Insights into natural products biosynthesis from analysis of 490 polyketide synthases from *Fusarium*

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Abstract

Species of the fungus *Fusarium* collectively cause disease on almost all crop plants and produce numerous natural products (NPs), including some of the mycotoxins of greatest concern to agriculture. Many *Fusarium* NPs are derived from polyketide synthases (PKSs), large multi-domain enzymes that catalyze sequential condensation of simple carboxylic acids to form polyketides. To gain insight into the biosynthesis of polyketide-derived NPs in *Fusarium*, we retrieved 488 PKS gene sequences from genome sequences of 31 species of the fungus. In addition to these apparently functional PKS genes, the genomes collectively included 83 pseudogenized PKS genes. Phylogenetic analysis resolved the PKS genes into 67 clades, and based on multiple lines of evidence, we propose that homologs in each clade are responsible for synthesis of a polyketide that is distinct from those synthesized by PKSs in other clades. The presence and absence of PKS genes among the species examined indicated marked differences in distribution of PKS homologs. Comparisons of *Fusarium* PKS genes and genes flanking them to those from other Ascomycetes provided evidence that *Fusarium* has the genetic potential to synthesize multiple NPs that are the same or similar to those reported in other fungi, but that have not yet been reported in *Fusarium*. The results also highlight ways in which such analyses can help guide identification of novel *Fusarium* NPs and differences in NP biosynthetic capabilities that exist among fungi.

Keywords: *Fusarium*; secondary metabolite; natural product; polyketide; evolution; polyketide synthase

Introduction

Fungi produce thousands of structurally diverse metabolites that are not essential for growth or reproduction (Cole et al., 2003). Such compounds are referred to as secondary metabolites or natural products (NPs), and collectively they have a broad range of biological activities (Keller, 2015; Keller et al., 2005). Some have beneficial pharmaceutical activities, some are toxic to animals and/or plants, others are plant hormones, and still others are pigments. Although the roles of most NPs in the ecology of fungi that produce them are not known, some contribute to virulence on plants or insects, while others enhance competitiveness with other microbes. Over the past two decades, there has been a tremendous effort to understand the genetic and biochemical pathways that lead to the formation of NPs, because of the potential for such information to contribute to drug discovery, efforts to control plant disease and toxin contamination problems caused by fungi, as well as development of effective biological control strategies for insect and microbial pests (Ahuja et al., 2012; Brown et al., 2014).

The majority of fungal NPs characterized to date are derived from polyketides (Brown et al., 2015; Wiemann and Keller, 2014; Wiemann et al., 2013). Fungal polyketide synthases (PKSs) are large, multi-domain proteins that catalyze sequential (or iterative) condensation of simple carboxylic acids, such as malonate (Hopwood and Khosla, 1992). The broad structural diversity of polyketides arises from: different carboxylic acids substrates used by PKSs; whether the β -carbonyl formed during each condensation cycle is reduced; and whether the polyketide is cyclized. The even greater structural diversity of polyketide-derived NPs results from post-PKS structural modifications, such as oxygenation, acylation, and further cyclization. In fungi, the genes encoding a PKS and polyketide-modifying enzymes required for synthesis of the same NP, are typically located next to one another in gene clusters (Brown et al., 1996). Such clusters also typically include a gene encoding a protein that transports the NP(s) across the cell membrane, as well as a gene encoding a transcription factor that regulates expression of the cluster genes.

Genomic studies indicate that fungi have the genetic potential to produce many more NPs than predicted based on chemical analyses of fermentation extracts (Cuomo et al., 2007; Ehrlich and Mack, 2014; Gaffoor et al., 2005; Georgianna et al., 2010). Significant effort has been invested to identify the polyketides and/or polyketide-derived NPs formed via the activities of PKSs identified through fungal genome sequencing. These efforts require a combination of molecular genetics and analytical chemistry approaches and, as a result, can be demanding of

time and resources. Fungal NP biosynthetic gene clusters are often not expressed under laboratory culture conditions. Thus, one approach to identify a polyketide product of a PKS is to overexpress the corresponding PKS gene and/or the transcription factor gene located within the same NP gene cluster (Ahuja et al., 2012; Connolly et al., 2013; Jorgensen et al., 2014). Although such efforts have achieved significant success, the polyketides synthesized by many fungal PKSs identified by genome sequencing have yet to be identified. For example, in *Aspergillus nidulans* the polyketide products of 11 of 27 PKSs and in *Fusarium graminearum* the products of seven of 15 PKSs have not been identified (Ahuja et al., 2012; Gaffoor et al., 2005; Hansen et al., 2015; Jorgensen et al., 2014; Sorensen et al., 2014).

Phylogenetic and other comparative analyses of PKS homologs from multiple fungi can guide efforts to identify novel NPs and gain insights into NP biosynthetic pathways (Throckmorton et al., 2015). Different genera of fungi as well as different species within the same genus can produce the same or structurally similar NPs and therefore have relatively closely related homologs of NP biosynthetic genes, including PKS genes. For example, some species of the relatively distantly related fungi *Fusarium* (Sordariomycetes) and *Aspergillus* (Eurotiomycetes) produce fumonisins, a group of structurally related polyketide-derived mycotoxins (Susca et al., 2014). Thus, it is often possible to gain significant insight into the genetic potential of a fungus to produce NPs by comparing its putative NP biosynthetic genes to those from other fungi in which the genetics of NP biosynthesis has been more thoroughly characterized and genetic tools are readily available.

Fusarium includes over 100 described species that collectively cause disease on an extraordinarily wide range of plant species, including most crops (Leslie and Summerell, 2006; Morgavi and Riley, 2007). *Fusarium* is also reported to produce over 50 structurally related families of NPs that vary tremendously in chemical structure and biological activity (Desjardins, 2006; Vesonder and P., 1989). Among these NPs are mycotoxins (e.g. fumonisins, trichothecenes and zearalenone) that can contaminate food and feed derived from *Fusarium*-infected crops. The economic impact of mycotoxin contamination via its effects on health of humans and livestock as well as on international trade is estimated to be in the 100s of millions of US dollars each year (Wu, 2007).

Comparative analyses of *Fusarium* PKS genes and associated gene clusters can provide important insights into the genetic potential of species of the fungus to produce NPs as well as

guide efforts to identify the corresponding polyketides and NPs (Brown et al., 2012a; Brown and Proctor, 2013; Hansen et al., 2015; Hansen et al., 2012; Ma et al., 2010; Wiemann et al., 2013). Such analyses can also provide insights into the distribution and evolution of NP biosynthetic genes. Most recently, 153 PKSs have been cataloged from analyses of genome sequences of 10 *Fusarium* species: *F. acuminatum*, *F. circinatum*, *F. culmorum*, *F. fujikuroi*, *F. graminearum*, *F. mangiferae*, *F. oxysporum*, *F. pseudograminearum*, *F. solani* f. sp. *pisi*, and *F. verticillioides* (Hansen et al., 2015). Phylogenetic analyses have resolved the PKSs into two major clades, or groups: a non-reducing PKS (NR-PKS) group responsible for synthesis of aromatic polyketides; and a reducing PKS (R-PKS) group responsible for synthesis of mostly nonaromatic polyketides (Brown et al., 2012a; Ma et al., 2010; Wiemann et al., 2013). In addition, the R-PKS group has been further resolved into three subclades, designated R-PKS I, II and III. The resolution of *Fusarium* PKSs into the NR-PKS and three R-PKS groups is consistent with results of similar analyses of PKSs from multiple fungal genera (Baker et al., 2006; Kroken et al., 2003; Riley et al., 2014). Hansen et al. (2015) classified the 153 PKSs from *Fusarium* into 52 orthology groups and summarized information on the polyketide-derived NP products formed via the activities of orthologs in 15 of the groups. Therefore, the NPs corresponding to the other 37 orthology groups have not been identified.

