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FUNGOS NEGROS DERIVADOS DE *Atta* spp.: DIVERSIDADE E FATORES DE VIRULÊNCIA

ANA PAULA MIRANDA DUARTE

Dissertação apresentada ao Instituto de Biociências do Campus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas (Área: Microbiologia Aplicada).

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TÍTULO: FUNGOS NEGROS DERIVADOS DE Atta spp.: DIVERSIDADE E FATORES DE VIRULÊNCIA

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Ao Santo Expedito,
das causas justas e urgentes,
pelo pedido atendido.
Dedico,

À minha família,
pelo apoio incondicional.
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“The Grasshopper, singing
All summer long,
Now found winter stinging,
And ceased in his song.
Not a morsel or crumb in his cupboard--
So he shivered, and ceased in his song.
Miss Ant was his neighbor;
To her he went:
"O, you're rich from labor,
And I've not a cent.
Lend me food, and I vow I'll return it,
Though at present I have not a cent."
The Ant's not a lender,
I must confess.
Her heart's far from tender
To one in distress.
So she said: "Pray, how passed you the summer,
That in winter you come to distress?"
"I sang through the summer,"
Grasshopper said.
"But now I am glummer
Because I've no bread."
"So you sang!" sneered the Ant. "That relieves me.
Now it's winter--go dance for your bread!"

Grasshopper and the Ant
Jean de La Fontaine

RESUMO

As formigas da tribo Attini são conhecidas como “cultivadoras de fungos” devido à complexa simbiose que mantém com um fungo basidiomiceto, cultivado como alimento. Além do fungo mutualista, cresce continuamente a lista de micro-organismos encontrados nos ninhos e na cutícula desses insetos. Recentemente, leveduras negras, relacionadas ao gênero *Phialophora*, foram consideradas componentes da simbiose formiga-fungo por comprometerem a habilidade das formigas em lidar com o micoparasita *Escovopsis*. Os fungos melanizados estão frequentemente relacionados a infecções humanas e, por isso, tem sido questionada a possibilidade desses insetos atuarem como vetores de dispersão de fungos negros patógenos no ambiente urbano. Esta dissertação marca o início de um esforço no qual procuramos investigar a diversidade de fungos negros presentes na cutícula de formigas cortadeiras, bem como verificar o grau de virulência das estirpes obtidas. Este último aspecto, ou seja, a possibilidade das formigas cortadeiras abrigarem e dispersarem micro-organismos patogênicos pode ser o início de um novo ramo de investigação. Analisando fêmeas aladas (içás) de *Atta capiguara* e *A. laevigata*, coletadas antes do início do voo nupcial, verificou-se que elas abrigam em suas cutículas uma comunidade diversa de fungos negros, como os gêneros *Cladophialophora*, *Cladosporium*, *Cochliobolus*, *Exophiala*, *Ochroconis*, *Phaeococcomyces*, *Phialophora*, *Penidiella* e *Pyrenochaeta*. Num segundo estudo, os resultados indicaram que leveduras negras do gênero *Exophiala*, presentes na cutícula de içás e em ambientes contaminados por derivados de petróleo, apresentam características que as tornam potenciais oportunistas humanos, como consistente crescimento a 40°C e produção de lacases. Espera-se que os resultados deste trabalho possam servir de ponto de partida para estudos mais detalhados visando examinar de que forma esses fungos chegam ao interior dos ninhos, como eles sobrevivem e se relacionam com os insetos, e, também, verificar se são propagados a partir desse ambiente.

Palavras chave: fungos melanizados, oportunista, *Exophiala*, formigas-cortadeiras.

ABSTRACT

Attini ants are known as fungus-growing ants due to a complex symbiosis with a basidiomycete fungus cultivated as a food source. Besides the mutualistic fungus, there is a continuous growth in the list of microorganisms found in nests and cuticles of these insects. *Phialophora*-related black yeasts have recently been considered as components of the ant-fungus symbiosis by compromising the ability of ants to deal with the mycoparasite *Escovopsis*. Melanized fungi are frequently related to human infections and, therefore, the ability of these insects act as vectors for dispersal of pathogenic black fungi in the urban environment has been questioned. This dissertation makes a start at assessing the diversity of black fungi derived from the cuticle of leaf-cutting ants, as well as verifying the virulence degree of the strains obtained. The latter, i.e., the possibility of ants sheltering and dispersing pathogenic microorganisms can be the start of a new research field. Our microbial profile of *Atta capiguara* and *A. laevigata* winged females, collected prior to the mating flight, revealed that the cuticle of ants can harbor a diverse community of black fungi, such as the melanized genera *Cladophialophora*, *Cladosporium*, *Cochliobolus*, *Exophiala*, *Ochroconis*, *Phaeococcomyces*, *Phialophora*, *Penidiella* and *Pyrenochaeta*. Moreover, we demonstrated that *Exophiala* species, associated with gynes' cuticle and hydrocarbon-polluted environments, have characteristics that make them potential human opportunists, like consistent growth at 40°C and laccase production. Our results will certainly serve as a basis for more detailed future studies to explore how these fungi reach the interior of the nest, how they survive and relate to insects, as well as check whether the fungi are propagated from this environment.

Key words: melanized fungi, opportunist, *Exophiala*, leaf-cutting ants.

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INTRODUÇÃO

1 Formigas-cortadeiras (Tribo Attini)

As formigas da tribo Attini (Hymenoptera: Formicidae), encontradas exclusivamente nas Américas (WEBER, 1972; MAYHÉ-NUNES; JAFFÉ, 1998; MEHDIABADI; SCHULTZ, 2009), estão envolvidas em um antigo e obrigatório mutualismo com fungos basidiomicetos do gênero *Leucoagaricus* (CHAPELA et al., 1994), cultivados como alimento (MUELLER; RABELING, 2008; SCHULTZ; BRADY, 2008).

A fungicultura das formigas Attini originou-se há aproximadamente 60-50 milhões de anos (SCHULTZ; BRADY, 2008) e desde então tem experimentado uma série de modificações evolutivas de modo que as formigas e os fungos não podem mais sobreviver isoladamente.

Embora todas as formigas Attini sejam conhecidas como cultivadoras de fungos, os dois gêneros mais derivados filogeneticamente (CURRIE, 2001), *Acromyrmex* e *Atta*, são mais comumente conhecidos como formigas-cortadeiras por possuírem o hábito de cortar elevadas quantidades de material vegetal (MARICONI, 1970), utilizadas como substrato para o crescimento do fungo simbionte (WEBER, 1972). Uma vez no interior do ninho, o substrato vegetal é processado e incorporado no "jardim de fungos", estrutura de coloração esbranquiçada constituída pelo micélio do fungo simbionte e material vegetal coletado.

Apesar de serem consideradas herbívoros generalistas, algumas espécies de formigas-cortadeiras se especializaram em cortar gramíneas, outras, dicotiledôneas, podendo também coletar flores e frutos (HÖLLDOBLER; WILSON, 1990; BEATTIE; HUGHES, 2003). A Quadro 1 apresenta as principais diferenças entre as duas espécies amostradas no presente estudo, *Atta capiguara* e *A. laevigata*.

A relação simbiótica entre o fungo e as formigas proporciona benefícios a ambos (SCHULTZ et al., 2005). O fungo mutualista produz enzimas, principalmente pectinases e celulases (BACCI et al., 1995; SIQUEIRA et al., 1998; SILVA et al., 2006), que degradam as folhas cortadas, resultando dessa hidrólise açúcares simples que servirão de alimento para o próprio fungo e para as operárias (SILVA et al., 2003). Adicionalmente, partes do fungo são

coletadas e manipuladas por operárias para alimentar as larvas (WEBER, 1972; WETTERER, 1994).

Quadro 1. Principais diferenças entre as espécies *A. capiguara* e *A. laevigata*.

	A. capiguara	***A. laevigata***	**Referências**
Distribuição	Região sudeste do Brasil	Venezuela e grande parte do território brasileiro	DELABIE et al., 2011
Localização ninhos	Áreas com grande insolação	Áreas ensolaradas e sombreadas	MARICONI, 1970; MOREIRA et al., 2004a; ANDRADE et al., 2005
Profundidade dos ninhos	Até 4,5 m	Até 7 m	MOREIRA et al., 2004b; ANDRADE et al., 2005
Material vegetal coletado	Preferencialmente monocotiledôneas	Mono- e dicotiledôneas, preferência por folhas secas	JUSTI et al., 1996; VASCONCELOS; CHERRETT, 1996; FORTI; BOARETTO, 1997

As formigas, por sua vez, fornecem ao fungo um ambiente favorável ao crescimento e protegem-no de diversos contaminantes e competidores (CHERRETT et al., 1980; WETTERER, 1994). Dentre as estratégias utilizadas pelas formigas para impedir ou diminuir o desenvolvimento de micro-organismos parasitas destacam-se: 1. Limpeza do substrato vegetal coletado, eliminando mecanicamente esporos fúngicos contaminantes e outros micro-organismos (CURRIE; STUART, 2001; VAN BAEL et al., 2009); 2. Uso de secreções glandulares com propriedades antissépticas (MARSARO-JÚNIOR et al., 2001; FERNÁNDEZ-MARÍN et al., 2006); 3. Remoção de partes infectadas dos jardins de fungo (CURRIE; STUART, 2001); e, 4. Interações com outros micro-organismos capazes de proteger os ninhos (CURRIE et al., 1999b). Além disso, esses insetos também promovem a dispersão do fungo mutualista para novas colônias através de um inóculo inicial levado na cavidade infrabucal de fêmeas aladas (içás) durante o período de reprodução, chamado de revoada (MUELLER, 2002).

A revoada ocorre após o amadurecimento sexual da colônia, entre outubro e dezembro para a região Sudeste do Brasil, geralmente em dias claros, quentes e úmidos (MARINHO et al., 2011). Antes de deixar o jardim de fungo, as içás retiram uma pequena porção do fungo *Leucoagaricus* e o aloja na cavidade infrabucal. No dia do voo nupcial um grande número de formas aladas, machos (bitús) e fêmeas (içás), abandonam os ninhos,

permanecem um curto período na superfície do solo e levantam voo, acasalando em pleno ar. Depois do acasalamento, as futuras rainhas dos formigueiros retornam ao solo para estabelecerem novas colônias.

2 Comunidade microbiana associada às formigas Attini

Historicamente, os jardins de fungos foram descritos como monoculturas puras, nas quais as formigas excluíam micro-organismos contaminantes (MÖLLER, 1893). Essa visão permaneceu por muito tempo, mas relatos da presença de outros micro-organismos nos jardins de fungos e exoesqueleto de formigas-cortadeiras foram publicados (PAGNOCCA et al., 2011, 2012).

Um século após a primeira descrição do fungo mutualista, Currie et al. (1999a) estabeleceram que os jardins também abrigam fungos parasitas especializados do gênero *Escovopsis* (Ascomycota: Hypocreales). Encontrado apenas em associação com colônias de formigas Attini (CURRIE et al., 1999a, 2001), o fungo *Escovopsis* sp. explora diretamente o cultivo das formigas secretando compostos que degradam a parede das hifas/micélio do fungo mutualista e absorvendo os nutrientes liberados (REYNOLDS; CURRIE, 2004).

Apesar da alta virulência em colônias de formigas Attini verificada por Currie et al. (1999a), análises moleculares recentes e evidências experimentais revelaram pouca especificidade entre o parasita *Escovopsis* sp. e as formigas-cortadeiras *Atta* e *Acromyrmex* (TAERUM et al., 2007).

Até o momento duas espécies do gênero estão descritas: *E. weberi* (MUCHOVEJ; DELLA LUCIA, 1990) e *E. aspergilloides* (SEIFERT et al., 1995). Augustin (2011) propõe em sua tese (dados ainda não publicados) a descrição de quatro novas espécies, obtidas de jardins de *Acromyrmex*: *E. moelleri*, *E. niveo-chlamydosporiformans*, *E. microsporum* e *E. lentecrescens*. Devido a grande diversidade morfológica apresentada por isolados deste gênero (CURRIE, 2001; GERARDO et al., 2006), novas espécies ainda devem ser encontradas.

Coincidentemente com o estabelecimento do fungo *Escovopsis* sp. como um parasita da interação formiga-fungo cultivado, um simbionte adicional foi descoberto por Currie et al.

(1999a). Localizadas na cutícula das formigas, bactérias filamentosas do gênero *Pseudonocardia* produzem metabólitos secundários com propriedades antimicrobianas, utilizados pela formiga para suprimir o crescimento do fungo *Escovopsis* sp. (CURRIE et al., 1999b; CALDERA et al., 2009).

A posição da bactéria *Pseudonocardia* sp. no exoesqueleto das operárias e sua distribuição nos vários gêneros da tribo Attini são variáveis, podendo ser rara ou ausente em *Atta* sp. (CURRIE et al., 1999b; ZHANG et al., 2007; MUELLER et al., 2008). Alguns trabalhos também demonstram a presença destas bactérias em jardins de fungo (POULSEN et al., 2003; MUELLER et al., 2008) e nos pellets da cavidade infrabucal de rainhas (MUELLER et al., 2008) e de operárias (LITTLE et al., 2006).

Contrariando os trabalhos de Currie et al. (1999b, 2001), que atribuíram à bactéria *Pseudonocardia* sp. o papel de produção de substâncias específicas para inibir o fungo parasita *Escovopsis* sp. e também estimular o desenvolvimento do fungo mutualista, existem relatos de algumas linhagens de *Pseudonocardia* sp. que não possuem atividade contra *Escovopsis* sp. e apresentam atividade antagonista ao fungo mutualista (SEN et al., 2009), questionando a teoria de que a bactéria é um terceiro simbionte das formigas Attini.

Outro micro-organismo foi identificado como parte dessa complexa relação simbiótica fungo-formiga. Pertencente à família Herpotrichiellaceae (Ascomycota, relacionada a *Phialophora*), a levedura negra cresce no mesossoma da formiga, na mesma região cuticular onde se encontra a bactéria *Pseudonocardia* sp. (LITTLE; CURRIE, 2007). Little e Currie (2008) propõem que a levedura negra compete por nutrientes com as bactérias filamentosas, suprimindo seu crescimento e indiretamente reduzindo a habilidade em evitar o crescimento do fungo parasita *Escovopsis* sp.

Encontrada até então apenas em formigas *Apterostigma* (tribo Attini), a levedura negra, classificada como um quinto simbionte, foi identificada apenas em gênero e mais detalhes dessa associação estão por ser desvendados.

Neste contexto, alguns trabalhos têm sido desenvolvidos na tentativa de preencher essa lacuna de informações a respeito de micro-organismos envolvidos na relação simbiótica das formigas Attini com seu fungo cultivado. A literatura relaciona aproximadamente 30

artigos mais específicos, sendo aproximadamente um terço voltado ao isolamento de leveduras associadas a formigas-cortadeiras (CRAVEN et al., 1970; ANGELIS et al., 1983; PAGNOCCA et al., 1996, 2008; CARREIRO et al., 1997, 2002, 2004; MIDDELHOVEN et al., 2003; RODRIGUES et al., 2009) e o restante voltado aos fungos filamentosos (MÖLLER, 1893; GOETSCH; STOPPEL, 1940; KREISEL, 1972; MUCHOVEJ et al., 1991; DIEHL-FLEIG, 1993; LUCIANO et al., 1995; SEIFERT et al., 1995; FISCHER et al., 1996; CURRIE et al.; 1999a; CURRIE, 2001; HUGHES et al., 2004, 2009; RODRIGUES et al., 2005a, 2005b, 2008, 2010, 2011; PAGNOCCA et al., 2008; CARLOS et al., 2011; GUEDES et al., 2012; RIBEIRO et al., 2012). No que diz respeito à riqueza, 64 táxons (correspondentes a 22 gêneros) de leveduras foram encontrados e, dentre eles, importantes representantes oportunistas como *Candida guilliermondii*, *C. parapsilosis*, *C. utilis*, *Cryptococcus albidus*, *Cryptococcus laurentii*, *Rhodotorula glutinis* e *R. mucilaginosa* (de HOOG et al., 2009).

Três novas espécies de leveduras: *Cryptococcus haglerorum* (MIDDELHOVEN et al., 2003), *Sympodiomyces attinorum* (CARREIRO et al., 2004), renomeada para *Blastobotrys attinorum* e *Trichosporon chiarellii* (PAGNOCCA et al., 2010), até o momento encontradas apenas em associação com formigas Attini, foram descritas durante projetos desenvolvidos no Laboratório de Microbiologia, Centro de Estudos de Insetos Sociais, UNESP (Rio Claro, SP).

Com relação aos fungos filamentosos, 179 táxons foram relatados, pertencentes a 85 gêneros, com destaque para os gêneros *Cladophialophora*, encontrado no exoesqueleto de fêmeas aladas de *A. laevigata* (PAGNOCCA et al., 2008), *Exophiala* e *Scolecobasidium*, isolados de jardins de *A. texana* (RODRIGUES et al., 2011). Os táxons citados acima foram observados pela primeira vez em formigas-cortadeiras e identificados apenas a nivel de gênero por análises moleculares, sugerindo a existência de espécies ainda não descritas.

