Diverse Lifestyles and Strategies of Plant Pathogenesis Encoded in the Genomes of Eighteen *Dothideomycetes* Fungi

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**Abstract**

The class *Dothideomycetes* is one of the largest groups of fungi with a high level of ecological diversity including many plant pathogens infecting a broad range of hosts. Here, we compare genome features of 18 members of this class, including 6 necrotrophs, 9 (hemi)biotrophs and 3 saprotrophs, to analyze genome structure, evolution, and the diverse strategies of pathogenesis. The *Dothideomycetes* most likely evolved from a common ancestor more than 280 million years ago. The 18 genome sequences differ dramatically in size due to variation in repetitive content, but show much less variation in number of (core) genes. Gene order appears to have been rearranged mostly within chromosomal boundaries by multiple inversions, in extant genomes frequently demarcated by adjacent simple repeats. Several *Dothideomycetes* contain one or more gene-poor, transposable element (TE)-rich putatively dispensable chromosomes of unknown function. The 18 *Dothideomycetes* offer an extensive catalogue of genes involved in cellulose degradation, proteolysis, secondary metabolism, and cysteine-rich small secreted proteins. Ancestors of the two major orders of plant pathogens in the *Dothideomycetes*, the *Capnodiales* and *Pleosporales*, may have had different modes of pathogenesis, with the former having fewer of these genes than the latter. Many of these genes are enriched in proximity to transposable elements, suggesting faster evolution because of the effects of repeat induced point (RIP) mutations. A syntenic block of genes, including oxidoreductases, is conserved in most *Dothideomycetes* and upregulated during infection in *L. maculans*, suggesting a possible function in response to oxidative stress.

**Introduction**

*Dothideomycetes* is the largest and most ecologically diverse class of fungi [1]. One or more members of this class infect almost every major crop, including those involved in the production of food, feed, fiber and biofuel. In addition to housing important plant pathogens, the class includes fungi with an unparalleled diversity of life history strategies and metabolic profiles. *Dothideomycetes* are present on every continent, including Antarctica, and are very important to ecosystem health and global carbon cycling as saprotrophs and degraders of plant biomass. Many are tolerant of environmental extremes including heat, cold, solar radiation and desiccation. Some produce enzymes that help degrade rocks [2] while others are associated with alcoholic vapors [3]. A few are

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Author Summary

**Dothideomycetes** is the largest and most ecologically diverse class of fungi that includes many plant pathogens with high economic impact. Currently 18 genome sequences of **Dothideomycetes** are available, 14 of which are newly described in this paper and in several companion papers, allowing unprecedented resolution in comparative analyses. These 18 organisms have diverse lifestyles and strategies of plant pathogenesis. Three feed on dead organic matter only, six are necrotrophs (killing the host plant cells), one is a biotroph (forming an association with and thus feeding on the living cells of the host plant cells) and 8 are hemibiotrophs (having an initial biotrophic stage, and killing the host plant at a later stage). These various lifestyles are also reflected in the gene sets present in each group. For example, sets of genes involved in carbohydrate degradation and secondary metabolism are expanded in necrotrophs. Many genes involved in pathogenesis are located near repetitive sequences, which are believed to speed up their evolution. Blocks of genes with conserved gene order were identified. In addition to this we deduce that the mechanism for mesosynteny, a type of genome evolution particular to **Dothideomycetes**, is by intra-chromosomal inversions.

Plant pathogens occur in at least six of the 12 orders. The two largest dothideomycete orders, **Pleosporales** and **Capnodiales**, each contain a large number of highly destructive plant pathogens. These include some of the most important diseases of the cereal crops wheat, barley and maize, trees such as pine and poplar, dictions including soybeans, canola and tomato, and tropical fruits including bananas. The allergens of *Daedalia tassiana* (aka *Cladosporium herbarum*) and *Alternaria alternata* are the most important of all known fungal allergens and represent two of the four allergens associated with these two orders.

Their high economic impact and intriguing biological diversity have stimulated much interest in genomic sequencing of **Dothideomycetes**. Key representatives have been sequenced through the Fungal Genome program at the U.S. Department of Energy Joint Genome Institute (JGI), which has had an emphasis on **Dothideomycetes** for several years [18]. The sequenced species are important to agriculture, especially those that are pathogens of bioenergy crops, or they represent phylogenetic and ecological diversity like AF-TOIL (Assembling the Fungal Tree of Life) targets [19], or they are of interest to bioenergy production because of their unusual physiology such as *Baudonia coniothyrea*, whose growth on outdoor surfaces is induced by fugitive ethanol vapor emissions from spirit maturation warehouses and bakeries [20]. These extensive efforts have yielded more sequences of fungi in the **Dothideomycetes** than any other class, providing an unparalleled opportunity for comparative genomics.

Here we report new genome sequences of 14 dothideomycete genomes and use them in comparative analyses with those of four **Dothideomycetes** published previously [21,22,23,24], plus representative outgroups from the *Ascomycota* and *Basidiomycota* (Table 1), a total of 39 genomes. Among the 18 sequenced dothideomycete genomes, nine are from species of the order **Pleosporales**, seven from the order **Capnodiales** and two from the order **Hysteriales**. From the perspective of lifestyle, fifteen are from species of plant pathogens (six necrotrophs, eight hemibiotrophs and one biotroph) and three are saprotrophs.

The order **Pleosporales** comprises the necrotroph *Cochliobolus heterostrophus* (isolates C4 and C5) and the hemibiotroph *Setosphaeria turcica* which infect corn (*Zea mays*), the hemibiotroph *Cochliobolus sativus* which infects barley, wheat and several other cereal crops, the necrotrophs *Pyrenophora tritici-repentis* and *Stagonospora nodorum* which infect wheat (*Triticum aestivum*), the necrotroph *Pyrenophora teres* forma teres which infects barley (*Hordeum vulgare*), and the necrotroph *Alternaria brassicicola* and the hemibiotroph *Leptosphaeria maculans* which infect plants in the *Brassicaceae*.

The order **Capnodiales** comprises the hemibiotroph *Mycosphaerella graminicola* (*Zymoseptoria tritici*) which infects wheat, the hemibiotroph *Dobsonia septosporum* (*Mycosphaerella pinus*) which infects more than 70 species of pine, the hemibiotrophs *Mycosphaerella populorum* (*Septoria muscoides*) and *Mycosphaerella populiocida* (*Septoria populiocida*) which infect species of poplar, the hemibiotroph *Mycosphaerella fijiensis* which infects bananas (*Musa spp*.), the biotroph *Cladosporium fulvum* (*Passalora fulva*) which infects tomato (*Solanum lycoctonum*), and the extremophilic saprotroph *Baudonia coniothyrea*. The latter’s primary known habitat is various exposed substrates near liquor maturation warehouses and commercial bakeries [20], where ambient ethanol vapors provoke its colonization.

The order **Hysteriales** comprises the two saprotrophs *Hysterium pulicace* and *Rhizhysterium rafuti*. Phylogenetically these species form a sister group to the plant pathogens in the **Pleosporales** [25], and are usually associated with dead or dying plant tissues.

Comparative genomic analysis of 18 **Dothideomycetes** provides valuable insights into fundamental questions regarding fungal lifestyles, evolution and adaptation to diverse ecological niches,
| Species                  | Taxonomy                  | Lifestyle | Host/substrate                      | Sequencing Center                  | Ref                  |
|-------------------------|---------------------------|-----------|------------------------------------|------------------------------------|----------------------|
| Cochliobolus heterostrophus C5 | Dothideomycetes           | Necrotrophic plant pathogen | Maize (*Zea mays*)               | JGI (USA) (unpublished data) a      |                      |
| Cochliobolus heterostrophus C4 | Dothideomycetes           | Necrotrophic plant pathogen | Maize (*Zea mays*)               | JGI (USA) (unpublished data) a      |                      |
| Cochliobolus sativus     | Dothideomycetes           | Hemibiotrophic plant pathogen | Wheat, barley and other grasses   | JGI (USA) (unpublished data) a      |                      |
| Setosphaeria turcica     | Dothideomycetes           | Hemibiotrophic plant pathogen | Maize (*Zea mays*)               | JGI (USA) (unpublished data) a      |                      |
| Alternaria brassicicola  | Dothideomycetes           | Biotrophic plant pathogen   | *Brassica* spp.                   | Washington State University (USA)   | [78]                 |
| Pyrenophora tritici-repentis | Dothideomycetes           | Necrotrophic plant pathogen | Barley (*Hordeum vulgare*)       | Curtin University, Australia        | [22]                 |
| Leptosphaeria maculans   | Dothideomycetes           | Hemibiotrophic plant pathogen | *Populus* spp.                    | Wageningen University & Research Centre, The Netherlands | [73]                 |
| Stagonospora nodorum     | Dothideomycetes           | Necrotrophic plant pathogen | Wheat (*Triticum* spp.)           | BROAD (USA) (unpublished data)      |                      |
| Mycosphaerella fijiensis | Dothideomycetes           | Hemibiotrophic plant pathogen | Banana (*Musa* spp.)             | JGI (USA) (unpublished data) a      |                      |
| Cladosporium fulvum     | Dothideomycetes           | Biotrophic plant pathogen   | Tomato (*Solanum lycopersicum*)   | Wageningen University & Research Centre, The Netherlands | [73]                 |
| Dothistroma septosporum | Dothideomycetes           | Hemibiotrophic plant pathogen | Pines and other conifers          | JGI (USA) (unpublished data) a      |                      |
| Mycosphaerella graminicola | Dothideomycetes           | Hemibiotrophic plant pathogen | *Triticum* spp.                   | JGI (USA) (unpublished data) a      |                      |
| Baudoinia compniacensis  | Dothideomycetes           | Saprotrophic, extremophile  | Ethanol vapor. Hard surfaces       | JGI (USA) (unpublished data) a      |                      |
| Outgroup Ascomycota      |                           |           |                                    |                                    |                      |
| Aspergillus nidulans     | Eurotiomycetes            | Saprotrophic                | Soil                               | AspGD consortium                  | [119]                |
| Botrytis cinerea         | Leotiomycetes             | Necrotrophic plant pathogen | Fruit and leaves of many species   | BROAD                              | [120]                |
| Chaetomium globosum      | Sordariomycetes           | Saprotrophic                | Dead plant material                | BROAD                              | [121]                |
| Fusarium graminearum     | Hypocreales               | Necrotrophic plant pathogen | *Triticum* spp./ *Hordeum vulgare* | BROAD                              | [122]                |
| Fusarium oxysporum       | Hypocreales               | Hemibiotrophic plant pathogen | *Triticum* spp./ *Hordeum vulgare* | Many plant hosts; soil             | [123]                |
| Magnaporthe grisea       | Sordariomycetes           | Hemibiotrophic plant pathogen | Endophyte/Saprotrophic             | Rice (*Oryza sativa*)              | [124]                |
| Neosartorya grisea       | Sordariomycetes           | Necrotrophic plant pathogen | *Triticum* spp./ *Hordeum vulgare* | Many plant hosts, soil             | [125]                |
| Neosartorya grisea       | Sordariomycetes           | Saprotrophic                | Dead plant material, particularly after fire | BROAD                              |                      |
| Neurospora crassa        | Sordariomycetes           | Saprotrophic                | Dead plant material                | JGI                                 | [126]                |

Table 1. Species used in this comparative study.
### Table 1. Cont.

| Species                  | Taxonomy         | Class          | Order            | Lifestyle                  | Host/substrate                  | Sequencing Center | Ref       |
|--------------------------|------------------|----------------|------------------|----------------------------|---------------------------------|-------------------|-----------|
| *Saccharomyces cerevisiae* | Saccharomycetes   | Saccharomycetales | Saprotrophic     | Fruit                      | SGD consortium                | [127]             |           |
| *Sclerotinia sclerotiorum* | Leotiomycetes     | Helotiales      | Necrotrophic plant pathogen | Many, primarily dicots     | BROAD                          | [120]             |           |
| *Trichoderma reesei*     | Sordariomycetes   | Hypocreales     | Saprotrophic     | Soil, woody surfaces       | JGI                            | [128]             |           |
| *Verticillium dahliae*   | Sordariomycetes   | Hypocreales     | Necrotrophic plant pathogen | Many dicots               | BROAD                          | [129]             |           |
| **Outgroup Basidiomycota** |                  |                |                  |                            |                                 |                   |           |
| *Coprinopsis cinerea*    | Agaricomycetes    | Agaricales      | Saprotrophic     | Dung, plant material       | BROAD                          | [130]             |           |
| *Cryptococcus neoformans var. grubii* | Tremellomycetes | Tremellales | Animal pathogen | Humans                   | BROAD                          | [131]             |           |
| *Laccaria bicolor*       | Agaricomycetes    | Agaricales      | Ectomycorrhizal  | Many tree species          | JGI                            | [35]              |           |
| *Melampsora larici-populina* | Urediniomycetes  | Uredinales      | Obligate biotrophic plant pathogen | Poplar trees (Populus spp.) | JGI                            | [132]             |           |
| *Phanerochaete chrysosporium* | Agaricomycetes | Polyporales    | Saprotrophic     | Wood                      | JGI                            | [133]             |           |
| *Postia placenta*        | Agaricomycetes    | Polyporales     | Saprotrophic     | Wood                      | JGI                            | [134]             |           |
| *Puccinia graminis*      | Urediniomycetes   | Uredinales      | Obligate biotrophic plant pathogen | Wheat (Triticum spp.)     | BROAD                          | [132]             |           |
| *Schizophyllum commune*  | Agaricomycetes    | Agaricales      | Saprotrophic     | Dead wood                 | JGI                            | [135]             |           |
| *Ustilago maydis*        | Ustilaginomycetes | Ustilaginales   | Biotrophic plant pathogen | Maize (Zea mays) and teosinte (Euchlena mexicana) | BROAD                          | [136]             |           |

*Additional genome-centric papers are planned.

