THE EVOLUTION OF SECONDARY METABOLISM REGULATION AND PATHWAYS IN THE ASPERGILLUS GENUS

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LIST OF ABBREVIATIONS

SM.....Secondary metabolism

SMGC.....Secondary metabolic gene cluster

VeA.....Velvet A

MtfA.....Master Transcription Factor A

LaeA.....Loss of AflR Expression A

VosA.....Viability of Spores A

VelB.....Velvet-like B

BrlA.....Bristle

AbaA.....Abacus

WetA.....Wet

PKS.....Polyketide synthase

NRPS.....Non-ribosomal peptide synthase

Anid.....*Aspergillus nidulans*

Afum.....*Aspergillus fumigatus*

Aory.....*Aspergillus oryzae*

Aniger.....*Aspergillus niger*

AspGD.....Aspergillus Genomes Database

Ogroup.....Orthogroup

SNV.....Single-nucleotide variant

GMM.....General minimal media

RPMI..... Roswell Park Memorial Institute

MFS.....General minimal media

MCL.....Markov cluster algorithm

DHN..... 1,8-dihydroxynaphthalene

BiNGO .....Biological Networks Gene Ontology

CHAPTER I

INTRODUCTION

Filamentous fungi produce a diverse array of bioactive organic small molecules known as secondary metabolites. Secondary metabolites are critical to the fungal lifestyle, with functions in defense, virulence, and communication (O'Brien and Wright 2011). Secondary metabolites also have large impacts on human health in a variety of ways. Many SMs function as toxins, as in the case of the highly carcinogenic secondary metabolite aflatoxin produced by *Aspergillus flavus* and *Aspergillus parasiticus*, which is found in contaminated food and causes acute aflatoxicosis and liver cancers (J. W. Bennett and Klich 2003). Alternatively, some secondary metabolites have been repurposed as effective medications, including the widely prescribed cholesterol lowering statins, the iconic antibiotic penicillin, and the immunosuppressant cyclosporine (Keller, Turner, and Bennett 2005).

Ecological roles of fungal secondary metabolites

Secondary metabolites play diverse ecological roles in the lifestyle of filamentous fungi, including enhancing virulence, facilitating communication, and self-protection. Secondary metabolites are so named because they are generally not required for basic cellular functions, distinguishing them from primary metabolites that are (J. Bennett and Bentley 1989). However, the line separating primary and secondary metabolism is sometimes blurred by metabolites that play roles in both primary and secondary processes. Some siderophores, for example, can be produced intracellularly for basic iron metabolism; or, as in the opportunistic human pathogen

Aspergillus fumigatus, secreted to enhance virulence by scavenging iron during infection of the animal lung (Schrettl et al. 2007). Nevertheless, most secondary metabolites share general ecological, chemical, and genetic features that separate them from primary processes.

Many filamentous fungi are saprotrophs that grow in soil environments containing other species of microbes, including fungi, bacteria and microbial eukaryotes. These environments are competitive and crowded, and secondary metabolites can give a competitive edge to the fungus as well as function to mediate signaling both within and between species (Macheleidt et al. 2016). The particular ecological role of a given secondary metabolite is highly dependent on context. Differences in concentrations of an SM, for example, can determine whether it functions as a broad spectrum antibiotic or functions as a signaling molecule to alter the behavior of other microbes (J. Bennett and Bentley 1989; Yim, Wang, and Davies 2007). In addition, secondary metabolites functioning as signaling molecules can be antagonistic or synergistic (Macheleidt et al. 2016); for example, fusaric acid produced by *Fusarium graminearum* represses the production of an antifungal compound in *Pseudomonas fluorescens* CHA0 (Notz et al. 2002), while several secondary metabolites produced by *Fusarium oxysporum* function as chemoattractants and promote hyphal colonization by *Pseudomonas fluorescens* WCS365 (de Weert et al. 2004).

Secondary metabolites also play roles in virulence in pathogenic filamentous fungi, both on animal and plant hosts. Plant-fungal interactions are complex and ancient, likely dating to the emergence of land plants (Krings, Taylor, and Dotzler 2012; Hibbett and Matheny 2009), and phylogenetic evidence suggests that plant pathogenic fungi are typically closely related to fungal endophytes, which colonize plants without causing disease (Delaye, García-Guzmán, and Heil 2013; Spatafora and Bushley 2015). In contrast to their endophytic relatives, pathogenic fungi

produce secondary metabolites that function as toxins with various roles in pathogenesis. *Fusarium graminearum*, a cereal pathogen, produces terpenes including the trichothecene deoxynivalenol that aid the fungus in colonizing barley and wheat (Kazan, Gardiner, and Manners 2012). In some cases, the secondary metabolites produced by plant pathogenic fungi specify host range and host-specific virulence. A classic example of this phenomenon is T-toxin, a polyketide secondary metabolite produced by *Cochliobolus heterostrophus* race T which is hypervirulent on corn varieties carrying the mitochondrial Texas cytoplasmic male sterility gene (Turgeon and Baker 2007). Secondary metabolites produced by plant pathogens are also targets of the plant immune system in the evolutionary arms race between plants and their pathogens; the rice blast pathogen *Magnaorthe grisea* contains a PKS-NRPS hybrid gene that is recognized by certain rice cultivars, rendering the fungus avirulent (Böhnert et al. 2004).

Filamentous fungi can also be animal pathogens, though are typically opportunistic rather than obligate animal pathogens. Members of the *Aspergillus* and *Mucor* genus of filamentous fungi, in particular *Aspergillus fumigatus*, can cause a variety of diseases in respiratory tracts of humans and other animals, ranging in severity from asthma and breathing related disorders to invasive fungal infections (Pfaller, Pappas, and Wingard 2006; Gibbons and Rokas 2012). These fungi cause infections beginning in the lung when their conidia are inhaled and germinate. Conidia and germlings are generally cleared efficiently by the host immune system, and these infections are much more likely to affect immune compromised individuals than healthy individuals (Roilides, Katsifa, and Walsh 1998). However, some fungi have defenses against the immune system that render them more effective pathogens. For example, the conidia of *Aspergillus fumigatus* contain a secondary metabolite derived melanin pigment required for virulence that prevents killing of conidia by phagocytes by interfering with acidification in

phagolysosomes and additionally inhibits apoptosis of macrophages (Heinekamp et al. 2012). This pigment also plays a role in protecting conidia from UV radiation. Other secondary metabolites with known roles in animal virulence include siderophores, which are produced by *A. fumigatus* to scavenge for iron in the iron-poor environment of the lung (Schrettl et al. 2007). Other fungi produce siderophores during animal infection, such as the entomopathogenic fungus *Metarhizium robertsii* (Giuliano Garisto Donzelli, Gibson, and Krasnoff 2015). In both fungi, siderophores are essential for virulence. Other secondary metabolites produced by animal pathogenic fungi have not been linked to *in vivo* biological mechanisms, though some are produced at higher levels during infection. While some of these metabolites are dispensable for virulence, the gliotoxin secondary metabolite in *Aspergillus fumigatus* enhances virulence during mouse infection and is produced at higher levels during infection (Sugui et al. 2008; Bignell et al. 2016).

There are many more known cases of secondary metabolite mediated specific host-pathogen interactions between plants and fungi than there are animals and fungi. One explanation for this is that most filamentous fungi, with the exception of insect-infecting fungi, are only rarely successful in infecting healthy individuals (Abad et al. 2010). The relatively recent increase in the immune compromised population through the spread of HIV/AIDS and through increased use of immunosuppressants correlates with the increased incidence of filamentous fungal infections (Latge 1999). While there are clear roles for some secondary metabolites in pathogenesis, it is likely that these metabolites have other ecological functions in the fungi that produce them.

Secondary metabolic pathways in fungal genomes

A unique genetic feature of secondary metabolism in filamentous fungi is that the biosynthetic genes that synthesize a given compound are found in contiguous gene clusters in the genome, which is atypical of biosynthetic pathways in other eukaryotes (Keller and Hohn 1997). These clusters contain backbone synthesis genes, such as non-ribosomal peptide synthases, polyketide synthases, dimethylallyl tryptophan synthases, and terpene synthases, as well as additional biosynthetic genes that modify the chemical product of the backbone enzyme. Backbone synthesis genes are often large, multi-domain and in the case of nonribosomal peptide synthases, multi-modular biosynthetic enzymes. Additional biosynthetic enzymes found in SM gene clusters include oxidoreductases, methyltransferases, acetylases, and esterases (Keller, Turner, and Bennett 2005).

In addition to biosynthetic genes, these clusters contain transporters involved in either secreting the metabolite or, in some cases, sequestering the metabolite in a vesicle-vacuole fusion derived organelle known as a toxisome (Keller 2015). Toxisomes play roles in biosynthesis and export of reactive or toxic metabolites. One example of this process occurs during aflatoxin biosynthesis, in which the penultimate metabolic intermediate is transported into a toxisome where it is converted to the final product and secreted via exocytosis (Chanda et al. 2009). Secondary metabolic gene clusters can also contain additional types of detoxifying genes that protect the fungus from reactive metabolites; for example, the gliotoxin gene cluster contains an oxidoreductase that reduces the reactivity of the metabolite (Dolan et al. 2015; Scharf et al. 2010). Finally, these clusters often contain a cluster-specific transcription factor that regulates the expression of the genes in the cluster. The most common type of cluster-specific transcription

factors are from the Zn_2Cys_6 zinc finger transcription factor family, but Cys_2His_2 transcription factors can also be found in SM gene clusters (Chang and Ehrlich 2013; Tsuji et al. 2002).

Because of this unique organization, the position of gene clusters in fungal genomes can be computationally predicted from genome sequences. The most widely used algorithms for gene cluster prediction are antiSMASH and SMURF (Medema et al. 2011; Khaldi et al. 2010), which scan fungal genomes using profile hidden Markov models trained on experimentally characterized gene clusters. Additional computational methods for gene cluster prediction rely on the assumption that secondary metabolic gene clusters are co-expressed and use gene expression data to scan fungal genomes for co-expressed gene clusters (Umemura et al. 2013; Andersen et al. 2013). While experimentally characterized SM gene clusters predate the genomic era by decades (Keller and Hohn 1997), genome sequencing revealed scores of previously unknown SM gene clusters and demonstrated that they are extremely common in fungal genomes (Nierman et al. 2005; Brakhage and Schroeckh 2011).

Secondary metabolites are extraordinarily diverse among filamentous fungi, reflecting their often highly specialized ecological roles. Individual metabolites are often only produced by one or a handful of species, and the taxonomic distribution of these metabolites can be either narrow (i.e., restricted to a small number of closely related species) or discontinuous (i.e., produced by a small number of unrelated fungi). Phylogenetic analyses have demonstrated that the genes producing these metabolites also often follow that discontinuous distribution (Bushley and Turgeon 2010). The discontinuous distribution of SM gene clusters is caused by gene and gene cluster loss as well as horizontal gene transfer of entire SM gene clusters from bacteria to fungi as well as between fungi (Bushley and Turgeon 2010; Slot and Rokas 2011; Wisecaver and Rokas 2015). In some characterized cases, horizontal transfer of SM gene clusters occurs

between fungi and other microbes with overlapping ecologies, likely reflecting a shared benefit of the metabolite (Greene et al. 2014).

Regulation of fungal secondary metabolites

Around 60% of experimentally characterized and computationally predicted SM gene clusters contain a transcription factor, which in many cases regulates the transcription of the remaining genes in the cluster (Brakhage 2013). Well-characterized examples of this phenomenon are the *aflR* transcription factor in the aflatoxin gene cluster in *Aspergillus flavus* and the zinc finger transcription factor *gliZ* in the gliotoxin gene cluster in *Aspergillus fumigatus* (Chang et al. 1995; Bok, Chung, et al. 2006). In both cases, presence of the transcription factor is required for metabolite production, and overexpression of the transcription factor leads to an increase in production of the metabolite. Some cluster-specific SM gene cluster regulators are located outside of the cluster that they regulate. For example, the asperfuranone cluster in *Aspergillus nidulans* is regulated by the ScpR transcription factor which is found in a different SM gene cluster (Bergmann et al. 2010).

In addition to pathway-specific regulation, secondary metabolism production is coordinated by environmentally responsive master SM regulators (Brakhage 2013). Most secondary metabolites are not produced constitutively throughout the filamentous fungal life cycle, and instead are produced under specific environmental conditions or certain stages of development (O. Bayram and Braus 2012). Abiotic environmental conditions that activate or repress certain secondary metabolites include light availability, carbon source, nitrogen source, ambient pH, redox status, presence of trace metals, among many others. The induction or repression of SM gene cluster expression in response to environmental conditions is controlled

by master SM regulators that regulate multiple SM gene clusters. In contrast to the pathways they regulate, these master regulators are generally well conserved across filamentous fungi. One such example is PacC, a pH-responsive master regulator activated at alkaline pH and initiates the transcription of the penicillin biosynthetic gene cluster and represses transcription of the sterigmatocystin gene cluster in *Aspergillus nidulans* (Bignell et al. 2005; Espeso and Penalva 1996; Then Bergh and Brakhage 1998). One ecological explanation for PacC's regulation of penicillin is that β-lactam antibiotics such as penicillin are generally more toxic against bacteria at alkaline pH (Arst 1996; Brakhage 2013).

Some other master regulators of secondary metabolism are also general environmentally responsive regulators with multiple roles in fungal biology. Master regulators of secondary metabolism that activate or repress the expression of SM gene clusters in response to different nutrient source also regulate primary metabolic pathways. For example, the master SM regulator AreA is activated by changes in nitrogen source and regulates nitrogen utilization genes as well as multiple SM gene clusters (Caddick and Arst 1998). The carbon catabolite repressor CreA is also a master regulator of SM gene clusters, including penicillin in *Aspergillus nidulans* (Dowzer and Kelly 1991). Other processes, including asexual or sexual development, can be bridged by master SM regulators. The Velvet regulatory complex, comprised of the Velvet domain proteins VeA and VelB and the putative methyltransferase LaeA, regulate both sexual development and many SM pathways across filamentous fungi (Ö. Bayram et al. 2008; O. Bayram and Braus 2012). This regulatory complex is itself regulated by light availability. When the fungus is exposed to light, the VeA/VelB dimer is inhibited from entering the nucleus, while under dark conditions VeA and VelB are both localized in the nucleus where they form the heterotrimeric

Velvet complex with LaeA and interact with other regulatory proteins to control secondary metabolism and start the sexual developmental cycle.

Chapter previews

In this dissertation, I address several central questions concerning the evolution of secondary metabolism and its regulation using species from the model filamentous fungal genus *Aspergillus*. Several unique features of secondary metabolism raise questions with respect to how its regulation evolves and changes in response to different environmental signals and different stages of the fungal life cycle. Here, I address how divergent and often species-specific secondary metabolic pathways are regulated by conserved master regulators alongside more conserved processes such as development, and also examine the genetic drivers behind the species-specificity of secondary metabolic gene clusters.

First, while secondary metabolites are fast evolving and often species-specific, the transcriptional regulators that control these metabolites are typically well conserved across filamentous fungi. In Chapter II, I examine the ways these regulatory networks have evolved across species through transcriptional rewiring. I quantify the conservation and divergence of SM gene clusters across the *Aspergillus* genus of fungi using four representative and well annotated genomes and sets of SM gene clusters. I next examine the genome-wide regulatory roles of two master regulators of SM and development, VeA and MtfA, in *Aspergillus nidulans* and *Aspergillus fumigatus* using RNA sequencing. This reveals if and to what extent transcriptional rewiring contributes to SM diversity, as well as how transcriptional rewiring impacts the conserved process of development.

In Chapter III, I address whether master SM regulators are able to regulate secondary metabolite production based on combinations of environmental signals. While most master regulators of secondary metabolism are described as responding to one environmental signal, it is likely that these regulators combinatorially control SM production to fine-tune the metabolic profile of a fungus to changing environments. This possibility is supported by work showing that multiple environmental cues and transcription factors can regulate production of the SM terrein in *Aspergillus terreus* (Gressler et al. 2015), that both the light-responsive regulator VeA and the nitrogen regulator AreA are required for wild-type levels of SM-producing gene transcription in *Fusarium oxysporum* (López-Berges et al. 2014) and that small changes in glucose concentration impact SM production in *A. nidulans* by changing VeA's subcellular location (Atoui et al. 2010). In chapter III, I examine whether the light-sensitive Velvet complex can respond to additional environmental signals by determining whether the complex is also sensitive to temperature. This work demonstrates that secondary metabolic regulators can fine-tune their regulatory programs based on multiple environmental signals.

Next, I investigate how development impacts secondary metabolism in filamentous fungi. The onset of asexual development and secondary metabolism production often occur at the same time in many species of filamentous fungi. Secondary metabolites that are produced during asexual development are typically localized in developmental tissues and can play roles in spore protection as well as virulence. In Chapter IV, I investigate how these processes are coordinated by determining the impact of central asexual regulatory pathways in *Aspergillus fumigatus* on secondary metabolism production. Early, intermediate, and late stages of asexual development are controlled by the three transcriptional regulators BrlA, AbaA, and WetA, respectively, in all *Aspergillus* species. These regulators are linked in a circuit where BrlA activates AbaA, which

activates WetA. Using RNA-sequencing, I determine the genome wide regulatory roles of these three transcription factors and the shared and regulator-specific targets of the circuit. I further dissect the individual contributions of each regulator in controlling spore-specific as well as non-developmentally linked metabolites. This work demonstrates how the asexual developmental program and secondary metabolism production are coordinated transcriptionally in filamentous fungi.

In Chapter V, I determine the genetic drivers that generate diversity in secondary metabolic gene clusters. In Chapter II, I demonstrate that secondary metabolic gene clusters are divergent and rapidly evolving across different species of *Aspergillus*. In order to determine the mechanisms that result in this rapid divergence, I examine the secondary metabolism clusters in a large population-based sample of *Aspergillus fumigatus* strains. I utilize novel whole-genome sequences of 8 strains of *A. fumigatus* as well as an additional 58 publicly available genome sequences. By looking at population-level changes rather than species-level differences, this analysis is able to catch diversity-generating mechanisms “in the act”. From these data, I propose likely mechanisms by which secondary metabolic gene clusters are evolving.

CHAPTER II

EXAMINING THE EVOLUTION OF THE REGULATORY CIRCUIT CONTROLLING SECONDARY METABOLISM AND DEVELOPMENT IN THE FUNGAL GENUS *ASPERGILLUS*

Authors

Abigail L. Lind, Jennifer H. Wisecaver, Timothy D. Smith, Xuehuan Feng, Ana M. Calvo, and Antonis Rokas

Introduction

Filamentous fungi produce diverse repertoires of small molecules known as secondary metabolites (SMs) (Keller, Turner, and Bennett 2005). SMs include widely used pharmaceuticals such the antibiotic penicillin (A. A. Brakhage 1998), the cholesterol-reducing drug lovastatin (Kennedy et al. 1999), and the immunosuppressant cyclosporine (Weber et al. 1994), as well as potent mycotoxins such as aflatoxin (P K Chang et al. 1995) and fumonisin (Daren W Brown et al. 2007; Robert H Proctor et al. 2003). SMs play key ecological roles in territory establishment and defense, communication, and virulence (Yim, Wang, and Davies 2007; Rohlfs et al. 2007; Vining 1990; Demain and Fang 2000; Rohlfs and Churchill 2011).

The genes involved in fungal SM pathways are often physically linked in the genome, forming contiguous SM gene clusters (Keller and Hohn 1997). These gene clusters are typically characterized by a backbone gene, such as those encoding nonribosomal peptide synthetases

(NRPSs), polyketide synthases (PKSs), hybrid NRPS-PKS enzymes, and prenyltransferases (PTRs), whose protein products are responsible for synthesizing the proto-SM. Additional genetic components of SM gene clusters include genes for one or more tailoring enzymes that chemically modify SM precursors, transporter genes responsible for exporting the final product, and transcription factors that drive expression of additional genes in the cluster. For example, the gene cluster responsible for the synthesis of the mycotoxin gliotoxin in the opportunistic human pathogen *Aspergillus fumigatus* contains 13 genes including a non-ribosomal peptide synthase (*gliP*), multiple tailoring enzymes (*gliI*, *gliJ*, *gliC*, *gliM*, *gliG*, *gliN*, *gliF*), a transporter gene (*gliA*), a transcription factor (*gliZ*), and a gliotoxin oxidase gene that protects the fungus from the harmful effects of gliotoxin (*gliT*) (Gardiner and Howlett 2005; Scharf et al. 2012).

Surveys of fungal genomes show that for any given fungus there are many more gene clusters than known SMs, suggesting that the currently characterized SMs might be only a small fraction of the SMs that a fungus can produce (Khaldi et al. 2010; Hoffmeister and Keller 2007). For example, 33 of the 37 putative SM gene clusters in *A. fumigatus* have no characterized products, despite evidence from metabolomics surveys suggesting that the fungus produces many SMs (Lin et al. 2013; Inglis et al. 2013; Frisvad et al. 2009). This is likely due to the fact that characterizing the function of SM gene clusters and matching them to specific SMs is a non-trivial task; most SM gene clusters contain many genes and are often only activated under specific ecological conditions such as the availability of different nutrients or the presence of other species (Axel a Brakhage 2013).

Filamentous fungi exhibit a huge amount of SM biochemical diversity. Individual SMs are often known to be produced by only one or a handful of species, and the SM chemotypic profiles of closely related fungi are typically non-overlapping (Keller, Turner, and Bennett 2005;

J. Bennett and Bentley 1989). For example, the meroterpenoid fumagillin, originally isolated from *Aspergillus fumigatus*, has only been detected in *Aspergillus fumigatus* and some isolates of *Penicillium raistrickii* (Christensen, Frisvad, and Tuthill 1999; McCowen, Callender, and Lawlis 1951). The gene cluster required for its production appears to be conserved in the *A. fumigatus* close relative, *Aspergillus fischerianus*, though only intermediate compounds have been detected from cultures of this and other closely related species (Lin et al. 2013; Asami et al. 2004; Wiemann, Guo, et al. 2013). In some genera, including *Aspergillus* (Frisvad, Andersen, and Thrane 2008), the extent of fungal SM distribution is so taxonomically narrow that SM chemotypic profiles have been used as unequivocal species-level identifiers.

As might be expected given their key roles in fungal ecology, SM production – and as a consequence SM gene cluster transcriptional activity – is tightly controlled by a complex network of master SM regulators triggered by a wide variety of environmental cues such as temperature, light, pH, and nutrient availability (Axel a Brakhage 2013). Among the master SM regulators identified to date are members of the fungal-specific Velvet protein family, which regulate SM production in response to dark conditions in the model filamentous fungus *Aspergillus nidulans* (Ö. Bayram et al. 2008; Kato, Brooks, and Calvo 2003; Stinnett et al. 2007; Purschwitz et al. 2008). The founding member of the Velvet family, VeA, stimulates production of diverse types of SMs in various fungal genomes under dark conditions, and has been shown to regulate gliotoxin, fumagillin, fumitremorgin G, and fumigaclavine C gene cluster expression and metabolite production in *A. fumigatus* (Sourabh Dhingra et al. 2013). Recently, a VeA-dependent regulator of secondary metabolism, MtfA, was identified in *A. nidulans*, which–unlike VeA–is localized in the nucleus regardless of light conditions (Ramamoorthy et al. 2013). MtfA regulates terrequinone, sterigmatocystin, and penicillin in *A. nidulans*; in *A. fumigatus*, MtfA is

necessary for normal protease activity, and virulence assays using the moth *Galleria mellonella* suggest it plays a role in pathogenicity (Smith and Calvo 2014).

In addition to regulating SM, both of these regulators have been linked to the regulation of asexual and sexual development. Timing of SM production with developmental changes is well established in filamentous fungi, and the presence/absence of certain SMs has been linked with developmental changes (Tsai et al. 1998; A. M. Calvo et al. 2002; A. M. Calvo 2008). It has been suggested that regulators that coordinate SM and development allow filamentous fungi to have more complex lifestyle and diverse natural products than their unicellular yeast relatives, which lack *veA* as well as backbone synthesis genes necessary for SM production (Roze, Chanda, and Linz 2011; Kroken et al. 2003; A. M. Calvo 2008).

Remarkably, both *veA* and *mtfA* appear to be broadly conserved in filamentous fungi with non-overlapping SM profiles (Ramamoorthy et al. 2013; O. Bayram and Braus 2012). We used four well-studied organisms from the fungal genus *Aspergillus*, a highly diverse genus and producer of some of the most iconic SMs, including gliotoxin and penicillin, to investigate the evolutionary variability in the distribution of SM gene clusters and its interaction with these two broadly conserved global transcriptional regulators that differ in their response to light, *veA* and *mtfA*. Our evolutionary analyses show that although both the SM gene clusters as well as their gene content are poorly conserved between *Aspergillus* species, explaining the narrow taxonomic distribution and distinctiveness of their SM profiles, the effects of the global transcriptional regulators on SM production in response to environmental cues are largely conserved across these same species. In contrast, examination of the role of *veA* and *mtfA* in development, a process that involves genes that are highly conserved between the two species and whose regulation is intimately linked to SM regulation, yields a very different pattern; whereas the role

of *veA* is conserved, *mtfA* regulates development in the homothallic *A. nidulans* but not in the heterothallic *A. fumigatus*.

Methods

Genome sequences and orthogroup definitions for A. nidulans, A. fumigatus, A. oryzae, and A. niger

All genome sequences and annotations for *A. nidulans* FGSC A4 s10-m02-r03, *A. fumigatus* AF293 s03-m04-r11, *A. oryzae* RIB40 s01-m08-r21 and *A. niger* CBS 513.88 s01-m06-r10 were taken from the Aspergillus Genomes Database (AspGD) (Arnaud et al. 2010). Orthogroups for these four genomes were taken from AspGD's orthology assignments for 16 *Aspergillus* species, which were generated using a Jaccard clustering approach (Crabtree et al. 2007). AspGD orthogroups contain groups of genes that are thought to have descended from the *Aspergillus* common ancestor; genes from the same species that are part of a given orthogroup are defined as in-paralogs that have duplicated at some later point after the species diverged from the *Aspergillus* common ancestor. Species-specific genes, which were absent from AspGD orthogroups, were organized into species-specific orthogroups using the MCL algorithm in combination with all-versus-all protein BLAST search (Enright, Van Dongen, and Ouzounis 2002). Proteins with BLAST hits with 60% query and subject coverage, an e-value of less than $1e^{-5}$, and a percent identity of greater than 60% were subsequently clustered in MCL with an inflation parameter of 2 and were considered species-specific orthogroups. Proteins that did not pass the BLAST cutoffs were considered single-gene, species-specific orthogroups.

Gene category definitions

Genes involved in secondary metabolism were taken from a previous study that expertly annotated secondary metabolic gene clusters in the four species under study (Inglis et al. 2013). Manually curated gene cluster boundaries were used when available. Primary metabolism genes were annotated using a previously described enzyme classification pipeline which utilizes KEGG Enzyme Commission annotations (J. H. Wisecaver, Slot, and Rokas 2014). Genes involved in development were determined from all genes in *A. fumigatus* and *A. nidulans* annotated to the GO term "developmental process" (GO:0032502) in AmiGO (Carbon et al. 2009). This data was accessed on 2014-07-19.

Strains and culture conditions

The strains used in this study include *A. fumigatus* CEA10, TSD1.15(Δ*veA*) and TTDS4.1(Δ*mtfA*) (S Dhingra, Andes, and Calvo 2012; Smith and Calvo 2014) and *A. nidulans* TRV50.2 (Ramamoorthy et al. 2012), TXFp2.1(Δ*veA*) generated in this study, and TRVpΔ*mtfA* (Ramamoorthy et al. 2013). Many *A. nidulans* studies have used a *veA* partial deletion (H.-S. Kim et al. 2002). For the present study, we generated a strain with a complete deletion of the *veA* coding region, TXFp2.1(Δ*veA*). This strain was generated as follows. First, The *veA* deletion cassette was obtained by fusion PCR as previously described (Szewczyk et al. 2006). A 1.4 kb 5' UTR and a 1 kb 3' UTR *veA* flanking regions were PCR amplified from wild type FGSC4 genomic DNA with primers veA_comF and AnidveA_p2, and ANVeASTagP3 and ANVeASTagP4 primers sets, respectively. The *A. fumigatus pyrG* ($pyrG^{A.fum}$) selectable marker was amplified with AnidveA_p5 and ANVeASTagP6 primers from plasmid p1439. The 5' and 3' UTR fragments were then PCR fused to $pyrG^{A.fum}$ to generate the *veA* replacement construct

using primers AnidveA_P7 and AnidveA_P8. The deletion cassette was transformed into *A. nidulans* RJMP1.49 strain (Shaaban et al. 2010). The resulting transformants were then transformed with the pSM3 plasmid containing the *A. nidulans pyroA* to generate prototrophs, obtaining the Δ*veA* strain. This strain was confirmed by DNA analysis (data not shown) and designated as TXFp2.1.

All strains were grown in liquid stationary cultures in Czapek-Dox medium (Difco) in the dark. The experiments were carried out with two replicates. After 72 hours of incubation at 37ºC mycelia samples were harvested, immediately frozen in liquid nitrogen and lyophilized.

RNA extraction

Total RNA was isolated from lyophilized mycelia using the directzol RNA MiniPrep Kit (Zymo) according to the manufacturer's instructions. RNA then was quantified using a nanodrop instrument. Expression patterns of *veA* and *mtfA* were verified in the *A. fumigatus* and *A. nidulans* wild types as well as in the deletion mutants by qRT-PCR prior to RNA sequencing (not shown), conforming the absence of transcripts in the deletion mutants.

RNA sequencing

RNA-Seq libraries were constructed and sequenced at Vanderbilt Technologies for Advanced Genomics using the Illumina Tru-seq RNA sample prep kit as previously described (Gibbons, Salichos, et al. 2012; Gibbons, Beauvais, et al. 2012; Sourabh Dhingra et al. 2013). In brief, total RNA quality was assessed via Bioanalyzer (Agilent). Upon passing quality control, poly-A RNA was purified from total RNA and the second strand cDNA was synthesized from mRNA. cDNA ends were then blunt repaired and given an adenylated 3' end. Next, barcoded adapters were ligated to the adenylated ends and the libraries were PCR enriched, quantified,

pooled and sequenced an on Illumina HiSeq 2500 sequencer. Two biological replicates were generated for each strain sequenced.

RNA-seq read alignment and differential gene expression

Raw RNA-seq reads were trimmed of low-quality reads and adapter sequences using Trimmomatic using the suggested parameters for single-end read trimming (Bolger, Lohse, and Usadel 2014). Trimmed reads were aligned to *A. nidulans* and *A. fumigatus* genomes using Tophat2 using the reference gene annotation to guide alignment and without attempting to detect novel transcripts (parameter –no-novel-juncs) (D. Kim et al. 2013). Reads aligning to each gene were counted using HTSeq-count with the intersection-strict mode (Anders, Pyl, and Huber 2014). Differential expression between Δ*veA* and WT and Δ*mtfA* and WT strains of *A. fumigatus* and *A. nidulans* were determined using DESeq2 (M. I. Love, Huber, and Anders 2014). Genes were considered differentially expressed if their adjusted *P*-value was less than 0.1 and their $\log_2$ fold change was greater than 1 or less than -1.

Statistical analyses

GO term enrichment was determined for over- and under-expressed genes in all four conditions tested (*A. nidulans* and *A. fumigatus* Δ*veA* vs. WT and Δ*mtfA* vs. WT) using the Cytoscape plugin BiNGO (Shannon et al. 2003; Maere, Heymans, and Kuiper 2005). To allow for a high-level view of the types of differentially expressed gene sets, the Aspergillus GOSlim term subset developed by AspGD was used. The Benjamani-Hochberg multiple testing correction was applied, and terms were considered significantly enriched if the adjusted *P*-value was less than 0.05.

Fisher's exact tests were performed using the R function fisher.test with a two-sided alternative hypothesis (R Core Team 2014). *P*-values were adjusted for multiple comparisons using the R function p.adjust with the Benjamini-Hochberg multiple testing correction (Benjamini and Hochberg 1995) Figures were created using the R plotting system ggplot2 (Wickham 2009) and circos (Krzywinski et al. 2009).

Results

The majority of SM gene clusters in Aspergillus *are species-specific*

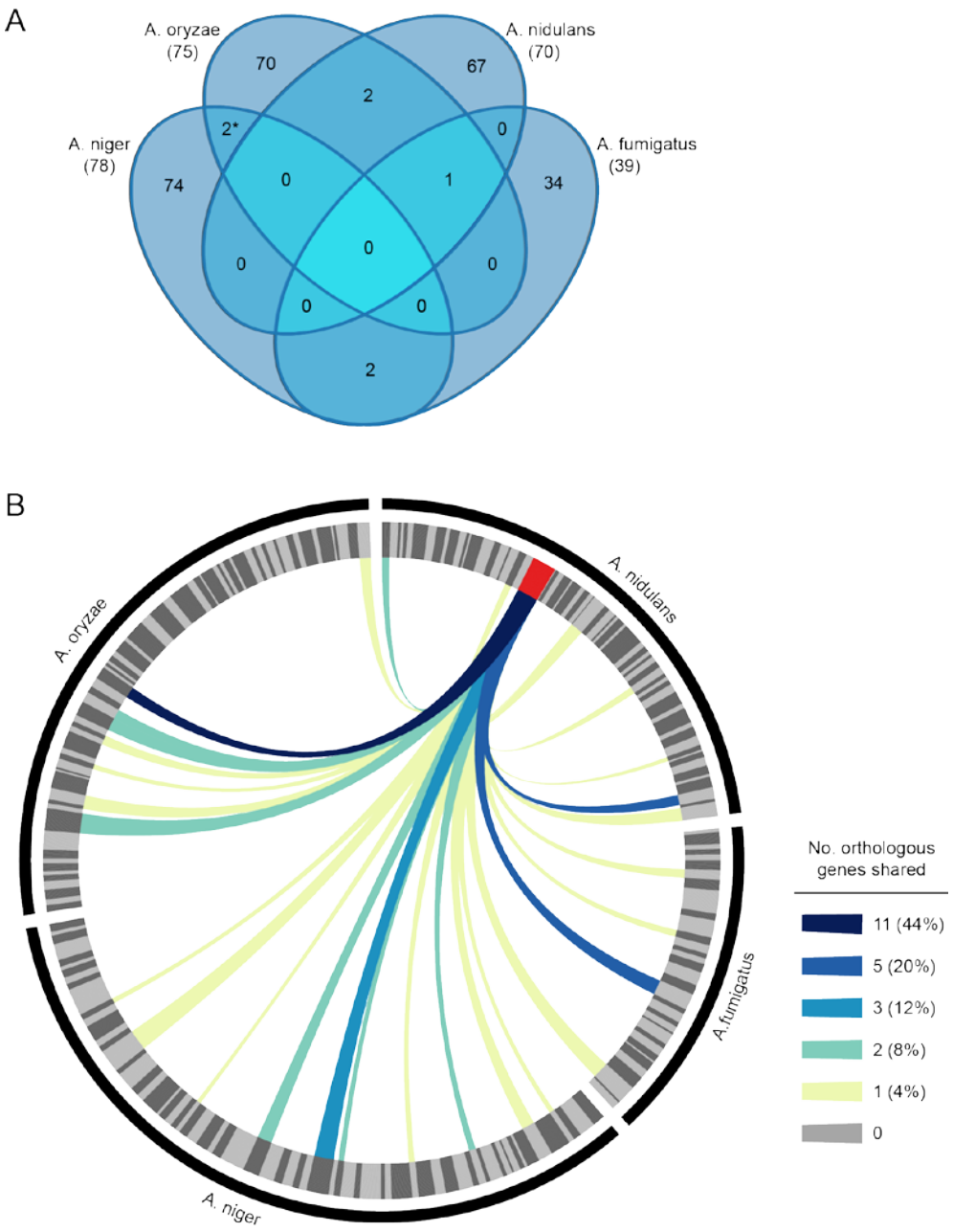

Figure 2-1. SM gene clusters in *Aspergillus* show minimal evolutionary conservation. **(A)** Venn diagram showing homologous SM gene clusters between *A. fumigatus*, *A. nidulans*, *A. niger*, and *A. oryzae*. Two SM gene clusters were considered homologous if greater than 50% of their genes were orthologs. Numbers in parenthesis indicate the total number of SM gene clusters present in each species. The asterisk (*) is to clarify that that two SM gene clusters in *A. oryzae* are homologous to one gene

cluster in *A. niger*. **(B)** Circos plot showing all SM gene clusters in *A. fumigatus, A. nidulans, A. niger,* and *A. oryzae*. The outer black track shows the relative SM gene counts in each of the four species. SM clusters are indicated by the alternating light and dark grey wedges of the inner track; wedge thickness is proportional to number of clustered genes. The sterigmatocystin gene cluster in *A. nidulans* is colored red. Links indicate SM clusters containing one or more genes assigned to the same orthogroup as gene(s) in the sterigmatocystin gene cluster; link color indicates the number of shared genes.

The genomes of *A. fumigatus, A. nidulans, A. oryzae,* and *A. niger* contain 317, 498, 725, and 584 secondary metabolic genes, respectively, which are organized in 37, 70, 75, and 78 corresponding secondary metabolic gene clusters (Inglis et al. 2013). We considered SM gene clusters to be conserved between species if greater than half of the genes in the larger gene cluster were orthologous to greater than half of the genes in the smaller gene cluster. Even with this very liberal definition of gene cluster conservation, we found that no SM gene clusters were conserved across all four species. Moreover, 91.9-96.1% of SM gene clusters were specific to each species, with only 7 SM gene clusters conserved between any species (Figure 2-1a). As none of these SM gene clusters have chemically characterized products, little can be inferred about the similarity or differences of the products of these conserved gene clusters.

While very few conserved SM gene clusters can be identified between these four species, SM gene clusters do contain genes whose orthologs are parts of other, non-homologous, SM gene clusters. For example, the 25 genes in the sterigmatocystin gene cluster in *A. nidulans*, one of the largest SM gene clusters present in the genomes analyzed, have orthologs in 25 SM gene

clusters in the other three species as well as inparalogs in 8 other *A. nidulans* SM gene clusters (Figure 2-1b). However, in all but one case, less than 20.0% (5 genes) of the sterigmatocystin gene cluster is present in the other gene cluster. The only exception is the truncated aflatoxin gene cluster of *A. oryzae*, which shares 11 orthologs with the ST gene cluster. Although the *A. oryzae* aflatoxin gene cluster is non-functional (Machida et al. 2005; Kiyota et al. 2011; Gibbons, Salichos, et al. 2012), the evolutionary conservation between the aflatoxin and sterigmatocystin gene clusters is reflected in the fact that sterigmatocystin is the penultimate precursor product of the aflatoxin biosynthetic pathway (D W Brown et al. 1996).