Fungos melanizados das ordens Chaetothyriales (p.e. *Cladophialophora* e *Exophiala*) e Capnodiales (p.e. *Cladosporium*) também foram observados por Mayer e Voglmayr (2009) em galerias construídas por formigas *Azteca brevis* (Formicidae, Dolichoderinae).

As observações desses fungos negros associados a formigas têm sido extremamente relevante, pois são micro-organismos não comumente isolados do meio ambiente e, para

muitas espécies, seu nicho na natureza ainda é desconhecido (VICENTE et al., 2008; SAUNTE et al., 2012).

Considerando que, até então, apenas um trabalho foi realizado para avaliar a biodiversidade fúngica melanizada associada às formigas-cortadeiras (GUEDES et al., 2012), esta dissertação visou ampliar o conhecimento sobre as interações entre fungos negros e tais insetos. Aspectos como a presença desses fungos no ambiente das formigas, a possibilidade de sua dispersão e um maior conhecimento da biodiversidade microbiana associada são apresentados no Capítulo **1**.

3 Componentes cuticulares em formigas-cortadeiras

Consideradas um dos grupos mais bem-sucedidos, as formigas apresentam grande diversidade de espécies, amplo domínio ecológico e numerosas interações com diversos organismos, atraindo a atenção de pesquisadores de diversas áreas. Tal sucesso deve-se principalmente pela sua complexa organização social sustentada em grande parte por um eficiente sistema de comunicação (HÖLLDOBLER, 1995).

As formigas, assim como outros insetos sociais, possuem dois grupos de substâncias utilizadas como mecanismo de comunicação: os feromônios, constituídos por moléculas pequenas e altamente voláteis (CALIMAN, 2008) utilizados para a dispersão rápida de sinais químicos e os hidrocarbonetos cuticulares, que constituem compostos não voláteis (HOWARD; BLOMQUIST, 2005).

Os feromônios são produzidos e armazenados em várias glândulas dispersas pelo corpo das formigas e podem ser encontrados por todo o exoesqueleto (VIANA-BAILEZ et al., 2011). Participam das interações rainha-operárias e operária-operária, mediando conflitos sociais e regulando as atribuições de tarefas dentro da colônia. Também são utilizados na construção de trilhas e recrutamento, marcação de território e comportamento de alarme (VIANA-BAILEZ et al., 2011).

Além disso, a cutícula das formigas contem hidrocarbonetos, inseridos em uma camada de lipídeos, que oferecem uma barreira de proteção contra a desidratação (GIBS,

1998) e também atuam como sinais de comunicação (VANDER MEER; MOREL, 1998) em mecanismos de reconhecimento intra e interespecíficos (BLOMQUIST et al., 1998).

O perfil de hidrocarbonetos em uma colônia pode variar com o tempo ou mesmo ao longo das estações do ano e, através do contato entre as antenas, os indivíduos são capazes de reconhecer o perfil de hidrocarbonetos um do outro (WAGNER et al., 2000; GREENE; GORDON, 2007).

Apesar de sua importância, poucos estudos são encontrados detalhando a composição cuticular de hidrocarbonetos. Soroker e Hefetz (2000) relataram a presença de n-alcanos, monometilalcanos e dimetilalcanos na cutícula de formigas *Cataglyphis niger*, típicas de ambientes secos e quentes como desertos. Para formigas *Solenopsis saevissima*, os principais hidrocarbonetos cuticulares encontrados foram: um pentacoseno, três alcanos (tricosano, tetracosano e pentacosano) e dois metil-alcanos (3-metil-tricosano e 3-metil-pentacosano) (FOX et al., 2007).

Em formigas da tribo Attini, as colônias possuem um número elevado de castas com diferentes morfologias e a composição da cutícula é típica de cada uma delas (WAGNER et al., 2000), variando também entre as espécies. No gênero *Atta*, existem relatos de marcação química de território a partir de alcanos e alcenos de cadeias lineares variando de 13 a 23 carbonos, (z)-9-tricoseno, nnonadecadieno e (z)-9-nonadeceno (EVERSHED; MORGAN, 1981). Adicionalmente, os principais componentes do feromônio de reconhecimento são hidrocarbonetos, sendo até então identificados 33 em *Acromyrmex subterraneus subterraneus* (RICHARD et al., 2004).

A presença de hidrocarbonetos voláteis e não-voláteis na cutícula de formigas-cortadeiras pode permitir o desenvolvimento de alguns fungos que utilizam esses compostos metabolicamente, como os gêneros *Cladophialophora* e *Cladosporium* (WEBER et al., 1995; BADALI et al., 2011), anteriormente relatados em associação com o exoesqueleto de fêmeas aladas e soldados de formigas-cortadeiras do gênero *Atta* (PAGNOCCA et al., 2008; GUEDES et al., 2012).

4 Os fungos como biodegradadores

Estudos envolvendo o levantamento da biodiversidade microbiana são de grande relevância por permitirem um melhor entendimento da dinâmica de populações e das funções que desempenham no ambiente, podendo ainda levar à identificação de novas espécies com capacidade de degradação de compostos xenobióticos, as quais podem ser objeto de grande interesse para a aplicação biotecnológica na recuperação de ambientes contaminados (OLIVEIRA, 2001).

Sohnger e Kaserer realizaram os primeiros estudos da utilização de hidrocarbonetos por micro-organismos em 1906. Dois anos mais tarde, Störmer isolou uma bactéria, por ele denominada *Bacillus hexcarbovorum*, capaz de utilizar tolueno, xilol e metano como fontes de carbono (BUSHNELL; HAAS, 1941).

Os fungos desempenham importante papel na degradação dos hidrocarbonetos no solo, pois utilizam esses compostos como substrato para crescimento, sendo, por isso, considerados adequados à processos de biorremediação (HARMS et al., 2011).

Em busca de formas de recuperação de áreas contaminadas com compostos poluentes e/ou tóxicos como os hidrocarbonetos aromáticos, agrotóxicos e outras substancias, surgiu a biorremediação, que compreende um conjunto de técnicas utilizadas para degradar, reduzir ou eliminar a periculosidade de vários contaminantes valendo-se para isso da atividade biológica de micro-organismos (VIDALI, 2001).

A utilização de fungos em processos de biorremediação tem aumentado devido à sua maior capacidade de adaptação a condições inóspitas (GOPINATH et al., 2005), como maior tolerância a estresse osmótico e variação de temperatura, capacidade de viver em meios com pH ácido e desenvolvimento em ambientes com altas concentrações de gás carbônico. Adicionalmente, não apresentam restrição quanto ao tamanho da cadeia do hidrocarboneto a ser degradado (ROSATO, 1997; TORTORA et al., 2003).

O recorrente isolamento de fungos negros da família Herpotrichiellaceae (ordem Chaetothyriales) em ambientes ricos em hidrocarbonetos voláteis, como tolueno (PRENAFETA-BOLDÚ et al., 2006), e não voláteis, como resíduos de petróleo (SATOW et

al., 2008) e madeira tratada com creosoto (SUDHADHAM et al., 2008), sugere que essas substâncias desempenham um papel importante na ecologia e habilidade competitiva desses fungos (ZHAO et al., 2010).

Prenafeta-Boldú e colaboradores (2005) em uma revisão bibliográfica sobre sistemática, ecologia e metabolismo de fungos assimiladores de hidrocarbonetos observaram que a maioria dos isolados são fungos negros da ordem Chaetothyriales. Dentre os trabalhos revisados, Woertz et al. (2001) utilizaram *Exophiala lecanii-corni*, uma espécie de levedura negra, em biofiltro para remoção de tolueno em um biorreator de fase gasosa. Prenafeta-Boldú et al. (2001) isolaram estirpes dos gêneros *Cladophialophora* e *Exophiala* capazes de crescer utilizando tolueno como única fonte de carbono. Adicionalmente, Prenafeta-Boldú et al. (2004) observaram que a cepa de *Cladophialophora* sp. T1 foi capaz de degradar etilbenzeno e tolueno, bem como metabolizar xileno apresentando assim, potencial de aplicação na biorremediação de solos.

Por muito tempo a busca por um micro-organismo com potencial para degradação de hidrocarbonetos teve como passo inicial o isolamento a partir de ambientes ricos desses compostos, como refinarias de petróleo, tanques de combustíveis e solos destinados ao tratamento de hidrocarbonetos (de HOOG et al., 2006; SATOW, 2005). Nesses ambientes, novas espécies de fungos negros foram descritas, como *Exophiala xenobiotica*, frequentemente encontrada em ambientes ricos em hidrocarbonetos aromáticos e alcanos (de HOOG et al., 2006), *Cladophialophora psammophila* (BADALI et al., 2011), isolada de solos contaminados com benzeno, tolueno, etilbenzeno e xileno (BTEX), e *Exophiala sideris* (SEYEDMOUSAVI et al. 2011), obtida de minas altamente poluídas com arsênio, metais pesados e hidrocarbonetos aromáticos.

Recentemente, o enfoque de muitos trabalhos tem sido a busca por novas fontes de isolamento, tendo como princípio a procura por ambientes que ofereçam condições similares àquelas encontradas em ambientes contaminados, como a presença de hidrocarbonetos.

Dentre as novas fontes de isolamento, fungos com potencial para biorremediação têm sido obtidos de ambientes marinhos e apresentam como diferencial a produção de novos metabólitos secundários e enzimas não encontradas nos seus homólogos terrestres (JENSEN; FENICAL, 2000; RAGHUKUMAR, 2008).

Passarini et al. (2011) indicam a utilização dos fungos *Aspergillus sclerotiorum* CBMAI 849 e *Mucor racemosus* CBMAI 847, obtidos de ambientes marinhos, em processos de biorremediação de ambientes poluídos com PAHs (Hidrocarbonetos Policíclicos Aromáticos), devido a habilidade dessas estirpes de transformar e detoxificar pireno e benzo[a]pireno a metabólitos mais solúveis em água e menos tóxicos.

No que diz respeito aos fungos negros, Zhao et al. (2010) relataram a presença de espécies de *Exophiala* em frutos silvestres. Com o avanço de técnicas analíticas, tem sido demonstrado que hidrocarbonetos aromáticos voláteis que tradicionalmente estão associados com ambientes poluídos são, na verdade, onipresentes na natureza, embora em concentrações muito baixas. O tolueno, por exemplo, é produzido biologicamente em diferentes ambientes naturais, incluindo frutos maduros (HORVAT; SENTER, 1984).

A partir da constatação da presença de hidrocarbonetos voláteis (feromônios) e não-voláteis na cutícula das formigas-cortadeiras e os relatos de biodegradação desses compostos por fungos negros, um dos objetivos do trabalho foi explorar a micobiota associada ao exoesqueleto desses insetos, com ênfase nos fungos negros da família Herpotrichiellaceae. A pesquisa teve como ponto de partida a prévia publicação de dois gêneros de fungos negros presentes em formigas Attini, *Phialophora* (LITTLE; CURRIE, 2007) e *Cladophialophora* (PAGNOCCA et al., 2008), capazes de metabolizar hidrocarbonetos (PRENAFETA-BOLDÚ et al., 2004; PEIYING FENG et al., 2012).

5 Fungos negros: características e fatores de virulência

Os fungos negros, também denominados dematiáceos, formam um grupo polifilético de reconhecimento mundial. Esse grupo de micro-organismos abrange ascomicetos das ordens Chaetothyriales, comumente conhecidos como *black yeast-like*, e Dothideales, e alguns representantes basidiomicetos pertencentes ao gênero *Moniliella* (de HOOG et al., 2009).

Fungos negros patógenos e oportunistas humanos, bem como espécies sapróbias e degradadoras de hidrocarbonetos, pertencem à ordem Chaetothyriales (PRENAFETA-BOLDÚ et al., 2005; MARQUES et al., 2006), e apresentam restrito conhecimento ecológico. *Exophiala*, *Cladophialophora*, *Fonsecaea*, *Phialophora*, *Rhinocladiella* e *Cyphellophora* são gêneros representantes desse grupo. A ordem Dothideales é representada por fungos negros

fito-associados e sapróbios, em sua maioria filamentosos, como *Mycosphaerella*, *Hormonema* e *Hortaea* (STERFLINGER et al., 2001).

Os fungos pertencentes à ordem Chaetothyriales são caracterizados pela coloração escura das colônias e crescimento lento em meios de cultura tradicionais. Algumas espécies, como *Exophiala*, são dimórficas, com crescimento leveduriforme ou filamentoso dependendo do meio de cultivo e período de incubação. Adicionalmente, apresentam duplo nicho ecológico: por um lado a habilidade de desenvolvimento em ambientes ricos em hidrocarbonetos como benzeno, tolueno e xileno (PRENAFETA-BOLDÚ et al., 2006) e por outro lado sendo agentes etiológicos de infecções subcutâneas e sistêmicas em humanos e outros vertebrados (de HOOG et al., 2003; ZENG et al., 2007).

Dados recentes sugerem que as duas ecologias não estão geralmente combinadas nas mesmas estirpes, mas são encontradas em semelhantes (*siblings*) ecologicamente divergentes (BADALI et al., 2011). Assim, durante o período evolutivo, houve uma diferenciação entre as estirpes ambientais e clínicas, ocasionada, por exemplo, pela preferência por ambientes inadequados para a maioria dos micro-organismos competitivos (SUDHADHAM et al., 2011). Vicente et al. (2008) na tentativa de estabelecer uma comparação entre estirpes de *Fosecaea* de fontes naturais e clínicas concluiu que patógenos altamente específicos não podem ser isolados diretamente do ambiente e requerem a utilização hospedeiros mamíferos.

Espécies de fungos negros apresentam boa adaptação a uma variedade de ambientes estressantes ou extremos (GOSTINČAR et al., 2011), como aqueles criados artificialmente pelos homens, devido à proteção conferida pela parece celular melanizada. Neste ambiente, espécies de *Exophiala* têm sido isoladas frequentemente em banheiros e saunas na Ásia e Europa (SUDHADHAM et al., 2008), ralo de pia (MATOS et al., 2002; HAMADA; ABE, 2010) e lava-louças (ZALAR et al., 2011).

Apesar da importância médica e ambiental dos fungos negros, pouco se sabe a respeito de sua distribuição, biodiversidade e ocorrência natural (ZHAO et al., 2010). Até o momento, grande parte das pesquisas que isolaram fungos negros da natureza tinha como objetivo descobrir a fonte natural dos agentes causadores de doenças infecciosas, como a cromoblastomicose (SUDHADHAM et al., 2008; VICENTE et al., 2008; NAJAFZADEH et al., 2010).

A compreensão do habitat natural dos fungos negros, principalmente da ordem Chaetothyriales, tem avançado pela utilização de parâmetros seletivos em isolamentos, como a temperatura e meios de cultivo seletivos (SUDHADHAM et al., 2008; GUEDES et al., 2012), além de enriquecimento com alquil benzeno (ZHAO et al., 2010) e agitação em óleo mineral (SATOW et al., 2008; VICENTE et al., 2008). Esses métodos baseiam-se na premissa de que os fungos negros possuem natureza oligotrófica e baixa competitividade devido ao crescimento lento e baixa produção de esporos.

A técnica de flotação em óleo mineral, utilizada no presente trabalho, foi desenvolvida por Iwatsu et al (1981) e modificada por Satow et al (2008) e têm se mostrado muito eficiente no isolamento de fungos negros. A partir dessa metodologia, de Hoog et al. (2006) isolaram e descreveram uma nova espécie, *Exophiala xenobiotica*, proveniente de solos contaminados com compostos aromáticos voláteis. Além da utilização de solos como fonte de isolamento, Marques et al. (2006) demonstraram que o método também foi eficiente na recuperação de fungos negros de coco, folhas e material vegetal de babaçu em decomposição.

A utilização do óleo mineral, constituído por uma mistura de hidrocarbonetos, favorece a concentração das células de fungos negros, que são assimiladores de hidrocarbonetos aromáticos, atuando como um fator de enriquecimento para esses fungos (DIXON; SHADOMY, 1980). Zhao et al (2010) isolaram abundantemente este grupo de fungos de amostras ambientais não poluídas, como frutos silvestres, apenas após a adição de hidrocarbonetos voláteis aromáticos na metodologia de isolamento.

Os fungos negros, conhecidos como agentes de severas doenças, sistêmicas ou subcutâneas (de HOOG et al., 2000; MORIO et al., 2012) possuem certas peculiaridades conferidas a eles pela presença da DHN-melanina (derivada do precursor 1,8-dihidroxinaftaleno) na parede celular (LANGFELDER et al., 2003). Dentre essas propriedades podemos citar: 1. Tolerância à dissecação (STERFLINGER, 2006); 2. Capacidade de sobreviver sob baixa disponibilidade de nutrientes e água (SELBMANN et al., 2005; SATOW et al. 2008); 3. Proteção contra estresse e substâncias tóxicas (BUTLER; DAY, 1998); 4. Crescimento em pH ácido (de HOOG et al., 1994) e; 5. Proteção contra ação oxigênica de fagócitos do hospedeiro (LIU; NIZET, 2009). Como exemplo dessa alta resistência dos fungos negros à condições adversas, espécies de *Chaetomium* (fungo

ascomiceto dematiáceo) foram isoladas de gramíneas congeladas em geleiras por mais de 5.000 anos (HASELWANDTER; EBNER, 1994).

