y col. 1999) para *rpb*2 (véase Anexo 3). Los programas y ciclos para la amplificación se detallan en el Anexo 4. Los productos de PCR se secuenciaron en ambos sentidos usando los mismos cebadores en Macrogen Europe (Macrogen Inc., Amsterdam, Holanda).

3.7.3. *Ensamblaje de secuencias*

Los electroferogramas de las secuencias obtenidas se revisaron visualmente para determinar la calidad y fiabilidad de estas. Las secuencias fueron ensambladas y editadas, usando SeqMan v. 7.0.0 (DNASTAR Lasergene, USA). Todas las secuencias generadas en los estudios fueron depositadas en la base de datos GenBank (https://www.ncbi.nlm.nih.gov/genbank) usando la plataforma Webin del European Bioinformatics Institute (EMBL-EBI; https://www.ebi.ac.uk/ena/submit/webin/login).

3.7.4. *Comparación de secuencias nucleotídicas*

La comparación preliminar de los aislados se realizó determinando el grado de similitud genética con las secuencias disponibles en las bases de datos públicas como la del CBS (https://www.westerdijkinstitute.nl), o la del GenBank (https//www.ncbi.nlm.nih.gov/genbank). Para la identificación presuntiva de las cepas de interés a nivel de especie, se consideraron aquellas provenientes de cepas tipo o de referencia de colecciones internacionales, cuyas secuencias mostraron una identidad ≥ 98% y una cobertura ≥ 99%. Para los resultados porcentuales inferiores a los anteriormente mencionados, se consideraron como una identificación parcial, a nivel género, familia u orden, según correspondía.

3.7.5. *Alineamiento de secuencias y análisis filogenético*

Los árboles se construyeron usando diferentes métodos: Inferencia bayesiana (BI) y Máxima Likelihood (ML). Las secuencias de las especies descritas en estudios previos fueron

obtenidas del GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/). Para los estudios filogenéticos, las secuencias fueron alineadas usando el software MEGA v. 6.06 (Tamura y col. 2013) y concretamente la aplicación ClustalW (Thompson y col. 1994) para el alineamiento inicia, refinadas mediante MUSCLE (Edgar 2004) y editadas visualmente con la misma plataforma. Las regiones ambiguas fueron excluidas del análisis. Las reconstrucciones filogenéticas fueron implementadas mediante análisis ML y BI con la plataforma RAxML v. 8.2.10 (Stamatakis 2014) y el programa MrBayes v. 3.2.6 (Ronquist y col. 2012), respectivamente. El mejor modelo de sustitución para cada matriz fue estimado usando MrModelTest v. 2.3 (Nylander 2004). El soporte de las ramas internas fue estimado por 1000 pseudoreplicas (ML). Los soportes de bootstrap (BS) ≥ 70 % se consideraron como significativos. Para el análisis BI, se realizaron muestreos *Markov chain Monte Carlo* (MCMC), con un millón de generaciones, tomando muestras cada mil generaciones. Los valores de probabilidad (PP) fueron calculados después de eliminar el primer 25 % de los árboles. Los valores de PP ≥ 0,95 fueron considerados como estadísticamente significativos para cada agrupamiento. Las secuencias generadas en este trabajo fueron depositadas en el GenBank (Tabla 1), y las matrices usadas para los análisis filogenéticos fueron depositados en el TreeBASE (http://www.treebase.org).

3.8. Registro de las novedades taxonómicas

Las nuevas propuestas taxonómicas (familias, géneros y especies) fueron depositadas en la base de datos Mycobank (http://www.mycobank.org/; Crous y col. 2004), siguiendo las recomendaciones del actual código de nomenclatura fúngica (McNeill y col. 2012).

4. RESULTADOS

4.1. Hongos aislados durante el desarrollo de la presente tesis doctoral

Las cepas fúngicas aisladas en los diferentes estudios realizados a lo largo de esta tesis se refieren en la Tabla 1, así como los datos correspondientes a su localización, números de colección y de acceso de las secuencias depositadas en el GenBank. Fueron aisladas un total de 194 cepas fúngicas, 104 provenientes de muestras de miel de España y una de Argentina (54 %), 27 de vinos espumosos y tapones de corcho expuestos a dichos vinos de España (13 %), 32 de suelos, procedentes de la Antártida, Armenia, Chile, Ecuador, España, Irán, México y Vietnam (17 %), 9 de superficies con alteraciones cromáticas en España (5 %), y finalmente 22 aislados clínicos provenientes de USA (11 %), identificando un total de 98 especies distribuidas en 57 géneros.

En los siguientes apartados se adjuntan los trabajos publicados, sometidos, así como los que se encuentran en fase de preparación que se han generado a raíz de las investigaciones realizadas en la presente tesis doctoral.

Tabla 1. Aislados fúngicos estudiados en la presente tesis, identificación, orígen y números de acceso de las diferentes colecciones y de las secuencias depositadas en el GenBank.

Taxón	Número de colección	Sustrato	Origen	Códigos de acceso del GenBank				
				BenA	***CaM***	***rpb2***	**ITS**	**LSU**
Akanthomyces muscarius	FMR 16048	Suelo	Antártida, Isla King George	–	–	–	LR812690	–
Alternaria alternata	FMR 15666	Corcho	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LS453304
Alternaria multiformis	FMR 16018	Miel	España, Castilla y León, Salamanca	–	–	–	LT963545	LT963546
Antarctomyces psychrotrophicus	FMR 16038	Suelo	Antártida, Isla King George	–	–	–	LR812688	–
Arachnomyces bostrychodes	FMR 17685 = CBS 834921 ^{T}	Aislado clínico	Estados Unidos, Texas	–	–	–	LR701765	LR701766
Arachnomyces graciliformis	FMR 17691 = CBS 834923 ^{T}	Aislado clínico	Estados Unidos, Massachusetts	–	–	–	LR743667	LR743668
Areolospora bosensis	FMR 17563	Suelo	Vietnam	–	–	–	LR812704	–
Arthrographis curvata	FMR 17507	Suelo	Armenia	–	–	–	LR812699	–
Arthrographis kalrae	FMR 17514	Suelo	Vietnam	–	–	–	LR812703	–
Ascosphaera atra	FMR 16318	Miel	España, Extremadura, Cáceres	–	–	–	LT964944	LT984552
Ascotricha lusitanica	FMR 17427	Suelo	Armenia	–	–	–	LR812698	–
Ascotricha lusitanica	FMR 17607	Suelo	Irán, Isfaham	–	–	–	–	LR812705
Aspergillus asperescens	FMR 16310	Miel	España, Castilla y León, Zamora	LT963510	–	–		LT986672
Aspergillus aureolatus	FMR 16664	Cava	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LS453306
Aspergillus aureolatus	FMR 16676	Cava	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LS453310

Aspergillus jensenii	FMR 16673	Cava	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LS453309
Aspergillus montevidensis	FMR 15994	Miel	España, Valencia, Castellón	LR027804	–	–	LT963466	LT984537
Aspergillus pseudoglaucus	FMR 9392	Miel	España, Valencia, Castellón	LT963512	–	–	–	LT984695
Aspergillus pseudoglaucus	FMR 15992	Miel	España, Valencia, Castellón	LT963513	–	–	–	LT984696
Aspergillus pseudoglaucus	FMR 15993	Miel	España, Valencia, Castellón	LT963514	–	–	–	LT984697
Aspergillus pseudoglaucus	FMR 16011	Miel	España, Castilla y León, Salamanca	LT963518	–	–	–	LT984701
Aspergillus pseudoglaucus	FMR 16112	Miel	España, Castilla-La Mancha, Ciudad Real	LT963515	–	–	–	LT984698
Aspergillus pseudoglaucus	FMR 16281	Miel	España, Castilla-La Mancha, Ciudad Real	LT963516	–	–	–	LT984699
Aspergillus pseudoglaucus	FMR 16317	Miel	España, Castilla y León, Zamora	LT963517	–	–	–	LT984700
Aspergillus puulaauensis	FMR 16672	Cava	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LS453308
Aspergillus xerophilus	FMR 16004	Suelo	Chile, Ayllu de Sequitor, Atacama	–	–	–	LR812697	–
Beauveria bassiana	FMR 16669	Cava	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LS453307
Bettsia alvei	FMR 15670	Miel	España, Valencia, Castellón	–	–	–	–	LT963566
Bettsia alvei	FMR 15672	Miel	España, Valencia, Castellón	–	–	–	–	LT963567
Bettsia alvei	FMR 15678	Miel	España, Valencia, Castellón	–	–	–	–	LT963568
Bettsia alvei	FMR 15681	Miel	España, Valencia, Castellón	–	–	–	–	LT963569
Bettsia alvei	FMR 15685	Miel	España, Valencia, Castellón	–	–	–	–	LT963570
Bettsia alvei	FMR 16111	Miel	España, Extremadura, Cáceres	–	–	–	–	LT963571
Bettsia alvei	FMR 16115	Miel	España, Castilla-La Mancha, Toledo	–	–	–	–	LT963572

Bettsia alvei	FMR 16305	Miel	España, Galicia, Ourense	–	–	–	–	LT963574
Bettsia alvei	FMR 16313	Miel	España, Galicia, Ourense	–	–	–	–	LT963575
Bettsia alvei	FMR 16568	Miel	España, Extremadura, Cáceres	–	–	–	–	LT963573
Bettsia alvei	FMR 16570	Miel	España, Galicia, Ourense	–	–	–	–	LT963576
Biscogniauxia mediterranea	FMR 17639	Suelo	España, Mallorca	–	–	–	–	LR812685
Cadophora antarctica	FMR 16056 = CBS 143035 ^{T}	Suelo	Antártida, Isla King George	–	–	–	MG385664	MG385663
Candida glaebosa	FMR 17650	Suelo	Antártida, Isla King George	–	–	–	–	LR812693
Candida magnoliae	FMR 16311	Miel	España, Galicia, Ourense	–	–	–	–	LT963487
Candida magnoliae	FMR 16314	Miel	España, Galicia, Ourense	–	–	–	–	LT963488
Candida magnoliae	FMR 16496	Miel	España, Galicia, Ourense	–	–	–	–	LT963486
Candida patagonica	FMR 15463	Cava	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LS479915
Candida patagonica	FMR 15475	Cava	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LS479916
Candida patagonica	FMR 16674	Cava	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LS479914
Candida sorbosivorans	FMR 16278	Miel	España, Galicia, Ourense	–	–	–	–	LT963489
Chromelosporium sp. nov.	FMR 17605	Suelo	España, Mallorca	–	–	–	–	–
Cladophialophora recurvata	FMR 16667 = CBS 143843 ^{T}	Cava	España, Barcelona, Sadurní d'Anoia	LT985894	–	–	LT985878	LT985879
Cladosporium cladosporioides	FMR 15660	Corcho	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LS453303
Coniolariella limoniispora	FMR 17635	Suelo	Armenia, Yerevan	–	–	–	–	LR812700
Coniothyrium fraxini	FMR 17094	Hisopado	España, Tarragona, Els Pallaresos	–	–	–	–	LR812684
Corynascus verrucosus	FMR 17512	Suelo	Vietnam	–	–	–	LR812701	LR812702

Cunninghamella bertholletiae	FMR 16008	Miel	España, Castilla y León, Salamanca	–	–	–	LT963490	LR215930
Currahmyces sparsispora	FMR 17683 ^{T}	Aislado clínico	Estados Unidos, Florida	–	–	–	LR723272	LR723273
Dactylodendron ebriosum	FMR 16677	Cava	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LT985881
Dactylodendron ebriosum	FMR 15658 = CBS 144321 ^{T}	Corcho	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LT985880
Dactylodendron pluriseptatum	FMR 16678 = CBS 143846 ^{T}	Cava	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LT985882
Dothiora infuscans	FMR 16326 = CBS 144317 ^{T}	Hisopado	España, Tarragona, Els Pallaresos	–	–	–	LT993342	LT993345
Dothiora infuscans	FMR 16336	Hisopado	España, Tarragona, Els Pallaresos	–	–	–	LT993343	LT993346
Dothiora infuscans	FMR 16329	Hisopado	España, Tarragona, Els Pallaresos	–	–	–	LT993344	LT993347
Eremascus albus	FMR 16116	Miel	España, Extremadura, Cáceres	–	–	–	–	LT964975
Eremascus albus	FMR 16118	Miel	España, Extremadura, Cáceres	–	–	–	–	LT964976
Eremascus albus	FMR 16119	Miel	España, Castilla-La Mancha, Toledo	–	–	–	–	LT964977
Eremascus albus	FMR 16493	Miel	España, Extremadura, Cáceres	–	–	–	–	LT964978
Exophiala frigidotolerans	FMR 17078 = CBS 146539 ^{T}	Suelo	Ecuador, Guayaquil	LR699568	–	–	LR699566	LR699567
Geosmithia xerotolerants	FMR 17085 = CBS 144969 ^{T}	Hisopado	España, Tarragona, Els Pallaresos	LS998791	–	–	LS998789	LS998790
Helicoarthrosporum mellicola	FMR 15673	Miel	España, Valencia, Castellón	–	–	–	–	LT978462
Helicoarthrosporum mellicola	FMR 16307	Miel	España, Castilla y León, León	–	–	–	–	LT978463
Helicoarthrosporum mellicola	FMR 16308	Miel	España, Castilla y León, Zamora	–	–	–	–	LT906536
Helicoarthrosporum mellicola	FMR 16315	Miel	España, Extremadura, Cáceres	–	–	–	–	LT906537

Helicoarthrosporum mellicola	FMR 15679 = CBS 143838 ^{T}	Miel	España, Valencia, Castellón	–	–	–	–	LT906535
Hypoxylon sp. nov.	FMR 17508	Suelo	Vietnam	–	–	–	–	–
Hypoxylon sp. nov.	FMR 17509	Suelo	Vietnam	–	–	–	–	–
Kirschsteiniothelia ebriosa	FMR 16665	Cava	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LT985885
Kirschsteiniothelia ebriosa	FMR 16666 = CBS 143842 ^{T}	Cava	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LT985884
Kirschsteiniothelia vinifera	FMR 16668	Cava	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LS453301
Kirschsteiniothelia vinifera	FMR 15668 = CBS 143837 ^{T}	Corcho	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LT985883
Malbranchea albolutea	FMR 17679	Aislado clínico	Estados Unidos, Texas	–	–	–	LR701834	LR701835
Malbranchea albolutea	FMR 17689	Aislado clínico	Estados Unidos, Texas	–	–	–	LR701836	LR701837
Malbranchea aurantiaca	FMR 17688	Aislado clínico	Estados Unidos, California	–	–	–	LR701824	LR701825
Malbranchea aurantiaca	FMR 17682	Aislado clínico	Estados Unidos, Texas	–	–	–	LR701826	LR701827
Malbranchea conjugata	FMR 17699	Aislado clínico	Estados Unidos, Florida	–	–	–	LR701828	LR701829
Malbranchea conjugata	FMR 17697	Aislado clínico	Estados Unidos, Texas	–	–	–	LR701830	LR701831
Malbranchea flocciformis	FMR 17698	Aislado clínico	Estados Unidos, Texas	–	–	–	LR701822	LR701823
Malbranchea gymnoascoidea	FMR 17681 = CBS 835212 ^{T}	Aislado clínico	Estados Unidos, Texas	–	–	–	LR701757	LR701758
Malbranchea multiseptata	FMR 17695 = CBS 835213 ^{T}	Aislado clínico	Estados Unidos, Texas	–	–	–	LR701759	LR701760
Malbranchea stricta	FMR 17680 = CBS 835219 ^{T}	Aislado clínico	Estados Unidos, Florida	–	–	–	LR701638	LR701639

Malbranchea umbrina	FMR 17700	Aislado clínico	Estados Unidos, Colorado	–	–	–	LR701814	LR701815
Malbranchea umbrina	FMR 17701	Aislado clínico	Estados Unidos, Colorado	–	–	–	LR701816	LR701817
Malbranchea umbrina	FMR 17693	Aislado clínico	Estados Unidos, Washinton DC	–	–	–	LR701820	LR701821
Malbranchea umbrina	FMR 17694	Aislado clínico	Estados Unidos, Baltimore	–	–	–	LR701818	LR701819
Malbranchea zuffiana	FMR 17690	Aislado clínico	Estados Unidos, Washinton DC	–	–	–	LR701832	LR701833
Microdochium lycopodinum	FMR 17646	Suelo	Antártida, Isla King George	–	–	–	–	LR812691
Monascus pilosus	FMR 16306	Miel	España, Castilla y León, Zamora	–	–	–	LT963491	LT984551
Monascus purpureus	FMR 16283	Miel	España, Castilla y León, Ávila	–	–	–	LT963492	LR215932
Monascus purpureus	FMR 16316	Miel	España, Extremadura, Cáceres	–	–	–	LT963493	LT984550
Monascus purpureus	FMR 16321	Miel	España, Extremadura, Cáceres	–	–	–	LT963494	LR215933
Monascus ruber	FMR 16284	Miel	España, Castilla y León, Zamora	–	–	–	LT963495	LT986673
Monodictys arctica	FMR 16037	Suelo	Antártida, Isla King George	–	–	–	LR812687	–
Mortierella antarctica	FMR 17647	Suelo	Antártida, Isla King George	–	–	–	–	LR812692
Mucor plumbeus	FMR 16012	Miel	España, Castilla-La Mancha, Ciudad Real	–	–	–	LT963539	LR215934
Mucor plumbeus	FMR 16013	Miel	España, Castilla y León, Salamanca	–	–	–	LT963540	LT984540
Mucor plumbeus	FMR 16017	Miel	España, Castilla y León, Salamanca	–	–	–	LT963541	LT984548
Neocucurbitaria keratinophila	FMR 17092	Hisopado	España, Tarragona, Els Pallaresos	–	–	–	–	LR812683
Neonectria candida	FMR 17643	Suelo	Antártida, Isla King George	–	–	–	–	LR794336
Nothophoma gossypiicola	FMR 15790	Suelo	Chile, Ayllu de sequitor, Atacama	LR812681	–	LR812725	LR812694	LR812695

Oidiodendron mellicola	FMR 15680	Miel	España, Valencia, Castellón	–	–	–	LT906540	LT978465
Oidiodendron mellicola	FMR 16023	Miel	España, Castilla y León, Salamanca	–	–	–	LT978506	LT978470
Oidiodendron mellicola	FMR 16031	Miel	España, Castilla-La Mancha, Ciudad Real	–	–	–	LT906541	LT978466
Oidiodendron mellicola	FMR 16117	Miel	España, Castilla-La Mancha, Ciudad Real	–	–	–	LT978503	LT978467
Oidiodendron mellicola	FMR 16120	Miel	España, Castilla-La Mancha, Toledo	–	–	–	LT978507	LT978471
Oidiodendron mellicola	FMR 16274	Miel	España, Castilla y León, Burgos	–	–	–	LT978509	LT978473
Oidiodendron mellicola	FMR 16282	Miel	España, Castilla-La Mancha, Toledo	–	–	–	LT978508	LT978472
Oidiodendron mellicola	FMR 16503	Miel	España, Castilla-La Mancha, Ciudad Real	–	–	–	LT978504	LT978468
Oidiodendron mellicola	FMR 16504	Miel	España, Galicia, Ourense	–	–	–	LT978505	LT978469
Oidiodendron mellicola	FMR 15683 = CBS 143839 ^{T}	Miel	España, Valencia, Castellón	–	–	–	LT906544	LT978464
Penicillium camemberti	FMR 16016	Miel	España, Castilla y León, Salamanca	LR027805	–	–	LT963578	LT984541
Penicillium citrinum	FMR 16028	Miel	España, Castilla y León, Salamanca	LT963451	–	–	–	LT984702
Penicillium corylophilum	FMR 16661	Cava	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LS453305
Penicillium corylophilum	FMR 15659	Corcho	España, Barcelona, Sadurní d'Anoia	–	–	–	–	LS453302
Penicillium corylophilum	FMR 16010	Miel	España, Asturias	LR027808	–	–	LT963581	LT984538
Penicillium corylophilum	FMR 16027	Miel	España, Asturias	LT963452	–	–	–	LT986674
Penicillium corylophilum	FMR 16030	Miel	España, Extremadura, Cáceres	LR027809	–	–	LT963582	LT984547
Penicillium cravenianum	FMR 16019	Miel	España, Castilla y León, Salamanca	LR027807	–	–	LT963580	LT984542

Penicillium cravenianum	FMR 16020	Miel	España, Extremadura, Cáceres	LR027806	–	–	LT963579	LT984549
Penicillium heteromorphum	FMR 17343	Hisopado	España, Tarragona, Els Pallaresos	LR812679	–	–	–	–
Penicillium melanosporum	FMR 17424 ^{T}	Suelo	España, Castilla y León, Riaza	LR655196	LR655200	LR655204	LR655192	–
Penicillium michoacanense	FMR 17612 ^{T}	Suelo	México, Michoacán, Villa Jiménez	LR655198	LR655202	LR655206	LR655194	–
Penicillium rubens	FMR 15996	Suelo	Chile, Ayllu de sequitor, Atacama	–	–	–	LR812696	–
Penicillium sexuale	FMR 17380 ^{T}	Suelo	España, Castilla y León, Riaza	LR655199	LR655203	LR655207	LR655195	–
Penicillium siccitolerans	FMR 17381 ^{T}	Suelo	España, Castilla y León, Riaza	LR655197	LR655201	LR655205	LR655193	–
Penicillium turbatum	FMR 17229	Suelo	España, Castilla y León, Riaza	LR812680	–	–	–	–
Phialemonium guarroi	FMR 17080 = CBS 145626 ^{T}	Suelo	España, Canarias, La Palma	–	–	–	LR535737	LR535738
Pseudoarthropsis crassispora	FMR 17692 = CBS 834930 ^{T}	Aislado clínico	Estados Unidos, Minnesota	–	–	–	LR701763	LR701764
Pseudomalbranchea gemmata	FMR 17684 = CBS 835221 ^{T}	Aislado clínico	Estados Unidos, Florida	–	–	–	LR701761	LR701762
Rasamsonia frigidotolerans	FMR 16670	Cava	España, Barcelona, Sadurní d'Anoia	LT985896	LT985898	–	LT985887	LS453295
Rasamsonia frigidotolerans	FMR 16675 = CBS 143845 ^{T}	Cava	España, Barcelona, Sadurní d'Anoia	LT985895	LT985897	–	LT985886	LS453294
Rhizopus oryzae	FMR 16022	Miel	España, Extremadura, Cáceres	–	–	–	LT963543	LR215931
Schizosaccharomyces octosporus	FMR 16279	Miel	España, Galicia, Ourense	–	–	–	–	LT963544
Skoua asexualis	FMR 16497	Miel	España, Extremadura, Cáceres	–	–	–	LT964664	LT964665
Skoua asexualis	FMR 16567	Miel	España, Extremadura, Cáceres	–	–	–	LT964666	LT964667
Skoua asexualis	FMR 16572 = CBS 144072 ^{T}	Miel	España, Castilla y León, León	–	–	–	LT964668	LT964669

Skoua fertilis	FMR 10812	Miel	España, Valencia, Castellón	LR585993	–	LR586005	LR585979	LT965019
Skoua fertilis	FMR 10813	Miel	España, Valencia, Castellón	LR585994	–	LR586006	LR585980	LT965023
Skoua fertilis	FMR 10814	Miel	España, Valencia, Castellón	LR585995	–	–	LR585981	LT965016
Skoua fertilis	FMR 10815	Miel	España, Valencia, Castellón	–	–	LR586007	LR585982	LT965015
Skoua fertilis	FMR 15671	Miel	España, Valencia, Castellón	LR585996	–	LR586008	LR585983	LT965014
Skoua fertilis	FMR 15676	Miel	España, Valencia, Castellón	LR585997	–	LR586009	LR585984	LT965017
Skoua fertilis	FMR 15682	Miel	España, Valencia, Castellón	LR585998	–	LR586010	LR585985	LT965018
Skoua fertilis	FMR 15686	Miel	España, Valencia, Castellón	LR585999	–	LR586011	LR585986	LT965020
Skoua fertilis	FMR 15687	Miel	España, Valencia, Castellón	LR586000	–	LR586012	LR585987	LT965021
Skoua fertilis	FMR 15689	Miel	España, Valencia, Castellón	LR586001	–	–	LR585988	LT965022
Skoua fertilis	FMR 16032	Miel	España, Asturias	–	–	–	LR585989	LT965024
Skoua fertilis	FMR 16320	Miel	España, Castilla y León, Zamora	–	–	–	LR585990	LT965025
Skoua fertilis	FMR 16492	Miel	España, Extremadura, Cáceres	–	–	–	LR585991	LT965026
Skoua fertilis	FMR 16571	Miel	España, Extremadura, Badajoz	LR586002	–	LR586013	LR585992	LT965027
Spiromastigoides geomyces	FMR 17686	Aislado clínico	Estados Unidos, Minnesota	–	–	–	LR701769	LR701770
Spiromastigoides geomyces	FMR 17696 = CBS 835222 ^{T}	Aislado clínico	Estados Unidos, Illinois	–	–	–	LR701767	LR701768
Strongyloarthrosporum catenulatum	FMR 16121 = CBS 143841 ^{T}	Miel	España, Castilla-La Mancha, Toledo	–	–	–	–	LT906534
Superstratomyces tardicrescens	FMR 17387	Hisopado	España, Tarragona, Els Pallaresos	–	–	LR812723	LR812722	LR812722
Talaromyces affinitatimellis	FMR 15674	Miel	España, Valencia, Castellón	LT965001	–	–	–	LT968852
Talaromyces affinitatimellis	FMR 15675	Miel	España, Valencia, Castellón	LT965002	–	–	–	LT968853

Talaromyces affinitatimellis	FMR 15677	Miel	España, Valencia, Castellón	LT965003	–	–	–	LT968854
Talaromyces affinitatimellis	FMR 15684	Miel	España, Valencia, Castellón	LT965004	–	–	–	LT968855
Talaromyces affinitatimellis	FMR 15688	Miel	España, Valencia, Castellón	LT906553	LT906550	LT906547	LT906538	LT964941
Talaromyces affinitatimellis	FMR 16029	Miel	España, Extremadura, Cáceres	LT965005	–	–	–	LT968856
Talaromyces affinitatimellis	FMR 16033	Miel	España, Castilla y León, Salamanca	LT906554	LT906551	LT906548	LT906539	LT964942
Talaromyces affinitatimellis	FMR 16114	Miel	España, Castilla y León, Salamanca	LT965006	–	–	–	LT968857
Talaromyces affinitatimellis	FMR 16125	Miel	España, Castilla y León, Zamora	LT965009	–	–	–	LT968860
Talaromyces affinitatimellis	FMR 16126	Miel	España, Castilla y León, Zamora	LT965012	–	–	–	LT968861
Talaromyces affinitatimellis	FMR 16276	Miel	España, Castilla y León, Zamora	LT965010	–	–	–	LT968862
Talaromyces affinitatimellis	FMR 16494	Miel	España, Castilla y León, Zamora	LT965011	–	–	–	LT968863
Talaromyces affinitatimellis	FMR 16499	Miel	España, Extremadura, Cáceres	LT965007	–	–	–	LT968858
Talaromyces affinitatimellis	FMR 16501	Miel	España, Extremadura, Cáceres	LT965008	–	–	–	LT968859
Talaromyces affinitatimellis	FMR 15690 = CBS 143840 ^{T}	Miel	España, Valencia, Castellón	LT906552	LT906549	LT906546	LT906543	LT964939
Talaromyces basipetosporus	FMR 9720 = CBS 143836 ^{T}	Miel	Argentina, Buenos Aires	LT906563	–	LT906545	LT906542	LT964940
Talaromyces brunneosporus	FMR 16566 = CBS 144320 ^{T}	Miel	España, Castilla y León, Salamanca	LT962483	LT962488	LT962485	LT962487	LT964943
Talaromyces speluncarum	FMR 16662	Cava	España, Barcelona, Sadurní d'Anoia	LT985902	LT985907	LT985912	LT985891	LS453297

Talaromyces speluncarum	FMR 16663	Cava	España, Barcelona, Sadurní d'Anoia	LT985903	LT985908	LT985913	LT985892	LS453298
Talaromyces speluncarum	FMR 16671 = CBS 143844 [T]	Cava	España, Barcelona, Sadurní d'Anoia	LT985901	LT985906	LT985911	LT985890	LS453296
Talaromyces subericola	FMR 15664	Corcho	España, Barcelona, Sadurní d'Anoia	LT985900	LT985905	LT985910	LT985889	LS453300
Talaromyces subericola	FMR 15656 = CBS 144322 [T]	Corcho	España, Barcelona, Sadurní d'Anoia	LT985899	LT985904	LT985909	LT985888	LS453299
Thelebolus microsporus	FMR 16035	Suelo	Antártida, Isla King George	–	–	–	LR812686	–
Thelebolus microsporus	FMR 16039	Suelo	Antártida, Isla King George	–	–	–	LR812689	–
Virgaria sp. nov.	FMR 17568	Suelo	Vietnam	–	–	–	–	–
Xenodidymella saxea	FMR 17089	Hisopado	España, Tarragona, Els Pallaresos	–	–	–	–	LR812682
Xerochrysium xerophilum	FMR 15669	Miel	España, Valencia, Castellón	–	–	–	LT986724	LT986675
Zygosaccharomyces gambellarensis	FMR 16277	Miel	España, Castilla y León, Salamanca	–	–	–	–	LT963549
Zygosaccharomyces gambellarensis	FMR 16569	Miel	España, Extremadura, Cáceres	–	–	–	–	LT963548
Zygosaccharomyces mellis	FMR 16280	Miel	España, Galicia, Ourense	–	–	–	–	LT963550
Zygosaccharomyces mellis	FMR 16312	Miel	España, Galicia, Ourense	–	–	–	–	LT963551
Zygosaccharomyces siamensis	FMR 16034	Miel	España, Castilla y León, Salamanca	–	–	–	LT963547	LT984543

[a] **FMR**: Número de la colección de los aislados de la *Facultat de Medicina de Reus*,Tarragona, Spain**.**

[b] **CBS**: Aislados de la colección del *Westerdijk Biodiversity Institute*, Utrech, Países bajos; **UTHSC**: Aislados de la colección del *Fungus Testing Laboratory, University of Texas Health Science Center*, San Antonio, Texas, USA.

[c] **T** indica que corresponde a una cepa tipo.

4.2. Diversity of xerotolerant and xerophilic fungi in honey

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Diversity of xerotolerant and xerophilic fungi in honey

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Abstract

Fungi can colonize most of the substrata on Earth. Honey, a sugary food produced by bees (and other insects) has been studied little in terms of its fungal diversity. We have surveyed and evaluated the presence of xerotolerant and xerophilic fungi in a set of honey bee samples collected from across Spain. From 84 samples, a total of 104 fungal strains were isolated, and morphologically and phylogenetically characterized. We identified 32 species distributed across 16 genera, most of them belonging to the ascomycetous genera *Aspergillus*, *Bettsia*, *Candida*, *Eremascus*, *Monascus*, *Oidiodendron*, *Penicillium*, *Skoua*, *Talaromyces* and *Zygosaccharomyces*. As a result of this survey, eight new taxa are proposed: i.e. the new family *Helicoarthrosporaceae,* two new genera, *Helicoarthrosporum* and *Strongyloarthrosporum* in *Onygenales*; three new species of *Eurotiales*, *Talaromyces affinitatimellis*, *T. basipetosporus*, and *T. brunneosporus*; and two new species of *Myxotrichaceae, Oidiodendron mellicola,* and *Skoua asexualis*.

Keywords: *Eurotiales*, Fungi, Honey, New taxa, *Onygenales*, Osmophiles, Xerophiles

INTRODUCTION

Honey is a natural sweetener produced by honey bees (insects of the genus *Apis* of the order *Hymenoptera*) from nectar (blossom honey or nectar honey) or from carbohydrate-rich secretions of living green parts of plants or excretions of plant-sucking phytophagous aphids (insects of the family *Aphidida*, order *Hemiptera*) (honeydew honey) after combination with the bee's specific substances, placement, dehydration, and storage in the honey comb to ripen and mature. Honey is mostly composed of monosaccharides (dextrose and fructose), at a concentration of not lower than 60% and a much lesser amount of oligosaccharides, organic acids, enzymes (amylases and α-glucosidase) and solid particles. Due to its particular physicochemical nature and biological origin, honey should be an ideal substratum for the development of xerotolerant and xerophilic fungi. However, little information has been gathered about these fungi and their relationships with honey and honey products. Nonetheless, most of the fungal species from honey had been reported as new for science. Representative ascomycetous yeasts found in honey are *Blastobotrys meliponae*, *Candida lundiana*, *C. magnoliae*, *C. sorbosivorans*, *C. suthepensis*, *Schizosaccharomyces octosporus*, *Trichosporon mucoides*, *Zygosaccharomyces favi*, *Z. mellis*, *Z. richteri*, *Z. rouxii,* and *Z. siamensis* (Lochhead & Farrell 1931; Ruiz-Argueso & Rodriguez-Navarro 1975; Carvalho et al. 2010; Saksinchai et al. 2012a, b; Čadež et al. 2015; Crous et al. 2016). The obligate xerophiles *Ascosphaera apis* and *Bettsia alvei* have been reported in honey, as well as several xerotolerant species of *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* and a few mucoralean fungi (Snowdon & Cliver 1996; Kačániová et al. 2009; Pettersson & Leong 2011; Kačániová et al. 2012; Sinacori et al. 2014; Grabowski & Klein 2015). Recently, *Monascus mellicola, Penicillium apimei*, *P. meliponae*, *P. mellis,* and *Talaromyces brasiliensis* were reported from honey produced by stingless bees (*Melipona scutellaris*, family *Apidae*, order *Hymenoptera*) inhabiting Brazilian forests (Barbosa et al. 2017, 2018). Common environmental and plant pathogenic species of fungi have been reported in samples of honey collected in Spain (Pérez-Sánchez et al. 1997; Seijo et al. 2011; Magyar et al. 2016; Terrab et al. 2019) and Portugal (Martíns et al. 2003). In another study, the yeast *Metschnikowia reukaufii* was,

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surprisingly, the only fungus reported for floral honey from Portugal and Spain (Magyar et al. 2005). Although honey should be a substratum amenable for the development of xerotolerant and xerophilic fungi, few studies have intentionally targeted these fungi. Therefore, the main objective of this study was to assess the diversity of honey-associated fungi, by employing a selective culture medium to a set of samples collected predominantly in Spain, and to characterize the morphology, physiology and phylogeny of new isolates and those considered of taxonomic interest.

MATERIALS AND METHODS

Fungal isolation

A total of 83 samples of honeydew and blossom (nectar) honey from different locations in Spain (Fig. 1), and one from Argentina (San Martín, Buenos Aires province), have been processed. All samples were of the harvest in 2014, stored in settling tanks, and after a variable period of time clarified by filtration (with one exception, which was by centrifugation). Seventy-two of the Spanish samples corresponded to honeydew honeys, 45 from trading companies and 27 collected and processed by beekeepers. A few of the samples provided by commercial companies were categorized (according to the nature of the honeydew) as oak, holm oak and forest honey. The 11 samples of blossom honey were provided by beekeepers, and these were classified as multifloral. All samples provided by commercial companies were subjected to a thermal treatment, subjecting the honey at 45–55 °C for a few hours up to 2 days, or pasteurized (2 min at 80 °C). The samples provided by beekeepers have not undergone any heat treatment. For each sample, 10 g of honey was dissolved into 90 mL of sterile water in a sterile disposable plastic container, and 1 mL of such dilution (1:10) was aseptically plated onto two 90 mm diam. plastic Petri dishes and mixed with 15 mL of molten (at 50–55 °C) 18% glycerol agar (G18; DG18 [Hocking & Pitt 1980] without dichloran: 5 g peptone, 10 g dextrose, 1 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 15 g agar-agar, 110 g glycerol, 1 L tap water, and supplemented with 250 mg/L of L-chloramphenicol). Once the medium had solidified, one of the Petri dishes was incubated in darkness at 15 °C and the other at 25 °C for up to 2 months. The colonies developed were examined under a stereomicroscope. Fungal structures from selected (representative of

Fig. 1 Locations of Spain where the samples were collected. Asturias (As), Ávila (Av), Badajoz (Ba), Burgos (Bu), Cáceres (Cac), Castellón (Cas), Ciudad Real (CR), Granada (Gra), León (Le), Ourense (Ou), Salamanca (Sa), Tarragona (Tarr), Toledo (To), Zamora (Zam), and Zaragoza (Zar)

all morphological variety) colonies were transferred to 50 mm diam. Petri dishes containing G18 by using a sterile insulin-type needle and incubated in the same conditions to obtain pure cultures.

Phenotypic study

For cultural characterization, suspensions of spores from the isolates were prepared in a semi-solid medium (0.2% agar; 0.05% Tween 80), and 0.5 μL of such suspension was inoculated onto malt extract agar (MEA; Difco, Detroit, USA; Samson et al. 2010), oatmeal agar (OA; 30 g of filtered oat flakes, 15 g agar-agar, 1 L tap water; Samson et al. 2010), Czapek yeast extract agar (CYA; 30 g sucrose, 3 g $NaNO_3$, 5 g yeast extract, 1 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeSO_4$, 15 g agar-agar, 1 L tap water; Pitt 1979), yeast extract sucrose agar (YES; 20 g yeast extract, 150 g sucrose, 0.5 g $MgSO_4{\cdot}7H_2O$, 20 g agar-agar, 1 L tap water; Frisvad 1981), creatine sucrose agar (CREA; 3 g creatine, 30 g sucrose, 1.6 g $K_3PO_4{\cdot}7H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.05 g bromocresol purple, 20 g agar-agar, 1 L tap water; Frisvad 1981), G18, potato dextrose agar (PDA; Pronadisa, Madrid, Spain; Hawksworth et al. 1995), 25% glycerol nitrate agar (G25 N; 7.5 g Czapek concentrate, 0.75 g K_2HPO_4, 3.7 g yeast extract, 250 mL glycerol, 12 g agar-agar, 1 L tap water; Pitt 1979), bromocresol purple milk solids glucose agar (BCP-MS-G; 80 g skim milk powder, 40 g glucose, 10 mL of 1.6% of bromocresol purple in 95% ethanol, 30 g agar-agar,1 L tap water; Kane & Smitka 1978), test opacity tween medium (TOTM; 10 g bacteriological peptone, 5 g NaCl, 1 g $CaCl_2$, 5 mL Tween 80, 15 g agar-agar, 1 L tap water; Slifkin 2000), phytone yeast extract agar (PYE; Becton, Dickinson & Co., Sparks, MD, USA; Carmichael & Kraus 1959), malt extract yeast extract 70% fructose-glucose (MY70FG; 6 g malt extract, 6 g yeast extract, 10 g peptone, 350 g fructose, 350 g glucose, 12 g agar-agar, 1 L tap water; Beuchat & Hocking 1990), and blood agar (Becton, Dickinson & Co., Sparks, MD, USA). Colonies were characterized after three wk. at 25 °C in darkness. G18 medium was used to determine the minimum, optimal and maximum temperatures of growth. Christensen's urea agar (EMD Millipore, Darmstadt, Germany; Christensen 1946) was inoculated and incubated during 4–7 days at 25 °C in darkness to detect the production of urease. Cycloheximide tolerance of the fungal strains was tested on Sabouraud dextrose agar (SDA; Pronadisa, Spain) supplemented with 0.2% of cycloheximide (Sigma, USA) after incubation at 30 °C for two wk. Fungal tolerance to NaCl was evaluated on SDA adding 3, 10 and 20% w/v NaCl, with the same incubation conditions as in the previous test. Colour notations were according to Kornerup & Wanscher (1978). The microscopic structures were characterized and measured from wet mountings of slide cultures, using water and 60% lactic acid. Photo micrographs were taken using a Zeiss AxioImager M1 light microscope (Oberkochen, Germany) with a DeltaPix Infinity X digital camera, using Nomarski differential interference contrast. The samples for scanning electron microscopy (SEM) were processed according to Figueras & Guarro (1988), and SEM micrographs were taken at 15 keV with a JEOL JSM 840 microscope.

DNA extraction, amplification and sequencing

Total deoxyribonucleic acid (DNA) was extracted according to Marimon et al. (2006), and a fragment of the 28S nrRNA gene (LSU) was amplified and sequenced using the primer pair LR0R (Rehner & Samuels 1994) and LR5 (Vilgalys & Hester 1990). For some isolates the following markers were amplified and sequenced: ribosomal internal transcribed spacers (ITS) (ITS5/ITS4; White et al. 1990); and fragments of the beta-tubulin (*BenA*) (Bt2a/Bt2b; Glass & Donaldson 1995), calmodulin (*CaM*) (Cmd5/Cmd6; Hong et al. 2005) and RNA polymerase II subunit 2 (*rpb2*) (RPB2-5F/RPB2-7cR; Liu et al. 1999) genes. Amplicons were sequenced at Macrogen Europe (Macrogen, Amsterdam, The Netherlands). Consensus sequences were obtained using the SeqMan software v. 7 (DNAStar Lasergene, Madison, WI, USA). Sequences we generated were deposited in GenBank (Table 1).

Phylogenetic analysis

A preliminary molecular identification of the isolates was carried out with LSU sequences using Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) and only the type sequences or reliable reference strains from GenBank were considered for identification, and a maximum level of identity (MLI) of ≥98% was used for identification at the rank of species and < 98% at the rank of genus. *BenA* for to the genera *Aspergillus*, *Penicillium,* and *Talaromyces*, and ITS for the genera *Monascus*, *Oidiodendron* and *Skoua* were used for identification at the rank of species. An LSU tree was built to determine the phylogenetic relationships of all our isolates. Phylogenetic trees of ITS and a combination of ITS-*BenA-CaM-rpb2* were also built to distinguish the members of *Myxotrichaceae* and the genus *Talaromyces,* respectively. *Cunninghamella bertholletiae* (CBS 693.68), *Mucor plumbeus* (DAOM 220743), *Mucor racemosus* (ATCC 42647), and *Rhizopus oryzae* (CBS 112.07 and CBS 130146) were used as outgroup for the LSU tree; *Aphanoascus keratinophilus* (IMI 319010) for the *Myxotrichaceae* taxa tree; and *Trichocoma paradoxa* (CBS 247.57) for the *Talaromyces* tree. The sequence alignments and the maximum-

Table 1 Fungal taxa recovered with their nucleotide sequence accession number, and the geographic origin of the honey samples processed

Taxon	Culture collection accession number	EMBL/GenBank nucleotide sequence accession number					Geographic origin (province, community)
		BenA	*CaM*	*rpb2*	ITS	LSU	
Alternaria multiformis	FMR 16018	–	–	–	LT963545	LT963546	Salamanca, Castilla y León
Ascosphaera atra	FMR 16318	–	–	–	LT964944	LT984552	Cáceres, Extremadura
Aspergillus asperescens	FMR 16310	LT963510	–	–		LT986672	Zamora, Castilla y León
Aspergillus montevidensis	FMR 15994	LR027804	–	–	LT963466	LT984537	Castellón, Valencia
Aspergillus pseudoglaucus	FMR 9392	LT963512	–	–	–	LT984695	Castellón, Valencia
Aspergillus pseudoglaucus	FMR 15992	LT963513	–	–	–	LT984696	Castellón, Valencia
Aspergillus pseudoglaucus	FMR 15993	LT963514	–	–	–	LT984697	Castellón, Valencia
Aspergillus pseudoglaucus	FMR 16011	LT963518	–	–	–	LT984701	Salamanca, Castilla y León
Aspergillus pseudoglaucus	FMR 16112	LT963515	–	–	–	LT984698	Ciudad Real, Castilla-La Mancha
Aspergillus pseudoglaucus	FMR 16281	LT963516	–	–	–	LT984699	Ciudad Real, Castilla-La Mancha
Aspergillus pseudoglaucus	FMR 16317	LT963517	–	–	–	LT984700	Zamora, Castilla y León
Bettsia alvei	FMR 15670	–	–	–	–	LT963566	Castellón, Valencia
Bettsia alvei	FMR 15672	–	–	–	–	LT963567	Castellón, Valencia
Bettsia alvei	FMR 15678	–	–	–	–	LT963568	Castellón, Valencia
Bettsia alvei	FMR 15681	–	–	–	–	LT963569	Castellón, Valencia
Bettsia alvei	FMR 15685	–	–	–	–	LT963570	Castellón, Valencia
Bettsia alvei	FMR 16111	–	–	–	–	LT963571	Cáceres, Extremadura
Bettsia alvei	FMR 16115	–	–	–	–	LT963572	Toledo, Castilla-La Mancha
Bettsia alvei	FMR 16305	–	–	–	–	LT963574	Ourense, Galicia
Bettsia alvei	FMR 16313	–	–	–	–	LT963575	Ourense, Galicia
Bettsia alvei	FMR 16568	–	–	–	–	LT963573	Cáceres, Extremadura
Bettsia alvei	FMR 16570	–	–	–	–	LT963576	Ourense, Galicia
Candida magnoliae	FMR 16311	–	–	–	–	LT963487	Ourense, Galicia
Candida magnoliae	FMR 16314	–	–	–	–	LT963488	Ourense, Galicia
Candida magnoliae	FMR 16496	–	–	–	–	LT963486	Ourense, Galicia
Candida sorbosivorans	FMR 16278	–	–	–	–	LT963489	Ourense, Galicia
Cunninghamella bertholletiae	FMR 16008	–	–	–	LT963490	LR215930	Salamanca, Castilla y León
Eremascus albus	FMR 16116	–	–	–	–	LT964975	Cáceres, Extremadura
Eremascus albus	FMR 16118	–	–	–	–	LT964976	Cáceres, Extremadura
Eremascus albus	FMR 16119	–	–	–	–	LT964977	Toledo, Castilla-La Mancha
Eremascus albus	FMR 16493	–	–	–	–	LT964978	Cáceres, Extremadura
Helicoarthrosporum mellicola	FMR 15673	–	–	–	–	LT978462	Castellón, Valencia
Helicoarthrosporum mellicola [T]	FMR 15679 = CBS 143838	–	–	–	–	LT906535	Castellón, Valencia
Helicoarthrosporum mellicola	FMR 16307	–	–	–	–	LT978463	León, castilla y León
Helicoarthrosporum mellicola	FMR 16308	–	–	–	–	LT906536	Zamora, Castilla y León
Helicoarthrosporum mellicola	FMR 16315	–	–	–	–	LT906537	Cáceres, Extremadura
Monascus pilosus	FMR 16306	–	–	–	LT963491	LT984551	Zamora, Castilla y León
Monascus purpureus	FMR 16283	–	–	–	LT963492	LR215932	Ávila, Castilla y León
Monascus purpureus	FMR 16316	–	–	–	LT963493	LT984550	Cáceres, Extremadura
Monascus purpureus	FMR 16321	–	–	–	LT963494	LR215933	Cáceres, Extremadura

Table 1 Fungal taxa recovered with their nucleotide sequence accession number, and the geographic origin of the honey samples processed *(Continued)*

Taxon	Culture collection accession number	EMBL/GenBank nucleotide sequence accession number					Geographic origin (province, community)
		BenA	*CaM*	*rpb2*	ITS	LSU	
Monascus ruber	FMR 16284	–	–	–	LT963495	LT986673	Zamora, Castilla y León
Mucor plumbeus	FMR 16012	–	–	–	LT963539	LR215934	Ciudad Real, Castilla-La mancha
Mucor plumbeus	FMR 16013	–	–	–	LT963540	LT984540	Salamanca, Castilla y León
Mucor plumbeus	FMR 16017	–	–	–	LT963541	LT984548	Salamanca, Castilla y León
Oidiodendron mellicola	FMR 15680	–	–	–	LT906540	LT978465	Tarragona, Catalonia
Oidiodendron mellicola [T]	FMR 15683 = CBS 143839	–	–	–	LT906544	LT978464	Castellón, Valencia
Oidiodendron mellicola	FMR 16023	–	–	–	LT978506	LT978470	Salamanca, Castilla y León
Oidiodendron mellicola	FMR 16031	–	–	–	LT906541	LT978466	Ciudad Real, Castilla-La mancha
Oidiodendron mellicola	FMR 16117	–	–	–	LT978503	LT978467	Ciudad Real, Castilla-La Mancha
Oidiodendron mellicola	FMR 16120	–	–	–	LT978507	LT978471	Toledo, Castilla-La Mancha
Oidiodendron mellicola	FMR 16274	–	–	–	LT978509	LT978473	Burgos, Castilla y León
Oidiodendron mellicola	FMR 16282	–	–	–	LT978508	LT978472	Toledo, Castilla-La Mancha
Oidiodendron mellicola	FMR 16503	–	–	–	LT978504	LT978468	Ciudad Real, Castilla-La Mancha
Oidiodendron mellicola	FMR 16504	–	–	–	LT978505	LT978469	Ourense, Galicia
Penicillium camemberti	FMR 16016	LR027805	–	–	LT963578	LT984541	Salamanca, Castilla y León
Penicillium citrinum	FMR 16028	LT963451	–	–	–	LT984702	Salamanca, Castilla y León
Penicillium corylophilum	FMR 16010	LR027808	–	–	LT963581	LT984538	Asturias
Penicillium corylophilum	FMR 16027	LT963452	–	–	–	LT986674	Asturias
Penicillium corylophilum	FMR 16030	LR027809	–	–	LT963582	LT984547	Cáceres, Extremadura
Penicillium cravenianum	FMR 16019	LR027807	–	–	LT963580	LT984542	Salamanca, Castilla y León
Penicillium cravenianum	FMR 16020	LR027806	–	–	LT963579	LT984549	Cáceres, Extremadura
Rhizopus oryzae	FMR 16022	–	–	–	LT963543	LR215931	Cáceres, Extremadura
Schizosaccharomyces octosporus	FMR 16279	–	–	–	–	LT963544	Ourense, Galicia
Skoua asexualis	FMR 16497	–	–	–	LT964664	LT964665	Cáceres, Extremadura
Skoua asexualis	FMR 16567	–	–	–	LT964666	LT964667	Cáceres, Extremadura
Skoua asexualis [T]	FMR 16572 = CBS 144072	–	–	–	LT964668	LT964669	León, castilla y León
Skoua fertilis	FMR 10812	LR585993	–	LR586005	LR585979	LT965019	Castellón, Valencia
Skoua fertilis	FMR 10813	LR585994	–	LR586006	LR585980	LT965023	Castellón, Valencia
Skoua fertilis	FMR 10814	LR585995	–	–	LR585981	LT965016	Castellón, Valencia
Skoua fertilis	FMR 10815	–	–	LR586007	LR585982	LT965015	Castellón, Valencia
Skoua fertilis	FMR 15671	LR585996	–	LR586008	LR585983	LT965014	Castellón, Valencia
Skoua fertilis	FMR 15676	LR585997	–	LR586009	LR585984	LT965017	Castellón, Valencia
Skoua fertilis	FMR 15682	LR585998	–	LR586010	LR585985	LT965018	Castellón, Valencia
Skoua fertilis	FMR 15686	LR585999	–	LR586011	LR585986	LT965020	Castellón, Valencia
Skoua fertilis	FMR 15687	LR586000	–	LR586012	LR585987	LT965021	Castellón, Valencia
Skoua fertilis	FMR 15689	LR586001	–	–	LR585988	LT965022	Castellón, Valencia
Skoua fertilis	FMR 16032	–	–	–	LR585989	LT965024	Asturias
Skoua fertilis	FMR 16320	–	–	–	LR585990	LT965025	Zamora, Castilla y León

Table 1 Fungal taxa recovered with their nucleotide sequence accession number, and the geographic origin of the honey samples processed *(Continued)*

Taxon	Culture collection accession number	EMBL/GenBank nucleotide sequence accession number					Geographic origin (province, community)
		BenA	*CaM*	*rpb2*	ITS	LSU	
Skoua fertilis	FMR 16492	–	–	–	LR585991	LT965026	Cáceres, Extremadura
Skoua fertilis	FMR 16571	LR586002	–	LR586013	LR585992	LT965027	Badajoz, Extremadura
Strongyloarthrosporum catenulatum [T]	FMR 16121 = CBS 143841	–	–	–	–	LT906534	Toledo, Castilla-La Mancha
Talaromyces affinitatimellis	FMR 15674	LT965001	–	–	–	LT968852	Tarragona, Catalonia
Talaromyces affinitatimellis	FMR 15675	LT965002	–	–	–	LT968853	Tarragona, Catalonia
Talaromyces affinitatimellis	FMR 15677	LT965003	–	–	–	LT968854	Tarragona, Catalonia
Talaromyces affinitatimellis	FMR 15684	LT965004	–	–	–	LT968855	Castellón, Valencia
Talaromyces affinitatimellis	FMR 15688	LT906553	LT906550	LT906547	LT906538	LT964941	Castellón, Valencia
Talaromyces affinitatimellis [T]	FMR 15690 = CBS 143840	LT906552	LT906549	LT906546	LT906543	LT964939	Castellón, Valencia
Talaromyces affinitatimellis	FMR 16029	LT965005	–	–	–	LT968856	Cáceres, Extremadura
Talaromyces affinitatimellis	FMR 16033	LT906554	LT906551	LT906548	LT906539	LT964942	Salamanca, Castilla y León
Talaromyces affinitatimellis	FMR 16114	LT965006	–	–	–	LT968857	Salamanca, Castilla y León
Talaromyces affinitatimellis	FMR 16125	LT965009	–	–	–	LT968860	Zamora, Castilla y León
Talaromyces affinitatimellis	FMR 16126	LT965012	–	–	–	LT968861	Zamora, Castilla y León
Talaromyces affinitatimellis	FMR 16276	LT965010	–	–	–	LT968862	Zamora, Castilla y León
Talaromyces affinitatimellis	FMR 16494	LT965011	–	–	–	LT968863	Zamora, Castilla y León
Talaromyces affinitatimellis	FMR 16499	LT965007	–	–	–	LT968858	Cáceres, Extremadura
Talaromyces affinitatimellis	FMR 16501	LT965008	–	–	–	LT968859	Cáceres, Extremadura
Talaromyces basipetosporus [T]	FMR 9720 = CBS 143836	LT906563	–	LT906545	LT906542	LT964940	Buenos Aires, Argentina
Talaromyces brunneosporus [T]	FMR 16566 = CBS 144320	LT962483	LT962488	LT962485	LT962487	LT964943	Salamanca, Castilla y León
Xerochrysium xerophilum	FMR 15669	–	–	–	LT986724	LT986675	Castellón, Valencia
Zygosaccharomyces gambellarensis	FMR 16277	–	–	–	–	LT963549	Salamanca, Castilla y León
Zygosaccharomyces gambellarensis	FMR 16569	–	–	–	–	LT963548	Cáceres, Extremadura
Zygosaccharomyces mellis	FMR 16280	–	–	–	–	LT963550	Ourense, Galicia
Zygosaccharomyces mellis	FMR 16312	–	–	–	–	LT963551	Ourense, Galicia
Zygosaccharomyces siamensis	FMR 16034	–	–	–	LT963547	LT984543	Salamanca, Castilla y León

FMR = Faculty of Medicine of Reus culture collection; CBS = Westerdijk Fungal Biodiversity Institute (ex Centraalbureau voor Schimmelcultures). [T] = ex type

likelihood (ML) and Bayesian-inference (BI) phylogenetic analyses were performed as described previously (Valenzuela-Lopez et al. 2018). The final matrices used for the phylogenetic analysis were deposited in TreeBASE (www.treebase.org; accession number: S23122).

Growth at different water activities (a_w)

To test the capacity of growth in different water activities, media containing malt extract (1% w/w), yeast extract (0.25% w/w) and agar-agar (1% w/w) at pH 5.3 were adjusted at six different a_w (0.97, 0.95, 0.93, 0.92, 0.88 and 0.82) by adding equal weights of fructose and glucose (corresponding to 22, 30, 40, 44, 48, and 55% w/w of sugars, respectively) (Pitt & Hocking 1977). Water activity was measured in duplicate by a water activity meter (Aqualab, Decagon Devices CX3 02734) with an accuracy of ±0.002 at 25 °C. Triplicate plates were inoculated at their centre with 5 μL of spore suspension of selected fungi, and incubated at 25 °C in darkness, with the exception of FMR 15880, FMR 15883 and FMR 16031, which were at 15 °C (because of their poor growth at 25 °C). The colony diam. was measured after 21 days.

RESULTS

Fungal diversity

All honey samples produced fungal colonies on G18 at 15 °C as well as at 25 °C. Table 1 summarizes the fungal strains identified phenotypically and molecularly. With

the exception of a few ascomycetous yeasts and of *Mucorales*, most of the fungi were filamentous *Ascomycota*. From the latter, the highest number of strains corresponded to *Skoua* (syn. *Eremascus*) *fertilis*, *Bettsia alvei,* and *Oidiodendron* sp., followed by an unknown arthrosporic fungus, *Eremascus albus* and *Skoua* sp. *Alternaria multiformis*, *Ascosphaera atra*, another unknown arthrospored fungus and *Xerochrysium xerophilum* were isolated only once. Obligate xerophilic species of *Aspergillus* were not found, but the xerotolerant *A. pseudoglaucus*, *A. asperescens* and *A. montevidensis* were isolated. Three species of *Monascus* were identified, i.e. *M. pilosus, M. purpureus,* and *M. ruber*. The isolates of *Penicillium* were classified as *P. camemberti*, *P. citrinum*, *P. corylophilum,* and *P. cravenianum*. Members of *Talaromyces* were classified at the rank of section, i.e. section *Trachyspermi* and section *Purpurei*. We only identified three species of *Mucoromycota*, viz. *Cunninghamella bertholletiae*, *Mucor plumbeus,* and *Rhizopus oryzae*. Regardless of their geographical origin, type of honey (nectar or honeydew) and if honey was or not thermally treated, *S. fertilis* and *B. alvei* were present in all honey samples.

Molecular phylogeny

Our first phylogenetic study included 206 LSU sequences with a total of 606 characters, including gaps, 352 of them being parsimony informative. The ML analysis was congruent with that obtained in the BI analysis, both displaying trees with similar topologies. The isolates were distributed across two main clades (Fig. 2a-c), the first (100% BS / 1 PP) corresponding to the *Ascomycota* and including 99 isolates, and the second (100% BS / 1 PP) involving the rest of the isolates and pertaining to the *Mucoromycota*. The first main clade was divided into six subclades: A (82% BS / 1 PP), which represents *Onygenales*; B (75% BS / 0.96 PP), *Eurotiales*; C (100% BS / 1 PP); *Pleosporales,* D (unssuported) as *incertae sedis*; E (100% BS / 1 PP), *Schizosaccharomycetales*, and F (94% BS / - PP), *Saccharomycetales*. Subclade A contains seven well-supported groups, six of which represent the known families of *Onygenales*, i.e. *Gymnoascaceae* (A1), *Arthrodermataceae* (A3), *Nannizziopsiaceae* (A4), *Eremascaceae* (A7), *Ascosphaeriaceae* (A8), and *Spiromastigaceae* (A9), and a seventh group (A5) composed of five of our strains probably representing a new family. The groups representing *Ajellomycetaceae* (A6) and *Onygenaceae* (A2) were unsupported. Strains in subclade A were distributed as follows: the five mentioned above into A5, FMR 16121 into a separate branch of the *Ajellomycetaceae* (A6), four strains conspecific with *Eremascus albus* (A7), and one (FMR 16318) identified as *Ascosphaera atra* (A8). Thirty-nine strains were placed in *Eurotiales* (Subclade B). One (FMR 16566) was placed together with *Talaromyces flavus* and *T. kabodanensis* in an unsupported branch, and 16 strains near to *T. minioluteus* into a well-supported sister clade (B1). Into B2 (unsupported), which includes species of *Aspergillus*, eight of the strains were placed in a branch (99% BS / 1 PP) together with *A. glaucus, A. montevidensis* and *A. pseudoglaucus* (sect. *Aspergillus*). For the final identification of these eight strains, we used *Ben*A sequence comparison, which were found to be *A. montevidensis* (one strain) and *A. pseudoglaucus* (seven strains). FMR 16310 was placed in a branch together with the ex-type sequence of *A. asperescens* (sect. *Nidulantes*). Seven strains grouped into the sister clade B3 (unsupported), representing five species of *Penicillium*. FMR 15669 was identified as *Xerochrysium xerophilum* (B4), and five strains were initially identified as *Monascus* spp. Based on the comparison of ITS sequences, these five strains were finally identified as *M. pilosus* (one strain), *M. purpureus* (three strains), and *M. ruber* (one strain). Strain FMR 16018 was located together with *Alternaria multiformis* (Subclade C, *Pleosporales*). Subclade D (unsupported) was divided into three groups: D1, representing the *Myxotrichaceae*; D2, the genus *Skoua*; and D3, the *Pseudeurotiaceae*. This group had 38 strains, 10 among the genera *Oidiodendron* and *Myxotrichum* (D1), 17 together with *Skoua fertilis* (D2), and 11 within *Bettsia alvei* (D3). Subclade E (*Schizosaccharomycetales*), grouped FMR 16279 together with the ex-type sequence of *Schizosaccharomyces octosporus*. Subclade F (*Saccharomycetales*), had nine strains belonging to *Zygosaccharomyces* spp. (five strains) and *Candida* spp. (four strains). Clade G had 5 strains, *Mucorales*, divided into three groups that comprised *Mucor* spp. (three strains), *Cunninghamella bertholletiae* (FMR 16008) and *Rhizopus oryzae* (FMR 16022), respectively. Figures 3, 4 show the trees resulting from the phylogenetic analyses of *Myxotrichaceae* and *Talaromyces*, respectively. The phylogenetic tree based on the analysis of the ITS (Fig. 3), included 67 sequences belonging to *Myxotrichaceae* and *Pseudeurotiaceae*, whose alignments encompassed a total of 547 characters, including gaps, 204 of which were parsimony informative. The ML and BI analyses showed a similar tree topology. It comprised a main clade of *Myxotrichaceae*, where 20 strains were located, 17 of *Skoua* (14 identified as *S. fertilis*), and the remaining three in a separate branch that might represent a new species of the genus. Finally, three strains phylogenetically distant from the others appeared in a separate branch close to *Myxotrichum setosum* and *Oidiodendron truncatum*. The tree based on four concatenated *loci* (*BenA*, *CaM*, *rpb2* and ITS; Table 2; Fig. 4) was built to resolve the phylogenetic relationships of the *Talaromyces* strains. The dataset contained 123 sequences with a total of 2265 characters, including gaps, (520 of them for ITS, 377 for *BenA*, 516 for *CaM* and 852 for *rpb*2), of which

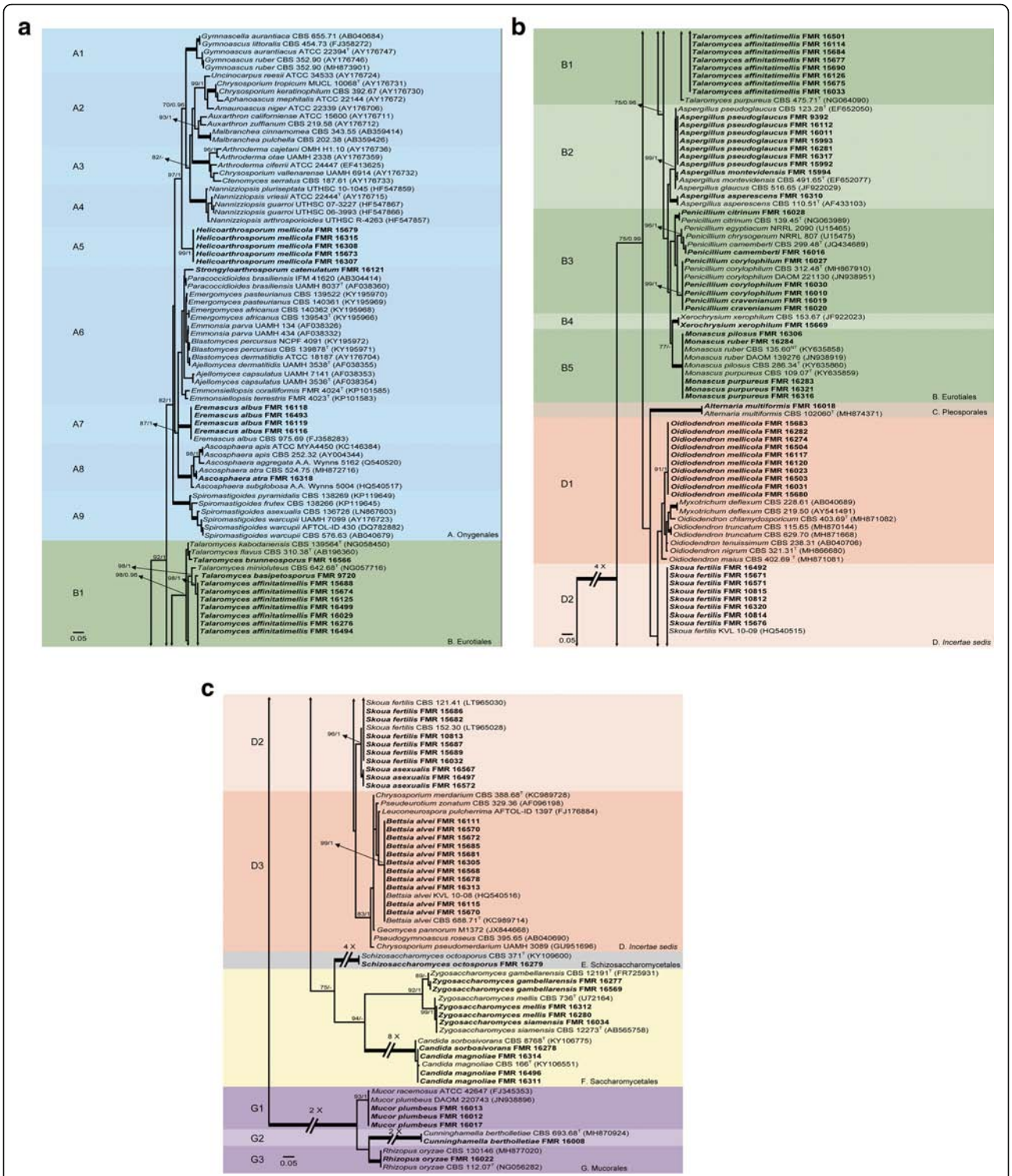

Fig. 2 a-**c** ML phylogenetic tree based on the analysis of LSU nucleotide sequences for all fungi isolated from honey. Members of Mucoromycota were chosen as out-group. Support in nodes is indicated above thick branches and is represented by posterior probabilities (BI analysis) of 0.95 and higher and/or bootstrap values (ML analysis) of 70% and higher. Fully supported branched (100% BS /1 PP) are indicated in bold. ^{T} = ex type. Alignment length 606 bp. The sequences generated by us are in Table 1

Fig. 3 ML phylogenetic tree based on the analysis of ITS nucleotide sequences of representative taxa of the families *Myxotrichaceae* (in grey balckground) and *Pseudeurotiaceae*. *Aphanoascus keratinophilus* IMI 319010 was chosen as out-group. Support in nodes is indicated above thick branches and is represented by posterior probabilities (BI analysis) of 0.95 and higher and/or bootstrap values (ML analysis) of 70% and higher. Fully supported branched (100% BS /1 PP) are indicated in bold. ^{T} = ex type. Alignment length 544 bp

