INSIGHT INTO PLOIDY VARIATION, THE COMPLICATED LIFE CYCLE, AND GENOMIC ARCHITECTURE OF *EXOBASIDIUM MACULOSUM*, CAUSE OF EXOBASIDIUM LEAF AND FRUIT SPOT OF BLUEBERRY

by

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(Under the Direction of Marin T. Brewer)

ABSTRACT

Exobasidium leaf and fruit spot emerged as a serious disease of blueberry in commercial production areas in the last decade. However, the fungal pathogen that causes the disease, *Exobasidium maculosum*, has high genetic diversity and population genetic structure indicating it is well established. The aim of this research was to investigate whether vectors play a role in transmission of the disease and identify factors that contribute to its high level of genetic diversity. Arthropod exclusion and trapping studies were conducted at two field sites in Alma and Toccoa, GA from 2018 to 2020. In each field, ten trap plants were covered with exclusion nets and paired with ten uncovered plants under diseased blueberry bushes for two weeks during the known infection window. The plants that were covered had 0 to 12 leaf spots while the uncovered plants developed up to 654, indicating

that we excluded an agent necessary for infection to occur and points to vector transmission of the disease. A fluctuation assay that was performed to compare the spontaneous mutation rate of six *E. maculosum* strains and two *Exobasidium rostrupii* strains revealed that *E. maculosum* has a higher mutation rate. CHEF electrophoresis performed to assess karyotypes of 15 *E. maculosum* strains showed five chromosomes with sizes at approximately 3, 3.5 and 5 Mb, one larger than 5.7 Mb and one varying among strains between 2.35 and 2.7Mb, resulting in genome size estimates of 19.6 to 19.9Mb. Ploidy variation among 14 strains was assessed using flow cytometry and a bioinformatics approach and revealed that *E. maculosum* has ploidy and cell size variation and a high incidence of aneuploidy. Analysis of copy number variation revealed that there was an average of 500 deletions and 5 duplications among strains, and that duplications were greater in the northeast North America population. Phylogenetic relationships of 14 *E. maculosum* strains from Georgia, North Carolina and northeast North America were evaluated using whole genome sequence data. Strains clustered based on geographic location of origin. These findings are important in the epidemiology of the disease and highlights new factors that should be considered in its management.

INDEX WORDS: Exobasidium leaf and fruit spot, blueberry, *Exobasidium maculosum* arthropod, fungal vectors, ploidy, copy number variation, mutation rate, comparative genomics, karyotype.

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DEDICATION

I dedicate this dissertation to my son, Emelio, whose beautiful smile is my motivation.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The *Exobasidium* genus

The genus *Exobasidium* was established in 1867 by Woronin and contains plant pathogens that affect members of the order Ericales. Exobasidium species are known to cause enlargement or growth abnormalities on different host plant tissues such as flowers, stems, shoots, buds, and leaves (Alexopolus et al. 1996). They also cause a wide range of symptoms such as galls, blisters, leaf or fruit spots, deformations, and witch's broom ((Brewer et al. 2014; Burt 1915; Nannfeldt 1981; Savile 1959; Sinclair and Lyon 2005). Host plants include many economically important crops such as cranberries, blueberries, tea, azaleas, rhododendrons and camelias. Broad taxonomic models have been suggested for *Exobasidium* because of this variation in symptoms which often overlapped or changed based on time and environmental factors (Burt 1915; Savile 1959). However, more recent studies have established a more rigid classification system with emphasis on host and symptom specificity and host associations (Begerow et al. 2002; Brewer et al. 2014; Kennedy et al. 2012; Nannfeldt 1981; Piatek et al. 2012). Different species can cause the same symptoms on different hosts or different symptoms on the same host. The evolutionary history of *Exobasidium* is not well characterized but Brewer et al. (2014) found through phylogenetic studies that species tend to cluster by disease phenotype and

not host species which could mean that genetic factors influencing pathogenicity may be similar among these closely related species.

The genus *Exobasidium* belong to the Basidiomycota and most species are dimorphic, growing saprophytically as single-celled yeasts and in filamentous form during its parasitic stage in the plant (Nannfeldt 1981). The yeast-like stage is easily cultured from germinating basidiospores and budding mycelium (Gadd and Loos 1948a; Graafland 1953; Sundström 1964; Wolf and Wolf 1952). The most characterized *Exobasidium* species is *E. vexans,* which causes blister blight of tea, but most do not have a defined disease cycle.

The Georgia blueberry industry

The United States is the top blueberry producer in the world followed by Canada, Peru, France and Poland (FAOSTAT 2019). After strawberries, blueberries are the second most produced berries in the United States (US). The amount of the fruit that was marketed, domestically consumed or exported in 2019 increased by 21% from 2018 and totaled 673 million pounds in the US (USDA-NASS 2019). Of that total, 373 million pounds went to fresh market and the remaining for processing. Georgia was the leading blueberry producer in the US as its acreage increased in 2019, with 21,700 harvested acres (USDA-NASS 2019). The other major blueberry-producing states are California, Florida, Indiana, Michigan, Mississippi, New Jersey, North Carolina, Oregon and Washington.

The demand for blueberries is year-round in the US and production and imports have significantly increased to meet consumer needs. Most of the US blueberry imports are from Peru, Chile and Mexico which together, accounted for approximately 80% of the total in 2019 (USDA-ERS 2019). Georgia blueberry production has surpassed that of peach in the

last decade, with a farm gate value growth from approximately $29.6 million in 2002 to $288.9 million in 2016, making it the state's most valuable fruit crop (Wolfe and Stubbs 2017). There are three types of blueberries grown in Georgia, namely rabbiteye (*Vaccinium virgatum*), southern highbush (*Vaccinium* interspecific hybrids) and northern highbush (*Vaccinium corymbosum*), with rabbiteye being the most productive and widely grown variety (Krewer 2006).

Among the major diseases of blueberries in Georgia are mummy berry disease caused by *Monolinia vaccinii-corymbosi*, Phytophtora root rot caused by *Phytophtora cinnamomi*, bacterial leaf scorch caused by *Xylella fastidiosa* (Brannen et al. 2016; Scherm et al. 2001; Scherm and Stanaland 2001) and Exobasidium leaf and fruit spot caused by *Exobasidium maculosum*.

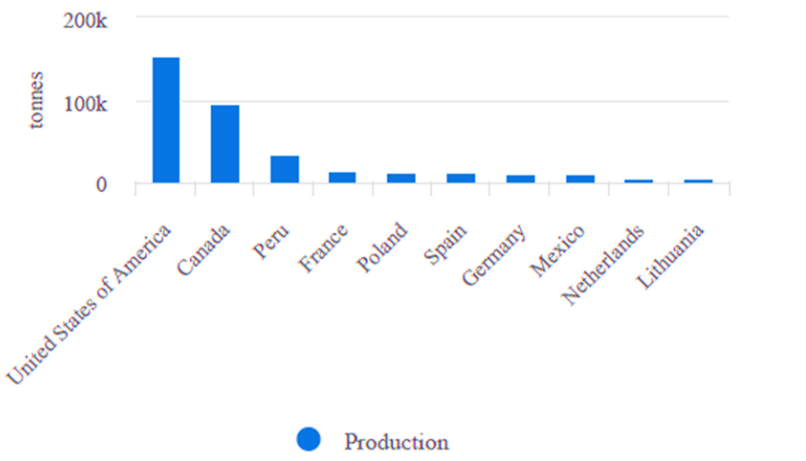

Figure 1.1. Top ten blueberry-producing countries in the world showing average production from 1994 to 2019. Source FAOSTAT 2019.

Exobasidium Leaf and Fruit Spot

Exobasidium leaf and fruit spot was first reported in 1997 on highbush blueberries in southeastern North Carolina (Cline 1998).This report was the first of an *Exobasidium* species infecting blueberry in the southeastern US (redleaf disease had been reported elsewhere in the US as *E. vaccinii* but is actually a different species that has not yet been named). A similar disease sporadically affecting lowbush blueberry (*Vaccinium angustifolium*) has been described in Nova Scotia, Canada (Nickerson et al. 1997). The disease is caused by the fungus *Exobasidium maculosum*, which was found to be a new species by Brewer et al. (2014). The disease has since emerged in the southeastern US with increased prevalence throughout commercial blueberry production areas in Mississippi, North Carolina and Georgia (Brannen et al. 2011; Cline and Brewer 2017). In the southeastern US, Exobasidium leaf and fruit spot has been reported on highbush blueberry (*Vaccinium corymbosum*), southern highbush blueberry (*Vaccinium* interspecific hybrid) and rabbiteye blueberry (*V. virgatum*), which seems to be most susceptible host species (Brewer et al. 2014).

Symptoms of the disease are restricted to leaves and fruits and are very distinct. Leaf and fruit symptoms first appear in early spring. In the early stages, leaf spots are circular and chlorotic with a pale yellow to pale green color which turn crimson to maroon as the spots get older. Leaf spot sizes range from 2-14 mm in diameter, are slightly raised, felt-like and consist of the hymenium on the lower surface and are smooth on the upper surface. Fruit spots are initially pale green and leathery, turning powdery and pale yellow to crimson or maroon over time (Brewer et al. 2014; Nickerson et al. 1997). Significant economic losses may be caused by the fruit stage of the disease with yield losses of up to

60% recorded on 'Premier' rabbiteye blueberry (Brannen et al. 2013; Brewer et al. 2014; Nickerson and Kloet 1996). Also, a major problem with this stage of the disease is the inability of color-sorters and packing-line inspectors to separate and remove affected berries during harvesting, sorting, and packing. Additionally, fruit spots are unsightly and may result in consumer rejection of product. Symptoms tend to be most severe in the lower canopy and do not usually occur on older leaves.

Ingram et al. 2019 found that the disease is monocyclic with a primary inoculum that is not aerially dispersed, and which overwinters epiphytically on host tissues. The authors also found that basidiospores produced on leaves function primarily in dispersal and does not cause leaf infections. Different fungicides such as Captan, Indar, Elevate, and Pristine, have been evaluated for control of the disease, which is currently managed by a single late dormant application of lime sulfur (Brannen et al. 2014, 2017; Cline and Bloodworth 2014; Scherm et al. 2014; Ingram et al. 2018). Resistance to Pristine has been observed in Georgia (Ingram et al. 2018).

To elucidate the genetic diversity and reason for disease emergence, Stewart et al. (2015) sequenced three conserved loci of 82 *E. maculosum* isolates from different plant tissues of the fungus collected from Georgia, North Carolina and Maine in the US and Nova Scotia, Canada. Within the conserved loci, there were 1775 sequenced nucleotides with 286 segregating sites and each isolate was found to be a unique multilocus haplotype. Although the nucleotide diversity and number of mutations were high, 94% of nucleotide substitutions were silent, suggesting that selection is acting to preserve the amino acid sequence of housekeeping genes. The authors suggested that this high genetic diversity does not support a recent introduction of *E. maculosum* or host shift, and emergence is

thought to be a result of either an environmental change or increase in the host population. One idea is that this environmental change involves a vector as other *Exobasidium* species produce colorful structures that are theorized to function in the attraction of insects for aid in disease transmission. Also, the conservation of amino acid sequence may not be observed in genes where a high level of genetic diversity provides a selective advantage, such as effectors which suppress host immunity and mating-type genes.

Another study using 90 loci from double digest restriction-site sequencing (ddRAD-seq) revealed that the high level of genetic diversity observed in the three conserved genes is maintained across the entire genome of the pathogen (Stewart et al. 2014). Bayesian cluster analysis using the multilocus genotype data detected minimal admixture. The authors also found that population structure was based on location and identified three genetic groups which correspond to three populations, namely Georgia, North Carolina and NE North America.

Life cycle of the smuts

The Basidiomycota is ordered in three sub-phyla, namely, Agaricomycotina, Pucciniomycotina and Ustilaginomycotina. Pucciniomycotina include the rust fungi while smut fungi belong to Ustilaginomycotina, many of which are pathogenic to many important agricultural crops. *Exobasidium* belongs to the subphylum Ustilaginomycotina and is related to *Ustilago maydis* which causes corn smut. Smut fungi are the second largest group of plant pathogens in the Basidiomycota and most have a yeast stage and produce dark, thick-walled teliospores, which germinate to form basidia. Members of the Exobasidiales,

which do not produce teliospores, are closely related to the smut fungi (Piepenbring 2011). Basidia of *Exobasidium* species are produced externally on infected host material. A typical member of Ustilaginomycotina is dimorphic, with a saprobic haploid stage and a dikaryotic parasitic stage. The saprobic stage begins with the production of haploid yeast cells which undergo plasmogamy and fuse to form the dikaryotic infectious stage. After infection occurs, the fungus extracts nutrients from its host. Karyogamy and the formation of diploid teliospores follows and the pathogen usually rests in this stage until conditions are favorable. The teliospores then germinate and form basidia, meiosis occurs, and haploid basidiospores are formed. Basidiospores bud to form yeast cells which restarts the cycle (Figure 1.2). *E. maculosum* does not form teliospores but it is filamentous within the leaf tissue and basidia, basidiospores and yeast cells are produced. The complete life cycle of the pathogen has not been fully described, mainly because artificial inoculation strategies have not been successful to date.

Mating in the Basidiomycota

The life cycle of Ustilaginomycotina includes both sexual and asexual stages. Mode of reproduction and mating systems are important evolutionary forces in many pathogens that may influence disease epidemiology. Sexual reproduction has been shown to be associated with virulence and the emergence of diseases (Byrnes et al. 2010; Fraser et al. 2005). Fungi in the Basidiomycota have some of the most complex mating systems in the fungal kingdom. They can either be homothallic (self-fertile) or heterothallic (self-sterile) with some species having both mating systems in their population (Kronstad and

Staben1997). Basidiomycota may also be bipolar or tetrapolar with one or two unlinked mating-type loci, respectively (Hsueh et al. 2008).