Além da melanização da parede celular, outros fatores têm sido considerados importantes na determinação da virulência em fungos negros e eles serão abordados a seguir.

5.1 Produção de exopolissacarídeos

Biofilmes, por definição, são comunidades microbianas multicelulares que, irreversivelmente, aderem-se a um substrato ou interface, e estão incorporadas em uma matriz de substâncias poliméricas extracelulares (exopolissacarídeos) produzidas pelos próprios micro-organismos constituintes do biofilme (DONLAN; COSTERTON, 2002). Individualmente, as células apresentam uma matriz extracelular em seu entorno, denominada glicocálice. Quando este material apresenta-se bem estruturado e fortemente ligado à parede celular é denominado cápsula.

Assim, os biofilmes microbianos são responsáveis pela fixação eficaz dos micro-organismos às superfícies, colonização dos tecidos do hospedeiro, expressão ou melhoramento de características de virulência, captura eficiente de nutrientes, comunicação célula-a-célula melhorada, dispersão e sobrevivência em condições adversas (HARDING et al., 2009). Adicionalmente, oferecem proteção a respostas do sistema imunológico de hospedeiros, agentes antimicrobianos (VAN BAARLEN et al., 2007; SZOMOLAY et al., 2005) e também estão envolvidos na patogênese (D'ENFERT, 2009).

No que diz respeito aos fungos negros, a produção de polissacarídeos extracelulares (EPS) por ascomicetos da ordem Dothideales tem recebido maior atenção, principalmente o gênero *Aureobasidium* (YURLOVA; de HOOG, 2002). Alguns fungos negros pertencentes a ordem Chaetothyriales também produzem cápsula protetora de exopolissacarídeos (SELBMANN et al., 2005) e estão frequentemente relacionados a infecções em humanos e outros vertebrados.

No gênero *Exophiala*, o material capsular já foi observado ao redor de células jovens de *Exophiala spinifera* e *E. jeanselmei* (MACKINNON et al., 1973). Adicionalmente, Nishimura e Miyaji (1983) relataram mucopolissacarídeos em células leveduriformes de

Exophiala dermatitidis. Segundo Yurlova e de Hoog (2002) os exopolissacarídeos produzidos por *Exophiala spinifera* e *E. dermatitidis* apresentam-se tanto na forma de cápsulas bem delimitadas ou como exopolissacarídeos difusamente exudados. Os polissacarídeos ainda não foram observados em outras espécies de *Exophiala*.

É notável que os exopolissacarídeos estão especificamente presentes nas espécies mais virulentas do gênero *Exophiala* (UIJTHOF et al., 1998) e, portanto, supõe-se que possuam um papel na patogenicidade para os seres humanos.

5.2 Termotolerância

A habilidade de adaptação de um fungo à temperatura do corpo humano é um dos fatores de virulência mais conhecidos entre os fungos patógenos (CASADEVALL et al., 2003). Micro-organismos patógenos que normalmente são encontrados no ambiente podem se adaptar para se desenvolver dentro de hospedeiros mamíferos, como o caso do fungo dimórfico *Sporothrix schenkii* (NEMECEK et al., 2006).

Na ordem Chaetothyriales, um dos principais fatores de virulência é a habilidade de crescer a 37-40°C, presente em espécies oportunistas de *Exophiala* e *Cladophialophora* (BADALI et al., 2008; de HOOG et al., 2011).

Espécies de *Exophiala* mostram crescimentos máximos diferentes a temperaturas mais ou menos coincidentes com a importância médica (ZENG et al., 2007). Assim, espécies encontradas no ambiente, como aquelas associadas a hidrocarbonetos, têm crescimento máximo em temperaturas por volta de 30-33°C. Por outro lado, espécies patógenas humanas, isoladas de casos clínicos, são capazes de crescer a elevadas temperaturas variando de 36 a 42°C (SEYEDMOUSAVI et al., 2011).

Nas últimas décadas, grande parte das estirpes de *Exophiala* isoladas apresentou baixa termotolerância e, no entanto, estavam envolvidas em infecções animais (de HOOG et al., 2011). Fungos com temperaturas máximas de crescimento abaixo de 33°C encontram-se causando doenças em animais de águas frias, que habitam o fundo do mar ou oceanos polares. Como exemplos podemos citar a levedura negra *Exophiala psychrophila* encontrada no salmão do Atlântico e *E. salmonis* encontrada em truta. Esta é uma característica notável, pois

em outras espécies do Reino dos fungos, a baixa termotolerância está presente em sapróbios sem habilidade de ocasionar infecções (de HOOG et al., 2011).

5.3 Produção de lacases

As lacases (benzenodiol: oxidorredutase oxigênio; EC 1.10.3.2) são enzimas que catalisam, por oxidação, uma grande variedade de substratos orgânicos e inorgânicos incluindo mono-, di- e polifenóis, aminofenóis, metoxifenóis e aminas aromáticas com concomitante redução de oxigênio para água (MAYER; STAPLES, 2002).

Lacases fúngicas tem sido demonstradas em diferentes grupos, principalmente em integrantes do filo Basidiomycota, conhecidos como fungos da podridão branca (KUNAMNENI et al., 2008). Apesar da produção já ter sido registrada também em certas bactérias (MARTINS et al., 2002) e actinomicetos (BERROCAL et al., 1997) existe pouca informação da produção de lacases por fungos negros.

A maioria das fungos negros, como espécies de *Exophiala*, produzem a dihidroxinaftaleno-melanina (DHN-melanina) via síntese de policetídeos e/ou fenoloxidases, como as lacases (BUTLER; DAY, 1998). No caso da eumelanina, sintetizada por alguns fungos basidiomicetos, evidências químicas diretas tem demonstrado que os polímeros da melanina são formados pela oxidação da catecolamina através da lacase (WILLIAMSON et al., 1998).

Tetsch et al (2005, 2006) já demonstraram a existência de lacases funcionais, provavelmente envolvidas na síntese da melanina, em *Hortaea acidophila* (Dothideales). Recentemente, foram publicados relatos da presença de lacases em espécies de *Fonsecaea* (SUN et al., 2012), *Cyphellophora* e *Phialophora* (PEIYING FENG et al., 2012), gêneros pertencentes a ordem Chaetothyriales.

A lacase tem sido descrita como um fator de virulência importante em muitos fungos. Essa enzima pode proteger o fungo de fitoalexinas e taninos (BRASIER; KIRK, 2001; SINGH, 2006), conferir resistência a esporos (WILLIAMSON et al., 1998) e pode ainda reduzir a atividade de lignificação do hospedeiro (VAN ETTEN et al., 1994). Adicionalmente, a atividade da lacase tem se mostrado um marcador de estresse, visto que a

indução dessa enzima está correlacionada com a inanição de substrato e presença de metais potencialmente tóxicos (ZHU; WILLIAMSON, 2004).

Adicionalmente, as lacases são de particular interesse em aplicações biotecnológicas e de biorremediação devido a sua capacidade de oxidar uma ampla variedade de substratos ambientalmente prejudiciais (VISWANATH et al., 2008), compostos fenólicos clorados (BOLLAG et al., 2003), corantes sintéticos (WESENBERG et al., 2003), pesticidas (TORRES et al., 2003) e hidrocarbonetos aromáticos policíclicos (POZDNYAKOVA et al., 2004).

Micro-organismos produtores de lacases têm sido triados tanto em meios sólidos contendo compostos indicadores colorimétricos que permitem a detecção visual da produção de lacase quanto em cultivos líquidos monitorados com medições da atividade enzimática (TETSCH et al., 2005). Como a lacase oxida vários tipos de substratos, diferentes compostos têm sido utilizados como indicadores; dentre eles os principais são os reagentes fenólicos sintéticos guaiacol e seringaldazina ou mediadores sintéticos não-fenólicos como o ABTS (2,2´- azinobis[3-etilbenzotiazolina-6-ácido sulfônico]) (BONUGLI-SANTOS et al., 2010).

A natureza extremofílica de fungos negros, em combinação com sua capacidade de metabolizar poluentes aromáticos a partir da produção de enzimas degradativas como a lacase, torna esses fungos candidatos ideais para processos de biorremediação. Considerando a potencial patogenicidade dos fungos negros, técnicas de recombinação genética podem permitir sua utilização como fonte de DNA para recombinação com outros micro-organismos que poderão ser utilizados na degradação de poluentes ambientais sem que haja risco à saúde e meio ambiente.

5.4 Diversidade molecular: classificação em genótipos

A identificação dos fungos negros apenas por critérios morfológicos é dificultada devido variações na expressão de características morfológicas durante ciclos de vida complexos e polimórficos ou pouca diferenciação de estruturas conidiais. Assim, métodos de biologia molecular são recomendados para uma classificação taxonômica confiável (ATTILI-ANGELIS et al., 1998; de HOOG, 1999; STERFLINGER; PRILLINGER, 2001; VICENTE, 2000).

Com o desenvolvimento de metodologias moleculares, Uijthof et al. (1994) notou uma diferença significante entre dois tipos principais de região ITS em *Exophiala dermatitidis*, mais tarde referidas como genótipos A e B por Matos et al. (2003). A classificação é baseada nas diferenças (deleções e substituições) entre as sequencias de ITS do rDNA quando comparadas.

Assim, o genótipo A, apresenta uma taxa maior de mutações e uma alta frequência em estirpes clínicas. Até o momento, todas as estirpes obtidas de casos fatais de infecções cerebrais foram classificadas como genótipo A, o que sugere maior virulência neste grupo (SUDHADHAM et al., 2009).

Por outro lado, o genótipo B compreende as estirpes obtidas do ambiente e menos virulentas (MATOS et al., 2003). Sudhadham et al. (2008) relataram a presença deste genótipo em *E. dermatitidis* isoladas de abacaxi e manga na Tailândia. Em ambientes artificiais, como banheiros e saunas, ambos os genótipos podem ser encontrados.

Até o momento, o genoma de *Exophiala dermatitidis* não foi sequenciado, e, portanto, pouca informação está disponível sobre os fatores de virulência. O Instituto BROAD (Cambridge, Inglaterra) possui um projeto em andamento para o sequenciamento do genoma de 15 espécies de fungos negros de importância clínica, incluindo *E. dermatitidis*. O sequenciamento poderá revelar características indicativas de versatilidade metabólica, estratégias de persistência no ambiente bem como os genes codificadores dos fatores de virulência (http://www.broadinstitute.org/).

Considerando a potencial patogenicidade e possibilidade de dispersão dos fungos negros no ambiente urbano pelas formigas-cortadeiras, um dos objetivos do presente estudo, apresentado no Capítulo **2**, foi avaliar supostos fatores de virulência para esses fungos, considerados aqui como sendo produção de lacases, tolerância à temperatura do corpo humano e a presença de mutações no DNA. Espécies de *Exophiala* foram escolhidas para os ensaios devido a alta frequência observada nos ninhos de formigas-cortadeiras amostrados e facilidade no preparo do inóculo, quando comparada a outros gêneros isolados.

6 Referências bibliográficas

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CAPÍTULO 1

Leaf-cutting ants: an environmental niche accommodating human opportunistic black fungi.

Abstract: Fungus-growing ants of the genus *Atta* are known for their leaf-cutting habit, a lifestyle they have maintained since their 50-million-year-old co-evolution with the symbiotic fungi (Agaricales: Lepiotaceae), which they cultivate as food. Recent studies have highlighted that, in addition to the symbiotic fungi, nests of ants harbor a great diversity of microbial communities. Such microorganisms include the dematiaceous fungi, which are characterized by their melanized cell walls. In order to contribute to the knowledge of fungal ecology, as well as opportunistic strains that may be dispersed by these social insects, we isolated and identified fungi carried by gynes of *A. capiguara* and *A. laevigata*, collected from colonies located in Fazenda Santana, Botucatu (São Paulo, Brazil). The isolation was carried out using the oil flotation technique, selective to black fungi. Inoculated plates were incubated at 25 and 35°C until black cultures were visible (20 - 45 days). Isolates were identified based on microscopic and molecular characteristics. Some isolated genera were: *Cladophialophora*, *Cladosporium*, *Cochliobolus*, *Exophiala*, *Ochroconis*, *Phaeococcomyces*, *Phialophora*, *Penidiella* and *Pyrenochaeta*. Hyaline species were also found. According to the literature, the genus *Penidiella* may cause *Eucalyptus* diseases. *Exophiala*, *Cladophialophora*, *Phialophora* and *Ochroconis* consist of human pathogenic genera, causing invasive subcutaneous, pulmonary and cerebral infections. The results obtained from this work highlighted that leaf-cutting gynes may contribute to the dispersal of pathogenic dematiaceous fungi. Therefore, more attention should be paid to this largely unknown subject.

Key words: Herpotrichiellaceous, microbial diversity, ant nests, oil flotation.

INTRODUCTION

Attini tribe ants (Hymenoptera: Formicidae) are involved in an ancient and compulsory mutualism with a basidiomycete fungus (family Lepiotaceae, genera *Leucoagaricus*) cultivated as food (MUELLER; RABELING, 2008; SCHULTZ; BRADY, 2008). Although all Attini ants are known as fungus-growing ants, the two most phylogenetically derivative genera (CURRIE, 2001), *Acromyrmex* and *Atta*, are commonly known as leaf-cutting ants, because they forage leaves and fresh flowers used as substrate for the growth of the symbiotic fungus (WEBER, 1972).

In this symbiotic relationship, the mutualistic fungus hydrolyzes the freshly harvested plant material incorporated by ants, thus obtaining energy for their own growth and providing nutrients for the colony (WEBER, 1972; BASS; CHERRETT, 1995; MUELLER et al., 2001; SILVA et al., 2006; HÖLLDOBLER; WILSON, 2008). In turn, ants provide a favorable environment for fungus growth, such as regulated temperature between 25 and 30°C, high humidity, slightly acid pH (QUINLAN; CHERRETT, 1978; POWELL; STRADLING, 1986; RIBEIRO; NAVAS, 2007) and establishment of mechanical and chemical barriers in order to protect their nests from harmful microbes (CURRIE; STUART, 2001; POULSEN; BOOMSMA, 2005; RODRIGUES et al., 2008a; CAFARO et al., 2011).

The mutualistic fungus is propagated to a new colony during the mating flight when winged females (known as gynes) carry a small portion of the fungus in the infrabuccal cavity (pellet), where it is protected from contamination until a new nest is found (MUELLER, 2002). In the state of São Paulo, the mating flight occurs at the beginning of the rainy season, usually in October, following significant rainfall (enough to soak the soil surface) (AUTUORI, 1941; MOSER, 1967).

Historically, fungus gardens were described as pure monocultures, in which the ants eliminated opportunistic microorganisms (MÖLLER, 1893). This vision remained for a long time, but reports of alien microorganisms in fungus gardens, waste deposits and ants' exoskeleton were described (PAGNOCCA et al., 2008, 2011, 2012). At the end of the 20th century, Currie et al. (1999a) established that fungus gardens also harbored a specialized parasitic fungus of the genus *Escovopsis* (Ascomycota: Hypocreales). Found only in association with ant colonies (CURRIE et al., 1999a, 2001), this parasitic fungus directly

exploits the cultivation of ants, secreting compounds that degrade the mutualistic fungal hyphae and absorbing the nutrients released (REYNOLDS; CURRIE, 2004).

Coincident with the establishment of *Escovopsis* sp. as a parasite of the ant-cultivated fungus interaction, an additional symbiont was discovered by Currie et al. (1999a). Located in the ants' cuticle, the filamentous bacteria of the genus *Pseudonocardia* produce secondary metabolites with antimicrobial properties, used by ants to suppress the growth of *Escovopsis* sp. (CURRIE et al., 1999b; CALDERA et al., 2009).

Due to advances in microbiological and molecular techniques, another microorganism was identified as part of this complex ant-fungus relation. Belonging to the Herpotrichiellaceae family (Ascomycota, *Phialophora*-related) this black yeast grows in the same region of the ants' cuticle where *Pseudonocardia* sp. could be found (LITTLE; CURRIE, 2007), competing directly with these bacteria for nutrients and indirectly reducing the ability to prevent the growth of *Escovopsis* sp. (LITTLE; CURRIE, 2008). Given the significant changes in the dynamics of microorganisms due to the presence of the black yeast, this finding illustrates the importance of further studies on symbiont communities (CALDERA et al., 2009).

Other recent works have described the presence of the black fungi *Cladophialophora*, associated with the exoskeleton of *A. laevigata* gynes (PAGNOCCA et al, 2008), *Exophiala* and *Scolecobasidium*, isolated from *A. texana* gardens (RODRIGUES et al, 2011). The taxa mentioned above were first observed in ants and identified only by genera level using molecular analyses, suggesting the existence of species not yet described. Melanized fungi of the orders Chaetothyriales (e.g. *Cladophialophora* e *Exophiala*) and Capnodiales (e.g. *Cladosporium*) were also observed by Mayer and Voglmayr (2009) in tunnels built by *Azteca brevis* ants (Formicidae).

