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ORIGINAL RESEARCH

Interkingdom Gene Transfer May Contribute to the Evolution of Phytopathogenicity in *Botrytis Cinerea*

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Abstract: The ascomycete *Botrytis cinerea* is a phytopathogenic fungus infecting and causing significant yield losses in a number of crops. The genome of *B. cinerea* has been fully sequenced while the importance of horizontal gene transfer (HGT) to extend the host range in plant pathogenic fungi has been recently appreciated. However, recent data confirm that the *B. cinerea* fungus shares conserved virulence factors with other fungal plant pathogens with narrow host range. Therefore, interkingdom HGT may contribute to the evolution of phytopathogenicity in *B. cinerea.* In this study, a stringent genome comparison pipeline was used to identify potential genes that have been obtained by *B. cinerea* but not by other fungi through interkingdom HGT. This search led to the identification of four genes: a UDP-glucosyltransferase (UGT), a lipoprotein and two alpha/beta hydrolase fold proteins. Phylogenetic analysis of the four genes suggests that *B. cinerea* acquired UGT from plants and the other 3 genes from bacteria. Based on the known gene functions and literature searching, a correlation between gene acquision and the evolution of pathogenicity in *B. cinerea* can be postulated.

Keywords: *Botrytis cinerea*, horizontal gene transfer, phylogeny, phytopathogenicity

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Introduction

Botrytis cinerea is an ascomycete responsible for gray mould on hundreds of dicot plants.[1–3] The broad host range of *B. cinerea* may be due to its numerous virulence factors and the ability to thrive on diverse host plants by subverting the resistance mechanisms acquired by plants to combat other pathogens.[4,5]

Horizontal gene transfer (HGT) has been regarded as a driving forces in the innovation and evolution of genomes in fungi and other eukaryotes.[6,7] In particular, HGT maybe bestow a clear selective advantage to fungi.[8] Indeed, Rosewich and Kistler suggested that HGT may play a greater role in fungal evolution than other eukaryotes although HGT in fungi there still remains "reasonable doubt".[9] Furthermore, Mallet et al estimated that the horizontally transferred genes account for about 2.2% of the total genes in the pathogenic fungus *Aspergillus fumigates*.[10] The only direct evidence for HGT in *B. cinerea* is that a nuclear rDNA intron might have been transferred from a hypothetical *Myriosclerotinia*-like ancestor to *Botrytis* species.[11,12] The contribution of HGT to the evolution and virulence of *B. cinerea* is largely unexplored.

The importance of HGT to extend the host range in plant pathogenic fungi has been recently appreciated,[4] which provides a clue to understand the broad host range of *B. cinerea*. However, recent data confirm that the gray mold fungus shares conserved virulence factors with other fungal plant pathogens with a narrower host range.[13] In addition, 20 genes participating in other fungal virulence have been identified in *B. cinerea* based on the method of gene inactivation.[13] In order to fully elucidate the evolution and virulence of *B. cinerea,* it will be crucial to identify potential genes that have been obtained by *B. cinerea* but not by other fungi through interkingdom HGT.

Many researchers have revealed that interkingdom HGT is widespread in fungi and may be important to the evolution of fungal metabolism, propagation and pathogenecity.[14,15] Indeed, the biotin formation associated genes acquired from bacteria are required for lipid and leucine metabolism in several fungi.[16,17] In addition, Gojkovic et al indicated that dihydroorotate dehydrogenase (DHODase) gene acquired from bacteria is important for yeast to propagate under anaerobic conditions. [18] However, it is still unclear for the role of interkingdom HGT in the evolution and virulence of *B. cinerea.*

Some parametric methods, which are based on the compositional characteristics, such as GC content and codon usage, have been applied in the detection of gene transfers, but these methods have been demonstrated to be unreliable without phylogenetic analysis.[7,19] Indeed, the detection of gene transfers is best achieved by generation of a strongly supported phylogenetic tree which contradicts the known species phylogeny.[6,7] Luckily, the genome of *B. cinerea* has been recently fully sequenced and deposited in NCBI.[20] This provides a strong basis for the overall understanding of the existence and functions of HGTs in *B. cinerea* based on phylogenetic analysis.

In this study, the interkingdom HGT events between *B. cinerea* and other distantly related species were investigated by sequence comparison of all predicted protein coding sequences from *B. cinerea* with the genome sequences of 87 eukaryotes and 295 prokaryotes (Supplementary Fig. 1), which represent a wide taxonomic diversity, and generated individual phylogenetic trees by different models in combination with other evidences (for example intronless in the transferred genes) for every gene that showed a higher sequence similarity to fungi than to any other species. This process identified four putative gene transfers. This study gives us a first glance to understand the role of interkingdom HGT in the evolution and virulence of *B. cinerea* by whole genome analysis.

