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Genome-based discovery of polyketide-derived secondary metabolism pathways in the barley pathogen *Ramularia collo-cygni*

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Abstract

*Ramularia collo-cygni* (Rcc) causes Ramularia leaf spot (RLS) disease of barley. The fungus develops asymptotically within its host until late in the growing season when necrotic lesions become visible on upper leaves. Fungal secondary metabolites (SMs) have been proposed as important factors in RLS lesion formation, but the biosynthetic pathways involved remain largely unknown. Mining the Rcc genome revealed the presence of ten polyketide synthases (PKSs), ten non-ribosomal peptide synthetases (NRPSs) and three hybrid PKS-NRPS (HPSs) identified within clusters of genes with predicted functions associated with secondary metabolism. SM core genes along with their predicted transcriptional regulators exhibited transcriptional co-expression during infection of barley plants. Moreover, their expression peaked during early stages of host colonisation and preceded or overlapped with the appearance of disease symptoms, suggesting that SMs may manipulate the host to promote colonisation or protect Rcc from competing organisms. Accordingly, Rcc inhibited the growth of several fungi *in vitro*, indicating it synthesised and excreted anti-fungal agents. Taken together, these findings demonstrate that the Rcc genome contains the genetic architecture to synthesise a wide range of SMs and suggests that co-expression of PKSs and HPSs is associated with competitive colonisation of the host and early symptom development.

Introduction

Ramularia leaf spot (RLS) is a late season disease of barley (*Hordeum vulgare*) occurring in temperate regions worldwide. Typical RLS symptoms are often reddish-brown in colour, delineated by the leaf vein, surrounded by a chlorotic halo and visible on both sides of infected leaves (Huss 2002). Symptoms can also be observed on stems, heads and awns in severely infected crops. As a result, RLS is considered a major disease of barley causing typical yield
penalties of approximately 20% but these losses can be as high as 70% (Havis et al. 2015). RLS can also reduce grain size by up to 4% which devalues the crop (Cromey et al. 2002; McGrann and Havis 2017).

RLS is caused by the filamentous fungus *Ramularia collo-cygni* (Rcc), a Dothideomycete within the order Capnodiales. Rcc was recently confirmed to be closely related to *Zymoseptoria tritici*, the fungus responsible for Septoria leaf blotch of wheat (McGrann et al. 2016; Crous et al. 2011). Rcc colonisation initially occurs asymptotically before turning to necrotrophic growth (Kaczmarek et al. 2017). The transition from asymptomatic to necrotrophic development appears linked with changes in environmental conditions, leading to breakdown of the plant antioxidant system as RLS symptoms generally develop concomitantly with leaf senescence (Schützendübel et al. 2008). For example, plants grown under high light conditions display higher disease levels than plants grown under normal light levels (Makepeace et al. 2008; Peraldi et al. 2014; McGrann and Brown, 2018). Moreover, barley plants with altered redox homeostasis or delayed senescence also appear to show differential expression of RLS symptoms (McGrann et al. 2014, 2015a, 2015b). The long latent period combined with the importance of seed transmission in Rcc dissemination (Havis et al. 2013) and the effects of abiotic stress on disease symptom formation has led to Rcc being described as an endophyte which can cause disease if it enters its necrotrophic stage (McGrann and Brown, 2018; McGrann and Havis 2017). The exact causes of the transition from endophytic to necrotrophic lifestyle are currently unknown but the action of phytotoxic secondary metabolites (SMs) has been proposed as a plausible mechanism (Walters et al. 2008; Heiser et al. 2003).
Many Dothideomycete fungi synthesise toxic SMs during disease development (Stergiopoulos et al. 2013). SMs such as the host specific toxin victorin, produced by the Victoria blight pathogen of oats Bipolaris victoriae (syn. Cochliobolus victoriae), are required for pathogenicity (Navarre and Wolpert 1999; Lorang et al. 2004), whereas others such as the non-host specific phytotoxin dothistromin produced by Dothistroma septosporum, responsible for Dothistroma needle blight of pine trees, act as virulence factors mediating disease severity (Kabir et al. 2015). Rcc is known to produce light-activated phytotoxic SMs called rubellins that have been isolated from Rcc-infected barley leaves (Miethbauer et al. 2003) and in vitro cultures (Heiser et al. 2004; Miethbauer et al. 2006). Six rubellin derivatives, named alphabetically from A to E plus 14-dehydro rubellin D have been found in Rcc cultures (Miethbauer et al. 2008). Rubellins are non-host specific toxins that induce fatty acid peroxidation in vitro upon light activation (Heiser et al. 2004). As light influences RLS symptom expression (Makepeace et al. 2008; Peraldi et al. 2014), the link between RLS and the action of rubellins in planta was presumed.

Most secondary metabolites produced by plant pathogenic fungi are derived from four core biosynthesis pathways that are controlled by polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs); terpene cyclases (TCs; syn. terpene synthases) and dimethylallyl tryptophan synthases (DMATs; Muria-Gonzalez et al. 2015). Rubellin is an anthraquinone-derived toxin structurally related to characterised fungal SMs such as cladofulvin, emodin and aflatoxin, respectively produced by Cladosporium fulvum, Aspergillus nidulans and Aspergillus flavus. Based on the structural similarity between rubellin and other anthraquinone SMs, rubellin biosynthesis has been proposed to occur through a polyketide pathway (Miethbauer et
Considering that SMs often mediate the interactions between a pathogen and its host; understanding the potential of Rcc for producing such compounds may provide insights into the nature of these interactions. Here, we investigated secondary metabolism in Rcc using the recently sequenced genome of this fungus (McGrann et al. 2016). We identified gene clusters involved in the synthesis of polyketide-derived SMs and demonstrate their transcriptional co-regulation during Rcc colonisation of the barley host and early stages of RLS symptom development.

Results

Identification of secondary metabolism-related core genes in *Ramularia collo-cygni*

Using Basic Local Alignment Search Tool (BLAST) combined with domain analysis, the Rcc genome was found to contain 21 genes encoding for proteins containing domains associated with PKSs and 25 genes encoding proteins exhibiting a domain associated with NRPSs. However, only ten putative PKSs possessed the three domains ketosynthase (KS), acyl-transferase (AT) and acyl carrier protein (ACP) required for prototypical PKS functionality. Of the ten putatively functional PKSs, three exhibited the domain organisation of non-reducing PKSs (NR-PKSs) and seven had the organisation of highly- and partially-reducing PKSs (Figure 1). A further three genes were classified as hybrid PKS/NRPS (HPS) genes as they encoded proteins containing a typical highly reducing PKS module fused to a single NRPS module consisting of condensation, adenylation and peptidyl carrier protein domains (Figure 1). The best BLAST hit for each Rcc PKS and HPS is given in Table 1. Furthermore, ten NRPSs appeared to have the
domain organisation found in functional type A and type B NRPS proteins. Three NRPS proteins were mono-modular, two bi-modular and five multi-modular (Supporting Information Figure S1). Corresponding GenBank accession numbers for the Rcc SM core genes are presented in Table S1. Compared with a previous analysis that identified only four TCs and no DMATSs in the Rcc genome (McGrann et al. 2016), it appears that Rcc possesses at least 27 SM core genes. A similar number of SM core genes was obtained using the alternative detection methods AntiSMASH and SMURF which identified 26 and 18 SM core genes, respectively (Supporting Information Table S2). The number of core genes identified in Rcc is similar to that found in other closely related Dothideomycetes such as Z. tritici, C. fulvum and Pseudocercospora fijiensis (McGrann et al. 2016; Ohm et al. 2012; de Wit et al. 2012; Noar and Daub 2016). Thus, the Rcc genome encodes for an appropriate number of SM core genes but these do not appear to reflect completely the lifestyle of the fungus as no correlation was apparent between fungi with different lifestyles in the Capnodiales (Supporting Information Table S3).

