Discovering the Secondary Metabolite Potential Encoded within Entomopathogenic Fungi

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Discovering the Secondary Metabolite Potential Encoded within Entomopathogenic Fungi

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This highlight discusses the secondary metabolite potential of the insect pathogens *Metarhizium* and *Beauveria*, including a bioinformatics analysis of secondary metabolite genes for which no products are yet identified.

Notes and references
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†**ABBREVIATIONS:** A, adenylation domain; FAS, fatty acid synthase; KO, knockout; KS, ketosynthase domain; NP, natural product; NRPS, nonribosomal peptide synthetase; NRP, nonribosomal peptide; NP, natural product; PKS, polyketide synthase; PK, polyketide; SM, secondary metabolite; TC, terpene cyclase; TS, terpene synthase; WT, wild type

I. Introduction

1.1. A brief review of insect pathogenic fungi

Arthropods constitute the most species-rich group of animals, and the most diverse arthropod subgroup, the insects, occupy innumerable niches and have immense impacts as pests, pollinators, and agents of control of injurious species. Aside from their biological roles and functions, their enormous biomass provides opportunities as hosts or nutrient sources for parasites, pathogens, and predators, ranging from bacteria and fungi to plants and animals. Of particular interest are the 700 species (from ~100 genera) of entomopathogenic (insect-pathogenic) fungi [1, 2] that constitute a unique, highly specialized trophic subgroup. Fungal pathogens of insects are found within every ecosystem and all major fungal lineages with the principal exception of the higher basidiomycetes [3-5]. Most entomopathogenic species are in the order Hypocreales (class Sordariomycetes, phylum Ascomycota). They associate with plants, animals and other fungi, and may subsist as pathogens, parasites, saprophytes, or in commensal and mutualistic relationships.

Fungi in the Hypocreales are thought to derive from plant-associated fungi [2, 3]. The entomogenous habit likely arose and spread concomitantly with the diversification of phytophagous insects that took place during the Cretaceous period [6]. These fungi have undergone repeated inter-kingdom host-shifts among plants, arthropods and fungal hosts, accompanied by switches in nutritional habits [2]. Such inter-kingdom host switching may have been facilitated in the soil or on aerial plant surfaces by the close proximity with sedentary insects feeding on the plants and saprobic fungi. The co-evolutionary arms race between the insects and their fungal pathogens (especially obligate pathogens) often results in fascinating physiological and behavioral changes in insects, including reduced feeding, diminished reproductive fitness, defensive behaviors such as heat seeking and increased social grooming, to more specific behavioral manipulations of the host by the fungus, such as elevation-seeking prior to death, which enhances spore dispersal from the cadaver [7, 8].

1.2. Scope of the highlight

For this highlight, we will primarily focus on the secondary metabolites and molecular genetics of biosynthesis in two major cosmopolitan insect pathogenic fungal species, *Beauveria bassiana* and *Metarhizium robertsii (anisopliae)*, with references to congeners where relevant. Both species have broad host-ranges encompassing over 1,000 insect species from > 50 different insect families, including pests of agricultural, veterinary and medical significance [2]. Consequently, both *Metarhizium* and *Beauveria* species have been

developed for use as biological control agents worldwide [9]. Besides being an effective pathogen, *B. bassiana* can exist as a saprophyte [10], and as a plant endophyte [11-13], and, in the latter case, can confer to its host-plant resistance against foraging insects and plant pathogens. *M. anisopliae* can also colonize plant roots [14] and protect against root-feeding insects [15, 16], and like *Beauveria*, produces a variety of cell types in culture, including conidia, hyphae, appressoria, unicellular blastospores, and multi-cellular hyphal bodies [17].

2. Current use of entomopathogenic fungi and the infection process

Interest in biological control agents has steadily grown due to concerns over environmental pollution and pest resistance resulting from the use of insecticides [18]. Unlike bacteria and viruses, insect pathogenic fungi infect their hosts by penetrating the cuticle and thus, with rare exceptions[19], need not be ingested, making them particularly well suited for broadcast application as contact biopesticides against a wide array of arthropods [2]. In many cases, these agents are also compatible with other control measures, including traditional pesticides, BT transgenic-plants, or other biological controls such as predators and parasitoids, and thus offer an alternative and complementary tool for use in integrated pest management programs. Although, at present, entomopathogens represent only a small niche in the pesticide market, market share continues to grow [9, 20]. Both *Beauveria* and *Metarhizium* products are generally considered to be safer than chemical pesticides for use based on extensive testing of effects on non-target organisms [21, 22]. In addition, there are no documented reports of field-evolved insect resistance to entomopathogens.

The process of insect infection by *B. bassiana* and *M. robertsii (anisopliae)* involves multiple steps. First complex mechanisms are deployed for breaching the formidable physiochemical barriers of the insect cuticle[23-31]. The spore germinates on the surface and forms an appressorium, that then generates a penetration peg which exerts physical pressure while a complex cocktail of enzymes including proteases (subtilisin-like, metallo-protease, trypsin, aminopeptidase and other classes), chitinases, and esterases is elaborated. The invading fungus must overcome antimicrobial lipids and phenols, enzyme inhibitors, proteins, and other defensive compounds present in the cuticle [32-34] and also cope with competition from the insect's microbial flora. The fungus then enters the hemocoel where it has to thwart infection-induced responses such as melanization and hemocyte activation [32, 35-37], specialized defense recognition mechanisms [38, 39], and anti-microbial peptides [40-43]. It then disperses as hyphal bodies and blastospores (budding yeast-like forms), quickly colonizes the insect fat body (the main producer of antifungal compounds) and proliferates throughout the host [44-46]. Ultimately aided by the continued production of a variety of hydrolases [47], the fungus re-emerges from the moribund or dead insect host, resumes filamentous growth**,** forms sporulation structures (conidiophores) on the insect surface, and produces primary conidia. The process described above can be viewed as a coordinated developmental program finely honed to exploit host resources for maximal production of propagules. Understanding how the energetically expensive production of secondary metabolites serves this end is a challenge we are just beginning to address.

3. Secondary metabolism in entomopathogenic fungi

Entomopathogenic fungi are a rich source of secondary metabolites, as reviewed previously [48-50]. Yet recent genomic analyses with *Metarhizium* and *Beauveria* [51, 52] indicate that over 80% of the putative secondary metabolite-associated genes have no identified specific products and have sequences that are unique to this group of organisms. Indeed, in a comparative study of sequenced fungi, secondary metabolite core clusters are better represented in the genomes of entomopathogenic species than in genomes of fungi with other trophic associations[53]. In addition to the obvious antibiotic service that fungal metabolites may perform in entomopathogens, they can play a role in pathogenicity and other interactions between a fungus and its insect host, [54-56] or they may mediate an inter- or intra-specific communicative function, or aid in mitigating abiotic and biotic stresses [57-60]. Also, surface host molecules, i.e. epicuticular waxes and lipids, may serve as substrates for enzymes, e.g. cytochrome P450s, involved in secondary metabolite biosynthesis and/or xenobiotic transformations [61-63].

One of the key discoveries in fungal genomics is the large number of gene clusters with predicted roles in biosynthesis of secondary metabolites (SM), relative to the comparatively small number of characterized products. The biosynthetic machinery used for their production is energetically expensive, suggesting that NP biosynthesis confers a greater fitness or survival advantage to the organism. The genomes of filamentous Ascomycete fungi are replete with SM gene clusters, and the entomopathogenic fungi sequenced to date rank highly for the number of predicted SM gene clusters. The majority of these SM gene clusters are silent under standard laboratory conditions [64-68], and require triggers, such as stress environments or intimate exposure to other organisms [60, 69-74], for some of their genes to be expressed. One interesting aspect observed in other Ascomycete genomes is that the core genes (*NPSs, PKSs*) encoding fungal secondary metabolite production are often organized in clusters with associated specific transcription factors and modifying enzyme genes. Many of these clusters are located in the fast-evolving, variable subtelomeric regions of chromosomes [75], which in some cases have been shown to contain species-specific genes correlated with adaptability to particular environmental niches [76-78].

