Phylogenomic analysis of polyketide synthase-encoding genes in *Trichoderma*

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Members of the economically important ascomycete genus *Trichoderma* are ubiquitously distributed around the world. The mycoparasitic lifestyle and plant defence-inducing interactions of *Trichoderma* spp. make them ideal biocontrol agents. Of the *Trichoderma* enzymes that produce secondary metabolites, some of which likely play important roles in biocontrol processes, polyketide synthase (PKSs) have garnered less attention than non-ribosomal peptide synthetases such as those that produce peptaibols. We have taken a phylogenomic approach to study the PKS repertoire encoded in the genomes of *Trichoderma reesei*, *Trichoderma atroviride* and *Trichoderma virens*. Our analysis lays a foundation for future research related to PKSs within the genus *Trichoderma* and in other filamentous fungi.

INTRODUCTION

Given its important roles in bioenergy-related enzyme production and plant and fungal health, the genus *Trichoderma* has a significant economic footprint (Schuster & Schmoll, 2010). This genus is well known for its opportunistic lifestyle that includes saprotrophy, mycoparasitism, endophytism, and interactions with plants and animals. Because of their ability to antagonize other fungi and stimulate plant defences against phytopathogens, *Trichoderma* strains have become prominent biocontrol agents (Schuster & Schmoll, 2010). Further highlighting their mycoparasitic lifestyle, sequencing and analysis of the genomes from two mycoparasitic *Trichoderma* species (*Trichoderma virens*, *Trichoderma atroviride*) have shown that these genomes encode a rich catalogue of fungal cell wall-degrading chitinases and a relative paucity of plant cell wall-degrading enzymes (Kubicek *et al.*, 2011; Martinez *et al.*, 2008).

As biocontrol agents and pathogens of fungal food crops, secondary metabolite production in *Trichoderma* spp. is of

particular importance. *Trichoderma* and other filamentous fungi are well known for the production of a diverse array of secondary metabolites. Non-ribosomal peptides and polyketides comprise a major portion of these products, which are synthesized by large proteins composed of various domains for the individual enzymic steps (Cox, 2007). Both broad and genus-specific phylogenomic and functional analysis of polyketide synthase (PKS)- and non-ribosomal peptide synthetase (NRPS)-encoding genes have been performed, which has accelerated the pace at which genes and pathways are being linked to specific secondary metabolites (Bushley & Turgeon, 2010; Bushley *et al.*, 2008; Chiang *et al.*, 2010; Cramer *et al.*, 2006; Gaffoor *et al.*, 2005; Kroken *et al.*, 2003; Kubicek *et al.*, 2011; Lee *et al.*, 2005).

Trichoderma spp. are probably best known for their production of peptaibols, which are non-ribosomal peptides with antimicrobial and plant defence-stimulating activity (Viterbo *et al.*, 2007). Relatively less studied in *Trichoderma* are PKSs. The recent analysis of the three available *Trichoderma* genome sequences has indicated that the genomes of these organisms encode only a small catalogue of PKSs: *T. atroviride* and *T. virens* each encode 18, and *Trichoderma reesei* only 11 predicted PKSs (Kubicek *et al.*, 2011; Martinez *et al.*, 2008). We have manually annotated and performed phylogenomic analysis of the catalogue of *Trichoderma* PKSs.

Abbreviations: CAI, codon adaptation index; HGT, horizontal gene transfer; KS domain, ketosynthase domain; MP analysis, maximum-parsimony analysis; MSAS, 6-methylsalicylic acid synthase; PKS, polyketide synthase.

Five supplementary figures and two supplementary tables are available with the online version of this paper.

Fig. 1. Genealogy of *Trichoderma* PKSs, inferred by MP analysis of the KS domain. Major clades and subclades are indicated by vertical bars, each of which shares a common organization of domains (those in parentheses are variable in their presence/absence within that clade). Numbers above branches indicate percentage bootstrap support for each clade (numbers below 50% are not shown). Abbreviations: Bf=*Botrytis fuckeliana*, Gm=*Gibberella moniliformis*, Gz=*Gibberella zeae*, Nc=*Neurospora crassa*, Ch=*Cochliobolus heterostrophus*. The accession numbers for all sequences obtained from GenBank are given by Kroken *et al.* (2003).