Aspergillus SM genes are significantly less conserved than genes for primary metabolism

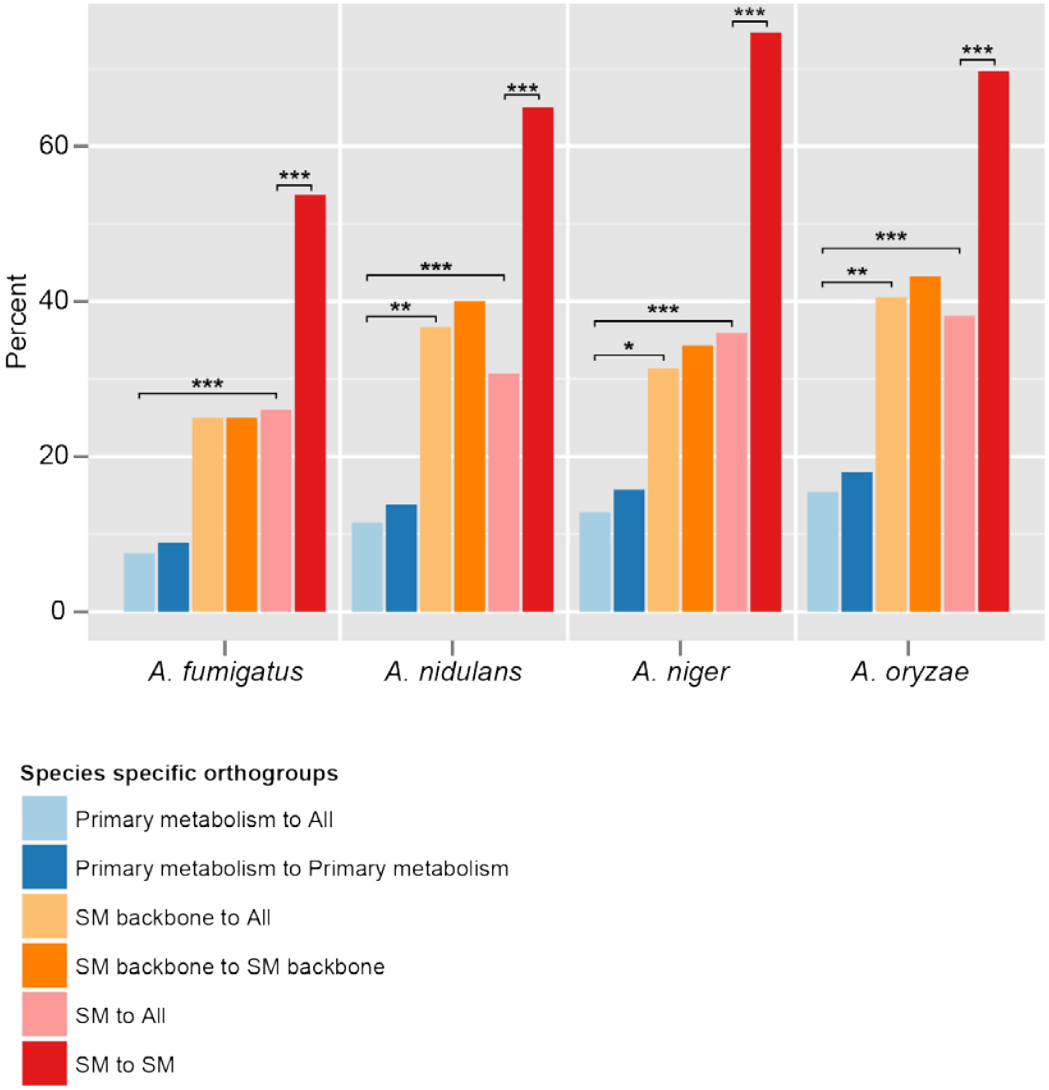

Figure 2-2. SM genes in *A. fumigatus, A. nidulans, A. niger,* and *A. oryzae* are less conserved than genes involved in primary metabolism. For each species, dark blue bars indicate the percentage of primary metabolism orthogroups that is species-specific when compared to all genes similarly annotated as participating in primary metabolism in the other three species; light blue bars indicate the percentage of each species' primary metabolism orthogroups that is species-specific compared to all genes, irrespective of their annotation, in the other three species. Similarly, light orange bars indicate the percentage

of each species' SM backbone synthesis orthogroups that is species-specific when compared to all genes similarly annotated as SM backbone synthesis genes in the other three species; dark orange bars indicate the percentage of each species' SM backbone synthesis orthogroups that is species-specific compared to all other genes. Light red bars indicate the percentage of each species' SM orthogroups that is species-specific when compared to all genes similarly annotated as SM genes in the other three species; dark red bars indicate the percentage of each species' SM orthogroups that is species-specific compared to all other genes. Asterisks indicate statistically significant differences based on a *P*-value ≤ 0.01 (*), ≤ 0.001 (**), or ≤ 0.0001 (***) in a two-tailed Fisher's exact test.

To determine the percentage of lineage-specific orthogroups, we determined the number of orthogroups annotated to a particular GOSlim term with at least one gene present in at least one other genome as well as the number of orthogroups with at least one gene annotated to the same functional category in at least one other genome. We found that SM orthogroups were significantly far less conserved than primary metabolic orthogroups in all four genomes examined (adjusted $P < 1e^{-10}$ for all combinations). No more than 18% of primary metabolic orthogroups were lineage-specific in any *Aspergillus* species; this low percentage was observed both for comparisons between just primary metabolic genes as well as across all genes (Figure 2-2). In contrast, SM orthogroups as well as orthogroups containing just SM backbone genes were more likely to be lineage-specific (Figure 2-2). For example, in *A. fumigatus,* the smallest genome in our analysis, 8.9% (117/1322) of primary metabolic orthogroups were lineage-specific versus 19.0% (4/21) of SM backbone orthogroups and 26.0% (73/281) of SM orthogroups. Strikingly, genes involved in secondary metabolism in at least 2 genomes were by

far the least conserved in our analysis. Between 53.7% (in *A. fumigatus,* with 37 SM gene clusters) to 74.7% (in *A. niger,* with 77 SM gene clusters) of SM orthogroups had no SM ortholog in any of the other species examined. SM backbone genes were conserved at a similar rate when compared to all genes and all SM backbone genes, which reflects the accurate prediction of polyketide synthase and non-ribosomal peptide synthase genes in the organisms under study.

VeA regulates the same biological processes as well as the same fraction of the genome in both A. nidulans and A. fumigatus

Table 2-1. Differentially expressed genes in Δ*veA* vs. WT and Δ*mtfA* vs. WT comparisons for *A. fumigatus* and *A. nidulans*

Condition	Species	Total dif. expressed[a]	Per. diff. expressed[a]	Under-expressed[a]	Over-expressed[a]
ΔveA	*A. fumigatus*	3,101	31.7%	1,555	1,546
	A. nidulans	2,836	26.5%	1,671	1,165
ΔmtfA	*A. fumigatus*	97	0.9%	63	34
	A. nidulans	968	9.0%	568	400

[a]Number of differentially expressed genes relative to wild type

We next examined the function of the conserved secondary metabolic regulator VeA by performing RNA sequencing (Lin et al. 2013; Gibbons, Salichos, et al. 2012; Gibbons, Beauvais, et al. 2012) of Δ*veA* and wild-type (WT) *A. fumigatus* strains TSD1.15 (S Dhingra, Andes, and Calvo 2012) and CEA10 and *A. nidulans* strains TXFp2.1 and TRV50.2 (Ramamoorthy et al. 2012) and analyzing the data to identify genes that are differentially regulated in Δ*veA* vs WT in the two species. Of the 9,783 transcribed genes in the *A. fumigatus* genome, 1,546 (15.8%) were over-expressed and 1,555 (15.9%) were under-expressed in the Δ*veA* vs WT analysis in *A. fumigatus* (Table 2-1). We observed very similar numbers of genes differentially regulated in the

A. nidulans Δ*veA* vs WT analysis; out of 10,709 genes in the *A. nidulans* genome, were 1,165 (10.9%) were over-expressed and 1,671 genes (15.6%) were under-expressed. In total, approximately 32% and 26% of protein coding genes were differentially regulated in Δ*veA* compared to WT in *A. fumigatus* and *A. nidulans*, respectively.

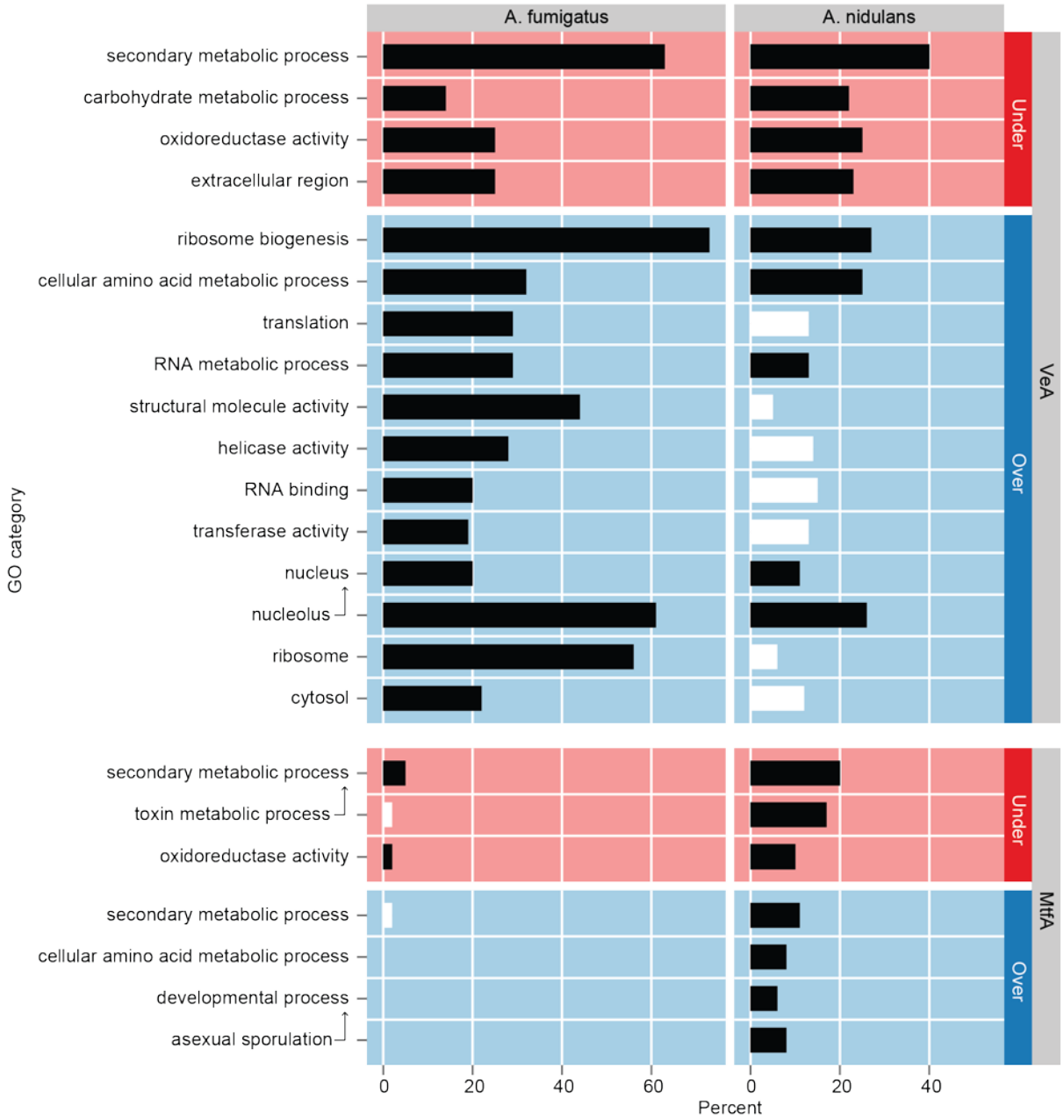

Figure 2-3. GO term enrichment analysis of genes differentially expressed in Δ*veA* and Δ*mtfA* in *A. fumigatus* and *A. nidulans*. Gene ontology (GO) categories statistically overrepresented in under-expressed (red) and over-expressed (blue) gene sets in Δ*veA* and Δ*mtfA* relative to wild type. Arrows point to GO term ancestors. Horizontal bars show the percentage of each gene set assigned to a particular GO term with black bars indicating significant enrichment (Benjamini & Hochberg adjusted *P*-value ≤ 0.05 in a hypergeometric test); white bars indicate no significant enrichment.

To characterize the broad functional categories of these differentially regulated genes, we performed GO term enrichment analysis using the Aspergillus GOSlim term hierarchy (Ashburner et al. 2000; Arnaud et al. 2010). Four GO terms, namely SECONDARY METABOLIC PROCESS, CARBOHYDRATE METABOLIC PROCESS, OXIDOREDUCTASE ACTIVITY, and - EXTRACELLULAR REGION, are significantly enriched in under-expressed genes in both *A. nidulans* and *A. fumigatus*, showing that VeA is a positive regulator of similar processes in both species (Figure 2-3). Over-expressed genes in *A. fumigatus* were significantly enriched for twelve GO terms potentially related to cell growth, namely RIBOSOME BIOGENESIS, CELLULAR AMINO ACID METABOLIC PROCESS, TRANSLATION, RNA METABOLIC PROCESS, STRUCTURAL MOLECULE ACTIVITY, HELICASE ACTIVITY, RNA BINDING, TRANSFERASE ACTIVITY, NUCLEUS, NUCLEOLUS, RIBOSOME, and CYTOSOL. Five of these twelve terms were also significantly enriched in *A. nidulans* (RIBOSOME BIOGENESIS, CELLULAR AMINO ACID METABOLIC PROCESS, RNA METABOLIC PROCESS, NUCLEUS, and NUCLEOLUS). Over-expressed genes were present in the remaining seven terms in *A. nidulans* but did not show statistically significant enrichment.

MtfA's regulatory role is smaller in scope in A. fumigatus *compared to* A. nidulans

We next examined the role of the recently identified SM regulator MtfA (Smith and Calvo 2014; Ramamoorthy et al. 2013) in *A. fumigatus* and *A. nidulans* by performing RNA sequencing and differential gene expression analysis of Δ*mtfA* vs WT strains of both species (*A. fumigatus* tTDS4.1 Δ*mtfA* (Smith and Calvo 2014) and CEA10, *A. nidulans* TRVp Δ*mtfA* and TRV50.2 (Ramamoorthy et al. 2013)). In contrast to our findings with *veA*, we found a striking difference in the percentage of genes regulated in both species (Table 2-1). Thirty-six genes were over-expressed (0.4%) and 63 (0.6%) were under-expressed in the *A. fumigatus* Δ*mtfA* vs WT analysis, whereas in the *A. nidulans* Δ*mtfA* vs WT analysis 400 genes were over-expressed (3.7%) and 568 were under-expressed (5.3%).

To determine the functional categories impacted by *mtfA* deletion in both species, we performed GO term enrichment analysis on the genes differentially expressed between Δ*mtfA* and WT strains. Under-expressed as well as over-expressed genes in *A. nidulans* were significantly enriched for SECONDARY METABOLIC PROCESS, TOXIN METABOLIC PROCESS and OXIDOREDUCTASE ACTIVITY, suggesting that MtfA is involved in positive and negative regulation of different secondary metabolites (Figure 2-3). Over-expressed genes in *A. nidulans* were also significantly enriched for asexual developmental processes, namely DEVELOPMENTAL PROCESS and ASEXUAL SPORULATION.

Under-expressed genes in *A. fumigatus* were significantly enriched for two of the three processes as in *A. nidulans*, namely SECONDARY METABOLIC PROCESS and OXIDOREDUCTASE ACTIVITY. However, over-expressed genes in *A. fumigatus* were not significantly enriched for any GO terms; some over-expressed genes were present in the SECONDARY METABOLIC PROCESS term, though this was not statistically significant.

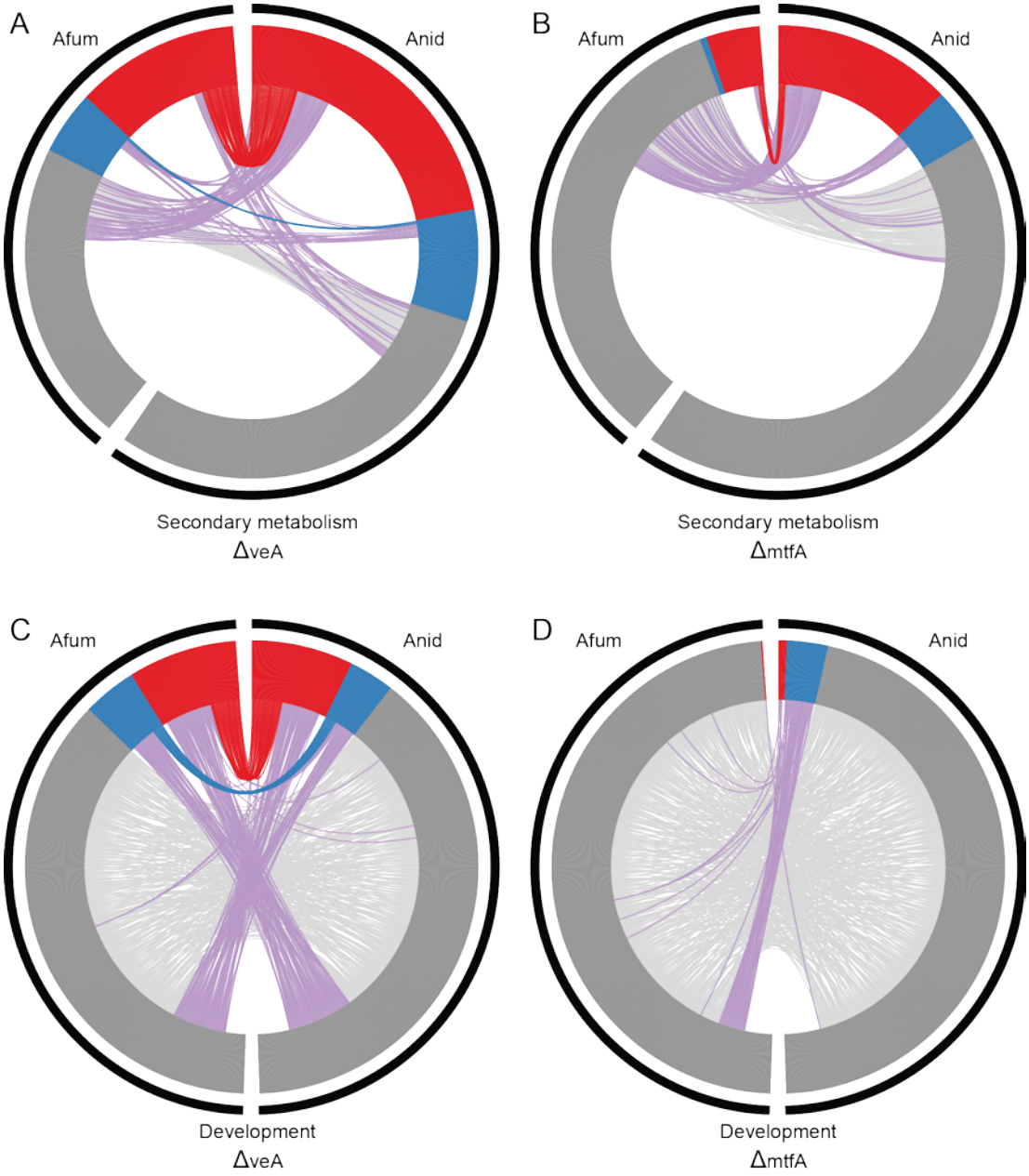

Figure 2-4. Orthology of SM and development genes differentially expressed in Δ*veA* and Δ*mtfA* in *A. fumigatus* and *A. nidulans*. Circos plots of SM genes **(A,B)** and developmental genes **(C,D)** showing change in gene expression patterns under Δ*veA* **(A,C)** and Δ*mtfA* **(B,D)** conditions. Outer black track shows the relative gene counts in *A.*

nidulans (right) and *A. fumigatus* (left). Inner track shows the relative number of under-expressed genes (red), over-expressed genes (blue) and not differentially expressed genes (grey). Links indicate orthologous genes between the two species that are both under-expressed (red links), both over-expressed (blue links) and both not differentially expressed (light grey links); purple links indicate that the orthologous genes have conflicting expression patterns.

To examine whether SM gene conservation correlated with conservation of regulation by VeA and MtfA, we examined whether orthologous genes in *A. nidulans* and *A. fumigatus* showed the same responses in Δ*veA* vs WT and Δ*mtfA* vs WT analyses (Figure 2-4). SM gene expression in Δ*veA A. nidulans* and *A. fumigatus* was similar in terms of numbers of differentially expressed genes despite the large number of genes without orthologs between these two species (Figure 2-4a; Figure 2-1a). Of the 184 under-expressed SM genes in Δ*veA A. nidulans,* only 64 genes (34.8%) had an ortholog in *A. fumigatus.* Of these 64 conserved genes, 45 (70.3%) had at least one differentially expressed ortholog in *A. fumigatus,* and 37 (57.8%) had at least one similarly under-expressed ortholog in *A. fumigatus*. Fewer SM genes were over-expressed in either Δ*veA A. nidulans* or *A. fumigatus*; of the 67 over-expressed genes in *A. nidulans,* 14 (20.9%) had orthologs in *A. fumigatus*. Of these 14 conserved genes, 5 had at least one differentially expressed ortholog in *A. fumigatus*, and 3 had at least one similarly over-expressed ortholog in *A. fumigatus*.

When *mtfA* was deleted, fewer SM genes were differentially expressed in *A. fumigatus* than in *A. nidulans.* Of the 107 under-expressed genes in Δ*mtfA A. nidulans,* 36 (33.6%) had an ortholog in *A. fumigatus* (Figure 4b). Unlike *veA*, however, only 6 of these conserved genes had

differentially expressed orthologs in *A. fumigatus*. Finally, of the 32 over-expressed genes in Δ*mtfA A. nidulans*, 2 of the 12 genes with orthologs in *A. fumigatus* had orthologs that were differentially expressed.

Apart from their involvement in the global regulation of SM, both VeA and MtfA are also involved in the regulation of asexual and sexual development. In contrast to genes involved in SM, genes involved in asexual and sexual development in *Aspergillus* have been shown to be highly conserved across the genus (Galagan et al. 2005). Of the 490 genes annotated to the GO term DEVELOPMENTAL PROCESS, 462 have at least one ortholog among the 478 genes annotated to this term in *A. fumigatus.* In Δ*veA* A. *nidulans*, 72 developmental genes are under-expressed and 32 are over-expressed. Of the 72 under-expressed genes, 66 (91.7%) have an ortholog in *A. fumigatus*, 30 of which have a differentially expressed ortholog (Figure 2-4C). There are fewer over-expressed developmental genes in Δ*veA A. nidulans*, but they show similar trends; 31 of the 32 over-expressed genes have an ortholog, 15 of which have differentially expressed orthologs in *A. fumigatus* and 11 of which have over-expressed orthologs. In contrast with *veA*, many more developmental genes were differentially expressed in *A. nidulans* Δ*mtfA* (35) than in *A. fumigatus* Δ*mtfA* (1). While 4 of the 6 under-expressed genes and 28 of the 29 over-expressed genes in *A. nidulans* had orthologs in *A. fumigatus*, none of these orthologs were differentially expressed (Figure 2-4D).

Discussion

Here, we examined the interplay between secondary metabolites with narrow taxonomic distributions and their broadly conserved SM global regulators in four *Aspergillus* species. We found remarkably few conserved SM gene clusters in *A. fumigatus*, *A. nidulans*, *A. oryzae*, and

A. niger (Figure 2-1a). Further, the genes comprising these clusters were significantly more species-specific than genes involved in primary metabolism (Figure 2-2), and those SM genes that were conserved were assigned to non-homologous pathways (e.g., genes in the sterigmatocystin gene cluster; Figure 2-1B). Despite the high level of divergence in SM pathways, regulators of SM production are conserved throughout filamentous fungi (Brakhage 2013). We assessed the conservation of roles of two of these regulators, *veA* and *mtfA*, in *A. fumigatus* and *A. nidulans* by comparing genome-wide gene expression of deletion mutants of *veA* and *mtfA* in both species with wild-type strains. We found that the role of *veA* in controlling secondary metabolism and development was conserved in both species (Figure 2-3), though the regulated genes were often different (Figure 2-4). In contrast, we found that while deleting *mtfA* negatively impacted SM gene expression in both *A. fumigatus* and *A. nidulans*, developmental genes were only impacted in *A. nidulans* (Figure 2-3).

Aspergillus *secondary metabolic genes and gene clusters are largely species-specific*

Previous studies have described relatively small numbers of conserved SM gene clusters between the closely related species *Aspergillus fumigatus, fisherianus*, and *clavatus* (Khaldi et al. 2010). These studies considered SM gene clusters to be conserved if 80% or more of their genes were shared. Since the four *Aspergillus* species under study here are much less closely related (Gibbons and Rokas 2012), we used a lower threshold of 50% for conservation. Even using this relaxed threshold, we found no clusters that were conserved in all four species, one cluster conserved in three species, and a small number conserved between pairs of species (Figure 2-1A). SM gene clusters have been described as not only species-specific but sometimes strain specific; two isolates of *A. fumigatus* differ in one putative SM gene cluster (Fedorova et al.

2008), and multiple SM gene clusters vary in their presence and absence in isolates of *A. niger* (Andersen et al. 2011).

In addition to the non-conservation of SM gene clusters, we found that the genes comprising SM genes are much more likely to be lineage-specific than those involved in primary metabolic functions (Figure 2-2). Surprisingly, many genes involved in SM clusters had orthologs in other *Aspergilli* that were not in an SM gene cluster themselves. This observation may support the hypothesis that SM gene clusters can be formed or altered by incorporating non-SM genes through genomic rearrangements and possible co-regulation with other genes in the biosynthetic pathway (Wong and Wolfe 2005). However, many of the SM genes were species-specific even when compared against all genes in the other four *Aspergilli* (Figure 2-1), and as many as 21.7% of SM genes were not present in any other sequenced *Aspergillus* species. This high number of species-specific genes involved in *Aspergilli* may be explained by extensive gene duplication and loss, *de novo* gene emergence, very high sequence divergence driven by selection, or horizontal gene transfer.

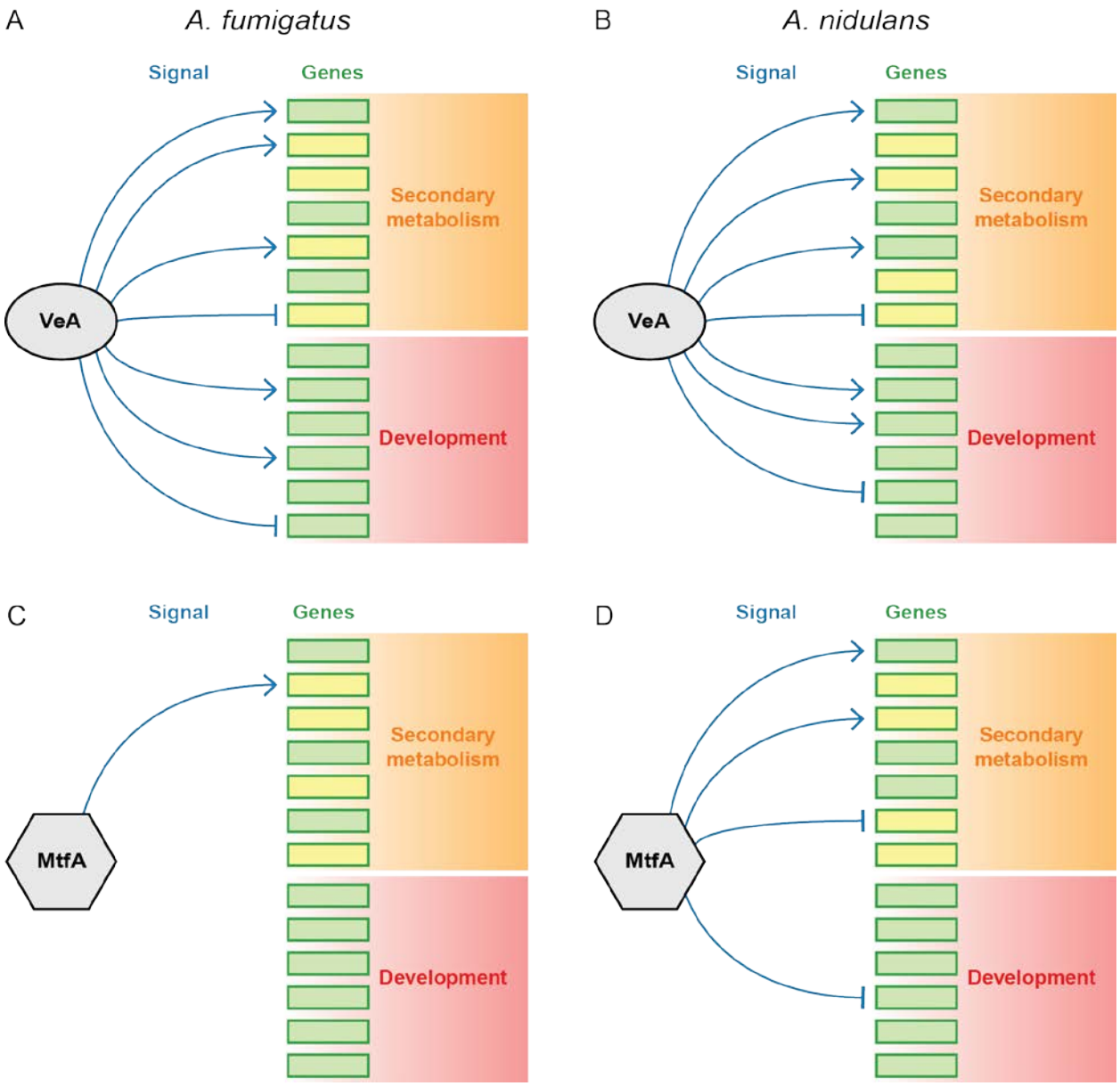

Figure 2-5. Model of gene regulatory network evolution in *Aspergillus*. Generalized gene regulatory networks for VeA **(A,B)** and MtfA **(C,D)** in *A. fumigatus* **(A,C)** and *A. nidulans* **(B,D)**. As master transcriptional regulators, VeA and MtfA can both promote the expression of a gene (indicated by an arrow →), or inhibit gene expression (indicated by a bar ⊣). Target genes are either present in both species (green fill) or species-specific (yellow fill).

Our findings with respect to the genes differentially expressed in the absence of *veA* support a conserved role for *veA* in regulating secondary metabolism and development in *A. fumigatus* and *A. nidulans*; however, the downstream genes regulated by VeA are different between the two species. Whether lineage-specific or conserved, SM genes are differentially expressed in *veA*'s absence in both *A. fumigatus* and *A. nidulans.* Interestingly, conserved genes differentially expressed in one species are often not differentially expressed in the other. We propose that the transcriptional circuit by which VeA regulates secondary metabolism and development in both fungi has diverged at the level of both the target genes and the regulatory signal (Figure 2-5). VeA is known to have many interacting partners (A. M. Calvo 2008); among these, it is responsible for transporting the Velvet family protein VelB from the cytoplasm to the nucleus, where both proteins interact with LaeA, forming a trimeric complex that regulates secondary metabolism production and development (Ö. Bayram et al. 2008). However, each protein has functions in the cell outside of this complex, which can be seen through the different effects of individual gene deletion on gene expression and morphogenesis (Ö. Bayram et al. 2008; Sourabh Dhingra et al. 2013; Perrin et al. 2007). VeA interacts with red light-sensing proteins in the nucleus, and it is speculated that VeA may act as a scaffold protein recruiting additional transcriptional regulators (O. Bayram and Braus 2012). Finally, recent analysis has shown that the Velvet domain is a DNA-binding domain, and that Velvet family proteins may act as direct transcriptional regulators (Ahmed et al. 2013). The number and complexity of VeA's interacting partners, and its putative transcription factor function, offers many degrees of freedom for changes in specific gene regulation in both *A. fumigatus* and *A. nidulans*, while preserving its important ecological role in coordinating sexual development and secondary metabolism production in response to dark conditions.

Much less is known about the regulatory partners of the putative C2H2 zinc finger transcription factor MtfA; however, we suggest that MtfA acts downstream of VeA in *A. nidulans*, but not in *A. fumigatus*, as its expression is decreased in Δ*veA A. nidulans* but not Δ*veA A. fumigatus*. Our results show that MtfA is involved in regulating secondary metabolism in both *A. fumigatus* and *A. nidulans*, though it regulates fewer clusters in *A. fumigatus* than in *A. nidulans*. As was the case with *veA*, deleting *mtfA* results in differentially expressed lineage-specific and conserved SM genes, though differentially expressed conserved genes were not necessarily differentially expressed in the other species, indicating a divergence in the signal that targets these genes for regulation by MtfA or its interacting partners. Unlike *veA*, however, deleting *mtfA* resulted in developmental gene expression changes exclusively in *A. nidulans*, suggesting that there has been a loss in *A. fumigatus* or gain in *A. nidulans* of the regulatory signal that directs MtfA or its downstream targets to regulate developmental processes. Taken together, our results suggest extensive rewiring in the regulatory circuit governing secondary metabolism and development between *A. nidulans* and *A. fumigatus*.

CHAPTER III

REGULATION OF SECONDARY METABOLISM BY THE VELVET COMPLEX IS TEMPERATURE-RESPONSIVE IN ASPERGILLUS

Authors

Abigail L. Lind, Timothy D. Smith, Timothy Saterlee, Ana M. Calvo, and Antonis Rokas

Introduction

Filamentous fungi produce a diverse array of small molecules collectively known as secondary metabolites (SMs). Much research on SMs has focused on their double-edged impact on humans (Keller, Turner, and Bennett 2005); while many are valued as pharmaceuticals, such as the antibiotic penicillin and the cholesterol-lowering drug lovastatin (Paláez 2004; Kennedy et al. 1999), others are potent toxins, such as the acutely carcinogenic aflatoxin (J. W. Bennett and Klich 2003). In the fungal natural environment, SMs have a variety of functions; they can operate as signaling molecules (Yim, Wang, and Davies 2007; Rodríguez-Urra et al. 2012), as virulence factors to aid pathogenic lifestyles (R H Proctor, Hohn, and McCormick 1995; Stanzani et al. 2005; Coméra et al. 2007), as microbial inhibitors to carve out a competitive advantage in environments crowded with other microbes (Losada et al. 2009; König et al. 2013), or as a defense against fungivorous predators (Rohlfs et al. 2007; A. M. Calvo and Cary 2015). SM production is closely linked with environmental signals (A. Brakhage et al. 2009; Keller 2015). For example, the SM aflatoxin is not produced by *Aspergillus parasiticus* at 37ºC, the organism's optimal temperature for growth, but is produced at 28ºC (Feng and Leonard 1998). Furthermore, the effects of specific environmental conditions on SM production can be varied;

for example, sterigmatocystin is produced in much higher quantities at 37ºC than at 28ºC in *Aspergillus nidulans*, a pattern of expression that is the reverse of its close chemical relative aflatoxin in *A. parasiticus* (Feng and Leonard 1998).

The expression of genes involved in the synthesis and secretion of SMs is governed by a hierarchical network of master regulators that respond to multiple environmental cues (Axel a Brakhage 2013). One such environmentally-responsive complex of master regulators is the Velvet protein complex, whose constituent proteins are broadly conserved regulators of fungal development and secondary metabolism (O. Bayram and Braus 2012; A. Calvo et al. 2016). In the absence of light in *A. nidulans*, two Velvet complex members, VeA and VelB, enter the nucleus where VeA interacts with the chromatin modifying protein LaeA (Ö. Bayram et al. 2008). The resulting heterotrimeric protein complex modulates expression of SM gene clusters and developmental processes in many fungi (Wiemann et al. 2010; Chettri et al. 2012; Hoff et al. 2010; Lind et al. 2015), including the opportunistic human pathogen *Aspergillus fumigatus* (S Dhingra, Andes, and Calvo 2012; Perrin et al. 2007; Sourabh Dhingra et al. 2013).

While most master regulators of secondary metabolism are known in the context of the individual environmental cues that activate them, it is likely that these regulators combinatorially control SM production to fine-tune the metabolic profile of a fungus to changing environments. The possibility of combinatorial regulation is supported by recent studies showing that multiple environmental cues can regulate production of the SM terrein in *Aspergillus terreus* (Gressler et al. 2015), that both the light-responsive regulator VeA and the nitrogen regulator AreA are required for wild-type levels of SM-producing gene transcription in *Fusarium oxysporum* (López-Berges et al. 2014), and that glucose concentration can impact SM production in *A. nidulans* through changes in the subcellular localization of VeA (Atoui et al. 2010).

The fungal genus *Aspergillus* is an excellent system to examine the influence of environmental variation in SM regulation, as the mechanisms for SM production have been widely studied in this group of organisms. The SM gene clusters (Inglis et al. 2013) and SM production profiles (Frisvad et al. 2009; Chiang et al. 2008) of several species are described in depth, and several master SM regulators are well characterized (Axel a Brakhage 2013). Furthermore, although variation of SM production in response to environmental cues including temperature (J. Yu et al. 2011; OBrian et al. 2007), pH (Tilburn et al. 1995; Bignell et al. 2005), light (Ö. Bayram et al. 2008), and hypoxia (Barker et al. 2012; Blatzer et al. 2011) has been observed, it has not been systematically characterized or mechanistically understood. For this study, we chose *A. fumigatus*, the most common cause of a suite of diseases known collectively as aspergillosis (Latge 1999). *A. fumigatus* produces a diverse array of SMs including the immune-suppressing SM gliotoxin, which is thought to promote its virulence (Scharf et al. 2012). Additionally, *A. fumigatus* is highly thermotolerant; it can grow at 55ºC and can survive at temperatures up to 75ºC (Ryckeboer et al. 2003; Beffa et al. 1998; Abad et al. 2010). It is unknown whether changes in temperature affect global patterns of gene expression in the secondary metabolic pathways of this opportunistic pathogen.

To test whether variation in environmental cues other than the known light response can influence Velvet complex based SM regulation in *A. fumigatus*, we examined global gene expression using RNA sequencing in response to different temperatures in wild-type, Δ*veA*, and Δ*laeA* backgrounds. We found that change in temperature had a marked impact on the expression of SM genes, and that VeA regulates the genes required for producing at least 4 SMs at 37ºC but not at 30ºC, suggesting a combinatorial interaction of temperature- and light-based regulation of secondary metabolism in *Aspergillus*.

Methods

Strains and culture conditions

Aspergillus fumigatus wild-type CEA10, Δ*veA* TDS1.15 (*pyrG1* Δ*veA*::*pyrG*$^{A.\ fum}$) (S Dhingra, Andes, and Calvo 2012) *and TSD62.1 ((pyrG1* Δ*veA*::*pyrG*$^{A.\ fum}$) (Sourabh Dhingra et al. 2013), were used in this study. Strains were stored as 30% glycerol stocks at -80°C. Conidia of *A. fumigatus* wild type, Δ*veA* and Δ*laeA* strains were inoculated in 25 ml Czapek-Dox medium (10^7/ml) and grown as stationary cultures for 72 h at either 30°C or 37°C in the dark.

RNA isolation

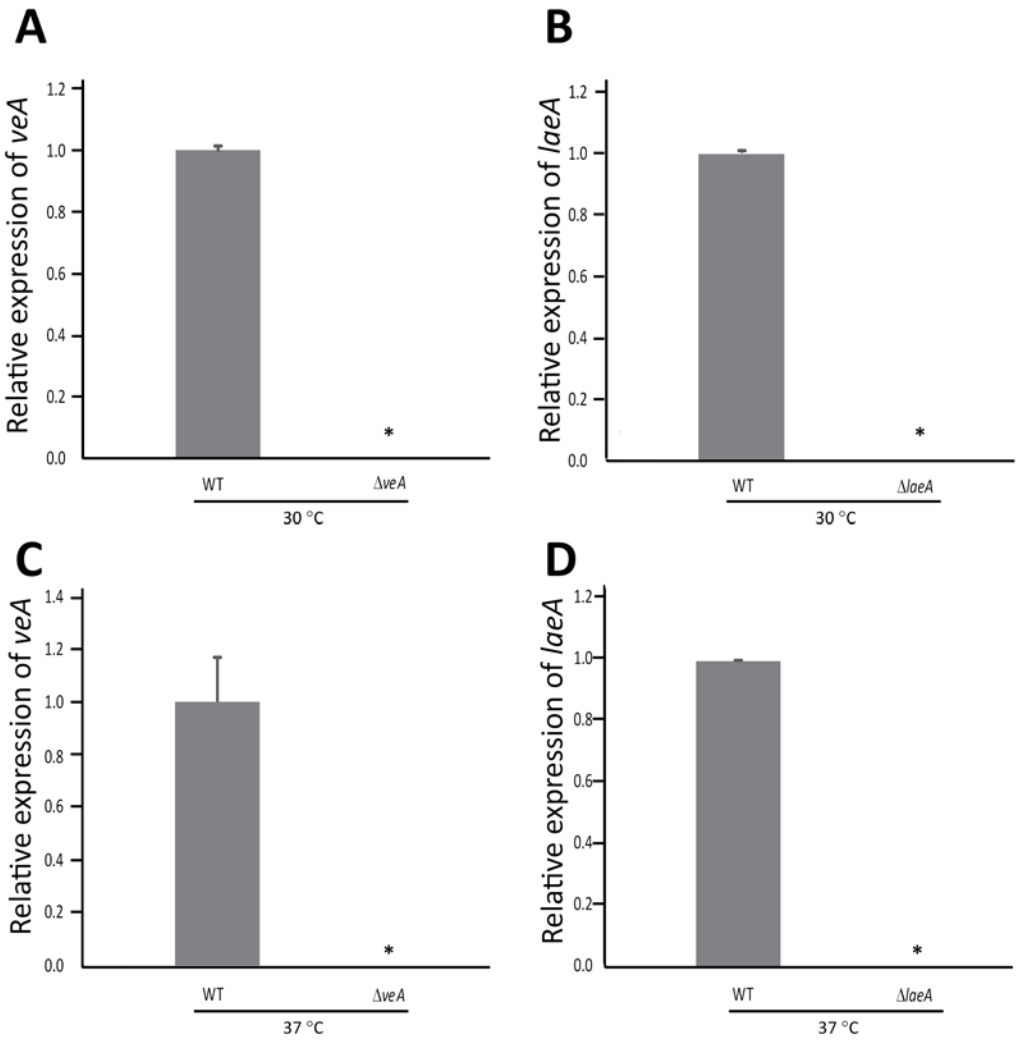

Figure 3-1. Expression analysis of veA and laeA in *A. fumigatus* wild type and respective mutants at 30° and 37° by qRT-PCR. Strains were grown on Czapek Dox medium. Expression was normalized to the wild-type. Bars represent standard error. Asterisks indicate no detection.

