Comparative Genomics of *Cochliobolus* Phytopathogens

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2.1 Introduction

2.1.1 Agricultural Biology of the Genus

Cochliobolus spp. are young, closely related species (<20 MYA, Ohm et al. 2012), which make them ideal for comparative studies (Fig. 2.1, Table 2.1). The genus divides phylogenetically into two groups each associated with a distinct anamorphic stage. The first group, which encompasses the majority of known aggressive pathogenic species with significant impact on host crops, has a *Bipolaris* asexual stage while the second group has a *Curvularia* asexual stage (Sivanesan 1987). To comply with the *International Code of Nomenclature for algae, fungi, and plants* (McNeil et al. 2012), a discussion is underway in the community as to whether the name *Bipolaris/Curvularia* or *Cochliobolus* should be used to align with the "one name one fungus" recommendation. Most contemporary genetic, molecular, and genomic research on virulence determinants and reproductive development of the group has employed the *Cochliobolus* designation. The first group of species includes the necrotrophic corn pathogens, *Cochliobolus heterostrophus* and *Cochliobolus carbonum,* the oat pathogen, *Cochliobolus victoriae,* the rice pathogen, *Cochliobolus miyabeanus*, the sorghum pathogen, *Bipolaris sorghicola*, and the sugarcane pathogen, *Bipolaris sacchari* (Figs. 2.1 and 2.2, Table 2.1). *Cochliobolus lunatus,* also a pathogen of sorghum, falls in the second group (Figs. 2.1 and 2.2). The only species with a known hemibiotrophic lifestyle is the generalized cereal and grass pathogen, *Cochliobolus sativus*, which belongs to the first group. Some of these species, i.e., *C. lunatus*, can act as opportunistic human pathogens.

The best-studied necrotrophic *Cochliobolus* spp. are notorious for their ability to evolve novel, highly virulent races producing host-selective toxins (HSTs) associated with the capacity of their producers to cause diseases on cereal crops that were bred, inadvertently, for susceptibility to the HST-producing pathogen (Yoder 1980; Turgeon and Baker 2007) (Table 2.1, Fig. 2.2). For example, in 1970, race T, a novel race of *C. heterostrophus* (*Bipolaris maydis*), caused a major epidemic of Southern Corn Leaf Blight (SCLB) that destroyed more than 15 % of the maize crop on the US eastern seaboard (Ullstrup 1970). Race T is genetically

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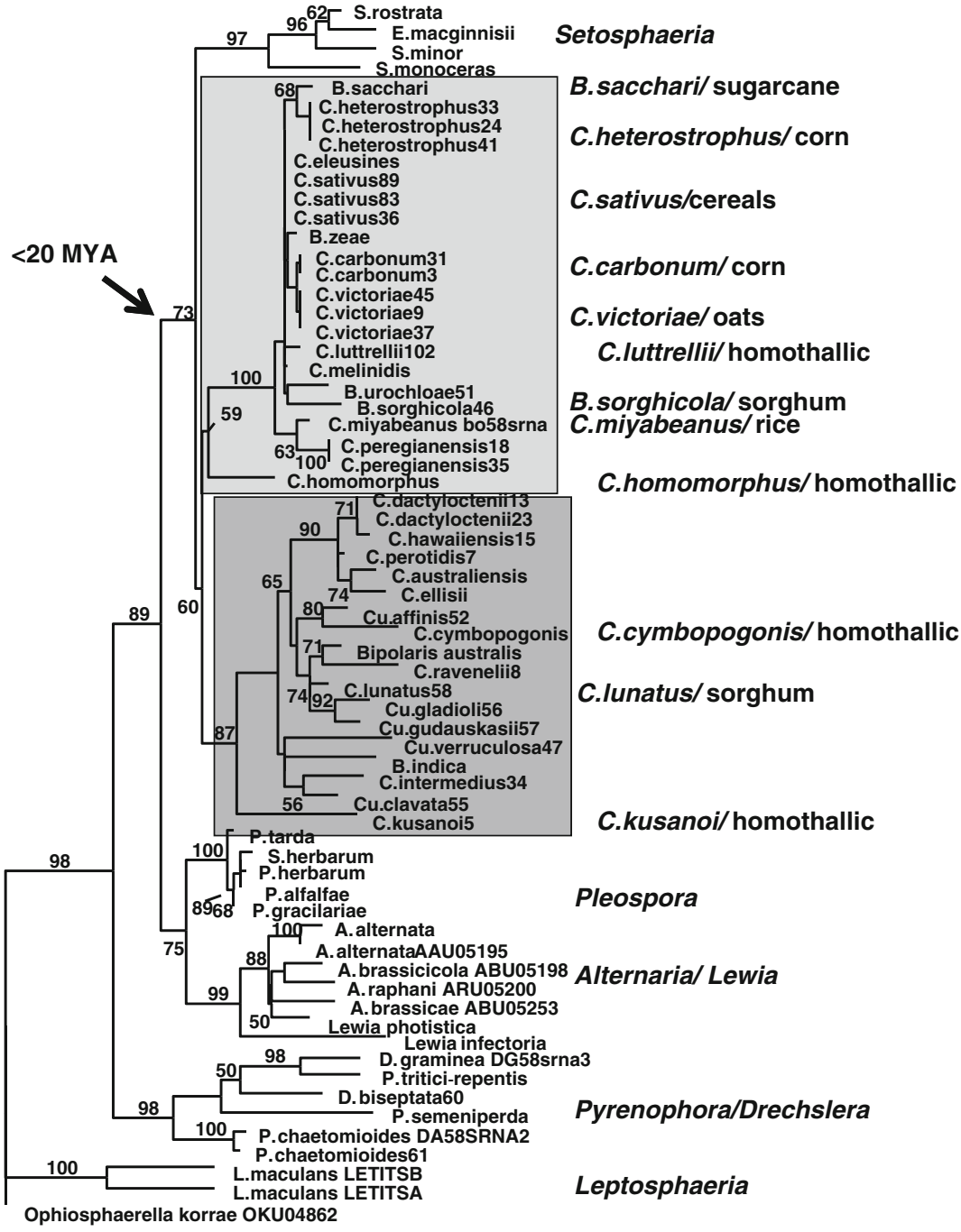


Fig. 2.1 Phylogenetic tree showing distribution of *Cochliobolus* species. *Cochliobolus* species fall into two distinct groups, boxed in two shades of gray. "*C*" *Cochliobolus*, "*B*" *Bipolaris*, "*Cu*" *Curvularia*. Host plants are indicated. All *Cochliobolus* species not designated as homothallic are heterothallic, while those indicated with a "*B*" or "*Cu*" have no known sexual stage. Numbers after species name are isolate designations. Tree constructed by M. Berbee, University of British Columbia, using GPD and ITS sequences. Genera sister to *Cochliobolus* are indicated

Table 2.1 *Cochliobolus*–host interaction biology

Species[a] (strains)	Host/tissue	Disease	HST/ effector?	HST/ effector target	Pathogen lifestyle
Ch race O (C5, Hm540)	Corn/leaves	Southern corn leaf blight	?	–	Necrotroph
Ch race T (C4, Hm338, PR1x412)	Corn with Tcms[b]/ leaves	Southern corn leaf blight	T-toxin	URF13 protein	Necrotroph
Cc race 1 (26-R-13)	*hm1hm1*[c] corn/ leaves	Northern leaf spot	HC-toxin	Histone deacetylase	Necrotroph
Cv (FI3)	*Vb*[d] oats/crown	Victoria blight	Victorin	Thioredoxin	Necrotroph
Cm (WK1C)	Rice/leaves	Brown spot	?	–	Necrotroph
Cs (ND90Pr)	Barley, wheat, cereals/leaves	Spot blotch, common root rot	?	–	Hemibiotroph
Cl (m118)	Sorghum, cereals, humans	Leaf spot, black kernel	?	–	?

[a] *Ch* = *C. heterostrophus, Cc* = *C. carbonum, Cv* = *C. victoriae, Cm* = *C. miyabeanus, Cs* = *C. sativus, Cl* = *C. lunatus*
[b] Tcms = Cytoplasmic male sterility
[c] *hm1hm1* = Homozygous recessive for carbonyl reductase
[d] *Vb* = Presumed to be the same as the *LOV1* (*Pc-2*) gene for resistance to *P. coronata*

distinct from race O, first described in 1925 (Drechsler 1925), in that it uniquely carries genes for biosynthesis of T-toxin, an HST essential for high virulence (Yoder 1980) to Texas male sterile cytoplasm (Tcms) maize (Turgeon and Lu 2000).

Cochliobolus victoriae (*Bipolaris victoriae*), causal agent of Victoria Blight of oats, produces the chlorinated cyclic pentapeptide HST, victorin, rendering it highly virulent to oats carrying the dominant *Vb* allele (Fig. 2.2, Table 2.1) (Litzenberger 1949). The *Vb*-associated trait, susceptibility to *C. victoriae,* and a *Pc-2*-associated trait, resistance to *Puccinia coronata,* cannot be separated genetically (Lorang et al. 2012). Recent work with Arabidopsis revealed an NB-LRR-type resistance protein (LOV1), guarding a thioredoxin protein target (TRX-h5), that when activated confers susceptibility to *C. victoriae* and victorin (Lorang et al. 2004, 2007). Victorin thus acts by co-opting effector triggered defenses against the biotroph, *P. coronata*, to promote susceptibility to a necrotroph.

In contrast to the dominant plant host genes required for susceptibility to *C. heterostrophus* and *C. victoriae,* susceptibility to Northern Corn Leaf Spot caused by *C. carbonum* (*Bipolaris zeicola*) is conferred by a homozygous recessive maize gene(s) (Johal and Briggs 1992; Multani et al. 1998). *C. carbonum* race 1 produces the cyclic-tetrapeptide HST, HC-toxin, which is specifically active, as is the fungus itself, against corn with the naturally occurring or mutant genotype *hmhm* (Fig. 2.2, Table 2.1) (Yoder 1980; Walton 1987, 1996). The site of action of HC-toxin in susceptible corn is histone deacetylase; it is hypothesized that HC-toxin acts to promote infection of maize of genotype *hm1hm1* by inhibiting this enzyme, resulting in the accumulation of hyperacetylated core histones. This then alters expression of genes encoding regulatory proteins involved in plant defense (Ransom and Walton 1997; Walton 2006). *C. carbonum* races 2 and 3 do not produce the toxin.

