MULTIFUNGICIDE RESISTANCE AND POPULATION GENOMICS OF *BOTRYTIS CINEREA* GREENHOUSE POPULATIONS

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ABSTRACT

Symptoms of Botrytis blight, a disease caused by the ubiquitous fungal plant pathogen *Botrytis cinerea*, include leaf spots, rot, damping off, and blight of petioles, flowers, leaves, and stems of ornamental crops. *B. cinerea* is regarded as the second most important fungal plant pathogen in the world partly due to its ability to cause extensive pre- and post- harvest losses in hundreds of economically valuable fruit, vegetable, and ornamental crops. Fungicides are important for Botrytis blight control and represent 10% of the global fungicide market. *B. cinerea* isolates (386) obtained from herbaceous ornamentals growing in Michigan greenhouses exhibited widespread multifungicide resistance; 63.5% were resistant to four or more site-specific fungicide classes and only 2.3% were sensitive to all fungicides. Resistance profiles of individual isolates were diverse (48 phenotypes). The most common profile (8.7%) demonstrated resistance to all site-specific fungicide classes including thiophanate-methyl (FRAC 1), fenhexamid (FRAC 17), iprodione (FRAC 2), fludioxonil (FRAC 12), boscalid/fluopyram (FRAC 7), cyprodinil (FRAC 9), and pyraclostrobin (FRAC 11). Resistance frequencies and profiles were not associated with location, year, or host.

Whole-genome sequencing was performed on 276 single-spored *B. cinerea* isolates from petunia, geranium, or poinsettia in 11 greenhouses during two growing cycles. *De novo* assembly and functional annotation were performed with isolates resistant (5) or sensitive (2) to all fungicides. Publicly available assemblies and corresponding annotation data are a resource for research on fungicide resistance, genetic relatedness among global crops and regions, and other *Botrytis* genomics-related studies. Reference-guided assembly of 276 isolate genomes enabled analyses of i) population structure stratified by multifungicide resistance, ii) novel genomic regions associated with multifungicide resistance, iii) mating type characterization, and iv) other

measurements of genetic differentiation based on fungicide resistance, crop, location, and other factors. Isolates with resistance to multiple fungicides (0-7) impacted population structure and were genetically differentiated as indicated by discriminant analyses of principal components, fixation index values, and analyses of molecular variance. A quantitative trait genome-wide association study identified genomic regions significantly associated with multifungicide resistance, including putative efflux pump genes. To date, this is the largest study of *B. cinerea* population genomics, prompting new questions and highlighting the need for novel management tools due to widespread fungicide resistance.

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CHAPTER 1: LITERATURE REVIEW

Introduction

Ornamental industry sales in the United States was worth an estimated $6.69 billion in 2022. Michigan's floriculture sales rank third in the nation and the state is a top producer of several major crops including begonia, geranium, impatiens, petunia, and poinsettia (USDA-NASS 2021). Plant categories with the greatest national contributions include annual bedding/garden plants, herbaceous perennial plants, and potted flowering plants (USDA-NASS 2023).

The fungal plant pathogen, *Botrytis cinerea* Pers. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is the causal agent of gray mold or Botrytis blight and can result in losses of many greenhouse-grown ornamentals (Williamson et al. 2007). This necrotrophic fungus can cause leaf spots, stem cankers, rots, damping off, and a blight of leaves, petioles, blossoms, and stems. Many valuable floriculture crops are susceptible to *B. cinerea*, including poinsettia, New Guinea impatiens, petunia, pansy, mums, and geranium (Hausbeck and Moorman 1996). Disease greatly impacts the esthetical value of floriculture crops and reduces their salability. The greenhouse environment is typically conducive for *B. cinerea* requiring that growers expend effort and money to control the pathogen. Disease control in the greenhouse is complicated by the wide variety of susceptible crops that are grown and the regular shifting of crops within the greenhouse that necessitate individualized pest control regimes (Bika et al. 2021). As *B. cinerea* is exposed to regular fungicide applications, resistant isolates are selected and may become widespread. As a result, certain fungicides may be unable to limit Botrytis blight and major losses can occur. The main objectives of this dissertation research are to assess the state of fungicide resistance in *B. cinerea* among ornamental greenhouse crops in Michigan and gain a better understanding of population genomics in relation to fungicide resistance and other factors.

Botrytis cinerea Biology and Epidemiology

The fungal genus *Botrytis* is comprised of several necrotrophic plant pathogenic species within the class Ascomycetes, order Helotiales, family Sclerotiniaceae (Hennebert 1973; Jarvis 1977). Although the genus was established in 1729, it was often originally confused with *Sclerotinia* spp. It was not until the 1940s that *Botrytis* was clearly distinguished, and the anamorphs were connected with their corresponding *Botryotinia* teleomorphs (Groves and Loveland 1953; Whetzel 1945).

The most notable species, *B. cinerea*, is a plant pathogen in temperate and tropical climates, and is even reported as a snow mold (Jarvis 1977; Williamson et al. 2007). The pathogen can infect a wide variety of hosts including nursery plants, vegetables, ornamentals, orchard crops, monocots, and other valuable plants comprising an estimated 586 genera (Elad et al. 2016). Due to its broad host range and ability to cause extensive pre- and post-harvest losses, *B. cinerea* is recognized as the second most important fungal plant pathogen in the world (Dean et al. 2012). Although damage by *B. cinerea* is difficult to measure, annual losses are thought to cost between $10 billion and $100 billion (Boddy 2015).

The fungus mostly infects above ground plant tissues including leaves, flowers, fruits, buds, and stems. Lesions start small but can progress quickly in optimal conditions and develop into large necrotic regions. Large, fluffy masses of dark conidia erupt from the lesions, giving rise to the disease name "gray mold" (Hausbeck and Moorman 1996). Macroconidia, the infectious propagule, measure an average of 10 x 8 μm and contain anywhere from 1 to 12 nuclei (Grindle 1979). Grape-like clusters of the conidia are released from straight, branched conidiophores inside lesions upon changes in relative humidity (RH) and are dispersed via wind, and sometimes via splash, to neighboring leaves or plants (Jarvis 1962a, b). Conidia may also be

disseminated by insects including thrips, moths, fungus gnats, and flies (Fermaud et al. 1994; Fermaud and Le Menn 1992; James et al. 1995; Louis et al. 1996). In greenhouse settings, conidia are aerially dispersed in association with grower activity events including irrigation, fertilization, movement of potted plants, and propagation (Hausbeck and Pennypacker 1991). Conidia serve an important role in dispersal and initial infection, but are short-lived, especially in soil (Coley-Smith 1980). The time length of conidial viability depends on the strain, tissue, and environmental conditions, among other factors. At room temperature, conidia may lose the ability to germinate after one month (Gindro and Pezet 2001) or germinate and form lesions after up to 14 months (Salinas et al. 1989).

B. cinerea conidia require at least 93% RH at room temperature, a film of water on the surface, and available nutrients for successful germination and subsequent infection (Carre 1984; Snow 1949). Flower petals often provide the initial nutrients in greenhouses, making them a common source of inoculum (Muñoz et al. 2019). Sometimes, solutes in leaf exudates contain sufficient nutrients for germination, enabling infection by *B. cinerea* (Brown 1922; Curtis 1943). On gerbera flowers, conidia swell and germinate under favorable conditions producing between one to five appressorium (Salinas and Verhoeff 1995). The germination speed varies across hosts and cultivars. On poinsettia leaves, conidia germinate within approximately 2h, then nutrient availability plays a large role in increasing germination on highly susceptible cultivars (Martínez-Fernández et al. 2004). Following germination, mucilage adheres the germ tube to the leaf as it grows along the surface (Doss et al. 1995). When an cell wall juncture is encountered, the tip of the germ tube forms an appressorium. As turgor pressure increases in the appressorium, a hyphal peg penetrates the cuticle for successful infection (McKeen 1974). Alternatively, conidial germ tubes can penetrate the host directly or form an infection cushion prior to entry

(Pie and De Leeuw 1991; Van den Heuvel and Waterreus 1983). Under high RH, lesions can appear following inoculation in less than 1 day on rose petals (Pie and Brouwer 1993; Williamson et al. 1995), 2 days on gerbera petals (Salinas and Verhoeff 1995), and 3 days on poinsettia leaves (Martínez-Fernández et al. 2004).

Cell-wall degrading enzymes, a range of toxins, and low-molecular-weight compounds contribute significantly to the initial penetration of the plant's surface. Botrydial, the most-studied phytotoxic metabolite of *B. cinerea* (Colmenares et al. 2002), induces the hypersensitive response in plant tissue and facilitates pathogen invasion (Rossi et al. 2011). Botrydial is considered an important virulence factor although its contribution in the infection process varies. Many strains of *B. cinerea* rely heavily on botrydial for infection, while the toxin has a minimal effect on virulence in other strains (Siewers et al. 2005). Toxin production is widely variable across strains and an increase in toxins is correlated with aggressiveness (Siewers et al. 2005). Some of the most aggressive strains produce botcinolide and/or its derivatives (Reino et al. 2004). Other phytotoxins include botcinins E and F (Tani et al. 2006) and sesquiterpenoid metabolites (Durán-Patrón et al. 2000). *B. cinerea* produces several enzymes including Cu-ZN-superoxide dismutase (Rolke et al. 2004), NADPH oxidases (Segmüller et al. 2008), endopolygalacturonases (Have et al. 1998), xylanase (Noda et al. 2010), and a cerato-platanin family protein (Frías et al. 2011) that aid in killing host cells. Genetic analyses have suggested that the species is under strong selection pressure for genes encoding phytotoxic products (Staats et al. 2007).

B. cinerea may overwinter as sclerotia which are melanized resting bodies measuring up to 4 mm in diameter (Hsiang and Chastagner 1992; Thomas et al. 1983). Sclerotia germinate to produce mycelium and conidiophores bearing macroconidia, which serve as primary inoculum

(Hsiang and Chastagner 1992). During the sexual cycle, sclerotia may also give rise to an apothecium, a cup-shaped structure bearing asci consisting of 8 ascospores, usually 1-5 mm in diameter. This form marks the teleomorph stage, historically referred to as *Botryotinia fuckeliana*, and is rarely observed in nature (Jarvis 1977). Chlamydospores, another survival structure, are thick hyaline cells, terminal or intercalary, with variable structure which form during periods of stress, remaining viable up to three months in drought conditions (Urbasch 1983). Chlamydospores have been shown to play a role in the survival of *B. cinerea* on greenhouse-grown fuchsia (Urbasch 1986). Hyphae and microconidia can arise from chlamydospores (Urbasch 1983). Microconidia are often observed in aging or contaminated cultures and can also survive harsh conditions, as supported by an ultrastructural analysis revealing a thick outer wall (Urbasch 1985). Averaging 3 µm in diameter, microconidia can also arise from hyphae, conidiophores, or macroconidia directly (Grindle 1979). After infection, mycelium of *B. cinerea* can enter a latent phase, also known as quiescent infection, which can serve as a third overwintering mechanism. This survival stage is characterized by non-progressive lesions until conditions become favorable and the mycelium becomes active again (Holz et al. 2007). Mycelium appears to be an especially important form of overwintering in greenhouse crops, where the environment is comparatively mild (Raposo et al. 2001). While mycelium of *B. cinerea* may enter a latent phase in some decaying plant tissues, it can also act as a saprophyte feeding on plant debris on surface soil. The pathogen can actively sporulate on dead tissue, serving as an important source of secondary inoculum (Johnson and Powelson 1983), especially in greenhouses where petals and leaves are common on soil surfaces.

Genetics and Diversity

The genetic architecture of *B. cinerea* is well-known for its extreme variability and complexity. Hyphal cells and conidia of *B. cinerea* are multinucleate, with an average of 3-6 nuclei but may contain significantly more (Grindle 1979). Microconidia and asci are uninucleate, giving rise to ascospores containing 4 nuclei (Lorenz and Eichorn, 1983). While generally regarded as a haploid, ploidy levels may vary (Büttner et al. 1994). The reference genome *B. cinerea* B05.10 contains 16 core chromosomes and 2 accessory chromosomes comprising an estimated genome length of 42.6 Mb (Staats and van Kan 2012; Van Kan et al. 2017). Many extrachromosomal elements including mitochondria, mitochondrial plasmids, transposable elements, and mycoviruses are readily transmitted via conidia, and sometimes ascospores. These features all have significant contributions to the genetic variation seen in *B. cinerea*. It is also likely that sexual recombination plays a key role in population diversity as evidenced by an even ratio of mating types (De Miccolis Angelini et al. 2016).

B. cinerea is regarded as a heterothallic fungus with an idiomorphic mating locus (*MAT1*) containing genes corresponding to either *MAT1-1* or *MAT1-2* mating types (Faretra et al. 1988). Mating types are commonly PCR identified in an approximate 1:1 ratio, regardless of crop or region, suggesting the regular occurrence of sexual recombination despite a lack of reported ascocarps (Kanetis et al. 2017; Wessels et al. 2016; Williamson et al. 2007; Zhang et al. 2018). Monoconidial isolates with the ability to form ascocarps with either mating type, also known as dual-mater (*MAT1-1/2*), are rarely reported and the genetic basis is unclear (Delcan and Melgarejo 2002; Faretra et al. 1988; Van der Vlugt-Bergmans et al. 1993). Low incidence may be biased due to the tedious processes involved in conducting sexual compatibility assays in strains of *B. cinerea* (Faretra and Grindle 1992). While the dual-mater phenotype has been

observed, there are no reports confirming the matching genotype (both alleles in a monoconidial isolate). Researchers have hypothesized that heterokaryosis, the presence of genetically distinct nuclei in the same cell, or heteroduplex DNA, are possible explanations for the observed cases of homothallic behavior (De Miccolis Angelini et al. 2016). In the classical parasexual cycle described for *Aspergillus niger*, hyphae and nuclei fuse to form a heterokaryon before the resulting diploid may either continue to divide as a diploid strain or resolve back to its original haploid form (Pontecorvo et al. 1953). Genomic regions controlling heterokaryon incompatibility in *B. cinerea* were recently described and may contribute to the theory explaining monoconidial dual-maters (Arshed et al. 2023).

