Enemy or ally: a genomic approach to elucidate the lifestyle of Phyllosticta citrichinaensis

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Abstract

Members of the fungal genus Phyllosticta can colonize a variety of plant hosts, including several Citrus species such as Citrus sinensis (orange), Citrus limon (lemon), and Citrus maxima (pomelo). Some Phyllosticta species have the capacity to cause disease, such as Citrus Black Spot, while others have only been observed as endophytes. Thus far, genomic differences underlying lifestyle adaptations of Phyllosticta species have not yet been studied. Furthermore, the lifestyle of Phyllosticta citrichinaensis is ambiguous, as it has been described as a weak pathogen but Koch’s postulates may not have been established and the presence of this species was never reported to cause any crop or economic losses. Here, we examined the genomic differences between pathogenic and endophytic Phyllosticta spp. colonizing Citrus and specifically aimed to elucidate the lifestyle of Phyllosticta citrichinaensis. We found several genomic differences between species of different lifestyles, including groups of genes that were only present in pathogens or endophytes. We also observed that species, based on their carbohydrate active enzymes, group independent of their phylogenetic association, and this clustering correlated with trophic prediction. Phyllosticta citrichinaensis shows an intermediate lifestyle, sharing genomic and phenotypic attributes of both pathogens and endophytes. We thus present the first genomic comparison of multiple citrus-colonizing pathogens and endophytes of the genus Phyllosticta, and therefore provide the basis for further comparative studies into the lifestyle adaptations within this genus.

Keywords: fungal plant pathogens; genomics; CAZymes; lifestyle adaptations; citrus; endophyte; pathogen

Introduction

Fungal and oomycete phytopathogens are a major threat to global food security (Fisher et al. 2012). Despite many technological and methodological developments, such as the development of disease-resistant crops, this threat remains a pressing concern for humankind due to emergence of new or adapted species, and a lack of in-depth understanding of disease mechanisms and their genomic basis (Fudal et al. 2009; Singh et al. 2011; Fisher et al. 2012).

Plant-associated fungi and oomycetes can be broadly classified as pathogens, endophytes, or saprotrophs, i.e. they are classified based on their capacity to cause disease symptoms on their host plants. Furthermore, these microbes can be linked to 5 different trophic classes based on their specific feeding behavior (Kabbage et al. 2015). Necrotrophs are characterized as pathogens that feed on dead tissue, biotrophs as pathogens that feed on living tissue, and hemibiotrophs are pathogens that go through an initial biotrophic phase before switching to a necrotrophic phase (Oliver and Ipcho 2004). In the same classification model, nonpathogenic species that live within a plant are classified as endophytes, while species that live only on decaying plant material are referred to as saprotrophs. This classification model, which is mainly based on observational data, clearly has limitations, for instance when one species is classified as a necrotroph when interacting with one host but as biotroph when interacting with another (Veloso and Van Kan 2018). Consequently, much research in recent years has focused on establishing the genomic basis underlying differences between species that exhibit different lifestyles. Uncovering these genomic signatures would provide a more reliable method of classification and an increased understanding of host colonization and disease mechanisms, which is of significant importance in developing more effective disease management strategies (O’Connell et al. 2012; Ohm et al. 2012; Spanu 2012; Möller and Stukenbrock 2017; Plissonneau et al. 2017; Haridas et al. 2020).

A common feature of various investigations into the genomic basis of pathogenicity is the identification of specific adaptations present in one lifestyle but absent or reduced in the other
A major current focus is the study of effectors, which are often defined as small secreted proteins that play an important role in establishing the interaction with the host, for instance by degrading the host cell wall or shielding the pathogen from detection by the host immune system. Effectors are often shared by strains and sometimes by species that colonize the same host. The identification of known effectors and other genes that are present only in species of a specific lifestyle can therefore provide useful information when studying the genomic basis of pathogenicity.

However, as hosts rapidly evolve mechanisms to recognize effectors to re-establish immunity, effectors frequently mutate resulting in rapid effector diversification to avoid detection by the host immune system. Thus, effector repertoires in separate fungal lineages may differ significantly.

Carbohydrate active enzymes (CAZymes) play diverse roles in degradation and biosynthesis of carbohydrates such as those found in plant cell walls. For example, plant pathogens can utilize CAZymes to penetrate the host cell wall to establish symbiosis and to liberate carbohydrates from host tissues for growth and reproduction. CAZymes can also contribute to virulence, and differences in CAZyme repertoires can mediate microbial lifestyle differences. Consequently, CAZymes have been used to propose new lifestyle classification models for oomycete and fungal species.

For instance, Hane and colleagues recently proposed 5 new trophic classes based on primary nutrient source preferences as approximated by the presence and abundance of CAZymes. These trophic classes correspond to primary nutrient sources as such as those found in plant cell walls. The phylogenetic diversity of these classes is reflected in the presence and abundance of CAZymes.

Previously, phylogenetic analyses between pathogenic and endophytic Phyllosticta spp. have been hampered by the quality of genomes and the availability of only a single endophyte genome (P. capitalesis), which was relatively distantly related to the species it was compared to, and consequently genomic adaptations toward these 2 broad lifestyles remained unclear. Therefore, a comparison of new and high-quality genomes which includes multiple species of different lifestyles could provide the necessary foundation to finally discovering the genomic underpinning for phytopathology in Phyllosticta, which is essential for the development of better disease management strategies.
first comparative genomics study using multiple complete genomes of 2 endophytic and 3 phytopathogenic Phyllosticta species and established genomic differences between species of different lifestyles within this genus. In addition, we use the genomes of 2 separate P. citrichinaensis isolates, one of which is newly sequenced, in an attempt to elucidate the lifestyle of the ambiguous P. citrichinaensis.