Taxonomy and lifestyle of the 18 Dothideomycetes used in this study, as well as the outgroups for comparative purposes.

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Figure 1. Estimated phylogeny and divergence times of Dothideomycetes, based on sequences of three protein-coding genes. Species with a sequenced genome that are included in this study are highlighted in dark blue. Vertical lines in blue and green indicate minimum and maximum ages for specific nodes, respectively. The age ranges for highlighted taxa are indicated by blocks with different shades of gray. Horizontal green lines indicate bootstrap recovery for specific nodes – thickened branches represent more than 70%, normal branches, 50–70% and less than 50% recovery.
especially as they relate to plant pathogenicity and biomass conversion.

**Results/Discussion**

**Dothideomycetes phylogeny and divergence time estimates**

The class *Dothideomycetes* comprises a huge diversity of fungi. To place the sequenced species in a broader evolutionary context, a three-gene phylogenetic tree was made representing 11 of 12 currently accepted orders in *Dothideomycetes* (Figure 1). This 67-taxon phylogeny is congruent with a tree made from 51 orthologous genes obtained from the 18 genome-sampled strains (Figure 2A) and previous phylogenies [17]. Divergence time estimates are indicated and age ranges of the taxonomic groups relevant for this paper are indicated in differently shaded gray blocks in Figure 1.

The class of *Dothideomycetes* last shared a common ancestor more than 280 million years ago (MYA). Genome sampling in this class is currently focused on two large and diverse orders, *Pleosporales* and *Capnodiales*, and to a lesser extent on *Hysteriales*. The main radiation of *Capnodiales* likely happened between 179 and 131 MYA, while a similar event likely occurred for the *Pleosporales* at a later date, between 133 and 97 MYA. This latter estimation is very likely influenced by our limited sampling of early-diverging lineages in *Pleosporales*. However, differences in divergence times become more pronounced in the two highlighted families with more representative sampling. *Mycosphaerella*, as defined currently, represents an ancient (diversifying at least 87 MYA) clade compared to *Pleosporales* (diversifying at least 17 MYA). The sampled *Hysteriales* shared a common ancestor at least 40 MYA. Figure 1 also illustrates that the strains currently labeled with the genus name *Mycosphaerella* diversified across a longer time than all species in *Pleosporaceae* and its several sister lineages. These lineages are included in suborder *Pleosporineae* which represents a well recovered phylogenetic node containing the four main families of plant pathogens in *Pleosporales*.

Additional considerations concerning phylogeny and nomenclature of *Dothideomycetes* are discussed in Text S1.

**Variation in genome sizes across diverse Dothideomycetes**

Genome sizes show dramatic variation among the *Dothideomycetes* (Figure 2B, Table S1), from 21.88 Mbp in *Baudoinia compniacensis* to 74.14 Mbp in *Mycosphaerella fijiensis*. The corre-

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**Figure 2. Phylogeny and genome characteristics of the 18 studied Dothideomycetes.** A. Genome-based phylogenetic tree of 18 Dothideomycetes computed using 51 conserved protein families. Bootstrap values are indicated on the branches. Lifestyles and strategies of pathogenesis (green circle for necrotrophs, orange circle for saprotrophs and blue circle for (hemi)biotrophs) are indicated. *Aspergillus nidulans* was used as an outgroup and its branch on the tree is not drawn to scale. B. Genome size and repeat content. Asterisks indicate genomes that were sequenced exclusively with Illumina technology. Repeat content in these genomes is likely an underestimate. C. Number of predicted genes, broken down by level of conservation. D. Gene counts of classes that have been implicated in plant pathogenesis. Members of *Capnodiales* have fewer genes in these classes than *Pleosporales* and *Hysteriales* (with the exception of *Cladosporium fulvum*). This trend is also illustrated by the estimated gene counts for the last common ancestors of the indicated taxa (below the x-axis), which correspond to the taxa in (A). See also Figure S3. Bars on all graphs (B, C, and D) correspond to the organisms on the tree in (A).

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lation between genome size and repeat content (0.91) is larger than the correlation between genome size and gene count (0.59) or between genome size and gene content (0.71), suggesting that the repeat content generally plays the largest role in determining genome size. When the genomes that have been sequenced exclusively using Illumina technology are excluded (see Table S1), the correlation between genome size and repeat content is even higher (0.94). Repeat content in Illumina-sequenced genomes is likely underestimated, since short repetitive reads are difficult to assemble into long repeat regions (as discussed in [26]). This underestimation is most apparent when C. heterostrophus strain C5 (Sanger assembly, 8.64% repeat content) is compared to strain C4 (Illumina assembly, 0.83% repeat content). To better estimate the repetitive content of Illumina-sequenced genomes an additional analysis was performed using the unassembled sequence reads and the assembler ALLPATHS-LG [26]. This analysis estimates the percentage of sequence reads that are repetitive. These percentages are considerably higher than those in Figure 2B (20% for P. teres f. teres, 9% for H. pulicare, 18% for R. rufidum, and 20% for C. heterostrophus C4), but it should be noted that they were obtained using fundamentally different methods, making direct comparisons difficult. It is clear, however, that repeat content is underestimated in Illumina assemblies (and possibly also in Sanger/454 assemblies).

The smallest of the 18 genomes is that of the extremophile B. campinanensis, which has four features consistent with its size compared to the other Dothideomycetes: lower repeat content; lower number of genes; fewer genes with an intron; and shorter intergenic space (Table S1). The largest genomes, those of M. fijensis and C. fulvum, contain 39.5% and 44.4% repeats, respectively, which are among the largest fractions reported in fungi.

From macro- to mesosynteny

The range of evolutionary distances among the members of this group of organisms offers a unique perspective on evolution of genome organization. It has been shown previously that filamentous Ascomycota and particularly Dothideomycetes display a phenomenon recently designated as mesosynteny [27]. Mesosynteny is characterized by conservation within chromosomes of gene content but not gene order or orientation, and this was demonstrated by whole-genome DNA comparisons. In organisms displaying mesosynteny most chromosomal rearrangements are intra- rather than inter-chromosomal.

When synteny analysis is extended from four [27] to 18 dothideomycete genomes, a range of syntenic relationships between organisms becomes apparent, from macro- to mesosynteny (Figure 3 and Table S2). Mesosynteny is found in the majority of genome-genome comparisons between species of Dothideomycetes.
Figure 4. Simulation of chromosome evolution leading to mesosynteny. 

A. Two identical sequences show perfect macrosynteny. 
B. This is also the case for scaffold_1 of Cochliobolus heterostrophus C4 and scaffold_2 of C. heterostrophus C5, reflecting their close relationship as progeny. 
C. The two sequences from (A) have each undergone one random inversion. 
D. Scaffold_4 of C. heterostrophus C5 and scaffold_9 of C. sativus show a very similar pattern as in (C). 
E. The two sequences in (A) have each undergone 25 random inversions. 
F. Scaffold_8 of Setosphaeria turcica and part of scaffold_10 of C. heterostrophus C5 show a pattern of syntenic regions progressively spreading across the scaffolds similar to that in (E) 
G. The two
In contrast, macrosynteny is observed only in pairwise comparisons of the most closely related organisms: the three Cochliobolus genomes and between M. populicola and M. populorum. Nearly perfect macrosynteny is observed when strains C4 and C5 of C. heterostrophus are compared (Figure 3A), reflecting their close relationship as progeny of a backcross series. Mostly macrosyntenic conservation also is seen when either of the C. heterostrophus strains is compared to C. sativus (last common ancestor estimated less than 1 MYA, Figure 1). Interestingly, however, large intrachromosomal inversions have taken place in several sequence pairs (Figure 3B). The same phenomenon is observed in a comparison between M. populicola and M. populorum. The signature of macrosynteny is less clear and the pattern of mesosynteny becomes stronger in a comparison between C. heterostrophus C5 and S. turcica (Figure 3C, last common ancestor estimated 5–6 MYA). Finally, when C. heterostrophus C5 is compared to the more distantly related S. nodorum a pattern of mesosynteny is observed (Figure 3D, last common ancestor estimated 45–61 MYA) that is very similar to that observed between observed between other pairs of Dothideomycetes [27]. We hypothesize that the intrachromosomal inversions observed between the genomes of C. heterostrophus and C. sativus are the first steps in the development of the mesosyntenic patterns observed between more distantly related Dothideomycetes.

To test whether inversions could generate the observed patterns of mesosynteny, we ran a simulation of the evolution of a diverging pair of chromosomes undergoing intrachromosomal inversions. Initially, the chromosome pairs are identical and therefore fully macrosyntenic (Figure 4A), similar to the pattern observed between C. heterostrophus strains C4 and C5 (Figure 4B). After one random intra-chromosomal inversion in each chromosome (Figure 4C), the pattern is very similar to that observed between C. heterostrophus C5 and C. sativus (Figure 4D). After 25 random inversions, syntenic regions are progressively spreading across the scaffolds (Figure 4E), similar to what is observed for C. heterostrophus C5 and S. turcica (Figure 4F). After 500 random inversions (Figure 4G), the pattern is very similar to that observed between D. septosporum and M. populorum (Figure 4H), which diverged from the same ancestor an estimated 74–100 MYA (Figure 1). This simulation shows that intra-chromosomal inversions alone are sufficient to obtain a pattern of mesosynteny between two genomes during evolution.

In most genomes all the chromosomes/scaffolds show mesosynteny with at least one chromosome/scaffold from another Dothideomycete. The exceptions are L. maculans [ln_SuperContig_22_v2], M. graminicola (chr_16 and chr_19), and M. fijiensis (scaffold_11, scaffold_15, scaffold_17, scaffold_18, and scaffold_20). Interestingly, all these scaffolds/chromosomes are predicted to be dispensable (see section ‘Putatively dispensable chromosomes’, below).

Interestingly, the inversion breakpoints are associated with simple repeats (i.e., low-complexity DNA such as dinucleotide repeats). Among relatively closely related mesosyntenic scaffold pairs, these simple repeats are over-represented in the 500 bp up- and downstream of these breakpoints, compared to the rest of the respective scaffolds (comparisons 1, 2 and 3 in Table S3). In more distantly related scaffold pairs this pattern is not observed (comparison 4 in Table S3), presumably because ancient inversion sites have since changed considerably.

Although the exact mechanism leading to mesosynteny is still unknown, this extended analysis using 18 genomes of Dothideomycetes was consistent with the simulations and added sufficient resolution to be able to show that frequent intra-chromosomal inversions most likely played a major role in the origin of this phenomenon. Whether the frequency and placement of simple repeats is different in the Dothideomycetes than organisms that do not show patterns of mesosynteny is not known.

