Fungal Biology 123 (2019) 397–407

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

Evolutionary relics dominate the small number of secondary metabolism genes in the hemibiotrophic fungus *Dothistroma septosporum*

I. Kutay Ozturk [a], Pierre-Yves Dupont [a, b], Pranav Chettri [a], Rebecca McDougal [c], Ole J. Böhl [d], Russell J. Cox [d], Rosie E. Bradshaw [a, *]

[a] Bio-Protection Research Centre, Institute of Fundamental Sciences, Massey University, Palmerston North, 4410, New Zealand
[b] Institute of Environmental Science and Research, Christchurch, 8041, New Zealand
[c] Scion, NZ Forest Research Institute Ltd, Rotorua, 3010, New Zealand
[d] Institut für Organische Chemie, Leibniz Universität Hannover, Schneiderberg 1b, Hannover, 30167, Germany

ARTICLE INFO

Article history:
Received 28 October 2018
Received in revised form
12 February 2019
Accepted 21 February 2019
Available online 27 February 2019

Corresponding Editor: Julian Rutherford

Keywords:
Dothideomycete
Fungal gene cluster
Nonribosomal peptide
Polyketide
Secondary metabolite
Squalestatin

ABSTRACT

Fungal secondary metabolites have important functions for the fungi that produce them, such as roles in virulence and competition. The hemibiotrophic pine needle pathogen *Dothistroma septosporum* has one of the lowest complements of secondary metabolite (SM) backbone genes of plant pathogenic fungi, indicating that this fungus produces a limited range of SMs. Amongst these SMs is dothistromin, a well-characterised polyketide toxin and virulence factor that is required for expansion of disease lesions in Dothistroma needle blight disease. Dothistromin genes are dispersed across six loci on one chromosome, rather than being clustered as for most SM genes. We explored other *D. septosporum* SM genes to determine if they are associated with gene clusters, and to predict what their likely products and functions might be. Of nine functional SM backbone genes in the *D. septosporum* genome, only four were expressed under a range of *in planta* and in culture conditions, one of which was the dothistromin PKS backbone gene. Of the other three expressed genes, gene knockout studies suggested that *DsPks1* and *DsPks2* are not required for virulence and attempts to determine a functional squalestatin-like SM product for DsPks2 were not successful. However preliminary evidence suggested that *DsNps3*, the only SM backbone gene to be most highly expressed in the early stage of disease, appears to be a virulence factor. Thus, despite the small number of SM backbone genes in *D. septosporum*, most of them appear to be poorly expressed or dispensable for virulence *in planta*. This work contributes to a growing body of evidence that many fungal secondary metabolite gene clusters might be non-functional and may be evolutionary relics.

1. Introduction

Fungal secondary metabolites (SMs) are a chemically diverse group of compounds that, while often dispensable, can exert various profound effects on fungal lifecycles (Keller et al., 2005). From a human perspective many SMs are important as toxins or pharmaceuticals and have been the subject of intensive research, but for the fungi that produce them, SMs can play essential roles in defence, communication, niche adaptation and development (Keller et al., 2005; Macheleidt et al., 2016). There are many examples of fungal SMs with prominent roles in plant disease (Macheleidt et al., 2016), such as victorin from *Cochliobolus victoriae* that induces host cell death (Tada et al., 2005) or fumonisin produced by *Fusarium verticillioides* that is required for lesion development on maize leaves (Glenn et al., 2008).

Secondary metabolites are classified as polyketides, nonribosomal peptides, hybrid polyketide-nonribosomal peptides, terpenes or indole alkaloids (Keller et al., 2005). Their biosynthesis depends on a backbone enzyme, such as a polyketide synthase (PKS) for polyketides which, in fungi, is often encoded in a gene cluster that also contains accessory/tailoring genes that modify the product of the backbone enzyme (Keller et al., 2005). The increasing

* Corresponding author. Fax: +64 6 355 7953.
E-mail address: r.e.bradshaw@massey.ac.nz (R.E. Bradshaw).

https://doi.org/10.1016/j.funbio.2019.02.006
1878-6146/

availability of fungal genome sequences enables prediction of the number of different types of SMs a fungus can potentially make based on the number of genes that encode backbone enzymes. The number of SM backbone genes can vary between fungal species (Ohm et al., 2012). Biotrophic fungi tend to contain fewer SM biosynthetic pathways compared to necrotrophs and saprobes (Spanu et al., 2010), but there are exceptions. The biotrophic tomato pathogen *Cladosporium fulvum* has 23 SM backbone genes, while the closely related hemibiotrophic pine pathogen *Dothistroma septosporum* was initially predicted to have only 11 (de Wit et al., 2012). Only three of the SM backbone genes were predicted to be common to these two species, revealing much lower concordance of their SM repertoires compared to their overall genetic similarities (de Wit et al., 2012). Intriguingly, of the *C. fulvum* SM backbone genes, some are pseudogenised and most are not expressed *in planta* (Collemare et al., 2014).

Among *D. septosporum* SMs, the polyketide dothistromin is the only one studied in detail so far. Dothistromin synthesis involves a backbone PKS gene *DsPksA* (Bradshaw et al., 2006) along with a suite of accessory genes that form a fragmented but co-regulated gene cluster distributed between six loci (Chettri et al., 2013, 2018). Studies comparing wild-type and dothistromin-deficient mutants of *D. septosporum* showed that dothistromin is a virulence factor in Dothistroma needle blight disease (Kabir et al., 2015a) and is produced mainly during the lesion expansion stage when the fungus is growing rapidly (Bradshaw et al., 2016; Kabir et al., 2015b). Two other SM backbone genes of *D. septosporum*, *DsPks1* and *DsPks2,* were recently studied. *DsPks1* was predicted to make the first precursor of dihydroxynaphthalene (DHN) melanin. *DsPks2* appeared to be novel in that its predicted PKS product appeared unrelated to any functionally characterised PKS (Ozturk et al., 2017), but the *DsPks2* gene cluster showed similarity with clusters involved in squalestatin biosynthesis in two ascomycetes (*Phoma* sp. C2932 and an unidentified fungus MF5453) (Bonsch et al., 2016).

Dothistroma needle blight is one of the most significant pine diseases worldwide, affecting 95 *Pinus* species and 14 species in other genera of the Pineaceae (Drenkhan et al., 2016). Recent increases in occurrence and severity of this disease have been associated with changes in climate (Woods et al., 2016). There is an urgent need to understand more about the biology of *D. septosporum* in order to develop new methods and molecular targets to control disease. Therefore, we analysed the SM gene complement of the *D. septosporum* genome to predict the types of metabolites this pathogen could produce and to look for evidence of genetic selection across a global strain population. We made predictions about physically associated genes to determine if there are gene clusters in *D. septosporum* or if the 'clusters' are all fragmented as in the case of dothistromin genes. Finally, we screened gene knockout mutants of two polyketide synthase genes, and a backbone non-ribosomal peptide synthase (NPS) gene that is highly expressed at an early stage of plant infection, to test the hypothesis that they have a role in virulence.

