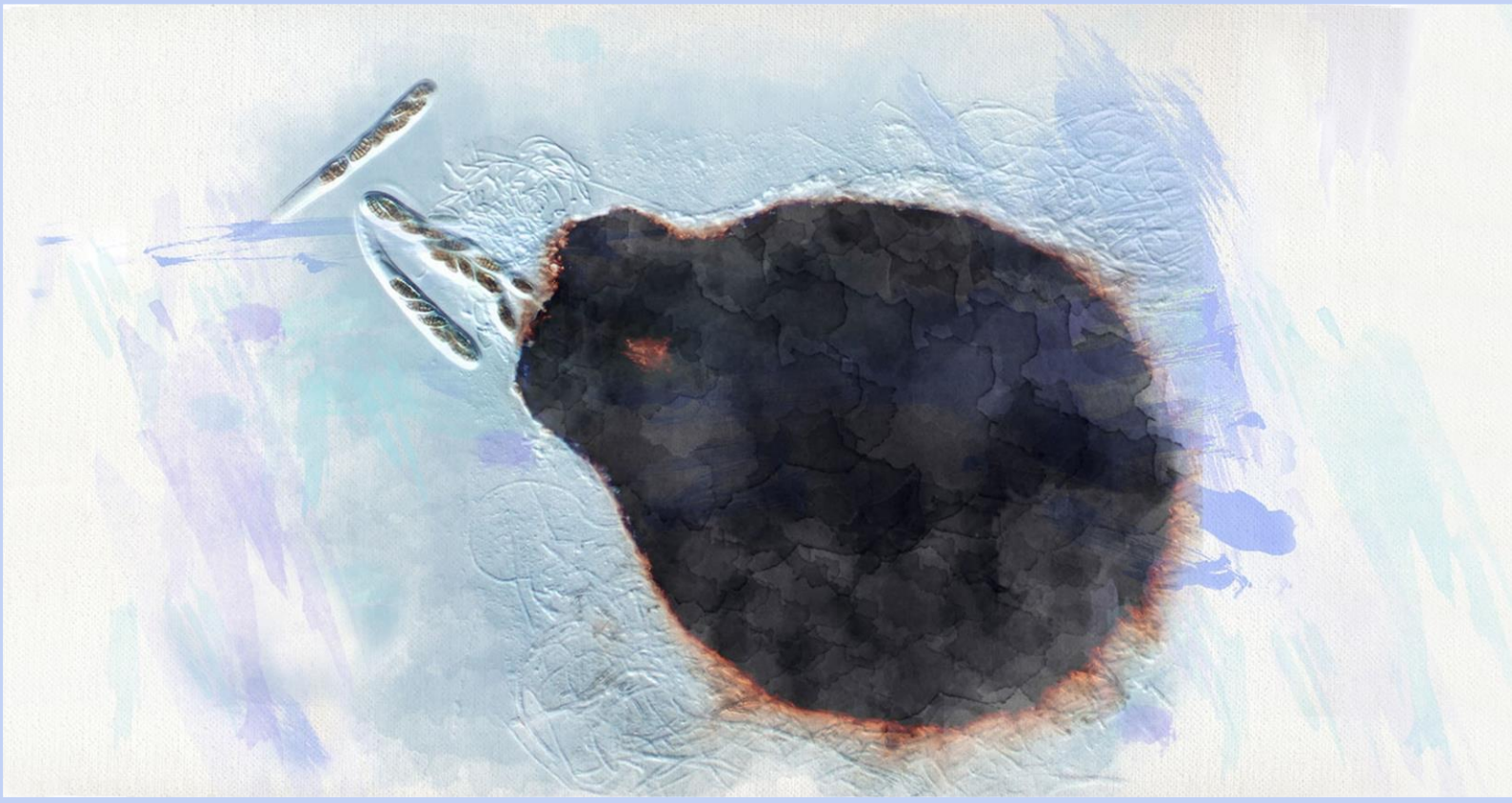
TAXONOMÍA Y FILOGENIA MOLECULAR DE HONGOS PROCEDENTES DE MATERIAL VEGETAL SUMERGIDO DE ESPAÑA

Viridiana Magaña Dueñas

UNIVERSITAT ROVIRA i VIRGILI

Taxonomía y filogenia molecular de hongos procedentes de material vegetal sumergido de España

VIRIDIANA MAGAÑA DUEÑAS



TESIS DOCTORAL
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TESIS DOCTORAL

Dirigida por los Doctores: José Francisco Cano Lira y
Alberto Miguel Stchigel Glikman

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

Reus
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HAGO CONSTAR que el presente trabajo, titulado **"Taxonomía y filogenia molecular de hongos procedentes de material vegetal sumergido de España",** que presenta **Viridiana Magaña Dueñas,** para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento de Ciencias Médicas Básicas de esta Universidad.

Reus, 21 de Febrero de 2022

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AGRADECIMIENTOS

En primer lugar, me gustaría agradecer a mis directores de tesis que han sido un apoyo fundamental para el desarrollo de ésta tesis. Dr Pep Cano porque más que mi padre científico has sido un gran amigo, por esa botella de cava que nunca faltó en mi cumpleaños, y que me hacía sentir confortada en un día tan duro para mi por estar tan lejos de mis seres queridos, por estar al pendiente de mi, no solo como tu becaria, si no como persona, por tener la cercanía de preguntar si estaba bien cuando no lo estaba. Dr. Alberto por también ser mi padre científico, por ésas charlas interminables, por apoyar y debatir las ideas que iban surgiendo a lo largo del tiempo y por todas las risas a causa del sentido del humor tan particular que compartimos, pero sobre todo por confiar en mí y hacerme saber mis errores, pero también mis aciertos. A ambos, infinitas gracias siempre les estaré muy agradecida.

Al Dr. Guarro, por permitirme ser parte del grupo de investigación. A la Dra. Danía García, por las risas, por la cercanía, por el té de las tardes, porque a pesar de todo siempre has tenido una alegría desbordante, porque en los momentos más duros estuviste ahí para darme palabras de aliento. Y a los demás integrantes de la unidad de micología, los Doctores Xavy, Beli, Josepa Gené, Katy, Ana, Marta, Emilio Mayayo y Loida, porque de una u otra forma fueron parte mi formación.

A Nuria por todas las conversaciones en los desayunos, por transmitir paz en un lugar de tanta locura. A mi bella pelirroja Carma, por las tardes British, por tener esa energía inmensa que me contagiaba de buen humor. A ambas, porque sin ustedes sería más complicada la labor en el laboratorio.

A Dany mi cari, por esas charlas sin hablar, por todos los ataques de risa de los cuales ni siquiera nosotros sabíamos el motivo, por ser esa lucesita que alumbró muchos días de oscuridad, por todos los apapachos, por ser un miembro más de mi familia al otro lado del charco. Gràcies amic del meu cor t'estimo molt. A Neto, por ser la conexión que me introdujo en éste sitio de locura, por tu amistad muchas gracias. A Isa, por las enseñanzas en el laboratorio y por todas las experiencias vividas fuera de el. A Nico por ser mi sensei en el mundo de los celomicetos. Fueron muchas las personas que han ido y venido a lo largo de todos estos años, algunos solo de estancia, otros por años, pero sin duda de cada uno me llevo una gran lección. Desde aquella única y fugaz charla con Jõao en la que me dijo disfruta intensamente cada minuto, porque cuando menos lo esperes estarás haciendo las maletas para volver. Pasando por Alba New que me dio una muestra magistral de madurez y fortaleza, me enseñó que

no importa las dificultades, siempre se puede salir adelante. A los que ya no están a los que están por irse y a los que acaban de llegar, muchas gracias a cada uno de ustedes, porque la experiencia del doctorado la hacemos los doctorandos.

A Ger mi gran amiga y compañera de viaje en éste lado del charco. Agradezco infinitamente a la vida por haberte puesto en mi camino. Has sido una pieza fundamental en la vida que cree en éste país, nunca terminaré de agradecerte por estar con mi versión más rota y estar ahí hasta que poco a poco me reconstruí. Leal y paciente muy paciente. Gracias por estar en momentos de infinita tristeza, de corazones rotos, de locura absurda y risas inagotables.

A mi núcleo duro, por todas las risas, por hacer mi estancia en éste continente más placentera, por la terapia grupal del grupo de ayuda que no ayuda, por ser buenos amigos. Porque a pesar de ser tan diferentes, de todas las maneras posibles, logramos un punto de unión y solo puedo decir que ha sido una delicia conocerlos.

A mis amigos de México, Elvia, Julio, Mary y Ruth. Porque a pesar del tiempo y la distancia siempre han estado ahí para mi. Porque nunca los sentí lejos, siempre encontraron la manera de hacerme saber que no estaba sola, que seguía siendo parte importante de sus vidas. Porque a la vuelta parecía que el tiempo nunca había pasado.

A mi tía Paty porque siempre creiste en mi. Cuando me fui esperaba encontrarte a mi regreso, no fue así y tu partida representó mi primer mayor reto. Me quedo con las ultimas palabras que nos dijimos. A disfrutar de la vida que solo hay una y siempre a sonreir, porque como decias un día sin reir es un día desperdiciado. Para ti y mi abuelo Dario infinitas gracias hasta el cielo. A mi tia Luci, Adi, Edgar, Pato, y a mis abuelas por estar presentes durante todos estos años, los quiero mucho.

A mi hermano, porque, aunque no eres muy expresivo, a tu manera me recordabas que estabas ahí y que me extrañabas. A mi Sofy, por ser tan paciente y por estar al pendiente más que cualquier persona, por todos los mensajes y fotografías que me daban la vida, porque a pesar de no ser madre, te quiero como si lo fuera. A mis padres, mi gran soporte, los que siempre apoyan todas mis locuras, por enseñarme que la nostalgia no debe de cortarme las alas, por amarme incondicionalmente y decirme siempre las palabras presisas para hacerme sentir mejor. Gracias infinitas.

ÍNDICE

Índice de abreviaturas

ADN Ácido desoxirribonucleico

ARN Ácido ribonucleico

ATCC Colección Americana de Cultivos Tipo (USA)

BI Inferencia Bayesiana

BLAST Herramienta Básica de Búsqueda de Alineación Local

BS Soporte de bootstrap

CBS Centraalbureau voor Schimmelcultures (oficina central de cultivos fúngicos), Westerdijk Fungal Biodiversity Institute, Utrecht, Paises Bajos.

CO_2 Dióxido de Carbono

comb nov Nueva combinación

cm Centímetro

D1-D2 Dominios D1 y D2 del gen 28S del rARN

diam Diámetro

EDTA Ácido etilendiaminotetraacético

ENA Archivo Europeo de Nucleótidos

et al. Y colaboradores

etc. Etcétera

EtOH Etanol

Fig. Figura

FMR Facultat de Medicina, Reus

g Gramo

gen. nov. Género nuevo

H_2O Agua

ITS Región espaciadora intergénica transcrita del ARNr

L Litro

LSU Subunidad mayor del ADN ribosomal

M Concentración molar

MCMC Cadena Markov-Monte Carlo

MEA Agar con extracto de malta

MEGA Análisis Genético Evolutivo Molecular

MFLUCC Colección de cultivos de la Universidad Mae Fah Luang, Chiang Rai, Tailandia

mg Miligramos

ML Máxima verosimilitud

mL Mililitro

mm Milimetro

mM Milimolar

MUSCLE Comparación de Secuencias Múltiples por Expectativa Logarítmica

NCBI Centro Nacional de Información Biotecnológica (EE.UU.)

OA Agar harina de avena

pb Pares de bases

P. ej Por ejemplo

PCR Reacción en cadena de la polimerasa

PDA Agar con extracto de patata-glucosa

pH Potencial de hidrógeno

PP Probabilidad posterior

RAxML Máxima Probabilidad Aleatorizada Axelerizada

RNAr ARN ribosomal

rpb2 Gen de la subunidad II de la ARN polimerasa

rpm Revoluciones por minuto

s Segundos

SDS Dodecilsulfato sódico

sp. nov. Especie nueva

SSU Gen 18S de la subunidad pequeña del ARN ribosomal

T Temperatura

tef-1 Factor de elongación 1-alfa

tub2 Beta-tubulina

UTHSC Laboratorio de Ensayos de Hongos, Centro de Ciencias de la Salud de la Universidad de Texas en San Antonio

v Versión

var. Variedad

µL	Microlitro
±	Más/ menos
º	Grados
ºC	Grados Celsius
%	Por ciento
≥	Mayor o igual que

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1.INTRODUCCIÓN

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1.1. Generalidades sobre los hongos

Los hongos representan uno de los grupos más diversos de organismos y, basados en análisis moleculares, se estima que evolucionaron como grupo monofilético hace unos 760 a 1,800 millones de años (Lücking et al. 2009). Los hongos se consideraron inicialmente como miembros del reino *Plantae* por poseer pared celular y ser inmóviles, por ello durante mucho tiempo se los clasificó dentro de la botánica de plantas criptogámicas. En 1821, el botánico sueco Elías Magnus Fries publicó *Systema Mycologicum,* la primera clasificación de los hongos basidiomicetos basada en las características morfológicas externas, utilizando criterios taxonómicos no valorados hasta entonces tales como: la forma y tamaño de los carpóforos, la consistencia de los mismos, el tipo de inserción de las láminas y el color de las esporas. La gran revolución de la taxonomía fúngica se produce en 1969, Whittaker propone la división de los seres vivos (Fig. 1) basándose en los niveles de organización celular (procariota, eucariota unicelular y eucariota pluricelular), y los modos de adquisición de nutrientes (fotosintético, absortivo o ingestivo), clasificándolos en cinco reinos: *Monera, Protista, Animalia, Plantae* y *Fungi* (Whittaker 1969)**.**

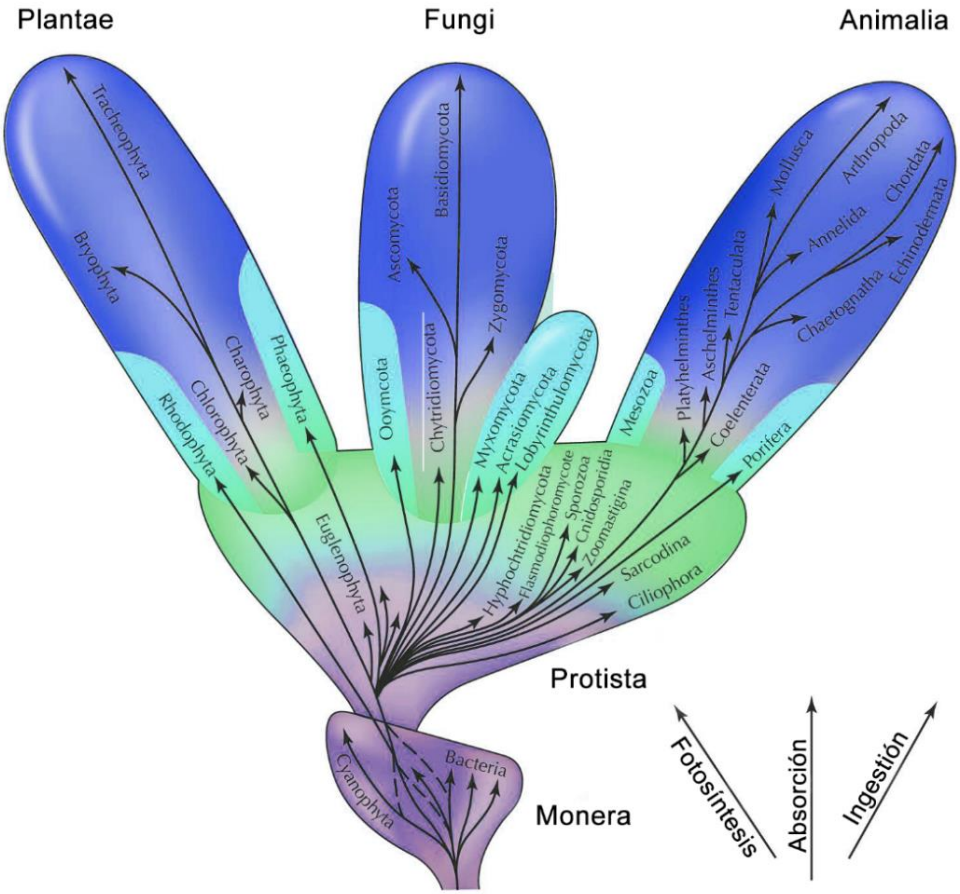

Figura 1. Árbol de los cinco reinos propuestos por Whittaker. (Adaptado y modificado de Barton 2007).

INTRODUCCIÓN

Tradicionalmente, en base a sus rasgos morfológicos, el reino de los hongos se dividió en cuatro divisiones: *Ascomycota, Basidiomycota, Chytridiomycota* y *Zygomycota* (Hawksworth et al. 1995). Sin embargo, los análisis moleculares revelaron que estos grupos de hongos son polifiléticos, y actualmente se reconocen nueve grupos filogenéticos bien definidos: *Opisthosporidia, Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Zoopagomycota, Mucoromycota, Glomeromycota, Ascomycota* y *Basidiomycota* (Fig. 2) (Spatafora et al. 2016).

Los hongos son organismos eucariotas, ubicuos, cuyo principal rol ecológico es degradar y reciclar la materia orgánica, contribuyendo así a regular el balance de carbono y nitrógeno, promoviendo la redistribución de nutrientes, mantenimiento de la estructura y fertilidad del suelo y ayudando al control biológico contra patógenos de las raíces (Frac et al. 2018). Los hongos del suelo pueden tener diferentes asociaciones con plantas, artrópodos, nematodos y otros hongos (Bridge & Spooner 2001). Son heterotróficos (porque al carecer de pigmentos fotosintéticos son incapaces de sintetizar moléculas orgánicas a partir de CO_2), por lo que, mediante la secreción de exoenzimas, hidrolizan diferentes macromoléculas presentes en el sustrato sobre el cual se desarrollan, generando así nutrientes que son absorbidos (alimentación osmótrofa) (Brandt & Warnock 2015). Los hongos actúan funcionalmente como saprobios o necrótrofos (nutriéndose de materia orgánica muerta), simbiontes (estableciendo una estrecha relación con otro/s organismo/s, con el/los que intercambian nutrientes), o parásitos (que viven en el exterior o interior de un huésped, del que obtienen beneficios sin proporcionar ninguna contribución útil a cambio); en el caso de los patógenos, esta relación es perjudicial para el huésped.

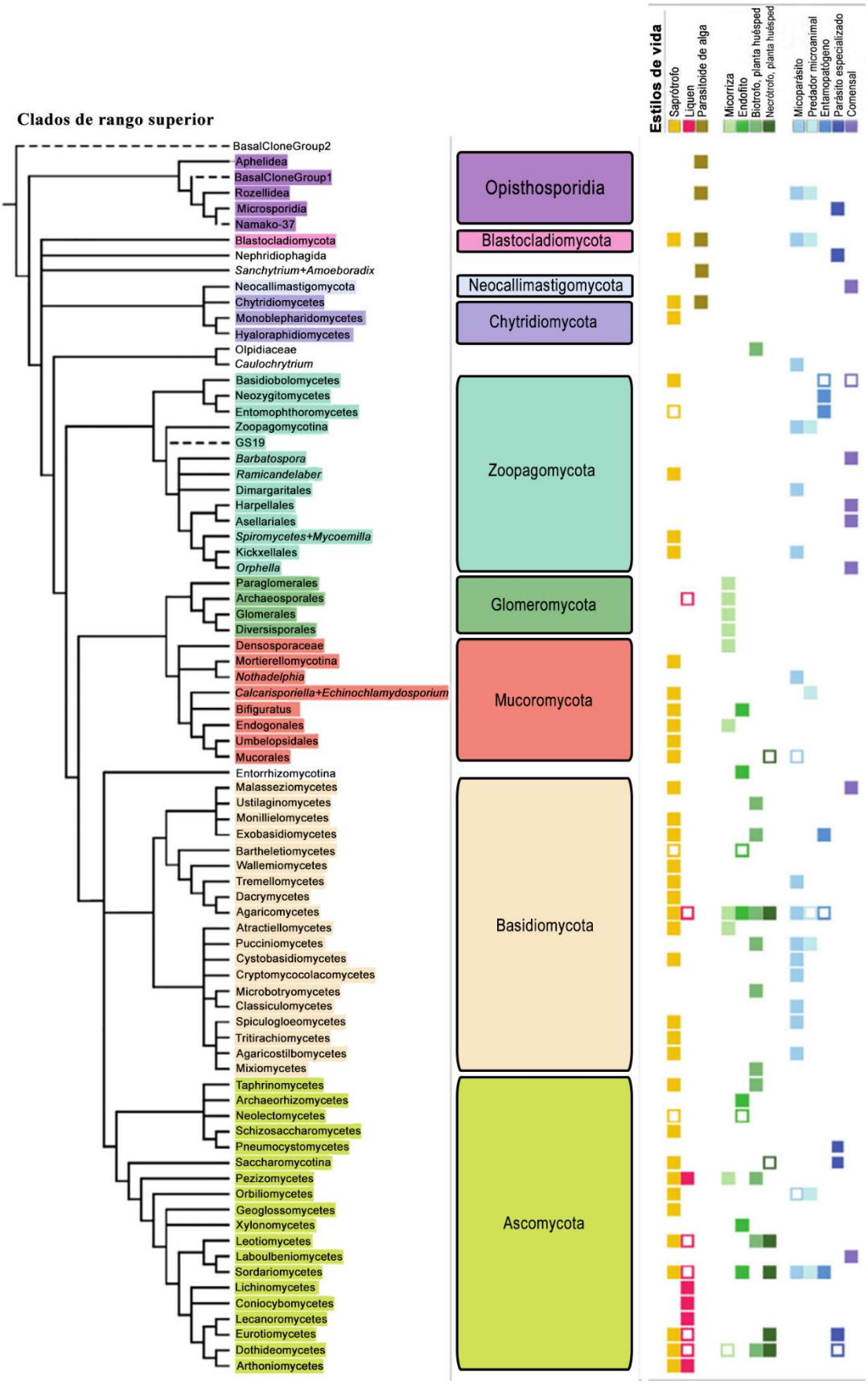

Figura 2: El árbol de la vida de los hongos. El árbol muestra los grupos descritos en el reino Fungi. La primera columna usa los colores para indicar los clados correspondientes. La segunda columna recopila los estilos de vida presentes en cada grupo. Los cuadros vacíos indican que el estilo de vida es hipotético (Adaptado y modificado de Naranjo-Ortiz & Gabaldón 2019).

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Los hongos pueden ser unicelulares o pluricelulares, denominándose hongos levaduriformes (levaduras) los primeros, y hongos filamentosos o miceliares (mohos) los segundos. Las levaduras se multiplican asexualmente mediante gemación o fisión, y producen colonias de tamaño restringido, mayoritariamente redondeadas, blanquecinas o coloreadas (debido a la producción de pigmentos carotenoides que le otorgan tonalidades amarillas, anaranjadas, asalmonadas o rojizas), y mucoides o butirosas en diferentes medios de cultivo. Los hongos filamentosos presentan estructuras vegetativas tubulares denominadas hifas, las que incrementan su longitud mediante un proceso conocido como extensión apical centrífuga (Brandt & Warnock 2015). Estas estructuras pueden ser cenocíticas (cuyas células no están individualizadas, consistente en un citoplasma multinucleado continuo) o septadas (divididas por invaginaciones de la pared celular denominadas septos). El conjunto de hifas que forma un individuo se denomina micelio. Existen tres tipos de micelio: sumergido en el sustrato (encargado de la fijación y la nutrición osmótrofa del individuo), superficial (micelio rampante), y aéreo (que es el responsablede la producción de estructuras reproductivas y de resistencia y de la textura de la colonia).

La velocidad o tasa de crecimiento de los hongos miceliares es más lento que el de las levaduras, y requiere tiempos de incubación de 7 a 30 días hasta obtener una colonia “madura” (que produce estructuras reproductivas fértiles). Algunos hongos tienen la capacidad de cambiar el tipo de talo, de levaduriforme a filamentoso (y/o viceversa), fenómeno conocido como dimorfismo. Este puede ser inducido por estímulos tales, como la variación de la concentración de oxígeno, el tipo y concentración de nutrientes, la actividad enzimática y/o la temperatura (Gauthier 2017).

En cuanto a la estrategia reproductiva (Fig 3), los hongos pueden multiplicarse asexualmente (forma, estado o fase asexual, anamorfo o forma imperfecta), en donde el hongo produce esporas cuyos núcleos provienen de la división mitótica de un núcleo preexistente, y/o sexualmente (forma, estado o fase sexual, teleomorfo o forma perfecta) caracterizada por la formación de esporas de origen meiótico. Las estructuras asociadas a ambos tipos de reproducción presentan una morfología muy diferente, por ello se los define como hongos pleomórficos (Hennebert 2003). Al hongo que presenta un ciclo de vida completo se le conoce como holomorfo. Algunos hongos pleomórficos son capaces de producir uno o más tipos de anamorfos, a los que se denomina sinanamorfos (Gams 1982). Sin embargo, para un importante número de hongos se desconoce su forma sexual, probablemente debido a las condicionales nutricionales, ambientales y genéticas, o porque a lo largo de la evolución han perdido la capacidad de reproducirse sexualmente (Hambleton & Sigler 2005).

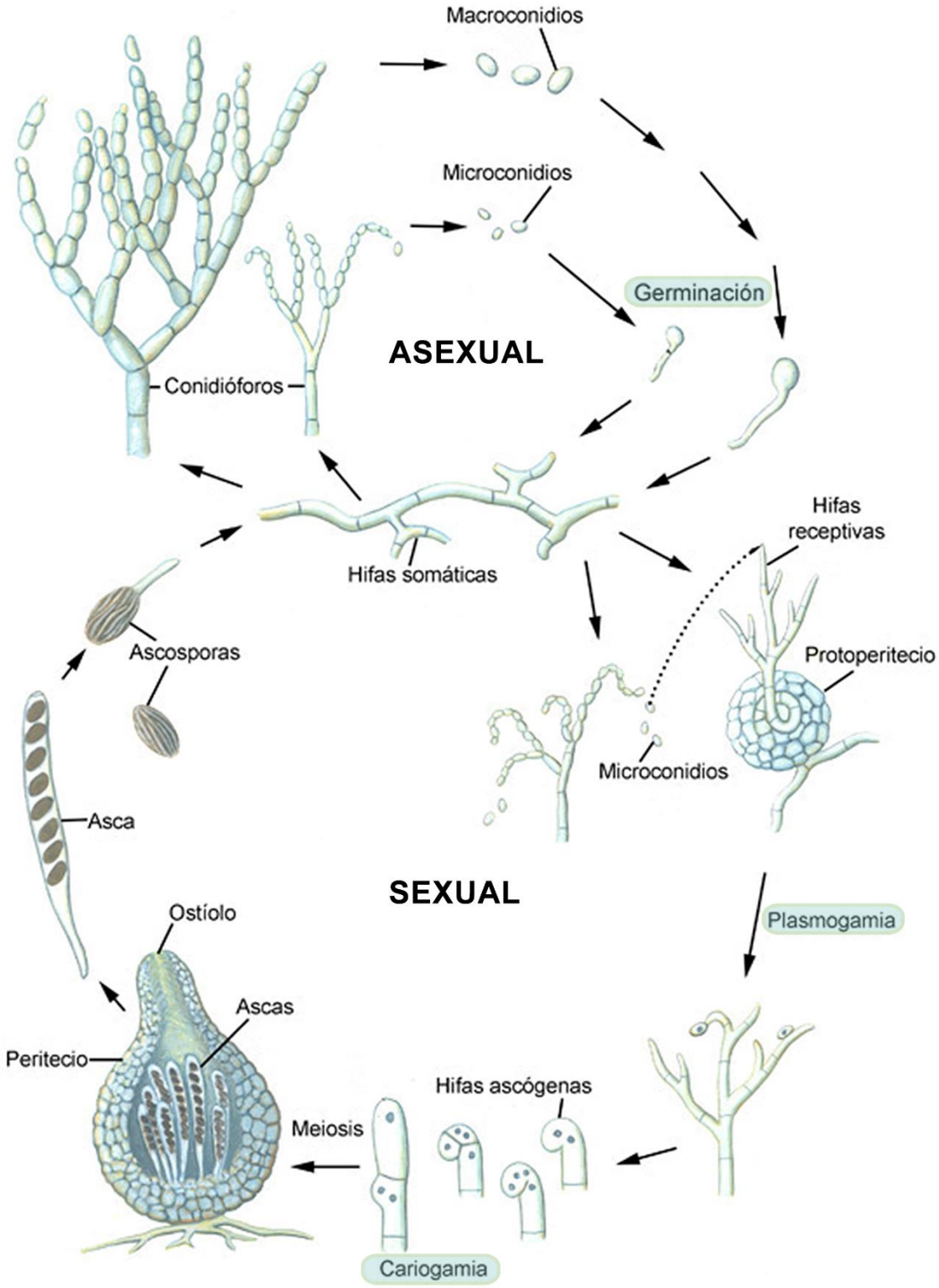

Figura 3. Fase de reproducción asexual (anamorfo) y sexual (teleomorfo) de un ascomiceto filamentoso (Adaptado y modificado de Herrera & Ulloa 1990).

1.2. Ecología fúngica

Los hongos son ubicuos, ocupan una amplia variedad de nichos ecológicos. Fisiológicamente los hongos se adaptan con relativa facilidad a diversas condiciones ambientes, recuperándose de una gran diversidad de sustratos como el suelo, la madera, el estiércol, los insectos, los tejidos vegetales/animales e incluso sobre otros hongos. El éxito en la colonización de un sustrato y de su persistencia a lo largo del tiempo en un determinado nicho ecológico dependerá de una serie de factores abióticos y bióticos tales como la disponibilidad de nutrientes, las características físicas del sustrato, la disponibilidad de agua, el pH, la humedad y la temperatura, entre otros (Mueller et al. 2004). Todos los hongos requieren de una fuente orgánica de carbono y energía, así como nitrógeno, fósforo, potasio, calcio y hierro, así como trazas de otros elementos químicos. Ante la escasez de fuentes de carbono orgánico asimilables, los hongos pueden utilizar el dióxido de carbono para complementar el suministro de carbono. En cuanto a los mecanismos de obtención de energía, los hongos preferentemente realizan respiración aeróbica en la cual el oxígeno es el aceptor final de electrones; en ausencia o déficit de oxígeno, tienen la capacidad de utilizar el nitrato como alternativa, así como la posibilidad de realizar diferentes tipos de fermentaciones (Watkinson 2016).

Los ambientes terrestres presentan una amplia diversidad de hábitats para la colonización fúngica, que van desde ambientes árticos, pasando por bosques templados de coníferas y bosques caducifolios y praderas, brezales y bosques tropicales hasta desiertos (Dighton 2007). El suelo, por su riqueza en materia orgánica, constituye un importante reservorio para propágulos fúngicos que son considerados el componente más abundante de la microbiota del suelo, que van desde levaduras hasta hongos filamentosos macroscópicos (Anderson & Domsch 1978).

El estiércol de animales representa otro medio ecológico rico para el desarrollo de hongos, ya que contiene elevadas y diversas fuentes de carbono disponibles, como celulosa, hemicelulosa y lignina, así como un alto contenido de nitrógeno. El estiércol de herbívoros puede contener un 4% de nitrógeno que es más que el del material vegetal originalmente consumido por los animales. También contiene vitaminas, minerales y alto contenido en agua, y con un pH alrededor de 6,5 , estás condiciones ofrecen un medio propicio para el crecimiento de hongos (Webster 1970). Los hongos coprófilos son un componente importante de los ecosistemas terrestres, son responsables de reciclar los nutrientes de las heces de los animales (Wicklow 1981).

Existe una gran diversidad de hábitats acuáticos charcas, estanques, lagos, pantanos, arroyos, ríos, lagos y lagunas salados de endorreicos, hielo, manantiales, aguas termales, mares y océanos. Los márgenes de estos ambientes están en contacto con rocas, tierra, barro y vegetación, los cuales representan fuentes ricas en carbono para la colonización fúngica (Dix & Webster 1995). A diferencia de los ecosistemas terrestres, que han sido estudiados ampliamente, los ecosistemas acuáticos han sido poco estudiados. Aunque, los hongos también pueden habitar ambientes de agua salada, en esta tesis hemos centrado los estudios en hongos que residen en hábitats de agua dulce (ambientes dulceacuícolas).

1.3. Hongos en aguas dulces

Los hongos que habitan las aguas dulces, también denominados dulceacuícolas, forman un grupo morfológica, filogenética y ecológicamente diverso. Su distribución es mundial, pudiendo estar restringidos a regiones con climas tropicales, templados o fríos, mientras que otros son cosmopolitas. Estos hongos dependen de los hábitats acuáticos la totalidad o parte de su ciclo de vida (Thomas 1996, Grossart et al. 2019). El principal rol de los hongos de agua dulce es en la degradación de material vegetal, tanto leñoso como herbáceo, que se encuentra en el agua, debido a la capacidad que tienen de degradar la celulosa y las lignocelulosas, como las hemicelulosas, el xilano y el almidón, macromoléculas cuantitativamente importantes en los dentritos vegetales, y fuente de carbono y energía para los primeros (Bäerlocher & Kendrick 1974, Zare-Maivan & Shearer 1988, Goh & Hyde 1996, Tsui et al. 2016). También están involucrados en la descomposición de partes de animales, como el pelo, las escamas de los peces y el exoesqueleto de los insectos. Otros hongos actúan como endófitos o patógenos de plantas (Wong et al. 1998).

En el proceso de descomposición son importantes la colonización, el crecimiento y la supervivencia del individuo en el sustrato. Las tasas de crecimiento se ven afectadas por factores tales como la temperatura y la interacción con otros organismos (Ogawa et al. 1996). Se ha reportado que los hongos de agua dulce muestran preferencia por determinados tipos de sustrato; en este sentido, el 60% de los mismos han sido aislados de trozos de madera sumergida, el 30% de restos herbáceos (incluidas las hojas y los tallos), y sólo el 10 % de ambos sustratos (Raja et al. 2009). Los investigadores sugieren que esto puede ser debido a diferentes motivos: **1)** los diferentes sustratos tienen variedad de nutrientes que favorecen el crecimiento de unos hongos sobre otros; **2)** a las diferentes sustancias inhibidoras presentes en los sustratos afectando la

germinación y esporulación de las esporas; **3)** a la diversidad en la estructura física del sustrato que afecta la eficiencia del anclaje y la capacidad de colonización por parte de algunos hongos, debido a que existe una variedad considerable en cuanto al tamaño y la forma de las esporas de los hongos mitospóricos acuáticos; **4)** a la variación en el tiempo del grado de descomposición de los sustratos, por lo que la exposición a las esporas y la colonización es mayor (Thomas el al. 1992).

1.3.1. Hábitats

Aproximadamente el 71% de la superficie del planeta Tierra está cubierta por agua. Sin embargo, sólo el 0,8 % es agua dulce. Los hábitats de agua dulce se caracterizan por tener un equilibrio de materia orgánica controlado por la superficie, ubicación y características de la cuenca (Wurzbacher et al. 2011). Los ecosistemas de agua dulce que albergan hongos pueden ser lénticos o lóticos.

Los ambientes lénticos son cualquier entorno acuático natural que carece de flujo constante de agua, tales como los lagos, los estanques, los pantanos, etc. (Thomas 1996). Estos proporcionan ambientes tranquilos para el desarrollo de los hongos. Sin embargo, tanto en los canales más amplios como en los lagos, se observa una baja proporción de materia orgánica proveniente de la vegetación, siendo mayoritaria la originada por la proliferación del fitoplancton (Bäerlocher 1992). La baja cantidad de flujo de agua comporta una deficiencia en el oxígeno disuelto, lo cual se traduce en una reducción significativa en la esporulación y en la producción de biomasa fúngica (Medeiros et al. 2009).

Los ambientes lóticos, comprenden ecosistemas acuáticos con flujo constante de agua (ríos, arroyos, manantiales, etc.) (Thomas 1996). El flujo constante permite que haya grandes cantidades de oxígeno disuelto. Estos hábitats suelen estar rodeados de vegetación, la cual proporciona grandes cantidades de materia orgánica en forma de hojarasca, madera, semillas y flores. Estas características crean un ambiente ideal para la colonización y esporulación de los hongos (Richardson & Danehy 2007).

Los seres humanos han transformado los paisajes naturales creando nuevos nichos acuáticos artificiales tales como las alcantarillas, tuberías de agua, plantas de tratamientos de aguas residuales, piscinas, fuentes, estanques o jardines, etc., los que han sido colonizados por los hongos (Grossart et al. 2019). Por otro lado, la descarga de efluentes industriales y domésticos, el uso indiscriminado de pesticidas y fertilizantes, y la eliminación de la vegetación ribereña ha causado efectos perjudiciales en la

comunidad fúngica dulceacuícola (Bärlocher 1992). Sin embargo, los efectos reales de la intervención humana en las comunidades de hongos acuáticos siguen siendo desconocidas (Grossart et al. 2019).

1.3.2. Grupos ecológicos

La manera en que un organismo entra en contacto con el agua desde un hábitat extra acuático puede tener un efecto significativo sobre el papel del mismo en este nuevo medio. Algunos hongos pueden ser transferidos como propágulos inactivos y entrar en contacto con el agua durante su dispersión, teniendo una menor probabilidad de asumir un papel ecológico activo que aquellos organismos que se transfieren al agua adheridos o incluidos dentro de un sustrato donde ya están metabólicamente activos (Park 1972). Es por esto que, considerando su grado de adaptación, actividad y dependencia de ambientes acuáticos, estos hongos se han clasificado en cuatro grupos ecológicos funcionales (Fig. 4).

- *Residentes o nativos:* totalmente adaptados a la vida en el agua, mostrando adaptaciones morfológicas y fisiológicas. Son capaces de mantener su biomasa a un nivel más o menos constante, en la medida que dispongan de nutrientes y sustrato. La mayoría de ellos son capaces de esporular en el agua (Park 1972). P. ej: *Aquaphila* y *Elegantimyces* (Goh et al. 1998a, 1998b).
- *Inmigrantes periódicos* o *anfibios:* son aquellos que habitan entornos acuáticos durante parte de su ciclo de vida (Park 1972). Diversos miembros de este grupo son conocidos como endófitos u hongos micorrícicos en ambientes terrestres. Tienen la capacidad de producir esporas tanto en ambientes terrestres como acuáticos (Vijaykrishna et al. 2006), un ejemplo de los mismos serían las especies del género *Spirosphaera* (Hennebert 1968).
- *Inmigrantes versátiles* o *acuáticos facultativos:* están poco adaptados al medio acuático y no esporulan bajo el agua. Sin embargo, son capaces de producir hifas para continuar sus funciones como saprobios, aunque de una manera menos eficiente que en un ambiente terrestre (Park 1972, Dix & Webster 1995, Goh & Hyde 1996). P. ej: *Lophiotrema rubi* (Fallah & Shearer 2001).

- *Transeúntes:* no están adaptados al medio acuático. Pueden llegar al medio e inmediatamente comenzar a reducir su actividad biológica como resultado de los cambios ambientales, tales como la disponibilidad de oxígeno y/o la pérdida de nutrientes por competencia con organismos mejor adaptados a la vida acuática. Dichos organismos pueden llegar activos al sustrato, pero ser incapaces de esporular y también de colonizar un nuevo sustrato (Park 1972). P. ej. *Volutella* spp. y *Stachybotrys* spp. (Velez et al. 2016).

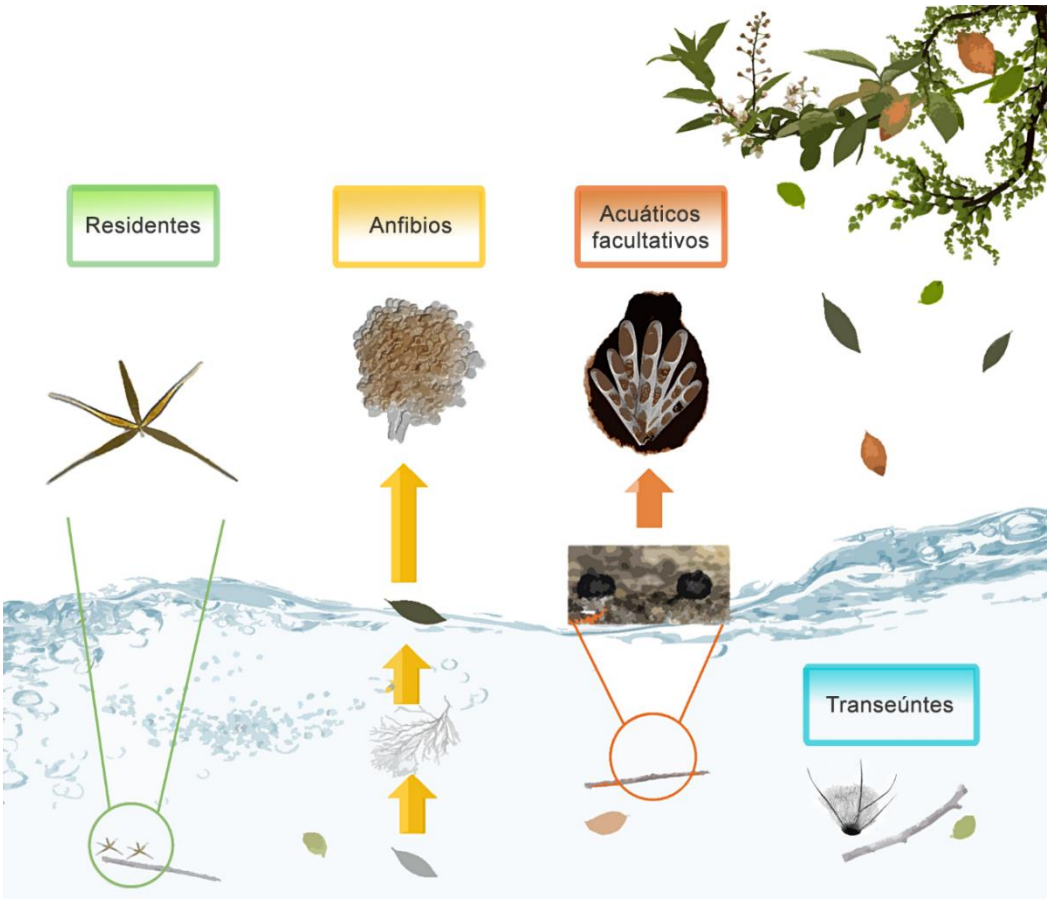

Figura 4. Grupos ecológicos de hongos acuáticos descritos por Park (1972).

1.3.3. Clasificación de los hongos dulceacuícolas y sus adaptaciones morfológicas.

Dentro de este grupo de hongos se pueden encontrar cuatro de los nueve filos del reino Fungi: *Chytridiomycota, Blastocladiomycota, Basidiomycota* y *Ascomycota*. El filo *Ascomycota* es el que cuenta con más representantes dentro del grupo. Se estima que alrededor de 740 especies de ascomicetos se han aislado en hábitats de agua dulce en estado sexual (http://fungi.life.illinois.edu/) y 900 especies en el estado asexual (El-Elimat et al. 2021). A pesar de lo limitado del número de especies de hongos dulceacuícolas descritos, en las últimas 3 décadas su número se viene incrementando paulatinamente hasta alcanzar a ser el 1 % del total de los miembros descritos para el

reino Fungi. En 1993 Shearer definió los ascomicetos de agua dulce como los hongos que se encuentran en sustratos sumergidos o parcialmente sumergidos en hábitats acuáticos.

1.3.3.1. Ascomicetos dulceacuícolas con reproducción sexual.

Este grupo se ha encontrado principalmente creciendo a expensas de restos de madera sumergida (Whong et al. 1998). Las estructuras reproductivas sexuales de este grupo de hongos han sufrido una serie de adaptaciones morfológicas para subsistir en los ambientes acuáticos. A menudo sus ascomas están total o parcialmente inmersos en el sustrato, lo que les permite permanecer adheridos incluso en aguas que se mueven rápidamente. Los ascos suelen tener aparatos apicales, y son fisitunicados o bitunicados, delimitados por una pared con una capa interna (endotúnica) unida estrechamente a la capa externa (exotúnica), son delicuescentes, característica que permite que las ascosporas sean liberadas al agua sin ejercer fuerza (Shearer 1993). Muchos hongos producen ascosporas con apéndices y/o capas de mucilago (Fig. 5), que facilitan su fijación a los sustratos y su posterior colonización a pesar de que la corriente de agua fluya rápidamente (Shearer 1993, Wong et al. 1998, Raja et al. 2018).

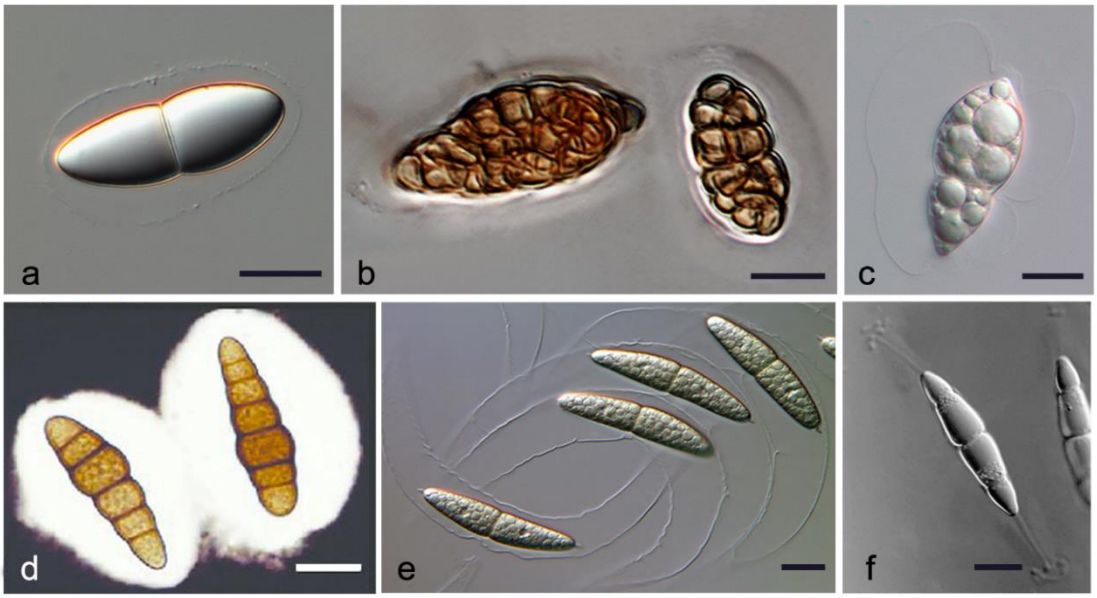

Figura 5. Ascosporas de Ascomicetos de agua dulce que muestran diversas adaptaciones a su forma de vida acuática (capas y apéndices mucilaginósos). **a**) *Lindgomyces lemonweirensis,* ascospora con un saco de mucilago oval. **b**) *Murispora fissilispora,* ascosporas maduras cubiertas de mucilago. **c**) *Alascospora evergladensis,* rodeada por una capa de mucílago con forma de alas. **d**) *Lolia aquatica,* ascosporas en tinta china mostrando la capa de mucilago. **e**) *Aliquandostipite crystallinis,* con ascosporas rodeadas por la capa de mucilago fusiforme*,* **f**) *Phaeosphaeria barriae,* cuyas ascosporas poséen apéndices bipolares. Escala= 10 µm. (Adaptado y modificado: Shearer Raja et al. 2010, Abdel-Aziz 2016, Magaña-Dueñas et al. 2020).

1.3.3.2. Ascomicetos dulceacuícolas con reproducción asexual.

Estos hongos han sido clasificados en tres tipos ecológicos, basados en sus adaptaciones a los hábitats de agua dulce. Dos de estos grupos tienen conidios adaptados para realizar su ciclo biológico o parte de el en los ambientes acuáticos: los hongos “ingoldianos" y los aeroacuáticos. Sin embargo, los propágulos de dispersión de las especies que componen el grupo de hongos mitospóricos diversos no muestran adaptaciones obvias para su persistencia en medios dulceacuícolas y se las denomina hongos acuáticos sumergidos (Raja et al. 2018, Eli-Elimat et al. 2021).

1.3.3.2.1. Hongos ingoldianos.

La primera especie de hongos acuáticos fue descrita por Saccardo en 1880. Sin embargo, no fue hasta 1942 que Ingold reconoció numerosas especies de “hifomicetes” acuáticos en hojas en descomposición, siendo posteriormente denominados como hongos ingoldianos en su honor (Webster & Descals 1981). Los hongos ingoldianos forman un grupo polifilético, con representantes de los filos Ascomycota y Basidiomycota (http://fungi.life.illinois.edu/about/mitosporic_fungi). Estos hongos realizan su ciclo de vida (incluidos el crecimiento vegetativo, la producción de esporas, la liberación de esporas y su diseminación) en sustratos sumergidos en aguas lóticas. Se caracterizan por producir estauroconidios o escolecoconidios (esporas asexuales con forma de estrella o alargados, respectivamente), mayoritariamente hialinos (Fig. 6). La forma de dichos conidios es una adaptación para poder sobrevivir y dispersarse en los hábitats acuáticos, principalmente en aquellos de aguas rápidas (Webster 1959). Las esporas ramificadas actúan como un ancla y permite su fijación en los sustratos y en la espuma que se produce en la superficie del agua (Ingold 1966). También se produce material mucilaginoso en los extremos de las esporas cruciformes, y al contacto con el sustrato el conidio se adhiere fuertemente (Tsui et al. 2016). Las morfologías de los hongos ingoldianos también son relativamente frecuentes en los hongos anamórficos terrestres que habitan hojarasca humedecida procedente de las plantas forestales (Shearer et al. 2007). La esporulación *in vitro* de dichos hongos puede ser inducida cuando un bloque

de agar inoculado con un cultivo puro se sumerge en agua y mejora considerablemente cuando es agitado con aire comprimido (Kegel 1906, Webster & Towfik 1972).

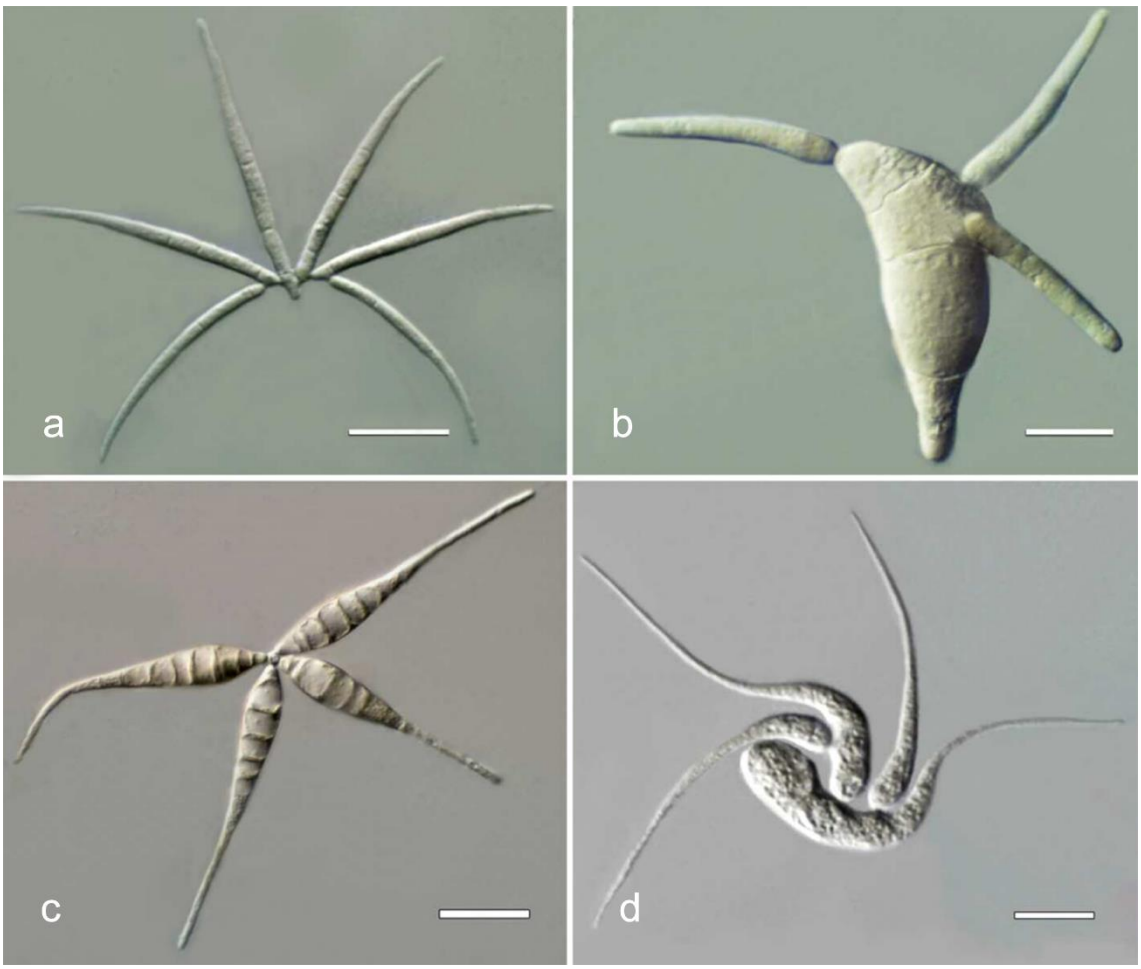

Figura 6. Conidios de hongos Ingoldianos. **a**) *Magdalanaea monograma*, **b**) *Culicidospora gravida*, **c**) *Flabellospora acuminata*, **d**) *Gyoerffyella rotula.* Escala= 10 µm. (Adaptado y modificado: Yeates 2019).

1.3.3.2.2. Hongos aeroacuáticos.

Dicha denominación se debe a los trabajos de van Beverwijk en 1951. Habitan en la hojarasca de plantas sumergidas en una variedad de cuerpos de agua dulce estancados y/o poco profundos. Como adaptación a este hábitat, muchos pueden soportar largos períodos de agotamiento de oxígeno disuelto, y son bastante tolerantes a los sulfuros (Field & Webster 1983, 1985). Son capaces de crecer en fase vegetativa en sustrato completamente sumergido, es decir en condiciones semi-anaeróbicas (Fisher & Webster 1978, Goh & Hyde 1996). Sin embargo, los hongos aeroacuáticos no pueden completar su ciclo de vida en sustratos sumergidos, ya que sus propágulos de dispersión solo se producen cuando el sustrato se encuentra expuesto en la interface aire-agua (Beverwijk 1951, Fisher & Webster 1977). Por lo tanto, dependen de la sequía

periódica de los hábitats. La naturaleza de sus propágulos de dispersión son la característica más destacable del grupo (Fig. 7). Consisten en estructuras complejas, generalmente multicelulares y morfológicamente diversas, pero todas tienen una característica en común: atrapan el aire en su interior, motivo por el cuál flotan en el agua (Webster & Descals 1981, Goh & Hyde 1996). El propágulo puede ser una espora espiralada (*Helicoon, Helicodendron*) (Abdullah et al. 1985, 1986), en forma de candelabro (*Hyaloscypha*) (Yamaguchi et al. 2020), una mórula compuesta por células globosas (*Pseudoaegerita*) (Abdullah & Webster 1983), una mórula de células encerradas por brazos espinosos curvados (*Peyronelina*) (Fisher et al. 1976), un sistema de ramas curvadas (*Fouskomenomyces cupreorufescens, Spirosphaera*) (Hennebert 1968, Voglmayr 2004), o un conjunto de células ramificadas dicotómicamente o tricotómicamente (*Brocchiosphaera*) (Yamaguchi et al. 2020). Frecuentemente, la flotabilidad se ve reforzada por la presencia de verrugas e incrustaciones hidrófobas en el propágulo, que a menudo solo son visibles con microscopía electrónica de barrido. La dispersión de los propágulos tiene lugar en la superficie del agua (Shearer et al. 2007).

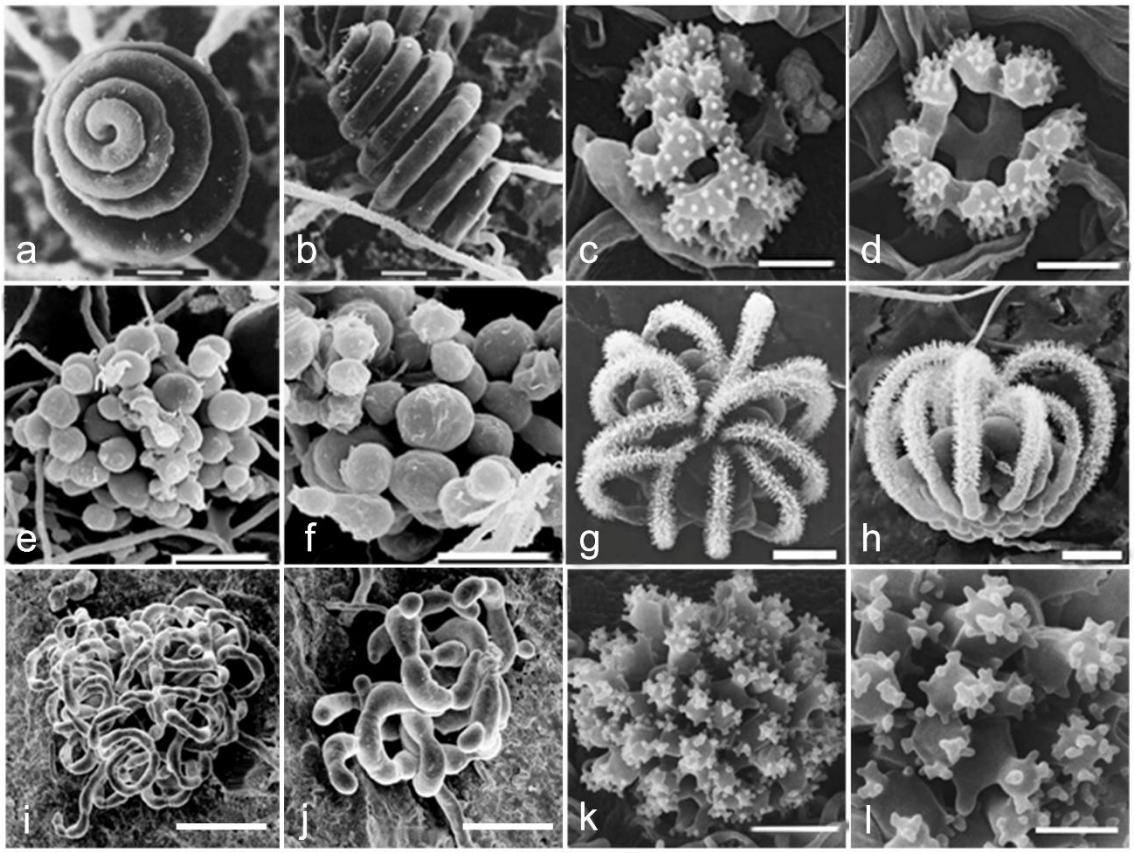

Figura 7. Diversidad de propágulos de dispersión de hongos aeroacuáticos. (**a,b**) *Helicoon maioricensis.* (**c,d**) *Hyaloscypha spinulosa.* (**e,f**) *Pseudaegerita confiera.* (**g,h**) *Peyronelina glomerulata.* (**i,j**) *Fouskomenomyces cupreorufescens* (**k,l**) *Brocchiosphaera bulbiformis.* Escala= 10 µm. (Adaptado y modificado: Abdullah et al. 1998, 2005, Voglmayr 2004, Yamaguchi et al. 2009, 2020).

1.3.3.2.3. Hongos mitospóricos diversos.

Este grupo de hongos fue abordado por primera vez por Ingold en 1975, siendo también denominados cómo acuáticos facultativos o lignícolas terrestres-acuáticos (Goh 1997). Estos incluyen hongos anamórficos dematiáceos y hialinos, y celomicetos (Fig. 8). Dichos hongos no poseen estructuras especializadas para sobrevivir en ambientes acuáticos como las que se observan en los ingoldianos o aeroacuáticos. Más bien es un conjunto heterogéneo de hongos anamórficos morfológica y filogenéticamente diversos (Goh & Hyde 1996).

Estos hongos se encuentran comúnmente en sustratos herbáceos y madera en hábitats acuáticos y semiacuáticos de todo el mundo (http://fungi.life.illinois.edu/about/mitosporic_fungi). Según Park (1972) pueden clasificarse en residentes e inmigrantes. Se han reportado especies aisladas solo de hábitats de agua dulce, mientras que otras provienen tanto de hábitats terrestres como de agua dulce (Raja et al. 2018).

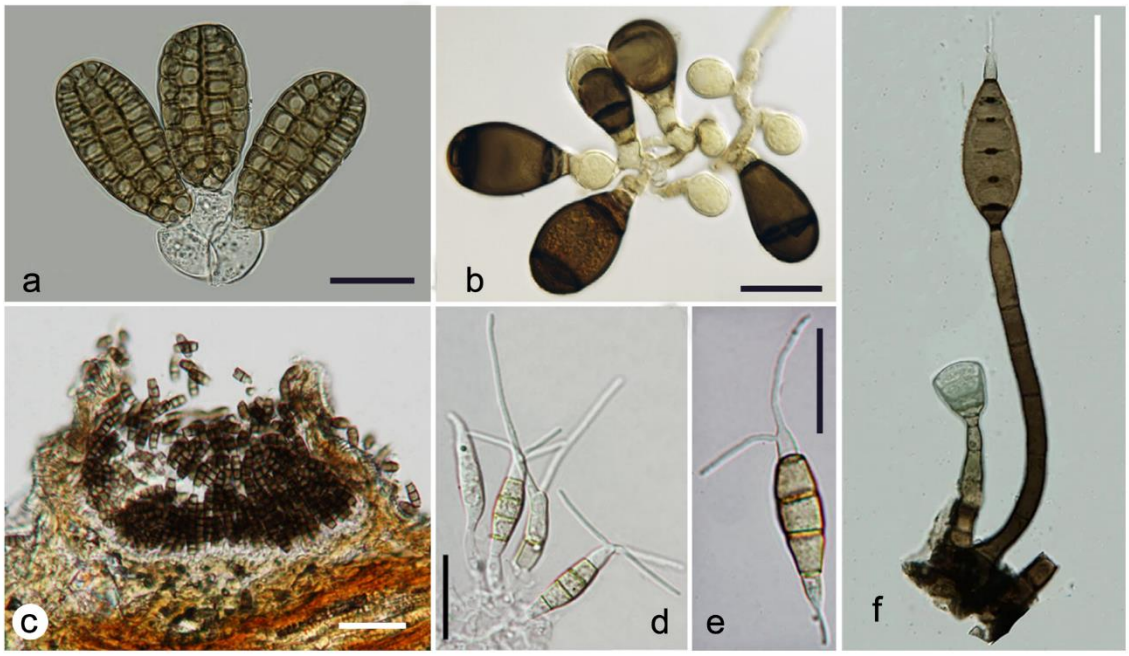

Figura 8. Hongos mitospóricos diversos. También denominados acuáticos facultativos o lignícolas terrestres. Estos hongos incluyen hongos dematiáceos y hialinos (**a, b, f**) y celomicetos (**c-e**). **a**) *Aquadictyospora lignicola.* **b**) *Bactrodesmium abruptum.* **c-e**) *Pestalotiopsis.* **f**) *Sporidesmium brachypus,* Barras de escala= 10 µm (Adaptado y modificado Maharachchikumbura et al. 2012, Luo et al. 2019, Réblová et al. 2020)

La mayoría de estos producen conidióforos conspicuos y colonizan sustratos leñosos sumergidos. La morfología de los conidióforos varía ampliamente. Muchos de ellos son mononematosos, algunos esporodoquiales y otros sinematosos. Los *loci*

conidiógenos pueden ser denticulados, con escaras, tréticos o fialídicos. Aunque algunas especies pueden esporular sumergidas en agua, la gran mayoría esporula cuando los sustratos están expuestos al aire (Hu et al. 2014). Los conidios son generalmente incoloros, y pueden variar en forma y color en diferentes etapas de crecimiento. La forma conidial varía ampliamente, de elipsoidales, globosos, subglobosos a cilíndricos o clavados (Goh & Hyde 1996, Hu et al. 2014).

Los celomicetos, también son conocidos como hongos picnidiales o acervulares (Seifert et al. 2011), pueden habitar una gran variedad de nichos ecológicos, pero en ambientes acuáticos se han reportado principalmente en plantas de aguas dulces y saladas, e incluso en aguas residuales. Los celomicetos dulceacuícolas suelen formar conidiomas (cuerpos fructíferos asexuales) de globosos a piriformes y abiertos al exterior a través de uno o varios ostíolos (picnidios), de color marrón a negruzco, los que suelen producir un gran número de conidios a partir de células conidiógenas dentro de los cuerpos fructíferos. Debido a gran similitud entre las estructuras reproductivas de taxones evolutivamente distantes, resultan difíciles de identificar morfológicamente, y por lo tanto han sido poco documentados (Goh & Hyde 1996). Hasta el año 2014 solo se habían reportado 16 especies de celomicetos de agua dulce (Hu et al. 2014).

1.3.4. Evolución de los hongos ascomicetos de agua dulce

Es probable que la mayoría de los hongos dulceacuícolas hayan evolucionado a partir de ancestros terrestres a través de varias vías evolutivas, una de las cuales podría haber sido como patógenos, endófitos y saprófitos en humedales y de plantas acuáticas, dado que estas invadieron hábitats de agua dulce, seguramente trayendo consigo sus microorganismos asociados. Por otra parte, los hongos capaces de sobrevivir y adaptarse a hábitats acuáticos pudieron haber sido ancestros de las especies actuales que se encuentran en macrófitas de agua dulce. Finalmente, pudieron llegar por medio de la vegetación de ribera, en los restos del material vegetal, ya que estos llevan consigo un considerable complejo de hongos capaces de adaptarse al agua dulce (Shearer 1993). Diversos estudios, utilizando las secuencias de nucleótidos del gen 18S del ARN ribosomal (Vijaykrishna et al. 2006, Shearer et al. 2009, Hu et al. 2014) demostraron que los hongos de agua dulce evolucionaron de hongos terrestres pertenecientes a varias clases del filo *Ascomycota* diferentes: *Arthoniomycetes, Chaetothyriomycetes*, *Dothideomycetes, Eurotiomycetes, Laboulbeniomycetes, Lecanoromycetes, Leotiomycetes, Lichinomycetes, Pezizomycetes* y *Sordariomycetes*.

1.3.5. Filogenia de los hongos ascomicetos de agua dulce.

Los ascomicetos de agua dulce se encuentran principalmente distribuidos entre las clases de los *Dothideomycetes, Sordariomycetes* y en menor medida *Leotiomycetes* y *Eurotiomycetes* (Shearer et al. 2009, Hu et al. 2014, Luo et al. 2019, Dong et al. 2020).

1.3.5.1. *Dothideomycetes* de agua dulce

Algunos miembros de los *Dothideomycetes* fueron de los primeros hongos en ser reportados en ambientes de agua dulce (Petrac 1925, Webster 1951, Ingold 1955), y desde entonces su cifra ha ido en continuo aumento. Aproximadamente el 30% de los ascomicetos de agua dulce reportados hasta la fecha (http://fungi.life.illinois.edu/about/mitosporic_fungi) pertenecen a dicha clase. Mayoritariamente han sido aislados de madera sumergida, y se distinguen de otros Ascomycota por producir ascostromas (o pseudotecios; ascoma similar a un estroma dentro del cual desarrolla el micelio reproductivo hasta formar ascos dentro de cavidades lisígenas [lóculos]) y ascos fisutinicados. Estos hongos también pueden multiplicarse asexualmente mediante la producción de conidios directamente sobre el micelio, producidos a partir de conidióforos o dentro de conidiomas (Shearer et al. 2014) (Fig. 9).