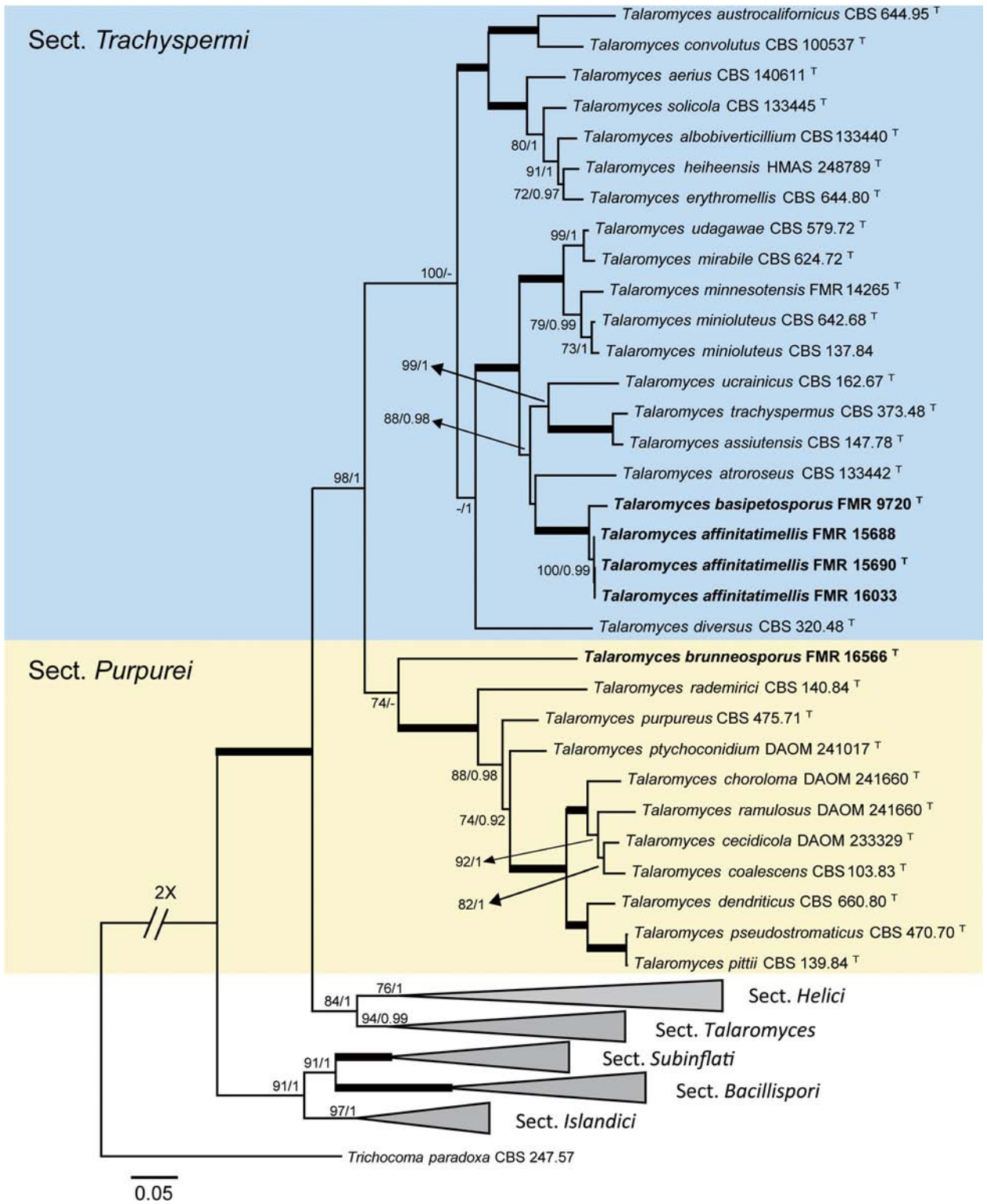

Fig. 4 ML phylogenetic tree built using the ITS, *BenA*, *CaM* and *rpb2* concatenated dataset for species of the genus *Talaromyces*. Species of the section *Trachyspermi* are indicated in a blue background and those of the section *Purpurei* in yellow. *Trichocoma paradoxa* CBS 247.57 was chosen as out-group. Support in nodes is indicated above thick branches and is represented by posterior probabilities (BI analysis) of 0.95 and higher and/or bootstrap values (ML analysis) of 70% and higher. Fully supported branched (100% BS /1 PP) are indicated in bold. ^{T} = ex-type strain. Alignment length 2265 bp

Table 2 Talaromyces spp. nucleotide sequences employed to build a phylogram to locate phylogenetically our strains from honey

Species name	Section	Strain no.	GenBank accession #			
			BenA	*CaM*	*rpb2*	*ITS*
Talaromyces aculeatus	*Talaromyces*	CBS 289.48 = IMI 040588 = NRRL 2129	KF741929	KF741975	KM023271	KF741995
Talaromyces adpressus	*Talaromyces*	CBS 140620 = CGMCC3.18211 = DTO 317-G4	KU866844	KU866741	KU867001	KU866657
Talaromyces alveolaris	*Talaromyces*	UTHSC DI16–146	LT559085	LT795594	LT795595	LT558968
Talaromyces amazonensis	*Talaromyces*	CBS 140373 = IBT 23215 = DTO 093-F9	KX011490	KX011502	–	KX011509
Talaromyces amestolkiae	*Talaromyces*	CBS 132696 = DTO 179-F5	JX315623	KF741937	JX315698	JX315660
Talaromyces angelicae	*Talaromyces*	KACC 46611	KF183640	KJ885259	–	KF183638
Talaromyces apiculatus	*Talaromyces*	CBS 312.59 = FRR 635 = IMI 068239	KF741916	KF741950	KM023287	JN899375
Talaromyces aurantiacus	*Talaromyces*	CBS 314.59 = IMI 099722 = NRRL 3398	KF741917	KF741951	–	JN899380
Talaromyces beijingensis	*Talaromyces*	CBS 140617 = CGMCC3.18200 = DTO 317-D8	KU866837	KU866733	KU866993	KU866649
Talaromyces calidicanius	*Talaromyces*	CBS 112002	HQ156944	KF741934	KM023311	JN899319
Talaromyces cnidii	*Talaromyces*	KACC 46617	KF183641	KJ885266	KM023299	KF183639
Talaromyces derxii	*Talaromyces*	CBS 412.89	JX494305	KF741959	KM023282	JN899327
Talaromyces duclauxii	*Talaromyces*	CBS 322.48 = IMI 040044 = MUCL 28672 = NRRL 1030	JX091384	KF741955	JN121491	JN899342
Talaromyces euchlorocarpius	*Talaromyces*	PF 1203 = DTO 176-I3 = DTO 176-I4	KJ865733	KJ885271	KM023303	AB176617
Talaromyces flavovirens	*Talaromyces*	CBS 102801 = IBT 27044	JX091376	KF741933	–	JN899392
Talaromyces flavus	*Talaromyces*	CBS 310.38 = IMI 197477 = NRRL 2098	JX494302	KF741949	JF417426	JN899360
Talaromyces francoae	*Talaromyces*	CBS 113134 = IBT 23221 = DTO 056-D9	KX011489	KX011501	–	KX011510
Talaromyces funiculosus	*Talaromyces*	CBS 272.86 = IMI 193019	JX091383	KF741945	KM023293	JN899377
Talaromyces fusiformis	*Talaromyces*	CBS 140637 = CGMCC3.18210 = DTO 317-F4	KU866843	KU866740	KU867000	KU866656
Talaromyces galapagensis	*Talaromyces*	CBS 751.74 = IFO 31796	JX091388	KF741966	–	JN899358
Talaromyces indigoticus	*Talaromyces*	CBS 100534 = IBT 17590	JX494308	KF741931	–	JN899331
Talaromyces intermedius	*Talaromyces*	CBS 152.65 = BDUN 267 = IFO 31752 = IMI 100874	JX091387	KJ885290	–	JN899332
Talaromyces kabodanensis	*Talaromyces*	DI16–149	LT559088	LT795598	LT795599	LT558971
Talaromyces liani	*Talaromyces*	CBS 225.66 = IMI 098480 = NRRL 3380 = VKM F-301	JX091380	KJ885257	–	JN899395
Talaromyces macrosporus	*Talaromyces*	CBS 317.63 = FRR 404 = IMI 197478	JX091382	KF741952	KM023292	JN899333
Talaromyces mangshanicus	*Talaromyces*	CGMCC 3.18013	KX447530	KX447528	KX447527	KX447531
Talaromyces marneffei	*Talaromyces*	CBS 388.87	JX091389	KF741958	KM023283	JN899344
Talaromyces muroii	*Talaromyces*	CBS 756.96 = PF 1153	KJ865727	KJ885274	–	JN899351
Talaromyces neofusisporus	*Talaromyces*	AS3.15415 = CBS 139516	KP765381	KP765383	–	KP765385
Talaromyces oumae-annae	*Talaromyces*	CBS 138208 = DTO 269-E8	KJ775213	KJ775425	–	KJ775720
Talaromyces panamensis	*Talaromyces*	CBS 128.89 = IMI 297546	HQ156948	KF741936	KM023284	JN899362
Talaromyces paucisporus	*Talaromyces*	PF 1150 = IFM 53616	–	–	–	AB176603
Talaromyces pinophilus	*Talaromyces*	CBS 631.66 = CECT 2809 = DSM 1944 = IAM 7013 = IMI 114933	JX091381	KF741964	KM023291	JN899382

Table 2 Talaromyces spp. nucleotide sequences employed to build a phylogram to locate phylogenetically our strains from honey *(Continued)*

Species name	Section	Strain no.	GenBank accession #			
			BenA	*CaM*	*rpb2*	*ITS*
Talaromyces primulinus	*Talaromyces*	CBS 321.48 = CBS 439.88 = FRR 1074 = IMI 040031 = MUCL 31321 = NRRL 1074	JX494305	KF741954	KM023294	JN899317
Talaromyces purgamentorum	*Talaromyces*	CBS 113145 = IBT 23220 = DTO 056-E1	KX011487	KX011500	–	KX011504
Talaromyces purpurogenus	*Talaromyces*	CBS 286.36 = IMI 091926	JX315639	KF741947	JX315709	JN899372
Talaromyces qii	*Talaromyces*	AS3.15414 = CBS 139515	KP765380	KP765382	–	KP765384
Talaromyces rapidus	*Talaromyces*	UTHSC DI16–148 = CBS 142382 T	LT559087	LT795600	LT795601	LT558970
Talaromyces ruber	*Talaromyces*	CBS 132704 = DTO 193-H6 = IBT 10703 = CBS 113137	JX315629	KF741938	JX315700	JX315662
Talaromyces rubicundus	*Talaromyces*	CBS 342.59 = IMI 099723 = NRRL 3400	JX494309	KF741956	KM023296	JN899384
Talaromyces sayulitensis	*Talaromyces*	CBS 138204 = DTO 245-H1	KJ775206	KJ775422	–	KJ775713
Talaromyces siamensis	*Talaromyces*	CBS 475.88 = IMI 323204	JX091379	KF741960	KM023279	JN899385
Talaromyces stipitatus	*Talaromyces*	CBS 375.48 = NRRL 1006 = IMI 39805	KM111288	KF741957	KM022380	JN899348
Talaromyces stollii	*Talaromyces*	CBS 408.93	–	JX315646	JX315712	JX315674
Talaromyces thailandensis	*Talaromyces*	CBS 133147 = KUFC 3399	JX494294	KF741940	KM023307	JX898041
Talaromyces verruculosus	*Talaromyces*	CBS 388.48 = DSM 2263 = IMI 040039 = NRRL 1050	KF741928	KF741944	KM023306	KF741994
Talaromyces viridis	*Talaromyces*	CBS 114.72 = ATCC 22467 = NRRL 5575	JX494310	KF741935	JN121430	AF285782
Talaromyces viridulus	*Talaromyces*	CBS 252.87 = FRR 1863 = IMI 288716	JX091385	KF741943	JF417422	JN899314
Talaromyces aerugineus	*Helici*	CBS 350.66 = BDUN 276 = IMI 105412	KJ865736	KJ885285	JN121502	AY753346
Talaromyces bohemicus	*Helici*	CBS 545.86 = CCF 2330 = IAM 14789	KJ865719	KJ885286	JN121532	JN899400
Talaromyces boninensis	*Helici*	CBS 650.95 = IBT 17516	KJ865721	KJ885263	KM023276	JN899356
Talaromyces cinnabarinus	*Helici*	CBS 267.72 = NHL 2673	AY753377	KJ885256	JN121477	JN899376
Talaromyces diversiformis	*Helici*	CBS 141931 = CGMCC3.18204 = DTO 317-E3	KX961216	KX961259	KX961274	KX961215
Talaromyces georgiensis	*Helici*	UTHSC DI16–145 = CBS 142380	LT559084	–	LT795606	LT558967
Talaromyces helicus	*Helici*	CBS 335.48 = DSM 3705 = IMI 040593 = NRRL 2106	KJ865725	KJ885289	KM023273	JN899359
Talaromyces reverso-olivaceus	*Helici*	CBS 140672 = CGMCC3.18195 = DTO 317-C3	KU866834	KU866730	KU866990	KU866646
Talaromyces ryukyuensis	*Helici*	NHL 2917 = DTO 176-I6	–	–	–	AB176628
Talaromyces varians	*Helici*	CBS 386.48 = IMI 040586 = NRRL 2096	KJ865731	KJ885284	KM023274	JN899368
Talaromyces cecidicola	*Purpurei*	CBS 101419 = DAOM 233329	FJ753295	KJ885287	KM023309	AY787844
Talaromyces chlorolomus	*Purpurei*	DAOM 241016 = CV 2802	GU385736	KJ885265	KM023304	FJ160273
Talaromyces coalescens	*Purpurei*	CBS 103.83	JX091390	KJ885267	KM023277	JN899366
Talaromyces dendriticus	*Purpurei*	CBS 660.80 = IMI 216897	JX091391	KF741965	KM023286	JN899339
Talaromyces pittii	*Purpurei*	CBS 139.84 = IMI 327871	KJ865728	KJ885275	KM023297	JN899325
Talaromyces pseudostromaticus	*Purpurei*	CBS 470.70 = FRR 2039	HQ156950	KJ885277	KM023298	JN899371
Talaromyces ptychoconidium	*Purpurei*	DAOM 241017 = CV 2808 = DTO 180-E7	GU385733	JX140701	KM023278	FJ160266
Talaromyces purpureus	*Purpurei*	CBS 475.71 = FRR 1731 = IMI 181546	GU385739	KJ885292	JN121522	JN899328

Table 2 Talaromyces spp. nucleotide sequences employed to build a phylogram to locate phylogenetically our strains from honey *(Continued)*

Species name	Section	Strain no.	GenBank accession #			
			BenA	*CaM*	*rpb2*	*ITS*
Talaromyces rademirici	*Purpurei*	CBS 140.84 = CECT 2771 = IMI 282406	KJ865734	–	KM023302	JN899386
Talaromyces ramulosus	*Purpurei*	DAOM 241660 = CV 2837 = DTO 184-B8	FJ753290	JX140711	KM023281	EU795706
Talaromyces aerius	*Trachyspermi*	CBS 140611 = CGMCC3.18197 = DTO 317-C7	KU866835	KU866731	KU866991	KU866647
Talaromyces albobiverticillius	*Trachyspermi*	CBS 133440 T = DTO 166-E5 = YMJ 1292	KF114778	KJ885258	KM023310	HQ605705
Talaromyces assiutesis	*Trachyspermi*	CBS 147.78 T	KJ865720	KJ885260	KM023305	N899323
Talaromyces atroroseus	*Trachyspermi*	CBS 133442 T = IBT 32470 = DTO 178-A4	KF114789	KJ775418	KM023288	KF114747
Talaromyces austrocalifornicus	*Trachyspermi*	CBS 644.95 T = IBT 17522	KJ865732	KJ885261	–	JN899357
Talaromyces convolutus	*Trachyspermi*	CBS 100537 T = IBT 14989	KF114773	–	JN121414	JN899330
Talaromyces diversus	*Trachyspermi*	CBS 320.48 T = DSM 2212 = IMI 040579 = NRRL 2121	KJ865723	KJ885268	KM023285	KJ865740
Talaromyces erythromellis	*Trachyspermi*	CBS 644.80 T = FRR 1868 = IMI 216899	HQ156945	KJ885270	KM023290	JN899383
Talaromyces heiheensis	*Trachyspermi*	CGMCC 3.18012	KX447525	KX447532	KX447529	KX447526
Talaromyces minioluteus	*Trachyspermi*	CBS 137.84	KF114798	–	–	NR138301
Talaromyces minioluteus	*Trachyspermi*	CBS 642.68 = IMI 089377 = MUCL 28666	KF114799	KJ885273	JF417443	JN899346
Talaromyces minnesotensis	*Trachyspermi*	FMR 14265 T = CBS 142381	LT559083	LT795604	LT795605	LT558966
Talaromyces mirabile	*Trachyspermi*	CBS 624.72	KF114797	–	–	NR138300
Talaromyces solicola	*Trachyspermi*	DAOM 241015 T = CV 2800 = DTO 180-D4	GU385731	KJ885279	KM023295	FJ160264
Talaromyces trachyspermus	*Trachyspermi*	CBS 373.48 T = IMI 040043	KF114803	KJ885281	JF417432	JN899354
Talaromyces ucrainicus	*Trachyspermi*	CBS 162.67 T = FRR 3462 = NHL 6086	KF114771	KJ885282	KM023289	JN899394
Talaromyces udagawae	*Trachyspermi*	CBS 579.72 T = FRR 1727 = IMI 197482	KF114796	–	–	JN899350
Talaromyces bacillisporus	*Bacillispori*	CBS 296.48 = IMI 040045 = NRRL 1025	AY753368	KJ885262	JF417425	KM066182
Talaromyces columbiensis	*Bacillispori*	CBS 113151 = IBT 23206 = DTO 058-F3	KX011488	KX011499	–	KX011503
Talaromyces emodensis	*Bacillispori*	CBS 100536 = IBT 14990	KJ865724	KJ885269	JN121552	JN899337
Talaromyces hachijoensis	*Bacillispori*	PF 1174 = IFM 53624	–	–	–	AB176620
Talaromyces mimosinus	*Bacillispori*	CBS 659.80 = FRR 1875 = IMI 223991	KJ865726	KJ885272	–	JN899338
Talaromyces proteolyticus	*Bacillispori*	CBS303.67 = NRRL 3378	KJ865729	KJ885276	KM023301	JN899387
Talaromyces unicus	*Bacillispori*	CBS 100535 = CCRC 32703 = IBT 18385	KJ865735	KJ885283	–	JN899336
Talaromyces palmae	*Subinflati*	CBS 442.88 = IMI 343640	HQ156947	KJ885291	KM023300	JN899396
Talaromyces subinflatus	*Subinflati*	CBS 652.95 = IBT 17520	KJ865737	KJ885280	KM023308	JN899397
Talaromyces acaricola	*Islandici*	CBS 137386 = DTO 183-B3 = DAOM 241025 = IBT 32387	JX091610	JX140729	KF984956	JX091476
Talaromyces allahabadensis	*Islandici*	CBS 304.63	KF984614	KF984768	KF985006	KF984873
Talaromyces atricola	*Islandici*	CBS 255.31 = NRRL 1052 = FRR 1052 = Thom 4640.439	KF984566	KF984719	KF984948	KF984859
Talaromyces brunneus	*Islandici*	CBS 227.60 = FRR 646 = IFO 6438 = IHEM 3907 = IMI 078259 = MUCL 31318	KJ865722	KJ885264	KM023272	JN899365
Talaromyces cerinus	*Islandici*	CBS 140622 = CGMCC3.18212 = DTO 318-A2	KU866845	KU866742	KU867002	KU866658
Talaromyces	*Islandici*	CBS 140635 = CGMCC3.18199 = DTO 317-D5	KU866836	KU866732	KU866992	KU866648

Table 2 Talaromyces spp. nucleotide sequences employed to build a phylogram to locate phylogenetically our strains from honey *(Continued)*

Species name	Section	Strain no.	GenBank accession #			
			BenA	*CaM*	*rpb2*	*ITS*
chlamydosporus						
Talaromyces columbinus	*Islandici*	NRRL 58811	KF196843	KJ885288	KM023270	KJ865739
Talaromyces crassus	*Islandici*	CBS 137381 = DTO 181-C5 = DAOM 241027 = IBT 32814	JX091608	JX140727	KF984914	JX091472
Talaromyces infraolivaceus	*Islandici*	CBS 137385 = DTO 182-I2 = DAOM 241024 = IBT 32487	JX091615	JX140734	KF984949	JX091481
Talaromyces islandicus	*Islandici*	CBS 338.48 = IMI 040042 = MUCL 31324 = NRRL 1036	KF984655	KF984780	KF985018	KF984885
Talaromyces loliensis	*Islandici*	CBS 643.80 = FRR 1798 = IMI 216901 = MUCL 31325	KF984658	KF984783	KF985021	KF984888
Talaromyces neorugulosus	*Islandici*	CBS 140623 = CGMCC3.18215 = DTO 318-A8	KU866846	KU866743	KU867003	KU866659
Talaromyces piceus	*Islandici*	CBS 361.48 = IMI 040038 = NRRL 1051	KF984668	KF984680	KF984899	KF984792
Talaromyces radicus	*Islandici*	CBS 100489 = FRR 4718	KF984599	KF984773	KF985013	KF984878
Talaromyces rotundus	*Islandici*	CBS 369.48 = IMI 040589 = NRRL 2107	KJ865730	KJ885278	KM023275	JN899353
Talaromyces rugulosus	*Islandici*	CBS 371.48 = IMI 040041 = MUCL 31201 = NRRL 1045	KF984575	KF984702	KF984925	KF984834
Talaromyces scorteus	*Islandici*	CBS 340.34 = NRRL 1129 = FRR 1129	KF984565	KF984684	KF984916	KF984892
Talaromyces subaurantiacus	*Islandici*	CBS 137383 = DTO 181-I2 = DAOM 241020 = IBT 32838	JX091609	JX140728	KF984960	LT558965
Talaromyces tardifaciens	*Islandici*	CBS 250.94	KC202954	KF984682	KF984908	JN899361
Talaromyces tratensis	*Islandici*	CBS 133146 = KUFC 3383	KF984559	KF984690	KF984911	KF984891
Talaromyces wortmannii	*Islandici*	CBS 391.48 = IMI 040047 = NRRL 1017	KF984648	KF984756	KF984977	KF984829
Talaromyces yelensis	*Islandici*	DTO 268E5	KJ775210	–	–	KJ775717
Trichocoma paradoxa	–	CBS 247.57	JF417468	JF417505	JF417421	JF417485

1069 were parsimony informative (195 for ITS, 217 for *BenA*, 308 for *CaM* and 349 for *rpb2*). The sequence datasets did not show conflict in the tree topologies for the 70% reciprocal bootstrap trees, which allowed the multi-locus analysis. The ML analysis showed similar tree topology and was congruent with the Bayesian analysis. In this tree (Fig. 4), the five *Talaromyces* strains we obtained were located in two different clades: one corresponding to the section *Trachyspermi* (100% BS / - PP), with four strains phylogenetically distant from *T. atroroseus*, one of them (FMR 9720) in a separate branch; and the second corresponding to the section *Purpurei* (74% BS / - PP), where the fifth strain (FMR 16566) was located in a distant branch.

TAXONOMY

Subclade A: *Onygenales*

Based on the above phylogenetic analyses, we suggest the following novel taxonomic arrangements: *Helicoarthrosporaceae* fam. nov. (Fig. 2; sister clade A5), phylogenetically close to the family *Gymnoascaceae,* with *Helicoarthrosporum* gen. nov. as type genus and *H. mellicola* sp. nov. as the type species; based on the strain FMR 16121, we introduce *Strongyloarthrosporum* gen. nov. with *S. catenulatum* sp. nov. as its type species. These new taxa are described and illustrated below.

Helicoarthrosporaceae Stchigel, Rodr.-Andr. & Cano, fam. nov. MycoBank MB 832226.

Diagnosis: Differing from other families of Onygenales by the production of long, sinuous to helical chains of arthroconidia (which are shorter, right, curved or contorted in other taxa).

Type genus: Helicoarthrosporum Stchigel et al. 2019.

Description: *Hyphae* hyaline, septate. *Asexual morph* reduced to sinuous, helical or zig-zag lateral branches, terminal part becoming fertile, disarticulating into conidia. *Conidia* hyaline, prismatic to cuboid, holo- and enteroarthric conidia. *Sexual morph* not observed.

Helicoarthrosporum Stchigel, Cano & Rodr.-Andr., gen. nov. MycoBank MB 823584.

Etymology. From Greek *έλικα-*, helix, *–άρθρωση-*, joint, and *-σπορά*, spore, referring to the morphology of the conidiophores.

Diagnosis: Distinguished from other phylogenetically related genera by its long, sinuous to helical chains of prismatic to cuboid arthroconidia, and by its extreme xerotolerance.

Type species: Helicoarthrosporum mellicola Stchigel et al. 2019.

Description: Mycelium composed by hyaline, septate hyphae. *Conidiophores* consisting in fertile lateral branches and terminal part of the hyphae, sinuous, helical or zigzag, disarticulating in hyaline, mostly prismatic to cuboid, holo- and enteroarthric conidia.
Helicoarthrosporum mellicola Stchigel, Cano & Rodr.-Andr., sp. nov. Fig. 5. MycoBank MB 823585.

Etymology: From Latin *mellis-*, honey, and *-cola*, to reside, referring to the habitat of the fungus.

Diagnosis: Helicoarthrosporum mellicola morphologically resembles *Scytalidium cuboideum* (syn. *Arthrographis cuboidea*), *S. ganodermophthorum,* and *S. sphaerosporum* in producing long chains of cuboid arthroconidia (Kang et al. 2010). *Helicoarthrosporum mellicola* grows slowly on PDA and shows a high xerotolerance, whereas *Scytalidium* spp. grow fast on PDA and do not show a xerotrophic habit; also, *S. ganodermophthorum* and S. *sphaerosporum* produce both asexual and sexual morphs, while *H. mellicola* only displays an asexual one.

Type: Spain: *Valencia community*: Castellón province, from decanted and filtered honey, 10 May 2014, *A. Gómez Pajuelo* (CBS H-23368 – holotype; CBS 143838 = FMR 15679 – ex-type cultures; LSU sequence GenBank LT906535).

Description: Colonies on G18 reaching 38–41 mm diam after 3 wk. at 25 °C, flattened, velvety, yellowish white (4A2) at the centre, margins regular, sporulation sparse; exudate absent; reverse pale yellow (4A3), diffusible pigment absent. *Mycelium* composed of hyaline to subhyaline, septate, smooth- and thin-walled hyphae, 1.5–4 µm wide; racquet hyphae present. *Conidiophores* reduced (mostly) to fertile side branches and to the terminal part

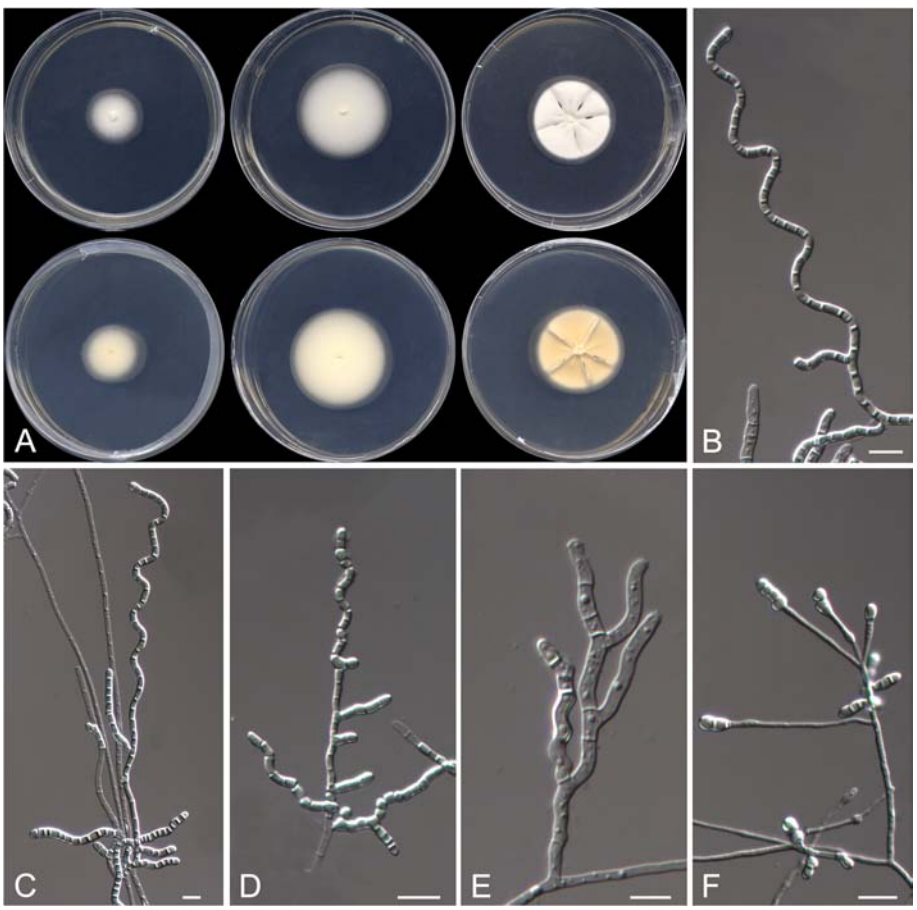

Fig. 5 *Helicoarthrosporum mellicola* CBS 143838 ^{T}. **a** Colonies on G18 at 15 °C and at 25 °C, and on PDA at 25 °C (from left to right), surface and reverse (from top to bottom). **b**–**e** Conidiophores. **d** Chlamydospores on OA. Scale bar = 10 µm

of a vegetative hyphae, sinuous to helical or in zig-zag, mostly simple, sometimes branched, 15–180 µm long, hyaline, disarticulating in conidia. *Conidia* mostly 1-celled, sometimes up to 4-celled, mostly holoarthric, occasionally enteroarthric, in chains of up to 30, mostly barrel-shaped, prismatic or cuboid, sometimes triangular and "Y"-shaped, smooth-walled, thicker than the hyphae, thickener at the ends, 2–8 × 2–5 µm, hyaline, disarticulating by schizolysis or rhexolysis from the conidiogenous hyphae. *Chlamydospores* produced on OA, terminally on or intercalary in the fertile hyphae hyaline, one to multicellular, smooth- and thick-walled, globose, ovoid, pyriform, clavate or irregularly-shaped, truncate at the base or at both ends, to 10 µm long and 3–5 µm wide.

Colonies on G18 reaching 22–27 mm diam after 3 wk. at 15 °C, flat, velvety, yellowish-white (4A2), margins regular, sporulation sparse, exudate absent; reverse pale yellow (4A3), diffusible pigment absent; no growth on G18 over 35 °C; on PDA reaching 31–35 mm diam after 3 wk. at 25 °C, slightly elevated, velvety, slightly sulcate, yellowish (3A2) at the centre and white (3A1) at the edge, exudate absent; reverse reddish yellow (4A6) at the centre and pale orange (5A3) at the edge, diffusible pigment absent; on OA at 25 °C after 3 wk. very small, 7–8 mm diam, velvety, white (4A1), sporulation sparse, exudate absent; reverse pale orange (5A3), diffusible pigment absent.
Minimum, optimal and maximum temperature of growth on G18 are 15 °C, 25 °C, and 30 °C, respectively; no hemolysis observed on blood agar at 25 °C, and on BCP-MS-G casein hydrolyzed without pH changes. Lipase negative, urease positive. Inhibited by cycloheximide and 20% NaCl, but tolerant to 3% and to 10% NaCl on Sabouraud dextrose agar.

Other specimens examined: Spain: *Valencia community*: Castellón province, from decanted and filtered honey, 10 May 2014, *A. Gómez Pajuelo* (FMR 15673). *Castilla y León community*: León province, from decanted, filtered and thermally treated honey, 20 May 2014, *A. Terrab* (FMR 16307). *Castilla y León community*: Zamora province, from decanted and filtered honey, 5 Oct. 2014, *A. Gómez Pajuelo* (FMR 16308). *Extremadura community*: Cáceres province, from decanted, filtered and thermally treated honey, 16 May 2014, *A. Terrab* (FMR 16315).
Strongyloarthrosporum Rodr.-Andr., Cano & Stchigel, gen. nov. MycoBank MB 823587.

Etymology: From Greek *στρογγυλός-*, globose, *–άρθρωση-*, joint, and *-σπορά*, spore, referring to the morphology of the conidia.

Diagnosis: Distinguished from other genera of Onygenales by the production of thick-walled globose arthroconidia, and because this fungus is an obligate xerophile.

Type species: Strongyloarthrosporum catenulatum Rodr.-Andr. et al. 2019.

Description: *Mycelium* of hyaline, septate hyphae. *Conidiophores* fertile lateral branches and part of the vegetative hyphae, disarticulating. *Conidia* enteroarthic, hyaline, mostly globose.
Strongyloarthrosporum catenulatum Rodr.-Andr., Cano & Stchigel, sp. nov. Fig. 6. MycoBank MB 823588.

Etymology: From Latin *catenulatus*, in chains, referring to the disposition of the conidia.

Diagnosis: *Strongyloarthrosporum catenulatum* is phylogenetically close to the *Ajellomycetaceae*, a family of non-xerophilic fungi characterized by their thermally dimorphic nature and, consequently, pathogenic for animals. By contrast, *S. catenulatum* is an obligate xerophilic fungus with globose conidia sometimes disposed in chains.

Type: Spain: *Castilla-La Mancha community*: Toledo province, from decanted, filtered and thermally treated honey, 12 May 2014, *A. Terrab* (CBS H- 23371 – holotype; CBS 143841 = FMR 16121 – ex-type cultures; LSU sequence GenBank LT906534).

Description: Colonies on G18 reaching 20–21 mm diam after 3 wk. at 25 °C, elevated, velvety, sulcate, sporulation sparse, exudate absent, yellowish white (4A2) at the centre and white (3A1) at the edge; reverse orange-grey (5B2), diffusible pigment absent. *Mycelium* composed of hyaline, septate, smooth, thin- to thick-walled, anastomosing hyphae, 1.5–4 µm wide. *Conidiophores* reduced mostly to single fertile side branches and to the terminal part of the vegetative hyphae, 5–60 µm long, hyaline, disarticulating in conidia. *Conidia* hyaline, mostly one-celled, occasionally two-celled, holo- and enteroarthric, solitary, disposed terminally, intercalary or sessile on the fertile hyphae, or produced in basipetal chains of up to ten conidia, smooth-walled, thicker than the hyphae, thickener at the ends, mostly globose, 3–6 µm diam, flattened or not at one or both ends, disarticulating by rhexolytic secession from the conidiogenous hyphae. *Chlamydospores* and racquet hyphae absent.

Colonies on G25 N reaching 19–20 mm diam after 3 wk. at 25 °C, elevated, velvety, sulcate, exudate absent, sporulation sparse, light orange (5A4) at the centre and grey (5B1) at the edge; reverse greyish orange (5B5), diffusible

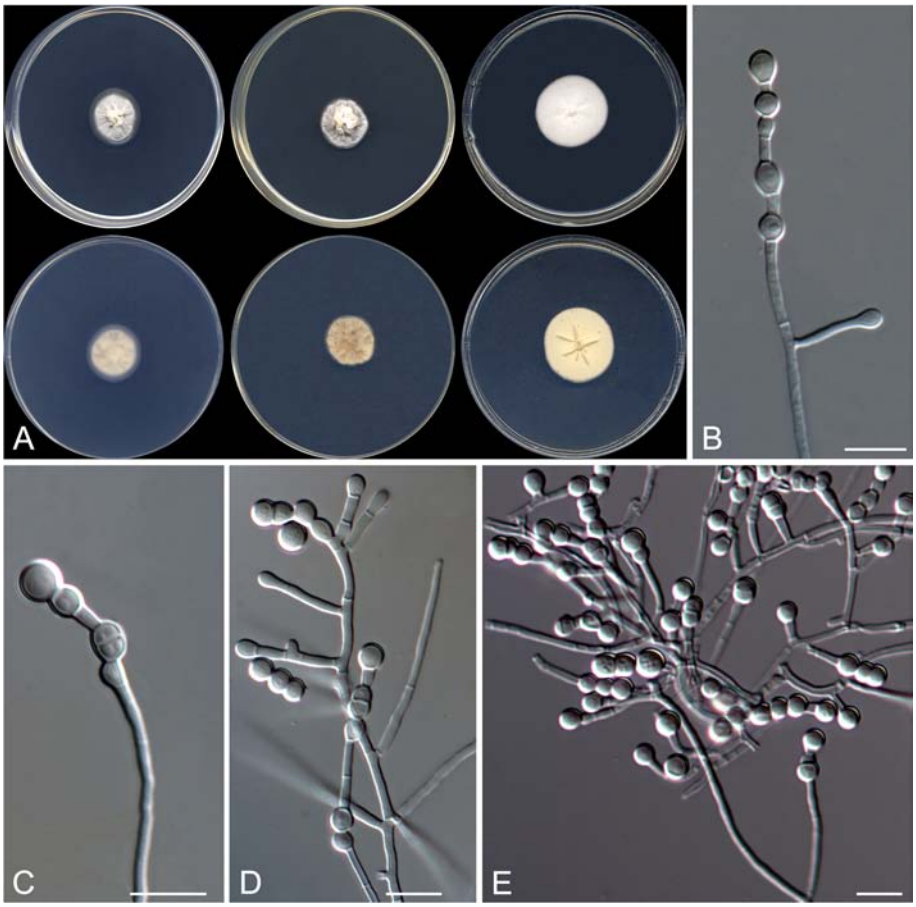

Fig. 6 *Strongyloarthrosporum catenulatum* CBS 143841 ^{T}. **a** Colonies on G18, G25 N and MY70FG at 25 °C (from left to right), surface and reverse (from top to bottom). **b**–**e** Conidiophores and conidia. Scale bar = 10 μm

pigment absent; on MY70FG reaching 29–30 mm diam after 3 wk. at 25 °C, flat, floccose, margins entire, sporulation sparse, white; reverse light yellow (4A4), diffusible pigments absent.

Minimum, optimal and maximum temperature of growth on G18 are 15 °C, 25 °C, and 35 °C, respectively, does not grow on blood agar, BCP-MS-G, Sabouraud dextrose agar with different NaCl concentrations, TOTM, OA, PYE nor on Christensen's urea agar.

Subclade B: *Eurotiales*

Due to both LSU-based (Fig. 2; sister clade B1) and ITS-*BenA-CaM-rpb*2-based (Fig. 4) phylogenetetic trees, four of our *Talaromyces* strains were placed in section *Trachyspermi* in a well-supported subclade divided in two branches, and one more strain was placed into the section *Purpurei* in a basal position (Fig. 4), phylogenetically distant and phenotypically different from other species of *Talaromyces* in this section, consequenly, we propose the recognition of three new species of the genus.

Talaromyces basipetosporus Stchigel, Cano & Rodr.-Andr., sp. nov. Fig. 7. MycoBank MB 823589.

Etymology: After the morphological similarity to the asexual morph of *Basipetospora* (formerly applied to the asexual morph of *Monascus*).

Diagnosis: Differs from other species in sect. *Trachyspermi* in that the conidiogenesis is very similar to that of *Monascus* (syn. *Basipetospora*), characterized by retrogressively produced conidia, which have not been previously described in *Talaromyces* (see diagnosis of *Talaromyces affinitatimellis*).

Type: Argentina: *Buenos Aires province*: San Martín, from decanted, filtered and thermally treated honey, 1 Oct. 2007, *M. A. Álvarez* (CBS H-23365 – holotype; CBS 143836 = FMR 9720 – ex-type cultures; LSU sequence GenBank LT964940).

Description: *Colonies* on MEA reaching 10–11 mm diam after 3 wk. at 25 °C, slightly elevated, velvety to floccose,

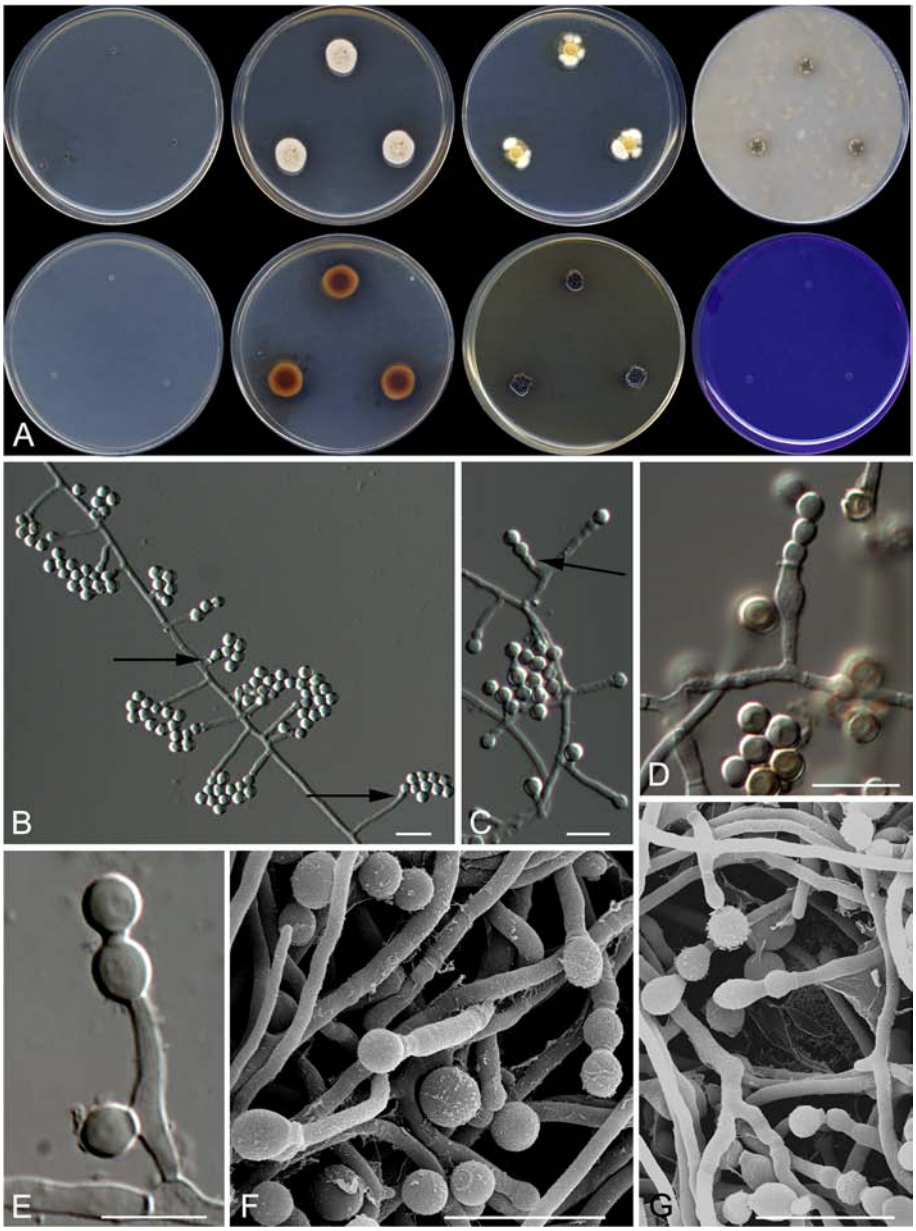

Fig. 7 *Talaromyces basipetosporus* CBS 143836 ^{T}. **a** Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES and CREA. **b**–**g** Conidiophores and conidia; the arrows shows the conidia formed basipetally. Scale bar = 10 μm

margins entire, yellowish grey (4B2) at the centre and white (4A1) at the edge, exudate absent, sporulation sparse; reverse brownish red (8C8) at the centre and greyish orange (5B6) at the edge, diffusible pigments absent. *Mycelium* abundant, composed of subhyaline to pale brown, smooth to echinulate, thin-walled, septate, anastomosing hyphae, of 2–3 μm wide. *Conidiophores* mostly reduced to a single conidiogenous cell, sometimes slender and with an additional conidiogenous locus near the base, arising alternately or oppositely at both sides of the vegetative hyphae, mostly separate from the vegetative hyphae by a basal septum. *Conidiogenous cells* smooth-walled to echinulate, mostly cylindrical and occasionally slightly slender towards the apex, sometimes broadening below the apex, but also flask- or barrel-shaped, very variable in length, 3–20(– 45) × 1–2.5 μm, conidiogenesis retrogressive. *Conidia* one-celled, hyaline and echinulate when young, becoming brown to dark brown and nearly smooth-walled with the age, formed basipetally, in false chains of up to ten conidia, mostly globose, 3.0–5.0 μm diam. Sexual morph not observed.

Colonies on DG18 reaching 13–14 mm diam after 3 wk. at 25 °C, colonies moderately elevated, texture floccose,

yellowish orange (4B7) with mycelium white (5A1) at edge, sporulation dense, exudate absent, diffusible pigments absent, reverse reddish golden (6C7) at centre and pale yellow (3A4) at edge; on G18 reaching 10–11 mm diam after 3 wk. at 25 °C, slightly elevated, velvety to floccose, margins regular, yellowish white (3A2), exudates uncolored, diffusible pigment absent, reverse pale orange (5A3) at the centre and white at the edge; on OA reaching 5–6 mm diam. After 3 wk. at 25 °C, flat, margins entire, mycelium grey, texture velvety to floccose, sporulation dense, diffusible pigments absent, exudate absent, colonies dark brown (5D4) at centre and grey with olive-brown (6B1-4E6) patches at edge; on PDA reaching 10–11 mm diam. After 3 wk. at 25 °C, elevated, velvety, brown (7E7) at the centre and brownish grey (4D2) at the edge, sporulation abundant, exudate absent, diffusible blackish olive (2G6) pigment present, reverse dark brown (7F4) at centre and brown (7E8) at the edge; on YES reaching 7–8 mm diam after 3 wk. at 25 °C, moderately elevated, sulcate, rough, sporulation strong, blackish brown (6G8), diffusible pigments absent, exudates absent, reverse yellowish brown (5E8).

Minimum, optimal and maximum temperature of growth on G18 are 15, 25, and 30 °C, respectively; does not grow on CYA, Czapek 20% sucrose, CREA, Starch agar, or MY70FG.

Talaromyces brunneosporus Rodr.-Andr., Cano & Stchigel, sp. nov.

Figure 8. MycoBank MB 823590.

Etymology: From Latin *brunneus-*, brown, and *-sporarum*, spore, in reference to the colour of the conidia.

Diagnosis: Distinguished from other species in sect. *Purpurei*, with the exception of *T. purpurei* (the type species of the section), by the production of solitary phialides and monoverticillate conidiophores (biverticillate conidiophores in the other species of the section). However, *T. brunneosporus* can be differentiated from *T. purpureus* because lack of a sexual morph (present in the latter species),

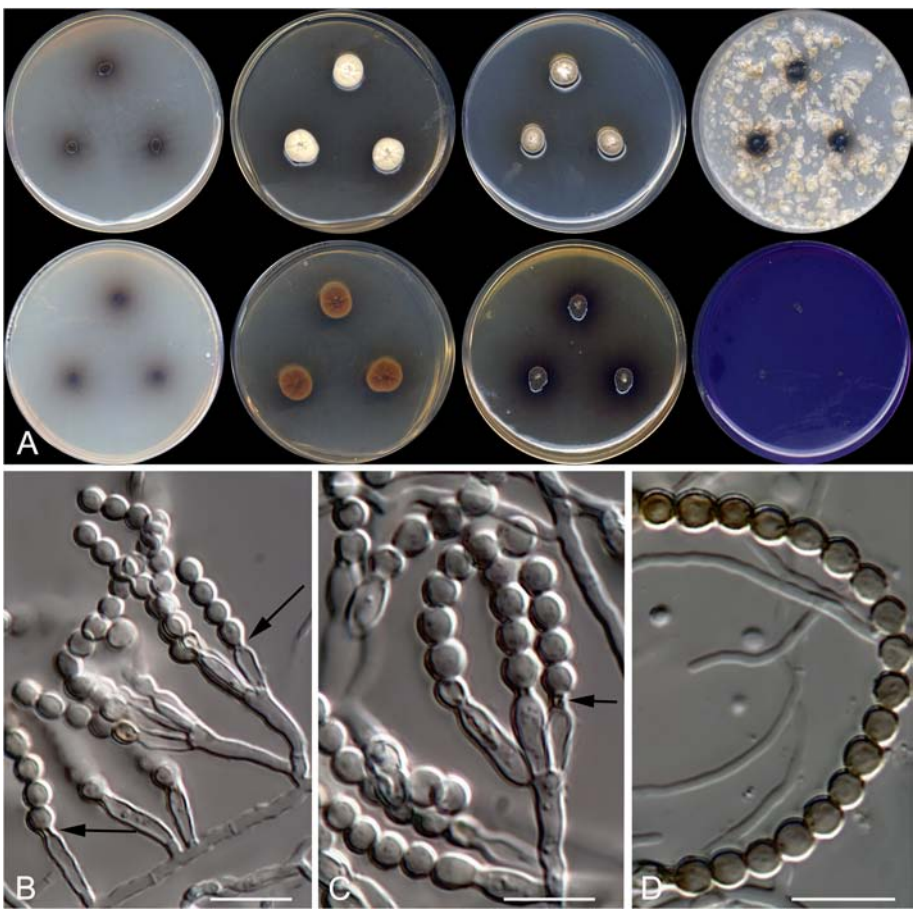

Fig. 8 *Talaromyces brunneosporus* CBS 144320^{T}. **a** Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES and CREA. **b**, **c** Poorly-developed (single phialide) and well-developed (monoverticillate) conidiophores; the arrows indicate the conspicuous collarette at the top of the phialides. **d** A chain of globose, dark brown, verrucose conidia. Scale bar = 10 μm

and produces penicillate conidiophores (having an aspergillate look in *T. purpureus*) and verrucose conidia (ornamented with spiral ridges in *T. purpureus*).

Type: Spain: *Castilla y León community*: Salamanca province, from decanted, filtered and thermally treated honey, 1 Oct. 2014, *A. Terrab* (CBS H-23375 – holotype; CBS 144320 = FMR 16566 – ex-type cultures; LSU sequence GenBank LT964943).

Description: Colonies on MEA reaching 13–14 mm diam after 3 wk. at 25 °C, slightly elevated, velvety to floccose, margins irregular, yellowish white (4A3), exudate absent, sporulation sparse, reverse light brown (6D8) at the centre and yellowish brown (5D6) at the edge, diffusible yellowish brown (5E6) pigment present. *Mycelium* abundant, composed of subhyaline, smooth- and thin-walled, septate, anastomosing hyphae 2–3 µm wide. *Conidiophores* mostly stalked, monoverticillate, smooth- and thin-walled, bearing one to four conidiogenous cells at the top, frequently arising oppositely at both sides of the vegetative hyphae, sometimes reduced to a single conidiogenous cell, sessile or integrated to the vegetative hyphae (= adelophialides). *Conidiogenous cells* phialidic, smooth-walled, mostly slender towards the apex, flask-shaped, 8–12 × 2.5–3.5 µm, with a darkened apical area when the conidiogenous cells have produced several conidia, conidiogenesis enteroblastic. *Conidia* one-celled, globose, hyaline and smooth-walled when young, becoming brownish-green to dark brown and verrucose with the age, 3–4 µm diam, in long false chains of up to 25 conidia. *Sexual morph* not observed.

Colonies on CYA reaching 4–5 mm diam after 3 wk. at 25 °C, elevated, velvety, dark brown (8F4) at the centre and greyish-brown (7E3) at the edge, exudate absent, sporulation abundant, reverse dark brown (8F6) at the centre and reddish brown (8E5) at the edge, diffusible brown (6E7) pigment present; on DG18 reaching 10–11 mm diam after 3 wk. at 25 °C, moderately elevated, floccose, margins irregular, yellowish white (4A2) at the centre and olive-brown (4D6) at the edge, exudate absent, sporulation strong, reverse light brown (5D7), diffusible yellowish brown (5D5) soluble pigment present; on OA reaching 9–10 mm diam after 3 wk. at 25 °C, flat, floccose, margins entire, exudate absent, sporulation strong, colonies blackish olive (2G6) at the centre and brown (6E6) at the edge, diffusible olive brown (4E8) pigment present; on YES reaching 8–9 mm diam after 3 wk. at 25 °C, flat, floccose, black at the centre and yellowish-brown (5E6) at the edge, exudate absent, sporulation sparse, reverse dark violet (8E8), diffusible blackish brown (6G8) pigment present.

Minimum, optimal and maximum temperature of growth on G18 are 15, 25, and 30 °C, respectively; no growth on CYA at 37 °C nor on CREA at 25 °C.

Notes: Talaromyces brunneosporus and *T. purpureus* grow more slowly on CYA and MEA than other species of the section. However, *T. brunneosporus* produces dark brown colonies with a dark brown diffusible pigment on CYA, while the colonies of *T. purpureus* are pale beige and without diffusible pigments. Also, the colonies on OA and MEA are purplish in *T. purpureus* and pale coloured and dark brown in *T. brunneosporus*.

Talaromyces affinitatimellis Rodr.-Andr., Stchigel & Cano, sp. nov. Fig. 9. MycoBank MB 823591.

Etymology: From Latin *affinitatis-*, affinity, and *-mellis*, honey, after the substrate from which the fungus was isolated.

Diagnosis: Differing from all other species in sect. *Trachyspermi* (with the exception of *T. basipetosporus*) by the production of conidia by retrogressive conidiogenesis. *Talaromyces affinitatimellis* differs from *T. basipetosporus* by the production cylindrical, smooth-walled to echinulate conidiogenous cells ending in a greenish brown, broad collarette-like structure (conidiogenous cells irregularly-shaped, smooth-walled, and without such apical structure in *T. basipetosporus*).

Type: Spain: *Valencia community*: Castellón province, from decanted and filtered blossom honey, 10 May 2014, *A. Gómez Pajuelo* (CBS H- 23370 – holotype; CBS 143840 = FMR 15690 – ex-type cultures; LSU sequence GenBank LT964939).

Description: Colonies on MEA reaching 29–30 mm diam. After 3 wk. at 25 °C, flat, floccose, not sulcate, margins entire, olive (3D3) at the centre and white (4A1) at edge, exudate absent, sporulation sparse; reverse pale orange (5A3) at centre and pale yellow (4A3) at edge, diffusible pigment absent. *Mycelium* abundant, composed of subhyaline to pale brown, smooth- and thin-walled, septate, anastomosing hyphae, of 2–4 µm wide. *Conidiophores* hyaline to pale brown, reduced to a single conidiogenous cell, occasionally with an additional conidiogenous locus near the base or lateraly disposed, or short-stalked and bearing two conidiogenous cells, sometimes with an additional lateral conidiogenous cell arising alternately at both sides of the vegetative hyphae, separate from them by a basal septum. *Conidiogenous cells* hyaline to pale brown, smooth-walled, mostly cylindrical and occasionally slightly slender towards the apex, sometimes ending in a greenish-brown, broad collarette-like structure, 3–20 × 1.5–3 µm, conidiogenesis retrogressive but

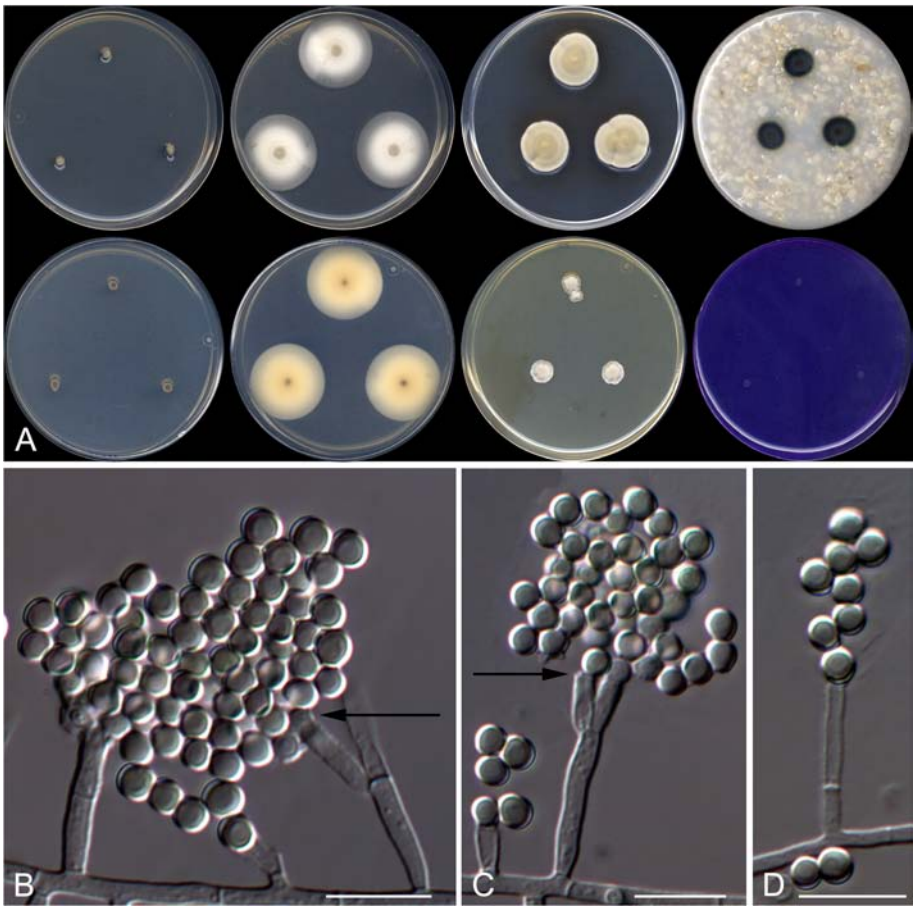

Fig. 9 *Talaromyces affinitatimellis* CBS 143840 ^{T}. **a** Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES and CREA. **b**–**d** Conidiophores and conidia; the arrows shows the conidia formed basipetally. Scale bar = 10 μm

enteroblastic. *Conidia* one-celled, hyaline and echinulate, becoming brown to dark brown and nearly smooth-walled with the age, produced basipetally in false chains of up to ten in number, mostly globose, 3.0–5.0 μm diam. Sexual morph not observed.

Colonies on DG18 reaching 13–14 mm diam after 3 wk. at 25 °C, moderately elevated, floccose, yellowish orange (4B7) with white (5A1) margins, exudates absent, sporulation strong; reverse reddish golden (6C7) at the centre and pale yellow (A4) at the edge, diffusible pigment absent; on G18 reaching 21–24 mm diam at 25 °C, slightly elevated, velvety to floccose, margins regular, yellowish white (4A4), exudates absent, sporulation abundant, reverse greyish orange (5B6), diffusible pigment absent; on OA reaching 12–13 mm diam after 3 wk. at 25 °C, flat, velvety to floccose, margins entire, black, exudates absent, sporulation abundant; colonies grey (7F1) at the centre and dark brown (6F4) to black at the edge, diffusible pigment absent; on PDA reaching 39–43 mm diam after 3 wk. at 25 °C, flat, velvety, margins slightly irregular, yellowish-brown (5F6) at the centre, grey (7F1) and yellowish brown (5E4) at the middle part, and light grey (5B1) at the edge, exudate absent, sporulation scarce, reverse dark brown (7F7) at the centre and brownish yellow (5C7) at the edge, diffusible pigment absent; on YES reaching 10–11 mm diam after 3 wk. at 25 °C, moderately elevated, floccose, white (4A1), exudate absent, sporulation sparse, reverse greyish orange (5B6), diffusible pigment absent.
Minimum, optimal and maximum temperature of growth on G18 are 15, 25, and 35 °C, respectively; no growth on CYA, Czapek 20% or CREA, or at 40 °C on all tested media.

Other specimens examined: Spain: *Catalonia community*: Tarragona province, from decanted and filtered blossom honey, 10 May 2014, *A. Gómez Pajuelo* (FMR 15674, FMR 15675, and FMR 15677); *Valencia community*: Castellón province, from decanted and filtered blossom honey, 10 May 2014, *A. Gómez Pajuelo* (FMR 15684 and FMR 15688); *Extremadura community*: Cáceres province, from decanted, filtered and thermally treated honeydew honey, 16 May 2014, *A. Terrab* (FMR 16029, FMR 16499, and FMR 16501); *Castilla y León community*: Salamanca province, from decanted, filtered and thermally treated

honeydew honey, 01 Oct. 2014, *A. Terrab* (FMR 16033 and FMR 16114); Zamora province, from decanted, filtered and thermally treated honeydew honey, 05 Oct 2014, *A. Terrab* (FMR 16125, FMR 16126, FMR 16276, and FMR 16494).

Subclade D: *Incertae sedis*

Based on both LSU-based (Fig. 2; sister clade D1) and ITS-based (Fig. 3) phylogenetic trees, ten of our strains were located in a well-supported and separated branch related to species of the genera *Oidiodendron* and *Myxotrichum*, and phylogenetically distant from the most similar taxa included in the study, *M. setosum* and *O. truncatum* (Fig. 3). Recognition of all of these distinct strains was also supported by unique phenotypic characteristics; therefore, we propose the recognition of the new species *Oidiodendron mellicola*. Furthermore, because three of our strains were placed near *Skoua fertilis* in both LSU-based (Fig. 2; sister clade D2) and ITS-based (Fig. 3) phylogenies and because they showed different phenotypic features and enough phylogenetic distance relative to *S. fertilis*, we also propose the introduction of a further new species, *Skoua asexualis*.

Oidiodendron mellicola Rodr.-Andr., Cano & Stchigel, sp. nov. Fig. 10. MycoBank MB 823586.

Etymology: From Latin *mellis-*, honey, and *-cola* dwelling on, referring to the habitat.

Diagnosis: Forming a terminal clade together with *O. truncatum* and *M. setosum* at a significant phylogenetic distance (5.3% from the other two species), and differing morphologically from other known species of *Oidiodendron* and the asexual morphs of *Myxotrichum* in the absence of well-differentiated conidiophores, and the slow growth.

Type: Spain: *Valencia community*: Castellón province, from decanted and filtered blossom honey, 10 May 2014, *A. Gómez Pajuelo* (CBS H-23369 – holotype; CBS 143839 = FMR 15683 – ex-type cultures; ITS sequence GenBank LT906544).

Description: *Colonies* on PDA at 15 °C reaching 15–16 mm diam after 3 wk., white (5A1), sporulation sparse

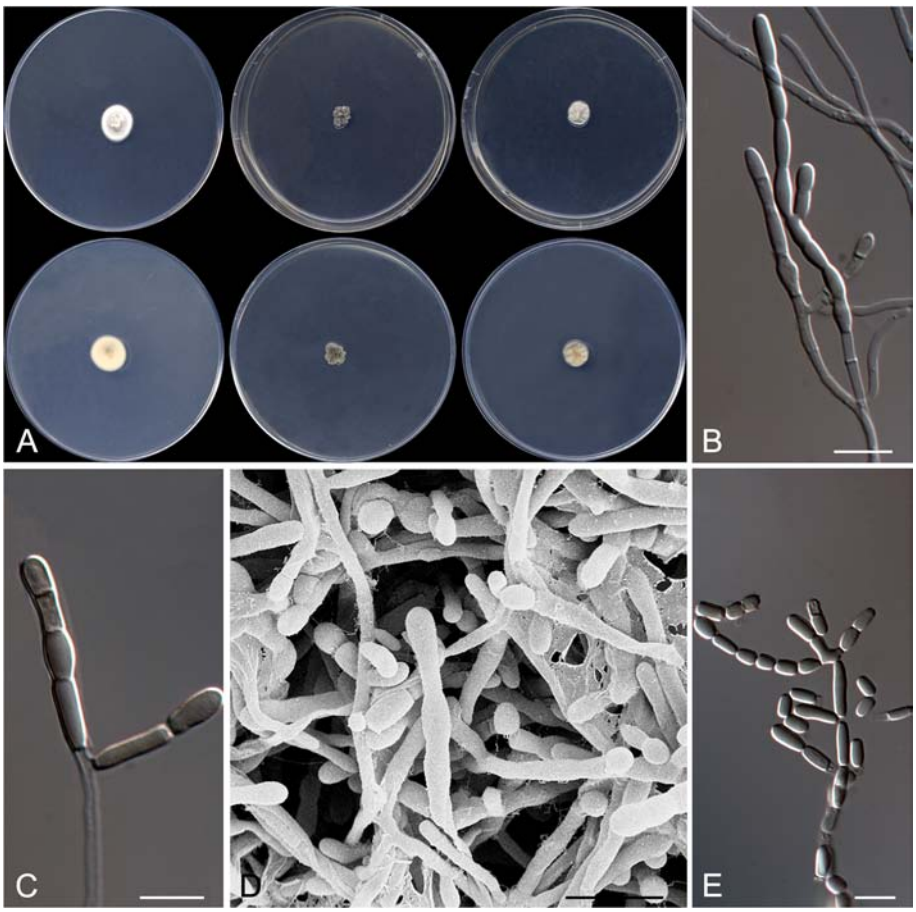

Fig. 10 *Oidiodendron mellicola* CBS 143839 ^{T}. **a** Colonies on PDA at 15 °C and at 25 °C, and on G18 at 25 °C (left to right), surface and reverse (from top to bottom). **b**–**d** Conidiophores. **e** Disarticulating chains of conidia. Scale bar = 10 μm

(seen after 6 wk. of incubation), exudate absent, reverse orange-white (6A2) at the centre and orange-grey (6B2) at the edge, diffusible pigment absent. *Mycelium* composed of hyaline, septate, smooth- and thin-walled hyphae, 1–3 μm wide. *Conidiophores* reduced to fertile side branches and the terminal part of a vegetative hyphae, mostly simple or once branched near or at the base, 10–40 μm long, pale olive, disarticulating in conidia. *Conidia* one-celled, mostly holoarthric, sometimes enteroarthric, mostly in chains of up to ten, occasionally solitary and sessile, mostly barrel-shaped, sometimes cylindrical, conical or "Y"-shaped, 5–14 × 2.5–5 μm, pale olive, disarticulating by schizolytic or rhexolytic secession from the hyphae. *Chlamydospores* absent. *Sexual morph* absent.

Colonies on PDA reaching 10–11 mm diam. After 3 wk. at 25 °C, elevated, compact, velvety, margins irregular, olive brown (4E3), exudates absent, sporulation abundant; reverse olive brown (4E5) at the center, grey (5D1) at the edge, diffusible pigment absent. Colonies on G18 reaching 11–12 mm diam after 3 wk. at 25 °C, elevated, velvety to floccose, yellowish white (4A2) at the centre and white (4A1) at the edge, margins regular, sporulation absent, reverse pale yellow (4A3), diffusible pigment absent; on G18 at 15 °C reaching 12–15 mm diam after 3 wk., similar in aspect than at 25 °C; on MY70FG and MEA 2% at 25 °C after 3 wk. reaching 1–3 mm diam.
Minimum, optimal and maximum temperature of growth on G18 are 5, 15, and 25 °C, respectively; no growth on OA or PCA at 25 °C.

Other specimens examined: Spain: *Catalonia community*: Tarragona province, from decanted and filtered blossom honey, 10 May 2014, *A. Gómez Pajuelo* (FMR 15680); *Castilla-La Mancha community*, Ciudad Real province, from decanted, filtered and thermally treated honeydew honey, 10 May 2014, *A. Terrab* (FMR 16031, FMR 16117, and FMR 16503); Toledo province, from decanted, filtered and thermally treated honeydew honey, 12 May 2014, *A. Terrab* (FMR 16120 and FMR 16282); *Galicia community*: Ourense province, from decanted, filtered and thermally treated honeydew honey, 03 May 2014, *A. Terrab* (FMR 16504); *Castilla y León community*: Salamanca province, from decanted, filtered and thermally treated honeydew honey, 01 Oct. 2014, *A. Terrab* (FMR 16023); Burgos province, from decanted, filtered and thermally treated honeydew honey, 23 May 2014, *A. Terrab* (FMR 16274).
Skoua asexualis Rodr.-Andr., Cano & Stchigel, sp. nov. Fig. 11. MycoBank MB 824092.

Etymology: From Latin *asexualis*, without sex, because of lack of a known sexual morph.

Diagnosis: Differing from the other known species of the genus, *S. fertilis*, in asexual reproduction, as the latter only produces ascospores within globose asci arising from the mycelium.

Type: Spain: *Castilla y León community*: León province, from decanted, filtered and thermally treated honeydew honey, 1 Oct. 2014, *A. Terrab* (CBS H-23397 – holotype; CBS 144072 = FMR 16572 – ex-type cultures; ITS sequence GenBank LT964668).

Description: *Colonies* on PDA reaching 6–7 mm diam after 3 wk. at 25 °C, elevated, velvety, sporulation abundant, exudates absent, diffusible pigment absent, colonies brown (7E6) at the centre and whitish at the edge, reverse brownish orange (6C5) at the centre and greyish orange (5B3) at the edge. *Mycelium* composed of hyaline, repeatedly septate, smooth- and thin-walled hyphae, 2–6 μm wide. *Conidiophores* absent. *Conidia* mostly one-celled, occasionally two- to three-celled, hyaline, solitary or in short chains, smooth- and thick-walled, mostly globose, occasionally broadly ellipsoidal, pyriform, or irregular-shaped, truncate at one or both ends, 3–7 μm diam, conidiogenesis holoblastic when sessile or terminal, and holothallic when intercalary, disarticulating by rhexolytic secession; the holoblastic and holothallic conidia produce a succession of secondary holoblastic conidia, forming a big, radiating mass of cells of up to 50 μm diam, which eventually detach as complex asexual propagules from the fertile hyphae. *Chlamydospores* similar to the conidia but thicker, mostly non- or occasionally one-septate, intercalary or terminal. *Sexual morph* unknown.