2. Materials and methods

2.1. Growth and RNA sequencing of wild type and DsLaeA mutant strains of Dothistroma septosporum

Co-expression of genes in putative gene clusters associated with each of the expressed SM backbone genes was assessed to support gene cluster predictions. In addition to analysis of published *in planta* expression data (Bradshaw et al., 2016), we analysed gene expression in a global regulator mutant *ΔDsLaeA* previously shown to affect expression of some of the *D. septosporum* backbone secondary metabolite genes (Chettri and Bradshaw, 2016). *D. septosporum* wild type (WT) strain NZE10 (de Wit et al., 2012) and the Δ*DsLaeA* gene knockout mutant (Chettri and Bradshaw, 2016) were grown, and spores produced, as outlined previously (Chettri and Bradshaw, 2016). Spores were inoculated into 25 mL broth at 10^5 spores mL^{-1} and incubated at 22 °C with shaking at 180 rpm under constant light conditions. To maximise possibilities for differential secondary metabolite gene expression two different growth conditions were used; condition 1 (C1) comprised a nutrient-rich LDB broth (Malt extract (Oxoid) 25 g/L; Nutrient broth (Oxoid) 20 g/L) and 9 d incubation while condition 2 (C2) comprised a nutrient-poor PMMG broth containing pine needle extracts (McDougal et al., 2011) and 16 d incubation. For each of the two strains (WT and Δ*DsLaeA*) grown under the two conditions, three independent biological replicates were grown for RNA extraction and sequencing. RNA was extracted from mycelium using a Spectrum plant total RNA kit (Sigma, CA, USA) then treated with DNase (Ambion, Austin, USA). TruSeq mRNA libraries were made and Illumina sequencing (HiSeq 2x125 PE, version 4 chemistry) was carried out by New Zealand Genomics Ltd. Reads were trimmed and mapped to the NZE10 genome using methods described earlier (Bradshaw et al., 2016) except using TopHat v2.2.1 (Kim et al., 2013) for read mapping. Reads are available at https://www.ncbi.nlm.nih.gov/sra/SRP126961.

2.2. Bioinformatic analyses of Dothistroma septosporum secondary metabolite genes

Nucleotide and predicted amino acid sequences of *D. septosporum* strain NZE10 SM backbone genes were from the Joint Genome Institute (JGI) (http://genome.jgi.doe.gov/Dotse1) (de Wit et al., 2012). Domains were predicted with the PKS/NRPS Analysis programme (Bachmann and Ravel, 2009), and confirmed using both Structure Based Sequence Analysis of Polyketide Synthases (SBSPKS) (Anand et al., 2010) and NCBI domain finder (Marchler-Bauer et al., 2015). The *DsPksA*, *DsPks1*, *DsPks2* and *DsNps2* SM products were previously predicted (de Wit et al., 2012; Ozturk et al., 2017). Identification of orthologous genes in other fungal species, and maximum likelihood phylogenetic analyses, were carried out as described previously (Ozturk et al., 2017) but with 100 bootstrap replicates to support branches.

Intraspecific analyses of *D. septosporum* SM backbone genes were done using genome sequences of 19 strains from different countries (Ozturk et al., 2017). Reads and assemblies for these strains are available at NCBI (BioProject accession number PRJNA381823). Reads were mapped to the *D. septosporum* NZE10 reference genome as described previously (Ozturk et al., 2017) then SNP identification and amino acid predictions done using Geneious v8.0.3 (Kearse et al., 2012). For each SM backbone gene, all SNPs from each sample were concatenated and aligned to use as input for building a phylogeny using PhyML (Guindon et al., 2010). The trees were then used as references for dN/dS ratio and multiple test-corrected P-value calculations for each site determined using the sitewise likelihood ratio (SLR) method (Massingham and Goldman, 2005), with P-values < 0.05 as a threshold for significance. The impact of nucleotide changes at four positively selected sites on DsNps3 was assessed by secondary structure predictions using HHPred (Zimmermann et al., 2018) for both the NZE10 sequence and a sequence containing nucleotide changes at all those four sites.

2.3. Gene cluster predictions

In order to predict putative SM gene clusters, 20 genes upstream and downstream of each SM backbone gene were analysed using antiSMASH v3.0 (Antibiotics & Secondary Metabolite Analysis

SHell) (Weber et al., 2015) to find genes with potential roles in secondary metabolism. Functions of these candidate genes were predicted by both manual InterProScan (Jones et al., 2014) and gene ontology (GO) analysis of *D. septosporum* NZE10 gene sequences (Bradshaw et al., 2016). The genes were added to the putative gene cluster if genes with similar predicted functions were found in other fungal SM gene clusters.

Gene expression analysis was carried out to help define the gene cluster contents and boundaries, using published *in planta* expression data (Bradshaw et al., 2016) along with the WT/*ΔDsLaeA* in culture expression data, by manual assessment of co-expression patterns. The gene expression data for the *DsPks2* cluster were further analysed using the FunGeneClusterS programme (Vesth et al., 2016) that predicts groups of co-expressed genes by using Pearson product–moment correlation coefficients for calculation of a gene expression correlation score (CS). These predictions were done using a window size of seven genes where a CS score was calculated for groups of seven genes and reported for the central gene, allowing up to two interspersed genes with lower co-expression correlation score (CS) within a gene cluster. Gene co-expression was also assessed by manual analysis of fold-changes in gene expression across all pairs of conditions, expressed in heatmaps.

2.4. Functional analysis of the DsNps3, DsPks2 and DsPks1 genes by targeted gene knockout

Genomic DNA was extracted from freeze-dried mycelia of *D. septosporum* using the Plant Genomic DNA Mini Kit (Geneaid, New Taipei, Taiwan). *D. septosporum* gene knockout constructs were made with One Step Construction of Agrobacterium-Recombination-ready-plasmids (OSCAR) using PCR-based methods described previously (Chettri and Bradshaw, 2016). PCR primer and probe sequences used in this study are shown in Table S1.

Generation of *DsNps3* and *DsPks2* mutant strains was carried out using methods described for the *DsPks1* mutant in a previous study (Ozturk et al., 2017). The plasmid construct (pR411) for *DsNps3* gene replacement was made so that a 1878 bp region of *DsNps3* (nucleotides 1,327,897–1,329,774 of *D. septosporum* NZE10 scaffold 4, containing the first adenylation and thiolation domains) was replaced by a hygromycin resistance gene with an *Aspergillus nidulans trpC* promoter, and flanked with 1004 bp (5′) and 1010 bp (3′) regions of *DsNps3* to enable targeted integration. The *DsPks2* gene replacement construct (pR415) was prepared so it would replace ~7100 bp of *DsPks2* coding region (nucleotides 910138–918247 of *D. septosporum* scaffold 8) with the same hygromycin resistance cassette, flanked with 1000 bp 5′ and 800 bp 3’ of *DsPks2*.

D. septosporum NZE10 was transformed with the gene deletion constructs using protoplast-based methods described previously (Chettri et al., 2013) and hygromycin-resistant transformants were single-spore purified. Targeted gene replacement mutants were confirmed by PCR and by Southern hybridization of EcoRV- or Pst1-digested DNA with a digoxigenin (DIG)-labelled probe as described in the Roche Molecular Biochemicals DIG Application Manual. For details see Fig. S1.

Radial growth rate analysis of WT and *ΔDsNps3 D. septosporum* was performed using inoculum from 7-day old cultures grown in DM agar (Bradshaw et al., 2000) at 22 °C. From each culture, 5 mm agar plugs were transferred to four replicate DM plates and colony diameters were measured every three days for 30 d. Colony growth was expressed as mean growth rate in mm/day.