The objective of this study was to gain further insight into the genetics of NP biosynthesis in *Fusarium* through comparative analysis of PKS genes retrieved from genome sequences of 31 species of the fungus. The results of this analysis provide evidence that the species examined have the collective genetic potential to produce 64 structurally distinct families of polyketide-derived NPs. The results also reveal extensive variability in patterns of distribution of PKS genes among the species and provide evidence for production of seven families of NPs that have not been reported previously in *Fusarium*. Insights gained from these results should help guide future studies aimed at identifying novel polyketides and polyketide-derived NPs produced by *Fusarium* and other fungi.

Materials and Methods.

***Fusarium* species and DNA sequence data generation.** The *Fusarium* species examined in this study are listed in Table 1. Genome sequence data for 11 *Fusarium* species were obtained from the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov).

Whole-genome sequencing was performed on DNA extracted from 20 *Fusarium* species grown in GYP (2% glucose, 1% peptone, and 0.3% yeast extract) liquid cultures using the ZR Fungal DNA MiniPrep™ kit as described by the manufacturer (Zymo Research, Irvine CA). Genome sequence data for seven *Fusarium* species were generated by the Beijing Genome Institute, Hong Kong) using a HiSeq 2000 sequencing platform. Genome sequence data for 13 *Fusarium* species were generated using a Life Technologies Ion Torrent PGM™ (12 genomes) or Illumina MiSeq® (one genome) sequencing platform at NCAUR-ARS. The genomic DNA libraries for sequencing with Ion Torrent were constructed from 1 µg of purified DNA using the New England Biolabs NEBnext Fast DNA Library prep set for Ion Torrent. The sequencing data was obtained using the Ion Torrent 400 bp template kit and the HiQ sequencing chemistry on a 318v2 chip. The genomic DNA library for sequencing with the Illumina MiSeq was constructed from 1 ng of purified DNA using the Nextera XT DNA Library Preparation Kit. The sequencing data was obtained using the MiSeq Reagent Kit v3. Low-quality reads were trimmed and filtered using CLC Genomics Workbench (version 8.0) and assembled into contigs.

Search for PKSs and housekeeping genes. Putative functional and non-functional PKSs (e.g. pseudogenes) and housekeeping genes were identified by Basic Local Alignment Search Tool (BLAST) analysis (Zhang et al., 1998) against BLAST databases for all 31 *Fusarium* genome sequence data sets created in CLC Genomics Workbench. The PKS BLAST query sequences were nucleotide sequences encoding 16 *F. fujikuroi* PKSs, three *F. verticillioides* PKSs, 10 *F. graminearum* PKSs, one *F. circinatum* PKS, four *F. oxysporum* PKSs and four *F. solani* f. sp. *pisi* PKSs, which collectively represent the 37 different groups or clades of *Fusarium* PKSs identified previously by phylogenetic analysis (Brown and Proctor, 2013; Wiemann et al., 2013). Nucleotide sequences with E values $< 1 \times 10^{-10}$ to the query sequence and flanking sequences were subjected to BLASTX analysis against NCBI-NR databases to identify the full-length PKSs and PKS pseudogenes (Supplemental Table 2). Sequence adjacent to select putative PKSs were also subjected to BLASTX analysis against NCBI-NR databases to identify potential NP biosynthetic genes. The housekeeping gene BLAST query sequences were 20 putative housekeeping genes from *F. graminearum* (Supplemental Table 3 and 4). Nucleotide sequences with E values $< 1 \times 10^{-100}$ to the query sequence and flanking sequences were subjected to BLASTX analysis against NCBI-NR databases to identify each full-length housekeeping gene.

Phylogenetic analysis. The evolutionary history of 490 *Fusarium* PKSs and 55 PKSs from other Ascomycetes (Supplemental Table 1 and 2) within and among species was inferred using the Maximum Likelihood (ML) method, based on the Tamura-Nei Model (Tamura and Nei, 1993) and the Maximum Parsimony (MP) method in MEGA6 (Tamura et al., 2013). Amino acid sequences corresponding to the 546 predicted proteins were aligned using Muscle and converted to nucleotide sequence (Supplemental Text) prior to constructing the phylogeny. All positions containing gaps and missing data were eliminated. The ML tree with the highest log likelihood is shown (Figure 1) and is rooted on the fatty acid synthase (FAS) from *Gallus gallus*. The percentage of trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Phylogenetic trees based on the coding sequences for the combined keto-synthase and acyltransferase/malonlytransferase (KS-AT/MAT) domains were very similar to the tree based on the entire PKS gene coding region (data not shown). A species phylogeny for the 31 *Fusarium* species was inferred from 20 housekeeping genes (Supplemental Table 3 and 4) using the same methods used for phylogenetic analysis of the PKS genes except that alignments were generated from nucleotide with ClustalW as implemented in MEGA6 (Figure 2).

Results and Discussion

Phylogenetic framework of species. To establish a phylogenetic framework of the 31 *Fusarium* species examined in this study, we retrieved the nucleotide sequences of coding regions of 20 housekeeping genes from genome databases of each species, aligned homologous sequences, and then subjected concatenated alignments of all 20 genes to maximum likelihood and maximum parsimony analysis. The resulting species phylogenies (Figure 2) were largely consistent with previously described phylogenies that included similar sets of species (Geiser et al., 2013; O'Donnell et al., 2013). The phylogeny inferred in the current study indicated that the species examined are distributed among 10 previously described species complexes (Figure 2) (Geiser et al., 2013; O'Donnell et al., 2013). Also consistent with the previous studies, *Fusarium*

sp. NRRL 25184 was not resolved within a species complex, but instead was resolved as a single-member lineage that is sister to the *F. fujikuroi*, *F. nisikadoi*, and *F. oxysporum* species complexes (Figure 2).

Resolution of *Fusarium* PKSs into 67 Clades. In this study, we examined 488 PKS gene sequences retrieved from 11 publically available *Fusarium* genome sequences (171 PKSs) and 20 newly generated *Fusarium* genome sequences (317 PKSs) (Supplemental Table 2). Our analysis also included two PKS gene sequences from *Fusarium heterosporum* (for a total of 490 *Fusarium* PKSs) and 55 PKS sequences (Supplemental Table 1) from other genera of the Ascomycota. The latter PKSs have been functionally characterized and the corresponding polyketides or polyketide-derived NPs have been identified.

The genealogies generated by maximum likelihood and maximum parsimony analysis of the nucleotide sequences of the full-length PKS coding regions (Figure 1) or the KS-AT/MAT domains (data not shown) were similar in topology as well as support for clades. Consistent with previous studies (Brown et al., 2012a; Ma et al., 2010; Wiemann et al., 2013), the current analysis resolved two well supported clades (100%) corresponding to the NR-PKS and R-PKS II groups of PKSs and a moderately supported clade (79%) corresponding to R-PKS III (Figure 1). In contrast to the previous studies, however, the current analysis did not resolve R-PKS I PKSs into a well supported clade, but instead resolved them into 21 smaller clades, each with >99% bootstrap support, and two single-member PKS clades. As a result, in the current study we assigned PKSs to the R-PKS I groups based on phylogenetic relationships to PKSs previously assigned to this group (Brown et al., 2012a; Wiemann et al., 2013). In previous studies, two PKSs, FCIRG_08230 from *F. circinatum* and FO47SC12 from *F. oxysporum* were present on separate branches sister to the NR-PKS clade (Brown et al., 2012a; Wiemann et al., 2013). In this study, a PKS from *F. redolens* and another from *F. foetens* were resolved within the same clade as FO47SC12. These four PKSs are distinct from all other R-PKSs in that they lack either a functional or pseudogenized methyltransferase (ME) domain and, therefore, will be referred to hereafter as Rm-PKSs.

