

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

**AGRUPAMENTOS GÊNICOS ENVOLVIDOS NA
BIOSSÍNTESE DE METABÓLITOS SECUNDÁRIOS NO
FUNGO ENTOMOPATOGÊNICO *Metarhizium anisopliae*:
IDENTIFICAÇÃO GÊNOMICA E PADRÕES DE
EXPRESSÃO EM CUTÍCULAS DO CARRAPATO**

Rhipicephalus microplus

Dissertação de Mestrado

Nicolau Sbaraini Oliveira

Porto Alegre, Outubro de 2016

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Dissertação submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do Grau de Mestre em Biologia Celular e Molecular.

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LISTA DE ABREVIATURAS

h	Horas
°C	Graus Celsius
pH	Potencial hidrogeniônico
MS	Metabólito Secundário
ITS	<i>Internal transcribed spacers</i>
EF-1α	<i>Elongation factor 1-alpha</i>
β-tub	Beta-tubulina
RPB1	<i>DNA-directed RNA polymerase II subunit RPB1</i>
RPB2	<i>DNA-directed RNA polymerase II core subunit RPB2</i>
SABIA	<i>System for Automated Bacterial Integration of Annotation</i>
Mya	<i>million years ago: milhões de ano atrás</i>
HGT	<i>horizontal gene transfer: transferência horizontal gênica</i>
MP	Metabólito primário
BGC	<i>Biosynthetic gene clusters: agrupamentos gênicos biossintéticos</i>
PKS	<i>Polyketide synthase: sintase envolvida na biossíntese de policetídeos</i>
NRPS	<i>Non-ribosomal peptide synthetase: sintetase envolvida na biossíntese de peptídeos não ribossomais</i>
TC terpênicos	<i>Terpene cyclase: ciclase envolvida na biossíntese de compostos terpênicos</i>
PT	Preniltransferases
Log	Logaritmo
GGPP sintase	Geranyl-geranyl difosfato sintase
RPKM	<i>Reads Per Kilobase per Million mapped reads</i>
MFS	<i>Major Facilitator Superfamily</i>

1 RESUMO

O gênero *Metarhizium* abriga fungos cosmopolitas que infectam hospedeiros artrópodes. Curiosamente, enquanto algumas espécies do gênero infectam um amplo espectro de hospedeiros (hospedeiro-generalistas), outras espécies infectam apenas alguns artrópodes (hospedeiro-especialistas). Esse traço evolutivo singular permite comparações únicas, a fim de determinar como patógenos e fatores de virulência surgem. Dentre os diversos fatores de virulência descritos, se destacam os metabólitos secundários que hipoteticamente desempenhem papéis essenciais na infecção fúngica. No entanto, a maioria dos genes para a produção de metabólitos secundários em *Metarhizium* spp. não foram ainda caracterizados, e pouco se sabe sobre a organização gênica, expressão e regulação destes genes. A fim de melhor compreender estes aspectos, nós realizamos uma análise e descrição detalhada de agrupamentos gênicos (*clusters*) envolvidos na biossíntese de metabólitos secundários em *M. anisopliae*, analisamos dados de um estudo transcritômico onde o fungo foi cultivado em cutículas de carrapato avaliando a expressão diferencial destes *clusters*, bem como avaliamos a conservação destes genes entre espécies do gênero *Metarhizium*. Ademais, nossa análise se estendeu avaliando aspectos evolutivos e filogenéticos para três *clusters*: MaPKS1 cujo produto é putativamente assemelhado a tropolonas e citrininas, MaNRPS-PKS2 cujo produto é putativamente assemelhado a pseurotina e MaTERP1 cujo produto putativo é o ácido helvólico. Dentre os 73 *clusters* identificados no genoma de *M. anisopliae*, 20 % estavam positivamente regulados na condição experimental de infecção inicial, com presumível papel na virulência do fungo. Dentre os *clusters* positivamente regulados estão genes já caracterizados envolvidos na biossíntese de destruxinas, NG39x e ferricrocina, em conjunto com genes putativamente envolvidos na biossíntese do ácido helvólico, produtos assemelhados a pseurotina e tropolonas e citrininas, além de genes envolvidos na biossíntese de compostos desconhecidos. Curiosamente, diversos *clusters* positivamente regulados na condição de infecção inicial não estão presentes em espécies hospedeiro-especialistas do gênero *Metarhizium*, indicando que existem diferenças nas estratégias metabólicas empregadas por espécies hospedeiro-generalistas e hospedeiro-especialistas no ciclo infeccioso. Estas diferenças no potencial metabólico podem ter sido parcialmente moldadas por eventos de transferência horizontal, conforme sugere nossa análise filogenética sobre a origem do o cluster putativamente envolvido na biossíntese do ácido helvólico em *Metarhizium* spp. Em conclusão, diversos *clusters* desconhecidos são descritos e aspectos da sua organização, regulação e origem são discutidos, fornecendo evidências sobre o impacto dos metabólitos secundários no ciclo de vida e infecção de espécies do gênero *Metarhizium*.

2 ABSTRACT

The *Metarhizium* genus harbors cosmopolitan fungi that infect arthropod hosts. Interestingly, while some species infect a wide range of hosts (host-generalists), other species infect only a few arthropods (host-specialists). This singular evolutionary trait permits unique comparisons to determine how pathogens and virulence determinants emerge. Among the several virulence determinants that have been described, secondary metabolites (SMs) are suggested to play essential roles during fungal infection. However, the majority of genes related to SM production in *Metarhizium* spp. are uncharacterized, and little is known about their genomic organization, expression and regulation. To better understand these aspects, we have performed a deep survey and description of SM biosynthetic gene clusters (BGCs) in *M. anisopliae*, analyzed RNA-seq data from fungi grown on cattle-tick cuticles, evaluated the differential expression of BGCs, and assessed conservation within the *Metarhizium* genus. Furthermore, our analysis extended to the construction of a phylogeny for the following three BGCs: a tropolone/citrinin-related compound (MaPKS1), a pseurotin-related compound (MaNRPS-PKS2), and a putative helvolic acid (MaTERP1). Among 73 BGCs identified in *M. anisopliae*, 20% were up-regulated during initial tick cuticle infection and presumably possess virulence-related roles. These up-regulated BGCs include known clusters, such as destruxin, NG39x and ferricrocin, together with putative helvolic acid and pseurotin- and tropolone/citrinin-related compound clusters, as well as uncharacterized clusters. Interestingly, several up-regulated BGCs were not conserved in host-specialist species from the *Metarhizium* genus, indicating differences in the metabolic strategies employed by generalist and specialist species to overcome and kill their host. These differences in metabolic potential may have been partially shaped by horizontal gene transfer (HGT) events, as our phylogenetic analysis provided evidence that the putative helvolic acid cluster in

Metarhizium spp. originated from an HGT event. In conclusion several unknown BGCs are described, and aspects of their organization, regulation and origin are discussed, providing evidence for the impact of SMs on the *Metarhizium* genus lifestyle and infection process.

3 REVISÃO BIBLIOGRÁFICA

3.1 Controle Biológico

O controle biológico é um fenômeno natural onde uma população de organismos alvo tem seu número controlado pela ação de uma outra população de organismos controladores. Tais organismos controladores mantêm relações de predação, parasitismo, herbivoria ou outros mecanismos, podendo ser empregados pelo homem, com o objetivo de controlar a população de uma praga alvo. O controle biológico clássico consiste na introdução, controlada e proposital, de uma nova espécie ou linhagem em um ambiente não ocupado, para o controle de determinado organismo, reestruturando assim este ambiente (Flint *et al.*, 1998).

Nos últimos anos, a dispersão de populações de pragas resistentes a múltiplos pesticidas tem sido uma das maiores preocupações, tanto de produtores rurais, quanto de órgãos públicos. O uso indiscriminado de defensivos agrícolas, além de danoso ao meio-ambiente, tem acelerado o surgimento de artrópodes multirresistentes, como pragas do campo, como a lagarta *Helicoverpa armigera* (Alvi *et al.*, 2012; Tay *et al.*, 2013), vetores de doenças humanas como mosquitos do gênero *Aedes*, *Anopheles* e *Culex* (Augusto *et al.*, 2016; Hemingway *et al.*, 2016; Wu *et al.*, 2016) e pragas domésticas, como percevejos do gênero *Cimex*, a mosca doméstica (*Musca domestica*) e baratas da espécie *Periplaneta americana* (Benoit *et al.*, 2016).

A utilização de organismos como controladores biológicos é uma alternativa ambientalmente menos impactante que pode ser utilizada em programas de manejo, para controlar pragas resistentes e não resistentes (Peshin e Dhawan, 2009). Por exemplo, o uso de vírus da família Baculoviridae, que naturalmente infectam artrópodes, se mostrou efetivo no controle biológico de *H. armigera* (Rowley *et al.*, 2011). Outra alternativa são

formulações de fungos e bactérias entomopatogênicas. Essas formulações também tem sido empregadas e testadas para o controle biológico de vetores de doenças e pragas domésticas (Barson *et al.*, 1994; Mohanty *et al.*, 2008; Hubner-Campos *et al.*, 2013; Ulrich *et al.*, 2014; Gomes *et al.*, 2015). O uso de controladores biológicos também tem se mostrado uma alternativa, a fim de contornar a dispersão e surgimento de insetos resistentes a plantas transgênicas (Liu *et al.*, 2014). De maneira geral, são diversos os relatos na literatura na qual a aplicação de tecnologias que envolvem controladores biológicos foram bem-sucedidas (Cory e Franklin, 2012).

3.1.1 O carrapato bovino *Rhipicephalus microplus* e seu Controle Biológico

A classe Arachnida abriga diversos artrópodes. Dentre os mais conhecidos se encontram as aranhas, ácaros, escorpiões e carrapatos. Os carrapatos são parasitas cosmopolitas que infectam um ampla gama de hospedeiros, de répteis a mamíferos, sendo um dos maiores problemas para pecuaristas de regiões tropicais e subtropicais (Jonsson, 2006). Seu parasitismo reduz o ganho de peso, podendo levar à anemia, anorexia e apatia, além de causar danos ao couro do animal, e, em casos mais extremos, à morte (Seixas *et al.*, 2012). Carrapatos também podem ser vetores de diversas doenças animais e humanas, como a doença de Lyme, a febre maculosa das montanhas, a erliquiose, a tularemia e a anaplasmoze (Minniear e Buckingham, 2009; Snowden e Stovall, 2011; Vayssier-Taussat, 2014; Sainz *et al.*, 2015).

No Brasil, um dos principais problemas na pecuária bovina são os carrapatos da espécie *Rhipicephalus microplus* (Evans *et al.*, 2000). Essa espécie se caracteriza por possuir apenas um hospedeiro (parasita monóxeno), e seu ciclo de vida compreende um período parasitário e outro de vida livre. O período parasitário dura em média 3 semanas (Webster, 2013). O ciclo de infecção de *R. microplus* se inicia com a adesão de larvas ao

couro de um animal suscetível (Barre e Uilenberg, 2010). As larvas conseguem detectar o hospedeiro através de um órgão sensorial especializado quimiorreceptor e termorreceptor (Órgão de Haller) que detecta, principalmente, dióxido de carbono (CO₂) exalado pelo hospedeiro (Stange e Stowe, 1999). Uma vez aderidas ao hospedeiro, as larvas se distribuem pelo corpo do animal, sendo encontradas em maior número na virilha, face interna das pernas, pescoço, cauda e pavilhão auricular, partes do corpo onde o hospedeiro possui menos acesso, dificultando a remoção mecânica do parasita pelo hospedeiro (Webster, 2013). Uma vez fixadas, as larvas se alimentam por cerca de uma semana, até sofrerem a primeira muda, atingindo o estágio de ninfa, que se alimentará por sete dias até sofrer a segunda muda, atingindo a idade adulta (Figura 1) (Webster, 2013). Os carrapatos atingem a idade sexual cinco dias após a ecdise para a fase adulta, realizando a cúpula. Após cúpula, a fêmea fertilizada aumenta a ingestão sanguínea, intumescendo (Webster, 2013). Normalmente, vinte e um dias após a adesão das larvas ao hospedeiro, a fêmea desprende-se, a fim de realizar a postura no solo. Uma vez no solo, a fêmea necessita amadurecer seu sistema reprodutivo (período pré-postura, com duração de aproximadamente 3 dias), para então gerar e ovipositar cerca de 3.000 ovos (Barre e Uilenberg, 2010; Webster, 2013). A oviposição dura aproximadamente 14 dias (Figura 1).

Atualmente, o tratamento químico é o mais empregado para o controle de surtos parasitários causados por *R. microplus* (Kunz e Kemp, 1994). Dentre os defensivos químicos mais utilizados, encontram-se os organofosforados, piretróides, amitraz, lactonas macrocíclicas, fipronil e fluazuron, muitos deles atuando sobre o sistema nervoso do artrópode (Klafke *et al.*, 2010; Webster, 2013; Rodriguez-Vivas *et al.*, 2014). Por diversos fatores, que vão desde a ausência de uma política oficial de controle de carrapatos, até a má utilização dos acaricidas, como quantidade inadequada, tratamentos em excesso e produtos

de qualidade não comprovada, o número de linhagens de carrapatos resistentes aos principais químicos tem aumentado significativamente (Pereira *et al.*, 2009; Webster, 2013), sendo diversos os relatos de populações resistentes a organofosforados, piretróides, amitraz, lactonas macrocíclicas e fipronil (Kunz e Kemp, 1994; Martins e Furlong, 2001; Castro-Janer *et al.*, 2010; Reck *et al.*, 2014; Webster *et al.*, 2015). No início de 2014, pesquisadores relataram, pela primeira vez, o isolamento de carrapatos resistentes a benzoil fenil ureia, o único dos acaricidas indicados para o controle de carrapato no Brasil que ainda não possuía populações resistentes, tal linhagem foi denominada Jaguar (Reck *et al.*, 2014). Assim, métodos alternativos e/ou sinérgicos aos convencionais são necessários, a fim de contornar possíveis resistências adquiridas por estas populações de artrópodes.

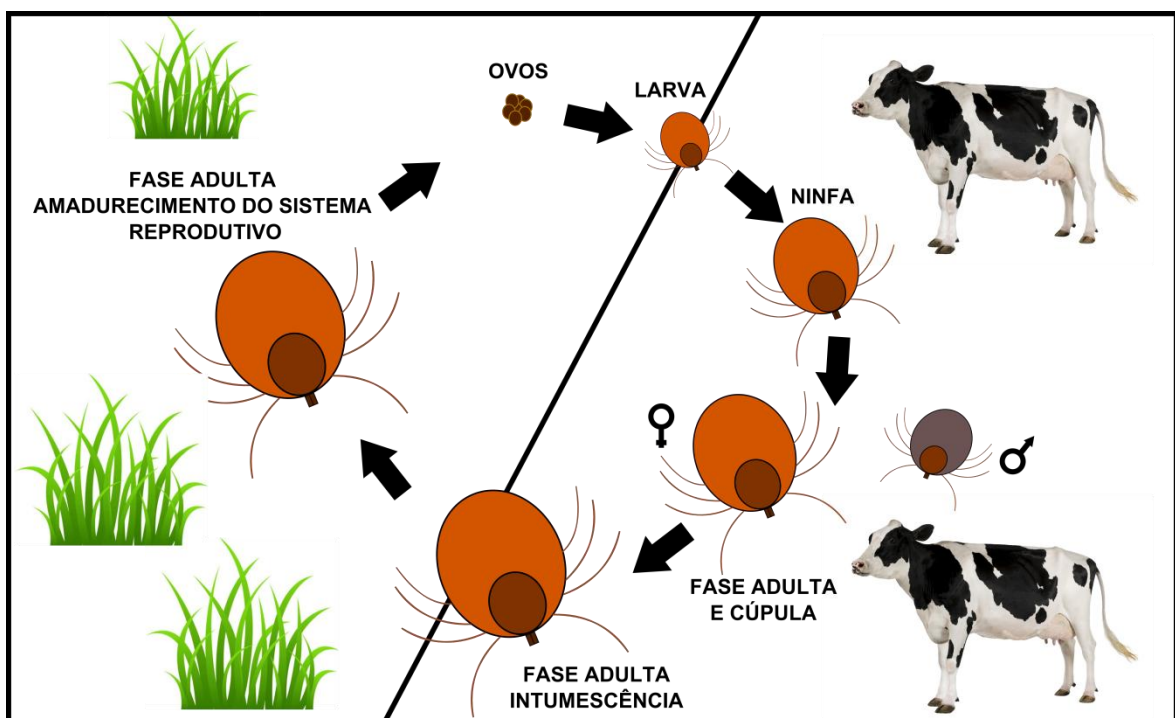


Figura 1: Ciclo de vida do carrapato bovino *Rhipicephalus microplus*. *R. microplus* se caracteriza por ser um exo-parasita monóxeno, com um ciclo de vida que compreende um período parasitário e outro de vida livre. O período parasitário dura em média 3 semanas, se iniciando com as larvas que se alimentam até chegar a fase adulta. Pós-cúpula a fêmea aumenta consideravelmente a ingesta de sangue, intumescendo e se despreendendo do animal. Uma vez no solo, a fêmea amadurece seu sistema reprodutivo a fim de ovipositar.

Métodos que empregam organismos no controle biológico de *R. microplus* têm sido desenvolvidos, uma vez que esses acaricidas biológicos são atóxicos para os animais e podem contornar possíveis resistências (Samish *et al.*, 2004; Fernandes *et al.*, 2012). Dentre os controladores biológicos mais empregados para o controle de *R. microplus*, se encontram os fungos entomopatogênicos dos gêneros *Metarhizium*, *Beauveria*, *Isaria* e *Lecanicillium* (Fernandes *et al.*, 2012). Destaca-se a espécie *Metarhizium anisopliae*, a qual causa altas taxas de mortalidade em todos os estágios de desenvolvimento de carrapatos, sendo efetivo contra diversas espécies, reduzindo também a oviposição de fêmeas ingurgitadas (Webster *et al.*, 2015). Sendo a utilização de *M. anisopliae* uma ferramenta promissora para o controle de *R. microplus*, conhecer a fundo as interações patógeno-hospedeiro, além de métodos de aplicação a campo de formulações de *M. anisopliae*, se fazem necessárias. Esse maior aprimoramento das metodologias visa auxiliar no desenvolvimento de programas de controle biológico mais eficientes (Fernandes *et al.*, 2012; Webster *et al.*, 2015).

3.2 O modelo de estudo *Metarhizium* spp.

3.2.1 Características e Taxonomia de *Metarhizium anisopliae* e do Gênero *Metarhizium*

Metarhizium anisopliae é um fungo do filo Ascomycota, da classe dos Sordariomycetes, da ordem Hypocreales e família Clavicipitaceae. *M. anisopliae* é a principal espécie do gênero de fungos entomopatogênicos *Metarhizium*. Isolado pela primeira vez em 1879 por Metschnikoff, o gênero foi estabelecido por Sorokin em 1883.

Este fungo é distinguido por apresentar micélio hialino e septado, dotado de conidióforos dos quais emergem conídios cilíndricos esverdeados, organizados em colunas (Figura 2) (Zimmermann, 2007).

Atualmente sessenta e sete entradas estão listadas para membros do gênero *Metarhizium* no *Index Fungorum*, o qual se caracteriza por ser um banco de dados de espécies fúngicas (Crous *et al.*, 2004). Embora nem todas as espécies listadas sejam reconhecidas como espécies, a filogenia do gênero tem sido alvo de grandes revisões nos últimos anos. A primeira revisão do gênero, baseada em características morfológicas, foi realizada por Tulloch em 1976 (Tulloch, 1976). Em seu trabalho, ele distinguiu duas espécies: *M. anisopliae* e *M. flavoviride*. Em 1994, outra tentativa de classificação, baseada em características morfológicas e coloração dos conídios, propôs três espécies: *M. anisopliae*, *M. flavoviride* e *M. album*, com duas variedades *M. anisopliae var. majus* e *M. flavoviride var. flavoviride* (Curran *et al.*, 1994). Em 2000, Curran e colaboradores foram os primeiros a aplicar abordagens moleculares na tentativa de descrever o gênero, utilizando sequências de ITS (*internal transcribed spacers*). Tal análise recomendou a manutenção das espécies já descritas, além de acrescentar mais duas variedades à espécie *M. anisopliae* e cinco variedades à espécie *M. flavoviride* (Driver *et al.*, 2000). Até então somente a fase anamórfica (reprodução assexuada) do fungo havia sido identificada. Em 2001, Liu e colaboradores mediante a comparação de sequências ITS de *Metarhizium spp.* com *Cordyceps spp.*, foi identificada à espécie *Cordyceps brittlebankisoides* como a forma teleomórfica (reprodução sexuada) de *M. anisopliae var. majus* e, com isso, o gênero *Metarhizium* passou a integrar o filo Ascomycota. Esses resultados foram suportados por trabalhos subsequentes, sendo a fase sexual de *Metarhizium* pertencente ao gênero *Metacordyceps*. A descrição da fase sexuada e assexuada em diferentes gêneros é uma

peculiaridade de fungos filamentosos ainda passível na filogenia destas espécies (Liu *et al.*, 2001; Sung, Hywel-Jones, *et al.*, 2007).

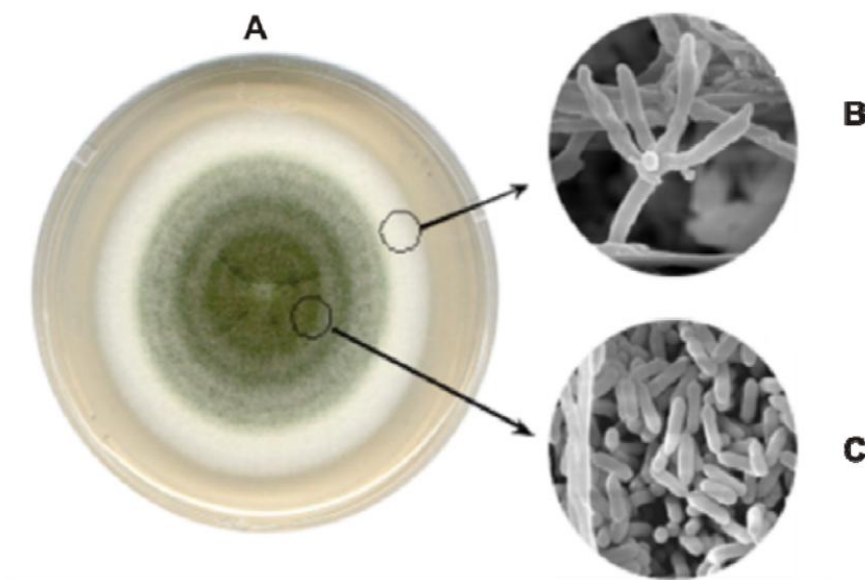


Figura 2: Aspecto morfológico da linhagem E6 de *Metarhizium anisopliae*. (A) Colônia em Meio de Cove Completo (MCC). (B) Do micélio hialino emergem os conidióforos que darão origem aos conídios de coloração verde-musgo (C). Imagem de autoria de Arruda e colaboradores (Arruda *et al.*, 2005).

Em 2009, Bischoff e colaboradores realizaram uma nova análise utilizando um número maior de marcadores moleculares, a qual dividiu o gênero em 12 espécies: *M. anisopliae*; *M. acridum*; *M. brunneum*; *M. globosum*; *M. guizhouense*; *M. lepidiotae*; *M. majus*; *M. pinghaense*; *M. robertsii*; *M. album*; *M. flavoviride*; *M. frigidum* (Bischoff *et al.*, 2006; 2009). O trabalho de Bischoff e colaboradores também evidenciou a impossibilidade em discriminar espécies crípticas utilizando ITS como marcador de gênero *Metarhizium* e aconselhou o uso do EF-1 α (*elongation factor 1-alpha*), um gene ubíquo, cujo produto atua na tradução. Este trabalho foi apoiado por trabalhos posteriores (Kepler e Rehner, 2013). Em 2014, Kepler e colaboradores realizaram um novo estudo, com base na utilização de fragmentos dos genes para a β -tub (beta-tubulina), RPB1 (*DNA-directed RNA polymerase*

II subunit RPB1), *RPB2 (DNA-directed RNA polymerase II core subunit RPB2)* e *EF-1 α* como marcadores moleculares, identificando mais 25 espécies: *M. atrovirens*; *M. brittlebankisoides*; *M. brasiliense*; *M. campostorni*; *M. carneum*; *M. cylindrosporum*; *M. granulomatis*; *M. guniujiangense*; *M. indigoticum*; *M. khaoyaiense*; *M. koreanum*; *M. kusanagiense*; *M. marquandii*; *M. martiale*; *M. minus*; *M. novozealandicum*; *M. owariense*; *M. owariense f. viridescens*; *M. pemphigi*; *M. pseudoatrovirens*; *M. rileyi*; *M. taii*; *M. yongmunense*; *M. viridulum*; *M. viride* (Kepler *et al.*, 2014). Em 2016, mais uma espécie foi identificada (*M. blattodeae*), contabilizando 38 espécies descritas no gênero atualmente (Montalva *et al.*, 2016).

Embora os trabalhos recentes tenham identificado um grande número de novas espécies, tal divisão pode ainda não refletir a grande biodiversidade existente. Esse gênero é notável por apresentar variações na virulência a diferentes artrópodes, na taxa de crescimento e esporulação, na capacidade de utilizar diferentes nutrientes, na produção de toxinas, entre outros aspectos (Hu *et al.*, 2006; Zimmermann, 2007; Lubeck *et al.*, 2008; Schrank e Vainstein, 2010; Nishi *et al.*, 2013) (Figura 3). Tais diferenças podem indicar um número maior de espécies crípticas ainda não identificadas.

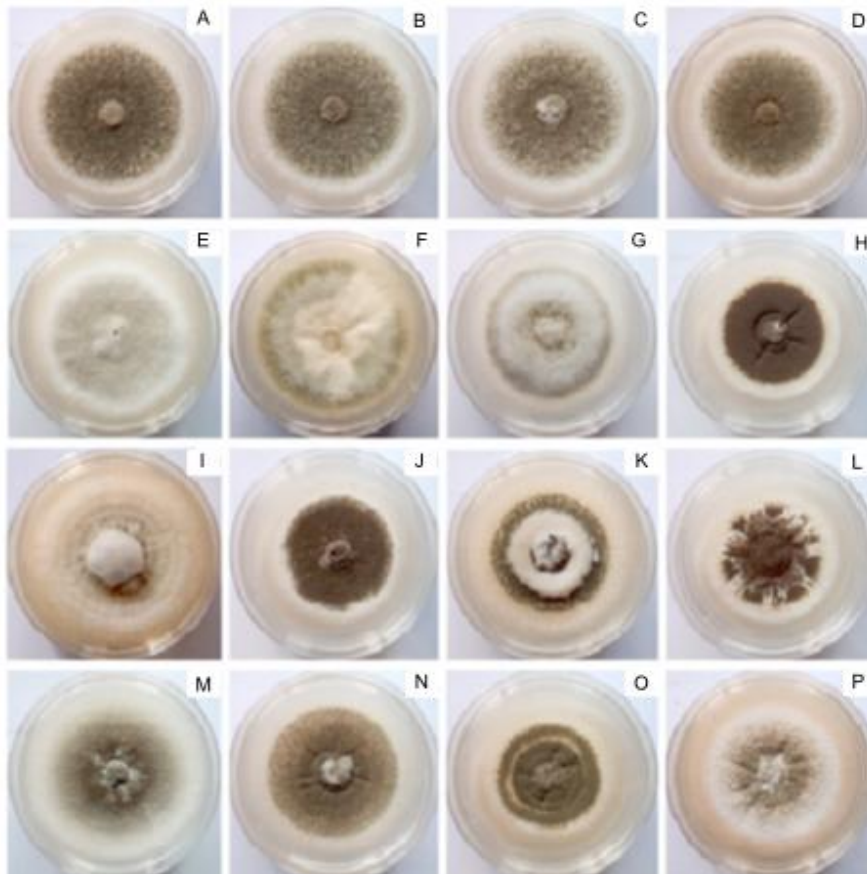


Figura 3: Diferenças morfológicas entre linhagens de *Metarhizium anisopliae*. (A) AL; (B) M5; (C) MT; (D) E6; (E) E9; (F) Rjd; (G) Nordeste; (H) CARO7; (I) CARO11; (J) CARO12; (K) CARO14; (L) CARO19; (M) CG27; (N) CG30; (O) CG31; (P) CG33. Modificado de Schrank & Vainstein (Schrank e Vainstein, 2010).

3.2.2 Sequenciamento do genoma da linhagem E6 de *Metarhizium anisopliae*

Poucos experimentos geram tanta informação como o sequenciamento do genoma de um organismo. Nos últimos 10 anos, diversos genomas fúngicos foram sequenciados, incluindo organismos modelo, como *Neurospora crassa* e *Aspergillus fumigatus* (Galagan *et al.*, 2003; Nierman *et al.*, 2005); fitopatógenos importantes, como *Magnaporthe grisea* e *Fusarium graminearum* (Dean *et al.*, 2005; Cuomo *et al.*, 2007); patógenos humanos, como *Cryptococcus neoformans* e *Sporothrix schenckii* (Loftus *et al.*, 2005; Teixeira *et al.*, 2014) e fungos entomopatogênicos, como *Beauveria bassiana* e *Cordyceps militaris*

(Zheng *et al.*, 2011; Xiao *et al.*, 2012). Até o presente momento, estão depositados na plataforma do NCBI os genomas de 972 fungos (dados de Agosto de 2016).

Em 2014, nosso grupo (Biologia Celular e Molecular de Fungos Filamentosos do CBiot-UFRGS) publicou o genoma da linhagem E6 de *M. anisopliae*, abrindo um nicho para explorar diversos aspectos do ciclo de vida deste fungo (Staats *et al.*, 2014). As etapas de sequenciamento, montagem do genoma e anotação foram realizados em colaboração com a Unidade de Genômica Computacional Darcy Fontoura de Almeida e do Laboratório de Bioinformática do LNCC. O genoma de *M. anisopliae* E6 tem um tamanho estimado de 38.5 Mb, tendo sido sequenciado pelo método de pirosequenciamento, utilizando a plataforma 454, com uma cobertura média de 19 vezes (Staats *et al.*, 2014). O genoma abriga 181 tRNAs, 10,817 proteínas preditas, com um conteúdo GC de 51 %, uma densidade gênica de 280 genes por Mbp, com uma média de 2,7 exons por gene. Diferindo de outros projetos genoma que utilizam somente métodos automáticos para a anotação de proteínas preditas, o genoma de *M. anisopliae* E6 foi anotado automaticamente e, posteriormente, curado manualmente utilizando a plataforma SABIA (*System for Automated Bacterial Integration of Annotation*) (Almeida *et al.*, 2004; Staats *et al.*, 2014). A anotação manual garante uma qualidade superior, uma vez que as proteínas preditas são avaliadas individualmente ao longo da anotação (Humphreys *et al.*, 2015). Adicionalmente, análises detalhadas a respeito de proteínas secretadas, aspectos filogenéticos e evolutivos e uma análise transcritômica em condições que mimetizam o processo de infecção de *M. anisopliae*, também foram conduzidas (Staats *et al.*, 2014). No entanto, assim como qualquer projeto genoma, cujo volume de dados e resultados gerados é muito grande, muitos aspectos não foram explorados, como diversas famílias de proteínas e fatores de virulência.

3.2.3 Evolução da ordem Hypocreales, família Clavicipitaceae e do gênero *Metarhizium*

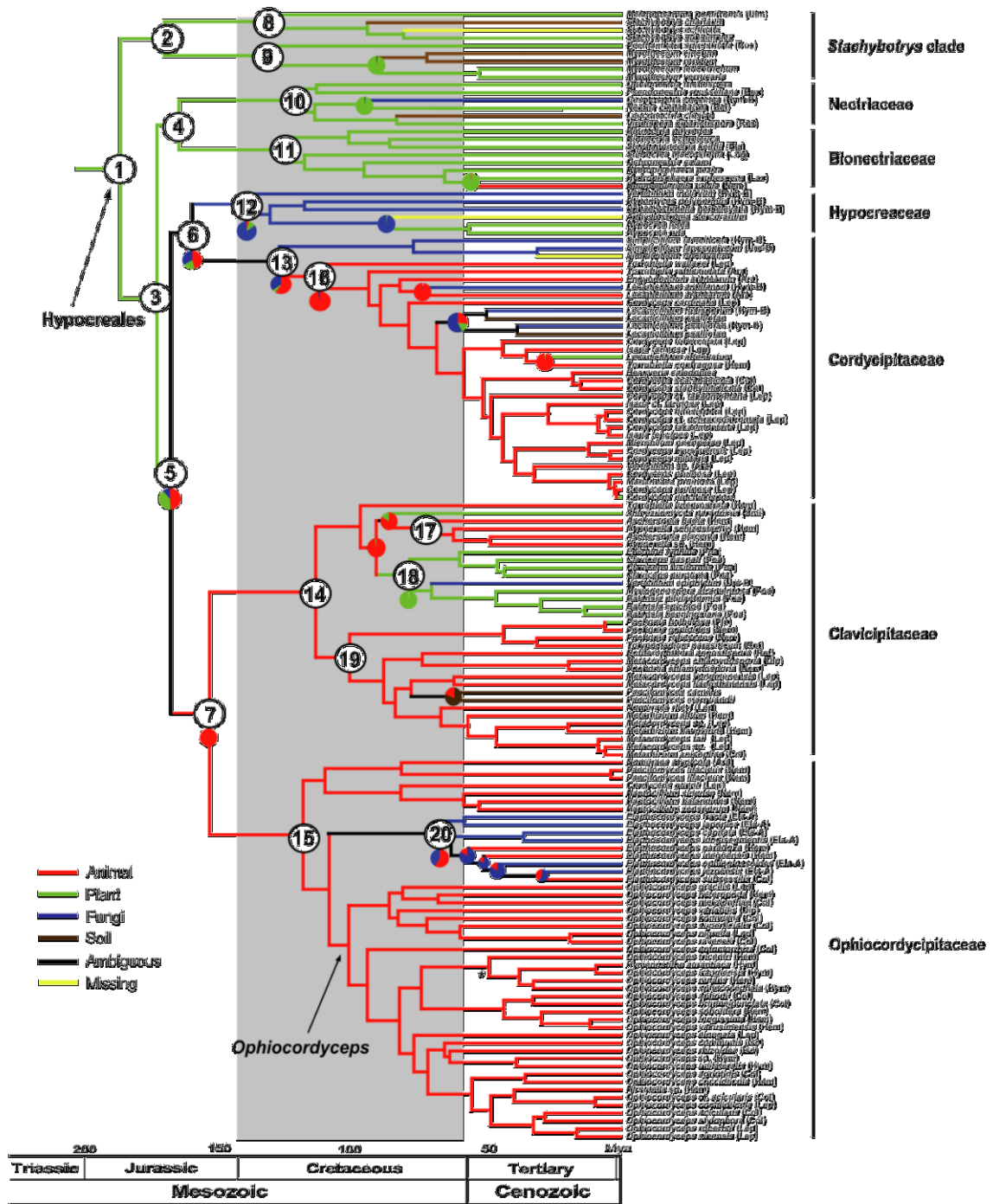


Figura 4: Divergência temporal das maiores famílias da ordem Hypocreales. Calibração das divergências foi baseada em um parasita fúngico (supostamente pertencente a ordem Hypocreales) encontrado aderido ao corpo de um inseto fossilizado em âmbar, do período Cretáceo. As cores agrupam os prováveis estilos de vida/ traços nutritivos das linhagens ancestrais e de espécies atuais. Modificado de Sung e colaboradores (Sung *et al.*, 2008).

Provavelmente uma das características mais singulares do gênero *Metarhizium* seja a sua história evolutiva. A ordem Hypocreales abriga espécies com traços patogênicos com estilos de vida bastante distintos. Fungos do gênero *Trichoderma*, por exemplo, são micoparasitas, com aplicações no controle biológico de fungos fitopatógenos (John *et al.*, 2010). Fungos do gênero *Claviceps*, *Epichlōe*, *Verticillium*, *Bionectria* e *Myrothecium* são fungos fitopatogênicos, epífitas ou endófitos, atuando tanto como causadores de doenças, quanto em mecanismos de defesa de plantas (Lorenz *et al.*, 2010; Guesmi-Jouini *et al.*, 2014; Guerre, 2015; Garibaldi *et al.*, 2016; Wheeler e Johnson, 2016). Fungos do gênero *Stachybotrys* por sua vez, são saprófitas, mas podem causar infecções oportunistas em humanos (Dosen *et al.*, 2016). Fungos do gênero *Beauveria*, *Torrubiela*, *Cordyceps*, *Hypocrella*, *Isaria*, *Aschersonia*, *Pochonia*, *Hirsutella* e *Ophiocordyceps*, em conjunto com fungos do gênero *Metarhizium* são entomopatogênicos, nematófagos e acaropatogênicos, infectando diversos invertebrados (Lee *et al.*, 2005; Sung, Sung, *et al.*, 2007; Schrank e Vainstein, 2010; Zheng *et al.*, 2011; Xiao *et al.*, 2012; Hubner-Campos *et al.*, 2013; Larriba *et al.*, 2014; Guo *et al.*, 2015; Ganaha *et al.*, 2016) (Figura 4).