To assess the virulence of *DsNps3, D. septosporum* wild type and *ΔDsNps3* strains were each inoculated on eight *Pinus radiata* seedlings (6–8 m old) all derived from one family with low Dothistroma needle blight resistance. The inoculations were performed using a pine seedling inoculation method described previously (Kabir et al., 2013). At three weeks post-inoculation (wpi), three needles per seedling were taken from each seedling for scanning electron microscopy (SEM) analysis. The needle samples were prepared as previously described (Kabir et al., 2015a) and visualised at the Manawatu Microscopy & Imaging Centre (MMIC) using a FEI Quanta 200 SEM (Hillsboro, OR, USA). At 10 wpi, all needles were harvested, and lesions were cut for biomass estimations. DNA was extracted from between 12 and 30 freeze-dried disease lesions (approx. 1 mm lesion length) for each biological replicate of each knockout strain using a Plant Genomic DNA Mini Kit (Geneaid, New Taipei, Taiwan). To estimate the biomass of *D. septosporum* in the infected needles, primer and TaqMan probe sets for real-time PCR assays (Table S1) were designed to detect the single-copy *D. septosporum* polyketide synthase *DsPksA* (target gene) and *P. radiata* cinnamyl alcohol dehydrogenase *CAD* (reference gene) using methods described earlier (Chettri et al., 2012). The relative amounts of fungal biomass in the samples were calculated as fungal target/plant reference and expressed as both fungal biomass per mg of dry weight of lesion and fungal biomass per lesion. Welch's t-test was used to determine significant differences based on the null hypothesis of no significant difference between wild-type and respective knockout strains.

2.5. Cyclopiazonic acid assay

To identify if cyclopiazonic acid (CPA) is produced, wild-type *D. septosporum* NZE10 was grown in 25 mL Low DB broth (2.5 % malt extract, 2 % nutrient broth) in 125 ml culture flasks for 14 d at 22 °C with continuous light, at 200 rpm. The combined mycelia and culture filtrate were acidified to pH 2 with 2 M HCl and harvested, homogenized and then extracted with chloroform:methanol (4:1) by shaking for 12 h (Hermansen et al., 1984). After filtration the organic phase was separated and dried in a rotary vacuum evaporator. Samples were analysed by reverse phase gradient HPLC as described previously (Chettri et al., 2012) except the mobile phases A and B were methanol:water (70:30) and distilled water respectively, each containing 300 mg $ZnSO_4 \cdot 7H_2O$/l. UV spectra were measured with a Dionex UVD 340S UV/VIS PDA Diode Array Detector (Thermo Fisher Scientific, Sunnyvale, CA, USA). The CPA standard (Sigma, Missouri, USA) was prepared at 100 μg/ml in methanol. The elution time of the samples was compared with that of the CPA standard.

2.6. Attempts to determine the DsPks2 cluster secondary metabolite

To identify if *D. septosporum* produces any squalestatin-type compounds, the wild type strain NZE10 and the Δ*DsPks2* mutant (as negative control) were grown for 14 d at 18 °C in a range of liquid and solid media including PMMG (pine minimal medium with glucose), PDB (potato dextrose broth) and DB (Dothistroma broth) previously used for *D. septosporum* (Bradshaw et al., 2000; McDougal et al., 2011) as well as in YMG and CM2 that were previously shown to support high production of squalestatin by the fungus MF5453 (Bonsch et al., 2016). Culture filtrates and/or mycelium were extracted and analysed by LC/MS using earlier methods (Bonsch et al., 2016) except that the LC/MS detector was operated between 150 and 1000 *m/z*, with a gradient starting at 10 % acetonitrile/90 % HPLC grade water (with 0.05 % formic acid) and ramping to 90 % acetonitrile over 15 min. Starter feeding experiments involved the use of a chemically synthesized benzoyl-CoA mimic (benzoyl-SNAC, provided by Karen Lebe); the compound was dissolved in DMSO, and added to growth media 1–2 d before extraction at a concentration of 1 mM (Böhl, 2018).

3. Results and discussion

3.1. Dothistroma septosporum *NZE10 has nine secondary metabolite backbone genes, of which only four were clearly expressed*

The *D. septosporum* NZE10 genome was initially predicted to have eleven SM backbone genes composed of five PKS, three non-ribosomal peptide synthase (NPS), two hybrid polyketide-nonribosomal peptide synthase (HPS) and one dimethylallyl tryptophan synthase (DMA) genes (de Wit et al., 2012). This number was subsequently reduced to nine, as the *DsPks4* gene (JGI ID 24654; truncated) and *DsPks3* (JGI ID 90367; multiple internal stop codons and frameshifts) were identified as pseudogenes (van der Burgt et al., 2014).

An overview of features of the nine intact *D. septosporum* NZE10 SM backbone genes is shown in Table 1. The nine genes are distributed across seven chromosomes, with only three (*DsPks1*, *DsNps1* and *DsNps3*) located within 130 kb of a telomere. All nine genes were present in the genome sequences of 18 other *D. septosporum* strains from different countries (Ozturk et al., 2017). Analysis of these genome sequences indicated that all the SM backbone genes were under negative evolutionary selection pressure (dN/dS < 1), with the three polyketide synthase genes showing the lowest dN/dS ratios (Table 1). This suggested the potential for important and conserved roles of some of the SMs in *D. septosporum*.

Based on published *in planta* gene expression data (Bradshaw et al., 2016), and additional in culture expression data from this study, only four SM backbone genes of *D. septosporum* (*DsPksA*, *DsPks1, DsPks2* and *DsNps3*) were expressed above an arbitrary threshold level of 40 RPMK (Fig. 1, Table S2). Of the expressed genes, *DsPksA*, *DsPks1* and *DsPks2* showed highest expression in the mid and/or late stages of infection *in planta* while *DsNps3* was most highly expressed at the early stage of plant infection. Gene expression was also studied in a Δ*DsLaeA* global regulator mutant of *D. septosporum* (Chettri and Bradshaw, 2016) under two different culture conditions. In the Δ*DsLaeA* mutant, *DsPksA* and *DsPks2* showed higher expression than in the wild type, while the expression of *DsNps3* was reduced, suggesting some control of

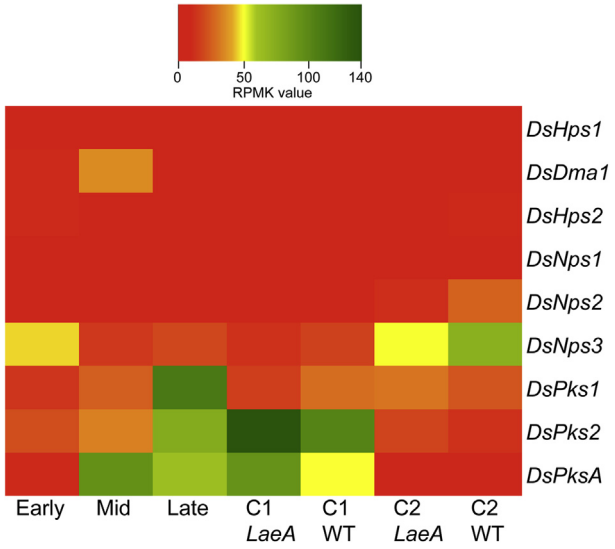

Fig. 1. Heatmap of *Dothistroma septosporum* SM backbone gene expression *in planta* and in culture. The colour key indicates RPMK expression values. Shown are *in planta* expression levels from early, mid and late stages of infection of *Pinus radiata* (from Bradshaw et al., 2016) and in culture expression of genes in wild type and *DsLaeA* mutant strains grown in two conditions: C1, 9 d in DM medium; C2, 16 d in PMMG medium. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Complete list of predicted *Dothistroma septosporum* NZE10 secondary metabolite backbone genes.