Materials and methods
Sequencing, annotation, genome quality, and availability

All non-Phyllosticta genomes were previously published (Haridas et al. 2020) and are available on MycoCosm (https://mycocosm.jgi.doe.gov/Dothideomycetes; last accessed: 22 March 2022); Grigoriev et al., 2014). The database identifiers (DBIDs) that are given by the Joint Genome Institute (JGI) to identify specific genomes, and which can be used to access the genome’s online portal (https://mycocosm.jgi.doe.gov/DBID, e.g. https://mycocosm.jgi.doe.gov/Aaao1; last accessed: 22 March 2022) are listed in Supplementary Table 1. Seven of the 8 Phyllosticta genomes included in our analyses were also previously published (Guarnaccia et al., 2019), and are available on MycoCosm (mycocosm.jgi.doe.gov/Phyllosticta; last accessed: 22 March 2022).

Phyllosticta citrichinaensis liquid cultures (250 ml Malt peptone broth in 500 ml Erlenmeyer flasks) were incubated at 25°C and 180 rpm for 10 to 14 days, after which genomic DNA was isolated using the Qiagen Genomic-tip 100/G kit and the Qiagen Genomic DNA Buffer Set. The genome assembly of P. citrichinaensis genome (CBS 129764) was generated by the JGI using the PacBio (>10kb with Blue Pippin Size Selection) long-read sequencing technology. Long-read sequencing data was assembled using Fl ye 2.3.6 and the genome assembly was annotated using the JGI Annotation pipeline (Grigoriev et al. 2014). The genome assembly and annotation are available via the MycoCosm platform (https://mycocosm.jgi.doe.gov/Pcit129764; last accessed: 22 March 2022). Quality assessments were performed using BUSCO 4.1.4 (Manni et al. 2021) and QUAST 5.0.2 (Gurevich et al. 2013) using default parameters. One-to-one whole-genome comparisons were performed using ProMer (default settings), which is part of the MUMmer 3.25 conda package (Marçais et al. 2018) and plotted with mummerplot (also part of MUMMer) using the –filter and –fat parameters. We used OrthoFinder 2.2.6 (Emms and Kelly 2019) to identify ortholog groups (OGs) across all 116 fungal genome annotations (Supplementary Table 2a). Ortholog groups unique to species of specific lifestyles were identified using UpSetR 1.4.0 (Supplementary Table 2b, Conway et al. 2017).

Secreted proteins and effectors

Predector 1.1.0 was used to predict secreted proteins and effectors in the predicted proteomes of all 116 fungal genomes. We used predicted secreted proteins with a manual secreted core > 4, and effectors with an effector score > 1 or > 2 in further analysis. We visualized the distribution of OGs of which 50% or more of the genes were predicted to be a secreted protein or an effector, by generating a clustered heatmap in R using the ComplexHeatmap 2.6.2 package (Gu et al. 2016).

Carbon utilization and CATAstrophy

Carbon growth studies were performed as described previously (Buijs et al. 2021). In short, 1-mm-diameter plugs from 2-week-old colony edges of Phyllosticta species were inoculated on 35 different carbon sources and incubated at 25°C until the largest colony reached the edge of a 35-mm-diameter plate. As different Phyllosticta species demonstrate different growth speeds, this moment fell on different days after inoculation (between 5 and 10 days). When the largest colony of a species reached the edge of a plate, colony diameters were measured on all sources and images were taken using a standard camera setup. This approach was chosen to be able to compare species with different growth speeds. All growth studies were performed in duplicate. Measurements were averaged and used to generate a clustered heatmap using the ComplexHeatmap 2.6.2 package (Gu et al. 2016) in R (R Core Team, 2021).

We used CATAstrophy to predict lifestyles from CAZyme repertoires (Hane et al. 2020). To this end, we first used hmmpress 3.3.2 to generate a local HMMER database of dBCAN 8 (Zhang et al. 2018). We then queried all 116 predicted proteomes with the local dBCAN HMMs database using hmsmscan 3.3.2 with the –domtblout parameter to create a domain table for each proteome. CATAstrophy 0.0.3 was then ran on all 116 domain tables using parameters –p, –c, –model v8 and –format hmmer_domtab. The heatmap was created by identifying all OGs (as created previously using OrthoFinder) that contained a CAZyme, counting the number of genes in each OG for each species, and then generating a heatmap using the ComplexHeatmap 2.6.2 package in R. Empty columns, e.g. CAZYme families for which no genes present in these species, were filtered out of the heatmap for visualization purposes, but are present in the original data (Supplementary Table 5).