Colonies on MEA reaching 3–4 mm diam after 3 wk. at 25 °C, colonies elevated, velvety to floccose, margins irregular, sporulation abundant, diffusible pigment absent, mycelium yellowish white (4A2), reverse pale yellow (4A3); on G18 reaching 4–5 mm diam after 3 wk. at 25 °C, elevated, floccose, margins irregular, sporulation sparse, diffusible pigment absent, exudates absent, colonies pale yellow (4A3) at the centre, reverse orange-grey (5B2).
Minimum, optimal and maximum temperature of growth on G18 are 15, 25, and 30 °C, respectively; no growth on CYA, CREA, OA, or YES at 25 °C.

Other specimens examined: Spain: *Extremadura community*: Cáceres province, from decanted, filtered and thermally treated honeydew honey, 16 May 2014, *A. Terrab* (FMR 16497 and FMR 16567).

DISCUSSION

This is the most comprehensive assessment of the diversity of the xerotolerant and xerophilic fungi of honey

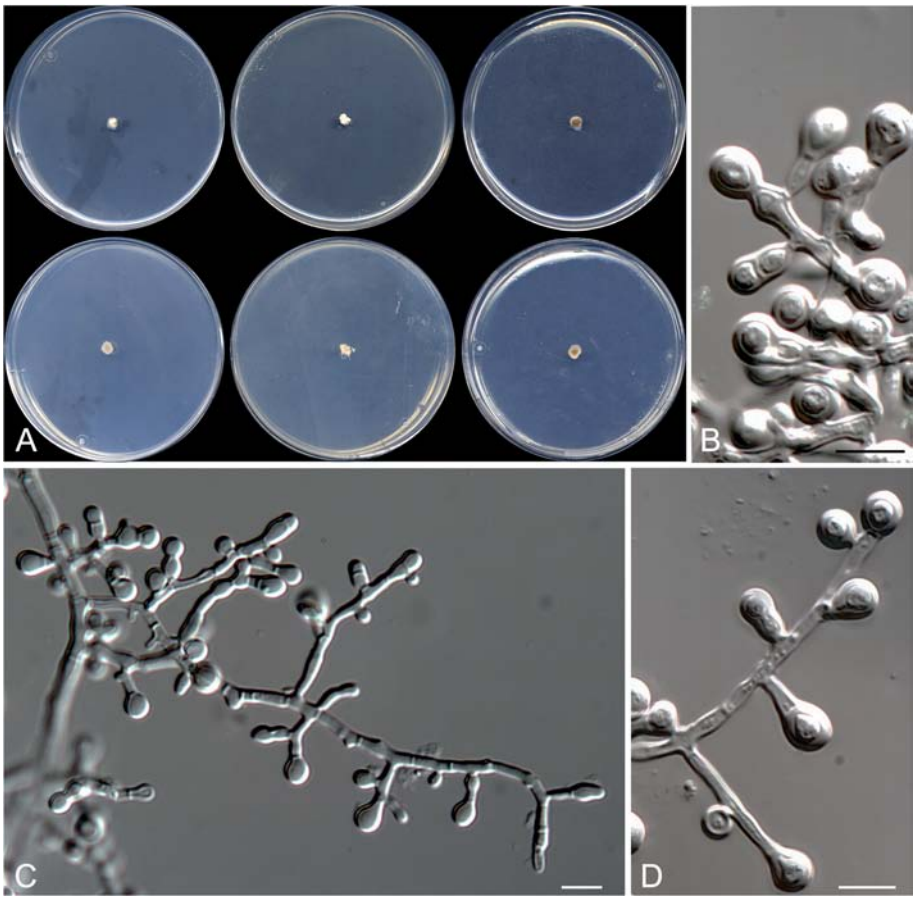

Fig. 11 *Skoua asexualis* CBS 144072 ^{T}. **a** Colonies on G18, MEA and PDA at 25 °C (left to right), surface and reverse (from top to bottom). **b**–**d** Conidiophores and conidia. Scale bar = 10 μm

intended for human consumption to date. We have isolated selectively and identified, by a polyphasic approach, six species of ascomycetous yeasts and 27 of filamentous ascomycetes, some representing new taxa, from honey samples. The yeasts, *Candida magnoliae*, *C. sorbosivorans*, *Schizosaccharomyces octosporus*, *Zygosaccharomyces barkeri*, Z. *mellis*, and *Z. gambellarensis*, had been reported from honey before, and *C. magnoliae* has also been associated with living honeybees (Gilliam et al. 1974b). All these yeasts have been described as osmophilic and able to grow at a_w of 0.80 or lower (Tilbury 1967; van Eck et al. 1993; Ganthala et al. 1994; Erickson & McKenna 1999; Torriani et al. 2011). We found *C. magnoliae* and *C. sorbosivorans* were phylogenetically closely related (see Fig. 2), and it was reported that both differ only in a few physiological characteristics (James et al. 2001). To our knowledge, none of the species of *Aspergillus* that we isolated (*A. asperescens*, *A. montevidensis*, and *A. pseudoglaucus*) have previously been reported from honey. *Aspergillus asperescens* was originally isolated from soil and bat dung (Stolk 1954), but also from rotten wood and soybean seeds; however, most of the isolates were from cave soil (probably linked to bat dung). *Aspergillus montevidensis* and *A. pseudoglaucus* have been reported as the most important food-spoilage species of the genus (Pitt & Hocking 1977; Kozakiewicz 1989), but are known from extreme environments such as salterns (Butinar et al. 2005). *Aspergillus montevidensis* has been reported from various environmental samples (air, soil, etc.), and even on honeybees and bee larvae (http://gcm.wfcc.info/; Talice & Mackinnon 1931; Gilliam et al. 1974a); *A. pseudoglaucus* has been reported in air, paper and soil (http://gcm.wfcc.info/; Blochwitz 1929). *Aspergillus montevidensis* and *A. pseudoglaucus* are able to grow at a_w values of 0.80 (Snow 1949; Armolik & Dickson 1956; Guynot et al. 2003). *Monascus* is a well-known genus with species (especially *M. purpureus* and *M. ruber*) of economic importance due to their use in production of foodstuffs, bioactive compounds, pigments and enzymes. Currently, *Monascus* is placed in *Aspergillaceae* (syn. *Trichocomaceae*) based on phylogenetic studies, and closely related to *Leiothecium ellipsoideum* and *Xeromyces bisporus* (Houbraken & Samson 2011; Pettersson et al. 2011). Recently, three new species

were added, all of them associated with stingless bees: *M. flavipigmentosus*, *M. mellicola,* and *M. recifensis* (Barbosa et al. 2017). We found a small number of isolates, including *M. pilosus*, *M. purpureus,* and *M. ruber*. These species have been frequently reported in fermented and spoiled foods (van Tieghem 1884; Hesseltine 1965; Lin 1975; Hawksworth & Pitt 1983). *Monascus ruber* has also been found in soil and human clinical specimens (Hawksworth & Pitt 1983). Species of *Monascus* have been previously reported in honey by Snowdon & Cliver (1996) and by Barbosa et al. (2017). *Monascus pilosus*, *M. purpureus,* and *M. ruber* were reported previously (Hawksworth & Pitt 1983) as able to grow well on G25 N ($a_w = 0.93$). The species of *Penicillium* we found in honey included *P. camemberti*, *P. citrinum, P. corylophilum,* and *P. cravenianum*. The most common source of isolation of *P. camemberti* is blue cheeses, but it can also be found on a wide variety of substrata (Thom 1906; http://gcm.wfcc.info/). *Penicillium citrinum* was originally reported in milk and bread in the USA (Thom 1910), but it is found globally and easy to recover from spoiled foods and diverse environmental sources (www.cabri.org/collections.html) including honey, pollen and bee nests (Barbosa et al. 2018). *Penicillium corylophilum* (Dierckx 1901) mostly occurs in damp buildings in North America and Western Europe, but also in foods and mosquitoes (Da Costa & De Oliveira 1998; McMullin et al. 2014), and honey (Sinacori et al. 2014). The minimum a_w reported for the growth of *P. camemberti*, *P. citrinum* and *P. corylophilum* was around 0.80 (Abellana et al. 2001; Fontana 2008; Kalai et al. 2017). *Penicillium cravenianum*, a species moderately xerotolerant (grows on G25 N), has only been reported in soil (Visagie et al. 2016). Notably, all the isolates of *Talaromyces* that we found in honey belonged to three unrecognized species. *Talaromyces basipetosporus* was recovered from a honey sample in Buenos Aires province, Argentina, and is characterized by simple conidiophores that mimic those of the asexual morph of *Monascus* (syn. *Basipetospora*), which develops conidia by a retrogressive mode of conidiogenesis, a feature not previously reported in *Talaromyces*. *Talaromyces affinitatimellis* displays a similar conidiogenesis to *T. basipetosporus* and both species are phylogenetically closely related but phenotypically differentiated as *T. affinitatimellis* grows faster and produces more complex conidiophores. *Talaromyces brunneosporus* differs from the other species of sect. *Purpurei*, apart from *T. purpureus*, in having monophialidic and monoverticillate conidiophores (they are biverticillate in the other species). However, both species are distinguishable because *T. brunneosporus* produces penicillate conidiophores (not aspergillate as in *T. purpureus*), longer phialides, and verrucose conidia with a flattened base (*T. purpureus* conidia are ornamented by spiral ridges). *Talaromyces basipetosporus* has a high xerotolerance, with similar growth rates on MEA with sugars up to a_w 0.82. Despite the decreasing growth rates of *T. brunneosporus* and *T. affinitatimellis* when sugar concentration increases, both fungi are able to grow at a_w 0.82 (Fig. 12). *Xerochrysium xerophilum* (Pitt et al. 2013; syn. *Chrysosporium xerophilum*, Pitt 1966), is an extreme xerophile with a minimum a_w for growth of 0.66 (Gock et al. 2003; Leong et al. 2011). This fungus, previously reported from chocolate, coconut, dried prunes, and stored corn (Pitt & Hocking 2009; Pitt et al. 2013), has not been found in honey until now. This species is phylogenetically close to *Monascus* (Pitt et al. 2013). Among the species of *Onygenales*, *Ascosphaera atra* and *Eremascus albus* were recovered once and four times, respectively. *Ascosphaera atra* (Skou & Hackett 1979) was originally reported from dead larvae of the alfalfa leafcutter bee covered in cysts of *Ascosphaera aggregata* (Skou 1975), and from pollen in the gut of healthy leafcutter larvae. This fungus was subsequently reported from grass silage (Skou 1986). *Ascosphaera atra* is homothallic and saprobic, probably being a common contaminant of pollen (Skou & Hackett 1979), which would explain its presence in honey samples. *Eremascus albus* is a well-known xerophilic fungus, with spores that can germinate at a_w as low as 0.70 (Pitt 1965). This fungus has been reported to spoil malt extract (Eidam 1883), chocolate cake, dried fruits, and mustard powder (Harrold 1950), but never previously from honey. We identified several isolates belonging to the newly described family *Helicoarthrosporaceae*, which only includes the new monotypic genus *Helicoarthrosporum*, and a single strain belonging to the new monotypic genus *Strongyloarthrosporum* (*Ajellomycetaceae*). The morphology of *Helicoarthrosporum mellicola* resembles species of *Scytalidium* (*S. cuboideum*, *S. ganodermophthorum,* and *S. sphaerosporum*) because of the production of cuboid arthroconidia in long chains. However, *Helicoarthrosporum* is phylogenetically distant from *Scytalidium*, as the latter is related to *Myxotrichaceae*. *Strongyloarthrosporum catenulatum* was found to be phylogenetically close to *Ajellomycetaceae*, whose members are thermally dimorphic and pathogenic to animals (including the humans), and has never been reported as xerotolerant. However, having features not seen in that family, *S. catenulatum* is unequivocally a xerophilic fungus, only growing on G18, G25 N and MY70FG, and producing globose arthroconidia, either singly or in chains. The sole xerophilic fungus phylogenetically close to *S. catenulatum* is *Eremascus albus* (*Eremascaceae*), but it only develops a sexual morph. Regarding the family *Myxotrichaceae*, *Skoua fertilis*, which was detected in all honey samples, resembles *Eremascus albus* (Eidam 1883) in having naked asci arising

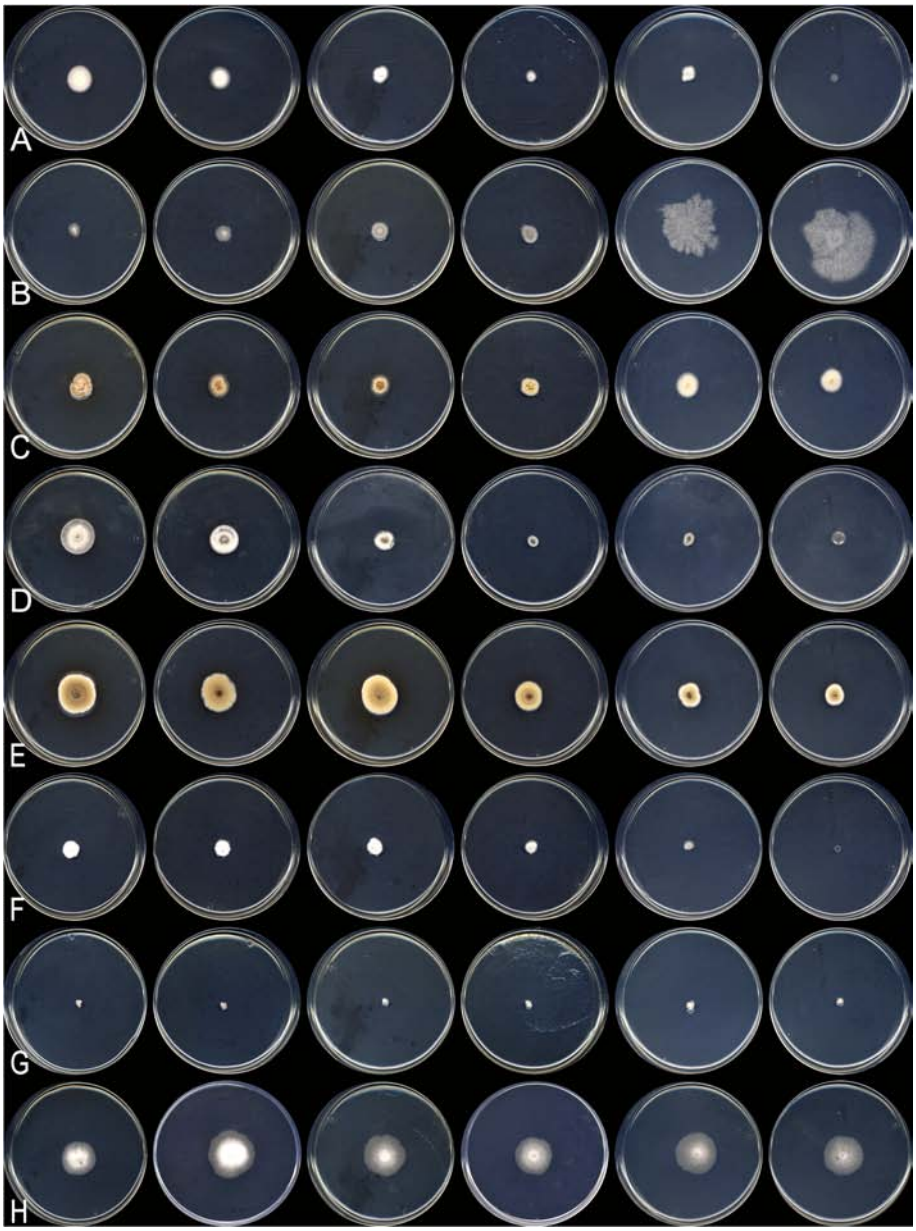

Fig. 12 Relatedness between the growth of the new fungal taxa and the decreasing water activity (a_w) of the culture medium. Surface of the colonies grown on MEA with a_w values of 0.97, 0.95, 0.93, 0.92, 0.88 and 0.82 (from left to right, respectively). **a** *Helicoarthrosporum mellicola*. **b** *Strongyloarthrosporum catenulatum*. **c** *Talaromyces basipetosporus*. **d** *Talaromyces brunneosporus*. **e** *Talaromyces affinitatimellis*. **f** *Oidiodendron mellicola*. **g** *Skoua asexualis*. **h** *Skoua fertilis* (as reference, highly xerotolerant fungus)

directly out of the mycelium and formed by the fusion of two equal cells borne on short entwined hyphae. Both taxa can be only morphologically differentiated by the shape of the ascospores and by sexual reproductive details. While *S. fertilis* (syn. *E. fertilis*) belongs to *Leotiomycetes*, closely related to *Myxotrichaceae* (Wynns 2015), *E. albus* is located in *Eurotiomycetes*, closely related to *Onygenales* (Cai et al. 1996; Berbee 2001; Wynns 2015). *Skoua* was introduced for *E. fertilis* (i.e. *Skoua fertilis*) and has been reported on bee bread, honeycomb, dried prunes and spoiled moist prunes, green compost, and shortcake (www.cabri.org/collections.html; http://gcm.wfcc.info/; Harrold 1950), but not so far on honey. The minimum a_w for growth and sporulation reported for *S. fertilis* was 0.77 (Pitt 1965; Wynns 2015), a similar value observed in all our strains (0.82). We isolated three strains of *Skoua* phylogenetically different from *S. fertilis*, and named them as *Skoua asexualis* because they form asexual spores instead of the sexual spores as observed in the type species of the genus. *Bettsia alvei* (Skou

1972, 1975), the other fungus identified in all honey samples, belongs to *Pseudeurotiaceae* and is characterized by dark, closed ascomata (usually called "spore cysts") and hyaline globose ascospores, forming a sticky mass. *Bettsia alvei* has been isolated from hives in Europe as well as the USA (Burnside 1929), and from bakery products, spoiled chocolate, desiccated coconut, honeycomb, concentrated jelly, dried and spoiled prunes, pollen, table jelly, bee wax, and wine starters (www.cabri.org/collections.html; http://gcm.wfcc.info/). It was also isolated from chocolate in Austria (a_w less than 0.3), but thus far had not been recorded from honey. The lowest a_w tested for growth of this species was 0.88 (Beuchat & Pitt 1990) and 0.89 (Udagawa & Toyazaki 2000), similar values to those we found. All our isolates of *B. alvei* developed the chrysosporium-like asexual morph but failed in the production of the sexual morph. Among the most frequent species we isolated was an undescribed species of *Oidiodendron*, *O. mellicola*. Species of this genus are mostly recovered from soil and other substrata rich in cellulose, and are found worldwide (Domsch et al. 1980; Calduch et al. 2004; Rice & Currah 2005). *Oidiodendron mellicola* is phylogenetically related to *O. truncatum* and *M. setosum,* the former characterized by well-differentiated dark conidiophores and barrel-shaped conidia with a dark scar at one or both ends (typical features of *Oidiodendron*), and the latter by hyaline conidiophores and conidia, and by dark brown to black, spinose, gymnothecial ascomata (typical of the genus *Myxotrichum*). Interestingly, *M. setosum* is reported as a common hive fungus in Europe (Burnside 1929). *Oidiodendron mellicola* is the only species of the genus reported from honey, and it can be distinguished morphologically from other species of the genus by its absence of stipitate conidiophores, and the production of long chains of conidia, which are pale, smooth, ellipsoidal to cylindrical, truncated (but not darkened, as in *O. truncatum*) at one or both ends, and by the slow growing colonies. Like most of the species of the genus, *O. mellicola* grows better at 15 °C than 25 °C. Other fungi rarely found in our study were *Alternaria multiformis*, previously only reported from soil (Simmons 1998), and the mucoralean *Cunninghamella bertholletiae*, *Mucor plumbeus,* and *Rhizopus oryzae*, all found worldwide. These probably represent environmental contaminants. Although all the new taxa that we propose displayed a high xerotolerance, only *Strongyloarthrosporum catenulatum* can be considered an obligate xerophile, because it was able to grow faster at the lowest a_w tested (Fig. 12).

CONCLUSION

The application of G18 as a selective culture medium for isolation of xerotolerant/xerophilic fungi from honey samples enabled the recovery and identification of 13 genera and 29 species of *Ascomycota*, and three genera (one species for each) of *Mucoromycota*. Many of these fungi have never reported from honey before. Among them, we proposed a new family (*Helicoarthrosporaceae*), two new genera (*Strongyloarthrosporum* and *Helicoarthrosporum*) and seven new species (*Strongyloarthrosporum catenulatum*, *Helicoarthrosporum mellicola*, *Oidiodendron mellicola*, *Skoua asexualis Talaromyces basipetosporus*, *T. brunneosporus*, and *T. affinitatimellis*). All fungal taxa that we isolated from honey were able to grow at low water activity (up to 0.82), but only *Ascosphaera atra*, *Bettsia alvei* (two fungi strongly associated to honeybees and their life-style), *Eremascus albus*, *Strongyloarthrosporum catenulatum* (one of the new taxa we described) and *Xerochrysium xerophylum* can be considered obligate xerophiles. Also, because several of the honey samples were thermally treated, these fungi can be considered as hot-resistant. Honey is evidently a reservoir of xerotolerant and xerophilic fungi, which survives to the thermal treatment used to make honey non-crystallisable. Some of these fungi are related to the honeybee life-style; however, as is in the case of the new taxa described here, the origin in nature remains unknown. In the latter case, flowers and aphids could play an important role as a source of such fungi. During the course of the study, the most important pathogenic fungi for honeybees, *Aspergillus flavus* and *Ascosphaera apis*, were not found. Several of the fungi found in honey samples (*Aspergillus* and *Penicillium* spp.) are potential producers of mycotoxins, but this does not mean that the honey may represent a risk to the health of the consumer, because (in general) the production of mycotoxins or the fungal growth are suppressed at water activities lower than 0.70 (Mannaa & Kim 2017), as is the case of honey (a_w of 0.60 or less). Honey should be considered as a "living food" and, consequently, its "normal" mycobiota merits more extensive study. It is expected that such "normal" mycobiota may vary qualitatively and quantitatively, depending on the geographic origin, the botanical type and water activity of the honey, among other physicochemical and biological parameters. Honey is clearly one of the relatively unexplored habitats for the missing fungal diversity, especially as the new taxa we found came from samples from just two countries.

Abbreviations

a_w: water activity; BCP-MS-G: Bromcresol purple milk solids glucose agar; BEA: Bile esculin agar; *BenA*: fragment of the beta-tubulin gene; BI: Bayesian-inference; BLAST: Basic Local Alignment Search Tool; *CaM*: fragment of the calmodulin gene; CREA: Creatine sucrose agar; CYA: Czapek yeast extract agar; DG18: Dichloran 18% glycerol agar; DNA: Deoxyribonucleic acid; G18 DG18: without dichloran; G25 N 25%: glycerol nitrate agar; ITS: Ribosomal internal transcribed spacers; LSU: Large sub unit of the ribosomal genes; MEA: Malt extract agar; ML: Maximum-likelihood; MLI: Maximum level of

identity; MY70FG: Malt extract yeast extract 70% fructose-glucose; nrRNA: Nuclear ribosomal ribonucleic acid; OA: Oatmeal agar; PDA: Potato dextrose agar; PYE: Phytone yeast extract agar; *rpb2*: fragment of the RNA polymerase II subunit 2 gene; SEM: Scanning electron microscopy; TOTM: Test opacity tween medium; TreeBASE: a repository of user-submitted phylogenetic trees and data used to build them; YES: Yeast extract sucrose agar

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Adherence to national and international regulations
The authors confirm that this manuscript respects the Nagoya Protocol to the Convention on Biological Diversity.

Authors' contributions
ER-A performed all the experimental work, culturing the samples, isolating in pure culture the fungi and performing their phenotypic characterization, as well as the DNA extraction and purification, gene sequencing and data processing for phylogenetic analysis, being one of the major contributors of this manuscript. AMS, because their experience on fungi from honey, supervised all steps of the experimental work by ER-A, collaborating in the description of the novel fungi and in the writing of chapters "Introduction" and "Discussion", reviewing of the draft several times. AT provided most of the samples analyzed in this work, gave useful suggestions to write the manuscript and reviewed the draft several times. JG contributed actively in the identification and taxonomy of the fungal strains, and reviewed the draft several times. JFC-L supervised the nucleotide sequence alignment and phylogenetic reconstruction, took the pictures that appear in the figures, contributed actively in the identification and taxonomy of the fungal strains, gave useful suggestions to write the manuscript and reviewed several times the draft. All authors read and approved the final manuscript.

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4.3. Fungal diversity of deteriorated sparkling wine and cork stoppers in Catalonia, Spain

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Fungal Diversity of Deteriorated Sparkling Wine and Cork Stoppers in Catalonia, Spain

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Abstract: Filamentous fungi are rarely reported as responsible for spoiling wine. Cork taint was detected in sparkling wine; therefore, we investigated fungal contamination as a possible cause of organoleptic alteration. Spoiled wine was filtered and membranes were plated onto potato dextrose agar (PDA). The cork stoppers used for sealing bottles were cut and also plated onto PDA. Fungal strains were phenotypically characterized and molecularly identified by sequencing of a fragment of the 28S nrRNA gene (LSU) and (occasionally) by other additional molecular markers. Twenty-seven strains were isolated and sixteen species were identified, all of them belonging to the phylum Ascomycota. The fungi isolated from wine were three species of *Aspergillus* section *Nidulantes*, a species of *Penicillium* section *Exicaulis* and *Beauveria bassiana*. *Candida patagonica* was isolated from both sort of samples, and the fungi isolated from cork stoppers were *Altenaria alternata* and *Cladosporium cladosporioides*. Surprisingly, most of the taxa recovered from the cork stoppers and/or wine were new to the science: a new genus (*Dactylodendron*) and seven new species belonging to the genera *Cladophialophora*, *Dactylodendron*, *Kirschsteiniothelia*, *Rasamsonia*, and *Talaromyces*. Future studies could let us know if these fungi would be able to produce compounds responsible for cork taint.

Keywords: Ascomycota; *cava*; cork taint; fungi; sparkling; spoilage; stoppers; wine

1. Introduction

Sparkling wine is one of the most economically important wine varieties in southern Europe. It is produced by the "champenoise" method, which consists of two steps: a primary alcoholic fermentation, in which the grape must is transformed to the wine base (cuvée); and a second alcoholic fermentation after the addition of sucrose, selected yeasts, and bentonite to the base wine, which is then bottled, closed with a metal cap or a cork stopper, and allowed to age in cellars for a longer period of time (at least 12 months for French champagne and 9 months in the case of the Spanish—mostly Catalonian— "cava") [1]. During fermentation, a certain diversity of environmental microorganisms, mainly bacteria and fungi, can produce organoleptic alterations that render the wine undrinkable. Some of these fungi can be present on the cork stoppers and/or be acquired by exposure of the must to bio-aerosols, perhaps because of poor environmental microbiological control at the cellar. Cork taint is a musty or mouldy off-odor in wine often caused by the presence of 2,4,6-trichloroanisole (2,4,6-TCA) among other chemical compounds [2], and between 0.5 and 7% of wines can be affected by cork taint. It is estimated that cost of cork-related wine spoilage can exceed several billions of dollars per year [3,4]. The metabolic effect of fungi living on cork in the production of 2,4,6-TCA has been described [5]. Among several fungi recovered from agglomerate cork stoppers, *Acremonium strictum*, *Chrysonilia sitophila*, *Cladosporium oxysporum*, *Fusarium oxysporum*, *Paecilomyces*

viridis, *Penicillium chrysogenum*, *Trichoderma longibrachiatum*, *Trichoderma viride*, and *Verticillium psalliotae* have displayed such an effect [5].

Surprisingly, little is known about the nature of the wine spoilage fungi, but there have been some reports about these organisms being isolated during and at the end of fermentation, such as *Cladosporium cucumerinum*, *Cryptococcus tephrensis*, *Hanseniaspora thailandica*, *Schizosaccharomyces japonicas*, and *Sporobolomyces coprosmae* [6–14]. Most of those studies focused on microorganisms present on cork stoppers. Some filamentous fungi have been reported on this substrate, such as *Alternaria alternata*, *Armillaria mellea*, *Aspergillus* spp., *Aureobasidium pullulans*, *Cladosporium* spp., *Fusarium* spp., *Mucor* spp., *Neurospora sithophila*, *Penicillium* spp., *Rhizopus arrhizus*, *Scopulariopsis candida*, and *Trichoderma* spp., and also yeasts such as *Debaryomyces hansenii*, *Lachancea thermotolerans*, *Rhodotorula* spp., *Sporidiobolus johnsonii*, several species of *Saccharomyces*, *Tausonia pullulans*, and *Trichomonascus ciferri* [15–27]. Interestingly, other yeasts, such as *Bullera* sp., *Cutaneotrichosporon mucoides*, *Cryptococcus albidus*, *D. hansenii*, *Rhodosporidium kratochviloae*, *Rhodotorula* spp., *Sporidiobolus* spp., and *Wickerhamomyces anomalus* have all been isolated during the manufacturing process of the cork stoppers [28]. In addition, the sordariaceous mold *Zopfiella ebriosa* has been found on cork stoppers exposed to wine [29].

We recently had the opportunity to study the fungal biota associated to wine deterioration when a local winery located in Sant Sadurní d'Anoia (Barcelona province, Spain) detected cork taint in some bottles of sparkling wine during an inspection of the cellars at its historic vineyards. We conducted a study to detect, isolate, and identify the fungi involved in the production of this sort of flavor alteration.

2. Materials and Methods

2.1. Fungal Isolation

Samples of sparkling wine and cork stoppers were obtained from a cellar in Sant Sadurní d'Anoia, Barcelona province, Spain. Approximately, 500 bottles of sparkling wine from five different batches were opened in situ to obtain a representative number of negative controls (without organoleptic alteration) and a panel of four experts detected taste defects in any samples that had a musty or mouldy off-odor and/or flavor. A total of 54 bottles of sparkling wine sealed by cork stoppers (15 negative control and 39 with deteriorated wines) were selected and processed. A sample of 100 mL of sparkling wine was filtered through a filter membrane of 0.45 µm diameter (Millipore SA, Molsheim, France). After filtering, the membrane was plated onto a 90 mm diameter Petri dish containing potato dextrose agar (PDA; Pronadisa, Madrid, Spain) plus 50 mg/L L-chloramphenicol. The Petri dishes were incubated at 25 °C for a time period ranging from 4 weeks to 2 months in darkness, and examined under a stereomicroscope to observe any production of mold colonies with reproductive structures. If bacteria and/or yeasts develop on the culture medium, these could be recognized by mucous to buttery colonies of reduced diameter, and by the absence of hyphae; also, slide mountings on water seen under bright field microscope allows detection of the bacterial/yeast cells. The cork stoppers were cut into small pieces using a sterile disposable scalpel and plated onto 90 mm diameter Petri dishes containing PDA, which were incubated in the same way as described above. For both sorts of samples, fungal structures from selected colonies (representative of all the morphological varieties) were transferred to 50-mm diameter Petri dishes containing PDA using a sterile insulin-type needle and incubated in the same conditions to obtain pure cultures.

2.2. Phenotypic Characterization of the Fungal Strains

For the isolates of *Rasamsonia* and *Talaromyces*, suspensions of conidia were prepared in a semi-solid agar (0.2% agar, 0.05% Tween 80) [30] and inoculated in three equidistant points onto 2% malt extract agar (MEA; Difco Inc., Detroit, USA), oatmeal agar (OA) [30], Czapek yeast extract agar (CYA) [31], yeast extract sucrose agar (YES) [32], creatine sucrose agar (CREA) [32], dichloran 18% glycerol agar (DG18) [33], and cork (cut in slices by a scalpel, placed into appropriate containers, and sterilized three times in alternative days at 121 °C during 15 minutes) onto tap water agar (TWA; 1.5% agar in

tap water) into disposable Petri dishes of 90 mm diameter, and incubated at 25 °C in darkness after 14 days. Cultures on CYA were also incubated at different temperatures (5, 15, 25, 30, 35, and 37 °C) to determine the minimum, optimal, and maximum temperatures of growth. The rest of the isolates were cultured and studied onto MEA, OA, PDA, and TWA with pieces of sterile cork, incubated at 25 °C in darkness after 14 days. The determination of the cardinal temperatures of growth of these strains was determined on PDA. Color notations in parentheses are from Kornerup and Wanscher [34]. The characterization and measurements of fungal structures were performed in water and 60% lactic acid from slide cultures by using the culture media cited before. Photographs were taken by a Zeiss Axio Imager M1 light microscope (Zeiss, Oberkochen, Germany) with a DeltaPix Infinity X digital camera, using Nomarski differential interference contrast. The samples for scanning electron microscopy (SEM) were processed according to Figueras and Guarro [35], and SEM micrographs were taken at 15 keV with a Jeol JSM 840 microscope. The taxonomic descriptions and names of the fungal novelties were introduced into MycoBank (www.mycobank.org) [36].

2.3. DNA Extraction, Amplification, and Sequencing

Total DNA, extracted by the modified protocol of Marimon et al. [37], was used to amplify and sequence a fragment of the 28S nrRNA gene (LSU) using the primer pair LR0R [38] and LR5 [39] for all isolates. For the phylogeny of the isolates of *Cladophialophora*, *Rasamsonia,* and *Talaromyces*, the following molecular markers were amplified and sequenced: i.e., ribosomal internal transcribed spacers (ITS) (ITS5/ITS4) [40] and fragments of the beta-tubulin (*BenA*) (Bt2a/Bt2b) [41]; calmodulin (*CaM*) (Cmd5/Cmd6) [42] and RNA polymerase II subunit 2 (*rpb*2) (RPB2-5F/RPB2-7cR) [43] genes. Sequencing of the amplicons was made in both directions with the same primer pair used for amplification at Macrogen Europe (Macrogen Inc., Amsterdam, The Netherlands). The consensus sequences were obtained using the SeqMan software v. 7 (DNAStar Lasergene, Madison, WI, USA). Sequences generated in the present work were deposited in GenBank (Table 1).

Table 1. Fungal taxa and their nucleotide sequences of the molecular markers used to build the *Cladophialophora* spp., *Rasamsonia* spp., and *Talaromyces* spp. phylogenetic trees.

Species Name	Strain	GenBank Accession #				
		BenA	***CaM***	***rpb2***	**ITS**	**LSU**
Cladophialophora abundans	CBS 126736^T	–	–	–	KC776592	KC812100
C. arxii	CBS 306.94^T	–	–	–	EU103986	KX822320
C. australiensis	CBS 112793^T	–	–	–	EU035402	EU035402
C. bantiana	CBS 173.52^T	–	–	–	EU103989	–
C. boppii	CBS 126.86^T	–	–	–	EU103997	FJ358233
C. carrionii	CBS 160.54^T	EU137201	–	–	EU137266	FJ358234
C. chaetospira	CBS 491.70	–	–	–	EU035405	EU035405
C. devriesii	CBS 147.84^T	–	–	–	EU103985	KC809989
C. emmonsii	CBS 979.96	–	–	–	EU103996	–
C. floridiana	NRRL 66282^T	–	–	–	AB986343	AB986343
C. immunda	CBS 834.96^T	EU137203	–	–	EU137318	KC809990
C. inabaensis	EUCL1^T	–	–	–	LC128795	LC128795
C. minourae	CBS 556.83^T	–	–	–	AY251087	FJ358235
C. multiseptada	CBS 136675^T	–	–	–	HG003668	HG003671
C. mycetomatis	CBS 12263^T	–	–	–	FJ385276	KX822321
C. parmeliae	CBS 129337	–	–	–	JQ342180	JQ342182
C. potulentorum	CBS 112222	–	–	–	EU035409	EU035409
C. samoensis	CBS 259.83^T	EU137174	–	–	EU137291	KC809992
C. saturnica	CBS 118724^T	–	–	–	EU103984	–
C. subtilis	CBS 122642^T	–	–	–	FJ385273	KX822322
C. tortuosa	NRRL 66284^T	–	–	–	AB986424	AB986424
C. recurvata	**FMR 16667^T**	**LT985894**	–	–	**LT985878**	**LT985879**

C. yegresii	CBS 114405^T	EU137209	–	–	EU137323	KC809994
Exophiala exophialae	CBS 668.76	EF551499	–	–	NR111130	KX712348
E. oligosperma	CBS 725.88	KF928550	–	–	NR111134	KF928486
Rasamsonia aegroticola	DTO 137A8^T	JX273020	JX272956	–	JX272988	–
R. argillacea	CBS 101.69^T	JF417456	JF417501	–	JF417491	–
R. brevistipitata	CBS 128785^T	JF417454	JF417499	–	JF417488	–
R. byssochlamydoides	CBS 413.71^T	JF417460	JF417512	–	JF417476	–
R. columbiensis	CBS 141097^T	LT548285	–	–	LT548281	–
R. composticola	CGMCC 3.13669^T	JF970183	JQ729688	–	JF970184	–
R. cylindrospora	CBS 275.58^T	JF417448	JF417493	–	JF417470	–
R. frigidotolerans	**FMR 16675^T**	**LT985895**	**LT985897**	**–**	**LT985886**	**LS453294**
R. frigidotolerans	**FMR 16670**	**LT985896**	**LT985898**	**–**	**LT985887**	**LS453295**
R. eburnea	CBS 100538^T	JF417462	JF417494	–	JF417483	–
R. emersonii	CBS 393.64^T	JF417463	JF417510	–	JF417478	–
R. piperina	CBS 408.73^T	JX273000	JX272936	–	JX272968	–
R. pulvericola	DAOM242435^T	KF242520	KF242522	–	KF242514	–
Talaromyces aerius	CBS 140611^T	KU866835	KU866731	KU866991	KU866647	–
T. albobiverticillius	CBS 133440^T	KF114778	KJ885258	KM023310	HQ605705	–
T. assiutesis	CBS 147.78^T	KJ865720	KJ885260	KM023305	N899323	–
T. atroroseus	CBS 133442^T	KF114789	KJ775418	KM023288	KF114747	–
T. austrocalifornicus	CBS 644.95^T	KJ865732	KJ885261	–	JN899357	–
T. basipetosporus	FMR 9720^T	LT906563	–	LT906545	LT906542	–
T. brasiliensis	CBS 142493^T	LT855560	LT855563	LT855566	MF278323	–
T. convolutus	CBS 100537^T	KF114773	–	JN121414	JN899330	–
T. dendriticus	CBS 660.80^T	JX091391	KF741965	JN121547	JN899339	–
T. diversus	CBS 320.48^T	KJ865723	KJ885268	KM023285	KJ865740	–
T. affinitatimellis	FMR 15690^T	LT906552	LT906549	LT906546	LT906543	–
T. erythromellis	CBS 644.80^T	HQ156945	KJ885270	KM023290	JN899383	–
T. flavus	NRRL 2098^T	EU021663	EU021694	–	EU021596	–
T. heiheensis	CGMCC 3.18012^T	KX447525	KX447532	KX447529	KX447526	–
T. minioluteus	CBS 642.68^T	KF114799	KJ885273	JF417443	JN899346	–
T. minnesotensis	FMR 14265^T	LT559083	LT795604	LT795605	LT558966	–
T. mirabile	CBS 624.72^T	KF114797	–	–	NR138300	–
T. rademirici	CBS 140.84^T	KJ865734	–	KM023302	JN899386	–
T. rubrifaciens	CGMCC 3.17658^T	KR855648	KR855653	KR855663	KR855658	–
T. samsonii	CBS 137.84^T	KF114798	–	–	NR138301	–
T. solicola	DAOM 241015^T	GU385731	KJ885279	KM023295	FJ160264	–
T. speluncarum	**FMR 16671^T**	**LT985901**	**LT985906**	**LT985911**	**LT985890**	**LS453296**
T. speluncarum	**FMR 16662**	**LT985902**	**LT985907**	**LT985912**	**LT985891**	**LS453297**
T. speluncarum	**FMR 16663**	**LT985903**	**LT985908**	**LT985913**	**LT985892**	**LS453298**
T. systylus	BAFCcult 3419^T	KR233838	KR233837	–	KP026917	–
T. trachyspermus	CBS 373.48^T	KF114803	KJ885281	JF417432	JN899354	–
T. ucrainicus	CBS 162.67^T	KF114771	KJ885282	KM023289	JN899394	–
T. udagawae	CBS 579.72^T	KF114796	–	–	JN899350	–
T. subericola	**FMR 15656^T**	**LT985899**	**LT985904**	**LT985909**	**LT985888**	**LS453299**
T. subericola	**FMR 15664**	**LT985900**	**LT985905**	**LT985910**	**LT985889**	**LS453300**

Trichocoma paradoxa	CBS 103.73	JF417469	JF417506	–	JF417486	–

BAFCcult: Culture collection of the Department of Biological Science, Faculty of Exact and Natural Sciences, Buenos Aires, Argentina; CBS: Culture collection of the Westerdijk Biodiversity Institute, Utrecht, the Netherlands; CGMCC: China General Microbiological Culture Collection Centre, Beijing, China; DAOM: Canadian Collection of Fungal Cultures, Ottawa, Canada; DTO: Applied and Industrial Mycology Department Collection, Utrecht, the Netherlands; FMR: Faculty of Medicine Reus culture collection, Spain; NRRL: ARS Culture Collection, Peoria, United States. ^{T}: ex-type strain. Sequences newly generated in this study are indicated in **bold**. ITS: internal transcribed spacer region 1 and 2 including 5.8S nrDNA; LSU: 28S large subunit of the nrRNA gene; *BenA*: β-tubulin; *CaM*: calmodulin; *rpb*2: partial RNA polymerase II, second largest subunit.

2.4. Preliminary Identification and Phylogenetic Analysis

Preliminary molecular identification was carried out by comparing of the LSU sequences of our isolates with those of the type or reliable GenBank reference strains using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). A maximum level of identity (MLI) of ≥98% was considered to allow for species-level identification. MLI values < 98% provided identification only at genus level. For identification of species of *Aspergillus* and *Penicillium*, sequences of a fragment of *BenA* gene were used. To determine the phylogenetic placement of all our isolates, an LSU tree was built. Additionally, three trees with a combined data set were built to distinguish among the species of *Talaromyces* section *Trachyspermi* (by using the combined dataset ITS-*BenA*-*CaM*-*rpb*2), the species of *Rasamsonia* (ITS-*BenA*-*CaM*), and the species of *Cladophialophora* (ITS-LSU-*BenA*). *Candida bituminiphila* and *Candida patagonica* for LSU tree, *Talaromyces rademirici* and *Talaromyces dendriticus* for *Talaromyces* section *Trachyspermi* tree, *Talaromyces flavus* and *Trichocoma paradoxa* for *Rasamsonia* spp. tree, and *Exophiala oligosperma* and *Exophiala exophialae* for *Cladophialophora* spp. tree were used as out-groups. For sequence alignment and to perform the maximum-likelihood (ML) and Bayesian-inference (BI) phylogenetic analyses, we followed the methodology described by Valenzuela-Lopez et al. [44]. The final matrices used for phylogenetic analysis were deposited in TreeBASE (www.treebase.org; accession number: S23148).

2.5. Growing at Different Ethanol Concentrations

Strains from cork stoppers and sparkling wine were grown on test tubes with 5 mL of 2% malt extract in tap water supplemented with different amounts of ethyl alcohol to reach 5, 10, 15, and 20% v/v final concentration. The tubes were closed with plastic caps, hermetically sealed by parafilm®, and incubated at 15 °C for up to 13 months in darkness without agitation, trying to simulate the method employed for resting/aging of wine. The tubes were examined every month for fungal growth. If growth was absent, 0.1 mL of the broth was plated onto PDA, and incubated during 2 weeks in darkness at 25 °C to confirm absence of fungal growth.

3. Results

3.1. Fungal Diversity of Cork Stoppers and Sparkling Wine Samples

None of the negative controls of sparkling wine developed fungal colonies. On the other hand, 24 out of 39 odor/flavor altered samples developed bacterial, yeasts, and/or mold colonies. All of the cork stopper samples developed fungal colonies. A total of 27 ascomycetes, representing all the morphological variability of the fungal colonies produced, were isolated from cork stoppers and from sparkling wine. Five of them were identified as *Talaromyces* spp., four as *Kirschsteiniothelia* spp., two as *Rasamsonia* spp. and one as *Cladophialophora* sp., three other strains belonged to an unknown arthrosporate fungus. Several *Aspergillus* spp. were recovered from sparkling wine: i.e., *Aspergillus aureolatus*, *Aspergillus jensenii*, and *Aspergillus puulaauensis* (all of them belonging to the section *Nidulantes*). *Penicillium corylophilum* (section *Exilicaulis*) and several other fungi were identified from both sparkling wine and cork stoppers. *Alternaria alternata* and *Cladosporium cladosporioides* were

identified on cork stoppers, but *Beauveria bassiana* and *Candida patagonica* were only found in sparkling wine.

3.2. Molecular Phylogeny

The first phylogenetic study included 64 LSU sequences, with a total of 517 characters including gaps, from which 273 were parsimony informative. The ML analysis was congruent with the BI analysis, both displaying a similar topology. In the LSU tree, our fungal isolates were distributed across two main clades (Figure 1), the first (100% BS / 1 PP), corresponding to the filamentous Ascomycota, included 24 of our isolates, and the second (100% BS / 1 PP), corresponding to the class Saccharomycetales (true yeasts), included the other isolates (three). The first main clade divided into six subclades: A (unsupported, including 13 isolates), corresponding to the order Eurotiales; B (100% BS / 1 PP, three isolates), representing the family Eremascaceae (of the order Onygenales); C (100% BS / 1 PP, one isolate), grouped the family Herpotrichiellaceae (order Chaetothyriales); D (unsupported, five isolates), which included the family Kirschteiniotheliaceae (sister clade D1; 100% BS / 1 PP) and the family Pleosporaceae (sister clade D2; 100% BS / 1 PP) (both pertaining to the order Pleosporales); E (100% BS / 1 PP, one isolate), with the family Cladosporiaceae (order Capnodiales); and F (100% BS / 1 PP, one isolate), with the family Cordycipitaceae (order Hypocreales). Subclade A has three well-supported sister clades, representing the genera *Rasamsonia* and *Talaromyces* (sister clade A1; 90% BS / 0.99 PP), *Penicillium* (sister clade A2; 100% BS / 1 PP), and *Aspergillus* (sister clade A3; 88% BS / 0.99 PP). In this context, seven of our isolate formed three well-supported branches within the sister clade A1: two within the genus *Talaromyces* and the third near to the species of *Rasamsonia*, but not closely related to any of the known species. Within the sister clade A2, two isolates grouped with *Penicillium corylophilum*. The sister clade A3 has two isolates placed together with *Aspergillus aureolatus* and another two together with *Aspergillus puulaauensis* and *Aspergillus jensenii*. Subclade B includes three isolates related to *Arthrographis pinicola* and *Eremascus albus* (Onygenales). Subclade C groups different species of *Cladophialophora*, the isolate FMR 16667 being phylogenetically closely related to the type strain of *Cladophialophora mycetomatis*. Sister clade D1 (*Kirschsteiniothelia* spp. and *Dendryphiopsis* spp.) includes four of our isolates, grouped within two fully supported branches. Sister clade D2 includes FMR 15666 and *Alternaria alternata*. Subclade E includes FMR 15660, *Cladosporium silenes*, *C. cladosporioides*, and *C. grevilleae*. Subclade F includes FMR 16669, *Beauveria brongniartii,* and *B. bassiana*. Finally, in the second main clade (Saccharomycetales), three of our isolates and *Candida patagonica* were placed into a well-supported sister branch (100% BS / 1 PP).

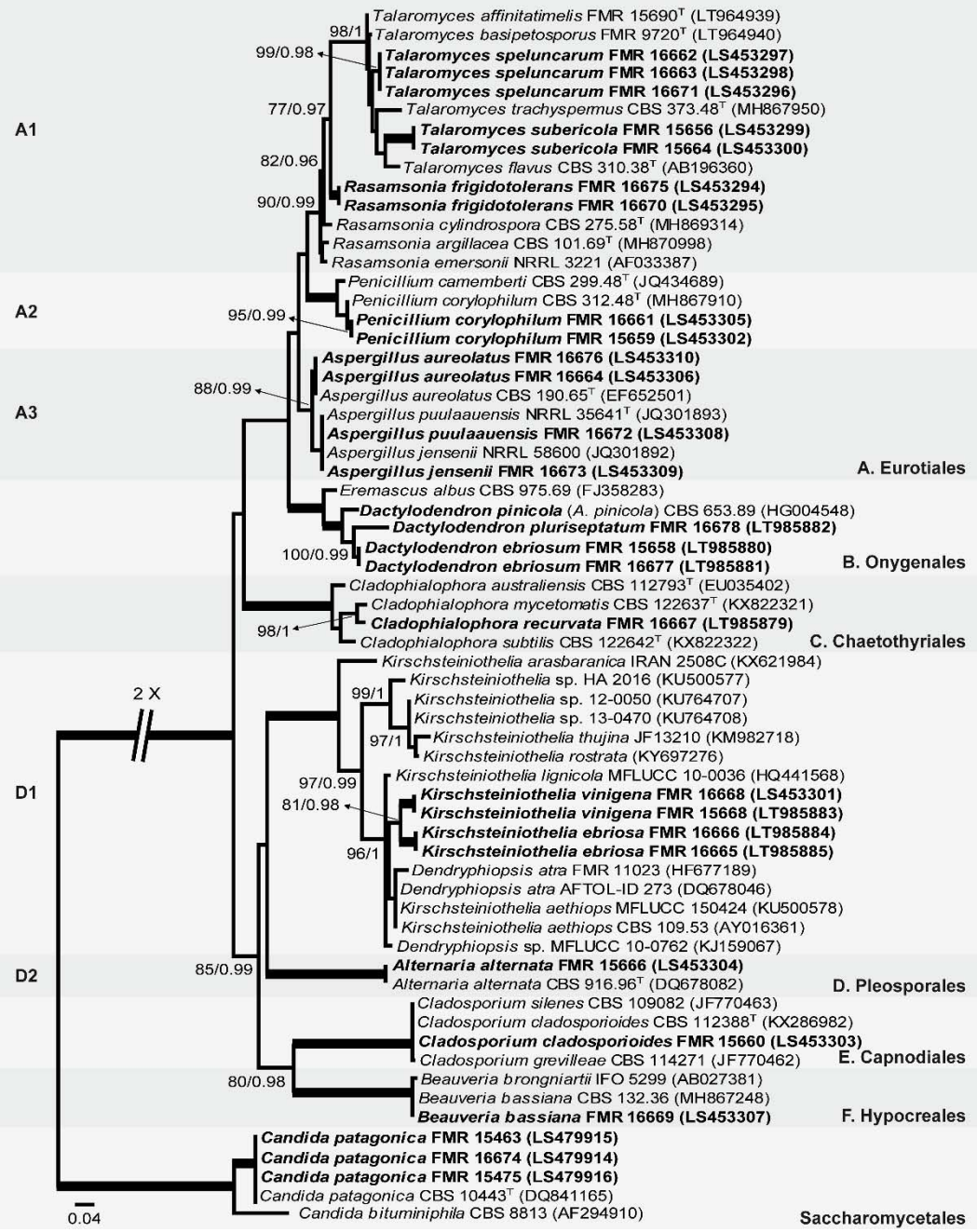

Figure 1. Maximum likelihood (ML) phylogenetic tree based on the analysis of LSU nucleotide sequences for all fungi isolated from sparkling wine and cork stoppers. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. Some branches were shortened; these are indicated by two diagonal lines with the number of times a branch was shortened. ^{T} = ex type. Alignment length 519 bp. The sequences not generated by us were retrieved from EMBL/GenBank.

Three additional phylogenies allowed the taxonomy of *Talaromyces*, *Rasamsonia*, and *Cladophialophora* to be resolved. The first (ITS, *BenA*, *CaM*, and *rpb*2) clarified the relationships among the species of *Talaromyces* section *Trachyspermi*, which included five of our isolates (Figure 2). The final concatenated dataset was obtained using both ML and Bayesian analyses. It contained 29 taxa with a total of 2270 characters including gaps (515 of them for ITS, 376 for *BenA*, 527 for *CaM*, and 852 for *rpb*2), 728 of which were parsimony informative (128 of them for ITS, 145 for *BenA*, 212 for *CaM*, and 243 for *rpb*2). The datasets did not conflict with the tree topologies for the 70% reciprocal bootstrap trees, which allowed the four genes to be combined for the multi-locus analysis. The support values were only slightly different between these two analyses. Within the main clade,

corresponding to *Talaromyces* section *Trachyspermi* (100% BS / 1 PP), three of our isolates were placed in a distinct branch (100% BS / 1 PP), related with *T. affinitatimellis* and *T. basipetosporus*; the other two isolates were located within another branch (100% BS / 1 PP) of a well-supported terminal clade (100% BS / 0.99 PP), which also included *Talaromyces brasiliensis*.

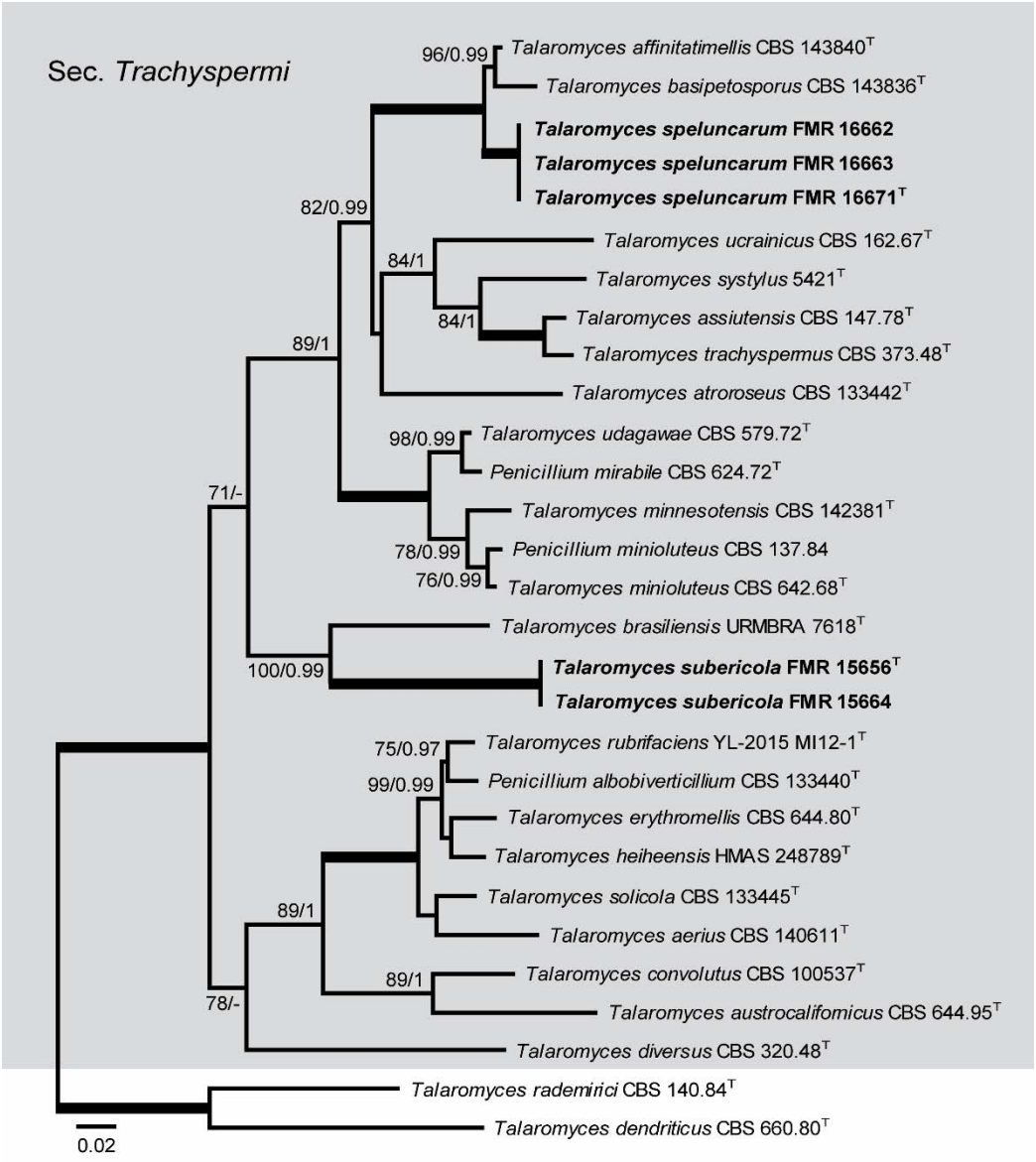

Figure 2. ML phylogenetic tree based on the analysis of internal transcribed spacers (ITS), *BenA*, *CaM,* and *rpb*2 concatenated dataset for species of the genus *Talaromyces* section *Trachyspermi* isolated from sparkling wine and cork stoppers. *Talaromyces rademirici* CBS 140.84 and *Talaromyces dendriticus* CBS 660.80 were chosen as out-group. ^{T} ex-type strain. Alignment length is 2270 bp. The sequences used in this analysis are in Table 1.

The second additional phylogenetic analysis was performed (ITS, *BenA*, and *CaM*) to resolve the taxonomical placement of two of our isolates between the genera *Talaromyces* and *Rasamsonia* (Figure 3). The final concatenated dataset contained 15 sequences with a total of 1657 characters including gaps (707 of them for ITS, 392 for *BenA*, and 558 for *CaM*), 424 of which were parsimony informative (120 of them for ITS, 115 for *BenA*, and 189 for *CaM*). The ML analysis showed a similar topology and was congruent with the Bayesian analysis. The phylogenetic tree distinguished a main clade corresponding to the genus *Rasamsonia* (92% BS / 1 PP), which was divided in three subclades; our

two isolates were located within one of them (83% BS / – PP) in a well-supported branch related to *R. cylindrospora*, *R. brevistipitata*, *R. columbiensis*, and *R. pulvericola*.

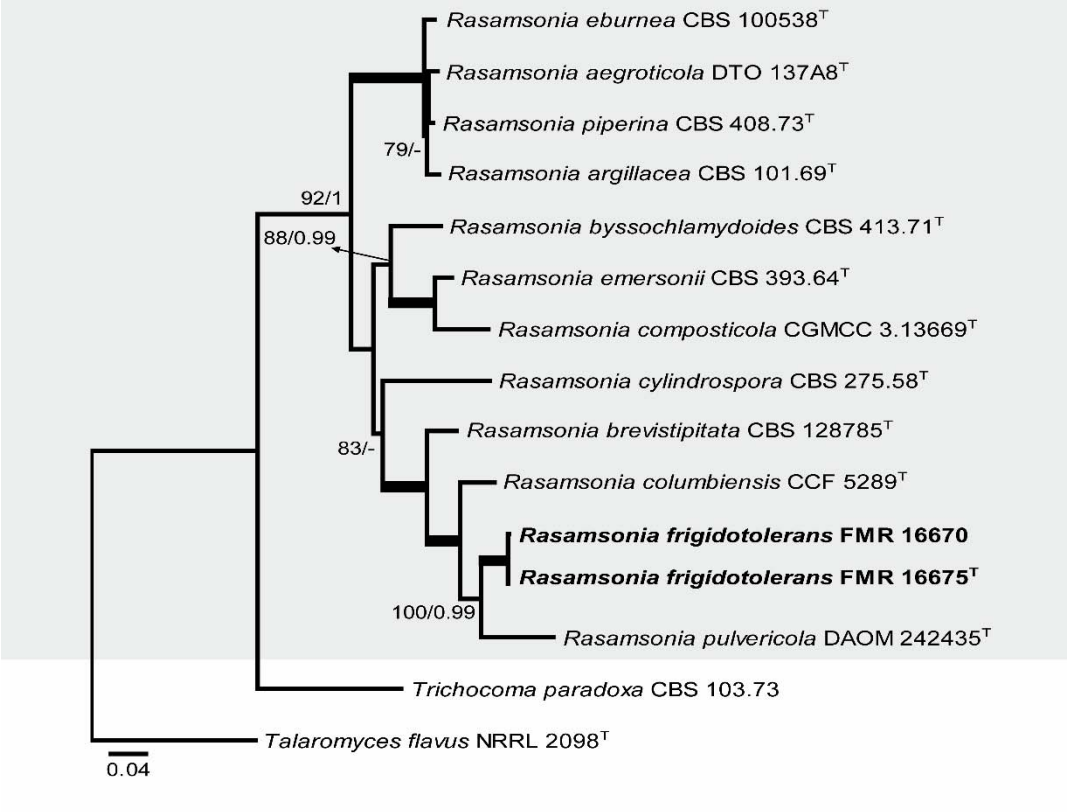

Figure 3. ML phylogenetic tree based on the analysis of ITS, *BenA*, and *CaM* concatenated dataset for species of the genus *Rasamsonia* isolated from sparkling wine. *Trichocoma paradoxa* CBS 103.73 and *Talaromyces flavus* NRRL 2098 were chosen as out-group. T ex-type strain. Alignment length is 1657 bp. The sequences used in this analysis are in Table 1.

ITS-LSU-*BenA* analysis included sequences from 23 taxa of *Cladophialophora*, with a total of 1554 characters including gaps (615 of them for ITS, 562 for LSU, and 377 for *BenA)*, 377 of which were parsimony informative (209 of them for ITS, 55 for LSU, and 113 for *BenA*). The topologies of both ML and Bayesian analyses showed similar topologies and so were congruent. In the phylogenetic tree (Figure 4), a main clade corresponding to *Cladophialophora* spp. (100% BS / 1 PP) was obtained. Within this clade, a terminal branch (100% BS / 1 PP) included *Cladophialophora mycetomatis* and FMR 16667.

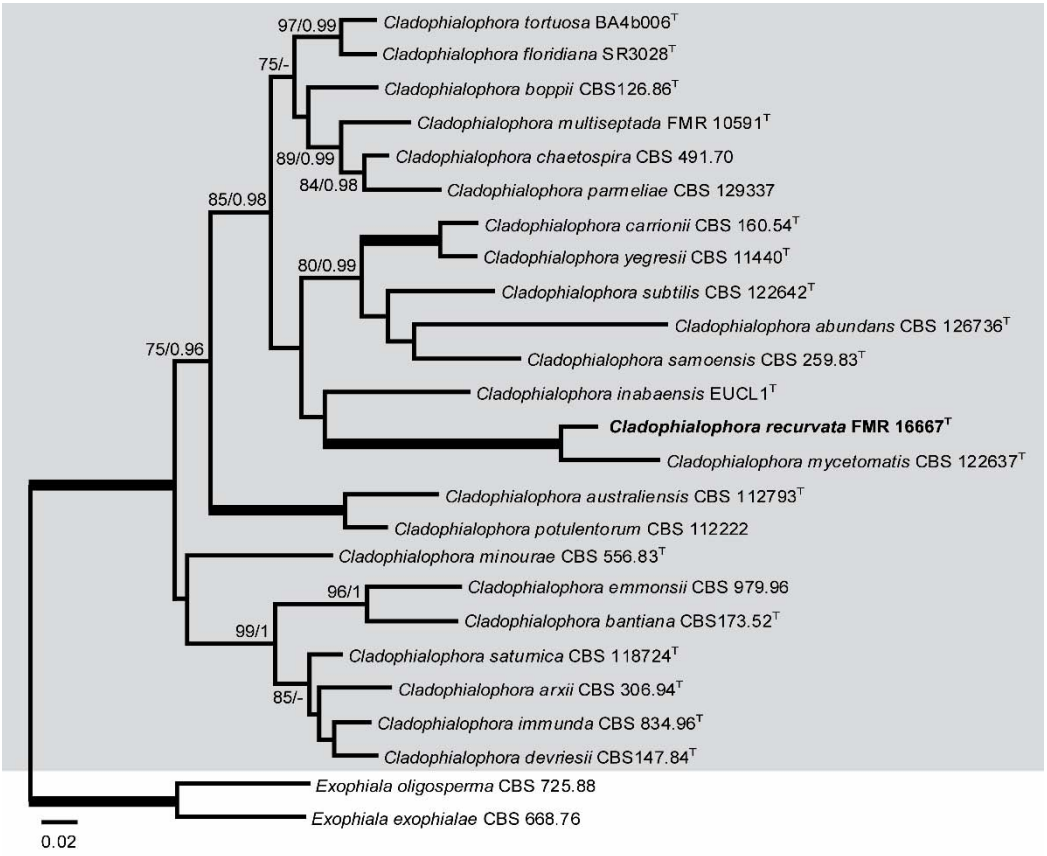

Figure 4. ML phylogenetic tree based on the analysis of ITS, LSU, and *BenA* concatenated dataset for species of the genus *Cladophialophora* isolated from sparkling wine. *Exophiala oligosperma* CBS 725.88 and *Exophiala exophialae* CBS 668.76 were chosen as out-group. ^{T} ex-type strain. Alignment length is 1554 bp. The sequences used in this analysis are in Table 1.

3.3. Alcohol Tolerance

All the isolates tested displayed good to excellent growth at 5% v/v ethanol, but failed to grow at higher concentrations of alcohol.

3.4. Taxonomy

3.4.1. Subclade A: Eurotiales

Because our strains FMR 16662, FMR 16663, and FMR 16667 form a separate branch into a terminal clade including *T. basipetosporus* and *T. affinitatimellis*, and strains FMR 15656 and FMR 15664 form another independent branch within a terminal clade that also includes *T. brasiliensis* (Figure 2), and because all of them display enough phenotypic and phylogenetic differences with respect to the other species of *Talaromyces* section *Trachispermi* to be considered two new species, we propose the erection of *Talaromyces speluncarum* and *Talaromyces subericola* as follows:

Talaromyces speluncarum **Rodr.-Andr., Cano et Stchigel, sp. nov.** MycoBank MB 830606. (Figure 5)

Etymology: From Latin speluncarum, of caves, the place where the wine is aged.

Diagnosis: *Talaromyces speluncarum* falls into a terminal clade which also includes *T. basipetosporus* and *T. affinitatimellis* (Rodríguez-Andrade et al., in press). *Talaromyces speluncarum* is easily distinguishable by its spinose enteroblastic conidia arising from phialides (smooth-walled and of retrogressive conidiogenesis in *T. basipetosporus* and *T. affinitatimellis*).

Type: Spain: Barcelona province: Sant Sadurní d'Anoia, from sparkling wine, 3 Apr. 2011, J. F. Cano-Lira & A. M. Stchigel (CBS H-23372 – holotype; CBS 143844 = FMR 16671 – ex-type cultures; LSU sequence GenBank LS453296).

Description: Colonies on CYA: reaching 2–3 mm diameter after 2 weeks at 25 °C, slightly elevate, velvety to floccose, margins regular, yellowish white (4A2), exudate absent, sporulation sparse; reverse greyish orange (5B4), diffusible pigment absent. Mycelium: abundant, composed of subhyaline to pale brown, smooth- and thin-walled, septate hyphae, 1.5–2 μm wide. Conidiophores: mostly biverticillate, less frequently monoverticillate or irregularly verticillate, stipitate, smooth- and thin-walled, 17–32×2.5–3 μm, bearing 2–3 branches at the top; branches 1-septate or non-septate, hyaline, smooth- and thin-walled, cylindrical, 8–17×2.5–3 μm, bearing 1–4 conidiogenous cells at the top. Conidiogenous cells: phialidic, smooth- and thin-walled, mostly cylindrical and slightly slender toward the apex, 7–10×1.5–2.5 μm, frequently with a hyaline, broad, and flattened collarette. Conidia: enteroblastic, one-celled, pale greenish when young, mid brown when mature, spinose to verrucose, globose, 3–4 μm diameter, in basipetal chains of up to 30. Sexual morphology: not observed.

