Universitat Autònoma de Barcelona

Estudio fenotípico y molecular de Malassezia pachydermatis y Malassezia furfur aisladas de animales

Laura Puig Carles

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Memoria presentada para optar al grado de doctor

Laura Puig Carles

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FRANCISCO JAVIER CABAÑES SAENZ, Catedrático de Universidad y GEMMA CASTELLÁ GÓMEZ y M. ROSA BRAGULAT ARARÀ, Titulares de Universidad, profesores del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona

CERTIFICAN:

que Doña **LAURA PUIG CARLES** ha realizado el presente trabajo sobre **"Estudio fenotípico y molecular de *Malassezia pachydermatis* y *Malassezia furfur* aisladas de animales"**, bajo nuestra dirección en el Departament de Sanitat i d'Anatomia Animals de la Universitat Autònoma de Barcelona.

Y para que conste, a efectos de ser presentada como Memoria de Tesis para optar al título de Doctora por la Universitat Autònoma de Barcelona, firmamos el presente certificado en Bellaterra a 22 de Junio de 2017.

F.J. Cabañes Saenz G. Castellá Gómez M.R. Bragulat Ararà

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Esta memoria de Tesis Doctoral se presenta como compendio de publicaciones. La relación de artículos es la siguiente:

- Cryptic diversity of *Malassezia pachydermatis* from healthy and diseased domestic animals. Puig L, Castellá G, Cabañes FJ. Mycopathologia 2016; 181: 681-688.

- Characterization of the species *Malassezia pachydermatis* and re-evaluation of its lipid dependence using a synthetic agar medium. Puig L, Bragulat MR, Castellá G, Cabañes FJ. 2017. Plos One 2017; 12: e0179148.

- Quantification of *Malassezia pachydermatis* by real-time PCR in swabs from the external ear canal of dogs. Puig L, Castellá G, Cabañes FJ (enviado para su publicación).

- Phenotypic and genetic diversity of *Malassezia furfur* from domestic and zoo animals. Puig L, Bragulat MR, Castellá G, Cabañes FJ (enviado para su publicación).

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ABREVIATURAS

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AFLP	*Amplified fragment length polymorphism* (polimorfismos de longitud de fragmentos amplificados)
CHS2	*Chitin sintase 2* (quitin sintasa 2)
DGGE	*Denaturing gradient gel electrophoresis* (electroforesis en gel con gradiente desnaturalizante)
DNA	*Deoxyribonucleic acid* (ácido desoxirribonucleico)
D1D2	*D1D2 domains* (dominios D1D2 de la LSU del rRNA)
ITS	*Internal transcribed spacer* (regiones espaciadoras internas transcritas del rRNA)
LNA	*Leeming and Notman agar* (agar de Leeming y Notman)
LSU	*Large subuni*t (subunidad grande del rRNA)
MAT	*Mating type* (tipo sexual)
mDA	*modified Dixon agar* (agar de Dixon modificado)
MLEE	*Multilocus enzyme electrophoresis* (electroforesis enzimática multilocus)
PFGE	*Pulsed-field gel electrophoresis* (electroforesis en gel de campo pulsado)
PCR	*Polymerase chain reaction* (reacción en cadena de la polimerasa)
qPCR	*quantitative PCR* (PCR cuantitativa)
RAPD	*Random amplification of polymorphic DNA* (polimorfismos de DNA amplificado aleatoriamente)
RNA	*Ribonucleic acid* (ácido ribonucleico)
rRNA	*Ribosomal ribonucleic acid* (RNA ribosomal)

RFLP	*Restriction fragment length polymorphism* (polimorfismos de longitud de fragmentos de restricción)
SGA	*Sabouraud glucose agar* (agar glucosado de Sabouraud)
tFLP	*Terminal fragment length polymorphism* (polimorfismos de longitud terminal)
SSCP	*Single strand conformation polymorphism* (polimorfismos de conformación de cadena simple)
YNBA	*Yeast nitrogen base agar* (agar base nitrogenado para levaduras)

1. INTRODUCCIÓN

1.1. El género *Malassezia* Baillon (1889)

El género *Malassezia* incluye en la actualidad 17 especies de levaduras lipófilas, tres de las cuales han sido propuestas recientemente [14, 50]. Estas levaduras poseen una morfología diversa, presentando formas ovales, globosas y alargadas. Frecuentemente se pueden observar las células en gemación unipolar de cuello ancho, particularidad que proporciona a estos microrganismos un aspecto característico [39].

Las levaduras del género *Malassezia* se consideran parte de la microbiota normal de varios animales, incluida la especie humana. Sin embargo, en determinadas condiciones, la población de estas levaduras puede proliferar en exceso y causar procesos patológicos[33]. En humanos, estas levaduras están implicadas en el desarrollo de enfermedades como la pitiriasis versicolor, dermatitis seborreica y dermatitis atópica, entre otras afecciones dérmicas. También se han documentado casos de fungemia por *Malassezia* spp., especialmente en pacientes inmunosuprimidos y neonatos [89]. En los animales, las especies de *Malassezia* causantes de enfermedad suelen ser distintas a las que causan procesos patológicos en humanos. Por ejemplo, en perros la especie más frecuentemente aislada es *Malassezia pachydermatis*, la cual se considera agente etiológico de otitis y dermatitis, mientras que esta especie en humanos se considera parte de la microbiota transitoria [13].

Una característica fisiológica particular de este género es la capacidad de utilizar lípidos como fuentes de carbono. Todas las especies son consideradas lipodependientes, ya que requieren suplementación con ácidos grasos de cadena larga para su cultivo. No obstante, *M. pachydermatis* es la única especie del género capaz de desarrollarse en medios generales, como el SGA [39]. Sin embargo, recientemente se ha demostrado por métodos moleculares que *M. pachydermatis* no posee el gen codificante para una enzima sintasa de ácidos grasos. Consecuentemente, se ha sugerido que esta especie también requiere el aporte de lípidos, como los ácidos grasos proporcionados por los componentes de medios complejos como el SGA [92].

La variabilidad morfológica y las dificultades de aislar y mantener estos exigentes microorganismos en condiciones de cultivo han originado controversia alrededor de la taxonomía de estas levaduras. Debido a que la clasificación fenotípica puede presentar

resultados dudosos, la introducción de métodos moleculares permitió clarificar este aspecto [39].

1.1.1. Antecendentes históricos

La primera descripción de levaduras de *Malassezia* corresponde a Eichstedt en 1846, cuando reconoció a los microorganismos en escamas dérmicas de lesiones de pitiriasis versicolor. En esta ocasión se describió la enfermedad, pero no fue hasta el 1853 que el microorganismo fue nombrado, por Robin, como *Microsporon furfur* [6]. Sin embargo, en 1889, Baillon creó el género *Malassezia*, y el binomio *Microsporon furfur* fue reemplazado por *Malassezia furfur* [52].

En 1925, Weidman aisló de la piel de un rinoceronte unas levaduras que aparentemente se desarrollaban en medios sin suplementación lipídica, a las cuales denominó *Pityrosporum pachydermatis*. En 1935, Dodge propuso que dicha levadura fuera nombrada *Malassezia pachydermatis*, aunque este cambio no fue aceptado hasta tiempo después [41]. En 1951, Gordon aisló levaduras con morfología esférica de piel sana y lesiones de pitiriasis versicolor y las denominó *Pityrosporum orbiculare*. En 1955, Gustafson aisló unas levaduras del conducto auditivo externo de perros con otitis externa, similares a *P. pachydermatis*, y las denominó *Pityrosporum canis* [38].

Durante años, se mantuvo la sinonimia entre *Pityrosporum* y *Malassezia*, hasta que en 1986 la Comisión Internacional para la Taxonomía de los Hongos aceptó *Malassezia* como único nombre válido para agrupar estas levaduras. Se aceptaron dos especies en el género, *M. furfur*, asociada a humanos, y *M. pachydermatis*, asociada a animales [52]. En 1990, Simmons y Guého describieron la especie *Malassezia sympodialis*, que se diferenciaba de *M. furfur* en aspectos morfológicos, el porcentaje de guanina-citosina y porcentaje de reasociación DNA/DNA [76]. En 1995, Guillot y Guého estudiaron la diversidad de estas levaduras por secuenciación de la región D1D2 y reconocieron ocho grupos de cepas genéticamente distintos [43]. El siguiente año, se realizó la revisión del género utilizando criterios morfológicos, fenotípicos y moleculares, y se describieron cuatro nuevas especies: *M. globosa* Midgley, Guého y Guillot, 1996, *M. obtusa* Guillot y

Guého, 1996, *M. restricta* Guého, Guillot y Midgley, 1996 y *M. slooffiae* Guillot, Midgley y Guého, 1996 [40].

Paralelamente, se desarrollaron técnicas prácticas para la identificación de las especies de *Malassezia* basadas en el estudio del fenotipo [45]. También se extendió el uso de métodos moleculares para este propósito, lo cual favoreció la descripción de nuevas especies. Así, se describieron las especies *M. dermatis* [81], *M. japonica* [80] y *M. yamatoensis* [78], aisladas de piel humana en Japón. Por otra parte, se identificaron nuevas especies relacionadas con la piel de animales, como *M. nana*, aislada de gato [49], *M. caprae*, de cabra [15], *M. equina*, de caballo [15] y *M. cuniculi*, de conejo [16]. Recientemente, se han descrito dos especies aisladas de aves de Brasil, *M. psittaci* y *M. brasiliensis* [14], y *M. arunalokei*, aislada de la piel de humanos sanos y con dermatitis seborreica en India [50].

1.1.2. Situación taxonómica

La taxonomía vigente sitúa el género *Malassezia* en el filo *Basidiomycota*, en una posición incierta dentro del subfilo *Ustilaginomycotina*, el cual incluye mayoritariamente hongos asociados a plantas, agrupados en las clases *Exobasidiomycetes* y *Ustilaginomycetes* [13]. Sin embargo, filogenéticamente, el género *Malassezia* forma un linaje monofilético diferenciado de estas clases.

Por otra parte, las levaduras de *Malassezia* poseen caracteres fenotípicos únicos y diferenciados en cuanto a la morfología, ultraestructura, fisiología y características bioquímicas respecto a los otros organismos del subfilo *Ustilaginomycotina* [91]. Paralelamente, en estudios de genómica comparativa se ha demostrado una gran diferencia a nivel enzimático entre las especies *Ustilago maydis*, el cual es un hongo patógeno de plantas, clasificado en el subfilo *Ustilaginomycotina*, y *Malassezia* [92, 93]. Por las razones mencionadas, se propuso la clase *Malasseziomycetes* para agrupar al género *Malassezia*, bajo el orden *Malasseziales*, en el subfilo *Ustilaginomycotina* [91].

1.1.3. Características microbiológicas

Las especies del género *Malassezia* presentan ciertas peculiaridades a nivel fenotípico, que hacen a estas levaduras únicas entre los hongos. Entre estas cualidades destacan la pared celular, su morfología, la dependencia de lípidos y su reproducción [39].

1.1.3.1. Ultraestructura

La pared celular de *Malassezia* es única entre los hongos. Esta tiene unos 0,12 μm de grosor y está formada por múltiples capas, que incluyen una lamela externa, una pared multicapa y una membrana plasmática con ondulaciones [39]. Estudios de microscopía electrónica han demostrado que *M. furfur* y *M. pachydermatis* tienen una pared celular gruesa, electrodensa y con múltiples capas. Esta pared típica de basidiomicetos aparece cruzada por hendiduras regulares de la membrana plasmática [27, 40, 67].

La proporción de lípidos en la membrana de estas levaduras es notablemente más alta que en otros géneros. Mientras que en *Saccharomyces* es aproximadamente del 1-2%, en *Malassezia* es aproximadamente del 15%. Este alto contenido en lípidos, así como las hendiduras de la membrana plasmática, se consideran que podrían conferir resistencia a fenómenos externos, como cambios osmóticos o fuerzas mecánicas [62, 67]. Por otra parte, los lípidos de la pared celular podrían ser un factor de virulencia, protegiendo las células de la fagocitosis y regulando la respuesta inmune del huésped [62].

Asimismo, es característico del género que, durante el proceso de gemación, la célula hija surge de la capa más interna de la pared celular, dejando una cicatriz en forma de collar en la célula madre después de su liberación. Esta cicatriz visible aumenta progresivamente de tamaño en las sucesivas gemaciones [39].

1.1.3.2. Morfología

Microscópicamente, las levaduras presentan un aspecto que puede variar de esférico, a oval o cilíndrico. La gemación monopolar les confiere un aspecto parecido a una huella de zapato [39].

Las colonias de *Malassezia* spp. en medio mDA, uno de los más utilizados para su aislamiento e identificación, son de color blanquecino a marronáceo, lisas o ligeramente rugosas, con textura cremosa o friable, brillantes o mates, planas o convexas, con márgenes lisos o lobulados [39].

1.1.3.3. Lipodependencia

Una de las características más relevantes corresponde a la dependencia de lípidos que presentan estas levaduras para su crecimiento. En cultivo, estos lípidos son proporcionados por los medios específicos como el agar Dixon o el LNA [39]. *Malassezia pachydermatis* es la única especie clásicamente considerada como no lipodependiente, ya que es capaz de crecer en medios generales como el SGA [39].

A nivel genómico, se ha corroborado la ausencia del gen codificante para una sintasa de ácidos grasos en todas las especies del género *Malassezia* [92]. Esta deficiencia enzimática es compensada por una abundancia de genes codificantes para hidrolasas, de modo que las levaduras son capaces de hidrolizar los lípidos de su entorno para obtener ácidos grasos [62]. Recientemente, también se ha demostrado la ausencia del gen codificante para la sintasa de ácidos grasos en el genoma de *M. pachydermatis* y se ha observado la ausencia de crecimiento en medios sin lípidos en dos cepas [92]. Consecuentemente, se ha postulado que *M. pachydermatis* también posee cierto grado de dependencia, y requiere los lípidos aportados por medios complejos como el SGA. Sin embargo, existen algunas cepas de *M. pachydermatis* más exigentes respecto al aporte de lípidos. Se ha documentado la presencia de cepas que crecen con dificultad en SGA [9], y también algunas cepas incapaces de crecer en este medio [18, 29, 54].

1.1.3.4. Reproducción

Las levaduras de este género poseen en general gemación monopolar, blástica y percurrente. Las células hijas emergen de la capa más interna de la pared celular, y dejan una cicatriz en forma de collar en la célula madre cuando estas se desprenden por formación de un septo con posterior fisión. Mientras en algunas especies como *M. restricta*, *M. sympodialis*, *M. nana* o *M. globosa* las células hijas emergen desde una base más estrecha, en otras, como *M. obtusa*, *M. pachydermatis*, *M. furfur* o *M. slooffiae* estas surgen de una base más amplia. *Malassezia sympodialis* también puede manifestar gemación simpodial [39].

Hasta el momento no se ha observado ciclo sexual. No obstante, la variabilidad intraespecífica detectada en algunas especies sugiere la existencia de recombinación en estas levaduras [15]. Asimismo, en estudios de secuenciación genómica, se han detectado genes MAT, relacionados con la reproducción sexual, en los genomas de algunas especies del género, que apoya la existencia de ciclo sexual en estas levaduras [25, 75, 92, 93]. La presencia de dicho ciclo podría ser un aspecto clave respecto a la virulencia de estas levaduras, ya que la variabilidad genética adquirida mediante recombinación podría facilitar la distribución de cepas patógenas [89].

1.2. Epidemiología y ecología

Las especies del género *Malassezia* se hallan frecuentemente como parte de la microbiota normal en la piel y el canal auditivo externo de animales homeotermos, incluyendo los humanos y una variedad de animales domésticos y salvajes [13]. No obstante, se ha detectado DNA con secuencias similares a *Malassezia* en hábitats marinos, sedimentos antárticos, gusanos de tierra y raíces de plantas [5, 35].

Aunque mayoritariamente las especies de *Malassezia* son microorganismos comensales de la piel de los animales que habitan, en condiciones concretas estas levaduras pueden proliferar en exceso y actuar como patógenos oportunistas, normalmente provocando afecciones dérmicas u otitis. La patogénesis de estas enfermedades se desconoce en

detalle, aunque se cree que involucra interacciones entre las levaduras, la piel y el sistema inmunitario del huésped [13].

1.2.1. *Malassezia* en humanos

La piel de los humanos está frecuentemente colonizada por especies lipodependientes de *Malassezia*. De hecho, *Malassezia* spp. forma parte de la microbiota cutánea desde el nacimiento, y su colonización se incrementa progresivamente en las primeras semanas de vida [7].

Las especies predominantes en la piel humana, tanto sana como con lesiones, son *M. globosa* y *M. restricta* [32], aunque también se han detectado otras especies, como *M. furfur*, *M. sympodialis*, *M. slooffiae*, *M. obtusa*, *M. dermatis*, *M. japonica*, *M. pachydermatis*, *M. yamatoensis* y *M. arunalokei* [32, 50]. Debido a que estas levaduras obtienen los lípidos por hidrólisis de triglicéridos de su entorno, normalmente se encuentran colonizando más intensamente áreas sebáceas de la piel, como la cara, el cuero cabelludo o la parte superior del tronco. En algunos casos, son directamente responsables de la aparición de enfermedades, como la pitiriasis versicolor y foliculitis, mientras en otras patologías, como la dermatitis seborreica y dermatitis atópica, actúan como factores agravantes de la enfermedad [47].

La fisiopatología de las enfermedades dérmicas con intervención de estas levaduras presenta aún grandes enigmas, debido a las complejas interacciones de estas levaduras con el huésped. En la piel sana, estas levaduras se nutren de sustancias de la piel sin causar enfermedad, pero cuando este proceso se altera por inmunosupresión o enfermedades concomitantes pueden adaptarse a las nuevas condiciones modificando la expresión de ciertas enzimas, como lipasas y fosfolipasas. En ese caso, pueden aparecer alteraciones leves en los melanocitos sin inflamación del tejido, como ocurre en la pitiriasis versicolor. En otros casos, como en la dermatitis seborreica y la caspa, puede aparecer inflamación de la piel sin inducción de la respuesta inmune específica o con inducción de inmunidad específica, como sucede en la dermatitis atópica. Las levaduras también pueden invadir e inflamar otros tejidos, provocando afecciones como foliculitis u onicomicosis [89].

La pitiriasis versicolor es una infección superficial de la piel normalmente más frecuente en las áreas tropicales que en climas templados. Esta enfermedad está directamente relacionada con un sobrecrecimiento de las levaduras de *Malassezia*, y los signos característicos son áreas de piel hipo e hiperpigmentadas con irritación, que suelen aparecer en el tronco, cuello y brazos [33]. En el caso de la foliculitis, las levaduras infectan los folículos pilosos, normalmente de la parte superior del tronco, donde aparecen lesiones pustulares y pruriginosas, con destrucción de folículos [30]. En otras enfermedades, como la dermatitis seborreica, *Malassezia* spp. actúa como factor agravante. Esta afección suele tener una presentación crónica y está caracterizada por la aparición de lesiones eritematosas con descamación de la piel, que suele afectar a zonas sebáceas, como la cara, el pecho y el cuero cabelludo. La dermatitis atópica es una enfermedad multifactorial, caracterizada por la aparición de eczemas, en la cual algunas especies de *Malassezia* exacerban la patología [32, 47]. Estas levaduras también pueden intervenir en la aparición de otras patologías, como psoriasis, acné, otitis y onicomicosis [30]. En las enfermedades mencionadas, las recidivas pueden ser frecuentes, ya que están estrechamente relacionadas con el estado inmunitario del huésped.

Aunque no es la afección más habitual, se han documentado fungemias iatrogénicas por levaduras de *Malassezia*, mayoritariamente causadas por *M. furfur* o *M. pachydermatis*[33]. Estas infecciones suelen ocurrir en pacientes immunodeprimidos y niños de corta edad, especialmente neonatos [23]. Estas fungemias están relacionadas con la nutrición parenteral, administrada a través de catéter, y en la que los lípidos presentes en estas fórmulas actúan como estimulante del crecimiento de las levaduras [55]. Los factores de riesgo para la aparición de esta patología incluyen estancias largas en el hospital, bajo peso al nacimiento, prematuridad, intubación endotraqueal y lesiones preexistentes en la piel de los pacientes [86, 89].

La mayor dificultad de las fungemias causadas por estas levaduras recae en su diagnóstico, ya que normalmente las muestras de sangre se procesan mediante métodos automáticos, y la detección de las especies de *Malassezia* no se suele incluir en el diagnóstico diferencial. Si no se utilizan medios con suplementación lipídica para detectarlas, estos casos pueden ser diagnosticados incorrectamente [69, 85]. Sin embargo, la mayoría de estos procesos suelen remitir con antifúngicos sistémicos y adecuadas medidas higiénicas [32].

Mientras que *M. furfur* está frecuentemente asociada a la piel de humanos, *M. pachydermatis* se considera una especie zoófila, habitualmente asociada a la piel de perros. Sin embargo, *M. pachydermatis* se considera un agente zoonótico y se podría transmitir a los pacientes a través de trabajadores sanitarios propietarios de perros domésticos [22]. Para prevenir la transmisión de estas levaduras las medidas higiénicas son un factor de gran importancia [41].

1.2.2. *Malassezia* en animales

Las levaduras de *Malassezia* son parte de la microbiota normal de la piel de muchos vertebrados homeotermos [77]. Su presencia se asocia frecuentemente con animales domésticos, aunque también se han detectado en algunos animales salvajes, como el elefante, el mono, el leopardo, el avestruz, el pelicano y los loros [13, 14]. En algunos estudios, se han aislado estas levaduras en distintos animales, como murciélagos [34] y grandes felinos en cautividad [26]. Sin embargo, la identificación en estos últimos casos no se hizo mediante la secuenciación de genes ribosomales como la región D1D2, considerado el método de elección para la identificación de estas levaduras, sino con otros métodos de PCR o técnicas fenotípicas. Como se puede observar en la **Tabla 1**, excluyendo el perro, en la mayoría de animales domésticos hay una dominancia de especies lipodependientes.

Tabla 1. Especies de *Malassezia* descritas y huéspedes habituales [13, 14, 50].

Especie	Principales huéspedes / otros*
M. pachydermatis (Weidman) Dodge 1925	Perro, gato / carnívoros, aves
M. furfur (Robin) Baillon 1889	Hombre / vaca, elefante, cerdo, mono, aves
M. sympodialis Simmons & Guého, 1990	Hombre / caballo, cerdo, oveja
M. globosa Guého et al., 1996	Hombre / leopardo, vaca
M. obtusa Guého et al., 1996	Hombre
M. restricta Guého et al., 1996	Hombre
M. slooffiae Guého et al., 1996	Hombre, cerdo / cabra, oveja
M. dermatis Sugita et al., 2002	Hombre
M. japonica Sugita et al., 2003	Hombre
M. nana Hirai et al., 2004	Gato, vaca / perro
M. yamatoensis Sugita et al., 2004	Hombre
M. caprae Cabañes et al., 2007	Cabra / caballo
M. equina Cabañes et al., 2007	Caballo / vaca
M. cuniculi Cabañes et al., 2011	Conejo
M. psittaci Cabañes et al., 2016	Loro
M. brasiliensis Cabañes et al., 2016	Loro
M. arunalokei Honnavar et al., 2016	Hombre

* Confirmados por secuenciación de rRNA.

