1 Phylogenomics and evolution of secondary metabolism in plant-associated fungi

2
3
4 Joseph W. Spatafora^a and Kathryn E. Bushley^b

5
6
7 ^aDepartment of Botany and Plant Pathology, Oregon State University, Corvallis, OR USA 97331

8
9 ^bDepartment of Plant Biology, University of Minnesota, St. Paul, MN USA 55108

10
11
12 Corresponding author: Joseph W. Spatafora (joseph.spatafora@oregonstate.edu)

13
14 **Abstract.**

15 Fungi produce a myriad of secondary metabolites, compounds that are not required for basic
16 cellular processes, but are thought to be central to ecological functions. Genomic sequencing of
17 fungi has revealed a greater diversity of secondary metabolism than previously realized,
18 including novel taxonomic distributions of known compounds and uncharacterized gene clusters
19 in well-studied organisms. Here we provide an overview of the major groups of metabolites,
20 their ecological functions, the genetic systems that produce them, and the patterns and processes
21 associated with evolutionary diversification of secondary metabolism in plant-associated
22 filamentous ascomycetes.

Introduction.

Fungi occupy all major environmental niches on the planet and provide ecological services associated with nutrient cycling in natural and managed ecosystems. In doing so, they establish numerous interactions with plants that range from beneficial to antagonistic. Although their morphological diversity is limited, their biochemical diversity is extraordinary. A significant portion of this biochemical diversity consists of secondary metabolites, small organic molecules that are not essential for an organism's survival. These have been shown to be involved in numerous biological functions including morphological development, environmental sensing, microbial interactions and pathogenicity [1]. Here we discuss significant examples of the diversification of secondary metabolism among plant-associated fungi as revealed by recent advances in genomics.

Diversity of fungal secondary metabolism.

Fungal secondary metabolites are produced by complex biosynthetic pathways that often result in homologous genomic machinery being involved in the production of chemically distinct compounds. The major groups of secondary metabolites produced by fungi include – but are not limited to – polyketides, peptides, alkaloids, and terpenes (Fig. 1). The core genes synthesizing these metabolites include polyketide synthases (PKS), non-ribosomal peptide synthetases

(NRPS), members of the DMATS/CymD prenyl transferase family (DMATS), and terpene synthases (TS) among others [1]. In fungi, these core genes are often found in gene clusters that include associated genes necessary for metabolite biosynthesis, regulation and transport [2]. These gene clusters are frequently found in subterminal regions of chromosomes and their expression is controlled by epigenetic regulation of heterochromatin [3]. Many clusters are considered “silent” in that their expression has not been observed in experimental settings and they have not been definitively linked to a metabolite [4]. While the majority of fungal secondary metabolism has been characterized through molecular genetics and chemical approaches, sequencing of fungal genomes has uncovered a greater potential of secondary metabolism, even well-characterized species [e.g., 5,6]. Although secondary metabolism exists across Kingdom Fungi (Fig 2), here we focus on the well-characterized plant endophytes and plant pathogens from the Ascomycota classes Sordariomycetes, Dothideomycetes and Eurotiomycetes.

Endophytes.

Fungal endophytes of plants reside inside of plant tissues (e.g., leaves, roots), but do not cause any significant disease on the host plant. They are categorized into multiple functional groups including the clavicipitaceous endophytes, which are frequently vertically transmitted in host seeds, and the non-Clavicipitaceae endophytes of angiosperms and gymnosperms, which are mostly horizontally transmitted by spores [7]. The clavicipitaceous endophytes (e.g., *Epichloë*) are the best studied with respect to their ecology, evolution, and genetics of secondary metabolism [8,9•]. These fungi produce a number of secondary metabolites including ergot alkaloids, indole-diterpenes, lolines and peramine, which have been demonstrated to elicit antiherbivory effects on insects and in some cases mammals. Some are known to function as

analogs to neurotransmitters, resulting in vasoconstriction, tremors, and severe disorientation or hallucinations in humans. In addition to endophytes, Clavicipitaceae (Sordariomycetes) also contains fungi that are pathogens of insects, including scale pathogens in the genus *Hypocrella* and biological control agents in the genus *Metarhizium* [10]. Genomic sequencing of both grass endophytes and insect pathogens within Clavicipitaceae revealed that several secondary metabolite genes and clusters are shared among these fungi including those homologous to the ergot alkaloids cluster, which includes a DMATS and several NRPS genes [9•,11,12•], and to peramine, which is produced by a bimodular NRPS with additional enzyme domains [12•,13•]. Clavicipitaceae is closely related to two other families of insect pathogenic fungi, Cordycipitaceae and Ophiocordycipitaceae [14]. It is hypothesized that grass endophytes in Clavicipitaceae represent a reversal from an insect pathogenic ancestral ecology to a plant-associated ecology, and that anti-insect metabolites of some grass endophytes may have evolved from insecticidal activity associated with the secondary metabolism of insect pathogenic fungi. This hypothesis is supported for Clavicipitaceae by the homology of gene clusters, such as the ergot alkaloid cluster (Figs. 3, 4), across closely related fungi with different ecologies (e.g. *Claviceps* and *Metarhizium*). Furthermore, some insect pathogenic fungi (e.g., *Metarhizium*) have been demonstrated to have a dual ecology and also function as endophytes [15•].