Gene[a]	JGI protein ID	Scaffold	Start position (direction)[b]	Intron number	Amino acid number	Domains[c]	Predicted final metabolite[d]	Best hit organism JGI ID[e]	% aa identity/ E value	Distance from telomere	dN/dS[f]
DsHps1	180045	11	965 798 (+)	0	3974	KS-AT-DH-CM-KR-C-A-T	CPA	*A. terreus* 325	47.6/0	581 kb	0.39
DsHps2	157678	9	927 111 (−)	7	4029	KS-AT-DH-CM-KR-T-C-A-T	Unknown	*K. oryzae* 424330	56.9/0	831 kb	0.22
DsDma1	28625	11	965 313 (−)	1	420	N/A	CPA	*C. cereale* 782051	40.4/2.54e^{-42}	593 kb	0.29
DsPksA	48345	12	605 210 (−)	2	2399	SAT-KS-AT-ACP-ACP-ACP-TE	Dothistromin	*C. fulvum* 194256	74.7/0	598 kb	0.17
DsPks1	47338	10	127 527 (+)	0	2189	SAT-KS-AT-ACP-ACP-TE	Melanin	*C. fulvum* 191425	89.7/0	127 kb	0.13
DsPks2	73814	8	910 134 (+)	10	2507	KS-AT-DH-ER-KR-ACP	Squalestatin	*L. palustris* 437929	59.2/0	910 kb	0.21
DsNps1	52251	3	2 664 965 (+)	2	5888	C-A-T-C-A-T-C-A-T-C-A-T-C-A-T-C	Unknown	*A. wentii* 24303	44.3/0	69 kb	0.23
DsNps2	90481	8	1 353 680 (−)	2	4883	A-T-C-A-T-C-T-C-A-T-C-T-C-T-C	Ferricrocin	*C. fulvum* 193954	76.6/0	574 kb	0.27
DsNps3	71189	4	1 327 192 (+)	0	2610	A-T-C-A-NM-T	Unknown	*P. expansum* 371848	52.4/8.5e^{-187}	129 kb	0.27

[a] Gene names as shown in JGI (http://genome.jgi.doe.gov/Dotse1/Dotse1.home.html). Pks: polyketide synthase, Nps: non-ribosomal peptide synthase, Hps: hybrid Pks/Nps, DMA: dimethylallyl tryptophan synthase.
[b] Position (nt) and orientation of predicted gene in scaffold.
[c] Predicted domain structures of the SM backbone gene proteins. KS: keto-synthase, AT: acyltransferase, ACP: acyl carrier protein, TE: thioesterase, DH: dehydratase, ER: enoylreductase, KR: ketoreductase, C: condensation, A: adenylation, T: thiolation, NM: N-methyltransferase, CM: C-methyltransferase, SAT: starter unit acyl-carrier protein transacylase.
[d] Predicted SM produced based on orthology and gene cluster analyses. *Dma1* is adjacent to and divergently transcribed from *Hps1*, therefore might be part of the Hps1 SM cluster.
[e] All hits were confirmed true with reciprocal BlastP analyses. Full species names are *Aspergillus terreus*, *Khuskia oryzae*, *Colletotrichum cereale*, *Cladosporium fulvum*, *Lepidopterella palustris*, *Aspergillus wentii*, *Penicillium expansum*.
[f] Pairwise dN/dS ratios were calculated using Phylogenetic Analysis by Maximum Likelihood (PAML) (See Materials and Methods).

expression by DsLaeA (Fig. 1, Table S2). Five SM backbone genes (*DsDma1, DsHps1, DsHps2, DsNps1, DsNps2*) were barely expressed under any of the conditions tested in either wild-type or Δ*DsLaeA* strains (Fig. 1, Table S2), although the possibility of increased expression under different conditions cannot be ruled out.

Down-regulation or silencing of SM genes has been reported in other fungi. The genome of *C. fulvum,* a close relative of *D. septosporum,* contains 23 SM backbone genes (de Wit et al., 2012). Of these, only 15 appear to be functional and only two are expressed during specific stages of growth *in planta* (Collemare et al., 2014). Down-regulation of the remaining genes was proposed as a mechanism for adaptation of *C. fulvum* to its biotrophic lifestyle (Collemare et al., 2014). The only characterised SM isolated from this fungus is cladofulvin, a toxic anthraquinone toxin which appears to be involved in stress tolerance and survival *ex planta* (Collemare et al., 2014; Griffiths et al., 2015). *C. fulvum* transformants engineered to over-express cladofulvin *in planta* resulted in host tissue necrosis, suggesting that cladofulvin production and biotrophy are indeed incompatible (Griffiths et al., 2018). In contrast, in the hemibiotrophic fungus *Zymoseptoria tritici*, another relative of *Dothistroma septopsorum*, 16 of 32 predicted SM gene clusters showed co-regulated expression of cluster genes, amongst which eight clusters showed patterns of stage-specific expression, indicative of potential biological function of the respective SMs (Cairns and Meyer, 2017). In the necrotrophic pathogen *Parastagonospora nodorum* nine of 38 SM genes were up-regulated *in planta* (Chooi and Solomon, 2014). Hence it is common for only a proportion of available SM genes to be expressed *in planta* in other fungi, but the numbers of expressed genes are often higher than those seen in *D. septosporum* and *C. fulvum.*

Despite the low levels of expression seen, we determined whether the *D. septosporum* SM backbone genes are associated with gene clusters. We also established whether metabolite predictions can be made based on orthology to other SM gene products, and whether any of the expressed putative SMs contribute to virulence functions *in planta*.

3.2. DsDma1, DsHps1 and DsHps2

The *DsDma1* and *DsHps1* genes are less than 0.5 kb apart on chromosome 11 in divergent transcript orientations and are predicted to be part of a common SM gene cluster. Reciprocal FASTA searches and phylogenetic analyses (Fig. 2 and Fig. S2) suggested that *DsHps1* is orthologous to the *Aspergillus flavus* neurotoxin cyclopiazonic acid (CPA) HPS gene *cpaA* (JGI ID 36768) (Seshime et al., 2009). The highest sequence similarity between the predicted *A. flavus* and *D. septosporum* HPS proteins was in the ketosynthase (KS), acetyltransferase (AT) and adenylation (A) domains (Fig. S3). In intraspecific comparisons between *D. septosporum* strains non-synonymous mutations were found in these and all other predicted functional domains (Fig. S4), although there was no evidence for positive selection at any codons. In contrast to the similarity of DsHps1 with *Aspergillus* (Eurotiomycete) CPA gene products, a phylogeny of the predicted dimethylallyl tryptophan synthase DsDma1 showed Sordariomycete gene products as the closest relatives (Table 2, Fig. S2) and no evidence for orthology (and less than 24 % amino acid identity) with the *A. flavus* dmaT that is involved in CPA production (Chang et al., 2009).

In *D. septosporum* several other genes were clustered with *DsHps1* and *DsDma1* that are similar to those in some known CPA gene clusters, although the identities of the predicted proteins were low (Fig. 3). These include genes predicted to encode an FAD-dependent oxidoreductase, cytochrome P450 and methyltransferase; the latter two are not present in all CPA-producing fungi but are involved in additional modifications that occur in biosynthesis

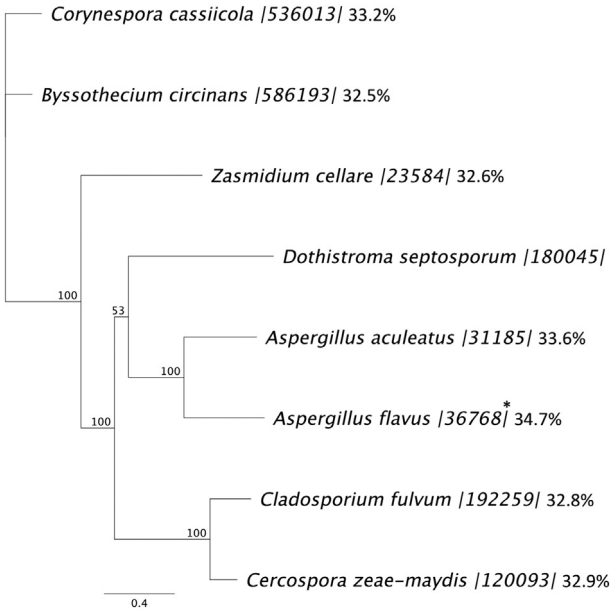

Fig. 2. Phylogenetic tree of amino acid sequences of putative DsHps1 orthologs, selected from the more extensive phylogeny shown in Fig. S2. Numbers next to the species names are JGI protein IDs, followed by amino acid identities to DsHps1. The asterisk indicates a functionally characterized HPS protein required for cyclopiazonic acid (CPA) biosynthesis. Numbers on branches show bootstrap scores. All species shown are Dothideomycetes except the two *Aspergillus* species (Eurotiomycetes).