We will focus on the secondary metabolite products of nonribosomal peptide synthetases (NRPSs) [79-81], polyketide synthases (PKSs)[66, 82], and hybrids of the two (NRPS-PKSs) since these chemistries are abundant in *Metarhizium* and *Beauveria* [50] and their overall biosynthetic pathways are well characterized.

3.1. The *Metarhizium* genomes and known SMs.

3.1.1. Genome comparisons among *Metarhizium* species.

Metarhizium robertsii (formerly *anisopliae*- ARSEF (ARS Collection of Entomopathogenic Fungi) Isolate 2575 has been sequenced (Genbank accession PRJNA230500), and there are two other *Metarhizium* genomes available - *M. anisopliae* and *M. acridum* (PRJNA38717; www.ncbi.nlm.nih.gov/assembly/243998/) [51]. Genome sizes are approximately 40 MB; predicted gene count for *M. robertsii, M. anisopliae*, and *M. acridum* is approximately 12,300, 10,600, and 9,900, respectively. In *M. robertsii* (ARSEF 2575), there are 85 core genes putatively involved in SM biosynthesis, encoding 16 NRPS, 24 PKS, 9 NRPS-like, 7 PKS-NRPS hybrids, and 28 involved in FAS/terpene or steroid-like biosynthesis. *M. anisopliae* was reported to have comparable numbers of SM gene clusters as *M. robertsii* as these genomes are highly similar [51]. *M. acridum* has been reported to contain a total of 57 SM core genes, including 13 NRPS, 13 PKS, 8 NRPS-like, 4 PKS-like, 1 NRPS-PKS, and 16 involved in FAS/terpene or steroid-like biosynthesis [51]. Of the SM gene clusters, 3 NPs (destruxins (**1**), NG391/393 (**2**), serinocyclin (**3**)) have been functionally linked to a specific gene cluster in *M. robertsii* and *M. anisopliae*, using gene deletion studies [83-85].

(Structures 1–3 here)

3.1.2. Known SMs and SM genes of *Metarhizium* species

Two groups [85, 86] identified *DXS* as encoding the candidate *NRPS* responsible for biosynthesis of destruxins (DTX) due to the number of ATC modules in the protein and the presence of two adjacent methylation domains, corresponding to the N-Me-valine - N-Me-alanine motif in the cyclic structure of the major destruxin analogues, DTX A, B, and E (**1**). Disruption of *DXS* results in a knockout (KO) mutant that lacks the ability to produce destruxins. In addition, the genome of *M. acridum*, which does not produce destruxins, lacks the gene encoding this *NRPS*. Virulence assays on larvae of *Spodoptera exigua* (beet armyworm) indicate that destruxins are not essential for pathogenicity of *M. robertsii*. DXS expression was seen at later log to stationary phases of liquid culture, and in vivo analyses revealed its expression in the later stages of infection [85]. *DXS* transcripts were also detected in conidia, hinting at either the presence of the metabolites in these propagules or the retention of transcripts produced during conidiation [87]. The similarity of *in vivo* and *in vitro* expression patterns of destruxins suggests developmental regulation of this gene as has been observed with other SM-associated genes [88-91].

Targeted gene disruption experiments of *NPS1* with *M. robertsii* ARSEF 2575 were used to test the role of serinocyclin (**2**), a spore-derived peptide, by comparing WT and several *NPS1*-KO deletion mutants [92]. No avirulence phenotype was observed when the mutants were tested on larvae of beet armyworm (lepidopteran) and Colorado potato beetle (coleopteran), and no differences in morphological or physiological characteristics could be demonstrated, although the compound produced a sub-lethal defect in the swimming ability of mosquito larvae [93]; the biological significance of this effect remains unclear.

Fusarin-like compounds, NG39x (**3a,b**), from *M. robertsii* and *M. acridum* were identified [94], and the *NGS1* gene cluster encoding the NRPS-PKS responsible for production of NG39x compounds (NG synthetase) was disrupted [84]. No significant change in virulence against larvae of beet armyworm were observed, nor were any significant morphological changes seen, including responses to oxidative stress. One surprising result was the discovery that *NGS1* and other genes within the cluster were expressed in the early stages of insect infection, and the expression was correlated with specific cell densities of the fungus; this is the first evidence of the expression of a secondary metabolite gene from *Metarhizium in vivo* during infection. A recent report that NG39x causes a dramatic decrease in transcriptional activity in human cell cultures at μM concentrations [95] presents a possible lead to follow to understand how these compounds might serve *M. robertsii*.

(Structures 4–8 here)

Several other SMs are reported from *Metarhizium* strains, but have yet to be linked to a specific SM gene cluster. Cytochalasins (C, D) (**4a,b**), macrocyclic lactone derivatives of perhydroindoles that inhibit actin and microtubule formation, were isolated from *M. anisopliae* [96], as well as other fungi; in *Penicillium expansum*, cytochalasin biosynthesis involves a hybrid NRPS-PKS, as suggested by RNA silencing [97]. Swainsonine (**5**), an indolizidine alkaloid that inhibits α-mannosidases and blocks complex carbohydrate production, is produced by some isolates of *M. anisopliae* [98-100]. A precursor forms pipecolic acid that is then derivatized with acetate or malonate, although the biosynthetic genes have not been described. The dipeptide tyrosine betaine (**6**) was identified from conidia of *Metarhizium* although its presumed NRPS has yet to be identified[101]. The terpenoid helvolic acid (**7**), an antimicrobial terpenoid identified from *Aspergillus fumigatus* [102] has also been isolated from *M. anisopliae* [103, 104], and a geranylgeranyl diphosphate synthetase putatively involved in synthesis has been identified [105]. Conidia of *M. acridum* contain metacridimides (**8**), two novel 17-membered macrocycles consisting of a phenylalanine unit condensed with a nonaketide, suggestive of a hybrid NRPS-PKS biosynthetic route [106].

3.2. The *Beauveria* genome and known SMs.

3.2.1. Genome analysis of *Beauveria*

Beauveria bassiana (ARSEF 2860) possesses a genome of approximately 34 MB, and contains approximately 10,400 protein-coding genes [52]. Sequence identity between *B. bassiana* and *M. anisopliae* is about 58%, and blast analysis indicates that *B. bassiana* is more closely related to the Chinese medicinal fungus *Cordyceps militaris* [107] than to *Metarhizium* sp. [52]. The *B. bassiana* genome contains 45 SM core genes, including 13 NRPS, 12 PKS, 7 NRPS-like, 1 PKS-like, 3 hybrid NRPS-PKS, and 12 genes related to FAS/terpene/steroid biosynthesis. Three of the SM genes are conserved among the 4 insect pathogenic genomes (1 NRPS, 1 PKS, and 1 terpene synthase (TS)), and 4 clusters are shared with *Trichoderma reesei* (2 NRPS-like, 1 NRPS, and 1 terpene cyclase (TC)) [52, 108].