METHODS

Phylogenetic analysis. The amino acid sequences of the ketosynthase (KS) domains were aligned using CLUSTAL W and subsequently manually adjusted. The alignment was then analysed by the COBALT multiple sequence alignment tool that finds, using RPS-BLAST, BLASTP and PHI-BLAST, a collection of pairwise constraints derived from a conserved domain database, protein motif database, and sequence similarity. Pairwise constraints are then incorporated into a progressive multiple alignment (Papadopoulos & Agarwala, 2007). The resulting alignment exported in FASTA format was phylogenetically analysed.

The genealogy of 196 PKSs on the basis of the alignment of the KS domain was inferred by neighbour-joining and maximum-parsimony (MP) analysis using MEGA version 4 (Tamura *et al.*, 2007). Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The majority rule consensus bootstrap tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. The MP trees were obtained using the Close-Neighbor-Interchange algorithm with search level 3, in which the initial trees were obtained with the random addition of sequences (10 replicates). The trees are drawn to scale, with branch lengths calculated using the average pathway method, and are in the units of the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). In addition, a more robust approach was used to infer phylogenomic relationship among the PKSs studied by performing a randomized bootstrap maximum-likelihood analysis using RAxML software (Stamatakis *et al.*, 2008), setting the bootstrap analysis to 1000 runs and the bootstrap random seed value to 12345. There were 736 characters and the Dayhoff mutation data matrix was used for the analysis of the alignment. Protein IDs are consistent with the comparative *Trichoderma* genome analysis presented in Kubicek *et al.* (2011); revisions to protein IDs between Joint Genome Institute (JGI) genome versions are noted in Supplementary Table S1.

K_a/K_s ratio. Tajima's D statistic (Tajima, 1989) was determined with DNAsp 5.0 (Librado & Rozas, 2009), using a sliding-window approach. The pairwise K_a/K_s ratio [the ratio of the number of non-synonymous substitutions per non-synonymous site (K_a) to the number of synonymous substitutions per synonymous site (K_s)] was determined on the respective phylogenetic tree with the aid of the K_a/K_s calculation tool (<http://services.cbu.uib.no/tools/kaks>), using pre-aligned sequence datasets and MP analysis. Codon-based Fisher's test and the codon-based Z-test, implemented in MEGA 4.0 (Tamura *et al.*, 2007), were used to directly test the hypotheses of evolutionary models.

Codon usage was estimated using the codon adaptation index (CAI), which is a measure of the relative adaptation of the gene codon usage towards the codon usage of highly expressed genes for that organism (Sharp & Li, 1987). To estimate this index, the EMBOSS (<http://emboss.bioinformatics.nl/cgi-bin/emboss>) program 'cai' was used (Rice *et al.*, 2000).

RESULTS

Phylogenetic analysis and manual annotation of clusters

T. reesei was previously shown to contain 11 PKSs (Martinez *et al.*, 2008). This number is exceeded by *T. atroviride* and *T. virens*, both of which have 18 PKS-encoding genes (Kubicek *et al.*, 2011). The organizational framework for fungal PKSs based on a phylogeny of fungal PKS KS domains is well established (Kroken *et al.*, 2003). We have therefore analysed the position of the *Trichoderma* PKS proteins in the phylogenetic tree (Fig. 1). The data revealed that *Trichoderma* contains PKS proteins that are members of the non-reducing clades I, II and III, and of the reducing clades I, III and IV. We have divided the PKS-encoding genes into orthologous groups (Table 1) and singlets (Table 2). The *T. reesei* genome has only two singlet PKS-encoding genes, while the *T. virens* and *T. atroviride* genomes have seven and nine singlet PKS-encoding genes, respectively (Fig. 2, Table 2). There are only minor differences between our MP (Fig. 1) and maximum-likelihood analyses (Supplementary Fig. S1), the most significant being the placement of the predicted fungal 6-methylsalicylic acid synthase (MSAS) PKSs.