Non-clavicipitaceous endophytes belong to multiple classes of Pezizomycotina, including Sordariomycetes (e.g., *Fusarium*), Dothideomycetes (e.g., *Bipolaris*), Eurotiomycetes (e.g., *Penicillium*) and Leotiomycetes (e.g., *Lophodermium*) and colonize a broad phylogenetic diversity of plant species [7]. They are a rich and relatively unsampled source of novel bioactive compounds [16,17•], with genomic sequencing of some species (e.g., *Pestalotiopsis* [18]) documenting some of the highest potential of secondary metabolism among fungi. Tens to

hundreds of different fungi can colonize a single leaf and form a mosaic of interacting and competing species [17•,19,20]. This ecology may favor synthesis of compounds with antimicrobial activity against other fungi [21], bacteria [17•], and herbivorous insects [22•]. Endophytes are also known to form symbioses with bacteria that function in the production of compounds that are biologically active in plants (e.g., indole acetic acid [23], rhizoxin [24]). One of the best known medicinal secondary metabolites is taxol, an anticancer terpenoid synthesized by yew trees and reportedly also by some endophytes of yew trees [25]. Molecular and genomic analyses, however, did not find evidence for the presence of the taxol biosynthetic pathway in endophytes of yew and its production by fungi remains controversial [26].

Plant Pathogens.

Phylogenetic analyses suggest that plant pathogens are often closely related to well-known endophytic species [7,27,28]. The genus *Fusarium*, for example, contains important pathogens of agronomic grasses and other angiosperms, but also includes closely related species that are endophytic on the same or alternative hosts [29,30]. Many pathogens produce toxins with roles in plant pathogenesis. *F. graminearum*, for example, synthesizes terpenes such as the trichothecene deoxynivalenol (DON), a mycotoxin that aids colonization of barley and wheat heads by the fungus [31]. *F. verticillioides*, causal agent of kernel and ear rot diseases of maize, produces polyketides including fumonisins [32]. While the role of fumonisins in plant pathogenesis is less clear, they are known to impact beta-1,3-glucanases involved in plant defense [33]. Fumonisins have also been identified by chemical means in closely related Sordariomycetes insect pathogens [34] and species of *Aspergillus* (Eurotiomycetes) [35]. Genome sequencing has uncovered secondary metabolite clusters involved in fumonisin

production in these taxa and in several Dothidiomycete plant pathogens (e.g. *Bipolaris maydis*) that harbor homologs of genes in the fumonisin biosynthetic cluster, although fumonisin production has never been detected in these species [36•].

Dothideomycetes plant pathogens are well known producers of host-selective toxins (HSTs), compounds that enable pathogenesis on specific plant hosts. These include metabolites synthesized by PKSs and NRPSs, as well as small protein toxins synthesized using ribosomes [37]. HSTs evolve rapidly and are by definition often unique to particular species or races within a species (e.g., Southern corn leaf blight caused by *B. maydis* race T [38]). In *Alternaria* species many of the metabolite clusters responsible for HSTs are carried on small dispensable chromosomes, facilitating their movement between strains [39]. Other Dothidiomycetes such as *Pyrenophora tritici-repentis* and *Stagonospora nodorum* synthesize protein toxins including ToxA and ToxB [40] and others [41]. Genome sequencing identified a homolog of *ToxA* gene in *S. nodorum* that has been interpreted as support for horizontal transfer of *ToxA* to *P. tritici-repentis* [42]. In contrast, the *ToxB* gene is widely distributed across ascomycete phylogeny [43]. Although most HSTs have restricted distributions, phylogenomic analyses have identified homologs of core genes in other Pezizomycotina, but their function as HSTs is only known for Dothideomycetes [36•,44,45].

Phylogenomics of secondary metabolism.

Comparative genomic studies have revealed that homologous genes and gene clusters that encode for secondary metabolism are distributed across a broader phylogenetic spectrum of species than previously realized. In some cases these distributions are consistent with inheritance from a common ancestor (e.g., ergot alkaloid gene cluster in *Claviceps* and *Metarhizium*), but in

other cases the possession of large, complex gene clusters in distantly related taxa has been hypothesized to be the result of horizontal gene transfer (HGT). Examples include sterigmatocystin from *Aspergillus* to *Podospora* [46], and hybrid NRPS/PKS genes from bacteria to fungi [47].