(Structures 9–12 here)

3.2.2. Known SMs and SM genes of *Beauveria* species

Beauveria species produce a number of cyclic peptides, of which the cyclooligomer depsipeptide beauvericin (**9**) and the octodepsipeptide bassionolide (**10**) are the best studied. The cyclooligomer depsipeptide beauvericin, an acyclic trimer of the dipeptidol monomer D-hydroxyisovaleric acid (D-Hiv) - *N*-methyl-L-phenylalanine (*N*-Me-Phe), is synthesized by multiple *Beauveria* spp., *Paecilomyces* and plant pathogenic *Fusaria*. Beauvericin has moderate antibacterial, antifungal, and insecticidal activities [109-111] as well as potent cytotoxic activity against human cell lines [112]. Through diffusion, it transports divalent cations across biological membranes and acts as an ionophore; beauvericin increases cytoplasmic Ca^{2+} concentration, causes ATP depletion, and activates calcium-sensitive cell apoptotic pathways [113, 114]. In *in vitro* studies, beauvericin reverses the multidrug-resistance (MDR) phenotype in yeast and potentiates the fungicidal activity of fluconazole against fluconazole-resistant *Candida albicans* at sub-cytotoxic concentrations [115-117] and known cytotoxic agents against multidrug-resistant (MDR) cancer cell lines [113, 115, 116]. Beauvericin hinders directional cell motility of cancer cells [118]. The gene cluster containing the beauvericin synthetase *NPS* has been identified and analyzed [56]; KO mutants showed small but measurable effects on virulence in comparison to WT strains when tested against *Spodoptera frugiperda*, *Helicoverpa zeae*, and *Galleria mellonella* larvae [56, 119].

The octodepsipeptide bassianolide is a cyclic tetrameric ester of the dipeptidol monomer D-hydroxyisovaleric acid-*N*-methylleucine, produced by *B. bassiana* and *Lecanicillum* sp. Bassianolide is insecticidal [120] and inhibits acetylcholine-induced smooth muscle contraction independent of ionophoric interactions [121]. The bassianolide *NPS* was described from the wood-decaying fungus *Xylaria* sp. [122] and from *B. bassiana* [119]. *B. bassiana* mutants with targeted disruptions of the corresponding genes were morphologically indistinguishable from WT isolates, but the KOs showed decreased virulence when tested against three different insect larvae [119]. These are the first examples of an alteration in virulence attributed to the loss of production of a secondary metabolite in *Beauveria*, although there is no evidence that these secondary metabolites are produced inside the insect during infection.

Tenellin (**11a**) and bassianin (**11b**) were first described as yellow pigments from *B. bassiana* [123, 124]; bassianin differs from tenellin by 1 chain extension in the ketide moiety. Eley et al. [125] described the production of the acyltetramic acid tenellin by a hybrid PKS-NRPS gene cluster that shows much similarity to the gene cluster encoding NG39x in *Metarhizium* and fusarins in *Fusarium* spp. The tenellin knockout mutants showed no loss of virulence in assays against *Galleria mellonella* suggesting that tenellin is not involved in pathogenesis. A highly similar gene cluster involved in desmethylbassianin biosynthesis has also been identified from *B. bassiana*[126].

(Structures 12–14 here)

Several *Beauveria* SMs have no identified gene cluster as yet. The beauverolides (**12**) are a family of cyclic tetradepsipeptides that have been reported from B. *bassiana* [127, 128] as well as other fungal species[129, 130] for which the biosynthetic cluster has not been identified. Oosporein (**13**) is a red pigment found in both *B. bassiana* [131] and *B. brongniartii* [132, 133] that has antifungal activity [134]. Earlier labelling studies with acetate and mevalonate suggest a PKS biosynthetic route [135], and it is chemically similar to orsellinic acid whose core PKS gene cluster has been identified in *Aspergillus nidulans* [136]. Bassiatin (**14**) is a cyclized monomeric unit of the trimer involved in beauvericin biosynthesis and thus it might be a shunt product of that pathway [137].

4. Comparison of predicted SM cluster genes of *Metarhizium and Beauveria*

NRPS and PKS genes from *B. bassiana* and *M. robertsii* were analyzed using two approaches. The first was a phylogenetic analysis in which the core protein in either NRPSs or PKSs from both fungi were compared to a collection of orthologous proteins of the same type which included proteins associated with a defined metabolite and proteins previously used in phylogenetic studies [138, 139]. This analysis had the objective to classify *B. bassiana* and *M. robertsii* SM core genes and broadly infer the structure of their natural products. To this end, either adenylation (A) domains (NRPSs and hybrid PKS/NRPSs) or ketoacyl synthase (KS) domains (PKSs and hybrid PKS/NRPSs) from target protein sequences were selected using their respective Hidden Markov Models obtained from PFAM (http://pfam.sanger.ac.uk/). Extracted fragments were aligned with Muscle 3.8.31 [140], clustered with PhyML 3.1 [141] and visualized with either Figtree (http://tree.bio.ed.ac.uk/software/figtree/) or TreeDyn [142] (Fig. 1 and 2). The second analysis was based on the web annotation service antiSMASH [143] and had the objective of identifying similarities among *B. bassiana* and *M. robertsii* gene clusters and those of other fungi or bacteria.

Overall, alignment of 81 reference sequences with 36 *Metarhizium* (EXU/EXV-series) and 18 *Beauveria* (EJP series) KS domains allowed for the segregation of PKSs into the broad structural classes of reducing, non-reducing and enzymes associated with bacterial type I PKSs as well as more narrowly defined groups (Fig. 1). While this analysis is speculative, it allows for classification and, to a certain extent, comparison of the PKSs found in *B. bassiana* and *M. robertsii* as well as very approximate predictions concerning putative backbone structure for the resultant products. This analysis indicates that both *B. bassiana* and *M. robertsii* have greater numbers of reducing (10 and 15, respectively, with hybrids not included) than non-reducing PKSs (2 and 10, respectively).

M. robertsii and *B. bassiana* were found to be each other's closest matches in 7 instances. Eight *M. robertsii* core PKS genes displayed greater similarity to sequences found in other fungi (besides *B. bassiana*) whereas three *B. bassiana* sequences displayed greater similarity to sequences outside those of *M. robertsii*. For *M. robertsii*, these included PKS genes involved in the syntheses of: (1) *Aspergillus* spp. ochratoxin A production, (EXV03186.1), (2) *A. nidulans* aspyridone synthesis (EXU98291.1), (3) *A. clavus* cytochalasin E synthesis (EXU97553.1), (4) *Alternaria soltani* aslanipyrone synthesis (EXU95862.1), (5) *A. fumigatus* pseurotin synthesis (EXU98505.1), (6) *A. nidulans* pkhB (EXU95974.1), (7) *A. alternata* AF-toxin synthesis (EXU98661.1), and (8) *Penicillium aethiopicum* viridicatumtoxin synthesis (EXU97310.1). Similarly, *B. bassiana* PKSs bear similarity to proteins involved in syntheses of: (1) *A. terreus* lovastatin nonaketide synthesis (Q9Y8A5.1, although in this case a closely related *Metarhizium* gene also clustered with this group, EXU96139.1), (2) *A. nidulans* emericellamide synthesis (EJP62832.1), and (3) *Cochliobolus heterostrophus* T-toxin synthesis (EJP70141.1). Neither *B. bassiana* nor *M. robertsii* appeared to contain any bacterial Type I or 6-MSAS-type PKSs. *M. robertsii* genome contains one NRPS-PKS (EXU97632.1), which is structurally similar to *C. heterostrophus* PKS24 [138, 144], while *B. bassiana* lacks this gene (Fig. 1, Bacterial PKS-associated clades). Additionally, within the non-reducing group, neither *B. bassiana* nor *M. robertsii* has PKSs clustering with either 1,3,6,8-tetrahydroxynaphthalene (T4HN) synthases or aflatoxin-like PKSs. Phylogenetic analysis show the presence of three *M. robertsii* and two *B. bassiana* PKSs divergent enough to be excluded from all the identified clades (Fig. 1).

