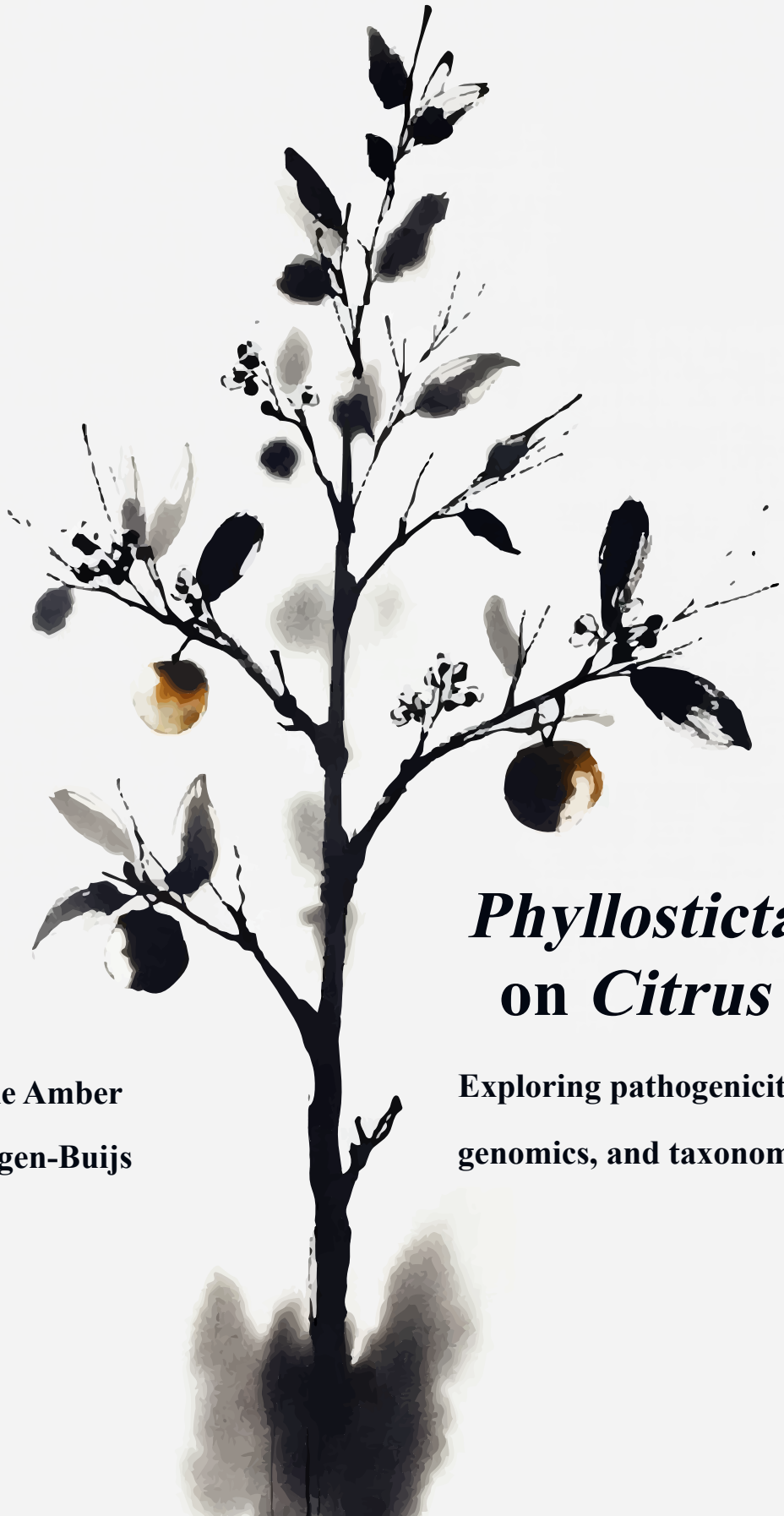


Phyllosticta on *Citrus*

Exploring pathogenicity, genomics, and taxonomy

Valerie Amber van Ingen-Buijs

Propositions

1. The idea that plant-colonizing fungal species adhere to rigid, categorizable lifestyles is outdated.
   (this thesis)

2. Descriptions of new fungal species solely on the basis of DNA are not reliable.
   (this thesis)

3. The hyper-competitive landscape of science undermines its quality by favoring fast work over the best work.

4. Failure is underappreciated among scientists because it cannot be published.

5. Divisive language in everyday situtations contributes to polarisation and with that, to extremism.

6. Plants have a healing power that surpasses that of their chemical constituents.

Propositions belonging to the thesis, entitled

Phyllosticta on *Citrus*
Exploring pathogenicity, genomics, and taxonomy

Valerie A. van Ingen-Buijs
Wageningen, 11 June 2026

Phyllosticta on *Citrus*

Exploring pathogenicity, genomics, and taxonomy

Valerie A. van Ingen-Buijs

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Valerie A. van Ingen-Buijs

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Chapter 1

General Introduction

Citrus and *Phyllosticta*

As the global population continues to expand, safeguarding a reliable food supply is a cornerstone for societal stability and human well-being. Recent insights highlight fungal and oomycete pathogens as the foremost biological adversary to our essential calorie-rich crops. A 2019 ranking of 137 key pests and pathogens, evaluated for their agricultural impact, revealed fungi claiming the leading six spots for diseases of the planet's five primary calorie crops (wheat, rice, maize, potato and soybean, Savary et al., 2019). Even with routine fungicide applications and the adoption of more disease resilient plant varieties, annual global harvest losses from fungal attacks range from 10% to 23%, compounded by 10% to 20% more in storage and transit (Fisher et al., 2012; Steinberg & Gurr, 2020). This vulnerability is clearly illustrated in staples like rice, wheat, maize, soybean, and potato, which succumb to threats such as rice blast, stem rust, corn smut, soybean rust, and late blight, respectively (Steinberg & Gurr, 2020). Strikingly, the yield losses from these pathogens alone could nourish anywhere from 600 million to 4 billion individuals with 2,000 daily calories over a full year (Fisher et al., 2012).

Citrus fruits are widely consumed worldwide, with global production reaching 158.5 –161 million metric tons in 2021 (Pereira Gonzatto & Scherer Santos, 2023; World Citrus Organisation, 2022). The bulk of this production comes from South America and Asia, led by top producers China, Brazil, and India (FAO, 2020). Citrus fruits are associated with a variety of health advantages, stemming from their nutrient-dense profile. They are rich in micronutrients like ascorbic acid, along with vitamins such as thiamine, pyridoxal phosphate, and folate, while also providing essential minerals including potassium (Aslan et al., 2024). Beyond these primary compounds, *Citrus'* secondary metabolites have drawn increasing scientific interest lately (Lu et al., 2023), featuring potent bioactive elements like flavonoids, alkaloids, terpenes, and carotenoids (Aslan et al., 2024). Such components offer diverse therapeutic effects, ranging from antioxidant and antidiabetic to cardioprotective, anticancer, and anti-inflammatory actions (Gandhi et al., 2020; Mahmoud et al., 2019; Zou et al., 2016). Past research has examined the health implications of *Citrus* and its compounds across multiple methods, encompassing *in vitro* experiments, *in silico* modeling, and *in vivo* trials with animals (Jahan et al., 2022; Kim et al., 2012; Muhtadi et al., 2015).

The genus *Phyllosticta* (*Phyllostictaceae*), represents a sister family to *Botryosphaeriaceae* in *Botryosphaeriales* (*Dothideomycetes*), encompassing a large group of fungi, with over 3,000 names recorded, and including saprophytes, endophytes, and plant pathogens (Index Fungorum, accessed June 2, 2023; Wikee, Lombard, Nakashima, et al., 2013). A revision by Van der Aa and Vanev in 2002 recognized 141 species, capable of colonizing a wide variety of plant hosts (van der Aa & Vanev, 2002). Several *Phyllosticta* species occur on *Citrus*. For instance, *Phyllosticta citriasiana* is a pathogen primarily affecting *Citrus maxima* (van Raak et al., 2025; Wulandari et al., 2009). *Phyllosticta citrichinaensis* is considered a weak pathogen across multiple *Citrus* species, though its ability to cause disease remains uncertain due to the lack of confirmation through Koch's postulates (Wang et al., 2012). *Phyllosticta citribraziliensis*, identified as an endophyte in *Citrus limon*, has to date only been reported in Brazil (Glienke et al., 2011). Lastly, *Phyllosticta capitalensis*, a common endophyte in *Citrus*, is found across numerous plant hosts and may act as a pathogen on non-*Citrus* species, such as orchids (Silva et al., 2008; Wikee et al., 2013).

Phyllosticta citricarpa: a threat to global *Citrus* production

A significant threat to *Citrus* production is posed by fungal plant pathogens such as *Phyllosticta citricarpa*, the causal agent of Citrus Black Spot (CBS). Citrus Black Spot, first identified in Australia, leads to approximately 80 million AUD in annual economic losses there (Drenth, 2018). The disease has since spread to most *Citrus*-growing regions globally (CABI, 2022), often causing significant yield reductions. For example, in Ghana, CBS has been linked to crop losses of up to 22% (Brentu et al., 2012). In the European Union, *P. citricarpa* is classified as an A1 quarantine pest (Annex IIA), and it is present on the European and Mediterranean Plant Protection Organization (EPPO) A1 Quarantine Pest List. The detection of CBS symptoms on even a single fruit can result in the rejection of entire shipments, potentially containing thousands of fruits, leading to substantial financial losses for exporters and their countries.

While *P. citricarpa* is widespread globally, it is considered absent from Europe according to the EPPO and the Centre for Agriculture and Bioscience International (CABI, 2022; EPPO, 2020). However, a study by Guarnaccia et al. identified 20 isolates of the fungus from

Citrus-growing sites in Italy, Malta, and Portugal, constituting two genetically distinct clonal populations and suggesting at least two separate introductions (Guarnaccia et al., 2017). Despite this, no CBS symptoms were observed. Follow-up surveys by national plant protection organizations also found no evidence of CBS symptoms (Jeger et al., 2018). The suitability of the Mediterranean climate for CBS development remains a topic of debate, with studies offering conflicting conclusions (Baker et al., 2009; Er et al., 2013; Fourie et al., 2017; Martínez-Minaya et al., 2015, 2017; Paul et al., 2005). A recent CBS outbreak in Tunisia, combined with climatic modeling, suggests that the Mediterranean climate may support CBS under rare, specific conditions (EPPO, 2019; Galvañ et al., 2022). Consequently, the presence and potential impact of *P. citricarpa* in Europe remain uncertain.

Phyllosticta citricarpa infects and damages a wide variety of *Citrus* species, including *Citrus limon*, *Citrus sinensis*, and *Citrus reticulata* (EPPO, 2020; Miles et al., 2019). Characteristic symptoms include small, dark, sunken lesions on fruit, sometimes with pycnidial conidiomata, and necrotic leaf spots featuring a pale center and darker margins (**Fig. 1**). Less common symptoms, such as virulent spot or lacy spot, may also appear infrequently (Miles et al., 2019; Truter, 2010). Disease intensity is determined by factors such as the availability of inoculum, the presence of conducive climatic conditions, the fruit stage, and the timely application of fungicides (Moyo et al., 2025). The fungus spreads asexually through conidia formed on infected tissues and leaf litter, and sexually via ascospores, which are produced



Fig. 1. Typical Citrus Black Spot symptoms on *Citrus sinensis*: small, dark sunken lesions, sometimes with pynidial conidiomata. Photo by Megan Dewdney.
Source: https://citrusindustry.net/2020/02/05/black-spot-scouting-and-control/

exclusively on leaf litter (**Fig. 2**; Guarnaccia et al., 2019). With its severe symptoms and widespread distribution, *P. citricarpa* proposes a significant challenge to *Citrus* production.

The necessary tools to study pathogenicity and taxonomy

To effectively control fungal plant pathogens like *Phyllosticta citricarpa*, a deep understanding of their disease mechanisms is crucial. Pinpointing traits that differentiate pathogenic strains from their non-pathogenic relatives can reveal targets for management strategies. A key aspect of fungal colonization is the breakdown of the plant cell wall, primarily composed of polysaccharides, lignin, and proteins (Mäkelä et al., 2014). This process relies on plant cell wall degrading enzymes (PCWDEs), secreted by fungi and often linked to pathogenicity (Douaiher et al., 2007; King et al., 2011; Kubicek et al., 2014; ten Have et al., 2002). PCWDEs belong to the broader category of Carbohydrate Active Enzymes (CAZymes), which degrade pol-

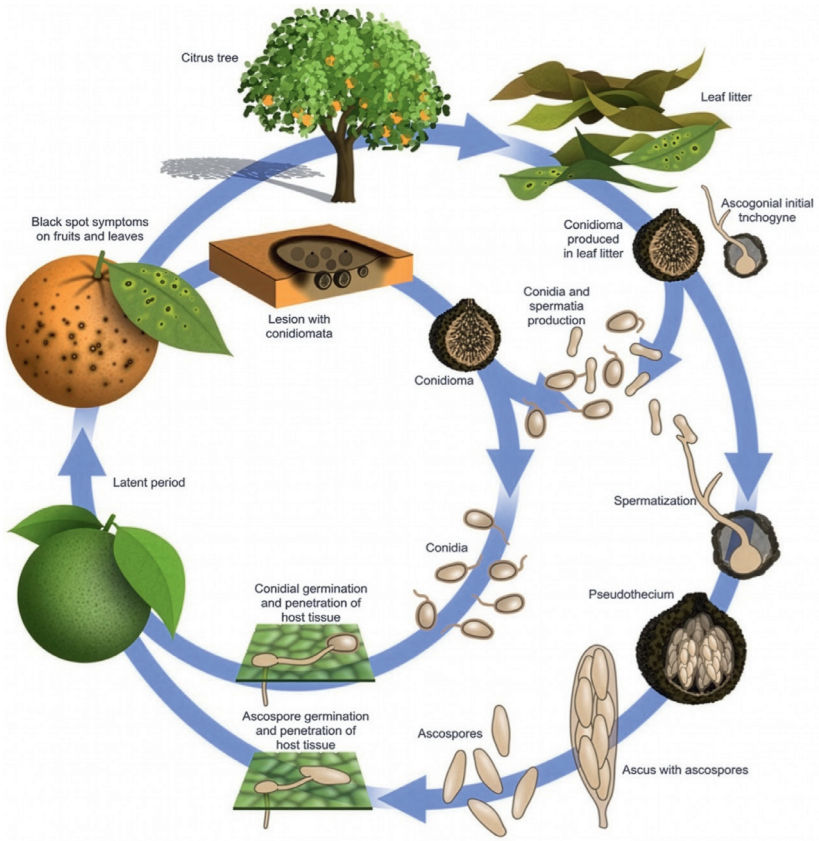


Figure 2. Life cycle of *Phyllosticta citricarpa* (Guarnaccia et al., 2017).

ymeric and oligomeric plant carbohydrates and have been well-characterized across fungal species (Lombard et al., 2014). Since plant cell wall composition varies by species and tissue, fungi require specialized repertoires of enzymes to degrade the specific cell wall of their hosts (Houston et al., 2016; van den Brink & de Vries, 2011). Consequently, PCWDE repertoires differ often even between closely related species. For instance, Brunner et al. demonstrate that PCWDE repertoires have become specialized in closely related *Zymoseptoria* species infecting different hosts (Brunner et al., 2013). In addition, King et al. showed that pathogens of monocots are better adapted for degradation of monocot cell walls while pathogens of dicots are better adapted for degradation of dicot cell walls (King et al., 2011). Notably, also the PCWDE repertoires of plant-pathogenic fungi differ from those of endophytic species, underscoring their role in disease. For example, Lo Presti et al. observed fewer PCWDEs in symbiotic and biotrophic fungi compared to saprotrophs, necrotrophs, and hemibiotrophs, while Choi et al. noted that pathogens possess more PCWDE-encoding genes and distinct gene types compared to saprotrophs (Choi et al., 2013; Lo Presti et al., 2015).

PCWDEs may be classified as effectors, which are intensely studied for their role in host-pathogen interactions. Effectors, typically small secreted proteins, facilitate host colonization, for instance by degrading cell walls or evading the host's immune defenses (Fouché et al., 2018; Lo Presti et al., 2015; Rovenich et al., 2014). These proteins may be conserved among strains or species targeting the same host (Chiapello et al., 2015; van Dam et al., 2017) and, in rare cases, may be transferred between species via horizontal gene transfer (Gardiner et al., 2012; van Dam & Rep, 2017). However, as hosts evolve to detect effectors, these proteins rapidly mutate, leading to diverse effector repertoires across fungal lineages to avoid immune recognition (Lo Presti et al., 2015; Rovenich et al., 2014). Even individuals of the same species may have diversified repertoires (de Vries et al., 2020; Fouché et al., 2018; Möller & Stukenbrock, 2017). In contrast, species with markedly different lifestyles can sometimes exhibit strikingly similar effector repertoires (Seidl et al., 2015). Nonetheless, identifying effectors and genes unique to specific fungal lifestyles can shed light on the genetic basis of pathogenicity (Gibriel et al., 2016).

Genome sequencing has emerged as an invaluable approach for studying plant pathogens and their effectors, enabling researchers to uncover critical insights into virulence

mechanisms, evolutionary dynamics, host-pathogen interactions, and diagnostic strategies (Nizamani et al., 2023). Traditionally, investigations relied on labor-intensive methods like morphological identification and limited genetic markers, which often fell short in resolving complex and fast-evolving pathogen genomes (Venbrux et al., 2023). However, advancements in sequencing technologies have revolutionized this field, including platforms like Illumina's high-throughput short-read sequencing, as well as long-read methods such as Oxford Nanopore and PacBio, which provide more complete, high-quality assemblies at reduced costs and faster turnaround times (Satam et al., 2023). These innovations now allow for rapid whole-genome analysis of fungal pathogens, facilitating precise taxonomy, virulence gene discovery, and ultimately, targeted disease management in agriculture (Fagundes et al., 2025; Plissonneau et al., 2016; Steenkamp et al., 2018). For *Phyllosticta*, few genomes have been sequenced to date, and knowledge of pathogenicity genes and disease mechanisms is limited (Guarnaccia et al., 2019; Rodrigues et al., 2019; Wang et al., 2020).

Once genes related to pathogenicity are identified, experimental confirmation of the function of these genes is necessary. One approach could be to disrupt the gene of interest and perform pathogenicity assays. Traditional methods encompassed transformation via homologous recombination, random mutagenesis with chemicals or UV radiation, and insertional approaches like restriction enzyme-mediated integration (Li et al., 2017). However, these strategies are often time-consuming, demand extensive screening to identify successful mutants, exhibit low efficiency and could lead to numerous unintended off-target effects (Wang et al., 2023). The introduction of CRISPR/Cas9 has fundamentally reshaped this field by providing a highly precise, efficient tool for targeted gene editing, often enabling marker-free modifications and simultaneous targeting of multiple genes, thereby speeding up functional analyses and enhancing insights into genetic functions in fungal pathogens (Wang et al., 2023). Although well developed in model organisms, development of CRISPR/Cas9 methodologies in non-model organisms may be challenging (Wilson & Wingfield, 2020). Although one *Agrobacterium*-mediated transformation protocol exists for *Phyllosticta* species, it comes with all the limitations of traditional transformation methods, and to date no CRISPR/Cas9 protocol has been reported for *Phyllosticta* species.

Species boundaries of *Citrus*-colonising *Phyllosticta* species

Ambiguous species delimitation can significantly impede researchers' ability to derive reliable and robust conclusions from their studies, while also complicating the implementation of effective legislation, quarantine measures, and disease control strategies (Carnegie & Pegg, 2018). A good example is the historical taxonomic confusion between *Phyllosticta citricarpa* and *Phyllosticta capitalensis*. The frequent co-occurrence and pronounced morphological similarity of these species have previously led to uncertainty regarding the pathogenicity of *P. capitalensis* (Baayen et al., 2002; Glienke et al., 2011). For a period, this ambiguity resulted in the erroneous assumption that pathogenic and non-pathogenic strains belonged to a single species, with no reliable method to differentiate them. Such taxonomic uncertainty complicates the development of effective management strategies as they must account for the ecological and pathological differences among multiple species rather than a single taxon.

Further challenges in species delimitation arise with *Phyllosticta citricarpa* and *Phyllosticta paracitricarpa*, which exhibit substantial morphological and genetic similarities (Guarnaccia et al., 2017, 2019). *Phyllosticta paracitricarpa* was first described from leaf litter in a *Citrus limon* orchard in Greece in 2017 (Guarnaccia et al., 2017). Its morphological characteristics closely resemble those of *P. citricarpa*, and although molecular differentiation was initially achieved using mainly the translation elongation factor 1-alpha (*tef1*) gene, subsequent studies have demonstrated that this marker is unreliable for consistently distinguishing between the two clades (Ioos et al., 2023). These challenges underscore the necessity for more precise taxonomic tools to ensure accurate species identification and inform effective disease management.

This thesis

Global food security is increasingly threatened by fungal and oomycete pathogens that devastate major crops, with *P. citricarpa* posing a particularly severe risk to *Citrus* production worldwide. *Phyllosticta citricarpa*, the causal agent of Citrus Black Spot (CBS), inflicts significant economic losses through yield reductions, fruit lesions, and trade restrictions. Taxonom-

ic ambiguities between *Phyllosticta* species, combined with limited (genomic) insights into virulence mechanisms, hinder effective disease management and diagnostics, and exacerbate vulnerabilities in *Citrus* supply chains. The precise genetic and molecular mechanisms that differentiate pathogenic *Phyllosticta* species like *P. citricarpa* from non-pathogenic relatives could be targeted for improved disease control and diagnostics, but are currently unknown. In **Chapter 2**, I therefore study carbon utilization capabilities of different *Phyllosticta* species and demarcate differences between pathogenic and endophytic species. Additionally, in **Chapter 3**, I study other genetic differences between *Phyllosticta* species with different lifestyles using newly generated whole-genome data and comparative genomics. The possibilities for experimental validation of identified pathogenicity genes in *Phyllosticta* are currently very limited, since no efficient CRISPR/Cas9 transformation protocols exist. In **Chapter 4**, I therefore describe the development of CRISPR/Cas9 methodology adapted for *Phyllosticta* species. Lastly, taxonomic uncertainties surrounding *P. citricarpa* and closely related species like *P. paracitricarpa* need to be resolved to be able to better study pathogenicity mechanisms, prevent misidentification and enhance quarantine measures. In **Chapter 5**, I therefore compare phylogenomics to the standard barcoding regions used for phylogenetic analyses to resolve the taxonomy of *P. citricarpa* and *P. paracitricarpa*. This thesis thus presents a comprehensive investigation into pathogenicity within the genus *Phyllosticta* occurring on *Citrus*, explored through multiple approaches.

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Chapter 2

Carbon utilization and growth inhibition of *Citrus*-colonizing *Phyllosticta* species

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ABSTRACT

The genus *Phyllosticta* includes both endophytic and phytopathogenic species that occur on a broad range of plant hosts, including *Citrus*. Some pathogenic species cause severe disease, such as *Phyllosticta citricarpa*, the causal agent of Citrus Black Spot (CBS). In contrast, other species, such as *Phyllosticta capitalensis*, have an endophytic lifestyle in numerous plant hosts. Carbon utilization capabilities are hypothesized to influence both host range and lifestyle, and are in part determined by the set of Carbohydrate Active Enzyme (CAZyme) encoding genes of a species. In this study, carbon utilization capabilities of five *Phyllosticta* species were determined, as well as the CAZyme repertoire (CAZome) encoded in their genomes. Little variation was found among species in terms of carbon utilization capabilities and CAZome. However, one of the tested carbon sources, sugar beet pulp (SBP), inhibited growth of the plant pathogens, also when combined with another carbon source, while endophytic species remained unaffected.

1. INTRODUCTION

Fungal plant pathogens form an increasing threat to the agricultural sector and to our society, as they are responsible for enormous global crop losses and drive the dependency on environmentally harmful fungicides (Savary et al., 2019). In order to reduce the impact of these destructive diseases, an increased understanding of the underlying mechanisms of pathogenicity and the basic biology of the responsible species is of vital importance. One such disease is Citrus Black Spot caused by the fungus *Phyllosticta citricarpa*, which results in various disease symptoms including leaf and fruit lesions, and leads to economic losses and in severe cases crop loss due to early fruit drop (Brentu et al., 2012; Kotzé, 1981, 2000). Disease control includes leaf litter removal which can be highly effective but is a very labour-intensive and costly process (Truter, 2010). Successful management using fungicides is highly dependent on proper timing, the latter of which can reduce crop loss up to 77% (Eustáquio Lanza et al., 2018). However, neither of these approaches is fully effective, and the use of fungicides increases the risk of antifungal resistance developing.

The genus *Phyllosticta* includes many other species colonizing a broad range of host plants globally (Glienke et al., 2011; Guarnaccia et al., 2017; Wang et al., 2012; Wikee et al., 2013a, 2013b; Wulandari et al., 2009). Several *Phyllosticta* spp. have often been found to co-occur on a single *Citrus* host plant, with some being endophytic, while others are pathogenic. A species often found together with *P. citricarpa* is *Phyllosticta capitalensis*, an endophyte with a very broad host range (Wikee et al., 2013a). The co-occurrence and high morphological similarity of these species have led to some confusion in the past as to whether *P. capitalensis* is also a pathogen (Baayen et al., 2002; Glienke et al., 2011). Another endophytic species is *Phyllosticta citribraziliensis*, which was relatively recently described from *Citrus* hosts in Brazil (Glienke et al., 2011). Other pathogenic species include *Phyllosticta citriasiana*, causing tan spot on pomelos (*Citrus maxima*), and the recently described *Phyllosticta paracitricarpa*, which is both morphologically and genetically very similar to *P. citricarpa* (Guarnaccia et al., 2017). Their close genetic relationship combined with their varying lifestyles make these species ideal candidates to study whether the ability to utilize carbon sources differentiates pathogens from endophytes. The aforementioned species were chosen based on their phylogenetic relationship and distinct lifestyles; other species of *Phyllosticta* on *Citrus* not used in this study include *P. citrichinaensis* and *P. citrimaxima*.

An important process during fungal colonization of a plant is the degradation of the plant cell wall, which consists largely of polymeric compounds (polysaccharides, lignin, protein, Mäkelä et al., 2014). The enzymes involved in this process are referred to as plant cell wall degrading enzymes (PCWDEs), which are secreted by the fungus and often involved in pathogenicity (Douaiher et al., 2007; King et al., 2011; ten Have et al., 2002, for a review see Kubicek et al., 2014). Differences occur between the PCWDE arsenals of plant-pathogenic and endophytic species, supporting their role in pathogenicity. For instance, Lo Presti et al. (2015) reported a reduction of PCDWEs in symbionts and biotrophs (both facultative and obligate) compared to saprotrophs, necrotrophs and hemibiotrophs. Choi et al. also reported a higher number of PCWDE-encoding genes in pathogens as compared to saprotrophs, as well a difference in the type of genes that are present (Choi et al. 2013). According to their study, plant pathogens generally contain several genes encoding pectin lyases (PL) and polygalacturonases (PG), whereas most or even all of these genes are missing in wood-decaying fungi

(Choi et al., 2013).

PCWDEs are a subset of the so-called Carbohydrate Active Enzymes (CAZymes), which have been extensively described for many fungal species, and perform the breakdown of the polymeric and oligomeric plant carbohydrates (Lombard et al., 2014). Plant cell wall differs in composition based on species and tissue, and its degradation therefore necessitates tailored sets of enzymes (van den Brink & de Vries, 2011; Houston et al., 2016). Fungi that colonize distinct hosts or employ dissimilar lifestyles possess varying CAZyme repertoires, which have even been proposed as a basis to classify phytopathogenic fungi into different lifestyles (Hane et al., 2020). Haridas et al. found that within the class *Dothideomycetes*, saprobes generally had a higher number of CAZyme encoding genes compared to pathogens (Haridas et al., 2020).

Studies into the presence of CAZyme encoding genes in *Phyllosticta* have been restricted to a limited number of species and typically detected highly variable and relatively low numbers of CAZyme encoding genes (Rodrigues et al., 2019; Wang et al., 2020). For instance, Rodrigues et al. found 44 and 23 CAZyme encoding genes in *P. capitalensis* and *P. citricarpa*, respectively (Rodrigues et al., 2019). Wang et al. studied the CAZome in *P. citriasiana* and found 267 genes, including many different CAZy families (Wang et al., 2020). Although this number is much higher than those in the study by Rodrigues et al. (2019), this number is still low compared to other *Dothideomycetes*, which on average contain between 300 and 400 CAZyme encoding genes (Ohm et al., 2012; Haridas et al., 2020). For *Phyllosticta*, Wang et al. hypothesized a correlation of the low number of CAZyme encoding genes with a relatively long infection time (Wang et al., 2020). However, an underestimation of gene numbers as a result of incompleteness of a genome assembly or the method used to detect CAZyme encoding genes cannot be excluded.

We hypothesize that there may be a difference in carbon utilization capabilities and CAZome content between pathogens and endophytes of the genus *Phyllosticta*. In this study, carbon utilization of five *Phyllosticta* species known to occur on *Citrus*, including pathogens and endophytes, are studied, and their CAZomes are compared to determine whether either or both of these factors play a role in determining lifestyle of *Phyllosticta* species on *Citrus*.

2. MATERIALS AND METHODS

2.1 Species and culture conditions

All *Phyllosticta* cultures (**Table 1**) were obtained from the Westerdijk Fungal Biodiversity Institute culture collection (CBS) or the working collection of Pedro Crous (CPC). Fungi were grown on malt extract agar (MEA, Crous et al., 2019) at room temperature for 14 days before a plug was taken to inoculate test media. Initial test conditions used *Aspergillus niger* minimal medium (MM) containing 6 g/L $NaNO_3$, 1.5 g/L KH_2PO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4$*$7H_2O$ and 0.2 ml/L Vishniac solution with a standard pH = 6.0 at 25 °C (de Vries et al., 2004). For testing the effect of pH on growth, the pH of media was lowered to 4.9–5.1 (low) and increased to 7 (high); Bromocresol purple (Merck Netherlands) was added to monitor pH levels. For testing the requirement of supplements for growth, 1 mg/L thiamine (Merck Netherlands) or 12 mg/mL inositol (Merck Netherlands) was added to the media.

2.2 General carbon utilization studies

Assays for carbon utilization essentially followed those of the Fungal Growth Database (fung-growth.org), with the exception that a modified *A. niger* MM (mMM) was used (with lowered pH and added thiamine as described in the previous section). All mono-, di- and oligosaccharides were added to mMM at a final concentration of 25 mM, all controls and polysaccharides were added to a final concentration of 1%, and all crude plant biomass substrates were added to a final concentration of 2% (the complete set of carbon sources is shown in **Suppl. Table S1**). All experiments were performed in duplicate. A 1-mm-diameter plug was taken from the edge of a colony grown for 14 days on MEA. Cultures were incubated at 25 °C and grown until the largest colony of a species reached the edge of the plate (plate diameter = 35 mm). Colony diameters were measured at 2–3 day intervals by taking two perpendicular measurements of the colony. For non-circular and irregularly shaped colonies, both the longstest and the shortest width of the colony were measured, and the average of both was used. When the biggest colony of a species reached the edge of the plate, the experiment was concluded for that species and photos were taken of on all 35 sources using a standard camera setup. This means that the experiment duration was shorter for species with higher growth rates.

2.3 Sugar beet pulp growth study

Modified minimal medium (mMM), mMM + Glucose (25 mM) and mMM + 1.5% WB (wheat bran) were used as control media. A range of SBP concentrations (0.25%, 0.5%, 1%, 2%, 3%) was added to mMM + 1.5% WB to test whether SBP inhibits growth on other carbon sources (plate diameter = 35 mm). Acidity of all media was adjusted to pH 5. A 1-mm-diameter plug was taken from the edge of a colony grown for 14–21 days, inoculated on the media and incubated at 25 °C. All species from **Table 1** were inoculated in triplicate. Culture diameters were measured after 3, 6, 8, and 10 days. All cultures were photographed after 10 days using a standard camera setup.

2.4 Statistical analysis sugar beet pulp tests

Measurements from day 8 were chosen for statistical analysis as none of the species had reached their maximum colony diameter on this day, but all had reached their maximum growth rate. A transformation was applied to all data to be able to compare species with different growth rates using the following method. For each species, the average of all colony diameter measurements on WB was calculated. Species with a higher growth speed will have a higher average colony diameter on WB (**Suppl. Fig. S1A**). The average of one species was then subtracted from all measurements taken for that species. The effect of this data transformation can be seen in **Suppl. Fig. S1B**. This method allows for comparison of species with different growth rates while leaving any other variation, whether they be biological or technical, intact. The same transformation was applied to measurements taken on the WB + SBP combined source. To determine any statistically significant differences between species of different lifestyles, species were assigned to one of two groups: endophytes or pathogens. *P. capitalensis* and *P. citribraziliensis* were assigned to endophytes, while *P. citriasiana*, *P. citricarpa*, and *P. paracitricarpa* were assigned to pathogens. All (transformed) measurements on WB were combined to use as reference. Colony sizes of the (transformed) lifestyle-specific measurements on WB + SBP and the (grouped) WB reference were then compared to determine any statistically significant difference using a t-test.

2.5 SBP fractionation studies

Differential centrifugations: 1.25 g SBP was added to 25 mL demineralized water, which was centrifuged at 100 g for 5 min. The supernatant was collected and centrifuged at 600 g for 10 min. The supernatant was again collected and centrifuged at 15,000 g for 20 min. All pellets created by the centrifugation steps, as well as the supernatant of the last centrifugation, were added to mMM + 1.5% WB to check for inhibitory properties. mMM, mMM + glucose (25 mM), mMM + 1.5% WB and mMM + 0.5% SBP were used as control media.

Autoclavation followed by centrifugation: 1.0 g of SBP was added to 80 mL of water,

Table 1. *Phyllosticta* species and strains used in this study, and their most commonly reported lifestyle. Strains in bold were used for establishing minimal growth requirements and for these strains genome assemblies were available and used for studying CAZyme genes.

Species	CBS collection number	Pathogen (P) or endophyte (E)
Phyllosticta capitalensis	**CBS 128856 (CPC 18848)**	E
	CBS 356.52	E
	CBS 111638	E
	CBS 117118	E
	CBS 123374	E
	CBS 173.77	E
	CBS 226.77	E
Phyllosticta citriasiana	**CBS 120486**	P
	CBS 120426	P
	CBS 123371	P
Phyllosticta citribraziliensis	**CBS 100098**	E
	CPC 17464	E
Phyllosticta citricarpa	**CBS 141350** (1)	P
	CBS 127454 (2)	P
	CBS 102373	P
	CBS 122482 (CPC 14848)	P
	CBS 122670	P
	CBS 131864	P
	CBS 141352	P
	CPC 16586	P
	CBS 111.20	P
Phyllosticta paracitricarpa	**CBS 141357 (CPC 27169)**	P
	CBS 141358 (CPC 27170)	P

autoclaved, and centrifuged at 100 g for 5 min. The pellet and supernatant were separated and added to mMM + 1.5% WB individually to check for inhibitory properties. mMM + 1.5% WB and mMM + 0.5% SBP were used as control media.

Ethyl acetate extraction: 1.0 g SBP was added to 20 mL demiwater and 20 mL ethyl acetate (VWR chemicals), mixed for 2 h at room temperature, followed by a centrifugation at 2500 rpm for 5 min. The upper ethyl acetate fraction, lower water fraction, and pellet were separated, and added to mMM + 1.5% WB to test for inhibitory properties. The ethyl acetate in the upper fraction was vaporized under constant N_2-flow prior to addition to the medium. mMM + 1.5% WB and mMM + 0.5% SBP were used as control media.

Hexane extraction: 1.0 g SBP was added to 20 mL demiwater and 20 mL n-hexane (Merck), mixed for 2 h at room temperature, followed by centrifugation at 2500 rpm for 5 min. The upper hexane fraction, lower water fraction, and the pellet were separated, and added to mMM + 1.5% WB to test for inhibitory properties. N-hexane in the upper fraction was vaporized under constant N_2-flow prior to addition to the medium. mMM +1.5% WB and mMM +0.5% SBP were used as control media.

Proteinase K digestion: 1.0 g SBP was added to 20 mL Tris EDTA (TE, pH 8.0) containing 4 mg proteinase K (Roche), incubated at 37 °C and 200 rpm for 3 h, and added to mMM + 1.5 WB to check for inhibitory properties. mMM + 1.5% WB, mMM + 0.5% SBP, and mMM + 1.5% WB + 20 mL TE (in 200 mL) were used as control media.

2.6 CAZyme analysis

Carbohydrate Active Enzyme annotation was performed as part of the JGI annotation pipeline by the Henrissat lab (Lombard et al., 2014). To compare specific CAZyme genes across species, Orthofinder was used to make Ortholog Groups (OG), using all annotated genes (Emms & Kelly, 2019). All *Phyllosticta* gene sequences used were downloaded from the genomes available on the JGI website (Guarnaccia et al., 2019). The number of CAZyme genes (as annotated by JGI) present in each OG from each species was then determined. CAZyme genes involved in biomass degradation were selected manually. To determine which CAZymes are involved in degradation of specific types of biomass, the same classification as Vesth et al. and de Vries et al. was used (Vesth et al., 2018; de Vries et al., 2017). CAZyme-containing OGs

comprising a different number of genes in pathogens compared to endophytes were assessed further. If genes distinctive to one lifestyle formed a separate clade in the phylogenetic trees automatically generated by Orthofinder, their nucleotide sequences were collected and blasted against both the assembled genomes as well as the raw sequence data of the species of the opposite lifestyle, to exclude the possibility that these genes were merely missing because of an assembly error.

3. RESULTS

3.1 Minimum growth requirements

To be able to study the effect of different carbon sources without the influence of other nutrients, a minimal medium first needed to be established for *Phyllosticta* spp. When provided with this minimal medium alone, the fungus should show only minimal or impaired growth, and upon addition of a common carbon source such as glucose this impairment should be lifted. *Aspergillus niger* MM (de Vries et al., 2004) was first tested, but appeared to be insufficient for growth of *Phyllosticta* spp (**Suppl. Fig. S2**). Several additional conditions and nutrients were tested using six *Phyllosticta* strains (**Table 1**, in bold). First, the pH of the media was stabilized at 7 (high) or 5 (low), and bromocresol purple was added to monitor pH levels. Interestingly, while the species showed improved growth at low pH, they had an alkalizing effect on the medium in almost all cases. One exception to this was *P. paracitricarpa* on MM + glucose at low pH, where it had an acidifying effect (**Fig. 1A**). The mechanisms or evolutionary advantages of this behaviour are unknown to us. Addition of inositol had no effect on growth of *Phyllosticta*. In combination with lowering the pH below 5, addition of thiamine to MM proved to be sufficient for growth of all tested *Phyllosticta* spp. (**Fig. 1B**, two representative species shown). Therefore, this modified MM (mMM) was used for the studies on carbon utilization.

3.2 Differences in carbon utilization by *Phyllosticta* species

On most of the 35 carbon sources tested, the different species reached comparable colony sizes (**Suppl. Fig. S3**, selected examples are shown in **Fig. 2**), with some variation in growth rate.

Growth rates on the carbon sources generally followed one of four patterns, for each of which an example is shown in **Fig. 2**. Colonies are of equal (small) size for all species on mMM (**Fig. 2**), with the two endophytic species (in green shades) growing faster than the pathogenic species (in brownish shades). This difference in growth speed can be observed quite clearly on wheat bran (WB, **Fig. 2**), on which the species reached maximum growth rates. The first two species to cover the plate containing WB were the endophytic *P. capitalensis* and *P. citribraziliensis*, followed 3 days later by the three pathogenic species *P. citriasiana*, *P. citricarpa* and *P. paracitricarpa*.