Prediction of *Ramularia collo-cygni* polyketide synthase and hybrid polyketide synthase-non-ribosomal peptide synthetase secondary metabolism gene clusters

The genomic locus around each putatively functional PKS and HPS gene was investigated by *in silico* genome walking. Eight of the identified PKSs and all three HPS core genes appeared to be located in a cluster containing several genes with a predicted function associated with secondary metabolism (Figure 2). *Pks8* and *Pks2* were not located within potential SM gene clusters but these PKS sequences were located at an end of their respective contig/scaffold so it is possible that SM-related genes are present near these core genes but these regions were not
completely assembled in the draft genome assembly. The largest cluster, located on contig 58 near Pks6, was over 45.2 kb in length and contained 16 putative SM-related genes and two predicted genes which currently have no transcript evidence based on RNAseq data (McGrann et al., 2016). The smallest cluster spanned 17 kb on contig 282 and contained five SM-related genes including the PKS core gene (Pks10) and a TC gene (named Tc1 in Figure 2) previously identified in the Rcc genome (McGrann et al., 2016). Out of the eight putative SM clusters located around PKSs, four appeared to have more than one predicted core gene present within the cluster. The cluster on contig 118 contained two genes with predicted PKSs domains; however, only Pks3 exhibited the typical domain organisation of functional PKSs. Two other clusters, located on contig 280 and contig 58 near Pks9 and Pks6, both contained an additional SM gene exhibiting a domain architecture found in non-functional NRPSs. These genes were designated Nps12 (contig 280) and Nps13 (contig 58). Six of the 11 clusters surrounding PKS or HPS cores genes possessed at least one gene with a predicted function associated with cellular transport from either the major facilitator superfamily (MFS) or ATP-binding cassette family, which are known to be involved in secondary metabolism processes including protecting fungi against their own toxic SMs (Gardiner et al. 2005). Two putative SM clusters surrounding a PKS core gene (Pks1 and Pks6 identified on scaffold m24 and contig 58, respectively) and two located near a HPS gene (Hps1 and Hps2 identified on contig 1 and contig 14, respectively) contained predicted transcription factors with GAL4 domains, also known as Zn(II)2Cys6 DNA binding domains that have been shown to regulate secondary metabolism in fungal pathogens (Brakhage 2013).
Three of the SM-related gene clusters found in Rcc exhibited high similarity to clusters involved in the biosynthesis of SMs previously characterised in another fungal species (Figure 3; Supporting Information Table S4). Four of the six genes identified in the cluster located on contig 30 containing Pks5 are similar to genes present in the betaenone biosynthesis cluster identified in the oilseed rape pathogen *Phoma betae* (syn. *Pleospora betae*) (Ugai et al. 2015; Figure 3.A). Pks1 was located in a cluster exhibiting similarity to an SM-cluster involved in monodictyphenone production in *Aspergillus nidulans* (Chiang et al. 2010; Figure 3.B). Similarly, the locus on contig 14 surrounding Hps2 comprises genes showing similarity to all seven genes present in the chaetoglobosin A biosynthetic cluster identified in *Penicillium expansum* as well as two genes not identified in the *P. expansum* cluster (Schumann and Hertweck 2007; Figure 3.C). The clusters identified on contigs 282, 118, 58, 1 and 38, surrounding Pks10, Pks3, Pks6, Hps1 and Hps3 respectively, all showed similarity to SM clusters present in other fungal species but the end SM products of these clusters are currently unknown (Supporting Information Figure S2). As all of the Rcc clusters that showed similarity to SM-gene clusters in other fungi typically exhibited gene inversions and rearrangements within the Rcc locus it remains to be determined whether or not Rcc can synthesise these SM products.

The relationship between Rcc core genes and core genes in other fungal species was further examined by a phylogenetic analysis of the protein sequences encoded by these genes (Supporting Information Figure S3). Amongst the NR-PKSs, the protein encoded by Pks1 appeared to be closely related to that encoded by *Acas, ClaG, EncA* and *MdpG*, respectively involved in atrochrysone carboxylic acid, cladofulvin, endocrocin and monodictyphenone biosynthesis in *Aspergillus terreus, C. fulvum, Aspergillus fumigatus* and *A. nidulans,*
respectively (Awakawa et al. 2009; Griffiths et al. 2016; Lim et al. 2012; Chiang et al. 2010). The HPS encoded by Hps2 was located on the same clade as the enzyme encoded by CheA, the core gene involved in the chaetoglobosin A biosynthesis pathway in P. expansum (Schumann and Hertweck 2007). Chaetoglobosins are alkaloid cytochalasans that contain an isoindoline backbone fused to a macrocyclic ring. HPSs involved in cytochalasin synthesis are known to utilise a specific range of amino acids. Chaetoglobosins are synthesised from tryptophan, cytochalasins from phenylalanine, alachalasins from alanine, scoparasins from tyrosine and aspochalasins from leucine (Scherlach et al. 2010). AntiSMASH-based predictions showed that the A-domain in Hps2 is likely to incorporate leucine, tryptophan or alanine suggesting that the product of Hps2 could be a cytochalasan, potentially belonging to the chaetoglobosin, aspochalasin or alachalasin family (data not shown). The in silico analysis of the Rcc genome demonstrates that Rcc possess genetic loci that may be able to synthesise a wide range of SMs.

Gene expression profiling of Ramularia collo-cygni SM-related genes during Ramularia leaf spot development

Changes in transcript levels of Rcc PKS and HPS SM core genes were investigated during Rcc colonisation and RLS development in artificially inoculated barley seedlings using quantitative reverse transcription PCR (qRT-PCR). Primers with amplification efficiency suitable for qRT-PCR were successfully designed for six PKSs, Pks5, Pks4, Pks10, Pks3, Pks2 and Pks1 and all three HPSs.

RLS symptoms developed significantly over time (P < 0.001) with small pepper spots typical of the earliest stages of the disease first visible from 7-10 days post inoculation (dpi)
onwards (Figure 4.A). Subsequently lesions expanded to form larger spots which eventually coalesced to cover larger portions of the leaf from 15-21 dpi onwards. As RLS symptoms developed green leaf area (GLA) retention significantly declined over time (P < 0.001; Figure 4.B). Loss of GLA was slower between 5-7 dpi (P < 0.05) but rapidly increased from 10 dpi onwards (P < 0.001). *R. collo-cygni* biomass assessed as accumulation of fungal DNA also increased significantly during the inoculation time course (P = 0.001; Figure 4.C). However, significant increases between consecutive time points were only noted between 15-18 dpi (P = 0.019) with the amount of fungal DNA plateauing at 18 and 21 dpi. Significant changes in *R. collo-cygni* colonisation stages were also observed over time. The number of stomata penetrated by fungal hyphae and subsequently developing conidiophore-like structures gradually increased over the duration of the time course (P < 0.001; Supporting Information Figure S4). Necrosis associated with stomata significantly increased from 10 dpi onwards (P = 0.006).

Transcripts for five of the six PKSs tested were most abundant at 5 and 7 dpi corresponding to the asymptomatic and the early symptom formation stage, respectively. Expression of *Pks5, Pks4, Pks2* and *Pks1* was highest at 5 dpi with transcript levels subsequently declining after 10 dpi. *Pks10* transcript levels were highest at both 5 and 7 dpi but decreased rapidly at 10 dpi and remained low thereafter (Figure 5). In contrast, the NR-PKS, *Pks3*, showed consistently low transcript levels that did not change over the infection time course. Transcript levels of all three HPSs were most abundant during the early symptom development phase with the expression of *Hps1* and *Hps3* peaking at 7 dpi and slowly decreasing to minimal levels at 21 dpi whereas *Hps2* was highly expressed at both 5 and 7 dpi before declining to a low level of
expression from 10 dpi onwards (Figure 5). The expression profile of several NRPSs indicated that these SM core genes were also most abundant at 5 and 7 dpi (Supporting Information Figure S5). SM core gene transcription was highest when foliar Rcc biomass was at its lowest.

Concomitant with the expression of RLS symptoms there is a decline in the hosts antioxidant system (Schützendübel et al. 2008) which is associated with elevated levels of reactive oxygen species hydrogen peroxide in the plant (McGrann and Brown, 2018). Therefore, Rcc catalase expression was measured over the time course of disease development as an experimental control gene that would be predicted to increase transcript abundance as disease develops, similar to the interaction between the related fungus Z. tritici and wheat (Keon et al. 2005). As Rcc biomass increased over the experiment time course along with RLS symptom expression, transcript levels of Rcc catalase, a hydrogen peroxide scavenger, also increased (Figure 5).

These results suggest that elevated Rcc SM core gene expression is distinct from fungal biomass accumulation and preceded or overlapped with early disease symptom expression.