Results

Phyllosticta genome assemblies are of high quality

Lifestyle differences are often driven by genomic adaptations (Ohm et al. 2012; Kabbage et al. 2015; Haridas et al. 2020), and we hypothesize that this also applies to Phyllosticta species with different lifestyles. Taxonomically, Phyllosticta belongs to Dothideomycetes, a fungal class with extensive genomic resources (Wikee et al. 2013b; Haridas et al. 2020). To enable studies in lifestyle differences in Phyllosticta, we made use of 8 Phyllosticta genome sequences, 7 of which were assembled and published previously (Guarnaccia et al. 2017, 2019). Here, we performed genome sequencing of P. citrichinaensis (CBS 129764), which is the second genome of this species to be sequenced, thereby enabling us to also evaluate intra-species variation. We included genome assemblies of 2 different P. citrichinaensis strains, as well as the genome assemblies of the endophyte P. citribraziliensis, the closest relative of P. citrichinaensis, those of the 2 pathogenic species P. citricarpa and P. paracitricarpa, which are very closely related (Fig. 1a, also see Guarnaccia et al. 2019), of the pathogenic species P. citriasiana, and of the endophyte P. capitulensis, which is phylogenetically the least related to the other Phyllosticta species (Fig. 1a). As these genome assemblies include multiple species of both lifestyles (pathogens and endophytes), comparative genomics may reveal the genomic underpinning for lifestyle adaptations within this genus, and ultimately aid in determining the lifestyle of P. citrichinaensis.

To determine genome assembly size, fragmentation, and annotation completeness of the Phyllosticta genomes, we used QUAST (Gurevich et al. 2013) and BUSCO (Manni et al. 2021), and compared the results to 100 previously published Dothideomycete genomes as well as 8 genomes of fungal species outside of Dothideomycetes (Fig. 1a; Supplementary Table 1, Haridas et al. 2020). Compared to the other fungal species, Phyllosticta genome assemblies are of a slightly smaller size (29–32 Mb) as opposed to
an average of 40 Mb in other Dothideomycetes (Fig. 1a). The Phyllosticta genome assemblies have a low number of contigs, namely 14–152 contigs compared with on average 471 contigs, and BUSCO scores between 93.3% and 95.8%, suggesting that the Phyllosticta genome assemblies are en par or above-average quality in terms of genome contiguity and completeness compared to the other Dothideomycetes genomes, which should facilitate further comparative analyses.

**Phyllosticta differ in gene number and functional annotation in a lifestyle dependent manner**

Species of similar lifestyles often share (groups of) genes (Lo Presti et al. 2015; Kim et al. 2016). Consequently, we hypothesized that the presence or absence of specific genes may provide information about the (predominant) lifestyle of *P. citrichinaensis*. To be able to compare gene content over different species and strains, we used OrthoFinder (Emms and Kelly 2019) to identify ortholog groups (OGs) across all 116 predicted proteomes. Orthofinder identified 35,379 OGs containing 88.1% of all genes (Supplementary Table 2a). The 11.9% of genes that were not assigned to any OG likely constitute species-specific genes, which is to be expected given the taxonomically diverse set of fungal species considered in our study (Fig. 1a). In total, 1,794 OGs (5.1%) contained genes from all species, representing a fungal core genome. Of all OGs, 32.2% (11,352) contained at least 1 gene from a Phyllosticta species, and of those, 57.8% (6,558, Fig. 2) contained genes from all *Phyllosticta* species and 33.2% (3,764) were unique to *Phyllosticta* (i.e. they only contained *Phyllosticta* genes). The latter percentage is quite high because of the close taxonomic relation of some of the genomes: a rather large fraction of the *Phyllosticta* unique OGs contain 2 or 3 genes (2,734, 72.6%), as these often contain 1 gene from each of the 2 *P. citricarpa* genomes and one more gene from *P. paracitricarpa*. Genes that are unique to a species, i.e. sequences that are sufficiently different from other sequences, do not form a separate OG on their own and consequently are not considered in these statistics. Since the *P. citricarpa* and *P. paracitricarpa* genomes are so closely related, many of their “unique genes” are assigned to an OG, which causes the fraction of *Phyllosticta* unique OGs to be quite large.

To discover lifestyle-associated genes in Phyllosticta, we compared OG content across the 8 Phyllosticta species and sought to identify differences between species of different lifestyles. The number of OGs that is shared by *Phyllosticta* species of a specific lifestyle (45–222, Fig. 2 and Supplementary Table 2b) is much...
smaller than the total number of OGs that all species in this genus share (6,558). The OGs shared by *Phyllosticta* spp. of a specific lifestyle might also contain genes from species outside of this genus. *Phyllosticta* pathogens shared 222 OGs that are not present in *Phyllosticta* endophytes, a much higher number than the total of 45 OGs that are shared by endophytes and not present in pathogens, which is likely due to the larger phylogenetic distance between the 2 endophytes compared with the pathogenic *Phyllosticta* species (Fig. 1a). The larger phylogenetic distance of *P. capitalensis* is also apparent through the fact that it has a group of 65 OGs that are not shared with any of the other *Phyllosticta* species. This is a rather large number when compared to the other *Phyllosticta* species, which all had less than 25 OGs that were not shared with the other *Phyllosticta* species. In addition to OGs that are unique to species of a specific lifestyle, OGs that are present in all species but differ in their abundance in species that share a lifestyle (e.g. there are more genes in species of 1 lifestyle compared to the others) may provide information on how species adapt to their lifestyles. We thus identified OGs for which the average number of genes in species of 1 lifestyle was higher than the average number of genes for species of the other lifestyle; we did not consider OGs that contained large outliers, i.e. one species having a much larger number of genes as compared to the other species. This resulted in a total of 87 OGs: 73 OGs that had more genes in endophytes and 14 OGs that had more genes in pathogens (Supplementary Table 2c), suggesting that achieving the endophytic lifestyle requires additional genes.