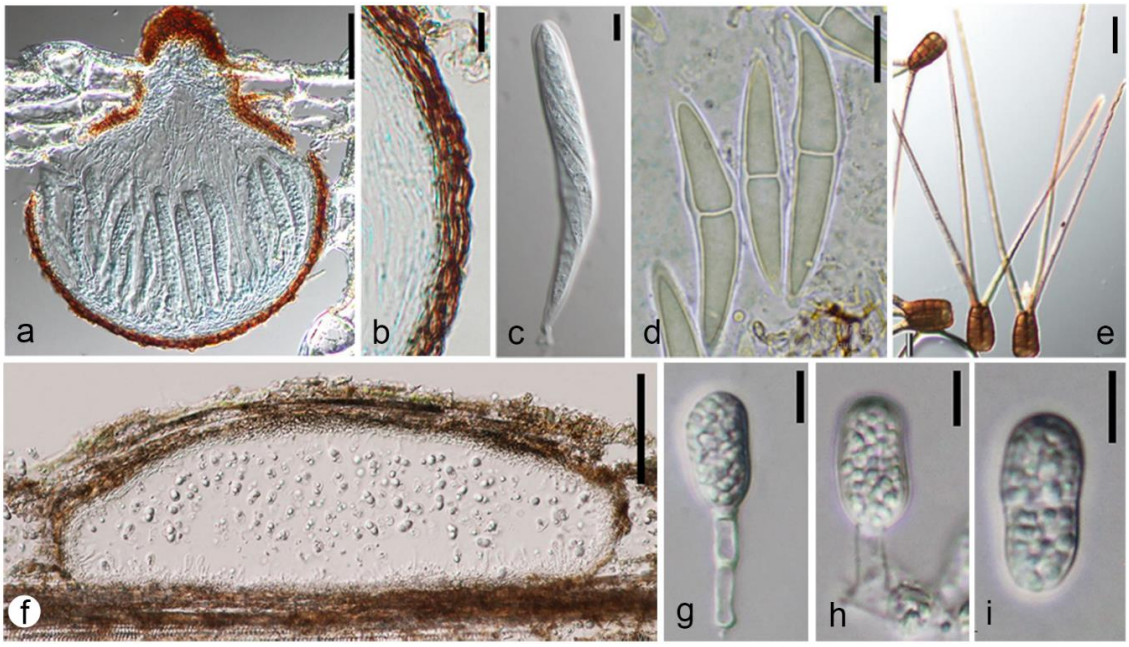

Figura 9. Características morfológicas de los *Dothideomycetes* de agua dulce. *Tetraploa* sp. **a**) Ascostroma (ascoma). **b**) Sección de ascoma. **c**) Asca. **d**) Ascosporas. **e**) Conidios; *Clohesyomyces aquaticus.* **f**) Picnidio. **g,h**) Célula conidiógena con conidio. **i**) Conidio. Escala a, b, f = 50 µm; c-e, g-i= 10 µm. (Adaptado y modificado Tanaka et al. 2009, Dong et al. 2020).

El estudio filogenético de los *Dothideomycetes* (aislados de agua dulce) realizado por Dong et al. (2020) divide la clase en seis ordenes, 43 familias y 145 géneros, de los cuales 46 géneros son exclusivos de agua dulce. Estos géneros se encuentran distribuidos principalmente entre los órdenes *Pleosporales* (225 especies, 29 géneros)*, Tubeufiales* (98 especies, 23 géneros), *Jahnulales* (32 especies, ocho géneros)*, Kirschsteiniotheliales* (seis especies, un género)*, Minutisphaerales* (18 especies, dos géneros) y *Natipusillales* (cuatro especies, un género).

1.3.5.2. *Sordariomycetes* de agua dulce

Los *Sordariomycetes* de agua dulce representan la segunda clase más grande de Ascomycota (Zhang et al. 2006, Luo et al. 2019). Sus especies se caracterizan principalmente por poseer ascomas periteciales y ascos unitarios inoperculados o no fisitunicados (Fig. 10).

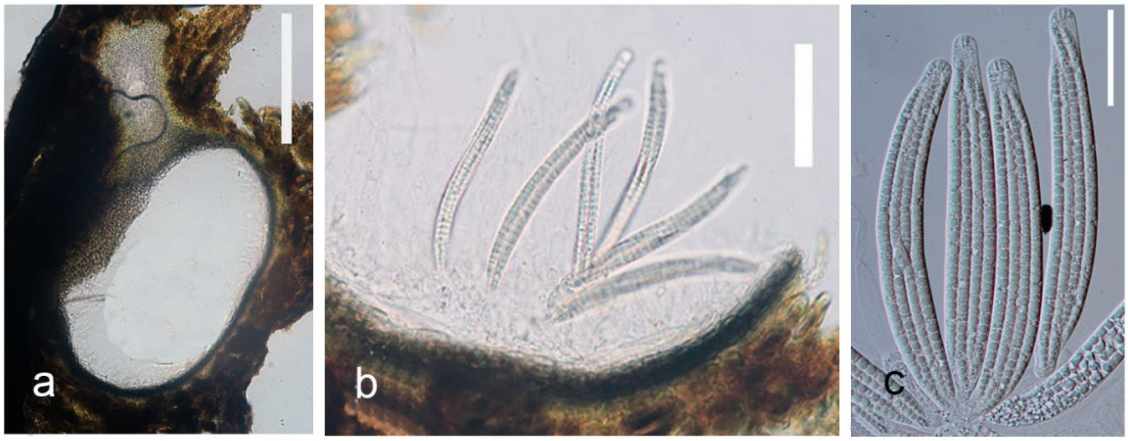

Figura 10. Características morfológicas de los *Sordariomycetes* de agua dulce*. Ceratosphaeria lignicola.* **a**) Ascoma). **b**) Ascoma y ascas **c**) Ascas. Barras de escala= a 100 µm; b 50 µm; c 30 µm. (Adaptado y modificado. Dong et al. 2020).

En 2019 Luo y colaboradores, realizaron un estudio filogenético y morfológico de los *Sordariomycetes* de agua dulce. Dichos hongos fueron ubicados en los órdenes: *Annulatascales* (32 especies, diez géneros), *Atractosporales* (seis especies, dos géneros), *Chaetosphaeriales* (61 especies, 16 géneros), *Coniochaetales* (seis especies, un género), *Conioscyphales* (seis especies, un género), *Cordanales* (cuatro especies, un género), *Coronophorales* (una especie, un género), *Diaporthales* (siete especies, cinco géneros), *Distoseptisporales* (32 especies, dos géneros), *Fuscosporellales* (once especies, seis géneros), Glomerellales (siete especies, dos géneros), *Hypocreales* (35 especies, 19 géneros), *Jobellisiales* (tres especies, un género), *Magnaporthales* (18

especies, cuatro géneros), *Microascales* (32 especies, diez géneros), *Myrmecridiales* (dos especies, un género), *Ophiostomatales* (una especie, un género), *Phomatosporales* (siete especies, un género), *Phyllachorales* (tres especies, dos géneros), *Pisorisporiales* (dos especies, dos géneros), *Pleurotheciales* (24 especies, cinco géneros), *Savoryellales* (26 especies, cuatro géneros), *Sordariales* (26 especies, trece géneros), *Sporidesmiales* (doce especies, un género), *Tirisporellales* (una especie, un género), *Togniniales* (dos especies, un género), *Torpedosporales* (tres especies, un género), *Trichosphaeriales* (tres especies, dos géneros), *Xenospadicoidales* (18 especies, cuatro géneros) y *Xylariales* (once especies, ocho géneros)

2. JUSTIFICACIÓN Y OBJETIVOS

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Los hongos de agua dulce son un grupo ecológico, morfológica y filogenéticamente diverso, que está ampliamente distribuido en una gran variedad de ambientes acuáticos, tanto lóticos como lenticos. Estos hongos interaccionan con otros micro- y macroorganismos acuáticos, actuando como saprobios, parásitos, patógenos o simbiontes. Además, los hongos dulceacuícolas juegan un rol destacable en la descomposición de la materia orgánica muerta, debido a la producción de exoenzimas capaces de hidrolizar la celulosa, la lignina y la hemicelulosa de la pared de las células vegetales, pero también la quitina y la queratina, macromoléculas de origen animal. A pesar de que los ambientes acuáticos representan una gran extensión en nuestro planeta, éstos no han sido explorados con la misma intensidad que los ecosistemas terrestres (en especial de los bosques lluviosos) en busca de biodiversidad fúngica. Jones (2014) estimó que han sido identificadas alrededor de unas 4.000 especies de hongos de agua dulce, lo que representa menos del 3% de los (aproximadamente) 135.000 hongos conocidos hasta el presente (Hibbett et al. 2016). Debido a que los trabajos más conservadores sobre la estimación del número de especies potenciales del reino Fungi ronda la cifra de 1,5 millones (Hawksworth 2001), se puede asumir que el número potencial de especies fúngicas en los ambientes acuáticos debería rondar las 45.000.

La diversidad climática, fitogeográfica e hidrográfica de España hacen que este país tenga un gran potencial de nichos ecológicos diferentes, algunos de los cuales son capaces de albergar una gran diversidad de hongos, incluidos los acuáticos. Muchos de estos ambientes, sin embargo, corren el riesgo de desaparecer o sufrir una importante pérdida de su biodiversidad a corto plazo debido a que están sometidos a una fuerte presión antrópica. Esta acción deletérea se ejerce a través de acciones tales como las descargas del drenaje de efluentes domésticos e industriales, la deforestación, la sobreexplotación de los acuíferos para su uso agrícola y ganadero, y el empleo indiscriminado de fertilizantes y fitosanitarios, entre otros.

En la actualidad existen un número muy limitado de trabajos sobre la diversidad de hongos dulceacuícolas de España capaces de reproducirse sexualmente mediante la producción de ascosporas y/o de conidios dentro de cuerpos fructíferos (ascomas y conidiomas, respectivamente).

Por lo antes expuesto, el objetivo general de la presente tesis fue:

Aislar e identificar los hongos, principalmente ascomicetos con reproducción sexual y/o asexual (formación de conidios dentro de conidiomas), a partir de material vegetal sumergido en aguas dulces en España, realizando una

pormenorizada caracterización fenotípica y evaluando su posición taxonómica y filogenia mediante el empleo de herramientas moleculares.

Para desarrollar dicho objetivo, se plantearon los siguientes objetivos específicos:

1. Realizar diferentes muestreos de material vegetal en descomposición sumergido en diversos ambientes dulceacuícolas en diferentes localizaciones de España.

2. A partir de los ascomas y/o conidiomas fértiles (conteniendo ascosporas y conidios, respectivamente), producidos sobre el material vegetal colocado dentro de cámaras húmedas, aislar en cultivo puro los hongos de interés, para posteriormente proceder a su identificación fenotípica presuntiva basada en la descripción exhaustiva y documentación gráfica de las estructuras reproductivas, y su comparación con las formadas por otros taxones descritos en la bibliografía de referencia.

3. Identificar los aislados mediante la amplificación y la secuenciación nucleotídica de los marcadores moleculares filogenéticamente más informativos para cada grupo.

4. Construir árboles filogenéticos para determinar la posición taxonómica de los hongos de interés, y elucidar su relación evolutiva con taxones previamente conocidos.

5. Publicar los hallazgos taxonómicos en revistas científicas especializadas, preferentemente aquellas indexadas en el JCR y con el mayor factor de impacto posible.

6. Contribuir al conocimiento de la biodiversidad de este grupo de hongos en nuestro país.

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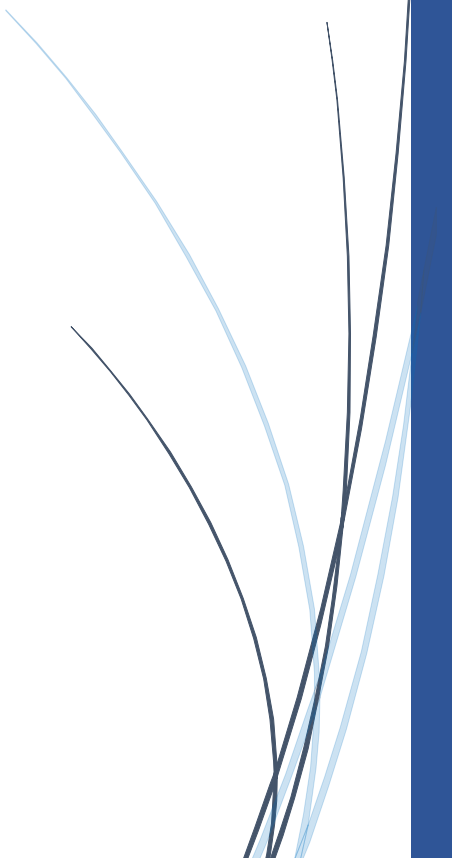
3. MATERIALES Y MÉTODOS



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3.1. Tipología, origen, recolección y transporte de las muestras

En la presente tesis doctoral se han examinado e identificado un total de 111 cepas (Anexo Tabla A1). Las cuales fueron aisladas a partir de un total de 167 muestras procedentes de diferentes localizaciones en España en el periodo comprendido entre los años 2018 y 2021 (Tabla 1). Dichas muestras consistían en restos vegetales en descomposición sumergidos en agua dulces, mayoritariamente hojas y ramas.

Tabla 1. Sitios de colecta y número de muestras obtenidas.

Comunidad autónoma	Sitio de colecta Localización, Provincia	Fecha	Número de muestras
Andalucía	Parque Natural Sierra Norte, Sevilla	Mayo 2019	50
Cataluña	Capafonts, Tarragona	Marzo 2019	22
	Les Guilleries, Barcelona	Noviembre 2017	3
	Pontons, Barcelona	Junio 2018	15
	Río Segre, Lérida	Diciembre 2019	2
	Roda de Ter, Barcelona	Septiembre 2020	3
	Serra del Montsant, Tarragona	Febrero 2018	30
Castilla y León	Riaza, Segovia	Mayo 2018	17
Islas Baleares	Cúber, Escorca, Mallorca	Noviembre 2018	10
Madrid	Miraflores de la Sierra, Madrid	Mayo 2019	10
País Vasco	Parque de Doña Casilda Iturriza, Bilbao, Vizcaya	Agosto 2020	2
	Parque de los pueblos de Europa, Gernika, Vizcaya	Agosto 2020	2
Comunidad Valenciana	Burriana, Castellón	Marzo 2021	3

Las muestras se recolectaban manualmente, y eran colocadas dentro de bolsas de plástico autosellables, las cuales eran rotuladas con la fecha de recolección y el nombre del sitio de la colecta con un marcador indeleble. Adicionalmente, gracias a la ayuda del *GPS* (*Global Positioning System*), se anotaban las coordenadas en las mismas. Las bolsas eran posteriormente introducidas en una bolsa isotérmica y transportadas al laboratorio para su posterior procesamiento en el menor tiempo posible desde su recolección. Caso contrario, las muestras se conservaban dentro de neveras domésticas (4-7 ºC) antes de su transporte.

3.2. Procesamiento de las muestras y obtención de las cepas fúngicas

Las muestras se enjuagaban enérgicamente dos veces con 500 mL de agua de grifo estéril dentro de bolsas estériles de 1000 mL de capacidad. Posteriormente, se cortaban (en caso que sus dimensiones así lo requerían) con hoja de bisturí estéril en fragmentos de un máximo de 3-4 cm de longitud, y se colocaban dentro de placas de Petri de 15 cm de diámetro, cuyo interior estaba cubierto con dos hojas de papel de filtro, las que eran humedecidas regularmente con agua con dieldrin (1 mL de una solución de 200 mg de Dieldrin (Sigma Aldrich) en 20 mL de dimetilcetona / 0,5 L de agua), para evitar su desecación y la proliferación de ácaros. Estas cámaras húmedas eran incubadas a temperatura ambiente, y examinadas periódicamente bajo el microscopio estereoscópico, durante un período máximo de dos meses. Varios propágulos y/o cuerpos fructíferos eran transferidos a placas Petri de 5,5 cm de diámetro que contenían agar avena (OA; 30 g de copos de avena hervidos en 500 mL de agua durante 15 a 20 minutos, y filtrados tras la ebullición, 6,5 g de agar-agar; completando con agua del grifo hasta volumen final de 1000 mL [Samson et al. 2010]) utilizando jeringuillas tipo tuberculina/insulina con sus respectivas agujas desechables, para luego ser incubadas a temperatura ambiente durante un período máximo de seis semanas. Una vez que se obtenían cultivos axénicos, las cepas se caracterizaban fenotípicamente, y una vez realizada la identificación presuntiva empleando la bibliografía de referencia, estas eran sometidas a estudios filogenéticos basados en la amplificación y secuenciación de uno o más marcadores moleculares filogenéticamente informativos.

3.3. Estudio fenotípico

3.3.1. Caracterización macroscópica (cultural)

La caracterización fenotípica de las cepas se llevaba a cabo en los medios de cultivo, temperaturas y tiempos de incubación recomendados en la bibliografía pertinente para cada uno de los géneros diferentes. Los medios de cultivo utilizados se detallan a continuación: agar con extracto de malta (MEA; 40 g de extracto de malta, 15 g de agar-agar, 1000 mL de agua destilada), agar con harina de avena, agar con extracto de patata y glucosa (PDA; Pronadisa, Madrid, España).

Después del periodo de incubación, se medía el diámetro de la colonia y se describían características tales como la textura, el tipo de borde, la presencia de pigmentos difusibles, de exudados, y el color anverso y reverso, para cuyo fin se empleaba el catálogo de Kornerup y Wanscher (1978). La capacidad de los aislados de crecer a temperaturas cardinales se determinaba en el medio de cultivo PDA después de siete días de crecimiento en oscuridad, con un rango de temperaturas de entre 5 y 35 °C, a intervalos de 5 °C, pero también a 37 °C. Las siembras se realizaban por triplicado y se informaba el rango de diámetros obtenidos para cada medio y condición de cultivo. Transcurrido el tiempo establecido, se medía el diámetro de las colonias, y con los datos obtenidos se establecían las temperaturas: mínima, optima y máxima de crecimiento.

3.3.2. Caracterización microscópica

Las mediciones y descripciones de las estructuras microscópicas (hifas, clamidosporas, conidiomas, conidióforos, células conidiogenas, conidios, ascomas, ascosporas, ascos, etc.) se realizaban a partir de montajes (entre porta y cubreobjetos) en medio de Shear (3 g de acetato de potasio, 60 mL de glicerol, 90 mL de etanol al 95 % y 150 mL de agua destilada (Chupp 1940). Para documentar los conidiomas y ascomas se realizaban cortes histológicos a mano alzada, con la ayuda de una aguja estéril de 0,3 x 13 mm y de un bisturí *Laseredge* de 30º de corte y 15 mm de ancho. El examen y toma de medidas se realizaba utilizando un microscopio de campo claro Olympus BH-2 (Olympus Corporation, Tokio, Japón). Las imágenes microscópicas se tomaban usando un microscopio Zeiss Axio-Imager M1 (Oberkochen, Alemania) con una cámara digital DeltaPix Infinity X usando contraste interferencial de Nomarski. La edición y procesamiento de las imágenes se realizó con el programa Adobe Photoshop CS6 v.13.0

3.4. Estudio molecular

3.4.1. Extracción de ADN, amplificación y secuenciación

Las cepas eran cultivadas en PDA durante siete días a 25 ºC en la obscuridad. En un microtubo con perlas de cristal estériles, se introducía material fúngico raspado de la superficie del cultivo con 500 µL de buffer de lisis (100 mM Tris pH 8,0, 50 mM EDTA, 1% SDS). Posteriormente se trituró el micelio utilizando el disruptor de células de alta velocidad FastPrep FP120 (Thermo Savat, Holbrook, Nueva York) durante 30 s. A continuación, se centrifugaron las muestras durante 10 min a 13.000 rpm. Una vez centrifugado, el sobrenadante se transfirió a un tubo nuevo, al cual se le agregaron 275 µL de 7 M acetato de amonio (27 g de acetato de amonio y agregar agua, hasta un volumen final de 50 mL) pH 7,0, incubando la mezcla a 65ºC por 5 min y luego 5 min en hielo. Después de agregaron 500 µL de cloroformo, la mezcla se centrifugó por 5 min a 13.000 rpm y la fase superior se llevó a un tubo nuevo. Posteriormente se agregó 1 mL de isopropanol, se refrigeró a 4ºC durante 15 min y se centrifugó 10 min a 13.000 rpm. Se descartó el sobrenadante y a continuación se realizaron dos lavados con 400 µL de EtOH 70%. El sobrenadante se dejó secar y se resuspendió en 50 µL de agua miliQ y 2 µL de RNasa, se incubó durante 30 min a 37ºC. El ADN se cuantificaba utilizando el Nanodrop 2000 (Thermo Scientific, Madrid, España) y se almacenaba a -4 ºC hasta la realización de la PCR.

El ADN total se utilizaba para realizar la amplificación de los marcadores genéticos filogenéticamente informativos seleccionados para cada grupo de hongos. Los *loci*, los pares de cebadores y las condiciones utilizadas en los diferentes estudios incluidos en la presente tesis, están resumidos en la Tabla 2. Para corroborar la eficacia de la amplificación, los amplicones se sometían a electroforesis en gel de agarosa al 1 %. Los productos de PCR se almacenaban a -20 ºC hasta su secuenciación, que se llevaba a cabo en Macrogen Europe (Macrogen Inc, Madrid, España) con los mismos pares de cebadores utilizados para la amplificación de cada gen.

Tabla 2. Marcadores genéticos, cebadores y condiciones de hibridación

Gen	Cebador	Dirección	Secuencia (5´-3´)	Tª annealing (ºC)	Referencia
ITS	ITS-5	*Forward*	GGAAGTAAAAGTC GTAACAAGG	53	White et al. 1990
	ITS-4	*Reverse*	TCCTCCGCTTATTG ATATGC		
LSU	LROR	*Forward*	GTACCCGCTGAAC TTAAGC	53	Rehner & Samuels 1994
	LR5	*Reverse*	TCCTGAGGGAAAC TTCG		Vilgalys & Hester 1990
tef-1	TEF1-983F	*Forward*	GCY*CCYGGHCAY CGTGAYTTYAT	57	Rehner 2001
	TEF1-2218R	*Reverse*	ATGACACCRACRG CRACRGTYTG		
tub2	TUB2Fd	*Forward*	GTBCACCTYCARA CCGGYCARTG	56	Woudenberg et al. 2009
	TUB4Rd	*Reverse*	CCRGAYTGRCCRA ARACRAAGTTGTC		
rpb2	fRPB2-5F	*Forward*	GAYGAYMGWGAT CAYTTYGG	56	Liu et al.1999
	fRPB2-7R	*Reverse*	CCCATWGCYTGCT TMCCCAT		

*IUPAC código; **Y**: C ó T, **H**: A ó C ó T, **R**: A ó G, **B**: C ó G ó T, **M**: A ó C, **W**: A ó T

3.4.2. Análisis filogenético

Las secuencias obtenidas se ensamblaban y editaban con el *software SeqMan* v. 7.0.0 (DNAStar Lasergene, Madison, WI, EE.UU.). Las secuencias obtenidas en la presente tesis eran depositadas en la base de datos *European Nucleotide Archive* (ENA; https://www.ebi.ac.uk/ena/browser/home). Las secuencias generadas eran comparadas con las del *National Center for Biotechnology Information* (NCBI; https://www.ncbi.nlm.nih.gov/) utilizando la herramienta *Basic Local Alignment Search Tool* (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi), así como con las de la base de datos del *Westerdijk Fungal Biodiversity Institute* (https://wi.knaw.nl/page/Pairwise_alignment). La alineación para cada *locus* eran realizadas con el software MEGAv.7.0. (Kumar et al. 2016) a través del algoritmo

ClustalW (Thompson et al. 1994) y refinadas con MUSCLE (Edgar 2004), o manualmente, si era necesario, en la misma plataforma.

Las reconstrucciones filogenéticas se realizaban mediante el método de máxima verosimilitud (ML) y de inferencia bayesiana (BI). El mejor modelo de sustitución de nucleótidos para cada *locus* se estimó mediante el programa MEGA y el jModelTest v. 2.1.10 (Posada 2008) para ML y BI, respectivamente. El análisis de ML se realizó con el mismo programa Mega o con RAxML-HPC2 en la versión XSEDE 8.2.12 (Stamatakis et al. 2014), ejecutado en la plataforma *Cipres Science Gateway* versión 3.3 (Miller et al. 2012). La robustez de las ramas internas se evaluaba por el método de *bootstrap* con 1.000 repeticiones, tomando un valor ≥70% como estadísticamente significativo. El análisis de BI se realizaba con el programa MrBayes versión 3.1.2 (Ronquist et al. 2012), y se calculaba a partir de la distribución de probabilidad posterior utilizando el teorema de Bayes con la técnica de simulación denominada cadena Markov–Monte Carlo (MCMC). Se realizaban dos series paralelas de 5.000.000 generaciones, cuatro MCMC y se almacenaban los árboles resultantes cada 1.000 generaciones. El árbol consenso de la regla de la mayoría del 50%, así como los valores de probabilidad posterior (PP), se calculaban después de descartar el primer 25% de las muestras. Se consideró estadísticamente significativo un valor de PP ≥ 0,95.

3.5. Registro y conservación de las cepas

Los nombres de los nuevos taxones, así como sus respectivas descripciones, se registraban en la base de datos MycoBank (https://www.mycobank.org/). Los cultivos vivos de las nuevas especies, así como los holotipos, fueron depositados en la colección y el herbario del *Westerdijk Fungal Biodiversity Institute* (Utrecht, Países Bajos), respectivamente.

Los cultivos puros de la totalidad de los hongos identificados en la presente tesis se depositaban en la colección de hongos de la Facultad de Medicina de Reus (FMR, URV). Se utilizaron tres métodos de conservación para asegurar la supervivencia de los aislados, lo cuales se detallan a continuación.

3.5.1. Conservación bajo aceite mineral

Los aislados se sembraban en tres tubos de cristal con tapa a rosca, los que contenían medio de cultivo (uno de PDA y dos de OA) inclinado en *slant* (inclinados en "pico de flauta"). Los tubos inoculados se incubaban a temperatura ambiente hasta obtener crecimiento y formación de cuerpos fructíferos. Posteriormente, se cubrían con aceite mineral estéril y se almacenaban en obscuridad a temperatura ambiente.

3.5.2. Conservación en agua estéril

A partir de colonias crecidas en medio OA ó PDA y con ayuda de un bisturí, se recortaban bloques de los medios agarizados conteniendo el hongo de aproximadamente 1 cm^2, y se introducían en dos viales de cristal, uno de ellos conteniendo agua de red y otro agua destilada, en ambos casos esterilizadas. Posteriormente, se cerraban los frascos herméticamente con tapón de caucho y se almacenaban en obscuridad a temperatura ambiente.

3.5.3. Liofilizados

A partir de colonias crecidas y fructificadas en OA ó PDA se raspaba la superficie con un bisturí estéril, intentando arrastrar la mayor cantidad posible de material fúngico, a ser posible cuerpos fructíferos (conidiomas y/o ascomas). La masa fúngica obtenida se depositaba en un tubo estéril que contenía 3 mL de solución *skim milk* (Difco) al 10 %, y después de homogenizar se distribuía en viales de vidrío estériles. Los viales se introducían en el liofilizador automatizado VirTis Advantage 2.0 ES (SP Scientific, USA). Una vez que se terminaba el proceso de liofilización, los frascos se cerraban herméticamente con un anillo metálico. Para evaluar la viabilidad y pureza de las cepas, el contenido de uno de los viales se empleaba como control de calidad, el mismo se sembraba en medio de cultivo PDA y se incubaba por un tiempo prudencial a temperatura ambiente. Los otros dos viales se almacenaban a temperatura ambiente en oscuridad.

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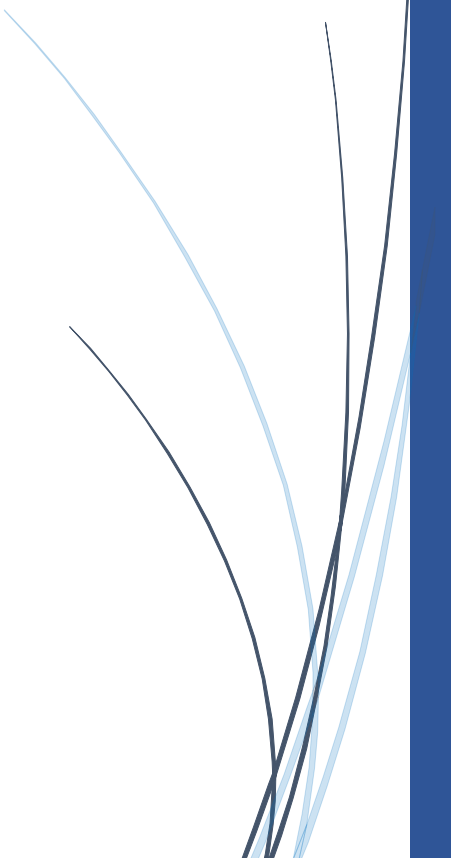
4. RESULTADOS



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4.1. New Taxa of the Family *Amniculicolaceae* (Pleosporales, *Dothideomycetes*, Ascomycota) from Freshwater Habitats in Spain

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Publicado en: Microorganisms 2020; 8: 1355.

DOI:10.3390/microorganisms8091355.

Área de la revista**:** Microbiología

Posición de la revista**:** 34/133 (Q2)

Factor de impacto**:** 4.128

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microorganisms

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Article

New Taxa of the Family Amniculicolaceae (Pleosporales, Dothideomycetes, Ascomycota) from Freshwater Habitats in Spain

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Received: 12 August 2020; Accepted: 3 September 2020; Published: 4 September 2020

Abstract: With the exception of the so-called Ingoldian fungi, the diversity and distribution of the freshwater aero-aquatic or facultative fungi are not well known in Spain. In view of that, we collected and placed into wet chambers 105 samples of submerged and decomposing plant debris from various places in Spain, looking for individuals belonging to these latter two morpho-ecological groups of fungi. As a result, we found and isolated in pure culture several fungi, the morphology of some of them belonging to the family *Amniculicolaceae* (order *Pleosporales*, class *Dothideomycetes*). After a careful phenotypic characterization and a phylogenetic tree reconstruction using a concatenated sequence dataset of D1-D2 domains of the 28S nrRNA gene (*LSU*), the internal transcribed spacer region (ITS) of the nrDNA, and a fragment of the translation elongation factor 1-alpha (*tef1*) gene, we report the finding of three new species of the genus *Murispora*: *Murispora navicularispora*, which produces cinnamon-colored, broadly fusiform to navicular ascospores; *Murispora fissilispora*, which has as a remarkable characteristic the production of both sexual and asexual morphs in vitro; and *Murispora asexualis*, the unique species of the genus that lacks a sexual morph. As a consequence of the phylogenetic study, we introduce the new aero-aquatic genus *Fouskomenomyces*, with a new combination (*Fouskomenomyces cupreorufescens*, formerly *Spirosphaera cupreorufescens* as the type species of the genus) and a new species, *Fouskomenomyces mimiticus*; we propose the new combinations *Murispora bromicola* (formerly *Pseudomassariosphaeria bromicola*) and *Murispora triseptata* (formerly *Pseudomassariosphaeria triseptata*); and we resurrect *Massariosphaeria grandispora*, which is transferred to the family Lopiostomataceae.

Keywords: Ascomycota; freshwater; fungi; plant debris; *Pleosporales*; Spain

1. Introduction

Fungi are a diverse group of ubiquitous organisms present in almost all ecosystems on Earth, including aquatic habitats [1]. Several fungal taxa have been isolated from freshwater environments, which offer a wide range of organic substrates for fungal colonization [2]. The most important role of fungi in freshwater is the recycling of dead organic matter, typically submerged plant debris [3]. Freshwater fungi complete (at least one part of) their life cycle into the water, and disperse their propagules through the water. Freshwater fungi are generally classified into different sorts of morphological and ecological groups: the "Ingoldian", producing submerged star-like (*stauro*-) or worm-like (*scoleco*-) asexual spores (or propagules) in lotic habitats (moving waters) [2,4]; the aero-aquatic, forming helical, net-like or globose conidia above the surface of lentic (standing) waters [5]; and members of the Ascomycota reproducing by the formation of conidia or sexual propagules (ascospores) into fertile bodies (conidiomata and ascomata, respectively). Members of

the Ascomycota reproducing by ascospores seem to be less exclusively adapted to life in aquatic environments than the other sort of previously cited fungal groups [6]. These fungi produce unitunicate asci with apical structures, or these are fissitunicate (bitunicate) and the ascospores are mostly ornate with mucilaginous sheaths or appendages which facilitate the attachment to submerged substrates [7].

The freshwater Ascomycota (FWA) are one of the least studied groups of fungi but they are taxonomically diverse and have representatives in a wide spectrum of families and orders. Approximately one third of the FWA with sexual reproduction belongs to the class *Dothideomycetes* [8]. Four lineages of the class *Dothideomycetes* have been recently described from this habitat: the order *Jahnulales* [9], the family *Lindgomycetaceae* [10,11], the family *Natipusillaceae* [12] and the family *Amniculicolaceae* [13,14]. The latter was established by Zhang in 2009 [15] to accommodate the genera *Amniculicola*, *Murispora* and *Pseudomassariosphaeria* [15]. Although all species of those three genera grow and produce a purple pigment on submerged wood, they differ in the morphology of the ascospores, which vary in color (hyaline in *Amniculicola*, and brown in *Murispora* and in *Pseudomassariosphaeria*) and septation (1-septate in *Amniculicola*, transversely multiseptate in *Pseudomassariosphaeria*, and with multiple transversal, longitudinal and oblique septa (muriform) in *Murispora*) [15,16]. Most of the *Amniculicolaceae* have been reported from freshwater habitats in Italy, France, Germany, Denmark and China [15–18].

During a survey on fungi living on decaying plant material in freshwater habitats in Spain, several strains that are morphologically compatible with members of the family *Amniculicolaceae* have been isolated in pure culture. The objectives of this study were to characterize phenotypically and to identify such fungi by phylogenetic analysis using nucleotide sequences of informative molecular markers.

2. Materials and Methods

2.1. Sample Collection and Specimen Examination

A total of 105 samples of submerged plant material were collected: three in *Les Guilleries* (Barcelona province), 50 in *Cascadas del Huéznar* (Cazalla de la Sierra, Sevilla province), 22 in Capafonts (Tarragona province) and 30 in *Serra del Montsant* (Tarragona province), Spain. These were placed into sterile self-sealing plastic bags to be transported to the lab and stored until processing. The specimens were rinsed twice with tap water, placed into Petri dishes or appropriate plastic containers lined inside with filter paper and moistened with sterile water with diehldrin® (20 drops of a solution of 20 mg diehldrin in 20 mL of dimethyl-ketone/L of water), incubated at room temperature (20–25 °C), and examined periodically under stereomicroscope for up to 2 months. Several ascomata or asexual propagules were taken and transferred using sterile disposable tuberculin-type needles to 55 mm diam. Petri dishes containing oatmeal agar (OA; 30 g of filtered oat flakes, 15 g agar-agar, 1 L tap water [19]), then incubated at room temperature. All isolates were stored in the culture collection of the Faculty of Medicine at Universitat Rovira i Virgili (FMR; Reus, Spain). Type specimens and ex-type cultures of the novel fungi were deposited in the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands (Table 1).

Table 1. Fungal taxa and their nucleotide sequences of the molecular markers used to build the phylogenetic tree.

Taxon	Strain	GenBank Accession No.		
		LSU	**ITS**	***tef1***
Amniculicola lignicola	CBS 123094^T	MH874798	—	GU456278
Amniculicola longissima	CB L22	GU266240	AY204596	—
Amniculicola lonsissima	CCM-F10304	JN673029	AY204594	—
Amniculicola parva	CBS 123092^T	FJ795497	MH863272	GU349065
Fouskomenomyces cupreorufescens	A20^T	AY616236	AY616232	—

Table 1. *Cont.*

Taxon	Strain	GenBank Accession No.		
		LSU	**ITS**	***tef1***
Fouskomenomyces mimiticus	**FMR 16,958^{T}**	**LR824585**	**LR824586**	**LR824584**
Fouskomenomyces mimiticus	**FMR 17,151 = CBS 146935**	**LR824588**	**LR824587**	**LR824589**
Leptosphaeria dolium	CBS 125979	JF740283	JF740208	—
Leptosphaeria dolium	CBS 505.75	GQ387576	JF740205	GU349069
Lindgomyces ingoldianus	ATCC 200398^{T}	AB521736	JF419898	—
Lindgomyces rotundatus	KT966	AB521739	JF419901	—
Lophiostoma macrostomum	CBS 122681	EU552141	EU552141	LC001753
Lophiostoma arundinis	KT 651	AB618999	JN942965	LC001738
Massariosphaeria grandispora	CBS 613.86	FJ795507	—	GU349036
Murispora aquatica	MFLU 19-0990^{T}	MN325075	MN325085	MN337969
Murispora asexualis	**FMR 17,248^{T} = CBS 146937**	**LR824596**	**LR824593**	**LR824590**
Murispora bromicola	MFLUCC 15-0031^{T}	NG_059595	NR_164235	KT305999
Murispora cardui	MFLUCC 13-0761^{T}	NG_059607	KT736082	KT709190
Murispora cicognanii	MFLUCC 14-0953^{T}	NG_059609	NR_155381	MK109804
Murispora fagicola	MFLUCC 13-0600^{T}	NG_060797	NR_155379	KT709188
Murispora fissilispora	**FMR 17,251^{T} = CBS 146936**	**LR824597**	**LR824594**	**LR824591**
Murispora galii	MFLUCC 13-0819^{T}	KT709175	NR_154629	KT709189
Murispora haswksworthii	MFLUCC 14-091^{T}	KT709180	NR_138414	KT709192
Murispora medicaginicola	MFLUCC 13-0762^{T}	NG_059609	NR_155380	KT709191
Murispora navicularispora	**FMR 17,838^{T}**	**LR824598**	**LR824595**	**LR824592**
Murispora rubicunda	IFRD 2017^{T}	FJ795507	—	GU456289
Murispora triseptata	MF1336^{T}	MK411002	—	—
Neomassariosphaeria typhicola	KT797^{T}	AB521747	JF419906	—
Preussia lignícola	CBS 264.69	MH878448	—	GU349027
Preussia minima	CBS 524.50	MH868263	MH856741	DQ677897
Quadricrura septentrionalis	CBS 125430	MH875152	NR_119402	—
Triplosphaeria máxima	KT 870	AB524637	NR_119407	—
Tetraplosphaeria sasicola	KT 563^{T}	AB524631	AB524807	—
Vargamyces aquaticus	CBS 636.9^{T}	KY853539	NR_154471	—
Vargamyces aquaticus	FMR 11587	KY853538	KY853475	—
Vargamyces aquaticus	**FMR 16,953**	**LR812096**	**LR812095**	—
Westerdykella ornata	CBS 379.55^{T}	NG_057861	NR_103587	GU349021

[1] A20: Hermann Volgmayr; ATCC: American Type Culture Collection, Virginia, USA; CB: Christiane Baschien; CBS: Culture collection of the Westerdijk Biodiversity Institute, Utrech, The Netherlands; CCM: Czech Collection on Microorganisms, Masaryk University, Faculty of Science, Brno, Czech Republic; FMR: Facultat de Medicina, Reus, Spain; IFRD: IFRDCC: Culture Collection, International Fungal Research & Development Centre, Chinese Academy of Forestry, Kunming, China; KT: Kazuaki Tanaka; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand. [2] Strains studied by us are indicated in bold. [T] Ex-type strain.

2.2. Phenotypic Study

Macroscopic characterization of the colonies was performed on OA, 2% malt extract agar (MEA; Difco, Detroit, MI, USA) [19] and potato dextrose agar (PDA; Pronadisa, Madrid, Spain) [20] into 90 mm diam. Petri dishes, after incubation for three weeks at 15 °C in the dark for species of the genus *Murispora* [16], and in similar conditions but at 20 °C for other taxa. Color notations were according to Kornerup and Wanscher (1978) [21]. The ability of the isolates to grow at cardinal temperatures was determined on PDA after 7 d in the dark, ranging from 5 to 35 °C, at 5 °C intervals, but also at 37 °C. Measurements and descriptions of microscopic structures were taken from specimens mounted in Shear's mounting medium (3 g potassium acetate, 60 mL glycerol, 90 mL ethanol 95% and 150 mL distilled water) [22], using an Olympus BH-2 bright field microscope (Olympus Corporation, Tokyo, Japan). Photomicrographs were taken using a Zeiss Axio-Imager M1 microscope (Oberkochen, Germany) with a DeltaPix Infinity × digital camera using Nomarski differential interference contrast.

2.3. DNA Extraction PCR Amplification and Sequencing

The strains were cultured on PDA for 7 days at 25 °C in the dark. Total DNA was extracted using the FastDNA kit protocol (Bio101, Vista, CA, USA), with a FastPrep FP120 instrument (Thermo Savant, Holbrook, NY, USA) according to the manufacturer's protocol. DNA was quantified by using Nanodrop 2000 (Thermo Scientific, Madrid, Spain). The following *loci* were amplified and sequenced: *LSU* (28S nrRNA gene), with the primer pair LR0R [23] and LR5 [24]; ITS (internal transcribed spacer region), with the primer pair ITS5 and ITS4 [25]; and *tef1* with EF1-983F and EF1-2218R [26]. The PCR amplifications were performed in a total volume of 25 µL containing 5 µL 10 × PCR Buffer (Invitrogen, CA, USA), 0.2 mM dNTPs, 0.5 µL of each primer, 1 U Taq DNA polymerase and 1–10 ng genomic DNA. PCR conditions for *LSU* and ITS were set as follows: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation, annealing and extension, and a final extension step at 72 °C for 10 min. For the *LSU* and ITS amplification, the 35 cycles consisted of 45 s at 95 °C, 45 s at 53 °C and 2 min at 72 °C; and for the *tef1* an initial denaturation at 94 °C for 2 min, followed by 30 cycles consisting of 30 s at 94 °C, 1 min 20 s at 57 °C and 1 min 30 s at 72 °C. PCR products were purified and stored at −20 °C until sequencing. The same pairs of primers were used to obtain the sequences at Macrogen Spain (Macrogen Inc., Madrid, Spain). The consensus sequences were obtained using the SeqMan software v. 7 (DNAStar Lasergene, Madison, WI, USA).

2.4. Phylogenetic Analysis

The sequences generated in this study were compared with those of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignment for each *locus* was performed with the MEGA (Molecular Evolutionary Genetics Analysis) software v. 7.0. [27], using the ClustalW algorithm [28] and refined with MUSCLE [29] or manually, if necessary, on the same platform. The alignment included our sequences, together with those available at the NCBI databases, of all genera and species belonging to the family *Amniculiculaceae*, and representatives of the families *Lindgomytaceae, Teratospharriaceae, Lophiostomataceae* and *Sporormiaceae* (Table 1). The phylogenetic analyses were carried out using Maximum-Likelihood (ML) and Bayesian Inference (BI) with RAxML v. 8.2.10 [30] using the Cipres Science gateway portal [31] and MrBayes v. 3.2.6 [32], respectively. For ML analyses, the best nucleotide substitution model was General Time Reversible with Gamma distribution. Support for internal branches was assessed by 1000 ML bootstrapped pseudoreplicates. For the BI phylogenetic analysis, the best nucleotide substitution model was determined using jModelTest [33]. For ITS, we used the symmetrical model with gamma distribution (SYM + G), for *LSU*, we used the symmetrical model with proportion of invariable sites and gamma distribution (SYM + I + G), and for *tef1*, we used the General Time Reverse with proportion of invariable sites and gamma distribution (GTR + I + G). The parameter settings were two simultaneous runs of 5M generations, four Markov chain Monte Carlo (MCMC), sampled every 1000 generations. The 50% majority-rule consensus tree and posterior probability values were calculated after discarding the first 25% of the samples. *Leptosphaeria dolium* (CBS 125,979 and CBS 505.75) served as outgroup taxa. Confident branch support was defined as Bayesian posterior probabilities (PP) ≥ 0.95 and ML bootstrap support (BS) ≥ 70%. Sequences generated in this study were deposited in European Nucleotide Archive (ENA).

3. Results

3.1. Phylogenetic Analyses

The final concatenated ITS-*LSU*-*tef1* sequence dataset using both ML and Bayesian analyses contained 37 ingroup strains from five families (*Amniculicolaceae*, *Lindgomytaceae*, *Lophiostomataceae*, *Sporormiaceae* and *Teratospharriaceae*). The alignment comprised a total of 1936 characters including gaps (815 for *LSU*, 399 for ITS and 722 for *tef1*), of which 435 were parsimony informative (125 for *LSU*, 173 for ITS and 137 for *tef1*). The individual sequence datasets did not show any conflicts in the tree

topologies for the 70% reciprocal bootstrap trees, which allowed the three genes for the multi-locus analysis to be combined. The ML analysis showed similar tree topology and was congruent with that obtained in the Bayesian analysis. For the BI multi-locus analysis, a total of 2706 trees were sampled after the burn-in with a stop value of 0.01. The support values were slightly different with the two analysis methods: with BI, posterior probabilities being higher than the ML bootstrap support values. In our phylogenetic analysis, the family *Amniculicolaceae* formed a well-supported main clade (99% BS/1 PP) (Figure 1). All taxa in this family were split into two well-supported clades. The first one (99% BS/1 PP) included two accepted genera (*Amniculicola*, 96% BS/1 PP and *Vargamyces*, 91% BS/1 PP), plus another genus (81% BS/0.98 PP). We propose this one as the new *Fouskomenomyces*, comprising *Fouskomenomyces cupreorufescens* (basionym *Spirosphaera cupreorufescens*) and two of our strains (FMR 17,151 and FMR 16,958). The second main clade, corresponding to the genus *Murispora* (94% BS/1 PP), was represented by all previously described species (including the type species of the genus, *M. rubicunda*). Our strains FMR 17,248, FMR 17,251 and FMR 17,838 were placed into independent terminal branches, each one representing a new species for the genus and two new combinations, *M. bromicola* (basionym *Pseudomassariosphaeria bromicola*) and *M. triseptata* (basionym *P. triseptata*). Surprisingly, *Pseudomassariosphaeria grandispora* (formerly included in the *Amniculicolaceae*) fell into the *Lopiostomataceae* (96% BS/1 PP).

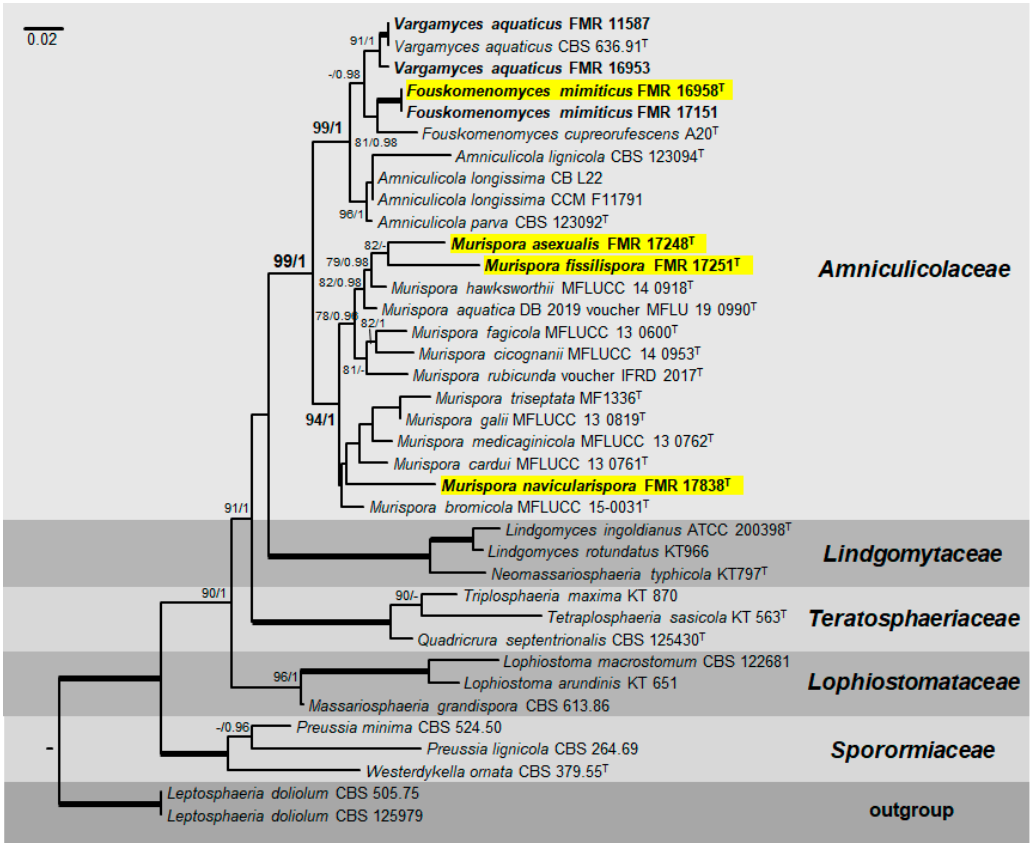

Figure 1. Phylogenetic tree inferred from a maximum likelihood analysis based on a concatenated alignment of D1-D2 domains of the 28S nrRNA gene (*LSU*), the internal transcribed spacer region (ITS) of the nrDNA, and a fragment of the translation elongation factor 1-alpha (*tef1*) gene sequences of 37 strains representing species in *Amniculicolaceae, Lindgomytaceae, Lophiostomataceae, Sporormiaceae* and *Teratosphaeriaceae.* The Bayesian posterior probabilities (PP) above 0.95 and the RAxML bootstrap support values (BS) above 70% are given at the nodes (PP/BS). Fully supported branches (1 PP/100 BS) are indicated in bold. Strains isolated during the developing of this work are in bold. Newly proposed taxa are highlighted in a yellow background. Type strains are indicated by a superscript "T". The tree was rooted with *Leptosphaeria dolium* (CBS 125,979 and CBS 505.75). Alignment length 1,936 bp.

3.2. Taxonomy

Amniculicolaceae Y. Zhang ter, C.L. Schoch, J. Fourn., Crous & K.D. Hyde, Studies in Mycology 64: 95 (2009). MycoBank 515469.

Type genus: Amniculicola Y. Zhang ter & K.D. Hyde, Mycol. Res. 112(10):1189 (2008).

Because *Spirosphaera cupreorufescens* was placed into a terminal clade in the *Amniculicolaceae*, and the type species of the genus, *Spirosphaera floriformis*, is phylogenetically distant (in the class *Leotiomycetes* [34]), and our strains FMR 16,958 and FMR 17,151 grouped together on a sister branch in the same terminal clade as *S. cupreorufescens*, we erect the new genus *Fouskomenomyces*, and recognize two species: *Fouskomenomyces cupreorufescens* comb. nov. (the type species of the genus) and *Fouskomenomyces mimiticus* sp. nov.

Fouskomenomyces V. Magaña-Dueñas, Cano & Stchigel, **gen. nov.** MycoBank MB835696.

Etymology. From Greek *φουσκωμένο-*, inflated, and *-μύκητα*, fungus, because of the nature of the propagules.

Description: Mycelium superficial to immersed composed by septate, smooth- and thin-walled, hyaline to pale brown, branching hyphae. *Conidiophores* micronematous to semi-macronematous, simple, pale brown, conidiogenous cells integrated, holoblastic, polyblastic. *Conidial propagules* brown to copper brown, more or less globose, scattered, composed by a compact branched system of globose to polyhedral cells, each one blown out successively to produce several daughter cells, detached by rhexolytic secession, or formed by branched, loosely spirally, interwoven septate filaments. *Chlamydospores* and *sexual morph* not observed.

Type species: Fouskomenomyces cupreorufescens (Voglmayr 2004) V. Magaña-Dueñas, Stchigel & Cano. MycoBank MB 835697.

Fouskomenomyces cupreorufescens (Voglmayr 2004) V. Magaña-Dueñas, Stchigel & Cano, **comb. nov.** MycoBank MB 835697.

Basionym: *Spirosphaera cupreorufescens* Voglmayr, Studies in Mycology 50:221–228. (2004).

Description: Voglmayr (2004).

Notes: The main distinctive features of *F. cupreorufescens* are its production of coppery-brown conidia in mass, irregularly globose and up to 150 μm diam. and its branched, loosely spiralled, interwoven, septate filaments.

Fouskomenomyces mimiticus V. Magaña-Dueñas, Cano & Stchigel, **sp. nov.** FMR 16,958. Mycobank MB 835698. (Figure 2)

Etymology. From Greek *μιμητικός*, mimetic, because the morphological resemblance to other genera such as *Pseudoagerita*.

Description: *Mycelium* superficial to immersed, composed by septate, smooth- and thin-walled, pale brown, branched, 2–3 μm wide hyphae. *Conidiophores* micronematous to semi-macronematous, simple, pale brown, conidiogenous cells integrated, polyblastic. *Conidial propagules* brown, globose to sub-globose, 55–150 μm diam., composed by a compact branched system of globose to polyhedral cells of 4–5 μm diam., each one successively budding out up 3–5 daughter cells, not breaking up into fragments when old, and detaching from the hyphae by rhexolytic secession. *Chlamydospores* and *sexual morph* absents.

Culture characteristics (after 3 weeks at 20 °C): Colonies on natural substratum not evident, appearing as scattered propagules. Colonies on MEA 2% reaching 25 mm diam., velvety, umbonate, margins regular, with abundant aerial mycelium, orange white to brownish-orange (6C4); reverse dark brown to brown (7F8/7E8), orange white (6C4) at the margins. Colonies on OA reaching 34–36 mm diam., flattened, slightly floccose, margins regular, with sparse aerial mycelium, dark purple to purplish grey (14F6/14B2); reverse violet to grey (15E8/14D1/15A3), margins white (1A1). Colonies on PDA reaching 28 mm diam., convex, cottony at the center, slightly floccose and velvety in the rest of the colony, margins regular, pinkish-white (9A2), margins orange grey (6B2); reverse brownish orange to reddish brown (6C3/8E7), margins orange white (5A2). Cardinal temperatures of growth: Optimum 20 °C, maximum 28 °C, minimum 15 °C.

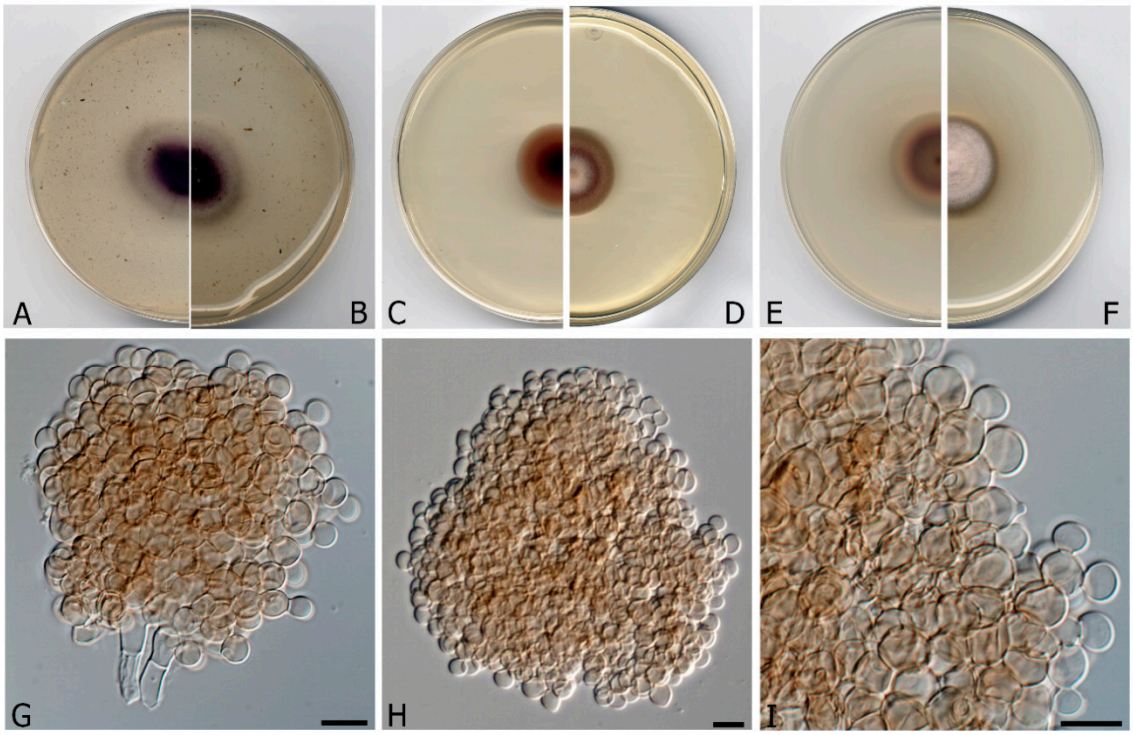

Figure 2. *Fouskomenomyces mimiticus* CBS H-24461. (**A**,**B**) Colonies on OA (oatmeal agar; reverse and front), (**C**,**D**) MEA (malt extract agar) 2% (reverse and front), (**E**,**F**) PDA (potato dextrose agar; reverse and front), at 20 °C after 3 weeks. (**G**) Conidial propagules with attached conidiogenous cells. (**H**) Free propagule. (**I**) Detail of a propagule showing the budding-like cells. Scale bars = 10 µm.

Material examined: Spain, Barcelona province, Les Guilleries, from freshwater submerged plant debris, Nov. 2017, Eduardo Jose de Carvalho Reis, holotype CBS H-24461, culture ex-type FMR 16,958. Spain, Barcelona province, Les Guilleries, from freshwater submerged plant debris, Nov. 2017, Eduardo Jose de Carvalho Reis, living cultures FMR 17,151 = CBS 146935.

Notes: Fouskomenomyces mimiticus produces brown to dark brown, globose to sub-globose propagules, composed of a compact branched system of globose to polyhedral cells, whereas the propagules of *Fouskomenomyces cupreorufescens* are formed by branched, loosely spiralled, interwoven filaments, which are coppery-brown in mass.

Because the genus *Murispora* now includes three new species and two new combinations (Figure 1) displaying novel morphological features, we have amended it as follows:

Murispora Y. Zhang ter, J. Fourn. & K.D. Hyde, in Zhang et al., Stud. Mycol. 64: 95 (2009). MycoBank MB 515472.

Saprobic fungi living in freshwater habitats. *Ascomata* scattered or in small groups, immersed, erumpent, or nearly superficial, dark brown to black, ostiolate, globose to subglobose, neck periphysate with an apex weakly papillate, conical or nearly so. *Peridium* 3–7-layered, outer layer of *textura angularis* or *textura intricata*. *Pseudoparaphyses* trabeculate, embedded in mucilaginous material. *Asci* (4–)8-spored, bitunicate, fissitunicate, short pedicellate, cylindrical to clavate, with an ocular chamber. *Ascospores* transversally septate or muriform, hyaline when young, mostly becoming pale brown to reddish brown with age, less commonly remaining hyaline, constricted at the septa, navicular to broadly ellipsoidal, usually surrounded by an irregular, hyaline, gelatinous sheath. Staining the substrate in purple. *Asexual morph* coelomycetous.

Type species: Murispora rubicunda (Niessl) Y. Zhang ter, J. Fourn. & K.D. Hyde, in Zhang et al., Stud. Mycol. 64: 96 (2009).

≡ *Pleospora rubicunda* Niessl, Notiz. Pyr.: 31 (1876).

= *Massariosphaeria rubicunda* (Niessl) Crivelli, Ueber die heterogene Ascomycetengattung pleospora rabh.; Vorschlag für eine Aufteilung (Diss. Eid genössischen technischen hochschule Zürich 7318): 144 (1983).

= *Karstenula rubicunda* (Niessl) M.E. Barr, N. Amer. Fl., Ser. 2 (New York) 13: 52 (1990).

Murispora bromicola (Phukhams., Ariyaw., Camporesi & K.D. Hyde) V. Magaña-Dueñas, Cano & Stchigel, **comb. nov.** MycoBank MB 835699.

Basionym: Pseudomassariosphaeria bromicola Phukhams., Ariyaw., Camporesi & K.D. Hyde, Ariyawansa et al., Fungal Diversity: 10.1007/s13225-015-0346-5, [2014] (2015).

Description: Ariyawansa et al. 2015.

Notes: Morphologically differing from the other species of *Murispora* by its production of hyaline ascospores (brown in the rest of the species of the genus), fusiform to lunate and narrower towards the apex (mostly ellipsoidal with rounded ends in other species), and not strongly constricted at the septa (although strongly constricted at septa in all other species of the genus).

Pseudomassariosphaeria triseptata, of marine origin, is a species recently introduced to the genus *Pseudomassariosphaeria* by Jones et al., in 2020 [35]. However, in our phylogenetic analysis, this species, as well as *P. bromicola*, was placed into the *Murispora* clade. Therefore, we propose the next new combination for this fungus.

Murispora trisepata (E.B.G. Jones & Abdel-Wahab) V. Magaña-Dueñas, Cano & Stchigel, **comb. nov.** MycoBank MB 836493.

Basionym: Pseudomassariosphaeria triseptata E.B.G. Jones et Abdel-Wahab. Botanica Marina 63(2):157 (2020)

Description: Jones et al. 2020.

Notes: Murispora triseptata differs from all other species of the genus by possessing hyaline, 3-septate, ellipsoidal big ascospores [35].

Based on phenotypic features and phylogenetic results, three new species of *Murispora* are proposed as follows:

Murispora fissilispora V. Magaña-Dueñas, Stchigel & Cano, **sp. nov.** FMR 17,251. MycoBank MB 835710 (Figure 3).

Etymology. From Latin *fissile-*, splitting, and *-sporarum*, spore, because the ascospores split at the middle when old.

Mycelium superficial to immersed, composed by septate, smooth- and thin-walled, pale brown, branched, 2–3 µm wide hyphae. *Ascomata* perithecial, immersed to semi-immersed, solitary, dark brown to black, ostiolate, papillate, *neck* conic-truncate, 105–108 × 60 µm, pyriform, 320–350 × 280–300 µm;, *peridial wall* 2–3-layered, 30–60 µm thick, outer wall of *textura intricata* composed of brown to dark brown hyphae 2–4 µm diam, inner wall layer hyaline and thin; *hamathecium* comprising numerous hyaline, filamentous, branched, septate *paraphyses* 1.5–2 µm wide;, periphysate; *asci* 4–8-spored, bitunicate, stipitate, cylindrical to cylindrical-clavate, 160–200 × 14–16 µm, stipe 20–25 µm long, without apical structures; *ascospores* hyaline when young, becoming brown at maturity, muriform, broadly fusiform to irregularly shaped, 15–27 × 6–8 µm, surrounded by a mucilaginous sheath, divided at the middle when old due to the narrowing of the medial septum. *Conidiomata* pycnidial, solitary, mainly immersed, pale brown to brown, ostiolate, subglobose, 65–70 × 85–90 µm; *conidiomata wall* of *textura angularis*, composed of pale brown to brown, flattened polygonal cells of 4–7 µm diam.; *conidiophores* reduced to the *conidiogenous cells*, which are phialidic, hyaline, smooth-walled, formed from the innermost layer of the pycnidial wall; *conidia* one-celled, hyaline, ovoid to ellipsoidal, 3–4 × 1.5–2.5 µm, guttulate.

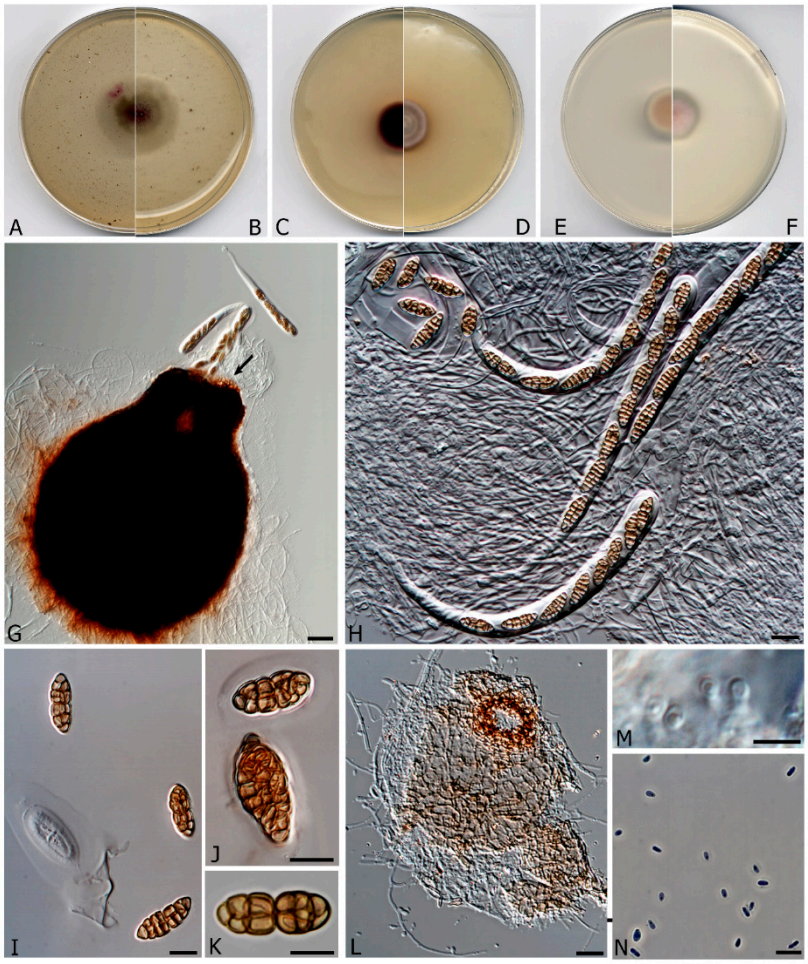

Figure 3. *Murispora fissilispora* CBS H-24462. (**A**,**B**) Colonies on OA (reverse and front), (**C**,**D**) MEA 2%(reverse and front), (**E**,**F**) PDA (reverse and front). All media at 15 °C after 3 weeks. (**G**) Ascomata expelling asci. (**H**) Asci. (**I**–**K**) Ascospores (Note the mucilaginous sheath in I and J). (**L**) Pycnidia. (**M**) Conidiogenous cells. (**N**) Conidia. Scale bars: G = 25 μm, H–N = 10 μm.

Culture characteristics (3 weeks at 15 °C). Colonies on PDA reaching 20–22 mm diam., convex, floccose, margin regular, with abundant aerial mycelium, surface purplish pink to white (6A2/1A1), border grey (14B1); reverse purplish pink to grey (14A3/14 B 1), diffusible pigment absent. Colonies on MEA 2% reaching 18–20 mm diam., flattened, velvety, margin regular, greyish brown to dull red (8E3/8C3); reverse reddish brown to greyish red (8F7/7B3), diffusible pigment reddish brown (8D5). Colonies on OA reaching 30–32 mm diam., flattened to slightly floccose, margins regular, with sparse aerial mycelium, deep magenta to purplish grey, with greyish magenta patches (13D8/13D1/13D5), borders white; reverse deep magenta to olive grey with greyish magenta patches (14D8/1E2/13D5), diffusible absent. Cardinal temperatures of growth: Optimum 15–20 °C, maximum 28 °C, minimum 5 °C.

Material examined: Spain, Tarragona province, *Serra del Montsant*, from freshwater submerged plant debris, February, 2018, collected by Eduardo Jose de Carvalho Reis, holotype CBS H-24462, culture ex-type FMR 17,251 = CBS 146936.

Notes: Murispora fissilispora, genetically distinct from its neighboring *Murispora asexualis*, is the only species of the genus that produces both sexual and asexual morphs in vitro.

Murispora asexualis V. Magaña-Dueñas, Cano & Stchigel, **sp. nov.** FMR 17,248. MycoBank MB 835711 (Figure 4).

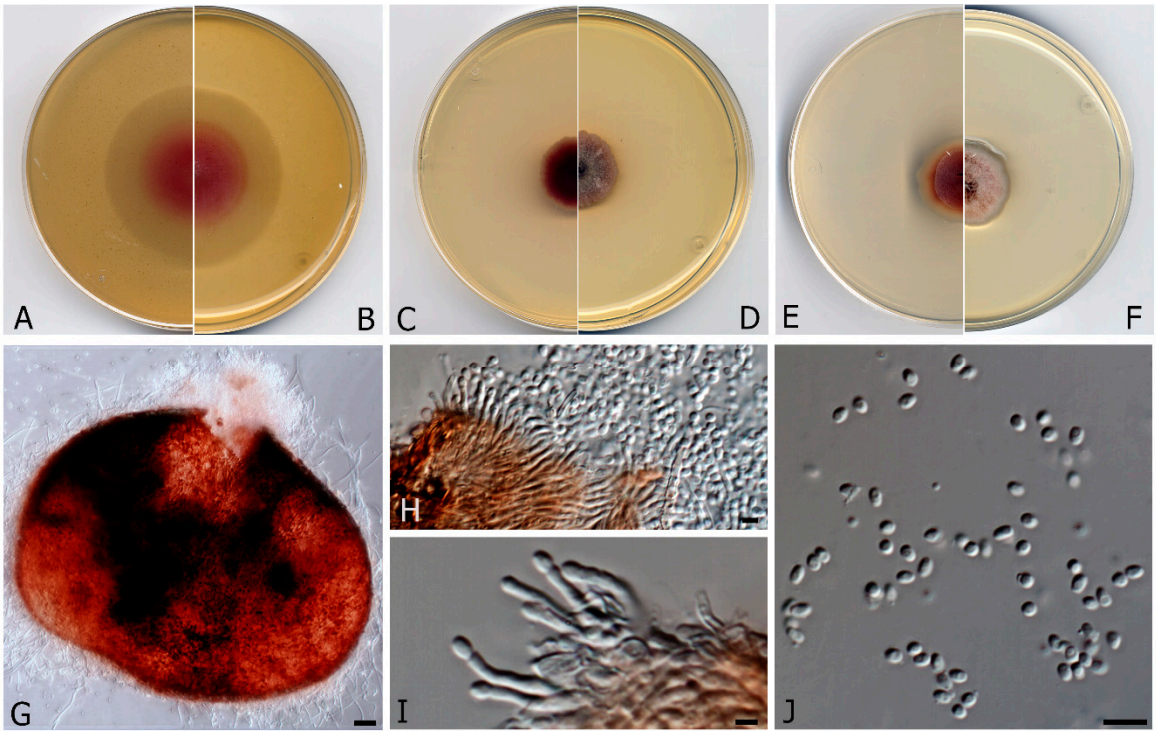

Figure 4. *Murispora asexualis* CBS H-24463. (**A**,**B**) Colonies on OA (reverse and front), (**C**,**D**) MEA 2% (reverse and front), (**E**,**F**) PDA (reverse and front). All media at 15 °C after 3 weeks. (**G**) Pycnidia (**H**,**I**) Conidiogenous cells (**J**) Conidia. Scale bars: (**G**) = 25 µm, (**H**,**J**) 10 µm, (**I**) = 2.5 µm.