Usando um fungo, hipoteticamente da ordem Hypocreales, fossilizado em âmbar e reconstrução filogenética, Sung e colaboradores (2008) propuseram que o traço nutritivo ancestral da ordem Hypocreales estaria relacionado a plantas (Sung *et al.*, 2008), diversificando-se ao longo da evolução por múltiplas mudanças de hospedeiro (Kepler *et al.*, 2012). Um suposto ancestral da ordem Hypocreales estaria presente no período Jurássico Inferior, que compreende o período de 199.6 Mya (*million years ago*: milhões de ano atrás) até 175.6 Mya (Figura 4) (Sung *et al.*, 2008). As mais numerosas linhagens familiares da ordem Hypocreales supostamente surgiram no período Jurássico Superior, que compreende o período de 161.2 Mya até 145.5 Mya, com a diversificação destas

famílias no período Cretáceo, de 145.5 Mya até 66 Mya (Sung *et al.*, 2008). Curiosamente, o período Cretáceo é descrito como o período no qual as Angiospermas se diversificaram (Percy *et al.*, 2004). A diversificação das Angiospermas acarretou em mudanças significativas nos ecossistemas terrestres e afetou, significativamente, a biodiversidade terrestre de outros organismos. Um dos mais bem documentados exemplos é a expansão e diversificação de diversas linhagens de artrópodes, intimamente relacionados a Angiospermas (Gaunt e Miles, 2002; Percy *et al.*, 2004). Assim, a diversificação de linhagens de fungos da ordem Hypocreales durante o período Cretáceo está supostamente relacionada à diversificação das Angiospermas e sua biota associada (Sung *et al.*, 2008). Embora ainda existam incertezas, se supõe que o primeiro ancestral da ordem Hypocreales a manter uma simbiose fungo-animal se originou no Jurássico Superior (Figura 4) (Sung, Hywel-Jones, *et al.*, 2007; Sung *et al.*, 2008). Atualmente, aqueles fungos que mantêm simbioses fungo-animal na ordem Hypocreales estão, quase que exclusivamente, restritos a três famílias (Clavicipitaceae, Cordycipitaceae and Ophiocordycipitaceae). A maioria das linhagens fúngicas dessas três famílias possui traços nutritivos relacionados à patogênese de artrópodes, existindo suporte filogenético para o surgimento e diversificação dessas famílias no período Cretáceo (Figura 4) (Sung *et al.*, 2008).

A família Clavicipitaceae, a qual abriga o gênero *Metarhizium*, surgiu e se diversificou no período Cretáceo Inferior, sendo seu nodo basal datado de no mínimo 117 Mya por Sung e colaboradores (2008). Esta família abriga também fungos endofíticos, como o gênero *Epichlöe* e *Balansia*; endofíticos/fitopatogênicos, como o gênero *Claviceps* e fitopatogênicos, como o gênero *Shimizuomyces* (Koroch *et al.*, 2006; Fleetwood *et al.*, 2007; Lorenz *et al.*, 2007; Sung *et al.*, 2010). Existem duas hipóteses em relação ao surgimento desses fungos da família Clavicipitaceae relacionados a plantas. Enquanto

Sung e colaboradores (2008) e Marcet-Houben & Gabaldón (2015), argumentam que essas espécies são derivadas de um ancestral que mantinha uma simbiose fungo-animal (Spatafora *et al.*, 2007; Sung *et al.*, 2008; Marcet-Houben e Gabaldon, 2016), Gao e colaboradores (2011) e Hu e colaboradores (2014) alegam que essas espécies relacionados a plantas são ancestrais em relação às espécies que mantêm simbioses fungo-animal (Gao *et al.*, 2011; Hu *et al.*, 2014). Embora ambas as hipóteses possuam suporte filogenético, nenhuma é conclusiva.

Em relação ao gênero *Metarhizium*, Hu e colaboradores (2014) desempenharam um papel essencial a fim de melhor entender a história evolutiva intra-gênero (Hu *et al.*, 2014). Como anteriormente citado, espécies do gênero *Metarhizium* possuem um espectro de hospedeiros suscetíveis à infecção bastante variado (Schrack e Vainstein, 2010). Existem espécies que são extremamente adaptadas a uma ordem de hospedeiros artrópodes, conhecidas como espécies "hospedeiro-especialistas", como *M. acridum* (adaptada para infectar insetos da ordem Orthoptera) e *M. album* (adaptada para infectar insetos da ordem Hemiptera) (Hu *et al.*, 2014). Outras espécies infectam um número intermediário de artrópodes, conhecidas como espécies "hospedeiro-intermediárias", como *M. majus* e *M. guizhouense* (adaptadas para infectar insetos das ordens Coleoptera e Lepidoptera) (Hu *et al.*, 2014). Ainda são descritas espécies "hospedeiro-generalistas", que infectam mais de sete ordens de insetos (incluindo as ordens Diptera, Lepidoptera, Coleoptera, Hymenoptera, Orthoptera, Blattodeae e Hemiptera), além de ácaros. Dentre as espécies hospedeiro-generalistas, se destacam *M. robertsii*, *M. brunneum* e a espécie mais estudada do gênero, *M. anisopliae* (Hu *et al.*, 2014). Outra característica das espécies hospedeiro-generalistas é a sua capacidade de colonizar as raízes de plantas, podendo contribuir na absorção de nitrogênio pelas plantas hospedeiras (Behie *et al.*, 2012).

O sequenciamento do genoma de *M. acridum*, *M. album*, *M. majus*, *M. guizhouense*, *M. robertsii*, *M. brunneum* e *M. anisopliae*, revelou que espécies hospedeiro-generalistas são derivadas de espécies hospedeiro-intermediárias, que por sua vez são derivadas de espécies hospedeiro-especialistas (Hu *et al.*, 2014). Em geral, os genomas de espécies hospedeiro-intermediárias e hospedeiro-generalistas se caracterizam por apresentar um maior número de fatores de virulência e de famílias de fatores de virulência, com perda de mecanismos relacionados à proteção do genoma contra ácidos nucleicos exógenos e um número maior de eventos de transferência horizontal, quando comparado aos genomas de espécies hospedeiro-especialistas (Hu *et al.*, 2014). Essas diferenças são demonstradas, por exemplo, pelo número de proteínas preditas e anotadas: enquanto *M. acridum* possui 9.849 proteínas anotadas, *M. anisopliae* possui 10.817 proteínas anotadas. Esse maior número de proteínas está refletido em um maior espectro de hospedeiros e em maior flexibilidade de espécies hospedeiro-generalistas (e.g., capacidade de ocupar diferentes nichos).

3.2.4 Interação *Metarhizium*-Hospedeiro

A interação entre *M. anisopliae* e seus hospedeiros artrópodes é um processo complexo, podendo ser didaticamente dividido em oito etapas (Figura 5). Entender detalhadamente estas etapas e quais são as proteínas envolvidas é um dos principais alvos de estudo em fungos entomopatogênicos (Zimmermann, 2007; Schrank e Vainstein, 2010).

Embora existam descrições na literatura de infecção pós-ingestão, esta não parece ser uma via comum de infecção na maioria dos hospedeiros de *M. anisopliae*, sendo a carapaça a via de entrada preferencial (Figura 6) (Leemon e Jonsson, 2012; Butt *et al.*, 2013; Boomsma *et al.*, 2014; Alkhaibari *et al.*, 2016). A adesão do conídio à superfície do hospedeiro, a sua germinação e posterior penetração são etapas críticas no processo de

infecção. Interações hidrofóbicas entre proteínas que recobrem o conídio e a camada lipídica dos artrópodes, e proteínas como hidrofobinas e adesinas desempenham um papel essencial no processo de adesão (Wang e St Leger, 2007; Sevim *et al.*, 2012). Além disso, a presença de aminoácidos livres, peptídeos e outras substâncias podem desencadear a adesão e a germinação (Pedrini *et al.*, 2007a; Zimmermann, 2007). As lipases produzidas pelo fungo também parecem desempenhar um papel essencial no processo de adesão e germinação dos conídios (Gindin *et al.*, 2009; Santi *et al.*, 2010), uma vez que o tratamento de *M. anisopliae* com um inibidor de lipases, preveniu a infecção do carrapato bovino *R. microplus* (Da Silva *et al.*, 2010).

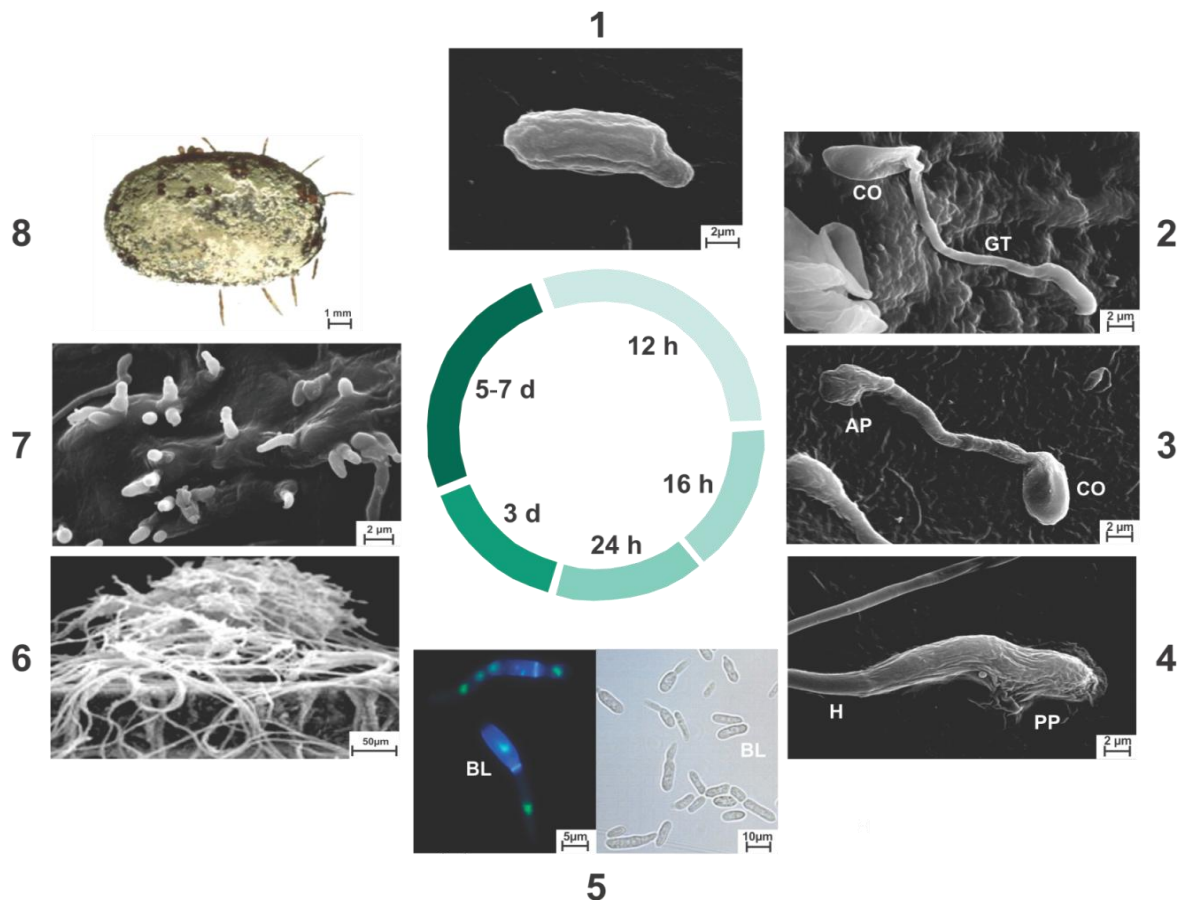


Figura 5: Ciclo infeccioso de *Metarhizium anisopliae*. (1, 2, 3) Adesão do conídio à superfície do hospedeiro, germinação e diferenciação em apressório; (4) Penetração; (5) Blastosporos na hemolinfa

do artrópode; (6) Colonização do artrópode; (7) Extrusão das hifas do cadáver mumificado do artrópode; (8) Conídios e conidióforos sobre a superfície do cadáver mumificado do artrópode. CO- conídio, GT- tubo germinativo, AP- apressório, H- hifa, PP- penetração. Modificado de Schrank & Vainstein (Schrank e Vainstein, 2010).

A germinação do conídio e posterior formação do apressório para a penetração são etapas metabolicamente ativas, onde o fungo deve utilizar reservas endógenas para o seu crescimento (Ment *et al.*, 2012). Fatores ambientais também são essenciais nessa etapa, uma vez que o conídio só irá germinar em condições de temperatura e umidade favoráveis (Pedrini *et al.*, 2007a). O apressório, uma estrutura de penetração especializada, conservada em fungos entomopatogênicos e fitopatogênicos, diferencia-se a partir de uma das extremidades da hifa germinada. Essa estrutura combina pressão mecânica e a secreção de diversas enzimas hidrolíticas para romper a cutícula (Schrank e Vainstein, 2010). Dentre as enzimas secretadas para a degradação de componentes da cutícula estão, entre outras, proteases, esterases, lipases, quitinases, aminopeptidases, dipeptidil peptidases e tripsina (Junges *et al.*, 2014). Algumas destas enzimas são determinantes para o sucesso da infecção (Zimmermann, 2007). As subtilisinas da família Pr1 e quitinases, por exemplo, tem sido alvo de diversos estudos como importantes fatores de virulência (St Leger *et al.*, 1996; St Leger *et al.*, 1998; Bagga *et al.*, 2004).

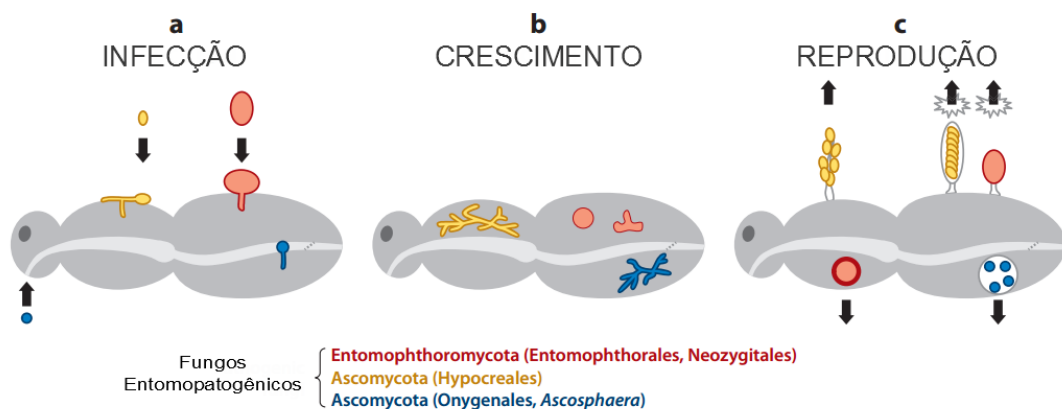


Figura 6: Vias de infecção, crescimento e reprodução de diferentes fungos entomopatogênicos. (a) Enquanto fungos das ordens Entomophthorales, Neozygitales e Hypocreales iniciam sua infecção

através da carapaça, fungos da ordem Onygenales desenvolvem a infecção pós-ingestão de conídios pelo hospedeiro. **(b)** Crescimento de hifas pós-penetração. **(c)** Liberação de conídios no ambiente. Modificado de Boomsma e colaboradores (Boomsma *et al.*, 2014).

Uma vez que as hifas do fungo atravessam a cutícula, *M. anisopliae* se diferencia em estruturas unicelulares leveduriformes, conhecidas como blastosporos, (Schrank e Vainstein, 2010). Estes blastosporos são importantes na evasão da resposta imune, aquisição de nutrientes, colonização e morte dos hospedeiros, sendo dispersos passivamente (Clarkson e Charnley, 1996; Wang e St Leger, 2007; Zhang e Xia, 2009; Alkhaibari *et al.*, 2016).

A hemolinfa dos insetos é rica em trealose, um dissacarídeo de difícil assimilação (Barraza e Sánchez, 2013). O gene *Mpk1*, cujo produto funcional é uma fosfocetolase envolvida na via das pentoses, importante para a assimilação da trealose do hospedeiro, foi adquirida via transferência horizontal gênica (HGT: *horizontal gene transfer*: transferência horizontal gênica) em *M. anisopliae* (Duan *et al.*, 2009). Recentemente, um estudo com o gene *Mr-npc2a* de *M. robertii*, envolvido em rotas de assimilação de esterol do hospedeiro, mostrou que esse gene também foi adquirido por HGT, via evento inseto-fungo. O produto funcional deste gene liga-se preferencialmente ao esterol do inseto, facilitando sua assimilação, não se ligando ao esterol fúngico. A deleção deste gene reduziu a quantidade de esterol nos blastosporos, sendo estes mais suscetíveis à resposta imune do hospedeiro (Zhao *et al.*, 2014). Os eventos de HGT que ocorreram durante a evolução de fungos do gênero *Metarhizium* são de vital importância na patogênese e na capacidade de assimilação de nutrientes do hospedeiro.

Quando o fungo atinge a hemolinfa o período de incubação e morte do inseto é variável. Quando exauridos os nutrientes do hospedeiro, ocorre a extrusão do fungo que passa a se desenvolver na superfície externa do artrópode de maneira saprofítica, com a

mumificação do cadáver e a produção de novos conídios que serão liberados no ambiente (Zimmermann, 2007).

Outro fator importante para o sucesso da infecção é a evasão da resposta imune do artrópode, tema que tem levantado interesse de diversos grupos de pesquisa (Vilcinskas, 2010; 2013). Os artrópodes são os animais mais bem sucedidos na Terra em termos de diversidade de espécies. Seu sucesso evolutivo se deve, em muito, a sua capacidade de adaptação a ambientes inóspitos, patógenos e parasitas (Vilcinskas, 2010; Evans e Schwarz, 2011; Vilcinskas, 2013). A resistência de hospedeiros a fungos entomopatogênicos já foi descrita por alguns pesquisadores (Broza *et al.*, 2001; Gindin *et al.*, 2002; Kirkland *et al.*, 2004; Vilcinskas, 2010; 2013). Essa resistência pode ser o resultado de diversos fatores, atuando de forma sinérgica ou independente. Acredita-se que alterações comportamentais em resposta à infecção podem contribuir para a resistência a fungos entomopatogênicos, sendo esta resposta observada em diversas ordens de insetos. Práticas de higienização do ninho e de indivíduos são um exemplo bastante descrito, observado em formigas (Yanagawa *et al.*, 2008; Tragust *et al.*, 2013). A febre comportamental é também uma alteração bastante comum. Definida como uma alteração aguda nas preferências térmicas do hospedeiro em resposta ao reconhecimento do patógeno, essa alteração tem sido relatada em diversos invertebrados e vertebrados ectotérmicos, podendo favorecer a resposta imune e dificultar o crescimento do patógeno (Boltana *et al.*, 2013). Em gafanhotos infectados, temperaturas corpóreas superiores a 44 °C já foram observadas. Esta elevada temperatura é inibitória ao crescimento da maioria das linhagens de *Metarhizium* (Inglis *et al.*, 1997; Ouedraogo *et al.*, 2003; Arruda, 2005; Elliot *et al.*, 2005). Outra forma de defesa é a carapaça/superfície do hospedeiro, pois além de ser uma barreira física, essa estrutura também pode atuar como uma barreira química.

Apresentando composições variáveis, mesmo entre artrópodes da mesma espécie (Pedrini *et al.*, 2007b), a superfície do hospedeiro pode estar recoberta com compostos tóxicos, inibindo a germinação da hifa, ou ser deficiente em nutrientes que suportem o desenvolvimento do fungo (Butt *et al.*, 1992; Wang e St. Leger, 2005; Pedrini *et al.*, 2007b; Golebiowski *et al.*, 2008; Ment *et al.*, 2012; Ment *et al.*, 2013). A resistência pode ser também adquirida devido a respostas imunes do hospedeiro não só durante a penetração, mas também após. A melanização, uma resposta imune primitiva que não está presente em todos os hospedeiros, consiste na hidroxilação de fenóis e o-fenóis, resultando na formação de melanina a partir de quinonas. Durante este processo, espécies reativas de oxigênio são geradas, sendo tóxicas tanto para o fungo quanto para o hospedeiro (Ment *et al.*, 2012). A melanização ocorre na endocutícula de zonas de penetração, e tem por objetivo interromper o crescimento do patógeno, além de evitar que o patógeno atinja a hemolinfa, sendo uma importante forma de defesa em artrópodes como *Galleria melonella* e carrapatos (Gunnarsson, 1988; Chouvenec *et al.*, 2009; 2011; Ment *et al.*, 2012). Uma vez que a penetração é bem-sucedida, o fungo deve encontrar meios de escapar dos hemócitos, células fagocitárias de invertebrados (Bogus *et al.*, 2007; Ebrahimi *et al.*, 2011). A resposta de hemócitos a infecções fúngicas é pouco estudada, porém um recente trabalho no gafanhoto *Locusta migratoria* identificou quatro tipos de hemócitos atuando na resposta ao fungo *M. acridum* (Yu *et al.*, 2016). Os hemócitos foram classificados como granulócitos, plasmócitos, pré-hemócitos e oenocitóides, de acordo com o seu tamanho, morfologia e propriedades de colaração. Esses hemócitos estavam envolvidos em processos de reconhecimento, encapsulação e digestão de hifas e blastosporos, sendo sugerido que plasmócitos e granulócitos são as principais células de defesa de *L. migratoria* contra infecções fúngicas (Yu *et al.*, 2016). No entanto, embora os hemócitos consigam fagocitar

algumas células fúngicas, uma vez que a penetração ocorre, em muitos casos o hospedeiro não consegue resistir à infecção.

3.2.5 Metabólitos secundários de fungos e sua importância no gênero *Metarhizium*

Durante a infecção dos hospedeiros artrópodes, além de diversas enzimas hidrolíticas que são produzidas e secretadas, e tipos celulares que facilitam a penetração e a dispersão, *M. anisopliae* também produz diversos metabólitos secundários (MSs) com provável atividade inseticida (Molnar *et al.*, 2010). A definição do que são MSs é complexa. Enquanto um metabólito primário (MP) é definido como uma molécula diretamente envolvida no crescimento, desenvolvimento e reprodução de um organismo em condições laboratoriais normais; metabólitos secundários são definidos como moléculas orgânicas de baixo peso molecular que, diferentemente de MPs, não estão diretamente envolvidos no crescimento, desenvolvimento e reprodução de um organismo em condições laboratoriais normais. No entanto, essas moléculas desempenham papéis ecológicos bastante importantes, provendo maior aptidão àquele organismo em determinado habitat (Keller, 2015).

Desde seu descobrimento, os MSs produzidos tanto por fungos, como por bactérias, tem revolucionado a medicina e a saúde humana (Gould, 2016). Antes mesmo dos experimentos de Alexander Fleming sobre a atividade antimicrobiana da penicilina, civilizações antigas já utilizavam pão bolorento para diversos tratamentos (Gould, 2016). Dentre os MSs isolados de fungos, se destacam as já citadas penicilinas e seus derivativos (Bennett e Chung, 2001), estatinas utilizadas em tratamentos para a hipercolesterolemia (Golomb e Evans, 2008), ciclosporinas com atividade imunossupressora e equinocandinas com atividade antifúngica (Dunn *et al.*, 2001; Morrison, 2006). Notavelmente, apenas uma

pequena parcela do potencial global de MSs já foi descoberta e uma parcela ainda menor já foi caracterizada, tendo sua atividade descrita (Yaegashi *et al.*, 2014).

Diversos MSs com atividades de interesse, tanto a nível científico, como comercial, já foram isolados de culturas de fungos entomopatogênicos (Molnar *et al.*, 2010). Alguns exemplos desses metabolitos são o depsipeptídeo beauvericina e o octodepsipeptídeo bassianolide isolados de *Beauveria bassiana* (Xu *et al.*, 2009; Wang e Xu, 2012); Cordicepina isolada de fungos do gênero *Cordyceps* (Tuli *et al.*, 2014) e o ácido hirsutélico isolado de fungos do gênero *Hirsutella* (Thongtan *et al.*, 2006). O depsipeptídeo beauvericina possui atividade moderada contra bactérias e fungos, além de apresentar uma moderada atividade inseticida e uma potente atividade citotóxica contra linhagens celulares humanas. Esse metabólito atua como um ionóforo, transportando Ca^{2+} através de membranas biológicas, aumentando sua concentração no citoplasma, causando a depleção de ATP e ativação de vias apoptóticas sensíveis ao cálcio (Molnar *et al.*, 2010; Gibson *et al.*, 2014). O octodepsipeptídeo bassianolide, por sua vez, possui potente atividade inseticida, além de inibir contrações do músculo liso induzidas por acetilcolina (Xu *et al.*, 2009; Gibson *et al.*, 2014). A cordicepina induz a ativação de múltiplas rotas apoptóticas em células cancerígenas, além de apresentar atividade antiproliferativa (Tuli *et al.*, 2014; Tian *et al.*, 2015), enquanto que o ácido hirsutélico exibe atividade contra o parasita causador da malária *Plasmodium falciparum*, sendo atóxico para linhagens celulares humanas (Thongtan *et al.*, 2006).

Em fungos do gênero *Metarhizium*, o MS mais bem caracterizado é a destruxina e seus derivativos, ciclodepsipeptídeos com diversas atividades biológicas (Liu e Tzeng, 2012). Até o momento, já foram isolados 39 derivativos de destruxinas, não apenas de culturas de fungos do gênero *Metarhizium*, mas também de culturas de *Aschersonia*

aleyrodis, *Alternaria brassicae*, *Beauveria felina* e *Nigrosabulum globosum* (Liu e Tzeng, 2012). Essas moléculas apresentam atividades antitumorais, antivirais, citotóxicas, imunossupressoras, fitotóxicas e antiproliferativas, embora a atividade inseticida seja a melhor caracterizada (Gibson *et al.*, 2014). Em artrópodes, as destruxinas se mostraram capazes de induzir estresse oxidativo, alterar a fosforilação de certas proteínas, causar alterações morfológicas e no citoesqueleto de células de defesa, bloquear a biossíntese de DNA, RNA e proteínas, regular negativamente a expressão de peptídeos antimicrobianos, modular a ativação de canais de cálcio, além de inibir a V-ATPase, enzima que desempenha diversas atividades biológicas em eucariotos (Molnar *et al.*, 2010; Gibson *et al.*, 2014).

Além das destruxinas, diversos outros MSs já foram isolados de culturas de *Metarhizium*, como: (i) metarhizina A, que inibe a proliferação celular (Katou *et al.*, 2014); (ii) swainsonina, metabólito com atividade imunomodulatória e inibidor de glicosil hidrolases (Singh e Kaur, 2014), (iii) citocalasinas, moléculas com diversas atividades: antibacteriana, antiviral, antiinflamatória e citotóxica (Vilcinskas *et al.*, 1997); (iv) aurovertinas, que possuem atividade antifúngica, atuando também como inibidores da ATPase mitocondrial F1F0, a qual catalisa o passo final na fosforilação oxidativa (Azumi *et al.*, 2008); (v) ovalicina, metabólito que tem sido testado para o tratamento da dermatite atópica (Yoon *et al.*, 2011); (vi) ácido helvólico, molécula cuja atividade é antibactericida e antifúngica (Lodeiro *et al.*, 2009); (vii) compostos com atividade mutagênica e efeitos antiproliferativos em células humanas, denominados NG-391 e NG-393 (Donzelli *et al.*, 2010); (viii) sideróforos, importantes para o transporte de ferro como metaquelina, e na virulência como a ferricrocina (Giuliano Garisto Donzelli *et al.*, 2015); além de outros MSs, alguns ainda pouco explorados e caracterizados (Molnar *et al.*, 2010).

Em fungos, os genes que codificam produtos envolvidos na biossíntese de um mesmo MS estão frequentemente agrupados no genoma (*clusters*) sendo co-regulados (Wisecaver e Rokas, 2015). Esses agrupamentos gênicos biossintéticos (BGCs: *biosynthetic gene clusters*) usualmente apresentam genes-chave para a biossíntese dos MSs (*backbone genes*) (Inglis *et al.*, 2013). Os genes-chave mais comumente encontrados são: (i) sintases envolvidas na biossíntese de policetídeos (PKSs: *polyketide synthases*); (ii) sintetases envolvidas na biossíntese de peptídeos não ribossomais (NRPSs: *non-ribosomal peptide synthases*); (iii) híbridos (PKS-NRPS); (iv) ciclases envolvidas na biossíntese de compostos terpênicos (TCs: *terpene cyclases*); e (v) preniltransferases (PTs). Além dos genes-chave, os BGCs apresentam genes adjacentes que auxiliam na regulação, transporte e maturação dos compostos (Inglis *et al.*, 2013). As técnicas de sequenciamento de nova geração, bem como os dados disponibilizados por projetos empregando essas técnicas, somado as ferramentas de bioinformática para a predição de BGCs, revelou que genomas fúngicos podem produzir um número muito maior de MSs do que se postulava anteriormente (Medema *et al.*, 2015). Essa diversidade de BGCs crípticos, cujos produtos não são produzidos em condições laboratoriais rotineiras, reflete a complexidade do habitat onde esses fungos se encontram (Keller, 2015; Netzker *et al.*, 2015). Quando em contato com diversos outros organismos e estresses ambientais presentes no habitat, os MSs são produzidos e desempenham papéis chave nestes processos (Netzker *et al.*, 2015). A ativação e a caracterização destes BGCs crípticos, são uma oportunidade tanto científica quanto comercial (Yaegashi *et al.*, 2014).

Ademais, esses BGCs também são interessantes em nível evolutivo. Tem sido proposto que os agrupamentos dos genes envolvidos na biossíntese de MSs favorece a sobrevivência destes genes, uma vez que BGCs dependem, em parte, de eventos de HGT

para sua dispersão (Walton, 2000). Em concordância com essa hipótese, diversos eventos de HGT envolvendo BGCs fúngicos foram postulados nos últimos anos. Por exemplo, o *cluster* envolvido na biossíntese de esterigmatocistina foi putativamente transferido de uma espécie relacionada ao gênero *Aspergillus* para *Podospora anserina* (Slot e Rokas, 2011). Um segundo exemplo é o cluster ACE1 presente em *Aspergillus clavatus*, o qual foi putativamente adquirido por HGT de um doador relacionado ao gênero *Magnaporthe* (Khaldi *et al.*, 2008). A aquisição de BGCs via HGT também pode estar relacionada ao sucesso de patógenos emergentes. O fungo fitopatógeno *Mycosphaerella populorum* putativamente adquiriu um *cluster* assemelhado ao da quetoglobosina, estando relacionado à infecção do fungo aos seus hospedeiros vegetais (Dhillon *et al.*, 2015). Outro exemplo é a transferência de cromossomos dispensáveis entre linhagens do fungo fitopatógeno *Alternaria alternata* (Mehrabi *et al.*, 2011). Esses cromossomos podem abrigar BGCs para a produção de toxinas, e a aquisição desses cromossomos pode auxiliar na infecção de *A. alternata* a diferentes hospedeiros vegetais (Mehrabi *et al.*, 2011).

Curiosamente, enquanto diversos MSs já foram isolados de culturas de fungos do gênero *Metarhizium*, pouco se conhece a respeito dos genes e seus produtos funcionais envolvidos nessas rotas biossintéticas, e como a ausência desses BGCs influenciam a infecção e o ciclo de vida do fungo. Dos poucos BGCs caracterizados, a ausência de cinco destes não afetou significativamente a virulência: o BGC para biossíntese de serinociclina, responsável por peptídeos cíclicos presentes nos conídios; o BGC para biossíntese de NG-39x; o BGC para biossíntese de metaquelina; e dois BGCs cujos gene-chave são PKSs (*MrPKs1* e *MrPKS2*) (Chen *et al.*; Moon *et al.*, 2008; Donzelli *et al.*, 2010; Giuliano Garisto Donzelli *et al.*, 2015). Até o presente momento, apenas a deleção de genes envolvidos na biossíntese das destruxinas e do sideróforo ferricrocina afetaram a virulência

(Wang *et al.*, 2012; Giuliano Garisto Donzelli *et al.*, 2015). Mutantes de *M. robertsii* cujo BGC para a produção de destruxinas estava ausente apresentaram reduzida virulência à lagarta *Bombyx mori* e ao gafanhoto *L. migratoria* (Wang *et al.*, 2012), da mesma forma que em mutantes cujo gene-chave para a biossíntese de ferricrocina estava ausente apresentaram reduzida virulência à lagarta *Spodoptera exigua* (Giuliano Garisto Donzelli *et al.*, 2015). Considerando que diversos metabólitos com atividade inseticida já foram isolados de culturas de fungos do gênero *Metarhizium*, e boa parte destes compostos ainda não foram caracterizados em nível genômico, sendo os genes para sua biossíntese ainda desconhecidos, análises globais para a identificação de BGCs ligados ao processo de infecção podem levar a descobertas importantes.

3.2.6 A interação *Metarhizium anisopliae*-*Rhipicephalus microplus* explorada por métodos transcritômicos

Como descrito anteriormente, o conhecimento profundo das interações patógeno-hospedeiro é essencial para a formulação de estratégias de controle biológico mais eficientes (Fernandes *et al.*, 2012). Nesse sentido, diversos trabalhos tem sido relatados examinando aspectos básicos da interação *M. anisopliae*-*R. microplus*, abordando desde ensaios microscópicos (como demonstrados na Figura 5) (Arruda, 2005; Schrank e Vainstein, 2010), até interações entre componentes do fungo e componentes da cutícula dos artrópodes (Da Silva *et al.*, 2010; Ment *et al.*, 2013), além de explorar aspectos ambientais que podem influenciar a infecção (Samish *et al.*, 2014) e construção de linhagens mutantes de *M. anisopliae* com genes deletados ou superexpressos. No entanto, um dos trabalhos mais notáveis na caracterização de novos fatores de virulência putativamente envolvidos na infecção de *M. anisopliae* a *R. microplus* foi o ensaio transcritômico (RNA-seq), anteriormente citado, quando *M. anisopliae* foi cultivado em

condições que mimetizavam a infecção (Staats *et al.*, 2014). No trabalho, o fungo foi cultivado em três condições: (I) cultivo durante 48 horas (h) em meio rico (48hC; condição que, teoricamente, não induziria a expressão de fatores de virulência); (II) cultivo durante 48 h em cutículas de carrapato como única fonte de carbono (48hI; condição que, teoricamente, induziria a expressão de fatores de virulência); (III) cultivo durante 144 h em cutículas de carrapato como única fonte de carbono (144hI; condição na qual, teoricamente, o fungo estaria crescendo de forma saprofítica, consumindo os nutrientes do artrópode). Por conseguinte, comparações pareadas foram realizadas a fim de identificar genes diferencialmente expressos: 48hC x 48hI (condição de infecção inicial) e 48hI x 144hI (condição de infecção tardia) (Staats *et al.*, 2014).

Na condição de infecção inicial, 1.237 genes estavam positivamente regulados e 1.062 genes negativamente regulados. Enquanto que na condição de infecção tardia, 564 genes estavam positivamente regulados e 644 genes estavam negativamente regulados. Dentre os genes positivamente regulados na condição de infecção inicial, estavam quatro subtilisinas, enzimas importantes para a degradação da cutícula do artrópode e nutrição, os genes *CASI* e *MASI*, putativamente envolvidos com a formação do apressório, assim como genes que codificam para catalases, quitinases e endopeptidases (Staats *et al.*, 2014). A expressão aumentada desses genes, com provável envolvimento no processo de infecção, indica que o cultivo de 48 h com cutículas de carrapato induziu a expressão de fatores de virulência. Adicionalmente, na condição de infecção tardia, boa parte destes genes com provável importância no processo infeccioso estavam negativamente regulados, enquanto que genes relacionados à biossíntese de proteínas, relacionados ao metabolismo e transporte de nucleotídeos, processos de interação proteína-proteína e que codificam produtos envolvidos na ligação, transporte e metabolismo de RNA estavam positivamente

regulados (Tabela 1) (Staats *et al.*, 2014). Esses resultados indicam que, ao longo do ciclo de infecção, o fungo altera seu perfil transcricional, demonstrado pelos genes relacionados a processos infecciosos, que se apresentavam positivamente regulados na condição de infecção inicial e, em sua maioria, passaram a estar negativamente regulados na condição de infecção tardia. Adicionalmente, na condição de infecção tardia, genes envolvidos na biogênese ribossomal e progressão do ciclo celular estavam positivamente regulados (Staats *et al.*, 2014). Além de comprovar que modelos de infecção mimetizada são úteis, podendo ser utilizados para avaliar e elucidar novos fatores de virulência, a disponibilização desses dados, por si só, já fornece um banco de sequências de possíveis fatores de virulência importantes na interação *M. anisopliae*-*R. microplus*.