As functions of genes in OGs that are unique to, or enriched in, species of a certain lifestyle may underly lifestyles adaptations, and this may help to uncover which lifestyle *P. citrichinaensis* has, we looked into the annotations of the lifestyle-related OGs. In addition to the annotations of *Phyllosticta* genes, we also included the annotations of 50 out of the 108 species outside the genus *Phyllosticta* for which annotation data was available on the JGI database. The number of OGs in which at least one gene was functionally annotated (other than as hypothetical or expressed protein) varied widely between different lifestyle-related groups: while nearly 50% of the OGs that had more genes in endophytes received an annotation, less than 3% of the pathogen-only group did (Supplementary Table 3, a–f). We further divided the individual functional annotations into KOG-classes (from the EuKaryotic Orthologous Groups tool, https://mycocosm.jgi.doe.gov/help/kogbrowser.jsf; last accessed: 22 March 2022), which provide a high-level classification system to group genes with comparable activities. Of all KOG-classes, the class “Secondary metabolites biosynthesis, transport, and catabolism” was most often found to be associated with lifestyle-related OGs: only 2 of 6 lifestyle-related groups did not contain genes in this class, suggesting this group of genes may be useful to distinguish species of different lifestyles (Supplementary Table 3i).

To study secondary metabolite biosynthesis genes in more detail we used antiSMASH (Blin et al. 2019) to identify biosynthetic gene clusters (BGCs) in the 8 *Phyllosticta* genomes. The total number of predicted BGCs varied from 20 in *P. paracitricarpa* to 24 in *P. citricarpa*, with no apparent differences in numbers between species of different lifestyles. Interestingly, one of the terpene clusters was predicted as a squalestatin in all pathogenic species, while it received no functional prediction in the endophytic species or in *P. citrichinaensis*, suggesting there is a difference in this cluster between species of different lifestyles. Squalestatins are predicted to be inhibitors of squalene synthase, which produces squalene, a sterol biosynthetic intermediate that is reported to
play a role in mediating interactions between fungi and their plant hosts (Lindo et al. 2020). Therefore, further characterization of this BGC and others in Phyllosticta will be worthwhile for future studies into pathogenicity of Phyllosticta species.

Ortholog groups with more genes in Phyllosticta endophytes were more often functionally annotated, suggesting that these are generally better characterized and likely evolutionary conserved. We did not find any particular KOG-class to be annotated in higher abundance in endophytes, but nonetheless found a few interesting annotated OGs, such as 6 OGs that were annotated to belong to the “carbohydrate transport and metabolism” class including a CAZyme family (GH55) gene and several transporters, suggesting a role for carbohydrate transport in lifestyle (Supplementary Table 3, a-f, l). One endophyte-only OG contained the MAT1-1 gene, which was the result of all sequenced pathogenic strains having MAT1-2 mating types (Petters-Vandresen et al. 2020). In addition, although the P. citrichinaensis MAT1-1 gene is not present in this OG, we did find the MAT1-1 gene in the P. citrichinaensis genome assembly and found it to be highly similar to that of P. citribraziliensis, its closest relative.

The ortholog-only group was poorly functionally annotated; out of 222 OGs, only 5 received a functional annotation. The fact that such a large fraction of OGs could not be assigned a functional annotation is of interest as some of the nonannotated OGs in the pathogen-only group could contain putative effectors or genes that are otherwise involved in virulence, as effectors often remain unannotated in standard annotation pipelines (Sperschneider et al. 2015). In addition, 2 OGs received functional annotations that have previously been implied to be virulence factors and could therefore be interesting targets for future functional studies; a pectin lyase fold (Yang et al. 2018), and a cytochrome p450 (Siewers et al. 2005; Shin et al. 2017).

**Phyllosticta citrichinaensis shares more lifestyle-specific OGs with endophytes, but follows an intermediate pattern in other lifestyle-associated OGs**

The lifestyle of P. citrichinaensis is currently ambiguous (Wang et al. 2012), but the number of OGs that it shares with species of either lifestyle may provide clarity not only about the lifestyle of P. citrichinaensis itself but also about the differences between species of different lifestyles within this genus. The 2 P. citrichinaensis strains share more lifestyle specific OGs with endophytes (50) than they do with pathogens (30). In addition, the number of OGs that is shared by only the 2 endophytes and P. citrichinaensis is larger than the number of endophyte-only OGs that are not shared with P. citrichinaensis (Fig. 2, green rectangles), suggesting that P. citrichinaensis indeed compared well to endophytic species. However, for 30 out of 87 OGs that contained more genes in either pathogenic or endophytic species, the P. citrichinaensis gene numbers corresponded best to the endophytic numbers, while in 32 OGs, they corresponded to the pathogenic numbers. In 25 OGs, the number of genes of P. citrichinaensis corresponded to neither lifestyle (Supplementary Table 2c). As opposed to the numbers of OGs specific to species of one lifestyle, where P. citrichinaensis shared more with endophytes (Fig. 2), the numbers of P. citrichinaensis genes in OGs that contained more genes in either pathogenic or endophytic species thus indicate that it compares equally well to species of either lifestyle. Thus, these data suggest that presence/absence and/or gene abundance differences are not sufficient to provide insights into the lifestyle of P. citrichinaensis.