Etymology. Because of the lack of a sexual morph, typical of the genus.

Mycelium composed of hyaline, smooth- and thin-walled, septate hyphae, 1.4–1.8 µm wide. *Conidiomata* pycnidial, solitary, brown to reddish brown, mainly immersed, glabrous, papillate, ostiolate, ovoid, 360–380 × 270–290 µm diam.; *peridial wall* of *textura angularis*, 4–6-layered, 20–40 µm thick, composed of brown to dark brown, flattened polygonal cells 3–4 µm diam.; *conidiophores* branched at the base, septate, hyaline to pale brown, straight or sinuous to slightly curved, 7.5–8.5 µm long; *conidiogenous cells* phialidic, hyaline, smooth- and thin-walled, ampulliform, slightly curved at the apex, 8–11 × 1–2 µm; *conidia* hyaline, non-septate, ovoid, 3–4 µm. *Sexual morph* unknown.

Culture characteristics (3 weeks at 15 °C). Colonies on PDA, reaching 30–32 mm diam., convex, velvety, margins irregular, with abundant aerial mycelium, surface reddish to white (12A2/1A1) margins grey (12C1); reverse violet brown to reddish brown (10E8/8D8), margins white, diffusible pigment absent. Colonies on MEA 2% reaching 24–28 mm diam., flattened, floccose, margins irregular, with abundant aerial mycelium, dark ruby to greyish ruby (12F3/12E6), margins reddish grey (12D2); reverse reddish brown to greyish red (8F7/7B3), diffusible pigment reddish brown (8D5). Colonies on OA reaching 38–42 mm diam., margins regular, mycelium mostly immersed, surface pink to yellowish white (12A4/4A2); reverse pink to yellowish white (12A4/4A2), diffusible pigment absent. Cardinal temperature for growth: Optimum 15–20 °C, maximum 30 °C, minimum 5 °C.

Material examined: Spain, Tarragona province, *Serra del Montsant*, from freshwater submerged plant debris, February, 2018, Eduardo Jose de Carvalho Reis, holotype CBS H-24463, culture ex-type FMR 17,248 = CBS 146937.

Notes: Murispora asexualis differs morphologically from the phylogenetically nearest species *M. fissilispora*, because it lacks a sexual morph. Furthermore, the conidiophores of *M. asexualis* are branched and slightly curved, while those of *M. fissilispora* are reduced to the conidiogenous cells.

Murispora navicularispora V. Magaña-Dueñas, Stchigel & Cano, **sp. nov.** FMR 17,838. MycoBank MB 835712 (Figure 5).

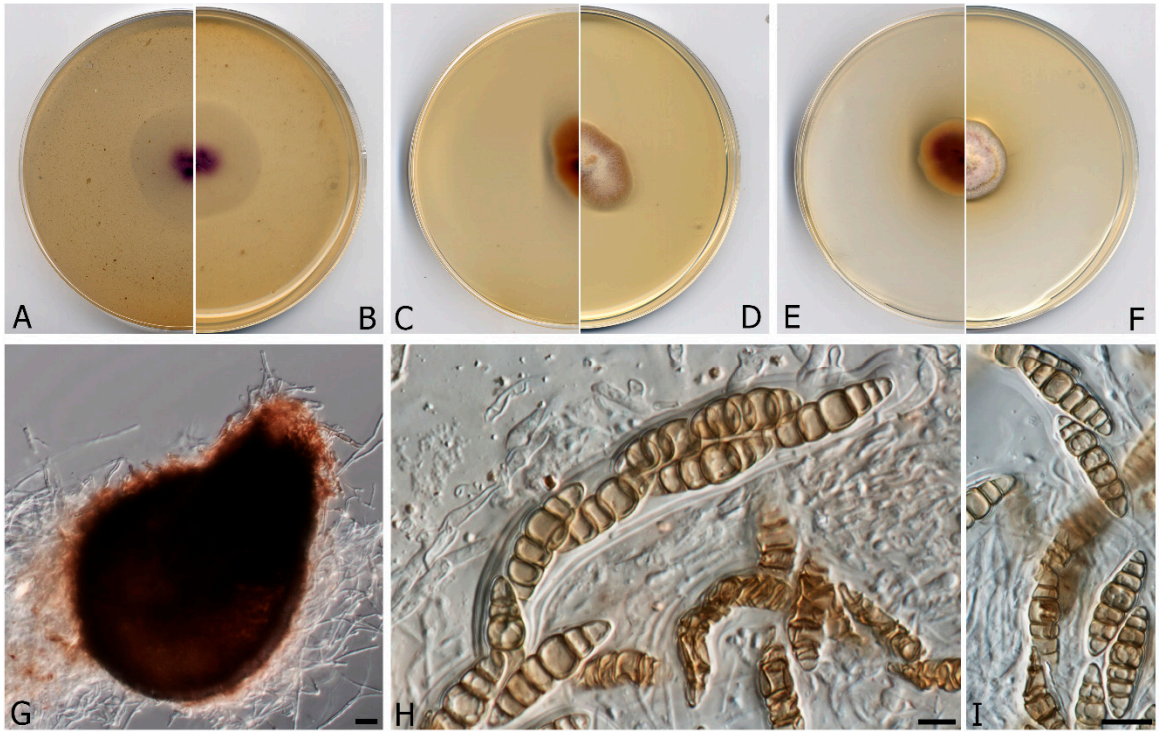

Figure 5. *Murispora navicularispora* CBS H-24464 (**A**,**B**) Colonies on OA (reverse and front), (**C**,**D**) MEA 2% (reverse and front), (**E**,**F**) PDA (reverse and front). All media at 15 °C after 3 weeks. (**G**) Ascomata. (**H**) Asci. (**I**) Ascospores. Scale bars: (**G**) = 25 µm, (**H**,**I**) = 10 µm.

Etymology. From Latin *navicularis*-, boat-shaped, and -*sporarum*, spore, because the shape of the ascospores.

Mycelium composed of hyaline, smooth- and thin-walled, septate hyphae, 1.4–1.8 µm wide. *Ascomata* perithecial, immersed to semi-immersed, solitary, brown to dark brown, ostiolate, papillate, *neck* conic-truncate, 100–90 × 60–70 µm, pyriform, 190–265 × 160–250 µm; *peridial wall* 2–4-layered, 20–50 µm thick, outer wall of *textura intricata*, composed of hyaline to brown hyphae 1.5–3 µm diam., inner wall composed by hyaline flattened cells; *hamathecium* comprising numerous hyaline, septate, filamentous, branched *paraphyses*, periphysate. *Asci* 8-spored, bitunicate, cylindrical to cylindrical-clavate, 115–120 × 15–20 µm, without apical structures. *Ascospores* 3–7-septate, cinnamon, broadly fusiform to navicular, 21–29 × 6–9 µm, narrowing towards the extremes, constricted at the septa, surrounded by a mucilaginous sheath. Natural substrate stained in purple. *Asexual form* unknown.

Culture characteristics (after 3 weeks at 15 °C). Colonies on PDA reaching 25–30 mm diam., umbonate, velvety, slightly cottony center, surface orange white to reddish white (5A2/6A2), pale orange (5A3) at the regular margins; reverse violet brown to yellowish white (10E4/4A2), diffusible pigment orange white (5A3). Colonies on MEA 2% reaching 26–28 × 17–20 mm diam, ellipsoidal, velvety, convex, white to reddish grey (8B2), with regular margins; reverse reddish brown (8F5), orange white (6A2) at the margins, diffusible pigment absent. Colonies on OA reaching 40–48 mm diam., flattened, with sparse aerial mycelium, surface and reverse deep violet (15E8), with yellowish white (4A2) regular margins; diffusible pigment absent. Cardinal temperatures of growth: Optimum 15–20 °C, maximum 30 °C, minimum 5 °C.

Material examined: Spain, Sevilla province, Cazalla de la Sierra, *Cascadas del Huéznar*, from freshwater submerged plant debris, May 2019, collected by José Francisco Cano Lira, holotype CBS H-24464, culture ex-type FMR 17838.

Notes: The fungus produces cinnamon, broadly fusiform to navicular ascospores, features never seen in the genus before.

Lophiostomataceae Sacc. Sylloge Fungorum, 2:672 (1883). MycoBanK 80966.

Type genus: Lophiostoma Ces. & De Not., Comm. Soc. crittog. Ital. 1(fasc. 4): 219 (1863). MycoBank MB 2933.

The genus *Pseudomassariosphaeria* was introduced by Phukhamsakda et al. in 2015 [17], to accommodate *Pseudomassariosphaeria bromicola,* found in a dead stem of *Bromus sterilis* L., transferring also *Massariosphaeria grandispora* to this genus (as *Pseudomassariospaheria grandispora*). However, in our phylogenetic study *P. bromicola* is clearly placed into the family *Amniculicolaceae* (transferred by us to the genus *Murispora* as *M. bromicola* earlier in this manuscript), whereas *P. grandispora* was located in the family *Lophiostomataceae,* phylogenetically close to *Lophiostoma macrostomum* and *L. arundinis.* The placement of *P. grandispora* into the *Lophiostomataceae* was previously suggested by Wang in 2007 [36], based on a molecular analysis using 28S rDNA, 18S rDNA and *rpb*2 gene. Consequently, we resurrected the name *Massariosphaeria grandispora* for this fungus.

Massariosphaeria grandispora (Sacc.) Leuchtm., Sydowia 37: 172 (1984). MycoBank MB 114956.

Description: Phukhamsakda et al. 2015.

Basionym: *Leptosphaeria grandispora* Sacc. Michelia 1(3): 341 (1878).

Synonyms: Lophiotrema grandispora (Sacc.) Shoemaker & C.E. Babc., Can. J. Bot. 67(5): 1580 (1989).

Metasphaeria grandispora (Sacc.) Sacc., Syll. fung. (Abellini) 2: 181 (1883).

Neomassariosphaeria grandispora (Sacc.) Y. Zhang ter, J. Fourn. & K.D. Hyde, in Zhang, Schoch, Fournier, Crous, Gruyter, Woudenberg, Hirayama, Tanaka, Pointing, Spatafora & Hyde, Stud. Mycol. 64: 96 (2009).

Pseudomassariosphaeria grandispora (Sacc.) Phukhams., Ariyaw. & K.D. Hyde, in Ariyawansa et al., Fungal Diversity: 10.1007/s13225-015-0346-5, [17] (2015).

4. Discussion

Of the three morpho-ecological groups of freshwater fungi (Ingoldian's, aero-aquatic and facultative) only the latter two were addressed in this study. In our phylogenetic analysis, all of the *Amniculicolaceae* species clustered in a distinct sister clade to *Lindgomycetae*, which is similar to previous studies [16–18]. Most *Aminiculicolaceae* species are reported from freshwater habitats and are widely distributed across Austria, Italy, France, Germany, Denmark, China, Hungary and Spain [15–18,34,37]. However, with exception of *Murispora aquatica* and *M. triseptata* (basionym *Pseudomassariospaheria triseptata*), all species of *Murispora* were isolated from terrestrial habitats such as dead terrestrial stems and dead and fallen twigs [14–18,35]. In this study, we have introduced three new species of *Murispora* collected from Spain in freshwater habitats. Thanks to the phenotypic characterization of several fungal isolates and to the subsequent phylogenetic analysis based on a concatenate database of the ITS-*LSU-tef1* sequences, we have erected three new species of *Murispora*: *M. asexualis,* the unique species of the genus because it lacks a sexual morph; *M. fissilispora,* the first species of this genus to produce a holomorph in vitro, and *M. navicularispora*, which produces cinnamon-colored, broadly fusiform to navicular ascospores, features never seen in the genus before. In addition, we have proposed the new combinations *M. bromicola* (formerly *P. bromicola*) and *M. triseptata* (formerly *P. triseptata*), demonstrating that this genus is monophyletic. Consequently, we have enlarged the current concept of *Murispora*, including species with hyaline, navicular and transversally septate ascospores, or lacking a sexual morph. Our results also indicate that some morphological features, such as the size and shape of the ascospores, have less phylogenetic significance than previously proposed by other authors. Despite *Spirosphaera cupreorufescens* displaying features considered as typical of that genus, it was phylogenetically distant in our phylogeny (in the class *Dothideomycetes*) from the type species of the genus (*Spirosphaera floriformis*, in the class *Leotiomycetes*), and because *S. cupreorufescens* formed a strongly supported clade together with two of our strains, we have proposed the erection of the new genus *Fouskomenomyces,* to include *Fouskomenomyces cupreorufescens* (the type species of the genus) and the new species *Fouskomenomyces mimiticus,* both aero-aquatic conidial fungi. Finally, we have also resurrected *Massariosphaeria grandispora*, because in our phylogeny it was placed into the *Lopiostomataceae* instead of the *Amniculicolaceae*. To date, there have been few reports of fungi isolated from freshwater habitats

in Spain, therefore this work represents an important contribution to the knowledge of the Spanish mycobiota in aquatic environments.

Author Contributions: V.M.-D. 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. A.M.S., because of their experience with fungal biology and taxonomy, supervised all steps of the experimental work by V.M.-D., collaborating in the description of the novel fungi and in the writing of chapters "Introduction" and "Discussion", reviewing the draft several times. J.F.C.-L. supervised the experimental work too, especially those steps related to nucleotide sequencing of the molecular markers employed in this study, but also 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 the draft several times. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Spanish *Ministerio de Economía y Competitividad*, grant CGL2017-88094-P.

Acknowledgments: We thank Juan Ramón García Martínez (a non-professional mycologist from Granja de Torrehermosa, Badajoz, Spain) for collaborating in the Sierra Norte de Sevilla sampling. We also thank Phil Hoddy for editing and proofreading the final text.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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TAXONOMÍA Y FILOGENIA MOLECULAR DE HONGOS PROCEDENTES DE MATERIAL VEGETAL SUMERGIDO DE ESPAÑA
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4.2. New Coelomycetous Fungi from Freshwater in Spain

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Publicado en: Journal of Fungi 2021; 7: 368.

DOI:10.3390/jof7050368.

Área de la revista**:** Micología

Posición de la revista**:** 5/129 (D1)

Factor de impacto**:** 5.816

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TAXONOMÍA Y FILOGENIA MOLECULAR DE HONGOS PROCEDENTES DE MATERIAL VEGETAL SUMERGIDO DE ESPAÑA
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New Coelomycetous Fungi from Freshwater in Spain

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Abstract: Coelomycetous fungi are ubiquitous in soil, sewage, and sea- and freshwater environments. However, freshwater coelomycetous fungi have been very rarely reported in the literature. Knowledge of coelomycetous fungi in freshwater habitats in Spain is poor. The incubation of plant debris, from freshwater in various places in Spain into wet chambers, allowed us to detect and isolate in pure culture several pycnidia-producing fungi. Fungal strains were phenotypically characterized, and a phylogenetic study was carried out based on the analysis of concatenated nucleotide sequences of the D1–D2 domains of the 28S nrRNA gene (LSU), the internal transcribed spacer region (ITS) of the nrDNA, and fragments of the RNA polymerase II subunit 2 (*rpb*2) and beta tubulin (*tub*2) genes. As a result of these, we report the finding of two novel species of *Neocucurbitaria*, three of *Neopyrenochaeta*, and one of *Pyrenochaetopsis*. Based on the phylogenetic study, we also transferred *Neocucurbitaria prunicola* to the genus *Allocucurbitaria*. This work makes an important contribution to the knowledge of the mycobiota of plant debris in freshwater habitats.

Keywords: Ascomycota; coelomycetous; freshwater fungi; phylogeny; plant debris; taxonomy

Citation: Magaña-Dueñas, V.; Stchigel, A.M.; Cano-Lira, J.F. New Coelomycetous Fungi from Freshwater in Spain. *J. Fungi* **2021**, *7*, 368. https://doi.org/10.3390/jof7050368

Academic Editor: Lei Cai

Received: 12 March 2021
Accepted: 6 May 2021
Published: 8 May 2021

1. Introduction

Coelomycetous fungi are characterized by the production of conidia within a cavity lined by fungal or fungal-host tissue called conidiomata [1]. Conidiomata can be acervular (open, cup-shaped asexual fruiting bodies developing below the epidermis of the plant host tissue and bearing a series of adpressed conidiophores), pycnidial (globose, pyriform to flask-shaped asexual reproductive structures whose conidia are liberated through an usually apical opening [ostiolum]), or stromatic (consisting of undifferentiated sclerotic tissues, ostiolate or not, in which one or more lysigenic cavities develops, upholstered inside by conidiophores/conidiogenous cells forming conidia). Coelomycetous fungi are mostly parasites of terrestrial vascular plants but are also saprobic, growing at the expense of dead organic matter on the ground, especially on plant debris. These are ubiquitous on soil, sewage, and in salt- and freshwater environments. [2]. Freshwater coelomycetous fungi occur on stream-side plants or on submerged wood litter, and their conidia can also be recovered from foam and water samples [3]. Usually, they produce brown to blackish pycnidial fruiting bodies on submerged woody debris and stems of herbaceous plants, and produce several conidia from the conidiogenous cells [4]. Identification of coelomycetous fungi has gone through dramatic changes over the last decade, and currently involves DNA sequencing of several (four to six) genetic markers and the building of phylogenetic trees [5]. In Spain, there have been a few reports of coelomycetous fungi recovered from freshwater habitats. In 1990, Roldán and Honrubia reported *Bartalinia robillardoides* and *Truncatella angustata* [6], and Giralt described *Diplolaviopsis ranula* [7]. Up to 2014, only 16 coelomycetous fungi had been reported from freshwater habitats [4,8–16].

The main objective of this work was to characterize phenotypically and to identify molecularly those coelomycetous fungi found in different freshwater habitats in Spain.

2. Materials and Methods

2.1. Sampling and Fungal Isolation

A hundred samples of decomposing plant material submerged in freshwater habitats in Spain were collected: 3 from "Les Guilleries" (Barcelona province), 50 from "Cascadas del Huéznar" (Cazalla de la Sierra, Sevilla province, Spain), 17 from Riaza (Segovia province, Spain), and 30 from "Serra del Montsant" (Tarragona province, Spain). The samples were placed into self-sealing sterile plastic bags, which were closed and transported to the laboratory, and stored at room temperature (20−25 °C) until they were processed. The specimens were rinsed twice with 500 mL tap water, placed into Petri dishes or appropriate plastic containers lined inside with two sheets of filter paper, and moistened with sterile water with diehldrin® (1 mL of a solution of 20 mg diehldrin® in 20 mL of dimethyl-ketone/L of water), incubated at room temperature, and examined periodically under stereomicroscope for up to 2 months. Several propagules and/or fruiting bodies were taken and transferred using sterile disposable tuberculin-type needles to 55 mm diameter Petri dishes containing oatmeal agar (OA; 30 g of filtered oat flakes, 15 g agar-agar, 1 L tap water; [17]), and then incubated at room temperature. Once obtaining an axenic culture of each isolate, these were stored in the culture collection of the Faculty of Medicine of University Rovira i Virgili (FMR; Reus, Spain). Type specimens and ex-type cultures of the novel fungi were deposited in the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands (Table S1).

2.2. Phenotypic Study

Macroscopic characterization of the colonies was performed on OA and on malt extract agar (MEA; Difco, Detroit, MI, USA) incubated for 14 d in the dark at 25 ± 1 °C [17]. Colony colour was determined according to Kornerup and Wanscher [18]. The ability of the isolates to grow at cardinal temperatures was determined on potato dextrose agar (PDA; Pronadisa, Madrid, Spain) after 7 d in the dark, ranging from 5 to 35 ± 1 °C at 5 °C intervals, plus 37 ± 1 °C [19]. Morphological characterization of vegetative and reproductive structures was performed growing the fungal strains on OA in the same conditions as for colony characterization, and examining at least 30 individuals of each structure [20,21] on Shear's mounting medium (3 g potassium acetate, 60 mL glycerol, 90 mL ethanol 95%, and 150 mL distilled water; [22]) using a Olympus BH-2 bright field microscope (Olympus Corporation, Tokyo, Japan). Photomicrographs were taken using a Zeiss Axio-Imager M1 microscope (Oberkochen, Germany) with a DeltaPix Infinity X digital camera using Nomarski differential interference contrast.

2.3. DNA Extraction, Amplification and Sequencing

Fungal strains were cultured on PDA for 7 days at 25 ± 1 °C in the dark. Total DNA was extracted using the FastDNA kit protocol (Bio101, Vista, CA, USA) with a FastPrep FP120 instrument (Thermo Savant, Holbrook, NY, USA) according to the manufacturer's protocol. DNA was quantified by using Nanodrop 2000 (Thermo Scientific, Madrid, Spain). The following *loci* were amplified and sequenced: LSU, with the primer pair LR0R [23] and LR5 [24]; ITS, with the primer pair ITS5 and ITS4 [25]; a fragment of the beta-tubulin gene (*tub*2) with the primers TUB2Fw and TUB4Rd [26]; and a fragment of the RNA polymerase II subunit 2 gene (*rpb*2) with RPB2-5F2 [27] and fRPB2-7cR primers [28]. The PCR amplifications were performed in a total volume of 25 µL containing 5 µL 10× PCR Buffer (Invitrogen, CA, USA), 0.2 mM dNTPs, 0.5 µL of each primer, 1 U Taq DNA polymerase, and 1−10 ng genomic DNA. PCR conditions for LSU, ITS, and *tub*2 were set as follows: an initial denaturation at 95 °C for 5 min; followed by 35 cycles of denaturation, annealing, and extension; and a final extension step at 72 °C for 10 min. For the LSU and ITS amplification, the 35 cycles consisted of 45 s at 95 °C, 45 s at 53 °C, and 2 min at 72 °C; and for the *tub*2 region 30 s at 94 °C, 45 s at 56°C, and 1 min at 72 °C. The PCR program for *rpb*2 amplification consisted of 5 cycles of 45 s at 94 °C, 45 s at 60 °C, and 2 min at 72 °C; then 5 cycles with 58 °C annealing temperature; and 30 cycles with a 54 °C annealing

temperature. PCR products were purified and stored at −20 °C until sequencing. The same pairs of primers were used to obtain the sequences at Macrogen Spain (Macrogen Inc., Madrid, Spain). The consensus sequences were obtained using the SeqMan software v. 7 (DNAStar Lasergene, Madison, WI, USA).

2.4. Phylogenetic Analysis

We made a preliminary molecular identification by comparing the LSU, ITS, *tub*2, and *rpb*2 sequences of our isolates with those of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 16 March 2021)). For *tub*2 sequences, a maximum level of identity (MLI) of <98% provides identification only at genus level, and a value >98% was considered to allow for species-level identification. Alignment for each locus was performed with the MEGA (Molecular Evolutionary Genetics Analysis) software v. 7.0. (Tamura et al. 2013), using the ClustalW algorithm [29] and refined with MUSCLE [30] or manually, if necessary, on the same platform. Individual and concatenated phylogenetic trees were built after a maximum likelihood (ML) analysis carried out using the RAxML v. 8.2.10 [31] software on the online Cipres Science gateway portal [32], and a Bayesian Inference (BI) analysis using MrBayes v. 3.2.6 [33]. For ML analyses, the best nucleotide substitution model was General Time Reversible with Gamma distribution. Support for internal branches was assessed by 1000 ML bootstrapped pseudoreplicates. For the BI phylogenetic analysis, the best nucleotide substitution model was determined using jModelTest [34]. For ITS we used the symmetrical model with gamma distribution (SYM + G), for LSU and *tub*2 the symmetrical model with proportion of invariable sites and gamma distribution (SYM + I + G), and for *rpb*2 the symmetrical model with gamma distribution (SYM + G). The parameter settings used were two simultaneous runs of 5 M generations and four Markov chain Monte Carlo (MCMC), sampled every 1000 generations. The 50% majority-rule consensus tree and posterior probability values (PP) were calculated after discarding the first 25% of the samples. *Pleospora herbarum* CBS 191.86 and *P. typhicola* CBS 132.69 served as outgroup taxa. Confident branch support is defined as Bayesian posterior probabilities (PP) >0.95 and maximum likelihood bootstrap support (BS) >70%. Sequences generated in this study were deposited in European Nucleotide Archive (ENA), the final matrix used for phylogenetic analyses in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S28077 (accessed on 16 March 2021)) and the novel taxonomic descriptions and nomenclature in MycoBank (www.mycobank.org (accessed on 16 March 2021)).

3. Results

3.1. Blast Search

Blast search results are shown in Table S2 (Supplementary Material).

3.2. Phylogenetic Relationships among Freshwater Fungi

The final concatenated dataset obtained with both ML and BI analyses contained 71 in-groups of strains with a total of 2252 characters including gaps (455 for ITS, 791 for LSU, 272 for *tub*2, and 734 for *rpb*2), of which 704 are parsimony informative (170 for ITS, 69 for LSU, 143 for *tub*2, and 322 for *rpb*2). The sequence datasets did not show conflict in the tree topologies for the 70% reciprocal bootstrap trees, which allowed us 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 BI. For the BI multi-locus analysis, a total of 11,663 trees were sampled after removal of the burn-in and reaching a stop value of 0.01. The support values were slightly different with the two analysis methods. In the phylogenetic tree (Figure 1), our strains were spread into three well-supported main clades, representing the families *Cucurbitariaceae* (99% BS/1 PP), *Neopyrenochaetaceae* (98% BS/1 PP), and *Pyrenochaetopsisaceae* (100% BS/1 PP). The *Cucurbitariaceae* clade was divided into four well-supported clades corresponding to the accepted genera (*Neocucurbitaria*, 100% BS/1 PP;

Paracucurbitaria, 100% BS/1 PP; *Cucurbitaria*, 100% BS/1 PP and *Allocucurbitaria*, 95% BS/1 PP). The *Neocucurbitaria* clade was represented by all previously described species and three of our strains, all placed in independent terminal branches. The clade corresponding to the genus *Allocucurbitaria* included the type species *A. botulispora* and the new combination *A. prunicola* (basionym *Neocucurbitaria prunicola*). The *Neopyrenochaetaceae* clade included 11 species of the genus *Neopyrenochaeta*. Five of our strains resulted as co-specific with *N. annellidica* and *N. maesuayensis*, whereas the other three strains were each placed into independent terminal branches. The family *Pyrenochaetopsisaceae* was divided in two clades, corresponding to the genera *Neopyrenochaetopsis* and *Pyrenochaetopsis* (100% BS/1PP). *Pyrenochaetopsis* encompassed 16 species and our strain FMR 17327, which is located in an independent branch. Single gene-based phylogenies are shown as Supplemental Material (Figures S1–S4) because they resulted in being less informative and resolutive than those based on the four-loci concatenated tree.

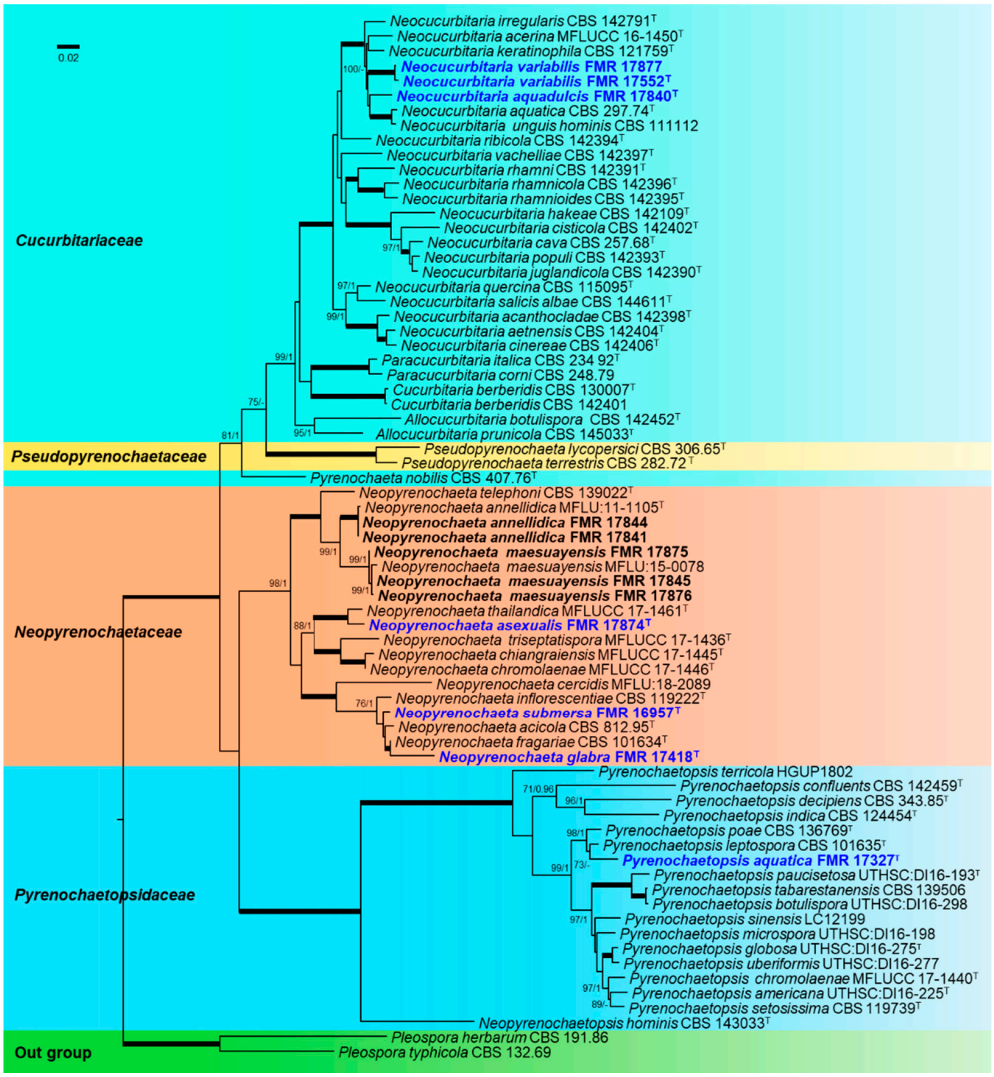

Figure 1. ML phylogenetic tree of *Cucurbitariaceae*, *Pseudopyrenochaetaceae*, *Neopyrenochaetaceae*, and *Pyrenochaetopsidaceae* inferred from the combined sequences of ITS, LSU, *tub*2, and *rpb*2 loci. Support in nodes is indicated above branches and is represented by posterior probabilities (BI analysis) of 0.95 and higher, and/or bootstrap values (ML analysis) of 70% and higher. Full-supported branches (100% BS/1 PP) are indicated by **thicker lines**. ^{T} =ex-type strains. New species are indicated in **blue**. New strains isolated during this study are indicated in **bold**. Alignment length 2252 bp. The sequences not generated by us were retrieved from EMBL/GenBank and are indicated in Supplementary Table S1.

3.3. Taxonomy

Dothideomycetes.

Cucurbitariaceae G. Winter (as *Cucurbitarieae*), Rabenh. Krypt.-Fl., Edn 2 (Leipzig) 1.2: 308 (1885). MycoBank MB 80667.

Type genus: *Cucurbitaria* Gray, Nat. Arr. Brit. Pl. (London) 1: 519 (1821).

Neocucurbitaria Wanas., E.B.G. Jones & K.D. Hyde, in Wanasinghe, Phookamsak, Jeewon, Li, Hyde, Jones, Camporesi & Promputtha, Mycosphere 8(3): 408 (2017). MycoBank MB 552832.

Type species: *Neocucurbitaria unguis-hominis* (Punith. & M.P. English) Wanas., E.B.G. Jones & K.D. Hyde, in Wanasinghe, Phookamsak, Jeewon, Li, Hyde, Jones, Camporesi & Promputtha, Mycosphere 8(3): 412 (2017). MB 552835.

= *Pyrenochaeta unguis-hominis* Punith. & M.P. English, Transactions of the British Mycological Society 64 (3): 539 (1975). MB 322137.

Neocucurbitaria variabilis V. Magaña-Dueñas, Stchigel & Cano, ***sp. nov.*** Figure 2. MycoBank MB 838833.

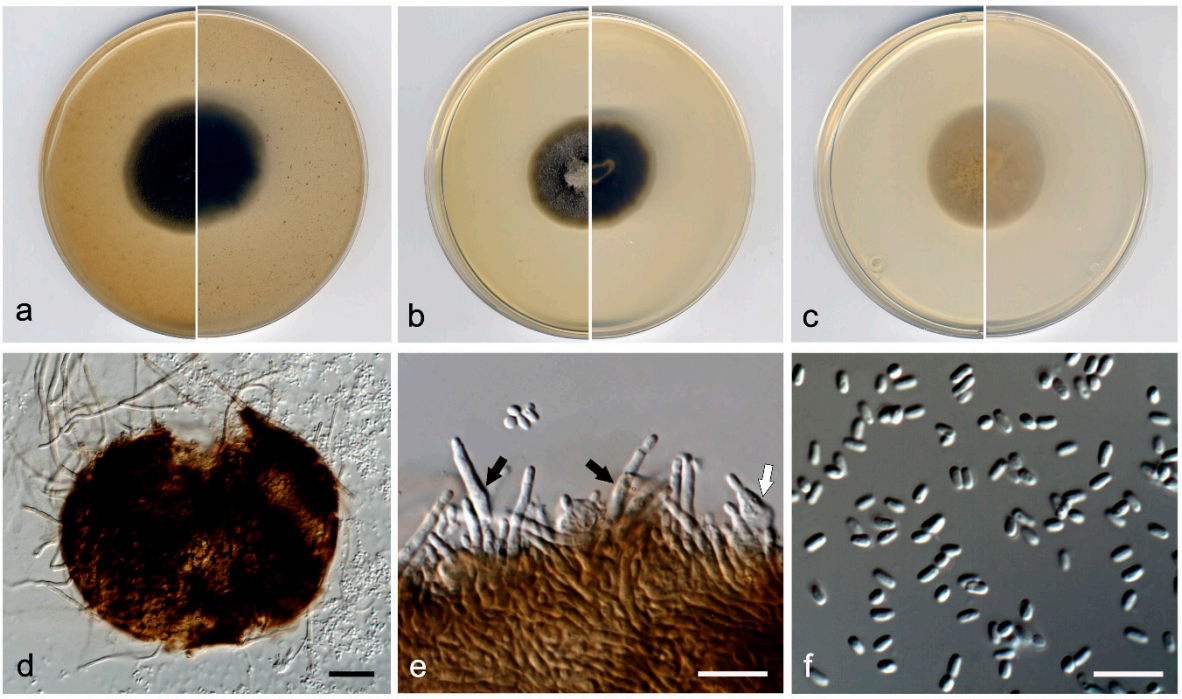

Figure 2. *Neocucurbitaria variabilis* FMR 17552 ^{T}. (**a**) Colonies on OA, (**b**) MEA, and (**c**) PDA, after 2 weeks at 25 ± 1 °C (surface, left; reverse, right); (**d**) pycnidium; (**e**) conidiogenous cells (black arrow, elongated cylindrical; white arrow, flask-shaped); (**f**) conidia. Scale bars: d = 50 µm, e,f = 10 µm.

Etymology: From Latin *variabilis*, due to the variable shape of the conidiogenous cells.

Type: Spain, Segovia province, Riaza, from plant debris in freshwater, May 2018, Viridiana Magaña Dueñas, holotype CBS H-24739, culture ex-type FMR 17552.

Description: Hyphae hyaline to pale brown; septate; branched; smooth- and thin-walled; 2–5 µm wide; with short, finger-like lateral projections; anastomosing. Conidiomata pycnidial, brown to dark brown, immersed to semi-immersed, solitary, scattered, setose, ostiolate, subglobose to globose, 110−120 µm × 120–140 µm, ostiole 40−50 µm diameter. Setae pale brown, erect, septate, smooth- and thick-walled, rounded at the tip, 40−80 µm × 3−4 µm. Conidiomata wall composed of three to five layers of cells, 15–25 µm thick, covered by a mass of interwoven, pale brown to brown hyphae; outer layer of *textura angularis*, composed of brown to dark brown, flattened polygonal cells of 3.5–4.5 µm diameter. Conidiophores absent. Conidiogenous cells phialidic, determi-

nate, hyaline and smooth-walled, flask-shaped, 5–6 µm × 2–3 µm, or elongate-cylindrical, straight, sinuous or slightly curved, 10−14 µm × 1.5−3 µm. Conidia one-celled, hyaline, smooth- and thin-walled, ellipsoidal, ovoid or kidney-shaped, 2.5–3.5 µm × 1.0–1.5 µm. Chlamydospores absent.

Culture characteristics: Colonies on PDA reaching 22 mm diameter after 7 days at 25 ± 1 °C, flattened, velvety, margin regular, surface and reverse yellowish grey (4B2). Colonies on OA reaching 20 mm diameter after 7 days at 25 ± 1 °C, flattened, floccose, margin regular, grey to brownish grey (4F1/4D2); reverse grey to yellowish grey (4F1/4B2). Colonies on MEA reaching 16 mm diameter after 7 days at 25 ± 1 °C, umbonate, velvety, margin regular, yellowish grey to olive brown (4B2/4D3); reverse brownish grey to yellowish grey (7F2/4B2). Exopigment absent. Cardinal temperatures of growth: minimum 5 °C, optimum 25 °C, and maximum 30 °C.

Other material examined: Spain, Sevilla province, Parque Natural Sierra Norte (37.994712, −5.668709), from plant debris in freshwater, May 2019, José F. Cano Lira, living cultures FMR 17877.

Diagnosis: In our phylogenetic tree, N. variabilis was placed in a terminal branch within the same subclade as *N. acerina*, *N. aquadulcis*, *N. aquatica*, *N. irregularis*, *N. keratinophila*, and *N. unguis-hominis*. *Neocucurbitaria variabilis* differs morphologically from all these species in having two kinds of enteroblastic conidiogenous cells, ampulliform and elongate-cylindrical, which are discrete; and doliiform in *N. acerina*, *N. aquatica* and *N. irregularis*, and integrated in acropleurogenous conidiophores (i.e., having terminal and lateral openings) in *N. keratinophila* and *N. unguis-hominis* [35].

Notes: Differences in nucleotide sequences (ITS-LSU-*tub*2-*rpb*2 concatenated dataset; 2252 bp) between *N. variabilis* and the species in the same terminal clade are: *N. aquadulcis*, 71 bp; *N. keratinophila*, 77 bp; *N. irregularis*, 78 bp; *N. acerina*, 80 bp; *N. aquatica*, 82 bp; and *N. unguis-hominis*, 83 bp.

Neocucurbitaria aquadulcis V. Magaña-Dueñas, Cano & Stchigel, *sp. nov*. Figure 3. MycoBank MB 838834.

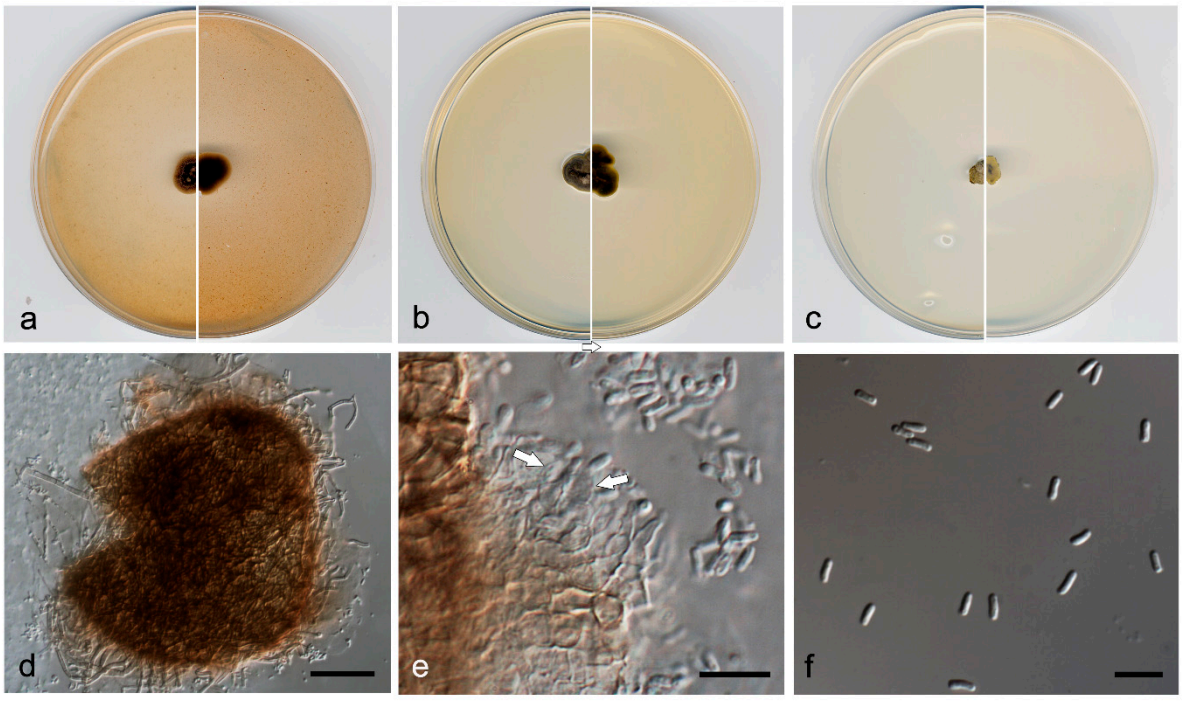

Figure 3. *Neocucurbitaria aquadulcis* FMR 17840 ^{T}. (**a**) Colonies on OA, (**b**) MEA, and (**c**) PDA, after 2 weeks at 25 ± 1 °C (surface, left; reverse, right); (**d**) pycnidium; (**e**) conidiogenous cells (white arrows); (**f**) conidia. Scale bars: d = 50 µm, e,f = 10 µm.

Etymology: From Latin *aqua-*, water; and *-dulcis*, sweet, because of the origin of the fungus.

Type: Spain, Sevilla province, Parque Natural Sierra Norte (37.931670, −5.704493), from plant debris in freshwater, May 2019, José F. Cano Lira, holotype CBS H-24740, culture ex-type FMR 17840 = CBS 147605.

Description: Hyphae hyaline to light brown, septate, branched, smooth- and thin-walled, 2–3 µm wide, anastomosing. Conidiomata pycnidial, brown to dark brown, immersed to semi-immersed, solitary or confluent, scattered, ostiolate, covered by a mass of interwoven, pale brown to brown hyphae, ovoid to globose, 120–150 µm × 130–170 µm, ostiole of 15–20 µm diameter. Conidiomata wall composed of three to six layers of cells, 15–35 µm thick, outer layer of *textura angularis*, composed of brown to dark brown, flattened polygonal cells of 3–5.5 µm diameter. Conidiophores absent. Conidiogenous cells phialidic, determinate, hyaline, smooth-walled, ampulliform, 4–6 µm × 2–3 µm. Conidia one-celled, hyaline, smooth- and thin-walled, bacillary, slightly curved, 2.5–4.5 µm × 1.0–2.0 µm. Chlamydospores absent.

Culture characteristics: Colonies on PDA reaching 6–9 mm diameter after 7 days at 25 ± 1 °C, convex, granular, margins irregular, surface, and reverse olive brown (4D5). Colonies on OA reaching 10–11 mm diameter after 7 days at 25 ± 1 °C, flattened, velvety, margin regular, surface and reverse yellowish brown (5F5) to brownish grey (5B2). Colonies on MEA reaching 10–15 mm diameter after 7 days at 25 ± 1 °C, umbonate, velvety, margins regular, brownish grey to orange grey (5E2/5B2) reverse yellowish brown to orange grey (5E4/5B2). Exopigment not produced. Cardinal temperatures of growth: minimum 5 °C, optimum 25 °C, and maximum 30 °C.

Diagnosis: *Neocucurbitaria aquadulcis*, unlike *N. variabilis*, produces solely ampulliform phialides (see before) and bigger conidia (2.5–4.5 µm × 1.1–1.9 µm vs. 2.5–3.5 µm × 1.2–1.7 µm). Also, the conidiomata wall of *N. aquadulcis* is covered by hyphae, whereas it is setose in *N. variabilis*.

Notes: Differences in nucleotide sequences (ITS-LSU-*tub*2-*rpb*2 concatenated dataset; 2252 bp) between *N. aquadulcis* and the species in the same terminal clade are: *N. keratinophila*, 54 bp; *N. irregularis*, 57 bp; *N. acerina*, 61 bp; *N. aquatica* and *N. unguis-hominis*, 62 bp; and *N. variabilis*, 71 bp.

Allocucurbitaria Valenz.-Lopez, Stchigel, Guarro & Cano, in Valenzuela-Lopez, Cano-Lira, Guarro, Sutton, Wiederhold, Crous & Stchigel, Stud. Mycol. 90:51 (2017). MycoBank MB 821455.

Type species: *Allocucurbitaria botulispora* Valenz.-Lopez, Stchigel, Guarro & Cano, in Valenzuela-Lopez, Cano-Lira, Guarro, Sutton, Wiederhold, Crous & Stchigel, Stud. Mycol. 90:51 (2017). MycoBank MB 819770.

Allocucurbitaria prunicola (Crous & Akulov) V. Magaña-Dueñas, Stchigel & Cano, *comb. nov.* MycoBank MB 838843.

Basionym: *Neocucurbitaria prunicola* Crous & Akulov, in Crous, Schumacher, Akulov, Thangavel, Hernández-Restrepo, Carnegie, Cheewangkoon, R; Wingfield, Summerell, Quaedvlieg, Coutinho, Roux, Wood, Giraldo & Groenewald, Fungal Systematics and Evolution 3:91 (2019).

Description: Crous & Akulov 2019.

Notes: In 2019, Crous & Akulov introduced *N. prunicola* to the genus *Neocucurbitaria*, based on morphological and nucleotide sequence data analysis [36]. However, in our phylogenetic study, *N. prunicola* is clearly placed in the genus *Allocucurbitaria*. Therefore, we propose a new combination for that species.

Neopyrenochaetaceae Valenz.-Lopez, Crous, Stchigel, Guarro & Cano, in Valenzuela-Lopez, Cano-Lira, Guarro, Sutton, Wiederhold, Crous & Stchigel, Stud. Mycol. 90:54 (2017). MycoBank MB 820416.

Type genus: *Neopyrenochaeta* Valenz.-Lopez, Crous, Stchigel, Guarro & Cano, in Valenzuela-Lopez, Cano-Lira, Guarro, Sutton, Wiederhold, Crous & Stchigel, Stud. Mycol. 90:54 (2017). MycoBank MB 820313.

Type species: *Neopyrenochaeta acicola* (Moug. & Lév.) Valenz.-Lopez, Crous, Stchigel, Guarro & Cano, in Valenzuela-Lopez, Cano-Lira, Guarro, Sutton, Wiederhold, Crous & Stchigel, Stud. Mycol. 90:54 (2017). MycoBank MB 820314.

Neopyrenochaeta asexualis V. Magaña-Dueñas, Stchigel & Cano, *sp. nov.* Figure 4. MycoBank MB 838835.

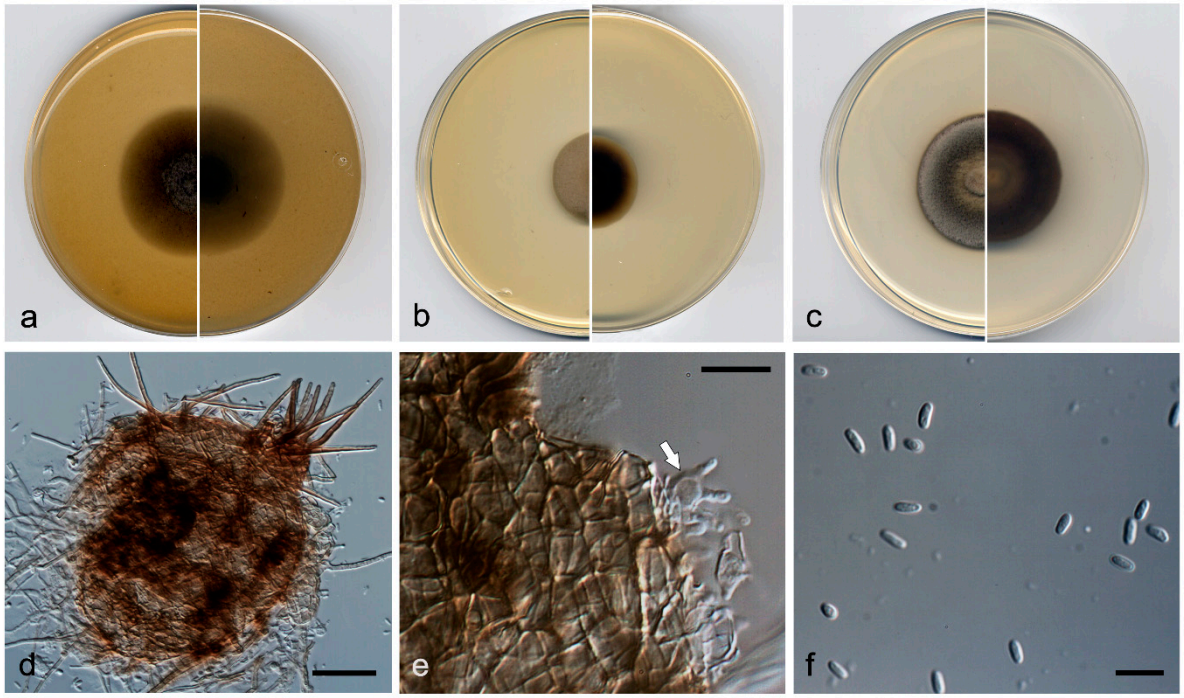

Figure 4. *Neopyrenochaeta asexualis* FMR 17874 ^{T}. (**a**) Colonies on OA, (**b**) MEA, and (**c**) PDA, after 2 weeks at 25 ± 1 °C (surface, left; reverse, right); (**d**) pycnidium; (**e**) conidiogenous cells (white arrow); (**f**) conidia. Scale bars: d = 50 µm, e,f = 10 µm.

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

Type: Spain, Sevilla province, Parque Natural Sierra Norte (37.994712, −5.668709), from plant debris in freshwater, May 2019, José F. Cano Lira, holotype CBS H-24741, culture ex-type FMR 17874 = CBS 147606.

Description: Hyphae hyaline to light brown, septate, branched, smooth-and thin-walled, 2–3 µm wide. Conidiomata pycnidial, brown to dark brown, immersed to semi-immersed, solitary or confluent, setose, ostiolate, globose to subglobose, 100–170 µm × 85–150 µm, ostiole 30–40 µm diameter. Setae pale brown to brown, septate, erect, nodose, narrowing towards the tip, thick-walled 40–80 µm × 2–4 µm, mainly disposed around the ostiole but also scattered, sometimes curved or recurved at the tip. Conidiomata wall composed of two to four layers of cells, 10–25 µm thick, outer layer of *textura angularis*, composed of brown to dark brown, flattened polygonal cells of 5–7 µm diameter. Conidiophores absent. Conidiogenous cells phialidic; determinate; hyaline; smooth-walled; doliiform; 5–6 µm × 4–5 µm; with mostly one, less frequently two conidiogenous *loci*. Conidia one-celled, hyaline, smooth- and thin-walled, ellipsoidal, 4–5 µm × 1.5–2.5 µm, sometimes slightly curved. Chlamydospores absent.

Culture characteristics: Colonies on PDA reaching 36 mm diameter after 7 days at 25 ± 1 °C, umbonate, velvety, margins regular, brownish grey (4D2), with patches of white; reverse olive brown (4D3). Colonies on OA reaching 40 mm diameter after 7 days at 25 ± 1 °C, flattened to slightly floccose, margin regular, with sparse aerial mycelium, grey (30C1); reverse greenish grey to yellowish grey (30E1/30F2). Colonies on MEA reaching 25 mm diameter after 7 days at 25 ± 1 °C, convex, velvety, margin regular, golden

grey (4C2); reverse brownish grey to beige (4F2/4C3). Exopigment absent. Cardinal temperatures of growth: minimum 5 °C, optimum 25 °C, and maximum 30 °C.

Diagnosis: *Neopyrenochaeta asexualis* is grouped in the same terminal clade as *N. thailandica*, but as a distinct taxon. Morphological comparison between *N. asexualis* and *N. thailandica* is not possible because only the former produces the asexual morph and only the latter one forms ascomata [37]. However, it is noteworthy that *N. asexualis* produces conidiomata with doliiform phialides with up two conidiogenous *loci*.

Notes: The difference in nucleotide sequences (ITS-LSU-*tub*2-*rpb*2 concatenated dataset) between *N. asexualis* and *N. thailandica* is 38 bp.

Neopyrenochaeta submersa V. Magaña-Dueñas, Cano & Stchigel, *sp. nov.* Figure 5. MycoBank MB 838840.

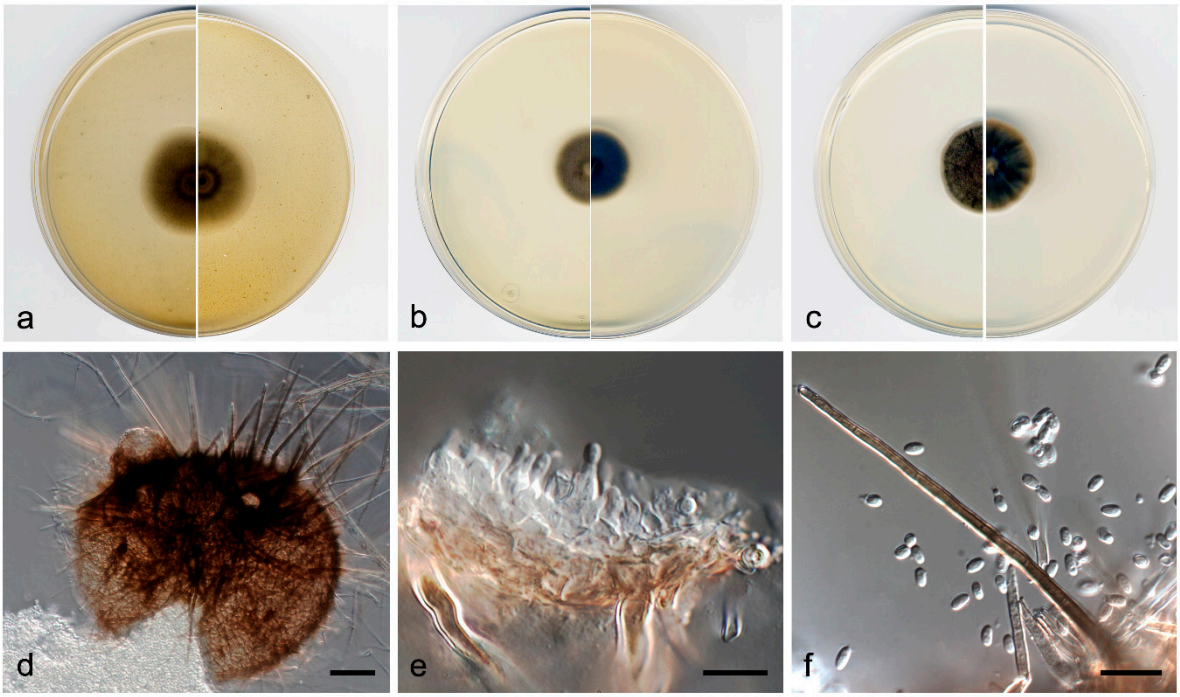

Figure 5. *Neopyrenochaeta submersa* FMR 16957 ^{T}. (**a**) Colonies on OA, (**b**) MEA, and (**c**) PDA, after 2 weeks at 25 ± 1 °C (surface, left; reverse, right); (**d**) pycnidium; (**e**) conidiogenous cells; (**f**) conidia and setae. Scale bars: d = 50 µm, e,f = 10 µm.

Etymology: From Latin *submersum*, submerged, because the fungus was recovered from plant debris in freshwater.

Type: Spain, Barcelona province, Les Guilleries (41.9362028, 2.4122862), from plant debris in freshwater, Nov 2017, Eduardo Jose de Carvalho Reis, holotype CBS H-24742, culture ex-type FMR 16957 = CBS 147607.

Description: Hyphae pale to dark brown, septate, branched, smooth- and thin-walled, 2–3 µm wide. Conidiomata pycnidial, brown to dark brown, semi-immersed, solitary or confluent, scattered, ostiolate, setose, globose to subglobose, 140–200 µm × 180–240 µm, one to three ostioles per conidioma, 60–85 µm diameter. Setae brown to dark brown, septate, erect, rounded at the tip, thick-walled, 75−160 µm × 2−3 µm, narrowing towards the tip, and mostly disposed around the ostiole. Conidiomata wall composed of three to five layers of cells, 10–20 µm thick, with an outer layer of *textura angularis*, composed of brown to dark brown, flattened polygonal cells of 3–4 µm diameter. Conidiophores absent. Conidiogenous cells phialidic, determinate, hyaline, smooth-walled, ampulliform, 6–8 µm × 2.5–3.5 µm. Conidia one-celled, hyaline, smooth- and thin-walled, ellipsoidal, 3–4 µm × 2–3 µm. Chlamydospores absent.

Culture characteristics: Colonies on PDA reaching 23 mm diameter after 7 days at 25 ± 1 °C, umbonate, velvety, rugose, margin regular, surface greyish green to greenish

grey (30E4/30C2), reverse greyish green to dull green (30B4/30E3), margin greenish grey (30C2). Colonies on OA reaching 28 mm diameter after 7 days at 25 ± 1 °C, convex, velvety, margin regular, surface and reverse grey (30C1). Colonies on MEA reaching 20 mm diameter after 7 days at 25 ± 1 °C, umbonate, velvety, margins regular, greyish green to dull green (30C3/30E3), margin white; reverse dark green (30F3), margins white. Exopigment absent. Cardinal temperatures of growth: minimum 5 °C, optimum 25 °C, and maximum 30 °C.

Diagnosis: In our phylogenetic analysis, *N. submersa* is located in the same terminal clade as *N. acicola*, *N. fragariae*, *N. inflorescentiae*, and *N. glabra*. With the exception of *N. glabra*, all these species are morphologically very similar. However, *N. submersa* grows faster than *N. fragariae* (reaching 14 mm and 11 mm diameter after 7 days at 25 °C on OA and MEA, respectively). Also, *N. submersa* does not produce exopigment on OA, which is lilac-rose in *N. acicola* [38] and orange in *N. fragariae*.

Notes: Differences in nucleotide sequences (ITS-LSU-*tub*2-*rpb*2 concatenated dataset) between *N. submersa* and the other species in the same terminal clade are: *N. fragariae* 28 bp; *N. acicula*, 33 pb; *N. inflorescentiae*, 51 pb; and *N. glabra*, 98 bp.

Neopyrenochaeta glabra V. Magaña-Dueñas, Stchigel & Cano, *sp. nov.* Figure 6. MycoBank MB 838841.

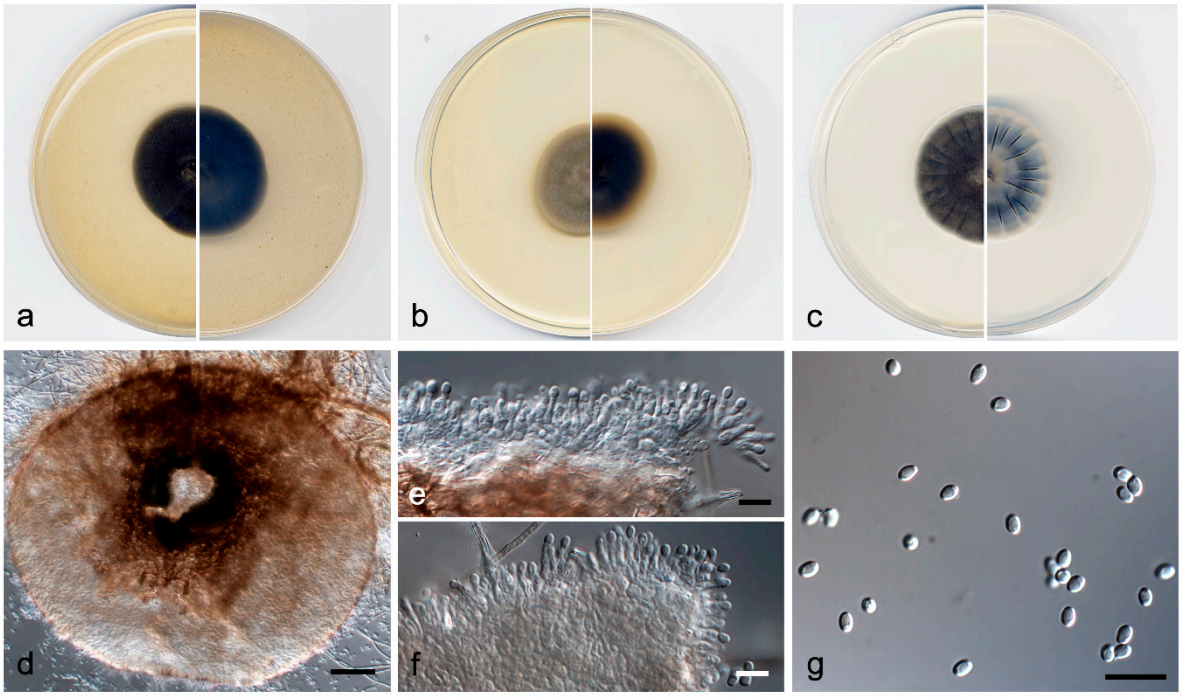

Figure 6. *Neopyrenochaeta glabra* FMR 17418 ^{T}. (**a**) Colonies on OA, (**b**) MEA, and (**c**) PDA, after two weeks at 25 ± 1 °C (surface, left; reverse, right); (**d**) pycnidium; (**e**,**f**) conidiogenous cells; (**g**) conidia. Scale bars: d = 50 µm, e–g = 10 µm.

Etymology: From Latin *glaber*, hairless, relating to absence of setae.

Type: Spain, Segovia province, Riaza (41.238863, -3.435258), from freshwater submerged plant debris, May 2018, Viridiana Magaña Dueñas, holotype CBS H-24743, culture ex-type FMR 17418 = CBS 147608.

Description: Hyphae hyaline to pale brown, septate, branched, smooth- and thin-walled, 1.5–2.5 µm wide. Conidiomata pycnidial immersed to semi-immersed, solitary or confluent, scattered, ostiolate, glabrous, translucent, pale brown to brown, but carbonaceous around the ostiole, mostly subglobose, 140–200 µm × 180–240 µm, ostiole 60–85 µm diam. Conidiomata wall composed of four to six layers of cells, 10–25 µm thick, with an outer layer of *textura angularis*, composed of pale brown to brown, flattened polygonal cells of 3–4 µm diameter. Conidiophores absent. Conidiogenous cells phialidic, determi-

nate, hyaline, smooth-walled, ampulliform, 7–9 µm × 3–4 µm. Conidia aseptate, hyaline, smooth- and thin-walled, ellipsoidal, 3–4 µm × 2–3 µm. Chlamydospores absent.

Culture characteristics: Colonies on PDA reaching 23 mm diameter after 7 days at 25 ± 1 °C, umbonate, velvety, rugose, margin regular, surface, and reverse grey to dark brown (8F1/8D1). Colonies on OA reaching 25 mm diameter after 7 days at 25 ± 1 °C, flattened, velvety, margin regular, surface and reverse grey (6F1). Colonies on MEA reaching 23 mm diam after 7 days at 25 ± 1 °C, convex, velvety, margins regular, grey to olive brown (4F1/4D3), margin yellowish grey (4B2); reverse brownish grey (5E2) margins orange grey (5B2). Exopigment absent. Cardinal temperatures of growth: optimum 25 °C, maximum 30 °C, minimum 5 °C.

Diagnosis: Morphologically, *N. glabra* differs from the phylogenetically closest species, *N. acicula*, *N. fragariae, N. inflorescentiae,* and *N. submersa* by lacking conidiomatous setae in the conidiomata walls.

Notes: Differences in nucleotide sequences (ITS-LSU-*tub*2-*rpb*2 concatenated dataset) between *N. glabra* and the other species of the same terminal clade are: *N. fragariae*, 89 bp; *N. submersa*, 98 bp; *N. acicola*, 108 bp; and *N. inflorescentiae*, 131 bp.

Pyrenochaetopsidaceae Valenz.-Lopez, Crous, Cano, Guarro & Stchigel, in Valenzuela-Lopez, Cano-Lira, Guarro, Sutton, Wiederhold, Crous & Stchigel, Stud. Mycol. 90:56 (2017). MycoBank MB 820308.

Type genus: *Pyrenochaetopsis* Gruyter, Aveskamp & Verkley, Mycologia 102 (5):1076 (2010). MycoBank MB 514653.

Type species: *Pyrenochaetopsis lep.tospora* (Sacc. & Briard) Gruyter, Aveskamp & Verkley, Mycologia 102 (5):1076 (2010). MycoBank MB 514654.

Pyrenochaetopsis aquatica V. Magaña-Dueñas, Cano & Stchigel, *sp. nov*. Figure 7. MycoBank MB 838842.

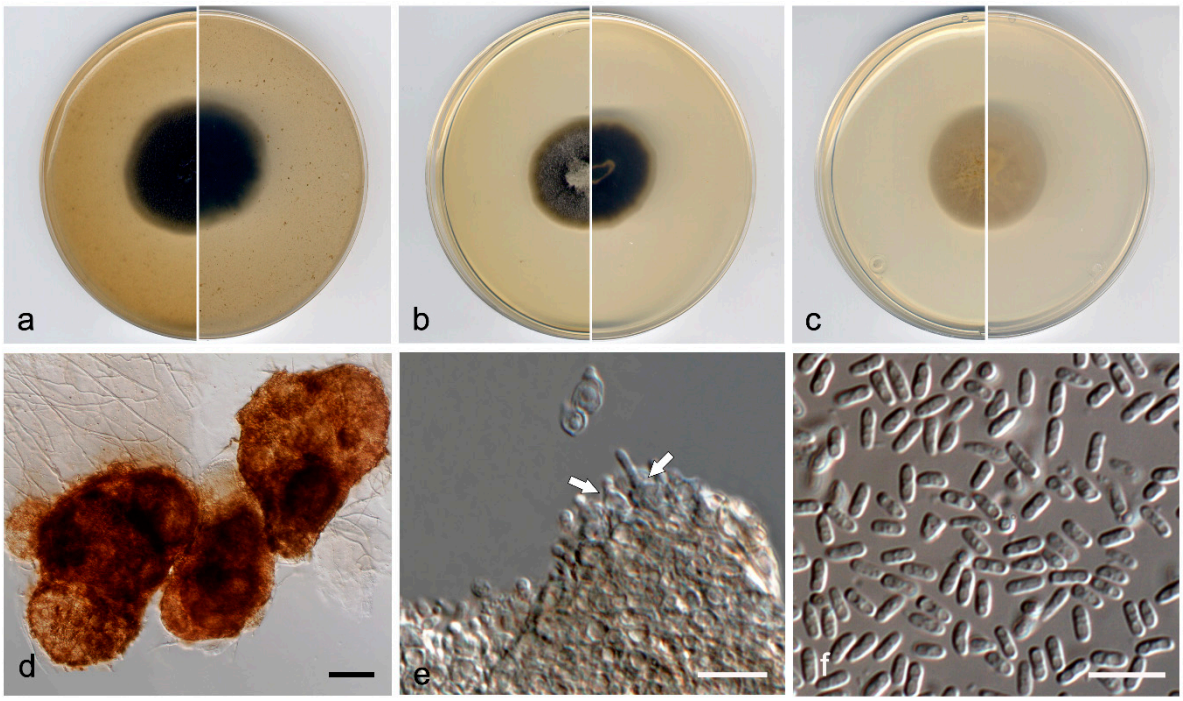

Figure 7. *Pyrenochaetopsis aquatica* FMR 17327 ^{T}. (**a**) Colonies on OA, (**b**) MEA, and (**c**) PDA, after 2 weeks at 25 ± 1 °C (surface, left; reverse, right); (**d**) pycnidium; (**e,f**) conidiogenous cells (white arrows).

Etymology: From Latin *aquaticus*, referring to the habitat from which the fungus was recovered (freshwater).

Type: Spain, Tarragona province, Serra del Montsant (41.32871, 0.87105), from plant debris in freshwater, February 2018, Eduardo Jose de Carvalho Reis, holotype CBS H-24744, culture ex-type FMR 17327 = CBS 147609.

