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Repeated loss of an anciently horizontally transferred gene cluster in *Botrytis*

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Abstract: At least five of the six genes of the bikaverin secondary metabolic gene cluster were shown to have undergone horizontal transfer (HGT) from a *Fusarium* donor to the *Botrytis* lineage. Of these five, two enzyme-encoding genes are found as pseudogenes in *B. cinerea* whereas two regulatory genes and the transporter remain intact. To reconstruct the evolutionary events leading to decay of this gene cluster and infer a more precise timing of its transfer, we examined the genomes of nine additional broadly sampled *Botrytis* species. We found evidence that a *Botrytis* ancestor acquired the entire gene cluster through an ancient HGT that occurred before the diversification of the genus. During the subsequent evolution and diversification of the genus, four of the 10 genomes appear to have lost the gene cluster, while in the other six the cluster is in various stages of degeneration. Across the *Botrytis* genomes, the modes of gene decay in the cluster differed between enzyme-encoding genes, which had higher rates of transition to or retention of pseudogenes and were universally inactivated, and regulatory genes (particularly the non-pathway-specific regulator *bik4*), which more frequently appeared intact. Consistent with these results, the regulatory genes *bik4* and *bik5* showed stronger evidence of transcriptional expression than other bikaverin genes under multiple conditions in *B. cinerea*. These results could be explained by pleiotropy in the bikaverin regulatory genes either through rewiring or their interaction with more central pathways or by constraints on the order of gene loss driven by the intrinsic toxicity of the pathway. Our finding that most of the bikaverin pathway genes have been lost or pseudogenized in these *Botrytis* genomes suggests that the incidence of HGT of gene cluster-encoded metabolic pathways might be higher than what is possible to be inferred from isolated genome analyses.

Key words: genome evolution, plant pathogens, rewiring, secondary metabolism

Introduction

Genes for the production of secondary metabolites often are physically clustered on the chromosomes of filamentous fungi (Keller et al. 2005). Several studies have now shown that these metabolic gene clusters undergo horizontal gene transfers (HGT) between fungi, which may provide ecological advantages to the recipient (Patron et al. 2007; Slot and Hibbett 2007; Khaldi et al. 2008; Slot and Rokas 2010, 2011). For example, the polyketide pigment bikaverin can provide protection against nematodes and microbes (Balan et al. 1970, Kwon et al. 2007, Son et al. 2008). We recently reported that homologs of five out of six genes for the production of bikaverin in *Botrytis cinerea* strains B05.10 and T4 were derived from *Fusarium oxysporum* by HGT (Campbell et al. 2012). The bikaverin gene cluster in *Fusarium* spp. contains three enzyme-encoding genes (*bik1*, *bik2*, *bik3*), two regulatory genes (*bik4*, *bik5*) and a transporter (*bik6*) (Wiemann et al. 2009). In *B. cinerea* strains B05.10 and T4, the three enzyme-encoding genes are either absent (*bik1*) or are pseudogenes (*bik2* and *bik3*), while the sequences of regulatory genes and the transporter appear intact and are presumably still functional. Analyses of rates of sequence evolution suggested that the regulatory genes, in particular the NMRA-like transcription factor enhancer, *bik4*, are being selectively conserved while other genes are under relaxed selection (Campbell et al. 2012).

The term ''taphonomy'' is used predominantly to describe the decay and possible fossilization of whole organisms, but it is also the only English word

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TABLE I. Evolutionary rates[a] of *Botrytis* vs. *Fusarium* bikaverin genes

Gene	H_0 lnL	H_1 lnL	2ΔL	*P* value	H_0 ω	H_1 ω (background)	H_1 ω (foreground)
BIK1	−8663.63	−8630.3	66.65	< 0.0001	0.0831	0.0506	0.2199
BIK2	−3693.55	−3643.71	99.69	< 0.0001	0.1722	0.0726	0.8188
BIK3	−1356.56	−1330.52	52.1	< 0.0001	0.0882	0.0153	0.5822
BIK4	−2330.48	−2315.92	29.12	< 0.0001	0.1491	0.081	0.454
BIK5	−4919.04	−4898.22	41.65	< 0.0001	0.1193	0.0664	0.334
BIK6	−1508.53	−1495.18	26.71	< 0.0001	0.1077	0.0326	0.2926

[a] $P < 0.05$ indicates *Botrytis* (foreground) bikaverin sequences are evolving significantly faster than *Fusarium* (background). H_0 = *Botrytis* and *Fusarium* sequences are constrained to equal rates of evolution. H_1 = Unconstrained rates of evolution.

designated to the study of decomposition and decay. Here we have conducted a genome-level taphonomic study, where the subject of decay is the gene cluster, and pseudogenes by analogy are fossils of genes. To reconstruct the sequence and timing of the evolutionary events associated with the HGT and decay of the bikaverin gene cluster in *B. cinerea*, we examined representative genomes from nine additional broadly sampled *Botrytis* species for the presence of homologous sequences. We then characterized the patterns of pseudogenization and loss of bikaverin genes since the ancestral transfer.