Consistent with previous results, the maximum likelihood analysis in the current study resolved PKSs within the NR-PKS and R-PKS groupings into smaller and well supported clades, which we have designated Clades 1 – 33, 35 – 40, 42 – 45 and 48 – 71. A subset of these clades correspond, for the most part, to the PKS orthology groups *PKS1* – *PKS33*, *PKS35* – *PKS40*,

219 *PKS42 – PKS45* and *PKS48 – PKS52* recently described by Hansen et al. (2015). For example,
 220 Clades 1 – 33 correspond to orthology groups *PKS1 – PKS33*. However, there are a few
 221 discrepancies between the clades resolved in the current analysis and the orthology groups
 222 described by Hansen et al. (2015). In addition, we also gave clade numbers to 15 *Fusarium*
 223 PKSs that were not resolved into clades with other PKSs: Clades 31, 49, 54 – 60, 62, 64, 67, 68,
 224 70 and 71. PKS Clades 1 – 33, 35 – 40, 42 – 45 and 48 – 71 were distributed across the larger
 225 NR-PKS and three R-PKS groups: the NR-PKS group was resolved into 11 smaller clades, and
 226 R-PKS groups I, II and III were resolved into 23, 14 and 17 smaller clades respectively. In
 227 addition, the four PKSs in the Rm-PKS group were resolved into two smaller clades. Even
 228 though the PKS genes used in our phylogenetic analysis included highly divergent sequences, the
 229 phylogenetic analysis with this large and divergent data set was effective at resolving a large
 230 number of clades. Phylogenetic analyses with smaller sets of more closely related sequences
 231 confirmed relationships resolved with the large data set, but provided a higher resolution for
 232 relationships between homologs within clades and, in some cases, between clades
 233 (Supplementary Figure 2).

234 Based on the PKS domain structures and knowledge of the polyketide products
 235 synthesized by some of the PKSs, we hypothesize that PKS homologs in each clade are
 236 responsible for synthesis of a polyketide that is structurally distinct from polyketides synthesized
 237 by PKSs in other clades. If this hypothesis is correct, the 490 *Fusarium* PKSs analyzed here are
 238 likely responsible for synthesis of 67 structurally distinct polyketides; i.e., one polyketide for
 239 each of the 67 PKS Clades. The hypothesis is consistent with the location of the PKS genes
 240 from the same clade within homologous gene clusters. For example, the five PKSs in Clade 24
 241 are orthologs of the enzyme (Fum1), which is required for biosynthesis of fumonisin mycotoxins
 242 (Proctor et al., 1999). In all five species, the *FUM1* gene is located in the 16-17 gene fumonisin
 243 biosynthetic gene (*FUM*) cluster (Proctor et al., 2013).

244 It is likely though that fewer than 67 polyketide-derived NPs, or families of NPs, are
 245 formed from the 67 putative polyketides. This is because some NPs are formed via the activity
 246 of pairs of PKSs consisting of an R-PKS and an NR-PKS where the R-PKS synthesizes a
 247 polyketide that serves as a starter unit for the NR-PKS to synthesize a larger polyketide. Such R-
 248 PKS/NR-PKS pairs are required for synthesis of zearalenone in *Fusarium* (Clade 13 and Clade
 249 4) and several other metabolites in *Aspergillus* (Ahuja et al., 2012; Gaffoor and Trail, 2006; Kim

et al., 2005). The genes encoding these R-PKS/NR-PKS pairs are typically located adjacent or close to one another on a chromosome. In *F. fujikuroi*, an R-PKS/NR-PKS pair of unknown function located 15 kb apart was found and proposed to be involved in the synthesis of a single polyketide (Wiemann et al., 2013). The 488 PKS genes that we retrieved from *Fusarium* genome sequences included four pairs of R-PKS/NR-PKS genes located adjacent to on another. The zearalenone pair, Clade 13 and Clade 4 is present in four *Fusarium* and the unknown metabolite putative pair from *F. fujikuroi*, Clade 36 and Clade 37, is present in 10 *Fusarium*. Two new R-PKS/NR-PKS pair include the R-PKS genes of Clade 48 and NR-PKS genes of Clade 53 in three *Fusarium* and the R-PKS gene of Clade 62 and the NR-PKS gene of Clade 54 in *F. thapsinum*. Based on their locations next to one another, these additional pairs of R-PKS/NR-PKS genes are likely required for synthesis of the same NP. Thus, although we propose that each of the 67 clades of *Fusarium* PKSs is responsible for synthesis of a structurally distinct polyketide, it is likely that 67 polyketides give rise to only 63 families of NPs.

Discrepancies between Clades and Orthology Groups. Comparison of the orthology groups described by Hansen et al. (2015) with clades resolved by our phylogenetic analysis indicated that most of the orthology groups correspond to well-supported clades. There were, however, four exceptions. The first exception involves orthology groups *PKS16*, *PKS46* and *PKS47* described by Hansen *et al.* (2015). *PKS46* and *PKS47* groups each consisted of two PKSs, one from *F. acuminatum* and another from *F. avenaceum*. Our phylogenetic analysis indicated that these PKSs are closely related to PKSs in orthology group *PKS16*, which consists of PKSs required for synthesis of the pigment bikaverin (Figure 3A). Analysis of genes flanking *PKS46* and *PKS47* in *F. acuminatum* and *F. avenaceum* revealed the presence of homologs of some or all of the genes in the bikaverin biosynthetic gene (*BIK*) cluster (Figure 3A) (Wiemann et al., 2009). These findings suggest that *F. acuminatum* and *F. avenaceum* have two *BIK* cluster-like clusters. Given that genes *BIK1*, *BIK2* and *BIK3* are the only enzyme-encoding genes required for bikaverin synthesis (Wiemann et al., 2009), and that these three genes are present in the *BIK* cluster-like clusters in *F. acuminatum* and *F. avenaceum*, we propose that PKSs in orthology groups *PKS46* and *PKS47* are likely functional orthologs of the bikaverin PKS and, therefore, we include them in the same grouping Clade 16. These findings also indicate that *F. acuminatum* and *F. avenaceum* have the genetic potential to produce bikaverin and/or a bikaverin-like

metabolite(s). Because *PKS46* and *PKS47* described by Hansen et al (2015) were included in Clade 16 in the current study, and in an attempt to avoid confusion, we have not included a Clade 46 or 47 in our numbering system. In a previous study, the bikaverin PKS gene (*BIK1*) was not detected in multiple strains of *F. avenaceum* and *F. acuminatum* by Southern blot analysis using a hybridization probe that consisted of an approximately 1-kb fragment that spanned the keto-synthase (KS)-coding domain of the *F. verticillioides BIK1* gene (O'Donnell et al., 2013). The failure to detect *BIK1* homologs in *F. acuminatum* and *F. avenaceum* is likely a result of the relatively low level of amino acid sequence identity (61 – 67%) of the *F. verticillioides BIK1* probe (KS domain) to the KS domain of the homologs in the other two species. Previously, based in part on phylogenetic analysis, it was proposed that the *bik1* from *Botrytis cinerea* was acquired from *Fusarium* through horizontal gene transfer (HGT) (Campbell et al., 2012; Schumacher et al., 2013). Our results are consistent with this hypothesis and further suggest that the donor was likely a species that arose after the *F. fujikuroi*, *F. nisikadoi* and *F. oxysporum* species complexes diverged from *F. redolens* (Supplemental Figure 3).