**Microsynteny is conserved across large groups of Dothideomycetes**

Chromosomal rearrangement events (such as those leading to mesosynteny) will theoretically result eventually in a random distribution of genes across chromosomes, except for certain clusters of genes associated with a common function for which physical clustering is beneficial (e.g., secondary metabolism). Although physical clustering of functionally related fungal genes does occur, it is considerably more rare than in prokaryotes. The physical clustering of genes across related organisms can therefore give insight into functional relationships between genes.

In the genomes of the 18 Dothideomycetes two blocks of genes were identified that were conserved in 15 and 14 of the 18 studied strains. Both blocks consist of at least 5 genes that are located in a block of at most 10 genes (Tables S4 and S5). Block 1 consists of genes with annotations that do not seem obviously related from a functional point of view. In contrast, block 2 contains genes encoding two dehydrogenases and two (oxido)reductases, which strongly suggests a functional connection. These two blocks were not present in any of the outgroups used in this study.

Interestingly, in L. maculans 3 of 6 genes in block 1 are at least 2-fold down-regulated and all 5 genes in block 2 are at least 2-fold up-regulated in leaves 7 or 14 days after infection, when compared to expression in mycelium (reanalyzed expression data obtained from previously published whole-genome microarray data [23]; see also ‘comparative transcriptomics during pathogenesis’ in Text S3, Tables S6 and S7). This apparent co-regulation in L. maculans may be an effect of the physical clustering on the chromosome, but it also suggests a related functional role where co-location may provide a fitness advantage. Because the genes in block 2 in L. maculans were up-regulated in infected leaves, they could play a role in pathogenesis in that organism. Since they are conserved in nearly all sequenced genomes of Dothideomycetes, these blocks may have been present in the common ancestor of all Dothideomycetes and were maintained throughout their evolutionary history.

The same microsynteny analysis was performed on two Dothideomycetes subsets: the Pleosporales (excluding C. heterostrophus strain C4, since it is very similar to strain C5) and the Mycosphaerellaceae (see Figure 1). This resulted in 502 and 58 syntenic blocks of genes present in at least 75% of the studied organisms in each group, respectively (Tables S8 and S9). This difference can be explained (at least in part) by the much shorter evolutionary distances among the 8 examined Pleosporales (last common ancestor estimated 41–61 MYA, Figure 1), compared to those among the 6 studied Mycosphaerellaceae (last common ancestor estimated 87–117 MYA). An analysis of functional annotation terms of the genes in syntenic blocks reveals enrichment of genes involved in a wide variety of biological processes in the Pleosporales (Table S10). In the Mycosphaerellaceae, however, genes enriched in
Table 2. Potential core and dispensable chromosomes in the genomes of Dothideomycetes.

| Organism                     | Category                        | Size excluding gaps (bp) | GC-content (%) | Gene count | Gene density (genes/Mbp) | Gene products with PFAM domain (%) | Repeat content (%) | Name                                      |
|------------------------------|---------------------------------|--------------------------|----------------|------------|--------------------------|-----------------------------------|-------------------|------------------------------------------|
| Mycosphaerella graminicola   | Core chromosomes                | 35077646                 | 52.33          | 10317      | 294.12                   | 50.01                             | 11.32            |                                          |
|                              | Dispensable chromosomes         | 4602608                  | 50.65          | 654        | 142.09                   | 3.36                              | 19.42            | chr_14, chr_15, chr_16, chr_17, chr_18, chr_19, chr_20, chr_21 |
| Mycosphaerella fijiensis     | Putative core scaffolds         | 63951705                 | 45.85          | 12699      | 198.57                   | 50.76                             | 37.12            |                                          |
|                              | Putative dispensable scaffolds  | 9779308                  | 40.71          | 408        | 41.72                    | 2.45                              | 56.72            | scaffold_11, scaffold_13, scaffold_14, scaffold_15, scaffold_16, scaffold_17, scaffold_18, scaffold_20, scaffold_21, scaffold_22, scaffold_23, scaffold_24, scaffold_25, scaffold_26 |
| Leptosphaeria maculans       | Putative core scaffolds         | 42453795                 | 45.55          | 12423      | 292.62                   | 45.79                             | 31.59            |                                          |
|                              | Putative dispensable scaffolds  | 919483                   | 35.42          | 42         | 45.68                    | 9.52                              | 89.36            | lm_SuperContig_22_v2, lm_SuperContig_29_v2 |
| Setosphaeria turcica         | Putative core scaffolds         | 38177662                 | 51.46          | 11695      | 306.33                   | 55.94                             | 14.46            |                                          |
|                              | Putative dispensable scaffolds  | 74746                    | 41.92          | 7          | 93.65                    | 14.29                             | 72.32            | scaffold_28                              |
| Stagonospora nodorum         | Putative core scaffolds         | 36907047                 | 50.52          | 12380      | 335.44                   | 51.61                             | 27.7             |                                          |
|                              | Putative dispensable scaffolds  | 142552                   | 28.14          | 0          | 0.00                     | 0.00                              | 32.97            | scaffold_46                              |
| Cochliobolus heterostrophus  | Putative core scaffolds         | 36190278                 | 49.81          | 13322      | 368.11                   | 53.51                             | 83.9             |                                          |
|                              | Putative dispensable scaffolds  | 134153                   | 40.04          | 14         | 104.36                   | 7.14                              | 85.38            | scaffold_27                              |

Mycosphaerella graminicola has been shown previously to contain dispensable (i.e., not necessary for survival) chromosomes [24]. These chromosomes are smaller, less gene-dense and more repeat-rich than the core chromosomes. Proteins encoded by genes on these chromosomes less frequently contain a PFAM domain. Scaffolds with similar characteristics are also present in five other Dothideomycetes. Additional statistics for these scaffolds are given in Table S12.

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conserved blocks are mostly involved in transcriptional regulation (Table S11).

It was shown previously that the MAT1 mating type loci of several Pleosporales show conservation of gene order [28]. In our microsynteny analysis the MAT1 locus corresponds to the adjacent syntenic blocks 141 and 142 (Table S8). In addition to the 3 genes described previously, our study reveals that at least 10 genes have been conserved in location across at least 6 Dothideomycetes.

Despite the progressive reshuffling of the chromosomes by the processes behind mesosynteny, many syntenic blocks of genes have remained intact. For closely related species, this can be explained by the short evolutionary time in which chromosomal rearrangements could occur. The syntenic gene blocks identified across Dothideomycetes, however, most likely were selected for during evolution. Molecular manipulation of the genes in these syntenic blocks should help reveal function and possible reasons for the conservation of their gene order.

Putatively dispensable chromosomes

The sequenced strain of M. graminicola has been shown previously to contain 8 dispensable chromosomes [24]. One or more of these chromosomes could be missing in progeny of sexual crosses and in field isolates. However, isolates missing one or more of these dispensable chromosomes show no obvious phenotypic changes compared to their parents or other progeny isolates [29]. Similarly dispensable chromosomes (in the literature also referred to as supernumerary chromosomes, B chromosomes or mini-chromosomes [30]) have been identified previously in the Dothideomycetes L. maculans [31], C. heterostrophus [32] and A. alternata [33], as well as in several other filamentous fungi (reviewed in [30]).

| Table 3. Summary of expansion and depletion of PFAM domains and multi-gene families in various comparisons based on phylogeny and lifestyle (Table 1). |
|---------------------------------------------------------------|
| **Comparison**                                               | **PFAM domain** | **Multi-gene families** |
|                                                             | Expanded (of which unique) | Depleted (of which absent) | Expanded (of which unique) | Depleted (of which absent) |
| Dothideomycetes versus ascomycete and basidiomycete outgroups | 233 (2)            | 37 (3)                     | 3358 (840)                | 280 (116)                 |
| Dothideomycete plant pathogens versus other plant pathogens  | 69 (10)            | 21 (9)                     | 2098 (1411)               | 1209 (1081)               |
| Pleosporales versus Capnodiales                               | 137 (39)           | 67 (31)                    | 2995 (2468)               | 2129 (1917)               |
| Necrotrophic versus (hemibiotrophic dothideomycete plant pathogens) | 4 (4)              | 21 (21)                    | 299 (299)                 | 1195 (1195)               |
| Dothideomycete cereal pathogens versus other Dothideomycetes  | 6 (6)              | 14 (14)                    | 492 (492)                 | 359 (359)                 |
| Dothideomycete tree pathogens versus other Dothideomycetes    | 4 (4)              | 77 (77)                    | 1220 (974)                | 2226 (2226)               |
| Dothideomycete saprotrophs versus dothideomycete plant pathogens | 7 (7)              | 25 (25)                    | 516 (511)                 | 551 (550)                 |

All expanded and depleted PFAM domains and multi-gene families (as well as the statistics) are given in Tables S14 and S15, respectively.

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Compared to the core chromosomes, the dispensable chromosomes of *M. graminicola* are generally smaller, have a lower GC content, have higher repeat content, are less gene dense, and the percentage of predicted proteins with a PFAM domain is lower (Table 2). Using these criteria, we screened the other *Dothideomycetes* for chromosomes or scaffolds that are potentially dispensable. Only scaffolds larger than 100 kbp were taken into account. Scaffolds containing long rDNA repeats (as determined by RNAmer [34]) were removed from the dataset as they probably represent unplaced contigs.

Genome scaffolds with the above mentioned characteristics of the *M. graminicola* dispensome were identified in five other *Dothideomycetes*: 14 in *M. fijensis*, 2 in *L. maculans*, and 1 each in *C. heterotrophus* C5, *S. turcica* and *S. nodorum* (Table 2, Table S12). *L. maculans* has been shown previously by pulsed-field gel electrophoresis to contain at least one dispensable chromosome of 650 to 950 kbp [31]. It was identified previously as supercontig 22 (730 kbp) [23], but it may also include supercontig 29 (200 kbp), since this supercontig shows very similar characteristics to supercontig 22 (Table S12). To our knowledge, no dispensable chromosomes have been identified previously in *S. nodorum* or *S. turcica*. In contrast to the *M. graminicola* sequence, none of these genomes is finished so it is possible that these potentially dispensable scaffolds are in fact part of larger core chromosomes, and additional dispensable chromosomes with other characteristics may also exist. Segregation patterns in progeny of a cross could determine whether these scaffolds indeed are dispensable.

The origin and evolutionary benefit of dispensable chromosomes is unknown, although horizontal transfer from other fungi has been suggested as a possible origin [24]. The observation that chromosomal rearrangements take place mostly within chromosomes (see above) causes these dispensable chromosomes to remain isolated and have a separate evolutionary history from the core chromosomes, regardless of whether they have a function.

**Gene content comparison across phylogeny and lifestyle**

Predicted gene complements within the *Dothideomycetes* range from 9,739 in *M. populicola* to 13,336 in *C. heterotrophus* C5 (Figure 2C and Table S1). There is considerably less variation in gene count than in repeat content (Figure 2B). The 18 gene sets allowed us to identify gene core families conserved in all sequenced *Dothideomycetes* as well as those evolving in species-specific manner. Identifying multi-gene families, we clustered all 215,225 predicted genes representing 233 PFAM domains are expanded in *Dothideomycetes* (Tables 3 and S14). Notable examples include a domain involved in signaling (response regulator receiver domain), metabolism (succinylglutamate desuccinylase/aspartoacylase domain), several glycoside hydrolases (see CAZY below) and a DNA photolyase domain.

Comparison of the dothideomycete plant pathogens to other fungal plant pathogens reveals 10 PFAM domains that are unique to *Dothideomycetes* pathogens and 69 PFAM domains that are expanded (Tables 3 and S14). Although these differences could be explained by phylogeny (most cereal pathogens analyzed except for *M. graminicola* belong to the *Pleosporales*) they are an interesting class of genes to investigate further. Tree pathogens are enriched in a specific hydrolase, whereas saprotrophs are enriched in a specific peptidase (Tables 3 and S14).