Mycelial mats were collected and immediately frozen in liquid nitrogen. Samples were then lyophilized and ground. Total RNA was extracted using Direct-zol™ RNA MiniPrep Kit from ZYMO following the manufacturer instructions. RNA was resuspended in autoclaved ddH_2O. Samples were stored at -80°C. Expected *veA* and *laeA* expression patterns in the wild type and corresponding deletion mutants were verified by qRT-PCR (Fig 3-1).

RNA sequencing

RNA-seq libraries were constructed and sequenced at the Vanderbilt Technologies for Advanced Genomics Core Facility at Vanderbilt University using the Illumina Tru-seq RNA sample prep kit as previously described (S Dhingra, Andes, and Calvo 2012; Lind et al. 2015). Briefly, total RNA quality was assessed via Bioanalyzer (Agilent). Upon passing quality control, poly-A RNA was purified from total RNA and second strand cDNA was synthesized from mRNA. cDNA ends were then blunt repaired and 3' ends adenylated. Barcoded adapters were ligated to the adenylated ends and the libraries were PCR enriched, quantified, pooled and sequenced an on Illumina HiSeq 2500 sequencer. Two biological replicates were generated for each strain sequenced.

Gene expression analysis

Raw RNA-seq reads were trimmed of low-quality reads and adapter sequences using Trimmomatic with the suggested parameters for single-end read trimming (Bolger, Lohse, and

Usadel 2014). After read trimming, all samples contained between 9.5-14.1 million reads, with the average sample containing 12 million reads. Trimmed reads were aligned to the *A. fumigatus* Af293 version s03_m04_r11 genome from the *Aspergillus* Genome Database (Arnaud et al. 2010; Arnaud et al. 2012). Read alignment was performed with Tophat2 using the reference gene annotation to guide alignment and without attempting to detect novel transcripts (parameter –no-novel-juncs) (D. Kim et al. 2013). Reads aligning to each gene were counted using HTSeq-count with the union mode (Anders, Pyl, and Huber 2014). Differential expression was determined using the DESeq2 R package (Michael I Love, Huber, and Anders 2014). Genes were considered differentially expressed if their Benjamini-Hochberg adjusted p-value was less than 0.1 and their log2 fold change was greater than 1 or less than -1.

Functional enrichment analysis

Functional category enrichment was determined for over- and under-expressed genes in all conditions tested using the Cytoscape plugin BiNGO (Shannon et al. 2003; Maere, Heymans, and Kuiper 2005). To allow for a high-level view of the types of differentially expressed gene sets, the Aspergillus GOSlim v1.2 term subset was used (The Gene Ontology Consortium 2014). The Benjamini-Hochberg multiple testing correction was applied and functional categories were considered significantly enriched if the adjusted p-value was less than 0.05.

Gene cluster expression

Aspergillus fumigatus secondary metabolic gene clusters were taken from a combination of computationally predicted and experimentally characterized gene clusters (Inglis et al. 2013; Lind et al. 2015). SM gene clusters were designated “differentially expressed” if half or more of the genes in the cluster were differentially expressed. As many gene clusters contained genes that were statistically significantly differentially expressed (adjusted p-value < 0.1) but did not meet

our cutoff of a $\log_2$ fold-change greater than 1 or less than -1, gene clusters with half or more genes meeting the statistical significance cutoff were considered "weakly differentially expressed". Clusters containing a mix of over- and under-expressed genes were considered to have "mixed" expression.

Data availability

All RNA sequence data files are available from the NCBI's Short Read Archive database (accession number: SRP080951).

Results

Temperature shift changes the expression of 10% of all genes and of more than half of the genes in SM gene clusters

To investigate the effect of temperature on gene expression, we compared the transcriptomes of *A. fumigatus* wild-type (WT) grown at 37ºC compared to WT grown at 30ºC. This comparison identified 1,101 differentially expressed genes ($|\log_2$ fold-change$| > 1$, adjusted p-value < 0.1), which corresponds to more than 10% of the *A. fumigatus* transcriptome. Of these genes, 402 were expressed at a higher degree (over-expressed) and 699 genes were expressed at a lower degree (under-expressed) at 37ºC than at 30ºC. Genes over-expressed at 37ºC were enriched (adjusted p-value < 0.05) for the functional categories CARBOHYDRATE METABOLIC PROCESS and EXTRACELLULAR REGION; genes under-expressed at 37ºC were enriched for the categories CELL ADHESION, SECONDARY METABOLIC PROCESS, TOXIN METABOLIC PROCESS, and OXIDOREDUCTASE ACTIVITY (Figure 3-2A).

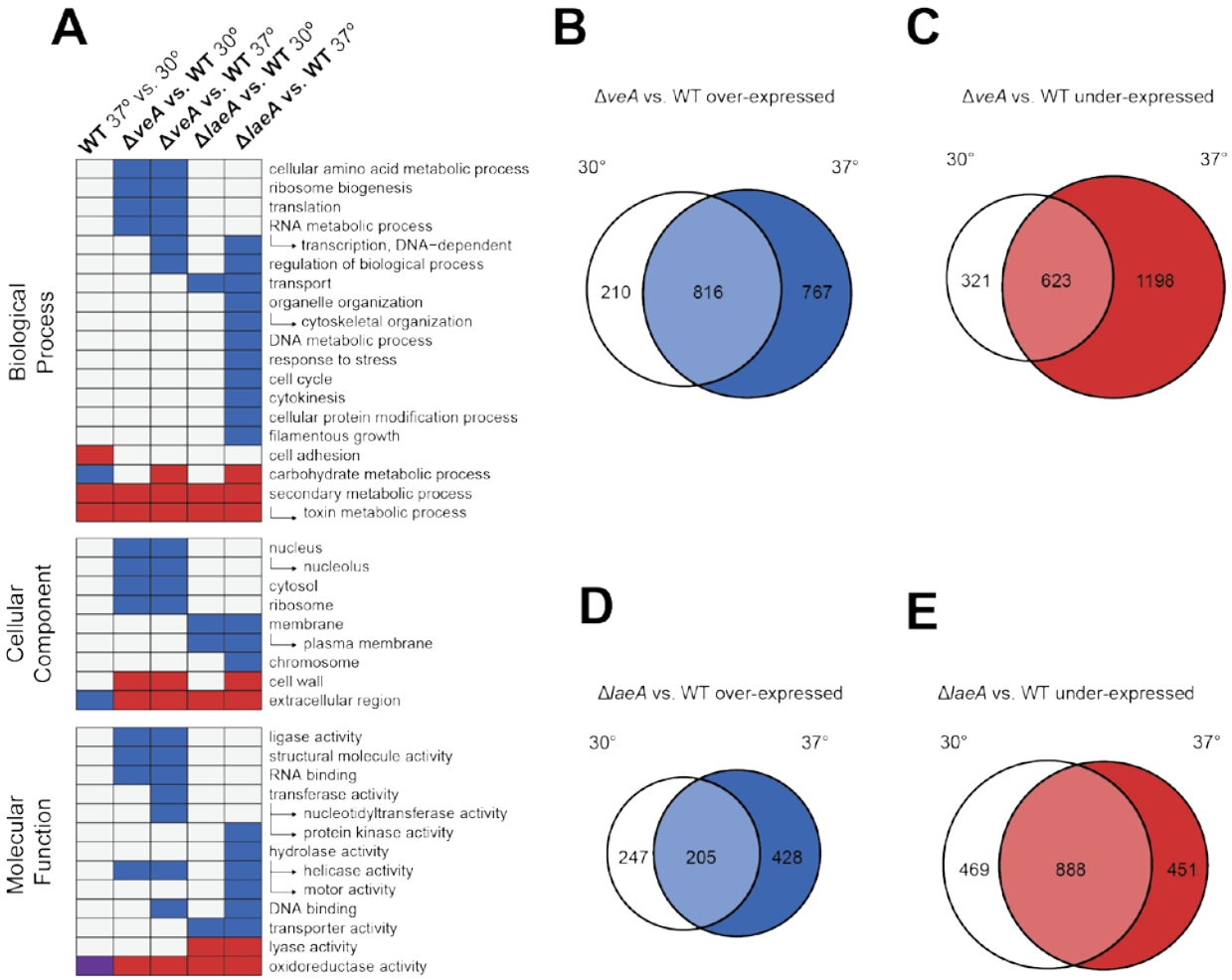

Figure 3-2. Comparison of enriched functional categories and overlapping differential gene expression. (A) Enriched functional categories for genes differentially expressed under variable temperature conditions. Red boxes indicate GOSlim terms enriched in under-expressed genes, blue boxes indicate categories enriched in over-expressed genes, and purple boxes indicate categories enriched in both over- and under-expressed genes. **(B,C)** Overlap between genes differentially expressed in ΔveA vs. WT at 30º and 37º. **(D,E)** Overlap between genes differentially expressed in ΔlaeA vs. WT at 30º and 37º.

As functional category enrichment analysis indicated that genes involved in secondary metabolism were expressed at lower levels in WT at 37ºC than at 30ºC, we next investigated the impact of temperature on expression of each of the 37 previously identified secondary metabolic gene clusters (Inglis et al. 2013; Lind et al. 2015). We found that half or more of the genes in 13 gene clusters were expressed at lower levels at 37ºC than at 30ºC, including the clusters encoding the conidial melanin pigment, fumigaclavine, endocrocin, trypacidin, fumipyrrole, gliotoxin, fumiquinazoline, fumitremorgin, fumagillin, pseurotin and three gene clusters that do not encode known products (cluster 15, cluster 30, and cluster 35) (Figure 3-4). As previous analysis has shown that endocrocin is not produced at temperatures above 35ºC, these results indicate that this is attributable to changes in gene expression (Berthier et al. 2013).Three other gene clusters that do not encode known products, namely cluster 21, cluster 25, and cluster 36, were over-expressed at 37ºC (Figure 3-4). Additionally, half or more genes in 6 gene clusters (cluster 5, cluster 6, cluster 18, cluster 23, cluster 28, and cluster 31) were differentially expressed, but contained a mixture of both over- and under-expressed genes; none of these gene clusters encode known products.

VeA regulates a much large number of genes at 37ºC than at 30ºC

To investigate how temperature influences VeA's role in controlling gene expression, we compared the transcriptomes of a Δ*veA* strain with WT grown at either 37ºC or 30ºC. In agreement with previous studies (Sourabh Dhingra et al. 2013; Lind et al. 2015), we found a very large number (3,404) of differentially expressed genes in Δ*veA* at 37ºC, with 1,821 over-expressed genes and 1,583 under-expressed genes. Many fewer genes were differentially expressed in the Δ*veA* strain at 30ºC. Specifically, 1,986 genes were differentially expressed in

Δ*veA*, with 1,026 genes over-expressed and 960 genes under-expressed. A comparison of the 3,404 differentially expressed genes at 37ºC with the 1,986 differentially expressed genes at 30ºC revealed that a subset of 1,439 genes was differentially expressed in Δ*veA* at both temperatures, suggesting that their regulation by VeA is temperature independent (Figure 3-2B). However, while 518 genes were differentially expressed solely at 30ºC, almost four times as many genes (1,935) were differentially expressed solely at 37ºC; these results indicate that the regulatory impact of VeA is much greater at 37ºC than at 30ºC.

To determine the functions of differentially expressed genes in Δ*veA* vs WT at 30ºC and 37ºC we performed functional category enrichment analyses. Over-expressed genes in Δ*veA* were enriched for functional categories relating to transcription and translation activity at both 30ºC and 37ºC, while the categories DNA-DEPENDENT TRANSCRIPTION, TRANSFERASE ACTIVITY, NUCLEOTIDYLTRANSFERASE ACTIVITY, REGULATION OF BIOLOGICAL PROCESS, and DNA BINDING were only enriched at 37ºC (Figure 3-2A). Further, the number of over-expressed genes in each category was higher for all significantly enriched categories at 37ºC, with the exceptions of LYASE ACTIVITY, CYTOSKELETAL ORGANIZATION, and MOTOR ACTIVITY which were unchanged.

Genes under-expressed in Δ*veA* were enriched for functional categories related to secondary metabolism, including SECONDARY METABOLIC PROCESS and TOXIN METABOLIC PROCESS. The only significantly enriched category for genes under-expressed in Δ*veA* at 37ºC that was not enriched for genes under-expressed at 30ºC was CARBOHYDRATE METABOLIC PROCESS (Figure 3-2A). The number of under-expressed genes annotated to each functional category was higher at 37ºC, with the exception of RIBOSOME, CYTOSKELETAL ORGANIZATION, and CELL ADHESION which remained unchanged. These enrichment analyses indicate that though

many more genes are differentially expressed in Δ*veA* at 37ºC, VeA is regulating similar categories of genes at both temperatures.

LaeA regulates similar numbers and types of genes at 30ºC and 37ºC

To investigate how temperature influences LaeA's role in gene regulation, we compared the transcriptomes of a Δ*laeA* strain with WT grown at either 37ºC or 30ºC. While Δ*veA* strains showed temperature-dependent differences in the number of differentially expressed genes, Δ*laeA* strains showed similar numbers of differentially expressed genes at both 30ºC and 37ºC. In total, 1,971 genes were differentially expressed in Δ*laeA* strains as compared to WT at 37ºC (632 over-expressed and 1,339 under-expressed), while 1,809 genes were differentially expressed at 30ºC (452 over-expressed and 1,357 under-expressed). There was moderate overlap of the sets of differentially expressed genes at the two temperatures; 1,109 genes were differentially expressed in Δ*laeA* at both temperatures, while 770 and 862 genes were only differentially expressed at 30ºC and 37ºC, respectively (Figure 3-2C).

To identify the functions of genes differentially expressed in the Δ*laeA* strain compared to WT at 37ºC and 30ºC, we performed functional category enrichment analyses. Genes over-expressed at both 37ºC and 30ºC in Δ*laeA* were enriched for the categories TRANSPORT, TRANSPORTER ACTIVITY, MEMBRANE, and PLASMA MEMBRANE. However, genes over-expressed at 37ºC were enriched for an additional 14 functional categories related to cell division, filamentous growth, and DNA metabolism that were not enriched in genes over-expressed at 30ºC (Figure 3-2A). Under-expressed genes at both 30ºC and 37ºC were enriched for categories relating to secondary metabolism, in agreement with LaeA's well-documented role as a master regulator of secondary metabolism (Bok, Hoffmeister, et al. 2006; O. Bayram and Braus 2012).

Two functional categories, CARBOHYDRATE METABOLISM and CELL WALL were enriched for under-expressed genes at 30ºC but not 37ºC.

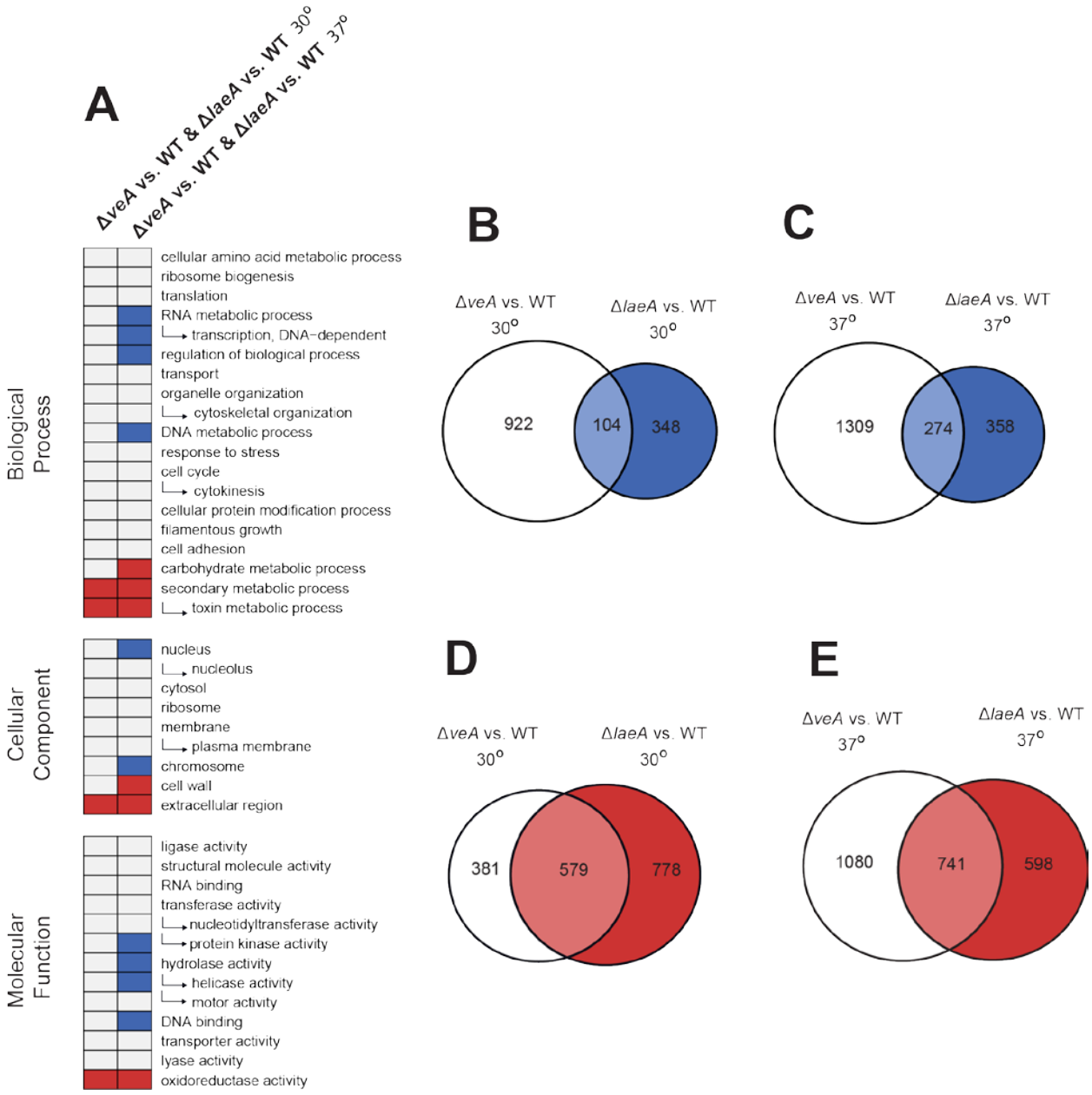

Figure 3-3. Comparison of enriched functional categories and differential gene expression in ΔveA and ΔlaeA strains at 30º and 37º. (A) Enriched functional categories for genes differentially expressed in both ΔveA vs. WT and in ΔlaeA vs. WT at either 30º

or 37º. Red boxes indicate GOSlim terms enriched in under-expressed genes, blue boxes indicate categories enriched in over-expressed genes, and purple boxes indicate categories enriched in both over- and under-expressed genes. **(B)** Overlap between genes over-expressed in ΔveA vs. WT and ΔlaeA vs. WT at 30º. **(C)** Overlap between genes over-expressed in ΔveA vs. WT and ΔlaeA vs. WT at 37º. **(D)** Overlap between genes under-expressed in ΔveA vs. WT and ΔlaeA vs. WT at 30º. **(E)** Overlap between genes under-expressed in ΔveA vs. WT and ΔlaeA vs. WT at 37º.

VeA and LaeA have greater regulatory overlap at 37ºC than at 30ºC

As VeA and LaeA are both members of the Velvet complex and are known to interact, it is very likely that they exhibit substantial overlap in the genes they regulate (A. M. Calvo 2008). To examine the effect of temperature on this regulatory overlap, we determined the intersection of genes differentially expressed in Δ*veA* versus WT and in Δ*laeA* versus WT at 30ºC and 37ºC. In total, 741 genes were under-expressed in both Δ*veA* and Δ*laeA* at 37ºC (this number corresponds to 41% of all under-expressed genes in Δ*veA* and to 55% of all under-expressed genes in Δ*laeA*) and 579 genes were under-expressed in both Δ*veA* and Δ*laeA* at 30ºC (41% of all under-expressed genes in Δ*veA* and 55% of all under-expressed genes in Δ*laeA*) (Figure 3-2B,C). The 741 genes under-expressed at 37ºC were significantly enriched for the functional categories SECONDARY METABOLIC PROCESS, OXIDOREDUCTASE ACTIVITY, EXTRACELLULAR REGION, TOXIN METABOLIC PROCESS, CELL WALL, and CARBOHYDRATE METABOLIC PROCESS (Figure 3-3A). The 579 genes under-expressed at 30ºC were also enriched for the functional categories SECONDARY METABOLIC PROCESS, OXIDOREDUCTASE ACTIVITY, EXTRACELLULAR

REGION, and TOXIN METABOLIC PROCESS, but not for the CELL WALL and CARBOHYDRATE METABOLIC PROCESS categories (Figure 3-3A).

In total, 274 genes were over-expressed in both Δ*veA* and Δ*laeA* at 37ºC (17% of all genes over-expressed in Δ*veA* and 43% of all genes over-expressed in Δ*laeA*), while 104 genes were over-expressed in both Δ*veA* and Δ*laeA* at 30ºC (10% of all genes over-expressed in Δ*veA* and 23% of all genes over-expressed in Δ*laeA*) (Figure 3-2C,D). Enrichment of functional categories in genes that were over-expressed in Δ*veA* and Δ*laeA* was strikingly different at 30ºC and 37ºC. Although the categories RNA METABOLIC PROCESS, DNA BINDING, HELICASE ACTIVITY, NUCLEUS, DNA-DEPENDENT TRANSCRIPTION, REGULATION OF BIOLOGICAL PROCESS, PROTEIN KINASE ACTIVITY, CHROMOSOME, HYDROLASE ACTIVITY, and DNA METABOLIC PROCESS were significantly enriched in genes over-expressed in both Δ*veA* and Δ*laeA* at 37ºC (Figure 3-2A), no functional categories were significantly enriched at 30ºC. Because fewer genes were over-expressed in both Δ*veA* and Δ*laeA* at either temperature than were under-expressed, and many more genes were over-expressed in VeA's absence than in LaeA's absence, these results suggest that LaeA may primarily function as a positive regulator of gene expression.

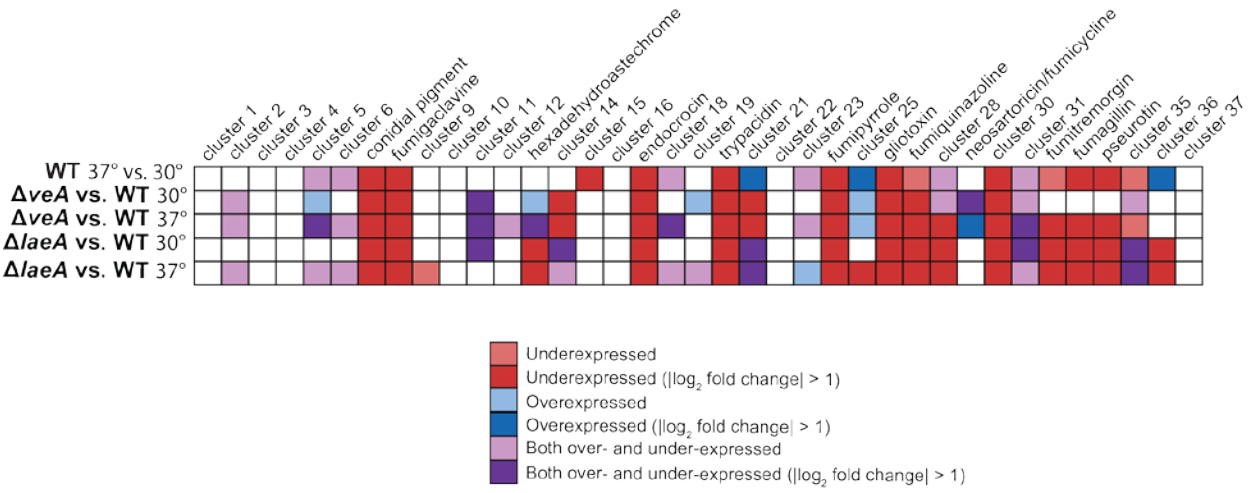

Figure 3-4. Differential expression of SM gene clusters in all conditions. Dark red boxes indicate half or more genes are underexpressed, dark blue boxes indicate half or more genes are overexpressed, and dark purple boxes indicate that half or more genes are a combination of overexpressed and underexpressed genes. Light-colored boxes indicate that half or more genes in that gene cluster meet the statistical significance cutoff for differential expression but have less than a twofold change in expression.

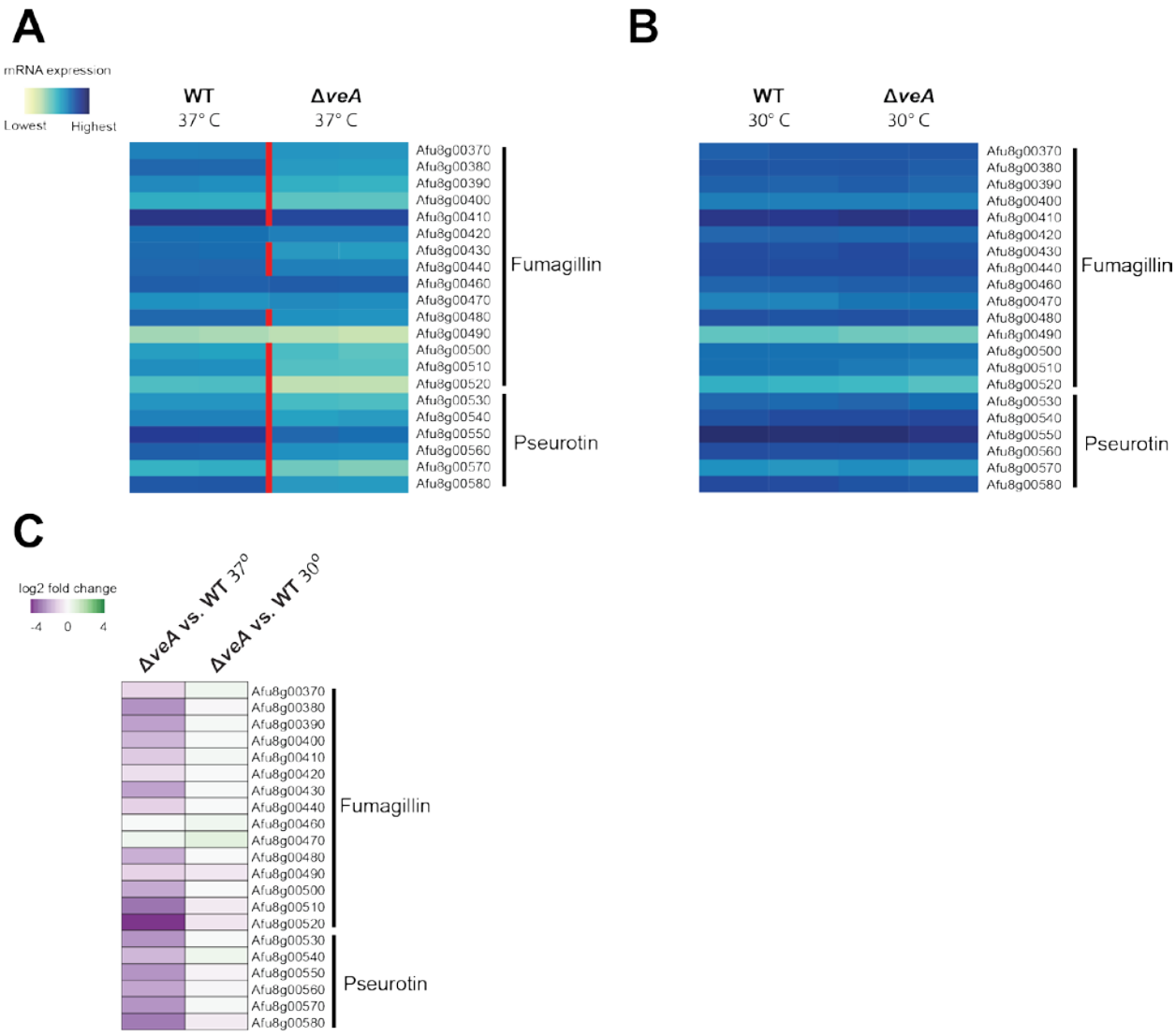

Figure 3-5. Expression and differential expression of the fumagillin and pseurotin clusters in wild-type and ΔveA at 37º and 30º. (A, B) Expression in panels A and B is shown as the regularized log transformation of the number of RNA-seq reads aligning to that gene as implemented in DESeq2. Genes that are differentially expressed between conditions are separated by a red line if under-expressed in ΔveA or yellow line if over-expressed in ΔveA. **(C)** log2 fold change of all genes in the fumagillin and pseurotin gene clusters between ΔveA vs. WT at 37º and 30º.

Many SM gene clusters are regulated by both VeA and LaeA at 37ºC, but only by LaeA at 30ºC

We expected that SM clusters regulated by the Velvet complex, comprised of the VelB, VeA, and LaeA proteins (Ö. Bayram et al. 2008), would require both VeA and LaeA for wild-type levels of expression. SM gene clusters not controlled by this protein complex, however, may not show differential gene expression in Δ*veA* or Δ*laeA* strains or may be differentially expressed in only one strain. At 37ºC, 12 SM gene clusters were under-expressed in both Δ*veA* and Δ*laeA*, suggesting that they may be regulated by the Velvet protein complex; these clusters include DHN melanin pigment, fumigaclavine, endocrocin, trypacidin, fumipyrrole, gliotoxin, fumiquinazoline, cluster 28, cluster 30, fumitremorgin, fumagillin, and pseurotin (Figure 3-4). Interestingly, 6 of these SM gene clusters were either normally expressed in Δ*veA* at 30ºC or had much less of a change from wild-type expression, suggesting that VeA's regulatory role may be temperature-dependent. These clusters include DHN melanin pigment, fumiquinazoline, cluster 28, fumitremorgin, fumagillin, and pseurotin (Figure 3-4; Figure 3-5; Figure 3-6). Furthermore, clusters expressed more highly at 37ºC than at 30ºC in WT *A. fumigatus* were also often under-

expressed in Δ*veA* and Δ*laeA* strains; of the 12 clusters under-expressed in both Δ*veA* and Δ*laeA* strains at 37ºC (Figure 3), 11 were expressed at higher levels in WT at 30ºC than at 37ºC. The exception was cluster 28, which was under-expressed in both Δ*veA* and Δ*laeA* strains at 37ºC but was expressed at similar levels in WT at 30ºC and 37ºC.

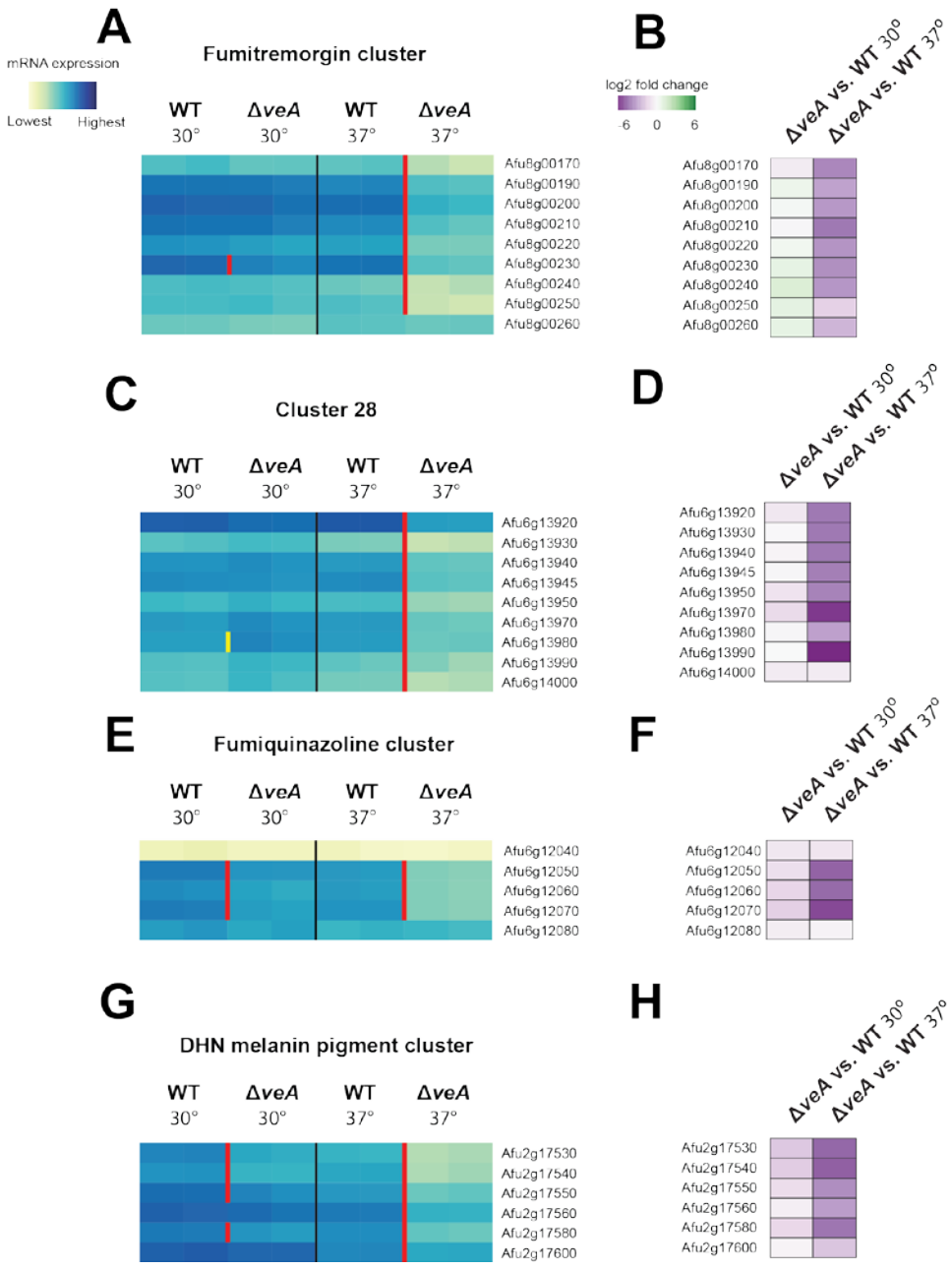

Figure 3-6. Expression and differential expression of gene clusters showing a higher change in gene expression in ΔveA 37º than at 30º. (A,B) Fumitremorgin cluster, **(C,D)**

cluster 28, **(E,F),** fumiquinazoline cluster, and **(G,H)** DHN melanin pigment clusters in wild-type and ΔveA at 37º and 30º. Expression **(A,C,E,G)** is represented as the regularized log transformation of the number of RNA-seq reads aligning to that gene as implemented in DESeq2. Genes that are differentially expressed between conditions are separated by a red line if under-expressed in ΔveA or yellow line if over-expressed in ΔveA. Differential expression between ΔveA and wild-type is also shown as log2 fold change **(B,D,F,H).**

Several clusters were differentially expressed in either Δ*laeA* or Δ*veA*, but not in both. The hexadehydroastechrome cluster, while under-expressed in Δ*laeA* at both 30ºC and 37ºC, was over-expressed in Δ*veA* at 30ºC and showed mixed expression in Δ*veA* at 37ºC (Figure 3-4). Two gene clusters, cluster 14 and cluster 21, were under-expressed in Δ*veA* at both temperatures but showed mixed expression in Δ*laeA*. Finally, the neosartoricin/fumicycline cluster, which was very lowly expressed in WT at both 30ºC and 37ºC, contained some up-regulated genes in Δ*veA* at both temperatures, but showed no change in expression in Δ*laeA* strains. The expression patterns of these gene clusters indicate that, although VeA and LaeA play roles in their regulation, these proteins may in some cases be acting independently of each other.

Discussion

Production of secondary metabolites in *A. fumigatus* and other filamentous fungi is triggered by diverse environmental cues such as temperature, pH, and nutrient sources, and several master SM regulators that respond to these cues have been identified. However, the extent to which master SM regulators can integrate multiple environmental cues to regulate SM production is not known. Considered together, our findings that temperature regulates global SM

production in *A. fumigatus* and that the light-responsive master SM regulator VeA is also responsive to changes in temperature, provide support for the hypothesis that regulation of SM production in response to environmental cues is combinatorial.

Growth at 37ºC compared to growth to 30ºC had a marked impact on gene expression in *A. fumigatus* WT, significantly changing the expression levels of ~10% of its genes. Importantly, genes involved in secondary metabolism were disproportionately affected (Fig 3-2A); 13 of the total 37 SM gene clusters were expressed at higher levels at 30ºC than 37ºC, while three clusters were expressed at lower levels at 30ºC (Fig 3-4A). These results are in accordance with studies in *A. flavus* that find a global pattern of higher SM cluster expression at 30ºC than at 37ºC, the optimal temperature for growth in both fungi (J. Yu et al. 2011). Our findings indicate that temperature plays a significant role in secondary metabolism expression, though as *A. fumigatus* grows more rapidly at 37ºC than at 30ºC we cannot rule out that other growth-related factors (e.g., differential cell density) might also play a role in the gene expression changes we observe.

To elucidate the possible interactions between environmental conditions including temperature change to light-responsive SM production, we exposed deletion strains of genes encoding two key members of the Velvet protein complex, *veA* and *laeA*, to different temperature conditions. At 37ºC, the optimal temperature for *A. fumigatus* growth, we find that VeA and LaeA are both involved in regulating genes in many SM gene clusters. While the lists of which genes are parts of the known SM gene clusters are not identical to the lists used in previous analyses of LaeA's regulatory role of controlling secondary metabolism, our RNA-seq results generally agree with previously published microarray data (Perrin et al. 2007). One notable difference from previous reports is our finding that a putative terpene-producing cluster on chromosome 5 (Afu5g00100-00135) is under LaeA regulation. Further, our findings that VeA

transcriptionally regulates many gene clusters agrees with chemical data that shows that VeA is required for the synthesis of fumagillin, fumitremorgin, and fumigaclavine at 37ºC (Sourabh Dhingra et al. 2013).

The sets of genes that are increased in VeA and LaeA's absence do not show broad overlap in their functions (Figure 3-1A), suggesting that VeA and LaeA's regulatory roles are distinct from each other. This inference is further supported by the observation of 6 SM gene clusters that are differentially regulated by VeA but not by LaeA at different temperatures. The Velvet protein complex formed by LaeA, VeA, and VelB has been implicated as a regulator of secondary metabolism in many fungi (O. Bayram and Braus 2012; Chettri et al. 2012; Wiemann et al. 2010; Ö. Bayram et al. 2008; A. M. Calvo 2008), and our findings provide additional evidence that the LaeA and VeA have functionally distinct roles in regulating SM clusters (O. Bayram and Braus 2012; Lin et al. 2013).

Our finding that VeA's regulation of SM gene clusters is temperature-dependent raises the hypothesis that in addition to its critical role in controlling dark-responsive secondary metabolism by localizing in the nucleus under dark conditions and to a lesser degree under light conditions (Ö. Bayram et al. 2008), VeA may also be involved in controlling the response to temperature. Interestingly, previous work in *A. nidulans* has shown that glucose concentration influences both VeA's subcellular localization and sterigmatocystin production, altering the effect of light on the biosynthesis of this mycotoxin (Atoui et al. 2010); thus, light and temperature might just be two of the many environmental cues that VeA responds to.

How might VeA, a single protein, mediate such a diversity of regulatory controls on multiple SM gene clusters in response to several different environmental cues? One possibility is that VeA's regulatory diversity is mediated through the protein's multiple interaction partners.

VeA forms a heterodimer with another velvet family protein, VelB, and both proteins are necessary for sexual fruiting body formation in *A. nidulans*. Many of VeA's interacting partners impact its subcellular localization. For example, in *A. nidulans* VeA interacts with the methyltransferases LlmF and the VipC-VapB heterodimer, which respectively increase and repress VeA's nuclear import (Palmer et al. 2013; O. Sarikaya-Bayram et al. 2014). VeA is also known to interact directly with the red light sensing protein FphA and therefore indirectly with the blue light sensing White Collar homologs LreA and LreB, which may modulate VeA's light responsive capabilities and subcellular location as well as potentially playing roles in glucose response (Purschwitz et al. 2008; Purschwitz, Müller, and Fischer 2009; Atoui et al. 2010; Ö. Sarikaya-Bayram et al. 2015). Another possible mechanism explaining VeA's multi-faceted role is offered by recent experiments in *A. nidulans* showing that phosphorylation of different combinations of residues of VeA generates distinct phenotypes, including changes in sterigmatocystin production (Rauscher et al. 2015).

Irrespective of what the precise molecular mechanism(s) contribute to VeA's diverse array of regulatory controls, the emerging picture from recent studies including this one is that VeA is integrating multiple environmental signals including light (Ö. Bayram et al. 2008), glucose (Atoui et al. 2010), nitrogen source (López-Berges et al. 2014), and temperature (this study), allowing filamentous fungi to modulate cellular processes including secondary metabolism in response to changing environments.