Colonies on MEA: reaching 12–13 mm diameter after 2 weeks at 25 °C, slightly elevate, velvety to floccose, irregular margins, greyish orange (5B6) at center, pale yellow (4A4) to the edge, exudate abundant, dark orange (5A8), sporulation abundant; reverse dark orange (5A8), diffusible pigment golden yellow (5B7). Colonies on DG18: reaching 7–8 mm diameter after 2 weeks at 25 °C, slightly elevated, velvety to floccose, yellowish-white (4A2) at center, white (4A1) to the edge, exudates absent, sporulation sparse; reverse pale orange (5A3), diffusible pigment absent. Colonies on OA: reaching 9–10 mm diameter after 2 weeks at 25 °C, elevated, velvety to floccose, margins entire, greyish yellow (4C4), exudates absent, sporulation abundant; reverse, diffusible pigment absent. Colonies on YES: reaching 5–6 mm diameter after 2 weeks at 25 °C, elevated, velvety, irregular margins, white (4A1), exudate absent, sporulation absent; reverse greyish orange (5B4), diffusible pigment absent. Colonies on TWA with sterile cork: olive brown (4D4), exudate absent, sporulation abundant; reverse olive brown (4D4), diffusible pigment absent. The fungus does not grow on CYA at 30 °C or on CREA at 25 °C.

Other specimens examined: Spain: Barcelona province: Sant Sadurní d'Anoia, from sparkling wine, 3 Apr. 2011, J. F. Cano-Lira & A. M. Stchigel (FMR 16662 and FMR 16663).

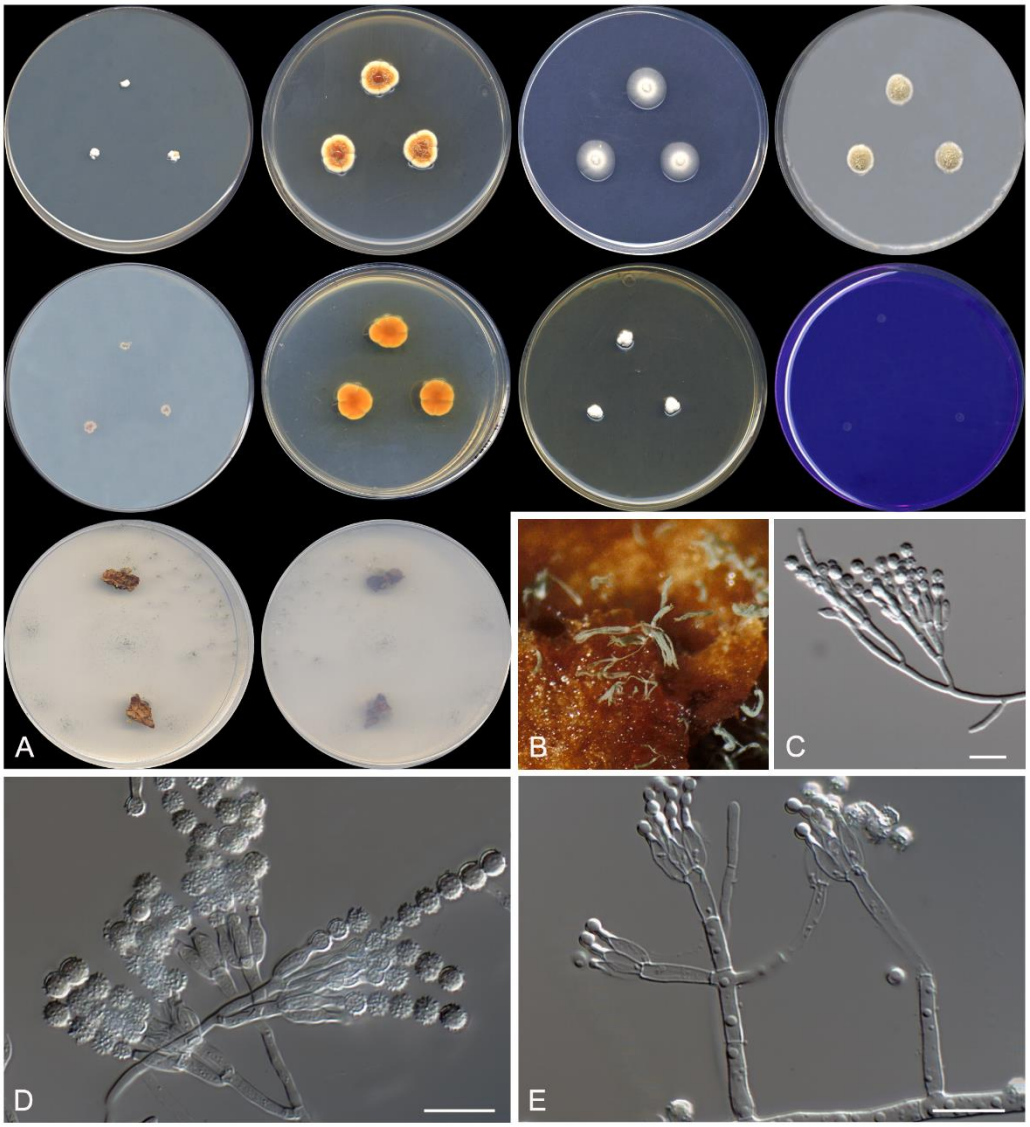

Figure 5. *Talaromyces speluncarum* CBS 143844. (**A**) Colonies on Czapek yeast extract agar (CYA), malt extract agar (MEA), dichloran 18% glycerol agar (DG18), and oatmeal agar (OA) after 14 days at 25°C, from left to right (top row); reverse of the colonies on CYA and MEA, and surface of the colonies on yeast extract sucrose agar (YES) and creatine sucrose agar (CREA), from left to right (medium row); surface and reverse of the colony on tap water agar (TWA) with cork, from left to right (bottom row). (**B**) Detail of the colony on TWA with cork. (**C**–**E**) Conidiophores and conidia. Scale bar = 10 μm.

Talaromyces subericola **Rodr.-Andr., Cano et Stchigel, sp. nov.** MycoBank MB 830607. (Figure 6)

Etymology: From Latin suber, cork, because of the origin of the fungus.

Diagnosis: *Talaromyces subericola* differs from *T. brasiliensis* [45] in faster growing rates of the colonies on all culture media tested, and by the production of smooth-walled to verruculose conidia (coarsely verrucose in *T. brasiliensis*).

Type: Spain: Barcelona province: Sant Sadurní d'Anoia, from sparkling wine, 3 Apr. 2011, J. F. Cano-Lira & A. M. Stchigel (CBS H-23366 – holotype; CBS 144322 = FMR 15656 – ex-type cultures; LSU sequence GenBank LS453299).

Description: Colonies on CYA: reaching 30–32 mm diameter after 2 weeks at 25 °C, slightly elevated, floccose, sulcate, margins entire, pale yellow (4A3), and dawn grey (4D1) at center, white (4A1) to the edge, exudate absent, sporulation sparse; reverse violet brown (10F7) at center and pale brown (7D7) to the edge, diffusible pigment absent. Mycelium: abundant, composed of subhyaline,

smooth- and thin-walled, septate, anastomosing hyphae, of 2-μm wide. Conidiophores: biverticillate, short-stipitate, smooth- and thin-walled, 30–45 μm×2–3 μm; branches hyaline, mostly non-septate, smooth- and thin-walled, 2–3 by stipe, bearing 2–4 conidiogenous cells at the top, cylindrical, 12–20 μm×2–3 μm. Conidiogenous cells: phialidic, smooth- and thin-walled, mostly cylindrical and occasionally slightly slender toward the apex, 7–10×2–3 μm. Conidia: enteroblastic, one-celled, broadly ellipsoidal to globose, pale green to pale brown when young but soon becoming mid brown, smooth-walled but verruculose with the age, 3 μm diameter, in basipetal chains of up to 20. Sexual morphology: not observed on the culture media tested.

Colonies on MEA: reaching 27–30 mm diameter after 2 weeks at 25 °C, flat, floccose, sulcate, irregular margins, yellowish white (4A2), exudate absent, sporulation sparse; reverse greyish yellow (4B5) at center and pale yellow (4A3) to the edge, diffusible pigment absent. Colonies on DG18: reaching 13–14 mm diameter after 2 weeks at 25 °C, flat, velvety to floccose, yellowish white (4A2), exudates absent, sporulation sparse; reverse golden yellow (5B8), diffusible pigment absent. Colonies on OA: reaching 44–46 mm diameter after 2 weeks at 25 °C, flat, velvety, margins entire, olive brown (4F4), exudates absent, abundant sporulation; reverse, diffusible pigment absent. Colonies on YES: reaching 31–32 mm diameter after 2 weeks at 25 °C, slightly elevated, velvety, irregular margins, yellowish white (4A2) at center, and white (5A1) to the edge, exudate absent, sporulation sparse; reverse orange (5A7), diffusible pigment absent. Colonies on CREA: reaching 24–26 mm diameter after 2 weeks at 25 °C, moderately elevated, cottony, grey (5B1), exudate absent, sporulation absent, and acid production absent. Colonies on TWA with sterile cork: olive brown (4E4), exudate absent, sporulation abundant; reverse olive brown (4E4), diffusible pigment absent. Colonies on CYA: at 30 °C, reaching 16–18 mm. The fungus does not grow on CYA at 37 °C.

Other specimens examined: Spain: Barcelona province: Sant Sadurní d'Anoia, from sparkling wine, 3 Apr. 2011, J. F. Cano-Lira & A. M. Stchigel (FMR 15664).

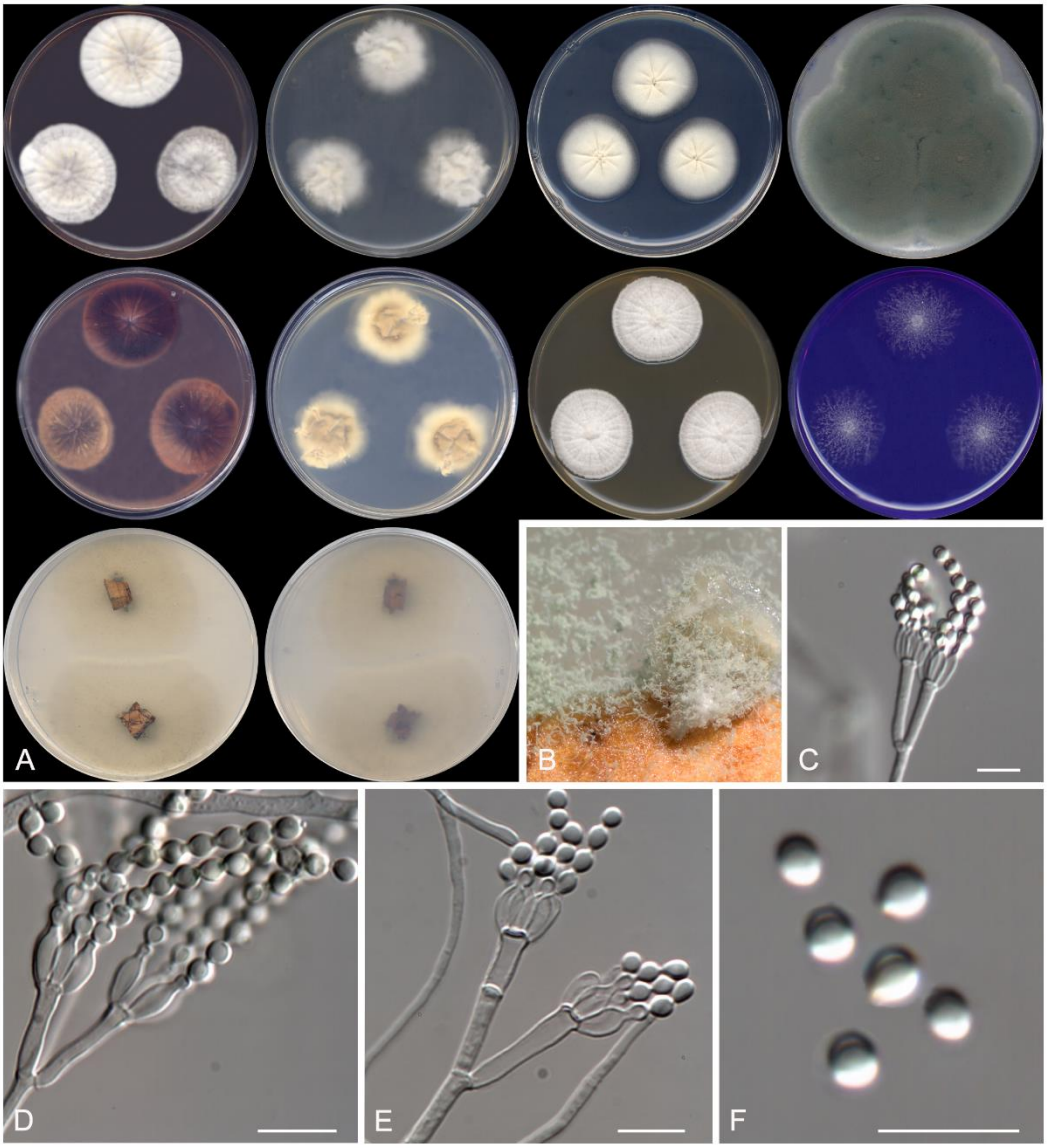

Figure 6. *Talaromyces subericola* CBS 144322. (**A**) Colonies on CYA, MEA, DG18, and OA after 14 days at 25°C, from left to right (top row); reverse of the colonies on CYA and MEA, and surface of the colonies on YES and CREA, from left to right (medium row); surface and reverse of the colony on TWA with cork, from left to right (bottom row). (**B**) Detail of the colony on TWA with cork. (**C**–**E**) Conidiophores. (**F**) Conidia. Scale bar = 10 μm.

Because FMR 16670 and FMR 16675 were placed together in a branch (Figure 3) that is phylogenetically distant from other species of the genus *Rasamsonia*, and due to their phenotypic differences with the other species, *Rasamsonia frigidotolerans* is therefore proposed as a new species.

***Rasamsonia frigidotolerans* Rodr.-Andr., Cano et Stchigel, sp. nov.** MycoBank MB 830608. (Figure 7)

Etymology: From Latin frigus-, cold, and -tolerans, tolerant, in reference to its ability to grow at relatively low temperatures.

Diagnosis: Differing notably from other species of the genus [46–50] by the absence of growth on CYA at 30 °C (after one week incubation, greater than 5 mm diameter in the other species), and by the production of globose conidia (ellipsoidal, ovoid to cylindrical in the rest of the species), with the exception of *R. pulvericola*. However, *R. frigidotolerans* can be easily differentiated from *R. pulvericola* by its production of smooth-walled stipes and branches (verrucose in *R. pulvericola*), and because the conidia are connected by disjunctors (absent in the rest of the species of the genus).

Type: Spain: Barcelona province: Sant Sadurní d'Anoia, from sparkling wine, 3 Apr. 2011, J. F. Cano-Lira & A. M. Stchigel (CBS H-23373 – holotype; CBS 143845 = FMR 16675 – ex-type cultures; LSU sequence GenBank LS453294).

Description: Colonies on CYA: reaching 3–4 mm diameter after 2 weeks at 25 °C, elevated, velvety to floccose, sulcate, margins irregular, pale yellow (4A3), exudate absent, sporulation sparse; reverse pale yellow (4A5), diffusible pigment absent. Mycelium: abundant, composed of hyaline, smooth- and thin-walled when young, becoming slightly verrucose with the age, and septate hyphae, 2 μm wide. Conidiophores: mostly monoverticillate, sometimes biverticillate, stipitate, smooth- and thin-walled, 12–50 μm×1.5–2.5 μm, bearing up to 2 branches at the top; branches hyaline, non-septate, smooth- and thin-walled, 10–15 μm×1.5–2 μm. Conidiogenous cells: phialidic, smooth- and thin-walled, in pressed verticils of 2–5 at the top of the stipe or of the branches, slender toward the apex, cylindrical, 8–14 μm×1.5–2 μm. Conidia: enteroblastic, one-celled, smooth-walled, pale brown, globose, 1–2 μm diameter, in basipetal chains of up to 20 and connected by disjunctors. Sexual morphology: not observed on the culture media tested.

Colonies on MEA: reaching 22–23 mm diameter after 2 weeks at 25 °C, slightly elevated, velvety, irregular margins, greyish yellow (4B4), exudate absent, sporulation abundant; reverse olive brown (4D8), diffusible pigment absent. Colonies on DG18: reaching 5–7 mm diameter after 2 weeks at 25 °C, elevated, velvety to floccose, pale yellow (4A3), exudates absent, sporulation sparse; reverse yellowish orange (4A6), diffusible pigment absent. Colonies on OA: reaching 10–11 mm diameter after 2 weeks at 25 °C, flat, velvety, margins entire, greyish yellow (4C7), exudates absent, abundant sporulation; reverse, diffusible pigment absent. Colonies on YES: reaching 7–9 mm diameter after 2 weeks at 25 °C, elevated, velvety to floccose, irregular margins, yellowish white (4A2) at center, and greyish yellow (4B3) at the edge, exudate absent, sporulation abundant; reverse orange yellow (4B8), diffusible pigment absent. Colonies on TWA with sterile cork: greyish yellow (3B7), exudate absent, sporulation abundant; reverse greyish yellow (3B7), diffusible pigment absent. Growth on CYA at 30 °C and on CREA at 25 °C: absent. Minimum, optimal, and maximum temperature of growth: 15 °C, 25 °C, and 35 °C, respectively.

Other specimens examined: Spain: Barcelona province: Sant Sadurní d'Anoia, from sparkling wine, 3 Apr. 2011, J. F. Cano-Lira & A. M. Stchigel (FMR 16670).

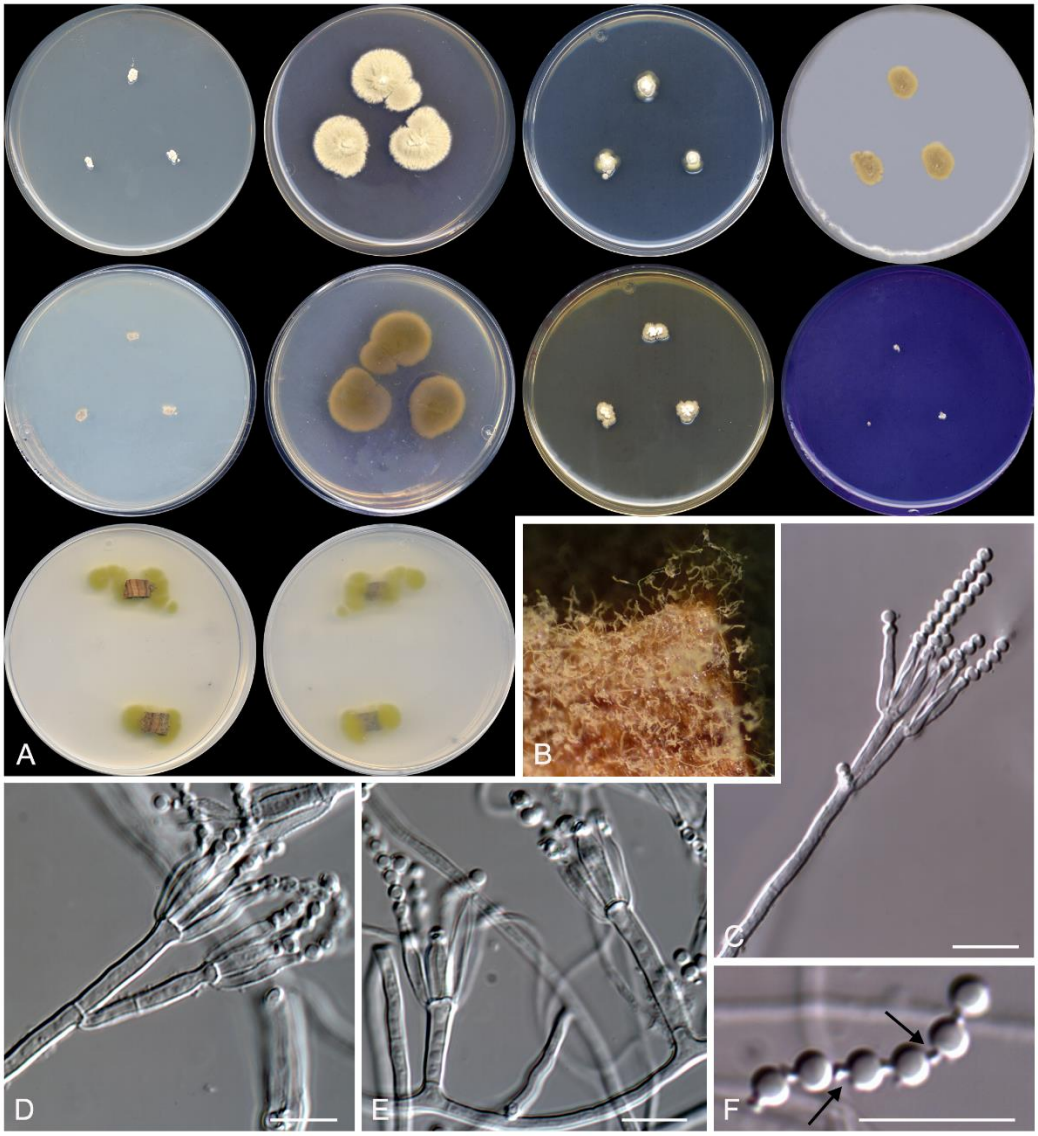

Figure 7. *Rasamsonia frigidotolerans* CBS 143845. (**A**) Surface of the colonies on CYA, MEA, DG18, and OA after 14 days at 25 °C, from left to right (top row); reverse of the colonies on CYA and MEA, and surface of the colonies on YES and CREA, from left to right (medium row); surface and reverse of the colony on TWA with cork, from left to right (bottom row). (**B**) Detail of the colony on TWA with cork. (**C**–**E**) Conidiophores. (**F**) A chain of conidia. The arrow shows a disjunctor between conidia. Scale bar = 10 μm.

3.4.2. Subclade B: Onygenales

Because *Arthrographis pinicola* is phylogenetically placed far from the type species of the genus *Arthrographis* (*Arthrographis kalrae*), which is located within the family Eremomycetaceae (class Dothideomycetes), and because of the morphological differences with *Eremascus albus*, we introduce the new genus *Dactylodendron* into the family Eremascaceae (order Onygenales, class Eurotiomycetes) (Figure 1), and design *Dactylodendron pinicola* (formerly *Arthrographis pinicola*) as the type species of the genus.

***Dactylodendron Stchigel*, Rodr.-Andr. et Cano, gen. nov.** MycoBank MB 827858.

Etymology: From Greek δάχτυλο-, finger, and -δένδρον, tree, due to the aspect of the conidiophores.

Diagnosis: Recognized by its hyaline, hyphae-like, successively branched conidiophores, or short-stalked conidiophores ending in a verticilate arrangement of fertile branches. In both cases, fertile branches produce hyaline, cylindrical, or cuboid arthroconidia.

Type species: *Dactylodendron pinicola* (Sigler & Yamaoka) Rodr.-Andr., Cano et Stchigel. Mycobank MB 827859.

Description: Colonies: slow-growing at room temperature, always with shades of yellow. Conidiophores semi-macronematous, hyphae-like, single or grouped in discrete dome-shaped or floccose conidiomata, erect, successively branched, or short-stalked, ending in a verticilate arrangement of fertile branches, fertile branches eventually producing arthroconidia. Arthroconidia: hyaline, smooth-walled, usually truncate at both ends, cylindrical or cuboid, produced by transverse septation in basipetal order, separated very late by schizolytic secession from the conidiogenous branches, without disjunctors or separating cells. Chlamydospores: occasionally seen. Sexual morphology: not observed.

***Dactylodendron pinicola* (Sigler & Yamaoka) Rodr.-Andr., Cano et Stchigel, comb. nov.** MycoBank MB 827859.

Basionym: Arthrographis pinicola Sigler & Yamaoka, Canadian Journal of Microbiology 36: 78 (1990) [MycoBank MB 126499].

Description: Hyphae: septate and hyaline, (0.5–) 0.8–2.5 μm wide, bearing narrow conidiophores which branch repeatedly to form floccose conidiomata. The fertile branches are initially sparsely septate and of uniformly narrow diameter, but, as arthroconidial development begins, the apical region broadens and septation occurs in basipetal sequence to form many small cells. Arthroconidia secede by schizolysis, often remaining connected in chains of 3 to 4, which then undergo further schizolysis. There are no disjunctors or separating cells. Mature arthroconidia are smooth, hyaline, tan in mass, cylindrical, but often broader than long, 1.5–4.0×1.5–2.5 μm wide. Teleomorph was not observed. No yeast stage was observed.

Notes: The habitat reported for such fungus is wood of *Pinus contorta* var. *latifolia*, especially in galleries and adult beetles of Ips latidens, and from galleries of *Dendroctonus ponderosae* in Alberta, Canada.

Because the genus *Dactylodendron* was placed into the subclade B (Figure 1), and was divided into three different sister branches comprising *D. pinicola*, FMR 16678, FMR 15658, and FMR 16677, and because of the morphological differences among them, the new species *Dactylodendron ebriosum* and *Dactylodendron pluriseptatum* are proposed as follows:

***Dactylodendron ebriosum* Rodr.-Andr., Cano et Stchigel, sp. nov.** MycoBank MB827862. (Figure 8)

Etymology: From Latin ebrios, drunk, due to the habitat of this fungus.

Diagnosis: Morphologically resembling to *Staheliella nodosa*, because of the aspect of the upper part of the conidiophores and the sort of conidiogenesis. However, *S. nodosa* clearly differs from *D. ebriosum* because the former is a dematiaceous fungus with longer and wider conidiophores, which also display a percurrent development, bearing several fertile loci along them (the unique fertile part of *D. ebriosum* conidiophores are at the tip of the same).

Type: Spain: Barcelona province: Sant Sadurní d'Anoia, from a cork stopper, 03 June 2011, J. F. Cano-Lira & A. M. Stchigel (CBS H-23367 – holotype; CBS 144321 = FMR 15658 – ex-type cultures; LSU sequence GenBank LT985880).

Description: Colonies on PDA: reaching 10–11 mm diameter after 2 weeks at 25 °C, velvety to floccose, slightly elevated, margins slightly irregular, reddish yellow (4A6) at center, vivid yellow (3A8) and white (3A1) to the edge, exudate absent, sporulation sparse; reverse yellowish-white (3A2), diffusible pigment absent. Mycelium: composed of septate, hyaline, smooth- and thin-walled, 1–2 μm wide hyphae. Conidiophores: macronematous, erect, hyaline to slightly yellow, 1–2-septate, smooth-walled to verrucose (especially at the base), 20–30×2–5 μm, slightly tapering toward the fertile apex, which consists in a verticillate arrangement of 5 to 15 fertile branches. Fertile branches: single, hyaline, smooth- and thin-walled, cylindrical but rounded at the end, up to 20 μm in length, 1–1.5 μm wide, forming septa basipetally to produce 3 to 5 arthroconidia released via schizolythic secession. Arthroconidia: hyaline, smooth- and thin-walled, 2–4 μm×1.25–2 μm. Chlamydospores and sexual morphology: not observed.

Colonies on MEA: reaching 6–7 mm diameter after 2 weeks at 25 °C, velvety, slightly elevated, margins regular, orange white (5A2) at center, and white (3A1) to the edge, exudate absent, sporulation sparse; reverse pale yellow (4A3), diffusible pigment absent. Colonies on tap water agar (TWA) with sterile cork: reddish yellow (4A6), exudate absent, sporulation abundant; reverse orange (6B7), diffusible pigment absent.

Other specimens examined: Spain: Barcelona province: Sant Sadurní d'Anoia, from a sparkling wine sample, 03 June 2011, J. F. Cano-Lira & A. M. Stchigel (FMR 16677).

Notes: Despite *S. nodosa* remains as incertae sedis, a Blast search using the ITS sequence available at the GenBank/EMBL databases (data not shown) has placed this fungus phylogenetically close to members of the order Helotiales (class Leotiomycetes), a taxon phylogenetically far from the order Onygenales (class Eurotiomycetes), where the genus *Dactylodendron* is located.

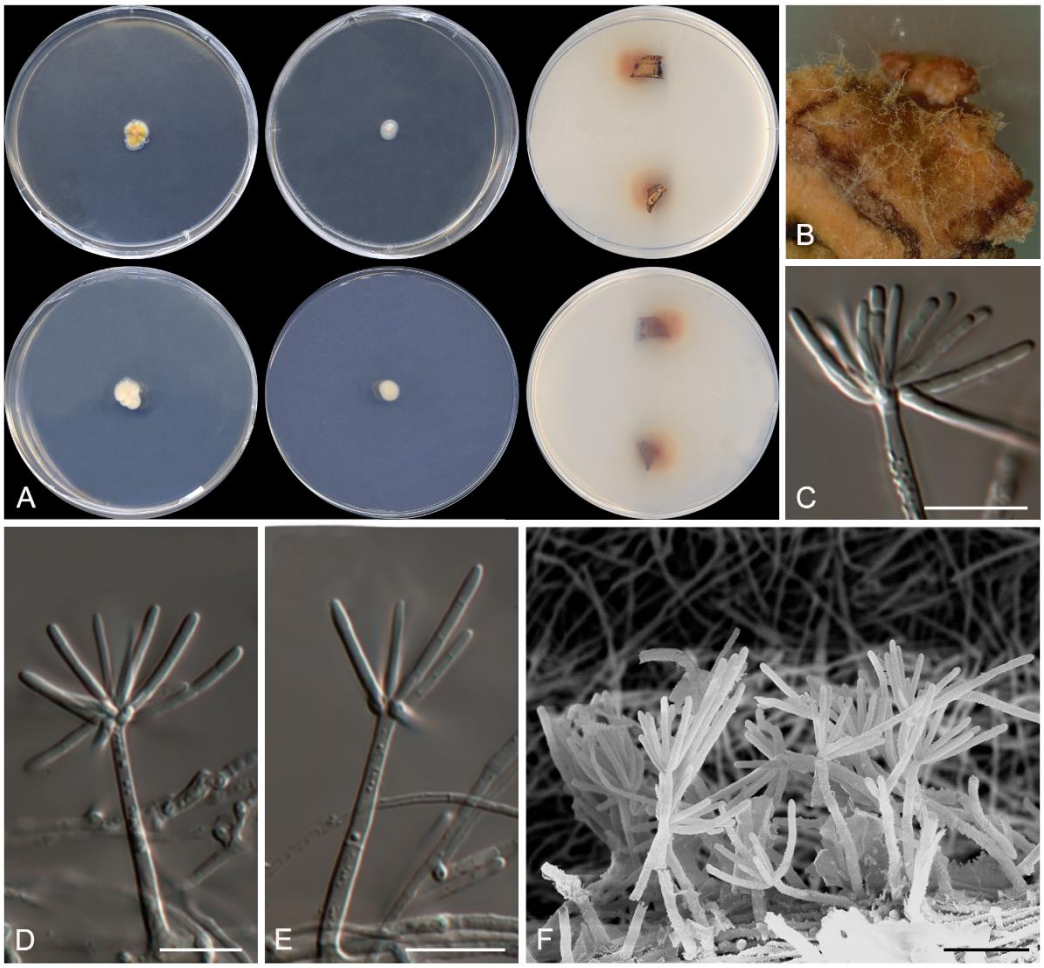

Figure 8. *Dactylodendron ebriosum* CBS 144321. (**A**) Colonies on potato dextrose agar (PDA), MEA, and TWA with cork after 14 days at 25 °C, from left to right (top row, surface; bottom row, reverse); (**B**) Detail of the colony on TWA with cork. (**C**–**E**) Conidiophores and conidia. (**F**) Conidiophores under SEM. Scale bar = 10 μm.

Dactylodendron pluriseptatum **Rodr.-Andr., Cano et Stchigel, sp. nov.** MycoBank MB 827863. (Figure 9)

Etymology: From Latin pluri-, many, and -septatum, septate, due to the presence of many septa along the fertile branches.

Diagnosis: Characterized by the production of successively branched conidiophores, whose verticillate arrangement of fertile branches develop long chains of disarticulating, prismatic arthroconidia. These conidiophores are similar to those of *D. pinicola*, but *D. pluriseptatum* never produce conidiomata, which is seen in *D. pinicola*.

Type: Spain: Barcelona province: Sant Sadurní d'Anoia, from sparkling wine, 03 June 2011, J. F. Cano-Lira & A. M. Stchigel (CBS H-23374 – holotype; CBS 143846 = FMR 16678 – ex-type cultures; LSU sequence GenBank LT985882).

Description: Colonies on PDA: reaching 9–10 mm diameter after 2 weeks at 25 °C, floccose, slightly elevated, margins slightly irregular, orange (5A7) at center and light orange (5A5) at edge, exudate absent, sporulation abundant; reverse deep orange (5A8), diffusible pigment absent. Mycelium: composed of septate, hyaline, smooth- and thin-walled, 1–1.5-µm wide hyphae. Conidiophores: semi-macronematous, erect, hyaline, smooth- and thin-walled, 50–75×1–1.5 µm, septate, repeatedly branched, fertile branches in terminal verticillate arrangements of 2–5. Fertile branches: hyaline, thin- and smooth-walled, cylindrical but rounded at the end, 15–45×1–1.5 µm, producing septa basipetally for delimitation of arthroconidia, which are very late released in chains or individually from the fertile branch via schizolythic secession. Arthroconidia: in long chains (up to 15 elements), hyaline, smooth- and thin-walled, cylindrical to almost cuboid, 2–4 µm×1–1.5 µm. Chlamydospores: hyaline, one-celled, smooth- and thick-walled, irregularly globose, 5–7 µm diameter, arising laterally on the vegetative hyphae. Sexual morphology: not observed.

Colonies on MEA: reaching 9–10 mm diameter after 2 weeks at 25 °C, floccose, elevated, margins irregular, pale yellow (4A4), exudate absent, sporulation sparse; reverse greyish orange (5B6), diffusible pigment absent. Colonies on TWA with sterile cork: hairy brownish orange (6C8), exudate absent, sporulation abundant; reverse orange (6B8), diffusible pigment absent.

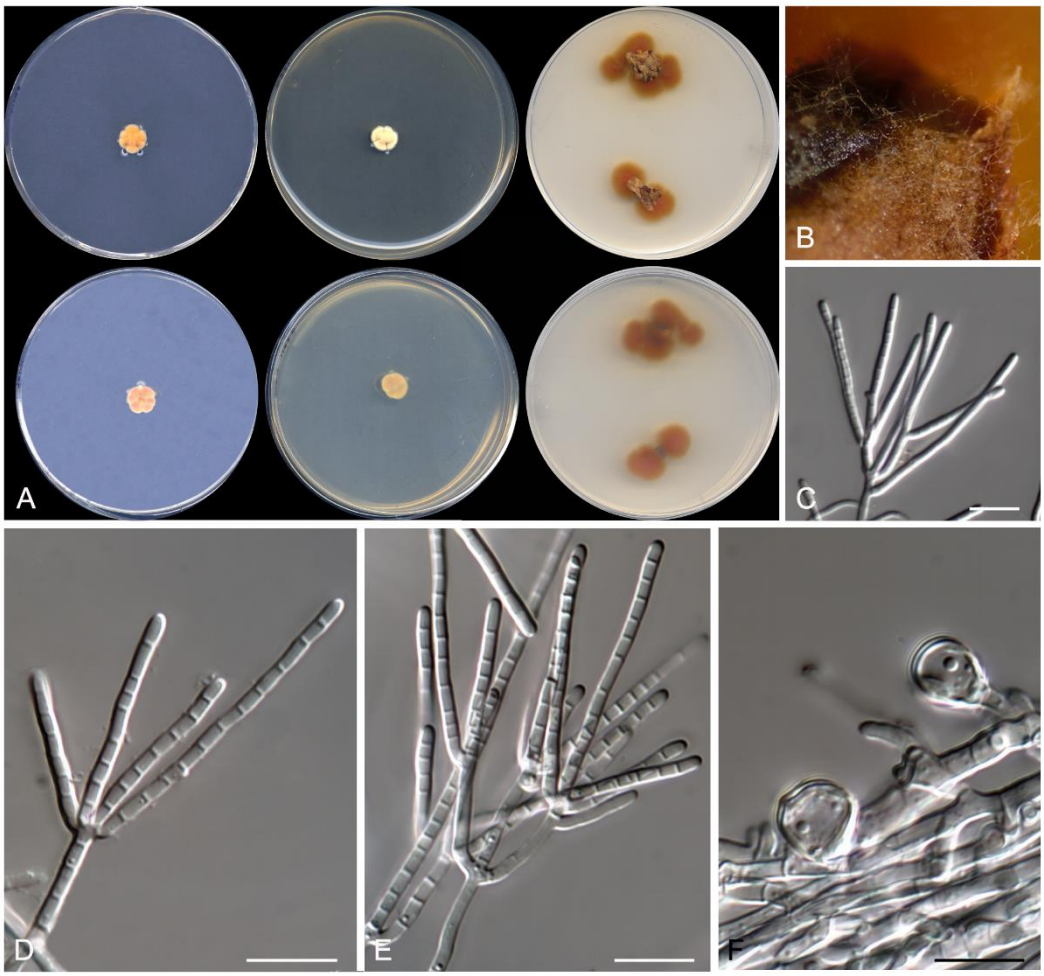

Figure 9. *Dactylodendron pluriseptatum* CBS 143846. (**A**) Colonies on PDA, MEA, and TWA with cork after 14 days at 25°C, from left to right (top row, surface; bottom row, reverse); (**B**) Detail of the colony on TWA with cork. (**C**–**E**) Conidiophores and conidia. (**F**) Chlamydospores. Scale bar = 10 µm.

3.4.3. Subclade C: Chaetothyriales

Because our strain FMR 16667 was placed within subclade C (Figure 1) corresponding to the species of the genus *Cladophialophora*, in a terminal branch (Figures 1 and 4) together with *Cladophialophora mycetomatis*, and because FMR 16667 displays enough phenotypic and phylogenetic differences from the latter and from other species of the genus, we propose *Cladophialophora recurvata* as a new species, described as follows.

Cladophialophora recurvata **Rodr.-Andr., Cano et Stchigel, sp. nov.** MycoBank MB 830605. (Figure 10)

Etymology: From Latin recurvatis, recurved, because the presence of coiled hyphae.

Diagnosis: Forming a terminal clade with *C. mycetomatis*, species placed within clade II of Cladophialophora s. str. (Figure 1) [51]. *Cladophialophora recurvata* differs from the latter by its production of bigger conidia (4–8×3–7 μm versus 2.5–4×2–3 μm), which are also broadly ellipsoidal to subglobose in the former and fusiform to broadly fusiform in *C. mycetomatis*, and by the inconspicuous flattened scars (much more evident in the rest of the species of the genus).

Type: Spain: Barcelona province: Sant Sadurní d'Anoia, from sparkling wine, 3 Apr. 2011, J. F. Cano-Lira & A. M. Stchigel (CBS H-23380 – holotype; CBS 143843 = FMR 16667 – ex-type cultures; LSU sequence GenBank LT985879).

Description: Colonies on MEA: reaching 21–22 mm diameter after 2 weeks at 25 °C, flat, felted, regular margins, greyish brown (5E3) at center, and olive (2F3) to the edge, exudate absent, sporulation abundant; reverse olive (2F3), diffusible pigment absent. Mycelium: composed by septate, smooth- and thin-walled, pale olivaceous hyphae, 3–4 μm wide, locally forming abundant coils from which arise most of the conidiophores. Conidiophores: micronematous, indistinguishable from the vegetative hyphae. Conidiogenous cells: mono- to polyblastic, determinate, integrated to the hyphae or discrete, in this case, ampuliform to barrel-shaped, 5–8×4–5 μm, arising from hyphae or the coils. Conidia: holoblastic, one-celled, pale olivaceous to pale olivaceous-brown, thin- and smooth-walled to verrucose, broadly ellipsoidal to subglobose, 4–8×3–7 μm, disposed in long, branched acropetal chains, with one or two inconspicuous, flattened scars of up to 3-μm wide, of the same color than the rest of the conidium; ramoconidia one-celled, cylindrical to nearly so, one-celled, in chains up to 3, 5–15×2–3 μm. Budding cells, chlamydospores, muriform cells, synanamorph, and sexual morphology: not observed.

Colonies on PDA: reaching 21–22 mm diameter after 2 weeks at 25 °C, flat, regular margins, greyish brown (5E3) at center, and olive (2F3) to the edge, exudate absent, sporulation abundant; reverse olive (2F3), diffusible pigment absent. Colonies on OA: reaching 20–21 mm diameter after 2 weeks at 25 °C, flat, felted, regular margins, olive grey (3D2) at center, and olive (2F3) at edge, exudate absent, sporulation sparse; reverse olive (2F3), diffusible pigment absent. Colonies on TWA with sterile cork: olive brown (4F3), exudate absent, sporulation abundant; reverse olive brown (4F3), and diffusible pigment absent.

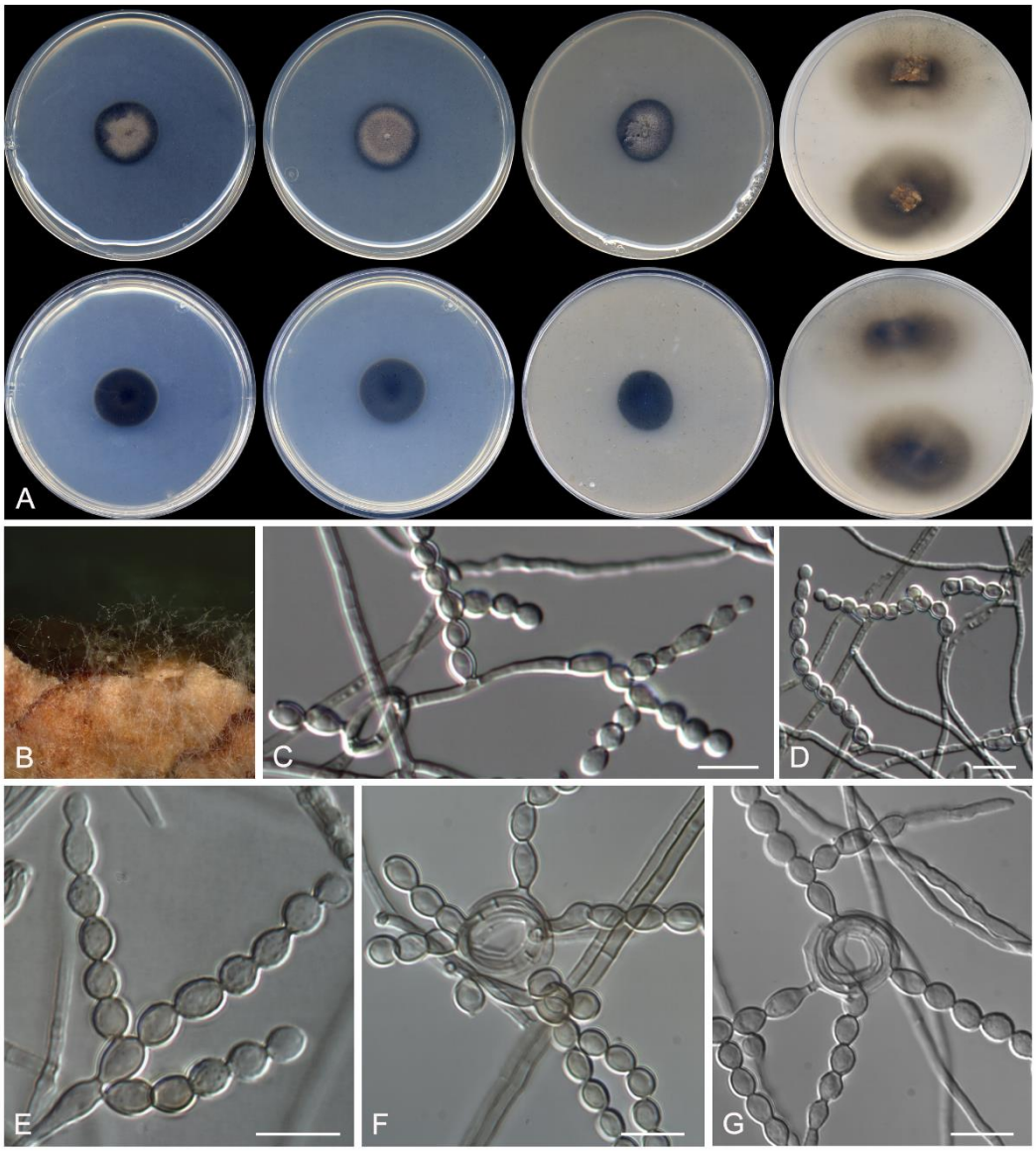

Figure 10. *Cladophialophora recurvata* CBS 143843. (**A**) Colonies on PDA, MEA, OA, and TWA with cork after 14 days at 25°C, from left to right (top row, surface; bottom row, reverse). (**B**) Detail of the colony on TWA with cork. (**C**–**G**) Conidiophores and conidia. Scale bar = 10 μm.

3.4.4. Subclade D: Pleosporales

Because FMR 15668 and FMR 16668, and FMR 16665 and FMR 16666, were placed within two independent sister branches (100% BS / 1 PP each one) in one terminal clade (81% BS / 0.98) of the sister clade D1 (Figure 1), and because these strains display enough morphological differences with respect to the other species of the genus, we propose the erection of the new species *Kirschsteiniothelia ebriosa* and *Kirschsteiniothelia vinigena*, which are described as follows:

Kirschsteiniothelia ebriosa **Rodr.-Andr., Cano et Stchigel, sp. nov.** MycoBank MB 830603. (Figure 11)

Etymology: From Latin ebrios, drunk, due to the habitat of this fungus.

Diagnosis: Resembling more a species of the genus *Diplococcium* than a *Kirschsteiniothelia*'s asexual morphology (= Dendryphiopsis), because of the acropetal chains of 1-septate conidia produced laterally along the conidiophore. (These are multiseptate and solitary on the branches arising at the top of the conidiophore in *Kirschsteiniothelia*.) However, *K. ebriosa* falls within the same clade as other *Kirschsteiniothelia* spp., which is placed far away from *Diplococcium* spp. (data not

shown). *Kirschsteiniothelia ebriosa* is distinguishable from all other species of the genus by its production of (mostly) 1-septate small conidia in branched acropetal chains, laterally on the stipe of the conidiophore.

Type: Spain: Barcelona province: Sant Sadurní d'Anoia, from sparkling wine, 03 June 2011, J. F. Cano-Lira & A. M. Stchigel (CBS H-23379 – holotype; CBS 143842 = FMR 16666 – ex-type cultures; LSU sequence GenBank LT985884).

Description: Colonies on MEA: reaching 28–31 mm diameter after 2 weeks at 25 °C, floccose, slightly elevated, regular margins, yellowish brown (5E4) at center, and blackish-olive (2G6) at edge, exudate absent, abundant sporulation; reverse blackish olive (2G6), diffusible pigment absent. Mycelium: abundant, composed of dark brown, septate, smooth-and thin-walled, 4-μm wide hyphae. Conidiophore: macronematous, consisting of a straight or slightly sinuous, erect, dark brown, septate, thin- and smooth-walled to slightly verrucose stipe, 40–150×4 μm, bearing a few lateral branches and occasionally one branch at the top; branches are brown, thin- and smooth-walled, 1–5-septate, cylindrical, 30–50×4 μm, with rounded ends. Conidiogenous cells: mono- to polytretic, integrated to the stype, to the branches and to the conidia, intercalary or terminal, determinate, cylindrical. Conidia: holoblastic, brown to dark brown, thin- and smooth-walled, 1–2(–5)-septate, sometimes solitary, mostly in branched acropetal chains of up to 5, cylindrical with rounded ends, 8–14×4–5 μm, sometimes slightly constricted at septum, produced laterally and terminally on the stipe, on the branches and on the conidia. Chlamydospores and sexual morphology: not observed.

Colonies on PDA: reaching 26–28 mm diameter after 2 weeks at 25 °C, floccose, slightly elevated, regular margins, pitch black (5H2) at center, grey (8E1) to the edge, exudate absent, sporulation sparse; reverse blackish olive (2G6), diffusible pigment absent. Colonies on OA: reaching 20–21 mm diameter after 2 weeks at 25 °C, floccose, slightly elevated, regular margins, black (18G2) at center, grey (5E1) to the edge, exudate absent, sporulation abundant; reverse blackish violet (15G8), diffusible pigment absent. Colonies on TWA with sterile cork after 2 weeks at 25 °C: blackish olive (2G6), exudate absent, sporulation abundant; reverse blackish olive (2G6), diffusible pigment absent.

Other specimens examined: Spain: Barcelona province: Sant Sadurní d'Anoia, from sparkling wine, 03 June 2011, J. F. Cano-Lira & A. M. Stchigel (FMR 16665).

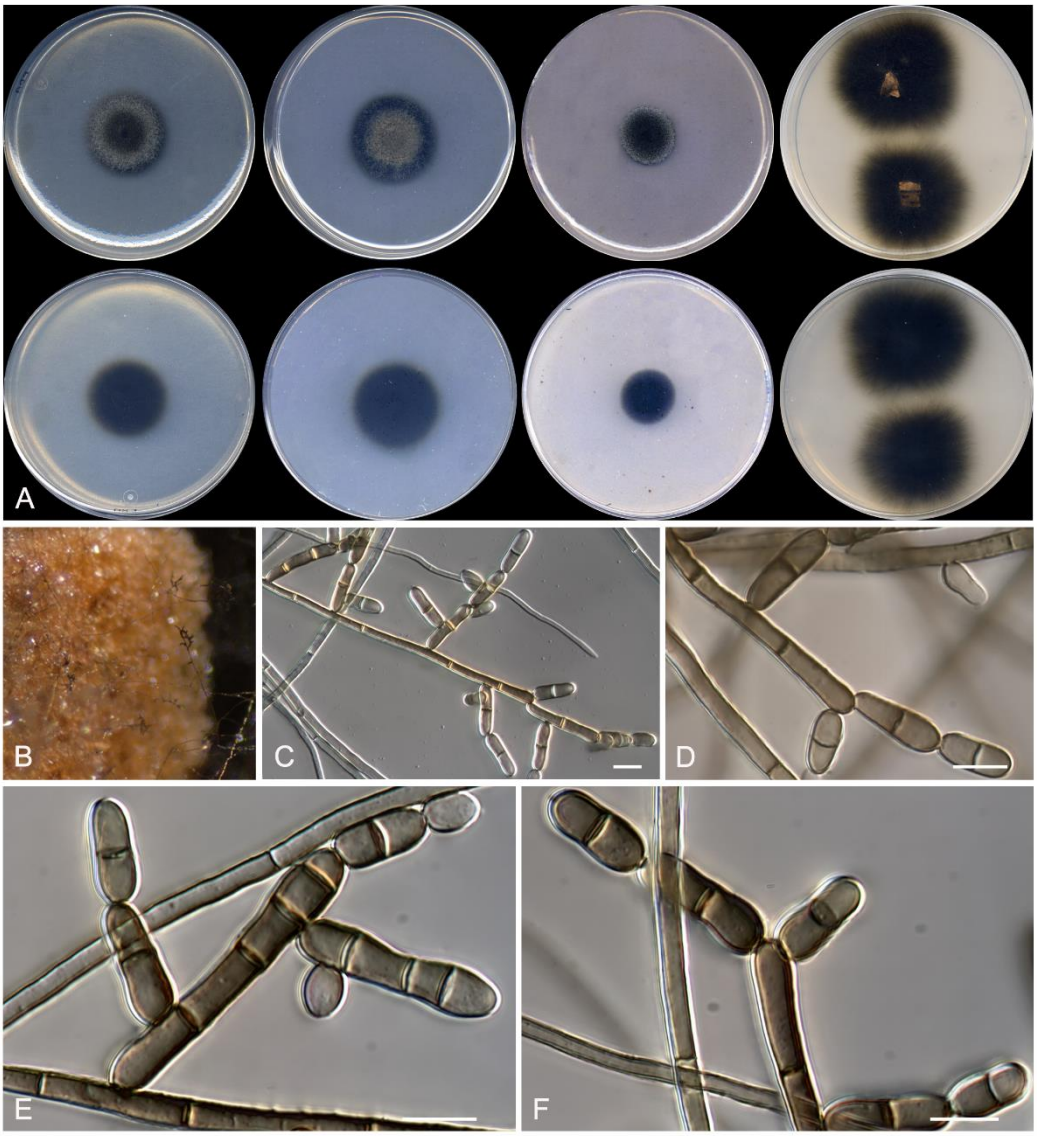

Figure 11. *Kirschteiniothelia ebriosa* CBS 143842. (**A**) Colonies on PDA, MEA, OA, and TWA with cork after 14 d at 25 °C, from left to right (top row, surface; bottom row, reverse). (**B**) Detail of the colony on TWA with cork. (**C**–**F**) Conidiophores and conidia. Scale bar = 10 μm.

Kirschsteiniothelia vinigena **Rodr.-Andr., Cano et Stchigel, sp. nov.** MycoBank MB 830604. (Figure 12)

Etymology: From Latin vinum, wine, because the origin of the fungus.

Diagnosis: Distinguished from other species of the genus because of its production of ornamented (verrucose) stipes, branches and conidia (smooth-walled or nearly so for the other of the species), and by the production of complex system of branches (not reported for the other species).

Type: Spain: Barcelona province: Sant Sadurní d'Anoia, from cork stopper, 03 June 2011, J. F. Cano-Lira & A. M. Stchigel (CBS H-23378 – holotype; CBS 143837 = FMR 15668 – ex-type cultures; LSU sequence GenBank LT985883).

Description: Colonies on MEA: reaching 23–24 mm diameter after 2 weeks at 25 °C, floccose, slightly elevated, regular margins, blackish grey (2G1) at center, blackish olive (2G6) at the middle part, and grey (5E1) at the edge, exudate absent, sporulation sparse; reverse blackish red (10H8), diffusible pigment absent. Mycelium: abundant composed of brown, smooth- and thin-walled, septate hyphae, 2–3 μm wide. Conidiophore: macronematous, consisting in a straight or slightly sinuous, erect, dark brown, septate, thin- and smooth-walled to coarsely verrucose (specially at the base) stipe, 100–150×3 μm, bearing several lateral branches, which also branch irregularly; branches

are abundant, brown, thin- and smooth-walled to coarsely verrucose (specially at the base), non-septate to 7-septate, barrel-shaped to cylindrical, 5–80×4 μm, with rounded ends. Conidiogenous cells: mono- to polytretic, integrated to the stype, to the branches and to the conidia, intercalary or terminal, determinate, and cylindrical. Conidia: holoblastic, dark brown, thin- and smooth-walled to coarsely verrucose, 1–2(–7)-septate, sometimes solitary, mostly disposed in branched acropetal chains of up to 4, cylindrical to scolecoid, 8–80 x× 4–5 μm, with rounded ends, sometimes slightly constricted at septa, produced laterally and terminally on the stipe, on the branches, and on other conidia. Chlamydospores and sexual morphology: not observed.

Colonies on PDA: reaching 24–25 mm diameter after 2 weeks at 25 °C, floccose, slightly elevated, regular margins, pitch black (5H2) at center, with consecutive blackish olive (2G6) and grey (5E1) rings, and pitch black (5H2) at the edge, exudate absent, sporulation sparse; reverse blackish olive (2G6), diffusible pigment absent. Colonies on OA: reaching 14–15 mm diameter after 2 weeks at 25 °C, floccose, slightly elevated, regular margins, ink black (18G2) at center, grey (5E1) to the edge, exudate absent, sporulation sparse; reverse blackish olive (2G6), diffusible pigment absent. Colonies on TWA: with sterile cork, blackish olive (2G6), exudate absent, sporulation sparse; reverse blackish-olive (2G6), diffusible pigment absent.

Other specimens examined: Spain: Barcelona province: Sant Sadurní d'Anoia, from sparkling wine, 03 June 2011, J. F. Cano-Lira & A. M. Stchigel (FMR 16668).

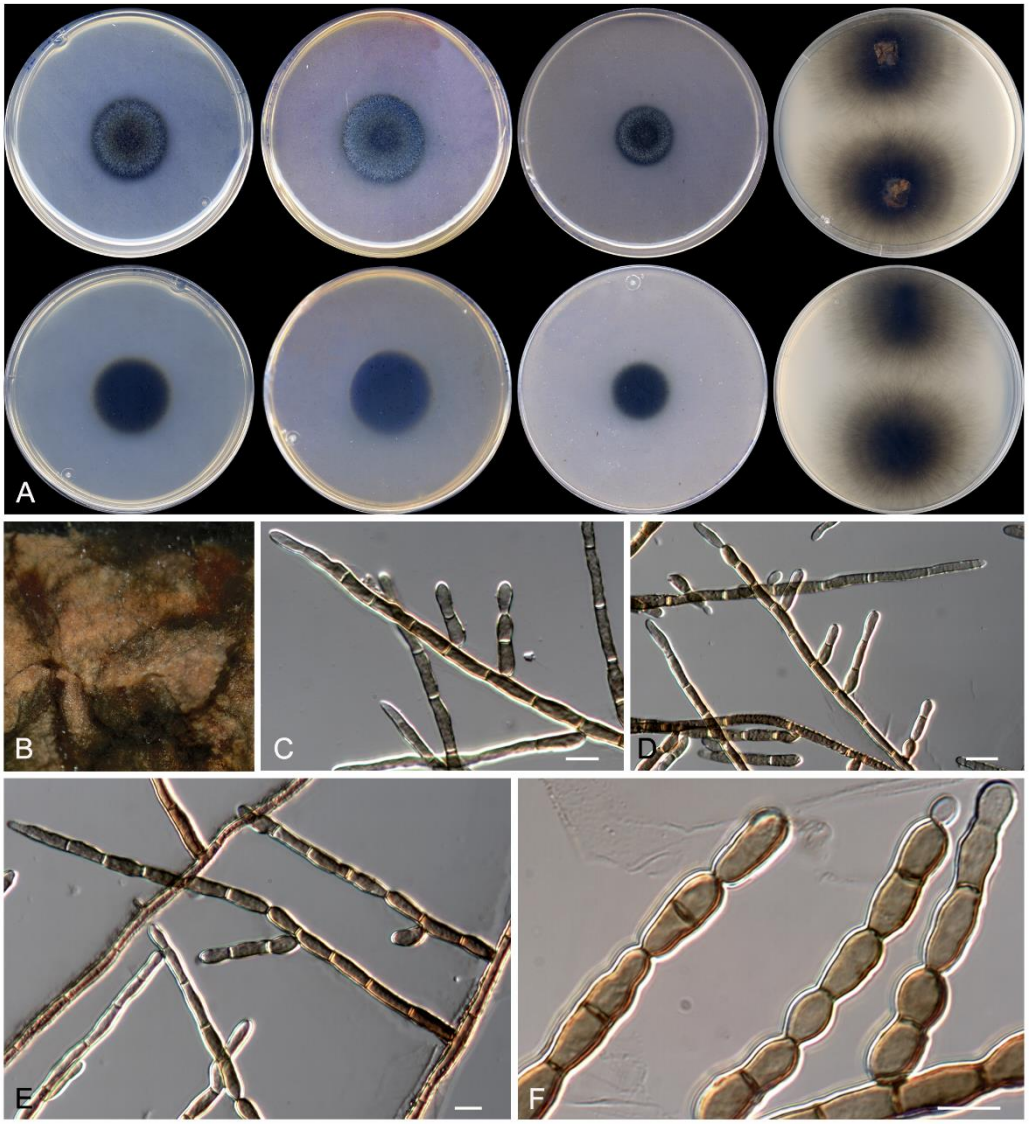

Figure 12. *Kirschteiniothelia vinigena* CBS 143837. (**A**) Colonies on PDA, MEA, OA, and TWA with cork after 14 days at 25 °C, from left to right (top row, surface; bottom row, reverse). (**B**) Detail of the colony on TWA with cork. (**C**–**F**) Conidiophores and conidia. Scale bar = 10 μm.

4. Discussion

Candida patagonica was the only yeast retrieved in our study. This fungus has previously been reported from fermentation vats and oak barrels in the cellars of North Patagonia, Argentina [52]. Despite the ascomycetous yeasts such as *Dekkera bruxellensis*, *Hanseniaspora uvarum*, *Issatchenkia orientalis*, *Metschnikowia pulcherrima*, and some species of the genera *Candida* and *Zygosaccharomyces* [53,54] having been involved in the deterioration of wines, *C. patagonica* has not been reported as a spoilage organism for these sorts of alcoholic beverages.

The isolates belonging to the order Eurotiales showed the broadest fungal diversity, being distributed among the genera *Aspergillus*, *Penicillium*, *Rasamsonia* and *Talaromyces*. This latter genus was the most frequently recovered from both sorts of substrata, and two of them, particularly from sparkling wine, are new species, i.e., *Talaromyces speluncarum*, characterized by mostly biverticillate conidiophores and brown, spinose to verrucose, globose conidia, and *Talaromyces subericola*, which grows faster than *T. speluncarum* and produces smooth-walled conidia (spinose to verrucose in *T. speluncarum*). *Aspergillus*, another common genus in sparkling wine, was represented by *A. aureolatus*, *A. jensenii*, and *A. puulaauensis*, all of them pertaining to the section *Nidulantes* [55]. *Aspergillus aureolatus* [56] was originally isolated from air in Serbia, *A. jensenii* [57] from soil in the USA, and *A. puulaauensis* [57] from dead hardwood in the Hawaiian archipelago. There have not been any reports of these three species found in wines. *Penicillium corylophilum* [58] was isolated from sparkling wine samples. This taxon was reported mostly in damp buildings in North America and West Europe, but also from foods and from mosquitoes [59,60], vineyards, grape must, fermentation wine, and fruit juices [11,61,62]. Previously, we had isolated this fungus from the environment of the cellars where the bottles containing the sparkling wine were aging (data not published). Consequently, finding *P. corylophilum* might be due to the bottle not being sufficiently sealed by the cork stopper. A new species of *Rasamsonia*, *Rasamsonia frigidotolerans*, was found in the wine samples. The genus is characterized by the production of ornamented, paecilomyces-like conidiophores and olive-brown conidia, and in four of the species, the production of ascomata have been reported. *Rasamsonia* spp. have been reported in Asia, Europe, and North America, from substrata such as compost, conifer wood chips, cow dung, house dust, indoor air, piles of peat, rice straw, seed of *Piper nigrum*, soil, and human and animal clinical specimens [47,48,63,64]. None of the previous studies have reported this genus either in wine or on cork stoppers. *Rasamsonia* species are thermotolerant or thermophilic, with an optimum growth temperature above 30 °C and a maximum above 45 °C [65,66]. *Rasamsonia frigidotolerans* is characterized by the production of smooth-walled conidiophores (verrucose in all other species of the genus), by an absence of growth on CYA at 30 °C (all other species are thermotolerant), and by the production of globose conidia connected by disjunctors (absent in the rest of the species of the genus).

The new genus *Dactylodendron*, phylogenetically closely related to the order Onygenales, is characterized by its branched conidiophores and the production of chains of arthroconidia. The type species, *Dactylodendron pinicola*, is an asexual fungus previously classified phenotypically within the genus *Arthrographis*. It was originally isolated from insect galleries and from adult beetles of *lps latidens*, and of *Dendroctonus ponderosae* in *Pinus contorta* var. *latifolia* in Canada [67]. *Dactylodendron pinicola* produces conidiomata (absent in the other two species of the genus), whereas *D. pluriseptatum* produces long chains of prismatic arthroconidia, and *D. ebriosum* forms conidiophores that produce fertile branches at the apex.

In our study, we found a few isolates morphologically similar to the genus *Dendryphiopsis*, which in a polyphasic study demonstrated to be new species of *Kirschsteiniothelia*. This genus demonstrated to be phylogenetically related to the anamorphic genus *Dendryphiopsis* [68,69]. Species of *Kirschsteiniothelia* / *Dendryphiopsis* have been isolated principally from decaying wood and leaves [70–72], and even in freshwater habitats [73–75], but never from sparkling wine or cork stoppers.

Kirschsteiniothelia ebriosa and *K. vinigena* differ from the other species of the genus by the absence of a sexual morphology, and the conidia arising in chains directly from the main axis of the conidiophore. These two species can be distinguished each from other by the number of septa and the length of the conidia (mostly two-celled and short in *K. ebriosa*, and multi-celled and long in *K. vinigena*).

We also isolated an interesting strain of *Cladophialophora* from sparkling wine. *Cladophialophora recurvata* produces aseptate, broadly ellipsoidal to subglobose, relatively large conidia, with inconspicuous flattened scars (more evident in the other species of the genus). The species of the genus *Cladophialophora* have been never reported from wine or cork.

Other species isolated during our study were *Alternaria alternata* and *Cladosporium cladosporioides*, both from cork stoppers. There are a few reports of *A. alternata* in grape must from the Priorat region in Spain and from Douro in Portugal [11,61]. The species of the genus *Alternaria* infects a broad variety of living plants, but can also be recovered from plant debris [76]. *Cladosporium oxysporum* (but not *C. cladosporioides*) has been isolated previously from cork stoppers [5]. *Cladosporium* species are found worldwide, and frequently occur as a secondary invader of necrotic parts of different sort of plants, but also are easily recovered from air, soil, textiles, and numerous other substrata [77]. *Beauveria bassiana*, a well-known entomopathogenic fungus [78], was isolated once from a sample of sparkling wine. This fungus is usually found in soil [19], but is also known to be endophytic in living plants including grapevine [79].

Because the new fungal species failed to proliferate at ethanol concentrations ≥ 10% v/v, we consider them to be from a different origin than the grape must, probably the cellar and/or the cork stoppers, despite some of them being recovered only from sparkling wine samples. We are hopeful that future studies will allow us to discover whether these fungal strains produce 2,4,6-TCA and other compounds responsible for cork taint.

5. Conclusions

The presence of yeasts and molds (and occasionally of bacteria) was detected in several (24 out of 39) samples of sparkling wine (Catalonian cava) affected by cork taint, with a musty, or mouldy, off-odor, and/or flavor alteration that makes the wine undrinkable. On the other hand, all negative controls (without appreciable organoleptic alteration) were free of fungi. All cork stoppers from negative controls and deteriorated wine developed fungal colonies. We isolated 27 different fungi from both substrata. Among them, we found a new genus (*Dactylodendron*) and eight new species (*Cladophialophora recurvata*, *Dactylodendron ebriosum*, *Dactylodendron pluriseptatum*, *Kirschteiniothelia ebriosa*, *Kirschteiniothelia vinigena*, *Rasamsonia frigotolerans*, *Talaromyces speluncarum* and *Talaromyces subericola*). All fungal taxa were able to grow on cork, but only at alcohol concentrations ≤ 10% v/v (which is lower than 11.5% strength of Catalan sparkling wines). We therefore conclude that the fungi present in sparkling wine were also present in turn on the cork stoppers and/or are part of the environment of the cellar. Although *Penicillium corylophylum* was found in wine samples, its presence does not represent per se a risk to the health of the consumer (this fungus is a mycotoxin producer), because all the fungi we found were unable to grow at the ethanol concentration of the sparkling wines. Future studies will allow us to find out whether these fungi form 2,4,6-TCA and/or other volatile organic compounds involved in the production of cork taint in wines.

Author Contributions: Conceptualization: A.M.S.; Data curation: E.R.-A.; Formal analysis: E.R.-A. and A.M.S.; Funding acquisition: J.F.C.-L.; Investigation: E.R.-A. and A.M.S.; Methodology: A.M.S. and J.F.C.-L.; Project administration: J.F.C.-L.; Resources: J.F.C.-L.; Software: E.R.-A. and J.F.C.-L.; Supervision: A.M.S. and J.F.C.-L.; Visualization: A.M.S.; Writing – original draft: E.R.-A. and A.M.S.; Writing – review & editing: E.R.-A., A.M.S., J.G. and J.F.C.-L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest. The authors confirm that this manuscript respects the Nagoya Protocol to the Convention on Biological Diversity. All data generated or analyzed during this study are included in this published article.

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4.4. Rare malbranchea-like fungal isolates from clinical specimens collected in the United States of America

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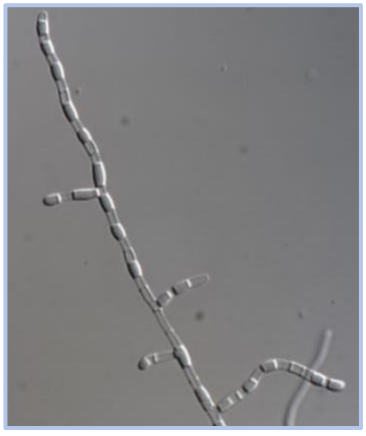

Rare malbranchea-like fungal isolates from clinical specimens collected in the United States of America.