Since the PFAM database only contains previously described domains, novel gene families can be missed. For this reason, the same comparisons as above were made for multi-gene families that were identified based on similarity followed by Markov clustering. The resulting numbers are higher than for the PFAMs (Tables 3 and S15). Frequently these multi-gene families have no functional annotations assigned to them. For example, 3,458 multi-gene families are expanded in *Dothideomycetes*, compared to the outgroups used. Of those, 1,360 (41%) have no PFAM domains assigned to them, meaning that they contain mostly novel proteins. This again shows that *Dothideomycetes* contain many unique and novel proteins that may be involved in their specific lifestyle and strategy of pathogenesis.

**Below we discuss specific classes of genes that have been shown to be involved in plant pathogenesis:** small secreted proteins, genes involved in secondary metabolism, carbohydrate-active enzymes, peptidases, and lipases. In addition to these, kinases are discussed in Text S2, Table S16 and Figure S2.
Small secreted proteins and candidate effectors

It is apparent that some small secreted proteins (SSPs) play an important role in plant-fungus interactions [35,36]. SSPs were identified in the genomes of the 18 Dothideomycetes and of the outgroups (Table S17). Counts varied from 67 in the saprotroph B. compniacensis to 251 in C. heterostrophus C4 and are within a similar range as other members of the Ascomycota (from 50 in Saccharomyces cerevisiae to 389 in Magnaporthe grisea) and Basidiomycota (from 40 in Cryptococcus neoformans to 540 in Melampsora laricis-populina) when using 200 amino acids as the upper limit for protein size. The three saprotrophs (B. compniacensis, R. rufulum and H. pulicare) are among the Dothideomycetes with the lowest number of predicted SSPs, confirming that SSPs are likely to be involved in plant-pathogen interactions. The Pleosporales generally have higher numbers of SSPs than the Capnodiales, which is also illustrated by the estimated numbers of SSPs in the last common ancestor of these respective taxonomic groups (189 and 134 for Pleosporales and Capnodiales, respectively [Figures 2D and S3]).

Of the predicted SSPs in Dothideomycetes, 8.3% had at least one PFAM domain. This is less than the 51.6% for all proteins, reflecting the fact that the function of SSPs is frequently unknown. The percentage of cysteine residues in the SSPs was higher than in the other proteins. Of all proteins, 9.8% resided in a singleton orthologous cluster (i.e., gene families with only one protein from only one organism). For the predicted SSPs this amount was 21.3%, reflecting the fact that this class of proteins is frequently species-specific.

Secondary metabolism

Secondary metabolites were among the first factors shown to be required for virulence and host specificity of necrotrophs in the Dothideomycetes [37,38]. Filamentous ascomycete genomes, including those of the Dothideomycetes, carry large numbers of genes encoding enzymes for secondary metabolite production (nonribosomal peptide synthetases (NPS), polyketide synthases (PKS) and terpene synthases (TPS) [39,40]), in contrast to genomes of early diverging ascomycetes (yeasts) and basidiomycetes (Figure S4 and Table S18). With this in mind, we screened the 18 genomes for counterparts of highly curated C. heterostrophus NPSs, PKSs and the less well studied TPSs and found that most were not conserved and thus there is extreme diversity among species (Tables S19, S20, S21). This distribution supports the hypothesis that the metabolites biosynthesized by these enzymes are good candidates for involvement in species diversification, virulence, and/or host-specificity.

Generally, the numbers of genes encoding enzymes for secondary metabolite production are more numerous in the Pleosporales and Hysteriales than in the Capnodiales (Figures 2D and S3), and this is especially the case for the PKSs. This is also illustrated by the estimated number of genes encoding enzymes for secondary metabolite production in the respective last common ancestors of the Pleosporales (40 genes), Hysteriales (46 genes), and Capnodiales (24 genes).

The numbers of NPSs were high in the 18 Dothideomycetes, ranging from a low of 2 in the saprotroph B. compniacensis to a high
of 44 in *P. teres f. teres*. These numbers were also high in the *Sordariomycetes*, *Eurotiomycetes*, and *Leotiomycetes*, in contrast to numbers in the yeasts and basidiomycetes (Figure S4 and Table S10). Numbers are higher in *Pleosporales* and *Hysteriales* than in *Capnodiales* (with the exception of *A. brassicicola*). In general, there are only a few fully conserved NPS genes/proteins across the fungi [39], including the 10 dothideomycete genomes examined here (Table S19). Only NPS10 (unknown product, mutants of *C. heterostrophus* are sensitive to oxidative stress) was perfectly conserved across the 18 dothideomycete genomes, in agreement with the earlier hypotheses [39] that NPS10 is among the more ancestral NPSs.

The next most highly conserved NPS (present in 17 of the 18 *Dothideomycetes*) is the counterpart of *C. heterostrophus* NPS2 (responsible for siderophore biosynthesis and intracellular iron storage). The latter is a critical cellular function, presumably required to prevent the Fenton reaction and comitant accumulation of reactive oxygen species. Note that *B. compniacensis*, *C. fulvum*, *D. septosporum* and *M. populorum* have only two NPS orthologs each and that these are NPS10 and NPS2, discussed above. NPS2 proteins in *Pleosporales* and *Hysteriales* have four adenylating (AMP) domains, while those in the *Capnodiales* have three, similar to the other major groups of ascomycetes and basidiomycetes [41]. Next in degree of conservation is NPS4 (present in 10 of the 18 genomes, unknown product, *C. heterostrophus*, *A. brassicicola* and *F. graminearum* nps4 mutant colonies are hydrophobic, rather than hydrophobic, like wild type [42]) and NPS6 (present in 11 of the 18 genomes; responsible for extracellular siderophore biosynthesis and thus competition for iron in the plant-fungal interaction). NPS6 has been shown to be involved in virulence of *C. heterostrophus* to corn, of *C. mycaceus* to rice, of *A. brassicicola* to *Arabidopsis thaliana* and of *Fusarium graminearum* to wheat and for oxidative stress management (in vitro) [43].

The remaining *C. heterostrophus* NPS representatives are discontinuously distributed across the 18 genomes. The greatest conservation was found for members of the *Pleosporales*, and the fewest for the *Capnodiales*. These genes are known to be rapidly evolving and thus highly diverse, with a tendency to "pop up" in disparate genomes. For example, the three-AMP-domain NPS for biosynthesis of *A. alternata* AM-toxin (Acc # BAI4739) has a perfect match in *M. graminicola* (JGI protein ID 56291) and the four-AMP-domain NPS, HTS1 (Acc # AAA33023), for *C. carbonum* HC-toxin biosynthesis, has orthologs in *S. boracca* and *P. tritici repens* [44].

The numbers of Type I PKSs ranged from two in the saprotroph *B. compressicaulis* to 34 in *R. rubiformis* (Figure S4 and Table S18). Type III PKSs, known to be rare in filamentous fungi, had no members in the *Capnodiales* (with the exception of *M. graminicola*) and one member in the *Pleosporales*. Only one PKS protein, responsible for melanin biosynthesis, was universally conserved in all 18 genomes (with the exception of *A. brassicicola*) (Table S20). For many fungi, melanin is a virulence determinant [45,46]. PKS1 and PKS2, required for T-toxin production and high virulence on maize, are found only in *C. heterostrophus* race T (strain C4). PKSs 4, 7, 20 and 25 are found only in all *C. heterostrophus* strains, while PKS11 and PKS24 were found in *C. heterostrophus* and *C. sativus* only. *C. heterostrophus* PKS24 is a hybrid NPS:PKS (NPS7:PKS24) and the entire protein is present in *C. sativus* (i.e., the NPS component is also present (Table S19). Some *C. heterostrophus* PKS orthologs (PKS6, 10, 13, 14, 16, 17, 21 and 22) were not present in *C. sativus*, yet were present in other species. With few exceptions (e.g., PKS17, which is present in *M. graminicola* and not other species in the *Pleosporales*), species that carried these genes tended to be those with a closer phylogenetic relationship.

The pattern of distribution of TPSs follows that of NPSs and PKSs in that few are conserved across the 18 *Dothideomycetes*. Most highly conserved is the *C. heterostrophus* protein ID 1098890, which shows >80% identity in all the genomes of *Pleosporales* and *Hysteriales*, but not of *Capnodiales* (Table S20). The best blast hit for this protein is lanosterol synthase, described as an integral membrane protein associated with the cytoplasmic side of the endoplasmic reticulum in eukaryotes. To our knowledge none of these TPSs has been functionally characterized in any of the 18 dothideomycete genomes and they thus represent untapped candidates for roles in species specificity, host specificity and/or virulence.

An example of a well described secondary metabolite pathway in *Dothideomycetes* is the biosynthesis of dothistromin [47,48,49,50,51]. Analyses with a core set of *D. septosporum* dothistromin genes suggested that only two of the other dothideomycete species, *C. fulvum* (sister species to *D. septosporum*) and *R. rubiformis*, have a putative orthologous gene set (Text S4 and Figure S5), showing that it is discontinuously present across relatively distantly related *Dothideomycetes*.

The power of availability of multiple genomes for comparison cannot be over emphasized for fast-evolving genes such as those involved in secondary metabolism. Given that PKS, NPS and TPS orthologs are discontinuously distributed across genomes [39,40] (Tables S19, S20, S21), a larger dataset is likely to uncover more orthologs in distantly related fungi. The debate continues regarding whether the tendency for duplication (gain) and loss, and recombination, coupled with the fast-evolving nature of these genes which erases evolutionary origin (for example due to RIP in the proximity of TE repeats, see above), are the basis of spotty distribution or whether there is support for the notion of horizontal transfer. We suggest both are likely.

**Carbohydrate-active enzymes**

Plant cell wall polysaccharides function both as a physical barrier to plant pathogens and as a carbon source for plant pathogens and saprotrophs alike. Because of the enormous structural and functional diversity of these complex carbohydrates, the enzymes involved in their breakdown show a remarkable functional diversity. Carbohydrate-active enzyme (CAZyme) families such as glycoside hydrolases (GH), polysaccharide lyases (PL) and carbohydrate esterases (CE), and CAZyme components such as the carbohydrate-binding modules (CBMs) therefore represent powerful reporters of the lifestyle of fungi, because (i) the latter achieve the digestion of complex carbohydrates extracellularly and (ii) sequence-based families of CAZymes correlate with structural and functional properties, although precise substrate specificities can be hard to predict [52]. In fact, whilst the sequence-based families of CAZymes frequently group together enzymes of varying substrate specificities, the functional correlation is often improved when considering broad substrate categories, especially among the different classes of plant polysaccharides (cellulose, hemicellulose, pectin). We have thus probed the CAZyme repertoires of the 18 *Dothideomycetes* to obtain clues to their digestive potential, especially against plant cell wall polysaccharides.

Table S22 shows that the genomes of the 18 examined *Dothideomycetes* encode almost 6,000 catabolic CAZyme catalytic domains (GHs, PLs, CEs) and CBMs but only 1,700 glycosyltransferases (GTs) involved in the assembly of fungal cell wall polysaccharides, N- and O-glycoproteins and reserve carbohydrates. The GTs, which assume roles that are not directly
Table 4. Summary of gene classes that are over-represented in repeat regions (i.e., the 2000 bp flanking predicted transposable elements).

| TE repeat content (%) | Over-representation of gene classes in repeat regions |
|------------------------|------------------------------------------------------|
|                        | Small secreted proteins | All secreted proteins | Secondary metabolism | Expanded orphan multi-gene families |
| Alternaria brassicicola | 5.58                    |                        |                      | *                                      |
| Baudoinia campanicensis | 0.4                     |                        |                      |                                        |
| Cladosporium fulvum     | 44.24                   |                        |                      |                                        |
| Cochliobolus heterostrophus C5 | 7.77             | *                     | *                    |                                        |
| Cochliobolus heterostrophus C4 (*) | 0               |                        |                      |                                        |
| Cochliobolus sativus     | 5.44                    | *                     | *                    |                                        |
| Dothistroma septosporum  | 0.67                    | *                     |                      |                                        |
| Hysterium pulicare (*)   | 0.57                    | *                     |                      |                                        |
| Leptosphaeria maculans   | 30.93                   |                        |                      | *                                      |
| Mycosphaerella fijensis  | 38.97                   | *                     |                      |                                        |
| Mycosphaerella graminicola | 11.66                | *                     |                      |                                        |
| Mycosphaerella papulicola | 20.81                | *                     |                      |                                        |
| Mycosphaerella populorum | 3.56                   | *                     | *                    | *                                      |
| Pyrenophora tritici-repentis | 11.44               | *                     | *                    | *                                      |
| Pyrenophora teres f. teres (*) | 1.98               |                        |                      | *                                      |
| Rhytidhysteron rufulum (*) | 0.18                |                        |                      |                                        |
| Setosphaeria turcica     | 11.16                   | *                     |                      |                                        |
| Stagonospora nodorum      | 2.37                    | *                     |                      |                                        |

See also Table S27 for more information. Genomes labeled with an asterisk (*) have been sequenced exclusively using Illumina technology.
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connected to the external environment show much less variation among the 18 genomes (min 83, max 110, average 96) than the digestive components: GHs (min 156, max 292, average 238); PLs (min 0, max 23, average 10); CEs (min 14, max 51, average 35); CBMs (min 17, max 101, average 48).