The second exception involved orthology group *PKS20* of Hansen et al. (2015), which consisted of a PKS from *F. avenaceum*, *F. fujikuroi*, *F. oxysporum* and *F. verticillioides*. In our phylogenetic analysis, the *F. fujikuroi*, *F. oxysporum* and *F. verticillioides* PKSs were resolved as part of a well-supported clade (Clade 20; bootstrap value 100) that did not include the *F. avenaceum* PKS (FAVG1_07658). In fact, FAVG1_07658 resolved as part of a clade (bootstrap value 97) that included the *F. heterosporum* PKS that is responsible for synthesis of the NP fusaridione (Figure 1). Analysis of the genes flanking the Clade 20, Clade 69 and FAVG1_07658 PKS genes revealed that two of the FAVG1_07658 flanking genes were closely related homologs of genes in the proposed five-gene fusaridione biosynthetic gene cluster (Figure 3B). In addition, none of the FAVG1_07658 flanking genes were closely related to genes flanking the Clade 20 PKS genes. Thus, we consider FAVG1_07658 to be a single-homolog lineage, which we have designated Clade 68 (Figure 1), and we hypothesize that it catalyzes synthesis of a molecule that is similar in structure to the fusaridione polyketide. Assuming FAVG1_07658 and some of the genes flanking it constitute a biosynthetic gene cluster, we further hypothesize that the corresponding NP is similar to but structurally distinct from fusaridione. Based on current information, however, we cannot rule out the possibility that FAVG1_07658 and Clade 69 PKSs synthesize the same polyketide that is further modified to

different NPs. Given differences in their sequences, it seems likely that FAVG1_07658 and the Clade 20 PKS homologs give rise to structurally distinct polyketides. Furthermore, given differences in the content of the putative gene clusters in which the PKS genes are located, it is likely that FAVG1_07658 and the Clade 20 PKSs are involved in distinct biosynthetic pathways that give rise to structurally distinct NPs.

The third exception involves orthology group *PKS14* described by Hansen et al. (2015), which consisted of a PKS from *F. avenaceum*, *F. culmorum*, *F. graminearum* and *F. pseudograminearum*. In our analysis, however, the *F. avenaceum* PKS, FAVG1_07922, was not resolved in the same clade as the other three *PKS14* homologs, but instead was resolved in a clade (Clade 53) with PKSs from *F. redolens* and *F. sacchari*. As mentioned previously, **all three Clade 53 PKSs are adjacent to an R-PKS present in Clade 48**. Thus, our results indicate that PKSs in orthology group *PKS14* have been partitioned into two groups: Clade 14 and Clade 53. Clade 14 consists of the *F. culmorum*, *F. graminearum*, and *F. pseudograminearum* PKSs from orthology group *PKS14* as well as *F. poae* PKS Fpo 93; and Clade 53 consists of FAVG1_07922 and homologs from *F. redolens* and *F. sacchari* (Figure 1).

The fourth exception involves orthology groups *PKS34* and *PKS43*. In the Hansen et al. study, group *PKS34* consisted of a single PKS from *F. solani*, JGI 123123, and group *PKS43* consisted of a PKS from *F. avenaceum* and FIESC 5 (*F. equiseti* in Hansen et al.). In our analysis, groups *PKS34* and *PKS43* were resolved in the same clade (Clade 43) along with six other PKS homologs. In addition, all eight PKSs of this clade are flanked by orthologs of the same gene, encoding a putative chalcone synthase, suggesting a two-gene cluster. Because *PKS34* described by Hansen et al (2015) was included in Clade 43 in the current study, and in an attempt to avoid confusion, we have not included a Clade 34 in our clade numbering system.

Distribution of PKSs in *Fusarium*. Examination of the occurrence of the 488 PKS genes in the 31 *Fusarium* species surveyed revealed multiple and disparate patterns of distribution (Figure 4). At one extreme, homologs of Clades 3, 7 and 8 occur in all 31 species. This finding is consistent with previous studies that reported these three PKS genes occurred in all species examined (Brown et al., 2012a; Ma et al., 2010; Wiemann et al., 2013). Homologs in Clade 3 are required for the synthesis of fusarubin (Gaffoor et al., 2005; Studt et al., 2012), while the polyketides and NPs formed via the activity of the PKS homologs in Clades 7 and 8 have not been identified. At

the other extreme in distribution, 15 PKS clades consist of only one *Fusarium* homolog, and therefore, such PKSs are unique to one of the 31 *Fusarium* species examined. For example, Clade 58 consists of only the *F. aywerte* PKS Fay 343, and Clade 59 consists of only the *F. bulbicola* PKS Fbu 946. *F. circinatum*, *F. miscanthi*, *F. redolens*, *F. solani* f. sp. *pisi* and *F. scirpi* each have one of the unique PKSs; *F. aywerte*, *F. bulbicola* and *F. thapsinum* each have two of the unique PKSs; and *F. avenaceum* has four unique PKSs. Eight PKS clades consist of two PKS homologs, and seven of these clades include PKSs that occur in species from only one species complex. Furthermore, five of the two-homolog clades (Clades 29, 30, 32, 33, and 35) occur only in the two representative members of the *F. solani* complex: *F. solani* f. sp. *pisi* and *F. euwallaceae*. This restricted distribution may reflect that this complex is relatively distantly related to the other complexes examined (Figure 2) (O'Donnell et al., 2013).

PKSs in some other clades (e.g. Clades 16, 20, 21 and 22) occur widely or even uniformly among members of closely related species complexes. Clade 16 homologs, for example, occur in all species examined from the *F. babinda*, *F. fujikuroi*, *F. nisikadoi*, *F. oxysporum*, and *F. redolens* complexes as well as in the closely related species *Fusarium* sp. NRRL 25184 and the two representative species of the *F. tricinctum* species complex. Although a Clade 16 homolog is present in both members of the *F. oxysporum* complex that were included in the current study (i.e. *F. foetens* and *F. oxysporum* strain 4287), a homolog of the gene was absent in two of 12 strains of *F. oxysporum* previously examined (Brown and Proctor, 2013). Thus, although our analysis indicated that PKSs from the same Clades occur uniformly among species of one or more species complexes, deeper sampling will likely reveal variation in occurrence within species and species complexes.

PKSs in some clades also occur widely in diverse species but exhibit a discontinuous distribution; that is, they are present in some species complexes or some members of a complex but absent in others. Examples of discontinuously distributed PKSs are those in Clades 2, 5 and 10. PKS homologs that occur in relatively few species also tend to exhibit discontinuous distribution, sometimes across diverse species complexes. PKS homologs in Clade 43, for example, occur in only eight species, but these species are dispersed across six species complexes (Figure 1).

The discontinuous distribution of PKS homologs and other NP biosynthetic genes in *Fusarium* has been reported previously (Proctor et al., 2013; Wiemann et al., 2013). Multiple

evolutionary processes, including horizontal transfer and loss/deletion of the genes, have been proposed to contribute to such discontinuity (Proctor et al., 2013; Rooney and Ward, 2005; Slot and Rokas, 2011; Wiemann et al., 2013). Determining the causes of discontinuity of PKS homologs observed in the current study (e.g., those in Clades 2, 5, 10, 15, 43 and 53) will require more detailed phylogenetic analyses of the genes/proteins, including comparison of levels of divergence of PKS genes and housekeeping genes used to infer species phylogenies.