The ecological ants-black fungi interaction needs further investigation due to some peculiarities of this group of microorganisms, such as pathogenic potential, involvement in a broad spectrum of human infections, and the ability to degrade hydrocarbons (de HOOG et al. 2000, 2006). Furthermore, the presence of black fungi associated with ants has been extremely relevant. Although the establishment of new isolation techniques show that these fungi are much more common in the environment than we thought (VOGLMAYR et al.,

2011), the niche in nature is still unknown for many species (VICENTE et al., 2008; SAUNTE et al., 2012).

Black fungi, known as agents of serious systemic or subcutaneous diseases (de HOOG et al., 2000; MORIO et al., 2012), can often infect healthy individuals (AL-TAWFIQ; AMR, 2009). Certain peculiarities are conferred to them by the presence of DHN-melanin in the cell wall (LANGFELDER et al. 2003), such as tolerance to temperatures above 37°C (de HOOG; HAASE, 1993), the ability to survive under low nutrient and water availability (STERFLINGER, 1998; SATOW et al. 2008), growth in acid pH (de HOOG et al., 1994) and protection against oxygenic action of phagocytes in hosts (LIU; NIZET, 2009).

Under common laboratory culture conditions, melanized fungi are seldom encountered due to their slow growth, oligotrophic nature and limited competitive abilities. Numerous protocols for the selective isolation of black yeasts have been developed to allow the recovery of black fungi both in nature and in artificial environments (PRENAFETA-BOLDÚ et al., 2006; SUDHADHAM et al., 2008; ZHAO et al., 2010). The oil flotation technique was previously applied to recover dematiaceous fungi in environmental samples, such as plant materials and soil form termite mounds (VICENTE, 2000; MARQUES et al., 2006), and it proved to be very efficient.

Hence, the purpose of this study was to extend the knowledge on the interaction among black fungi and leaf-cutting gynes of *A. capiguara* and *A. laevigata*. Aspects such as the presence of these fungi in ants' environment, the possibility of dispersion by these insects and a better understanding of microbial biodiversity associated were discussed. Therefore, we applied the selective isolation technique named "oil flotation," combined with identification methods based on phenotypic and molecular features.

MATERIAL AND METHODS

Nest location and gyne sampling

Collections were performed during the mating flight, right at the moment gynes and drones appeared at the nest entrance, moments before they started the flight, in three adult

nests of *A. capiguara* (Gonçalves) and one of *A. laevigata* (Smith), located at Fazenda Santana, São Paulo State, Brazil (GPS location: 22°50.6‟ S; 48°26.1‟ W; elevation798 m).

Two collections were performed in the course of this study: (i) in October 2008 – a total of 70 gynes (30, 10, and 30 of *A. capiguara* nests #1, # 2 and #3, respectively) were taken; and (ii) in October 2009 – 50 gynes were collected of each *A. capiguara* nests #1 and #2, and 50 of *A. laevigata*. In 2009 alates were not observed in nest #3 of *A. capiguara* on collection day. All gynes were picked up with sterile forceps, put into individual sterile plastic plates and transported to the laboratory within the next four hours.

Fungal isolation

In order to isolate black fungi associated with gynes, we used a culture-dependent selective method, the oil flotation technique (SATOW et al., 2008). Initially, the pellet containing the symbiotic fungus inoculum was aseptically removed from the infrabuccal pocket by immobilizing the gynes with pins using a magnifying glass. For the isolation, both pellet and bodies were used as a source and immersed separately in 1 mL and 25 mL of saline solution (NaCl 0.85%, 200 U penicillin, 200 μg/mL of chloramphenicol, 200 μg/mL of streptomycin and 500 μg/mL of cycloheximide), respectively. Tubes were shaken and incubated at room temperature for 30 min. Then, 0.5 mL and 5 mL of sterile mineral oil were added, respectively, followed by vigorous agitation for 5 minutes on vortex. After resting for 20 min, 50μL aliquots were removed from the oil-solution interface containing the pellets, deposited in the form of drops on the surface of Mycosel agar. 100 μL aliquots were taken off the interface of the solution containing bodies and plated on Mycosel agar using a Drigalsky loop. The plates were incubated at 35°C and monitored daily for up to 45 days until black colonies appeared. After removing the aliquots, the tubes were also incubated at 25°C for 45 days.

Fungal identification

Morphology

The yeasts and filamentous fungi isolated from gynes of leaf-cutting ants were maintained on MEA (2% Malt Extract Agar) slants at 4ºC (CROUS et al., 2009a) and cryopreserved at -80°C in 15% glycerol. Due to some difficulties in reactivate the

cryopreserved black fungi, melanized strains were additionally preserved with Castellani's method (CAPRILER et al., 1989).

Preliminary identification was carried out based on macro- and microscopic features of the colonies after slide culturing on MEA or PDA (Potato Dextrose Agar) at room temperature (CROUS; SAMSON, 2010). Slides were prepared with lactophenol cotton blue and observations were done using a Leica® DM 1000 microscope. For cultures with insufficient phenotypic markers for identification, sequencing of ribossomal DNA (rDNA) was performed, using the techniques described below.

DNA extraction

Strains were transferred to fresh MEA plates and incubated at 25°C for 3-7 d. About 1cm^2 of mycelium was transferred to a 2-mL Eppendorf tube containing 490 μL of CTAB (cetyltrimethylammonium bromide) buffer [(Tris-HCl 100 mM, EDTA 20 mM, NaCl 1.4 M, CTAB 2% (w/v), b-mercaptoethanol 0.2% (v/v), pH 8.0)] and 6–10 acid washed glass beads (Sigma G9143). 10 μL of proteinase K (50mg/mL) was added and vortexed for 10 min. The mixture was then incubated at 60°C for 2 hours. Subsequently 500 μL of chloroform:isoamyl alcohol (24:1) was added to the solution, shortly mixed by shaking it for 2 min and centrifuged for 10 min at 14000 r.p.m. The supernatant was transferred to a new tube with a 0.55 volume of cold isopropanol, carefully mixed by flipping, kept at -20°C overnight and spun for 10 min at 14.000 r.p.m. The pellet was washed with ice-cold 70% ethanol. After drying at room temperature, it was re-suspended in 50 μL of TE buffer (Tris 0,12% w/v, Na-EDTA 0.04% w/v). DNA quality was verified by NanoDrop®ND- 1000 Spectrophotometer using ND-1000 v. 3.3.0 software (Coleman Technologies, Wilmington, DE, U.S.A.). DNA extracts were stored at −20 °C prior to use.

Sequencing

In order to identify the isolated fungal strains, a fragment of rDNA including the internal transcribed spacer (ITS) region was amplified using primers V9G and LS266 (GERRITS VAN DEN ENDE; de HOOG, 1999), and sequenced using primers ITS1 and ITS4 (WHITE et al., 1990). PCR reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) in 25 μL volumes containing 10 ng of template DNA, 2.5 μL of reaction buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M KCl, 15 mM MgCl2, 0.1 % gelatin, 1 % Triton X-100), 1 mM dNTP, 1 μL of each primer (10 ρmol/μL) and 0.5 U Taq

DNA polymerase (ITK Diagnostics, Leiden, The Netherlands). Amplifications for ITS were performed as follows: 94°C for 5 min, followed by 35 cycles consisting of 94°C for 45s, 52°C for 30s and 72°C for 1 min, and post elongation step at 72°C for 7 min. Amplicons were purified using GFX PCR DNA and a gel band purification kit (GE Healthcare, Buckinghamshire, UK). BigDye terminator cycle sequencing kits were used in sequence reactions (Applied Biosystems, Foster City, CA, USA). Cycle sequencing was performed as follows: 95°C for 1 min, followed by 30 cycles of 95°C for 10s, 50°C for 5s and 60°C for 2 min. Reactions were purified with Sephadex G-50 fine (GE Healthcare, Uppsala, Sweden) and sequencing was done on an ABI 3730XL automatic sequencer (Applied Biosystems).

Sequence data obtained in this study were adjusted using the BioEdit Sequence Alignment Editor v. 7.0.5.3 (HALL, 1999) and compared with homologous sequences deposited in the database of Centraalbureau voor Schimmelcultures (CBS) - Fungal Biodiversity Centre (Utrecht, The Netherlands) (http://www.cbs.knaw.nl/databases) and the National Center for Biotechnology Information (NCBI) - GenBank (http://www.ncbi.nlm.nih.gov/genbank; BENSON et al., 2011) using BLASTn (ALTSCHUL et al., 1997). CBS sequences were chosen as the closest sequences at GenBank.

Fungal sequences that shared similarities higher or equal to 99% with the homologous sequences from the databases were considered as conspecific. For sequences below this similarity percentage, the fungi were identified only to the genus level.

Statistics

In order to evaluate differences in species diversity carried by gynes of different nests and collection times, Chao1 species richness estimators and diversity indices (Fisher's alpha, Shannon, and Simpson) were calculated. For the rarefaction curve, we used Mao Tau, 95% Lower Bound and 95% Upper Bound confidence intervals (CI) to create a confidence limit (CL). For the species estimator, we used Chao1 Mean, Chao1 95% Lower Bound and Chao1 95% Upper Bound confidence intervals (CI). Furthermore, individual-based rarefaction curves were computed to compare species richness; Sorensen and Jaccard's shared species estimator were calculated to compare the communities of each sampled nest. Indices were calculated using EstimateS v.8.2.0 software (COLWELL, 2006) and the graphs were created in OriginPro 8.5 software (OriginLab, OriginPro 8.5, USA).

RESULTS

Prevalence and diversity of fungal isolates from gynes' exoskeleton and pellet

Of the 220 gynes used in this study, 84 gynes (38%) showed fungi in the exoskeleton (Fig. 1). The highest frequency of gynes with fungi occurred in *A. capiguara* #1 nest (2009 collection), with the presence of fungi in 31 gynes (62%); thus contradicting the 2008 collection in the same nest, which showed the presence of fungi in only 2 gynes (6.7%). We observed that the occurrence of fungi on the gynes' exoskeleton from *A. laevigata*, *A. capiguara* #1 and *A. capiguara* #2 nests was similar in samples collected in 2009. The number of positive gynes in 2008 was significantly lower ($p < 0.1$, nonparametric Kruskal–Wallis and Dunn tests) than those collected in 2009, proving that the presence of these micro-organisms in gynes varied considerably between the two years sampled.

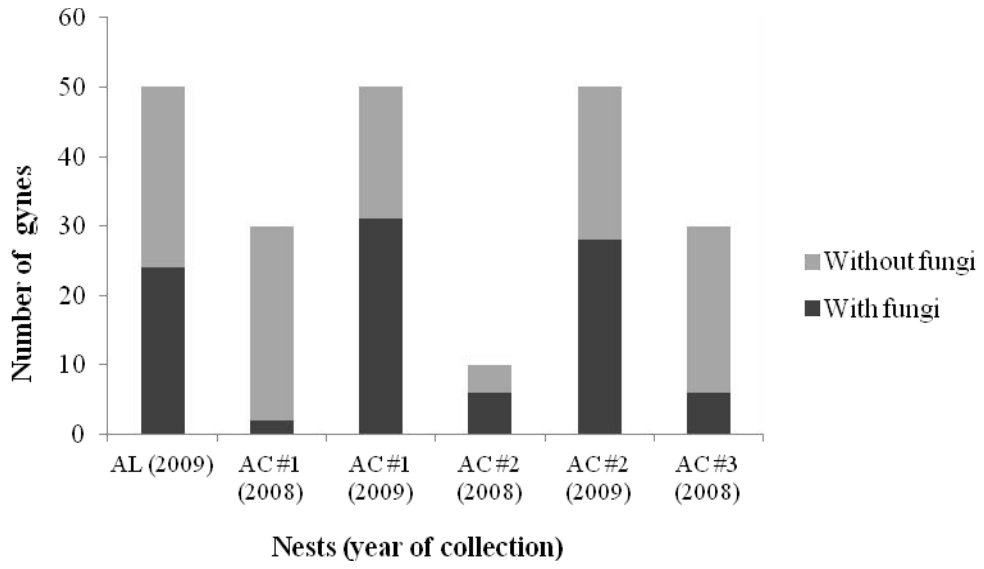

Fig. 1 – Proportion of gynes with fungal occurrence. AL: *A. laevigata*; AC: *A. capiguara*; #1, 2, 3: nests identification.

A total of 440 isolation attempts were made, in which 120 fungal isolates were recovered from pellets and gynes' exoskeleton of all ant nests (*A. capiguara* #1, 2 and 3; and *A. laevigata*) studied in two years (2008-2009). In general, the number of black fungi recovered was relatively high, 82 of which are melanized, i.e., approximately 68% (Table 1). Considering the 2008 material, a total of 19 fungi were recovered from 140 isolation attempts (12.9% success) and from the 2009 material, a total of 101 strains were recovered from 300 isolation attempts (33.7% success).

Table 1. Number of gynes, isolation trials and isolates in nests of leaf-cutting ants.

Year	Parameter	Ant Nest								Total
		A laevigata		*A. capiguara* #1		*A. capiguara* #2		*A. capiguara* #3		
2008	Gynes collected	†		30		10		30		70
		P	B	P	B	P	B	P	B	
	Isolation trials	-	-	30	30	10	10	30	30	140
	Black isolates	-	-	1	1	2	5	0	5	14
	Hyaline isolates	-	-	0	1	0	3	1	0	5
2009	Gynes collected	50		50		50		†		150
		P	B	P	B	P	B	P	B	
	Isolation trials	50	50	50	50	50	50	-	-	300
	Black isolates	8	7	1	27	1	24	-	-	68
	Hyaline isolates	4	13	0	9	0	7	-	-	33

Filamentous fungi and yeasts isolated by: P = fungal pellet were dissected from gynes.; B = gynes were immersed in saline-oil solution.
1, 2, 3: nests identification.
† unsampled nests (see methods for details).
-: no data.

Regarding the two incubation temperatures used in the isolation methodology, 25 and 35°C, we noted that only 15.3% of the isolates were obtained from 35°C, including 14 melanized strains.

The pellets exhibited a low recovery --18 strains--, mostly isolated from *A. laevigata* gynes (66.7%) (Table 2). Of the isolates, 13 strains are melanized and comprise 7 species (highlighted in Table 2). Five hyaline fungi were also found and 1 unidentified ascomycete.

Table 2. Fungal species isolated from pellet of leaf-cutting gynes prior to their mating flight. Molecular identification was based on BLASTn results of rDNA ITS1/ITS4 regions of isolates regarding GenBank homologous sequences.

Isolate ID		Nest	Year	GenBank Best Hit (Similarity, Accession #)
dH 22888	176	*A. capiguara* #1	2008	*Pyrenochaeta* sp. (93%, EU750693.1)
dH 23007	156	*A. capiguara* #3	2008	*Aspergillus versicolor* (99%, GQ229082.1)
dH 22941	160	*A. capiguara* #2	2008	*Cochliobolus lunatus* (100%, HQ607991.1)
dH 22884	154	*A. capiguara* #2	2008	*Cladosporium perangustum* (100%, HM148147.1)
dH 22887	311	*A. capiguara* #2	2009	Chaetothyriales sp. (93%, FJ538964.1)
dH 22963	423	*A. laevigata*	2009	*Cladophialophora* sp. strain 1 (93%, EU035403.1)
dH 23001	299	*A. laevigata*	2009	*Cladophialophora* sp. strain 1 (94%, EU035403.1)
dH 22883	347	*A. capiguara* #1	2009	*Cladosporium cladosporioides* (100%, HM148049.1)
dH 23072	425	*A. laevigata*	2009	*Exophiala dermatitidis* (100%, AY663828.1)
dH 22927	426	*A. laevigata*	2009	*Exophiala dermatitidis* (99%, EU910264.1)
dH 23048	427	*A. laevigata*	2009	*Exophiala dermatitidis* (99%, FJ387565.1)
dH 23029	323	*A. laevigata*	2009	*Exophiala dermatitidis* (100%, AB498925.1)
dH 22919	429	*A. laevigata*	2009	*Exophiala dermatitidis* (99%, DQ826738.1)
dH 22920	431	*A. laevigata*	2009	*Penicillium citrinum* (100%, JF266706.1)
dH 23030	325	*A. laevigata*	2009	*Penicilium citrinum* (100%, HM469428.1)
dH 23075	406	*A. laevigata*	2009	*Penicillium citrinum* (nd)
dH 23076	334	*A. laevigata*	2009	*Penicillium citrinum* (nd)
dH 23010	310	*A. laevigata*	2009	n.i. ascomycetous (87%, GU934604.1)

nd: no molecular data; fungi were identified only by phenotypic similarity.
n.i.: not identified.
#1, 2, 3: nests identification.
Melanized fungi.

The 102 isolates recovered from the exoskeleton, comprise 12 genera, 23 species and 2 unidentified ascomycetes (Table 3). Overall, gynes of *A. capiguara* #1 nest (2009 collection) allowed the isolation of the highest abundance of strains (36 strains) and higher species richness, 17 species (74% of all species found). Remarkably, gynes sampled in the same nest in 2008 resulted in the isolation of the lowest abundance (2 strains) and the lowest species richness (2 species, 8.7%) of all nests.