1.2.2.1. Carnívoros domésticos

En el perro, la especie predominante es *M. pachydermatis*, aunque se han aislado con menor frecuencia algunas especies lipodependientes. En gato, por el contrario, predominan las especies lipodependientes, siendo mucho menos frecuente la presencia de *M. pachydermatis* [13, 77].

Los factores que intervienen en la proliferación de las levaduras son diversos, incluyendo la raza y características morfológicas del animal. Algunas razas caninas con

predisposición a afecciones causadas por *M. pachydermatis* son dálmata, pastor alemán, basset hound, cocker spaniel y west highland white terrier. En gatos, algunas razas como la devon rex también padecen predisposición [77]. Las alteraciones en el microambiente de la piel también juegan un papel clave en su proliferación. En este aspecto son de importancia cambios en el pH de la piel, presencia de bacterias, hongos y enfermedades coexistentes, humedad localizada, y producción de cerumen y sebo. Estos cambios pueden ser debidos a tratamientos con antibióticos o glucocorticoides, endocrinopatías, defectos en la queratinización, alergias, déficits higiénicos, infecciones bacterianas o inmunodeficiencias [77].

1.2.2.1.1. Dermatitis

Las causas primarias de dermatitis por *Malassezia* en animales domésticos pueden ser diversas, como hipersensibilidad, atopia, endocrinopatías, etc. En estas condiciones, la población de estas levaduras puede incrementarse hasta 100-10.000 veces los valores normales en la piel sana [11]. La patogenia de estas afecciones se desconoce en gran medida, aunque se relaciona con cambios en el microambiente de la piel [89]. Las lesiones en los animales afectados pueden ser generalizadas o localizadas, presentando eritema y exudados grasos. Secundariamente, puede aparecer excoriación, liquenificación y placas seborreicas. Comúnmente, aparecen en la cara, espacio interdigital, axila, ingle y periné. Los signos clínicos de esta enfermedad no son patognomónicos, de modo que se debe sospechar de *Malassezia* spp. como agente etiológico en casos de aparición de sintomatología compatible [10].

Hasta el momento, no se han establecido criterios diagnósticos estandarizados para la dermatitis por *Malassezia*. Normalmente, se diagnostica en base a los signos clínicos, el recuento de levaduras con morfología compatible en observación directa al microscopio y la respuesta al tratamiento antifúngico. El tratamiento habitual consiste en antifúngicos tópicos o sistémicos, derivados de los azoles, como ketoconazol o itraconazol. Normalmente hay buena respuesta al tratamiento que lleva a la remisión de la enfermedad[41].

1.2.2.1.2. Otitis externa

La aparición de otitis externa en perros y gatos por levaduras de *Malassezia* está directamente relacionada con alteraciones en los mecanismos físicos, químicos e inmunológicos que controlan la población microbiana del conducto auditivo [89]. Aunque la patogénesis de estas afecciones es prácticamente desconocida, parece ser que estas levaduras actúan como patógeno oportunista [70, 74].

La otitis externa por *Malassezia* puede adquirir distintas presentaciones clínicas. En primer lugar, puede presentarse eritema generalizado con presencia de cerumen en el canal auditivo externo y si la infección se cronifica, suele aparecer exudado marrón oscuro. En caso de infecciones mixtas bacterianas, puede aparecer exudado purulento amarillento con eritema y ulceración. En todas las presentaciones de otitis externa, los animales afectados presentan signos clínicos similares, que corresponden a movimientos de cabeza, rascado de las orejas, acumulación de exudado, olor desagradable en la oreja y dolor a la palpación [41].

Actualmente, el diagnóstico de las otitis se basa en la observación de los signos clínicos, examen otoscópico, estudio citológico y análisis microbiológico, conjuntamente con la respuesta al tratamiento. El tratamiento, tanto en perros como gatos, consiste en limpiezas frecuentes del canal auditivo externo, aplicación de solución iodada tópica y antifúngicos derivados de los azoles, a veces asociados a corticosteriodes [10].

1.3. Métodos de detección, aislamiento e identificación

1.3.1. Métodos tradicionales

Debido a los requerimientos nutricionales de estas especies, no se pueden aplicar las técnicas de cultivo estándar para levaduras. La mayoría de pruebas tradicionales para su identificación se basan en el crecimiento en diferentes medios de cultivo, así como pruebas bioquímicas y fisiológicas. No obstante, estos métodos requieren un tiempo considerable, a menudo los resultados no son de fácil interpretación y pueden presentar una baja reproducibilidad [39].

1.3.1.1. Medios y condiciones de cultivo

El primer cultivo de *Malassezia* se atribuye a Panja, en 1927, quien fue capaz de cultivar las levaduras en un medio con base de huevo [48]. Posteriormente, se obtuvieron cultivos añadiendo ácido oleico al SGA, aunque el crecimiento era inconsistente. Otras formulaciones de medios de cultivo incluían componentes para mejorar su crecimiento, como leche, infusiones de carne o aceite de oliva [39]. No obstante, el cultivo de levaduras de *Malassezia* experimentó una mejoría notable con el desarrollo del medio de Dixon [88], y su posterior modificación por Midgley [64]. Paralelamente, se formuló el medio de LNA[59], siendo ambos medios los más utilizados actualmente para el cultivo de *Malassezia* spp.[39].

Para detectar estas levaduras a partir de la piel o conducto auditivo externo, se suelen obtener muestras con hisopos o apósitos adhesivos. Para determinar su presencia y abundancia se suele realizar un frotis de la muestra y se observa al microscopio [39]. En paralelo, las muestras se suelen inocular en medios de cultivo específicos, como mDA o LNA, y se incuban a 32ºC, hasta dos semanas [39]. Si tiene lugar crecimiento de colonias de morfología compatible con *Malassezia*, se puede proseguir con su identificación a nivel de especie mediante distintas pruebas.

1.3.1.2. Pruebas bioquímicas y fisiológicas

La diferenciación fenotípica de las especies de *Malassezia* se basa en distintas pruebas bioquímicas y fisiológicas. Estas incluyen la actividad catalasa, crecimiento a diferentes temperaturas (32, 37 y 40ºC), actividad β-glucosidasa por hidrólisis de la esculina y asimilación de los Tweens 20, 40, 60, 80 y Cremophor EL [40, 45]. *Malassezia pachydermatis* se diferencia del resto de especies por su capacidad de crecer generalmente en medio SGA. Las características fenotípicas principales de las especies descritas de *Malassezia* se resumen en la **Tabla 2**.

1.3.1.2.1. Actividad catalasa

La actividad del enzima catalasa se determina poniendo en contacto una gota de peróxido de hidrógeno con una colonia de levadura, en un portaobjetos. La enzima descompone el peróxido de hidrógeno en agua y oxígeno, que se observa en forma de efervescencia [39].

1.3.1.2.2. Crecimiento a diferentes temperaturas

Se considera que la temperatura óptima para el crecimiento de levaduras de *Malassezia* es 32ºC. Sin embargo, *M. pachydermatis* presenta mejor crecimiento a 37ºC. Por el contrario, otras especies como *M. globosa* y *M. restricta* son menos termotolerantes. Normalmente, se valora el crecimiento a 32, 37 y 40ºC, inoculando por agotamiento las cepas de estudio en placas de medio mDA o LNA, y se incuban a la temperatura correspondiente hasta diez días [39].

1.3.1.2.3. Actividad β-glucosidasa

Algunas especies de *Malassezia* poseen el enzima β-glucosidasa, que hidroliza el enlace glucosídico de la esculina, liberando glucosa y esculetina. Para valorar la presencia de dicha enzima, se inoculan las levaduras en picadura en agar esculina en tubo, y se incuban a 32ºC hasta cinco días. La reacción positiva se observa por un ennegrecimiento del medio, debido a la reacción del grupo fenólico de la esculetina con el hierro del medio [39].

1.3.1.2.4. Test de difusión de Tweens y Cremophor EL

En esta prueba se valora la capacidad de las cepas de crecer en SGA, suplementado individualmente con diferentes esteres de polisorbitano, que corresponden a los Tweens 20, 40, 60 y 80, y Cremophor EL [39].

Para realizar este test, se realiza una siembra por inclusión de las levaduras en SGA. Cuando el medio está solidificado, se realizan cinco pocillos y cada uno se rellena con

Tween 20, 40, 60, 80 o Cremophor EL. Las placas se incuban a 32ºC y se observan los patrones de crecimiento cada 24 horas, hasta diez días de incubación. Mediante esta prueba, se pueden observar distintos patrones de asimilación de estos componentes, que pueden variar desde la presencia de crecimiento hasta su inhibición completa [39].

Tabla 2. Características fenotípicas principales de las especies de *Malassezia* [14, 16, 39, 50].

Especies	Morfología celular	Crecimiento en SGA	Crecimiento a 37ºC	Crecimiento a 40ºC	Actividad catalasa	Actividad β–glucosidasa	Test de difusión de Tweens				
							T 20	T 40	T 60	T 80	CrEL
M. caprae	Globosa, elipsoidal	-	-, (L)	-	+	+, (-)	-[2]	+[1]	+[1]	+[1], (-)	-
M. dermatis	Elipsoidal, globosa	-	+	+	+	-	+	+	+	+	L, (+)
M. equina	Elipsoidal	-	l	-	+	-	L[2]	+	+[1]	+[1]	-
M. furfur	Globosa, elipsoidal	-	+	+	+, (-)	-, (L)	+, (-)	+, (-)	+, (-)	+, (-)	+, (-)
M. globosa	Globosa	-	-, (L)	-	+	-	-	-[2]	-[2]	-	-
M. japonica	Globosa, elipsoidal	-	+	-	+	+	-	L	+	-	L
M. nana	Elipsoidal	-	+	v	+	-	v	+	+	L	-
M. obtusa	Elipsoidal, cilíndrica	-	-, (L)	-	+	+	-	-	-	-	-
M. pachydermatis	Elipsoidal	+	+	+	+, L	+, (-)	+[1]	+	+	+	+[1]
M. restricta	Globosa, elipsoidal	-	v	-	-	-	-	-[3]	-[3]	-	-
M. slooffiae	Elipsoidal, cilíndrica	-	+	+	+	-	+, L, (-)	+	+	-, (L)	-
M. sympodialis	Elipsoidal	-	+	+	+	+	-, L[2]	+	+	+	-, (L)
M. yamatoensis	Elipsoidal	-	+	-	+	-	+	+	+	+	L
M. cuniculi	Globosa	-	+	+	+	+	-	-	-	-	-
M. psittaci	Globosa, ovoidal	-	-	-	+	-	+	+	+	+	+
M. brasiliensis	Ovoide, elipsoidal	-	+	+	+	-	+	+	+	+	+
M. arunalokei	Ovoide, globosa	-	+	-	-	-	-	-	-	v	-

SGA, agar glucosado de Sabouraud. Crecimiento a 37ºC y 40ºC en agar Dixon modificado.
Test de difusión de Tweens [39, 40]; +, positivo; -, negativo; v, variable; L, leve; (), desviaciones ocasionales del patrón principal; +[1], el crecimiento puede ser inhibido cerca del punto donde se coloca el sustrato; -[2], puede aparecer crecimiento a distancia del punto con el sustrato; -[3], puede aparecer una zona opaca.

1.3.2. Métodos moleculares

La introducción de los métodos moleculares permitió la descripción de nuevas especies de *Malassezia*, estudiar su variabilidad intraespecífica a nivel genético y analizar su distribución, así como desarrollar métodos rápidos para su detección [77].

1.3.2.1 Métodos de identificación

Para identificar las levaduras a partir de cultivo se han usado distintos métodos moleculares, aunque la metodología más ampliamente usada consiste en la secuenciación de diferentes genes [77].

1.3.2.1.1. Secuenciación

Se considera que la secuenciación de la región D1D2 es la técnica de elección para la identificación de las especies [12, 40], aunque la región ITS también es de gran utilidad. Este gen tiene una longitud variable entre las especies de *Malassezia* e incluye tres regiones, la región 5.8S que es la más conservada y las regiones ITS-1 y ITS-2 que son más variables [77]. En varios estudios, se han usado estos marcadores moleculares para identificar estas levaduras, especialmente secuenciando la región ITS-1 [31, 61]. Además de los genes ribosomales, se han utilizado genes codificantes de proteínas para identificar especies de *Malassezia*, como el gen de la β-tubulina [19, 20] y de CHS2 [1, 15, 18, 56].

1.3.2.1.2. Otros métodos moleculares

Alternativamente, se han usado otras técnicas para este fin, como cariotipificación y métodos dependientes de la amplificación del DNA [77].

La técnica de PFGE se ha usado para analizar el cariotipo de distintas especies de *Malassezia* [8]. Sin embargo, esta técnica tiene una utilidad limitada, debido a la costosa instrumentación requerida, así como el tiempo y la dificultad que puede implicar la

interpretación de los resultados [77]. Otras técnicas empleadas para la identificación de especies son AFLP [15, 46, 84], RFLP [42, 53, 57, 58, 66] y RAPD [8].

Otros métodos dependientes de PCR que se han ensayado para el estudio de especies de *Malassezia* son tFLP [36] y SSCP [18], así como DGGE [84]. No obstante, estos métodos han sido menos utilizados para la identificación de estas levaduras, ya que requieren un equipamiento específico y los resultados pueden ser de difícil interpretación [77].

1.3.2.1.3. Estudio de la variabilidad intraespecífica

Algunas especies de *Malassezia* poseen una notable heterogeneidad intraespecífica, como *M. pachydermatis* y *M. furfur*. En *M. pachydermatis*, se ha demostrado mediante secuenciación de distintos marcadores moleculares [2, 17, 44, 60], y otros métodos, como RAPD [1, 2, 21, 51] y MLEE [65].

Respecto a *M. furfur*, también se ha determinado la existencia de variabilidad dentro de esta especie por diferentes métodos, como secuenciación de genes ribosomales [61], PFGE[8], AFLP [46] y RAPD [8, 84].

1.3.2.2 Métodos de detección

Las técnicas moleculares han sido de gran utilidad para detectar especies de *Malassezia* en muestras clínicas, especialmente la PCR. Varios autores, a partir de muestras de escamas de piel de humanos, han desarrollado técnicas de PCR para diferenciar especies de *Malassezia*, amplificando genes ribosomales [4, 36, 68, 82], así como PCRs múltiplex, para la detección simultánea de diversas especies [90].

Mediante qPCR, técnica que permite detectar y cuantificar el DNA amplificado, se ha podido estimar la población de especies de *Malassezia* en la piel humana [3, 71]. Este método también ha permitido demostrar el aumento de la población de estas levaduras en casos de enfermedades como dermatitis atópica [79], pitiriasis versicolor [73] y caspa [24].

Las técnicas desarrolladas de secuenciación masiva han permitido detectar estas levaduras en diferentes ambientes, así como estimar su abundancia. Usando esta metodología, se ha comprobado que *Malassezia* es de los géneros fúngicos más abundantes en la piel humana [31, 83]. En perros, también se ha comprobado mediante secuenciación masiva que *Malassezia* es de los géneros fúngicos predominantes en la piel[63]. Con estas herramientas, incluso se han podido detectar secuencias similares a las de *Malassezia* en ambientes tan distintos como corales, intestino de larvas de langosta, sedimentos antárticos, raíces de plantas, y esponjas de aguas hawaianas [5].

1.3.2.3. Estudios genómicos

Los métodos de secuenciación genómica han permitido obtener los genomas de especies de *Malassezia*, abriendo las puertas a una gran cantidad de conocimientos a nivel genético de los cuales anteriormente no se tenía información. Actualmente, se dispone del genoma de 14 de las 17 especies del género [92].

Varios estudios de secuenciación masiva han determinado que estas levaduras poseen los genomas más pequeños existentes en hongos, desde 7.2 Mbp correspondiente al genoma de *M. restricta* a 9.0 Mbp en el caso de *M. globosa* [28]. Excepcionalmente, se han evidenciado duplicaciones en el genoma de algunas cepas de *M. furfur*, con genomas de 13.4-14.8 Mbp. La existencia de estos genomas tan reducidos sugiere que contienen la información mínima para su supervivencia en nichos muy específicos [92].

Comparado con otros basidiomicetos, se ha demostrado la pérdida de más de 700 genes en *Malassezia* spp., afectando especialmente a genes relacionados con el metabolismo de carbohidratos. Por el contrario, se ha constatado la expansión de familias de genes de lipasas, fosfolipasas y proteasas [92], algunos de los cuales están altamente expresados en el cuero cabelludo humano [72]. Estos eventos evolutivos son concordantes con la adaptación de *Malassezia* a ambientes en los cuales hidroliza los lípidos de su entorno para usarlos como fuentes de carbono. Este hecho podría haberse potenciado por la transferencia horizontal de genes de origen bacteriano, que conferirían ciertas ventajas selectivas a estas levaduras [92].

Diversos estudios genómicos han demostrado la ausencia del gen de la sintasa de ácidos grasos en diferentes especies de *Malassezia* [25, 37, 72, 92] incluso en *M. pachydermatis*, la especie clásicamente considerada no lipodependiente [87, 92]. Este hecho sugiere que todas las especies requieren un mínimo de lípidos para su crecimiento, al ser incapaces de sintetizarlos.

La secuenciación de genomas de especies de *Malassezia* también ha desvelado información sobre su reproducción, ya que se ha constatado la presencia de genes involucrados en la reproducción sexual en algunas especies, como *M. globosa*, *M. restricta* y *M. sympodialis*, que podría indicar la existencia de ciclo sexual en estas levaduras [25, 37, 93].

Aunque aún permanecen muchas incógnitas alrededor de las levaduras de *Malassezia,* la secuenciación de sus genomas ha permitido entender un poco más su adaptación en los nichos que ocupan, así como clarificar su papel en el desarrollo de enfermedades. Probablemente, futuros estudios permitirán establecer los roles concretos de estos genes en la colonización y patogénesis de estas levaduras.

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2. OBJETO DEL ESTUDIO

El género *Malassezia* incluye en la actualidad 17 especies de levaduras lipófilas que presentan una morfología diversa, gemación unipolar y son consideradas parte de la microbiota normal de varios animales, incluyendo la especie humana. Sin embargo, en determinadas condiciones, la población de estas levaduras puede proliferar en exceso y causar procesos patológicos, como otitis y dermatitis. Una de las patologías más frecuentes que se diagnostican en las clínicas veterinarias son las otitis del canal auditivo externo en perro asociadas a *M. pachydermatis*. Cabe destacar que, en animales, las especies de *Malassezia* causantes de enfermedad suelen ser distintas a las que causan procesos patológicos en humanos.

Además de la morfología, la composición de la pared celular y el tipo de gemación, una característica fisiológica particular de estas levaduras es la capacidad de utilizar lípidos como fuentes de carbono. Las especies de *Malassezia* son consideradas lipodependientes y, aunque con distintos grados de dependencia, requieren de la adición de ácidos grasos de cadena larga para su cultivo. Debido a esta característica, no es posible aplicar las pruebas de identificación estándar para levaduras, motivo por el que existen pocos estudios sobre su actividad metabólica.

Si bien inicialmente los métodos fenotípicos han sido utilizados para su clasificación, debido a su lipodependencia y difícil cultivo, actualmente los métodos moleculares han permitido clarificar la taxonomía del género. Entre ellos, la metodología más utilizada se basa en la amplificación y secuenciación de marcadores moleculares como genes ribosomales y genes codificantes de distintas proteínas.

Algunas especies de *Malassezia* poseen una notable heterogeneidad intraespecífica, como *M. pachydermatis* y *M. furfur*. Esta variabilidad sugiere un fenómeno de adaptación de estas levaduras a nuevos ambientes, que podría originar la formación de nuevas especies.

Asimismo, los métodos moleculares han sido de gran utilidad para detectar especies de *Malassezia* en muestras clínicas. Entre estas técnicas podemos destacar la qPCR, que además de la detección posibilita la cuantificación del DNA amplificado. Con esta técnica algunos autores han demostrado el aumento de la población de estas levaduras en casos de dermatitis en humanos.

Por todo ello, el objeto del presente trabajo ha sido:

- Estudiar el fenotipo de la especie *M. pachydermatis* utilizando pruebas específicas para levaduras adaptadas a su lipodependencia.

- Caracterizar mediante técnicas moleculares la especie *M. pachydermatis,* a partir de aislamientos de distintas especies de animales, utilizando marcadores genéticos que permitan analizar la diversidad intraespecífica y su relación filogenética.

- Diseñar una técnica de qPCR, que permita la detección y cuantificación en el laboratorio de levaduras de *M. pachydermatis* a partir de hisopos procedentes del canal auditivo externo de perros.

- Estudiar el fenotipo de la especie *M. furfur* utilizando pruebas específicas para levaduras adaptadas a su lipodependencia.

- Caracterizar mediante técnicas moleculares la especie *M. furfur,* a partir de aislamientos de distintas especies de animales, utilizando marcadores genéticos que permitan analizar la diversidad intraespecífica y su relación filogenética.

3. RESUMEN DE LOS RESULTADOS Y DISCUSIÓN

En la presente Memoria de Tesis Doctoral se ha realizado un estudio de cepas del género *Malassezia* pertenecientes a las especies *M. pachydermatis* y *M. furfur* aisladas de distintas especies animales mediante métodos fenotípicos y moleculares.

Parte del trabajo realizado se ha centrado en el estudio de la diversidad intraespecífica en *M. pachydermatis*, tanto desde el punto de vista genético como fenotípico. Para el estudio molecular (**apartado 5.1.**), se seleccionaron 16 cepas representativas de animales domésticos en los que se puede encontrar esta levadura. Se seleccionaron diez cepas previamente aisladas de la piel y conducto auditivo de perros, tres cepas de gatos, una de caballo, una de cerdo y una de cabra. De todas ellas, se extrajo el DNA y se amplificaron y secuenciaron las regiones ITS y D1D2, juntamente con el gen de la β-tubulina y CHS2. Después de analizar las secuencias resultantes, se obtuvieron cinco genotipos de D1D2, once de ITS, nueve de CHS2 y ocho de β-tubulina. Con los cuatro genes, se realizó un estudio multilocus, y el árbol filogenético resultante reveló 15 genotipos, agrupados en dos clados principales. En el árbol filogenético se apreció una asociación de algunos genotipos con determinadas especies animales, sin embargo, no se observó ningún tipo de asociación respecto a su estado de salud. Los resultados indicaron que *M. pachydermatis* está sufriendo un proceso de adaptación, en el cual las levaduras se estarían diferenciando para vivir en distintos huéspedes animales.