Cochliobolus sativus (*Bipolaris sorokiniana*), a hemibiotroph and less specialized cereal pathogen, causes diseases of roots (common root rot), leaves (spot blotch), and spikes (black point or kernel blight) of cereals (mainly barley and wheat) (Fig. 2.2, Table 2.1) (Mathre 1997; Weise 1987). Three *C. sativus* pathotypes (0, 1, and 2) have been described (Valjavec-Gratian and Steffenson 1997) based on differential virulence

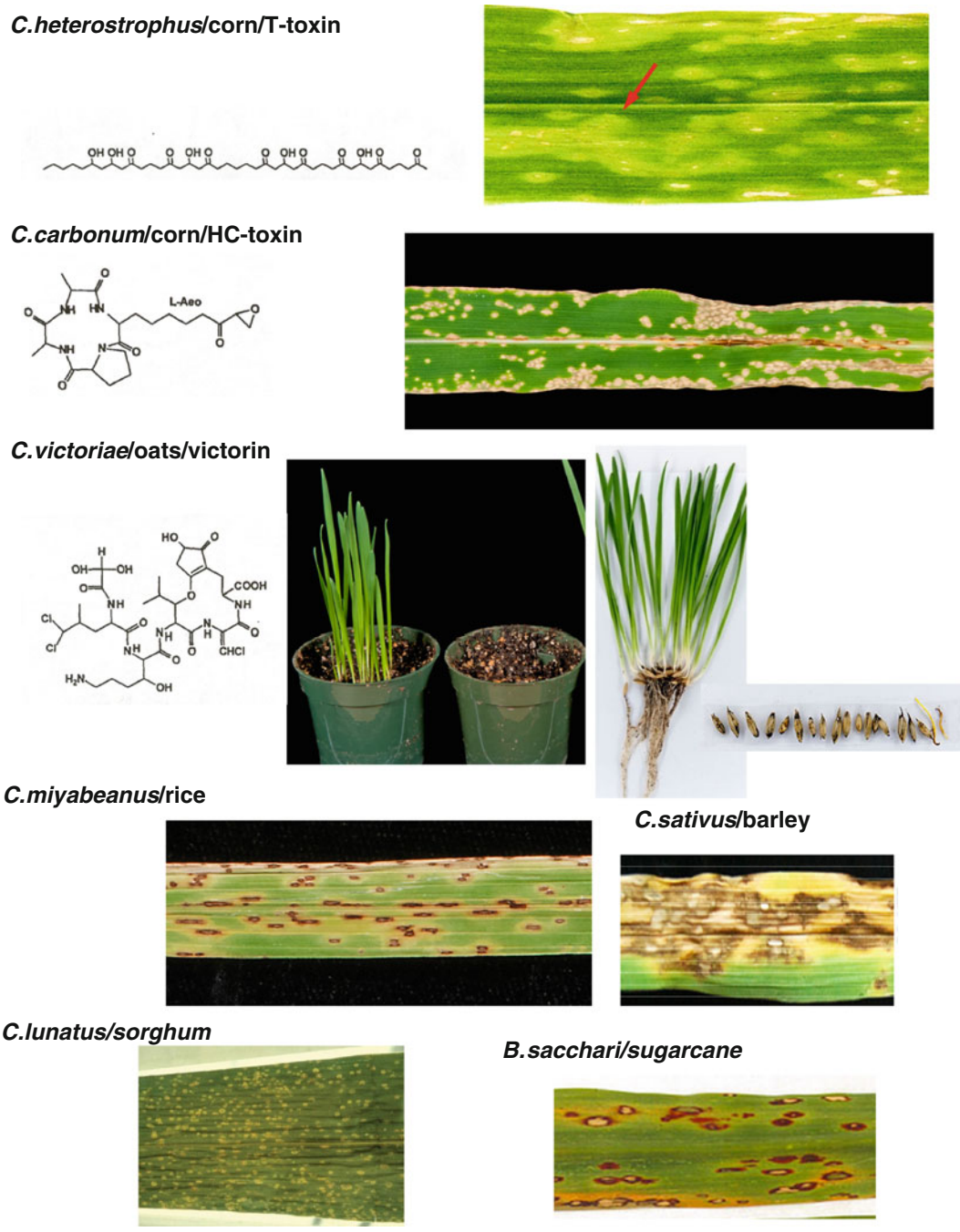


Fig. 2.2 *Cochliobolus* species and their disease phenotypes. All species, except *C. victoriae,* cause lesions on host leaves and in some cases on other plant tissues. *C. heterostrophus* race T produces T-toxin (*arrow*) which increases virulence on Tcms corn; *C. carbonum* race 1 and *C. victoriae* produce the HSTs HC-toxin and victorin, respectively, which are required for pathogenicity on the host. Pots contain resistant (*left*) and susceptible (*right*) oat seeds inoculated with a slurry of *C. victoriae—Note* none of the susceptible oat seeds germinated (*extreme right*). *Image* of *C. lunatus* from maizedoctor.cimmyt.org/index.php?option=com_content&t

Table 2.2 *Cochliobolus* spp. mating type characteristics

Isolate	Mating type	Lifestyle	Comments
Ch C5	*MAT1-1*	Heterothallic	Inbred line
Ch C4	*MAT1-2*	Heterothallic	Inbred line
Ch Hm540	*MAT1-1*	Heterothallic	Field isolate
Ch Hm338	*MAT1-2*	Heterothallic	Field isolate
Ch PR1x412	*MAT1-1*	Heterothallic	Progeny of cross between strain PR1 and strain 412
Cv FI3	*MAT1-2*	Heterothallic	All known isolates are *MAT1-2* and female sterile (Christiansen et al. 1998)
Cc 26-R-13	*MAT1-1*	Heterothallic	(Christiansen et al. 1998)
Cs ND90Pr	*MAT1-2*	Heterothallic	Pathotype 2
Cm WK1C	*MAT1-2*	Heterothallic	(Arie et al. 1997)
Cl m118	*MAT1-2*	Heterothallic	
C. ellisii	*MAT1-2*	Heterothallic	(Yun et al. 1999)
C. luttrellii	*MAT1-1*:*MAT1-2*	Homothallic	−115 aa 3′*MAT1-1*, −49 aa 5′ *MAT1-2* (Yun et al. 1999)
C. homomorphus	*MAT1-2*:*MAT1-1*	Homothallic	−9 aa 3′*MAT1-2*, −7 aa 5′ *MAT1-1* (Yun et al. 1999)
C. kusanoi	*MAT1-1*:*MAT1-2*	Homothallic	(Yun et al. 1999)
C. cymbopogonis	*MAT1-1*, *MAT1-2*	Homothallic	*MAT1-1* and *MAT1-2* are unlinked (Yun et al. 1999)

patterns on three barley genotypes (ND5883, Bowman, and NDB112). Pathotype 0 isolates show low virulence on all three barley genotypes. Pathotype 1 isolates show high virulence on ND5883 but low virulence on other barley genotypes. Pathotype 2 isolates show high virulence on Bowman but low virulence on ND5883 and NDB112. Genetic analysis and molecular mapping indicates that a single locus, *VHv1*, controls high virulence of the pathotype 2 isolate ND90Pr on Bowman (Valjavec Gratian and Steffenson 1997; Zhong et al. 2002). The *VHv1* locus is unique to pathotype 2 and encodes two nonribosomal peptide synthetases (NRPSs), one of which when deleted, drastically reduces virulence of pathotype 2 on cultivar Bowman (Condon et al. 2013).

Cochliobolus miyabeanus (*Bipolaris oryzae*) is the causal agent of brown spot of rice which contributed, along with a cyclone and tidal waves, to the Bengal rice famine of 1942/1943 that resulted in starvation of more than two million people (Dasgupta 1984) (Fig. 2.2, Table 2.1). The interaction between rice and *C. miyabeanus* is inadequately understood from the perspective of genetic and molecular mechanisms and no HST has been correlated with the ability of *C. miyabeanus* to cause disease.

Cochliobolus lunatus (*Curvularia lunata*) is a pathogen of sorghum (Fig. 2.2, Table 2.1) (Thakur et al. 2006) and is also known to be an opportunistic human pathogen (Thakur et al. 2006; Manamgoda et al. 2011, 2012). The sequenced strain (m118, MUCL 38696) was selected originally as a pilot organism for steroid biotransformation (Vitas et al. 1994, 1995) in the laboratories of Friedrich Schiller University, Jena, Germany. This, and another strain, *C. lunata* AT46, have been utilized widely for steroid transformation (Rozman et al. 1996).

2.1.2 Reproductive Biology

Sexual *Cochliobolus* species can be self-sterile (heterothallic, requiring genetically distinct partners) or self-fertile (homothallic, no partner required) (Fig. 2.1, Table 2.2). As in most ascomycetes, a single mating type locus (*MAT*) controls the ability to reproduce sexually and in

Cochliobolus, all heterothallic species have either *MAT1-1* or *MAT1-2* (but never both) in different individuals whereas all homothallic species carry both *MAT1-1* and *MAT1-2* in the same nucleus of an individual (Turgeon et al. 1993). Asexual species (i.e., those with no known sexual cycle), such as *B. sacchari,* also are found in the group. It is well documented that asexual species also carry *MAT* genes (Sharon et al. 1996). Thus, *Cochliobolus* spp. are an excellent choice for comparisons of reproductive mechanisms in asexual, heterothallic and homothallic species within a closely related group of species in the same genus (Fig. 2.3) (Turgeon and Debuchy 2007; Debuchy and Turgeon 2006; Yun et al. 1999).

The coexistence of heterothallic and homothallic species in the same genus is common to many classes of ascomycete and coexistence of both mating type genes in the same nucleus is common to most homothallic species, including *Cochliobolus*. For *Cochliobolus* spp., a self-sterile to self-fertile evolution is well supported since all homothallic *Cochliobolus* spp. are polyphyletic, their *MAT* genes are diverse in structure and arose independently, while all heterothallic *MAT* genes are conserved in structure; plus, molecular evidence exists for recombination mechanisms (Yun et al. 1999; Inderbitzin et al. 2005). Functional analyses (Yun et al. 1999; Lu et al. 2011) indicate that *MAT* genes can be transferred from heterothallic to homothallic species or vice versa and retain function, although there are yet to be understood nuances associated with fertility. Where causes of asexuality have been studied functionally, asexual species such as *B. sacchari* have been found to be asexual for reasons not associated with the *MAT* genes themselves; their *MAT* genes are fully functional in *mat* null strains of *C. heterostrophus* (Sharon et al. 1996).

C. carbonum and *C. victoriae* are capable of crossing to each other (Scheffer et al. 1967; Christiansen et al. 1998). We have hypothesized that *C. victoriae* may have evolved from a *MAT1-2 C. carbonum* strain (Christiansen et al. 1998). This is supported by our finding that all extant strains of *C. victoriae* are *MAT1-2* and female sterile. As noted below, *C. victoriae* and *C. carbonum* share an intermediate number of SNPs at the whole-genome level compared to *C. heterostrophus* inter- and intra-species comparisons, in support of this close relationship. Table 2.2 is a summary of mating attributes for the *Cochliobolus* species with sequenced mating type loci.

2.1.3 Genetic Tools

Cochliobolus spp. are easily grown in culture, produce abundant asexual spores (except *C. lunatus*), and can be stored for long periods of time in glycerol or silica gels (Yoder 1988). They also have an efficient sexual stage readily produced in the laboratory in 3 weeks (Fig. 2.3) (Leach et al. 1982), and are easily transformed (Turgeon et al. 2010). Targeted gene deletion using PCR fragments is highly efficient (Turgeon et al. 2010; Wirsel et al. 1996; Catlett et al. 2003a). Chromosomes can be resolved using pulsed-field gel electrophoresis (Kodama et al. 1999; Tzeng et al. 1992).

In this review, we compare genome similarities and differences among sequenced *Cochliobolus* pathogens, with particular emphasis on strain and species-unique sequences, virulence determinants (secondary metabolites, iron and oxidative stress), mechanisms of reproduction, and signaling.