Giraud et al. (1997) used restriction fragment length polymorphism (RFLP) markers to show genetic recombination within *B. cinerea*, leading to the discovery of restricted gene flow of transposable elements between two groups. Strains were often classified based on their presence of two transposons: *Boty* (Diolez et al. 1995) and *Flipper* (Levis et al. 1997). The presence of both elements is labeled *transposa*, whereas the lack of both is known as *vacuma*. Previously, *B. cinerea* "group I" included *vacuma* strains only and "group II" included *Boty* only, *Flipper* only, and *transposa*. "Group I" was eventually identified as *B. pseudocinerea*, but because some strains of this species are *transposa*, the group classification system is no longer useful (Walker et al. 2011). Several other cryptic species have since been discovered living in sympatry with *B. cinerea* (Garfinkel 2021).

Numerous molecular techniques have been utilized to characterize *B. cinerea* populations. Commonly-used tools include RAPD fingerprinting (Kerssies et al. 1997; Moyano et al. 2003; Van der Vlugt-Bergmans et al. 1993) and PCR detection of transposable elements and microsatellites (Albertini and Leroux 2004; Albertini et al. 2002; Diolez et al. 1995;

Fournier et al. 2002; Levis et al. 1997; Ma and Michailides 2005). With the recently available gapless genome sequence of *B. cinerea*, future research can now perform in-depth analyses focused on genetic variation and functional genomics on a larger scale (Van Kan et al. 2017).

Diversity is more often found within populations, regardless of stratification, as opposed to between. Factors found to impact *B. cinerea* population structure include crop, location, and fungicide resistance, though their individual impact is highly inconsistent (Mercier et al. 2021; Naegele et al. 2022; Walker et al. 2015). It is difficult to compare the diversity and impact of individual factors among different studies because of variable methodology and tools, in addition to apparent disparities in standing diversity. Some tomato greenhouse studies report that more diversity exists within greenhouses, suggesting a low degree of *B. cinerea* inoculum spread among sites (Adjebli et al. 2015; Bardin et al. 2014; Karchani-Balma et al. 2008). However, ornamental systems are unique for their host diversity, plant movement among locations, and restricted climatic environment (Bika et al. 2021), which could result in different population structure.

Cultural Disease Management

As a ubiquitous pathogen, successful control of *B. cinerea* requires an integrated approach. Effective cultural practices are especially important due to its propensity for developing fungicide resistance. Greenhouses offer variable climate and crop diversity, requiring highly specialized disease management plans (Daughtrey and Buitenhuis 2020; Dik and Wubben 2007). These programs can be adjusted based on each crop's level of susceptibility, lighting and temperature requirements throughout growth stages, and the registered fungicides.

Greenhouse sanitation is one of the most important practices because *B. cinerea* infects dead tissue, surviving as mycelia, conidia, or sclerotia in crop debris for extended periods.

Removing dead and infected plant material from production areas can help reduce disease incidence rates (Jacometti et al. 2007).

Greenhouse environmental manipulation is a key cultural tool to reduce disease pressure in greenhouses. Maintaining low RH and reducing the leaf wetness period can prevent spore germination and includes watering early to allow quick evaporation, avoiding overhead irrigation, venting and heating, and maintaining air flow within the canopy by properly spacing pots (Dik and Wubben 2007). Light quality significantly effects multiple life stages of *B. cinerea* and has potential as a disease management tool. Monochromatic lighting differentially affects conidiation and sclerotia development (Schumacher 2017), but has a limited effect on growth rate (Meng et al. 2020). Near-ultraviolet light and far-red light increase conidia production, while wavelengths in between, within the visible light range, appear to have the opposite effect (Tan 1975). In addition, blue light inhibits sclerotia development (Tan 1975) and green light slows mycelial growth (Zhu et al. 2013). There are conflicting data regarding the impact of lighting on growth rate (Meng et al. 2020; Schumacher 2017; Zhu et al. 2013) which may be explained by lighting intensity and/or other experimental differences, the impact of the crop's response to lighting, and individual phenotypes of *B. cinerea* isolates. There is notable diversity in the phenotypic responses among *B. cinerea* isolates, complicating the ability of monochromatic light a disease management tool. Recently, Schumacher (2017) described 11 putative photoreceptors that aid in regulating morphogenesis, tropism, entrainment and stress responses, underlining the complex relationship between lighting and *B. cinerea*. In the future, research on the association between disease pressure and greenhouse lighting may yield novel control practices. Recommended practices must also consider the significant impact that lighting has on the growth and flower development of ornamental crops (Paradiso and Proietti 2022).

Breeding for resistance is difficult due to the need to maintain the aesthetic features of ornamentals and the lack of resistant germplasm. Differences in ornamental cultivar susceptibility has been described among geranium (Shrestha and Hausbeck 2021), petunia (Shrestha and Hausbeck 2023), cut roses (Pie and Brouwer 1993), lisianthus (Wegulo and Vilchez 2007), and peony (Tian et al. 2019). While there are no ornamental cultivars with complete resistance to *B. cinerea*, choosing less susceptible cultivars can be helpful in an integrated control program. Transgenic approaches, including some targeting chitinase genes, are more promising than conventional breeding, but more research is needed (Bika et al. 2021).

Biological Control

As multifungicide resistance concerns rise, biological control options have been increasingly valuable for both disease management and fungicide resistance management programs. Biorational fungicides targeting *B. cinerea* may contain active ingredients derived from fungi, bacteria, or plants. While biorational products are generally regarded as a safer option than traditional synthetic fungicides, the unintended consequences and hidden risks have been an important recent discussion (Feldmann and Carstensen 2018; Haddi et al. 2020). Mechanisms behind biological control options can be based on competition for nutrients and space, antibiosis, mycoparasitism, induced resistance in the host, and other direct or indirect methods that target pathogen viability (Elad 1996).

There is a wide range of biocontrol options available for Botrytis blight on greenhouse ornamentals with varying degrees of efficacy. Greenhouse trials comparing the efficacy of biorational products for Botrytis blight on petunia found that *Gliocladium catenulatum* was able to provide a level of control similar to fenhexamid, a conventional fungicide (Shrestha and Hausbeck 2023). Suspensions of other fungi such as *Ulocladium atrum* and *Gliocladium roseum*

can effectively reduce disease pressure in greenhouses via colonization of dead leaves, a common source of pathogen inoculum (Köhl et al. 1998). Botrytis blight on petunia and geranium was suppressed after regular applications of bacterial suspensions including *Pseudomonas* spp. (Shrestha and Hausbeck 2021; South et al. 2020). De Meyer et al. (1998) demonstrated the ability of *Trichoderma harzianum* strain T-39 as a gray mold antagonist. Although the commercialized product (TRICHODEX, produced by Makhteshim Chemical Works Ltd, Be'er Sheva, Israel) is marketed for grapes, it has been used in greenhouses with mixed reports of disease control (Barakat and Al-Masri 2017; Hjeljord et al. 2000). Greenhouse-grown tomatoes had reduced disease severity following applications of essential oils from a variety of aromatic plants, with origanum essential oil yielding promising results (Soylu et al. 2010).

In contrast to synthetic fungicides, the efficacy of biological control depends on environmental conditions including temperature, lighting, RH, previous fungicide applications, irrigation timing, host microbiome, and host growth stage and structure. Due to variation in levels of disease control, biological products are often recommended for application in a rotational program alongside synthetic fungicides (Bika et al. 2021).

Fungicides and Fungicide Resistance

Paired with good sanitation practices and environmental management, fungicides can aid in preventing infection and growth of *B. cinerea*. An estimated €1 billion is spent on chemicals and other tools to mitigate diseases incited by *B. cinerea* on an annual basis (Dean et al. 2012). There are several chemical classes available for disease control via foliar application, consisting of both broad-spectrum and site-specific fungicides. Several products have been used for decades and as a result, fungicide resistance is an increasingly concerning problem in cropping systems

that battle diseases caused by *B. cinerea*. The fungicide resistance action committee (FRAC) classifies *B. cinerea* as a 'high-risk' pathogen due to its ability to develop widespread resistance quickly (Brent and Hollomon 1998). Growers are encouraged to incorporate fungicide resistance management programs, often involving the practice of rotating chemical classes, to slow the spread of resistance in populations of *B. cinerea*.

Multisite inhibitors, including dithiocarbamates (thiram and mancozeb) and phthalimides (captan), have been used again *Botrytis* since the 1940s (Hahn 2014). Because these fungicides have a non-specific mode of action, the risk of resistance development is relatively low (Brent and Hollomon 1998). Nonetheless, stable and inheritable cross resistance has been reported against multisite inhibitors with minimal fitness costs (Barak and Edgington 1984; Hsiang and Chastagner 1992). Multisite inhibitors are considered less efficacious against Botrytis blight than site-specific fungicides and are not commonly used alone to target *B. cinerea* today (Hahn 2014).

The first widely used site-specific fungicides for *Botrytis* control were benzimidazoles (e.g. thiophanate-methyl) beginning in 1968. Benzimidazoles target β-tubulin assembly and inhibit cytoskeleton formation in most ascomycete fungi (Leroux et al. 2002). Resistance in an isolate of *B. cinerea* from greenhouse-grown cyclamen was reported 2 years after its introduction (Bollen and Scholten 1971). Single-nucleotide polymorphisms (SNPs) in a β-tubulin gene conferring resistance are exceedingly stable, persisting in populations that have not been exposed to benzimidazoles for extended periods of time (Yourman et al. 2001). Today, caution is advised when choosing to use thiophanate-methyl to target *B. cinerea* (Mobasher Amini et al. 2023).

Dicarboximides (e.g. iprodione) were the next available site-specific fungicide in 1975. This class of fungicides interferes with the osmotic signal transduction pathway (Cui et al. 2004). There are several mutations within the *bos1* gene that confer resistance (Grabke et al. 2014;

Oshima et al. 2002), though resistant isolates tend to be unstable and are shown to have decreased sporulation and reduced sclerotia viability inside and outside of greenhouses (Hsiang et al. 1991; Raposo et al. 2000).

The anilinopyrimidines fungicides (e.g., cyprodinil), available since 1994, prevent elongation of the germ tube and inhibit mycelial growth preventing infection. Biochemical analyses suggest that these compounds inhibit methionine synthesis (Fritz et al. 1997), but the exact mechanism has not been elucidated. Anilinopyrimidines also prevent *B. cinerea* from secreting extracellular proteases associated with symptoms (Milling and Richardson 1995, Miura et al. 1994). High resistance is a genetically stable trait in *B. cinerea* and is correlated with hypersensitivity to osmotic stressors (Bardas et al. 2008).

Fludioxonil, the only registered chemical belonging to the phenylpyrrole class, is a non-systemic and protective foliar fungicide that was first used in 1995. The mode of action is not entirely understood, but it inhibits conidial germination, germ tube elongation, and mycelial growth. Few isolates have been reported to demonstrate high resistance due to mutations in *Bos1* (Dowling et al. 2021; Ren et al. 2016).

Fenhexamid, a hydroxyanilide, is a locosystemic fungicide that has effectively controlled *B. cinerea* since 1999 (Rosslenbroich 2000). Like the anilinopyrimidines, this fungicide stops developmental stages of the pathogen such as germ tube elongation and mycelial growth. Specifically, fenhexamid works by inhibiting 3-ketoreductase of the C-4-demethylation enzyme complex during ergosterol biosynthesis (Albertini and Leroux 2004). Mutations in *erg27*, the gene encoding 3-ketoreductase, are common and confer varying levels of resistance (Amiri and Peres 2014; Fillinger et al. 2008). Recent work has shown that applying a mixture of a low

dosage of fenhexamid and captan may be an effective tool in a fungicide resistance management program (Boushell and Hu 2024).

Quinone outside inhibitors (QoI) were introduced in 1996 and include the strobilurins (e.g., azoxystrobin, pyraclostrobin, and trifloxystrobin). These chemicals kill *B. cinerea* and other fungi by binding to a cytochrome complex within the electron transport chain and inhibiting mitochondrial respiration (Bartlett et al. 2002). Resistant isolates have the same fitness as sensitive isolates (Karaoglanidis et al. 2010; Veloukas et al. 2014) and are often attributed to the G143A mutation in the mitochondrial gene *cytb* (Banno et al. 2009).

Succinate dehydrogenase inhibitors (SDHI; e.g., boscalid and fluopyram) were first used against *Botrytis* in 2004. Several newer efficacious fungicides have been developed with varying degrees of cross-resistance including, but not limited to, pydiflumetofen (He et al. 2020), penthiopyrad, isofetamid, and benzovindiflupyr (Zuniga et al. 2020). SDHIs target subunits of the succinate dehydrogenase enzyme, thus inhibiting respiration. There are several mutations in genes encoding succinate dehydrogenase subunits, especially *sdhB*, that confer resistance. Unlike other fungicide classes, isolates of *B. cinerea* can develop resistance to one SDHI (boscalid) and remain sensitive to another (fluopyram) depending on the mutation (Lalève et al. 2014).

Multifungicide resistance has been reported in greenhouse populations of *B. cinerea* around the world and, in some instances, includes resistance to all common site-specific fungicide classes (Fan et al. 2017; Malandrakis et al. 2022; Raposo et al. 1995; Rupp et al. 2017; Samarakoon et al. 2017). Hu et al. (2016) proposed the idea of 'selection by association' in *B. cinerea*, a theory that isolates with resistance to a specific fungicide (namely, thiophanate-methyl) are more likely to develop resistance to a second specific fungicide. Over time, an accumulation of SNPs conferring resistance to individual fungicides may lead to the

multifungicide resistant phenotypes that are commonly observed today. Alternatively, efflux-based mechanisms conferring resistance to multiple fungicides at once has been described in field populations of *B. cinerea* (Kretschmer et al. 2009; Leroch et al. 2011). There are two classes of efflux pumps that are known to confer multifungicide resistance in *B. cinerea*. The first mechanism is a mutation in *mrr1* (*m*ultidrug *r*esistance *r*egulator 1) that results in the constitutive overexpression of an ABC (ATP synthase-binding cassette) transporter. The other mechanism, likely to have occurred in a single European isolate, is a rearrangement in the promoter region of an MFS (major facilitator superfamily) gene (Kretschmer et al. 2009). Both phenotypes, MDR1 and MDR2, respectively, demonstrate reduced sensitivity to several site-specific fungicides including, but not limited to, iprodione, cyprodinil, and fludioxonil. Efflux-mediated multifungicide resistance mechanisms present a new challenge for fungicide resistance management programs.