MATERIALS AND METHODS

The presence of the bikaverin cluster in the genus *Botrytis* was determined through tBLASTn (Altschul et al. 1997) queries for homologous regions in the genomes of nine additional *Botrytis* species (Staats and van Kan unpubl) with nucleotide sequences from *B. cinerea* (*bik2–6*) and *Fusarium oxysporum* (*bik1*) as queries. Procedures for Illumina sequencing, read-quality trimming and genome assembly of *B. cinerea* strains B05.10 and T4 are described in Staats and van Kan (2012). For *B. tulipae*, Illumina Hiseq2000, paired-end sequencing libraries with average insert sizes of 150 bp (DNAVision) and 3.5 kb (Macrogen) were sequenced. De novo assembly (SUPPLEMENTARY TABLE I) was performed with the short-read assembler Velvet 1.2.05 (Zerbino and Birney 2008) with a *k*-mer length of 33. For all other *Botrytis* species, 150 bp paired-end Illumina sequencing libraries (DNAVIsion) were generated and scaffolds were assembled de novo with a *k*-mer length of 43. New sequences annotated for this study are deposited in GenBank (accession numbers KC244360–KC244373, KC295241–KC295243).

Nucleotide sequences of loci in which the bikaverin gene homologs were found were manually aligned with MacClade 4 (Maddison and Maddison 2003) or with progressive mauve in Mauve 2.3.1 (Darling, Mau and Perna 2010). Alignments are deposited in TreeBASE (Study 14 010, www.treebase.org). Maximum likelihood gene phylogenies were inferred for alignments containing four or more taxa with RAxML 7.3.1 under the GTRGAMMA model of sequence evolution (Stamatakis 2006) and internode support was assessed with 100 bootstrap replicates. A species phylogeny for the 10 *Botrytis* genomes and the outgroup, *Monilinia fructigena*, was constructed with a concatenated alignment (species alignment) of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), heat-shock protein 60 (HSP60) and DNA-dependent RNA polymerase subunit II (RPB2) nucleotide sequences, as in Staats, van Baarlen and van Kan (2005) and was analyzed in the same way as the bikaverin gene phylogenies.

To test for changes in the evolutionary rate of the bikaverin genes, we examined the ratio of nonsynonymous to synonymous substitutions (dN/dS) with PAML (Yang 2007). The evolutionary rate of each gene tree was tested with a null hypothesis that the entire tree (the *Botrytis* bikaverin genes plus their *Fusarium* homologues, based on the species tree) had a single rate of evolution. This was compared to the alternative hypothesis that the dN/dS rate for the *Botrytis* species was different from the dN/dS rate of the *Fusarium* species. We used a likelihood ratio test to determine which of the two models better fit the data.

Relative divergence times of the *Botrytis* species were calculated with BEAST 1.7.1 (Drummond and Rambaut 2007). All branches were estimated in units of substitution/site scaled by evolutionary rate. We assumed the SRD06 model of sequence evolution (Shapiro, Rambaut and Drummond 2006), allowing for rate heterogeneity across sites through the gamma distribution, and the uncorrelated log-normal relaxed clock model. We chose the Yule process as our tree prior. Using the species alignment, we launched 10 independent runs for 10 000 000 generations, sampling parameters every 10 000 generations. We verified the convergence of all runs by examining the effective sample size of the likelihood and posterior probability parameters for each analysis (> 100 parameters) and by visually inspecting the likelihood and posterior probability distributions across independent runs. We discarded the first 10% of sampled data points from each run as burn-in. We mapped the character states of each bikaverin gene (present or absent) onto the *Botrytis* species tree with Dollo parsimony, which assumes that a gene that is absent cannot be regained. We then ran a correlation test to determine whether there was a significant correlation between divergence times and the presence or absence of the bikaverin genes.

We used the BEAST posterior distribution of trees retained following burn-in to determine transition rates in the Bayes multistate module of the BayesTraits package (Pagel et al. 2004). Each gene was coded as either functional (F), pseudogene (Ψ) or absent (A) for each of