As the expression profile of most of the Rcc SM core genes investigated here showed similar patterns of transcription, the expression of previously characterised fungal transcriptional regulators as well as SM pathway-specific transcription factors (TFs) was investigated to determine possible co-regulation of these genes. Putative Rcc homologues of the zinc-finger TFs PacC, CreA and AreA involved in responses to pH, carbon and nitrogen conditions respectively; VeA and LaeA, two components of the light-activated velvet complex which regulates diverse biological processes including asexual reproduction and secondary metabolism; and pathway-specific TFs AflR and AflJ, both known to regulates genes involved in the aflatoxin and dothistromin biosynthetic cluster in A. flavus and D. septosporum, were
identified in the Rcc genome using BLASTp (Supporting Information Table S5). Transcript profiles of PacC and AreA were similar to that observed for most of the SM core genes with highest transcript levels at 5 and 7 dpi before a general decline in gene expression from 10 dpi onwards (Figure 6.A). The pathway specific TFs AflR and AflJ were also most highly expressed during the early stages of Rcc colonisation (Figure 6.C). Both VeA and LaeA exhibited slightly different expression profiles with the highest transcription of these genes between 5-10 dpi (Figure 6.B). These results indicate that several transcription regulators with known roles in SM regulation in plant pathogenic fungi appear co-regulated with most of the Rcc SM core genes examined. The increased expression of SM core genes during the early stages of Rcc colonisation suggests that if these SMs are indeed produced by Rcc the SM products of the genes investigated here could play a role in early plant-pathogen interactions such as manipulating the host to aid fungal colonisation or to protect the fungus from competing organisms.

**In vitro antifungal activity of *Ramularia collo-cygni***

To assess antifungal activity of SMs produced by Rcc, *in vitro* dual culture competition assays between Rcc and common fungal pathogens of barley were used. The presence of Rcc significantly inhibited the growth of all the tested competing fungi examined (Figure 7). Growth of *Pyrenophora teres*, the causative agent of net blotch, was inhibited by 66% (*P* = 0.041) and 80% (*P* = 0.002) on AEA and PDA media, respectively. *Fusarium poae* growth was inhibited on both media by more than 50% in the presence of Rcc (AEA *P* < 0.001; PDA *P* < 0.001) and that of *Fusarium graminearum* by more than 60% (AEA *P* < 0.001; PDA *P* < 0.001). Rcc also had an...
antagonistic effect on *Rhynchosporium commune*, responsible for barley leaf scald, growth ($P < 0.001$) on PDA. A red coloration of the medium surrounding Rcc cultures which was particularly visible on PDA when grown in competition with *P. teres* and *R. commune* (Figure 7.B), suggests that Rcc releases compounds into the growing medium that inhibits the growth of other fungi. Previous reports have demonstrated that the one characterised SM family synthesised by Rcc, the rubellins, can produce a red colouration in culture media (Heiser et al. 2003). Together these data illustrate that SMs could provide Rcc with a competitive advantage over other microorganisms during host colonisation.

**Discussion**

Genomic analysis of the *Ramularia collo-cygni* genome reveals the potential to produce several different secondary metabolites. Genomes of filamentous Ascomycetes and in particular of Dothideomycetes possess a large number of SM-related genes which result in the ability to produce a wide range of chemical compounds (Kroken et al. 2003; Bushley and Turgeon 2010). The Rcc genome contains at least 23 potentially functional SM core genes belonging to the PKS, NRPS and HPS families. This finding contrasts with McGrann et al. (2016) which reported 19 PKSs, fourteen NRPSs and four terpene cyclases but did not report any dimethylallyl tryptophan synthases nor HPSs. This variation highlights the importance of analysing domain structure rather than relying on BLAST similarity alone to identify SM core genes which can lead to mis-identification of putatively non-functional genes as well as excluding HPSs. Core genes tend to be organised within the Rcc
The cluster located at the Pks5 loci contained putative homologues to all the genes involved in betaenone biosynthesis in *Phoma betae* (Ugai et al. 2015). Betaenones are phytotoxins associated with leaf spot disease of sugar beet and induce leaf chlorosis (Ichihara et al. 1983). The cluster located near the core gene Hps2 contained putative homologues of the seven genes involved in the biosynthesis of chaetoglobosin A in *Penicillium expansum* and two additional genes, a putative O-methyltransferase and a MFS transporter. Chaetoglobosins are known to have cytotoxic properties (Ohtsubo et al. 1978) and to exhibit antifungal activity (Zhang et al. 2013). Therefore, although to date only rubellins were isolated from Rcc, it appears that Rcc has the genetic capacity to produce a wide range of SMs including several phytotoxins and antifungal agents. Rcc gene clusters with similarity to uncharacterised SM biosynthetic clusters, where the function of the SM has not yet been determined in other species were also discovered; further highlighting the potential diversity of the Rcc SM arsenal.

The pathway responsible for rubellin biosynthesis has yet to be determined but the chemical structure of this SM may provide clues towards better understanding its production. Chemically rubellins resemble several fungal polyketide-derived SMs such as endocrocin, emodin and cladofulvin that all exhibit a dioxaanthracene backbone characteristic of anthraquinones and are synthesised by the NR-PKSs *EncA*, *MdpG* and *ClaG*, in *A. fumigatus*, *A. nidulans* and *C. fulvum*, respectively (Chiang et al. 2010; Lim et al. 2012; Griffiths et al. 2016). Three NR-PKSs, *Pks3*, *Pks1* and *Pks2* were identified in the Rcc genome. Of these Rcc NR-PKSs, *Pks3* can probably be excluded from the biosynthesis of rubellin or other anthraquinone-
derived metabolites as the domain organisation of Pks3, which includes a methyltransferase
domain and lacks a product template domain, differs significantly from the SAT-KS-AT-PT-ACP
organisation of EncA, MdpG and ClaG. Furthermore, the phylogenetic analysis suggests that the
protein encoded by Pks3 is not related to proteins involved in anthraquinone biosynthesis. The
domain organisation of Pks2 is similar to that of other NR-PKSs including AfPksA and DsPksA
involved in the production of the anthraquinone-derived A. flavus aflatoxin and D. septosporum
dothistromin, as well as the PKSs encoded by CTB1, Pks1 and Cppks1 involved in the
biosynthesis of the perylenequinone toxins cercosporin, elsinochrome and phleichrome,
isolated from Cercospora nicotianae, Elsinoë fawcettii and Cladosporium phlei, respectively
(Choquer et al. 2005; Liao and Chung 2008; So et al. 2015). Of these NR-PKSs the phylogenetic
analysis suggests that Pks2 is more closely related to the elsinochrome synthase Pks1 and the
phleichrome synthase Cppks1 than AfPksA and DsPksA, which are recovered in a different
clad. These results suggest that Pks2 may either be involved the production of an
anthraquinone-derived SM or in the biosynthesis of SMs potentially belonging to the
perylenequinone family.

Pks1 encodes a protein exhibiting the same domain organisation as the proteins encoded
by MdpG, EncA and ClaG. Rcc PKS Pks1 was also closely related to MdpG, EncA, ClaG as well as
Acas which is responsible for the biosynthesis of atrochrysone carboxylic acid, a precursor of
A putative role for Pks1 in emodin or chrysophanol biosynthesis was further supported by the
co-localisation of Pks1 with genes exhibiting sequence similarity to those identified in the
monodictyphenone biosynthetic cluster involved in emodin and chrysophanol production
Considering that the rubellins reported from Rcc, especially rubellin A and B, and uredinorubellins (Miethbauer et al. 2008) exhibit a chemical structure resembling that of a dimer of chrysophanol, Pks1 appears to be a strong PKS candidate involved in the rubellin biosynthetic pathway. Functional characterisation of this gene and its genetic locus may provide further insights into the pathways that regulate rubellin biosynthesis.

*Ramularia collo-cygni* secondary metabolism core gene transcripts are most abundant during the early stages of *R. collo-cygni* colonisation

Transcript levels of core genes associated with potential secondary metabolism pathways in Rcc were most abundant during the asymptomatic and the earliest lesion formation phases following Rcc colonisation (Figure 5). Transcription of SM-related genes in *Colletotrichum orbiculare, C. higginsianum* and *C. gloeosporioides* were most abundant during the early stages of infection (O’Connell et al. 2012; Gan et al. 2013; Alkan et al. 2015). In the Rcc sister species *Z. tritici*, SM gene transcripts were most abundant during the transition phase from biotrophic to necrotrophic growth (Rudd et al. 2015). SMs produced during this transition phase could be involved in protecting the fungus from plant defence mechanisms or inducing host cell death to facilitate fungal development during necrotrophic colonisation. However, some secondary metabolism-associated transcripts were found to be most abundant during later stages of disease development in *Z. tritici* including sporulation (Palma-Guerrero et al. 2016; Cairns and Meyer 2017). Similarly, in the closely related *D. septosporum*, four core genes including three PKSs were up-regulated during the later stages of infection (Bradshaw et al. 2016). The variation in SM-transcription profiles between different Dothideomycetes highlights
the complex nature of SM functions in these fungi and may provide insights into how SM
affects the unique pathology of the associated diseases.