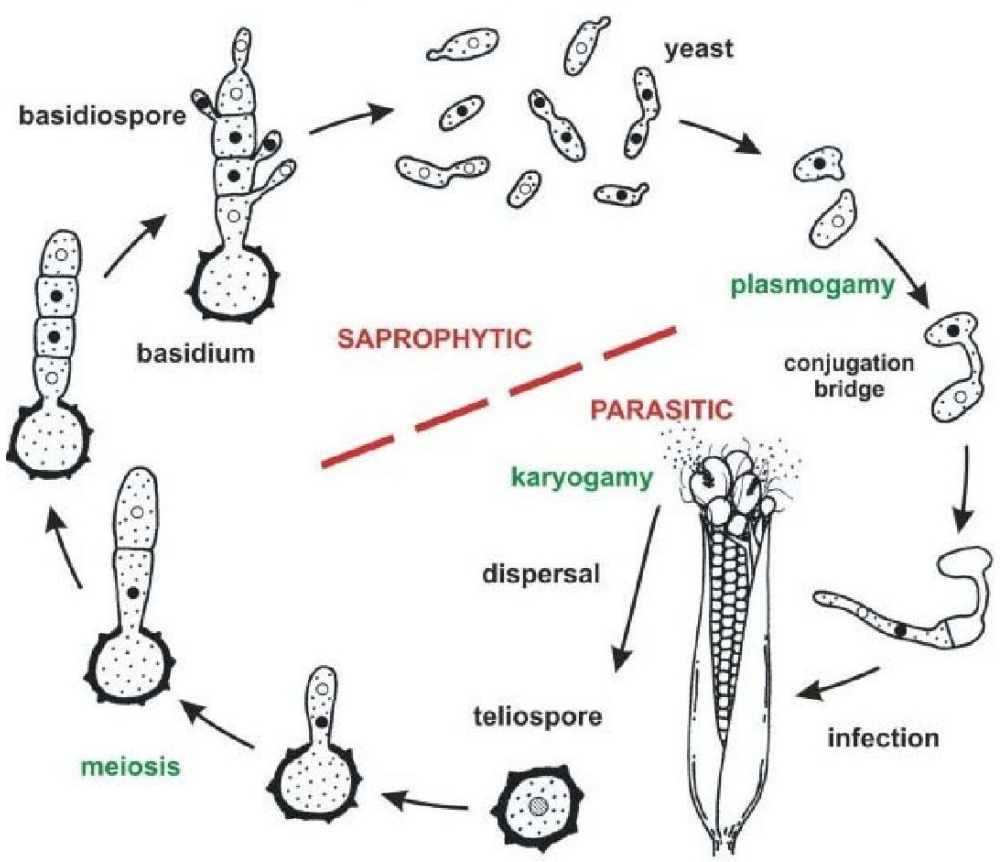

Figure 1.2. Life cycle of *Ustilago maydis* and typical smut fungi in Ustilaginomycotina. Source Piepenbring (2011).

Most Basidiomycota however are heterothallic and have a tetrapolar mating system. In the smuts MAT loci are characterized as an "*a* locus" which houses the pheromone and pheromone receptor genes and a "*b* locus" which houses the homeodomain transcription factors. There is some variation in the structure of mating type loci within and among each

subphylum of Basidiomycota. For example, in the Ustilaginomycotina, there are species that have bipolar and tetrapolar mating systems. *Ustilago hordei* has a bipolar mating system where the *a* and *b* loci are linked and segregate together, and two alternate alleles (MAT-1 and MAT-2) at the MAT locus (Bakkeren et al. 2008; Hsueh and Heitman 2008; Kronstad and Staben 1997). *Ustilago maydis* on the other hand, is tetrapolar with the biallelic *a* and multiallelic *b* loci on different chromosomes (Banuett 1995). In the Pucciniomycotina, *Microbotryum violaceum* is bipolar with linked *a* and *b* loci (Fraser et al. 2008). In the Agaricomycotina, *Cryptococcus neoformans* is bipolar and heterothallic (Hsueh and Heitman 2008). However, there are a few species such as *Coprinopsis cinerea*, *Laccaria bicolor* and *Schizophyllum commune*, that are tetrapolar with multiallelic *A* or *B* loci, or multiple subloci (Casselton and Olesnicky 1998; Niculita-Hirzel et al. 2008; Raper 1966).

Vector transmission of fungal diseases

Understanding the life and disease cycles of plant pathogens is important for management but some are more complex than others. Though Ingram et al. (2019) studied the epidemiology of Exobasidium leaf and fruit spot, there are still some gaps in our knowledge of how these cycles are completed. One of the key elements is the infection process, which cannot be reproduced by artificial inoculation, suggesting that there may be some form of vector involvement. Many plant pathogens have insect vectors, some of which are economically important, but are most known for viruses since they rely on them for dissemination (Bragard et al. 2013). There are only a few examples of fungi being directly transmitted by vectors because most agriculturally important species are wind or splash

dispersed (Brown and Hovmøller 2002). Many of the fungi that are transmitted by insects do so by either creating wounds which facilitates the entry of the pathogen or by unintentional transport on their bodies while moving between diseased and healthy host plants (Agrios 2007). The more specialized insect-fungus interactions involve transmission through insect feeding structures or by fungal mimicry of various sorts to attract insects, which aid in dispersal (Ngugi and Scherm 2006; Ngugi et al. 2006; Roy 1993). Insect-pathogen relationships depend on variations in vector feeding habits, the population dynamics of both and the biology of the fungus. These factors set the stage for important coevolutionary dynamics and adaptation strategies employed by fungi. Moreover, evolutionary patterns suggest that selection favors pathogens which have better dissemination capabilities (Eigenbrode et al. 2018). Many of these fungi have evolved to manipulate their host plant to increase the likelihood of being transmitted.

Some plant-pathogenic fungi have developed the ability to induce the production of pseudoflowers which are flower-like structures induced by the fungus and produced by the plant upon infection during vegetative growth (Roy 1993). These specialized structures serve to facilitate sexual outcrossing or transport of reproductive structures by pollinator insects that are attracted to them. They tend to look different from host flowers in the former but similar in the latter and is especially important in fungi who complete their life cycles on host flowers (McArt et al. 2016). Flower mimicry in this form is most widely documented in different species of rust fungi in *Puccinia* and *Uromyces*, where heterothallic strains are efficiently outcrossed by pollinator insects (Ngugi and Scherm 2006; Roy 1993; Pfunder et al. 2000; Naef et al., 2002). It is shown in the anther smut fungus *Microbotryum violaceum,* however, that mimicry does not always involve the whole flower but can result

in manipulation of individual parts. In this system the fungus causes sterility of the anthers but deposits teliospores on them for insect transmission (Ngugi 2006). Pollinators that have been implicated in dispersal of this fungus include bees, syrphid flies and moths (Altizer et al., 1998).

While these pseudoflowers attract insects visually, other insect vectors are attracted to floral scent mimicry. In these cases, fungal infection induces the production of volatiles that are similar to those found in flowers, attracting pollinator insects that aid in their dispersal. Some of these volatiles have sweet smells that are a made up of alcohols, aldehydes and esters that are aromatic (Raguso et al., 1998). This phenomenon was shown using the economically important mummy berry-blueberry pathosystem, where leaves infected with *Monilinia vaccinii-corymbosi* produced the volatiles cinnamyl alcohol and cinnamic aldehyde as found in flowers but not in uninfected leaves (McArt et al 2016). Another study of floral mimicry in *Fusarium xyrophilum* affecting yellow-eyed grass (*Xyris* spp.), showed that pseudoflowers were completely fungal tissue, mimicked host floral traits, were ultraviolet reflective and produced pigment that matched the spectral sensitivity of trichromatic insects (Laraba et al. 2020). The authors also found that *Xyris* flowers and *F. xyrophilum* cultures produced a volatile that attracts pollinators, 2-ethylhexanol.

Other fungal pathogens manipulate the physiology of the host plant. Some of them that also depend on pollinator insects cause changes in the plant such as timing of flowering. An example of this is in reported by Jennerson (1988), where *Viscaria vulgaris* plants that were infected by the anther smut fungus, *Microbotryum violaceum* (*Ustilago violacea*), bloomed earlier and stayed open longer than healthy plants. Presumably, this

lengthens the time available for pollinator insects to visit these plants and transmit the disease.

Fungal hyphae and conidia have also evolved to resemble pollen tubes and pollen in some instances where they also imitate movements to allow evasion of host defenses. This is demonstrated explicitly in *Monilinia vaccinii-corymbosi* (Ngugi et al., 2002). Fungi which complete their life cycles on flowers are more likely to depend on insects for dispersal (Kaiser 2006; McArt et al. 2016), but examples of plant pathogenic yeast-insect interactions are most common in the literature. They are often recovered from stylets, receptacles, hindgut and heads of insects (mainly adults) with viability of spores differing in each structure. These yeasts are mainly transmitted by insects in the *Heteroptera* also called the "true bugs", some of which also vector other types of fungal plant pathogens. Some examples include: *Nematospora coryli* and *Nematospora gossypii* which causes pecky rice disease after stink bug damage (Mitchell 2004). Other fungi isolated from stink bugs affecting rice include *Curvularia lunata, Alternaria alternata, Fusarium oxysporum, Cochliobolus miyabeanus* and *Bipolaris oryzae*, all of which only caused pecky rice disease symptoms if inoculated with a wire that imitates the stylets of these insects (Purcell et al., 2005). *Nematospora coryli* also causes yeast-spot disease of soybeans and is transmitted by at least four different species of stink bugs (Daugherty, 1967). Mealworm beetles have also been shown to transmit various *Fusarium* species to wheat grains (Guo et al. 2018). Burgess et al. showed in 1991, that the yeast *Nematospora sinecauda* infects mustard crops through the false chinch bug, *Nysius niger.* Dried-fruit Beetles were found to transmit three species of yeasts causing the souring of figs (Caldis, 1930).

There are other well-documented fungal plant pathogens that rely on insect vectors to cause disease, while others are implicated through circumstantial evidence. Of those that are conclusive, many are important in agriculture and forestry. Dutch Elm Disease is a disease of elm species, and more significantly American elm, caused by the fungus *Ophiostoma ulmi* and *O. novi-ulmi* which are vectored by the European elm bark beetle (*Scolytus multistriatus*) and the native elm bark beetle (*Hylurgopinus rufipes*). The vectors are necessary for the transmission of the fungus from diseased to healthy elm trees (Agrios, 2005). Black stain root disease affects pine, hemlock and douglas fir trees and is caused by the fungal pathogen *Leptographium wageneri,* which is vectored by *Hilastes nigrinus*, a root feeding bark beetle, as well as the crown and root weevils *Steremnius carinatus* and *Pissodes fasciatus* (Agne et al. 2018). The perennial canker of apple is caused by the fungus *Neofabraea malicorticis* which is transmitted by *Eriosoma lanigerum*, also known as the wooly aphid (Aguilar et al. 2019). A few vascular wilt diseases are caused by fungi and transmitted by insects. Some of these include persimmon wilt caused by *Cephalosporium diospyri* and transmitted by both the powder-post beetle (*Xylobiops basilaris*) and the twig girdler beetle (*Oncider cingulatus*) (Crandall and Baker 1950), mango wilts caused by the fungi *Diplodia recifensis* and *Ceratocystis fimbriata* and transmitted by the beetles *Xyleborus affinis* and *Hypocryphalus mangiferae* respectively (Harris and Maramorosch 2013). One of the most destructive forest diseases, oak wilt, is caused by the fungus *Ceratocystis fimbriata* and vectored by nitidulid, scotylid, buprestid, and brentid beetles and the flat-headed borer (Hayslett et al. 2008). Some foliar diseases caused by fungi and transmitted by insects include: red pine needle blight caused by *Pullularia pullulans* and transmitted by cecidomyiid midge (Phillips and Burdekin 1982) and cucurbit anthracnose caused by

Colletotrichum lagenarium and vectored by the spotted cucumber beetle (Agrios 2007). Blossom blight of red clover is caused by *Botryitis anthophila* and transmitted by pollinating bees (Leach 1935). Flower spot of azalea is transmitted by various species of ants, thrips and bees and is caused by the fungus *Ovulina azaleae* (Agrios 2007). Coffee bean rot is caused by the fungi *Nematospora corylii* and *Nematospora gosypii* and enabled through feeding wounds by the insects *Antestia lineaticolis* and *Antestia faceta*. Other plant-pathogenic fungi are transmitted through wounds made by insects during oviposition. One such disease is the boll rot of cotton which is caused by fungi such as *Fusarium monoliforme, Alternaria tenuis, Aspegillus flavus* and *Rhizopus nigricans* and transmitted in this way by the boll weevil, cotton bollworm and tarnished plant bug (Agrios 2007).

The evolution and structural variations in fungal genomes

Exobasidium maculosum has a high level of genetic diversity (Stewart et al. 2015). This study was done on a few conserved loci and does not cover genome-scale structural variations. The plethora of fungal genome sequences that are now available for comparative genomic studies has shed light on the incredible variation that exists in genome size and architecture within and among species. These types of studies in *E. maculosum* can help us to better understand how it evolves and adapts.

The coevolutionary dynamics in the arms race between plant defense response and pathogen evolution have helped to shape this variation which can be seen at the genomic level and by assessing evolutionary histories (Barrett et al. 2009, Bergelson et. al 2001, Dodds et al. 2006, Kanzaki et al. 2012). Sustainable management of plant diseases is constantly challenged by the ability of certain pathogens to rapidly adapt to varying

environments and overcome control strategies using genetic arsenals such as transposable elements and remarkable genome plasticity (de Jonge et al. 2013, Farrer et al. 2011, Galazka and Freitag 2014, Raffaele and Kamoun 2012, Seidl and Thomma 2014, Selmecki et al. 2010, Stuckenbrock et al. 2011). For example, the introduction of a new wheat and rye hybrid resulted in the emergence of a new pathogen from the hybridization of two *Blumeria graminis* formae speciales (Menardo et al. 2016). In many cases there are specific regions of pathogen genomes that have high mutation or recombination rates, rapidly providing the material on which selection can act. Genomic studies have shown that these regions usually host virulence genes, are rich in transposable elements or clusters of tandem duplicated genes or in accessory chromosomes (Badouin et al. 2016, Chuma et. al 2011, Faino et al. 2016, Ma et al. 2010, Rouxel et al. 2011). Accessory chromosomes define host range or are lineage specific in certain pathogens such as *Fusarium oxysporum* and *Nectria haematococca* (*Fusarium solani*) where they sometimes move horizontally or have effectors which determine pathogenicity on certain hosts or varieties (Chuma et al. 2011, Temporini et al. 2002, van der Does et al. 2016). Structural rearrangements of faulty DNA repair mechanisms may lead to these regions that are rich in transposable elements or the formation of accessory chromosomes (Faino et al. 2016, Plissonneau and Stürchler 2016). Genomic regions with tandem gene duplications and repetitive DNA but low in transposable elements are also drivers of rapid evolution in some plant-pathogenic fungi and is shown in the smut fungi *Ustilago maydis*, *Sporisorium scitamineum* and *Sporisorium reilianum*, where these regions are associated with virulence gene clusters (Dutheil et al. 2016, Kämper et al. 2006, Schirawski et al. 2010).