CHAPTER IV

TRANSCRIPTIONAL REGULATORS OF ASEXUAL DEVELOPMENT CONTROL BOTH SPORE AND HYPHAL SECONDARY METABOLIC PATHWAYS IN ASPERGILLUS FUMIGATUS

Authors

Abigail L. Lind, Fang Yun Lim, Nancy P. Keller, Antonis Rokas

Introduction

Filamentous fungi produce a diverse array of small molecule secondary metabolites that with diverse ecological roles in fungal pathogenicity, communication, and defense. These secondary metabolites (SMs) are typically produced by pathways organized into contiguous gene clusters (SMGCs), which is atypical of metabolic pathways in most eukaryotes (Keller, Turner, and Bennett 2005). Secondary metabolites are fast evolving, and they are often shared by only a small number of fungal species (J. Bennett and Bentley 1989). Transcriptional regulators of secondary metabolism, in contrast, are typically well conserved. Master secondary metabolic regulators respond to a variety of environmental signals including pH, temperature, light, and nutrient sources to transcriptionally regulate SMGCs (Axel a Brakhage 2013).

Secondary metabolism and development in *Aspergillus* species and other fungi are tightly linked (A. M. Calvo et al. 2002). In fact, many of the environmental signals that regulate SM production including temperature, pH, and carbon or nitrogen sources, also trigger the onset of asexual and sexual development (A. M. Calvo et al. 2002). Several SMs are specifically

localized or produced in differentiated asexual tissues, such as the DHN melanin pigment integrated in the cell wall of *Aspergillus fumigatus* conidia (Keller 2015).

Asexual developmental tissues in *A. fumigatus* are linked with multiple secondary metabolites with spore-protective properties, including the DHN melanin pigment, endocrocin, and fumiquinazoline (Keller 2015; Lim et al. 2014). In *Aspergillus*, the central asexual development pathway is controlled by three central regulatory genes expressed at specific time points in developing tissues (J.-H. Yu 2010). The founding member of this regulatory cascade, BrlA, accumulates in vegetative cells shortly before asexual development (Park and Yu 2016). In the middle stages of conidiation, BrlA activates expression of AbaA, which controls conidiophore development. AbaA then activates WetA in late stages of development, which is required for critical cell wall components of conidia (Park and Yu 2016). Recent evidence suggests that these regulators may play roles in the expression of both spore-specific, and in one case vegetative-cell specific, SM gene clusters (Lim et al. 2014; Shin, Kim, and Yu 2015; Twumasi-Boateng et al. 2009). Further, evidence suggests all three regulators are critical for vegetative growth, including hyphal branching and cell death (Tao and Yu 2011).

To determine whether the central asexual developmental pathway BrlA→AbaA→WetA is involved in regulating secondary metabolism generally in *Aspergillus fumigatus*, we performed RNA sequencing and metabolomic analyses of on Δ*brlA*, Δ*abaA*, Δ*wetA* and wild-type *A. fumigatus*. Further, we demonstrate a role for BrlA in regulating secondary metabolites associated with asexual tissues as well as those that are produced primarily by vegetative cells. This work adds to the evidence that the fast evolving and species-specific process of secondary metabolism is regulated in tandem with conserved processes by ancient, highly conserved regulatory networks.

Methods

Growth conditions and RNA isolation

All strains were inoculated into liquid YPD media and incubated at 37 ^{o}C for 18 hours to ensure developmental competence and synchronization. Mycelia were harvested, washed, and transferred to either liquid GMM or liquid RPMI media and incubated at 29 ^{o}C. Tissue was harvested after 48 hours post transfer and RNA was extracted using the Qiagen RNeasy Plant Mini Kit.

RNA sequencing

RNA-seq libraries were constructed and sequenced at the Genomic Services Lab of Hudson Alpha (Huntsville, Alabama). Libraries were constructed with the Illumina TruSeq Stranded mRNA Library Prep Kit (Illumina) and sequenced on an Illumina HiSeq 2500 sequencer. Two biological replicates were generated for each strain sequenced.

Differential gene expression analysis

Raw RNA-seq reads were trimmed of low-quality reads and adapter sequences using Trimmomatic with the suggested parameters for paired-end read trimming (Bolger, Lohse, and Usadel 2014). After read trimming, all samples contained between 19-49 million read pairs, with the average sample containing 28 million reads. Trimmed reads were aligned to the *A. fumigatus* Af293 version s03_m04_r11 genome from the *Aspergillus* Genome Database (Arnaud et al. 2010; Arnaud et al. 2012). Read alignment was performed with Tophat2 using the reference gene annotation to guide alignment and without attempting to detect novel transcripts (parameter –no-

novel-juncs) (D. Kim et al. 2013). Reads aligning to each gene were counted using HTSeq-count with the union mode (Anders, Pyl, and Huber 2014). Differential expression was determined using the DESeq2 software (Michael I Love, Huber, and Anders 2014). Genes were considered differentially expressed if their Benjamani-Hochberg adjusted p-value was less than 0.1.

Functional enrichment analysis

Functional category enrichment was determined for differentially expressed genes in all conditions tested using the Cytoscape plugin BiNGO (Shannon et al. 2003; Maere, Heymans, and Kuiper 2005). To allow for a high-level view of the types of differentially expressed gene sets, the Aspergillus GOSlim v1.2 term subset was used (The Gene Ontology Consortium 2014). The Benjamini-Hochberg multiple testing correction was applied and functional categories were considered significantly enriched if the adjusted p-value was less than 0.05.

Gene cluster expression

A. fumigatus secondary metabolic gene clusters were taken from a combination of computationally predicted and experimentally characterized gene clusters (Inglis et al. 2013; Bignell et al. 2016) SM gene clusters were designated differentially expressed if half or more of the genes in the cluster were differentially expressed. Gene clusters were designated over-expressed if half or more of the genes in the cluster were over-expressed or were designated under-expressed if half or more of the genes in the cluster were under-expressed. Gene clusters where half or more of the genes in the cluster were differentially expressed but did not have half or more genes being either over-expressed or under-expressed were designated mixed expression.

Results

Genome-wide transcriptional impact of BrlA, AbaA, and WetA

Table 4-1. Differential gene expression in Δ*brlA*, Δ*abaA*, Δ*wetA* and wild-type during growth on GMM and RPMI media

Expression	WT RPMI vs. GMM	ΔbrlA GMM	ΔbrlA RPMI	ΔabaA GMM	ΔabaA RPMI	ΔwetA GMM	ΔwetA RPMI
Over-expressed	3305	3358	2391	1148	309	1192	1241
Under-expressed	3429	3380	2474	747	208	966	996

To determine the genome-wide regulatory roles of asexual developmental regulators, RNA sequencing was performed on Δ*brlA*, Δ*abaA*, Δ*wetA A. fumigatus* strains grown on both GMM and RPMI media. Growth on these different media sources caused dramatic differences in gene expression, particularly with respect to secondary metabolism, in wild-type fungi. Strains grown on GMM grow in the vegetative state for a prolonged period, while strains grown on RPMI begin producing spores faster than strains grown on GMM. In total, 3305 genes were expressed more highly during RPMI growth and 3429 genes were expressed more highly during GMM growth, comprising 69% of all protein-coding genes in *A. fumigatus* (Table 4-1). Genes expressed more highly during growth on GMM than on RPMI were significantly enriched for 45 functional categories related to processes including growth, development, and lipid metabolism. Genes expressed more highly during growth on RPMI than on GMM were enriched for significantly enriched for six functional categories, including oxidoreductase activity, peroxisome, hydrolase activity, carbohydrate metabolic process, lipase activity, and DNA binding.

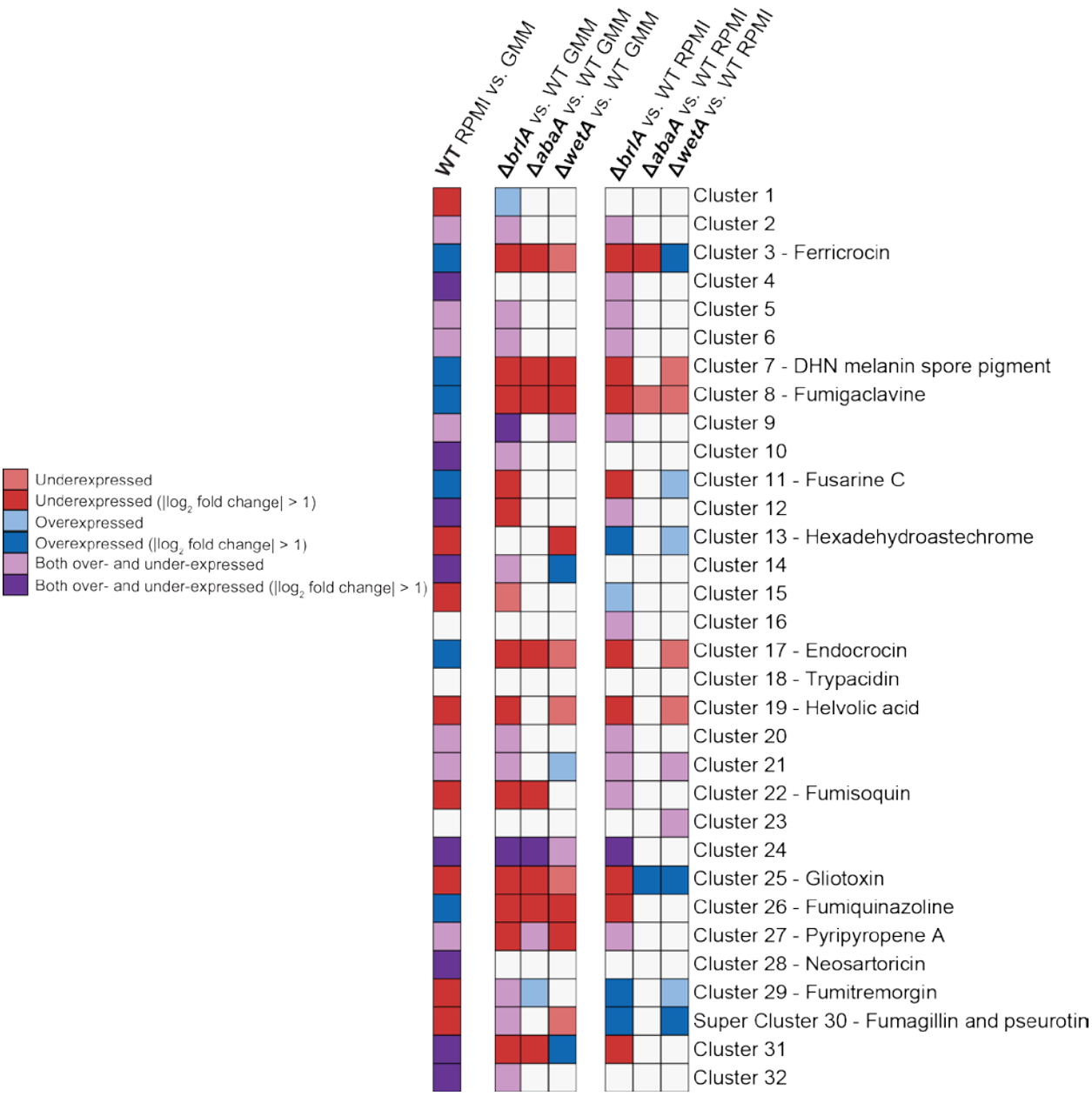

Figure 4-1. Expression of all secondary metabolic gene clusters in Aspergillus fumigatus in all conditions tested.

A large portion of the 32 secondary metabolic gene clusters (SMGCs) were expressed at different levels on different media conditions (Figure 4-1). Several SMGCs associated with asexual reproduction, including DHN melanin, fumigaclavine, and endocrocin, were expressed more highly during growth on RPMI. As *A. fumigatus* sporulates rapidly on RPMI media, these

differences are likely caused by a higher amount of conidia present in strains grown on RPMI. Other metabolites, such as gliotoxin and fumisoquin, are expressed at much higher levels on GMM than on RPMI media. During growth on GMM, all genes in the gliotoxin and fumisoquin gene clusters are expressed on average 32-fold more highly than they are on RPMI media.

A large number of the 9,784 genes in the *A. fumigatus* genome were differentially expressed in Δ*brlA* strains during growth on GMM and during growth on RPMI, comprising 6,738 differentially expressed genes (3,358 over-expressed and 3,380 under-expressed) and 4,865 genes (2,391 over-expressed and 2,474 under-expressed) respectively (Table 4-1). Fewer genes were differentially expressed in the Δ*abaA* and Δ*wetA* strains, where roughly 20% of all protein-coding genes were differentially expressed. The Δ*abaA* RPMI biological replicates were less similar to each other than all other pairs of biological replicates (Figure 4-2), and it is an outlier with only 517 differentially expressed genes due to lower statistical power (Figure 4-2**)**.

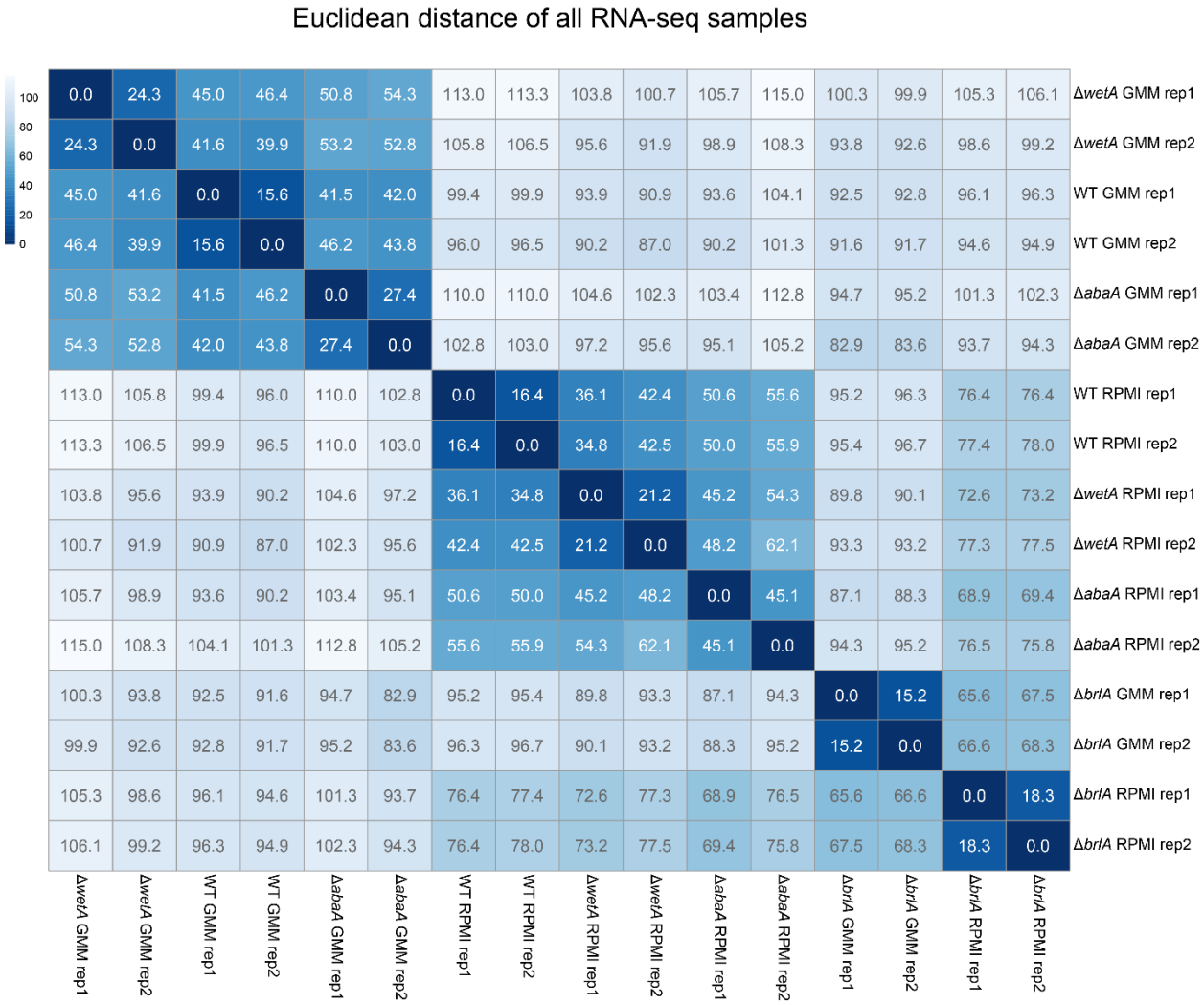

Figure 4-2. Heatmap of Euclidean distance between all RNA-seq samples.

Genes under-expressed in Δ*brlA* during growth on GMM and on RPMI were enriched for similar functional categories, including SECONDARY METABOLISM, DEVELOPMENTAL PROCESS, and ASEXUAL SPORULATION (Figure 4-3A). Categories only enriched during growth on GMM included RIBOSOME BIOGENESIS, TRANSLATION, CELLULAR RESPIRATION, RIBOSOME, and STRUCTURAL MOLECULE ACTIVITY. Categories only enriched during growth on RPMI included PEROXISOME and SIGNAL TRANSDUCER ACTIVITY. Genes under-expressed in Δ*abaA* during growth on GMM and on RPMI were enriched for SECONDARY METABOLIC PROCESS and

OXIDOREDUCTASE ACTIVITY (Figure 4-3B). No additional functional categories were enriched for genes under-expressed in Δ*abaA* during growth on RPMI, but four additional categories were enriched during growth on GMM (CELLULAR AMINO ACID METABOLIC PROCESS, RESPONSE TO CHEMICAL STIMULUS, TOXIN METABOLIC PROCESS, and TRANSFERASE ACTIVITY). Three categories, including SECONDARY METABOLIC PROCESS, TOXIN METABOLIC PROCESS, and OXIDOREDUCTASE ACTIVITY were enriched for genes under-expressed in Δ*wetA* during growth on GMM, but no functional categories were enriched for genes under-expressed during growth on RPMI (Figure 4-3C).

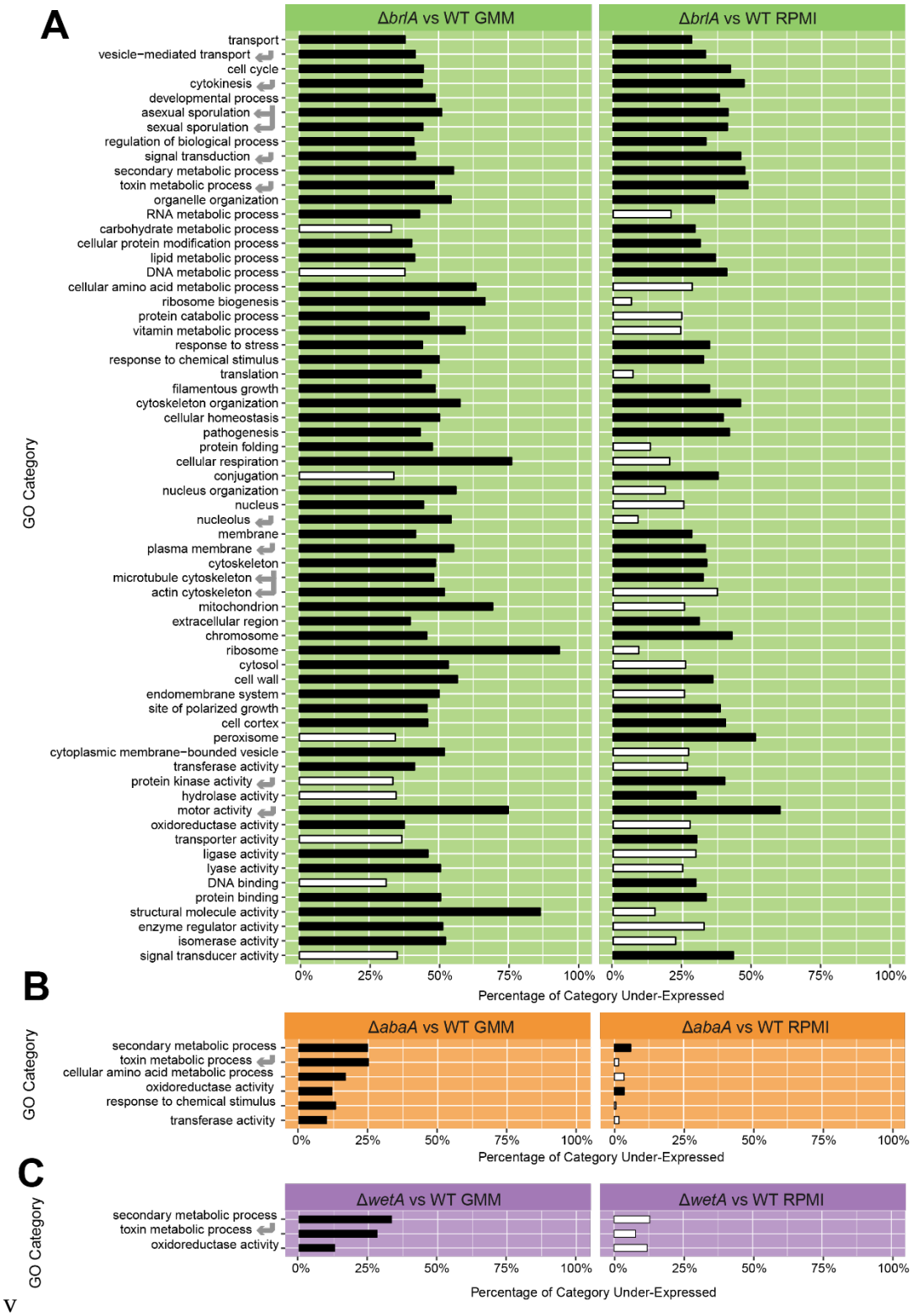

Figure 4-3. Gene Ontology enrichment analysis for genes under-expressed in **(A)** Δ*brlA*, **(B)** Δ*abaA*, and **(C)** Δ*wetA* strains as compared to wild-type.

Media-specific effects of asexual regulators

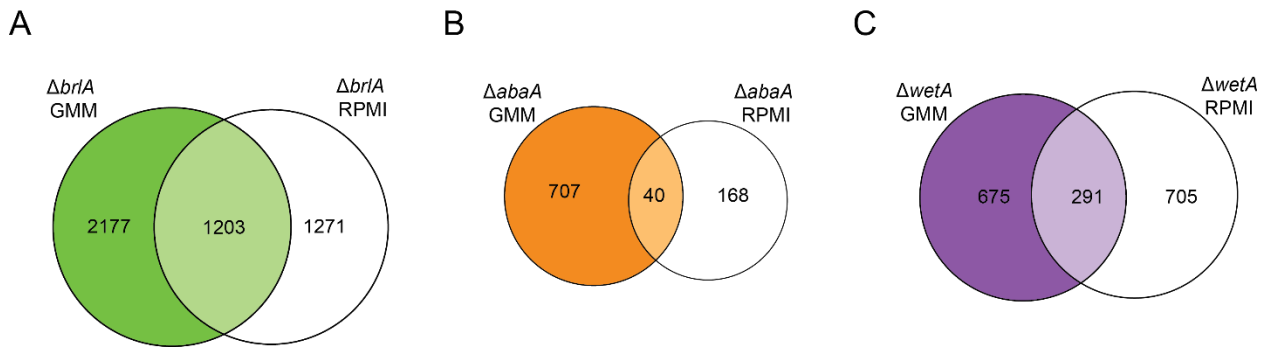

Figure 4-4. Overlap of genes under-expressed in A) ΔbrlA, (B) ΔabaA, and (C) ΔwetA as compared to wild-type during growth on GMM media versus growth on RPMI media.

To determine the extent that BrlA, AbaA, and WetA gene regulation is dependent on media conditions, we compared under-expressed genes in both GMM and RPMI conditions. In total, 1,203 genes were under-expressed in Δ*brlA* during growth on both GMM and RPMI (Figure 4-4A). However, many genes were only under-expressed relative to wild-type during growth on GMM (2,177 genes) or on RPMI (1,271 genes). Similar results were observed for Δ*abaA* and Δ*wetA* (Figure 4-4B,C). In total, 40 genes were under-expressed in Δ*abaA* during growth on RPMI and on GMM, while 707 were only differentially expressed during growth on GMM and 168 were only differentially expressed during growth on RPMI. Finally, 291 genes were under-expressed in Δ*wetA* during growth on both types of media, while 675 were only under-expressed on GMM and 705 were only under-expressed during growth on RPMI.

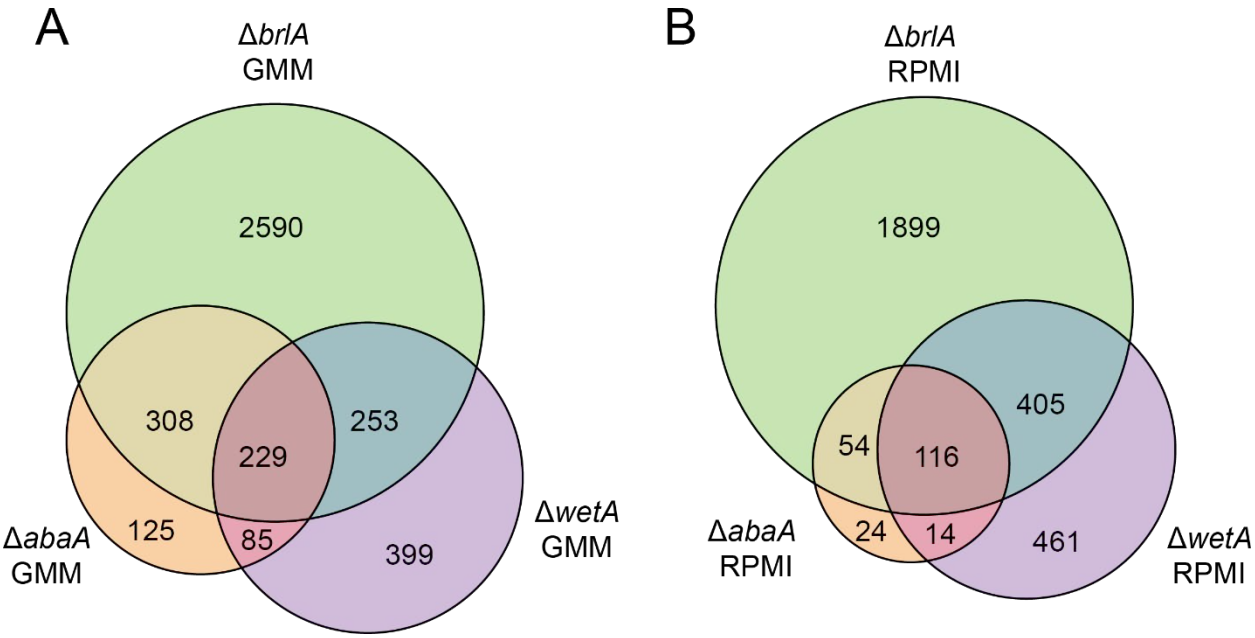

Figure 4-5. Shared and specific targets of BrlA, AbaA, and WetA. (A) Genes under-expressed in Δ*brlA*, Δ*abaA*, and Δ*wetA* compared to wild-type on GMM media. (B) Genes under-expressed in Δ*brlA*, Δ*abaA*, and Δ*wetA* compared to wild-type on RPMI media.

As BrlA, AbaA, and WetA constitute a core transcriptional cascade that controls asexual development in *Aspergillus* species, we expect that some genes will be under-expressed in the absence of each regulator. We found that 229 genes were under-expressed in all strains during growth on GMM and 116 genes were under-expressed in all strains during growth on RPMI (Figure 4-5). Relatively few genes were only differentially expressed in Δ*abaA*, where 125 genes (16.7%) were under-expressed exclusively in the Δ*abaA* strain during growth on GMM and 24 genes (11.5%) during growth on RPMI. More genes were uniquely under-expressed in Δ*wetA* strains, with 399 genes (41.3%) were under-expressed exclusively in Δ*wetA* during growth on GMM and 461 genes (46.3%) were under-expressed exclusively in Δ*wetA* during growth on RPMI. However, a strikingly large number of genes were only differentially expressed in Δ*brlA*;

2,590 genes (76.6%) were differentially expressed only on Δ*brlA* GMM and 1,899 genes (76.7%) were differentially expressed only on Δ*brlA* RPMI.

Secondary metabolic gene clusters and their products are regulated by BrlA, AbaA, and WetA

Nine SMGCs were under-expressed in Δ*brlA* strains during growth on both GMM and RPMI media, including ferricrocin, fusarine C, DHN melanin, fumigaclavine, endocrocin, helvolic acid, gliotoxin, fumiquinazoline, and a cluster encoding an unknown product (Cluster 31) (Figure 4-1). Several SMGCs showed media-dependent changes in expression; the hexadehydroastechrome cluster, the fumitremorgin cluster, and the supercluster encoding the fumagillin and pseurotin gene clusters were up-regulated in the Δ*brlA* strain during growth on RPMI but not on GMM. Additionally, the fumisoquin gene cluster and the pyripyropene gene cluster were under-expressed on GMM media but showed mixed regulation on RPMI media.

SMGCs were also differentially expressed in Δ*abaA* and Δ*wetA* strains, though fewer than in the Δ*brlA* strain. The SMGC encoding ferricrocin was under-expressed in Δ*abaA* on both GMM and RPMI media, and the gene clusters encoding DHN melanin, fumigaclavine, endocrocin, and helvolic acid were under-expressed in Δ*wetA* on both media types.
As seen for BrlA, several SMGCs showed media-dependent regulation by AbaA and by WetA. The gene clusters encoding DHN melanin, endocrocin, fumisoquin, fumiquinazoline, and a cluster encoding an unknown product (Cluster 31) were under-expressed in Δ*abaA* on GMM but not RPMI, and the clusters encoding fumiquinazoline and pyropyripene A were only under-expressed in Δ*wetA* on GMM and not RPMI.

Some SMGCs showed patterns of repression by WetA and AbaA on RPMI media but activation on GMM; the gliotoxin gene cluster was under-expressed in Δ*abaA* and Δ*wetA* on

GMM but over-expressed in Δ*abaA* and Δ*wetA* on RPMI. Additionally, as for Δ*brlA*, the supercluster encoding fumagillin and pseurotin was under-expressed in Δ*wetA* on GMM but over-expressed on RPMI.

BrlA regulates secondary metabolites both associated with asexual tissues and associated with hyphal growth

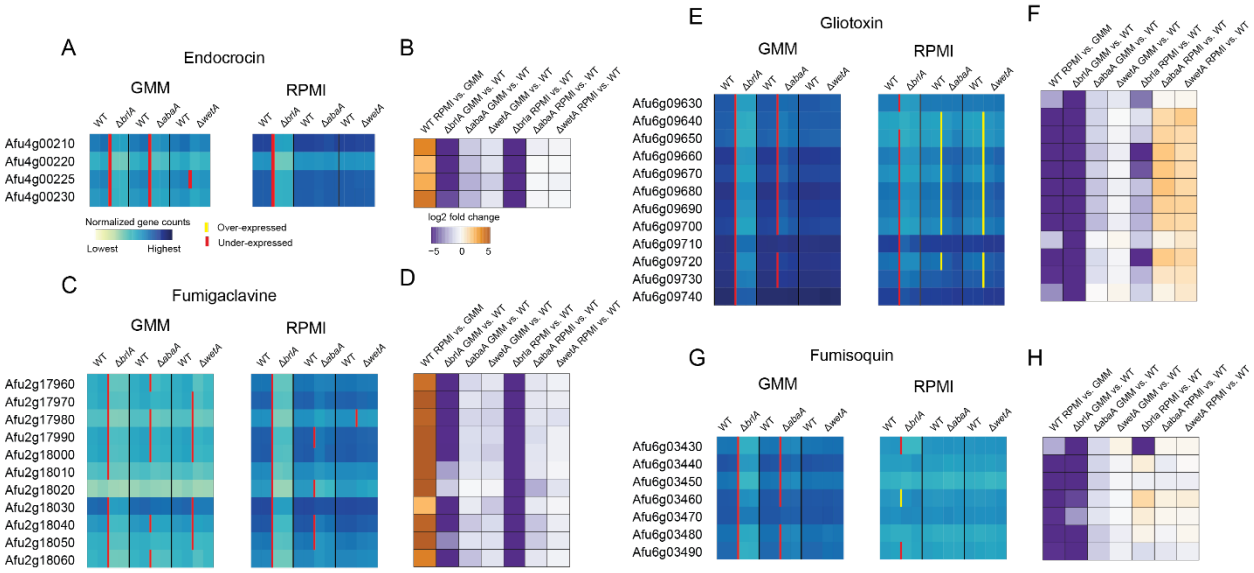

Figure 4-6. Gene expression and fold changes of spore and hyphal associated secondary metabolic gene clusters in all strains and conditions. **(A,B)** Gene expression and log2 fold change of the endocrocin gene cluster, a spore-associated secondary metabolite. **(C,D)** Gene expression and log2 fold change of the fumigaclavine gene cluster, a spore-associated ergot alkaloid. **(E, F)** Gene expression and log2 fold change of the gliotoxin gene cluster, a metabolite produced throughout filamentous growth. **(G,H)** Gene expression and log2 fold change of the fumisoquin gene cluster, a metabolite produced throughout filamentous growth.

The small molecule products of many of the SM gene clusters under-expressed in Δ*brlA*, Δ*abaA*, and Δ*wetA* are known to be spore-associated and are either only produced in asexual tissues or are produced at higher levels during the onset of asexual development. These include endocrocin, DHN melanin, fumigaclavine, and others (Berthier et al. 2013; Tsai et al. 1999; Coyle et al. 2007). The Δ*brlA* strain does not undergo any form of asexual development while the Δ*abaA* and Δ*wetA* strains are halted at later points in asexual development. Accordingly, SM gene clusters that are associated with asexual tissues are under-expressed in Δ*brlA*, Δ*abaA*, and Δ*wetA* strains in at least one of the media conditions tested, but the largest change in gene expression compared to wild-type is seen in the Δ*brlA* strain (Figure 4-6A-D).

In addition to these developmentally associated SMs, BrlA shows a pattern of influencing non-developmental associated secondary metabolites. These include ferricrocin, helvolic acid, gliotoxin, and fumisoquin. These non-developmental associated secondary metabolites were typically produced at higher levels in the wild-type when grown on GMM media, and in some cases were very lowly expressed on RPMI media. These gene clusters were strongly under-expressed in the Δ*brlA* mutant on GMM and somewhat under-expressed in Δ*abaA* and Δ*wetA* (Figure 4-6 E-H).

Discussion

The onset of asexual development and changes in secondary metabolite production occur simultaneously in filamentous fungi. Here we investigate how these processes are transcriptionally linked by analyzing the genome wide transcriptional roles of the central regulators of asexual development. The three regulators, BrlA, AbaA, and WetA, are required for the early, middle, and late stages of asexual development, respectively. These regulators

collectively coordinate structural changes as the fungus moves from the vegetative growth stage to the spore-producing and dispersing stage. Here, we find significant overlap in the identities and the types of genes controlled by these regulators across different environmental conditions. However, we find a distinct role for BrlA in regulating secondary metabolism, and notably in regulating secondary metabolites associated with spore protection as well as those associated with vegetative growth. These results suggest that BrlA plays a central role in coordinating both the physical and chemical changes associated with secondary metabolism.

Remarkably, BrlA regulates the highly conserved process of asexual development as well as the fast evolving and often species-specific process of secondary metabolism. How this regulator bridges that gap is unclear, though transcriptional rewiring may play a role, as other regulators of secondary metabolism and development undergo extensive transcriptional rewiring both of their developmental and transcriptional targets (Lind et al. 2015).

Interestingly, while many other secondary metabolic regulators have been lost in yeasts that lack true hyphae as well as secondary metabolism (Roze et al. 2010), both BrlA and AbaA are conserved. In yeast, the AbaA homologue TEC1 retains part of its developmental role, controlling the expression of genes involved in filamentation (Chou, Lane, and Liu 2006). The BrlA homologue MSN2, however, controls parts of the stress response and does not play an identified role in development (Gorner et al. 1998). Further elucidation of the transcriptional network controlled by BrlA and comparative studies will be required to understand how this asexual regulatory network has changed and been rewired across fungi.

CHAPTER V

DRIVERS OF GENETIC DIVERSITY IN SECONDARY METABOLIC GENE CLUSTERS IN A FUNGAL POPULATION

Authors

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Introduction

Filamentous fungi produce a diverse array of small molecules that function as toxins, antibiotics, and pigments (Vining 1990). Though by definition secondary metabolites (SMs) are not strictly necessary for growth and development, they are critical to the lifestyle of filamentous fungi (Schimek 2011). For example, antibiotic SMs give their fungal producers a competitive edge in environments crowded with other microbes (Fox and Howlett 2008). SMs can additionally mediate communication between and within species, as well as contribute to virulence on animal and plant hosts in pathogenic fungi (Scharf, Heinekamp, and Brakhage 2014; Yim, Wang, and Davies 2007).

A genomic hallmark of SMs in filamentous fungi is that the biosynthetic pathways that produce them are typically organized into contiguous gene clusters in the genome (Keller, Turner, and Bennett 2005). These gene clusters contain the chemical backbone synthesis genes whose enzymatic products produce a core metabolite, such as non-ribosomal peptide synthases (NRPS) and polyketide synthases (PKS), tailoring enzymes that chemically modify the metabolite, transporters involved in product export, and transcription factors that control the

expression of the clustered genes (Keller, Turner, and Bennett 2005). Filamentous fungal genomes, particularly those in the phylum Ascomycota (Keller, Turner, and Bennett 2005), typically contain dozens of SM gene clusters. However, most individual SM gene clusters appear to be either species-specific or narrowly taxonomically distributed in only a handful of species (J. Bennett and Bentley 1989; Keller, Turner, and Bennett 2005). SM gene clusters that are more broadly distributed show discontinuous taxonomic distributions and are often highly divergent between species. Consequently, the identity and total number of SM gene clusters can vary widely even between very closely related species whose genomes exhibit very high sequence and synteny conservation (Khaldi et al. 2010; Lind et al. 2015).

In the last decade, several comparative studies have described macroevolutionary patterns of SM gene cluster diversity. For example, studies centered on genomic comparisons of closely related species, such as members of the same genus, have identified several different types of inter-species divergence, including single nucleotide substitutions, gene gain / loss events, and genomic rearrangements. Single nucleotide substitutions in one gene result in structural differences in fumonisins produced by *Fusarium* species (Robert H. Proctor et al. 2008)), while gene gain and loss events have altered trichothecene gene clusters in *Fusarium* species and aflatoxin family SM gene clusters in *Aspergillus* species (Robert H Proctor et al. 2009; Berry et al. 2011; Slot and Rokas 2011; Ehrlich et al. 2004; Carbone et al. 2007; J. Yu et al. 2004). Additionally, trichothecene gene clusters in *Fusarium* have been altered by genome rearrangement events (Robert H Proctor et al. 2009). Genetic and genomic comparisons across fungal orders and classes have further identified several instances of gene gain or loss (Campbell, Rokas, and Slot 2012; Kroken et al. 2003; Bushley and Turgeon 2010) and horizontal gene transfer (Slot and Rokas 2011; Patron et al. 2007; Khaldi et al. 2008; Khaldi and Wolfe

2011; Reynolds et al. 2017) acting on individual genes or on entire gene clusters, providing explanations for the diversity and discontinuity of the taxonomic distribution of certain SM gene clusters across fungal species.

Although inter-species comparative studies have substantially contributed to our understanding of SM diversity, the high levels of evolutionary divergence of SM clusters make inference of the genetic drivers of SM gene cluster evolution challenging. Simply put, it has been difficult to "catch" the mechanisms that generate SM gene cluster variation "in the act". Several previous studies have examined intra-species or population-level differences in individual SM gene clusters, typically focusing on the presence and frequency of non-functional alleles of clusters involved in production of mycotoxins. Examples of clusters exhibiting such polymorphisms include the gibberellin gene cluster in *Fusarium oxysporum* (Wiemann, Sieber, et al. 2013), the fumonisin gene cluster in *Fusarium fujikuroi* (Chiara et al. 2015), the aflatoxin and cyclopiazonic gene clusters in *Aspergillus flavus* (P.-K. Chang, Horn, and Dorner 2005), and the bikaverin gene cluster in *Botrytis cinerea* (Schumacher et al. 2013). While these studies have greatly advanced our understanding of SM gene cluster genetic variation and highlighted the importance of population-level analyses, studies examining the entirety of SM gene cluster polymorphisms in fungal populations are so far lacking. We currently do not understand the types and frequency of SM gene cluster polymorphisms in populations, whether these polymorphisms affect all types of SM gene clusters, as well as the genetic drivers of SM gene cluster evolution.