Predicted pigment genes

The genomes of all three *Trichoderma* genomes encode PKS genes that group in the non-reducing fungal PKS clade I, which includes the genes associated with *Fusarium graminearum* aurofusarin (Frandsen *et al.*, 2011; Kim *et al.*, 2005; Malz *et al.*, 2005), *Fusarium fujikuroi* bikaverin (Linnemannstöns *et al.*, 2002; Wiemann *et al.*, 2009) and *Aspergillus* spp. DHN melanin (Baker, 2008; Chiang *et al.*, 2011; Jørgensen *et al.*, 2011; Langfelder *et al.*, 1998; Tsai *et al.*, 1998, 2001; Watanabe *et al.*, 1999, 2000). In addition, all three genomes encode putative multi-copper oxidases that are clustered with the PKS genes and distantly related to *Aspergillus fumigatus* brown2/Arp2 and *F. graminearum* gip1 pigment pathway-associated genes (Fig. 3). The third gene in the putative pigment clusters shows amino acid sequence similarity to the recently characterized protein product of *F. graminearum* aurZ, which has been shown to convert the compound YWA1 to nor-rubofusarin (Frandsen *et al.*, 2011). None of the *Trichoderma* genomes has genes that are related to known hydroxynaphthalone (HN) reductases, which catalyse the conversion of 1,3,6,8-tetrahydroxynaphthalene to scytalone and the reduction of 1,3,8-trihydroxynaphthalene to vermeline. It is therefore intriguing that both the *T. atroviride* and the *T. virens* genomes contain

Table 1. *Trichoderma* PKS orthologous groups

Orthologous group or singlet designation	Protein ID – percentage identity	Clade
PKS group 1	Tr 65172 81% / 85% Ta 45485 – 83% – Tv 51044	Reducing clade I: lovastatin/citrinin diketide
PKS group 2	Tr 65891 68% / 74% Ta 51909 – 72% – Tv 42255	Reducing clade I: lovastatin/citrinin diketide
PKS group 3	Tr 105804 67% / 70% Ta 31567 – 76% – Tv 10060	Non-reducing fungal PKS clade III
PKS group 4	Tr 82208 73% / 79% Ta 79 – 77% – Tv 77826	Non-reducing fungal PKS clade I
PKS group 5	Tr 59482 80% / 83% Ta 34066 – 80% – Tv 53926	Reducing PKS clade III: t-toxin
PKS group 6	Tr 60118 70% / 75% Ta 146945 – 73% – Tv 47828	Reducing PKS clade I: lovastatin/citrinin diketide
PKS group 7	Tr 65116 82% / 87% Ta 45973 – 84% – Tv 47407	Reducing PKS clade IV: fumonisins
PKS group 8	Tr 81964 75% Tv 49800	Non-reducing fungal PKS clade I–II
PKS group 9	Tr 106272 77% Tv 35498	Reducing clade I: lovastatin/citrinin diketide
PKS group 10	Ta 83179 77% Tv 41840	Reducing PKS clade IV: fumonisins
PKS group 11	Ta 33843 77% Tv 29151	Reducing clade I: lovastatin/citrinin diketide

genes, not clustered with the putative pigment PKS genes, which are predicted to encode scytalone dehydratases.

Evolution of the PKS genes

In order to examine whether the *Trichoderma* PKS-encoding genes would indeed be products of horizontal gene transfer (HGT) or be subject to other evolutionary

mechanisms, we first used Tajima's D test (Tajima, 1989). To this end, we grouped the *Trichoderma* PKS-encoding genes according to their position in the phylogenetic tree into five datasets: (a) non-reducing PKS clades I and II; (b) non-reducing PKS clade III; (c) reducing PKS clade I and II (lovastatin-type); (d) reducing PKS clade III (fumonisins-type); and (e) a small clade that occupied a basal position to the reducing PKS clades. Tajima's D was calculated with a