Single nucleotide polymorphisms (SNPs) and copy-number variations (CNVs) are increasingly being recognized for the role that they play in important human diseases, though not studied much in plant disease systems (Ionita-Laza et. al 2009). The complexity of genomes due to gene CNV which arise by duplications or deletions, has a profound effect on the fitness and evolutionary and adaptive potential of organisms (Katju et. al. 2013, Bailey et. al. 2006, Conrad et. al. 2010). It is therefore important to understand the rates at which copy-number variation is introduced and removed from populations to gain insight into the role that these play in genomic structuring and mechanisms of evolution and pathogenesis. In fact, ploidy, mating and serotype together have been shown to impact the virulence and fitness of the human pathogen *Cryptococcus neoformans* (Lin et. al. 2008).

Ploidy is the number of sets of chromosomes in a cell. Ploidy variation is implicated in adaptation and has been shown to have a dynamic influence on the genetic restructuring of many yeast species (Todd et. al. 2017, Zhu et. al. 2016, Selmecki et. al. 2015, Storchova et. al 2006, Gerstein et. al. 2008). Various species have extensive ploidy variation among strains while others exhibit ploidy switching as a response to environmental stressors (Ezov et. al. 2006, Suzuki et. al. 1982, Ford et. al. 2015). The human pathogen *Crytoccocus neoformans* has even been shown to have cells within the same strain with different ploidies (Gerstein et. al. 2015). Ploidy changes can cause genome instability and is a dynamic evolutionary force (Storchova 2014). It has also been shown to produce increased fitness and the evolution of antifungal drug resistance in *Candida albicans* (Selmecki et. al. 2009).

Justification and Objectives

Exobasidium leaf and fruit spot is an economically important blueberry disease but there is an incomplete understanding of the biology and genetic architecture of the pathogen which causes the disease, *Exobasidium maculosum.* As blueberry production continues to increase in Georgia the disease may become a greater economic concern. This is compounded by the fact that the pathogen seems to have the genetic machinery for rapid evolution of resistance to the current approach of pesticide management. Currently, little is known about the basic biology of *E. maculosum*, the reason for disease emergence, the specific stages in the life and disease cycles and the genetic basis for adaptation in populations of the fungus. Although major steps have been made in defining how the pathogen causes disease in the field (Ingram et al. 2019), there are still gaps in our knowledge of precise stages of disease transmission. There are many evolutionary processes that allow organisms to adapt to varying environments. Changes at the genomic level can be deleterious but, in many cases, provide increased fitness. For many fungal plant pathogens, pathogenicity or virulence is determined by specific virulence factors, effectors or other variations that exists within their genomes. Genomes of *E. maculosum* are extremely diverse and understanding the mechanisms that lead to this diversity may provide some insight into its emergence, biology and how it adapts to different environments. A thorough understanding will help in designing and ensuring durable management protocols.

The objectives of this PhD project are to:

1. Determine if *E. maculosum* is transmitted by a vector
2. Determine the reasons for the high level of genetic diversity seen in *E. maculosum*

3. Assess structural variations among genomes of *E. maculosum*
4. Identify signatures of adaptation through comparative genomics

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CHAPTER 2

EVIDENCE FOR VECTOR TRANSMISSION OF THE BLUEBERRY PATHOGEN *EXOBASIDIUM MACULOSUM* WHICH CAUSES EXOBASIDIUM LEAF AND FRUIT SPOT

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ABSTRACT

Exobasidium leaf and fruit spot of blueberry, which is caused by the fungal pathogen *Exobasidium maculosum*, results in significant yield losses in commercial production if not properly managed. To determine if an arthropod vector is involved in transmission of the disease, we conducted arthropod exclusion and trapping studies at two field sites in Alma and Toccoa, GA, from 2018 to 2020. In each field, ten trap plants were covered with exclusion nets and paired with ten uncovered plants under diseased blueberry bushes for two weeks during the known infection window in late-March and April. The plants that were covered had 0 to 12 leaf spots while the uncovered plants developed up to 654, indicating that we excluded an agent necessary for infection to occur and points to vector transmission of the disease. To identify possible vectors of the fungus, a variety of traps were used to collect arthropods in both fields during the known infection window. A total of 181 insects were selected from traps, then crushed and plated on potato dextrose agar to assess for growth of *E. maculosum*. A species-specific PCR assay was developed and conducted to test for *E. maculosum* in the 181 insect samples. We recovered *E. maculosum* from one insect that was plated on PDA and detected it in three additional samples with the PCR assay. Preliminary growth chamber bioassays with spotted-wing drosophila (*Drosophila suzukii*) and brown marmorated stink bugs (*Halyomorpha halys*) did not show transmission of the disease by these insect species. Although we did not conclusively identify specific vectors in our study, we provide preliminary evidence of vector involvement in the transmission of this pathogen in what is a complex pathosystem.

Keywords: Exobasidium leaf and fruit spot, blueberry, *Exobasidium maculosum*, arthropod, fungal vectors

INTRODUCTION

Fungi represent the largest, most diverse, and important group of plant pathogens in agriculture and forestry, yet there are few examples of them being directly transmitted by arthropods. Most interactions between plant-pathogenic fungi and their arthropod vectors are either indirect or speculative in nature (Agrios 2008; Fermaud and Le 1992; Prom et al. 2003; Scarpino et al. 2015). Indirect transmission is incidental, and often involves vectors picking up fungal inoculum and transporting it from diseased to healthy plants, or vectors creating wounds during oviposition or feeding allowing the entrance of fungi. Direct transmission, on the other hand, is active acquisition and transmission of the pathogen, which usually requires circulation or retention of inoculum in vector body parts. While insect transmission of viruses and bacteria have been well characterized (Hogenhout et al. 2008; Huang et al. 2020; Purcell 1982; Whitfield et al. 2015), there is a lack of information on these relationships with fungi, mainly because they are uncommon. Those that have been studied show plant-pathogenic fungi employ unique strategies to adapt to a vector-transmitted lifestyle, such as floral structure and scent mimicry and the manipulation of host physiology (Ngugi et al. 2004, 2006; Raguso et al. 1998; Roy 1993). For example, some plant-pathogenic fungi induce the production of pseudoflowers by their host upon infection (Laraba et al. 2020; McArt et al. 2016). These specialized flower-like structures facilitate sexual outcrossing of the fungus or the transport of fungal reproductive structures by pollinator insects attracted to the pseudoflowers (Pfunder et al.

2000). Some fungi also induce the production of volatiles that mimic floral scent to attract these insects. This was shown in the mummy berry-blueberry pathosystem, where leaves infected with *Monilinia vaccinii-corymbosi* produced the volatiles cinnamyl alcohol and cinnamic aldehyde found in flowers but not in uninfected leaves (McArt et al. 2016). Some fungal species are also luminescent and emit light that may attract arthropods. Sivinski (1981) showed that more of certain arthropods were captured in baits with light emitting fungal mycelia and fruiting bodies than those without. There are also instances where infected tissue seem to reflect ultraviolet light in the same wavelength as flowers to attract pollinators (Bunyard 2007; Laraba et al. 2020). Fungi which complete their life cycles on flowers are more likely to depend on insects for dispersal, but usually in a passive or indirect way. Direct transmission of fungal plant pathogens often involves yeast species (Caldis 1930; Daugherty 1967; Purcell et al. 2005). They are usually recovered from stylets, receptacles, hindgut, and heads of insects (mainly adults) with the viability of spores differing in each structure. These yeasts are mainly transmitted by Hemipteran insects or "true bugs", some of which also vector other types of plant pathogens, such as aphids, whiteflies and psyllids.

Exobasidium leaf and fruit spot is a disease of blueberry caused by the fungus *Exobasidium maculosum* (Brewer et al. 2014). Symptoms of the disease first appear on leaves in early spring as small, green leaf spots that expand and become white as they age, and as green spots on fruits that become more apparent, white and velvety as berries ripen. Diseased berries are usually tough or misshapen and have an undesirable appearance, taste and texture. They must be manually removed at the packing line because they are not automatically detected and can lead to the rejection of fruit loads. The disease is now

prevalent in commercial blueberry production fields in the southeastern United States and northeastern North America where it can cause significant yield losses if not properly managed (Brannen et al. 2013, Nickerson and Kloet 1996). The disease is mainly a problem in the production of rabbiteye blueberry (*Vaccinium virgatum*), highbush blueberry (*Vaccinium corymbosum*) and southern highbush blueberry (*Vaccinium* interspecific hybrids), but lowbush blueberry (*Vaccinium angustifolium*) is also affected. Interestingly, the genus *Exobasidium* (class Exobasidiomycetes, subphylum Ustilaginomycotina, phylum Basidiomycota) includes species that cause a wide range of plant deformities, including some that induce flower-shaped galls that may function in attracting insects (Halsted 1893; Kennedy et al. 2012; Tattar 1989). Members of *Exobasidium* are biotrophs on hosts in the Ericales,Theales including the Ericacea and Theaceae and cause other symptoms such as blisters, leaf spots and witches' brooms (Callan and Carris 2004).

The causal fungus of Exobasium leaf and fruit spot, *E. maculosum,* is dimorphic with a filamentous infective stage in the plant and a single-celled yeast stage that is saprotrophic and culturable (Brewer et al. 2014). Since the fungus was first identified there has been no success with artificial infection of blueberry plants despite repeated attempts, including with mixed cultures. Placement of young, potted blueberry plants during the brief infection period in early spring near bushes severely infected the previous season with *E. maculosum* in the field, however, results in infection followed by leaf and fruit spot symptoms (Ingram et al. 2019). Ingram et. al (2019) conducted epidemiological field studies in southern Georgia to characterize the disease cycle and concluded that the pathogen overwinters epiphytically on plants in the field, the infection window was from late-March into April, and that basidiospores are produced and dispersed after spot development and are

therefore unlikely as a source of primary inoculum. Additionally, they found that disease progresses in a monocyclic pattern with fruit spots having the same inoculum source as leaf spots. The authors proposed that initial infection occurs through splashing of overwintered inoculum on the surface of the plant; therefore, we were interested in testing this in greenhouse assays with young plants and branches collected from infected field sites. Alternatively, we considered that an arthropod vector may be involved and even necessary for infection and disease development. Moreover, Stewart et al. (2015) studied the population genetic structure of *E. maculosum* when the disease was first emerging in Georgia, North Carolina, Maine and eastern Canada, and found very high levels of genetic diversity and that populations were structured by geographic location (Stewart et al. 2015); populations of pathogens that are recently introduced, changed hosts, or evolved aggressive strains are not usually genetically diverse or geographically structured. The authors concluded that disease emergence was likely caused by an increasing host population or environmental change. It is possible that this environmental change involves shifting vector dynamics. The brown marmorated stink bug or BMSB (*Halyomorpha halys*) and spotted wing drosophila or SWD (*Drosophila suzukii)* started becoming a problem in stone fruit and soft summer fruit production in the southeastern United States around the same time that Exobasidium leaf and fruit spot emerged (Asplen et al. 2015; Ludwick et al. 2020; Wiman et al. 2015).

We were interested in investigating the potential vector transmission of Exobasidium leaf and fruit spot. Our objectives were to (i) investigate if infections are initiated by splash-dispersed inoculum from blueberry stems (ii) determine if arthropod exclusion reduces disease, (ii) determine if *E. maculosum* fluoresces in ultraviolet light, (iii)

develop a PCR-based assay for detection of *E. maculosum*, (iv) identify if *E. maculosum* is present on arthropods collected from the field at the time of disease onset and, if so, identify the body part where the fungus is localized, and (v) determine if SWD or BMSB are involved in transmission.

MATERIALS AND METHODS

Artificial inoculations

To test if diseased plant material collected from the field would cause disease on healthy trap plants away from the field, cuttings were separated into stems, stems with leaves, or stems with leaves and buds. They were then hung on top of three trap plants each in the greenhouse at 22°C to 28°C for 14 days (March 29 to April 13, 2019). One plant per treatment was also placed outside the greenhouse. A hose with a 5.7cm nozzle was used to water plants from atop in an attempt to mimic rainfall.

Young and old diseased leaves were incubated on wet paper towels in glass petri plates to induce sporulation. Leaf spots were checked for fungal sporulation under a stereoscope and those that were sporulating were cut from leaves with a razor blade. One leaf spot was placed on 10 separate leaves of 2 healthy trap plants for a total of 20 leaf spots. After 24hrs, leaf spots were removed from leaves. One trap plant was placed in the greenhouse at 22°C to 28°C. The other was placed in a dew chamber for 2 days at 100% relative humidity, then in the greenhouse with the other plant. Plants were checked daily for the development of disease symptoms until 28 days had passed.

To test if plant injury would facilitate infection, leaves were wounded with a razorblade or a 0.8mm gauged hypodermic needle. A mixed yeast cell suspension of 1×10^6 cells/ml was sprayed with an atomizer onto injured leaves. The mixed population included a combination of three isolates from different locations derived from basidiospores of single spots that should include individuals of compatible mating types, which may be required for the filamentous, infectious stage similar to the related *Ustilago maydis*. The plant was then placed in the greenhouse at 22°C to 28°C and checked daily for disease symptoms until 28 days had passed.

To test if direct introduction of spores inside leaf tissue would cause infection, the spore suspension was also injected into three detached leaves using 0.5 mm, 0.8 mm and 1.1mm gauged hypodermic needles. They were then placed in glass petri plates in an incubator at 23°C and checked daily for symptom development. These injections were also done on leaves of a healthy plant which was then placed in the greenhouse as described above. Vacuum infiltration at 250 mbar for 2min was also used to introduce the spore suspension inside a detached leaf.