On another rich medium such as citrus pulp, growth rates were more comparable, but the difference in growth speed could still be observed (**Fig. 2**). A striking difference between endophytes and pathogens was observed on sugar beet pulp (SBP, **Fig. 2**). All pathogenic species showed reduced growth on SBP compared to the endophytic species (**Fig. 3**). To confirm this, and to gain further understanding of this effect, tests using more strains of the aforementioned species and another carbon source (wheat bran) as reference were performed.

3.3 Growth inhibition by sugar beet pulp

The decreased growth rates of pathogenic species of *Phyllosticta* on SBP could have two underlying causes. Firstly, the pathogenic species could lack the genes necessary for utilization

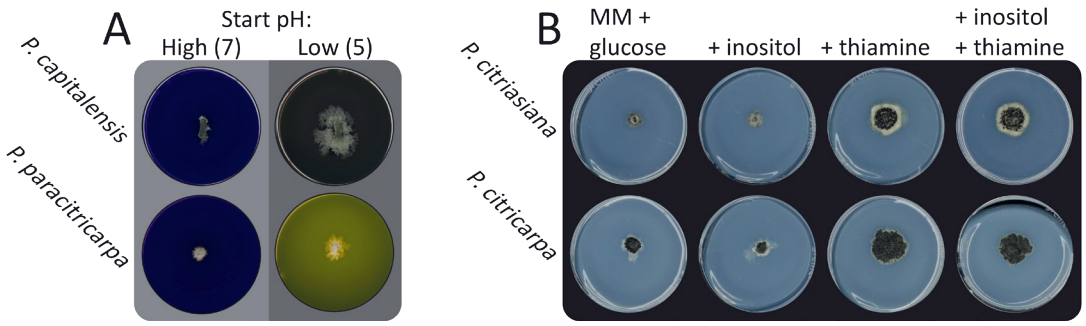


Fig. 1. Minimum growth requirements for *Phyllosticta* (A). One-mm-diam plugs were inoculated on *A. niger* MM + glucose with a pH = ~7 (high) or pH = ~5 (low). Bromocresol purple was added to monitor pH (yellow = pH < 5.5, purple/blue = pH > 6.6). Photos were taken after 7 days **(B).** One-mm-diam plugs were put on *A. niger* MM + glucose with pH = ~5, and indicated ingredients were added (1 mg/L thiamine, 12 mg/mL inositol). Photos were taken after 11 days. The two species shown are representative of all *Phyllosticta* spp. tested in this study.

of SBP, in which case the effect should be lifted after addition of a source on which the species can grow (such as wheat bran). Alternatively, growth of pathogenic species is somehow inhibited by a component of SBP, in which case the effect would not be lifted after addition of another rich source such as wheat bran. To test which scenario is more likely to be true, SBP was added at different concentrations to wheat bran (WB) on which all species show a steady and reproducible growth. Growth on WB + SBP was then compared to growth on WB as a sole source to assess the presence of potential inhibitory effects of SBP.

The colony diameters of endophytic species on WB and SBP hardly differ from their colony diameters on WB alone (blue boxes in **Fig. 4**, **Suppl. Fig. S4**). At higher concentrations, a slightly but significantly larger colony diameter can be observed compared to WB

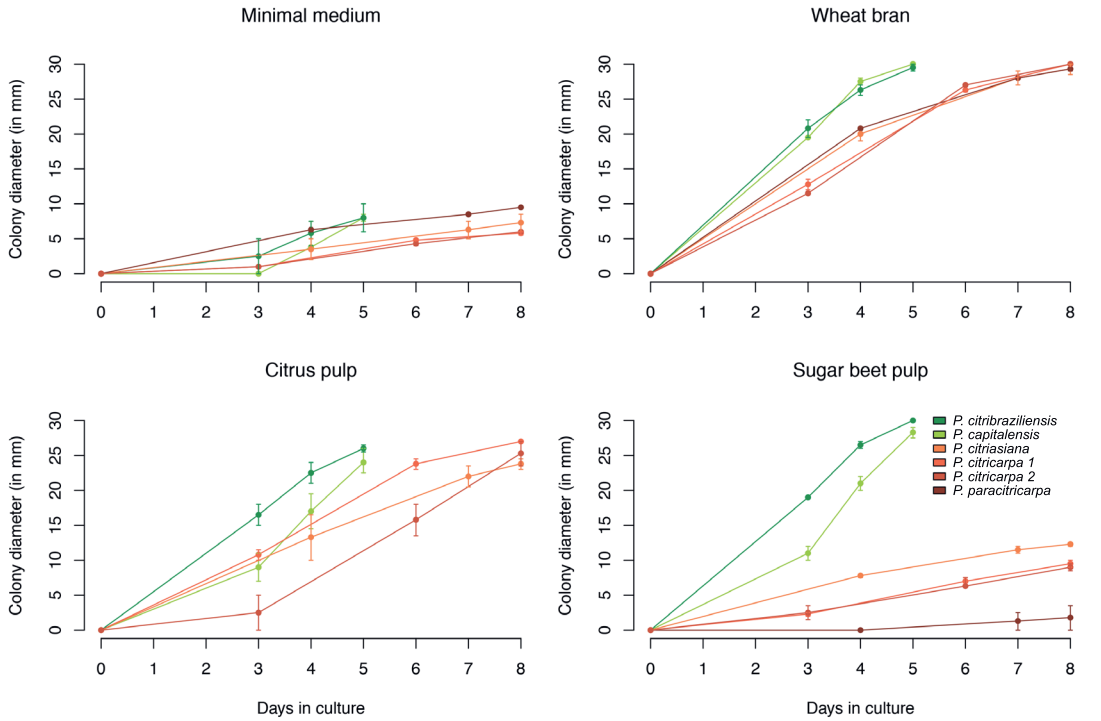


Fig. 2. Colony diameters of *Phyllosticta* spp. illustrated on four carbon sources. When the biggest colony of a species reached the edge of the plate, the experiment was concluded for that particular species (on all carbon sources). As a result, because endophytes grew faster, their experiments were concluded earlier. All measurements were done in duplicate. However, one of the *P. paracitricarpa* cultures on citrus pulp failed to grow. As we considered this to be due to technical reasons, *P. paracitricarpa* measurements on this source were omitted from the figure. Lines show averages with variance indicated with bars. All sources shown in the figure were added to an end concentration of 3%.

alone, which is in line with expectations as addition of SBP leads to a higher nutrient availability. In contrast, a large negative effect on the colony diameters of pathogenic species can be observed on SBP and WB compared to on WB alone (red boxes in **Fig. 4**, **Suppl. Fig. S4**). Colony sizes are significantly smaller even at the lowest concentrations of SBP, with the effect seemingly becoming larger at higher concentrations. An exception to this is the highest concentration, where the effect seems to be decreasing. These results support an inhibitory effect of SBP on the growth of pathogenic species of *Phyllosticta*, while no effect is observed with endophytic species.

In an attempt to determine the nature of the inhibitory substance, several experiments were performed. Firstly (autoclaved) SBP was subjected to differential centrifugation, after which the separate fractions were tested for inhibitory activity. The substance always remained in the first (heaviest) fraction, and remained active, indicating that it is either heat-tolerant or somehow protected. Secondly, two methods commonly used for extraction of bio-active com-

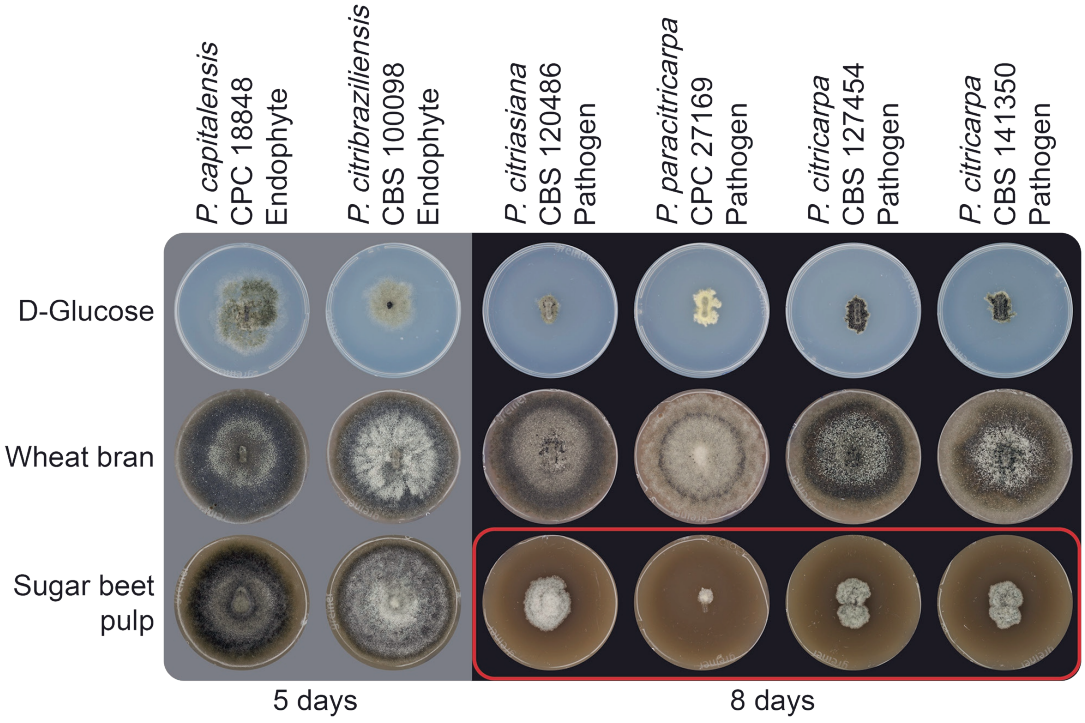


Fig. 3. Variable growth of *Phyllosticta* spp. on sugar beet pulp. Photos were taken after the number of days indicated below the figure. Species are ordered by lifestyle: endophytic on the left (grey background), pathogenic on the right (black background). Plate diameter = 35 mm.

pounds, namely ethyl-acetate extraction and n-hexane extraction, were used in an attempt to isolate the inhibitory substance. Again, the separate fractions were tested for inhibitory activity, which was detected for the pellet only, indicating that these extractions were not successful. Thirdly, a proteinase K digestion was performed, with no effect, indicating that the substance is not a protein. As these results provided only limited information and further attempts would require advanced chemical testing, a genetic approach was taken next.

3.4 CAZymes

In an attempt to find genes underlying the difference in growth rates on SBP, the presence of CAZyme encoding genes in the genomes of these species was examined. Two other descriptions of the CAZyme repertoire of *Phyllosticta* have previously been published (Rodrigues et al., 2019; Wang et al., 2020), but in both cases relatively few CAZyme genes were found (see Introduction), which could be due to the fact that the genome assemblies used were generally

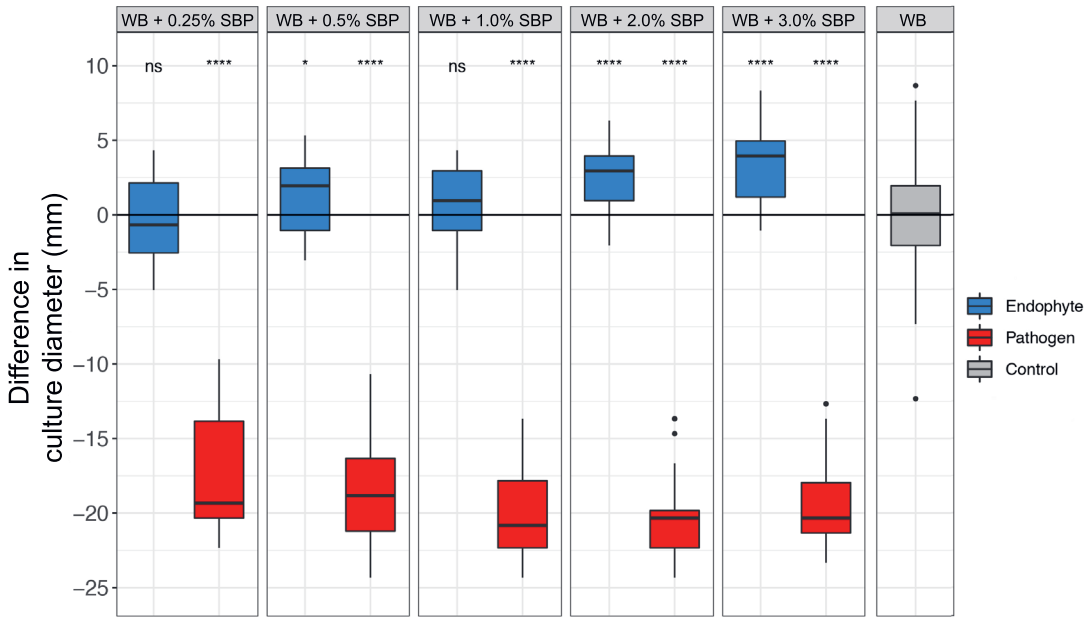


Fig. 4. Effect of sugar beet pulp (SBP) on growth of *Phyllosticta* spp. Culture diameters were measured on day 8. Per species, average culture diameter on 1.5% wheat bran (WB) was subtracted from each measurement on WB + SBP. Species were grouped together based on lifestyle (endophyte = blue, pathogen = red). The adjusted diameters were compared for a significant difference with growth on WB (both lifestyles together, grey). ns = not significant, * = $p < 0.05$, **** = $p < 0.00005$.

relatively fragmented (92–5748 contigs in one study, Wang et al., 2020, and 11,080–19143 contigs in the other, Rodrigues et al., 2019) or due to the method used to identify the CAZyme encoding genes. In our study, CAZyme genes were annotated as part of the standard JGI annotation pipeline by the Henrissat lab (Lombard et al., 2014) for all tested species, except for one of the *P. citricarpa* genomes as no CAZyme annotation was performed for that genome. Between 381 and 396 CAZyme genes were found, with higher numbers of genes for the species with better genome assemblies. Most genes were in the Glycoside Hydrolase (GH) family (174–180 genes), followed by genes in the Glycosyl Transferase (GT) family (79–82 genes), Auxiliary Activity family (54–59 genes), Carbohydrate-Binding Module (CBM) family (34–45 genes), Carbohydrate Esterase (CE) family (15–17 genes), Polysaccharide Lyase (PL) family (8 genes), and genes distantly related to plant expansins (3–6 genes). The CAZyme genes found in this study were blasted to those found by Wang et al. (2020), and for 111 genes, no significant hits were found. This number corresponds well to the fact that we found on average 118 genes extra in this study. The 111 missing genes included genes from all six different types of CAZyme families (e.g. GH, GT, AA, CBM, CE and PL). Most striking was the difference in number found for the CBM family: while Wang et al. (2020) found a single gene in this family, we identified 34–45 genes. From the pool of genes without hits, 14 were randomly selected and blasted against the genome used by Wang et al. (2020). As no significant hits were found, the absence of these genes in earlier studies can likely be attributed to assembly quality.

Table 2. Number of genes containing CAZy family modules in *Phyllosticta* (obtained from JGI MycoCosm).

	P. capitalensis	*P. citri-braziliensis*	*P. citriasiana*	*P. para-citricarpa*	*P. citricarpa*
Total	**396**	**382**	**382**	**382**	**381**
Auxiliary Activity	58	59	55	54	54
CBM*	45	34	39	42	42
Carbohydrate Esterase	15	17	16	16	15
Distantly related to PE**	6	5	3	4	3
Glycosyl Hydrolase	180	178	177	174	175
Glycosyl Transferase	82	79	82	82	82
Polysaccharide Lyase	8	8	8	8	8

*Carbohydrate-Binding Module **Distantly related to plant expansins

Generally, all species seem to have similar CAZyme repertoires with some small differences in specific families (**Table 2**). CAzyme genes were filtered manually for those involved in plant biomass breakdown as described by Vesth et al. (2018) and de Vries et al. (2017). *Phyllosticta* genomes contain a lower number of genes in most categories (xylan, xyloglucan, mannan, pectin, starch) when compared to the numbers in several *Aspergillus* spp. as described by Vesth et al. (2018), except for cellulose, for which they contain a similar number of genes (**Suppl. Table S2**). However, *Phyllosticta* spp. were able to grow on most carbon sources, albeit at a lower rate (**Suppl. Figure 3**). The only source on which growth was severely diminished for all species is D-xylose. This is surprising, as the enzymes required for D-xylose utilization are also required for L-arabinose utilization (Khosravi et al., 2015), on which growth was observed for most species.

Interestingly, significant differences were observed for growth in different substrates, but these could in most cases not be attributed to differences in genome content. Relative growth (compared to D-glucose) of *P. citribraziliensis* and *P. citriasiana* on xylan was better than that of the other species, but the genome content of known xylan-acting enzyme encoding genes was nearly identical between all species. Similarly, relative growth on calcium lignin was particularly good for *P. paracitricarpa* without any obvious difference in the number of laccases (CAZy family AA1) or other putative genes involved in lignin degradation. Also, the relative growth of *P. paracitricarpa* and both *P. citricarpa* strains on pectin is low compared to the other three species, without an obvious reduction in the number of putative pectinolytic genes. This suggests that the growth differences on these substrates are not due to differences in gene content, but rather to differences in gene expression. This has also been observed previously for various *Aspergillus* species (Mäkelä et al., 2018; de Vries et al., 2017; Benoit et al., 2015), indicating that genome content gives only a partial picture of the ability of a fungal species.

To gain a more precise understanding of the CAZyme repertoire of these species and to assess whether there are differences between endophytes and pathogens, all gene annotations from the available *Phyllosticta* genomes were grouped into Ortholog Groups (OGs) using orthofinder. Ortholog Groups containing annotated CAZyme genes were selected, and the number of genes annotated as CAZyme in each OG was counted for each species (for details

see methods). Nearly all OGs contained similar or even the exact same number of CAZyme genes for each species (**Suppl. Table S2**). In six cases, an OG contained consistently more CAZyme genes in pathogens than in endophytes, or vice versa. However, as CAZyme genes were annotated for only one of the two *P. citricarpa* genomes, but both *P. citricarpa* genomes were included in the OG analysis, it was necessary to check whether the second *P. citricarpa* genome contained a similar number of genes in these OGs. In two OGs, this was not the case; the other four OGs and the putative function of the genes contained in them are listed in **Table 3**. These OGs included genes from the CBM18 family, which contain a chitin-binding domain, the GT31 family potentially involved in cell wall remodeling and the GH3 family which might play a role in cellulose degradation. The genes specific to one lifestyle formed a distinct clade from the other genes of the OG when assembled into a phylogenetic tree (**Suppl. Fig. S5**). The missing genes were not found when blasted against both the assembled genomes as well as the raw sequence data, indicating they are not missing due to an assembly error.

4. DISCUSSION AND CONCLUSION

In this study, carbon utilization of *Phyllosticta* spp. occurring on *Citrus* was examined with particular attention to differences between endophytic and pathogenic species. Firstly, the minimum growth requirements were established; *Phyllosticta* spp. have a thiamine dependency and only grow properly on a minimal medium supplemented with thiamine and at a low pH (~5). Wang et al. (2019) studied the effect of combinations of several nutrients on conidial germination and appressorium formation of *Phyllosticta citricarpa*. Their results suggest that

Table 3. CAZyme genes in OGs with consistent differences between pathogens and endophytes.

OG nr	Predicted function	Gene nr. in endophytes	Gene nr. in pathogens
OG2	GH3*	9	8
OG3	AA1/3, CBM18**	8	7
OG36	GT31***	3	4
OG468	CBM18****	1	0

* Glycoside Hydrolase family 3

** Auxiliary activity family 1, subfamily 3 and Carbohydrate Binding Module family 18

*** Glycosyl Transferase family 31

**** Carbohydrate Binding Module family 18

absence of thiamine reduces appressorium formation with approximately 20%, an effect that becomes much stronger in the absence of salts (40%). They found no significant effect of solely thiamine on conidial germination, but in absence of citric acid the addition of thiamine had a positive effect on both conidial germination and appressorium formation. These results suggest that thiamine is involved in several complex processes in *Phyllosticta*, but what role it exactly plays in mycelial growth is unknown. *Ustilago esculenta*, a phytopathogenic species inducing edible galls in its host *Zizania latifolia*, has been reported to exhibit an obligate requirement for thiamine (Chung & Zheng, 2009), but further reports on the requirement of phytopathogenic species are very limited. A thiamine dependency of *Phyllosticta* spp. is not entirely surprising; many citrus fruits including oranges contain high levels of thiamine (Bailey & Thomas, 1942). We therefore hypothesize that the fungus has thiamine at its disposal in its natural surroundings and has lost the need and ability to produce it. The most important genes in thiamine biosynthesis seem to be present in the genomes of *Phyllosticta* spp. The inability to produce thiamine is therefore likely either the result of an unidentified mutation or due to altered transcriptional regulation.

All the species show improved growth at low pH (pH 5), while they are able to grow at a broader range. All species have been isolated from multiple host tissues, e.g. citrus leaves and citrus fruits. Citrus fruits are known to be acidic as they contain both citric acid and ascorbic acid. Therefore, it is quite possible that the preference for a low pH is an adaptation to growing in the acidic host fruit. These results are also in line with those of Wang et al. (2019), who observed that a pH between 3 and 5 is optimal for conidial germination and appressorium formation. Interestingly, despite a growth preference for low pH, *Phyllosticta* spp. generally alkalinized the surrounding media. Many phytopathogenic fungi are known to either acidify or alkalize their host environment (Alkan et al., 2013; Vylkova, 2017). Some species, such as *Botrytis cinerea*, release specialized sets of pathogenicity factors depending on the pH of the surroundings (Manteau et al., 2003). Furthermore, studies have shown that for certain species, such as *Plenodomus lingam* (*Leptosphaeria maculans*) and *Sclerotinia sclerotiorum*, the observed alkalinisation or acidification is directly related to canker length (Bousset et al., 2019). Several species of *Colletotrichum* are also known for preferring an environment with low pH, but have an alkalising effect on the surrounding host tissue (Alkan et al., 2013; Shnaiderman

et al., 2013). For some of these, this alkalising effect has been shown to be directly correlated to pathogenicity (Alkan et al., 2008; Miyara et al., 2010). Whether a similar mechanism occurs *in planta* with *Phyllosticta* spp. needs to be addressed in future studies.

In general, all species show similar growth on 35 carbon sources, although a few differences were observed. Endophytic species appear to have higher growth rates than pathogenic species on all carbon sources, which is in line with the earlier finding that *P. capitalensis* grows faster than *P. citricarpa* (Baayen et al., 2002; Glienke et al., 2011). Small differences in growth, which were observed on several carbon sources, could be due to strain variation rather than species variation. A much broader sampling using a much larger number of globally distributed strains is needed to test whether these small differences are significant. The results of the growth profiles generally correspond well to what the species would be expected to rely on in their natural habitat. The species are often found on both the citrus fruit peel as well as citrus leaves, and sometimes twigs. On rich sources, such as citrus pulp, wheat bran, and cotton seed pulp, all species grew well, as expected. An exception is sugar beet pulp, which is discussed extensively in a later paragraph. Citrus peel contains large amounts of pectin, hemicellulose and cellulose, the latter two of which are present in smaller amounts than the first (Ting & Deszyck, 1961; Shan, 2016). All species grew well on pectin, and better on citrus pectin compared to apple pectin, which is to be expected for species with *Citrus* as host. On cellulose as a sole source, most species grew poorly. Citrus leaves contain large amounts of starch, as well as sucrose, glucose, and fructose (the latter three to a lesser extent than the first, Arbona et al., 2005; Iglesias et al., 2002, 2006). All species grew well on starch, with pathogens showing a slightly decreased growth rate compared to endophytes, and medium to well on glucose, fructose and sucrose.

The most closely related species for which a similar growth profiling has been performed is *Sphaeropsis sapinea* (www.funggrowth.org). Interestingly, similarly to *Phyllosticta*, this species shows reduced growth on D-xylose, while growth on L-arabinose was unaffected. This is surprising as utilization of both sources depends on nearly the same set of enzymes. The first step of the conversion of L-arabinose and D-xylose is mediated by a set of three pentose reductases in *A. niger* (Chroumpi et al., 2021), which however have different affinities for the two pentose sugars (Terebieniec et al., unpublished data). Possibly the reductase in

Phyllosticta has a low affinity for D-xylose, causing the reduced growth on D-xylose. Alternatively, *Phyllosticta* may have a poor uptake ability for D-xylose. It could be that the molecular basis of the reduced D-xylose growth is shared within the *Botryosphaeriales* and is not specific to the genus *Phyllosticta*. However, to be able to determine the extent of this effect, wider and more extensive sampling within the fungal order is required.

An inhibitory effect of SBP on pathogenic species was observed, also in presence of another carbon source (WB) and at low concentrations (0.25%). Which compound is responsible for this effect is unknown. Although a slight decrease in growth rate is observed for pathogens on starch, we consider this substance an unlikely candidate for causing the inhibitory effect, as SBP generally contains less starch compared to wheat bran (Onipe et al., 2015; Palmgren Karlsson et al., 2002) and a strong inhibitory effect is even observed on 0.25% SBP compared to 1% starch (data not shown). As the inhibitory effect was not observed on citrus pulp, it is not likely to be caused by pectin or cellulose, but rather a trace component of SBP. Trace elements such as iron (Fe), zinc (Zn), copper (Cu) and manganese (Mn) can have inhibitory effects when present in higher concentrations than optimum for the tested fungi (Thind & Mandahar, 1968). Sugar beet pulp has been shown to contain relatively high levels of Fe as well as several other trace elements (Škrbić et al., 2010; Organisation for Economic Co-operation and Development, 2002). However, the aforementioned trace elements have also been detected in wheat bran (Yu et al., 2018), which implies that the effect would be dose-dependent, and it is also very likely that the set and concentration of trace elements is strongly batch-dependent. To determine whether these or other elements are responsible for the inhibitory effect would therefore require an exact determination of the levels of the complete set of trace elements in both substances, and a detailed comparison to identify candidate compounds that may be responsible for the growth inhibition. This is a challenging task that requires specific expertise, but it could prove very worthwhile in future studies. Furthermore, optimum concentrations of the trace elements differ widely for different fungi and have not been determined for *Phyllosticta* spp.

Several experiments were performed in an attempt to identify the inhibitory substance, but with only limited results. Proteinase K digestion had no effect on the activity of the substance, indicating that it is not a protein. As the substance remains active after autoclaving,

it is likely either thermotolerant, or protected by, for instance, embedment in rigorous cell wall components. Ethyl acetate and hexane extractions were not effective at extracting the inhibiting substance. After differential centrifugation, the substance consistently remains in the first fraction, even after spinning down at only 100 g. This indicates that the substance is likely either bound to something of substantial mass, or highly hydrophobic; perhaps it is associated with the cell wall or cell membrane. To determine the exact nature of the inhibitory substance, a precise analysis of the components present in SBP should first be performed in the future, after which the different components could be tested separately and in varying combinations.

At the highest concentration tested (3%), the inhibitory effect seemed to be decreasing. It is possible that the large increase in nutrient availability starts compensating for the inhibitory effect at this concentration. Interestingly, sugar cane residue was also reported to have an application in disease management of *P. citricarpa* (van Bruggen et al., 2017). Whether this is due to a similar mechanism is unclear.

Furthermore, we studied the presence of genes related to the carbon utilization capabilities of *Phyllosticta* spp. by analysing the CAZome of these species. Previous studies reported CAZyme encoding gene counts in *Phyllosticta* between 23 and 267 genes, which is low compared to other *Dothideomycetes*, which generally contain between 300 and 400 CAZyme genes (Haridas et al., 2020; Ohm et al., 2012; Rodrigues et al., 2019; Wang et al., 2020). In this study, we found much higher numbers of CAZyme encoding genes (381–396), which is in line with what is reported for other *Dothideomycetes*. The genome assemblies used in this study seem to be more complete than those used in previous studies. Several of the genes found in this study were blasted to the genome used by Wang et al. (2020), and no significant hits were found. We therefore think these data on CAZyme genes provide a more accurate account of what is present in these species.

Some notes on the method of generating gene counts in this study should be taken into account when comparing to gene counts generated by other pipelines. Genes are annotated as CAZyme when they possess a certain signature; as genes can possess more than one signature, some genes are included in more than one annotation. In fact, the numbers on the JGI MycoCosm table reflect the number of genes carrying a specific CAZy module, not the unique number of genes that have any CAZy module, which may cause differences with other approaches

for CAZyme annotation. For instance, in the Auxiliary Activity family of *P. capitalensis* CBS 128856, two genes received a double annotation: 299,199 and 255,817, as they each contain the modules belonging to two different families. Functional research into these genes could provide further knowledge about their functions.

In contrast with earlier studies, we did not find a difference in the number of putative PCWDE genes between species with different lifestyles within this genus. The different *Phyllosticta* species assessed in this study possess similar CAZyme gene repertoires, with only small differences. *Phyllosticta* genomes contained fewer CAZyme genes for breakdown of plant biomass categories when compared to the numbers in several *Aspergillus* spp. as described by Vesth et al. (2018), with the exception of cellulose, for which they contain a similar number of genes. The species of *Aspergillus* studied by Vesth et al. (2018) are very diverse, including strains isolated from several different kinds of biomass, but *Aspergillus* species typically have a broad substrate range, which likely explains their larger set of CAZymes. This is not clearly reflected in the ability of *Phyllosticta* spp. to grow on a range of carbon sources, as the only source on which growth was severely diminished for all species was xylose. Other small but significant differences in growth on several sources were observed, but none could be attributed to a change in gene number. The ability to degrade biomass is therefore likely transcriptionally regulated.

In four cases, an OG was found which consistently contained a different number of genes in endophytes as compared to pathogens (**Table 3**). The lifestyle-specific genes formed a separate clade from other genes in the OG when assembled into a phylogenetic tree, indicating they are different to some extent from the other genes in the OGs (**Suppl. Fig. S5**). Although further research is necessary to confirm a role in pathogenicity for these genes, it is interesting to speculate about their putative functions.

Two of these OGs contain genes belonging to the CBM18 family (OG3 and OG468). CBM18 consists of enzymes containing a chitin-binding domain. Genes from this family have been hypothesized to prevent the activation of a host defence response by disguising fungal chitin in the two pathogenic fungal species *Batrachochytrium dendrobatidis* and *Verticillium nonalfalfae* (Liu & Stajich, 2015; Volk et al., 2019). A similar mechanism has been described for an endophytic *Pestalotiopsis* sp. by Landwehr et al. (2016). Determining whether such a

mechanism is also present in *Phyllosticta* would be an interesting subject for future studies. Genes in OG36 belong to the GT31 family, and are likely important for cell wall remodelling. However, nothing is known about a potential role in pathogenicity for this gene family. Genes in OG2 belong to the GH3 family and are predicted to be β-glucosidases, important in cellulose degradation.

Overall, very few differences were found in the CAZome of *Phyllosticta* species with different lifestyles. Only four families showed consistent differences between pathogens and endophytes in this study, which are therefore interesting subjects for future studies. In general, *Phyllosticta* species with different lifestyles also showed only small variations in their ability to utilize different carbon sources, with two exceptions. Firstly, all endophytes grew faster than all pathogens. Secondly, growth of all pathogens was significantly inhibited by addition of sugar beet pulp to the medium. Despite attempts to identify the specific substance that is responsible for the inhibitory effect of sugar beet pulp, the substance and the mechanism associated with its inhibitory effect remain unknown. It is important to note that, while few differences in gene repertoire were found, variations could very well occur on the transcriptional level. Future studies on transcriptomic data of *Phyllosticta* spp. could be of great value in creating a better understanding of the difference in lifestyles between these species, and could provide important clues into further understanding their pathogenicity.

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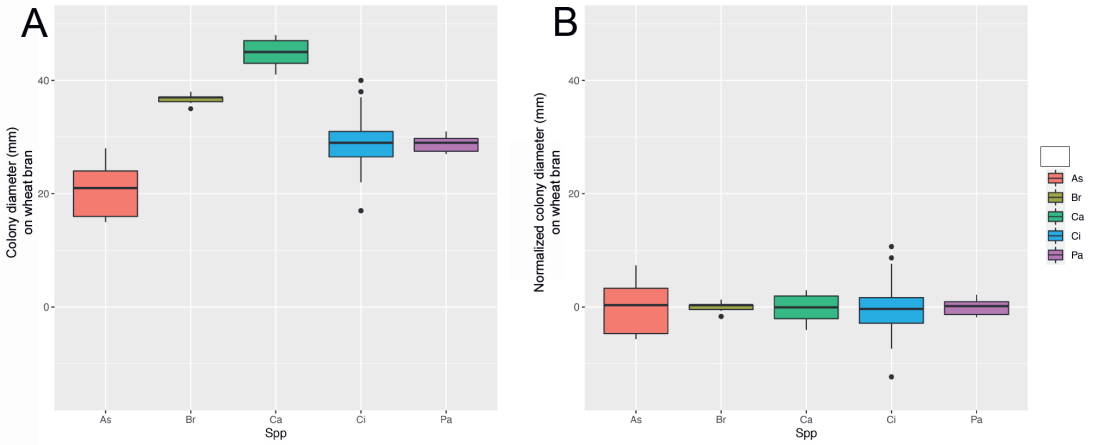
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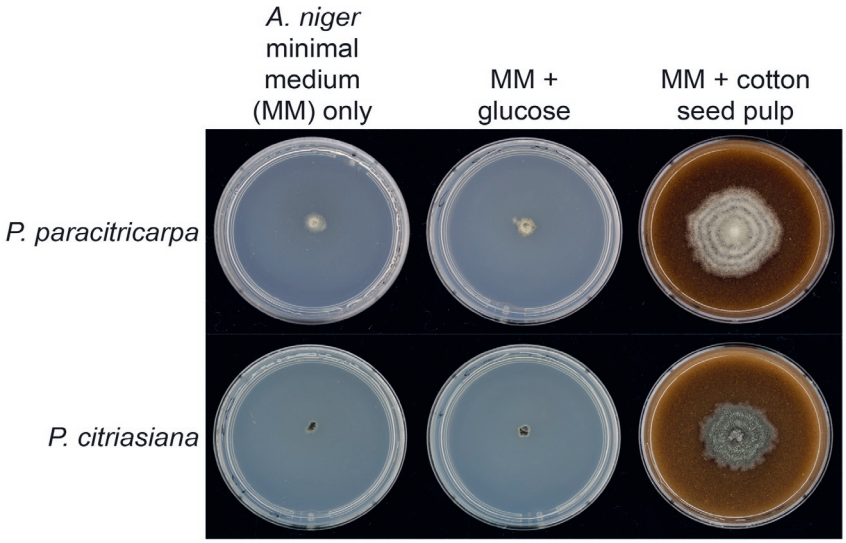
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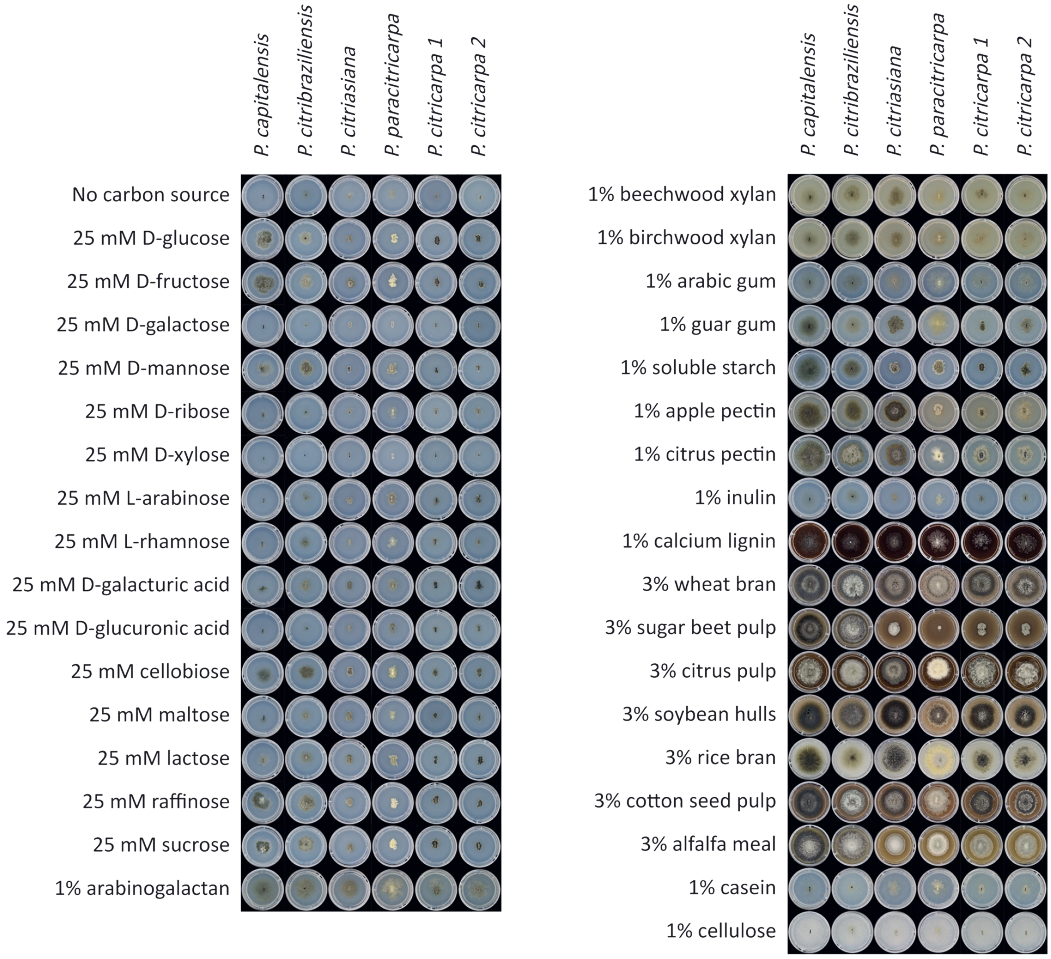
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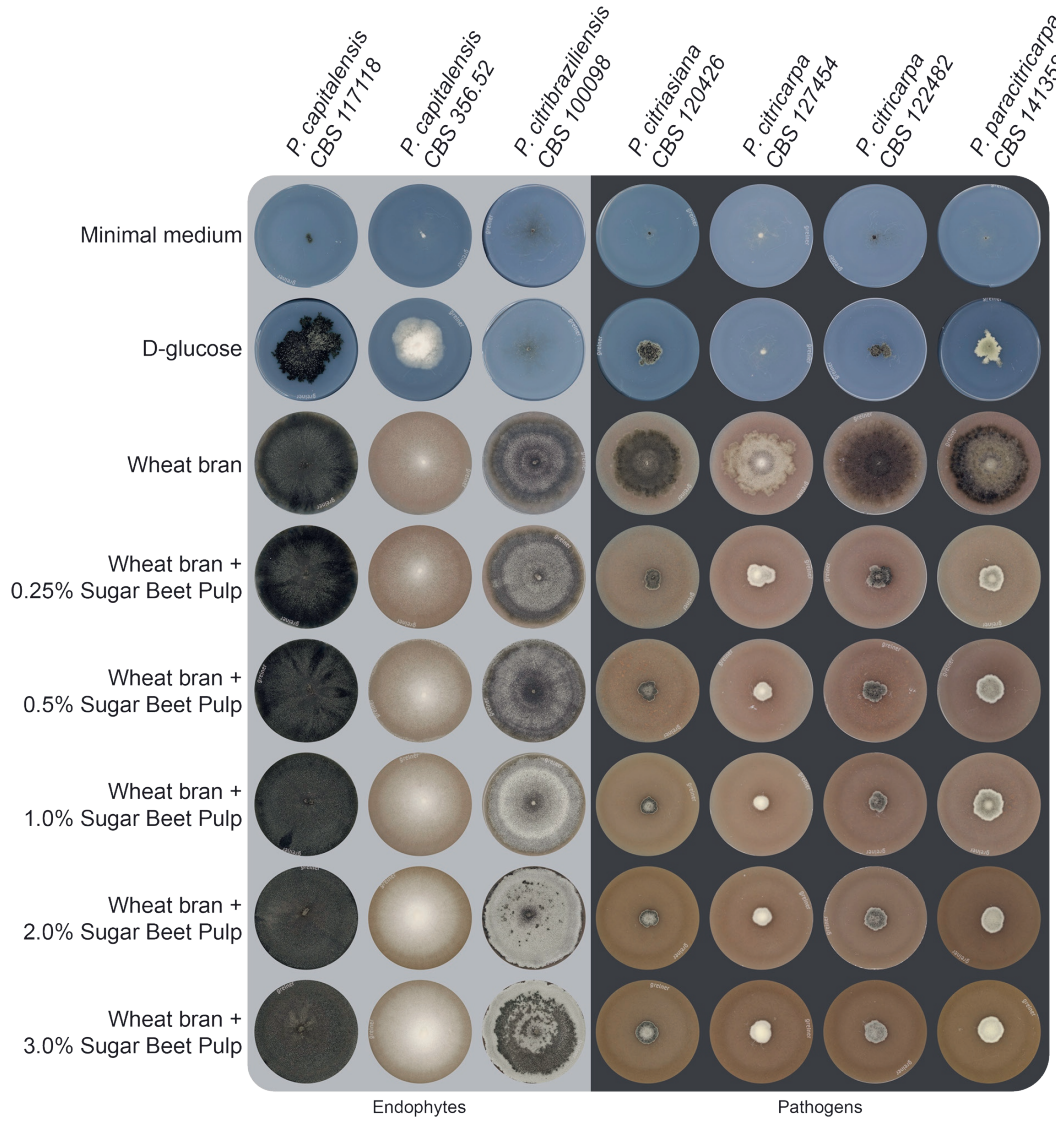
Suppl. Fig. S1. Normalization of wheat bran + suger beet pulp growth measurements. Measurements were taken on day 8. As = *P. citriasiana*, Br = *P. citribraziliensis*, Ca = *P. capitalensis*, Ci = *P. citricarpa*, Pa = *P. paracitricarpa*.