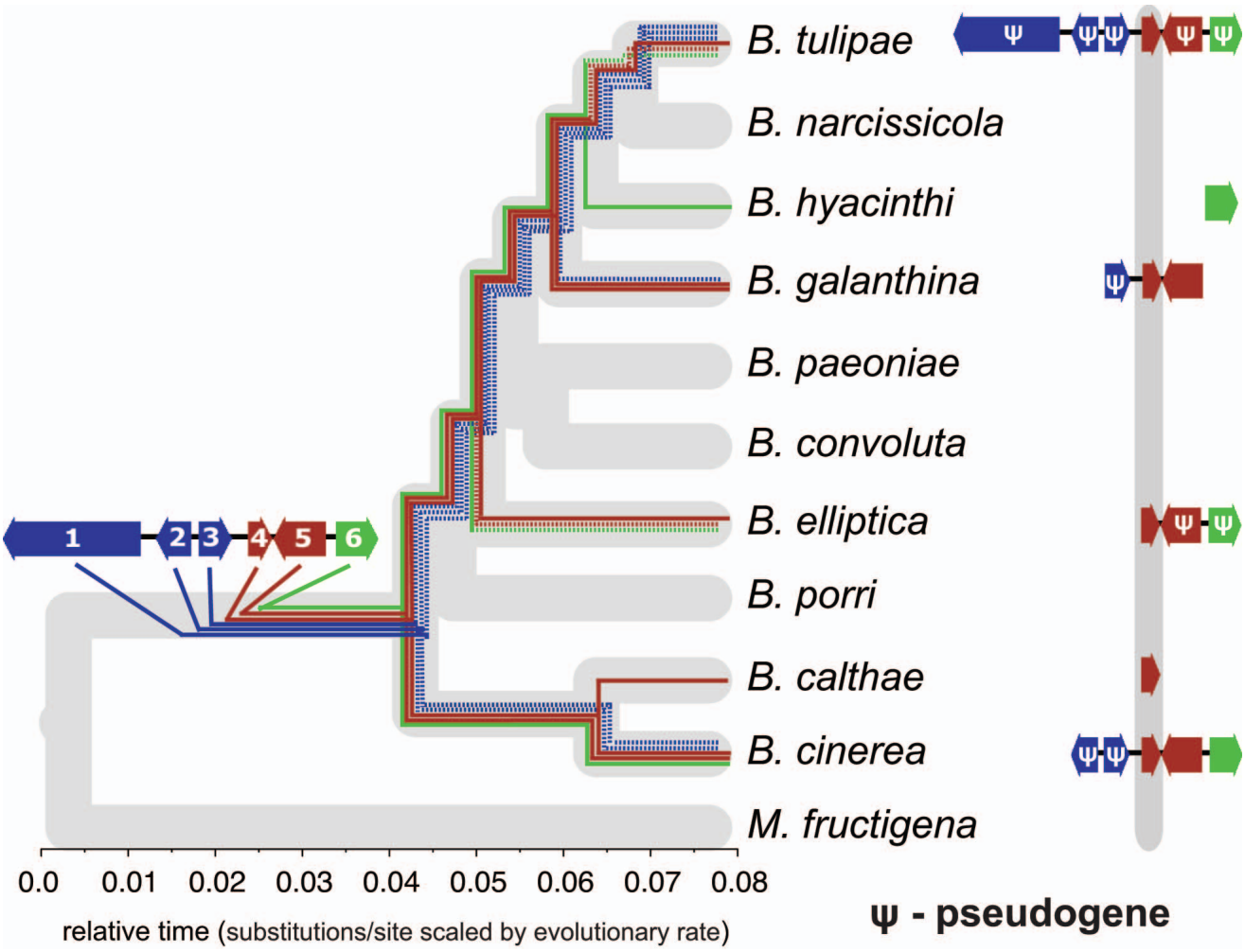

FIG. 1. Decay of the bikaverin cluster in *Botrytis*. Genes are depicted by arrows to show gene clustering. Lines in a *Botrytis* species tree trace the most parsimonious reconstruction of gene states over time since divergence from a common ancestor. Solid lines indicate inferred functional genes and dashed lines indicate inferred pseudogenes. Blue, red and green arrow and line colors represent enzyme-encoding, regulatory and transporter genes respectively. The species phylogeny was derived from a BEAST analysis of a concatenated alignment of G3PDH, HSP60 and RPB2 amino acid sequences (Staats, van Baarlen and van Kan 2005). The status of bikaverin genes in *Monilinia* is not known, however we assume HGT occurred after the divergence of *Botrytis* for the purposes of this illustration.

the 10 *Botrytis* species. All *M. fructigena* genes were coded A. The ancestral state of each cluster gene was constrained to F at the most recent common ancestor (MRCA) of all 10 *Botrytis* species to represent the donor, and rates of transition from non-functional (Ψ, A) to F were constrained to be zero. The average log likelihood of a null model, in which the rates (F → Ψ, F → A, Ψ → A) were constrained to be equal, was compared to that of the optimal model with a likelihood ratio test assessed on a chi-square distribution with 599 986 degrees of freedom corresponding to 100 000 rates from each gene, minus any rates equal to zero. We then combined the F → Ψ and F → A transitions rates for each gene and compared rates of loss among genes with a Model II ANOVA test and Tukey's HSD test, adjusting significance for multiple testing by the Bonferroni method (Sokal 1995) as implemented in JMP 10.0.0 (SAS Institute Inc., Cary, North Carolina).

Expression of the bikaverin genes in *B. cinerea* were determined by RNAseq with Illumina sequencing (200–700 bp insert size libraries, 100 bp paired-end reads; BGI, Hong Kong), in five RNA samples collected from mycelium grown in liquid glucose-containing medium, mycelium grown in liquid sodium polygalacturonate-containing medium and two batches of infected tomato leaves at 16 or 40 h post inoculation (in strain B05.10) and in a mixture of sclerotia and apothecia in various developmental stages (in strains SAS405, SAS56). Expression was calculated as reads per kilobase per million reads (RPKM).

RESULTS

The bikaverin gene cluster has been repeatedly lost in Botrytis.—We detected bikaverin cluster gene

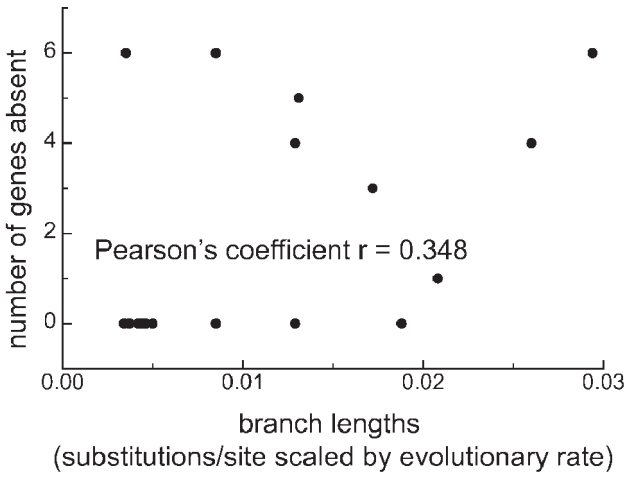

FIG. 2. Graph of branch lengths (representing relative divergence times) versus number of genes absent for the *Botrytis* and *Fusarium* species. Branch lengths were derived from a BEAST analysis of a concatenated alignment of G3PDH, HSP60 and RPB2 amino acid sequences.