While we did not observe clear patterns in the types of functions that P. citrichinaensis shares with either endophytes or pathogens, we nevertheless could observe some interesting functional patterns in P. citrichinaensis (Supplementary Table 3, g and h). For instance, 2 groups were annotated as heat shock proteins: an Hsp40 (DNAJC17) that had more genes in pathogens (3 in pathogens vs 1–2 in endophytes) as well as an Hsp70 that had more genes in endophytes (4–5 in pathogens vs 6–7 in endophytes). Phyllosticta citrichinaensis contains fewer genes in both groups, suggesting it may respond differently to stress. Furthermore, 1 group that had more genes in pathogens as well as in P. citrichinaensis (1 in endophytes vs 2–3 in pathogens) contains genes annotated as peroxiredoxin-1 and peroxiredoxin-6. Peroxiredoxins are necessary for full virulence in several fungal pathogens such as Magnaporthe oryzae and Aspergillus fumigatus as they offer an antioxidant defense against reactive oxygen species produced by the host as part of host defense responses (Mirt et al. 2015; Rocha et al. 2018). It is thus possible that these additional genes in Phyllosticta pathogens and in P. citrichinaensis contribute to their virulence.

**There is little difference between Phyllosticta endophytes and pathogen in the numbers of putative secreted proteins and putative effectors**

Secreted proteins, including effectors, play an important role in lifestyle and virulence (de Wit et al. 2009; Lo Presti et al. 2015; Plissonneau et al. 2017). Based on the comparison of OGs in species of different lifestyles, we concluded that pathogenic species contain a large number of unannotated genes in pathogen-specific OGs (217 OGs). We hypothesized that some of these genes may be effectors or other secreted proteins, and that the presence of putative secreted proteins and effectors in the genomes of species may be an indicator for lifestyle differences. We therefore assessed the presence of OGs that contain 50% or more putative secreted or effector proteins in all 116 species used in this study. We used Predector (Jones et al. 2021) with a manual secretion score >4 to predict secreted proteins and an effector score >1 to predict effectors. A total of 3,537 OGs (10% of 35,379 OGs) consisted of at least 50% secreted proteins (Fig. 3b, Supplementary Fig. 1, Supplementary Table 4a) and of these, 1,315 OGs consisted of at least 50% proteins with an effector score > 1 (Fig. 3, c and d, Supplementary Table 4b). Increasing the effector score threshold to >2 resulted in a large decrease in predicted effectors. Notably, only 34 OGs containing putatively secreted genes were present in all 116 species, and only 1 OG containing putative effectors contained genes from all 116 species, corroborating that secreted proteins and effectors are typically not shared between different species and especially effectors are rather species and/or strain specific (Stergiopoulos et al. 2012).

Phyllosticta genes were present in a total of 674 putatively secreted OGs, with 322 of those containing genes from all Phyllosticta species included in this study. With an average of 642 genes per species, Phyllosticta species contain less genes in OGs encoding secreted proteins compared with other Dothideomycetes (an average of 848 genes, Supplementary Table 4). In addition, Phyllosticta species contained on average 152 putatively secreted genes that were not in an OG or that were the only protein predicted to be secreted in an OG (singleton), which is slightly more than we observed for the overall average of 144 singleton secreted genes (Supplementary Table 4). When assessing the total number of predicted secreted genes (both in OGs and singletons), Phyllosticta endophytes have more putative secreted proteins (average of 814) as compared to Phyllosticta pathogens (average of 795). However, when considering these as a percentage of the total predicted proteome size, this difference becomes negligible, with endophytes having a slightly smaller percentage of proteins that is secreted
(7.03%) as compared to pathogens (7.10%, Supplementary Table 4c). We found 288 putatively secreted OGs to be unique to *Phyllosticta*, none of which contained functionally annotated genes, as is often the case for putative effectors (Lo Presti et al. 2015).

*Phyllosticta* species contain less genes in OGs encoding effectors with an average of 116 genes compared to the overall average of 177 genes. In addition, *Phyllosticta* species contained on average 27 putative effector genes that were not in an OG or that were the only effector in an OG (singletons), which is lower than the average of 46 singleton effector genes for the other Dothideomycetes. Comparable to secreted proteins, when assessing the total number of predicted effector genes (both in OGs and singletons), *Phyllosticta* endophytes appear to have slightly more putative effector genes (average of 149) as compared to *Phyllosticta* pathogens (average of 144), but when taken as percentage of the total number of predicted proteomes per species, this difference is negligible (1.28% vs 1.29%) (Fig. 3, c and d, Supplementary Table 4d). Effector genes are often hypothesized to be species and/or strain specific (Lo Presti et al. 2015; Sperschneider et al. 2015). We identified in total 63 OGs containing effectors that are unique to *Phyllosticta*, 3 of which were present in all pathogens but not in endophytes, 2 were present in all endophytes but not in pathogens, and 1 had higher gene numbers in endophytes (Supplementary Table 4e). None of these unique effector genes were functionally annotated, suggesting that these have yet undescribed functions.

The occurrence of *P. citrichinaensis* effector genes in lifestyle-associated OGs could provide further evidence for its lifestyle. One of the 3 effector OGs that only occurred in pathogens contained a gene from one of the *P. citrichinaensis* strains. In contrast, both endophyte-only effector OGs contained genes from *P. citrichinaensis*, in one case only from one strain, and in the other case from both strains. In the effector OG that had more genes in endophytes, *P. citrichinaensis* followed an intermediate pattern: one strain contained the same number of genes as pathogens, while the other contained the same number as endophytes. These data thus suggest that *P. citrichinaensis* follows an intermediate lifestyle.