Table 3. Fungal species isolated from gynes' exoskeleton prior to the mating flight in 2008 and 2009.

Fungal species	**Number of isolates**						GenBank*
	A. laevigata 2009 (n=50)	*A. capiguara* #1 2008 (n=30)	*A. capiguara* #1 2009 (n=50)	*A. capiguara* #2 2008 (n=10)	*A. capiguara* #2 2009 (n=50)	*A. capiguara* #3 2008 (n=30)	
Aspergillus fumigatus			2		1		100%, HQ026746.1
Aspergillus sclerotiorum	1						100%, FR733826.1
Chaetothyriales sp.			2		2		93%, FJ538964.1
Cladophialophora sp. strain 2	1		6		4		92%, EU137293.1
Cladophialophora sp. strain 3			1				90%, EU035403.1
Cladosporium perangustum	1					1	100%, HM148147.1
Cladosporium cladosporioides	2	1	3	1	6	2	100%, GQ458030.1
Cladosporium phaenocomae			1				100%, JF499837.1
Exophiala dermatitidis	1		1		1	1	100%, AB498925.1
Exophiala spinifera			1			1	100%, AY156975.1
Exophiala sp.			1	1	2		95%, HQ631063.1
Metarhizium anisopliae	2						100%, FJ609307.1
Neosartorya aureola			1				100%, JN093268.1
Ochroconis sp. strain 1				1			88%, AB161066.4
Ochroconis sp. strain 2			1				87%, AB161064.3
Paecilomyces lilacinus	5		5	3	2		100%, AB103380.1
Penicillium citrinum	5		1		4		100%, JF266706.1
Penicillium ochrochloron		1					99%, GU565138.1
Phaeococcomyces sp.	1						91%, AJ507323.4
Phialophora sp. strain 1			2				88%, JF436949.1
Phialophora sp. strain 2	1		1	1			91%, HQ608107.1
Penidiella sp. strain 1			4		9		92%, EU167572.1
Penidiella sp. strain 2			2				92%, EU167583.1
n.i. ascomycetes			1		1		86%, HQ608155.1
Total	20	2	36	7	32	5	

n.i.: not identified. #1, 2, 3: nests identification. Number in parenthesis: number of gynes used in each sample.
* Similarity (%) and access number of the closest sequence found in GenBank.
Melanized fungi.

In order to perform statistical analyses, the pellet isolates were added to exoskeleton isolates of each nest. Besides, the results of 2008 collection were not considered due to the low number of strains obtained (*A. capiguara* #1, n=3; *A. capiguara* #2, n=9; *A. capiguara* #3, n=6). Figure 2 denotes the species richness of fungi in the 2009 nests sampled. Although the abundance of strains obtained in each nest was relatively similar (*A. laevigata*, n=32; *A. capiguara* #1, n=37; *A. capiguara* #2, n=32), *A. capiguara* #1 nest showed the highest species richness compared to other nests. Significant differences could not be found in species richness among nests sampled in 2009. However, the 2008 collection was significantly lower ($p < 0.1$, nonparametric Kruskal–Wallis and Dunn tests), demonstrating the need for sampling different nests at different times in order to study the diversity of microorganisms in these microhabitats.

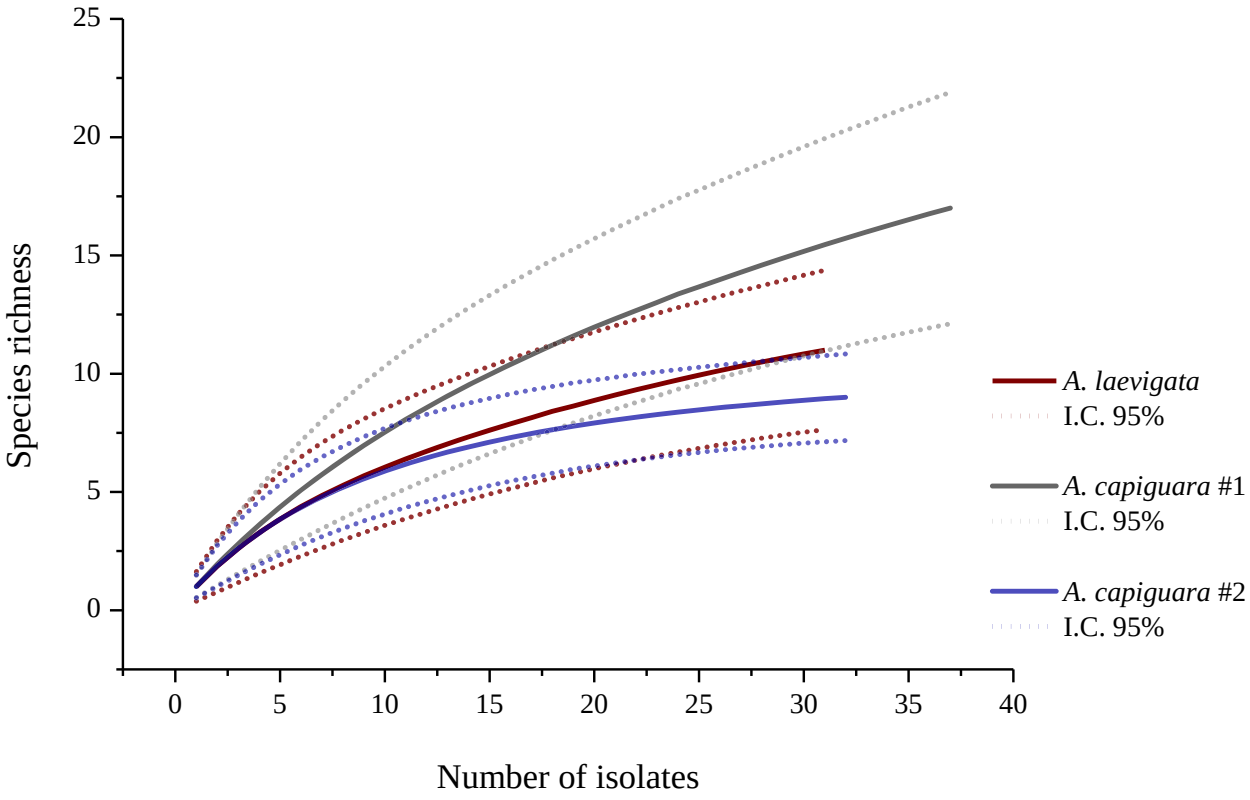

Fig. 2 – Rarefaction curve of fungal species richness from gynes of *A. capiguara* and *A. laevigata* nests, 2009 collection. I.C. = confidence intervals.

Chao 1 index, used to statistically estimate the number of species that can be found by changing the sampling effort, is illustrated in Figure 3. According to rarefaction analyses, the Chao 1 richness estimator predicted a total 9.74 ± 0.93 taxa (mean ± SD) in gynes of *A. capiguara* #2 (2009 collection), which indicates that sampling effort was enough to cover the

diversity, considering that 9 species (32 strains) were obtained from this nest. On the other hand, for *A. laevigata* (10 species, 32 strains) and *A. capiguara* #1 (17 species, 37 strains) nests, the species estimates were 18.63 and 30.13, respectively, which indicate that more sampling effort is necessary to cover the full diversity of fungal species associated with leaf-cutting gynes from these nests. Since the sampling effort was remarkably the same for all three nests in question (n=50 gynes of each nest), despite the wide difference between estimated species (n=37, Chao 1 estimator) and the ones actually found (n=17), *A. capiguara* #1 nest showed higher species richness compared to other nests.

Overall, Fisher's alpha, Shannon, and Simpson diversity indices had similar values (Table 4). All three indices confirmed that *A. capiguara* #1 gynes harbored a more diverse community.

Table 4. Diversity indices for fungi isolates from gynes of 2009 collection.

Sampled nest	**Simpson**	**Shannon**	**Fisher's alpha**
A. capiguara #2	7.29	1.98	4.16
A. laevigata	7.27	2.06	6.09
A. capiguara #1	14.17	2.58	12.18

#1, 2: nests identification.

Additionally, according to Jaccard and Sorensen's similarity between gyne nests collected in 2009, *A. capiguara* #1 and #2 nests shared the highest number of fungal species (Jaccard: 0.556; Sorensen: 0.714). Since values closer to 1 indicates that more species are shared between samples, the major values achieved by the two *A. capiguara* species can be explained by the proximity of nests and shared plant material which enters the nest through the workers, contributing to the formation of a more similar mycobiota. In addition, *A. laevigata* gynes showed a composition of species more similar to *A. capiguara* #2 gynes sampled (Jaccard: 0.333; Sorensen: 0.5).

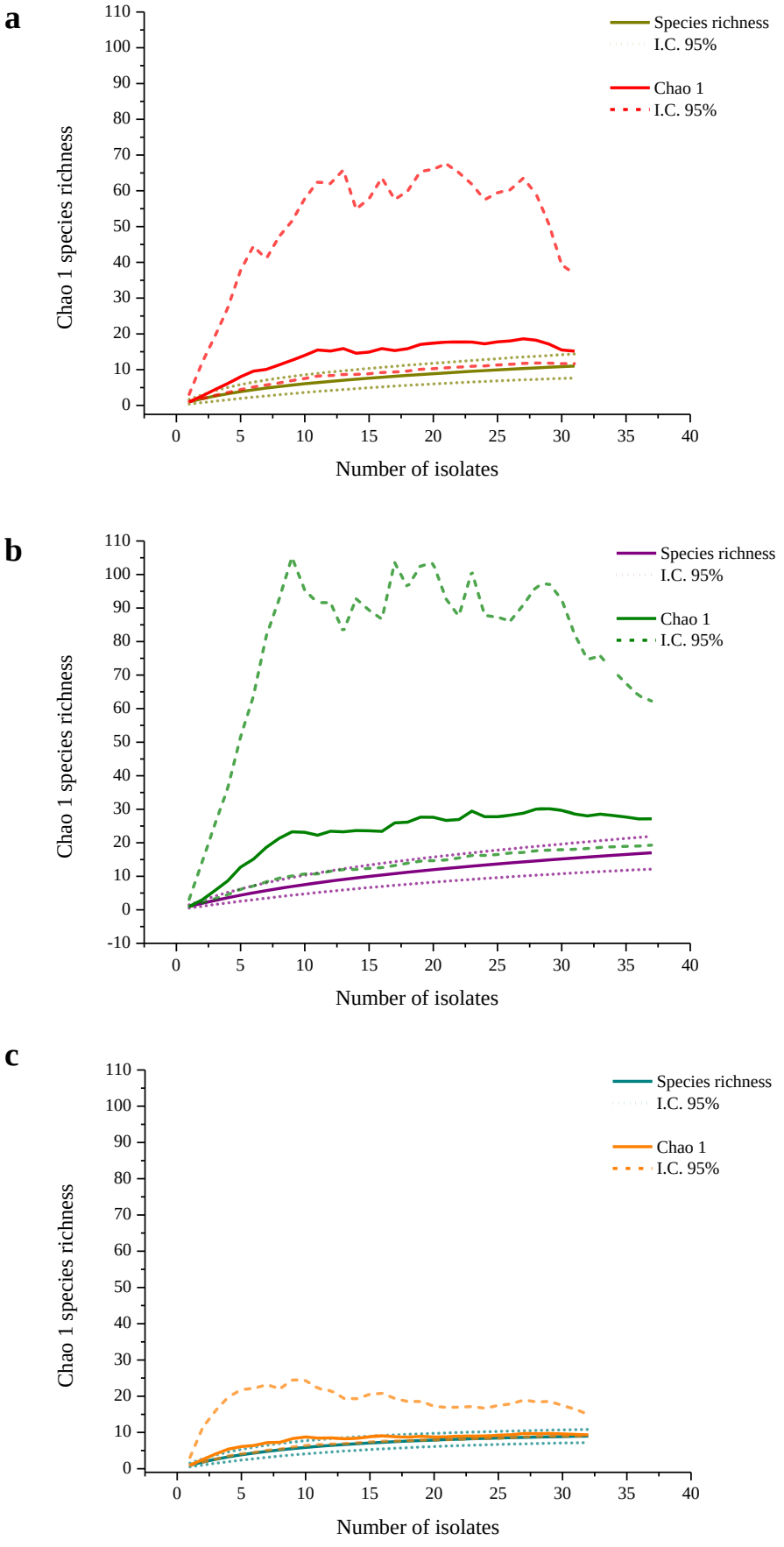

Fig. 3 – Individual-based rarefaction curves and Chao 1 richness estimators of fungal species in gynes of three leaf-cutting ant nests (2009 collecting): **a.** *A. laevigata*; **b.** *A. capiguara* #1; **c.** *A. capiguara* #2. I.C.= confidence intervals.

Fungal species carried by A. laevigata *and* A. capiguara *gynes*

Only 18 strains, corresponding to 9 species (7 melanized species) and 1 unidentified ascomycete (Table 2), were isolated from the infrabuccal pellets. Featuring a more diverse mycobiota, the isolation from the gynes' exoskeleton resulted in 102 strains pooled in 23 species and 2 unidentified ascomycetes (Table 3), among which the melanized genera *Cladosporium* (n=18) and *Cladophialophora* (n=12) were the most common. Although the focus of the study was the black fungi, all isolates were identified, and *Paecilomyces* (n=15) and *Penicillium* (n=11) were the most common hyaline filamentous fungi in the exoskeleton of gynes.

Regarding the melanized fungi, the following genera were found: *Cladophialophora* (n=14), *Cladosporium* (n=20), *Cochliobolus* (n=1), *Exophiala* (n=15), *Ochroconis* (n=2), *Phaeococcomyces* (n=1), *Phialophora* (n=5), *Penidiella* (n=15) and *Pyrenochaeta* (n=1). Table 5 provides information about the occurrence of the main melanized fungal genera found in this study (some already reported in leaf-cutting ants) and classifies them according to their pathogenic or phytopathogenic character.

Interestingly enough, *Cladosporium cladosporioides* (n=15) was found in the exoskeleton of gynes from all nests over two collection years, and one occurrence in *A. laevigata* pellet. Some species showed lower specificity about the isolation source, such as *Exophiala spinifera* isolated from *A. capiguara* #3 (2008 collection) and *A. capiguara* #1 (2009 collection) gynes.

From the biannual (2008, 2009) collection in *A. capiguara* nests #1 and #2, we observed that the genera *Cladosporium* and *Penicillium* were obtained in both sampled years for nest #1; and *Exophiala*, *Cladosporium* and *Paecilomyces* for nest #2, suggesting that these fungi can remain in latent form in the colonies. Interestingly, the specialized parasite *Escovopsis* sp. was not isolated from any of the nests sampled in this study over the 2-year collection.

Table 5. Main taxa obtained from gynes' exoskeleton and fungal pellet, report in other studies based on the fungal isolation from leaf-cutting ants and host classification.

Taxa (Biosafety Level)	Report in leaf-cutting ants	Host (Reference)
Cladophialophora sp. (BSL-2)	PAGNOCCA et al., 2008	Human (Frank et al., 2011)
Cladosporium sp. (BSL-1)	PAGNOCCA et al., 2008; GUEDES et al., 2012	Human (de Hoog et al., 2000); Plants (Huang et al., 2012)
Cochliobolus lunatus (BSL-1)	RODRIGUES et al., 2011	Human (Carter; Boudreaux, 2004); Plants (Cui; Sun, 2012)
Exophiala dermatitidis (BLS-2)	–	Human (Michel-Nguyen et al., 2009)
Exophiala spinifera (BLS-2)	–	Human (Badali et al., 2012)
Exophiala sp. (BSL-1/2)	RODRIGUES et al., 2011	Human (de Hoog et al., 2000)
Ochroconis sp. (BSL-1/2)	–	Human (Qureshi et al., 2012)
Penidiella sp. (BSL-1)	–	Plants (Crous; Groenewald, 2011)
Phialophora sp. (BSL-1/2)	–	Human (Kimura et al., 2003)
Pyrenochaeta sp. (BLS-2)	–	Human (Ferrer et al., 2009)

-: no data.

DISCUSSION

Given the paucity of information about melanized fungi from natural sources and the recent observation of occurrence of these microorganisms associated with leaf-cutting ants, waste deposits and gardens (LITTLE; CURRIE, 2007, 2008; PAGNOCCA et al., 2008; DEFOSSEZ et al., 2009; de HOOG et al., 2009), black fungi were the main focus of this study.

Developed by Iwatsu et al. (1981) and modified by Satow et al. (2008), the isolation methodology using mineral oil to recover melanized fungi was applied in this study and, despite being selective for black fungi, it also allowed the recovery of non-melanized fungi also. As reported by Guedes et al. (2012), this methodology, coupled with agar Mycosel and incubation at 35°C, was effective for the selective isolation of black fungi and to avoid high-sporulation fungi.