Selection and preparation of arthropod exclusion nets

Fabric netting for exclusion nets of three different mesh sizes were tested to ensure that passage of yeast cells was achievable and that nets were not excluding *E. maculosum* inoculum. The three fabrics were cut into 10-cm squares, sterilized in an autoclave, and secured on the openings of 500 ml beakers containing 20 ml potato dextrose agar (PDA). Two beakers of PDA were tested per mesh size. A 200 µl cell suspension (1×10^6 cells/ml)

with a mixed population of *E. maculosum* spores (described above) was sprayed onto each net. The netting was removed from the beakers, which were covered with parafilm and incubated at 23°C for 7 days with a 12-h photoperiod at which point they were checked for growth of *E. maculosum* colonies (Figure 2.1). The material with the smallest mesh size that did not exclude *E. maculosum* was used for the study. Fabric was cut to the appropriate size and sewn at the edges to entirely cover young blueberry trap plants from the top in their 2.8 L pots (Fig. 2.2)

Arthropod exclusion field study

Dormant, 60 to 90 cm in height 'Premier' rabbiteye blueberry plants were potted in 2.8L plastic pots and placed in a cold room at 4°C until needed. Plants were placed in the greenhouse at 22°C to 28°C for 10 days to break dormancy before taking them to the field. Field studies were conducted between March 28 and April 30, 2019 on two commercial blueberry farms in Alma (southern Georgia) and Toccoa (northern Georgia), with recurring Exobasidium leaf and fruit spot outbreaks. Southeast Georgia is the center of blueberry production in the state and Exobasidium leaf and fruit spot epidemics while northeast Georgia has less production and incidence of the disease. Trap plants were placed in pesticide-untreated sections of each field with 'Premier' and 'Tifblue' rabbiteye blueberry plants in Alma and Toccoa, respectively. They were placed in each field for a two-week period near the time of year previously determined to have the highest infection levels for the disease (Ingram et al. 2019). The dates were March 28 to April 12 in Alma and April 10 to 23 in Toccoa. Disease onset in northern Georgia is usually later than southern Georgia because of cooler temperatures causing later blueberry development. Ten plants were

covered with nets before being taken to the field while another 10 were left uncovered for a total of 20 plants per field site. Plants were placed in pairs of covered and uncovered underneath diseased blueberry bushes and fitted with watering stakes and jugs. Water levels were checked and replenished, if necessary, after 7 days. After a 14-day period, the plants were removed from the field and placed in the greenhouse to observe symptom development. After 14 days in the greenhouse, when no additional spots developed, the number of leaf spots on each plant was counted and recorded. A Wilcoxon Signed Rank Test was used to compare paired data in each location using the R statistical computing software (R Core Team)

Ultraviolet fluorescence

Lamps with ultraviolet (UV) bulbs in the wavelengths within the UVB (280-315nm) and UVA(315-400nm) spectra were shone onto ten Exobasidium leaf spots from Alma, GA, on two freshly collected leaves to determine if they were fluorescent, which could attract arthropods for dispersal of spores. Laundry detergent and a cotton ball, which both fluoresce under UV light, and water, which does not fluoresce, were used as controls. This procedure was also followed with two single-spored isolates of *E. maculosum* yeast colonies from Maine and Georgia.

Insect trapping and processing

In 2019, two sets of three 12.7 × 17.8 cm yellow and 7.6 × 15.2 cm clear sticky cards (Great Lakes IPM, Vestaburg, MI) were placed among diseased bushes for 7 days each at the same time that trap plants were placed in the fields. Yellow sticky cards are broad spectrum

insect attractants and are useful for trapping aphids, fungus gnats, whiteflies, thrips, and leaf miners, among others. Clear sticky cards are mainly used in the field to monitor stink bugs. Apple cider vinegar traps are used to attract insects such as aphids and fruit flies and were made by drilling two adjacent holes in plastic jars for arthropod access and attaching wire loops for hanging on blueberry bushes. Four jars were filled half-waywith apple cider vinegar and hung on diseased bushes in each field. Stink bug pheromone traps (Dead-Inn Stink Bug Trap, Ecogreen Warehouse,) which attract brown marmorated stink bug (*Halyomorpha halys),* the green stink bug (*Acrosternum hilare*), and the native brown stink bug (*Euschistus servus, E. conspersus, E. heros, E. obscurus, E. tristigmus*) and (Rescue! Reusable Stink Bug Trap, Harris Seeds, Rochester, NY) were used to trap stink bugs in Toccoa. They were placed in the field when trap plants were being removed and left for 3 days.

Arthropods were recovered from traps systematically to represent all the morphological variation that was observed (Table 2.1), and catalogued with field name, trap type and an insect number. They were then dipped in 70% ethanol and allowed to air dry before being plated on fungal growth media. To determine if the fungus was localized to a specific body part, their heads were separated from abdomens, and both were crushed on PDA containing streptomycin (100 μg/ml) in 100-mm Petri dishes. Plates were incubated at 23°C with a 12-h photoperiod and checked daily for growth of *E. maculosum*. A total of 46 insects were processed and plated on PDA.

In 2020, three each of net-covered an uncovered 60-90 cm trap plants with 4×4 cm squares of yellow, blue, or clear sticky cards on leaves, were placed under diseased blueberry bushes in the same fields in Alma and Toccoa, GA. Openings were cut in nets of

covered plants to allow arthropod entry (Figure 2.3) so that we could catch arthropods more likely to be involved in transmission. Eight each of 10.1× 15.2 cm yellow-blue, and 20.3× 25.4 cm clear sticky traps were also placed among bushes for 7 days. A fluorescent universal black light bucket trap (BioQuip, Rancho Dominguez, CA) which is used to trap nocturnal insects, was placed in the field for 4 days . An insect vacuum (Serene Life), beating sheets and sweep nets (BioQuip) were also used to actively sample insects from blueberry bushes with high disease incidence in previous years. Active sampling involved shaking bushes to dislodge arthropods that may not be trapped using other techniques and collecting them. Clear sticky traps were placed on beating sheets to immobilize organisms that were dislodged from bushes. Traps were placed in the field when active sampling was conducted on April 4 and 8 in Alma and Toccoa, respectively, and left for 7 days.

Insects were selected from traps to represent all types that were present but mainly those that seemingly had piercing or sucking mouthparts. This was because we noticed piercings in the centers of leaf spots consistent with this type of insect. Large sticky cards were cut into small squares and they along with 4×4 cm square sticky cards were placed under a stereoscopic microscope. Insects were then selected, photographed, and numbered. They were then removed with a flame-sterilized dissecting needle. Smaller insects (less than 6 mm) were crushed with a micropestle in 50 µl of sterile water and larger insects (6 mm or larger) in 100 µl in 2ml Eppendorf tubes. Insects that were collected using non-stick methods were also photographed under a stereoscopic microscope and transferred to tubes using flame sterilized forceps. Twenty-five microliters of the suspension was spread on PDA amended with chloramphenicol (50 µg/ml), gentamicin (5 µg/ml) and streptomycin (100 µg/ml) in 100-mm Petri dishes.

Plates were incubated at 23°C with a 12-h photoperiod and checked daily for growth of *E. maculosum*. A total of 181 insects (Table 2.1) were processed from Alma and Toccoa, GA.

Exobasidium maculosum-specific PCR-based detection assay

To determine if *E. maculosum* was associated with any of the collected insects, we developed and used an *E. maculosum*-specific PCR-based detection method. An *E. maculosum*-specific forward primer, ExoITSF (5'-CGCTTTCGAGCTCTTCCTCTA-3'), was designed for the internal transcribed spacer (ITS) region of fungal ribosomal DNA (rDNA) of *E. maculosum*. The region of ITS1 used for primer design was conserved among isolates of *E. maculosum* (Stewart et al. 2016), which can be genetically diverse, but different from other *Exobasidium* species (Brewer et al. 2014). PCR was conducted with the ExoITSF and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (White et al. 1990). Each reaction contained 2.5 µl of 10× ExTaq buffer (Takara), 2.5 µl of dNTP mixture (Takara), 1.25 µl of 10 µM ExoITSF primer, 1.25µl of 10 µM ITS4 primer, 0.15 µl ExTaq DNA polymerase (Takara) and 1 µl genomic DNA suspension for a total of 25 µl. A Mastercycler Pro thermal cycler (Eppendorf, USA) was used to perform all PCR reactions. The initial denaturation step of 94°C for 2 min was followed by 39 cycles of amplification (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s), and a final elongation step of 72°C for 5 min. All PCR products were visualized with a UV transilluminator on a 1% (wt/vol) agarose gel containing 10× gel red made with 1× Tris-borate-EDTA (TBE) buffer after electrophoretic separation at 88 V for 2 h. Positive controls included *E. maculosum* DNA, DNA extracts of *E. maculosum* yeast cells in varying quantities from 1 to 20 total cells, and DNA extracts of blueberry buds with 1 to 20 *E. maculosum* yeast cells. Negative controls included water and DNA extracted from

blueberry buds, *Ustilago maydis*, *Exobasidium* spp. (syn. *Exobasidium vaccinii*, causal agent of red leaf disease of blueberry (Brewer et al. 2014)), and *Exobasidium rostrupii*. All DNA was extracted with Qiagen Plant DNeasy miniprep kit (Qiagen, Germantown) following manufacturer's directions.

Crude genomic DNA was collected from the remaining volume of the 181 insect suspensions using a fast prep protocol. Briefly, insects were ground using a micropestle, tubes were incubated at 95°C for 20 mins, vortexed for 10 s after 10 min of incubation, briefly centrifuged and 1 µl of the suspension from each used in the PCR assay. The PCR-based assay was conducted as described above. DNA from two *E. maculosum* isolates were used as positive controls, and sterile water and DNA from *Corynespora cassiicola* were used as negative controls.

Live insect vector assays

Young 60-90 cm blueberry plants potted in 2-gallon plastic pots were removed from the cold room and placed in the greenhouse to break dormancy. A mixed population of *E. maculosum* as explained above was used to prepare a yeast cell suspension (1×10^6 cells/ml) which was sprayed on plants using an atomizer to drip-off. Mixed cultures on PDA in two 100-mm Petri dishes were placed at the base of each plant. Plants were covered with exclusion netting (described above) to keep insects trapped inside. Live Spotted Wing Drosophila (SWD) and Brown Marmorated Stink Bug (BMSB) colonies were obtained (Sial lab) and in separate trials, a single colony of up to 20 SWD flies was released inside each of three netted plants and 10 stink bugs each in three netted plants were released. Blueberry fruit obtained from the supermarket were hung with safety pins on plants that housed

SWD because we speculated that fruit would attract flies and increase the chances of *E. maculosum* transmission to leaves. A cotton ball in water was also placed inside nets for insect hydration. One plant from each trial was placed in the greenhouse at 22°C to 28°C, the second in a growth chamber at 23°C with a 12 hr photoperiod and the third in an incubator at 23°C with a 12 hr photoperiod all for 14 days. Plants were monitored daily for signs of Exobasidium leaf and fruit spot disease.

RESULTS

Artificial inoculations.

Disease symptoms were not observed on plants that were artificially inoculated by injury, infiltration or from using diseased plant material from the field.

Arthropod exclusion field study

Inoculum of *E. maculosum* could penetrate all exclusion nets and grow on PDA (Figure 2.1), so we used the netting with the finest mesh. The first Exobasidium leaf spots were seen six- and eight-days after transfer of trap plants to the greenhouse from Alma and Toccoa, respectively, and no new spots developed after 13-14 days in the greenhouse. The greatest number of leaf spots on a net-covered plant in Alma, GA, was 5 with a mean of 2 spots, while those that were uncovered had 21 to 654 leaf spots with a mean of 232 spots (Table 2.1). In Toccoa, the number of leaf spots ranged from 0 to 12 leaf spots on net-covered plants with a mean of 2 spots, and 0 to 109 leaf spots on uncovered plants with a mean of 24 spot. The Wilcoxon Signed Ranks Test indicated that the number of leaf spots on

uncovered plants was significantly higher than covered plants in both locations with p = 0.002 and 0.02 for Alma and Toccoa, respectively. Excluding arthropods with nets significantly reduced the number of leaf spots (Fig. 2.4). Leaf spots that did occur on net-covered plants were on leaves that were in contact with the nets and were usually aggregated on one to a few leaves. When leaf spots were scrutinized, needle-sized piercings were observed in the center of the spots (Fig. 2.5). White halos with various degrees of intensity were observed around these injuries which seemingly reflected the age of the spot.

Ultraviolet fluorescence

None of the Exobasidium leaf spots or yeast colonies on PDA fluoresced under UVA or UVB light.

Isolation in culture or PCR-based detection of *E. maculosum* from collected insects

Of the 181 insects (Table 2.2) that were removed from traps, crushed, and plated, *E. maculosum* was only recovered from one on a 4×4 cm yellow sticky card on an uncovered plant in Alma. The fungus was recovered from the head of the insect. The PCR-based assay was specific to *E. maculosum* and amplified a 550 bp fragment for all samples with *E. maculosum* DNA or yeast cells (4 or more cells) but did not amplify any fragment for other fungal or plant samples that did not contain *E. maculosum*. It was consistently sensitive to 4 yeast cells and became inconsistent when less than 4 cells were used in DNA extractions. The 550 bp ITS amplicon was obtained for 3 of the samples subjected to the PCR-based assay. The sample that the fungus was cultured from did not produce a band. Interestingly,

the four insects from which *E. maculosum* was cultured or detected were from 4×4cm sticky cards on uncovered plants in Alma (Fig. 2.6). There was no success in capturing arthropods with a sweep net or insect vacuum.

Live insect vector assays

There were no Exobasidium leaf spots observed on blueberry plants in live insect assays with SWD and BMSB.

DISCUSSION

In this study we show that using exclusion nets to prevent arthropod access to blueberry plants results in no disease or a significant reduction in Exobasidium leaf spot severity. We presume that we excluded an agent that is necessary for disease to occur and provide evidence to support vector transmission although the exclusion of dew as a source of leaf wetness in the canopies of net-covered plants cannot be excluded. This study negates the simple splash-dispersal theory proposed by Ingram et al. (2019) as we showed suspensions of yeast cells in water can pass through the netting and grow although our conclusion was based on sprayed inoculum and not on natural dripping of water (large drops) from plants in the field. Moreover, studies with stems, buds and infected leaves collected from the field did not result in disease transmission. While basidiospores were not tested in our beaker and net assay, we suppose they can also penetrate nets because they are approximately the same size as yeast cells. Additionally, Ingram et al. (2019) showed that basidiospores were produced and dispersed after leaf spots had formed in the

field, so they are not likely the primary leaf inoculum. It is also important to note that our insect transmission studies with SWD and BMSB were unsuccessful, so the conclusion of involvement an insect vector remains tenuous at this time.Insect exclusion netting is used in organic production of many agricultural crops to limit insect access and damage to plants by insect themselves or the pathogens that they carry. It has been explored as a method to manage SWD in blueberry and raspberry and other pests of thornless blackberry and grape (Strang et al. 1992; Cormier et al. 2015; Link 2014; McDermott et al. 2014; Leach et al. 2016; Rogers et al. 2016). Exobasium leaf and fruit spot emerged around the same time that SWD became established in the United States and caused significant losses in small fruit production. Although SWD only causes fruit damage we thought that there could be a connection with E*. maculosum* and SWD, since an environmental shift is one of the factors that possibly caused the emergence of Exobasidium leaf and fruit spot, and fruit flies are known to consume or have natural yeast symbionts (Hamby et al. 2012; Hoang et al. 2015). Piercings in the center of leaf spots, however, pointed to disease being directly transmitted by an insect in the Hemipteran order which includes insects that use piercing and sucking mouthparts to extract plant sap while feeding. We considered another likely candidate to be BMSB because this pest also shared a similar pattern of establishment across the US in commercial fruit production (Roush 2010; Wallner et al. 2014). The damage BMSB causes to leaves is characteristic of that observed in leaf spots. Although our studies with these insects did not yield any positive results, it is possible that there are physiological or environmental conditions that were not met, so they cannot be excluded as vectors. Since artificial puncturing of leaf tissue to simulate insect damage did not yield infection by the fungus, we conclude that there may be a more intimate

association between the pathogen and its vector that is not easily reproduced. For example, fungal mating, fungal priming, and insect physiology are considerations (Dietrich et al. 2013; Ghanim et al. 2016; Heck 2018). Other than stink bugs, another Hemipteran that is prominent in blueberry fields is the leaf-footed bug (Stafne 2013). Both insects are known to carry yeast in their mouthparts that is introduced into fruit when they feed (Brust 2011; Morgan 2019). The only true bug that was recovered and processed from traps in our studies were stink bugs that were lured with pheromones (Table 1). By using different types of traps, we targeted a wide range of insects, but a more precise approach with timing and trap type may be necessary for capturing more true bugs.