Por otro lado, se estudió la diversidad intraespecífica en *M. pachydermatis* con métodos fenotípicos (**apartado 5.2.**). Esta especie es la única de su género capaz de crecer en SGA sin suplementación lipídica, por ello, clásicamente se la ha considerado una especie no lipodependiente. Se analizaron fenotípicamente las 16 cepas de *M. pachydermatis* previamente estudiadas con métodos moleculares, juntamente con tres cepas atípicamente lipodependientes. Para detectar estas tres cepas se estudiaron más de 400 cepas lipodependientes aisladas de animales. La identidad de estas tres cepas lipodependientes como *M. pachydermatis*, se confirmó mediante secuenciación de los genes ITS y D1D2, β-tubulina y CHS2. La secuenciación de estas tres cepas reveló que algunas de sus secuencias correspondían a nuevos genotipos. Así, se definieron dos nuevos genotipos de ITS y un nuevo genotipo de β-tubulina. Todas las cepas fueron analizadas con la metodología clásicamente usada para el estudio de levaduras de *Malassezia*, incluyendo las pruebas fisiológicas de crecimiento a distintas temperaturas de incubación (32, 37, 40

y 42ºC), la actividad catalasa y β-glucosidasa, y el test de difusión de Tweens y Cremophor EL en el medio de cultivo SGA. En paralelo, se modificó esta prueba sustituyendo el medio SGA por el medio YNBA. Esta modificación resultó muy útil para la diferenciación de cepas de *M. pachydermatis*, ya que facilitó la interpretación de los resultados y proporcionó una mayor reproducibilidad. Paralelamente, se confirmó la naturaleza lipodependiente de *M. pachydermatis*, con el medio sintético sin lípidos YNBA. Usando este medio, se comprobó que *M. pachydermatis* es incapaz de desarrollarse y asimilar la glucosa sin aporte de lípidos. Consecuentemente, se confirmó el requerimiento de cierto aporte de lípidos para el desarrollo de esta especie, como aquellos presentes en medios complejos como el SGA.

También se ha realizado otro estudio (**apartado 6.1.**) centrado en la detección molecular de *M. pachydermatis* en muestras procedentes del conducto auditivo externo del perro, donde esta levadura forma parte de la microbiota normal. Sin embargo, bajo condiciones concretas, la población de estas levaduras puede experimentar un sobrecrecimiento y provocar otitis externa. El diagnóstico de estas afecciones se efectúa mediante métodos no estandarizados y poco sensibles, como la observación de sintomatología compatible, la observación de levaduras con morfología típica y la respuesta al tratamiento. Para mejorar este aspecto, se diseñó y validó una técnica de qPCR para detectar y cuantificar la población de *M. pachydermatis* a partir de muestras de conducto auditivo externo de perros. Para este trabajo se recogieron muestras de 13 hisopos del conducto auditivo externo de perros con otitis externa por *M. pachydermatis* y 11 hisopos de perros sanos, con la colaboración de los responsables de la perrera de la UAB, así como del Hospital Veterinario de la UAB y varias clínicas veterinarias. Para desarrollar la técnica, se diseñaron unos cebadores específicos para amplificar el DNA de *M. pachydermatis*, y se desarrolló y optimizó una qPCR para obtener un método específico, sensible y preciso para cuantificar estas levaduras. La técnica se validó para detectar y cuantificar *M. pachydermatis* a partir de cultivo puro, así como de muestras de hisopos del canal auditivo externo de perros. Aplicando la qPCR diseñada, se observaron diferencias cuantitativas en la población de *M. pachydermatis* en perros sanos y en aquellos con otitis externa. La utilización de este método en casos de otitis externa por *M. pachydermatis* podría mejorar notablemente el diagnóstico de estas afecciones, así como ser de utilidad en la monitorización de la población de estas levaduras en perro.

Asimismo, se ha estudiado la diversidad intraespecífica en *M. furfur*, mediante métodos moleculares y fenotípicos (**apartado 6.2.**). *Malassezia furfur* es una especie aislada frecuentemente de humanos, tanto como integrante de la microbiota normal como de casos de dermatitis. Por el contrario, esta especie sólo se ha aislado de forma ocasional en algunos animales. Para llevar a cabo este estudio, se seleccionaron 21 cepas aisladas de distintas especies animales, incluyendo cepas de nuestra colección, así como cepas obtenidas de animales del zoo de Barcelona. En este estudio se incluyeron las dos cepas neotipo de *M. furfur* de origen humano, siete cepas de caballo, cuatro de aves tresquiornítidas, dos cepas de loros, una de avestruz, una de elefante, una de oveja, una de gato, una de cerdo y una de cabra. De todas ellas, se extrajo el DNA y se amplificaron y secuenciaron las regiones D1D2 y ITS, juntamente con el gen de la β-tubulina. Las secuencias obtenidas fueron analizadas y se construyeron árboles filogenéticos para cada gen. Este análisis permitió observar una importante variabilidad genética dentro de esta especie. Tras analizar las secuencias se obtuvieron cuatro genotipos de LSU, seis de ITS y ocho de β-tubulina. En los árboles filogenéticos resultantes, las secuencias de humanos y animales domésticos se mostraron diferenciadas de las secuencias de los animales del zoo. Asimismo, las secuencias de animales domésticos presentaban una mayor variabilidad genética que las de animales salvajes en cautividad. Paralelamente, estas cepas se estudiaron con métodos fenotípicos. Estos incluyeron los métodos utilizados clásicamente para estudiar especies de *Malassezia*, previamente mencionados (crecimiento a diferentes temperaturas, actividad catalasa, actividad β-glucosidasa y test de difusión de Tweens y Cremophor EL en SGA), y también se realizó el test de difusión de Tweens y Cremophor EL en medio YNBA. Mediante estas pruebas, se demostró una notable variabilidad fenotípica dentro de esta especie. Asimismo, como ocurrió con *M. pachydermatis*, se observó que el test de difusión en YNBA resultaba de gran utilidad para observar diferencias entre las cepas estudiadas. Con este medio de cultivo se pudo observar que las cepas aisladas de animales salvajes en cautividad presentaron un mismo perfil, mientras que las cepas aisladas de animales domésticos mostraron una mayor variabilidad de perfiles de asimilación de los lípidos ensayados. En este estudio se ha determinado una importante variabilidad en *M. furfur*, a la vez que se ha constatado su presencia en especies animales de las cuales se desconocía.

4. CONCLUSIONES

Como resumen de los resultados obtenidos y a modo de conclusiones podemos señalar:

1. Existe una importante variabilidad intraespecífica en cepas de *M. pachydermatis* aisladas de diferentes animales, que se ha corroborado tanto mediante el estudio del fenotipo como del genotipo.

2. Se ha demostrado la naturaleza lipodependiente de la especie *M. pachydermatis*. Por una parte, se ha confirmado que requiere el aporte de lípidos proporcionados por medios complejos, como el SGA y, por otra parte, se han aislado y caracterizado cepas de *M. pachydermatis* incapaces de crecer en este medio de cultivo.

3. La modificación realizada del test de difusión de Tweens y Cremophor EL, que consiste en la sustitución del medio complejo SGA por el medio sintético YNBA, resulta útil para el estudio de la variabilidad intraespecífica en la especie *M. pachydermatis*, ya que permite una mejor visualización de los resultados, así como una mayor reproducibilidad.

4. Mediante el estudio genético realizado en cepas de *M. pachydermatis* se han determinado cinco genotipos de D1D2, 13 de ITS, nueve de CHS2 y nueve de β-tubulina.

5. Se ha podido comprobar una asociación de ciertos genotipos de *M. pachydermatis* con determinadas especies animales, como gatos y perros, independientemente del estado de salud de los animales.

6. Se ha diseñado una qPCR con cebadores específicos para la amplificación y cuantificación de DNA de *M. pachydermatis*, tanto a partir de cultivo como de muestras de hisopos de perros.

7. La aplicación de la qPCR desarrollada en muestras de hisopos del conducto auditivo externo de perros permitió constatar diferencias cuantitativas en la población de *M. pachydermatis* en perros sanos y perros con otitis externa. Esta técnica puede ser de utilidad en el diagnóstico de otitis externas en animales domésticos.

8. Por primera vez, se ha descrito el aislamiento de *M. furfur* del canal auditivo externo de aves tresquiornítidas, concretamente en las especies ibis escarlata (*Eudocimus ruber*) y espátula rosada (*Platalea ajaja*).

9. Existe una importante variabilidad intraespecífica en cepas de *M. furfur* aisladas de diferentes animales, que se ha corroborado tanto mediante el estudio del fenotipo como del genotipo.

10. La modificación realizada del test de difusión de Tweens y Cremophor EL, que consiste en la sustitución del medio complejo SGA por el medio sintético YNBA, resulta útil para el estudio de la variabilidad intraespecífica en la especie *M. furfur*, ya que permite una mejor visualización de los resultados, así como una mayor reproducibilidad.

11. Mediante el estudio genético realizado en cepas de *M. furfur* se han determinado cuatro genotipos de D1D2, seis de ITS y ocho de β-tubulina.

12. En la especie *M. furfur* existe una mayor variabilidad genética entre las cepas de animales domésticos que entre las cepas estudiadas de fauna salvaje en cautividad.

5. ARTÍCULOS

5.1. Cryptic diversity of *Malassezia pachydermatis* from healthy and diseased domestic animals. Puig L, Castellá G, Cabañes FJ**. Mycopathologia 2016; 181: 681-688.**

Mycopathologia
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Cryptic Diversity of *Malassezia pachydermatis* from Healthy and Diseased Domestic Animals

Laura Puig · Gemma Castellá · F. Javier Cabañes

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Abstract *Malassezia pachydermatis* is part of the normal cutaneous microbiota of wild and domestic carnivores. However, under certain conditions this yeast can overproliferate and cause several diseases in its host, mainly otitis and dermatitis in dogs. The aim of this study was to conduct a molecular characterization of *M. pachydermatis* isolates from healthy and diseased domestic animals, in order to assess the molecular diversity and phylogenetic relationship within this species. The large subunit (LSU) and the internal transcribed spacer (ITS) of ribosomal RNA, chitin synthase 2 (CHS2) and β-tubulin genes from sixteen strains isolated from dogs, cats, a goat, a pig and a horse were sequenced. A different number of types of sequences were identified for each target gene, including some types described for the first time. Five sequence types were characterized for the LSU, eleven for the ITS region, nine for CHS2 and eight for β-tubulin. A multilocus analysis was performed including the four genes, and the resulting phylogenetic tree revealed fifteen genotypes. Genotypes were distributed in two well-supported clades. One clade comprised strains isolated from different domestic animals and a strongly supported cluster constituted by strains isolated from cats. The second clade included strains isolated mainly from dogs and an outlier strain isolated from a horse. No apparent association could be observed between the health status of the animal hosts and concrete strains. The multilocus phylogenetic analysis is a useful tool to assess the intraspecific variation within this species and could help understand the ecology, epidemiology and speciation process of *M. pachydermatis*.

Keywords *Malassezia pachydermatis* · Sequencing · Multilocus · β-Tubulin · Domestic animals

Introduction

Members of the genus *Malassezia* are lipophilic basidiomycetous yeasts, which are part of the normal cutaneous microbiota of humans and other warm-blooded animals. Currently, this genus consists of 14 species that have been characterized by phenotypic and molecular methods. Among them, *M. pachydermatis* is the only member that does not require lipid supplementation for growth on Sabouraud glucose agar [1]. Recently, it has been shown that the gene encoding the fatty acid synthase is missing in *M. pachydermatis* [2] and fatty acid requirement for

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L. Puig · G. Castellá (✉) · F. J. Cabañes
Veterinary Mycology Group, Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
e-mail: Gemma.Castella@uab.cat

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growth in a defined medium has been reported for this species [3].

Malassezia pachydermatis is frequently found on wild and domestic carnivores and has been considered to be zoophilic [1]. Particularly, this yeast is present on the skin and in the external ear canal of healthy dogs, whereas lipid-dependent *Malassezia* are more frequently isolated in cats, horses and domestic ruminants [1, 4]. *Malassezia pachydermatis* is usually associated with otitis externa and different kinds of dermatitis in dogs although its pathogenicity is poorly understood at the moment [4, 5]. Several typing methods have been used as epidemiological tools to understand the diversity of *M. pachydermatis*, including techniques as karyotyping [6] and random amplified polymorphic DNA [7–12]. Nowadays, sequencing has become the most widely used method for these purposes. In particular, sequencing of the large subunit (LSU), internal transcribed spacer 1 region (ITS-1), the intergenic spacer 1 (IGS-1) of ribosomal RNA and chitin synthase 2 gene (CHS2) has been performed for taxonomic and epidemiological purposes, mainly using strains isolated from dog skin [8, 9, 13–19]. All those studies revealed a considerable genetic diversity among *M. pachydermatis*, although the relationship between genotypes and pathogenicity has not yet been resolved.

The aim of the present study was to conduct a detailed molecular characterization of *M. pachydermatis* isolates. The molecular diversity and phylogenetic relationship among *M. pachydermatis* isolates from various animal species and health status were analyzed using a multilocus sequence approach based on two rRNA regions (ITS and LSU rRNA) and two protein-encoding genes (CHS2 and β-tubulin).

Materials and Methods

Strains

Fifteen *M. pachydermatis* strains and the neotype strain CBS 1879 were studied. Strains were selected from different domestic animals and were representative of the animal species from which they were recovered, the health status of those animals and their RAPD profile. These strains were selected from more than 1000 isolates obtained from 1994 to 2014 and were isolated mainly from dogs, where this species is predominantly found and from few different domestic animals (horses, goat, pigs and cats) where this species is relatively infrequently isolated.

Nine strains were recovered from eight different dogs, three of which were isolated from healthy animals while the rest were obtained from animals with otitis. These strains were representative of the four RAPD patterns described in a previous study [11]. Three strains were recovered from three different cats, one from a healthy animal, one from an animal with otitis and one from the skin of a cat with atopic dermatitis. One strain from a healthy horse was included, as well as a strain from a healthy goat and one from a healthy pig. The source and origin of the strains investigated are listed in Table 1. The strains were stored at −80 °C [20].

DNA Extraction, Gene Amplification, Sequencing and Phylogenetic Analysis

DNA was extracted and purified directly from 5 day-old cultures grown on Sabouraud glucose agar (Oxoid S.A., Madrid, Spain) according to the FastDNA Spin kit protocol with the FastPrep FP-24 instrument (MP Biomedicals, Biolink, Barcelona, Spain). The DNA was stored at −20 °C until used as a template for PCR.

The ITS gene, including the regions ITS1, 5.8S rRNA and ITS2, along with the LSU rRNA gene, CHS2 and β-tubulin genes were amplified and sequenced, using the primers and the protocols described previously [21]. The resulting sequences were aligned using Clustal X v2.0.12 [22], and regions of ambiguous alignment were removed with Gblocks [23]. For the phylogenetic analyses, sequences of representative strains from the rest of the *Malassezia* species and those determined in a previous study [21] were also included.

Maximum likelihood analysis of the β-tubulin sequences was conducted using MEGA 6 software [24]. Maximum likelihood and bayesian inference phylogenetic analyses of the combined data matrices (LSU, ITS, CHS2 and β-tubulin) were conducted. Maximum likelihood trees were inferred with the server version of RAxML-HPC2 v8 [25], as implemented on the Cipres portal, using the GTRGAMMA model. The robustness of the trees was estimated by a bootstrap analysis with 1000 replicates. Bayesian inference was conducted using MrBayes v.3.2.2 [26]. Bayesian analyses were run for 1,000,000

Table 1 Strains of *Malassezia pachydermatis* used in the study, including original animal host, pathology, RAPD profile and corresponding type of sequence for each gene sequenced

Strain	Host	Location	Pathology	Genetic type by RAPD[a]	D1D2 sequence type (GenBank accession no.)	ITS sequence type (GenBank accession no.)	CHS2 sequence type (GenBank accession no.)	β-Tubulin sequence type (GenBank Accession no.)
CBS1879	Dog-7	Ear	Otitis	I	I (AY743605)	I (AY743637)	I (EF140657)	I (KC573803)
CBS1884	Dog-8	Ear	Otitis	–[b]	I	I	I	II (KU313727)
CBS6535	Dog	Ear	Healthy	–[b]	I	I	II (KU313719)	I
MA13	Dog-1	Ear	Healthy	I	I	I	III (KU313720)	I
MA52	Dog-2	Ear	Healthy	III	I	II (KU313709)	I	II
MA56	Dog-2	Ear	Healthy	I	I	II	I	II
MA94	Horse	Skin	Healthy	I	I	III (KU313710)	I	I
MA107	Goat	Ear	Healthy	I	II (KU313705)	IV (KU313711)	IV (KU313721)	III (KU313728)
MA140	Cat-1	Ear	Healthy	I	I	V (KU313712)	V (KU313722)	IV (KU313729)
MA195	Dog-3	Ear	Otitis	I	I	VI (KU313713)	I	I
MA280	Dog-4	Ear	Otitis	IV	III (KU313706)	VII (KU313714)	IV	III
MA312	Cat-2	Ear	Otitis	I	IV (KU313707)	VIII (KU313715)	VI (KU313723)	V (KU313730)
MA356	Dog-5	Ear	Otitis	II	III	IV	VII (KU313724)	VI (KU313731)
MA475	Pig	Ear	Healthy	I	II	IX (KU313716)	VIII (KU313725)	VII (KU313732)
MA579	Cat-3	Skin	Atopic dermatitis	I	IV	X(KU313717)	V	IV
MA1382	Dog-6	Ear	Otitis	–[b]	V (KU313708)	XI (KU313718)	IX (KU313726)	VIII (KU313733)

The same accession number was given to identical type of sequences

Prefixes KU of accession numbers correspond to the sequences generated in this study

CBS Centraalbureau voor Schimmelcultures, *MA* culture collection of the Veterinary Mycology group

[a] Genetic type determined by RAPD in a previous study [11]

[b] Strain not tested by RAPD

generations in two independent analyses with a sample frequency of 1 in 1000. To obtain the consensus tree and Bayesian inference posterior probabilities, 10 % of the generations were removed to discard trees sampled before likelihood values had reached a plateau. Clades that were supported by bootstrap values (bs) of ≥70 % and posterior probability (pp) values of ≥0.95 were regarded as strongly supported.

Results

With the primers used, we were able to amplify and sequence 580, 712–737, 489 and 952 bp of the LSU, ITS, CHS2 and β-tubulin genes, respectively. Representative nucleotide sequences of the different sequence types determined in this study have been deposited at the GenBank database and are identified by the accession number listed in Table 1.

A different number of sequence types were identified for each gene. Regarding the LSU region, five types of sequences were identified (I–V). Sequence I was isolated from dogs, a horse and a cat, type II from a goat and a pig, types III and V from dogs, and type IV was obtained from a cat. The pairwise differences among these sequences were 0.3–1.0 %.

From the amplified ITS region, eleven sequence types could be described, named I to XI. Types I, II, VI, VII and XI were retrieved from isolates from dogs, types V, VIII and X from cats, sequence type III was obtained from a horse, type IV from an isolate from a goat and a dog, and type IX was obtained from a strain from a pig. The pairwise differences among these ranged from 0.1 to 6.8 %.

Nine sequence types were identified for the CHS2 region. Type I was isolated on dogs and a horse, types II, III, VII and IX were retrieved from dogs, types V and VI from cats, type IV from a goat and type VIII from a pig. The pairwise differences among them were 0.2–3.3 %.

As for the β-tubulin gene, eight sequence types were identified. Sequence type I was found in strains from dogs and a horse, type II, VI and VIII was identified from strains from dogs, types IV and V from isolates from cats, type III from a strain from a goat and a dog, and type VII from an isolate recovered from a pig. Pairwise comparisons among the sequences obtained for each strain revealed a nucleotide variation ranging from 0.3 to 3.4 % (online supplementary Table 1).

Maximum likelihood and Bayesian analyses of the β-tubulin data are shown in Fig. 1. *M. furfur* appeared as a basal lineage while *M. pachydermatis* strains grouped in a strongly supported clade (100 % bs, 1 pp). Strains isolated mainly from dogs formed a strongly supported subclade (99 % bs, 0.96 pp) and included strains with sequence types I and II. The rest of the strains defined another poorly supported subclade (71 % bs, 0.6 pp) and comprised sequence types from III to VIII, including the strains from cats, grouped in a very well-supported cluster (100 % bs, 1 pp).

When the four loci (LSU, ITS, CHS2 and β-tubulin) were combined, the data set included 2274 characters. Each gene contributed to that length as follows: LSU, 554 characters; ITS, 513 characters; CHS2, 364 characters and β-tubulin, 843 characters. Maximum likelihood and Bayesian analyses produced phylogenetic trees with the same topology (Fig. 2). In the phylogenetic analysis, *M. cuniculi* appeared as a separate lineage. The five species of the *M. sympodialis* complex (*M. nana, M. dermatis, M. sympodialis, M. equina* and *M. caprae*) formed a separate clade (100 % bs, 1 pp), as well as *M. globosa* and *M. restricta* (100 % bs, 1 pp). The two representative strains of *M. furfur* grouped with *M. japonica* and *M. obtusa* in the same clade, while *M. slooffiae* and *M. yamatoensis* formed a separated clade (100 %, 1 pp).

A well-supported node (100 % bs, 1 pp) separated *M. pachydermatis* isolates from the rest of the species. The combination of the sequences from the four studied genes revealed fifteen genotypes. A different genotype was obtained for each strain, with the exception of strains MA52 and MA56. The genotypes were distributed in two well-supported clades. The first clade (79 % bs, 0.99 pp) comprised a strongly supported cluster (97 % bs, 1 pp), constituted by three genotypes isolated from healthy and diseased cats. The rest of the genotypes of this clade included one isolate from a pig, one from a goat and three from dogs. A second well-supported clade (100 % bs, 1 pp) was defined that contained six genotypes from strains from dogs and one from a horse.

Discussion

Malassezia pachydermatis is a known skin opportunistic pathogen, whose intraspecific variability has

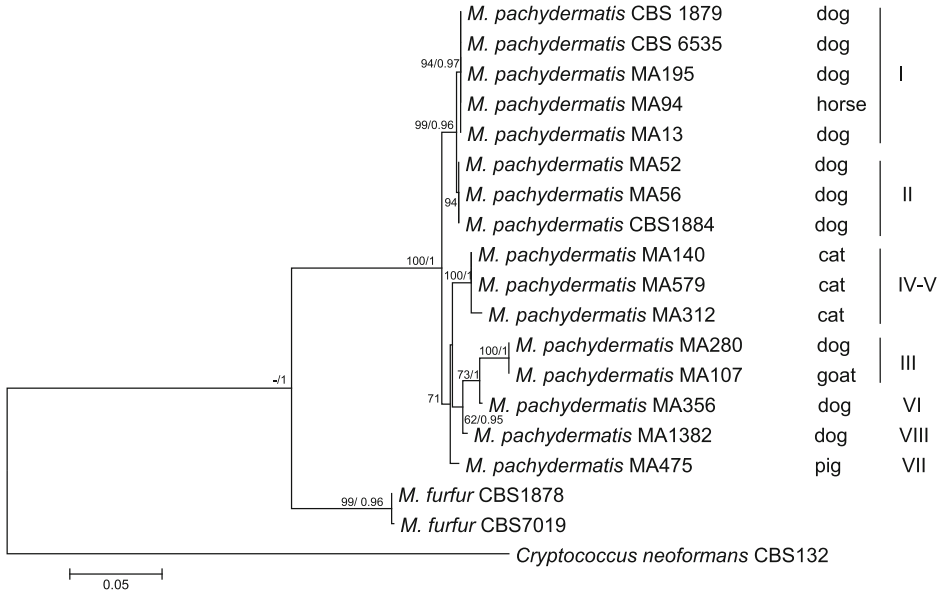

Fig. 1 Molecular phylogenetic tree inferred from maximum likelihood and Bayesian inference analyses of β-tubulin sequences of *Malassezia pachydermatis*. Bootstrap values > 50 % in 1000 replications and Bayesian posterior probability > 0.95 are shown at the nodes. The tree is rooted with *Cryptococcus neoformans*

been studied by sequencing different genes, mainly from strains isolated from dogs [9, 17–19, 27]. We characterized five LSU sequence types. Previously, seven different LSU sequence types were defined, named Ia–Ig, from strains from distinct animals [13]. Five of the sequences described by Guillot et al. [13] were also identified in our study. However, the host from which they were recovered differed in some occasions. For example, type Ie (V in our study) was isolated from dogs, cats, a horse, a rabbit and a macaque by Guillot et al. [13], while we retrieved it only from a dog. Type If (II in our study) recovered from a pig, we also recovered it from a goat. Type Ia, found on dogs, cats and wild animals, corresponded to our sequence type I, and we also found it in strains from dogs, a cat and a horse. Type Ig (our genotype IV) was identified in isolates from ferrets, while in our study it was found in strains from cats. The two genotypes identified by Guillot et al. [13] that did not correspond to any of ours were both identified in isolates from wild animals: Ib, being recovered from strains from bears, fennecs, a seal and a wallaby, and Ic from isolates from rhinoceros. In a study conducted only with strains from dogs, three sequences types, named A to C, were identified by Cafarchia et al. [27]. These were also found in our study, most of them isolated from dogs although we also detected sequence type A in a cat and a horse.