Pagnocca et al. (2008) performed the first study of yeasts and filamentous fungi carried by gynes of *A. capiguara* and *A. laevigata*, using the same nests of this study. The authors sampled 290 gynes and used three less selective isolation methods for melanized fungi: (1) gynes walked in culture medium; (2) gynes immersed in liquid medium; and (3) fungal pellet immersed in liquid medium. Despite the higher abundance of black fungi isolated by Pagnocca et al. (2008), the species richness found (n=4) was lower than the one in current study (n=18), showing that the specificity of the oil flotation technique used for black fungi allowed the isolation of species not so commonly found in nature, such as *Exophiala* and *Ochroconis* species.

Results showed that black fungi are abundantly present in the ants' exoskeleton. *A. capiguara* #1 gynes had proportionally more fungal species richness than other sampled nests. Although the sampling effort has resulted in the isolation of 27 species, from infrabuccal pellets and gynes' exoskeleton, according to Chao 1 species estimator, at least 13 other species not found in this study should be present in *A. capiguara* #1 nest, for instance.

Furthermore, according to the Jaccard and Sorensen's similarity, *A. capiguara* #1 and #2 nests showed a more similar mycobiota composition. Although they are located in the same region (Fazenda Santana, Botucatu, SP), the difference in fungal species diversity

between *A. capiguara* and *A. laevigata* nests may be related to different microorganisms associated with different plants harvested by leaf-cutting ants. Thus, *A. capiguara* ants have a preference for monocotyledons, whereas *A. laevigata* ants collect mono- and dicotyledonous (JUSTI et al., 1996; VASCONCELOS; CHERRETT, 1996; FORTI; BOARETTO, 1997).

Some black fungi isolated from *A. capiguara* and *A. laevigata* gynes are being mentioned for the first time: *Exophiala dermatitidis*, *E. spinifera*, *Ochroconis* sp., *Penidiella* sp., *Phaeococcomyces* sp., *Phialophora* sp. and *Pyrenochaeta* sp. *Phialophora* related-species were also found in specialized cuticular structures of *Apterostigma* ants (LITTLE; CURRIE, 2007) and compete for nutrients with the symbiont bacteria symbiont *Pseudonocardia* sp., suppressing the bacterial growth and indirectly reducing the ability to avoid the growth of the parasite *Escovopsis* sp. (LITTLE; CURRIE, 2008). It is still unknown whether the antagonism between *Phialophora* sp. and *Pseudonocardia* sp. can also occur between other species of black yeasts, as those found in this study, and the symbiotic bacteria.

The maximum identity percentage of *Cladophialophora* sp. strain 1 (94%), *Cladophialophora* sp. strain 2 (92%), *Cladophialophora* sp. strain 3 (90%), *Pyrenochaeta* sp. (93%), *Exophiala* sp. (95%), *Ochroconis* sp. strain 1 (88%), *Ochroconis* sp. strain 2 (87%), *Phaeococcomyces* sp. (91%), *Phialophora* sp. strain 1 (88%), *Phialophora* sp. strain 2 (91%), *Penidiella* sp. strain 1 (92%) and *Penidiella* sp. strain 2 (92%) suggest that these strains are unknown. These putative new and undescribed species should be better investigated with the aim of confirming this finding.

Comprising some non-ubiquitous melanized fungi, such as *Cladophialophora* and *Ochroconis*, remains unclear how these fungi enter the nest and survive in ants' exoskeleton. The proximity of the nest to soil and intensive foraging behavior with great flow of workers facilitate the entrance of microorganisms into the nests (PAGNOCCA et al., 2011). Furthermore, Rodrigues et al. (2011) suggest that the type of plant substrate collected by the ants may influence the mycobiota present inside the nest. Since *A. laevigata* and *A. capiguara* species collect different plant materials, it was expected that nests of *A. capiguara* shared more species between themselves than with *A. laevigata*, which in fact occurred. Thus, we suggest that the leaves collected are an important factor in the composition of nest-associated microorganisms.

Also, hydrocarbons present in the ants' cuticle may serve as a trigger for black fungal colonization on this substrate, and several studies have demonstrated the affinity of Chaetothyriales fungi with these molecules (de HOOG et al., 2006; PRENAFETA-BOLDÚ et al., 2006). For ants, hydrocarbons are important for the communication signals (VANDER MEER; MOREL, 1998) and providing a barrier of protection against dehydration (GIBS, 1998). Prenafeta-Boldú et al. (2001) isolate strains of *Cladophialophora* and *Exophiala* genera able to grow using toluene, a volatile hydrocarbon, as the only carbon source. Therefore, we suggest that the hydrocarbons present on the cuticle of gynes may allow the development of dematiaceous fungi that use this compound as an energy source.

During the mating flight, reproductive forms can be vehicles for the dispersion of microorganisms present inside the colony (PAGNOCCA et al., 2008) and in the close environment (AUGUSTIN et al., 2011). Although microorganisms can be accidentally transported by gynes, they will probably compose the initial microbiota of the new colony. Indeed, some fungi isolated in our samples are mentioned as soil inhabitants, such as *Cladosporium*, *Penicillium* and *Paecilomyces*, indicating that the ants might come into contact with these fungi during foraging.

Pagnocca et al. (2008) found the genera *Cladosporium* (in 78% of cases), *Aureobasidium*, *Candida*, *Cryptococcus* and other yeasts in fungal pellets of *A. capiguara* and *A. laevigata* gynes. Rodrigues et al (2008b) noted that some taxa, such as *Cladosporium cladosporioides*, *Paecilomyces lilacinus* and *Penicillium* species, occurred in the fungus gardens in all seasons, suggesting that these fungi can remain in the colonies over long periods of time.

Despite the selectivity of the isolation technique for black fungi, hyaline fungi, such as *Aspergillus*, *Paecilomyces* and *Penicillium* genera, were also found. These highly sporulating fungi are common soil inhabitants (CROUS; SAMSON, 2010) and are probably carried into the nest during foraging. Interestingly, gynes harbor the entomopathogenic fungi *Metarhyzium anisopliae*, known for the use in biological control against insect pests.

Among the melanized genera isolated, *Cladophialophora*, *Exophiala*, *Ochroconis*, *Phialophora* and *Pyrenochaeta* are groups frequently reported in cases of human infection (de HOOG et al., 2000; QURESHI et al. 2012; SAUNTE et al., 2012). Some cases of infections

by *Exophiala* species refer to farm workers that were infected from fungal inoculation in skin lesions (NAJAFZADEH et al., 2011; HOFFMANN et al., 2011). Whether these opportunistic fungi may be transferred or not to the plant under attack, or to the environment close to the nest during foraging, is still an unanswered issue.

Although several authors reported that most fungi isolated from nature offer less virulence than their pathogen siblings, the association of these species with ants may be considered a problem to the society due to the ants' great capacity for adapting to the urban environment and possible dispersion of these microorganisms (BUENO; CAMPOS-FARINA, 1999).

Our data also demonstrated that in addition to nests harboring human pathogens fungi, they can also accommodate phytopathogenic species. In this respect, important fungal phytopathogens were found in the exoskeleton of gynes, such as *Penidiella* genera (CROUS et al., 2009b, 2009c; CROUS; GROENEWALD, 2011). *Penidiella* species were already reported in the literature as being responsible for the brown spots on leaves of *Eucalyptus globulus* and *Eucalyptus tenuiramis* (CROUS et al., 2009c). The presence of this fungus in leaf-cutting ants is mentioned for the first time in this study. In principle, a connection between phyto-associated fungi isolated from leaf-cutting ants and plant diseases was not the aim of this study, but the results encourage future investigations.

In conclusion, we have shown that gynes' exoskeleton and pellets provides a habitat for potential human pathogenic fungi. *Phialophora, Ochroconis, Exophiala* and *Cladophialophora,* first observed in association with leaf-cutting ants, are known to be opportunistic human pathogens and require further studies to better understand their role in the complex ant-symbiont fungus interaction. Although our results show open questions to be elucidated, the fungal biodiversity found in the ants' exoskeleton has not been fully explored and, according to the low similarity of some DNA sequences, new species might be described.

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CAPÍTULO 2

Molecular diversity and virulence factors in *Exophiala* species from environmental samples

Abstract: The opportunistic black yeast *Exophiala* species are known as etiologic agents of fatal and disseminated infections in humans. In the natural environment, these chaetothyrialean fungi are rarely found, and their presence in the ants' exoskeleton has recently been reported. In contrast, in the human-made environment, the species is a very frequent colonizer of bathing facilities and hydrocarbon-polluted environments. Many studies have recently been conducted to clarify whether environmental strains differ from known etiologic disease agents according to morphology, genetic and pathology. Thus, given the potential pathogenicity of chaetothyrialean fungi, some virulence factors have been studied, mainly for environmental strains. Among the strains tested in the present study, two *E. dermatitidis* deserve more attention. Isolated from the exoskeleton of leaf-cutting ants, dH 22927 and dH 23048 were classified as genotype A. Also, they exhibited high growth rates at 40°C and consistently produced laccase, characteristics found mostly in pathogenic fungi. In the present study, we found that the environmental strains have characteristics that make them potential opportunistic human pathogens, and the possible spread of these fungi by ants in urban areas may have clinically relevant consequences.

Key words: black yeasts, Chaetothyriales, genotype, lacase activity, thermotolerance.

INTRODUCTION

Dematiaceous, or black fungi, comprise a polyphyletic group recognized worldwide. This group of microorganisms includes ascomycetes of the orders Chaetothyriales, commonly known as black yeast, and Dothideales, and some basidiomycetes belonging to the genus *Moniliella* (de HOOG et al., 2009).

Black yeasts and their filamentous relatives belonging to the Chaetothyriales order are characterized by their heavily melanized cell walls and polymorphic life cycle, with yeast or filamentous growth, depending on the culture or environmental conditions. Furthermore, they exhibit a double ecological niche: on the one hand, for the ability to develop in extreme environments, such as those rich in the hydrocarbons benzene, toluene and xylene (PRENAFETA-BOLDÚ et al., 2006); on the other hand, for being etiologic agents of fatal and disseminated infections in humans and other vertebrates (de HOOG et al., 2003; ZENG et al., 2007).

One example of black yeasts with a complex life cycle and an unusual type of ecology is the genus *Exophiala* (Herpotrichiellaceae: Chaetothyriales). *Exophiala* species shows high adaptation to extreme environments, due to the protection provided by melanized wall cells, and have been frequently isolated from several types of human-made environments, such as bathing facilities (SUDHADHAM et al., 2008), sinks (MATOS et al., 2002; HAMADA; ABE, 2010), washing machines (ZALAR et al., 2011) and petroleum-contaminated railway ties in the tropics (SUDHADHAM et al., 2008).

Recurrent isolation of the *Exophiala* species from volatile hydrocarbon-rich environment, such as toluene (PRENAFETA-BOLDÚ et al., 2001), and non-volatile, such as petroleum residues (SATOW et al., 2008) and creosote-treated oak (SUDHADHAM et al., 2008), suggests that these substances play an important role in the ecology and competitive ability of these fungi (ZHAO et al., 2010).

With the establishment of new specific techniques for the isolation of black fungi, it has been found that the *Exophiala* species are more common in the natural environment than previously thought. Sudhadham et al. (2008) reported the presence of *Exophiala dermatitidis* in mango (*Mangifera indica*) and pineapple (*Ananas comosus*). Similarly, Zhao et al. (2010)

isolated an undescribed *Exophiala* species in *Sorbus aucuparia*, a wild berry. Furthermore, it has been shown that volatile aromatic hydrocarbons, traditionally associated with polluted environments, are, in fact, ubiquitous in nature, although at very low concentrations (ZHAO et al., 2010). For example, toluene is biologically produced in natural environments, such as mature fruits (HORVAT; SENTER, 1984).

Moreover, Chaetothyriales black fungi, such as *Phialophora*, *Cladophialophora* and *Exophiala* species, have been reported in the exoskeleton of Attini ants (LITTLE; CURRIE, 2007; PAGNOCCA et al., 2008; GUEDES et al., 2012). It is suggested that the presence of hydrocarbons in the cuticle of these insects can determine the associated mycobiota, selecting fungi capable of using these compounds metabolically (WEBER et al., 1995; BADALI et al., 2011).

Given the potential pathogenicity of the chaetothyrialean fungi, some virulence factors have been studied, mainly for environmental strains (SUDHADHAM et al., 2008; de HOOG et al., 2011; ZALAR et al., 2011). Among the items studied is the ability to produce melanin pigments and to tolerate extreme temperatures. The production of melanin was shown to be involved in the virulence trigger of black fungi, providing protection against the oxygenic action of phagocytes (LIU; NIZET, 2009) and sustained the growth of fungi at 37°C infection temperature (JACOBSON, 2000; LIU et al., 2004). In addition, the consistent ability to grow at temperatures above 37°C (PADHYE et al., 1978; SUDHADHAM et al., 2009) and the production of extracellular polysaccharide capsules (YURLOVA; de HOOG, 2002) are regarded as virulence factors.

In many organisms, laccase is associated with melanin production and has been described as a virulence factor in black fungi. This phenoloxidase enzyme participates in the melanin synthesis (BUTLER; DAY, 1998), provides protection for fungal pathogen against tannins and phytoalexins (BRASIER; KIRK, 2001) and confers resistance to spores (WILLIAMSON et al., 1998).

Additionally, for *Exophiala dermatitidis* isolates, molecular genotypes within the species have been described, which might indicate strains subject to divergent types of stress and selection over time. Due to the development of molecular methods, Uijthof et al. (1994) reported a significant difference between two main types of ITS (Internal Transcribed Spacer)

region, based on the comparison and differences (deletions and substitutions) of the ITS sequences, later referred to as genotype A and B by Matos et al. (2003). So far, all strains obtained from fatal cerebral infections have been classified as genotype A, suggesting higher virulence in this genotype.

Given the nature of the fungus as an opportunistic agent of potentially fatal infections in humans, the presence of *Exophiala* species in ant-associated and human-made environments poses a significant public health risk, since these insects can greatly adapt to the urban environment and carry fungal propagules, acting as reservoirs of microorganisms.

In this paper, we will analyze putative virulence factors for black yeast-like fungi isolated from environmental samples, considered in this study as thermotolerance, laccases activity and DNA genotype classification.

MATERIAL AND METHODS

Fungal strains and culture conditions

A total of 28 environmental *Exophiala* isolates were used in the study, which were obtained from winged leaf-cutting ants (gynes, n=11; and drones, n=2) and hydrocarbon contaminated garage soil samples (n=15), using the oil flotation technique (SATOW et al., 2008). Soil contaminated strains were collected by D. Attili-Angelis (Campinas, Brazil). Stock cultures were maintained on MEA (2% Malt Extract Agar) slants at 4°C (CROUS et al., 2009). All cultures in this study are maintained in the culture collection of the Microbial Resource Center (UNESP, Brazil).

DNA extraction

Strains were transferred to fresh MEA plates and incubated at 25°C for 3-7 d. Approximately 1 cm^2 of mycelium was transferred to a 2-mL Eppendorf tube containing 490 µL of CTAB (cetyltrimethylammonium bromide) buffer [(Tris-HCl 100 mM, EDTA 20 mM, NaCl 1.4 M, CTAB 2% (w/v), b-mercaptoethanol 0.2% (v/v), pH 8.0)] and 6–10 acid washed glass beads (Sigma G9143). 10 µL of proteinase K (50mg/mL) was added and vortexed for 10 min. The mixture was then incubated at 60°C for 2 hours. Subsequently 500 µL of

chloroform:isoamyl alcohol (24:1) was added to the solution and was shortly mixed by shaking it for 2 min and centrifuged for 10 min at 14000 r.p.m. The supernatant was transferred to a new tube with 0.55 vol of cold isopropanol, mixed carefully by flipping, kept at -20°C overnight and spun for 10 min at 14000 r.p.m. The pellet was washed with ice cold 70% ethanol and after drying at room temperature, it was re-suspended in 50 μL of TE buffer (Tris 0,12% w/v, Na-EDTA 0.04% w/v). DNA quality was verified by NanoDrop®ND- 1000 Spectrophotometer using ND-1000 v. 3.3.0 software (Coleman Technologies, Wilmington, DE, USA). DNA extracts were stored at −20°C prior to use.

Molecular characterization

For the identification of *Exophiala* strains, a fragment of rDNA, including internal transcribed spacer (ITS) region, was amplified using primers V9G and LS266 (GERRITS VAN DEN ENDE; de HOOG, 1999), and sequenced using primers ITS1 and ITS4 (WHITE et al., 1990). PCR reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) in 25 μL volumes containing 10 ng of template DNA, 2.5 μL of reaction buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M KCl, 15 mM MgCl2, 0.1 % gelatin, 1 % Triton X-100), 1 mM dNTP, 1 μL of each primer (10 ρmol/μL) and 0.5 U Taq DNA polymerase (ITK Diagnostics, Leiden, The Netherlands). Amplifications for ITS were performed as follows: 94°C for 5 min, followed by 35 cycles consisting of 94°C for 45s, 52°C for 30s and 72°C for 1 min; and the post elongation step, at 72°C for 7 min. Amplicons were purified using GFX PCR DNA and the gel band purification kit (GE Healthcare, Buckinghamshire, UK). BigDye terminator cycle sequencing kits were used in sequence reactions (Applied Biosystems, Foster City, CA, USA). Cycle sequencing was performed as follows: 95°C for 1 min, followed by 30 cycles of 95°C for 10s, 50°C for 5s and 60°C for 2 min. Reactions were purified with Sephadex G-50 fine (GE Healthcare, Uppsala, Sweden) and sequencing was done on an ABI 3730XL automatic sequencer (Applied Biosystems). Sequence data obtained in this study were adjusted using the BioEdit Sequence Alignment Editor v. 7.0.5.3 (HALL, 1999) and compared with homologous sequences deposited in the database of the National Center for Biotechnology Information (NCBI) - GenBank (http://www.ncbi.nlm.nih.gov/genbank; BENSON et al., 2011).