Due to the modular nature of NRPSs, the phylogenetic analyses of these proteins from *B. bassiana* and *M. robertsii* are significantly more complex. Each NRPS module carries information concerning both function and evolutionary history; however neither aspect is well understood. In particular, the "nonribosomal code" of amino acid selection has not been precisely identified for fungal genomes [145-148]. The evolutionary history of these modules as well as each NRPS as a whole appear to be a layering of vertical, possibly horizontal transmission events, and intricate genetic rearrangements which have led to a staggering complexity of module assortments [139]. Despite these limitations, phylogenetic analysis based on 187 A domains identified from predicted *M. robertsii* and *B. bassiana* NRPS, NRPS-like and other adenylating enzymes and 188 reference A domains from other organisms provides several useful clues (Fig. 2). In contrast to PKS phylogeny, direct grouping of *B. bassiana* and *M. robertsii* A domains occurs frequently for NRPS-like proteins but only in 7 instances for NRPSs, of which three were from siderophore biosynthesis NRPSs. Candidate proteins involved in intracellular and extracellular (ferricrocin and coprogens, respectively) siderophore biosynthesis are found in distinct clades as well as the respective α-aminoadipate reductases (AARs), involved in lysine biosynthesis. The additional A domains found in these clades belong to proteins that differ in their structure from canonical siderophore or AARs NRPSs and their possible role in either iron metabolism or lysine biosynthesis is difficult to speculate. Characterized *M. robertsii* NRPS DXS (destruxin) and NPS1 (serinocyclin) have a complex clustering pattern. The first 4 DXS A domains group with both A domains found in perA (peramine biosynthesis NRPS). The last 2 A domains group with the second A domain of both *C. heterostrophus* NPS3 and NPS1 and all are adjacent to a N-methyltransferase domain. For the serinocyclin synthetase NPS1, 6 out of 7 A domains are found in the ergot clade and the A domain from module #2 is clustered with EXV04526.1, an uncharacterized *M. robertsii* monomodular NRPS. Characterized *B. bassiana* bassianolide and beauvericin NRPSs have a more straightforward clustering pattern with each one of their A domains falling within either enniatin module 1 or cyclosporine/enniatin module 2 groups (Fig. 2). No *B. bassiana* or *M. robertsii* NRPS is included in well-defined clades

such as penicillin, peptaibol, or echinocandin. On the other hand, a clade composed of A domains from *B. bassiana* NRPS EJP64345.1 and two *M. robertsii* NRPSs (EXU95985.1, 8 modules; EXU97071, 8 modules) form a separate group. This clade coincides with the "Insect Pathogen Expanded Clade" identified by others [149].

Besides differing greatly in the number of predicted core genes, *M. robertsii* and *B. bassiana* secondary metabolism appears highly specific in terms of the nature of the natural products potentially produced (see Supplemental Tables 1, 2, 3, 4). Some PKSs from *B. bassiana* and *M. robertsii* share significant overall structural similarities (examples are EJP68806.1 and EXU96285.1; EJP67836.1 and EXU97187.1), but the respective predicted gene clusters are quite divergent, hinting at dissimilar natural products. Similarities between *M. robertsii* and *B. bassiana* NRPS metabolism are even less pronounced. The overlapping set includes essentially only siderophore and conidial pigment biosynthesis, both of which tend to be conserved among Ascomycota. Thus, whether or not a specific secondary metabolite has a role in pathogenicity or any interaction with other organisms, our analyses indicate that these two entomopathogens rely on a quite different set of natural products, a likely reflection of their different life styles and functional environments, and consistent with their entomopathogenicity being an example of convergent evolution in which one could predict a similar overall process mediated by unique mechanisms.

5. Predicting novel pathways

Phylogenetic analysis coupled with comparison of entire gene clusters to those characterized in other fungi can be used to approximately predict the structure of natural products associated with some of the *B. bassiana* and *M. robertsii* PKSs and NRPSs. Here, we give 4 examples.

B. bassiana harbours 2 nonreducing PKSs (EJP64619.1 and EJP62792.1) structurally similar to those involved in conidial pigment production in other fungal species [150]. Together with *M. robertsii* EXU96629.1, EJP64619.1 clusters with YWA-like PKSs and is co-localized with genes similar to those involved in melanin biosynthesis (EJP64622.1: tetrahydroxynaphthalene reductase; EJP64623.1: scytalone dehydratase), which makes it a likely heptaketide naphthopyrone synthase. Despite its structural similarity to EJP64619.1 and other YWA-like enzymes, the second nonreducing PKS (EJP62792.1) does not group with either (Fig 1). EJP62792.1 gene is co-localized with a transporter (EJP62793.1) and a laccase (EJP62796.1), which suggests secretion and dimerization of its product, respectively (Fig. 3A). This scenario is similar to that described for the biosynthesis of aurofusarin [151] and consistent with a pathway leading to oosporein production.

A second prediction can be proposed for a mixed PKS-NRPS pathway. Both phylogenetic and AntiSMASH analyses indicate that the PKS EJP62832.1 and the NRPS EJP62835.1 may participate in the biosynthesis of a compound similar to emericellamide [65]. In *A. nidulans* four proteins (NRPS, PKS, acyltransferase, acyl-CoA ligase) are required for that pathway and all are found in close proximity in *B. bassiana* (Fig. 3B). This gene cluster is conserved in *C. militaris*, but appears to be absent in *M. robertsii* and *M. acridum*.

M. robertsii also contains several recognizable pathways beyond those dedicated to the biosynthesis of melanin (EXV02536.1), ferricrocin (EXV05490.1) and a coprogen-type siderophore (EXV04699.1). One includes two NRPSs (EXU97504.1 and EXU97506.1) in which phylogenetic analyses associate with NRPSs involved in alkaloid biosynthesis and co-localize with genes also similar to those involved in ergot alkaloid biosynthesis in *C. purpurea* (Fig. 3C) [152]. This cluster is conserved in *M. acridum*. No clear ergot-like cluster was identified in *B. bassiana* although the NRPSs EJP66334.1, EJP67097.1, and EJP68775.1, respectively, have 2 out of 3, 3 out of 3 and 1 out of 1 adenylation domains within the ergot clade (Fig. 3). A further predicted pathway in *M. robertsii* is that of the PKS EXU97310.1. This non-reducing PKS is structurally similar to that responsible for the biosynthesis of the tetracycline-like antibiotic viridicatumtoxin in *P. aethiopicum* [153]. EXU97310.1 is flanked by homologs responsible for the majority of the tailoring steps and secretion of this antibiotic, except for genes corresponding to VrtC, VrtD, VrtE and VrtK (Fig. 3D). These proteins are presumably responsible for the addition and modification of a geranyl group to the tetracyclic polyketide in *P. aethiopicum*, which is likely to be absent in the corresponding metabolite in *M. robertsii*.

6. Specific biochemical transformations mediated by entomopathogenic fungi

The above sections summarize aspects of the phylogenetics of some of the secondary metabolite biosynthetic clusters present in *M. ansiopliae* and *B. bassiana*. However, these organisms (*Beauveria* spp. in particular) have been extensively used in chemical compound transformations. While little is known about the specific enzymes involved in these reactions, select genes found in secondary metabolite clusters (e.g. especially cytochrome P450s) are likely to be involved in modification of exogenously supplied chemical compounds. Biotransformation involves the use of the enzymatic repertoire of an organism to catalyse the chemical modification of a compound. Reactions involving biotransformation can be separated into two broad categories. Xenobiotic transformations occur when an organism acts on a completely unknown (i.e. does not occur endogenously) substrate. In contrast, biosynthetically patterned transformations involve modification(s) of substrates related to compounds found in intrinsic biochemical pathways present in the catalysing organism. In the case of microbial agents, the use of the whole-cells as biocatalytic units has been considered a sustainable, "green chemistry" approach for large scale biosynthesis, novel compound discovery, biosynthetic pathway probing, as well as models that can be used to examine the metabolic fate of drug candidates [154].