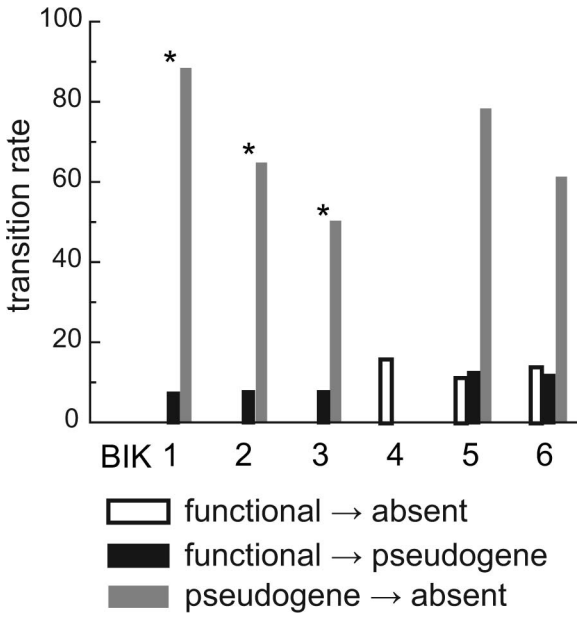

FIG. 3. Transition rates of bikaverin cluster genes. Transitions were reconstructed over a concatenated three-gene alignment (Staats, van Baarlen and van Kan 2005) in Bayes multistate. Asterisks denote significant differences ($P < 0.05$, see METHODS) between optimal and constrained models. No constrained analysis was possible for *bik4* due to a lack of evidence for pseudogenes.

homologs in six of 10 *Botrytis* genomes representing 10 species (FIG. 1). One species, *B. tulipae*, contained homologs of all six bikaverin genes, five species (*B. hyacinthi*, *B. galanthina*, *B. elliptica*, *B. calthae*, *B. cinerea*) contained some genes of the cluster, while the remaining four (*B. paeoniae*, *B. convoluta*, *B. narcissicola*, *B. porri*) contain no evidence of having had the bikaverin gene cluster. A new assembly of *B. cinerea* (Staats and van Kan 2012) also revealed a small fragment of *bik1* (391/7741 bp) on the same scaffold as *bik2–6* (Supercontig 13) but displaced by 138 kb (SUPPLEMENTARY FIG. 1). All enzyme-encoding genes detected were inferred to be pseudogenes due to stop codons or point deletions interrupting open reading frames. The NMRA-like regulatory gene, *bik4*, is always intact at the sequence level when present, while the $Zn(II)_2Cys_6$ transcription factor, *bik5* and the major facilitator superfamily (MFS) transporter gene, *bik6,* can be found either intact or pseudogenized. Gene phylogenies made from each of the bikaverin genes (SUPPLEMENTARY FIG. 2A–F) show a well supported clade of *Botrytis* sequences. Conflict in gene-tree topology is observed among *bik3, bik5* and *bik6* and the *Botrytis* species phylogeny (SUPPLEMENTARY FIG. 2G), but branch lengths are short, bootstrap support is generally low and there is no consistent pattern of conflict among the *Botrytis* bikaverin genes. The *bik4* phylogeny is unresolved in *Botrytis*, and too few sequences exist to determine relationships among *bik1 and bik2*. A 3.5 kb locus containing two hypothetical genes adjacent to *bik6* in *B. cinerea* (bcin_15243, bcin_15244) on the 3′ flank is also partially present in *B. tulipae*, *B. hyacinthi* (bcin_15244 homolog only) and *B. elliptica* (intergenic only) in the same location. The most similar sequences to these 3′-flank sequences are found adjacent to *bik6* in *F. oxysporum* in reverse order compared to the *Botrytis* homologs (SUPPLEMENTARY FIG. 1H, 2).

Bikaverin regulatory genes in Botrytis *are more often retained than enzyme-encoding genes.*—All *Botrytis* bikaverin genes show accelerated evolution when compared to their *Fusarium* counterparts, indicating a relaxation of selection pressures on all bikaverin genes (TABLE I). Furthermore, a comparison of the relationship between gene loss and amount of evolutionary time elapsed indicates no correlation between divergence times and gene presence or absence, consistent with episodic pathway decay and individual gene loss versus gradual pathway decay over time (FIG. 1, Pearson's coefficient $r = 0.348$). By mapping the character states of the bikaverin genes (present and intact, absent or pseudogenized) we found evidence of at least three independent non-functionalization events ($F \rightarrow \Psi$ or $F \rightarrow A$) of the bikaverin cluster (FIG. 1), which also are supported by non-overlapping stop codons and indels among the pseudogenes of each gene family. A likelihood ratio test supported that the optimal rates of non-functionalization estimated in a Bayes multistate analysis (FIG. 3) are significantly more likely than the rates

TABLE II. Bikaverin transcript accumulation[a] data for *B. cinerea*[b] grown under different conditions and/or at different developmental stages