Exobasidium leaf and fruit spot is currently managed by a single late-dormant application of lime sulfur (Brannen et al. 2017). This application is thought to subdue the surface population of *E. maculosum* on plants that is responsible for primary infection, thereby causing less overall disease. Although this strategy is effective in disease management, it is important to understand the dynamics of the system to predict its longevity and appropriately inform future recommendations. Exclusion nets, though not commonly used to manage fungal diseases, could be an alternative to pesticides in managing Exobasidium leaf and fruit spot since they are already employed to control insect pests in small fruit production (Leach et al. 2016; Link 2014; McDermott and Nickerson 2014; Strang et al. 1992).

The results of this study call for additional experimentation to identify the potential vector(s) of the disease. It is important to understand all aspects of the life cycle of the pathogen and how they fit into the disease cycle. Having insect vectors is likely to impact

pathogen dispersal and thereby, disease epidemiology. This work highlights a complex life cycle of *E. maculosum* and identifies new considerations for disease management.

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Table 2.1. Disease severity of Exobasidium leaf and fruit spot on net-covered and uncovered *Vaccinium virgatum* cultivar 'Premier' trap plants placed in two commercial blueberry fields in Georgia. The number of leaf spots on net-covered and uncovered plants are ordered by pair in both locations.

Field Location	Treatment	No. of plants	Number of leaf spots per plant	Mean no. of leaf spots (±SD[a])
Alma, GA	net-covered	10	0, 0, 5, 0, 0, 2, 2, 4, 1, 2	1.6 (±1.7)
	uncovered	10	21, 138, 364, 183, 91, 135, 455, 654, 63, 215	231.9 (±199.8)
Toccoa, GA	net-covered	10	2,12, 3, 2, 0, 2, 0, 2, 0, 1	2.4 (±3.4)
	uncovered	10	7, 12, 1, 13, 8, 27, 0, 8, 109, 51	23.6 (±31.8)

p-values, , Wilcoxon signed rank test (Alma:0.002, Toccoa:0.02)

[a] Standard deviation

Table 2.2. Number of insects processed from different trap types. Traps were placed in blueberry fields with a known incidence of Exobasidium leaf and fruit spot in Alma and Toccoa, GA. Traps were placed in March or April of 2019 and 2020.

Trap type	Number of insects processed	
	Alma, GA	Toccoa, GA
Large sticky cards	88	21
4×4 cm sticky cards (covered plants with holes)	23	3
4×4 cm sticky cards (uncovered plants)	39	3
Apple cider vinegar	3	2
Stink bug pheromone trap	0	12
Beating sheet	15	2
Insect vacuum	0	0
Sweep net	0	0
Black light bucket trap	13	3
Total	181	46

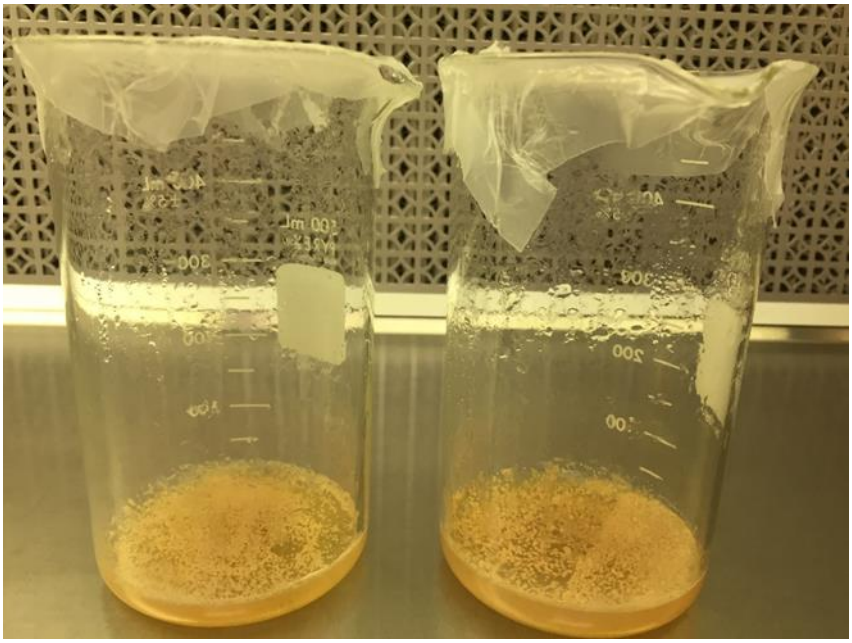

Figure 2.1. *Exobasidium maculosum* yeast cells can penetrate exclusion netting and grow on PDA.

Figure 2.2. Pair of uncovered and net-covered trap plants of *Vaccinium virgatum* cultivar 'Premier' fitted with an automatic watering system and placed together and under a diseased bush of the same cultivar in a blueberry field in Alma, GA on March 28, 2019.

Figure 2.3. 4×4 cm square sticky cards arbitrarily placed on uncovered and covered trap plants with small openings of *Vaccinium virgatum* cultivar 'Premier' to allow arthropod access.

Figure 2.4. Exobasidium leaf and fruit spot severity on uncovered (left) and net-covered (right) trap plants of *Vaccinium virgatum* cultivar 'Premier' placed together under a diseased bush in a blueberry field in Alma, GA from March 28, 2019, to April 12, 2019. Plants were moved to the greenhouse to allow for symptom development. Photos were taken April 26, 2019.

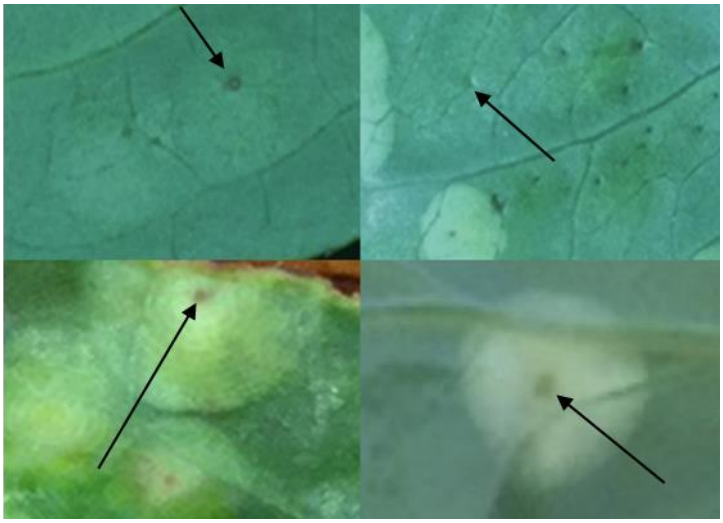

Figure 2.5 Exobasidium leaf spots with apparent insect damage in center of spots and other insect piercings on leaves. Leaves collected in Alma or Toccoa, GA on *Vaccinium virgatum* cultivars 'Premier' and 'Tifblue' respectively. Photos were taken between March 28 and April 26, 2019.

Figure 2.6. Insects from which *Exobasidium maculosum* was cultured (58) and detected with PCR-based assay (48,74,76).

CHAPTER 3

VARIATION IN PLOIDY, MUTATION RATE, KARYOTYPE AND NUCLEI PER CELL IN *EXOBASIDIUM MACULOSUM* POPULATIONS

[1]Abrahams, A.D., and Brewer M.T. To be submitted to BMC Genomics

ABSTRACT

The high level of genetic diversity in *Exobasidium maculosum* suggests that the pathogen can rapidly adapt to different environments, which has important implications for its management in commercial blueberry production. We hypothesized that ploidy variation, a high spontaneous mutation rate and karyotype variation may contribute to this high level of genetic diversity. We assessed ploidy variation among 14 strains of *E. maculosum* using flow cytometry and found that 1 was haploid, 4 were diploid and 9 were aneuploid. Aneuploids were close to diploidy and tetraploidy. We also estimated the spontaneous mutation rate among 6 strains of *E. maculosum* and 2 strains of the closely related species *Exobasidium rostrupii* using a fluctuation assay. We found that the spontaneous mutation rate of the haploid *E. maculosum* strain (16.9×10^{-7} mutations/generation) is ten times higher than that of *E. rostrupii* (1.7×10^{-7} mutations/generation). Aneuploid strains had lower mutation rates (3.8×10^{-7} to 10.1×10^{-7} mutations/generation) than the haploid strain and no mutants grew on selective media for diploid strains. Contour-clamped homogenous electric field (CHEF) electrophoresis was used to assess the number and size of chromosomes among 15 strains of *E. maculosum.* Four chromosomal bands at 3, 3.5, 5 and > 5.7 Mb were observed for the 9 strains that showed separation. For these nine strains, one chromosomal band was variable in size between 2.35 and 2.7 Mb for a total of five bands per strain. To understand the lifecycle of *E. maculosum* we used various staining protocols to assess the number of nuclei in yeast and hyphal cells, and whether there are clamp connections in hyphae by staining the nuclei, hyphae and septae. We found that the yeast cells are uninucleate and preliminary evidence that hyphal cells are multinucleate.

These findings suggest that there are multiple factors at the cellular and genetic level that contribute to the population-level genetic variation observed in the fungus.

INTRODUCTION

Understanding the basic biology of a pathogen, including their cellular structure, how they survive, propagate and cause disease, is important for better managing the diseases they cause. Plant disease prediction models, which are based on various factors of a disease cycle such as dormancy, reproduction, dispersal, and pathogenesis, are often used in disease epidemiology to inform management decisions such as whether and when to apply pesticides and the varieties to be planted (De Wolf and Isard 2007). For example, Fusarium head blight of wheat epidemics are largely correlated with weather conditions and multiple forecasting models have been developed which aid in informing pesticide application timing to reduce mycotoxin contaminated grain (De Wolf et al. 2003; Del Ponte et al. 2005; Hooker et al. 2002). Some models also use stages in the life cycle of a pathogen to predict disease likelihood. Manter et al. (2005) developed a model to predict Swiss needle cast of Douglas fir epidemics by linking the mean winter temperature to ascocarp development in *Pheaocryptous gaeumannii*. Therefore, it is beneficial if the life and disease cycles of plant pathogens are clearly defined.

Many members of the Ustilaginomycotina are dimorphic and have two stages in their life cycles. These usually include a saprobic haploid yeast stage and a plant-parasitic dikaryotic stage that is filamentous. The subphylum consists of many plant pathogens including some of the smut fungi. Some of these fungi, such as *Ustilago maydis,* which

causes corn smut, affect important agricultural crops. *Exobasidium maculosum* is a member of the subphylum Ustilaginomycotina that does not produce teliospores making it and other members of the genus distinct from the smut fungi. However, *E. maculosum* is dimorphic and produces basidia, basidiospores, filamentous hyphae and yeast cells (Brewer et al. 2014). The fungus causes Exobasidium leaf and fruit spot of blueberry (*Vaccinium* spp.) and is filamentous in the host plant tissue but grows as a yeast in culture. In studying the epidemiology of the disease, Ingram et al. (2019) found that it is monocyclic with a primary inoculum that is not aerially dispersed, and that the fungus overwinters epiphytically on host tissues. They also found that basidiospores produced on leaves function primarily in dispersal and do not appear to cause leaf infections. Artificial infection of plants has not been successful and insect exclusion studies have provided preliminary evidence that a vector may be involved in disease transmission (Chapter 2). Apparent piercing in the center of leaf spots also supports this theory.

Exobasidium vexans which causes blister blight on *Camellia sinensis* (L.) Kunze (tea) is the only species of *Exobasidium* in the literature that has a well-defined and complete disease cycle. *E vexans* completes its life cycle on the tea plant and is polycyclic. The pathogen is not dimorphic and produces basidiospores which are dispersed by wind that also serves as the primary inoculum. The pathogen survives in necrotic lesions in the off-season (Mouli 2003). The fungus forms an appressorium upon infection which penetrates young leaves and stems. However, there is no mention in the literature of the nuclear state of *E. vexans* structures or those of other *Exobasidium* species. Clamp connections are hook like structures formed during mitosis in dikaryotic hyphae in some Basidiomycota that ensure that each cell receives a set of both daughter nuclei (Brandt 2013). Clamp

connections only occur within Basidiomycota and usually indicate that the fungus is a dikaryon; however, not all dikaryons have clamps (Webster and Weber 2007). We are interested in better understanding the lifecycle of this important plant pathogen, but it is difficult because it does not grow hyphae in culture and artificial inoculation of the blueberry host plants has not been achieved.

E. maculosum has a high level of nucleotide and haplotype diversity which Stewart et al. (2015) suggested is due to high recombination and population mutation rates. The authors sequenced three conserved loci of 82 *E. maculosum* isolates from different plant tissues of the fungus collected from Georgia, North Carolina and Maine in the US and Nova Scotia, Canada. Within the conserved loci, there were 1775 sequenced nucleotides with 286 segregating sites and each isolate was found to be a unique multilocus haplotype. We predicted that a high spontaneous mutation rate may also be influencing the high level of genetic diversity that is seen in *E. maculosum*. Twenty percent of the genes detected in the reference genome (DOE-JGI Project ID 1150418) are segmental duplications (Stephen Mondo, personal communication). We also observed variations in copy number of housekeeping and DNA repair genes, leading us to believe that there may be variations in ploidy and chromosome number and size among strains of the pathogen.