The results of the current and previous studies suggest that recent duplication events have not made a large contribution to variability in the occurrence of PKS genes among *Fusarium* species. Of the 490 *Fusarium* PKSs analyzed here, only two, FOXG_15886 and FOXG_14850 (Clade 22), appear to be the result of a recent gene duplication event. These two PKS gene paralogs occur in *F. oxysporum* strain 4287 but not in any of the other 11 *F. oxysporum* strains for which genome sequences were available (Brown and Proctor, 2013). These two PKSs exhibit 98% nucleotide sequence identity. Further analysis in the current study indicated that *F. commune* PKS Fco 3239 is more similar to FOXG_14850 than to FOXG_15886 (Figure 1). One possible explanation for the relationships of these three homologs to one another is that FOXG_14850 is the progenitor homolog and FOXG_15886 was derived from it by a duplication event. Alternatively, FOXG_15886 could have been obtained by horizontal gene transfer from a closely related *Fusarium* donor. A number of other Clades contain two PKSs from the same *Fusarium* species but, the sequence identity shared by such paralogs is much lower than the identity shared by FOXG_14850 and FOXG_15886. For example, the *F. acuminatum* PKSs paralogs, Fac 2643 and Fac 676, which were both resolved within Clade 22, share 64% nucleotide identity. The lower degree of shared identity indicates that if the presence of these two PKSs in *F. acuminatum* is the result of duplication, the duplication event was almost certainly more ancient than the putative duplication event leading to the presence of FOXG_14850 and FOXG_15886 in a single strain of *F. oxysporum*. However, as with FOXG_14850 and FOXG_15886, horizontal transfer also provides a possible explanation for the presence of the two relatively closely related PKSs in *F. acuminatum*.

Functional predictions. Clades that include a PKS of known function, either a previously characterized *Fusarium* PKS or a PKS from another Ascomycete, also support the hypothesis that PKSs in the same clade are involved in the synthesis of the same or structurally similar

metabolites. Twenty-four clades include at least one PKS for which the polyketide product (or polyketide-derived NP) has been identified (Table 2). The 175 PKSs that make up these 24 clades represent 36% of the *Fusarium* PKSs examined. Sixteen clades include a functionally characterized PKS from a *Fusarium*, while eight clades include a functionally characterized PKS from other Ascomycetes (Table 2).

Our analysis of PKSs indicates that the genetic potential for production of some polyketide-derived metabolites is more widely distributed than previously reported. For example, fusaridione production has been reported only in *F. heterosporum* (Table 3) (Kakule et al., 2013). However, orthologs of the fusaridione PKS (Clade 69, Figure 1 and Figure 3B) occur in four other species. Likewise, although equisetin production has been previously reported only in a limited number of *Fusarium* species (Hansen et al., 2015; Kakule et al., 2013), the results of the current study indicate that the equisetin PKS occurs in 18 species (Clade 18, Figure 1). This in turn suggests that a greater diversity of species have the genetic potential to produce equisetin than previously reported.

Clades 17, 44, 45 and 52 include PKS homologs from fungi other than *Fusarium* and are encoded in proposed biosynthetic gene clusters for which the corresponding polyketide and/or polyketide-derived NP has been identified. Examination of genes flanking the *Fusarium* PKS genes in these clades revealed the presence of homologs of the cluster genes described in the other fungi (Figure 5). These findings provide evidence for the potential production of NPs that have not yet been reported in *Fusarium*. For example, the alternapyrone PKS (*alt1*) from *Alternaria solani* was resolved as a basal lineage of Clade 52 (Figure 1). In *A. solani*, the proposed alternapyrone biosynthetic gene cluster consists of five genes (*alt1* – *alt5*) (Fujii et al., 2005). All six fusaria with PKS homologs in Clade 52 also have homologs of *alt2*, *alt3* and *alt4*, but none have a homolog of *alt1*. Except for the absence of *alt1*, the order and orientation of *alt* genes is the same in *Fusarium* and *Alternaria* (Figure 5A). Likewise, the solanapyrone PKS gene (*sol1*) from *A. solani* was resolved in Clade 44 along with *F. avenaceum* PKS FAVG1_08719 and *F. acuminatum* PKS Fac 3328. In *A. solani*, the proposed solanapyrone biosynthetic cluster consists of six genes (*sol1* – *sol6*) (Kasahara et al., 2010). The region adjacent to FAVG1_08719 and Fac 3328 genes includes homologs of *sol2*, *sol3*, *sol4*, *sol5* and *sol6* (Figure 5B).

The *Beauveria bassiana* tenellin PKS gene, *tenS*, was resolved as a basal lineage of Clade 45 (Figure 1). The proposed tenellin biosynthetic gene cluster consists of five genes: *ORF0*, *tenA*, *tenB*, *tenC*, *tenS* (Figure 5C) (Eley et al., 2007). A homologous cluster responsible for synthesis of the tenellin-like NP fumosorinone has also been characterized in *Isaria fumosorosea* (Figure 5C) (Liu et al., 2015). The three fusaria with Clade 45 PKSs also have homologs of tenellin cluster genes in addition to the PKS gene, but all lack a *tenB* homolog (Figure 5C). In addition, the *tenA* homolog in *F. poae* is located on a different contig than the other *ten* genes, suggesting fragmentation of the cluster. All three fusaria have two additional genes (designated as *F* and *M* for the purposes of this discussion) next to or between the *ten* genes. We also identified tenellin-like gene cluster in *Trichoderma reesei* that includes homologs of *F* and *M*. The location of *F* and *M* next to the *ten* genes in *Fusarium* and *Trichoderma* suggests that they are involved in synthesis of a tenellin/fumosorinone-like metabolite in both genera.

The occurrence of homologs of the alternapyrone, solanapyrone and tenellin/fumosorinone clusters indicates a genetic potential for synthesis of these or structurally similar metabolites that, to our knowledge, have not previously been reported in *Fusarium*. Because the *F. avenaceum* homolog of the solanapyrone cluster includes homologs of all six *sol* genes described in *A. solani*, it is possible that if the cluster were active in *F. avenaceum*, it would result in production of solanapyrone. In contrast, because the gene content of the *Fusarium* homologs of the tenellin cluster differs from the content in the cluster in *B. bassiana*, it is possible that the metabolic products of the *Fusarium* clusters differ in structure from both tenellin and fumosorinone. Differences in gene content of homologous gene clusters among fungal genera can lead to analogs of metabolites that have the same core structure but differ markedly in modifications to the structure, as is the case for the trichothecene biosynthetic gene cluster in *Fusarium* and *Stachybotrys* (Brown et al., 2002; Semeiks et al., 2014). However, more subtle differences in production can occur in different fungi even when there are marked differences in gene content of homologous clusters, as observed with the production of different numbers of fumonisin analogs mycotoxins in *Aspergillus* and *Fusarium* (Susca et al., 2014).

Five other PKS clades (Clades 55, 64, 54, 17 and 56) also include functionally characterized PKSs from other fungi that provide insight into the functions of the *Fusarium* PKSs. For example, PKS Clade 55 and 64 each consist of two PKSs: one from *F. thapsinum* and the other from *Aspergillus nidulans*. In both fungi, and as mentioned previously, the Clade 55

and 64 PKS genes are located adjacent to one another, and in *A. nidulans* both PKSs are required for synthesis of the polyketide 2,4-dihydroxy-6-[(3*E*, 5*E*, 7*E*)-2-oxonona-3,5,7-trienyl]benzaldehyde (Ahuja et al., 2012). Thus, we propose that the *F. thapsinum* Clade 55 and 64 PKS function in concert during the synthesis of the same NP, as described above for zearalenone: i.e., the Clade 55 R-PKS synthesizes a polyketide that is used as a starter unit for the Clade 64 NR-PKS to synthesis a larger polyketide.