Currently, fungicide resistance management research today is often focused on developing new chemistries or employing alternative disease management tools. Strategies include inducing plant resistance, using novel biological control options, and optimizing environmental manipulation (Shao et al. 2021). Monitoring fungicide resistance frequencies and increasing current knowledge of *B. cinerea* resistance mechanisms and spread can improve Botrytis blight disease management in greenhouses.

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CHAPTER 2: RESISTANCE TO SEVEN SITE-SPECIFIC FUNGICIDES IN *BOTRYTIS CINEREA* FROM GREENHOUSE-GROWN ORNAMENTALS

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Summary

The fungal pathogen *Botrytis cinerea* is a notorious problem on many floriculture greenhouse hosts including petunia, geranium, and poinsettia; these key crops contribute to the $6.43 billion U.S. ornamental industry. While growers use cultural strategies to reduce relative humidity and free moisture to limit Botrytis blight, fungicides remain a primary component of control programs. Isolates (n = 386) of *B. cinerea* sampled from symptomatic petunia, geranium, and poinsettia in Michigan greenhouses from 2018 to 2021 were screened for resistance to eight fungicides belonging to seven Fungicide Resistance Action Committee (FRAC) groups. Single-spored isolates were subjected to a germination-based assay using previously defined discriminatory doses of each fungicide. Resistance was detected to thiophanate-methyl (FRAC 1; 94%), pyraclostrobin (FRAC 11; 80%), boscalid (FRAC 7; 67%), iprodione (FRAC 2; 65%), fenhexamid (FRAC 17; 38%), cyprodinil (FRAC 9; 38%), fludioxonil (FRAC 12; 21%), and fluopyram (FRAC 7; 13%). Most isolates (63.5%) were resistant to at least four FRAC groups, with 8.7% of all isolates demonstrating resistance to all seven FRAC groups tested. Resistance frequencies for each fungicide were similar among crops, production regions, and growing cycles but varied significantly for each greenhouse. Phenotypic diversity was high, as indicated by the 48 different fungicide resistance profiles observed. High frequencies of resistance to multiple fungicides in *B. cinerea* populations from floriculture hosts highlight the importance of sustainable and alternative disease management practices for greenhouse growers. Sample collection, subsequent research, data analysis, and writing for this publication was performed by Nicole Taylor Lukasko.

CHAPTER 3: WHOLE-GENOME RESOURCE OF SEVEN *BOTRYTIS CINEREA* SENSU LATO ISOLATES WITH RESISTANCE OR SENSITIVITY TO SEVEN SITE-SPECIFIC FUNGICIDES

SOURCE:

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Summary

Botrytis cinerea is among the most important fungal pathogens in ornamental greenhouses due to its ability to cause severe Botrytis blight on various crops throughout the year. Fungicides are a key component in disease management programs, but frequencies of resistance in pathogen populations are notoriously high, leading to its role as a model system for fungicide resistance research. Here, we present seven high-quality genomes of North American *B. cinerea* sensu lato isolates collected from ornamental greenhouses with resistance (five) or sensitivity (two) to seven chemical classes representative of all commonly used site-specific fungicides. This resource can contribute to the exploration of multifungicide resistance mechanisms, host specificity, and geographic relatedness in populations of *B. cinerea*. Sample collection, data analysis, and writing for this publication was performed by Nicole Taylor Lukasko.

CHAPTER 4: NOVEL GENOMIC REGIONS ASSOCIATED WITH MULTIFUNGICIDE RESISTANCE IN *BOTRYTIS CINEREA* AND FACTORS IMPACTING GREENHOUSE POPULATION STRUCTURE

Abstract

Fungal plant pathogen *Botrytis cinerea* affects hundreds of valuable crops including fruits, vegetables, and ornamental plants. Greenhouse growers rely heavily upon fungicides as a key tool in disease management programs, though multifungicide resistance is a widespread phenomenon. An improved understanding of *B. cinerea* populations can contribute to the development of resistance management strategies. Isolates (276) of *B. cinerea* were collected from ornamental greenhouses and whole-genome resequenced in an effort to evaluate genetic differentiation among hosts, locations, growing cycles, and number of fungicides that an isolate is resistant to. Discriminant analyses of principal components and analyses of molecular variance revealed limited genetic differentiation among isolates from different hosts, greenhouses, and years. In contrast, the same analyses alongside pairwise fixation indexes and an evaluation of population structure indicated significant genetic differentiation among isolates based on the number of fungicides that it was resistant to. There are two described mechanisms that confer resistance to multiple fungicides at the same time, both of which are mediated by efflux pumps. Results from a quantitative trait genome-wide association study revealed novel genomic regions associated with multifungicide resistance, including two genes that encode putative efflux pumps.

Introduction

Botrytis cinerea is a notorious fungal pathogen of many high-value crops including strawberry, grape, and ornamental crops in addition to over 500 other species (Williamson et al. 2007). Petunia, geranium, and poinsettias are valuable ornamental commodities in the United States and are especially susceptible to *B. cinerea* (Hausbeck and Moorman 1996). Global losses attributed to diseases caused by *B. cinerea* are estimated to exceed $10 billion annually (Hua et al. 2018), with an estimated €1 billion spent on exclusively disease management tools (Dean et al. 2012). While environmental manipulation practices can effectively decrease disease pressure, greenhouse growers still rely heavily on chemical control measures to reduce disease levels. A high rate of multifungicide resistance as well as resistance to all available site-specific fungicides has been detected in isolates from ornamental greenhouses (Lukasko and Hausbeck 2024a), highlighting a need for greater understanding of pathogen behavior, improved resistance management strategies, and alternative disease control tools.

Population diversity and structure in *B. cinerea* is historically difficult to assess, partly due to the highly polymorphic nature of the species. Genotyping methods used to assess population structure and diversity among *B. cinerea* populations often include characterizing microsatellites, transposable elements, and mating types (DeLong et al. 2020; Fournier and Giraud 2008; Ma and Michailides 2005; Naegele et al. 2022). Recent technological advancements have provided the ability to whole-genome sequence (WGS) multiple isolates of the same species for a more comprehensive analysis of plant pathogen populations (Grünwald et al. 2016). To date, there are few studies that have employed WGS to investigate populations of *B. cinerea*, each with less isolates than this research (Atwell et al. 2015a; Mercier et al. 2021).

A signification proportion of the genetic variation is often found within *B. cinerea* populations, as opposed to between (Alfonso et al. 2000; Ma and Michailides 2005). Genetic differentiation among samples from different hosts plants has been found comparing isolates of *B. cinerea* from grape and tomato (Mercier et al. 2021; Muñoz et al. 2002) and grapevine and bramble (Fournier and Giraud 2008). Host was found to have a minimal impact on population structure in isolates from California small fruits, while significant genetic differentiation was detected among multifungicide resistant isolates (Naegele et al. 2022). Previous research has demonstrated that while many factors including host, time, location, and fungicide resistance impact the population structure and overall diversity in *B. cinerea* populations, their individual weight is highly inconsistent. It is likely that the cropping system and practices are important structuring factors, which could explain the differences detected among previous reports (Walker et al. 2015).

Ornamental greenhouse practices differ significantly from most other cropping systems. Single greenhouses often grow a variety of ornamentals plants, most of which are susceptible to *B. cinerea* (Hausbeck and Moorman 1996). Diverse plants are added, removed, and moved around within greenhouses on a regular basis, contributing to pathogen dispersal (Daughtrey and Buitenhuis 2020). Del Castillo Múnera et al. (2019) found that there was a common source of soilborne *Pythium* inoculum among Michigan floriculture greenhouses, suggesting that infected plant material may be spread among greenhouses or arrive on a commodity purchased by many growers. Human-mediated activity may have a larger weight on pathogen dispersal in greenhouse settings compared to other crops, such as strawberries or grapes.

The goals of this research are to utilize whole-genome sequencing data to i) confirm *B. cinerea* is the dominant pathogen of Botrytis blight in ornamental greenhouses, ii) evaluate

genetic differentiation and population structure among hosts, greenhouses, growing cycles, and fungicide response phenotypes, and iii) identify genomic regions associated with multifungicide resistance.

Materials and Methods

A total of 276 single-spored *Botrytis cinerea* isolates were analyzed in this research. Samples were collected as described in Lukasko and Hausbeck (2024a). Isolates were collected from symptomatic petunia (n=110), geranium (n=118), or poinsettia (n=48) in Michigan ornamental greenhouses (11) between 2018 and 2021 (**Table 1**). Growing cycle 1 includes isolates from poinsettia in 2018 and geranium and petunia in 2019. Growing cycle 2 includes isolates from poinsettia in 2019 and geranium and petunia in 2021. Isolates were not collected in 2020 due to the COVID-19 pandemic. Approximately half of the isolates (n=163) were included in the previous study where they were screened for resistance to thiophanate-methyl, boscalid, fluopyram, iprodione, fenhexamid, fludioxonil, pyraclostrobin, and cyprodinil in a discriminatory dose germination-based assay (Lukasko and Hausbeck 2024a).

Table 1. Isolates of *Botrytis cinerea* collected from greenhouses and whole-genome sequenced.

		Greenhouse											
Crop	Growing Cycle	B	E	I	L	M	O	Q	R	W	X	Y	Total
Geranium	1	2	12	20	16	4	1	3	--	--	--	--	58
	2	4	18	17	14	4	--	3	--	--	--	--	60
Petunia	1	12	--	10	--	--	3	--	4	14	4	17	64
	2	9	--	6	--	--	--	--	4	4	3	20	46
Poinsettia	1	10	12	--	--	--	--	--	--	--	--	--	22
	2	11	15	--	--	--	--	--	--	--	--	--	26
Total	--	48	57	53	30	8	4	6	8	18	7	37	276

High-quality genomic DNA was extracted and sequenced as previously described (Lukasko and Hausbeck 2024b). In brief, single-spored isolates were grown on V8 agar (16 g/L agar, 840 mL/L water, 5.5 fl oz/L V8 juice (Campbell Soup Company, Camden, NJ), 3 g/L calcium carbonate) for 12-14 days. DNA was extracted from fungal tissue with the Applied Biosystems MagMAX Plant DNA Isolation Kit (ThermoFisher Scientific, Waltham, MA, U.S.) and submitted to the Michigan State University (MSU) Genomics Core for library preparation and sequencing. Libraries were prepared using the Illumina TruSeq Nano DNA Library Preparation Kit and sequenced using an Illumina NovaSeq 6000 SP or S4 flow cell in 150 bp paired end format. Quality was analyzed with MultiQC (Ewels et al. 2016) followed by filtering and trimming of reads with fastp (Chen et al. 2018), removing reads with a phred score below 30.

Filtered reads were mapped to the *MAT1-2* locus in *B. cinerea* SAS405 (KF944386.1) (De Miccolis Angelini et al. 2016), while the B05.10 reference genome MAT locus served as the reference for *MAT1-1* (Van Kan et al. 2017). Isolates were classified as *MAT1-1* if they had zero missing bases within the reference genome region 1:813500-815500 and an average read depth of at least 3x. This region and read depth were chosen to avoid depth bias due to a truncated form of *MAT1-1-1* found in isolates regardless of mating type (De Miccolis Angelini et al. 2016). Isolates were classified as *MAT1-2* if SAS405 reference sites 1000-3000 contained 10 or fewer missing bases and had an average read depth of 3x or greater. Isolates were classified as *MAT1-1/2* if the criteria for both mating types were met. Additionally, isolates that were questionably both mating types had fewer than 1500 missing bases for the opposing mating type. A subset of isolates (5 *MAT1-1/2*, 6 *MAT1-1*, and 4 *MAT1-2*) were tested using an established PCR reaction with the mating type primer pairs Y (*MAT1-1*) and HMG.SP (*MAT1-2*) (De Miccolis Angelini et

al. 2016). ITS primer pair ITS1 and ITS4 served as a control. Reaction conditions were 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 20 s, and extension at 72°C for 1 min, followed by the final extension at 72°C for 7 min. PCR products were run on a 1.5% agarose gel and visualized with BioRad GelDoc XR+.

Reads were aligned to the *Botrytis cinerea* B05.10 reference genome (Ensembl assembly ASM83294v1) (Van Kan et al. 2017) using BWA-MEM2 (Vasimuddin et al. 2019). Sam files were converted to bam files, then sorted and indexed using SAMtools v1.16.1 (Li et al. 2009). Variants were called with -ploidy 1 using Genome Analysis Tool Kit (GATK) V4.2.5.0 HaplotypeCaller (HC) (Poplin et al. 2018) and combined into a single gVCF and genotyped with GATK (McKenna et al. 2010). Hard filtration of single-nucleotide variants (SNVs) and indels was performed using GATK SelectVariants and VariantFiltration following GATK recommendations (https://gatk.broadinstitute.org/hc/en-us/articles/360035531112--How-to-Filter-variants-either-with-VQSR-or-by-hard-filtering). In brief, filter names for SNVs were "QD2", "QUAL30", "SOR3", "FS60", "MQ40", "MQRankSum-12.5", and "ReadPosRankSum-8". Indel filter names included "QD2", "QUAL30", "FS200", and "ReadPosRankSum-20". Functional variant annotation for biallelic SNVs and indels was performed using snpEFF 5.1d (Cingolani et al. 2012). The filtered variant call format **(**VCF) file containing SNVs was converted to a bed file and filtered further using PLINK 2.0 (Chang et al. 2015). Variants were filtered for missingness and minor allele frequency using the flags --geno 0.1, --mind 0.1, and --maf 0.05. A SNV subset in approximate linkage equilibrium was selected using --indep-pairwise 50 5 0.5 and used in subsequent analyses.