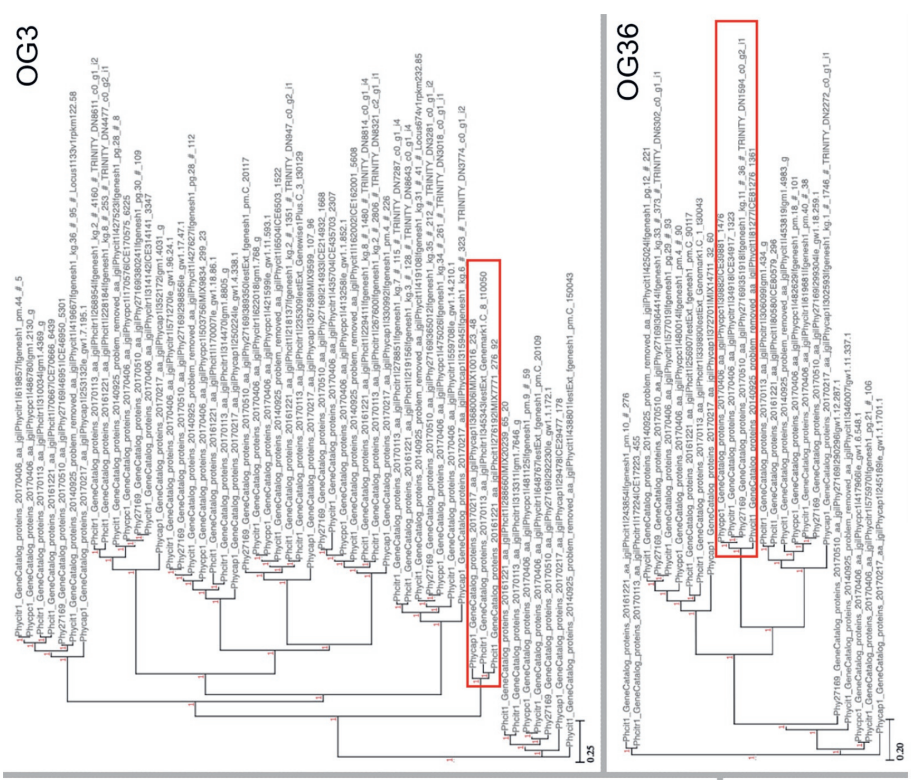
Suppl. Fig. S2. Effect on growth of different modications to *Aspergillus niger* minimal medium. *A. niger* minimal medium (MM) alone is not sufficient for growth of *Phyllosticta* spp. The medium MM + glucose does not show improved growth compared to MM, whereas MM + cotton seed hulls shows good growth. The addition of a rich carbon source alleviates the inability to grow, while the addition of a simple carbon source such as glucose does not. Plate diameter = 35 mm.



Suppl. Fig. S3. Overview of growth profiling of *Phyllosticta* spp. on 35 different carbon sources.



Suppl. Fig. S4. Growth profiles of different *Phyllosticta* species to test the effect of different concentrations of sugar beet pulp (SBP). Photos were taken on day 10. All plates with SBP also contain the same concentration of wheat bran (WB) as used for the WB control plates. The modified *A. niger* miminal medium is shown at the top and glucose was included as monosaccharide reference. Not all species tested are shown. Plate diameter = 35 mm.



Supplementary Fig. S5. Ortholog Groups (OGs) with lifestyle-specific CAZyme gene clades. In OG2, OG3, and OG468, endophytes contain an extra CAZyme gene (indicated with red rectangle). In all cases, this gene is also present in *Phyllosticta citrichinaensis*, for which the lifestyle is unclear. In OG36, pathogens contain an extra CAZyme gene. OG2 includes a clade with a misannotated gene, indicated with a dotted black rectangle. Trees were generated using Orthofinder. Phy27169: *P. paracitricarpa*. Phcit1: *P.citribraziliensis*. Phycitr1: *P. citricarpa*. Phycap1: *P. capitalensis*. Phycit1: *P. citriasiana*.

Supplementary Table S1. Carbon sources used in growth studies. pH was adjusted to pH 6.

No carbon source	25 mM D-glucose	25 mM D-fructose	25 mM D-galactose
25 mM D-mannose	25 mM D-ribose	25 mM D-xylose	25 mM L-arabinose
25 mM L-rhamnose	25 mM D-galacturic acid	25 mM D-glucuronic acid	25 mM cellobiose
25 mM maltose	25 mM lactose	25 mM raffinose	25 mM sucrose
1 % arabinogalactan	1 % beechwood xylan	1 % birchwood xylan	1 % arabic gum
1 % guar gum	1 % soluble starch	1 % apple pectin	1 % citrus pectin
1 % inulin	1 % calcium lignin	3 % wheat bran	3 % sugar beet pulp
3 % citrus pulp	3 % soybean hulls	3 % rice bran	3 % cotton seed pulp
3 % alfalfa meal	1 % casein	1 % cellulose	

Supplementary Table S2. Ortolog Group and CAZyme gene counts.
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Chapter 3

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 trophy 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.

1. 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 & 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 & 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 & 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 (Klosterman et al., 2011; Gardiner et al., 2012; Kim et al., 2016). 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 (Rovenich et al., 2014; Lo Presti et al., 2015; Fouche et al., 2018). Effectors are often shared by strains and sometimes by species that colonize the same host (Chiapello et al., 2015; van Dam et al., 2017), and on rare occasions are even passed on to a separate species through horizontal gene transfer (Gardiner et al., 2012; van Dam & Rep, 2017). 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 (Gibriel et al., 2016). 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 (Rovenich et al., 2014; Lo Presti et al., 2015).

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 (van den Brink & de Vries, 2011; Kubicek et al., 2019). Thus, CAZymes can also contribute to virulence, and differences in CAZyme repertoires can mediate microbial lifestyle differences (ten Have et al., 2002; King et al., 2011; Hane et al., 2020). Consequently, CAZymes have been used to propose new lifestyle classification models for oomycete and fungal species (Hane et al., 2020). 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 directly predicted from genome assemblies (Hane et al., 2020): polymertrophs correspond best to necrotrophs, and have received their name due to a preference for polymeric carbohydrates as primary nutrient sources. In contrast, monomertrophs, which correspond best to symbionts and biotrophs, prefer monomeric primary nutrient sources. Mesotrophs are an intermediate group utilizing both monomeric and polymeric nutrient sources, and correspond best to hemibiotrophs. Vasculartrophs are similar to hemibiotrophs, but also include species commonly classified as wilts, anthracnoses, and rots. The saprotrophic class remains a separate group, encompassing species that feed mainly on decaying plant material. These new trophic classes were proposed based on a broad and phylogenetically diverse set of phytopathogenic fungi and oomycetes (Hane et al., 2020), including several members of *Dothideomycetes*, a diverse class including many plant-associated fungi as well as fungi adapted to other lifestyles such as marine or soil environments (Haridas et al., 2020).

Within the *Dothideomycetes*, members of the genus *Phyllosticta* are particularly well suited to study the genomic basis of lifestyle adaptation and phytopathogenicity. *Phyllosticta* contains at least 50 species that are able to associate with a broad range of plant hosts (Wikee et al., 2013b), but species that colonize *Citrus* are of particular interest as they comprise both endophytes and pathogens while being phylogenetically closely related (Wikee et al., 2013b; Guarnaccia et al., 2019). The most well-known species of this genus is *Phyllosticta citricarpa*

which causes Citrus Black Spot, a disease causing significant economic losses worldwide and which therefore has a quarantine status in Europe (Kotzé, 2000; European Food Safety Authority, 2014; Eustáquio Lanza et al., 2018). *Phyllosticta paracitricarpa* is closely related to *P. citricarpa*, bears a strong morphological resemblance and appears to cause similar disease symptoms on *Citrus* (Guarnaccia et al., 2017). Other pathogens include *P. citriasiana*, described from several *Citrus* hosts in Asia, *P. citrimaxima*, described from *Citrus maxima* in Thailand, and *Phyllosticta citrichinaensis*, described as a weak pathogen from several *Citrus* hosts in China (Wulandari et al., 2009; Wang et al., 2012; Wikee et al., 2013b). Endophytic species within the genus are *P. capitalensis* with a very broad host range and present on all continents, *P. paracapitalensis*, known from *Citrus* in Europe, and *P. citribraziliensis*, currently known only from *Citrus* in Brazil (Glienke et al., 2011; Wikee et al., 2013a; Guarnaccia et al., 2017).

Genomes were recently published for *P. capitalensis, P. citriasiana, P. citribraziliensis, P. citricarpa, P. citrichinaensis*, and *P. paracitricarpa*, with genome sizes ranging between 29 and 32 Mb (Guarnaccia et al., 2019). These genomes pave the way for comparative genomic studies aimed to disentangle lifestyle differences within the genus *Phyllosticta* (Guarnaccia et al., 2019). Although both mating types (MAT1-1 and MAT1-2) are reported to exist for both endophytic and pathogenic species (Guarnaccia et al., 2019; Petters-Vandresen et al., 2020), the pathogenic strains for which genomes have been published are all heterothallic and only of the MAT1-2 mating type. In contrast, the sequenced strain of the endophytic *P. citribraziliensis* is heterothallic and of the MAT1-1 mating type, while the other sequenced endophyte, *P. capitalensis*, is homothallic and therefore contains both mating types genes (Guarnaccia et al., 2017, 2019; Petters-Vandresen et al., 2020). *Phyllosticta citrichinaensis* is also homothallic, but the MAT1-2 idiomorph in *P. citrichinaensis* is not present at the mating-type locus but at another genomic region (Petters-Vandresen et al., 2020). This phenomenon sets *P. citrichinaensis* apart from the other species in the genus as the configuration of the mating-type locus is typically very conserved amongst *Phyllosticta* species (Petters-Vandresen et al., 2020).

Previous comparative 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. capitalensis*), which was relatively distantly related to the species it was compared

to, and consequently genomic adaptations toward these two broad lifestyles remained unclear (Wikee et al., 2013b; Rodrigues et al., 2019; Wang et al., 2020). 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.

Phyllosticta citrichinaensis was originally described as a weakly aggressive pathogen of several *Citrus* hosts in China as it was isolated from freckles or spots on fruits or leaves of *Citrus* (Wang et al., 2012). However, lesions never exhibited typical pycnidia, the presence of this species was never reported to cause any crop or economic losses and Koch's postulates may not have been established (Wang et al., 2012), and thus its lifestyle remains ambiguous. *P. citribraziliensis* is a very close relative of *P. citrichinaensis* and has been described only as an endophyte from Brazil. Therefore, if these two species were ascertained to be of two different lifestyles, they would be ideal species to study pathogenicity in *Phyllosticta*. As the genome of *P. citrichinaensis* has not been included in earlier comparative work focused on lifestyle differences (Rodrigues et al., 2019; Wang et al., 2020), a thorough study of its genome and comparison to the genomes of the other species in this genus could provide valuable information on this species' lifestyle as well as genomic underpinning of disease mechanisms of other species in this genus. Here, we present the first comparative genomics study using multiple complete genomes of two endophytic and three phytopathogenic *Phyllosticta* species and established genomic differences between species of different lifestyles within this genus. In addition, we use the genomes of two separate *P. citrichinaensis* isolates, one of which is newly sequenced, in an attempt to elucidate the lifestyle of the ambiguous *P. citrichinaensis*.

2. MATERIALS AND METHODS

2.1 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/Aaoar1; last accessed: 22 March 2022) are listed in **Supplementary Table 1**. Seven of the eight *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 the *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 Flye 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 & Kelly, 2019) to identify ortholog groups (OGs) across all 116 fungal genome annotations (**Supplementary Table 2A**). OGs unique to species of specific lifestyles were identified using UpSetR 1.4.0 (**Supplementary Table 2B**, Conway et al., 2017).

2.2 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 score > 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).

2.3 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 hmmscan 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 are present in a species, were filtered out of the heatmap for visualization purposes, but are present in the original data (**Supplementary Table 5**).

3. RESULTS

3.1 *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 eight *Phyllosticta* genome sequences, seven 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 two different *P. citrichinaensis* strains, as well as the genome assemblies of the endophyte *P. citribraziliensis*, the closest relative of *P. citrichinaensis*, those of the two pathogenic species *P. citricarpa* and *P. paracitricarpa*, which are very closely related (**Fig. 1**, also see Guarnaccia et al., 2019), of the pathogenic species *P. citriasiana*, and of the endophyte *P. capitalensis*, which is phylogenetically the least related to the other *Phyllosticta* species (**Fig. 1**). 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 eight genomes of fungal species outside of *Dothideomycetes* (**Fig. 1, Suppl. 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. 1**). 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.

3.2 *Phyllosticta* species 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 & Kelly, 2019) to identify ortholog groups (OGs) across all 116 predicted proteomes. Orthofinder identified 35,379 OGs containing 88.1% of all genes (**Suppl. 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. 1**). 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 one 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

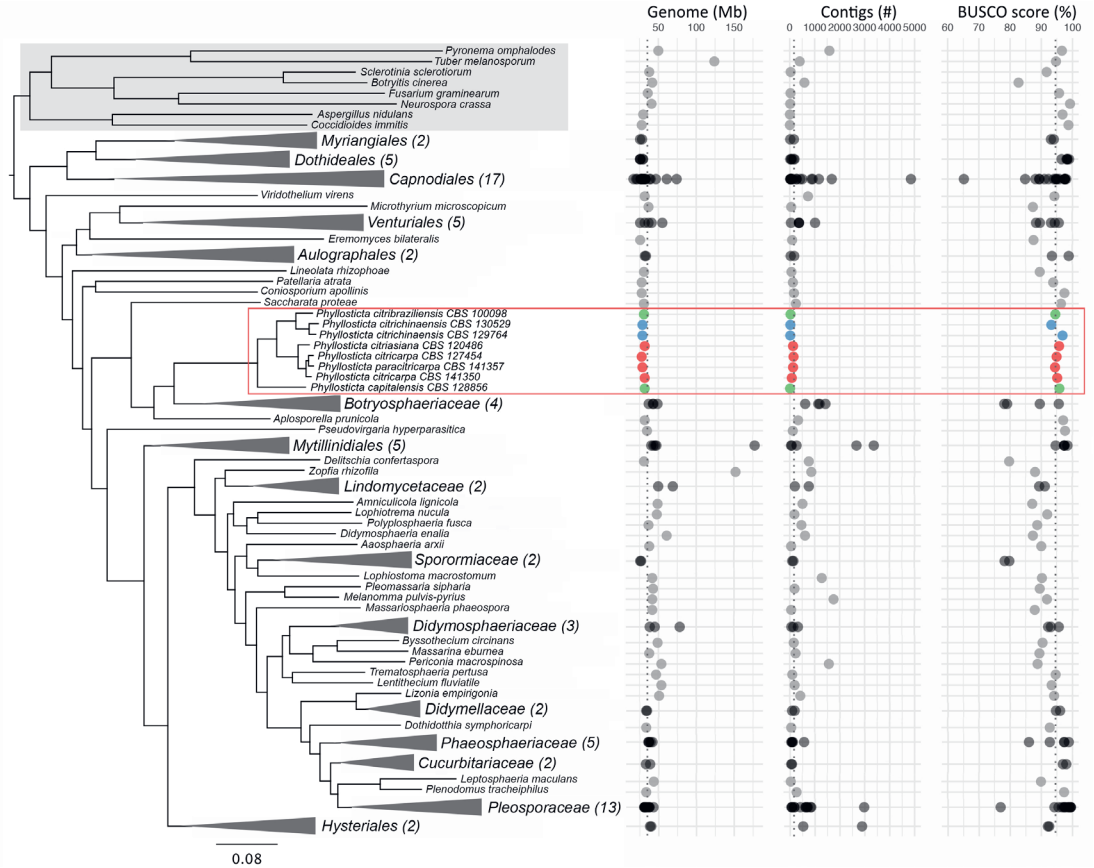


Fig. 1. *Phyllosticta* genomes are of high quality. The phylogenetic relationship of more than 100 fungal species (116 genomes) is shown. The phylogenetic tree was generated using OrthoFinder, and subtrees were collapsed manually to enhance readability. Gray box indicates species outside the *Dothideomycetes*, red rectangle indicates the genus *Phyllosticta*. Red orbs = *Phyllosticta* pathogens, green orbs = *Phyllosticta endophytes*, blue orbs = *P. citrichinaensis*.

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 two or three genes (2,734, 72.6%), as these often contain one gene from each of the two *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 eight *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**, **Suppl. Table 2B**) is much smaller than the total number of OGs that all species

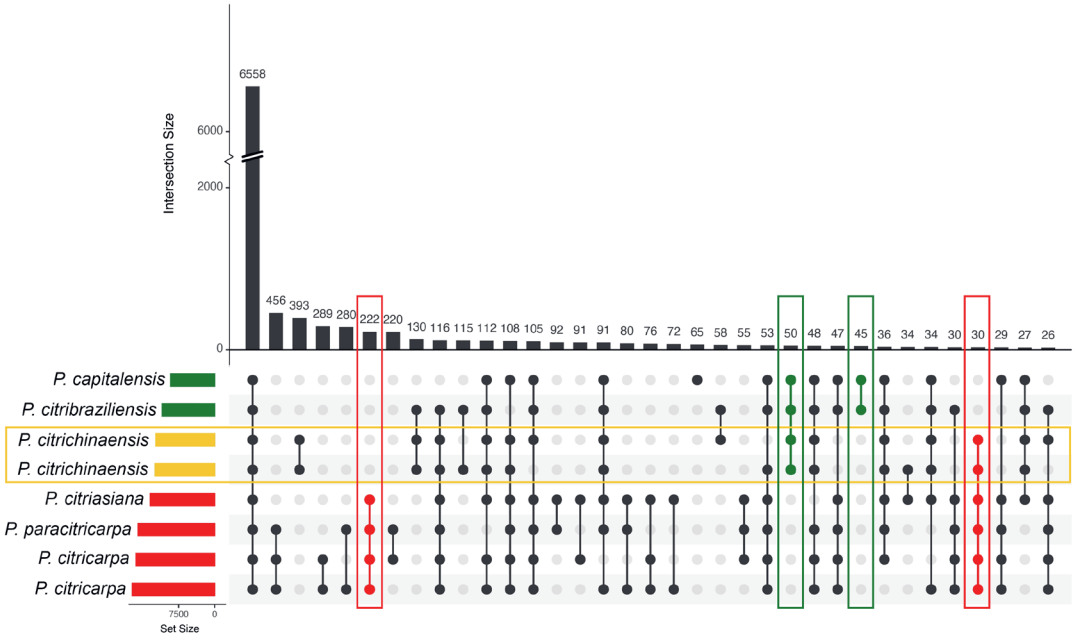


Fig. 2. *Phyllosticta citrichinaensis* shares more unique OGs with endophytes than with pathogens, but pathogens share more OGs with each other. Yellow rectangles = *P. citrichinaensis*, red rectangles = pathogens, green rectangles = endophytes. Figure generated using UpSetR. Only the largest 35 groups are shown.

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 two endophytes compared with the pathogenic *Phyllosticta* species (**Fig. 1**). 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 (**Fig. 2**). This is a rather large number when compared to the other *Phyllosticta* species, which all have less than 25 OGs that are 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 one 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 one 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 (**Suppl. 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 lifestyle 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 (**Suppl. 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 two of six lifestyle-related groups did not contain genes in this class, suggesting this group of genes may be useful to distinguish species of different lifestyles (**Suppl. 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 eight *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 six 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 (**Suppl. Table 3, A–F, I**). 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 pathogen-only group was poorly functionally annotated; out of 222 OGs, only five 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 non-annotated 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).

3.3 *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 two *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 two 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 (**Suppl. 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* (**Suppl. Table 3G, H**). For instance, two groups were annotated as heat shock proteins: an Hsp40 (DNAJC17) that had more genes in pathogens

(three in pathogens vs one or two 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* (one in endophytes vs two or three in pathogens) contains genes annotated as peroxiredoxin-1 or 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 (Mir 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.

3.4 There is little difference between *Phyllosticta* endophytes and pathogens 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, Suppl. Fig. 1, Suppl. Table 4A**) and of these, 1,315 OGs consisted of at least 50% proteins with an effector score > 1 (**Fig. 3C, D, Suppl. 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 one 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, **Suppl. 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 (singletons), which is slightly more than we observed for the overall average of 144 singleton secreted genes (**Suppl. 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

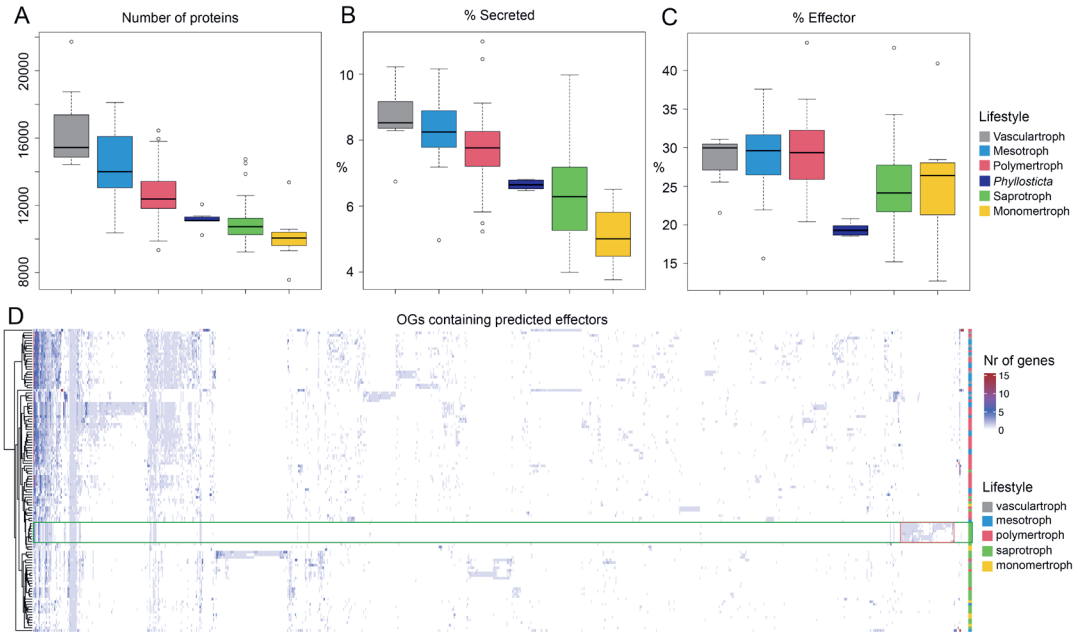


Fig. 3. Species cluster independent of their taxonomic relationship based on presence of effector proteins. A. The total number of proteins. **B.** The percentage of proteins that is predicted to be secreted. **C.** The percentage of secreted proteins that is predicted to be an effector, in species of different life-styles (as predicted by CATAstrophy, Fig. 5). **D.** Clustered heatmap of the number of genes in OGs that were predicted to contain effectors. Lifestyles as predicted by CATAstrophy (see Fig. 5). *Phyllosticta* species are highlighted with green rectangle, *Phyllosticta*-unique OGs are highlighted in red rectangle.

compared to pathogens (7.10%, **Suppl. 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. 3C, D, Suppl. 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*, three of which were present in all pathogens but not in endophytes, two were present in all endophytes but not in pathogens, and one had higher gene numbers in endophytes (**Suppl. 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 three 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.

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

CAZymes enable fungi to utilize different carbon sources (van den Brink & 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 seven *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.

3.6 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 & 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 tropic 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 , Suppl. 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

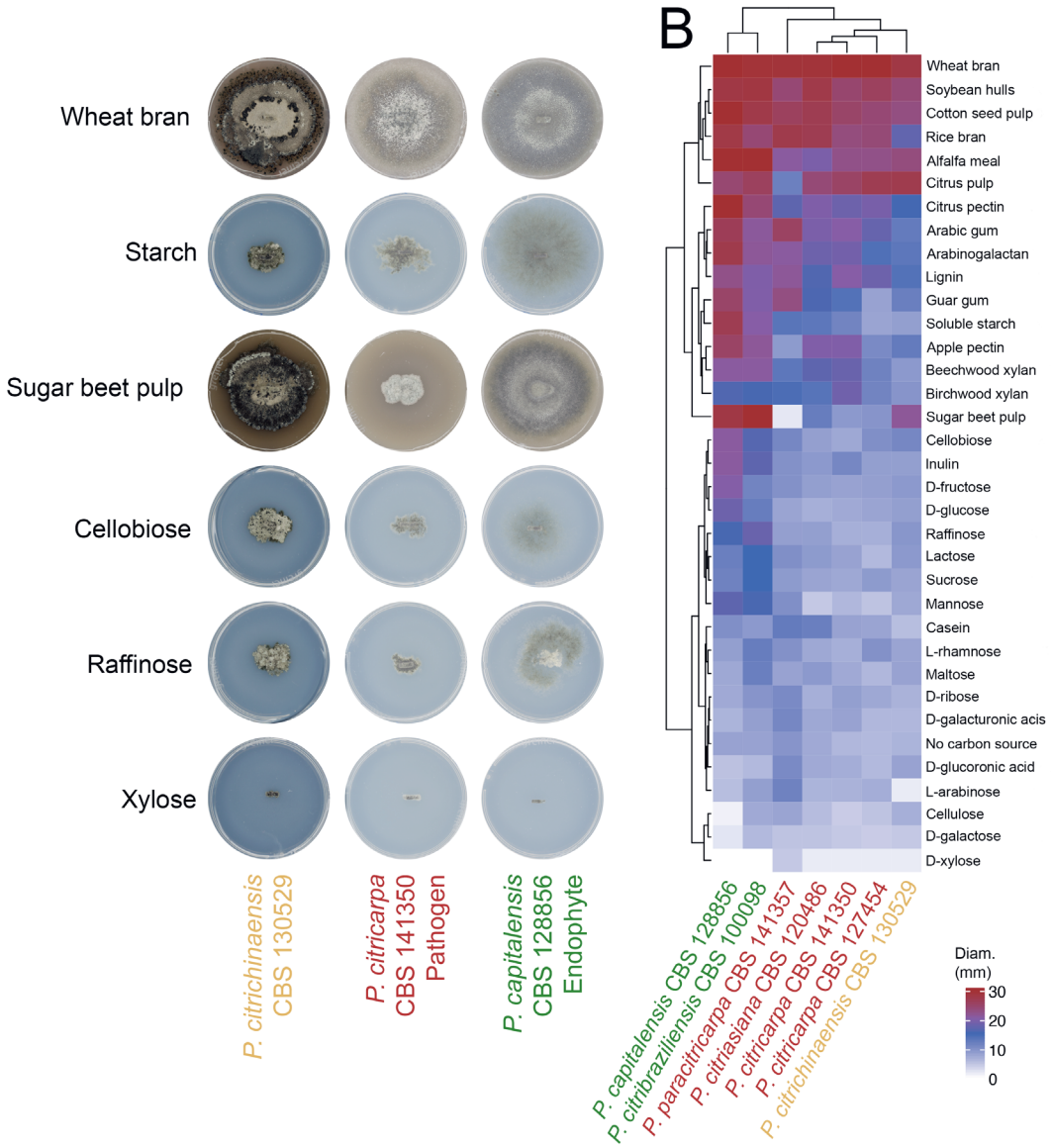


Fig. 4. *Phyllosticta citrichinaensis* clusters with pathogens based on growth on 35 carbon sources, but behaves like an endophyte in the presence of sugar beet pulp. A. Images of *Phyllosticta* species growing on a selection of different carbon sources. **B.** Clustering of *Phyllosticta* species based on their growth on different carbon sources. All species were grown on 35 different carbon sources, colony diameters were measured, and images takes on all sources when the biggest colony of a species reached the edge of its plate. All species grew fastest on wheat bran.

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, Suppl. 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 three clusters as was observed for CAZyme genes (**Figs. 3, 5C, Suppl. Fig. 1**).

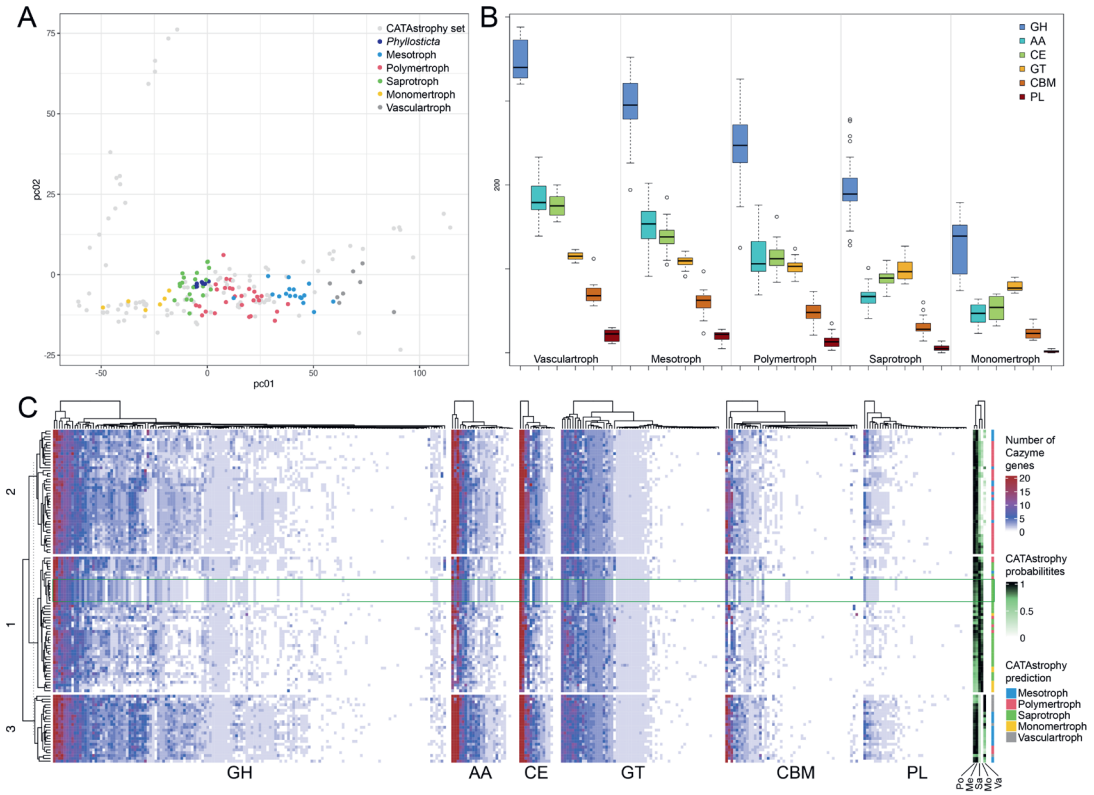


Fig. 5. Separation of species into different trophy classes based on presence of CAZyme genes in their genomes. A. PCA plot of PC1 vs PC2. PC1 separates species of different trophy classes, PC2 separates species on phylogeny. The CATAstrophy dataset published by Hane et al. (2020) is shown in light gray for comparison. **B.** Number of CAZyme genes in each CAZy family per CATAstrophy class. **C.** Clustered heatmap of the number of CAZyme-gene containing OGs per species. GH, glycosyl hydrolase; AA, auxiliary activity; CE, carbohydrate esterase; GT, glycosyl transferase; CBM, carbohydrate binding module; PL, pectin lyase; Po, polymertroph; Me, mesotroph; Sa, saprotroph; Mo, monomertroph; Va, vasculartroph.

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 (**Suppl. Table 5**). Consequently, *P. citrichinaensis* clustered most closely to *P. citribraziliensis*, an endophyte, as this is its closest relative (**Fig. 1**). We nevertheless found six 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.

Table 1. CATAstrophy predictions for the *Phyllosticta* species analyzed in this study.

	Final	Trophy scores:				
Species name	**Prediction**	**Meso**	**Monomer**	**Polymer**	**Sapro**	**Vascular**
P. capitalensis	Saprotroph	0.88	0.80	**0.95**	**1.00**	0.00
P. citribraziliensis	Saprotroph	0.84	0.82	0.90	**1.00**	0.00
P. citrichinaensis (CBS 129764)	Saprotroph	0.82	0.85	0.88	**1.00**	0.00
P. citrichinaensis (CBS 130529)	Saprotroph	0.78	0.88	0.84	**1.00**	0.00
P. citriasiana	Saprotroph	0.77	0.86	0.84	**1.00**	0.00
P. citricarpa (CPC 27913)	Saprotroph	0.82	0.83	0.89	**1.00**	0.00
P. citricarpa (CBS 127454)	Saprotroph	0.75	0.88	0.82	**1.00**	0.00
P. paracitricarpa	Saprotroph	0.75	0.87	0.82	**1.00**	0.00

Bold values indicate scores of 0.95 or higher.

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

Species name	**Lifestyle**	**CBM67**	**AA1_3**	**CBM18**	**GH3**	**PL22**	**AA3**
P. capitalensis	E	0	10	10	15	1	2
P. citribraziliensis	E	0	10	9	15	1	2
P. citrichinaensis (CBS 129764)	?	0	10	9	16	1	1
P. citrichinaensis (CBS 130529)	?	0	10	9	15	1	1
P. citriasiana	P	1	9	7	13	0	1
P. citricarpa (CPC 27913)	P	1	9	6	14	0	1
P. citricarpa (CBS 127454)	P	1	9	7	14	0	1
P. 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 = Pathogen, ? = Lifestyle ambiguous.

4. DISCUSSION

Lifestyle adaptations are thought to be driven by differences in gene content, and especially CAZymes are assumed to be crucial (ten Have 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 *P. 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 as 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 four CAZyme families showed a consistent difference between endophytic and pathogenic species (Buijs et al., 2021), one of which contained AA1_3/CBM18 (seven in pathogens vs eight in endophytes), which were mixed in one orthologue group, and another one contained family CBM18 only. In this study, we found in total six CAZyme families with a consistent difference between endophytes and pathogens. These included two separate OGs that contained the AA1_3 and CBM18 CAZyme families, 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 active enzyme 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.

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 & Bradshaw, 2010; for a review see Hüttel & 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 two trophies, such as for *Sclerotinia sclerotiorum*,

which received very high scores for both the polymertroph and the saprotroph class (**Suppl. 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 & 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 three classes (Oliver & 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 & Van Kan, 2018). Within the genus *Phyllosticta*, some obscurity with respect to lifestyle is present for instance for *P. capitalensis*, 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 *Neocosmospora solani*, which is dependent on the presence of only a few genes, or with pathogenicity of *Fusarium oxysporum* on cucurbit species, which is determined by the absence or presence of a mobile pathogenicity chromosome (Temporini & Vanetten, 2002; Dong et al., 2015; Möller & 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 species of 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 & Ipcho, 2004; Veloso & 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 & 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.

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Conflicts of interest

None declared.

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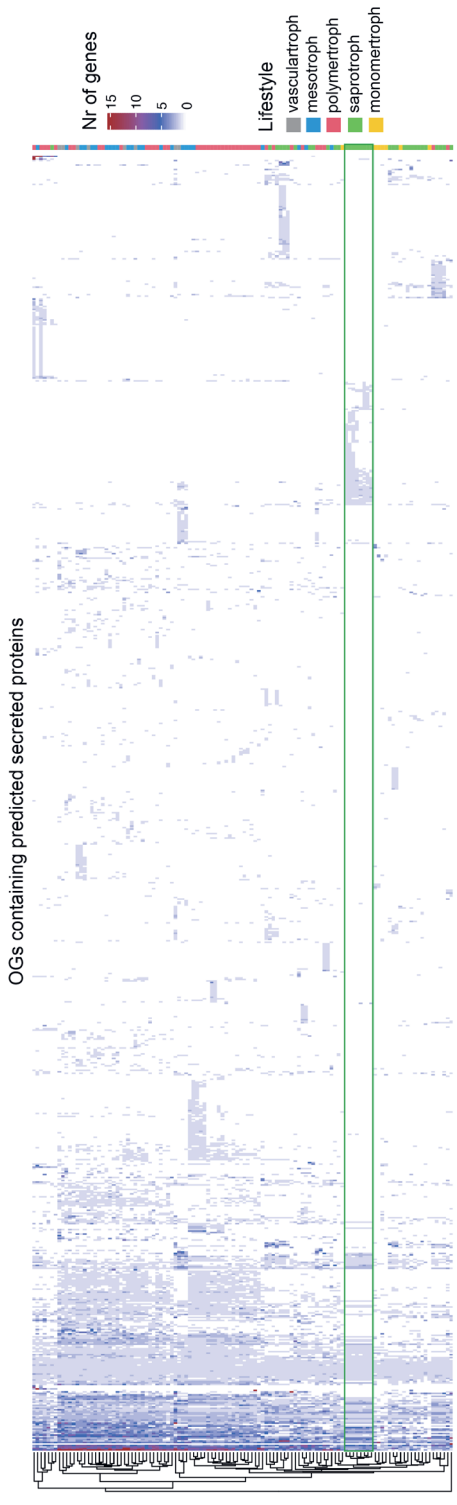
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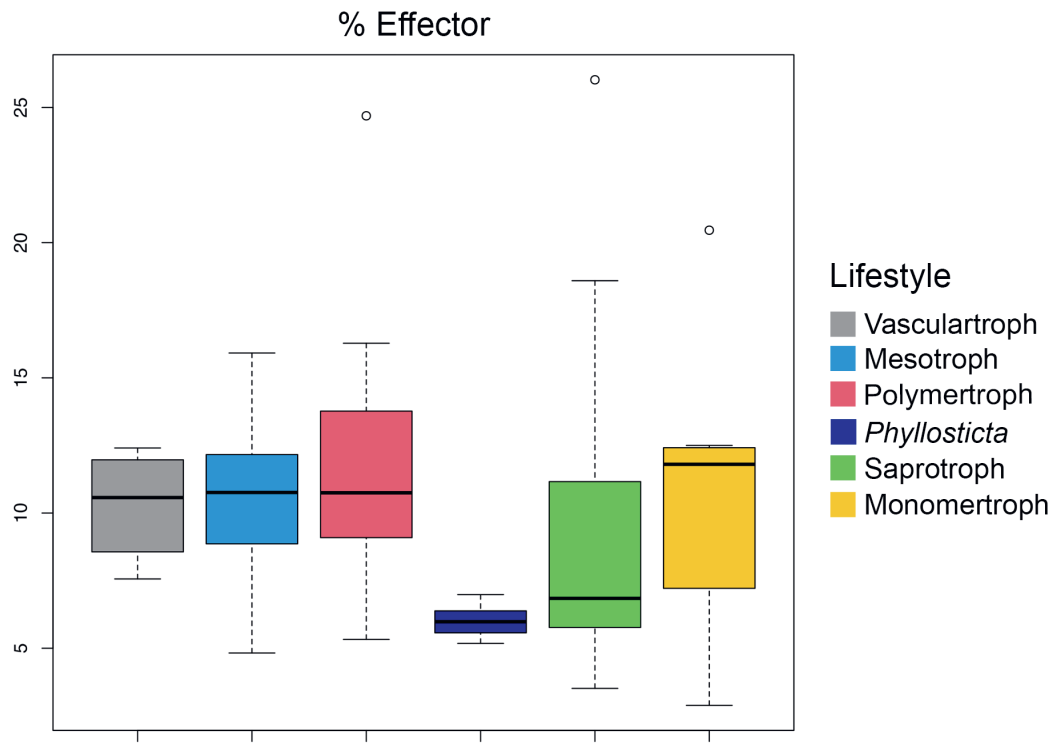
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Supplementary Materials



Supplementary Fig. S1. Species clustering differently based on the presence of predicted secreted proteins in their genomes. Heatmap was generated using the ComplexHeatmap R-package. *Phyllosticta* species are highlighted with a green rectangle. Not all collumns (= OGs) are plotted because there are too many to visually distinguish.