Generally, the numbers of CAZymes are higher in the Pleosporales and Hysteriales than in the Capnodiales (Figure 2D). At the individual family level (Table S22) the differences are even more striking and hierarchical clustering based on CAZyme family numbers (Figure 6) divides the 18 genomes into two major groups, with the Capnodiales on one side and the Hysteriales and Pleosporales on the other. The division into these two groups is dominated by differences in the number of CAZymes acting on cellulose. The strongest difference is found with family GH61 (enzymes performing oxidative cleavage of cellulose [53]), where Hysteriales and Pleosporales have an average of 24 genes (min 20, max 30) but Capnodiales have between one and three only (Table S22). GH61 is not the sole cellulytic family affected as families GH6, GH7, GH5 and CBM1 also show a clear expansion in Pleosporales and Hysteriales compared to Capnodiales, suggesting that the latter order of Dothideomycetes (containing mostly hemibiotrophs) does not extensively digest cellulose or that it employs another strategy for its digestion. This situation is reminiscent of the white rot/brown rot dichotomy ([54,55].

The difference between Capnodiales and other Dothideomycetes extends to the digestive enzymes directed against the other plant cell wall polysaccharides, specifically xylan and pectin. For instance, the two xylanase families GH10 and GH11 and the two acetylxylan esterase families CE1 and CE3 are significantly expanded in Pleosporales and Hysteriales compared to Capnodiales (Table S22). Patterns of enzymes involved in pectin digestion show a similar pattern, as the pectate lyases (families PL1 and PL3) and pectin methylesterases (family CE8) are expanded in Pleosporales (average 14.1 genes) compared to Capnodiales (average 6.0 genes) (Table S22). Also, Capnodiales encode fewer proteins with family CBM18 chitin-binding domains, than Pleosporales and Hysteriales. Chitin being produced by fungi and not plants, the multiplication of these domains perhaps reflects different strategies of Dothideomycetes to evade recognition by plant defence mechanisms as shown for C. fulvum [36,56,57] and proposed for M. graminicola [24].

Not all CAZymes are under-represented in the Capnodiales. For example, family GH64 is more abundant in Capnodiales (av. 4.7) than in Pleosporales and Hysteriales (av. 1), and family GH114 is more abundant in the two Hysteriales saptrotrphs (av. 7) than Pleosporales (av. 1.6) and Capnodiales (av. 0.4). No fungal enzyme from these families has been characterized so far.

Altogether, genome mining revealed that the overall distribution of genes encoding enzymes for plant cell wall digestion globally follows the taxonomical division of Capnodiales and Pleosporales, and that it probably corresponds to different strategies for (or extents of) the breakdown of cellulose, as well as xylan and pectin. Constraints perhaps just as important as the precise composition of plant cell walls may well have shaped the carbohydrate-active enzyme profile of Dothideomycetes, such as the strategy of penetration through the outer layers of plant tissues, the strategy to break down crystalline cellulose and the strategy to evade plant defense mechanisms.

Peptidases

Peptidases are important hydrolytic enzymes in plant pathogens that may have roles in signaling, nutrition, degradation of host
plant tissues and digestion of proteins involved in the plant response against pathogens [58,59,60,61]. Peptidase-encoding genes were catalogued in the predicted proteomes of the 18 genomes of Dothideomycetes and those of 21 other Ascomycota and Basidiomycota species according to the MEROPS database (Table S23, Figure S6). Secreted peptidases were studied separately, since these are more likely to be involved in pathogen-host interactions.

Dothideomycetes have a larger range of different exo- and endopeptidases than plant pathogens found in other fungal classes (Figure S7A) [62]. These proteins include several secreted peptidases of the MEROPS subfamilies A01, S08, S09 and S10 expected to efficiently digest proteins and/or with an acidic optimum able to work in inhospitable environments of the extracellular matrix (A01, C13, G01, M35, M20 and S10) (Table S23, Figure S7B) [62]. The genomes of the Dothideomycetes contain fewer non-secreted and secreted aspartic peptidases (A01) than those of the plant-pathogenic necrotrophs of the Leotiomycetes (Botrytis cinerea and Sclerotinia sclerotiorum) and the saprotrophs and ectomycorrhizal symbionts of the Agaricales (C. cinereus, P. chrysosporium, P. placenta, S. commune and L. bicolor), but this is compensated for by having the highest content in secreted metallo-(carboxypeptidases of the M14 subfamily and exopeptidases M28) and serine-peptidases of the carboxypeptidases S10 subfamily (Table S23, Figure S7AB). Within the Dothideomycetes the Pleosporales are specifically enriched in zinc-metallopeptidases of the M14 (5–9 models vs. 0–4 in Capnodiales and Hysteriales) and M28 subfamilies (10–13 models in Pleosporales vs. 6–9 in the others). Among the M14 secreted carboxypeptidases, three that are found in all members of the Pleosporales (and also found in the Sordariomycetes) have been lost in all of the fungi belonging to the orders Hysteriales and Capnodiales (Table S23, Figure S8).

Secreted zinc-metallopeptidases as well as trypsin (S01) and subtilisin (S01 and S53) serine-peptidases are known to have a potential role in pathogenicity and to be putatively involved in direct cell wall degradation by plant pathogens, as hydroxyproline-rich glycoproteins are possible targets of these enzymes [63,64,65]. Trypsin-like peptidases are limited in many fungal genomes to only one to five models found in each species, whereas plant pathogens are generally enriched in subtilisin-like proteins. Interestingly, within Dothideomycetes genes encoding secreted S08 subtilisin-like proteins is lower in the Capnodiales and Hysteriales (average 4.8 models vs. 7.3 in Pleosporales), whereas genes encoding aorin and grifolisin-like peptidases of the S53 subfamily are higher (average 5.7 models vs. 2.3 models in Pleosporales). The selection of specific subfamilies of peptidases in each of these fungal orders suggests that differences in the properties of the enzymes could have provided functional advantages to their respective common ancestors.

Another notable difference within the Dothideomycetes is that the genomes of the wheat pathogen M. graminicola and poplar pathogens M. populorum and M. populicola encode more oligopeptidases of the M03B subfamily (six for M. populorum, four for M. populicola and 18 for M. graminicola) than any other fungus analyzed.

Lipases

Several lipases are known to play important roles in plant pathogenicity. Fungal pathogens secrete lipases and cutinases that catalyze the hydrolysis of ester bonds from fatty acid polymers, facilitating fungal penetration through the cuticle [66,67]. A genome-wide analysis of lipase-encoding genes among the Dothideomycetes revealed that 14 families are conserved among these fungi, with considerable variations between species and taxonomical groups (Table S24, Figure S9). Secreted lipases are more likely to be involved in pathogen-host interactions than non-secreted lipases. Seven families of secreted lipases are conserved among the Dothideomycetes. Generally, Pleosporales and Hysteriales have higher numbers of lipases and secreted lipases than the Capnodiales (Figure 2D). This difference is most apparent in the cutinases, which are esterases capable of breaking the thick cutin protection of external plant tissues. While the examined pathogens have an average of 8.9 and 4.5 genes encoding secreted cutinases...
in the Pleosporales and Capnodiiales, respectively, the saprotrophs have 0 to 3 secreted cutinases each. The same distribution pattern is observed for plant pathogens versus non-pathogens in the outgroup, and can be explained by the fact that cutinases serve to break through the plant surface. Although the role of cutinases in fungal pathogenicity stayed controversial for a long time, the relationship with pathogenicity has been proven in several knockout studies [67,68]. Furthermore, there are several examples of cutinases playing various roles in the establishment of infection by being involved in spore attachment [69,70], surface signaling [71], and dissolution of the plant cuticle during penetration [72].

Enrichment of potential effector genes in proximity to Transposable Elements

As mentioned above, there is a large variation in numbers of transposable elements among species, from approximately 40% in C. fulvum [73] and M. fijiensis to almost no repeats in B. compositae (Figure 2B, Tables 4 and S25). Repeats are under-represented in genomes sequenced exclusively using Illumina technology due to limitations of the technology so are not directly comparable to those sequenced by other means. The majority of TEs (over 40%) of repeat content) DNA transposons and non-LTR retrotransposons are observed in smaller proportions, with predominantly C. fulvum, L. maculans and M. fijiensis showing a considerable percentage of their genomes being comprised of repeats of these types. The most frequently identified family of transposable elements is Gypsy (Table S25). All 18 Dothideomycetes have the same components of the silencing machinery encoded in their genomes (Table S26), so this does not offer an explanation for the differences in numbers of TEs.

Repetitive sequences in fungal genomes have been shown previously to be a target of the Repeat Induced Point mutation (RIP) machinery [74,75]. To analyze the effect of proximity of a gene to a repeat region, the RIP index of these genes was calculated as a function of distance to the repeat sequences. Only repeats that belonged to a known family of transposable elements were taken into account (Figure 2B).

Overall, the closer a gene was located to a repeat, the more likely a RIP signature was detected (Figure 7). The RIP index of a repeat family [A/p/ and Tp/a/Ap and Tp A/RIP products, correcting for false positives due to AT-rich regions. Higher values of the Tp/a/Ap and Tp A/RIP index indicate a stronger RIP response [74,76]. Based on this index the effect is strongest within the first 500 bp nearest the repeat and then drops more slowly and disappears at approximately 2000 bp from the repeats.

Next, we determined what genes are over-represented in the 2000 bp around the repeats. Only genes that (at least partially) overlap this region, but that do not overlap a repeat were taken into account. This was done to exclude the pseudogenes that are frequently found inside TE repeat sequences, but were either included in gene sets or not depending on annotation strategy of the different sequencing centers. Interestingly, in several genomes the genes encoding small secreted proteins, proteins involved in secondary metabolism, or members of expanded orphan multi-gene families are over-represented in the regions flanking repeats compared to the rest of the genome (Tables 4 and S27). Most genomes with a TE content of at least 2% have at least one of these functional annotation terms over-represented in the flanking regions around repeats with the exception of C. fulvum, in which however several genes in the proximity of TEs were reported as affected by RIP [73]. An expanded orphan multi-gene family is defined here as a gene family with at least 2 members that is present in only one Dothideomycete and in none of the outgroups. Family members frequently include small secreted proteins, but relatively few PFAM domains (Table S20).

In L. maculans, AT-rich blocks composed of transposable elements were previously shown to occasionally harbor genes encoding small secreted proteins, and those genes were more subjected to RIP than other genes [23]. We show here that this is a widely occurring phenomenon among Dothideomycetes, although not universal. Our analysis shows that not only genes encoding small secreted proteins, but also genes involved in secondary metabolism are preferentially located in the vicinity of transposable elements. The products of some members of these classes of genes have been implicated as effectors in pathogenesis [36]. The potential evolutionary benefit of co-localization of repeat elements and effector genes is a higher rate of mutation due to RIP, which in turn may lead to a higher rate of evolution. This would allow the pathogen to adapt more quickly to the host plant’s defenses. Furthermore, the observation that members of expanded orphan multi-gene families are over-represented near TEs suggests that TEs may have a function in species-specific gene family expansion in these organisms, presumably due to TE mobility.