Sequencing the ITS region, eleven types of sequences were differentiated. Cafarchia et al. [16] identified eight sequence types, named A1 to A4, B and C1 to C3, sequencing the ITS-1 gene of strains from dogs. The discrepancy with our results could be explained by the fact that we also included the 5.8S and ITS-2 fragments and we analyzed strains from more animal species. Nevertheless, considering only the ITS-1 fragments of our sequences, nine different types of sequences could be recognized, and four of them were in agreement with the ones described by Cafarchia et al. [16]. We detected sequence type A1, B and C2 in strains from dogs, while type A2 was isolated from a horse. We identified five new ITS-1 sequences types, two from dogs (corresponding to ITS sequence types IV, VI), two from cats (corresponding to ITS sequence types V, VIII and X), and one recovered from a pig (corresponding to ITS sequence type IX).

Comparison of the CHS2 sequences revealed nine sequence types. In previous studies, three sequence types were found in strains isolated from dogs [16, 27]. These sequence types defined previously were also identified in our study, although we could detect these sequence types not only in isolates from dogs and cats, but also in an isolate from a horse or a goat. We identified three new CHS2 sequence types from strains from dogs (II, III and VII), two from isolates from cats (V, VI), and one from a strain from a pig (VIII).

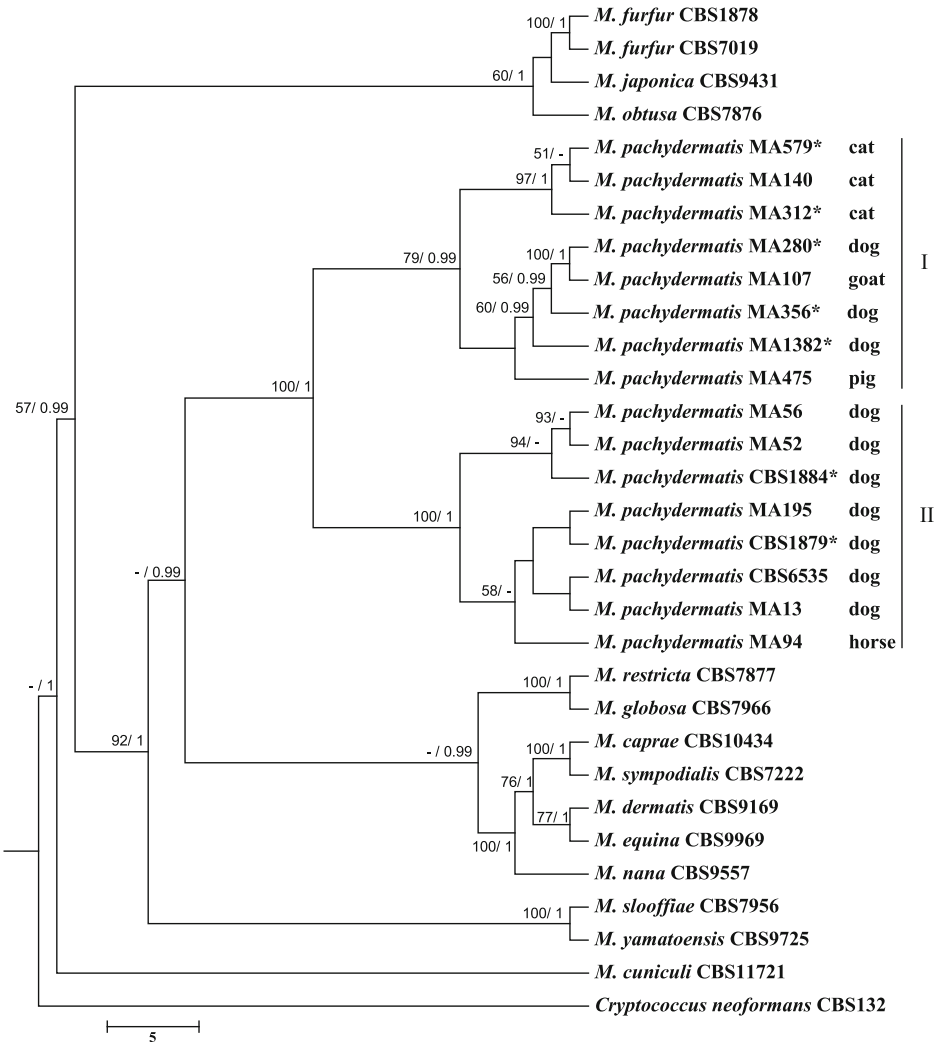

Fig. 2 Molecular phylogenetic tree inferred from maximum likelihood and Bayesian inference analyses of LSU, ITS, CHS2 and β-tubulin sequences of members of the genus *Malassezia*. Bootstrap values > 50 % in 1000 replications and Bayesian posterior probability > 0.95 are shown at the nodes. The tree is rooted with *Cryptococcus neoformans*. * Strains from animals with otitis or dermatitis

In the present study, we have characterized the β-tubulin gene in *M. pachydermatis* isolates for the first time. Sequence differences in the β-tubulin gene among strains ranged from 0.3 to 3.4 %, which is similar to those described with CHS2, ranging from 1.9 to 3.4 % [27]. With this gene, we observed eight different sequence types and most of them were host-specific. Three sequences types were found on strains recovered from only dogs, two from strains only from cats, and one from a strain from a pig. Two sequences types were recovered from strains isolated mainly from dogs but also included a strain from a horse and from a goat, confirming the usefulness of the β-tubulin gene as a genetic marker to assess diversity within this species.

In previous studies, three main genotypes and eight subgenotypes were described in *M. pachydermatis* isolates from dogs when combining sequences of LSU, CHS2 and ITS-1 genes [16]. In our study, when the sequences from the four loci were combined, fifteen genotypes were differentiated within *M. pachydermatis*. A different genotype was obtained for each strain, with the exception of those strains isolated from the same dog but showed a different RAPD pattern (MA52 and MA56). Of these genotypes, nine were recovered from strains from dogs, showing a greater

variability than in previous studies. This greater variability could be explained by the description of new sequence types in ITS-1 and CHS2, the inclusion of the ITS-2 region and the use of β-tubulin as a new genetic marker. The rest of the genotypes were recovered from strains from animal species that had not been investigated before (cats, a goat, a pig and a horse).

The combined analysis of the four genes showed that *Malassezia* species formed a group with *M. cuniculi* as a basal lineage. The tree topology obtained was slightly different and with lower support than a previously reported tree [21], although the clustering of some species was the same. *M. pachydermatis* appeared clearly differentiated from the rest of the species by a strongly supported node. Fifteen genotypes were identified, grouped in two well-supported clades. One clade included strains isolated from different domestic animals, and a strongly supported cluster contained the three genotypes retrieved from cats. The second clade included strains isolated mainly from dogs and an outlier strain isolated from a horse. These findings could represent the adaptation of different *M. pachydermatis* genotypes to distinct conditions. As a matter of fact, it has been noted that some *Malassezia* genotypes could have a distinct affinity for certain biochemical compositions, due to diverse specific requirements, linked to the existent microbiota of the skin, pH, salts and its concentrations, etc. [5]. It seems to be a reality hence that *M. pachydermatis* is undergoing a process of host adaptation, although its knowledge is nowadays a controversial matter that requires further investigation.

In the present study, it has become clear that some isolates appear to be more strongly associated to some animal species than others, in most cases regardless of the health status of its host, since there is no apparent link between phylogenetic association and disease, confirming the opportunistic nature of these yeasts. In the multilocus tree, no apparent association could be observed between the health status of the animal hosts and concrete strains, since they seem to be clustered more accordingly to the animal species from which they were recovered. In previous studies, a relationship between particular *M. pachydermatis* genotypes and the health status of the host was intended to be established, by sequencing the LSU, ITS-1 and CHS2 genes, although no specific genotypes could be associated to a particular condition of the animals [16, 17, 19, 27].

In conclusion, this study demonstrates that *M. pachydermatis* species shows a high genetic variability. The multilocus sequence analysis showed two phylogenetically delineated clades, which would reflect intraspecific populations or potentially cryptic species. Nonetheless, what appears to be a reality is the adaptation process that *M. pachydermatis* is undergoing, in which different genotypes would be differentiating to thrive in a variety of distinct hosts. Further studies are required to investigate whether the divergence among *M. pachydermatis* is sufficient to resolve them as individual species or whether it only indicates that this species is in the course of differentiation and adaptation to specific animal hosts.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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5.2. Characterization of the species *Malassezia pachydermatis* and re-evaluation of its lipid dependence using a synthetic agar medium. Puig L, Bragulat MR, Castellá G, Cabañes FJ. **PLOS One 2017; 12: e0179148.**

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RESEARCH ARTICLE

Characterization of the species *Malassezia pachydermatis* and re-evaluation of its lipid dependence using a synthetic agar medium

Laura Puig, M. Rosa Bragulat*, Gemma Castellá, F. Javier Cabañes

Veterinary Mycology Group, Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Bellaterra, Catalonia, Spain

* rosa.bragulat@uab.cat

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Abstract

The genus *Malassezia* includes lipophilic yeasts, which are part of the skin microbiota of various mammals and birds. Unlike the rest of *Malassezia* species, *M. pachydermatis* is described as non-lipid-dependent, as it is able to grow on Sabouraud glucose agar (SGA) without lipid supplementation. In this study we have examined the phenotypic variability within *M. pachydermatis* and confirmed its lipid-dependent nature using a synthetic agar medium. We used a selection of representative non-lipid-dependent strains from different animal species and three atypical lipid-dependent strains of this species, which were not able to grow after multiple passages on SGA. More than 400 lipid-dependent *Malassezia* isolates from animals were studied in order to detect the three lipid-dependent strains of *M. pachydermatis*. The identity of the atypical strains was confirmed by DNA sequencing. On the other hand, we have modified the Tween diffusion test, which is widely used in the characterization of these yeasts, by using a synthetic agar-based medium instead of SGA. This modification has proved to be useful for differentiation of *M. pachydermatis* strains, providing reproducible results and a straightforward interpretation. The finding of these peculiar lipid-dependent strains exemplifies the large variability within the species *M. pachydermatis*, which involves rare atypical strains with particular growth requirements.

Introduction

The genus *Malassezia* includes lipophilic yeasts, which are part of the skin microbiota of various mammals and birds. Currently, the genus includes 17 species [1, 2], three of which have been recently proposed [3, 4]. Of all these species, *M. pachydermatis* is a zoophilic yeast frequently isolated from the skin of wild and domestic carnivores. Although *M. pachydermatis* is part of the normal microbiota of the skin and ear canal of these animals, under some predisposing factors it can overgrow and lead to the development of dermatitis and otitis. These diseases are common in dogs, and occur less frequently in other animals [5].

Unlike the rest of *Malassezia* species, *M. pachydermatis* is described as non-lipid dependent, as it is able to grow on Sabouraud glucose agar (SGA) without lipid supplementation. The

remaining species of the genus (e.g. *Malassezia furfur*), require fatty acid supplementation for growth in culture, and consequently they are named lipid-dependent species. Complex culture media, such as modified Dixon agar (mDA) and Leeming and Notman agar provide a variety of fatty acids, required by these fastidious lipid-dependent species [2]. Although *M. pachydermatis* is the less lipid-demanding species of the genus, it has been shown that it requires the peptone components of SGA, which are highly complex and undefined, but provide fatty acids essential for this species [1].

However, some *M. pachydermatis* isolates from dogs have shown some inconsistent lipid dependence [6]. They were reported as markedly lipid-dependent isolates. Some of these isolates grew poorly when sub-cultured onto SGA. Nevertheless, most of them were able to form colonies typical of this species on SGA after some subsequent transfers on this medium. On the other hand, the isolation of *M. pachydermatis* strains unable to grow on SGA has been rarely reported [7–9].

More recently, the use of massive sequencing methods has allowed a deeper understanding of the genome of these yeasts. For instance, a typical fungal fatty acid synthase was not detected in the genome of the neotype strain of *M. pachydermatis* [10]. More interestingly, it has been also proved that the gene encoding for the fatty acid synthase is missing in the genomes of all *Malassezia* species [11]. Furthermore, these authors also mentioned that two *M. pachydermatis* strains were only able to grow with lipid supplementation in the synthetic yeast nitrogen base broth, confirming the unique lipid-dependent nature of all *Malassezia* species.

Different standard physiological tests used in the identification of yeasts have been proposed in order to characterize phenotypically *M. pachydermatis* (e.g. assimilation of carbon compounds, fermentation of carbohydrates) [12, 13]. However, due to their essential requirements for lipids standard assimilation tests are not applicable to these yeasts [14]. Nowadays, the physiological characterization of *M. pachydermatis* is based mainly on the evaluation of its ability to grow on SGA and on its ability to use certain polyoxyethylene sorbitan esters (Tweens 20, 40, 60 and 80) and Cremophor EL using a glucose/peptone agar-based medium (SGA) [1, 15].

The aim of the present study was to examine the phenotypic variability within the species *M. pachydermatis* and to confirm its lipid-dependent nature using a synthetic agar medium. To do this, SGA medium used in the Tween diffusion technique [1, 15] was replaced by a synthetic agar-based medium. Moreover, in this study, we have included three atypical lipid-dependent *M. pachydermatis* strains and confirmed their identity by DNA sequencing.

Materials and methods

Strains

A total of 19 strains of *M. pachydermatis* were studied. These were selected from our collection in order to obtain representative strains from different animal species with different health status and genetic types (**Table 1**). Swabs from the skin and the external ear canals of various animals were obtained for microbiologic examination. All samples were inoculated onto SGA and mDA with 0.05% of chloramphenicol and 0.05% of cycloheximide. These strains were obtained during routine veterinary procedures and with the verbal owner consent. Most of the strains selected for this study had been recovered from dogs, where *M. pachydermatis* is most frequently isolated, but we also included strains from animals where this species is more rarely isolated. Three atypical lipid-dependent strains of this species (MA-366, MA-374 and MA-380) were also included. More than 400 lipid-dependent *Malassezia* isolates from animals were studied in order to detect the three lipid-dependent strains of *M. pachydermatis*. The

Table 1. *Malassezia pachydermatis* studied, including original animal host, pathology, and LSU rRNA, ITS rRNA, CHS2 and beta-tubulin genotypes.

Strain	Host	Location	Pathology	LSU /ITS/CHS2/beta-tubulin genotypes [a]
CBS 1879	Dog-9	Ear	Otitis	I/I/I/I
CBS 1884	Dog-10	Ear	Otitis	I/I/I/II
CBS 6535	Dog	Ear	Healthy	I/I/II/I
MA-13	Dog-1	Ear	Healthy	I/I/III/I
MA-52	Dog-2	Ear	Healthy	I/II/I/II
MA-56	Dog-2	Ear	Healthy	I/II/I/II
MA-94	Horse	Skin	Healthy	I/III/I/I
MA-107	Goat	Ear	Healthy	II/IV/IV/III
MA-140	Cat-1	Ear	Healthy	I/V/V/IV
MA-195	Dog-3	Ear	Otitis	I/VI/I/I
MA-280	Dog-4	Ear	Otitis	III/VII/IV/III
MA-312	Cat-2	Ear	Otitis	IV/VIII/VI/V
MA-356	Dog-5	Ear	Otitis	III/IV/VII/VI
MA-366 [c]	Dog-6	Ear	Healthy	V/XII/IX/VIII [b]
MA-374 [c]	Cow	Ear	Healthy	V/XII/IX/VIII [b]
MA-380 [c]	Dog-7	Ear	Healthy	III/XIII/IV/IX [b]
MA-475	Pig	Ear	Healthy	II/IX/VIII/VII
MA-579	Cat-3	Skin	Dermatitis	IV/X/V/IV
MA-1382	Dog-8	Ear	Otitis	V/XI/IX/VIII

[a] Genotypes determined in a previous sequencing study [18].

[b] Genotypes determined in the present study.

[c] Lipid-dependent strains.

Naming source: CBS, Centraalbureau voor Schimmelcultures; MA, culture collection of the Veterinary Mycology group.

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identity of all strains was confirmed by DNA sequencing. The neotype strain of *M. pachydermatis* CBS 1879 was also included. Strains were stored at -80˚C [16].

Morphological and physiological characterization

M. pachydermatis strains were streaked on mDA (36 g of malt extract (Oxoid S.A., Madrid, Spain), 10 g of bacteriological peptone (Oxoid S.A., Madrid, Spain), 20 g of desiccated ox bile (Sigma-Aldrich S.L., Madrid, Spain), 15 g of agar bacteriological (Oxoid S.A., Madrid, Spain), 10 ml of Tween 40 (Sigma-Aldrich S.L., Madrid, Spain), 2 ml of glycerol (Sigma-Aldrich S.L., Madrid, Spain) and 2 g of oleic acid (MP Biomedicals LLC., Illkrich, France) per liter; pH 6.0) [1] and incubated at 32˚C. When fully developed colonies were observed (after 3–4 days of incubation), these were streaked on SGA (Oxoid S.A., Madrid, Spain) and incubated at 32˚C. Strains that did not grow on SGA after 4 days were repeatedly inoculated, up to five times, to confirm their lipid dependence [6]. Morphological characteristics were observed after 7 days of incubation at 32˚C on mDA. Physiological characterization was based on the splitting of esculin due to beta-glucosidase activity, catalase reaction and growth at 37˚C, 40˚C, 42˚C and 45˚C on mDA [1].

The ability to assimilate Tween 20 (MP Biomedicals LLC., Illkrich, France), Tween 40, Tween 60 (Merk KGaA, Madrid, Spain), Tween 80 (MP Biomedicals LLC., Illkrich, France) and Cremophor EL (Sigma-Aldrich S.L., Madrid, Spain) was tested with the Tween diffusion test on SGA [1]. On the other hand, the Tween diffusion test was also performed on yeast nitrogen base agar (YNBA). The composition of the medium was 6.7 g yeast nitrogen base

(BD Difco S.A., Madrid, Spain), 20g agar bacteriological per liter (pH 5.4). For each strain, 18 ml of YNBA were melted and allowed to cool to about 50˚C. Three ml of a yeast suspension were added to the medium. The suspension was obtained by inoculating two loopfuls of growing yeast in 3 ml of sterile distilled water. The agar mixture was poured onto a petri dish and when the medium was solidified, five wells of 2 mm in diameter were punched on the surface and filled with 15 microlitres of Tween 20, 40, 60, 80 and Cremophor EL, respectively. Plates were incubated for 10 days at 32˚C, and growth was checked every 24 hours. Glucose assimilation as a unique carbon source was tested using also YNBA, following the same technique. When the medium was solidified, three equidistant wells of 2 mm in diameter were punched on the agar. Afterwards, each well was filled with 15 microlitres of a glucose (VWR International Eurolab S.L., Barcelona, Spain) dilution in sterile distilled water at different concentrations (1%, 2% and 4%). Plates were incubated at 32˚C for 10 days and growth was checked every 24 hours. A *Rhodotorula glutinis* strain (RH-2) from our collection was used as control. All tests were performed by duplicate.

All strains were also streaked on YNBA supplemented with 10 g/1000 ml peptone and 40 g/1000 ml glucose, on YNBA with 10 g/1000 ml peptone, on YNBA with 40 g/1000 ml glucose and on YNBA with palmitic acid (MP Biomedicals LLC., Illkrich, France) at different concentrations (12 g/1000 ml, 6 g/1000 ml, 0.6 g/1000 ml and 0.06 g/1000 ml).

DNA extraction, amplification, sequencing and phylogenetic analyses

DNA was extracted from 4-day old cultures on mDA of strains MA-366, MA-374 and MA-380, according to the FastDNA Spin kit protocol with the FastPrep FP-24 instrument (MP Biomedicals, Biolink, Barcelona, Spain). DNA was stored at -20˚C until used as a template for PCR. Internal transcribed spacer (ITS) region (including the genes ITS1, 5.8S rRNA and ITS2), large subunit of the ribosomal RNA (LSU rRNA) region, chitin synthase 2 (CHS2) and beta-tubulin genes were amplified and sequenced, using the primers and the protocols described previously [17]. Sequences of the four genes of the remaining strains had been characterized previously [18].

For the phylogenetic analyses, LSU rRNA sequences of *M. pachydermatis* strains studied were aligned using Clustal X v2.0.12 [19], and regions of ambiguous alignment were removed with Gblocks [20]. A maximum likelihood analysis was conducted using MEGA 6 software [21] with 1,000 bootstrap replicates. A phylogenetic tree was constructed using the maximum likelihood method based on the Kimura 2-parameter model. The initial tree for heuristic search was obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The rate variation model allowed for some sites to be evolutionarily invariable. Clades that were supported by bootstrap values (bs) of $\geq$70% were regarded as strongly supported. Sequences of *M. furfur* CBS 1878 and CBS 7019, *Ustilago maydis* ATCC MYA-4924 and *Cryptococcus neoformans* CBS 132 were selected as outgroup for the tree construction.

Results

Morphology and physiology

Differential phenotypic characteristics of the studied strains are summarized in **Table 2**. All strains were able to grow on mDA and on SGA at 32˚C, except for the strains MA-366, MA-374 and MA-380, which were unable to grow on SGA, confirming their lipid dependence. The microscopic examination of the lipid-dependent strains showed ellipsoidal yeast cells with buds formed on a broad base, characteristic of *M. pachydermatis*.

Table 2. Main differential phenotypic characteristics of the studied *M. pachydermatis* strains.