Supplementary Fig. S2. The percentage of proteins that is predicted to be an effector is similar between species of different lifestyles when using a Predector effector score threshold of >2.

Supplementary Table S1. Genome data of all species used in this study and related publication details.

Supplementary Table S2. Orthofinder statistical data, list of ortholog groups (OGs) that are unique to specific lifestyles, and list of ortholog groups (OGs) that have more genes in species of one lifestyle

Supplementary Table S3. Overview of ortholog group presence in species with specific lifestyles.

Supplementary Table S4. Overview of secreted and effector ortholog groups.

Supplementary Table S5. CATAstrophy results.

All Supplementary Tables are available online at https://doi.org/10.1093/g3journal/jkac061



Chapter 4

A protoplasting and PEG-mediated CRISPR/Cas9 transformation method for *Phyllosticta*

In preparation

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ABSTRACT

The fungal genus *Phyllosticta* contains a large number of species, many of which are considered to be plant pathogens. However, very little is known about which genes are important for pathogenicity and how these contribute to this fungal lifestyle. To be able to study candidate genes, the first important step is establishing a good transformation protocol. In this study, we characterize the pH modulation capabilities of *P. capitalensis* and *P. paracitricarpa*, two related species that have been characterized as an endophyte and a pathogen, respectively. The ambient pH signal transduction pathway PAL/RIM is well described in several fungi and has been reported to play a role in pathogenicity of several phytopathogenic fungi. Using a protoplasting and CRISPR/Cas9 transformation method adapted specifically to *Phyllosticta*, we targeted two genes that are thought to be important for pH modulation and phytopathogenicity, namely *pacC* and *palH*. Transformation was successful in *P. capitalensis* for *palH*, but not *pacC* nor for *P. paracitricarpa*. Δ*palH* knockout mutants showed a decreased growth rate, but no changes were observed in pH modulation abilities. The newly developed protoplasting and CRISPR/Cas9 transformation method provides the foundation for further development and optimization to effectively study pathogenicity genes in *Phyllosticta*.

1. INTRODUCTION

Fungal plant pathogens pose a serious threat to global food security with an estimated average annual loss of 17–30% caused by pathogens on the five major crops: wheat, rice, maize, potato and soybean (Savary et al., 2019). The large fungal genus *Phyllosticta* comprises 141 recognized species that colonize a variety of different plant hosts and cause disease on many of them (van der Aa & Vanev, 2002; Wikee et al., 2013a). An important example is *Phyllosticta citricarpa*, the causative agent of citrus black spot, which has quarantine status in Europe and the EPPO region (EPPO, 2020). *Phyllosticta citricarpa* is present in all citrus-growing regions worldwide except for Europe, although on rare occasions the species has been isolated from *Citrus* leaf litter in Italy, Malta, and Portugal (CABI, 2022; Guarnaccia et al., 2017). Typical infection symptoms in orchards include dark sunken lesions on fruit and necrotic spots on

leaves. The disease renders fruit unfit for sale and in severe cases may lead to early fruit drop, both of which may lead to large economic losses. In Australia alone this amounts to 80M AUD annually (Brentu et al., 2012; Drenth, 2018; Kotzé, 2000, 1981). The species can cause symptoms on a broad range of *Citrus* hosts such as *C. limon*, *C. sinensis*, and *C. reticulata* (EPPO, 2020). A recently described close relative of *P. citricarpa*, *P. paracitricarpa*, may cause similar symptoms (Guarnaccia et al., 2017), however, we show through phylogenomic analyses that these two species are in fact synonymous (Chapter 5, van Ingen-Buijs et al., 2024).

Next to *P. citricarpa*, several other species of *Phyllosticta* may also colonize *Citrus* (Glienke et al., 2011; Guarnaccia et al., 2019; Petters-Vandresen et al., 2020). For example, *P. capitalensis* colonizes many species of *Citrus* as an endophyte. *Phyllosticta capitalensis* can also colonize many other non-*Citrus* hosts, and although endophytic on *Citrus*, it may act as a pathogen in other hosts such as orchids (Silva et al., 2008; Wikee et al., 2013b). Other species of *Phyllosticta* that can be found on *Citrus* are the pathogenic species *P. citriasiana*, which is a relatively close relative of *P. citricarpa*, but is predominantly found on a different host (*C. maxima*), the weak pathogen *P. citrichinaensis*, and the endophytic species *P. citribraziliensis* (Glienke et al., 2011; van Raak et al., 2025; Wang et al., 2012; Wulandari et al., 2009). In an earlier study, we characterized the carbon-utilization abilities of five *Phyllosticta* species and assessed whether differences exist between endophytic and pathogenic strains (Chapter 2, Buijs et al., 2021). Interestingly, species were able to modulate environmental pH, with notable differences between the endophytic *P. capitalensis* and the pathogenic *P. paracitricarpa*.

The ambient pH signal transduction pathway PAL/RIM is well described in several fungi and has been reported to play a role in pathogenicity of several phytopathogenic fungi (Alkan et al., 2013; Rascle et al., 2018; Rollins, 2003). The pathway consists of the transcription factor *pacC* and six *pal*-genes that are involved in pH sensing and *pacC* processing (Peñalva et al., 2008). *PacC* is important for virulence in many important plant pathogenic fungi, such as *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Penicillium digitatum*, *Pyricularia oryzae* and *Sclerotinia sclerotiorum* (Landraud et al., 2013; Miyara et al., 2008, 2010; Rascle et al., 2018; Rollins, 2003; Zhang et al., 2013). In addition to an effect on pathogenicity, gene deletions of *pacC* also lead to a decreased ability to grow at high pH (Barda et al., 2020; Luo et al., 2017; Rascle et al., 2018; Rollins, 2003; Tilburn et al., 1995; You & Chung, 2007; Zhang

et al., 2013). Deletions of the other *pal*-genes, such as *palH*, may lead to a significant decrease in *pacC* expression and may thus lead to a similar phenotype *in vitro* (Dohmoto et al., 2010). Thus, deletions in any of the genes in this pathway may lead to detectable phenotypic changes, making them compelling candidates for knock-out.

To aid in developing effective management strategies and prevent further spread of citrus black spot, understanding the pathogenicity of *P. citricarpa* and its relatives in *Citrus* is essential. The development of CRISPR/Cas9 transformation protocols has aided many plant pathologists in deciphering the genes that are essential for pathogenicity in their species of choice, e.g. *Fusarium oxysporum* (Wang et al., 2018), *Sclerotinia sclerotiorum* (Li et al., 2018), or *Ustilago maydis* (Schuster et al., 2018). However, in contrast to the fast adaptation of CRISPR/Cas9 protocols for model organisms such as *Aspergillus niger* (Jin et al., 2022), the development of such protocols in lesser studied organisms can be challenging and time-consuming (Wilson & Wingfield, 2020). To our knowledge, while for members of the genus *Phyllosticta*, only an *Agrobacterium tumefaciens*-mediated transformation protocol exists (Gomes-Figueiredo et al., 2010), no CRISPR/Cas9 transformation protocol has yet been developed. Here, we characterized the *in vitro* pH modulating capabilities of the endophytic *P. capitalensis* and the pathogenic *P. paracitricarpa*. Subsequently, two pH-modulating genes, *PacC* and *palH*, are targeted for gene deletion using a newly adapted protoplasting and PEG-mediated transformation protocol, and the phenotypic impact of the knock-out of *palH* in *P. capitalensis* is evaluated. We thus provide, to our knowledge, the first CRISPR/Cas9 protocol developed for *Phyllosticta*, which will be invaluable for further studies in the genus.

2. MATERIALS AND METHODS

2.1 Characterization of pH modulation

For the purpose of this study, we use the name *P. paracitricarpa* for CBS 141357, the name under which it was originally identified and deposited at the onset of the study. *Phyllosticta capitalensis* CBS 128856 and *P. paracitricarpa* CBS 141357 were inoculated in triplicate on six different media: 1. *Aspergillus niger* minimal medium (MM) + 25 mM glucose; 2. MM + 25 mM glucose + 0.6% thiamine; 3. MM + 1.5% citrus pulp + 0.6% thiamine; 4. MM +

sucrose + 0.6% thiamine; 5. potato dextrose agar (PDA) + 0.6% thiamine; and 6. Yeast Mold Agar (YMA) + 0.6% thiamine. Each medium contained 0.005% (m/v) bromocresol purple as pH indicator. In addition, each medium was prepared at five different pH levels: 4, 5, 6, 7 and 8. Inoculation was achieved by transferring a 1-mm-diam plug from an approx. 2-week-old culture on PDA. Plates were incubated at 25 °C and photos of the developing colonies were taken on day 2, 4, 7, 9, and 11 post inoculation.

2.2 Candidate genes and sgRNA and dDNA design

Two genes involved in pH modulation, *pacC* and *palH*, were targeted for mutation in *P. capitalensis* and *P. paracitricarpa*; their gene identifiers from the Joint Genome Institute (JGI) can be found in **Table 1**. We selected these two genes from the PAL/RIM pathway because they were expressed in both species and yielded constructs of the correct size following RNA extraction, cDNA synthesis and a PCR assay.

Table 1. Target genes and their JGI identifier codes.

Gene	*P. capitalensis*	*P. paracitricarpa*
pacC	Phycap1|245085	Phy27169|331386
palH	Phycap1|354423	Phy27169|319868

The design and synthesis of the single guide RNA (sgRNA) and donor DNA (dDNA) was performed as described by Wilson & Wingfield (2020) using Geneious v. 11.1.5 (Kearse et al., 2012). In short, 20 bp sequences upstream of a PAM sequence (5'-NGG-3') within the target genes were selected and binding probabilities were assessed to select the best protospacer sequences. To synthesize the sgRNA, 5 µL of each protospacer was combined with 10 µL reaction buffer (2× sgRNA Reaction Mix, BioLabs, #N3320A), 2 µL nuclease-free water (Promega, #P1193), 2 µL transcriptase enzyme (sgRNA Enzyme Mix, BioLabs, #M3322A), and 0.2 µL DTT (0.1 M, BioLabs, #B1222A). The mixture was incubated at 37 °C for 30 minutes, after which it was transferred to ice. 30 µL nuclease-free water (Promega, #P1193) and 2 µL DNase I (BioLabs, #M0303AA) were added and the mixture was incubated at 37 °C for 15 minutes. The synthesized sgRNAs were then visualized on a 1.2% agarose gel. Cleaving activity of the sgRNA was tested by combining an amplicon with the target sequence and the

sgRNA/RNP. These were incubated for 15 minutes at 37 °C as described by Wilson and Wingfield (2020). The (un)cleaved products were visualized on a 1.2% agarose gel.

The donor DNA (dDNA) sequence consisted of the hygromycin resistance gene from the pAN7.1 plasmid (Punt et al., 1987), flanked on both sides with 600–900 nt of sequence directly adjacent to the target site in the DNA of the target.

2.3 Protoplasting of *P. capitalensis*

The steps of this protocol are adapted from (Liu & Friesen, 2012).

(1) To generate sufficient fungal mycelium for protoplasting for one transformation round (controls + one condition), four PDA plates were inoculated with *P. capitalensis* 15 days before protoplasting.

(2) Six days before protoplasting, 4 mL of MiliQ was added to the PDA plates, and the plates were subsequently scraped using a blue disposable inoculation loop. The solution from the plates was then collected in 2 mL Eppendorf tubes, and the solution of two plates was used to inoculate one 250 mL flask with 60 mL Fries Medium (for 1 L: 5 g ammonium tartrate, 1.0 g ammonium nitrate, 0.5 g magnesium sulfate, 1.3 g KH_2PO_4, 2.6 g K_2HPO_4, 30.0 g sucrose, 1.0 g yeast extract, 2 mL trace element stock solution (1 L water containing 167 mg LiCl, 107 mg $CuCl_2 \cdot 2H_2O$, 34 mg H_2MoO_4, 72 mg $MnCl_2 \cdot 4H_2O$, and 80 mg $CoCl_2 \cdot 4H_2O$)). The flasks were incubated at 25–27 °C, 160 rpm for three days.

(3) Subsequently (three days before protoplasting), the contents of the flasks were poured into blender cups (one flask at a time, Waring Laboratory Variable Speed Blender, autoclaved stainless-steel cups), and blended 3× at high speed (11) for 10 seconds, and 3× at low speed (7) for 10 seconds.

(4) Each 60 mL culture was then poured in 200 mL Fries Medium in a 600 mL flask. The flasks were incubated for another three days at 25–27 °C, 160 rpm.

On the day of protoplasting, 40 mL MWS-based CWDE-solution (molecular wash solution-based cell wall degrading enzyme-solution) was made by adding 400 mg lysing enzyme from *Trichoderma harzanium* (Merck) and 100 mg Driselase (Merck) to 40 mL MWS (0.7 M KCl and 10 mM $CaCl_2$ in water, autoclaved) in a 50 mL tube. The solution

was allowed to shake for approx. 30 minutes on a rocking shaker or in a shaking incubator, and subsequently spun down at 3,500 rpm, room temperature for 5 minutes, to spin down large crystals that did not solubilize, after which supernatant was taken and filter sterilized using a 0.45 µm filter.

(5) The *Phyllosticta* liquid cultures in the flasks had formed small, sometimes quite sturdy (caviar-like) balls at this stage. The cultures were therefore blended again as described previously (see step 3).
(6) The blended cultures were filtered through two layers of autoclaved Miracloth (Millipore) on top of a small funnel. The liquid flowthrough was disposed, and the mycelium, while still on the Miracloth, was washed 3× with approx. 30 mL sterile water, and then 3× with approx. 30 mL MWS.
(7) All mycelium was then transferred into the 40 mL MWS-based CWDE-solution (using a small, sterilized spoon) and incubated at 25–27 °C for approx. 3 hours. The suspension was pipetted up and down using a 10 mL pipette to help release protoplasts every 30–40 minutes.
(8) To check for the formation of protoplasts, 10 µl of the suspension was put on a slide and checked under a microscope.
(9) Once the slide is full of protoplasts (after approx. 3 hours), the suspension was filtered through four layers of autoclaved Miracloth on top of a small funnel. Large mycelial fragments remained on the Miracloth, while the protoplasts flowed through.
(10) However, the flow-through still contained quite some large mycelial debris. Therefore, the suspension was centrifuged at 1,000 rpm for 5 minutes at 4 °C.
(11) Afterwards, the pellet contained most of the debris, and the supernatant containing the protoplasts was transferred to a new 50 mL tube.
(12) The new tubes were then centrifuged again at 3,500 rpm for 5 minutes at 4 °C.
(13) From here on, all work was performed on ice. The pellet now contained the protoplasts, so the supernatant was removed carefully. One mL of MWS was added to the pellet, very gently resuspended, and subsequently moved to a 2 mL tube. MWS was added to make an end volume of 2 mL and briefly mixed by gently pipetting up and down (P1000).

(14) The tubes were once more centrifuged at 3,500 rpm for 5 minutes at 4 °C.
(15) The supernatant was carefully removed.
(16) 500 µl STC/PEG (4:1, STC: 1.2 M Sorbitol, 10 mM pH 7.5 Tris–HCl, 10 mM $CaCl_2$; PEG: 50% PEG 3350, 10 mM $CaCl_2$, 10 mM Tris–HCl (pH 7.5)) was added to the pellet.

Subsequently, 10 µl of protoplasts were taken and diluted in one mL MWS, and 10 µl of the diluted solution was used to count protoplasts under the microscope. A minimum of 10^7, but preferably 10^8 protoplasts/mL was used for transformation. Protoplasts were kept on ice until used for transformation (not stored in freezer or fridge but used the same day).

2.4 PEG-mediated CRISPR/Cas9 transformation of *P. capitalensis*

The following protocol was adapted from Wilson & Wingfield (2020).

(1) To make the ribonucleoprotein (RNP) complex, 20 nM sgRNA was combined with 30 nM Cas9 protein (New England Biolabs), 10× reaction buffer and 10 µl of sterile water and incubated at 25–27 °C for 10 minutes.
(2) Next, 100 µL of protoplast solution was pipetted into 15 mL tubes (two for controls, and one per condition) on ice. Subsequently, the RNP complex, as well as 6 µg of DNA, were added to the protoplast mixture, and mixed by very gently stirring the solution. For controls, either 5 µL of STC/PEG (negative control) or 5 µL pAN7.1 plasmid (2 ng/µL, positive control) were added instead of RNP. The mixtures were incubated on ice for 20 minutes.
(3) Then, the tubes were placed in a rack at room temperature. 100 µL of PEG solution (50% PEG 3350, 10 mM $CaCl_2$, 10 mM Tris–HCl (pH 7.5)) was added to each tube, stirred very gently using the pipet tip to mix, and incubated at room temperature for 20 minutes.
(4) Subsequently, 600 µL of PEG solution was added, stirred very gently to mix, and incubated at room temperature for 20 minutes.
(5) Afterwards, 1 mL of STC was added and mixed by gently stirring and tapping the bottom of the tubes.
(6) Next, 3 mL of STC was added and mixed by gently stirring and tapping the bottom of the tubes.

(7) Subsequently, 4 mL of STC was added, and the suspension was mixed well by gently inverting the tubes.
(8) The tubes were centrifuged at 3,000 rpm for 5 minutes at room temperature.
(9) The supernatant was carefully removed, and the pellet containing the protoplasts was re-suspended in 800 µL regeneration medium (1.0 M sucrose, 0.1% yeast extract, and 0.1% tryptone) by gently pipetting up and down with a P1000 pipet.
(10) Another 800 µL of regeneration medium was added, and the suspension was incubated at 25–27 °C with gentle shaking at 70 rpm for 1–2 hours.
(11) The protoplast suspension was then moved to a 50 mL tube and 20 mL of regeneration agar (60 °C, not hotter) was added.
(12) The resulting solution was poured into two sterile empty Petri dishes per sample, 10 mL per Petri dish. The plates were incubated at 25–27 °C overnight.
(13) The next day, one plate per sample received an overlay of 10 mL regeneration medium, while the other plate received an overlay of 10 mL regeneration medium with hygromycin (100 µg/mL). The plates were left to dry before being incubated at 25–27 °C for approx. 14 days (or until colonies became visible). Only colonies that reached through the upper hygromycin-containing layer were used for further analyses.

Potential mutant colonies were transferred to plates containing regeneration medium with 100 µg/mL hygromycin and incubated at 25–27 °C for approx. 14 days. Colonies that still displayed active growth were transferred to OA + hygromycin (100 µg/mL) plates and DNA was isolated to confirm the presence of the marker in the mutant. Mutant colonies that showed the presence of the insert were passed on selective medium (OA +hygromycin, 100 µg/mL) three times using a 1-mm-diam plug, with two weeks between each passing, and stored in the Westerdijk Fungal Biodiversity Institute's CBS culture collection before being used for pH growth studies (cultures CBS 149177, CBS 149178, CBS 149179, and CBS 149180).

2.5 Analysis of mutants

The wild type *P. capitalensis* strain (CBS 128856) and the four mutants (CBS 149177, CBS 149178, CBS 149179, and CBS 149180) were subjected to whole genome sequencing at

the Utrecht Sequencing Facility (USEQ) using Illumina NextSeq500, Mid Output: 2 × 150 bp (paired-end). Trimming as well as quality filtering and checking were performed using FastQC and a custom python script.

For analyses, we used Breseq v. 0.38.3 (Deatherage & Barrick, 2014) to map the new sequences to the reference genome (sequenced previously by JGI using PacBio, Grigoriev et al., 2014; Guarnaccia et al., 2019)) using standard settings, and results were visualized using the Integrative Genomics Viewer (IGV) Web app v. 2.2.7 (Robinson et al., 2011). Because the reference genome was sequenced using PacBio and the mutants were sequenced using Illumina, we expected technical differences to be present between the two datasets. In addition, the Illumina-sequenced strain was cultured for a few weeks before being used for transformation and subsequently sequencing, which may also lead to the introduction of differences in the genomic sequences that are not attributable to transformation. To be able to exclude these differences from the analyses of the mutant strains, Illumina sequences from the wild type strain were mapped to the reference genome, and any observed differences were then excluded from subsequent analyses of the mutant strains. After mapping the mutant sequencing reads to the reference genome, the presence of Read Alignment (RA) evidence, indicative of small mutations such as indels, was assessed, as well as areas with missing coverage (MC), which is indicative of larger insertions or mutations. The target location should show missing coverage, as the hygromycin gene should be inserted here, and this gene is not present in the reference genome. Any RA or MC evidence was manually checked using the IGV. In addition to mapping the mutant reads to the reference genome, we also generated an *in silico* construct consisting of the donor DNA (the hygromycin gene with 600–900 nt reference genome flanks) with 4000 nt reference genome flanks to be able to compare coverage of the target area of the genome. We expected that a correct mutant should display a smooth coverage of the entire *in silico* construct with no differences in coverage anywhere on the construct.

To check for a phenotypic effect, the wild type and four mutants were inoculated in triplicate on four different media: 1. *Aspergillus niger* minimal medium (MM) + 25 mM glucose; 2. MM + 25 mM glucose + 0.6% thiamine; 3. potato dextrose agar (PDA) + 0.6% thiamine; and 4. Yeast Mold Agar (YMA) + 0.6% thiamine. Each medium contained 0.005% (m/v) bromocresol purple as pH indicator. In addition, each medium was prepared at four

different pH levels: 5, 6, 7 and 8. Inoculation was achieved by transferring a 1-mm-diam. plug from an approx. 2-week-old culture on PDA. Plates were incubated at 25 °C and photos of the developing colonies were taken on day 2, 5, 7, 9, 12, and 14 post inoculation.

3. RESULTS

3.1 Differences in pH modulation of media between wild type *P. capitalensis* and wild type *P. paracitricarpa*

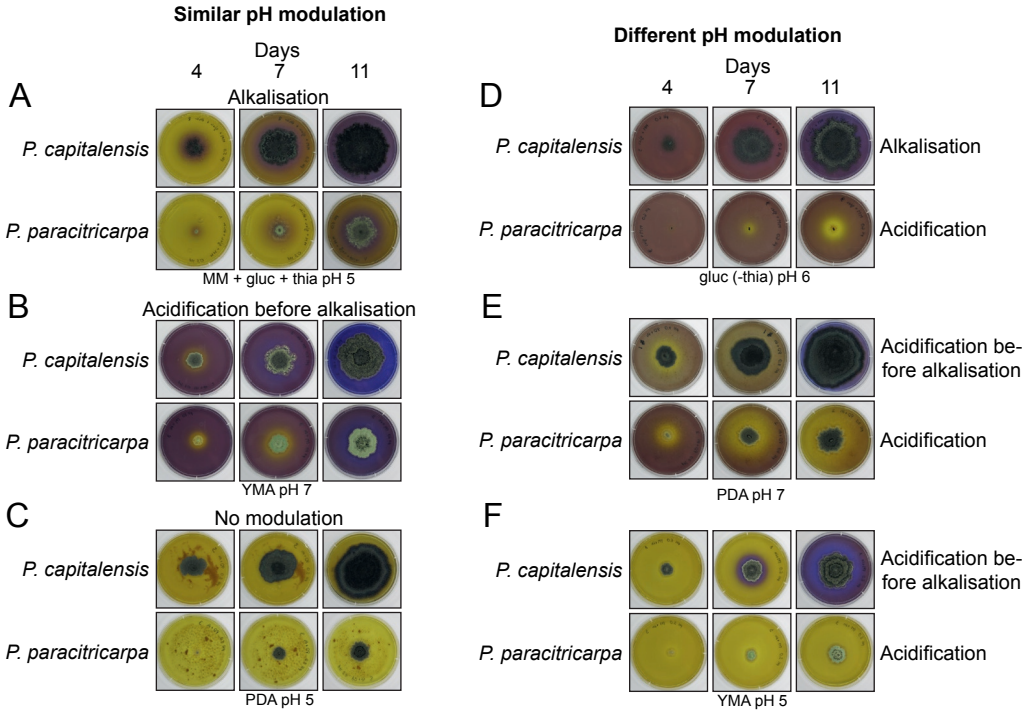
To characterize the pH modulation capabilities of *P. capitalensis* and *P. paracitricarpa*, wild type strains of both species were grown on five different media at five different pH levels (25 different conditions), and bromocresol purple was added to monitor pH. On most media, the species showed similar responses. Both species alkalize their surroundings on two media (all tested pH): MM + glucose + thiamine and MM + sucrose + thiamine (**Fig. 1A**). On YMA (pH 6–8) both species acidify, then alkalize their surroundings (**Fig. 1B**). The species do not modulate the pH of their surroundings on YMA (pH 4) and PDA (pH 4–5, **Fig. 1C**).

On three media, the species differently modulate the pH. On MM + glucose, *P. capitalensis* alkalizes at all pH levels. In contrast, *P. paracitricarpa* shows no growth on most pH levels except for pH 6, where it acidifies the media (**Fig. 1D**). On PDA (pH 6–8), *P. capitalensis* first acidifies, and later alkalizes, while *P. paracitricarpa* only acidifies the medium (**Fig. 1E**). On YMA (pH 5) we observed a similar pattern where *P. capitalensis* first acidifies and then alkalizes the medium, while *P. paracitricarpa* only acidifies (**Fig. 1F**). Thus, there are 16 conditions on which the endophytic *P. capitalensis* and the pathogenic *P. paracitricarpa* behave similarly, while they differently modulate the pH of their surroundings on nine conditions (**Fig. 1G**), suggesting differences in genes involved in pH modulation.

3.2 A protoplasting and PEG-mediated CRISPR/Cas9 method for *Phyllosticta* species

The observed differences in pH modulation, especially at higher pH, could be due to the PAL/RIM pathway. To be able to study the function of genes in *Phyllosticta*, it is essential to have a suitable transformation system available. Inspired by successes in other phytopathogens, we therefore here sought to develop a protoplasting method for *Phyllosticta* species (**Fig.**

2), based on an adjusted protocol by Liu and colleague (Liu & Friesen, 2012). This protocol focuses on the generation of sufficient starting material (**Fig. 2**, steps 1–4), the generation of protoplasts using cell wall degrading enzymes (CWDE, **Fig. 2**, steps 5–8), and finally, the purification and concentration of the protoplasts to be able to be used for transformation (**Fig 2**, steps 9–16). These protoplasts were then used to perform a polyethylene glycol (PEG)-mediated CRISPR/Cas9 transformation, based on the method described by Wilson & Wingfield (2020). In this method, a ribonucleoprotein (RNP) complex is first generated, which is then



G

pH	4		5		6		7		8	
	ca	pa	ca	pa	ca	pa	ca	pa	ca	pa
MM + gluc	alka	no growth	alka	no growth	alka	acid	alka	no growth	alka	no growth
MM + gluc + thia	alka	alka	alka	alka	alka	alka	alka	alka	alka	alka
MM + suc + thia	alka	alka	alka	alka	alka	alka	alka	alka	alka	alka
PDA	none	none	none	none	ac-alk	acid	ac-alk	acid	ac-alk	acid
YMA	none	none	ac-alk	acid	ac-alk	ac-alk	ac-alk	ac-alk	ac-alk	ac-alk

Fig. 1. pH modulation of growth media by *P. capitalensis* and *P. paracitricarpa*. Bromocresol purple was added to monitor pH: pH < 5.5 = yellow/green, pH > 5.5 and < 6.6 = brown, pH > 6.6 = purple. **A–C.** Examples of media on which the two species show similar modulation. **D–F.** Examples of media on which the two species show different modulation. **G.** Summary of the observed pH modulation by two different *Phyllosticta* species. Ca = *P. capitalensis*. Pa = *P. paracitricarpa*, alka = alkalization, acid = acidification, ac-alk = acidification followed by alkalization, none = no modulation.

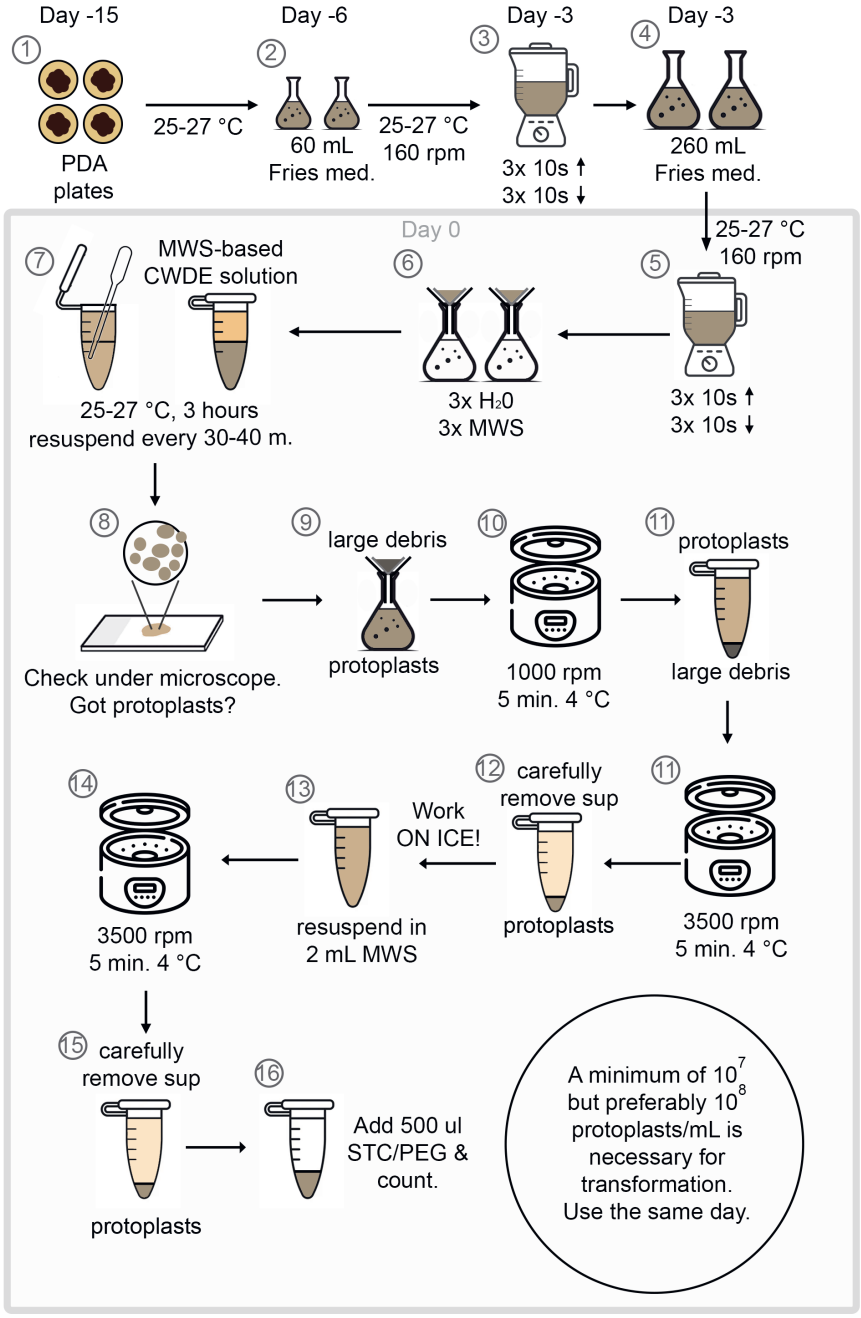


Fig. 2. Protoplasting protocol developed for *Phyllosticta* species. Steps 1–4 serve to generate sufficient starting material, and during steps 5–8 protoplasts are generated using CWDE. During steps 9–16 protoplasts are filtered through Miracloth and subjected to several rounds of centrifugation in order to purify and concentrate the protoplasts to be able to use them for transformation effectively. Detailed description of the protocol can be found in the M&M.

added to the protoplasts together with PEG (**Fig. 3**). A detailed description of the method is given in the Materials and Methods sections 2.3 and 2.4. Notably, we successfully generated sufficient quantities of protoplasts (10^7–10^8/mL) for both *P. capitalensis* and *P. paracitricarpa*.

3.3 Sequencing reveals that all mutant strains contain the insert, but only one exactly as intended, and that there are only few off-target mutations

To generate mutants of two pH modulation genes, *pacC* and *palH*, we aimed to insert a hygromycin resistance gene into the target site for both *P. capitalensis* and *P. paracitricarpa* using two different sgRNA constructs per species (**Fig. 4A**). The sgRNAs were designed as described by Wilson & Wingfield (2020). The target sequences are located early in the genes to increase chances of successful disruption of the gene, and two sgRNA constructs were used per species and per gene to see if there were differences in effectivity. Any colonies growing through the hygromycin-containing overlay after transformation were used for further analyses. We were able to generate four Δ*palH* mutants for *P. capitalensis* (Δ*palH-1*, *-2*, *-3*, and *-5*, **Suppl. Fig. S1**), but were unable to generate any mutants for *pacC*, or for *P. paracitricarpa*. Three of the mutants were generated using sgRNA-587 (Δ*palH-1*, *-2*, and *-3*) and one was generated using sgRNA-314 (Δ*palH-5*), suggesting that sgRNA-587 was more effective.

To confirm whether the dDNA construct had indeed inserted into *palH*, we PCR amplified 1569 nt surrounding the target site in the *palH* gene. The size of the amplicon is normal in the wild type (wt) and a hygromycin-resistant control strain (hyg+), and larger (i.e., 4000–5000 bp) in the derived mutants (**Fig. 4B**). However, for Δ*palH-1* and *-2*, the size is larger than expected (~5000 bp compared with 4262 bp), while the size of the palH gene in Δ*palH-3* and *-5* is as expected (**Fig. 4B**). To check whether indeed the hygromycin gene was inserted, we PCR amplified a construct starting in the *palH* flanking region and into the hygromycin gene, thus covering the junction that could only be present when the dDNA has inserted. The expected junction was present in all mutants, but in conjunction with the larger amplicon size of the target site in Δ*palH-1* and *-2*, the amplicon of the junction region was larger than expected in Δ*palH-1* and *-2* (**Fig. 4C**).

To be able to exactly determine how the dDNA inserted into the target site, and to be able to assess the presence of any off-target effects, the mutant strains were then subjected to

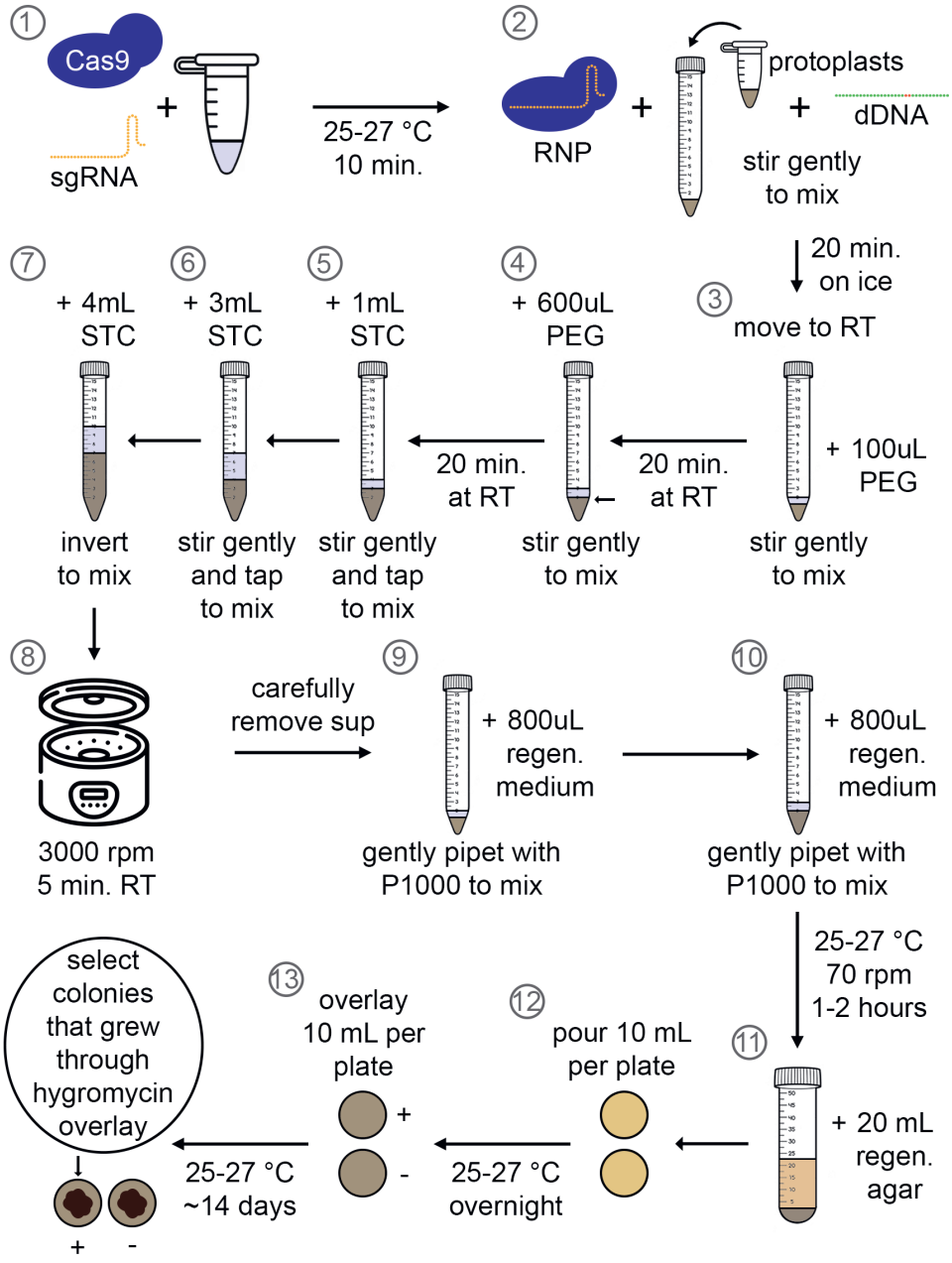


Fig. 3. PEG-mediated CRISPR/Cas9 transformation protocol of *Phyllosticta* species. Step 1 serves to generate an RNP-complex, which is added to the protoplasts in step 2, followed by PEG-mediated transformation in steps 3–4. Steps 5–8 serve to gently recover protoplasts while removing PEG, while in steps 9–12 any putative mutants are plated in regeneration agar. A selective overlay is added in step 13. Detailed description of the protocol can be found in the M&M.

whole-genome Illumina short-read sequencing. Quality-checked mutant and control sequences were then mapped to the reference genome, and to an *in silico* generated construct of the dDNA sequence with 4,000 nt flanks of reference sequence on both sides. Mapping the mutant sequences to the latter was done to determine whether the dDNA construct has inserted as anticipated. In two mutants (Δ*palH-1* and *-2*), coverage of the dDNA area was very high, suggesting a tandem insertion of the dDNA (**Suppl. Fig. S2, S3**). This is in line with earlier PCR results where the amplicons were too large. In one mutant (Δ*palH-5*) coverage of one flank was double, suggesting double insertion of the flank (**Suppl. Fig. S4**). In the last mutant (Δ*palH-3*), sequencing results suggest that the construct inserted perfectly (**Fig. 4D**). When the Δ*palH-3* mutant sequence is mapped to the reference sequence, a single gap is present where the construct should have inserted. When mapping the mutant sequence to the dDNA construct with 4,000 nt flanks, coverage is even and continuous, corroborating a single insertion in the target locus (**Fig. 4D**).