HGT is the genetic transfer from one species to another and has been suggested to explain the discontinuous distribution of metabolic gene clusters between distantly related species [48•]. Standard evidence for HGT is phylogenetic analyses that result in gene tree topologies being in topological conflict with the organismal tree, and thus inconsistent with inheritance from a common ancestor [49]. Although HGT has been suggested for many fungal species and gene systems, a well-defined genetic mechanism for HGT is lacking for fungi. It is assumed to involve fusion of hyphae or spores between distinct species and the genetic transfer of secondary metabolite gene clusters, most likely through some form of mitotic recombination [49, 50•]. Other mechanisms that likely explain the distribution of some fungal secondary metabolite genes and gene clusters are complex patterns of gene gains and losses, neofunctionalization, incomplete lineage sorting and trans-species polymorphisms [51,52•], all of which can also lead to incongruent gene-species evolutionary histories. The current sampling of fungal genomes is still relatively sparse and the current observation of discontinuous distributions of some secondary metabolite genes and gene clusters may be an artifact of insufficient sampling, including within species. Much genomic sampling to date has focused on plant pathogenic fungi, but these species have evolved from endophytes and decomposers associated with pre-agricultural landscapes [53], and a greater sampling of these ecologies is required to better understand the evolution of fungal secondary metabolism.

Phylogenomic analyses of fungal secondary metabolism have revealed that the modular nature of some core genes, namely PKS and NRPS, promote diversification and evolution of novel metabolites. Fungal polyketides are synthesized by the action of Type I PKSs, which are multidomain proteins related to eukaryotic fatty-acid synthetases [54]. PKSs assemble small bioactive molecules through iterative condensation of acetyl-CoA and malonyl-CoA. In fungal PKSs, the ketoacyl CoA synthase (KS), acyltransferase (AT) and acyl carrier (ACP) domains are essential for polyketide synthesis, whereas additional domains that are not found in all PKSs can catalyze methylation, reduction and cyclization of the molecule [55]. Phylogenomic analyses of KS domains have revealed that PKS evolution is strongly shaped by gene duplication and loss, gain-of-function from domain acquisition, and loss-of-function from point mutations [56,57].

NRPSs are large (5 to >100 KDa), multimodular enzymes that assemble short peptides without the use of a nucleic acid template [58]. NRPSs contain a basic modular structure that comprises an adenylation (=AMPylation [52•]) domain (A), which recognizes and activates an amino acid residue (often nonproteinogenic amino acid and occasionally hydroxy acid), a thiolation domain (T), which covalently binds the residue to the NRPS enzyme via a thioester linkage, and a condensation domain (C), which grows the peptide by adding residue “n” to residue “n+1”. NRPS A-domains show varying levels of specificity with some A-domains activating a single residue, whereas others are less discriminating and may activate multiple residues. NRPSs can contain several ATC modules with some being co-linear with their peptides (e.g., 11-module *simA* producing the 11-residue cyclic peptide cyclosporin) whereas others are iterative (e.g., 2-module *esynI* producing the 6-residue cyclic depsipeptide enniatin).

Phylogenetic analyses of NRPS A-domains have revealed two main patterns and mechanisms of diversification. First, A-domains with varying specificities from the same NRPS gene

frequently group in the same clade [12•,36•,59]. This finding supports A-domain duplication and divergence of substrate specificity as being an important mechanism in NRPS diversification (e.g., ergot alkaloids, Fig. 4). Second, a single NRPS gene may possess A-domains from distantly related clades, consistent with gene fusion being an important diversifying mechanism (Fig. 4; [12•,44,59]). Phylogenomic analyses of NRPS A-domains have also revealed numerous undiscovered cases of homology between NRPS A-domains that produces chemically distinct secondary metabolites that were not suspected to have a common evolutionary origin (e.g., cyclosporin, destruxin).

Module fusions have also occurred between PKS and NRPS genes resulting in PKS-NRPS hybrid enzymes, which catalyze synthesis of polyketide amino acids (e.g., lovastatins) that are incapable of being produced by either system alone [60]. Phylogenetic analyses of fungal NRPSs resolve a monophyletic group of A-domains from PKS-NRPS hybrids and these enzymes possess a PKS domain structure that is common for highly reducing PKSs of filamentous ascomycetes, supporting a common origin of fungal PKS-NRPS hybrids within Pezizomycotina [60].

Concluding Remarks.

Phylogenomic analyses have revealed a greater diversity and complexity of fungal secondary metabolism than previously detected through chemical screening. They have also exposed unexpected patterns of taxonomic distribution of secondary metabolite gene clusters and homologies of genetic machinery involved in the biosynthesis of chemically distinct compounds. The modular nature of secondary metabolite core genes facilitates their diversification through duplication and divergence and fusion of non-orthologous genes/modules. Future phylogenomic

studies should emphasize greater sampling within and across species of Pezizomycotina to better understand the distribution of secondary metabolite gene clusters and their mechanisms of inheritance.

Acknowledgements.