Tabela 1: Exemplos de genes diferencialmente expressos nas comparações pareadas do ensaio transcritômico conduzido por Staats e colaboradores (2014) para *M. anisopliae*.

<i>Locus tag</i>	<i>Log-2 fold change</i> 48hCx48hI	<i>Log-2 fold change</i> 48hIx144hI	Descrição	Número de Acesso no NCBI
MANI_010000	3.9	-3.6	Arginil tRNA sintetase	KFG79893.1
MANI_021586	2.5	-4.5	Isoleucil-tRNA sintetase putativa	KFG80873.1
MANI_029315	5	-3.3	Glutamil-tRNA sintetase	KFG84681.1
MANI_003773	9.2	-3.2	Proteína de biogênese ribossomal Rial	KFG82737.1
MANI_019956	3.4	-2.9	Serina/Treonina cinaseRIO1	KFG84633.1
MANI_017853	3.3	-4.4	Gliceraldeido-3-fosfato desidrogenase	KFG83731.1
MANI_008633	2.6	-2.7	Triosefosfato isomerase	KFG86238.1
MANI_009461	*	3.3	Subunidade 1 da ATP-citrato sintase	KFG79391.1
MANI_021563	*	2.2	Protease assemelhada a tripsina	KFG79123.1
MANI_122550	1.5	2.5	Protease assemelhada a tripsina	KFG77596.1
MANI_010008	*	2.4	Tripsina protease	KFG79895.1

MANI_014555	-1.4	2.3	Aspergillopepsina putative	KFG81121.1
MANI_009142	*	-1.2	Aspergillopepsina putative	KFG86315.1
MANI_010753	*	-1.3	Aspartil protease	KFG84389.1
MANI_002103	*	-1.1	Proteinase aspartica secretada	KFG85128.1
MANI_019967	6.1	-5.6	Proteinase aspartica	KFG84632.1
MANI_013010	2.9	-7.7	Endopeptidase aspartica putativa	KFG80419.1
MANI_012760	-6.3	*	Quitinase chimaA4 do subgrupo A	KFG87333.1
MANI_013004	-5.1	*	Proteína que contém o domínio HsbA	KFG80436.1
MANI_013481	-7.6	*	Quitina sintase 4	KFG86503.1
MANI_017353	1,8	*	Nitrato redutase	KFG84561.1
MANI_005230	5.3	-2.3	Proteína ssgA assemelhada a hidrofobina	KFG84331.1
MANI_018952	*	2.2	Proteína assemelhada a metiltransferase SirN	KFG79720.1
MANI_012831	3,5	1,3	Superoxido dismutase	KFG87283.1
MANI_006661	1,2	*	Catalase peroxissomal	KFG85449.1
MANI_010895	5.1	-2.4	Catalase peroxidase bifuncional Cat2	KFG80386.1
MANI_010603	1.3	*	Proteína assemelhada a Glutathiona-S-transferase Ure2	KFG78516.1
MANI_018943	-10.5	*	Glutathiona-S-transferase putativa	KFG79728.1
MANI_028221	8.7	-6.4	Thioredoxina redutase	KFG77993.1
MANI_005169	12.3	-1.7	Proteína antioxidante especifica para thiol putativa	KFG78728.1

As alterações relativas nos níveis de expressão dos genes foram estimadas em 48 horas para a condição controle (hC) e em 48 e 144 horas na condição de infecção mimetizada (hI). *Diferença não significativa (FDR < 0.05 e Log-2 *Fold Change* ≥ 1 or ≤ -1).

4 JUSTIFICATIVA

Metabólitos secundários são moléculas de interesse devido às suas diversas atividades biológicas. Fungos filamentosos são prolíficos produtores de metabólitos secundários, com uma vasta gama de compostos de interesse isolados e caracterizados. Não obstante, o sequenciamento de diversos genomas de fungos filamentosos revelou que o número de compostos isolados e caracterizados é pequeno, quando comparado ao número de genes potencialmente envolvidos na biossíntese desses compostos. Fungos entomopatogênicos, como *Metarhizium anisopliae*, são utilizados no controle biológico de diversos artrópodes, desde vetores de doenças humanas até pragas da agropecuária. O principal foco de estudo em *M. anisopliae* reside em caracterizar genes e produtos funcionais relacionados ao ciclo de infecção, sendo postulado, e parcialmente caracterizado, que metabólitos secundários podem desempenhar papéis de destaque na interação *M. anisopliae*-hospedeiro. No entanto, pouco se conhece das rotas biossintéticas destes compostos, tampouco como esses genes são organizados e regulados em *M. anisopliae*, e no gênero *Metarhizium* como um todo.

A grande diversidade de genes envolvidos na biossíntese de metabólitos secundários (vale ressaltar que esses genes podem atuar na biossíntese de compostos com atividades diversas, não necessariamente somente sobre o ciclo infeccioso) também acrescenta mais um nível de dificuldade na caracterização de possíveis metabólitos secundários que atuem como fatores de virulência. A fim de melhor entender estes aspectos, metodologias que empregam ferramentas de biologia molecular e bioquímica devem ser empregadas. No entanto, estas metodologias demandam tempo, sendo extremamente laboriosas. Adicionalmente, tendo em vista a grande diversidade destes genes, a chance de se caracterizar genes não envolvidos no ciclo infeccioso é grande, como

bem demonstrado por Moon e colaboradores (2008), Donzelli e colaboradores (2010) e Chen e colaboradores (2014). A fim de melhor compreender alguns destes aspectos e encontrar genes com um provável envolvimento na infecção, métodos *in silico* são de grande valia. A utilização conjunta da análise *in silico* e das análises transcritômicas pode resultar na descrição de alvos interessantes para futuras análises funcionais.

O sequenciamento do genoma da linhagem E6 de *M. anisopliae*, acrescido ao estudo transcritômico conduzido em condições que mimetizavam a infecção, torna propício realizar estudos *in silico* para a caracterização de novos fatores de virulência nesta linhagem, principalmente de genes envolvidos na biossíntese de metabólitos secundários.

Adicionalmente, estes genes podem estar envolvidos em eventos de transferência horizontal, sendo passível de explorar a origem e a história evolutiva dos mesmos. A história evolutiva singular do gênero *Metarhizium* também permite avaliar a conservação destes genes intra-gênero e postular hipóteses sobre a importância dos produtos desses genes no espectro de hospedeiros suscetíveis à infecção.

A proposta é identificar e analisar genes envolvidos na biossíntese de metabólitos secundários no fungo *M. anisopliae*, explorando aspectos evolutivos, regulatórios e transcricionais. Essas análises permitem identificar genes de interesse que podem estar fortemente relacionados ao processos infecciosos, sendo estes determinantes para o sucesso do ciclo de vida e infecção do fungo em seus hospedeiros.

5 OBJETIVOS

5.1 Objetivo Geral

Identificar, descrever e avaliar a expressão de genes envolvidos na biossíntese de metabólitos secundários no fungo entomopatogênico *M. anisopliae*.

5.2 Objetivos Específicos

- Identificar genes possivelmente envolvidos na biossíntese de metabólitos secundários no fungo *M. anisopliae*, pelo emprego de ferramentas para a sua predição e identificação;
- Avaliar a expressão dos genes com potencial envolvimento na biossíntese de metabólitos secundários em um estudo transcritômico, previamente publicado pelo grupo, onde foram utilizadas condições de cultivo que mimetizavam a infecção;
- Acessar a conservação dos genes envolvidos na biossíntese de metabólitos secundários em outros fungos do gênero *Metarhizium* que possuem genoma sequenciado (*M. acridum*, *M. album*, *M. majus*, *M. guizhouense*, *M. robertsii* e *M. brunneum*);
- Avaliar evolutiva e filogeneticamente alguns genes identificados;
- Identificar possíveis reguladores globais do traço fúngico que sabidamente influenciam a biossíntese de metabólitos secundários em outras espécies, e avaliar a expressão destes genes em um estudo transcritômico, previamente publicado, onde o fungo foi cultivado em condições que mimetizavam a infecção.

6 RESULTADOS

A coleção de resultados obtidos durante o período do Mestrado propiciou a publicação de um artigo científico no periódico BMC Genomics, intitulado “Secondary metabolite gene clusters in the entomopathogen fungus *Metarhizium anisopliae*: Genome identification and patterns of expression in a cuticle infection model”. O artigo foi aceito para publicação no dia 28 de julho de 2016 e publicado no dia 25 de outubro de 2016, com doi: 10.1186/s12864-016-3067-6.

6.1 Manuscrito: Secondary metabolite gene clusters in the entomopathogen fungus *Metarhizium anisopliae*: Genome identification and patterns of expression in a cuticle infection model

RESEARCH

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Secondary metabolite gene clusters in the entomopathogen fungus *Metarhizium anisopliae*: genome identification and patterns of expression in a cuticle infection model

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Abstract

Background: The described species from the *Metarhizium* genus are cosmopolitan fungi that infect arthropod hosts. Interestingly, while some species infect a wide range of hosts (host-generalists), other species infect only a few arthropods (host-specialists). This singular evolutionary trait permits unique comparisons to determine how pathogens and virulence determinants emerge. Among the several virulence determinants that have been described, secondary metabolites (SMs) are suggested to play essential roles during fungal infection. Despite progress in the study of pathogen-host relationships, the majority of genes related to SM production in *Metarhizium* spp. are uncharacterized, and little is known about their genomic organization, expression and regulation. To better understand how infection conditions may affect SM production in *Metarhizium anisopliae*, we have performed a deep survey and description of SM biosynthetic gene clusters (BGCs) in *M. anisopliae*, analyzed RNA-seq data from fungi grown on cattle-tick cuticles, evaluated the differential expression of BGCs, and assessed conservation among the *Metarhizium* genus. Furthermore, our analysis extended to the construction of a phylogeny for the following three BGCs: a tropolone/citrinin-related compound (MaPKS1), a pseurotin-related compound (MaNRPS-PKS2), and a putative helvolic acid (MaTERP1).

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Results: Among 73 BGCs identified in *M. anisopliae*, 20 % were up-regulated during initial tick cuticle infection and presumably possess virulence-related roles. These up-regulated BGCs include known clusters, such as destruxin, NG39x and ferricrocin, together with putative helvolic acid and, pseurotin and tropolone/citrinin-related compound clusters as well as uncharacterized clusters. Furthermore, several previously characterized and putative BGCs were silent or down-regulated in initial infection conditions, indicating minor participation over the course of infection. Interestingly, several up-regulated BGCs were not conserved in host-specialist species from the *Metarhizium* genus, indicating differences in the metabolic strategies employed by generalist and specialist species to overcome and kill their host. These differences in metabolic potential may have been partially shaped by horizontal gene transfer (HGT) events, as our phylogenetic analysis provided evidence that the putative helvolic acid cluster in *Metarhizium* spp. originated from an HGT event.

Conclusions: Several unknown BGCs are described, and aspects of their organization, regulation and origin are discussed, providing further support for the impact of SM on the *Metarhizium* genus lifestyle and infection process.

Keywords: *Metarhizium* spp, Secondary metabolite biosynthetic gene clusters, Infection process, Transcriptome analysis, Biological control, Cattle tick

Background

The genus *Metarhizium* comprises entomopathogenic fungi that have been employed for the biological control of crop plagues and vector-borne diseases since these species were first described [1]. The wide range of arthropod hosts infected by *Metarhizium* spp. has resulted in a need to better understand the infection process and to improve its modulation for biocontrol. *Metarhizium* spp. are models for host-pathogen interaction studies and virulence factor discovery [2–4] as well as for the development of potential novel applications [5–7]. Additionally, this genus comprises unique evolutionary traits, harboring well-characterized transitional species with varying degrees of host specificity. Some species are host-specialists (*M. acridum* and *M. album*), some demonstrate an intermediate host range (*M. guizhouense* and *M. majus*) and some are host-generalists (*M. anisopliae*, *M. robertsii*, and *M. brunneum*) (Table 1) [8]. Comparative genomic analyses have suggested that generalists evolved from specialists via transitional species with intermediate host ranges [8].

Metarhizium spp. infection begins when fungal conidia adhere to the surface of a suitable host. Host cuticle composition and fungal characteristics determine the host specificity [8, 9]. Under appropriate humidity and temperature conditions, conidia germination gives rise to the germ-tube and to a specialized infection structure, the appressorium. This structure assists the fungus in breaching the host cuticle to reach the hemocoel, where host colonization and sepsis commence, ultimately resulting in host death. During infection, several hydrolytic enzymes, such as chitinases, proteases and lipases, act as important virulence determinants [2]. These enzymes not only facilitate nutritional processes but also morphogenesis and autolytic processes in fungal development [10]. In addition to hydrolytic enzymes, secondary

metabolites (SMs) are also produced by *Metarhizium* to overcome and kill the host [11].

SMs are small molecules with diverse biological activities and applications. Numerous SMs of interest have been isolated from entomopathogenic fungi in recent years (reviewed by [11]), such as beauvericin from *Beauveria bassiana*, which possesses insecticidal, antifungal, antibacterial and potent cytotoxic activities against human cells [11]. Cordycepin, an SM product from *Cordyceps militaris*, exhibits apoptotic and anti-proliferative activities against cancer cells [12], and hirsutellin acid A from *Hirsutiella* spp. demonstrates activity against the malarial parasite *Plasmodium falciparum* [13]. Additionally, many subclasses of destruxins, which exert insecticide, antiviral and cytotoxic effects, have been isolated from *Metarhizium* spp. [11]. The various biotechnological applications of such compounds have aroused great interest in *Metarhizium* spp. as sources of novel control drugs [14, 15].

In fungi, genes for the biosynthesis of SMs are often arranged in clusters and are co-regulated. These biosynthetic gene clusters (BGCs) usually contain backbone genes such as polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS), hybrids (PKS-NRPS), terpene cyclases (TCs) and prenyltransferases (PTs) as well as adjacent genes that assist in regulation, transport and metabolite trimming [15, 16]. Massive sequence data availability, combined with tools to predict BGCs, have revealed that fungal genomes encode far greater numbers of SMs than previously estimated. This diversity of silent metabolites, which are not accessible under normal laboratory culture conditions, reflects habitat complexity [17] and represents great scientific and commercial opportunities [14]. Furthermore, these BGCs are also evolutionarily interesting. It has been proposed that clustering favors the survival of SM genes, and BGCs partially

Table 1 Host range of *Metarhizium* species

<i>Metarhizium</i> species	Host range	Hosts
<i>Metarhizium album</i>	Specialist	Hemiptera;
<i>Metarhizium acridum</i>	Specialist	Orthoptera;
<i>Metarhizium majus</i>	Intermediate	Coleoptera and Lepidoptera;
<i>Metarhizium guizhouense</i>	Intermediate	Coleoptera and Lepidoptera;
<i>Metarhizium brunneum</i>	Generalist	More than seven orders of insects, as well as arachnids;
<i>Metarhizium robertsii</i>	Generalist	More than seven orders of insects, as well as arachnids;
<i>Metarhizium anisopliae</i>	Generalist	More than seven orders of insects, as well as arachnids;

depend on horizontal gene transfer (HGT) for their dispersal [18]. In fact, several horizontally transferred BGCs have been described. For example, the sterigmatocystin cluster was transferred from *Aspergillus* spp. to *Podospira anserina* [19], and the homologous *ACE1* gene cluster in *Aspergillus clavatus* originated via HGT from a donor related to *Magnaporthe* spp. [20]. HGT events for BGCs have also been linked with the success of emergent pathogens, such as *Mycosphaerella populorum*, which acquired a chaetoglobosin-like cluster from an unknown donor that is potentially involved in poplar tree infection [21].

Although a vast array of SM compounds has been isolated from *Metarhizium* species [11, 22–25], few BGCs have been examined at the gene level employing functional mutants [26–30]. The deletion of five genes, specifically a gene from the serinocyclin BGC (synthesis of cyclic peptides in conidia), a gene from the NG39x BGC (synthesis of mutagenic fusarin-like compounds NG391 and NG393), a gene from metachelin BGC (synthesis of siderophore) and two PKS genes (*MrPKs1* and *MrPKs2*), did not significantly affect virulence. Until now, only the deletion of the destruxin and siderophore ferricrocin synthesis genes has been shown to affect virulence. An *M. robertsii* mutant lacking destruxin demonstrated reduced infection efficiency against *Bombyx mori* and *Locusta migratoria* [28], and an *M. robertsii* mutant lacking the siderophore ferricrocin exhibited reduced virulence in *Spodoptera exigua* [30]. Furthermore, it has been suggested that the retention of the destruxin BGC is evolutionarily related to the host range, given that host-specialists do not possess a fully functional destruxin synthesis cluster [28]. This was also suggested in reports predicting BGCs using bioinformatics tools, which indicated that host-specialist species of *Metarhizium* have a different set of BGCs than host-generalist species [8, 31]. In general, these results suggest that the presence of a different range of SMs may be related to the narrowed virulence and specialization of host-specialist species.

Therefore, it is reasonable to assume that many BGCs, including clusters that are not expressed under normal laboratory conditions, participate in the *Metarhizium* spp. infection process. However, the activation of silent clusters, and functional gene analysis methods are laborious and time-consuming. Alternatively, to investigate genes related to infection in a genome-wide strategy, in this work we have deepened the existing knowledge of SMs in the genus *Metarhizium*. We have performed an exhaustive survey and description of BGCs in *M. anisopliae* and assessed the conservation of BGCs and related genes within the *Metarhizium* genus. To validate some of these BGCs, we analyzed RNA-seq data from *M. anisopliae* grown on cattle-tick (*Rhipicephalus microplus*) cuticles to evaluate their differential expression. In addition, we selected three up-regulated BGCs (*Ma-PKS1*, *MaNRPS-PKS2*, and *MaTERP1*) and applied phylogeny and comparative genomic analyses to predict their metabolic pathways and evolutionary history.

Methods

Genomes and RNA-seq data

All fungal genomes were downloaded from the NCBI Genome Database, and the descriptions and accession numbers are displayed in Additional file 1. For RNA-seq experiments, briefly, cattle tick *R. microplus* cuticles were sterilized and used as the sole carbon source for *M. anisopliae* E6 growth. Spore suspensions (5×10^6 spores per ml) were used to inoculate the cuticles by immersion for 30 s. The inoculated cuticles were dispersed over 1 % water agar plates and maintained for 48 h (48hI) and 144 h (144hI) at 28 °C. As a control, the fungus was cultivated in 100 mL of liquid Cove's complete medium (MCc) for 48 h (48hC) at 28 °C. The detailed RNA-seq experimental procedure, sequencing and data management have been previously described [32], and sequencing data are available under accession number PRJNA257269.

Normalization and expression analysis

For expression analysis, we considered RPKM values > 2 to indicate detectable expression. Genes were considered differentially expressed if the corresponding log₂-fold change ratios were > 1 or < -1 , with a 5 % false discovery rate (FDR ≤ 0.05) [32].

BGCs and related gene predictions

Putative BGCs in the *M. anisopliae* genome were identified with the antiSMASH 3.0 [33], SMURF [34], and SMIPS [35] algorithms and previous results from the literature were also examined [31, 36, 37]. The borders of each cluster were initially detected based on the antiSMASH 3.0 prediction [33] and subsequently confirmed with CASSIS, which assumes the

existence of common regulatory patterns in cluster promoters for cluster delimitation [35]. The conservation of predicted clusters among *Metarhizium* spp. was assessed with MultiGeneBlast [38], based primarily on backbone gene conservation (e -value $< 1 \times 10^{-5}$, query coverage > 60 % and identity > 60 %). Afterward, BLASTP (non-redundant protein sequences database, recovering the best 500 hits) was used to search and curate orthologous clusters among other filamentous fungi genomes, and to select putative orthologous backbone genes for the phylogenetic analysis of MaPKS1, MaNRPS-PKS2, and MaTERP1 (e -value $< 1 \times 10^{-5}$, query coverage > 50 and identity > 45 %, ignoring more than one sequence under the same species) [39]. To further confirm that the collected genes were truly orthologous, the backbone genes of MaPKS1, MaNRPS-PKS2, and MaTERP1 were blasted against the MetaPhOrs database [40], and complete genomes were subjected to OrthoMCL curation, a Markov-based algorithm (clustering thresholds: e -value $< 1e-05$ and identity ≥ 30 %) (Additional file 2) [41]. Forty species with complete annotated genomes representing each taxon shown in this study were selected for OrthoMCL analysis (Additional file 1). Additionally, several fungal genomes from the Clavicipitaceae family were deposited at NCBI as raw or incomplete assemblies from projects that generally employed whole genome shotgun (WGS) strategies [42–44]. The contents of several unannotated genomes (*Epichl e festucae*, *Balasia oblecta*, *Epichl e baconii*, *Pochonia chlamydosporia*, *Periglandula ipomoeae*, *Claviceps fusiformis*, *Aciculosporium take*, *Epichl e sylvatica*, *Neotyphodium gansuense*, *Hypocrella siamensis* and *Atkinsonella hypoxylon*) were accessed using BLASTN against the WGS database and MultiGeneBlast. The putative orthologous genes were annotated with FGENESH (gene-finding parameters for *Metarhizium* spp. or *Claviceps* spp.) and aligned with the backbone genes from *M. anisopliae* [45]. Genes that satisfied the previously fixed cutoffs were added to the phylogenetic analysis and cluster curation. Moreover, known global regulators that affect SM biosynthesis were also identified in *M. anisopliae* and their expression was evaluated.

Phylogenetic analysis

A special procedure was adopted for the MaPKS1 phylogeny: given that the phylogeny of PKS genes can be chaotic, particularly for ortholog definition as it is difficult to differentiate orthologous from non-orthologous genes, we generated a tree that included collected entries comprising putative orthologs of MaPKS1, all PKS from *M. anisopliae* E6, and all characterized PKS from MIBiG, a database of characterized biosynthetic gene clusters [46]. An amino acid alignment was built using PRANK, and the evolutionary history was inferred using the

Maximum Parsimony method with 1,000 bootstrap replicates and MrBayes [47–49] for 10^7 generations (sampled every 100 steps), applying an average standard deviation of split frequencies < 0.01 as the convergence criterion. Parameters and trees obtained through the Bayesian approach were summarized by applying a 25 % burn-in (these trees are contained in Additional file 3) [50, 51].

After this confirmation, amino acid (BLAST collected entries for MaPKS1 and MaNRPS-PKS2) and nucleotide alignments (*tefl* gene, detailed below) were built and trimmed with GUIDANCE using PRANK as an MSA algorithm with default parameters [50, 52] to generate the phylogenetic analysis (these alignments are contained in Additional files 4, 5, 6). The best-fit evolutionary model for each alignment was assessed using Prottest 3.4 [53] for proteins and jmodeltest-2.1.9 [54] for nucleotides (Additional file 7). Phylogenetic trees were constructed using PhyML 3.1 [55] with 100 bootstrap replicates, and MrBayes with the same parameters as described above.

To generate the phylogeny of the putative helvolic acid cluster (MaTERP1; possibly obtained via HGT), protein sequences for each orthologous gene (excluding genes involved in fusidic acid biosynthesis; for a detailed explanation see Additional file 8) that belong to this cluster were recovered and processed as described above. An matrix representation parsimony (MRP) supertree was constructed based on the inferred trees with CLANN 3.1.3 with 100 bootstrap replicates [56]. Alternatively, the alignments were concatenated into a supermatrix using SeaView (supermatrix alignment is contained in Additional file 9) [57]. Evolutionary model analysis and phylogenetic inference for this supermatrix followed the procedures described above, and the tree was inferred using PhyML 3.1 (run with 1,000 bootstrap replicates). The cluster trees were rooted at species from the *Aspergillus* genus, and the topology was similar when the trees were unrooted or rooted at the midpoint. Both the supertree and the supermatrix tree were compared with the species tree to highlight possible HGT events implicated in the evolutionary history of this BGC [21, 58]. The species tree was based on the translation elongation factor 1-alpha (*tefl*) barcode and rooted at fungal species that did not belong to the Pezizomycotina class. The *tefl* gene is the current barcode pattern for species delimitation and classification in the *Metarhizium* genus and Clavicipitaceae family [59, 60]. The inferred species tree was analyzed manually for conflicts and incongruities with the current fungi and Clavicipitaceae evolutionary history [61, 62].

Results

BGC predictions and boundaries delimitation

The genome survey predicted 73 putative BGCs, comprising to twenty-two PKS, thirteen NRPS, nine terpenes

(TERP), seven NRPS-PKS, three indoles (IND), two IND-NRPS, 1 IND-TERP, 1 TERP-PKS, 1 siderophore (SID), and fourteen BGCs, classified by antiSMASH as "OTHER", a generic class of clusters encoding unusual BGCs (Additional file 10). Our survey found more BGCs than any other survey previously published for *Metarhizium* spp. [8, 31].

To refine the BGC boundaries, the predicted backbone genes were subjected to CASSIS, which assumes the presence of common regulatory patterns among genes from the same cluster. CASSIS was chosen because other tools only predict backbone genes (e.g., SMIPS), ignoring accessory genes, or overrating cluster boundaries (e.g., SMURF and antiSMASH). Based on the CASSIS prediction, 49 BGC boundaries were reassigned when compared to the previous antiSMASH prediction (Additional file 11; BGCs with reassigned boundaries are marked). However, the CASSIS results must be carefully analyzed, because other layers of regulation may be present, and some accessory genes may not exhibit the same regulation patterns found in the rest of the cluster [35].

Conservation of BGCs in the *Metarhizium* genus and host range

The majority of BGCs (> 83 %) found in *M. anisopliae* are well conserved in host-generalists (*M. robertsii* and *M. brunneum*) and intermediate-host-range species (*M. guizhouense* and *M. majus*); including *M. robertsii* ARSEF23 (69 conserved clusters [cc]), *M. brunneum* ARSEF3297 (70 cc), *M. guizhouense* ARSEF977 (64 cc) and *M. majus* ARSEF297 (61 cc) (Additional file 10). Some SM clusters were also found to be conserved in host-specialist species, such as *M. acridum* CQMa102 (35 cc) and *M. album* ARSEF1941 (30 cc); however, this conservation was present to a lesser degree (Additional file 10).

Comparative genomic analysis of BGCs and phylogeny

Comparative genomic analysis was employed to clarify the predictable final products of the assigned BGCs, integrating these data with BGCs previously characterized in *Metarhizium* spp. These comparisons revealed certain interesting clusters, which are listed in Additional file 10. MaPKS2 (MANI_004781) was predicted to be responsible for the biosynthesis of aurovertins, which are metabolites that have already been isolated from *Metarhizium* spp. cultures [63] but lack a characterized BGC. MaPKS2 exhibited 42–77 % identity with the BGC responsible for aurovertin biosynthesis in *C. arbuscula* (Additional file 12) [64]. MaTERP2 (MANI_002110) was assigned as a lanosterol cyclase, exhibiting 79 % identity with the partially characterized lanosterol cyclase from *Trichoderma harzianum* [65]. The final product of

MaIND-NRPS1 (MANI_029655) was predicted to be an elymoclavine/ergovaline-related compound. This generic classification took into account the conservation between MaIND-NRPS1 and both elymoclavine and ergovaline characterized clusters (Additional file 12). MaIND-NRPS1 (MANI_029655) exhibited 57–77 % identity with a portion of the elymoclavine BGC from *C. fusiformis* [66]. Furthermore, the NRPS gene (MANI_029666) internal to the cluster exhibited 59 % identity with the biosynthetic ergovaline NRPS from *Neotyphodium lolii* [67] (Additional file 12). A generic classification was also applied for MaIND-TERP1 (MANI_011022) and MaNRPS-PKS3 (MANI_023437), which were designated as a terpendole E/lolitrems-related compound, and xenolozoyenone-related compound, respectively. MaIND-TERP1 exhibited 60–75 % identity with the biosynthetic terpendole E BGC characterized in *Chaunopycnis (Tolypocladium) alba* [68], and 59–77 % identity with the biosynthetic lolitrems BGC characterized in *E. festucae* [69, 70] (Additional file 12). However, MaIND-TERP1 cluster contains additional genes that are not conserved in the terpendole E and lolitrems clusters; these genes could potentially participate in the biosynthesis of the resulting terpendole E/lolitrems-related compound. MaNRPS-PKS3 exhibited 31–50 % identity with the xenolozoyenone BGC characterized in *Glarea lozoyensis* [71] (Additional file 12). Despite the low identity, both clusters were phylogenetically related as determined by Yue and coworkers (2015), further supporting our proposed assignment.

For the MaNRPS-PKS2 (MANI_018878), MaTERP1 (MANI_010527/MANI_010530/MANI_010531/MANI_010532), and MaPKS1 (MANI_014762) clusters, we deepened the comparative genomic analysis by performing a phylogeny. This phylogeny was performed because these three clusters are up-regulated during early infection (48hC x 48hI; following section), have a narrow cluster distribution among fungi (differing from MaTERP2, for example, which is also up-regulated in early infection, but is ubiquitous among Ascomycota), may have originated in *Metarhizium* spp. via HGT events and are located in singular genomic regions.

MaNRPS-PKS2 (MANI_018878) matched the characterized pseurotin BGC from *A. fumigatus* with considerable identity (63–81 %) (Fig. 1a and b) [72]. Furthermore, the search for orthologs and phylogenetic analysis revealed a restricted cluster distribution among filamentous fungi, with conservation observed only in host-generalist *Metarhizium* spp. (Fig. 1a). In *A. fumigatus*, this BGC is located in a singular genomic region with intertwined biosynthetic genes involved in the formation of fumitremorgin, fumagillin, and pseurotin. This region is under the control of the global regulator LaeA, and fumagillin and pseurotin are co-regulated by a supercluster-embedded regulatory

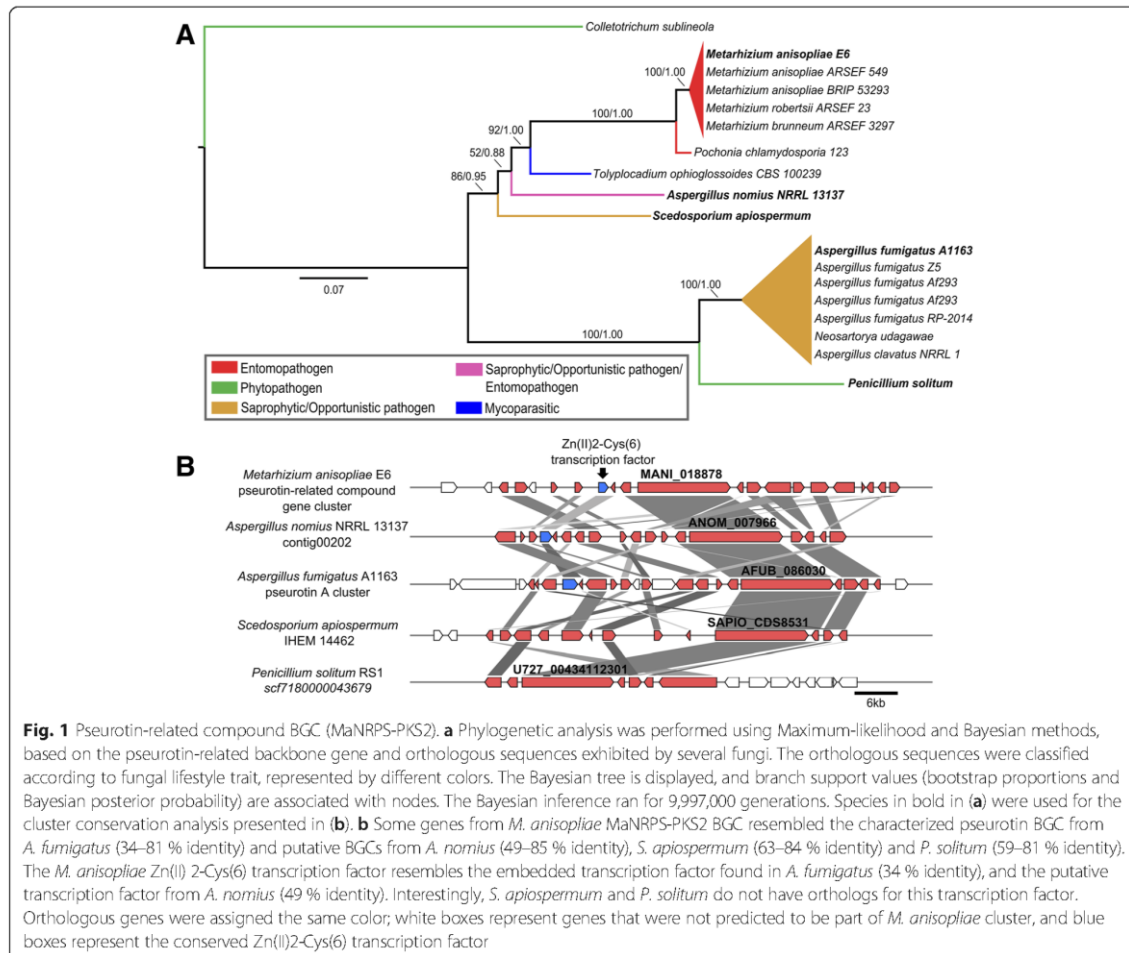
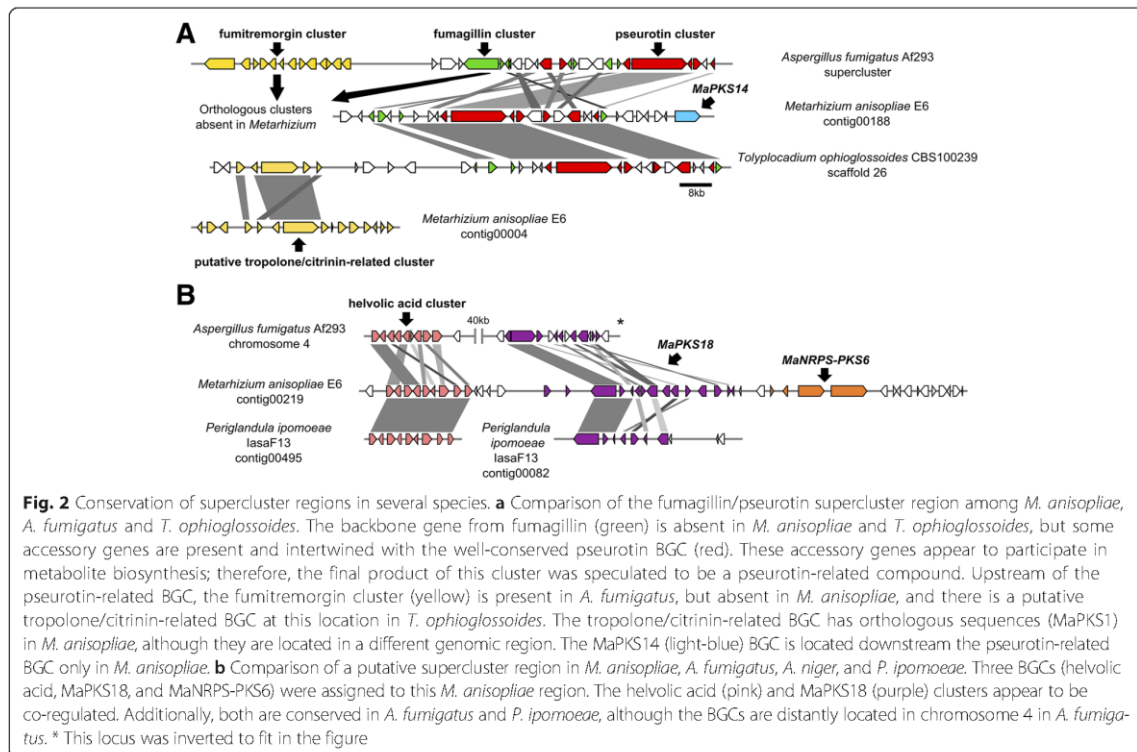


Fig. 1 Pseurotin-related compound BGC (MaNRPS-PKS2). **a** Phylogenetic analysis was performed using Maximum-likelihood and Bayesian methods, based on the pseurotin-related backbone gene and orthologous sequences exhibited by several fungi. The orthologous sequences were classified according to fungal lifestyle trait, represented by different colors. The Bayesian tree is displayed, and branch support values (bootstrap proportions and Bayesian posterior probability) are associated with nodes. The Bayesian inference ran for 9,997,000 generations. Species in bold in **(a)** were used for the cluster conservation analysis presented in **(b)**. **b** Some genes from *M. anisopliae* MaNRPS-PKS2 BGC resembled the characterized pseurotin BGC from *A. fumigatus* (34–81 % identity) and putative BGCs from *A. nomius* (49–85 % identity), *S. apiospermum* (63–84 % identity) and *P. solitum* (59–81 % identity). The *M. anisopliae* Zn(II) 2-Cys(6) transcription factor resembles the embedded transcription factor found in *A. fumigatus* (34 % identity), and the putative transcription factor from *A. nomius* (49 % identity). Interestingly, *S. apiospermum* and *P. solitum* do not have orthologs for this transcription factor. Orthologous genes were assigned the same color; white boxes represent genes that were not predicted to be part of *M. anisopliae* cluster, and blue boxes represent the conserved Zn(II)2-Cys(6) transcription factor

gene [36]. In the genus *Metarhizium*, this BGC appears to have been horizontally acquired from an unknown donor, and fumitremorgin and fumagillin backbone genes are absent, although it is likely that some fumagillin accessory genes were also horizontally acquired (Fig. 2a). These accessory genes are strongly up-regulated, similar to the remaining pseurotin cluster (Table 2), indicating their likely participation in compound biosynthesis and leading us to classify the final product of this cluster as a pseurotin-related compound. Although CASSIS was unable to detect similar regulatory regions in the promoters of the pseurotin-related compound BGC, the embedded Zn(II) 2-Cys(6) transcription factor highlighted in *A. fumigatus* is conserved in *M. anisopliae* (MANI_018928; 34 % identity). This transcription factor can regulate the entire cluster and genes in the vicinity, analogous to the regulation that occurs in *A. fumigatus*. In addition, there is one other up-regulated backbone gene (MaPKS14;

MANI_018879; Additional file 11) that is located near the pseurotin-related compound in *Metarhizium*, indicating possible co-regulation (Fig. 2a). Similarly, a pseurotin-related cluster located near an orthologous for MaPKS1 (although MaPKS1 is located in another genomic region in *Metarhizium* spp. genomes) was detected in *Tolylocadium ophioglossoides* (Fig. 2a). These results suggest that pseurotin and pseurotin-related compound clusters can be embedded in superclusters in different vicinities and configurations. Furthermore, these differences in pseurotin cluster location favor the proposed explanation that this cluster is located in highly variable regions in different genomes.