PKS Clade 54 includes a single *Fusarium* PKS, *F. bulbicola* Fbu 86 as well as the *Acremonium strictum* PKS MosA, which is required for synthesis of 3-methylorsellinic acid (Bailey et al., 2007). Based on this close phylogenetic relationship, Fbu 86 is likely involved in the synthesis of 3-methylorsellinic acid or a structurally similar polyketide. PKS Clade 17 includes seven *Fusarium* PKSs as well as the *Alternaria brassicicola* PKS Dep5, which is required for depudecin synthesis (Wight et al., 2009). Based on their close phylogenetic relationship to Dep5, these seven *Fusarium* PKSs likely synthesize the polyketide that serves as the first committed intermediate in the depudecin biosynthetic pathway. PKS Clade 56 includes *F. circinatum* FCIR_08230, the *Alternaria solani* PKS (MlnS) required for synthesis of mellein, and the *Aspergillus terreus* PKS (AtX) required for synthesis of 6-methylsalicylic acid (MSAS). Because FCIR_08230 and MlnS are more closely related to one another than either is to AtX, FCIR_08230 is likely required for synthesis of a polyketide that is more similar in structure to mellein than MSAS.

Non-reducing PKSs and pigments. Many fungal pigments consist of two or more fused aromatic rings that are formed via the activity of an NR-PKS. The NR-PKSs responsible for synthesis of four such *Fusarium* pigments have been characterized. The four pigments and the corresponding PKS clades resolved in our analysis are: fusarubins – Clade 3 (Studt et al., 2012); aurofusarin – Clade 12 (Gaffoor et al., 2005; Malz et al., 2005); bikaverin – Clade 16 (Linnemannstons et al., 2002); an uncharacterized red pigment of *F. solani* f. sp. *pisi* – Clade 35 (Graziani et al., 2004). The *Fusarium* NR-PKSs responsible for pigment production are part of a large well-supported clade (bootstrap value 92) within the NR-PKS group and that includes homologs from other fungi (Figure 1).

The distribution of PKS homologs in Clades 3, 12, 16 and 35 indicates that 28 of the 31 species examined have the genetic potential to produce two or more PKS-derived pigments. The

fusarubin NR-PKS (Clade 3) is the most wide-spread, being present in all 31 species, while the bikaverin NR-PKS (Clade 16) is present in 22 species across the *F. tricinctum*, *F. babinda*, *F. fujikuroi*, *F. nisikadoi*, *F. oxysporum* and *F. redolens* complexes. Of the remaining six species examined, four in the *F. sambucinum* species complex have a Clade 12 NR-PKS homolog, which is required for aurofusarin synthesis, and two in the *F. solani* species complex have a Clade 35 NR-PKS homolog which is required for synthesis of an uncharacterized red pigment. *F. acuminatum* and *F. avenaceum*, the two representative members of the *F. tricinctum* species complex, are unique in that they have homologs of four pigment biosynthetic NR-PKSs: the fusarubin, aurofusarin, and two bikaverin-like NR-PKSs.

Aromatic pigments produced by *Fusarium* can impart color to mycelia, perithecia and/or culture media. Bikaverin can impart a reddish color to the mycelia and culture media of fusaria that have PKS orthologs from Clade 16 (Wiemann et al., 2009). Fusarubin can also impart a reddish color to the mycelia and culture media of fungi with a homolog from Clade 3 (Studt et al., 2012). Although the identity of the compound(s) responsible for the dark purple pigmentation of perithecia of the *F. fujikuroi*, *F. sambucinum*, and *F. tricinctum* species complexes has not yet been reported, it is almost certainly a fusarubin analog or derived from a fusarubin(s), because deletion of the NR-PKS gene required for fusarubin production results in colorless perithecia (Brown et al., 2012b; Gaffoor et al., 2005; Proctor et al., 2007; Studt et al., 2012). The two *F. solani* complex species (*F. euwallaceae* and *F. solani* f. sp. *pisi*) have the fusarubin PKS gene as well as other genes in the fusarubin biosynthetic gene cluster (Proctor et al., 2007; Studt et al., 2012; Wiemann et al., 2009). However, perithecia produced by members in this species complex are red. This pigmentation is most likely imparted by a compound synthesized by the Clade 35 NR-PKSs rather than by the Clade 3 (fusarubin) NR-PKSs. This conclusion is based on the fact that deletion of the Clade 35 NR-PKS gene in *F. solani* f. sp. *pisi* results in colorless perithecia (Graziani et al., 2004).

PKS pseudogenes. BLASTn analysis of the 31 *Fusarium* genome sequences revealed that in addition to the 488 apparently functional PKS genes, the genomes collectively have 81 pseudogenized PKS genes (Table 1 and Supplemental Table 5). In some cases, pseudogenization resulted from single-nucleotide mutations (transitions, transversions, insertions or deletions) that introduced frame shifts and/or internal stop codons into coding regions. In

other cases, pseudogenization resulted from larger deletions of 10s – 1000s of nucleotides. The PKS pseudogenes constitute almost 15 % of the combined functional and nonfunctional PKS genes in the species examined. The levels of identity were sufficient to determine the PKS Clades to which 93% of the pseudogenes were most closely related. This analysis indicates that the pseudogenes were nonfunctional members of 27 Clades from across the NR-PKS and R-PKS groups (Figure 6). Although no pseudogenes were noted in the two Rm-PKS Clades, this could be due to the limited number of PKSs of this type present in *Fusarium*. The number of PKS pseudogenes per clade ranged from one to 10 (Figure 6), while the number of PKS pseudogenes per species ranged from zero to six (Table 1). The obvious, immediate consequence of pseudogenization of a PKS gene is loss of potential to synthesize the corresponding polyketide, any intermediates, or final NP(s) of the biosynthetic pathway for which the polyketide serves as a precursor. Pseudogenization could also indirectly influence the synthesis of other polyketides because loss of function of one PKS has the potential to increase availability of carboxylic acids used as substrates for polyketide synthesis by other PKSs.

We found two examples where strains of the same species differed in the presence of a functional versus pseudogenized PKS gene. The first example is the *F. avenaceum* PKS gene FAVG1_08496 (Clade 49). Sequence comparisons of this gene in three *F. avenaceum* strains indicate that it is functional in strain 05001 but pseudogenized in strains LH03 and LH27. In both strains LH03 and LH27, the pseudogenized FAVG1_08496 is missing nucleotide sequence corresponding to the first 3206 predicted AAs. The second example is the fusarin mycotoxin PKS gene (Clade 10) in *F. proliferatum*. A sequence comparison indicates that the gene is functional in *F. proliferatum* strain ITEM 2336 but pseudogenized in strain NRRL 62905. In addition to missing sequence corresponding to the first 2080 predicted AA of the PKS, NRRL 62905 is also missing sequence corresponding to eight other genes that comprise the fusarin biosynthetic gene cluster (Brown et al., 2012b).

PKS Clade 17 has the largest number of pseudogenes. Functional PKSs of this clade are likely involved in synthesis of depudecin, a linear polyketide that was first isolated from *Alternaria brassicicola* (Isaka et al., 2000) and that inhibits histone deacetylase (Wight et al., 2009). The distribution of functional and pseudogenized homologs of the depudecin PKS gene among members of the *F. babinda*, *F. chlamydosporum*, *F. fujikuroi*, *F. nisikadoi*, *F. oxysporum* and *F. redolens* species complexes as well as *Fusarium* sp. NRRL 25184 (Figure 2) suggests that

a functional homolog of the gene was present in the common ancestor of these fusaria and that pseudogenization of the gene occurred independently multiple times as species diverged from the ancestor.