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ABSTRACT

The fungi of the order *Onygenales* can cause important human infections; however, their taxonomy and worldwide occurrence is still little known. We have studied and identified a representative number of clinical fungi belonging to that order from a reference laboratory in the USA. A total of twenty-two strains isolated from respiratory tract (40 %) and human skin and nails (27.2 %) showed a malbranchea-like morphology. Six genera were phenotypically and molecularly identified, i.e. *Auxarthron/Malbranchea* (68.2 %), *Arachnomyces* (9.1 %), *Spiromastigoides* (9.1 %), and *Currahmyces* (4.5 %), and two newly proposed genera (4.5 % each). Based on the results of the phylogenetic study, we synonymysed *Auxarthron* to *Malbranchea*, and erected two new genera: *Pseudoarthropsis* and *Pseudomalbranchea*. New species are proposed: *Arachnomyces bostrychodes*, *A. graciliformis*, *Currahmyces sparsispora*, *Malbranchea gymnoascoidea*, *M. multiseptata*, *M. stricta*, *Pseudoarthropsis crassispora*, *Pseudomalbranchea gemmata* and *Spiromastigoides geomyces*, along with a new combination for *Malbranchea gypsea*. The echinocandins showed the highest *in vitro* antifungal activity against the studied isolates, followed by terbinafine and posaconazole; in contrast, amphotericin B, fluconazole, itraconazole and 5-fluorocytosine were less active or lacked *in vitro* activity against these fungi.

Key words: Antifungals, *Arachnomycetales, Auxarthron*, Clinical fungi, *Malbranchea*, *Onygenales*, Mycosis, Taxonomic novelties.

Taxonomic novelties: new genera: *Pseudoarthropsis* Stchigel, Rodr.-Andr. & Cano, *Pseudomalbranchea* Rodr.-Andr., Cano & Stchigel; **New species**: *Arachnomyces bostrychodes* Rodr.-Andr., Cano & Stchigel, *Arachnomyces graciliformis* Rodr.-Andr., Stchigel and Cano, *Currahmyces sparsispora* Rodr.-Andr., Cano & Stchigel, *Malbranchea gymnoascoidea* Rodr.-Andr., Stchigel & Cano, *Malbranchea multiseptata* Rodr.-Andr., Cano & Stchigel, *Malbranchea stricta* Rodr.-Andr., Stchigel & Cano, *Pseudoathropsis crassispora* Rodr.-Andr., Stchigel & Cano, *Pseudomalbranchea gemmata* Rodr.-Andr., Cano & Stchigel, *Spiromastigoides geomyces* Stchigel, Rodr.-Andr. & Cano; **New combinations:** *Malbranchea californiense* (G.F. Orr & Kuehn) Rodr.-Andr., Stchigel & Cano, *Malbranchea chlamydospora* (M. Solé, Cano & Guarro) Rodr.-Andr., Cano & Stchigel, *Malbranchea compacta* (G.F. Orr & Plunkett) Rodr.-Andr., Cano & Stchigel, *Malbranchea concentrica* (M. Solé, Cano & Guarro) Rodr.-Andr., Stchigel & Cano, *Malbranchea conjugata* (Kuehn) Rodr.-Andr., Cano & Stchigel, *Malbranchea indica* (Kuehn) Rodr.-Andr., Cano & Stchigel, *Malbranchea longispora* (Stchigel, Y. Marín, Guarro & Cano) Rodr.-Andr., Stchigel & Cano, *Malbranchea ostraviense* (Hubka, Dobiášová & M. Kolařík) Rodr.-Andr., Cano & Stchigel, *Malbranchea pseudauxarthron* (G.F. Orr & Kuehn) Rodr.-Andr., Stchigel & Cano, *Malbranchea reticulata* (Arx) Rodr.-Andr., Stchigel & Cano, *Malbranchea umbrina* (Boud.) Rodr.-Andr., Cano & Stchigel, *Malbranchea zuffiana* (Morini) Rodr.-Andr., Stchigel & Cano, *Pseudoarthropsis cirrhata* (Oorschot & de Hoog) Stchigel, Rodr.-Andr. & Cano, *Spiromastigoides gypsea* (Sigler & Carmichael) Stchigel, Rodr.-Andr. & Cano.

INTRODUCTION

The order *Onygenales* includes medically important fungi, such as the dermatophytes and the thermally dimorphic systemic pathogens (*Histoplasma*, *Coccidioides* and related fungi), which are naturally present in keratinous substrates, in soil, and in freshwater sediments (Currah 1985, 1994, Doveri *et al*. 2012, Dukik *et al*. 2017, Hubálek 2000, Hubka *et al*. 2013, Sharma & Shouche 2019). The genus *Malbrachea*, which is characterized by the production

of alternate arthroconidia in branches from the vegetative hyphae, is one of the genus-form of this order; however, it's pathogenic role in human infections is little known. Only a few cases of fungal infections by species of this genus have been described: *Malbranchea dendritica* has been recovered from lungs, spleen and liver of mice (Sigler & Carmichael 1976), *Malbranchea pulchella* has been suggested as a possible cause of sinusitis (Benda & Corey 1994), and *Malbranchea cinnamomea* was recovered from dystrophic nails in patients with underlying chronic illnesses (Lyskova 2007, Salar & Aneja 2007). More recently, *Malbranchea* spp. have been proposed as one of the causative agents of Majocchi's granuloma (Govind *et al*. 2017; Durdu *et al.* 2019). In a study of 245 patients with fungal saprophytic infections of nails and skin, *Malbranchea* spp. were isolated in 1% of skin samples (Lyskova 2007). Other studies have demonstrated the coexistence (0.3% of the cases) of *Malbranchea* spp. together with the primary pathogen in patients with tuberculosis (Benda & Corey 1994, Yahaya *et al*. 2015).

Malbranchea was erected by Saccardo in 1882 for a single species, *Malbranchea pulchella.* It is characterized by alternate arthroconidia originating in curved branches from the vegetative hyphae, which had developed on the surface of wet cardboard collected by A. Malbranche in Normandy, France (Fig. 1). Cooney and Emerson reviewed the genus in 1964, providing an appropriate description for mesophilic (*M. pulchella*) and thermophilic (*Malbranchea sulfurea*) species. In a more recent revision by Sigler and Carmichael (1976) twelve species were accepted, while a close relationship with the genus *Auxarthron* (family *Onygenaceae*, order *Onygenales*) was reported, i.e. the species *Auxarthron conjugatum* forms a malbranchea-like asexual morph, and *Malbranchea albolutea* produces a sexual morph related to *Auxarthron*. Also, Sigler and co-workers (2002) connected *Malbranchea filamentosa* with *Auxarthron* based on molecular studies, and also reported the production of fertile ascomata after an *in vitro* mating of several sexually compatible strains of *M. filamentosa*. The genus *Auxarthron* produces reddish brown, appendaged gymnothecial ascomata with globose prototunicate 8-spored asci, and globose or oblate, reticulate ascospores (Solé *et al.* 2002). Some species of this genus, such as *Auxarthron ostraviense* and *A. umbrinum* have been reported as producing onychomycosis in humans (Hubka *et al*. 2013), and *Auxarthron brunneum*, *A. compactum* and *A. zuffianum* were also isolated from

the lungs of kangaroo rats, *A. conjugatum* from lungs of rodents, and *A. umbrinum* from lung of dogs, bats and rodents (Orr *et al*. 1963, Kuehn *et al.* 1964).

Fig. 1. *Malbranchea pulchella* Sacc. & Penzig. Holotype and lectotype. Black ink drawings by A. Malbranche, and pencil drawings by P. A. Saccardo (credits: Rosella Marcucci, erbario micologico di Pier Andrea Saccardo, Università di Padova, Italy).

Malbranchea-like asexual morphs are also present in other taxa of ascomycetes. The genus *Arachnomyces* (family *Arachnomycetaceae*, order Arachnomycetales; Malloch & Cain 1970, Guarro *et al.* 1993), characterized by the production of brightly coloured cleistothecial ascomata bearing setae, and by the production of an onychocola-like (Sigler *et al.* 1994) or a malbranchea-like (Udagawa & Uchiyama 1999) asexual morph, have been also implicated in animal and human infections. Specifically, *Arachnomyces nodosetosus* and *Arachnomyces*

kanei have been reported as causing nail and skin infections in humans (Sigler & Congly 1990, Sigler *et al.* 1994, Campbell *et al.* 1997, Contet-Audonneau *et al.* 1997, Kane *et al.* 1997, Koenig *et al.* 1997, Gupta *et al.* 1998, Erbagci *et al.* 2002, Gibas *et al.* 2002, Llovo *et al.* 2002, O'Donoghue *et al.* 2003, Gibas *et al*. 2004, Stuchlík *et al.* 2011, Järv 2015, Gupta *et al.* 2016). More recently, *Arachnomyces peruvianus* has been reported to cause cutaneous infections (Brasch *et al*. 2017) and *Arachnomyces glareosus* was isolated from nail and skin samples (Gibas *et al*. 2004; Sun *et al*. 2019).

The recently described species *Spiromastigoides albida,* isolated from human lung in the USA (Stchigel *et al.* 2017), also produces a malbranchea-like asexual morph. This genus (family *Spiromastigaceae*, Onygenales) produces orange gymnothecial ascomata with contorted to coiled appendages and pitted and lenticular ascospores (Huehn & Orr 1962, Uchiyama *et al.* 1995, Unterainer *et al.* 2002, Hirooka *et al.* 2016).

Due to the limited knowledge of *Malbranchea* and their relatives on human infections, we have studied phenotypically and molecularly a set of malbranchea-like fungal strains from clinical specimens received in a fungal reference centre in the USA. Phylogenetic analysis and an antifungal susceptibility testing were also carried out.

MATERIALS AND METHODS

Fungal strains

Twenty-two malbranchea-like fungal strains (nineteen from human specimens and three from animals) from different locations in the USA were included in this study. The strain number, anatomical source, and geographic origin of the specimens are listed in Table 1. They were provided by the Fungus Testing Laboratory at the University of Texas Health Science Centre at San Antonio (UTHSC; San Antonio, Texas, USA).

Phenotypic study

For cultural characterization, suspensions of conidia were prepared in a semi-solid medium (0.2 % agar; 0.05 % Tween 80) and inoculated onto phytone yeast extract agar (PYE; Becton, Dickinson & Company, Sparks, MD, USA; Carmichael & Kraus 1959), potato dextrose agar (PDA; Pronadisa, Madrid, Spain; Hawksworth *et al*. 1995), oatmeal agar (OA; 30 g of filtered oat flakes, 15 g agar-agar, 1 L tap water; Samson *et al.* 2010), bromocresol purple-

milk solids-glucose agar (BCP-MS-G; 80 g skim milk powder, 40 g glucose, 10 mL of 1.6 % of bromocresol purple in 95 % ethanol, 30 g agar-agar,1 L tap water; Kane & Smitka 1978), and test opacity tween medium (TOTM; 10 g bacteriological peptone, 5 g NaCl, 1 g $CaCl_2$, 5 mL Tween, 5 mL Tween 80, 15 g agar-agar, 1 L tap water; Slifkin 2000). Colonies were characterized after 14 days at 25°C in the dark. Potato dextrose agar (PDA) was used to determine the cardinal temperatures of growth. Colour notations were taken according to Kornerup & Wanscher (1978). Christensen's urea agar (EMD Millipore SA, Darmstadt, Germany; Christensen 1946) was inoculated and incubated for 4 days at 25 °C in the dark to detect the production of urease. Cycloheximide tolerance was tested growing the fungal strains on Sabouraud dextrose agar (SDA; Pronadisa S.A., Spain) supplemented with 0.2 % cycloheximide (Sigma, USA) at 30 °C for two weeks. Fungal tolerance to NaCl was evaluated on SDA at 3, 10 and 20 % w/w NaCl, with the same incubation conditions as previously described. The microscopic structures were characterized and measured from wet mounts of slide cultures, using water and 60% lactic acid. Photo micrographs were taken using a Zeiss Axio-Imager M1 light microscope (Oberkochen, Germany) with a DeltaPix Infinity X digital camera using Nomarski differential interference contrast. The descriptions of the taxonomical novelties were submitted to MycoBank (www.mycobank.org; Crous *et al*. 2004).

DNA extraction, amplification and sequencing

Total DNA was extracted as previously described (Valenzuela-Lopez *et al*. 2018), and the following phylogenetic markers were amplified: the internal transcribed spacers (ITS) (ITS5/ITS4 primers; White *et al*. 1990), and a fragment of the large subunit (LSU) gene (LR0R/LR5 primers; Vilgalys & Hester 1990; Rehner & Samuels 1994) of the nrDNA. Amplicons were sequenced at Macrogen Europe (Macrogen Inc., Madrid, Spain) using the same pair of primers. Consensus sequences were obtained by SeqMan software v. 7 (DNAStar Lasergene, Madison, WI, USA). Sequences generated in this work were deposited in GenBank (Table 1).

Phylogenetic analyses

A preliminary molecular identification of the isolates was carried out with ITS and LSU nucleotide sequences using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and only the sequences of ex-type or reference strains from GenBank were included for identification. A

maximum level of identity (MLI) ≥ 98% was used for species-level and < 98% for genus-level identification. Maximum-likelihood (ML) and Bayesian-inference (BI) phylogenetic analyses of the concatenated ITS-LSU sequences were performed in order to determine the phylogenetic placement of our clinical strains. Species of the order Arachnomycetales were used as outgroups. The sequence alignments and ML / BI analyses were carried out according to Valenzuela-Lopez *et al.* (2018). The final matrices used for the phylogenetic analysis were deposited in TreeBASE (www.treebase.org; accession number: 25068).

Antifungal susceptibility testing

In vitro antifungal susceptibility testing was carried out following the broth microdilution method from the Clinical and Laboratory Standards Institute (CLSI) protocol M38 (CLSI, 2017) with some modifications. The antifungal drugs tested were amphotericin B (AMB), fluconazole (FLC), voriconazole (VRC), itraconazole (ITC), posaconazole (PSC), anidulafungin (AFG), caspofungin (CFG), micafungin (MFG), terbinafine (TRB), and 5-fluorocytosine (5FC). Briefly, incubation media, temperature and time were set to the sporulation requirements of every strain, and conidia suspensions were inoculated into the microdilution trays after being adjusted by haemocytometer counts. Incubation was set at 35 ºC (without light or agitation) until the drug-free well displayed visible fungal growth (minimum 48 h; maximum 10 days) for quantification of the Minimal Effective Concentrations (MEC) for the echinocandins and the Minimal Inhibitory Concentrations (MIC) for the other tested antifungals. The MEC value was established as the lowest drug concentration at which short, stubby and highly branched hyphae were observed, while the MIC value was defined as the lowest concentration that completely inhibited fungal growth. *Candida parapsilosis* ATCC 22019 was used as the quality control strain in all experiments.

RESULTS

Fungal diversity

Table 1 shows the identity of the twenty-two fungal strains studied. The highest number of strains corresponded to *Auxarthron umbrinum* (4), followed by *Auxarthron alboluteum* (2), *Auxarthron conjugatum* (2), and *Malbranchea aurantiaca* (2). *Auxarthron zuffianum*, *Currahmyces indicus* and *Malbrancea flocciformis* were represented by one strain each. Eight

strains were only identified at genus-level (three belonging to *Malbranchea*, two to *Spiromastigoides*, two to *Arachnomyces*, one to *Arthropsis*, and one to *Currhamyces*), one strain (FMR 17684) only at family-level (*Onygenaceae*).

Molecular phylogeny

Our phylogenetic study included 92 sequences corresponding to 75 species with a total of 1,213 characters (700 ITS and 513 LSU) including gaps, of which 579 were parsimony informative (402 ITS and 177 LSU). The ML analysis was congruent with that obtained in the BI analysis, both displaying trees with similar topologies. The datasets did not show conflict with the tree topologies for the 70% reciprocal bootstrap trees, which allowed the two genes to be combined for the multi-locus analysis.

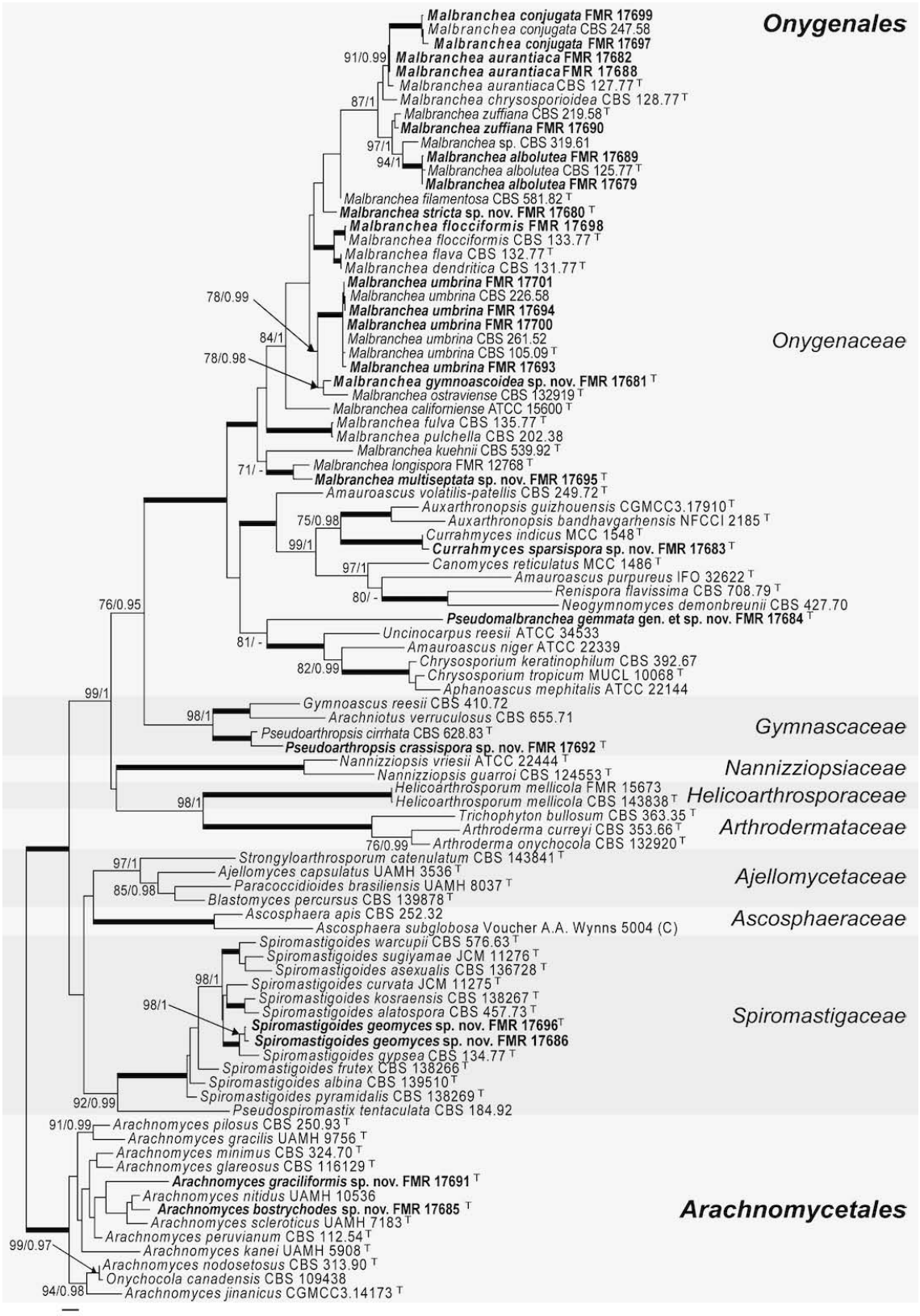

Fig. 2 ML phylogenetic tree based on the analysis of ITS-LSU nucleotide sequences for the twenty-two clinical fungi from the USA. Bootstrap support values/Bayesian posterior probability scores of 70/0.95 and higher are indicated on the nodes. ^{T} = ex type. Fully supported branches (100% BS /1 PP) are indicated in bold.

Strains identified by us are in bold. *Arachnomyces* spp. were chosen as out-group. The sequences used in this analysis are shown in Table 1

Twenty of the strains were placed into a main clade corresponding to the members of the *Onygenales* (100% BS / 1 PP), while two were placed in the *Arachnomycetales* (100% BS / 1 PP) (Fig. 2). The *Onygenales* clade was divided into eight clades corresponding to the families *Onygenaceae* (100% BS / 1 PP), *Gymnascaceae* (98% BS / 1 PP), *Nannizziopsiaceae* (100% BS / 1 PP), *Helicoarthrosporaceae* (100% BS / 1 PP), *Arthrodermataceae* (100% BS / 1 PP), *Ajellomycetaceae* (97% BS / 1 PP), *Ascosphaeraceae* (100% BS / 1 PP), and *Spiromastigaceae* (92% BS / 0.99 PP), which included a basal terminal branch for *Pseudospiromastix tentaculata*. Most of our strains (17/22) were distributed into several subclades of the *Onygenaceae*: 15/22 into *Auxarthron*/*Malbranchea* subclade (100% BS / 1 PP), one into a terminal branch (FMR 17683) together *Currahmyces indicus* (100% BS / 1 PP), and another one (FMR 17684) into a distant, independent terminal branch. One strain (FMR 17692) was placed into the *Gymnascaceae*, in a terminal branch together with *Arthropsis cirrhata* (100% BS / 1 PP). The *Spiromastigaceae* included the last two strains (FMR 17686 and FMR 17696), placed into a terminal branch together *Malbranchea gypsea* (100% BS / 1 PP).

Taxonomy

Since the strains FMR 17685 and FMR 17691 represented two species of *Arachnomyces* that were different from the other species of the genus*,* they are proposed as new, i.e. *Arachnomyces bostrychodes* and *Arachnomyces graciliformis*, respectively.

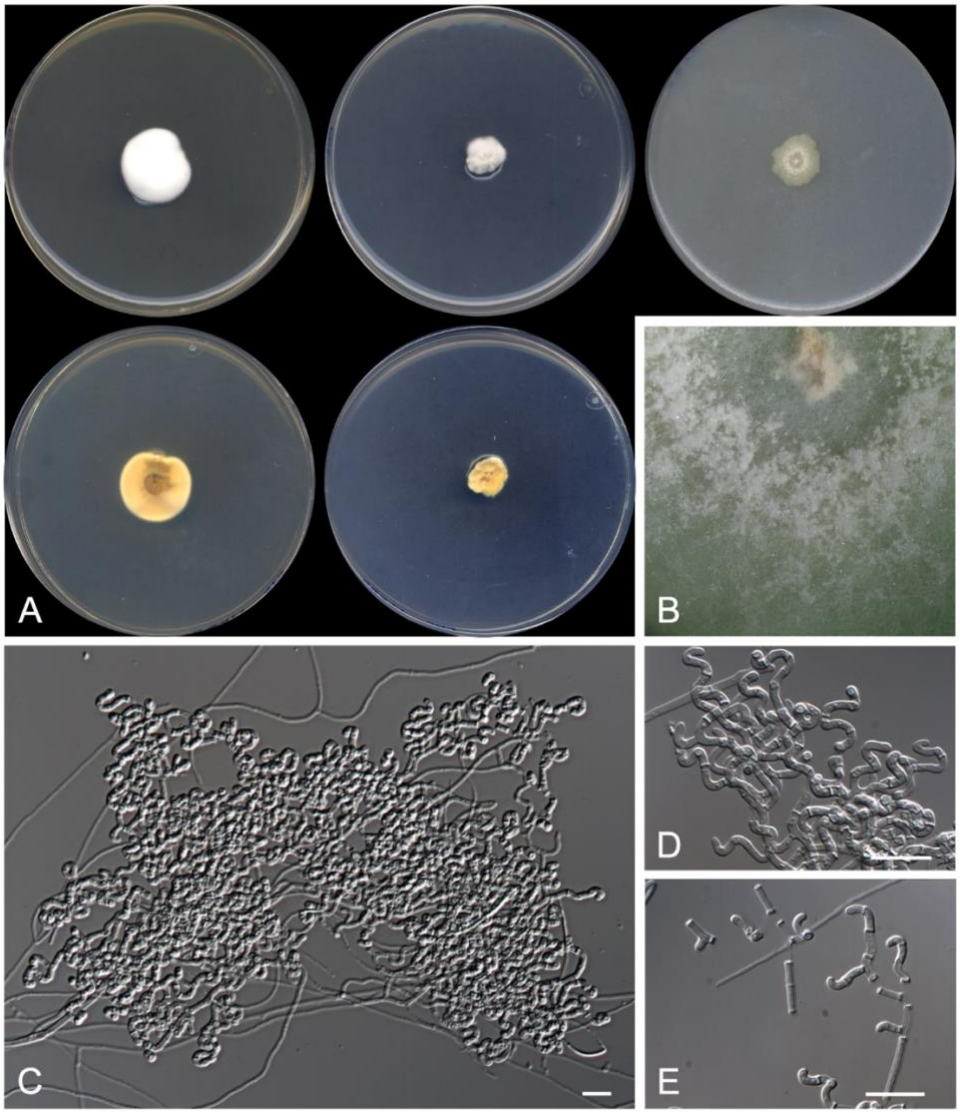

Fig. 3 *Arachnomyces bostrychodes* CBS 834921^{T}. A. Colonies on PYE, PDA and OA after 14 d at 25°C, from left to right (top row, surface; bottom row, reverse). B. Detail of the colony on OA. C, D. Sinuous, contorted to coiled fertile hyphae. E. Arthroconidia. Scale bar = 10 µm.

Arachnomyces bostrychodes Rodr.-Andr., Cano & Stchigel, **sp. nov.** MycoBank MB 834921. Fig. 3.

Etymology: From Greek *βοστρυχος-*, curl, due to the appearance of the reproductive hyphae.

Micromorphology: Vegetative hyphae hyaline, septate, branched, smooth- and thin-walled, 1.0–2.0 µm wide. *Fertile hyphae* well-differentiated, arising as lateral branches from the vegetative hyphae, successively branching to form dense clusters, arcuate, sinuous, contorted or tightly curled, 1.0–2.0 µm wide, forming randomly intercalary and terminally

arthroconidia. *Conidia* enteroarthric, hyaline, one-celled, smooth-walled, cylindrical, barrel-shaped, and finger-like-shaped when terminal, 4.0–8.0 × 1.0–2.0 µm, mostly curved and truncated at one or (mostly) both ends, separated from the fertile hyphae by rhexolysis. *Chlamydospores, racquet hyphae*, *setae*, and s*exual morph* not observed.

Culture characteristics: Colonies on PYE reaching 19–20 mm diam. after 2 weeks at 25 °C, elevated, cottony, margins regular, white (5A1), sporulation absent; reverse light orange (5A4). Colonies on PDA reaching 11–12 mm diam. after 2 weeks at 25 °C, elevated, velvety with floccose patches, margins regular, yellowish white (4A2), sporulation abundant; reverse greyish yellow (4B6). Colonies on PDA reaching 13–14 mm diam. after 2 weeks at 30 ºC, slightly elevated, velvety to floccose, regular margins, white (4A1), sporulation sparse; reverse, greyish yellow (4B6). Colonies on OA researching 13–14 mm diam. after 2 weeks at 25 °C, flattened, smooth and granulose, irregular margins, yellowish white (2A2) at centre and light yellow (2A5) at edge, sporulation abundant. Exudate and diffusible pigment absent. Minimum, optimal and maximum temperature of growth (on PDA): 10 °C, 30 °C, and 37 °C, respectively. Non-haemolytic. Casein not hydrolyzed. Not inhibited by cycloheximide. Urease and esterase (TOTM) tests positive. Growth occurs at NaCl 10 % w/w, but not at 20 % w/w.

Material examined: United States of America (USA), Texas, from a human scalp, XX-XX-2008, collected by N. Wiederhold (holotype CBS H-XXXX, cultures ex-type CBS XXXX = FMR 17685 = UTHSCSA DI18-91).

Notes: The phylogenetically closest species to *Arachnomyces bostrychodes* is *A. peruvianum* (Fig. 2). Nevertheless, *Arachnomyces botrychodes* lacks a sexual morph and racket hyphae (both present in *A. peruvianum*), and produces longer conidia than *A. peruvianum* (4.0–8.0 × 1.0–2.0 µm vs. 4.0–5.0 × 1.0–3.0 µm); also, *A. bostrychodes* grows more slowly on OA (13–14 mm diam. after 14 days at 25 ºC) than *A. peruvianum* (30 mm diam.) (Cain 1957, Brasch *et al*. 2016). *Arachnomyces bostrychodes* resembles morphologically *Arachnomyces gracilis*, but the former grows faster, and produces more twisted branches and lacks a sexual morph.

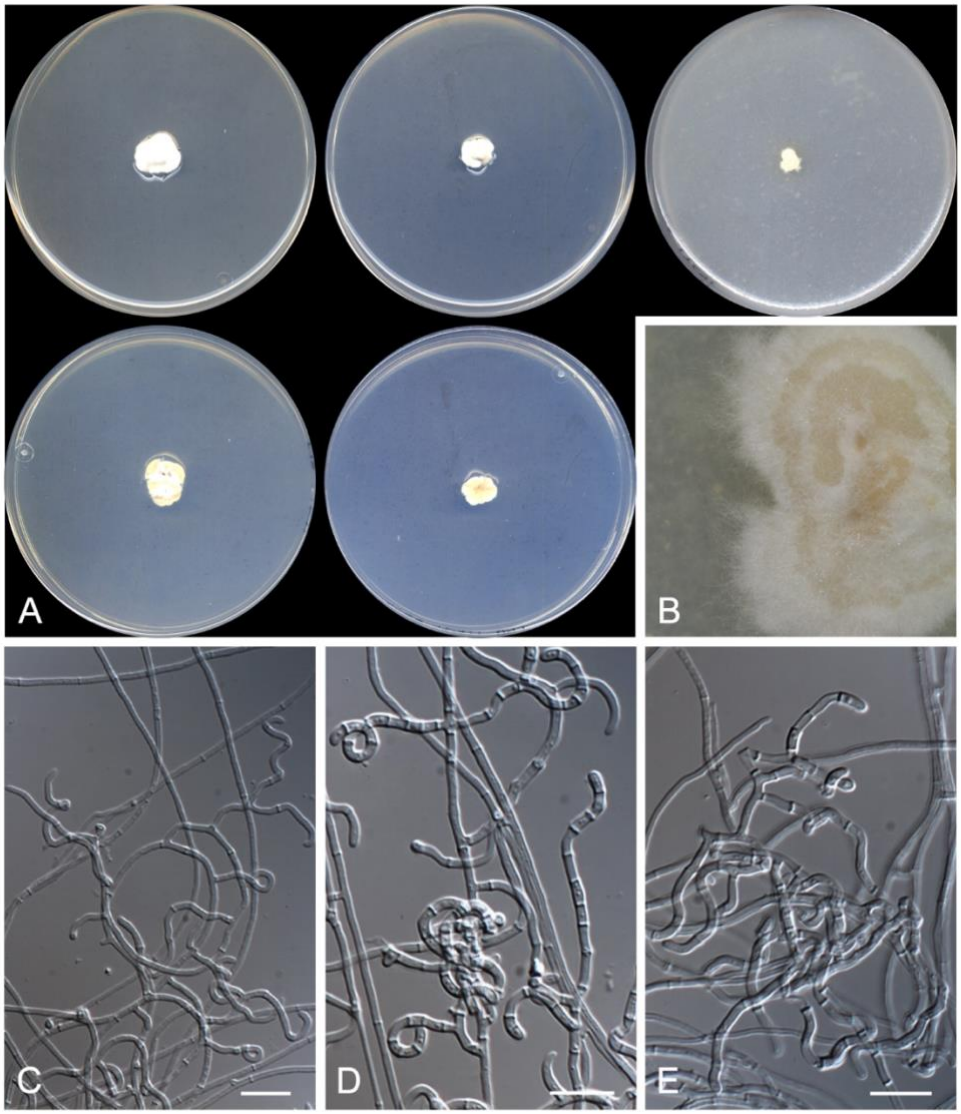

Fig. 4 *Arachnomyces graciliformis* CBS 834923^{T}. A. Colonies on PYE, PDA and OA after 14 d at 25°C, from left to right (top row, surface; bottom row, reverse). B. Detail of the colony on OA. C–E. Contorted, apically coiled fertile hyphae bearing arthroconidia. Scale bar = 10 µm.

Arachnomyces graciliformis Rodr.-Andr., Stchigel & Cano, **sp. nov.** MycoBank MB 834923. Fig. 4.

Etymology: Because the morphological similarity with *Arachnomyces gracilis*.

Micromorphology: Vegetative hyphae hyaline, septate, branched, smooth- and thin-walled, 1.0–2.0 µm wide. *Fertile hyphae* well-differentiated, arising as lateral branches from the vegetative hyphae, branching repeatedly, sinuous to arcuate or apically coiled, 1.5–2.0 µm wide, forming randomly intercalary and terminally arthroconidia. *Conidia* enteroarthric, hyaline, unicellular, smooth- and thin-walled, cylindrical or finger-like-shaped when

terminal, 4.0–10.0 × 1.5–2.0 µm, mostly curved, detached from the fertile hyphae by rhexolysis. *Chlamydospores*, *racquet hyphae*, *setae*, and *sexual morph* not observed.

Culture characteristics: Colonies on PYE reaching 12–13 mm diam. after 2 weeks at 25 °C, elevated, velvety to floccose, margins regular, slightly furrowed, yellowish white (3A2), sporulation absent; reverse greyish orange (5B3). Colonies on PDA reaching 9–10 mm diam. after 2 weeks at 25 °C, slightly elevated, velvety to floccose, margins regular, slightly furrowed, yellowish white (1A2), sporulation absent; reverse greyish yellow (4B3). Colonies on PDA reaching 3–4 mm diam. after 2 weeks at 30 ºC, slightly elevated, velvety to floccose, margins regular, slightly furrowed, yellowish white (1A2), sporulation absent; reverse, greyish yellow (4B3). Colonies on OA reaching 6–7 mm diam. after 2 weeks at 25 °C, flattened, velvety and granulose, margins irregular, pale yellow (4A3), sporulation absent (conidia appear after 5-6 weeks incubation). Exudate and diffusible pigment absent. Minimum, optimal and maximum temperature of growth (on PDA): 10 °C, 25 °C, and 30 °C, respectively. Non-haemolytic. Casein not hydrolysed. Not inhibited by cycloheximide. Urease and esterase tests positive. Growth occurs at NaCl 10 % w/w, but not at 20 % w/w.

Material examined: USA, Massachusetts, from an animal´s bone, XX-XX-2012, collected by N. Wiederhold (holotype CBS H-XXXX, cultures ex-type CBS XXXX = FMR 17691 = UTHSCSA DI18-97).

Notes: Arachnomyces graciliformis is phylogenetically close to *A. glareosus* and to *A. minimus* (Fig. 2). These three species forma common clade together with *A. nodosetosus* and *A. jinanicus* (84 BS / 1 PP). Unlike *A. glareosus* and *A. minimus*, *A. graciliformis* does not produce racquet hyphae nor a sexual morph (Gibas *et al*. 2004), but produces longer conidia than *A. glareosus* (4.0–10.0 × 1.5–2.0 µm vs. 2.5–4.5 × 1.5–2.0 µm), which are not produced by *A. minimus*. *Arachnomyces graciliformis* resembles morphologically *Arachnomyces gracilis*, but the former grows more slowly, produces more twisted fertile branches and does not form a sexual morph (Udagawa & Uchiyama 1999).

Dichotomic key to *Arachnomyces* (adapted from Sun *et al.* 2019).

1a. Homothallic; asexual morph present or not..... 2

1b. Heterothallic; asexual morph present..... 6

2a. Peridial setae coiled or circinate; asexual morph absent..... 3

2b. Peridial setae straight, tapering towards the apex; asexual morph arthroconidia ..... *A. gracilis*

3a. Peridial setae slightly nodose; ascospores mostly < 3.5 µm diameter..... 4

3b. Peridial setae smooth-walled; ascospores mostly > 3.5µm diameter..... 5

4a. Ascospores smooth-walled..... *A. minimus*

4b. Ascospores echinulate..... *A. peruvianus*

5a. Ascomata 100–300 µm diameter..... *A. nitidus*

5b. Ascomata 500–700 µm diameter..... *A. sulphureus*

6a. Arthroconidia alternate..... 7

6b. Arthroconidia in persistent chains..... 12

7a. Arthroconidia cylindrical or barrel-shaped; sclerotia present..... 8

7b. Arthroconidia distinct; sclerotia absent..... 9

8a. Colonies becoming greyish brown, not growing at 35 ºC..... *A. glareosus*

8b. Colonies white to pale brown, growing at 35 ºC..... *A. scleroticus*

9a. Arthroconidia subglobose to pyriform..... 10

9b. Arthroconidia cylindrical to finger-like-shaped..... 11

10a. Arthroconidia smooth-walled to finely asperulate; setae (produced on the vegetative mycelium) smooth-walled to slightly nodose..... *A. kanei*

10b. Mature arthroconidia coarsely verrucose; setae (produced on the vegetative mycelium) strongly nodose..... *A. pilosus*

11a. Fertile hyphae successively branching to form dense clusters, arcuate, sinuous, contorted or tightly curled..... ***A. bostrychodes***

11b. Fertile hyphae branching but not in clusters; branches only apically coiled..... ***A. graciliformi***

12a. Setae (produced on the vegetative mycelium) strongly nodose, circinate or loosely coiled at the apex..... *A. nodosetosus*

12b. Setae (produced on the vegetative mycelium) strongly nodose, tip straight..... *A. jinanicus*

Since the strain FMR 17692 was placed into the same terminal clade as *Arthropsis cirrhata*, while the type species of the genus (*Arthropsis truncata*) is phylogenetically far

away (into the order *Sordariales*; Giraldo et al. 2013), we propose the erection of the new genus *Pseudoarthropsis* for *A. cirrhata*, and the new species *Pseudarthropsis crassispora*.

Pseudoarthropsis Stchigel, Rodr.-Andr. & Cano, **gen. nov.** MycoBank MB 834925.

Etymology: From Greek *ψευδής-*, resembling, because the morphological semblance to *Arthropsis*.

Mycelium composed by hyaline to orange, septate hyphae. Conidiophores consisting in fertile lateral branches and a part of the main hyphae, which disarticulate in yellowish orange, thin-walled, cylindrical to cuboid enteroarthric conidia, or in hyaline, thick-walled, ellipsoidal, globose to barrel-shaped holoarthric conidia.

Type species: Pseudoarthropsis cirrhata (Oorschot & de Hoog) Stchigel, Rodr.-Andr. & Cano.

Pseudoarthropsis cirrhata (Oorschot & de Hoog) Stchigel, Rodr.-Andr. & Cano, **comb. nov.** MycoBank MB 834928.

Basionym: *Arthropsis cirrhata* Oorschot & de Hoog, Mycotaxon 20: 130 (1984).

Description: Vegetative hyphae septate, pale yellowish orange, smooth- and thin-walled, dichotomously branched, 2–3 µm wide. *Fertile hyphae* well-differentiated, arising at right angles as recurved lateral branches of the vegetative hyphae, forming septa basipetally to produce chains of enteroarthric conidia. *Arthroconidia* yellowish orange, smooth- and thin-walled, cylindrical to cuboid, often broader than long, 2.5–4.0 × 2–3 µm, truncated at both ends, separated by trapezoid connectives, secession rhexolytic. *Colonies* on PYE reaching 4–5 mm diam. after 10 days at 25 °C, powdery, fealty, slightly raised, orange (5A7), pale orange (5A5) at centre; reverse brownish orange (7C8), diffusible pigment brown.

Typus: CBS 628.83, 1984, from a wall near Schiphol, The Netherlands, collector C.A.N. van Oorschot.

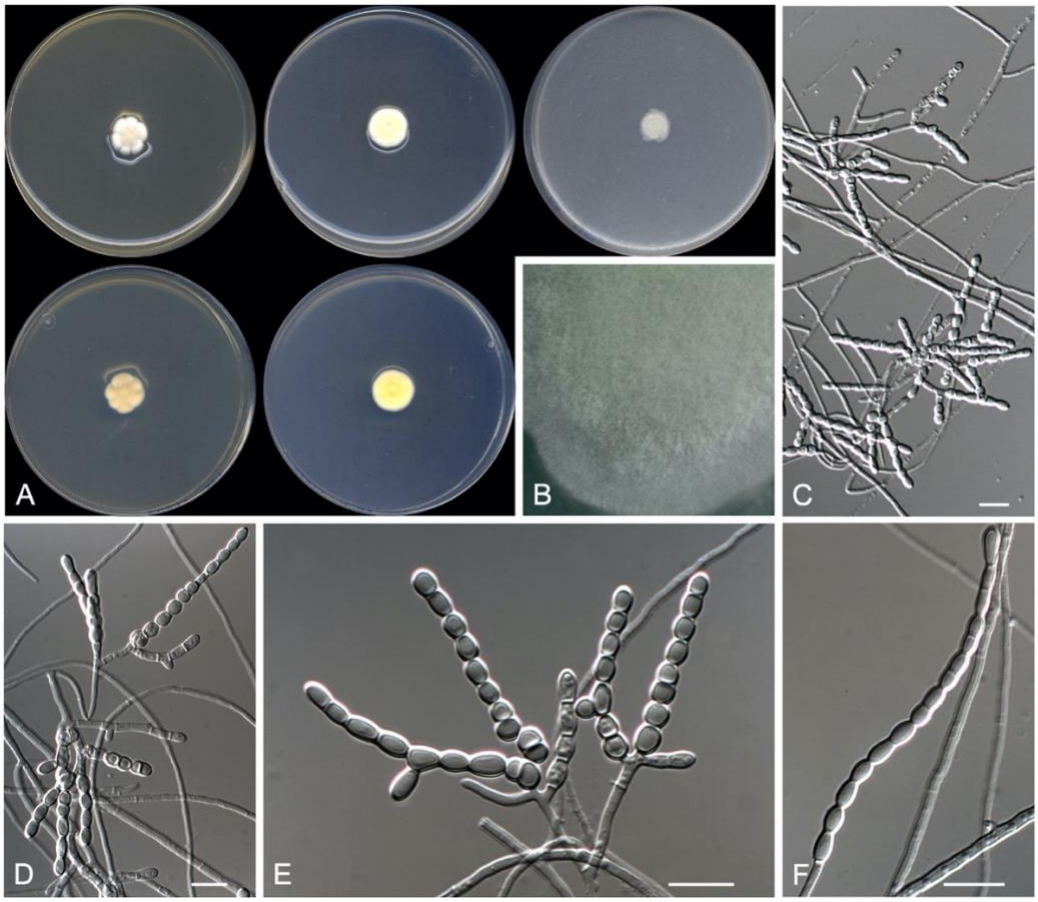

Fig. 5 *Pseudoarthropsis crassispora* CBS 834930^{T}. A. Colonies on PYE, PDA and OA after 14 d at 25°C, from left to right (top row, surface; bottom row, reverse). B. Detail of the colony on OA. C–E. Bi- to trichotomously-branched fertile hyphae. F. A large chain of holoarthric conidia. Scale bar = 10 µm.

Pseudoathropsis crassispora Rodr.-Andr., Stchigel & Cano**, sp. nov.** MycoBank MB 834930. Fig. 5.

Etymology: From Latin *crassus*-, thick, and -*sporarum*, spore, because of the thick wall of the conidia.

Micromorphology: Vegetative hyphae septate, hyaline, smooth- and thin-walled, mostly straight, occasionally branched, 1.5–2.0 µm wide. *Fertile hyphae* well-differentiated, arising as lateral branches of the vegetative hyphae, hyaline, septate, smooth- and thin-walled, erect, simple or branched up to 3 times at the apex, stipe 10–20 × 1.5–2.0 µm, branches 10–70 × 1.5–2.0 µm, forming septa basipetally to produce chains of arthroconidia. *Conidia* holoarthric, unicellular, hyaline, smooth- and thick-walled, ellipsoidal, globose or barrel-shaped, transiently presents as bi-cellular conidia, 2.5–3.5 × 4.5–5.5 µm, in chains of up to

20, separate from the fertile hyphae by schizolysis, rarely by rhexolysis. *Chlamydospores*, *racquet hyphae*, *setae*, and *sexual morph* not observed.

Culture characteristics: Colonies on PYE reaching 13–14 mm diam. after 2 weeks at 25 °C, slightly elevated, velvety, margins regular, furrowed, yellowish white (3A2) and yellowish grey (4B2) at centre, sporulation abundant; reverse pale yellow (4A3. Colonies on PDA reaching 14–15 mm diam. after 2 weeks at 25 °C, flattened, velvety, margins regular, greenish white (30A2) and pastel green (30A4) at centre, sporulation abundant; reverse pastel yellow (3A4). Colonies on PDA reaching 15–16 mm diam. after 2 weeks at 30 ºC, slightly elevated, velvety, margins regular, furrowed, yellowish white (3A2), sporulation sparse; reverse yellow (3A6), with a scarce production of yellowish diffusible pigment. Colonies on OA researching 10–11 mm diam. after 2 weeks at 25 °C, flattened, velvety to floccose, margins irregular, greenish white (30A2) and pale green (28A3) at centre, sporulation abundant. Exudate and diffusible pigment absent, except on PDA. Minimum, optimal and maximum temperature of growth on PDA: 10 °C, 30 °C, and 37 °C, respectively. Non-haemolytic. Casein hydrolyzed without pH change. Not inhibited by cycloheximide. Urease and esterase tests positive. Growth occurs at NaCl 10 % w/w, but not at 20 % w/w.

Material examined: United States, Minnesota, from a human´s bronchial washing, XX-XX-2012, collected by N. Wiederhold (holotype CBS H-XXXX, cultures ex-type CBS XXXX = FMR 17692 = UTHSCSA DI18-98).

Notes: Pseudoarthropsis crassispora is phylogenetically close to *P. cirrhata*. Nevertheless, the former produces holoarthric conidia, while they are enteroarthric in the latter. Also, the conidia of *P. crassispora* are ellipsoidal, globose or broadly barrel-shaped, while these are cylindrical to cuboid (often wider than they are long) in *P. cirrhata* (van Oorschot & de Hoog 1984). Moreover, the conidia are bigger in *P. crassispora* than in *P. cirrhata* (4.5–5.5 × 2.5–3.5 µm vs. 2.5–4.0 × 2.0–3.0 µm). Also, *P. crassispora* grows faster than *P. cirrhata* (on PYE at 25 ºC), and the maximum temperature of growth is at 37 ºC and 30 ºC, respectively.

Due to the strain FMR 17683 being placed into a terminal branch of the *Onygenaceae* together with *Currahmyces indicus* (Sharma & Shouche 2019), and because they differ molecularly and phenotypically, we propose the erection of the new species *Currahmyces sparsispora*.

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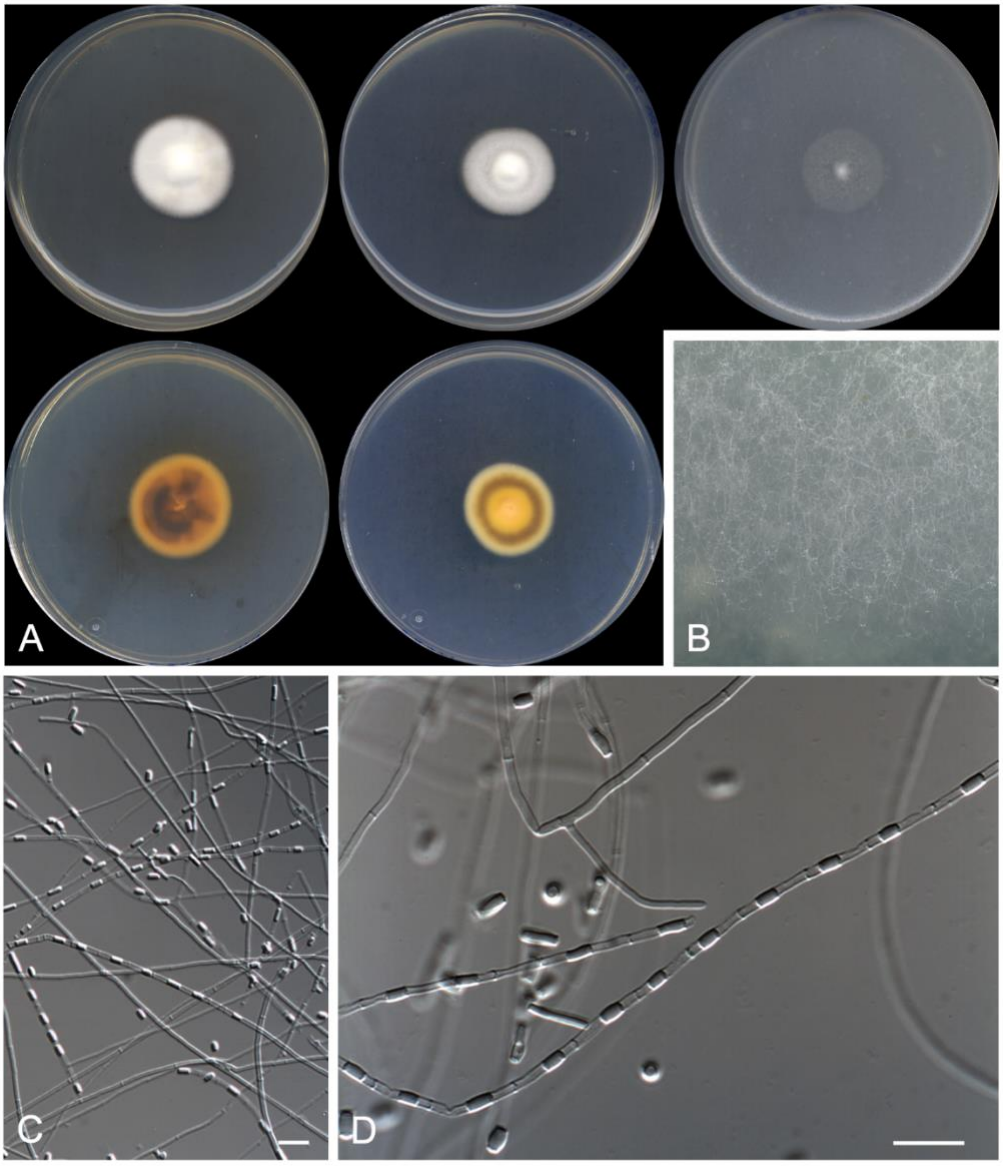

407

408 **Fig. 6** *Currahmyces sparsispora* CBS XXXXX^{T}. A. Colonies on PYE, PDA and OA after 14 d at 25°C, from left to
409 right (top row, surface; bottom row, reverse). B. Detail of the colony on OA. C–D. Intercalary arthroconidia
410 along the fertile hyphae. Scale bar = 10 µm.

411 ***Currahmyces sparsispora*** Rodr.-Andr., Cano & Stchigel, **sp. nov.** MycoBank MB XXXX. Fig.
412 6.

413 *Etymology:* From Latin *sparsa-*, splashed, *-sporarum*, spore, due to the disposition of the
414 conidia along the hyphae.

415 *Description: Vegetative hyphae* septate, hyaline, smooth- and thin-walled, mostly
416 straight, rarely branched, 1.5–2.0 µm wide. *Fertile hyphae* undifferentiated from the

vegetative hyphae. *Conidia* enteroarthric, hyaline, unicellular, smooth- and thin-walled, disposed relatively far from each other along the fertile hyphae, separated by 1–2 evanescent connective cells, cylindrical to slightly barrel-shaped, 3.0–12.0 × 1.0–2.0 µm, separated by rhexolysis. *Chlamydospores*, *racquet hyphae*, *setae*, and *sexual morph* not observed.

Culture characteristics: Colonies on PYE reaching 27–28 mm diam. after 2 weeks at 25 °C, slightly elevated, velvety to floccose, margins regular, pale orange (5A3) at centre and white (5A1) at edge, sporulation sparse; reverse orange (5A6). Colonies on PDA reaching 23–24 mm diam. after 2 weeks at 25 °C, slightly elevated, velvety, margins regular, light orange (5A5) at centre and orange white (5A2) at edge, sporulation sparse; reverse deep orange (6A8). Colonies on PDA reaching 30–31 mm diam. after 2 weeks at 30 ºC, slightly elevated, velvety, slightly furrowed, margins regular, orange (5A6), sporulation sparse; reverse brownish orange (6C8). Colonies on OA reaching 20–21 mm diam. after 2 weeks at 25 °C, slightly elevated, velvety, margins regular, orange white (5A2) at centre and white (5A1) at edge, sporulation sparse. Exudate and diffusible pigment absent in all culture media tested. Minimum, optimal and maximum temperature of growth on PDA: 10 °C, 30 °C, and 37 °C, respectively. Haemolytic. Casein not hydrolysed. Not inhibited by cycloheximide. Urease and esterase tests positive. Growth occurs at NaCl 3 % w/w and 10 % w/w, but not at 20 % w/w.

Typus: United States, Florida, from human sputum, XX-XX-2007, collected by N. Wiederhold (holotype CBS H-XXXX, cultures ex-type CBS XXXX = FMR 17683 = UTHSCSA DI18-89)

Notes. Currahmyces sparsispora is phylogenetically close to *Currahmyces indicus*; however, they can be differentiated because the former has broader hyphae (1.5–2.0 µm vs. 0.7–1.1 µm) and lacks a sexual morph (typical gymnothecial ascomata are produced on hair-baited soil plates by *C. indicus*).

Taking into account that *Auxarthron* and *Malbranchea* are congeneric, as has been in previous studies (Sigler *et al.* 2002, Sarroco *et al.* 2015) and here (Fig. 2), and that *Malbranchea* (Saccardo 1882) has historical priority (International Code of Nomenclature for algae, fungi, and plants; Turland *et al.* 2018) over *Auxarthron* (Orr, Kuehn and Plunkett 1963), we transfer the species of *Auxarthron* (Orr *et al.* 1963) to *Malbranchea* as follows:

Malbranchea californiense (G.F. Orr & Kuehn) Rodr.-Andr., Stchigel & Cano, **comb. nov.** MycoBank MB 835229.

Basionym: *Auxarthron californiense* G.F. Orr & Kuehn, *Can. J. Bot.* 41: 1442 (1963).

Synonym: *Gymnoascus californiensis* (G.F. Orr & Kuehn) Apinis, *Mycol. Pap.* 96: 12 (1964).

Malbranchea chlamydospora (M. Solé, Cano & Guarro) Rodr.-Andr., Cano & Stchigel, **comb. nov.** MycoBank MB 835230.

Basionym: *Auxarthron chlamydosporum* M. Solé, Cano & Guarro, *Stud. Mycol.* 47: 108 (2002).

Malbranchea compacta (G.F. Orr & Plunkett) Rodr.-Andr., Cano & Stchigel, **comb. nov.** MycoBank MB 835231.

Basionym: *Auxarthron compactum* G.F. Orr & Plunkett, *Can. J. Bot.* 41: 1453 (1963).

Malbranchea concentrica (M. Solé, Cano & Guarro) Rodr.-Andr., Stchigel & Cano, **comb. nov.** MycoBank MB 835232.

Basionym: *Auxarthron concentricum* M. Solé, Cano & Guarro, *Stud. Mycol.* 47: 106 (2002).

Malbranchea conjugata (Kuehn) Rodr.-Andr., Cano & Stchigel, **comb. nov.** MycoBank MB 835233

Basionym: *Myxotrichum conjugatum* Kuehn, *Mycologia* 47: 883 (1956) [1955].

Synonym: *Auxarthron conjugatum* (Kuehn) G.F. Orr & Kuehn, *Mycotaxon* 24: 148 (1985).

Malbranchea indica (Kuehn) Rodr.-Andr., Cano & Stchigel, **comb. nov.** MycoBank MB 835234.

Basionym: *Auxarthron indicum* M.S. Patil & A.B. Pawar [as 'indica'], *Geobios New Reports* 6: 194 (1987).

Malbranchea longispora (Stchigel, Y. Marín, Guarro & Cano) Rodr.-Andr., Stchigel & Cano **comb. nov.** MycoBank MB 835235.

Basionym: *Auxarthron longisporum* Stchigel, Y. Marín, Guarro & Cano, *Persoonia* 31: 267 (2013)

Malbranchea ostraviense (Hubka, Dobiášová & M. Kolařík) Rodr.-Andr., Cano & Stchigel, **comb. nov.** MycoBank MB 835236.

Basionym: *Auxarthron ostraviense* Hubka, Dobiášová & M. Kolařík, *Med. Mycol.* 50: 619 (2012).

Malbranchea pseudauxarthron (G.F. Orr & Kuehn) Rodr.-Andr., Stchigel & Cano, **comb. nov.** MycoBank MB 835237.

Basionym: *Auxarthron pseudauxarthron* G.F. Orr & Kuehn, *Mycologia* 64: 67 (1972).

Malbranchea umbrina (Boud.) Rodr.-Andr., Cano & Stchigel, **comb. nov.** MycoBank MB 835238.

Basionym: *Gymnoascus umbrinus* Boud., *Bull. Soc. mycol. Fr.* 8: 43 (1892).

Synonym: *Auxarthron brunneum* (Rostr.) G.F. Orr & Kuehn, *Can. J. Bot.*41: 1446 (1963).

Auxarthron umbrinum (Boud.) G.F. Orr & Plunkett, *Can. J. Bot.* 41: 1449 (1963).

Auxarthron thaxteri (Kuehn) G.F. Orr & Kuehn, *Mycologia* 63: 200 (1971).

Gymnoascus subumbrinus A.L. Sm. & Ramsb., *Trans. Br. Mycol. Soc*. 5: 424 (1917) [1916].

Gymnoascus umbrinus var. *thaxteri* (Kuehn) Apinis, *Mycol. Pap.* 96: 14 (1964).

Myxotrichum brunneum Rostr., *Bot. Tidsskr.* 19: 216 (1895).

Myxotrichum thaxteri Kuehn, *Mycologia* 47: 878 (1956) [1955].

Malbranchea zuffiana (Morini) Rodr.-Andr., Stchigel & Cano, **comb. nov.** MycoBank MB 835239.

Basionym: *Gymnoascus zuffianus* Morini, Mem. R. Accad. Sci. Ist. Bologna, Ser. 4 10: 205 (1889).

Synonym: *Auxarthron zuffianum* (Morini) G.F. Orr & Kuehn, *Can. J. Bot.* 41: 1445 (1963).

We also revalidate the *Malbranchea* species listed below:

Malbranchea albolutea Sigler & J.W. Carmich., *Mycotaxon* 4(2): 416 (1976).

Synonym: *Auxarthron alboluteum* Sigler, Hambl. & Flis, *Stud. Mycol.* 47: 118 (2002).

Malbranchea filamentosa Sigler & J.W. Carmich., Mycotaxon 15: 468 (1982).

Synonym: *Auxarthron filamentosum* Sigler, Hambl. & Flis, *Stud. Mycol.* 47: 116 (2002).

Because in a Blast search using the ITS and LSU nucleotide sequences from the ex-type strains, *Malbranchea circinata* and *Malbranchea flavorosea* match with taxa into the family *Myxotrichaceae*, both species are excluded to the genus.

Consequently, an emended description of the genus *Malbranchea* is provided as follows:

Malbranchea Sacc. MycoBank MB 8833.

Vegetative hyphae septate, hyaline, smooth- and thin-walled, straight or branched. *Asexual morph* consisting in undifferentiated fertile hyphae, and/or well-differentiated lateral branches, curved or not, which form randomly or basipetally terminal and intercalary arthroconidia. *Conidia* enteroarthric, rarely holoarthric, unicellular, hyaline, smooth- and thin-walled, mostly cylindrical, barrel-shaped, or irregularly shaped, sometimes cylindrical, detached from the fertile hyphae by rhexolysis. *Sexual morph* (when present) consisting in ascomata formed by of an anastomosing network of orange to brown, ornamented or not thick-walled hyphae (gymnothecia), bearing elongate appendages and/or spine projections, within there are small, evanescent, inflated asci which forms eight globose to oblate ascospores, whose cell wall is ornamented with a (coarse or thin) reticulate pattern. Species homothallic or heterothallic, thermotolerant or thermophilic, keratinolytic, chitinolytic or cellulolytic.

Despite the strain FMR 17681 being placed phylogenetically close to *Malbranchea ostraviense* and *Malbranchea umbrina*, it differs genetically and phenotypically from both species, therefore we propose the new species *Malbranchea gymnoascoidea* as follows:

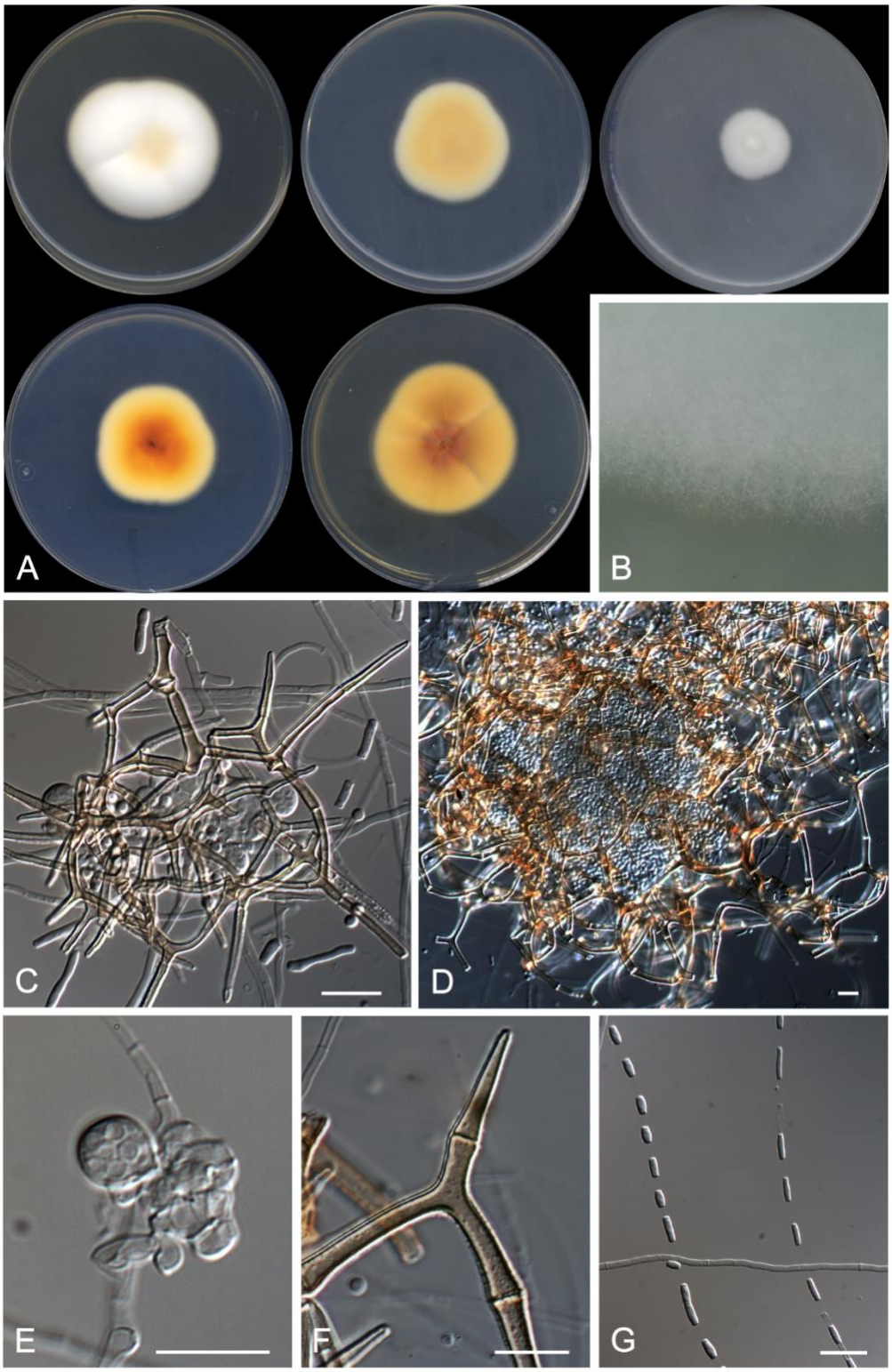

Fig. 7 *Malbranchea gymnoascoides* CBS 835212^{T}. A. Colonies on PYE, PDA and OA after 14 d at 25°C, from left to right (top row, surface; bottom row, reverse). B. Detail of the colony on OA. C–D. Young and mature ascomata. E. Young ascus on fertile hyphae. F. Peridial spine-like appendage. G. Intercalary arthroconidia along the fertile hyphae. Scale bar = 10 µm.

Malbranchea gymnoascoides Rodr.-Andr., Stchigel & Cano, **sp. nov.** MycoBank MB 835212. Fig. 7.

Etymology: Because to the ascomata are morphologically similar to those of *Gymnoascus reessii.*

Micromorphology: Vegetative hyphae septate, hyaline, smooth- and thin-walled, mostly straight, rarely branched, 1.5–2.5 µm wide. *Asexual morph* consisting in undifferentiated *fertile hyphae* which form randomly intercalary and terminally arthroconidia. *Conidia* enteroarthric, unicellular, hyaline, smooth- and thin-walled, mostly barrel-shaped, sometimes cylindrical or irregularly-shaped, 6.0–10.0 × 1.5–2.0 µm, detached by rhexolysis. *Ascomata* gymnothecial, solitary or in clusters, hyaline at first, becoming orange brown with the age, globose or nearly so, 130–250 µm diam. excluding the appendages, which cover entirely the surface. *Peridial hyphae* septate, orange brown, branching and anastomosing to form a reticulate network, asperulate, very thick-walled, 3.5–5.5 µm wide, fragmenting by the septa when ageing, with lateral appendages. *Appendages* 0–1-septate, orange brown, asperulate, thick-walled, progressively tapering towards the apex, apex sinuous, 250–400 µm long, connected by basal knuckle joints. *Asci* 8-spored, globose or nearly so, 4–7 µm diam., soon deliquescent. *Ascospores* unicellular, hyaline at first, yellowish in mass when mature, smooth-walled under bright field microscope, globose, 2.5–3.5 µm diam.

Culture characteristics: Colonies on PYE reaching 46–47 mm diam. after 2 weeks at 25 °C, slightly elevated, velvety to floccose, margins regular, pale orange (5A3) at centre and white (5A1) at edge, sporulation sparse; reverse orange (5A6). Colonies on PDA reaching 36–37 mm diam. after 2 weeks at 25 °C, slightly elevated, velvety, margins regular, light orange (5A5) at centre and orange white (5A2) at edge, sporulation sparse; reverse deep orange (6A8). Colonies on PDA reaching 31–32 mm diam. after 2 weeks at 30 °C, slightly elevated, velvety, margins regular, slightly furrowed, orange (5A6), sporulation sparse; reverse brownish orange (6C8). Colonies on OA reaching 21–22 mm diam. after 2 weeks at 25 °C, slightly elevated, velvety, margins regular, orange white (5A2) at centre and white (5A1) at edge, sporulation sparse. Exudate and diffusible pigment absent in all culture media tested. Minimum, optimal and maximum temperature of growth on PDA: 10 °C, 25 °C, and 35 °C, respectively. Non-haemolytic. Casein hydrolyzed without pH change. Not inhibited by

cycloheximide. Urease and esterase tests positive. Growth occurs at NaCl 10 % w/w, but not at 20 % w/w.

Material examined: United States, Texas, from bronchial washing, XX-XX-2005, collected by N. Wiederhold (holotype CBS H-XXXX, cultures ex-type CBS XXXX = FMR 17681 = UTHSCSA DI18-87).

Notes. Malbranchea gymnoascoides is phylogenetically close to *M. ostraviense* and *M. umbrina* (Fig. 2). Nevertheless, *M. gymnoascoides* produces smaller ascomata (up to 250 µm diam. in *M. gymnoascoides vs*. up to 450 and up to 600 µm diam. in both, *M. ostraviense* and *M. umbrina*, respectively) (Orr *et al*. 1963, Hubka *et al.* 2013). Also, the peridial appendages of *M. gymnoascoides* are longer than those of *M. umbrina* (250–400 µm *vs.* 5–72 µm), but shorter than those of *M. ostraviense* (of 350–600 µm long). The ascospores of *M. gymnoascoides* are like those of *M. ostraviense* (smooth-walled under the bright field microscope, oblate to globose, 2.5–3.5 µm diam), whereas those of *M. umbrina* are lenticular and measure 2.8–4.0 × 2.1–2.6 µm. Moreover, the arthroconidia of *M. gymnoascoides* are larger than those of *M. umbrina* (6.0–10.0 × 1.5–2.0 µm and 2.6–7.0 × 1.4 µm, respectively). *Malbranchea ostraviense* also produces a pinkish to red diffusible pigment on MEA, PDA and SDA, a feature not observed in *M. gymnoascoides* nor in *M. umbrina*. Both *Malbranchea gymnoascoides* and *M. umbrina* can grow slowly at 35 ºC, whereas the maximum temperature of growth for *M. ostraviense* is 32 ºC.