Given that supercluster arrangements are misleading when performing BGC predictions using search algorithms [36], we identified another putative supercluster in intermediate- and generalist-host-range *Metarhizium* spp. This putative supercluster is located in *M. anisopliae*



contig 219, and is comprised of three clusters: MaPKS18 (MANI_010451), MaTERP1 (putatively enrolled in helvolic acid biosynthesis, as detailed below) and MaNRPS-PKS6 (MANI_010456/ MANI_121659) (Fig. 2b). This sequence region misleads the antiSMASH prediction, being the BGCs delimited by CASSIS and previous results from the literature. Furthermore, there is an apparent co-regulation of both the MaPKS18 and putative helvolic acid BGCs, which are up-regulated in early infection (48hC x 48hI) and down-regulated in late infection (48hI x 144hI), supporting the supercluster hypothesis (Table 3; Additional file 11). The putative helvolic acid (MaTERP1) and MaPKS18 BGCs have orthologs in *A. fumigatus* and *P. ipomoeae* (Fig. 2b). In *A. fumigatus* Af293, both clusters are located in the same chromosome and are separated by 40 kb, but the quality of the *P. ipomoeae* lasaF13 genome did not permit a synteny comparison. This set of results reinforces the notion that BGCs are located in rapidly evolving genomic regions and suggests that superclusters can be widespread in multiple fungal genomes.

The putative helvolic acid BGC (MaTERP1) showed considerable identity (41–65 %) with the helvolic acid cluster from *A. fumigatus* [37] (Fig. 3; Table 3). The helvolic acid cluster is organized around the prostadienol

synthase gene AFU4G14770 in *A. fumigatus*. This cluster is unique, containing four backbone paralogous genes. As already suggested, this cluster evolved by gene duplication and differentiation from an ancestral monooxygenase, a transferase and two dehydrogenases [37]. This configuration is also observed in *M. anisopliae*, with four backbone genes (MANI_010527/MANI_010530/MANI_010531/MANI_010532). Furthermore, in addition to the backbone genes, all accessory genes highlighted in *A. fumigatus* are conserved in *M. anisopliae* (Fig. 3). Additionally, the isolation of helvolic acid from *Metarhizium* cultures supports the suggestion that MaTERP1 is responsible for metabolic biosynthesis [25].

Additionally, the phylogenetic analysis revealed a narrow cluster distribution (Fig. 4). This BGC is only found in intermediate- and generalist-host range *Metarhizium* spp., *P. ipomoeae* (Hypocreales order), and species from the *Aspergillus* genus (Eurotiales order), and is absent in *Metarhizium* host-specialist species and other members of the Clavicipitaceae family. The phylogenetic trees presented in this work, the strong gene conservation and uncommon cluster origin/formation, suggesting that this cluster may have been originated in *Metarhizium* species via an HGT event from a donor species closely related to the Eurotiales order (Fig. 4). This

Table 2 Expression profiling of the *M. anisopliae* cluster related to the biosynthesis of a pseurotin-related compound

NCBI gene locus ID	Expression (RPKM)			Differential expression (log ₂ -fold change)		Gene product
	48hC	48hl	144hl	48hCx48hl	48hx144hl	
MANI_029058	0.00	0.34	0.34	NA	NA	Hypothetical protein
MANI_018942	1.75	0.00	5.75	NA	NA	Hypothetical protein
MANI_029071	0.00	209.91	134.67	9.77	ND	Integral membrane protein
MANI_018916	0.55	492.48	212.39	9.64	-1.28	Cytochrome P450
MANI_018955	1.03	37.39	59.92	5.02	ND	Hypothetical protein
MANI_018958	2.21	355.01	165.40	7.30	-1.10	Phytanoyl-CoA dioxygenase
MANI_018941	1.92	422.89	226.54	7.76	ND	Phytanoyl-CoA dioxygenase
MANI_018928	0.00	63.24	30.65	8.67	-1.06	C6 finger transcription factor
MANI_018959	1.84	986.40	323.46	8.90	-1.57	Hypothetical protein
MANI_018934	0.65	268.46	62.03	8.51	-2.11	Alpha/beta hydrolase
MANI_018878	0.44	736.13	182.13	10.78	-1.96	Hybrid NRPS/PKS enzyme
MANI_018952	0.00	1129.61	347.42	12.26	-1.68	Methyltransferase
MANI_029062	0.00	286.33	99.09	11.23	-1.50	Cytochrome P450
MANI_120428	31.50	117.64	50.96	2.02	-1.25	Methionine aminopeptidase
MANI_029068	109.68	117.21	86.21	ND	ND	Methionine aminopeptidase
MANI_018894	0.00	131.20	48.31	10.56	-1.38	Acetate-CoA ligase
MANI_111428	0.98	599.02	205.75	9.29	-1.51	Steroid monooxygenase
MANI_018962	0.00	269.62	67.44	9.55	-1.94	Hypothetical protein
MANI_018945	0.00	15.06	4.25	NA	NA	Methyltransferase
MANI_018943	0.00	296.14	80.09	10.19	-1.81	Glutathione S-transferase
MANI_018919	0.74	889.71	213.01	10.07	-2.08	O-methyltransferase

The relative changes in expression levels were estimated at 48 h for the control condition (hC) and both 48 and 144 h for infection conditions (hl). NA Not Available, ND No Difference

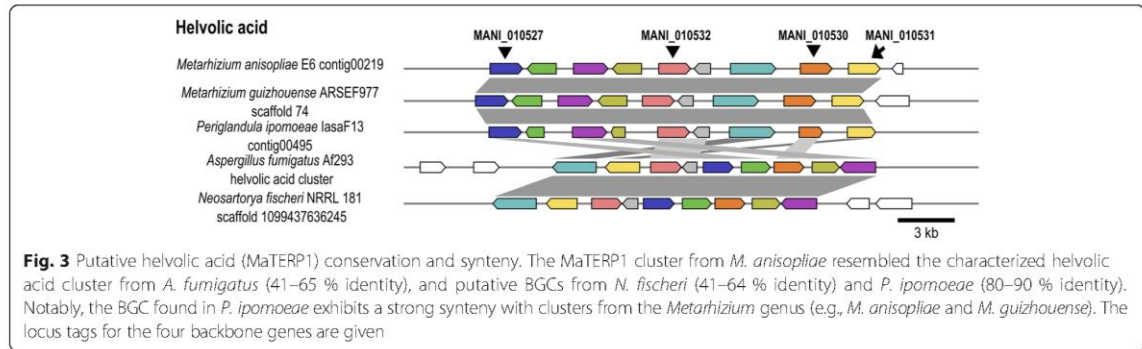
hypothesis is supported by the large evolutionary distance between Eurotiomycetes and Sordariomycetes (which diverged approximately 400 million years ago [MyA]) [73] and by the absence of a complete helvolic acid BGC in other species of the Hypocreales order (Fig. 3; Additional file 4).

Another contribution of functional genomics coupled with phylogenetic analysis was the partial elucidation of the first steps in the biosynthesis of up-regulated MaPKS1. The *MaPKS1* backbone gene (MANI_014762) is orthologous to several characterized backbone genes: tropolone/stipitatic acid [74], citrinin [75], phomenoic

Table 3 Expression profiling of the *M. anisopliae* cluster related to the biosynthesis of a helvolic acid compound

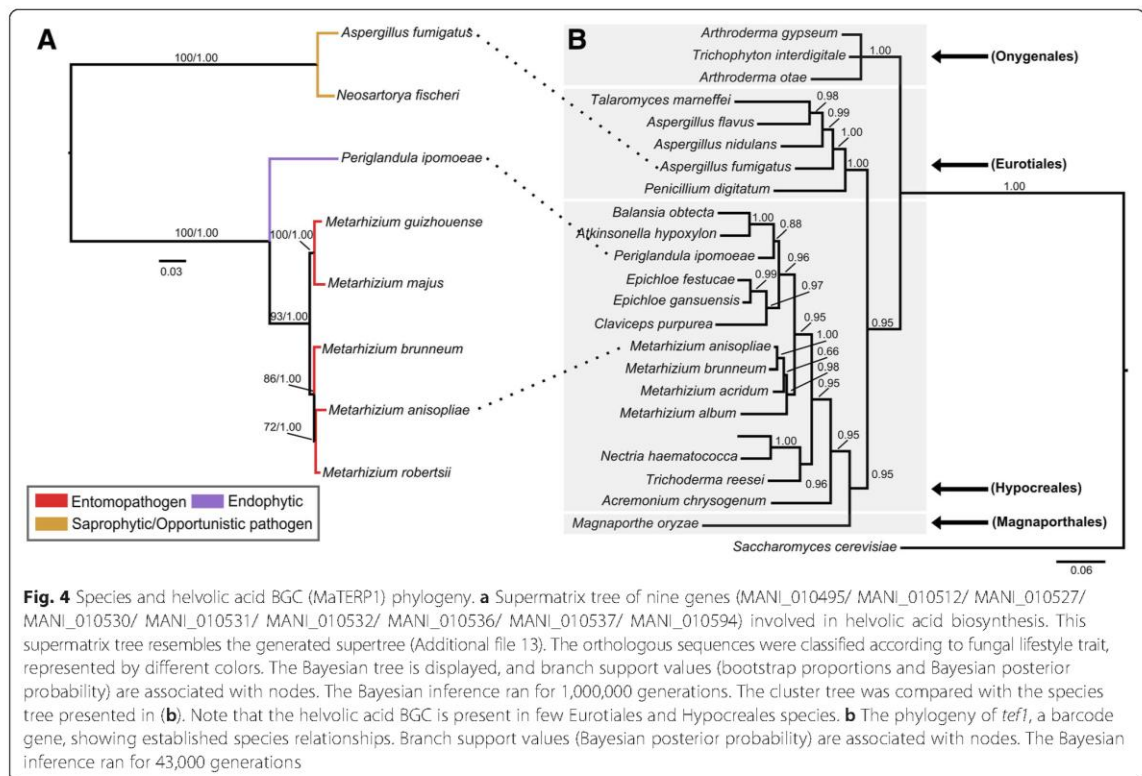
NCBI gene locus ID	Expression (RPKM)			Differential expression (log ₂ -fold change)		Gene product
	48hC	48hl	144hl	48hCx48hl	48hx144hl	
MANI_010527	0.54	13.59	2.67	4.51	-2.27	Cytochrome P450
MANI_010536	8.44	36.91	1.48	2.22	-4.50	Transferase family protein
MANI_010512	4.59	25.08	0.42	2.56	-5.58	FAD binding domain-containing
MANI_010537	3.03	30.78	4.47	3.42	-2.72	Transferase family protein
MANI_010532	0.00	12.57	0.91	6.73	-3.60	Cytochrome P450
MANI_010594	3.34	25.10	6.37	2.94	-1.90	3-oxoacyl-reductase 1
MANI_010495	9.06	87.20	9.34	3.44	-3.17	Squalene-hopene-cyclase
MANI_010530	11.58	48.40	11.28	2.21	-2.09	Cytochrome P450
MANI_010531	84.51	57.83	17.62	NA	-1.67	Cytochrome P450

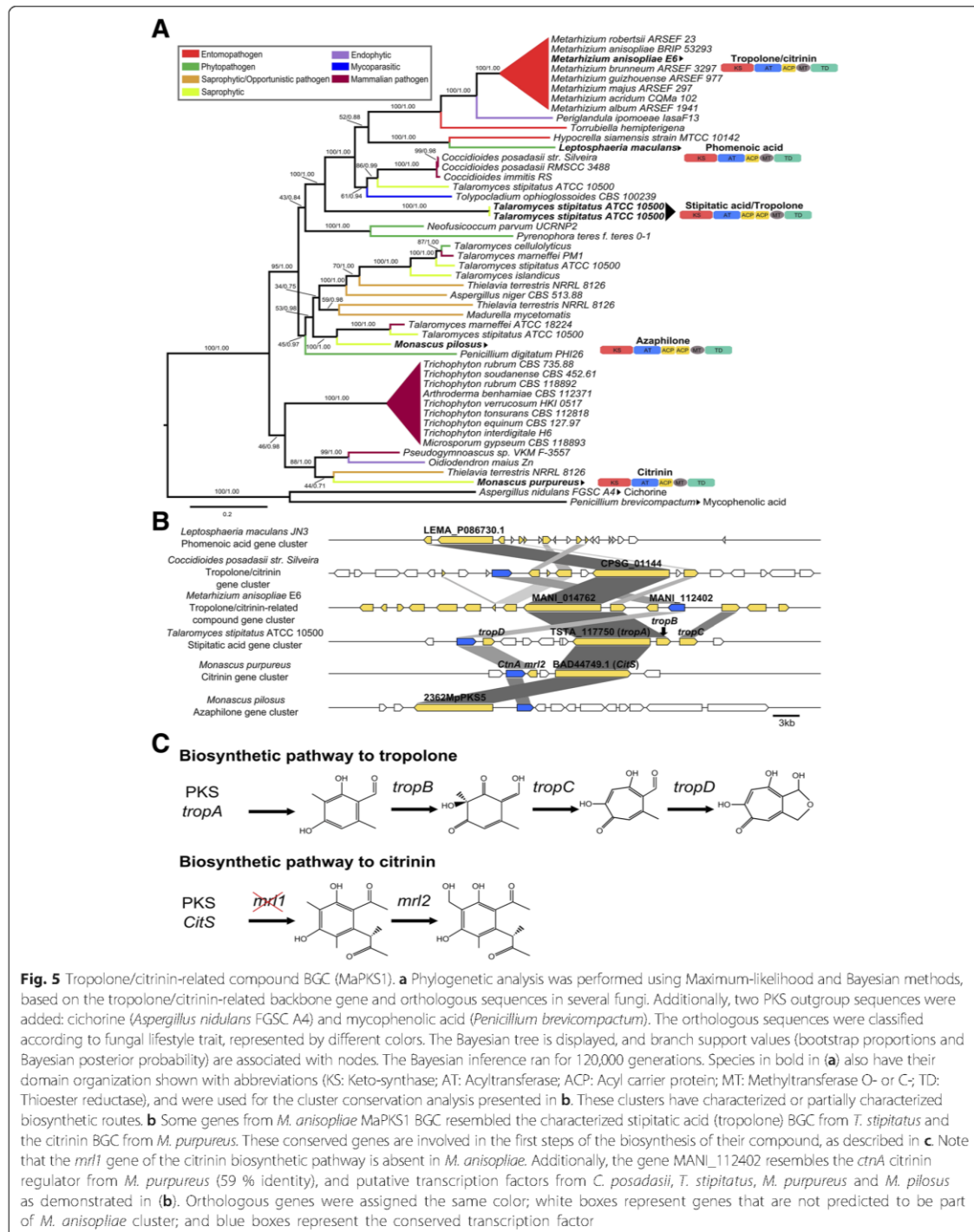
The relative changes in expression levels were estimated at 48 h for the control condition (hC) and both 48 and 144 h for infection conditions (hl). NA Not Available, ND No Difference



acid [76] and azaphilone [77]. These orthologs suggest that a similar biosynthetic route is partially shared between MaPKS1 and these characterized metabolites (Fig. 5a e b). The conserved genes between MaPKS1 and the tropolone/stipitatic acid route are involved in the first steps of metabolic biosynthesis. The stipitatic acid backbone gene *tropA* exhibits 46 % identity with the *MaPKS1* backbone gene (MANI_014762), *tropB* exhibits 47 % identity with MANI_014847, *tropC* exhibits 60 % identity with MANI_112407 and *tropD* exhibits 64 % identity with MANI_014887 (Fig. 5c). Similarly, two

genes conserved between MaPKS1 and the citrinin biosynthetic route are also involved in the first steps of metabolic biosynthesis. The citrinin backbone gene *CitS* exhibits 45 % identity with the *MaPKS1* backbone gene (MANI_014762), and *mrl2* exhibits 37 % identity with MANI_014887, although *mrl1* is absent (Fig. 5c). The potential conservation of these first metabolic steps supports the hypothesis that the final product of this BGC has, at minimum, the same biosynthetic origin and is related to tropolones and citrinins. However, the CASSIS prediction delineates a BGC comprised of 15 genes





(several genes are not conserved in the tropolone or citrinin routes), leading us to classify the product of this cluster as a potential and generic tropolone/citrinin-related compound.

Our analysis also suggested that the self-transcription factor (MANI_112402) is involved in MaPKS1 regulation (Table 4). This gene exhibits strong expression (>6-fold difference with $P < 0.05$) and 59 % identity with the transcription factor *ctnA* from the *M. purpureus* citrinin cluster (Fig. 5b). In *M. purpureus*, the deletion of *ctnA* caused a large decrease in citrinin production [78]. Moreover, a similar gene is also conserved in the *Coccidioides posadasii* putative tropolone/citrinin BGC, in the *T. stipitatus* tropolone BGC, and in the *Monascus pilosus* azaphilone BGC, suggesting a similar, widespread regulatory strategy among these BGCs (Fig. 5b; genes marked in blue). It would be simple to characterize this BGC in *Metarhizium* by constructing a knockout strain for this gene.

Expression of BGCs

To validate some of our predictions regarding *M. anisopliae* BGCs, we conducted RNA-seq analysis using a system mimicking host infection. The fungus was cultured in Cove's Complete Medium (C: Control condition) and in the presence of tick cuticles (I: Infection condition)

for 48 or 144 h, as described in the Materials and Methods. Two pairwise comparisons were performed: 48hC x 48hI (early infection conditions) and 48hI x 144hI (late infection conditions). Nearly half of the predicted SM clusters, specifically 49 (36/73), were expressed (RPKM ≥ 2) under the analyzed culture conditions, and 20 % (15/73) were up-regulated under early infection conditions (48hI x 48hC), highlighting their potential relevance in the initial steps of infection. Conversely, 7 % (5/73) of the predicted BGCs were down-regulated when comparing 48hI versus 48hC. No up-regulated cluster was detected when 48hI was compared with 144hI; however, 14 BGCs were down-regulated. Moreover, of those 14 down-regulated BGCs, 9 were up-regulated under early infection conditions (Additional file 10).

The 15 BGCs up-regulated under early infection conditions included the already-cited tropolone/citrinin-related compound BGC (MaPKS1) (Table 4), the pseurotin-related compound BGC (MaNRPS-PKS2) (Table 2), the lanosterol cyclase BGC (MaTERP1), and the putative helvolic acid BGC (MaTERP2) (Table 3), in addition to the already-characterized destruxin (MaNRPS1) (Table 5), NG39x (MaNRPS-PKS1) (Table 6) and ferricrocin (MaNRPS9) (Additional file 11) clusters; Additionally, there were eight putative clusters of uncharacterized

Table 4 Expression profiling of the *M. anisopliae* cluster related to the biosynthesis of a tropolone/citrinin-related compound

NCBI gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)		Gene product
	48hC	48hI	144hI	48hCx48hI	48hIx144hI	
MANI_014850	37.70	8.46	12.14	-1.95	ND	Mercuric reductase
MANI_014940	0.00	13.82	33.93	ND	1.36	Hypothetical protein
MANI_014941	0.00	0.00	1.19	NA	NA	Hypothetical protein
MANI_014846	0.55	3.11	0.00	NA	NA	Major Facilitator Superfamily protein
MANI_014815	1.74	1.05	0.71	NA	NA	Hypothetical protein
MANI_028157	23.92	1.45	0.98	-3.76	ND	Pantothenate transporter
MANI_014957	2.35	70.02	49.98	4.76	ND	YCII-domain protein
MANI_014867	21.19	3.01	4.08	-2.63	ND	Major Facilitator Superfamily protein
MANI_014762	0.56	29.70	34.13	5.77	ND	Polyketide synthase
MANI_014847	2.36	8.57	32.88	ND	1.92	Salicylate 1-monooxygenase
MANI_014887	0.00	86.51	86.40	8.80	ND	Leucoanthocyanidin Dioxygenase
MANI_112402	0.00	9.21	8.88	6.25	ND	Citrinin biosynthesis transcriptional activator CtnR
MANI_112407	0.57	17.13	28.22	4.71	ND	Cytochrome P450
MANI_014818	1.86	23.25	28.56	3.68	ND	Siderophore iron transporter
MANI_014903	0.00	56.20	81.77	8.09	ND	Nucleoside-diphosphate-sugar epimerase

The relative changes in expression levels were estimated at 48 h for the control condition (hC) and both 48 and 144 h for infection conditions (hI). NA Not Available, ND No Difference

Table 5 Expression profiling of the *M. anisopliae* cluster related to destruxin biosynthesis

NCBI gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)		Gene product
	48hC	48hI	144hI	48hCx48hI	48hIx144hI	
MANI_024443	2.67	138.02	14.72	5.80	-3.14	ABC multidrug transporter
MANI_024450	2.78	88.43	8.54	5.06	-3.34	Hypothetical protein
MANI_131037	0.00	119.36	8.75	9.88	-3.71	Glutamate decarboxylase
MANI_130923	3.06	100.47	14.43	5.11	-2.80	Aldo-keto reductase
MANI_024448	4.58	480.60	52.47	6.81	-3.08	Cytochrome P450
MANI_024437	9.41	261.50	15.65	4.92	-3.96	Destruxin synthetase

The relative changes in expression levels were estimated at 48 h for the control condition (hC) and both 48 and 144 h for infection conditions (hI). NA Not Available, ND No Difference

products (MaNRPS7; MaPKS14; MaPKS17; MaPKS18; MaIND1; MaOTHER8; MaOTHER12; MaOTHER13) (Additional file 11).

The five down-regulated BGCs under early infection conditions included: the xenolozoyenone-related compound (MaNRPS-PKS3) [71] and four putative clusters of uncharacterized products (MaNRPS8; MaNRPS10; MaOTHER1; MaOTHER11) (Additional file 11). The fourteen down-regulated BGCs under late infection conditions included the destruxin (MaNRPS1), serinocyclin (MaNRPS2), NG39x (MaNRPS-PKS1), helvolic acid (MaTERP1), xenolozoyenone-related compound (MaNRPS-PKS3), and pseurotin-related compound (MaNRPS-PKS2) BGCs, in addition to six putative clusters of uncharacterized products (MaNRPS11; MaPKS10; MaPKS14; MaPKS17; MaPKS18; MaTERP9; MaIND1; MaOTHER12) (Tables 2;3;5;6 and Additional file 11). Together, 30 % (22/73) of the predicted BGCs were differentially expressed in at least one of the pairwise comparisons.

Expression of global regulators of fungal traits

Many BGCs contain self-transcription factors integrated into the cluster organization [35, 79], but global regulators of fungal traits also influence the expression of BGCs [80, 81]. These global regulators extend from single transcription factors and histone-modifying enzymes (e.g., CreA, PacC, StuA, nscC, AreA, AreB, MeaB, GcnE and hdaA) to protein complexes (e.g., Velvet, and CCAAT-binding complexes) [80–85]. Our RNA-seq analysis detected the expression of all of the aforementioned global regulators at significant levels (RPKM >= 2) (Table 7).

Under early infection condition, global regulators linked to carbon (CreA) and nitrogen (AreA) metabolism, pH (PacC), light stimuli (VeA), asexual development (nsdC) and sporulation (StuA) were up-regulated (Table 7). Conversely, the best characterized global regulator, the LaeA methyltransferase, demonstrated lower expression. Additionally, global regulators linked to iron (HapB; HapC; HapE; HapX) and nitrogen (AreB; MeaB)

Table 6 Expression profiling of the *M. anisopliae* cluster related to NG39x biosynthesis

NCBI gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)		Gene product
	48hC	48hI	144hI	48hCx48hI	48hIx144hI	
MANI_020814	6.73	6.92	7.65	ND	ND	Monophenol monooxygenase
MANI_020801	0.48	0.00	0.00	NA	NA	Ankyrin repeat protein
MANI_020903	0.00	1.63	0.00	NA	NA	Eukaryotic aspartyl protease
MANI_020934	0.00	2.15	0.00	NA	NA	eEF-1B gamma subunit-like protein
MANI_020906	0.00	2.23	0.00	NA	NA	Alpha/beta hydrolase
MANI_020791	0.00	14.72	0.37	9.80	-5.22	Hybrid PKS-NRPS protein
MANI_020948	6.83	45.86	3.73	ND	ND	Integral membrane protein
MANI_020870	4.31	19.98	2.65	2.24	-2.81	Major facilitator superfamily protein
MANI_121064	0.00	6.23	0.53	NA	NA	Hypothetical protein
MANI_020865	2.30	13.40	1.88	2.58	-2.72	P450 monooxygenase
MANI_020911	5.66	7.82	5.96	ND	ND	Carboxyl methyltransferase
MANI_121074	3.99	11.79	6.53	1.65	ND	Hypothetical protein

The relative changes in expression levels were estimated at 48 h for the control condition (hC) and both 48 and 144 h for infection conditions (hI). NA Not Available, ND No Difference

Table 7 Expression profiling of the *M. anisopliae* global regulators of fungal traits

Global regulator	Metabolism	NCBI gene locus ID	Expression (RPKM)			Differential expression (log ₂ -fold change)	
			48hC	48hI	144hI	48hCx48hI	48hIx144hI
Velvet Complex							
VeA	Light	MANI_008143	30.53	71.74	45.95	1.32	ND
VelB		MANI_013601	25.91	29.21	18.58	ND	ND
LaeA		MANI_030399	3.64	6.84	4.97	NA	NA
CCAT-binding complex							
HapB	Iron	MANI_001834	38.29	29.90	55.14	ND	ND
HapC		MANI_119196	23.11	27.92	41.43	ND	ND
HapE		MANI_018323	41.24	22.68	30.49	ND	ND
HapX		MANI_009173	30.15	16.19	19.09	ND	ND
PacC	pH	MANI_008549	20.37	69.23	100.18	1.88	ND
CreA	Carbon	MANI_015776	9.31	65.57	70.54	2.91	ND
AreA	Nitrogen	MANI_016951	17.45	34.12	39.49	1.07	ND
AreB		MANI_028387	22.46	15.89	28.44	ND	ND
MeaB		MANI_019405	40.46	54.10	50.67	ND	ND
StuA	Sporulation	MANI_024223	38.24	75.85	92.09	1.02	ND
GcnE	Chromatin remodeling	MANI_000421	13.86	13.51	15.54	ND	ND
hdaE		MANI_025732	17.80	17.52	14.26	ND	ND
nsdC	Asexual development	MANI_013461	9.74	20.40	18.76	1.19	ND

The relative changes in expression levels were estimated at 48 h for the control condition (hC) and both 48 and 144 h for infection conditions (hI). NA Not Available, ND No Difference

metabolism, light stimuli (VeB), the chromatic remodeling histone acetyltransferase (GcnE) and deacetylase (hdaA), did not exhibit significant changes in gene expression (Table 7). In addition to demonstrating that many of these global regulators are active (a significant amount remain unexplored in the *Metarhizium* genus), expression analysis provides clues regarding how infection conditions can be regulated and which genes may perform this regulation.

Discussion

Although a vast number of interesting metabolites have been isolated from *Metarhizium* cultures, little is known regarding how BGCs are organized, expressed and regulated as well as their potential functions during host infection and their evolutionary history. To this end, a deep survey of BGCs was performed in the *M. anisopliae* genome and combined with a transcriptional profile analysis from an infection model in the economically important cattle-tick *R. microplus*, which is responsible for a variety of livestock infections and is a promising candidate for biological control by *Metarhizium* [86–89].

Our survey predicted and delimited 73 BGCs, of which 20 % were up-regulated under early infection conditions (48hC x 48hI), and a subset of these (9 out of 15) were down-regulated under late infection conditions

(48hI x 144hI) (Additional file 10). These results point to a drastic change between the metabolic profiles of early and late infection. The *Metarhizium* infection process is dynamic and may end in arthropod or fungal death [90]. Once the fungus adheres to a host, rapid morphological and transcriptional changes occur, including the expression of several virulence factors [91]. Ment and coworkers (2012) showed that after 3 or 4 days of attachment to a suitable host, *Metarhizium* kills the host [90], switching to a saprophytic state, and virulence determinant expression is attenuated [2]. Conversely, in an unsuccessful infection scenario, the fungus will exhaust the endogenous spore nutrient reserves at 3 or 4 days post-cuticle adhesion while attempting to circumvent host defenses, resulting in the demise of the pathogen [90]. We suggest that some of the identified up-regulated BGCs participate in the first scenario (successful infection), given that this hypothesis corroborated with the observed strong expression of the destruxin cluster, a well-known virulence factor [92], and ferricrocin [30]. Additionally, we hypothesize that other BGCs are also expressed during early and late infection, such as BGCs for the production of antifungal and antibacterial compounds that help the fungus to circumvent competition with opportunistic and symbiotic microorganisms. These BGCs may be induced by direct interactions between *M. anisopliae* and other microorganisms,

independent of the host interaction. Moreover, our experimental design employing tick cuticles may have blocked the expression of these antibiotic BGCs, as the natural tick gut microbiota was excluded [32]. Indeed, the antibacterial viridicatumtoxin BGC (MaPKS9) proposed by Gibson and coworkers (2014) was silent under our mimicked infection conditions, supporting this notion (Additional file 11) [31].

Some BGCs that were previously characterized in *Metarhizium* were silent or did not alter their expression under at least one of the tested conditions. This was the case for MaNRPS2 BGC (serinoclycin; MANI_020119), which was down-regulated under late infection conditions, and MaNRPS8 (metachelin; MANI_003049), which was down-regulated under early infection conditions. The MaPKS8 (MrPKS2; MANI_028434), already-cited MaPKS9, (viridicatumtoxin; MANI_003768) and MaPKS20 (MrPKS1; MANI_122426) BGCs did not demonstrate detectable expression. The reduced participation of these BGCs in the infection process is expected in accordance with previous literature [26, 29–31].

Similarly, the up-regulation of the destruxin cluster under early infection conditions was in accordance with the described insecticidal effects and phenotypic analysis of destruxin mutants. Similarly, the up-regulation of the ferricrocin cluster highlights the already-described importance of this siderophore in the *Metarhizium* lifestyle and infection process [30]. However, while destruxin metabolites directly affect the host defenses [92], the reduced infection resulting from the absence of ferricrocin is linked to delayed germination and alterations in endogenous fungal iron content [30].

Some putative clusters highlighted by the comparative genomic analysis do not appear to affect the virulence of *Metarhizium* species (Additional files 10 and 11). Such is the case for the putative BGCs for aurovertin (MaPKS2; MANI_004781), elymoclavine/ergovaline-related compound (MaIND-NRPS1; MANI_029655) and terpendole E/lolitrein-related compound (MaIND-TERP1; MANI_011022). Aurovertin metabolites have been isolated from *M. anisopliae*, *P. chlamydosporia*, and *C. arbuscula* [63, 64, 93]. These compounds exhibit potent inhibition of adenosine triphosphate synthase [64], and aurovertin D, which was isolated from *P. chlamydosporia*, induced the death of the free-living nematode *Panagrellus redivivus* [93]. The non-expression (RPKM < 2, under the three conditions) of this cluster is intriguing, particularly in light of the reports regarding *P. chlamydosporia*, a species closely related to the *Metarhizium* genus. Similarly, elymoclavine, ergovaline, terpendole E and lolitrein are ergot alkaloids produced by fungi from the *Claviceps*, *Epichl e* and *Tolyposcladium* genera, which are closely related to the *Metarhizium* genus [66, 67]. Ergot alkaloids are potent toxic alkaloids

whose intake can lead to several effects ranging from poor weight gain to gangrene and death [67]. Similar to aurovertin, it could be postulated that these metabolites play a role in infection, although our results suggested the opposite. Certainly, the construction of gene knockouts for these BGCs will help to understand their importance in the *Metarhizium* lifestyle and interactions.

The xenolozoyenone-related compound (MaNRPS-PKS3; MANI_023437) was another cluster that was down-regulated, indicating decreased participation in the infection process. Xenolozoyenone is a pyrrolidinedione-containing compound isolated from *G. lozoyensis*. Although no biological activity or possible function has been linked to this compound, the backbone gene from this cluster (*glpks3-glnrps7*) was the first described classical fungal protein-coding operon [71]. The conservation of orthologs of these genes highlights the possibility that protein-coding operons are widespread among fungal genomes and BGCs and represent a new and virtually unexplored level of regulation.

The results of the transcriptomic analysis may also help to redefine the importance of NG39x (MaNRPS-PKS1; MANI_020791) in the infection process. The RNA-seq results suggested that the NG39x BGC might play a role in infection, as demonstrated by the differential expression (> 9.5-fold difference with $P < 0.05$ for the backbone gene MANI_020791) observed under early infection conditions (Table 6). Although NG39x cluster expression has been observed in vivo [27], a previous study performed RT-PCR analysis to evaluate the transcripts of the NG39x cluster while comparing in vitro fungal growth with growth in infected *S. exigua* larvae and did not reveal clear differences between the two conditions. It was postulated that the expression of the NG39x cluster is developmentally regulated, given that there was an increase in BGC expression related to biomass augmentation. Additionally, the knockout mutant for the backbone gene responsible for NG39x biosynthesis did not lead to diminished fungal virulence, indicating minor or no participation in the insect infection process [27]. NG39x compounds were recently reported to exert antiproliferative effects in human cell cultures, via a mechanism that involves impairment of the integrity of nucleic acid biosynthesis. However, it has not been established if these compounds directly interact with DNA or RNA or interact with some protein [94]. Therefore, it is possible that the toxicity of NG39x compounds varies according to the host and may exemplify cases of host specificity, which explains the increased expression of this cluster under early infection condition.

We expanded the comparative genomic analysis by constructing a phylogeny to investigate the metabolic pathways of three interesting up-regulated clusters: a

pseurotin-related compound BGC (MaNRPS-PKS2), a putative helvolic acid BGC (MaTERP1) and a tropolone/citrinin-related compound BGC (MaPKS1).