2.2 Genome Structure

2.2.1 Genome Sequence Comparisons

Five strains of *C. heterostrophus* and one strain each of *C. victoriae, C. carbonum*, *C. miyabeanus, C. sativus,* and *C. lunatus* were sequenced by the Joint Genome Institute (JGI). Two *C. heterostrophus* strains and the *C. sativus* strain were fully sequenced, while the remaining genomes were sequenced using Illumina and assembled de novo using Velvet or AllPathsLG (http://genome.jgi.doe.gov/programs/fungi/index.jsf). The highly

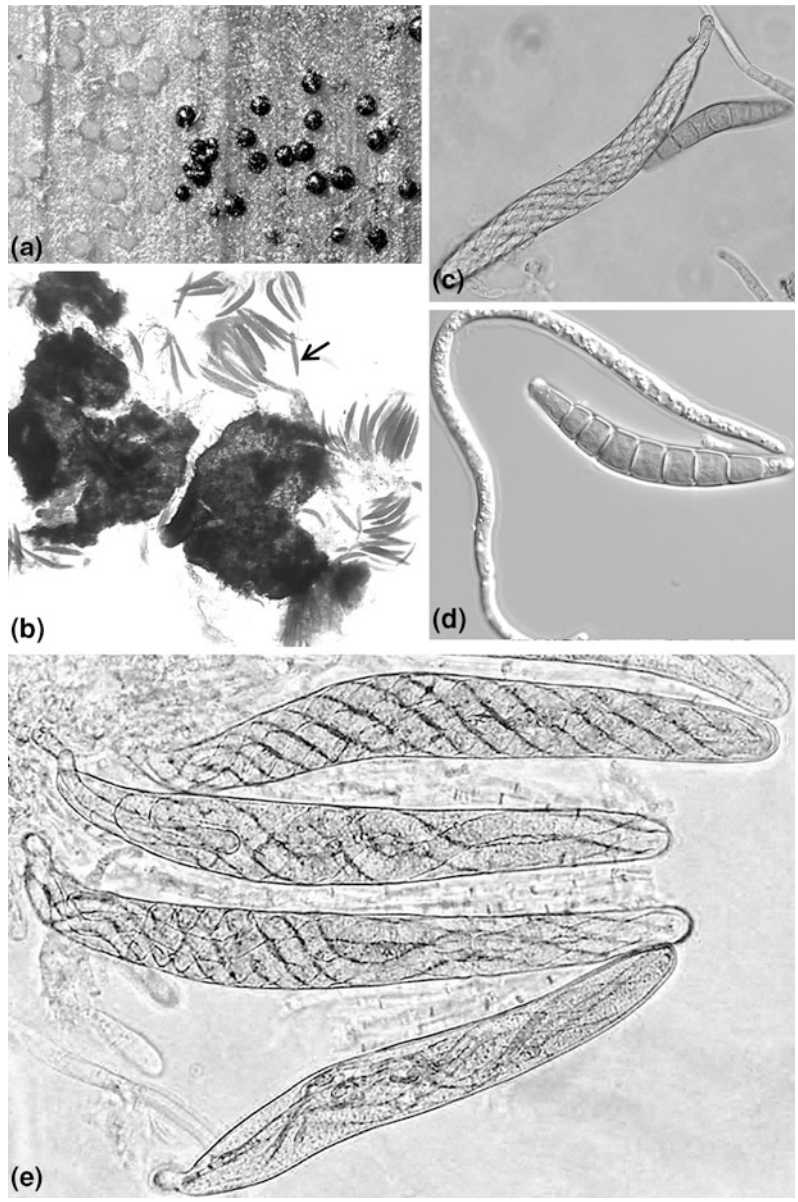


Fig. 2.3 Reproductive stages of *C. heterostrophus*. **a** Portion of a mating plate containing a senescent corn leaf inoculated with a pigmented *MAT1-1* strain and an albino *MAT1-2* strain. Both *black* and *white* pseudothecia are formed indicating both strains are hermaphroditic. **b** Two pseudothecia that have been squeezed to release asci (*arrow*). **c** A single ascus containing ascospores (*tetrad*) and a single conidium. **d** A single ascospore and a single conidium. **e** Several asci containing tetrads with varying numbers of ascospores

inbred *C. heterostrophus* race O lab strain C5 was used as the reference sequence for all comparisons, as it is the most complete, consisting of only 68 scaffolds. Three additional *C. sativus* strains have been sequenced recently, but are not discussed here (Zhong unpublished).

Overall sequence assembly and annotation statistics are presented in Table 2.3. All *Cochliobolus* genomes are in the 31–37 Mb range with an estimated gene content of 12,000–13,300. Gene content and genome organization are highly similar within this group of fungi, although less so for *C. lunatus*. In contrast, comparative analysis of *C. heterostrophus* and *C. sativus* in the context of 15 more distantly related Dothideomycetes genomes (Ohm et al. 2012) revealed significant variation.

The relative scale of conservation at the nucleotide level, compared to *C. heterostrophus* C5, was used as an estimation of similarity.

Table 2.3 Genome statistics

Species (strain)[a]	Genome characteristics							
	Assembly size (Mb)	Scaffold #	Scaffold N50/L50 (Mb)	# Predicted genes	# NPS genes	# PKS genes	# P450 genes	# SSP genes
Ch (C5)	36.46	68	7/1.84	13,336	14	23	156	180
Ch (C4)	32.93	207	13/0.96	12,720	14	25	149	171
Cv (F13)	32.83	676	47/0.23	12,894	18	21	138	160
Cc (26-R-13)	31.27	844	82/0.11	12,857	20	27	143	153
Cm (WK1C)	31.36	619	68/0.13	12,007	11	21	124	143
Cs (ND90Pr)	34.42	157	7/1.79	12,250	25	18	127	289
Cl (m118)	31.17	171	10/1.53	12,131	9	15	106	230

[a] For species designations, *see* footnotes, Table 2.1

Most of the *C. heterostrophus* race T strain C4 assembly could be aligned and only 1,584 SNPs were found between the two strains (Fig. 2.4). This remarkable level of conservation is molecular verification of the highly inbred nature of the two strains, achieved through generations of successive backcrossing. Note, since race O strain C5 is the reference to which all sequences are aligned, alignment of C4–C5 excludes the 1.2 Mb of *Tox1* DNA that is unique to race T. In contrast to results with the inbred strains, comparison of each *C. heterostrophus* field strain to C5 revealed roughly 10 times more SNPs than the same comparison with C4 and comparison of each *Cochliobolus* species revealed roughly 10 times more SNPs than did any *C. heterostrophus* field strain. Thus, there is a clear diminishing gradation of similarity at the whole-genome level as comparisons move from inbred strains to field strains within a species, to across species. As expected, based on phylogenetic distance (Fig. 2.1), *C. lunatus* appears to be the most diverged species, as only 20 % of its genome could be aligned to reference C5, compared to ~75 % for other *Cochliobolus* species (Fig. 2.4).

Most significantly, at the species level, a total of 11.76 Mb present in all *C. heterostrophus* genomes was missing from *C. victoriae, C. carbonum, C. sativus,* and *C. miyabeanus* (*C. lunatus* was excluded from this analysis). Only 1.6 Mb of this was in segments larger than 5 kb in the alignment to C5. Most of the sequence that separates *C. heterostrophus* from other species, therefore, is not the result of large wholesale insertions or deletions of DNA, but from a more piecewise gain and loss. We and others (Hane et al. 2011; Goodwin et al. 2011; Rouxel et al. 2011) have recently coined the term mesosynteny (Ohm et al. 2012) to describe organizational conservation between species. Genetic content is conserved across chromosomes, but not colinearly. It seems possible that our findings with *Cochliobolus*, showing that many small, scattered differences sum to significant quantitative differences (i.e., 25 % dissimilar), could be the product of the same mechanisms.

Pathogens of the same host (e.g., *C. carbonum* and *C. heterostrophus* on maize) were not more similar to each other than those with different hosts. Instead, overarching genetic patterns followed phylogenetic lines. A telling example of this is our finding that *C. carbonum* and *C. victoriae* have fewer SNPs between them than revealed in comparisons between other pairs of *Cochliobolus* species. These comparisons support our previously reported hypothesis that *C. victoriae* arose from a *MAT1-2* strain of a non-HC-toxin-producing strain of *C. carbonum* and is expected therefore to be more closely related to it than to other species (Christiansen et al. 1998). Given that the Pleosporaceae arose as a group less than 23–17 MYA (*see* Fig. 2.1 in Ohm et al.

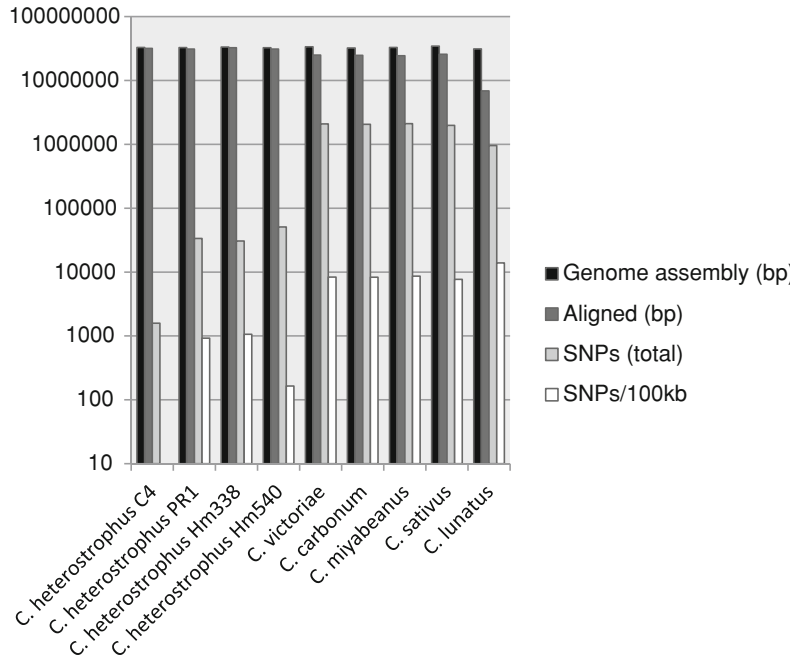


Fig. 2.4 Relative conservation of *Cochliobolus* species and *C. heterostrophus* strains to *C. heterostrophus* C5 reference. Each genome in this study was aligned, pairwise, to the *C. heterostrophus* C5 assembly using the MUMmer DNAdiff tool (Kurtz et al. 2004), and data were plotted logarithmically. The majority of each *Cochliobolus* species genome could be aligned (*dark gray bars*) to *C. heterostrophus* C5, except for *C. lunatus.* SNPs called between aligned regions (*light gray bars*) demonstrate that the inbred *C. heterostrophus* C5 and C4 strains are highly similar and *C. heterostrophus* field strains are more similar to *C. heterostrophus* strain C5 than to any other *Cochliobolus* species. SNPs/100 kb of aligned sequence (*white bars*) support this trend and show *C. lunatus* is the most dissimilar to *C. heterostrophus* of the *Cochliobolus* species, which fits with phylogenetic placement (Fig. 2.1). Data are displayed relative to the total query assembly size (*black bar*)

(2012)) and the genus *Cochliobolus* is young in the Pleosporaceae group, genome comparisons provide us with an overall picture of a timeline of how genome diversity varies with speciation.