Materials and Methods

Genome

The genome sequence of *B. cinerea* was downloaded from NCBI, which has 38.87 million base pairs (Mbp) distributed in 4,537 contigs with 16,389 identified protein coding genes.[20]

Local database generation

In order to construct the phylogenetic trees for each sequence identified with the target taxon/similarity profile automatically, a local database was constructed using a 300-core blade server in the IBM Biocomputing Laboratory, Zhejiang University. The constructed database contains predicted protein sequences (2,385,947 coding sequences in total) from 382 species representing a wide diversity of eukaryote and prokaryote taxa including 87 eukaryotes (from insects, plants,

fungi and animals) and 295 prokaryotes (Supplementary Fig. 1).

Search procedure

Each candidate sequence in *B. cinerea* was compared against sequences in the local database using BLASTp and the the highest similar sequences from each species were extracted for further analysis.[21] Homologs were searched in the genome with BLASTp and selected when E-value $<10^{-5}$. We considered two genes as homologs if they aligned through more than 80% of their sequence and were >40% similar.[22] OrthoMCL[23] was used to generate groups of orthologous proteins from homologs with default parameters. We used the local database first to exclude the genes that have homologous genes in *Sclerotinia sclerotiorum*, a closely related fungus with *B. cinerea* (Fig. 1). Furthermore, the remaining sequences that have a higher BLASTp similarity score to fungi than to any other species were removed (Fig. 1). In addition, sequences that have no hits except themselves were also excluded from further analysis. The detailed procedure was showed in Figure 1.

Phylogenetic analysis

The remaining candidate HGT genes were selected to search against GenBank non-redundant protein database (nr). Search strategy was the same as described above. These sequences were aligned using Clustal W,[24] and the conserved region of each alignment was trimmed using Gblocks with stringent settings described previously.[25,26] Maximum Likelihood (ML) phylogenies were constructed by Phyml[27] using the JTT model as suggested by ModelGenerator,[28] and a gamma distribution with eight rate categories. The proportion of invariant sites was estimated from the data. For Bayesian phylogenies generated by MrBayes,[29] two independent Metropolis-coupled Monte Carlo Markov Chain (MCMC) runs, each with one cold and three heated chains (heat parameter = default), were analyzed for one million generations after a burn-in of 25,000 samples and allowed mixed models of amino acid substitution. For Maximum Likelihood phylogenies, 1,000 bootstraps were performed to gain the branch support values. To be considered as being indicative of a potential HGT event, the ML branch support about *B. cinerea* should be ≥80% while Bayesian posterior probability should be ≥85%.[30]

To test the support for contentious topology, we performed nonparametric branch support tests based on a Shimodaira-Hasegawa-like procedure using Tree-Puzzle.[31] In addition, we used this software to test the statistical significance (5%) of specific topology over a collapsed version of the same branching relationship.

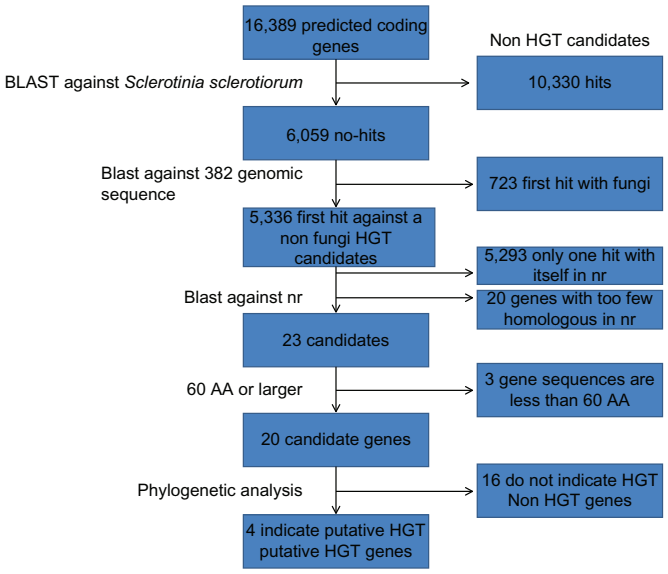

Figure 1. Flowchart of the methodology used to search for HGT genes in *Botrytis cinerea* and of the results of each step.

Results

HGT candidates search

This study used a pipeline with several filters to search for *B. cinerea* predicted coding genes that are candidates obtained by HGT as summarized in Figure 1. In the first filter, the 16,389 predicted coding genes of *B. cinerea* were compared with genomic sequences of *S. sclerotiorum* available in GenBank, which excluded 10,330 genes as possible interkingdom HGT candidates since they have homologs in this closely related fungus (Fig. 1). In the second filter, every predicted *B. cinerea* protein coding sequence that showed a higher BLAST similarity score to a gene from any other species other than fungi was retained for subsequent analysis, while sequences that had no hits except themselves were excluded from further analysis. This filter screen resulted in 23 clusters which were considered to be candidate HGT genes in *B. cinerea*. In the third filter, we removed three candidate HGT genes encoding predicted proteins shorter than 60 amino acids (AA) as too short sequences to contain enough phylogenetically information sites.