**Phyllosticta citrichinaensis clusters with pathogens based on carbon growth data**

CAZymes enable fungi to utilize different carbon sources (van den Brink and de Vries 2011; Lombard et al. 2014), and are thought to be involved in fungal pathogenicity (ten Have et al. 2002; King et al. 2011; Kubicek et al. 2014; Hane et al. 2020). We have previously shown that carbon utilization capabilities differ between *Phyllosticta* spp. and uncovered a clear distinction in the ability of pathogens and endophytes to grow in the presence of sugar beet pulp; while the growth of endophytes was unchanged, pathogens were strongly inhibited (Buijs et al. 2021). To assess if *P. citrichinaensis* displays similar growth behavior to pathogens or endophytes, we grew *P. citrichinaensis* on 35 different carbon sources including sugar beet pulp (Fig. 4). Interestingly, growth of *P. citrichinaensis* is not inhibited by the presence of sugar beet pulp (Fig. 4a), suggesting that *P. citrichinaensis* behaves comparable to endophytic *Phyllosticta* spp. To further substantiate this observation, we performed hierarchical clustering of 7 *Phyllosticta* strains based on their growth on all 35 carbon sources. Unexpectedly, *P. citrichinaensis* clustered together with the pathogenic species...
rather than with the endophytes (Fig. 4b). Thus, although P. citrichinaensis is not inhibited by sugar beet pulp, it generally displays carbon utilization capabilities comparable with pathogens.

**Genomes of Dothideomycetes can be clearly distinguished based on CAZyme content, but this does not correlate well with lifestyles described in literature**

The genetic basis for the ability to utilize different carbon sources is often caused by differences in CAZymes repertoires (van den Brink and de Vries 2011; Lombard et al. 2014). Interestingly, the abundance and diversity of CAZymes encoded in a genome is also related to lifestyle and consequently enables to predict the trophic classification of a species (Lo Presti et al. 2015; Hane et al. 2020). Our dataset of 116 genomes included 27 species whose trophy classification was previously predicted by Hane et al. using CATAstrophy. We used CATAstrophy to annotate CAZyme genes for the other 89 predicted proteomes and to perform a principal component analysis (PCA) to distinguish species with different trophic classes based on their CAZyme repertoire. CATAstrophy clearly separated species of different trophic classes based on the first principal component (PC1) (Fig. 5a and Supplementary Table 5). As the second principal component (PC2) mainly separates oomycetes from fungi (Hane et al. 2020), we did not observe much separation based on PC2 as the here analyzed genomes did not include any oomycetes. For these 116 species, the different trophic classes differ considerably in the numbers of genes per CAZyme family (Fig. 5b). For example, GH families differ clearly between trophy class, while almost no difference can be observed in the PL family. The CATAstrophy gene predictions were used to identify CAZyme-containing OGs, for which we then obtained the number of genes present in each species to generate a clustered heatmap (Fig. 5c). We observed 3 distinct clusters that differ in their CAZyme repertoires, which typically correlate well with the CATAstrophy trophy predictions (Fig. 5c and Supplementary Table 5). The CATAstrophy trophy predictions also correlate well with the numbers of secreted proteins (predictor threshold >4) and effectors (predictor threshold >1 and >2), although we did not observe such a strict separation into 3 clusters as was observed for CAZyme genes (Figs. 3 and 5c, Supplementary Fig. 1).

All Phyllosticta species were predicted to be saprotrophs by CATAstrophy (Table 1, Fig. 5c) and clustered according to their phylogenetic relationship, suggesting that they are generally very similar in terms of CAZymes (Supplementary Table 5). Consequently, P. citrichinaensis clustered most closely to P. citribraziliensis, an endophyte, as this is its closest relative (Fig. 1a). We nevertheless found

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Fig. 4. Phyllosticta citrichinaensis clusters with pathogens based on growth on 35 carbon sources, but behaves like an endophyte in the presence of sugar beet pulp. a) Images of Phyllosticta species growing on a selection of different carbon sources. b) Clustering of Phyllosticta species based on their growth on different carbon sources. All species were grown on 35 different carbon sources, colony diameters were measured, and images taken on all sources when the biggest colony of a species reached the edge of its plate. All species grew fastest on wheat bran.
6 CAZyme families that show consistent differences in gene number between pathogens and endophytes: AA1_3, AA3, CBM18, CBM67, GH3, and PL22 (Table 2). In all cases except AA3, *P. citrichinaensis* follows the endophytic pattern. Together, these results indicate that in terms of the presence of CAZyme genes, the *P. citrichinaensis* genomes compare best to those of endophytes.

**Discussion**

Lifestyle adaptations are thought to be driven by differences in gene content, and especially CAZymes are assumed to be crucial (ten Hove et al. 2002; King et al. 2011; Hane et al. 2020). Here, we aimed to elucidate the genomic differences between endophytes and pathogens within the *Phyllosticta* genus occurring on Citrus,
and aimed to determine the lifestyle of *Phyllosticta citrichinaensis*. Based on the results, we uncovered several differences between species with different lifestyles. For instance, endophytes more frequently contain higher numbers of genes in OGs, and these genes are more often annotated than in pathogenic species. In addition, pathogenic species share more un-annotated lifestyle-specific OGs compared to endophytic species. Furthermore, we show that species cluster independently of phylogeny based on the CAZyme content of their genomes, and this clustering correlated well with trophic lifestyle prediction by CATAstrophy. The ambiguous species *P. citrichinaensis* showed characteristics that matched with endophytes in some cases, with pathogens in other cases, and sometimes it did not match with either lifestyle, suggesting it may exhibit an intermediate lifestyle not accounted for in the current definitions.