Despite the strain FMR 17695 being phylogenetically close to *Malbranchea longispora*, it differs phylogenetically and morphologically from it. Consequently, we propose the erection of the new species *Malbranchea multiseptata*.

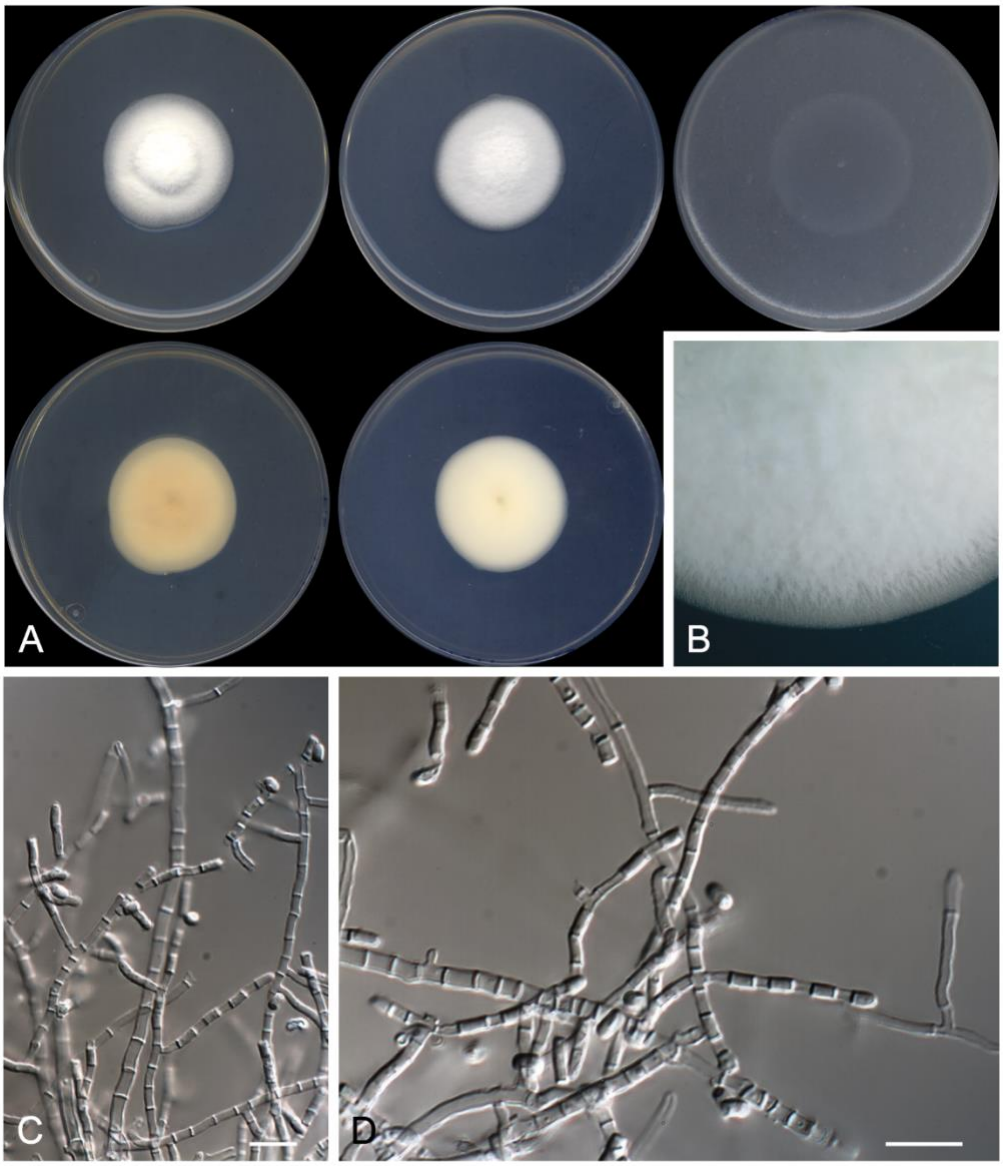

Fig. 8 *Malbranchea multiseptata* CBS 835213^{T}. A. Colonies on PYE, PDA and OA after 14 d at 25°C, from left to right (top row, surface; bottom row, reverse). B. Detail of the colony on PDA. C–D. Highly septate fertile hyphae and arthroconidia. Scale bar = 10 µm.

Malbranchea multiseptata Rodr.-Andr., Cano & Stchigel, **sp. nov.** MycoBank MB 835213. Fig. 8.

Etymology: From Latin *multi-*, many, and *–septatae*, septa, because the vegetative hyphae are multiseptate.

Micromorphology: Vegetative hyphae hyaline, smooth- and thin-walled straight to sinuous, sparsely branched, 1.0–2.0 µm wide, becoming highly septate with age, septa

thickened. *Fertile hyphae* arising as lateral branches (sometimes arranged opposite each other) from the vegetative hyphae, unbranched, straight or slightly sinuous, 1.5–2.0 µm wide, forming randomly intercalary and terminally arthroconidia. *Conidia* enteroarthric, unicellular, hyaline, smooth- and thin-walled, separated by evanescent connective cells, cylindrical, 3.0–9.0 × 1.5–2.0 µm, rounded at the end when terminal, rhexolytic secession. *Chlamydospores*, *racquet hyphae*, *setae*, and *sexual morph* not observed.

Culture characteristics: Colonies on PYE reaching 35–36 mm diam. after 2 weeks at 25 °C, elevated, velvety to floccose, margins regular, white (5A1), sporulation sparse; reverse greyish yellow (4B4). Colonies on PDA reaching 34–35 mm diam. after 2 weeks at 25 °C, slightly elevated, velvety to floccose, margins regular, white (5A1), sporulation absent; reverse yellowish white (3A2). Colonies on PDA reaching 27–28 mm diam. after 2 weeks at 30 ºC, slightly elevated, velvety to floccose, margins regular, white (5A1), sporulation absent; reverse pale yellow (3A3). Colonies on OA researching 37–38 mm diam. after 2 weeks at 25 °C, flattened, barely perceptible growth, not distinguishable colour, sporulation sparse. Exudate and diffusible pigment absent in all culture media tested. Minimum, optimal and maximum temperature of growth on PDA: 10 °C, 25 °C, and 35 °C, respectively. Haemolytic. Casein hydrolyzed without pH change. Not inhibited by cycloheximide. Urease positive. The fungus grows at NaCl 3 % w/w, but not at 10 %w/w. Neither grow on TOTM.

Material examined: United States, Texas, from human bronchial washing, XX-XX-2014, collected by N. Wiederhold (holotype CBS H-XXXX, cultures ex-type CBS XXXX = FMR 17695 = UTHSCSA DI18-101).

Notes: Malbranchea multiseptata is phylogenetically linked to *M. longispora*. Nevertheless, *M. multiseptata* does not form chlamydospores nor a sexual morph as in *M. longispora* (Crous *et al*. 2013). Also, *M. multiseptata* produces shorter conidia (3.0–9.0 × 1.5–2.0 µm) than those of *M. longispora* (4.0–24.0 × 1.0–5.5 µm).

Because the strain FMR 17680 was placed phylogenetically close to *Malbranchea filamentosa* but in a separate terminal branch, and because both differ morphologically and genotypically, the new species *Malbranchea stricta* is proposed.

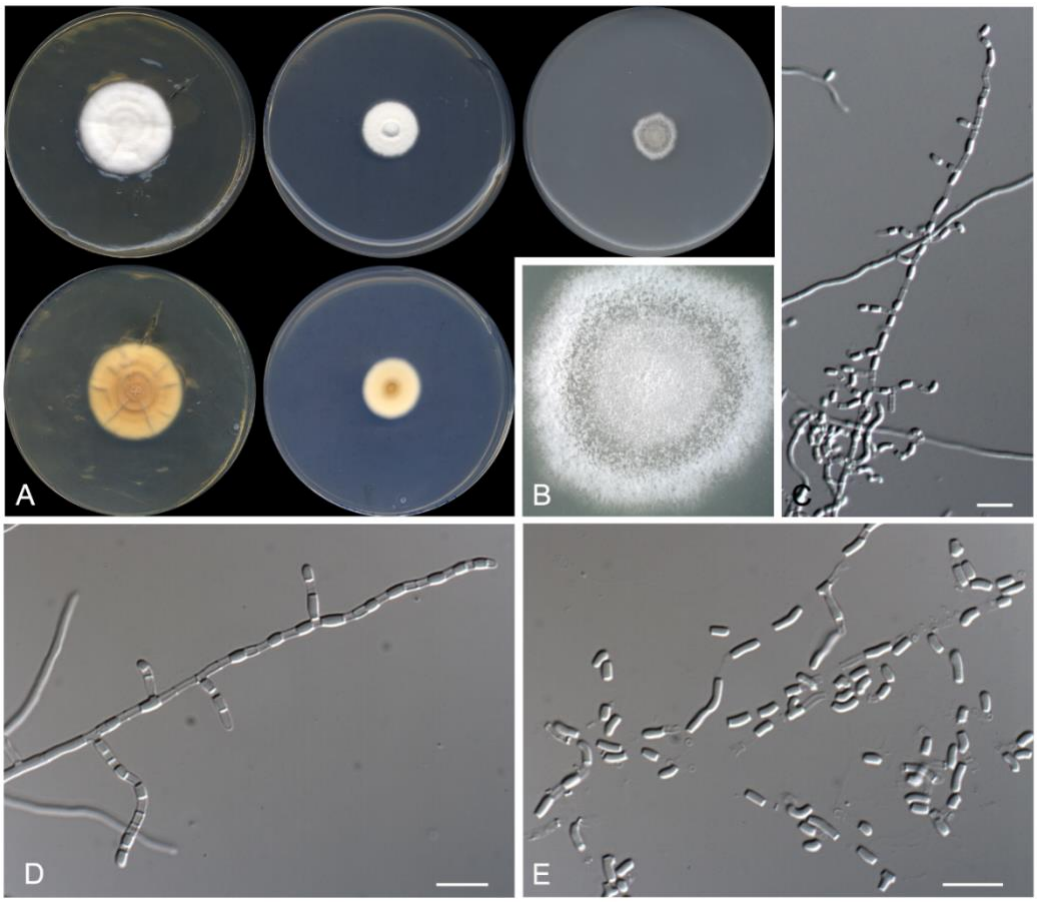

Fig. 9 *Malbranchea stricta* CBS 835219^{T}. A. Colonies on PYE, PDA and OA after 14 d at 25°C, from left to right (top row, surface; bottom row, reverse). B. Detail of the colony on OA. C–E. Alternate arthroconidia on primary hyphae and lateral branches. Scale bar = 10 µm.

Malbranchea stricta Rodr.-Andr., Stchigel & Cano, **sp. nov.** MycoBank MB XXXX. Fig. 9.

Etymology: Latin *stricta*, strict, due to the production of the typical reproductive structures of the genus.

Micromorphology: Vegetative hyphae hyaline, smooth- and thin-walled, straight to sinuous, sparsely branched, 1.5–2.0 µm wide. *Fertile hyphae* well-developed, arising as lateral branches from the vegetative hyphae, mostly unbranched, right or slightly sinuous, contorted or arcuate at the end, up to 25 µm long, 1.5–2.0 µm wide, or developing at the extremes of the vegetative hyphae, in both cases forming arthroconidia randomly intercalary and terminally. *Arthroconidia* enteroarthric, hyaline, becoming yellowish with the age, barrel-shaped, “T”-shaped, “Y”-shaped, finger-shaped or irregularly-shaped 2.0–6.0 × 1.0–2.0 µm, with rhexolytic secession. *Chlamydospores*, *racquet hyphae*, and *sexual morph* not observed.

Culture characteristics: Colonies on PYE reaching 32–33 mm diam. after 2 weeks at 25 °C, flattened, velvety, regular margins, furrowed, white (4A1), sporulation sparse; reverse pale orange (5A3). Colonies on PDA reaching 20–21 mm diam. after 2 weeks at 25 °C, slightly elevated, velvety to floccose, regular margins, white (3A1), sporulation abundant; reverse pale yellow (4A3). Colonies on PDA reaching 20–21 mm diam. after 2 weeks at 30 ºC, slightly elevated, velvety to floccose, margins regular, white (3A1), sporulation abundant; reverse yellowish brown (5E8) at centre and greyish yellow (4B5) at the margins. Colonies on OA researching 16–17 mm diam. after 2 weeks at 25 °C, flattened, granulose, white (3A1), margins regular, sporulation sparse. Exudate and diffusible pigment absent. Minimum, optimum and maximum temperature of growth (on PDA): 10 °C, 30 °C, and 37 °C, respectively. Colonies haemolytic (on BA), and casein hydrolyzed without pH changes at 25 °C (on BCP-MS-G). Not inhibited by cycloheximide. Urease and esterase tests positive. Growth occurs at NaCl 10 % w/w, but not at 20 % w/w.

Material examined: United States, Florida, human nail, XX-XX-2003, collected by N. Wiederhold (holotype CBS H-XXXX, cultures ex-type CBS XXXX = FMR 17680 = UTHSCSA DI18-86).

Notes: Malbranchea stricta is phylogenetically close to *M. filamentosa*. Also, both species lack a sexual morph (Sigler *et al*. 2002). However, *M. filamentosa* produces more regularly shaped conidia than *M. stricta*, and forms thick-walled brown setae, structures absent in *M. stricta*.

Dichotomous key to *Malbranchea* spp. (adapted from Sigler & Carmichael 1976, Solé *et al.* 2002, and Hubka *et al.* 2013).

1a. Homothallic species..... 2

1b. Heterothallic species..... 13

2a. Peridial appendages longer than 150 µm long..... 3

2b. Peridial appendages shorter or absent..... 8

3a. Appendages 350–600 µm in length; diffusible pigment pinkish to reddish; not growing at 35 ºC..... *M. ostraviensis*

3b. Those features not combined..... 4

663 4a. Ascospores smooth-walled under bright field microscope..... ***M. gymnoascoides***

664 4b. Ascospores reticulate..... 5

665 5a. Peridial cells short, 4–12 µm in length; peridial projections with truncate
666 ends..... *M. compacta*

667 5b. Peridial cells longer; peridial projections with mostly acute ends..... 6

668 6a. Ascospores usually exceeding 4 µm diameter..... *M. californiensis*

669 6b. Ascospores ≤ 4 µm diameter..... 7

670 7a. Species growing at 37 °C..... *M. conjugata*

671 7b. No growth at 37 °C..... *M. umbrina*

672 8a. Asexual morph not produced..... *M. pseudoauxarthron*

673 8b. Malbranchea-like asexual morph present..... 9

674 9a. Ascomata with spine-like peridial projections, 27–40 µm in length..... *M. zuffiana*

675 9b. Ascomata without peridial projections..... 10

676 10a. Colonies on PDA brown..... *M. kuehnii*

677 10b. Colonies on PDA otherwise..... 11

678 11a. Peridial hyphae smooth-walled..... *M. concentrica*

679 11b. Peridial hyphae strongly ornamented; chlamydospores present.....12

680 12a. Arthroconidia 2–10 × 2.5–3.5 µm; growing above 30 ºC..... *M. chlamydospora*

681 12b. Arthroconidia 4–24 × 1.0–5.5 µm; not growing above 30 ºC..... *M. longispora*

682 13a. Fertile hyphae arcuate or curved..... 14

683 13b. Fertile hyphae straight to sinuous, branched or not..... 21

684 14a. Fertile hyphae coiled..... 15

685 14b. Fertile hyphae curved or arcuate..... 16

686 15a. Thermophilic; conidia 2.5–4.5 µm wide..... *M. cinnamomea*

687 15b. Not thermophilic; conidia narrower..... *M. pulchella*

688 16a. Colonies orange..... 17

689 16b. Colonies different..... 18

690 17a. Aleuroconidia laterally or terminally dispersed..... *M. chrysosporoidea*

691 17b. Aleuroconidia absent..... *M. aurantiaca*

692 18a. Colonies golden yellow, exudate brown, diffusible pigment yellow ..... *M. graminicola*

693 18b. Features are not combined.....19

19a. Sexual morph produced by *in vitro* mating of compatible strains..... *M. albolutea*

19b. Sexual morph not formed.....20

20a. Thick-walled brown setae produced on OA from the vegetative mycelium ..... *M. filamentosa*

20b. Setae not produced ..... *M. arcuate*

21a. Fertile hyphae unbranched or scarcely branched.....22

21b. Fertile hyphae branched.....23

22a. Arthroconidia cylindrical; becoming many septate with the age..... ***M. multiseptata***

22b. Arthroconidia barrel-shaped, "T"-shaped, "Y"-shaped, finger-shaped or irregular; vegetative hyphae regularly septate..... ***M. stricta***

23a. Fertile hyphae branching acutely, displaying a tree-like appearance..... *M. dendritica*

23b. Fertile hyphae branching pattern otherwise.....24

24a. Fertile hyphae repeatedly branched, in dense tufts..... *M. flocciformis*

24b. Fertile hyphae more restrictedly branched.....25

25a. Colonies buff or tan..... *M. fulva*

25b. Colonies lemon yellow..... *M. flava*

Despite the strain FMR 17684 being placed phylogenetically into the *Onygenaceae*, is paraphyletic and distant from the other members of the family, therefore this fungus is proposed as the type species of the new genus *Pseudomalbranchea*.

Pseudomalbranchea Rodr.-Andr., Cano & Stchigel, **gen. nov.** MycoBank MB 835220.

Etymology. Because the morphological similarity with *Malbranchea*.

Micromorphology: Mycelium sparse, composed of hyaline, smooth- and thin-walled septate hyphae. *Asexual morph* consisting of mostly enteroarthric –occasionally holoarthric– conidia, intercalary disposed along unbranched vegetative hyphae, solitary or in short chains, with rhexolytic or rarely schizolysic secession. *Arthroconidia* one-celled, hyaline, smooth- and thick-walled, cylindrical but becoming globose with age. *Chlamydospores, racquet hyphae* and s*exual morph* not observed.

Type species: Pseudomalbranchea gemmata Rodr.-Andr., Cano & Stchigel. MycoBank MB 835221.

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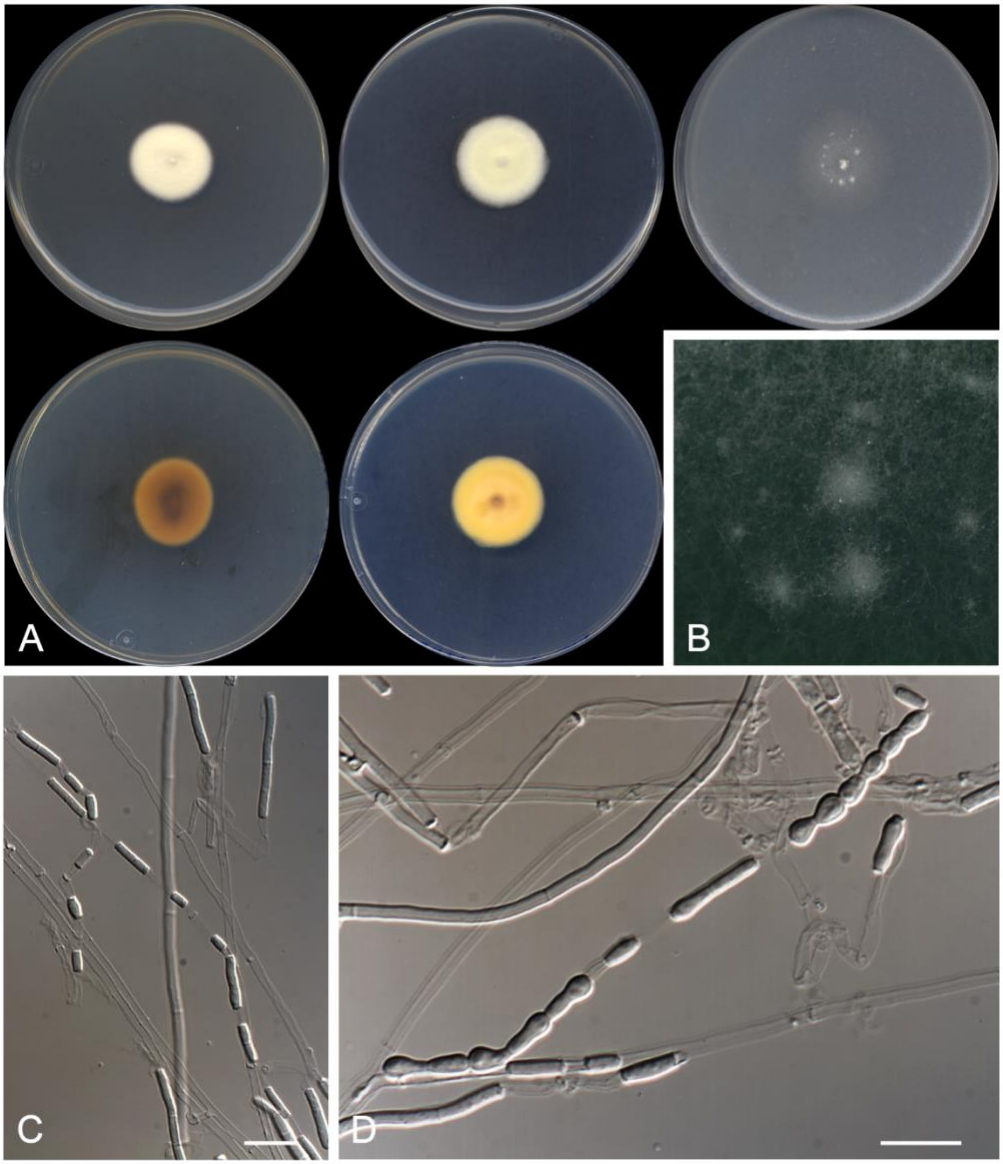

725

726 **Fig. 10** *Pseudomalbranchea gemmata* CBS 835221^{T}. A. Colonies on PYE, PDA and OA after 14 d at 25°C, from
727 left to right (top row, surface; bottom row, reverse). B. Detail of the colony on OA. C–D. Large, intercalary,
728 irregularly-shaped arthroconidia disposed singly or in chains along the fertile hyphae. Scale bar = 10 µm.

729 ***Pseudomalbranchea gemmata*** Rodr.-Andr., Cano & Stchigel, **sp. nov.** MycoBank MB
730 835221. Fig. 10.

731 *Etymology:* From the Latin *gemmatum*, jewelled, because the swollen conidia disposed in
732 chains.

733 *Micromorphology: Mycelium* sparse, composed of hyaline, smooth- and thin-walled,
734 sparsely septate hyphae, 1.0–2.0 µm wide. *Conidia* enteroarthric (occasionally holoarthric),

intercalary disposed along unbranched vegetative hyphae, one-celled, solitary or in short chains of up to 7, one-celled, hyaline, smooth- and thick-walled, cylindrical but becoming globose with the age, 4.0–11.0 × 2.0–3.5 µm, liberated from the fertile hyphae by rhexolysis (rarely by schizolysis). *Chlamydospores*, *racquet hyphae* and *sexual morph* not observed.

Culture characteristics: Colonies on PYE reaching 22–23 mm diam. after 2 weeks at 25 °C, slightly elevated, velvety, margins regular, pale yellow (3A3), sporulation sparse; reverse brown (6E6). Colonies on PDA reaching 24–25 mm diam. after 2 weeks at 25 °C, slightly elevated, velvety, margins regular, pale yellow (3A3), sporulation sparse; reverse light yellow (4A5). Colonies on PDA reaching 25–26 mm diam. after 2 weeks at 30 ºC, flattened, radially folded, velvety, margins regular, pale yellow (3A3), sporulation sparse; reverse light yellow (4A5). Colonies on OA reaching 28–29 mm diam. after 2 weeks at 25 °C, flattened, velvety to granulose, irregular margins, white (6A1), sporulation sparse. Exudate and diffusible pigment lacking. *Minimum, optimum and maximum temp*erature of growth: 10 °C, 30 °C, and 37 °C, respectively. Colonies haemolytic, casein not hydrolyzed. The fungus was not inhibited by cycloheximide. Urease and esterase tests positive. Growth occurs at NaCl 3 % w/w, but not higher concentrations.

Material examined: United States, Florida, from human bronchial washing, XX-XX-2014, collected by N. Wiederhold (holotype CBS H-XXXX, cultures ex-type CBS XXXX = FMR 17684 = UTHSCSA DI18-90).

Notes: Pseudomalbranchea gemmata is phylogenetically close to *Uncinocarpus reesii* and *Amauroascus volatilis-patellis*. However, it does not produce a sexual morph and it differs from *U. reessi* and *A. volatilis-patellis* by the production of longer arthroconidia (4.0–11.0 × 2.0–3.5 µm in *P. gemmata* vs. 3.5–6.0 × 2.5–3 µm in *U. reessi*, and 4.0–5.4 × 2.0–3.0 in *A. volatilis-patellis*; Orr & Kuehn 1972, Sigler & Carmichael 1976, Currah 1985). As well as *A. volatilis-patellis*, *P. gemmata* lacks appendages, which are present and similar to the asexual morph in *U. reessi* (Currah 1985).

Because the strains FMR 17686 and FMR 17696 were placed together into a terminal branch closely related to the ex-type strain of *M. gypsea* in the *Spiromastigaceae* clade (Fig. 2), *M. gypsea* is renamed as *Spiromastigoides gypsea*, and the former strains are proposed as belonging to the new species *Spiromastigoides geomycoides*.

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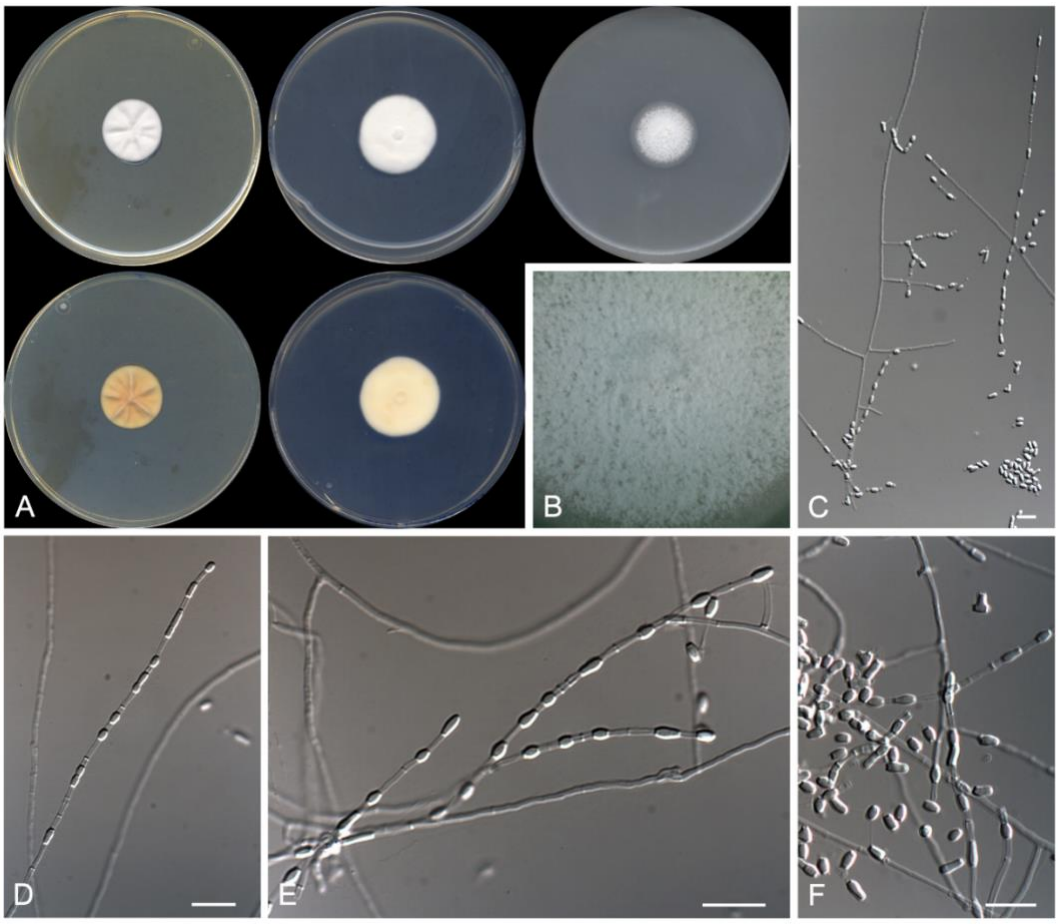

766

767 **Fig. 11** *Spiromastigoides geomyces* CBS 835222[T]. A. Colonies on PYE, PDA and OA after 14 d at 25°C, from
768 left to right (top row, surface; bottom row, reverse). B. Detail of the colony on OA. C. Fertile lateral branches
769 mimicking *Geomyces* spp. conidiophores. D–E. Fertile hyphae with intercalary, barrel-shaped arthroconidia. F.
770 Morphological diversity of arthroconidia. Scale bar = 10 µm.

771

772 ***Spiromastigoides geomycoides*** Stchigel, Rodr.-Andr. & Cano, **sp. nov.** MycoBank MB
773 835222. Fig. 11.

774 *Etymology:* Because of the production of conidiophores morphologically similar to those
775 of *Geomyces*.

776 *Micromorphology: Mycelium* abundant, composed of hyaline, smooth- and thin-walled,
777 septate, branched, 1.0–2.0 µm wide hyphae, septa thickened with age. *Fertile hyphae* arising
778 as lateral branches, straight or slightly curved, unbranched or, rarely, with a branching
779 pattern similar to that of the conidiophores of *Geomyces*, septate, hyaline, smooth- and thin-

780 walled, producing intercalary and terminally arthroconidia separated by 1–2 empty
781 intermediary cells. *Conidia* enteroarthic, unicellular, hyaline, mostly barrel-shaped, less
782 frequently "T"-shaped or cylindrical, 1.5–2.5 × 1.0–2.0 µm, rhexolytic dehiscence.
783 *Chlamydospores*, *racquet hyphae* and *sexual morph* not observed.

784 *Culture characteristics*: Colonies on PYE reaching 24–25 mm diam. after 2 weeks at 25 °C,
785 flattened, velvety, furrowed, regular margins, white (4A1), abundant sporulation; reverse,
786 pale orange (5A3). Colonies on PDA reaching 26–27 mm diam. after 2 weeks at 25 °C,
787 flattened, velvety, regular margins, white (4A1), abundant sporulation; reverse, yellowish
788 white (4A2). Colonies on PDA reaching more than 90 mm diam. after 2 weeks at 30 ºC,
789 flattened, velvety, regular margins, yellowish white (4A2), sporulation absent; reverse, pale
790 yellow (4A3). Colonies on OA researching 20–21 mm diam. after 2 weeks at 25 °C, flattened,
791 granulose, regular margins, white (4A1), abundant sporulation. Exudate and diffusible
792 pigment absent in all culture media tested. Minimum, optimum and maximum temperature
793 of growth on PDA: 5 °C, 30 °C, and 37 °C, respectively. Colonies non-haemolytic. Casein not
794 hydrolyzed. Resistent to cycloheximide. Urease negative and esterase positive. The fungus
795 grows at NaCl 10 % w/w, but not at 20 % w/w.

796 *Material examined:* United States, Illinois, from human foot skin, XX-XX-2014, collected
797 by N. Wiederhold (holotype CBS H-XXXX, cultures ex-type CBS XXXX = FMR 17696 = UTHSCSA
798 DI18-102).

799 *Other specimens examined*.:FMR 17686, United States, Minnesota, from blood, XX-XX-
800 2009, collected by N. Wiederhold.

801 *Notes: Spiromastigoides geomycoides* is phylogenetically close to *S. gypsea*. However, *S.*
802 *geomyces* produces smaller conidia (1.5–2.5 × 1.0–2.0 µm) than *S. gypsea* [(2.5)3–6(9) × 2–
803 2.5 µm] (Sigler & Carmichael 1976). Also, *S. geomyces* grows faster than *S. gypsea* on PYE at
804 35 ºC.

805 ***Spiromastigoides gypsea*** *(Sigler & Carmichael) Stchigel, Rodr.-Andr. & Cano* ***comb. nov.***
806 *MycoBank MB 835228.*

807 *Basionym*: *Malbranchea gypsea* Sigler & Carmichael, *Mycotaxon* 4: 455 (1976) [MycoBank
808 MB 317129].

Description (adapted from the original work): Arthroconidia produced intercalary or terminally along of straight primary hyphae, or on short or long lateral branches, separated each one by one or more alternate empty cells, or, rarely, formed immediately adjacent to each other. *Arthroconidia* unicellular, hyaline, smooth- and thin-walled, cylindrical or slightly barrel-shaped, (2.5) 3–6 (9) × 2–2.5 µm, slightly broader than the interconnecting cells. No sexual morph obtained by matting. Colonies on PYE reaching 17–39 mm after three weeks at room temperature, chalky white to creamy white, downy to velvety, slightly raised, surface folded to convoluted, umbonated at centre, reverse buff. Optimum temperature of growth 25–30 ºC. Maximum temperature of growth 37 ºC (but strain dependent).

Dichotomous key to *Spiromastigoides* spp. (adapted from Hirooka *et al.* 2016).

1a. Homothallic..... 2

1b. Heterothallic..... 6

2a. Ascospores globose to subglobose, reticulate..... *S. sphaerospora*

2b. Ascospores oblate, equatorial thickening present or not..... 3

3a. Ascospores with equatorial thickening..... 4

3b. Ascospores without such equatorial thickening..... 5

4a. Ascomata appendages straight or slightly undulate; ascospores yellow, smooth-walled, pitted under SEM..... *S. alatospora*

4b. Ascomata appendages slightly undulate or wavy; ascospores pale yellowish brown, minutely punctate under SEM..... *S. saturnispora*

5a. Ascospores punctate, sometimes with a few fine grooves in the polar region, 2.5–2.9 × 2.0–2.5 µm..... *S. warcupii*

5b. Ascospores lens-shaped, regularly pitted, 3.0 × 2.0 µm..... *S. sugiyamae*

6a. Asexual morph chrysosporium-like; sterile ascomata present..... *S. asexualis*

6b. Asexual morph not so..... 7

7a. Asexual morph malbranchea-like..... 8

7b. Asexual morph more complex..... 11

8a. Fertile hyphae straight, branched..... ***S. gypsea***

8b. Fertile hyphae curved..... 9

9a. Fertile hyphae successively branched to form sporodochia-like structures..... *S. albida*

9b. Fertile hyphae unbranched or scarcely branched..... 10

10a. Fertile hyphae unbranched or sparsely branched, curved, up to 28 µm long; chlamydospores present..... *S. curvata*

10b. Fertile hyphae unbranched, slightly curved, up to 15 µm long; chlamydospores absent..... *S. minimus*

11a. Conidiophores unbranched or scarcely branched..... ***S. geomycoides***

11b. Conidiophores branched several times..... 12

12a. Conidiophores up to 300 µm in length, verticillate..... *S. kosraensis*

12b. Conidiophores 100–150 µm in length, with pyramidal or bush-like branching..... 13

13a. Conidiophores up to 150 µm long, with pyramidal branching..... *S. pyramidalis*

13b. Conidiophores up to 100 mm long, with bush-like branching..... *S. frutex*

In vitro **antifungal susceptibility testing**

The results of the antifungal susceptibility testing are summarized in Table 2. In general, the echinocandins (AFG, CFG and MFG) displayed the most potent *in vitro* antifungal activity, whileTRB and PSC also demonstrated good activity against these fungi. In contrast, limited to no inhibition of growth was observed with AMB, FLC, ITC and 5-FC. Antifungal activity was evaluated against all strains with the exception of FMR 17691, due to the scarce production of conidia and because this strain does not grow in RPMI medium, even after two weeks of incubation.

DISCUSSION

To our knowledge, this is the main study of malbranchea-like fungi from a clinical origin. We have shown that several of these fungi have not been reported previously from human specimens, and although the pathologic role remains uncertain, their diversity is of interest since some represent new species.

Morphological and physiological characterization and phylogenetic analysis has allowed us to identify fifteen strains as belonging to the genus *Malbranchea* (syn. *Auxarthron*), of which three of them are proposed as new species. These results indicate a

high diversity of onygenalean fungi in these sort of specimens, which may be difficult to differentiate only by phenotypic characteristics.

All strains belonging to *Malbranchea* displayed thermotolerance, suggesting the potential pathogenicity of this genus in animals, including humans, as has been previously noted by others (Saccardo 1908, Saccardo & Trotter 1913, Cooney & Emerson 1964, Sigler & Carmichael 1976). In fact, all them were able to grow at 30 ºC, and most of them at 35-37 ºC.

Malbranchea-like fungi were most commonly isolated from the respiratory tract (40 %), followed by nails and skin (27.2 %). *Currahmyces sparsispora*, *Malbranchea albolutea*, *M. conjugata*, *M. gymnoascoides*, *M. multiseptata*, *Pseudoarthropsis crassispora* and *Pseudomalbranchea gemmata* were all recovered from respiratory tract specimens (mostly obtained by bronchial-alveolar washing), while those of *M. umbrina* were isolated from the widest variety of anatomical sites. The rest of the taxa isolated were mostly from skin and annexes.

Regarding to the antifungal susceptibility of malbranchea-like fungi, limited data are available. However, in a previous study of onychomycosis-causing strains of *Auxarthron ostraviense* and *Auxarthron umbrinum* (transferred to the genus *Malbranchea* in the present study) reduced susceptibility to AMB, ITC and PSC was reported, but a high susceptibility to TRB was observed (Hubka *et al*. 2013). Another study (Gupta & Kohli 2003) showed that strains of *Arachnomyces nodosetosus* (syn. *Onychocola canadensis*) where highly susceptible to cicciclopirox and TRB. Our results are consistent with such previous studies, but we also demonstrated the enhanced susceptibility of the malbranchea-like fungi to the echinocandins.

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Table 1. DNA barcodes used to build the phylogenetic tree.

Species	Strains[1]	GenBank accession #[2]		Geographic origin and source
		ITS[3]	LSU[3]	
Ajellomyces capsulatus	UAMH 3536 ^{T}	AF038354	AF038354	Alberta, Canada; woman, 25-years-old, biopsy of right middle lobe lung
Amauroascus niger	ATCC 22339	MH869547	AY176706	California, U.S.A.; soil
Amauroascus purpureus	IFO 32622^{T}	AJ271564	AY176707	Japan; soil
Amauroascus volatilis-patellis	CBS 249.72^{T}	MH860467	MH872189	Utah, U.S.A.; soil
Aphanoascus mephitalis	ATCC 22144	MH859941	AY176725	Ontario, Canada; wolf dung
Arachniotus verruculosus	CBS 655.71	NR_145221	AB040684	Utah, U.S.A.; soil
***Arachnomyces bostrychodes* sp. nov.**	**UTHSCSA DI18-91 = FMR 17685 = CBS XXXXX ^{T}**	**LR701765**	**LR701766**	**Texas, U.S.A.; human scalp**
Arachnomyces glareosus	CBS 116129^{T}	AY624316	FJ358273	Alberta, Canada; man, 30-years-old, thumb nail
***Arachnomyces graciliformis* sp. nov.**	**UTHSCSA DI18-97 = FMR 17691 = CBS XXXXX ^{T}**	**LR743667**	**LR743668**	**Massachusetts, U.S.A.; animal bone**
Arachnomyces gracilis	UAMH 9756^{T}	AY123779	-	Uganda; termitarium soil
Arachnomyces jinanicus	CGMCC3.14173^{T}	KY440749	KY440752	Jinan, China; pig farm soil

Arachnomyces kanei	UAMH 5908^{T}	AY123780	-	Toronto, Canada; human nail
Arachnomyces minimus	CBS 324.70 ^{T}	AY123783	FJ358274	Ontario, Canada; decaying wood
Arachnomyces nitidus	UAMH 10536	-	AB075351	Israel; twigs
Arachnomyces nodosetosus	CBS 313.90^{T}	AY123784	AB053452	Saskatchewan, Canada; woman, 67-years-old, onychomycosis
Arachnomyces peruvianus	CBS 112.54 ^{T}	MF572315	MH868792	Peru; *Globodera rostochiensis* cyst
Arachnomyces pilosus	CBS 250.93^{T}	MF572320	MF572325	Catalonia, Spain; river sediment
Arachnomyces scleroticus	UAMH 7183^{T}	AY123785	-	Sulawesi, Indonesia; poultry farm soil
Arthroderma curreyi	CBS 353.66^{T}	MH858822	MH870459	UK; unknown
Arthroderma onychocola	CBS 132920^{T}	KT155794	KT155124	Prague, Czech Republic; human nail
Ascosphaera apis	CBS 252.32	-	AY004344	København, Denmark; *Apis mellifera*
Ascosphaera subglobosa	A.A. Wynns 5004 (C)^{T}	NR_137060	HQ540517	Utah, U.S.A.; pollen provisions of *Megachile rotundata*
Auxarthronopsis bandhavgarhensis	NFCCI 2185 ^{T}	HQ164436	NG_057012	Bandhavgarh, India; soil
Auxarthronopsis guizhouensis	CGMCC3.17910^{T}	KU746668	KU746714	Guizhou, China; air

Blastomyces percusus	CBS 139878^{T}	NR_153647	KY195971	Israel; human granulomatous lesions
Canomyces reticulatus	MCC 1486^{T}	MK340501	MK340502	Maharashtra, India; soil
Chrysosporium keratinophilum	CBS 392.67	MH859002	AY176730	New Zealand; soil
Chrysosporium tropicum	MUCL 10068^{T}	MH858134	AY176731	Guadalcanal, Solomon islands; woollen overcoat
Currahmyces indicus	MCC 1548^{T}	MK340498	MK340499	Maharashtra, India; hen resting area
***Currahmyces sparsispora* sp. nov.**	**UTHSCSA DI18-89 = FMR 17683 = CBS XXXXX ^{T}**	**LR723272**	**LR723273**	**Florida, U.S.A.; human sputum**
Gymnoascus reesii	CBS 410.72	MH860507	MH872224	California, U.S.A.; soil
Helicoarthrosporum mellicola	CBS 143838^{T}	LR761645	LT906535	Granada, Spain; honey
Helicoarthrosporum mellicola	FMR 15673	LR761646	LT978462	Valencia, Spain; honey
Malbranchea albolutea	**UTHSCSA DI18-85 = FMR 17679**	**LR701834**	**LR701835**	**Texas, U.S.A.; human BAL**
Malbranchea albolutea	**UTHSCSA DI18-95 = FMR 17689**	**LR701836**	**LR701837**	**Texas, U.S.A.; human BAL**
Malbranchea albolutea	CBS 125.77 ^{T}	MH861039	MH872808	Utah, U.S.A.; soil
Malbranchea aurantiaca	**UTHSCSA DI18-94 = FMR 17688**	**LR701824**	**LR701825**	**California, U.S.A.; animal**
Malbranchea aurantiaca	**UTHSCSA DI18-88 = FMR 17682**	**LR701826**	**LR701827**	**Texas, U.S.A.; animal skin lesion**

Malbranchea aurantiaca	CBS 127.77 ^{T}	NR_157447	AB040704	Utah, U.S.A.; culture contaminant
Malbranchea californiensis	ATCC 15600 ^{T}	MH858121	NG_056947	California, U.S.A.; dung of pack rat
Malbranchea chrysosporioidea	CBS 128.77 ^{T}	AB361632	AB359413	Arizona, U.S.A.; soil
Malbranchea circinata	ATCC 34526 ^{T}	MN627784	MN627782	Utah, U.S.A.; soil
Malbranchea conjugata	**UTHSCSA DI18-105 = FMR 17699**	**LR701828**	**LR701829**	**Florida, U.S.A.; human lung tissue**
Malbranchea conjugata	**UTHSCSA DI18-103 = FMR 17697**	**LR701830**	**LR701831**	**Texas, U.S.A.; human BAL**
Malbranchea conjugata	CBS 247.58	NR_121475	HF545313	Arizona, U.S.A.; soil
Malbranchea dendritica	CBS 131.77^{T}	AY177310	AB359416	Utah, U.S.A.; soil
Malbranchea filamentosa	CBS 581.82^{T}	NR_111136	AB359417	Argentina; soil
Malbranchea flava	CBS 132.77 ^{T}	AB361633	AB359418	California, U.S.A.; soil
Malbranchea flavorosea	ATCC 34529 ^{T}	NR 158362	AB359419	California, U.S.A.; soil
Malbranchea flocciformis	**UTHSCSA DI18-104 = FMR 17698**	**LR701822**	**LR701823**	**Texas, U.S.A.; human skin**
Malbranchea flocciformis	CBS 133.77 ^{T}	AB361634	AB359420	France; saline soil
Malbranchea fulva	CBS 135.77 ^{T}	NR_157444	AB359422	Utah, U.S.A.; air

***Malbranchea gymnoascoides* sp. nov.**	**UTHSCSA DI18-87 = FMR 17681 = CBS XXXXX ^{T}**	**LR701757**	**LR701758**	**Texas, U.S.A.; human BAL**
Malbranchea kuehnii	CBS 539.72 ^{T}	NR_103573	NG_056928	Unkown; dung
Malbranchea longispora	FMR 12768 ^{T}	HG326873	HG326874	Beija, Portugal; soil
***Malbranchea multiseptata* sp. nov.**	**UTHSCSA DI18-101 = FMR 17695 = CBS XXXXX ^{T}**	**LR701759**	**LR701760**	**Texas, U.S.A.; human BAL**
Malbranchea ostraviense	CBS 132919^{T}	NR_121474	-	Ostrava, Czech Republic; fingernail sample
Malbranchea pulchella	CBS 202.38	AB361638	AB359426	Italy; unknown
***Malbranchea stricta* sp. nov.**	**UTHSCSA DI18-86 = FMR 17680 = CBS XXXXX ^{T}**	**LR701638**	**LR701639**	**Florida, U.S.A.; human nail**
Malbranchea sp.*	CBS 319.61	MH858065	MH869635	California, U.S.A.; soil
Malbranchea umbrina	**UTHSCSA DI18-106 = FMR 17700**	**LR701814**	**LR701815**	**Colorado, U.S.A.; human BAL**
Malbranchea umbrina	**UTHSCSA DI18-107 = FMR 17701**	**LR701816**	**LR701817**	**Colorado, U.S.A.; human sinus**
Malbranchea umbrina	**UTHSCSA DI18-100 = FMR 17694**	**LR701818**	**LR701819**	**Baltimore, U.S.A.; human wound**
Malbranchea umbrina	**UTHSCSA DI18-99 = FMR 17693**	**LR701820**	**LR701821**	**Washington DC, U.S.A.; human nail**
Malbranchea umbrina	CBS 105.09 ^{T}	MH854591	MH866116	UK; soil

Malbranchea umbrina	CBS 226.58	MH857765	MH869296	Unknown
Malbranchea umbrina	CBS 261.52	MH857026	MH868556	UK; soil
Malbranchea zuffiana	**UTHSCSA DI18-96 = FMR 17690**	**LR701832**	**LR701833**	**Washington DC, U.S.A.; human wound**
Malbranchea zuffiana	CBS 219.58 ^{T}	MH869293	AY176712	Texas, U.S.A.; prairie dog lung
Nannizziopsis guarroi	CBS 124553 ^{T}	MH863384	MH874904	Barcelona, Spain; iguana skin
Nannizziopsis vriesii	ATCC 22444 ^{T}	AJ131687	AY176715	The Netherlands; Ameiva (lizard) skin and lung
Neogymnomyces demonbreunii	CBS 427.70	AJ315842	AY176716	Missouri, U.S.A.; unknown
Onychocola canadensis	CBS 109438	-	KT154998	Italy; nail and skin scrapings
Paracoccidioides brasiliensis	UAMH 8037^{T}	AF038360	AF038360	Alberta, Canada; man, 59-years-old, lung biopsy
Pseudoarthropsis cirrhata	CBS 628.83 ^{T}	-	NG_060792	Schiphol, The Netherlands; wall sample
***Pseudoarthropsis crassispora* sp. nov.**	**UTHSCSA DI18-98 = FMR 17692 = CBS XXXXX ^{T}**	**LR701763**	**LR701764**	**Minnesota, U.S.A.; human BAL**
***Pseudomalbranchea gemmata* gen. nov. et sp. nov.**	**UTHSCSA DI18-90 = FMR 17684 = CBS XXXXX ^{T}**	**LR701761**	**LR701762**	**Florida, U.S.A.; human BAL**
Pseudospiromastix tentaculata	CBS 184.9210536	AY527406	LN867603	Hiram, Somalia; soil
Renispora flavissima	CBS 708.79^{T}	AF299348	AY176719	Kansas, U.S.A.; soil in barn housing *Myotis velifer*

Spiromastigoides alatosporus	CBS 457.73^{T}	MH860740	AB075342	Madras, India; *Vigna sinensis* rhizosphere
Spiromastigoides albina	CBS 139510 ^{T}	LN867606	LN867602	Texas, U.S.A.; human lung biopsy
Spiromastigoides asexualis	CBS 136728^{T}	KJ880032	LN867603	Phoenix, U.S.A.; discospondylitis material from a German shepherd dog
Spiromastigoides curvata	JCM 11275 ^{T}	KP119631	KP119644	México; contaminant of a strain of *Histoplasma capsulatum*
Spiromastigoides frutex	CBS 138266 ^{T}	KP119632	KP119645	Nayarit, Mexico; house dust, rental studio
***Spiromastigoides geomycoides* sp. nov.**	**UTHSCSA DI18-92 = FMR 17686**	**LR701769**	**LR701770**	**Minnesota, U.S.A.; human blood**
***Spiromastigoides geomycoides* sp. nov.**	**UTHSCSA DI18-102 = FMR 17696 = CBS XXXXX ^{T}**	**LR701767**	**LR701768**	**Illinois, U.S.A.; human skin foot**
Spiromastigoides gypsea	CBS 134.77^{T}	KT155798	NG_063935	California, U.S.A.; soil
Spiromastigoides kosraensis	CBS 138267 ^{T}	KP119633	KP119646	Kosrae, Micronesia; house dust
Spiromastigoides pyramidalis	CBS 138269 ^{T}	KP119636	KP119649	Australia; house dust
Spiromastigoides sugiyamae	JCM 11276^{T}	LN867608	AB040680	Japan; soil
Spiromastigoides warcupii	CBS 576.63^{T}	LN867609	AB040679	Australia; soil

Strongyloarthrosporum capsulatus	CBS 143841[T]	LR760230	LT906534	Toledo, Spain; honey
Trichophyton bullosum	CBS 363.35[T]	NR_144895	NG_058191	Unkown
Uncinocarpus reesii	ATCC 34533	MH861035	AY176724	Australia; feather

[1]**ATCC**: American Type Culture Collection, Virginia, USA; **BCCM/MUCL**: Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; **CBS**: Culture collection of the Westerdijk Biodiversity Institute, Utrech, The Netherlands; **CGMCC**: China General Microbiological Culture Collection Center, Beijing, China; **FMR**: Facultat de Medicina, Reus, Spain; **IFO**: Institute for Fermentation Culture Collection, Osaka, Japan; **JCM**: Japan Collection of Microorganisms, Tsukuba, Japan; **MCC**: Microbial Culture Collection, Universite of Pune Campus Ganeshkhind, India; **NFCCI**: National Fungal Culture Collection of India, Maharastra, India; **UAMH**: University of Alberta Microfungus Collection and Herbarium, Alberta, Canada; **UTHSC**: Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, San Antonio, Texas, United States.

[2]Strains studied by us are indicated in **bold**.

[3]ITS: internal transcribed spacer region 1 and 2 including 5.8S nrDNA; LSU: large subunit of the nrRNA gene.

[T]Ex-type strain.

*Strain formerly assigned to *Auxarthron thaxteri* (a species synonymized with *Malbranchea umbrina*).

Table 2. Antifungal susceptibility of malbranchea-like strains studied.

Taxon	Strain	MIC/MEC (µg/mL)									
		AMB	**FLC**	**VRC**	**ITC**	**PSC**	**AFG**	**CFG**	**MFG**	**TRB**	**5-FC**
Arachnomyces bostrychodes	FMR 17685	16	16	2	16	16	0,03	0,06	0,06	.5	16
Currahmyces sparsispora	FMR 17683	16	16	4	16	2	16	8	16	0,03	16
Malbranchea albolutea	FMR 17679	8	16	1	1	0,25	0,03	0,06	0,06	0,25	16
	FMR 17689	8	16	2	16	1	0,12	0,06	0,25	0,25	16
M. aurantiaca	FMR 17682	16	16	1	16	0,25	0,12	1	0,12	4	16
	FMR 17688	16	16	2	16	0,5	0,5	0,06	1	2	16
M. conjugata	FMR 17697	8	16	0,5	0,25	0,03	0,06	0,25	0,25	1	16
	FMR 17699	16	16	0,5	2	0,5	0,12	0,25	0,25	1	16
M. flocciformis	FMR 17698	16	16	1	16	0,5	0,12	0,03	0,12	0,5	16
M. gymnoascoidea	FMR 17681	16	16	8	16	1	0,03	0,03	0,12	0,5	16
M. multiseptata	FMR 17695	6	16	0,12	0,5	0,25	0,03	0,5	2	1	16

M. stricta	FMR 17680	8	16	0,25	0,12	0,12	0,03	0,25	0,25	0,12	16
M. umbrina	FMR 17693	4	16	2	16	0,5	0,06	0,06	0,12	0,25	16
	FMR 17694	16	16	4	16	0,5	0,06	1	0,12	0,25	16
	FMR 17700	16	16	16	16	16	0,5	1	0,5	16	16
	FMR 17701	16	16	4	16	0,12	0,03	0,03	0,03	0,12	16
M. zuffiana	FMR 17690	16	16	1	16	0,5	0,05	1	4	0,25	16
Pseudomalbranchea gemmata	FMR 17684	2	16	0,25	0,25	0,25	6	1	6	0,03	16
Spiromastigoides geomyces	FMR 17686	16	16	2	1	1	16	2	16	0,12	16
	FMR 17696	16	16	2	0,5	0,5	2	6	16	0,06	16

AMB, amphotericin B; FLC, fluconazole; VRC, voriconazole; ITC, itraconazole; PSC, posaconazole; AFG, anidulafungin; CFG, caspofungin; MFG, micafungin; TRB, terbinafine; 5-FC, 5-fluorocytosine. ND*: Non-determined due to no fungal growth under the conditions stablished by the CLSI protocol.

UNIVERSITAT ROVIRA I VIRGILI
CARACTERIZACIÓN FENOTÍPICA Y FILOGENIA MOLECULAR DE HONGOS EXTREMÓFILOS
Ernesto Rodríguez Andrade

4.5. New xerophilic species of *Penicillium* from soil

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Article

New xerophilic species of *Penicillium* from soil.

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Abstract: During a suvey on biodiversity of soil-borne fungi in Spain and Mexico several strains of *Penicillium* were isolated. A phylogenetic analysis based on ITS, *BenA*, *CaM* and *rpb*2 nucleotide sequences showed that some these fungi were previously unknown for science. Consequently, we propose the erection of four new species: *Penicillium melanosporum*, *Penicillium michoacanense* and *Penicillium siccitolerans*, belonging to the section *Lanata-Divaricata*, and *Penicillium sexuale* of section *Torulomyces*. *Penicillium melanosporum* is characterized by its asexual morph, having divaricate monoverticillate conidiophores, the formation of brownish conidia covered by a brown sheath, and the latter of the produced conidia, which is remarkably ornate and dark, remains attached to the phialide. *Penicillium michoacanense* and *Penicillium siccitolerans* produce sclerotia and their asexual morph is similar to those species in the section *Aspergilloides*; however, whereas the sclerotial cells are thick-walled in *P. michoacanense*, these are thin-walled in *P. siccitolerans*. *Penicillium sexuale* differs from all species in its section in that it does not produce an asexual morph, and the sexual morph is characterized by the production of ascostromata of thick-walled polygonal cells and broadly lenticular ascospores with two widely separated equatorial ridges by a furrow. All four *Penicillium* species were able to grow at a water activity (a_w) of 0.76, reason why these species are considered as xerophilic.

Keywords: Ascomycota; Eurotiales; *Penicillium*; phylogeny; soil; taxonomy; xerophiles

1. Introduction

Soil is the natural reservoir of numerous organisms, such as algae, archaea, bacteria, fungi, protozoa, helminths and arthropods, forming populations that are in a dynamic ecological balance. Many of them are responsible for degradation of dead plants and animal remains to less complex molecules, contributing to the formation of humus and the maintenance of soil fertility [1]. The fungi grow in the space between soil particles, and organic matter and/or living roots provide of nutrients for their suitable development. They attack insoluble substrates that persisted after remosion of most of the soluble nutrients by early colonizers, by production of a broad spectrum of hydrolytic enzimes [2]. The products of enzymatic degradation are readily available, generating competition between different

organisms present in the same ecological niche. Many of these fungi produce substances with antibiotic activity, which help to them to compete more effectively against other microorganisms for shortly available nutritional resources. Among them, species of the genus *Penicillium* are always present. The genus *Penicillium*, erected by Link [3] to place three previously unknown fungi (*Penicillium candidum*, *Penicillium glaucum* and *Penicillium expansum*, the latter one corresponding to the type species of the genus), typically producing brush-like structures (conidiophores) responsible for the formation of the asexual spores (conidia). At the present, more than 350 species of *Penicillium* have been described [4, 5]; however, this genus includes more than 1,300 species names (Index of Fungi; http://www.indexfungorum.org/Names/Names.asp). Soil-borne *Penicillium* species are ubiquitous geographically, occurring in a wide range of environmental conditions [4]. Several of these are extremophilic, and able to proliferate at low or high temperatures, low pH values and at high salt or sugar concentrations [6, 7, 8, 9, 10, 11]. Regarding the latter group, only a few species have been reported as xerophilic, and consequently able to grow at or below 0.85 water activity (a_w) [12].

The aim of the present study was to resolve the taxonomical position and to identity several xerophilic isolates of *Penicillium* from soils in Spain and Mexico, based on the morphology and a multilocus sequence data phylogenetic analysis (ITS, *BenA*, *CaM* and *rpb*2).

2. Materials and Methods

2.1. Sampling and fungal strains studied

Soil samples were collected in Jiménez (Michoacán state, Mexico) and Riaza (Castilla y León community, Spain). Riaza village (41°16′59″N, 3°28′00″O) is at 1190 meters altitude. The climate is cool temperate Mediterranean, the average annual temperature is between 8 and 12°C, the average annual rainfall is above 700 mm, and soils are based on meteorized blackboards, quartzites and schists. The vegetation of Riaza is characterized by oaks (*Quercus pirenaica*), and includes a rich undergrowth formed by bushes and shrubs such as holly, maillo, rowan, blackthorn, hawthorn, bramble, wild rose, juniper, heather, steppe, rockrose, black and white broom, hornbill, heather, ferns, bearberry, pennyroyal, wild strawberry, raspberry, oregano, gammon, common chamomile, Benasque tea, bilberry, thyme, marjoram, violet, daisy, magarza, poppy, dandelion, digitalis, aconite, among others. Along the river and streams, species such as country elm, white poplar, black poplar, aspen, sagra, the common ash and white salguera are dominant. The most common bushes are wicker, arraclán, elderberry and honeysuckle. Mushrooms, mosses and lichens are abundant. El Zapote (19°57'14.9"N, 101°38'34.8"W) is a small village at 2100 meters above sea level. Its climate is temperate sub-humid with rains during summer, with an average annual temperature of 16-18 ºC and an average annual rainfall between 800 and 1.000 mm, and soils are of extrusive volcanic origin, the vast majority of which are destined to the cultivation of corn and sorghum. Samples were placed into plastic bags and sealed. Once in the lab, soil samples were stored at room temperature in the dark until be processed. The methodology used for fungal isolation was described by Stchigel et al. [13]. Approximately, 1 g of soil was placed into a test tube, mixed by shaking with 5 mL 2% (p/v) phenol (phenol crystal, Panreac, Barcelona, Spain), and leaving it to settle for for 10 min. Then, the supernatant was discarded and the sediment was resuspended in 10 mL of sterilized water. The suspensions (1.6 mL) were poured into 90 mm diam. dischartable Petri dishes and mixed with 15 mL of molten (at 50-55ºC) sterile potato-carrot agar medium (PCA; potatoes, 20 g; carrot, 20 g, 1 L tap water). After jellification of the culture medium, Petri dishes

were incubated at room temperature (22-25ºC) in the dark until 4-5 weeks. Cultures were examined periodically under stereomicroscope and, when the formation of reproductive structures was observed, these were transferred to 5 cm diam. Petri dishes containing potato dextrose agar (PDA; Pronadisa, Spain) [14] supplemented with L-chloramphenicol (100 mg / L), and after that incubated at 22-25 ºC in the dark.

2.2. Phenotypic study

Cultural characterization was performed following the recommendations of Visagie et al. [4]. Briefly, the isolates were grown using spores suspensions in a semi-solid agar (0.2 % agar; 0.05 % Tween 80) onto malt extract agar (MEA; Difco, Detroit, USA) [15], oatmeal agar (OA; 30 g of filtered oat flakes, 15 g agar-agar, 1 L tap water) [15], czapek yeast extract agar (CYA; 30 g sacarose, 3 g $NaNO_3$, 5 g yeast extract, 1 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4$-7·H_2O, 0.01 g $FeSO_4$, 15 g agar-agar, 1 L tap water) [16], yeast extract sucrose agar (YES; 20 g yeast extract, 150 g saccharose, 0.5 g $MgSO_4$-7·H_2O, 20 g agar-agar, 1 L tap water) [17], creatine sucrose agar (CREA; 3 g creatine, 30 g saccharose, 1.6 g K_3PO_4-7·H_2O, 0.5 g $MgSO_4$-7H_2O, 0.5 g KCl, 0.01 g $FeSO_4$-7·H_2O, 0.05 g bromocresol purple, 20 g agar-agar, 1 L tap water) [17], dichloran® 18 % glycerol agar (DG18; 31.5 g dichloran, 5 g peptone, 10 g dextrose, 1 g KH_2PO_4, 0.5 g $MgSO_4$-7·H_2O, 15 g agar-agar, 110 g glycerol, 1 L tap water; supplemented with 250 mg/L of L-chloramphenicol) [18], 25 % glycerol nitrate agar (G25N; 250 mL glycerol, 7.5 g Czapek concentrate, 3.7 g yeast extract, 0.75 g KH_2PO_4, 15 g agar-agar, 1 L tap water) [19] and malt extract yeast extract 70 % fructose-glucose (MY70FG; 6 g malt extract, 6 g yeast extract, 10 g peptone, 350 g fructose, 350 g glucose, 12 g agar-agar, 1 L tap water) [20] incubated at 25 ºC for 14 days in the dark. Colony diameters were measured after 14 days at 5, 15, 25, 30, 37 and 40 ºC on CYA to determine the minimum, optimum and maximum temperature of growth. Colour notations were according to Kornerup and Wanscher [21]. Microscopic characterization was performed after 14 days of growth on MEA at 25 ºC and mounting the fungal structures on a drop of lactophenol between slide and coverslide. Photomicrographs were taken by a Zeiss Axio-Imager M1 bright field microscope (Oberkochen, Germany) with a DeltaPix Infinity X digital camera, using Nomarski differential interference contrast and phase contrast optics. The novel taxonomic descriptions and the proposed names were deposited in MycoBank (http://www.mycobank.org) [22].

2.3. DNA extraction, amplification and sequencing

Total DNA was extracted directly from colonies on MEA after 7-10 days incubation at 25 ºC in the dark, through the modified protocol of Marimon et al. [23]. The DNA were used to amplify internal transcribed spacer (ITS) (ITS5/ ITS4) [24], a fragment of the beta-tubulin (*BenA*) (T10/Bt2b) [25], a fragment of the calmodulin (*CaM*) (Cmd5/Cmd6) [26] and a fragment of the RNA polymerase II subunit 2 gene (*rpb*2) (RPB2-5F/RPB2-7cR) [27]. Single band PCR products were purified and sequences at Macrogen Europe (Macrogen Inc., Madrid, Spain). Sequence assembly and editing were performed using SeqMan software v. 7.0 (DNAStar Lasergene, Madison, WI, USA). GenBank accession numbers for the sequences newly generated in this study are listed in Table 1

Table 1. *Penicillium* EMBL/GenBank codes used in this study.