To assess the presence of any off-target effects, we used Breseq (Deatherage & Barrick, 2014) to map the reads to the reference genome. Since a different sequencing technology was used to sequence the reference genome (PacBio), we also re-sequenced the wild type using Illumina sequencing technology. Any differences found between the re-sequenced wild-type and the reference genome are expected to be of technical origin and are thus excluded from subsequent analyses. Using this approach, we assessed the presence of two types of alterations; the presence of small mutations such as single nucleotide mutations and indels, and the presence of larger insertions, which present themselves as areas with missing coverage when mapping the genome sequences to the reference genome assembly. We expected that the target location would always show missing coverage in the mutant strains as there should be a large insert present, and indeed we found that this was the case in all mutants (**Fig. 4D, Suppl. Fig. S2–S4**). We found no evidence of off-target insertions of the resistance cassette based on missing coverage in any of the mutants we examined. We did, however, find small mutations in two of the mutants. In Δ*palH-2*, we observed one transition mutation (C>T, scaffold 14 at position 574,498), and in Δ*palH-3* two transition mutations (C>T, scaffold 1 at position 646,747, C>G, scaffold 3 at position 2,145,636) were identified. Thus, we found only very few off-target mutations that could be the result of the transformation process.

Unanticipatedly, we also observed (identical) mutations in the hygromycin gene in all mutants (6 inserts, 2 shifts, and 1 deletion). The sequence used for generation of the in silico construct matches 100% with the online sequence of pAN7.1, and thus the mutations must have been present in our laboratory stock. However, the mutations do not seem to have influenced the function of the gene, as we were successfully able to select the mutant based on their hygromycin resistance.

3.4 No clear phenotypic effect of knockout of *palH* in *P. capitalensis*

To assess the effect of the *palH* knockout, the wild type and the four mutants were inoculated on four different media at four different pHs (in total 16 conditions), and growth speed and the

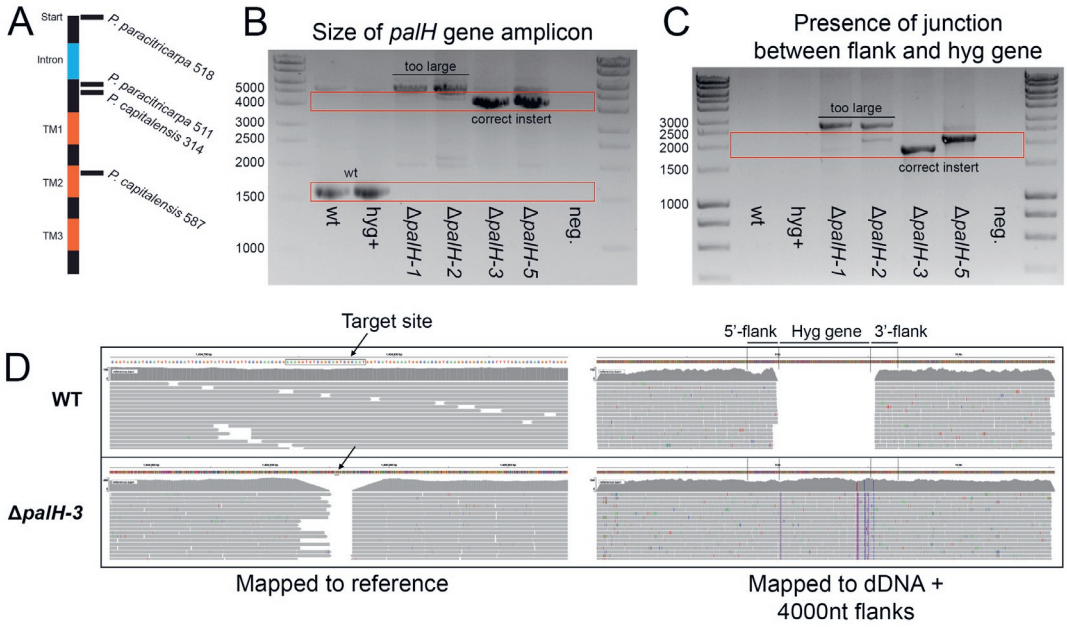


Fig. 4. The hygromycin gene inserted in all mutants, but only in the Δ*palH-3* mutant exactly as intended. A. Location of sgRNA targets in the *palH* gene. Only the start of the gene including an intron and three transmembrane (TM) domains is shown. **B.** Size of the *palH* gene amplicon in wildtype and Δ*palH* strains, with an increase in size for the mutant strains due to insertion of the hygromycin gene. **C.** Presence of the junction between the hygromycin insert and the flanking region in the genome, to assess whether the hygromycin gene inserted in the intended location. **D.** Mapping results of the wildtype reads (upper rectangle) and Δ*palH-3* mutant reads (lower rectangle) to the reference genome (left) and an *in silico* construct of the hygromycin gene and the intended flanking regions in the genome (right). A gap in the mapping to the reference genome indicated a large insertion, while smooth coverage of the mapping to the *in silico* construct indicates insertion of the hygromycin gene at the intended location.

ability to alkalize or acidify the media were assessed.

On most media, a decrease in growth speed can be observed, but at varying levels (**Fig. 5**). On MM + glucose, the decrease in growth speed was most notable at high pH, where colony sizes were on average 61% smaller compared to wild type at pH 8 while only 19% smaller at pH 5 **(Fig. 5A, Suppl. Figs. S5**, **S6**). On MM + glucose + thiamine, the decrease in growth speed was less pronounced (average of 20% smaller colony) but still present for all mutants except Δ*palH-5* at high pH (**Fig. 5B, Suppl. Figs. S5**, **S7**). In contrast to MM +

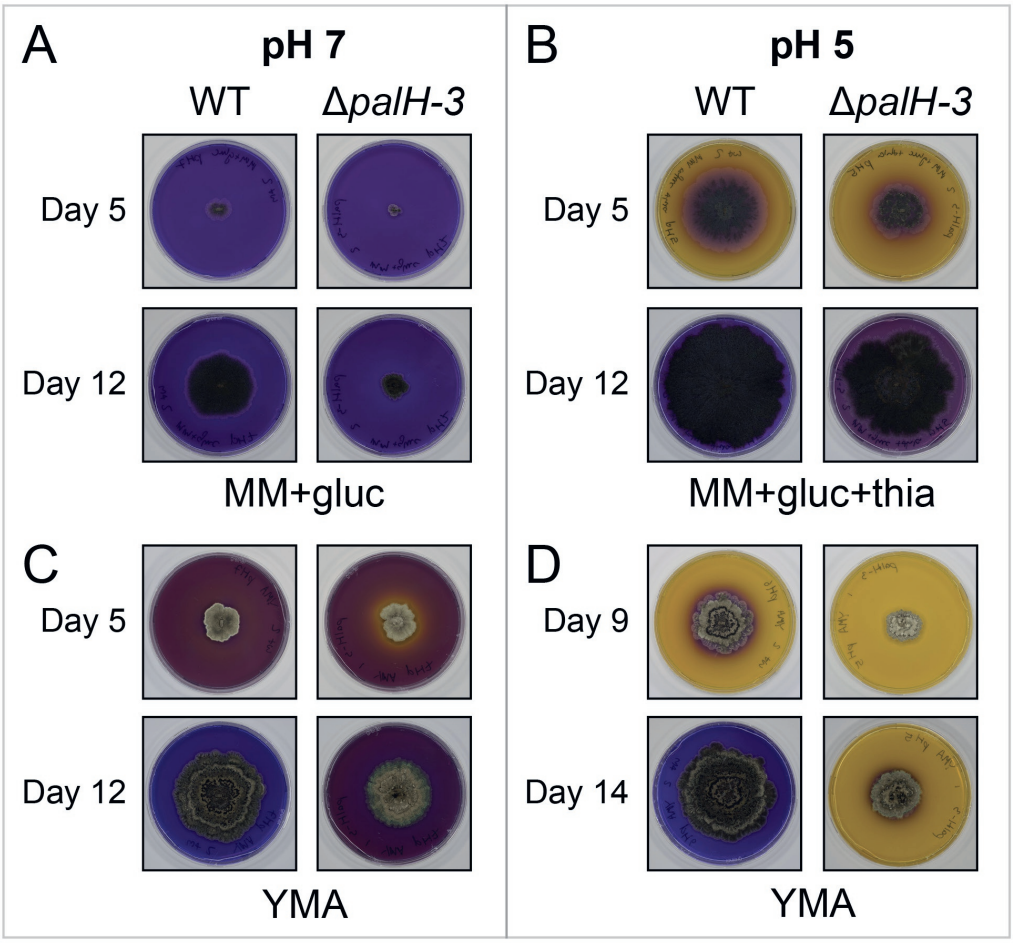


Figure 5. Slower growth rates of transformed *P. capitalensis* strains. Bromocresol purple was added to monitor pH: pH < 5.5 = yellow/green, pH > 5.5 and < 6.6 = brown, pH > 6.6 = purple. **A.** Strongly decreased growth on MM + glucose pH 7, **B.** Slightly decreased growth on MM + glucose + thiamine pH 5, **C.** Slightly decreased growth on YMA pH 7 and **D.** Decreased growth on YMA pH 5.

glucose, we observed a smaller effect on growth speed on YMA at higher pHs, where colonies were on average 17% smaller at pH 8 compared to wild type (**Fig. 5C, Suppl. Figs. S5**, **S8**) while 49% smaller at pH 5 (**Fig. 5D, Suppl. Figs. S5**, **S8**). On PDA, a difference in growth speed is smaller with an average of 23% smaller compared to wild type (**Suppl. Fig. S9**).

In contrast to these changes, we did not observe any changes in the ability to acidify or alkalize the medium. However, in some cases, the onset of alkalization appears to be delayed. This difference is most notable on YMA at low pH (**Fig. 5D**). While the wild type starts alkalizing the medium on day 9, the mutants only start on day 14. This effect can also be seen to a lesser extent at high pH (**Fig. 5C**). Thus, in contrast to studies in other fungi, in *P. capitalensis* we did not find an effect of *palH* knockout on the ability to modulate the pH of the media.

4. DISCUSSION AND CONCLUSION

Although pH modulation has been shown to be important for virulence in several phytopathogenic fungi, no studies have been done on the ability to modulate environmental pH in the genus *Phyllosticta*, which contains the important phytopathogenic species *P. citricarpa*. In addition, no CRISPR/Cas9 protocol was available for this genus. In this study, we characterized the pH modulating abilities of two *Phyllosticta* species and used an adapted protoplasting and CRISPR/Cas9 transformation protocol to target genes potentially involved in pH modulation. We succeeded in generating four Δ*palH* mutant strains in *P. capitalensis*, but only in one mutant (Δ*palH-3*) the insertion occurred exactly as intended. Thus, we here present to our knowledge, the first protoplasting and PEG-mediated CRISPR/Cas9 transformation protocol in *Phyllosticta*, which could provide opportunity to disentangle virulence in this important pathogen and adds to the growing literature of successful CRISPR/Cas9 protocols (Schuster & Kahmann, 2019).

Unfortunately, we were not able to generate any *pacC* mutants. We speculate that a *pacC* knockout may have a strongly negative effect on the vitality of *Phyllosticta* mutant strains, thereby making recovery of mutant strains challenging. Indeed, knockout of *pacC* has been shown to decrease vitality at differing degrees in several fungi such as *Beauveria bas-*

siana, *Botrytis cinerea*, *Pyricularia oryzae* and *Sclerotia sclerotiorum* (Landraud et al., 2013; Li et al., 2021; Luo et al., 2017; Rascle et al., 2018; Rollins, 2003). Further optimization of the protocol, especially regeneration conditions, may increase the possibility to generate *pacC* mutants. In addition, we were not able to generate any mutant strains for *P. paracitricarpa*, but this may be a matter of further optimizing the protocol to this species. The slower growth rate of *P. paracitricarpa* may render it less resilient than *P. capitalensis*, and, given the inherent fragility of protoplasts and the fact that their recovery sometimes hinges on subtle, fungus-specific factors, this could explain our inability to generate mutants in *P. paracitricarpa*.

Adapting CRISPR/Cas9 protocols to a new species may be challenging. We chose to use an approach using a ribonucleoprotein (RNP) due to its relative simplicity compared to plasmid-based strategies, such as those commonly used in *Aspergillus* (Garrigues et al., 2023; Nødvig et al., 2015; Song et al., 2018), which we attempted but could not establish in *Phyllosticta*. The plasmid-based strategy may prove difficult to establish in a non-model organism, since problems may arise in many different parts of the system, such as transcription or translation of the Cas9 gene, nuclear retention of the sgRNA or nuclear import of the Cas9 protein, and it is difficult to discern which component is not functional (Schuster & Kahmann, 2019). Thus, we chose to use an RNP-based strategy, which proved effective at least for one of the two fungal species. Nonetheless, many steps of the protocol presented here may be further optimized. Adjustment such as a shorter or longer CWDE-incubation time, using different enzymes, or gentler centrifuging during protoplast purification may lead to more (resilient) protoplasts (Coca-Ruiz et al., 2024; Wilson et al., 2025). Thus, the method presented here should be seen as a proof-of-principle and can be further developed and optimized for higher efficiency and accuracy.

Whole-genome sequencing data shows that in one of four mutants, the construct inserted exactly as intended, while in the other three mutants, (parts of) the construct inserted in tandem. This has also been demonstrated in other studies and organisms, such as in *Sclerotinia sclerotiorum* and in human and mouse cell lines (Hermantara et al., 2024; Li et al., 2018; Skryabin et al., 2020). This kind of tandem insertions may not always be detected using conventionally applied PCR analyses, and thus the number of cases where tandem insertions happened may be underestimated (Skryabin et al., 2020). We also assessed the presence of

off-target effects and found in total three transition mutations in two of the mutants. It is not possible to conclude whether these off-target mutations are due to effects of the CRISPR/Cas9 method or of the transformation process in general. Indeed, solely culturing or going through the transformation treatment has been shown to introduce mutations in several fungi (Abdallah et al., 2018; Álvarez-Escribano et al., 2019; Garrigues et al., 2023). However, it is worth noting that the number of off-target effects found is this study is relatively low, as the number of off-target effects in some fungi is reported to be much higher, reaching up to an average of 400 mutations per strain (Abdallah et al., 2018; Garrigues et al., 2023). Thus, the few unintended on-target and off-target effects caused by the protocol presented in this study are fewer than anticipated and within generally accepted limits.

We have previously shown that *Phyllosticta* species differ in how they modulate pH of their surroundings (Buijs et al., 2021). We sought to apply our newly developed protoplasting and PEG-mediated CRISPR/Cas9 transformation protocol and assessed the effect of knockout of *palH*, a gene involved in pH modulation, on the ability of *Phyllosticta* species to grow at high pH and modulate environmental pH. Previous work on *palH* showed that, upon *palH* knockout, expression of *PacC* at high pH was strongly inhibited, and growth was completely inhibited at high pH (Dohmoto et al., 2010). In contrast, we observed that all mutants exhibited decreased growth rates at all pH levels, and on YMA the effect seems to be even stronger at lower pH levels. In addition, studies have shown that the *Pal* pathway is involved in environmental pH modulation, specifically in relation to fungal virulence (Peñalva et al., 2008; Vylkova, 2017). We did not observe any difference in the ability to alkalize or acidify the media upon *palH* knockout. On some media, onset of alkalization was delayed, but this could be an effect of the decreased growth rate, as all strains seem to start alkalization at roughly the same colony size. In addition, responses to and modulation of environmental pH may also be dependent on other pathways (Peñalva et al., 2008). Based on the current results it is challenging to conclude whether the decreased growth rate is an effect of the *palH* knockout, or rather an effect of the strain going through the somewhat harsh transformation conditions. We did originally include an additional control that went through the transformation process but in which *palH* was not targeted. However, the hygromycin gene insertion site in this strain is unknown even after whole-genome sequencing. It is possible that the gene inserted in a repetitive area

and therefore was not present in our whole-genome sequence. In addition, the strain showed an aberrant phenotype with a strongly decreased growth rate and changes in pH modulation, therefore it was not included in this study. Thus, we cannot conclude from data presented here that knockout of *palH* in *Phyllosticta* had any phenotypic effect.

In summary, we have successfully adapted a protoplasting and PEG-mediated CRISPR/Cas9 transformation protocol that opens new doors for genetic editing in *Phyllosticta* — although our initial *palH* knockout results remain ambiguous. While the method demands additional fine-tuning to boost its reliability beyond a handful of successful trials, it holds great promise for unraveling the genetic drivers of pathogenicity in *Phyllosticta*.

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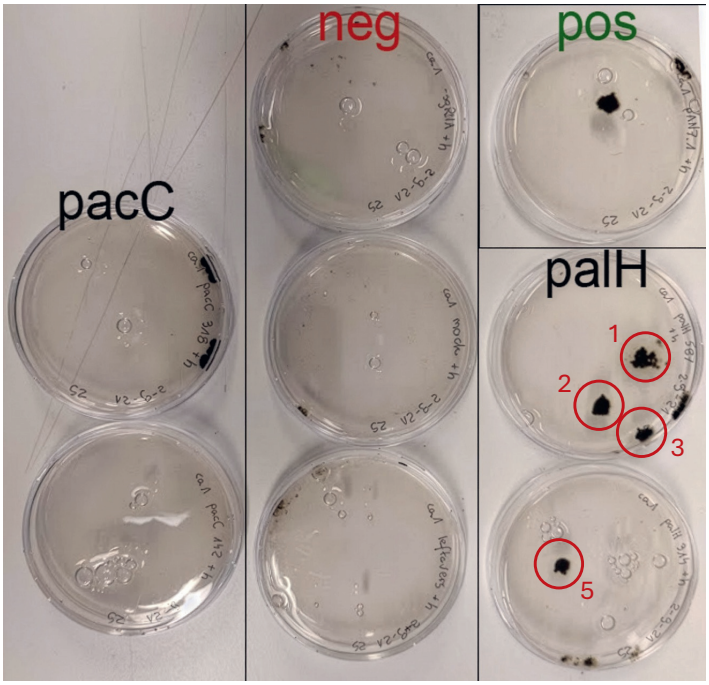
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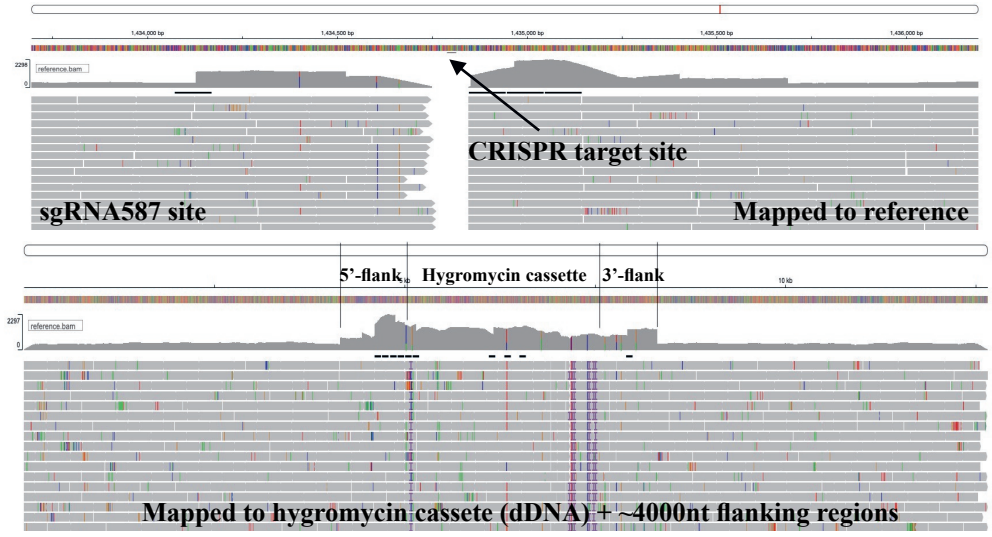
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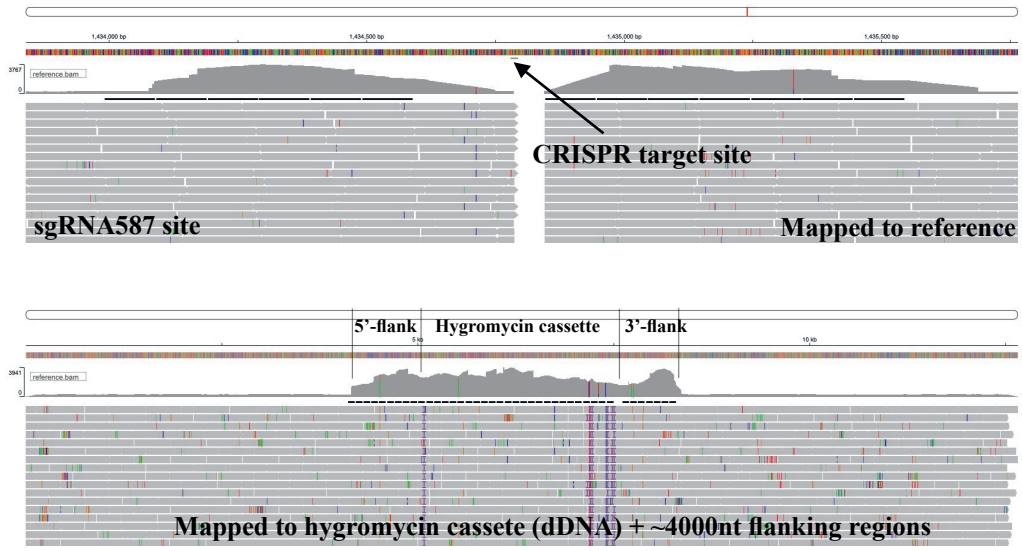
Supplementary figures



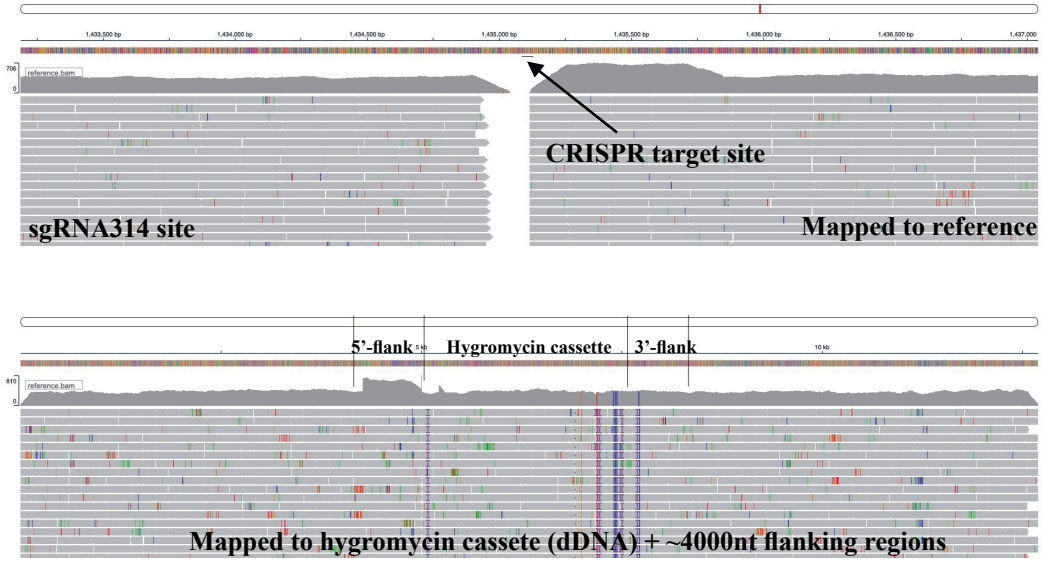
Suppl. Fig. S1. Photo of the generated mutants on selective media.



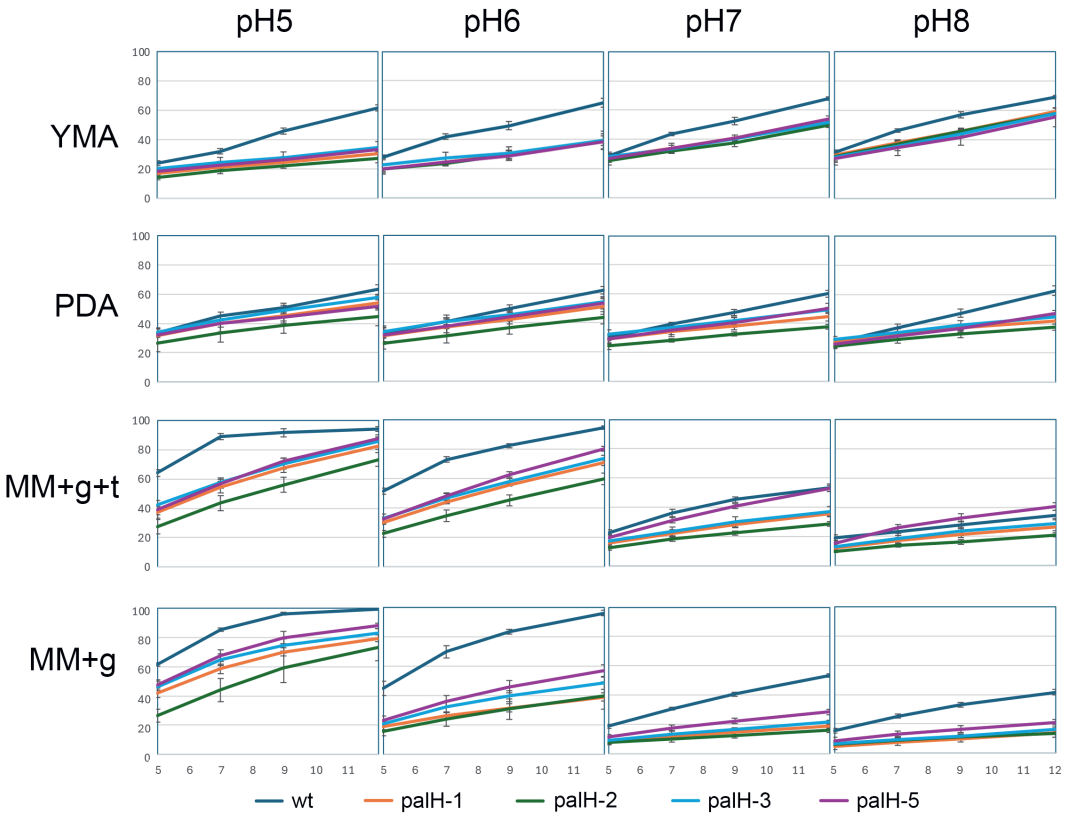
Suppl. Fig. S2. Results of mapping Δ*palH-1* reads to the reference genome (upper) and the *in silico* construct (lower).



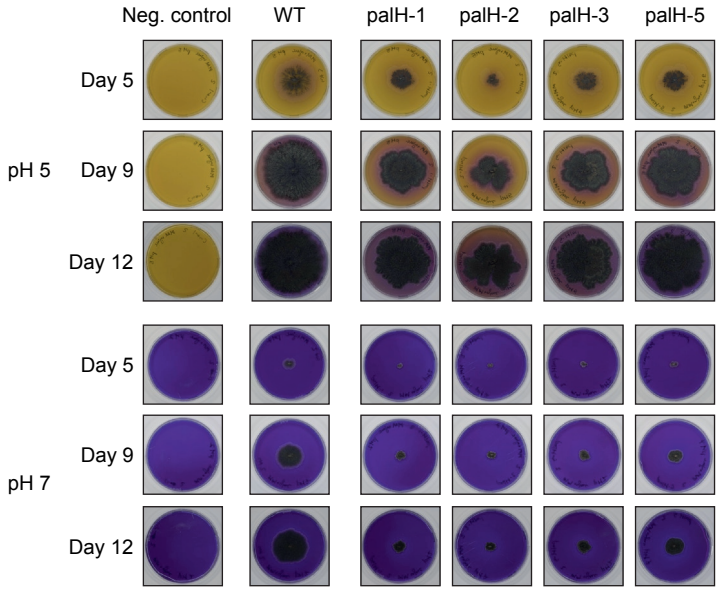
Suppl. Fig. S3. Results of mapping Δ*palH-2* reads to the reference genome (upper) and the *in silico* construct (lower).



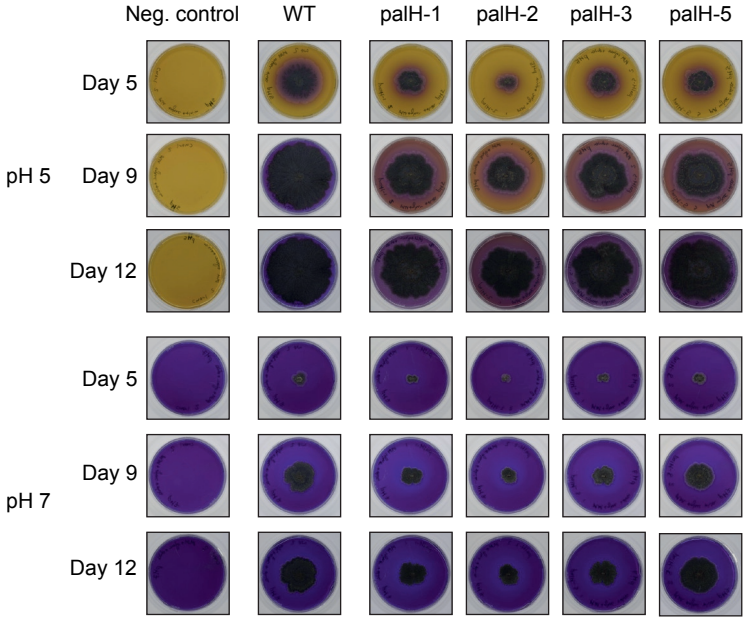
Suppl. Fig. S4. Results of mapping Δ*palH-5* reads to the reference genome (upper) and the *in silico* construct (lower).



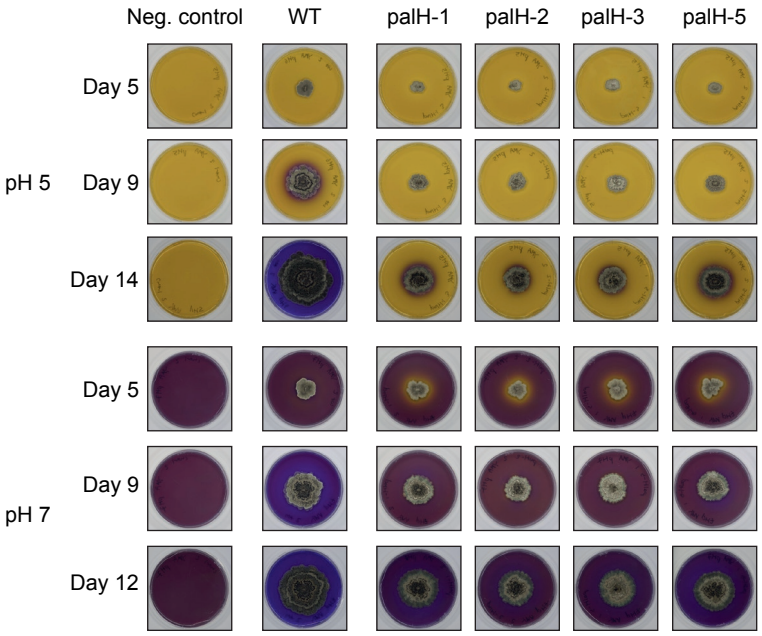
Suppl. Fig. S5. Average colony size (percentage of plate covered) of wild type (wt) and mutants. Error bars show standard deviation.



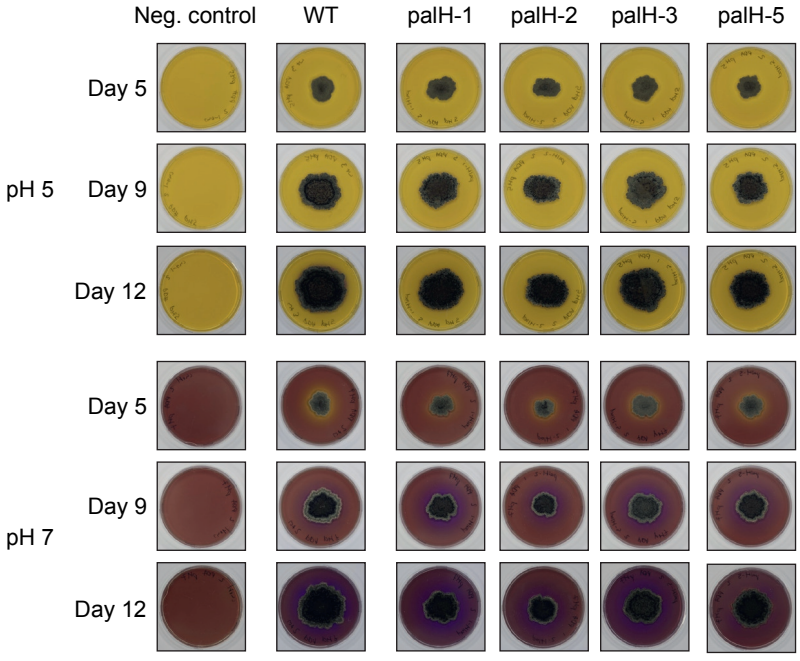
Suppl. Fig. S6. Summary of the growth test of the mutants on MM + glucose.



Suppl. Fig. S7. Summary of the growth test of the mutants on MM + glucose + thia.



Suppl. Fig. S8. Summary of the growth test of the mutants on YMA.



Suppl. Fig. S9. Summary of the growth test of the mutants on PDA.



Chapter 5

Phyllosticta paracitricarpa is synonymous with the EU quarantine fungus *P. citricarpa* based on phylogenomic analyses

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ABSTRACT

Phyllosticta citricarpa is an important *Citrus* pathogen and a quarantine organism in the European Union. Its recently described relative, *P. paracitricarpa*, is very closely related and not listed as a quarantine organism. *P. paracitricarpa* is very difficult to distinguish from *P. citricarpa*, since its morphological features overlap and the barcoding gene sequences that were originally used to delimit them as distinct species have a low number of species-specific polymorphisms that have subsequently been shown to overlap between the two clades. Therefore, we performed extensive genomic analyses to determine whether the genetic variation between *P. citricarpa* and *P. paracitricarpa* strains should be considered to represent infraspecific variation within *P. citricarpa*, or whether it is indicative of distinct species. Using a phylogenomic analysis with 3,000 single copy ortholog genes and whole-genome comparisons, we determined that the variation between *P. citricarpa* and *P. paracitricarpa* can be considered as infraspecies variation within *P. citricarpa*. We also determined the level of variation in mitochondrial assemblies of several *Phyllosticta* species and concluded there are only minimal differences between the assemblies of *P. citricarpa* and *P. paracitricarpa*. Thus, using several orthogonal approaches, we here demonstrate that variation within the nuclear and mitochondrial genomes of other *Phyllosticta* species is larger than variation between genomes obtained from *P. citricarpa* and *P. paracitricarpa* strains. Thus, *P. citricarpa* and *P. paracitricarpa* should be considered as conspecific.

1. INTRODUCTION

Citrus fruits are popular worldwide and have a multitude of uses and applications. *Citrus* species are however threatened by plant pathogens that may severely limit or even destroy harvests, resulting in great economic losses. One of the most serious pathogens of *Citrus* is *Phyllosticta citricarpa*, the fungus that causes Citrus Black Spot (CBS; Kotzé, 2000). CBS was originally described from Australia, where it causes an estimated ~ 80 M AUD in economic losses annually (Drenth, 2018). It has subsequently spread to nearly all citrus growing areas in the world (CABI, 2022), where it may cause severe crop losses. For instance, in Ghana, CBS

was reported to be responsible for up to 22% crop losses (Brentu et al., 2012). Typical symptoms include small and dark sunken lesions that may develop pycnidial conidiomata on fruit, and necrotic spots with a light centre and darker edge on leaves. Atypical symptoms such as virulent spot and lacy spot may also occur infrequently (Miles et al., 2019; Truter, 2010). *Phyllosticta citricarpa* can colonize and cause disease on a broad range of *Citrus* species such as *C. limon*, *C. sinensis*, and *C. reticulata* (EPPO, 2020; Miles et al., 2019). *Phyllosticta citricarpa* is a quarantine organism in the European Union, which means that if even one fruit contains CBS symptoms, an entire shipment containing up to thousands of fruits is rejected, potentially with severe economic consequences for the exporting company and country of origin.

Phyllosticta citricarpa is present worldwide, but is considered absent from Europe by EPPO (European Plant Protection Organisation) and CABI (Centre for Agriculture and Bioscience International, 2022; EPPO, 2020; Guarnaccia et al., 2017). However, Guarnaccia et al. (2017) collected material from 95 different sites throughout *Citrus*-growing areas in Europe, and found 20 isolates in three countries (Italy, Malta, and Portugal) representing two genetically distinct clonal populations, suggesting two separate introduction events of *P. citricarpa* into Europe, yet no CBS symptoms were observed. As stated by EFSA (The European Food Safety Authority, Jeger et al., 2018), later surveys conducted by the respective countries' NPPOs confirmed the absence of CBS symptoms. The suitability of the Mediterranean climate for development of CBS symptoms is a much-debated subject with no apparent conclusive answer (Baker et al., 2009; Er et al., 2013; Fourie et al., 2017; Martínez-Minaya et al., 2017, 2015; Paul et al., 2005). Nonetheless, a recent outbreak of CBS in Tunisia and further climatic modelling based on data from this outbreak of CBS in Tunisia seems to indicate that the Mediterranean climate may be suitable during specific conditions that may rarely present themselves (EPPO, 2019; Galvañ et al., 2022). Thus, the presence of *P. citricarpa* in Europe remains uncertain.