Pseurotins are a group of compounds containing phenylalanine coupled to a polyketide with a spiro ring structure, and have been isolated from *Aspergillus* spp. and *Pseudeurotium ovalis* cultures [95]. Anti-angiogenic activities as well as IgE and chitin synthase inhibitory activities have been reported for pseurotins [96]. Although this metabolite has never been isolated from *Metarhizium* cultures, a recent report revealed a putative pseurotin cluster in *M. robertsii* [36] and served as the starting point for analysis. The expression and comparative genomic analysis suggested that the final product of this cluster is not pseurotin, but a related compound. Wiemann and coworkers (2013) speculate that the product of this BGC could be 12-hydroxy-ovalicin (Mer- β). Mer- β exhibits inhibitory and immunosuppressive activities against certain tumor cell lineages, and ovalicin-related compounds isolated from *M. anisopliae* cultures have been tested for the treatment of atopic dermatitis in mice [97, 98].

The pseurotin-related compound BGC from *M. anisopliae* may also be embedded in a supercluster, analogous to the pseurotin BGC from *Aspergillus* species. This macro regulation exerted by a supercluster is an interesting and virtually unexplored area of research. Comparative genomic analysis and the transcription profile suggested the presence of another supercluster in *M. anisopliae*. In view of the apparent co-regulation of MaPKS18 and the putative helvolic acid BGC (MaTERP1) as well as the fact that the sequence region containing both clusters misled the antiSMASH prediction we suggest that this region is a supercluster. However, more investigation is needed to prove this hypothesis.

The aforementioned helvolic acid is another interesting secondary metabolite. Helvolic acid is a well-known fusadine triterpene antibiotic that is active against Gram-positive bacteria [11]. In *A. fumigatus*, this SM is suggested to play an important role in human pathogenesis, exerting inhibitory effects on macrophages and inducing epithelial damage [99]. This compound also exhibits antifungal activity against phytopathogens and demonstrates antifeedant properties in the armyworm *Mythimna separata* [100]. Our phylogenetic analysis supports the notion that this cluster was horizontally acquired from a species closely related to Eurotiales, given that this BGC is restricted to only a few species. Moreover, not only helvolic acid but its derivative (1,2-dihydrohelvolic acid) have been isolated from *M. anisopliae* cultures. Furthermore, 1,2-dihydrohelvolic acid was isolated from fungus grown in insect-derived material, suggesting that this cluster is active under infection conditions [25]. Conversely, purified helvolic acid from

M. anisopliae cultures does not appear to be toxic to some insects [101]. Although expression analysis suggested a role in infection and antifeedant properties for this metabolite have been reported in the literature, the true importance of helvolic acid in the *Metarhizium* lifestyle must be assessed.

Our analysis also suggested that the final product of MaPKS1 is a tropolone/citrinin-related compound. Tropolones and citrinins are structurally very similar and are chemically grouped by the local suffering oxidation [74, 75]. Furthermore, MaPKS1 contains several genes that are not conserved in the characterized tropolone/stipitatic acid and citrinin biosynthetic routes. Thus, while some genes in the MaPKS1 BGC are conserved in the tropolone and citrinin routes, and these genes likely perform similar functions in *M. anisopliae*, the final metabolic function of this cluster is still unknown. Better classification of the final product of this BGC will emerge with the functional analysis of this cluster.

Our analysis also uncovered global regulators of fungal development, nutrition and niche adaptation that are up-regulated under early infection conditions and can govern the expression of both up- and down-regulated BGCs, as observed in other fungal species [82]. The up-regulation of PacC under early infection conditions (>1.88-fold difference with $P < 0.05$; MANI_008549) is in agreement with the ability of *M. anisopliae* to modulate pH during the infection process by alkalizing infected cuticles [102, 103]; under alkaline pH conditions, PacC serves as a positive regulator promoting the transcription of alkaline-expressed genes, and it has been suggested that PacC simultaneously represses acid-expressed genes [104]. The importance of PacC in *Metarhizium* infection and fungal growth has already been established via the construction of knockouts [105]. However, it has not been determined how this gene influences secondary metabolism in *Metarhizium* spp. PacC may potentially influence the BGC for destruxin production, as it was verified that destruxin production is favored by an alkaline pH [106]. In accordance with the central role of PacC in secondary metabolism regulation, there have been reports in *Aspergillus* spp., indicating that this gene regulates penicillin and sterigmatocystin BGCs [107, 108]. Another gene linked to secondary metabolism regulation is the carbon catabolite repressor (CreA), which is also up-regulated under early infection conditions (> 2.91-fold difference with $P < 0.05$; MANI_015776). CreA is a global repressor that ensures the utilization of preferred carbon sources, preventing the expression of genes linked to assimilatory traits of non-preferred carbon sources [109]. CreA is known to negatively influence the production of penicillin at high carbon concentrations and was suggested to act in ochratoxin A regulation [82, 110]; however, it still must be determined how this gene influences *Metarhizium* spp. secondary

metabolism. Interestingly, in filamentous fungi, carbon and pH metabolisms appear to be related [111]. Recently, Bi and coworkers (2015) showed that many organisms acidify media under conditions of carbon excess, while alkalization occurs under carbon deprivation. Mutants for glutamate dehydrogenase 2 (*gdh2*), which catalyzes the deamination of non-preferred carbon sources, resulting in ammonia production, exhibited reduced virulence and alkalization potential, and *gdh2* expression was negatively correlated with CreA in *Colletotrichum gloeosporioides*, *Penicillium expansum*, *Aspergillus nidulans*, and *Fusarium oxysporum* [111]. This similar regulation in distantly related species (e.g., *Fusarium* spp. and *Aspergillus* spp.) suggests a widespread form of regulation that can be present in *Metarhizium* species. However, our observation of both PacC and CreA up-regulation is contrary to this notion. The positive regulation of these global regulators under early infection conditions may indicate a switch from infection to a saprophytic state, which could lead to the down-regulation of BGCs involved in infection. Notably, the orthologs for the *laeA* gene in *M. anisopliae* exhibited lower expression (difference not available under both early and late infection conditions with $P < 0.05$; MAN1_030399) (Table 7). *LaeA* demonstrates central importance in SM metabolism and virulence in *Aspergillus* spp. and *Fusarium* spp. [112, 113]. In *Aspergillus carbonarius*, the deletion of *laeA* led to a drastic decrease in ochratoxin A production [114], and the deletion of this gene in *A. fumigatus* blocks the expression of sterigmatocystin, penicillin, lovastatin and helvolic acid BGCs [115]. Future studies should be performed to assess the role of *laeA* in the *Metarhizium* lifestyle and virulence.

Finally, the conservation of the BGCs found among *Metarhizium* species is yet another fundamental topic. The absence of the destruxin BGC in the host-specialists *M. acridum* and *M. album* led to the conclusion that this SM could play a pivotal role in the host-generalist lifestyle [28]. The comparative genomic analyses of BGCs in the *Metarhizium* genus indicate that only one half of these BGCs are conserved in host-specialist species. In addition to this, host-specialist species also encode BGCs that not conserved in host-generalist species, which represents an important difference in the metabolic profile and niche adaptation potential of these species. Entomopathogenic fungi may retain some BGCs related to the infection process, and SM acquisition can act as a driving force towards host generalization. Accordingly, our results showed that only 6 out of the 15 up-regulated clusters are conserved in *M. acridum* and *M. album* (Additional file 10). The results suggest that the plethora of SM produced by host-generalist and host-intermediate species exert an underestimated impact in the infection process, and destruxins as well as

many other SMs may be essential for fungal adaptation to new hosts.

Conclusions

Although considerable progress has been made in understanding the *Metarhizium* infection process, unanswered questions remain, in particular those related to the definition of host specificity, which is a central and still not fully understood topic in the study of entomopathogenic fungi. Different fungal species, and even different strains, are able to synthesize different combinations of SM compounds that may result in distinct adaptation strategies [73]. In general, the results point to a fundamental role for SM in initial infection, with a notable difference in the BGCs present in host-specialist versus host-generalist *Metarhizium* species. Transcript analysis of samples obtained under conditions mimicking tick infection, showed the activation of several BGCs, with some up-regulated only during the early steps of infection. The conservation and expression of these genes can actively support *M. anisopliae* as a successful generalist pathogen. The origin and evolution of SM clusters is another interesting topic, and the results suggest that HGT events may have shaped the metabolic potential of generalist species, but this hypothesis still must be explored.

Several BGCs characterized by other groups, which were shown to participate in the infection process only to a minor degree, as well as several putative BGCs highlighted by this work were silent under mimicked conditions, reaffirming the hypothesis regarding their participation in the infection process. In this work, we took the first steps toward the characterization of several previously unexplored BGCs and examined their regulation, focusing on their potential participation in infection. These are important issues to investigate, not only to acquire basic knowledge regarding fungal lifestyles and adaptation, but also to explore for future biological control applications, and the identified genes and global regulators represent valuable targets for further experimental study.

Additional files

- Additional file 1:** Fungal genomes used in this work. (PDF 158 kb)
- Additional file 2:** OrthoMCL clustering results. (PDF 157 kb)
- Additional file 3:** Phylogenetic trees constructed with putative orthologs of MaPKS1, all PKS from *M. anisopliae* E6, and all characterized PKS from MIBiG. (PDF 600 kb)
- Additional file 4:** Amino acid alignment for the pseurotin-related backbone gene and related orthologs. (FASTA 67 kb)
- Additional file 5:** Amino acid alignment for the tropolone/citrinin-related backbone gene and related orthologs. (FASTA 125 kb)
- Additional file 6:** Nucleotide alignment for the *ref1* gene. (FASTA 40 kb)
- Additional file 7:** Best-fit evolutionary models predicted with Prottest 3.4 or jmodeltest-2.1.9 for each alignment. (PDF 12 kb)

Additional file 8: The partially conserved cluster found in *A. chrysogenum* and species from the *Arthroderma* genus is putatively linked to fusidic acid biosynthesis. (PDF 307 kb)

Additional file 9: Amino acid alignment for helvolic acid supermatrix. (FASTA 40 kb)

Additional file 10: Overview of the BGCs in *M. anisopliae* E6, their domain structures, and conservation among six *Metarhizium* species. (PDF 256 kb)

Additional file 11: Expression profile of predicted BGCs. (PDF 934 kb)

Additional file 12: Comparative genomic analysis and synteny for the aurovertin, elymoclavine/ergovaline-related compound, terpendole E/lolitrem-related compound, and xenolozoyenone-related compound BGCs. (PDF 161 kb)

Additional file 13: Additional phylogenetic trees. (PDF 463 kb)

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Declarations

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Availability of data and materials

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Authors' contribution

Conceived and designed the experiments: NS, RLMG, MHV, ATRV, and AS. Transcriptome analysis: NS, RLMG, and GLM. BGCs curation and comparative analyses: NS, AJ, and RLMG. Phylogenetic analyses: NS and FCA. Contributed reagents/materials/analysis tools: MHV, ATRV, and AS. Wrote the article: NS, AJ, RLMG, FCA, MHV and AS. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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6.2 Arquivos Suplementares do Manuscrito:

Additional File 1: Fungal genomes used in this work.

Genome	NCBI's Accession Number
Genomes from <i>Metarhizium</i> species	
<i>Metarhizium anisopliae</i> E6	PRJNA245858
<i>Metarhizium robertsii</i> ARSEF23	PRJNA38717
<i>Metarhizium acridum</i> CQMa102	PRJNA38715
<i>Metarhizium majus</i> ARSEF297	PRJNA184757
<i>Metarhizium guizhouense</i> ARSEF977	PRJNA184755
<i>Metarhizium brunneum</i> ARSEF3297	PRJNA184756
<i>Metarhizium album</i> ARSEF1941	PRJNA72731
Unannotated genomes	
<i>Epichloë festucae</i> F11	PRJNA51625
<i>Epichloë baconii</i> ATCC 200745	PRJNA221976
<i>Balansia obtecta</i> B249	PRJNA221345
<i>Epichloë sylvatica</i> GR10156	PRJNA275112
<i>Neotyphodium gansuense</i> E7080	PRJNA67299
<i>Pochonia chlamydosporia</i> 123	PRJNA68669
<i>Periglandula ipomoeae</i> IasaF13	PRJNA67303
<i>Aciculosporium take</i> MAFF-241224	PRJNA67241
<i>Hypocrella siamensis</i> MTCC 10142	PRJNA242986
<i>Atkinsonella hypoxylon</i> B4728	PRJNA221544
<i>Claviceps fusiformis</i> PRL 1980	PRJNA67243
Genomes used for OrthoMCL clustering	
<i>Metarhizium anisopliae</i> E6	PRJNA245858
<i>Metarhizium robertsii</i> ARSEF23	PRJNA38717
<i>Metarhizium acridum</i> CQMa102	PRJNA38715
<i>Metarhizium majus</i> ARSEF297	PRJNA184757
<i>Metarhizium guizhouense</i> ARSEF977	PRJNA184755
<i>Metarhizium brunneum</i> ARSEF3297	PRJNA184756
<i>Metarhizium album</i> ARSEF1941	PRJNA72731
<i>Metarhizium anisopliae</i> E6	PRJNA245858
<i>Acremonium chrysogenum</i> ATCC 11550	PRJNA248608
<i>Aspergillus fumigatus</i> Af293	PRJNA131
<i>Aspergillus nomius</i> NRRL 13137	PRJNA246595
<i>Coccidioides posadasii</i> str. <i>Silveira</i>	PRJNA17787
<i>Leptosphaeria maculans</i> JN3	PRJNA171003
<i>Penicillium digitatum</i> PHI26	PRJNA157541
<i>Scedosporium apiospermum</i> IHEM 14462	PRJNA244532

<i>Talaromyces marneffe</i> PM1	PRJNA251717
<i>Talaromyces stipitatus</i> ATCC 10500	PRJNA19557
<i>Thielavia terrestris</i> NRRL 8126	PRJNA32847
<i>Tolylocadium ophioglossoides</i> CBS 100239	PRJNA91059
<i>Trichophyton soudanense</i> CBS 452.61	PRJNA186831
<i>Arthroderma benhamiae</i> CBS 112371	PRJNA30573
<i>Aspergillus clavatus</i> NRRL 1	PRJNA15664
<i>Aspergillus fischeri</i> NRRL 181	PRJNA15672
<i>Aspergillus niger</i> CBS 513.88	PRJNA19275
<i>Arthroderma otae</i> CBS 113480	PRJNA30939
<i>Aspergillus udagawae</i>	PRJDB3949
<i>Coccidioides immitis</i> RS	PRJNA12883
<i>Coccidioides posadasii</i> RMSCC 3488	PRJNA17783
<i>Colletotrichum sublineola</i>	PRJNA262370
<i>Microsporum gypseum</i> CBS 118893	PRJNA20599
<i>Madurella mycetomatis</i>	PRJNA267680
<i>Neofusicoccum parvum</i> UCRNP2	PRJNA187491
<i>Oidiodendron maius</i> Zn	PRJNA74727
<i>Pseudogymnoascus</i> sp. VKM F-3557	PRJNA74727
<i>Pyrenophora teres</i> f. <i>teres</i> 0-1	PRJNA50389
<i>Talaromyces cellulolyticus</i>	PRJDB3250
<i>Torrubiella hemipterigena</i>	PRJEB7402
<i>Trichophyton interdigitale</i> H6	PRJNA186827
<i>Talaromyces islandicus</i>	PRJEB8788
<i>Trichophyton rubrum</i> CBS 118892	PRJNA38221
<i>Trichophyton verrucosum</i> HKI 0517	PRJNA39693

Additional File 2: Phylogenetic trees constructed with putative orthologs of MaPKS1, all PKS from *M. anisopliae* E6, and all characterized PKS from MIBiG

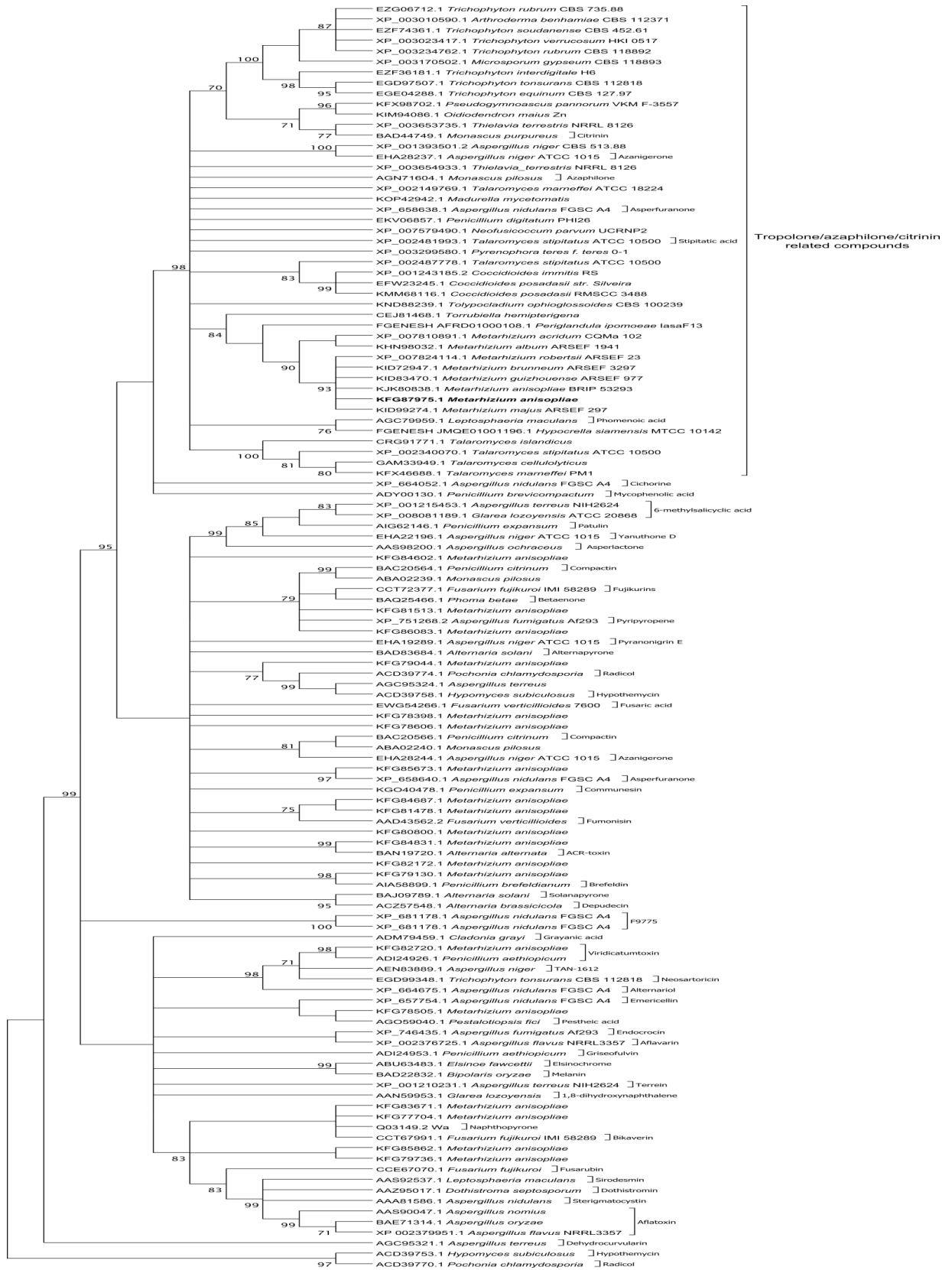
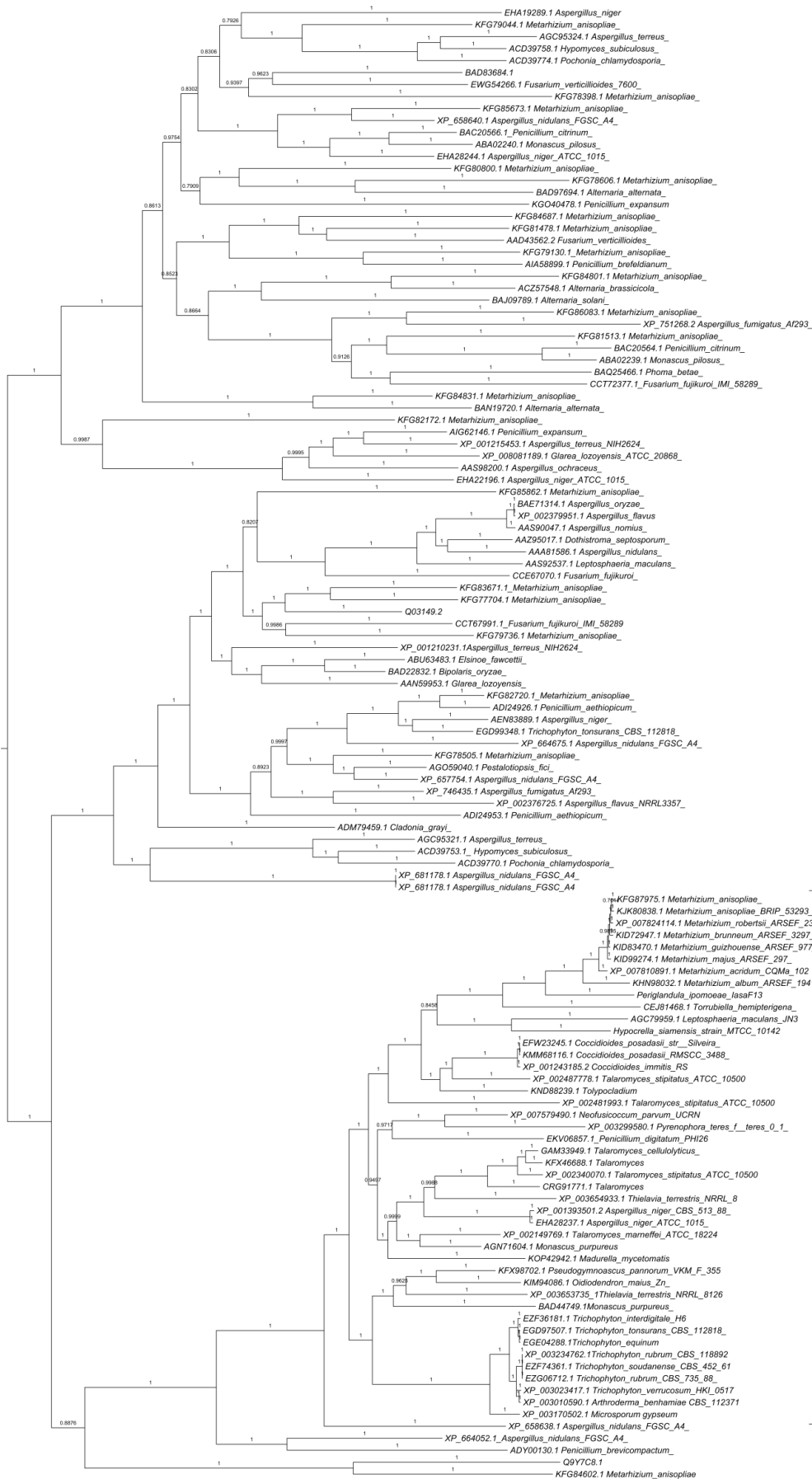


Figure 1: Maximum Parsimony analysis of taxa. The evolutionary history was inferred using the Maximum Parsimony method. The most parsimonious tree with length = 3580 is shown. The consistency index is (0.311236), the retention index is (0.658923), and the composite index is 0.207616 (0.205081) for all sites and parsimony-informative sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The Maximum Parsimony tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 120 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 168 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.



Tropolone/azaphilone/citrinin related compounds

0.4

Figure 2: Bayesian analysis of taxa. Branches support values (Bayesian posterior probability) are associated with nodes. The Bayesian inference ran for 3,125,600 generations.

Additional File 3: Best-fit evolutionary models predicted with Prottest 3.4 or jmodeltest-2.1.9 for each alignment.

Locus tag/Gene/Tree	Model
MaNRPS-PKS2 (MANI_018878)	JTT+I+G+F
MaPKS1 (MANI_014762)	LG+I+G+F
MaTERP1 (MANI_010532)	LG+G
MaTERP1 (MANI_010531)	LG+I+G+F
MaTERP1 (MANI_010536)	LG+I+G+F
MaTERP1 (MANI_010537)	JTT+G+F
MaTERP1 (MANI_010495)	LG+I+G+F
MaTERP1 (MANI_010530)	LG+I+G+F
MaTERP1 (MANI_010594)	LG+G+F
MaTERP1 (MANI_010512)	LG+G+F
MaTERP1 (MANI_010527)	LG+G+F
<i>tef1</i>	GTR+I+G
supermatrix	JTT+G
All PKS tree	LG+I+G+F

Additional File 4: The partially conserved cluster found in *A. chrysogenum* and species from the *Arthroderma* genus is putatively linked to fusidic acid biosynthesis.

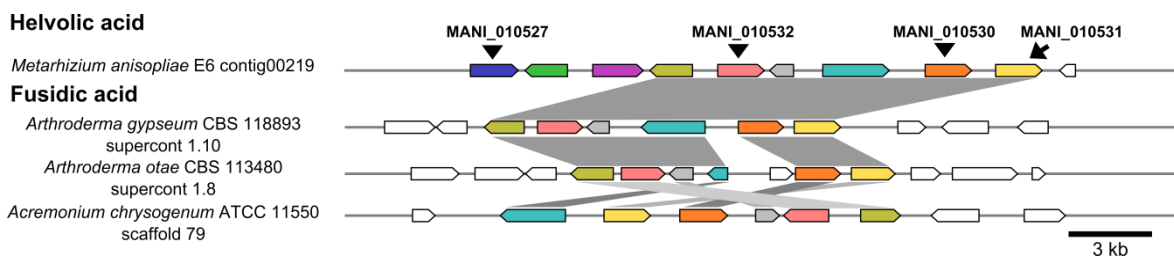





Figure 1: The search for orthologous of MaTERP1 (helvolic acid cluster) has found that this cluster is partly conserved in species from *Arthroderma* genus and *Acremonium chrysogenum* (Hypocreales: Hypocreaceae). However, this partially conserved cluster was suggested to produce fusidic acid, a molecule similar to helvolic acid that is supported by isolation of fusidic acid-like compounds from *Acremonium spp* (Evans *et al.*, 2006; Lodeiro *et al.*, 2009).

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Additional File 5: Overview of the BGCs in *M. anisopliae* E6, their domain structures, and conservation among six *Metarhizium* species.

Cluster/ Backbone gene	MAR	MAC	MAM	MAJ	MGU	MBR	Product/Alt. names	Locus tag	48hCx48hI	48hIx144hI	Backbone gene domains
<i>MaNRPS1</i>							Destruxin	MANI_024437	Up	Down	A C A E C A C A C A M C A M C
<i>MaNRPS2</i>							Serinocyclin	MANI_020119	ND	Down	A C C A C A C A C A E C A C A E C A C
<i>MaNRPS3</i>								MANI_011572	NA	NA	C A E C A
<i>MaNRPS4</i>								MANI_020971	NA	NA	A C A E C
<i>MaNRPS5</i>								MANI_004659	NA	NA	C A C
<i>MaNRPS6</i>								MANI_004647	ND	ND	A C A C A E C A C A E C A C A E C A C
<i>MaNRPS7</i>								MANI_010631	Up	ND	C A C
<i>MaNRPS8</i>							Metachelin	MANI_003049	Down	NA	A C C
<i>MaNRPS9</i>							Ferricrocin	MANI_117325	Up	ND	C A C C C
<i>MaNRPS10</i>								MANI_003902	NA	NA	A C A E C C
<i>MaNRPS11</i>								MANI_006947	ND	Down	A C A C A E C A C A E C A E C A C A C C
<i>MaNRPS12</i>								MANI_006155	NA	NA	A E C A C A C A E C
<i>MaNRPS13</i>								MANI_008843	NA	NA	A C A C A C A
<i>MaPKS1</i>							Tropolone /citrinin-related compound	MANI_014762	Up	ND	KS AT AC MT TD
<i>MaPKS2</i>							Aurovertin	MANI_004781	NA	NA	KS AT DH MT KR AC
<i>MaPKS3</i>								MANI_011575	NA	NA	KS AT DH AC TE
<i>MaPKS4</i>								MANI_110051	NA	NA	KS AT DH MT ER KR
<i>MaPKS5*</i>								MANI_027199	NA	NA	KS DH AT DH
<i>MaPKS6*</i>								MANI_010100	NA	NA	KS AT DH
<i>MaPKS7</i>								MANI_020125	NA	NA	KS AT DH MT ER KR AC
<i>MaPKS8</i>							MrPKS2	MANI_028434	NA	NA	KS AT DH AC AC TE
<i>MaPKS9</i>							Viridicatumtoxin	MANI_003768	NA	NA	KS AT AC

<i>MaPKS10</i>								MANI_019155	Down	Down	A AC KS AT KR AC TD
<i>MaPKS11</i>								MANI_006783	NA	NA	KS AT DH MT KR AC
<i>MaPKS12</i>								MANI_110978	NA	NA	KS AT DH MT ER KR
<i>MaPKS13</i>								MANI_006250	ND	ND	KS AT DH MT ER KR AC
<i>MaPKS14</i>								MANI_018879	Up	Down	KS AT DH AC
<i>MaPKS15</i>								MANI_021529	NA	NA	KS AT DH ER KR AC
<i>MaPKS16</i>								MANI_029455	NA	NA	KS AT DH ER KR AC
<i>MaPKS17</i>								MANI_025650	Up	Down	KS AT DH ER KR
<i>MaPKS18</i>								MANI_010451	Up	Down	KS AT AC
<i>MaPKS19</i>								MANI_012054	ND	ND	KS AT DH ER KR AC
<i>MaPKS20</i>							MrPKS1	MANI_122426	NA	NA	KS AT AC AC TE
<i>MaPKS21</i>								MANI_006397/ MANI_006388	NA	NA	ER KR AC DH ER
<i>MaPKS22</i>								MANI_017297	ND	ND	AT KS AC
<i>MaNRPS-PKS1</i>							NG39x	MANI_020791	Up	Down	KS AT DH MT KR AC C A TD
<i>MaNRPS-PKS2</i>							Pseurotin-related compound	MANI_018878	Up	Down	KS AT DH KR C A TD
<i>MaNRPS-PKS3</i>							Xenolozoyenone-related compound	MANI_023437	Down	Down	KS AT DH ER KR C A TD
<i>MaNRPS-PKS4</i>								MANI_022470/ MANI_022473	ND	ND	KS AT DH MT ER KR AC C A C
<i>MaNRPS-PKS5</i>								MANI_006158	NA	NA	KS AT DH MT KR C A
<i>MaNRPS-PKS6</i>								MANI_010456/ MANI_121659	NA	NA	A C A KS AT DH MT ER
<i>MaNRPS-PKS7</i>								MANI_001951/ MANI_001950	NA	NA	AT DH MT KR C A TD
<i>MaTERP1</i>							Helvolic acid	MANI_010532/ MANI_010527/ MANI_010530/ MANI_010531	Up	Down	
<i>MaTERP2</i>							Lanosterol cyclase	MANI_002110	Up	ND	
<i>MaTERP3</i>								MANI_027216	NA	NA	
<i>MaTERP4</i>								MANI_116096	ND	ND	

<i>MaTERP5</i>							GGPP synthase	MANI_024139	ND	ND	
<i>MATERP6</i>								MANI_020049	ND	ND	
<i>MaTERP7</i>								MANI_020253	NA	NA	
<i>MaTERP8</i>								MANI_027397	NA	NA	
<i>MaTERP9</i>								MANI_022959	ND	Down	
<i>MaIND1</i>								MANI_023817	Up	Down	
<i>MaIND2</i>								MANI_004342	NA	NA	
<i>MaIND3</i>								MANI_024963	NA	NA	
<i>MaSID1</i>								MANI_004303	NA	NA	
<i>MaIND-TERP1</i>							Terpendole E/ Lolitreml-related compound	MANI_011022	NA	NA	
<i>MaIND-NRPS1</i>							Elymoclavine/ Ergovaline-related compound	MANI_029655	NA	NA	
<i>MaIND-NRPS2</i>								MANI_122478	NA	NA	
<i>MaTERP-PKS1</i>								MANI_006324	NA	NA	
<i>MaOTHER1</i>								MANI_007147	Down	ND	
<i>MaOTHER2</i>								MANI_109737	ND	ND	
<i>MaOTHER3</i>								MANI_004951	ND	ND	
<i>MaOTHER4</i>								MANI_019946	ND	ND	
<i>MaOTHER5</i>								MANI_018009	NA	NA	
<i>MaOTHER6</i>								MANI_117972	ND	ND	
<i>MaOTHER7</i>								MANI_000865	NA	NA	
<i>MaOTHER8</i>								MANI_000852	Up	ND	
<i>MaOTHER9</i>								MANI_024071	NA	NA	
<i>MaOTHER10</i>								MANI_011653	NA	NA	
<i>MaOTHER11</i>								MANI_026624	Down	ND	
<i>MaOTHER12</i>								MANI_010482	Up	Down	
<i>MaOTHER13</i>								MANI_025168	Up	ND	

<i>MaOTHER14</i>								MANI_122490	NA	NA	
TOTAL= 73	69	35	30	61	64	70					
Adenylation				N-Methylation			Acyltransferase		Enoylreductase		Thioester reductase
Condensation				Keto-synthase			Acyl carrier protein		Methyltransferase O- or C-		Ketoreductase
Epimerization							Dehydratase		Thioesterase		

M. robertsii ARSEF23 (MAR), *M. acridum* CQMa102 (MAC), *M. album* ARSEF1941 (MAM), *M. majus* ARSEF297 (MAJ), *M. guizhouense* ARSEF977 (MGU) and *M. brunneum* ARSEF3297 (MBR). Known products as well as alternative names and locus tags of the backbone genes are also listed. Relative changes in expression levels were estimated after 48 hours for the control condition (hC) and both 48 and 144 hours for infection conditions (hI). NA: Not Available. ND: No Difference. * The backbone gene appears to be truncated.

Additional File 6: Expression profile of predicted BGCs.

Table 1: Expression profiling of the MaNRPS2 cluster (Serinocyclin).

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_020146	39.27	20.86	16.60	NA	NA
MANI_020153	3.28	0.00	0.00	NA	NA
MANI_020163	83.85	2.50	13.55	-4.78	2.46
MANI_020161	0.00	0.90	2.74	NA	NA
MANI_020136	5.00	11.18	2.27	1.25	-2.21
MANI_020119	14.72	10.71	4.77	NA	-1.09
MANI_115536	0.00	7.42	0.00	NA	NA
MANI_020154	0.00	1.66	0.00	NA	NA
MANI_020156	0.88	0.00	0.00	NA	NA
MANI_020147	1.69	0.91	0.00	NA	NA
MANI_020164	0.00	1.92	0.00	NA	NA
MANI_020155	9.37	37.12	2.56	2.09	-3.75
MANI_020139	33.07	36.39	33.99	NA	NA
MANI_020162	24.84	15.84	8.47	NA	NA
MANI_020151	32.58	91.15	163.66	1.58	NA
MANI_029315	5.81	15.61	92.76	1.54	2.52

Table 2: Expression profiling of the MaOTHER1 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_007159	17.87	34.95	64.73	NA	NA
MANI_007149	39.73	20.00	28.13	NA	NA
MANI_026611	96.44	23.48	18.35	-1.90	NA
MANI_007147	24.95	5.81	4.43	-1.99	NA
MANI_026610	9.99	10.45	10.62	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 3: Expression profiling of the MaOTHER2 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_109737	0.00	4.81	0.98	NA	NA
MANI_007750	0.00	0.00	0.84	NA	NA
MANI_131937	0.00	0.00	1.71	NA	NA
MANI_007754	136.01	214.67	220.69	NA	NA
MANI_007753	285.59	207.31	336.46	NA	NA

Table 4: Expression profiling of the MaNRPS-PKS3 cluster (Xenolozoyenone-related compound).

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_023523	40.29	10.67	4.07	-1.74	-1.35
MANI_023437	11.23	1.59	0.62	-2.68	-1.30

MANI_030051	6.43	6.62	4.10	NA	NA
MANI_030058	0.69	0.00	0.00	NA	NA
MANI_023546	4.74	3.82	1.67	NA	NA
MANI_023568	3.27	1.32	1.34	NA	NA
MANI_023497	0.00	0.00	0.00	NA	NA
MANI_023527	2.24	2.25	6.42	NA	1.48

The BGC boundaries were delimited by CASSIS.

Table 5: Expression profiling of the MaIND1 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_023817	0.74	13.71	6.06	4.04	-1.13
MANI_030140	4.11	3.49	3.90	NA	NA
MANI_113953	39.08	0.95	1.45	-5.03	NA
MANI_023814	1.07	0.29	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 6: Expression profiling of the MaPKS2 cluster (Aurovertin).