Bayesian clustering analysis was conducted with ADMIXTURE v1.3 following cross-validation for *k*-values 1-25, where *k* represents the number of putative ancestral populations,

before population structure was estimated with *k*=4. This was chosen based on cross-validation error values calculated with ADMIXTURE and where the Bayesian Information Criterion (BIC) began to decrease minimally (Alexander and Lange 2011). Discriminant analysis of principal components (DAPC), a function of R v4.2.2 package *adegenet* 2.1.5 that maximizes separation between populations and minimizes separation within populations, was performed on populations stratified by crop, greenhouse, and chemical class resistance (CCR; the number of chemical classes that an isolate is resistant to) (Jombart 2008). Weir and Cockerham's weighted Fst estimator, a measure of population differentiation due to genetic structure, were calculated using VCFtools v0.1.15 (Danecek et al. 2011). Analyses of Molecular Variance (AMOVA) was calculated with the R package *poppr* (v2.9.3) following a tutorial (https://grunwaldlab.github.io/Population_Genetics_in_R/AMOVA.html) (Kamvar et al. 2014).

A genome-wide association study (GWAS) was conducted using PLINK to identify genomic regions associated with multifungicide resistance. Isolates (163) were included in the quantitative trait association analysis, with phenotypes ranging from 0-7 in accordance with the number of fungicides each isolate was resistant to. Fasta files for individual loci with were derived from VCF files with GATK FastaAlternateReferenceMaker after ensuring regional coverage (McKenna et al. 2010). Loci were aligned to the reference genome and visualized in Geneious Prime 2022.2.2.

Results

An average of 95.43% of reads were mapped to the reference genome, ranging from 85.78% to 98.41% (**Table 2**). On average, 1.9% of reads mapped to multiple locations throughout the genome. The average error rate was 0.0096, with a low of 0.0079 and high of 0.0142. Read duplication averaged 0.30 with a minimum of 0.22 and maximum of 0.39.

Chromosomes 1-16 had high average percent coverage values ranging from 97.73 to 99.59. Chromosomes 17 and 18 are regarded as accessory chromosomes; isolates had varying degrees of coverage for both (**Figure 1**). 18 isolates had less than 50% coverage of chromosome 17, 88 isolates had less than 50% coverage of chromosome 18, and another 21 isolates had less than 50% coverage of both. Genome-wide read depth averaged 35.43 with a minimum of 10.57 and maximum of 64.64.

Table 2. Sequencing and alignment metrics from Picard and SAMtools for 276 isolates of *B. cinerea*.

	Average	Maximum	Minimum
Unmapped reads	555359	2012926	98834
Estimated library size	13676500	27982181	6690096
Read pair optical duplicates	584904	1540211	277252
Read pairs examined	8098449	15886714	2214721
Unpaired reads examined	34123	72961	9598
Percent duplication	0.30	0.39	0.22
Read pair duplicates	2477345	5649548	532570
Secondary or supplementary reads	70633	122649	32320
Sequences	11815941	21400393	3523206
Bases mapped	1519241404	2771442019	454937914
Mismatches	14391222	26532564	4261525
Error rate	0.010	0.014	0.008
Average length	134	134	134
Average quality	36	36	36
Insert size average	353	412	301
Reads mapped and paired percent	95.28	98.30	85.52
Reads MQ0 percent	1.90	3.37	0.35
Average read depth	35.43	64.64	10.57
Chromosomes 1-16 coverage	98.61	99.99	93.61
Chromosome 17 coverage	84.64	100.00	11.03
Chromosome 18 coverage	62.81	99.76	7.18

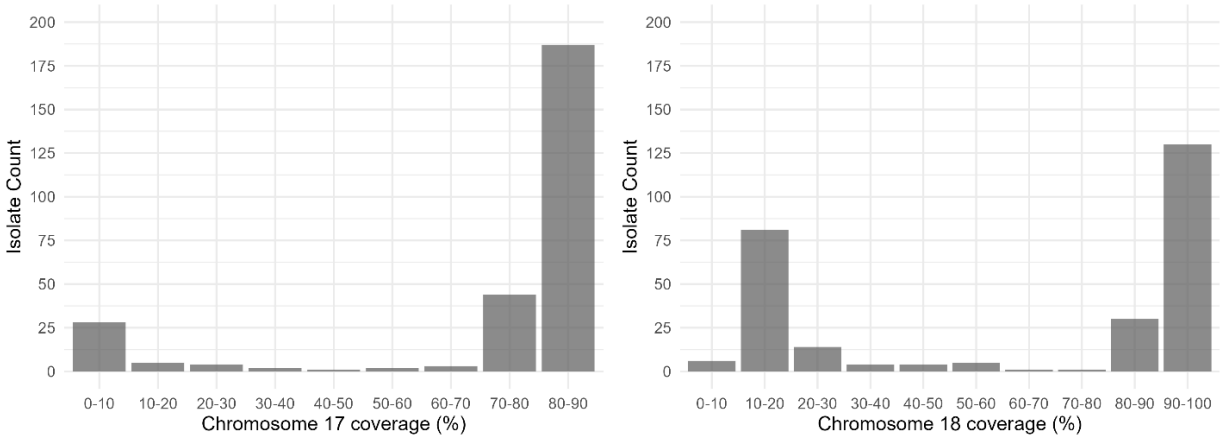

Figure 1. Percent coverage of accessory chromosomes 17 and 18 for all isolates (n=276).

All sequenced *B. cinerea* isolates in this study formed a clade containing the *B. cinerea* reference genome (B05.10), other *B. cinerea* isolates, *B. pelargonii*, and *B. eucalypti* in a PhyML phylogenetic tree using a concatenation of partial gene sequences of *G3PDH*, *HSP60*, and *RPB2* (**Figure 2**). When excluding the reference genome, all *B. cinerea* isolates within and outside this study form a monophyletic clade excluding other *Botrytis* spp. A large subset of isolates from this research (205) shared 100% sequence homology within the three genes extracted. *B. cinerea* Group B, a genetically distinct group lacking botcinic acid biosynthesis genes, was not identified based on a diagnostic 15-bp deletion in Bcin11g00620 (Plesken et al. 2021). Analysis of *Bcmrr1* sequences revealed the presence of *B. cinerea* Group S, as determined by a characteristic 21-bp insertion, in 82 isolates (29.7%) (Leroch et al. 2013). High genotypic diversity in *Bcmrr1*was revealed in 6 *B. cinerea* sensu stricto genotypes and 20 different Group S genotypes containing a total of 118 unique variants (**Figure 3**). Group S isolates were identified from all crops and both growing cycles spanning 11 greenhouses. Fungicide resistance frequencies in Group S isolates did not differ significantly from *B. cinerea* sensu stricto (**Table 3**). Group S isolates comprised all levels of multifungicide resistance: 7 CCR0, 5 CCR1, 4 CCR2, 4 CCR3, 7 CCR4, 4 CCR5, 7 CCR6, and 4 CCR7 for a total of 42 previously phenotyped Group S isolates.

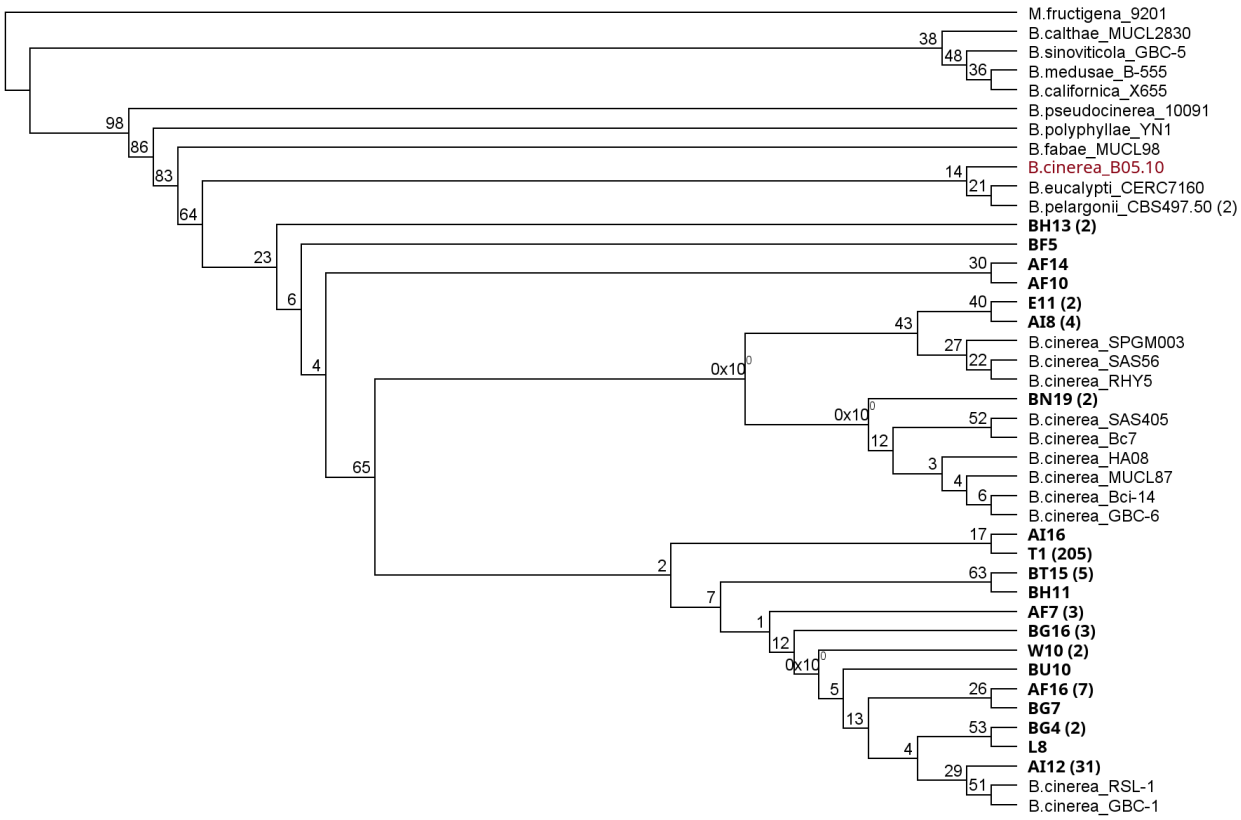

Figure 2. Maximum-likelihood phylogenetic tree of *Botrytis* spp. in clade 1 using a concatenation of partial gene sequences of *G3PDH*, *HSP60*, and *RPB2*. Numbers in parentheses are isolates from this research with identical 3-gene sequences, except for *B. pelargonii* representing another isolate of the same species. Bolded isolates are from this research. The *B. cinerea* reference genome is red. Numbers on branches correspond to bootstrap support (n=1000).

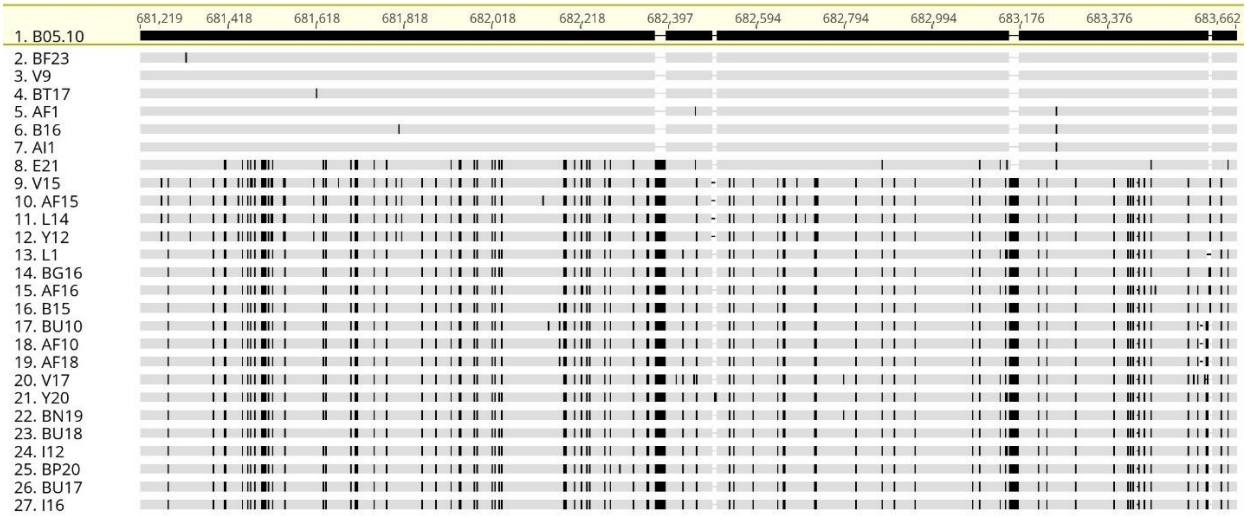

Figure 3. Diversity of *Bcmrr1* genotypes in *B. cinerea* sensu stricto isolates (2-7) and Group S isolates (8-27) representative of all isolates. Dark regions and dark vertical lines represent insertions and single nucleotide polymorphisms while horizontal lines represent missing bases. All variants are relative to positions on chromosome 5 of the highlighted reference genome.

Table 3. Frequencies of resistance to each fungicide in isolates of *Botrytis* Group S (n=42) and *B. cinerea* sensu stricto (n=122).

	Fen*	Ipr	Flud	Thi	Bos	Fluo	Cyp	Pyr
Botrytis cinerea sensu stricto	41.0%	65.6%	27.0%	98.4%	65.6%	11.5%	41.0%	80.3%
Botrytis Group S	35.7%	52.4%	19.0%	81.0%	52.4%	14.3%	38.1%	61.9%

*Fen = fenhexamid; Ipr = iprodione; Flud = fludioxonil; Thi = thiophanate-methyl; Bos = boscalid; Fluo = fluopyram; Cyp = cyprodinil; Pyr = pyraclostrobin.

Isolates were identified as *MAT1-1* (124) or *MAT1-2* (127) with an approximate 1:1 ratio overall and at greenhouse-level. A subset of 25 isolates contained genes for both mating types. Additionally, 25 isolates classified as a single mating type had less than 1,500 missing sites of the opposing genotype. The regions with missing bases were not shared among isolates. 13 of 14 isolates subjected to PCR validation resulted in the expected mating type based on coverage and read depth criteria. The single isolate classified as *MAT1-1* with unexpected amplification of genes for *MAT1-1/2* had an average genome-wide read depth of 43.9, an average *MAT1-1* read depth of 39.5 with zero missing bases, and an average *MAT1-2* read depth of 5.1 with 69 missing bases.