We previously observed that only 4 CAZyme families showed a consistent difference between endophytic and pathogenic species (Buijs et al. 2021), one of which contained AA1_3/CBM18 (7 in pathogens vs 8 in endophytes), which were mixed in one orthologue group, and another one contained family CBM18 only. In this study, we found in total 6 CAZyme families with a consistent difference between endophytes and pathogens. These included 2 separate OGs that contained the AA1_3 and CBM18 family, which both consistently contained more genes in endophytes compared to pathogens. CATAstrophy predicts on average about 90 CAZyme genes extra compared to our previous results (Buijs et al. 2021). The biggest difference can be found in the CE (carbohydrate esterase) family, where between 83 and 89 genes are predicted instead of 15–17. Most of these are in the CE10 family (47–53). The CE10 family is no longer listed as carbohydrate esterase by the cazy.org database (used by JGI) because most of the members of this family act on noncarbohydrate substances (Lombard et al. 2014). If we manually remove these, CATAstrophy still predicts 20 extra genes in the CE family. In addition, there are some 15–20 extra genes predicted in the AA family, 5 to 10 more in the GH family, and approximately 5 more in the GT family. Numbers in the PL family are practically identical. In the CBM family, CATAstrophy predicts about 5 to 10 genes less. In the JGI annotation pipeline, many of the genes in the CBM family contained multiple domains and were therefore counted multiple times, which might have led to an overestimation. However, for most families it seems that CATAstrophy predicts more genes compared to JGI. As most of the CAZyme genes predicted by JGI are based on some experimental validation (cazy.org), it would be wise to experimentally validate the CAZyme genes that were predicted by CATAstrophy and not by JGI.

**Table 2.** CAZyme families with gene abundance differences between endophytes and pathogens.

| Species name                        | Traditional lifestyle | CBM67 | AA1_3 | CBM18 | GH3 | PL22 | AA3 |
|-------------------------------------|-----------------------|-------|-------|-------|-----|------|-----|
| Phyllosticta capitansis             | E                     | 0     | 10    | 10    | 15  | 1    | 2   |
| Phyllosticta citribrasilensis       | E                     | 0     | 10    | 9     | 15  | 1    | 2   |
| Phyllosticta citrichinaensis (CBS 129764) | ?                   | 0     | 10    | 9     | 16  | 1    | 1   |
| Phyllosticta citrichinensis (CBS 130529) | ?                   | 0     | 10    | 9     | 15  | 1    | 1   |
| Phyllosticta citrusiana             | P                     | 1     | 9     | 7     | 13  | 0    | 1   |
| Phyllosticta citricarpa (CBS 27913) | P                     | 1     | 9     | 6     | 14  | 0    | 1   |
| Phyllosticta citricarpa (CBS 127454) | P                   | 1     | 9     | 7     | 14  | 0    | 1   |
| Phyllosticta paracitricarpa          | P                     | 1     | 9     | 7     | 14  | 0    | 1   |

Columns with a darker fill color indicate the family which is more abundant in pathogens, while the lighter color indicates those which are more abundant in endophytes. E = Endophyte, P = Pathogens, ? = Lifestyle ambiguous.

Genes related to secondary metabolite biosynthesis but not the number of BGCs as predicted by antiSMASH differ between species. It is important to note here that BGC prediction by antiSMASH is based primarily on the presence of a gene to produce the metabolite ‘backbone’, such as a polyketide synthase (PKS) (Blin et al. 2019). Once such a gene is found, antiSMASH takes an area in the genome of up to 20 kb (depending on the type of backbone gene) on either side of the gene and checks for the presence of tailoring genes, which are then all automatically included in the BGC. This means that tailoring genes that are altered or inactive are still included in the predicted BGC, and that alterations in genes may not result in an altered BGC prediction. In contrast, although the cluster may look very similar, alterations in tailoring genes may lead to the production of a very different compound: a good example is the synthesis of the toxins dothistromin, aflatoxin, and sterigmatocystin, which are all synthesized in a very similar manner, with only the very last tailoring steps being different (Schwelm and Bradshaw 2010, for a review see Hüttel and Müller 2021). We therefore conclude that although antiSMASH did not detect differences in BGC numbers, alterations in biosynthetic genes may be responsible for the differences between species of different lifestyles in the genus *Phyllosticta*, and this will be an interesting subject for future studies.

CATAstrophy was able to clearly separate species based on the number of CAZyme genes present in the genomes and was able to separate species into different trophy predictions. Apart from closely related species that cluster together, a phylogenetic pattern cannot be observed in the clustering of the heatmap or in the trophy predictions, which suggests that there is a strong signal that links genome content to lifestyle. CATAstrophy also allows for trophy-overlap for species that are bordering 2 trophies, such as for *Sclerotinia sclerotiorum*, which received very high scores for both the polymertroph and the saprotroph class (Supplementary Table 5). This is consistent with literature, as *S. sclerotiorum* has been described to exhibit a necrotrophic phase that is followed by a saprotrophic phase (Hegedus and Rimmer 2005). *Sclerotinia sclerotiorum* is a much-researched model organism, and the descriptions in literature of its lifestyle are therefore well-developed. However, for many fungal species, this is not the case, and circumscriptions in literature are often limited or conflicting. Indeed, we see that the trophy predictions by CATAstrophy do not always correlate well with lifestyles described (or supposed) in literature: each trophy class includes species that are described as pathogens, endophytes, symbionts, or saprotrophs, or which have been described to exhibit multiple of these lifestyles. An underlying cause for this fact is that lifestyles that are described in literature may be inaccurate as the
border between species of different lifestyles such as necrotrophs, hemibiotrophs, or biotrophs is not very strict, or need very specific conditions to manifest. For instance, Phytophthora infestans has been placed in all 3 classes (Oliver and Ipcho 2004). Similarly, Botrytis cinerea has been placed in different classes as the symptoms it causes differ widely in their severity, depending on the exact interaction with the host (Veloso and Van Kan 2018).