Phyllosticta is a large genus with more than 3,000 names listed, and species in this genus can occur as endophytes, saprophytes or plant pathogens (Index Fungorum, 2023; Wikee et al., 2013b). In 2002, the genus was revised by Van der Aa and Vanev, who accepted 141 different species colonizing a broad range of plant hosts (Index Fungorum, 2023; van der Aa & Vanev, 2002; Wikee et al., 2013b). *Phyllosticta* contains several species that can colonize

Citrus. This includes the pathogen *Phyllosticta citriasiana*, which is mainly found on *Citrus maxima* (Wulandari et al., 2009). *Phyllosticta citrichinaensis* has been described as a weak pathogen of several *Citrus* species, but since Koch's postulates have never been demonstrated, it is uncertain to what extent this species can cause disease (Wang et al., 2012). In an attempt to elucidate its lifestyle, we recently performed genomic analyses and concluded that its genome shares attributes with both endophytes and pathogens, suggesting that it has an intermediate lifestyle (Buijs et al. 2022). *Phyllosticta citribraziliensis* is an endophyte described from *Citrus limon* in Brazil (Glienke et al., 2011). Lastly, *Phyllosticta capitalensis* can be found on many plant hosts, and although it is found in *Citrus* as an endophyte, it may cause disease on other hosts such as orchids (Silva et al., 2008; Wikee et al., 2013a).

Phyllosticta paracitricarpa was recently described from *Citrus*, and is morphologically and genetically highly similar to *P. citricarpa* (EPPO, 2020; Guarnaccia et al., 2017). For example, conidiophores of *P. paracitricarpa* are longer and slightly narrower, and conidiogenous cells and conidia are larger than those of *P. citricarpa* (Guarnaccia et al. 2017); conidial sizes are (9–)11–13(−15) x 7–8(− 9) for *P. paracitricarpa* versus (10−)11–12(−14) x 7(− 8) for *P. citricarpa*. Thus, although the averages are higher, the range shows considerable overlap, which could represent infraspecific variation (Guarnaccia et al., 2017; van der Aa, 1973). In addition, *P. paracitricarpa* strains produce a yellow pigment on MEA, while *P. citricarpa* strains do not (Guarnaccia et al. 2017).

As *P. citricarpa* has a quarantine status, and *P. paracitricarpa* currently does not, the ability to indisputably distinguish the two species is of great importance, especially for the National Plant Protection Organisations that are tasked with performing phytosanitary border controls at the European Union borders. Molecularly, the two phylogenetic clades were originally distinguished based on partial DNA sequence data of the *tef1* gene, and to a much lesser extent the partial 28S nrRNA (LSU) gene (Guarnaccia et al., 2017). A method to distinguish *P. citricarpa* from *P. paracitricarpa* based on three SNPs and two indels in the *tef1* sequence was recently proposed (Zajc et al., 2023). However, Ioos et al. showed that some strains that displayed *tef1* sequence polymorphisms, which are considered to be typical for *P. paracitricarpa,* clustered with *P. citricarpa* in their phylogenetic analyses when more genes (51) were used (Ioos et al., 2023). In addition, *P. paracitricarpa tef1* sequences contained infraspecific

polymorphisms. Together, these data demonstrate that *tef1* is not a good genetic marker to distinguish these two clades. Furthermore, the authors assigned strains into separate clades using a phylogenetic analysis based on 51 single-copy genes, and performed a microsatellite analysis on a subset of the strains to test their method. Of the 51 genes, only 14 (27.5 %) were polymorphic and useful to distinguish between *P. citricarpa* and *P. paracitricarpa* strains, 28 genes (54.9 %) were identical between the two species, and nine genes (17.6 %) exhibited polymorphisms that were not specific to *P. citricarpa* or *P. paracitricarpa*. Based on this separation, a genomic region that allows for distinction of two clades has been identified and subsequently a new qPCR-based marker to distinguish *P. citricarpa* and *P. paracitricarpa* has been developed. The authors described the polymorphisms in this distinctive genomic region as two 7-bp insertions/deletions and two SNPs. However, upon closer inspection the variation in this region appears to consist of a single 35-bp inversion (from base 516,826 to and including 516,860 on scaffold_23). Although useful for distinction of the clades, this variation thus appears to be the result of a single mutation event, indicating a close relation of the two clades. These data do therefore not resolve whether these clades should be considered as one species or two.

Phyllosticta paracitricarpa was described from leaf litter in one *Citrus limon* orchard in Greece (Guarnaccia et al., 2017). However, Wang et al. (2012) reported that *P. citricarpa* strains isolated from spots on *Citrus sinensis* fruits grouped into two subclades, and sequence data from two of their strains from "subclade II" were included in the study by Guarnaccia et al. (2017). As these strains grouped with *P. paracitricarpa*, all strains in this subclade (a total of 16) could also be considered as *P. paracitricarpa*. However, as the identity of these strains is based on *tef1* sequence data, which has subsequently been shown to be unreliable (Ioos et al., 2023), these strains could also represent *P. citricarpa*. In addition, several *P. paracitricarpa* strains isolated from symptomatic fruit were recently reported from China by both Guarnaccia et al. (2017) and Wang et al. (2023). However, there are some inconsistencies as to the identity of the Chinese *P. citricarpa*/*P. paracitricarpa* strains from Wang et al., as some of the *P. paracitricarpa* strains grouped within the *P. citricarpa* clade based on their *tef1* sequence phylogeny (Suppl. Fig. S4 in Wang et al. 2023), suggesting that these are in fact not *P. paracitricarpa* strains, at least based on the *tef1* species definition. Lastly, Ioos et al. (2023) reported one new

P. paracitricarpa strain from China and one from Bangladesh, the latter of which could also be considered *P. citricarpa* based on the *tef1* sequence. In conclusion, a limited number of *P. paracitricarpa* strains have been reported and because nearly all of their identifications as *P. paracitricarpa* are based on *tef1* sequence data, it is uncertain whether these strains are actually belonging to *P. paracitricarpa* and not in fact to *P. citricarpa*.

With such closely related clades, one cannot help but question whether they should be regarded as separate species. The original species delimitation was largely based on differences in the *tef1* gene sequence, which has now been shown not to hold up as species-specific with larger sampling (Ioos et al., 2023). Generally, species delimitation for fungi is based mostly on either morphological features and/or DNA barcoding genes. As *P. citricarpa* and *P. paracitricarpa* are nearly identical in these aspects, another method is required. One approach to determine whether these strains are conspecific, is to study their genomes and those of closely related species. Ioos et al. (2023) showed that, based on their analyses using 51 concatenated genes (84,155-bp alignment), average percentage relatedness was extremely high at 99.26 % (35 species-specific polymorphic sites), suggesting the two clades might be lineages within one species rather than two separate species. However, further genomic analysis is necessary to provide more information on infra- and inter-species relatedness and to aid in putting these data in perspective, as has been done for species of *Alternaria* (Dettman & Eggertson, 2021). If one were to describe the genomic variation that is present within other well identified and accepted species within the genus *Phyllosticta*, it should be possible to determine whether the variation between *P. citricarpa* and *P. paracitricarpa* should be considered as infra- or interspecific variation. Until now, no analysis of infraspecific genomic variation of citrus-colonizing *Phyllosticta* species has been performed. In this study, we make use of 3,000 single copy ortholog genes to generate a phylogenomic tree. Next, we also generated whole-genome comparisons to determine genomic variation within *Phyllosticta* species. Lastly, we determined how much variation is present in the mitochondrial assemblies of these species. Thus, in this study, we performed an extensive analysis to determine the levels of variation within and between species of *Phyllosticta*, and we used these data to determine whether *P. paracitricarpa* should be considered as synonym of *P. citricarpa*.

2. MATERIALS AND METHODS

2.1 Genome sequencing

For this study, 16 genomes were sequenced and assembled, and seven previously published genomes available at the time (June 2022) and sequenced with similar technology, were also used. The total number of genomes per species used for the majority of analyses in this study is six for *P. capitalensis*, three for *P. citriasiana,* two for *P. citribraziliensis*, eight for *P. citricarpa*, two for *P. citrichinaensis*, and two for *P. paracitricarpa*. All information on the genomes can be accessed at MycoCosm: https://mycocosm.jgi.doe.gov/Phyllosticta. The JGI database identifiers (DBIDs), collection numbers, genome size and BUSCO scores can also be found in **Suppl. Table S1**. For the 12 gene-region analyses, two additional, previously published, *P. paracitricarpa* genomes were used. DNA isolation was performed as described previously (Buijs et al., 2022). In short, liquid cultures (250 mL Malt peptone broth) were grown for 10–14 days, after which DNA was isolated using the Qiagen Genomic-tip 100/G kit and the Qiagen Genomic DNA buffer set. Library preparation was performed in one of two ways: either 5 µg genomic DNA was sheared to > 10 kb using Covaris g-tubes, or to 15–20 kb using the Megaruptor 3 (Diagenode). The sheared DNA was treated with exonuclease to remove single-stranded ends and DNA damage repair mix followed by end repair and ligation of blunt adapters using SMRTbell Template Prep Kit 1.0 (Pacific Biosciences) or SMRTbell Express Template Prep Kit 2.0 (PacBio). The libraries were purified with AMPure PB Beads (PacBio). PacBio Sequencing primer was then annealed to the SMRTbell template library and sequencing polymerase was bound to them using the Sequel II Binding kit 1.0. The prepared SMRTbell template libraries were then sequenced on a Pacific Biosciences' Sequel II sequencer using 8 M v1 SMRT cells and Version 1.0 sequencing chemistry with 1x900 sequencing movie run times. Assembly was performed using either Falcon (Chin et al., 2016) or Flye (Kolmogorov et al., 2019). For more details on assembly, and on which approach was used for which genome specifically, see **Suppl. Table S2**. Annotations were performed using the JGI fungal annotation pipeline (Grigoriev et al., 2014). Quality assessment was performed using Quast v5.2 (Gurevich et al., 2013) to assess parameters such as GC content and number of contigs, and BUSCO v5.4.2 (Manni et al., 2021) was used to assess genome completeness

using the *Dothideomycetes* odb10 dataset and otherwise default parameters.

2.2 Phylogenetic analyses using ten barcoding genes

To assess if the newly sequenced genomes challenge the established phylogeny of *Phyllosticta* species, we generated a phylogenetic tree using the sequences of 12 full-length barcoding genes of which partial gene sequences are commonly used in molecular phylogenetic analyses of diverse groups of fungi (Crous et al., 2021). The complete gene sequences (nucleotide) of ten protein-coding barcode marker loci as well as two regions of the nuclear ribosomal RNA gene operon, namely the internal transcribed spacers and intervening 5.8S nrDNA (ITS) and the nuclear large subunit nrDNA (28S; LSU) were retrieved from the 23 *Phyllosticta* genomes above as well as two Chinese *P. paracitricarpa* strains that were published in 2023. The LSU of *P. citriasiana* CBS 120486 (Phycit1) was missing from the genome assemblies and could therefore not be included in the analyses. The genome of *Botryosphaeria dothidea* was used as outgroup (JGI identifier Botdo1_1). The protein-coding loci are: actin gene (*actA*), calmodulin gene (*CaM*), chitin synthase 1 gene (*chs1*), glyceraldehyde-3-phosphate dehydrogenase gene (*gapdh*), histone H3 gene (*his3*), DNA replication licensing factor (*mcm7*), DNA-directed RNA polymerase II largest subunit gene (*rpb1*), DNA-directed RNA polymerase II second largest subunit gene (*rpb2*), translation elongation factor 1-alpha gene (*tef1*), and beta-tubulin gene (*tub2*). The sequences were individually aligned using the online interface of MAFFT v7 (https://mafft.cbrc.jp/alignment/server/index.html; Katoh et al., 2019; Kuraku et al., 2013), after which they were concatenated using SequenceMatrix v1.9 (Vaidya et al., 2011). The concatenated alignment consisted of the 25 *Phyllosticta* isolates and the outgroup taxon and the partitions were as follows: *CaM*: 1–1,040; *actA*: 1,041–2,736; *chs1*: 2,737–6,053; *gapdh*: 6,054–7,529; *his3*: 7,530–8,160; *mcm7*: 8,161–10,850; *rpb1*: 10,851–16,439; *rpb2*: 16,440–20,329; *tef1*: 20,330–22,502; *tub2*: 22,503–24,275; ITS: 24,276–24,860; LSU: 24,861–28,215.

Maximum parsimony phylogenetic trees were generated for each partition using PAUP v4a168 (Swofford, 2003) with tree bisection and reconstruction (TBR) branch swapping as algorithm. Each parsimony analysis consisted of heuristic searches with 100 random taxon additions and were performed on parsimony-informative, unordered, and equally

weighted characters. Gaps were treated as new character states and a maximum of 1,000 equally most parsimonious trees were allowed. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Other statistical measures calculated and recorded were tree length, consistency index, retention index and rescaled consistency index (TL, CI, RI and RC, respectively). The robustness of the obtained trees was evaluated by 1,000 bootstrap replications using the same heuristic searches as in the original analyses. Resulting trees were viewed in Geneious Prime v2022 (https://www.geneious.com, Kearse et al., 2012) and prepared for layout using Adobe Illustrator vCC 2023. The resulting trees can be found in **Suppl. Fig. S1**, with the exception of *tef1* phylogeny which is presented in **Fig. 2**. Statistics for the maximum parsimony phylogenetic analyses are available in **Suppl. Table S3**. The alignment and phylogenetic trees were deposited in figshare (https://doi.org/10.6084/m9.figshare.25187918).

2.3 Phylogenetic analysis based on BUSCO genes

The phylogenetic relatedness of 23 isolates was determined based on 2,996 conserved single copy orthologs identified using BUSCO v5.3.2 using the *Dothideomycetes* odb10 database (Manni et al., 2021). Protein sequences were aligned using MAFFT v7.453 (Katoh et al., 2002) and the maximum-likelihood phylogeny was determined using IQ-TREE v2 (Minh et al., 2020b) with 1,000 bootstraps and the setting rcluster 5 and the TESTMERGE modelfinder. Because bootstrap supports reach a maximum relatively easily with large datasets, we sought to provide a more robust support and therefore calculated gene concordance factors (gCF) and site concordance factors (sCF); site concordance was calculated based on maximum likelihood (-scfl) for 100 quartets. The phylogenetic tree was visualized using iTol (Letunic & Bork, 2021). The SplitsTree network was created for the BUSCO gene alignment using SplitsTree v4.17.0 (Huson & Bryant, 2006).

2.4 Whole-genome analyses

To calculate the amount of shared material between the strains, all-vs-all whole-genome alignments were made using PROmer v3.0 (Kurtz et al., 2004). For each pairwise alignment, bedtools coverage was used to calculate the coverage per window of 500 bp. Isolates were clus-

tered based on pairwise identity using the hierarchical clustering and visualized using seaborn v0.11.2 (Waskom, 2021).

2.5 Mitochondrial data

Mitochondrial assemblies were generated and annotated using the JGI pipeline (**Suppl. Table S2**, Haridas et al., 2018). We were able to generate complete and high-quality mitochondrial assemblies for 13 of the strains. To generate a phylogenetic tree based on the amino acid sequence alignments of mitochondrial genes, 13 genes were selected based on their presence in all genomes and the quality of the annotation data, these are: *atp6, atp9, cob, cox1, cox2, nad1, nad2, nad3, nad4, nad4L, nad5, nad6*, and *rps3*. The concatenated alignment consisted of the 13 *Phyllosticta* isolates and the outgroup taxon and the partitions were as follow: *atp6*: 1–263; *atp9*: 264–326; *cob*: 327–620; *cox1*: 621–1,199; *cox2*: 1,200–1,484; *nad1*: 1,485–1,863; *nad2*: 1,864–2,542; *nad3*: 2,543–2,659; *nad4*: 2,660–3,164; *nad4L*: 3,165–3,257; *nad5*: 3,258–3,967; *nad6*: 3,968–4,189; *rps3*: 4,190–4,703. The maximum-likelihood phylogeny was determined using IQ-TREE v2.1.3, with 1,000 bootstrap replicates and the default MFP modelfinder (Kalyaanamoorthy et al., 2017; Minh et al., 2020b; Nguyen et al., 2015). The phylogenetic tree was visualized using Geneious Prime v2022 (Kearse et al., 2012, https://www.geneious.com) and prepared for layout using Adobe Illustrator vCC 2023.

2.6 Mitochondrial genome alignment

Clinker v0.0.28 (Gilchrist & Chooi, 2021) was used to align the mitochondrial protein (amino acid) sequences of *Phyllosticta* species, using those alignments as ankers to align the mitochondrial genomes, and show the best match and the identity between genes.

3. RESULTS

3.1 Pigment production is inconsistent across *P. paracitricarpa* strains and 16 newly sequenced genome assemblies are of good quality for comparative genomics.

One of the characteristics proposed to distinguish *P. citricarpa* and *P. paracitricarpa* is the production of a yellow pigment on MEA by *P. paracitricarpa* strains (Guarnaccia et al., 2017).

However, when we grew *P. citricarpa* and *P. paracitricarpa* for two weeks on MEA, some *P. paracitricarpa* strains did not produce a yellow pigment (**Suppl. Fig. S2**), which is not in line with earlier reports. These results demonstrate that this morphological feature is not a reliable basis to distinguish the two clades. To determine the relationship between *Citrus*-colonizing

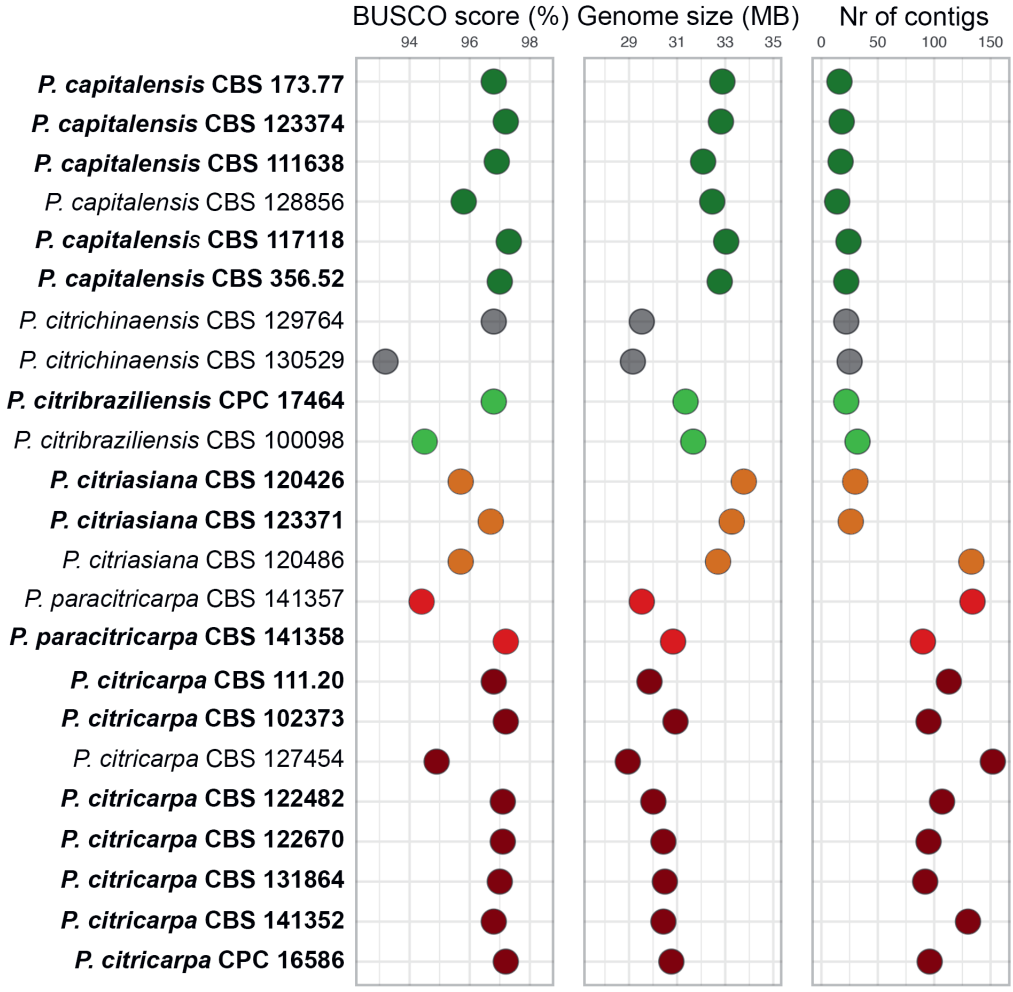


Fig. 1. Genome assembly quality statistics of 23 *Phyllosticta* genomes. The genome assemblies of 23 *Phyllosticta* strains were compared. The genomes of 16 strains that were newly sequenced and assembled are indicated in bold, and these data were supplemented with seven publicly available genome assemblies (see **Suppl. Table S1** for an overview). For each sequenced *Phyllosticta* strain, the BUSCO score, genome size, and number of contigs are shown. Strains in green are considered to be endophytic, strains in red and orange are pathogenic, and strains in grey are ambiguous or intermediate (Buijs et al., 2022; Glienke et al., 2011; Wang et al., 2012; Wikee et al., 2013).

Phyllosticta species, and to be able to assess infraspecific variation, eight new genomes of *P. citricarpa* and *P. paracitricarpa* were sequenced, assembled, and compared to those of four related *Phyllosticta* species (*P. capitalensis*, *P. citriasiana*, *P. citribraziliensis*, and *P. citrichinaensis*), for which we also generated eight additional genome assemblies. The assembled genomes varied in size from 29 to 34 MB, and genome completeness assayed with BUSCO genes yielded BUSCO scores between 93 and 98 %, indicating adequate quality for comparative genomics (**Fig. 1**). The number of contigs varied between 14 and 152, with the genomes of *P. citricarpa* and *P. paracitricarpa* generally being much more fragmented compared to the other species' assemblies. With the exception of *P. citriasiana* culture CBS 120486, which was sequenced using short-read sequencing technology (Illumina), all strains were sequenced using the same PacBio long-read sequencing platform at the Joint Genome Institute (JGI).

3.2 Phylogenetic and phylogenomic analysis show that interspecific variation between *P. paracitricarpa* and *P. citricarpa* is less than infraspecific variation in other *Phyllosticta* species

To assess to which extent the addition of the newly sequenced genomes challenges the established phylogeny of *Phyllosticta* species, we initially constructed a maximum parsimony phylogenetic tree based on 12 full-length gene regions. Of the genes used, *tef1* is most relevant, as it was previously used to distinguish *P. citricarpa* from *P. paracitricarpa* (**Fig. 2A**, left tree; Guarnaccia et al., 2017; Zajc et al., 2023). The other genes included were actin, *CaM*, *chs1*, *gapdh*, *his3*, ITS, LSU, *mcm7*, *rpb1*, *rpb2*, and *tub2*, which are all commonly used in phylogenetic analyses (**Suppl. Fig. S1**; Crous et al., 2021). To test whether including *P. paracitricarpa* strains from another geographic origin would affect the phylogeny, we also included two Chinese *P. paracitricarpa* strains that were made available online in 2023 (**Suppl. Table S1**, Guarnaccia et al. 2017, Ioos et al. 2023). Both the *tef1* as well as the 12-gene phylogenetic tree clearly separate at least five species: *P. capitalensis*, with a rather high level of infraspecific variation, *P. citrichinaensis*, *P. citribraziliensis*, and *P. citriasiana* with a lower level of infraspecific variation, and the *P. citricarpa*/*P. paracitricarpa* clade (**Fig. 2A**). The four *P. paracitricarpa* strains form a stable clade within the larger *P. citricarpa* clade, which is consistent with earlier phylogenetic studies (Guarnaccia et al., 2019, 2017). The level of variation between

the *P. citricarpa* and *P. paracitricarpa* clades is similar to the variation within the *P. citriasiana* clade, and even smaller than the variation within the *P. capitalensis* clade. To quantify the amount of variation within a species, we determined the number of sequence changes from

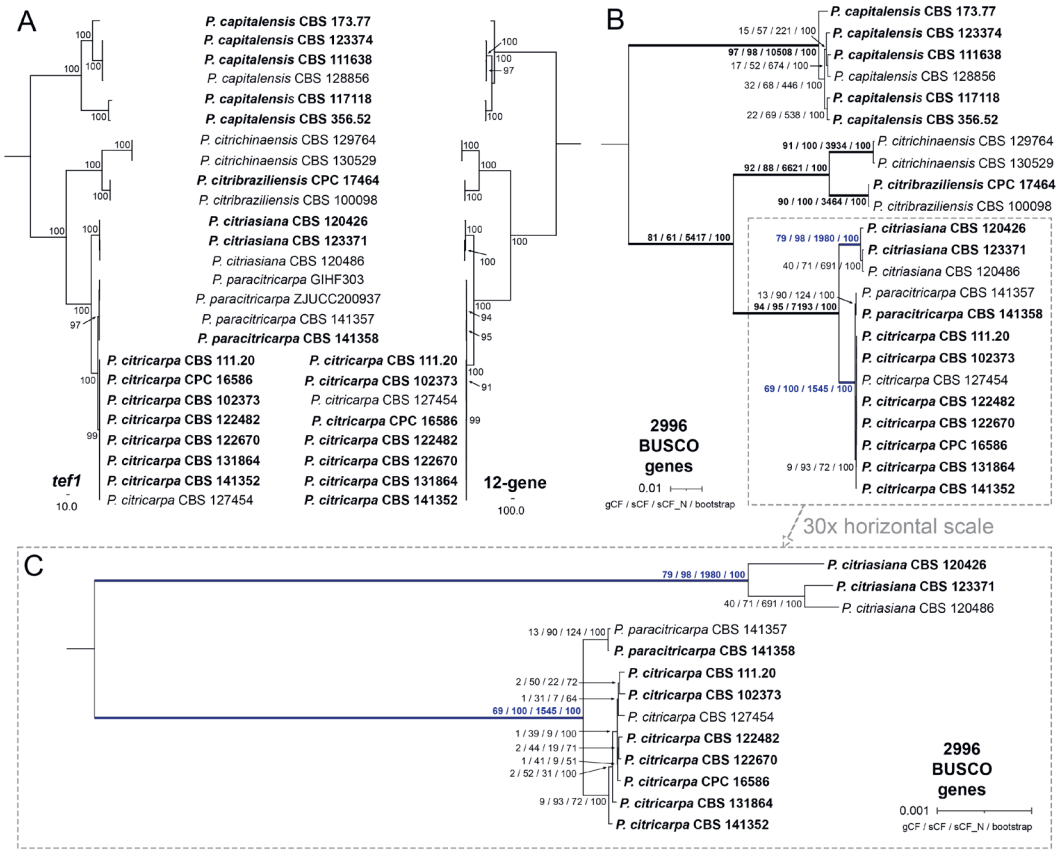


Fig. 2. Phylogenetic and phylogenomic analyses demonstrate that *Phyllosticta* infraspecific variation is larger than the interspecific variation between *P. citricarpa* and *P. paracitricarpa*. Newly sequenced genomes are shown in bold. **A.** Phylogenetic trees based on full-length barcoding genes. The left-hand tree is based on *tef1* only, the right-hand tree is based on a concatenated alignment of 12 barcoding gene regions: *tef1, actin, CaM, chs1, gapdh, his3,* ITS, LSU, *mcm7, rpb1, rpb2,* and *tub2.* For details on the analysis see **Suppl. Table S3**. The most parsimonious trees were generated using PAUP (Swofford, 2003). Only bootstraps above 80% are shown. The two trees are nearly identical with the exception of branch supports and some strains swapping positions within the *P. citricarpa* clade. **B.** Phylogenomic tree based on 2,996 BUSCO genes (1,905,363 aligned amino acids) from the class *Dothideomycetes*. Support values of clades are: gene concordance factor (gCF) / site concordance factor (sCF) / number of informative sites for sCF (sCF_N) / bootstrap. Branches with a gCF above 80% are shown in bold, branches with a gCF 60 – 80% are shown in bold and dark blue. **C.** Part of the tree from (B) but at 30x horizontal scale to allow visualization of leaves within the *P. (para)citricarp*a clade.

the most recent common ancestor (MRCA) to each strain within that species. For instance, the highest number of changes in a strain of *P. citriasiana* compared to the MRCA of *P. citriasiana* is 28 (**Suppl. Fig S3**). Within *P. capitalensis*, this number is a ten-fold higher reaching up to 261 changes. The highest number of changes of any *P. citricarpa* strain as compared the MRCA of both *P. citricarpa* and *P. paracitricarpa* is 12, and for *P. paracitricarpa* it is 13. The variation between *P. citricarpa* and *P. paracitricarpa* is thus lower than the infraspecies variation within *P. citriasiana* or *P. capitalensis*.

To include more data in the phylogenetic analysis, and thereby provide a more robust basis, we made use of nearly 3,000 conserved BUSCO genes to construct a phylogenomic tree (**Fig. 2B**). In addition to bootstrap support values which reach the maximum of 100% relatively easily with large datasets, we also generated gene concordance factors (gCF) and site concordance factors (sCF, Minh et al., 2020a). The gCFs indicate the percentage of BUSCO genes that are in concordance with the position of each clade in the tree. The clades that contain the species *P. capitalensis*, *P. citrichinaensis* and *P. citribraziliensis* have gCFs of 97, 91 and 90 %, respectively, while the clade that contains the three supposed species *P. citricarpa*, *P. paracitricarpa* as well as *P. citriasiana* has a similar (high) gCF of 94% (**Fig. 2B**). The clades of *P. citriasiana*, and *P. citricarpa*/*P. paracitricarpa* have lower gCFs of 79 and 69 %, respectively, which means there is lower support to separate these clades, suggesting that these species might be more closely related than the others (**Fig. 2B, C**). The clades of *P. citricarpa* and *P. paracitricarpa* separately have extremely low gCFs of 9 and 13% respectively, which means that the gCFs do not support the separation of these strains into distinct clades, while gCFs do support the separation of all other species. The low gCFs of the *P. citricarpa* and *P. paracitricarpa* clades can be explained by a high similarity: sCFs are relatively high (93 and 90%), but the sCF_Ns (number of informative sites) are only 124 and 72 (**Fig. 2B, C**). This is very low compared to the number of informative sites for other species clades such as 1,980 for *P. citriasiana*, 3,464 for *P. citribraziliensis*, and even 10,508 for *P. capitalensis*. In addition, the sCF_Ns within other species clades are higher, such as 691 within *P. citriasiana*, and up to 674 within *P. capitalensis*.

In addition, we assessed the presence of phylogenetic inconsistencies by performing a SplitsTree analysis (**Suppl. Fig. S4**, Huson & Bryant, 2006), which generates a split net-

work to visualize inconsistencies in phylogenetic data caused by for instance hybridization or recombination events. This tree again confirmed the 12-gene tree topology; no inconsistencies are present and five clearly separated species can be observed (**Suppl. Fig. S4**), with *P. citricarpa* and *P. paracitricarpa* showing a level of variation that is comparable to infraspecies variation such as in *P. capitalensis* and *P. citriasiana.*

3.3 Genome-wide sharedness indicates variation within *Phyllosticta citricarpa* is larger than variation between *P. citricarpa* and *P. paracitricarpa.*

To include variation throughout the entire genome, including noncoding regions, in our analysis, we established the percentage of shared genetic material ('sharedness') between the different strains using all-vs-all whole-genome alignments. To do so, for each pairwise alignment the percentage of shared windows (500 bp) was calculated.

Whole-genome sequences within *Phyllosticta* species generally share between 81 and 95% genetic material, with the least infraspecific shared genetic material between two strains observed within *P. capitalensis* (81%) and *P. citriasiana* (83%). In contrast, the highest sharedness between two strains was observed within *P. citricarpa* (95%), *P. citribraziliensis* (94%) and, interestingly, between *P. citricarpa* and *P. paracitricarpa* (94%, **Fig. 3A, Suppl. Table S4**). Noticeably, whole-genome sharedness within *P. citricarpa* lies between 87 and 95%, while sharedness between *P. citricarpa* and *P. paracitricarpa* lies between 88 and 94%, indicating that the percentage of material shared between *P. paracitricarpa* and *P. citricarpa* lies entirely within the range of material shared within *P. citricarpa.* Moreover, the shared material of 94% between *P. citricarpa* and *P. paracitricarpa* is higher than the highest observed shared genetic material within *P. paracitricarpa* (92%), suggesting that one of the *P. paracitricarpa* strains is more similar to a *P. citricarpa* strain than to another *P. paracitricarpa* strain. In addition, hierarchical clustering based on the genome-wide shared genetic material clearly separated all species except for *P. citricarpa* and *P. paracitricarpa* strains that were intermixed within a single clade (**Fig. 3C**). To exclude the possibility that small, fragmented scaffolds and/or contigs that are only present in some genomes are distorting our analyses, we performed the same analysis but with the small scaffolds (<1,000 bp) removed. We did not observe any significant differences to the previous results (**Suppl. Fig. S5**).

We also performed a similar genome-wide analysis, but excluded unique windows, thereby only preserving informative windows in our analysis. On average, the percentage of genome-wide shared genetic material was much higher when compared to the previous analyses, with the least amount of infraspecies shared genetic material again observed within *P. capitalensis* (96.8%) and *P. citriasiana* (98.3%), and the highest sharedness within *P. citribraziliensis* (99.9%, **Fig. 3B, Suppl. Table S5**). The percentage of shared genetic material within *P. citricarpa* and *P. paracitricarpa* was between 99.3 and 99.8%, and 99.7 and 99.8%, respectively, while the percentage of shared genetic material between *P. citricarpa* and *P. paracitricarpa* ranged from 99.1 to 99.5%. In other words, the highest sharedness between a *P. citricar-*

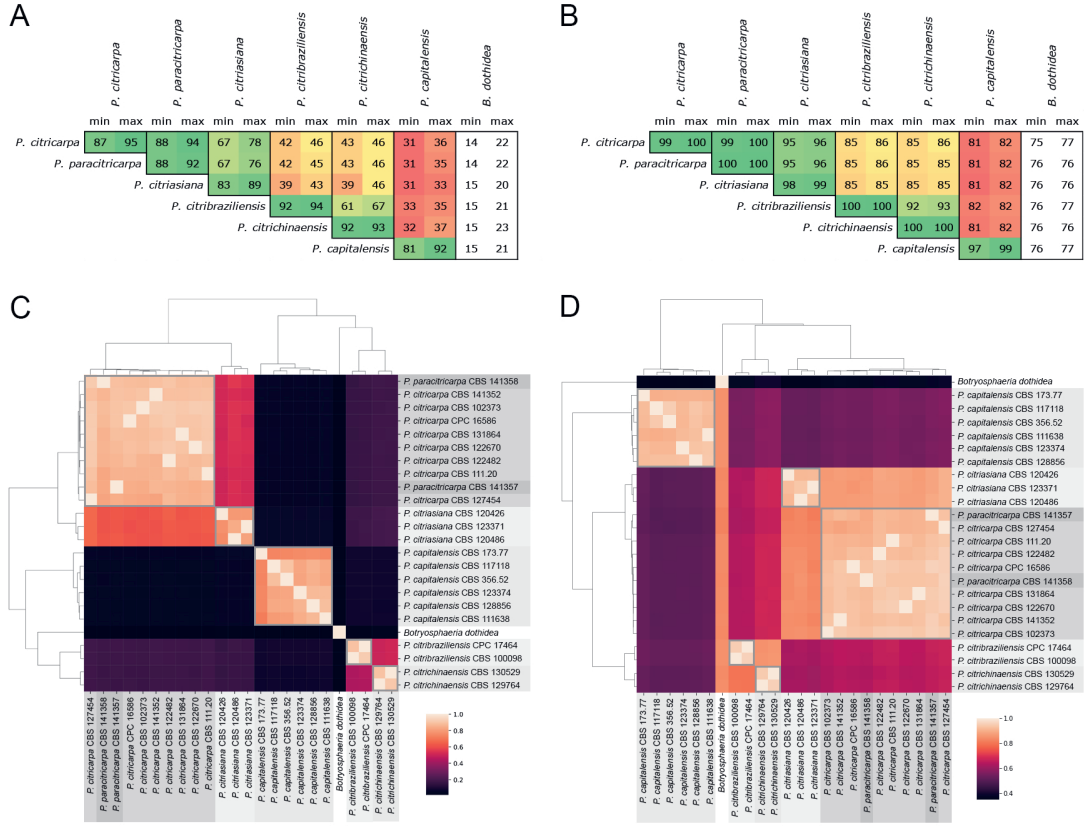


Fig. 3. Genome-wide sharedness indicates more variation within *Phyllosticta* species than between *P. citricarpa* and *P. paracitricarpa*. Minimum and maximum percentages of genetic material shared (i.e. 'sharedness') between different *Phyllosticta* species based on: **A.** whole genome sequences, and **B.** only the informative windows excluding unique windows. Heatmaps and hierarchical clustering based on sharedness of **C.** whole-genome sequences, and **D.** only the informative parts of the genomes.

pa and a *P. paracitricarpa* strain is 99.5%, while the lowest sharedness within *P. citricarpa* is 99.3%, and thus there are strains within the *P. citricarpa* clade that are more divergent from *P. citricarpa* than some *P. paracitricarpa* strains. In addition, using hierarchical clustering based on the sharedness, all strains are assigned to distinct clusters based on their species, while *P. citricarpa* and *P. paracitricarpa* were intermixed in a single clade (**Fig. 3D**), again demonstrating that *P. citricarpa* and *P. paracitricarpa* should be considered as one species.

3.4 All *Phyllosticta* species show large variations in their mitochondrial assemblies, but differences between *P. citricarpa* and *P. paracitricarpa* are minimal

Our analyses did not yet consider mitochondrial (mt)DNA, which is also used as a molecular marker in phylogenetic analyses since it is structurally well conserved (several genes will be found in practically any species), yet may have high mutation rates, meaning variation between species may be observed in mtDNA even if genomic DNA is highly similar (Kouvelis et al., 2008; Rubinoff & Holland, 2005; Sarma et al., 2017). We were able to generate high-quality single-contig mitochondrial genome assemblies using the JGI pipeline for several strains; strains for which we were not able to do so were excluded from the subsequent analyses.

Strikingly, we observed considerable differences in mitochondrial assembly size between different *Phyllosticta* species, with the largest assembly of 213 kb for *P. citriasiana* CBS 123371 being nearly twice as large as the smallest assembly of 118 kb for *P. capitalensis* CBS 173.77 (**Fig. 4A, B**). Difference in assembly length seems to correlate with lifestyles of the different species (**Fig. 1**), as species that are considered endophytic (*P. capitalensis*, *P. citribraziliensis*) have an average assembly length of 125.8 kb, whereas species that are considered pathogenic (*P. citriasiana*, *P.* (*para*)*citricarpa*) have an average assembly length of 209 kb (**Fig. 4A**). Notably, the difference in length is mainly caused by differences in gene length, as the total gene length of some species is also almost twice as long as those of other species (**Fig. 4A**). However, the total coding-sequence length across all species is very similar. It thus appears that this difference in length is almost entirely the result of differences in intron number and length (**Fig. 4A**), which can be clearly observed in *cob*, *nad1*, *nad5*, *cox3*, and especially for *cox1* (**Fig. 4C**). The difference in intron length may be explained by the presence of LAGLIDADG homing endonuclease genes (HEGs). Whereas endophytes contain on average 3.4

HEGs in the mitochondrial genome, pathogenic species contain on average 10.9 HEGs.