Conclusions

Dothideomycetes is one of the largest groups of fungal plant pathogens, the genomic sequences of which were largely unknown until now. Here we described 14 newly sequenced genomes of Dothideomycetes and compared them with each other, the four previously published Dothideomycetes and with 21 other previously sequenced fungal genomes. The 18 sequenced dothideomycete genomes are members of the three major orders of Capnodiiales, Pleosporales, and Hysteriales, and represent a range of evolutionary distances within over 280 MYA since their common ancestor, as well as a variety of lifestyles and plant host associations. This added resolution makes it possible for the first time at such a large scale to explore genome organization, evolution, and differences between saprotrophic and the various modes of pathogenic lifestyles in Dothideomycetes.

There are large variations in genome size between the Dothideomycetes, which can be largely explained by the repetitive content of the individual genomes. Chromosome structural evolution in this class of fungi proceeds largely by intrachromosomal rearrangements. A gradient of synteny from macro- to mesosynteny was observed in comparisons between species depending on evolutionary distance and agreed with simulation analyses of chromosomal evolution by frequent inversions. The high rate of inversions may be facilitated by the occurrence of simple repeats at the boundaries of inverted segments. Whether this phenomenon of frequent inversions is fortuitous or has been selected for to allow for rapid rates of evolution is not known. Gene order has not been completely reshuffled by these inversions, since blocks of genes with conserved order have been identified across Dothideomycetes. Their function and the reason for their conservation are currently unknown, but the observation that in one case in L. maculans all the genes in one conserved block of genes are up-regulated during plant infection suggests that co-regulation may be an important factor in pathogenesis.

A structural feature of the Dothideomycetes is the presence of seemingly dispensable chromosomes with no obvious function [24,31,32,33]. Although dispensable chromosomes are known in other fungi, they usually are very few in number and have clear roles in niche adaptation, usually conditioning host specificity. Analyses of the 18 genomes of Dothideomycetes identified one to many scaffolds in multiple species that have the characteristics of dispensable chromosomes, so this phenomenon may occur commonly among the fungi in this class. Why and how these
putatively dispensable chromosomes are maintained through long periods of evolutionary history is not known. However, the intra-
chromosomal rearrangements leading to mesosynteny could keep
dispensable chromosomes intact and may at least in part explain
their apparent longevity.

The 18-genome comparative analysis also identified several
functional adaptations of Dothideomycetes to their specific lifestyles. Genes encoding protein classes that were shown previously to play
important roles as effectors in pathogenicity (e.g., enzymes for
secondary metabolite production, carbohydrate-active enzymes,
small secreted proteins, peptidases, and lipases) were found in all
Dothideomycetes. However, large variations in these numbers exist
between the different fungi. Generally, the Pleosporales and
Hysteriales have higher numbers of these genes than the Capnodiaceae.
This is also illustrated by the estimated gene counts of the
respectively last common ancestors for each of these groups. Possibly
these last common ancestors each had different lifestyles or modes
of pathogenesis. In the current set of organisms, necrotrophs
are found exclusively in the Pleosporales, whereas six out of seven
Capnodiaceae are (hem)biotrophs. For a necrotroph, having a large
arsenal of different types of effector genes presumably allows it to
efficiently attack and kill the host plant in various ways. In contrast,
(hem)biotrophs spend an extended part of their life cycle in a
stealth mode of pathogenicity, evading the host plant’s
defenses. In such a situation expressing a large arsenal of effectors
could be detrimental, as it could lead to detection by the host plant
and triggering of its defenses. The smaller set of effectors in
members of the Capnodiaceae presumably allows them to evade this
detection, as proposed previously for M. graminicola [24]. Another
method would be to efficiently down-regulate these genes during
stealth pathogenesis, which also may be the case for the three
hemibiotrophs in the Pleosporales. An analysis of gene expression
during the various stages of the life cycle should further light
on this. For saprotrophs such as those in the Hysteriales having a
large arsenal of these genes would be beneficial to efficiently obtain
nutrients from their environment, and this is reflected in their gene
complement. The extremophilic saprotroph B. compniacensis
appears to have adopted a different strategy than other
Dothideomycetes by reducing its genome size and complement of
effectors.

In addition to the various modes of pathogenicity, we have
identified numerous protein domains and multi-gene families that
are expanded in pathogens of cereal or trees, when compared to
the other Dothideomycetes. Their role is generally unknown,
however. It should be noted that host plant specificity may be
determined by a small set of genes and may therefore not show up
in genome-wide comparisons. The exact role of these domains and
of effectors in general cannot be predicted from large-scale
comparative studies and require genome- or gene-focused analyses
and experiments. However, an initial comparison of in planta
transcriptomes can already suggest genes that may be important
for pathogenicity.

We have shown that genes for effector proteins, previously
shown to occur in AT-rich and gene-poor regions in the genome of
L. maculans [23], occur often in close proximity to transposable
elements (TEs) in several Dothideomycetes. TEs are frequently a
target of Repeat Induced Point (RIP) mutations, and we have shown
that RIP also occurs in the flanking regions surrounding these TEs.
Co-localization of TEs and effector genes therefore exposes these genes to a higher rate of point mutations. This could
possibly accelerate their rates of evolution and thereby provide an
advantage in the arms race against their hosts. We also have shown
that orphan multi-gene families (i.e. gene families with at
least two members, but only found in one Dothideomycete) frequently
co-localize with TEs. A possible explanation for this is that the
high TE mobility rate functions as a driving force behind the
duplication of these genes, allowing rapid species-specific gene
family expansion and diversification.

As demonstrated by these results, the power of comparative
genomics is huge and will become increasingly important as more
genomes are sequenced. For fungi in general and Dothideomycetes
in particular the field is ascending rapidly. Several other Dothideomy-
cetes have been sequenced or are in progress and will provide
greater representation of the extensive pathogenic and ecological
diversity in this largest class of fungi. Completion of the 1000 Fungal Genomes project (http://jgi.doe.gov/fungi) at the US
DOE, Joint Genome Institute plus numerous genomes sequenced
through other initiatives provide a huge wealth of virtually
untapped resources for future progress in understanding fungal
biology and evolution.

Materials and Methods

Data availability

All genome assemblies and annotations can be interactively
accessed through the JGI fungal genome portal MycoCosm [77] at
http://jgi.doe.gov/fungi. The 14 new Dothideomycete genomes
discussed here are also deposited to DDBJ/EMBL/GenBank
under the following accessions numbers Alternaria brassicicola ATCC
96836: ACIW00000000, Baudonia comnii AUMH 10762:
AEIF00000000, Cladosporium fulum CBS131901 (race 0WU):
AMRR00000000, Cochliobulus heterostrophus ATCC 48331 (race T,
strain C4): AHU00000000, Cochliobulus heterostrophus ATCC 48331
(race O, strain C5): AIDY00000000, C. sativus ND90Pr:
AEIN00000000, D. septosporum CBS128990 (NZE10):
AEN00000000, H. pulicare CBS 123377: PRJNA181797,
Mycosphaerella fijiensis CIRAD86: AHZ00000000, Mycosphaerella
populicola P02.02b: AIDU00000000, Mycosphaerella populorum
SO2202: AEFD00000000, Pyrenophora tritici-repentis Pt-1C-BFP:
AAX00000000, Rhytidioblastus radiculatus CBS 306.38: PRJNA181799,
S. turcica Et28A: AHT00000000. The four additional
genomes of Leptosphaeria maculans [N3] [25], Mycosphaerella graminicola
IO323 [24], Pyrenophora teres f. teres 0–1 [22], and S. turcica
nodorum SN15 [21] used in the comparative analyses were
published earlier.

Genome sequencing and assembly

The genomes of the 14 new Dothideomycetes discussed in this
report were sequenced by several sequencing centers (Table 1)
using various sequencing platforms and analyzed using different
strategies, most of which are discussed in detail in genome-centric
publications [44,73,78]. Additional genome-centric papers are
planned for M. fijiensis, C. heterostrophus C5, C. heterostrophus C4, C.
sativus, S. turcica, M. populicola, M. populorum, and B. compniacensis.
The genomes of A. brassicicola, C. heterostrophus C5, M. fijiensis and P.
tritici-repentis were sequenced using Sanger technology, assembled
using A. arachne [79], and improved using a genetic linkage map (M.
fijiensis), optical map (P. tritici-repentis), or targeted finishing (C.
heterostrophus C5). The genomes of C. fijiensis and M. populicola were
sequenced using 454 reads and assembled with Celera Assembler
[73] and Newbler [80], respectively. The genomes of C.
heterostrophus C4, H. pulicare, and R. radiculatus were sequencing using
Illumina technology only and assembled using ALLPATHS-LG
(C4, [26]) and Velvet (both Hysteriales, [81]). The rest are hybrid
assemblies sequenced using combinations of 454, Illumina and
optionally Sanger reads, all assembled with Newbler [80].
Assembly characteristics are summarized in Figure 2B and Table
S1.
Genome annotation

Genomes sequenced by different organizations were annotated using different gene prediction pipelines. The *P. tritici-repentis* genome was annotated using the Broad Institute’s pipeline [44], the *A. brassicicola* genome was annotated using an ENSEMBL annotation pipeline [78] and the *C. fulvum* assembled scaffolds were annotated using the Cyrille2 workflow management system [73]. For *H. papuana* and *R. rubalum*, contigs greater than 300 bp were used for *ab initio* gene prediction in the software package Augustus [82] using the *Aspergillus fumigatus* gene predictions [83] for model guidance. The remaining 9 genomes were annotated using the JGI annotation pipeline, which combines multiple tools for gene prediction, annotation, and analysis, and deposits the results in the JGI Fungal Genome Portal MycoCosm (http://jgi. doe.gov/fungi) [77]. The assembled genomic scaffolds were masked using RepeatMasker [84] with the RepBase fungal library of 234 fungal repeats [85] and genome-specific libraries derived using RepeatScout [86] (see Repeat content below). Multiple sets of gene models were predicted for each assembly, and automated filtering based on homology and EST support was applied to produce a final non-redundant GeneCatalog representing the best gene model found at each genomic locus. The gene-prediction methods were: *EST-based predictions* with EST map (http://softberry.com) using raw ESTs and assembled EST contigs for each genome; *homology-based predictions* with Fgenesh+ [87] and GeneWise [88], with homology seeded by BLASTx alignments of the GenBank non-redundant sequence database (NR: http://www.ncbi.nlm.nih.gov/BLAST/) to the genomic scaffolds; and *ab initio predictions* using Fgenesh [87] and GeneMark [89]. GeneWise models were extended to include 5’ start and/or 3’ stop codons when possible. Additional EST-extended sets were generated using BLAT-aligned [90] EST data to add 5’ UTRs, 3’ UTRs, and CDS regions that were supported by ESTs but had been omitted by the initial prediction methods.

The predicted gene models from the genomes of Dothideomycetes and the outgroups were functionally annotated using the same pipeline for each genome, allowing comparison across species. Functional annotation by similarity to genes from the GenBank non-redundant set using BLASTp alignments [91] and hardware-accelerated double-affine Smith-Waterman alignments (http://www.timelogic.com) against SwissProt (http://www.expasy.org/sprot), the Kyoto Encyclopedia of Genes and Genomes (KEGG) [92], and eukaryotic orthologous groups of proteins (KOG) [93]; analyzed for signal sequences and transmembrane domains with SignalP [94] and TMHMM [95]; and functional domains were predicted using InterProScan [96]. Enzyme commission (EC) numbers (http://www.expasy.org/enzyme) were assigned based on KEGG hits, and Gene Ontology (GO) terms [97] were assigned based on Interpro and SwissProt hits. Multi-gene families in all the species in Table 1 were predicted using the JGI clustering pipeline. First, an all-versus-all blastp analysis is performed using an E-value of 1e-5 as cut off value. Next, for each blastp hit pair a modified blast score is calculated: blast score * cov1 + cov2. Here, cov1 and cov2 are the alignment coverages for protein 1 and 2 of the pair, respectively. This alignment coverage is a fraction of 1. This modified blast score is used as input for the MCL Markov clustering program [98,99], using an inflation parameter of 4. Each resulting cluster is considered a multi-gene family.