Experimental conditions		Gene				
		bik2	*bik3*	*bik4*	*bik5*	*bik6*
Glucose	RPKM	N/A	N/A	2.69	3.26	0.37
	Coverage	N/A	N/A	84.4	81.74	24.24
	Unique reads	N/A	N/A	19	47	3
Polygalacturonic acid	RPKM	0.2	N/A	3.23	1.89	0.71
	Coverage	11.41	N/A	86.14	65.42	32.32
	Unique Reads	2	N/A	20	24	5
Apothecia/sclerotia	RPKM	1.24	1.02	3.99	5.12	1.17
	Coverage	42.97	48.3	75.84	75.75	37.52
	Unique reads	12	9	24	63	8
Tomato leaves (16 hpi[c])	RPKM	N/A	N/A	N/A	N/A	N/A
	Coverage	N/A	N/A	N/A	N/A	N/A
	Unique reads	N/A	N/A	N/A	N/A	N/A
Tomato leaves (40 hpi)	RPKM	N/A	N/A	N/A	1.58	2.13
	Coverage	N/A	N/A	N/A	9.18	18.76
	Unique reads	N/A	N/A	N/A	4	3

[a] RPKM = sequence reads per kilobase of transcriptome per million reads; coverage = percent transcript length represented by at least one read; unique reads = those that can be mapped to only one genomic locus.

[b] All RNA sequence data were collected in strain B05.10 except apothecia/sclerotia data, which were collected in strains SAS405 and SAS56.

[c] hpi = hours post inoculation.

estimated when all rates are constrained to be equal ($F \rightarrow \Psi = F \rightarrow A = \Psi \rightarrow A$), for *bik1* ($P < 0.001$), *bik2* ($P < 0.001$) and *bik3* ($P < 0.01$). Transition rates for *bik5* and *bik6* show no significant differences in the constrained and unconstrained models of evolution, and no constrained analysis was possible for *bik4* due to a lack of evidence of transitions between functional and pseudogene.

Bikaverin regulatory gene expression is low in B. cinerea *B05.10.*—A small number of transcripts was detected for bikaverin genes in *B. cinerea* B05.10 under several experimental conditions (and in SAS405 and SAS56 for resting stages and fruiting bodies) through RNA sequencing (TABLE II). Transcript accumulation of *bik4* and *bik5* was highest, both in terms of unique reads as well as the percent of the gene length represented by at least one read, while other genes were poorly represented. *bik4* and *bik5* transcript accumulation was detected during growth on glucose or polygalacturonic acid medium, resting stages (sclerotia) and fruiting bodies (apothecia), but there was only limited evidence of transcript accumulation in *bik5* during infection of tomato leaves.

DISCUSSION

Gene cluster HGT events may be underestimated due to their repeated loss.—The horizontal transfer of the bikaverin cluster was a single event involving all six genes, which was not shown in analyses of the genomes of *B. cinerea* isolates B05.10 and T4 only (Campbell et al. 2012). The ancestral presence of all six genes has been corroborated very recently by the identification of an entire, functional bikaverin cluster in rare *B. cinerea* isolates (Schumacher et al. 2013). An alternative hypothesis of multiple horizontal transfers of bikaverin genes from a common donor is not likely due to shared flanking sequences across the *Botrytis* lineage, the relative rarity of HGT and the divergent niches among the *Botrytis* species with highly similar bikaverin gene sequences. If these same genomes were analyzed in isolation, only one out of nine additional genomes investigated here would show horizontal transfer of the whole cluster; four would suggest no HGT has occurred, two would suggest only partial transfer and two would suggest HGT of a single gene. The decay of the bikaverin cluster suggests that horizontal transfer of clusters may be underestimated in isolated genome analyses (Khaldi and Wolfe 2008, Morris et al. 2009, Mallet et al. 2010, Pombert et al. 2012). This is consistent with the hypothesis that an individual gene's fitness is improved if it is part of a gene cluster, which can confer a more complete function to the host following HGT (Lawrence 1999, Walton 2000). In fact, recent work has highlighted the role of self-contained modules that can be made of single genes

(Moran et al. 2012) or gene clusters (Hittinger et al. 2004; Slot and Hibbett 2007; Khaldi et al. 2008; Slot and Rokas 2010, 2011) in fungal evolution.

Multiple lines of evidence suggest different modes of evolution for the regulatory and enzyme-encoding genes in the bikaverin cluster.—Our results suggest that a complete, functional bikaverin cluster was horizontally transferred to the ancestor of the 10 sequenced *Botrytis* genomes. Evidence of independent pseudogenization involving the same gene in different lineages suggests the ancestral genes were intact, and retention of multiple genes through speciation events following HGT is consistent with selection for a conferred phenotype. However the distribution of genes in extant species is highly biased, with regulatory genes and the transporter more frequently found intact, while all enzyme-encoding genes are non-functionalized. Furthermore, the estimated dN/dS values for bikaverin genes found in multiple *Botrytis* species are greater in enzyme-encoding than in regulatory genes, which suggests different levels of selection. There also appears to be bias in the rates of transition between different states of functionality, which suggests either that metabolic genes have a greater propensity to persist as pseudogenes or that regulatory genes are more likely to be lost by deletion than by pseudogenization. The existence of a functional bikaverin cluster in some strains of *B. cinerea* suggests that this study has actually underestimated the rates of bikaverin enzyme-encoding gene loss of function. The lack of a correlation between time and gene absence further suggests gene loss is not a neutral process but instead affects different genes or classes of genes differently in the bikaverin cluster. Finally, preferential expression of regulatory genes in *B. cinerea* suggests an underlying mechanism for the differences in evolutionary trajectories among the different genes in the cluster. Two potential explanations for continued expression of regulatory genes could be (i) repurposing of these genes to a novel function, for which we have not seen additional supportive evidence, or (ii) an effect of the degree of pleiotropy on pathway loss (Martchenko et al. 2007, Rokas and Hittinger 2007). It has been hypothesized, for example, that enzyme-encoding genes of gene clusters could be lost more rapidly during the degradation of a gene cluster due to selection against mutated enzymes that result in toxic novel metabolites or accumulation of toxic metabolic intermediates (Hittinger et al. 2004, Campbell et al. 2012). Taken together the distinct patterns of evolution within the bikaverin cluster in *Botrytis* could indicate evolutionary modularity of cluster components.