Regulation of fungal SM-related genes is the result of a finely tuned balance between
requirements for fungal development and secondary metabolism (Calvo et al. 2002). As a
result, SM-related genes are often co-regulated, facilitating rapid control of SM production. Rcc
SM core gene expression appeared to be co-regulated with several well characterised fungal
transcriptional regulators, including the pH dependent transcription regulator PacC as well as
components of the light-regulated velvet complex. However, the similar expression profile of
Rcc PacC (Figure 6.A) with SM gene transcription in Rcc does not conclusively indicate that this
gene is involved in regulating SM gene expression in Rcc. The observation that PacC specific 5’-
GCCARG-3’ binding sites (Tilburn et al. 1995) were present within 1000 base pairs of the start
codon of all the tested Rcc PKS genes (data not shown), except Pks3 which has a slightly
different expression profile, provides further evidence that may indicate that PacC could be
involved in PKS core gene regulation in Rcc. Further studies on the possible role of PacC and
other well characterised fungal SM gene regulators are required to ascertain the true role of
such genes in SM gene transcription in Rcc.

The velvet complex differentially regulates fungal SM biosynthesis in a light-dependent
manner (Wiemann et al. 2010). This complex which is formed by the three proteins VelB, VeA
and LaeA is involved in regulating SM-related gene expression (Bayram et al. 2008). Most of the
Rcc core genes examined showed higher transcript levels at the earliest stages of fungal
colonisation concomitant with higher levels of VeA and LaeA transcription (Figure 6.B). Similar
results were found in other fungal species such as *A. nidulans, F. fujikuroi, F. oxysporum* and *B. maydis* where the velvet complex regulates SM biosynthesis (Park et al. 2012; Wiemann et al. 2010; López-Berges et al. 2013; Wu et al. 2012). These data indicate that the velvet complex may be involved in SM gene expression regulation in Rcc; however, the elevated transcription of *VeA* and *LaeA* at 10 dpi when transcription of most Rcc SM core genes was declining either suggests that control of secondary metabolism in Rcc may be under the control of other transcriptional regulators or that the velvet complex may have another function in Rcc. Obtaining Rcc mutants impaired in *LaeA* or *VeA* expression may provide useful insights into the role played by the velvet complex in this fungus.

The two NR-PKSs *Pks2* and *Pks1* with the highest sequence similarity to *D. septosporum PksA*, the core gene involved in dothistromin biosynthesis, and *Pks5*, which was identified in a betaenone-like cluster (Figure 3), were most abundant at 5 and 7 dpi suggesting that, if the SMs synthesised by these PKSs are phytotoxins, then these SMs could be produced early during Rcc colonisation and stored. A red compound was observed inside vesicles within Rcc hyphae indicating that metabolites such as rubellin could be stored within the fungus until needed (McGrann et al. 2016). However, the presence of SM in vesicles does not necessarily correspond to a storage phase. In *Aspergillus parasiticus*, several steps in the biosynthesis of the highly toxic SM aflatoxin are carried out in specialised vesicles termed aflatoxisomes (Chanda et al. 2009). Therefore, the previous observations of red coloured compounds stored in vesicles within Rcc hyphae may correspond to a specific phase in Rcc SM biosynthesis rather than a storage phase. Alternatively, the necrotic lesions observed as early as 7 dpi (Figure 4) during RLS development indicate the action of a cell death inducer. It is possible that it is these
early colonisation phase cell death inducers which are being observed in hyphal vesicles although as cell death can be induced through a number of pathways it is unclear whether the observed cell death is linked with the action of a phytotoxin, SM or some other product.

Transcript levels of the three HPSs identified were also the highest during the early stages of colonisation. If the chaetoglobosin A-like cluster located at the Hps2 locus is functional, the expression profile of Hps2 suggests this HPS may synthesise an antifungal agent early during the infection of the host plant. Considering the slow growing nature of Rcc (Kaczmarek et al. 2017) which initially develops as an endophyte, chaetoglobosin production could act to eliminate fungi competing for the same ecological niche as Rcc. In vitro antifungal activity of Rcc was observed against common fungal pathogens of barley (Figure 7). The antimicrobial activity of rubellin toxins produced by Rcc is reported by Miethbauer et al. (2008) as being light-dependent. The assay used here was undertaken in constant darkness, suggesting that the antimicrobial effect of Rcc on in vitro fungal growth may not be linked with rubellin activity. Therefore, other SMs, such as chaetoglobosin, or other non-SM compounds may be responsible for the observed antifungal activity and function to confer an ecological advantage to Rcc. Obtaining Rcc mutants in which SM core genes have been deleted may provide useful insights into the role played by SMs in the biology of this fungus.

In silico analysis of the Rcc genome reveals that this fungus like many other Dothideomycetes has the genetic potential to produce several classes of SMs including hybrid polyketide/non-ribosomal peptide such as chaetoglobosin or polyketides such as betaenones or the previously characterised anthraquinone rubellin (Miethbauer et al. 2003; Heiser et al. 2004). Although the identification of the complete Rcc secondary metabolome may be difficult
as several SMs may only be produced under specific conditions relating to their unique functions in the biology of this fungus; our work suggest that Rcc has the genetic architecture to produce several SMs potentially involved in the infection process. Therefore, characterising the SMs produced by Rcc during disease development would open new opportunities to understand the interaction between the fungus and its environment.

Materials and Methods

In silico identification of *Ramularia collo-cygni* secondary metabolism core genes

The genome of Rcc isolate DK05 Rcc001, collected from the susceptible spring barley cultivar Braemar in Denmark in 2005 was recently sequenced and estimated to be 30.3 Mb with 90x coverage and 11,617 predicted gene models (McGrann et al. 2016). RNAseq data were generated from total RNA extracted from mycelium of DK05 grown *in vitro* on PDA plates for 10-12 days and confirmed the expression of 8,514 of the predicted 11,617 genes under these conditions. A database containing the corresponding 11,617 Rcc protein models was created in GeneiousR9 software (Biomatters Ltd, Auckland, NZ). Protein sequences of PKSs and NRPSs involved in the synthesis of model fungal SMs were downloaded from the NCBI GenBank database ([https://www.ncbi.nlm.nih.gov/genbank/](https://www.ncbi.nlm.nih.gov/genbank/); Supporting Information Table S6). These sequences were used to interrogate the Rcc genome using protein BLAST (BLASTp) (Altschul et al. 1990) to identify putative homologues in Rcc. Significant Rcc matches were retained if the BLASTp search with at least one of the fungal query proteins had an e-value <1.e^-10. The protein sequence of the corresponding Rcc gene models was then used to interrogate InterproScan.
DUSSART, F. (www.ebi.ac.uk) and the PKS/NRPS analysis website (http://nrps.igs.umaryland.edu/) to identify typical PKS or NRPS domains. Results from these analyses were combined and proteins that did not contain at least one of the PKS or NRPS domains eliminated. Genes encoding the selected proteins were positioned on contigs/scaffolds within in the Rcc genome assembly using tBLASTn. For comparison purposes the SM core gene complement of the Rcc genome was also analysed using the programmes AntiSMASH (https://fungismash.secondarymetabolites.org) and SMURF (http://www.jcvi.org/smurf).

Identification of putative secondary metabolism clusters

To identify putative SM clusters in silico genome walking was carried out both up- and downstream along Rcc contigs/scaffolds where each core gene was located. StarOrf (http://star.mit.edu/orf/; Massachusetts Institute of Technology, MA, USA) was used to determine open reading frames (ORFs) along each contig/scaffold with a minimal ORF size of 120 base pairs. The protein sequences of each ORF predicted in the genetic space surrounding each putative core gene were used to interrogate the NCBI non-redundant protein database with BLASTp with an e-value cut-off <1.e^-10. Each protein sequence of the best match fungal BLAST hit was downloaded from GenBank and used to interrogate the Rcc protein models by BLASTp and the annotation of each gene in every cluster was confirmed. Borders of a gene cluster were delineated by a 5 kb region of non-coding DNA or when three consecutive genes with no predicted function associated with SM production were identified. Comparative analysis of predicted Rcc SM clusters with SM clusters in other fungal species was performed with the Antibiotics & Secondary Metabolite Analysis Shell (antiSMASH) website.
Phylogenetic analysis of *Ramularia collo-cygni* polyketide synthase and hybrid polyketide synthase-non-ribosomal peptide synthetase proteins

Protein sequences of PKSs and HPSs involved in the biosynthesis of characterised fungal SMs used in previous phylogenetic studies (Fisch 2013; Collemare et al. 2014) as well as sequences from fungi related to Rcc showing sequence similarity, based on BLASTp, to known SMs core genes were downloaded from the GenBank database (Supporting information Table S7). Full length sequences of PKSs and HPSs were aligned using MAFFT (http://www.ebi.ac.uk), poorly aligned sequences were manually removed in Aliview (Larsson 2014). Phylogenetic trees were built using the RAxML version 8.2.4 (Stamatakis et al. 2005) with 100 rapid bootstrap replicates and the protein model PROTGAMMAAUTO, where model testing is part of the algorithm.