Sequences were automatically aligned using ClustalX (THOMPSON et al., 1997). Alignments were adjusted manually using Molecular Evolutionary Genetics Analysis

(MEGA) software version 5.0 (TAMURA et al., 2011). Genotypes of *Exophiala dermatitidis* were determined according to Matos et al. (2003).

Physiology

Exophiala strains were cultured in triplicate. Temperature tests were performed on MEA plates, onto which 1 mm fungal diameter was inoculated in the center. Plates were incubated in the dark for 3 weeks at the following temperatures: 25, 30, 33, 36, 37 and 40°C. In order to evaluate whether the high temperature was fungicidal or not, apparently inhibited cultures were set back to 25°C and incubated for an additional two weeks. Radial growth diameter was measured weekly and averages were determined.

Laccase assay activity was performed in triplicate with ABTS-agar medium (TETSCH et al., 2005) (glucose 20g/L, peptone 10g/L, agar 20g/L and 0.03% ABTS [2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt]). Each plate was inoculated with 1 mm diameter obtained from an actively growing mycelium of each fungal strain and incubated at 25°C in the dark for 10 d. *Trametes versicolor* CBMAI 871 served as positive control, and *Candida albicans* as negative control. A positive laccase reaction was indicated by a colored zone around the fungal colony. The width spread was considered to be directly related to the amount of extracellular laccase produced.

Statistics

Metabolite and colony diameters were statistically analyzed by the nonparametric test of Mann-Whitney (NOETHER, 1983), using BioEstat software (AYRES et al., 2007). Mean diameters of each colony are the result of triplicate experiments. Furthermore, $p < 0.1$ was considered to indicate a significant difference.

RESULTS AND DISCUSSION

Genotyping

Nuclear rDNA ITS sequencing of 24 *Exophiala dermatitidis* strains confirmed the existence of two major genotypes differing in three positions in ITS1 (Table 1), which had

uneven prevalence in the compared habitats. Genotype **A** was more common than **B** for hydrocarbon contaminated strains (**A:B** = 11:4) (Table 2), whereas for leaf-cutting ants strains the genotype **B** was prevalent (**A:B** = 2:7) (Table 3). Additionally, we observed that genotype **A** has variations (A2 and A3) due to a nucleotide substitution on positions 63 and 125 of rDNA ITS1.

Table 1. Intraspecific variability in rDNA ITS 1 of *Exophiala dermatitidis*.

	ITS 1		
Nucleotides positions	162	184	196
Genotype **A**	T	-	A
Genotype **B**	C	T	C

Abbreviations used: ITS = Internal Transcribed Spacer; - = incompatible positions.

In human-made habitats (e.g., steam baths, dishwasher), both genotypes have been found (MACHOUART et al., 2011), being in accordance with our data obtained for hydrocarbon contaminated soil. Besides, we found a higher frequency of genotype A in this niche, which seems to be promoted in this habitat due to extreme conditions, such as poor nutrients and poisonous environment. Sudhadham et al. (2008) suggests that artificial human-made environments have selected some strains, providing opportunities for evolutionary adaptations. Thus, the genotypes A and B seem to be specialized in different niches.

It is remarkable that two strains from leaf-cutting ants belong to ITS-genotype A, dH 22927 and dH 23048. This is the same genotype as the one found in all cerebral infection cases (HIRUMA et al., 1993). In natural environments (feces of tropical, fruit-eating warm-blooded animals) genotype B seems to be more common, comprising less virulent isolates, while A is practically absent (MATOS et al. 2003; SUDHADHAM et al. 2010). Zeng et al. (2007) noted that *Exophiala* strains from different sources or continents had the same or very similar genotype, suggesting that environmental strains could exhibit potential for opportunism.

Table 2. Characteristics of *E. dermatitidis* from hydrocarbon contamined soil, growth at different temperatures, laccase activity and genotyping.

Isolate		Species	Source	GenBank Best Hit acession, similarity	Genotype	Laccase	Halo diam (mm)*	Ratio of halo diam/ colony diam (mm)	Temperature tests (mm)★	
									37 graus	40 graus
dH 23012	D17	*E. dermatitidis*	Hydrocarbon contamined soil	AB498927.1, 100%	A	+	17	1.8	17.8	11.3
dH 22983	D20	*E. dermatitidis*	Hydrocarbon contamined soil	AF050269.1, 99%	A	+	12	1.3	19	13.3
dH 23003	D22	*E. dermatitidis*	Hydrocarbon contamined soil	JN391328.1, 100%	A3	+	12	1.3	21	5.0
dH 22978	D26	*E. dermatitidis*	Hydrocarbon contamined soil	GQ911541.1, 100%	B	+	12	1.2	30.5	18
dH 22985	D33	*E. dermatitidis*	Hydrocarbon contamined soil	AB498927.1, 100%	A	+	12	1.2	21	10.5
dH 23056	D36	*E. dermatitidis*	Hydrocarbon contamined soil	FJ974060.1, 99%	A3	+	11	1.2	34.8	22.8
dH 23059	D37	*E. dermatitidis*	Hydrocarbon contamined soil	DQ826738.1, 99%	A3	+	12	1.1	30	16.8
dH 22984	D38	*E. dermatitidis*	Hydrocarbon contamined soil	GU942305.1, 100%	B	+	12	1.1	27	4.3
dH 22986	D42	*E. dermatitidis*	Hydrocarbon contamined soil	AB498927.1, 100%	A	+	11	1.3	26	13
dH 22977	D43	*E. dermatitidis*	Hydrocarbon contamined soil	JX669015.1, 100%	B	+	11	1.1	31	20.3
dH 22995	D46	*E. dermatitidis*	Hydrocarbon contamined soil	AY213651.1, 99%	A3	+	12	1.1	31.3	9.3
dH 22993	D52	*E. dermatitidis*	Hydrocarbon contamined soil	FJ387565.1, 99%	A	+	17	1.5	20.5	12.8
dH 22975	D57	*E. dermatitidis*	Hydrocarbon contamined soil	AB498925.1, 100%	B	-	0	0	30.8	10.5
dH 22974	D61	*E. dermatitidis*	Hydrocarbon contamined soil	AF050269.1, 99%	A3	+	12	1.1	31.3	19.5
dH 22989	D64	*E. dermatitidis*	Hydrocarbon contamined soil	JN391328.1, 100%	A3	+	13	1.1	13.8	6.3

+ laccase production; - no production; * Halo diam, colored zone around the colony with positive laccase reaction on ABTS-agar after 10 d.

★ temperature tests measure was performed after 21 d.

Table 3. Characteristics of *E. dermatitidis* and *E. spinifera* from leaf-cutting ants, growth at different temperatures, laccase activity and genotyping.

Isolate		Species	Source	GenBank Best Hit acession, similarity	Genotype	Laccase	Halo diam (mm)*	Ratio of halo diam/ colony diam (mm)	Temperature tests (mm)★	
									37 graus	40 graus
dH 22899	AP147	*E. dermatitidis*	Gyne cuticle (*A. capiguara* #3)	AB498925.1, 100%	B	+	12	1.2	23.3	10
dH 22997	AP318	*E. dermatitidis*	Gyne cuticle (*A. capiguara* #1)	JN675372.1, 100%	B	+	12	1.1	21	10
dH 23029	AP323	*E. dermatitidis*	Gyne pellet (*A. laevigata* #1)	AB498925.1, 100%	B	+	11	1.2	23.3	12
dH 23065	AP402	*E. dermatitidis*	Gyne cuticle (*A. capiguara* #2)	AB498925.1, 100%	B	+	12	1.2	32.3	9.8
dH 22929	AP424	*E. dermatitidis*	Gyne cuticle (*A. laevigata* #1)	DQ826738.1, 99%	B	+	13	1.3	23.3	4.5
dH 23072	AP425	*E. dermatitidis*	Gyne pellet (*A. laevigata* #1)	AY663828.1, 100%	B	+	11	1.1	24.8	10.5
dH 22927	AP426	*E. dermatitidis*	Gyne pellet (*A. laevigata* #1)	EU910264.1, 99%	A3	+	11	1.1	29	18.5
dH 23048	AP427	*E. dermatitidis*	Gyne pellet (*A. laevigata* #1)	FJ387565.1, 99%	A3	+	12	1.2	31.8	20.8
dH 22919	AP429	*E. dermatitidis*	Gyne pellet (*A. laevigata* #1)	DQ826738.1, 99%	B	+	14	1.5	23.5	14.8
dH 23008	AP128	*E. spinifera*	Gyne cuticle (*A. capiguara* #3)	AY484985.1, 98%		+	13	1.5	2.0	0
dH 23057	AP359	*E. spinifera*	Gyne cuticle (*A. capiguara* #1)	AY156975.1, 100%		+	15	1.6	4.5	0
dH 22925	AP449	*E. spinifera*	Drone cuticle (*A. laevigata* #2)	AY484985.1, 97%		+	18	1.9	7.8	0
dH 22900	AP472	*E. spinifera*	Drone cuticle (*A. laevigata* #2)	AY156975.1, 100%		+	16	2.0	10.8	0

+ laccase production; - no production; * Halo diam, colored zone around the colony with positive laccase reaction on ABTS-agar after 10 d.

★ temperature tests measure was performed after 21 d.

#1, 2 and 3 indicate the nests identification. Pellet is the fungal portion that gynes carry out in the infra-bucal cavity during the matting flight.

Physiology

The cardinal growth temperature test showed that all cultures used in this study had their optimal development at 30-33°C, indicating that the isolates are mesophylls. *Exophiala dermatitidis* strains were consistently able to grow at 37°C and 40°C, whereas for *E. spinifera* strains the maximum growth temperature on MEA was 37°C, although at a low rate (Appendix). After 2 weeks, two *E. spinifera* strains (dH 22925 and dH 22900) incubated at 40°C started to regrow when transferred to 25°C, indicating the high temperature was fungistatic for these strains rather than fungicidal. The remaining *E. spinifera* strains (dH 23008 and dH 23057) remained without growth.

Remarkably, five of seven *E. dermatitidis* strains with the highest growth at 40°C (dH 23056, dH 23059 and dH 22974, from soil contaminated; dH 22927 and dH 23048, from ants' cuticle) were classified as genotype A, and considered as the most virulent group (Table 3). There was no significant difference ($p > 0.1$) between the two isolation sources as regards the temperatures tested for *E. dermatitidis*.

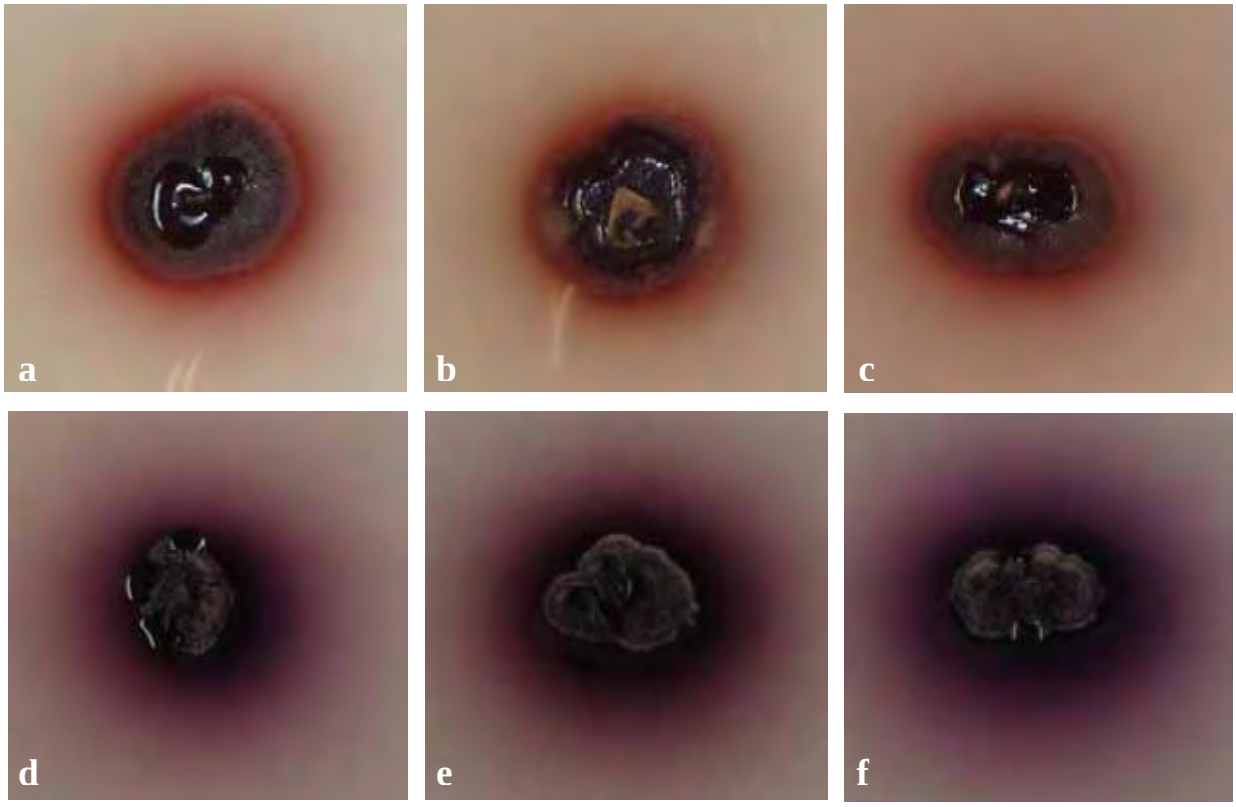
According to Zeng et al. (2007), *Exophiala* species show different maximum growth at temperatures more or less coinciding with the clinical predilection. Thus, while species found in association with hydrocarbons have maximum growth at temperatures around 30-33°C, human pathogenic species are able to grow at elevated temperatures ranging from 36 to 42°C (SEYEDMOUSAVI et al., 2011). The species that cause systemic infections are generally able to grow at 40°C and saprobic species grow up to 37°C (STERFLINGER, 2006), as was also observed in our data to *E. dermatitidis* and *E. spinifera*.

Contrary to our data, Satow (2008) also isolated *Exophiala* species from hydrocarbon contaminated soils, but the strains were not able to grow at 40°C. Still in human-made environments, active growth for *Exophiala* was recorded with up to 47°C for strains isolated from dishwasher (ZALAR et al., 2011). For representing more stressful environments, black yeasts developed mechanisms to colonize adverse environments and compete with other microorganisms. According to Sudhadham et al. (2011), the evolutionary origin of virulence in black yeasts has been supposed to have an ecological trigger in the preference for environments that are unsuitable for most competing microorganisms.

Twenty eight strains of *Exophiala* species were cultured on ABTS-agar medium. A positive result was verified when the colorless medium turned purple/red due to the oxidation of ABTS to ABTS-azine in the presence of laccase (Fig. 5). Most of the strains (96%) were found to be positive for laccase, with different halo sizes (Tables 2 and 3). Colored metabolites were observed in both species, *E. dermatitidis* and *E. spinifera*, but statistical analysis showed that *E. spinifera* had higher laccase activity ($p < 0.1$).

Notably, the ABTS oxidation resulted in different colors for *E. dermatitidis* and *E. spinifera* strains (Fig. 5). Laccases are enzymes with several varieties (isoenzymes) and some fungus secrete different laccases at the same time. So, the explanation for the different colors can be related to the biological species in question, suggesting that each species produces a pool of isoenzymes slightly different in relation to its action mode and, consequently, its structure.

Fig. 5. Laccase activity assay. Colored zones around the colonies were measured from 10-day-old cultures at 25°C on ABTS-agar medium. **a–c**. Growth of *Exophiala dermatitidis* strains dH 22993, dH 22986 and dH 22919. **d-f**. Growth of *E. spinifera* strains dH 22900, dH 22925 and dH 23057.



Laccase studies on black yeasts are recent and started with the work of Tetsch et al. (2005, 2006). These authors showed that *Hortaea acidophila* (Dothideales) has two laccase types highly stable in low pH. Laccase type I (extracellular) contains an N-terminal signal sequence for protein export and seems to be involved in the solubilization of brown coal and other recalcitrant polyphenolic-rich substrates in order to access carbon and energy sources. Moreover, lacase type II (extra- and intracellular) might be involved in the synthesis of melanin, by mediating the polymerization of dihydroxynaphthalene monomers.