Table 2. PKS singlets

Singlet	Protein ID	Clade
PKS singlet 1	Ta 79482	Non-reducing fungal PKS clade III
PKS singlet 2	Ta 32458	Reducing PKS clade IV fumonisins
PKS singlet 3	Ta 52962	Reducing clade I: lovastatin/citrinin diketide
PKS singlet 4	Ta 85006	Reducing clade I: lovastatin/citrinin diketide
PKS singlet 5	Ta 134224	Reducing clade I: lovastatin/citrinin diketide
PKS singlet 6	Ta 51503	Reducing clade I: lovastatin/citrinin diketide
PKS singlet 7	Ta 51726	Non-reducing fungal PKS clade II–III
PKS singlet 8	Ta 80219	Non-reducing fungal PKS clade I–II
PKS singlet 9	Ta 45540	Fungal MSAS PKS
PKS singlet 10	Tr 73621	Non-reducing fungal PKS clade III
PKS singlet 11	Tr 73618	Reducing clade I: lovastatin/citrinin diketide
PKS singlet 12	Tv 53518	Reducing clade I: lovastatin/citrinin diketide
PKS singlet 13	Tv 53633	Reducing PKS clade IV: fumonisins
PKS singlet 14	Tv 70876	Reducing PKS clade IV: fumonisins
PKS singlet 15	Tv 62549	Reducing clade I: lovastatin/citrinin diketide
PKS singlet 16	Tv 64883	Reducing clade I: lovastatin/citrinin diketide
PKS singlet 17	Tv 20101	Non-reducing fungal PKS clade III
PKS singlet 18	Tv 39352	Non-reducing fungal PKS clade II–III

sliding-window approach over the length of the KS domain-encoding portion of the gene. The data are shown in Fig. 4: Tajima's D was between 0 and -0.15 throughout in the genes in clades (a) to (d), and only the basal branch (e) showed three areas of slightly positive values. P values were all >0.05 , however, and we therefore conclude that the evolution of the PKS-encoding genes is neutral. We also used the K_a/K_s ratio as an alternative means to test whether the PKS-encoding genes are under any evolutionary selection. To this end, we calculated the K_a/K_s ratio for all pairwise combinations of genes. With all five groups, we

only obtained ratios that were significantly less than 1, which indicates the operation of negative or purifying selection, the actual ratios varying between 0.02 and 0.6. When the K_a/K_s ratios were plotted on the nodes of the phylogenetic tree of the five PKS clades, the lowest values were observed in most (albeit not all) of the terminal clades, whereas the highest values were associated with internal nodes that represented ancient splitting events (Supplementary Figs S2–S5).

We further used the codon-based Z-test and the codon-based Fisher's exact test to compare the relative abundance of synonymous and non-synonymous substitutions that have occurred in the gene sequences. The first test did not reject the null hypothesis of purifying selection, whereas that of neutral and positive selection was rejected; in Fisher's exact test, a p of 1.0 also supported purifying selection, rather than positive selection. We therefore conclude that the PKS-encoding genes of *Trichoderma* evolve under purifying selection.

CAI

To estimate deviations in codon usage, the CAI was calculated for all five datasets. CAI is a measure of the relative adaptiveness of the codon usage of a gene towards the codon usage of highly expressed genes for that organism: the higher the index value, the greater the codon usage bias (Sharp & Li, 1987). CAI was previously determined for 100 orthologous and syntenic genes in *T. reesei*, *T. virens* and *T. atroviride*, and the mean value was around 0.72 (Kubicek *et al.*, 2011). We also checked CAI for the strongly expressed fungal chitinase gene CHI18-5 (Seidl *et al.*, 2005) and arrived at values between 0.65 and 0.8. The corresponding values for the five PKS clades of this study were between 0.65 and 0.7 (Supplementary Table S2). Therefore, there is a moderate codon usage bias in

Fig. 2. Venn diagram illustrating distribution of orthologous *T. reesei*, *T. virens* and *T. atroviride* PKS-encoding genes.