The 20 HGT candidates produced 16 phylogenies that did not support the HGT hypothesis (Fig. 1). For example, a phylogenetic tree was constructed based on BC1G_01701, a hypothetical gene sequence (Supplementary Fig. 2). However, the low branch support values failed to support the validity of this phylogeny, indicating that the 16 genes should be excluded from further analysis. The remaining four genes were considered reliable HGT candidates, which were validated by Bayesian and Maximum Likelihood phylogenetic methods (both methods inferred the same topology). In all these data sets, the SH test also showed the HGT topology at the 5% significance level.[32] The general information about the four genes was presented in Table 1 and the phylogenetic trees of these genes were shown in Supplementary Figures 3–5.

Putative functional assignment of four HGTs

The potential identities and biological functions of the 4 HGT candidates were investigated based on the similarity of their putatively encoded products to known proteins. Results from this study indicated that the 3 putative transferred genes are enzymes, which suggested a strong functional trend among these HGTs in *B. cinerea*. The first putative horizontally transferred gene found in *B. cinerea* is a UDP-glucosyltransferase (UGT) (BC1G_03266, PF00201, e-value $3e^{-5}$), which catalyzes the transfer of glucuronic acid to a wide variety of exogenous and endogenous lipophilic substrates.[33] The second and third HGT are two putative alpha/beta hydrolase fold proteins (BC1G_05874, PF00561, e-value $4.4e^{-11}$ and BC1G_15488, PF00561, e-value $5.1e^{-5}$). The fourth HGT candidate showed sequence similarity to a lipoprotein (BC1G_10872, PF12006, $2.06e^{-74}$), which is a biochemical assembly that contains both proteins and lipids water-bound to the proteins.

Phylogenetic Analysis of Four HGTs

Phylogenomic analysis revealed three candidate HGTs from bacteria to *B. cinerea* and one HGT from plant to *B. cinerea*. The phylogeny of UGT indicated that *B. cinerea* gene was grouped with those of *Hieracium pilosella* and *Ricinus communis* with 100% ML branch support and Bayesian posterior probability while the sister group is also a group of plant species (Fig. 2), which constitutes very strong evidence for this phylogeny. Thus, this phylogeny supports one HGT event from *Hieracium pilosella* or unsampled plant species to *B. cinerea* (Fig. 2).

Two putative alpha/beta hydrolase fold proteins (BC1G_05874 and BC1G_15488) were encoded by an HGT candidate from *Burkholderia* species (Supplementary Fig. 3) and *Nakamurella multipartite* (Supplementary Fig. 4) to *B. cinerea*, respectively. The enzymes are believed to have diverged from a common ancestor, preserving the arrangement of the catalytic residues.[34] In general, homologs for BC1G_05874 and BC1G_15488 were found in a diverse range of bacteria while *B. cinerea* grouped with *Burkholderia* species with 100% ML branch support and Bayesian posterior probability in BC1G_05874 phylogeny, grouped with *Nakamurella multipartita* with 95% ML branch support and Bayesian posterior probability in BC1G_15488 phylogeny, indicating very strong evidence for the two phylogenies.

Another candidate HGT was a gene transferred from *Acetobacter pasteurianus* to *B. cinerea* (Supplementary Fig. 5). Homologs for this gene were found in both

Table 1. HGT candidates in the *Botrytis cinerea* genome.

Gene name	Gene ID	Top hit organism	Accession number	E-value*	SH test[$]
UDP-glucosyltransferase	BC1G_03266	*Hieracium pilosella*	ACB56927.1	$3e^{-92}$	1.000
Alpha/beta hydrolase	BC1G_05874	*Burkholderia* sp. H160	ZP_03266005.1	$1e^{-88}$	0.983
Alpha/beta hydrolase	BC1G_15488	*Nakamurella multipartita* DSM 44233	YP_003199962.1	$1e^{-51}$	1.000
Lipoprotein	BC1G_10872	*Acetobacter pasteurianus* IFO 3283-01	YP_003188039.1	$3e^{-66}$	0.947

Notes: *Values to the top hits; [$]5% confidence level was used in SH test.