Species name	Section	Strain no.	GenBank accession			
			ITS[3]	*BenA*	*CaM*	*rpb2*
Penicillium abidjanum	*Lanata-Divaricata*	CBS 246.67[1] = ATCC 18385 = FRR 1156 = IMI 136244 [T]	GU981582	GU981650	KF296383	JN121469
Penicillium amphipolaria	*Lanata-Divaricata*	DAOMC 250551 = CBS 140997 [T]	KT887872	KT887833	KT887794	-
Penicillium annulatum	*Lanata-Divaricata*	CBS 135126 = DTO 180-G7 [T]	JX091426	JX091514	JX141545	KF296410
Penicillium araracuaraense	*Lanata-Divaricata*	CBS 113149 = IBT 23247 [T]	GU981597	GU981642	KF296373	KF296414
Penicillium ausonae	*Lanata-Divaricata*	FMR 16948 [T]	LR655808	LR655809	LR655810	LR655811
Penicillium austrosinense	*Lanata-Divaricata*	CGMCC 3.18797 = CBS 144505 [T]	KY495007	KY495116	KY494947	KY495061
Penicillium bissettii	*Lanata-Divaricata*	DAOMC 167011 = CBS 140972 [T]	KT887845	KT887806	KT887767	-
Penicillium brasilianum	*Lanata-Divaricata*	CBS 253.55 = ATCC 12072 = FRR 3466 [T]	GU981577	GU981629	AB667857	KF296420

Penicillium brefeldianum	*Lanata-Divaricata*	CBS 235.81 = NRRL 710 = FRR 710 = IFO 31731 = IMI 216896 ^{T}	AF033435	GU981623	AB667857	KF296421
Penicillium camponotum	*Lanata-Divaricata*	DAOMC 250557 = CBS 140982 ^{T}	KT887855	KT887816	KT887777	-
Penicillium caperatum	*Lanata-Divaricata*	CBS 443.75 = ATCC 28046 ^{T}	KC411761	GU981660	KF296392	KF296422
Penicillium cataractum	*Lanata-Divaricata*	DAOMC 250534 = CBS 140974 ^{T}	KT887847	KT887808	KT887769	-
Penicillium cluniae	*Lanata-Divaricata*	CBS 326.89 ^{T}	KF296406	KF296471	KF296402	KF296424
Penicillium coeruleum	*Lanata-Divaricata*	CBS 141.45 ^{T}	GU981606	GU981655	KF296393	KF296425
Penicillium cremeogriseum	*Lanata-Divaricata*	CBS 223.66 = ATCC 18320 = ATCC 18323 = FRR 1734 = IMI 197492 = NRRL 3389 ^{T}	GU981586	GU981624	KF296403	KF296426
Penicillium curticaule	*Lanata-Divaricata*	CBS 135127 = DTO 180-D3 = DAOM 241159 ^{T}	FJ231021	JX091526	JX141536	KF296417
Penicillium daleae	*Lanata-Divaricata*	CBS 211.28 = ATCC 10435 = FRR 2025 = IFO 6087 = IFO 9072 ^{T}	GU981583	GU981649	KF296385	KF296427
Penicillium echinulonalgiovense	*Lanata-Divaricata*	CBS 328.59 = ATCC 18314 = FRR 638 = IFO 6229 = IMI 068213 ^{T}	GU981587	GU981631	KX961269	KX961301

Penicillium ehrlichii	*Lanata-Divaricata*	CBS 324.48 = ATCC 10442 = IMI 039737 = NRRL 708 ^{T}	AF033432	GU981652	KF296395	KF296428
Penicillium elleniae	*Lanata-Divaricata*	CBS 118135 = IBT 23229 ^{T}	GU981612	GU981663	KF296389	KF296429
Penicillium excelsum	*Lanata-Divaricata*	ITAL7572 = IBT 31516 ^{T}	KR815341	KP691061	KR815342	-
Penicillium flaviroseum	*Lanata-Divaricata*	CGMCC 3.18805 = CBS 144479 ^{T}	KY495032	KY495141	KY494972	KY495083
Penicillium glaucoroseum	*Lanata-Divaricata*	CBS 138908 = NRRL 908 (Not type)	KF296407	KF296469	KF296400	KF296430
Penicillium globosum	*Lanata-Divaricata*	CGMCC 3.18800 = CBS 144639 ^{T}	KY495014	KY495123	KY494954	KY495067
Penicillium griseoflavum	*Lanata-Divaricata*	CGMCC 3.18799 = CBS 144525 ^{T}	KY495011	KY495120	KY494951	KY495064
Penicillium griseopurpureum	*Lanata-Divaricata*	CBS 406.65 = ATCC 22353 = FRR 3429 = IFO 9147 = IMI 096157 ^{T}	KF296408	KF296467	KF296384	KF296431
Penicillium guangxiense	*Lanata-Divaricata*	CGMCC 3.18793 = CBS 144526 ^{T}	KY494986	KY495095	KY494926	-
Penicillium hainanense	*Lanata-Divaricata*	CGMCC 3.18798 = CBS 144527 ^{T}	KY495009	KY495118	KY494949	-
Penicillium infrabuccalum	*Lanata-Divaricata*	DAOMC 250537 = CBS 140983 ^{T}	KT887856	KT887817	KT887778	-

Penicillium janthinellum	*Lanata-Divaricata*	CBS 340.48 = ATCC 10455 = IMI 040238 = NRRL 2016 ^{T}	GU981585	GU981625	KF296401	JN121497
Penicillium javanicum	*Lanata-Divaricata*	CBS 341.48 = ATCC 9099 = IFO 31735 = IMI 039733 = MUCL 29099 = NRRL 707 ^{T}	GU981613	GU981657	KF296387	JN121498
Penicillium jianfenglingense	*Lanata-Divaricata*	CGMCC 3.18802 = CBS 144640 ^{T}	KY495016	KY495125	KY494956	KY495069
Penicillium koreense	*Lanata-Divaricata*	KACC 47721 ^{T}	KJ801939	KM000846	-	-
Penicillium laevigatum	*Lanata-Divaricata*	CGMCC 3.18801 = CBS 144481 ^{T}	KY495015	KY495124	KY494955	KY495068
Penicillium levitum	*Lanata-Divaricata*	CBS 345.48 = ATCC 10464 = IFO 6101 = IFO 8849 = IMI 039735 ^{T}	GU981607	GU981654	KF296394	KF296432
Penicillium limosum	*Lanata-Divaricata*	CBS 339.97 ^{T}	GU981568	GU981621	KF296398	KF296433
Penicillium lineolatum	*Lanata-Divaricata*	CBS 188.77 ^{T}	GU981579	GU981620	KF296397	KF296434
Penicillium ludwigii	*Lanata-Divaricata*	CBS 417.68 = FRR 559 ^{T}	KF296409	KF296468	KF296404	KF296435
Penicillium malacosphaerulum	*Lanata-Divaricata*	CBS 135120 = DTO 180-E6 = DAOM 241161 ^{T}	FJ231026	JX091524	JX141542	KF296438

Penicillium mariae-crucis	*Lanata-Divaricata*	CBS 271.83 = IMI 256075 [T]	GU981593	GU981630	KF296374	KF296439
Penicillium melanosporum	***Lanata-Divaricata***	**FMR 17424 [T]**	**LR655192**[2]	**LR655196**	**LR655200**	**LR655204**
Penicillium meloforme	*Lanata-Divaricata*	CBS 445.74 = ATCC 28049 = IMI 216903 [T]	KC411762	GU981656	KF296396	KF296440
Penicillium michoacanense	***Lanata-Divaricata***	**FMR 17612 [T]**	**LR655194**	**LR655198**	**LR655202**	**LR655206**
Penicillium ochrochloron	*Lanata-Divaricata*	CBS 357.48 = ATCC 10540 = IMI 039806 = NRRL 926 [T]	GU981604	GU981672	KF296378	KF296445
Penicillium onobense	*Lanata-Divaricata*	CBS 174.81 = ATCC 42225 [T]	GU981575	GU981627	KF296371	KF296447
Penicillium ortum	*Lanata-Divaricata*	CBS 135669 = DTO 180-I9 [T]	JX091427	JX091520	JX141551	KF296443
Penicillium oxalicum	*Lanata-Divaricata*	CBS 219.30 = ATCC 1126 = FRR 787 = IMI 192332 = MUCL 29047 [T]	AF033438	KF296462	KF296367	JN121456
Penicillium panissanguineum	*Lanata-Divaricata*	DAOMC 250562 = CBS 140989 [T]	KT887862	KT887823	KT887784	-
Penicillium paraherquei	*Lanata-Divaricata*	CBS 338.59 = ATCC 22354 = ATCC 46903 = FRR 3454 = IFO 6234 = IMI 068220 = NRRL 3454 [T]	AF178511	KF296465	KF296372	KF296449

Penicillium pedernalense	*Lanata-Divaricata*	CBS 140770 [T]	KU255398	KU255396	-	-
Penicillium penarojense	*Lanata-Divaricata*	CBS 113178 = IBT 23262 [T]	GU981570	GU981646	KF296381	KF296450
Penicillium piscarium	*Lanata-Divaricata*	CBS 362.48 = ATCC 10482 = FRR 1075 = IFO 8111 = IMI 040032 [T]	GU981600	GU981668	KF296379	KF296451
Penicillium pulvillorum	*Lanata-Divaricata*	CBS 280.39 = IFO 7763 = NRRL 2026 [T]	AF178517	GU981670	KF296377	KF296452
Penicillium raperi	*Lanata-Divaricata*	CBS 281.58 = ATCC 22355 = IFO 8179 = IMI 071625 = NRRL 2674 [T]	AF033433	GU981622	KF296399	KF296453
Penicillium reticulisporum	*Lanata-Divaricata*	CBS 122.68 = ATCC 18566 = IFO 9024 = IMI 136700 [T]	AF033437	GU981665	KF296391	KF296454
Penicillium rolfsii	*Lanata-Divaricata*	CBS 368.48 = ATCC 10491 = FRR 1078 = IFO 7735 [T]	JN617705	GU981667	KF296375	KF296455
Penicillium rubriannulatum	*Lanata-Divaricata*	CGMCC 3.18804 = CBS 144641 [T]	KY495029	KY495138	KY494969	KY495080
Penicillium simplicissimum	*Lanata-Divaricata*	CBS 372.48 = ATCC 10495 = FRR 902 = IFO 5762 = IMI 039816 [T]	GU981588	GU981632	KF296368	JN121507
Penicillium singorense	*Lanata-Divaricata*	CBS 138214 = DTO 133-C6 [T]	KJ775674	KJ775167	KJ775403	-

Penicillium skrjabinii	*Lanata-Divaricata*	CBS 439.75 = NRRL 13055 = FRR 1945 = IMI 196528 ^{T}	GU981576	GU981626	KF296370	EU427252
Penicillium soliforme	*Lanata-Divaricata*	CGMCC 3.18806 = CBS 144482 ^{T}	KY495038	KY495147	KY494978	-
Penicillium spinuliferum	*Lanata-Divaricata*	CGMCC 3.18807 = CBS 144483 ^{T}	KY495040	KY495149	KY494980	KY495090
Penicillium subrubescens	*Lanata-Divaricata*	CBS 132785 = DTO 188-D6 ^{T}	KC346350	KC346327	KC346330	KC346306
Penicillium svalbardense	*Lanata-Divaricata*	CBS 122416 = IBT 23856 ^{T}	GU981603	KC346325	KC346338	KF296457
Penicillium tanzanicum	*Lanata-Divaricata*	DAOMC 250514 = CBS 140968 ^{T}	KT887841	KT887802	KT887763	-
Penicillium vanderhammenii	*Lanata-Divaricata*	CBS 126216 = IBT 23203 ^{T}	GU981574	GU981647	KF296382	KF296458
Penicillium vasconiae	*Lanata-Divaricata*	CBS 339.79 = ATCC 42224 ^{T}	GU981599	GU981653	KF296386	KF296459
Penicillium viridissimum	*Lanata-Divaricata*	CGMCC 3.18796 = CBS 144484 ^{T}	KY495004	KY495113	KY494944	KY495059
Penicillium wotroi	*Lanata-Divaricata*	CBS 118171 = IBT 23253 ^{T}	GU981591	GU981637	KF296369	KF296460
Penicillium siccitolerans	***Lanata-Divaricata***	**FMR 17381 ^{T}**	**LR655193**	**LR655197**	**LR655201**	**LR655205**

Penicillium yunnanense	*Lanata-Divaricata*	CGMCC 3.18794 = CBS 144485 [T]	KY494990	KY495099	KY494930	KY495048
Penicillium zonatum	*Lanata-Divaricata*	CBS 992.72 = ATCC 24353 [T]	GU981581	GU981651	KF296380	KF296461
Penicillium aeris	*Torulomyces*	CBS 135897 = DTO 207D4 [T]	KF303654	KF303614	KF303627	KF303681
Penicillium austricola	*Torulomyces*	CBS 135900 = DTO 183E6 = DAOM 241066 [T]	JX091466	JX091579	JX141600	KF303705
Penicillium cantabricum	*Torulomyces*	CBS 120415 = DTO 76I9 = FMR 9121 [T]	KF303655	KF303615	KF303646	KF303682
Penicillium catalonicum	*Torulomyces*	CBS 110532 = DTO 78H5 [T]	KF303650	KF303609	KF303644	KF303683
Penicillium cryptum	*Torulomyces*	CBS 271.89 = DTO 122C9 = ATCC 60138 = IMI 296794 = NRRL 13460 [T]	KF303647	KF303608	KF303628	JN121478
Penicillium lagena	*Torulomyces*	CBS 185.65 = DTO 77I8 = MUCL 8221 [T]	KF303665	KF303619	KF303634	JN121450
Penicillium lassenii	*Torulomyces*	CBS 277.70 = DTO 95D6 = NRRL 5272 = ATCC 22054 = FRR 858 = IMI 148395 [T]	KF303648	KF303607	KF303629	JN121481
Penicillium marthae-christenseniae	*Torulomyces*	CBS 129213 = DTO 201B5 [T]	KF303651	KF303613	KF303645	KF303711

Penicillium oregonense	*Torulomyces*	CBS 129775 = DTO 208A5 [T]	KF303668	KF303623	KF303640	KF303710
Penicillium porphyreum	*Torulomyces*	CBS 382.64 = DTO 78G7 [T]	KF303666	KF303621	KF303636	KF303677
Penicillium riverlandense	*Torulomyces*	CBS 135896 = DTO 182F6 = DAOMC 241060 [T]	JX091457	JX091580	JX141593	KF303685
Penicillium sexuale	***Torulomyces***	**FMR 17380 [T]**	**LR655195**	**LR655199**	**LR655203**	**LR655207**
Penicillium tubakianum	*Torulomyces*	CBS 287.66 = DTO 138D9 = MUCL 8519 = IFO 8315 [T]	KF303652	KF303611	KF303637	KF303712
Penicillium variratense	*Torulomyces*	CBS 337.97 = DTO 137C8 [T]	KF303649	KF303610	KF303630	KF303675
Penicillium williamettense	*Torulomyces*	CBS 129774 = DTO 208A4 [T]	KF303667	KF303622	KF303639	KF303709
Penicillium wisconsinense	*Torulomyces*	CBS 128279 = DTO 198H7 [T]	KF303670	KF303624	KF303641	KF303706
Penicillium wollemiicola	*Torulomyces*	CBS 137177 = DTO 297E3 [T]	KJ174314	KJ174315	KJ174316	KJ174313
Penicillium corylophilum	*Exilicaulis*	CBS 330.79 [T]	GU944557	GU944519	GU944607	JN406569
Penicillium restrictum	*Exilicaulis*	CBS 367.48 = ATCC 11257 = FRR 1748 = IMI 040228 = NRRL 1748 [T]	AF033457	KJ834486	KP016803	JN121506

Penicillium toxicarium	*Exilicaulis*	NRRL 6172 [T]	EF198650	EF198620	EF198631	EF198499

[1]**ATCC**: American Type Culture Collection, Virginia, USA; **BCCM/MUCL**: Mycothèque de l'Université catholique de Louvain, Louvain-la-Neuve, Belgium; **CBS**: Culture collection of the Westerdijk Biodiversity Institute, Ultrech, the Netherlands; **CGMCC**: China General Microbiological Culture Collection Center, Beijing, China; **DAOM**: Canadian Collection of Fungal Cultures, Ottawa, Canada; **DTO**: Applied and Industrial Mycology Department Collection, Ultrecht, the Netherlands; **FRR**: Food Science Australia, Ryde; **FMR**: Facultad de Medicina, Universitat Rovira i Virgili, Reus, Spain; **IBT**: IBT Culture Collection of Fungi, Lyngby, Denmark; **IFO**: Institute for Fermentation, Osaka, Japan, now NBRC; **IMI**: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K.; **ITAL**: Instituto de Tecnologia de Alimentos, Sao Paulo, Brazil; **KACC**: Korean Agricultural Culture Collection, Suwon, Republic of Korea; **NRRL**: ARS Culture Collection, Peoria, United States.

[T]: ex-type strain.

[2]Sequences newly generated in this study are indicated in **bold**.

[3]ITS: internal transcribed spacer region 1 & 2 including 5.8S nrDNA; *BenA*: β-tubulin; *CaM*: calmodulin; *rpb2*: partial RNA polymerase II, second largest subunit.

2.4. Phylogenetic analysis

The sequences generated in this study were compared with those of the National Center for Biotechnology Information using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). To determine the phylogenetic relationship of all isolates, a combined of ITS-*BenA*-*CaM*-*rpb*2 was built to distinguish among other species of *Penicillium* belong to the sections *Lanata-Divaricata* and *Torulomyces* (Fig. 1). *Penicillium toxicarium* NRRL 6172, *Penicillium restrictum* NRRL 1748 and *Penicillium corylophilum* CBS 330.79 (section *Exilicaulis*) were selected as outgroup. The sequence alignments and the maximum-likelihood (ML) and Bayesian-inference (BI) phylogenetic analyses were performed as was described by Valenzuela-Lopez et al. [28]. The final matrices used for phylogenetic analyses were deposited in TreeBASE (www.treebase.org; accession number: 25066).

3. Results

3.1. Molecular phylogeny

The Blast search gave the following results: FMR 17424 matched with *Penicillium meloforme* CBS 445.74 (similarity: ITS, 97 %; *BenA*, 93.3 %; *CaM*, 88.2 %; *rpb*2, 93.9 %); FMR 17381 with *P. meloforme* CBS 445.74 (similarity:ITS, 99.2 %; *BenA*, 98.1 %; *CaM*, 96.8 %; *rpb*2, 98.8 %); FMR 17612 with *P. limosum* CBS 339.97 (similarity: ITS, 99.6 %; *BenA*, 97.2 %; *CaM*, 96.3%; *rpb*2, 99 %); FMR 17380 with *P. wisconsinense* CBS 128279 (similarity: ITS, 93.8 %) and with *P. cryptum* CBS 271.89 (similarity: *BenA*, 86 %; *CaM*, 85.6 %; *rpb*2, 89.7 %).

We carried out individual and combined phylogenetic analyses with ITS, *BenA*, *CaM* and *rpb*2 sequences to resolve the taxonomical position of our strains using the sequences of type strains of the accepted species of *Penicillium* into the sections *Lanata-Divaricata* and *Torulomyces*. A concatenated dataset from 91 sequences contained a total of 2,425 characters including gaps (570 of them for ITS, 439 for *BenA*, 660 for *CaM* and 756 for *rpb*2), from which 961 were parsimony informative (96 for ITS, 248 for *BenA*, 347 for *CaM* and 270 for *rpb*2). The sequence datasets did not show conflict in the tree topologies for the 70 % reciprocal bootstrap trees, which allowed to combine the four genes for the multi-locus analysis. The ML analysis showed similar tree topology and was congruent with that obtained in the Bayesian analysis. The phylogenetic tree (Fig. 1) was divided into two main clades representing the sections *Lanata-Divaricata* (100 % BS / 1 PP) and *Torulomyces* (100 % BS / 1 PP). Four of our strains were placed into the section *Lanata-Divaricata* clade: FMR 17424 in a sister branch of a terminal clade containing FMR 17381 and *Penicillium meloforme* (100 % BS / 1 PP); and FMR 17612 formed a terminal clade (100 % BS / 1 PP) with *P. brefeldianum* and *P. limosum* CBS 339.97. On the other hand, FMR 17380 was located into the section *Torulomyces*, into a terminal clade (100 % BS / 1 PP) together *P. cryptum*.

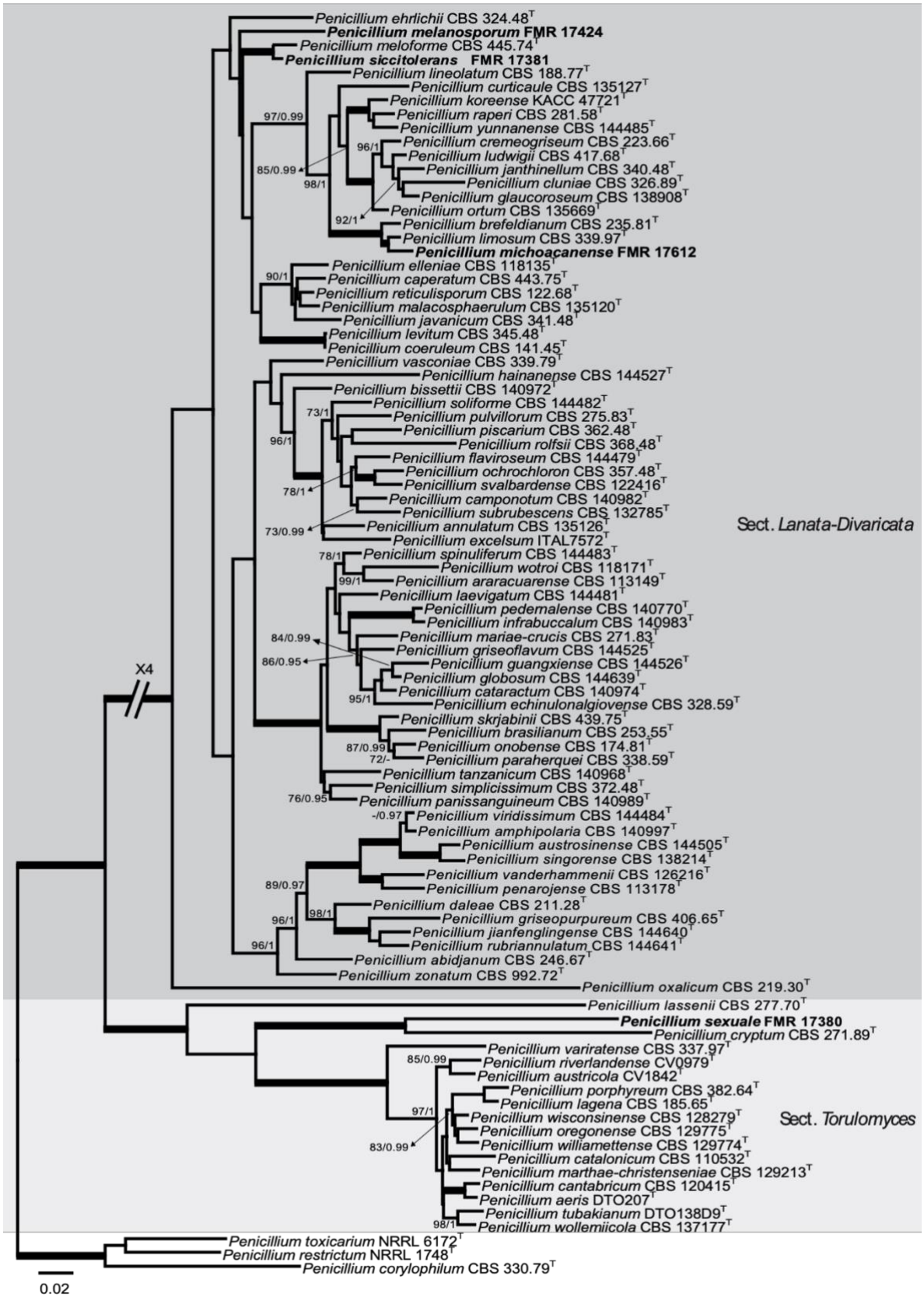

Figure 1. ML phylogenetic tree of *Penicillium* section *Lanata-Divaricata* and section *Torulomyces* inferred from the combined ITS, *BenA*, *CaM* and *rpb*2 loci. Support in nodes is indicated above thick branches and is

represented by bootstrap values (ML analysis) of 70 % and higher and/or posterior probabilities (BI analysis) of 0.95 and higher. Some branches were shortened, these are indicated by two diagonal lines with the number of times a branch was shortened. Fully supported branched (100 % BS /1 PP) are indicated in **bold**. ^{T} =ex type. Alignment length 2,425 bp. The sequences not generated by us were retrieved from EMBL/GenBank and are indicated in Table 1.

3.2. Taxonomy

Because FMR 17424 forms a sister branch distant from the nearest terminal clade composed by FMR 17381 and *Penicillium meloforme*, and because FMR 17381 differs phylogenetically and phenotypically from *Penicillium meloforme*, both strains are proposed as two new species of sect. *Lanata-Divaricata* as follows.

Penicillium melanosporum Rodr.-Andr., Cano & Stchigel, ***sp. nov.*** MycoBank MB 835938. Fig. 2.

Etymology: From Greek *μελανό-*, black, and *-σπόριο*, spore, referring to the production of dark pigmented conidia.

Section *Lanata-Divaricata*.

Type: Spain, Castilla y León, Riaza, from a soil sample, 12 May 2018, E. Rodríguez-Andrade & J. F. Cano (holotype CBS H-XXXX, cultures ex-type CBS XXXX = FMR 17424).

Description: *Mycelium* composed of hyaline, septate, smooth- and thin-walled hyphae, 2–4 μm wide. *Conidiophores* divaricate, monoverticillate or reduced to a single phialide arising directly from the vegetative hyphae; *stipes* hyaline, non- to 1–2-septate, mostly not septate at the base, smooth- and thin-walled, 15–70 × 2–3 μm; *phialides* 1 to 3 at the top of the stipe, hyaline, smooth- and thin-walled, ampuliform with a ventricose base, 9–10 × 2.5–3 μm, sometimes with a dark coloured collarette when old; *conidia* at first subhyaline becoming olive green to dark brown with the age, smooth–walled to verruculose, subglobose, 4–5 μm, several of them covered by a dark brown sheath with the age, the last of the conidia produced remaining attached to the phialide, thick-walled and coarsely ornamented, globose, 6–7 μm of diam, always surrounded by a dark brown sheath. *Sclerotia* not produced. *Sexual morph* not observed.

Culture characteristics (14 d at 25 ºC). Colonies on CYA reaching 60–65 mm diam., raised at the centre, velvety, sulcate, margins regular, yellowish white (4A2), sporulation absent to sparse, exudates absent; reverse olive brown (4D4), soluble pigment absent. On MEA reaching 53–55 mm diam., slightly raised, velvety to floccose, whitish, sulcate, margins regular, sporulation sparse, exudates absent; reverse light yellow (4A4), soluble pigment absent. On YES reaching 68–71 mm diam., raised and fluffy, margins regular, grey (24D1) at the centre and white (4A1) at the edge, sporulation abundant, exudates absent; reverse brownish orange (5C4), soluble pigment absent. On OA reaching 65–68 mm diam., slightly raised, floccose and fluffy, whitish with turquoise grey (24D2) spots, sporulation abundant, exudates absent; soluble pigment absent. On DG18 reaching 8–11 mm diam., raised, olive brown (4D4) and yellowish grey (4B2), margins regular, sporulation sparse, exudates absent; reverse greyish yellow (3C3) at the centre and yellowish white (4A2) at the edge, soluble pigment absent. On G25N reaching 25–26 mm diam., slightly elevated, cottony, sulcate, margins regular, white (4A1) at the centre and greyish beige (4C2) at the edge, sporulation abundant, exudates absent; reverse greyish yellow (4C5),

soluble pigment absent. On MY70FG reaching 22–24 mm diam., slightly elevated, sulcate, cerebriform, margins regular, greyish orange (5B3), sporulation sparse, exudates absent; reverse orange white (5A2), soluble pigment absent. On CREA reaching 58–59 mm diam., flattened, slightly powdery, whitish, exudates absent, sporulation sparse, margins irregular; no acid production. Cardinal temperatures of growth: minimum 15 ºC, optimum 30 ºC, maximum 40 ºC.

Barcodes: ITS barcode LR655192 (alternative markers: *BenA* = LR655196; *CaM* = LR655200; *rpb*2 = LR655204).

Notes: *Penicillium meloforme* and *Penicillium siccitolerans* sp. nov., which form a well-supported terminal clade in our tree (Fig. 1), are the species most phylogenetically related to *P. melanosporum*. *Penicillium melanosporum* differs from *P. meloforme*, because the former produces an asexual morph and laks of a sexual morph, while the second one forms a sexual morph and the asexual morph is only produced on MY70FG. *Penicillium siccitolerans* differs from *P. melanosporum* by the production of sclerotia. *Penicillium melanosporum* also produces shorter stipes than those of *P. meloforme* (15–70 × 2–3 μm vs. 150–500 × 2–3 μm) [29], and bigger conidia (4–5 μm diam.) than those of *P. meloforme* (2–3 × 1–2.5 μm) and of *P. siccitolerans* (2–3 × 1–2.5 μm). Moreover, *P. melanosporum* differs from *P. meloforme* and *P. siccutolerans* by the production of a mucilaginous brown to dark brown exopigment surrounding the conidia, and because the last of the conidia produced remains attached to the phialide. Nevertheless, *P. melanosporum* and *P. siccitolerans* are capable to grow on CYA at 40 °C while *P. meloforme* does not growth at 37 °C [29].

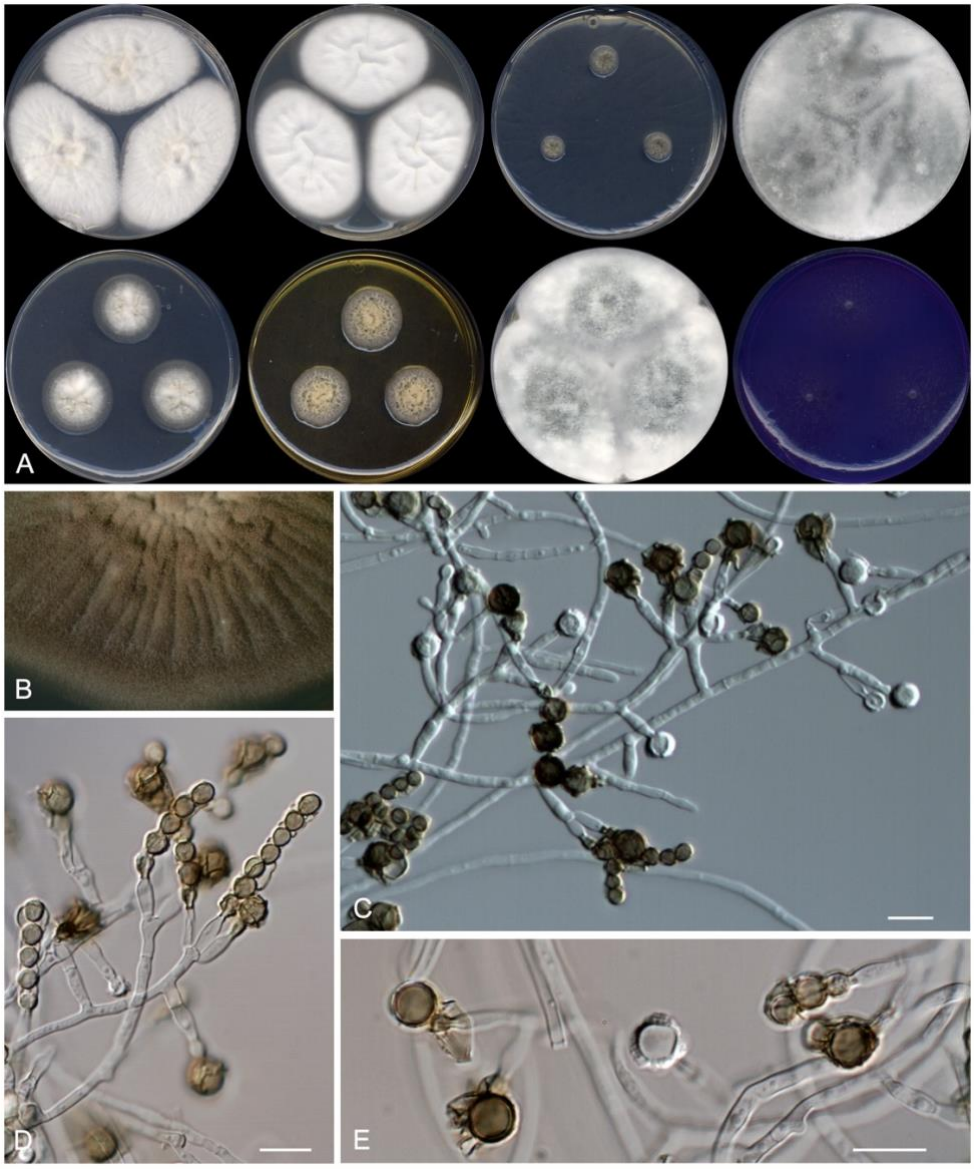

Figure 2. Morphological characters of *Penicillium melanosporum* CBS XXXX [T]. A. Colonies on CYA, MEA, DG18 and OA, from left to right (top row); and on G25N, MY70FG, YES, and CREA (bottom row), 14 days at 25 ºC. B. Detail of the colony on CYA under the stereomicroscope. C–E. Conidiophores and conidia. Scale Bar = 10 μm.

Penicillium siccitolerans Rodr.-Andr., Stchigel & Cano ***sp. nov.*** MycoBank MB 835939. Fig. 3.

Etymology: From Latin *siccus-*, dry, and *-tolerans*, tolerance, due to the ability of this fungus to grow at a low water activity.

Section *Lanata-Divaricata*.

Type: Spain, Castilla y León, Riaza, from a soil sample, 12 May 2018, E. Rodríguez-Andrade & J. F. Cano (holotype CBS H-XXXX, cultures ex-type CBS XXXX = FMR 17381).

Description: *Mycelium* superficial to immersed, composed of hyaline, septate, smooth–walled hyphae, 1.5–2 μm wide. *Conidiophores* monoverticillate; *stipes* smooth–walled, 30–80 × 1.5–3 μm; *phialides* (1) 2–6 (–10) per stipe, ampuliform to flask-shaped, smooth–walled, 9–11 × 1.5–2 μm; *conidia* smooth–walled, olive green when mature, broadly limoniform, 2.5–3.5 × 2.5–3 μm. *Sclerotia* white, tansluscent, slightly tomentose, mostly globose, 120–190 μm diam., composed of hyaline, thin-walled, polygonal cells of 5–8 μm diam. *Sexual morph* not observed.

Culture characteristics (14 d at 25 ºC). Colonies on CYA reaching 59–61 mm diam., flattened, sulcate, with regular margins, white (5A1), sporulation sparse, hyaline exudates scarce; reverse greyish orange (5B5), soluble pigment absent. On MEA reaching 47–50 mm diam., slightly raised, velvety, margins regular, yellowish white (4A2), sporulation absent, exudates absent; reverse light yellow (4A5), soluble pigment absent. On YES reaching 65–69 mm diam., raised sulcate, margins regular, white (4A1) at the center and pastel yellow (2A4) at the edge, sporulation sparse, exudates absent; reverse orange (5A6), soluble pigment absent. On OA reaching 73–75 mm diam., flattened, granulose, margins irregular, olive brown (4D4) at the centre and pale yellow (3A3) at the edge, sporulation sparse, exudates dark yellow (4C8); soluble pigment absent. On DG18 reaching 13–16 mm diam., flattened, white (4A1), margins regular, sporulation sparse, exudates absent; reverse yellowish white (3A2), soluble pigment absent. On G25N reaching 25–26 mm diam., slightly elevated, velvety, margins regular, white (4A1) with reddish yellow (4A6) spots, sporulation sparse, exudates absent; reverse orange (5A7), soluble pigment absent. On MY70FG reaching 24–25 mm diam., slightly elevated, velvety, regular margins, white (1A1), sporulation sparse, exudates absent; reverse pale-yellow (4A3), soluble pigment absent. On CREA reaching 49–53 mm diam., flattened, granulose, margins irregular, white (4A1), sporulation sparse, exudates absent; acid production weak. Cardinal temperatures of growth: minimum 15 ºC, optimum 30 ºC, maximum 40 ºC.

Barcodes: ITS barcode LR655193 (alternative markers: *BenA* = LR655197; *CaM* = LR655201; *rpb*2 = LR655205).

Notes: *Penicillium siccitolerans* is phylogenetically close related to *P. meloforme*. Nevertheless, *P. siccitolerans* does not produce a sexual morph, which is present in *P. meloforme*. For additional differences between these species see *P. melanosporum* notes (above).

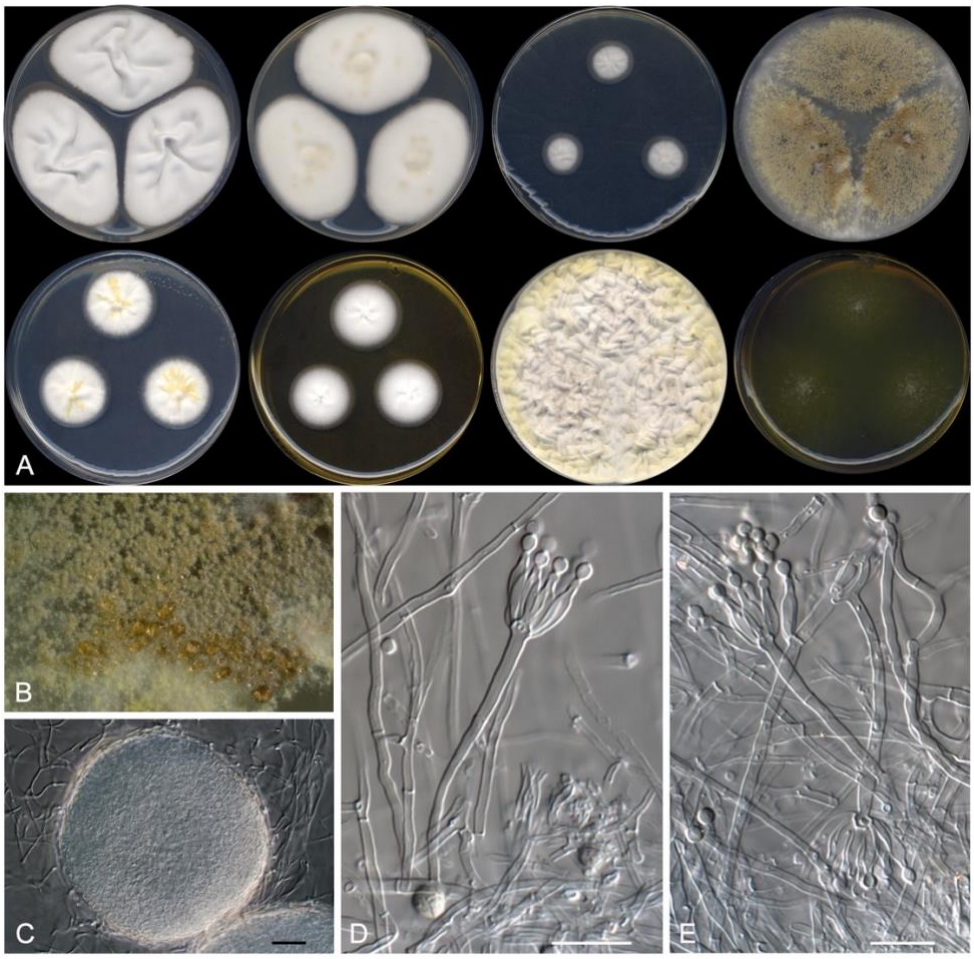

Figure 3. Morphological characters of *Penicillium siccitolerans* CBS XXXX [T]. A. Colonies on CYA, MEA, DG18 and OA, from left to right (top row); and on G25N, MY70FG, YES, and CREA (bottom row), 14 days at 25 ºC. B. Detail of the colony on OA under the stereomicroscope. C. Sclerotia. D–E. Conidiophores. Scale Bar: C = 25 μm. D–E = 10 μm.

Penicillium michoacanense Rodr.-Andr., Cano & Stchigel, ***sp. nov.*** MycoBank MB 835940. Fig. 4.

Etymology: The species name refers to Michoacán state, México, the geographical area where the soil sample was collected.

Section *Lanata-Divaricata*.

Type: México, Michoacán state, Jiménez, El Zapote, from soil intended for corn cultivation, 03 January 2010, E. Rodríguez & A. González (holotype CBS H-XXXX, cultures ex-type CBS XXXX = FMR 17612).

Description: *Mycelium* superficial to immersed, composed of septate, smooth-walled, hyaline hyphae of 1–2 μm wide. *Conidiophores* divaricate, monoverticillate; *stipes* hyaline, smooth–walled, 15–60 × 1–1.5 μm; *phialides* (1–) 4–6 (7) per stipe, hyaline, smooth–walled, flask-shaped, 4–5 × 1.5 μm; *conidia*

olive green, mostly smooth-walled with a few warts, broadly limoniform, 1.5–2.5 × 1–2 μm. *Sclerotia* hyaline to light brown, globose, 70–120 × 90–140 μm, composed by very thick–walled, hightly refringent, polyhedral to globose cells of 5–12 μm diam. *Sexual morph* not observed.

Culture characteristics (14 d at 25 ºC). Colonies on CYA reaching 62–64 mm diam., flattened, margins regular, pastel yellow (1A4) at the center and white (1A1) at the edge, sporulation sparse, with a little production of hyaline exudates; reverse yellowish brown (5D5), soluble pigment absent. On MEA reaching 55–57 mm diam., flattened, velvety, margins regular, yellowish grey (3C2), sporulation sparse, exudates absent; reverse greyish yellow (4B5), soluble pigment absent. On YES reaching 63–67 mm diam., raised, sulcate, velvety to floccose, margins regular, brownish grey (4D2) at the center and white (4D1) with pastel yellow (3A4) spots at the edge, sporulation abundant, with a little production of brownish orange (5C6) exudates; reverse, greyish orange (5B4), soluble pigment absent. On OA reaching 69–70 mm diam., flattened, granulose, margins irregular, brownish grey (5D2) at the center and greyish yellow (4B3), sporulation abundant, exudates production pale yellow (4A3); soluble pigment absent. On DG18 reaching 10–11 mm diam., slighty raised, margins regular, velvety to floccose, greenish grey (26B2) at the center and white (6A1) at the edge, sporulation abundant, exudates absent; reverse yellowish white (4A2), soluble pigment absent. On G25N reaching 23–24 mm diam., slightly elevated, velvety, margins regular, yellowish white (4A2) at the center, then orange-grey (5B2) and turquoise-white (24A2) at the edge, sporulation sparse, exudates absent; reverse pale-yellow (4A3), soluble pigment absent. On MY70FG reaching 25–27 mm diam., slightly elevated, sulcate, velvety, margins regular, white (1A1), exudates absent, sporulation sparse; reverse light-yellow (4A4), soluble pigment absent. On CREA reaching 25–26 mm diam., flattened, granulose, margins irregular, yellowish white (3A2), sporulation abundant, exudates absent; soluble pigment absent, acid production weak. Cardinal temperatures of growth: minimum 15 ºC, optimum 30 ºC, maximum 37 ºC.

Barcodes: ITS barcode LR655194 (alternative markers: *BenA* = LR655198; *CaM* = LR655202; *rpb*2 = LR655206).

Notes: *Penicillium michoacanense* is phylogenetically close to *P. limosum*. Nevertheless, *P. michoacanense* has shorter and thinner stipes (15–60 × 1–1.5 μm) and smaller conidia (1.5–2.5 × 1–2 μm) than those of *P. limosum* ([62–]75–225 × 2–3[–3.5] μm, and 2.8–3.3 × 2.5–3 μm, respectively) [30]. In addition, *P. michoacanense* does not produce the sexual morph on any media tested, which is produced by *P. limosum* on MEA and OA [30].

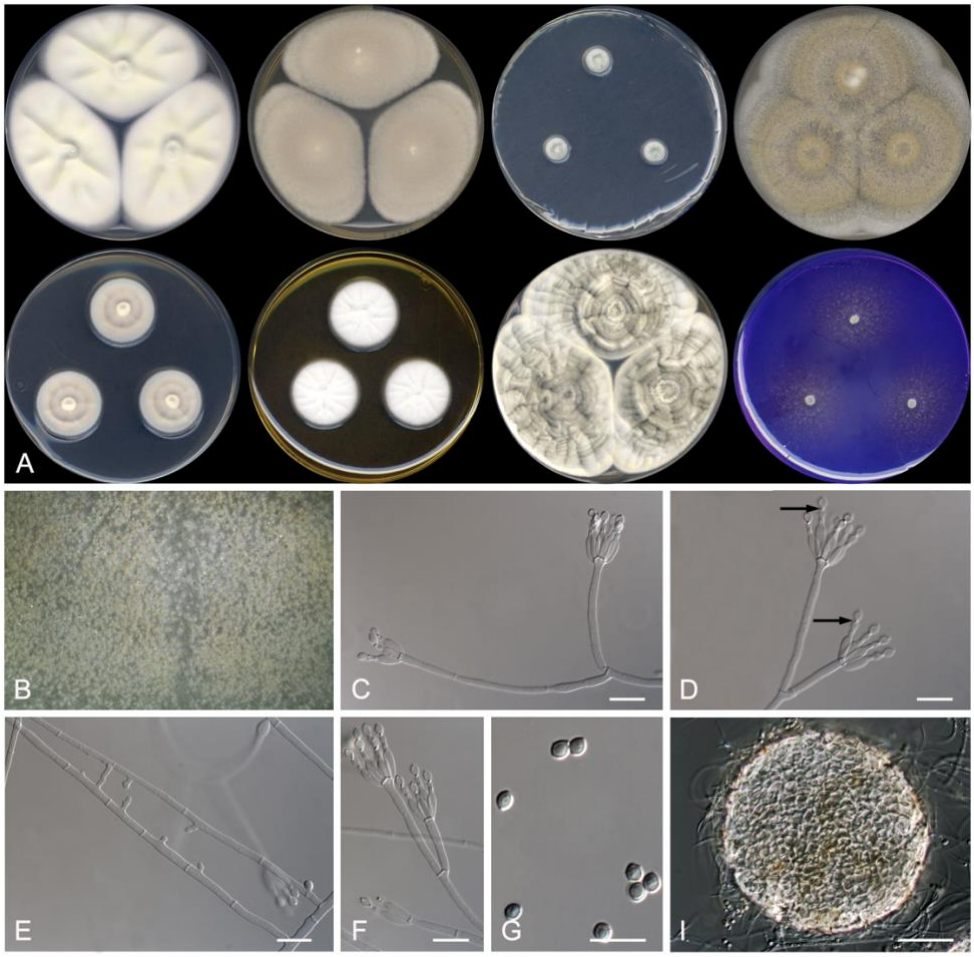

Figure 4. Morphological characters *Penicillium michoacanense* CBS XXXX [T]. A. Colonies on CYA, MEA, DG18 and OA, from left to right (top row); and on G25N, MY70FG, YES, and CREA (bottom row), 14 days at 25 ºC. B. Detail of the colony on OA under the stereomicroscope. C–F. Conidiophores and hyphae in anastomosis. G. Conidia. I. Sclerotia. Scale Bar: C–G = 10 μm. I = 50 μm.

Penicillium sexuale Rodr.-Andr., Stchigel & Cano ***sp. nov.*** MycoBank MB 835941. Fig. 5.

Etymology: Refering to the fact that only presents a sexual morph.

Section *Torulomyces*

Type: Spain, Castilla y León, Riaza, from a soil sample, 12 May. 2018, E. Rodríguez-Andrade & J. F. Cano (holotype CBS H-XXXX, cultures ex-type CBS XXXX = FMR 17380).

Description: *Mycelium* superficial to immersed, composed of septate, smooth–walled, hyaline hyphae of 1–2 μm wide. *Ascostromata* cream to tan coloured, more or less globose, 30–50 μm diam., composed of transluscent, refringent, thick-walled polygonal cells of 5–12 μm diam, peridial wall becoming 1–2-layered when the ascospores are produced, outer peridial layer of *textura angulata*. *Asci* 8-spored, borne singly, globose, 7–10 μm diam. *Ascospores* hyaline, smooth-walled under the brightfield

322 microscope, broadly lenticular, 2.5–3 × 2–2.5 μm, with two widely separated equatorial ridges and with
323 an equatorial furrow. *Asexual morph* not observed.

324 **Culture characteristics (14 d at 25 ºC).** Colonies on CYA reaching 4–5 mm diam., slightly raised,
325 velvety, margins regular, white (4A1), exudates absent, sporulation absent; reverse yellowish white
326 (4A2), soluble pigment absent. On MEA reaching 21–22 mm diam., slightly raised, velvety, margins
327 regular, white (3A1), sporulation absent, exudates absent; reverse pale yellow (4A3), soluble pigment
328 absent. On YES reaching 11–12 mm diam., raised, sulcate, velvety, magins regular, yellowish white
329 (4A2), sporulation absent, exudates absent; reverse pastel yellow (4A4), soluble pigment absent. On
330 DG18 reaching 14–15 mm diam., slightly raised, velvety, magins regular, white (4A1), sporulation
331 absent, little production of hyaline exudates; reverse pale yellow (4A3), soluble pigment absent. On
332 G25N reaching 5–6 mm diam., slightly elevated, velvety, margins regular, white (4A1), sporulation
333 absent, exudates absent; reverse pale yellow (4A3), soluble pigment absent. On MY70FG reaching 6–8
334 mm diam., slightly elevated, velvety, margins regular, white (1A1), exudates absent, sporulation absent;
335 reverse pale-yellow (4A3), soluble pigment absent. On CREA reaching 4–5 mm diam., flattened, velvety
336 to floccose, margins irregular, white (4A1), sporulation absent, exudates absent; acid production absent.
337 Cardinal temperatures of growth: minimum 15 ºC, optimum 25 ºC, maximum 37 ºC.

338 **Barcodes:** ITS barcode LR655195 (alternative markers: *BenA* = LR655199; *CaM* = LR655203; *rpb*2 =
339 LR655207).

340 **Notes:** *Penicillium sexuale* differs significantly from *Penicillium cryptum* [31], the phylogenetically
341 nearest species (see Fig. 1), by a very a late production of ascospores into the ascostromata (after 2
342 months growing on PDA; after two weeks in *Penicillium cryptum*), and because does not produce an
343 asexual morph in any of the culture media tested.

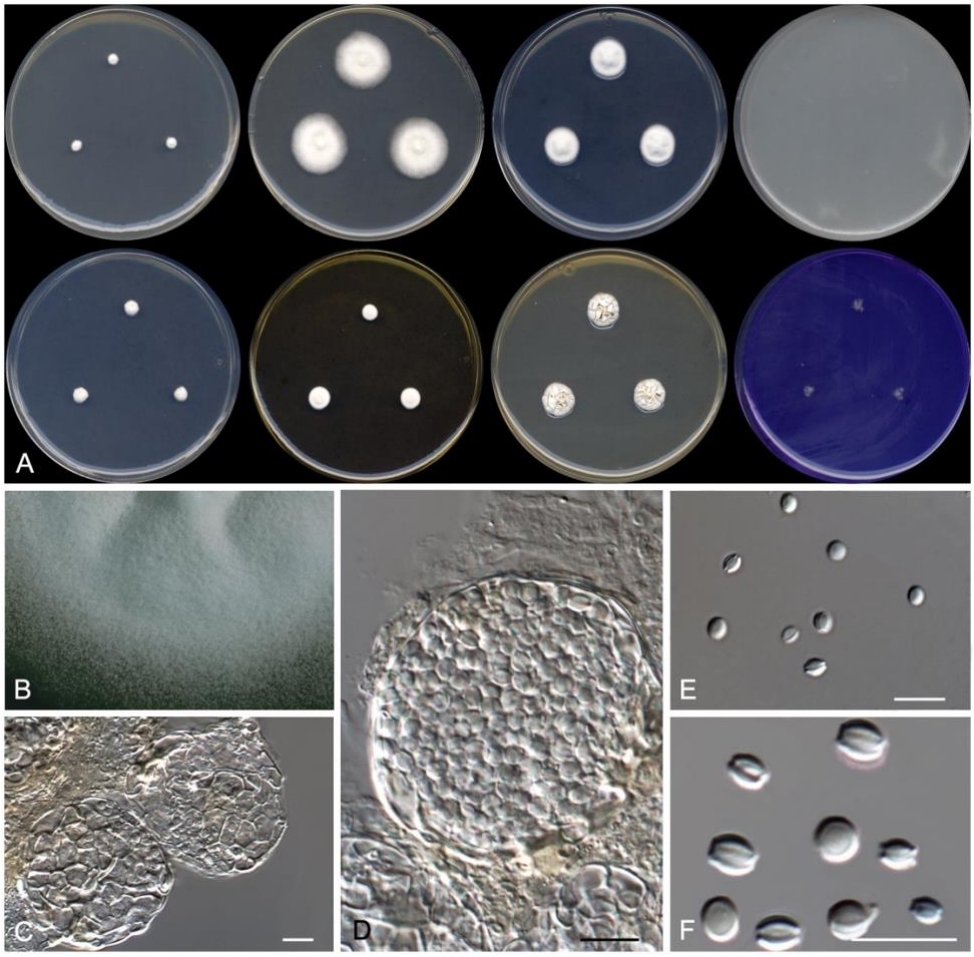

Figure 5. Morphological characters *Penicillium sexuale* CBS XXXX ^{T}. A. Colonies on CYA, MEA, DG18 and OA, from left to right (top row); and on G25N, MY70FG, YES, and CREA (bottom row), 14 days at 25 ºC. B. Colony on MEA under stereomicroscope. C–D. Asci. E-F. Ascospore. Scale Bar = 10 μm.

4. Discussion

Species of *Penicillium* living in soils, inhabitant of caves and of buildings, and causing food spoilage, such as *Penicillium brevicompactum*, *Penicillium chrysogenum*, *Penicillium cinnamopurpureum*, *implicatum* and *P. janczewskii* grow at a minimum a_w of 0.78; *Penicillium corylophilum*, *Penicillium fellutanum*, *Penicillium viridicatum* and *Penicillium verrucosum* develop at a_w as low 0.80; and *Penicillium aurantiogriseum*, *Penicillium citrinum*, *Penicillium expansum*, *Penicillium griseofulvum*, and *Penicillium restricum* do it at a_w of 0.81–0.82 [12, 32, 33, 34, 35, 36, 37]. Because these species grow at a_w lower than 0.85, all of them must be considered as xerophilic organisms [36]. Very recently, *Penicillium apimei*, *Penicillium meliponae*, and *Penicillium mellis* have been described in honey produced by stingless bees in Brazil [10]. Despite these species were isolated from a sugar-rich substrate, whose a_w is usually lower than 0.60, and as well in other recent taxonomic studies on *Penicillium* species, the ability to grow at low water activity was not tested.

In the present study, the multigene-based phylogeny (using ITS, *BenA*, *CaM*, and *rpb*2 sequences) allowed us to recognize four new species of *Penicillium* from soil in Spain and Mexico:

Penicillium melanosporum, *P. michoacanense*, and *P. siccutolerans*, of the section *Lanata-Divaricata*, and *P. sexuale*, of the section *Torulomyces*. All four *Penicillium* spp. were capable to grow at 0.76 a_w (on MY70FG culture medium) forming colonies, thus demonstrating xerophyly. The asexual morph of *P. melanosporum* reseambles to those of *Penicillium brunneoconidiatum* and of *Penicillium tsitsikammaense* [38], of the section *Aspergilloides*. However, the strongly ornamented dark brown conidia are restricted to the last one of those produced by the phialides in *P. melanosporum*, being all conidia equally ornamented in the other two species. The asexual morph of *Penicillium michoacanense* and *P. siccitolerans* is also reminiscent of the species of the genus within the section *Aspergilloides* [38], than those of the section *Lanata-Divaricata*. *Penicillium michoacanense* and *P. siccitolerans* produce sclerotia, but in the former species these are composed by thick-walled polygonal cells, whose are thin-walled in *P. siccitolerans*. Finally, *P. sexuale* differs from all the species of the section *Torulomyces* because not forms the typical asexual morph consisting in (mostly) conidiophores with solitary phialides, presenting only a sexual morph.

Results paradoxical the relatively large number of studies about the xerophyly and on the physiological mechanisms involved in this topic for the genus *Aspergillus*, but conversely the scarcity of this sort of studies for species of *Penicillium*. Due to our findings, results evident that more studies are needed to understand the diversity of extremophilic species of *Penicillium*, and the mechanisms involved in the adaptation to extreme environments.

Author Contributions: ER-A performed the experimental work, the phenotypic characterization of the isolates, as well as the DNA extraction and purification, gene sequencing and data processing for phylogenetic analysis, being one of the major contributors of this manuscript. AMS, because their experience on fungal biology and taxonomy, supervised all steps of the experimental work by ER-A, collaborating in the description of the novel fungi and in the writing of chapters "Introduction" and "Discussion", reviewing the draft several times. JFC-L supervised the nucleotide sequence alignment and the phylogenetic reconstructions, took the pictures that appear in the figures, contributed actively in the identification and the taxonomy of the fungal strains, gave useful suggestions to write the manuscript and reviewed several times the draft. All authors read and approved the final manuscript.

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Conflicts of Interest: The authors declare no conflict of interest. The authors confirm that this manuscript respects the Nagoya Protocol to the Convention on Biological Diversity. All data generated or analyzed during this study are included in this published article.

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4.6. Nuevas especies de hongos extremófilos en muestras de suelos y otros sustratos

Persoonia, Fungal Planet description sheets, 2017

4.6.1 *Cadophora antarctica* sp. nov. (39: 286-287)

Persoonia, Fungal Planet description sheets, 2018

4.6.2 *Dothiora infuscans* sp. nov. (40: 276-277)

4.6.3 *Geosmithia xerotolerans* sp. nov. (41: 368-369)

Persoonia, Fungal Planet description sheets, 2019

4.6.4 *Phialemonium guarroi* sp. nov. (42: 450-452)

Persoonia, Fungal Planet description sheets, 2020

4.6.5 *Exophiala frigidotolerans* sp. nov. (44: 301-459)

Fungal Planet 627 – 20 December 2017

Cadophora antarctica Rodr.-Andrade, Stchigel, Mac Cormack & Cano, *sp. nov.*

Etymology. Named after the locality where it was collected, Antarctica.

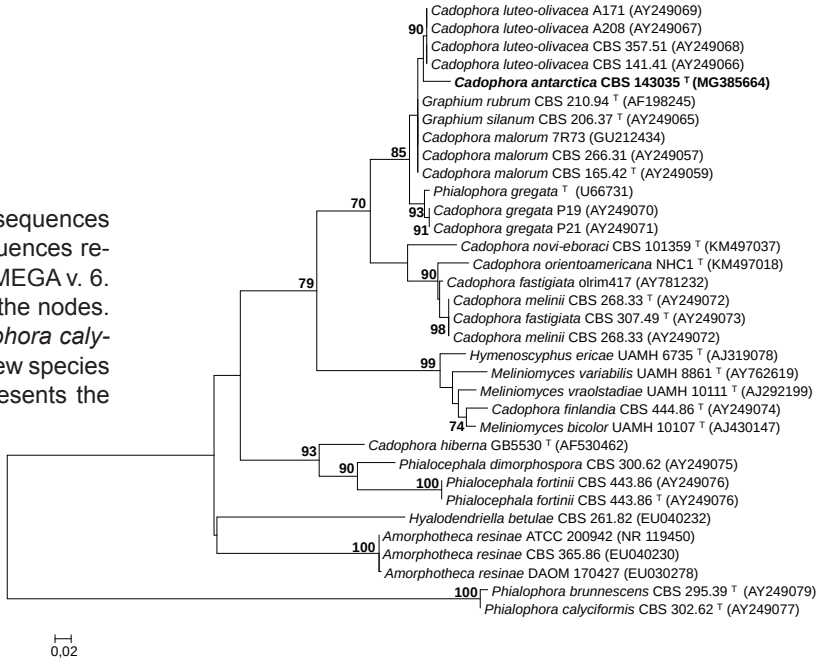
Classification — *Incertae sedis*, *Helotiales*, *Leotiomycetes*, *Pezizomycotina.*

Mycelium composed of hyaline to olivaceous brown, smooth to verrucous, thin- to thick-walled, septate, anastomosing hyphae, 2–4 µm wide. *Conidiophores* mostly reduced to a short chain of ramoconidia on a scar, laterally or terminally disposed on a recurved or compressed coiled hyphae, rarely well-developed, simple, stalked, erect or decumbent, up to 200 µm long, up to 4 µm broad. *Ramoconidia* holoblastic, 0(–1)-septate, brown to dark brown, sometimes inequilaterally coloured, with one side darker than the opposite, in longitudinal chains of up to six, smooth- and thick-walled, lemon-shaped, flask-shaped, clavate or nearly cylindrical, 5–13 × 2–4 µm, with one basal and up to four apical scars. *Conidia* holoblastic, aseptate, brown to dark brown, inequilaterally coloured, with one side darker than the other, disposed in long, simple or ramified chains, with up to four small-sized scars, smooth- and thick-walled, mostly broadly lens-shaped but inequilateral due to one side being more flattened than the other, 4–5 × 3–4 µm.

Culture characteristics — Colonies on MEA after 3 wk at 15 °C reaching 52–54 mm diam, velvety, zonate, successively pale grey (1D1), dark brown (6F4), medium grey (1E1), dark grey (1F1), pale grey (1B1) and greyish brown (5E3) from the centre towards the edge; exudates absent; diffusible pigment absent; sporulation abundant; reverse successively greyish orange (5B3), greyish brown (5E2), grey (7E1) and orange white (5A2) from the centre towards the edge. *Colonies* on OA after 3 wk of incubation at 15 °C 44–45 mm diam, flat, floccose at the centre, greyish brown (6D3) at the centre and brownish orange (5C4) at the edge; exudates absent; diffusible pigment absent; sporulation sparse; reverse greyish brown (6D3) at the centre and pale grey (1D1) at the edge. Minimum temperature of growth, 5 °C; optimum temperature of growth, 15 °C; maximum temperature of growth, 25 °C.

Typus. Antarctica, South Shetland archipelago, King George Island, near to Carlini's Argentinean scientific base, from a diesel-contaminated soil sample, 11 Jan. 2011, *A.M. Stchigel* (holotype CBS H-23211, cultures ex-type CBS 143035 = FMR 16056; ITS and LSU sequences GenBank MG385664 and MG385663, MycoBank MB822232).

Notes — *Cadophora antarctica*, recovered from a soil sample contaminated with diesel in King George Island (Antarctica), displays the typical features of a psychrotrophic organism: it has an optimal temperature of growth at 15 °C and is not able to grow above 25 °C. *Cadophora antarctica* differs from all previously known species of the genus (Gramaje et al. 2011, Travadon et al. 2014), displaying holoblastic conidiogenesis, forming conidiophores morphologically similar to cladosporium-like taxa. Based on a megablast search of NCBIs GenBank nucleotide database, the closest hit using the ITS sequence is with the ex-type strain of *Cadophora luteo-olivacea* (CBS 141.41, GenBank AY249066; Identities = 493/513 (97 %), Gaps 2/513 (0 %)); and using the LSU sequence it is with the same strain of *Cadophora luteo-olivacea* (GenBank AY249081; Identities = 533/541 (98 %), no gaps). Our ITS phylogenetic tree corroborated the placement of our isolate as a new species of the genus *Cadophora*, phylogenetically closely related to *Cadophora luteo-olivacea*.



Maximum likelihood tree obtained from the DNA sequences dataset from the ITS region of our isolate and sequences retrieved from GenBank. The tree was built by using MEGA v. 6. Bootstrap support values ≥ 70 % are presented at the nodes. *Phialophora brunnescens* CBS 295.39 and *Phialophora calyciformis* CBS 302.62 were used as outgroup. The new species proposed in this study is indicated in **bold**. T represents the ex-type strains.

Colour illustrations. Typical landscape of King George Island (South Shetland archipelago, Antarctica); colonies growing on different culture media (OA, PDA and MEA at 15 °C, and MEA at 5 °C; top picture); conidiophores and conidia. Scale bars = 10 µm.

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Fungal Planet 727 – 13 July 2018

Dothiora infuscans Rodr.-Andrade, Stchigel, Guarro & Cano*, sp. nov.*

Etymology. From Latin *infusco*, to make dark, referring to the black fungal growth on the substrate it was isolated from.

Classification — *Dothioraceae, Dothideales, Dothideomycetes*.

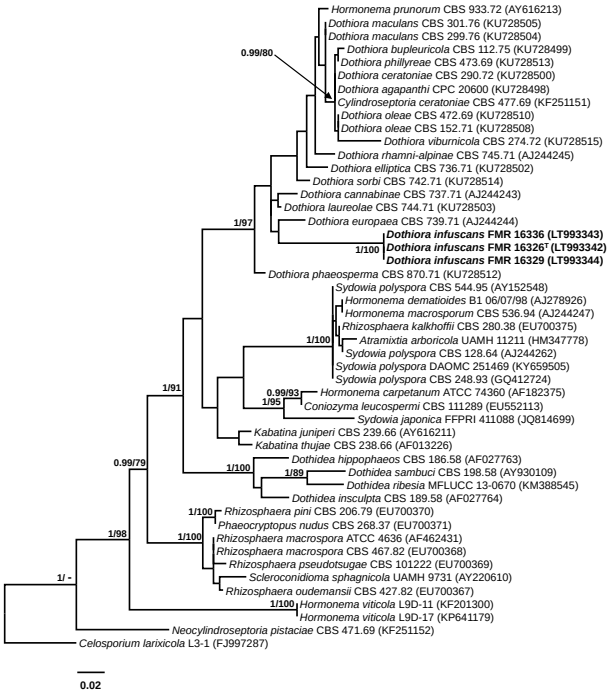
Mycelium composed of subhyaline, smooth-, thin-walled, septate hyphae, 5–7 µm wide, later becoming thick-walled, increasing the number of septa and the volume of their cells to give them a moniliform appearance, and finally the hyphae turn dark brown and produce chains of holothallic (chlamydospore-like) conidia of up to 20 µm diam, which also develop longitudinal/oblique secondary septa over time, giving consequently a 'muriform' aspect to these propagules. *Conidiophores* micronematous, reduced to conidiogenous cells, mostly intercalary, producing conidia on lateral, short to long conic-truncate denticles, with 1–3 per conidiogenous cell. *Conidia* holoblastic, solitary, but attached to one another by a mucilaginous substance; mostly aseptate, smooth- and thin- to thick-walled, hyaline, becoming dark brown, thick-walled, roughened and mostly 1-septate, occasionally 2–3-septate, globose, ellipsoid or irregularly-shaped, prominently constricted at septa when old; unicellular conidia 8–9 × 4–5 µm; 2-celled conidia 10–13 × 6–7 µm; multi-celled conidia 18–19 × 5–7 µm. *Microcyclic conidia* produced by budding of the hyaline or pigmented conidia, solitary or in chains of up to 5 elements on inconspicuous denticles when the conidiogenous cell is young, but on protruding conical-truncate denticles when old, at one or both ends but also laterally, being smaller than the primary conidia. *Endoconidia*, *conidiomata* and *sexual morph* not observed.

Culture characteristics — Colonies on MEA reaching 27–29 mm diam after 3 wk at 25 °C, flattened, light yellow (4A5; Kornerup & Wanscher 1978) at centre, and successively greyish yellow (4B5), pale yellow (4A3) and reddish yellow (4A7) towards the edge, exudates absent, sporulation sparse; reverse light yellow (4A4), diffusible pigment absent. Colonies on PDA reaching 28–29 mm diam after 3 wk at 25 °C, flat and slimy at centre and sulcate at edge, yellowish brown (5D8) at centre, brownish black (6H8) at edge and light yellow (3A5) at the margins, exudates absent, sporulation abundant; reverse light orange (5A4) at centre, brownish grey (5E2) at the edge, and a pale yellow (4A3) margin, diffusible pigment absent. Colonies on OA 6–7 mm diam after 3 wk of incubation at 25 °C, slightly elevated, compact, margins irregular, blackish blue (20F8), exudates absent, abundant yeast-like conidia; reverse blackish brown (6G8) at centre and brownish orange (5C3) at edge, diffusible pigment absent. Colonies on PCA reaching 18–19 mm diam after 3 wk at 25 °C, flat and slimy at centre and filamentous (because of the submerged mycelium) at edge, black (18G2) at centre and olive brown (4E6) at edge, exudates absent, yeast-like conidia abundant; reverse orange white (5A2) at centre, brownish grey (6D2) at the edge, and yellowish white (4A2) at the margins, diffusible pigment absent. Minimum, optimal and maximum temperature of growth: 15 °C, 25 °C and 30 °C, respectively.

Colour illustrations. Wall with chromatic alteration in Els Pallaresos village, Tarragona province, Spain (background picture); colonies growing on different culture media (MEA, PDA, OA and PCA at 25 °C; upper picture); conidia, conidiogenous cells and denticles (black arrows), and 'muriform' propagules (inner pictures); detail of the wall with chromatic alterations (picture inside the black box). Scale bars = 10 µm.

Typus. Spain, Tarragona province, Els Pallaresos village, isolated from the blackened wall of an industrial warehouse, 10 July 2017, *J. Cano* & *A.M. Stchigel* (holotype CBS H-23480, cultures ex-type FMR 16326 = CBS 144317; ITS and LSU sequences GenBank LT993342 and LT993345; MycoBank MB824999).

Notes — *Dothiora infuscans* was recovered by a wall surface swab taken in Els Pallaresos village, Tarragona province, Catalonia, Spain. Species of *Dothiora* produce a dothichiza-like asexual morph, as well as a hormonema-like synasexual morph (Crous & Groenewald 2016, 2017). *Dothiora infuscans* can be distinguished from other *Dothiora* spp. with a hormonema-like sexual morph by the production of 'muriform' thalloconidia. Based on a megablast search of NCBIs GenBank nucleotide database, the closest hit using the ITS sequence is with the ex-type strain of *Dothiora europeae* CBS 739.71 (GenBank NR_145339; Identities = 445/470 (95 %), Gaps 5/470 (1 %)); and using the LSU sequence it is with *Dothiora oleaea* (GenBank KU728549; Identities = 834/842 (99 %), no gaps). Our ITS phylogenetic tree corroborated the placement of our isolate as a new species of the genus *Dothiora*, being phylogenetically close to *Dothiora europeae*.



Maximum likelihood tree obtained from the ITS alignment of our isolate and sequences retrieved from GenBank. The tree was built by using RAxML CIPRES (http://www.phylo.org/sub_sections/portal/) and the analysis of probability was run in MrBayes v. 3.2.1 (Huelsenbeck & Ronquist 2001). Bootstrap support values ≥ 70 % and Bayesian posterior probability values ≥ 0.95 are presented at the nodes. *Neocylindroseptoria pistaciae* CBS 471.69 and *Celosporium larixicola* L3-1 were used as outgroups. The new species proposed in this study is indicated in **bold**. ^{T} represents the ex-type strain of the novel species.

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Geosmithia xerotolerans

Fungal Planet 845 – 13 December 2018

Geosmithia xerotolerans Rodr.-Andrade, Cano & Stchigel, *sp. nov.*

Etymology. From Greek *ξερός*-, dry, and Latin *-tolerans*, tolerance, due to the ability of this fungus to grow on culture media with a low water activity.