To address these questions, we investigated the genetic diversity of all 36 known and predicted SM gene clusters in whole genome sequence data from 66 strains of the opportunistic human pathogen *Aspergillus fumigatus*, 8 of which were sequenced in this study. We found that

13 SM gene clusters were generally conserved and harbored low amounts of variation. In contrast, the remaining 23 SM gene clusters were highly variable and contained one or more of five different types of genetic variation: single-nucleotide polymorphisms including nonsense and frameshift variants, individual gene gain and loss polymorphisms, entire cluster gain and loss polymorphisms, polymorphisms associated with changes in cluster genomic location, and clusters with non-homologous alleles resembling the idiomorphs of fungal mating loci. Many clusters contained interesting combinations of these different polymorphisms, such as pseudogenization in some strains and entire cluster loss in others. The types of variants we find are likely generated by a combination of DNA replication and repair errors, recombination, genomic insertions and deletions, and horizontal transfer. We additionally find an enrichment for transposable elements (TEs) around horizontally transferred clusters, clusters that change in genomic locations, and idiomorphic clusters. Taken together, our results provide a guide to both the types of polymorphisms and the genetic drivers of SM gene cluster diversification in filamentous fungi. As most of the genetic variants that we observe have been previously associated with SM gene cluster diversity across much larger evolutionary distances and timescales, we argue that population-level processes influencing SM gene cluster diversity are sufficient to explain SM cluster macroevolutionary patterns.

Methods

Strains analyzed

Eight strains of *A. fumigatus* were isolated from four patients with recurrent cases of aspergillosis in the Portuguese Oncology Institute in Porto, Portugal. Each strain was determined to be *A. fumigatus* using macroscopic features of the culture and microscopic morphology

observed in the slide preparation from the colonies with lactophenol solution (de Hoog et al. 2001). All clinical strains were classified as *A. fumigatus complex*-Fumigati based on morphology. The genomes of all eight strains were sequenced using 150bp Illumina paired-end sequence reads at the Genomic Services Lab of Hudson Alpha (Huntsville, Alabama, USA). Genomic libraries were constructed with the Illumina TruSeq library kit and sequenced on an Illumina HiSeq 2500 sequencer. Samples of all eight strains were sequenced at greater than 180X coverage or depth.

We retrieved 58 additional *A. fumigatus* strains with publicly available whole genome sequencing data, resulting in a population genomics dataset of 66 strains (Appendix B). The strains used included both environmental and clinical strains and were isolated from multiple continents. Genome assemblies for 10 of these strains were available for download from GenBank (Paul et al. 2017; Liu et al. 2011; Nierman et al. 2005; Fedorova et al. 2008; Knox et al. 2016; Abdolrasouli et al. 2015). For 6 of these strains, short read sequences were also available from the NCBI Short Read Archive (SRA), which were used for variant discovery only (see Single nucleotide variant (SNV) and indel discovery) and not for genome assembly. Short read sequences were not available for the remaining 4 strains. Short read sequences were downloaded for an additional 48 strains from the Short Read Archive if they were sequenced with paired-end reads and at greater than 30x coverage.

Single nucleotide variant (SNV) and indel discovery

All strains with available short read data (62 of 66 strains) were aligned to both the Af293 and A1163 reference genomes using BWA mem version 0.7.12-r1044 (Li and Durbin 2009). Coverage of genes present in the reference genome was calculated using bedtools v2.25.0

(Quinlan and Hall 2010). SNV and indel discovery and genotyping was performed relative to the Af293 reference genome and was conducted across all samples simultaneously using the Genome Analysis Toolkit version 3.5-0-g36282e4 with recommended hard filtering parameters (McKenna et al. 2010; Van der Auwera et al. 2013; DePristo et al. 2011) and annotated using snpEff version 4.2 (Cingolani et al. 2012).

De novo *genome assembly and gene annotation*

All 56 strains without publicly available genome assemblies were *de novo* assembled using the iWGS pipeline (Zhou et al. 2016). Specifically, all strains were assembled using SPAdes v3.6.2 and MaSuRCA v3.1.3 and resulting assemblies were evaluated using QUAST v3.2 (Bankevich et al. 2012; Zimin et al. 2013; Gurevich et al. 2013). The average N50 of assemblies constructed with this strategy was 463 KB (Appendix B). Genes were annotated in these assemblies as well as in five GenBank assemblies with no predicted genes using augustus v3.2.2 trained on *A. fumigatus* gene models (Stanke and Morgenstern 2005). Repetitive elements were annotated in all assemblies using RepeatMasker version open-4.0.6 (Smit, Hubley, and Green, n.d.).

Secondary metabolic gene cluster annotation and discovery

Secondary metabolic gene clusters in the Af293 reference genome were taken from two recent reviews, both of which considered computational and experimental data to delineate cluster boundaries (Inglis et al. 2013; Bignell et al. 2016). The genomes of the other 65 strains were scanned for novel SM gene clusters using identified using antiSMASH v3.0.5.1 (Medema et al. 2011). To prevent potential assembly errors from confounding the analysis, any inference

about changes in genomic locations of genes or gene clusters was additionally verified by manually inspecting alignments and ensuring that paired end reads supported an alternative genomic location (see SNV and indel discovery). Cases where paired end reads did not support the change in genomic location or where mapping was ambiguous or low quality were discarded.

Phylogenetic analysis

To construct a SNP-based strain phylogeny, biallelic SNPs with no missing data were pruned using SNPRelate v1.8.0 with a linkage disequilibrium threshold of 0.8 (Zheng et al. 2012). A phylogeny was constructed using RAxML v8.0.25 using the ASC_BINGAMMA substitution model (Stamatakis 2014). The tree was midpoint rooted and all branches with bootstrap support less than 80% were collapsed.

To understand the evolutionary histories of specific SM gene clusters showing unusual taxonomic distributions, we reconstructed the phylogenetic trees of their SM genes. Specifically, SM cluster protein sequences were queried against a local copy of the NCBI non-redundant protein database (downloaded May 30, 2017) using phmmer, a member of the HMMER3 software suite (Eddy 2009) using acceleration parameters --F1 1e-5 --F2 1e-7 --F3 1e-10. A custom perl script sorted the phmmer results based on the normalized bitscore (nbs), where nbs was calculated as the bitscore of the single best-scoring domain in the hit sequence divided by the best bitscore possible for the query sequence (i.e., the bitscore of the query aligned to itself). No more than five hits were retained for each unique NCBI Taxonomy ID. Full-length proteins corresponding to the top 100 hits (E-value < 1 × 10-10) to each query sequence were extracted from the local database using esl-sfetch (Eddy 2009). Sequences were aligned with MAFFT v7.310 using the E-INS-i strategy and the BLOSUM30 amino acid scoring matrix (Katoh and

Standley 2013) and trimmed with trimAL v1.4.rev15 using its gappyout strategy (Capella-Gutierrez, Silla-Martinez, and Gabaldon 2009). The topologies were inferred using maximum likelihood as implemented in RAxML v8.2.9 (Stamatakis 2014) using empirically determined substitution models and rapid bootstrapping (1000 replications). The phylogenies were midpoint rooted and branches with less than 80% bootstrap support were collapsed using the ape and phangorn R packages (Paradis, Claude, and Strimmer 2004; Schliep 2011). Phylogenies were visualized using ITOL version 3.0 (Letunic and Bork 2016).

Results

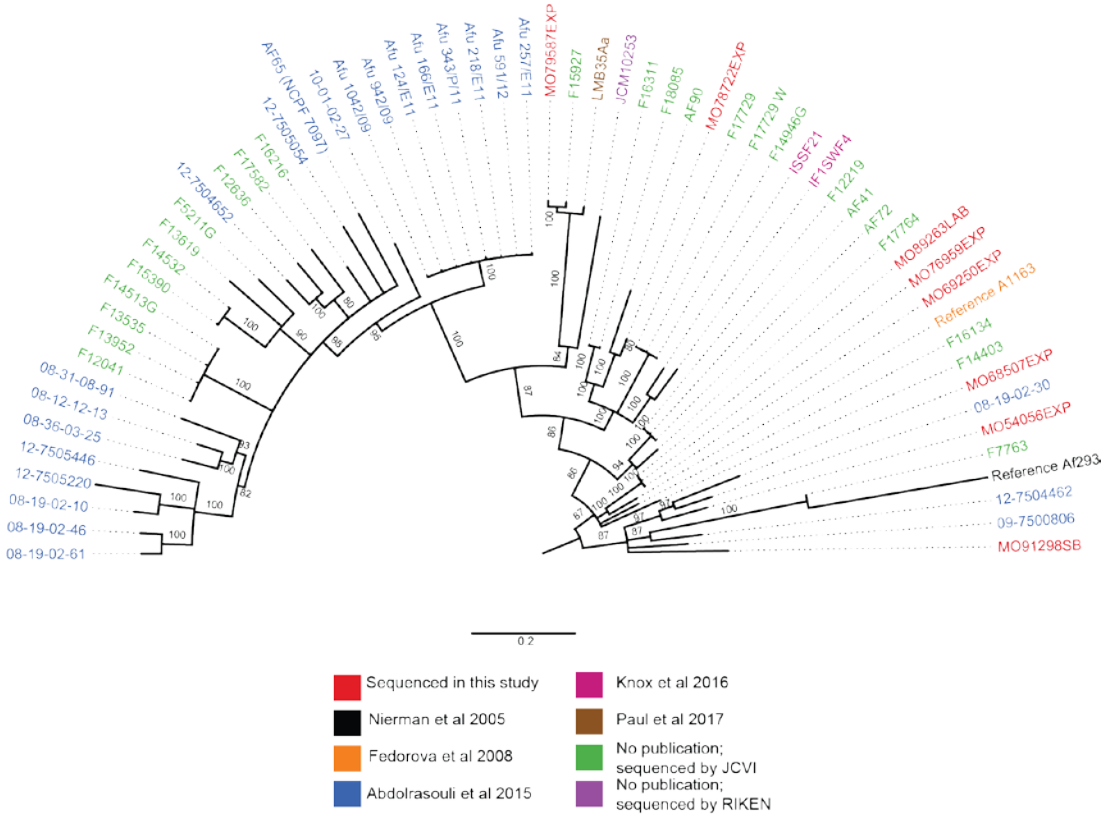

Figure 5-1. SNP-based phylogeny of *A. fumigatus* strains. The phylogeny was constructed using biallelic SNPs with no missing data. The tree is midpoint rooted and all branches with bootstrap support less than 80% are collapsed.

We analyzed the genomes of 66 globally distributed strains of *Aspergillus fumigatus* for polymorphisms in SM gene clusters. We performed whole-genome sequencing on 8 strains, and collected the remaining 58 strains from publicly available databases including NCBI Genome and the NCBI Short Read Archive (Figure 5-1, Appendix B) (Nierman et al. 2005; Fedorova et al. 2008; Abdolrasouli et al. 2015; Knox et al. 2016; Paul et al. 2017). We analyzed all strains for polymorphisms in 33 curated SM gene clusters present in the reference Af293 genome and additionally searched for novel SM gene clusters (see Methods). These examinations revealed five distinct types of polymorphisms which influence SM gene cluster variation (Table 5-1):

a) Single nucleotide and short indel polymorphisms. 33 / 33 SM gene clusters (present in the reference Af293 strain) contained multiple genes with missense SNPs and short indel variants in at least one strain in the population. 23 / 33 SM gene clusters contained one or more genes with frameshift or nonsense variants in the population.
b) Gene content polymorphisms involving loss or gain of one or more genes. 6 / 33 SM gene clusters contained a gene content polymorphism in the population.
c) Whole SM gene cluster gain and loss polymorphisms. 3 / 33 SM gene clusters present in the genome of the reference Af293 strain were absent in at least one strain in the population and an additional 3 previously unknown SM gene clusters were present in the population.
d) Idiomorphic polymorphisms. One locus contained multiple non-homologous SM gene cluster alleles in different strains of the population.
e) Genomic location polymorphisms. 2 / 33 SM gene clusters were found in different genomic locations (e.g., different chromosomes) between strains.

Table 5-1. Types and rates of SM gene cluster variants in *A. fumigatus* strains.

Description	Phenotype	Drivers	Frequency at cluster level	Frequency in strains	Previous reports
Single-nucleotide polymorphisms and indels	Potential for protein function change (missense); abrogation of protein function (nonsense and frameshift)	DNA replication errors; relaxation of purifying selection	100% (33/33 clusters; missense); 70% (23/33 clusters; nonsense and frameshift)	Every strain affected	Bikaverin in *Botrytis* (Schumacher et al. 2013; Campbell, Rokas, and Slot 2012), aflatoxin in *Aspergillus oryzae* and *Aspergillus flavus* (P.-K. Chang, Horn, and Dorner 2005), fumonisins in *Fusarium* (Robert H. Proctor et al. 2008), many others
Gene content polymorphisms	Loss of gene cluster function; structural changes in the metabolite; change in cluster expression or metabolite transport	Deletion and insertion events; recombination; transposable elements	6 clusters	27 / 66 strains	Trichothecene in *Fusarium*, aflatoxin and sterigmatocystin in *Aspergillus* (Robert H Proctor et al. 2009; Berry et al. 2011; Slot and Rokas 2011; Ehrlich et al. 2004; Carbone et al. 2007)
Whole gene cluster polymorphisms	Loss or gain of novel metabolites	Deletion and insertion events; horizontal gene transfer; transposable elements	6 clusters	13 / 66 strains	Gibberellin and fumonisin in *Fusarium* (Chiara et al. 2015; Wiemann, Sieber, et al. 2013)
Cluster idiomorphs	Changes in metabolites produced or structure of metabolites	Transposable elements; recombination; other mechanisms?	1 gene cluster	8 unique identified alleles	Putative SM gene clusters in dermatophytes; putative SM gene cluster in *Aspergillus*

					flavus and *Aspergillus oryzae* (Zhang, Rokas, and Slot 2012; Gibbons, Salichos, et al. 2012)
Mobile gene clusters	Potential for change in gene regulation	Transposable elements; horizontal gene transfer; other mechanisms?	2 gene clusters	8 / 66 strains	None

Single-nucleotide and indel polymorphisms

It is well established that single nucleotide polymorphisms (SNPs) and short indel polymorphisms are caused by errors in DNA replication and repair, and are a major source of genomic variation (Roberts and Kunkel 1996). Non-synonymous SNPs and indels with missense, frameshift, and nonsense effects were widespread across the 33 SM reference gene clusters. Every strain contained numerous missense mutations and at least one nonsense or frameshift mutation in its SM gene clusters. Although missense mutations are likely to influence SM production, the functional effects of nonsense and frameshift mutations are comparatively easier to infer from genomic sequence data because they often lead to truncated proteins lacking a significant portion of their amino acid sequence. For example, a frameshift mutation in the polyketide synthase (PKS) of the trypacidin gene cluster in the A1163 strain results in loss of trypacidin production (Throckmorton et al. 2015). Interestingly, we identified a premature stop codon (Gln273*) in a transcription factor required for trypacidin production, *tpcD*, in a strain sequenced in this study (MO79587EXP). These data suggest that function of this SM gene cluster has been lost through at least two independent genetic events in *A. fumigatus*.

Individual nonsense or frameshift variants ranged from very common in the population to rarer variants present in one or a handful of strains. For example, the non-ribosomal peptide synthase (NRPS) *pes3* gene (Afu5g12730) in SM gene cluster 21 harbors 16 nonsense or frameshift polymorphisms in 55 strains. Seven of these polymorphisms are common and present in 10 or more strains, while seven are rarer and found in 5 or fewer strains. Strains with lab-mutated null alleles of the *pes3* gene are more virulent than strains with functional copies (O'Hanlon et al. 2011), which may explain the widespread occurrence of null *pes3* alleles in the *Aspergillus* population.

Gene content polymorphisms

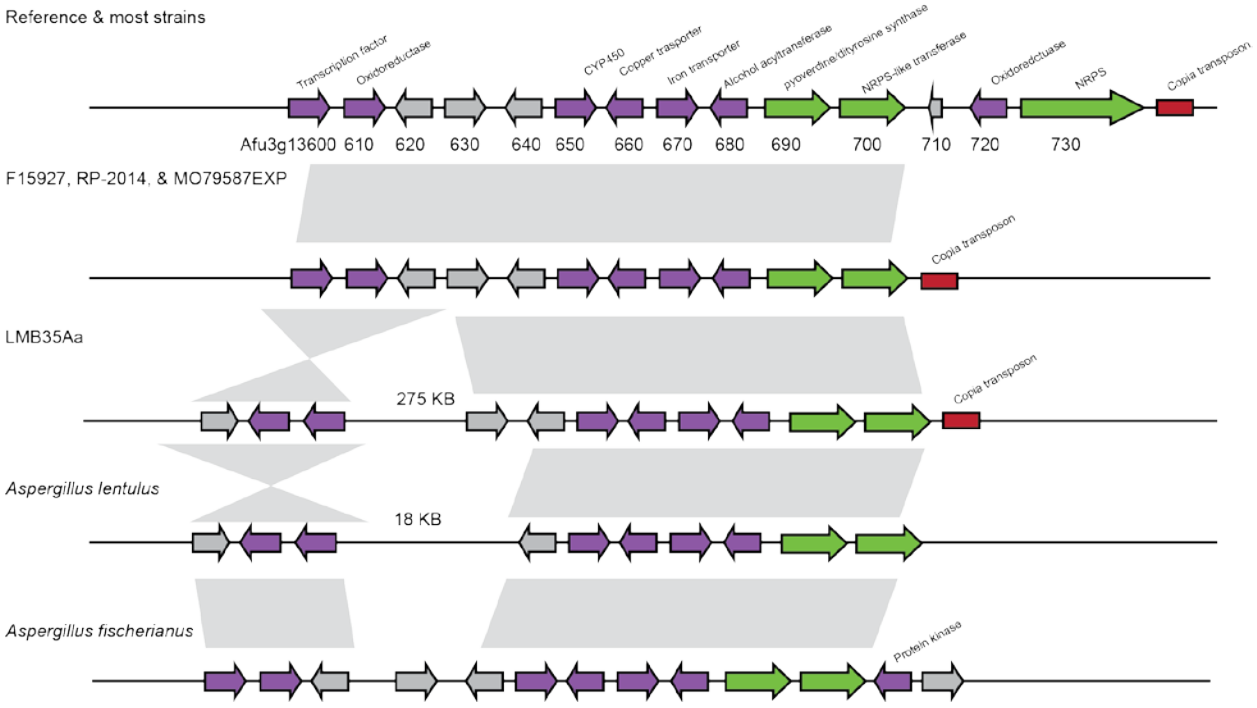

Figure 5-2. Differences in gene content in SM gene cluster 14 in *A. fumigatus* strains and closely related species. Four *A. fumigatus* strains lack an 11-Kb region in this cluster,

including an NRPS backbone gene. Regions upstream and downstream of this cluster are syntenic. LMB35Aa also contains a large inversion that moves a transcription factor, oxidoreductase, and hypothetical protein 275 kb away from the cluster. *Aspergillus fischerianus* and *Aspergillus lentulus*, close relatives of *A. fumigatus*, contain a cluster lacking the 11-kb region.

We additionally identified several SM gene clusters that gained or lost genes in some strains. These gene content polymorphisms were most likely generated through genomic deletion or insertion events and were often present in high frequencies in the population (Table 5-1). In three cases, these polymorphisms impact backbone synthesis genes, rendering the SM gene cluster non-functional. One example involves SM gene cluster 14, whose standard composition includes a pyoverdine synthase gene, an NRPS-like gene, an NRPS backbone gene, and several additional modification genes (Figure 5-2). We discovered that 4 / 66 strains lack an 11-kb region on the 3' end of the cluster which normally contains an NRPS gene and two additional cluster genes, and the first non-SM genes on the 3' end flanking the cluster. All *A. fumigatus* strains contain a *copia* family transposable element (Kapitonov and Jurka 2008) at the 3' end of the cluster, suggesting that transposable elements may have been involved in the generation of this polymorphism. While this polymorphism could have arisen through a deletion event, a homologous cluster lacking the 11-kb region is also present in the reference genomes of *Aspergillus lentulus* and *Aspergillus fischerianus,* close relatives of *A. fumigatus* (Figure 5-2). The most parsimonious explanation is that the genome of the *A. fumigatus* ancestor contained an SM gene cluster that lacked the 11-kb region, and that this genomic region was subsequently gained and increased in frequency in the *A. fumigatus* population.

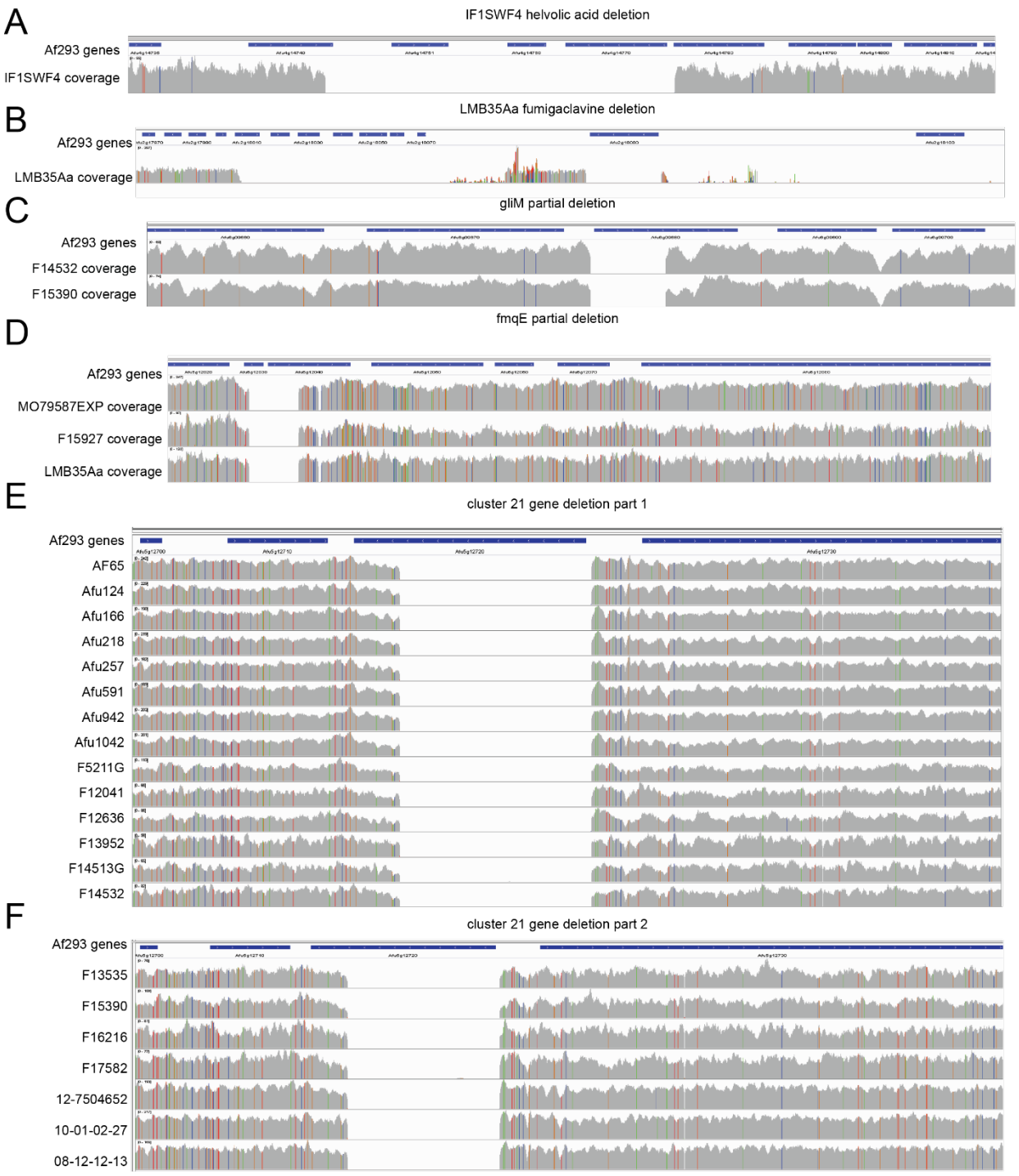

Figure 5-3. Alignments showing deletion of genes in SM gene clusters. (A) Deletion of helvolic acid genes in IF1SWF4. **(B)** Deletion of fumigaclavine genes in LMB35Aa. **(C)** Partial deletion of gliM in the gliotoxin gene cluster in two strains. **(D)** Partial deletion of

fmqE in the fumiquinazoline gene cluster in 3 strains. **(E)** Partial deletion of Afu5g12720 in SM gene cluster 21 in 14 strains. **(F)** Partial deletion of Afu5g12720 in SM gene cluster 21 in 7 strains.

Two additional gene content polymorphisms affecting SM backbone genes were restricted to one strain each and appear to have arisen through genomic deletion events. Specifically, strain IF1SWF4 lacks an 8-Kb region near the helvolic acid SM gene cluster, resulting in the loss of the backbone oxidosqualene cyclase gene as well an upstream region containing two non-SM genes (Figure 5-3A). Strain LMB35Aa lacks a 54-kb region on the end of chromosome 2, which includes five genes from the telomere-proximal fumigaclavine C cluster (Figure 5-3B).

In three other cases, gene content polymorphisms involved gene loss or truncation events of non-backbone structural genes. We found that the second half of the ORF of the *gliM O*-methyltransferase gene in the gliotoxin gene cluster has been lost in 2 / 66 strains (Figure 5-3C), that the first half of the permease *fmqE* in the fumiquinazoline gene cluster has been lost in 4 / 66 strains (Figure 5-3D), and that an ABC transporter gene in SM cluster 21 has been almost entirely lost in 21 / 66 strains (Figure 5-3E,F).

Whole gene cluster gain and loss polymorphisms

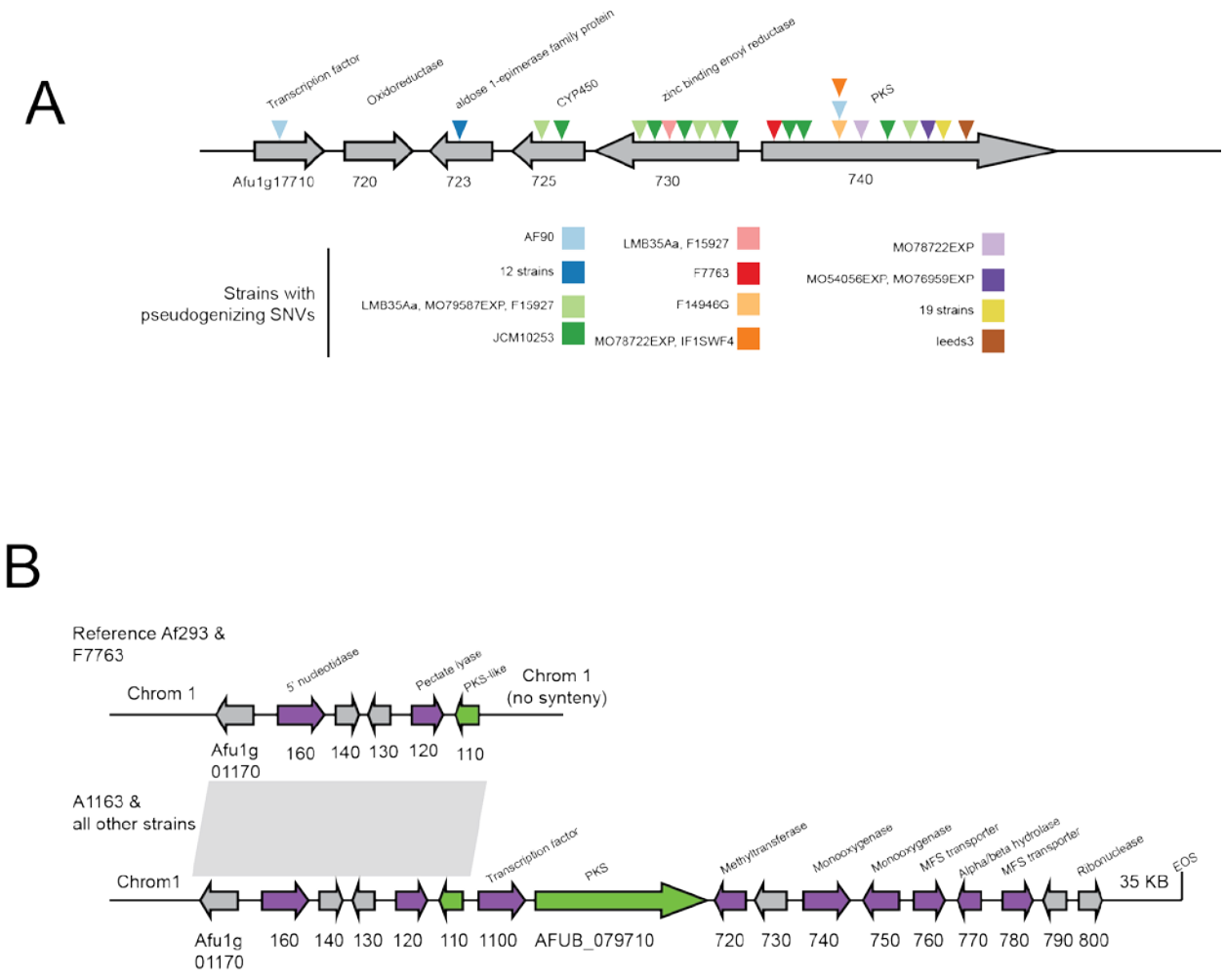

Figure 5-4. Pseudogenization and gene loss in SM gene clusters. (A) SM gene cluster found in most *A. fumigatus* strains but absent from the Af293 reference and from the F7763 strain. EOS denotes end of scaffold. **(B)** Positions of frameshift variants and nonsense variants in the fusarielin-like SM gene cluster 4.

Several SM gene clusters were gained or lost entirely in 13 / 66 strains. We observed instances where a cluster present in the genome of the reference Af293 strain was absent or pseudogenized in other strains as well as cases in which SM clusters present in other strains were absent from the reference Af293 strain.

The most notable example of an SM gene cluster that was present in the Af293 reference genome but absent or pseudogenized in others was SM cluster 4. This cluster contains 5 genes on the tip of the Af293 chromosome 1 and contains orthologs to five of the six genes in the fusarielin gene cluster in *Fusarium graminearum* (Sørensen et al. 2012). This cluster is also present in several other *Aspergillus* species, including *A. clavatus* and *A. niger* (Sørensen et al. 2012). Phylogenetic analysis of the genes in this SM gene cluster is consistent with horizontal gene transfer between fungi in the class Sordariomycetes and fungi in the class Eurotiomycetes, or alternatively with extensive gene loss in both Sordariomycetes and Eurotiomycetes (Appendix C). This gene cluster is entirely absent in 4 / 66 strains, and its genes are undergoing pseudogenization in an additional 44 strains via multiple independent mutational events (Figure 5-4A). Specifically, 19 strains shared a single frameshift variant in the polyketide synthase gene (4380_4381insAATGGGCT; frameshift at Glu1461 in Afu1g17740) and an additional 13 strains shared a single frameshift variant (242delG; frameshift at Gly81) in an aldose 1-epimerase gene (Afu1g17723). Twelve other strains each contained one to several frameshift or nonsense polymorphisms involving nine unique mutational sites, suggesting that this pathway is undergoing multiple independent pseudogenization events. Five of these strains contained multiple distinct frameshifts and premature stop codons in more than one gene in the cluster, indicating that the entire pathway is pseudogenized in these strains.

By searching for novel SM gene clusters in the genomes of the other 65 *A. fumigatus* strains, we found three SM gene clusters that were absent from the genome of the Af293 reference strain. As SM gene clusters are often present in repeat-rich and subtelomeric regions that are challenging to assemble (Treangen and Salzberg 2011; Palmer and Keller 2010), these strains might harbor additional novel SM gene clusters.

One of the novel SM gene clusters that we identified, cluster 34, was present in all but two of the strains (Af293 and F7763). This cluster contains a PKS backbone gene, one PKS-like gene with a single PKS associated domain, nine genes with putative biosynthetic functions involved in secondary metabolism, and six hypothetical proteins (Figure 5-4B). The two strains that lack this cluster contain a likely non-functional cluster fragment that includes the PKS-like gene, two biosynthetic genes, and three hypothetical proteins. Interestingly, the 3' region flanking this cluster is syntenic across all 66 strains but the 5' region is not, suggesting that a recombination or deletion event may have resulted in the loss of this cluster in the Af293 and F7763 strains.

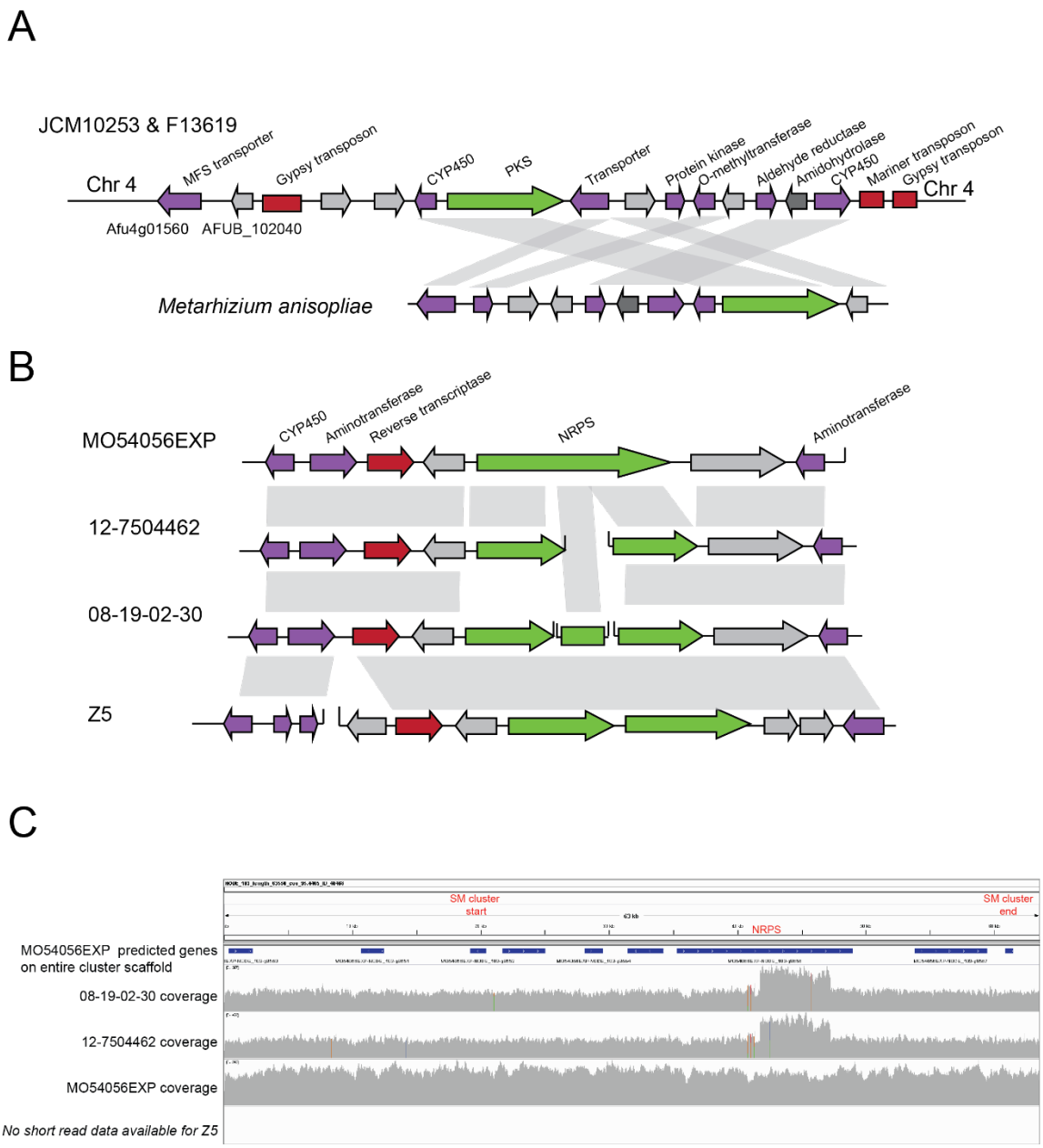

Figure 5-5. Novel SM gene clusters in *A. fumigatus* strains. (A) Synteny between a novel PKS containing cluster in two strains with an SM gene cluster in *Metarhizium anisopliae*. This novel PKS cluster is located between transposable elements in a region syntenic with the reference Af293 chromosome 4. **(B)** Novel SM gene cluster in MO54056EXP and

3 additional strains. This cluster is only located on one scaffold in MO540556EXP and is fragmented across the other strains (ends of scaffolds are denoted). **(C)** Coverage data from short read alignments for MO54056EXP, 12-7504462, and 08-19-02-30 relative to the MO54056EXP scaffold containing the novel SM gene cluster.

The other SM gene clusters that were absent from the Af293 genome are present at lower frequencies in the population; cluster 35 is present in 2 / 66 strains and cluster 36 in 4 / 66 strains. Cluster 35 is located in a region syntenic with an Af293 chromosome 4 region and is flanked on both sides by transposable elements (Figure 5-5A). Eight of the 14 genes in this SM gene cluster are homologous to genes in an SM gene cluster in the genome of the insect pathogenic fungus *Metarhizium anisopliae* (Figure 5-5A). Phylogenetic analysis of these 8 genes is consistent with a horizontal transfer event (Appendix C). Cluster 36 is an NRPS containing cluster located on genomic scaffolds that lack homology to either the Af293 or A1163 genomes, making it impossible to determine on which chromosome this cluster is located (Figure 5-5B,C). The evolutionary histories of the genes in the cluster are consistent with vertical inheritance and are present in multiple *Aspergillus* species.

Idiomorph polymorphisms

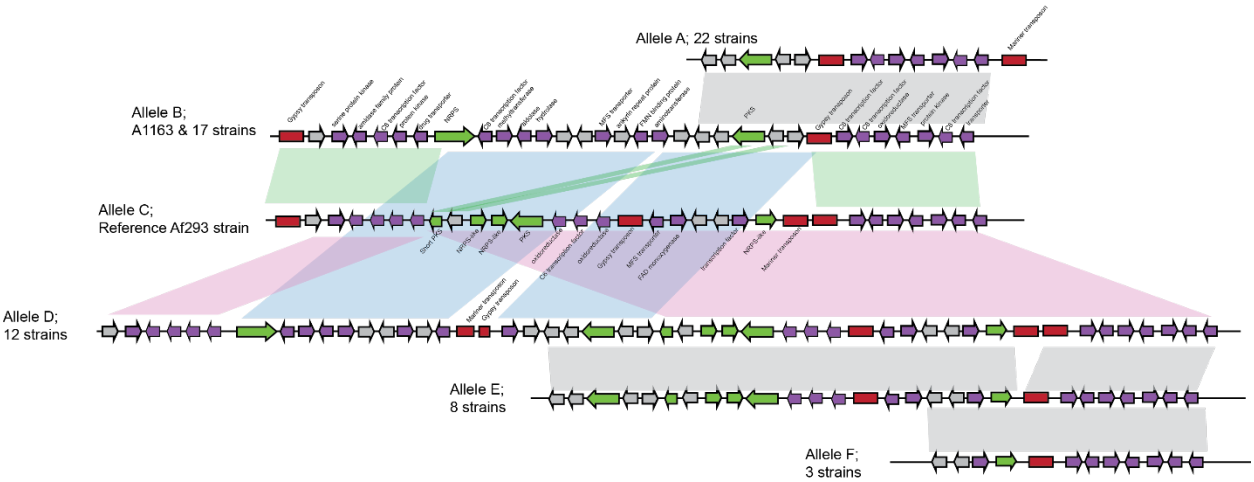

Figure 5-6. Six alleles of an idiomorphic SM gene cluster. Alleles of SM gene cluster 10 on chromosome 3. Red boxes denote transposable elements. Green arrows denote backbone genes (PKS or NRPS) or genes containing domains associated with PKS or NRPS genes. Purple arrows denote genes likely involved in SM biosynthesis, transport, or cluster regulation. Gray arrows denote genes without a putative function or with functions unrelated to SM.

One of the most peculiar types of polymorphisms that we identified is a locus containing different unrelated alleles of SM gene clusters, reminiscent of the idiomorph alleles at the fungal mating loci (Metzenberg and Glass 1990). This locus, which resides on chromosome 3 and corresponds to cluster 10 in the Af293 genome (Figure 5-6), was previously described as being strain-specific in a comparison between Af293 and A1163 (Fedorova et al. 2008) and is thought to reside in a recombination hot spot (Abdolrasouli et al. 2015). Our analysis showed that this locus contained at least 6 different alleles present in two or more of the 66 strains as well as 2 additional alleles that were each only present in one strain (Figure 5-7).