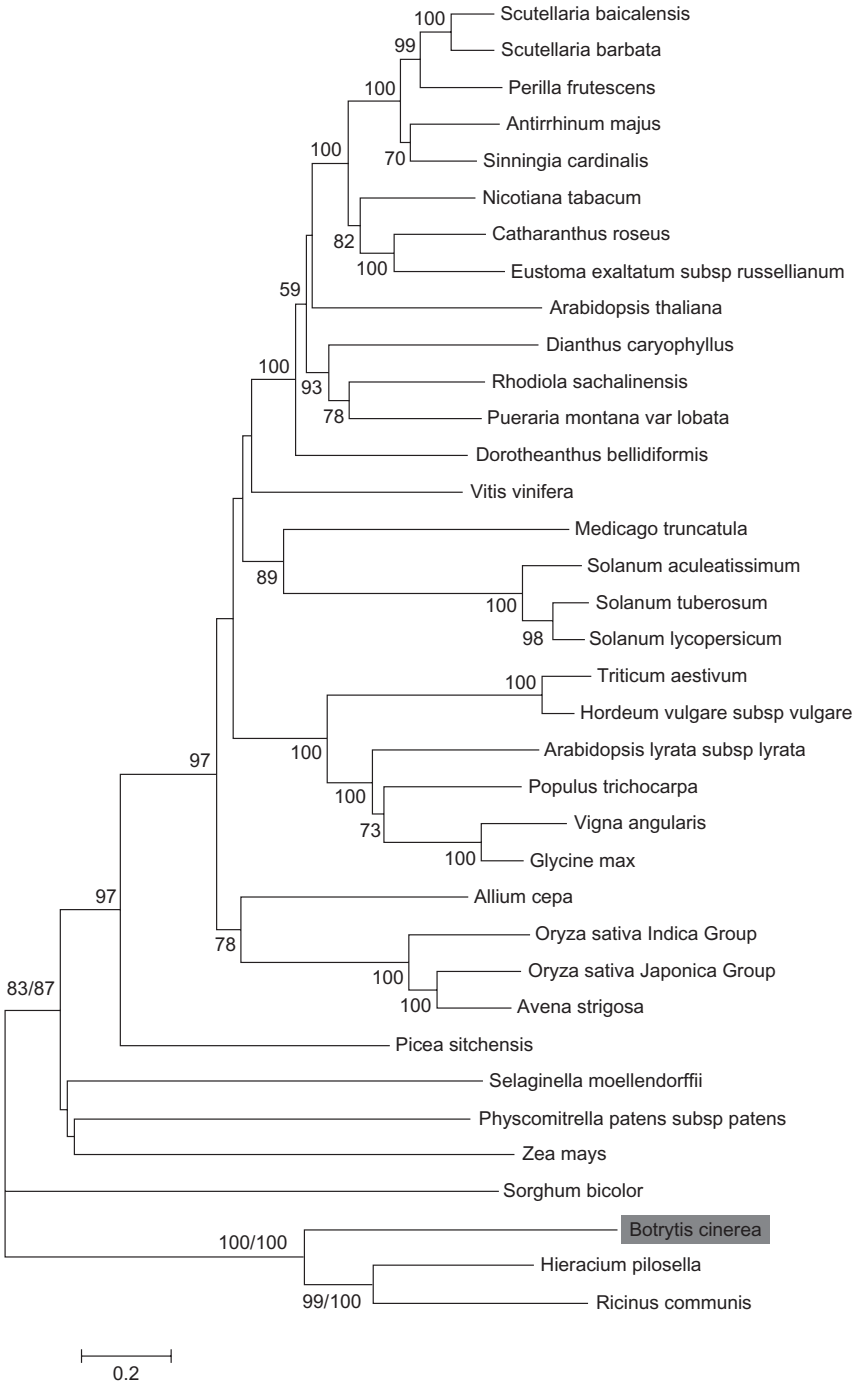

Figure 2. A phylogeny of the putative UDP-glucosyltransferase (UGT) (BC1G_03266). The phylogenetic tree shown was calculated using the Maximum Likelihood (ML) program PhyML and Bayesian program Mr. bayes (detailed parameters were described in the main text of paper). Only the values in ML ≥ 80% and Bayesian posterior probability ≥85% were shown. For key nodes the actual support values are shown in the order ML bootstraps/ Bayesian posterior probability.
Note: Bar, 0.2 substitution per site.

bacteria and fungi. The phylogeny of this gene indicated that *B. cinerea* was grouped with *Acetobacter pasteurianus* and other bacteria with 82% ML branch support and 85% Bayesian posterior probability, while other fungi were grouped in a different clade apart from *B. cinerea* with 100% ML branch support and Bayesian posterior probability (Supplementary Fig. 5). The result suggested that a HGT event from *Acetobacter pasteurianus* or unsampled bacteria to *B. cinerea* is evident.

Discussion

HGT detection method based on phylogenetic analysis is believed to be the most robust if gene phylogenies can be reconstructed with reliability.[30] However, it may take several months to construct all the phylogenies based on all the genes in the genome. Therefore, it is necessary to reduce the number of potential HGT candidates before performing phylogenetic analysis since computing resources required to reconstruct the phylogeny of all genes found in a complete genome is usually very extensive.[30] In order to reduce the number of candidate genes, in this study we used a local database instead of NCBI NR as first filter, which includes 382 genomes from a wide diversity of eukaryotic and prokaryotic species.

Choquer et al (2007) revealed that *B. cinerea* shares conserved virulence factors with other fungal plant pathogens with a narrower host range based on the method of gene inactivation.[13] However, this may be because these gene inactivation studies of *B. cinerea* were carried out based on the known virulence-related genes in other fungi. In order to find the new pathogenicity related genes, which only exists in *B. cinerea* but not in other fungi, this research excluded all *B. cinerea* genes that have homologous genes in other fungi and focused on this role of interkingdom HGT in the evolution and virulence of *B. cinerea*.

The potential HGT events between *B. cinerea* and other fungi were neglected in this study. In general, it may be a little arbitrary to rule out the possibility that *B. cinerea* acquired pathogenic genes from other fungi. However, recent studies highlight specific roles in the pathogenicity of some signaling components of *B. cinerea* when compared with other fungal pathogens.[13] In particular, some genes such as *BMP*3 and *bcSak*1 are involved in pathogenicity of *B. cinerea*, but not in other fungi,[13] indicating the complex in the evolution of phytopathogenicity in *B. cinerea.* These results make it difficult to understand the role of the potential transferred genes from other fungi in pathogenicity of *B. cinerea*.