Less than 1 year after the comprehensive analyses of 18 genomes in Ohm et al. (2012) and Condon et al. (2013) were published, the number of sequenced Dothideomycete genomes has doubled in the JGI Mycocosm (http://genome.jgi.doe.gov/programs/fungi/index.jsf). As more genera are sequenced to the same depth as *Cochliobolus*, the close similarity seen among *Cochliobolus* species can be compared to relationships among suites of species taxa within other genera. Attempting to align separate *Aspergillus* species, using our methodologies, for example, would yield poor alignments, as they are much more distantly related to each other. Indeed—studies that identify syntenic genomic regions between *Aspergillus* species do so with a much lower threshold for similarity and conservation (Fedorova et al. 2008).

2.2.2 Insights from Genome Comparisons

2.2.2.1 Secondary Metabolism

Armed with the knowledge that most of the best known aggressive *Cochliobolus* pathogens are necrotrophs and that high virulence/pathogenicity of the most devastating of these is associated with secondary metabolite production in the form of HSTs biosynthesized by NRPS and PKSs, we extracted all NRPS and PKS encoding genes from all 6 species (10 strains). Number of *NPS*s per genome, ranged

from 9–25, while number of *PKS*s ranged from 15–27 (Table 2.3). Comparative analyses revealed that the suites of these genes are astoundingly diverse among species but remarkably conserved among isolates of the same species, whether inbred or field strains, except for defining examples that generally map to unique genomic regions. Functional analysis of several of these strain-unique *PKSs* and *NPSs* reveals a strong correlation with a role in virulence as hinted at decades earlier with, e.g., the *PKS* genes for T-toxin production in *C. heterostrophus* race T and the genes for HC-toxin production by race 1 of *C. carbonum,* which are not found in any other *Cochliobolus* species.

Comparing the inventories of secondary metabolism genes across several closely related species yields key insights (Figs. 2.5 and 2.6). The first insight is that broadly conserved NRPSs or PKSs are most likely to produce metabolites of biological function central to the fungal cell itself. *NPS2*, *NPS6*, *NPS4, NPS10,* and *PKS18* are *C. heterostrophus NPS* and *PKS* genes conserved across all *Cochliobolus* species (Figs. 2.5, 2.6 and 2.7). Functional studies of *C. heterostrophus* mutants deleted for these genes demonstrate that the metabolites produced by the conserved biosynthetic enzymes affect developmental processes such as sexual and asexual development, morphology, hydrophobicity of colony surfaces, as well as stress (oxidative, iron, etc.) management (Figs. 2.6 and 2.7). Properly defining the scope of inclusion for this inference is essential—across the 18 Dothideomycetes examined in Ohm et al. (2012), only *NPS10* is conserved in all, despite the importance of these metabolites in *Cochliobolus* species. This finding is in agreement with the earlier hypotheses (Bushley and Turgeon 2010) that *NPS10* is among the more ancestral NRPSs. The product of NPS10 is not known, however, *C. heterostrophus* mutants are sensitive to oxidative stress. *C. heterostrophus* NPS2 is responsible for siderophore biosynthesis and intracellular iron storage and is conserved in 17 out of the 18 Dothideomycetes examined in Ohm et al. (2012, Table S19) (Fig. 2.6). NPS6 is present in 11 of the 18 genomes and is responsible for extracellular siderophore biosynthesis and thus competition for iron in the plant–fungal interaction (Fig. 2.6). NPS6 has been shown to be involved in virulence of *C. heterostrophus* to corn, of *C. miyabeanus* to rice, of *A. brassicicola* to *Arabidopsis thaliana,* and of *Fusarium graminearum* to wheat. It is also required for in vitro oxidative stress management (Oide et al. 2006). NPS4 makes an unknown product, but is present in 10 of the 18 genomes. *C. heterostrophus*, *A. brassicicola*, and *F. graminearum nps4* mutant colony surfaces are hydrophilic, rather than hydrophobic like wild type (Fig. 2.7) (Turgeon et al. 2008). PKS18, responsible for melanin biosynthesis, is conserved in all *Cochliobolus* species and was reported as conserved in 17 of 18 genomes in the study of Ohm et al. (2012). We have subsequently observed that *A. brassicicola,* the species missing PKS18, does in fact posses the gene (Fig. 2.6).

The second key insight is that genes encoded by genes "unique" to a particular species or strain of a species, encode enzymes that are likely biosynthesizing secondary metabolites involved in virulence (Fig. 2.8). A canonical example is the identification of a group of *C. sativus* pathotype 2-specific AMP domains (Fig. 2.5), one of which (ID 115356) when deleted, drastically reduces virulence on cultivar Bowman (Fig. 2.8). Another example is the *C. heterostrophus* race T- specific *PKS1* and *PKS2* genes (Fig. 2.6). These two polyketide synthases are responsible for production of T-toxin in race T and high virulence to Tcms maize and have long been described as unique to race T based on DNA–DNA hybridization blots. Phylogenetic analyses of PKS KS domains confirmed that they are not found in any other *Cochliobolus* species (Fig. 2.6).

A third example is the genes encoding *C. carbonum* HC-toxin (Fig. 2.5). *C. carbonum* race 1 is the only *Cochliobolus* species to possess HTS1, the NRPS (4 AMP domains) responsible for producing HC-toxin (Fig. 2.8). Wider genome resources, however, uncover candidate orthologs for all 4 AMP domains plus other genes associated with biosynthesis of HC-toxin, in

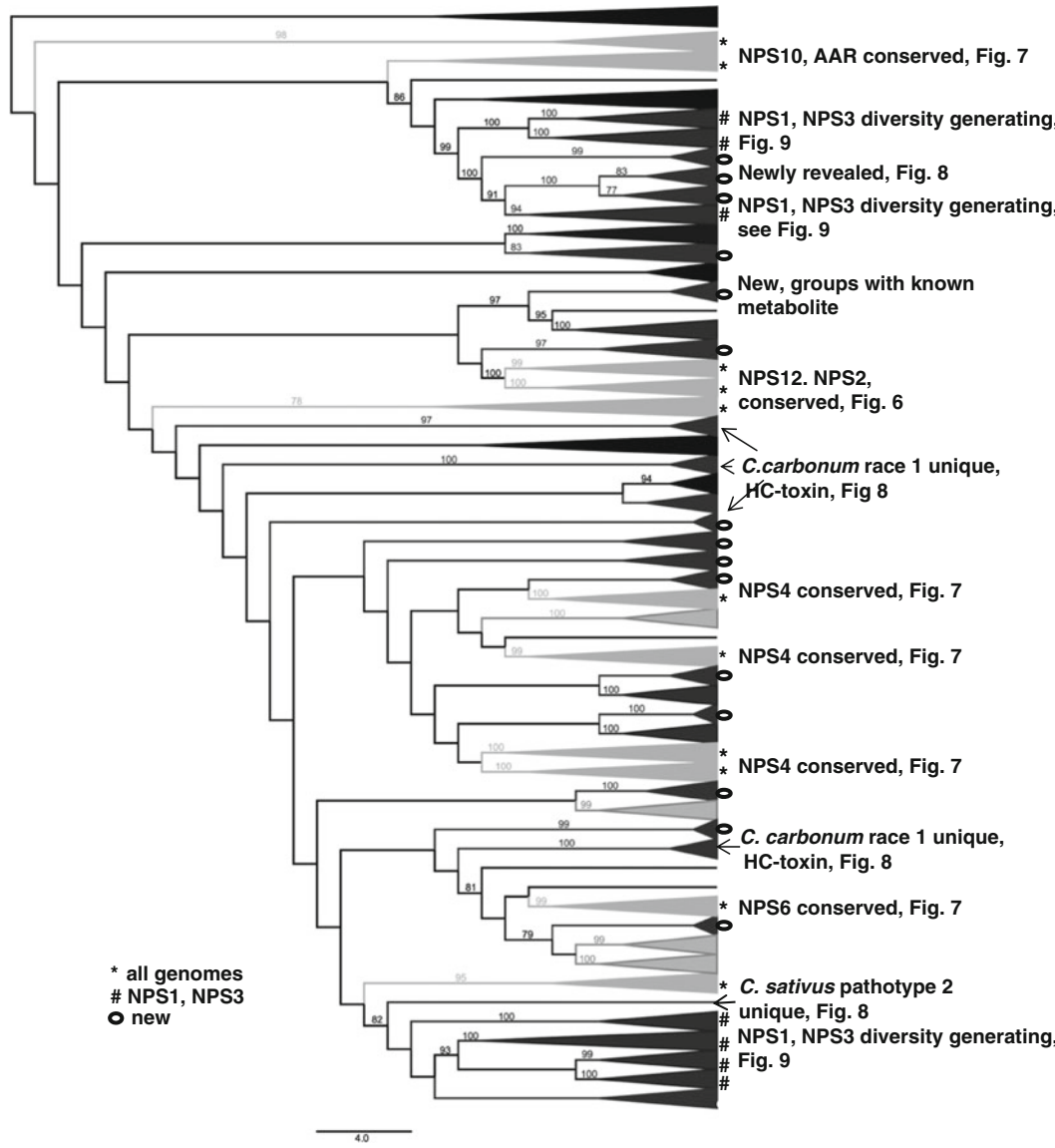


Fig. 2.5 Cartoon of cross-species phylogenomic analyses of individual AMP binding domains from NRPS proteins. NRPS AMP domains were extracted from all five *C. heterostrophus* and from the *C. victoriae, C. carbonum, C. miyabeanus*, *C. sativus,*, and *Setosphaeria turcica* genomes. Members of the reference set of previously annotated *C. heterostrophus* NRPS AMP domains (Lee et al. 2005; Bushley and Turgeon 2010) were used as benchmarks for branches. Branches of the full phylogenetic tree are collapsed according to clustering with the reference set of *C. heterostrophus* AMP domains. Presence in each of the five *C. heterostrophus* strains, *Cochliobolus* species, and *S. turcica* is noted by "*", AMP domains not grouping with the previously annotated *C. heterostrophus* set are labeled as "newly revealed" or "new, groups with known metabolite." NPS1, NPS3 AMPs are labeled as diversity generating (Fig. 2.9). *C. carbonum* HTS1 AMPs are indicated, as is the *C. sativus* pathotype 2 NRPS discussed in text (Fig. 2.8)

Setosphaeria turcica, Alternaria jesenkae, and *Pyrenophora tritici-repentis* and *Fusarium semitectum* (Manning et al. 2013; Condon et al. 2013). The metabolites produced by the first three of these *HTS1* orthologous clusters have not been identified and they may not be HC-toxin