*Ramularia collo-cygni* inoculation of barley seedlings

The RLS susceptible spring barley cv. Century was grown until growth stage 12/13 (Zadoks et al. 1974) in a controlled environment cabinet (Snijders Scientific, Tilburg, Netherlands) at 18°C, with a photoperiod of 16h/8h light/dark supplemented with 250 µmol m⁻² s⁻¹ light and 80% relative humidity. Rcc isolate Rcc001 DK05 ss2 was maintained and inoculum prepared as previously described (Makepeace et al. 2008; Peraldi et al. 2014). Inoculum was applied at a rate of 0.5 mL per seedling as previously reported (Makepeace et al. 2008).
Inoculated plants were placed in a plastic tray (50×40×5 cm), covered with a clear plastic lid to ensure maximum humidity and incubated in the dark for 48h at 18°C. Following incubation the light regime was restored and 72 hours later the clear plastic lids were removed. Development of RLS was assessed at 5, 7, 10, 12, 15, 18 and 21 dpi. Visual assessment was carried out on the prophyll leaf and disease severity was estimated as the percentage of the leaf area covered by RLS lesions. The area under disease progress curve (AUDPC; Shaner and Finney 1977) was calculated to report the development of the disease over time. Green leaf area retention was also visually assessed at each time point as RLS development has been associated with leaf senescence (Schützendübel et al. 2008). Data shows the mean ± standard error calculated on six independent inoculation experiments each of them containing two biological replications of 20 leaves each.

Microscopic assessment of *Ramularia collo-cygni* colonisation

Colonisation by Rcc during RLS development was followed using light microscopy (Leica DMRBE, Leica Microsystems, Wetlzar, Germany). Leaves were harvested at 5, 7, 10, 12, 15, 18 and 21 dpi and cleared in 70% ethanol for seven days prior to staining with 0.05% trypan blue in lacto-glycerol and briefly de-staining with 100% ethanol. For each leaf a minimum of 120 stomata were observed over its full length. Fungal development stages and the associated host response was scored in three categories based on those described previously (McGrann and Brown, 2018) (1) proportion of stomata penetrated by fungal hyphae, (2) proportion of stomata surrounded by necrosis and (3) the proportions of stomata with fungal conidiophore-like
structures emerging. Data show the mean ± standard error calculated on six independent
experiments each of them containing two biological replications of two leaves each.

Ramularia collo-cygni DNA quantification during RLS development

Two leaves were harvested from cv. Century plants at each time point between 5 and 21
dpi, snap frozen in liquid nitrogen and DNA extracted using the DNeasy Plant Mini Kit (QIAGEN
Ltd, Hilden, Germany). Rcc DNA quantification based on an internal transcribed spacer (ITS)
region was carried out using qPCR as described by Taylor et al. (2010). Each reaction volume of
25 µL consisted of 1 × iQ™ supermix (BioRad, Hercules, CA, USA), 400 nmol L⁻¹ forward primer
RamF6 (5’-CGTCATTTCACCACCTCAAG-3’) and reverse primer RamR6 (5’-CCTCTGGAATAGTTGCC-
3’), 5 µL DNA template (5 ng µL⁻¹), 150 nmol L⁻¹ Rcc probe Ram6 (5’-
GCGATTCCGGCTGAGCGGTTCGTCATCGCG-3’), and molecular grade water to the final volume.
Reactions were performed in a Mx3000P thermocycler (Agilent Technologies, Santa Clara, CA,
USA) using the following programme: initial denaturation of 10 min at 95°C followed by 50
cycles of 95°C for 20 s, 55°C for 20 s and 72°C for 20 s, followed by a final extension of 95°C for
1 min. Potential primer-dimer formation and confirmation of single gene-specific product
amplification was monitored at the end of each reaction by melt curve analysis. Data show the
mean quantity (pg) of Rcc DNA calculated from a standard curve produced by a 10-fold dilution
series of Rcc DNA. Rcc DNA levels were assessed in five independent inoculation experiments.

Expression profiling of Ramularia collo-cygni SM-related genes
Transcript levels of putative Rcc SM-related genes were assessed as RLS developed 5-21 dpi with Rcc001 DK05 ss2. For each biological replicate two leaves were harvested and pooled prior to snap freezing in liquid nitrogen. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN Ltd, Hilden, Germany) following the manufacturer’s instructions. Contaminating genomic DNA was removed from RNA samples using TURBO DNase I (Ambion®, Thermo Fisher Scientific, Austin, TX, USA). cDNA was synthesised from 1 µg total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). cDNA was diluted 10-fold in sterile distilled water and used in qRT-PCR using the SybrGreen Jump Start™ Taq system (Sigma, Dorset, UK) and a Mx3000P thermocycler (Agilent Technologies, Santa Clara, CA, USA). The stability of four Rcc reference genes, elongation factor 1 α; glyceraldehyde-3-phosphate dehydrogenase (GAPDH); α-tubulin and actin, was assessed under the conditions of this experiment using GeNorm (Vandesompele et al. 2002). The two most stable reference genes GAPDH and α-tubulin were used for cDNA normalisation. Gene specific primers (Supporting information Table S8) for Rcc genes of interest were designed using Primer3 (http://primer3.ut.ee/). Primers with amplification efficiency ranging from 80% to 110% were used for transcript quantification based on the E^ΔΔCt method (Livak and Schmittgen 2001). Data show the mean normalised expression values ± standard error of three independent experiments each of them containing two biological replicates.

**In vitro fungal growth inhibition assays**

Growth inhibition of commonly occurring barley pathogens *Fusarium poae*, *F. graminearum*, *Pyrenophora teres* and *Rhynchosporium commune* by Rcc isolate Rcc001 DK05 ss2 was assessed.
in dual culture plate assays. The assay was carried out in 9 cm-Petri dishes (Sarstedt, Nümbrecht, Germany), on two different media, alkyl ester agar (AEA) and PDA (Sigma, Dorset, UK) containing 5 µg mL\(^{-1}\) streptomycin. Growth inhibition of \textit{R. commune} was only tested on PDA due to the slow growing nature of both \textit{R. commune} and \textit{Rcc} on AEA. To compensate for the differential \textit{in vitro} growth rate between \textit{Rcc} and \textit{F. poae}, \textit{F. graminearum} and \textit{P. teres}, an 8 mm plug of \textit{Rcc} mycelium was extracted from a 14 day-old culture plate, placed 2 cm from the border of the Petri dish and allowed to grow for 14 days in isolation. Mycelial plugs from competing fungal species were taken from 14 day-old cultures and placed 3 cm away from the \textit{Rcc} culture. Considering the slow growing nature of both \textit{Rcc} and \textit{R. commune}, the mycelial plug of the two fungi were added to the culture plate at the same time and were placed 3 cm apart. All fungal cultures were grown in a Sanyo MIR-254 incubator (Sanyo Electric Ltd, Osaka, Japan) in constant darkness at 15°C. Fungal radial growth was measured after 10 days for \textit{P. teres} and \textit{F. poae}, 7 days for \textit{F. graminearum} and after 21 days for \textit{R. commune}. The assay was repeated at least three times for each fungus, with experimental replicates consisting of at least three individual plates per assay.

Data analysis

All statistical tests were performed in GenStat v.16 (Payne et al. 2009). Data were analysed by analysis of variance (ANOVA) using general linear models (GLM) unless stated otherwise. Each GLM assessed how the various factors that contributed to the design of the different experiments affected variation in the scored phenotype. Disease score data from the \textit{R. collo-cygni} time course inoculation experiment were Logit+ transformed (McGrann et al., 2014) prior
to analysis. GLA retention during RLS development was assessed with a linear mixed model of repeated measures using the uniform correlation/split plot in time covariance matrix (McGrann et al., 2015a). The factors day, experiment, and tray were fixed in the model whether as the leaf x day interaction term was a random factor. The microscopy experiment was analysed using a generalized linear model where each score category was analysed separately as a proportion of the total number of stomata scored on each leaf using a binomial distribution as the link function. Growth inhibition effects of *R. collo-cygni* on each pathogen were assessed separately using a GLM assessing variation attributable to experimental replicates and the direction of fungal growth from the point of inoculation either towards the *R. collo-cygni* culture or towards the edge of the culture plate. Data are presented as growth inhibition calculated as the ratio (\%) of radial fungal growth towards *R. collo-cygni* culture from point of inoculation/ radial fungal growth towards the edge of the culture plate from point of inoculation. Growth on either PDA or AEA was assessed separately. After modelling, treatments were compared within each experiment using t-test probabilities and applying Fisher’s least significant difference to unplanned comparisons.