The results of this study indicated that four out of 16,389 identified protein coding genes were identified as HGT candidates based on the phylogenetic method, which is best worked with the genes from phylogenetically distant species.[35,36] The proportion of transferred genes is really less than that of other fungi,[10] which may be due to the stringent filters used to identify HGT events in this study. Indeed, the application of this procedure in this study may cause the neglect of several potential HGT events. First, the HGT in the common ancestor of fungi may be considered as vertical inheritance in this study if none of the other sampled taxons other than fungi has homolog. However, we cannot exclude the possibility of an HGT event from an extinct linkage.[37] Second, our filter could also remove genes from other fungi to *B. cinerea* by multiple HGT events as we only retained the genes which showed a higher BLAST similarity score to a gene from any other species other than fungi (Material and Methods).

Marcet-Houben and Gabaldón described the transfers of a bacterial catalase to three important plant pathogens: the Dothideomycetes *Stagonospora nodorum* and *Mycosphaerella fijiensis* and *B. Cinerea*.[14] The large evolutionary distance between the first two species and *B. cinerea* and phylogenetic analysis suggests that *B. cinerea* acquired the bacterial catalase from a Dothideomycetes. Although catalases can help pathogens overcome host defense mechanisms, we failed to detect the HGT event in this study because the genes showed a higher BLAST similarity score to a gene from fungi were excluded.

In this study, the direction of transfer from prokaryote and plant to *B. cinerea* is strongly suggested. As shown in the phylogenetic trees, *B. cinerea* was nested within bacterial or plant clades, while many bacterial or plant species formed basal branches. Although this can be explained by "loss" hypothesis, which suggests an ancient HGT from the common ancestor of fungi to *B. cinerea* while loss in the other fungal species. However, this is highly unlikely for three reasons. First, it requires massive independent losses to explain the presence of these genes only in *B. cinerea*. Second, this hypothesis has difficulty in explaining the remarkable sequence identity between *B. cinerea* and its candidate donors (Table 1). Further, all of these transferred genes are intronless. Based on these reasons, the direction of transfer from prokaryote and plant to *B. cinerea* is mainly confirmed.

In agreement with the result in this study, most of interkingdom HGT events were plastids-eukaryote transfer, while only a few instances of potential HGTs

between eukaryotes are known.[6,38] Furthermore, interkingdom HGTs from prokaryotes to eukaryotes are important for the evolution of fungi. Interestingly, Marcet-Houben and Gabaldón recently detected seven transferred genes in *B. cinerea* by searching for genes present in few (<10) fungi and absent in other eukaryotes,[14] but which could be found in a relatively high number of prokaryotic genomes (>30). This is inconsistent with our results that *B. cinerea* acquired three genes from bacteria. This contrary result may be due to the difference in the stringency of the pipeline.

The function of transferred genes in *B. cinerea* need to be fully elucidated based on the method of gene inactivation. However, the four HGT candidates in this study may be all related to the pathogenicity of *B. cinerea*. UGT is able to detoxify deoxynivalenol produced by phytopathogens, which is a kind of pathogenetic factor, and can inhibit protein biosynthesis and presumably interfere with the expression of plant defense-response genes.[39,40] Therefore, the transferred gene may be involved in the interaction between *B. cinerea* and plant hosts. Furthermore, Alpha/beta hydrolase fold proteins are rapidly becoming one of the largest groups of structurally related enzymes with diverse catalytic functions.[41] These enzymes have been reported to regulate the interactions between pathogenic bacteria and their hosts,[42,43] indicating the potential importance of both HGT candidate genes in the pathogenecity of *B. cinerea*. In addition, the lipoprotein is a major component of the outer membrane of bacteria while Zhang et al revealed the released lipoprotein is an important virulence factor.[44]

In general, result from this study indicated that interkingdom HGT events between *B. cinerea* and bacteria or plants may be the most consistent explanation for either the taxonomic distribution or the phylogenies of the four genes using comparative genomics and phylogenetic analysis based on ML and Bayesian analysis as well as comparative topology tests. However, some studies have showed that high support values for a clade encompassing distantly related organisms do not necessarily indicate HGT, because stochastic variations on nucleotide and amino acid composition can happen to cause well-supported topology, which has little biological meaning.[45] In addition, we also could not rule out the possibility that the HGT candidates with the highest BLASTP score is not necessarily the donor species because the true donor may not have been sequenced. However, as shown in this study, *B. cinerea* is the only fungus species in these phylogenetic trees. Therefore, it could be concluded that these genes were transferred from bacterial or plant species.