Description: Hyphae hyaline to pale brown, septate, branched, smooth- and thin-walled, 1.5–2 µm wide. Conidiomata pycnidial, brown, immersed to semi-immersed, solitary or confluent, scattered, ostiolate, mostly glabrous or covered with few short setae, pyriform, 200–300 µm × 130–180 µm, ostiole 60–80 µm diameter. Setae pale brown to brown, septate, erect, nodose, thick-walled, of 10–20 µm × 3–4 µm, tapering towards the apex, mainly disposed around the ostiole. Conidiomata wall composed of four to six layers of cells, 15–30 µm thick, with an outer layer of *textura angularis*, composed of brown to dark brown, flattened polygonal cells of 3.5–4.5 µm diameter. Conidiophores absent. Conidiogenous cells phialidic, determinate, hyaline, smooth-walled, ampulliform, 6–7 µm × 2.5–3 µm. Conidia aseptate, hyaline, smooth- and thin-walled, mostly long ellipsoidal, 3.5–5 µm × 1–1.8 µm, slightly constricted at the middle, sometimes slightly curved and irregularly shaped, biguttulate. Chlamydospores absent.

Culture characteristics: Colonies on PDA reaching 15 mm diameter after 7 days at 25 ± 1 °C, flattened, velvety, margin regular, grey to yellowish white (A1C/4A2); reverse greyish green to yellowish white (4B3/4A2). Colonies on OA reaching 20 mm diameter after 7 days at 25 ± 1 °C, flattened, velvety, margin regular, yellowish grey to yellowish white (4B2/4A2). Colonies on MEA reaching 20 mm diameter after 7 days at 25 ± 1 °C, umbonate, velvety, margins regular, yellowish grey to yellowish brown (4B2/5F4), margin orange grey (5B2); reverse orange grey (5B2). Exopigment absent. Cardinal temperatures of growth: optimum 25 °C, maximum 30 °C, minimum 5 °C.

Diagnosis: *Pyrenochaetopsis aquatica* differs morphologically from the phylogenetically nearest species *P. leptospora* and *P. poae*, because it is mostly glabrous or covered with few short setae, while the pycnidia of *P. leptospora* and *P. poae* are abundantly covered with long setae [39].

Notes: Differences in nucleotide sequences (ITS-LSU-*tub*2-*rpb*2 concatenated dataset) between *P. aquatica* and the other species of the same terminal clade are: *P. leptospora*, 57 bp; and *P. poae*, 67 bp.

4. Discussion

The genus *Neocucurbitaria* was introduced by Wanasinghe et al. [40] to accommodate *N. acerina*, *N. quercina* and *N. unguis-hominis* (the type species of the genus). Twenty-two species are currently accepted (Index of Fungi; http://www.indexfungorum.org/names/Names.asp (accessed on 16 March 2021)). *Neocucurbitaria* spp. has been isolated from human corneal and skin lesions, seawater, and trees and shrubs [5,40,41]. We described two new species for the genus, *N. aquadulcis* and *N. variabilis*, from submerged plant debris in freshwaters, the first report for this sort of habitat. It is remarkable that *N. variabilis* produces two sorts of conidiogenous cells (flask-shaped and long cylindrical) and that *N. aquadulcis* only produces ampulliform phialides, whereas the other species in the same subclade (*N. acerina*, *N. aquatica*, *N. irregularis*, *N. keratinophila* and *N. unguis-hominis*) produce doliiform phialides or well-developed conidiophores. In 2019, Crous & Akulov introduced *N. prunicola* to that genus [36]. However, in our phylogenic analysis, *N. prunicola* was located far from the type species of *Neocucurbitaria* (*N. unguis-hominis*), being located within the genus *Allocucurbitaria*. Consequently, we propose the new combination *Allocucurbitaria prunicola*.

A molecular study by Valenzuela-López et al. [5] allowed recognition of four new families of coelomycetous fungi included previously in the family *Cucurbitariaceae*: *Neopyrenochaetaceae*, *Parapyrenochaetaceae*, *Pseudopyrenochaetaceae,* and *Pyrenochaetopsidaceae*. In the latter family, the authors recognized four species belonging to the genus *Neopyrenochaeta*: *N. acicola* (basionym: *Vermicularia acicola*; originally described on decaying leaves of *Pinus sylvestris*, Vosges, France), *N. fragariae* (originally identified as *Pyrenochaeta acicola*; isolated from *Fragaria* (×) *ananassa*, The Netherlands), *N. inflorescentiae* (basionym: *Pyrenochaeta in-*

florescentiae; from style of senescent flowerhead of *Protea neriifolia*, Western Cape Province, South Africa), and *N. thelephonii* (basionym: *Pyrenochaeta telephonii*; from surface of cell phone, Maharashtra, India) [40,42,43]. During 2019 and 2020, eight more species were described [37,44], three of them (*Neopyrenochaeta annellidica*, *Neopyrenochaeta chiangraiensis* and *Neopyrenochaeta maesuayensis*) from submerged decaying wood in Thailand. Interestingly, we also identified two of these latter three species in Spain (Figure 1). This implies that the geographical distribution of *N. annellidica* and *N. maesuayensis* is much broader than would be expected, since their original report was from tropical areas of Southeastern Asia. In the present study, we report the finding of three novel additional species from submerged plant debris in Spain: *Neopyrenochaeta glabra*, *N. asexualis,* and *N. submersa*. *Neopyrenochaeta glabra* is easily recognized by the absence of setae and the darker conidiomata wall around the ostiole. *Neopyrenochaeta asexualis* is distinguished from other species of the genus because it produces doliiform phialides with one or two conidiogenous loci. Otherwise, *N. submersa* is difficult to discriminate morphologically from *N. acicola*, *N. fragariae,* and *N. inflorescentiae* species phylogenetically related but differing molecularly.

The fungal genus *Pyrenochaetopsis* was introduced by De Gruyter et al. [45] to accommodate: *P. decipens, P. indica, P. leptospora* (type species of the genus), *P. microspore,* and *P. pratorum*. Currently 16 species are accepted (Index Fungorum 2020). The members of this genus have been found in terrestrial and marine environments, human dermatitis, sputum, and blood human samples, [5,37,46–49]. In our phylogenetic analysis, the strain FMR 17337, named here as *P. aquatica*, clustered within the *Pyrenochaetopsis* clade, is distant from other species of this genus, with the exception of *P. leptospora* and *P. poae*, which forms a sister clade. Both species differ phylogenetically and morphologically from *P. aquatica* in having more abundant and longer setae.

5. Conclusions

In the present study, we have isolated several coelomycetous fungi from submerged plant debris collected in different freshwater habitats in Spain by incubation of the samples in wet chambers. After a phenotypic characterization and a phylogenic study based on the analysis of nucleotide sequences of the ITS, LSU, *tub*2, and *rpb*2 loci, six new species have been described: *Neocucurbitaria aquadulcis* and *N. variabilis*; *Neopyrenochaeta glabra*, *N. asexualis* and *N. submersa*; and *Pyrenochaetopsis aquatica*. Also, thanks to the phylogenetic analysis, *Neocucurbitaria prunicola* was transferred to the genus *Allocucurbitaria*. In our opinion, the present study makes an important contribution to the knowledge of the coelomycetous fungi growing on decomposing plant material in aquatic habitats.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jof7050368/s1, Table S1: coelomycetous fungi sequences used in this study; Table S2: Results of blast search of the new proposed species. Figure S1. ML phylogenetic tree of Cucurbitariaceae, Neopyrenochaetaceae, Pseudopyrenochaetaceae, and Pyrenochaetopsidaceae inferred from the ITS sequences (455 bp). Support in nodes is indicated above by bootstrap values of 70% or higher. T = ex-type strains. New species are indicated in blue. New strains isolated during this study are indicated in bold; Figure S2. ML phylogenetic tree of Cucurbitariaceae, Neopyrenochaetaceae, Pseudopyrenochaetaceae, and Pyrenochaetopsidaceae inferred from the LSU sequences (791 bp). Support in nodes is indicated above branches by bootstrap values of 70% or higher. T = ex-type strains. New species are indicated in blue. Strains isolated during this study are indicated in bold; Figure S3. ML phylogenetic tree of Cucurbitariaceae, Neopyrenochaetaceae, Pseudopyrenochaetaceae, and Pyrenochaetopsidaceae inferred from rpb2 sequences (734 bp). Support in nodes is indicated above branches by bootstrap values of 70% or higher. T = ex-type strains. New species are indicated in blue. New strains isolated during this study are indicated in bold; Figure S4. ML phylogenetic tree of Cucurbitariaceae, Neopyrenochaetaceae, Pseudopyrenochaetaceae, and Pyrenochaetopsidaceae inferred from tub2 sequences (272 bp). Support in nodes is indicated above branches by bootstrap values of 70% and higher. T = ex-type strains. New species are indicated in blue. Strains isolated during this study are indicated in bold. Alignment length.

Author Contributions: Conceptualization, A.M.S. and J.F.C.-L.; methodology, V.M.-D.; software, V.M.-D. and J.F.C.-L.; validation, A.M.S. and J.F.C.-L.; formal analysis, V.M.-D., A.M.S. and J.F.C.-L.; investigation, V.M.-D.; resources, J.F.C.-L.; data curation, A.M.S. and J.F.C.-L.; writing—original draft preparation, V.M.-D.; writing—review and editing, V.M.-D., A.M.S. and J.F.C.-L.; visualization, A.M.S. and J.F.C.-L.; supervision, A.M.S. and J.F.C.-L.; project administration, J.F.C.-L.; funding acquisition, J.F.C.-L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Spanish Ministerio de Economía y Competitividad, grant CGL2017-88094-P.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Juan Ramón García Martínez (a non-professional mycologist from Granja de Torrehermosa, Badajoz, Spain) for collaborating in the Sierra Norte de Sevilla sampling and to the Sociedad Ibérica de Micología (SIM) for the organization of the sampling into Riaza. We also thank Phil Hoddy for editing and proofreading the final text.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Table S1. Fungal taxa sequences used in this study.

Taxon	**Strain**	**GenBank Accession Number**			
		LSU	**ITS**	***tub2***	***rpb2***
Allocucurbitaria botulispora	CBS 234.92 T	LN907416	LT592932	LT593001	LT593070
Allocucurbitaria prunicola	**CBS 145033 T**	**MK442534**	**NR_166273**	**MK442737**	**MK442668**
Cucurbitaria berberidis	CBS 142401	MF795756	MF795756	MF795886	MF795798
Cucurbitaria berberidis	CBS 130007 T	KC506793	LT717673	LT717676	LT854936
Neocucurbitaria acanthocladae	CBS 142398 T	MF795766	NR_156354	MF795894	MF795808
Neocucurbitaria acerina	MFLUCC 16-1450 T	NG_059784	NR_154254	---	---
Neocucurbitaria aetnensis	CBS 142404 T	MF795769	NR_156355	MF795897	MF795811
Neocucurbitaria aquatica	CBS 297.74 T	EU754177	LT623221	LT623238	LT623278
Neocucurbitaria cava	CBS 257.68 T	EU754199	JF740260	KT389844	LT717681
Neocucurbitaria cinereae	CBS 142406 T	MF795771	NR_156356	MF795899	MF795813
Neocucurbitaria cisticola	CBS 142402 T	MF795772	NR_156357	MF795900	MF795814
Neocucurbitaria aquadulcis	**FMR 17840 T**	**LR897771**	**LR897770**	**LR897794**	**LR897793**
Neocucurbitaria hakeae	CBS 142109 T	KY173526	KY173436	KY173613	KY173593
Neocucurbitaria irregularis	CBS 142791 T	LN907372	LT592916	LT592985	LT593054
Neocucurbitaria juglandicola	CBS 142390 T	MF795773	NR_156358	MF795901	MF795815
Neocucurbitaria keratinophila	CBS 121759 T	LT623215	EU885415	LT623236	LT623275

Neocucurbitaria populi	CBS 142393 T	MF795902	NR_156359	MF795902	MF795816
Neocucurbitaria prunicola	CBS:145033 T	MK442534	MK442594	---	MK442668
Neocucurbitaria quercina	CBS 115095 T	GQ387619	LT623220	LT623237	LT623277
Neocucurbitaria rhamni	CBS 142391 T	MF795775	---	---	MF795817
Neocucurbitaria rhamnicola	CBS 14239 6T	MF795780	NR_156360	MF795906	MF795822
Neocucurbitaria rhamnioides	CBS 142395 T	MF795782	NR_156361	MF795908	MF795824
Neocucurbitaria ribicola	CBS 142394 T	MF795785	NR_156362	MF795911	MF795827
Neocucurbitaria salicis albae	CBS 144611 T	MK442535	NR_16336	MK442738	MK442669
Neocucurbitaria unguis hominis	CBS 111112	GQ387623	LT623222	LT623239	LT623279
Neocucurbitaria vachelliae	CBS 142397 T	MF795787	NR_156363	MF795913	MF795829
Neocucurbitaria variabilis	**FMR 17877**	**LR897785**	**LR897784**	**LR897807**	**LR897806**
Neocucurbitaria variabilis	**FMR 17552 T**	**LR897769**	**LR897768**	**LR897792**	**LR897791**
Neopyrenochaeta acicola	CBS 812.95 T	GQ387602	LT623218	LT623232	LT623271
Neopyrenochaeta annellidica	**FMR 17841**	**LR897773**	**LR897772**	**LR897796**	**LR897795**
Neopyrenochaeta annellidica	**FMR 17844**	**LR897775**	**LR897774**	**LR897798**	**LR897797**
Neopyrenochaeta annellidica	MFLU 11-1105 T	MT183502	MT185538	---	---
Neopyrenochaeta cercidis	MFLU 18-2089	MK347932	MK347718	---	MK434908
Neopyrenochaeta chiangraiensis	MFLUCC 17-1445 T	MT214468	NR_168875	---	---
Neopyrenochaeta chromolaenae	MFLUCC 17-1446 T	MT214469	NR_168876	---	MT235824

Neopyrenochaeta submersa	**FMR 16957 T**	**LR897765**	**LR897764**	**LR897787**	**LR897786**
Neopyrenochaeta glabra	**FMR 17418 T**	**LR897767**	**LR897766**	**LR897790**	**LR897789**
Neopyrenochaeta neothailandica	**FMR 17874 T**	**LR897779**	**LR897778**	**LR897802**	**LR897801**
Neopyrenochaeta fragariae	CBS 101634 T	GQ387603	LT623217	LT623231	LT623270
Neopyrenochaeta inflorescentiae	CBS 119222 T	EU552153	EU552153	LT623233	LT623272
Neopyrenochaeta maesuayensis	**FMR 17845**	**LR897777**	**LR897776**	**LR897800**	**LR897799**
Neopyrenochaeta maesuayensis	**FMR 17875**	**LR897781**	**LR897780**	**LR897804**	**LR897803**
Neopyrenochaeta maesuayensis	**FMR17876**	**LR897783**	**LR897782**	**---**	**LR897805**
Neopyrenochaeta maesuayensis	MFLU:15-0078	MT183504	MT185540	---	---
Neopyrenochaeta telephoni	CBS 139022 T	KM516290	KM516291	LT717678	LT717685
Neopyrenochaeta thailandica	MFLUCC 17-1461 T	NG_068716	MT214376	---	MT235825
Neopyrenochaeta triseptatispora	MFLUCC 17-1436 T	MT214471	MT214377	---	MT235826
Neopyrenochaetopsis hominis	CBS 143033 T	LN907381	LT592923	LT592992	LT593061
Paracucurbitaria corni	CBS 248.79	GQ387608	LT903672	LT900365	LT903673
Paracucurbitaria italica	CBS 234.92 T	EU754176	LT623219	LT623235	LT623274
Pleospora herbarum	CBS 191.86 T	JX681120	NR_111243	---	KC584471
Pleospora typhicola	CBS 132.69	JF740325	---	KT389843	KC584505
Pseudopyrenochaeta lycopersici	CBS 306.65 T	EU754205	NR_103581	LT717674	LT717680
Pseudopyrenochaeta terrestris	CBS 282.72 T	LT623216	LT623228	LT623246	LT623287

Pyrenochaeta nobilis	CBS 407.76 T	EU754206	EU930011	KT389845	LT623276
Pyrenochaetopsis americana	UTHSC:DI16-225 T	LN907368	LT592912	LT592981	LT593050
Pyrenochaetopsis botulispora	UTHSC:DI16-298	LN907432	LT592941	LT593010	LT593080
Pyrenochaetopsis chromolaenae	MFLUCC 17-1446 T	MT214469	NR_168876	---	MT235824
Pyrenochaetopsis confluents	CBS 142459 T	LN907446	LT592950	LT593019	LT593089
Pyrenochaetopsis decipiens	CBS 343.85 T	GQ387624	LT623223	LT623240	LT623280
Pyrenochaetopsis aquatica	**FMR 17327 T**	**LR216649**	**LR216648**	**LR897788**	**LR216647**
Pyrenochaetopsis globosa	UTHSC:DI16-275 T	LN907418	LT592934	LT593003	LT593072
Pyrenochaetopsis indica	CBS 124454 T	GQ387626	LT623224	LT623241	LT623281
Pyrenochaetopsis leptospora	CBS 101635 T	GQ387627	JF740262	LT623242	LT623282
Pyrenochaetopsis microspora	UTHSC:DI16-198	LN907341	LT592899	LT592968	LT593037
Pyrenochaetopsis paucisetosa	UTHSC:DI16-193 T	LN907336	LT592897	LT592966	LT593035
Pyrenochaetopsis poae	CBS 136769 T	KJ869175	KJ869117	KJ869243	LT623286
Pyrenochaetopsis setosissima	CBS 119739 T	GQ387632	LT623227	LT623245	LT623285
Pyrenochaetopsis sinensis	LC12199	MK348581	MK348586	MK348221	MK355077
Pyrenochaetopsis tabarestanensis	CBS 139506	KF803343	KF730241	KX789523	---
Pyrenochaetopsis terricola	HGUP1802	MH697393	MH697394	MH697392	MH697395
Pyrenochaetopsis uberiformis	UTHSC:DI16-277 T	LN907420	LT592935	LT593004	LT593074

[1]**CBS**: Culture collection of the Westerdijk Biodiversity Institute, Utrech, The Netherlands; **FMR**: Facultat de Medicina, Reus, Spain; **HGUP**: Corresponding author's personal collection deposited in laboratory, housed at Guizhou, China; **LC**: Corresponding author's personal collection deposited in laboratory, housed at CAS, China; **MFLU** : Mae Fah Luang University Herbarum, Chiang Rai, Thailand; **MFLUCC** : Mae Fah Luang University Culture Collection, Chiang Rai, Thailand ; UTHSC, Fungus Testing Laboratory at the University of Texas Health Science Center, San Antonio, Texas, USA. [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; *rpb2*: RNA polymerase II second subunit; *tub2*: ß-tubulin. [T]Ex-type strain.

Supplementary Table S2. Closest hits to our strains (FMR) of interest after a blast search of NCBI's GenBank nucleotide database.

Accession number	ITS / identity	LSU / identity	*tub*2 / identity	*rpb*2 / identity
FMR 17552	*Neocucurbitaria unguis-hominis* CNRMA 4.1112 / 96 %	*Neocucurbitaria vachelliae* CBS 142397 / 99 %	*Neocucurbitaria acerina* CBS 142397 / 94 %	*Neocucurbitaria acerina* CBS 142403 / 95 %
FMR 17840	*Neocucurbitaria unguis-hominis* CBS 111112 / 97 %	*Neocucurbitaria salis-albae* CBS 144611 / 99 % *Neocucurbitaria keratinophila* CBS 121759 / 99 % *Neocucurbitaria quercina* CBS 297.74 / 99 %	*Neocucurbitaria keratinophila* CNM-CM 8674 / 94 %	*Neocucurbitaria acerina* CBS 142403 / 96 % *Neocucurbitaria keratinophila* CNM-CN8674 / 96 %
FMR 16957	*Neopyrenochaeta acicola* MUT<ITA>:4382 / 100 %	*Neopyrenochaeta acicola* MUT<ITA>:4382 / 100 %	*Neopyrenochaeta acicola* CBS 101634 / 98 %	*Neocucurbitaria acicola* CBS 812.95 / 98 %
FMR 17418	*Neopyrenochaeta acicola* CBS 101634 / 100 %	*Neopyrenochaeta acicola* MUT<ITA>:4382 / 100 %	*Neopyrenochaeta acicola* CBS 101634 / 98 %	*Neopyrenochaeta acicola* CBS 101634 / 99%
FMR 17874	*Neopyrenochaeta thailandica* MFLUCC 17-1461 / 98 %	*Neopyrenochaeta thailandica* MFLUCC 17-1461 / 100 %	*Neopyrenochaeta acicola* CBS 101634 / 92 %	*Neocucurbitaria thailandica* MFLUCC 17-1461 / 96 %
FMR 17327	Pyrenochaetopsis leptospora P6589* / 100 %	*Pyrenochaetopsis leptospora* CBS 101635 / 99 %	*Pyrenochaetopsis leptospora* CBS 101635 / 95 %	*Pyrenochaetopsis leptospora* CBS 122787 / 95 %

CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; ; CNM-CM: National Centre for Microbiology, Instituto Carlos III, Madrid, Spain; CNRMA: National Reference Center for Invasive Mycoses and Antifungals; Institut Pasteur, Paris, France; FMR: Faculty of Medicine culture collection, Reus, Spain; MUT: Mycotheca Universitatis Taurinensis, Turin, Italy; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand. * Sequence deposited by J. G. Macia-Vicente at NCBI databases.

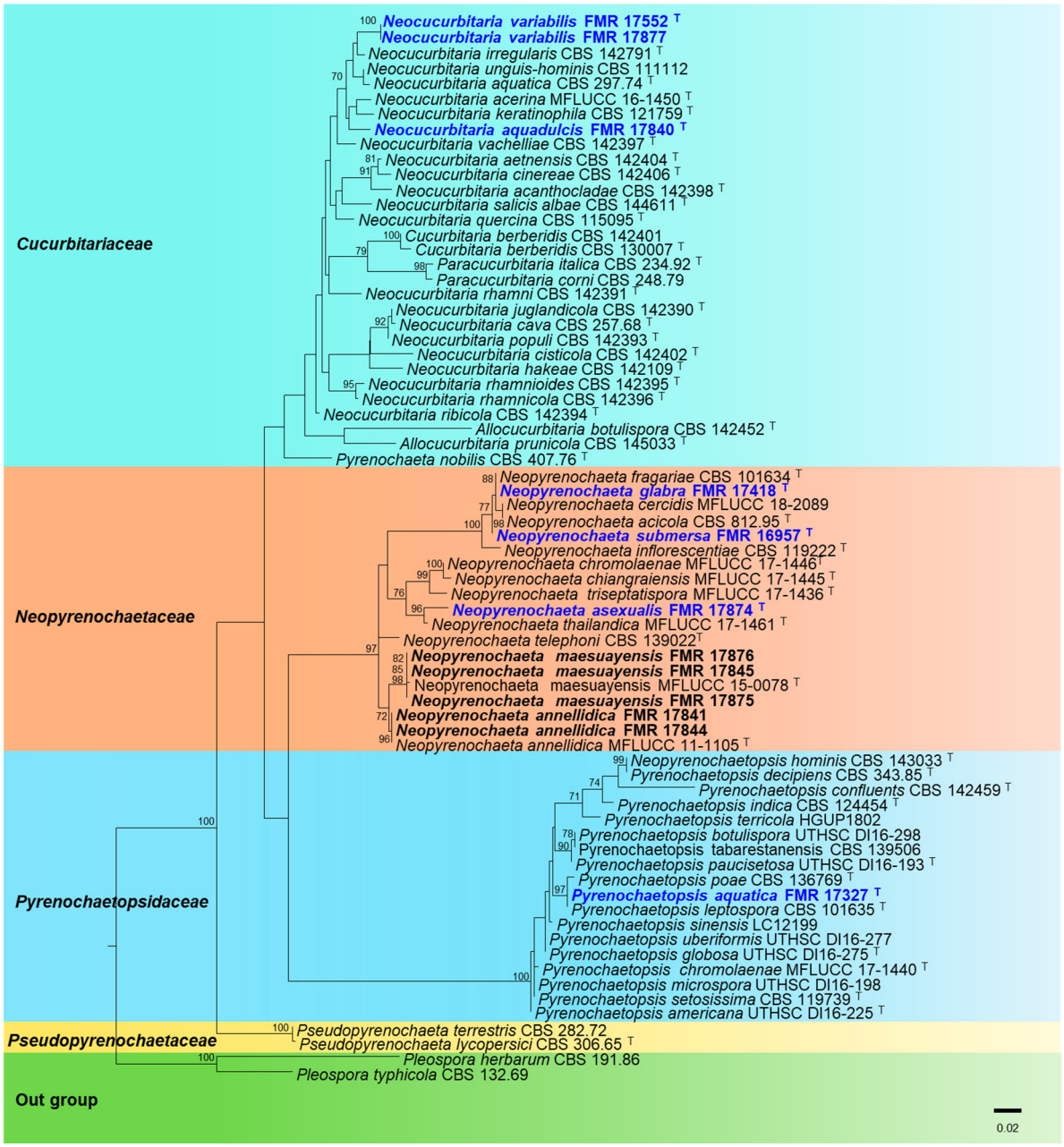

Figure S1. ML phylogenetic tree of *Cucurbitariaceae, Neopyrenochaetaceae, Pseudopyrenochaetaceae,* and *Pyrenochaetopsidaceae* inferred from the ITS sequences (455 bp). Support in nodes is indicated above by bootstrap values of 70 % or higher. T = ex-type strains. New species are indicated in **blue**. New strains isolated during this study are indicated in **bold**.

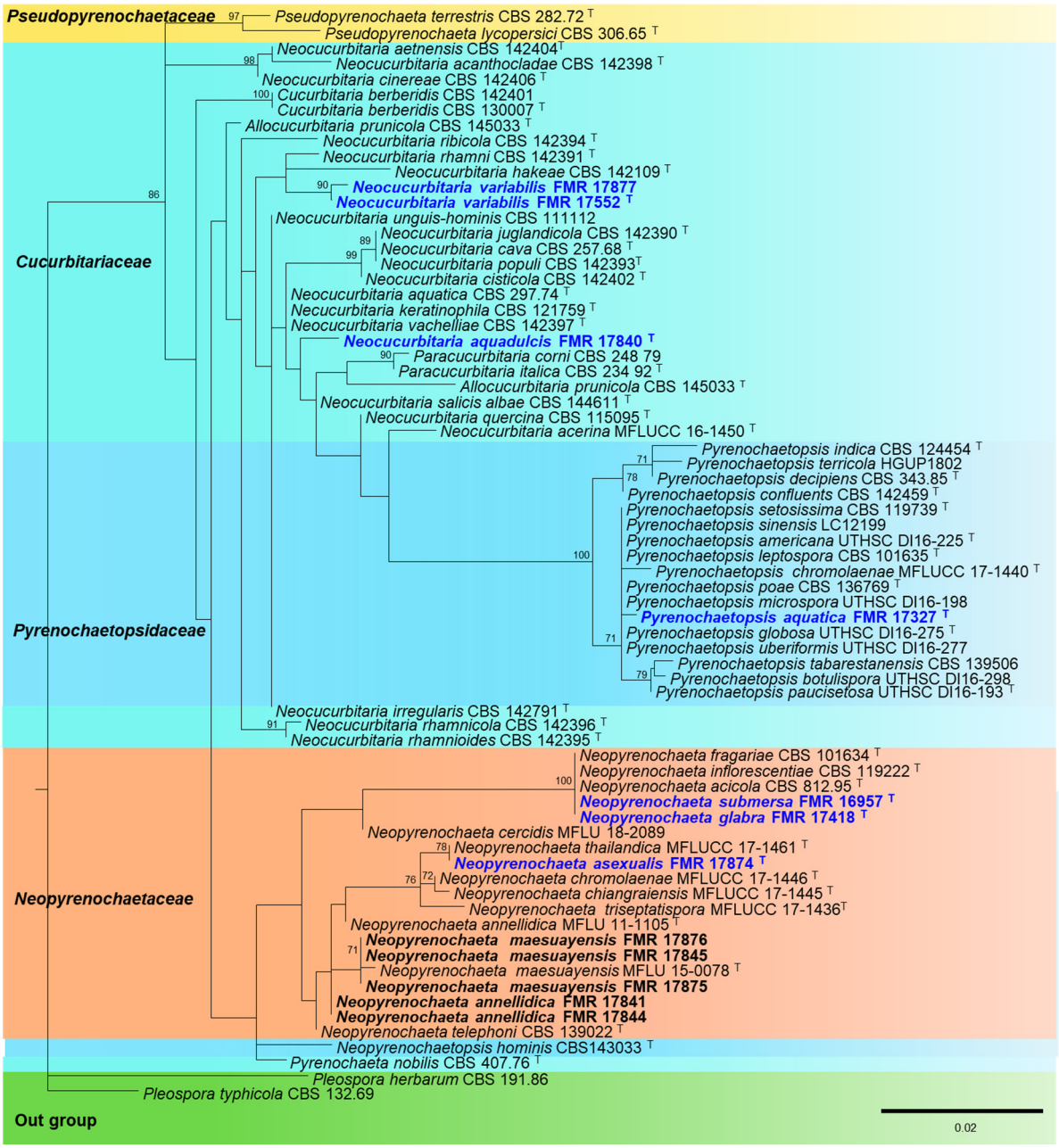

Figure S2. ML phylogenetic tree of *Cucurbitariaceae*, *Neopyrenochaetaceae*, *Pseudopyrenochaetaceae*, and *Pyrenochaetopsidaceae* inferred from the LSU sequences (791 bp). Support in nodes is indicated above branches by bootstrap values of 70 % or higher. T = ex-type strains. New species are indicated in **blue**. Strains isolated during this study are indicated in **bold**.

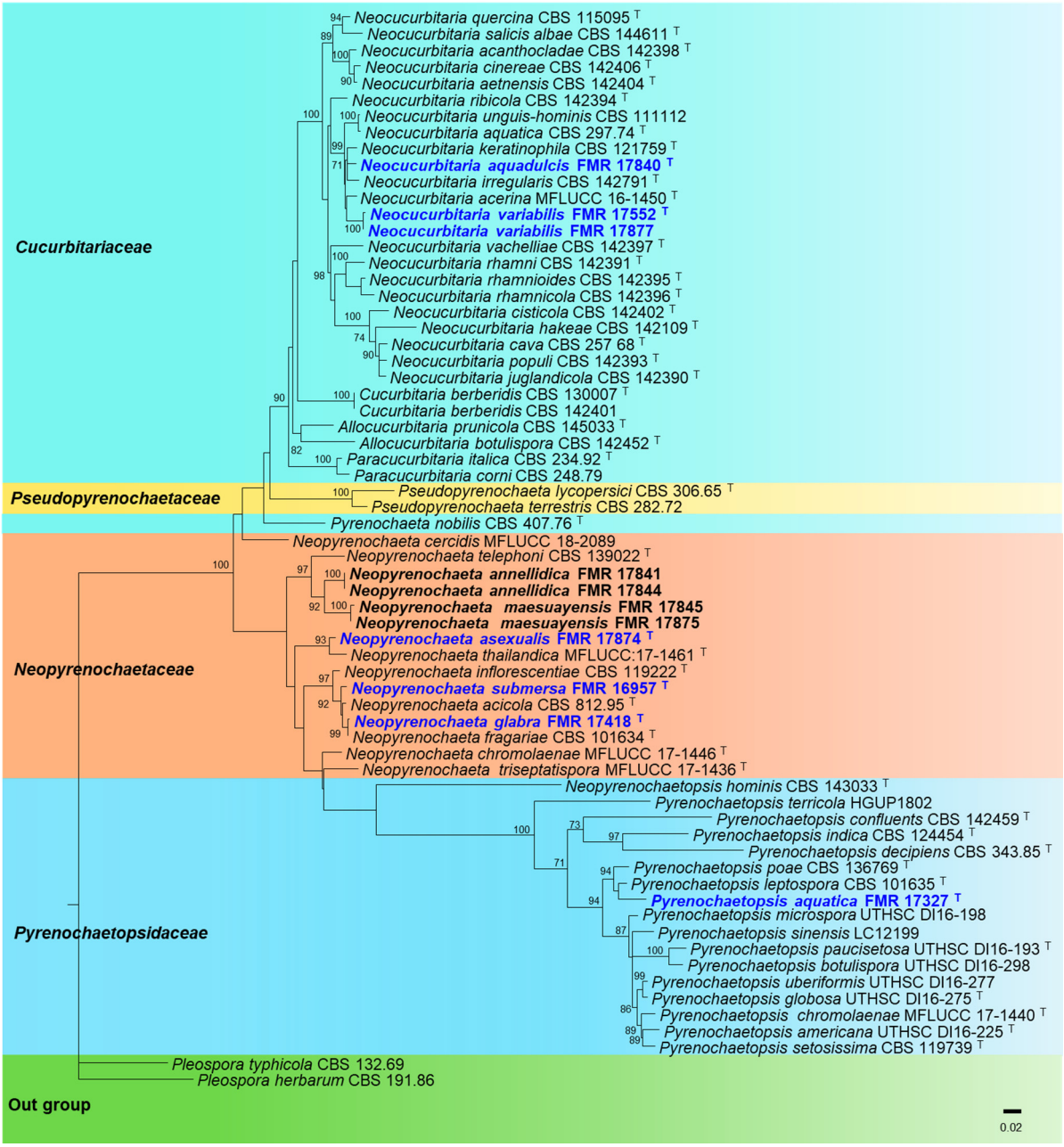

Figure S3. ML phylogenetic tree of *Cucurbitariaceae*, *Neopyrenochaetaceae*, *Pseudopyrenochaetaceae*, and *Pyrenochaetopsidaceae* inferred from *rpb*2 sequences (734 bp). Support in nodes is indicated above branches by bootstrap values of 70 % or higher. ^{T} = ex-type strains. New species are indicated in **blue**. New strains isolated during this study are indicated in **bold**.

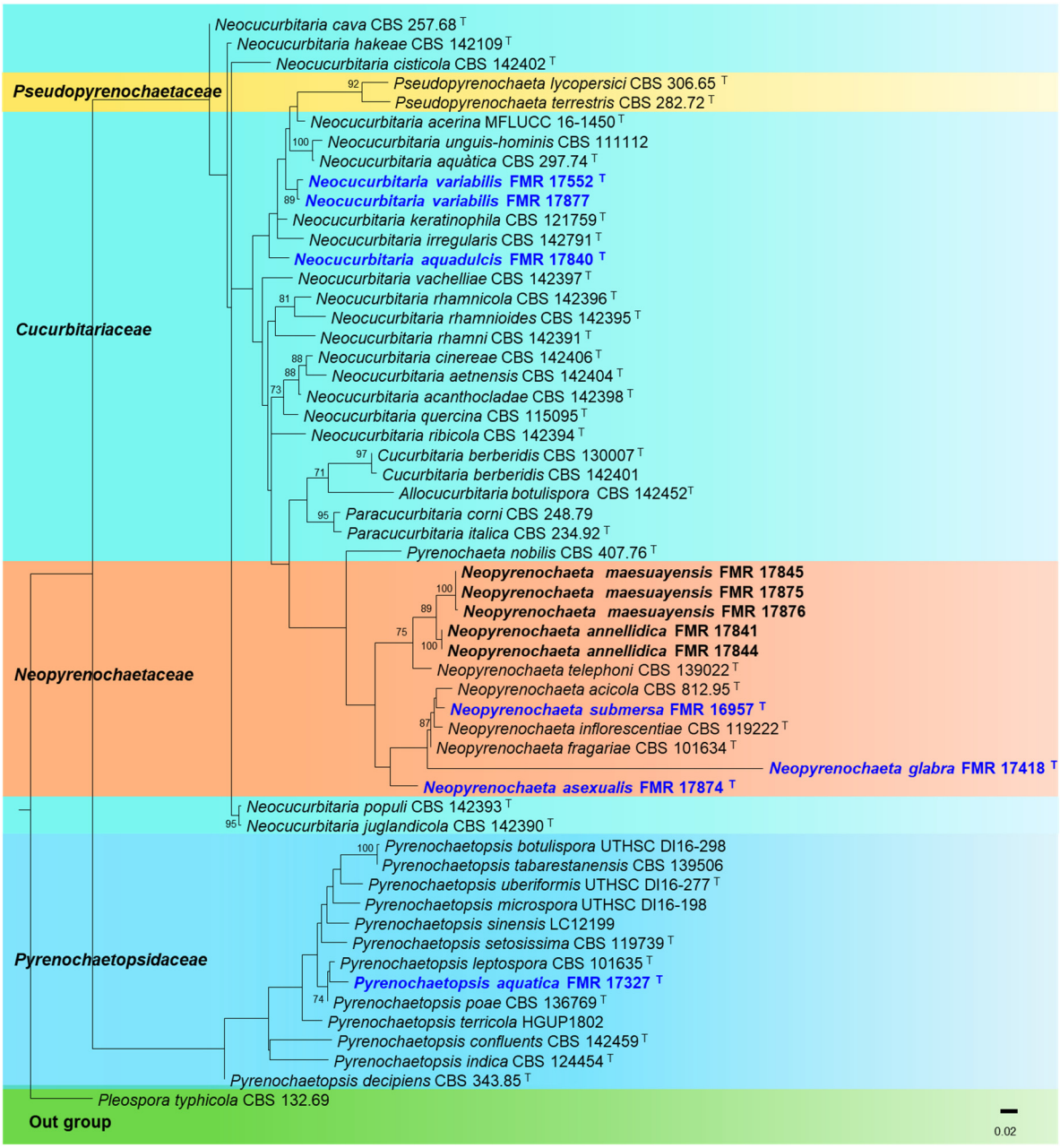

Figure S4. ML phylogenetic tree of *Cucurbitariaceae, Neopyrenochaetaceae, Pseudopyrenochaetaceae,* and *Pyrenochaetopsidaceae* inferred from *tub*2 sequences (272 bp). Support in nodes is indicated above branches by bootstrap values of 70 % and higher. T = ex-type strains. New species are indicated in **blue**. Strains isolated during this study are indicated in **bold**. Alignment length.

4.3. New *Dothideomycetes* from Freshwater Habitats in Spain

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Publicado en: Journal of Fungi 2021; 7: 1102.

DOI:10.3390/jof7121102

Área de la revista**:** Micología

Posición de la revista**:** 5/129 (D1)

Factor de impacto**:** 5.816

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMÍA Y FILOGENIA MOLECULAR DE HONGOS PROCEDENTES DE MATERIAL VEGETAL SUMERGIDO DE ESPAÑA
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Journal of Fungi

MDPI

Article

New *Dothideomycetes* from Freshwater Habitats in Spain

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Abstract: The *Dothideomycetes* are a class of cosmopolitan fungi that are present principally in terrestrial environments, but which have also been found in freshwater and marine habitats. In the present study, more than a hundred samples of plant debris were collected from various freshwater locations in Spain. Its incubation in wet chambers allowed us to detect and to isolate in pure culture numerous fungi producing asexual reproductive fruiting bodies (conidiomata). Thanks to a morphological comparison and to a phylogenetic analysis that combined the internal transcribed spacer (ITS) region of the nrDNA with fragments of the RNA polymerase II subunit 2 (*rpb*2), beta tubulin (*tub*2), and the translation elongation factor 1-alpha (*tef*-1) genes, six of those strains were identified as new species to science. Three belong to the family *Didymellaceae*: *Didymella brevipilosa*, *Heterophoma polypusiformis* and *Paraboeremia clausa*; and three belong to the family *Phaeosphaeriaceae*: *Paraphoma aquatica*, *Phaeosphaeria fructigena* and *Xenophoma microspora*. The finding of these new taxa significantly increases the number of the coelomycetous fungi that have been described from freshwater habitats.

Keywords: *Dothideomycetes*; freshwater fungi; taxonomy; phylogeny

Citation: Magaña-Dueñas, V.; Cano-Lira, J.F.; Stchigel, A.M. New *Dothideomycetes* from Freshwater Habitats in Spain. *J. Fungi* **2021**, *7*, 1102. https://doi.org/10.3390/jof7121102

Academic Editor: Lei Cai

Received: 29 November 2021
Accepted: 19 December 2021
Published: 20 December 2021

1. Introduction

Freshwater fungi are a taxonomically heterogeneous ecological group of organisms of cosmopolitan distribution playing an important ecological role in the recycling of dead organic matter [1], where some of them are restricted to tropical or temperate areas, and others are present in cold-water habitats [2] Most freshwater fungi belong to the phylum Ascomycota (the ascomycetes), whose biodiversity depends on their geographical location and substrates [3]. Around 740 species of ascomycetes have been reported in freshwater habitats (http://fungi.life.illinois.edu/search/world_records, accessed on 10 November, 2021), and approximately one-third of these belong to the class *Dothideomycetes* [4,5].

The *Dothideomycetes* is among the earliest fungi that were reported in freshwater environments [5]. They are a group of fungi characterized by the production of fissitunicate (bitunicate) asci in unilocular and polylocular ascomata [6,7], currently classified into two subclasses: the *Dothideomycetidae*, comprising the orders *Capnodiales, Dothideales,* and *Myriangiales;* and the *Pleosporomycetidae*, comprising the orders *Gloniales, Hysteriales, Jahnulales, Mytilinidiales,* and *Pleosporales* [8,9]. The majority of freshwater *Dothideomycetes* belong to the *Pleosporales* and to the *Jahnulales* [5].

The *Pleosporales* is the largest order of the *Dothideomycetes*, comprising a quarter of its species [6,10]. Taxa in this order have been found in diverse habitats, and can act as saprobes, endophytes, pathogens, or parasites. Most of the *Pleosporales* are plant pathogens with a wide range of hosts and mainly cause leaf and stem lesions [6,9,11,12].

The diverse hydrography, topology, and climatology of Spain led to the formation of several well-defined ecological regions that have a wide spectrum of scarcely explored habitats potentially rich in fungal populations. During this study, we isolated several

fungi living on plant debris in freshwater habitats. The objective was to perform phylogenetic analyses using the nucleotide sequences of informative molecular markers that can clarify the taxonomy of these fungal isolates, and to describe any noteworthy taxa.

2. Materials and Methods

2.1. Samples Collection and Fungal Isolation

A total of 119 samples of plant debris submerged in freshwater in Spain were collected as follows: two in *Parque de Doña Casilda Iturriza* (Vizcaya province), three in *Les Guilleries* (Barcelona province), ten in *Cúber* (Escorca, Mallorca), 22 in *Capafonts* (Tarragona province), 15 in *Pontons* (Barcelona province), 17 in *Riaza* (Segovia province), and 50 in *Cascadas del Huéznar* (Cazalla de la Sierra, Sevilla province). Samples were placed into sterile self-sealing plastic bags for transport to the laboratory and stored at 5 °C. Samples were processed and examined following the method described previously by Magaña-Dueñas et al. [13]. All isolates were stored in the culture collection at the Faculty of Medicine and Health Sciences (FMR), Reus (Spain), and herborized materials and living cultures of novel fungi were deposited in the fungal collection at Westerdijk Fungal Biodiversity Institute (CBS; Utrecht, The Netherlands). The nomenclature and descriptions were registered in MycoBank (https://www.mycobank.org/page/Registration%20home, accessed on 4 October 2021).

2.2. Phenotypic Study

The phenotypic studies were carried out according to Magaña-Dueñas et al. [14], except for strain FMR 17808, in which the measurements of the structures were carried out after 30 days, due to the slow production of fertile fruiting bodies.

2.3. DNA Extraction, Amplification, and Sequencing

DNA extraction, amplification, and sequencing were carried out following the protocols outlined by Magaña-Dueñas et al. [13,14]. SeqMan software v. 7.0.0 (DNAStar Lasergene, Madison, WI, USA) was used to obtain and edit the consensus sequences. Sequences generated in this study were deposited at the European Nucleotide Archive (ENA) (Table S1).

2.4. Phylogenetic Analysis

The sequences obtained were compared with other fungal sequences deposited at the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 20 July, 2021). For the phylogenetic study, an alignment for each locus was made using the MEGA (Molecular Evolutionary Genetics analysis) program v. 7.0 [15], using the Clustal W algorithm [16] and refined with MUSCLE [17], or manually, when necessary, on the same platform. Phylogenetic analyses were made by maximum-likelihood (ML) and Bayesian interference (BI) with RAxML v. 8.2.12 [18] software on the online Cipres Science gateway portal [19] and MrBayes v.3.2.6 [20], respectively. The final matrix used for phylogenetic analyses were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S295050 (accessed on 25 November 2021)).

For the *Didymellaceae*, the phylogenetic reconstructions were performed using the concatenated nucleotide sequences of three phylogenetic markers (ITS, *rpb2,* and *tub2*), while for the *Phaeosphaeriaceae* four phylogenetic markers (ITS, *rpb2, tub2,* and *tef-1*) were used. The best nucleotide substitution model for the BI analysis of the family *Didymellaceae* was the Kimura 2-parametrer with proportion of Invariable sites and Gamma distribution (K80 + I + G) for *rpb2*, and the Symmetrical model with proportion of Invariable sites and Gamma distribution (SYM + I + G) for ITS and *tub2*. For the *Phaeosphaeriaceae*, the best model was General Time Reversible with proportion of Invariable sites and Gamma distribution (GTR + I + G) for ITS, K80 + I+G for *rpb2*, Hasegawa–Kishino–Yano with Gamma

distribution (HKY + G) for *tub2* and General Time Reversible with Gamma distribution (GTR + G) for *tef-1*, all estimated using the program jModelTest [21]. The parameter settings used in BI analyses were two simultaneous runs of 5,000,000 generations, and Markov chain Monte Carlo (MCMC), with samples taken every 1000 generations. The 50% majority rule consensus tree and posterior probability values (PP) were calculated after discarding the first 25% of the resulting trees. A PP value ≥ 0.95 was considered as significant [22]. For ML analysis, support for internal branches was assessed by 1000 ML bootstrapped pseudo replicates. Bootstrap support value (BS) $\geq 70\%$ was considered significant.

3. Results

3.1. Phylogeny

For the Didymellaceae, the alignment comprised 31 ingroups of strains with a total of 1389 characters, including gaps (476 bp for ITS, 301 for *tub2,* and 612 *rpb2*), which included 493 bp variable sites (111 for ITS, 114 for *tub2,* and 268 for *rpb2*) and 399 bp phylogenetically informative sites (91 for ITS, 81 for *tub2,* and 227 for *rpb2*). *Vacuiphoma oculihominis* UTHSC: DI16-308 and *V. bulgarica* CBS 357.84 were used as outgroup. The BI analysis showed similar tree topology and was congruent with that obtained in the ML analysis. For the BI multilocus analysis, a total of 1352 trees were sampled after the burn-in with a stop value of 0.01. In the phylogenetic tree (Figure 1), our strains were placed in three well-supported clades. The *Paraboeremia* clade (100% BS/1 PP) included all the described species of the genus together with two of our strains (FMR 18598 and FMR 18597), which were placed in a fully supported independent terminal branch. The *Didymella* clade (95% BS/1 PP) comprised the seven previously described species of the genus *Didymella* (including the type species *D. exigua*) together with our strain FMR 17415, distant from the rest in an independent terminal branch. The *Heterophoma* clade (100% BS/1 PP) included all six previously described species together with our strain FMR 17837, and was placed in the same terminal clade as *H. verbasci-densiflori*.

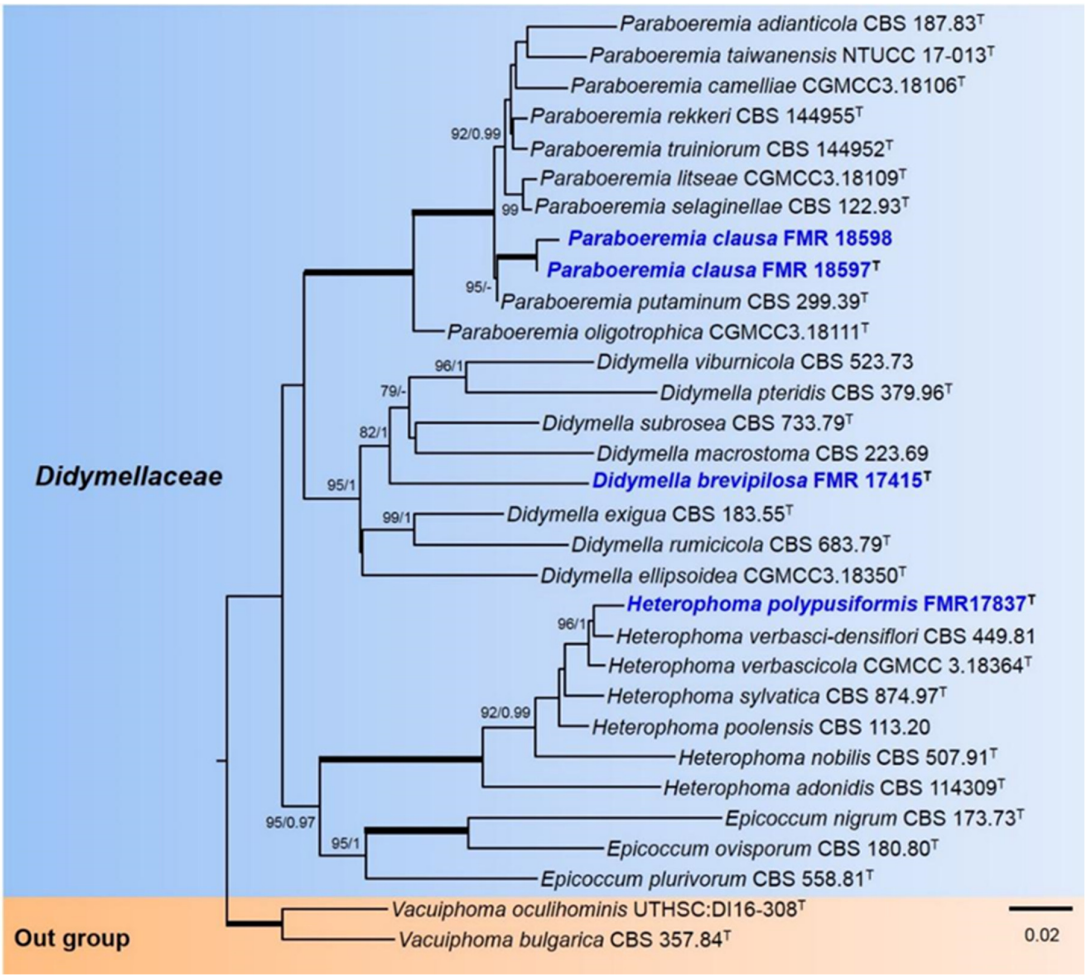

Figure 1. Phylogenetic tree inferred from a ML analysis based on a concatenated alignment of ITS, *rpb2*, and *tub2* sequences of 31 strains representing 31 species of *Didymellaceae*. The Bayesian posterior probabilities (PP) above 0.95 and the RAxML bootstrap support values (BS) above 70% are given at the nodes (PP/BS). Fully supported branches (1 PP/100 BS) are indicated in thicker lines. Newly proposed taxa are given in blue. Type strains are indicated by a superscript T. The tree was rooted with *Vacuiphoma bulgarica* CBS 357.84 and *V. oculihominis* UTHSC:DI16-308. Alignment length 1389 bp.

For the *Phaeosphaeriaceae*, the alignment included 32 ingroups of strains, with a total of 2256 characters including gaps (422 bp for ITS, 699 for *rpb2*, 282 for *tub2*, and 853 for *tef-1*), which comprised 715 bp variable sites (161 for ITS, 288 for *rpb2*, 94 for *tub2*, and 172 for *tef-1*) and 507 bp phylogenetically informative sites (130 for ITS, 72 for *tub2*, 219 for *rpb2*, and 86 for *tef-1*). *Neophaeosphaeria filamentosa* CBS 102202 and *N. agaves* CBS 136429 were included as outgroup. For the BI multi-locus analysis, 1378 trees were sampled after the burn-in with a stop value of 0.01. The phylogenetic tree included nine genera of the *Phaeosphaeriaceae* (Figure 2). To resolve the phylogenetic placement of our strain FMR 17808, a phylogenetic tree was constructed using the LSU sequences of *Phaeosphaeria*, since for most species of the genus the sequences of other markers (*rpb2*, *tef-1*, and ITS) are not available (Figure S1). In the *Phaeosphaeriaceae* tree (Figure 2), the *Phaeosphaeria* clade (91% BS/1 PP) included eight previously described species together with FMR 17808, which was placed in a terminal independent branch distant from the rest of the species. The *Xenophoma* clade (100% BS/1 PP) included the type species *X. puncteliae* together with our strain FMR 17947. The *Paraphoma* clade (70% BS/0.90 PP) comprised all the described species of the genus and our strain FMR 16956, which was placed in a full supported terminal branch together with *P. radicina.*

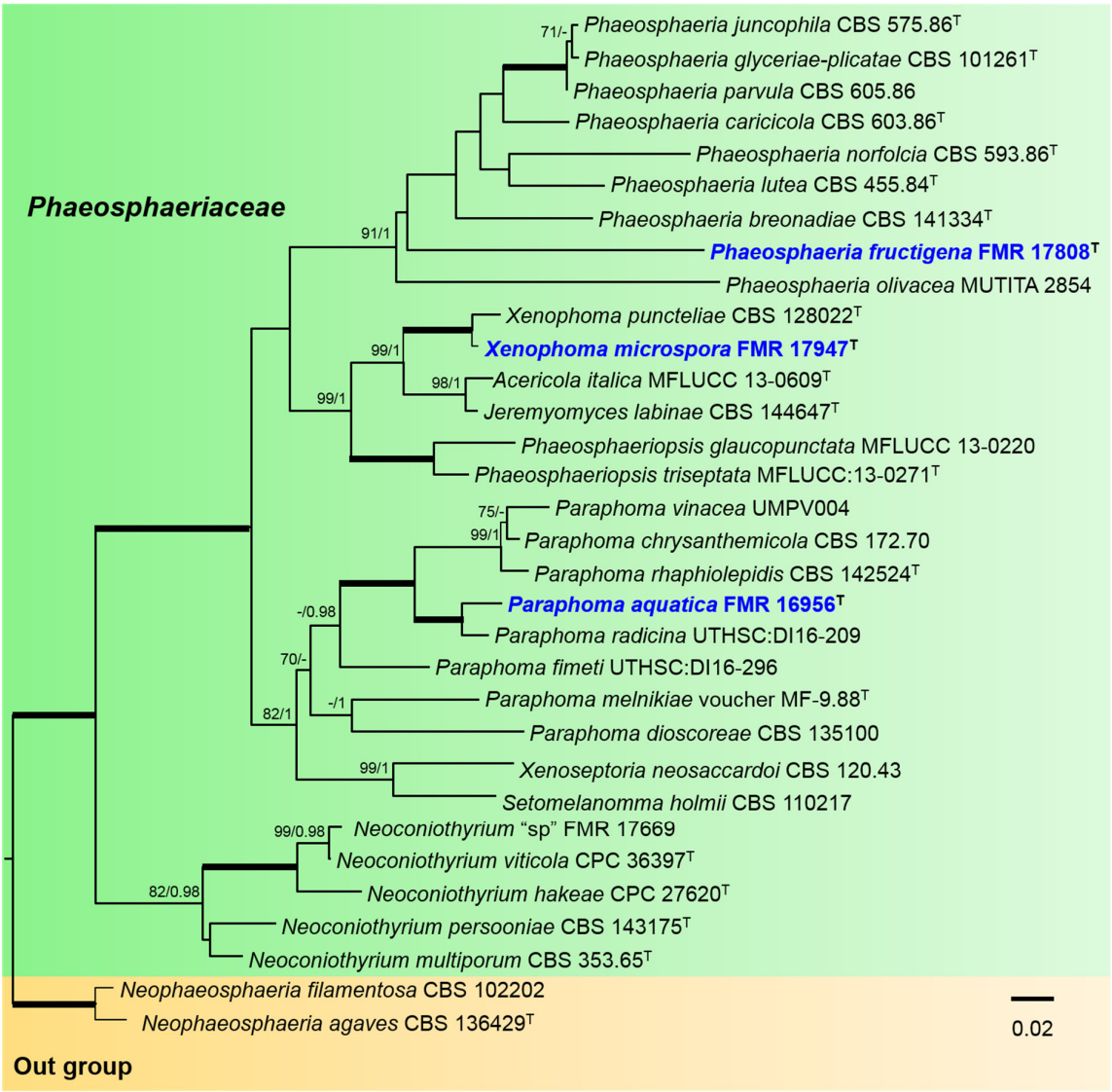

Figure 2. Phylogenetic tree inferred from a Maximum likelihood analysis based on a concatenated alignment of ITS, *rpb2*, *tub2*, and *tef-1* sequences of 32 strains representing 30 species of *Phaeosphaeriaceae*. The Bayesian posterior probabilities (PP) above 0.95 and the RAxML bootstrap support values (BS) above 70% are given at the nodes (PP/BS). Fully supported branches (1 PP/100 BS) are indicated thicker lines. Newly proposed taxa are given in blue. Type strains are indicated by a superscript T. The tree was rooted with *Neophaeosphaeria agaves* CBS 136429 and *N. filamentosa* CBS 102202. Alignment length 2256 bp.

3.2. Taxonomy

Didymellaceae Gruyter et al., Mycol. Res. 113: 516. 2009.

Type genus: *Didymella* Sacc.

Basionym: *Peyronellaea* Goid. ex Togliani, Ann. Sperim. Agrar. II 6: 93. 1952.

Didymella Sacc. ex Sacc., Syll. Fung. 1: 545. 1882. Emend. Chen et al., Stud. Mycol. 82: 173. 2015.

Type species: *Didymella exigua* (Niessl) Sacc.

Didymella brevipilosa V. Magaña-Dueñas, Stchigel and Cano, sp. nov. MycoBank MB841361 (Figure 3).

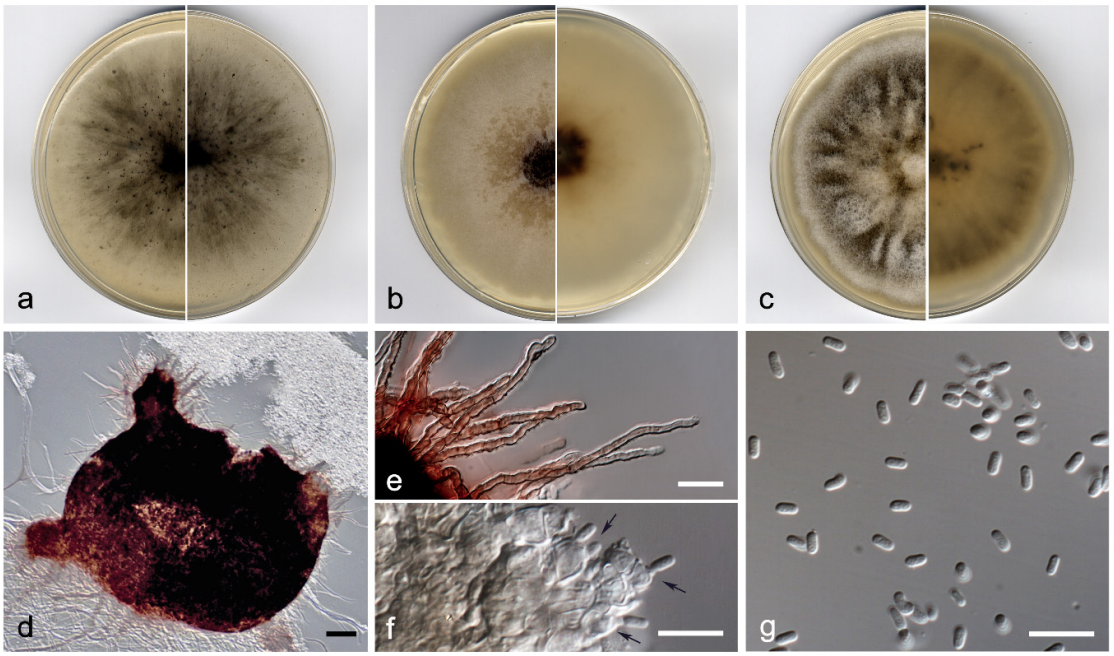

Figure 3. *Didymella brevipilosa* FMR 17415: (**a**) colonies on OA; (**b**) MEA; and (**c**) PDA after two weeks at 25 ± 1 °C (surface, left; reverse, right); (**d**) pycnidium; (**e**) setae; (**f**) conidiogenous cells (black arrows); (**g**) conidia. Scale bars: d = 50 µm, e,f = 10 µm.

Etymology. From Latin *brevivus*-, short, and *-pilosae*, hairy, because the short setae surrounded the neck.

Type: Spain, Segovia province, *Riaza* (41.238863, −3.435258), from plant debris submerged in freshwater, May. 2018, col. Viridiana Magaña Dueñas, holotype CBS H-24906. living cultures FMR 17415 = CBS 148654.

Description: Hyphae hyaline to pale brown, septate, branched, smooth- and thin-walled, 1.5–2.5 µm wide. Conidiomata pycnidial, brown to dark brown, immersed to semi-immersed, solitary, scattered, setose, ostiolate, mostly subglobose, 200–400 × 160–410 µm; 1 to 3 ostiolar necks, 50–60 × 60–75 µm, ostiole 50–60 µm diam. Setae subhyaline to brown, septate, slightly sinuous, nodose and verrucose, 15–100 µm, tapering towards the apex, which is slightly apiculate, mostly arranged around the neck. Conidiomata wall 4–6-layered, 15–30 µm thick, with an outer layer of *textura angularis*, composed of light brown to dark, flattened polygonal cells of 5–8 µm diam. Conidiophores absent. Conidiogenous cells phialidic, determinate, hyaline, smooth-walled, ampulliform to globose, 5–8 × 4–6 µm. Conidia aseptate, hyaline, smooth- and thin-walled, bacilliform to kidney-shaped, 4–5 × 2–3 µm. Chlamydospores absent.

Culture characteristics: Colonies on PDA reaching 38–41 mm diam after 7 days at 25 ± 1 °C, flattened, slightly floccose, margin undulate, olive brown (4F7) with white patches, border yellowish grey (4B2); reverse brownish grey to greyish yellow (8F2/4B3). Colonies on OA reaching 40–43 mm diam after 7 days at 25 ± 1 °C, flattened, granular due to abundant pycnidia, margin regular, surface and reverse grey (5F1). Colonies on MEA reaching 37–39 mm diam after 7 days at 25 ± 1 °C, flattened, velvety, margins undulate, dark brown to greyish yellow (6F7/4B4), border yellowish grey (4B2); reverse dark brown to greyish orange (6F7/4B4) border greyish yellow (4B4). Exopigment absent. Cardinal temperatures for growing—optimum 25 °C, maximum 30 °C, minimum 5 °C.

Diagnosis: Morphologically, *Didymella brevipilosa* differs from the rest of the species located in the same clade in having slightly sinuous, nodose and verrucose setae mainly on the neck and around the pycnidial ostiole, while the other species produce glabrous

conidiomata [23,24]. In the submerged plant material, from which the fungus was isolated, only the asexual stage was observed.

Notes: Regarding the ITS-*rpb2*-*tub2* concatenated sequences alignment, the nucleotide differences between *D. brevipilosa* and the other species in the same terminal clade were: *D. macrostoma,* 110 bp; *D. pteridis,* 120 bp; *D. subrosea,* 97 bp; and *D. viburnicola,* 101 bp.

Heterophoma Qian Chen and L. Cai, Stud. Mycol. 82: 165. 2015.

Type species: Heterophoma sylvatica (Sacc) Qian Chen and L. Cai.

Because the sexual stage of *Heterophoma* has not been previously reported and described, we emend the generic description as next:

Description: Sexual stage: Ascomata superficial, solitary, non-ostiolate, dark brown, opaque, lens shaped to subglobose; *hamathecium* comprising numerous hyaline, septate, filamentous paraphyses; asci 8-spored, cylindrical, with an apical annular apparatus; ascospores two-celled, hyaline and biconic when young, becoming muriform and brown to dark brown at maturity, with 5 transversal septa (frequently developing 1–2 additional transverse septa) and 2–4 longitudinal and oblique septa, broadly fusiform, frequently constricted at the middle septum, occasionally constricted at other septa, surrounded by a mucilaginous sheath. Asexual stage: Conidiomata pycnidial, globose to subglobose, superficial on or immersed into the agar, solitary or confluent, ostiolate; pycnidial wall pseudoparenchymatous, 5–12-layered; conidiogenous cells phialidic, hyaline, smooth, ampulliform to doliiform; conidia variable in shape and size, hyaline, smooth- and thin-walled, 0–1(–2)-septate, i.e., ellipsoidal, oblong, cylindrical, reniform, or slightly allantoid, mostly guttulate. Chlamydospores unicellular, globose, intercalary in chains, olivaceous.

Heterophoma polypusiformis V. Magaña-Dueñas, Cano and Stchigel, sp. nov. MycoBank MB841362 (Figure 4).

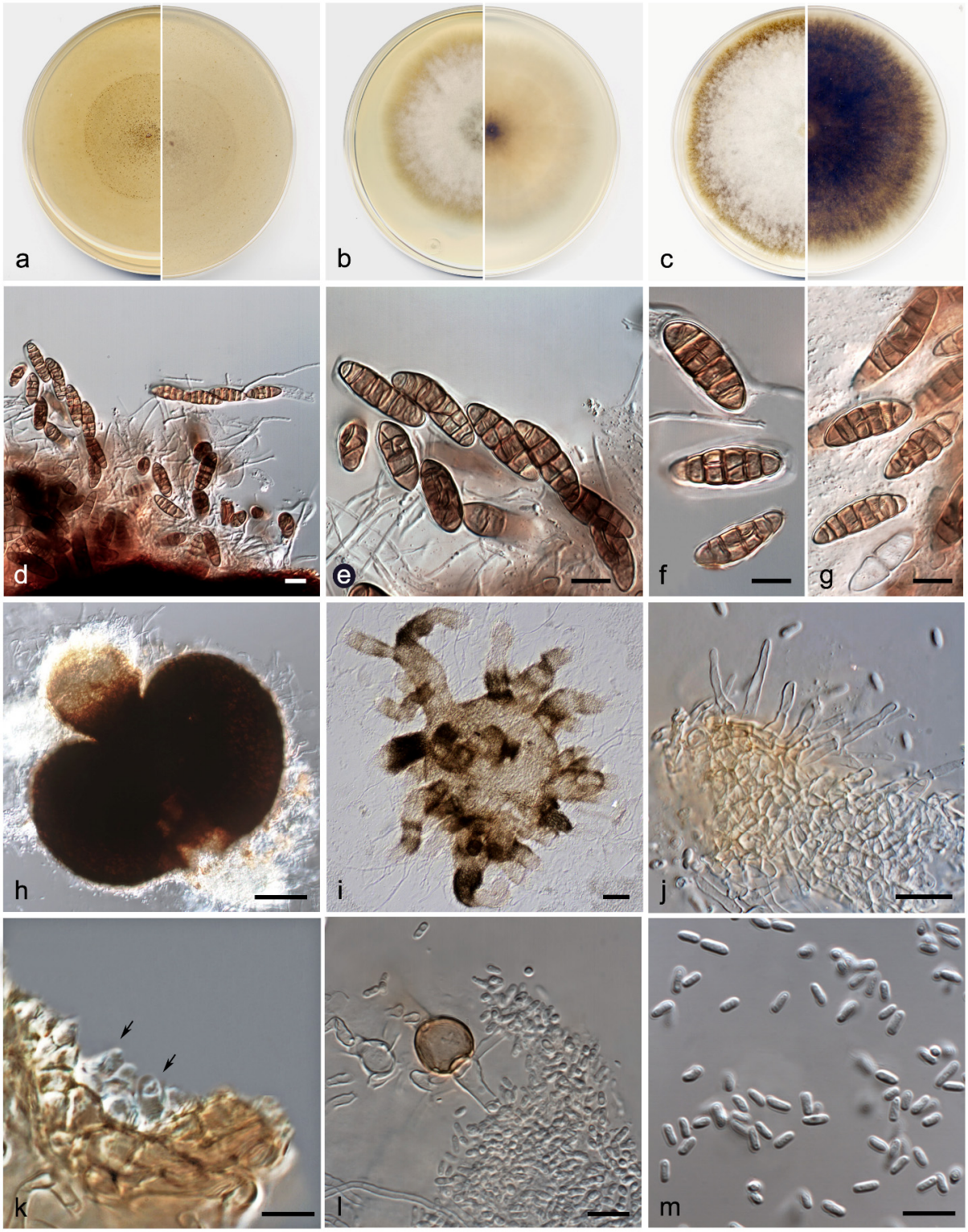

Figure 4. *Heterophoma polypusiformis* FMR 17837: (**a**) colonies on OA; (**b**) MEA; and (**c**) PDA after two weeks at 25 ± 1 °C (surface, left; reverse, right); (**d**,**e**) asci and ascospores; (**f**,**g**) ascospores (note the mucilaginous sheath in f); (**h**,**i**) pycnidium; (**j**) setae; (**k**) conidiogenous cells (black arrows); (**l**) chlamydospores and conidia; (**m**) conidia. Scale bars: h,i = 50 μm; d–g, j–m = 10 μm.

Etymology. From Latin *polypus-*, octopus, *-formis*, shape, because the morphological resemblance of immersed conidiomata to an octopus.

Type: Spain, Sevilla province, *Cascadas del Huéznar* (37.9935997, −5.6718387), from plant debris in freshwater, May. 2019, José F. Cano Lira, holotype CBS H-24907, living cultures FMR 17837 = CBS 148655.