Filtration resulted in 1,402,115 variants, corresponding to 1 variant for every 30 bases, distributed across all chromosomes. Variant types analyzed included SNPs (1,281,167), insertions (57,800), and deletions (63,148). The average size of indels was 3.4 bases, with a standard deviation of 10 bases. High impact variants were identified at a rate of 0.158%, low impact variants at 3.956%, moderate impact variants at 2.93%, and modifiers at 92.96%. Functional classes of variants were analyzed revealing 171,462 (44.87%) missense mutations, 208,064 (54.45%) silent mutations, and 2,590 (0.68%) nonsense mutations. Most variants were located upstream of genes (35.06%), downstream of genes (38.96%), or within intergenic regions (13.07%). A raw transition/transversion (TsTv) ratio of 3.002 was calculated.

A total of 81,220 filtered SNPs (SNVs present in at least 5% of the population) in approximate linkage equilibrium were used for downstream analyses (**Table 4**). AMOVA indicated that most of the genetic variation was found within populations, as opposed to between (**Table 5**). A small amount of variation was attributed to crop (1.94%), growing cycle (0.49%), mating type (2.23%), and most individual fungicides. Slightly more variation was explained by

greenhouse (4.52%) and Group S (4.44%). Populations that explained most of the variation in this research were chemical class resistance (CCR), also known as resistance to a specific number of fungicides (5.53%), or individual resistance to thiophanate-methyl (14.15%)

Table 4. Distribution of SNP subset across all chromosomes.

Chromosome	Length	Variants	Variants rate
1	4,109,373	6,758	608
2	3,341,473	4,516	739
3	3,226,611	4,923	655
4	2,468,882	7,021	351
5	2,959,378	5,298	558
6	2,725,906	5,505	495
7	2,652,353	5,075	522
8	2,617,329	4,392	595
9	2,547,566	5,563	457
10	2,419,276	4,754	508
11	2,359,939	4,782	493
12	2,352,958	4,938	476
13	2,257,609	4,909	459
14	2,138,025	4,376	488
15	2,027,721	4,147	488
16	1,969,743	4,121	477
17	247,158	97	2,548
18	208,766	45	4,639
Total	42,630,066	81,220	524

Table 5. Results from AMOVA among populations and significance testing based on 999 permutations.

Strata	Source of Variation	Degrees of freedom	Sum of squares	Mean sum of squares	% variation[a]	*P* value
Greenhouse	Between	10	241079.7	24107.97	**4.52**	0.001
	Within	265	3019364.2	11393.83	95.48	
	Total	275	3260443.9	11856.16	100.00	
Growing Cycle	Between	1	19847.76	19847.76	**0.49**	0.001
	Within	274	3240596.12	11826.99	99.51	
	Total	275	3260443.88	11856.16	100.00	
Crop	Between	2	63577.09	31788.54	**1.94**	0.001
	Within	273	3196866.79	11710.13	98.06	
	Total	275	3260443.88	11856.16	100.00	
Group S	Between	1	73952.65	73952.65	**4.44**	0.001
	Within	274	3186491.23	11629.53	95.56	
	Total	275	3260443.88	11856.16	100.00	
Mating type[b]	Between	1	45634.4	45634.4	**2.23**	0.001
	Within	249	2941783.9	11814.39	97.77	
	Total	250	2987418.3	11949.67	100.00	
CCR[c]	Between	7	171775.5	24539.36	**5.53**	0.001
	Within	155	1761393.7	11363.83	94.47	
	Total	162	1933169.2	11933.14	100.00	
Thiophanate -methyl	Between	1	44597.04	44597.04	**14.15**	0.001
	Within	161	1888572.16	11730.26	85.85	
	Total	162	1933169.2	11933.14	100.00	
Fenhexamid	Between	1	40131.54	40131.54	**2.99**	0.001
	Within	161	1893037.66	11758	97.01	
	Total	162	1933169.2	11933.14	100.00	
Iprodione	Between	1	16212.03	16212.03	**0.47**	0.035
	Within	161	1916957.17	11906.57	99.53	
	Total	162	1933169.2	11933.14	100.00	
Boscalid	Between	1	56757.54	56757.54	**4.82**	0.001
	Within	161	1876411.66	11654.73	95.18	
	Total	162	1933169.2	11933.14	100.00	
Fluopyram	Between	1	27824.14	27824.14	**3.71**	0.001
	Within	161	1905345.06	11834.44	96.29	
	Total	162	1933169.2	11933.14	100.00	
Pyraclostro -bin	Between	1	44377.78	44377.78	**4.48**	0.001
	Within	161	1888791.42	11731.62	95.52	
	Total	162	1933169.2	11933.14	100.00	

Table 5 (cont'd)

Cyprodinil	Between	1	28316.16	28316.16	**1.74**	0.001
	Within	161	1904853.03	11831.39	98.26	
	Total	162	1933169.2	11933.14	100.00	
Fludioxonil	Between	1	20633.56	20633.56	**1.19**	0.002
	Within	161	1912535.64	11879.1	98.81	
	Total	162	1933169.2	11933.14	100.00	

[a]Percentage of variation n explained by stratified populations.
[b]Isolates classified as both mating types excluded.
[c]Isolates without fungicide sensitivity data were excluded from CCR and individual fungicide analyses.

Populations exhibited a high degree of admixture with only 34% of isolates having greater than 90% membership to a single cluster. No correlations were observed when populations were stratified by growing cycle, crop, mating type, or Group S (data not shown). Apart from one greenhouse visit (greenhouse I, growing cycle 1, geranium) associated with Cluster 2, proportions of cluster membership among greenhouses were similar. Similarly, DAPC did not indicate strong genetic separation among crops (**Figure** 4) nor greenhouses (**Figure** 5). Trends in cluster membership were observed in isolates based on chemical class resistance (CCR), or the number of fungicides they were resistant to (**Figure 6**). Cluster 4 was most prevalent in isolates resistant to 0-3 fungicides, cluster 3 was dominant in isolates resistant to 4-6 fungicides, and cluster 1 was most prevalent in isolates resistant to all 7 fungicides. Pairwise Fst values were highest when comparing CCR0, isolates sensitive to all fungicides, to isolates with any number of fungicide resistances (**Figure 7**). The lowest Fst values were detected between CCR1/CCR2, CCR3/CCR4, and CCR6/CCR7. Isolates with resistance to all 7 fungicides were substantially differentiated from isolates with resistance to 5 or less fungicides. DAPC revealed extreme separation between isolates sensitive to all fungicides compared to those with resistance to one or more fungicides (**Figure 8**).

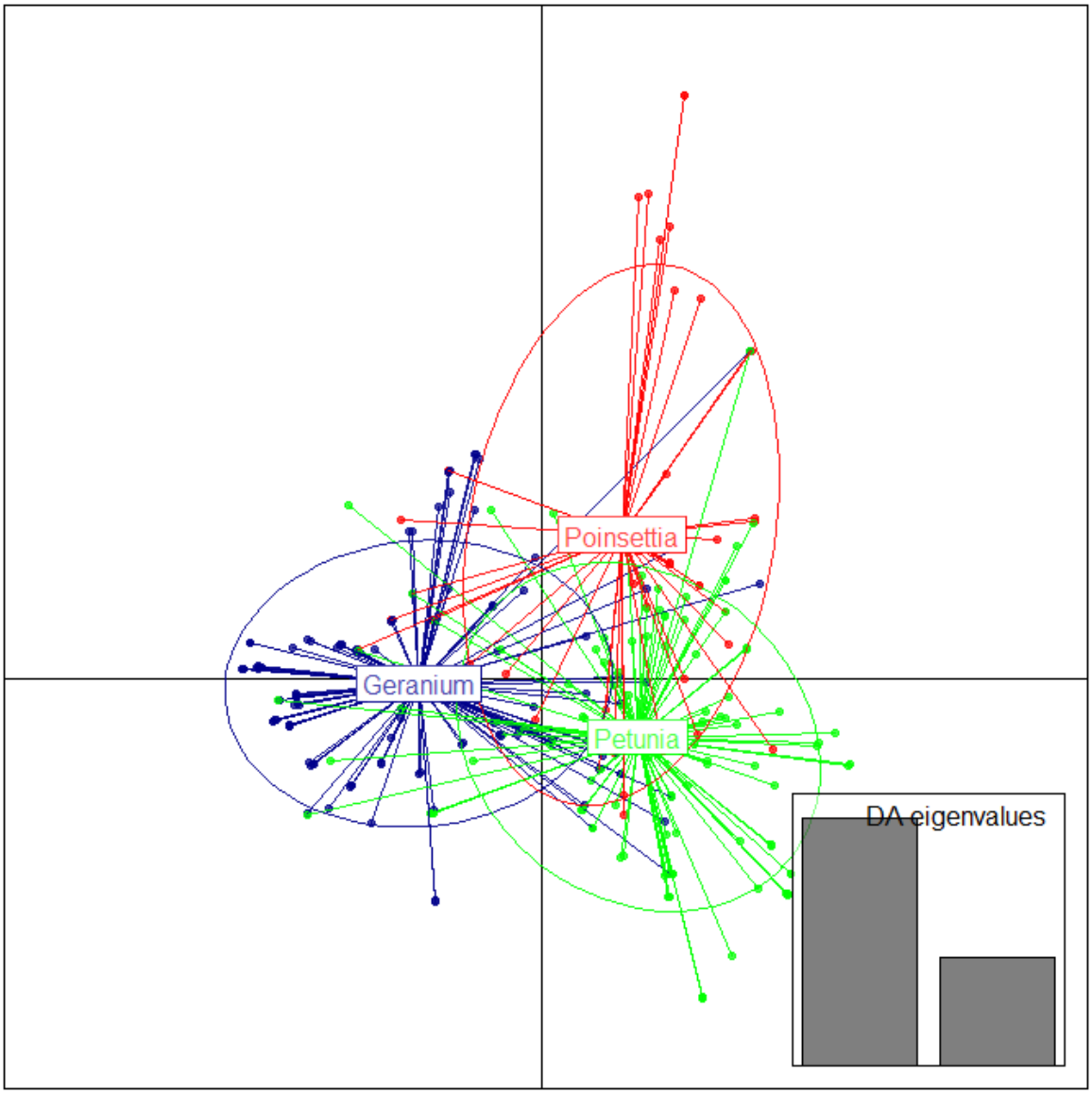

Figure 4. Discriminant analysis of principal components (DAPC) among isolates collected from geranium, petunia, or poinsettia.

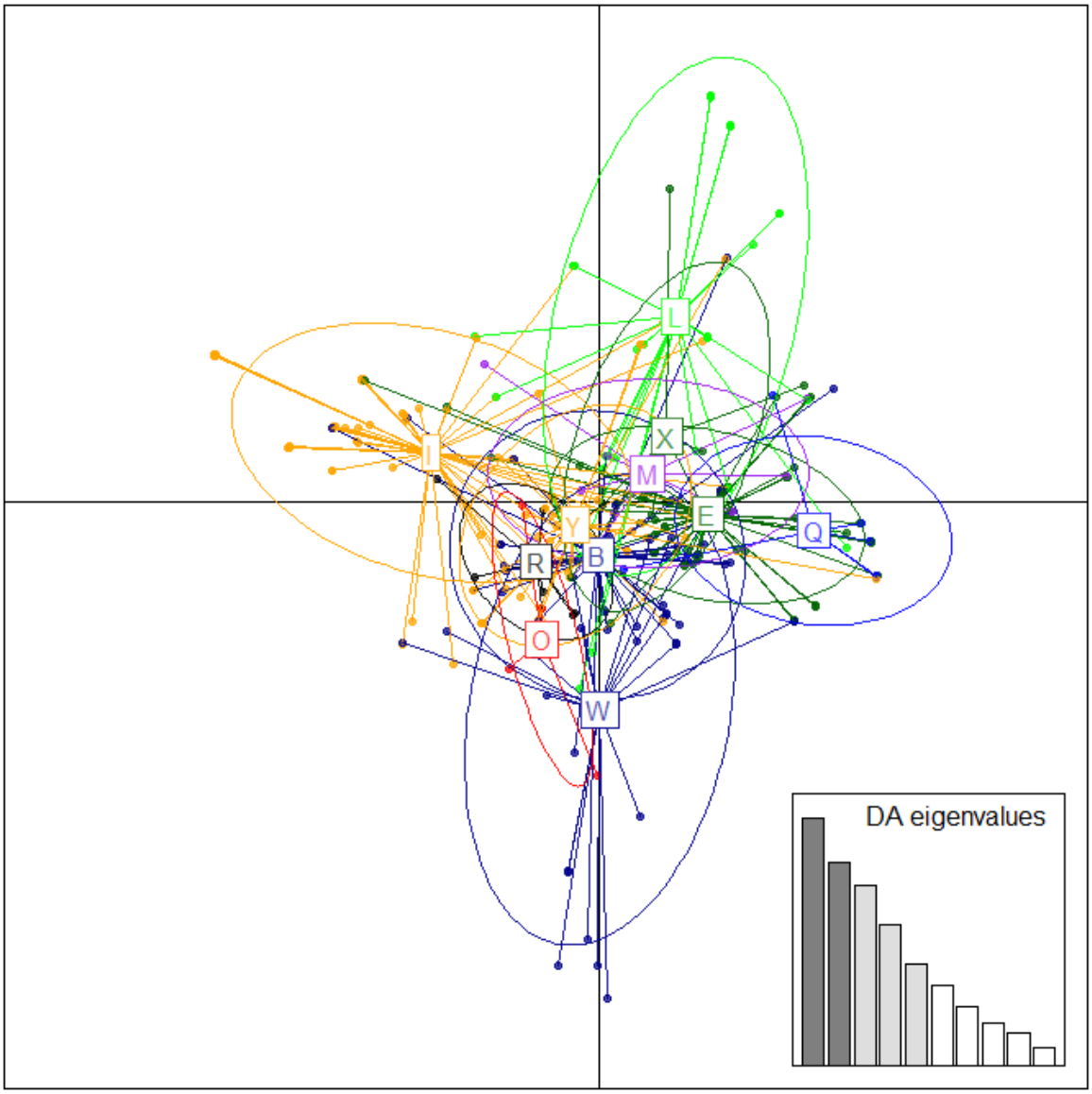

Figure 5. Discriminant analysis of principal components (DAPC) among isolates collected from 11 ornamental greenhouses.