It is well known that fungal pathogens were able to acquire genes from bacterial pathogens. Furthermore, Martínez suggested that pathogens can acquire pathogenecity-related genes from natural environments,[46] which may explain the result of this study that *B. cinerea* was grouped with bacteria of environmental origin in the phylogeny of the lipoprotein gene. The phylogenetic positions of several bacteria of environmental origin based on the lipoprotein gene were in contrast with their taxonomy position, indicating massive HGTs between bacteria.[47] In addition, McClure has reported that pathogens can acquire genes from their hosts to enhance their replication in host tissues.[48] Interestingly, *Hieracium pilosella* is a kind of chrysanthemum, which is the host species of *B. cinerea*.[49] This provided strong support for plant-to-*B. cinerea* HGT event in our study.

In contrast with the other transferred genes, homologs for the lipoprotein gene were also found in fungi other than *Sclerotinia sclerotiorum* while this gene showed a higher BLASTp similarity score to bacteria than to fungi. The *B. cinerea*-donor hypothesis was clearly excluded because *B. cinerea* is not clustered with fungal group. The phylogeny of this gene indicated that *B. cinerea* was grouped with bacteria, while other fungi were grouped in a different clade apart from *B. cinerea*. In addition, the phylogenetic positions of these other fungi based on the lipoprotein gene are nearly consistent with the phylogeny based on the beta-tubulin sequences, which has been widely used in fungal phylogeny.[50] This result constitutes very strong evidence in this phylogeny.

In summary, results from this study clearly indicated that interkingdom HGTs have occurred between *B. cinerea* and bacteria or plants based on large-scale genome sequence data analysis using a strict and highly conservative set of ML and Bayesian methods in combination with topology tests. In addition, function analysis of four transferred genes revealed these interkingdom

HGT events may at least partially contribute to the evolution of phytopathogenicity in *B. cinerea*.

Author Contributions

BZ conceived of the study. All authors contributed to data collection. BZ, SLZ, QZ and GQZ analyzed the data and prepared the report. All authors provided critical review of the draft and approved the final version.

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Disclosures

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Supplementary Materials

Figure S1. Genomes used for HGT identification pipeline.

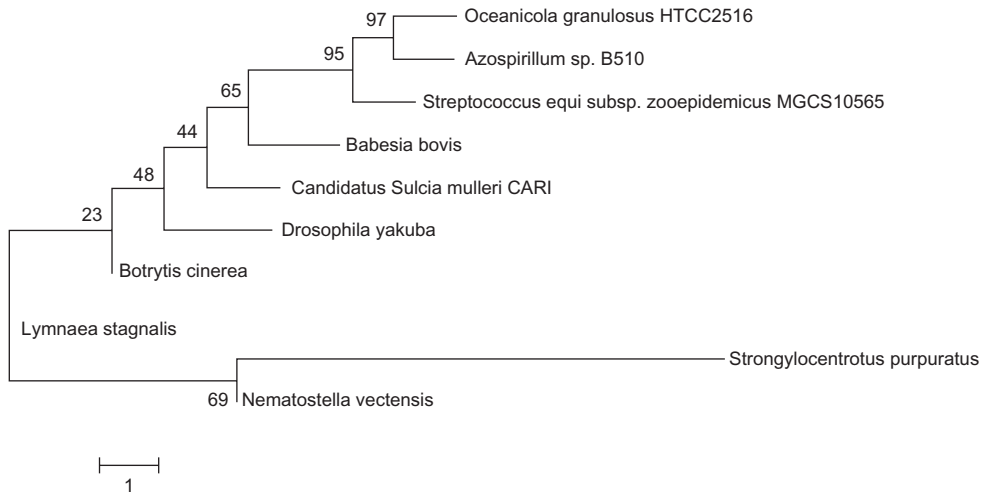

Figure S2. An example of incompatible phylogeny with the HGT hypothesis.