Conclusions. In the pre-genomics era, *Fusarium* served as a rich source of metabolites in numerous NP characterization programs (Cole et al., 2003). Initial genomic studies indicated that fungi have the genetic potential to synthesize many more NPs than expected based on chemical analysis. The results of the current study suggest that the *Fusarium* species examined have the genetic potential to produce 64 structurally distinct polyketide-derived NPs. Individual species of *Fusarium* tend to have similar numbers of PKS and nonribosomal peptide synthetase (NRPS) genes (Hansen et al., 2015; Wiemann et al., 2013). Thus, it is likely that the 31 species examined here have the potential to produce a number of structurally distinct nonribosomal peptide-derived NPs that is similar to the number of polyketide-derived NPs that they produce. *Fusarium* species also have terpene synthase and prenyltransferase genes, which catalyze synthesis of molecules that serve as core structures for other classes of NPs. However, in individual species, the numbers of these other synthase genes are typically much lower than the numbers of PKS and NRPS genes (Ma et al., 2010; Wiemann et al., 2013). Regardless, mining of genomic sequences of *Fusarium* for NP biosynthetic genes suggests that this genus has a tremendous capacity to produce a structurally diverse array of NPs and that remarkable variation in production can exist between species and species complexes.

Together, the discontinuous distribution of multiple PKS genes, the presence of multiple PKSs that are unique to a single species or species complex, and the relatively high percentage of PKS pseudogenes indicate that dynamic changes in production of polyketide-derived NPs have occurred during the evolution of *Fusarium*. Variation in PKS gene content within single species (e.g., the fusarin PKS in *F. proliferatum*) further suggests that some changes have occurred relatively recently. Most *Fusarium* isolates are recovered from agricultural plants. Therefore, the variation in *Fusarium* PKS content observed here and in other studies leads to questions of whether agricultural practices contribute to variation in NP production in this fungus; and further whether understanding how such variation arises could contribute to strategies that reduce crop diseases and mycotoxin contamination problems caused by *Fusarium*.

In the current study, we have provided evidence that *Fusarium* has the genetic potential to produce seven NPs that are the same or structurally similar to NPs reported in other fungi. Thus, this study and others like it (Throckmorton et al., 2015) can guide efforts to identify NPs produced by *Fusarium* (Chiang et al., 2010; Niehaus et al., 2014; von Barga et al., 2015) that have the potential to contribute to the ability of the fungus to occupy a wide range of habitats, including a diversity of crop plants.

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Figure 1. Genealogy of 490 *Fusarium* PKSs and 55 PKSs from other Ascomycetes inferred from maximum likelihood analysis of nucleotide sequences of PKS coding regions. The NR-PKS and R-PKS I, II and III groups are delineated with red horizontal lines, and the 67 clades are indicated with a unique number (in red text) to the right of the tree. Statistical support for branches within the phylogenetic tree were generated by bootstrap analysis with 500 pseudoreplicates and are indicated by numbers above the internodes (black text). The protein/gene designations for PKSs corresponding to gene model designations at NCBI and predicted for this study are listed in Table 1. The gene name of the 55 PKSs from other Ascomycetes as well as the name of the organism and polyketide/NP are also indicated to the right of the tree. The names of 16 *Fusarium* metabolites are indicated adjacent to each PKS Clade number. Representative PKSs that have been functionally characterized are indicated in red text. A representative chemical structure for a functionally characterized PKS is indicated for eight Clades that include a *Fusarium* PKS. In the chemical structures, thick lines represent the region of a structure that is synthesized by a PKS. The *Gallus gallus* fatty acid synthase genes served as the outgroup.

Figure 2. Species phylogeny of the 31 *Fusarium* species examined in the current study. The tree was generated by maximum likelihood analysis of the concatenated sequences of the coding regions of 20 housekeeping genes (Supplemental Table 3). Numbers above internodes represent bootstrap support values based on 500 pseudoreplicates. Taxa are color coded according to species complex (O'Donnell et al., 2013). The three subclades within *F. fujikuroi* species complex are indicated: American, African and Asian. The tree and aligned sequence data from which it was inferred have been submitted to TreeBASE:S18497 (URL: <http://purl.org/phylo/treebase/phylows/study/TB2:S18497>). The presence (+), absence (--) or pseudogenization (Ψ) of homologs of the depudecin PKS (Clade 17) are indicated to the right.

Figure 3. Comparison of *Fusarium* genes flanking PKSs in Clade 16 (A) and Clade 20, Clade 68 and Clade 69 (B). The relevant portion of the phylogram from Figure 1 is depicted on the left. The arrowheads indicate direction of transcription, and the blue arrows represent cluster genes. Bikaverin cluster genes are labeled *BIK1* through *BIK6*. Putative fusaridione cluster genes are labeled *fsdS*, *K*, *D*, *H*, *G* and *C*. Green arrows represent genes with a putative function associated with NP biosynthesis. C6 = transcriptional regulator, mono = monooxygenase, red = reductase, hyd = hydrolase, dhy = dehydrogenase, and mfs = MFS type transporter. Black arrows represent genes with an unknown function or genes generally not associated with NP biosynthesis. The shaded area indicate homologous genes in the different *Fusarium* species.

Figure 4. Distribution of PKSs among the *Fusarium* species examined in the current study. The total height of each bar (both black and gray) represents the total number of Clades with the number of PKSs indicated on the horizontal axis. The height of the black portion of each bar represents the number of Clades in which the function of at least one PKS has been determined.

Figure 5. Comparison of *Fusarium* genes flanking PKSs that are closely related homologs of PKSs from other Ascomycetes and for which the polyketide/or polyketide-derived NP has been identified. The arrowheads indicate direction of transcription, and the blue arrows represent cluster genes. A. Alternapyrone cluster homologs. The proposed alternapyrone cluster genes from *Alternaria solani* are labeled *alt1* through *alt5*. The green arrow represents the *alt* cluster gene not conserved in *Fusarium*. B. Solanapyrone cluster homologs. The proposed solanapyrone cluster genes from *Alternaria solani* are labeled *sol1* through *sol6*. C. Tenellin cluster homologs. The proposed tenellin cluster genes are labeled *tenS*, *A*, *B*, *C* and *I*. The orange arrows represent putative genes in *Trichoderma reesii* and *Fusarium* not present in the *ten* cluster in *B. bassiana*.

Figure 6. Distribution of functional and pseudogenes across PKS Clades. The PKS Clades identified by phylogenetic analysis are indicated on the horizontal axis. The total number of functional (black) and pseudogenes (grey) in each Clade is indicated on the vertical axis. The total number of functional and pseudogenes per PKS Clade is indicated along the horizontal axis.

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Table 1. Information on strains, species and species complexes of *Fusarium*, PKS gene/enzyme designations, numbers of PKS genes and pseudogenes per species, and sources of genome sequence data used in this study.