Description: Hyphae hyaline, septate, branched, smooth- and thin-walled, 2–3 μm wide. Sexual stage: Ascomata superficial, solitary, non-ostiolate, dark brown, opaque, lens shaped to subglobose, up to 600 × 400 μm, thin-walled; hamathecium comprising numerous hyaline, septate, filamentous paraphyses, 1–3 μm wide; asci 8-spored, cylindrical, 90–100 × 10–15 μm, with an apical, annular apparatus; ascospores two-celled, hyaline and biconic when young, becoming muriform and brown to dark brown at maturity, with 5 transversal septa (frequently developing 1–2 additional transverse septa) and 2–4 longitudinal and oblique septa, broadly fusiform, 10–35 × 5–10 μm, frequently constricted at the middle septum, occasionally constricted at other septa, surrounded by a mucilaginous sheath. Asexual stage: Conidiomata pycnidial, semi-immersed, brown to dark brown, solitary, scattered, ostiolate, setose, subglobose, 160–180 × 170–200 μm, developing one to a few necks turning paler towards the ostiole, cylindrical 60–150 μm long, ostiole 60–85 μm diam; when the conidiomata grow immersed in the medium (OA) develop numerous necks that branch out, giving them a cephalopod look to the pycnidia; setae hyaline to sub-hyaline, septate, nodose, thick-walled, 15–40 μm long, mainly disposed around the ostiole, rounded and curved at the tip, conidiomata wall 4–6-layered, 10–25 μm thick, with an outer layer of *textura angularis* composed of brown to dark brown, flattened polygonal cells of 4–8 μm diam; conidiogenous cells phialidic, determinate, hyaline, smooth-walled, doliiform, 4–6 × 3–4 μm; conidia aseptate, hyaline, smooth- and thin-walled, cylindrical, 4–5 × 1.5–2 μm. Chlamydospores aseptate, intercalary, smooth-walled, brown, globose, 11–15 μm diam.

Culture characteristics: Colonies on PDA reaching 59–60 mm diam after 7 days at 25 + 1 °C, flattened, velvety, margin regular, orange grey to yellowish (6B2/5D4); reverse dark brown (6F7), margins orange white (6A2). Colonies on OA reaching 39–42 mm diam after 7 days at 25 + 1 °C, flattened, slightly cottony, margin regular, surface and reverse orange white (6A2). Colonies on MEA reaching 43–45 mm diam after 7 days at 25 + 1 °C, flattened, velvety, margins undulate, white to orange white (6A2); reverse greyish orange (5B3). Exopigment absent. Cardinal temperatures for growing—optimum 25 °C, maximum 35 °C, minimum 5 °C.

Diagnosis: *Heterophoma polypusiformis* is easily distinguishable from the other species of the genus because it produces a sexual stage, which is morphologically related to the genus *Ascochyta*, a member of the *Didymellaceae* [25]. Moreover, *H. polypusiformis* develops into OA pycnidia with numerous ostiolar necks that branch out giving them a cephalopod appearance, a feature never seen in the other species of the genus. Unlike *H. polypusiformis,* its phylogenetically closer species, *H. verbasci-densiflori*, produces in the same culture conditions pycnidia bearing 1-6 papillated to short (of less than 60 μm long, whereas these can reach up to 200 μm in *H. polypusiformis*) unbranched necks (branched in *H. polypusiformis*), with a 'potato-like' appearance. The sexual stage of *H. polypusiformis* was found on the submerged plant material, whereas its asexual stage was observed once the fungus was grown in pure culture.

Notes: Differences between the nucleotide sequences (ITS-*rpb2*-*tub2*concatened dataset) of *H. polypusiformis* and *H. verbasci-densiflori* were of 16 bp.

Paraboeremia Q. Chen and L. Cai, Stud. Mycol. 82: 183. 2015.

Type species: Paraboeremia selaginellae (Sacc.) Q. Chen & L. Cai.

Paraboeremia clausa V. Magaña-Dueñas, Stchigel and Cano, sp. nov. MycoBank MB841363 (Figure 5).

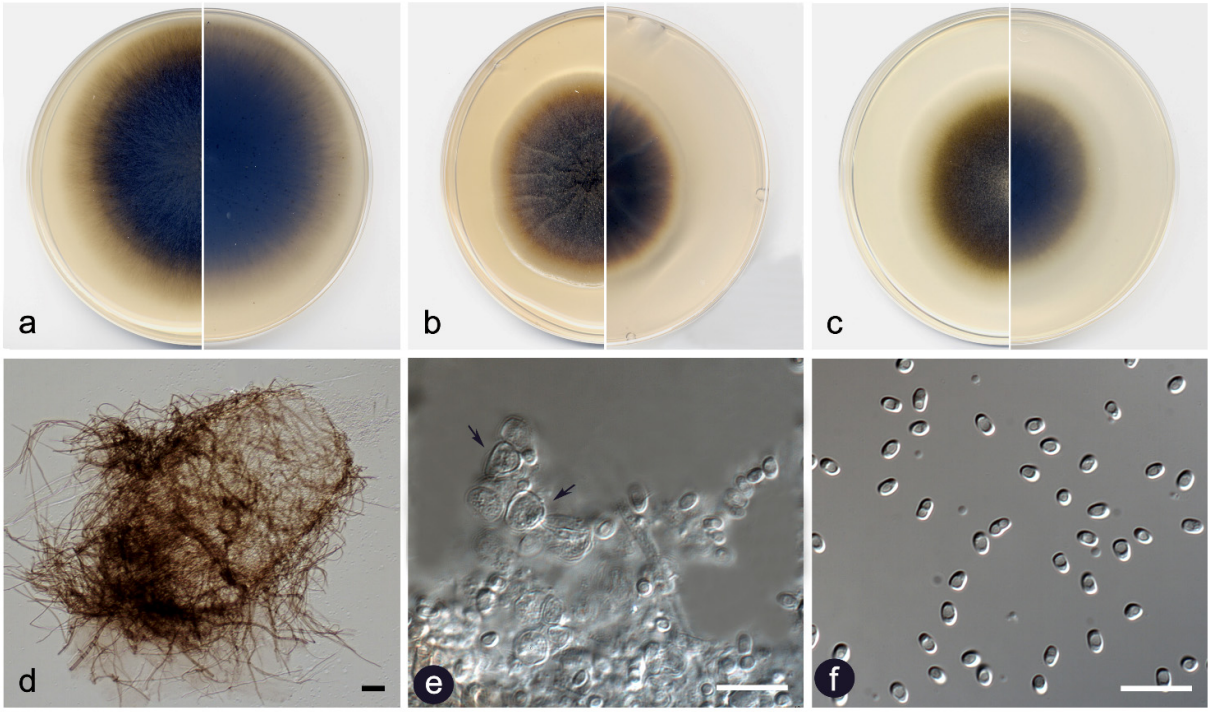

Figure 5. *Paraboeremia clausa* FMR 18597: (**a**) colonies on OA; (**b**) MEA; and (**c**) PDA after two weeks at 25 ± 1 °C (surface, left; reverse, right); (**d**) pycnidium; (**e**) conidiogenous cells (black narrows); (**f**) conidia. Scale bars: d = 50 µm, e,f = 10 µm.

Etymology. From Latin *clausa*, closed, because the absence of conidiomata ostioles.

Type: Spain, Vizcaya province, Bilbao, *Parque de Doña Casilda Iturriza* (43.2658246, −2.942885), from freshwater submerged plant debris, Aug 2020, Viridiana Magaña Dueñas, holotype CBS H-24908, living cultures FMR 18597 = CBS 148656.

Other material examined: Spain, Vizcaya province, Bilbao, *Parque de Doña Casilda Iturriza* (43.2658246, −2.942885), from freshwater submerged plant debris, Aug. 2020, Viridiana Magaña Dueñas, living cultures FMR 18598.

Description: Hyphae pale brown to brown, septate, branched and smooth-walled, 2.5–5 µm wide. Conidiomata pycnidial, sub-hyaline to pale brown, translucent, immersed to semi-immersed, solitary, scattered, barrel-shaped to pyriform, 270–480 × 270–300 µm, covered by brown, septate, smooth to asperulate, thin-walled anastomosing hyphae; neck absent or rarely present, conical-truncate, 120–130 × 180–200 µm, ostiole indistinguishable. Conidiomata wall 4–6-layered, 25–35 µm thick, with an outer layer of *textura intricata*, composed of pale brown to brown hyphae of 2–4 µm wide. Conidiophores absent. Conidiogenous cells phialidic, determinate, hyaline, smooth-walled, globose, 5.5–6.5 × 5.5–7 µm. Conidia aseptate, hyaline, smooth- and thin-walled, broadly ellipsoidal to ovoid, 3–3.5 × 1.5–2.5 µm, one- or biguttulate. Chlamydospores absent.

Culture characteristics: Colonies on PDA reaching 37–40 mm diam after 7 days at 25 + 1 °C, flattened, velvety, margin regular, yellowish brown (5F4) with a yellowish white (4A2) border; reverse with the same colour than the surface. Colonies on OA reaching 55–57 mm diam after 7 days at 25 + 1 °C, flattened, granular due to abundant production of pycnidia, margin filamentous, brown (6F4), reverse greyish brown (6F3). Colonies on MEA reaching 41–44 mm diam after 7 days at 25 + 1 °C, flattened, radiate, velvety, margins regular, dark brown to greyish brown to (8F8/8F3), border orange white (6A2); reverse brownish grey to greyish brown (8F2/7E3) border orange white (6A2). Exopigment light

orange (5A4). Cardinal temperatures for growing—optimum 25 °C, maximum 30 °C, minimum 5 °C.

Diagnosis: *Paraboeremia clausa* is phylogenetic close to *P. putaminum*, but differs from the latter because the pycnidia lack ostioles, and the conidia are hyaline in mass, while those of *P. putaminum* are greenish [26]. Both strains of *P. clausa* displayed similar phenotypic features in pure culture, and the asexual stage of *P. clausa* was originally detected on the freshwater submerged plant debris.

Notes: Differences between ITS-*tub2*-*rpb2* concatenated nucleotide sequences of *P. clausa* and *P. putaminum* were 15 bp.

Phaeosphaeriaceae M. E. Barr Mycologia 71(5): 948. 1979.

Type genus: Phaeosphaeria I. Miyake.

Paraphoma Morgan-Jones and J.F. White, Mycotaxon 18 (1): 58. 1983.

Type species: *Paraphoma radicina* (McAlpine) Morgan-Jones and J.F. White, Mycotaxon 18 (1): 60. 1983.

Paraphoma aquatica V. Magaña-Dueñas, Stchigel and Cano, sp. nov. MycoBank MB841364 (Figure 6).

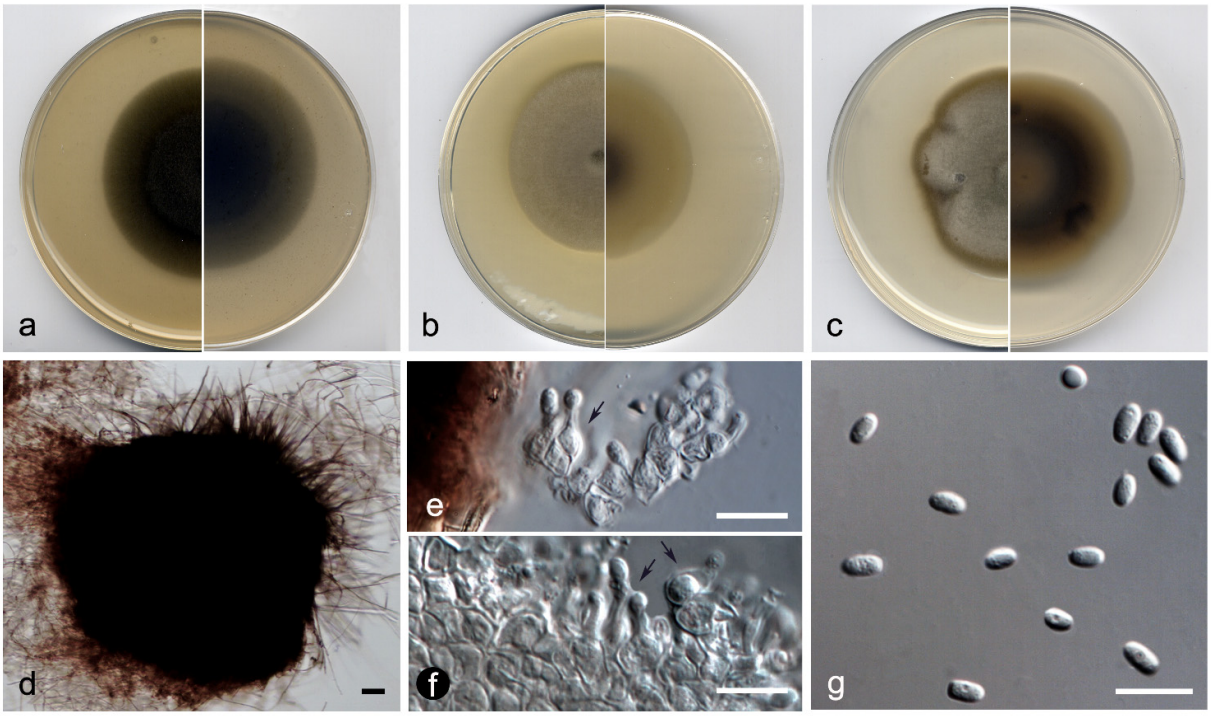

Figure 6. *Paraphoma aquatica* FMR 16956: (**a**) colonies on OA; (**b**) MEA; and (**c**) PDA after two weeks at 25 ± 1 °C (surface, left; reverse, right); (**d**) pycnidium; (**e**,**f**) conidiogenous cells (black arrows); (**g**) conidia. Scale bars: d = 25 µm, e–g = 10 µm.

Etymology. From Latin *aquaticus*, referring to the habitat from which the fungus was recovered.

Type: Spain, Barcelona province, *Les Guilleries* (41.9362028, 2.4122862), from freshwater submerged plant debris, Nov. 2017, Eduardo Jose de Carvalho Reis, holotype CBS H-24909, living cultures FMR 16956 = CBS 148657.

Description: Hyphae pale brown to brown, septate, branched, smooth- and thin-walled, 1.5–2.5 µm wide. Conidiomata pycnidial, dark brown, semi-immersed, solitary, scattered, setose, globose to subglobose 380–570 × 400–570 µm, non-ostiolate. Setae brown,

septate, smooth- and thick-walled, rounded at the tip, 90–150 μm. Conidiomata wall 5–7-layered, 15–30 μm thick, with an outer layer of *textura angularis*, composed of brown to dark brown, flattened polygonal cells of 5–8 μm diam, covered by a mass of interwoven, brown hyphae. Conidiophores absent. Conidiogenous cells phialidic, determinate, hyaline, smooth-walled, ampulliform to globose, 4–6 × 5–8 μm. Conidia aseptate, hyaline, smooth- and thin-walled, ellipsoidal, 5–8 × 2–3 μm. Chlamydospores absent.

Culture characteristics: Colonies on PDA reaching 54–55 mm diam after 7 days at 25 + 1 °C, flattened, velvety, margin regular, greyish brown to brownish grey (5D3/5F2); reverse yellowish brown to orange grey (5D5/5B2). Colonies on OA reaching 56–59 mm diam after 7 days at 25 + 1 °C, umbilicate, velvety, margin regular, dull green (30E4); reverse greenish grey to dull green (30F2/30E4), border white. Colonies on MEA reaching 45–48 mm diam after 7 days at 25 + 1 °C, flattened, velvety, margins regular, olive grey to greenish grey (3D2/3B4), reverse grey to greyish yellow (3F1/3B4), border yellowish white (3A2). Exopigment absent. Cardinal temperatures for growing—optimum 25 °C, maximum 30 °C, minimum 5 °C.

Diagnosis: *Paraphoma aquatica* differs from the phylogenetically closest species, *P. radicina*, because its pycnidia lack of a neck and are non-ostiolate. The asexual stage of *P. aquatica* was also observed in the submerged substrate.

Notes: *Paraphoma aquatica* is located in the clade with a weak support; however, it forms a fully supported clade with *Paraphoma radicina*. The concatenated ITS-*rpb2*-*tub2*-*tef-1* nucleotide sequences of both species differs in 59 bp.

Phaeosphaeria I. Miyake, Bot. Mag., Tokyo 23: 93. 1909.

Type species: *Phaeosphaeria oryzae* I. Miyake, Bot. Mag., Tokyo 23: 93. 1909.

Phaeosphaeria fructigena V. Magaña-Dueñas, Cano and Stchigel, sp. nov. MycoBank MB841365 (Figure 7).

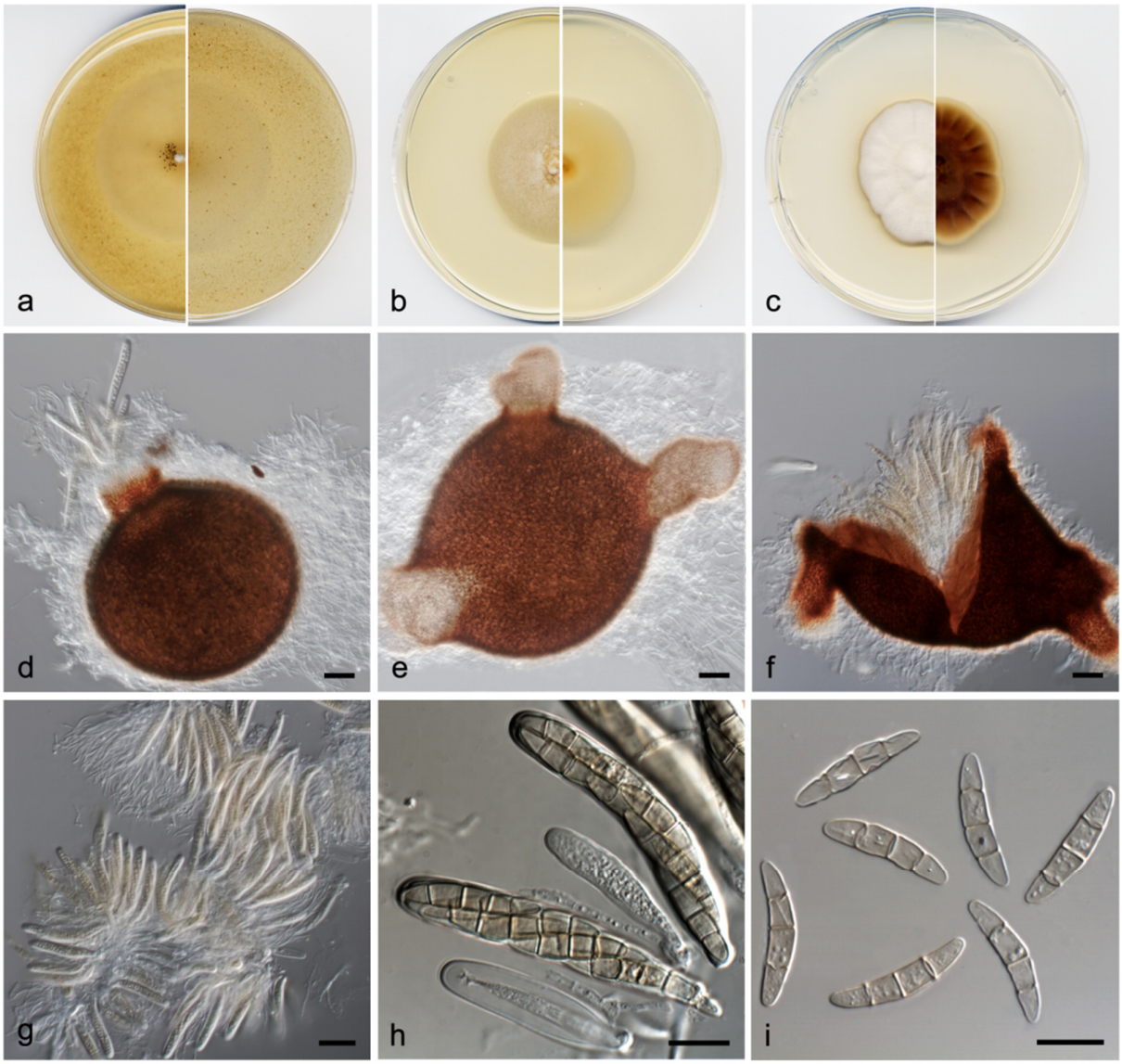

Figure 7. *Phaeosphaeria fructigena* FMR 17808: (**a**) colonies on OA; (**b**) MEA; and (**c**) PDA after two weeks at 25 ± 1 °C (surface, left; reverse, right); (**d**–**f**) ascomata (d expelling asci); (**g**,**h**) asci; (**i**) ascospores. Scale bars: d–f = 50 μm, g–i = 10 μm.

Etymology. From Latin *fructi-*, fruits, and *-genes*, because of the production of ascomata *in vitro*.

Type: Spain, Tarragona province, *Capafonts* (41.29598, 1.02753), from freshwater submerged plant debris, Mar. 2019, Viridiana Magaña Dueñas and Isabel Iturrieta González, Holotype CBS H-24910, living cultures FMR 17808 = CBS 148658.

Description: Mycelium superficial to immersed, composed by septate, hyaline, smooth- and thin-walled, branched hyphae, 2–3 μm wide. Ascomata perithecial, immersed to semi-immersed, solitary, ostiolate, with up to three necks, reddish-brown to dark brown, becoming paler towards the top of the neck, pyriform, globose to irregularly-shaped, 230–370 × 210–330 μm; neck conic-truncate, 70–145 × 40–80 μm, ostiole 30–70 μm diam.; peridial wall 2–4-layered, 35–60 μm thick, outer wall of *textura intricata*, composed

of brown to dark brown hyphae of 3–4 µm wide. *Hamathecium* comprising numerous, filamentous, septate, branched paraphyses, and pseudoparaphyses of 1.5–2 µm wide. Asci 6–8-spored, bitunicate, cylindrical to cylindrical-clavate, 80–120 × 10–12 µm, without apical structures. Ascospores hyaline when young, becoming pale brown at maturity, three-septate, fusiform, 22–28 × 4–5 µm, narrowly rounded at the ends.

Culture characteristics: Colonies on PDA reaching 40–41 mm diam after 7 days at 25 + 1 °C, umbonate, velvety, margin undulate, with abundant aerial mycelium, surface white (6A1), border orange white (6A2); reverse pale brown (6D4), border orange white (6A2). Colonies on OA reaching 46–48 mm diam, flattened to slightly floccose, margins regular, with sparse aerial mycelium, surface and reverse yellowish grey (4B2). Colonies on MEA reaching 39–40 mm diam, flattened, velvety, margin regular, with abundant aerial mycelium, orange grey (5B2); reverse orange white (5A2). Cardinal temperature for growing—optimum 25 °C, maximum 30 °C, minimum 5 °C.

Diagnosis: Morphologically, *Phaeosphaeria fructigena* is characterized by the production in vitro of ascomata with up to three necks, and of fusiform ascospores. Only the sexual stage of *P. fructigena* has been observed in both original material and pure culture.

Notes: In our phylogenetic analysis *P. fructigena* is located at an independent branch, thus revealing itself as a new species.

Xenophoma Crous & Trakunyingcharoen, IMA fungus. 5(2): 404. 2014.

Type species: *Xenophoma pucteliae* Crous & Trakunyingcharoen, IMA fungus. 5(2): 404. 2014.

Basionym: *Phoma puncteliae* Diederich & Lawrey, Fungal Div. 55: 207 (2013).

Xenophoma microspora V. Magaña-Dueñas, Stchigel and Cano, sp. nov. FMR 17947. MycoBank MB841366 (Figure 8).

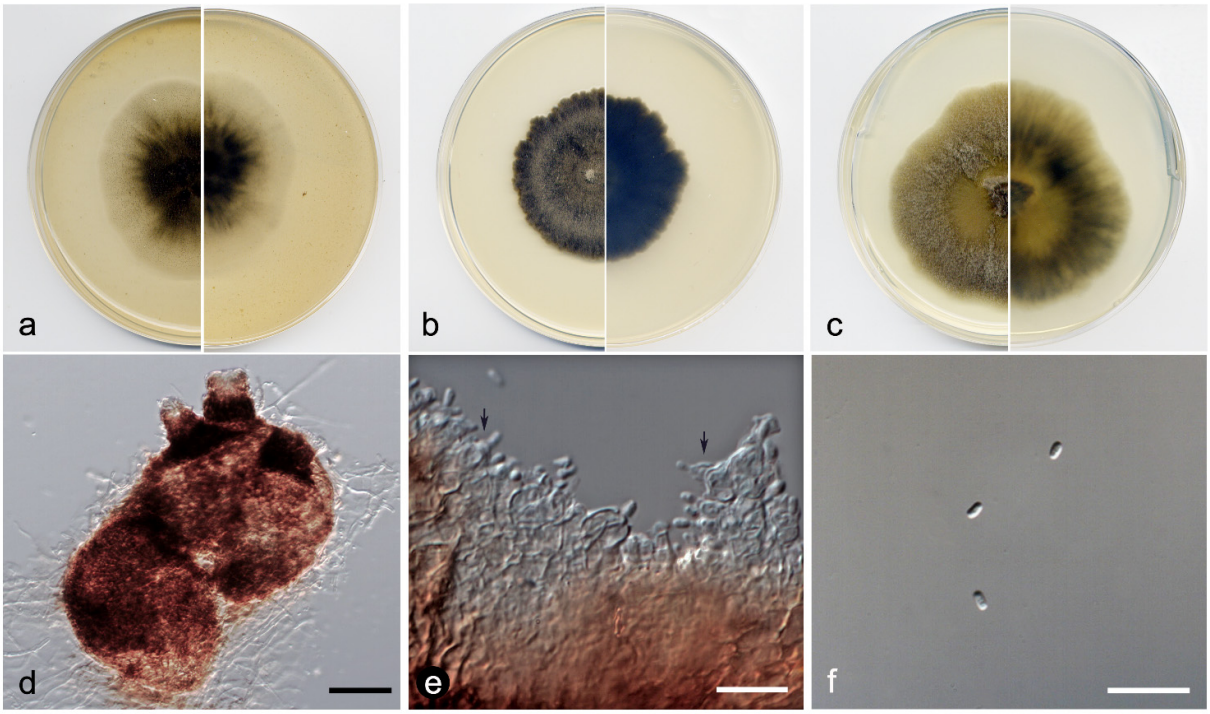

Figure 8. *Xenophoma microspora* FMR 17947: (**a**) colonies on OA; (**b**) MEA; and (**c**) PDA after two weeks at 25 ± 1 °C (surface, left; reverse, right); (**d**) pycnidium; (**e**) conidiogenous cells (black arrows); (**f**) conidia. Scale bars: d = 50 µm, e,f = 10 µm.

Etymology. From Greek *μικρο-*, small, *-σπόριο*, spore, due to the small size of the conidia.

Type: Spain, Barcelona province, *Pontons* (41.41397, 1.52678), from freshwater submerged plant debris, Jun. 2018, Viridiana Magaña Dueñas, Holotype CBS H-24911 living cultures FMR 17947 = CBS 148659.

Description: Hyphae hyaline to subhyaline, septate, smooth- and thin-walled, branched, 2.5–3 μm wide. Conidiomata pycnidial, brown to dark brown, immersed to semi-immersed, solitary, scattered, ostiolate, globose to subglobose, 160–180 × 200–250 μm, with up to 3 conic-truncate ostiolar necks of 30–40 × 35–45 μm. Conidiomata wall 4–6-layered, 15–25 μm thick, with an outer layer of *textura angularis*, composed of light brown to brown, flattened polygonal cells of 4–6 μm diam. Conidiophores absent. Conidiogenous cells phialidic, determinate, hyaline, smooth-walled, ampulliform to globose, 2.5–4 × 3–3.5 μm. Conidia aseptate, hyaline, smooth- and thin-walled, bacilliform, 1.5–2.5 × 1–1.2 μm Chlamydospores absent.

Culture characteristics: Colonies on PDA reaching 59–60 mm diam after 7 days at 25 + 1 °C, flattened, floccose, margin irregular, surface and reverse olive brown (4D4). Colonies on OA reaching 49–51 mm diam after 7 days at 25 + 1 °C, flattened, velvety, margin regular, surface and reverse yellowish grey with patches olive brown (4B2/4F3). Colonies on MEA reaching 42–44 mm diam after 7 days at 25 + 1 °C, flattened, velvety, margins lobate, olive brown (4F4); reverse brownish grey (4F2). Cardinal temperatures for growing—optimum 25 °C, maximum 30 °C, minimum 5 °C.

Diagnosis: Morphologically, *Xenophoma microspora* is distinguished from *Xenophoma puncteliae* by the production of up to three ostiolar necks, and also by producing smaller conidia than *X. puncteliae* (1.5–2.5 × 1–1.2 μm vs. 2.5–3 × 2–2.5 μm). The asexual stage of *X. microspora* was observed in plant debris submerged in freshwater.

Notes: Among the concatenated sequences (ITS-*rpb2*-*tub2*-*tef-1*), the difference in nucleotides between *X. punctileae* and *X. microspora* is 27 bp.

4. Discussion

Recently, molecular biology helped to clarify the phylogenetic relationships between the members of the *Dothideomycetes*, especially among several phoma-like fungal taxa. Multilocus analyses based on LSU, ITS, *rpb2, tef-1,* and *tub2* sequences have been widely used to define the species boundaries for the *Didymellaceae*, the *Phaeosphaeriaceae* and other families of the *Dothideomycetes* [23,24,27–30]. However, *rpb2* alone provides a phylogenetic tree with a similar topology to those obtained with more phylogenetic markers [24,30,31].

During the development of the present study, we isolated several fungi from submerged wood in certain freshwater habitats of Spain. We carried out phylogenetic analyses with concatenated of three loci (ITS-*rpb2*-*tub2*) for the *Didymellaceae* members, and of four loci (ITS-*rpb2*- *tub2*-*tef-1*) for those taxa in the *Phaeosphaeriaceae*. Thus, we report six new species to science: *Didymella brevipilosa*, *Heterophoma polypusiformis*, *Paraboeremia clausa, Paraphoma aquatica, Phaeosphaeria fructigena,* and *Xenophoma microspora*.

The species of *Didymella*—genus established by Saccardo [32] to accommodate *D. exigua*—are saprobes commonly found in living and dead parts of herbaceous and woody plants but are also important phytopathogens. Several species have also been isolated from inorganic substrates, such as asbestos, cement, and paint [23,28,33]. In 2015, Chen et al. carried out a multilocus phylogenetic analysis and the genus was defined as monophyletic and encompassing 37 species [28]. Approximately 30 new species have recently been included in the genus [23,24,29,30,34–36]. In our phylogenetic analysis, *Didymella brevipilosa* was placed in an independent branch separated from the rest of *Didymella* spp. In addition, this species is characterized by having short, sinuous and asperulate setae mainly located around the ostioles, unlike most of the species of the genus, which lack these structures.

The genus *Heterophoma* was introduced by Chen et al. [28] to accommodate *H. adonidis, H. nobilis, H. novae-verbascicola, H. poolensis,* and *H. sylvatica*. Seven species are

currently recognized (http://www.indexfungorum.org/names/Names.asp, accessed on 25 October 2021). Species of this genus are saprobes and plant pathogens (especially on members of the families *Brassicaceae* and *Scrophulariaceae*) with a cosmopolitan distribution [23,28,30]. In our study, we report the finding of *Heterophoma polypusiformis*, the first species of the genus isolated from wood submerged in freshwater. Moreover, *H. polypusiformis* produces both asexual and sexual stages, being the first species of the genus reported to have sexual reproduction. The main features of the ascospores (smooth-walled, muriform, brown, and surrounded by a gelatinous sheath) correspond to those reported for other genera of the family *Didymellaceae*, such as *Ascochyta* and *Neomicrosphaeropsis* [25]. *Heterophoma polypusiformis* is easily distinguishable from the other species of the genus because the pycnidia submerged in the culture medium have an 'octopus' appearance.

Chen et al. [28] introduced the genus *Paraboeremia* into the *Didymellaceae* to accommodate *P. adianticola, P. putaminum,* and *P. selaginellae*. Nine species are currently accepted in the genus (http://www.indexfungorum.org/Names/Names.asp, accessed on 25 October 2021). The majority of the species of the genus are plant parasites, causing leaf or stem spots [28,31]. Moreover, *Paraboeremia* spp. have been isolated from the rhizosphere, soil, and healthy and dead plants [23,28,30,31]. *Paraboeremia clausa* is the first species reported in plant material submerged in freshwater and is characterized by the production of barrel-shaped to pyriform, translucent, very pale colored pycnidia covered by dark brown anastomosing hyphae and lacking ostioles.

The genus *Phaeosphaeria* was introduced by Miyake [37] to accommodate its type species, *P. oryzae*. Later, the lectotype was designated by Eriksson [38], and due to the morphological similarities with the *Leptosphaeria*, both genera were for a long time considered synonyms. Barr [39] subsequently established a new family *Phaeosphaeriaceae,* designating *Phaeosphaeria* as its type genus. *Phaeosphaeria* species have a cosmopolitan distribution, they are saprobic but also pathogenic stems, flowers, and leaves of monocotyledons, and hyperparasites of other fungi [27]. There are 219 species currently listed in the Index Fungorum (http://www.indexfungorum.org/Names/Names.asp, accessed on 25 October 2021). *Phaeosphaeria fructigena* was isolated from plant debris submerged in freshwater, and its sexual stage shares several features with other species of the genus (e.g., *P. musae, P. oryzae,* and *P. thysanolaenicola*), such as the fissitunicate asci and 3-septate, hyaline to pale yellowish ascospores [27,40]. In our phylogenetic analysis *P. fructigena* was placed in and independent branch in the clade.

In 1983, Morgan-Jones introduced the new genus *Paraphoma* in order to accommodate phoma-like species with setose conidiomata [41]. However, the genus was later treated at section level within *Phoma* by Boerema [42]. De Gruyter [43] reinstated the genus, and placed it into the family *Phaeosphaeriaceae* based on a phylogenetic analysis. Twelve species are currently accepted in the genus (http://www.indexfungorum.org/names/Names.asp, accessed on 25 October 2021). *Paraphoma* spp. have been reported mainly as soil-borne phytopathogens, causing root and crown rot diseases [27,43,44]. *Paraphoma aquatica* differs from the other species of the genus because the ascomata lacks of ostiolar necks.

In 2012, Lawrey and Driederich introduced the new species *Phoma puncteliae*, isolated from the parasitized thalli of *Punctelia rudecta* [45]. Based on a phylogenetic analysis, Trakunyingcharoen and Crous [46] erected the new genus *Xenophoma* and placed it in the Phaeosphaeriaceae, designating *X. puncteliae* as its type species. The morphology of *Xenophoma* is similar to that of *Phoma*, differing by the production of cauliflower-shaped, unito multilocular conidiomata, and of the subspherical to ellipsoid conidia. Our new species, *X. microspora* differs from *X. puncteliae* (the phylogenetically nearest species) by the production of more than one ostiole per conidiomata and by the smaller bacilliform conidia.

The sexual stages of the freshwater ascomycetes have undergone a series of morphological adaptations to survive in aquatic environments. Many of them produce ascospores

with appendages and/or mucilaginous sheaths, which facilitate their attachment to substrates into the water [47–49]. In this study, the sexual stage of *H. polypusiformis*, found on plant debris submerged in freshwater, produces ascospores with a mucilaginous sheath, a feature also found in other genera living in similar environments, such as *Murispora* and *Lolia* [48,50]. On the other hand, some coelomycetous fungi exclusively reported in freshwater habitats, such as *Aquasubmersa mircensis*, *Coelomyces aquaticus*, and *Lolia aquatica*, are characterized by the production of conidia with mucilaginous appendages [50,51], a feature not observed in our fungal strains, nor in the terricolous counterparts.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/jof7121102/s1, Figure S1: ML phylogenetics tree of *Phaeosphaeriaceae* inferred from the LSU sequences of 59 strains. Support in nodes is indicated above by bootstrap values of 70% or higher. The tree was rooted with *Neophaeosphaeria agaves* CBS 136429 and *Neophaeosphaeria filamentosa* CBS 102202. Alignment length 713 b.p. Newly proposed taxa are given in blue. Type strains are indicated by a superscript T. Table S1: Sequences used in this study.

Author Contributions: Conceptualization, A.M.S. and J.F.C.-L.; methodology, V.M.-D.; software, V.M.-D. and J.F.C.-L.; validation, A.M.S. and J.F.C.-L.; formal analysis, V.M.-D., A.M.S., and J.F.C.-L.; investigation, V.M.-D.; resources, J.F.C.-L.; data curation, A.M.S. and J.F.C.-L.; writing—original draft preparation, V.M.-D.; writing—review and editing, V.M.-D., A.M.S., and J.F.C.-L.; visualization, A.M.S. and J.F.C.-L.; supervision, A.M.S. and J.F.C.-L.; project administration, J.F.C.-L.; funding acquisition, J.F.C.-L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Spanish *Ministerio de Economía y competitividad*, grant CGL2017-88094-P.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We are grateful to the Iberian Society of Mycology (SIM) for organizing the sampling in Riaza, to Juan Ramón García Martínez, (non-professional mycologist of Granja de Torrehermosa, Badajoz, Spain) for participating in the sampling of the Sierra Norte de Sevilla and to Phil Hoddy for reviewing and correcting the final text.

Conflicts of Interest: The authors declare no conflict of interest.

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Table S1. Fungal taxa and their nucleotide sequences of the molecular markers used to build the phylogenetic trees.

Taxon	Strain	GenBank Accession No.			
		ITS	***tub2***	***rpb2***	***tef1***
Acericola italica	MFLUCC:13-0609	NR_156344			
Didymella brevipilosa	**FMR 17415**	**OU612373**	**OU612358**	**OU612359**	
Didymella ellipsoidea	CGMCC3.18350	KY742060	KY742302	KY742145	
Didymella exigua	CBS 183.55	GU237794	GU237525	EU874850	
Didymella macrostoma	CBS 223.69	GU237801	GU237623	KT389608	
Didymella pteridis	CBS 379.96	KT389504	KT389801	KT389624	
Didymella rumicicola	CBS 683.79	KT389503	KT389800	KT389622	
Didymella subrosea	CBS 733.79	NR_170787	MT005643	MT018174	
Didymella viburnicola	CBS 523.73	GU237879	GU237667	MH872477	
Epicoccum nigrum	CBS 173.73	FJ426996	FJ427107	KT389631	
Epicoccum ovisporum	CBS 180.80	FJ427068	FJ427174	LT623252	
Epicoccum plurivorum	CBS 558.81	GU237888	GU237647	KT389634	
Heterophoma adonidis	CBS 114309	MH862963	KT389803	KT389637	
Heterophoma nobilis	CBS 507.91	NR_170721	GU237603	KT389638	
Heterophoma polypusiformis	**FMR17837**	**OU612367**	**OU600611**	**OU600610**	
Heterophoma poolensis	CBS 113.20	MH854684	GU237638	MT018056	

Heterophoma sylvatica	CBS 874.97	GU237907	GU237662	MT018052	
Heterophoma verbascicola	CGMCC 3.18364	NR_158268	GU237650	KY742187	
Heterophoma verbasci-densiflori	CBS 449.81	MN973474	MT005573	MT018049	
Jeremyomyces labinae	CBS:144647	NR_163362	MK442733	MK442665	**OU600603**
Neoconiothyrium hakeae	CPC 27620	NR_154839	KY173600	KY173583	
Neoconiothyrium multiporum	CBS 353.65	MH858605			
Neoconiothyrium persooniae	CBS 143175	MG386041			
Neoconiothyrium sp.	**FMR 17669**	**OU641117**	**OU641013**	**OU641014**	
Neoconiothyrium viticola	CPC 36397	MN562123		MN556804	
Neophaeosphaeria agaves	CBS:136429	NR_137833			
Neophaeosphaeria filamentosa	CBS 102202	JF740259		GU357803	GU349084
Paraboeremia adianticola	CBS 187.83	GU237796	GU237576	KP330401	
Paraboeremia camelliae	CGMCC3.18106	KX829034	KX829058	KX829050	
Paraboeremia clausa	**FMR 18597**	**OU612369**	**OU600598**	**OU600597**	
Paraboeremia clausa	**FMR 18598**	**OU612371**	**OU600600**	**OU600599**	
Paraboeremia litseae	CGMCC3.18109	KX829029	KX829053	KX829045	
Paraboeremia oligotrophica	CGMCC3.18111	KX829031	KX829055	KX829047	
Paraboeremia putaminum	CBS 299.39	MN823454	MN824628	MN824480	
Paraboeremia rekkeri	CBS 144955	MN823511	MN824685	MN824537	

Paraboeremia selaginellae	CBS 122.93	GU237762	GU237656	LT623255	
Paraboeremia taiwanensis	NTUCC 17-013	MK840826	MK839232	MK839234	
Paraboeremia truiniorum	CBS 144952	MN823495	MN824669	MN824521	
Paraphoma aquatica	**FMR 16956**	**OU612361**	**OU612355**	**OU612357**	**OU612356**
Paraphoma dioscoreae	CBS 135100	KF251167	KF252662		
Paraphoma chrysanthemicola	CBS 172.70	KF251165	KF252660		
Paraphoma fimeti	UTHSC:DI16-296	LT796872	KF252665	LT797032	LT797112
Paraphoma melnikiae	MF-9.88	MG764063	MG779456	MG779466	
Paraphoma radicina	UTHSC:DI16-209	LT796835	KF252667	LT796995	LT797075
Paraphoma rhaphiolepidis	CBS 142524	KY979758	KY979924	KY979851	
Paraphoma vinacea	UMPV004	KU176887	KU176895		
Phaeosphaeria breonadiae	CBS 141334	NR_155675			
Phaeosphaeria caricicola	CBS 603.86	KF251182	KF252676		
Phaeosphaeria fructigena	**FMR 17808**	**OU612363**	**OU600608**	**OU600607**	**OU600609**
Phaeosphaeria glyceriae-plicatae	CBS 101261	MH862724			
Phaeosphaeria juncophila	CBS 575.86	AF439488			GU456283
Phaeosphaeria lutea	CBS 455.84	MH861760			
Phaeosphaeria norfolcia	CBS 593.86	MH861997			
Phaeosphaeria olivacea	MUTITA 2854	MG813228			

Phaeosphaeria parvula	CBS 605.86	MH862001			
Phaeosphaeriopsis glaucopunctata	MFLUCC:13-0220	KJ522473	KF252693		MG520918
Phaeosphaeriopsis triseptata	MFLUCC:13-0271	KJ522475		KJ522485	MG520919
Setomelanomma holmii	CBS 110217	KT389542		GU371800	GU349028
Vacuiphoma bulgarica	CBS 357.84				
Vacuiphoma oculihominis	UTHSC:DI16-308				
Xenophoma microspora	**FMR 17947**	**OU612365**	**OU600605**	**OU600604**	**OU600606**
Xenophoma puncteliae	CBS 128022	JQ238617	KP170711	**OU600601**	**OU600602**
Xenoseptoria neosaccardoi	CBS 120.43	KF251280	KF252761		

[1]**CBS**: Culture collection of the Westerdijk Biodiversity Institute, Utrech, The Netherlands; **CGMCC**: China General Microbiological Culture Collection Center; **FMR**: Facultat de Medicina, Reus, Spain; **CPC** :Culture Collection of Pedro Crous; **MFLUCC** : Mae Fah Luang University Culture Collection, Chiang Rai, Thailand ; **NTUCC**: National Taiwan University Culture Collection; **UMPV**: University of Melbourne, *Paraphoma vinacea* strain **UTHSC**, Fungus Testing Laboratory at the University of Texas Health Science Center, San Antonio, Texas, USA

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

[T]Ex-type strain.

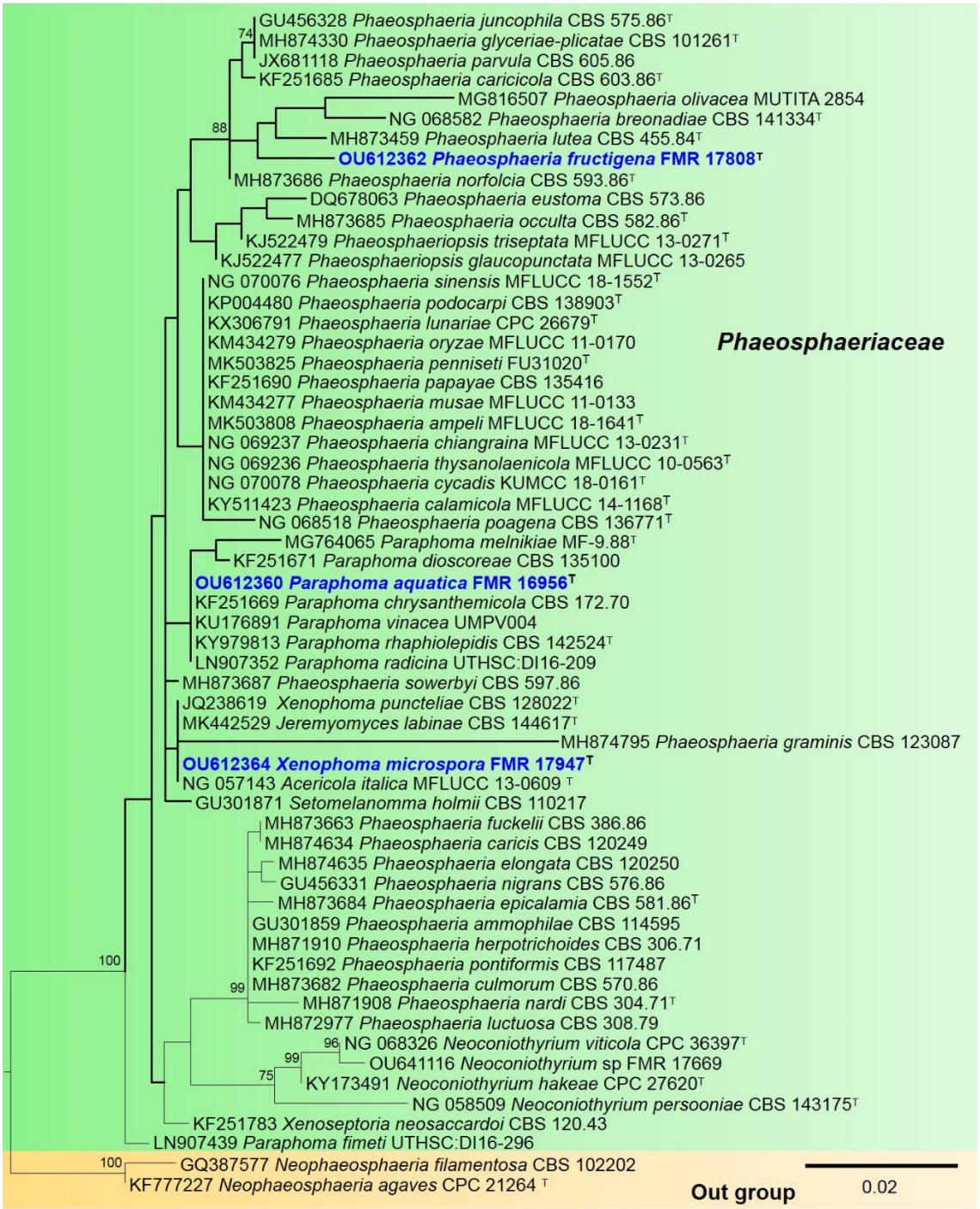

Figure S1 ML phylogenetics tree of *Phaeosphaeriacea* inferred from the LSU sequences of 59 strains. Support in nodes is indicated above by bootstrap values of 70% or highter. The tree was rooted with *Neophaeosphaeria agaves* CBS 136429 and *Neophaeosphaeria filamentosa* CBS 102202. Alignment length 713 b.p. Newly proposed taxa are given in blue. Type strains are indicated by a superscript ^{T}.

4.4. Novel Freshwater Ascomycetes from Spain

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En redacción para: Persoonia

Área de la revista**:** Ecología, Evolución, Comportamiento y sistemáticas

Posición de la revista**:** 12/647 (Q1)

Factor de impacto**:** 11.051

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMÍA Y FILOGENIA MOLECULAR DE HONGOS PROCEDENTES DE MATERIAL VEGETAL SUMERGIDO DE ESPAÑA
Viridiana Magaña Dueñas

Novel freshwater ascomycetes from Spain.

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Abstract

Freshwater ascomycetes is a group of fungi with a great ecological importance, because they are involved in processes of decomposition and recycling of organic matter in aquatic ecosystems. These fungi are taxonomically diverse, with representatives in various orders and families of the phylum Ascomycota. In this and study, we collected and placed into wet chambers ninety-two samples of plant material submerged in freshwater from several places in Spain. As a result, several fungi belonging to various families such as *Amniculicolaceae*, *Chaetomellaceae*, *Lophiostomataceae*, *Pyrenochaetopsidaceae*, *Roussoellaceae* and *Sympoventuriaceae* have been isolated in pure culture. After its phenotypic characterization, we performed a multilocus phylogenetic analysis using nucleotide sequences of D1–D2 domains of the 28S nrRNA gene (LSU), the internal transcribed spacer (ITS) region of the nrDNA, and fragments of the RNA polymerase II subunit 2 (*rpb2*), of the beta tubulin (*tub2*) and of the translation elongation factor 1-alpha (*tef1*) genes. In the present study we report the finding of a new species of *Amniculicola* producing a coelomycetous asexual stage, fact never reported before for that genus; a new species of *Elongatopedicellata* producing a previously unknown asexual stage; a new species of *Neovaginatispora* producing both sexual and asexual stages *in vitro*; and two new species of *Pyrenochaetopsis*, whose sexual stage

is described for the first time. In addition, we describe a new species for each of the genera *Pillidium* and *Pseudosigmoidea*.

Introduction

The phylum Ascomycota (the ascomycetes) is a monophyletic group of fungi containing several taxa recorded usually from freshwater habitats, where they complete a part or the whole of their life-cycle (Shearer 1993; Grossart et al. 2019). The substrates that they decompose in such environments comprise mostly stems, rotten wood and decaying leaves falling into bodies of water from adjacent vegetation (Wong et al. 1998). The main role of these fungi in freshwater habitats is the degradation of plant debris as result of their ability to produce a rich array of enzymes able to degrade cellulose, hemicellulose and lignin. In this way, they provide assimilable nutrients for themselves but also to other organisms in the same ecological niches (Zare-Maivan & Shearer 1988).

Freshwater habitats are characterized by having a balance of organic matter controlled by the surface, location and characteristics of the watershed (Wurzbacher et al. 2011). Freshwater ecosystems that host fungi can be lentic or lotic. The lotic environments include aquatic ecosystems with constant flow of water (rivers, streams, springs, etc.). Lentic environments are any body of water that lacks a constant flow of water such as lakes, ponds, swamps, etc. (Thomas 1996).

The freshwater ascomycetes can be divided in four main groups, based on their occurrence in aquatic environments: 1) genera whose species are exclusively found in freshwater habitats; 2) genera with freshwater and terrestrial species; 3) genera with freshwater and marine species; and 4) genera containing species in all three habitats (Vijaykrishna et al. 2006). Considering their degree of adaptation, activity and dependence on aquatic environments, these fungi have been classified into various groups. Resident or native, fully adapted to aquatic life, showing morphological and physiological adaptations, most are capable of sporulating under water (Park 1972). Periodic immigrants or amphibians, inhabiting aquatic environments during part of their

life cycle, have the ability to produce spores in both terrestrial and aquatic environments (Park 1972, Vijaykrishna et al. 2006). Versatile or facultative aquatic immigrants: they are poorly adapted to water and do not sporulate under water; transenuents, they are not adapted to the aquatic environment, they can reach the substrate active, but be unable to sporulate and also to colonize a new substrate (Park 1972).

In recent years, the knowledge about the taxonomy and phylogeny of these fungi has been increasing thanks to the use of molecular techniques (Luo et al. 2019; Dong et al. 2020; Hyde et al. 2021). About 738 species of ascomycetes has been reported from freshwater environments, most of them producing sexual reproductive structures alone (http://fungi.life.illinois.edu/world_records). Freshwater ascomycetes have representatives in several orders and families scattered throughout three main classes: the *Leotiomycetes* (112 species), the *Sordariomycetes* (342 species) and the *Dothideomycetes* (282 species) (http://fungi.life.illinois.edu/world_records).

The spanish hydrographical system presents has many peculiarities and strong contrasts. The variety of regimes of runoff is due to a rich and diverse waterways, environmental and landscape (https://hispagua.cedex.es/en/datos/hidrografia#1). There are four drainage areas in Spain: north, east, west, and south. The Spanish rivers are very variable due to the climate of the country. In terms of flow, except for the rivers of the north, the Duero and the Ebro, they are rivers with a relatively low flow due to low rainfall. Their profiles vary compared, with fast rivers in the Cantabrian slope and in the Pyrenees and slow in the center. In addition to the Ebro, the rivers of the Atlantic slope are the longest (Tapiador F.J. 2020)

Despite Spain is a country where the hyphomycetous fungi (producing only asexual stages where the conidia borne from conidiophores or from undifferentiated hyphae) have been studied during decades (Abdullah et al. 1985, 1998, Roldán & Honrubia 1990). The sexual stages of freshwater ascomycetes and their coelomycetous

asexual stages have begun to be investigated with a greater intensity only in recent years (Magaña-Dueñas et al. 2020, 2021a, 2021b).

In this study, we report the finding of several new species belonging to different families of the Ascomycota, all of them isolated from submerged plant debris in several freshwater habitats in Spain. The main objective of this study was characterized the morphology and phylogeny of new isolates considered of taxonomic interest. In addition, we described sexual and asexual states previously unknown to some genus.

Materials and methods

Sample collection and fungal isolation

Ninety-two samples of plant material submerged in freshwater, consisting in small branches, leaves and bark, were manually collected in various lotic environments (whose waters flow “rapidly” in a single direction) in Spain: 50 from *Cascadas del Huéznar* (Cazalla de la Sierra, Sevilla province, Spain), 17 from *Riaza* river (near Riaza, Segovia province, Spain), 22 from *de les Hortes* river (on the outskirts of Capafonts, Tarragona province, Spain), and three from *Clot de la Mare de Déu* (Burriana, Castellón province, Spain). *Cascadas del Huéznar* (37.993824, -5.668985) forms a part of the course of the *Rivera de Huesna* river*,* in the Sierra Norte de Sevilla natural park. The climate is Csa (hot-summer Mediterranean climate) according to the Köppen-Geiger climate classification (Rubel, F., and M. Kottek, 2010. Observed and projected climate shifts 1901-2100 depicted by world maps of the Köppen-Geiger climate classification. Meteorologische Zeitschrift Vol. 19 No. 2, p. 135 - 141 DOI: 10.1127/0941-2948/2010/0430). The average annual temperature is 16 °C and the average annual rainfall is 540 mm (https://es.climate-data.org/europe/espana/andalucia/san-nicolas-del-puerto-828536/). The altitude is around 600 m.a.s.l. Impressive travertine formations stand out in this waterfall. This place has abundant riverside vegetation, that forms a dense gallery forest of elms, ash trees, willows and alders

(https://www.juntadeandalucia.es/medioambiente/portal/web/ventanadelvisitante/detalle-buscador-mapa/-/asset_publisher/Jlbxh2qB3NwR/content/cascadas-del-huesna-2/255035). The *Riaza* river (41.28368, -3.47187) also present a riverside forest with abundance of elms, poplars, alders and ash trees. The altitude is around 1,190 m.a.s.l. The climate is Cfb (temperate oceanic climate without dry season), the average annual temperature is 10.8 °C, and the total annual rainfall is around 690 mm (https://es.climate-data.org/europe/espana/castilla-y-leon/riaza-188771/). Soils are acidic, with a pH around 5, conditioned by the presence of siliceous materials (Hernando Costa J., Hernando Massanet I., Ares Mateo A. 2002. Formaciones edáficas del tramo alto de la cuenca del río Riaza. Observatorio Medioambiental Vol. 5 (2002): 149-162). The *de les Hortes* river (41.28664, 1.04033) runs on a calcareous soil and is surrounded by a forest of boxwoods of considerable dimensions, pines, holm oaks and poplars, among other trees and shrubs (http://www.valldecapafonts.com/capafonts_racons_llodriga.html). The sampling place is located at approx. 830 m.a.s.l., has a Csa climate, the average annual temperature is of 13 ° C, and the average annual rainfall of 525 mm (https://es.climate-data.org/europe/espana/cataluna/capafonts-662465/). The *Clot de la Mare de Déu* municipal natural area (39.88043, -0.05954) originates from springs in the last part of the *Sec* river (or *Anna* river), which passes through the Burriana city. It is a representative example of the riparian forest, composed of elms poplars, poplars, hackberries and willows, which together with abundant shrubs, herbaceous and aquatic vegetation including reeds and rushes (https://turisme.burriana.es/listing/el-clot-de-la-mare-de-deu/). The soil is made up of ochre-colored clays and silts (https://www.burriana.es/ayuninf/tablon/ORDENACION/EVALUACION-AMBIENTAL-PGOU/ESTUDIO-DE-PAISAJE/MEMORIA_PDF/MEMORIA.pdf). The local climate is BSk (cold semi-arid), the average annual temperature is around 17 ºC, and the average annual precipitation of 400 mm (https://es.climate-data.org/europe/espana/comunidad-valenciana/burriana-56924/).

RESULTADOS

The samples were placed into self-sealing sterile plastic bags, closed and transported to the laboratory, and stored at room temperature (20−25 ºC) until they were processed. The plant debris was rinsed twice with 500 mL tap water, and subsequently placed into moist chambers consisitng in 15 cm diam. disposable Petri dishes lined inside with two layers of filter paper and moistened with sterile water (added of 1 ml of solution of 20 mg diehldrin® in 20 mL of dimethyl-ketone / L of water), incubated at room temperature (20-25 ºC), and examined periodically under stereo microscope, during a minimum of four weeks to a maximum of 2-months, since the development of reproductive (asexual or/and sexual) structures. Several of the reproductive structures (asexual or sexual bodies, or conidia) were transferred using sterile disposable needles to 55 mm diam. disposable Petri dishes containing oatmeal agar (OA; 30 g of filtered oat flakes, 15 g agar-agar, 1 L tap water; Samson et al. 2010), being then incubated at 25ºC. After successive passages (in the same conditions than previously mentioned) until obtaining a pure culture, the fungal strains of interest were preserved at the culture collection of the Faculty of Medicine (FMR, Reus, Spain) in three different ways: slant cultures on OA and PDA under a layer of liquid vaseline, OA blocks (where the strain had grown) immersed in sterile water in caramel-colored self-sealing vials, and lyophilized. Holotypes and cultures ex-type of the novel fungi were deposited in the Westerdijk Fungal Biodiversity Institute (CBS; Utrecht, The Netherlands). The names and descriptions were deposited in MycoBank.

Phenotypic study

Morphological features were obtained from fungi growing on the natural substrate or on OA agar (at 25± 1ºC for 14 days). After the incubation period, the diameter of the colony was measured and characteristics such as texture, border, presence of diffusible pigments, exudates, and front and back color were described using the Kornerup & Wansche catalog (1978). The cardinal temperatures were determined on PDA agar after 7 days in darkness, ranging from 5 to 35ºC at 5ºC intervals. To document the structures

of the coelomycetes, histological sections of the pycnidia were made freehand, with the help of a sterile 0.3x13mm needle and a 30º cut and 15mm wide Laseredge Scalpel. Measurements and descriptions of microscopic structures were taken from specimens mounted in Shear´s mounting media using an Olympus BH-2 light microscope (Olympus Corporation, Tokyo, Japan). Photomicrographs were captured using a Zeiss Axio-Imager M1 microscope (Oberkochen, Germany) with a DeltaPix Infinity X digital camera using Nomarski differential interference contrast.

DNA extraction, amplification and sequencing

For the extraction of genomic DNA, the mycelium of axenic cultures grown in PDA for 7 days at 25±1 ºC in the dark was scraped with a sterile scalpel. Genomic Subsequently the DNA was extracted through the FastaDNA kit protocol (Bio101, Vista, CA, USA) with a Fast Prep FP120 instrument (Thermo Savant, Holbrook, NY, USA) according to the manufacturer´s protocol. DNA was quantified using Nanodrop 2000 (Thermo Scientific, Madrid, Spain). The following loci were amplified and sequencing:, internal transcribed spacer region (ITS), with the primer pair ITS5 and ITS4 (White et al. 1990); a fragment of the 28S nrRNA gene (LSU) with the primer pair LR0R (Rehner & Samuels 1994) and LR5 (Vilgalys & Hester 1990); fragment of the RNA polymerase II subunit 2 gene (*rpb2*) with RPB2-5F2 (Sung et al. 2007) and fRPB2-7cR (Liu et al. 1999); a fragment of the beta-tubulin gene (*tub2*) with the primers TUB2Fw and TUB4Rd (Woudenberg et al. 2009); and Translation elongation factor 1-alpha (*tef1*) with the primers EF1-983F and EF1-2218R (Rehner et al. 2021). Sequencing of the amplicons was made in both directions with the same primer pair used for amplification at Macrogen Spain (Macrogen Inc., Madrid, Spain). The sequences obtained were edited and contigs were assembled using SeqMan software v. 7.0.0 (DNAStar Lasergene, Madison, WI, USA). Sequences generated in this study were deposited in European Nucleotide Archive (ENA).

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Phylogenetic analysis

Each sequence generated in this study was subjected to an individual blast search to verify its identity in the database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence-based identities with a maximum level of identity (MLI) of ≥98% for identification in the species range and <98% in the genus range were considered significant in this study. ITS for the genera *Pilidium* and *Pseudosigmoidea*; LSU for the genus *Elongatopendicellata*; *rpb2* for *Neovaginatispora* and *Pyrenochaetopsis* genera; and *tef-1* for *Amniculicola* genus were used for identification at the rank of species. Alignment for each locus was performed with the MEGA (Molecular Evolutionary Genetics Analysis) software v. 7.0 (Kumar et al. 2016), using the ClustalW algorithm (Thompson et al. 1994) and refined with MUSCLE (Edgar 2004) or manually, if necessary, on the same software. Phylogenetic analysis was made by maximum-likelihood (ML) and Bayesian interfer-ence (BI) with RAxML v. 8.2.12 (Stamatakis 2014) software on the online Cipres Science gateway portal (Miller et al. 2021) and MrBayes v.3.2.6 (Ronquist et al. 2012).

The phylogenetic tree for the family *Amniculicolaceae* was built using the concatenated nucleotide sequences of the ITS region and a fragment of *tef1* gene; for family *Roussoellaceae* only the LSU was employed; for the family *Lophiostomataceae* three concatenated markers —ITS, *rpb2* and *tef1*— were used; for members of the families *Chaetomellaceae* and *Sympoventuriaceae* the concatenated markers employed were LSU and ITS; and for the phylogenetic analysis of the family *Pyrenochaetopsidaceae* the concatenated sequences of ITS, *rpb2* and *tub2* were employed. The best nucleotide substitution model for BI analysis were estimated using the program jModelTest (Posada 2008). The best model used for the *Amniculicolaceae* was the Symmetrical model with proportion of Invariable sites and Gamma distribution (SYM+I+G) for ITS; and the General Time Reversible with Gamma distribution (GTR+G)

for LSU and *tef1*. The best model used for the *Roussoellaceae* was the General Time Reversible with proportion of Invariable sites and Gamma distribution (GTR+I+G) for LSU. For the *Lophiostomataceae,* the best model was Kimura 2-parametrer with proportion of Invariable sites and Gamma distribution (K80+I+G) for ITS and *rpb2;* and GTR+G for *tef1.* The best model substitution for the *Chaetomellaceae* was SYM+I+G for ITS and K80+I+G for LSU. For the *Sympoventuriaceae* it was K80+I for ITS and LSU. For the *Pyrenochaetopsidaceae*, the Kimura 2-parametrer with proportion of Invariable sites (K80+I) was used for ITS, the SYM+I+G for *rpb2,* and the Hasegawa-Kishino-Yano with Gamma distribution (HKY+G) for *tub2.* The parameters used in Bayesian analysis were two simultaneous runs of 5,000,000 generations. The 50% majority rule consensus tree and posterior probability values (PP) were calculated after discarding the first 25% of the resulting trees. A PP value ≥ 0.95 was considered as significant (Hespanhol 2019). For ML analysis, support for internal branches was assessed by 1,000 ML bootstrapped pseudo replicates. Bootstrap support value (BS) ≥ 70% was considered significant. The novel nomenclature and novel taxonomic descriptions in MycoBank (http://www.mycobank.org).

Results

Phylogeny

First, a phylogenetic study was made including 91 LSU sequences, with 776 bp characters including gaps, of which 266 were parsimony informative sites, *Saccharomyces cerevisiae* NRRLY12632 and M16 were used as outgroup. The ML analysis was congruent with that obtained in the BI analysis, both displaying trees with similar topologies. The isolates were distributed across to two classes *Dothideomycetes* and *Leotiomycetes.* Within *Dothideomycetes,* a total of twelve families:

RESULTADOS

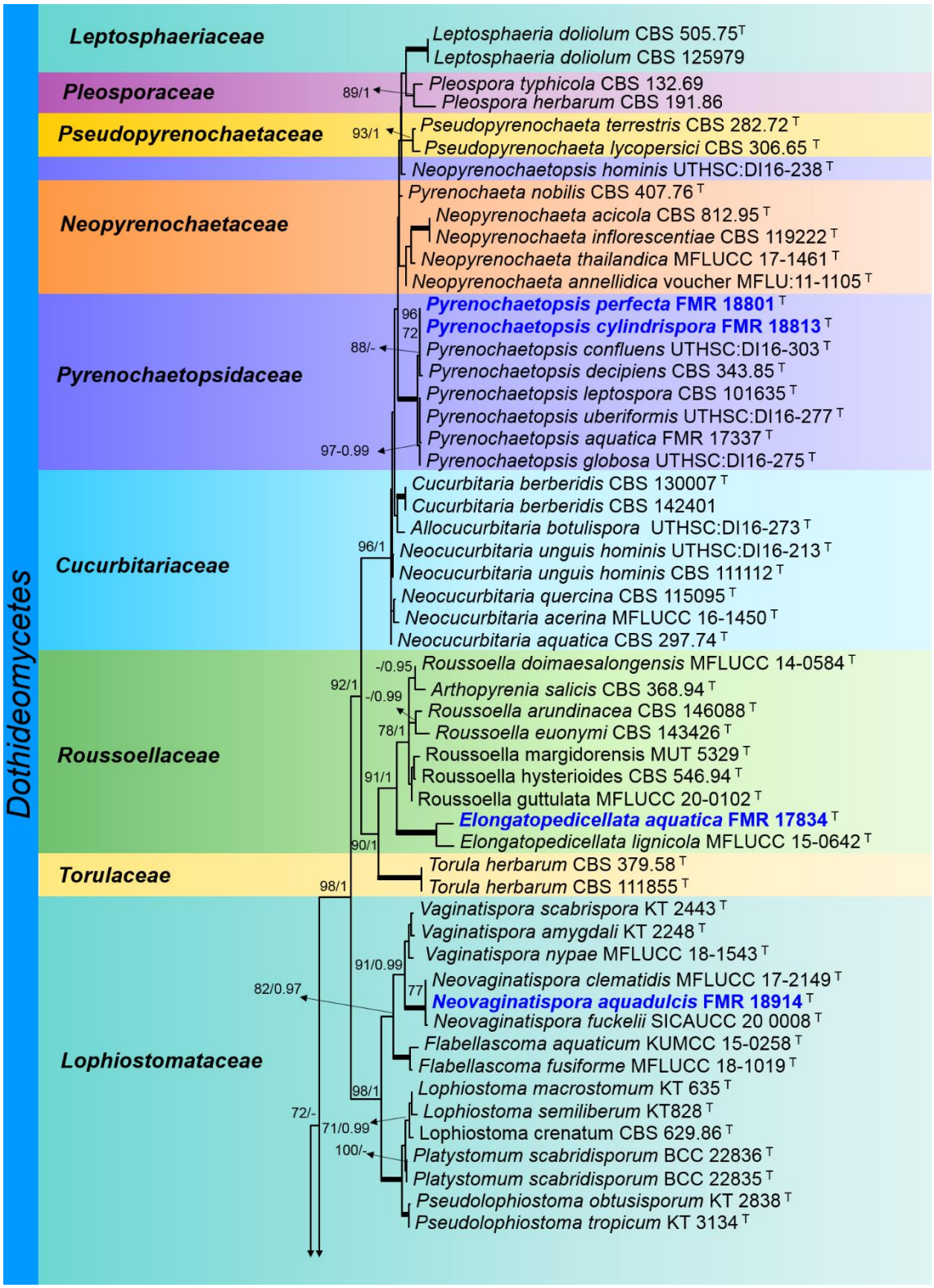

Figure 1. Phylogenetic tree inferred from a ML analysis inferred from LSU sequences of 91 strains representing thirteen in *Amniculicolaceae,* fifteen in *Chaetomellaceae,* eight in *Cucurbitariaceae,* five in *Neopyrenochaetaceae,* two in *Leptosphaeriaceae,* fifteen in *Lophiostomataceae,* two in *Pleosporaceae,* two in *Pseudopyrenochaetaceae*, nine in *Pyrenochaetopsidaceae,* nine in *Roussoellaceae,* six in

Sympoventuraceae, two in *Torulaceae* and one in *Venturaceae*. Support in nodes is indicated above by bootstrap values of 70% or higher. Newly proposed taxa are given in **blue**. Type strains are indicated by a superscript T. The tree was rooted with *Saccharomyces cerevisiae* NRRLY12632 and M16. Alignment length 776 bp.