of a range of CPA and CPA-like products (Uka et al., 2017). The *D. septosporum Hps1-Dma1* cluster did not contain a transporter gene. The predicted zinc cluster transcription factor had only low identities (14–16 %) to those in the CPA clusters (Fig. 3). Instead, the *Hps1-Dma1* cluster transcription factor showed slightly higher identity to aflatoxin AflR-type pathway regulators such as *A. flavus* AflR (accession number AAM02991.1, 17.4 % amino acid identity), and AflR-like proteins from *Talaromyces cellulolyticus* (GAM33609.1; 22.4 %), *Rasamsonia emersonii* (XP_013325168.1, 23.2 %) and *Aspergillus udagawae* (GAO83066.1, 25.2 %). Interestingly deletion of the zinc cluster regulatory gene *ctfR1* in *A. flavus,* which was expected to be required for transcriptional regulation of the CPA cluster genes, did not affect CPA production (Chang et al., 2009). Overall the genetic evidence suggests that the *DsHps1-DsDma1* cluster may be responsible for an unknown derivative of CPA based on the low identity of all cluster genes except for *DsHps1* with *CpaA*. HPLC assays showed that wild type *D. septosporum* NZE10 did not produce any detectable quantity of CPA under the conditions tested, while a sharp single peak was observed at retention time of 29 min for the CPA standard (Fig. S5). However, a range of other growth conditions would need to be tested to rule out production of CPA by *D. septosporum*.

Although the *DsHps1-DsDma1* cluster has similarities to CPA clusters in other fungi, to the best of our knowledge there are no reported examples of CPA production in any Dothideomycete fungi. Amongst Eurotiomycete fungi known to produce CPA, it is worth noting that aflatoxin and CPA clusters are physically next to each other in the *A. flavus* genome (Chang et al., 2009). Aflatoxin and dothistromin gene clusters share an evolutionary history (Bradshaw et al., 2013) so the presence of a CPA-like cluster in *D. septosporum* might also reflect common ancestry of these associated gene clusters. Although the predicted CPA (*DsHps1-DsDma1*) cluster in *D. septosporum* is on a different chromosome to the dothistromin genes, the presence of a regulatory gene fragment with similarity to AflR-like regulatory proteins lends support to a hypothesis of shared ancestry. This gene may be a vestige of the

Table 2
Genes within *DsHps1* and *DsNps2* putative gene clusters.

JGI Protein ID[a]	Gene cluster	Predicted protein[b]	Best hit organism/JGI ID[c]	E value/% aa identity
*Ds83206	*DsHps1*	Zinc cluster transcription factor	*Cladosporium fulvum*/197225	1.6e-163/79.5
*Ds180045	***DsHps1***	**Hybrid PKS-NRPS**	*Aspergillus aculeatus*/31185	0/39.9
*Ds28625	***DsHps1***	**Dimethylallyl tryptophan synthase**	*Colletotrichum falcatum*/703187	3.2e-64/44.7
*Ds28624	*DsHps1*	Oxidoreductase	*Zymoseptoria pseudotritici*/799177	8.0e-139/61.7
*Ds75219	*DsHps1*	Cytochrome P450	*Thozetella* sp./746229	5.9e-80/45.9
*Ds139328	*DsHps1*	Methyltransferase	*Patellaria atrata*/1013025	4.2e-72/33.7
+Ds73932	*DsNps2*	Zinc cluster transcription factor	*Cladosporium fulvum*/193955	1.0e-54/63.5
+Ds90481	***DsNps2***	**Non-ribosomal peptide synthetase**	*Cladosporium fulvum*/193954	0/76.6
+Ds55453	*DsNps2*	ABC transporter related protein	*Cladosporium fulvum*/193953	0/79.7
+Ds73935	*DsNps2*	Lysine/ornithine N-monooxygenase	*Cladosporium fulvum*/193952	0/90.0

Asterisk indicates that genes with similar predicted functions are present in other fungal HPS gene clusters.
Plus indicates that genes with similar predicted functions are present in other fungal NPS gene clusters.
All BlastP hits were confirmed true with reciprocal blast analyses.
Bold font indicates the secondary metabolite backbone genes.
[a] http://genome.jgi.doe.gov/Dotse1/Dotse1.home.html.
[b] Predicted proteins according to antiSMASH and InterProScan analyses.
[c] Joint Genome Institute protein ID numbers.

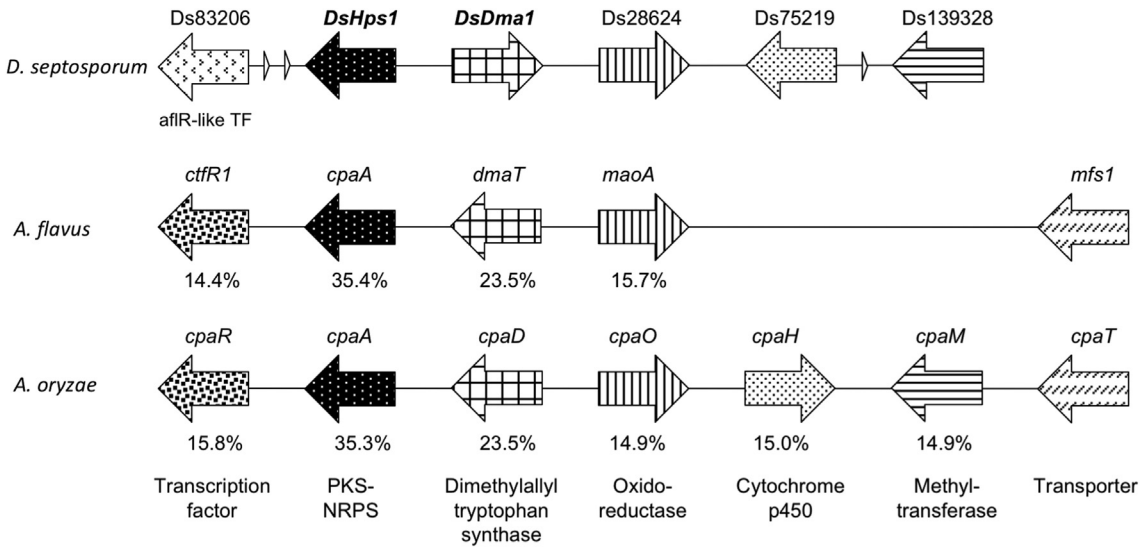

Fig. 3. Synteny of the *D. septosporum DsHps1-DsDma1* putative gene cluster with CPA clusters of *A. flavus* NRRL 3357 and *A. oryzae* NBRC 4177. Matching arrow patterns indicate genes with similar predicted roles (listed under arrows). *D. septosporum* JGI IDs and *Aspergillus spp.* gene names are indicated above arrows. Percentage amino acid identities to the respective *D. septosporum* peptide sequences are indicated below arrows.

evolutionary process and provides a hint that the CPA-like cluster in *D. septosporum* might be a non-functional relic.