Strain	Growth on SGA	Growth on mDA	Growth at 42˚C	beta–glucosidase activity	Tween diffusion test									
					with SGA					with YNBA				
					T 20	T 40	T 60	T 80	CrEL	T 20	T 40	T 60	T 80	CrEL
CBS 1879	+	+[1]	w	-	+	+	+	+	+	+[a]	+	+	+[a]	-
CBS 1884	+	+[1]	w	-	+[b]	+	+	+	+	+[a]	+	+	-	-
CBS 6535	+	+[1]	w	-	+	+	+	+	+	+[b]	+	+	+[a]	-
MA-13	+	+[1]	w	-	v	+[b]	+	+	+[b]	+[b]	+	+	w	w
MA-52	+	+[1]	w	-	-	+	+	+	+[b]	+[a]	+	+	w	w
MA-56	+	+[1]	+	-	-	+	+	+	+[b]	w	+	+	+[a]	+[a]
MA-94	+	+[1]	+	-	-	+	+	+	+[b]	w	+	+	+[a]	-
MA-107	+	+[2]	-	-	+	+	+	+	+[b]	w	+	+	+[a]	w
MA-140	+	+[1]	+	-	-	+[b]	+	+	+[b]	+[b]	+	+	+[a]	+[a]
MA-195	+	+[1]	+	-	v	+[b]	+	+	+[b]	+[a]	+	+	w	w
MA-280	+	+[2]	w	-	+	+	+	+	+[b]	+[b]	+	+	w	-
MA-312	+	+[1]	w	-	+[b]	+	+	+	+	+[b]	+	+	+	-
MA-356	+	+[1]	w	-	v	+[b]	+	+	+[b]	+	+	+	+[a]	-
MA-366	-	+[1]	-	+	+[a]	+	+	+	w	+	+	+	+	w
MA-374	-	+[1]	-	+	+[a]	+	+	+	w	+	+	+	+	w
MA-380	-	+[1]	-	+	+[a]	+	+	+	w	+	+	+	+	w
MA-475	+	+[1]	+	-	-	+[b]	+	+	+[b]	+[b]	+[b]	+	+[a]	-
MA-579	+	+[2]	-	-	w	+	+	+	+	+[a]	+	+	w	+[a]
MA-1382	+	+[1]	w	-	w	+[b]	+	+	+[b]	+	+	+	-	-

Growth on SGA (Sabouraud Glucose Agar) at 32˚C; Growth on mDA (modified Dixon Agar) after 7 days of incubation at 32˚C: +[1] colony diameter of 2–5 mm

+[2] colony diameter of <1 mm; Growth at 42˚C on mDA after 7 days of incubation.

Tween diffusion test [1, 15] with SGA and YNBA (Yeast nitrogen base agar): +, good growth; w, weak growth; +[a], growth at a distance of the well where the substrate was placed; +[b], ring of growth inhibition at a distance of the well; -, growth inhibition; v, variable results between replicates.

Naming source: CBS, Centraalbureau voor Schimmelcultures; MA, culture collection of the Veterinary Mycology group.

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After 7 days of incubation on mDA at 32˚C two types of *M. pachydermatis* colonies were observed. Most of the strains formed colonies that were 2–5 mm in diameter, while strains MA-107, MA-280 and MA-579 formed colonies <1 mm in diameter. All strains were also able to grow on mDA at 37˚C and 40˚C, while at 42˚C growth of strains MA-107, MA-366, MA-374, MA-380 and MA-579 was inhibited. All strains failed to grow at 45˚C. None of the strains showed beta-glucosidase activity, except for the three lipid-dependent strains. The catalase reaction was positive for all strains.

The Tween diffusion test was performed on SGA and on the synthetic medium without lipids YNBA. Using SGA, all strains except for MA-366, MA-374 and MA-380 grew on the entire surface of the agar, while on YNBA growth was only observed around the lipid supplements. On both media, although some intermediate growth patterns were found, five main assimilation patterns were observed around the lipid supplements (Fig 1). These patterns were defined as good growth (+), weak growth (w), growth at a distance of the well where the substrate was placed (+[a]), ring of growth inhibition at a distance of the well (+[b]), and growth inhibition (-).

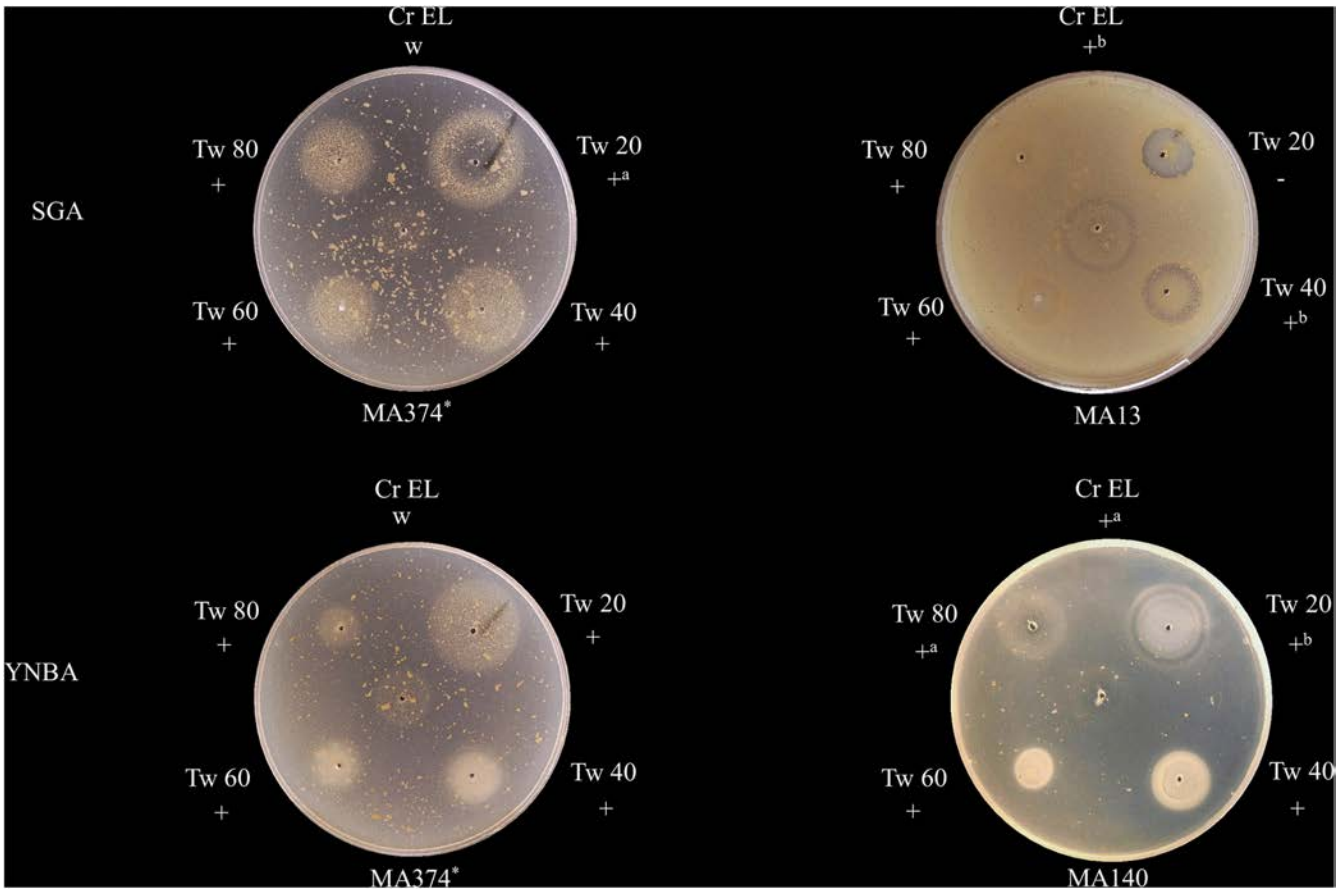

Fig 1. Growth patterns of *M. pachydermatis* strains in the Tween diffusion test with SGA and YNBA, after 7 days of incubation at 32˚C. The growth patterns were defined as good growth (+); weak growth (w); growth at a distance of the well where the substrate was placed (+[a]); ring of growth inhibition at a distance of the well (+[b]); growth inhibition (-); *: lipid-dependent strain.

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Using YNBA, growth profiles between replicates were identical. However, variable growth patterns were observed in some strains with Tween 20 on SGA.

None of *M. pachydermatis* strains was able to assimilate glucose as a unique carbon source on YNBA after 10 days of incubation. *Rhodotorula glutinis* RH-2 used as control presented good growth at 2% and 4% glucose concentrations. Glucose assimilation profiles were identical between replicates. All strains were able to grow both on YNBA supplemented with peptone and glucose, and on YNBA with peptone only at 32˚C, except for the lipid-dependent strains MA-366, MA-374 and MA-380 (Fig 2). The growth of the non-lipid-dependent strains was better on the peptone and glucose containing medium than on the medium containing only peptone. None of the strains grew on YNBA supplemented with palmitic acid at the different concentrations tested.

DNA sequencing and phylogenetic analysis

LSU rRNA region was successfully amplified for the three lipid-dependent strains (MA-366, MA-374 and MA-380), resulting in a product of 580 bp. A search on GenBank database using BLAST [22] revealed that the sequences of these strains had a percent identity of 99% to the

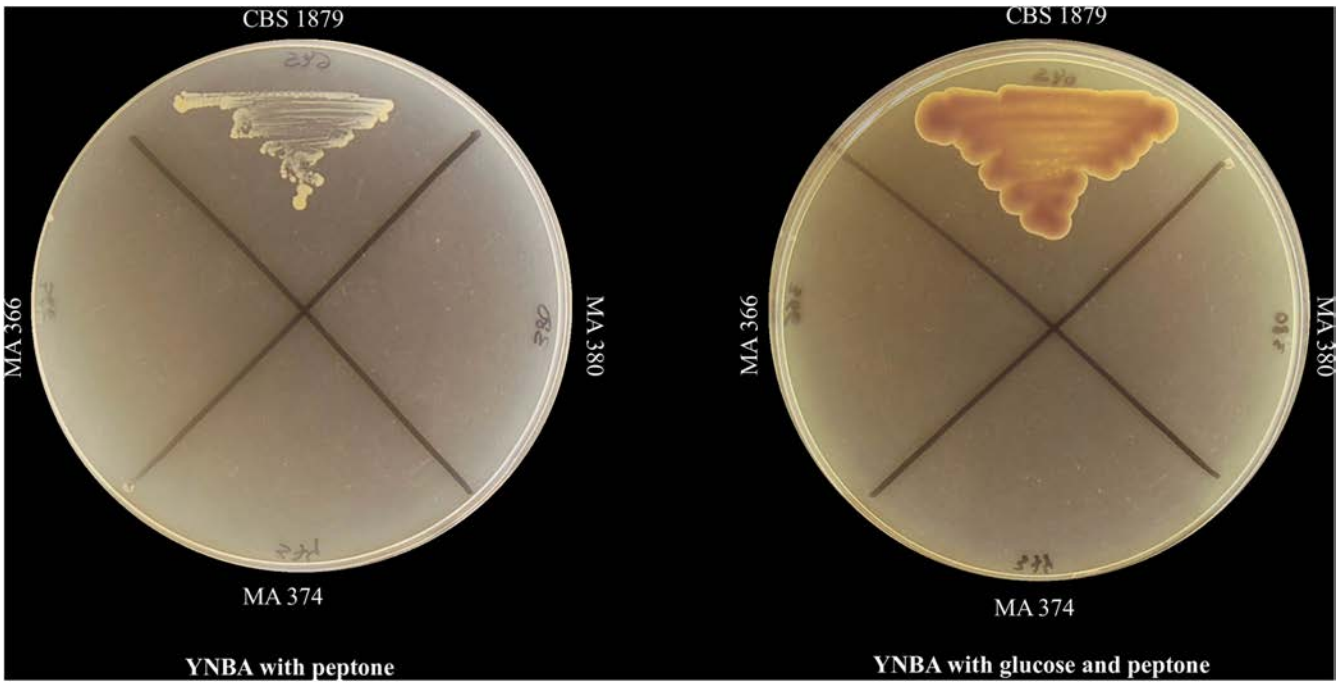

Fig 2. Growth of the neotype strain of *M. pachydermatis* (CBS 1879) on YNBA supplemented with peptone (10 g/1000 ml) and on YNBA supplemented with glucose (40 g/1000 ml) and peptone (10 g/1000 ml). None of the lipid-dependent strains (MA-366, MA-374 and MA-380) was able to grow on this media.

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sequence of the neotype strain of *M. pachydermatis* CBS 1879. Strains MA-366 and MA-374 showed identical LSU sequences and had an identity of 100% to *M. pachydermatis* MA-1382, while strain MA-380 had an identity of 100% to *M. pachydermatis* MA-280 (Table 1). The phylogenetic tree of LSU rRNA sequences revealed that *M. pachydermatis* strains formed a well-supported cluster, with 100% bootstrap support (Fig 3).

ITS rRNA, CHS2 and beta-tubulin genes were also amplified and sequenced for the lipid-dependent strains. Strains MA-366 and MA-374 showed identical ITS, CHS2 and beta-tubulin sequences. Length of the ITS region was 730 bp and constituted a new genotype (genotype XII), whose sequence has been deposited in GenBank under the accession number KY655274. Sequences of CHS2 (489 bp) and beta-tubulin (952 bp) genes matched previously described genotypes IX and VIII, respectively. Sequences of ITS (720 bp) αvd beta-tubulin (952 bp) of strain MA-380 constituted new genotypes namely genotype XIII (accession no. KY655275) and genotype IX (accession no. KY655276), respectively, whereas CHS2 sequence matched genotype IV previously described. The pairwise differences among sequences of the new genotypes from lipid-dependent strains and the previously described genotypes ranged from 0.1 to 7.0% and 0.1 to 3.4% for ITS and beta-tubulin genes, respectively. These genetic analyses confirmed the identification of the lipid-dependent strains as *M. pachydermatis*.

Discussion

Guillot et al. [15] proposed the first practical approach for phenotypic characterization of *Malassezia* species. It is based mainly on the ability to utilize certain lipid compounds (e.g. Tweens) using a diffusion test on SGA. This method is still currently used for differentiation of

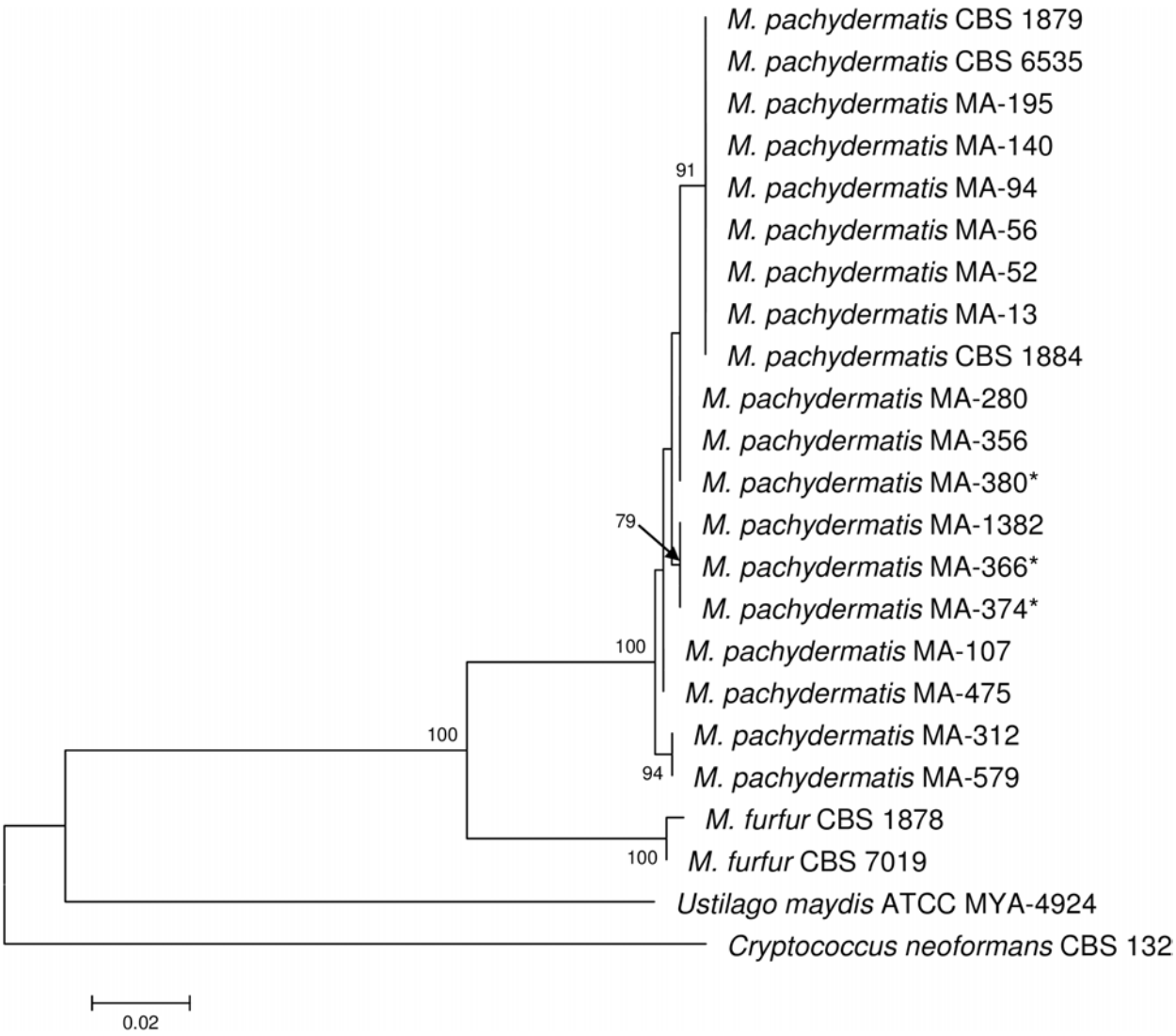

Fig 3. Molecular phylogenetic tree inferred from maximum likelihood analysis of LSU sequences of *Malassezia pachydermatis* strains. Bootstrap values > 70% in 1,000 replications are shown at the nodes. Sequences of *M. furfur* CBS 1878 and CBS 7019, *Ustilago maydis* ATCC MYA-4924 and *Cryptococcus neoformans* CBS 132 as outgroup were selected for the tree construction.* Lipid-dependent strains.

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Malassezia species [1]. Nonetheless, in some cases it may not be accurate enough to achieve a correct identification of atypical strains [7, 9, 23–25]. Therefore, some molecular methods (e.g. rDNA sequencing) are necessary to confirm the identification to species level of *Malassezia* yeasts [1, 17, 26].

In routine fungal identification, isolates of *M. pachydermatis* are usually identified by microscopic morphology and by its ability to grow on SGA. In the first steps of the identification scheme of *Malassezia* species, it is considered that if growth on SGA is observed, the yeast is *M. pachydermatis* [1, 15]. Although in most cases this assumption is correct, in our study three *M. pachydermatis* strains did not grow on SGA. Previous studies have reported the existence of atypical lipid-dependent *M. pachydermatis* isolates [7–9].

In routine *Malassezia* spp. identification, SGA is widely used. In *M. pachydermatis*, the term "non-lipid-dependent species" is clearly linked to the use of this medium and it means that this yeast is able to grow on SGA. Interestingly, most *M. pachydermatis* isolates grow on this medium. However, this term, used in a wide sense, would not be correct since recently it has been proved that the gene encoding for the fatty acid synthase is missing in the genomes of all *Malassezia* species [11] and atypical lipid-dependent *M. pachydermatis* isolates have been described in the present study. Nowadays, in the case of this species, we think that it would be more suitable to use the term "traditionally described as non-lipid dependent".

In the present study, all strains were able to grow on mDA from 32˚C to 40˚C, and showed catalase activity, in agreement with previous studies [1]. Most of the strains were able to grow at 42˚C, except for the three lipid-dependent strains and strains MA-107, isolated from a goat, and MA-579, recovered from a cat. Almost all the strains formed similar colonies of normal size and appearance at 32˚C. However, a few strains showed small colonies at this temperature. Similarly, previous studies reported the presence of *M. pachydermatis* strains that grew poorly on SGA and had a smaller colony diameter [6, 27, 28]. All studied strains were unable to split esculin, except for the three lipid-dependent strains. In this species, variable test results for beta-glucosidase activity have been reported [1].

In our study, the Tween diffusion test was performed on SGA and on YNBA. On SGA, all isolates except for the lipid-dependent strains were able to grow on the entire surface of the agar. All strains were able to assimilate Tweens 40, 60 and 80 and Cremophor EL, showing distinct growth patterns. Most of the strains also assimilated Tween 20. However, some strains showed different assimilation patterns using this lipid source. Following this technique, different assimilation patterns have been reported for *M. pachydermatis* strains [1]. For instance, growth inhibition around the four Tweens was reported by Guillot et al. [15], while other strains have been reported to assimilate Tweens 40, 60 and 80 [13]. We observed a particular assimilation pattern on both culture media, corresponding to a ring of growth inhibition at a distance of the well where the substrate was placed ($+^{b}$). Previously, a similar pattern on SGA had been described as secondary or delayed growth, after the diffusion of the lipid supplements through the medium [1]. Nonetheless, in our study this pattern was observed within the first 24 hours of incubation. Therefore, this pattern could be due to the interaction of lipid supplements and components of the medium, but in depth studies should be performed in order to confirm this hypothesis.

We think, that the Tween diffusion test could be improved substituting SGA for YNBA. The lack of intra- and interlaboratory reproducibility of this technique are due, in part, to the peptone components of SGA, which are highly complex and undefined, and may vary from batch to batch [29]. In our study, using YNBA, the assimilation patterns were identical between replicates, showing a higher reproducibility. On the other hand, growth was only observed around the lipid supplements, facilitating the visualization of growth patterns. Moreover, more assimilation patterns were observed among strains, allowing the visualization of differences that could not be detected using SGA.

In the present study, all *M. pachydermatis* strains were unable to assimilate glucose as a sole source of carbon in YNBA, which confirmed that a minimum amount of lipid is required for *M. pachydermatis* growth in this medium. Recently, Wu et al [11], using genomic analyses, revealed that a larger set of genes involved in carbohydrate metabolism had been lost in *Malassezia* species, concordant with adaptation to skin's carbohydrate-deficient environment. Moreover, these authors pointed out that, at the same time, a wide expansion of lipid hydrolases occurred in these yeasts.

All strains examined in our study, with the exception of the lipid-dependent strains, grew on YNBA with peptone. Therefore, some components of peptone promoted the growth of

these yeasts. However, their growth was more abundant on YNBA containing glucose and peptone. Thus, glucose increased the growth of the non-lipid-dependent strains in these conditions. However, this was not the case for the atypical lipid-dependent strains of *M. pachydermatis*, which did not grow under any of these conditions tested. On the other hand, it has been reported that the peptone in SGA contains palmitic acid and lesser amounts of other fatty acids, and it has been suggested that those lipids are required for *M. pachydermatis* growth [11]. In our work, none of the strains was able to grow on YNBA supplemented with palmitic acid at various concentrations. Possibly, the other fatty acids contained in peptone, among other compounds, are required for the growth of these yeasts. In fact, Wu et al [11] showed that the number of lipases varies among the different *Malassezia* species. These authors hypothesized that the more lipases these species had, the more lipids they could use, and consequently, they could live in more diverse ecosystems. Due to the genetic diversity observed in *M. pachydermatis* strains [18, 30–32], we also hypothesize that the same could happen within this species.