The level of conservation of mitochondrial sequences determines how well these sequences can be used to distinguish species. While most exons show a high level of conser-

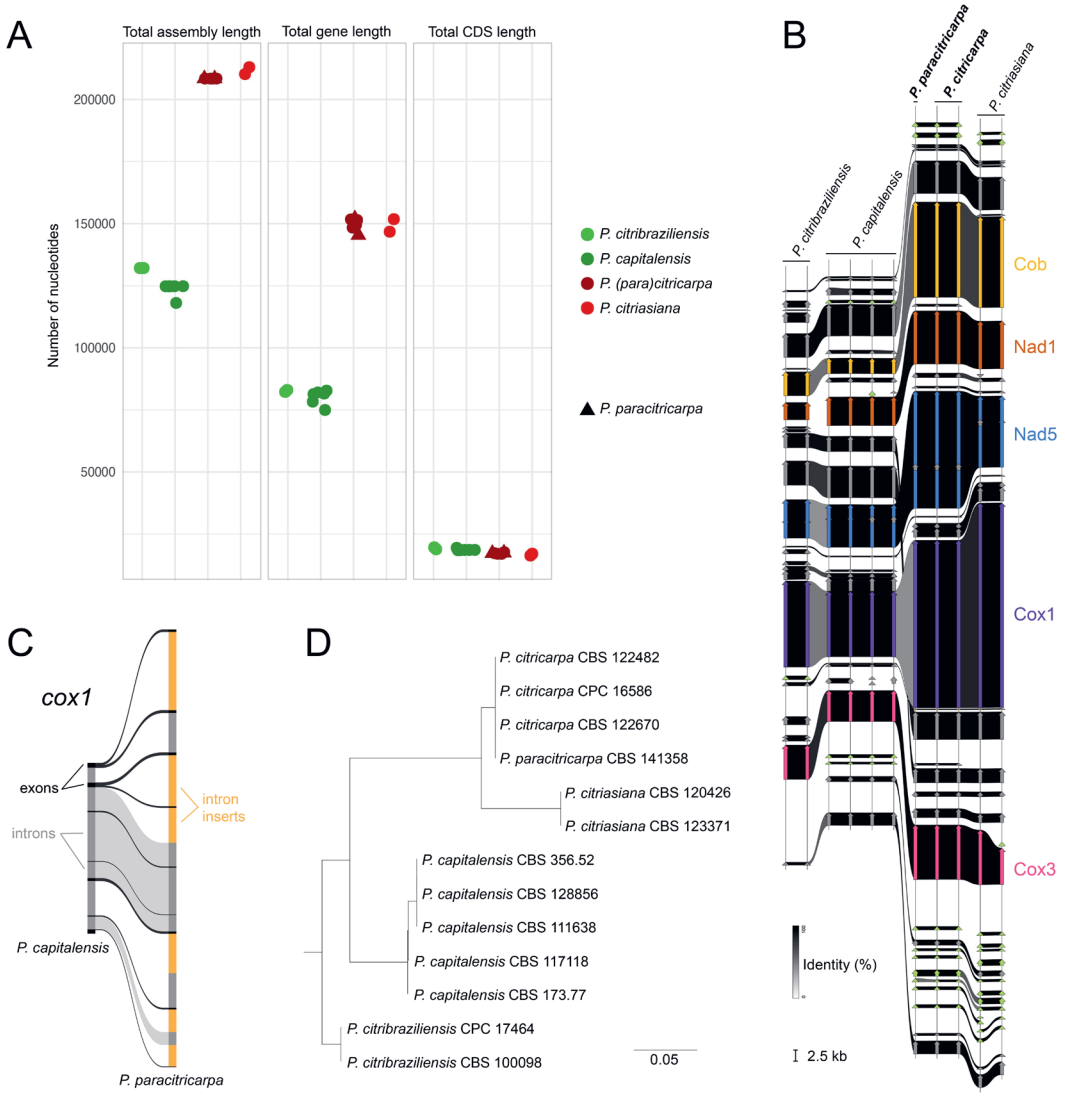


Fig. 4. *P. citricarpa* and *P. paracitricarpa* cannot easily be distinguished based on mitochondrial assemblies, while other *Phyllosticta* species can. A. Graph showing mitochondrial assembly statistics such as assembly length, gene length and coding sequence length. **B.** Clinker (Gilchrist & Chooi, 2021) alignment between mitochondrial assemblies of different *Phyllosticta* species. Connections indicate the best match between genes based on protein sequence identity (white 0% to black 100%). **C.** Schematic representation of an alignment between the *cox1* genes of *P. capitalensis* (left) and *P. paracitricarpa* (right). **D.** Phylogenetic tree obtained from the amino acid alignment of 13 mitochondrial genes.

vation between different species, the introns may thus contain large insertions, sometimes up to several thousands of nucleotides long. Although these insertions vary between species, they seem to be conserved within species. In fact, mitochondrial assemblies within a species only show small variations such as SNPs or gaps up to a few nucleotides (mostly within *P. capitalensis*), and most species can easily be distinguished based on their mitochondrial intron content (**Fig. 4B**). However, unlike other *Phyllosticta* species, *P. citricarpa* and *P. paracitricarpa* were not easily distinguishable based on their mitochondrial assemblies as the sequences showed only minimal differences, and their intron structures were identical (**Fig. 4B**). We used the predicted mitochondrial annotations to generate a phylogenetic tree based on the amino acid alignment of 13 mitochondrial core genes (**Fig. 4D**). All species clearly clustered separately, with some variation present within *P. capitalensis*, but the *P. citricarpa* and *P. paracitricarpa* strains all clustered within one clade having completely identical amino acid sequences. Thus, the mitochondrial genome data corroborates the nuclear genome data, and suggest that *P. citricarpa* and *P. paracitricarpa* should be considered as one species.

3.5 Taxonomy

Phyllosticta citricarpa (McAlpine) Aa, Stud. Mycol. 5: 40. 1973.

Basionym: Phoma citricarpa McAlpine, Fungus Diseases of *Citrus* trees in Australia: 21. 1899.

New synonym: Phyllosticta paracitricarpa Guarn. & Crous, Stud. Mycol. 87: 177. 2017.

Additional synonyms: See MycoBank (https://www.mycobank.org/).

Note: Phyllosticta paracitricarpa is reduced to synonymy with *P. citricarpa* based on the data presented here.

4. DISCUSSION AND CONCLUSION

To be able to effectively manage plant diseases, it is of vital importance to properly distinguish dangerous plant pathogens from less threatening ones. This is particularly important for quarantine organisms, since fast and correct identification may prevent further spread. In the case of the quarantine fungus *P. citricarpa* and its close non-quarantine relative *P. paracitricarpa*, discrimination between the two clades is challenging, albeit not impossible (Ioos et al., 2023).

Although these two clades were described as distinct species based on some morphological and a few nucleotide differences (Guarnaccia et al., 2017), their high degree of similarity raised the question as to whether these are indeed two distinct species. We therefore sought to determine how much variation is present in the genomes of the *Phyllosticta* species that colonize *Citrus*, and whether the interspecific variation between *P. citricarpa* and *P. paracitricarpa* falls within the limits of such infraspecific variation or not. Using several different genomic methods, such as phylogenomics using nearly 3,000 conserved single-copy ortholog genes, whole-genome comparisons, and a study of the mitochondrial assemblies, we determine that infraspecific variation within *Phyllosticta* species is larger than the variation between *P. citricarpa* and *P. paracitricarpa*. Based on these data, we therefore conclude that *P. citricarpa* and *P. paracitricarpa* should be considered as one species.

A phylogenetic analysis based on nearly 3,000 genes gave results highly similar to an analysis using only 12 gene regions, which demonstrates the sufficiency of these genes for phylogenetic and taxonomic purposes. The desired parts of the genome for such analyses are those that are somewhat conserved so that they can be found in different strains or species, but which are also variable enough to be able to distinguish species or strains from each other. Many conserved genes, including the commonly used barcoding genes, fit these qualifications. These are often belonging to the so-called housekeeping genes, i.e. genes involved in essential functions such as cell division or cell wall structure and thus by definition required for normal functioning of the cell. In cases where established barcoding genes may not work because of, for instance, conflicting signal or too little signal, supplementing with additional conserved genes may be necessary and useful (Balasundaram et al., 2015; Lücking et al., 2020; Pino-Bodas et al., 2013). However, our data shows that, when barcoding genes provide sufficient resolution and there are no conflicts, adding more genes may not necessarily be more informative, except for providing a stronger statistical branch support signal. Specifically, the use of gene concordance and site concordance factors (gCF/sCF) when using larger datasets provides a deeper insight into the potential presence of discordant signals that may be overseen when using a smaller set of genes. In conclusion, it is important to consider at least several (barcoding) genes and the resolution they provide before reaching conclusions on species delimitations or phylogenies.

Our data also showed the potential of mitochondrial assemblies for taxonomic purposes. Although *P. citricarpa* and *P. paracitricarpa* only showed minimal variations, other *Phyllosticta* species showed very large differences and could easily be distinguished. Rather than differences in gene sequence, differences in assembly length and gene structure, mainly intron presence and length, provided a valuable basis on which to distinguish species. Indeed, mitochondrial sequences are regularly used in taxonomic studies, most often as barcoding genes (Kouvelis et al., 2008; Kulik et al., 2020; Sarma et al., 2017). In the present study, we chose to perform the phylogenetic analyses on amino acid alignments as the high variability between species and absence/presence of introns made it impossible to obtain robust alignments for an analysis based on a nucleotide alignment of the mitochondrial genes. In addition, other studies have shown that fungal mitochondrial genomes can be highly dynamic in gene order and, as was shown here for *Phyllosticta*, may contain many and/or large introns (Paquin et al., 1997; Sandor et al., 2018). Our observation that HEGs contribute to mitochondrial genome size by increasing intron size is not unusual and has been described previously in other fungi (Fonseca et al., 2021; Megarioti & Kouvelis, 2020). However, whether the presence of extra HEGs in pathogenic *Phyllosticta* may have contributed to the emergence of a pathogenic lifestyle is unknown, and could be a subject for further study. Moreover, a detailed exploration of *Phyllosticta* mitochondrial assemblies could provide interesting insight into the development and evolution of the genus.

The presence of *P. citricarpa* in Europe remains a debated subject. Although Guarnaccia et al. (2017) collected multiple isolates from several *Citrus*-growing areas in Europe, limited information is provided in respect to their methodology; however they do state that CBS symptoms were not observed. Later surveys conducted by the NPPOs of Italy, Greece, Malta and Portugal confirmed absence of CBS symptoms and the absence of *P. citricarpa* in leaf litter and asymptomatic fruit. Unfortunately, no detailed reports were published of these surveys, and thus it is difficult to conclude on how their methodologies might have differed from those of Guarnaccia et al. (2017). Remarkably, the guideline for surveys of *Phyllosticta citricarpa* as published by EFSA (Lázaro et al., 2020) does not include the sampling of leaf litter, even though all the European *P. citricarpa* strains reported by Guarnaccia et al. (2017) were isolated from leaf litter. This may be because the purpose of these surveys is to determine the presence

of the disease Citrus Black Spot, which is a markedly different objective than determining the presence of the causal agent *P. citricarpa*, which can indeed be present asymptomatically in fruit, but may also survive and reproduce in leaf litter (Guarnaccia et al., 2019). Importantly, the presence of *P. citricarpa* may only lead to development of Citrus Black Spot symptoms under certain climatic conditions. The publication of the methodologies and results of surveys that include sampling of leaf litter could provide much clarity on the presence of *P. citricarpa* in Europe.

Although differences can be observed between *P. citricarpa* and *P. paracitricarpa*, these differences are smaller than the average *Phyllosticta* infraspecific variation, and thus these two species could be considered as one species. However, this only becomes apparent when considering multiple species of the genus, and multiple strains per species in an analysis, and thus it is very important to always place species in the context of a broader phylogenetic lineage by including more distant relatives in a genus or family. In our analysis, *P. capitalensis* showed the most infraspecific variation. In fact, based on our data, one could argue that *P. capitalensis* should not be considered as a single, but in fact several species. In other words: if we were to consider the degree of infraspecies variation of *P. capitalensis* as a 'threshold' for all *Phyllosticta* species, *P. citricarpa*, *P. paracitricarpa*, and *P. citriasiana* should be considered as a single species as well. However, *P. citriasiana* appears to have a particularly distinct host range and causes slightly different symptoms as compared to *P. citricarpa*, meaning there are notable biological distinctions between these two species. Indeed, including biological data in taxonomic studies is not uncommon (Chethana et al., 2021; Lücking et al., 2020). In addition, the interspecific variation between *P. citricarpa* and *P. citriasiana* is much larger than the infraspecific variation of both species, e.g. there is a barcoding gap present (Meyer & Paulay, 2005). It therefore appears unwise at present to consider them as a single species. If we were to apply a single 'threshold' of infraspecific variation that is allowed to exist in all species within *Phyllosticta*, and *P. citriasiana* should be considered a separate species, by the same threshold, *P. capitalensis* can then not be considered as one species. Several previous authors have in fact reported cryptic species in *P. capitalensis* in the past (see review by Norphanphoun et al., 2020), but even these are often difficult to distinguish using the standard multilocus phylogenies. However, a significant difference between *P. capitalensis* and the other *Phyllosticta* spe-

cies is that *P. capitalensis* has a very broad host range of which it is frequently an ubiquitous, cosmopolitan endophyte and the larger variation within this species may therefore represent different host specializations within one species. Indeed, the strains used in this study span different continents and hosts, and the high variability within *P. capitalensis* may therefore indicate its broad range in geographic origin and host (**Suppl. Table S1**). Current data are insufficient to resolve whether *P. capitalensis* should be considered as one, or perhaps multiple species. Future studies involving phylogenomic analyses of accepted species in the *P. capitalensis* species complex are needed to identify better barcoding genes with a higher resolving power to identify which species are truly distinct and which might be synonyms.

It is important to realize that the question we pose here is not whether we are ultimately able to distinguish *P. citricarpa* and *P. paracitricarpa*, because if one really wants to, it should be possible to distinguish even the closest genetic relatives based on small species-specific genetic differences such as SNPs, as demonstrated by Ioos and colleagues (Ioos et al., 2023). A very good application of such lineage-specific differences is the work done on the different lineages of the oomycete pathogen *Phytophthora ramorum*, for which is it useful to distinguish lineages originating from different parts of the world (Gagnon et al., 2014; Søndreli et al., 2023). However, with *P.* (*para*)*citricarpa* the question should first be whether it is meaningful to distinguish strains from each other at the species level. Species concepts for fungi are complicated, and over 30 different concepts have been proposed (Lücking et al., 2020). However, essentially there are three different types of properties of a species that can be considered to delimit it from another species; biological, morphological, and molecular differences.

Biological differences may for instance be the ability to mate or to colonize certain hosts. To use the (in)ability to mate in species delimitation for fungi can be challenging, as two lineages that cannot mate are not necessarily different species. For instance, mating may be very rare, could be dependent on environmental cues that may be unknown, and two distinct mating types may be necessary for successful reproduction (Li et al., 2020; Nagel et al., 2018; Yurkov et al., 2015). In addition, phylogenetically distinct species may hybridize (Samarasinghe et al., 2020). For *P. citricarpa* and *P. paracitricarpa*, biological differences do not provide a basis to delimit the species. Specifically, they can be found on the same host plants; *P. paracitricarpa* was isolated from *C. sinensis* and *C. limon* in China (Wang et al., 2023, 2012),

while in this study we used *P. citricarpa* strains that were isolated from the same hosts from Argentina, Australia, Portugal, the USA and Zimbabwe. Although it is likely that they can mate due to their high similarity, this has not been demonstrated. However, it was shown that both species are heterothallic and that their mating-type loci are highly similar (Petters-Vandresen et al., 2020). The ability of *P. paracitricarpa* to cause disease symptoms similar to those caused by *P. citricarpa* remains uncertain. Wang et al. (2023) performed detached fruit assays using a single representative strain per species to prove Koch's postulates, and concluded that *P. paracitricarpa* strains are able to cause symptoms similar to those of *P. citricarpa*. However, it is unclear to which *tef1* clade (*citricarpa* or *paracitricarpa*) the used representative strain belongs. Guarnaccia et al. (2017) also used a detached fruit assay to show that artificially inoculated sweet orange fruits develop similar symptoms when inoculated with *P. citricarpa* and *P. paracitricarpa*. Whether disease symptoms of *P. paracitricarpa* exist in nature is unclear; Wang et al. (2012) lists numerous strains isolated from spots on *Citrus sinensis* fruits in China as "*P. citricarpa* subclade-II" (Wang et al., 2012), some of which could also be considered as *P. paracitricarpa* based on the *tef1* definition. This would imply that this species causes symptoms in nature. Considering the genetic similarity between *P. citricarpa* and *P. paracitricarpa* it seems logical that *P. paracitricarpa* would be able to cause similar symptoms, but to the authors' knowledge there are currently no data available to unequivocally support this claim. To be able to separate the two clades based on a difference in pathogenicity, more studies would have to be performed.

A second property that is often used in fungal species delimitation is morphology. However, fungal morphology is not always straightforward either as fungi may demonstrate several phenotypically distinct forms, often related to different parts of their life cycle (Bruckart et al., 2010; Wingfield et al., 2012). Morphological characteristics do not provide a criterium on which to distinguish *P. citricarpa* and *P. paracitricarpa* either, as their morphology is highly similar. Although average sizes may differ, such as those of conidiophores and conidia, sizes do overlap and may represent variation within a species. Culture characteristics are also frequently employed to help separate species. On MEA, fungal colonies of *P. paracitricarpa* appear yellow, becoming leaden-grey in the centre and yellow at the margin, while *P. citricarpa* colonies are olivaceous-grey (Guarnaccia et al., 2017). However, we show in this publication

that not all *P. paracitricarpa* strains share this characteristic. Thus, these characteristics currently do not provide a basis to separate these clades either.

The third property on which to distinguish species is molecular characteristics. However, fungal genomes may be very diverse, with some species having much higher levels of infraspecific variation than others. Barcoding gaps, the absence of an overlap between levels of infra- and interspecific variation, may be present between species (Meyer & Paulay, 2005). We show in this publication that other *Phyllosticta* species have higher infraspecific variation than there is variation between *P. citricarpa* and *P. paracitricarpa*, indicating they should be considered one species. We included a variety of *P. citricarpa* strains from different geographical origins and hosts, thus variation of *P. citricarpa* is well represented in this study. In addition, we included the ex-type strain for *P. paracitricarpa* and included strains from different geographical origins in our 12-gene analyses, showing that addition of more diverse strains of *P. paracitricarpa* does not change the tree topology. In addition, results from both Ioos et al. (2023) and Wang et al. (2023) also suggest that an increased sampling does not dramatically increase the resolution between the two clades. On the contrary, the increased genetic variation being introduced with more strains results in less clade-specific polymorphisms and thus even more diffused clade boundaries. The genomic data shown here therefore provides a solid basis to combine *P. citricarpa* and *P. paracitricarpa* into one species, and does not provide any arguments to keep them separate at the species level. Thus, there is currently no basis – whether it be biological, morphological, or molecular – to distinguish them as separate species, and we should consider these strains to represent the same pathogen.

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Data statement

All genomes that were newly sequenced and assembled in this study are available at https://mycocosm.jgi.doe.gov/Phyllosticta. All supplementary data have been deposited at figshare (doi: 10.6084/m9.figshare.25187918).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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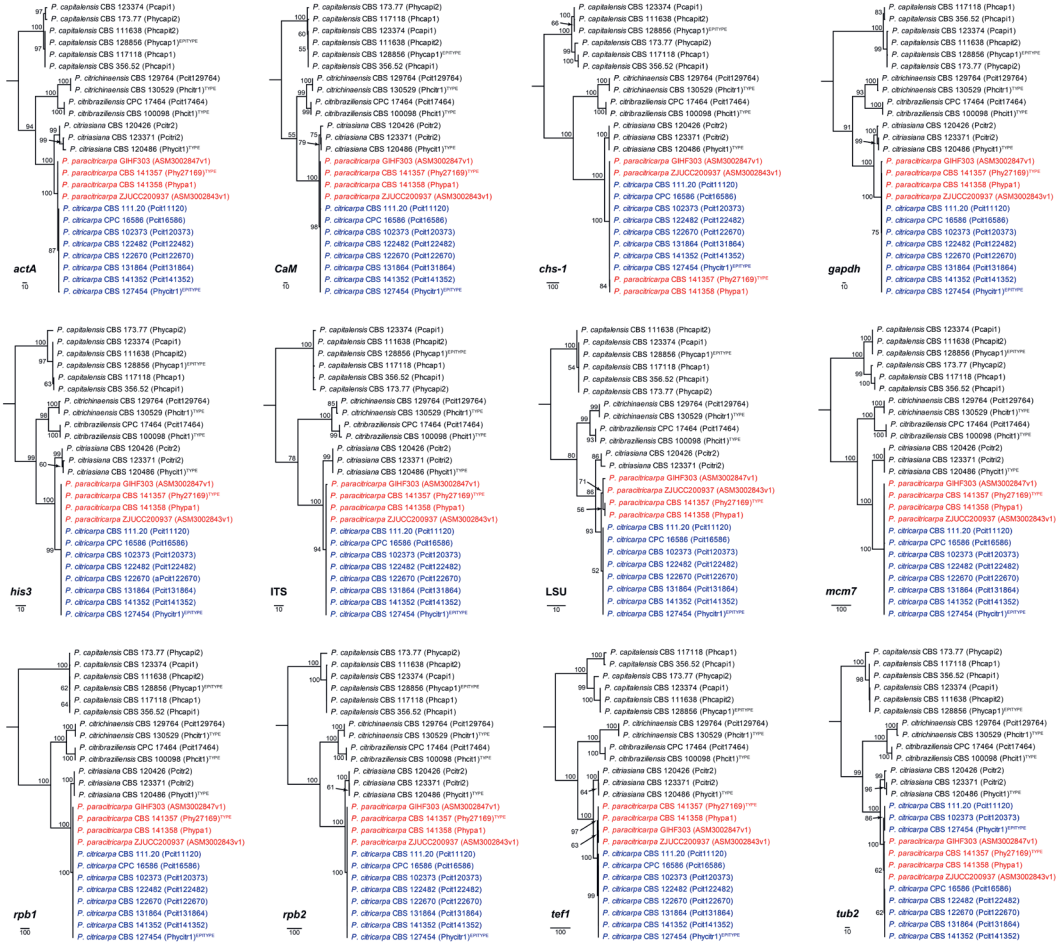
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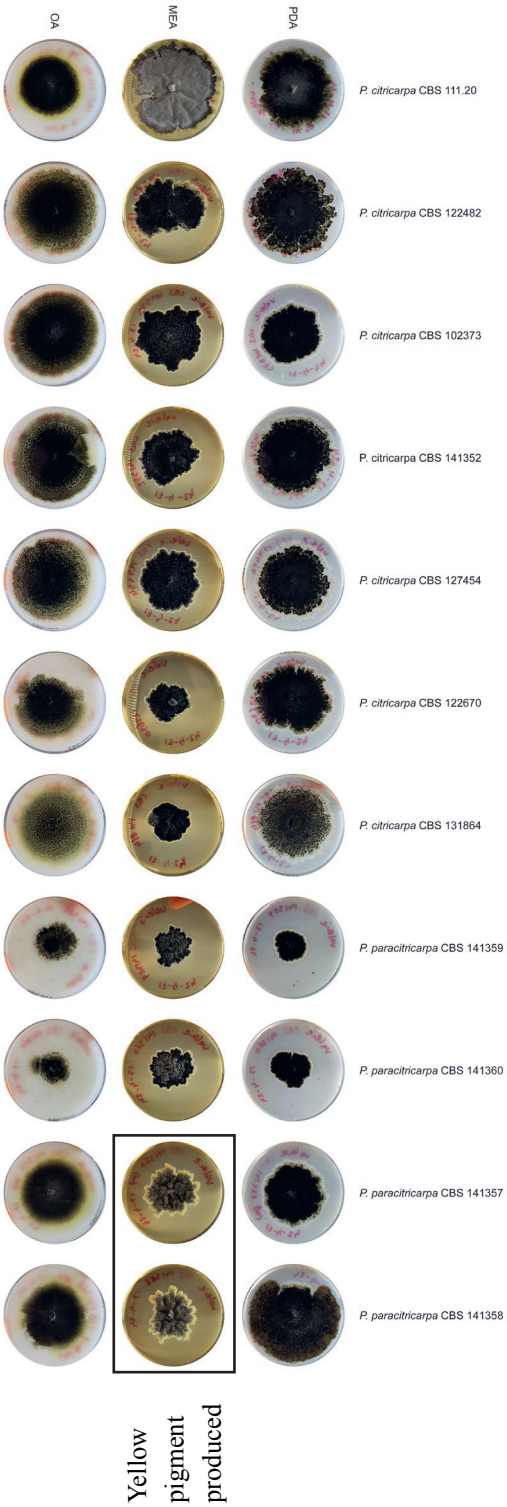
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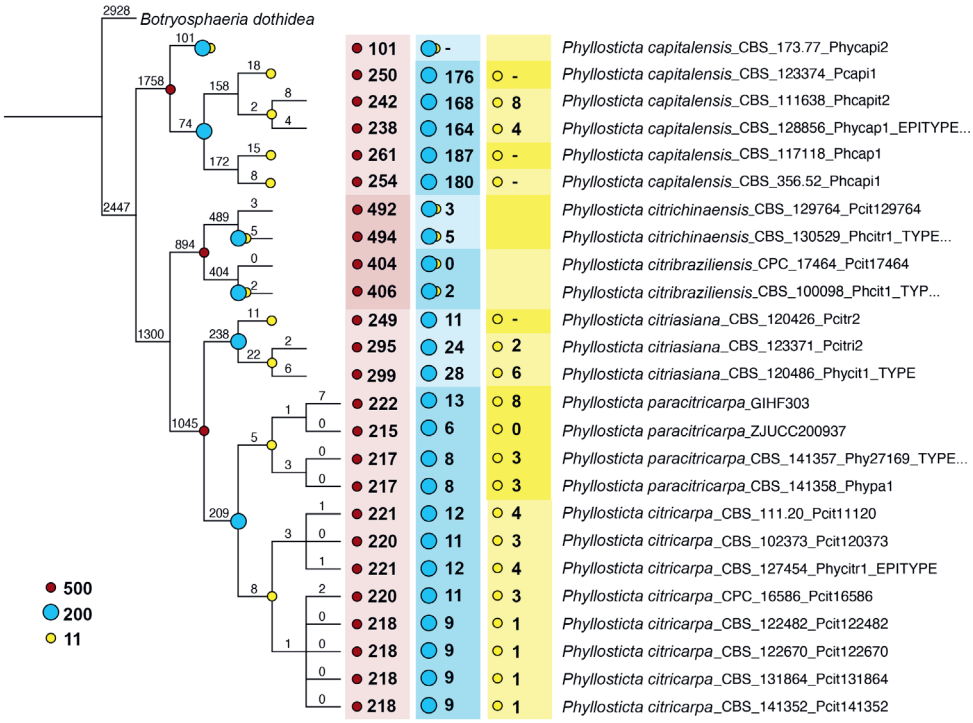
Supplementary Figures



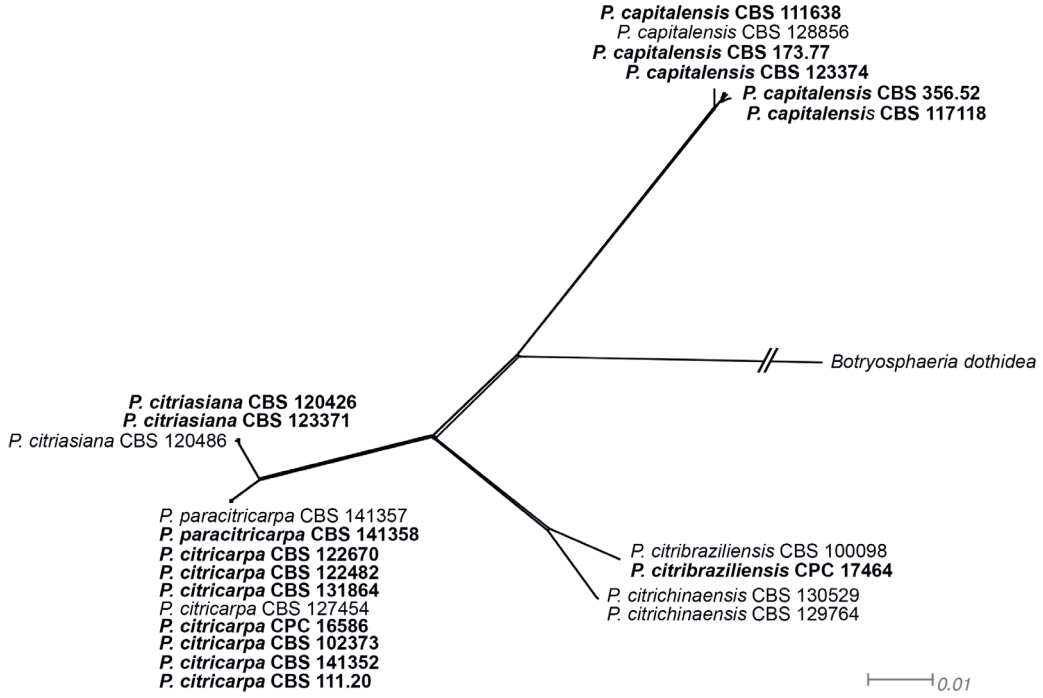
Suppl. Fig S1. Single gene trees (12x). Phylogenetic trees of twelve marker gene loci: actin gene (*actA*), chitin-synthase 1 gene (*chs1*), calmodulin gene (*CaM*), glyceraldehyde-3-phosphate dehydrogenase gene (*gapdh*), histone H3 gene (*his3*), internal transcribed spacer (ITS), large subunit (LSU), DNA replication licensing factor (*mcm7*), DNA-directed RNA polymerase II largest subunit gene (*rpb1*), DNA-directed RNA polymerase II second largest subunit gene (*rpb2*), translation elongation factor 1-alpha gene (*tef1*), and beta-tubulin gene (*tub2*).



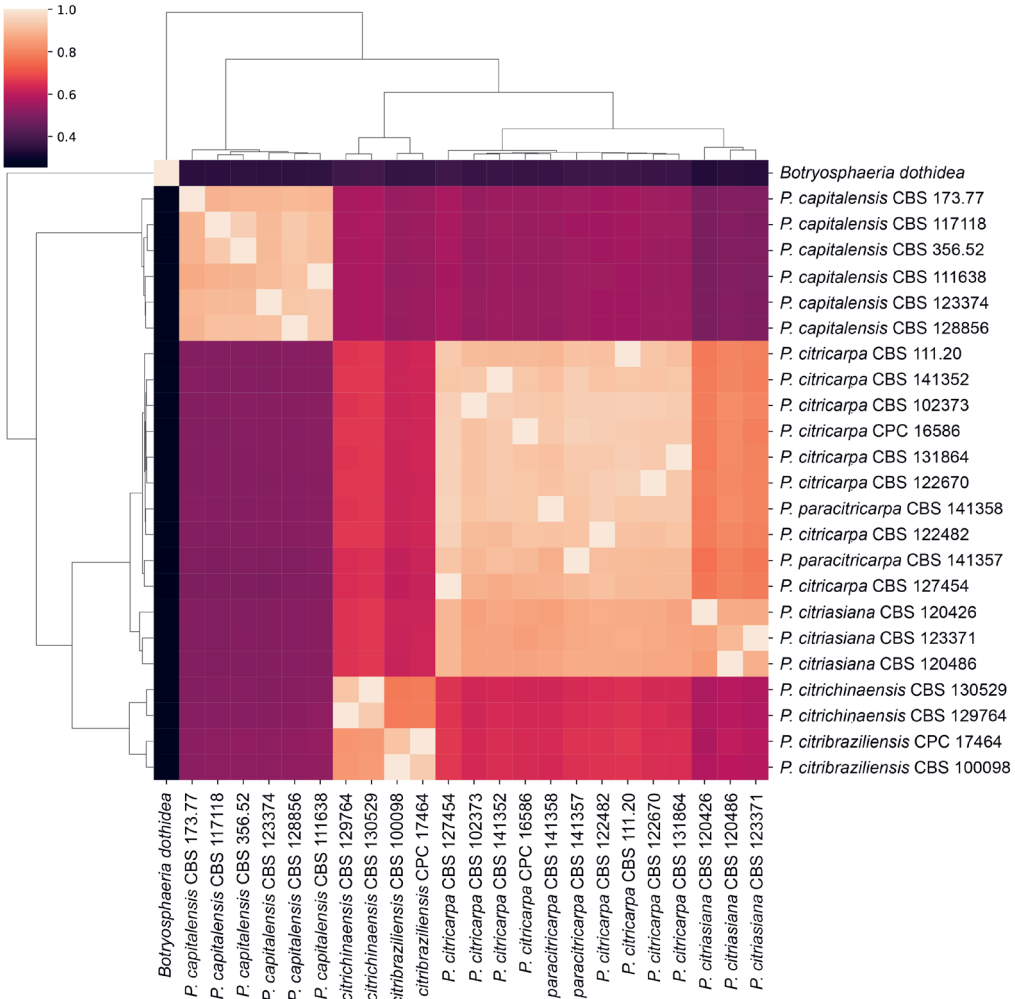
Suppl. Fig. S2. Pigment production of *Phyllosticta (para)citricarpa* on PDA, MEA and OA. Two strains of *P. paracitricarpa* produce a yellow pigment on MEA (the two left-most strains), the others do not.



Suppl. Fig S3. Quantification tree. Phylogenetic tree based on the concatenated 12 full-length barcoding marker nucleotide sequences. The number of nt changes in a strain as compared to a most recent common ancestor (MRCA) were calculated, and different MRCA 'thresholds' were compared: red MRCAs allow for up to 500 nt changes, blue MRCAs allow for up to 200 nt changes, and yellow MRCAs allow for up to 11 nt changes in a strain as compared to the MRCA. The positions of the different colors of MRCA are indicated in the tree. The yellow threshold was chosen to allow for separation of the *P. citricarpa* and *P. paracitricarpa* clades. Using the yellow threshold, *P. citricasiana* is split up into two clades, and *P. capitalensis* is split up into five clades. If the threshold is set at 200 nt changes, strains that are considered to be of one species all fall within one clade, with the exception of *P. capitalensis*, which is split into two clades, and *P. citricarpa* and *P. paracitricarpa*, which fall into one clade. The red MRCA allows for all *P. capitalensis* strains to fall into one clade, but *P. citrichinaensis* and *P. citribraziliensis* also form one clade, and *P. citriasiana*, *P. citricarpa* and *P. paracitricarpa* all group into one clade as well.



Suppl. Fig S4. SplitsTree analysis. The SplitsTree network reconstruction was created for the BUSCO gene alignment using v4.17.0 (Huson & Bryant, 2006). No indication of phylogenetic inconsistenties is present, and strains of *P. citricarpa* and *P. paracitricarpa* group into one intermixed clade.



Suppl. Fig. S5. Heatmap with small scaffolds (<1,000 bp) removed. Heatmap and hierarchical clustering based on the percentage of whole genome material shared, small scaffolds (<1,000 bp) excluded.

Supplementary Table S1. Overview of genomes and strains used in this study.

Strain	BUSCO score*	Size (MB)	# Contigs	DBID	Geographic origin	Host origin	Reference
***P. capitalensis* CBS 111638**	96,9	32,08	17	Phcapit2	Dominican Republic	*Capsicum* sp. (Solanaceae), fruit	this study
***P. capitalensis* CBS 117118**	97,3	33,04	24	Phcap1	Indonesia	*Musa acuminata*	this study
***P. capitalensis* CBS 123374**	97,2	32,82	18	Pcapi1	Thailand	*Citrus aurantium* (Rutaceae)	this study
P. capitalensis CBS 128856	95,8	32,46	14	Phycap1	Brazil	*Stanhopea* sp.	Guarnaccia et al. 2019
***P. capitalensis* CBS 173.77**	96,8	32,88	16	Phycapi2	New Zealand	*Citrus aurantiifolia* (Rutaceae), fruit	this study
***P. capitalensis* CBS 356.52**	97	32,77	22	Phcapi1	Unknown	*Ilex* sp.	this study
***P. citriasiana* CBS 120426**	95,7	33,77	30	Pcitr2	China	*Citrus maxima* (Rutaceae)	this study
P. citriasiana CBS 120486	95,7	32,7	133	Phycit1	Thailand	*Citrus maxima* (Rutaceae)	Guarnaccia et al. 2019
***P. citriasiana* CBS 123371**	96,7	33,27	26	Pcitri2	Vietnam	*Citrus maxima* (Rutaceae)	this study
P. citribraziliensis CBS 100098	94,5	31,67	32	Phcit1	Brazil	*Citrus limon*, healthy leaves	Guarnaccia et al. 2019
***P. citribraziliensis* CPC 17464**	96,8	31,35	22	Pcit17464	Brazil	*Citrus* sp.	this study
***P. citricarpa* CBS 102373**	97,2	30,93	95	Pcit120373	Brazil	*Citrus aurantium* (Rutaceae), black spot on fruit	this study
***P. citricarpa* CBS 111.20**	96,8	29,85	113	Pcit11120	China	*Citrus* peel	this study
***P. citricarpa* CBS 122482**	97,1	30,01	107	Pcit122482	Zimbabwe	*Citrus sinensis* (Rutaceae), lesions on fruit	this study
***P. citricarpa* CBS 122670**	97,1	30,43	95	Pcit122670	South Africa	hardspot on *Citrus* cv. Valencia	this study
P. citricarpa CBS 127454	94,9	28,95	152	Phycitr1	Australia	*Citrus limon*	Guarnaccia et al. 2019
***P. citricarpa* CBS 131864**	97	30,49	92	Pcit131864	USA	Infected *Citrus sinensis* fruit	this study
***P. citricarpa* CBS 141352**	96,8	30,43	130	Pcit141352	Portugal	*Citrus sinensis* leaf litter	this study
***P. citricarpa* CPC 16586**	97,2	30,76	96	Pcit16586	Argentina	*Citrus limon*	this study
P. citrichinaensis CBS 129764	96,8	29,53	22	Pcit129764	China	*Citrus reticulata* leaves	Buijs et al. 2022
P. citrichinaensis CBS 130529	93,2	29,16	25	Phcitr1	China	*Citrus maxima*	Guarnaccia et al. 2019
P. paracitricarpa CBS 141357	94,4	29,53	134	Phy27169	Greece	*Citrus limon*, leaf litter	Guarnaccia et al. 2019
***P. paracitricarpa* CBS 141358**	97,2	30,83	90	Phypa1	Greece	*Citrus limon*, leaf litter	this study
P. paracitricarpa ZJUCC 200937	-	31,7	5690	-	China	*Citrus sinensis*	Guarnaccia et al. 2017, Ioos et al. 2023
P. paracitricarpa GIHF 303	-	32,2	6096	-	China	*Citrus sinensis*	Ioos et al. 2023

*busco lineage used: Dothideomycetes odb10

Strains in **bold** are newly sequenced in this study

Supplementary Table S2. Sequencing and assembly methods. Available online at https://doi.org/10.1016/j.fgb.2024.103925

Supplementary Table S3. Statistics for the maximum parsimony phylogenetic analyses of the 10 full-length barcoding gene nucleotide sequences. The LSU alignment contains some missing data due to incomplete sequences available in the genomes, with the LSU of *P. citriasiana* CBS 120486 (Phycit1) completely missing. All missing data were coded as missing characters in the analyses.

	actA	***CaM***	***chs1***	***gapdh***	***his3***	***mcm7***	***rpb1***	***rpb2***	***tef1***	***tub2***	**ITS**	**LSU**
Number of alignment positions (incl. gaps)	1696	1040	3317	1476	631	2690	5589	3890	2173	1773	585	3355
Constant characters	1247	607	1912	897	457	1707	3519	2649	1373	1379	369	3252
Parsimony-uninformative characters	228	249	451	233	74	368	728	475	225	186	93	46
Parsimony-informative characters	221	184	954	346	100	615	1342	766	575	208	123	57
Number of equally parsimonious trees saved	1	1	1	2	1	2	1	2	1	3	3	1
Tree length	567	558	2058	838	241	1388	2754	1692	1237	516	313	127
Consistency index	0.929	0.937	0.896	0.896	0.942	0.880	0.927	0.907	0.874	0.909	0.939	0.898
Retention index	0.966	0.968	0.963	0.957	0.975	0.952	0.974	0.965	0.947	0.959	0.972	0.960
Rescaled consistency index	0.898	0.907	0.863	0.858	0.918	0.838	0.903	0.876	0.828	0.872	0.913	0.862
Homoplasy index	0.071	0.063	0.104	0.104	0.058	0.120	0.073	0.093	0.126	0.091	0.061	0.102

actA: actin gene; *CaM*: calmodulin gene; *chs1*: chitin-synthase 1 gene; *gapdh*: glyceraldehyde-3-phosphate dehydrogenase gene; *his3*: histone H3 gene; *mcm7*: DNA replication licensing factor; *rpb1*: DNA-directed RNA polymerase II largest subunit gene; *rpb2*:DNA-directed RNA polymerase II second largest subunit gene; *tef1*: translation elongation factor 1-alpha gene; *tub2*: beta-tubulin gene; ITS: internal transcribed spacers and intervening 5.8S nrDNA; LSU: partial large subunit (28S) of the nrRNA gene operon.