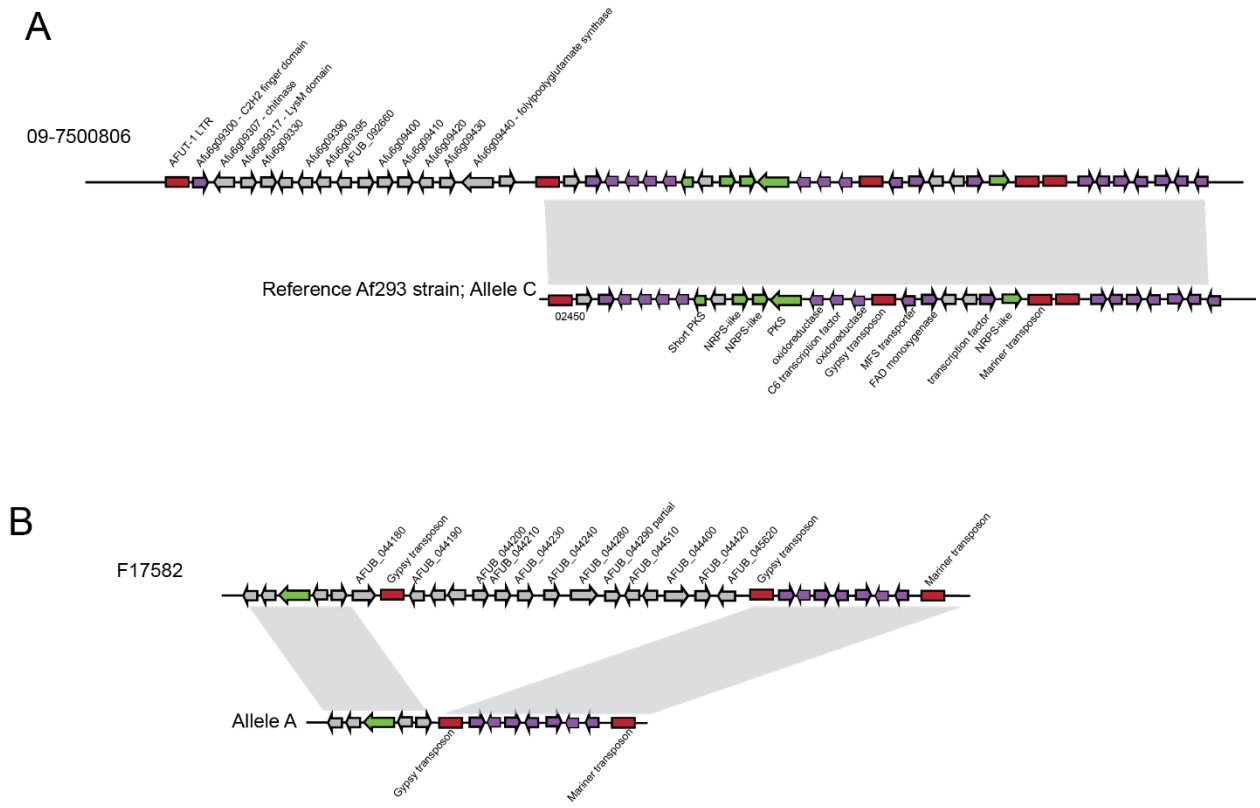

Figure 5-7. Two alleles of the idiomorphic SM gene cluster 10 present in one strain each. (A) This allele contains an insertion of genes from Chromosome 6 immediately upstream of Allele C (see Figure 5-6). None of these genes are likely SM gene cluster backbone genes. An additional transposable element is found flanking this insertion. **(B)** This allele contains an insertion of genes present in the A1163 reference but not in the Af293 reference in the middle of Allele A (see Figure 5-6). None of these genes are likely SM gene cluster backbone genes. One additional transposable element is contained in this insertion.

In the Af293 reference genome, the cluster present at this locus contains one full-length PKS gene along with multiple genes that contain NRPS- or PKS-associated domains (Allele C). In the A1163 reference genome and 17 other strains, there is a full-length NRPS and a full-length

PKS (Allele B). These alleles show an almost complete lack of sequence similarity except for a conserved hypothetical protein and a fragment of the full-length A1163 PKS in the Af293 allele; in contrast, the upstream and downstream flanking regions of the two alleles, which do not contain any backbone genes, are syntenic. Remarkably, another allele, present in 12 strains, contains all of the genes from both the Af293 and A1163 clusters (Allele D). The remaining three alleles contain various combinations of these genes. One allele found in 22 strains contains some A1163-specific genes and no Af293-specific genes (Allele A), while another allele found in 3 strains contains some Af293-specific genes but no A1163 genes (Allele F). The final allele, present in 8 strains, contains the entire Af293 allele as well as part of the A1163 allele (Allele E). Every allele is littered with long terminal repeat sequence fragments from *gypsy* and *copia* TE families as well as with sequence fragments from DNA transposons from the *mariner* family (Kapitonov and Jurka 2008). In some cases, these TEs correspond with breakpoints in synteny between alleles, suggesting that the diverse alleles of this SM gene cluster may arise via TE-driven recombination. Further, both of the alleles that are restricted to a single strain had an insertion event of several genes near a TE, though the rest of the locus is highly similar to one of the more common alleles (Figure 5-7). The evolutionary history of this highly diverse locus is unclear. While it is tempting to speculate that the largest allele containing all observed genes represents the ancestral state, it does not explain the presence of a shared hypothetical protein and PKS gene fragment between the Af293 locus (Allele C) and the A1163 locus (Allele B).

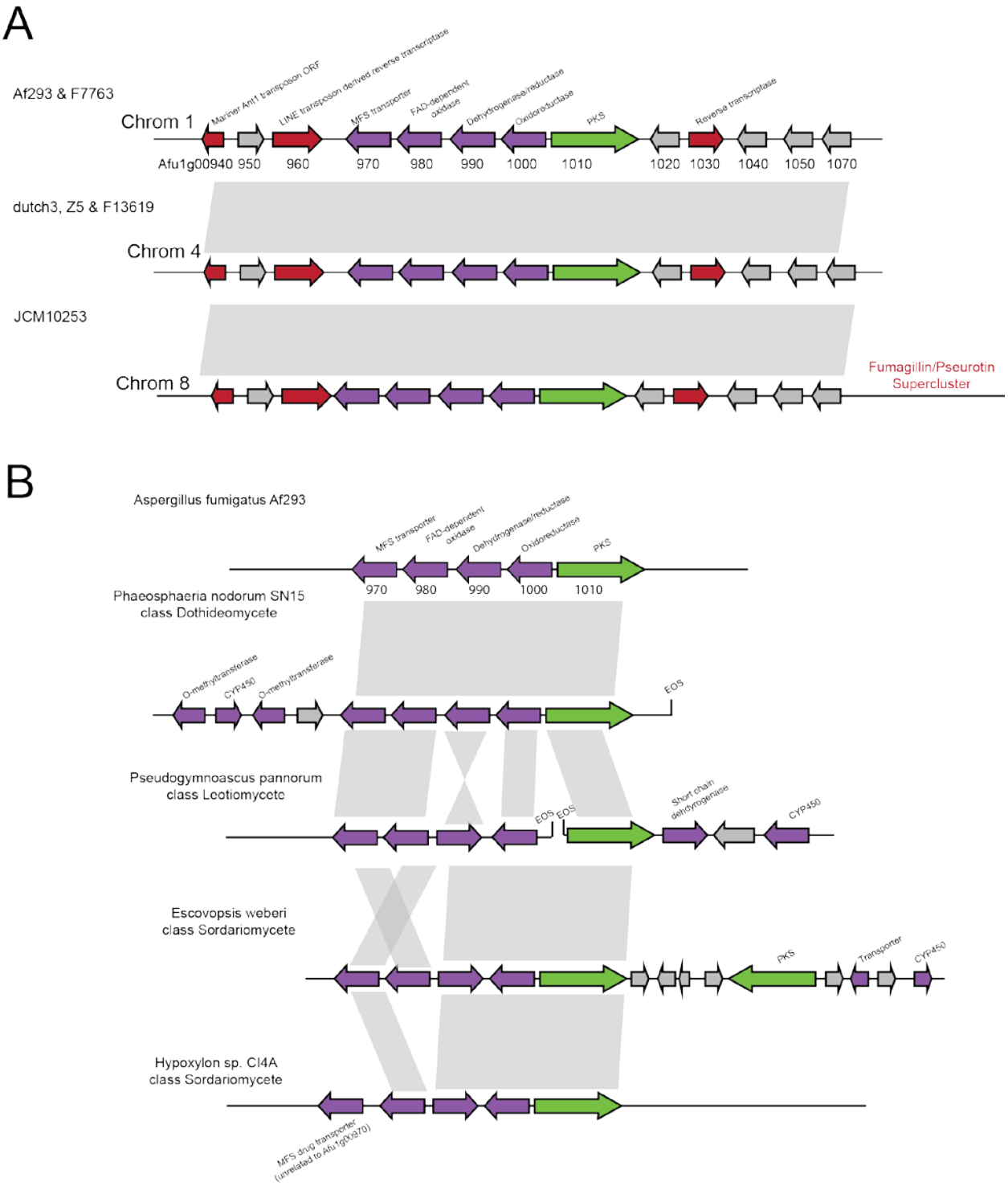

Figure 5-8. Multiple genomic locations of a horizontally transferred SM gene cluster.

(A) Genomic location of SM gene cluster 1 (Afu1g00970-01010) and flanking region in all strains. This cluster is on chromosome 1 in two strains, chromosome 4 in three strains, and adjacent to the intertwined fumagillin and pseurotin supercluster on chromosome 8 in one strain. The flanking regions contain transposon-derived open reading frames including two putative reverse transcriptases. **(B)** Synteny of *A. fumigatus* SM gene cluster 1 with clusters in *Phaeosphaeria nodorum*, *Pseudogymnoascus pannorum*, *Escovopsis weberi*, and *Hypoxylon* sp. CI4A. EOS denotes end of scaffold. All species contain non-syntenic genes predicted by antiSMASH to be part of a biosynthetic gene cluster.

The final type of polymorphism that we observed is associated with SM gene clusters that are located in different genomic locations in different strains, suggesting that these SM gene clusters are behaving like mobile elements. This type of polymorphism was observed in SM gene clusters 1 and 33, both of which produce as yet to be identified products, and are present at low frequencies in the population.

SM gene cluster 1, which is present in six strains at three different genomic locations (Figure 5-8A), consists of a PKS and four other modification genes that are always flanked by a 15 Kb region (upstream) and a 43 Kb region (downstream) containing TEs. In the reference Af293 strain and in strain F7763, this SM gene cluster and its flanking regions are located on chromosome 1, while in strains dutch3, F13619, and Z5 they are located between Afu4g07320 and Afu4g07340 on chromosome 4. In contrast, in strain JCM_10253, the cluster and flanking regions are located on chromosome 8 immediately adjacent to the 3’ end of the intertwined fumagillin and pseurotin SM gene supercluster (Wiemann, Guo, et al. 2013).

In 5 / 6 strains, the cluster appears to be functional and does not contain nonsense SNPs or indels. However, the cluster found on chromosome 1 in strain F7763 contains two stop codons in the oxidoreductase gene (Gln121* and Gln220*) and two premature stop codons in the polyketide synthase (Gln1156* and Gln1542*), suggesting this strain contains a null allele.

This "jumping" gene cluster is not present in any other sequenced *Aspergillus* genus, and phylogenetic analysis of its constituent genes is consistent with horizontal gene transfer between fungi (Appendix C). Specifically, this gene cluster is also present in *Phaeosphaeria nodorum*, a plant pathogen from the class Dothideomycetes, *Pseudogymnoascus pannorum*, a fungus isolated from permafrost from the Leotiomycetes, *Escovopsis weberi*, and a fungal parasite of fungus-growing ants from the Sordariomycetes (Figure 5-8B). One additional species, the endophyte *Hypoxylon* sp. CI4A from the class Sordariomycetes, contains four of the five cluster genes but is missing Afu1g00970, an MFS drug transporter. However, this species contains an unrelated gene annotated as an MFS drug transporter immediately adjacent to this cluster, so this species may be using a different transporter (Figure 5-8B). None of these fungi contain the upstream or downstream TE-rich flanking regions present in *A. fumigatus*, and each fungus contains additional unique genes with putative biosynthetic functions adjacent to the transferred cluster. The most likely explanation for this change in flanking regions is that this SM gene cluster was transferred into *A. fumigatus* once and has subsequently moved its location in the genome.

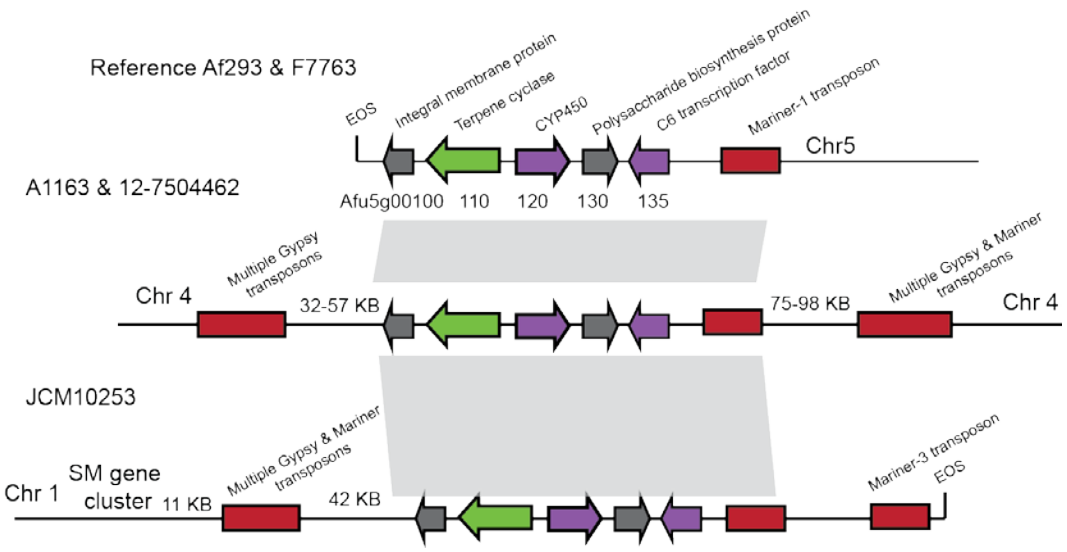

Figure 5-9. Genomic contexts of mobile SM gene cluster 5. This gene cluster is located on Chromosome 5 in two strains, on Chromosome 4 in two strains, and on Chromosome 1 in one strain adjacent to a putative SM gene cluster. Multiple transposable elements flank the cluster in each strain.

The second SM gene cluster that shows variation in its genomic location across strains, cluster 33, contains a terpene synthase. This cluster is present in only 5 strains at 3 distinct locations (Figure 5-9). Similar to cluster 1, this cluster is also flanked by TEs and in one strain the cluster is located 58 Kb from another SM gene cluster. In contrast to cluster 1, this cluster does not appear to have been horizontally transferred between fungi and its genes are present in other sequenced *Aspergillus* species. As this mobile cluster was not horizontally transferred, it is possible that the horizontal transfer of cluster 1 and its mobile nature throughout the *A. fumigatus* genome are driven by different mechanisms.

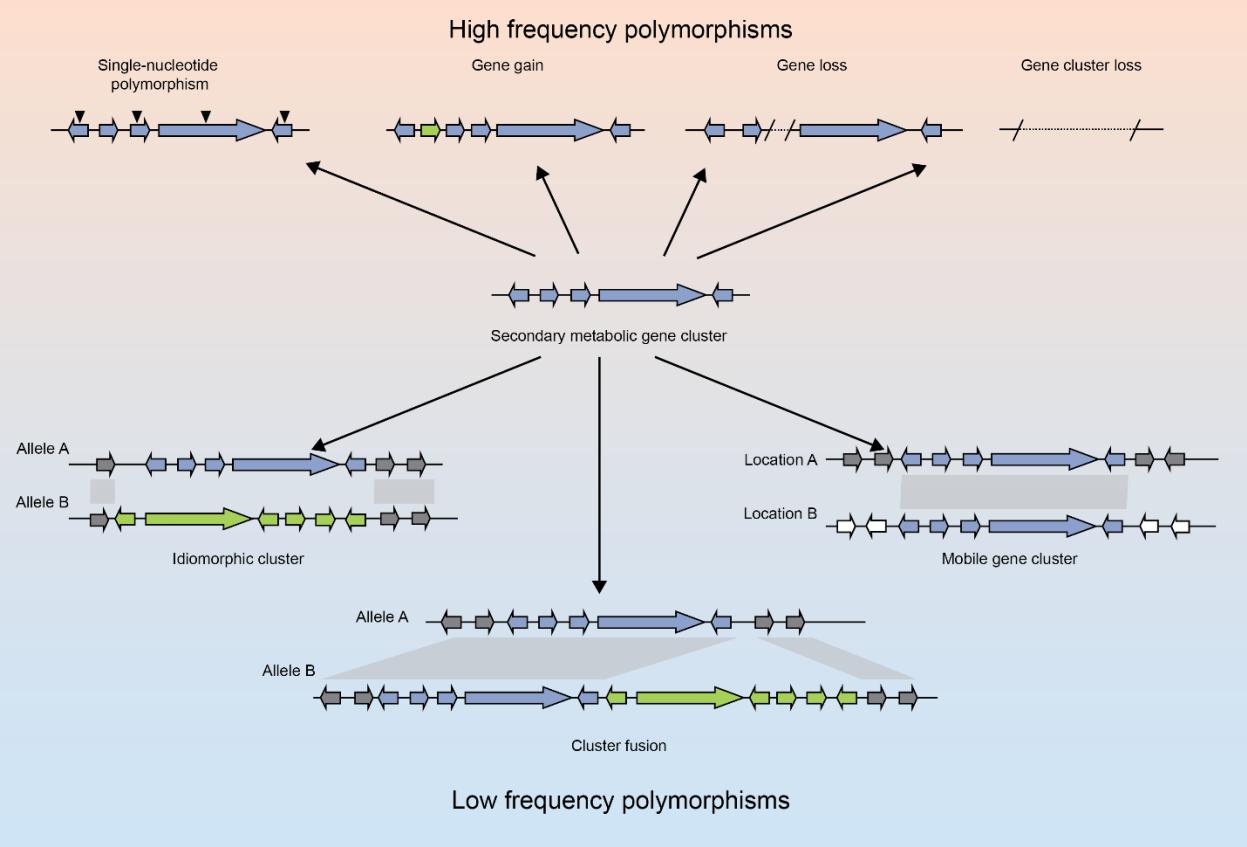

Figure 5-10. Types and frequencies of all SM gene cluster variants in the *A. fumigatus* population.

Our examination of 66 genomes from strains of *Aspergillus fumigatus* revealed that five general types of polymorphisms describe variation in SM gene clusters in a fungal population. These polymorphisms include variation in single nucleotides, gene and gene cluster gains and losses, non-homologous clusters at the same genomic position, and changes in genomic locations of clusters (Figure 5-10). In several cases, the genetic mechanisms that gave rise to these polymorphisms and their functional consequences are clear. Polymorphisms in single-nucleotides most likely arose from errors in DNA replication or repair, while gene loss

polymorphisms likely arose from genomic deletion or recombination events. Others, such as SM gene clusters that exist as non-homologous alleles or that entered the population through horizontal transfer, could arise through multiple genetic mechanisms. Transposable elements are common in polymorphic SM gene clusters and are found flanking mobile and horizontally transferred clusters, as well as in regions adjacent to non-homologous alleles and where gene gain has occurred, suggesting they contribute to SM gene cluster diversity. Using a population genomics approach to identify SM gene cluster variants allowed us to also capture and describe novel polymorphisms, including mobile gene clusters and idiomorphic clusters, which are difficult to identify in comparative genomic studies between species whose conservation of genome-wide synteny is low. Strikingly, the variants and genetic drivers we observe at the population level are also implicated as driving SM gene cluster variation between fungal species, suggesting that the observed microevolutionary processes are sufficient to explain macroevolutionary patterns of SM gene cluster evolution. Below, we discuss our key results and place them in the broader context of SM gene cluster evolution.

The first novel type of polymorphism was observed in SM gene clusters 1 and 33, which by occupying different genomic locations in different strains, appear to behave in a manner similar to mobile genetic elements. Interestingly, both clusters are located near or immediately adjacent to other SM gene clusters in some strains. For example, cluster 1 is located immediately adjacent to the intertwined fumagillin and pseurotin supercluster (Wiemann, Guo, et al. 2013) in one strain. This supercluster is regulated by the transcriptional factor *fapR* and is located in a chromosomal region controlled by the master SM regulators *laeA* and *veA* (Wiemann, Guo, et al. 2013; Lin et al. 2013), raising the hypothesis that these mobile gene clusters might be co-opting the regulatory machinery already in place. Previous work has hypothesized that the fumagillin

and pseurotin supercluster formed through genomic rearrangement events placing the once-independent gene clusters in close proximity to each other (Wiemann, Guo, et al. 2013). Our observation that this mobile gene cluster is located in this same region not only supports this hypothesis but also implicates transposable elements as one of the mechanisms by which such genomic rearrangements are formed. These superclusters may also represent an intermediate stage in the formation of new SM gene clusters. Supercluster formation, potentially mediated by mobile gene clusters, and followed by gene loss, could explain macroevolutionary patterns of SM gene clusters have shown that clustered genes in one species can be dispersed over multiple gene clusters in other species (Robert H Proctor et al. 2009; Lind et al. 2015).

The second novel type of polymorphism was the presence of multiple non-homologous alleles at the cluster 10 locus, echoing the structure of the idiomorphic mating type locus (Metzenberg and Glass 1990). This region has previously been reported to be a recombination hotspot in *A. fumigatus* (Abdolrasouli et al. 2015) and all alleles contain numerous transposable elements. Thus, it is possible that polymorphism at this locus originated via SM gene cluster fusion or splitting events driven by transposable elements. Interestingly, two other previously described instances of SM gene cluster variation bear close resemblance to the *A. fumigatus* idiomorphic SM gene cluster 10 locus. The first is the presence of two non-homologous *Aspergillus flavus* alleles, where some strains contain a 9-gene sesquiterpene-like SM gene cluster and others contain a non-homologous 6-gene SM gene cluster at the same genomic location (Gibbons, Salichos, et al. 2012). The second is the presence of two non-homologous SM gene clusters at the same, well-conserved, locus in a comparison of six species of dermatophyte fungi (Zhang, Rokas, and Slot 2012). Based on these results, we hypothesize that idiomorphic

clusters may be common in fungal populations and contribute to the broad diversity of SM gene clusters across filamentous fungi.

The remaining types of SM polymorphism in the *A. fumigatus* population have previously been described at the species level. For example, null alleles have been reported for many individual SM gene clusters across diverse fungal species (Schumacher et al. 2013; Campbell, Rokas, and Slot 2012; P.-K. Chang, Horn, and Dorner 2005; Robert H. Proctor et al. 2008) and our results show that it is a widespread phenomenon, affecting two-thirds of the *A. fumigatus* SM gene clusters. Consistent with previous literature reporting the presence of additional SM gene clusters not present in the reference strain (Wiemann, Sieber, et al. 2013; Chiara et al. 2015), we also identify four low-frequency SM gene clusters in the *A. fumigatus* population, two of which are horizontally transferred from distantly related fungi and two that appear vertically inherited. We find numerous cases of gene loss and relatively fewer cases of gene gain; previous studies have implicated these processes across numerous types of SM gene clusters and reported their effects on metabolite production (Schardl et al. 2013). Previous work has indicated that gene and gene cluster gain and loss tends to occur near telomeres, suggesting that higher rates of genetic events like recombination lead to loss (Young et al. 2015). While we do find several cases of gene and gene cluster loss in subtelomeric clusters, including the fumigaclavine gene cluster and the helvolic acid gene cluster, we also find gene loss in clusters that are not located near telomeres, such as the gliotoxin gene cluster and the fumiquinazoline gene cluster. These findings suggest that while gene and gene cluster loss can occur in telomeric regions, clusters in other genomic regions also experience high rates of loss.

Previous work has demonstrated that SM gene clusters, like the metabolites that they produce, are highly divergent between fungal species. Our examination of genome-wide

variation shows that these SM gene clusters are correspondingly diverse within individual strains of a single fungal species. Furthermore, the observed genetic changes in SM gene clusters are widespread across different types of gene clusters and are caused by many underlying genetic drivers, including gene and gene cluster gain, loss, non-homologous cluster alleles, and mobile gene clusters. The net effect of these substitutions, gains, losses, and rearrangements raises the hypothesis that fungal SM gene clusters are likely in a state of evolutionary flux, constantly altering their SM gene cluster repertoire, and consequently modifying and diversifying their chemodiversity.

CHAPTER VI

SUMMARY

In this dissertation, I examine how secondary metabolism regulation changes across species, across different stages of the fungal lifestyle, and across environmental conditions. This work reveals that secondary metabolism regulation has been extensively rewired across different fungi, that regulators of development also play roles in controlling secondary metabolism production, and that environmentally responsive regulators can integrate multiple environmental signals to fine tune the metabolites they regulate. I additionally examine the genetic drivers of secondary metabolic gene cluster diversity at the population level and discover numerous types of genetic variants that explain species-level distributions of these clusters. This dissertation contributes to our understanding of how transcriptional networks controlling species-specific processes evolve, as well as genetic factors that drive the diversification of fast evolving, clustered metabolic pathways.

In Chapter II, I examined how the transcriptional networks controlling secondary metabolism and development have evolved in *Aspergillus*. I demonstrated that no secondary metabolic gene clusters are conserved across the *Aspergillus* genus and that when any genes in these clusters are conserved, they are fragmented across different clusters and chromosomes in other species. By examining the genome-wide regulatory role of two master regulators of secondary metabolism, VeA and MtfA, in *Aspergillus nidulans* and *Aspergillus fumigatus*, I showed that VeA has been extensively transcriptionally rewired both with respect to secondary metabolism and development, and that MtfA's regulatory role has changed between *A. fumigatus* and *A. nidulans*. This works shows that conserved secondary metabolic regulatory networks

control highly species-specific secondary metabolic pathways through extensive transcriptional rewiring. Under this bottom-up model of regulatory evolution, fast-evolving and novel secondary metabolic gene clusters are "plugged in" to an existing, environmentally responsive regulatory network.

Future directions. While this study demonstrated that the VeA regulator has been rewired across *Aspergillus* species, it is not clear how the structure of the regulatory network itself has changed. Studies of regulatory rewiring in other organisms, primarily yeast, have collectively demonstrated that different regulatory networks often experience common evolutionary constraints and share similar trajectories (Sorrells and Johnson 2015). However, these studies focus on the evolution of core biological processes that tend to be well conserved. Because the targets of secondary metabolic regulatory networks are so fast evolving, the structure and evolution of these regulatory networks may have unique features that have not yet been described. In particular, it is not clear how horizontally transferred gene clusters, which occur at relatively high frequencies in fungal genomes, are integrated into the regulatory machinery of the accepting fungus. These questions could be addressed in the future using methods such as ChIP-seq and regulatory network reconstruction.

In Chapter III, I hypothesized that master regulators of secondary metabolism may be able to sense and respond to multiple different environmental cues. To test this, I examined the transcriptome-wide regulatory role of two members of the light-responsive Velvet regulatory complex, VeA and LaeA, at different temperatures. The regulatory role of LaeA, a putative methyltransferase, did not change at different temperatures, but the regulatory role of VeA changed at different temperatures. Notably, VeA regulated three SM gene clusters on

Chromosome 8 at 37 ºC but not at 30 ºC. These results, along with previous work that shows carbon source concentration can affect VeA's subcellular localization (Atoui et al. 2010), show that this regulator responds to environmental stimuli other than light and can integrate multiple environmental signals into its regulation of secondary metabolism.

Future directions. This work demonstrates that the VeA master regulator of secondary metabolism is capable of responding to multiple environmental signals. It is unclear if this is a general phenomenon of master secondary metabolic regulators, as LaeA showed no change in function at different temperatures. Further work could be done to test different VeA with different environmental conditions, or on testing different master regulators at different temperatures. Understanding cross-talk between different master regulators as well as combinatorial effects of environmental signals will likely reveal interesting links between the ecological roles of fungal secondary metabolites and their regulation.

In Chapter IV, I investigated how asexual development and secondary metabolism are coordinated at the transcriptional level. In *Aspergillus fumigatus* and many other fungi, asexual development and secondary metabolism production occur simultaneously. I examined how these processes are coordinated by determining the impact of central asexual regulatory pathways in *Aspergillus fumigatus* on secondary metabolism production. I demonstrated that the asexual developmental regulator BrlA plays previously unknown roles in regulating both spore-specific secondary metabolites as well as secondary metabolites with no roles in development.

Future directions. BrlA is a highly conserved regulator across filamentous and non-filamentous fungi, and it was previously unknown that this regulator contributed broadly to secondary metabolism production. It is not clear whether BrlA regulates secondary metabolism

in other filamentous fungi or whether this is restricted to *Aspergillus fumigatus*. Examining BrlA's role in secondary metabolism regulation in other fungi and gaining a more complete understanding of the regulatory network it controls will contribute to our understanding of secondary metabolism regulation and its evolution.

I determined in Chapter II that secondary metabolic gene clusters are not conserved across different species of *Aspergillus.* Attempting to understand the genetic mechanisms that changed these clusters over time at this species-level comparison was difficult, as there was almost no overlap in clusters. To understand what genetic factors drive variation in secondary metabolic gene clusters, in Chapter V I compared the genomes of 66 strains of *Aspergillus fumigatus*. This analysis revealed that secondary metabolic gene clusters vary dramatically at the population level, but by using a population based analysis I was able to determine the likely genetic events that gave rise to this variation. I found that SNVs and indels were extremely common in secondary metabolic gene clusters, including nonsense and frameshift variants that render the gene product nonfunctional. Other common types of genetic variation in SM gene clusters included gene deletion and insertion events and entire gene cluster deletion and gain events. I was further able to identify two mobile gene clusters that change location throughout the genome, and a cluster with non-homologous alleles that resemble the "idiomorphic" mating type locus in fungi. These types of genetic variants are sufficient to explain species-level differences in secondary metabolic gene clusters. The extent of this genetic variation within a single species suggests that these secondary metabolic gene clusters and metabolites that they produce are in a state of evolutionary flux where change and variation are the rule rather than the exception.

Future directions. The major limitation to this analysis is that the effects of these genetic variants on fitness is not clear, as the majority of secondary metabolites in *Aspergillus fumigatus* lack well understood ecological roles. While some contribute to the virulence of the fungus, others likely play roles in other common secondary metabolite functions like self-protection and microbial communication. Future work on understanding the contributions of these variants to fitness and what constraints exist on secondary metabolite evolution will require more functional work characterizing the metabolites themselves. Other experiments that would elucidate the fitness costs and benefits of variants in secondary metabolic gene clusters would be experimental evolution tests and fitness tests of strains containing different variants.

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APPENDIX A

ROLE OF THE STUDENT

I was the primary author on all of the text in this dissertation and carried out the majority of the research presented in Chapters II-V. In Chapter II, I performed all RNA-seq data analysis and all downstream analyses, and determined the level of conservation of metabolic gene sets and SM gene clusters in all genomes analyzed. In Chapter III and IV, I performed all RNA-seq data analysis as well as all downstream analysis involving Gene Ontology enrichment analysis and SM gene cluster expression analyses. In Chapter V, I performed *de novo* assembly, gene prediction, SM gene cluster prediction, short read alignment, polymorphism discovery, and SM gene conservation analyses.

APPENDIX B

STRAINS USED IN CHAPTER V

Official name	Pubmed ID	Source	Publicly available assembly	Publicly available gene predictions	Publicly available short reads
IF1SWF4	27830189	Environmental	Y	N	Y
ISSF21	27830189	Environmental	Y	N	Y
AF41	N/A; sequenced by JCVI	Clinical	N	N	Y
AF72	N/A; sequenced by JCVI	Clinical	N	N	Y
AF90	N/A; sequenced by JCVI	Clinical	N	N	Y
F12041	N/A; sequenced by JCVI	Clinical	N	N	Y
F12219	N/A; sequenced by JCVI	Clinical	N	N	Y
F12636	N/A; sequenced by JCVI	Clinical	N	N	Y
F13535	N/A; sequenced by JCVI	Clinical	N	N	Y
F13619	N/A; sequenced by JCVI	Clinical	N	N	Y
F13952	N/A; sequenced by JCVI	Clinical	N	N	Y
F14403	N/A; sequenced by JCVI	Clinical	N	N	Y
F14513G	N/A; sequenced by JCVI	Clinical	N	N	Y
F14532	N/A; sequenced by JCVI	Clinical	N	N	Y
F14946G	N/A; sequenced by JCVI	Clinical	N	N	Y
F15390	N/A; sequenced by JCVI	Clinical	N	N	Y
F15927	N/A; sequenced by JCVI	Clinical	N	N	Y

F16134	N/A; sequenced by JCVI	Clinical	N	N	Y
F16216	N/A; sequenced by JCVI	Clinical	N	N	Y
F16311	N/A; sequenced by JCVI	Clinical	N	N	Y
F17582	N/A; sequenced by JCVI	Clinical	N	N	Y
F17729	N/A; sequenced by JCVI	Clinical	N	N	Y
F17729W	N/A; sequenced by JCVI	Clinical	N	N	Y
F17764	N/A; sequenced by JCVI	Clinical	N	N	Y
F18085	N/A; sequenced by JCVI	Clinical	N	N	Y
F7763	N/A; sequenced by JCVI	Clinical	N	N	Y
F5211G	N/A; sequenced by JCVI	Clinical	N	N	Y
AF65 (NCPF 7097)	26037120	Clinical	N	N	Y
Afu 1042/09	26037120	Clinical	N	N	Y
Afu 124/E11	26037120	Environmental	N	N	Y
Afu 166/E11	26037120	Environmental	N	N	Y
Afu 218/E11	26037120	Environmental	N	N	Y
Afu 257/E11	26037120	Environmental	N	N	Y
Afu 343/P/11	26037120	Clinical	N	N	Y
Afu 591/12	26037120	Clinical	N	N	Y
Afu 942/09	26037120	Clinical	N	N	Y
08-12-12-13	26037120	Clinical	N	N	Y
08-36-03-25	26037120	Clinical	N	N	Y
08-31-08-91	26037120	Clinical	N	N	Y
08-19-02-61	26037120	Environmental	N	N	Y

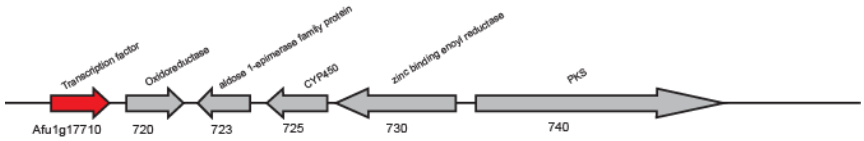
08-19-02-30	26037120	Environmental	N	N	Y
10-01-02-27	26037120	Clinical	N	N	Y
08-19-02-46	26037120	Environmental	N	N	Y
08-19-02-10	26037120	Environmental	N	N	Y
12-7505446	26037120	Clinical	N	N	Y
12-7505220	26037120	Clinical	N	N	Y
09-7500806	26037120	Clinical	N	N	Y
12-7504652	26037120	Clinical	N	N	Y
12-7504462	26037120	Clinical	N	N	Y
12-7505054	26037120	Clinical	N	N	Y
A1163	18404212; N/A, sequenced by JCVI (resequencing)	Clinical	Y	Y	Y
Af293	16372009; 26037120 (resequencing)	Clinical	Y	Y	Y
JCM10253	N/A; sequenced by RIKEN Center for Life Science Technologies	Unclear	Y	Y (on RIKEN website, not available through Genbank)	Y
LMB35Aa	N/A; sequenced by Universidad Nacional Agraria La Molina, Peru	Environmental	Y	N	Y
Af10	N/A; sequenced by JCVI	Clinical	Y	N	N
Af210	N/A; sequenced by JCVI	Clinical	Y	N	N

Z5	26076650	Environmental	Y	Y	N
Niveus	N/A; Sequenced at Oklahoma State University	Unclear	Y	N	N
MO54056EXP	N/A; sequenced in this study	Clinical	N	N	Y; this study
MO68507EXP	N/A; sequenced in this study	Clinical	N	N	Y; this study
MO69250EXP	N/A; sequenced in this study	Clinical	N	N	Y; this study
MO76959EXP	N/A; sequenced in this study	Clinical	N	N	Y; this study
MO78722EXP	N/A; sequenced in this study	Clinical	N	N	Y; this study
MO79587EXP	N/A; sequenced in this study	Clinical	N	N	Y; this study
MO89263LAB	N/A; sequenced in this study	Clinical	N	N	Y; this study
MO91298SB	N/A; sequenced in this study	Clinical	N	N	Y; this study

APPENDIX C

GENE PHYLOGENIES FROM CHAPTER V

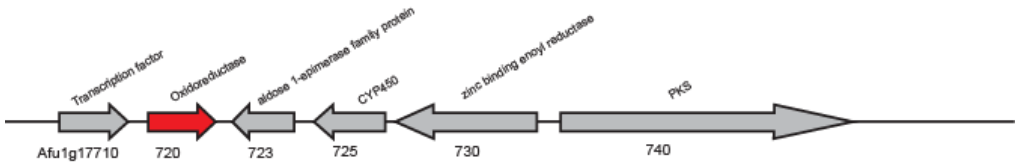
Gene phylogenies of the fusarielin-like SM gene cluster 4. These phylogenies are consistent with horizontal transfer between Eurotiomycete and Sordariomycete fungi or with extensive gene loss.