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_004840	0.00	0.00	0.00	NA	NA
MANI_004781	1.66	0.19	0.00	NA	NA

MANI_114236	0.00	0.00	0.00	NA	NA
MANI_004795	0.54	0.44	0.00	NA	NA
MANI_004789	2.46	3.30	2.01	NA	NA
MANI_114238	0.00	0.00	0.00	NA	NA
MANI_124919	0.00	0.00	0.00	NA	NA
MANI_004822	0.00	5.32	0.68	NA	NA
MANI_004785	0.00	0.00	0.00	NA	NA
MANI_004787	0.00	0.00	0.00	NA	NA
MANI_026081	9.41	1.17	0.30	-2.79	NA
MANI_004841	96.96	44.17	50.18	NA	NA
MANI_004827	3.27	3.30	6.04	NA	NA
MANI_124927	1.83	1.47	0.00	NA	NA
MANI_004821	1.52	0.00	0.62	NA	NA
MANI_114246	0.00	0.00	0.00	NA	NA
MANI_004807	68.73	28.78	12.16	-1.12	-1.21
MANI_004844	22.42	61.91	34.12	1.59	NA
MANI_004776	53.60	30.98	20.79	NA	NA
MANI_004777	22.95	6.88	8.48	-1.57	NA
MANI_004778	133.95	80.25	79.03	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 7: Expression profiling of the MaNRPS3 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log ₂ -fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI

MANI_027530	0.00	0.00	0.00	NA	NA
MANI_027532	0.00	0.00	0.00	NA	NA
MANI_011622	0.00	0.00	0.00	NA	NA
MANI_011572	0.00	0.37	0.00	NA	NA
MANI_027526	0.00	1.09	0.19	NA	NA
MANI_011609	3.60	18.83	1.47	2.46	-3.51
MANI_114418	0.00	0.00	0.00	NA	NA
MANI_011644	0.00	0.00	0.00	NA	NA
MANI_011593	0.00	0.00	0.00	NA	NA

Table 8: Expression profiling of the MaPKS3 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_027534	0.59	39.58	9.20	5.86	-2.05
MANI_027538	0.00	132.34	210.52	8.68	NA
MANI_011630	0.00	64.38	38.76	6.69	NA
MANI_027536	0.00	6.93	0.00	NA	NA
MANI_011575	0.68	0.22	0.22	NA	NA
MANI_011615	7.99	747.98	415.37	6.65	NA
MANI_011585	0.00	0.00	0.00	NA	NA
MANI_011587	0.00	0.00	0.00	NA	NA
MANI_011645	0.00	0.00	0.00	NA	NA
MANI_011623	0.98	0.79	0.00	NA	NA

Table 9: Expression profiling of the MaNRPS4 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_029521	0.00	0.00	0.00	NA	NA
MANI_021152	2.00	130.76	239.85	5.82	0.97
MANI_021040	1.51	10.53	0.00	2.81	-6.68
MANI_021047	0.00	0.49	3.01	NA	NA
MANI_021015	0.83	2.33	4.40	NA	NA
MANI_021096	6.12	4.93	0.84	NA	NA
MANI_029504	8.13	14.32	5.41	NA	-1.39
MANI_029514	0.00	0.00	0.00	NA	NA
MANI_021147	3.57	0.00	0.00	NA	NA
MANI_021114	0.00	0.00	0.00	NA	NA
MANI_021108	0.00	0.00	0.00	NA	NA
MANI_021072	0.00	0.00	0.00	NA	NA
MANI_021085	1.73	0.70	0.00	NA	NA
MANI_021143	0.00	0.00	0.00	NA	NA
MANI_021102	0.00	0.00	0.00	NA	NA
MANI_021077	0.00	0.00	0.00	NA	NA
MANI_020971	0.51	0.17	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 10: Expression profiling of the MaNRPS5 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI

MANI_026060	5.57	1.34	10.47	-1.90	2.83
MANI_110048	44.93	29.69	12.25	NA	-1.20
MANI_026063	0.00	0.00	0.00	NA	NA
MANI_004685	0.00	0.00	0.00	NA	NA
MANI_004679	12.37	0.00	0.00	NA	NA
MANI_026064	5.69	1.53	1.55	NA	NA
MANI_004691	6.25	0.00	1.02	NA	NA
MANI_004698	4.93	14.57	0.00	NA	NA
MANI_004659	0.87	0.14	0.28	NA	NA
MANI_004676	2.14	17.24	1.17	NA	NA
MANI_004665	0.00	0.00	0.00	NA	NA
MANI_026062	0.00	0.00	0.00	NA	NA
MANI_004683	0.00	0.00	0.00	NA	NA
MANI_004696	0.00	0.00	0.00	NA	NA
MANI_004669	0.00	0.00	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 11: Expression profiling of the MaNRPS6 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_004647	9.24	4.92	2.81	NA	NA
MANI_026065	28.51	8.83	1.79	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 12: Expression profiling of the MaPKS4 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_029451	0.00	0.00	0.00	NA	NA
MANI_020788	0.00	0.00	0.00	NA	NA
MANI_020787	0.00	1.14	0.00	NA	NA
MANI_020781	12.56	16.04	14.53	NA	NA
MANI_020786	0.00	0.00	0.00	NA	NA
MANI_020789	1.84	2.96	0.00	NA	NA
MANI_110051	0.23	0.09	0.19	NA	NA
MANI_029463	0.00	0.41	0.00	NA	NA
MANI_020891	0.00	0.00	0.00	NA	NA
MANI_020915	0.00	2.12	0.72	NA	NA
MANI_020808	2.70	8.25	9.71	NA	NA
MANI_020807	6.01	5.25	15.16	NA	1.51

Table 13: Expression profiling of the MaOTHER3 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_005013	0.81	0.00	0.00	NA	NA
MANI_005019	6.30	4.44	0.00	NA	NA
MANI_004943	0.00	0.00	0.00	NA	NA
MANI_005067	21.84	0.00	0.00	NA	NA
MANI_004951	5.08	0.77	2.08	NA	NA

MANI_026138	0.71	0.00	0.00	NA	NA
MANI_005008	0.00	0.00	0.00	NA	NA
MANI_004982	0.00	0.00	0.00	NA	NA
MANI_005015	0.00	0.00	0.00	NA	NA
MANI_005038	0.00	0.00	0.00	NA	NA
MANI_005061	0.00	0.00	0.00	NA	NA

Table 14: Expression profiling of the MaTERP2 cluster (Lanosterol cyclase).

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_025532	6.98	5.62	18.85	NA	1.71
MANI_002110	27.78	65.85	123.06	1.37	NA
MANI_002183	28.69	22.06	16.01	NA	NA
MANI_002164	4.64	14.96	4.27	1.80	-1.73
MANI_002157	86.77	55.48	61.95	NA	NA
MANI_002152	62.42	20.46	20.34	-1.46	NA

The BGC boundaries were delimited by CASSIS.

Table 15: Expression profiling of the MaNRPS-PKS4 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_022500	0.00	0.00	0.00	NA	NA
MANI_029817	8.86	0.59	5.44	NA	NA

MANI_022499	0.00	0.00	1.06	NA	NA
MANI_022470	7.87	4.92	1.25	NA	-1.95

The BGC boundaries were delimited by CASSIS.

Table 16: Expression profiling of the MaPKS5 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log ₂ -fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_010111	0.00	0.00	0.00	NA	NA
MANI_010152	0.00	0.00	0.00	NA	NA
MANI_010132	2.88	2.32	0.79	NA	NA
MANI_010133	0.00	0.00	0.00	NA	NA
MANI_010116	0.00	0.00	0.71	NA	NA
MANI_010145	0.00	0.00	0.00	NA	NA
MANI_010135	0.00	0.00	2.31	NA	NA
MANI_010096	0.85	16.35	18.69	4.12	NA
MANI_010114	14.06	16.99	18.53	NA	NA
MANI_027208	0.28	0.23	0.00	NA	NA
MANI_027199	0.00	0.24	0.00	NA	NA
MANI_115496	0.00	0.00	0.00	NA	NA
MANI_010167	0.00	0.00	0.00	NA	NA
MANI_010121	0.00	0.00	0.00	NA	NA
MANI_010120	0.00	0.69	2.09	NA	NA
MANI_010092	0.00	0.00	0.00	NA	NA
MANI_010168	0.00	0.00	0.00	NA	NA

MANI_010063	0.74	0.00	0.00	NA	NA
MANI_010140	0.00	0.00	0.00	NA	NA
MANI_027224	0.00	1.25	0.00	NA	NA

Table 17: Expression profiling of the MaTERP3 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_027210	0.00	0.00	0.00	NA	NA
MANI_027216	0.00	0.00	0.00	NA	NA
MANI_010143	3.46	1.86	10.37	NA	NA
MANI_125771	0.00	0.00	1.37	NA	NA
MANI_115504	0.00	0.00	0.00	NA	NA

Table 18: Expression profiling of the MaPKS6 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_115504	0.00	0.00	0.00	NA	NA
MANI_010070	0.00	0.31	0.00	NA	NA
MANI_010106	0.00	0.00	0.00	NA	NA
MANI_010080	0.00	0.00	0.52	NA	NA
MANI_027206	0.00	0.00	0.00	NA	NA
MANI_010100	0.00	0.00	0.00	NA	NA
MANI_115518	0.00	0.00	0.00	NA	NA
MANI_010062	0.00	0.00	0.00	NA	NA

MANI_027223	0.00	0.00	0.00	NA	NA
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Table 19: Expression profiling of the MaPKS7 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_020133	1.32	12.52	51.84	3.33	2.00
MANI_115550	0.00	0.00	1.21	NA	NA
MANI_020132	0.00	0.00	0.00	NA	NA
MANI_020152	0.00	0.00	0.00	NA	NA
MANI_020166	0.00	0.00	0.00	NA	NA
MANI_020125	0.12	0.00	0.00	NA	NA
MANI_020170	0.00	0.00	0.00	NA	NA
MANI_029300	1.96	2.37	0.00	NA	NA
MANI_020096	0.00	0.00	0.00	NA	NA
MANI_115552	1.09	1.53	9.11	NA	2.57
MANI_020099	4.06	11.77	118.42	NA	3.35
MANI_020114	0.00	1.84	0.00	NA	NA
MANI_029303	0.00	0.00	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 20: Expression profiling of the MaOTHER4 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI

MANI_019978	130.30	139.93	1338.01	NA	3.22
MANI_019961	14.31	18.73	10.98	NA	NA
MANI_019967	3.47	2.24	10.80	NA	2.22
MANI_019956	228.68	81.62	415.42	-1.35	2.26
MANI_029277	15.97	6.27	6.70	-1.23	NA
MANI_029279	5.81	1.25	0.32	NA	NA
MANI_019946	59.12	52.64	43.27	NA	NA
MANI_019963	66.63	32.66	58.30	NA	NA
MANI_019984	31.07	0.00	0.00	NA	NA
MANI_019966	0.80	0.00	0.00	NA	NA
MANI_019955	19.35	26.54	9.84	NA	-1.43
MANI_029283	0.00	0.00	0.00	NA	NA

Table 21: Expression profiling of the MaNRPS7 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_027353	8.11	6.53	0.00	NA	NA
MANI_010645	17.79	17.30	12.08	NA	NA
MANI_010631	6.41	20.32	12.50	1.79	NA
MANI_115821	50.55	62.04	55.14	NA	NA
MANI_010634	33.25	36.79	55.35	NA	NA
MANI_010656	12.92	13.68	18.73	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 22: Expression profiling of the MaTERP4 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_021634	25.52	20.56	12.53	NA	NA
MANI_021633	5.19	7.32	13.81	NA	NA
MANI_021625	7.97	27.80	13.03	1.93	-1.05
MANI_116096	32.26	46.37	47.62	NA	NA
MANI_021624	2.34	4.71	0.96	NA	NA
MANI_021637	7.16	1.92	0.00	NA	NA

Table 23: Expression profiling of the MaPKS8 cluster (MrPKS2).

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_028483	0.00	0.00	0.00	NA	NA
MANI_016007	0.00	0.00	0.00	NA	NA
MANI_116665	0.00	1.72	0.00	NA	NA
MANI_016082	0.00	0.00	0.00	NA	NA
MANI_028434	1.24	1.22	0.22	NA	NA
MANI_016169	0.00	2.67	0.00	NA	NA
MANI_016181	1.59	6.41	0.00	NA	NA
MANI_016065	2.32	0.00	0.00	NA	NA
MANI_110628	0.00	2.70	0.00	NA	NA
MANI_016229	0.00	0.00	0.00	NA	NA
MANI_016226	0.00	0.00	0.00	NA	NA

MANI_016231	0.00	0.00	0.00	NA	NA
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The BGC boundaries were delimited by CASSIS.

Table 24: Expression profiling of the MaOTHER5 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_116947	236.85	15.37	9.15	-3.82	NA
MANI_018049	74.38	45.22	64.43	NA	NA
MANI_028870	132.40	21.86	20.18	-2.45	NA
MANI_018046	1.25	35.29	13.83	4.82	-1.34
MANI_018106	2.62	3.17	1.07	NA	NA
MANI_028863	16.63	10.42	1.51	NA	-2.67
MANI_018104	37.83	10.60	22.88	-1.66	1.13
MANI_018111	105.18	36.08	28.68	-1.37	NA
MANI_018053	44.82	12.58	9.44	-1.71	NA
MANI_018009	52.57	17.96	34.76	-1.42	1.01
MANI_110662	12.83	12.72	19.38	NA	NA
MANI_017975	15.21	8.31	11.11	NA	NA
MANI_028845	14.53	27.80	16.35	NA	NA
MANI_028842	97.33	60.97	71.89	NA	NA
MANI_017970	13.37	13.08	35.16	NA	1.48
MANI_017982	21.66	7.75	23.63	NA	1.58
MANI_017985	24.41	23.08	24.31	NA	NA

Table 25: Expression profiling of the MaNRPS8 cluster (Metachelin).

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_003049	5.59	1.72	0.54	-1.55	NA
MANI_003143	14.88	6.20	2.52	-1.16	NA
MANI_025764	3.83	0.00	0.00	NA	NA
MANI_003177	22.45	9.61	4.59	-1.10	-1.06
MANI_003060	5.47	5.11	3.94	NA	NA
MANI_003059	28.13	17.17	17.20	NA	NA
MANI_003078	12.29	16.20	15.08	NA	NA
MANI_003226	11.23	2.26	18.40	NA	NA
MANI_003103	12.66	13.78	11.22	NA	NA
MANI_003099	16.46	31.92	34.02	1.07	NA

The BGC boundaries were delimited by CASSIS.

Table 26: Expression profiling of the MaNRPS9 cluster (Ferricrocin).

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_008252	5.97	1.92	0.00	NA	NA
MANI_127150	15.49	22.86	18.33	NA	NA
MANI_008246	39.97	36.56	15.34	NA	-1.22
MANI_117323	17.81	44.82	39.16	1.42	NA
MANI_117325	10.64	28.26	21.38	1.51	NA
MANI_008248	2.57	6.21	3.36	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 27: Expression profiling of the MaPKS9 cluster (Viridicatumtoxin).

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_003872	0.00	0.00	1.05	NA	NA
MANI_003860	0.00	0.00	0.00	NA	NA
MANI_003846	3.52	3.41	2.88	NA	NA
MANI_003816	7.62	6.14	3.12	NA	NA
MANI_003811	1.21	0.00	0.33	NA	NA
MANI_003842	0.00	0.00	0.00	NA	NA
MANI_003768	0.66	0.00	0.00	NA	NA
MANI_003787	3.60	1.45	0.00	NA	NA
MANI_025884	2.33	4.37	3.80	NA	NA
MANI_003838	23.66	1.03	1.05	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 28: Expression profiling of the MaNRPS10 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_003979	1.00	4.01	0.00	NA	NA
MANI_003960	7.40	2.98	0.61	NA	NA
MANI_003945	0.00	0.45	0.00	NA	NA

MANI_003902	0.17	0.87	0.34	NA	NA
MANI_004003	0.00	0.00	0.00	NA	NA
MANI_003995	0.00	1.43	0.00	NA	NA
MANI_003990	0.00	52.01	5.98	7.65	-3.02
MANI_003985	0.00	0.00	0.00	NA	NA
MANI_003934	0.70	0.57	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 29: Expression profiling of the MaPKS10 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log ₂ -fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_019165	299.73	28.05	6.75	-3.31	-1.98
MANI_019170	231.24	21.04	8.71	-3.34	-1.24
MANI_019155	262.76	22.90	8.86	-3.52	-1.34
MANI_019168	430.71	96.96	32.83	-2.12	-1.49

The BGC boundaries were delimited by CASSIS.

Table 30: Expression profiling of the MaOTHER6 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log ₂ -fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_127795	0.00	32.13	143.38	NA	2.15
MANI_006107	47.10	38.84	70.65	NA	NA

MANI_006104	3.96	1.60	0.81	NA	NA
MANI_006102	8.04	0.00	1.20	NA	NA
MANI_110861	2.65	3.56	1.45	NA	NA
MANI_117972	9.17	8.72	8.86	NA	NA
MANI_006066	2.22	12.50	9.98	NA	NA
MANI_006043	55.72	11.33	6.91	-2.17	NA
MANI_006072	10.62	62.02	11.95	2.62	-2.31
MANI_127783	5.46	5.49	4.47	NA	NA
MANI_026371	7.27	6.51	3.97	NA	NA
MANI_006080	0.00	0.00	0.00	NA	NA
MANI_026382	372.43	218.58	42.29	NA	-2.40
MANI_026375	6.64	14.97	9.23	1.26	NA

Table 31: Expression profiling of the MaOTHER7 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_000880	0.00	0.00	0.00	NA	NA
MANI_118212	0.00	0.00	0.00	NA	NA
MANI_000883	7.91	4.25	3.59	NA	NA
MANI_025256	9.30	2.50	5.07	NA	NA
MANI_000863	5.37	1.44	0.73	NA	NA
MANI_000865	0.32	0.00	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 32: Expression profiling of the MaOTHER8 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_000878	1012.15	626.02	73.40	NA	-3.14
MANI_110901	11.64	136.93	74.57	3.63	NA
MANI_000858	12.25	54.27	24.50	2.26	-1.13
MANI_000852	0.00	207.98	129.69	10.09	NA
MANI_118224	9.55	204.61	60.95	4.50	-1.78
MANI_000859	8.82	278.69	146.05	5.09	-1.00
MANI_000861	6.11	180.78	190.77	4.96	NA

The BGC boundaries were delimited by CASSIS.

Table 33: Expression profiling of the MaTERP-PKS1 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_118227	0.00	0.00	0.00	NA	NA
MANI_026441	1.02	23.73	0.83	NA	NA
MANI_006343	0.00	0.00	0.00	NA	NA
MANI_127977	0.00	0.00	0.00	NA	NA
MANI_006355	0.00	0.00	1.46	NA	NA
MANI_006367	1.21	0.00	0.00	NA	NA
MANI_006324	0.00	0.22	0.00	NA	NA
MANI_006356	0.00	0.00	0.00	NA	NA
MANI_006353	0.00	0.00	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 34: Expression profiling of the MaPKS11 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_006805	13.56	6.95	7.07	NA	NA
MANI_006801	17.98	12.35	9.08	NA	NA
MANI_006859	15.96	18.56	26.11	NA	NA
MANI_006835	32.66	26.31	18.96	NA	NA
MANI_006858	43.49	28.29	67.06	NA	1.26
MANI_026542	0.00	0.00	0.00	NA	NA
MANI_006811	0.00	0.55	0.00	NA	NA
MANI_006808	0.00	0.00	0.00	NA	NA
MANI_006856	0.00	0.00	0.00	NA	NA
MANI_006827	0.00	0.00	0.00	NA	NA
MANI_006783	0.93	0.37	0.00	NA	NA
MANI_006831	0.00	0.00	0.00	NA	NA
MANI_006795	0.79	0.63	0.00	NA	NA
MANI_006836	0.00	0.00	0.00	NA	NA
MANI_118628	1.60	1.72	1.31	NA	NA
MANI_006798	1.75	4.69	4.77	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 35: Expression profiling of the MaPKS12 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_002711	0.00	0.51	0.00	NA	NA
MANI_002718	1.15	0.00	0.00	NA	NA
MANI_002715	0.91	0.73	0.00	NA	NA
MANI_002710	1.58	0.85	2.16	NA	NA
MANI_110978	0.12	0.00	0.10	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 36: Expression profiling of the MaTERP5 cluster (GGPP synthase).

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_024154	56.33	19.06	14.72	-1.42	NA
MANI_024125	30.14	45.84	60.37	NA	NA
MANI_024126	45.84	32.23	36.97	NA	NA
MANI_024161	42.10	33.90	31.26	NA	NA
MANI_030188	3.93	3.54	5.12	NA	NA
MANI_030184	0.48	0.39	1.57	NA	NA
MANI_118863	13.35	13.35	15.50	NA	NA
MANI_024131	19.90	29.06	41.22	NA	NA
MANI_024139	12.34	9.94	19.06	NA	NA
MANI_024162	6.06	1.63	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 37: Expression profiling of the MaOTHER9 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_024160	0.00	0.00	0.00	NA	NA
MANI_024071	0.00	0.22	0.00	NA	NA
MANI_024095	5.57	0.37	0.38	NA	NA
MANI_024163	19.00	13.70	4.09	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 38: Expression profiling of the MaNRPS11 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_006972	0.00	3.22	0.00	NA	NA
MANI_006947	10.67	7.60	1.95	NA	-1.97
MANI_006962	13.24	19.43	23.60	NA	NA
MANI_006985	14.84	15.21	32.00	NA	1.08
MANI_026573	6.23	4.02	0.00	NA	NA
MANI_006959	60.29	41.06	51.73	NA	NA
MANI_006969	11.71	3.14	1.60	NA	NA
MANI_118897	8.95	5.90	14.01	NA	1.31

The BGC boundaries were delimited by CASSIS.

Table 39: Expression profiling of the MaNRPS-PKS5 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_006242	2.06	0.00	0.00	NA	NA
MANI_128792	0.00	0.00	0.00	NA	NA
MANI_006238	4.25	0.00	0.00	NA	NA
MANI_006214	2.36	0.95	0.00	NA	NA
MANI_026405	6.57	4.91	1.54	NA	NA
MANI_006237	9.55	0.00	0.00	NA	NA
MANI_006185	1.88	1.21	0.31	NA	NA
MANI_006236	5.57	0.00	0.00	NA	NA
MANI_006158	0.16	0.00	0.00	NA	NA
MANI_006235	0.00	0.00	0.00	NA	NA
MANI_006219	3.16	0.00	0.00	NA	NA
MANI_026410	0.00	0.00	0.00	NA	NA
MANI_026395	0.12	0.00	0.00	NA	NA

Table 40: Expression profiling of the MaNRPS12 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_006235	0.00	0.00	0.00	NA	NA
MANI_006219	3.14	0.00	0.00	NA	NA
MANI_026410	0.00	0.00	0.00	NA	NA
MANI_026395	0.12	0.00	0.00	NA	NA

MANI_006234	0.00	0.00	0.00	NA	NA
MANI_026399	0.40	0.16	0.33	NA	NA
MANI_006155	0.38	0.04	0.04	NA	NA
MANI_006174	19.31	31.10	33.75	NA	NA
MANI_006224	2.22	2.98	2.42	NA	NA
MANI_006172	0.37	0.00	0.00	NA	NA
MANI_119390	0.00	0.46	0.00	NA	NA
MANI_006208	11.80	0.79	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 41: Expression profiling of the MaPKS13 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_006263	0.00	0.00	0.00	NA	NA
MANI_006262	21.65	6.60	4.31	-1.56	NA
MANI_006250	17.29	13.39	8.52	NA	NA
MANI_006260	15.58	14.53	6.04	NA	-1.22
MANI_006261	77.75	3.89	1.80	-4.20	NA
MANI_111163	68.91	73.35	79.32	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 42: Expression profiling of the MaIND-TERP1 cluster (Terpendole E/lolitrein-related compound).

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_027395	1.49	6.01	8.54	NA	NA
MANI_027395	1.49	6.01	8.54	NA	NA
MANI_111286	0.00	7.49	1.38	NA	NA
MANI_027412	0.00	0.00	0.00	NA	NA
MANI_027413	0.00	0.00	0.00	NA	NA
MANI_119883	1.37	2.21	13.49	NA	2.60
MANI_011022	0.00	0.00	0.00	NA	NA
MANI_011010	0.00	0.00	0.74	NA	NA
MANI_011011	0.00	0.00	0.00	NA	NA
MANI_011024	0.87	0.00	0.00	NA	NA
MANI_119895	0.00	0.00	0.00	NA	NA
MANI_011015	0.00	0.00	0.56	NA	NA
MANI_027409	0.00	0.00	0.00	NA	NA
MANI_011023	0.00	1.23	0.62	NA	NA

Table 43: Expression profiling of the MaNRPS13 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_008887	87.51	40.93	72.24	NA	NA
MANI_008916	34.75	12.44	14.76	-1.29	NA
MANI_008932	50.81	57.28	47.19	NA	NA
MANI_008883	3.06	3.95	5.01	NA	NA
MANI_026956	23.71	37.62	34.69	NA	NA

MANI_026948	0.37	1.20	1.22	NA	NA
MANI_008854	0.00	4.49	0.00	NA	NA
MANI_008852	0.86	0.00	0.00	NA	NA
MANI_008843	0.15	0.00	0.00	NA	NA
MANI_008835	0.00	0.00	0.00	NA	NA
MANI_008820	3.25	5.57	1.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 44: Expression profiling of the MaPKS14 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_018961	0.00	6.48	0.00	NA	NA
MANI_029060	0.92	0.74	0.75	NA	NA
MANI_018925	0.00	1.95	0.49	NA	NA
MANI_018949	0.99	7.15	1.61	NA	NA
MANI_018915	0.51	7.81	7.93	NA	NA
MANI_018966	2.15	6.91	3.51	NA	NA
MANI_018879	2.82	6.81	2.58	1.42	-1.37

Table 45: Expression profiling of the MaIND-NRPS1 cluster (Elymooclavine/ergovaline-related compound).

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_021859	0.00	0.00	0.00	NA	NA

MANI_029655	0.00	0.00	0.00	NA	NA
MANI_021853	0.00	0.00	0.00	NA	NA
MANI_029666	0.00	0.00	0.00	NA	NA
MANI_029677	0.00	0.00	0.00	NA	NA
MANI_021832	2.48	0.50	0.51	NA	NA
MANI_021880	0.00	0.00	0.00	NA	NA
MANI_120630	0.39	1.26	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 46: Expression profiling of the MaOTHER10 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_011655	7.77	6.57	10.81	NA	NA
MANI_011669	10.08	13.19	5.15	NA	-1.33
MANI_011674	6.85	1.38	0.70	NA	NA
MANI_011690	0.00	112.28	107.00	8.53	NA
MANI_011679	1.18	1.43	19.78	NA	3.68
MANI_011680	5.09	8.72	59.38	NA	2.75
MANI_011687	14.26	58.09	22.64	2.14	-1.32
MANI_011653	0.00	0.00	0.00	NA	NA

Table 47: Expression profiling of the MaPKS15 cluster.

NCBI's gene locus ID	Expression (RPKM)	Differential expression (log2-fold change)
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	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_021560	6.78	4.96	19.70	NA	1.93
MANI_021544	0.00	3.49	0.00	NA	NA
MANI_021563	16.13	1022.48	19.79	6.09	-5.64
MANI_021550	6.02	16.57	44.34	1.58	1.42
MANI_021547	2.17	10.06	12.00	2.27	NA
MANI_021569	1.26	1.01	1.03	NA	NA
MANI_021559	0.60	2.92	0.00	NA	NA
MANI_029610	0.73	0.00	1.80	NA	NA
MANI_021580	0.00	0.00	0.00	NA	NA
MANI_021529	0.98	0.79	0.20	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 48: Expression profiling of the MaPKS16 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_020896	0.00	0.00	0.00	NA	NA
MANI_029455	0.24	0.19	0.00	NA	NA
MANI_020910	0.00	0.00	0.00	NA	NA
MANI_020904	0.00	0.00	0.00	NA	NA
MANI_121029	0.00	0.57	0.58	NA	NA
MANI_020797	0.00	0.00	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 49: Expression profiling of the MaOTHER11 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_121318	32.80	17.12	10.18	NA	NA
MANI_007223	10.35	18.63	17.43	NA	NA
MANI_007317	19.62	6.70	16.05	-1.36	1.25
MANI_121326	60.49	25.00	29.63	-1.15	NA
MANI_026683	157.67	74.92	48.26	NA	NA
MANI_111648	86.50	52.01	42.86	NA	NA
MANI_026624	12.59	3.59	3.49	-1.68	NA
MANI_007232	41.21	26.04	26.71	NA	NA
MANI_121338	46.45	14.76	12.15	-1.53	NA
MANI_121345	22.65	16.91	46.09	NA	1.49
MANI_136963	22.73	12.20	12.42	NA	NA
MANI_007164	17.88	26.56	25.27	NA	NA
MANI_130057	39.08	15.38	12.52	-1.24	NA

Table 50: Expression profiling of the MaNRPS-PKS7 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_002005	0.00	0.00	0.00	NA	NA
MANI_001951	0.22	0.17	0.71	NA	NA
MANI_001950	0.00	0.16	0.00	NA	NA
MANI_121458	0.00	0.00	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 51: Expression profiling of the MaPKS17 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_002833	0.00	0.00	0.00	NA	NA
MANI_002790	0.00	0.00	0.00	NA	NA
MANI_002789	13.10	5.63	10.00	NA	NA
MANI_002808	0.00	3.00	1.52	NA	NA
MANI_025650	1.54	5.05	1.58	1.75	-1.55
MANI_002819	0.80	13.61	2.63	NA	NA
MANI_002768	1.37	23.87	9.65	4.22	-1.32
MANI_002813	0.84	0.67	0.00	NA	NA
MANI_002850	2.43	1.95	0.00	NA	NA
MANI_002828	0.00	0.00	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 52: Expression profiling of the MaOTHER12 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_010565	0.00	0.00	0.00	NA	NA
MANI_010519	83.27	258.33	140.03	1.75	NA
MANI_010516	0.00	11.80	1.89	NA	NA

MANI_010482	0.59	8.37	2.43	3.81	-1.74
MANI_010462	1.05	16.85	3.42	NA	NA
MANI_010460	0.00	2.70	0.00	NA	NA
MANI_010583	1.91	65.38	35.94	5.09	NA
MANI_027314	0.54	6.06	3.08	NA	NA
MANI_010548	0.78	69.21	56.88	6.31	NA
MANI_027320	0.00	21.77	7.04	7.38	-1.66

The BGC boundaries were delimited by CASSIS.

Table 53: Expression profiling of the MaPKS18 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_027325	279.12	15.82	12.19	-4.04	NA
MANI_010559	0.00	0.00	0.00	NA	NA
MANI_010451	2.14	296.21	8.90	7.21	-5.01
MANI_010561	7.70	427.29	28.00	5.90	-3.98
MANI_027340	1.85	44.71	0.00	4.43	-6.89
MANI_010595	5.59	243.85	4.57	5.52	-5.65
MANI_010577	0.91	237.72	14.91	7.86	-3.95
MANI_010494	5.23	394.22	2.44	6.35	-7.27
MANI_010469	0.00	35.23	1.38	8.20	-4.52
MANI_027305	0.00	60.56	6.04	8.75	-3.32
MANI_121654	0.83	105.53	15.61	6.85	-2.78
MANI_010508	2.57	456.62	2.10	7.54	-7.67

MANI_010524	0.65	216.07	1.59	8.20	-6.92
MANI_010603	7.89	1844.05	101.19	7.94	-4.13
MANI_121656	0.00	179.64	3.23	8.75	-5.62

The BGC boundaries were delimited by CASSIS.

Table 54: Expression profiling of the MaNRPS-PKS6 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_010576	0.00	0.72	0.00	NA	NA
MANI_010456	0.00	0.00	0.00	NA	NA
MANI_121659	0.00	0.00	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 55: Expression profiling of the MaPKS19 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_012057	32.23	35.24	28.70	NA	NA
MANI_012116	35.65	16.36	21.78	NA	NA
MANI_012094	24.60	6.60	26.09	-1.74	2.01
MANI_012096	25.33	16.58	66.06	NA	1.85
MANI_012206	2.03	4.08	5.81	NA	NA
MANI_121697	10.57	57.48	12.98	2.50	-2.05

MANI_130428	0.00	0.82	0.00	NA	NA
MANI_012054	2.89	4.95	7.29	NA	NA
MANI_012122	34.25	11.69	14.72	-1.46	NA

The BGC boundaries were delimited by CASSIS.

Table 56: Expression profiling of the MATERP6 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_020045	4.98	9.12	10.37	NA	NA
MANI_020046	9.79	8.35	11.31	NA	NA
MANI_020049	1.81	3.65	8.89	NA	NA
MANI_020053	58.05	21.51	35.15	-1.30	NA

The BGC boundaries were delimited by CASSIS.

Table 57: Expression profiling of the MaOTHER13 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_000542**	23.17	48.52	50.97	1.14	NA
MANI_025166**	2.95	3.57	0.60	NA	NA
MANI_000548**	0.00	0.00	0.00	NA	NA
MANI_000544**	0.00	8.10	0.00	NA	NA
gene fragmented between two contigs	X	X	X		

MANI_000554***	4.34	23.00	31.48	2.51	NA
MANI_025168***	0.18	7.82	10.94	5.23	NA
MANI_000583****	8.03	9.70	1.64	NA	-2.43
MANI_000620****	16.39	7.92	10.73	NA	NA
MANI_000601****	17.74	6.90	19.52	-1.24	1.47
MANI_000573****	3.17	2.98	8.20	NA	1.48
MANI_000623****	0.00	0.00	0.00	NA	NA
MANI_000569****	0.00	0.00	0.00	NA	NA

contig00046 *contig00234 ****contig00235

The BGC boundaries were delimited by comparative genomic.

Table 58: Expression profiling of the MaIND2 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_004366	23.11	1503.88	224.45	6.15	-2.69
MANI_004339	8.07	6.99	2.03	NA	NA
MANI_004371	4.99	18.77	23.19	NA	NA
MANI_111866	27.97	55.51	49.94	1.11	NA
MANI_004296	603.43	29.95	15.52	-4.23	NA
MANI_004325	6.92	4.71	2.18	NA	NA
MANI_004330	62.93	283.97	119.47	2.30	-1.23
MANI_004315	149.01	74.86	43.86	NA	NA
MANI_004356	17.98	86.01	27.68	2.38	-1.66

MANI_004327	1.15	26.91	25.45	4.51	NA
MANI_004359	0.00	0.00	0.00	NA	NA
MANI_004342	0.00	2.15	0.00	NA	NA
MANI_004337	2.88	2.32	1.88	NA	NA
MANI_025992	3.17	0.85	0.00	NA	NA
MANI_122213	6.38	7.19	2.09	NA	NA
MANI_025981	4.80	3.48	2.36	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 59: Expression profiling of the MaSID1 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_004337	2.88	2.32	1.88	NA	NA
MANI_025992	3.17	0.85	0.00	NA	NA
MANI_122213	6.38	7.19	2.09	NA	NA
MANI_025981	4.80	3.48	2.36	NA	NA
MANI_025985	5.19	2.09	6.38	NA	NA
MANI_122223	3.64	0.00	0.00	NA	NA
MANI_004345	13.90	16.23	7.97	NA	NA
MANI_025995	34.27	23.91	9.36	NA	NA
MANI_004301	0.00	0.00	0.00	NA	NA
MANI_004302	0.00	0.00	0.00	NA	NA
MANI_004303	0.00	0.00	0.00	NA	NA
MANI_025984	0.00	0.00	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 60: Expression profiling of the MaTERP7 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_020268	7.86	0.00	1.07	NA	NA
MANI_020207	0.59	0.48	0.00	NA	NA
MANI_020272	0.00	0.00	0.00	NA	NA
MANI_020253	0.00	0.00	0.00	NA	NA
MANI_020226	3.26	1.97	1.33	NA	NA
MANI_130866	1.39	0.00	0.00	NA	NA
MANI_020201	3.17	22.04	15.93	2.86	NA

The BGC boundaries were delimited by CASSIS.

Table 61: Expression profiling of the MaPKS20 cluster (MrPKS1).