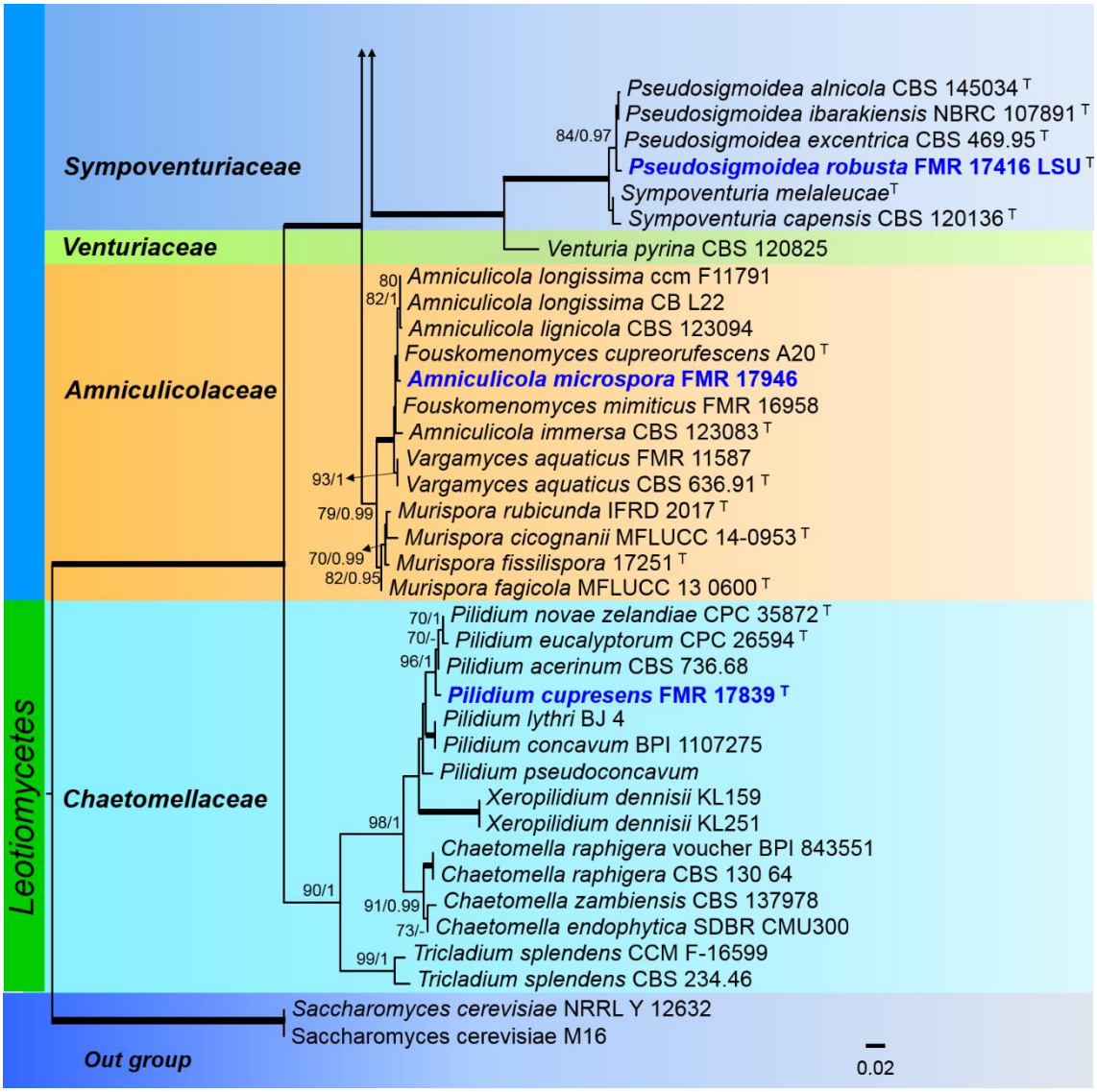

Figure 1. (Continued)

The concatenated phylogenetic tree for the members of the *Amniculicolaceae* comprised 20 ingroups of species with a total of 1,117 characters including gaps, from which 187 bp were parsimony informative (113 for ITS and 74 for *tef1*). The BI analysis showed similar tree topology and was congruent with that obtained in the ML analysis. For the BI multi-locus analysis, a total of 1,141 trees were sampled after the burn-in with

a stop value of 0.01. In the phylogenetic tree (Fig. 1) the *Amniculicolaceae* formed a full-supported clade (100% BS/1 PP) including all genera accepted in the family: *Amniculicola*, *Fouskomenomyces*, *Murispora* and *Vargamyces*. Our strain FMR 17946 was placed in the same terminal clade than *A. guttulatta.*

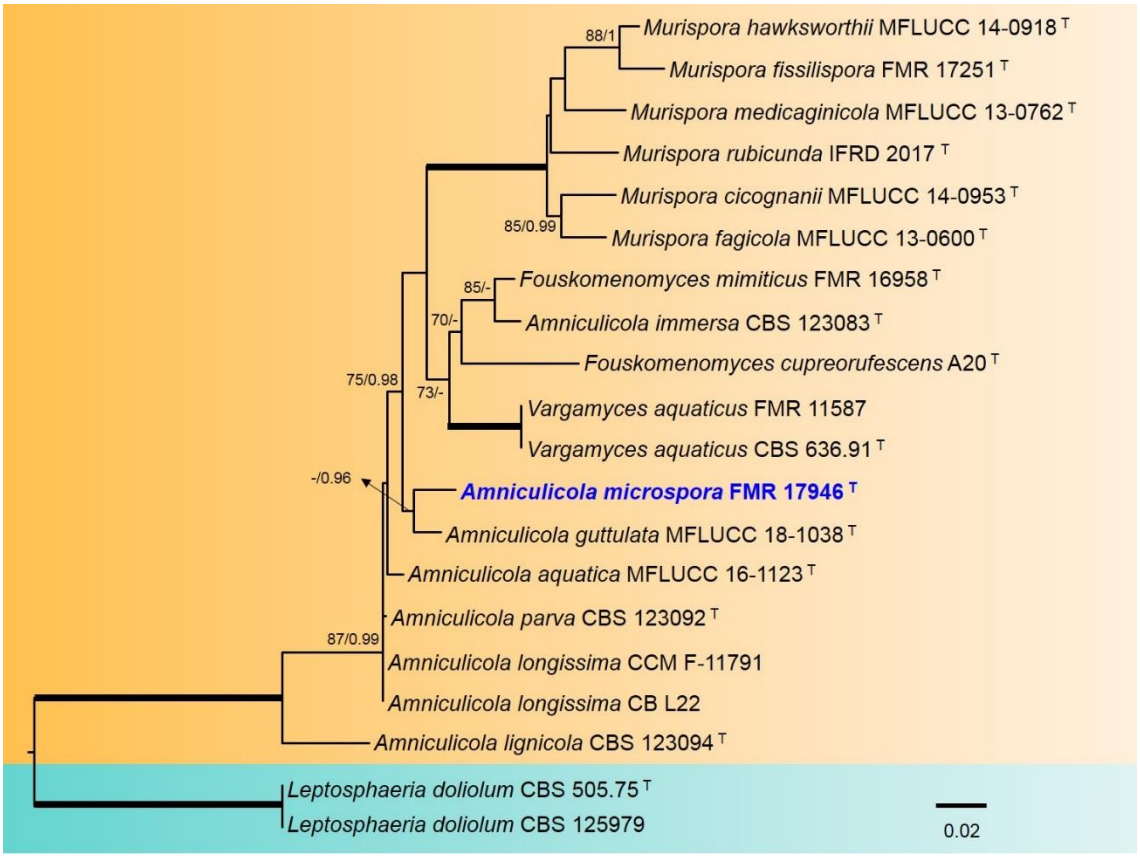

Figure 2. Phylogenetic tree inferred from a ML analysis based on a concatenated alignment of ITS and *tef-1* sequences of 20 strains representing species in *Amniculicolaceae*. The Bayesian posterior probabilities (PP) above 0.95 and the RAxML bootstrap support values (BS) above 70% are given at the nodes (PP/BS). Fully supported branched (1 PP/100 BS) are indicated in thicker lines. Newly proposed taxa are given in **blue**. Type strains are indicated by a superscript T. The tree was rooted with *Leptosphaeria dolium* CBS 505.75 and CBS 125979. Alignment length 1,117 bp.

The phylogenetic analysis of the members of the *Roussoellaceae* included nucleotide sequences from 18 species with a total of 845 characters including gaps, from which 80 bp were parsimony informative. In the phylogenetic tree (Fig. 2) our strain FMR 17834 was placed in the same full-supported terminal clade than the type species of the genus *Elongatopedicellata* (*E. lignicola*).

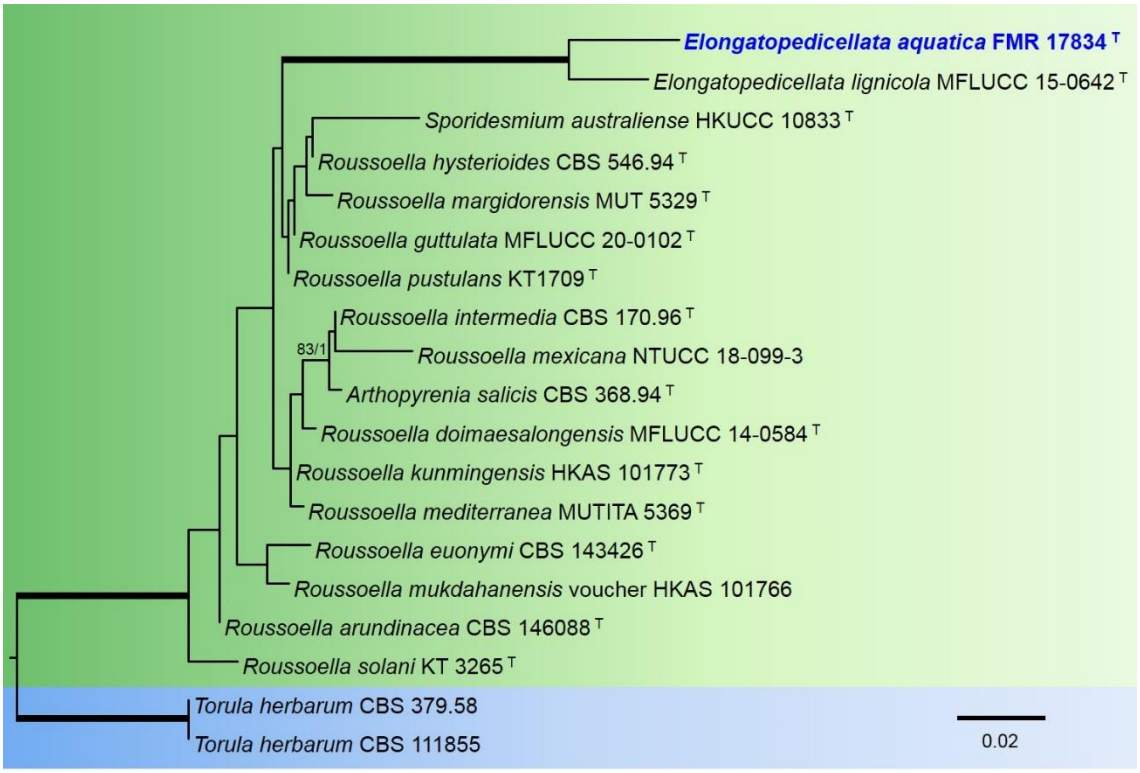

Figure 3. Phylogenetic tree inferred from a ML analysis inferred from LSU sequences of 18 strains representing species in *Roussoellaceae*. Support in nodes is indicated above by bootstrap values of 70% or higher. Newly proposed taxa are given in **blue**. Type strains are indicated by a superscript T. The tree was rooted with *Torula herbarum* CBS 379.58 and CBS 111855. Alignment length 845 bp.

For the *Lophiostomataceae,* the alignment included 15 ingroups of species with a total of 2,265 characters including gaps, from which 609 bp parsimony informative sites (178 for ITS, 298 for *rpb2,* 129 for *tub2*). Both BI and ML analyses displayed a similar tree topology. For the BI multi-locus analysis, a total of 402 trees were sampled after the burn-stop value of 0.01. Into the *Lophiostomataceae* (Fig. 3), the genus *Neovaginatispora* (100% BS/1 PP) included the two previously accepted species plus our strain FMR 18914.

RESULTADOS

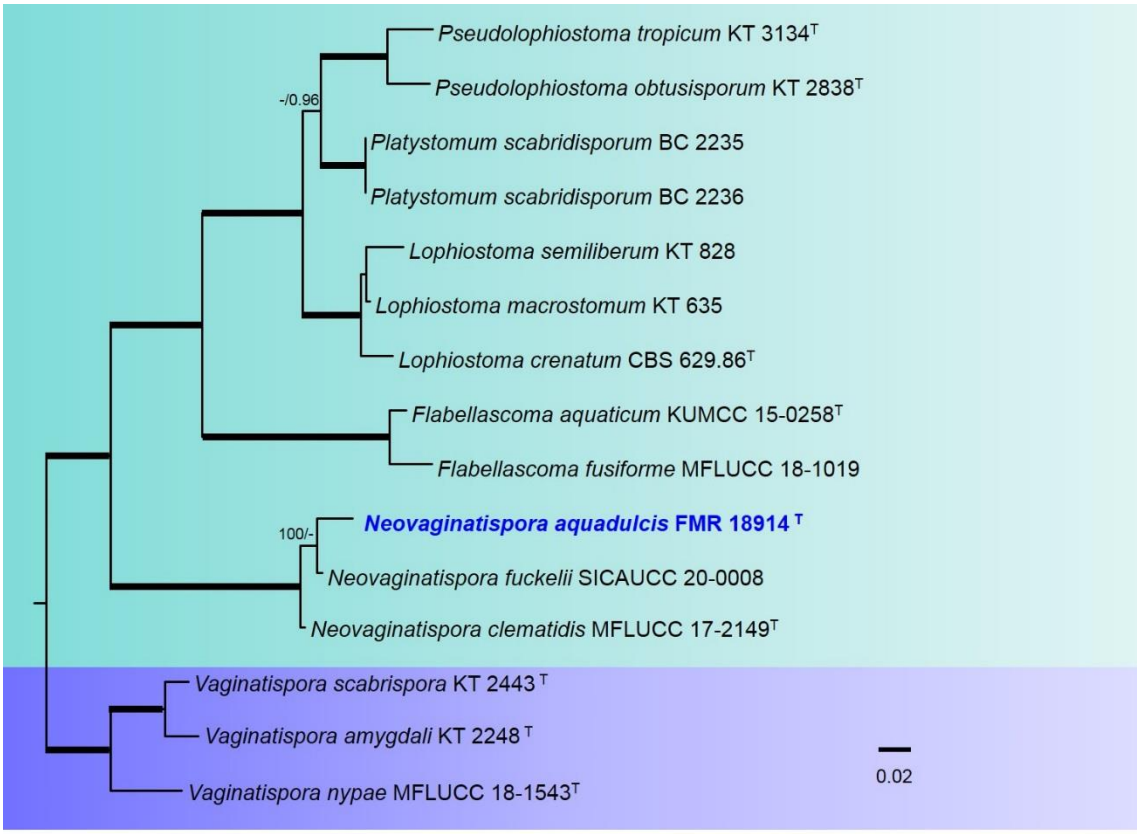

Figure 4. Phylogenetic tree inferred from a ML analysis based on a concatenated alignment of ITS, *rpb2* and *tef-1* sequences of 15 strains representing species in *Lophiostomataceae*. The Bayesian posterior probabilities (PP) above 0.95 and the RAxML bootstrap support values (BS) above 70% are given at the nodes (PP/BS). Fully supported branched (1 PP/100 BS) are indicated in thicker lines. Newly proposed taxa are given in **blue**. Type strains are indicated by a superscript T. The tree was rooted with *Vaginatispora amygdali* KT 2248, *V. nypae* MFLUCC 18-1543 and *V. scabrispora* KT 2443. Alignment length 2,265 bp.

To build the phylogenetic tree of the *Chaetomellaceae* 18 ITS_LSU concatenated sequences with a total of 1,246 characters, from which 277 bp parsimony informative sites (172 for ITS and 105 for LSU), were included. Both ML and BI analyses showed similar tree topology. For the BI multilocus analysis, a total of 134 trees were sampled after the burn-stop value of 0.01. In that phylogenetic tree (Fig. 4), the genus *Pilidium* (100%BS/1PP) included all species with sequences available as well as our strain FMR 17839, which was solely placed in a basal terminal branch.

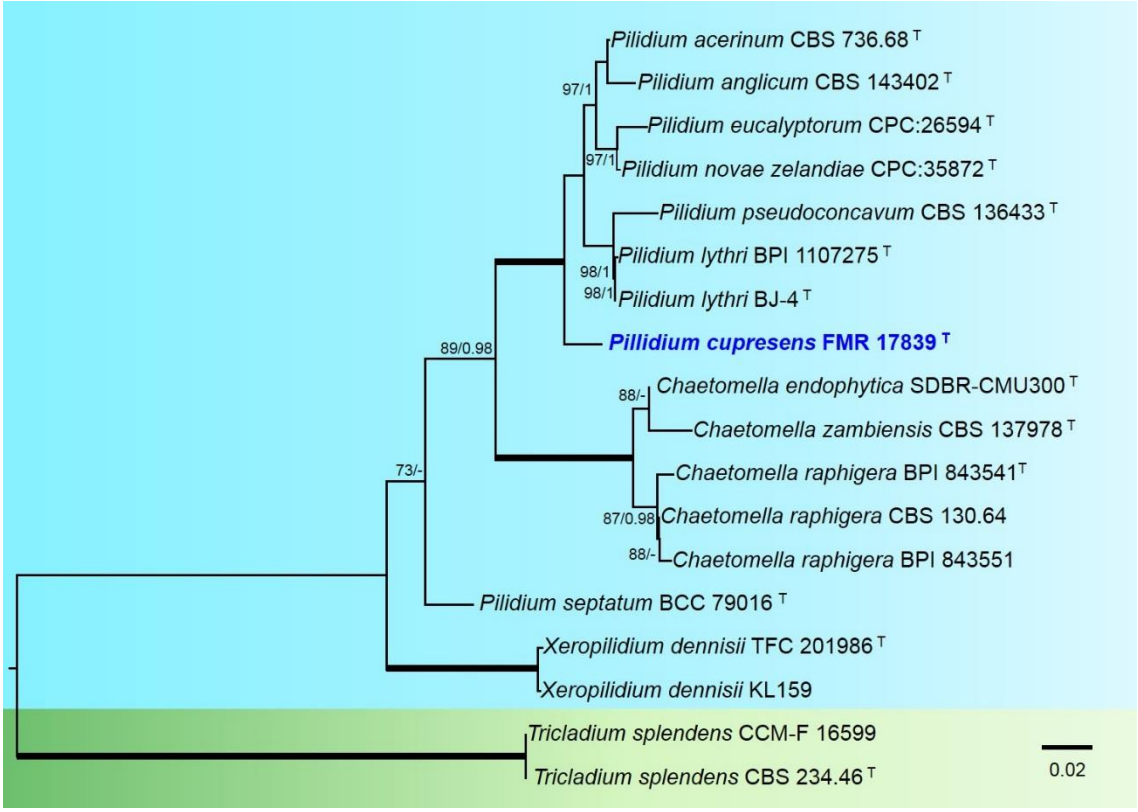

Figure 5. Phylogenetic tree inferred from a ML analysis based on a concatenated alignment of ITS, and LSU sequences of 18 strains representing species in *Chaetomellaceae*. The Bayesian posterior probabilities (PP) above 0.95 and the RAxML bootstrap support values (BS) above 70% are given at the nodes (PP/BS). Fully supported branched (1 PP/100 BS) are indicated in thicker lines. Newly proposed taxa are given in **blue**. Type strains are indicated by a superscript T. The tree was rooted with *Tricladium splendens* CBS 234.46 and CCM-F 16599. Alignment length 1,246 bp.

The phylogenetic analysis of the *Sympoventuriaceae* included sequences from eight species, with 1,325 characters including gaps, from which 161 bp parsimony informative sites (93 for ITS and 68 for LSU). Both BI and ML analyses displayed a similar tree topology. For the BI multi-locus analysis a total of 29 trees were sampled after the burn-stop value of 0.01. In our phylogenetic tree (Fig. 5), the genus *Pseudosigmoidea* formed a well-supported clade (99% BS/1 PP), and included all accepted species (except from the type species *P. cranei)* plus our strain FMR 17416.

RESULTADOS

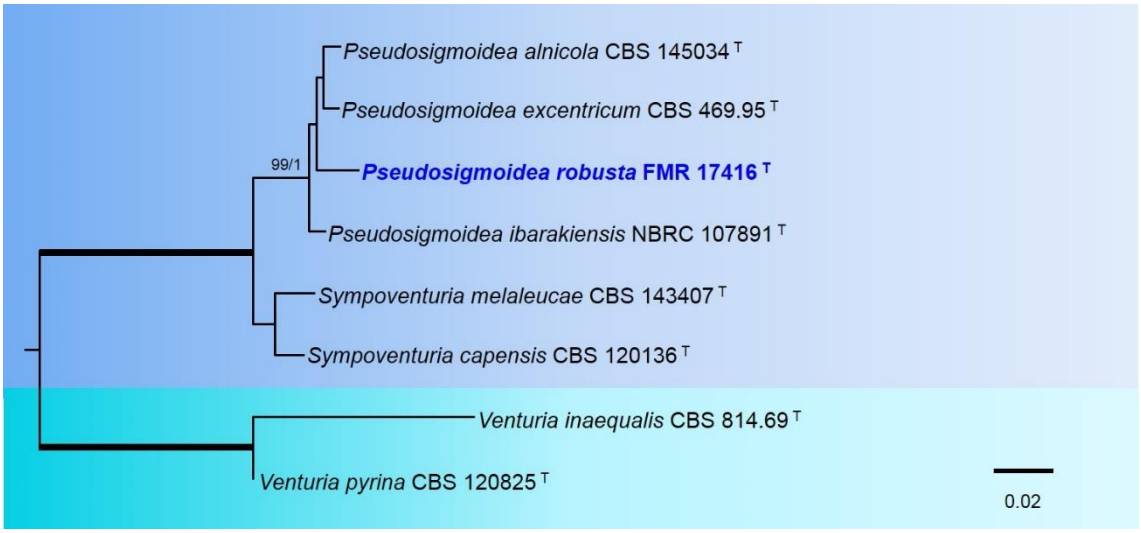

Figure 6. Phylogenetic tree inferred from a ML analysis based on a concatenated alignment of ITS, and LSU sequences of eight strains representing species in *Sympoventuriaceae*. The Bayesian posterior probabilities (PP) above 0.95 and the RAxML bootstrap support values (BS) above 70% are given at the nodes (PP/BS). Fully supported branched (1 PP/100 BS) are indicated in thicker lines. Newly proposed taxa are given in **blue**. Type strains are indicated by a superscript T. The tree was rooted with *Venturia inaequalis* CBS 814.69 and *V. pyrina* CBS 120825. Alignment length 1,325 bp.

The tree for the *Pyrenochaetopsidaceae* was built using the concatenated sequences from 24 species, totalizing 1,488 characters including gaps, from which 471 bp were parsimony informative (82 for ITS, 281 for *rpb2*, and 107 for *tub2*). The BI analysis showed similar tree topology and was congruent with that obtained by the ML analysis. A total of 1,702 trees were sampled after the -stop value 0.01. In the phylogenetic tree (Fig. 6), the genus *Pyrenochaetopsis* formed a full-supported clade including all previously accepted species and our strains FMR 18801 and FMR 18913, which were placed in two different and full-supported terminal branches, FMR 18913 together *P. globosa* and *P. uberiformis*, and FMR 18801 jointly to *P. confluens*.

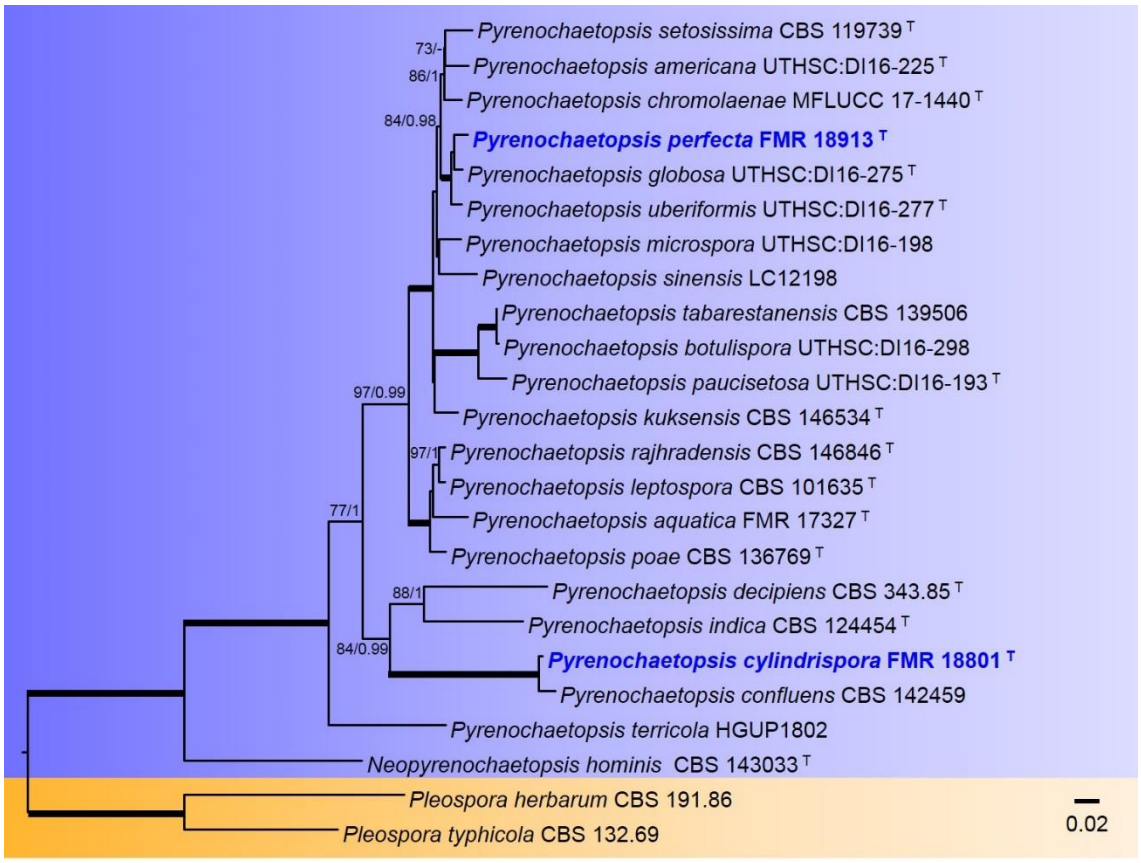

Figure 7. Phylogenetic tree inferred from a ML analysis based on a concatenated alignment of ITS, *rpb2* and *tub2* sequences of 24 strains representing species in *Pyrenochaetopsidaceae*. The Bayesian posterior probabilities (PP) above 0.95 and the RAxML bootstrap support values (BS) above 70% are given at the nodes (PP/BS). Fully supported branched (1 PP/100 BS) are indicated in thicker lines. Newly proposed taxa are given in **blue**. Type strains are indicated by a superscript T. The tree was rooted with *Pleospora herbarum* CBS 191.86 and *P. typhicola* CBS 132.69. Alignment length 1,488 bp.

Taxonomy

Amniculicolaceae Y. Zhang ter, C.L. Schoch, J. Fourn., Crous & K.D. Hyde, Studies in Mycology 64: 95 (2009a). MycoBank MB515469.

Type genus: Amniculicola Y. Zhang ter & K.D. Hyde, Mycol. Res. 112: 1189 (2008). MycoBank MB 511328.

Type species: Amniculicola lignicola Y. Zhang ter & K.D. Hyde, Mycological Research 112 (10): 1189 (2008). MycoBank MB 511330.

Because the synaasexual stage of the genus *Amniculicola* has not been reported and descripted yet, we emended the generic description.

Amniculicola Y. Zhang & K.D. Hyde, emended by V. Magaña-Dueñas, Cano and Stchigel.

Saprobic on wood in freshwater habitats. *Sexual stage*: *Ascomata* solitary to gregarious, immersed to nearly superfcial, black, uniloculate, subglobose to conical, glabrous, ostiolate, with or without two tuberculate fared lips surrounding a slit-like ostiole, sometimes with a fattened base not easily removed from the substrate, usually staining the woody substrate in purple tinge. *Peridium* 2-layered, outer layer composed of heavily pigmented thick-walled cells of *textura angularis*, inner layer composed of hyaline thin-walled cells of *textura angularis*. *Pseudoparaphyses* dense, trabeculate, filiform, persistent, hyaline, embedded in mucilage, anastomosing between and above the asci. *Asci* 8-spored, bitunicate, fissitunicate, cylindrical to narrowly fusoid, short pedicellate. *Ascospores* mostly uniseriate, fusoid, hyaline, septate, symmetrical, smooth- and thin-walled, surrounded by a hyaline, gelatinous sheath. *Asexual stages*: anguillospora-like, *conidiophores* usually simple, *conidia* septate, hyaline, curved or sigmoid, tapering to the extreme; phoma-like, *conidiomata* pycnidial, dark brown, semi-immersed, solitary, scattered, pycnidial wall of *textura angularis*, globose to subglobose, *conidiogenous cells* phialidic, determinate, hyaline, globose to ampulliform, flask-shaped or cylindrical, *conidia* aseptate, hyaline, smooth- and thin-walled, globose to kidney-shaped or bacilliform.

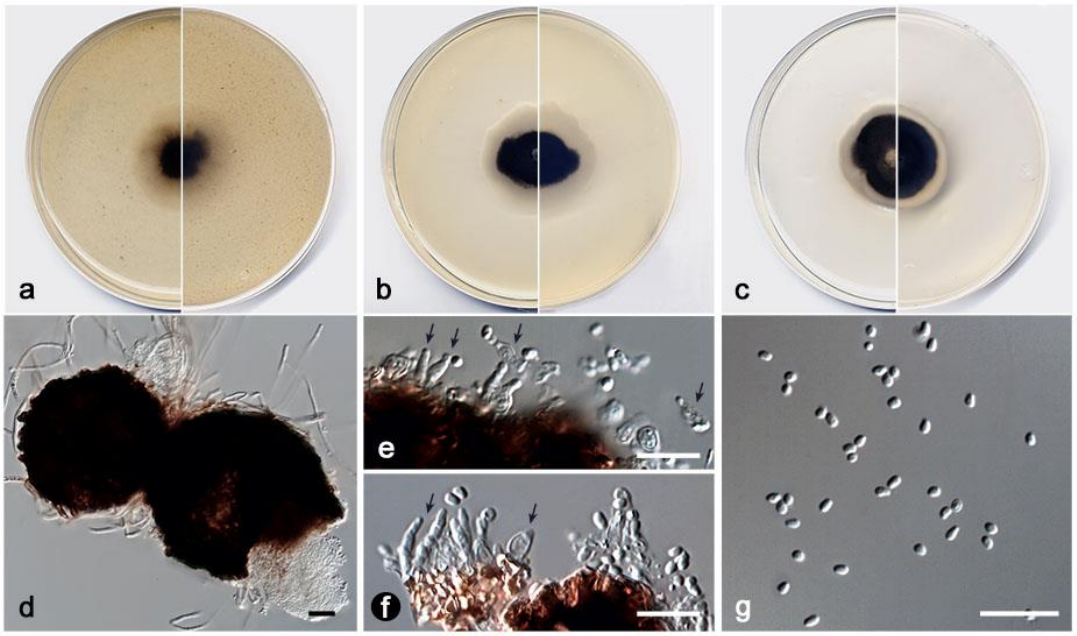

Figure 7. *Amniculicola microspora* FMR 17946. a. Colonies on OA; b. on MEA; c. on PDA (surface, left; reverse, right); d. Pycnidia; e, f. Conidiogenous cells (black narrows); g. Conidia. Scale bars = 10 µm.

Amniculicola microspora V. Magaña-Dueñas, Stchigel & Cano, sp. nov. FMR 17946. MycoBank MB842769 (Fig. 7).

Etymology: Referring to the small size of the conidia produced.

Type: Spain, Sevilla province, Parque Natural Sierra Norte, Cascadas del Huéznar, from plant debris into freshwater, May 2019, José F. Cano Lira, holotype ___, culture ex-type FMR 17946

Hyphae hyaline to pale brown, septate, branched, smooth- and thin-walled, 2–2.5 µm wide. *Conidiomata* pycnidial, dark brown, semi-immersed, solitary, scattered, globose to subglobose 75–120 × 70–130 µm; *conidiomata wall* 4–6-layered, 10–20 µm thick, with an outer layer of *textura angularis*, composed of brown to dark brown, flattened polygonal cells of 4–6 µm diam.; *conidiophores* absent; *conidiogenous cells* phialidic, determinate, hyaline, smooth- and thin-walled walled, flask-shaped, ampulliform or cylindrical, 4–6 × 4–8 µm; *conidia* aseptate, hyaline, smooth- and thin-walled, bacilliform, globose to kidney-shaped, 2–3 × 2–3 µm. *Chlamydospores* absent.

Culture characteristics: Colonies on PDA reaching 17 mm diam after 7 days at 25±1 ºC, flattened, velvety, margin regular, brownish grey to grey, border orange grey

(6C2/6E1/6B2); reverse brownish grey, border orange grey (6F2/6A2). Colonies on OA reaching 14 mm diam after 7 days at 25±1 ºC, flattened, velvety, margin regular, surface and reverse brownish beige to grey (6F3/6B1). Colonies on MEA reaching 10x15 mm diam after 7 days at 25±1 ºC, flattened, velvety, margins undulate, surface and reverse greyish brown to grey (6F3/6B1). Exopigment absent. Cardinal temperatures for growing: optimum 25 ºC, maximum 30 ºC, minimum 5 ºC.

Diagnosis: Morphologically, *A. microspora* differs from the rest of the species of the genus because is the only species that has a coelomycetous asexual stage.

Notes: Amniculicola microspora is phylogenetically close to *A. gutulata.* The differences in nucleotides between both concatenated (ITS-*tef1*) sequences is of 27 bp.

Key to freshwater *Amniculicola* species (modified from Dong et al. 2020)

1. Asexual stage present……..……..……2

1. Sexual stage only present……..……..……...3

2. Conidia elongate to sigmoidal, produced from conidiophores…….....*A. longissima*

2. Conidia bacilliform, globose to reniform, produced into asexual reproductive bodies (= pycnidia)……..…..*A. microspora*

3. Ascomata superficial……...……...4

3. Ascomata immersed……..*A. immersa*

4. Asci longer than 130 µm……...*A. lignicola*

4 Asci shorter than 130 µm……...……5

5. Substrate stained purple……..……*A. parva*

5. Substrate natural color……..……..6

6. Peridium 35–50 µm thick, ascospores 24–32 × 6–8 µm……*A. aquatica*

6. Peridium 27–35 µm thick, ascospores 23–27 × 5–7 µm……*A. guttulata*

Roussoellaceae J.K. Liu ,Phookamsak, D.Q. Dai & K.D. Hyde, Phytotaxa 181 : 7 (2014) MycoBank MB 804651.

Type genus Roussoella Sacc., Atti dell´Istituto Veneto Scienze 6: 410 (1888) MycoBank MB 4799.

Elongatopedicellata J.F. Zhang, J.K. Liu, K.D. Hyde & Z.Y. Liu, Fungal Diversity 75: 118 (2015) MycoBank MB 551484

Type species: Elongatopedicellata lignicola J.F. Zhang, J.K. Liu, K.D. Hyde & Z.Y. Liu, Fungal Diversity 75: 118 (2015) MycoBank MB 551485.

Because the asexual stage of the genus *Elongatopedicellata* has not been reported and descripted yet, we emended the generic description at next.

Elongatopedicellata J.F. Zhang, J.K. Liu, K.D. Hyde & Z.Y. Liu, emended by V. Magaña-Dueñas, Cano and Stchigel.

Hyphae hyaline to brown, septate, branched, smooth- and thin-walled. *Sexual stage*: *Ascomata* solitary to gregarious, scattered, immersed or erumpent, uniloculate, subglobose to obpyriform, coriaceous, with papillate ostiole; *peridial wall* 14–21 µm thick, composed of several layers of brown to dark brown, thick-walled cells, arranged in a *textura angularis*; *hamathecium* composed of 1–2 µm wide, filiform pseudoparaphyses, anastomosing between and above the asci, embedded in a gelatinous matrix; *asci* 8-spored, bitunicate, fissitunicate, fusiform-clavate, with a long pedicel, apically rounded, with a well-developed ocular chamber; *ascospores* 1–3 overlapping seriate, hyaline, fusiform, 1-septate, constricted at the septum, upper cell shorter and wider, lower cell long and narrow, surrounded by a mucilaginous sheath. *Asexual stage*: *conidiomata* pycnidial, semi-immersed, solitary, scattered, ostiolate, setose; *conidiomata wall* of *textura intricata*, pale brown from the base to the middle part of the fruiting body, then darkening to the top, globose; *conidiogenous cells* phialidic, determinate, hyaline, smooth-walled, flask-shaped to ampulliform; *conidia* aseptate, smooth-walled, hyaline to

pale brown, clavate, ovoid or kidney-shaped

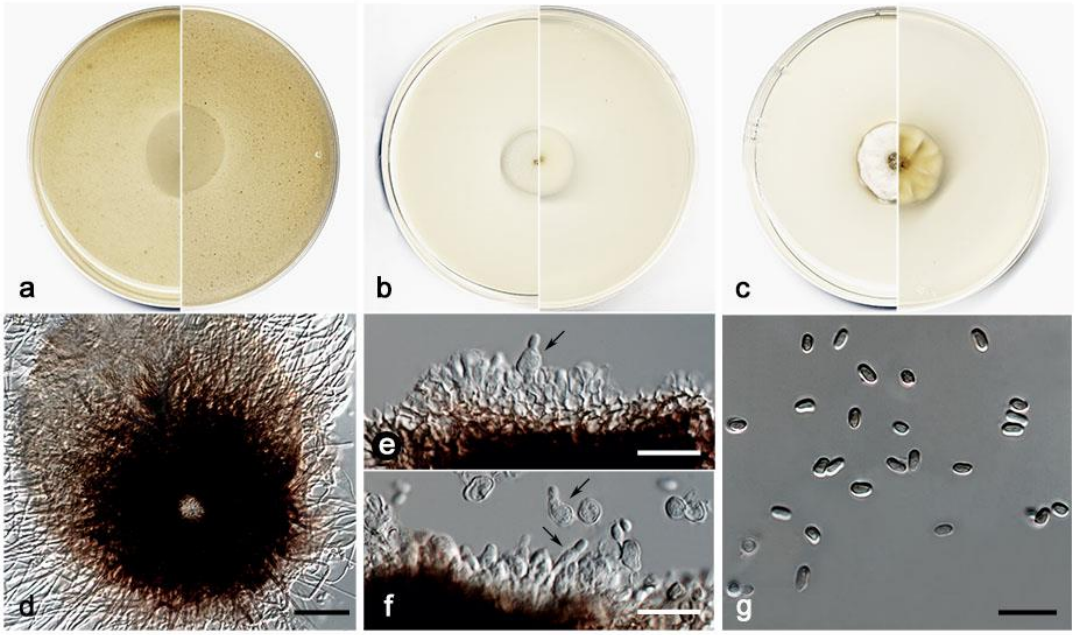

Figure 9. *Elongatopedicellata aquatica* FMR 17834. a. Colonies on OA; b. on MEA; c. and on PDA (surface, left; reverse, right); d. Pycnidium; e, f. Conidiogenous cells (black narrows); f. Conidia. Scale bars: d=50 µm, e-g=10 µm.

Elongatopedicellata aquatica V. Magaña-Dueñas, Cano & Stchigel, ***sp. nov.*** **FMR 17834** MycoBank MB842770. (Fig. 9).

Etymology. : From Latin *aquaticus*, referring to the habitat from which the fungus was isolated (freshwater).

Type: Spain, Tarragona province, Capafonts, de les Hortes river, from plant debris submerged in freshwater, Mar 2019, Viridiana Magaña Dueñas and Isabel Iturrieta González, holotype ___, culture ex-type FMR 17834.

Hyphae hyaline to light brown, septate, branched, smooth- and thin-walled, 1.0–2.5 µm wide. *Conidiomata* pycnidial, semi-immersed, solitary, scattered, ostiolate, setose, subglobose, 160–190 × 165–220 µm; *ostiole* 15–20 µm diam.; *setae* hyaline to brown, septate, erect, nodose, curved at the tip, sometimes narrowing towards the tip, 60–90 µm long, mainly disposed around the ostiole; *conidiomata wall* 6–8-layered, 15–25 µm thick, with an outer layer of *textura intricata* composed of hyaline to dark brown hyphae

1.5–2.5 µm diam., and an inner wall composed by hyaline flattened cells, the basal part of the pycnidium is pale brown towards the middle part, then darkener towards the tip; *conidiophores* absent; *conidiogenous cells* phialidic, determinate, hyaline, smooth- and thin-walled, ampulliform to globose, 5–6 x 4–5 µm; *conidia* aseptate, smooth- and thin-walled, hyaline to pale brown, brown in mass, clavate, ovoid or reniform, 3.5–5.5 × 1.5–3.5 µm. *Chlamydospores* absent.

Culture characteristics: Colonies on PDA reaching 20 mm diam after 7 days at 25±1 ºC, convex, cotton, border regular, brownish grey (6D2) to white, border orange grey (6B2), reverse orange white (5A2), border orange grey (6B2). Colonies on OA reaching 22 mm diam after 7 days at 25±1 ºC, flattened, matte, margin regular, surface and reverse orange white (6A2). Colonies on MEA reaching 18 mm diam after 7 days at 25±1 ºC, flattened, matte, border regular, surface and reverse yellowish white (3A2). Exopigment absent. Cardinal temperatures for growing: optimum 25 ºC, maximum 35 ºC, minimum 5 ºC.

Diagnosis: Since the present work, the genus *Elongatopedicellata* was monospecific. *Elongatopedicellata lignicola,* its type species, was described only producing asexual stage on wood (Ariyawansa et al. 2015). In our study, we report a new species, *E. aquatica*, characterized by the production of an asexual coelomycetous stage on the natural substrate as well as *in vitro.*

Notes: The difference nucleotides between LSU sequences of *E. lignicola* and *E. aquatica* is of 27 bp.

Lophiostomataceae Sacc, Sylloge Fungorum 2: 672 (1883) MycoBank MB 80966.

Type genus: Lophiostoma Ces. & De Not., Commentario della Società Crittogamologica Italiana 1: 219 (1863). MB 2933.

Neovaginatispora A. Hashim., K. Hiray. & Kaz. Tanaka, Stud. Mycol. 90: 167 (2018). MycoBank MB 823147.

Type species: *Neovaginatispora fuckelii* (Sacc.) A. Hashim., K. Hiray. & Kaz.

Tanaka, Stud. Mycol. 90: 167 (2018). MycoBank MB 823148.

≡ *Lophiostoma fuckelii* Sacc., Michelia 1(no. 3): 336 (1878)

=*Vaginatispora fuckelii*(Sacc.) Thambug.et al., Fungal Diversity 74: 243. 2015.

Because the asexual stage of *Neovaginatispora* has not been reported and descripted yet, we emend the description of this genus as next.

Neovaginatispora A. Hashim., emended by V. Magaña-Dueñas, Cano and Stchigel.

Mycelium superficial to immersed, composed of septate, smooth- and thin-walled, pale brown, branched hyphae. *Sexual stage*: *Ascomata* solitary, semi-immersed to erumpent, black, coriaceous, ostiolate, subglobose; *ostiole* rounded or slit-like, central, with a pore-like opening; *peridium* uneven in width, thinner at the base, two-layered, outer layer fusing with the host cells, inner layer comprising hyaline cells of *textura angularis; hamathecium* composed of numerous, cellular, hypha-like, septate pseudoparaphyses; *asci* 8-spored, bitunicate, fissitunicate, cylindric-clavate, with a short, bulbous pedicel, with an indistinct ocular chamber; *ascospores* uni- to biseriate, two- to multi-celled, constricted at the septa, hyaline, smooth- and thin-walled, fusiform with acute ends, with globose appendages at both ends*. Asexual stage*: *Conidiomata* pycnidial, brown, towards the ostiole dark brown, semi-immersed, solitary, scattered, globose, ostiolate; *conidiomata wall* of *textura angularis*, composed of brown to dark brown polygonal cells; *conidiogenous cells* phialidic, determinate, hyaline, smooth- and thin-walled, globose; *conidia* aseptate, hyaline, smooth- and thin-walled, guttulate. *Chlamydospores* abundant, aseptate, terminal and intercalary, sometimes in chains, thick wall, brown, globose to clavate.

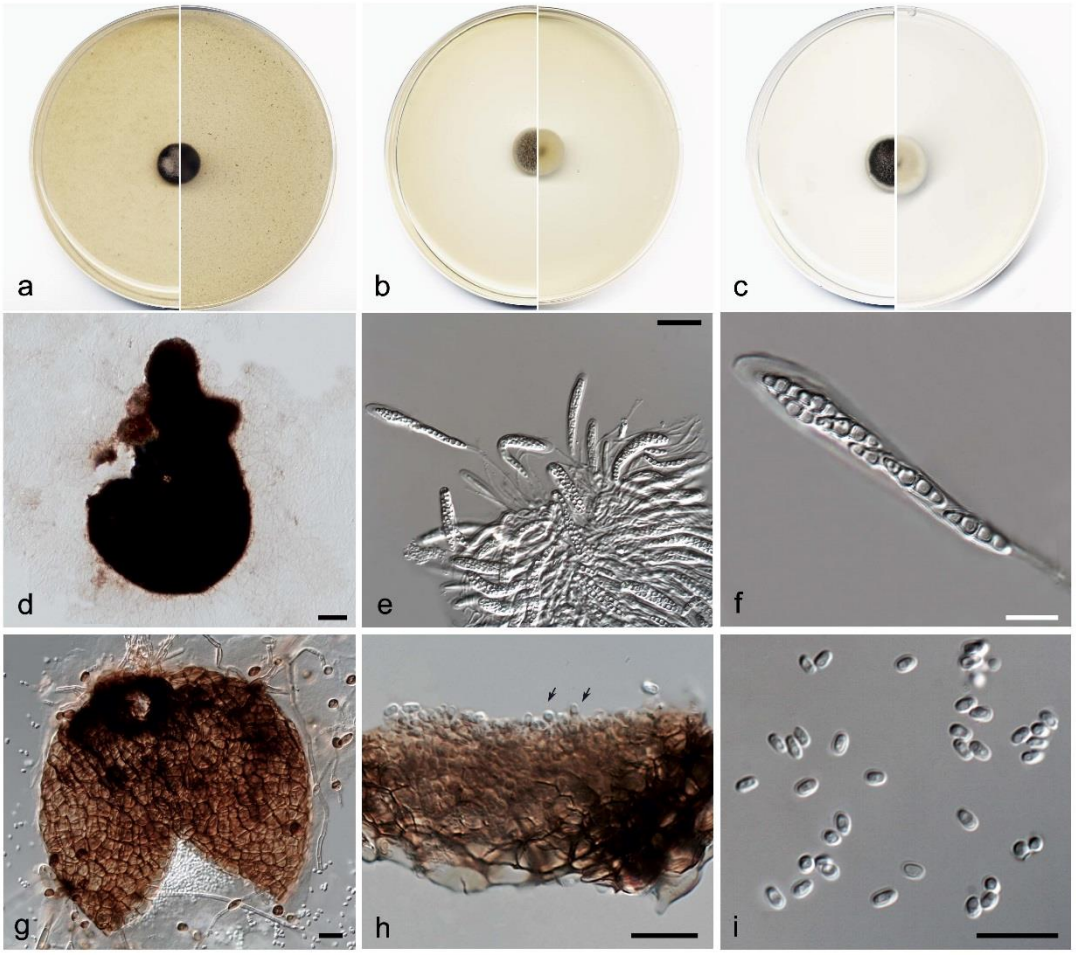

Figure 10. *Neovaginatispora aquadulcis* FMR 18914. a. Colonies on OA; b. on MEA; c. and on PDA (surface, left; reverse, right); d. Ascomata, e, f. Asci; g. Pycnidium; h. Conidiogenous cells (black narrows); i. Conidia. Scale bars: d, g=50 µm, e=25 µm, f, h, i=10 µm.

Neovaginatispora aquadulcis V. Magaña-Dueñas, Cano & Stchigel, sp. nov. FMR 18914. MycoBank MB842771 (Fig. 10).

Etymology. From Latin *aqua*-, water; and -*dulcis*, sweet, because of the origin of the fungus.

Type: Spain, Castellón province, Burriana, Clot de la Mare de Déu, from plant debris submerged into freshwater, Mar 2021, Alan Omar Granados Casas and Ana Fernández Bravo, holotype ___, culture ex-type FMR 18914.

Hyphae septate, pale brown, branched, smooth- and thin-walled, 2–3 µm wide. *Sexual stage*: *ascomata* perithecial, immersed to semi-immersed, solitary, dark brown, papillate,

glabrous, pyriform, 400–550 × 300–325 µm, neck conic-truncate, 100–125 × 90–100 µm; *peridial wall* 3–4-layered, 40–60 µm thick, outer wall of *textura angularis* composed of dark brown flattened polygonal cells 6-8 µm diam; *hamathecium* comprising numerous hyaline, filamentous, septate, branched paraphyses at the base, 1–1.5 µm wide; *asci* 8-spored, bitunicate, cylindrical to cylindrical-clavate, 50–70 × 8–10 µm, without apical structures; *ascospores* 3-septate, hyaline, fusiform, 15 × 4–4.5 µm, with elongated appendages at end. *Asexual stage: conidiomata* pycnidial, brown, towards the ostiole dark brown, semi-immersed, solitary, scattered, ostiolate, globose, 165–180 × 160–170 µm; *ostiole* 20–30 µm diam.; *conidiomata wall* 4–6-layered, 15–20 µm thick, with an outer layer of *textura angularis* composed of brown to dark brown polygonal cells 5–10 µm diam.; *conidiophores* absent; *conidiogenous cells* phialidic, determinate, hyaline, smooth- and thin-walled, globose, 5–6 × 4–5 µm; *conidia* aseptate, hyaline, smooth- and thin-walled, guttulate, 2.5–3.5 × 1.5–2.5 µm. *Chlamydospores* abundant, aseptate, terminal and intercalary, sometimes in chains, thick wall, brown, globose to clavate 6–8 × 5–6 µm.

Culture characteristics: Colonies on PDA reaching 8–10 mm diam. after 7 days at 25±1 ºC, convex, velvety, border regular, grey (30E1) border white, reverse orange grey (6B2). Colonies on OA reaching 6–9 mm diam. after 7 days at 25±1 ºC, convex, velvety, border regular, surface and reverse brownish grey (5F2). Colonies on MEA reaching 7–10 mm diam after 7 days at 25±1 ºC, convex, velvety, border regular, surface and reverse grey border orange grey (6D1/6B2). Exopigment absent. Cardinal temperatures for growing: Optimum 25 ºC, maximum 35 ºC, minimum 5 ºC.

Diagnosis: Neovaginatispora aquadulcis is the only species of the genus that produces both asexual and sexual morphs *in vitro.* Furthermore, our species is characterized by the production of ascospores 1–3-septate, unlike the reported species (that are only 1-septate).

Notes: Differences in nucleotides between *N. aquadulcis* and *N. fuckelii* and *N. clematidis* ITS-*rpb2-tef1* concatenated sequences are 47 and 25 bp, respectively.

Chaetomellaceae Baral, P.R. Johnst. & Rossman, Index Fungorum 225: 1 (2015). MycoBank MB 551076

Type genus: Chaetomella Fuckel, Fungi Rhenani Exsiccati. Supplementi Fasc. 5: no. 1962 (1867). MycoBank MB 7575.

Pilidium Kunze in Kunze & Schmidt, Mykol. Hefte 2: 92 (1823). MycoBank MB 9395

≡ *Sclerotiopsis* Speg., An. Soc. Cient. Argent. 113: 14 (1882).

= *Hainesia* Ellis & Sacc., in Saccardo, Syll. fung. (Abellini) 3: 698 (1884).

= *Discohainesia* Nannf., Nova Acta Regiae Soc. Sci. Upsal., Ser. 48: 88 (1932).

Type species: Pilidium acerinum Kunze in Kunze & Schmidt, Mykol. Hefte 2: 92 (1823). MycoBank MB 178919.

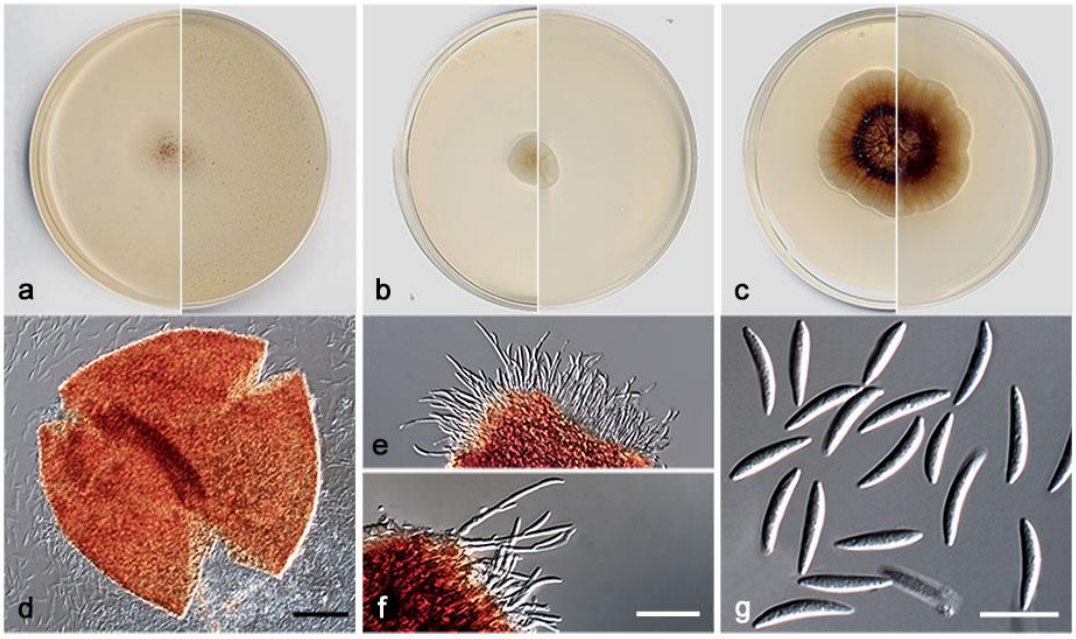

Figure 11. *Pillidium cuprescens* FMR 17839. a. Colonies on OA; b. MEA; c. and PDA after two weeks at 25±1 ºC (surface, left; reverse, right); d. Pycnidium; e, f. Conidiophores; g. Conidia. Scale bars: d=50 µm, e-g=10 µm.

Pilidium cuprescens V. Magaña-Dueñas, Cano & Stchigel, sp. nov. FMR 17839. MycoBank MB842772 (Fig. 11).

Etymology. Referring to the copper-colored conidiomata.

Type: Spain, Sevilla province, Parque Natural Sierra Norte, Cascadas del Huéznar, from plant debris in freshwater, May 2019, José F. Cano Lira, holotype ___, culture ex-type FMR 17839.

Hyphae hyaline to subhyaline, septate, branched, smooth- and thin-walled, 2–2.5 µm wide. *Conidiomata* pycnidial, copper-colored, semi-immersed, solitary, scattered, non-ostiolate, globose to subglobose 300–360 × 280–350 µm; *conidiomata wall* 4–6-layered, 10–20 µm thick, with an outer layer of *textura angularis*, composed of copper-colored, flattened polygonal cells of 4–6 µm diam; c*onidiophores* hyaline, septate, straight or sinuous to slightly curved, some branching at the base, 15–30 × 1–2 µm; *conidiogenous cells* phialidic, integrated to the conidiophore, terminal and lateral, hyaline, smooth- and thin-walled; *conidia* aseptate, hyaline, smooth- and thin-walled, fusiform, 10–15 × 2–3 µm. *Chlamydospores* absent.

Culture characteristics: Colonies on PDA reaching 25 mm diam after 7 days at 25±1 ºC, convex, concentrically radiate, margin lobed, greyish orange to brownish orange with a ring reddish brown (6B3/6C4/8F7), border orange grey (6B2); reverse brown to brownish orange (6E6/6C3), border orange grey (6B2). Colonies on OA reaching 10 mm diam after 7 days at 25±1 ºC, flattened, whit abundant production of pycnidia that provide a granular texture, margin regular, surface and reverse orange white (6A2). Colonies on MEA reaching 12 mm diam after 7 days at 25±1 ºC, flattened, matte, border regular, surface and reverse greyish yellow to yellowish white (4B4/4A2). Exopigment absent. Cardinal temperatures for growing: optimum 25 ºC, maximum 35 ºC, minimum 5 ºC.

Diagnosis: Species of *Pilidium* are characterized by producing two sort of conidiomata, pycnidial (pale brown when young, dark brown to black at maturity and uniloculate) and sporodochial (stalked, pale brown near base, becoming dark brown at apex, or reddish brown) ones (Rossman et al. 2004; Crous et al. 2013, 2017, 2019a; Marin-Felix et al. 2017). *Pilidium cuprescens* is easily distinguished from the rest of the species of that

genus by the production of closed copper-colored pycnidia.

Notes: The differences among the nucleotide sequences of *P. cuprescens* and the rest of the species of the genus are: from *P. acerinum*, 34 bp; from *P. anglicum*, 42 bp; from *P. eucalyptorum*, 42 bp*;* from *P. lythri*, 35 bp*;* from *P. novae-zelandie*, 38 bp; and from *P. pseudoconcavum*, 51 bp.

Sympoventuriaceae Y. Zhang ter et al., Fungal Diversity 51: 255. 2011. MycoBank MB 563117.

Type genus: Sympoventuria Crous & Seifert, Fungal Diversity 25: 31. 2007. MycoBank MB 501002.

Pseudosigmoidea K. Ando & N. Nakam., J. Gen. Appl. Microbiol., Tokyo 46: 55. 2000. MycoBank MB 28418.

Type species: *Pseudosigmoidea cranei* K. Ando & N. Nakam., J. Gen. Appl. Microbiol., Tokyo 46: 55. (2000). MycoBank MB 464825.

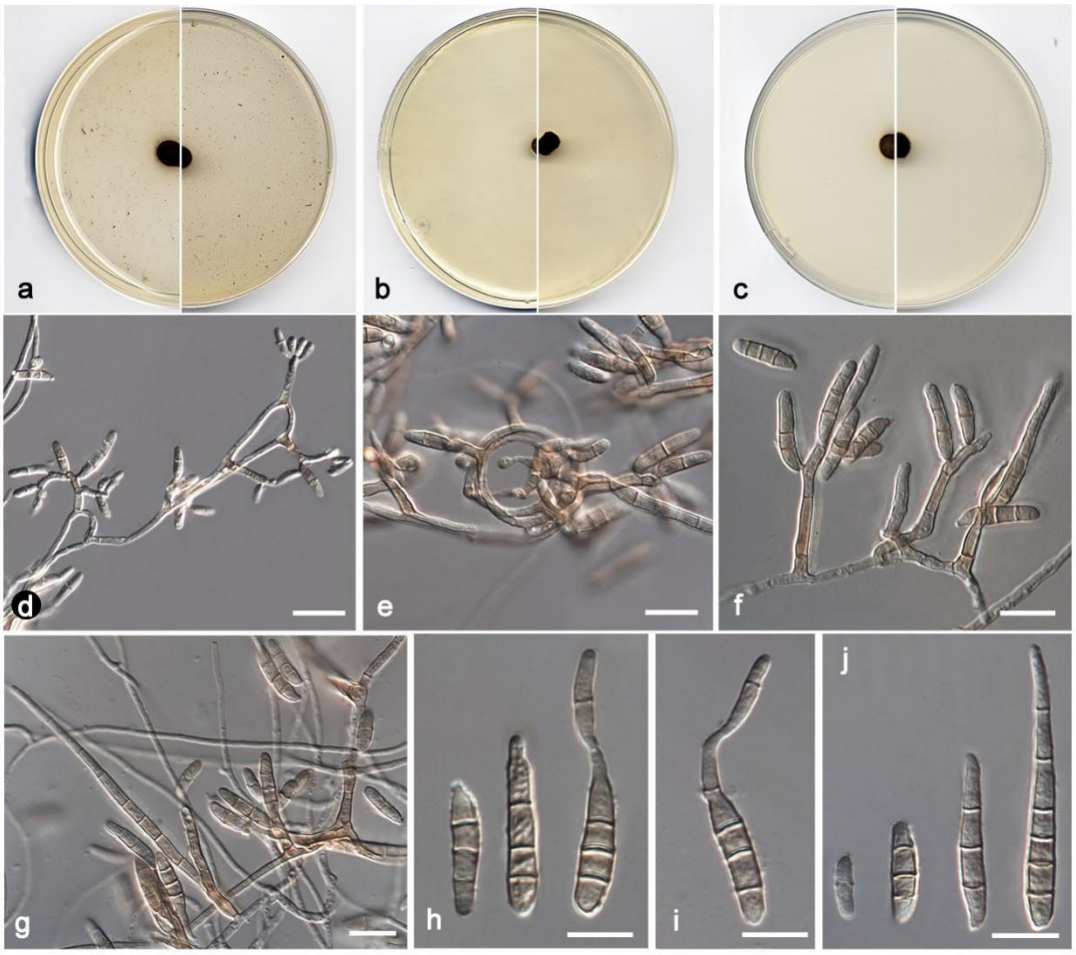

Figure 12. *Pseudosigmoidea* robusta FMR 17416. a, b, c. Colonies on OA, MEA and PDA, respectively, after two weeks at 25±1 ºC (surface, left; reverse, right); d–g. conidiophores; h, i. conidia displaying a secondary conidiogenesis; j. conidia showing variation in size. Scale bars: d-j = 10 µm.

Pseudosigmoidea robusta V. Magaña-Dueñas, Cano & Stchigel, *sp. nov.* FMR 17416. MycoBank MB842773 (Fig. 12).

Etymology. From Latin *robustum*, robust, due to the nature of the conidiophores and the conidia.

Type: Spain, Segovia province, Riaza river, from plant debris submerged in freshwater, May 2018, Viridiana Magaña Dueñas, holotype ___, culture ex-type FMR 17416 and CBS.

Hyphae hyaline to subhyaline, septate, branched, smooth- and thin-walled, 2.0–2.5 µm wide. *Conidiophores* pale brown, macronematous, mononematous, erect, straight or

curved, geniculate, cylindrical, rounded at the tip, septate, 8–15 × 2–3 µm; *conidiogenous cells* polyblastic, developing sympodially, terminal and intercalary, integrated to the conidiophore, 8–15 × 3–3.5 µm, denticulate; *conidia* holoblastic, 1–9-septate, mostly solitary, subhyaline to pale brown, smooth- and thin-walled, ovoid to obclavate, 8–65 × 2.5–3 µm, bearing a protruding foot-like scar due to a rhexolytic release from the conidiophore; in addition, the apical part of elongate conidia can develop a new conidiogenous *locus*, producing similar sort of conidia.

Culture characteristics: Colonies on PDA reaching 8–9 mm diam after 7 days at 25±1 ºC, umbonate, velvety, border regular, surface and reverse greyish brown (7F3). Colonies on OA reaching 7–9 mm diam after 7 days at 25±1 ºC, flattened, velvety, margin regular, surface and reverse dark brown (7F3). Colonies on MEA reaching 5–7 mm diam after 7 days at 25±1 ºC, flattened, floccose, border regular, surface and reverse greyish brown (7F3). Exopigment absent. Cardinal temperatures for growing: Optimum 20 ºC, maximum 30 ºC, minimum 5 ºC.

Diagnosis: Pseudosigmoidea robusta is easily morphologically recognizable because produces well-developed conidiophores in comparison than the other species of the genus. Also, the conidiophores have widely separated conidiogenous *loci* and produce conidia very variable in shape. Furthermore, the previously accepted species of *Pseudosigmoidea* produce larger conidia (80–250 × 3–4 µm in *P. alnicola*, 26–116.5 × 1.5–2.5 µm in *P. cranei*, and 68–133 × 4–8 µm in *P. ibarakiensis*) than *P. robusta* (8–65 × 2.5–3 µm).

Notes: Differences between the ITS-LSU nucleotide sequences of *P. robusta* and the other species of the genus are: from *P. alnicola*, 28 bp; from *P. excentricum*, 25 bp; and from *P. ibarakiensis*, 27 bp.

Pyrenochaetopsidaceae Valenzuela-Lopez, Cano-Lira, Guarro, Sutton, Wiederhold, Crous & Stchigel, Stud. Mycol. 90: 56 (2017). MycoBank MB 820308.

Because the sexual stage of the family has not been reported and descripted, we

emend the description of this family at next.

Sexual stage: ascomata immersed to semi-immersed, brown to dark-brown, ostiolate, outer wall of *textura angularis*; *hamathecium* comprising hyaline, septate, filamentous paraphyses; *asci* 8-spored, bitunicate, stipitate, cylindrical to clavate; *ascospores* 3–6 septate, hyaline, fusiform. *Asexual stage: conidiomata* pycnidial, pale brown to brown, solitary or confluent; *pycnidial wall* of *textura angularis*, glabrous or setose, subglobose to ovoid, with a non-papillate or papillate ostiolar neck; *conidiogenous cells* phialidic, hyaline, discrete or integrated in the septate, acropleurogenous conidiophores, *conidia* aseptate, hyaline, smooth- and thin-walled, ovoid, cylindrical to allantoid, guttulate.

Type genus: *Pyrenochaetopsis* Gruyter, Aveskamp & Verkley, Mycologia 102: 1076 (2010). MycoBank MB 514653.

Since the sexual stage of the *Pyrenochaetopsis* genus has not been reported, below we emend the generic description.

Sexual stage: ascomata immersed to semi-immersed, brown to dark-brown, ostiolate, outer wall of *textura angularis*; *hamathecium* comprising hyaline, septate, filamentous paraphyses; *asci* 8-spored, bitunicate, stipitate, cylindrical to clavate; *ascospores* 3–6 septate hyaline, fusiform. *Asexual stage*: *conidiomata pycnidial*, honey to citrine or olivaceous to olivaceous black, solitary to confluent, superficial or submerged, with a non-papillate or papillate ostiolar neck; *pycnidial wall* pseudoparenchymatous, setose, globose to subglobose; *conidiogenous cells* phialidic, hyaline, discrete and integrated in septate, acropleurogenous conidiophores; *conidia* aseptate, cylindrical to allantoid, guttulate.

Type species: *Pyrenochaetopsis leptospora* (Sacc. & Briard) Gruyter, Aveskamp & Verkley, Mycologia 102: 1076 (2010). MycoBank MB 514654.