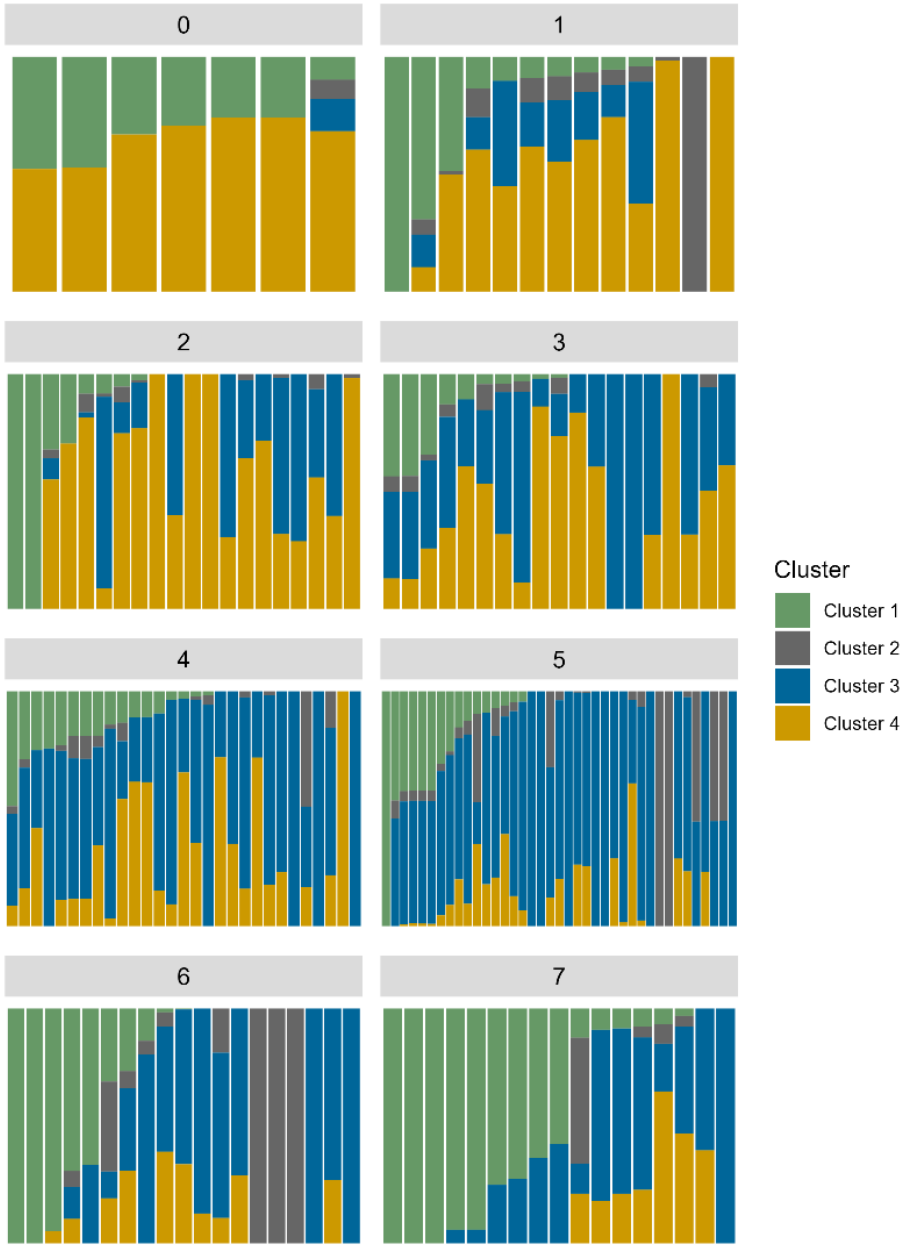

Figure 6. Population structure for *B. cinerea* isolates grouped by the number of fungicides they demonstrated resistance to.

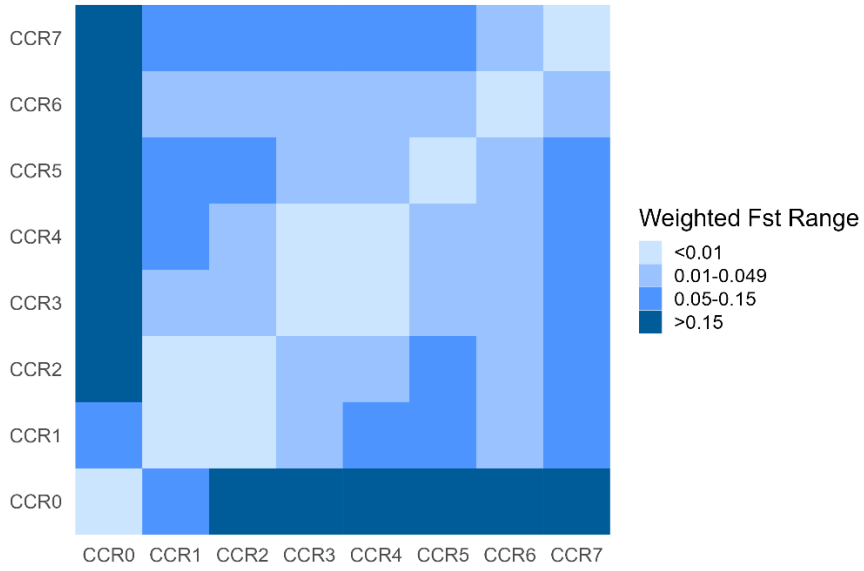

Figure 7. Genetic differentiation as determined by pairwise fixation index (F_{ST}) values among multifungicide resistant isolates.

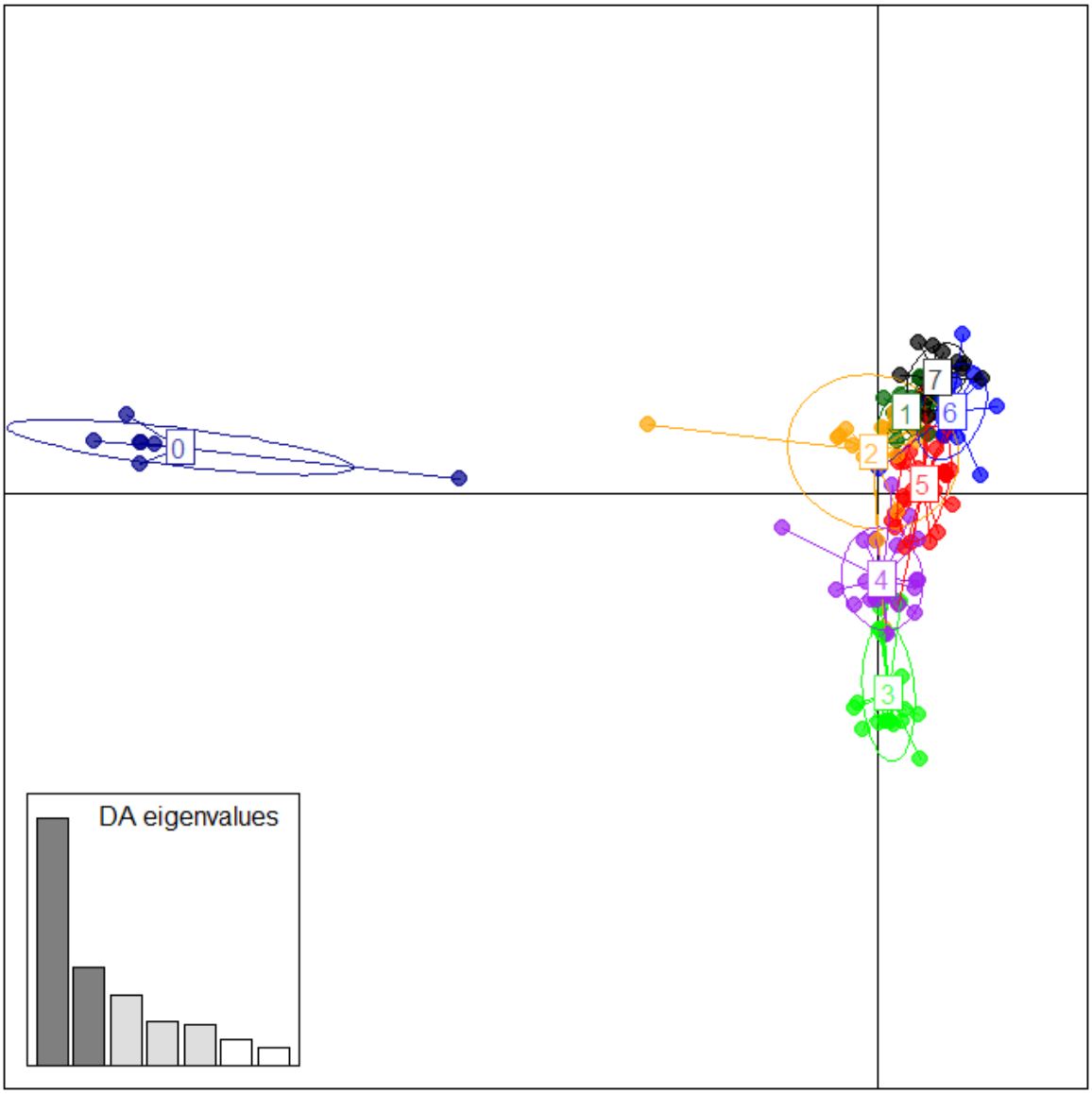

Figure 8. Discriminant analysis of principal components (DAPC) among isolates resistant to 0-7 fungicides.

Quantitative trait nucleotides (QTNs) associated with multifungicide resistance were located throughout the genome (**Figure 9**). Top QTNs were located on chromosomes 1, 2, 3, 11, and 14. These were located within or less than 1,000 bases from genes associated with mutations conferring fungicide resistance, genes associated with pleckstrin homology, copper transport, major facilitator superfamily genes, peptidases, permeases, and reductases of various predicted functions (**Table 5**).

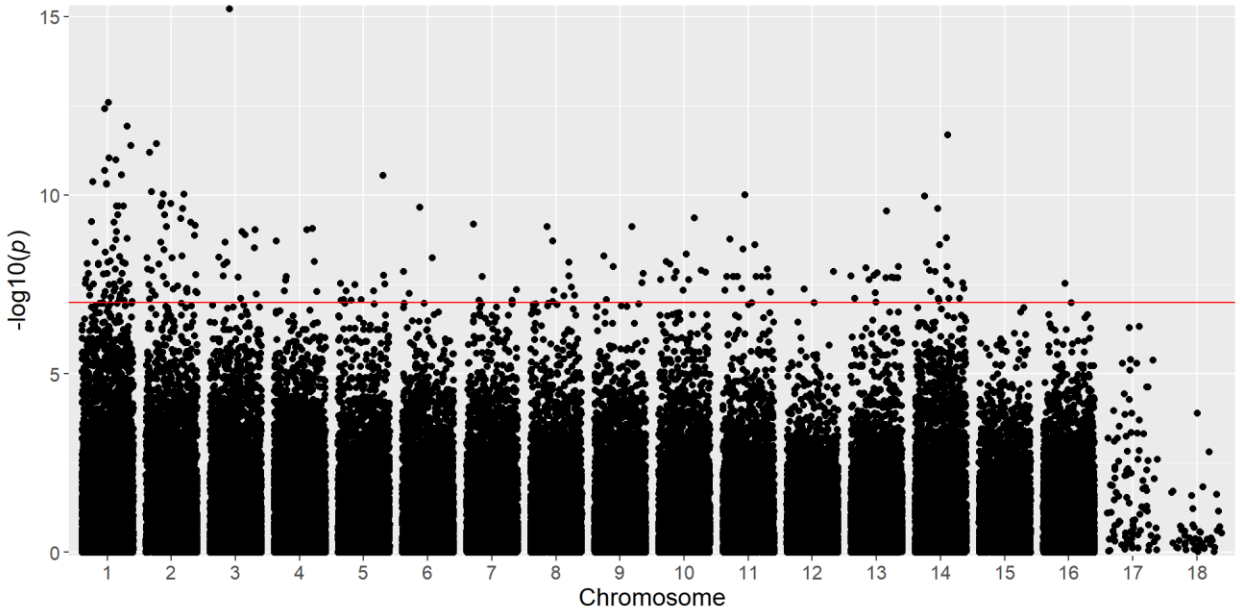

Figure 9. Manhattan plot showing the significance of single-nucleotide polymorphisms associated with multifungicide resistance (n=163). Points above the red line are statistically significant.

Table 5. Top QTN positions associated with multifungicide resistance and annotations.