This material is based upon work supported by the National Science Foundation under Grant No. DEB-1258162. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. The authors thank Dr. Kerry McPhail for assistance with preparation of Figure 1.

References.

1. Keller NP, Turner G, Bennett JW: **Fungal secondary metabolism - from biochemistry to genomics.** *Nat Rev Micro* 2005, **3**:937–947.
2. Andersen MR, Nielsen JB, Klitgaard A, Petersen LM, Zachariasen M, Hansen TJ, Blicher LH, Gottfredsen CH, Larsen TO, Nielsen KF: **Accurate prediction of secondary metabolite gene clusters in filamentous fungi.** *Proc Natl Acad Sci USA* 2013, **110**:E99–E107.
3. Brakhage AA: **Regulation of fungal secondary metabolism.** *Nat Rev Micro* 2013, **11**:21–32.
4. Brakhage AA, Schroeckh V: **Fungal secondary metabolites—strategies to activate silent gene clusters.** *Fungal Genet Biol* 2011, **48**:15–22.
5. Hansen FT, Gardiner DM, Lysøe E, Fuertes PR, Tudzynski B, Wiemann P, Sondergaard TE, Giese H, Brodersen DE, Sørensen JL: **An update to polyketide synthase and non-ribosomal synthetase genes and nomenclature in *Fusarium*.** *Fungal Genet Biol* 2015, **75**:20–29.
6. Inglis DO, Binkley J, Skrzypek MS, Arnaud MB, Cerqueira GC, Shah P, Wymore F, Wortman JR, Sherlock G: **Comprehensive annotation of secondary metabolite biosynthetic genes and gene clusters of *Aspergillus nidulans*, *A. fumigatus*, *A. niger* and *A. oryzae*.** *BMC Microbiol* 2013, **13**:91.
7. Rodriguez RJ, White JFJ, Arnold AE, Redman RS: **Fungal endophytes: diversity and**

- 241 **functional roles.** *New Phytol* 2009, **182**:314–330.
- 242 8. Tanaka A, Takemoto D, Chujo T, Scott B: **Fungal endophytes of grasses.** *Curr Opin*
243 *Plant Biol* 2012, **15**:462-468.
- 244 9. Schardl CL, Young CA, Hesse U, Amyotte SG, Andreeva K, Calie PJ, Fleetwood DJ,
245 Haws DC, Moore N, Oeser B, et al.: **Plant-symbiotic fungi as chemical engineers:**
246 **multi-genome analysis of the Clavicipitaceae reveals dynamics of alkaloid loci.** *PLoS*
247 *Genet* 2013, **9**:e1003323.
- 248 Comparative analyses of 10 Clavicipitaceae genomes showed that alkaloid clusters are
249 located in highly repeat rich regions of the genome. While the interior genes in the
250 alkaloid loci tend to harbor conserved cores that specify the skeleton structures of
251 alkaloids, genes located in the cluster peripheries often encoded enzymes that contribute to
252 chemical variation known to cause pharmacological differences. The location of
253 periphery genes near transposon-derived AT-rich repeat blocks likely contributes to
254 processes such as gene loss, duplication, and neofunctionalization.
- 255
- 256 10. Kepler RM, Sung G-H, Harada Y, Tanaka K, Tanaka E, Hosoya T, Bischoff JF, Spatafora
257 JW: **Host jumping onto close relatives and across kingdoms by *Tyrannicordyceps***
258 **(Clavicipitaceae) gen. nov. and *Ustilaginoides* (Clavicipitaceae).** *Am J Bot* 2012,
259 **99**:552–561.
- 260 11. Gao Q, Jin K, Ying S-H, Zhang Y, Xiao G, Shang Y, Duan Z, Hu X, Xie X-Q, Zhou G, et
261 al.: **Genome sequencing and comparative transcriptomics of the model**
262 **entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*.** *PLoS Genet* 2011,
263 **7**:e1001264.
- 264 12. Bushley KE, Raja R, Jaiswal P, Cumbie JS, Nonogaki M, Boyd AE, Owensby CA, Knaus
265 BJ, Elser J, Miller D, et al.: **The genome of *Tolypocladium inflatum*: evolution,**
266 **organization, and expression of the cyclosporin biosynthetic gene cluster.** *PLoS Genet*
267 2013, **9**:e1003496.
- 268 Genome sequencing and comparative genomic analysis of *Tolypocladium inflatum*
269 identified the cluster responsible for the production of the immunosuppressant drug
270 cyclosporin and revealed previously unsuspected homology of cyclosporin and other
271 NRPSs such as beauvericins and destruxins. RNA-seq analyses defined the boundaries of
272 the cluster and demonstrated that cluster genes, including a cyclophilin gene, were
273 upregulated in media containing insect components.
- 274 13. Berry D, Takach JE, Schardl CL, Charlton ND, Scott B, Young CA: **Disparate**
275 **independent genetic events disrupt the secondary metabolism gene *perA* in certain**
276 **symbiotic *Epichloë* species.** *Appl Environ Microb* 2015, **81**:2797–2807.
- 277 The authors document a novel mechanism of gene inactivation by which the terminal R
278 domain of the bidomain NRPS responsible for producing the insect anti-feedant *perA* is
279 deleted through insertion of MITE transposable elements. It is hypothesized that this
280 mutation arose once and became widespread throughout the *Epichloë* through horizontal