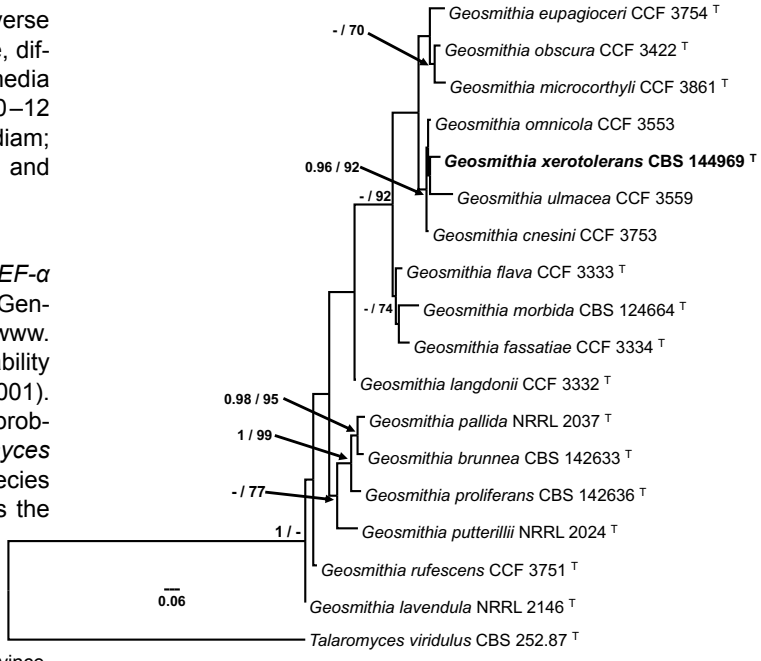
Classification — *Incertae sedis*, *Hypocreales*, *Sordariomycetes.*

Mycelium composed of hyaline, septate, funiculose hyphae, 2–3 µm wide. *Conidiophores* borne on vegetative mycelium, determinate, erect, septate, penicillate, bi- to terverticillate, mostly solitary, sometimes funiculose; stipes hyaline, 25–155 × 2–3 µm, septate, smooth-walled to verrucose, asymmetrically branched; primary branch (= rami) cylindrical, 20–40 × 2–3 µm, mostly septate, smooth-walled to verrucose; terminal branch (= metulae) cylindrical, 7–15 × 2 µm, rarely 1-septate, with smooth to verrucose walls, in whorls of 2–3; phialides cylindrical, 8–10 × 1.5–2 µm, abruptly tapering at the apex, with smooth to verrucose walls, in whorls of 2–5. *Conidia* hyaline, aseptate, ellipsoid to ovoid, 3–4 × 1.5–2 µm, rounded at both ends, smooth-walled, disposed in chains of up to 20 conidia. *Sexual morph* not observed.

Culture characteristics — *Colonies* on MEA reaching 38–39 mm diam after 2 wk at 25 °C, slightly elevated, powdery, margins irregular, orange white (5A2; Kornerup & Wanscher 1978) at centre and white (5A1) at edge, exudates absent, sporulation abundant; reverse orange (6A6), diffusible pigment absent. Colonies on CYA reaching 49–51 mm diam after 2 wk at 25 °C, slightly elevated, powdery, margins regular, white (4A1) at centre and pale yellow (4A3) at edge, exudates absent, sporulation abundant; reverse reddish orange (7B7) at centre and pale orange (6A5) at edge, diffusible pigment absent. Colonies on CZD 62–63 mm diam after 2 wk at 25 °C, cottony, margins irregular, white (3A1), exudates absent, sporulation abundant; reverse yellowish white (3A2), diffusible pigment absent. Colonies on YES reaching 62–63 mm diam after 2 wk at 25 °C, slightly elevated with radial waves, reddish grey (12B2) and white (4A1), exudates absent, sporulation abundant; reverse reddish brown (9E7) at centre and orange (6A6) at edge, diffusible pigment absent. This fungus grows on culture media with a low water activity (on DG18 after 2 wk at 25 °C, 10–12 mm diam; on G25N in the same conditions, 27–29 mm diam; on MY70S, 39–40 mm diam; and on MEA with 30, 40 and 50 % (glucose 50 % / fructose 50 %), 23–24 mm diam, 18–19 mm diam and 12–13 mm diam, respectively). In these culture media the fungal sporulation is abundant. Minimum, optimal and maximum temperature of growth: 15 °C, 25 °C and 35 °C, respectively.

Typus. Spain, Tarragona province, Els Pallaresos, isolated from a darkened wall of a house, 19 Apr. 2018, *J. Cano* & *A.M. Stchigel* (holotype CBS H-23734, cultures ex-type FMR 17085 = CBS 144969; *BenA*, *EF1-α*, ITS and LSU sequences GenBank LS998791, LS998792, LS998789 and LS998790; MycoBank MB827825).

Notes — *Geosmithia xerotolerans* was recovered from the surface of a darkened house wall taken in Els Pallaresos, Tarragona province, Spain. The genus *Geosmithia* was erected to accommodate species previously placed in *Penicillium*, with the following differentiable combination of characters: colonies in colours other than greyish blue or greyish green, penicillate and roughened conidiophores, with both phialides and conidia cylindrical (Pitt 1979). Based on a megablast search of NCBIs GenBank nucleotide database, the closest hit using the ITS sequence is the ex-type strain of *Geosmithia cnesini* MK 1820 (GenBank AM947671; Identities = 965/978 (99 %), 1 gap (0 %)); using the LSU sequence it was *Geosmithia microcorthyli* CCF3861 (GenBank MG954241; Identities = 809/815 (99 %), no gaps); using the *EF1-α* sequence, it was *Geosmithia omnicola* CNR8 (GenBank KR135476; Identities = 238/280 (85 %), 13 gaps (4 %)); and using the *BenA* sequence it matched with *Geosmithia omnicola* CNR43 (GenBank KP990575; Identities = 429/460 (93 %), 9 gaps (1 %)). Our ITS-*BenA*-*EF-α* phylogenetic tree corroborated the placement of our isolate as a new species of *Geosmithia*, being phylogenetically close to *Geosmithia omnicola.*



Maximum likelihood tree obtained from the ITS-*BenA*-*EF-α* alignment of our isolate and sequences retrieved from GenBank. The tree was built by using RAxML CIPRES (http://www.phylo.org/sub_sections/portal/) and the analysis of probability was run in MrBayes v. 3.2.1 (Huelsenbeck & Ronquist 2001). Bootstrap support values ≥ 70 % and Bayesian posterior probability values ≥ 0.95 are presented at the nodes. *Talaromyces viridulus* CBS 252.87 was used as outgroup. The new species proposed in this study is indicated in **bold**. T represents the ex-type strain of the novel species.

Colour illustrations. Darkened wall in Els Pallaresos, Tarragona province, Spain; colonies growing on different culture media (MEA, CYA, CZD and YES at 25 ºC) and conidiophores. Scale bars = 10 µm.

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Phialemonium guarroi

Fungal Planet 941 – 19 July 2019

Phialemonium guarroi Rodr.-Andr., Cano & Stchigel, *sp. nov.*

Etymology. In honour of the mycologist Josep Guarro Artigas.

Classification — *Cephalothecaceae*, *Sordariales*, *Sordariomycetes.*

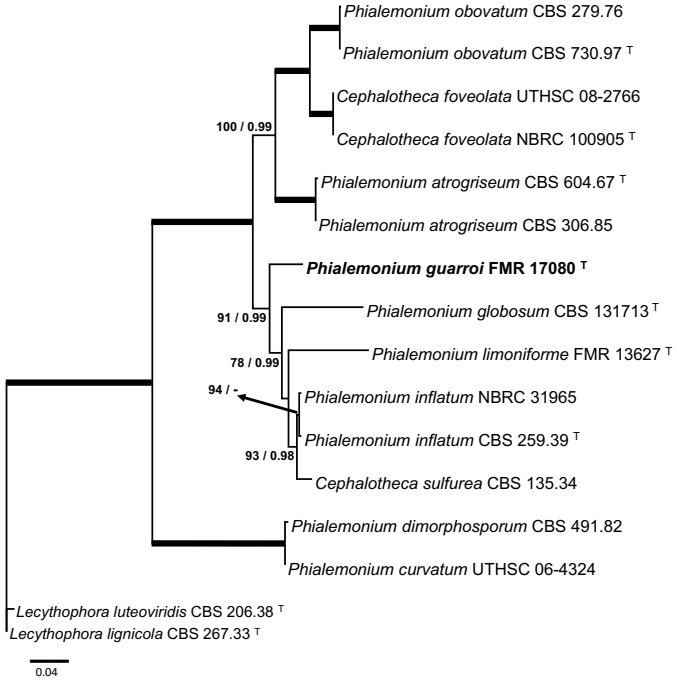
Mycelium composed of septate, hyaline, smooth- and thin-walled hyphae, 1.5–2 µm wide, becoming cinnamon and moniliform in old cultures, whose cells reach up to 10 µm diam. *Conidiophores* absent or poorly differentiated, often consisting in single lateral phialides and adelophialides borne directly from aerial hyphae, occasionally composed of a short stipe of up to 15 µm long and bearing 1–3 phialides in an irregular arrangement. *Phialides* abundant, hyaline, smooth-walled, flask-shaped, with more or less inflated at the base and tapering towards the top, 12–15 × 1.5–2 µm, percurrently proliferating to form long chains in old cultures. *Adelophialides* hyaline, smooth-walled, cylindrical but slightly tapering towards the top, 12–15 × 1.5–2 µm. *Conidia* hyaline, aseptate, lemon-shaped, 3–3.5 × 1.5–2 µm, smooth-walled, produced in chains of up to 25 conidia, with a cylindrical-truncate scar at both ends. *Chlamydospores* and *sexual morph* not observed.

Culture characteristics — *Colonies* on OA reaching 9–10 mm diam after 2 wk at 25 °C, flattened, velvety, grey (6B1; Kornerup & Wanscher 1978), margins regular, sporulation sparse, exudate absent; reverse pale yellow (3A3), diffusible pigment absent. *Colonies* on PCA attaining 10–11 mm diam after 2 wk at 25 °C, flattened, velvety, white (4A2), margins regular, sporulation abundant, exudate absent; reverse yellowish grey (3B2), diffusible pigment absent. *Colonies* on PDA of 12–13 mm diam after 2 wk at 25 °C, elevated, velvety to floccose, margin irregular, yellowish brown (5E4) at centre and yellowish grey (3B2) at edge, exudate absent, sporulation abundant; reverse olive brown (4E6) at centre and white (4A1) at edge, diffusible pigments absent. Minimum, optimal and maximum temperature of growth (on PDA): 15 °C, 25 °C and 30 °C, respectively.

Typus. Spain, Canarias, Santa Cruz de Tenerife province, La Palma, Punta Gorda, isolated from soil, Aug. 2009, *A.M. Stchigel & M. Calduch* (holotype CBS H-23924, cultures ex-type FMR 17080 = CBS 145626; ITS and LSU sequences GenBank LR535737 and LR535738, MycoBank MB830182).

Colour illustrations. Typical vegetation of La Palma island, Canary Islands archipelago, Spain (Photo credit: A. DeCort). Moniliform cells, adelophialides, phialides and conidia. Scale bars = 10 µm.

Notes — *Phialemonium guarroi* was recovered from a soil sample collected in Punta Gorda, La Palma, Canary Islands, Spain. The genus *Phialemonium* was established by Gams & McGinnis (1983). *Phialemonium* contains seven accepted species, mostly isolated from environmental sources and human specimens (Rivero et al. 2009, Perdomo et al. 2011, Guarro 2012, Crous et al. 2015b). *Phialemonium guarroi* is morphologically similar to *Phialemonium inflatum*. However, the new species can be distinguished from the latter due to the production of phialides which proliferate percurrently to form long chains (feature not reported in *P. inflatum*) and the production of smaller conidia than those of *P. inflatum*. Based on a megablast search of NCBIs GenBank nucleotide database, the closest hit using the **ITS** sequence is the ex-type strain of *P. inflatum* CBS 259.39 (GenBank LT633912; Identities = 490/535 (92 %), 10 gaps (1 %)); using the **LSU** sequence was the same ex-type strain of *P. inflatum* (GenBank LT633912; Identities = 845/857 (99 %), no gaps). The ITS-LSU phylogenetic tree corroborated the placement of our isolate as a new species of *Phialemonium*, being located phylogenetically close to *P. inflatum.*



Maximum likelihood tree obtained from the ITS-LSU alignment of our isolate and sequences retrieved from GenBank. The tree was built by using RAxML CIPRES (http://www.phylo.org/sub_sections/portal/) and the analysis of probability was run in MrBayes v. 3.2.6 (Ronquist et al. 2012). Bootstrap support (BS) values ≥ 70 % and Bayesian posterior probability (PP) values ≥ 0.95 are presented at the nodes. Fully supported branches (100 % BS / 1 PP) are indicated in **bold**. *Lecythophora luteoviridis* CBS 206.38 and *Lecythophora lignicola* CBS 267.33 were used as outgroup. The new species proposed in this study is indicated in **bold**. TRepresents the ex-type strains of the taxa employed in this analysis.

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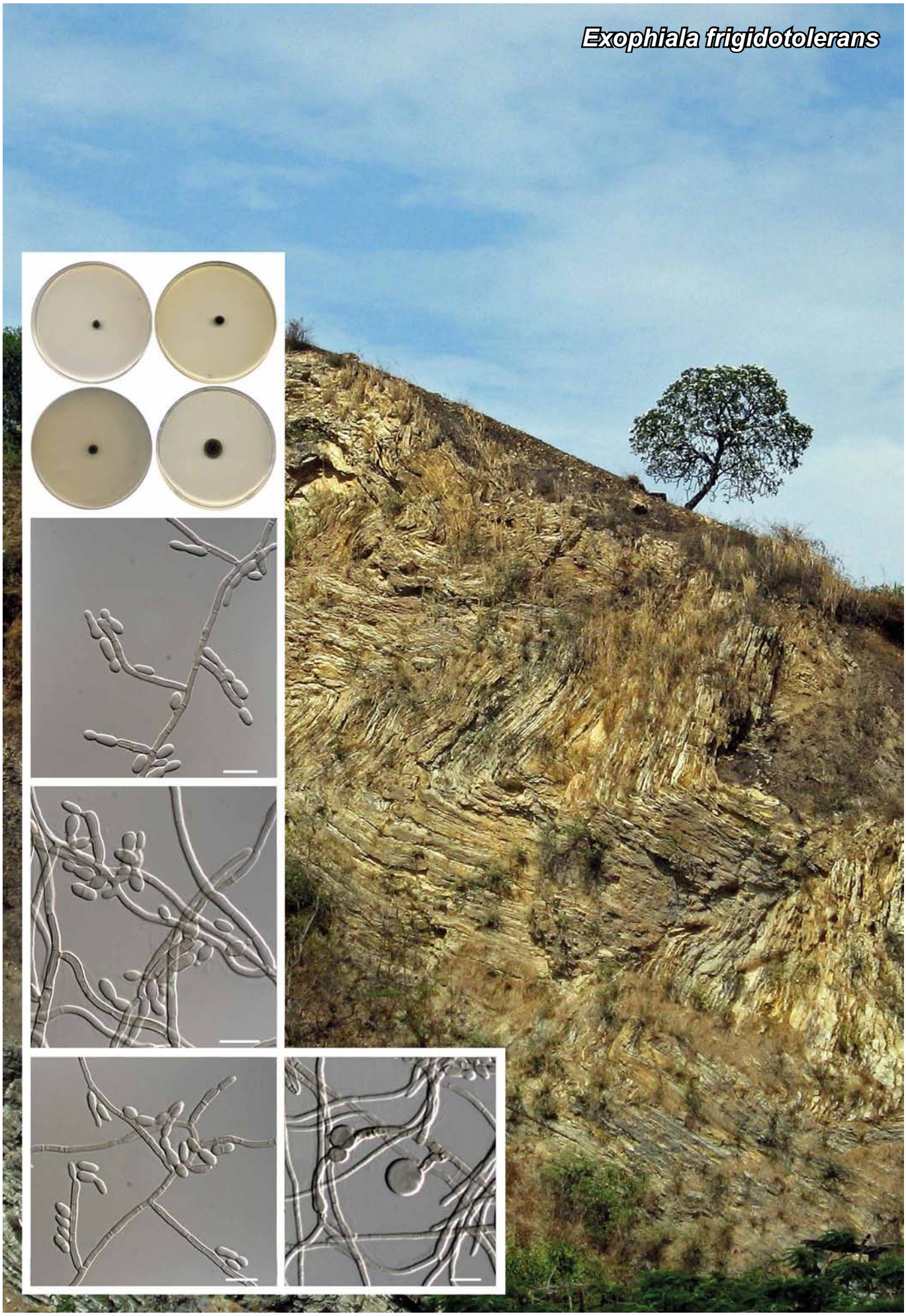


Fungal Planet 1079 – 29 June 2020

Exophiala frigidotolerans Rodr.-Andr., Cano & Stchigel, *sp. nov.*

Etymology. From Latin *frigus*-, cold, and - *tolerans*, tolerant, referring to its ability to grow fast at lower temperatures than 20 °C.

Classification — *Herpotrichiellaceae*, *Chaetothyriales*, *Chaetothyriomycetidae*, *Eurotiomycetes*.

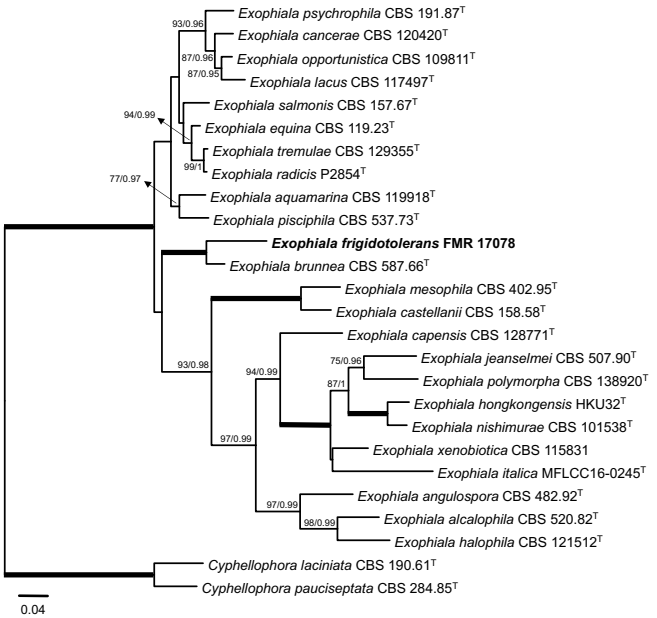
Mycelium composed of pale olivaceous brown, septate, branched, smooth- and thin-walled hyphae, 1–3 µm wide; older hyphae being more strongly pigmented. *Spirally twisted hyphae* present. *Moniliform cells* scarce, globose to ellipsoidal, in short chains (–5 cells). *Conidiophores* semi-micronematous, pale olivaceous brown, smooth- and thin-walled, mostly laterally disposed on the vegetative hyphae, sometimes terminally disposed, erect, rarely once branched near the base, cylindrical, with a rounded or pointed apex, 0–4-septate, with a terminal conidiogenous locus, sometimes with additional conidiogenous loci, 8–85 × 2–4 µm. *Conidiogenous cells* enteroblastic, mono- or polyblastic, integrated to the conidiophores, on vegetative hyphae or well-developed, in the latter case ellipsoidal, ovoid or flask-shaped, 5–11 × 2–3 µm, conidiogenous loci cylindrical or conic-cylindrical, with small percurrent proliferations. *Conidia* aseptate, occasionally 1-septate, pale olivaceous brown, smooth- and thin-walled, ellipsoidal to reniform, 4–7 × 2–4 µm, sometimes with a truncate base, solitary. *Budding cells* scarce, ellipsoidal, ovoid or barrel-shaped, 7–11 × 3–4 µm, in chains up to 5 elements. *Chlamydospores* scarce, olivaceous, globose, 5–15 µm diam.

Culture characteristics — *Colonies* on potato dextrose agar (PDA) reaching 5–6 mm diam after 2 wk at 25 °C, slightly raised, velvety, margins regular, brownish grey (M. 5E2; Kornerup & Wanscher 1978), sporulation absent, exudate absent; reverse brownish grey (M. 5E2), diffusible pigment absent. *Colonies* on oatmeal agar (OA) reaching 6–7 mm diam after 2 wk at 25 °C, morphologically similar to those on PDA, with sparse sporulation. *Colonies* on malt extract agar (MEA) reaching 5–7 mm diam after 2 wk at 25 °C, slightly raised, velvety, margins regular, olive brown (M. 4E4), sporulation absent, exudate absent; reverse olive brown (M. 4F3), diffusible pigment absent. *Colonies* on potato carrot agar (PCA) reaching 4–6 mm diam after 2 wk at 25 °C, slightly raised, velvety, margins regular, olive brown (M. 4E4), sparse sporulation, exudate absent; reverse brownish grey (M. 4F2), diffusible pigment absent. *Colonies* on PDA reaching 10–11 mm diam after 2 wk at 15 °C slightly raised velvety, margins regular, brownish grey (M. 5E2), sporulation absent, exudate absent; reverse brownish grey (M. 5E2), diffusible pigment absent. Minimum, optimal and maximum temperature of growth, 10 °C, 15 °C, and 25 °C, respectively.

Typus. Ecuador, Guayaquil, isolated from soil, Nov. 1996, *L. Zaror* (holotype CBS H-24326, cultures ex-type FMR 17078 = CBS 146539; ITS, LSU and *BenA* sequences GenBank LR699566, LR699567 and LR699568, MycoBank MB832466).

Notes — *Exophiala frigidotolerans* was recovered from a soil sample collected in Guayaquil, Ecuador. The genus *Exophiala* pertains to a group of fungi known as 'black yeasts', because of the production of yeast-like colonies and budding cells with dark, melanised cell walls. The genus *Exophiala* is characterised by an annellidic conidiogenesis and the production of solitary conidia grouping in slimy masses, and its phylogenetic affiliation to the ascomycete order *Chaetothyriales* (De Hoog et al. 2011). This genus contains numerous potential opportunists or pathogens of immunocompetent humans (Sudhadham et al. 2008, Li et al. 2008, 2009) and are isolated from a broad spectrum of substrata, environments and geographic areas (De Hoog et al. 2011, Ferrari et al. 2011). As in *E. psychrophila*, *E. frigidotolerans* exhibited the ability to grow at low temperatures. However, *E. frigidotolerans* presents more developed conidiophores than *E. psychrophila* (which are reduced to a unique discrete conidiogenous cell in this latter species), and produces shorter chains of moniliform cells (scarce and of up to 5 cells in the former species, and very abundant and of up to several hundred of cells in the latter).

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hit using the **ITS** sequence is the ex-type strain of *Exophiala brunnea* CBS 587.66 (GenBank JF747062; Identities = 539/560 (96 %), 6 gaps (1 %)); and using the **LSU** sequence the ex-type strain of *Exophiala brunnea* CBS 587.66 (GenBank MH870554; Identities = 868/876 (99 %), 1 gap (0 %)). The ITS-LSU-*BenA* phylogenetic tree corroborated the placement of our isolate as a new species of *Exophiala*, being located phylogenetically close to *E. brunnea*. *Exophiala brunnea* is easily distinguished from *E. frigidotolerans* by the production of 2-celled conidia (mostly 1-celled in *E. frigidotolerans*) and absence of budding cells (formed in *E. frigidotolerans*).



Maximum likelihood tree obtained from the ITS-LSU-*BenA* alignment of our isolate and sequences retrieved from GenBank. The tree was built by using RAxML CIPRES (http://www.phylo.org/sub_sections/portal/) and the analysis of probability was run in MrBayes v. 3.2.1 (Ronquist et al. 2012). Bootstrap support values ≥ 70 % and Bayesian posterior probability values ≥ 0.95 are presented at the nodes. Fully supported branches (100 % BS / 1 PP) are thickened. *Cyphellophora laciniata* CBS 190.61 and *Cyphellophora pauciseptata* CBS 284.85 were used as outgroup. The new species proposed in this study is indicated in **bold**. T represents the ex-type strains of the taxa employed in this analysis.

Colour illustrations. Guayaquil, Ecuador (image credit Doug Moyer). Colonies growing on different culture media (PCA, MEA, OA at 25 °C and PDA at 15 °C; upper pictures); conidiogenous cells, conidia, budding cells and inflated cells. Scale bars = 10 µm.

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5. DISCUSIÓN GENERAL

Durante el desarrollo de la presente tesis doctoral, nuestro estudio abarcó la caracterización fenotípica y molecular de 194 cepas fúngicas, de las cuales 104 fueron aisladas de muestras de miel (España y Argentina), 27 de vinos espumosos y tapones de corcho expuestos a dichos vinos (España), 32 de muestras de suelo (procedentes de la Antártida, Armenia, Chile, Ecuador, España, Irán, México y Vietnam), 9 de muestras de superficies (paredes de una planta industrial y casas colindantes) con alteraciones cromáticas (España), y finalmente 22 aislados clínicos (USA), identificando un total de 98 especies distribuidas en 57 géneros (véase Tabla 1).

Un escaso número de cepas (15/192, 8 %) pertenecían a hongos unicelulares (levaduras y levaduras "negras"), todas ubicadas dentro de la división *Ascomycota*. Originarias de la miel fueron identificadas: *Candida magnoliae*, *C. sorbosivorans*, *Zygosaccharomyces gambellarensis*, *Z. mellis* y *Z. siamensis* (clase *Saccharomycetales*), y *Schizosaccharomyces octosporus* (clase *Schizosaccharomycetes*) (Rodríguez-Andrade y col. 2019a), levaduras osmófilas/xerófilas anteriormente reportadas para dicho sustrato, las cuales son capaces de crecer a una $a_w \leq 0,8$ (Tilbury 1967; van Eck y col. 1993; Ganthala y col. 1994; Erickson y McKenna 1999; Torriani y col. 2011). A partir de muestras de cava se aislaron tres cepas de la especie *Candida patagonica* (Rodríguez-Andrade y col. 2019b), una levadura recientemente descrita y aislada a partir de cubas de fermentación y barriles de roble en bodegas del norte de la Patagonia argentina (Sangorrín y col. 2007). El presente es el segundo reporte mundial de dicha levadura, y el primero para dicho sustrato. Únicamente pudimos recuperar *Candida glaebosa* a partir de una muestra de suelo procedente de la Antártida. Esta especie, originalmente descrita a partir de sepia congelada en Japón (Komagata y Nakase 1965), ha sido posteriormente aislada de ambientes marinos y suelos de la Antártida (Duarte y col. 2013; Bueno y col. 2019). *Candida glaebosa* es una levadura con metabolismo exclusivamente oxidativo (incapaz de fermentar azúcares), mesofílica y psicrotolerante, incapaz de crecer en medios para hongos osmófilos y xerófilos o sin vitaminas (Komagata y Nakase 1965) y productora de moléculas tensoactivas a partir del glicerol (Bueno y col. 2019). Finalmente, a partir de varias muestras de alteraciones cromáticas de muros de una nave industrial se aisló *Dothiora infuscans* sp. nov., una nueva levadura negra extremadamente xerotolerante (Crous y col.

2017b) capaz de vivir a expensas de ciertos xenobióticos (moléculas que no forman parte de los componentes naturales de un ser vivo; datos no publicados).

Noventa de las 194 cepas (46 %) estudiadas en la presente tesis doctoral pertenecieron a los órdenes *Eurotiales* y *Onygenales*. El orden *Eurotiales* ha estado ampliamente representado en las muestras estudiadas en la presente tesis doctoral. A partir de las muestras de miel se recuperaron cuatro especies pertenecientes al género *Penicillium*: *P. camemberti*, *P. citrinum*, *P. corylophilum* y *P. cravenianum.* Estas cuatro especies son capaces de crecer a una a_w mínima de alrededor de 0,8 (Abellana y col. 2001; Fontana 2008; Kalai y col. 2017), y *P. camemberti* y *P. cravenianum* no habían sido previamente reportadas en dicho sustrato (Sinacori y col. 2014; Barbosa y col. 2018). Cuatro de las cepas de *Penicillium* de muestras de suelo de España y México pudieron ser identificadas tan solo a nivel de género y sección (tres pertenecientes a la sección *Lanata-Divaricata* y una a la sección *Torulomyces*). Estas cepas fueron capaces de crecer en los medios DG18, G25N y MY70FG con a_w de 0,95, 0,93 y 0,76, respectivamente (Pitt 1979; Hocking y Pitt 1980; Beuchat y Hocking 1990), demostrando así su extrema xerotolerancia. Gracias al estudio polifásico de las mismas se ha concluido de que se tratan de cuatro especies nuevas para la ciencia: *P. melanosporum*, *P. michoacanense*, *P. sexualis* y *P. siccutolerans* (Rodríguez-Andrade y col. {En preparación-b}). *Penicillium corylophilum* ha sido aislado de muestras de cava afectadas de "*cork taint*", y de tapones de corcho en contacto con dicha bebida (Rodríguez-Andrade y col. 2019b), pero también de muestras de aire y superficies de la bodega donde las botellas de vino eran sometidas a un proceso de "crianza" (datos no publicados), por lo que se considera un contaminante de origen ambiental. Esta especie ha sido reportada mayoritariamente en edificios húmedos del hemisferio norte, pero también en diversos alimentos y en mosquitos (da Costa y de Oliveira 1998; McMullin y col. 2014).

Varias cepas del género *Talaromyces* fueron aisladas a partir de las muestras de mieles pero, sorprendentemente, éstas solo pudieron ser identificadas a nivel de género. Basándonos en las diferencias fenotípicas y filogenéticas con respecto a las demás especies aceptadas para el género, se han propuesto tres nuevas especies: *T. affinitatimellis*. y *T. basipetosporus* (de la sección *Trachyspermi*), y *T. brunneosporus* (sección *Purpurei*) (Rodríguez-Andrade y col. 2019a). *Talaromyces basipetosporus* mostró una alta xerotolerancia, con una velocidad de crecimiento en

MEA constante hasta una a_w de 0,82. Además, *T. affinitatimellis* y *T. brunneosporus* crecieron hasta una a_w de 0,82 (Rodríguez-Andrade y col. 2019a). También *Talaromyces* fue uno de los géneros más frecuentemente aislados a partir de las muestras de vino "tranquilo" y de tapones de corcho. A pesar de que las dos nuevas especies: *T. speluncarum* y *T. subericola* pertenecientes a la sección *Trachyspermi*, fueron aisladas de muestras de vino, ninguna fue capaz de crecer en concentraciones de etanol superiores al 5 % v/v (Rodríguez-Andrade y col. 2019b), por lo que se asume que su presencia en el vino se debe a su papel como contaminante de la bodega.

Las especies *Aspergillus asperescens* (sección *Nidulantes*), *A. montevidensis* y *A. pseudoglaucus* (ambos de la sección *Aspergillus*), aisladas de varias muestras de mieles, no habían sido reportadas anteriormente para dicho sustrato. Sin embargo, *A. montevidensis* y *A. pseudoglaucus* han sido previamente aislados de aguas saturadas de sales (Butinar 2005), y *A. montevidensis* está asociado al estilo de vida de las abejas melíferas (http://gcm.wfcc.info/; Talice y Mackinnon 1931; Gilliam y col. 1974a). *Aspergillus montevidensis* y *A. pseudoglaucus* son capaces de crecer a a_w de 0,8 (Snow 1949; Armolik y Dickson 1956; Guynot y col. 2003). A partir de las muestras de vino y de los tapones de corcho se ha podido aislar *A. aureolatus*, *A. jensenii* y *A. puulaauensis*, pertenecientes a la sección *Nidulantes* (Chen y col. 2016), las que hasta el presente no habían sido reportadas para estos sustratos (Rodríguez-Andrade y col. 2019b). Y a partir de una muestra de suelo del desierto de Atacama (Chile) se ha podido aislar *A. xerophilus*, originalmente aislada a partir de una muestra de suelo del desierto de Egipto. *Aspergillus xerophilus* es un hongo xerófilo extremo, logrando crecer hasta a_w de 0,75 (Samson y Mouchacca 1975).

Tres especies del género *Monascus*, *M. pilosus*, *M. purpureus* y *M. ruber*, fueron aisladas de muestras de mieles. Este género contiene especies de importancia económica tales como *M. purpureus* y *M. ruber* (Hawksworth y Pitt 1983), empleadas en la producción (pero también implicadas en el deterioro) de alimentos fermentados y de pigmentos alimentarios (van Tieghem 1884; Hesseltine 1965; Lin 1975; Hawksworth y Pitt 1983). *Monascus pilosus*, *M. purpureus* y *M. ruber* tienen la habilidad de crecer en el medio G25N, con una a_w de 0,93 (Hawksworth y Pitt 1983). Recientemente, *M. mellicola* ha sido descrita como una nueva especie presente en miel de abejas sin aguijón en Brasil (Barbosa y col. 2017).

El género *Rasamsonia* contiene especies termotolerantes y termófilas cuya temperatura óptima de crecimiento es de alrededor de 30 ºC y una máxima de 45 ºC (Cooney y Emerson 1964; Maheshwari y col. 2000). Dos cepas aisladas de muestras de cava fueron capaces de crecer a 15 ºC y tener una temperatura máxima de crecimiento de 35 ºC, basados en sus capacidades psicrotolerantes, no antes reportadas para otra especie de este género y a las diferencias morfológicas y moleculares, proponemos a la especie *R. frigidotolerans* sp. nov. (Rodríguez-Andrade y col. 2019b).

Finalmente, se aisló a la especie *Xerochrysium xerophilum*, anteriormente conocido como *Chrysosporium xerophilum*, obtenido de una muestra de miel, siendo este un xerófilo extremo pudiendo crecer en a_w de 0,66 (Gock y col. 2003; Leong y col. 2011). Este hongo fue previamente aislado de chocolate, coco, ciruelas pasas y maíz almacenado (Pitt y Hocking 2009; Pitt y col. 2013), pero este es el primer reporte para esta especie en miel (Rodríguez-Andrade y col. 2019a).

El segundo orden más frecuentemente aislado fue el de los *Onygenales*, presentando una importante diversidad fúngica. Provenientes de las muestras de miel, se aisló *Ascosphaera atra* y *Eremascus albus*. El primero de estos taxones fue originalmente reportado sobre larvas muertas de la abeja cortadora de hojas de alfalfa cubiertas de quistes de *Ascosphaera aggregata*, y también del polen en el intestino de dichas larvas, pero sanas (Skou 1975. *Ascosphaera atra* es un contaminante habitual del polen (Skou y Hackett 1979), lo cual podría explicar su presencia en las muestras de miel. Por otro lado, *E. albus* es una especie xerófila, la cual produce propágulos de dispersión que pueden germinar a una a_w inferior a 0,7 (Pitt 1968). *Eremascus albus* fue originariamente reportado contaminando extracto de malta (Eidam 1883), y posteriormente aislado de pastel de chocolate, frutos secos y mostaza en polvo (Harrold 1950).

También a partir de varias muestras de miel aislamos varias cepas de un hongo artrosporado desconocido, el cual logramos identificar molecularmente solo a nivel de orden. El estudio filogenético de dichas cepas, así como su caracterización fenotípica ha permitido proponer que dicho hongo, *Helicoarthrosporum mellicola*, no era tan solo un taxón desconocido para la ciencia, sino que también pertenecía a una nueva familia *Helicoarthrosporaceae*. *Helicoarthrosporum mellicola* se mostró como un hongo

extremadamente xerotolerante, capaz de crecer a una a_w < 0,82 (Rodríguez-Andrade y col. 2019a).

Del mismo sustrato fue aislado otro hongo artrosporado desconocido, el que fue filogenéticamente ubicado en la familia *Ajellomycetaceae*, siendo por dichos motivos propuesto como *Strongyloarthrosporum catenulatum* gen. nov. et sp. nov. La familia *Ajellomycetaceae* incluye el grupo más diverso y clínicamente importante de hongos patógenos animales y humanos térmicamente dimórficos. A pesar de compartir ciertos rasgos morfológicos con los miembros de dicha familia, tal como multiplicarse asexualmente mediante la producción de artroconidios, *Strongyloarthrosporum catenulatum* es un hongo xerófilo estricto, característica nunca reportada para los miembros de esta familia (Rodríguez-Andrade y col. 2019a).

Por otro lado, provenientes de muestras de cava y de tapones de corcho se han aislado tres cepas pertenecientes filogenéticamente a dos especies de hongos previamente desconocidas, pero que están estrechamente relacionadas con *Arthrographis pinicola*. Debido a que la especie tipo del género *Arthrographis, A. kalrae*, pertenece a la familia *Eremomycetaceae* (clase *Dothideomycetes*), se ha propuesto establecer el nuevo género *Dactylodendron*, formado por las especies *D. pinicola* (la especie tipo, originariamente *A. pinicola*), *D. ebriosum* y *D. pluriseptatum*, ambas especies aisladas del vino y tapones de corcho fueron incapaces de crecer a concentraciones de etanol ≥ 10 % v/v, pero lograron mantener su viabilidad en dichas condiciones (Rodríguez-Andrade y col. 2019b).

Finalmente, se analizaron veintidós aislados género-forma *Malbranchea*, aisladas a partir de muestras clínicas y procedentes del “Fungus Testing Laboratory” (UTHSCsa San Antonio, Texas, USA). Algunas de dichas cepas fueron identificadas morfológicamente como pertenecientes al género *Auxarthron*, la principal forma teleomorfica de *Malbranchea* (Sigler y Carmichael 1976; Sigler y col. 1982; Sigler y col. 2002). Una de las principales características de todas las cepas estudiadas fue la de crecer a una temperatura superior a los 30 ºC, mayoritariamente a 37 ºC y algunas inclusive hasta 40 ºC, mostrando su carácter termotolerante, uno de los factores de virulencia necesarios para expresar su potencial patogenicidad en animales de sangre caliente, humanos incluidos (Saccardo 1908; Saccardo y Trotter 1913; Cooney y Emerson 1964; Sigler y Carmichael 1976). No obstante, una vez realizado el análisis filogenético de dichos aislados, se observó que no todas las cepas se agrupaban en

el clado correspondiente a las especies de los géneros *Auxarthron* y *Malbranchea* (Rodríguez-Andrade y col. {En preparación-a}). Por dicho motivo, en la presente tesis se propone la sinonimia de ambos géneros, lo que implica, que por prioridad histórica, transferir todas las especies de *Auxarthron* a *Malbranchea*, realizando las pertinentes nuevas combinaciones (*Malbranchea californiense*, *Malbranchea chlamydospora*, *Malbranchea compacta*, *Malbranchea concentrica*, *Malbranchea conjugata*, *Malbranchea longispora*, *Malbranchea ostraviense*, *Malbranchea pseudauxarthron*, *Malbranchea reticulata*, *Malbranchea umbrina* y *Malbranchea zuffiana*). Además, luego de examinar el lectotipo de *Malbranchea indica*, se concluye que morfológicamente guarda una escasa similitud con el resto de las especies del género (ascomas sin apéndices verdaderos y ascosporas elipsoidales y de paredes lisas), motivo por el cual debe ser excluida como una especie del género *Malbranchea*. En base al estudio filogenético y fenotípico se ha podido identificar entre los aislados estudiados a: *M. albolutea*, *M. aurantiaca, M. conjugata*, *M. flocciformis, M. umbrina* y *M. zuffiana*. Tres de las cepas solo pudieron ser identificadas a nivel género, por lo que en base a las diferencias moleculares y fenotípicas con respecto al resto de las especies del género, se proponen como las nuevas especies: *M. gymnoascoidea*, *M. multiseptata* y *M. stricta* (Rodríguez-Andrade y col. {En preparación-a}). Por otro lado, basados en las relaciones filogenéticas y en las características fenotípicas se han propuesto dos nuevas especies para el orden *Onygenales*, *Currahmyces sparsispora* y *Spiromastigoides geomycoides*, y dos géneros nuevos (con sus respectivas especies): *Pseudoarthropsis crassispora* y *Pseudomalbranchea gemmata*, todos termotolerantes. La creación del género *Pseudoarthropsis* implicó realizar una combinación para *Arthropsis cirrhata*, la que es designada como la especie tipo del género.

Paralelamente, se estudió la sensibilidad *in vitro* de todas las especies de *Malbranchea* y los nuevos taxones de origen clínico frente a diversos antifúngicos empleados en terapéutica humana, debido a que existen pocos datos a este respecto. El presente estudio demostró que la mayoría de ellos tenían una elevada sensibilidad frente a las equinocandinas, y las siguientes opciones terapéuticas fueron la TRB y el PSC, finalmente la AMB, el FLC, el ITC y la 5-FC no mostraron actividad sobre estas cepas.

El resto de las cepas (89; 46 %) pertenecieron a otros grupos taxonómicos. Dos de las cepas de origen clínico, morfológicamente identificadas como pertenecientes *Malbranchea* en el laboratorio de origen (FTL, UTHSC, USA), fueron filogenéticamente ubicadas dentro del orden *Arachnomycetales* (próximo a *Onygenales*, clase *Eurotiomycetes*): *Arachnomyces bostrychodes* y *A. graciliformis*, estas especies presentan temperaturas máximas de crecimiento de 37 ºC y 30 ºC, respectivamente. Ambas producen una mayor cantidad de ramas retorcidas respecto a las especies más cercanas filogenéticamente (Rodríguez-Andrade y col. {En preparación-a}).

Cladosporium cladosporioides, del orden *Capnodiales*, fue aislado de una muestra de tapón de corcho expuesto al "cava", siendo el primer reporte para esta especie; tan solo *C. oxysporum* había sido reportado para el mismo sustrato (Álvarez-Rodríguez y col. 2002). *Cladosporium cladosporioides*, dado de que se trata de una especie ampliamente distribuida, puede considerarse como un potencial contaminante del ambiente de la bodega.

Una nueva especie fúngica, *Cladophialophora recurvata,* perteneciente al orden *Chaetothyriales* (clase *Eurotiomycetes*), fue aislada de una muestra de cava afectada de "cork taint", lo que representa el primer reporte del género para este tipo de sustrato. Esta especie es filogenéticamente cercana a *M. mycetomatis*, difiere de la misma por la producción de conidios de mayor tamaño y por las cicatrices aplanadas discretas, además, presenta una alta producción de hifas en espiral (Rodríguez-Andrade y col. 2019b).

Exophiala frigidotolerans, aislada a partir de una muestra de suelo colectada en Ecuador, es una nueva especie caracterizada por tener una temperatura óptima de crecimiento de 15 ºC, un hecho insólito para el género, exceptuando *E. psychrophila* (Pedersen y Langvad 1989). También sorprende que *E. frigidotolerans* sea un hongo psicrotrófico, dado que el sitio geográfico de donde procede la muestra a partir de la cual fue aislada, la ciudad de Guayaquil, tiene una temperatura media anual por encima de los 26 ºC y una mínima absoluta en 15,8 ºC.

Cadophora antarctica, cuyo género pertenece al orden *Helotiales*, es una nueva especie psicrófila aislada a partir de una muestra de suelo contaminado con diesel de

la Antártida. *Cadophora antarctica* tiene un crecimiento óptimo de 15 ºC y es incapaz de crecer a 25 ºC (Crous y col. 2017a).

Beauveria bassiana, un hongo típicamente entomopatógeno del orden *Hypocreales* (Xiao y col. 2012), fue aislado de una muestra de cava con alteración organoléptica. Perteneciente al mismo orden, una nueva especie del género *Geosmithia*, *G. xerotolerans*, fue aislada de una muestra de pared con manchas oscuras, probablemente de origen biológico. *G. xerotolerans* es un hongo altamente xerotolerante, capaz de crecer a una a_w de 0,76 (Crous y col. 2018a).

Una cepa de *Alternaria alternata* (orden *Pleosporales*) fue aislada de un tapón de corcho expuesto al "cava", y junto con *A. multiformis*, a partir de muestras de miel, representando el primer reporte de ambas para dichos sustratos. Provenientes de las superficies con alteraciones cromáticas ha sido aislada una cepa de *Neocucurbitaria keratinophila*, un raro patógeno que fue originalmente aislado de una muestra de cornea humana, anteriormente clasificado en el género *Pyrenochaeta*, teniendo una similitud con *P. unguis-hominis*, pero fácilmente distinguible por la coloración de las colonias, así como la localización de las setas en el picnidio (Verkley y col. 2010; Valenzuela-Lopez y col. 2018).

Phialemonium guarroi, aislada de una muestra de suelo de las islas Canarias (España), es una nueva especie del orden *Sordariales* (clase *Sordariomycetes*), morfológicamente similar a *P. inflatum*, pero distinguible por la producción de fiálides, dispuestas percurrentemente en forma de cadena, así como conidios de menor tamaño (Crous y col. 2019).

Superstratomyces tardicrescens (orden *Superstratomycetales*) fue descrita a partir de un aislado clínico proveniente de Estados Unidos, esta especie se encuenta en la clase *Dothideomycetes* y se distingue del resto de las especies por la producción de pequeños conidios en conidioforos bien diferenciados (Crous y col. 2018a).

Pertenecientes al orden *Thelebolales* fueron identificados, a partir de muestras de suelo de la Antártida, *Antarctomyces psychrotrophicus* y *Thelebolus microsporus* (Stchigel y col. 2001), siendo esta última una de las especies psicrófilas más extendidas a nivel mundial y dominante en diferentes ecosistemas antárticos terrestres (Kobayasi y col. 1967; Marshall 1998; Godinho y col. 2013; Minnis y Lindner 2013).

Entre los hongos pertenecientes a la clase *Leotiomycetes*, una de las especies más frecuentemente aisladas de las muestras de miel fue *Bettsia alvei*, reportada generalmente para diversos sustratos con baja a_w (www.cabri.org/collections.html; http://gcm.wfcc.info/). *Bettsia alvei* tiene una a_w óptima de crecimiento de 0,89, y es capaz de crecer y germinar hasta una a_w de 0,73 y 0,70, respectivamente (Pitt y col. 2013). *Skoua fertilis*, perteneciente a la familia *Myxotrichaceae*, también fue detectado en todas las muestras de miel analizadas. Este hongo es capaz de crecer y producir propágulos de dispersión a una a_w mínima de 0,77 (Pitt 1965; Wynns 2015), una habilidad similar a la mostrada por nuestras cepas (a_w mínima testada de 0,82). A partir del mismo tipo de sustrato aislamos varias cepas de una especie filogenéticamente cercana, pero diferente a *S. fertilis*: *S. asexualis*. *Skoua asexualis* se diferencia morfológicamente de *S. fertilis* en que es incapaz de producir el morfo sexual, presentando tan solo un mecanismo de reproducción asexual, por otro lado, *S. asexualis* tiene una capacidad xerotolerante al crecer ligeramente a a_w de 0,82. *Oidiodendron mellicola*, una nueva especie de la familia *Myxotrichaceae* aislada de muestras de mieles, muestra, al igual que las demás especies del género, características psicrotróficas, creciendo de forma óptima a 15 ºC que a 25 ºC, además de características xerotolerantes al poder crecer en medio MY70FG con a_w de 0,76 (Rodríguez-Andrade y col. 2019a).

A partir de muestras de “cava” y de tapones de corcho que sellaban las botellas fueron aisladas varias cepas morfológicamente asimilables al género *Diplococcium*. Como resultado de un posterior estudio filogenético, dichas cepas se agruparon con otras del género *Kirschsteiniothelia*, cuya género-forma asexual era anteriormente denominado *Dendryphiopsis*, y con el cual nuestros aislados mostraban similitud morfológica. Debido a que estas cepas tenían características morfológicas lo suficientemente distintivas con respecto al resto de las especies aceptadas para el género, y a la distancia genética con respecto a las mismas, se han propuesto dos nuevas especies: *Kirschsteiniothelia ebriosa* y *K. vinigena*, distinguidos morfológicamente de las demás especies del género, por la producción de conidios con 1–2 septos que forman cadenas ramificadas (Rodríguez-Andrade y col. 2019b). Hay que destacar que ninguno de los hongos aislados de “cava” fue capaz de crecer a una concentración de etanol ≥ 10% v/v, concentración cercana al contenido alcohólico medio del vino espumoso (D. O. cava), de un 11,5% v/v; sin embargo, la

mayoría de estos hongos lograron mantener la viabilidad en dichas condiciones (Rodríguez-Andrade y col. 2019b).

Monodictys arctica (*incertae sedis*), originalmente aislada de raíces de *Saxifraga oppositifolia* en el alto ártico canadiense y descrita como una especie psicrófila (Day y col. 2006), fue recuperada de una muestra de suelo de la Antártida (datos no publicados).

Finalmente, a partir de las muestras de miel se obtuvieron seis aislados pertenecientes a cuatro especies de la división *Mucoromycota*: *Cunninghamella bertholletiae*, *Mortierella antarctica*, *Mucor plumbeus* y *Rhizopus oryzae*. Se sabe que estas especies están ampliamente distribuidas a nivel geográfico, siendo capaces de desarrollar sobre un amplio espectro de sustratos especialmente ricos en azúcares.

6. CONCLUSIONES

En la presente tesis doctoral se realizaron estudios polifásicos (morfológico, fisiológico y de filogenia molecular) a diversos aislados fúngicos obtenidos a partir de diversos sustratos y ambientes considerados como extremos para la mayoría de formas de vida: **mieles** (con una extremadamente baja a_w y una elevada pOsm); **cava** (con un pH alrededor de 4,0, casi sin azúcares residuales, con una concentración alcohólica cercana a 11,5 % v/v, y sometido a una elevadísima pCO_2)/tapones de corcho que sellaban las botellas; **suelos antárticos** (sometidos a largos períodos de tiempo a temperaturas bajo cero y una extrema desecación, seguido de breves períodos con temperaturas hasta los +11 ºC y HRA cercanas a 100%); **biopelículas polimicrobianas** sobre superficies metálicas (aleaciones de zinc e hierro), concreto (pH alcalinos, por encima de 8,5) y pinturas sintéticas en una nave industrial y viviendas vecinas (todas superficies sometidas a irradiación solar durante varias horas/día, a una extrema desecación debido a los vientos dominantes de la zona, y a xenobióticos producidos por la industria); y también especímenes clínicos procedentes de pacientes de todo USA y de diversas localizaciones anatómicas (mayoritariamente sometidos a una temperatura constante de 35-37 ºC y una pO_2 reducida). En el caso de los aislados de origen clínico, se realizó además un estudio de sensibilidad *in vitro* frente a las drogas antifúngicas más comúnmente empleadas en terapéutica humana. Como resultados de dichos estudios:

1. El empleo del medio de cultivo G18 con muestras de mieles ha permitido aislar una gran diversidad de hongos xerófilos y xerotolerantes, demostrando así que este sustrato es un hábitat poco explorado en cuanto a la diversidad fúngica.

   Un total de 104 cepas fúngicas han sido aisladas a partir de 83 muestras de miel de España (y de una procedente de Argentina), las que fueron distribuidas en 32 especies de los géneros *Alternaria*, *Ascosphaera*, *Aspergillus*, *Bettsia*, *Candida*, *Cunninghamella*, *Eremascus*, *Helicoarthrosporum*, *Monascus*, *Mucor*, *Oidiodendron*, *Penicillium*, *Rhizopus*, *Schizosaccharomyces*, *Skoua*, *Strongyloarthrosporum*, *Talaromyces*, *Xerochrysium* y *Zygosaccharomyces*.

   Varias de dichas cepas no pudieron ser clasificadas por debajo de nivel de orden o de familia, motivo por el cual se han propuesto los siguientes nuevos táxones: familia

Helicoarthrosporaceae Stchigel, Rodr.-Andr. & Cano, la cual tan solo incluye dos géneros monoespecíficos: ***Helicoarthrosporum*** Stchigel, Cano & Rodr.-Andr., con ***Helicoarthrosporum mellicola*** Stchigel, Cano & Rodr.-Andr. y ***Strongyloarthrosporum*** Rodr.-Andr., Cano & Stchigel, con ***Strongyloarthrosporum catenulatum*** Rodr.-Andr., Cano & Stchigel, como especies tipo respectivamente. Las especies: ***Oidiodendron mellicola*** Rodr.-Andr., Cano & Stchigel, ***Skoua asexualis*** Rodr.-Andr., Cano & Stchigel, ***Talaromyces basipetosporus*** Stchigel, Cano & Rodr.-Andr., ***Talaromyces brunneosporus*** Rodr.-Andr., Cano & Stchigel y ***Talaromyces affinitatimellis*** Rodr.-Andr., Stchigel & Cano.

2. A partir de las muestras de vino espumoso (D. O. cava) y tapones de corcho que sellaban las botellas, fueron aisladas un total de 27 cepas fúngicas, distribuidas en 16 especies correspondientes a los siguientes géneros: *Alternaria*, *Aspergillus*, *Beauveria*, *Candida*, *Cladophialophora*, *Cladosporium*, *Dactylodendron*, *Kirschsteiniothelia*, *Penicillium*, *Rasamsonia* y *Talaromyces*.

   A partir de estas cepas, se ha propuesto crear un género nuevo, ***Dactylodendron*** Stchigel, Rodr.-Andr. & Cano, el que incluye la nueva combinación ***Dactylodendron pinicola*** (Sigler & Yamaoka) Rodr.-Andr., Cano & Stchigel (la especie tipo del género) y las nuevas especies ***Dactylodendron ebriosum*** Rodr.-Andr., Cano & Stchigel y ***Dactylodendron pluriseptatum*** Rodr.-Andr., Cano & Stchigel; y las nuevas especies ***Kirschsteiniothelia ebriosa*** Rodr.-Andr., Cano & Stchigel, ***Kirschsteiniothelia vinigena*** Rodr.-Andr., Cano & Stchigel ***Rasamsonia frigidotolerans*** Rodr.-Andr., Cano & Stchigel, ***Talaromyces speluncarum*** Rodr.-Andr., Cano & Stchigel y ***Talaromyces subericola*** Rodr.-Andr., Cano & Stchigel.

   La presencia de estos hongos en las muestras de “cava” con alteración organoléptica compatibles con “*cork taint*” y en los tapones de corcho que sellaban las botellas tienen, muy probablemente, origen en el ambiente de la bodega donde los vinos sufren el proceso de fermentación secundaria y envejecimiento. Ninguna de las especies fúngicas recuperadas de dichos sustratos fue capaz de crecer a concentraciones de etanol similares a las del cava en estudio (aprox. 11,5 % v/v), pero mantuvieron su viabilidad a dichas concentraciones alcohólicas, de acidez fija y de elevadas presiones de CO_2 generadas dentro de las botellas. Sin embargo, queda por

evaluar la capacidad de dichos hongos de producir el TCA y/u otros compuestos volátiles orgánicos involucrados en la producción del "*cork taint*" detectado en las muestras de vino.

3. La caracterización fenotípica y el estudio de filogenia molecular de 22 cepas de origen clínico procedentes de USA y que habían sido identificadas presuntivamente como pertenecientes al género-forma *Malbranchea*, permitió asignar varias de ellas a los géneros *Arachnomyces* (2 cepas), *Currahmyce*s (1 cepa), *Malbranchea* (15 cepas) y *Spiromastigoides* (2 cepas).

   Dos de las cepas no pudieron ser clasificadas más allá del nivel de familia, motivo por el cual se han propuesto como especies tipo de dos nuevos géneros monoespecíficos, ***Pseudoarthropsis*** Stchigel, Rodr.-Andr. & Cano (***Pseudoathropsis crassispora*** Rodr.-Andr., Stchigel & Cano) y ***Pseudomalbranchea*** Rodr.-Andr., Cano & Stchigel (***Pseudomalbranchea gemmata*** Rodr.-Andr., Cano & Stchigel). También se han propuesto las siguientes nuevas especies para la ciencia: ***Arachnomyces bostrychodes*** Rodr.-Andr., Cano & Stchigel, ***Arachnomyces graciliformis*** Rodr.-Andr., Stchigel and Cano, ***Currahmyces sparsispora*** Rodr.-Andr., Cano & Stchigel, ***Malbranchea gymnoascoidea*** Rodr.-Andr., Stchigel & Cano, ***Malbranchea multiseptata*** Rodr.-Andr., Cano & Stchigel, ***Malbranchea stricta*** Rodr.-Andr., Stchigel & Cano y ***Spiromastigoides geomyces*** Stchigel, Rodr.-Andr. & Cano. En base a los resultados obtenidos del estudio filogenético, se ha propuesto sinonimizar el género *Auxarthron* con el género *Malbranchea*, lo que comporta la propuesta de las siguientes nuevas combinaciones: ***Malbranchea californiense*** (G.F. Orr & Kuehn) Rodr.-Andr., Stchigel & Cano, ***Malbranchea chlamydospora*** (M. Solé, Cano & Guarro) Rodr.-Andr., Cano & Stchigel, ***Malbranchea compacta*** (G.F. Orr & Plunkett) Rodr.-Andr., Cano & Stchigel, ***Malbranchea concentrica*** (M. Solé, Cano & Guarro) Rodr.-Andr., Stchigel & Cano, ***Malbranchea conjugata*** (Kuehn) Rodr.-Andr., Cano & Stchigel, ***Malbranchea indica*** (Kuehn) Rodr.-Andr., Cano & Stchigel, ***Malbranchea longispora*** (Stchigel, Y. Marín, Guarro & Cano) Rodr.-Andr., Stchigel & Cano, ***Malbranchea ostraviense*** (Hubka, Dobiášová & M. Kolařík) Rodr.-Andr., Cano & Stchigel, ***Malbranchea pseudauxarthron*** (G.F. Orr & Kuehn) Rodr.-Andr., Stchigel & Cano, ***Malbranchea reticulata*** (Arx) Rodr.-Andr., Stchigel & Cano, ***Malbranchea***

umbrina (Boud.) Rodr.-Andr., Cano & Stchigel, ***Malbranchea zuffiana*** (Morini) Rodr.-Andr., Stchigel & Cano, ***Pseudoarthropsis cirrhata*** (Oorschot & de Hoog) Stchigel, Rodr.-Andr. & Cano, ***Spiromastigoides gypsea*** (Sigler & Carmichael) Stchigel, Rodr.-Andr. & Cano.

Debido a que las 22 cepas fueron capaces de crecer por encima de los 30 ºC, 20 de estas pudieron hacerlo a los 35 ºC, y una hasta los 40 ºC, se ha demostrado su termotolerancia. Con respecto al estudio de sensibilidad *in vitro* de estos hongos, las equinocandinas (AFG, MFG y CFG) mostraron tener la mayor actividad antifúngica, seguida de la TRB y el PSC. Por el contrario, la AMB, la FLC, el ITC y la 5-FC mostraron tener una baja o nula actividad sobre estos hongos.

4. De los aislados del género *Penicillium* procedentes de muestras de suelo de México y España, después de su estudio taxonómico polifásico, cuatro resultaron pertenecer a nuevas especies para la ciencia: ***Penicillium melanosporum*** Rodr.-Andr., Cano & Stchigel, ***Penicillium michoacanense*** Rodr.-Andr., Cano & Stchigel, ***Penicillium sexualis*** Rodr.-Andr., Stchigel & Cano y ***Penicillium siccutolerans*** Rodr.-Andr., Stchigel & Cano. Las cuatro nuevas especies han mostrado ser extremadamente xerotolerantes, siendo capaces de crecer a a_w de 0,76.
5. De un total de 37 cepas aisladas a partir de sustratos (mayoritariamente suelo) colectados en la Antártida, Armenia, Chile, Ecuador, España, Irán y Vietnam, obtuvimos cinco nuevas especies para la ciencia: ***Cadophora antarctica*** Rodr.-Andr., Stchigel, Mac Cormack & Cano, ***Dothiora infuscans*** Rodr.-Andr., Stchigel, Guarro & Cano, ***Exophiala frigidotolerans*** Rodr.-Andr., Cano & Stchigel, ***Geosmithia xerotolerans*** Rodr.-Andr., Cano & Stchigel y ***Phialemonium guarroi*** Rodr.Andr., Cano & Stchigel.

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8. ANEXOS

ANEXO 1. Medios de cultivo

Los medios de cultivo fueron preparados con agar bacteriológico (Oxoid, España), esterilizados a 121 ºC durante 15'.

Algunos medios de cultivo, como aquellos destinados al aislamiento primario de los hongos, necesitaron la adición de 5 mL de una solución de L-cloranfenicol 1,5 % p/v en etanol absoluto por cada 500 mL de medio de cultivo estéril, volumen que era adicionado al medio de cultivo líquido en bañomaría a 50-55 ºC.

Agar agua

Agua de red, c.s.p. 1L; agar-agar, 8 g

Agar con extracto de patata y glucosa (PDA)

Agua destilada, c.s.p. 1L; agar patata dextrosa (Pronisa, España), 19,5 g

Agar con extracto de patata y zanahoria (PCA)

Agua destilada, c.s.p. 1L; agar-agar, 20 g; patata, 20 g; zanahoria, 20 g

Agar con extracto de malta (MEA)

Agua destilada, c.s.p. 1L; agar-agar, 7,5 g; L-cloranfenicol, 0,1 g; extracto de malta, 10 g; glucosa anhidra, 10 g; peptona bacteriológica, 0,5 g

Agar con harina de avena (OA)

Agua de red, c.s.p. 1L; agar-agar, 13 g; harina de avena, 30 g

Agar de Czapek con extracto de levadura (CYA)

Agua destilada, c.s.p. 1L; agar-agar, 7,5 g; cloruro de potasio, 0,5 g; extracto de levadura, 5 g; fosfato de potasio, 1 g; nitrato de sodio, 3 g; sacarosa, 30 g; sulfato de hierro $7H_2O$, 0,01 g; sulfato de magnesio $7H_2O$, 0,5 g

Agar con extracto de levadura y sacarosa (YES)

Agua destilada, c.s.p. 1L; agar-agar, 10 g; extracto de levadura, 10 g; sacarosa, 75 g

Agar con dicloran y glicerol 18 % (DG18)

Agua destilada c.s.p. 1L; agar-agar, 15 g; L-cloranfenicol, 0,1 g; dextrosa, 10 g; dicloran, 31,5 g; fosfato monopotásico, 1 g; glicerol, 220 g; peptona, 5 g; sulfato de magnesio $7H_2O$, 0,5 g; pH = 5,6 ± 0,2

Agar con glicerol 18 % (G18)

La preparación del G18 es igual a la de DG18, pero sin agregarle el dicloran.

Agar con creatina y sacarosa (CREA)

Agua destilada c.s.p. 1L; agar-agar, 10 g; cloruro de potasio, 0,5 g; creatina, 3 g; fosfato de potasio $3H_2O$, 1,6 g; purpura de bromocresol, 0,05 g; sacarosa, 30 g; sulfato de hierro $7H_2O$, 0,01 g; sulfato de magnesio $7H_2O$, 0,5 g

Agar con nitrato y 25 % glicerol (G25N)

Agua destilada c.s.p. 1L; agar-agar, 12 g; concentrado de Czapek, 7,5 g; extracto de levadura, 3,7 g; fosfato dipotásico, 0,75 g; glicerol, 250 mL

Agar glucosasado con sólidos de leche y púrpura de bromocresol (BCP-MS-G)

Agua destilada c.s.p. 1L; agar-agar, 30 g; glucosa, 40 g; leche desnatada en polvo, 80 g; purpura de bromocresol, 10 mL de 1,6 %/etanol 95 %

Agar para prueba de opacidad con Tween 80 (TOMT)

Agua destilada c.s.p. 1L; agar-agar, 15 g; cloruro de calcio, 1 g; cloruro de sodio, 5 g; peptona bacteriológica, 10 g; Tween, 5 mL; Tween, 80, 5 mL

Agar con fitona y extracto de levadura (PYE)

Agua destilada c.s.p. 1L; agar-agar, 20 g; extracto de levadura, 5 g; glucosa, 40 g; peptona papainica, 10 g

Agar con extractos de malta y de levadura y 70 % de fructosa/glucosa (MY70FG)

Agua destilada c.s.p. 1L; agar-agar, 12 g; extracto de malta, 6 g; extracto de levadura, 6 g; fructosa, 350 g; glucosa, 350 g; peptona, 10 g

Medios de montaje empleados para la observación microscópica

Las preparaciones microscópicas permanentes se realizaron con PVA en lactofenol (ácido láctico 20 g, fenol 20 g, glicerol 40 g y agua destilada 20 mL) (Barnett y col. 1974), y para las temporales con ácido láctico al 85 % (85 mL ácido láctico y 25 mL de agua destilada).

ANEXO 2. Almacenamiento y conservación de las cepas

Conservación en tubo con aceite mineral

Se realizaron subcultivos en varios tubos de ensayo de polietileno con tapón del mismo material y de cristal con tapa rosca, los cuales contenían el medio de cultivo más adecuado para favorecer el crecimiento y la esporulación del hongo (G18, para los aislados de mieles y PDA, para los aislados del cava, clínicos, de corcho, biopelículas y suelos), inclinados en pico de flauta. Estos se incubaron a 15 ºC para los psicrófilos, 25 ºC en el caso de los mesófilos y 35 ºC para los termófilos, hasta obtener colonias esporuladas. Las cepas en tubos de ensayo de cristal se conservaron en nuestra colección cubriendo totalmente el cultivo con aceite mineral estéril. Estos cultivos fueron almacenados a temperatura ambiente, mientras que los tubos de ensayo de plástico fueron enviados para su depósito en la colección del CBS.

Conservación en agua

Se cortaron varios bloques de 0,5 cm de lado a lado a partir de las colonias esporuladas/fructificadas de los hongos de interés crecidas en los medios de cultivo gelificados adecuados, y se introdujeron de forma aséptica en dos viales de cristal, uno con agua destilada y otro con agua de red estéril. Posteriormente, fueron cerrados herméticamente con un tapón de caucho sintético y se almacenaron a temperatura ambiente.

Liofilización

Del mismo modo que en la técnica anterior, a partir de colonias esporuladas de las cepas de interés con la ayuda de un asa de siembra se obtuvo una suspensión de estructuras reproductivas y vegetativas fúngicas en 3 mL de skim milk (Difco, USA) al 10%, un crioprotector previamente esterilizado a 115 ºC durante 15'. Se agitó ligeramente y 1 mL de la suspensión se colocó en viales de vidrio estériles de 3 mL. Dichos viales se colocaron en el liofilizador (Advantage 2.0 Series; Virtis Company Gardiner, USA) y se procedió a la sublimación de la muestra. Cuando el condensador llegó a la temperatura de -45 ºC y después de que obtuvo un vacío a 200 mTorr, se programó al siguiente ciclo de liofilización: -30 ºC (240'), -10 ºC (240'), +10 ºC (300'), y +30 ºC

(300'). Una vez finalizado el proceso, se sellaron los viales en condiciones de vacío. El éxito de la liofilización se determinó comprobando la viabilidad del hongo mediante la selección al azar de un vial, rehidratando la muestra y sembrándola en placa Petri con medio agarizado para poder observar el crecimiento del hongo deseado. Los viales con el hongo liofilizado se conservaron a temperatura ambiente.

ANEXO 3. Cebadores utilizados en la amplificación y secuenciación

Locus	**Cebador**	**Dirección**	**Secuencia**	**Referencia**
LSU	LR0R	Directo	GTA CCC GCT GAA CTT AAG C	Rehner & Samuels 1994
	LR5	Inverso	TCC TGA GGG AAA CTT CG	Vilgalys & Hester 1990
ITS	ITS5	Directo	GGA AGT AAA AGT CGT AAC AAG G	White y col. 1990
	ITS4	Inverso	TCC TCC GCT TAT TGA TAT GC	White y col. 1990
BenA	T10	Directo	ACG ATA GGT TCA CCT CCA GAC	O´Donnell y Cigelnik 1997
	Bt2b	Inverso	ACC CTC AGT GTA GTG ACC CTT GGC	Glass y Donaldson 1995
CaM	Cmd5	Directo	CCG AGT ACA AGG ARG CCT TC	Hong y col. 2005
	Cmd6	Inverso	CCG ATR GAG GTC ATR ACG TGG	Hong y col. 2005
*rpb*2	RPB2-5F	Directo	GAY GAY MGW GAT CAY TTY GG	Liu y col. 1999
	RPB2-7cR	Inverso	CCC ATW GCY TGC TTM CCC AT	Liu y col. 1999

De acuerdo con el comité de nomenclatura de la unión internacional de bioquímica (IUPAC), los siguientes simbolos representan a las bases nitrogenadas; **M**: Adenina o Citocina; **Y**: Citocina o Timina; **W**: Adenina o Timina.

ANEXO 4. Programas y ciclos térmicos utilizados para la amplificación

Gen	**Desnaturalización inicial**	**Ciclos**	**Desnaturalización**	**Anillaje**	**Elongación**	**Elongación final**
ITS, LSU	94 °C, 5'	35	94 °C, 30''	53 °C, 1'	72 °C, 2'	72 °C, 7'
BenA			95 °C, 30''	55 °C, 1'	72 °C, 1' 30''	
CaM			94 °C, 30''	55 °C, 1'	72 °C, 1' 30''	
*rpb*2			95 °C, 45''	56 °C, 1'	72 °C, 1' 45''	

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