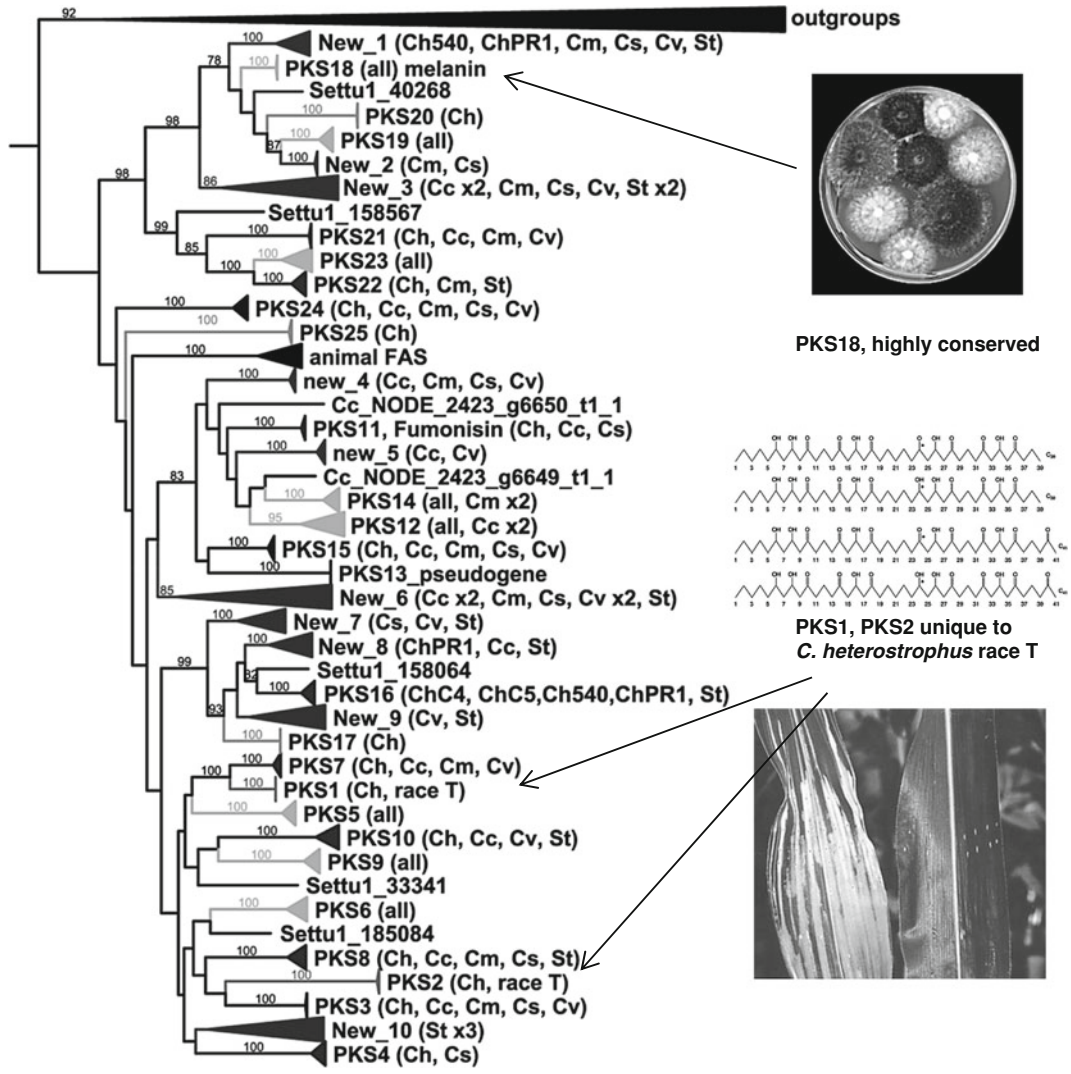
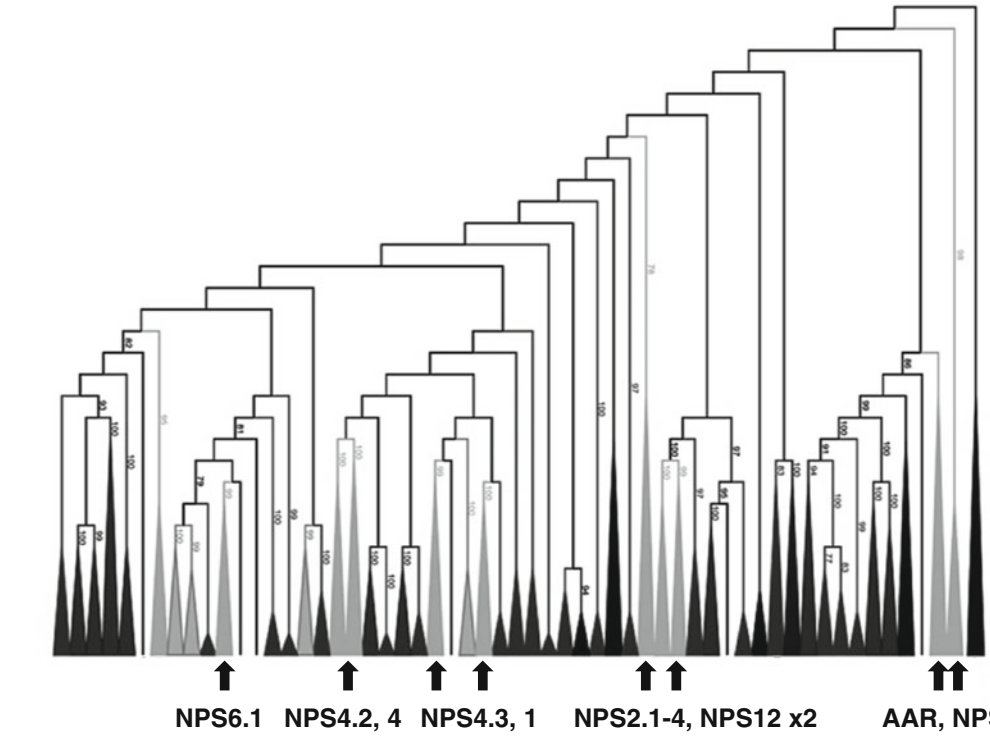


Fig. 2.6 Cartoon of cross-species phylogenomic analyses of individual ketosynthase (KS) domains from PKS proteins. The KS domains were extracted from all five *C. heterostrophus* and from the *C. victoriae, C. carbonum, C. miyabeanus*, *C. sativus* and *S. turcica* genomes. PKS designations match the *C. heterostrophus* set. KS domains not grouping with the previously annotated *C. heterostrophus* set are labeled as "New_1 through _10." Highly conserved PKS18, encoding the PKS for melanin biosynthesis and the unique PKSs (PKS1, PKS2) for *C. heterostrophus* race T T-toxin production are indicated

($C_{22}H_{34}N_4O_6$). *Fusarium semitectum,* for example, has the HTS1 ortholog, APS1, however this is the core NRPS for biosynthesis of apicidin ($C_{34}H_{49}N_5O_6$), a structurally different metabolite with the same biological activity as HC-toxin (both are histone deacetylase inhibitors) (Jin et al. 2010). Whether or not the other species produce HC-toxin, the discovery of these *HTS1* orthologs furthers our understanding of evolution of genes associated with HSTs in the fungal–plant interaction. Like apicidin and HC-toxin, orthologs may have profound medicinal application (Jin et al. 2010; Han et al. 2000). Thus, HST genes that were originally thought to be unique to the producer, like those for HC-toxin in race 1 of *C. carbonum* may prove not to be. As more and more genome sequences become available, it is even likelier that genes, such as *HTS1,* are not


NPS6.1
NPS4.2, 4
NPS4.3, 1
NPS2.1-4, NPS12 x2
AAR, NPS10
- H_2O_2
+ H_2O_2
nps6
WT
WT
nps4
WTx WT
nps2 x nps2
WT
nps2
nps6
nps2nps6
coprogen
ferricrocin

◀ **Fig. 2.7** The NRPS AMP domain tree (Fig. 2.5) and highly conserved AMPs. *See text* NPS2 consists of four AMP domains that group together and produce the hexapeptide intracellular siderophore, ferricrocin, responsible for iron storage within cells. When deleted, sexual reproduction (ascus formation, *right*) is absent. NPS4 consists of four AMP domains, only two of which group together. Product is unknown but lack of NPS4 converts colony surfaces from hydrophobic to hydrophilic (*middle*). NPS6 consists of one complete and one incomplete AMP domain for production of the tripeptide extracellular siderophore, coprogen, which when absent impacts ability to acquire iron, resist oxidative stress (*left*), and reduces wild-type virulence (*bottom*). There are two copies of NPS12 which has no known phenotype. AAR is alpha-aminoadipate reductase responsible for lysine biosynthesis in fungi. For each NPS, the number after the period refers to a particular AMP domain in the protein, starting from the N terminal end

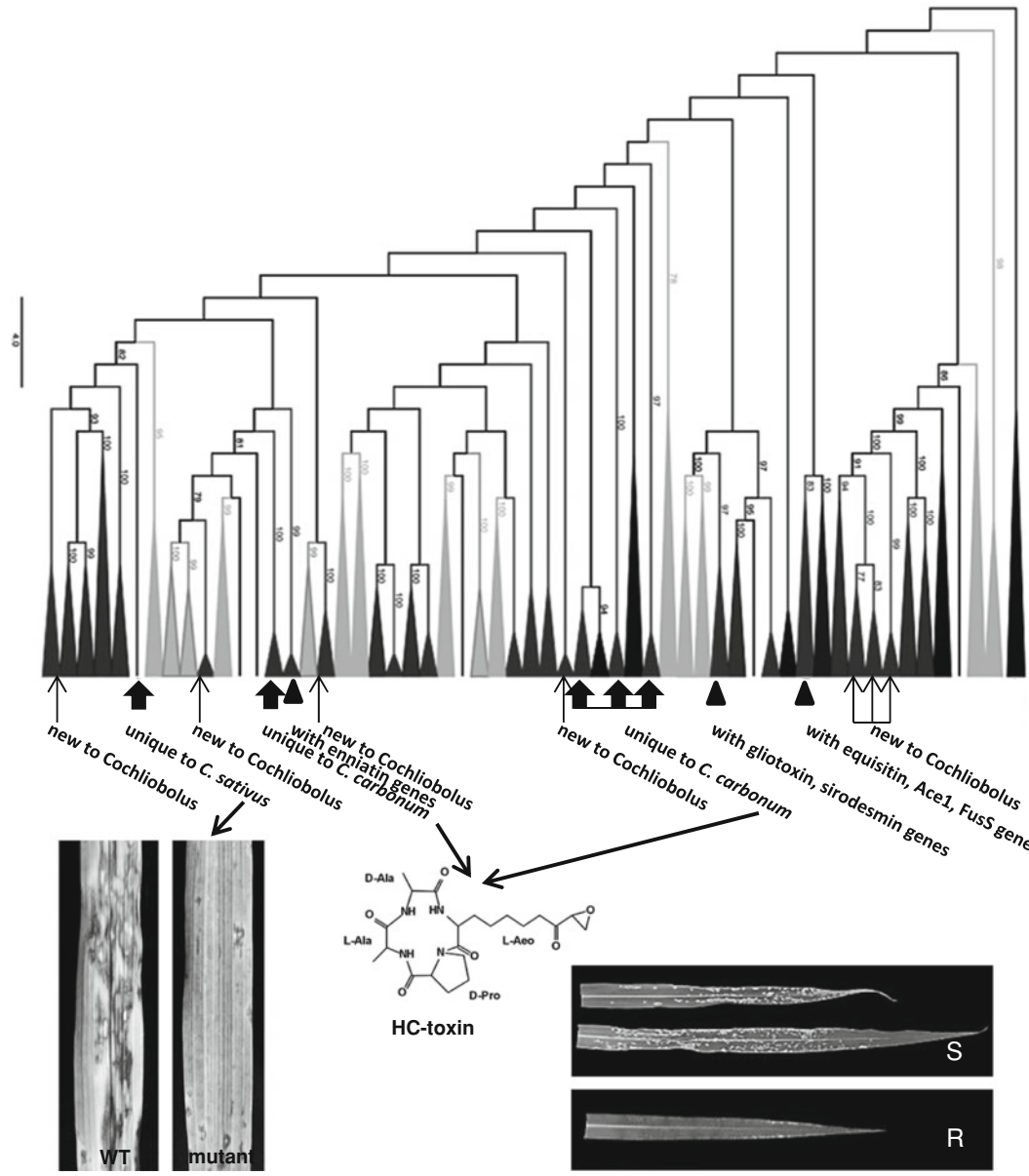


Fig. 2.8 The NRPS AMP domain tree (Fig. 2.5) and unique AMPs. *See* Figs. 2.5 and 2.7 for labeling. An example of a unique NRPS in *C. sativus,* associated with virulence of the strain on a particular cultivar of the host is shown (*left*). Barley cv. Bowman was inoculated with wild type (ND90Pr) and a mutant lacking the gene corresponding to protein ID 115356 shows reduced virulence. *Right* Susceptible (*S*) and resistant (*R*) maize inoculated with *C. carbonum* race 1, which produces the HST HC-toxin

unique but are spottily distributed with candidate orthologs in distant and/or isolated branches of the fungal phylogenetic tree.