Acknowledgments

SRUC receives funding from the Scottish Government RESAS. S.H.S. was funded by Royal Society University Research Fellowships (UF090321 and UF140600).

Literature cited


Table 1: *R. collo-cygni* secondary metabolite core polyketide synthase and hybrid polyketide/non-ribosomal peptide synthase gene complement.

<table>
<thead>
<tr>
<th><em>R. collo-cygni</em> gene</th>
<th>Best BLAST hit</th>
<th>Fungal organism</th>
<th>Accession number</th>
<th>e-value</th>
<th>Amino acid identity</th>
<th>Potential product</th>
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<td>Pks1 Polyketide synthase</td>
<td>Umbilicaria pustulata</td>
<td>SLM39055.1</td>
<td>0.0</td>
<td>67%</td>
<td>Rubellin&lt;sup&gt;a&lt;/sup&gt; / Uredinorubellin&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Pks4 Hypothetical protein</td>
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<td>Betaenone&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Pks6 Putative polyketide synthase</td>
<td>Zymoseptoria tritici</td>
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<td>Pks7 Lovastatin diketide synthase (LovF)</td>
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<td>Pks8 Polyketide synthase</td>
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<td>Pks9 Polyketide synthase like</td>
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<td>Pks10 Putative polyketide synthase</td>
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<td>Hps1 Polyketide synthase-non-ribosomal peptide synthetase</td>
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<sup>a</sup> Reported in Miethbauer et al. 2003  
<sup>b</sup> Reported in Miethbauer et al. 2008  
<sup>c</sup> Potential product reported in this study based on gene synteny of the Rcc biosynthetic cluster
Figure legends

Figure 1: Domain organisation of the proteins encoded by \textit{R. collo-cygni} polyketide synthase and hybrid polyketide synthase/non-ribosomal peptide synthetase genes.

Polyketide synthase and hybrid PKS/NRPS domains are labelled from N-terminus to C-terminus: starter unit acyl carrier transacylase (SAT), keto-synthase (KS), acyl transferase (AT), product template (PT), dehydratase (DH), methyl transferase (MT), enoyl reductase (ER), acyl carrier protein (ACP), condensation (C), adenylation (A), peptidyl carrier protein (PCP), thiolesterase (TE), reductase (R). Only PKS/NRPS genes with domain structures found in functional SM core proteins are shown.

Figure 2: Organisation of the genetic loci around \textit{R. collo-cygni} secondary metabolism core genes.

Arrow heads indicate predicted direction of transcription for each open reading frame. The end of a cluster is marked by a double oblique bar and the end of a contig/scaffold is marked by a single vertical bar. Brackets indicate the size of the cluster. Each cluster is drawn to scale. SM core genes with domain structures which would indicate that the resultant protein is non-functional are marked with an asterisk.

Figure 3: Synteny between selected \textit{R. collo-cygni} secondary metabolism clusters and gene clusters in other fungi.

Syntenic relationship between predicted \textit{R. collo-cygni} secondary metabolism clusters and gene clusters from \textit{Phoma betae} (A), \textit{Aspergillus nidulans} (B) and \textit{Penicillium expansum} (C) are shown. Arrow heads indicate ORFs predicted transcription direction. Putative orthologous genes identified by reciprocal BLAST (Supporting Information Table S4) are marked by dotted lines. The end of a cluster is marked by a double oblique bar and the end of a contig/scaffold is marked by a single vertical bar.

Figure 4: Development of Ramularia leaf spot (RLS) in barley cv. Century seedlings.

(A) Representative images of RLS symptoms on barley seedlings over a 21 day time course following inoculation with \textit{R. collo-cygni} DK05 Rcc001 ss2. (B) Decline in green leaf area (GLA) retention (black line) and increase in RLS symptom development (grey bars) measured over the 21 day inoculation time.
course as the area under the disease progress curve (AUDPC). AUDPC is presented as a proportion of the maximum AUDPC possible over the entire inoculation course. Time points that showed significantly different development of RLS symptoms (top row) or differences in GLA retention (bottom row) from each other (P < 0.05) are indicated by different letters. (C) *R. collo-cygni* DNA levels (pg) in barley prophyll leaves measured by qPCR. Fungal DNA levels (pg) were calculated using a standard curve produced from a dilution series of Rcc DNA. Time points that showed significantly different (P < 0.05) amounts of fungal DNA are indicated by different letters.

**Figure 5:** Expression of selected *R. collo-cygni* polyketide synthases and hybrid polyketide synthase/non-ribosomal peptide synthetase genes during Ramularia leaf spot development in barley seedlings.

Data are presented as mean normalised expression values ± standard error over a 21 day time course post inoculation. The expression of Rcc *Catalase* gene is shown as a control.

**Figure 6:** Expression of putative *R. collo-cygni* homologues of characterised fungal secondary metabolism regulators during Ramularia leaf spot development in barley seedlings.

(A) Transcriptional regulators *PacC*, *CreA* and *AreA* (B) Components of the velvet complex *LaeA* and *VeA* (C) Transcription factors *AflR* and *AflJ*. Data are presented as mean normalised expression values ± standard error over a 21 day time course post inoculation.

**Figure 7:** Growth inhibition assays between *R. collo-cygni* and common foliar fungal pathogens of barley.

(A) Growth inhibition of fungal pathogens in the presence of *R. collo-cygni*. Data are presented as the average growth inhibition (%) of each fungal pathogen ± standard error. ***P < 0.001, **P < 0.01, *P < 0.05, (B) Representative images of the dual plate assay between *R. collo-cygni* (placed in top position in each plate) and *F. poae, F. graminearum, P. teres* and *R. commune* (bottom position) after 10, 7, 10 and 21 days, respectively when grown on alkyl ester agar (i) and potato dextrose agar (ii).
Supporting information legends

Table S1: Accession number of the *R. collo-cygni* SM core genes in the NCBI database.

* SM core genes with domain structures which would indicate that the resultant protein is non-functional.

Table S2: Comparison of the number of SM core genes identified in *R. collo-cygni* using BLASTp, SMURF and AntiSMASH.

* Gene complement identified in McGrann et al. 2016.

Table S3: Comparison of the number of secondary metabolism core genes between *R. collo-cygni* and selected Dothideomycetes of the order of the Capnodiales.

* Based on Cairns and Meyer 2017, Chang et al. 2016, Collemare et al. 2014, Ohm et al. 2012, respectively. Data obtained from the JGI website (http://genome.jgi.doe.gov).

Table S4: BLAST hit matches between *R. collo-cygni* genes in SM gene cluster and other fungi.

* Data from BLASTp carried out in the *R. collo-cygni* (taxid:112498) database. Data from BLASTp carried out in the *P. betae* (taxid: 137527), *A. nidulans* (taxid: 162425) and *P. expansum* (taxid: 1208580) database.

Table S5: Top *R. collo-cygni* BLASTp hits to recognised regulators of secondary metabolism.

* Data from BLASTp carried out in the *R. collo-cygni* (taxid:112498) database.

Table S6: Fungal proteins used as query sequences for the identification of core genes in *R. collo-cygni*.

Table S7: Accession numbers for protein sequences of fungal secondary metabolism core genes used for phylogenetic comparison with those of *R. collo-cygni*.

A) Polyketide synthases. B) hybrid polyketide synthase/non-ribosomal peptide synthetases.

Table S8: Primer sequences used for the analysis of *R. collo-cygni* secondary metabolism-related transcription during Ramularia leaf spot development.
Figure S1: Domain organisation of the proteins encoded by *R. collo-cygni* non-ribosomal peptide synthetase genes.

Non-ribosomal peptide synthetase domains are labelled from N-terminus to C-terminus: adenylation (A), peptidyl carrier protein (PCP), condensation (C) and thiolesterase (TE). Only NRPS genes with domain structures found in functional SM core proteins are shown.

Figure S2: Synteny between *R. collo-cygni* secondary metabolism clusters and gene clusters in other fungi.

Syntenic relationship between predicted *R. collo-cygni* secondary metabolism clusters and gene clusters from (A) *Neofusicoccum parvum* and *Macrophomina phaseolina* (B) *Zymoseptoria tritici*, (C) *Scedosporium apiospermum*, (D) *Sphaerulina musiva* and (E) *Podospora anserina* are shown. Arrow heads indicate ORFs predicted transcription direction. Putative homologous genes are marked by dotted lines. Genes with domain structures found in non-functional SM core proteins are marked with an asterisk. The end of a cluster is marked by a double oblique bar and the end of a contig/scaffold is marked by a single vertical bar.

Figure S3: Phylogenetic analysis of *R. collo-cygni* secondary metabolism core genes.