- 281 transfer.
- 282 14. Spatafora JW, Sung G-H, Sung JM, Hywel-Jones NL, White JF: **Phylogenetic evidence**
 283 **for an animal pathogen origin of ergot and the grass endophytes.** *Mol Ecol* 2007,
 284 **16:1701–1711.**
- 285 15. • Behie SW, Jones SJ, Bidochka MJ: **Plant tissue localization of the endophytic insect**
 286 **pathogenic fungi *Metarhizium* and *Beauveria*.** *Fungal Ecol* 2015, **13:112–119.**
- 287 The authors demonstrated the endophytic ability of the insect pathogenic fungi
 288 *Metarhizium* and *Beauveria* and their preferential colonization of plant tissues. They use
 289 the term endophytic insect pathogenic fungi (EIPF) to denote the dual ecology of these life
 290 histories.
- 291 16. Kusari S, Hertweck C, Spiteller M: **Chemical ecology of endophytic fungi: origins of**
 292 **secondary metabolites.** *Chem Biol* 2012, **19:792–798.**
- 293 17. • Higginbotham SJ, Arnold AE, Ibañez A, Spadafora C, Coley PD, Kursar TA: **Bioactivity**
 294 **of fungal endophytes as a function of endophyte taxonomy and the taxonomy and**
 295 **distribution of their host plants.** *PLoS ONE* 2013, **8:e73192.**
- 296 The authors report results of bioactivity screening of a large and diverse endophyte
 297 collection from Panama. Analyses suggest that specific bioactivities are correlated with
 298 both host-plant association and fungal taxonomy, leading the way to more targeted
 299 searches for bioactive compounds in endophytes of tropical forests.
- 300 18. Wang X, Zhang Z, Liu L, Xiang M, Wang W, Sun X, Che Y, Guo L, Liu G, Guo L, Wang
 301 C, Yin W-B, Stadler M, Zhang X, Liu X: **Genomic and transcriptomic analysis of the**
 302 **endophytic fungus *Pestalotiopsis fici* reveals its lifestyle and high potential for**
 303 **synthesis of natural products.** *BMC Genomics* 2015, **16:28.**
- 304 19. Arnold AE, Maynard Z, Gilbert GS: **Fungal endophytes in dicotyledonous neotropical**
 305 **trees: patterns of abundance and diversity.** *Mycol Res* 2001, **105:1502–1507.**
- 306 20. Lau M, Johnson NC, ARnold AE: **Factors influencing communities of foliar fungal**
 307 **endophytes in riparian woody plants.** *Fungal Ecol* 2013, **6:365–378.**
- 308 21. Arnold AE, Mejía LC, Kyllö D, Rojas EI, Maynard Z, Robbins N, Herre EA: **Fungal**
 309 **endophytes limit pathogen damage in a tropical tree.** *Proc Natl Acad Sci USA* 2003,
 310 **100:15649–15654.**
- 311 22. • Estrada C, Weislo WT, Van Bael SA: **Symbiotic fungi alter plant chemistry that**
 312 **discourages leaf-cutting ants.** *New Phytol* 2013, **198:241–251.**
- 313 The authors demonstrate that the fungal endophyte community of tropical plants can
 314 change the chemistry of leaves and thus decrease herbivory by leaf-cutting ants.
- 315 23. Hoffman MT, Gunatilaka MK, Wijeratne K, Gunatilaka L, Arnold AE: **Endohyphal**

316 **bacterium enhances production of indole-3-acetic acid by a foliar fungal endophyte.**
317 *PLoS ONE* 2013, **8**:e73132. doi:10.1371/journal.pone.0073132.

318 24. Seo Y-S, Lim JY, Park J, Kim S, Lee H-H, Cheong H, Kim S-M, Moon JS, Hwang I:
319 **Comparative genomic analysis of rice-pathogenic *Burkholderia* provides insight into**
320 **capacity to adapt to different environments and hosts.** *BMC Genomics* 2015, **16**:349.

321 25. Soliman SS, Raizada MN: **Interactions between co-habiting fungi elicit synthesis of**
322 **taxol from an endophytic fungus in host *Taxus* plants.** *Front Microbiol.* 2013, **4**:3.

323 26. Heinig W, Scholz S, Jennewein S: **Getting to the bottom of taxol biosynthesis by fungi.**
324 *Fungal Divers* 2013, **60**:161-170.