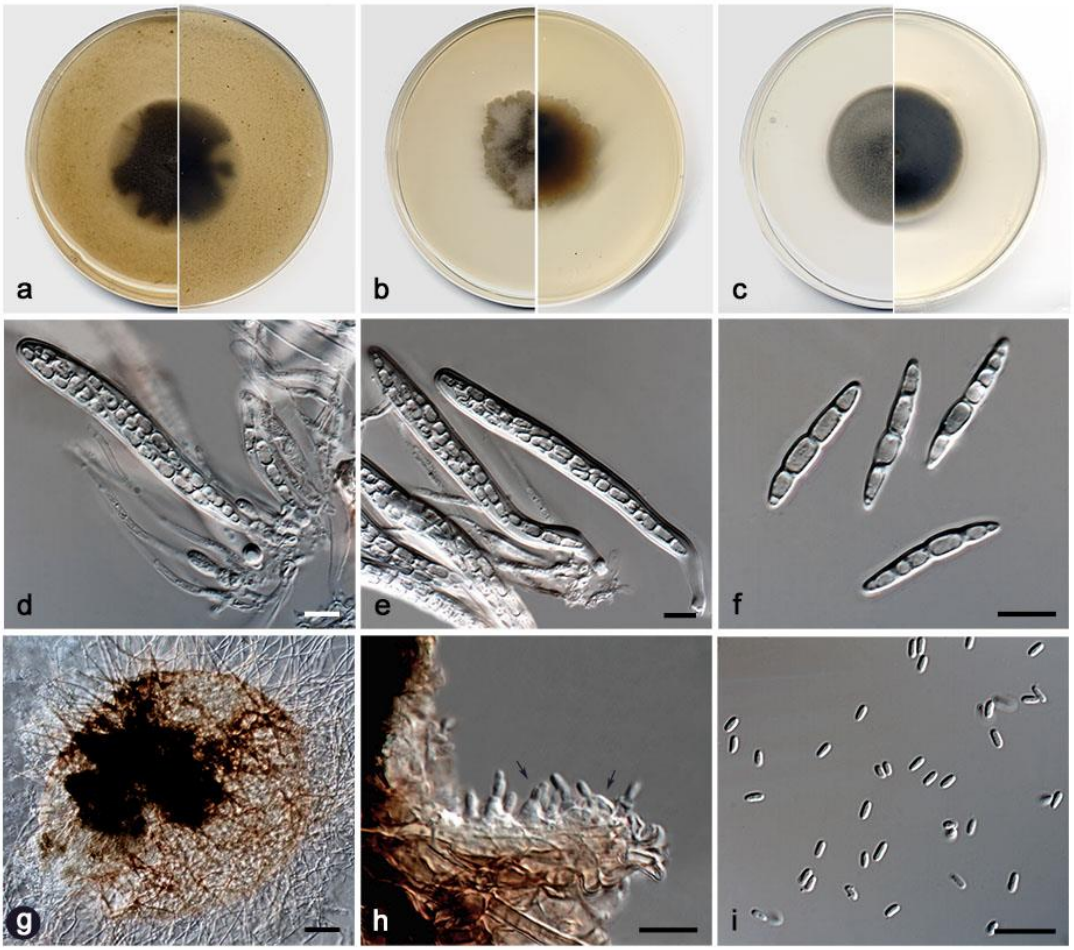

Figure 13. *Pyrenochaetopsis perfecta* FMR 18913.: a. Colonies on OA; b. on MEA; c. and on PDA (surface, left; reverse, right); d, e. Asci within ascospores; f. Ascospores; g. Pycnidium; h. Conidiogenous cells (black narrows); i. Conidia. Scale bars: g=25 µm, d-f, h, i=10 µm.

Pyrenochaetopsis perfecta V. Magaña-Dueñas, Stchigel & Cano, *sp. nov.* FMR 18913. MycoBank MB842774 (Fig. 13).

Etymology: From Latin *perfectus*, perfect, because the fungus produces both sexual and asexual stages.

Type: Spain, Castellón province, Burriana, Clot de la Mare de Déu, from freshwater submerged plant debris, Mar 2021, Alan Omar Granados Casas and Ana Fernández Bravo, holotype ___, culture ex-type FMR 18913.

Sexual stage: Ascomata immersed to semi-immersed, brown to dark-brown, ostiolate, outer wall of *textura angularis*; *hamathecium* comprising hyaline, septate, filamentous paraphyses, 1–3 µm wide; *asci* 8-spored, bitunicate, stipitate, cylindrical to clavate, 70–

100 × 8–10 µm, stipe 10–15 µm long; ascospores 3–6 septate hyaline, fusiform, 25–26 × 4–5 µm. *Asexual stage: conidiomata* pycnidial, semi-immersed, light-brown to brown with patches dark brown, solitary, scattered, setose, subglobose, 240–380 × 260–300 µm; *setae* pale brown to brown, septate, sinuous, thick-walled, 30–150 µm, rounded and curved at the tip; *conidiomata wall* 4–6-layered, 15–20 µm thick, outer layer of *textura angularis* composed of light brown to dark brown, flattened polygonal cells of 8–10 µm diam., wrapped in a framework of nodose, branching, sinuous and thick-walled hyphae; *conidiogenous cells* phialidic, determinate, hyaline, ampulliform, smooth-walled, 3.5–4 × 2.5–3.5 µm; *conidia* aseptate, hyaline, smooth- and thin-walled, ellipsoidal, 4.5–5 × 1.5–2 µm. *Chlamydospores* absent.

Culture characteristics: Colonies on PDA reaching 33–35 mm diam after 7 days at 25±1 ºC, flattened, velvety, margin regular, surface and reverse grey (6D1) border orange white (6A2). Colonies on OA reaching 33–35 mm diam after 7 days at 25±1 ºC, flattened, velvety, margin regular, surface and reverse grey (5B1). Colonies on MEA reaching 28–30 mm diam after 7 days at 25±1 ºC, flattened, velvety, margins undulate, grey to orange grey (5C1/5B2); reverse grey to yellowish brown (5F1/5E4). Exopigment absent. Cardinal temperatures for growing: Optimum 25 ºC, maximum 35 ºC, minimum 5 ºC.

Diagnosis: Pyrenochaetopsis perfecta is easily distinguishable from the other species of the genus because is the only one that produces a sexual stage. Unlike of its closest species, *P. globosa, P. perfecta* produces bigger pycnidia (240–380 × 260–300 µm vs. 50–220 × 140–190 µm) covered by abundant long setae (absent in *P. globosa*).

Notes: Differences in nucleotides between *P. globosa* and *P. uberiformis* ITS-*rpb2*-*tub2* concatenated sequences are 29 bp and 32 bp, respectively.

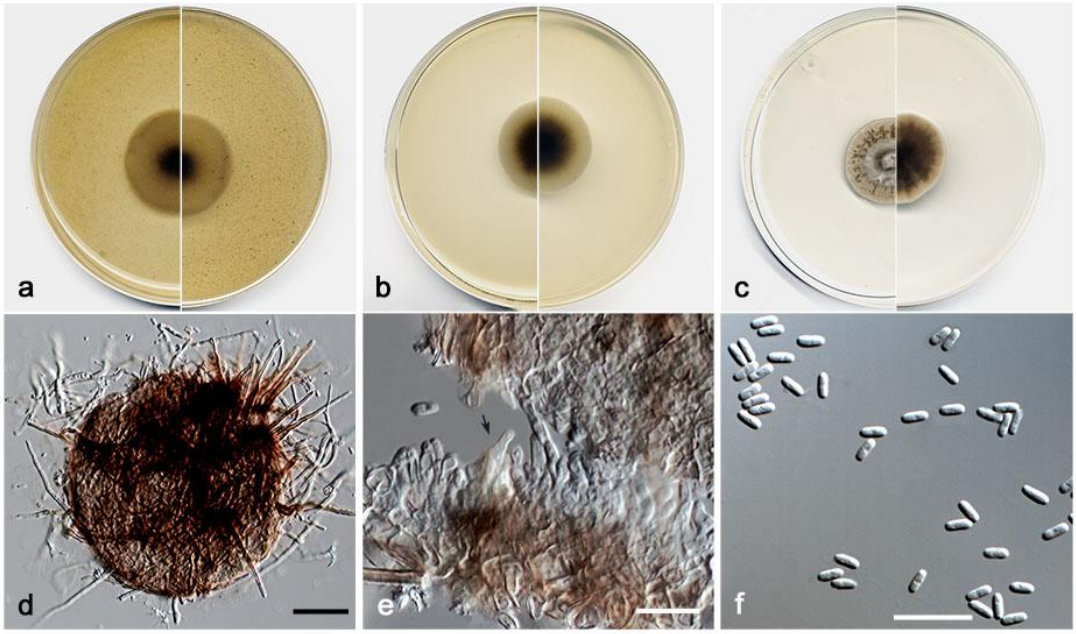

Figure 14. *Pyrenochaetopsis cylindrispora* FMR 18801. a. Colonies on OA; b. MEA; c. and PDA after two weeks at 25±1 ºC (surface, left; reverse, right); d. Pycnidium; e. Conidiogenous cells (black narrow); f. Conidia. Scale bars: d=20 µm, e,f=10 µm.

Pyrenochaetopsis cylindrospora V. Magaña-Dueñas, Stchigel & Cano, *sp. nov.* FMR 18801**.** MycoBank MB842778 (Fig. 14).

Etymology: From Greek *κυλινδρικός*-, cylindrical, and -*σπόριο*, spore, because the shape of the conidia.

Type: Spain, Castellón province, Burriana, Clot de la Mare de Déu, from freshwater submerged plant debris, Mar 2021, Alan Omar Granados Casas and Ana Fernández Bravo, holotype ___, culture ex-type FMR 18801.

Hyphae hyaline to pale brown, septate, branched, smooth- and thin-walled, 2–3 µm wide. *Conidiomata* pycnidial, brown to dark brown, immersed to semi-immersed, solitary, scattered, setose, ostiolate, subglobose, 100–120 × 110–130 µm; *ostiole* 10–20 µm diam.; *setae* brown, septate, slightly warty, sinuous, rounded at the tip, 35–75 µm long 2-2,5 µm wide; *pycnidial wall* 4–6-layered, 15–25 µm thick, with an outer layer of *textura angularis*, composed of pale brown to dark, flattened polygonal cells of 4–8 µm diam.; *conidiophores* absent; *conidiogenous cells* phialidic, determinate, hyaline, smooth- and

thin-walled, cylindrical, 4–5 × 2–3 µm; *conidia* aseptate, hyaline, smooth- and thin-walled, guttulate, bacilliform, 5–6 × 1.5–2 µm. *Chlamydospores* absent.

Culture characteristics: Colonies on PDA reaching 15 mm diam after 7 days at 25 ±1 ºC, umbonate, slightly velvety, margin regular, grey to orange grey (6C1/6C2), border white; reverse brownish grey to orange grey (6D2/6C2). Colonies on OA reaching 12 mm diam after 7 days at 25±1 ºC, flattened, slightly floccose, margin regular, surface and reverse grey to orange grey (5F1/6B2). Colonies on MEA reaching 13 mm diam after 7 days at 25±1 ºC, flattened, velvety, margins regular, brown to orange grey surface and reverse (5F6/5B2). Exopigment absen. Cardinal temperatures for growing: optimum 25 ºC, maximum 30 ºC, minimum 5 ºC.

Diagnosis: The closest species to *P. cylindrospora* is *P. confluens.* Morphologically, *P. cylindrospora* differs from *P. confluents* because the former produces lager setae (35–75 µm vs. 5–22.5(–35) µm) and bigger conidia (5–6 × 1.5–2 µm vs. 2–4 × 2–2.5 µm).

Notes: The nucleotide differences between *P. cylindrospora* and *P. confluens* ITS-*rpb2-tub2* concatenated sequences is of 24 bp.

Discussion

At the present work, we repot the finding of seven new species of presumptively facultative freshwater ascomycetes belonging to the families *Amniculicolaceae (Amniculicola microspora), Chaetomellaceae (Pilidium cuprescens), Lophiostomataceae (Neovaginatispora aquadulcis), Pyrenochaetopsidaceae (Pyrenochaetopsis cylindrospora* and *P. perfecta), Roussoellaceae (Elongatopenicillata aquatica)* and *Sympoventuriaceae (Pseudosigmoidea robusta*), all them isolated from decomposing plant material into lotic freshwater environments.

The genus *Amniculicola* was introduced by Zhang et al. (2008) to accommodate *Amniculicola lignicola*. Until now, there were six accepted species in the genus (http://www.indexfungorum.org/names/Names.asp), all them recovered from freshwater

habitats. Most of these species stain the substrate in purple tinges (Zhang et al. 2008, 2009), with exception for *A. aquatica, A. guttulata* (Hyde et al. 2019, Dong et al. 2020) and the new species *A. microspora*. *Amniculicola longissima* was the only species previously reported as producing an asexual stage, characterized by the formation of long curved or sigmoid conidia on short coniodiophores of sympodial development (Hyde et al. 2019). *Amniculicola microspora* is the unique species producing a coelomycetous asexual stage and lacking of a sexual stage.

Elongatopedicellata was introduced by Zhang et al. (Ariyawansa et al. 2015) to accommodate *E. lignicola. Elongatopedicellata* only comprises the type species, isolated from a dead tree branch in Mae Chang Hot Spring, Thailand. *Elongatopedicellata lignicola* produces in the natural substrate papilate ascomata, filiform pseudoparaphyses, bitunicate, fissitunicate and fusiform-clavate asci, and hyaline, 1-septate, fusiform ascospores constricted at the septum. The authors did not report the production of any fertile structure in pure culture (Ariyawansa et al. 2015). The new species, *E. aquatica*, is the former one reported from aquatic habitats and characterized by the production of *pyrenochaeta*-like or *pyreneochaetopsis*-like conidiomata, an undescribed feature for the genus.

Hashimoto et al. (2018) performed a phylogenetic study of the *Lophiostomataceae*, and introduced the genus *Neovaginatispora* to accommodate *N. fuckelii*. This genus differs from *Vaginatispora* in having thinner, sub-carbonaceous peridium of uniform thickness (Thambugala et al. 2015; Hashimoto et al. 2018). Actually, *Neovaginatispora* comprises two species, *N. clematidis* and *N. fuckelii*, both isolated from decaying plants (*Clematis viticella* and *Mangifera indica*, respectively) (Thambugala et al. 2015; Hashimoto et al. 2018; Phukhamsakda et al. 2020). *Neovaginatispora fuckelii* has also been reported from wood submerged in freshwater (Bao et al. 2019). The asexual stage in both species is unknown. *Neovaginatispora aquadulcis*, recovered from plant debris

into freshwater produce bot sexual and asexual stages in vitro. The formation of conidia into pycnidia by *N. aquadulcis* is a novel feature for that genus.

Pilidium is a genus introduced by Kunze (1823). Species of *Pilidium* are commonly found as plant-associated fungi or isolated from soil, and they are known to produce two kinds of conidiomata, sporodochia and pycnidia (Sutton 1980). Until recent years, the genus includad *P. acerinum, P. eucalyptorum, P. lythri, P. pseudoconcavum* and *P. septatum* (Rossman et al. 2004, Crous et al. 2013 and Marin-Felix et al. 2017). Subsequently, Crous et al. (2017, 2019) introduced *P. anglicum* and *P. novae-zelandie*. From all known species, only *P. septatum* has been reported in freshwater environments (Marin-Felix et al. 2017). The new species, *Pilidium cuprescens*, was also found in a freshwater habitat, is easily distinguished from other species of the genus by the production of closed, copper-colored globose pycnidia.

The genus *Pseudosigmoidea* was erected by Ando and Nakamura in 2000 to place the fungal strain 85B-65 (= ATCC 16660) isolated from freshwater in Maryland (USA). Originally, this fungus was identified as *Sigmoidea* (≡ Flagellospora) prolifera. Thereby, Ando and Nakamura (2000) distinguished *P. cranei* (the type species of the new genus) from S. prolifera because the former produces enteroblastic conidia from polyphialides, and the second one holoblastic conidia on a sympodialy proliferative conidiogenous cell. Jones et al. (2009), in a phylogenetic study based on the nucleotide sequences of the small subunit (SSU) of the ribosomal DNA, confirmed *P. cranei* as a distinct taxon than S. prolifera, despite both fungi were placed in the family *Phaeosphaeriaceae*. Later, Diene et al. (2013) described the second species, *P. ibarakiensis*, from forest soil in Honshu (Japan). Despite the SSU sequences displayed a high similarity (99-100 %) with those of *Troposporella fumosa*, *Helicoma monilipes* and *H. olivaceum*, the fungus was placed as a new species of *Pseudosigmoidea* because showed a high alignment score (100%) and 97 % of similarity with the SSU sequence of *P. cranei*, but also based on morphological similarities (*P. cranei* produce long subcylindrical to obclavate conidia and

P. ibarakiensis scolecoid conidia, whereas these spores are helical in *T. fumosa, H. monilipes* and *H. olivaceum*). However, the authors (Diene et al. 2013) did not built a phylogenetic tree to show the phylogenetic relationships among these taxa. *Pseudosigmoidea alnicola*, isolated from leaf litter near Berlin (Germany), was the third species of the genus. Despite a phylogenetic tree was built, only the ITS and LSU nucleotide sequences of the new species were used, and consequently *P. alnicola* falls in the same terminal clade than *T. fumosa*, *Scolecobasidium excentricum*, and *Sympoventuria capensis* and *S. melaleucae*. However, the authors mentioned that the ITS sequence of *P. alnicola* displays 95 % similarity with that of *P. ibarakiensis* (Crous et al. 2019). Probably, the morphological similarity between of the reproductive structures of *P. alnicola* and *P. cranei* and *P. ibarakiensis* was responsible for the final placement at genus level. The fourth species, *P. excentrica* (Shen et al. 2020), was originally erected as *Scolecobasidium excentricum*, being isolated from leaf litter in Santiago de las Vegas (Cuba) (Castañeda-Ruiz et al. 1997). Morphologically, *P. excentrica* differs from the other species of the genus because produce larger conidiophores bearing sympodially proliferating conidiogenous cells (short and polyphialidic in the other species), and by the production of shorter, cylindrical to allantoid conidia with rounded ends (scolecoid in the remaining species). Our new species, *P. robusta*, is morphologically similar to *P. excentrica*, because produce short cylindrical to obclavate conidia and conidiophores bearing integrated sympodially proliferating conidiogenous cells. However, *P. robusta* produces longer and narrower conidia than *P. excentrica* (8–65 × 2.5–3 µm vs. 14–22 × 3–5 µm), and because in the apical part of the conidia can develop a conidiogenous locus (feature not reported for *P. excentrica*).

Pyrenochaetopsis was introduced to accommodate *P. decipiens, P. indica, P. leptospora, P. microspora* and *P. pratorum*, result of a molecularly-based phylogenetic study performed by Gruyter el at. (2010) to resolve the taxonomical placement of several phoma-like (pycnida forming) fungi. Valenzuela-Lopez et al. (2018), thanks to a

multilocus phylogenetic analysis, transferred *Pyrenochaetopsis* from the family *Cucurbitariaceae* to the new family *Pyrenochaetopsidae*. Currently the genus contains 19 species (http://www.indexfungorum.org/names/Names.asp). The members of this genus have been isolated from terrestrial, marine, freshwater environments, and from human clinical specimens (superficial tissue, bronchial washing and blood) (De Gruyter et al. 2010, Papizadeh et al. 2017, Valenzuela-Lopez et al. 2018, Mapook et al. 2020, Magaña-Dueñas et al. 2021). Up to now, only the asexual (coelomycetous) reproductive stage has been reported for the members of the family. In the present study, we report two new species of *Pyrenochaetopsis*. *Pyrenochaetopsis perfecta*, one of the two new species described by us, is the former reported as producing the sexual reproductive stage, characterized by the production perithecial ascomata, cylindrical to clavate bitunicate asci and hyaline, 3–6 septate fusiform ascospores. In comparison with the other new species of the genus, *P. globosa, P. perfecta* produces bigger pycnidia, which are covered by abundant long setae (absent in those of *P. globosa*).

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5. DISCUSIÓN

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMÍA Y FILOGENIA MOLECULAR DE HONGOS PROCEDENTES DE MATERIAL VEGETAL SUMERGIDO DE ESPAÑA
Viridiana Magaña Dueñas

En los últimos años se ha incrementado el interés sobre la taxonomía de los hongos de agua dulce, particularmente de aquellos taxones que se reproducen a través de la generación de ascosporas y/o conidios dentro de cuerpos fructíferos, sexuales y asexuales, respectivamente (Yang et al. 2018, Bao et al. 2019a, 2019b, Luo et al. 2019, Dong et al. 2020, Magaña-Dueñas et al. 2020, 2021a, 2021b, Magaña-Dueñas et al. [en redacción]). Dichos estudios confirman que la diversidad de este grupo ecológico de hongos es significativamente mayor a la estimada previamente (Gessner & Van Ryckegem 2003). Actualmente se conocen alrededor de 740 especies de hongos ascomicetos de agua dulce con estado reproductivo exclusivamente sexual y unas 900 especies que solo presentan el estado asexual. Los ascomicetos de agua dulce están principalmente distribuidos en tres de las clases del filo Ascomycota: los *Dothideomycetes*, los *Leotiomycetes* y los *Sordariomycetes.* A pesar de estos recientes avances, el conocimiento sobre la fisiología y la diversidad de estos hongos sigue siendo muy escasa. A medida que se estudien más de estos organismos podremos comprender mejor su rol en los ambientes dulceacuícolas, y el conocimiento de su genética nos permitirá, entre otros objetivos, obtener nuevas moléculas bioactivas con una amplia gama de aplicaciones biotecnológicas y médicas.

Nuestro estudio implicó la caracterización fenotípica y molecular de 111 cepas, todos ellos provenientes de material vegetal sumergido en hábitats de agua dulce de España. El 62% de estas se ubicaron taxonómicamente en la clase *Dothideomycetes* (distribuidos entre las familias *Amniculicolaceae, Cucurbitariaceae, Dictyosporiaceae, Dydimellaceae, Lentitheciaceae, Lindgomycetae, Lophiostomataceae, Neopyrenochaetaceae, Pleosporaceae, Pyrenochaetopsidaceae, Roussoellaceae, Sporormiaceae, Sympoventuriaceae* y *Torulaceae.* Siendo las más comunes *Amniculicolaceae* [diez cepas]*, Didymellaceae* [quince cepas] y *Neopyrenochaetopsidae* [catorce cepas] y el 95% de ellas eran miembros del orden *Pleosporales.* El resto de las cepas se distribuyó de la siguiente manera: un 30% en los *Sordariomycetes*: distribuidos entre las familias: *Beltrinaceae, Bionectriaceae, Cerastotomataceae, Chaetosphaeriaceae, Diaporthaceae, Glomerellaceae, Hypocreaceae, Hypocreomycetidae, Sporocadaceae, Sordariaceae, Stachybotryaceae, Reticulascaceae* y *Valsaceae*, siendo las más comunes las incluidas en *Hypocreomycetidae* [doce cepas] *y Chaetosphaeriaceae* [cuatro cepas]) un 4.5% en los *Leotiomycetes* (distribuidas entre las familias *Chaetomellaceae, Leotiomycetidae, Microdochiaceae*, y *Tricladiaceae*) el 3.5 % restante en los *Eurotiomycetes* (distribuidas entre las familias *Eurotiomycetidae, Herpotrichiellaceae*y *Mycocaliciaceae*). Los géneros más frecuentemente representados fueron *Neopyrenochaeta* (14 cepas), *Didymella* (6

cepas), *Murispora* (4 cepas), *Pyrenochaetopsis* (4 cepas), y *Neocucurbitaria, Paraboeremia*, *Codinea* y *Vargamyces* (3 cepas cada uno). Estos resultados demuestran que los ascomicetos de aguas dulces en España son un grupo con una elevada diversidad, dentro del cual podemos encontrar a integrantes de los diferentes grupos de hongos acuáticos descritos por Park (1972) cómo ingoldianos (o residentes), inmigrantes periódicos (o anfibios) e inmigrantes versátiles (o acuáticos facultativos).

Muchas de estas cepas presentaban un estado asexual correspondiente a los hongos celomicetos, caracterizados por producir conidios dentro de conidiomas, en nuestro caso los denominados picnidios. El estudio taxonómico de los hongos celomicetos representa un verdadero desafío para el micólogo, debido a la escasez de caracteres morfológicos discriminatorios (incluso a nivel de género), y a la lenta (cuando no nula) fructificación y/o esporulación en las condiciones ambientales y en los medios de cultivo habitualmente empleados en el laboratorio. Tan solo el estudio taxonómico polifásico ha permitido ubicar las cepas de interés en distintos géneros. Algunas de ellas fueron identificadas como especies no descritas anteriormente, siendo por lo tanto descritas en el presente trabajo de tesis doctoral como nuevas para la ciencia.

5.1. *Dothideomycetes*

La clase *Dothideomycetes* es la taxonómicamente más numerosa de entre los Ascomycota, representando aproximadamente el 30% de los hongos reportados en ambientes de aguas dulces (http://fungi.life.illinois.edu/about/mitosporic_fungi). Son un grupo ecológicamente diverso, siendo en su mayoría saprobios, aunque también se pueden encontrar muchos patógenos vegetales y animales importantes. Se pueden encontrar en hábitats terrestres, marinos y de agua dulce, con una distribución geográfica muy amplia. Son importantes descomponedores de desechos leñosos y de hojas (Dong et al. 2020). Dentro de la clase de los *Dothideomycetes*, las especies de agua dulce se localizan exclusivamente en la familia *Pleosporomycetidae* pero no en la *Dothideomycetidae*; Shearer y colaboradores (2009) especulaban sobre diversas razones que podrían justificar éste particular patrón de distribución: la primera es que la familia *Dothideomycetidae* incluye patógenos vegetales especializados de los órdenes, *Botryosphaeriales, Capnodiales* y *Myriangiales*, muchos de los cuales crecen en las hojas, y es posible que tales hongos especializados hayan perdido el potencial para adaptarse a un estilo de vida saprobio en habitas acuáticos; otra razón podría ser que la ausencia de pseudoparáfisis en los taxones de *Dothideomycetidae* puede limitar la supervivencia de los mismos en hábitats acuáticos con niveles de agua fluctuantes. Las pseudoparáfisis de especies acuáticas en *Pleosporomycetidae* son a menudo

abundantes y rodeadas de mucilago, que pueden proteger a las ascas y ascosporas de la desecación durante condiciones de sequía. Las familias que contienen más miembros presentes en hábitats de agua dulce son: *Amniculicolaceae,* la mayoría de las especies han sido aisladas de agua dulce, aunque hay algunas que se han aislado a partir de hábitats terrestres y *Lentitheciae* y *Lindgomycetaceae* que incluyen taxones exclusivos de agua dulce (Shearer et al. 2009, Zhang et al. 2009).

El orden *Pleosporales* es el más diverso de entre los *Dothideomycetes*, comprendiendo una cuarta parte de todas las especies descritas para dicha clase. Tienen una distribución cosmopolita como saprobios, endófitos, patógenos, y parásitos de otros hongos e insectos. (Wanasinghe et al. 2018, Hongsanan et al. 2020, Mapook et al. 2020). Se caracterizan por producir ascomas peritecioides y ascos bitunicados, y sus ascosporas presentan gran diversidad de formas y tamaños, así como en el número de células. Las formas asexuales usualmente son conidiomas (hongos celomicetos), pero también pueden producir conidióforos (hongos hifomicetos) (Zhang et al. 2012, Hongsanan et al. 2020, Mapook et al. 2020).

5.1.1. *Amniculicolaceae*

La familia *Amniculicolaceae* fue establecida por Zhang y colaboradores (2009) para acomodar tres géneros de *Dothideomycetes*, en su mayoría procedentes de aguas dulces: *Amniculicola, Murispora* y *Neomassariosphaeria*. Los miembros de esta familia se caracterizan por producir ascomas solitarios de color negruzco, con formas que van desde globosas a lenticulares, de superficie rugosa, con una pared peridial delgada, en cuyo interior se encuentran paráfisis y ascos bitunicados, estos últimos de cilíndricos a claviformes, pedicelados, en cuyo interior se producen ascosporas fusiformes incoloras, amarronadas o rojizas, con uno o más septos transversales, o bien muriformes, estrechándose a la altura del septo medio y rodeadas de una capa mucilaginosa, y por teñir, usualmente, la superficie del sustrato en el cual desarrollan de color púrpura (con septos transversales, longitudinales y/u oblicuos) (Zhang et al. 2008, 2009; Hyde et al. 2013, Wanasinghe et al. 2015, Magaña-Dueñas et al. 2020). Los estados asexuales de la familia son poco conocidos. En su filogenia se incluían especies asexuales tales como *Anguillospora longissima,* un hongo ingoldiano con conidios sigmoidales incoloros (Ingold 1942); *Spirosphaera cupreorufescens* (actualmente *Fouskomenomyces cupreorufescens*), otro miembro de hongos aero-acuáticos que produce propágulos de dispersión formados por hifas ramificadas entrelazadas en forma de espiral (Voglmayr 2004); *Repetophragma ontariense* (actualmente *Vargamyces aquaticus*, del cual hemos aislado tres cepas), un hongo dematiáceo que se caracteriza por la producción de

conidios marrones y fusiformes a partir de conidióforos poco diferenciados (Castañeda-Ruíz et al. 2011) y *Murispora hawksworthii*, un ascomiceto picnidial (Wanasinghe et al. 2015).

Durante el desarrollo de la presente tesis doctoral se hallaron cuatro nuevas especies de la familia *Amniculicolaceae*, caracterizadas por la producción, de forma exclusiva, de esporas asexuales. Con la ayuda de un análisis filogenético se delimitó el nuevo género *Fouskomenomyces*, el cual incluyó la especie tipo *F. cupreorufescens* y la nueva especie *F. mimiticus* (Magaña-Dueñas et al. 2020). *Fouskomenomyces mimiticus* produce propágulos de dispersión característicos del grupo ecológico de los hongos aero-acuáticos (globosos, formados por hifas anastomosadas). Por otra parte, las nuevas especies *Amniculicola microspora, Murispora asexualis* y *M. fissilispora* se caracterizan por la producción de conidios dentro de conidiomas. *Amniculicola microspora*, además de ser una nueva especie, es el primer taxón del género que produce conidios dentro de picnidios (Magaña-Dueñas et al. 2020, Magaña-Dueñas et al. [en redacción]).

El género *Murispora* fue introducido por Zhang y colaboradores (2009) basado en *Pleospora rubicunda* Niessl. Posteriormente fueron introducidas otras seis especies, todas ellas aisladas de material vegetal en descomposición en ambientes terrestres, reportándose tan solo una especie con estado asexual (un hongo picnidial): *M. hawksworthii* (Wanasinghe et al. 2015). Cuatro de las especies han sido reportadas también para ambientes dulceacuícolas: *M. aquatica, M. cicognanii, M. fagicola* y *M. rubicunda* (Bao et al. 2019, Hyde et al. 2019). El estado sexual de las especies del género *Murispora* se caracteriza por producir ascosporas muriformes oscuras rodeadas de una envoltura mucilaginosa, estructura que favorece su anclaje a los sustratos sumergidos. Además, los estados sexuales de las especies del género han sido encontrados en el sustrato natural, mientras que el estado asexual de *M. hawksworthii* tan solo ha sido reportado en cultivo (Zhang et al. 2009, Wanasinghe et al. 2015, Bao et al. 2019a). Más recientemente, Akhmetova y colaboradores (en Crous, et al. 2021) han descrito una nueva especie para el género, *M. kazachstanica*, aislada de trigo. Esta nueva especie no produce estructuras reproductivas en cultivo, ni fueron reportadas en el material vegetal original, por lo que su inclusión en el género *Murispora* se basa en los resultados del estudio filogenético. En la presente tesis, la reconstrucción filogenética resultante del análisis de las secuencias nucleotídicas de los marcadores ITS, LSU y *tef-1* concatenados, permitió discriminar tres nuevas especies para el género: *M. asexualis, M. fissilispora* y *M. navicularispora. Murispora asexualis* se caracteriza por la ausencia de estado sexual y la formación pincidios tanto en el sustrato natural como *in*

vitro. *Murispora fissilispora* es la primera de las especies del género que produce el holomorfo (individuo que presenta todos los estados reproductivos, el sexual y el/los asexual/es) *in vitro;* y *M. navicularispora* produce ascomas tanto en sustrato natural como en cultivo puro, en cuyo interior se forman ascosporas de forma naviculares, septadas y de color marrón pálido*.* Por otro lado, los resultados de los análisis filogenéticos permitieron reubicar dentro del género *Murispora* a dos especies anteriormente ubicadas en el género *Pseudomassariosphaeria: P. bromicola* y *P. triseptata* (Magaña-Dueñas et al. 2020). Estos mismos resultados permiten afirmar que ciertas características morfológicas de los miembros de la familia, tales como el tamaño y la forma de las ascosporas, tienen menos peso taxonómico de lo que otros autores le habían atribuido (Zhang et al. 2009, Wanasinghe et al. 2015, Bao et al. 2019).

5.1.2. *Cucurbitariaceae*

La familia *Cucurbitariaceae* fue establecida por Winter (1885), con *Cucurbitaria* como género tipo. Dicho género se caracteriza por producir ascomas dispersos o gregarios, en o sobre una porción elevada o en un pseudoestroma, peritecioides, globosos, subglobosos, turbinados, lenticulares o piriformes, de marrones a negros, rugosos, ostiolados o papilados, con un canal ostiolar perifisado, con pared peridial pseudoparenquimatosa, parafisados, ascos cilíndricos a oblongos, bitunicados, con una cámara ocular y estípite corto, con 4 a 8 ascosporas elipsoides, fusoides u oblongas, marrones, muriformes, rara vez con una cubierta mucilaginosa, a veces con células anexas; sus estados asexuales son celomicetos similares a *Phoma* o *Pyrenochaeta*. Posteriormente, varios géneros asexuales, caracterizados por la formación de picnidios con setas y conidios sin septos fueron aceptados en la familia en base a estudios moleculares (Doilom et al. 2013, De Gruyter et al. 2010, 2013). Más tarde, Valenzuela-López y colaboradores (2018) realizaron análisis multilocus y reordenaron la familia, agregando un género nuevo (*Allocucurbitaria*) y reubicando otros tres previamente conocidos (*Cucurbitaria, Neocucurbitaria* y *Paracucurbitaria*)*.* Durante el desarrollo de la presente tesis doctoral se aislaron un total de cinco cepas pertenecientes a la familia *Cucurbitariaceae,* dos de ellas pertenecientes a *Parafenestella pseudoplatani* y el resto al género *Neocucurbitaria.* El género *Neocucurbitaria* fue introducido por Wanasingue y colaboradores (2017) para acomodar *N. acerina, N. quercina* y *N. unguis-hominis*. Las especies de este género han sido aisladas de diferentes sustratos, tales como material vegetal, muestras procedentes de lesiones en piel y córnea humana, y de agua de mar (Chen et al. 2017, Wanasingue et al. 2017, Valenzuela-Lopez et al. 2018). En la presente tesis se describen dos nuevas especies, *N. aquadulcis* y *N. variabilis*, y el primer reporte de *N. salicis-albae* en ambientes de agua dulce, todas ellas en su estado

asexual. Basándose en su estudio morfológico y en análisis filogenéticos, Crous y colaboradores (2019) introdujeron una nueva especie al género: *N. prunicola*. Sin embargo, los resultados obtenidos en la presente tesis, soportados por un análisis filogenético multilocus (ITS, LSU, *tub2* y *rpb2*), ubicó claramente *N. prunicola* dentro del género *Allocucurbitaria,* motivo por el cual ha sido propuesta como la nueva combinación *A. prunicola* (Magaña-Dueñas et al. 2021a).

5.1.3. *Didymellaceae*

Las especies de la familia *Didymellaceae* tienen una distribución cosmopolita y han sido aisladas de una gran diversidad de sustratos, tales como suelo, aire, agua, sedimentos marinos y quistes de nematodos (Aveskamp et al. 2008, 2010, Chen et al. 2015, 2017, Valenzuela-Lopez et al. 2018). Más del 50% de las especies reportadas son patógenas de plantas, causando lesiones en hojas y tallos, y generando grandes pérdidas económicas (Aveskamp et al. 2008). Inicialmente dentro de ésta familia se ubicaron los géneros *Ascochyta, Didymella* y otros géneros similares a *Phom a* (De Gruyter et al. 2009). Es una de las familias más numerosas del reino Fungi, habiendo sidas descritas hasta la fecha más de 5.400 especies en 31 géneros (Hou et al. 2020a). Durante el desarrollo de la presente tesis se han identificado 15 aislados como pertenecientes a la familia *Didymellaceae,* los cuales se distribuyeron de la siguiente manera: *Didymella* spp, seis cepas; *Epicocum nigrum*, una cepa; *Paraboeremia* spp.*,* tres cepas; *Phoma herbarum*, dos cepas; y *Phomatodes nebulosa*, tres cepas.

Las especies del género *Didymella* (Saccardo 1880) son mayoritariamente saprobias, y se encuentran comúnmente en partes vivas y muertas de plantas, pero también son importantes fitopatógenos. Además, han sido aisladas de sustratos artificiales tales como pintura, asbestos y cemento (Aveskamp et al. 2008, Chen et al. 2015, 2017). Chen y colaboradores (2015), realizaron un análisis filogenético multilocus y observaron que el género era monofilético. Desde entonces, aproximadamente 30 nuevas especies han sido introducidas al mismo (Chen et al. 2017, Crous et al. 2017, 2018, Valenzuela-Lopez et al. 2 018, Hou et al. 2020a, 2020b, Hyde et al. 2020). El género *Didymella* fue el segundo más aislado en el presente trabajo de tesis doctoral con un total de seis cepas. Tres de estas cepas fueron identificadas como *D. glomerata*, un importante fitopatógeno causante de plagas como el tizón de la hoja de pistacho (Moral et al. 2018); una cepa de *D. macrostoma*, hongo previamente aislado de hojas de *Ace r pseudoplatanus,* madera de *Malus sylvestris* y semillas

de *Pinus nigra* (Chen et al. 2015), y que recientemente ha pasado a engrosar las filas de los hongos patógenos oportunistas, produciendo onicomicosis (Kukhar et al. 2020)*;* y una cepa de *D. viburnicola*, previamente reportada desarrollando sobre restos vegetales de *Viburnum oxycoccus* y *V. cassioides* (Valenzuela-López et al. 2018). Estas cinco cepas corresponden a los primeros reportes para hábitats dulceacuícolas. La sexta cepa correspondió a una especie nueva para la ciencia: *D. brevipilosa* (Magaña-Dueñas et al. 2021b). El hallazgo de especies de hongos típicamente fitopatógenas o saprobias de plantas terrestres en ambientes acuáticos nos indica que, probablemente, estas ya estaban creciendo a expensas del sustrato antes de que cayera al agua, por lo que puede deducirse que estas corresponden a hongos acuáticos facultativos.

El género *Heterophoma* contiene también especies saprobias y fitopatógenas, especialmente de plantas de las familias *Brassicaceae* y *Scrophulariaceae*, y cuya distribución es cosmopolita (Chen et al. 2015, 2017, Hou et al. 2020a). En base a los resultados de un análisis multilocus (ITS-*rpb2*-*tub2*) se ha propuesto en el presente trabajo de tesis doctoral una nueva especie para dicho género: *H. polypusiformis*. Este hongo representa el primer hallazgo de una especie de *Heterophoma* en ambientes de aguas dulces. *Heterophoma polypusiformis* es una especie fácilmente reconocible, ya que es la primera que produce ambos estados reproductivos, el sexual y el asexual. Sus ascosporas, cuya forma coincide con las reportadas previamente para otros miembros de la familia *Didymellaceae*, tales como *Ascochyta* y *Neomicrosphaeropsis* (Wanasingue et al. 2018), son muriformes y están rodeadas de una capa de mucilago. El estado sexual de *H. polypusiformis* se encontró sobre el sustrato original, mientras que el estado asexual desarrolló en cultivo puro (Magaña-Dueñas et al. 2021b). Este último es extremadamente peculiar, consistiendo en picnidios con un número variable de cuellos, algunos de los cuales se ramifican, dando en conjunto a los conidiomas el aspecto de un cefalópodo (de ahí su nombre científico).

El género *Paraboeremia* fue introducido por Chen y colaboradores (2015) para acomodar *P. adianticola, P. putaminum* y *P. selaginellae.* La mayoría de las especies del género son parásitas de plantas, generando manchas necróticas en sus hojas y tallos. Las especies del género han sido aisladas de la rizosfera, del suelo y de diversas plantas (Jiang et al. 2016, Chen et al. 2017, 2018, Hou et al. 2020a). En la presente tesis se han aislado e identificado tres cepas de este género: una identificada como *P. putaminum*, mientras que las dos restantes pertenecieron a una especie nueva para la ciencia: *P. clausa,* se caracteriza por desarrollar conidiomas picnidiales que carecen de ostiolos*,* las hifas presentan anastomosis, tiene células

conidiogenas globosas y produce conidios elipsoidales a ovoides bi-gotulados (Magaña-Dueñas et al. 2021b). Ambos reportes representan los primeros hallazgos para ambientes de agua dulce.

5.1.4. *Lophiostomataceae*

Esta familia fue introducida por Saccardo (1883), con *Lophiostoma* como género tipo. La mayoría de las especies son saprobias y se encuentran principalmente en ramas, tallos, y cortezas de plantas leñosas y herbáceas en descomposición, tanto en ambientes terrestres como acuáticos (Ariyawansa et al. 2015, Liu et al. 2015, Hyde et al. 2017, Hashimoto et al. 2018). Hashimoto y colaboradores (2018), gracias a un estudio filogenético, introdujeron en la familia el nuevo género *Neovaginatispora* para acomodar *N. fuckelii* (anteriormente *Vaginatispora fuckelii*). El género comprende solo dos especies: *N. fuckelii,* aislada de *Magnifera indica* y posteriormente de agua dulce; y *N. clematidis, a*islada de *Clematis viticella* (Thambugala et al. 2015, Hashimoto et al. 2018, Bao et al. 2019b, Phukhamsakda et al. 2020). Durante el desarrollo de la presente tesis se reporta el hallazgo de una nueva especie, *N. aquadulcis,* la que produce su estado sexual tanto sobre el sustrato natural como *in vitro*, con ascomas piriformes con cuellos ostiolares en forma de cono truncado, ascas bitunicadas y cilíndricas, conteniendo ocho ascosporas con tres septos, hialinas y fusiormes. Su estado asexual corresponde a conidiomas de tipo picnidial (Magaña-Dueñas et al. en redacción).

5.1.5. *Neopyrenochaetaceae*

La familia *Neopyrenochaetaceae* fue establecida por Valenzuela-López y colaboradores (2018) para acomodar cuatro especies en el también nuevo género *Neopyrenochaeta*: *N. acicola,* aislada a partir de acículas de *Pinus sylvestris*; *N. fragariae*, aislada de *Fragaria ananassa*; *N. inflorescentiae*, aislada de la flor de *Protea neriifolia*; y *N. telephoni*, procedente de la superficie de la pantalla táctil de un *smartphone* (Crous et al. 2015, Chen at al. 2017, Wanasinghe et al. 2017). Más tarde se incorporaron al género nuevas especies: *N. cercidis*, de vainas de *Cercis chinensis* (Jayasiri et al. 2019); y *N. chiangraiensis*, *N. chromolaene, N. thailandica* y *N. triseptatispora*, aisladas de tallos aéreos muertos de *Chromolaena adorata*, (Mapook et al. 2020). Li y colaboradores (2020) reportaron el hallazgo de tres nuevas especies (*N. annellidica, N. chiangraiensis,* y *N. maesuayensis*) a partir de madera sumergida en agua dulce en Tailandia*.* Durante el desarrollo de la presente tesis doctoral, el género *Neopyrenochaeta* fue el más aislado (catorce cepas) a partir de material vegetal en descomposición sumergido en agua dulce. Las especies más predominantes fueron *N. annellidica* (siete cepas) y *N. maesuayensis*

(tres cepas), lo cual indicaría que dichas especies tienen una distribución geográfica más amplia que la originalmente reportada (solo restringida a Tailandia). Otra de las especies aisladas fue *N. telephoni*, constituyendo el primer reporte para este tipo de hábitats. Además, gracias a un estudio polifásico, se han podido describir tres nuevas especies procedente de ambientes dulceacuícolas: *N. asexualis, N. glabra* y *N. submersa* (Magaña-Dueñas et al. 2021a). Es difícil encontrar diferencias morfológicas en éste grupo de hongos y se suele recurrir a herramientas moleculares para poder diferenciarlas. Sin embargo, *N. glabra* es fácilmente reconocida por la ausencia de setas alrededor de los conidiomas; y *N. asexualis* se distingue de otras especies del género, debido a que produce células conidiogenas fialídicas doliformes con uno o dos *loci* conidiógenos.

5.1.6. *Phaeosphaeriaceae*

La familia *Phaeosphaeriaceae*, perteneciente al orden Pleosporales, incluye especies fúngica s de gran importancia económica, dado que infectan (mayoritariamente) a plantas monocotiledóneas tales como el arroz, la cebada, el trigo y la avena (Phookamsak et al. 2014). Los miembros de esta familia, sin embargo, han sido aislados en una amplia variedad de nichos ecológicos terrestres, marinos y de aguas dulces, sobre diversos tipos de sustratos, comportándose como saprobios y, mucho menos frecuentemente, como endófitos. Algunos de estos hongos son capaces de causar infecciones oportunistas en humanos (Phookamsak et al. 2014, 2017, Ahmed et al. 2017, Valenzuela-López et al. 2017, Maharachchikumbura et al. 2019, Tennakoon et al. 2020). *Phaeosphaeriaceae* fue establecida por Barr (1979) y tipificada por el género *Phaeosphaeria*. Tradicionalmente, la identificación de sus miembros se basaba en el tipo de huésped vegetal al que infectaban, y en las características morfológicas y estructurales de sus estados sexuales (más raramente de los asexuales). Sin embargo, su identificación actual se basa en la aplicación de análisis de secuencias nucleotídicas de marcadores filogenéticamente informativos (Phookamsak et al. 2014, Tennakoon et al. 2020). En la presente tesis doctoral se reporta el hallazgo de cuatro cepas pertenecientes a los géneros *Paraphoma* (1), *Phaeosphaeria* (2) y *Xenophoma* (1).

Gracias a un estudio filogenético multilocus, hemos podido identificar una especie nueva para el género *Phaeosphaeria*: *P. fructigena*. Esta especie se reproduce exclusivamente mediante la formación de ascosporas dentro de ascomas periteciales, los que se forman tanto en el sustrato natural como *in vitro*. *Phaeosphaeria fructigena* comparte características morfológicas y estructurales con otras especies del

género (tales como *P. musae, P. oryzae* y *P. thysanolaenicola*), como la producción de ascos bitunicados y ascosporas con tres septos, de incoloras a color amarillo pálido (Magaña-Dueñas et al. 2021b).

En 1982 Morgan-Jones introduce el género *Paraphoma* para acomodar las especies similares a *Phoma* cuyos picnidios están ornamentados con setas. Posteriormente, Boerema (1997) trató el género a nivel de sección dentro del género *Phoma.* Basándose en un análisis filogenético, De Gruyter y colaboradores (2010) reestablecieron el género *Paraphoma* dentro de la familia *Phaeosphaeriaceae*. Las especies de *Paraphoma* han sido reportadas como patógenas de plantas, causando enfermedades en sus raíces. En el presente trabajo se reporta el hallazgo de una nueva especie, *P. aquatica,* la segunda del género aislada de ambientes dulceacuícolas. *Paraphoma aquatica* se distingue fácilmente de otras especies del género por la producción de picnidios no ostiolados.

A partir de un análisis filogenético Trakunyingcharoen y colaboradores (2014) erigieron el nuevo género *Xenophoma*, designando como la especie tipo del género a *X. puncteliae*, aislada a partir de los tallos parasitados de *Punctelia rudecta*. En la presente tesis se describe una especie nueva especie de este género, *X. microspora*, la primera especie del género aislada de ambientes acuáticos (Magaña-Dueñas et al. 2021b). *Xenophoma microspora* se distingue de otras especies del género por la producción de picnidios con hasta tres cuellos ostiolados, y por sus conidios de pequeño tamaño.

5.1.7. *Pyrenochaetopsidaceae*

Valenzuela-López y colaboradores (2018) realizaron un estudio multilocus (empleando las secuencias nucleotídicas concatenadas de los *loci* ITS, LSU, *rpb2* y *tub2*) de varios miembros de la clase *Dothideomycetes*, y como consecuencia de sus resultados propusieron la nueva familia *Pyrenochaetopsidaceae* formada por los géneros *Pyrenochaetopsis* (género tipo), *Neopyrenochaetopsis* y *Xenopyrenochaetopsis*. Los miembros del género *Pyrenochaetopsis* han sido aislados de diferentes ambientes terrestres, marinos y de agua dulce; también se han encontrado en muestras clínicas de origen humano, tales como sangre y piel, y en el líquido procedente del lavado bronquio-alveolar (De Gruyter et al. 2010, Valenzuela-López et al. 2018, Mapook et al. 2020). Todas las especies reportadas hasta el momento para el género se multiplican asexualmente, produciendo conidiomas picnidiales setosos en cuyo interior se generan conidios hialinos a partir de células conidiógenas fialídicas. Por otro lado, el estado sexual del género

era desconocido. Durante el desarrollo del presente trabajo, se han aislado un total de cuatro cepas pertenecientes al género *Pyrenochaetopsis.* Gracias a un análisis multilocus, una de estas fue identificada como *P. confluens*, mientras que las otras tres representaron nuevas especies para la ciencia: *P. aquatica*, *P. cylindrispora* y *P. perfecta*. *P. perfecta* resultó ser la primera de las especies de la familia con un ciclo reproductivo completo. El estado sexual de *P. perfecta* se caracteriza por formar ascomas sumergidos o semi-inmersos marrones, ostiolados, cuya pared presenta una textura *angularis*, conteniendo y ascos bitunicados conteniendo 8 ascosporas, cilíndricos a claviformes, y ascosporas 3–6 septadas, incoloras y fusiformes (Magaña-Dueñas et al., en redacción).

5.1.8. *Roussoellaceae*

La familia *Roussoellaceae* fue introducida por Liu y colaboradores en 2014, y se caracteriza por producir ascostromas dispersos e inmersos, con una pared peridial de color marrón oscuro formada por varias capas, con numerosas pseudoparáfisis, ascos conteniendo de 4 a 8 ascosporas en su interior, bitunicados, cilíndricos a claviformes, con un corto o largo pedicelo, a menudo de paredes relativamente delgadas, apicalmente redondeados, con o sin cámara ocular, y ascosporas fusiforme-elipsoidales, rectas, bicelulares, constreñidas a nivel del septo, de color marrón, ornamentadas y rodeadas por una gruesa capa mucilaginosa. Los miembros de la familia se aíslan principalmente de plantas monocotiledóneas como el bambú y las palmas. Los estados asexuales están vinculados a hongos celomicetos pertenecientes a los géneros *Cytoplea, Melanconiopsis*, *Neomelanconium* y *Roussoella* (Liu et al. 2014, Ariyawansa et al. 2015).

El género *Elongatopedicellata* fue erigido por Zhang (Ariyawansa et al. 2015) para acomodar *E. lignicola*, aislada de madera muerta. El género se caracteriza por producir ascomas papilados, ascos bitunicados y fusiformes, y ascosporas incoloras, fusiformes, con un septo y constreñidas a la altura del mismo, sin estado asexual conocido. Como resultado de la presente tesis se ha descrito una nueva especie para el género, *E. aquatica*, la primera aislada de ambientes de agua dulce y que produce el estado asexual *in vitro.* El mismo está caracterizado por la producción de conidiomas picnidiales marrones semi-inmersos, solitarios, dispersos, ostiolados, setosos, cuya pared tiene una textura *intrincata*, globosos, conteniendo células conidiógenas fialídicas, determinadas, con forma de matraz ó ampuliformes, las que producen conidios unicelulares, de paredes lisas, de incoloras a marrón pálido, claviformes, ovoides o en

forma de riñón. La morfología del estado asexual de *E. aquatica* difiere significativamente de las de otras especies descritas previamente para la familia. Sin embargo, y a pesar de que el estado sexual de *E. aquatica* continúa siendo desconocido, su ubicación filogenética dentro del género es inequívoca.

5.1.9. *Sympoventuriaceae*

Basados en un estudio taxonómico polifásico Zhang y colaboradores (2011) introdujeron la nueva familia *Sympoventuriaceae* para acomodar los géneros *Sympoventuria* (género tipo), *Veronaeopsis* y especies similares a *Fusicladium*. Se pueden distinguir de otros miembros del orden *Venturiales* por su estilo de vida saprofito. Sus especies mayoritariamente han sido aisladas a partir de hojarasca, suelo, aguas termales e incluso de animales (Shen et al. 2020). El género *Pseudosigmoidea* fue introducido basándose en caracteres morfológicos de *P. cranei* como, la producción de conidios enteroblásticos y conidiogénesis fialídica, con estás observaciones los autores excluyeron *P. cranei* del género *Sigmoidea* (Ando & Nakamura 2000). Posteriormente, Jones y colaboradores (2009) realizaron un análisis filogenético utilizando el gen SSU para descifrar la relación taxonómica de *P. cranei* y *P. ibarakiensis*. Sin embargo, estás especies se clasificaron como *"incertae sedis"*. A continuación, Crous y colaboradores (2019) realizaron un estudio filogenético utilizando los genes LSU e ITS, este estudio mostró que el género se ubica dentro de la familia *Sympoventuriaceae.* Las especies de ése género han sido aisladas de agua dulce, hojas muertas y suelo forestal (Castañeda-Ruiz et al. 1997, Ando & Nakamura 2000, Diene et al. 2013, Crous et al. 2019). En la presente tesis doctoral se describe la nueva especie *P. robusta*, en base al análisis filogenético de las secuencias de los marcadores moleculares ITS y LSU, y a sus características morfológicas. *Pseudosygmoidea robusta* se distingue fácilmente del resto de las especies del género por la producción de conidióforos bien diferenciados y conidios holoblásticos a partir de la transformación del ápice de ciertos conidios en células conidiógenas, cuyos locus conidiógenos proliferan simpodialmente, al igual que en las especies del género *Alternaria*.

5.2. *Eurotiomycetes*

Constituyen una clase de hongos cosmopolitas de gran interés biotecnológico (a modo de ejemplo, muchas de las especies de los géneros *Aspergillus* y *Penicillium*,) y médico (varios de los géneros de los órdenes Arachnomycetales y Onygenales), los cuales se aíslan de una gran variedad de sustratos presentes en diversos ambientes (Domsch et al. 2007). Sin embargo, los miembros de esta clase han sido muy raramente

reportados en ambientes acuáticos (Wang et al. 2019). En la presente tesis tan solo el 3.5 % (una cepa de cada uno de los siguientes géneros: *Cladophialophora, Mycocalicium, Phialophora* y *Penicillium*) de las cepas fue ubicada taxonómicamente en esta clase.

5.3. *Letiomycetes*

Eriksson & Winka (1997) introdujeron la clase *Leotiomycetes* para ubicar a los discomicetos inoperculados. El concepto tradicional de los *Leotiomycetes* incluye solo ascomicetos con ascomas apoteciales productores de ascos unitunicados carentes de opérculos, los que se abren por perforación apical o a través de un poro para liberar sus ascosporas (Pfister y Kimbrough 2001). Son un grupo ecológicamente diverso, la mayoría son saprobios, aunque también incluyen algunos patógenos de plantas. Se pueden aislar de una gran diversidad de sustratos, pero preferentemente de material vegetal y estiércol (Takamatsu et al. 2015). Los miembros de esta clase aislados de aguas dulces incluyen especies pertenecientes a los órdenes *Helotiales, Pezizales* y *Rhytismatales* (Hibbett et al. 2007, Basshien et al. 2013). En la presente tesis se han aislado cinco cepas pertenecientes a dicha clase, distribuidas en *Clathrosporium intricatum* y *Spirosphaeria floriformis* (pertenecientes a los hongos aero-acuáticos); los hongos *Idriella lunata* y *Cadophora* sp., cuyos conidios (holoblásticos y enteroblásticos, respectivamente) se forman a partir de conidióforos más o menos diferenciados; y la nueva especie de hongo celomiceto *Pilidium cuprescens*, cuyos conidiomas no ostiolados son de color cobrizo, mientras que sus conidios son fusiformes y curvados.

5.4. *Sordariomycetes*

Sordariomycetes es la segunda clase más rica en especies del filo Ascomycota (Hyde et al. 2013; Luo et al. 2019). Los miembros de esta clase producen ascomas periteciales y ascos unitunicados o no fisitunicados inoperculados. (Zhang et al. 2006; Kirk et al. 2008). Tienen una distribución cosmopolita y viven principalmente en hábitats terrestres, pero varias de sus especies se han reportado también en ambientes acuáticos (Hyde and Wong 2000, Raja et al. 2009, Réblová et al. 2010, Hu et al. 2010, Su et al. 2016, Luo et al. 2019). En la presente tesis doctoral la clase *Sordariomycetes* fue en frecuencia la segunda más aislada, representando al 30% del total de las cepas. Dichas cepas pudieron ser clasificadas como pertenecientes a los géneros *Bactrodesmium, Bartalinia, Beltrania, Chaetomidium, Codinea, Colletotrichum, Dendroclathra, Diaporte, Discosia, Diversimediispora, Fusarium, Gliomastix, Melanospora, Menispora, Myrmecridium,*

Neonectria, Neurospora, *Phaeosaria, Stachybotryna, Trichoderma, Volutella* y *Waydora.* A excepción de las especies de los géneros *Bactrodesmium, Bartalinia* y *Stachybotryna*, el resto de los géneros representan los primeros reportes para ambientes dulceacuícolas de España.

La gran mayoría de los hongos hallados durante el desarrollo de la presente tesis doctoral se pueden clasificar dentro del grupo de los acuáticos facultativos, ya que los géneros a los que pertenecen tienen al menos un reporte en hábitats terrestres. Una de las características que llama poderosamente la atención es que sus ascosporas presentan adaptaciones que les permiten adherirse a sustratos presentes en el medio acuático, tal como es la presencia de una envoltura mucilaginosa en la ascospora. Sin embargo, los conidios producidos en el interior de los conidiomas carecen de tales adaptaciones, con algunas excepciones. *Lolia aquatica*, perteneciente a la familia *Lindgomycetae* y aislada exclusivamente de agua dulce, se caracteriza por producir ascosporas rodeadas de una capa de mucílago, mientras que sus conidios presentan apéndices apicales de la misma naturaleza (Abdel-Aziz 2016). Ambos estados presentan características que permiten la mejor adaptación a los hábitats acuáticos.

Cabe destacar que en la presente tesis se han hecho grandes contribuciones al conocimiento de la taxonomía de los hongos presentes en hábitats de aguas dulces de España (sobre todo de ambientes loticos), y muy especialmente en aquellos individuos que se multiplican asexualmente por formación de conidios dentro de conidiomas (los anteriormente conocidos como hongos celomicetos). La gran mayoría de las muestras fueron recolectadas de hábitats loticos, sería interesante explorarlos más a fondo e incluir más muestras procedentes de ambientes lenticos. Otra de las grandes aportaciones científicas de la presente tesis doctoral es el hallazgo de los estados sexuales para géneros de hongos tan solo conocidos por su estado asexual (*Heterophoma* y *Pyrenochaetopsis*), y viceversa (*Amniculicola, Elongatopedicellata* y *Neovaginatispora*). Sin embargo, a pesar de los aportes aquí realizados, todavía queda un enorme potencial de taxones fúngicos de aguas dulces por descubrir y caracterizar dentro del territorio español, dado la existencia de diferentes microhábitats y la gran diversidad de sustratos presentes en los mismos.

6. CONCLUSIONES

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMÍA Y FILOGENIA MOLECULAR DE HONGOS PROCEDENTES DE MATERIAL VEGETAL SUMERGIDO DE ESPAÑA
Viridiana Magaña Dueñas

1. A pesar de lo limitado del número de muestras procesadas de material vegetal en descomposición colectadas en ecosistemas lóticos durante el desarrollo de la presente tesis doctoral (debido a las restricciones en la movilidad dentro del territorio español impuestas por las autoridades competentes para contener la expansión de la epidemia de SARS-Cov-2), estas han mostrado ser la fuente de una gran diversidad de hongos dulceacuícolas facultativos (mayoritariamente) y aero-acuáticos.

2. Tras el estudio polifásico de las 111 cepas fúngicas recuperadas en cultivo puro a partir de muestras procedentes de diferentes cuerpos de agua dulce de España, las mismas fueron ubicadas taxonómicamente en los órdenes *Capnodiales* (una cepa)*, Chaetosphaeriales* (cuatro cepas), *Chaetothyriales* (dos cepas), *Diaporthales* (dos cepas)*, Eurotiales* (una cepa)*, Glomerellales* (tres cepas), *Helotiales* (dos cepas)*, Hypocreales* (quince cepas)*, Melanosporales* (una cepa)*, Mycocaliciales* (una cepa) *Myrmecridiales* (una cepa), *Pleosporales* (sesenta y cinco cepas)*, Pleurotheciales* (una cepa)*, Phyllachorales* (una cepa)*, Sordariales* (tres cepas)*, Tubeufiales* (una cepa)*, Xylariales* (cinco cepas), en dos de las cepas el orden fue en *Incertae sedis.* Entre estos, el orden *Pleosporales* fue el más abundante y biodiverso, con el 59% del total de las cepas aisladas.

3. La caracterización morfológica y fisiológica, junto con el análisis de las secuencias nucleotídicas de diversos marcadores filogenéticos, permitieron describir un género y veintitrés especies de hongos dulceacuícolas nuevos para la ciencia.

4. Los resultados de los estudios fenotípico y filogenético de las cepas pertenecientes a la familia *Amniculicolaceae* permitieron el establecimiento del nuevo género *Fouskomenomyces* así como la propuesta de cinco nuevas especies: *Amniculicola microspora, Fouskomenomyces mimiticus*, *Murispora asexualis, M. fissilispora* y *M. naviculispora*. A consecuencia de la reordenación taxonómica de la familia, *F. cupreorufescens* (= *Spirosphaera cupreorufescens*), *M. bromicola* (= *Pseudomassariosphaeria bromicola*) y *M. triseptata* (*Pseudomassariosphaeria triseptata*) fueron propuestas como nuevas combinaciones.

5. Tres nuevas especies fueron introducidas en la familia *Didymellaceae*: *Didymella brevipilosa, Heterophoma polypusiformis* y *Paraboeremia clausa*.

6. *Neocucurbitaria aquadulcis*, *N. variabilis* y *Neovaginatispora aquadulcis* fueron descritas como nuevas especies de la familia *Lophiosthomataceae.*

7. La nueva combinación *Allocucurbitaria prunicola* (= *Neocucurbitaria prunicola*) fue propuesta en base a la reordenación taxonómica de la familia *Cucurbitariaceae*.

8. *Neopyrenochaeta asexualis, N. glabra* y *N. submersa* fueron descritas como nuevas especies para la familia *Neopyrenochaetaceae*.

9. La familia *Pyrenochaetopsidaceae* incrementó su número de especies gracias a la incorporación de *Pyrenochaetopsis aquatica, P. perfecta* y *P. cylindrispora*.

10. *Paraphoma aquatica, Phaeosphaeria fructigena* y *Xenophoma microspora* fueron reportadas como nuevas especies de la familia *Phaeosphaeriaceae.*

11. Finalmente, *Elongatopedicellata aquatica*, *Pilidium cupresens* y *Pseudosigmoidea robusta* fueron descritas como nuevas especies de las familias *Chaetomellaceae*, *Roussoellaceae* y *Sympoventuriaceae*, respectivamente.

12. Los miembros de la clase Sordariomycetes representaron el 30% del total de las cepas aisladas. Dichas cepas pudieron ser identificadas como pertenecientes a los géneros *Bactrodesmium*, *Bartalinia*, *Beltrania*, *Chaetomidium*, *Codinea*, *Colletotrichum*, *Dendroclathra*, *Diaporte*, *Discosia*, *Diversimediispora*, *Fusarium*, *Gliomastix*, *Melanospora*, *Menispora*, *Myrmecridium*, *Neonectria*, *Neurospora*, *Phaeosaria*, *Stachybotryna*, *Trichoderma*, *Volutella* y *Waydora*. A excepción de las especies de los géneros *Bactrodesmium*, *Bartalinia* y *Stachybotryna*, el resto de los géneros representan los primeros reportes para ambientes dulceacuícolas de España.

13. Es de destacar que alrededor de un 20 % de las cepas fúngicas aisladas correspondieron a nuevas especies para la ciencia.

14. Los resultados expuestos en la presente memoria evidencian que los ecosistemas loticos del territorio español albergan una gran diversidad de hongos dulceacuícolas facultativos, sobre todo de hongos comúnmente conocidos con el nombre de celomicetos, muchos de los cuales eran desconocidos para la ciencia. Por tal motivo, es de prever que futuros trabajos pongan en evidencia que las aguas dulces del territorio español albergan una insospechada hasta el momento, elevada diversidad fúngica.

15. Estos resultados constituyen un motivo más para proteger estos ecosistemas frente a la acción humana, en especial de la ligada a la actividad agrícola y ganadera extensivas, así como para promover una serie de acciones tendentes a minimizar el impacto del cambio climático global.

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Viridiana Magaña Dueñas

8. ANEXOS

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMÍA Y FILOGENIA MOLECULAR DE HONGOS PROCEDENTES DE MATERIAL VEGETAL SUMERGIDO DE ESPAÑA
Viridiana Magaña Dueñas

Otros estudios realizados durante el desarrollo de la presente tesis doctoral

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Tabla A1. Hongos de material vegetal en descomposición sumergido en aguas dulces reportados durante el desarrollo de la presente tesis doctoral.