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_122426	0.43	0.11	0.00	NA	NA
MANI_030118	0.00	0.00	0.00	NA	NA
MANI_122440	0.00	0.00	0.00	NA	NA
MANI_024459	0.00	0.00	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 62: Expression profiling of the MaPKS20 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_009961	0.00	0.00	0.00	NA	NA
MANI_009911	0.00	0.00	0.00	NA	NA
MANI_009948	0.00	0.00	0.00	NA	NA
MANI_009931	0.00	0.00	0.00	NA	NA
MANI_122478	0.33	0.00	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 63: Expression profiling of the MaOTHER14 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_009929	8.46	2.62	0.00	NA	NA
MANI_027167	0.00	0.00	0.00	NA	NA
MANI_009917	0.00	0.00	0.00	NA	NA
MANI_122490	0.60	0.00	0.00	NA	NA
MANI_027170	0.62	0.00	1.01	NA	NA
MANI_009926	1.56	0.00	0.00	NA	NA
MANI_009964	0.00	0.00	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 64: Expression profiling of the MaTERP8 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_010996	0.00	142.69	29.99	8.44	-2.19
MANI_027397	0.00	0.00	0.00	NA	NA
MANI_010966	12.38	17.56	15.94	NA	NA
MANI_010962	1.85	1.46	3.03	NA	NA
MANI_010993	0.00	0.00	0.00	NA	NA
MANI_010967	0.00	0.00	0.54	NA	NA
MANI_027387	10.14	11.92	11.13	NA	NA
MANI_027402	3.12	6.71	6.83	NA	NA
MANI_119837	1.88	0.00	1.54	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 65: Expression profiling of the MaPKS21 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_110367	0.00	0.33	0.00	NA	NA
MANI_006393	0.00	0.00	0.00	NA	NA
MANI_006390	0.00	2.05	0.00	NA	NA
MANI_006399	0.70	0.00	0.00	NA	NA
MANI_006397	0.00	0.42	0.00	NA	NA
MANI_006388	1.13	0.61	0.00	NA	NA

MANI_006380	4.53	0.37	0.93	-3.34	NA
MANI_025247	13.04	7.88	8.01	NA	NA
MANI_110374	16.10	4.82	1.23	-1.61	NA
MANI_025248	0.94	0.75	1.53	NA	NA
MANI_000835	10.20	58.41	32.51	2.63	NA

The BGC boundaries were delimited by CASSIS.

Table 66: Expression profiling of the MaPKS22 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log ₂ -fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_017305	11.98	4.70	2.27	-1.21	NA
MANI_028697	32.25	19.25	11.85	NA	NA
MANI_017308	44.68	42.33	54.92	NA	NA
MANI_028694	9.95	7.16	1.17	NA	NA
MANI_017329	5.44	0.00	0.64	NA	NA
MANI_017326	14.64	33.41	15.00	1.34	-1.17
MANI_028690	100.76	59.02	47.20	NA	NA
MANI_017297	76.21	53.97	58.30	NA	NA
MANI_028699	16.10	7.98	15.22	NA	1.01
MANI_017319	28.36	15.67	19.14	NA	NA
MANI_014258	0.00	0.00	0.00	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 67: Expression profiling of the MaTERP9 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_022954	5.42	0.55	1.67	NA	NA
MANI_022919	19.28	6.79	2.57	-1.38	-1.39
MANI_029935	19.16	23.51	9.72	NA	-1.20
MANI_022959	11.49	12.03	5.18	NA	-1.15
MANI_022941	2.43	18.77	6.76	3.04	-1.44
MANI_022963	4.64	9.60	6.51	NA	NA
MANI_022973	0.00	4.29	1.45	NA	NA

The BGC boundaries were delimited by CASSIS.

Table 68: Expression profiling of the MaIND3 cluster.

NCBI's gene locus ID	Expression (RPKM)			Differential expression (log2-fold change)	
	48hC	48hI	144hI	48hCx48hI	48hIx144hI
MANI_024998	1.92	0.77	2.36	NA	NA
MANI_030435	0.00	0.00	0.00	NA	NA
MANI_024979	0.00	0.00	0.00	NA	NA
MANI_024963	2.58	0.26	2.90	NA	NA
MANI_030430	3.77	0.00	1.93	NA	NA
MANI_024985	1.68	0.90	3.22	NA	NA
MANI_112059	25.12	17.53	18.52	NA	NA

MANI_024957	2.32	22.89	6.89	3.42	-1.69
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For all tables, forty-eight hours for control condition (hC) and both 48 and 144 hours for infection condition (hI) were used to estimate the relative changes in expression levels. NA: Not Available.

Additional File 7: Comparative genomic analysis and synteny for the aurovertin, elymoclavine/ergovaline-related compound, terpendole E/lolitrem-related compound, and xenolozoyenone-related compound BGCs.

Elymoclavine/ergovaline related compound biosynthetic gene cluster

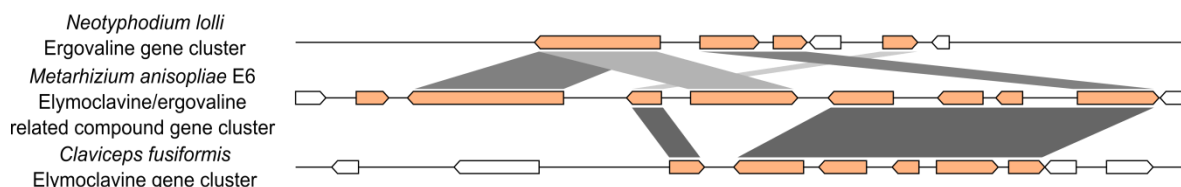


Figure 1: Comparative genomic analysis and synteny for elymoclavine/ergovaline related compound BGC (MaIND-NRPS1).

Terpendole E/lolitrem related compound biosynthetic gene cluster

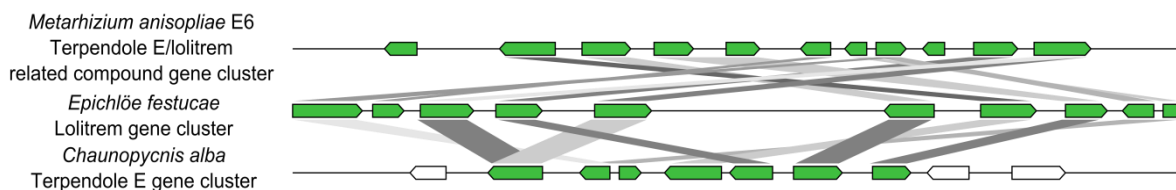


Figure 2: Comparative genomic analysis and synteny for terpendole E/lolitrem related compound BGC (MaIND-TERP1).

Xenolozoyenone related compound biosynthetic gene cluster

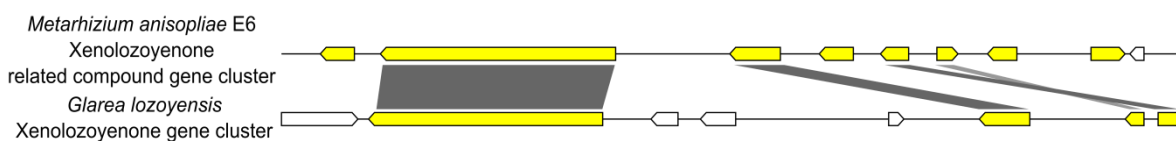


Figure 3: Comparative genomic analysis and synteny for xenolozoyenone related compound BGC (MaNRPS-PKS3).

Table 1*: Identity between aurovertin BGC from *C. arbuscula* and the putative aurovertin BGC (MaPKS2) from *M. anisopliae*.

<i>C. arbuscula</i> gene	<i>M. anisopliae</i> gene	Identity
<i>Aura</i>	MANI_004781	77%
<i>aurB</i>	MANI_004840	76 %
<i>aurC</i>	MANI_026083	77 %
<i>aurD</i>	MANI_026090	71 %
<i>aurE</i>	MANI_026098	53 %
<i>aurF</i>	MANI_120612	40%
<i>aurG</i>	x	X

*No nucleotide sequence found for aurovertin BGC from *C. arbuscula*, not allowing synteny analysis.

Additional File 8: Additional phylogenetic trees.

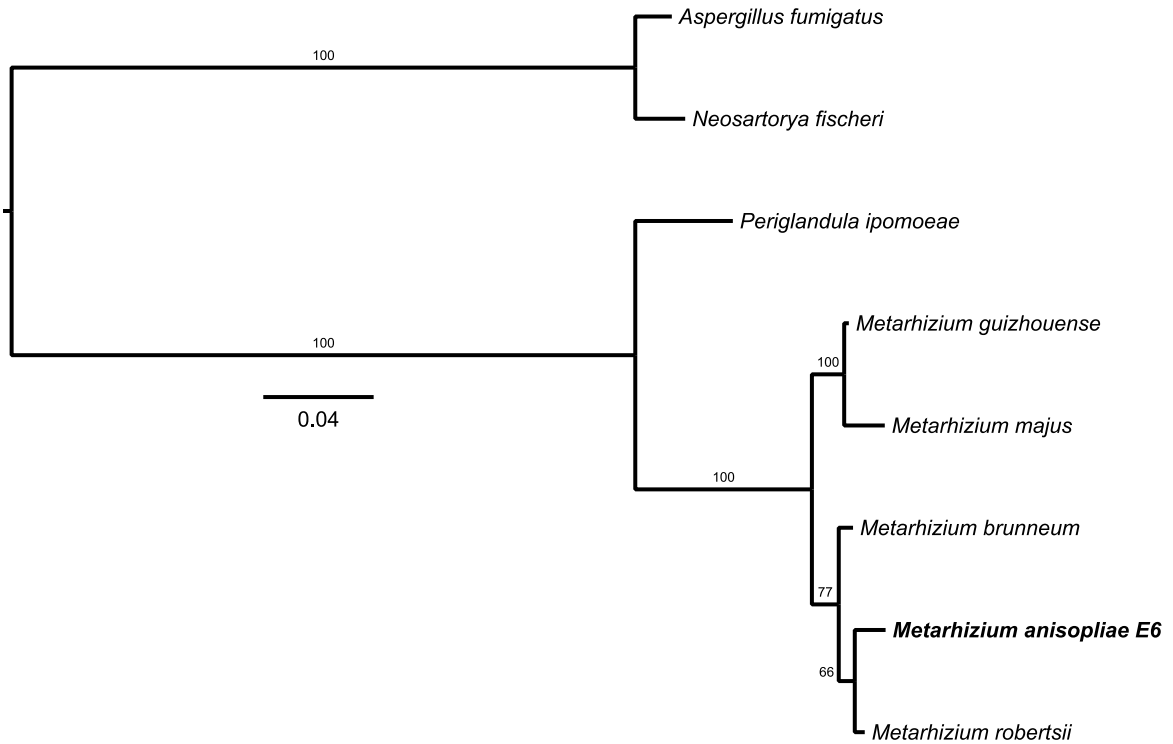


Figure 1: Maximum-likelihood tree for MANI_010495. The gene MANI_010495 assists in helvolic acid biosynthesis. Branches support values (bootstrap proportions) are associated with nodes.

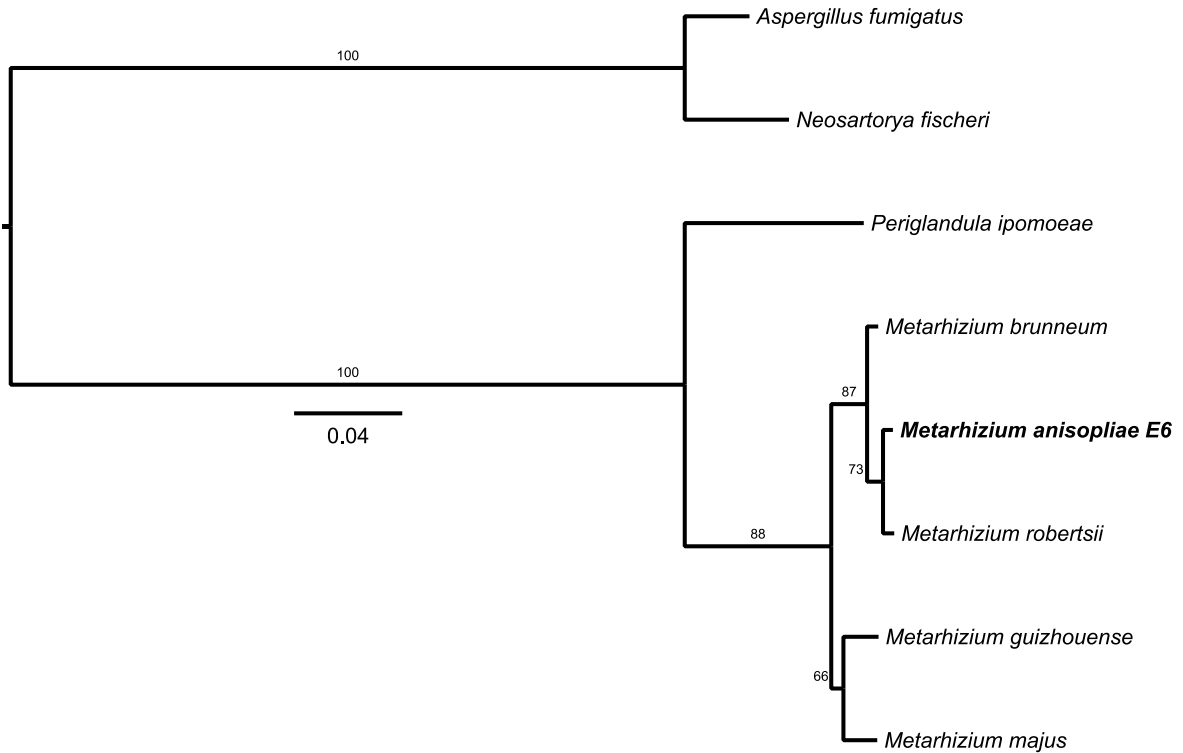


Figure 1: Maximum-likelihood tree for MANI_010512. The gene MANI_010512 assists in helvolic acid biosynthesis. Branches support values (bootstrap proportions) are associated with nodes.

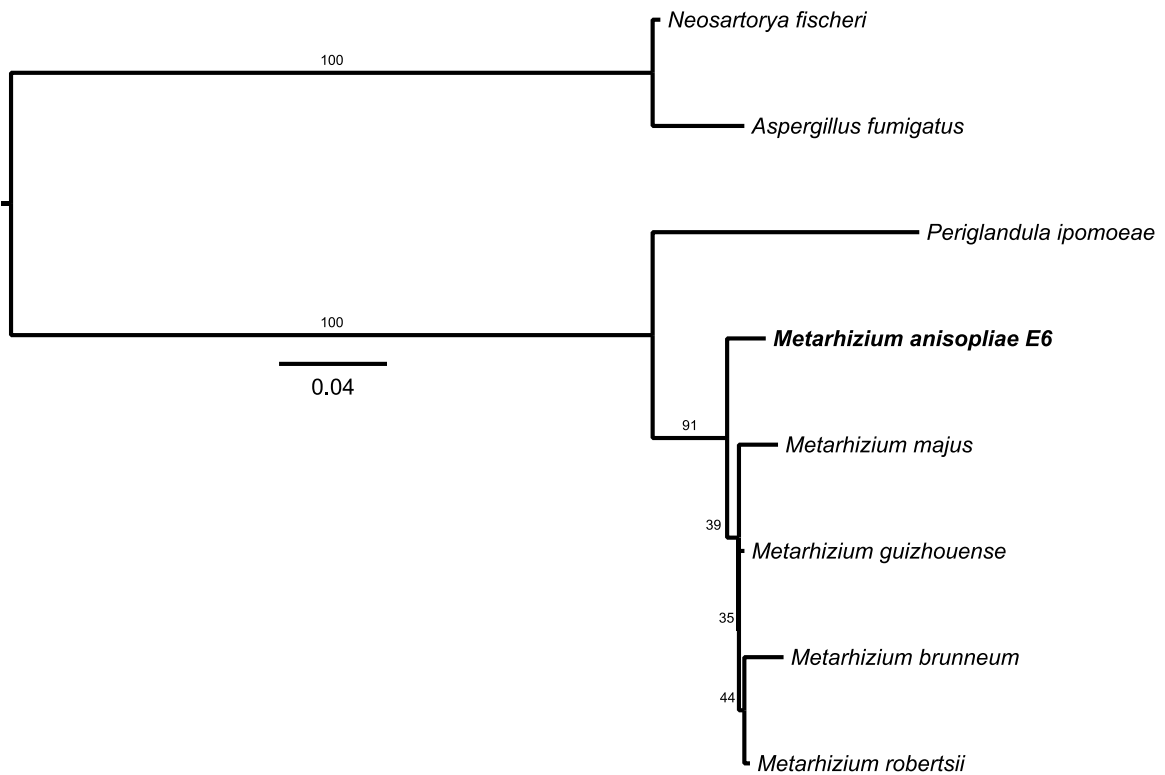


Figure 2: Maximum-likelihood tree for MANI_010527. The gene MANI_010527 assists in helvolic acid biosynthesis. Branches support values (bootstrap proportions) are associated with nodes.

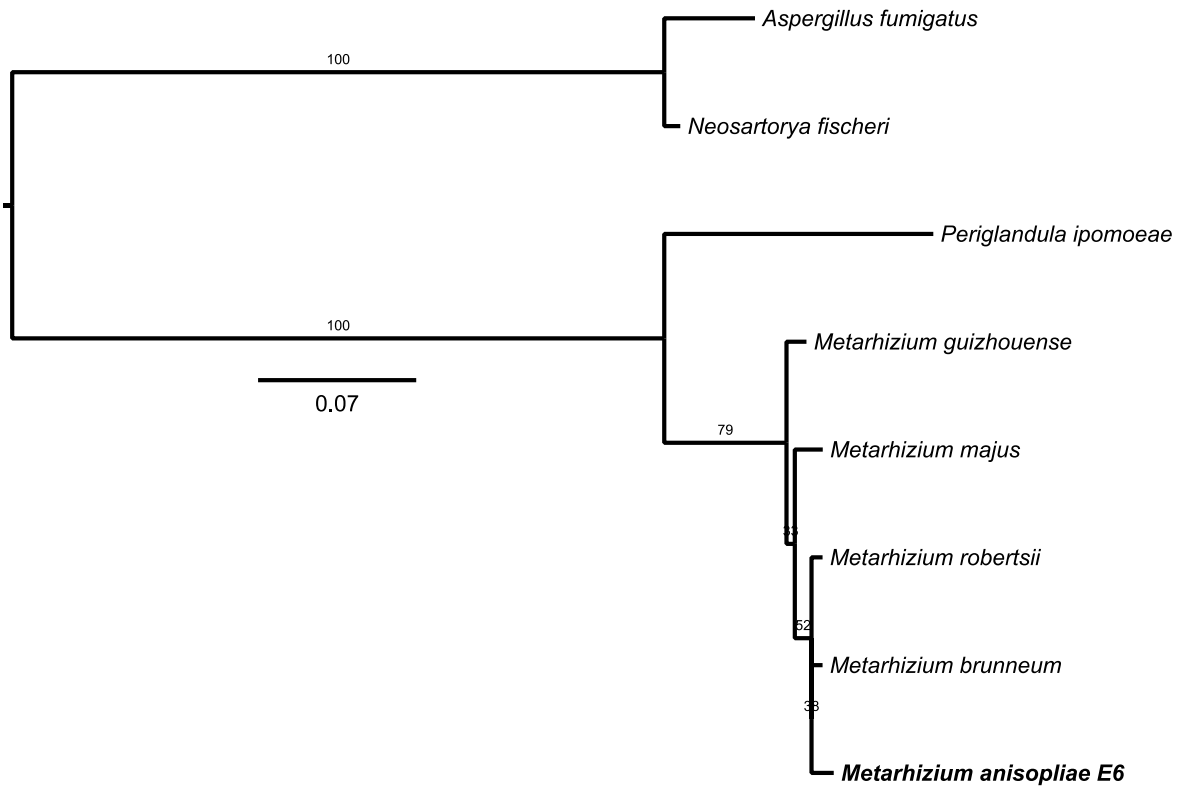


Figure 3: Maximum-likelihood tree for MANI_010530. The gene MANI_010530 assists in helvolic acid biosynthesis. Branches support values (bootstrap proportions) are associated with nodes.

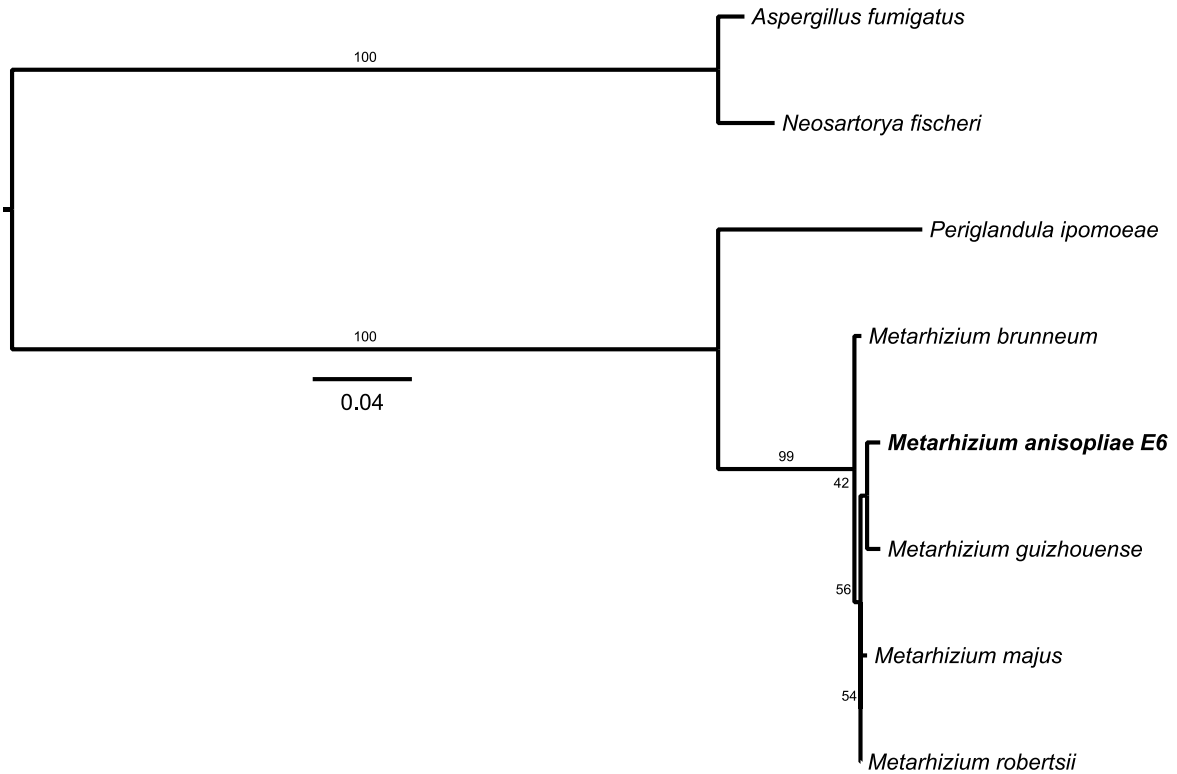


Figure 4: Maximum-likelihood tree for MANI_010531. The gene MANI_010531 assists in helvolic acid biosynthesis. Branches support values (bootstrap proportions) are associated with nodes.

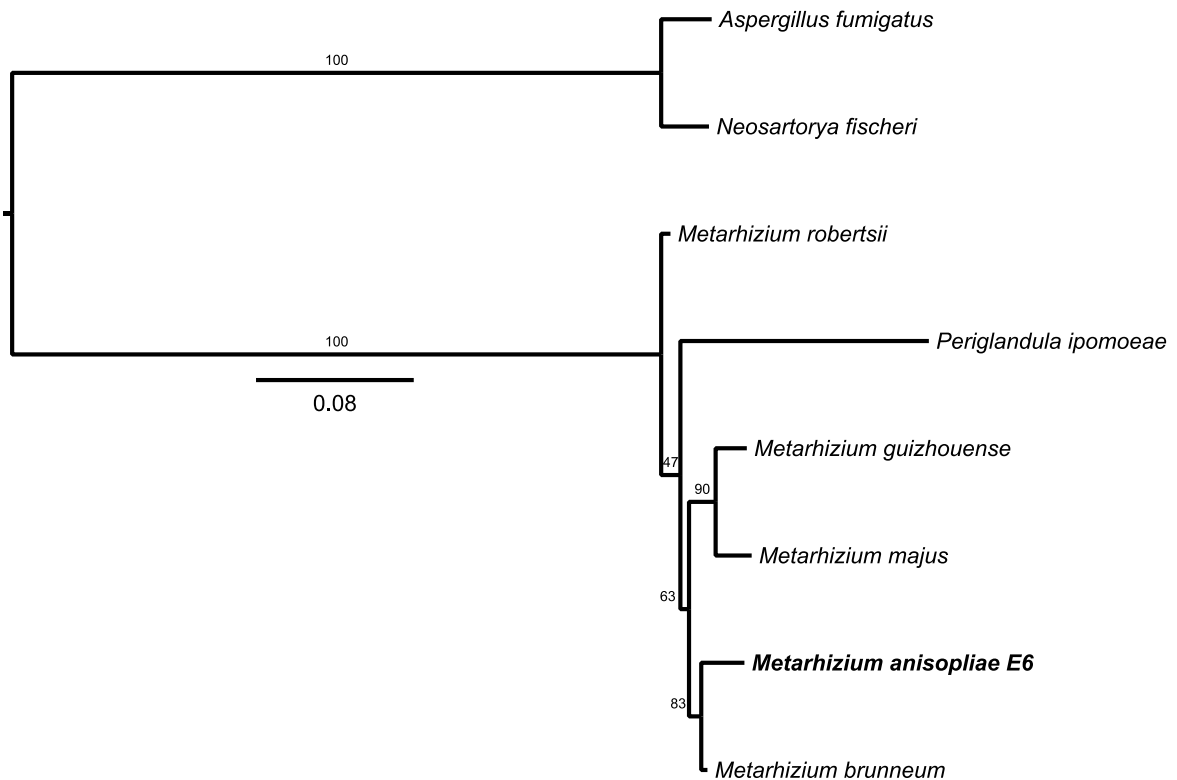


Figure 5: Maximum-likelihood tree for MANI_010532. The gene MANI_010532 assists in helvolic acid biosynthesis. Branches support values (bootstrap proportions) are associated with nodes.

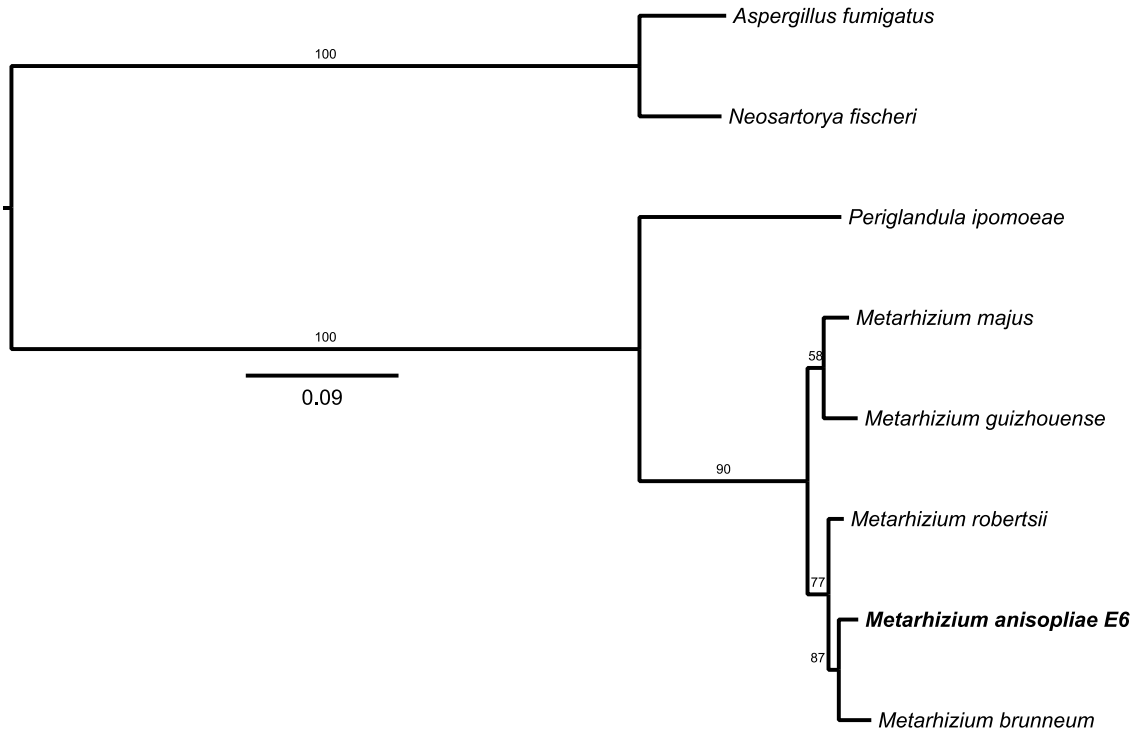


Figure 6: Maximum-likelihood tree for MANI_010536. The gene MANI_010536 assists in helvolic acid biosynthesis. Branches support values (bootstrap proportions) are associated with nodes.

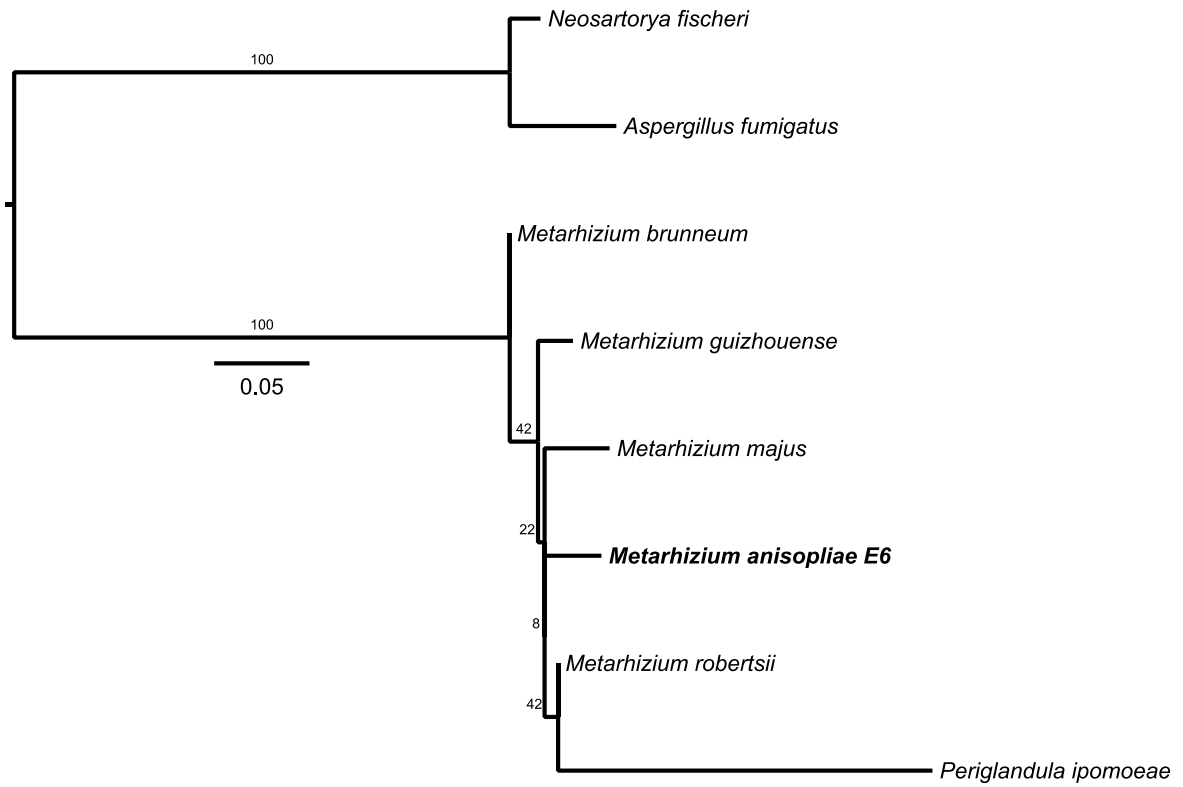


Figure 7: Maximum-likelihood tree for MANI_010537. The gene MANI_010537 assists in helvolic acid biosynthesis. Branches support values (bootstrap proportions) are associated with nodes.

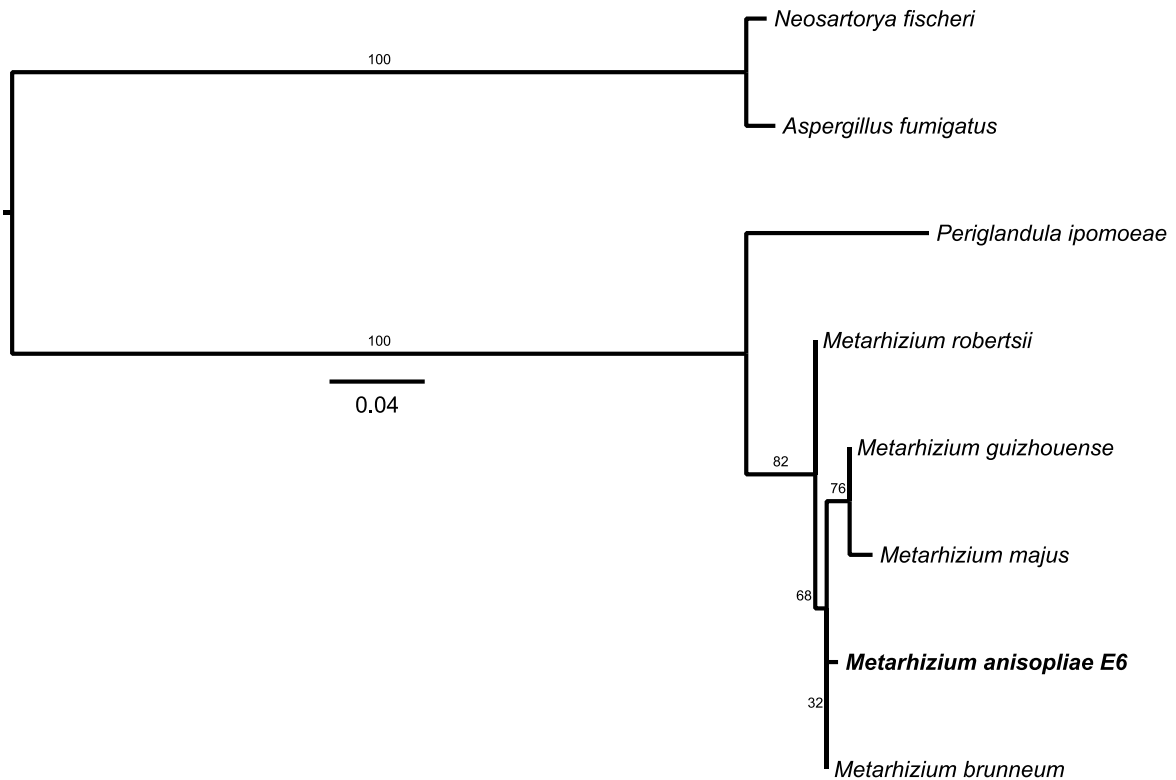


Figure 8: Maximum-likelihood tree for MANI_010594. The gene MANI_010594 assists in helvolic acid biosynthesis. Branches support values (bootstrap proportions) are associated with nodes.

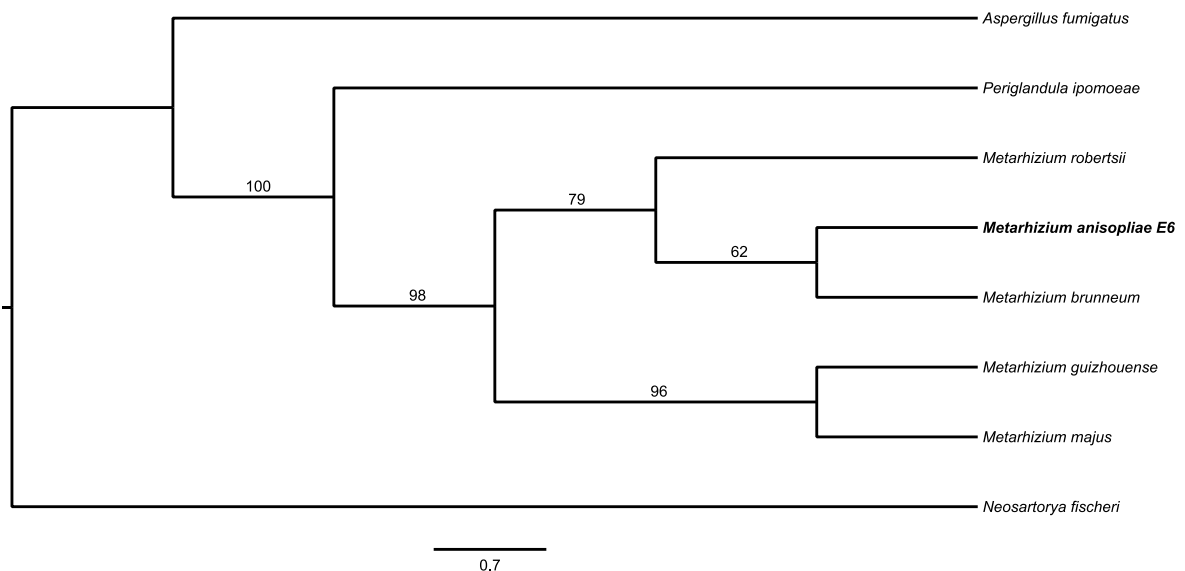


Figure 9: Supertree. MRP supertree constructed based on the inferred trees for helvolic acid cluster with CLANN 3.1.3. Branches support values (bootstrap proportions) are associated with nodes.