<i>Fusarium</i> species	Strain	Species complex ^a	PKS Designation ^x	PKSs	Pseudo ^b	Sequence Data ^c
<i>F. graminearum</i>	PH-1 and CS3005 ^d	<i>F. sambucinum</i>	FGSG #	16	0	NCBI
<i>F. pseudograminearum</i>	CS3069	<i>F. sambucinum</i>	FPSE #	14	0	NCBI
<i>F. poae</i>	NRRL 26941	<i>F. sambucinum</i>	Fpo #	11	2	this study ^I
<i>F. culmorum</i>	CS7071	<i>F. sambucinum</i>	Fcu #	13	1	NCBI
<i>F. aywerte</i>	NRRL 25410	<i>F. chlamydosporum</i>	Fay #	16	4	this study ^I
<i>F. scirpi</i>	NRRL 06979	<i>F. incarnatum-equiseti</i>	Fsc #	11	3	this study ^M
FIESC 5 ^e	CS3069	<i>F. incarnatum-equiseti</i>	Fsp3 #	11	2	NCBI
<i>F. acuminatum</i>	CS5907	<i>F. tricinctum</i>	Fac #	15	5	NCBI
<i>F. avenaceum</i>	Fa05001 and FaLH27 ^f	<i>F. tricinctum</i>	FAVG1 # ^g	27	2	NCBI
<i>F. sacchari</i>	FRC M-6865	<i>F. fujikuroi</i> (As) ^h	Fsa #	20	0	this study ^B
<i>F. fujikuroi</i>	IMI58289	<i>F. fujikuroi</i> (As)	FFUJ #	17	1	NCBI
<i>F. proliferatum</i>	NRRL 62905	<i>F. fujikuroi</i> (As)	Fpr #	19	2	this study ^B
<i>F. verticillioides</i>	FRC M-3125	<i>F. fujikuroi</i> (Af)	FVEG #	16	0	NCBI
<i>F. thapsinum</i>	FRC M-6563	<i>F. fujikuroi</i> (Af)	Fth #	16	3	this study ^B
<i>F. dlamini</i>	NRRL 13164	<i>F. fujikuroi</i> (Af)	Fdl #	16	5	this study ^B
<i>F. circinatum</i>	Fsp34	<i>F. fujikuroi</i> (Am)	FCIR #	15	3	NCBI
<i>F. succisae</i>	NRRL 13298	<i>F. fujikuroi</i> (Am)	Fsuc #	15	3	this study ^B
<i>F. subglutinans</i>	NSM 107	<i>F. fujikuroi</i> (Am)	Fsub #	18	2	this study ^B
<i>F. anthophilum</i>	NRRL 25214	<i>F. fujikuroi</i> (Am)	Fan #	19	3	this study ^I
<i>F. bulbicola</i>	NRRL 13618	<i>F. fujikuroi</i> (Am)	Fbu #	16	3	this study ^I
<i>Fusarium</i> sp. 52700	NRRL 52700	<i>F. fujikuroi</i>	Fsp5 #	15	6	this study ^B
<i>F. miscanthi</i>	NRRL 26231	<i>F. nisikadoi</i>	Fmi #	16	3	this study ^I
<i>F. gaditjirri</i>	NRRL 45417	<i>F. nisikadoi</i>	Fga #	14	6	this study ^I
<i>F. commune</i>	NRRL 28387	<i>F. nisikadoi</i>	Fco #	15	6	this study ^I
<i>F. oxysporum</i>	Fol4287 ⁱ	<i>F. oxysporum</i>	FOXG #	17	0	NCBI
<i>F. foetens</i>	NRRL 38302	<i>F. oxysporum</i>	Ffo #	14	3	this study ^I
<i>Fusarium</i> sp. 25184	NRRL 25184	not specific	Fsp2 #	19	3	this study ^I
<i>F. redolens</i>	NRRL 22901	<i>F. redolens</i>	Fre #	18	4	this study ^I
<i>F. babinda</i>	NRRL 25539	<i>F. babinda</i>	Fba #	15	4	this study ^I
<i>F. solani</i> f. sp. <i>pisi</i>	FGSC 9596	<i>F. solani</i>	JGI #	13	1	NCBI
<i>F. euwallaceae</i>	ATTC 74349	<i>F. solani</i>	Feu #	11	1	this study ^I
<i>F. heterosporum</i>		<i>F. heterosporum</i>	<i>fsdS</i> and <i>eqxS</i>	2	-	NCBI
Total	31 ^j			490	81	

^a Species complex as described by O'Donnell et al. (2013) and Geiser et al. (2013).

^x See Supplemental Table 2 for PKS GenBank Accessions and Gene Designations.

^b Pseudo refers to pseudogenes or remnant PKSs.

^c NCBI: National Center for Biotechnology Information. The I and M superscripts indicate that sequencing was performed at NCAUR using the Ion-Torrent (^I) or Illumina MiSeq (^M) platforms. The B superscript (^B) indicates that sequencing was performed by the Beijing Genome Institute-Hong Kong. See Supplemental Table X for GenBank Accession numbers for 317 new *Fusarium* PKSs and designations for all of the other PKSs examined.

^d *F. graminearum* strain CS3005 has a PKS that is not present in strain PH-1.

^e In Hansen et al. (2015), strain CS3069 is referred to as *F. equiseti*. In the NCBI database, however, this strain is referred to as FIESC 5: i.e., multilocus sequence type 5 of the *F. incarnatum-equiseti* species complex. Our analysis also indicated that strain CS3069 is

Table 2. Clades of *Fusarium* PKSs and corresponding polyketides or polyketide-derived NPs identified in *Fusarium* or Ascomycetes other than *Fusarium*.

Clade #	NP	No. PKS homologs ^a	Reference
Polyketide/NP identified in <i>Fusarium</i>			
3	fusarubins	31	Studt et al., 2012
35	perithecial pigment	2	Gaffoor et al., 2005
12	aurofusarin	6	Gaffoor et al., 2005
16	bikaverin	24	Wiemann et al., 2009
13/4 ^b	zearalenone	4/4	Gaffoor and Trail, 2006
14	orsellinic acid	4	Jorgensen et al., 2014
24	fumonisin	5	Proctor et al., 1999
21	fusaric acid	21	Brown et al., 2012
6	fusaristatin	4	Sorensen et al., 2014
40	W493 B	3	Sorensen et al., 2014
9	fusarielins	3	Sorensen et al., 2012
69	fusaridione	4	Kakule et al., 2013
18	equisetin	18	Kakule et al., 2013
39	fujikurin	3	von Barga et al., 2015
10	fusarins	18	Song et al., 2004
Polyketide/NP identified in Ascomycete other than <i>Fusarium</i>			
54	3-methylorsellinic acid	1	Bailey et al., 2007
55/64 ^b	oxononal benzaldehyde	1/1	Ahuja et al., 2012
56	mellein	1	Chooi et al., 2015
17	depudecin	7	Wight et al., 2009
44	solanapyrone	1	Kasahara et al., 2010
52	alternapyrone	6	Fujii et al., 2005
45	tenellin/fumosorinone	3	Eley et al., 2007; Liu et al., 2015

^a Number of PKS homologs in the clade. 4/4 and 1/1 indicate the number of PKS homologs in the two clades required for synthesis of zearalenone and oxononal benzaldehyde respectively.

^b Two PKSs are involved or likely involved in synthesis of NP.

most closely related to FIESC 5 (data not shown). In the current study, we refer to strain CS3069 as FIESC 5, because FIESC 5 and *F. equiseti* are not equivalent (O'Donnell et al., 2009).

^f *F. avenaceum* strain FaLH27 has a PKS that is not present in strain Fa05001.

^g FAVG1 #s refer to 24 *F. avenaceum* PKS gene names at NCBI. We identified three additional PKSs, labeled Faven#, in the *F. avenaceum* genomic DNA in the NCBI database that were not assigned FAVG1 #s.

^h The *F. fujikuroi* species complex includes three clades: Asian (As), African (Af) and American (Am).

ⁱ 14 *F. oxysporum* PKS were retrieved from strain Fol4287. One PKS each was retrieved strain Fo5176, FOOSC 3-a and Fo47.

^j This value refers to the number of *Fusarium* genomes screened for PKS genes and pseudogenes. The genome of *F. heterosporum* was not examined in this study. The two *F. heterosporum* PKSs examined were recovered from the NCBI database.

Non-Reducing PKS Clade

Reducing PKS Clade I

Reducing PKS Clade II

Figure 2.

Figure 3.

A.

B.

Figure 4. Distribution of PKSs across *Fusarium*

Figure 5.

A. Alternapyrone cluster homologs: PKS52

B. Solanapyrone cluster homologs: PKS44

C. Tenellin cluster homologs: PKS45

Figure 6. Distribution of Pseudogenes across PKS Clades