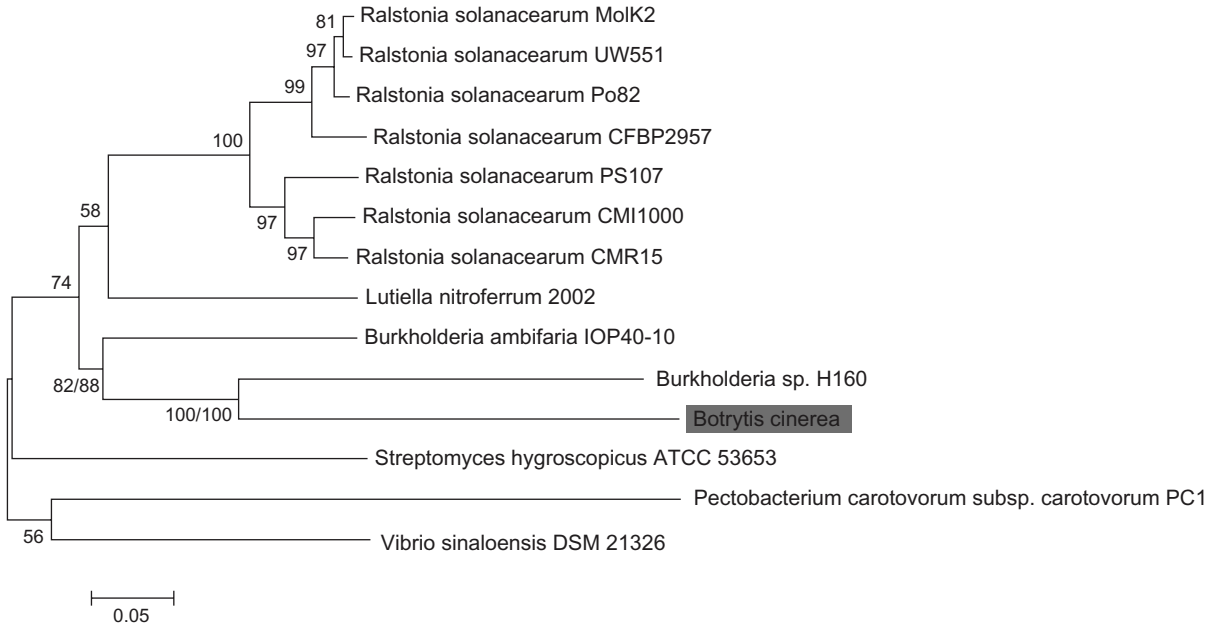

Figure S3. Phylogenies of putative two putative alpha/beta hydrolase fold proteins and lipoprotein genes. The phylogenetic tree shown was calculated using the Maximum Likelihood (ML) program PhyML and Bayesian program Mr. bayes (detailed parameters were described in the main text of paper). Only the values in ML ≥ 80% and Bayesian posterior probability ≥85% were shown. For key nodes the actual support values are shown in the order ML bootstraps/Bayesian posterior probability.

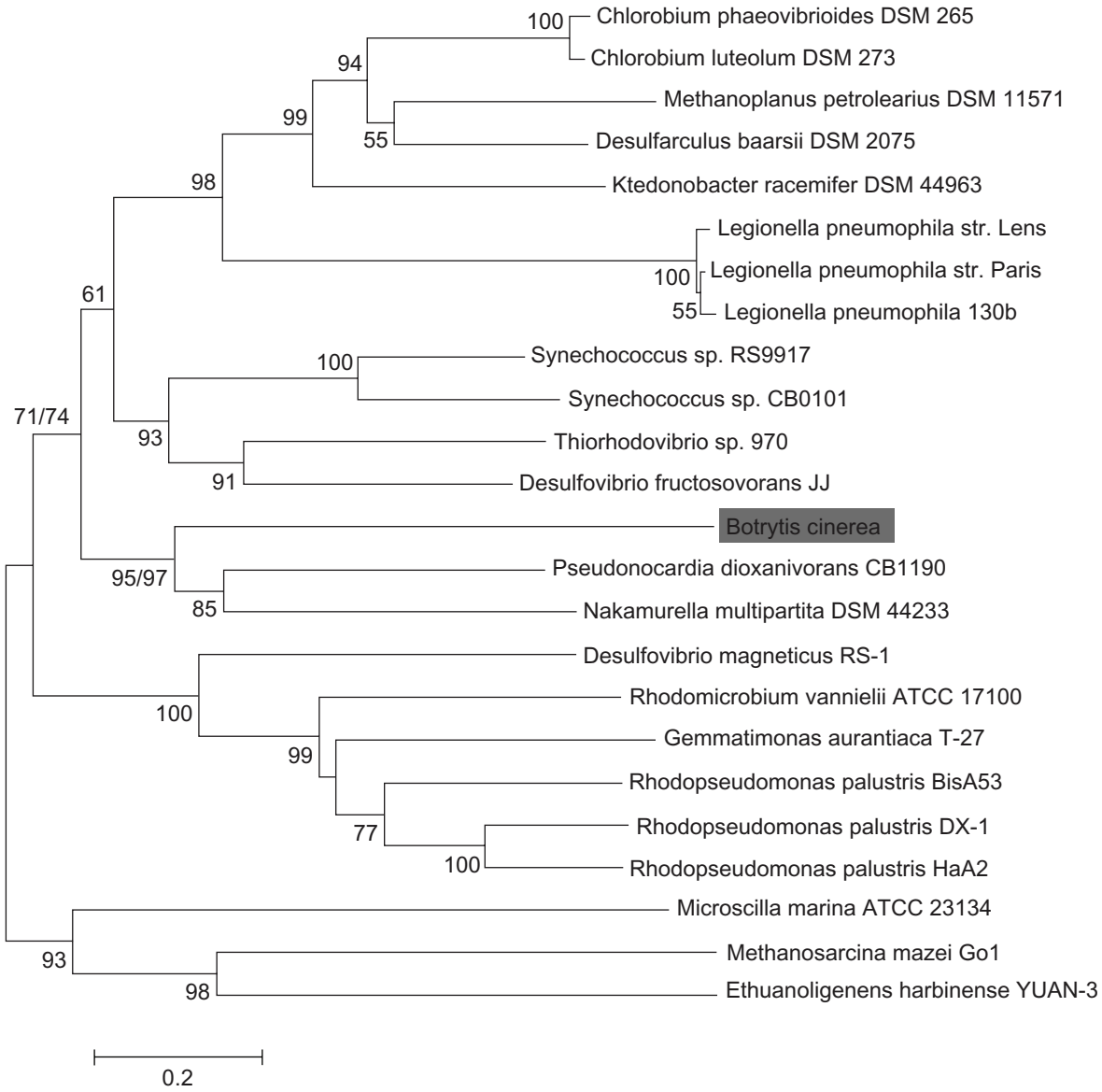

Figure S4. Phylogenies of putative two putative alpha/beta hydrolase fold proteins and lipoprotein genes. The phylogenetic tree shown was calculated using the Maximum Likelihood (ML) program PhyML and Bayesian program Mr. bayes (detailed parameters were described in the main text of paper). Only the values in ML ≥ 80% and Bayesian posterior probability ≥85% were shown. For key nodes the actual support values are shown in the order ML bootstraps/Bayesian posterior probability.