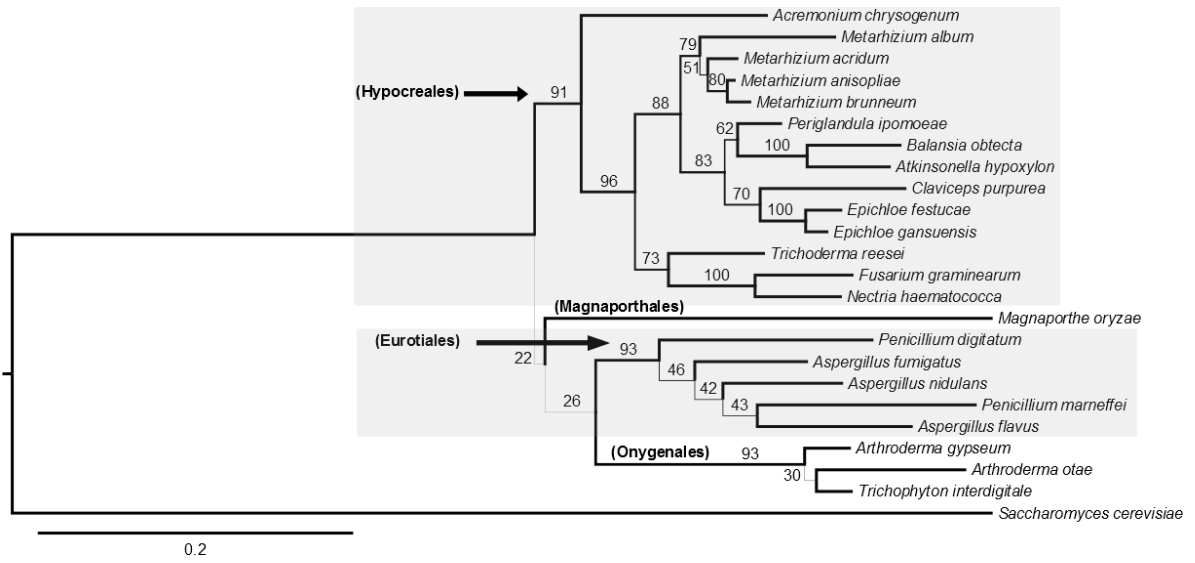


Figure 10: Species tree. Maximum-likelihood phylogeny for *tef1*, a barcode gene, showing the established species relationships. Branches support values (bootstrap proportions) are associated with nodes.

Additional File 9: Amino acid alignment for the pseurotin-related backbone gene and related orthologs.

Additional File 10: Amino acid alignment for the tropolone/citrinin-related backbone gene and related orthologs (.fasta).

Additional File 11: Nucleotide alignment for the *tef1* gene (.fasta).

Additional File 12: Amino acid alignment for helvolic acid supermatrix (.fasta).

Additional File 13: OrthoMCL clustering results (.pdf).

<p style="text-align: center;">MaPKS1 (MANI_014762) Backbone gene*</p>	<p> Acl EAW12049.1 Acl EAW12177.1 Afi EAW17018.1 Afi EAW19983.1 Afu EAL84933.1 Afu EAL86540.1 Ani XP001390084.2 Ani XP001393501.2 Ani XP001395291.2 Ano KNG83914.1 Ano KNG86665.1 Ano KNG91356.1 Aot XP002843683.1 Aot XP002846669.1 Aot XP002848394.1 Aud GAO81768.1 Aud GAO86048.1 Aud GAO89462.1 Aud GAO90476.1 Cim EAS31602.3 Cim EAS32138.2 Cim EAS33665.3 Cim EAS34547.3 Cpo EFW13280.1 Cpo EFW17356.1 Cpo EFW20973.1 Cpo EFW23245.1 Cps KMM68116.1 Cps KMM70187.1 Cps KMM71002.1 Cps KMM72476.1 Csu KDN60752.1 Csu KDN67932.1 Csu KDN68948.1 Csu KDN72002.1 Mac EFY84249.1 Mac EFY85737.1 Mac EFY89365.1 MANI_006324 MANI_020781 Mbr KID60724.1 Mbr KID72947.1 Mbr KID74158.1 Mgu KID81974.1 Mgu KID82773.1 Mgu KID83470.1 Mgu KID84064.1 Mma KID95125.1 Mma KID96266.1 Mma KID99274.1 Mmy KXX79183.1 Mmy KXX79217.1 Mro EFY94576.2 Mro EFY96642.1 Mro EFY96950.1 Oma KIM92947.1 Oma KIM94086.1 Oma KIM97742.1 Oma KIN05207.1 Pdi EKV06857.1 Pdi EKV16250.1 Pte EFQ90032.1 Pte EFQ91438.1 Pte EFQ92321.1 Tce GAM33949.1 Tce GAM33954.1 Tce GAM33973.1 Tce GAM35873.1 Tce GAM41193.1 Tce GAM41694.1 The CEJ81468.1 The CEJ90349.1 The CEJ91591.1 Tis CRG86486.1 Tis CRG88297.1 Tis CRG91771.1 Tis CRG91775.1 Tis CRG92018.1 Tma KFX42009.1 Tma KFX41537.1 Tma KFX45541.1 Tma KFX45542.1 Tma KFX46688.1 Tma KFX46693.1 Top KND87339.1 Top KND88239.1 Top KND93702.1 Top KND93710.1 Top KND93709.1 Tst EED12110.1 Tst EED12124.1 Tst EED18001.1 </p>
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	<p>Tst EED22580.1 Tst EED22651.1 Tst EED22678.1 Tst EED22683.1 Tte AEO64805.1 Tte AEO67399.1 Tte AEO68597.1 Tte AEO67410.1 Abe EFE29950.1 Ach KFH44362.1 Ani XP001393524.2 Lma XP003842913.1 Mal KHN98032.1 Mgy EFR04739.1 Npa EOD53036.1 Psp KFX98702.1 Sap KEZ43375.1 Tin EZF36181.1 Tru EGD89109.2 Tso EZF74361.1 Tve EFE42799.1 Ano KNG83518.1 Lma XP003842914.1 Psp KFX99638.1</p>
<p>MaNRPS-PKS2 (MANI_018878) Backbone gene*</p>	<p>Abe EFE30182.1 Abe EFE31625.1 Abe EFE33181.1 Abe EFE33214.1 Abe EFE33589.1 Abe EFE34630.1 Abe EFE36394.1 Ach KFH40504.1 Ach KFH40639.1 Ach KFH40913.1 Ach KFH41335.1 Ach KFH42117.1 Ach KFH43187.1 Ach KFH43887.1 Ach KFH43961.1 Ach KFH44143.1 Ach KFH44396.1 Ach KFH44962.1 Ach KFH45102.1 Ach KFH45393.1 Ach KFH45702.1 Ach KFH46614.1 Ach KFH47519.1 Ach KFH44074.1 Ach KFH42871.1 Ach KFH45364.1 Ach KFH40930.1 Ach KFH44047.1 Ach KFH44869.1 Acl EAW07064.1 Acl EAW07624.1 Acl EAW08895.1 Acl EAW09019.1 Acl EAW09117.1 Acl EAW11760.1 Acl EAW12170.1 Acl EAW12336.1 Acl EAW12416.1 Acl EAW13625.1 Acl EAW14856.1 Acl EAW08950.1 Acl EAW10019.1 Acl EAW13520.1 Acl EAW13531.1 Acl EAW15062.1 Afi EAW16387.1 Afi EAW16886.1 Afi EAW17052.1 Afi EAW17483.1 Afi EAW19759.1 Afi EAW21094.1 Afi EAW23638.1 Afi EAW23793.1 Afi EAW16804.1 Afi EAW20415.1 Afu EAL85113.2 Afu EAL86424.2 Afu EAL89230.2 Afu EAL91103.2 Afu EAL85129.1 Afu EAL87227.2 Afu EAL92117.1 Afu EAL87813.1 Ani XP001388555.2 Ani XP001389118.2 Ani XP001390513.2 Ani XP001391193.2 Ani XP001393447.2 Ani XP001393508.2 Ani XP001394029.2 Ani XP001394423.2 Ani XP001394543.2</p>

	<p> Ani XP001395346.2 Ani XP001395352.1 Ani XP001397040.2 Ani XP001398521.2 Ani XP001400073.2 Ani XP001389116.2 Ani XP001390395.2 Ani XP001394387.1 Ani XP001394579.1 Ani XP001396949.2 Ani XP001392496.2 Ani XP001394581.2 Ani XP001396381.2 Ani XP001396752.1 Ani XP001397313.2 Ani XP001401800.2 Ani XP001402359.2 Ani XP001393844.2 Ani XP003188554.1 Ani XP001394625.2 Ani XP001396431.2 Ani XP001399961.2 Ani XP001400817.2 Ani XP003188850.1 Ani XP001395761.2 Ano KNG81069.1 Ano KNG81092.1 Ano KNG81162.1 Ano KNG82131.1 Ano KNG83279.1 Ano KNG83443.1 Ano KNG83684.1 Ano KNG84539.1 Ano KNG85249.1 Ano KNG87291.1 Ano KNG88285.1 Ano KNG88668.1 Ano KNG88685.1 Ano KNG90368.1 Ano KNG90587.1 Ano KNG90606.1 Ano KNG90940.1 Ano KNG91513.1 Ano KNG89430.1 Ano KNG91625.1 Ano KNG84699.1 Ano KNG85583.1 Ano KNG90546.1 Ano KNG91681.1 Aot XP002842735.1 Aot XP002843368.1 Aot XP002843704.1 Aot XP002844003.1 Aot XP002846667.1 Aot XP002846808.1 Aot XP002847329.1 Aot XP002847685.1 Aot XP002850361.1 Aot XP002842777.1 Aot XP002843932.1 Aot XP002847170.1 Aud GAO81200.1 Aud GAO81231.1 Aud GAO81330.1 Aud GAO81771.1 Aud GAO81964.1 Aud GAO82999.1 Aud GAO83635.1 Aud GAO85953.1 Aud GAO85988.1 Aud GAO86083.1 Aud GAO86285.1 Aud GAO87672.1 Aud GAO87681.1 Aud GAO88286.1 Aud GAO90373.1 Aud GAO90494.1 Aud GAO90565.1 Aud GAO81540.1 Aud GAO86271.1 Cim EAS31150.3 Cim EAS34545.3 Cim EAS31990.3 Cim EAS37044.3 Cpo EFW14129.1 Cpo EFW17358.1 Cpo EFW16137.1 Cpo EFW22349.1 Cps KMM64104.1 Cps KMM71000.1 Cps KMM67662.1 Cps KMM72316.1 Csu KDN60538.1 Csu KDN64005.1 Csu KDN68160.1 Csu KDN68596.1 Csu KDN71930.1 Csu KDN72402.1 Csu KDN61564.1 Csu KDN62563.1 Csu KDN65093.1 Csu KDN68946.1 Csu KDN65693.1 Csu KDN67074.1 Csu KDN70595.1 Csu KDN72279.1 Csu KDN68224.1 Csu KDN70032.1 Csu KDN66389.1 Csu KDN70949.1 Csu KDN71389.1 Csu KDN66474.1 Csu KDN67432.1 Csu KDN68757.1 Csu KDN69972.1 Csu KDN70187.1 Csu KDN70914.1 Csu KDN71859.1 Csu KDN72352.1 Csu KDN72099.1 </p>
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	<p>Sap KEZ40857.1 Tin EZ34624.1 Tru EGD86700.2 Tso EZ72989.1 The CEJ93554.1 Mac EFY92579.1 Oma KIM92884.1 Psp KFY00590.1 Mac EFY84245.1 Mma KID96522.1 Mbr KID61232.1 Tce GAM41182.1 Mma KID96523.1 Top KND87976.1 MANI_001951 Aud GAO90371.1 Aot XP002847311.1 Afu EAL86536.1 Ani XP003188799.1 Ani XP003188800.1 Afi EAW19771.1 Csu KDN72130.1 Afi EAW24573.1 Afu EAL86636.1 Ani XP003188601.1 Ani XP003188851.1 Ano KNG83926.1 Aot XP002845180.1 Npa EOD45514.1 Mac EFY88214.1 Lma XP003841875.1 Npa EOD51963.1 Mac EFY88213.1 Pdi EKV13493.1 Mac EFY91835.1 Mma KID95486.1 Tst EED13941.1 MANI_0115518 Mro KHO10625.1 Mbr KID73295.1 Mbr KID75468.1 Mro EFY94354.1 Pdi EKV14022.1 Pdi EKV18176.1 Tce GAM36740.1</p>
MaTERP1 (MANI_010532) Backbone gene*	<p>Ach KFH43011.1 Afi EAW24654.1 Afu EAL89316.1 Aot XP002843072.1 Aud GAO81617.1 Mbr KID65418.1 Mgu KID82793.1 Mgy EFQ96702.1 Mma KID93904.1 Mro EFY97806.1</p>
MaTERP1 (MANI_010531) Backbone gene*	<p>Ach KFH43021.1 Afi EAW24653.1 Afu EAL89317.1 Aot XP002843067.1 Aud GAO81618.1 Mbr KID65414.1 Mgu KID82789.1 Mgy EFQ96706.1 Mma KID93897.1 Mro EFY97802.2</p>
MaTERP1 (MANI_010536)*	<p>Ach KFH40562.1 Afi EAW24657.1 Afu EAL89313.1 Aud GAO81614.1 Mbr KID65421.1 Mgu KID82796.1 Mma KID93907.1 Mro EFY97809.1</p>
MaTERP1 (MANI_010527) Backbone gene*	<p>Ach KFH40555.1 Afi EAW24656.1 Afu EAL89314.2 Aud GAO81615.1 Mbr KID65422.1 Mgu KID82797.1 Mma KID93908.1 Mro EFY97810.1</p>
MaTERP1 (MANI_010537)*	<p>Ach KFH43032.1 Afi EAW24659.1 Afu EAL89311.1 Aot XP002843073.1 Aud GAO81612.1 Mbr KID65419.1 Mgu KID82794.1 Mgy EFQ96701.1 Mma KID93905.1 Mro EFY97807.1</p>
MaTERP1 (MANI_010530)	<p>Ach KFH43043.1 Afi EAW24658.1 Afu EAL89312.1 Aot XP002843068.1</p>

Backbone gene*	Aud GAO81613.1 Mbr KID65415.1 Mgu KID82790.1 Mgy EFQ96705.1 Mma KID93898.1 Mro EFY97803.1
MaTERP1 (MANI_010495)*	Ach KFH43040.1 Afi EAW24652.1 Afu EAL89318.1 Aud GAO81619.1 Mbr KID65416.1 Mgu KID82791.1 Mgy EFQ96704.1 Mma KID93899.1 Mro EFY97804.1
MaTERP1 (MANI_010512)*	MANI_025019 Mbr KID63604.1 Mbr KID65420.1 Mgu KID82795.1 Mgu KID85770.1 Mro EFY95587.1 Mro EFY97808.1 Abe EFE36095.1 Ach KFH43984.1 Afi EAW24660.1 Afu EAL89310.1 Aot XP002846965.1 Aud GAO81611.1 Mac EFY86899.1 Mal KHN94024.1 Mgy EFQ99030.1 Mma KID93906.1 Npa EOD52809.1 Oma KIM93336.1 The CEJ93288.1 Tin EZF34275.1 Top KND92088.1 Tso EZF72233.1 Tve EFE43788.1 Aud GAO90117.1 Oma KIM93571.1 Mma KID80879.1 Top KND92083.
MaTERP1 (MANI_010594)*	Ach KFH43025.1 Afi EAW24655.1 Afu EAL89315.1 Aot XP002843071.1 Aud GAO81616.1 Mbr KID65417.1 Mgu KID82792.1 Mgy EFQ96703.1 Mma KID93903.1 Mro EFY97805.1
Transcription factor embedded in MaPKS1 cluster (MANI_112402)	Tma KFX45543.1 Tma KFX45544.1 Tma KFX45545.1 Tma KFX45546.1 Tma KFX45547.1 Ani XP001395290.1 Ach KFH44401.1 Acl EAW15012.1 Ano KNG91357.1 Cim EAS31607.3 Cpo EFW23250.1 Cps KMM68122.1 Lma XP003843017.1 Mal KHN98029.1 Mma KID99277.1 Mmy KXX77532.1 Npa EOD53046.1 Oma KIM94093.1 Psp KFY01609.1 Tce GAM33907.1 Tis CRG91749.1 Top KND87343.1 Tst EED13248.1 Tte AEO67302.1 Ani XP001395307.2 Mac EFY89368.1 Mbr KID72944.1 Mgu KID83467.1 Mro EFY96639.1 Pdi EKV19054.1 Pte EFQ92323.1 The CEJ92460.1 Tma KFX46639.1 Top KND88237.1 Ani XP001393502.1 Ano KNG85002.1 Aud GAO81772.1 The CEJ91586.1 Tst EED12116.1 Tce GAM33972.1 Tst EED22652.1
Transcription factor	Afi EAW19778.1 Afu EAL85124.1

embedded in MaNRPS- PKS2 cluster (MANI_018928)	Aud GAO86281.1 AnojKNG84526.1 Mbr KID73871.1 Mro EFY96238.2 Top KND88287.1 Tte AEO64233.1
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Table 1: Abe| *Arthroderma benhamiae* CBS 112371, Ach| *Acremonium chrysogenum* ATCC 11550, Acl| *Aspergillus clavatus* NRRL 1, Afi| *Aspergillus fischeri* NRRL 181, Afu| *Aspergillus fumigatus* Af293, Ani| *Aspergillus niger* CBS 513.88, Anoj| *Aspergillus nomius* NRRL 13137, Aot| *Arthroderma otae* CBS 113480, Aud| *Aspergillus udagawae*, Cim| *Coccidioides immitis* RS, Cpo| *Coccidioides posadasii* str. *Silveira*, Cps| *Coccidioides posadasii* RMSCC 3488, Csu| *Colletotrichum sublineola*, Lma| *Leptosphaeria maculans* JN3, Mac| *Metarhizium acridum* CQMa 102, Mal| *Metarhizium album* ARSEF 1941, MANI *Metarhizium anisopliae* E6, Mbr| *Metarhizium brunneum* ARSEF 3297, Mgu| *Metarhizium guizhouense* ARSEF 977, Mgy| *Microsporium gypseum* CBS 118893, Mma| *Metarhizium majus* ARSEF 297, Mmy| *Madurella mycetomatis*, Mro| *Metarhizium robertsii* ARSEF 23, Npa| *Neofusicoccum parvum* UCRNP2, Oma| *Oidiodendron maius* Zn, Pdi| *Penicillium digitatum* PHI26, Psp| *Pseudogymnoascus* sp. VKM F-3557, Pte| *Pyrenophora teres* f. *teres* 0-1, Sap| *Scedosporium apiospermum*, Tce| *Talaromyces cellulolyticus*, The| *Torribiella hemipterigena*, Tin| *Trichophyton interdigitale* H6, Tis| *Talaromyces islandicus*, Tma| *Talaromyces marneffeii* PM1, Top| *Tolypocladium ophioglossoides* CBS 100239, Tru| *Trichophyton rubrum* CBS 118892, Tso| *Trichophyton soudanense* CBS 452.61, Tst| *Talaromyces stipitatus* ATCC 10500, Tte| *Thielavia terrestris* NRRL 8126, Tve| *Trichophyton verrucosum* HKI 0517. *Only genes with ≥ 45 % identity were used for phylogeny;

7 DISCUSSÃO

Diversas atividades biológicas são exercidas por MSs. O complexo de genes que define o metabolismo secundário em fungos tem uma longa história evolutiva e atingiu alta complexidade. A busca por genes envolvidos em processos de interesse, como a patogenicidade por exemplo, parte de estratégias baseadas em análises direcionadas ou tentam identificar genes com expressão diferencial em determinado processo. Até o presente momento, genes envolvidos na biossíntese de MSs tem sido pouco investigados utilizando estratégias como as recém descritas. Um dos obstáculos no estudo de MSs é a dificuldade em produzi-los em condições laboratoriais padrão (Netzker *et al.*, 2015). Por outro lado, genes envolvidos na biossíntese de MSs ocorrem em agrupamentos no genoma dos fungos. Do ponto de vista de análises *in silico* de genomas isso representa uma vantagem pois encontrados alguns genes é possível identificar os agrupamentos. Neste trabalho iniciamos a descrição, organização e avaliamos alguns aspectos da regulação da expressão de genes envolvidos na biossíntese de MSs no entomopatógeno *M. anisopliae* na perspectiva de identificar a sua participação na sua patogênese e na possível definição da amplitude de hospedeiros de determinadas linhagens.

Utilizando métodos *in silico*, foram identificados 73 BGCs possivelmente envolvidos na produção de MSs na linhagem E6 de *M. anisopliae*. Dezesete dos 73 (~ 23 %) possuem ortólogos caracterizados em outras espécies, o que nos levou a sugerir que o produto final destes BGCs de *M. anisopliae* são compostos idênticos ou assemelhados. Alguns destes BGCs já foram caracterizados em espécies do gênero *Metarhizium*, embora não tenham sido caracterizados na espécie *M. anisopliae*. Este é o caso dos BGCs para produção das destruxinas (MaNRPS1; MANI_024437), serinociclina (MaNRPS2; MANI_020119), metaquelina (MaNRPS8; MANI_003049), ferricrocina (MaNRPS9; MANI_117325),

NG39x (MaNRPS-PKS1; MANI_020791), MrPKS1 (MaPKS20; MANI_122426) e MrPKS2 (MaPKS8; MANI_028434) (Chen *et al.*; Moon *et al.*, 2008; Donzelli *et al.*, 2010; Wang *et al.*, 2012; Giuliano Garisto Donzelli *et al.*, 2015). Os BGCs putativos de *M. anisopliae* apresentam a mesma configuração gênica e grande identidade com os BGCs caracterizados em outras espécies do gênero *Metarhizium*, indicando que, muito provavelmente, os compostos produzidos são os mesmos.

Um destes 17 BGCs possui um gene caracterizado em *M. anisopliae* (geranil-geranil difosfato sintase: GGPP sintase; MANI_024139; MaTERP5). O produto deste gene se mostrou importante na regulação de outros metabólitos, embora o resto do *cluster* não tenha sido caracterizado. Dentre os 17, ainda existem casos em que o produto final sugerido pelo nosso trabalho já foi isolado de culturas de fungos do gênero *Metarhizium*, embora o BGC para produção do composto não tenha sido caracterizado. Este é o caso das aurovertinas (MaPKS2; MANI_004781) e do ácido helvólico (MaTERP1; MANI_010527/ MANI_010530/ MANI_010531/ MANI_010532), metabólitos já isolados de culturas de *Metarhizium*. Em outros casos, os BGCs não foram caracterizados em *Metarhizium*, mas metabólitos próximos aos produto finais dos *clusters* ortólogos já foram isolados. Este o caso do BGC que contém uma lanosterol ciclase (MaTERP2; MANI_002110), o BGC cujo produto putativo é assemelhado a pseurotina (MaNRPS-PKS2; MANI_018878) e o BGC para produção de viridicatutoxina (este último BGC sugerido por Gibson e colaboradores (2014)).

Por fim, através de genômica comparativa e, em alguns casos, filogenia, nós conseguimos relacionar alguns BGCs, não caracterizados, de *M. anisopliae*, com *clusters* caracterizados em outras espécies. No entanto, o produto final putativo proposto por nosso trabalho nunca foi isolado de culturas do fungo. Este é o caso do BGC cujo produto

putativo é assemelhado a tropolonas e citrininas, do BGC cujo produto putativo é assemelhado a xenolozoyenona, do BGC cujo produto putativo é assemelhado a terpendola E e lolitrem e do BGC cujo produto putativo é assemelhado a elimoclavina e ergovalina. Embora boa parte dos BGCs ainda não tenha função atribuída (hipotéticos), a descrição parcial destes 17 *clusters* que possuem ortólogos caracterizados em outras espécies, em conjunto com o detalhamento da descrição e predição dos mesmos, certamente auxiliará trabalhos futuros.

É importante destacar que aproximadamente metade, 49 % (36/73), dos BGCs preditos estava expresso em pelo menos uma das condições testadas (RPKM \geq 2). A grande maioria dos genes eucarióticos está sujeita a um forte controle transcricional, com poucas condições capazes de induzir a expressão destes genes. Esse controle é especialmente importante para os BGCs envolvidos na biossíntese de MSs. Além de facilitar possíveis eventos de HGT, a ligação física entre os genes envolvidos na produção de MSs minimiza o número de passos regulatórios necessários para regular toda uma rota biossintética, assim contribuindo para a otimização fisiológica (Gacek e Strauss, 2012). A detecção de transcritos cognatos a trinta e seis destes BGCs indica que esses genes codificam produtos e portanto sejam funcionais, e não apenas remanescentes da evolução dos genomas.

Duas comparações pareadas foram realizados, a fim de identificar genes diferencialmente expressos. Aproximadamente 30 % (22/73) dos BGCs tiveram sua expressão fortemente alterada, tanto negativa quanto positivamente, em alguma das condições. Embora os BGCs caracterizados, ou que possuem ortólogos caracterizados, sejam mais fáceis de explorar e descrever, 11 dos 22 BGCs nunca foram explorados.

Dentre os BGCs positivamente regulados na condição experimental de infecção inicial

(48hI), foram selecionados três para análise em maior detalhe, acrescentando análises evolutivas o BGC para produção de ácido helvólico (MaTERP1; MANI_010527/ MANI_010530/ MANI_010531/ MANI_010532), o BGC cujo produto putativo é assemelhado a pseurotina (MaNRPS-PKS2; MANI_018878), e o BGC cujo produto putativo é assemelhado a tropolonas e citrininas (MaPKS1; MANI_014762).

Utilizando filogenia, nós sugerimos que MaTERP1 foi adquirido pelo gênero *Metarhizium* por de um evento de HGT, estando esse BGC presente em poucas espécies: nas espécies hospedeiro-intermediário e hospedeiro-generalista de *Metarhizium*, em *P. ipomoeae* e em algumas espécies do gênero *Aspergillus*. *P. ipomoeae* é uma espécie da família Clavicipitaceae, sendo filogeneticamente próxima a fungos do gênero *Metarhizium*. Isso pode indicar que MaTERP1 foi adquirido por um ancestral comum a fungos dos gêneros *Periglandula* e *Metarhizium*, tendo sido perdido em diversas linhagens, incluindo fungos hospedeiro-especialistas do gênero *Metarhizium*.

Outra hipótese é a de que esse BGC foi inicialmente adquirido por uma linhagem de *Metarhizium* ou *Periglandula*, através de um evento de HGT, e, posteriormente, através de um segundo evento de HGT, transferido para uma linhagem de *Metarhizium* ou *Periglandula*.

Uma terceira hipótese seria a ocorrência de dois eventos de HGT independentes, um onde o BGC foi adquirido por uma linhagem de *Periglandula*, e outro onde o BGC foi adquirido por uma linhagem de *Metarhizium*. No entanto, a terceira hipótese parece menos provável, tendo em vista a grande sintonia e identidade (> 80 %) exibida entre os BGCs de *Periglandula* e *Metarhizium*. Contudo, análises evolutivas e filogenéticas mais aprofundadas são necessárias a fim de melhor compreender detalhadamente a história

evolutiva destes genes em *Metarhizium* e na família Clavicipitaceae.

A expressão do BGC para produção do ácido helvólico também é influenciada em *Metarhizium* por um gene localizado em outro BGC (GGPP sintase; MaTERP5). Mutantes nulos para a GGPP sintase apresentaram reduzida produção de ácido helvólico e virulência atenuada (Singkaravanit *et al.*, 2010). Esta seria uma linha de evidência indicando que o ácido helvólico pode apresentar importância na virulência de *Metarhizium*. No entanto, como os autores sugerem, essa GGPP sintase pode estar influenciando a biossíntese de diversos MSs, assim como outros processos celulares. Assim, a virulência atenuada apresentada por mutantes nulos para esse gene não pode ser diretamente relacionada à baixa produção de ácido helvólico (Singkaravanit *et al.*, 2010). Nossos resultados estão em concordância com essa sugestão, uma vez que a expressão da GGPP sintase se manteve constante nas três condições de cultivo analisadas no estudo transcritoômico, indicando que a expressão deste gene não está correlacionada com a expressão do BGC para a produção do ácido helvólico. Ainda, este gene está conservado em todas as linhagens de *Metarhizium* analisadas, incluindo espécies hospedeiro-especialista que não contenham o BGC para produção do ácido helvólico. Assim, a importância do ácido helvólico na infecção de *Metarhizium* ainda precisa ser avaliada, por métodos adicionais, como a construção de mutantes nulos para genes destes *clusters*.

Outro aspecto é que um mesmo BGC pode atuar na biossíntese de uma plethora de compostos. Como já descrito, além do ácido helvólico, um derivativo (ácido 1,2-dihidrohelvólico) já foi isolado de culturas de *Metarhizium* (Lee *et al.*, 2008). Embora a rota biossintética para biossíntese do ácido 1,2-dihidrohelvólico seja desconhecida (i.e., genes que atuam catalisando modificações que fazem esse composto ser diferente do ácido helvólico) este composto certamente é derivado do *cluster* MaTERP1. Compostos

derivativos podem ser, especialmente, abundantes para BGCs que abrigam um grande número de genes, como o BGC cujo produto putativo é assemelhado à pseurotina (21 genes preditos) e o BGC cujo produto putativo é assemelhado às tropolonas e citrininas (15 genes preditos). Alguns genes, dos cluster que abrigam um grande número de genes, podem estar sujeitos a regulações diferenciais, só sendo expressos com o restante do BGC em situações específicas, gerando compostos com características e atividades únicas. De fato, isso pode estar acontecendo nas condições de cultivo avaliadas no ensaio transcritômico para o BGC cujo produto putativo é assemelhado a tropolonas e citrininas. Na condição de infecção inicial (48hI), sete genes estavam positivamente regulados, três genes negativamente regulados e dois genes não tiveram sua expressão alterada. Enquanto que na condição de infecção tardia (144hI), dez genes não tiveram sua expressão significativamente alterada (incluindo genes que estavam positiva e negativamente regulados na condição de infecção inicial), enquanto dois genes tiveram sua expressão aumentada. Adicionalmente, três genes apresentaram expressão muito residual (RPKM < 2). Dentre os genes que apresentaram expressão diferenciada em relação ao resto do BGC, estão: (i) o gene MANI_014847, provável responsável pela catálise de uma das etapas da biossíntese inicial da rota metabólica proposta, estando esse gene positivamente regulado na condição tardia (144hI); (ii) o gene MANI_014850, anotado como uma mercúrio redutase, que possui um domínio de oxireductase predito, encontrado negativamente regulado na condição de infecção inicial; além de mais dois genes (MANI_028157 e MANI_014867) com um domínio de transportador do tipo MFS (*Major Facilitator Superfamily*) predito, sendo ambos negativamente regulados na condição de infecção inicial (48hI). Proteínas com o domínio MFS geralmente atuam no transporte de MSs. Em concordância com a hipótese de que um mesmo BGC pode gerar um ou mais derivativos

está um cluster de *Talaromyces marneffei* (Woo *et al.*, 2014). Esse *cluster* atua na biossíntese de citrininas podendo atuar na biossíntese de outros compostos, dentre eles a monascorubrina, um pigmento, e a ankaflavina, uma molécula que inibe a lipogênese (Woo *et al.*, 2014; Chang *et al.*, 2015; Chen *et al.*, 2016).

Outros aspectos ainda são pouco explorados em relação a MSs de fungos filamentosos. Por exemplo, a existência de *superclusters* físicos (assunto que recentemente passou a ser explorado pelo nosso grupo em fungos do gênero *Metarhizium*) e metabólitos *cross-chemistry* (entre clusters separados fisicamente), que podem aumentar significativamente o repertório de compostos gerados (Andersen *et al.*, 2013; Wiemann *et al.*, 2013).

Outro exemplo é a biotransformação de MSs produzidos por outros organismos e seu papel e impacto no ambiente (Vanhoutte *et al.*, 2016). Tem sido descrito que diversos organismos são capazes de modificar e degradar MSs, no entanto, muitas vezes, os produtos dessas modificações ou degradações são mais tóxicos que o MSs originais (Vanhoutte *et al.*, 2016). Outra situação onde a biotransformação se mostra revelante é na interação entre fungos entomopatogênicos e artrópodes. Artrópodes podem produzir MSs com diversas atividades, existindo relatos descrevendo (e sugerindo) que fungos entomopatogênicos podem modificar estes compostos (Molnar *et al.*, 2010). Essas possíveis biotransformações podem render moléculas completamente desconhecidas, com atividades biológicas e possíveis aplicações ainda não testadas. A caracterização destes compostos é uma linha de pesquisa totalmente nova, que pode resultar em moléculas inéditas de interesse biotecnológico.

8 CONCLUSÕES E PERSPECTIVAS

Embora a a função de MSs no ciclo de infecção de fungos do gênero *Metarhizium*

ainda seja pouco conhecida, se supõe que essas moléculas desempenhem atividades importância como fatores de virulência. Métodos que visam caracterizar funcionalmente genes envolvidos na biossíntese de MSs ou expressar heterologicamente esses genes, tem sido cada vez mais utilizados. No entanto, um questionamento importante seria: qual gene (ou genes) caracterizar ou expressar? Assim, um panorama mais amplo do potencial metabólico do organismo de interesse é necessário. Nosso trabalho buscou proporcionar essa visão para fungos do gênero *Metarhizium*, em especial a espécie *M. anisopliae*. Com a aplicação conjunta de dados transcritômicos, as ferramentas *in silico* e os dados da literatura (principalmente de outros organismos), foi possível identificar, definir e descrever diversos genes envolvidos diretamente na biossíntese de MSs. Ainda, foi possível descrever melhor a organização gênica, identificar genes com um provável envolvimento no ciclo infeccioso, explorar aspectos evolutivos destes genes e hipotetizar sobre possíveis reguladores globais envolvidos na regulação transcricional destes. Claramente outros estudos são necessários antes que se tenha uma visão completa da participação dos MSs no processo de infecção e de seleção de hospedeiros.

Nosso laboratório tem experiência na construção e manutenção de mutantes funcionais, sendo este, um método importante na descoberta de novos fatores de virulência. Ademais, um dos focos do trabalho do grupo, nos últimos anos, tem sido a padronização de ferramentas moleculares que visam facilitar a geração destes mutantes. Dentre estas ferramentas, mutantes com defeitos nas vias de recombinação não-homóloga e o sistema CRISPR/Cas9 são destaques.

No presente trabalho foram explorados em maiores detalhes os genes putativamente envolvidos na biossíntese do ácido helvólico (MaTERP1), de metabólitos assemelhados a pseurotina (MaNRPS-PKS2) e de metabólitos assemelhados a tropolona e citrinina

(MaPKS1), sendo estes genes postulados como prováveis fatores de virulência. Assim, a construção de mutantes funcionais para genes destas três rotas metabólicas putativas irá permitir a melhor compreensão da importância dos mesmos e de seus produtos no ciclo de vida e infecção do fungo.

Nossos resultados demonstram que, ao longo do ciclo de infecção, a expressão de genes envolvidos na biossíntese de MSs se altera, exemplificado pelos BGCs putativamente envolvidos no ciclo de infecção positivamente regulados nas condições de infecção inicial e negativamente regulados nas condições de infecção tardia. Assim, outro objetivo é caracterizar quando, ao longo do ciclo de infecção, estes genes começam a ser expressos e quando sua expressão passa a ser reprimida. Outro ponto de destaque foram os reguladores globais do trato fúngico que podem estar influenciando a expressão dos genes envolvidos na biossíntese de MSs, em especial os genes *PacC* e *CreA*. Assim, outro objetivo futuro é melhor caracterizar como estes genes podem influenciar o ciclo de vida e a produção de MSs em *Metarhizium*. Para tanto, a construção de mutantes funcionais para estes genes também está prevista. Em conjunto, nossos estudos futuros devem auxiliar e aprofundar os conhecimentos a respeito da biossíntese de MSs em *Metarhizium*.

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