Tree scale: 0.1

Taxonomy

- Leotiomycetes
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses



Tree scale: 0.1

Taxonomy

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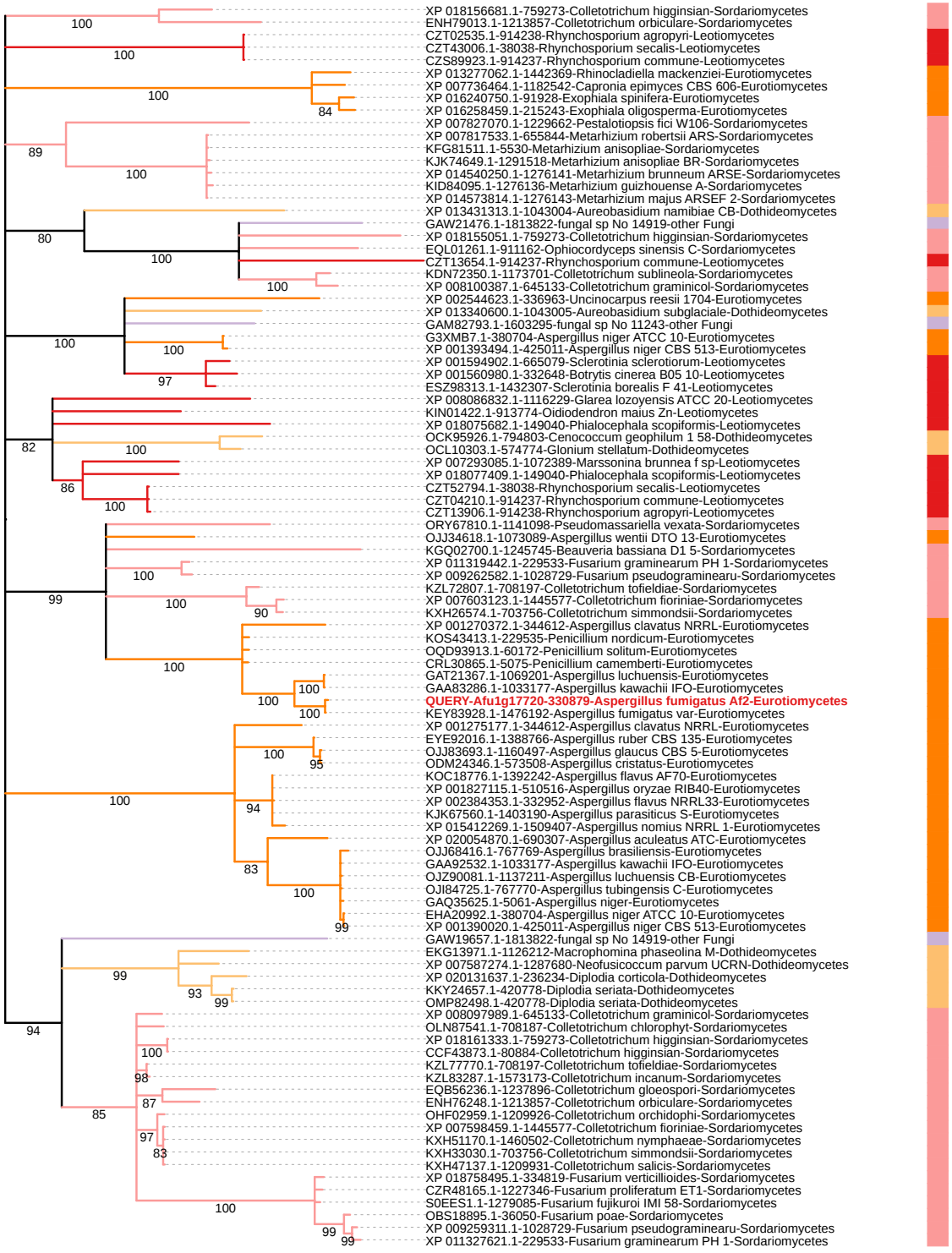
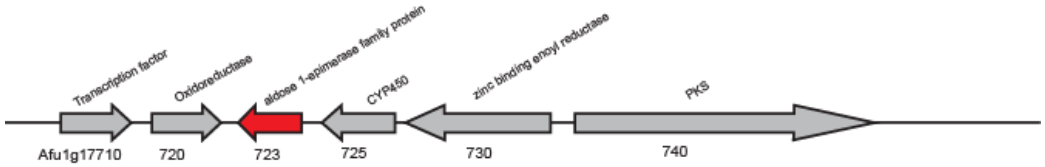
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses

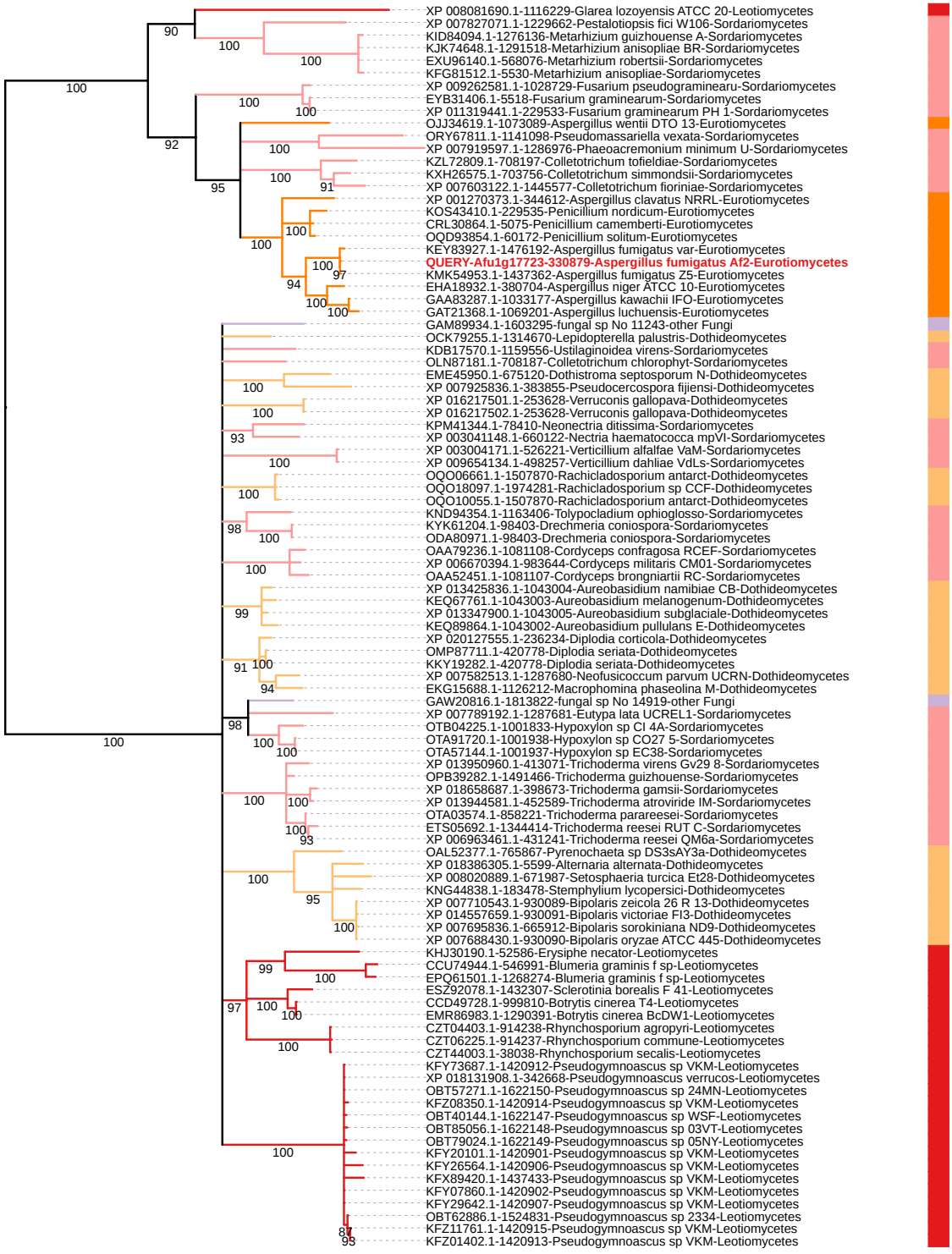


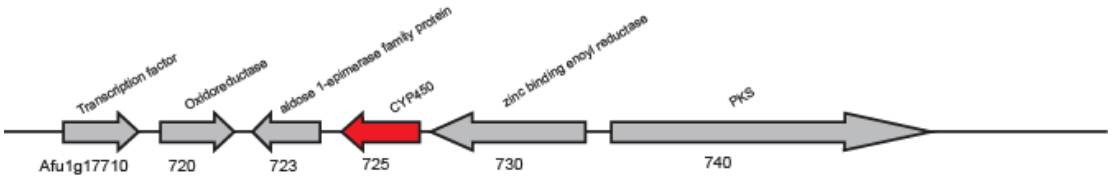


Tree scale: 0.1

Taxonomy

- Leotiomycetes
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses

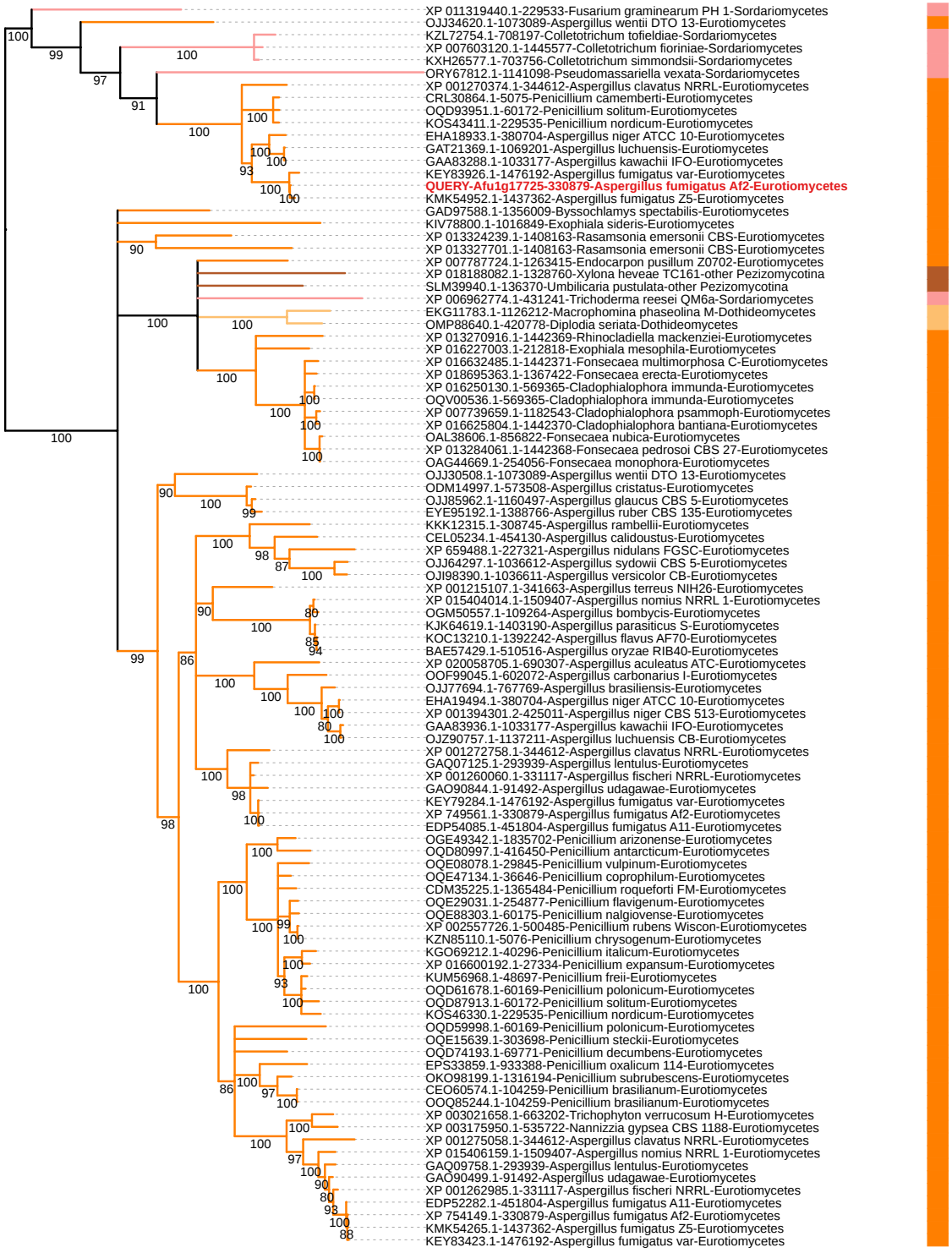


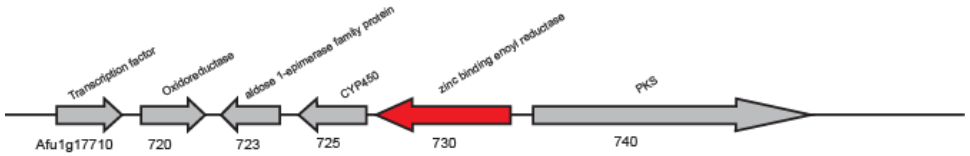


Tree scale: 0.1

Taxonomy

- Leotiomycetes
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses





Tree scale: 0.1

Taxonomy

- Leotiomycetes
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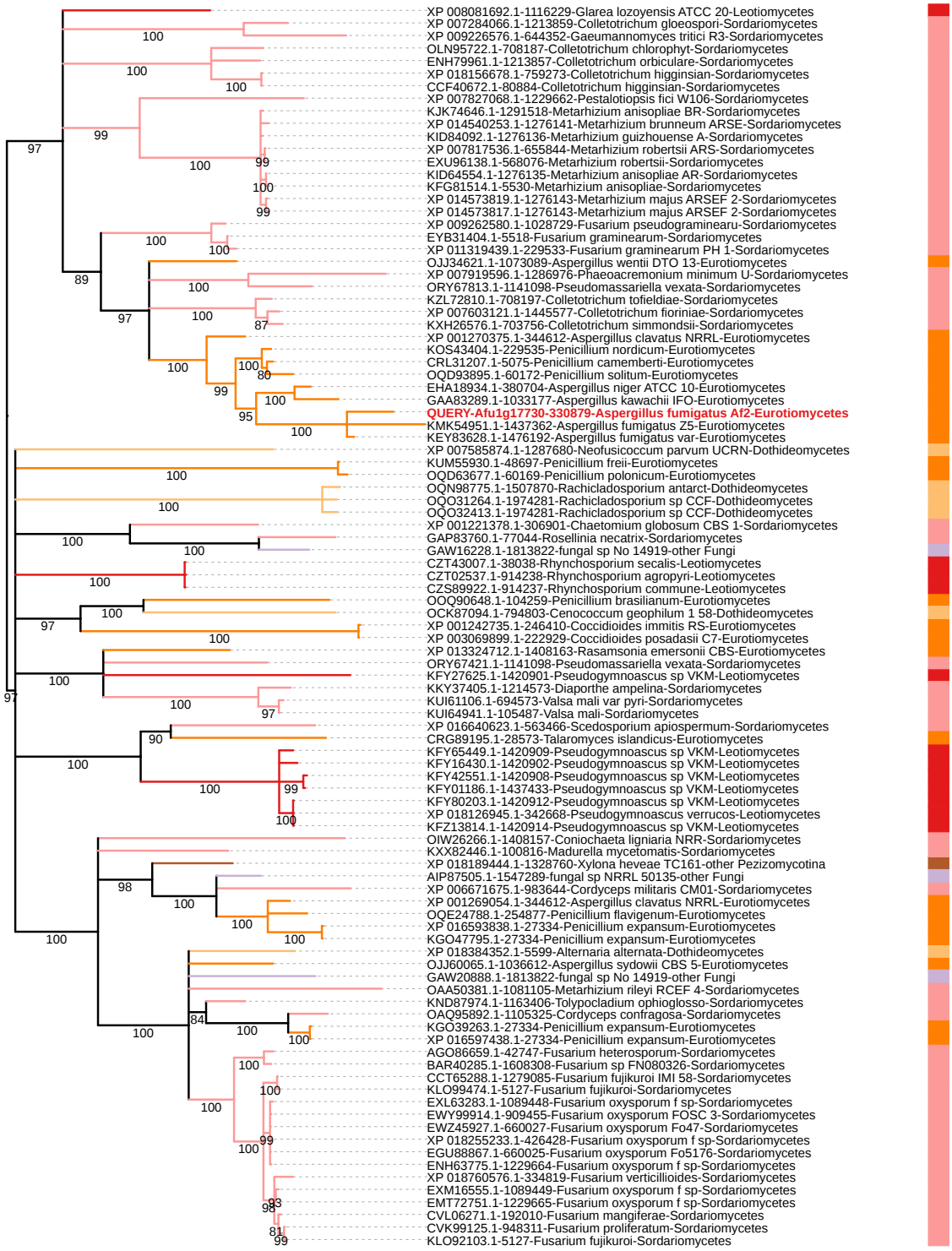
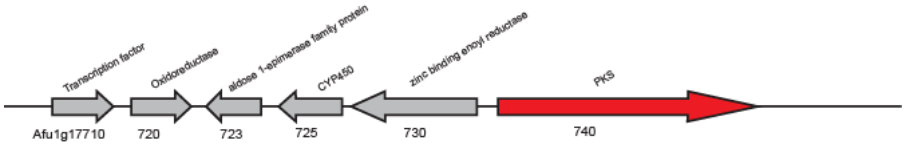
- Archaea
- Viruses





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Taxonomy

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- Dothideomycetes
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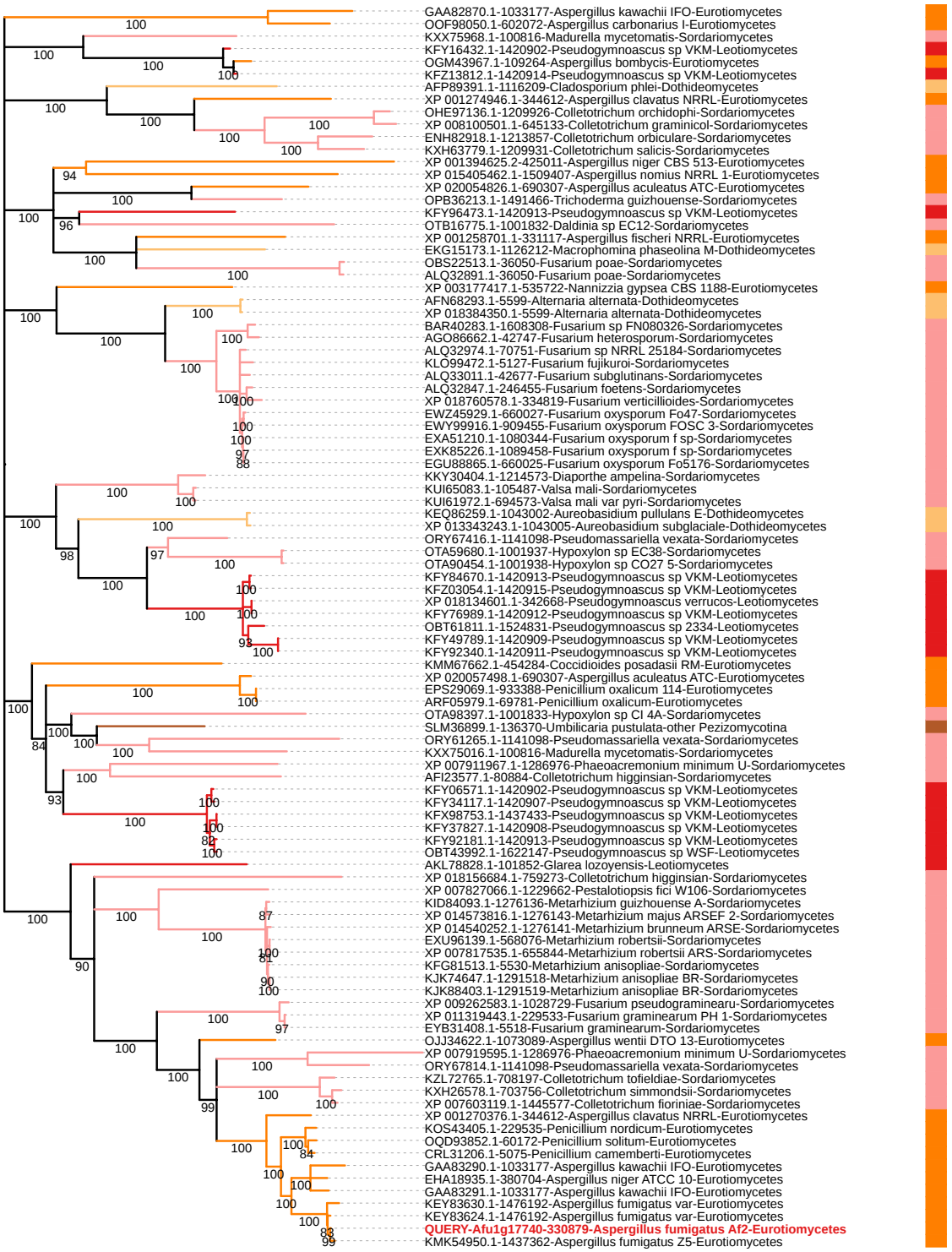
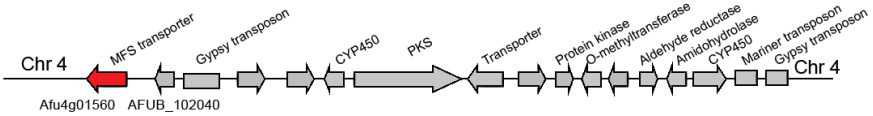
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses



Gene phylogenies of SM gene cluster 24. The phylogenies of several genes in this cluster are consistent with horizontal transfer between *Aspergillus fumigatus* and *Metarhizium* fungi.



Tree scale: 0.1

Taxonomy

- Leotiomycetes
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- Dothideomycetes
- other_Pezizomycotina
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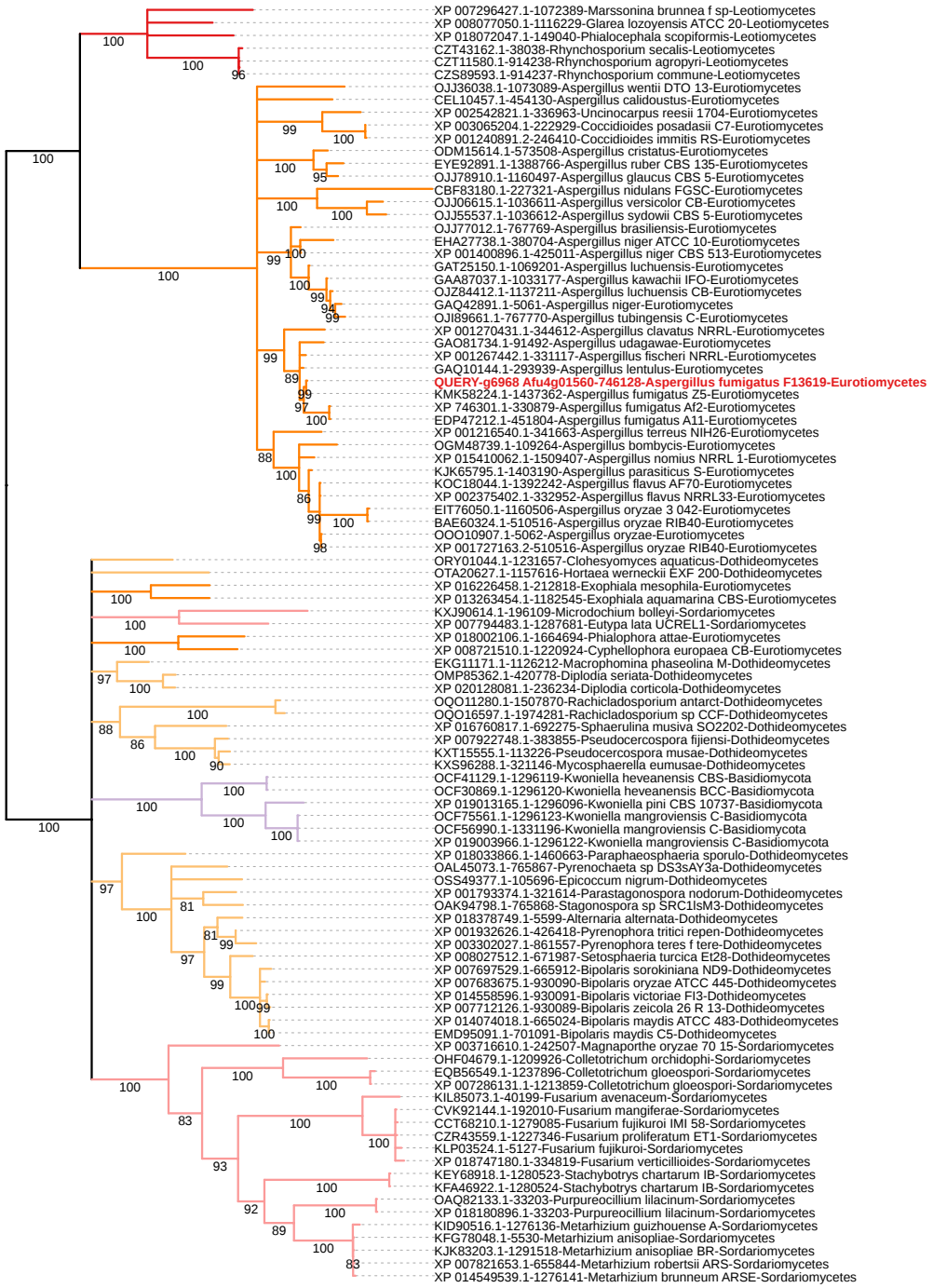
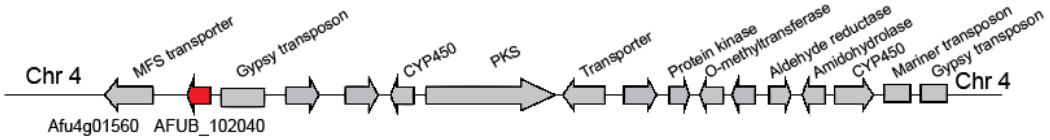
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses





Tree scale: 0.1

Taxonomy

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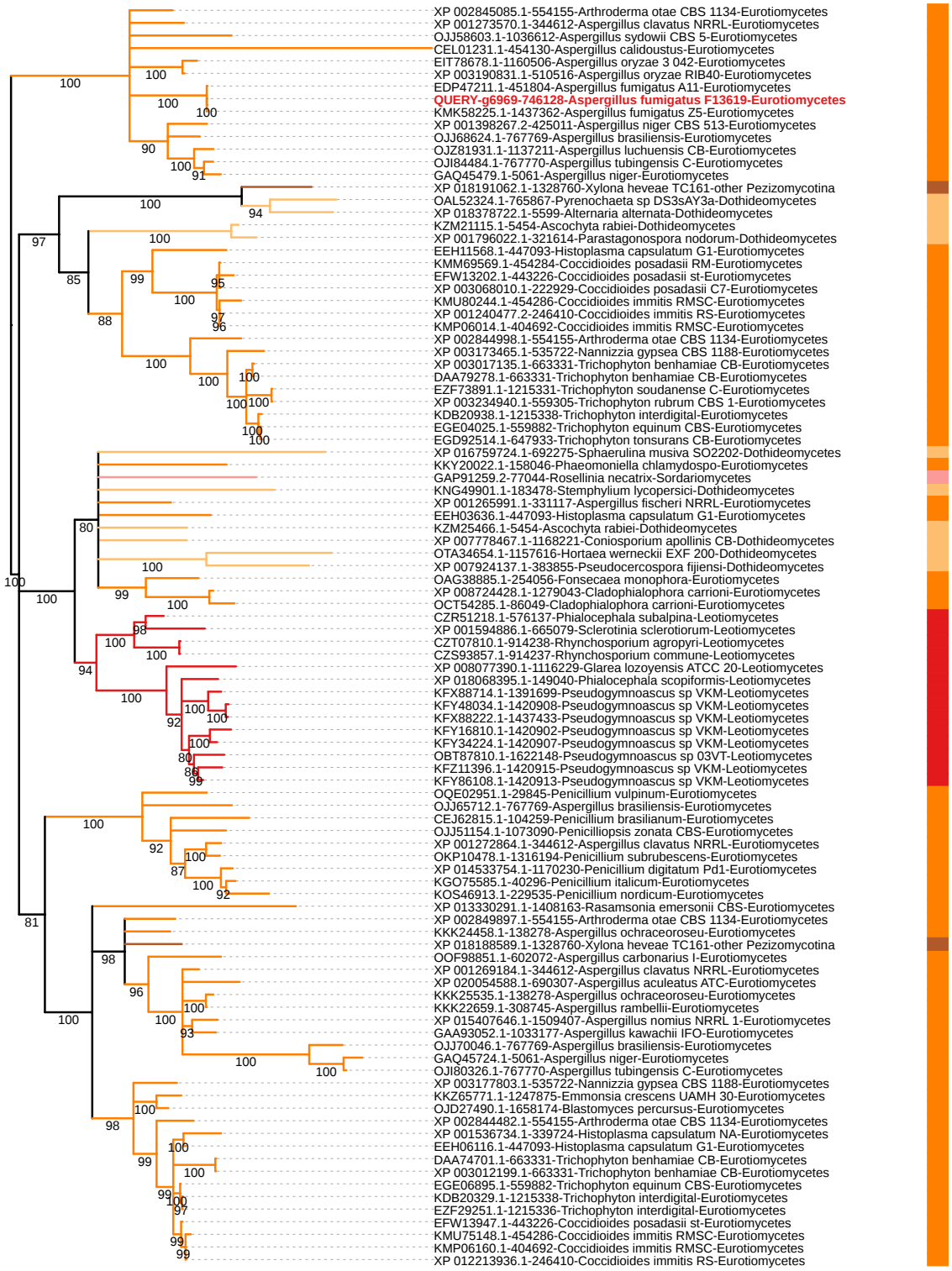

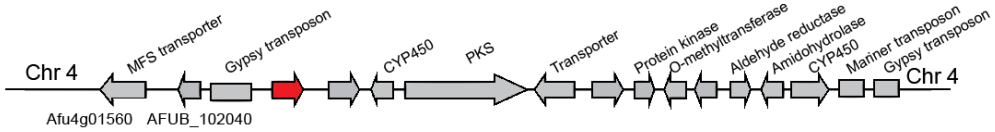

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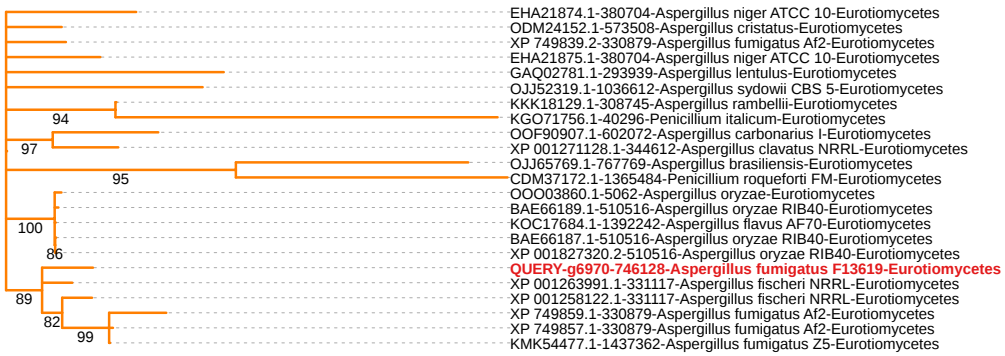
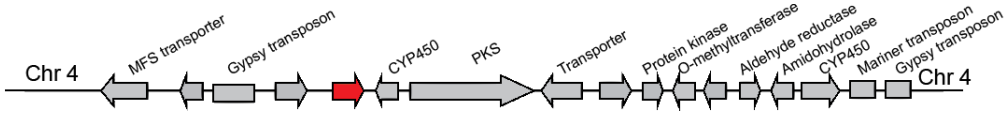


Taxonomy

- Leotiomycetes
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses



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Taxonomy

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- other_Eukaryota
- Bacteria
- Archaea
- Viruses

KFH70213.1-1069443-Mortierella verticillata-other Fungi
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OAL68712.1-34388-Trichophyton violaceum-Eurotiomycetes
XP 001215419.1-341663-Aspergillus terreus NIH26-Eurotiomycetes
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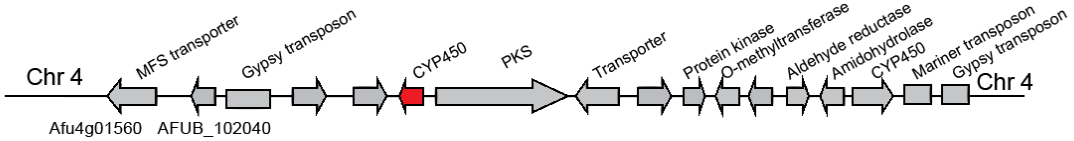
XP 002566875.1-500485-Penicillium rubens Wiscon-Eurotiomycetes
KZN87748.1-5076-Penicillium chrysogenum-Eurotiomycetes
CDM32672.1-1365484-Penicillium roqueforti FM-Eurotiomycetes
CDM32673.1-1365484-Penicillium roqueforti FM-Eurotiomycetes
XP 002566745.1-500485-Penicillium rubens Wiscon-Eurotiomycetes



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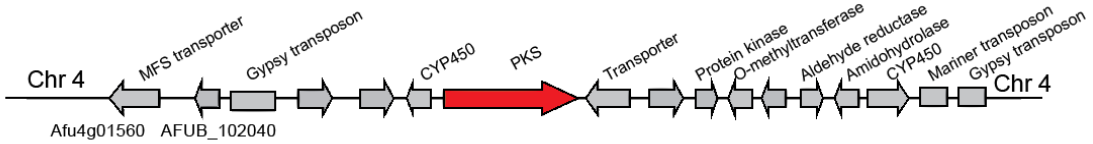
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses

KKP06687.1-5544-Trichoderma harzianum-Sordariomycetes
EKG13590.1-1126212-Macrophomina phaseolina M-Dothideomycetes
OCK74025.1-1314670-Lepidopterella palustris-Dothideomycetes
QUERY-g6972-746128-Aspergillus fumigatus F13619-Eurotiomycetes
KJK94812.1-1291519-Metarhizium anisopliae BR-Sordariomycetes
KJK74886.1-1291518-Metarhizium anisopliae BR-Sordariomycetes
CCD56079.1-999810-Botrytis cinerea T4-Leotiomycetes
EMR80975.1-1290391-Botrytis cinerea BcDW1-Leotiomycetes
XP 001550804.1-332648-Botrytis cinerea B05 10-Leotiomycetes
KJZ75226.1-1043627-Hirsutella minnesotensis-Sordariomycetes
KXX79210.1-100816-Madurella mycetomatis-Sordariomycetes
OCW41141.1-158607-Diaporthe helianthi-Sordariomycetes
XP 016643173.1-563466-Scedosporium apiospermum-Sordariomycetes
KIM92948.1-913774-Oidiodendron maius Zn-Leotiomycetes
GAW20844.1-1813822-fungal sp No 14919-other Fungi
KFA69530.1-1283841-Stachybotrys chlorohalona-Sordariomycetes
KEY72234.1-1280523-Stachybotrys chartarum IB-Sordariomycetes
KFA52277.1-1280524-Stachybotrys chartarum IB-Sordariomycetes
KKY16571.1-420778-Diplodia seriata-Dothideomycetes
XP 020125956.1-236234-Diplodia corticola-Dothideomycetes
XP 008027816.1-671987-Setosphaeria turcica Et28-Dothideomycetes
XP 003300446.1-861557-Pyrenophora teres f tere-Dothideomycetes
XP 001937751.1-426418-Pyrenophora tritici repen-Dothideomycetes
XP 007689110.1-930090-Bipolaris oryzae ATCC 445-Dothideomycetes
XP 014073566.1-665024-Bipolaris maydis ATCC 483-Dothideomycetes
EMD94542.1-701091-Bipolaris maydis C5-Dothideomycetes
KYK57378.1-98403-Drechmeria coniospora-Sordariomycetes
XP 014539081.1-1170230-Penicillium digitatum Pd1-Eurotiomycetes
KUM59361.1-48697-Penicillium freii-Eurotiomycetes
XP 018075919.1-149040-Phialocephala scopiformis-Leotiomycetes
OCK82927.1-1314670-Lepidopterella palustris-Dothideomycetes
OCK74982.1-1314670-Lepidopterella palustris-Dothideomycetes
OCK73526.1-1314670-Lepidopterella palustris-Dothideomycetes
XP 018157389.1-759273-Colletotrichum higginsian-Sordariomycetes
KZZ96510.1-1081109-Aschersonia aleyrodis RCE-Sordariomycetes
KDN69202.1-1173701-Colletotrichum sublineola-Sordariomycetes
XP 008098964.1-645133-Colletotrichum graminicol-Sordariomycetes
XP 007286629.1-1213859-Colletotrichum gloeospori-Sordariomycetes
EQB52534.1-1237896-Colletotrichum gloeospori-Sordariomycetes
KDN60187.1-1173701-Colletotrichum sublineola-Sordariomycetes
KXH47916.1-1209931-Colletotrichum salicis-Sordariomycetes
OCW42213.1-158607-Diaporthe helianthi-Sordariomycetes
KXH50180.1-703756-Colletotrichum simmondsii-Sordariomycetes
OIW33137.1-1408157-Coniochaeta ligniaria NRR-Sordariomycetes
KIM94742.1-913774-Oidiodendron maius Zn-Leotiomycetes
KXJ85092.1-196109-Microdochium bolleyi-Sordariomycetes
KXJ85939.1-196109-Microdochium bolleyi-Sordariomycetes
XP 001260852.1-331117-Aspergillus fischeri NRRL-Eurotiomycetes
OJJ40738.1-1073089-Aspergillus wentii DTO 13-Eurotiomycetes
XP 001271874.1-344612-Aspergillus clavatus NRRL-Eurotiomycetes
XP 001267016.1-331117-Aspergillus fischeri NRRL-Eurotiomycetes
KMK54731.1-1437362-Aspergillus fumigatus Z5-Eurotiomycetes
XP 751828.1-330879-Aspergillus fumigatus Af2-Eurotiomycetes
EME39031.1-675120-Dothistroma septosporum N-Dothideomycetes
AAC49196.1-162425-Aspergillus nidulans-Eurotiomycetes
XP 681087.1-227321-Aspergillus nidulans FGSC-Eurotiomycetes
KKK21817.1-308745-Aspergillus rambellii-Eurotiomycetes
XP 020053364.1-690307-Aspergillus aculeatus ATC-Eurotiomycetes
KND91002.1-1163406-Tolypocladium ophioglosso-Sordariomycetes
OAL39677.1-856822-Fonsecaea nubica-Eurotiomycetes
OAG34162.1-254056-Fonsecaea monophora-Eurotiomycetes
XP 013280246.1-1442368-Fonsecaea pedrosoi CBS 27-Eurotiomycetes
OAG44384.1-254056-Fonsecaea monophora-Eurotiomycetes
KXT06680.1-321146-Mycosphaerella eumusae-Dothideomycetes
XP 018159597.1-759273-Colletotrichum higginsian-Sordariomycetes
OLN90223.1-708187-Colletotrichum chlorophyt-Sordariomycetes
KZL71764.1-1573173-Colletotrichum incanum-Sordariomycetes
KZL63447.1-708197-Colletotrichum tofieldiae-Sordariomycetes
XP 008098516.1-645133-Colletotrichum graminicol-Sordariomycetes
XP 003717434.1-242507-Magnaporthe oryzae 70 15-Sordariomycetes
OOQ86043.1-104259-Penicillium brasilianum-Eurotiomycetes
OCK77653.1-1314670-Lepidopterella palustris-Dothideomycetes
GAD91552.1-1356009-Byssochlamys spectabilis-Eurotiomycetes
OQD73863.1-69771-Penicillium decumbens-Eurotiomycetes
CEJ57600.1-104259-Penicillium brasilianum-Eurotiomycetes
EPS31372.1-933388-Penicillium oxalicum 114-Eurotiomycetes
KMU74509.1-454286-Coccidioides immitis RMSC-Eurotiomycetes
KMM66554.1-454284-Coccidioides posadasii RM-Eurotiomycetes
EFW13723.1-443226-Coccidioides posadasii st-Eurotiomycetes
XP 003069072.1-222929-Coccidioides posadasii C7-Eurotiomycetes
XP 001240202.2-246410-Coccidioides immitis RS-Eurotiomycetes
KMP02584.1-404692-Coccidioides immitis RMSC-Eurotiomycetes
KFZ09804.1-1420914-Pseudogymnoascus sp VKM-Leotiomycetes
OBT43052.1-1622147-Pseudogymnoascus sp WSF-Leotiomycetes
XP 012740108.1-658429-Pseudogymnoascus destruct-Leotiomycetes
KFY12939.1-1420901-Pseudogymnoascus sp VKM-Leotiomycetes
KFY74280.1-1420912-Pseudogymnoascus sp VKM-Leotiomycetes
XP 018132394.1-342668-Pseudogymnoascus verrucos-Leotiomycetes
KFY92053.1-1420913-Pseudogymnoascus sp VKM-Leotiomycetes
KFZ12582.1-1420915-Pseudogymnoascus sp VKM-Leotiomycetes
OQE06029.1-29845-Penicillium vulpinum-Eurotiomycetes
OQE43268.1-36646-Penicillium coprophilum-Eurotiomycetes
OGM48142.1-109264-Aspergillus bombycis-Eurotiomycetes
KJK61356.1-1403190-Aspergillus parasiticus S-Eurotiomycetes
KOC10931.1-1392242-Aspergillus flavus AF70-Eurotiomycetes
OOO07101.1-5062-Aspergillus oryzae-Eurotiomycetes
EIT78191.1-1160506-Aspergillus oryzae 3 042-Eurotiomycetes
BAE65144.1-510516-Aspergillus oryzae RIB40-Eurotiomycetes
BAJ04387.1-5062-Aspergillus oryzae-Eurotiomycetes
XP 001826277.2-510516-Aspergillus oryzae RIB40-Eurotiomycetes



Tree scale: 0.1

Taxonomy

- Leotiomycetes
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
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- Bacteria
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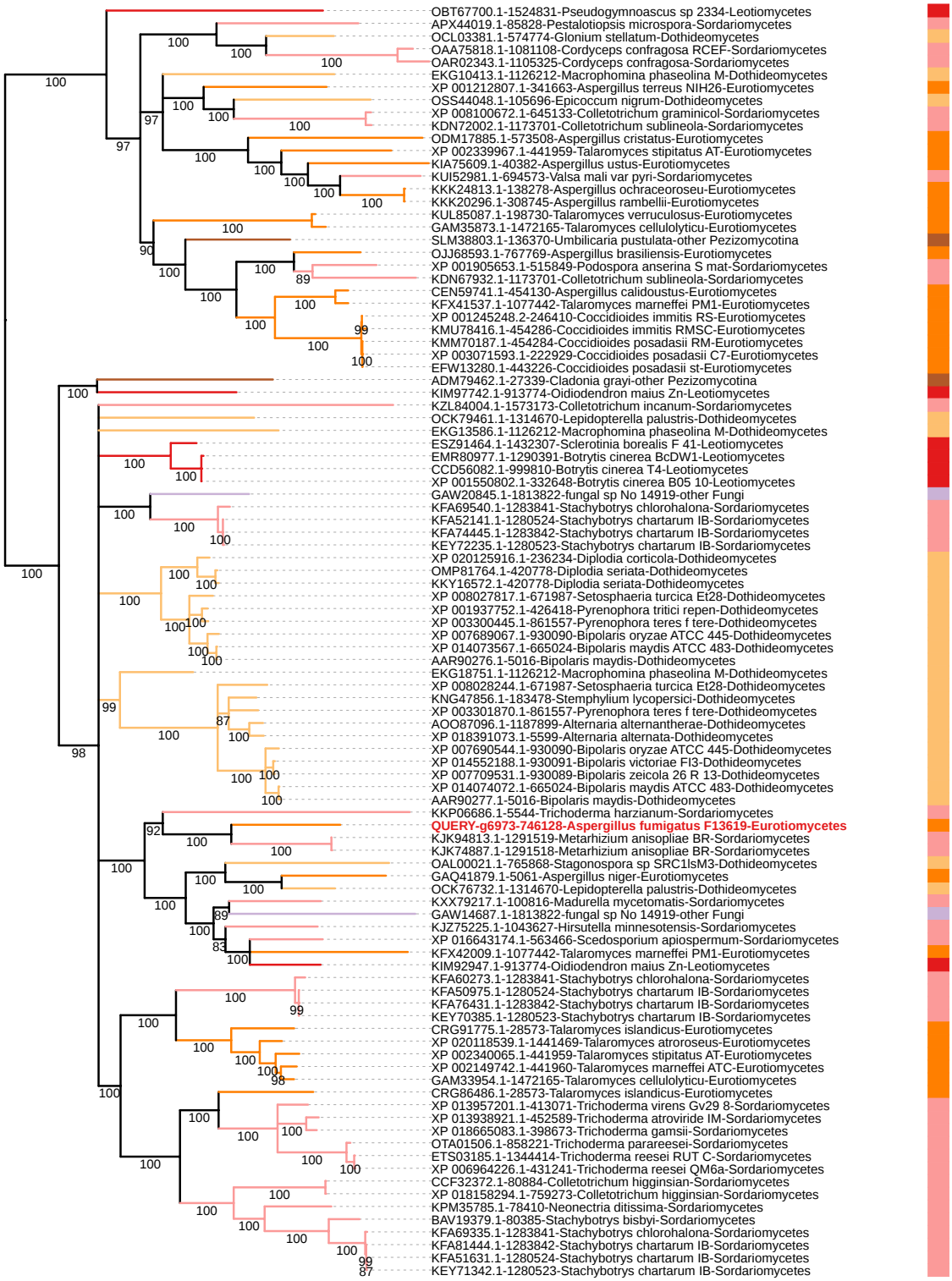
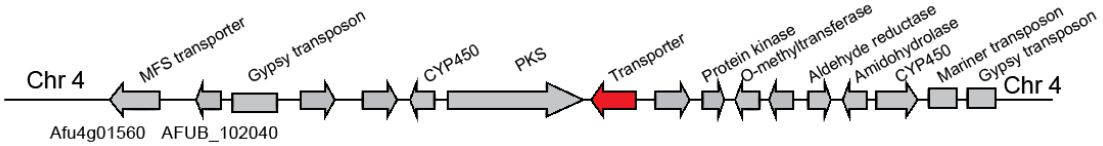
- Viruses