The other HPS gene in the *D. septosporum* genome, *DsHps2,* was also expressed at very low levels. *DsHps2* was predicted to have only three putative orthologs amongst Dothideomycete species based on phylogenetic and reciprocal FASTA analyses (Fig. S2) but none of these had been functionally characterized and on this basis no predictions could be made about the SM produced. Although the Hps2 protein sequences showed a high level of conservation across all functional domains, DsHps2 was predicted to have a 300 amino acid C-terminal extension compared to its orthologs (Fig. S3). Using antiSMASH, the *DsHps2* gene cluster was suggested to include five biosynthetic and two transporter protein genes (Fig. S6). Although most of the gene types present are commonly associated with HPS gene clusters (Uka et al., 2017), the Ds74346 gene was predicted to encode a carbamoyl phosphate synthase that has only been reported in bacterial HPS gene clusters to date, such as the saxitoxin cluster from *Anabaena circinalis* (Neilan et al., 2008). Further investigation is required to determine the potential SM products of both the *DsHps1* and *DsHps2* gene clusters.

3.3. DsPks1 and DsPks2

The polyketide synthase gene *DsPks1* was predicted to be involved in biosynthesis of 1,8- dihydroxynaphthalene (DHN)-melanin (de Wit et al., 2012; Ozturk et al., 2017). However, a recent study showed that *D. septosporum* NZE10 produces melanin mainly through the PKS-independent 4-dihydroxyphenylalanine (DOPA) melanin biosynthetic pathway (Ozturk et al., 2017), shedding some doubt on the biological function of DsPks1. Putative gene clusters for *DsPksA* (Chettri et al., 2013), *DsPks1* and *DsPks2* (Ozturk et al., 2017) were shown previously.

Another PKS encoded in the *D. septosporum* genome, DsPks2, was proposed to be a hexaketide synthase involved in synthesis of a squalestatin (Bonsch et al., 2016). This was based on similarity of the predicted gene cluster to that of the squalestatin S1 (SQS1)-producing fungus MF5453 and 67 % amino acid identity between the hexaketide synthases (Bonsch et al., 2016). Here, a revised gene cluster is presented for *DsPks2* (Table 3) based on similarity to the squalestatin gene cluster (Bonsch et al., 2016) and gene co-expression patterns determined by FunGeneClusterS (Vesth et al.,

Table 3
DsPks2 putative gene cluster based on FunGeneCluster analysis and similarity with the squalestatin (SQS1) gene cluster of unidentified fungus MF5453.

JGI ID[a]	Bonsch et al. (2016) ID (dir.)[b]	Putative function	MF5453 homolog	% amino acid identity	Correlation Score (CS)[c]
Ds36893	*dsL12* (−)	Copper dependent oxygenase	*mfM1*	32	2.22
Ds73802	*dsL11* (−)	Acyltransferase	*mfM4*	47	2.98
Ds73804	*dsL10* (+)	[MFS transporter]	*mfR5*	49	1.43
Ds156415	*dsL9* (−)	Non-heme iron oxygenase	*mfR1*	73	4.67
Ds73806	*dsL8* (+)	Unknown	*mfR2*	61	4.20
Ds73807	*dsL7* (−)	Citrate synthase	*mfR3*	66	4.22
Ds73808	*dsL6* (−)	Nucleolar GTPase/ATPase	*mfM5*	13	4.33
Ds73810	*dsL5* (−)	MFS transporter	*mfM6*	61	3.27
Ds55316	*dsL4* (+)	Short-chain dehydrogenase	*mfM3*	57	5.08
Ds176708	*dsL3* (−)	MFS transporter	*mfM2*	64	4.72
Ds64818	*dsL2* (−)	Acyl-CoA synthetase/ligase	–	–	5.25
Ds73813	*dsL1* (−)	Hydrolase	–	–	5.11
Ds73814	*dsPks2* (+)	Polyketide synthase	*mfpks2*	67	4.63
Ds90348	*dsM1* (+)	Esterase/lipase	*mfM8*	62	4.08
Ds36906	*dsM2* (−)	Phenylalanine ammonia-lyase	*mfM7*	17	2.86
Ds36907	*dsM3* (+)	[Acyltransferase]	*mfR4*	37	1.23
Ds36908	*dsM4* (−)	Squalene/phytoene synthase	*mfR6*	56	3.08
Ds73815	*dsM5* (−)	Acyl-CoA synthetase/ligase	*mfM9*	61	2.66
Ds73816	*dsM6* (+)	Hydrolase	*mfM10*	53	2.73

Table adapted from Bonsch et al. (2016).
[a] Genes that weren't identified in Ozturk et al. (2017) as part of the *DsPks2* gene cluster are shown in bold.
[b] Directions of each *Dothistroma septosporum* (Ds) gene, as named by Bonsch et al. (2016), are indicated with plus or minus in the brackets.
[c] The CS threshold was determined by FunGeneClusterS analysis as 1.81 – i.e. values higher than this are considered to show significantly correlated expression profiles. The *dsL10* and *dsM3* genes [with functions shown in square brackets] were below the CS threshold.

2016) using both *in vitro* and *in planta* gene expression data. The revised gene cluster is an expanded version of the previously published *DsPks2* cluster (Ozturk et al., 2017). While the borders of the larger gene cluster predicted by Bonsch et al. (2016) appear correct (*dsL12* to *dsM6*), two genes - *dsL10* (MFS transporter) and *dsM3* (acyltransferase) - were not co-regulated with the rest of the gene cluster (Table 3). The *dsL10* and *dsM3* genes can be excluded from the cluster on this basis, but also on the grounds that their functions are redundant, as there are genes for two other MFS transporters (*dsL5* and *dsL3*) and an acyltransferase (*dsL11*) in the predicted squalestatin gene cluster.

Squalestatins are produced by many ascomycete fungi (Bills et al., 1994) and inhibit the first step of cholesterol biosynthesis by targeting squalene synthase (Sidebottom et al., 1992). Because of this property they have antifungal activity and are also used in the development of drugs for hypercholesterolaemia (Bergstrom et al., 1995; Sidebottom et al., 1992), hence it was of interest to determine if a squalestatin-like compound could be detected in cultures of *D. septosporum*. Metabolite production by the wild type (WT) strain was compared to that of the *ΔDsPks2* mutant. In all the media conditions tested, LC-MS chromatogram peaks seen from the WT samples were also present from the mutant, with the exception of an 8.8 min peak in the WT in CM2 medium that was unreproducible and unrelated to the expected product size (Fig. S7a).

Because a squalestatin-like compound could not be detected under the range of conditions used, we next investigated whether feeding of a starter unit would facilitate production by *D. septosporum*. The SQS1 hexaketide chain has a benzoate starter unit for which phenylalanine ammonia lyase (PAL), encoded by a gene in the MF5453 SQS1 cluster, is thought to be required (Bonsch et al., 2016). Because expression of the corresponding PAL gene (Ds36906) in the *D. septosporum DsPks2* gene cluster is very low (<7 RPMK) (Bradshaw et al., 2016); the *D. septosporum* cultures were fed with a chemically synthesized benzoyl-CoA mimic as a starter unit, but again there was no evidence for squalestatin production (Fig. S7b). Our analysis was sensitive enough to observe consistent production of secondary metabolites above ~0.1 mg/L regardless of whether they are squalestatin-related or not, so the evidence suggests that related compounds are either not made or made in very low concentrations. In summary we obtained no evidence to support the involvement of DsPks2 in production of a squalestatin-like compound in *D. septosporum*.