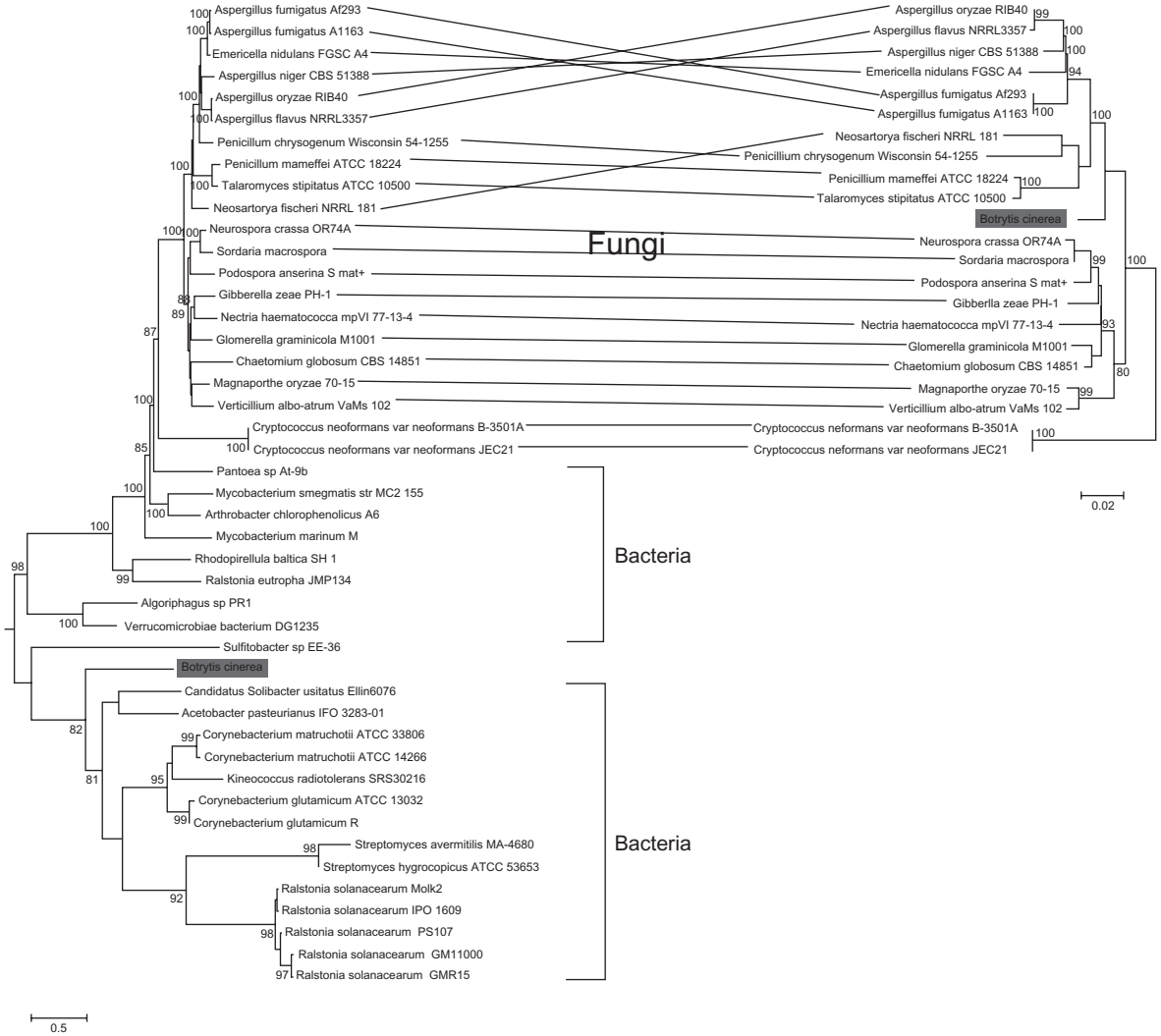

Figure S5. Phylogenies of putative two putative alpha/beta hydrolase fold proteins and lipoprotein genes. The phylogenetic tree shown was calculated using the Maximum Likelihood (ML) program PhyML and Bayesian program Mr. bayes (detailed parameters were described in the main text of paper). Only the values in ML ≥ 80% and Bayesian posterior probability ≥85% were shown. For key nodes the actual support values are shown in the order ML bootstraps/Bayesian posterior probability.

Supplementary Files are available from 8486Supplementaryfiles.zip