In our study, the three lipid-dependent strains of *M. pachydermatis* showed some differential phenotypic characteristics. Besides their inability to grow on SGA, these strains showed beta-glucosidase activity and unique Tween assimilation profiles in SGA and YNBA. Sequencing of the ITS and LSU rRNA regions, beta-tubulin and CHS2 genes confirmed that the three lipid-dependent strains belonged to the species *M. pachydermatis*. LSU rRNA and CHS2 sequences from the lipid-dependent strains matched previously characterized *M. pachydermatis* genotypes and the new ITS and beta-tubulin genotypes from these lipid-dependent strains did not exceed the variation generally observed to occur in *M. pachydermatis* [18]. Besides, in the phylogenetic tree of the LSU rRNA sequences, the lipid-dependent strains were grouped and interspersed with the non-lipid dependent *M. pachydermatis* strains.

In this study we have demonstrated the significant intraspecific diversity within the species *M. pachydermatis*. On the other hand, we have modified the Tween diffusion test for *M. pachydermatis* study using the synthetic medium YNBA, which has proved to be useful for differentiation of *M. pachydermatis* strains, providing reproducible results and a straightforward interpretation. Further studies are needed to assess the usefulness of this modified technique to distinguish the species of *Malassezia*. Moreover, testing glucose assimilation in YNBA we demonstrated that *M. pachydermatis* requires a minimum amount of lipid for growth in culture, as those provided by the complex medium SGA. On the other hand, we have characterized three lipid-dependent *M. pachydermatis* strains isolated from domestic animals. The finding of these peculiar strains exemplifies the huge variability within *M. pachydermatis*, which involves atypical strains with particular growth requirements.

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Author Contributions

Conceptualization: FJC.

Formal analysis: LP MRB GC FJC.

Funding acquisition: MRB GC FJC.

Investigation: LP MRB GC FJC.

Methodology: MRB FJC.

Project administration: MRB FJC.

Resources: MRB GC FJC.

Supervision: FJC.

Validation: LP MRB GC FJC.

Visualization: LP MRB GC FJC.

Writing – original draft: LP MRB GC FJC.

Writing – review & editing: MRB FJC.

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6. ANEXOS

6.1. Quantification of *Malassezia pachydermatis* by real-time PCR in swabs from the external ear canal of dogs. Puig L, Castellá G, Cabañes FJ. (enviado para su publicación)

Quantification of *Malassezia pachydermatis* by real-time PCR in swabs from the external ear canal of dogs

Laura Puig, Gemma Castellá* and F. Javier Cabañes

Veterinary Mycology Group, Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Bellaterra, Catalonia, Spain.

Short Title: Quantification of *M. pachydermatis* in samples by qPCR

*Corresponding author:

Gemma Castellá

Grup de Micologia Veterinària. Departament de Sanitat i Anatomia Animals

Facultat de Veterinària, Universitat Autònoma de Barcelona

E-08193 Bellaterra (Barcelona), Spain

Tel.: +34 93 581 1089

Fax: +34 93 581 2006

E-mail: Gemma.Castella@uab.cat

Keywords: *Malassezia pachydermatis*, real time PCR, β-tubulin, otitis, dogs.

Abstract

M. pachydermatis is part of the normal cutaneous microbiota of canine skin and external ear canal and it is associated with otitis externa in dogs. Laboratory diagnosis of *Malassezia* otitis relies on the presence of elevated number of the yeast on cytological examination of otic exudate and microbiological culture is required only when direct microscopy is negative. Cytological examination has a good specificity but a low sensitivity and microbiological culture is time consuming. The objective of the present work was to develop a real time PCR (qPCR) to detect and quantify *M. pachydermatis* yeasts and validate the method with swabs from external ear canal of dogs. A qPCR was developed using the β-tubulin gene as a target. Fifteen *M. pachydermatis* strains were tested with the developed assay, in order to ensure amplification of all genotypes of the target gene. Also, 16 strains from different *Malassezia* species were tested to assess the specificity of the qPCR. To validate the assay, 24 swabs from the external ear canal of dogs were analysed. The primers developed consistently amplified the DNA from all tested *M. pachydermatis* strains. The limit of quantification was established in 0.18 ng/reaction, equivalent to $1.8 \cdot 10^4$ gEq. Swabs from healthy dogs yielded quantification values of $\leq 2.0 \cdot 10^4$ gEq in the qPCR while swabs from dogs with otitis yielded quantification values of $\geq 2.2 \cdot 10^5$ gEq. We developed for the first time a qPCR assay that provides accurate quantification of *M. pachydermatis* yeasts from swab samples from dogs.

Introduction

The lipophilic yeast *Malassezia pachydermatis* is a normal inhabitant of healthy canine skin and mucosae [1]. Under determined circumstances, the skin population of *M. pachydermatis* on dogs can overgrow and act as an opportunistic pathogen, causing dermatitis and otitis externa in the affected animals [2]. Factors that favour proliferation of *M. pachydermatis* and its transition from commensal to pathogen organism on canine skin have not been fully clarified, although they are related with skin disturbances of physical, chemical and immunological mechanisms [3].

Otitis externa associated with *M. pachydermatis* is often characterized by the presence of a waxy, moist and dark exudate, with erythema and pruritus, and lesions that are often colonized by an increased number *M. pachydermatis* yeasts [1]. Diagnosis of otitis externa caused by *M. pachydermatis* is currently based on the observation of compatible lesions on the animal, the response to antifungal therapy and the presence of elevated numbers of the yeast by direct observation on the microscope [2]. Microbiological culture is required only when direct microscopy is negative in animals with suspected infections. Comparisons of cytological examination and fungal culture as the gold standard concluded that cytological examination has a good specificity but a low sensitivity [4] and consequently there is a need for a specific, sensitive, precise and rapid method to detect and quantify *M. pachydermatis* yeasts from dogs with otitis externa.

Advances in molecular biology have allowed great improvements in the diagnostics of diseases, both in human and animal health. Indeed, an increasing number of real time PCR (qPCR) assays for clinical purposes have been developed. Several qPCR assays have been developed to detect and quantify the most frequently isolated *Malassezia* species

from human skin, mainly *M. globosa* and *M. restricta* [5-9]. Those assays usually amplified ribosomal genes, which have multiple copies in the genome, and were designed to study the distribution of *Malassezia* species on human skin rather than the absolute quantification of these yeasts.

The aim of this study was to develop a fast, sensitive and accurate technique to reliably detect and quantify *M. pachydermatis* yeasts from the external ear canal of dogs. We developed and validated a qPCR using absolute quantification with SYBR Green chemistry, based on the amplification of the β-tubulin gene. With this assay, we were able to identify and quantify *M. pachydermatis* yeasts from samples consisting of swabs rubbed on the external ear canal of dogs.

Materials and Methods

Strains and samples

Strains used in this study are listed in Table 1. Fifteen *M. pachydermatis* strains representative of the eight β-tubulin genotypes [10] of this species were tested with the developed assay, in order to ensure amplification of all genotypes of the target gene. Also, 16 strains from different *Malassezia* species were tested to assess the specificity of the qPCR.

To validate the assay, 24 swabs from the external ear canal of 17 different dogs were analysed. Eleven swabs were collected from the external ear canal of 7 healthy dogs and 11 swabs were obtained from the external ear canal of 9 different dogs in which otitis caused by *M. pachydermatis* was diagnosed by veterinary clinicians. Also, 2 swabs from one dog with otitis externa caused by *M. pachydermatis* that had been treated for 15 days

with antifungal (miconazole) and antibiotic (polymyxin B) were processed. Samples from healthy dogs without otitis were obtained from the dog pound of the Autonomous University of Barcelona, and samples from cases of otitis externa were obtained with the collaboration of veterinary clinicians from the Autonomous University of Barcelona Veterinary Teaching Hospital and various veterinary clinics from the regions of Barcelona and Girona. The swabs were obtained during routine veterinary procedures and with the verbal owner consent. There was no "animal experiment" according to the legal definitions in Spain, and approval by an ethical committee was not necessary.

Microbiology

In all cases, two swabs were obtained from the affected ear of the same dog. One swab was maintained at -20ºC and used for qPCR analysis. The second swab was used for classical microbiological processing. Briefly, it was streaked on a Sabouraud glucose agar (SGA) plate (Oxoid S.A., Madrid, Spain) with 0.05g of chloramphenicol and on a blood agar plate (BD Difco S.A., Madrid, Spain). Plates were incubated at 37ºC with 5% of carbon dioxide for 5 days, and colony forming units (CFU) were counted. *M. pachydermatis* was identified by its ability to grow on SGA [11].

A cytological examination of all swabs was performed. Each swab was rolled on a clean glass slide and stained using Diff-Quick stain. The stained slides were examined on the microscope at 1000x magnification, with immersion oil. Cells with compatible morphology to *M. pachydermatis* were counted on ten observation fields, and presence of other fungi and bacteria were also noted.

DNA extraction

M. pachydermatis strains were grown on SGA whereas the rest of the strains used were grown on modified Dixon agar [11]. After 5 days of incubation at 32ºC, DNA was extracted from yeast colonies according following the FastDNA Spin kit protocol with the FastPrep FP-24 instrument (MP Biomedicals, Biolink, Barcelona, Spain). For DNA extraction from swabs the QIAmp UCP Pathogen kit (Qiagen, Madrid, Spain) was used, according to the protocol recommended for swab samples with pre-treatment with mechanical disruption. A sterile swab was processed in the same conditions as the external ear canal samples in order to control cross-contamination. DNA was stored at -20ºC until used as template in the qPCR.

Primer design

Primers were designed to amplify the β-tubulin gene of *M. pachydermatis*. Sequences used for primer design were obtained in a previous study where eight genotypes of this gene were described among *M. pachydermatis* strains [10]. Specific primers MPFRT-2 5'-CGGACGAGACGTTCTGCATT-3' and MPRRT-2 5'-TTGAGTGTGCGGAAGCAGAT-3' were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA). Primer dimers and secondary structure formation were evaluated with the Primer Express software and with IDT UNAFold online tool (https://eu.idtdna.com/Unafold/Home/Index). Both primers were searched against the NCBI database using Primer-BLAST to check their specificity.

qPCR development

The qPCR was developed following the defined criteria of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [12]. Real-

time PCRs were performed on the Applied Biosystems® 7500 Real-Time System, using SYBR Green chemistry with absolute quantification method.

Optimal concentrations of the forward and reverse primers were determined by testing different concentrations of each, between 100 and 800 nM. Concentrations giving the lowest quantification cycle (Cq) values for the standards were selected, being 300 nM for the forward primer and 500 nM for the reverse primer. The optimized reaction mix (20 µl final volume) contained 10 µl of 2x SYBR® Green PowerUp PCR Mastermix (Applied Biosystems, Foster City, CA), 0.45 µl of forward primer, 0.75µ of reverse primer, 3.8 µl of deionized DNase, RNase-free water and 5 µl of template DNA. Runs were performed using the following thermal cycling conditions: 1 cycle of 50ºC for 2 min, 1 cycle of 95ºC for 2 min, 30 amplification cycles of 95ºC for 15 secs (denaturing step), and 60ºC for 1 min (annealing-extension step and data collection). Next, a melting curve analysis was performed, with a gradual increase of temperature from 60ºC to 95ºC.

To achieve absolute quantification of the samples the standard curve method was used. In each plate, a standard curve of genomic DNA (gDNA) extracted from the neotype strain of *M. pachydermatis* CBS1879 was constructed, including 8 ten-fold dilutions, from 180 ng to 0.018 pg of DNA. Quantity and purity of the gDNA were determined with Nanodrop 2000 (Thermo Scientific, Barcelona, Spain). The amount of gDNA was extrapolated to genome equivalents (gEq), considering that β-tubulin is a single-copy gene in *M. pachydermatis* genome, the size of which is approximately 7.8 Mbp [13].

Efficiency was assessed in every run, based on the correlation coefficient (r2) and slope values from the standard curve, and sensitivity was evaluated with the limit of

quantification (LOQ), considered as the lowest concentration of DNA amplified from the standard curve. The linear dynamic range was determined, considered as the highest to the lowest gDNA dilution amplified from the standard curve [12].

To test for inhibition of the qPCR, gDNA standards were spiked with randomly selected samples from dogs. Cq values were compared to the Cq obtained from the same non-spiked standards to ensure that amplification was equivalent. Non-template controls (NTCs), in which DNA was substituted by water in the reaction mix, allowed verification that no contamination occurred and no primer dimers were formed. In each run all samples were run for triplicate, including the standards and NTCs.

Results

Primer design and specificity

The primers amplified a 61 bp amplicon with no similarity to the dog genome. A BLAST search against the NCBI database showed no significant similarity to other commensal and pathogen bacteria or fungi that can be found on the ear canal of dog. Besides β-tubulin gene from *M. pachydermatis*, next sequence matches belonged to organisms that are not related with domestic carnivores, such as a mushroom (*Coprinopsis argentea*), animals (*Drosophila navojoa*, *Cyprinus carpio, Esox lucius*) or a plant (*Nelumbo nucifera*).

Primers consistently amplified the DNA from all tested *M. pachydermatis* strains. Melting curve analyses yielded a single peak in all *M. pachydermatis* strains at 79.3-80.4ºC of melting temperature (Tm) depending on the genotype (Table 1). No detectable amplification of other *Malassezia* species was observed, except for *M. globosa*, *M. slooffiae* and *M. obtusa*, which provided Tm values of 78.6-80.1ºC, obtaining a single

dissociation peak in the cases of *M. globosa* and *M. slooffiae*, while *M. obtusa* also presented an additional smaller peak at approximately 70ºC of melting temperature.

qPCR performance

The β-tubulin gene from *M. pachydermatis* was consistently amplified in a linear dynamic range of 4 ten-fold dilutions of gDNA, from $1.8 \cdot 10^7$ to $1.8 \cdot 10^4$ gEq. The standard curve yielded r2 values superior to 0.990 in all runs, and slope values of approximately -3.40. All replicates were amplified with high precision, with less than 1 Cq of variation among them and standard deviation values lower than 0.4. The LOQ was established in 0.18 ng/reaction, equivalent to $1.8 \cdot 10^4$ gEq, which corresponds to the latest standard dilution amplified and accurately quantified ($Cq \geq 28$).

Microbiology and validation of the qPCR using clinical samples

The presence and amount of *M. pachydermatis* yeasts of samples from the external ear canal of dogs were also assessed by plate counting and cytological examination (Table 2). Samples from dogs without otitis yielded no growth of *M. pachydermatis* or plate counts ranging from 1 to 8 CFU/plate and negative cytological examination in most of the samples. In samples from dogs with otitis, 1-5 *M. pachydermatis* cells/field were observed in the cytological examination while ≥10 CFU/plate were obtained in culture. In most of the samples where a high number of *M. pachydermatis* colonies were obtained, bacterial colonies also grew on blood agar plates, mostly Gram-positive cocci.

In the developed qPCR, quantification values of up to $2.4 \cdot 10^4$ gEq were obtained from samples from healthy dogs, while values of $2.2 \cdot 10^5$ - $1.7 \cdot 10^6$ gEq were obtained from samples from dogs with otitis by *M. pachydermatis*. Melting curve analyses provided a

single peak in all cases, with Tm values of 79.4-80.4ºC (Figure 2). Amplification of NTCs was not detected, and inhibition was not detected in gDNA standards spiked with samples from dogs.

Discussion

In this study we developed a fast, precise and efficient qPCR assay that detects and quantifies *M. pachydermatis* yeasts, through amplification of a β-tubulin gene fragment. The external ear canal of dogs can be colonized by a variety of bacterial and fungal species. In recent massive sequencing studies, it was found that the predominant bacteria of the external ear canal of dogs are members of the phyla *Proteobacteria*, *Actinobacteria* and *Firmicutes* [14], while some of the predominant fungi correspond to members of the genera *Alternaria*, *Cladosporium*, *Epicoccum*, and *Cryptococcus*, besides *Malassezia* spp. [15]. A BLAST search against NCBI database showed that our primers did not match any of the deposited sequences of such organisms. The designed primers showed amplification of all tested *M. pachydermatis* strains with consistent Tm values within 1ºC of variation. Although a slight range of variation can occur in melting curve analyses depending on different factors like SYBR Green or DNA concentrations, an interval variation of ±1ºC among Tm values is considered to be acceptable for specificity determination [16-18]. Amplification of the other sixteen *Malassezia* species was not detected, except for *M. globosa*, *M. slooffiae* and *M. obtusa*. Although these three species provided very similar Tm values to *M. pachydermatis* strains, a double melting peak was observed in *M. obtusa*, which allowed its differentiation. However, amplification of *M. globosa* and *M. slooffiae* could not be differentiated from *M. pachydermatis*, since all of them presented very similar Tm values. Nonetheless, amplification of these species should not be considered as a disadvantage of the technique. *M. globosa* and *M. slooffiae*

have never been isolated from dogs [19-21], whereas there is one report of *M. obtusa* associated with canine otitis externa [22]. However, in this study the isolated was identified by phenotypic characteristics and no molecular confirmation was performed.

Our qPCR assay has a LOQ of 0.18 ng of *M. pachydermatis* DNA, which corresponded to $1.8 \cdot 10^4$ gEq/reaction. Some qPCR methods have been developed for quantification of different *Malassezia* species from human skin. In these methods, the LOQ ranged from 10^2 to 10^5 amplicon copies per reaction depending on the *Malassezia* species detected [5-6]. All these qPCR assays are based on multicopy genes and therefore they are more sensitive than single-copy genes but quantification is less accurate.

Quantitative differences were determined by all the methods used (culture, cytological examination, and qPCR) in samples from dogs according to their health status (healthy/otitis by *M. pachydermatis*). Samples from healthy dogs yielded plate counts ≤ 8 CFU/plate, a maximum of 2 *M. pachydermatis* cells/field at the cytological examination and quantification values of $\leq 2.0 \cdot 10^4$ gEq in the qPCR. Samples from dogs with otitis yielded plate counts ≥10 CFU/plate, 1-5 *M. pachydermatis* cells/field at the cytological examination and quantification values of $\geq 2.2 \cdot 10^5$ gEq in the qPCR. No colonies were grown on plates from the swabs obtained from a dog that had been treated for otitis externa by *M. pachydermatis* and quantification values similar to the LOQ were obtained with these samples on the qPCR. For some authors, the observation of more than 5 to 10 cells of compatible morphology with *M. pachydermatis* in several microscopic fields or more than 70 CFU per sample from ear specimens of dogs should be considered as indicative of an abnormal increase of *Malassezia* [1, 4, 23, 24]. However, these proposed guidelines are not generally accepted because a small population of the yeast might cause

disease in sensitized animals and variations in population sizes have been observed between different breeds [2]. Although it is not known whether there is a threshold population density needed for infection, population of *M. pachydermatis* in dogs with skin disease can be increased 100-10,000 fold. An accurate quantification of *M. pachydermatis* yeasts from swab samples from dogs can be achieved with the designed qPCR method. Correlation between the CFU obtained in culture and qPCR values was not exact. Regarding culture, diverse factors can affect the number of *M. pachydermatis* colonies grown on the plate, like the fact that *M. pachydermatis* has a tendency to form clumps, and colonies could have been originated from a clump of cells instead of a single yeast cell.

We developed for the first time a qPCR assay that provides accurate quantification of *M. pachydermatis* yeasts in swabs from the external ear canal of dogs. The kind of sample required for the developed assay is non-invasive, handling of the samples does not require any particular transportation condition and can be stored at -20ºC until DNA is extracted. With the described method, in a few hours it is possible to achieve accurate quantification of *M. pachydermatis*. The application of the developed method on clinical cases could improve the diagnosis of otitis by *M. pachydermatis* on dogs, and consequently lead to a more accurate treatment that would enhance the prognosis of the affected animals. On the other hand, this technique would also be useful to assess the response to treatment through quantification of the amount of *M. pachydermatis* yeasts of affected dogs.

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Conflict of interest statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure captions

Figure 1. Amplification plot and melting curve analysis of standard 3 (1.8 ng gDNA/reaction, equivalent to $1.8 \cdot 10^5$ gEq) and four samples from dogs, after 30 cycles. Swabs 12 and 16 were obtained from dogs in which otitis externa by *M. pachydermatis* was diagnosed, and swabs 8 and 11 were obtained from healthy dogs.

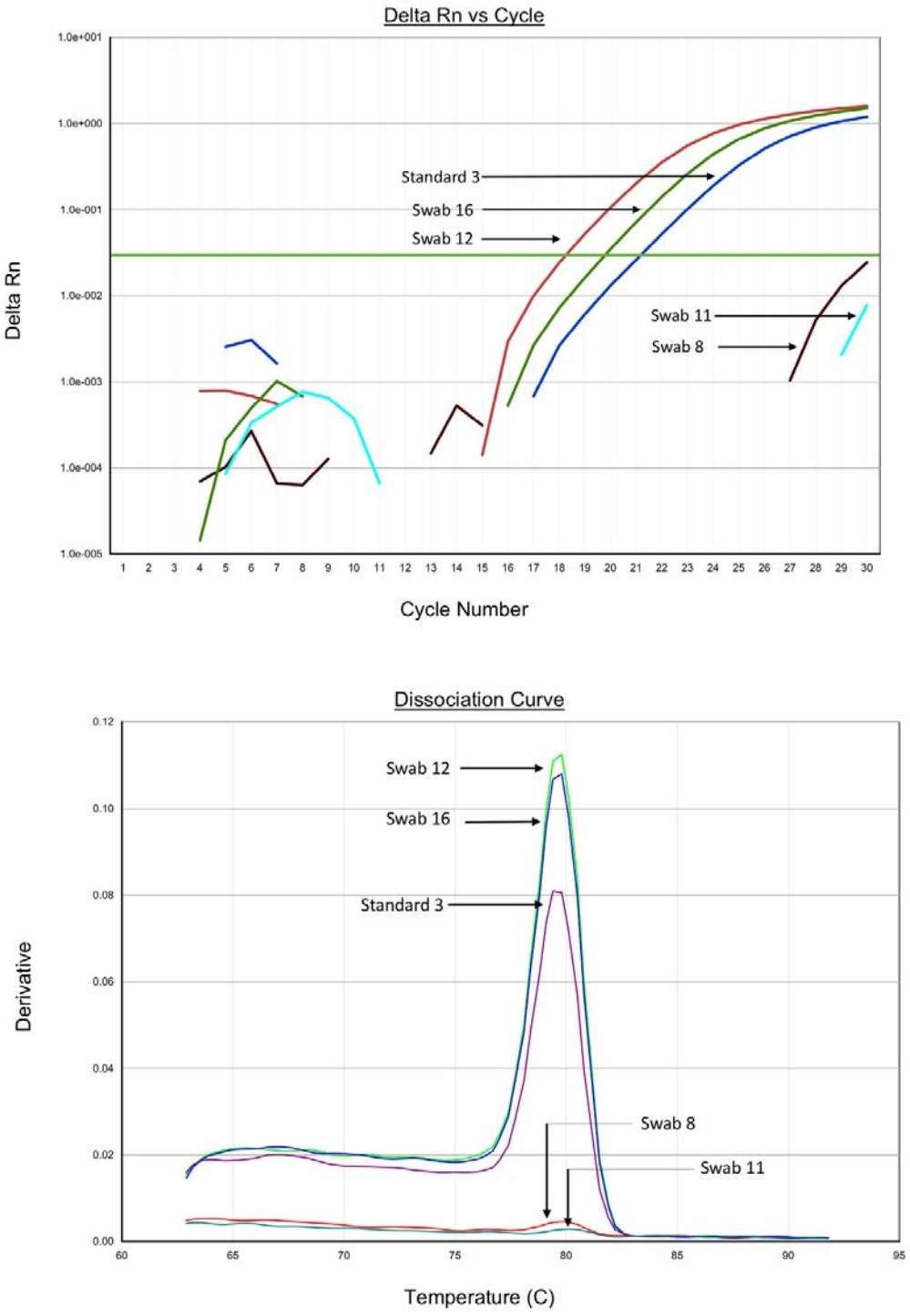
Delta Rn vs Cycle
Delta Rn
Cycle Number
Standard 3
Swab 16
Swab 12
Swab 11
Swab 8
Dissociation Curve
Derivative
Temperature (C)
Swab 12
Swab 16
Standard 3
Swab 8
Swab 11

Figure 1.