Ploidy is the number of sets of chromosomes in a cell. Eukaryotic genomes exist in multiple ploidy states, with many having one (haploid), two (diploid), more than two (polyploid) or abnormal (aneuploid) sets of chromosomes. Ploidy is shown to be important in adaptation to novel or adverse environments such as high temperatures, exposure to antifungals and salt, and oxidative stress (Dhar et al. 2011; Selmecki et al. 2015). For

example, ploidy increases were observed in the adaptation of *Saccharomyces cerevisiae* to saline stress in experiments conducted by Dhar et al. (2011). Ploidy variation has also been implicated in pathogenicity and evolution in a few fungal species, some of which cause important human diseases. The human fungal pathogen *Cryptococcus neoformans*, which causes meningoencephalitis in immunocompromised individuals, naturally occurs in the haploid state but has diploid infectious basidiospores and produces polyploid titan cells during infection (Lindell et al. 2005; Velagapudi et al. 2009; Ni et al. 2013; Feldmesser et al. 2001). Titan cells vary widely in size and ploidy; ploidy greater than 64N has been observed (Ene and Bennett 2014). Ploidy variation has also been found in *Candida albicans,* the common human pathogen that was previously thought to be an obligate diploid (Riggsby et al. 1982; Jones et al. 2004; Suzuki et al. 1982; Ford et al. 2015; Selmecki et al. 2010; Hickman et al. 2013; Abbey et al. 2014). The plant-pathogenic fungi *Ashbya gossipii* and *Fusarium oxysporum* also have alternate ploidy states which are important for growth, reproduction, and pathogenicity (Anderson et al. 2015; Vlaardingerbroek et al. 2016; Kasuga et al. 2016). Polyploidy is often a transient state towards aneuploidy because of its impact on genome stability and negative effects on cell physiology (Storchova 2014). Aneuploid states are known to decrease fitness under certain optimal growth conditions but can also be beneficial in stress response and rapid adaptation in other environments (Torres et al. 2008; Pavelka et al. 2010). Many drug-resistant phenotypes in clinically important fungi are a result of specific aneuploidies (Selmecki et al. 2006, 2008; Sionov et al. 2010, 2013; Ngamskulrungroj et al. 2012a). Moreover, it has been suggested that tetraploid-derived aneuploidy can drive fungicide resistance (Storchova 2014). Harrison et al. (2014) also showed that treating *C. albicans* with fluconazole-based drugs triggered

genome duplication and instability by interfering with cytokinesis and could increase the production of resistant aneuploid karyotypes.

Haldane (1932, 1933, 1937) provided many accounts of the impact that ploidy has on the accumulation of deleterious mutations and the rate of evolution and adaptation. Diploidy, for example, may provide a fitness advantage when there is a need to mask recessive deleterious mutations. The link between ploidy and mutation may not be as passive as previously thought however, and ploidy may be a major driver in the mutation process. A recent study that directly compared the genome-wide spectrum of spontaneous mutations arising in haploid and diploid forms of the budding yeast *Saccharomyces cerevisiae,* found that haploids were more prone to single-nucleotide and mitochondrial mutations, while larger structural changes were more common in diploids (Sharp et al. 2018). The genomic architecture and ploidy, therefore, may be influencing the types and rate of spontaneous mutations that arise in yeasts.

Mutation is the basic source of genetic variation, and they directly influence the rate at which organisms can respond to selective pressures. Spontaneous mutations are those which occur in the absence of extrinsic agents (Foster 2006). An elevated spontaneous mutation rate has been shown to amplify evolutionary adaptation (de Visser 2002). For example, an increasing number of clinically important human pathogens have mutations in DNA repair genes which increases their mutation rates and allows them to rapidly change and develop resistance to antifungals or be genetically unstable (Billmyre et al. 2017; Boyce et al. 2017; Harfe et al. 2000; Tran et al. 1997). Billmyre et al. (2017) found that a nonsense mutation in the mismatch repair component *MSH2* in *Cryptococcus deuterogattii* causes an increased mutation rate in genes with coding homopolymer runs which encode

the target for the antifungals FK506 and rapamycin. Boyce et al. (2017) also compared mutation rates of *Cryptococcus neoformans* DNA mismatch repair mutants and found that the loss of *MSH2, MLH1*, and *PMS1* results in increased mutation rates and rapid resistance to antifungals. There is evidently a link between genetic hyperdiversity, elevated mutation rates, microevolution and adaptation (Magditch et al. 2012; Weilgoss et al. 2013). For plant pathogens, this can be key in pathogenicity, virulence, or the ability to rapidly adapt to varying environments. There are many instances in agriculture where beneficial mutations in the genome of a pathogen have led to pesticide resistance (Carter et al. 2014; Camps et al. 2012; Cools & Fraaije 2013; Chen et al. 2007; Frenkel et al. 2015; Torriani et al. 2009), perpetuating the evolutionary arms race and longstanding efforts to find new pesticide formulations. Amaradasa and Everhart (2016) showed that exposure of *Sclerotinia sclerotiorum* to sublethal doses of certain fungicides increased its mutation rate and genetic variation and the acquisition of fungicide resistance.

Chromosomal rearrangements in fungi are known to affect pathogenicity and virulence, leading to host jumps, expanded host ranges and increased virulence (De Jonge et al. 2013; Ma et al. 2010). Core chromosomes are conserved and are essential for housekeeping functions while accessory chromosomes, also called B-chromosomes or supernumary chromosomes, are additional to the regular karyotype and conditionally dispensable to normal cell function. Fungal accessory chromosomes have become a major area of interest because they gain mutations and structural variations at a higher rate than core chromosomes and function in host-pathogen interactions (Chuma et al. 2011; Faino et al. 2016; Ma et al. 2010). Ma et al. 2010 demonstrated that the transfer of two lineage-specific chromosomes in *Fusarium oxysporum* converted a strain from non-pathogenic to

pathogenic. Karyotype diversity may also impact pathogenic traits, and lead to reproductive barriers, population structure and speciation in some instances (Bravo Ruiz et al. 2019; Galazka and Freitag 2014; Ma et al. 2010; Mehrabi et al. 2011; Selmecki et al. 2010). For example, the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* has lineage specific chromosomes that confer host specificity and virulence (Chuma et al. 2011). Chromosomal rearrangements can lead to genetic incompatibilities that become fixed in diverging lineages resulting in population structure (Stuckenbrock 2013). The maintenance of high levels of divergence in linked or isolated genes can also lead to reproductive isolation which over time also causes speciation (Rieseberg 2001).

The objectives of this study were to (i) estimate the ploidy of yeast cells of *E. maculosum* (ii) determine if *E. maculosum* has a high spontaneous mutation rate by comparing it with *E. rostrupii* (iii) to identify if there is variation in chromosome number and size among strains of *E. maculosum* (iv) estimate the number of nuclei per cell in yeast and hyphae and (v) determine if clamp connections are present in hyphae.

MATERIALS AND METHODS

Ploidy analyses using flow cytometry

Yeast cells of 14 *E. maculosum* strains (Table 3.1) and *Ustilago maydis* strain FB1 (a1b1) (generously provided by Dr. Karen Snetselaar, Saint Joseph's University) were grown on potato dextrose broth (PDB) for 7 days, then centrifuged at 3000 × *g* for 10 min at 4°C to form a pellet. *U. maydis* was used as a control because its genome is haploid and 20 Mb and that of the haploid reference genome of *E. maculosum* strain E12A7-4 is 23 Mb. The pellet

was washed with 1× phosphate buffered saline (PBS), centrifuged briefly, and fixed with 70% ethanol for 2 hr at 25°C. Cells were then centrifuged at 3000 × *g* at 4°C for 15 min, and resuspended in PBS to a concentration of 1 × 10^8 cells per 50 µl. Fifty microliters of the cell suspension was treated with 200 µl of 1 mg/ml RNAse A at 37°C for 2 hr and stained for flow cytometry using a modified Krishan hypotonic sodium citrate staining buffer (Krishan 1975). Briefly, cells were resuspended in the buffer (5 µg/ml propidium iodide, 4 mM sodium citrate, 0.1% Triton X-100) for 1 hr, centrifuged at 3000 × *g* at 4° C for 10 min to form a pellet and resuspended in 50 µl of PBS. A Millipore Sigma ImageStream Mark II imaging cytometer (Millipore Sigma, Massachusetts, USA) was used to assess the nuclear fluorescence intensity and cell area of 10,000 cells of each isolate. The IDEAS 6.2 Analysis Software Package (Luminex) was used to measure the median fluorescence intensity (MFI) and mean cell sizes of each strain. The experiment was conducted twice.

Spontaneous mutation rate estimation by fluctuation analysis

Fluctuation assays are used to estimate the rate at which cells mutate using a selective marker that can be assessed phenotypically. A fluctuation assay was performed to determine the rate at which cells mutated and became resistant to 5-fluoroorotic acid (5-FOA). 5-FOA in the presence of a functional URA3 is converted to the toxic 5-fluorouracil causing cell death (Längle-Rouault and Jacobs 1995). If there is a mutation in URA3 which causes it to become non-functional, cells will grow on selective media containing 5-FOA. Six previously sequenced *E. maculosum* strains and two *E. rotrupii* strains (Table 3.1) were randomly selected and grown on yeast peptone dextrose (YPD) agar at 23°C for 5 days. A 100 µl suspension from each culture was used to inoculate each of 10 parallel 30 ml YPD

broth cultures of each isolate in 50 mL Falcon conical tubes. The tubes were placed on a rotary shaker at 120 rpm at 23°C for 5 days. These parallel cultures allowed the cells to be grown for multiple generations in the absence of the phenotypic marker. One milliliter of each parallel culture was then diluted in water 1:10,000 in 2.0 mL Eppendorf tubes and 100 µl of each cell suspension was spread on a 100-mm diameter Petri plates with YPD agar. The same volume of undiluted suspensions was spread onto YPD-5FOA agar (1 mg/ml 5-FOA, 50 µg/ml uracil). After 14 days at 23°C with a 12-hr light and dark cycle, the number of colonies on YPD and the number of mutant colonies on YPD-5FOA plates was counted. The spontaneous mutation rate of each strain was calculated using the web tool Fluctuation AnaLysis CalculatOR (FALCOR) (Hall et al. 2009), using the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) method for analysis. The experiment was conducted twice.

Estimation of chromosome number and size by CHEF electrophoresis

Contour-clamped homogeneous electric field (CHEF) electrophoresis was used to compare the chromosome size and number among 15 strains of *E. maculosum* (Table 3.1). Agarose embedded yeast DNA was prepared by inoculating a 50 ml potato dextrose broth (PDB) medium in a 250 mL flask with each isolate and shaking at 160 rpm at 23°C for 5 days on a rotary shaker. The cells were centrifuged at 500 × *g* at 4°C for 10 min. The supernatant was removed, and cells resuspended in 10 ml cold 50 mM EDTA, pH 8. The cell concentration was determined by adding 10 µl of cells to 990 µl of water and analyzed on a Nexcelom Bioscience Cellometer X2 (Nexcelom, Lawrence, MA). The CHEF Yeast Genomic DNA Plug Kit (Bio-Rad, California, USA) was used to make agarose plugs. Briefly, plug

molds were used to make 0.75% agarose plugs with a cell concentration of 6×10^8 cells/ml of plugs. Plugs were treated with Lyticase and Proteinase K enzymes according to the manufacturer's recommendations (BioRad, California, USA). Agarose plugs were inserted and sealed in wells of a 0.8% (w/v) agarose gel (Certified Megabase Agarose; Bio-Rad, California, USA). Chromosomal size markers from *Hansenula wingei* with 7 chromosomes ranging from 1-3.1 Mb and *Schizosaccharomyces pombe* with 3 chromosomes ranging from 3.5-5.7 Mb (Bio-Rad, California, USA) were used to estimate the length of separated chromosomes. Pulsed-field gel electrophoresis was performed with the CHEF-DR III system (Bio-Rad, California, USA) at 4°C as follows; ramped switch times of 900-1300 s for 144 hr and 500-800 s for 24 hr, 2 V/cm, 106° angle. After electrophoresis, the gel was stained by diluting GelRed® (Biotium, California, USA) 10,000× stock solution 3,300-fold to make a 3× staining solution in water. The gel was submerged in 200 ml 3× staining solution and gently agitated at room temperature for 1 hr, then visualized with a UV transilluminator. The experiment was repeated 12 times using the same protocol for making plug molds and preparing gels but varying CHEF system parameters to optimize conditions to enable visualization of the bands.

Staining of yeast cells and filamentous hyphae to determine number of nuclei per cell

A preliminary study was done to determine the number of nuclei per cell in yeast cells. Yeast cells of two *E. maculosum* strains were cultured on PDA for 7 days, harvested and fixed with 70% ethanol. The cells were centrifuged at 500 × g at 4°C for 10 min, ethanol removed with a 1000 µl pipette and 1 µg/ml Hoechst 33528 (Biotium, California, USA) was

added to cells to stain nuclei. Cells were incubated at room temperature for 10 min and diluted to a concentration that provided enough separation for better visibility under the microscope. They were placed on a microscope slide and covered with a cover slip. A Zeiss Axio Scope A1 fluorescence microscope (Zeiss, California, USA) was used to visualize cells. Approximately 100 to 200 cells were observed per isolate.

To observe hyphae, leaves with whole leaf spots were cut into disks approximately 5 mm × 5 mm so that they could be placed into Eppendorf tubes. One hundred percent ethanol was used to cover leaf disks, which were left for 2 days at room temperature. The ethanol was removed with a pipette and 10% potassium hydroxide was added to cover leaves. Tubes were placed on a heating block at 85°C for 2 hr. Leaf disks were washed with 1× phosphate buffered saline (PBS) five times for neutralization. Staining solution (20 µg/ml propidium iodide, 10 µg/ml Wheat Germ Agglutinin Alexa Fluor™ 488 (ThermoFisher Scientific, Massachusetts, USA), 0.1% Tween 20 in 1× PBS) was added to cover leaf disks. Leaf disks were vacuum infiltrated at 250 mbar (7.3 mercury units) 4 times for 5 min each with 5 min resting intervals. Leaf disks were washed twice with PBS and analyzed using a Zeiss LSM 880 Confocal Microscope and a Zeiss Axio Scope A1 fluorescence microscope (Zeiss, California, USA). Leaf disks that were cleared with potassium hydroxide and washed with PBS were also crushed on a microscope slide, stained with 3% (w/v) Giemsa dye to stain nuclei for 30 min and visualized under a compound light microscope. Three to four leaf spots per treatment were examined for nuclei and clamp connections.

RESULTS

Ploidy analyses

Flow cytometry when used to measure ploidy, employs fluorescence intensity as a measure of DNA content within a cell and these parameters are directly proportional. The fluorescence intensity is then compared to that of a reference with known ploidy. The a1b1 strain of *U. maydis* is haploid and 20 Mb (Kamper et al. 2006), while the reference genome of *E. maculosum* is haploid and 23 Mb determined by sequence data (DOE-JGI). They were both used as references for flow cytometry. The median fluorescence intensity of *U. maydis* and *E. maculosum* was compared in each experiment because they have similar genome sizes, which means that these intensity values should be similar if they are the same ploidy. The ploidy of *E. maculosum* reference strain was estimated as haploid in both experiments. The median fluorescence intensity of all other strains was compared to that of the *E. maculosum* haploid reference strain by dividing their values (Table 3.2). All North Carolina strains were aneuploid. Strains 88-9, 89-5 and 92-50 from NE North America were diploid and Me1-13S1 was aneuploid near tetraploidy. (Figure 3.1). In Georgia, E12C1-4 was diploid, E12D1-1 was aneuploid and E12F2-81S and E13A1-S were aneuploid near diploidy. There was variation in cell size which mostly correlated with ploidy level. For example, the mean cell size of haploid strain E12A7-4 is 24 μm in rep 1 and that of aneuploid strain close to tetraploidy is 42 μm.