The functional annotations for all eighteen genomes are summarized in Figure 2 and in Table S1. In addition to this, repeats, lipases, CAZymes, peptidases, small secreted proteins, genes involved in secondary metabolism, and kinases in all 18 genomes were more extensively annotated as described below. The estimated gene counts of the last common ancestors of the taxa indicated in Figure 2D have been inferred using CAFÉ [100].

Repeat content

For all 18 genomes RepeatScout [86] was used to generate *de novo* predictions of transposable elements (TE). The output of RepeatScout is a library of consensus sequences corresponding to each family of identified repeats. We selected all repeat families which had a Blastn (and Blastx) hits to Repbase sequences [85] and also included families with a copy number of more than 150 in the genome. All potential repeats were searched for structural elements usually found at termini of TE, such as LTRs (long terminal repeats), TIRs (terminal inverted repeats) and TSDs (target site duplications). TEs were classified by a combination of similarity to Repbase sequences [85] and availability of structural repeats according to the procedures outlined previously [101]. Simple repetitive sequences (i.e., low-complexity DNA regions) were identified using RepeatMasker [84].

For the Illumina-only genomes an additional analysis was performed to estimate repeat content, using the FindErrors module of the ALLPATHS-LG assembler [36]. Briefly, a k-mer histogram using K = 24 bp is constructed and partitioned into bins corresponding to likely sequencing error, unique genome sequence, possible polymorphism, possible repetitive genome sequence, and highly represented k-mers based on the locations of peaks in the k-mer histogram. The sum of the counts corresponding to the bin associated with repetitive content is reported as the estimate.

Over-representation of functional annotation terms in genes overlapping a 2-kpb flank upstream or downstream of a TE repeat (but that did not overlap any TE repeat) was calculated as described below. Genes overlapping TE repeats were excluded from this analysis because their predictions varied widely among the genomes; they were actively removed from some gene sets whereas they were left untouched in others. It was outside the scope of this paper to re-predict genes for each genome.

Representation analysis

Custom scripts were developed in Python and R to analyze over- and under-representation of functional annotation terms in sets of genes using the Fisher Exact test. The Benjamini-Hochberg correction was used to correct for multiple testing using a *p*-value of 0.05, where applicable.

Estimating phylogenetic relationships and divergence times

The broad phylogeny in Figure 1 was performed with partial sequences of translation elongation factor-1 alpha (*TEF1*) and the largest and second-largest subunits of DNA-directed RNA polymerase II (*RPB1, RPB2*). DNA sequences were downloaded from GenBank as indicated in Table S29 or obtained from genome data available at the Fungal Genome portal at JGI. Each of the individual genes was conceptually translated in BioEdit [102] after the introns were removed. The amino acid sequences were aligned in SATE [103] using MAFFT [104] as the external sequence alignment tool and RAxML [105] as the tree estimator. The final data matrix had 67 taxa and 1129 characters with 31% missing and gap characters. Two isolates of *Arthrinomycetes* (*Simonyella variegata* and *Opegrapha dolomontica*), the sister class to *Dothideomycetes*, were used as outgroups. The three protein sequence alignments were individually subjected to model testing with ProtTest v.2.4, using the Akaike information criterion (AIC) and default settings at http://darwin.uvigo.es/software/
protest2_server.html [106]. This resulted in a choice of the following models: TEF1: LG; RPB1: RTREV; and RPB2: LG. A maximum-likelihood phylogenetic analysis of the concatenated alignment with partitioned models for each gene marker was performed with a gamma model of rate heterogeneity at the CIPRES web portal [107] using RAxML v. 7.2.8 [105,108]. Fifty maximum-likelihood (ML) searches were done, each one starting from a separate randomized tree, and the best-scoring tree was selected with a final likelihood score of −24058.182470. One thousand non-parametric bootstrap iterations were run and the resulting replicates plotted onto the best-scoring tree obtained previously. The RAxML tree was used to apply a penalized likelihood analysis in the program r8s v1.7 [109] to produce a chronogram. This meant that phylogenetic uncertainty was not incorporated in this analysis. We used two dates suggested in a more comprehensive analysis in previous work [110]. The root of the tree (and the split between Dothideomycetes and Arthrinomycetes) was set to ages of 420 and 309 MYA, respectively. These represent the upper and lower bound dates of a 95% confidence interval determined using a Bayesian approach in BEAST [111] as applied previously [110]. In our analysis the Langley-Fitch method and a truncated Newton method with bound constraints were applied following Taylor and Berbee [112].

For the genome-based tree in Figure 2, orthologous groups of genes (having exactly one gene for each organism in Table 1) were identified from the multi-gene family set described above. There were 51 of these orthologous groups and the corresponding proteins for the organisms belonging to the Dothideomycetes were concatenated. The sequences were aligned using MAFFT 6.717b [113] and well-aligned regions were extracted using Gblocks 0.91b [114]. The parallelized version of RAxML 7.2.8 [105,108] with the PROTGAMMAWAG model with 100 rapid bootstrap partitions was used to calculate a species tree. The tree was visualized using Dendroscope 2.7.4 [115].

Whole-genome DNA synteny

Whole-genome DNA synteny was calculated using VISTA [116] and visualized using the DotPlot function as implemented in the JGI Genome Portals [77]. Significance of whole-genome synteny was determined using the methods described previously [27], with modifications. For all combinations of genomes (genome A and genome B), all combinations of their sequences (sequence A from genome A and sequence B from genome B) were tested for significant sequence conservation. Only sequences of at least 500 kbp were used in these analyses. The probability of synteny (Psyn) between sequence A of genome A and sequence B of genome B was calculated using a one-tailed cumulative binomial test described previously [27], with the modification that n = 100; x = length conserved in sequence A * length conserved in sequence B/length of sequence A * length of sequence B) - 1/2 and rounded to the nearest integer; p = (Total length conserved in genome A * Total length conserved in genome B/Total length genome A * Total length genome B)/number of sequence pair combinations. A sequence pair was considered to have significant amounts of sequence conservation if Psyn ≥ 0.999. The determination of the whole-genome synteny and its type (mesosynteny or macrosynteny) were made using the significant pair ratio formula and the 20-kbp cut off, respectively [27]. The level of synteny degradation was computed using the pair exclusivity ratio [(Total length of conserved regions between sequences A and B)/(Total length of conserved regions for sequence A and all sequences of genome B + Total length of conserved regions for sequence B and all sequences of genome A)]. This formula is similar to that described previously [27] with modifications and the result is a fraction of 1. If the maximum value of all pair exclusivities of a genome pair was less than 0.75, synteny was classified as degraded.

Gene order conservation

Gene order conservation across the 18 Dothideomycetes was studied using a custom script written in Python. Multi-gene families were determined as described above. Each gene of all organisms was thus assigned to a multi-gene family and orthologous relations between the genes could be determined. These relations can be one-to-one, one-to-many or many-to-many. The location of the first codon of the genes was used to determine the order of those genes on a given scaffold. Next, a sliding window with the size of 10 genes on a given scaffold of a given organism was compared to all possible windows on all scaffolds in all organisms. If a scaffold contained fewer than 10 genes, then all genes on that scaffold were considered in one window. For each comparison, the number of represented orthologous groups that was present in both windows was determined. If this number was at least 5, then these two windows were considered to be syntenic. If this syntenic window was present at least once in at least 70% of the studied organisms, then it was considered to be a conserved syntenic window. This analysis was done for all possible windows in all studied organisms. Next, overlapping conserved syntenic windows were combined into conserved syntenic blocks.

Expansion and depletion of PFAM domains and multi-gene families

The expansion and depletion of PFAM domains and multi-gene families was determined by comparing genomes that were grouped according to phylogeny, host or lifestyle. In each comparison, an in-group of organisms was compared to an out-group. PFAM domains and multi-gene families were only included if they were present with at least one count in at least 50% of the organisms in at least one of these groups. If a PFAM domain or multi-gene family was unique to either the in-group or the out-group, or if it was expanded in either the in-group or the out-group (as determined by t-test and Wilcoxon rank test), then it was reported.

Small secreted proteins

Small secreted proteins (SSPs) are defined here as proteins that are smaller than 200 aa, have a secretion signal as determined by SignalP 3.0 [94] and have no transmembrane domain (TMM) as determined by TMHMM 2.0 [117]. However, one transmembrane domain is allowed when present in the N-terminal 40 amino acids, since this often corresponds to the secretion signal. An SSP was labeled as ‘high cysteine’ when the percentage of cysteine residues in the protein was at least twice as high as the average percentage of cysteine residues in all predicted proteins of that organism.

Secondary metabolism

The Hidden Markov Model (HMM) signatures previously described for the AntiSMASH pipeline [118] were used to identify and annotate putative polyketide synthase (PKS) and terpene synthase (TPS) genes in all 18 genomes after validation on the previously manually curated C. heterostrophus set. The same cut-off values and logic were applied. Nonribosomal peptide synthetase (NPS) encoding genes were identified using the method described by Bushey and Turgeon [39], since AntiSMASH [118] performed poorly on the previously manually curated set of C. heterostrophus NPS genes. In all cases we used the annotated C. heterostrophus proteins to query NPS, PKS, and TPS protein datasets extracted
from the 18 dothideomycete genomes using a best-hit Blastp search.

CAZyme annotation

The detection, module composition and family assignment of all carbohydrate-active enzymes was performed just as for the daily updates of the CAZy database (http://www.cazy.org) and described previously [52]. Briefly, the method combines BLAST and HMMER searches conducted against sequence libraries and HMM profiles made of the individual functional modules featured in the CAZy database. All positive hits were manually examined by human curators for final validation. For the heat map only the GH, PL, and CE families were considered. Hierarchical clustering of both families and organisms was performed with the program MeV, which is part of the TM4 Software Suite [83]. Euclidian distance was used as distance metric and complete linkage clustering as linkage method. In Figure 6 only CAZymes with more than 1 member in at least one organism were included, for clarity.

Peptidases

Peptidases were predicted from the protein model catalogs of 40 fungi (Table 1). For each fungal genome considered, the protein models were used as blastp query against full-length sequences of the MEROPS database (e-value = 1e-04) [release 9.5; http://merops.sanger.ac.uk]. False positives were eliminated following unsuccessful searches against peptidase units and peptidase domains of the MEROPS (e-value = 1e-04) and the Pfam (V. 26.0; HMMER searches, e-value = 1.0) databases, respectively. Similarity of the models to putative peptidases was finally cross checked by parsing hits obtained following a blastp search (e-value = 1e-04) on the NCBI nr protein database. Prediction of putative secreted peptidases was then carried out using a combination of the SignalP 4.0 and TargetP 1.1 servers (http://www.cbs.dtu.dk/services/).

Lipases

Putative lipases were classified according to BLASTp (E-value cut-off of 1e-04) results obtained against the Lipase Engineering Database (http://www.led.uni-stuttgart.de/). False positives were eliminated by parsing hits obtained for the presence of a lipase-specific domain. Prediction of putative secreted lipases was processed as described above for secreted peptidases.