There are several possible reasons why a squalestatin-like compound was not detected in *D. septosporum* despite the presence of the *DsPks2* gene cluster. For example, although a wide range of media and growth conditions were tested, it is possible that other conditions are required to initiate squalestatin production by up-regulation of cluster genes such as the low-expressed Ds36906 PAL gene which is needed for generation of the benzoyl-CoA starter. In our experiments the benzoyl-CoA mimic used as a starter to compensate for low PAL expression may not have been accepted by DsPks2, which is likely to be highly substrate-specific. It is also possible that other enzymes in the cluster might be inactive through mutation and that the *DsPks2* cluster is dispensable. Finally, it is possible that a different type of squalestatin is produced by *D. septosporum* that we did not detect using our assay. Squalestatin S1 (SQS1) is composed of both hexaketide and tetraketide chains, produced by independent PKS enzymes. Although the *D. septosporum DsPks2* gene is homologous to the MF5453 mfPks2 hexaketide synthase, there is no corresponding *D. septosporum* gene for the tetraketide synthase. However there are different types of squalestatins with different side chains (Bergstrom et al., 1995) that could potentially be produced by *D. septosporum*.

The *DsPks2* gene knockout mutant and a *DsPks1* knockout mutant described previously (Ozturk et al., 2017) were tested for virulence on pine seedlings. Mutants of both *DsPks1* and *DsPks2* were able to infect and colonise *P. radiata* needles to a similar extent as the wild type strain, as shown by qPCR-based fungal biomass estimations (Table 4). Thus, we found no evidence that either DsPks1 or DsPks2 are required for full virulence of *D. septosporum* on *P. radiata*.

Of the four expressed SM backbone genes (Fig. 1), three encoded PKS enzymes, establishing this class of SMs as being of key importance in *D. septosporum*. The production of the virulence factor dothistromin by DsPksA and other pathway gene products has been well established (Bradshaw et al., 2006; Chettri et al., 2013; Kabir et al., 2015a). Our investigations with gene knockout mutants suggest that neither *DsPks1* nor *DsPks2* are required for

Table 4
qPCR estimation of *D. septosporum* fungal biomass *in planta*.

Strains[a]	Fungal/pine Target/Ref[b]	Fungal DNA (ng/mg DW sample)[c]	Fungal DNA (ng per lesion)[d]
NZE10 WT	0.05 ± 0.01	4.45 ± 0.63	2.60 ± 0.44
DsPks1 KO1	0.05 ± 0.01	6.35 ± 1.51	3.09 ± 0.69
DsPks1 KO2	0.06 ± 0.02	4.53 ± 0.49	2.86 ± 0.16
DsPks2 KO	0.05 ± 0.02	4.62 ± 1.41	2.72 ± 0.45
DsNps3 KO	0.02 ± 0.01[e]	2.62 ± 0.38[e]	1.51 ± 0.35[e]

[a] WT, wild-type; KO knockout mutants of SM genes DsPks1, DsPks2, DsNps3 (n = 4, except *DsPks* KO1 n = 3); *DsPks1* had two independent mutants (KO1, KO2).
[b] Fungal biomass relative to pine, determined by qPCR of fungal *DsPksA* gene target/pine *CAD* reference, (mean ± SD).
[c] Fungal DNA (ng) per mg dry weight of infected needle lesion sample (mean ± SD).
[d] Fungal DNA (ng per lesion) (mean ± SD).
[e] Significant difference to WT ($P < 0.05$).

virulence of *D. septosporum* but further replications are required to confirm this. Production of DHN melanin by DsPks1 is probably not essential because *D. septosporum* melanin was mainly made by the alternative DOPA melanin pathway under the conditions tested (Ozturk et al., 2017). Similarly, for DsPks2 no evidence for biosynthesis of squalestatin was found in our experiments, however, an alternative form of squalestatin might be produced. Further work is required to determine if this is the case, and to discern if such a compound might have a role in competition against other fungi and other microorganisms, such as oomycetes, in the forest environment.

3.4. Nps1 and Nps2

DsNps1 and *DsNps2* were amongst the SM backbone genes that showed very low expression under all conditions tested. For DsNps1, reciprocal FASTA searches and phylogenetic analysis of closest matching proteins from across the Dothideomycetes, Eurotiomycetes and Sordariomycetes (Fig. S2) suggested that there are only two putative orthologs. Therefore, the metabolite associated with *DsNps1* may be unique. *DsNps1* was predicted to be part of a seven gene cluster, including three transporter genes and three other biosynthetic genes (Fig. S6). Although having three transporter proteins seems redundant, there are up to four transporters within single predicted NPS gene clusters of *Trichoderma* spp. (Bansal and Mukherjee, 2016). Two of the biosynthetic genes associated with *DsNps1* are not amongst gene classes usually found in other fungal NPS clusters, making prediction of the DsNps1 SM product difficult. Within *D. septosporum*, the *DsNps1* gene structure was conserved in the genomes of 18 other strains. However, the patterns of polymorphism suggested a possible ancestral recombination event in *DsNps1;* strains from Ecuador and Chile shared sequence similarity with other South American and Central American strains in the downstream region and with strains from Europe and other regions in the upstream region of the gene (Fig. S4), suggestive of intermixing of some strains from these diverse groups.

DsNps2 is orthologous to *C. fulvum Nps2* (*CfNps2*) which was previously predicted to be involved in biosynthesis of a ferricrocin-type siderophore, associated with intracellular storage of iron (Collemare et al., 2014; Oide et al., 2007). The phylogeny (Fig. S2) suggested that many other Dothideomycete fungi contain similar genes. A functionally characterized ferricrocin protein encoded by *A. nidulans sidC* (Eisendle et al., 2003) was also a close relative and among the best reciprocal FASTA hits of *DsNps2* (Fig. S2). The *DsNps2* cluster was predicted to have only four genes, including transcription factor, transporter and monooxygenase genes that are similar to, and in the same arrangement as, the *C. fulvum CfNps2* cluster (Fig. 4, Table 2). The Ds73935 monooxygenase gene is predicted to encode an L-ornithine N5 oxygenase which is required for biosynthesis of extracellular iron-scavenging ferrichromes as well as ferricrocin (Winterberg et al., 2010). The predicted *DsNps2* gene cluster lacks an acyltransferase which is required for both ferricrocin and ferrichrome biosynthesis (Silva-Bailão et al., 2014; Winterberg et al., 2010), but there are other acyltransferase genes elsewhere in the *D. septosporum* genome that could potentially fulfil this role. The presence of a transporter gene clustered with *DsNps2* further suggests the possibility that ferricrocin siderophores that might be made under certain conditions could be secreted to assist in scavenging iron, as previously reported in *Aspergillus fumigatus* (Hissen et al., 2004).

3.5. Nps3 is a potential virulence factor in Dothistroma needle blight

DsNps3 appears to be orthologous to functionally characterized beauvericin synthetases of *Beauveria bassiana* (Xu et al., 2008) and *Fusarium oxysporum* (López-Berges et al., 2013) and bassionalide synthetase of *Xylaria* sp. BCC 1067 (Jirakkakul et al., 2008) (Fig. 5, Fig. S2). Amongst these orthologs the first adenylation (A) and thiolation (T) domains were poorly conserved (Fig. S3), suggesting that the SM produced by DsNps3 may be different from beauvericin or bassianolide. Analysis of *DsNps3* sequences amongst strains of *D. septosporum* showed it to be unique amongst the SM backbone genes of this species in showing positively selected codons, including one in the first A domain (Fig. S4) although no effect on secondary structure of the protein was predicted (Fig. S8).

Unlike the other *D. septosporum* SM backbone genes, *DsNps3* did not appear to be associated with a gene cluster. There were no expected types of SM-related genes within 9 genes and 25 kb distance from *DsNps3*, apart from a predicted alcohol dehydrogenase gene (Ds71190) that had a very different expression pattern from *DsNps3* and thus was not co-regulated. It might be that DsNps3 alone is sufficient for product formation, as for peramine which is synthesised by the peramine synthease (PerA), a multifunctional NPS of the grass endophyte *Epichloë festucae* (Tanaka et al., 2005). Beauvericin, bassinolides and related metabolites in the enniatin family are usually also made by large multifunctional NRPS enzymes (Liuzzi et al., 2017), although some associated genes have been reported. Next to the *B. bassiana* NPS gene is a *kivr* (2-ketoisovalerate reductase) gene that is essential for production of both beauvericin and bassianolide and proposed to be required to produce the NPS substrate D-hydroxyisovalerate (D-Hiv) (Xu et al., 2008). However, this enzymatic activity appears to be incorporated into the multifunctional NPS for bassianolide in *Xylaria* sp. (Jirakkakul et al., 2008). An orthologue of the *kivr* gene was not found in the *D. septosporum* genome.