Supplementary Table S4. Percentages of whole genome shared material.

	ci1	ci2	ci3	ci4	ci5	ci6	ci7	ci8	pa1	pa2	as1	as2	as3	ch1	ch2	br1	br2	ca1	ca2	ca3	ca4	ca5	ca6	do
P. citricarpa CBS 102373 (**ci1**)		0,94	0,94	0,94	0,93	0,94	0,93	0,94	0,93	0,92	0,69	0,70	0,71	0,46	0,45	0,43	0,42	0,32	0,32	0,32	0,32	0,33	0,32	0,15
P. citricarpa CBS 141352 (**ci2**)	0,93		0,92	0,92	0,92	0,92	0,93	0,92	0,93	0,91	0,68	0,69	0,70	0,46	0,46	0,43	0,43	0,32	0,32	0,32	0,32	0,33	0,32	0,15
P. citricarpa CBS 111.20 (**ci3**)	0,90	0,90		0,91	0,90	0,92	0,91	0,93	0,91	0,89	0,68	0,69	0,70	0,46	0,46	0,43	0,43	0,32	0,32	0,32	0,32	0,33	0,32	0,15
P. citricarpa CBS 122482 (**ci4**)	0,90	0,90	0,92		0,92	0,91	0,92	0,94	0,91	0,91	0,69	0,69	0,70	0,46	0,46	0,43	0,43	0,32	0,32	0,32	0,32	0,33	0,32	0,15
P. citricarpa CPC 16586 (**ci5**)	0,92	0,93	0,94	0,94		0,93	0,93	0,94	0,94	0,92	0,68	0,69	0,71	0,46	0,46	0,43	0,43	0,32	0,32	0,32	0,32	0,33	0,32	0,15
P. citricarpa CBS 122670 (**ci6**)	0,92	0,93	0,95	0,94	0,93		0,93	0,94	0,92	0,91	0,69	0,70	0,71	0,46	0,46	0,43	0,43	0,32	0,32	0,32	0,32	0,33	0,32	0,15
P. citricarpa CBS 131864 (**ci7**)	0,92	0,93	0,94	0,93	0,92	0,93		0,95	0,92	0,91	0,68	0,70	0,71	0,46	0,46	0,43	0,42	0,32	0,32	0,32	0,32	0,33	0,32	0,15
P. citricarpa CBS 127454 (**ci8**)	0,88	0,87	0,90	0,91	0,88	0,90	0,90		0,90	0,88	0,67	0,69	0,69	0,45	0,44	0,42	0,42	0,31	0,32	0,32	0,31	0,32	0,31	0,14
P. paracitricarpa CBS 141357 (**pa1**)	0,89	0,91	0,90	0,91	0,90	0,89	0,90	0,92		0,88	0,67	0,68	0,69	0,45	0,44	0,43	0,42	0,31	0,32	0,32	0,31	0,32	0,32	0,14
P. paracitricarpa CBS 141358 (**pa2**)	0,92	0,92	0,92	0,93	0,92	0,92	0,92	0,94	0,92		0,68	0,69	0,71	0,46	0,46	0,43	0,42	0,32	0,32	0,32	0,32	0,33	0,32	0,15
P. citriasiana CBS 120426 (**as1**)	0,75	0,75	0,76	0,77	0,75	0,76	0,75	0,78	0,76	0,74		0,86	0,85	0,46	0,45	0,42	0,42	0,32	0,32	0,32	0,32	0,33	0,32	0,15
P. citriasiana CBS 123371 (**as2**)	0,75	0,75	0,77	0,76	0,74	0,76	0,76	0,78	0,75	0,75	0,84		0,89	0,46	0,45	0,43	0,42	0,32	0,32	0,32	0,31	0,33	0,32	0,15
P. citriasiana CBS 120486 (**as3**)	0,75	0,75	0,76	0,76	0,75	0,75	0,76	0,78	0,76	0,75	0,83	0,87		0,46	0,45	0,43	0,42	0,32	0,32	0,32	0,31	0,33	0,32	0,15
P. citrichinaensis CBS 130529 (**ch1**)	0,43	0,44	0,45	0,44	0,43	0,44	0,44	0,45	0,44	0,43	0,39	0,40	0,41		0,92	0,62	0,61	0,32	0,32	0,33	0,32	0,33	0,32	0,15
P. citrichinaensis CBS 129764 (**ch2**)	0,43	0,44	0,45	0,45	0,44	0,45	0,44	0,45	0,44	0,44	0,40	0,40	0,41	0,93		0,62	0,62	0,32	0,32	0,33	0,32	0,33	0,32	0,15
P. citribraziliensis CPC 17464 (**br1**)	0,44	0,45	0,45	0,45	0,44	0,45	0,45	0,46	0,45	0,44	0,39	0,40	0,41	0,67	0,66		0,92	0,33	0,33	0,34	0,33	0,34	0,33	0,15
P. citribraziliensis CBS 100098 (**br2**)	0,44	0,45	0,45	0,45	0,44	0,45	0,44	0,46	0,45	0,44	0,40	0,40	0,41	0,67	0,66	0,94		0,33	0,33	0,34	0,33	0,34	0,33	0,15
P. capitalensis CBS 173.77 (**ca1**)	0,34	0,35	0,35	0,35	0,35	0,35	0,35	0,36	0,35	0,35	0,31	0,32	0,32	0,36	0,36	0,34	0,34		0,82	0,83	0,81	0,82	0,82	0,15
P. capitalensis CBS 123374 (**ca2**)	0,34	0,35	0,35	0,35	0,34	0,35	0,35	0,36	0,35	0,35	0,31	0,32	0,32	0,36	0,36	0,35	0,34	0,82		0,91	0,87	0,91	0,87	0,15
P. capitalensis CBS 128856 (**ca3**)	0,34	0,35	0,35	0,35	0,34	0,35	0,34	0,36	0,35	0,34	0,31	0,32	0,32	0,36	0,36	0,35	0,35	0,82	0,90		0,88	0,92	0,88	0,15
P. capitalensis CBS 117118 (**ca4**)	0,34	0,35	0,35	0,35	0,34	0,35	0,35	0,36	0,35	0,35	0,31	0,31	0,32	0,37	0,36	0,35	0,35	0,82	0,87	0,90		0,88	0,93	0,15
P. capitalensis CBS 111638 (**ca5**)	0,34	0,34	0,35	0,35	0,34	0,35	0,34	0,36	0,35	0,34	0,31	0,31	0,32	0,36	0,36	0,35	0,34	0,80	0,88	0,91	0,85		0,86	0,15
P. capitalensis CBS 356.52 (**ca6**)	0,34	0,34	0,35	0,35	0,34	0,35	0,34	0,36	0,35	0,35	0,31	0,32	0,32	0,36	0,36	0,35	0,35	0,82	0,87	0,89	0,92	0,87		0,15
B. dothidea (**do**)	0,21	0,22	0,22	0,22	0,21	0,22	0,22	0,22	0,22	0,21	0,19	0,20	0,20	0,23	0,22	0,21	0,21	0,20	0,20	0,20	0,20	0,21	0,20	

Supplementary Table S5. Percentages of whole genome shared material, excluding unique windows.

	ci1	ci2	ci3	ci4	ci5	ci6	ci7	ci8	pa1	pa2	as1	as2	as3	ch1	ch2	br1	br2	ca1	ca2	ca3	ca4	ca5	ca6	do
P. citricarpa CBS 102373 (**ci1**)		0,997	0,997	0,997	0,997	0,997	0,997	0,996	0,995	0,994	0,96	0,96	0,96	0,86	0,85	0,85	0,85	0,81	0,82	0,81	0,81	0,82	0,81	0,76
P. citricarpa CBS 141352 (**ci2**)	0,997		0,997	0,997	0,997	0,997	0,997	0,996	0,995	0,995	0,96	0,96	0,96	0,85	0,85	0,85	0,85	0,81	0,82	0,81	0,81	0,82	0,81	0,76
P. citricarpa CBS 111.20 (**ci3**)	0,997	0,997		0,997	0,997	0,997	0,997	0,995	0,995	0,994	0,96	0,96	0,96	0,86	0,85	0,86	0,85	0,81	0,82	0,81	0,81	0,82	0,81	0,76
P. citricarpa CBS 122482 (**ci4**)	0,997	0,997	0,997		0,997	0,997	0,997	0,996	0,995	0,995	0,96	0,96	0,96	0,85	0,85	0,85	0,85	0,81	0,82	0,81	0,81	0,82	0,81	0,76
P. citricarpa CPC 16586 (**ci5**)	0,997	0,997	0,997	0,998		0,997	0,997	0,996	0,995	0,994	0,96	0,95	0,96	0,85	0,85	0,86	0,85	0,81	0,82	0,81	0,81	0,82	0,81	0,76
P. citricarpa CBS 122670 (**ci6**)	0,997	0,997	0,998	0,998	0,997		0,997	0,996	0,995	0,994	0,96	0,96	0,96	0,85	0,86	0,85	0,85	0,81	0,82	0,81	0,81	0,82	0,81	0,75
P. citricarpa CBS 131864 (**ci7**)	0,997	0,998	0,998	0,997	0,997	0,998		0,996	0,995	0,994	0,96	0,96	0,96	0,86	0,85	0,85	0,85	0,81	0,82	0,81	0,81	0,82	0,81	0,76
P. citricarpa CBS 127454 (**ci8**)	0,993	0,993	0,993	0,993	0,993	0,994	0,993		0,994	0,991	0,96	0,96	0,96	0,85	0,85	0,85	0,85	0,81	0,82	0,81	0,81	0,82	0,81	0,76
P. paracitricarpa CBS 141357 (**pa1**)	0,994	0,994	0,994	0,994	0,993	0,993	0,994	0,994		0,997	0,96	0,96	0,96	0,85	0,85	0,86	0,85	0,81	0,82	0,82	0,81	0,82	0,82	0,76
P. paracitricarpa CBS 141358 (**pa2**)	0,994	0,995	0,994	0,995	0,994	0,995	0,994	0,995	0,998		0,95	0,96	0,96	0,85	0,85	0,85	0,85	0,81	0,82	0,81	0,81	0,82	0,81	0,76
P. citriasiana CBS 120426 (**as1**)	0,96	0,96	0,96	0,96	0,96	0,96	0,96	0,96	0,96	0,96		0,99	0,98	0,85	0,85	0,85	0,85	0,81	0,81	0,81	0,81	0,81	0,81	0,76
P. citriasiana CBS 123371 (**as2**)	0,96	0,96	0,96	0,96	0,96	0,96	0,96	0,96	0,96	0,96	0,99		0,99	0,85	0,85	0,85	0,85	0,81	0,81	0,81	0,81	0,81	0,81	0,76
P. citriasiana CBS 120486 (**as3**)	0,96	0,96	0,96	0,96	0,96	0,96	0,96	0,96	0,96	0,96	0,98	0,99		0,85	0,85	0,85	0,85	0,81	0,82	0,82	0,81	0,81	0,81	0,76
P. citrichinaensis CBS 130529 (**ch1**)	0,85	0,85	0,85	0,85	0,85	0,86	0,85	0,85	0,85	0,85	0,85	0,85	0,85		0,995	0,93	0,93	0,81	0,82	0,82	0,82	0,82	0,82	0,76
P. citrichinaensis CBS 129764 (**ch2**)	0,85	0,85	0,85	0,85	0,85	0,85	0,85	0,85	0,85	0,85	0,85	0,85	0,85	0,995		0,92	0,93	0,82	0,81	0,82	0,82	0,82	0,81	0,76
P. citribraziliensis CPC 17464 (**br1**)	0,85	0,86	0,86	0,85	0,85	0,85	0,86	0,85	0,86	0,85	0,85	0,85	0,85	0,93	0,93		0,999	0,82	0,82	0,82	0,82	0,82	0,82	0,76
P. citribraziliensis CBS 100098 (**br2**)	0,85	0,86	0,85	0,85	0,85	0,86	0,85	0,85	0,85	0,85	0,85	0,85	0,85	0,93	0,93	0,998		0,82	0,82	0,82	0,82	0,82	0,82	0,76
P. capitalensis CBS 173.77 (**ca1**)	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,81	0,82	0,82	0,82	0,82		0,97	0,97	0,97	0,97	0,97	0,76
P. capitalensis CBS 123374 (**ca2**)	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,81	0,82	0,81	0,82	0,82	0,82	0,82	0,97		0,99	0,98	0,99	0,98	0,76
P. capitalensis CBS 128856 (**ca3**)	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,81	0,82	0,82	0,82	0,82	0,97	0,99		0,99	0,99	0,98	0,76
P. capitalensis CBS 117118 (**ca4**)	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,81	0,82	0,82	0,82	0,82	0,97	0,98	0,99		0,98	0,99	0,76
P. capitalensis CBS 111638 (**ca5**)	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,81	0,82	0,82	0,82	0,82	0,97	0,99	0,99	0,98		0,98	0,76
P. capitalensis CBS 356.52 (**ca6**)	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,82	0,81	0,82	0,81	0,82	0,82	0,82	0,82	0,97	0,98	0,98	0,99	0,98		0,76
B. dothidea (**do**)	0,77	0,76	0,76	0,76	0,77	0,77	0,76	0,76	0,76	0,76	0,76	0,76	0,76	0,76	0,76	0,77	0,77	0,76	0,77	0,76	0,76	0,77	0,76	



Chapter 6

General Discussion

Phyllosticta citricarpa causes Citrus Black Spot (CBS), thereby inflicting significant economic losses. Taxonomic ambiguities between different *Phyllosticta* species, combined with limited (genomic) insights into virulence mechanisms, hinder effective disease management and diagnostics, and exacerbate vulnerabilities in *Citrus* supply chains. This thesis provides a multifaceted exploration of pathogenicity and species delineation within the genus *Phyllosticta*, addressing key aspects of carbon utilization, comparative genomics, genetic modification, and taxonomic relationships. **Chapter 2** reveals that *Phyllosticta* species, regardless of lifestyle, exhibit highly similar carbon utilization profiles. Interestingly, we demonstrated that Sugar Beet Pulp (SBP) selectively inhibits growth of pathogenic species *in vitro*. **Chapter 3** investigates genomic differences across lifestyles, identifying small distinctions in the number of genes, secreted proteins and effectors between pathogens and endophytes, and classifying *P. citrichinaensis* as having an intermediate lifestyle. In addition, our data shows that clear CAZyme variations among *Dothideomycetes* fungi seem unrelated to lifestyle classifications in literature. **Chapter 4** establishes a proof-of-principle CRISPR/Cas9 protocol for *Phyllosticta*, demonstrating its application through the knockout of the pH-modulation gene *palH*, which did not result in any distinct phenotypic changes while the presence of the intended mutation was confirmed with whole-genome sequencing. Finally, **Chapter 5** resolves the debate surrounding the taxonomic status of *P. citricarpa* and *P. paracitricarpa*, demonstrating through phylogenetic and phylogenomic analyses that their interspecific variation is less than infraspecific variation in other *Phyllosticta* species, thereby showing that they should be treated as synonymous. These findings collectively advance our understanding of *Phyllosticta* and lay the groundwork for further research and development of refined disease management strategies.

Sugar beet pulp as a potential disease management tool

A key finding from **Chapter 2** is the pronounced inhibitory effect of sugar beet pulp (SBP) on the growth of pathogenic *Phyllosticta* species, observed even at low concentrations (0.25%) and in the presence of other carbon sources such as wheat bran. This effect, absent in non-pathogenic species, suggests a selective mechanism that could be harnessed for disease management. Indeed, other plant-derived compounds exist that have been shown to inhibit growth of

(plant-pathogenic) fungi (Eksteen et al., 2001; Savoia, 2012; Yazdani et al., 2011). Our efforts to characterize the inhibitory substance have yielded limited insights. The possibility that the effect arises not from a single agent, but from synergistic interactions among multiple SBP components, could complicate obtaining a purified effective compound. The implications of identifying this substance(s) are significant, potentially enabling targeted biocontrol methods for *Phyllosticta* pathogens using a low-cost by-product of the sugar beet industry, potentially overcoming the need for fungicide application. To overcome current limitations, future research should employ advanced analytical techniques, such as mass spectrometry or NMR spectroscopy, to profile SBP's chemical constituents comprehensively. Additionally, testing SBP derivatives under varied environmental conditions could clarify whether the inhibitory effect is consistent across pathogenic *Phyllosticta* strains and citrus hosts, ensuring practical applicability in disease management. The development of SBP-based strategies could transform *Phyllosticta* management, but limitations in pinpointing the active compound highlight the need for further research.

Studying the emergence of pathogens

Uncovering genomic signatures that underpin lifestyle differences can enhance insights into host colonization and pathogenesis (Haridas et al., 2020; Möller & Stukenbrock, 2017; O'Connell et al., 2012; Ohm et al., 2012; Plissonneau et al., 2017; Spanu, 2012). In **Chapter 3**, we sought to clarify the lifestyle of *Phyllosticta citrichinaensis* by examining genomic differences among *Phyllosticta* species with varying lifestyles. The identified genetic differences could offer promising targets for developing novel disease management strategies that could effectively target multiple pathogenic *Phyllosticta* species. Our findings show that endophytes tend to possess greater numbers of genes within certain orthologous groups compared to pathogens, implying that endophytic lifestyles may require extra genetic capabilities. Ancestral state reconstructions indicate that the common ancestor of *Dothideomycetes* was probably saprotrophic, while the progenitor of the *Botryosphaeriales*, encompassing the genus *Phyllosticta*, likely functioned as a plant pathogen (Abdollahzadeh et al., 2020; Haridas et al., 2020). Consequently, the transition from pathogenic to endophytic *Phyllosticta* species could have occurred through gene expansions, enhancing their functional repertoire. Additionally, patho-

genic genomes were found to harbour a higher number of lifestyle-specific orthogroups (OGs) with unknown functions, where none of the associated genes received any form of annotation. Such uncharacterized genes may represent virulence genes, for instance effectors involved in the manipulation of host defences, and thus represent compelling candidates for in-depth investigation in future research.

Classifying fungi according to distinct lifestyle categories presents, however, notable challenges, as emerging research increasingly demonstrates the plasticity of lifestyle boundaries, highlighting how lifestyles can shift dynamically in response to host interactions and environmental cues (Oliver & Ipcho, 2004; Sauters & Rokas, 2025; Temporini & Vanetten, 2002; van Dam & Rep, 2017; Veloso & Van Kan, 2018). In addition, many novel species were described based on a very small number of strains, and without proper information about host range, distribution, and population variation, and thus one can only extrapolate a supposed lifestyle based on very limited observations. Importantly, when we attempt to determine to what lifestyle category a species may belong, we are intrinsically running behind the facts: species constantly emerge as new pathogens, switch hosts, adjust to climatic shifts, or lose pathogenicity. Long after these changes occur, we are still piecing together the puzzle of how these adaptations unfold. A more effective approach might be to focus on understanding the mechanisms of adaptation that drive these lifestyle transitions, and classifying fungi based on their adaptive processes, which will allow for a better estimation of their potential to develop towards a specific lifestyle, rather than static lifestyle categories.

Understanding the mechanisms driving fungal lifestyle transitions requires examining the evolutionary trade-offs between endophytic and pathogenic states. An endophytic lifestyle may appear advantageous, as it offers stability, long-term survival, reduced stress, and access to abundant nutrients from a healthy host. In contrast, some pathogenic species seem to have greater adaptability, enabling adjustment to changing or stressful conditions, exploitation of new and underutilized niches (such as monoculture crop systems), and access to nutrients that are otherwise retained with the host (de Jonge et al., 2013; Möller & Stukenbrock, 2017; Plissonneau et al., 2017; Rouxel et al., 2011). Ultimately, the advantages of a specific lifestyle depend on environmental conditions and a species' adaptive capacity, shaping the trajectory of these evolutionary shifts.

Some fungal genera show only minimal genetic differences between species with varying lifestyles, enabling swift and frequent shifts between endophytic and pathogenic states (Hill et al., 2022). These species harbour genomic elements for both lifestyles, requiring only the activation or suppression of specific genes (Fouché et al., 2018; Kabbage et al., 2015; O'Connell et al., 2012). In contrast, some species maintain consistent ecological roles, such as those that never cause disease, even across diverse hosts or climates (Saleem et al., 2022). In addition, the ability of fungi to adapt to different hosts is also highly diverse among species. The ability to colonize a single host differs significantly from infecting multiple hosts, whether endophytic or pathogenic. From a phytosanitary perspective, the most adaptive species, both in terms of lifestyle- and host-adaptation, are of greatest concern, as their adaptability drives rapid invasion of new environments, posing significant risks for disease emergence and spread (**Fig. 1**). Therefore, prioritizing research and monitoring of these highly adaptive fungal species is essential to mitigate the risks of disease emergence and protect agricultural and ecological systems.

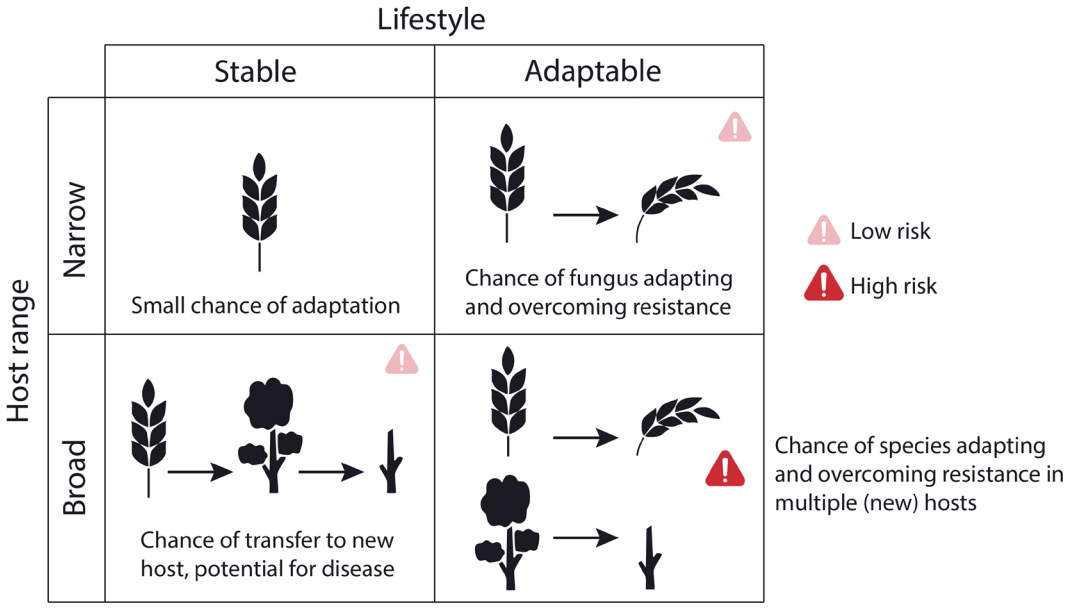


Fig. 1. Risk associated with lifestyle adaptation capability and host range.

Studying *Phyllosticta*

Despite the global significance of *P. citricarpa* as a citrus pathogen, its disease mechanisms remain poorly understood. A critical step toward unravelling these mechanisms is identifying key virulence genes. Through this work, we have made substantial contributions to genomic resources by providing high quality genomes of key *Citrus*-associated *Phyllosticta* species (including from their ex-type cultures where available) and developed CRISPR/Cas9-based gene-editing tools for this genus, significantly facilitating future virulence gene identification. We generated a set of genomes that can be used for future studies of virulence genes in **Chapter 3** and **Chapter 5**, while we introduce the first protoplasting and PEG-mediated CRISPR/Cas9 transformation protocol for *Phyllosticta* in **Chapter 4**. While an *Agrobacterium tumefaciens*-mediated transformation protocol was already available for *Phyllosticta* (Gomes-Figueiredo et al., 2010), our novel approach provides a faster and more efficient tool for uncovering virulence mechanisms in this genus while contributing to the expanding body of effective CRISPR/Cas9 applications (Schuster & Kahmann, 2019). However, numerous aspects of the protocol outlined here still hold potential for refinement. For instance, modifications like varying the CWDE incubation duration, selecting alternative enzymes, or applying milder centrifugation during protoplast isolation could yield greater numbers of robust protoplasts (Coca-Ruiz et al., 2024; Wilson et al., 2025). Consequently, the technique described should be regarded as an initial demonstration of feasibility, amenable to additional improvements aimed at boosting overall efficiency and precision.

In **Chapter 3**, we identified several genes with unknown functions that could be interesting targets for future research, which can be knocked out using our protocol developed in **Chapter 4**. However, conducting a knock-out screen to test candidate genes is hindered by the lack of efficient pathogenicity assays, which are currently time-consuming and logistically challenging. During the course of this study, we attempted to develop a modified detached fruit assay, inspired by previously described methods while incorporating reliable negative and positive controls which lacked in earlier studies (Guarnaccia et al., 2017; Miles et al., 2019; Wang et al., 2023). However, in most instances, when symptoms appeared, they did so not only with pathogenic species but also with those typically regarded as endophytic, such as *P. capitalensis* — likely attributable to excessive weakening of the fruit. The challenges in

establishing an effective detached fruit assay for *P. citricarpa* likely stem primarily from the extended latent period, which typically spans 4 to 6 months before symptoms manifest on fruit (Kiely, 1948; Kotzé, 1981). While protocols involving attached fruit exist (Baldassari et al., 2009; Miles et al., 2019; Moran Lemir et al., 2000), they hold substantial limitations, including the same prolonged latency, the annual fruiting cycle of many *Citrus* crops (Iglesias et al., 2007), and the considerable size of *Citrus* trees. Furthermore, as *P. citricarpa* is classified as a quarantine organism in the EU, it must be managed in a high-security (BPL-3) greenhouse, rendering experiments using whole plants logistically complex due to the limited availability and high costs of these facilities. An alternative experimental model was suggested by Tran et al. (2018), involving symptom induction on leaves of 2-month-old Troyer seedlings. However, leaf symptoms do not fully replicate those on fruit. Ultimately, the blotting method employed by Miles et al. (2019) appears to remain the best option, although it demands significant time investment, as fruits must remain on the tree until maturity for symptom evaluation. The development of a more efficient pathogenicity assay for *Phyllosticta* would be an invaluable tool for future studies into its disease mechanisms and should be a priority for anyone wishing to further study pathogenicity of *P. citricarpa*.

DNA, taxonomy and collaboration

Fungal taxonomy, the science of classifying, naming, and organizing fungi, plays a foundational role in multiple fields, from ecology and medicine to agriculture and conservation. While it may seem esoteric, investing effort in fungal taxonomy yields practical benefits that extend far beyond academic curiosity. It enables precise identification of pathogenic species, aiding in disease management for crops, animals, and humans, thereby reducing economic losses and health risks. Take for example the case of myrtle rust (*Austropuccinia psidii*) and its introduction and spread in Australia; the early identification of the rust as *Ustilago rangelii* and the introduction of the term “myrtle rust” (versus “Eucalyptus rust”) created confusion, leading to an underestimation of the incursion’s severity (Carnegie & Cooper, 2011; Carnegie & Pegg, 2018). In addition, by cataloguing fungal biodiversity, which has been estimated at millions of undescribed species (Hawksworth & Lücking, 2017), taxonomy helps increase our knowledge of ecosystem conservation, nutrient cycling, and symbiotic relationships critical

for soil and plant health. Ultimately, robust taxonomy provides evolutionary insights, may foster interdisciplinary research, and prevents nomenclatural confusion, ensuring effective communication, and global strategies for sustainability and emerging threats.

Fungal species concepts are inherently complex, with over 30 distinct frameworks proposed to date (Lücking et al., 2020). Essentially, species delimitation relies on three primary categories of properties (**Fig. 2**): biological (e.g., reproductive isolation), morphological (e.g., observable phenotypic traits), and molecular (e.g., genetic divergence, Lücking et al., 2020). In recent years, considerable debate has emerged regarding the permissibility of describing novel fungal species solely on the basis of DNA sequence data as the type specimen. The principal argument in favour is that this approach enables the formal recognition of unculturable or environmentally derived fungi (dark taxa), which constitute a substantial portion of undescribed biodiversity and are otherwise inaccessible through traditional methods (Hongsanan et al., 2018; Nilsson et al., 2023).

However, this strategy is not without significant drawbacks. DNA sequences are susceptible to sequencing artifacts, such as PCR chimeras or homopolymer errors, which may be erroneously interpreted as evidence of novel species in the absence of stringent validation protocols (Hongsanan et al., 2018). Although these errors are typically unintentional, the framework inherently permits fabrication. For instance, manipulated or fictitious sequences could be deposited in public repositories like GenBank, and without a physical voucher specimen, disproving them becomes exceedingly difficult (Thiele et al., 2023). The absence of a physical

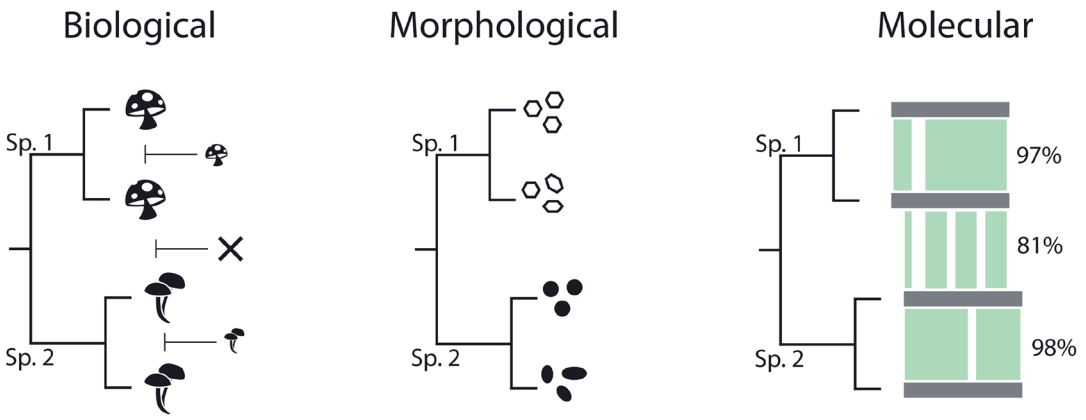


Fig. 2. Different properties on which species concepts may be based.

specimen also renders DNA-based descriptions intrinsically less reproducible (Thines et al., 2018). Sequences obtained from environmental samples, such as eDNA, cannot be re-examined in the manner comparable to that of a preserved fungarium voucher or deposited in a biobank as a metabolically inactive culture, and subsequent investigators might be unable to retrieve identical material to generate additional genomic loci (Hongsanan et al., 2018).

Compounding these issues is the lack of agreement on critical parameters, including appropriate gene regions, sequence lengths, or divergence thresholds for species recognition, resulting in varying methodologies and outcomes that are difficult to reproduce or compare across different research efforts. Consequently, some mycologists argue that voucher-less, or DNA-only types violate core scientific principles of testability and falsifiability, potentially turning aspects of mycology into a "pseudoscience" (Thines et al., 2018). Advocates argue that verifiability can be attained through rigorous quality assurance measures, including the deposition of raw data in public repositories, the application of multi-locus phylogenomics, or simply thorough peer-review scrutiny (Nilsson et al., 2023). Progress in sequencing technologies and bioinformatics tools is likely to mitigate certain artifact-related challenges. Still, even if consensus is reached, quality measures may fall short without physical vouchers, risking nomenclatural chaos and a flood of untestable names (Smith, 2024).

The mycological community must prioritize collaboration to reach consensus on critical issues, such as the integration of DNA-based nomenclature. Reaching such consensus, however, does not always seem to be the forte of mycologists. For instance, long-standing debates such as those surrounding the nomenclature of *Phytophthora* or the *Fusarium oxysporum* species complex have persisted for years, perpetuating confusion (Brasier et al., 2025; Lombard et al., 2019; O'Donnell et al., 2020). While some might argue that species renaming itself impedes advancement, and while we are not afraid to criticize the 'splitting' of some clades (such as *P. paracitricarpa* in this thesis), it seems to us that the true obstacle for these long-lasting discussions lies in an inability to truly connect and listen to one another. In an increasingly polarized world, we should set a positive example by uniting to resolve differences.

Agreeing on which names to apply to taxa forms the foundation of effective collaboration in mycology. Most countries maintain lists of regulated fungal species, such as those

on the EU quarantine list, to safeguard agriculture and trade. In this context, the ability to confidently identify these species is paramount—after all, it is impossible to exclude threats that cannot be reliably recognized. Thus, distinguishing harmful fungi from closely related species or even unrelated "look-alikes" is of vital importance, both to protect local growers from the potential damage caused by these organisms and to ensure the continuation of safe, effective trade. This thesis provides a compelling example: differentiating the regulated *P. citricarpa* from its non-regulated close relative *P. paracitricarpa* proved highly challenging, and our research described in **Chapter 5** revealed that they are, in fact, one and the same species. As a direct result of our work, both clades are now classified as *P. citricarpa* and subject to regulation. Combined with the other contributions outlined in this thesis, including a substantial expansion of genomic resources, detailed descriptions of carbon utilization patterns and genetic elements that differentiate pathogenic from endophytic species, and a proof-of-principle CRISPR/Cas9 protocol, this body of work advances fungal taxonomy, and lays important groundwork for future studies in *Phyllosticta* pathogenicity.

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SUMMARY

Citrus Black Spot, caused by the fungus *Phyllosticta citricarpa*, causes significant economic losses to global *Citrus* production. This thesis presents a comprehensive investigation into species of the fungal genus *Phyllosticta* that colonize *Citrus*. Through four experimental chapters, it addresses carbon utilization, genomic distinctions related to lifestyle variations, CRISPR/Cas9 applications, and taxonomy.

After a general introduction in **Chapter 1**, **Chapter 2** examines carbon utilization profiles across *Phyllosticta* species, revealing high similarity regardless of pathogenic or endophytic lifestyles. A notable finding is the selective inhibition of pathogenic species by sugar beet pulp (SBP) at low concentrations (0.25%), even when mixed with other substrates like wheat bran. This effect, absent in non-pathogens, suggests SBP's potential as a sustainable biocontrol agent. However, the specific inhibitory compound responsible for this effect remains unidentified. In addition, we assessed the CAZyme repertoires of species with different lifestyles but found only little variation.

Chapter 3 explores genomic differences between pathogens and endophytes, identifying minor variations in gene numbers, secreted proteins, and effectors. We found several genomic differences between species of different lifestyles, where unannotated pathogen-specific orthogroups present compelling targets for future virulence studies. We classified *Phyllosticta citrichinaensis* as having an intermediate lifestyle as it shares genomic and phenotypic attributes with both pathogens and endophytes. We also used CATAstrophy to elucidate CAZyme profiles across 116 *Dothideomycetes* genomes, and found no clear correlation between CAZyme repertoire and lifestyle reported in literature.

Chapter 4 develops a proof-of-principle CRISPR/Cas9 protocol for *Phyllosticta* via protoplasting and PEG-mediated transformation. We knocked out the pH-modulation gene *palH*, which has been well described in several fungi and reported to play a role in pathogenicity. We observed a decreased growth rate in Δ*palH* knockout mutants, but found no difference in pH modulation abilities. This tool provides the foundation to effectively study pathogenicity genes in *Phyllosticta*, though refinements could improve efficiency.

Chapter 5 resolves the *P. citricarpa* and *P. paracitricarpa* taxonomic debate. Through phylogenomic analyses using 3,000 single-copy ortholog genes and whole-genome comparisons, we established that the differences between *P. citricarpa* and *P. paracitricarpa* constitute infraspecific variation within *P. citricarpa*. We further assessed variation in mitochondrial genome assemblies across multiple *Phyllosticta* species and identified only minimal differences between the assemblies of *P. citricarpa* and *P. paracitricarpa*. Together with the fact that morphological features overlap, this data indicates that these species should be treated as synonyms.

The discussion (**Chapter 6**) reviews the previous chapters, notes lifestyle classification challenges, and debates DNA-only types. Overall, this thesis advances our understanding of *Phyllosticta* by uncovering genomic distinctions between lifestyles, presenting CRISPR tools, and resolving the *P. citricarpa*/*P. paracitricarpa* taxonomic debate, paving the way for refined disease strategies.

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Appendix

Authorship statement

PhD candidate's name : Valerie A. van Ingen-Buijs
First promotor : Prof. Dr. Pedro W. Crous
Title of PhD thesis : *Phyllosticta* on *Citrus:* exploring pathogenicity, genomics, and taxonomy
Date of public defence: 11 June 2026

Chapter 1. General Introduction. I contributed to refining the general research questions and wrote the text. I used AI (Grok) to improve the flow and linguistic clarity of some parts of the text. I revised the text after comments from my promotor and co-promotors.

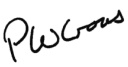
Chapter 2. Carbon utilization and growth inhibition of citrus-colonizing Phyllosticta *species.* I defined the research question, methodology and experimental design with the help of Prof. Dr. Ir. Ronald de Vries. I carried out the experiments together with an MSc student which I supervised. I performed the data analysis and wrote the text, which I revised after comments from the co-authors.

Chapter 3. Enemy or ally: a genomic approach to elucidate the lifestyle of Phyllosticta citrichinaensis. I defined the research questions. Experimental design and methodology were defined in collaboration with my (co-)promotors. With the exception of the genome sequencing, which was performed by the Joint Genome Institute, I carried out the experiments and performed the data analysis. I wrote the text, and revised it after comments from the co-authors.

Chapter 4. A protoplasting and PEG-mediated CRISPR/Cas9 transformation method for Phyllosticta. The research questions were defined together with my (co-)promotors. I defined the experimental design and methodology and performed the experiments together with an MSc student which I supervised. I performed the data analysis and wrote the text, which I revised after comments from the co-authors.

Chapter 5. Phyllosticta paracitricarpa *is synonymous with the EU quarantine fungus* P. citricarpa *based on phylogenomic analyses.* I defined the research questions. The methodology was defined together with my co-promotors. With the exception of the genome sequencing, which was performed by JGI, I carried out the experimental work and data-analysis together with an MSc student which I supervised. The generation of the BUSCO tree and genome-wide sharedness tree was done in collaboration with Anouk van Westerhoven. The generation of the clinker-alignment was done in collaboration with Petros Skiadas. I wrote the text, which I revised after comments from the co-authors.

Chapter 6. General Discussion. I defined the overall structure and drafted the text after an initial discussion with one of my co-promotors. I used AI (Grok) to improve the flow and linguistic clarity of some parts of the text. In response to feedback from my (co-)promotors, I revised the text.

Signature PhD candidate:  Signature promotor: 

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