Chromosome	Position	Gene ID	RefSeq protein annotations	*P*
3	1724265	*Bcerg27*	Short-chain dehydrogenases/ reductases (SDR) (cl25409)	6.12E-16
1	1793010	*Bcsdh2*	Succinate dehydrogenase/fumarate reductase, Fe-S protein subunit (cl28420)	2.46E-13
1	2465408	*BCIN_01g07050*	RAM signaling pathway protein (pfam10428), Rap1-interacting factor 1 N terminal (pfam12231)	3.81E-13
1	2264727	*Bcage1*	Pleckstrin homology-like domain (cl17171), Putative GTPase activating protein for Arf (pfam01412), Bin/Amphiphysin/Rvs (BAR) domain of uncharacterized fungal Arf GAP proteins (cd07608)	1.17E-12
14	1735425	*BCIN_14g04430*	--	2.01E-12
2	2324155	*BCIN_02g06630*	Slm1 pleckstrin homology (PH) domain (cd13311)	3.54E-12
1	2304156	*BCIN_01g06560*	Major facilitator superfamily (pfam07690)	4.01E-12
2	675876	*BCIN_02g01740*	PQ loop repeat (pfam04193)	6.38E-12
1	2251985	*BCIN_01g06380*	--	9.15E-12
1	2474358	*BCIN_01g07080*	Ctr copper transporter family (pfam04145)	1.03E-11
1	2225466	--	--	2.00E-11
1	2326726	*BCIN_01g06610*	--	2.69E-11
5	719698	*BCIN_05g01880*	Oligosaccharyltransferase subunit 5 (pfam05251)	2.74E-11
1	2366481	*BCIN_01g06740*	Major facilitator superfamily (cd06174)	4.14E-11
1	2313186	--	--	4.69E-11
1	2472787	*BCIN_01g07080*	Ctr copper transporter family (pfam04145)	4.97E-11
2	2295020	*BCIN_02g06550*	SGT1 protein (pfam07093)	7.75E-11
2	578104	*Bcspc3*	Signal peptidase subunit (pfam04573)	9.37E-11
2	617644	*BCIN_02g01570*	Amino acid permease (cl26159), phenylcoumaran benzylic ether reductase (PCBER) like, atypical	9.37E-11
11	2242988	*BCIN_11g06310*	SDRs (cd05259)	9.61E-11

Discussion

We resequenced the whole genome of 276 *B. cinerea* isolates collected from petunia (n=110), geranium (n=118), or poinsettia (n=48) in eleven Michigan greenhouses between 2018 and 2021 (**Table 1**). A subset of the isolates (163) was previously screened for resistance to eight fungicides in a germination-based assay (Lukasko and Hausbeck 2024a). This is the first large-scale whole-genome analysis of *Botrytis cinerea* populations. All isolates in this research were identified as *Botrytis cinerea* based on three conserved genes commonly used for species-level differentiation (Staats et al. 2005). Isolates formed a clade with the *B. cinerea* reference genome (B05.10), other *B. cinerea* isolates, *B. pelargonii*, and *B. eucalypti*. To our knowledge, there are only three isolates of *B. pelargonii* in existence. The species was originally described as a pathogen of geranium (Røed 1949). *B. cinerea* is well-known to have an extensive host range, and pathogenicity testing of *B. pelargonii* on other potential hosts was not conducted to rule out *B. cinerea* as the causal agent. The other two existing isolates were collected from ginseng and identified as *B. pelargonii* because they shared 100% nucleotide sequence homology in the partial gene sequences of *G3PDH*, *HSP60*, and *RPB2* (Lu et al. 2019). A single nucleotide polymorphism in *RPB2* thought to be exclusive to *B. pelargonii* was identified in an isolate (BF5) from geranium in this research. However, this isolate shared greater overall sequence homology (*G3PDH*, *HSP60*, and *RPB2*) with isolates of *B. cinerea* from previous research and this study (**Figure 2**). It seems likely that *B. pelargonii* is conspecific with *B. cinerea*, as previously suggested (Garfinkel 2021; Plesken et al. 2021). There is no available type species for *B. pelargonii* to sequence further or conduct pathogenicity assays. Similarly, *B. eucalypti* was identified on the basis on single host pathogenicity, which alone, is not valid for species-level identification within the *Botrytis* genus, as most species have multiple hosts that are

phylogenetically associated (Staats et al. 2005). *B. eucalypti*, first isolated from eucalyptus in China (Liu et al. 2016), was also identified as the causal agent of fruit rot on citrus (Liu et al. 2019) and fruit blight of cucumber (Ziedan et al. 2018). Due to the lack of morphological differences, sequence availability, and pathogenicity assays conducted for *B. pelargonii* and *B. eucalypti*, all isolates from this research were classified as *B. cinerea*. While other studies have identified cryptic species such as *B. pseudocinerea* living in sympatry (Walker et al. 2011), *B. cinerea* was the only species identified in our research.

Variant annotation by snpEff identified 1,281,167 SNPs, 57,800 insertions, and 63,148 deletions for a total of 1,402,115 unique variants among the 276 isolates. Results were similar to a previous study using 11 isolates of *B. cinerea* from diverse hosts, where they identified 980,329 SNPs, 87,489 insertions, and 70,447 deletions (Atwell et al. 2015b). While the 276 isolates from this research were sampled from less diverse hosts and environments, variant annotation statistics were similar overall. 2,872 genes were found to house at least one high impact variant, defined by snpEff as "probably causing protein truncation, loss of function, or triggering nonsense mediated decay". These variants likely have a significant impact on over 25% of the annotated protein-coding genes in *B. cinerea*. At least one high or moderate impact variant was identified in 11,029 genes, meaning most of the annotated protein-coding genes were found to have variants that likely impact protein activity.

A search for 44 established secondary metabolism key enzymes (KEs) in *B. cinerea* (da Silva Ripardo-Filho et al. 2023) found that 21 KEs had between 1 and 68 unique high impact variants. Fungal polyketide synthase, non-ribosomal peptide synthetase (PKS-NRPS) hybrids are KEs believed to have descended from a single origin (da Silva Ripardo-Filho et al. 2023). Interestingly, isolates from this study contained high impact variants in 2 of the 3 PKS-NRPS KE

genes; 258 isolates contained 68 unique variants in *Bcpks7*, a gene exclusive to *B. cinerea* (Valero-Jiménez et al. 2019), and 5 unique *Bcpks3* variants in 44 isolates. Future analyses of these genes may provide evolutionary insight. Other genes of interest with 10 or more unique high impact variants included *Bchol1*, *Bcboa13*, *Bcpkp1*, *Bcboa12*, *Bcmfs1*, *Bcpie2*, *Bcprd11*, and *Bcbik3*. These genes either share homology with multifacilitator superfamily genes (*Bchol1*, *Bcmfs1*), are involved in secondary metabolism (*Bcbik3*, *Bcboa13*, *Bcboa12*), or are uncharacterized in *B. cinerea* (*Bcprd11*, *Bcpie2*, *Bcpkp1*).

TsTv ratios are variable in fungi, but the value of 3.0015 reported in this research is higher than what has been observed in many ascomycetes and basidiomycete yeasts (Long et al. 2016) and *Aspergillus* spp. (Álvarez-Escribano et al. 2019). It is comparable to values reported in populations of *Magnaporthe oryzae*, which have been as high as 4.04 (Reddy et al. 2021). This high ratio could be explained by high pressure selection on genomic regions associated with virulence, host adaptation, fungicide resistance, or other factors. It could also be a result of genomic instability due to transposable elements, tandem repeats, DNA repair pathways, or epigenetic modifications (Bourgeois and Boissinot 2019; Habig et al. 2021).

The high level of diversity found in this research is parallel to previous work. One hypothesis that explains high rates of small-scale evolution is hypermutation as a result of mutations in DNA repair genes (Priest et al. 2020). A specific example of this can be found in genes encoding Ku70 and Ku80, key enzymes involved in the nonhomologous end-joining pathway (Choquer et al. 2008). Our research identified 14 unique mutations in *Bcku70* with a moderate impact in 266 isolates, and 13 unique mutations in *Bcku80* with a moderate impact in 199 isolates. Isolates with mutations in these genes may have increased susceptibility to mutagenic events. Mutations in the mismatch repair gene *MSH2* have been reported to result in

increased mutation rates and as a result, isolates can rapidly adapt to environmental stressors including antifungals (Billmyre et al. 2017; Healey et al. 2016). Nearly all isolates (271) from our research housed one or more of 30 unique variants in *Bcmsh2*. Other notable genes involved in DNA repair with multiple unique moderate-impact variants found in this research include homologous recombination pathway genes *Bcrad51* and *Bcrad52* (Symington 2002), the key light-driven DNA repair gene *BcCRY1* (Cohrs and Schumacher 2017), and genes encoding checkpoint kinases such as *Bcchk1* (Jung et al. 2019), some of which have been associated with resistance to antifungals. It is possible that mutations in these genes result in an increased rate of small-scale evolution and contribute to the development of fungicide resistance. To our knowledge, mutations in DNA repair enzymes have not been explored in field strains of *B. cinerea*. Future analysis of DNA repair enzymes may provide insight regarding *B. cinerea* diversity, evolution, and acquisition of fungicide resistance.

B. cinerea is regarded as a heterothallic fungus with an idiomorphic mating locus consisting of either *MAT1-1* or *MAT1-2* genes with a truncated form of an opposing mating type gene (De Miccolis Angelini et al. 2016; Faretra et al. 1988). The mating types were identified in an approximate 1:1 ratio with 124 *MAT1-1* and 127 *MAT1-2*, congruent with previous research suggesting the regular occurrence of sexual recombination (Faretra et al. 1988).

An additional 25 isolates possessed *MAT1-1* and *MAT1-2* genes. To improve our confidence that a single genotype was sequenced, we investigated a *de novo* assembly of BU9 (ASM3219752v1) (Lukasko and Hausbeck 2024b), an isolate possessing both mating types. If multiple genotypes were present, we would have anticipated a larger than average genome size with gene duplications, decreased mapping quality due to reads being mapped to multiple regions, and the SNP rate to be greater than the rate of sequencing errors when mapping reads

back to the genome (Wilson et al. 2023). Instead, the genome size was 43 Mbp with 11,454 genes, very close to other assemblies and the reference genome (Lukasko and Hausbeck 2024b). Filtering reads mapped back to the *de novo* assembly, following the same methods as previously, yielded 2,662 unfiltered SNPs located throughout the genome. This low proportion of SNPs aligns with expected single-genotype data (Wilson et al. 2023). When mapping reads to the reference genome, 96.97% of reads had a mapping quality of 60, which was similar among isolates regardless of *MAT1-1*, *MAT1-2*, or both mating types. The factors highlighted here suggest that isolates with both mating types likely represent a single isolate.

Read depth of the flanking genes in isolates with both mating types was close to the combined read depth of both mating types, supporting an interpretation that the locus is not present at another location in the genome. For example, genome read depth of isolate J3 averaged 24.4x, while the regional read depth of *MAT1-1* genes was 15.7x and *MAT1-2* genes was 11.4x. This was observed in other isolates and is a genomic signature of heterokaryotic fungi (Ropars et al. 2016). While the ratio of read depth within the MAT locus to the flanking genes was not consistently 0.5, this could indicate a need for greater sequencing depth among other possible explanations.

PCR amplification of an isolate subset validated the presence of single or both mating type genes in 13 of 14 single-spored isolates. To our knowledge, this is the first report of both mating type genes being successfully PCR amplified in the same isolate of *B. cinerea*. Based on our read depth and coverage criteria, the single unexpected isolate was predicted to be *MAT1-1*, but both mating types were PCR amplified. This indicates that the classification criteria are imperfect and it is possible that the presence of *MAT1-1/2* isolates is underestimated. Mating

type compatibility assays and PCR validation of more isolates would be helpful in defining criteria for predicting the mating types of WGS isolates in the future.

There have been reports of homothallic behavior in a small proportion of *B. cinerea* isolates, including those collected from greenhouses (Delcan and Melgarejo 2002; Lorenz and Eichhorn 1983). The mechanism for this phenomenon is unclear, but the phenotype may be a result of single multinucleate conidia containing nuclei with both mating types (Faretra et al. 1988). While the number of nuclei in hyphal cells and conidia is known to be highly variable, there is evidence of heterokaryotic behavior, or genetically distinct nuclei in the same cytoplasm, in some isolates (Büttner et al. 1994; Faretra et al. 1988; Grindle 1979). It is possible that this subset of isolates contains nuclei with *MAT1-1* genes and separate nuclei with *MAT1-2* genes. This behavior is known as "pseudohomothallism" and has been reported in other ascomycetes including *Neurospora tetrasperma* and *Cryphonectria parasitica* (McGuire et al. 2004; Merino et al. 1996). A high degree of homoallelism in genomic regions that are not linked to the mating type locus was detected in *N. tetrasperma* (Powell et al. 2001). This is consistent with the investigation of isolate BU9, where there was no evidence of gene duplications or an above average proportion of reads mapping to multiple locations of the de novo assembly. Powell et al. (2001) proposed that this could be a result of repeated selfing or inbreeding among strains of *N. tetrasperma*. Compared to most other *B. cinerea* studies in outdoor settings, greenhouse populations are geographically restricted and may factor into the existence of potentially pseudohomothallic isolates. While high diversity was observed at most individual greenhouses in this research, a conscious effort was made to sample from isolates in different spaces. This may partially explain why location did not have a large impact on population structure. Other greenhouse studies employing microsatellites have found lower standing diversity in *B. cinerea*

populations (Bardin et al. 2014; Walker et al. 2015). Future analyses of heterokaryon incompatibility loci and research focused on parasexual activity in *B. cinerea* could contribute to this hypothesis.

An investigation of allelic depth using the haploid VCF file revealed an even distribution of heteroallelic sites throughout the genome. This indicates that aneuploidy, or an uneven number of chromosomes, does not explain the observed behavior. The proportion of heteroallelic sites (to all variants) following variant filtration using varied from 0.01 to 0.83. If these sites were explained by difficulties mapping to repetitive regions or sequencing-related errors, the proportion of heteroallelic variants would be similar across isolates. Most isolates (63%) had less than 1% heteroallelic variants and are most likely homokaryotic. However, 13% of isolates possessed heteroallelic variants at more than half of the variant sites. It is important to note that these heteroallelic sites were reported from a haploid-generated VCF file and are likely missing a substantial number of variants. Future analyses should call isolates as diploid for an accurate count of heteroallelic sites.

Ascospore progeny of isolates with homothallic behavior have been shown to house *MAT1-1*, *MAT1-2*, or both mating type genes (Faretra et al. 1988; Soliman and van Kan 2012). The crossing of *MAT1-2* strains has resulted in progeny with the *MAT1-1* idiomorph, supporting the case for pseudohomothallism in *B. cinerea* (Soliman and van Kan 2012).

Previous reports of pseudo-homothallic isolates report amplification of genes corresponding to a single mating type, more often *MAT1-2* (De Miccolis Angelini et al. 2016; Soliman and van Kan 2012; Terhem and van Kan 2014). It is possible that additional isolates classified as *MAT1-2* in this study have homothallic behavior, but confirmation would require additional sexual compatibility assays.

It is important to note that another subset of isolates (25) classified as a single mating type also had a small number of reads that mapped to the opposing mating type. The missing bases were not shared among isolates and were located throughout the locus, indicating that no other truncated mating type gene forms were identified. Instead, this finding could be due to the presence of the opposite mating type at a very low ratio. This idea is supported by PCR amplification of both mating type genes in BR20, despite the isolate having a *MAT1-2* read depth of 5 and 69 missing bases. The amplified fragment was the same size as other isolates classified at *MAT1-2*, suggesting a need greater sequencing depth to confidently detect both mating type genes. This could be the result of the hyphal and/or conidial cells used in the DNA extraction harboring a ratio of a single nucleus with *MAT1-1* genes and the rest of the nuclei containing *MAT1-2* genes. Alternatively, the conidium used for single-sporing may have originally contained nuclei with both mating types, then nuclei segregated unevenly resulting in most hyphae/conidia containing *MAT1-1* genes and a small portion with *MAT1-2* genes. Mating type validation via PCR should be conducted for more isolates in the future.