Although a number of entomopathogenic fungi have been used as whole cell biocatalysts for various modification of chemical substrates, often to isolate new bioactive compounds, *Beauveria* sp have by far been the most frequently utilized organisms for such purposes [155]. Reactions catalyzed in such usages have included oxidations (hydroxylations), reductions, transglycosidations, and hydrolytic transformations. The major impetus for such conversions has been to produce (novel) compounds that display enhanced biological activities centered on providing human health benefits. Thus the feed chemical compounds typically start by being considered to possess various anti-microbial, anti-inflammatory, immune-stimulatory, anti-proliferative, and/or tissue-protective activities, often with a particular compound or various derivatives professed to display a remarkably wide range of the activities listed above. Here we will summarize the main chemical transformation reactions catalyzed by *B. bassiana* whole cells by providing recent examples of such work.

(Structures 15–15a here)

Hydroxylation

Silybin (**15**) is a flavonolignin that is the major constituent of herbal preparations derived from the seed extract of the milk thistle (*Silybum marianum*) used in pharmaceutical products for promotion of liver health. In addition to its hepatoprotective effects, silybin has been reported to display antioxidant (perhaps the mechanism behind

its liver health benefits?) and antiproliferative effects, the latter against a wide range of human cancer cell types including prostate, breast, cervix, lung, and liver. Silybin may also act as a neuro-protective, and offer treatment for heart and gastroinstestinal problems. Silybin was transformed to 8-hydroxysilybin (**15a**) by *B. bassiana*, and the resultant product displayed 8-9 fold greater free radical scavenging activity than that the parent compound (silybin) [156].

Lactone derivatives of steroids are of interest due to their potential for displaying anticancer, antiandrogenic, and/or cholesterol-reducing activities. A number of microorganisms including *B. bassiana* are capable of catalyzing Baeyer-Villiger (BV) oxidations that result in the conversion of ketones to lactones or esters. The steroids, epi- and dehydroepi- androsterone (**16, 17**), androstenedione (**18**), androstenediol (**19**), and progesterone (**20**) were first transformed to 11-α hydroxy-derivatives (**16-20a, 18b**) which then subsequently underwent BV oxidation reactions to form a series of 11α-hydroxy ring-D δ-lactones. For some substrates, prolonged incubation with the fungal cells also resulted in reductions to corresponding (17β)-alcohols [157, 158]. The consequences of these modifications on the activities of the various steroids have yet to be reported.

(Structures 16–20 here (*Biotransformations_1Final*))

Lignans represent a class of structurally diverse plant-derived polypropenoids with a wide range of bio-therapeutic applications. Aryltetralins and aryltetralones, in particular, are of interest as precursors for the synthesis of the anticancer drugs etiposide and teniposide. Incubation of the arylteralin lignan (-)-isogalbulin (**21**) with *B. bassiana* resulted in the formation of (-)-8-hydroxyisogalbulin (**21a**) as the only isolatable product [159].
Biotransformation of various steroid compounds by entomopathogenic fungi has also been investigated. The cardiovascular drug, mexrenone (**22**), was modified to two new derivatives, 11α- and 12β-hydroxymexrenone (**22a, 22b**), as well as the known metabolite 6β-hydroxymexrenone (**22c**) by *B. bassiana* [160].

(Structures 21–22 here)

Glycosidation
Flavonoids represent a class of pigmented ketone-containing plant metabolites characterized by a three-cycle (ring) backbone. These compounds are of particular interest due to potential beneficial effects as phytoestrogens, as part of hormone replacement therapies, as cancer chemo-preventive agents, as well as due to variously described antimicrobial, antiviral, and antioxidant activities. A number of hop (beer) derived flavonoids have been transformed to glycosylated products after incubation with *B. bassiana* whole cells, including xanthohumol (**23**), isoxanthohumol (**24**), and 8-prenylnaringenin (**25**). Xanthohumol was transformed by *B. bassiana* via regioselective C-4'-glycosylation to xanthohumol 4'-O- β -D-(4"'-O-methyl) glucopyranoside [161](**23a**), isoxantholhumol was converted to isoxanthohumol 7-*O*-β-D-(4"'-O-methyl)glucopyranoside [162] (**24a**), and 8-prenylnaringenin was converted to 8-prenylnaringenin 7-O- β -D-(4"'-O-methyl)glucopyranoside (**25a)** and 8-prenylnaringenin 7-O-β-D-glucopyranoside (**25b**) [163]. The former product (**24a**) was reported to display higher anti-proliferative activity (against a human colon cancer cell line) than the parent chemical, and the latter two compounds (**24a, 25a**) were considered novel, reported for the first time by the authors. These data indicate the high probability of novel compound production, with flavonoids of tricyclic structure undergoing the highly selective 4'-O-methyl- β -D-glucosidation at the C7'-OH and for chalcones at C4'-OH.

(Structures 23–25 here)

Selective glycosidation of anthraquinones, polyketide natural products being developed as leads for novel antimicrobial, antiproliferative, and anti-inflammatory compounds discovery, has also been reported [164]. In addition, *B. bassiana* has been shown to transform 1-aminoanthracene (**26**), a potent carcinogen, to various metabolites via acetylation (**26a**), oxidation (**26b,c**), and hydroxylation and O-methylglucosylation reactions (**26c,d,e**) [165].

(Structure 26 reaction scheme here (*Biotransformations_2Final*))

Demethylation
Donepezil (2,3-dihydro-5,6-dimethoxy-2-[[1-(phenylmethyl)-4-piperidinyl]methyl]-1*H*-inden-1-one (**27)**, is among a small handful of chemical compounds used in the treatment of severe Alzheimer's disease, acting as a reversible inhibitor of acetylcholinesterase. Donepezil transformation by *B. bassiana* produced trace amounts of 6-O-desmethyl donepezil (5-ODD) (**27a**) while the predominant product was 5-O-desmethyl donepezil (5-ODD) (**27b**), representing O-demethylation products [166].

(Structure 27 reaction scheme here (*Biotransformations_2Final*))

(Structure 28 reaction scheme here (*Biotransformations_2Final*))

Phenylurea derivatives have been extensively used as herbicides for prevention of the growth of undesirable plants and the environmental fate and/or microbial transformation of these products is of significant interest. Diuron, N(3,4-dichlorophenyl)N',N'-dimethylurea (**28**), is employed for total weed control particularly on non-cultivated areas, e.g. roads, where it can accumulate in the soil. Incubation of diuron in the presence of *B. bassiana* resulted in the formation of two terminal nitrogen atom demethylated products, 3,4-dichlorophenylurea (**28a**) and *N*-3,4-dichlorophenyl-*N'*-methylurea (**28b**). Of note, chemical syntheses and testing of both (N-demethylated) degradative products revealed that they possessed greater toxicity in some assays than the parent compound [167].

Redirection of intrinsic biosynthetic pathways
Fungi produce a host of small peptides synthesized using NRPS enzymatic mechanisms. This class of compounds includes the β-lactams and in *B. bassiana*, beauvericins (cyclooligomeric depsipeptides) that have various biological functions. Strategies that have yielded combinatorial biosynthesis of novel compounds via redirection of the beauvericin biosynthesis pathway have recently been reviewed [168]. Briefly, using both wild type and mutants deficient in the supply of the precursor compound, D-hydroxisovalerate, feeding of substrate mimics, i.e. replacement of D-hydroxyisovalerate or L-phenylalanine with 2-hydroxybutyrate, 2-hydroxy3-methylvalerate, or 3-fluorophenylalanine, resulted in novel compounds that displayed altered biological activities [169, 170].

Use of biotransformations in general
Whole-cell biotransformations seek to exploit enzymatic regio- and stereo- specificity to selectively modify chemical compounds. In many instances, use of *B. bassiana* as a whole cell catalyst has resulted in regioselective transformation of substrates into a small number of defined products. However, it should be noted that depending upon the substrate sequential reactions, i.e. demethylation, hydroxylation, and glycosylations could occur. Thus attempts in the

synthesis of the alkaloid epibatidine, a potent analgesic [171], and derivatives using *B. bassiana* resulted in the formation of O-demethylated compounds, which were further metabolized to β-4-methylglucosides, as well as their corresponding C-hydroxylated products [172].