As orthologs are discovered in more and more species, horizontal gene transfer may be a less enticing hypothesis—or, it may be the best explanation, depending on the distribution. The alternative hypothesis for rare distribution among species is rapid selective duplication and loss (Kroken et al. 2003; Bushley and Turgeon 2010). By diversifying inventories of HSTs or effectors, pathogens prevent hosts from developing a single resistant genotype. It is possible that uncharacterized members of the pool of uniquely distributed secondary metabolism genes act as HSTs in undiscovered contexts. Their anonymity may relate more to the fact that the corresponding host target, or host itself, is not widely deployed in agriculture, and therefore, the pathogenic potential of these metabolites is not known to us.

The third insight comes from our species inventory of NRPS genes and may weigh on the last point above. The AMP domains comprising *C. heterostrophus* NPS1, NPS3, NPS13 NRPS proteins indicate a complex evolutionary history (Condon et al. 2013) (Fig. 2.5). On the whole protein level, the complete *C. heterostrophus* NPS1 (trimodular) and NPS3 (tetramodular) domain sets are either present or absent in other species (Fig. 2.9). NPS1 is intact in *C. victoriae, C. carbonum,* and *C. lunatus,* while NPS3 is intact in *C. miyabeanus* and *C. sativus,* but absent from the other genomes. Mono-modular *C. heterostrophus* NPS13 is found only in *C. heterostrophus*. NPS1, NPS3, and NPS13 protein AMP domains are expanded discontinuously resulting in a suite of novel proteins which may be mono- or multi-modular (Fig. 2.9). All of the AMP domains that comprise these proteins form two separate clades in the phylogenetic tree of *Cochliobolus* AMP domains (Figs. 2.5 and 2.9).

We speculate that this group of AMP domains is a hotbed of evolutionary activity. Domains are rapidly duplicated, swapped, recombined, and genes are gained and lost. Future studies on the evolutionary signatures of different clades could help support this hypothesis. If the idea holds, it could explain how some NRPS are found in such a patchwork distribution throughout a phylogeny.

2.2.2.2 Iron and Oxidative Stress

Among the NRPSs involved in running the fungal cell itself are those biosynthesizing intracellular and extracellular siderophores for iron chelation. Iron is indispensable for virtually all organisms (Winkelmann 1991) and is involved in many fundamental biochemical reactions (respiration, the TCA cycle). It is also required for success as a pathogen. Iron can occur either in reduced ferrous (Fe^{2+}) or oxidized ferric (Fe^{3+}) form; this capacity to gain or lose electrons makes iron a major redox mediator. Iron has the potential to catalyze the Fenton/Haber Weiss reactions (Fenton 1894) generating highly cytotoxic ROS. Hence, mechanisms that sequester iron in cells are critical for survival. Paradoxically, although iron is essential, bioavailable forms are very limited in aerobic environments (Neilands and Leong 1986; Lesuisse and Labbe 1994; Haas 2003). Therefore, efficient and competitive iron-uptake mechanisms are also critical to survival of all organisms, including fungi during infection of plants. For this, fungi employ a variety of strategies, including two high-affinity uptake mechanisms, siderophore-assisted mobilization, and non-siderophore reductive iron assimilation (RIA) (Schrettl et al. 2004; Oide et al. 2006; Wolpert et al. 2011). As noted, with their strong iron-binding activity, siderophores function both in acquisition and in storage/sequestration of iron (Neubauer et al. 2000; Oide et al. 2006). Fungal (and bacterial) siderophores are biosynthesized by multi-modular NRPSs (encoded by *NPS2* and *NPS6*, previous section) (Fig. 2.7) (Oide et al. 2006). The alternative high-affinity iron-chelating mechanism in fungi, RIA, is a three step process in which ferric iron is reduced by a metalloreductase (*Fre*1p) extracellularly, and then the ferrous iron is oxidized by an iron multicopper oxidase (*Fet*3p) that is coupled to a high-affinity iron permease (*Ftr*1p) for transport

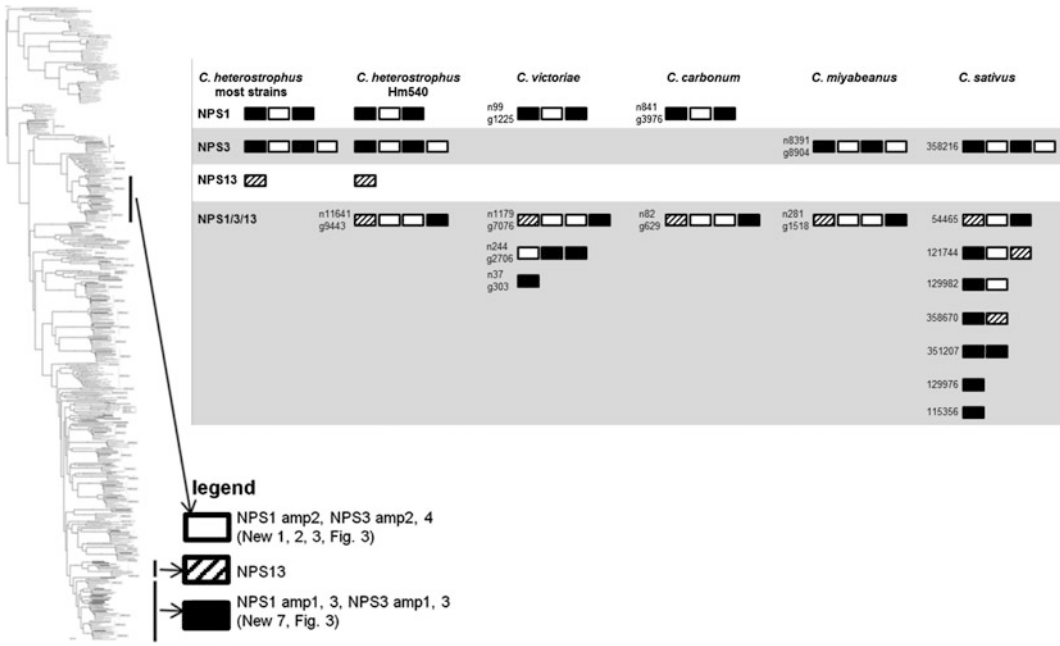


Fig. 2.9 NPS1, NPS3, and NPS13 are examples of NRPS proteins encoded by highly recombinogenic and expanded *NPS* genes. Full AMP domain phylogenetic tree (Condon et al. 2013) is cartooned at *left*. The reference NPS1, NPS3, and NPS13 proteins are cartooned *bottom left*. AMP domains corresponding to these proteins are completely conserved in the five strains of *C. heterostrophus,* but show discontinuous presence in all other *Cochliobolus* species (Fig. 2.5) and *Setosphaeria.* *Note* some AMP domains from NPS1 to NPS3 group at the *top* of the tree (AMPs 2 and 4, *white box*), while the rest group at the *bottom* of the tree (AMPs 1 and 3, *hatched box*); NPS13 AMP1 (*black*) also groups at the *bottom* of the tree. *Branches* correspond to individual AMP domains which group together and the particular corresponding AMP domain is depicted on the *right* of the diagram. *Note* collection of novel NRPSs composed of NPS1, NPS3, and NPS13 AMPs, at *bottom*

across the plasma membrane to the cytosol (Haas 2003). To a first approximation, necrotrophs, such as most *Cochliobolus* species, rely on extracellular siderophores for in planta iron acquisition, while (hemi)biotrophs tend to use the RIA mechanism of iron gathering.

We have generated many *C. heterostrophus* mutants lacking iron or oxidative stress related genes (Fig. 2.7). Associated phenotypes are shown in Table 2.4. NPS6 is a virulence factor for several pathogens (Oide et al. 2006). *nps6* mutants still have the RIA route available and also still produce the intracellular siderophore, ferricrocin, made by the product of the NRPS encoding gene, *NPS2*. Ferricrocin is not required for virulence of *C. heterostrophus,* but is required for sexual reproduction (Fig. 2.7). *nps2nps6* double mutants exhibit a greater reduction in virulence and impairment in sexual development than single *nps6* or *nps2* mutants (Fig. 2.7). Triple iron acquisition and storage mutants (*nps2nps6ftr1*) are almost avirulent, but do attach to and penetrate the host (Condon, Turgeon unpublished). *nps6* mutants are also hypersensitive to oxidative stress (Fig. 2.7) and there is a gradation of sensitivity of the single, double, and triple mutants, with the latter being the most sensitive.