Spontaneous mutation rate

The haploid reference strain of *E. maculosum* had the highest spontaneous mutation rate (16.9×10^{-7} mutations/generation) of all strains that were tested. Aneuploid strains

also had high spontaneous mutation rates (3.8×10^{-7} to 10.1×10^{-7} mutations/generation) while diploid strains produced no colonies on 5-FOA plates. Haploid and aneuploid *E. maculosum* strains had higher mutation rates than *E. rostrupii* strains with one showing no growth on selective media and one with a mutation rate of 1.7×10^{-7} mutations/generation (Fig. 3.2).

Separation of chromosomes

Electrophoretic separation of *E. maculosum* chromosomes showed 5 distinct bands. Three of these bands had approximate sizes of 3, 3.5 and 5 Mb. There was 1 band larger than the 5.7 Mb size marker and 1 smaller band varying across strains that is between 2.35 and 2.7 Mb (Fig. 3.3). The 4 larger bands had a consistent pattern across all gels and populations of the fungus. The smallest band was variable within and among populations of strains that had clear bands. Bands were not visible for strains E12A7-4, D1-1, E12C1-4, E12F2-81S, 92-50 and NCLC1-33. The 5 bands totaled a minimum of approximately 19.7 Mb as it was hard to estimate the size of the band above the 5.7 Mb size marker. The banding pattern could be recognized in 3 of the other 12 gels used in optimization, but there was not enough separation to clearly distinguish them. This banding pattern was also observed in at least one other gel for strains that were not visible on this gel but without good separation.

Staining of yeast cells and hyphae

Staining with Hoechst 33528 showed that *E. maculosum* yeast cells have one nucleus per cell (Fig. 3.4). This was consistent across numerous visualized cells in two strains of *E.*

maculosum. and was confirmed by flow cytometry of 14 strains (Fig 3.1). Hyphae from blueberry plants stained with Giemsa showed variation in number of nuclei within each cell ranging from 1 to 4 (Fig. 3.5A,B). Hyphae that were stained with Wheat Germ Agglutinin Alexa Fluor™ 488 showed protrusions that look like clamp connections (Fig. 3.5C). Counterstaining *E. maculosum* hyphae in the blueberry plant cell showed disruption of the plant cell and branching of hyphae from what appear to be clamp connections, but there was no success in staining the nuclei using this procedure (Fig. 3.6)

DISCUSSION

Here, we confirmed that there is variation in ploidy in the yeast stage of the blueberry pathogen *E. maculosum.* We observed haploid, diploid and aneuploid strains and some cultures derived from single spore strains showed within colony variation in ploidy. Aneuploidy was very common in *E. maculosum*, with 9 of the 14 tested strains determined to be likely aneuploids. Yeast genomes are dynamic with extensive ploidy variation observed in clinically important species (Zhu et al. 2016). While some fungi have stable ploidy states, others make shifts in ploidy based on their environment, using it as an adaptive mechanism (Selmecki et al. 2015). Aneuploidy is very common in fungal pathogens of humans such as *Candida albicans* and *Crytoccocus neoformans* (Ni et al. 2013; Selmecki et al. 2006). In fact, aneuploidy has a major influence on drug resistance in clinical settings. For example, Yang et al. (2017) showed reduced susceptibility of *C. albicans* cells to mainline echinocandin (ECN) drugs to aneuploidies of chromosomes 5 and 2. Aneuploidy has been reported in plant-pathogenic oomycetes such as *Phytophtora*

ramorum and *Phytophtora infestans* (Kasuga et al. 2016; Van der Lee et al. 2004). It seems likely that ploidy variation is an adaptive strategy in *E. maculosum*.

Exobasidium maculosum has a high level of nucleotide and haplotype diversity which is an order of magnitude higher than most other plant-pathogenic fungi (Stewart et al. 2015). There are different factors which contribute to genetic diversity including sexual reproduction, recombination, and spontaneous mutation rate. Spontaneous mutations provide the basic changes at the DNA level for selection to act on, thus pathogens with a higher rate are likely to more rapidly evolve mechanisms to evade host defenses, become resistant to control strategies, thereby causing greater economic damage in agricultural systems by killing plants and reducing yields (Hawkins et al. 2018). The spontaneous mutation rate of *E. maculosum* was higher than that of *E. rostrupii*. It was also high when compared to that of model fungal organisms in the literature (Table 3.3), which suggests that it may be a factor influencing the high level of genetic variation observed for this pathogen. Diploid strains of *E. maculosum* did not have colonies on selective media. This may be because with two copies of every gene, diploids mask mutations occurring in one copy (Otto and Goldstein 1992). Aneuploids may only have one copy of a gene at certain loci allowing mutations to be phenotypically assessed. This could explain why aneuploid strains did not have as high a mutation rate as the haploid strain but higher than diploid strains. The ploidy of *E. rostrupii* strains is not known so it cannot be ruled out as a factor influencing the lower mutation rate observed in the pathogen.

Genome structures are maintained by the interaction of cell cycle checkpoints and DNA repair mechanisms. When these functions are dysregulated, the result can be the loss

of genomic integrity (Hakem 2008) or an increase in mutation rates (Broustas and Lieberman 2014). There is growing evidence that faulty DNA repair can lead to hypermutator phenotypes of clinically important fungi, that cause them to rapidly adapt to varying environments, leading to difficult management in clinical settings (Billmyre et al. 2017; Boyce et al. 2017; Harfe et al. 2000; Tran et al. 1997). The mutation rate of *E. maculosum* was comparable to hypermutator strains of *C. neoformans* with faulty mismatch repair of DNA replication errors (Boyce et al. 2017).

Three DNA repair pathways have been identified in yeasts and include the nucleotide excision repair (NER), postreplication repair (PRR), and recombination-mediated repair (Girard and Boiteux 1997). The rad21 gene was found to be fast evolving when populations of *E. maculosum* from three different regions (Georgia, North Carolina, and Maine/Canada) were compared which means that it may be involved in adaptation of the pathogen (Stewart et al. 2014). The rad21 gene is implicated in DNA double-strand break (DSB) repair and is a component of the sister chromatid cohesion complex that functions in the alignment of sister chromatids during the cell cycle. Deletion of rad21 results in the abnormal separation of sister chromatids during interphase and improper alignment during metaphase (Sonoda et al. 2001). The authors also found an increased incidence of spontaneous chromosomal breaks. DNA repair genes may therefore play a part in *E. maculosum* adaptation.

Chromosome size and number can vary widely among and within fungal species. For example, Talbot et al. (1993) analyzed the karyotype among clonal lineages of the rice blast fungus *Magnaporthe grisea* and found that there were chromosome length polymorphisms

though chromsosome number was largely invariant. The authors also found from 1 to 3 minichromosomes in some strains ranging in size from 470kb to 2.2Mb. Electrophoretic separation of *E. maculosum* chromosomes was not successful for all strains that were assessed but those that had bands showed a consistent pattern among the 4 largest of the 5 bands that were observed. The largest band was larger than 5.7 Mb size marker so the size could not be accurately estimated. The other three larger bands were approximately 5, 3.5 and 3 Mb. The smallest band was variable in size among strains, ranging from approximately 2.35 to 2.7 Mb, and were not population or ploidy specific. The genome size of *E. maculosum* strains based on sequencing data range from 17.5 to 22.5 Mb and without knowing the size of the largest chromosomal band from CHEF electrophoresis, the minimum approximate sum of chromosomal bands is 19.7 Mb. The correlation between genome sizes based on sequence data and electrophoretic karyotyping suggests that there are 5 chromosomes in *E. maculosum*. The fact that the smallest chromosome was variable while others were consistent among strains could possibly mean that it is a minichromosomes, accessory chromosome or unstable. It could also simply be a chromosome length polymorphism, but more in-depth studies are necessary to determine what this band represents. Accessory chromosomes are usually enriched with repetitive DNA, have higher rates of non-synonymous substitutions and a lower gene density than core chromosomes (Möller and Stukenbrock 2017). Genome based studies have shown that accessory chromosomes can define host range or are lineage specific in certain pathogens such as *Fusarium oxysporum* and *Nectria haematococca* where they sometimes move horizontally or have effectors which determine pathogenicity on certain hosts or varieties (Chuma et al. 2011; Temporini and VanEtten 2002; van der Does et al. 2016). For example,

an accessory chromosome in the pea pathogen *Nectria haematococca* (anamorph *Fusarium solani*) encodes pea pathogencity (PEP) genes that are required for pathogenicity (Temporini and VanEtten 2002).

Many steps of the life cycle of *E. maculosum* are unclear. Brewer et al. (2014) showed that *E. maculosum* is filamentous within the plant tissue and produces basidia on the lower epidermis of leaves, which give rise to basidiospores that are often transversely one-septate. Basidiospores germinate to form aseptate conidia which are culturable. Ingram et al. (2019) suggested that basidiospores are not infectious propagules as no new spots formed when basidiospores were abundant in the air and that yeast cells grow epiphytically on plants. However, we previously did not know nuclear state or number of nuclei per cell for many stages of the cycle. Other somewhat closely related plant pathogens, such as *Ustilago maydis*, have uninucleate, haploid yeast cells in the saprophytic stage. Compatible yeast cells conjugate and form the infectious and filamentous dikaryotic stage. Here, we showed that *E. maculosum* yeast cells are also uninucleate and vary in ploidy, but it is still unclear which stage of the pathogen penetrates and causes infection. We showed that an arthropod vector may be involved in transmission and infection (Chapter 2). Structures similar to clamp connections are visible on hyphae and preliminary studies show multinucleated cells with observed variations from one to four nuclei between septa. Clamp connections only occur within Basidiomycota and usually indicate that the fungus is a dikaryon; however, not all dikaryons have clamps (Webster and Weber 2007). In *E. maculosum*, there is frequent branching from these structures. This kind of branching was also observed from light and electron microscopy of *Ustilago maydis* hyphae in maize (Snetselaar and Mims 1994) . The authors used leaf clearings to show that hyphal

branches often originated at clamp connection structures in the hyphae. There were also similar observations in the white rot fungus, *Panus tigrinus*, and the fossil fungus *Palaeancistrus martini* where hyphae branch from clamp connections (Dennis 1970). Butler (1972) also showed that clamp connections can be binucleate or trinucleate in *Coprinus disseminates*. The authors found that irregularities in nuclear distribution were associated with the presence of pseudoclamps which were of three types: clamp arm septate, nucleate, and not fused with the parent hypha nor grown on, clamp arm septate, nucleate, not fused with the parent hypha but grown on, with nuclear division, to form a 'tail', i.e., backward branch and clamp arm aseptate and anucleate, not fused with the parent hypha. They also found that apparent dikaryons were actually heterokaryotic di or trikaryons. The edible shiitake mushroom, *Lentinula edodes,* has both homokaryotic and heterokaryotic hyphae with varying number and size of nuclei per cell, with the latter also having clamp connections (Gao et al. 2019). If this is the case in *E. maculosum*, it may also be a factor influencing the genetic variation that is observed, as variation acquired in the heterokaryotic stage may be transferred to the yeast stage that is cultured and sequenced. Further studies will be needed for better resolution and confirmation of clamp connections and nuclear state of hyphae in *E. maculosum.*

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Table 3.1. Geographic origin of *Exobasidium maculosum*, *Exobasidium rostrupii* and *Ustilago maydis* strains used in this study. Strains that were used in flow cytometry, CHEF electrophoresis and fluctuation assay are denoted by (+) and those that were not used in each study as (-).

Species	Geographic location of origin	Strain	Flow cytometry	CHEF electrophoresis	Fluctuation assay
E. maculosum	Georgia	E12A7-4 [a]	+	+	+
E. maculosum	Georgia	E12C1-4	+	+	-
E. maculosum	Georgia	E13A1-S	+	+	-
E. maculosum	Georgia	E12D1-1	+	+	-
E. maculosum	Georgia	E12-FS2-8S1	+	+	+
E. maculosum	Maine	Me1-13S1	+	+	+
E. maculosum	Maine	E14MEB-2S1	-	+	-
E. maculosum	Nova Scotia	88-9	+	+	+
E. maculosum	Nova Scotia	89-5	+	+	+
E. maculosum	Nova Scotia	92-50	+	+	-
E. maculosum	North Carolina	NCLC1-14	+	+	-
E. maculosum	North Carolina	NCLC1-23	+	+	+
E. maculosum	North Carolina	NCLC1-33	+	+	-
E. maculosum	North Carolina	NCPC1-8	+	+	-
E. maculosum	North Carolina	NCLC1-44	+	+	-
E. rostrupii	New Jersey	1-1	-	-	+
E. rostrupii	New Jersey	2-3	-	-	+
U. maydis		FB1	+	-	-

[a] Reference *E. maculosum* strain that was sequenced, assembled, and annotated by DOE-JGI.
+ Strain was used in the experiment.
- Strain was not used in the experiment

Table 3.2. Ploidy estimation and cell size variation of *Exobasidium maculosum* strains using flow cytometry. Ploidy estimates were based on the median fluorescence intensity (MFI) of yeast cell nuclei stained with propidium iodide and compared with reference haploid *Ustilago. maydis* and *E. maculosum* strains. Numbers in parentheses are the MFI of that isolate divided by the MFI of the haploid reference strain E12A7-4, which was used to estimate the euploid state closest to aneuploid estimates. The cell size is represented by the area of the cell.

Population	Strain	Flow cytometry MFI rep 1	Mean cell size (μm) rep 1	Flow cytometry MFI rep 2	Mean cell size (μm) rep 2	Flow cytometry ploidy estimation
	FB1(*U. maydis*)	12974	36	20865 (n)	49	haploid
Georgia	E12A7-4 (ref)	13983 (n)	24	23222 (n)	35	haploid
	E12C1-4	26294 (1.9n)	30	47245 (2.0n)	31	diploid
	E13A1-S	34465 (2.5n)	23	39961 (1.7n)	31	aneuploid (diploid)
	E12D1-1	36891 (2.6n)	29	26329 (1.1n)	34	aneuploid
	E12-FS2-8S1	25185 (1.8n)	22	37383 (1.6n)	32	aneuploid (diploid)
NE North	Me1-13S1	71139 (5.1n)	42	86696 (3.7n)	54	aneuploid (tetraploid)
America	88-9	27817 (2.0n)	37	40439 (1.7n)	46	diploid
	89-5	30012 (2.1n)	33	50669 (2.2n)	40	diploid
	92-50	28416 (2.0n)	16	57701 (2.4n)	32	diploid
North Carolina	NCLC1-14	38163 (2.7n)	35	82214 (3.5n)	35	aneuploid
	NCLC1-23	53536 (3.8n)	38	74302 (3.1n)	39	aneuploid
	NCLC1-33	38437 (2.5n)	32	73928 (3.1n)	30	aneuploid
	NCPC1-8	35264/73536 (2.5n/5.2n)	27	78108 (3.4n)	34	aneuploid
	NCLC1-44	33265/66733 (2.4n/4.7n)	30	80643 (3.5n)	34	aneuploid

Table 3.3. Mutation rates per base pair per generation (μ_b) of *Exobasidium maculosum* and model fungal organisms.