Within the genus Phyllosticta, some obscurity with respect to lifestyle is present for instance for P. capitaleans, which is a widespread endophyte of many hosts including Citrus (Wikee et al. 2013a), but may cause disease in other hosts such as guava (Arafat 2018). In addition, nonpathogens can evolve a pathogenic lifestyle, and vice-versa, as can be observed with pathogenicity on pea by Nectospora solani, which is dependent on the presence of only a few genes, or with pathogenicity of Fusarium oxy- sporum on cucurbit species, which is determined by the absence or presence of a mobile pathogenicity chromosome (Temporini and Vanetten 2002; Dong et al. 2015; Möller and Stukenbrock 2017; van Dam et al. 2017). The possibility for species to be categorized into multiple trophies in the CAZyme-based classification system therefore presents an advantage over the traditional classification system as it allows for a more correct, double classification of species that exhibit multiple lifestyles depending on the host and other environmental parameters.

We compared genomes of species with different lifestyles within the genus Phyllosticta specifically, and found several distinctions. We observed that endophytes more often have higher numbers of genes in specific OGs as compared to pathogens. This suggests that the ability to be an endophyte necessitates the presence of additional genes. The ancestral Dothideomycete was likely a saprotroph, however, the most common ancestor of the Botryosphaeriales, the order in which Phyllosticta resides, was probably a plant pathogen, as determined by ancestral state reconstruction (Abdollahzadeh et al. 2020; Haridas et al. 2020). The evolution of Phyllosticta endophytes from Phyllosticta pathogens through a gain of genes and thereby gain of abilities is therefore a plausible scenario. With respect to lifestyle definition in Phyllosticta, P. citrichinaensis is the most ambiguous citrus-related species within the genus. By comparing the genome of this species with those of other species with different lifestyles within the genus Phyllosticta, we aimed to elucidate the lifestyle of this species. In several aspects, we found P. citrichinaensis to be most similar to endophytes. For instance, P. citrichinaensis shares more OGs specific to one lifestyle with endophytic species (50%) than it does with pathogenic species (30%), and none of its BGCs was predicted to produce a squalestatin, which was also the case for all of the endophytes, but not for the pathogens. In addition, CAZyme families that had more genes in endophytes than in pathogens, often also had more genes in P. citrichinaensis. Furthermore, growth of P. citrichinaensis was not inhibited by the presence of sugar beet pulp, similarly to endophytic species. In contrast, its broader carbon utilization capabilities were more comparable to those of pathogenic species. Another aspect in which P. citrichinaensis was comparable to pathogens, was the presence of more putative peroxiredoxin genes in pathogens and P. citrichinaensis as compared to endophytes. On other aspects, P. citrichinaensis did not compare well with species of either lifestyle, such as the number of OGs that had more genes in species of either lifestyle: in almost a third of the cases, P. citrichinaensis did not match the gene numbers of either lifestyle. The number of effector genes that P. citrichinaensis shared with species of either lifestyle also suggests an intermediate pattern. The lifestyle of P. citrichinaensis cannot be univocally determined without performing pathogenicity assays, but as these are currently not available for this species, the data presented here give a good estimation that shows that P. citrichinaensis is an intermediate taxon, not perfectly fitting into any of the currently defined lifestyle definitions.

Research performed in recent years has shown with increasing confidence that borders between lifestyles simply are not very strict and in fact are subject to constant change. Examples such as B. cinerea and Phytophthora infestans, which both have been placed in multiple lifestyle classes depending on the host and other environmental parameters, demonstrate that our current classification systems are not always adequate to separate species into different lifestyles (Oliver and Ipcho 2004; Veloso and Van Kan 2018). In addition, the ability of species to be pathogenic to a specific host may change with the gain of only a few genes (Temporini and Vanetten 2002; van Dam et al. 2017). Classifying plant-associated microbes into different lifestyles is an important area of research as it allows for the identification of genomic parameters that are required for pathogenicity, and therefore aids in the search of a remedy against such pathogens. However, a classification system is only valuable if it allows for the accurate separation of species; an incorrectly classified organism may lead to incorrect conclusions and could cause much confusion. A classification such as the one proposed by Hane et al. which is based on the number of CAZyme genes in a species’ genome, is a significant improvement since it allows for overlap between lifestyles. Further development of such classifications for instance by the addition of other genomic parameters such as the presence of effectors could lead to the development of a more accurate and useful classification system in the future.

Data availability

The whole-genome sequencing data including annotations for the newly sequenced Phyllosticta citrichinaensis genome are publicly available at the JGI genome portal: https://mycocosm.jgi.doe.gov/Pcit129764 (last accessed: 22 March 2022). The authors affirm that all other data necessary for confirming the conclusions of the article are present within the article, figures, and (supplementary) tables.

Supplemental material is available at G3 online.

Acknowledgments

The authors would like to thank Sofia Riera Vidal for her help with the Predictor data.

Funding

This work was funded by the Dutch Applied Science division (TTW) of NWO and the Technology Program of the Ministry of Infrastructure and Water Management under project 15807 of the Research Programme I&W Biotechnology and Safety. The work conducted by the U.S. Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

Conflicts of interest

None declared.
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