325 27. Chen K-H, Miadlikowska J, Molnár K, Arnold AE, U'Ren JM, Gaya E, Gueidan C,
326 Lutzoni F: **Phylogenetic analyses of eurotiomycetous endophytes reveal their close**
327 **affinities to Chaetothyriales, Eurotiales, and a new order - Phaeomoniellales.** *Mol*
328 *Phylogenet Evol* 2015, **85C**:117–130.

329 28. Delaye L, García-Guzmán G, Heil M: **Endophytes versus biotrophic and necrotrophic**
330 **pathogens—are fungal lifestyles evolutionarily stable traits?** *Fungal Divers* 2013,
331 **60**:125–135.

332 29. Demers JE, Gugino BK, Jiménez-Gasco MDM: **Highly diverse endophytic and soil**
333 ***Fusarium oxysporum* populations associated with field-grown tomato plants.** *Appl*
334 *Environ Microb* 2015, **81**:81–90.

335 30. Swett CL, Gordon TR: **Endophytic association of the pine pathogen *Fusarium***
336 ***circinatum* with corn (*Zea mays*).** *Fungal Ecol* 2015, **13**:120–129.

337 31. Arunachalam C, Doohan FM: **Trichothecene toxicity in eukaryotes: cellular and**
338 **molecular mechanisms in plants and animals.** *Toxicol Lett* 2013, **217**:149–158.

339 32. Proctor RH, Brown DW, Plattner RD, Desjardins AE: **Co-expression of 15 contiguous**
340 **genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*.**
341 *Fungal Genet Biol* 2003, **38**:237–249.

342 33. Sánchez-Rangel D, Sánchez-Nieto S, Plasencia J: **Fumonisin B1, a toxin produced by**
343 ***Fusarium verticillioides*, modulates maize β -1,3-glucanase activities involved in**
344 **defense response.** *Planta* 2012, **235**:965–978.

345 34. Mogensen JM, Møller KA, Freiesleben von P, Labuda R, Varga E, Sulyok M, Kubátová A,
346 Thrane U, Andersen B, Nielsen KF: **Production of fumonisins B2 and B4 in**
347 ***Tolypocladium* species.** *J Ind Microbiol Biotechnol* 2011, **38**:1329–1335.

348 35. Frisvad JC, Larsen TO, Thrane U, Meijer M, Varga J, Samson RA, Nielsen KF:
349 **Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains.** *PLoS*
350 *ONE* 2011, **6**:e23496.

- 351 36. • Condon BJ, Leng Y, Wu D, Bushley KE, Ohm RA, Otilar R, Martin J, Schackwitz W,
352 Grimwood J, MohdZainudin N, et al.: **Comparative genome structure, secondary**
353 **metabolite, and effector coding capacity across *Cochliobolus* pathogens.** *PLoS Genet*
354 2013, **9**:e1003233.
- 355 The authors compare SNP densities and secondary metabolite and effector gene content
356 across 10 *Cochliobolus* isolates (5 species) and the closely related Dothideomycete
357 *Setosphaeria turcica*. Results demonstrated that secondary metabolism varied widely
358 between species but was highly conserved within species with the exception of a few
359 defining examples that mapped to species unique regions.
- 360 37. Stergiopoulos I, Collemare J, Mehrabi R, De Wit PJGM: **Phytotoxic secondary**
361 **metabolites and peptides produced by plant pathogenic Dothideomycete fungi.** *FEMS*
362 *Microbiol Rev* 2013, **37**:67–93.
- 363 38. Baker SE, Kroken S, Inderbitzin P, Asvarak T, Li B-Y, Shi L, Yoder OC, Turgeon BG:
364 **Two polyketide synthase-encoding genes are required for biosynthesis of the**
365 **polyketide virulence factor, T-toxin, by *Cochliobolus heterostrophus*.** *Mol Plant*
366 *Microbe In* 2006, **19**:139–149.
- 367 39. Tsuge T, Harimoto Y, Akimitsu K, Ohtani K, Kodama M, Akagi Y, Egusa M, Yamamoto
368 M, Otani H: **Host-selective toxins produced by the plant pathogenic fungus *Alternaria***
369 ***alternata*.** *FEMS Microbiol Rev* 2013, **37**:44–66.
- 370 40. Pandelova I, Figueroa M, Wilhelm LJ, Manning VA, Mankaney AN, Mockler TC,
371 Ciuffetti LM: **Host-selective toxins of *Pyrenophora tritici-repentis* induce common**
372 **responses associated with host susceptibility.** *PLoS ONE* 2012, **7**:e40240.
- 373 41. Oliver RP, Friesen TL, Faris JD, Solomon PS: ***Stagonospora nodorum*: from pathology**
374 **to genomics and host resistance.** *Annu Rev Phytopathol* 2012, **50**:23–43.
- 375 42. Friesen TL, Stukenbrock EH, Liu Z, Meinhardt S, Ling H, Faris JD, Rasmussen JB,
376 Solomon PS, McDonald BA, Oliver RP: **Emergence of a new disease as a result of**
377 **interspecific virulence gene transfer.** *Nat Genet* 2006, **38**:953–956.
- 378 43. Andrie RM, Schoch CL, Hedges R, Spatafora JW, Ciuffetti LM: **Homologs of ToxB, a**
379 **host-selective toxin gene from *Pyrenophora tritici-repentis*, are present in the genome**
380 **of sister-species *Pyrenophora bromi* and other members of the Ascomycota.** *Fungal*
381 *Genet Biol* 2008, **45**:363–377.
- 382 44. Bushley KE, Turgeon BG: **Phylogenomics reveals subfamilies of fungal nonribosomal**
383 **peptide synthetases and their evolutionary relationships.** *BMC Evol Biol* 2010, **10**:26
- 384 45. Wight WD, Labuda R, Walton JD: **Conservation of the genes for HC-toxin biosynthesis**
385 **in *Alternaria jesenskae*.** *BMC Microbiol* 2013, **13**:165.
- 386 46. Slot JC, Rokas A: **Horizontal transfer of a large and highly toxic secondary metabolic**
387 **gene cluster between fungi.** *Curr Biol* 2011, **21**:134–139.