FMR	CBS	Taxón	Lugar de aislamiento	Fecha
		Dothideomycetes		
16952		*Pleurotheciopsis bramleyi*	Les Guilleries, Barcelona	Noviembre/ 2017
16953		*Vargamyces aquaticus*	Les Guilleries, Barcelona	Noviembre/ 2017
16955		*Didymella viburnicola*	Les Guilleries, Barcelona	Noviembre/ 2017
16956	**CBS 148657**	***Paraphoma aquatica***	Les Guilleries, Barcelona	Noviembre/ 2017
16957	**CBS 147607**	***Neopyrenochaeta submersa***	Les Guilleries, Barcelona	Noviembre/ 2017
16958		***Fouskomenomyces mimiticus***	Les Guilleries, Barcelona	Noviembre/ 2017
16959		*Didymella macrostoma*	Les Guilleries, Barcelona	Noviembre/ 2017
16962		*Lentithecium aquaticum*	Les Guilleries, Barcelona	Noviembre/ 2017
16963		*Stomiopeltis betulae*	Les Guilleries, Barcelona	Noviembre/ 2017
16964		*Paraboeremia putaminum*	Les Guilleries, Barcelona	Noviembre/ 2017
17151	**CBS 146935**	***Fouskomenomyces mimiticus***	Les Guilleries, Barcelona	Noviembre/ 2017
17153		*Jalapriya pulchra*	Les Guilleries, Barcelona	Noviembre/ 2017
17245		*Vargamyces aquaticus*	Les Guilleries, Barcelona	Noviembre/ 2017
17248	**CBS 146937**	***Murispora asexualis***	Serra del Montsant, Tarragona	Febrero/ 2018
17250		Clohesyomyces sp.	Serra del Montsant, Tarragona	Febrero/ 2018
17251	**CBS 146936**	***Murispora fissilispora***	Serra del Montsant, Tarragona	Febrero/ 2018

17253		*Phomatodes nebulosa*	Serra del Montsant, Tarragona	Febrero/ 2018
17254		*Phomatodes nebulosa*	Serra del Montsant, Tarragona	Febrero/ 2018
17327	**CBS 147609**	***Pyrenochaetopsis aquatica***	Serra del Montsant, Tarragona	Febrero/ 2018
17334		*Phoma herbarum*	Serra del Montsant, Tarragona	Febrero/ 2018
17335		*Vargamyces aquaticus*	Serra del Montsant, Tarragona	Febrero/ 2018
17336		*Torula herbarum*	Serra del Montsant, Tarragona	Febrero/ 2018
17415	**CBS 148654**	***Didymella brevipilosa***	Riaza, Segovia	Mayo/ 2018
17416		***Pseudosigmoidea robusta***	Riaza, Segovia	Mayo/ 2018
17418	**CBS 147608**	***Neopyrenochaeta glabra***	Riaza, Segovia	Mayo/ 2018
17435		*Parafenestella pseudoplatani*	Pontons, Barcelona	Junio/ 2018
17436		*Parafenestella rosacearum*	Pontons, Barcelona	Junio/ 2018
17437		*Didymella glomerata*	Pontons, Barcelona	Junio/ 2018
17552	**CBS H-24739**	***Neocucurbitaria variabilis***	Riaza, Segovia	Mayo/ 2018
17669		*Neoconiothyrium* sp.	Cúber, Escorca, Mallorca	Noviembre/ 2018
17805		*Epicocum nigrum*	Capafonts, Tarragona	Marzo/ 2019
17806		*Phaeosphaeria* sp.	Capafonts, Tarragona	Marzo/ 2019
17807		*Didymella glomerata*	Capafonts, Tarragona	Marzo/ 2019
17808	**CBS 148658**	***Phaeosphaeria fructigena***	Capafonts, Tarragona	Marzo/ 2019
17809		*Didymella glomerata*	Capafonts, Tarragona	Marzo/ 2019
17811		*Murispora* sp.	Capafonts, Tarragona	Marzo/ 2019
17833		*Forliomyces uniseptata*	Capafonts, Tarragona	Marzo/ 2019

17834		***Elongatopedicellata aquatica***	Capafonts, Tarragona	Marzo/ 2019
17835		*Chlamydotubeufia* sp.	Capafonts, Tarragona	Marzo/ 2019
17837	**CBS 148655**	***Heterophoma polipusiformis***	Parque Natural Sierra Norte, Sevilla	Mayo/ 2019
17838	**CBS H-24464**	***Murispora navicularispora***	Parque Natural Sierra Norte, Sevilla	Mayo/ 2019
17840	**CBS 147605**	***Neocucurbitaria aquadulcis***	Parque Natural Sierra Norte	Mayo/ 2019
17841		*Neopyrenochaeta annellidica*	Parque Natural Sierra Norte, Sevilla	Mayo/ 2019
17843		*Preussia* sp.	Miraflores de la Sierra, Madrid	Mayo/ 2019
17844		*Neopyrenochaeta annellidica*	Parque Natural Sierra Norte, Sevilla	Mayo/ 2019
17845		*Neopyrenochaeta maesuayensis*	Parque Natural Sierra Norte, Sevilla	Mayo/ 2019
17874	**CBS 147606**	***Neopyrenochaeta asexualis***	Parque Natural Sierra Norte, Sevilla	Mayo/ 2019
17875		*Neopyrenochaeta maesuayensis*	Parque Natural Sierra Norte, Sevilla	Mayo/ 2019
17876		*Neopyrenochaeta maesuayensis*	Parque Natural Sierra Norte, Sevilla	Mayo/ 2019
17877		***Neocucurbitaria variabilis***	Parque Natural Sierra Norte, Sevilla	Mayo/ 2019
17879		*Neopyrenochaeta telephoni*	Parque Natural Sierra Norte, Sevilla	Mayo/ 2019
17946		***Amniculicola microspora***	Parque Natural Sierra Norte, Sevilla	Mayo/ 2019
17947		***Xenophoma microspora***	Pontons, Barcelona	Junio/ 2018
18486		*Pleospora herbarum*	Río Segre, Lérida	Diciembre/ 2019
18499		*Neopyrenochaeta annellidica*	Parque de los pueblos de Europa, Gernika	Agosto/ 2020
18500		*Neopyrenochaeta annellidica*	Parque de Doña Casilda Iturriza, Bilbao	Agosto/ 2020
18501		*Neopyrenochaeta annellidica*	Parque de los pueblos de Europa, Gernika	Agosto/ 2020
18502		*Neopyrenochaeta annellidica*	Parque de los pueblos de Europa, Gernika	Agosto/ 2020

18505		*Epicoccum* sp.	Roda de Ter	Septiembre/ 2020
18506		*Neopyrenochaeta annellidica*	Roda de Ter	Septiembre/ 2020
18597	**CBS 148656**	***Paraboeremia clausa***	Parque de Doña Casilda Iturriza, Bilbao	Agosto/ 2020
18598		***Paraboeremia clausa***	Parque de Doña Casilda Iturriza, Bilbao	Agosto/ 2020
18799		*Lentithecium varaginerpora*	Burriana, Castellón	Marzo/ 2021
18800		*Pyrenochaetopsis confluens*	Burriana, Castellón	Marzo/ 2021
18801		***Pyrenochaetopsis cylindrispora***	Burriana, Castellón	Marzo/ 2021
18913		***Pyrenochaetopsis perfecta***	Burriana, Castellón	Marzo/ 2021
18914		***Neovaginatispora aquadulcis***	Burriana, Castellón	Marzo/ 2021
18915		*Wettsteinina* lacustris	Burriana, Castellón	Marzo/ 2021
		Eurotiomycetes		
16968		*Phialophora cyclaminis*	Les Guilleries, Barcelona	Noviembre/ 2017
17152		*Penicillium citrinum*	Les Guilleries, Barcelona	Noviembre/ 2017
17601		*Mycocalicium* sp.	Cúber, Escorca, Mallorca	Noviembre/ 2018
17836		*Cladophialophora multiseptata*	Cúber, Escorca, Mallorca	Noviembre/ 2018
		Letiomycetes		
17432		*Clathrosporium intricatum*	Riaza, Segovia	Mayo/ 2018
17433		*Spirosphaeria floriformis*	Riaza, Segovia	Mayo/ 2018
17553		*Cadophora sp.*	Riaza, Segovia	Mayo/ 2018
17839		***Pilidium cuprescens***	Parque Natural Sierra Norte, Sevilla	Agosto/ 2020
17842		*Idriella lunata*	Miraflores de la Sierra, Madrid	Agosto/ 2020

Sordariomycetes

16954	*Neonectria lugdunensis*	Les Guilleries, Barcelona	Noviembre/ 2017
16960	*Neonectria lugdunensis*	Les Guilleries, Barcelona	Noviembre/ 2017
16961	*Neonectria lugdunensis*	Les Guilleries, Barcelona	Noviembre/ 2017
16965	*Diaporthe eres*	Les Guilleries, Barcelona	Noviembre/ 2017
16966	*Fusarium ciliatum*	Les Guilleries, Barcelona	Noviembre/ 2017
16967	*Menispora ciliata*	Les Guilleries, Barcelona	Noviembre/ 2017
16969	*Trichoderma atroviride*	Les Guilleries, Barcelona	Noviembre/ 2017
17154	*Stachybotrys dichroa*	Serra del Montsant, Tarragona	Febrero/ 2018
17155	*Fusarium ciliatum*	Serra del Montsant, Tarragona	Febrero/ 2018
17156	*Reticulascus clavatus*	Serra del Montsant, Tarragona	Febrero/ 2018
17157	*Myrmecridium schulzeri*	Serra del Montsant, Tarragona	Febrero/ 2018
17158	*Volutella rosae*	Serra del Montsant, Tarragona	Febrero/ 2018
17246	*Codinaea setosa*	Les Guilleries, Barcelona	Noviembre/ 2017
17247	*Reticulascus clavatus*	Serra del Montsant, Tarragona	Febrero/ 2018
17249	Chaetomidium arxii	Serra del Montsant, Tarragona	Febrero/ 2018
17252	*Volutella rosae*	Serra del Montsant, Tarragona	Febrero/ 2018
17328	*Bactrodesmium obovatum*	Les Guilleries, Barcelona	Noviembre/ 2017
17329	*Codinaea setosa*	Serra del Montsant, Tarragona	Febrero/ 2018
17330	*Stachybotryna excentrica*	Serra del Montsant, Tarragona	Febrero/ 2018

17331	*Dendroclathra lignicola*	Serra del Montsant, Tarragona	Febrero/ 2018
17332	*Gliomastix murorum*	Serra del Montsant, Tarragona	Febrero/ 2018
17333	*Codinaea setosa*	Serra del Montsant, Tarragona	Febrero/ 2018
17414	*Stachybotryna excentrica*	Serra del Montsant, Tarragona	Febrero/ 2018
17417	*Gliomastix masseei*	Riaza, Segovia	Mayo/ 2018
17419	*Phaeoisaria clematidis*	Pontons, Barcelona	Junio/ 2018
17551	*Melanospora zamiae*	Pontons, Barcelona	Junio/ 2018
17602	*Waydora typica*	Cúber, Escorca, Mallorca	Noviembre/ 2018
17603	*Bartalinia robillardoides*	Cúber, Escorca, Mallorca	Noviembre/ 2018
17668	*Discosia artocreas*	Cúber, Escorca, Mallorca	Noviembre/ 2018
17810	*Beltrania querna*	Capafonts, Tarragona	Marzo/ 2019
18489	*Gelasinospora tetrasperma*	Parque de Doña Casilda Iturriza, Bilbao	Agosto/ 2020
18498	*Gelasinospora tetrasperma*	Parque de Doña Casilda Iturriza, Bilbao	Agosto/ 2020
18503	*Diversimediispora humicola*	Roda de Ter, Barcelona	Septiembre/ 2020
18504	*Colletotrichum truncatum*	Roda de Ter, Barcelona	Septiembre/ 2020

Los nombres en **negrita** son nuevas especies para la ciencia.

FMR: Colección de hongos de la Facultad de Medicina de Reus, Tarragona, España.

CBS: Centraalbureau voor Schimmelcultures (oficina central de cultivos fúngicos), Westerdijk Fungal Biodiversity Institute, (Utrecht, Paises Bajos).

Two new species of *Gloniopsis* (Hysteriales, Ascomycota) from clinical specimens: Morphological and molecular characterisation

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Publicado en: Mycoses, 2019, 00: 1-10

DOI:10.1111/myc.13006

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMÍA Y FILOGENIA MOLECULAR DE HONGOS PROCEDENTES DE MATERIAL VEGETAL SUMERGIDO DE ESPAÑA
Viridiana Magaña Dueñas

Received: 25 June 2019 | Revised: 6 September 2019 | Accepted: 9 September 2019

DOI: 10.1111/myc.13006

ORIGINAL ARTICLE

mycoses | WILEY

Two new species of *Gloniopsis* (Hysteriales, Ascomycota) from clinical specimens: Morphological and molecular characterisation

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Funding information
Spanish Ministerio de Economía y Competitividad,, Grant/Award Number: CGL2017-88094-P

Summary

Background: The coelomycetes comprise a wide range of fungal species distributed in at least three different classes of the phylum Ascomycota. These are morphologically characterised by producing their conidia inside of fruiting bodies called pycnidia or acervuli, and only a reduced number of species are able to cause human infections. However, their identification in the clinical laboratory is often difficult, due to their few morphological features or because they remain sterile.

Materials and Methods: In the present study, three isolates of coelomycetes of clinical origin were phenotypically and molecularly studied, by sequencing the D1-D2 fragment of the 28S nuclear ribosomal RNA (nrRNA) (LSU), the internal transcribed spacer region (ITS1-5.8S-ITS2) and a fragment of the translation elongation factor 1-alpha (*tef*1) genes.

Results and Conclusions: As result of the molecular analysis, the isolates were identified as belonging to the genus *Gloniopsis* (order *Hysteriales*, *Dothideomycetes*) but without the characteristics of any of the species described so far. Therefore, we propose the new species *Gloniopsis percutanea* and *Gloniopsis pneumoniae*. Furthermore, this study revealed that some isolates from clinical specimens identified previously as *Rhytidhysteron* spp. were misidentified, and considering the few studies in the order *Hysteriales* and the scarce number of sequences of phylogenetic markers, future revisions of this order should be performed to clarify their taxonomy and obtain a better identification from isolates involved in human mycoses.

KEYWORDS
coelomycetes, *Gloniopsis*, *Hysteriales*, mycosis, *Rhytidhysteron*

1 | INTRODUCTION

The coelomycetes comprise a wide range of fungal species distributed in at least three different classes of the phylum Ascomycota.[1] Despite the fact that the term "Coelomycetes" is invalid in the current taxonomic classification, it is still used for practical purposes for those fungi that are morphologically characterised by producing conidia inside of fruiting bodies called pycnidia or acervuli. These are mostly saprobic or parasites of terrestrial plant debris, but can also be isolated from soil or aquatic environments, and only a few species are able to cause human infections.[2,3] In addition, their identification in the clinical laboratory is often difficult, due to their few morphological features or because they remain sterile.[2,4] Recently, several studies in this group of fungi have been performed using isolates

TABLE 1 Isolates used in this study and their GenBank accession numbers. Newly generated sequences are indicated in bold. Type strains are indicated by superscript T

Taxa	Culture collection	Country	Host, substrate	GenBank accession numbers		
				LSU	ITS	TEF-1α
Graphyllium caracolinensis	HUEFS 42838^{T}	Brazil	Twig of unidentified plant	KF914914	—	—
Gloniopsis arciformis	GKM L166A^{T}	Kenya	Decorticated hardwood	GU323211	—	—
Gloniopsis calami	MFLUCC 15-0739^{T}	Thailand	*Calamus* sp	NG_059715	KX669036	KX671965
Gloniopsis kenyensis	GKM 1010T	Kenya	Decorticated hardwood	GQ221891	—	—
Gloniopsis percutanea (formerly *Rhytidhysteron rufulum*)	810 001	India	Unknown human sample	—	KY310737	—
Gloniopsis percutanea (formerly *Rhytidhysteron rufulum*)	B 124 10.2013	India	Nail residuals	—	KM052345	—
Gloniopsis percutanea (formerly *Rhytidhysteron rufulum*)	DD1	India	Subcutaneous phaeohyphomycosis	—	KP162180	—
Gloniopsis percutanea (formerly *Rhytidhysteron rufulum*)	DD2	India	Subcutaneous phaeohyphomycosis	—	KP162181	—
Gloniopsis percutanea (formerly *Plectophomella* sp.)	**FMR 8713^{T}**	Brazil	Human biopsia	**LS997561**	AM286786	**LS997569**
Gloniopsis percutanea (formerly *Rhytidhysteron rufulum*)	FMR 8743	India	Human biopsia	—	AM711974	—
Gloniopsis percutanea (formerly *Rhytidhysteron rufulum*)	IM 02	India	Pus sample	—	KJ787018	—
Gloniopsis percutanea	LVPEI H246 10	India	Ocular tissue	—	JX868651	—
Gloniopsis percutanea	UTHSC DI16-254	USA	Human aspirate sample	LN907397	**LS997559**	**LS997570**
Gloniopsis percutanea (formerly *Rhytidhysteron rufulum*)	VPCI 315 P 15	India	Right foot lipoma	—	KU236375	—
Gloniopsis percutanea (formerly *Rhytidhysteron rufulum*)	VPCI 319 P 15	India	Left knee swelling	—	KU236376	—
Gloniopsis pneumoniae	**UTHSC DI16-353^{T}**	USA	Lung tissue	LN907496	**LS997560**	**LS997571**
Gloniopsis praelonga	CMW 19 983	South Africa	*Leucadendron rubrum*	FJ161193	—	—
Gloniopsis praelonga	CBS 112 415	South Africa	*Staberoha distachyos*	FJ161173	—	FJ161090
Gloniopsis praelonga	CBS 123 337	USA	*Rubus* sp	FJ161195	—	—
Gloniopsis praelonga	CMW 18 053	South Africa	*Ischyrolepis subverticellata*	FJ161191	—	—
Gloniopsis sp.	MFLUCC 14-0581	Thailand	Unknown	KU377563	—	KU497491
Gloniopsis sp.	MFLUCC 12-0010	Thailand	Dead wood	KJ418114	KJ418115	
Gloniopsis subrugosa	CBS 123 346	South Africa	Decorticated hardwood	FJ161210	—	—
Gloniopsis subrugosa	SMH557	Cuba	Unknown	GQ221896	—	—

(Continues)

TABLE 1 (Continued)

Taxa	Culture collection	Country	Host, substrate	GenBank accession numbers		
				LSU	ITS	TEF-1α
Hysterium angustatum	CBS 236.34	USA	Betula sp	FJ161180	—	FJ161096
Hysterium angustatum	CBS 123 334	USA	*Pinus rigida*	FJ161207	—	—
Hysterium barrianum	ANM1442	USA	Unknown	GQ221884	—	—
Hysterium barrianum	ANM1495	USA	Unknown	GQ221885	—	—
Hysterium pulicare	ANM1455	USA	Unknown	GQ221904	—	GQ221932
Hysterium pulicare	ANM85	USA	Unknown	GQ221898	—	GQ221934
Hysterobrevium constrictum	SMH5211.1	New Zealand	Unknown	GQ221905	—	GQ221923
Hysterobrevium constrictum	GKM426N	Kenya	Unknown	GQ221901	—	GQ221913
Hysterobrevium mori	GKM 1013	Kenya	Unknown	GU397344	—	GU397338
Hysterobrevium mori	CBS 123 335	USA	Decorticated hardwood	FJ161202	—	—
Hysterobrevium mori	SMH5273	USA	Unknown	GQ221910	—	GQ221936
Hysterobrevium smilacis	CBS 200.34	USA	*Ulmus* sp	FJ161177	—	—
Hysterobrevium smilacis	CBS 114 601	Sweden	*Ulmus carpinifolia*	FJ161174	—	FJ161091
Hysterodifractum partisporum	HUEFS 42865^{T}	Brazil	Twig of unidentified plant	KF914916	—	—
Mytilinidion andinense	CBS 123 562	Argentina	*Austrocedrus chilensis*	FJ161199	—	FJ161107
Mytilinidion resinicola	CBS 304.34^{T}	USA	*Larix laricina*	FJ161185	—	FJ161101
Oedohysterium insidens	CBS 238.34	USA	*Quercus* sp	FJ161182	—	FJ161097
Oedohysterium insidens	ANM1443	USA	Unknown	GQ221882	—	—
Oedohysterium sinense	CBS 123 345	South Africa	Decorticated hardwood	FJ161209	—	—
Oedohysterium sinense	EB 0339	USA	Unknown	GU397348	—	GU397339
Ostreichnion centramurum	chuni 70	Thailand	Decaying bark	KM272256	—	KM277819
Ostreichnion sassafras	CBS 322.34	USA	*Quercus* sp	FJ161188	—	—
Psiloglonium araucanum	CMW 17 941	South Africa	*Cannomois virgata*	FJ161190	—	—
Psiloglonium araucanum	CBS 112 412	South Africa	*Rhodocoma capensis*	FJ161172	—	FJ161089
Psiloglonium clavisporum	CBS 123 340	USA	*Quercus rubrum*	FJ161205	—	—
Psiloglonium clavisporum	CBS 123 341	USA	*Quercus rubrum*	FJ161206	—	—
Psiloglonium macrosporum	MFLUCC 13-0448^{T}	Thailand	Dead twig	KU243049	KU243048	—
Rhytidhysteron neorufulum	CBS 306.38	Unknown	*Pistacia chinensis*	FJ469672	—	GU349031
Rhytidhysteron neorufulum	MFLUCC 13-0216^{T}	Thailand	Dead stem	KU377566	KU377561	KU510400
Rhytidhysteron neorufulum	MFLUCC 13-0221	Thailand	Dead stem	KU377567	KU377562	—
Rhytidhysteron neorufulum	MFLUCC 17-2236	Thailand	*Hevea brasiliensis*	MH063266	MH062956	—

(Continues)

TABLE 1 (Continued)

Taxa	Culture collection	Country	Host, substrate	GenBank accession numbers		
				LSU	ITS	TEF-1α
Rhytidhysteron opuntiae	GKM1190	Kenya	Unknown	GQ221892	–	–
Rhytidhysteron rufulum	EB 0384	Ghana	Unknown	GU397354	–	–
Rhytidhysteron rufulum	MFLUCC 12-0013	Thailand	Dead wood	KJ418111	KJ418112	-
Rhytidhysteron rufulum	MFLUCC 14-0577	Thailand	Dead stem	KU377565	KU377560	KU510399
Rhytidhysteron thailandicum	MFLUCC 14-0503^{T}	Thailand	Dead twig	NG_059648	KU377559	KU497490
Rhytidhysteron thailandicum	MFLUCC 12-0530	Thailand	Woody litter	KJ526125	KJ546123	–

recovered from clinical specimens,[4-6] and these have shown a great diversity after analysing molecularly, being distributed mainly in the classes *Dothideomycetes* and *Sordariomycetes*.[3]

In previous work performed by Valenzuela-Lopez *et al*,[4] we found two clinical isolates from the USA belonging to the order *Hysteriales*, but those remained in an ambiguous taxonomic position. Currently, the latter order has been taxonomically revised and comprises only one family (*Hysteriaceae*) with nine accepted genera.[7] Morphologically, the hysteriaceous fungi are characterised by having lirelliform (long-narrow) ascomata, generally called hysterothecia,[7] which correspond to their sexual morph description. Unfortunately, little is known about their asexual morph relatives, being only aposphaeria-like and pyrenochaeta-like the coelomycetes associated.[8]

In the literature, there are few reports of human infections caused by species of hysteriaceous fungi; one of these corresponds to the genus *Rhytidhysteron*, which was introduced by Spegazzini and currently includes 50 accepted species (Index Fungorum, 2019),[9,10] morphologically characterised by producing carbonaceous, immersed to erumpent to entirely superficial hysterothecia, and bitunicate asci, hyaline to pigmented, with one to multi-septate or muriform (spore divided in more than one plane) ascospores.[11] This genus has been reported mainly in superficial or subcutaneous mycoses can affect immunodeficient as many as immunocompetent patients, with clinical manifestations of chromoblastomycosis and phaeohyphomycosis, both having in common the presence of melanised fungal structures. The clinical laboratory identification of these fungi was mainly based on DNA sequence of the internal transcribed spacer region (ITS1-5.8S-ITS2). As the isolates remained sterile in the culture media, morphological comparisons were not performed.[12-16]

The present work revises the taxonomy of three isolates recovered from clinical specimens related with those in the order *Hysteriales* by combined phenotypic and molecular data. A multilocus analysis was performed using three molecular markers: the D1-D2 fragment of the 28S nuclear ribosomal RNA (nrRNA) (LSU), the internal transcribed spacer region (ITS) and a fragment of the translation elongation factor 1-alpha (*tef*1) gene.

2 | MATERIALS AND METHODS

2.1 | Fungal isolates

We studied three isolates of coelomycetous fungi recovered from clinical specimens, two were provided by the Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio (UTHSC; San Antonio, Texas, USA), and the other one was provided by the Facultat de Medicina at the Universitat Rovira i Virgili (FMR; Reus, Spain).

2.2 | Morphological characterisation

For morphology studies, the isolates were cultured on malt extract agar (MEA; 40 g of malt extract, 15 g of agar-agar, 1 L distilled water), oatmeal agar (OA; 30 g of filtered oat flakes, 15 g of agar-agar, 1 L

tap water) and potato dextrose agar (PDA; Pronadisa) at 20 ± 1°C for 14 days in darkness. The morphological features of the vegetative and reproductive structures were studied using an Olympus CH2 bright-field microscope (Olympus Corporation) in Shear`s wet mount of the fungal isolates. Colour standards by Kornerup and Wanscher[17] were used in colony description. Photomicrographs were taken with an Axio Imager M1 microscope (Zeiss).

2.3 | Molecular identification and phylogenetic analysis

Total genomic DNA was extracted following the protocols by Valenzuela-Lopez *et al*[4] The following genes were amplified and sequenced: a fragment of the 28S nrRNA gene (LSU) with the primer pair LR0R and LR5, internal transcribed spacer region (ITS1-5.8S-ITS2) with the primer pair ITS5 and ITS4, and a fragment of the translation elongation factor 1-alpha gene (*tef*1) with the primers TEF1-983F and TEF1-2218R.[18-21] The PCR amplifications were performed in a total volume of 25 μL containing 5 μL 10 × PCR Buffer (Invitrogen), 0.2 mM dNTPs, 0.5 μmol/L of each primer, 1 U Taq DNA polymerase and 1-10 ng genomic DNA. PCR conditions for LSU, ITS and *tef*1 were set as follows: for LSU and ITS an initial denaturation at 95°C for 5 minutes, followed by 35 cycles consisted of 45 seconds at 95°C, 45 seconds at 53°C and 2 minutes at 72°C; and for the *tef*1 region an initial denaturation at 94°C for 2 minutes, followed by 30 cycles consisted of 30 seconds at 94°C, 1 minutes 20 seconds at 57°C and 1 minutes 30 seconds at 72°C.

Sequences of type or reference strains described in previous studies were obtained from GenBank and are listed in Table 1. DNA sequences generated in this study were deposited in GenBank (accession numbers are given in Table 1).

For the phylogenetic studies, sequences were aligned using the ClustalW application of the MEGA 6.06 computer program and manually adjusted using the same software platform.[22,23]

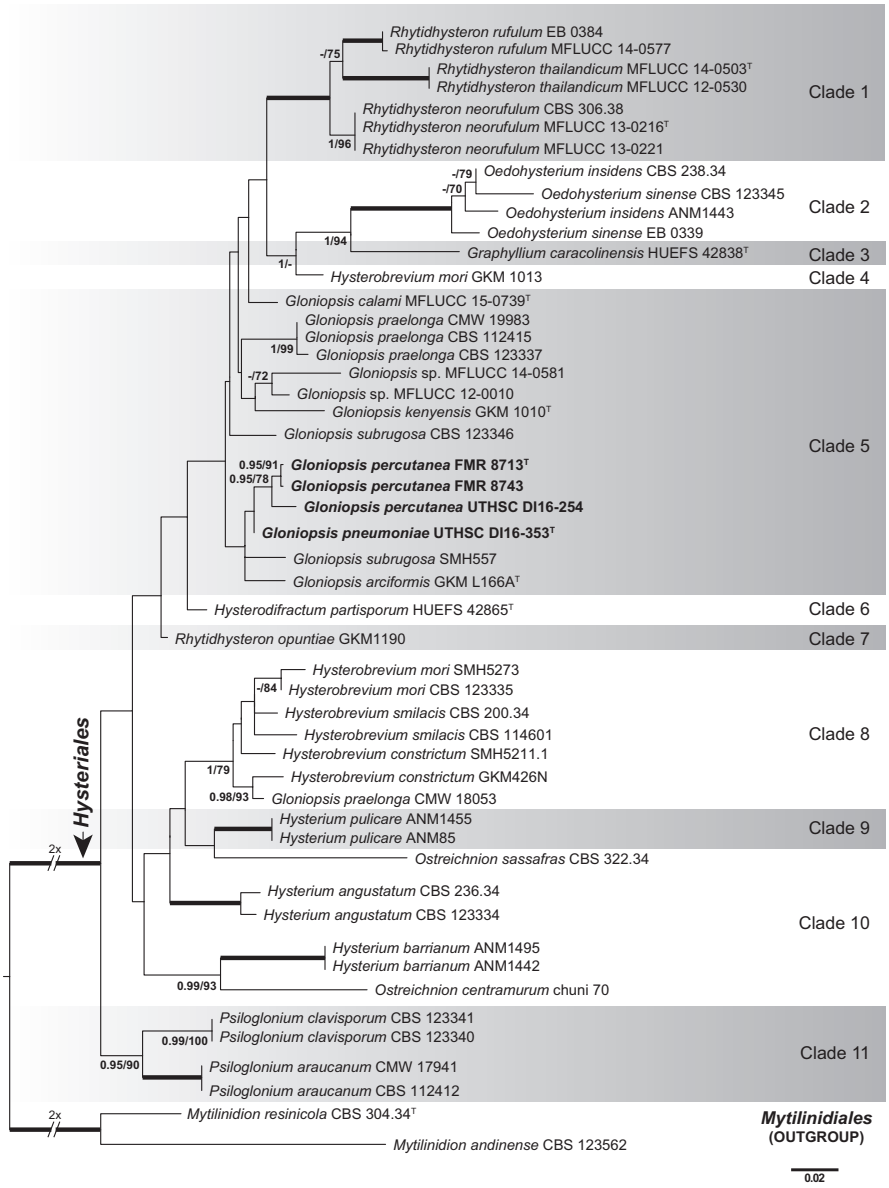

FIGURE 1 Phylogenetic tree inferred from a maximum-likelihood analysis based on a concatenated alignment of LSU and *tef*1 sequences of 50 strains representing species in *Hysteriales*. The Bayesian posterior probabilities (PP) above 0.95 and the RAxML bootstrap support values (BS) above 70% are given at the nodes (PP/BS). Some branches were shortened to fit them to the page; these are indicated by two diagonal lines with the number of times a branch was shortened. Fully supported branched (1 PP/100 BS) are indicated in bold. Newly proposed taxa are given in bold. Type strains are indicated by a superscript T. The tree was rooted with a reference strain of *Mytilinidion andinense* (CBS 123 562) and the type strain of *Mytilinidion resinicola* (CBS 304.34) of the order *Mytilinidiales*

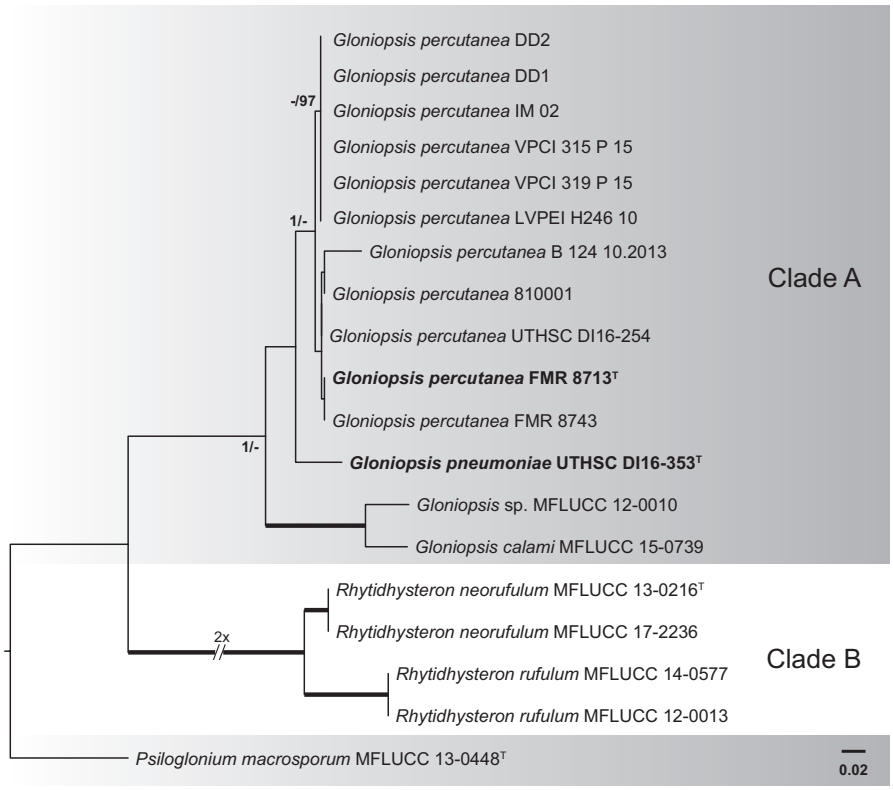

FIGURE 2 Phylogenetic tree inferred from a maximum-likelihood analysis based on a concatenated alignment of ITS sequences of 19 strains representing species in *Hysteriaceae*. The Bayesian posterior probabilities (PP) above 0.95 and the RAxML bootstrap support values (BS) above 70% are given at the nodes (PP/BS). One branch was shortened to fit it to the page, these is indicated by two diagonal lines with the number of times a branch was shortened. Fully supported branched (1 PP/100 BS) are indicated in bold. The new type strains FMR 8713 and UTHSC DI16-353 are given in bold. Type strains are indicated by a superscript ^{T}. The tree was rooted with the type strain of *Psiloglonium macrosporum* (MFLUCC 13-0448)

Phylogenetic reconstructions were made by maximum likelihood (ML) and Bayesian inference (BI) with MEGA 6.06 and MrBayes 3.2.6, respectively.[24] The best substitution model for each gene matrix was estimated using MrModelTest v. 2.3.[25] For ML analyses, nearest-neighbour interchange was used as the heuristic method for tree inference. Support for internal branches was assessed by 1,000 ML bootstrapped pseudoreplicates. Bootstrap support (BS) of ≥70 was considered significant. For BI analyses, Markov chain Monte Carlo (MCMC) sampling was carried out with four million generations, with samples taken every 1,000 generations. The 50% majority rule consensus trees and posterior probability values (PP) were calculated after removing the first 25% of the resulting trees for burn-in. A PP value of ≥0.95 was considered significant. A reference strain of *Mytilinidion andinense* (CBS 123562), and the type strains of *Mytilinidion resinicola* (CBS 304.34) and *Psiloglonium macrosporum* (MFLUCC 13-0448) were used as outgroups.

The novel taxonomic descriptions and nomenclature were deposited in MycoBank database (www.mycobank.org).[26]

3 | RESULTS

3.1 | Phylogenetic analyses

Two phylogenetic studies were performed. The first analysis included 50 strains combining their LSU and *tef*1 sequences with a total of 1096 characters including gaps (563 for LSU and 533 for *tef*1), of which 244 were parsimony informative (133 for LSU and 111 for *tef*1). The second analysis included 19 ITS sequences distributed among the genera *Gloniopsis*, *Psiloglonium* and *Rhytidhysteron*, with a total of 435 characters including gaps, of which 137 were parsimony informative. For each study, the ML analysis showed similar tree topology and was congruent with that obtained in the BI analysis.

Figure 1 shows the tree of first phylogenetic analysis, which distinguished two main well-supported clades corresponding to the orders *Hysteriales* and *Mytilinidiales* (1 PP/ 100% BS). The *Hysteriales* encompassed eleven clades that contain the nine genera currently accepted, of which six of them were phylogenetically congruent with their previous taxonomic description, forming six distinct clades (1, 2, 3, 6, 8 and 11) for the genera *Rhytidhysteron*, *Oedohysterium*, *Graphyllium*, *Hysterodifractum*, *Hysterobrevium* and *Psiloglonium*, respectively. However, the other five clades (4, 5, 7, 9 and 10) remain taxonomically ambiguous being phylogenetically polyphyletic such as the genera *Gloniopsis* (clade 5), *Hysterium* and *Ostreichnion* (clade 9 and 10). The strain GKM 1013 (clade 4) identified as *Hysterobrevium mori* and GKM 1190 (clade 7) as *Rhytidhysteron opuntiae* form two distinct clades that probably correspond to new taxa.

The second phylogenetic tree (Figure 2) was performed based on ITS sequences for those clinical strains previously reported in human mycoses identified as *Rhytidhysteron* spp., and our strains. This result showed that all previous strains associated with the latter genus were misidentified, and correspond to the genus *Gloniopsis*. In addition, the strains FMR 8713 and UTHSC DI16-254 as well as the strain UTHSC DI16-353 that formed a distinct clade in *Gloniopsis* are proposed here as the new species *Gloniopsis percutanea* and *Gloniopsis pneumoniae*, respectively.

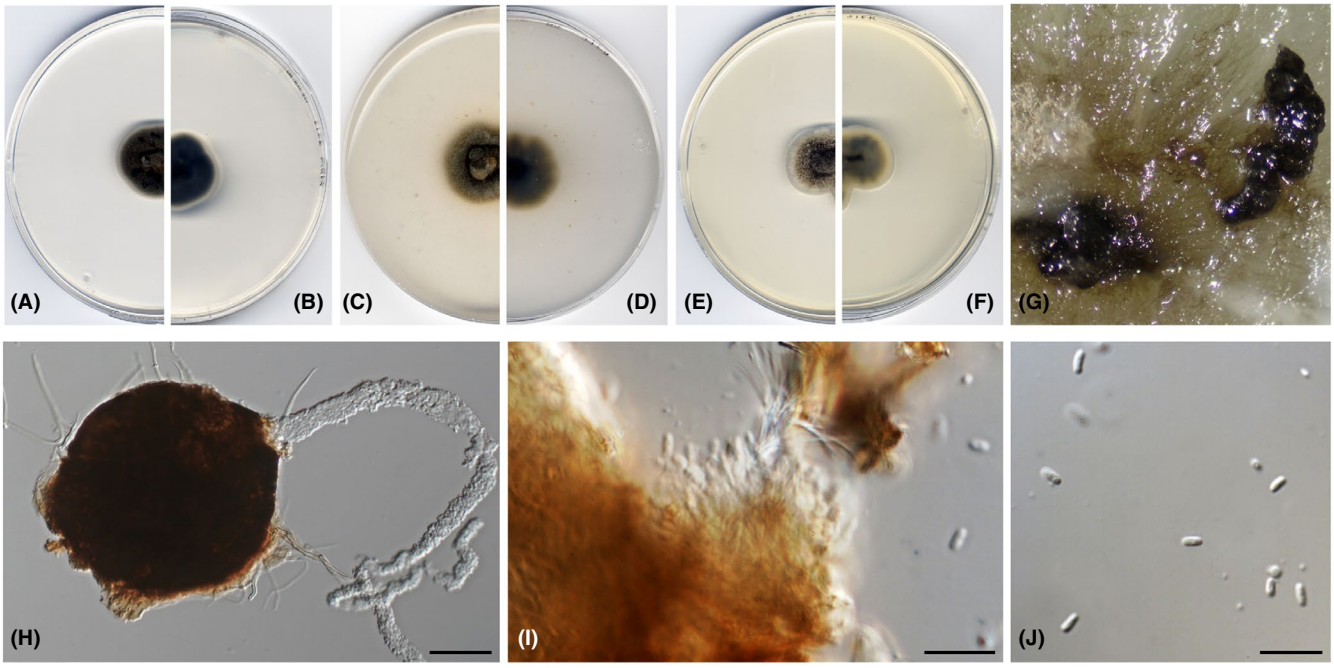

FIGURE 3 *Gloniopsis percutanea* (FMR 8713). A, B. Colony on MEA (front and reverse). C, D. Colony on OA (front and reverse). E, F. Colony on PDA (front and reverse). G. Pycnidia forming on OA. H. Pycnidium. I. Conidiogenous cells. J. Conidia. Scale bars: H = 25 µm, I, J = 10 µm

3.2 | Taxonomy

Gloniopsis percutanea Valenzuela-Lopez, Cano, Guarro & Stchigel sp nov., MycoBank MB 830898. Figure 3.

Etymology: The species name refers to the cutaneous tissue, from which the fungus was isolated.

Description: *Hyphae* hyaline to pale brown, 2-4 µm wide, smooth- and thin-walled, septate. *Conidiomata* pycnidial, brown to dark brown, superficial or immersed, aggregated, glabrous, very variable in shape, lenticular, subglobose, globose to pyriform, 100-190 × 90-250 µm, ostiolate; pycnidial wall 3-5-layered, 10-15 µm thick, covered by a compact mass of interwoven, brown to dark brown hyphae, followed by an outer-layer of *textura angularis* of brown to dark brown, flattened polygonal cells of 4.5-8 µm diam, incrusted by a dark brown to carbonaceous material especially around the papillate, slightly protruding ostiole of 15-35 µm diam. *Conidiogenous cells* phialidic, ampulliform, hyaline, 3.5-5.5 × 2-3 µm. *Conidia* aseptate, hyaline to pale brown, smooth- and thin-walled, bacilliform, 2.5-4 × 1-2 µm, sometimes slightly curved, guttulate.

Culture characteristics: Colonies on MEA reaching 11-12 mm diam after 7 days at 25 ± 1°C, velvety, olive brown (M. 4F3); reverse dark grey (M. 4F1). Colonies on OA reaching 14 mm diam, velvety to slightly floccose, curry yellow (M. 4C7) to olive brown (M. 4F3); reverse greyish yellow (M. 4B4) to olive brown (M. 4F3). Colonies on PDA reaching 10 mm diam, slightly floccose, light blond (M. 4C3) to olive brown (M. 4F5); reverse light blond (M. 4C3) to olive brown (M. 4E7). NaOH spot test negative. Crystals absent. Optimal, minimum and maximum temperatures of growth: 25, 15 and 37°C, respectively.

Holotype: Brazil, from biopsy of subcutaneous nodules at the right arm of a 31-year-old man with chronic myeloid leukaemia, 2004, J. Guarro (holotype CBS H-24013, ex-holotype living cultures CBS 119963 = FMR 8713).

Material examined: USA, from human aspirate sample, 2008, DA Sutton (living cultures UTHSC DI16-254 = FMR 13744).

Notes: The strain FMR 8713, which was isolated from subcutaneous arm nodules of a patient with leukaemia in Brazil, was at first identified as *Plectophomella* sp due to its coelomycetous morphology.[27] Despite sequencing the ITS region (GenBank # AM286786), the match with other coelomycetous fungi sequences was very low due to a lack of deposits in public databases at the time. Later, de Gruyter *et al*[28] placed the strain into the genus *Chaetophoma*. However, in our study this strain is phylogenetically related to the genus *Gloniopsis*, a sort of ascomycetous fungus characterised by the production of navicular carbonaceous ascomata with a longitudinal invaginated slit (= hysterothecia) and of hyaline to yellowish, muriform ascospores. Very recently, it was reported that a strain of *Gloniopsis subrugosa*, isolated from dead wood in Thailand, was capable of producing a coelomycetous asexual morph in vitro.[7] However, the coelomycetous asexual morph of *G subrugosa* possesses a thicker pycnidial wall than in FMR 8713 and FMR 13744, the conidiogenous cells measure 10-12 × 2-3 µm (3.5-5.5 × 2-3 µm in our strains), and the conidia are larger (4-6 × 2-3 µm vs 2.5-4 × 1-2 µm), ellipsoidal to allantoid (bacilliform in FMR 8713 and FMR 13744) and greenish-brown (hyaline to pale brown in our strains).

Gloniopsis pneumoniae Valenzuela-Lopez, Magaña-Dueñas, Cano, Guarro & Stchigel sp nov., MycoBank MB 830899. Figure 4.

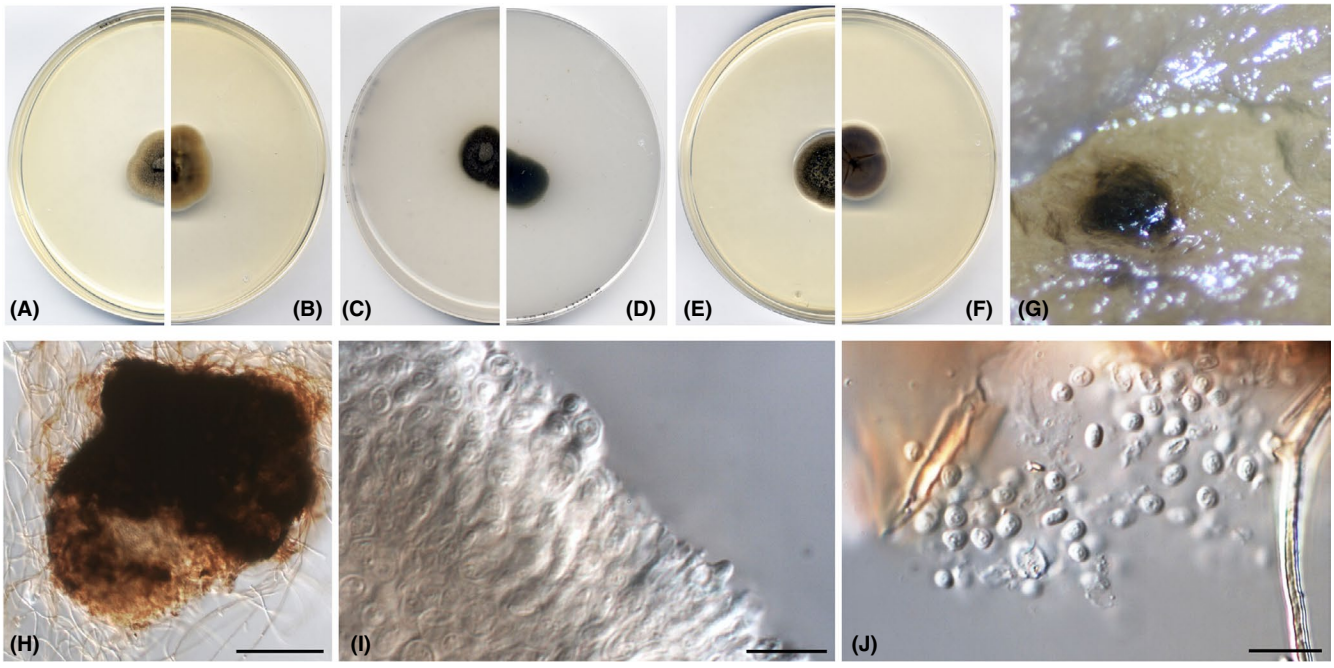

FIGURE 4 *Gloniopsis pneumoniae* (UTHSC DI16-353). A, B. Colony on MEA (front and reverse). C, D. Colony on OA (front and reverse). E, F. Colony on PDA (front and reverse). G. Pycnidium forming on OA. H. Pycnidium. I. Conidiogenous cells. J. Conidia. Scale bars: H = 100 μm. I, J = 10 μm

Etymology: The species name refers to the sort of infected tissues of where the fungus was isolated.

Description: *Hyphae* hyaline to brown, 1.5-5 μm wide, thin-walled, smooth to verrucose or tuberculate, septate, anastomosing. *Conidiomata* pycnidial, brown to dark brown, solitary, superficial or immersed, ovoid to ellipsoidal, 320-350 × 250-290 μm, hairy, ostiolate; pycnidial wall 2-4 layered, 15-25 μm thick, covered by a mass of interwoven, brown to dark brown hyphae, followed by an outer-layer of *textura angularis* of pale to dark brown flattened polygonal cells of 10-20 μm diam, incrusted of a dark brown to carbonaceous material especially around the neck; neck dark brown to carbonaceous, cylindrical to conical-truncate, 60-84 × 60-120 μm, with a few dark brown, thick-walled, sinuose, verrucose to nodulose setae of 20-35 × 3-5 μm, bearing an apical ostiole of 15-35 μm diam. *Conidiogenous cells* phialidic, hyaline, ampulliform, 7.5-11 × 2-3 μm. *Conidia* aseptate, hyaline, smooth- and moderately thick-walled, ellipsoidal to subglobose, 2.5-3.5 × 1.5-2.5 μm, guttulate.

Culture characteristics: Colonies on MEA reaching 10-11 mm diam after 7 days at 25 ± 1°C, velvety, cream (M. 4B3) to olive brown (M. 4E8); reverse maize (M. 4A6) to brownish grey (M. 4E2). Colonies on OA reaching 7 mm diam, flattened, olive grey (M. 3F2); reverse dark grey (M. 3F1). Colonies on PDA reaching 11 mm diam, velvety, white (M. 3D3) to olive grey (M. 3F2); reverse yellowish brown (M. 5F6). NaOH spot test negative. Crystals absent. Optimal, minimum and maximum temperatures of growth: 25, 15 and 35°C, respectively.

Material examined: USA, from human lung tissue, 2012, DA Sutton (holotype CBS H-24014, ex-holotype living cultures CBS 145808 = UTHSC DI16-353 = FMR 13846).

Notes: *Gloniopsis pneumoniae* is morphologically distinct from *G percutanea* by producing larger pycnidia (320-350 × 250-290 vs 100-190 × 90-250 μm) and by the shape of the conidia (ellipsoidal to globose versus bacilliform). The asexual morph recently described to *G subrugosa* is quite different from the clinical isolates, forming a thick peridial wall (thin-walled in our new species) and pigmented (greenish-brown) conidia (hyaline in *G pneumoniae* and hyaline to pale brown in *G percutanea*).[7] The most informative molecular markers for discriminating among *Goniopsis* species were ITS and *tef*1.

4 | DISCUSSION

The present study revises the taxonomy of *Hysteriaceae* the unique family associated with the order *Hysteriales*, this family was introduced by Chevallier as "*Hysterineae*" and later corrected by Corda as we currently know, being *Hysterium* its type genus.[29,30] Despite that Mycobank (2019) has associated 27 genera to this family, currently, nine genera have been accepted according to the study by Jayasiri *et al*,[7] which was based on molecular data and morphological characterisation of hysteriform *Dothideomycetes*. In addition, molecular studies on hysteriform ascomycetes are practically recent, showing that those fungi are phylogenetically polyphyletic being distributed in four different orders (*Hysteriales*, *Mytilinidiales*, *Pleosporales* and *Stigmatodiscales*), leaving the doubt about their real taxonomic placement.[7,8,11,31-33] However, the current phylogenetic markers sequenced for this group of fungi are not enough to fully understand its taxonomy as sequences are mainly available for the nrRNA genes as LSU, 18S (SSU) and less for ITS, and only a reduced number of species have sequenced structural

genes such as the translation elongation factor 1-alpha. Thus, sequences of more informative markers such as beta-tubulin gene (*tub2*) and/or RNA polymerase II subunit 2 gene (*rpb2*) are needed.[3]

The genus *Gloniopsis* is still a controversial fungus that appears with 68 epithets in Index Fungorum (2019). It was introduced by De Notaris without a designated type species. Consequently, posterior studies designated *Gloniopsis praelonga* as the type, including only *G curvata* as an additional species within the genus.[34,35] This genus is morphologically characterised by producing hyaline to yellowish asymmetric obovoid dictyospores (its sexual morph description) and produces aposphaeria-like asexual morph.[31] However, the coelomycetous fungus *Aposphaeria* is currently included in *Melanommataceae*, a different family within the order *Pleosporales*.[36] The molecular studies regarding *Gloniopsis* reveal that is a polyphyletic fungus of unclear taxonomy,[7,8,31] and our study confirms the previous state after analysing two phylogenetic markers.

In this study, we revised the clinical strain FMR 8713, that was first mentioned by Guarro *et al*[27]; it was isolated from an immunocompromised patient with a subcutaneous infection coming from Brazil and was identified as *Plectophomella* sp (currently *Plenodomus*) based on morphological characterisation, which resembled a coelomycetous fungus as the genus mentioned before, and by ITS sequence analysis, of which there were few sequences for coelomycetes available at that time. To our knowledge this was the first clinical association of clinical disease with a hysteriaceous fungus, although it was misidentified. Subsequently, in 2008 the second clinical case of a hysteriaceous fungus (strain FMR 8743) was reported in an Indian patient with chromoblastomycosis. In this report, the identification was closer to *Hysteriales* as it was identified as *Rhytidhysteron rufulum* based on sequencing the ITS and SSU, and comparisons with sequences available in GenBank. However, morphological characterisation was not possible since the isolate remained sterile, highlighting growth only at 28°C and 37°C.[12] Since then, more clinical cases mainly from India have been reported in patients with subcutaneous phaeohyphomycosis, and all of them caused by *Rhytidhysteron rufulum* identified only by ITS sequence analysis and without morphological descriptions.[13-16]

The present work provides two more isolates from clinical samples in the USA. Based on the morphological characterisation and molecular data, a new classification is given, amending the identification of Indian strains and proposing two novel species. Our phylogenetic study shows that former clinical strains identified previously as belonging to *Rhytidhysteron* corresponded to the genus *Gloniopsis*. Therefore, the new species *Gloniopsis percutanea* that have clinical isolates recovered from Brazil, India and North America is here proposed, rectifying former identifications as *Rhytidhysteron rufulum*. In addition, the American strain UTHSC DI16-353, initially identified as *Chaetophoma* sp, is here proposed as the new species *Gloniopsis pneumoniae*.

In the last few years, coelomycetous fungi have become of greater clinical importance as opportunistic pathogens and being more commonly identified in clinical laboratories.[5,37-39] Although identification by molecular assays is now easier and more available for clinical laboratories, misidentification of these fungi still occurs since they do not sporulate and there are not enough ITS sequences in the public databases for comparisons. For this reason, more taxonomic studies providing further molecular data are necessary so that the clinicians have a more accurate identification when morphological analysis is not possible, as often occurs with the genera *Phoma* and *Pyrenochaeta*, for example.[40,41] Recent studies have been conducted to determine which phylogenetic marker should be utilised for clinical identification, as ITS is not helpful by itself especially for coelomycetes, where LSU is more useful. Additional molecular markers may then be needed depending on the genera.[3,4,42]

Finally, regarding the order *Hysteriales*, its taxonomy still remains complex. The current phylogenetic analyses are not conclusive, and many species have previously been described for hysteriaceous fungi. Therefore, more molecular markers should be used for phylogenetic purposes, and new material should be collected in order to have a more stable phylogeny for this group.

ACKNOWLEDGEMENTS

Unfortunately, Dr Sutton died in the course of these investigations. This work is dedicated in memoriam. This work was supported by the Spanish Ministerio de Economía y Competitividad, grant CGL2017-88094-P.

CONFLICT OF INTEREST

No conflict of interest declared.

AUTHORS CONTRIBUTION

NW collected clinical isolates; N.V-L and J.C-L. molecular studies; AS, N.V-L and V.M-D morphological studies; and N.V-L, J.C-L, NW, AS and JG led the writing.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as the research in this article related to microorganisms.

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How to cite this article: Valenzuela-Lopez N, Magaña-Dueñas V, Cano-Lira JF, Wiederhold N, Guarro J, Stchigel AM. Two new species of *Gloniopsis* (Hysteriales, Ascomycota) from clinical specimens: Morphological and molecular characterisation. *Mycoses*. 2019;00:1–10. https://doi.org/10.1111/myc.13006

***Trichophoma* gen. et sp. nov.**

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Publicado en: Peersonia (Fungal Planet description sheets: 1042-111), 2020, 44: 444-445

DOI:10.3767/persoonia.2020.44.11

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMÍA Y FILOGENIA MOLECULAR DE HONGOS PROCEDENTES DE MATERIAL VEGETAL SUMERGIDO DE ESPAÑA
Viridiana Magaña Dueñas


Trichophoma cylindrospora

Fungal Planet 1106 – 29 June 2020

Trichophoma Magaña-Dueñas, Cano & Stchigel, *gen. nov.*

Etymology. From Greek τρίχες-, hairs, due to the abundant setae on the sporocarps.

Classification — *Sporormiaceae*, *Pleosporales*, *Dothideomycetes*.

Conidiomata pycnidial, solitary, subglobose to pyriform, ostiolate, covered by brown to dark brown, septate, nodose setae. *Conidioma wall* of *textura angularis*, composed of brown to dark brown, flattened polygonal cells. *Conidiogenous cells* phialidic, hyaline, smooth walled, ampulliform. *Conidia* 0–1-septate, hyaline, guttulate, long cylindrical, with a narrowly flattened base and rounded at the end.

Type species. Trichophoma cylindrospora Magaña-Dueñas, Cano & Stchigel.

MycoBank MB833525.

Trichophoma cylindrospora Magaña-Dueñas, Cano & Stchigel, *sp. nov.*

Etymology. From Greek κυλινδρικό-, cylindrical, due to the shape of the conidia.

Hyphae hyaline to brown, septate, branched, thin-walled, smooth to tuberculate, 1.5–2 µm wide. *Conidiomata* pycnidial, brown to blackish brown, immersed to semi-immersed, solitary, scattered, subglobose to pyriform, 300–390 × 300–410 µm, ostiolate, setose. *Conidioma wall* 4–6-layered, 15–30 µm thick, covered by a mass of interwoven, brown to dark brown hyphae, followed by an outer layer of *textura angularis*, composed of brown to dark brown, flattened polygonal cells of 5–8 µm diam, incrusted with a dark brown to carbonaceous material around the neck; neck dark brown to carbonaceous, cylindrical, 130–145 × 100–145 µm, covered by brown to dark brown, septate, erect, nodose, thick-walled setae 100–180 × 2–4.5 µm, tapering towards the apex, mainly disposed around the ostiole. *Conidiophores* absent. *Conidiogenous cells* phialidic, determinate, hyaline, smooth-walled, ampulliform, 3–5 × 8–14 µm. *Conidia* 0–1-septate, hyaline, smooth- and thin-walled, long cylindrical, 18–20 × 2–3 µm, guttulate, with a narrowly flattened base and rounded at the end.

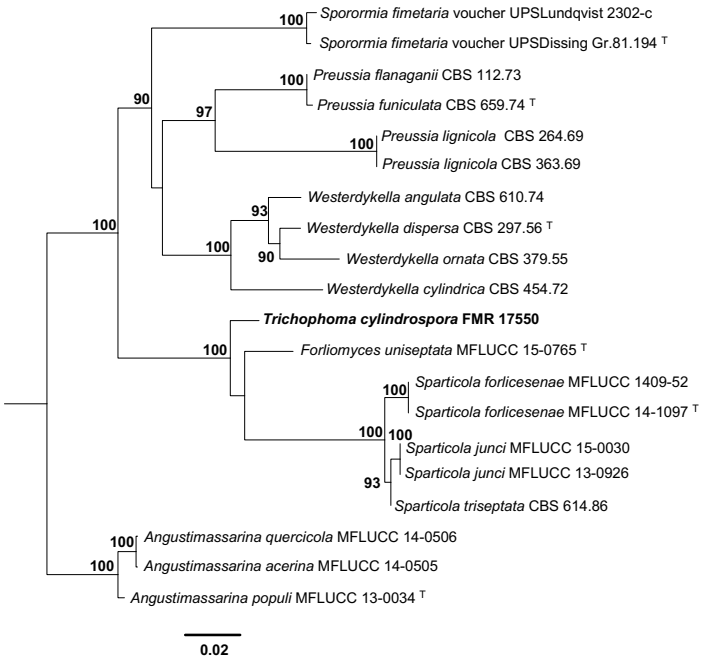
Culture characteristics — (7 d at 25 °C). Colonies on potato dextrose agar (PDA) reaching 24 mm diam, flattened, velvety, margins regular, yellowish white to reddish brown (M. 4A2 / 8D8; Kornerup & Wanscher 1978); reverse reddish brown to dark brown (M. 8E8 / 8F8), exopigment reddish brown to golden yellow (M. 8D8 / 5B7). Colonies on oatmeal agar (OA) reaching 40 mm diam, flattened, slightly floccose, margin regular, grey (M. 8F1); reverse grey (M. 8F1). Colonies on malt extract agar (MEA) 2 % reaching 25–29 mm diam, flattened, velvety to slightly floccose, margins lobate, dark brown to greyish yellow (M. 8F8 / 4C5); reverse greyish brown to greyish orange, with yellowish brown patches (M. 8F3 / 5B5 / 5F7).

Cardinal temperatures for growing — Optimum 30 °C, maximum 37 °C, minimum 15 °C.

Typus. Spain, Castilla y León community, Riaza, from plant debris, 4 May 2018, *I.A. Iturrieta-González* (holotype CBS H-24327; cultures ex-type CBS 146340 = FMR 17550; ITS, LSU and *tef-1α* sequences European Nucleotide Archive LR732023, LR732024 and LR732025, MycoBank MB833526).

Colour illustrations. Riaza, Spain. Colonies on PDA and OA after 14 d at 25 ± 1 °C; conidiomata; mycelial network on the pycnidial wall; conidiogenous cells and conidia. Scale bars = 50 µm (conidioma) and 10 µm (all others).

Notes — Based on the phylogenetic analysis of the ITS, LSU and *tef-1α* combined dataset, the closest relative of *T. cylindrospora* is *Forliomyces uniseptata*. *Forliomyces uniseptata* differs from *T. cylindrospora* in producing shorter and broader conidia (10–15 × 5–8 µm vs 18–20 × 2–3 µm), which are brown-coloured when mature (hyaline in *T. cylindrospora*) (Phukhamsakda et al. 2016). Based on a megablast search of NCBIs GenBank nucleotide database, the closest hit using the LSU sequence was *Forliomyces uniseptata* MFLUCC 15-0765 (GenBank NG_059659, Identities = 814/827 (98 %), no gaps); the closest hits using ITS was *Forliomyces uniseptata* MFLUCC 15-0765 (GenBank NR_154006, Identities = 440/458 (96 %), 3 gaps (0 %)); and the closest hits using the *tef-1α* sequence was *Forliomyces uniseptata* MFLUCC 15-0765 (GenBank KU727897, Identities = 420/438 (96 %), no gaps).



Maximum likelihood tree obtained from the combined DNA sequences dataset from three loci (ITS, LSU and *tef-1α*) of our isolate and sequences retrieved from the GenBank. Alignment and tree building were performed by MEGA v. 6.06 (Tamura et al. 2013). Type strains of the different species are indicated with T. The new taxon proposed in this study is indicated in **bold**. The RAxML bootstrap support values (> 70 %) are provided at the nodes. *Angustimassarina quercicola*, *Angustimassarina acerina* and *Angustimassarina populi* were use as outgroup.

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***Paraphoma variabilis* sp. nov.**

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Publicado en: Peersonia (Fungal Planet description sheets: 1284-1382),

2021, 47: 344-345

DOI:10.3767/persoonia.2021.47.06

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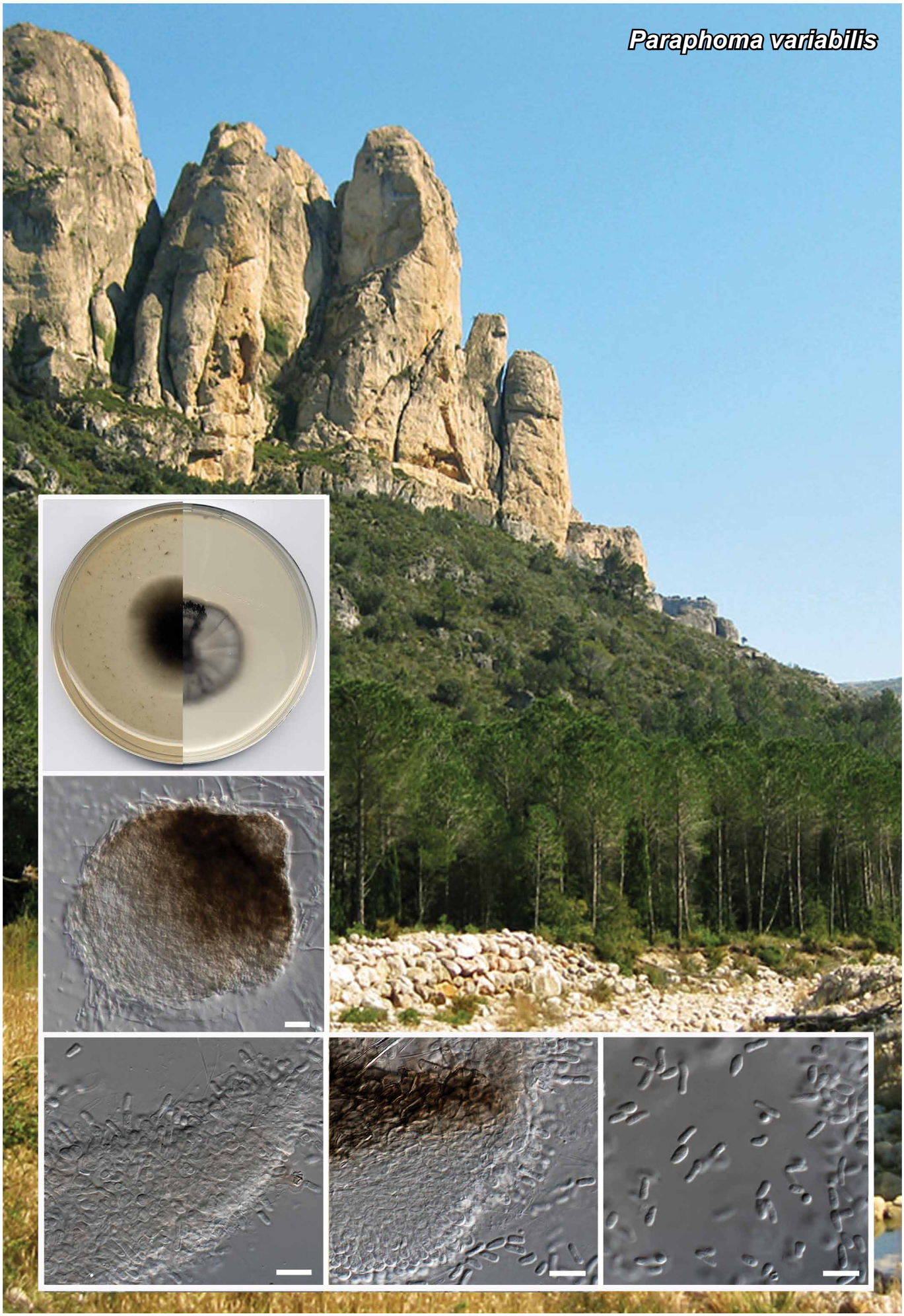
TAXONOMÍA Y FILOGENIA MOLECULAR DE HONGOS PROCEDENTES DE MATERIAL VEGETAL SUMERGIDO DE ESPAÑA
Viridiana Magaña Dueñas


Paraphoma variabilis

Fungal Planet 1370 – 24 December 2021

Paraphoma variabilis Magaña-Dueñas, Cano-Lira & Stchigel, *sp. nov.*

Etymology. From Latin *variabilis*, due to the variable shape of the conidia.

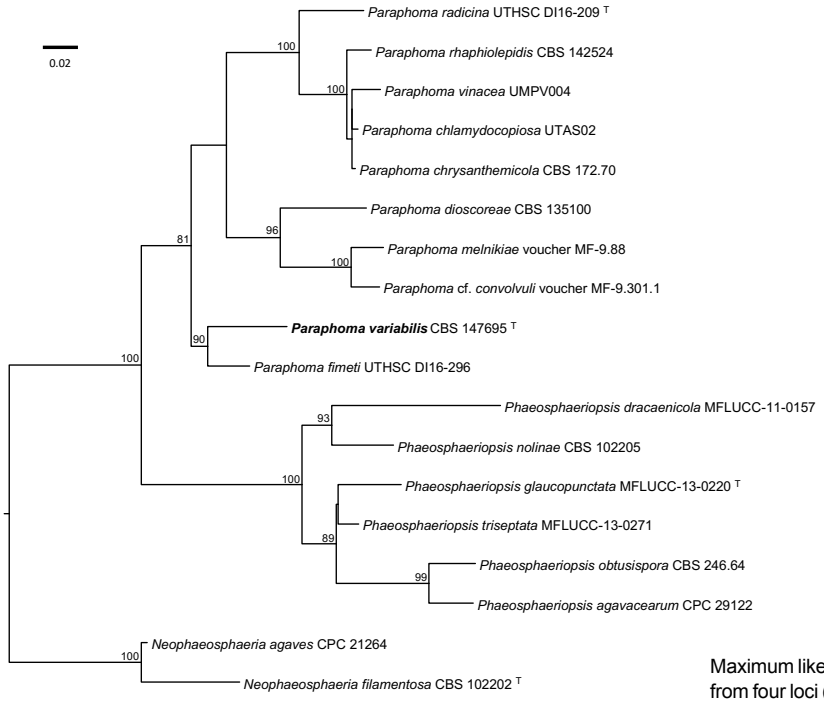
Classification — *Didymellaceae*, *Pleosporales*, *Dothideomycetes*.

Hyphae hyaline, septate, smooth- and thin-walled, 1.5–3 µm wide. *Conidiomata* pycnidial, immersed to semi-immersed, solitary, scattered, pale brown at the base, with an ostiolate neck, subglobose to pyriform, 150–180 × 175–200 µm, covered with very short setae. *Conidiomatal wall* 3–5-layered, 10–20 µm wide, translucent, of *textura angularis*, composed by an outer layer of very pale brown to dark brown, flattened polygonal cells of 4–6 µm diam; setae subhyaline, rounded at the tip, finger-like, 7–25 × 2.5–3.0 µm, ostiolar neck brown to dark brown, conical-truncate, 55–60 × 25–30 µm. *Conidiophores* absent. *Conidiogenous cells* phialidic, ampulliform to cylindrical, determinate, hyaline, smooth-walled, 5–8 × 1.5–3 µm. *Conidia* aseptate, hyaline, smooth- and thin-walled, variable in shape, cylindrical, ellipsoidal, ovate, reniform, sigmoid, 4–8 × 2–3 µm, truncate at the base.

Culture characteristics — (after 7 d at 25 °C). Colonies on PDA reaching 37–41 mm diam, radiated, velvety, margin undulate, with abundant aerial mycelium, grey to yellowish grey (29F1/29C1; Methuen Handbook of Colour); reverse bluish grey to yellowish grey (23F3/4B2), diffusible pigment not produced. Colonies on OA reaching 40 mm diam, convex, velvety, margin regular, with abundant aerial mycelium, surface and reverse grey (20A1), diffusible pigment not produced. Colonies on MEA 2 % reaching 25 mm diam, flattened, velvety, margin lobate, with abundant aerial mycelium, grey to orange grey (30E1/5B1); reverse grey to dark grey (30D1/30F1), diffusible pigment not produced. Cardinal growing temperatures: optimum 25 °C, maximum 30 °C, minimum 5 °C

Typus. Spain, Tarragona Province, Els Ports de Tortosa-Beseit, from dung, Oct. 2017, coll. *I.A. Iturrieta-González*, isol. *V. Magaña-Dueñas* (holotype CBS H-24765, cultures ex-type CBS 147695 = FMR 17160; ITS, LSU, *rpb2* and *tub2* sequences GenBank LR993310, LR993311, LR993313 and LR993314, MycoBank MB 839143).

Notes — Based on a megablast search of NCBIs GenBank nucleotide database, the closest hit using the **LSU** sequence was *Phaeosphaeriopsis nolinae* (strain CBS 102205, GenBank KY090667; Identities = 552/552 (100 %), no gaps). The closest hit using the **ITS** sequence was *Paraphoma fimeti* (strain UTHSC DI16-296, GenBank LT796872; Identities = 528/585 (90 %), eight gaps (1 %)). The closest hit using the ***rpb2*** sequence was *Paraphoma fimeti* (strain UTHSC DI16-296, GenBank LT797032; Identities = 842/905 (93 %), no gaps). The closest hit using the ***tub2*** sequence was *Paraphoma fimeti* (strain UTHSC DI16-296, GenBank LT796952; Identities = 294/320 (92 %), five gaps (1 %)). Based on the combined analysis of ITS, LSU, *rpb2* and *tub2* sequences, the closest relative of *Paraphoma variabilis* is *Paraphoma fimeti*. The latter differs from our novel species in that it produces smaller (3–5 × 2–3 µm vs 4–8 × 2–3 µm) ellipsoidal conidia (Boerema et al. 2004), which are cylindrical, ellipsoidal, ovate, reniform and sigmoid, and truncated at the base, in *P. variabilis*. Also, *P. fimeti* produce a yellowish diffusible pigment, which is absent in *P. variabilis*.



Maximum likelihood tree obtained from the combined DNA sequences dataset from four loci (ITS, LSU, *rpb2* and *tub2*) of our isolate and sequences retrieved from the GenBank database. Ex-type strains of the different species are indicated with superscript T. The new species proposed in this study is indicated in **bold**. The RAxML v. 8.2.10 (Stamatakis 2014) bootstrap support values (> 70 %) are provided at the nodes. *Neophaeosphaeria agaves* CPC 21264 and *Neophaeosphaeria filamentosa* CBS 102202 were used as outgroup.

Colour illustrations. Ports de Totosa-Beseit natural park, Tarragona, Spain. Colony on PDA and OA after 14 d at 25 + 1 °C; pyriform conidioma; conidiogenous cells; conidia. Scale bars = 25 µm (conidioma) and 10 µm (all others).

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