Supporting Information

**Figure S1** Ratio of predicted core and non-core proteins in the 18 genomes of Dothideomycetes. (TIFF)

**Figure S2** Phylogeny and gene count of predicted casein kinase 1 (CK1) genes in the genomes of 18 Dothideomycetes. (TIFF)

**Figure S3** Gene counts of classes that have been implicated in plant pathogenesis. Gene counts in extant species (indicated right of the tree) and estimated gene counts in inferred last common ancestors (indicated on the tree nodes) are given. A. Genes encoding small secreted proteins. B. Genes encoding proteins involved in secondary metabolic production. C. Genes encoding carbohydrate-active enzymes (CAZymes). D. Genes encoding secreted peptidases. E. Genes encoding secreted lipases. (TIFF)

**Figure S4** Predicted genes involved in secondary metabolism. Genomes of organisms belonging to the Pleosporales and Hypocreales generally contain more genes involved in this process than the Capnidiaceae, especially in the case of the Polyketide synthase Type I. (TIFF)

**Figure S5** Presence of aflatoxin-like genes in Dothideo-

mycetes and other fungi. Aflatoxin-like genes were found using BLASTp (e-value of 10^-5) of predicted amino acid sequences of Dothistroma septosporum dothistromin genes (main block of 14 Ds genes) or Aspergillus flavus sterigmatocystin (ergT [afM] and omtB [afO]) or aflatoxin genes (omtB [afP], omtA [afQ]) [Yu et al 2004], against gene catalogues for all genomes shown, with reciprocal BLAST back to the D. septosporum or A. flavus genomes. Dark gray boxes indicate presence of a reciprocal best hit, light gray indicates one-directional best hit, and white indicates no hit. Dothideomycetes are D. septosporum (Ds), Cladosporium fulcum (Cf), Mycosphaerella fijiensis (Mf), Mycosphaerella graminicola (Mg), Septoria musica (Sm), Septoria populicola (Sp), Boudaudina compacticeps (Bc), Leptosphaeria maculans (Lm), Pyrenopeziza teres f. teres (Pt), Cochliobolus sativus (Cs), Cochliobolus heterostrophus (Ch), Septoria turcica (St), Sargassospora nodorum (Sn), Pyrenophora tritici-repentis (Pr), Alternaria brassicicola (Ab), Rhytidistroma veyhuana (Rv), Hysterium pulicare (Hp), Eurotiomycetes (Euro) are Aspergillus nidulans (An), A. flavus (Af). Leotiomycetes (Leo) are Sclerotinia sclerotiorum (Ss), Botrytis cinerea (Bc), Sordariomycetes (Sord) are Verticillium dahliae (Vd), Magnaporthe grisea (Mg), Puccinia psidii (Pp), Pustula pyri (Pg), Puccinia graminis (Pg), Magnaporthe oryzae (Mo), Ustilaginoidea virens (Us), Malassezia globosa (Mg), Saccharomyces cerevisiae (Sc), Basidiomycetes are the Agraricozyma (Agar) Pusta placaeta (Pp), Laccaria bicolor (Lb), Cryptosporiaceae nonformans var. grubii (Cn), Coprinopsis cinerea (Cc), Schizopyllum commune (Sc), Phanerochaete chrysosporium (Ph), Pucciniomycotina (Puc), Puccinia graminis (Pg), Melampsora larici-populina (Mp), Ustulaginozyma ustulata (Us), Malassezia globosa (Mg), Ustulaginozyma (Um). Protein ID numbers of query genes are 66976, 181128, 192192, 192193, 75691, 57312, 48495, 75546, 75609, 139960, 75656, 75692, 75547, 75566, 192555, 181278, 139960, 192193, 75691, 57312, 48495, 75546, 75609, 139960, 75656, 75692, 75547, 75566 (D. septosporum genome) and AFL2G_07219.2, 07216.2, 07215.2, 07214.2 (A. flavus genome). Note only Ds-HexA, PksA, DotA, AdhA, VbsA and AflR were to a paralogous pair hit the same gene in each genome. AflP and AflQ were to a paralogous pair hit the same gene in each Agaricozyma species showing gray shading. In contrast, the top matches of Ds-HexA and Ds-HexB both hit the same gene in each Agaricozyma species showing gray shading. In contrast, the top matches of Ds-HexA and Ds-HexB were to a paralogous divergent pair of putative fatty acid synthase genes in all Dothideomycetes except Be, Pr, St, Sn, Pr and all Sordariomycetes except Fo. See also Text S4. (TIFF)

**Figure S6** Principal component analysis and hierarchical clustering of protease family assignments of 18 Dothideomycetes and outgroups. A matrix was constructed containing the number of proteases assigned to each MEROPS family in each fungal genome. Analyses were performed with the Rcmdr package of R, and the results graphed along the first two components. Colored squares represent the centroid of each cluster of species, indicated with the same color. Taxonomy: Ag., Agaricales; Cap., Capnidae; Dia., Diaporthales; Hel., Helotiales; Hyp., Hypocreales; Hys., Hypocreales; Mag., Magnaporthales; Mal., Malasseziales; Pat., Patellariales; Plc., Pleosporales; Pol., Polyporales; Sac., Saccharomycetales; Urc., Uredinales;
Ust., Ustilaginales; Tre., Tremellales. Lifestyles: B., Biotroph; Ec., Ectomycorrhizal; En., Endophyte; OB., Obligate biotroph; S., Saprotroph; N., Necrotroph; H., Hemi-biotroph; U., Undetermined.

(TIFF)

Figure S7 Distribution of peptidase families in Dothideomycetes and other fungal classes. Putative peptidases were classified based on the MEROPS database. A. Non-secreted and secreted peptidases. B. Secreted peptidases. Letters above columns indicate significant differences between peptidase content means as determined by a Tukey's HSD test after significant one-way ANOVA.

(TIFF)

Figure S8 Number of genes encoding peptidases in the genomes of Capnodiales, Pleosporales and Hysteriales fungi.

(TIFF)

Figure S9 Principal component analysis and hierarchical clustering of lipase family assignments of 18 Dothideomycetes and outgroups. Colored squares represent the centroid of each cluster of species, indicated with the same color. Taxonomy: Ag., Agaricales; Cap., Capnodiales; Dia., Diaporthales; Hel., Helotiales; Hyp., Hypocreales; Hys., Hysteriales; Mag., Magnaporthales; Mal., Malasseziales; Pat., Patellariales; Plo., Pleosporales; Pol. Polyporales; Sac., Saccharomycetales; Ure., Uredinales; Ust., Ustilaginales; Tre., Tremellales. Lifestyles: B., Biotroph; Ec., Ectomycorrhizal; En., Endophyte; OB., Obligate biotroph; S., Saprotroph; N., Necrotroph; H., Hemi-biotroph; U., Undetermined.

(TIFF)

Table S1 Assembly and annotation statistics of the genomes of 18 Dothideomycetes described in this report.

(XLS)

Table S2 Classification of synteny type in comparisons of 18 Dothideomycetes. The genome of Aspergillus nidulans was used as an outgroup.

(XLS)

Table S3 Simple repeats are over-represented near the inversion breakpoints in relatively closely related mesosyntenic scaffold pairs. The number of breakpoints that has at least one simple repeat within 500 bp distance is significantly higher than can be explained by chance.

(XLS)

Table S4 Two syntenic blocks of genes are identified in the genomes of the Dothideomycetes. Genes are located sequentially on the scaffolds. Each protein ID represents one predicted gene. The cluster ID represents the ID of the multi-gene family to which each gene belongs. Each gene that is part of a syntenic block has a syntenic ID.

(XLS)

Table S5 Annotations of the genes in the two syntenic blocks from Table S4. Each syntenic block contains 3 multi-gene families.

(XLS)

Table S6 Orthologous pairs of genes between Mycosphaerella graminicola and Leptosphaeria maculans that are up- or down-regulated in both organisms when expression during relatively early infection is compared to expression relatively late in infection. Each row represents an orthologous pair of genes. The protein IDs, expression ratio, GO annotation and PFAM annotation of each gene are indicated.

(XLS)

Table S7 Functional annotation terms that are over- or under-represented in genes that are part of an orthologous pair that is co-regulated in Mycosphaerella graminicola and Leptosphaeria maculans. See also Table S6.

(XLS)

Table S8 Syntenic blocks of genes are identified in the genomes of eight Pleosporales. Genes are located sequentially on the scaffolds. Each protein ID represents one predicted gene. The cluster ID represents the ID of the multi-gene family to which each gene belongs. Each gene that is part of a syntenic block has a syntenic ID.

(XLS)

Table S9 Syntenic blocks of genes are identified in the genomes of six Mycosphaerellaceae. Genes are located sequentially on the scaffolds. Each protein ID represents one predicted gene. The cluster ID represents the ID of the multi-gene family to which each gene belongs. Each gene that is part of a syntenic block has a syntenic ID.

(XLS)

Table S10 Functional annotation terms that are over- or under-represented in genes that are located in at least one syntenic block (described in Table S8). For each species the over-represented terms are indicated.

(XLS)

Table S11 Functional annotation terms that are over- or under-represented in genes that are located in at least one syntenic block (described in Table S9). For each species the over-represented terms are indicated.

(XLS)

Table S12 Additional statistics on the putatively dispensable chromosomes.

(XLS)

Table S13 Functional annotation terms that are over- or under-represented in genes of which the product is not part of the core proteome.

(XLS)

Table S14 PFAM domains that are expanded or depleted in the various comparisons in Table 3. In each comparison the organisms are marked as part of either the in-group or the out-group. Note that each comparison has its own tab.

(XLS)

Table S15 Multi-gene families that are expanded or depleted in the various comparisons in Table 3. In each comparison the organisms are marked as part of either the in-group or the out-group. Note that each comparison has its own tab.

(XLS)

Table S16 Predicted kinases in the genomes of the 18 Dothideomycetes and in those of the outgroups. For each kinase type in each genome, the absolute number is indicated, as well as the percentage of total kinases in that genome. CAMK (calmodulin-regulated kinases); CMGC family (cyclin-dependent kinases, mitogen-activated protein kinases, CDK-like kinases, and glycogen synthase kinase); STE (including many kinases functioning upstream of the MAPKs); AGC (including cyclic-nucleotide and calcium-phospholipid-dependent kinases, ribosomal S6-phos-
phorylating kinases, G protein-coupled kinases, and related kinases; CK1 family (casein kinase 1, and close relatives); the TK family (tyrosine kinases); and the TKL family (tyrosine kinase-like kinases (TKLs), which resemble TK but are actually serine-threonine kinases).

Table S17 Statistics of predicted small secreted proteins (SSPs) in the genomes of the 18 Dothideomycetes and in those of the outgroups.

Table S18 Predicted genes involved in secondary metabolism.

Table S19 Distribution of nonribosomal peptide synthetases across the 18 Dothideomycetes relative to the highly curated set in Cochliobolus heterostrophus set [39].

Table S20 Distribution of polyketide synthases across the 18 Dothideomycetes relative to the highly curated set in Cochliobolus heterostrophus set [40].

Table S21 Distribution of terpene synthases across the 18 Dothideomycetes relative to the Cochliobolus heterostrophus JGI set. Cutoff is greater than 70% identity at the amino acid level.

Table S22 Individual carbohydrate-active enzyme (CA-Zyme) family counts in the genomes of 18 Dothideomycetes and in those of the outgroups.

Table S23 Predicted peptidases in the genomes of 18 Dothideomycetes and in those of the outgroups used. Both the full set of peptidases and the subset of secreted peptidases are indicated.

Table S24 Predicted lipases in the genomes of Dothideomycetes. Both the full set of lipases and the subset of secreted lipases are indicated.

Table S25 Repetitive content and transposable element (TE) annotation for the 18 Dothideomycetes.

Table S26 Presence of components of the repeat induced point mutations (RIP) and silencing machineries in the genomes of Dothideomycetes. All Dothideomycetes contain the same complement of components. For each component in each genome the protein ID of the best blastp hit and the corresponding E-value are indicated. In some cases the gene was missed during gene prediction, and in these cases the genomic location of the best blastn hit and the corresponding E-value are indicated.

Table S27 Functional annotation terms that are over- or under-represented in genes that are located in regions up to 2 kbp from Transposable Elements.

Table S28 Statistics on members of expanded orphan multi-gene families. An expanded orphan multi-gene family is defined here as a gene family with at least 2 members that is present in only one Dothideomycete and in none of the outgroups. Family members frequently include small secreted proteins, but relatively few PFAM domains.

Table S29 Accession numbers of sequences that were used to estimate the phylogenetic relationships between the 67 organisms in Figure 1.

Text S1 Additional considerations concerning Dothideomycetes phylogeny and divergence time estimates.

Text S2 Predicted kinases in the genomes of 18 Dothideomycetes and in those of the outgroups used.

Text S3 Comparative transcriptomics during pathogenesis.

Text S4 Dothistromin and aflatoxin biosynthetic pathways in Dothideomycetes.

Author Contributions
Conceived and designed the experiments: RAO REB LC SBG IVG RCH GHJK CL JAS JWS BGT PJGMDW SZ. Performed the experiments: RAO REB BJC BD NF FG BH BAH IK AAS CLS BGT ACC KL EAL. Contributed reagents/materials/analysis tools: KWB SL. Wrote the paper: RAO REB NF SBG IVG BH BAH CLS BGT ACC. Sequenced and assembled genome(s) and/or ESTs: WBBC ACC BD CNH KL EAL SL. Annotated genome(s): CNH RAO.

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