Double mutants lacking *ChAP1* (Lev et al. 2005), a gene encoding a redox-regulated transcription factor and *NPS6* (*Chap1nps6*), or lacking *ChAP1* and the iron-sensitive transcription factor *Sre*1 (*Chap1sre1*) have been constructed (Table 2.4) and tested for oxidative stress and virulence. *Chap1nps6* mutants are more sensitive to oxidative stress than either parent, while *Chap1sre1*

Table 2.4 Iron- and ROS-related *C. heterostrophus* genes and mutants available

Gene (Acc.#)	Protein	Function/phenotype of mutant (References)
Iron-related genes deleted singly or in multiples		
NPS2 (77609)	NRPS	Intracellular sid/reduced sex, (Oide et al. 2007)
NPS6 (33171)	NRPS	Extracellular sid/reduced virulence, reduced resistance to ROS, hypersensitive to low iron, (Oide et al. 2006)
NPS2;NPS6	*See* above	Augmented in all above phenotypes
FTR1 (104817)	Iron permease	Reductive Fe assimilation/WT in virulence and sensitivity to ROS, and low iron
FTR1;NPS2	*See* above	nps2 phenotypes; otherwise WT as for ftr1
FTR1;NPS6	*See* above	Greater virulence/sensitivity phenotypes compared to nps6. Requires supplemental iron for growth/ conidiation on CM
FTR1;NPS2;NPS6	*See* above	Decreased virulence, sensitivity to ROS, more severe phenotypes than nps6;ftr1
SRE1 (109473)	Transcription factor	GATA-type Zn-finger/ regulator of iron metabolism, slow radial growth, less resistant to ROS than WT
NPS6;CHAP1	*See* above	Virulence = nps6, very reduced resistance to ROS, > either single mutant
ROS-related genes deleted singly or in multiples		
CHAP1 (130082)	bZIP tf	More sensitive to ROS than nps6 (Lev et al. 2005)
CHAP1;SRE1	*See* above	Virulence = nps6, reduced resistance to ROS, but < Chap1 mutant
CAT1 (115312)	Catalase	Decomposition of H_2O_2 to H_2O and O_2/non secreted, WT (Robbertse 2003)
CAT2 (110605)	Catalase	Decomposition of H_2O_2 to H_2O and O_2/non secreted, WT (Robbertse 2003)
CAT3 (109994)	Catalase	Decomposition of H_2O_2 to H_2O and O_2/secreted, sensitive to ROS (Robbertse 2003)
CAT1;CAT2	*See* above	WT (Robbertse 2003)
CAT1;CAT3	*See* above	Sensitive to ROS, WT virulence (Robbertse 2003)
CAT2;CAT3	*See* above	*See* above
CAT1;2;3	*See* above	*See* above
SOD1 (24548)	sup ox (Cu/ Zn)	Catalyzes dismutation of superoxide into O_2 and H_2O_2/WT
SOD2 (30814)	sup ox (Mn/ Fe)	*See* above, mitochondrial/WT
SOD3 (90570)	sup ox (Mn/ Fe)	*See* above/possibly essential (*see* footnote)
NOXA (95484)	NADPH oxidase	Membrane-bound enzyme complex; generates superoxide/WT in virulence, delayed conidiation, reduced somewhat decrease pigmentation
NOXB (95158)	As above	*See* above/reduced virulence
NOXC (117226)	As above	*See* above/decreased conidiation and somewhat decreased pigmentation
NOXR (28914)	Regulator	*See* above/reduced virulence, decreased conidiation

For oxidative stress and virulence phenotypes *see* Fig. 2.7; *sid* siderophore; *L-orn mono* L-ornithine monooxygenase; *sup ox* superoxide dismutase; *tf* transcription factor; attempts to delete *SOD3* have failed, suggesting it is essential. Acc.# = JGI

mutants partially rescue the *Chap1* oxidant-sensitive phenotype. Double mutant phenotypes are consistent with a model in which sequestering of iron by the NPS6 siderophore defends the fungal pathogen against oxidative stress.

2.2.2.3 The CYPome of *Cochliobolus* spp.

The published *Cochliobolus* genome manuscript (Condon et al. 2013) did not include *C. lunatus*, a species, as indicated in the

Introduction, that has been used as a workhorse for steroid biosynthesis centered on the activity of cytochromes P450 (CYPs). CYPs, a superfamily of heme-containing monooxygenases, are ubiquitously present in all kingdoms of life with fungi having the second largest number after plants. Some are involved in primary metabolism and are indispensable for normal development and homeostasis or in allowing fungi to live on particular carbon sources. Others are involved in xenobiotic metabolism and provide defense against natural products, while still others are associated with genes for secondary metabolite production and the biosynthesis of pigments, antioxidants, defense compounds, and toxins.

Despite the fact that CYPs play roles in hydroxylation and oxidation processes leading to degradation, detoxification, and syntheses of compounds crucial for life or for niche survival, the substrates on which they act are largely unknown. To identify P450s and annotate those associated with secondary metabolite gene clusters across *Cochliobolus* species, we searched gene models for annotations with the PF00067 (P450 superfamily) domain. Almost one thousand predicted P450s (943) were identified across six *Cochliobolus* species, averaging ~135 P450s per species and represents ~1 % of the total gene catalog (Table 2.3). This tally is comparable to the number in *Aspergillus nidulans* (version AN.3, CADRE (Kelly et al. 2009)) and other *Aspergillus* species (~125 P450s per species). The CYPome of the Dothideomycete *Mycosphaerella graminicola* has fewer (82 P450s plus one pseudogene) (Newsome et al. 2013). P450s in close proximity to secondary metabolism backbone genes (such as *NPS* or *PKS* genes) may be involved in secondary metabolite biosynthesis. *NPS* or *PKS* genes were located near 13–17 % of *C. heterostrophus* P450s, slightly lower than when this analysis was done for *A. nidulans*, 29 % (32 of 111 functional P450s) (Kelly et al. 2009). No preference was observed in the association of P450s with mono- or multi-modular *NPS* genes.

It is difficult and in most instances impossible, to predict the specific functions of the CYPs from their sequence similarities or even their association with *PKS*s or *NPS*s in gene clusters, as it is known that a single amino acid change can significantly alter metabolic capabilities. These difficulties, in combination with the abundance of P450s, make phylogenetic analyses an essential first step for studying these crucial genes.

2.2.2.4 Small Secreted Proteins (SSP)

A search for candidate effector proteins that are cysteine rich (>2 % cysteine), small (<200 amino acids), predicted to be secreted (using Phobius (Kall et al. 2007)), and without transmembrane domains revealed between 143 and 289 SSPs per *Cochliobolus* (Table 2.3) (Condon et al. 2013). An all-versus-all BLAST analysis to determine if SSPs were strain or species-unique revealed that few candidate *C. heterostrophus* SSPs were unique to any particular strain within the species. Among species, *C. sativus* had the most isolate-unique SSPs, containing 167 candidates (Condon et al. 2013). As this is the only *Cochliobolus* strain thought to act as a hemibiotroph, it is interesting that it contains more SSPs, and more unique SSPs, than the necrotrophic isolates, although this is only a correlation at this point. As is typical with candidate effectors, functional domain predictions were lacking, with only 37 candidates having some predicted function, generally involved in cell wall or extracellular matrix function. An additional 23 candidates were conserved in other fungi outside of the Dothideomycetes. The remaining 120 predicted candidates were featureless and seemingly unique to the Dothideomycetes (Condon et al. 2013). *Cochliobolus heterostrophus* strain C5 SSP predicted candidates were rich in SNP calls to other *Cochliobolus* genomes: 101 candidate SSPs had SNPs with at least one other *Cochliobolus* genome (Condon et al. 2013).

In our all-versus-all BLAST analysis, only 6 of the 180 *C. heterostrophus* C5 SSPs were found in all 10 strains examined and 14 were unique to strain C5 (Condon et al. 2013). The presence or absence of most SSPs did not fall

into easily categorized bins such as *C. heterostrophus*-specific, or maize-pathogens only. Instead, SSPs were present and absent in no particular pattern across the genomes. 115 SSPs were present in at least one other species (*C. victoriae, C. miyabeanus, C. carbonum*), with seven found in all species, and 27 in all *Cochliobolus* species. Unlike those in some phytopathogens, such as *Leptosphaeria maculans* (Rouxel et al. 2011), SSP-encoding genes did not occur in clusters; candidates seldom were located within 10 kb of each other. It has become clear in recent years that necrotrophs, like (hemi)biotrophs, also use effectors to manipulate specific targets in the host cell for the benefit of the pathogen. Unlike (hemi)biotrophs, however, the aim seems to be to *trigger* host defenses or cell death, rather than circumvent these processes. Two clear examples are victorin produced by the necrotroph *C. victoriae* and ToxA produced by the necrotrophs *Pyrenophora tritici-repentis* (Ciuffetti et al. 1998) and *Stagonospora nodorum* (Friesen et al. 2008). The extent to which necrotrophs employ effectors is an exciting and unknown frontier. Do necrotrophic effector molecules always aim to trip host defenses, or do some act more according to (hemi)biotrophic principles, quelling host defense response and intercepting signaling? As for their metabolic origins, are (presumably) secondarily encoded molecules like victorin the norm, or do necrotrophs utilize small cysteine rich ribosomally encoded effector proteins typical of hemi(biotrophic) interactions? The bioinformatics analysis described above is an earnest attempt to break ground answering these questions. The limitations of this approach, however, cannot be overstated. Bioinformatically predicted SSPs require in planta expression or protein secretion data, or functional knockout data, before they can be considered *bona fide* effectors. SSPs are small, and typically lack predicted functional domains—a trait they share with miscalled ORFs. Secretion prediction is also an imprecise technique with many false-positive and false-negative predictions. That our SSP inventories are larger for known hemibiotrophs than necrotrophs seems to suggest that we are indeed including at least some effectors in our prediction.

2.2.2.5 Signaling

As for P450s, the published *Cochliobolus* genome manuscript (Condon et al. 2013) did not include a comprehensive analyses of genes associated with signaling. Because signaling mechanisms are the centerpiece of interaction biology, we have included a brief summary of annotation of relevant genes in *Cochliobolus* species.

Conserved signaling pathways. If two closely related pathogens infect different hosts, one might conclude that they respond to different signals and hypothesize that comparison of the genomes of *Cochliobolus* pathogens of different hosts would identify critical response differences. There is, however, no simple correlation. Genes encoding heterotrimeric G protein subunits, MAP kinases, and histidine kinase response regulators have been studied in *C. heterostrophus* since the 1990s and these studies are now facilitated by the genome projects (Horwitz et al. 1999; Lev and Horwitz 2003; Lev et al. 2009; Oide et al.; Degani et al. 2004). Two signaling pathways (MAP kinase and heterotrimeric G protein) are shown schematically in Fig. 2.10. As in other pathogens, the *C. heterostrophus* core signaling proteins could be considered virulence factors because mutants are unsuccessful pathogens, but additional developmental alterations, obfuscate how exactly, signaling impacts virulence.

One way that host specificity could be attained is for cell surface receptors in each species to recognize host-specific ligands, which then transmit the signal via a conserved intracellular cascade. *C. heterostrophus,* for example, has 21 genes predicted to encode histidine kinase sensors but only four downstream response regulators (Catlett et al. 2003b; Oide et al. 2010). The two-component pathways initiated by histidine kinase sensors in *C. heterostrophus* have central functions in morphogenesis, stress response, and virulence (Oide et al. 2010). Comparing histidine kinase sensors suites across