Intriguingly, the pattern of gene loss in *Botrytis* also corresponds to the timing and level of expression of functional bikaverin genes in *Fusarium verticillioides* (Brown et al. 2012). This is significant because the relative expression pattern among genes can be expected to be conserved after successful HGT because the bikaverin cluster contains internal regulatory elements (Wiemann et al. 2009). Three significant correspondences between bikaverin cluster expression and decay can be drawn. In *F. verticillioides*, transcription of enzyme-encoding genes is (i) inversely correlated with the inferred rate of gene loss in *Botrytis*, (ii) inversely related to order of metabolism and (iii) occurs later than that of regulatory and transport genes. Consistent with these trends, the first gene to be expressed in *F. verticilioides* (*bik4*) is also the most often retained in *Botrytis* (and never found pseudogenized), and the last gene to be expressed (*bik1*) is the least often retained. We hypothesize that the pattern of bikaverin cluster expression reflects selection for precise order, which entails construction of the regulatory and secretory components before metabolite processing, and the capacity to process each metabolic intermediate before the production of the polyketide precursor compound. Fitness costs of toxic secondary metabolites and their precursors could drive this selection (Hurst et al. 2004, Slot and Rokas 2010), which also would impose constraints on the order in which gene inactivations are tolerated. An inverse relationship between the time of gene expression and the rate of gene loss also could reflect transcription-linked bias in DNA repair (Hanawalt and Spivak 2008). We are intrigued by the possibility that the non-random order of gene cluster decay could point to selective mechanisms rather than neutral processes driving the formation and persistence of gene clusters in fungi.

The loss of bikaverin clusters may reflect the emergence of virulence in some strains of Botrytis.—The transfer of the bikaverin cluster from *Fusarium* to *Botrytis* might have occurred in soil by the uptake of *Fusarium* DNA into (damaged) *Botrytis* sclerotia, which was shown to facilitate stable genetic transformation (Ish Shalom et al. 2011). The acquisition of the bikaverin cluster may have given such *Botrytis* ''transformants'' a selective advantage to survive as a saprotroph in soil in the presence of fungivore nematodes and microbes. It is becoming more apparent that lineages of agriculturally important necrotrophic pathogens, including *Botrytis*, are derived from larger populations and communities of asymptomatic endophytes (Shipunov et al. 2008, Sowley et al. 2010) that may provide protection to plants against pests or pathogens through the production of specialized metabolites such as bikaverin (Balan et al. 1970, Kwon et al.

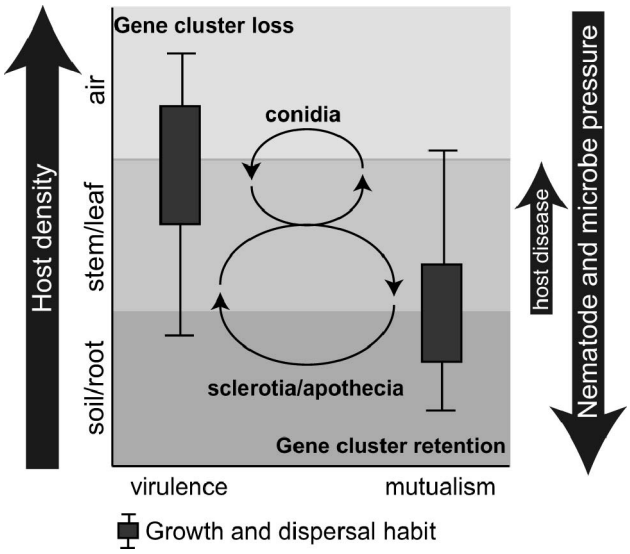

FIG. 4. Environmental factors influencing bikaverin gene evolution: a hypothesis. Fitness benefits conferred by bikaverin to *Botrytis* spp. and the host plant are more pronounced in soil and root tissue due to pressure from soil-borne pathogens. A prolonged endophytic life cycle followed by a limited necrotrophic phase, the development of overwintering structures and subsequent sexual reproduction in the soil therefore favors retention of a functional bikaverin cluster. Conversely, monocultures could act to favor more aerial, infectious growth and thereby relax selection to maintain a functional bikaverin cluster through prolonged clonal pathogenic growth.