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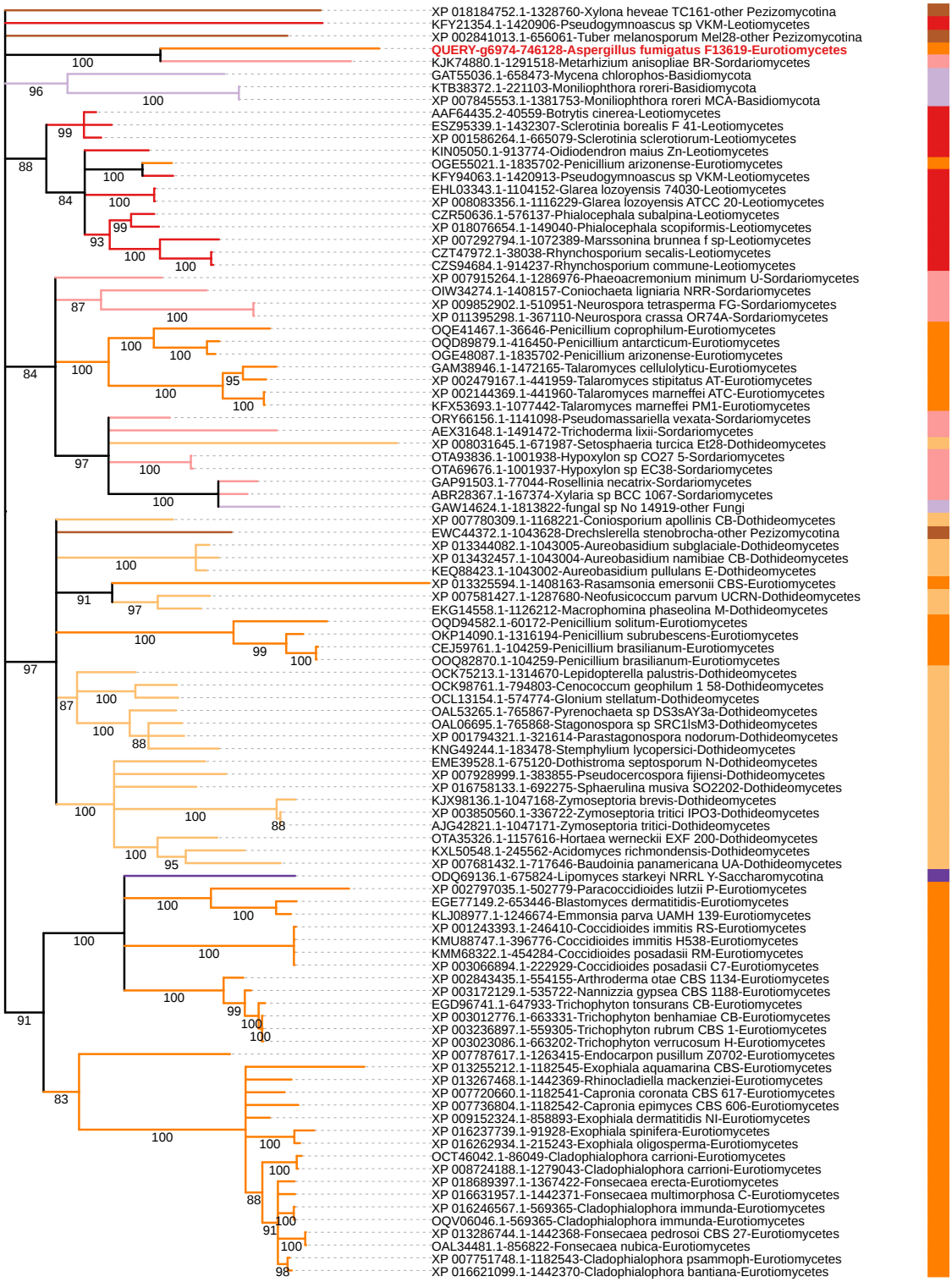

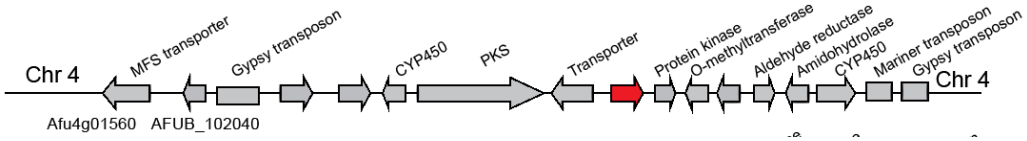

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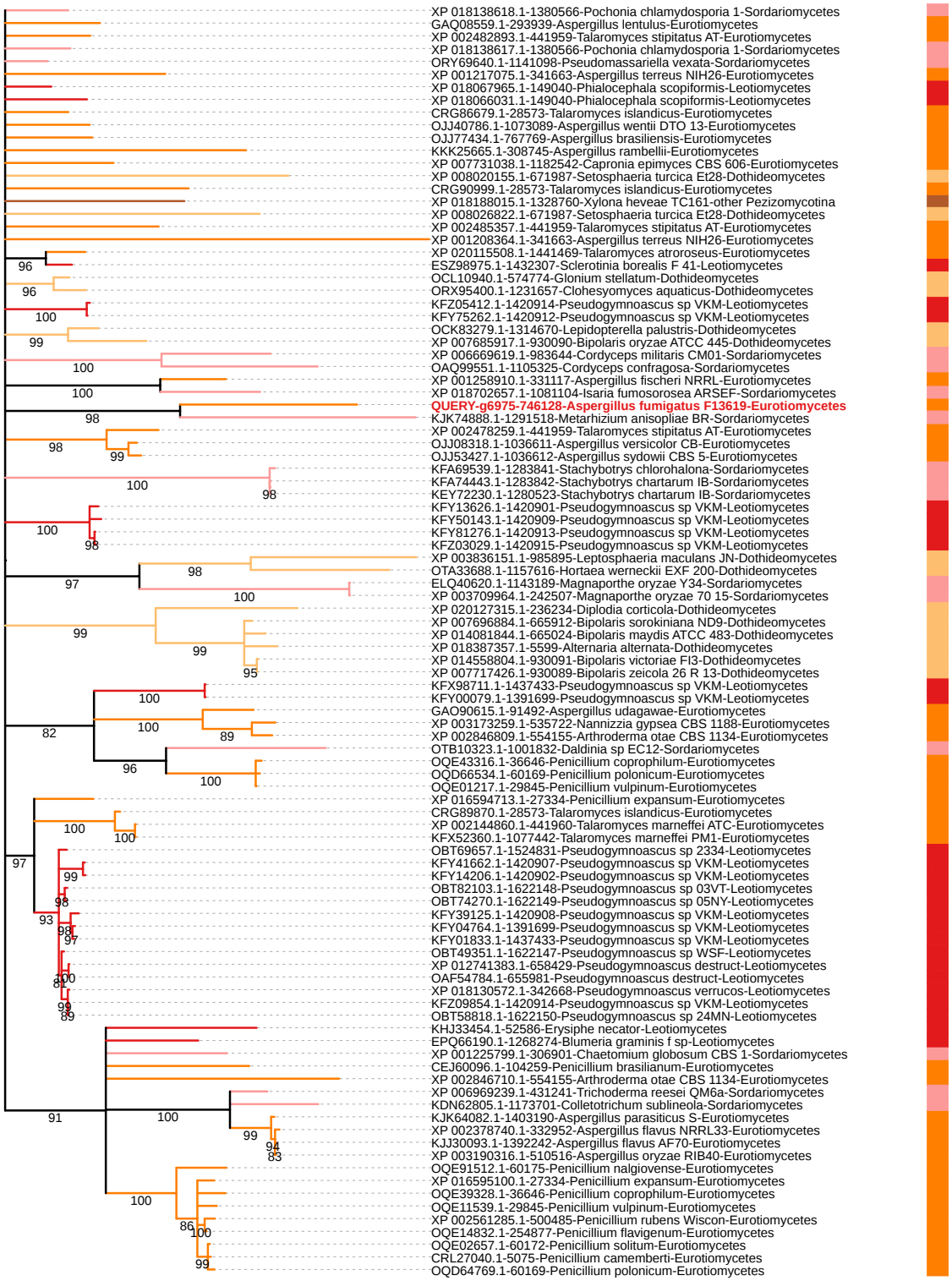

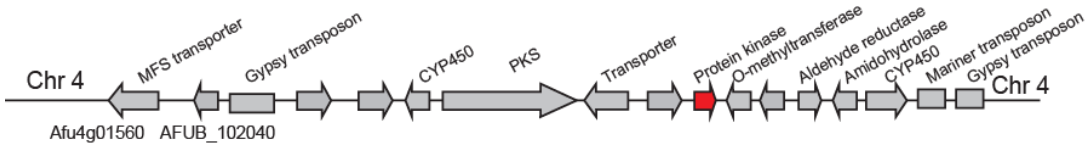

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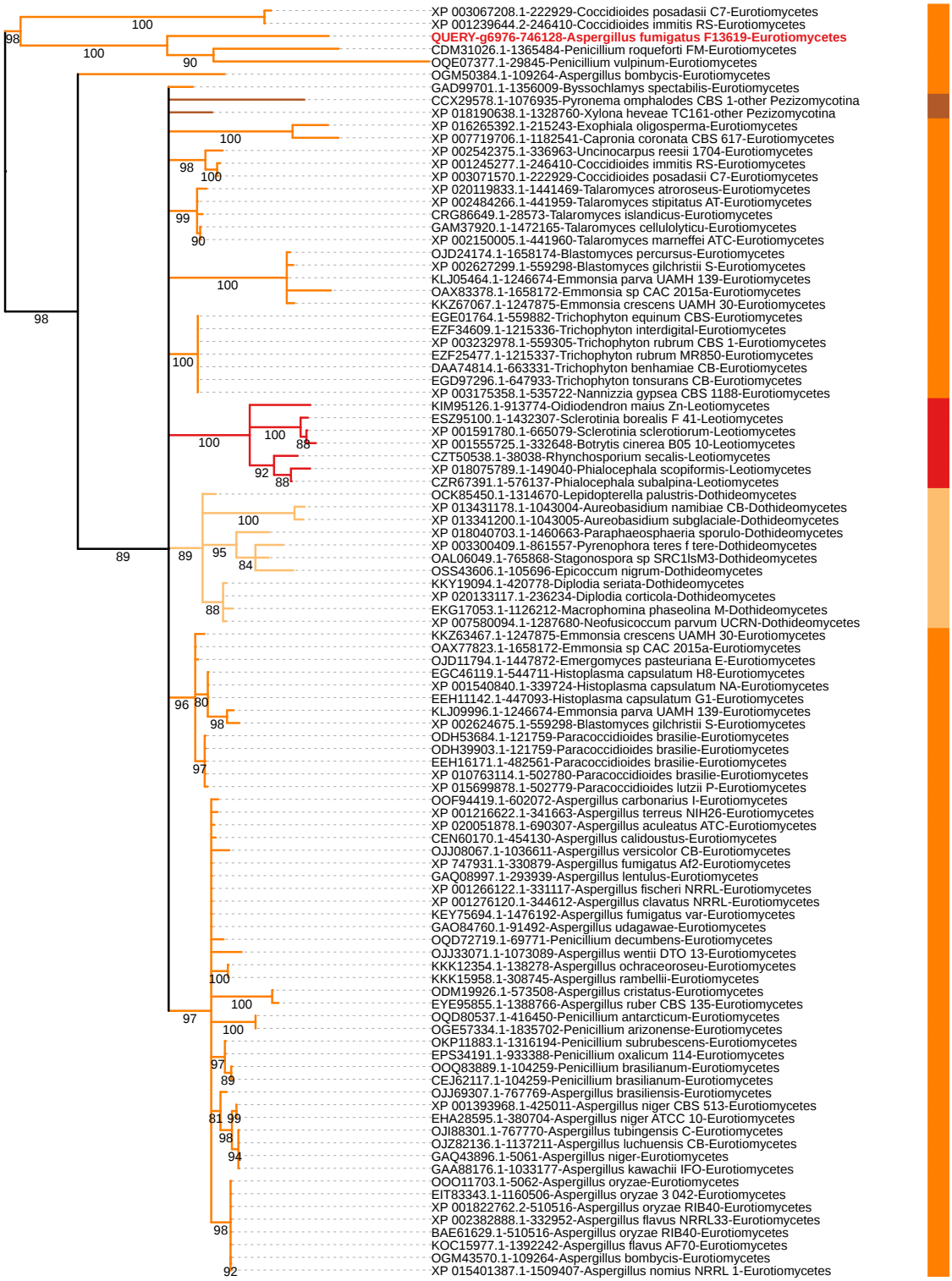

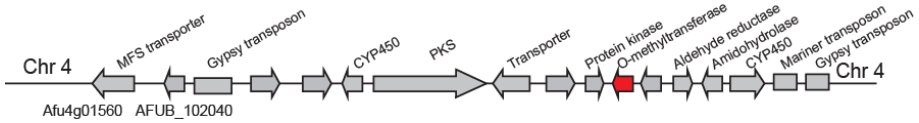

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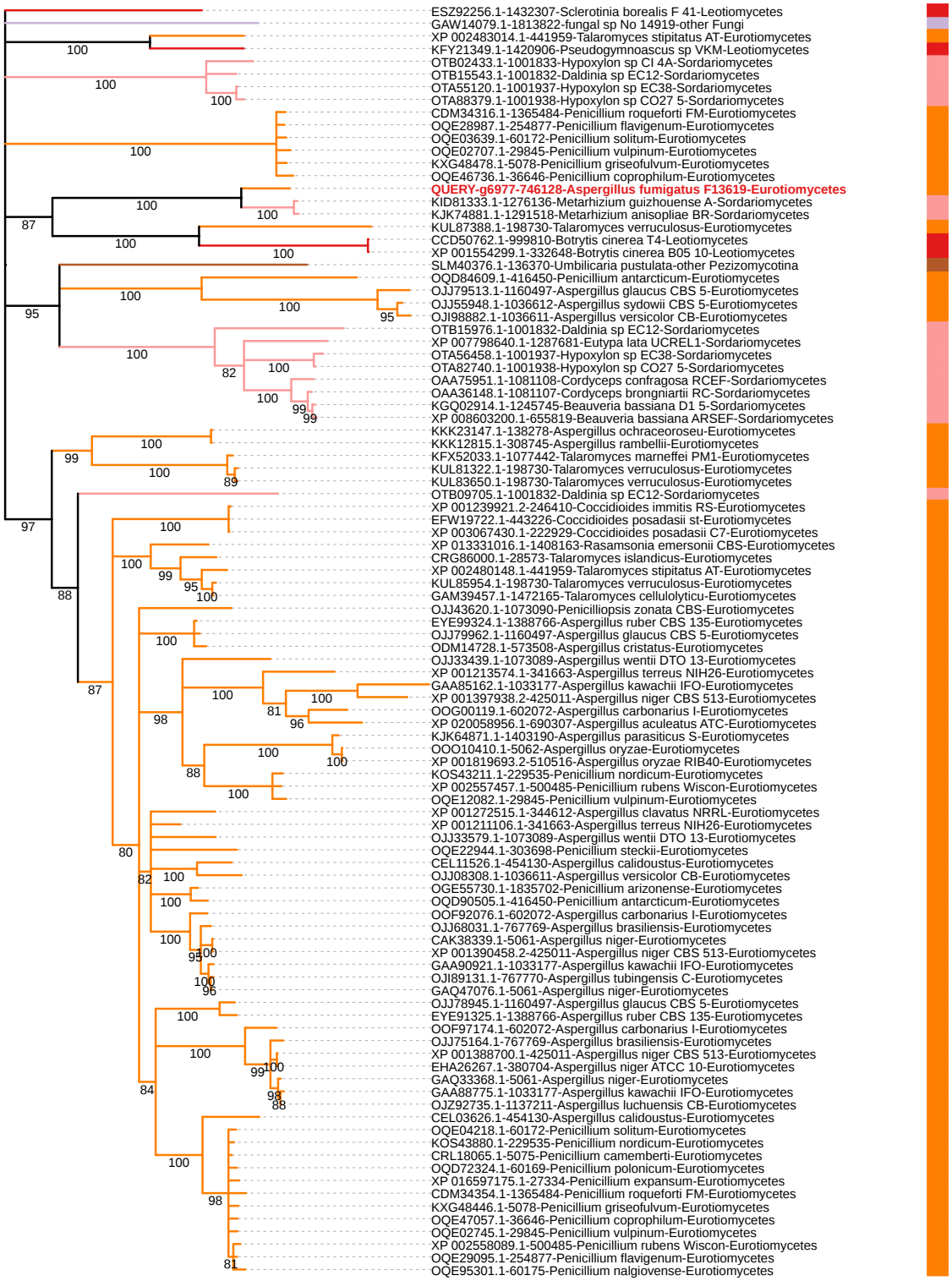

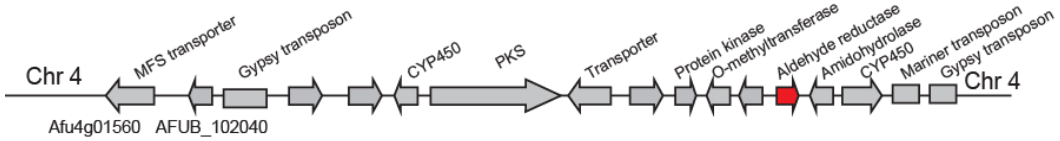

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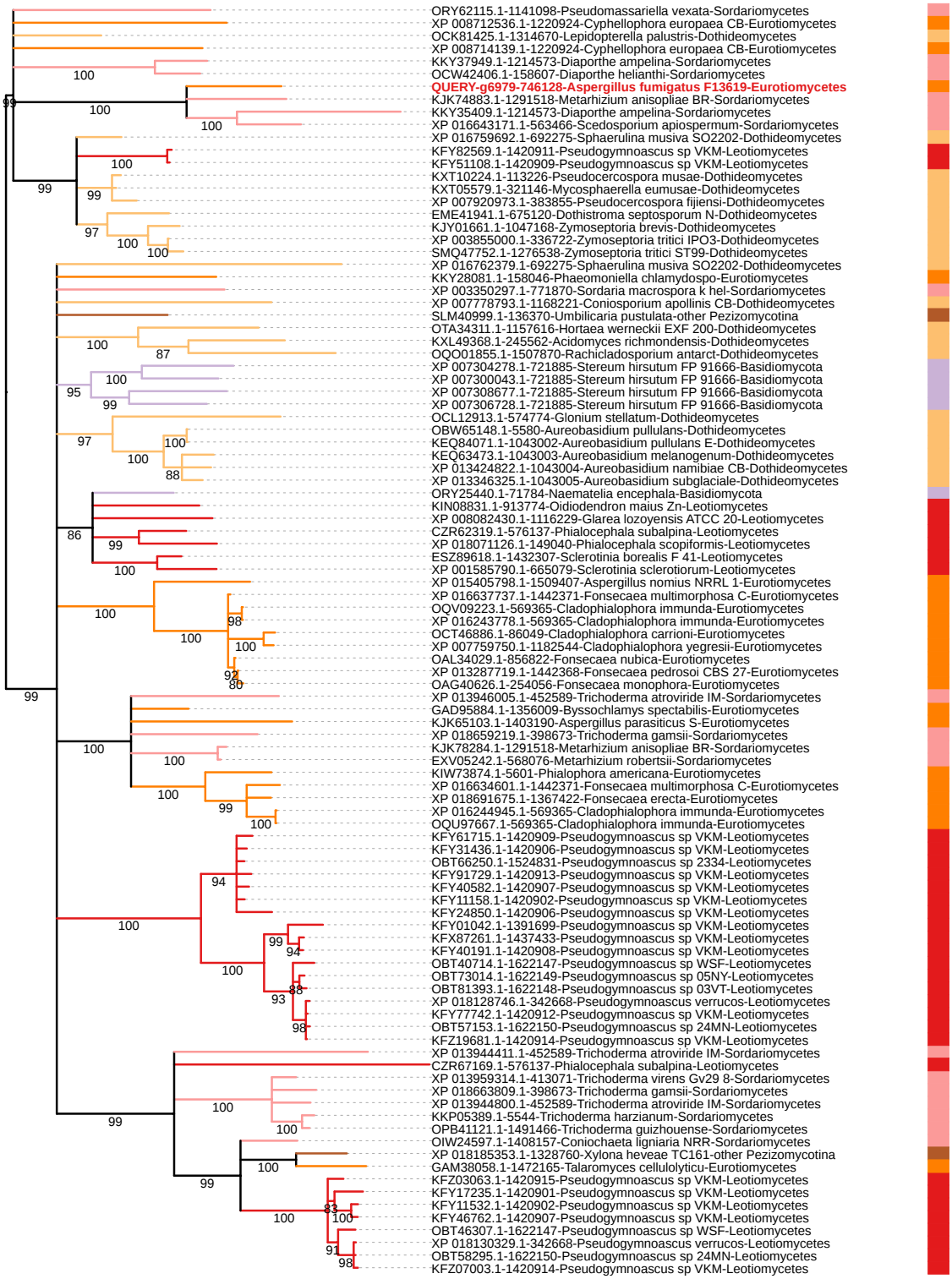

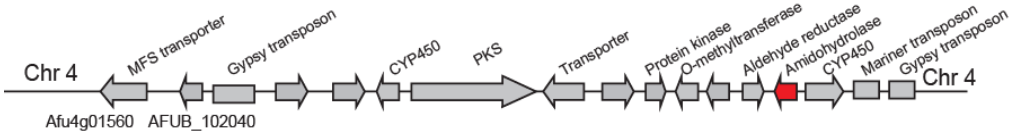

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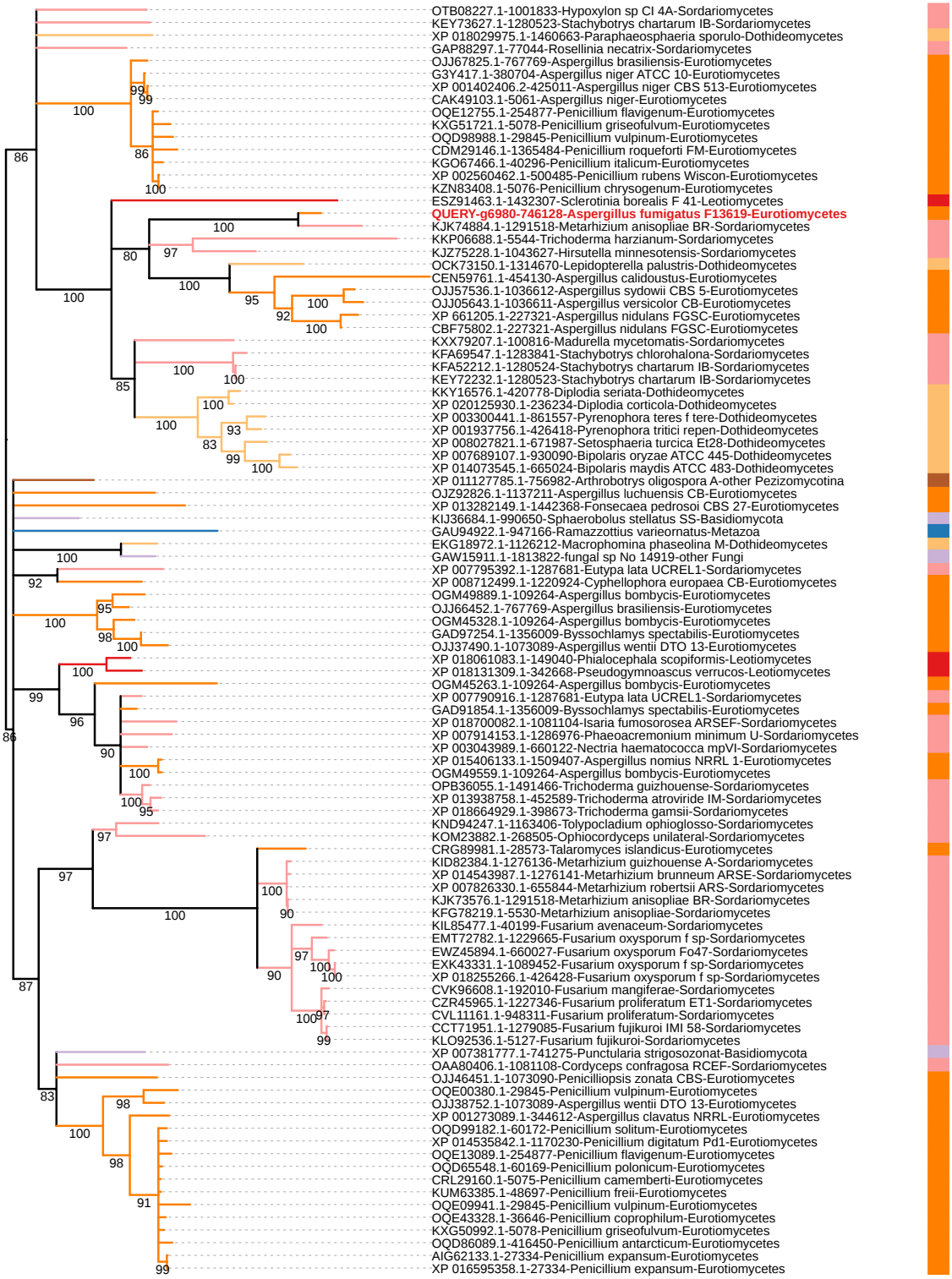

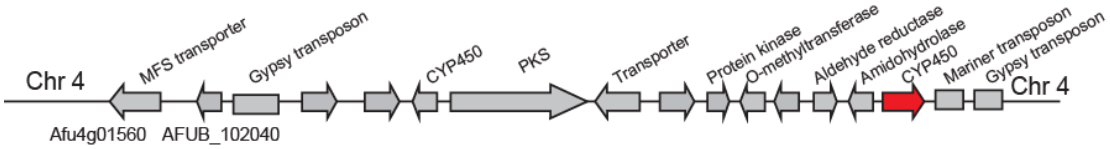

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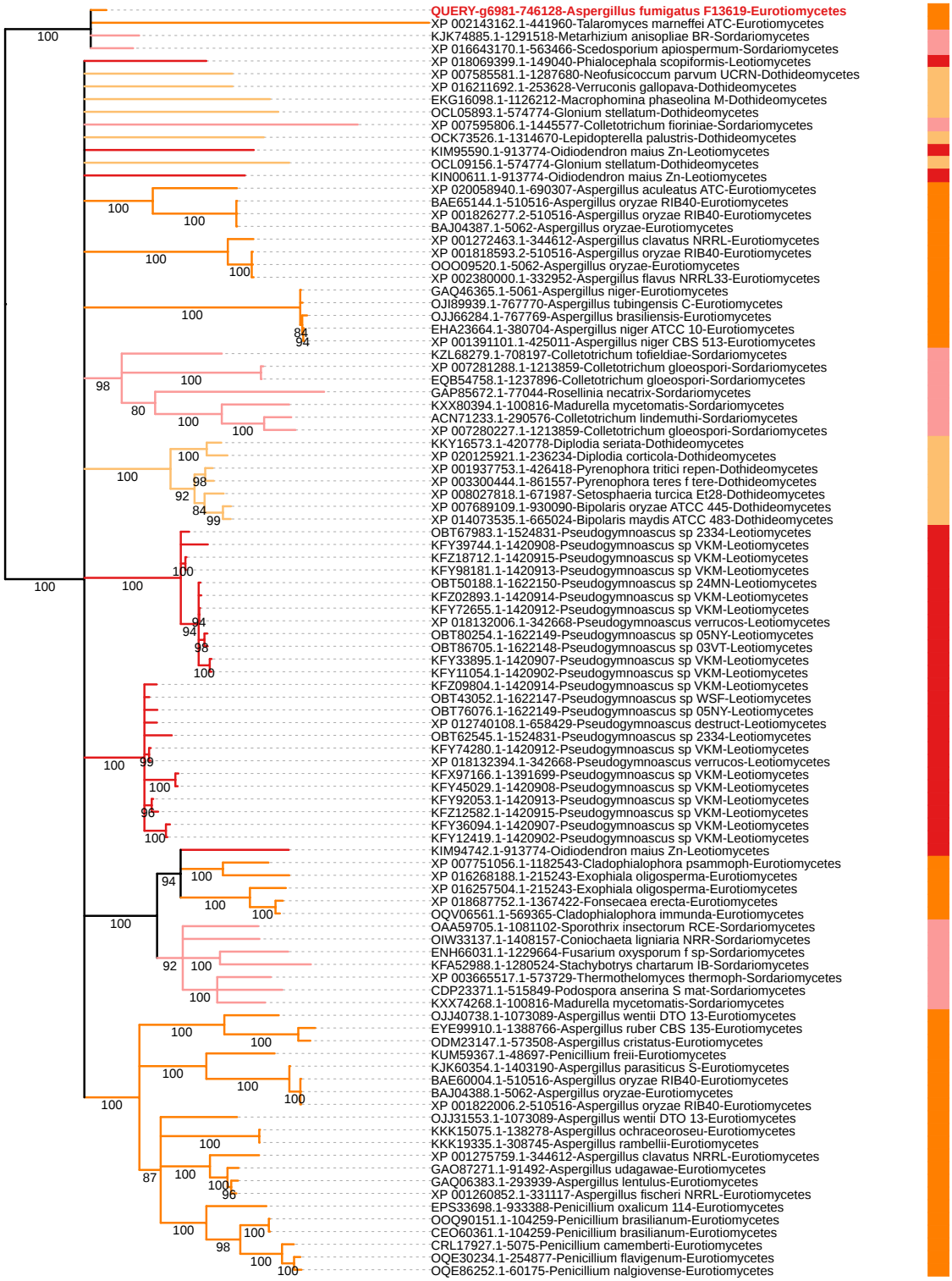
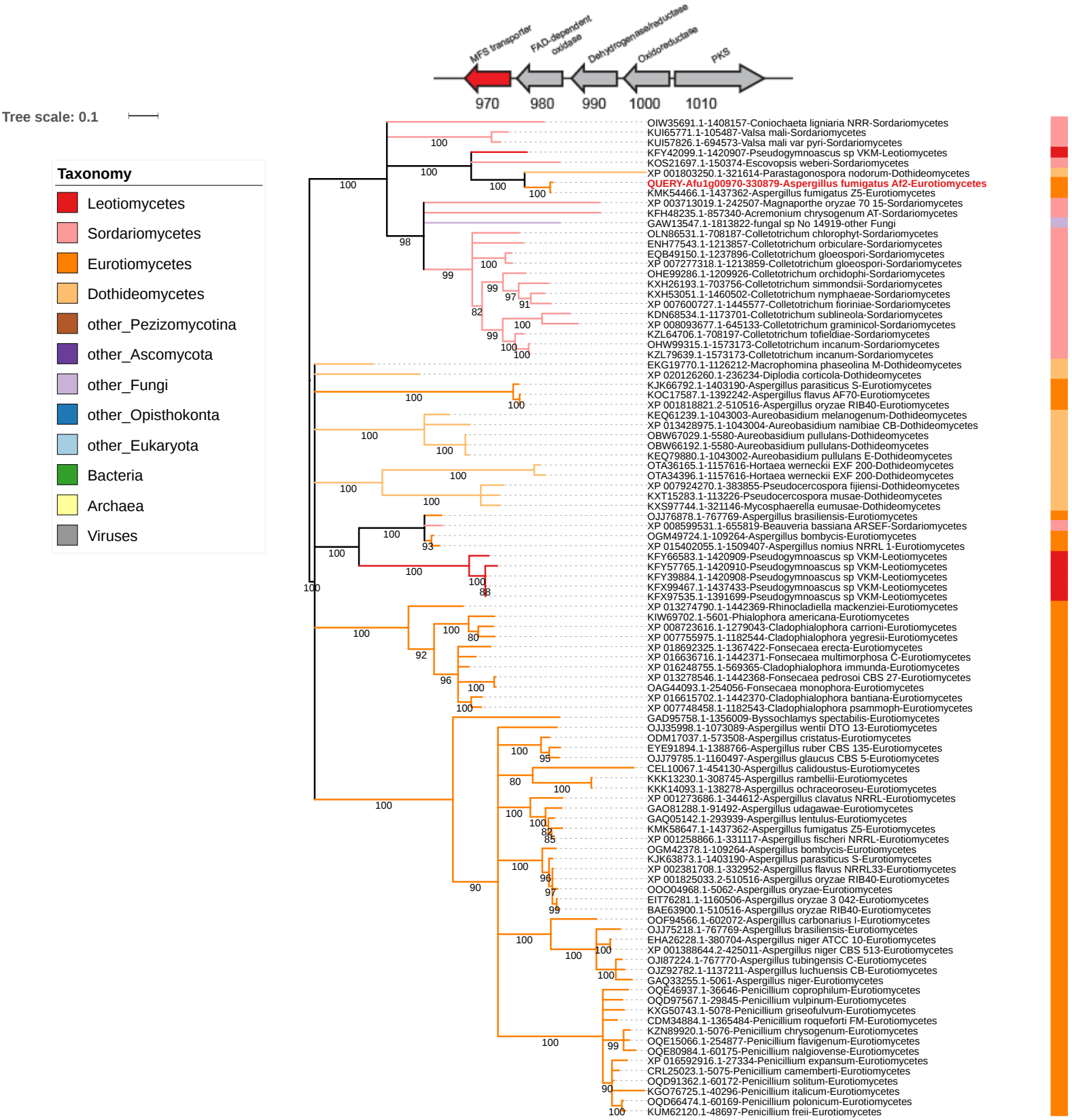
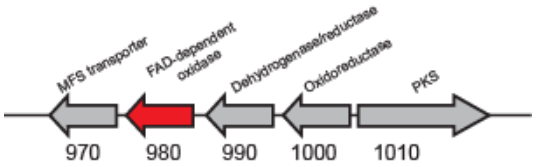


Gene phylogenies of the mobile SM gene cluster 1. These phylogenies are consistent with horizontal transfer between Eurotiomycete, Dothidiomycete, Leotiomycete, and Sordariomycete fungi.





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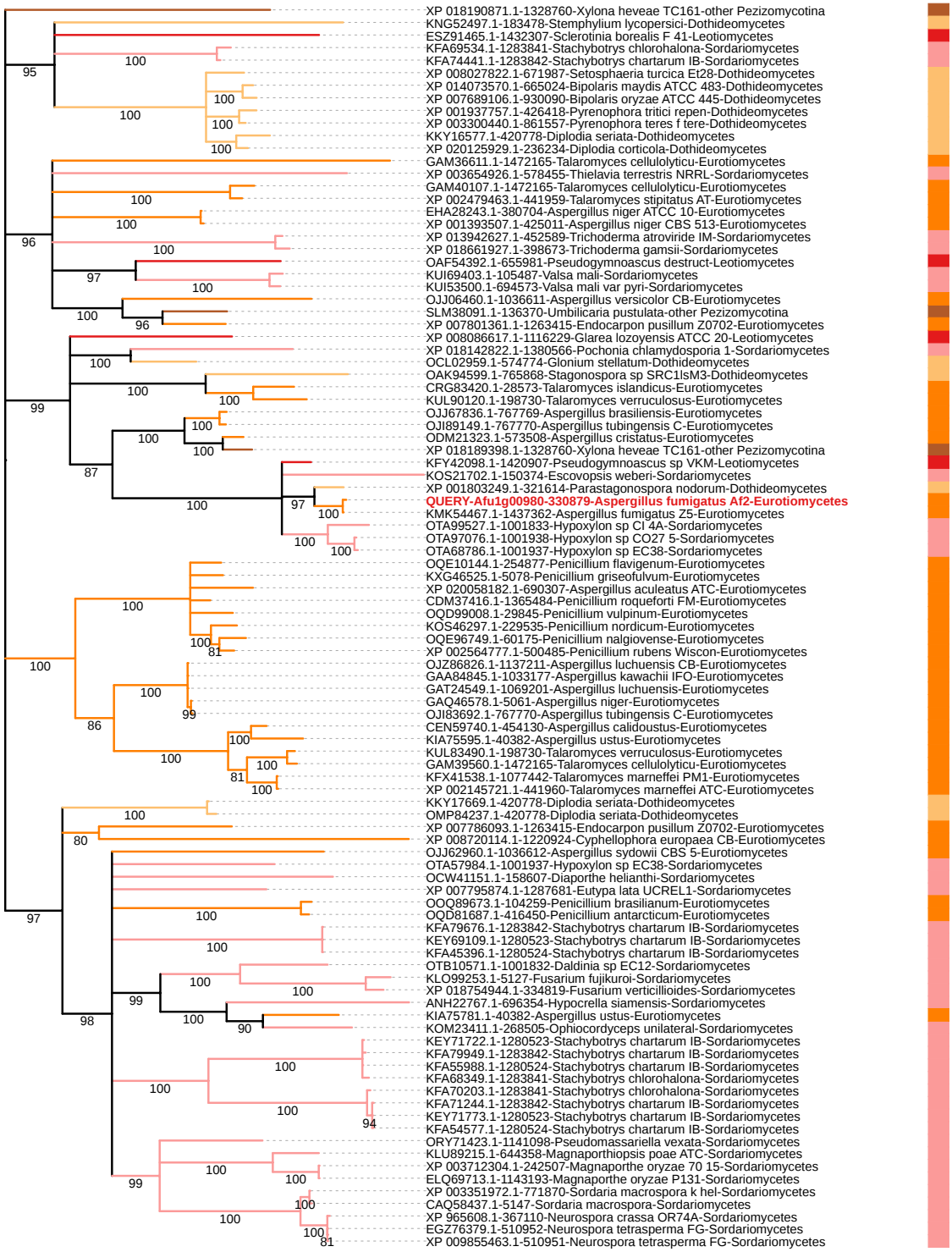

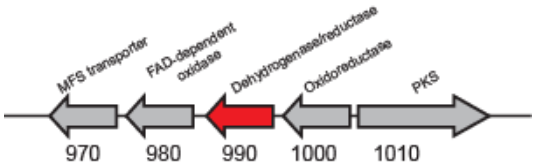

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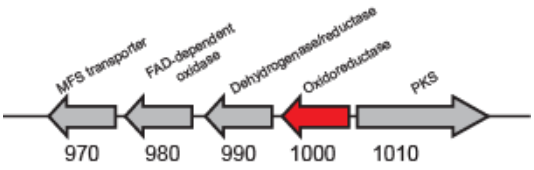

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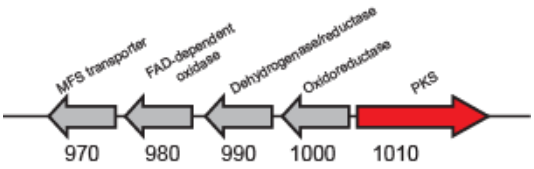

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