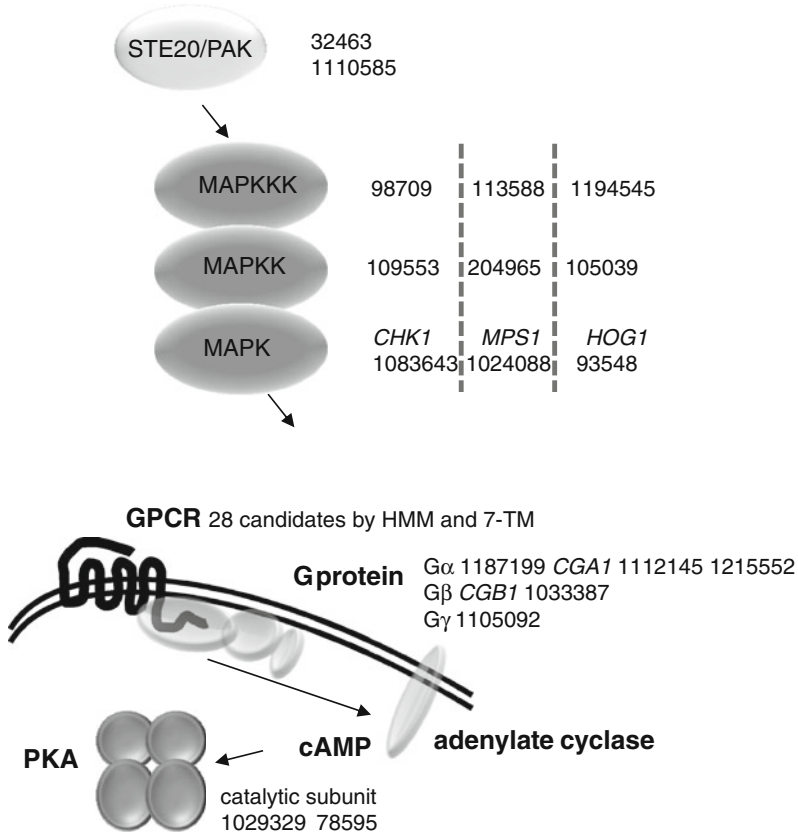


Fig. 2.10 Illustration of two conserved signaling pathways, with corresponding gene models from *C. heterostrophus.* The following genes have been studied by constructing deletion mutants: MAP kinases *ChHK1*, *MPS1*, and *HOG1*; G protein Gα subunit *CGA1*, and G protein G*β* subunit *CGB1*. The gene models identified by reciprocal BLASTP search and/or other methods (*see text*) are indicated as *C. heterostrophus* strain C5 v2.0 protein ID numbers. Two examples of signaling pathways are shown: *above*, MAP kinase cascade; the *vertical dashed lines* indicate a tentative association into MAPK modules, by homology (there is no functional information to support this, as yet) *below*, a model of heterotrimeric G protein signaling in which activation of adenylyl cyclase produces cAMP, which activates protein kinase A (PKA). Heterotrimeric G protein signaling could lower or raise cAMP levels

Cochliobolus pathogens may reveal nuances not readily apparent when core signaling components are compared.

For heterotrimeric G protein pathways, the capacity for signals from multiple receptors to converge on a few downstream transducers may be even greater than for the two-component pathways. G protein-coupled receptors (GPCRs) are more difficult to identify bioinformatically, than the highly conserved signal transducers, but methods are improving (Xue et al. 2008; Lafon et al. 2006; Omann et al. 2012; Kim et al. 2012). To estimate the number of GPCRs in *C. heterostrophus*, an initial analysis was done as part of the annotation effort (Horwitz lab unpublished; Ohm et al. 2012): filtered protein models were searched with an HMM tool designed to identify GPCRs (Wistrand et al. 2006), then those with seven transmembrane segments as predicted by PHOBIUS (Kall et al. 2007) selected. An initial phylogenetic tree was constructed. The candidate sequences were used to query the NCBI database to identify those with convincing homology to transporters, or having a conserved domain indicating that they may be transporters. These sequences, as well as

sequences falling on branches of the initial phylogeny together with annotated transporters or ATPases, were removed and the phylogeny was then recalculated. The analysis indicates orthologs of pheromone receptors *Ste*3 (1203184) and *Ste*2 (1215526), whose function in mating could be tested by gene deletion experiments. 20 candidates group with sequences annotated as related to the CFEM/Pth11 family in other fungi. Of these 20 candidates, three contain CFEM domains detected by Pfam, and in addition, have similarity with annotated CFEM-containing sequences. Three sequences show similarity to annotated Pth11-like sequences. These classes are proposed to be involved in pathogenicity (DeZwaan et al. 1999; Kulkarni et al. 2003). It would be of interest to compare these among *Cochliobolus* species for species-specific associations and, where possible, to test their function by gene deletion. This analysis provided no obvious orthologs of fungal opsins, even though two candidates were recognized previously by homology (C5 protein IDs 1195154 and 1139038, Oide and Turgeon unpublished). No members of the GPCR classes represented by *Neurospora* Gpr1-1, Gpr-5, and Gpr-4 (*see* Xue et al. 2008) were identified either, supporting our statement above that GPCRs are difficult to extract bioinformatically.

Light regulation. Not all signals are transduced from the cell surface to the nucleus by G protein and protein kinase pathways. In particular, dedicated fungal transcription factors relay information about light, pH, oxidants, and hypoxia. Once activated by the primary stimulus, these transcription factors may rely on additional regulators in order to produce the physiological output. The Neurospora circadian clock is a good example: the stress-activated MAPK (Hog1, Fig. 2.10) is activated rhythmically by the circadian oscillator (Vitalini et al. 2007). Circadian rhythmicity has not been studied in detail in any *Cochliobolus* species, but *C. heterostrophus* shows a clear banding pattern when grown under light/dark (L/D) cycles (Wu et al. 2012). A *C. heterostrophus* mutant lacking the ortholog of *N. crassa WC1* shows defective banding with a weaker, residual banding pattern suggesting that additional photoreceptors are active. Initial evidence that the circadian clock controls Hog1 phosphorylation via the response regulator Ssk1 (*N. crassa* RRG-1) in *C. heterostrophus* comes from our finding that the L/D banding pattern is defective in *hog1* and *ssk1* mutants (Oide et al. 2010), similar to that of *wc1* mutants (Turgeon and Horwitz labs unpublished).

Light regulation is of particular interest because it couples environmental sensing and secondary metabolism. *C. heterostrophus* mutants lacking key components of the velvet complex (*VEL1* or *LAE1*), which controls reproduction and secondary metabolism produce much less T-toxin than WT in the dark (Wu et al. 2012). Light conditions could be particularly relevant because light, as the source of energy, is a critical environmental factor for the host plant. Light could synchronize gene expression of the pathogen to match the host; thus, photocontrol of plant–pathogen interactions has received recent attention (Kim et al. 2011a, b; Lee et al. 2006a, b).

Redox signals. As noted above, *ChAp*1, an ortholog of yeast Yap1, senses oxidants in *C. heterostrophus*. Yap1 homolog-mediated oxidative stress tolerance is crucial for pathogenicity of the necrotrophic fungus *Alternaria alternata* on citrus (Lin et al. 2009; Kim et al. 2009) and *M. oryzae* on rice (Guo et al. 2011). *C. heterostrophus Chap1* mutants, although hypersensitive to oxidants, retain wild-type (Lev et al. 2005) or moderately reduced (Zhang et al. submitted) virulence on maize. The *Botrytis cinerea* Yap1 ortholog is required to resist peroxide stress in vitro, yet, Yap1 is not a virulence factor on bean, Arabidopsis, apple or tomato fruits, and its target genes are not induced on bean although H_2O_2 was detected (Temme and Tudzynski 2009).

There is strong genetic evidence for the involvement of multiple pathways in sensing oxidants. Loss of *C. heterostrophus* Hog1 (Fig. 2.10), its upstream response regulator Ssk1, or the response regulator Skn7, all result in hypersensitivity to oxidants (Oide et al. 2010). Although the oxidative burst is considered key to plant defense, it is worth noting that the ability

to cope with hypoxic stress is important for pathogens of animals. Neutrophils in the mammalian immune system produce ROS, yet *Aspergillus fumigatus* needs the hypoxic stress response for virulence (Blatzer et al. 2011; Grahl and Cramer 2010; Willger et al. 2012). Hypoxic stress has received less attention in plant pathogens.

Signaling pathways cannot be studied in isolation and the study of oxidants provides a good example. Loss of *HOG1*, *ChAP1*, *SKN7* as well as the NRPS responsible for extracellular siderophore production (Fig. 2.7), all diminish the ability of the pathogen to resist oxidative stress. Light signals are directly detected by the white collar transcription factors, but once again, the pathway is not a linear one because the global regulators Vel1 and Lae1 also participate. The light-sensing complex could be similar to that of other fungi but the details, and the genes regulated, likely hold surprises specific to *Cochliobolus*.

2.3 Applications from the Genome and Future Perspectives

The genomic resources available for comparative studies across the *Cochliobolus* genus are legion, thanks to the generous contributions of the JGI, with sequences and resources available for many different *Cochliobolus* species (and, for many species, multiple strains). The long history of using *Cochliobolus* species as model organisms allows an exciting marriage of functional work and in silico comparative genomics. As discussed in the Introduction, *Cochliobolus* taxa vary in their biology, host specificity, and developmental pathways. Whole-genome comparisons were startling vis-*a*-vis the incredible homology between most *Cochliobolus* species. Attempts to characterize "species-unique" sequence, found in all five *C. heterostrophus* strains, but no other *Cochliobolus* species, did not result in identification of large *C. heterostrophus* unique regions, but smaller differences. Uncovering the core identity of each species as it relates to their biology, therefore, is not as easy as identifying and characterizing a large obvious patch of genome. To address questions of differential biology, more refined approaches are necessary, facilitated by the history of molecular-genetic work for each species.

Molecular investigations into virulence factors have run the gamut from discovery of highly specific HSTs, to more general mechanisms involving iron and oxidative stress. Initially, secondary metabolism as a source of HSTs was considered the most compelling type of functional investigation for *Cochliobolus* pathogens (Lee et al. 2005; Turgeon et al. 2008). Extensive bioinformatic analyses of secondary metabolism genes occupy a large share of this chapter and these studies coupled with experimental research support our reasoning in this regard. The observation that phenotypes associated with secondary metabolism gene mutants follow phylogenetic distribution signatures provides a strong hypothesis and platform for further work. Conserved secondary metabolite clusters are likely to biosynthesize metabolites that broker basic cellular metabolism (iron gathering, oxidative stress management, etc.), while discontinuous and severely restricted gene distribution suggests niche-specific/virulence-specific function.

The second major aim of our comparative genomics study was to consider the role small secreted proteins may play in *Cochliobolus* species. Unlike secondary metabolites, this was not done against the backdrop of years of genetic characterization, but rather in the broader context of plant–microbe interactions. It has long been understood that biotrophic pathogens secrete effectors, which are often small and cysteine-rich proteins that elegantly subvert host defenses and prevent cell death. The traditional necrotroph, on the other hand, was thought to use a combination of toxins (including HSTs) and "brute force" methods (cell wall-degrading cellulases, pectinases) to overpower hosts. Recent work suggests that many necrotrophic virulence factors should truly be classified as effectors. An example of this is *C. victoriae*'s HST, victorin, which in the presence of an NB-LRR-type protein results in host susceptibility, instead of resistance. In light of these and similar observations, the obvious question is to what

extent do necrotrophs utilize effectors and do they employ small secreted proteins, as biotrophs do? *Cochliobolus* is a wonderful system to ask these questions, as it contains both hemibiotrophic and necrotrophic pathogens. Bioinformatic searches found SSPs in all species examined, although the number of predicted SSPs, and species-unique SSPs, was higher in hemibiotrophic species. The set of SSPs identified serves as a toehold for identifying candidate SSP effectors in necrotrophs and concomitant functional analysis has the capacity to greatly alter our perception of such pathogens.

We have also sought to discuss the functional work conducted in different *Cochliobolus* species on other topics pertinent to pathogenic and reproductive development, including iron metabolism, oxidative stress management, P450s, signaling components, and mating determinants. Extrapolation of rich molecular work within a single species, such as *C. heterostrophus*, to other closely related species, results in new hypotheses. Genomic differences among species can unearth biological phenomena that might go unnoticed examining one system alone. Each analysis, of course, must be taken with a digital grain of salt, until functional work can support a given hypothesis. Comparative bioinformatics offers us a tentative and highly valuable glimpse into the inner workings of an entire genus.

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