The Non-Ribosomal Peptide Synthase substrate predictor (NRPSsp) tool (Prieto et al., 2012) was used to predict the substrate specificities for the A domains of DsNps3. The first A domain was predicted to be D-2-hydroxyisovalerate and the second A domain prediction was leucine; the same predictions were also made for

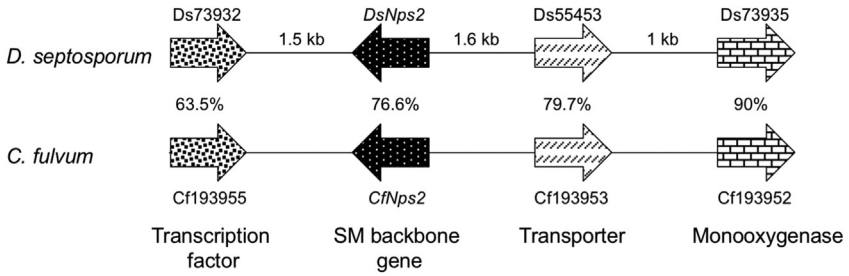

Fig. 4. Synteny of *DsNps2* and *CfNps2* putative ferricrocin-like gene clusters. Arrows representing the genes indicate relative directions of transcription. JGI identification numbers are indicated above *D. septosporum* and below *C. fulvum* genes. Percentage amino acid identities to the respective *D. septosporum* peptide sequences are indicated.

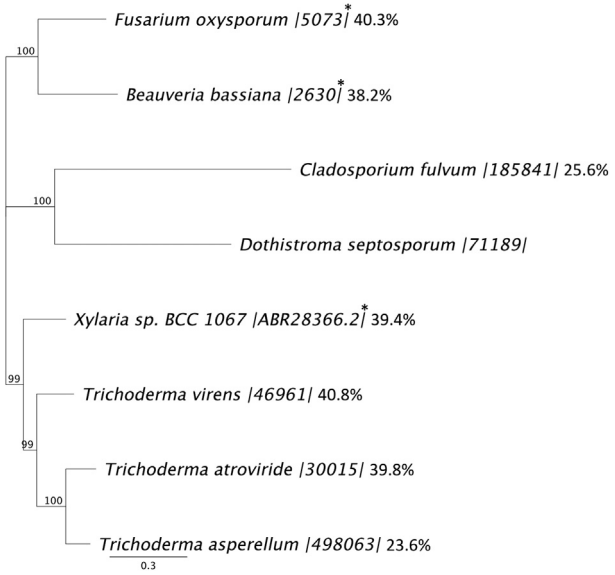

Fig. 5. Phylogenetic tree of amino acid sequences of putative DsNps3 orthologs, selected from the more extensive phylogeny shown in Fig. S2. Numbers next to the species names are JGI protein IDs, or the GenBank accession number for *Xylaria sp*., followed by amino acid identity to DsNps3. Asterisks indicate functionally characterized proteins. Numbers on branches show bootstrap scores. Apart from *C. fulvum* and *D. septosporum* (Dothideomycetes) all species shown are Sordariomycetes. The scale bar represents the number of substitutions per site.

beauvericin and bassianolide, however the substrate specificity for the beauvericin multifunctional NPS is not high (Peeters et al., 1988). Attempts were made to identify the product of DsNps3 from extracts of wild type *D. septosporum* cultures compared to those of the Δ*DsNps3* mutant. Although a possible additional product was detected in the wild type strain (Fig. S9) not enough metabolite could be made for further analysis.

Because of the high level of *DsNps3* gene expression at an early stage of infection *in planta*, we looked at whether this gene is important for *D. septosporum* to cause disease on *P. radiata*. A *ΔDsNps3* mutant strain was obtained (Fig. S1), although we only found one mutant amongst 38 potential gene knockout transformants screened. The growth rate of the mutant in culture was not compromised (*ΔDsNps3* 0.68 ± 0.03 mm/day; WT 0.64 ± 0.05 mm/day; $P = 0.12$, $n = 7$). When pine seedlings were sprayed with spores, no difference could be seen in the abilities of the Δ*DsNps3* mutant and wild type strains to form a network of mycelium across the needle surface, or to penetrate stomata (Fig. S10). After ten weeks, quantitative PCR assays showed a significant difference in the fungal biomass in disease lesions on plants infected with wild-type or the Δ*DsNps3* mutant (Table 4), indicative of lower virulence in the mutant. When repeated, a similar result was obtained, with Δ*DsNps3* biomass less than 30 % of that of the wild type.

Our results provide preliminary evidence that the SM produced by Nps3 might have a virulence function. Further work with more independent mutants or a strain in which the mutation has been complemented with a working copy of *DsNps3* are required. There are precedents for virulence functions of SMs made by DsNps3 orthologs: beauvericins produced by *B. bassiana* and *F. oxysporum* were shown to be virulence factors on their respective hosts (López-Berges et al., 2013; Xu et al., 2008). If the SM produced by DsNps3 is a virulence factor, it is likely to have a role in the early stage of infection due to the highest level of *DsNps3* expression during that period (Bradshaw et al., 2016). Although the Δ*DsNps3* mutant did not appear to differ in growth rate in culture, or in the type of surface growth and penetration through stomata on the pine needle, the plant surface growth may have had quantitative differences that we overlooked. Both beauvericin and bassionalide are insecticidal secondary metabolites (Vega et al., 2008), and beauvericin also has antifungal activity (Zhang et al., 2007). We propose that the *DsNps3* SM may provide a competitive advantage for *D. septosporum* against competitors such as fungi and plant-feeding insects in its early biotrophic stage, enabling more efficient invasion of the plant and consequently more growth *in planta*.

4. Conclusion

The pine needle pathogen *D. septosporum* appears to have a very limited arsenal of secondary metabolites, although it does have SM gene clusters, despite the fragmented nature of the well-characterised dothistromin 'cluster'. Of the nine intact SM backbone genes in the genome, five were only expressed at very low levels, two appear to be important for virulence (*DsPksA, DsNps3*) while two are dispensable (*DsPks1, DsPks2*). We found no evidence for production of cyclopiazonic acid or squalestatin-like compounds by *D. septosporum* despite the similarity of gene clusters. It is possible that expression of the 'silent' genes could be induced under other conditions, such as on dead needles on the forest floor, or on unknown alternative hosts, in which such SMs might play a role. We suggest that many of the SM gene clusters may be non-functional evolutionary relics. However, there is a need to study the chemical ecology of *D. septosporum* to determine if and when metabolites are produced by these gene clusters and what their roles might be.

Acknowledgements

This work was supported by Massey University and the Tertiary Education Commission (NZ) through the Bio-Protection Research Center. We acknowledge the assistance of Karen Lebe (University of Hannover) for the synthesis of benzoyl SNAC, and DFG (grant INST 187/621) for the provision of LCMS equipment, Matthew Savoian

(Massey University) for SEM imaging, Carla Eaton (Massey University) and Scott Griffiths (Westerdijk Fungal Biodiversity Institute) for early project planning, Paul Maclean (AgResearch NZ) for bioinformatics, Austen Ganley and Margaret Coe (University of Auckland) for preliminary LCMS analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.02.006.

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