Most chemical transformation studies have focused on *B. bassiana*, although several *Isaria* strains have also been used. Aside from direct biotranformations, these systems have provided approaches for biomimetic syntheses. It is intriguing to speculate that the diverse range of chemical transformations already demonstrated, presumably mediated by an arsenal of fungal enzymes, is a consequence of the interactions that entomopathogens have with plants, insects, and other organisms, and hence the need for detoxification and/or assimilation of xenobiotic compounds. Given that there are several other tractable entomopathogenic fungi, i.e. *Metarhizium* and *Cordyceps sp* for which genomes and molecular tools are available, there is likely a significant potential for further exploitation of these organisms as whole cell biocatalysts.

Conclusions

Genomic data show that entomopathogenic fungi are rich in SM gene clusters, revealing the potential to yield a wealth of chemical compounds. Comparative genomics has allowed for the prediction of several of these orphan pathways, pointing to likely scaffolds for the final products, although these predictions await experimental validation. A number of SM pathways in entomopathogenic fungi have been genetically characterized; however, despite these initial findings, and as seen in most fungi, the vast majority of SM gene clusters, their biological role(s), and the compounds produced remain to be uncovered. Specific genes in SM clusters are also likely to be responsible for the wide range of biotransformations mediated by entomopathogenic fungi in drug discovery and remediation research. With the availability of sequenced genomes, future research in the field is now poised to unravel the SMs produced by these gene clusters and the specific enzymes involved in transformations of chemical compounds.

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Figure legends

Figure 1. Phylogenetic\structural analysis of polyketide synthases (PKSs) and hybrid polyketide synthases-nonribosomal peptide synthetase (hybrid PKS-NRPSs) from *Beauveria bassiana* ARSEF 2860 and *Metarhizium robertsii* ARSEF 2575 inferred using Maximum-Likelihood (PhyML 3.1). Fifty three ketoacyl synthase (KS) domains identified in proteins from these two fungi were aligned with 82 KS domains from PKSs and hybrid PKS-NRPSs for which the natural product is known. Animal fatty acid synthases were used as the outgroup. Clades all have a bootstrap support >50%. T4HN: 1,3,6,8-tetrahydroxynaphthalene. Orph_00006 indicates a sequence found in a *M. robertsii* orphan contig not deposited in GenBank. Reference proteins used for the analysis were: *Alternaria alternata* AFT9-1*; A. solani* alt5. PKSF, sol1*; Aspergillus fumigatus* YWA1*; A. oryzae* PksL1*; A. terreus* LovF, LovB, AtX; *A. clavatus* CcsA, MsaS*; A. fumigatus* fma-PKS, psoA; *A. nidulans* stcA, WA, afoE, afoG, pkha, pkhB, easB, pkfA, AptA, cicF, pkgA, stcJ, orsA, ausA, apdA, pkdA, mdpG; *A. niger* An15g07920, YWA1, An14g04850, FUM1-like; *A. nomius* PksA*; A. ochraceoroseus* AflC*; A. ochraceus* LC35-12*; A. parasiticus* PksL1*; A. sojae* PksL1*; Bombyx mori* FAS_P270*; Cercospora nicotianae* CTB1*; Cochliobolus carbonum* PKS24*; C. heterostrophus* PKS1, PKS2; *Colletotrichum lagenaria* PKS1*; Elsinoe fawcettii* PKS1*; Fusarium heterosporum* eqxS, fsdS; *F. oxysporum* FUM1*; F. pseudograminearum* PKS10*; G. fujikuroi* Pks4, FusA; *G. moniliformis* FusS, FUM1; *G. zeae* PKS12, PKS13, PKS4; *Glarea lozoyensis* pks1*; Hypomyces subiculosus* hpm3*; Monascus purpureus* PksCT*; Mus musculus* FAS*; Mycobacterium sp.* NC_009077.1*; Mycobacterium ulcerans* PpsB*; Mycosphaerella pini* PksA*; M. zeae-maydis* PKS1*; Nodulisporium sp.* PKS1*; Nostoc sp.* all1648*; Penicillium aethiopicum* gsfA, VrtA; *P. brevicompactum* mpaC*; P. expansum* cheA*; P. griseofulvum* 6-MSAS*; P. nordicum* otapksPN*; Saccharomyces cerevisiae* fas2*; Saccharopolyspora erythraea* AM420293.1*; Salinispora arenicola* pks3a*; Sordaria macrospora* pks*; Streptomyces avermitilis* AVES 3*;Wangiella dermatidis* PKS1. *M. robertsii* accessions are reported in green color; *B. bassiana* accessions are reported in red color. Included are examples of metabolites produced by a PKS found within a specific clade.

Figure 2. Phylogenetic\structural analysis of nonribosomal peptide synthetases (NRPSs) and hybrid polyketide synthases-nonribosomal peptide synthetases (hybrid PKS-NRPSs) from *Beauveria bassiana* ARSEF 2860 and *Metarhizium robertsii* ARSEF 2575 inferred using Maximum-Likelihood (PhyML 3.1). One hundred eighty seven adenylation domains (As) identified in proteins from these two fungi were aligned with 375 AMP domains from NRPS, NRPS-like, acyl-CoA ligases and hybrid PKS-NRPSs for which respective products are known. Acyl-CoA ligases were used as the outgroup. Clades all have a bootstrap support >50%. Notations next to GenBank accession indicate which A domain was found in that particular clade. For instance, "EXU95985.1 [1, 2]/8" means that accession EXU95985.1 had A domains #1 and #2 clustering in that clade and that it contains a total of 8 A domains. Chet: *C. heterostrophus* [139]. Orph_00007 indicates a sequence found in a *M. robertsii* orphan contig not deposited in GenBank. Reference proteins used for the analysis were: *Alternaria alternata* AMT; *A. brassicae* Nps1; *Aspergillus clavatus* ccsA; *A. fumigatus* aarA, Afu5g10120, CPS1, FacA, gliP, nps11, pes1, psoA, sidC, sidD, sidI; *A. nidulans* acvA, cicB, easA, micA, TdiA; *A. terreus* apvA, btyA; *Claviceps purpurea* CPPS, CPPS2, CPPS3. CPPS4; C*ochliobolus carbonum* HTS1; *C. heterostrophus* CPS1, NPS1, NPS2, NPS3, NPS4, NPS5, NPS6, NPS7, NPS8, NPS9, NPS10, NPS11, NPS12; *Emericella rugulosa* EcdA ; *Epichloe festucae* PerA; *Fusarium equiseti* ESYN1; *F. graminearum* CPS1; *F. pseudograminearum* CPS1; *Gibberella moniliformis* FusS; *G. zeae* AAR, FG03589.1, FG10617.1, NPS6. NPS10; *Glarea lozoyensis* GLNRPS4; *Leptosphaeria maculans* maa1, SirP; *Magnaporthe oryzae* SSM1, SSM2_NPS6, syn2; *Metarhizium robertsii* PesA; *Neurospora crassa* AAR, NCU00608.7, NPS6; *Omphalotus olearius* Fso1; *Penicillium chrysogenum* ACVS1; *P. expansum* cheA; *Pyrenophora tritici-repentis* PTRG_06150; *Saccharomyces cerevisiae* ACS2, CPS1-like, LYS2; *Schizosaccharomyces pombe* AAR, sib1; *Tolypocladium inflatum* SimA; *Trichoderma virens* tex1; *Ustilago maydis* fer3, sid2, UM01434.1, UM04803.1. *M. robertsii* accessions are reported in green color; *B. bassiana* accessions are reported in red color. Included are examples of metabolites produced by an NRPS found within a specific clade.