Fig. 3. Predicted pigment biosynthetic gene clusters for three *Trichoderma* species compared with the aurofusarin biosynthetic cluster from *F. graminearum*. Numbers indicate JGI protein IDs associated with the gene model and are consistent with the numbering in Kubicek *et al.* (2011).

the PKS-encoding genes, probably indicative of moderate levels of expression. However, the values are high enough to conclude that none of these genes was recently acquired as the result of a horizontal transfer event.

DISCUSSION

Phylogenetic analysis of protein family coding sequences is a valuable aid for gene annotation and hypothesis generation. We have performed such an analysis on PKS-encoding genes from three genome-sequenced *Trichoderma*

species and those examined by Kroken *et al.* (2003). We find that the majority of PKS-encoding genes (29 out of a total of 47) fall into an orthologous group. It may be that *Trichoderma* as a genus has a limited catalogue of PKSs and that diversity in the respective metabolites may be low or is expanded at the level of 'accessory enzymes' versus the backbone synthesizing enzyme. However, as the number of sequenced *Trichoderma* genomes increases, the diversity of the PKS-encoding gene family within this genus will expand as well.

Our analysis has also allowed us to predict the PKS-encoding gene most likely responsible for the characteristic *Trichoderma* yellow-green pigment. Indeed, the value of this study and others like it is in the generation of hypotheses to be tested. Ultimately, our prediction must be validated by genetic and biochemical characterization of the *Trichoderma* pigment biosynthesis system. It is also interesting to note that there is synteny only at the level of the putative pigment biosynthetic cluster; the surrounding genes are not conserved. Most of the PKS-encoding genes present in *T. reesei* are also found in *T. virens* and *T. atroviride*, but about half of the genes remaining in the latter two are novel for the respective species, and similar to the case of the putative pigment cluster, form non-syntenic islands (C. P. Kubicek & S. E. Baker, unpublished data) in the genome, suggesting transfer by recombination.

Most of the *Trichoderma* PKS genes occur as orthologues in all three species, implying that several nearly identical polyketides are produced by *T. reesei*, *T. virens* and *T. atroviride*. In this regard, the almost complete lack of duplicated copies was remarkable: this could be due to repeat-induced point mutation (RIP) in *Trichoderma*, which occurs during sexual development and alters

Fig. 4. Tajima's D for the KS domains of each *Trichoderma*-containing PKS clade plotted against nucleotide position.

nucleotides in both copies of duplicated genes and renders them nonfunctional. Evidence for the operation of RIP in *Trichoderma* has been presented (Kubicek *et al.*, 2011). The operation of RIP would also be consistent with the much lower number of PKSs in *T. reesei* than in *T. atroviride* and *T. virens*, because *T. reesei* is almost always observed as a teleomorph while the other two species are frequently sampled as anamorphs. RIP may therefore also explain the – in comparison with other Pezizomycota – low number of PKS genes; indeed, only *Neurospora crassa* is known to have a lower number (Galagan *et al.*, 2003), for which strong RIP is known (Selker & Garrett, 1988; Selker *et al.*, 1987a, b).

There is also a still-ongoing debate about whether or not HGT contributes or has contributed to the evolution of PKS genes. Modular PKS genes appear to be transferred horizontally between bacteria similarly to iterative PKS genes (Ridley *et al.*, 2008). Kroken *et al.* (2003), however, demonstrated that there is little evidence for HGT with the fungal PKS genes (an exception being MSAS-encoding genes, which represent an HGT event from bacteria to fungi), and stressed the major importance of gene duplications eventually followed by gene loss. Our evolutionary analysis is consistent with the latter scenario. First, examination of the codon usage pattern showed that the codon bias is similar to that of other moderately expressed genes of *Trichoderma*. Further, the overall substitution ratios of the *Trichoderma* PKS ketoacyl synthase module revealed that it is under strong purifying selection. Similar findings have also been reported for PKS from bacteria (Zucko *et al.*, 2011) and lichenized fungi (Muggia *et al.*, 2008).

Our analysis lays the groundwork for future polyketide studies in *Trichoderma*. As more PKSs are linked to metabolite structures, the predictive value of this type of analysis will increase.

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