Despite being initial works, the presence of laccases has also been demonstrated in *Fonsecaea* (SUN et al., 2012), *Cyphellophora* and related *Phialophora* species (PEIYING FENG et al., 2012), genera belonging to Chaetothyriales order. It is important to emphasize that this is the first report of laccase production by *Exophiala* species and there is evidence of the presence of different isoforms associated with each species.

The present study was an attempt at verifying putative virulence factors in environmental strains of *Exophiala* species. Although several authors reported that most fungi isolated from nature may be less virulent than their siblings pathogens (VICENTE et al. 2008), we found that the environmental strains have characteristics that make them potential opportunistic human pathogens, such as growth at 40°C and laccase production. Knowing that these fungi coexist in human-made environments or could be spread by ants in urban areas, further research is necessary to clarify whether the presence of *Exophiala* species in these environments poses any threat to human health.

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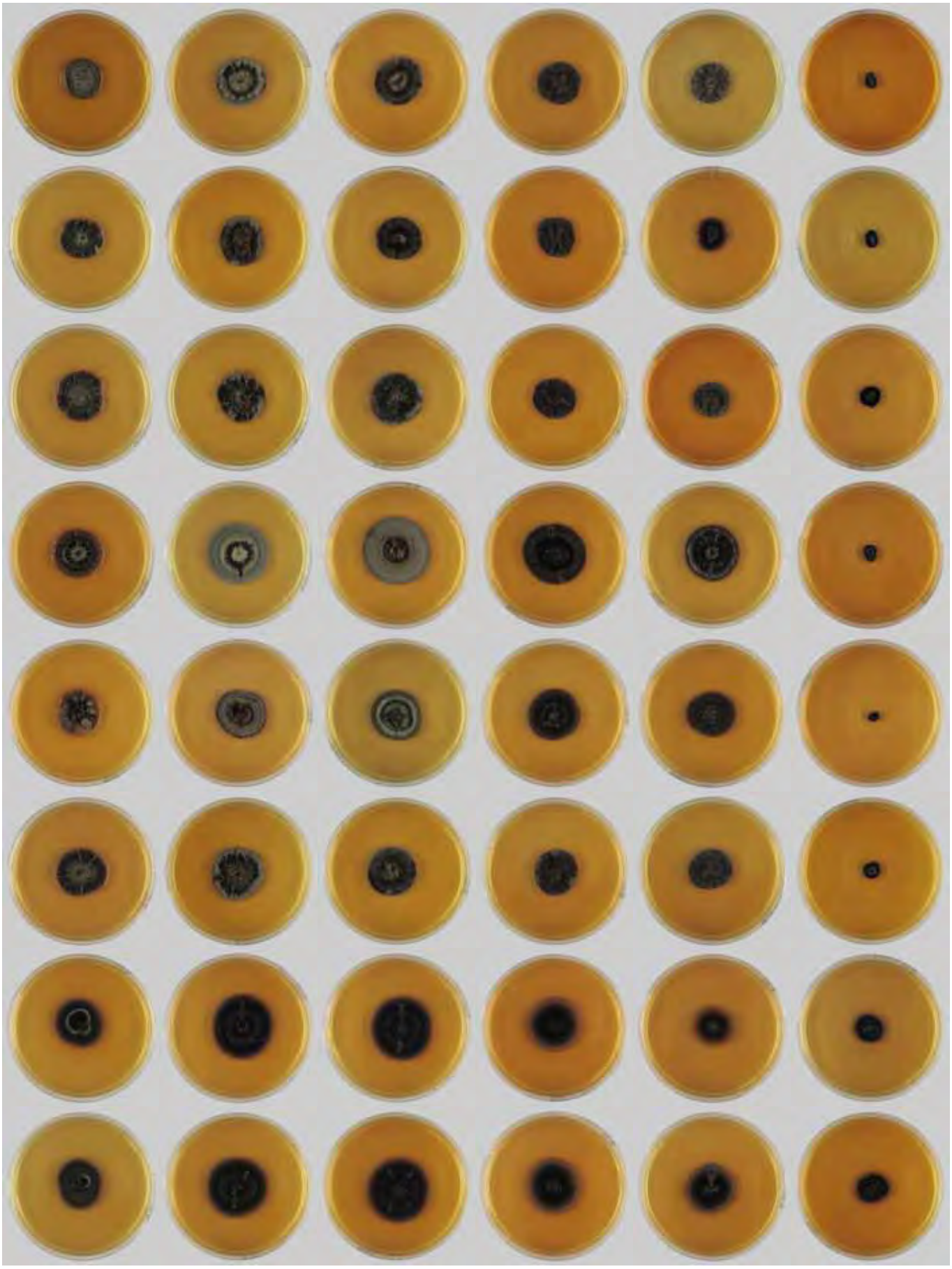
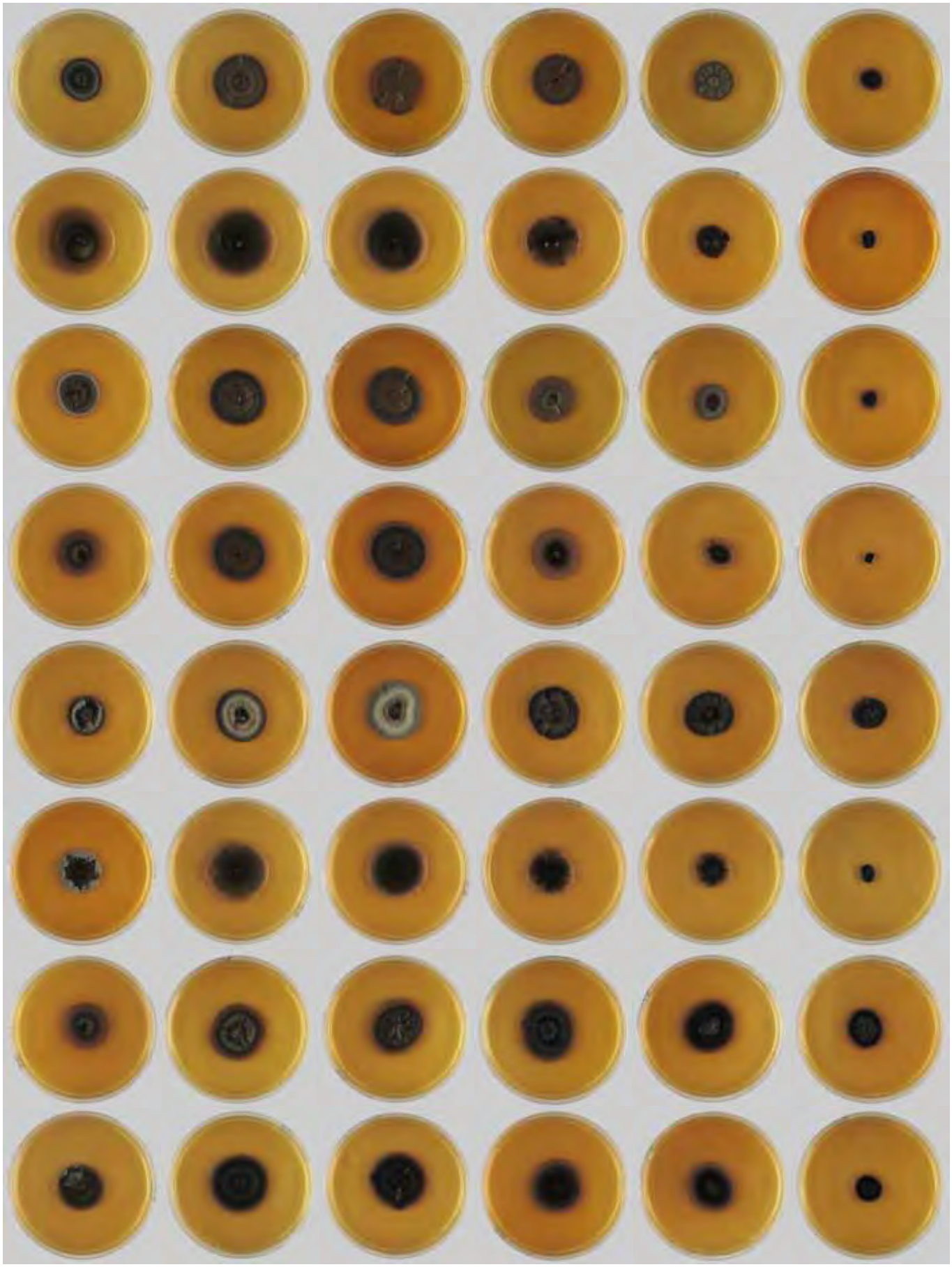
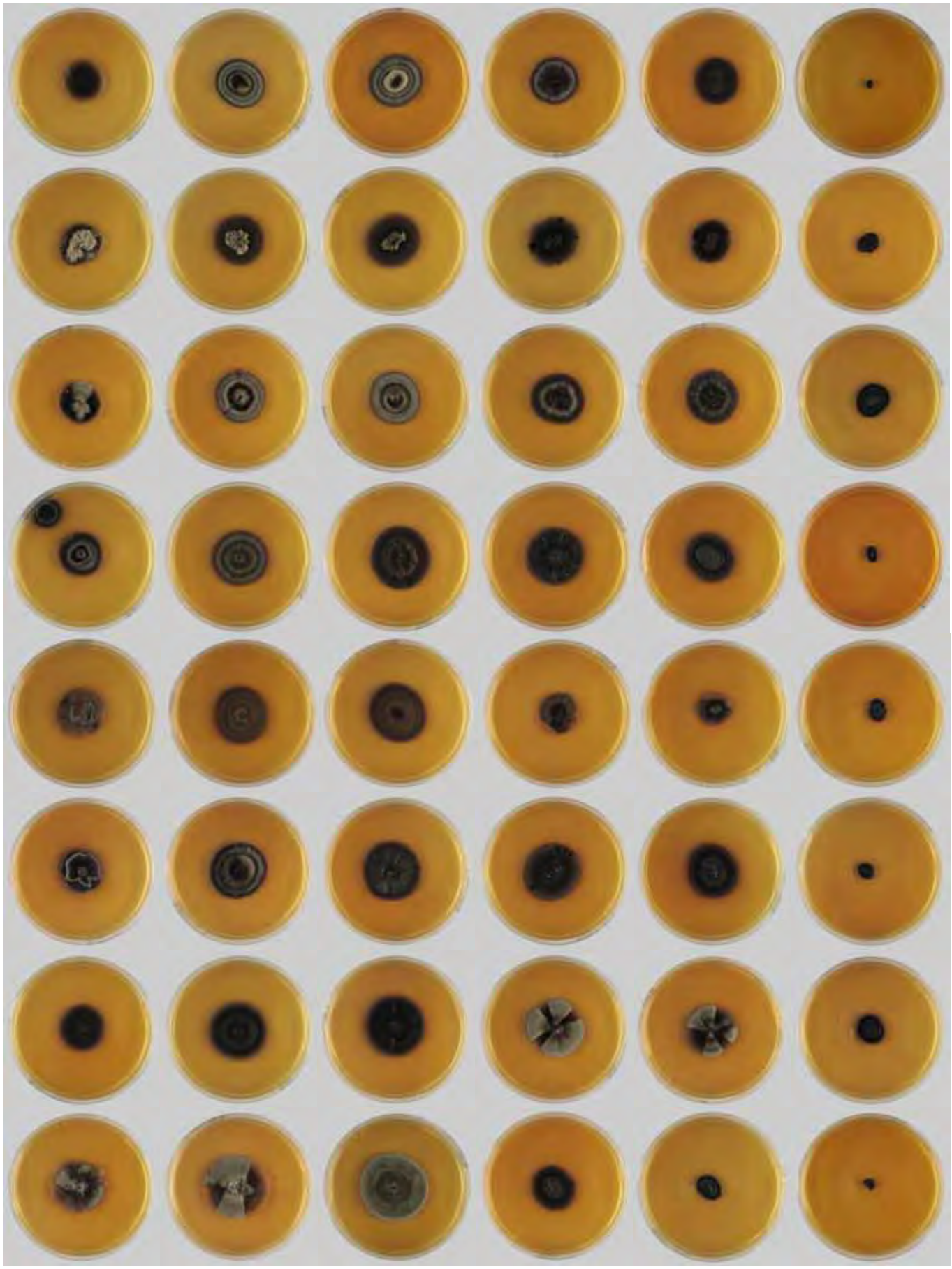
APÊNDICE



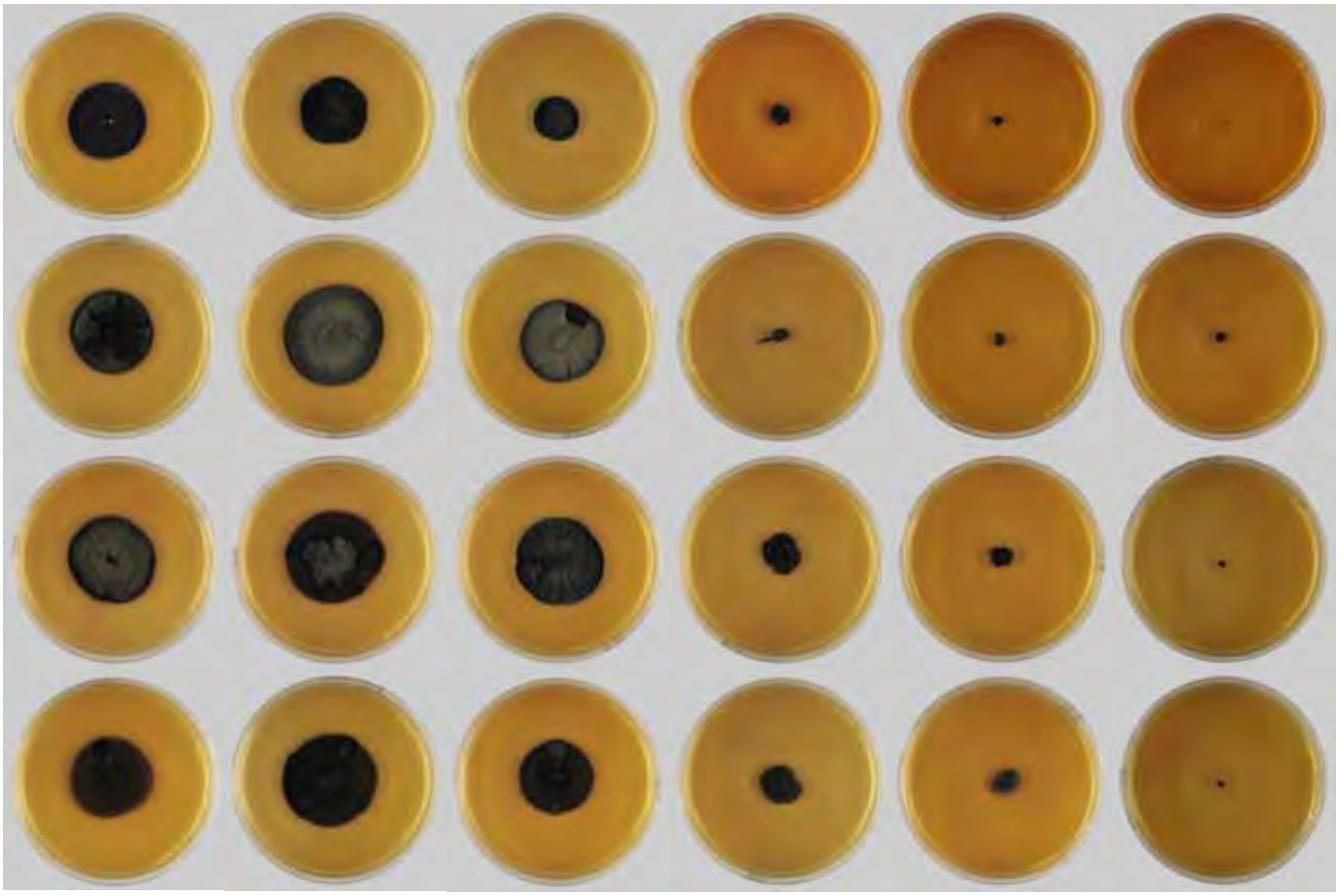
Overview of *Exophiala dermatitidis* growth rates on MEA after 21 d at various temperatures. Row, left to right: 25, 30, 33, 36, 37, 40°C; columns, top to bottom: dH 22899, dH 22997, dH 23029, dH 23065, dH 22929, dH 23072, dH 22927 e dH 23048.



Overview of *E. dermatitidis* growth rates on MEA after 21 d at various temperatures. Row, left to right: 25, 30, 33, 36, 37, 40°C; columns, top to bottom: dH 22919, dH 23012, dH 22983, dH 23003, dH 22978, dH 22985, dH 23056 e dH 23059.



Overview of *E. dermatitidis* growth rates on MEA after 21 d at various temperatures. Row, left to right: 25, 30, 33, 36, 37, 40°C; columns, top to bottom: dH 22984, dH 22986, dH 22977, dH 22995, dH 22993, dH 22975, dH 22974 e dH 22989.



Overview of *E. spinifera* growth rates on MEA after 21 d at various temperatures. Row, left to right: 25, 30, 33, 36, 37, 40°C; columns, top to bottom: dH 23008, dH 23057, dH 22925 e dH 22900.