Figure 3. Comparison of known gene clusters to similar clusters in *Beauveria bassiana* and *Metarhizium robertsii.* A. Organization of the predicted oosporein biosynthesis gene cluster in *Beauveria bassiana*; B. Comparison of the emericellamide synthase gene cluster in *Aspergillus nidulans* to those found in *Beauveria bassiana* and *Cordyceps militaris*; C. Comparison of the ergot biosynthesis gene cluster in *Claviceps purpurea* to the ergot-like gene cluster identified in *Metarhizium robertsii;* D. Comparison of the viridicatumtoxin biosynthesis gene cluster in *Penicillium aethiopicum* to a similar gene cluster identified in *Metarhizium robertsii*.

Figure 1.

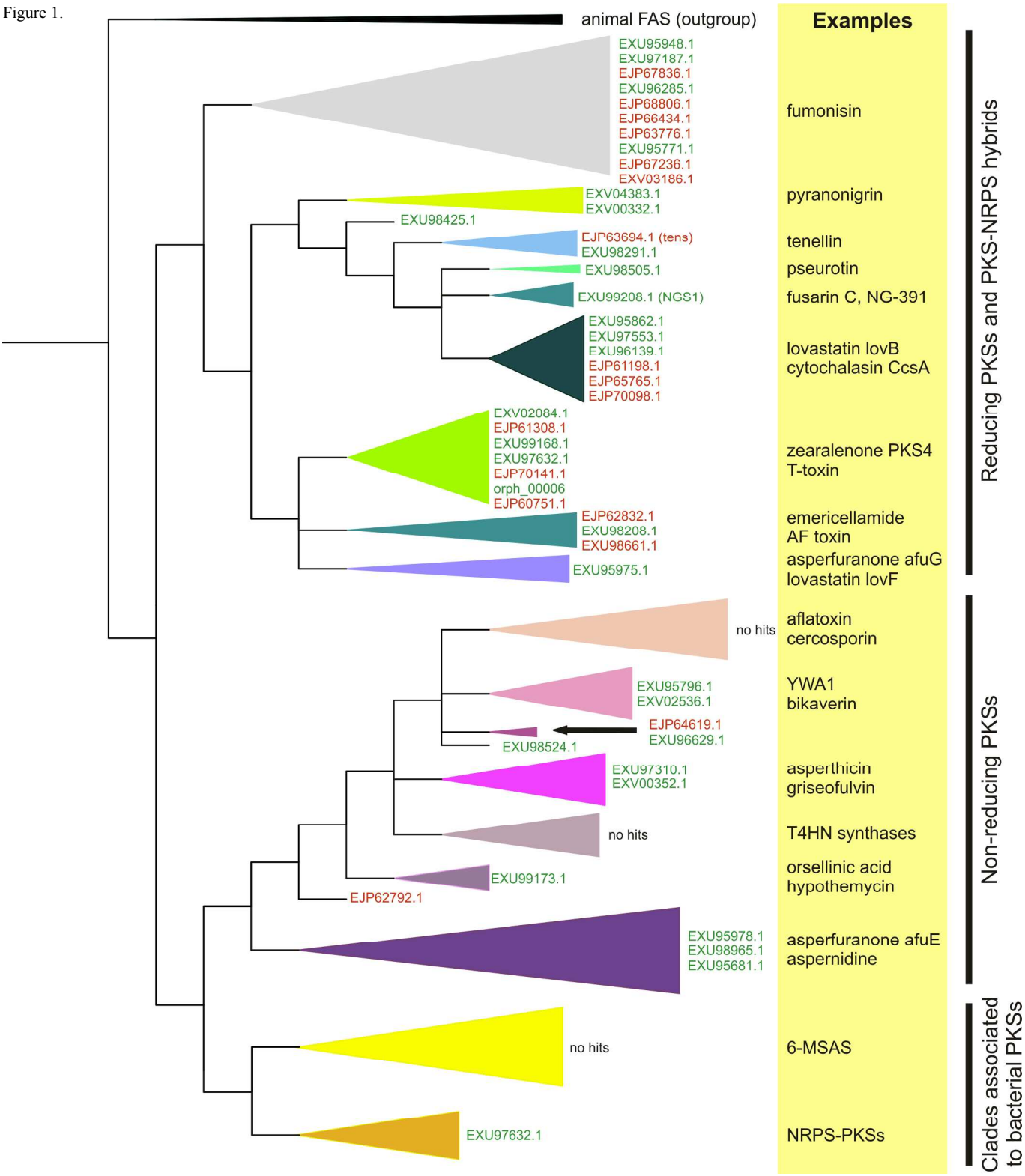

animal FAS (outgroup)
Examples
EXU95948.1
EXU97187.1
EJP67836.1
EXU96285.1
EJP68806.1
EJP66434.1
EJP63776.1
EXU95771.1
EJP67236.1
EXV03186.1
fumonisin
EXV04383.1
EXV00332.1
pyranonigrin
EXU98425.1
EJP63694.1 (tens)
EXU98291.1
tenellin
EXU98505.1
pseurotin
EXU99208.1 (NGS1)
fusarin C, NG-391
EXU95862.1
EXU97553.1
EXU96139.1
EJP61198.1
EJP65765.1
EJP70098.1
lovastatin lovB
cytochalasin CcsA
EXV02084.1
EJP61308.1
EXU99168.1
EXU97632.1
EJP70141.1
orph_00006
EJP60751.1
zearalenone PKS4
T-toxin
EJP62832.1
EXU98208.1
EXU98661.1
emericellamide
AF toxin
EXU95975.1
asperfuranone afuG
lovastatin lovF
Reducing PKSs and PKS-NRPS hybrids
no hits
aflatoxin
cercosporin
EXU95796.1
EXV02536.1
YWA1
bikaverin
EJP64619.1
EXU96629.1
EXU98524.1
EXU97310.1
EXV00352.1
asperthicin
griseofulvin
no hits
T4HN synthases
EXU99173.1
orsellinic acid
hypothemycin
EJP62792.1
EXU95978.1
EXU98965.1
EXU95681.1
asperfuranone afuE
aspernidine
Non-reducing PKSs
no hits
6-MSAS
EXU97632.1
NRPS-PKSs
Clades associated to bacterial PKSs

Figure 2.