Parasexual reproduction, the act of two isolates fusing hyphae and nuclei before resolving back to a haploid state, has been reported in filamentous ascomycetes and suggested in *B. cinerea* (Atwell et al. 2015a; Beever and Weeds 2007). The allorecognition process for parasexual behavior is controlled by heterokaryon compatibility loci, or vegetative incompatibility (VI) loci, which stimulate programmed cell death when isolates are incompatible. Two VI loci, *Bcvic1* and *Bcvic2*, were recently characterized in *B. cinerea* as behaving similar to other systems (Arshed et al. 2023). Deletion of both genes simultaneously resulted in a loss of vegetative incompatibility, enabling isolates to fuse and form heterokaryons regardless of the second isolate's genotype, albeit with slower and irregular growth. Variant

annotation from this research revealed high impact variants, which are predicted to result in a loss-of-function, in both described VI genes. 202 isolates contained a total of 25 unique high impact variants in *Bcvic1-1*, while 197 isolates contained a total of 15 unique high impact variants in *Bcvic1-2*. 139 isolates had high impact variants in both VI genes, suggesting the ability to form heterokaryons regardless of another isolate's VI genotypes. When considering moderate impact variants, all isolates contained at least one in *Bcvic2* and almost all isolates (261) had at least one in *Bcvic1*. It is currently unclear whether *B. cinerea* is able to maintain a stable heterokaryotic state without severe fitness costs *in vivo*. If possible, it may be able to partially explain the presence of both mating types in this research among other major implications.

A subset of isolates (n=163) from this research were previously screened for fungicide resistance to thiophanate-methyl, boscalid, fluopyram, fenhexamid, iprodione, fludioxonil, cyprodinil, and pyraclostrobin. Phenotypic results were combined with WGS data to evaluate genetic differentiation among multifungicide resistant isolates and identify novel regions associated with multifungicide resistance.

Previous research identified a genetically distinct subpopulation of *B. cinerea*, known as Group S, possessing increased frequencies of multifungicide resistance (Leroch et al. 2013) based on *Bcmrr1* sequences. Group S was identified in 81 isolates (42 phenotyped) from this research, but fungicide resistance frequencies were similar to *B. cinerea* sensu stricto (**Table 3**). It is possible that the variants from this research, including the diagnostic insertions, are a product of hitchhiking selection and do not confer fungicide resistance.

Previous work hypothesized that an increase in the number of chemistries that an isolate is resistant to can be explained by "selection by association" (Hu et al. 2016; Li et al. 2014b). In

other words, an isolate with resistance to a single, specific fungicide is more likely to develop resistance to another fungicide than an isolate without any fungicide resistance. The data here supports the possibility of stepwise accumulation; there is evidence of genetic differentiation between isolates with different numbers of resistances. Pairwise Fst values indicated that isolates with resistance to 0 fungicides were genetically distinct from all others, isolates resistant to 1 or 2 fungicides were most similar to each other, as were those resistant to 3 or 4 and 6 or 7, while isolates resistance to all chemical classes (CCR7) were genetically distinct from all others (**Figure 7**). The observed population structure was similar to previous analysis conducted in *B. cinerea* populations from small fruits in California (DeLong et al. 2020). It is likely that fungicide resistance has an important role in population structure in other cropping systems that battle *B. cinerea*. DAPC results provided a similar picture; isolates sensitive to all fungicides grouped separately while isolates resistant to specific numbers of fungicides were grouped together (**Figure 8**). AMOVA also revealed that among the factors evaluated in this research, the greatest amount of variation between populations was isolates resistant or sensitive to thiophanate-methyl (14.15%). This result may be bias because only 9 (6%) isolates were sensitive to the fungicide. However, the second highest values of between population variance was 5.53% explained by CCR (chemical class resistance), or the number of chemical classes that an isolate is resistant to. CCR also appeared to have an impact on population structure, as isolates with specific ratios of cluster memberships were associated with resistance to a specific number of fungicides (**Figure 6**).

A GWAS was conducted using all phenotyped isolates to search for genomic regions associated with multifungicide resistance. Quantitative trait nucleotides (QTNs), SNPs significantly associated with a phenotype, were located across the genome (**Figure 9**, **Table 5**).

The SNP with the greatest statistical significance was located within *Bcerg27*, a gene often containing mutations that confer resistance to fenhexamid (Amiri and Peres 2014). Similarly, the second most significant QTN was located in *Bcsdh2*, where mutations are known to result in resistance to succinate dehydrogenase inhibitors, such as boscalid and fluopyram (De Miccolis Angelini et al. 2014). Domains that appeared more than once in the top 20 QTNs included those with Pleckstrin homology and major facilitator superfamily (MFS) genes. MFS genes encode efflux transporters that are known to impact virulence, stress tolerance, morphology, fungicide resistance, and other functions in plant pathogenic fungi including *B. cinerea* (Hu and Chen 2021). A previous report of a promoter rearrangement in *mfsM2* resulted in increased levels of resistance to 9 fungicides, also known as the MDR2 (multidrug resistant 2) phenotype (Kretschmer et al. 2009). Researchers hypothesized that the unique event took place in a single isolate of *B. cinerea* from a European wine-growing region before spreading outward. While this genotype has not been reported in the United States, it is possible that the QTNs with MFS domains in this research are indicative of a similar occurrence. Large indels may not be identified due to the sequencing and alignment methods used in this research, but the QTNs may be in linkage disequilibrium with mutation(s) that have a more direct impact on fungicide resistance, such as a promoter rearrangement. The third most significant QTN was found in a gene encoding a putative RAM (regulation of Ace2 and morphogenesis) signaling pathway protein with a Rap1-interacting factor 1 (Rif1) N terminal. The QTN is located within the Rif1 domain. In yeast, an N-terminal truncated form of the Rap1 protein, the binding partner of Rif1, leads to hypersensitivity towards temperature and cell-wall perturbing agents (Azad et al. 2015).

Isolates with multifungicide resistance likely have a longer lineage in greenhouses environments and have therefore been under selection for traits related to general stress

tolerance, virulence, fitness, and other aspects that improve survival in a greenhouse setting. As a result, QTNs identified in this GWAS may also be associated with off-target traits. While regions of interest were identified, it would be helpful to perform a replicate GWAS with a different population and functional validation to further support causality of genes associated with multifungicide resistance (Genissel et al. 2017).

Data from this research, in combination with previous fungicide resistance phenotyping, indicates that multifungicide resistance is widespread and likely present in ornamental greenhouses regardless of recent fungicide applications (Lukasko and Hausbeck 2024a). Another study focused on *B. cinerea* fungicide resistance in greenhouse crops discussed similar results, as greenhouses demonstrated high resistance frequencies despite a lack of recent fungicide applications (LaMondia and Douglas 1997). If true, rotating chemical classes, a practice commonly recommended to growers, may have a minimal short-term impact on resistance management programs in ornamental greenhouses. Efforts to control Botrytis blight should be heavily focused on environmental manipulation and utilizing biological control tools.

This research is the first investigation of *B. cinerea* populations employing WGS with a high number of isolates (276). While Group S was identified, fungicide resistance phenotypes did not appear correlated with the subpopulation. The overall high level of diversity is parallel to previous work, where most genetic diversity is detected within populations, as opposed to between. Crops, growing cycles, and individual greenhouses had a minor impact on the population structure, while fungicide resistance appeared to have a larger impact. This interpretation was supported through DAPC, AMOVA, and Fst values. This study includes the first quantitative GWAS with populations stratified by CCR resulting in several genomic regions, including putative MFS genes, associated with multifungicide resistance. The presence of both

mating type genes in single-spore isolates of *B. cinerea* being reported for the first time alongside heteroallelic data is in support of the potential for parasexual behavior and stable heterokaryon formation. Moreover, parasexual behavior would explain the approximately even ratio of mating types and the lack of ascocarps observed in nature. Sequencing data and results from this work serve as a valuable resource for future *Botrytis* research and represent a turning point where mass quantities of isolates can be whole-genome sequenced to expand our knowledge of plant pathogen genetics.

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CHAPTER 5: CONCLUSIONS AND FUTURE WORK

Conclusions

The research detailed in this dissertation reveals the status of fungicide resistance among greenhouse populations of *Botrytis cinerea*, the fungus that incites Botrytis blight in ornamental crops. Germination-based fungicide sensitivity screening was conducted using 386 isolates of *B. cinerea* collected from symptomatic petunia, geranium, or poinsettia within the major production regions of Michigan between 2018 and 2021. Results showed high frequencies of resistance to several site-specific fungicides, including thiophanate-methyl, pyraclostrobin, boscalid, and iprodione. Intermediate or low frequencies of resistance were reported for cyprodinil, fenhexamid, fludioxonil, and fluopyram. Frequencies were similar among isolates regardless of host, production region, or growing cycle. Isolates with resistance to four or more FRAC groups were widespread, comprising 63.3% of the population. Isolates resistant to seven chemical classes were collected from all production regions, hosts, and growing cycles. Widespread multifungicide resistance is another factor to consider when developing integrated disease management programs for Botrytis blight.

De novo genome assemblies were created for a subset (7) of *B. cinerea* isolates spanning all crops, production regions, and growing cycles in this research. Five isolates previously demonstrated resistance to all commonly used site-specific FRAC groups but retained fluopyram sensitivity, and two isolates were sensitive to all 8 fungicides. Prior to this research, availability of *B. cinerea* whole genomes was limited with most isolates collected from small fruit in Europe. This increases the diversity of publicly available genomes and corresponding functional annotation data, serving as a valuable resource for future *B. cinerea* research.

Whole-genome resequencing was performed for 276 single-spored isolates of *B. cinerea*, including a subset previously screened for fungicide sensitivity. Populations of *B. cinerea* are

well-known to contain high levels of diversity, complicating comparative studies and population structure analyses. Methods used to assess genetic differentiation included discriminant analyses of principal components (DAPC), fixation index values (Fst), analyses of molecular variance, and a genome-wise association study (GWAS). This research found limited evidence of genetic differentiation when stratified by host, location (production region and individual greenhouses), or growing cycle. While temporal and geographic factors had a minimal impact on population structure, there was significant genetic differentiation detected among isolates resistant to a specific number of fungicides (0-7). DAPC and Fst values showed the greatest separation between isolates that were sensitive to all fungicide classes and isolates resistant to 1-7 fungicides. STRUCTURE analysis revealed a highly admixed population, with an association among cluster membership proportions and individual isolates' number of fungicide resistances. A quantitative trait GWAS revealed several genomic regions significantly associated with multifungicide resistance. While the two most significant quantitative trait nucleotides (QTNs) were located with genes known to contain single nucleotide polymorphisms that confer fungicide resistance, other top QTNs have no known relationship to fungicide resistance. Notably, two significant QTNs were located within putative multifacilitator superfamily genes that share homology with genes containing mutations that confer multifungicide resistance in one other European population. *B. cinerea* Group S, a genetically distinct subgroup containing two diagnostic insertions in the gene *Bcmrr1*, was identified throughout Michigan ornamental greenhouses. While this subgroup is historically associated with increased frequencies of fungicide resistance, *B. cinerea* Group S isolates from this study demonstrated fungicide resistance frequencies similar to *sensu stricto* isolates. There was limited evidence of significant genetic differentiation among Group S isolates, leaving unclear biological significance of the

subgroup. Mating type characterization is commonly included in *B. cinerea* population analyses. Mating is controlled by an idiomorphic mating type locus that contains either MAT1-1 genes or MAT1-2 genes. This is the first study to report the presence of both mating type genes in single-spored isolates of *B. cinerea*, prompting questions about karyogamy within the species.

This study incorporates the largest number of whole-genome sequenced *B. cinerea* isolates to date and revealed some of the highest reported fungicide resistance frequencies. Data and genomic resources provided in this dissertation have improved knowledge of *B. cinerea* population genomics in ornamental greenhouses.

Future Work

The rise of multifungicide resistance in greenhouse populations of *Botrytis cinerea* presents a new challenge in the development of integrated disease management plans. The frequency of resistance to each fungicide class should be considered when employing a disease management plan. Future strategies may employ heavier usage of cultural disease management practices, biological options, and a curated rotational fungicide approach to slow the spread of fungicide resistance.

This dissertation provided seven annotated *de novo* genome assemblies, greatly increasing the diversity of publicly available *B. cinerea* genomes. Genomic comparisons within the species, including the reference genome, may reveal genetic differentiation on a larger geographic scale and/or hosts. Data from this research showed highly variable coverage and read depth of accessory chromosomes 17 and 18, among other regions. The biological significance of most genes on the accessory chromosomes are currently unknown, but their presence and genetic variability may be correlated with phenotypic data. The genome-wide association study (GWAS) conducted in this dissertation served as a preliminary look into novel regions that may be

associated with multifungicide resistance. A replicate GWAS could be conducted with another population to validate the association between select genomic regions and fungicide resistance, as long-term greenhouse survival may be a confounding variable. There are only two known mechanisms that confer resistance to multiple fungicides in *B. cinerea*, both of which are mediated by efflux pumps. Two significant regions from the GWAS containing genes that encode putative efflux pumps were of special interest due to their similarity to an established resistance mechanism conferring the MDR2 phenotype (reduced sensitivity to several fungicides). Future work could delve deeper into the novel regions associated with multifungicide resistance. In addition, novel SNPs in target genes associated with resistance to individual fungicides could be derived using a combination of phenotypic and genotypic data from this research. This is the first report of both mating type genes present in single spored isolates of *B. cinerea*. This could be a result of heterokaryotic behavior, which has been previously suggested in *B. cinerea*, but validation would require more work. Recalling variants as a diploid may provide a better picture of heteroallelic sites and the ability to compare genome-wide variability among genotypes. It would be helpful to perform mating type compatibility assays, PCR validation on a larger subset of isolates and their single-spored progeny, evaluate heterokaryon formation and stability, and if possible, karyotyping of isolates with both mating type genes. The availability of pre-processed genomic data for 276 *B. cinerea* isolates provides several avenues for future research.