Table 1. Strains used in the study, including the host species and location from which they were retrieved , β-tubulin sequence and melting temperature (Tm).

Species	Strains	Host	Location	β-tubulin GenBank Accession no. (genotype)	Tm (ºC)
M. pachydermatis	CBS1879	Dog	Ear	KC573803 (I)	79.4
M. pachydermatis	CBS1884	Dog	Ear	KU313727 (II)	79.6
M. pachydermatis	CBS6535	Dog	Ear	KC573803 (I)	79.5
M. pachydermatis	MA 13	Dog	Ear	KC573803 (I)	79.6
M. pachydermatis	MA 52	Dog	Ear	KU313727 (II)	80.3
M. pachydermatis	MA 56	Dog	Ear	KU313727 (II)	80.3
M. pachydermatis	MA 107	Goat	Ear	KU313728 (III)	79.8
M. pachydermatis	MA 140	Cat	Ear	KU313729 (IV)	79.3
M. pachydermatis	MA 195	Dog	Ear	KC573803 (I)	79.3
M. pachydermatis	MA 280	Dog	Ear	KU313728 (III)	80.4
M. pachydermatis	MA 312	Cat	Ear	KU313730 (V)	79.8
M. pachydermatis	MA 356	Dog	Ear	KU313731 (VI)	80.1
M. pachydermatis	MA 475	Pig	Ear	KU313732 (VII)	80.1
M. pachydermatis	MA 579	Cat	Skin	KU313729 (IV)	79.4
M. pachydermatis	MA 1382	Dog	Ear	KU313733 (VIII)	80.2
M. arunalokei	CBS13387	Human	Scalp		-
M. brasiliensis	CBS 14135	Parrot	Beak	KR872311	-
M. caprae	CBS10434	Goat	Ear	KC573795	-
M. cuniculi	CBS11721	Rabbit	Skin	KC573808	-
M. dermatis	CBS9169	Human	Skin	KC573796	-
M. equina	CBS9969	Horse	Skin	KC573798	-
M. furfur	CBS1878	Human	Skin	KC573799	-
M. globosa	CBS7966	Human	Skin	KC573806	78.6
M. japonica	CBS9431	Human	Skin	KC573801	-
M. nana	CBS9557	Cat	Ear	HM594270	-
M. obtusa	CBS7876	Human	Skin	KC573802	70-79.3*
M. psittaci	CBS 14136	Parrot	Beak	KR872310	-
M. restricta	CBS7877	Human	Skin	KC573807	-
M. slooffiae	CBS7956	Pig	Skin	KC573805	80.1
M. sympodialis	CBS7222	Human	Skin	KC573797	-
M. yamatoensis	CBS9725	Human	Skin	KC573804	-

CBS, Centraalbureau voor Schimmelcultures; MA, culture collection of the Veterinary Mycology group.
- no melting peak detected
* two melting peaks detected

Table 2. Results obtained from samples from dogs with otitis externa by *M. pachydermatis* and healthy dogs, including cytological examination, plate counts and qPCR quantification values, expressed as genome equivalents (gEq).

Sample	Animal	Otitis[a]	Cells/field[b]	CFU/plate	gEq
Swab 1	Dog 1	No	0	3	$<1.8\cdot10^4$*
Swab 2	Dog 1	No	0	8	$<1.8\cdot10^4$*
Swab 3	Dog 2	No	0	0	$<1.8\cdot10^4$*
Swab 4	Dog 3	No	0	6	$<1.8\cdot10^4$*
Swab 5	Dog 3	No	0	3	$<1.8\cdot10^4$*
Swab 6	Dog 4	No	0	0	$<1.8\cdot10^4$*
Swab 7	Dog 4	No	0	0	$<1.8\cdot10^4$*
Swab 8	Dog 5	No	1	1	$<1.8\cdot10^4$*
Swab 9	Dog 5	No	2	4	$2.4\cdot10^4$
Swab 10	Dog 6	No	1	4	$<1.8\cdot10^4$*
Swab 11	Dog 7	No	0	0	$<1.8\cdot10^4$*
Swab 12	Dog 8	Yes	-[d]	179	$1.7\cdot10^6$
Swab 13	Dog 8	Yes	-[d]	91	$6.5\cdot10^5$
Swab 14	Dog 9	Yes	1	10	$3.0\cdot10^5$
Swab 15	Dog 10	Yes	1	57	$3.4\cdot10^5$
Swab 16	Dog 11	Yes	3	>250	$4.8\cdot10^5$
Swab 17	Dog 12	Yes	2	134	$7.1\cdot10^5$
Swab 18	Dog 12	Yes	1	40	$1.0\cdot10^6$
Swab 19	Dog 13	Yes	2	223	$1.0\cdot10^6$
Swab 20	Dog 14	Yes	5	210	$2.40\cdot10^5$
Swab 21	Dog 15	Yes	2	>250	$2.20\cdot10^5$
Swab 22	Dog 16	Yes	4	>250	$2.70\cdot10^5$
Swab 23[c]	Dog 17	Yes	1	0	$6.0\cdot10^4$
Swab 24[c]	Dog 17	Yes	1	0	$<1.8\cdot10^4$*

[a] Diagnosed otitis externa by *M. pachydermatis* when the sample was taken from the external ear canal of the dog; [b] Average number of cells/field of compatible morphology to *M. pachydermatis*, counted in 10 fields by direct observation, at 1000x magnification with immersion oil; [c] Samples from diferent ears of the same dog, which had been treated for 2 weeks with antifungals due to an otitis process caused by *M. pachydermatis*; [d] No available data; CFU, colony-forming units, obtained by plate counting; gEq, genome equivalents, obtained on the developed qPCR; *, quantification value below the limit of detection of the developed assay.

6.2. Phenotypic and genetic diversity of *Malassezia furfur* from domestic and zoo animals. Puig L, Bragulat MR, Castellá G, Cabañes FJ. (enviado para su publicación)

Phenotypic and genetic diversity of *Malassezia furfur* from domestic and zoo animals

Laura Puig, M. Rosa Bragulat*, Gemma Castellá and F. Javier Cabañes

Veterinary Mycology Group, Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Bellaterra, Catalonia, Spain.

Short Title: *Malassezia furfur* diversity from animals

*Corresponding author:

M. Rosa Bragulat

Grup de Micologia Veterinària. Departament de Sanitat i Anatomia Animals

Facultat de Veterinària, Universitat Autònoma de Barcelona

E-08193 Bellaterra (Barcelona), Spain

Tel.: +34 93 581 1089

Fax: +34 93 581 2006

E-mail: Rosa.Bragulat@uab.cat

Keywords: *Malassezia furfur*; animals; diversity; phenotype; sequencing

Abstract

Malassezia furfur is traditionally associated to human skin, although more recent studies have been revealing its presence in a variety of animals. The aim of this study was to analyse phenotypically and genetically the diversity among strains isolated from animals of this species. We have examined different strains from domestic and wild animals held in captivity. On the one hand, their phenotypic characteristics were studied, by assessing its growth at different incubation temperatures, their catalase and β-glucosidase activities and the Tween diffusion test on Sabouraud glucose agar (SGA), and on yeast nitrogen base agar (YNBA), a synthetic medium without lipids. On the other hand, the large subunit (LSU) and the internal transcribed spacer (ITS) of ribosomal RNA and the β-tubulin gene were sequenced. Different sequence types were identified for each target gene, and fourteen genotypes were revealed. While several genotypes were obtained from the strains from domestic animals, the strains from zoo animals appeared to be genetically more stable. With ITS and β-tubulin gene, *M. furfur* strains grouped in two clades. One clade included the strains from domestic animals and the other clade included the strains from zoo animals. The phenotypic tests also revealed a remarkable diversity within this species, which appeared to be more significant among strains from domestic animals. Moreover, the Tween diffusion test using YNBA was more useful to observe differences among strains, which could not be perceived using SGA.

Introduction

The genus *Malassezia* was created by Baillon in 1889 and remained limited to only two species for over a century: *M. furfur* (Robin) Baillon 1889 and *M. pachydermatis* (Weidman) Dodge 1935 [1]. Traditionally, the lipid-dependent species *M. furfur* (*sensu lato*) was thought to occur only on human skin, being the causal agent of different skin disorders. On the contrary, the classically considered non-lipid-dependent species *M. pachydermatis*, the only species in the genus that does not usually require lipid supplementation for development in Sabouraud glucose agar (SGA) [2], was restricted to animal skin. This species is usually associated with otitis externa and different kind of dermatitis in domestic animals [3].

Based mainly on molecular data and lipid requirements, the genus was revised and expanded [4] to include seven species comprising the former taxon *M. pachydermatis* and the splitting of *M. furfur* (*sensu lato*) into five new lipid-dependent species, *M. furfur* (*sensu stricto*), *M. sympodialis* [5], *M. globosa*, *M. obtusa*, *M. restricta* and *M. slooffiae*.

In the present century, ten new lipid-dependent species, *M. arulanokei* [6], *M. brasiliensis* [7], *M. caprae* [8], *M. cuniculi* [9], *M. dermatis* [10], *M. equina* [8], *M. japonica* [11], *M. nana* [12], *M. psittaci* [7], and *M. yamatoensis* [13] have been described. Of these last species, *M. brasiliensis*, *M. caprae*, *M. cuniculi*, *M. equina*, *M. nana* and *M. psittaci* were isolated from animals.

Although *M. furfur* (*sensu stricto*) is a common member of the human skin microbiota, it has been also associated with various human skin diseases (e.g. pityriasis versicolor, seborrheic dermatitis) and systemic diseases (e.g. catheter-associated sepsis) [14, 15].

This species has been also reported from the skin of various animal species but very little is known about their pathogenic role in animal skin [14]. However, in several studies, some yeasts have been identified as *M. furfur* on the basis of phenotypic characteristics (e.g. Tween assimilation) and/or using some PCR techniques, but without rDNA sequencing confirmation [16-26]. Only in few studies the identity of isolates as *M. furfur* has been confirmed by rDNA sequence analysis. Using this gold standard technique for identification of these yeasts, *M. furfur* has been reported from ostriches, a chimpanzee, a cow, an elephant, a horse, a pelican and a pig [4, 27], from cattle and a dog [28] and recently from parrots [7].

Consequently, *M. furfur* may be found in a wide diversity of animals other than human beings. The aim of this work was to isolate *M. furfur* from other animal species, mainly from zoo animals, and to study their phenotypic and genotypic variability. In order to examine the phenotypic variability within this species a synthetic agar medium was used to evaluate their lipid requirements. To do this, the SGA medium used in the Tween diffusion technique [1, 29] was replaced by a yeast nitrogen base agar medium (YNBA). Besides, the strains were studied sequencing the LSU, ITS and β-tubulin gene to understand their phylogenetic relationships and to analyse their specific genetic variation.

Material and Methods

Strains

A total of twenty-one strains of *M. furfur* were studied (**Table 1**). These were selected from our collection to obtain representative strains from different animal species. Also,

we included four strains recently isolated from the ear of four different dead birds from the zoo of Barcelona: MA-1555, MA-1567 and MA-1569 from scarlet ibis (*Eudocimus ruber*) and MA-1575 from roseate spoonbill (*Platalea ajaja*). These isolates were recovered using swabs introduced in the external ear canal, during the necropsy of the animals. The neotype strains of *M. furfur* (CBS 1878 and CBS 7019) [1] and two strains from the CBS collection were also included (CBS 7984, from an elephant and CBS 7985, from an ostrich). The strains were stored at -80 ºC [30].

Morphological and physiological characterization

Strains were streaked on modified Dixon agar (mDA) (36 g of malt extract [Oxoid S.A., Madrid, Spain], 10 g of bacteriological peptone [Oxoid S.A., Madrid, Spain], 20 g of desiccated ox bile [Sigma-Aldrich S.L., Madrid, Spain], 15 g of agar bacteriological [Oxoid S.A., Madrid, Spain[, 10 ml of Tween 40 [Sigma-Aldrich S.L., Madrid, Spain], 2 ml of glycerol [Sigma-Aldrich S.L., Madrid, Spain] and 2 g of oleic acid [MP Biomedicals LLC., Illkrich, France] per liter; pH 6.0) [1] and incubated at 32ºC. Morphological characteristics of the colonies and the cells were observed after 7 days of incubation.

Strains were physiologically characterized based on the splitting of esculin due to β-glucosidase activity, catalase reaction and growth at 37ºC, 40ºC and 42ºC on mDA. The ability to assimilate Tween 20 [MP Biomedicals LLC., Illkrich, France], Tween 40, Tween 60 [Merk KGaA, Madrid, Spain], Tween 80 [MP Biomedicals LLC., Illkrich, France] and Cremophor EL [Sigma-Aldrich S.L., Madrid, Spain] was evaluated with the Tween diffusion test on SGA [1]. Besides, the Tween diffusion test was performed on

YNBA (6.7 g yeast nitrogen base [BD Difco S.A., Madrid, Spain], 20g agar bacteriological per liter (pH 5.4), following the procedure previously described [2]. All tests were performed by duplicate.

DNA extraction, amplification, sequencing and phylogenetic analysis

DNA of all strains (except for CBS 1878, CBS 7019, MA1453, and MA1456 whose sequences were already deposited in GenBank) was extracted from 7-day old cultures on mDA, according to the FastDNA Spin kit protocol with the FastPrep FP-24 instrument [MP Biomedicals, Biolink, Barcelona, Spain]. DNA was stored at -20ºC until used as a template for PCR. The LSU, ITS and β-tubulin gene were amplified and sequenced, using the primers and the protocols described previously [31]. The β-tubulin gene of *M. arunalokei* CBS13387 was also sequenced.

Sequence alignments were carried out using MUSCLE implemented in MEGA 6 software [32]. Maximum likelihood analysis of the individual genes were conducted using MEGA 6 software with 1,000 bootstrap replicates. A suitable substitution model was determined for each gene. The initial tree for heuristic search was obtained by applying the Neighbour-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. Clades that were supported by bootstrap values (bs) of ≥70 % were regarded as strongly supported. Sequences of *Cryptococcus neoformans* CBS 132 were selected as outgroup for the tree construction.

Results

Morphology and physiology

The main differential phenotypic characteristics of the studied strains are summarized in **Table 2**. Colonies on mDA were mat, smooth and soft, of 4-5 mm in diameter approximately. Microscopic examination of the colonies showed cylindrical to ovoid cells, with buds forming on a more or less broad base, compatible with *M. furfur* [1, 4]. All strains were able to grow on mDA from 32 to 40ºC. At 42ºC, only two strains were unable to grow at this temperature (MA-105 and MA-128). The catalase reaction and the β-glucosidase activity were positive for all strains, although some presented a weak reaction.

The Tween diffusion test was performed on SGA and on the synthetic medium without lipids, YNBA (**Figure 1**). The characterization of the assimilation profiles obtained was performed according to the previously described methodology [2]. On SGA, although some intermediate assimilation patterns were found, all strains grew with the different lipids assayed. Three main assimilation patterns were observed around de lipid supplements, corresponding to good growth (+), weak growth (w) and growth at a distance of the well where the substrate was placed ($+^{a}$). On YNBA, five main assimilation patterns were observed around the lipid supplements, corresponding to good growth (+), weak growth (w), growth at a distance of the well where the substrate was placed ($+^{a}$), ring of growth inhibition at a distance of the well ($+^{b}$), and growth inhibition (-). Using SGA and YNBA, growth profiles between replicates were identical.

DNA sequencing and phylogenetic analysis

With the primers used we were able to amplify and sequence 582 bp, 733-735 bp and 905-906 bp of the LSU, the ITS and the β-tubulin gene, respectively. The new nucleotide sequences determined in this study have been deposited at the GenBank database under accession numbers MF185809-MF185816, including the β-tubulin sequence of *M. arunalokei* CBS 13387.

Different sequences types were identified for each gene (**Table 1**). Regarding the LSU, four types of sequences were identified. Sequence type I was isolated from strain CBS 1878 and various domestic and wild animals. Sequence type II was retrieved from strain CBS 7019 and domestic animals, mainly horses. Type III was obtained from wild birds and an elephant, while sequence type IV contained only one sequence, isolated from a sheep. Pairwise comparisons among them revealed a nucleotide variation of 0.17-0.51%.

For the amplified ITS region, six types of ITS sequences were identified. Type I was retrieved mainly from horses and CBS 1878 whereas type II included only one sequence, from CBS 7019. Type III also contained one sequence, from CBS 7984, and type IV was recovered only from birds. Sequence type V was obtained from different domestic animals and type VI contained one sequence, from a pig. Pairwise differences among the strains were 0.13-1.76%.

For the β-tubulin gene, eight sequence types were identified (I-VIII), and pairwise differences were 0.11-3.98%. Sequence type I was obtained from horses, a cat and CBS 1878, while type II was recovered only from CBS 7019. Type III included only strains

from birds and an elephant, while type IV included only sequences from domestic animals. Types V and VI were obtained from horses and type VII from a pig, while type VIII was recovered from a parrot.

The molecular phylogenetic trees based on the maximum likelihood analysis of the sequences of LSU, ITS and β-tubulin gene are shown in **Figures 2**, **3** and **4**, respectively. The phylogenetic tree of LSU sequences revealed that *M. furfur* strains were grouped together in a cluster with low bootstrap support. The neotype strain CBS 7019 and strains isolated form horses and a goat formed a well supported clade (86% bs) whereas the rest of strains defined another poorly supported clade. The phylogenetic tree of ITS sequences of *M. furfur* strains revealed two strongly supported clades. One clade included strains isolated from humans and domestic animals (98% bs) and the other clade comprised the strains from wild animals including parrots, ibis, a spoonbill, an ostrich and an elephant (99% bs). Full concordance was observed with the distribution of *M. furfur* strains in two clades using β-tubulin sequences, with a high bootstrap support (90% bs and 83% bs).

Discussion

Although the species *Malassezia furfur* was classically associated to human skin, several studies have related these yeasts to different animal species [3]. In the present study, we have demonstrated the presence of *M. furfur* in an ever broader spectrum of animal hosts, including four strains from wild birds held in captivity.

The identity of the isolates as *M. furfur* was demonstrated by the observation of macro and micromorphology of the yeasts and by sequencing of two ribosomal genetic regions

(LSU and ITS). Besides, strains were studied phenotypically [1, 29]. Using these methods, minor differences were observed among strains. Only two strains were unable to grow at 42ºC, and few differences were observed in the intensity of the β-glucosidase activity. Nonetheless, it is recognized that this reaction can be more or less marked in *M. furfur* [1].

Malassezia furfur is considered to be one of the most robust of the lipid dependent species of the genus. In SGA, this species shows more or less growth with the four Tweens and Cremophor EL as lipid sources, although with the latter it can be weaker [1]. Among the studied strains, in the Tween diffusion test up to three assimilation patterns were recognized. All strains showed good growth with Tweens 20, 40, 60 and 80, while with Cremophor EL different assimilation patterns were observed (+, w, or $+^{a}$).

Few studies reported the isolation of atypical *M. furfur* strains. An isolate of *M. furfur* from the external ear canal of a healthy dog was reported to be unable to use Cremophor EL [28], and few isolates from healthy human skin and with skin disorders were unable to assimilate Tween 20, 40 and 60, and also did not grow at 40ºC on Dixon agar [33]. In those cases, the identity of the atypical strains of *M. furfur* was demonstrated by LSU sequencing. Other authors reported *M. furfur* strains with atypical assimilation patterns with Cremophor EL, but these strains were not confirmed by DNA sequencing [17, 22].

Recently, it has been reported that the Tween diffusion test performed in YNBA instead of SGA resulted more useful in order to observe differences among *M. pachydermatis* strains [2]. Consequently, in the present study we also performed this test using YNBA with *M. furfur* strains. Certainly, this modification resulted in a higher variability of

growth profiles. With this synthetic medium, all strains were able to assimilate Tweens 40 and 60, showing different patterns, and a wider variability of assimilation patterns was observed with Tweens 20 and 80 and Cremophor EL.

In general, more variability among assimilation profiles was observed among strains from domestic animals, while the same assimilation profile was obtained in all the strains from zoo animals (including CBS 7984, from an elephant, and CBS 1985, from an ostrich), which coincided with the profile of the *M. furfur* neotype strain CBS 7019. This profile was very similar with SGA and YNBA, although with the latter, these strains assimilated Cremophor EL weakly.

In the LSU phylogenetic tree, *M. furfur* strains appear grouped in two clades with low bootstrap support. Sequences differences among *M. furfur* strains were less than 1%, not exceeding the within-species variation generally observed to occur in *Malassezia* [14, 34]. The closest species was *M. brasiliensis*, a recently described species [7]. Using the ITS rRNA and β-tubulin, *M. furfur* strains were grouped in two well-supported clades. One clade included the sequences from humans and domestic animals, while the second clade included the strains from wild animals held in captivity.

On the other hand, a higher genetic variability was observed among the strains from domestic animals. While sequences from zoo animals formed a consistent cluster, regarding the ITS and β-tubulin gene, they also showed the same genotype with the exception of strains MA-1555 and MA-1453. In contrast, more genotypes were identified among sequences from domestic animals.

In previous studies, the genetic variability within *M. furfur* was analysed by various molecular techniques. For instance, genetic heterogeneity among *M. furfur* strains was observed by RAPD and AFLP [35, 36]. Using AFLP, up to eight types of *M. furfur* were detected, five of which included only samples from humans and two included samples from animals (elephant, elk and ostrich) [34]. Sequencing the LSU rRNA gene, four *M. furfur* genotypes were detected [27], and heterogeneity was found among strains mostly recovered from human skin [34].

In the present study, we have demonstrated a remarkable phenotypic and phylogenetic variability among *M. furfur* strains from different animal species. Moreover, with both methodologies we observed a higher variability among the strains from domestic animals than among the strains from zoo animals. On the other hand, we have confirmed the usefulness of the modification of the Tween diffusion test using YNBA instead of SGA in *M. furfur*. As it occurred previously with *M. pachydermatis* [2], this modification enabled the observation of more differences than using the classic methodology.

As it has been demonstrated in other *Malassezia* species, these yeasts seem to be undergoing a diversification process in which they would be adapting to different hosts. In this study, we have proven that *M. furfur* can be found on a broad spectrum of animals, like wild birds held in captivity. Future studies will probably widen even more the number of niches that are colonized by these yeasts.

Acknowledgements

The authors thank to the Biological Bank- Parc Zoològic de Barcelona for providing some samples from zoo animals and Carolina Gómez from the Veterinary Mycology Group of the Universitat Autònoma de Barcelona (UAB) for their valuable technical assistance. Financial support came from Servei Veterinari de Bacteriologia i Micologia of the UAB.