2007, Son et al. 2008). In the case of bikaverin production in *Botrytis*, we hypothesize that cluster loss is associated with the emergence of virulence. While our sample of *Botrytis* genomes is biased toward virulent strains, a more comprehensive investigation of *Botrytis* genomes from natural environs may reveal less optimization toward pathogenicity (Nielsen and Heitman 2007) in general and retention of pathways that are beneficial to the host plant. It is instructive therefore that a number of isolates of *B. cinerea* maintain functional bikaverin clusters (Schumacher et al. 2013); recent loss of the bikaverin cluster could be expected given our finding of multiple independent losses over a relatively short time. Bikaverin may benefit *Botrytis* strains by facilitating survival of the sexual and resting states in the soil, under selective pressure from nematodes and microbes. Selection to retain bikaverin production may be reduced in the pathogenic stage, when *Botrytis* grows in aerial plant parts and disperses predominantly by wind or insect vectors. We support a model (FIG. 4) of the sporadic transition from a reservoir of endophytes to (facultative) necrotrophy. We speculate that high host density, such as in agricultural monocultures, could act to favor prolonged pathogenic growth and reduce the fitness cost of early host death because the fungus would be able to readily disperse to nearby hosts with highly similar genotypes. In this model, the combination of a prolonged asexual phase on aboveground plant parts, recurrent aerial dispersal of conidia during the entire growing season and a reduced pressure on soil-borne resting stages (sclerotia) and fruiting bodies (apothecia) by fungivore nematodes and microbes is expected to result in the loss of pathways that are more relevant to endophytic growth in the host and saprotrophic survival in the soil than to pathogenic growth. We therefore propose investigations of the genomic diversity of asymptomatic *Botrytis* endophytes to test this hypothesis.

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LITERATURE CITED

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST : a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402, doi:10.1093/nar/25.17.3389

Balan J, Fuska J, Kuhr I, Kuhrova V. 1970. Bikaverin, an antibiotic from *Gibberella fujikuroi*, effective against *Leishmania brasiliensis* Springer. Folia Microbiol 15: 479–484, doi:10.1007/BF02880192

Brown DW, Butchko RAE, Busman M, Proctor RH. 2012. Identification of gene clusters associated with fusaric acid, fusarin and perithecial pigment production in *Fusarium verticillioides*. Fungal Genet Biol 49:521–532, doi:10.1016/j.fgb.2012.05.010

Campbell MA, Rokas A, Slot JC. 2012. Horizontal transfer and death of a fungal secondary metabolic gene cluster. Genome Biol Evol 4:289–293, doi:10.1093/gbe/evs011

Darling AE, Mau B, Perna NT. 2010. Progressive mauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS ONE 5:e11147, doi:10.1371/journal.pone.0011147

Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214, doi:10.1186/1471-2148-7-214

Hanawalt PC, Spivak G. 2008. Transcription-coupled DNA repair: two decades of progress and surprises. Nat Rev Mol Cell Biol 9:958–970, doi:10.1038/nrm2549

Hittinger C, Rokas A, Carroll S. 2004. Parallel inactivation of multiple GAL pathway genes and ecological diversifi-

cation in yeasts. Proc Natl Acad Sci USA 101:14144–14149, doi:10.1073/pnas.0404319101

Hurst L, Pal C, Lercher M. 2004. The evolutionary dynamics of eukaryotic gene order. Nat Rev Genet 5:299–310, doi:10.1038/nrg1319

Ish Shalom S, Gafni A, Lichter A, Levy M. 2011. Transformation of *Botrytis cinerea* by direct hyphal blasting or by wound-mediated transformation of sclerotia. BMC Microbiol 11:266, doi:10.1186/1471-2180-11-266

Keller NP, Turner G, Bennett JW. 2005. Fungal secondary metabolism—from biochemistry to genomics. Nat Rev Microbiol 3:937–947, doi:10.1038/nrmicro1286

Khaldi N, Collemare J, Lebrun M-H, Wolfe KH. 2008. Evidence for horizontal transfer of a secondary metabolite gene cluster between fungi. Genome Biol 9:R18, doi:10.1186/gb-2008-9-1-r18

———, Wolfe KH. 2008. Elusive origins of the extra genes in *Aspergillus oryzae*. PLoS ONE 3:e3036, doi:10.1371/journal.pone.0003036

Kwon HR, Son SW, Han HR, Choi GJ, Jang KS, Choi YH, Lee S, Do Sung N, Kim J-C. 2007. Nematicidal activity of bikaverin and fusaric acid isolated from *Fusarium oxysporum* against pine wood nematode, *Bursaphelenchus xylophilus*. Plant Pathol J 23:318–321, doi:10.5423/PPJ.2007.23.4.318

Lavoie H, Hogues H, Whiteway M. 2009. Rearrangements of the transcriptional regulatory networks of metabolic pathways in fungi. Curr Opin Microbiol 12:655–663, doi:10.1016/j.mib.2009.09.015

Lawrence J. 1999. Selfish operons: the evolutionary impact of gene clustering in prokaryotes and eukaryotes. Current Opin Genet Dev 9:642–648, doi:10.1016/S0959-437X(99)00025-8

Maddison WP, Maddison DR. 2003. MACCLADE 4.06: analysis of phylogeny and character evolution. Sunderland, Massachusetts: Sinauer Associates.