Phylogenetic trees were built with full length protein sequences using maximum likelihood with 100 replications. Branches highlighted in bold show support value of 100%, cut-off value for support shown is 70%. Full names of fungal species and protein accession numbers used in this analysis are given in Table S7. (A) Polyketide synthases (PKS): Highly-reducing PKSs (HR-PKS), partially-reducing PKSs (PR-PKS) and non-reducing PKSs (NR-PKS) are shown. Characterised secondary metabolites (SM) are shown in parentheses. Chemical family for the corresponding SM is also indicated: 1: anthraquinone (tri- and tetra-hydroxyanthraquinone), 2: anthraquinone derivative, 3: pigments (naphtoquinone, naphtopyrone), 4: elsinochrome derivative, 5: naphthalene (tetra- and di-hydroxynaphtalene). (B) Hybrid polyketide synthase/non-ribosomal peptide synthetase: known SMs are shown in parentheses.
Chemical family for the corresponding SM is also described: 1: tryptophan-derived alkaloids, 2: tyrosine-derived pyridones.

**Figure S4**: Microscopic assessment of *R. collo-cygni* colonisation during Ramularia leaf spot development.

(A) Percentage of stomata penetrated by *R. collo-cygni* hyphae. (B) Percentage of stomata with conidiophore-like structures emerging. (C) Percentage of necrotic stomata. (D) Light microscopy images of penetrated stomata (red arrowheads) (i), conidiophore-like structure (red arrowhead) emerging from stomata (ii) and necrotic stomata (red arrowhead) (iii). Bar = 50 µm. Data are presented as mean values ± standard error. Time points that showed a significantly different (P < 0.05) amount of fungal colonisation for each a stage scored are indicated by different letters.

**Figure S5**: Expression selected *R. collo-cygni* non-ribosomal peptide synthetase genes during Ramularia leaf spot development in barley seedlings.

* NRPS genes predicted to be non-functional due to an atypical protein domain structure. Data are presented as mean normalised expression values ± standard error over a 21 day time course post inoculation.
Figure 1: Domain organisation of the proteins encoded by *R. collo-cygni* polyketide synthase and hybrid polyketide synthase/non-ribosomal peptide synthetase genes.

Polyketide synthase and hybrid PKS/NRPS domains are labelled from N-terminus to C-terminus: starter unit acyl carrier transacylase (SAT), keto-synthase (KS), acyl transferase (AT), product template (PT), dehydratase (DH), methyl transferase (MT), enoyl reductase (ER), acyl carrier protein (ACP), condensation (C), adenylation (A), peptidyl carrier protein (PCP), thiolesterase (TE), reductase (R). Only PKS/NRPS genes with domain structures found in functional SM core proteins are shown.

190x142mm (300 x 300 DPI)
Figure 2: Organisation of the genetic loci around *R. collo-cygni* secondary metabolism core genes.

Arrow heads indicate predicted direction of transcription for each open reading frame. The end of a cluster is marked by a double oblique bar and the end of a contig/scaffold is marked by a single vertical bar. Brackets indicate the size of the cluster. Each cluster is drawn to scale. SM core genes with domain structures which would indicate that the resultant protein is non-functional are marked with an asterisk.
Figure 3: Synteny between selected *R. collo-cygni* secondary metabolism clusters and gene clusters in other fungi.

Syntenic relationship between predicted *R. collo-cygni* secondary metabolism clusters and gene clusters from *Phoma betae* (A), *Aspergillus nidulans* (B) and *Penicillium expansum* (C) are shown. Arrow heads indicate ORFs predicted transcription direction. Putative orthologous genes identified by reciprocal BLAST (Supporting Information Table S4) are marked by dotted lines. The end of a cluster is marked by a double oblique bar and the end of a contig/scaffold is marked by a single vertical bar.

167x109mm (300 x 300 DPI)
Figure 4: Development of Ramularia leaf spot (RLS) in barley cv. Century seedlings.

(A) Representative images of RLS symptoms on barley seedlings over a 21 day time course following inoculation with R. collo-cygni DK05 Rcc001 ss2. (B) Decline in green leaf area (GLA) retention (black line) and increase in RLS symptom development (grey bars) measured over the 21 day inoculation time course as the area under the disease progress curve (AUDPC). AUDPC is presented as a proportion of the maximum AUDPC possible over the entire inoculation course. Time points that showed significantly different development of RLS symptoms (top row) or differences in GLA retention (bottom row) from each other (P < 0.05) are indicated by different letters. (C) R. collo-cygni DNA levels (pg) in barley prophyll leaves measured by qPCR. Fungal DNA levels (pg) were calculated using a standard curve produced from a dilution series of Rcc DNA. Time points that showed significantly different (P < 0.05) amounts of fungal DNA are indicated by different letters.
Figure 5: Expression of selected *R. collo-cygni* polyketide synthases and hybrid polyketide synthase/non-ribosomal peptide synthetase genes during Ramularia leaf spot development in barley seedlings.

Data are presented as mean normalised expression values ± standard error over a 21 day time course post inoculation. The expression of Rcc Catalase gene is shown as a control.
Figure 6: Expression of putative *R. collo-cygni* homologues of characterised fungal secondary metabolism regulators during *Ramularia* leaf spot development in barley seedlings.

(A) Transcriptional regulators *PacC*, *CreA* and *AreA* (B) components of the velvet complex *LaeA* and *VeA* (C) Transcription factors *AflR* and *AflJ*. Data are presented as mean normalised expression values ± standard error over a 21 day time course post inoculation.
Figure 7: Growth inhibition assays between *R. collo-cygni* and common foliar fungal pathogens of barley.

(A) Growth inhibition of fungal pathogens in the presence of *R. collo-cygni*. Data are presented as the average growth inhibition (%) of each fungal pathogen ± standard error. *** P <0.001, ** P <0.01, * P <0.05, (B) Representative images of the dual plate assay between *R. collo-cygni* (placed in top position in each plate) and *F. poae, F. graminearum, P. teres* and *R. commune* (bottom position) after 10, 7, 10 and 21 days, respectively when grown on alkyl ester agar (i) and potato dextrose agar (ii).
Figure S1: Domain organisation of the proteins encoded by R. collo-cygni non-ribosomal peptide synthetase genes.

Non-ribosomal peptide synthetase domains are labelled from N-terminus to C-terminus: adenylation (A), peptidyl carrier protein (PCP), condensation (C) and thiolesterase (TE). Only NRPS genes with domain structures found in functional SM core proteins are shown.
Figure S2: Synteny between *R. collo-cygni* secondary metabolism clusters and gene clusters in other fungi.

Syntenic relationship between predicted *R. collo-cygni* secondary metabolism clusters and gene clusters from (A) *Neofusicoccum parvum* and *Macrophomina phaseolina* (B) *Zymoseptoria tritici*, (C) *Scedosporium apiospermum*, (D) *Sphaerulina musiva* and (E) *Podospora anserina* are shown. Arrow heads indicate ORFs predicted transcription direction. Putative homologous genes are marked by dotted lines. Genes with domain structures found in non-functional SM core proteins are marked with an asterisk. The end of a cluster is marked by a double oblique bar and the end of a contig/scaffold is marked by a single vertical bar.

263x250mm (296 x 296 DPI)
Figure S3: Phylogenetic analysis of *R. collo-cygni* secondary metabolism core genes.

Phylogenetic trees were built with full length protein sequences using maximum likelihood with 100 replications. Branches highlighted in bold show support value of 100%, cut-off value for support shown is 70%. Full names of fungal species and protein accession numbers used in this analysis are given in Table S7. (A) Polyketide synthases (PKS): Highly-reducing PKSs (HR-PKS), partially-reducing PKSs (PR-PKS) and non-reducing PKSs (NR-PKS) are shown. Characterised secondary metabolites (SM) are shown in parentheses. Chemical family for the corresponding SM is also indicated: 1: anthraquinone (tri- and tetra-hydroxyanthraquinone), 2: anthraquinone derivative, 3: pigments (napthoquinone, naphtopyrone), 4: elsinochrome derivative, 5: naphthalene (treta- and di-hydroxynaphthalene). (B) Hybrid polyketide synthase/non-ribosomal peptide synthetase: known SMs are shown in parentheses. Chemical family for the corresponding SM is also described: 1: tryptophan-derived alkaloids, 2: tyrosine-derived pyridones.

254x299mm (300 x 300 DPI)
Figure S3: Phylogenetic analysis of *R. collo-cygni* secondary metabolism core genes.

Phylogenetic trees were built with full length protein sequences using maximum likelihood with 100 replications. Branches highlighted in bold show support value of 100%, cut-off value for support shown is 70%. Full names of fungal species and protein accession numbers used in this analysis are given in Table S7.