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Table 1. *Malassezia furfur* strains studied, including original animal host and LSU, ITS, and β-tubulin genotypes.

Strain	Host	Location	LSU / ITS / β-tubulin genotypes (Genbank acc. no.)
CBS 1878	Human	Scalp	I (AY743602) / I (AY743634) / I (KC573799)
CBS 7019	Human	Skin	II (AY743603) / II (AY743635) / II (KC573800)
CBS 7984	Elephant	Ear	III (AY387226) / III (AY387130) / III (KC573811)
CBS 7985	Ostrich	Wing	III (AY387225) / IV (AY387129) / III (KC573811)
MA-74	Sheep	Skin	IV (MF185809)[a] / V (MF185810)[a] / IV (MF185812)[a]
MA-86	Horse	Ear	II / I / I
MA-105	Horse	Ear	II / I / I
MA-121	Horse	Skin	II / I / IV
MA-128	Horse	Skin	II / I / IV
MA-136	Goat	Skin	II / I / IV
MA-157	Cat	Ear	I / V / I
MA-176	Horse	Skin	I / V / IV
MA-242	Horse	Skin	II / I / V (MF185813)[a]
MA-456	Horse	Skin	II / I / VI (MF185814)[a]
MA-474	Pig	Skin	I / VI (MF185811)[a] / VII (MF185815)[a]
MA-1453	Parrot	Beak	III / IV / VIII (KR872309)
MA-1456	Parrot	Oropharynx	III / IV / III
MA-1555	Ibis	Ear	I / IV / III
MA-1567	Ibis	Ear	III / IV / III
MA-1569	Ibis	Ear	III / IV / III
MA-1575	Spoonbill	Ear	III / IV / III

Naming source: CBS, Centraalbureau voor Schimmelcultures; MA, culture collection of the Veterinary Mycology group.
[a] New genotypes determined in the present study

Table 2. Main differential phenotypic characteristics of the studied *M. furfur* strains.

Strain	Growth at			Catalase activity	β–glucosidase activity	Tween diffusion test									
						with SGA					with YNBA				
	37ºC	40ºC	42ºC			T 20	T 40	T 60	T 80	CrEL	T 20	T 40	T 60	T 80	CrEL
CBS 1878	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CBS 7019	+	+	+	+	w	+	+	+	+	+	+	+	+	+	w
CBS 7984	+	+	w	+	w	+	+	+	+	+	+	+	+	+	w
CBS 7985	+	+	w	+	w	+	+	+	+	+	+	+	+	+	w
MA-74	+	+	+	w	w	+	+	+	+	w	+	+	+	+	w
MA-86	+	+	+	+	+	+	+	+	+	+[a]	+	+	+	+	w
MA-105	+	+	-	+	w	+	+	+	+	+[a]	+	+	+	+	+[a]
MA-121	+	+	+	+	+	+	+	+	+	+[a]	-	+[a]	+[b]	-	-
MA-128	+	+	-	+	+	+	+	+	+	+[a]	-	+[a]	+[b]	-	-
MA-136	+	+	+	+	+	+	+	+	+	+[a]	w	w	w	w	w
MA-157	+	+	+	+	+	+	+	+	+	w	w	+	+[b]	w	-
MA-176	+	+	+	+	+	+	+	+	+	w	w	+[b]	+[b]	w	-
MA-242	+	+	+	+	w	+	+	+	+	+[a]	w	w	w	w	-
MA-456	+	+	+	+	+	+	+	+	+	+[a]	w	w	w	w	-
MA-474	+	+	+	w	+	+	+	+	+	w	w	+[b]	+[a]	w	-
MA-1453	+	+	w	w	+	+	+	+	+	+[a]	+	+	+	+	w
MA-1456	+	+	+	+	+	+	+	+	+	w	+	+	+	+	w
MA-1555	+	+	+	+	w	+	+	+	+	w	+	+	+	+	w
MA-1567	+	+	w	+	w	+	+	+	+	+	+	+	+	+	w
MA-1569	+	+	w	+	w	+	+	+	+	+	+	+	+	+	w
MA-1575	+	+	w	+	w	+	+	+	+	+	+	+	+	+	w

Growth at 37ºC, 40ºC and 42ºC on mDA (modified Dixon Agar) after 7 days of incubation.
Tween diffusion test [1, 29] with SGA (Sabouraud glucose agar) and YNBA (Yeast nitrogen base agar) [2]: +, good growth; w, weak growth; +[a], growth at a distance of the well where the substrate was placed; +[b], ring of growth inhibition at a distance of the well; -, growth inhibition.
Naming source: CBS, Centraalbureau voor Schimmelcultures; MA, culture collection of the Veterinary Mycology group.

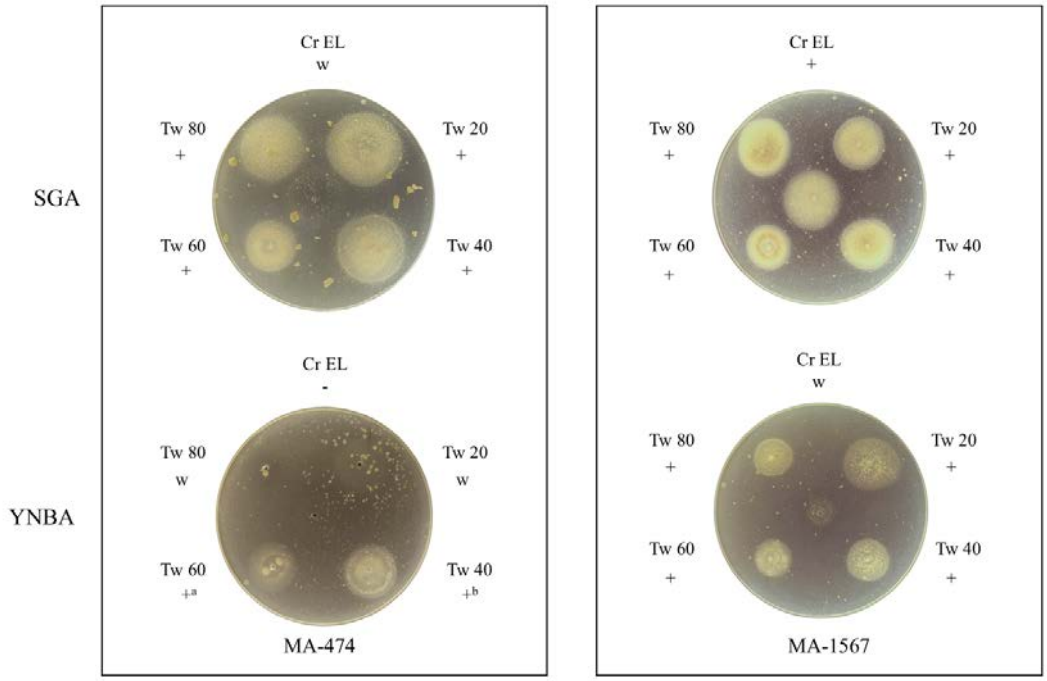
Figure captions

Figure 1. Growth patterns of *M. furfur* strains MA-474 and MA-1567 in the Tween diffusion test with SGA and YNBA, after 7 days of incubation at 32ºC.

Figure 2. Molecular phylogenetic tree inferred from maximum likelihood analysis of LSU sequences of members of the genus *Malassezia*. Bootstrap values > 70% in 1,000 replications are shown at the nodes. Sequences of *Ustilago maydis* ATCC MYA-4924 and *Cryptococcus neoformans* CBS 132 were selected as outgroup for the tree construction.

Figure 3. Molecular phylogenetic tree inferred from maximum likelihood analysis of ITS sequences of members of the genus *Malassezia*. Bootstrap values > 70% in 1,000 replications are shown at the nodes. Sequence of *Cryptococcus neoformans* CBS 132 was selected as outgroup for the tree construction.

Figure 4. Molecular phylogenetic tree inferred from maximum likelihood analysis of β-tubulin sequences of members of the genus *Malassezia*. Bootstrap values > 70% in 1,000 replications are shown at the nodes. Sequence of *Cryptococcus neoformans* CBS 132 was selected as outgroup for the tree construction.


SGA
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MA-474
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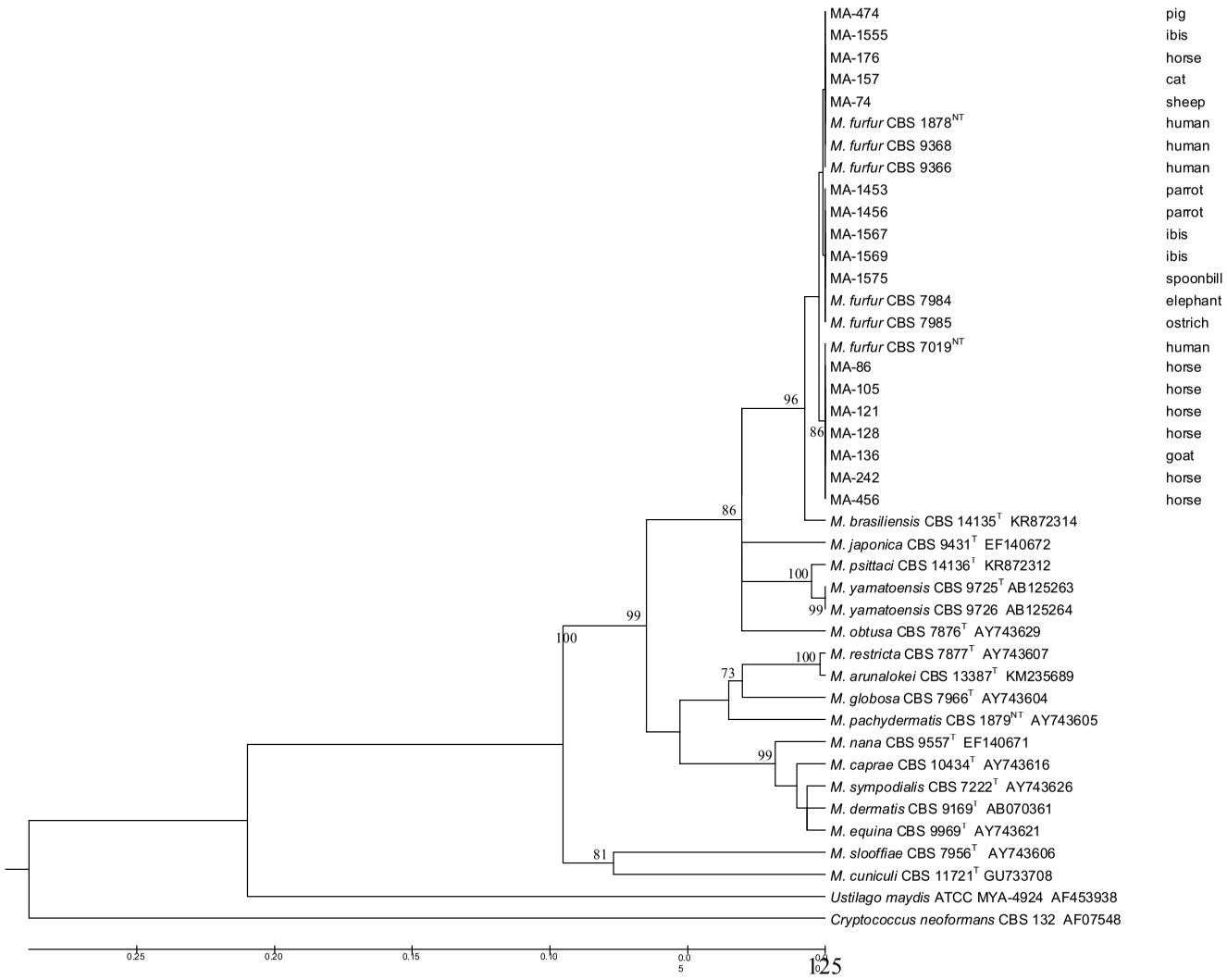
MA-1567

Figure 1.

Figure 2.


MA-474 pig
MA-1555 ibis
MA-176 horse
MA-157 cat
MA-74 sheep
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M. furfur CBS 9368 human
M. furfur CBS 9366 human
MA-1453 parrot
MA-1456 parrot
MA-1567 ibis
MA-1569 ibis
MA-1575 spoonbill
M. furfur CBS 7984 elephant
M. furfur CBS 7985 ostrich
M. furfur CBS 7019NT human
MA-86 horse
MA-105 horse
MA-121 horse
MA-128 horse
MA-136 goat
MA-242 horse
MA-456 horse
M. brasiliensis CBS 14135T KR872314
M. japonica CBS 9431T EF140672
M. psittaci CBS 14136T KR872312
M. yamatoensis CBS 9725T AB125263
M. yamatoensis CBS 9726 AB125264
M. obtusa CBS 7876T AY743629
M. restricta CBS 7877T AY743607
M. arunalokei CBS 13387T KM235689
M. globosa CBS 7966T AY743604
M. pachydermatis CBS 1879NT AY743605
M. nana CBS 9557T EF140671
M. caprae CBS 10434T AY743616
M. sympodialis CBS 7222T AY743626
M. dermatis CBS 9169T AB070361
M. equina CBS 9969T AY743621
M. slooffiae CBS 7956T AY743606
M. cuniculi CBS 11721T GU733708
Ustilago maydis ATCC MYA-4924 AF453938
Cryptococcus neoformans CBS 132 AF07548
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Figure 3.

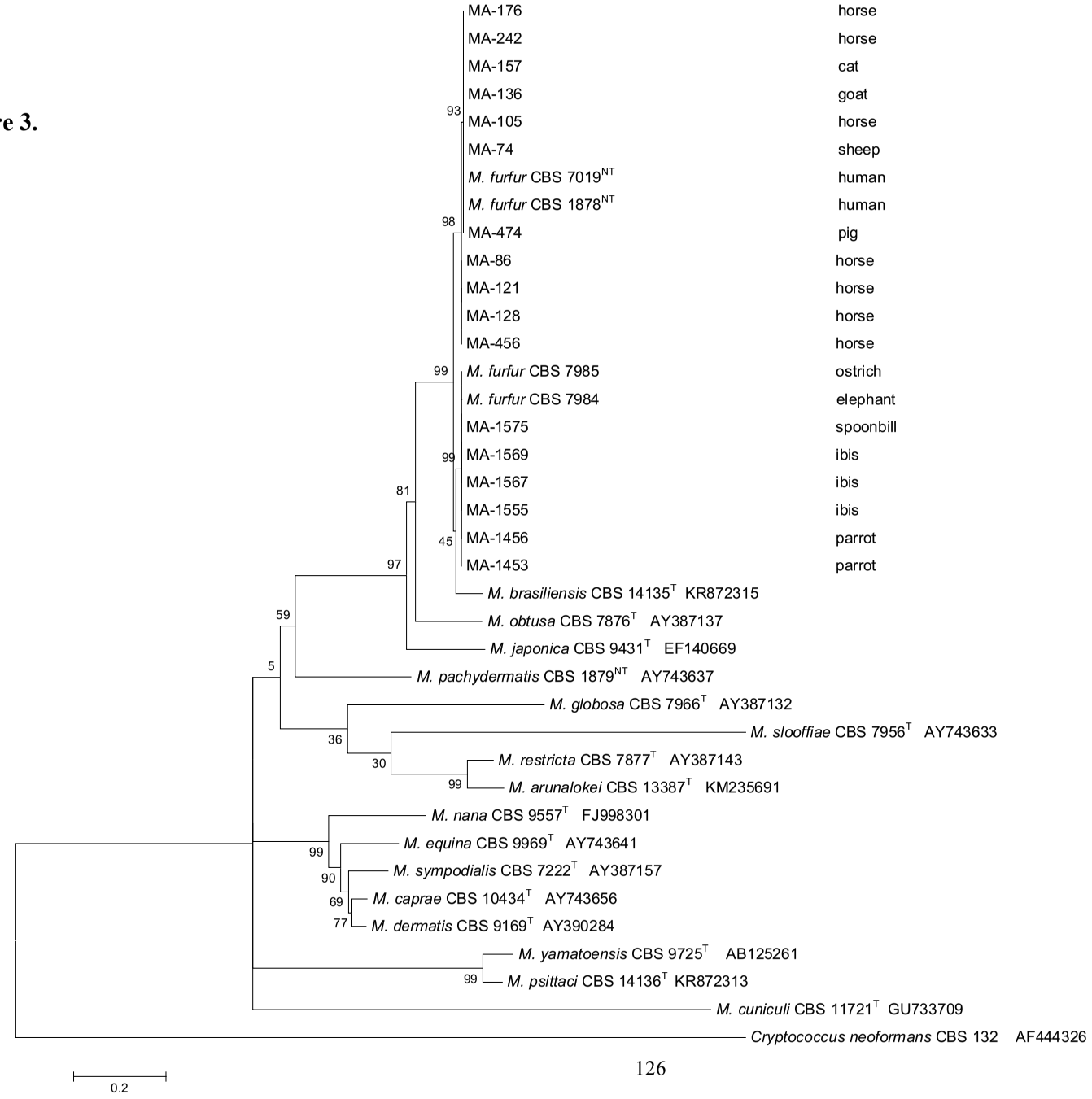
MA-176 horse
MA-242 horse
MA-157 cat
MA-136 goat
MA-105 horse
MA-74 sheep
M. furfur CBS 7019NT human
M. furfur CBS 1878NT human
MA-474 pig
MA-86 horse
MA-121 horse
MA-128 horse
MA-456 horse
M. furfur CBS 7985 ostrich
M. furfur CBS 7984 elephant
MA-1575 spoonbill
MA-1569 ibis
MA-1567 ibis
MA-1555 ibis
MA-1456 parrot
MA-1453 parrot
M. brasiliensis CBS 14135T KR872315
M. obtusa CBS 7876T AY387137
M. japonica CBS 9431T EF140669
M. pachydermatis CBS 1879NT AY743637
M. globosa CBS 7966T AY387132
M. slooffiae CBS 7956T AY743633
M. restricta CBS 7877T AY387143
M. arunalokei CBS 13387T KM235691
M. nana CBS 9557T FJ998301
M. equina CBS 9969T AY743641
M. sympodialis CBS 7222T AY387157
M. caprae CBS 10434T AY743656
M. dermatis CBS 9169T AY390284
M. yamatoensis CBS 9725T AB125261
M. psittaci CBS 14136T KR872313
M. cuniculi CBS 11721T GU733709
Cryptococcus neoformans CBS 132 AF444326
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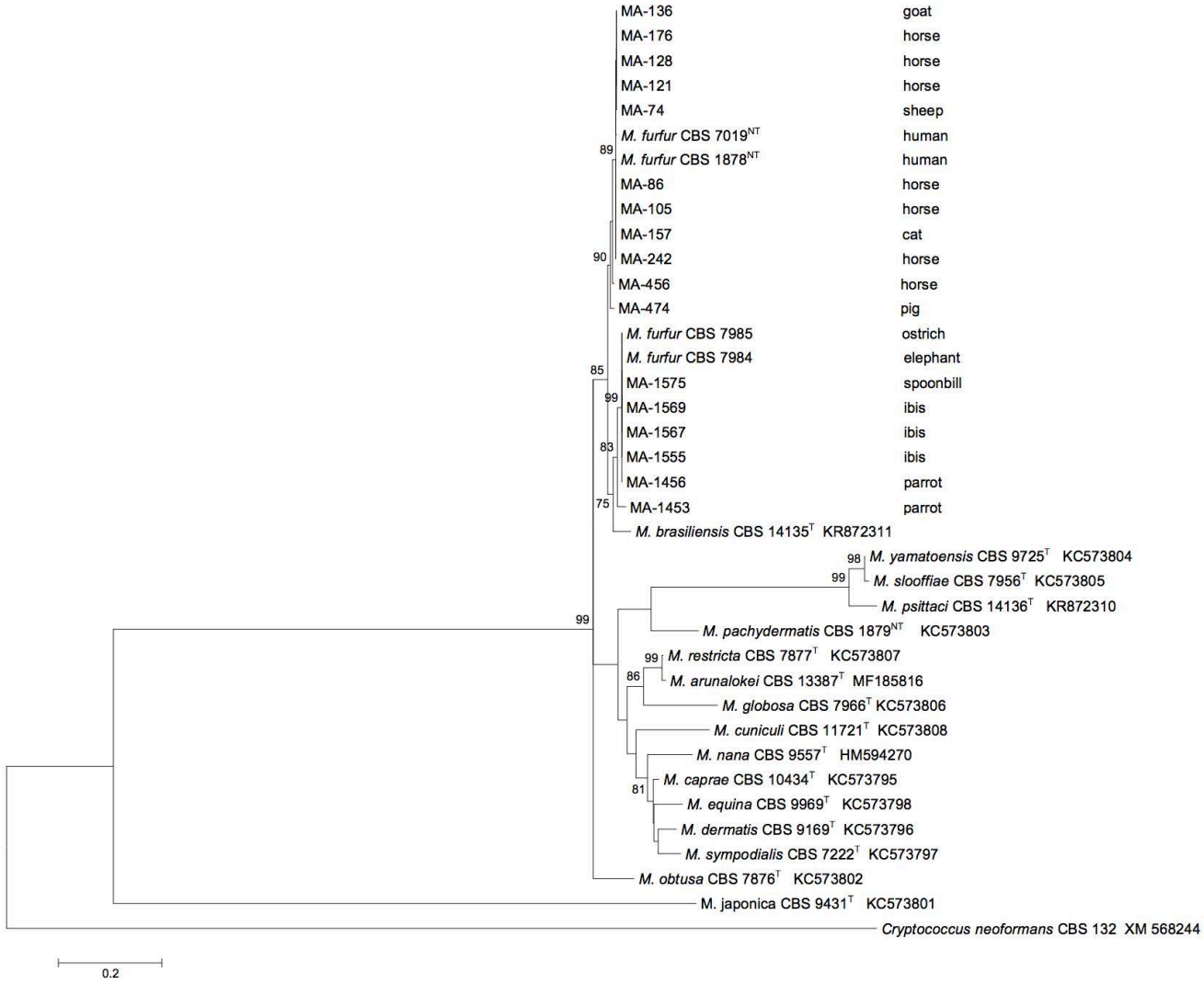
Figure 4.


MA-136 goat
MA-176 horse
MA-128 horse
MA-121 horse
MA-74 sheep
M. furfur CBS 7019NT human
M. furfur CBS 1878NT human
MA-86 horse
MA-105 horse
MA-157 cat
MA-242 horse
MA-456 horse
MA-474 pig
M. furfur CBS 7985 ostrich
M. furfur CBS 7984 elephant
MA-1575 spoonbill
MA-1569 ibis
MA-1567 ibis
MA-1555 ibis
MA-1456 parrot
MA-1453 parrot
M. brasiliensis CBS 14135T KR872311
M. yamatoensis CBS 9725T KC573804
M. slooffiae CBS 7956T KC573805
M. psittaci CBS 14136T KR872310
M. pachydermatis CBS 1879NT KC573803
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M. globosa CBS 7966T KC573806
M. cuniculi CBS 11721T KC573808
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M. equina CBS 9969T KC573798
M. dermatis CBS 9169T KC573796
M. sympodialis CBS 7222T KC573797
M. obtusa CBS 7876T KC573802
M. japonica CBS 9431T KC573801
Cryptococcus neoformans CBS 132 XM 568244
89
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