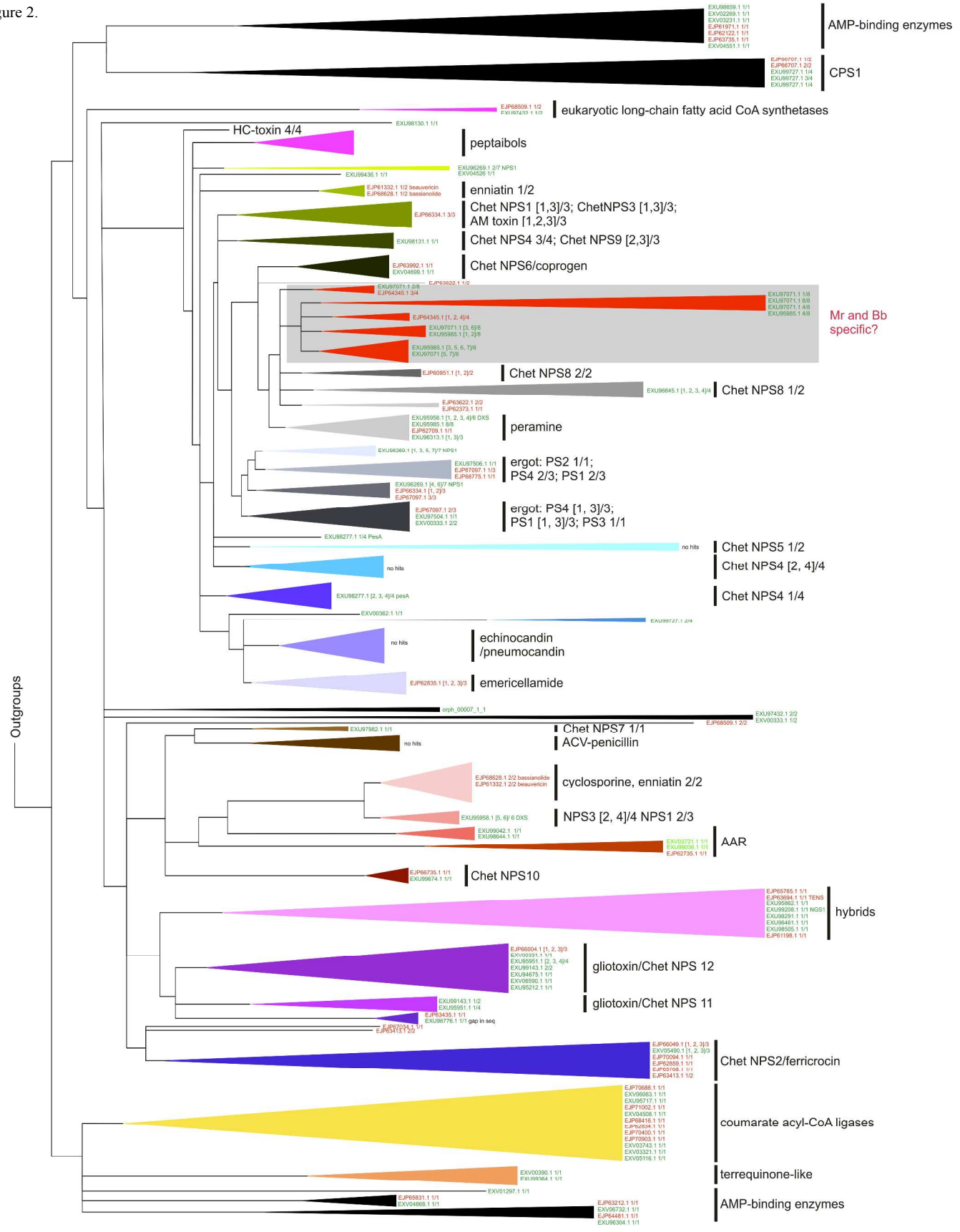
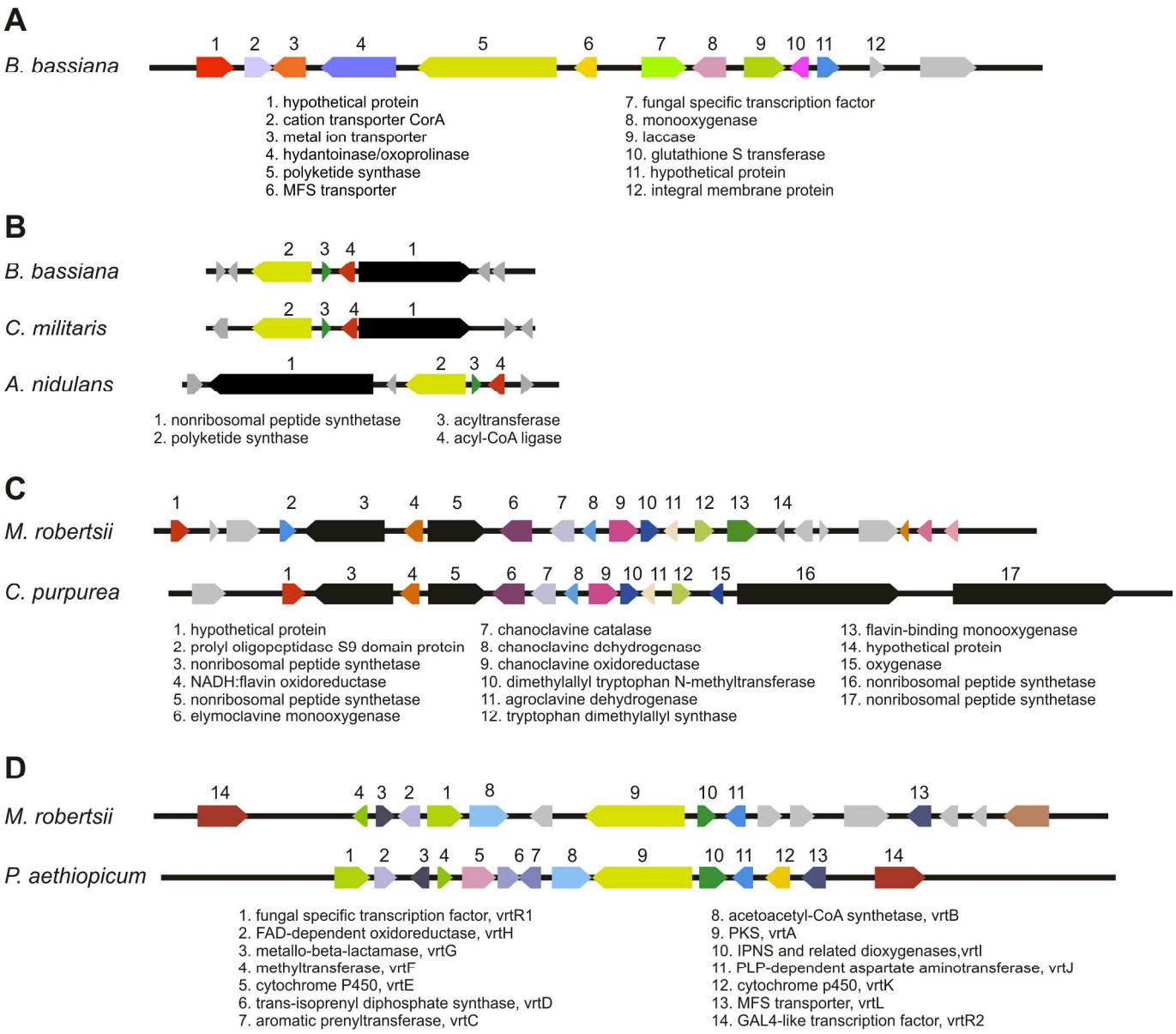


Figure 3.


A
B. bassiana
1 2 3 4 5 6 7 8 9 10 11 12
1. hypothetical protein
2. cation transporter CorA
3. metal ion transporter
4. hydantoinase/oxoprolinase
5. polyketide synthase
6. MFS transporter
7. fungal specific transcription factor
8. monooxygenase
9. laccase
10. glutathione S transferase
11. hypothetical protein
12. integral membrane protein
B
B. bassiana
C. militaris
A. nidulans
1. nonribosomal peptide synthetase
2. polyketide synthase
3. acyltransferase
4. acyl-CoA ligase
C
M. robertsii
C. purpurea
1. hypothetical protein
2. prolyl oligopeptidase S9 domain protein
3. nonribosomal peptide synthetase
4. NADH:flavin oxidoreductase
5. nonribosomal peptide synthetase
6. elymoclavine monooxygenase
7. chanoclavine catalase
8. chanoclavine dehydrogenase
9. chanoclavine oxidoreductase
10. dimethylallyl tryptophan N-methyltransferase
11. agroclavine dehydrogenase
12. tryptophan dimethylallyl synthase
13. flavin-binding monooxygenase
14. hypothetical protein
15. oxygenase
16. nonribosomal peptide synthetase
17. nonribosomal peptide synthetase
D
M. robertsii
P. aethiopicum
1. fungal specific transcription factor, vrtR1
2. FAD-dependent oxidoreductase, vrtH
3. metallo-beta-lactamase, vrtG
4. methyltransferase, vrtF
5. cytochrome P450, vrtE
6. trans-isoprenyl diphosphate synthase, vrtD
7. aromatic prenyltransferase, vrtC
8. acetoacetyl-CoA synthetase, vrtB
9. PKS, vrtA
10. IPNS and related dioxygenases,vrtI
11. PLP-dependent aspartate aminotransferase, vrtJ
12. cytochrome p450, vrtK
13. MFS transporter, vrtL
14. GAL4-like transcription factor, vrtR2