- 388 47. Lawrence DP, Kroken S, Pryor BM, Arnold AE: **Interkingdom gene transfer of a**
389 **hybrid NPS/PKS from bacteria to filamentous Ascomycota.** *PLoS ONE* 2011,
390 **6:e28231.**
- 391 48. • Wisecaver JH, Slot JC, Rokas A: **The evolution of fungal metabolic pathways.** *PLoS*
392 *Genet* 2014, **10:e1004816.**
393
394 The authors review the organization of metabolic gene clusters, their roles in specialized
395 metabolism, including virulence, and their propensity for serving as candidates for
396 horizontal gene transfer between fungi.
- 397 49. Richards TA, Leonard G, Soanes DM, Talbot NJ: **Gene transfer into the fungi.** *Fungal*
398 *Biol Rev* 2011, **25:98–110.**
- 399 50. • Soanes D, Richards TA: **Horizontal gene transfer in eukaryotic plant pathogens.** *Annu*
400 *Rev Phytopathol* 2014, **52:583–614.**
- 401 The authors provide a critiqued review of horizontal gene transfer (HGT) between fungi,
402 discuss analytical tests and possible mechanisms for HGT, and argue that while HGT is
403 relatively rare compared to gene birth/death events of the genome, it can have a profound
404 effect on fungal ecology.
- 405 51. Liu L, Xi Z, Davis CC: **Coalescent methods are robust to the simultaneous effects of**
406 **long branches and incomplete lineage sorting.** *Mol Biol Evol* 2015, **32:791–805.**
- 407 52. • Young CA, Schardl CL, Panaccione DG, Florea S, Takach JE, Charlton ND, Moore N,
408 Webb JS, Jaromczyk: **Genetics, genomics and evolution of ergot alkaloid diversity.**
409 *Toxins* 2015, **7:1273–1302.**
- 410 Ergot alkaloid biosynthesis is presented as a model system to study the evolutionary
411 diversification of fungal secondary metabolites. Three mechanisms are emphasized
412 including gene gains and losses and neofunctionalization of existing genes/domains.
413 Chromosomal location and propensity for rearrangements is presented as an important
414 mechanism for facilitating gene gains and losses.
- 415 53. Schoch CL, Sung G-H, López-Giráldez F, Townsend JP, Miadlikowska J, Hofstetter V,
416 Robbertse B, Matheny PB, Kauff F, Wang Z, et al.: **The Ascomycota tree of life: a**
417 **phylum-wide phylogeny clarifies the origin and evolution of fundamental**
418 **reproductive and ecological traits.** *Syst Biol* 2009, **58:224–239.**
- 419 54. Crawford JM, Townsend CA: **New insights into the formation of fungal aromatic**
420 **polyketides.** *Nat Rev Micro* 2010, **8:879–889.**
- 421 55. Weissman KJ: **Introduction to Polyketide Biosynthesis.** *Method Enzymol* 2009, **459:3–**
422 **16.**
- 423 56. Baker SE, Perrone G, Richardson NM, Gallo A, Kubicek CP: **Phylogenomic analysis of**
424 **polyketide synthase-encoding genes in Trichoderma.** *Microbiology* 2012, **158:147–154.**