Mallet LV, Becq J, Deschavanne P. 2010. Whole genome evaluation of horizontal transfers in the pathogenic fungus *Aspergillus fumigatus*. BMC Genomics 11:171, doi:10.1186/1471-2164-11-171

Martchenko M, Levitin A, Hogues H, Nantel A, Whiteway M. 2007. Transcriptional rewiring of fungal galactose-metabolism circuitry. Curr Biol 17:1007–1013, doi:10.1016/j.cub.2007.05.017

Moran Y, Fredman D, Szczesny P, Grynberg M, Technau U. 2012. Recurrent horizontal transfer of bacterial toxin genes to eukaryotes. Mol Biol Evol 29:2223–2230, doi:10.1093/molbev/mss089

Morris PF, Schlosser LR, Onasch KD, Wittenschlaeger T, Austin R, Provart N. 2009. Multiple horizontal gene transfer events and domain fusions have created novel regulatory and metabolic networks in the oomycete genome. PLoS ONE 4:e6133, doi:10.1371/journal.pone.0006133

Nielsen K, Heitman J. 2007. Sex and virulence of human pathogenic fungi. Adv Genet 57:143–173, doi:10.1016/S0065-2660(06)57004-X

Pagel M, Meade A, Barker D. 2004. Bayesian estimation of ancestral character states on phylogenies. Syst Biol 53:673–684, doi:10.1080/10635150490522232

Patron NJ, Waller RF, Cozijnsen AJ, Straney DC, Gardiner DM, Nierman WC, Howlett BJ. 2007. Origin and distribution of epipolythiodioxopiperazine (ETP) gene clusters in filamentous ascomycetes. BMC Evol Biol 7:174, doi:10.1186/1471-2148-7-174

Pombert J-F, Selman M, Burki F et al. 2012. Gain and loss of multiple functionally related, horizontally transferred genes in the reduced genomes of two microsporidian parasites. Proc Natl Acad Sci USA 109:12638–12643, doi:10.1073/pnas.1205020109

Rokas A, Hittinger C. 2007. Transcriptional rewiring: The proof is in the eating. Curr Biol 17:626–628, doi:10.1016/j.cub.2007.06.025

Schumacher J, Gautier A, Morgant G et al. 2013. A functional bikaverin biosynthesis gene cluster in rare strains of *Botrytis cinerea* is positively controlled by VELVET. PLoS ONE 8:e53729, doi:10.1371/journal.pone.0053729

———, Pradier J-M, Simon A, Traeger S, Moraga J, Collado IG, Viaud M, Tudzynski B. 2012. Natural variation in the VELVET gene *bcvel1* affects virulence and light-dependent differentiation in *Botrytis cinerea*. PLoS ONE 7:e47840, doi:10.1371/journal.pone.0047840

Shapiro B, Rambaut A, Drummond AJ. 2006. Choosing appropriate substitution models for the phylogenetic analysis of protein-coding sequences. Mol Biol Evol 23:7–9, doi:10.1093/molbev/msj021

Shipunov A, Newcombe G, Raghavendra AKH, Anderson CL. 2008. Hidden diversity of endophytic fungi in an invasive plant. Am J Bot 95:1096–1108, doi:10.3732/ajb.0800024

Slot J, Hibbett D. 2007. Horizontal transfer of a nitrate assimilation gene cluster and ecological transitions in fungi: a phylogenetic study. PLoS ONE 2:e1097, doi:10.1371/journal.pone.0001097

———, Rokas A. 2010. Multiple GAL pathway gene clusters evolved independently and by different mechanisms in fungi. Proc Natl Acad Sci USA 107:10136–10141, doi:10.1073/pnas.0914418107

———, ———. 2011. Horizontal transfer of a large and highly toxic secondary metabolic gene cluster between fungi. Curr Biol 21:134–139, doi:10.1016/j.cub.2010.12.020

Sokal, Robert R. 1995. Biometry: the principles and practice of statistics in biological research/Robert R. Sokal And F. James Rohlf.

Son SW, Kim HY, Choi GJ, Lim HK, Jang KS, Lee SO, Lee S, Sung ND, Kim JC. 2008. Bikaverin and fusaric acid from *Fusarium oxysporum* show antioomycete activity against *Phytophthora infestans*. J Appl Microbiol 104:692–698, doi:10.1111/j.1365-2672.2007.03581.x

Sowley ENK, Dewey FM, Shaw MW. 2010. Persistent, symptomless, systemic and seed-borne infection of lettuce by *Botrytis cinerea*. Eur J Plant Pathol 126:61–71, doi:10.1007/s10658-009-9524-1

Staats M, van Baarlen P, van Kan JAL. 2005. Molecular phylogeny of the plant pathogenic genus *Botrytis* and the evolution of host specificity. Mol Biol Evol 22:333–346, doi:10.1093/molbev/msi020

———, van Kan JAL. 2012. Genome update of *Botrytis cinerea* strains B05.10 and T4. Eukaryot Cell 11:1413–1414, doi:10.1128/EC.00164-12

Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690, doi:10.1093/bioinformatics/btl446

Walton J. 2000. Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: a hypothesis. Fungal Genet Biol 30:167–171, doi:10.1006/fgbi.2000.1224

Wiemann P, Willmann A, Straeten M, Kleigrewe K, Beyer M, Humpf H-U, Tudzynski B. 2009. Biosynthesis of the red pigment bikaverin in *Fusarium fujikuroi*: genes, their function and regulation. Mol Microbiol 72:931–946, doi:10.1111/j.1365-2958.2009.06695.x

Yang Z. 2007. PAML 4: phylogenetic analysis by maximum likelihood. Mol Biol Evol 24:1586–1591, doi:10.1093/molbev/msm088

Zerbino, Daniel R, Ewan, Birney. 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res 18:821–829, doi:10.1101/gr.074492.107