(A) Polyketide synthases (PKS): Highly-reducing PKSs (HR-PKS), partially-reducing PKSs (PR-PKS) and non-reducing PKSs (NR-PKS) are shown. Characterised secondary metabolites (SM) are shown in parentheses. Chemical family for the corresponding SM is also indicated: 1: anthraquinone (tri- and tetra-hydroxyanthraquinone), 2: anthraquinone derivative, 3: pigments (naphtoquinone, naphtopyrone), 4: elsinochrome derivative, 5: naphthalene (tetra- and di-hydroxynaphthalene).

(B) Hybrid polyketide synthase/non-ribosomal peptide synthetase: known SMs are shown in parentheses. Chemical family for the corresponding SM is also described: 1: tryptophan-derived alkaloids, 2: tyrosine-derived pyridones.
Figure S4: Microscopic assessment of *R. collo-cygni* colonisation during Ramularia leaf spot development.

(A) Percentage of stomata penetrated by *R. collo-cygni* hyphae. (B) Percentage of stomata with conidiophore-like structures emerging. (C) Percentage of necrotic stomata. (D) Light microscopy images of penetrated stomata (red arrowheads) (i), conidiophore-like structure (red arrowhead) emerging from stomata (ii) and necrotic stomata (red arrowhead) (iii). Bar = 50 µm. Data are presented as mean values ± standard error. Time points that showed a significantly different (P < 0.05) amount of fungal colonisation for each a stage scored are indicated by different letters.

160x156mm (300 x 300 DPI)
Figure S5: Expression selected *R. collo-cygni* non-ribosomal peptide synthetase genes during *Ramularia* leaf spot development in barley seedlings.

- NRPS genes predicted to be non-functional due to an atypical protein domain structure. Data are presented as mean normalised expression values ± standard error over a 21 day time course post inoculation.
Table S1: Accession number of the *R. collo-cygni* SM core genes in the NCBI database.

<table>
<thead>
<tr>
<th><em>R. collo-cygni</em> gene</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pks1</td>
<td>XP 023622379.1</td>
</tr>
<tr>
<td>Pks2</td>
<td>XP 023630039.1</td>
</tr>
<tr>
<td>Pks3</td>
<td>XP 023624502.1</td>
</tr>
<tr>
<td>Pks4</td>
<td>XP 023631364.1</td>
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<td>Pks5</td>
<td>XP 023629487.1</td>
</tr>
<tr>
<td>Pks6</td>
<td>XP 023628073.1</td>
</tr>
<tr>
<td>Pks7</td>
<td>XP 023621615.1</td>
</tr>
<tr>
<td>Pks8</td>
<td>XP 023621178.1</td>
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<td>Pks9</td>
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<td>XP 023628544.1</td>
</tr>
<tr>
<td>Pks11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>XP 023624495.1</td>
</tr>
<tr>
<td>Hps1</td>
<td>XP 023625913.1</td>
</tr>
<tr>
<td>Hps2</td>
<td>XP 023628578.1</td>
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<td>Hps3</td>
<td>XP 023624371.1</td>
</tr>
<tr>
<td>Nps1</td>
<td>XP 023622299.1</td>
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<tr>
<td>Nps2</td>
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<tr>
<td>Nps3</td>
<td>XP 023623198.1</td>
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<td>XP 023621041.1</td>
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<tr>
<td>Nps11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>XP 023622531.1</td>
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<tr>
<td>Nps12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>XP 023630609.1</td>
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<tr>
<td>Nps13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>XP 023628081.1</td>
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</table>

<sup>a</sup> SM core genes with domain structures which would indicate that the resultant protein is non-functional
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<tr>
<th></th>
<th>PKS</th>
<th>HPS</th>
<th>NRPS</th>
<th>DMATS</th>
<th>TC</th>
<th>Total</th>
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<td>3</td>
<td>10</td>
<td>0*</td>
<td>4*</td>
<td>27</td>
</tr>
<tr>
<td>SMURF</td>
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<td>1</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>18</td>
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<tr>
<td>AntiSMASH</td>
<td>9</td>
<td>3</td>
<td>10</td>
<td>0</td>
<td>4</td>
<td>26</td>
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</tbody>
</table>

* Gene complement identified in McGrann et al. 2016
Table S3: Comparison of the number of secondary metabolism core genes between *R. collo-cygni* and selected Dothideomycetes of the order of the Capnodiales.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Lifestyle</th>
<th>PKS</th>
<th>HPS</th>
<th>NRPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ramularia collo-cygni</td>
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<td>10</td>
<td>3</td>
<td>10</td>
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<tr>
<td>Zymoseptoria tritici&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Hemibiotroph</td>
<td>10</td>
<td>1</td>
<td>16</td>
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<tr>
<td>Pseudocercospora fijiensis&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Hemibiotroph</td>
<td>7</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Cladosporium fulvum&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Biotroph</td>
<td>8</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Dothistroma septosporum&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Hemibiotroph</td>
<td>5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Sphaerulina musiva&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Hemibiotroph</td>
<td>9</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Septoria populicola&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Hemibiotroph</td>
<td>20</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>Baudoinia compniacensis&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Saprotroph/extremophile</td>
<td>2</td>
<td>0</td>
<td>2</td>
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<sup>a,b,c</sup> Based on Cairns and Meyer 2017, Chang et al. 2016*, Collemare et al. 2014, Ohm et al. 2012, respectively

<sup>d</sup> Data obtained on the jgi website (http://genome.jgi.doe.gov)

*a,* Chang, T.-C., Salvucci, A., Crous, P. W., and Stergiopoulos, I. 2016. Comparative genomics of the Sigatoka disease complex on banana suggests a link between parallel evolutionary changes in *Pseudocercospora fijiensis* and *Pseudocercospora eumusae* and increased virulence on the banana host. PLOS Genet. 12:e1005904
**Table S4: BLAST hit matches between *R. collo-cygni* genes in SM gene cluster and other fungi.**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene</th>
<th>Accession number</th>
<th>e-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Identity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reciprocal blast e-value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Reciprocal blast identity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Rcc gene accession number&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Phoma betae</td>
<td>bet1</td>
<td>BAQ25466.1</td>
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<td>0.0</td>
<td>46%</td>
<td>XP_023629487.1</td>
</tr>
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<td>2e-67</td>
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<tr>
<td>Aspergillus nidulans</td>
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<td>0.0</td>
<td>56%</td>
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<td>-</td>
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<td>-</td>
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<tr>
<td>Penicillium expansum</td>
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<td>9e-109</td>
<td>46%</td>
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<td>65%</td>
<td>3e-107</td>
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<td>CAO91861.1</td>
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<td>XP_023628583.1</td>
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<sup>a</sup>Data from BLASTp carried out in the *R. collo-cygni* (taxid:112498) database.

<sup>b</sup>Data from BLASTp carried out in the *P. betae* (taxid: 137527), *A. nidulans* (taxid: 162425) and *P. expansum* (taxid: 1208580) database.
Table S5: Top *R. collo-cygni* BLASTp hits to recognised regulators of secondary metabolism.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>Protein ID</th>
<th>Corresponding <em>R. collo-cygn</em> gene</th>
<th>e-value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>PacC</td>
<td><em>A. nidulans</em></td>
<td>CAA87390.1</td>
<td>XP_023622497.1</td>
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<td>CreA</td>
<td><em>A. nidulans</em></td>
<td>AAR02858.1</td>
<td>XP_023626414.1</td>
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<tr>
<td>AreA</td>
<td><em>A. nidulans</em></td>
<td>CAA36731.1</td>
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<td>LaeA</td>
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<td>VeA</td>
<td><em>A. nidulans</em></td>
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<td>XP_023625862.1</td>
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<td>AflR</td>
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</tbody>
</table>

* Data from BLASTp carried out in the *R. collo-cygn* (taxid:112498) database.
Table S6: Fungal proteins used as query sequences for the identification of core genes in *R. collo-cygni*.

<table>
<thead>
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<th>Gene name</th>
<th>Accession number</th>
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<th>Organism</th>
<th>Metabolite</th>
<th>Role</th>
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<td>PKS</td>
<td>Dothistroma septosporum</td>
<td>Dothistromin</td>
<td>Phytotoxin (virulence factor)</td>
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<tr>
<td>PksA</td>
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Table S7: Accession numbers for protein sequences of fungal secondary metabolism core genes used for phylogenetic comparison with those of *R. collo-cygni*.

A) Polyketide synthases

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*: protein ID in the jgi database
Table S7: Accession numbers of protein sequences of fungal secondary metabolism core genes used for phylogenetic comparison with those of *R. collo-cygni*.

B) Hybrid polyketide synthase/non-ribosomal peptide synthetases

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