57. Brown DW, Butchko RAE, Baker SE, Proctor RH: **Phylogenomic and functional domain analysis of polyketide synthases in *Fusarium***. *Fungal Biol* 2012, **116**:318–331.
58. Strieker MS, Tanovic A, Marahiel MA: **Nonribosomal peptide synthetases: structures and dynamics**. *Curr Opin Struc Biol* 2010, **20**:234–240.
59. Wang B, Kang Q, Lu Y, Bai L, Wang C: **Unveiling the biosynthetic puzzle of destruxins in *Metarhizium* species**. *Proc Natl Acad Sci USA* 2012, **109**:1287–1292.
60. Boettger D, Hertweck C: **Molecular diversity sculpted by fungal PKS-NRPS hybrids**. *ChemBioChem* 2013, **14**:28–42.
61. Jones MDM, Forn I, Gadelha C, Egan MJ, Bass D, Massana R, Richards TA: **Discovery of novel intermediate forms redefines the fungal tree of life**. *Nature* 2011, **474**:200–203.
62. James TY, Pelin A, Bonen L, Ahrendt S, Sain D, Corradi N, Stajich JE: **Shared signatures of parasitism and phylogenomics unite Cryptomycota and microsporidia**. *Curr Biol* 2013, **23**:1548–1553.
63. White MM, James TY, O'Donnell K, Cafaro MJ, Tanabe Y, Sugiyama J: **Phylogeny of the Zygomycota based on nuclear ribosomal sequence data**. *Mycologia* 2006, **98**:872–884.

Figure 1. Examples of fungal secondary metabolites. A) Trichothecene: 4-Deoxynivalenol (DON). B) Alkaloid: Ergotamine. C) NRPS peptide: AM-Toxin I. D) Polyketide amino acid: Lovastatin. E) Polyketide: T-toxin.

Figure 2. Fungal Tree of Life. Phylogeny of Kingdom Fungi (small insert tree) depicting major phyla. The earliest diverging branches of Kingdom Fungi comprise the flagellated (zoosporic) Cryptomycota [57], the nonflagellated Microsporidia [58], and the zoosporic Blastocladiomycota and Chytridiomycota. The remaining major groups of Kingdom Fungi lack a flagellated stage, typically possess a well-developed filamentous thallus, and are hypothesized to have evolved and diversified in terrestrial habitats. Zygomycete Group 1 includes mainly plant-associated fungi (e.g., plant decomposers, root endophytes, mycorrhizae), whereas Zygomycete Group 2 includes

mainly nonplant-associated species (e.g., animals pathogens and commensals, mycoparasites) [59]. Ascomycota and Basidiomycota are dominant members of terrestrial ecosystems where they interact with plants in all forms of associations and symbioses. Green box denotes major plant-associated lineages. While all groups of fungi possess some form of secondary metabolism, it has diversified to its greatest extent within Ascomycota and Basidiomycota. The large insert tree depicts the three subphyla of Ascomycota and classes of Pezizomycotina. The numbers to the right of taxon names show the average number of genes for KOG annotations of “secondary metabolites biosynthesis, transport and catabolism” from published genomes available from the JGI Mycocosm (May 13, 2015).

Figure 3. Ergot gene cluster synteny. Homology of a portion of the ergot gene cluster between *Claviceps purpurea* and *Metarhizium* species, adapted from [9,11]. Grey box shows conserved synteny of the core ergot alkaloid gene cluster including *dmaW* and unimodular NRPS genes *lpsB* and *lpsC*. Trimodular NRPS genes *lpsA1* and *lpsA2* are downstream of the core cluster in *Claviceps*. *Metarhizium robertsii* and *M. acridum* contain a 7-module NRPS (MAA_06559, MAC_08899) that is not physically located near the core gene cluster, but six of the seven A-domains also members of the clade containing all ergot alkaloid NRPS A-domains. Domain 2 (tan) of MAA_06559 and MAC_08899 falls outside the ergot A-domain clade in a different A-domain clade than remaining six A-domains (green).

Figure 4. Phylogenomic tree of NRPS A-domains. 696 A-domain tree inferred from amino acid sequences using RAxML with a PROTGAMMAWAG model of evolution. Clades that contain A-domains from ergot alkaloid, peramine and cyclosporin synthetases are emphasized

477 with colored triangles. Ergot alkaloid A-domain clade is expanded to show evolutionary
478 relationships and homologies of related A-domains in *Claviceps*, *Fusarium*, *Metarhizium* and
479 *Tolypocladium*. Individual NRPS genes in each species are color coded (green = *Claviceps*
480 *purpurea*, red=*Metarhizium robertsii*, dark pink = *Metarhizium acridum*, orange =
481 *Tolypocladium inflatum*, and black = *Fusarium graminearum*) and number of domains or
482 modules listed. Of the seven A-domains present in *Metarhizium*, six are members of the ergot
483 clade and one (domain 2) is located outside of the ergot clade. A-domains are labeled as follows:
484 species (e.g., Cp), gene (e.g., LpsA1), A-domain (e.g., A1/3 is domain 1 out of 3). Numbers
485 above nodes are bootstrap values >50%.

486

A) Trichothecene (DON)

B) Ergotamine

C) Lovastatin

D) AM-Toxin I

E) T toxin