Organism	Genome size (Mb), ploidy	μ_b	Reference
Saccharomyces cerevisiae	12, haploid	2.2×10^{-10}	Goffeau et al. 1996
Neurospora crassa	42, haploid	7.2×10^{-11}	Drake 1991
Cryptococcus neoformans WT	19, haploid	5.0×10^{-8}	Boyce et al. 2017
Cryptococcus neoformans C45 hypermutator	18, haploid	1.6×10^{-6}	Boyce et al. 2017
Exobasidium maculosum E12A7-4	23, haploid	1.5×10^{-6}	this study

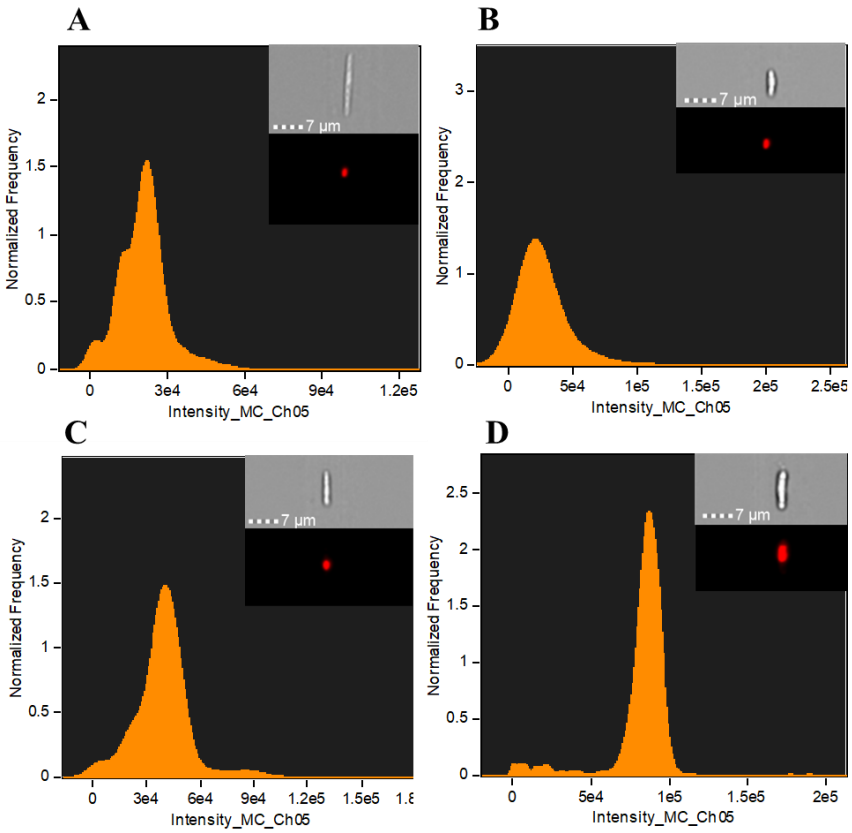

Figure 3.1. Fluorescence intensity of *Ustilago maydis* and *Exobasidium maculosum* cells stained with propidium iodide and representing the DNA content in nuclei. The axes show the fluorescence intensity (x) and cell frequency (y). Ten thousand cells were analyzed per strain. DNA content is proportional to ploidy. The median fluorescence intensity was compared to that of the reference *U. maydis* FB1 (A) and *E. maculosum* E12A7-4 (B) haploid strains to estimate ploidy. Insets show cell size (gray) and fluorescence intensity of the nucleus (red dot) among cells of each strain that represent the median value. A representative diploid strain, 89-5 (C) and an aneuploid strain near tetraploidy Me1-13S1 (D) are also shown.

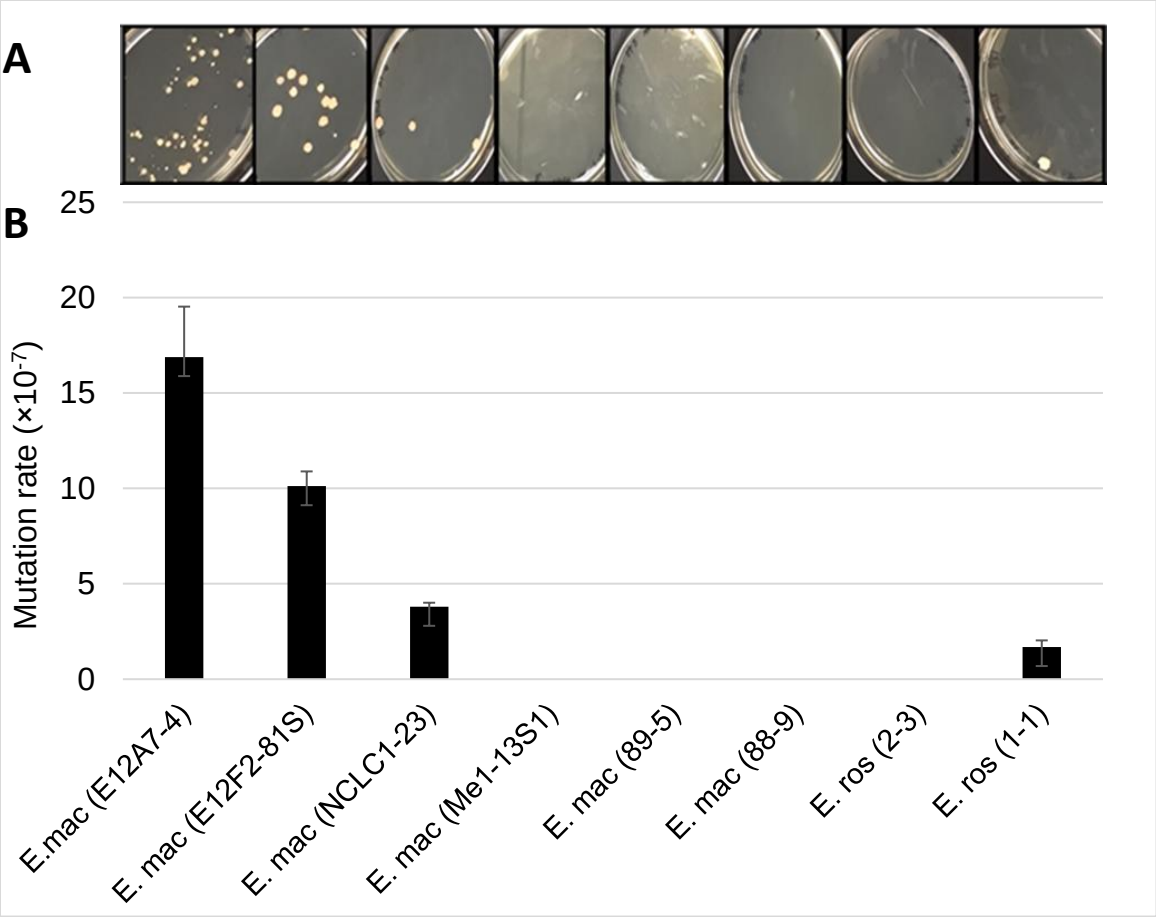

Figure 3.2. Haploid and aneuploid strains of *Exobasidium maculosum* have higher spontaneous mutation rates than *Exobasidium rostrupii*. (A) Spontaneous mutant colonies resistant to 5-FOA in the *E. maculosum* haploid reference strain (E12A7-4), aneuploid strains (E12F2-81S, NCLC1-23), diploid strains (Me1-13S1, 89-5, 88-9) and *E. rostrupii* strains (2-3, 1-1). Photos are of representative plates of each isolate on YPD-5-FOA agar. (B) The spontaneous mutation rate was quantified using a fluctuation analysis from the observed distribution of mutants on 10 parallel cultures of each isolate and calculated using the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE). *E. rostrupii* strain 2-3 and *E. maculosum* strains Me1-13S1, 89-5 and 88-9 did not have mutant colony growth on 5-FOA selective plates. The mean spontaneous mutation rate of the two experiments is represented in the figure with error bars showing the standard deviation. The spontaneous mutation rate of strains without mutant colonies were probably either too low for analysis with the concentration of cells plated or mutations masked in diploid strain

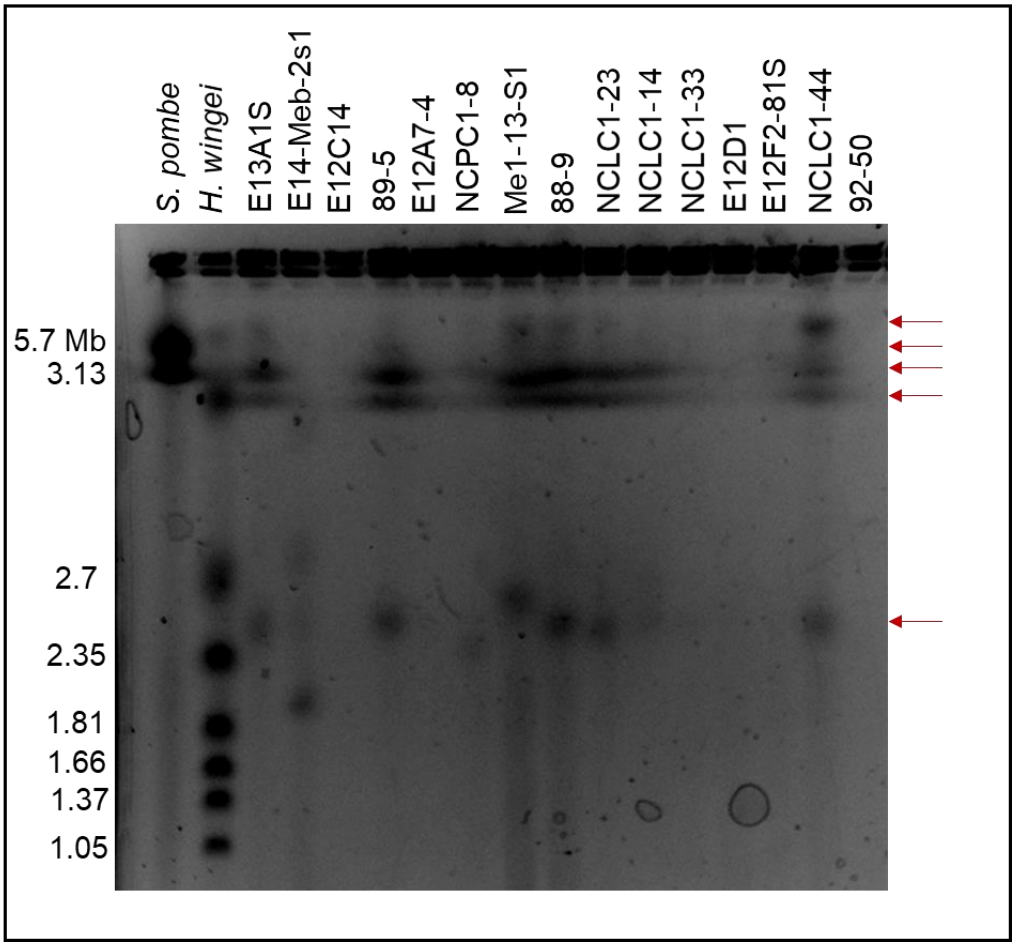

Figure 3.3. Electrophoretic separation of *Exobasidium maculosum* chromosomes of 15 strains. *S. pombe* and *H. wingei* size markers were used as references. There appears to be 3 chromosomes with approximate sizes of 3, 3.5 and 5 Mb, 1 larger than 5.7 Mb and 1 smaller chromosome varying between 2.35 and 2.7Mb indicated by red arrows. The experiment was repeated 12 times using different CHEF parameters, but this gel shows the best separation of chromosomes.

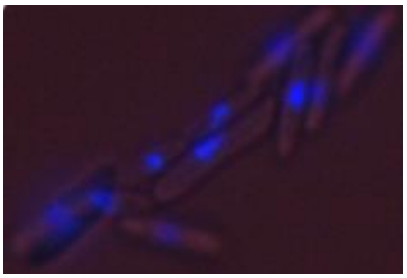

Figure 3.4. *Exobasidium maculosum* yeast cells stained with Hoechst 33528 showing one nucleus per cell. Approximately 100 to 200 cells were observed of two strains. Images were taken at 400× magnification.

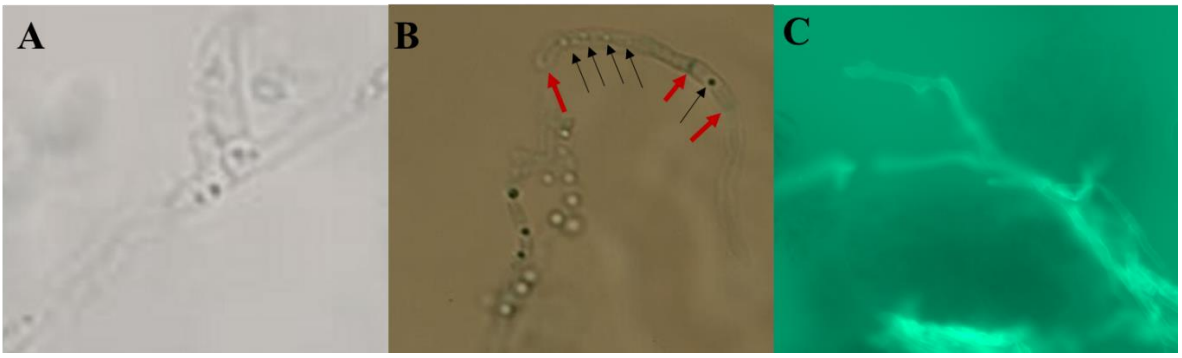

Figure 3.5 . Features of *Exobasidium. maculosum* hyphae stained with Giemsa and visualized under a light microscope (A-B) and stained with wheat-germ agglutinin under a fluorescent microscope. Two nuclei can be seen in each cell near septa in (A) while variation in number of nuclei can be seen in (B). Black arrows indicate nuclei and red arrows indicate septa. Protrusion from hyphae that look similar to clamp connections are visible in (C). Images were taken at 400× magnification.

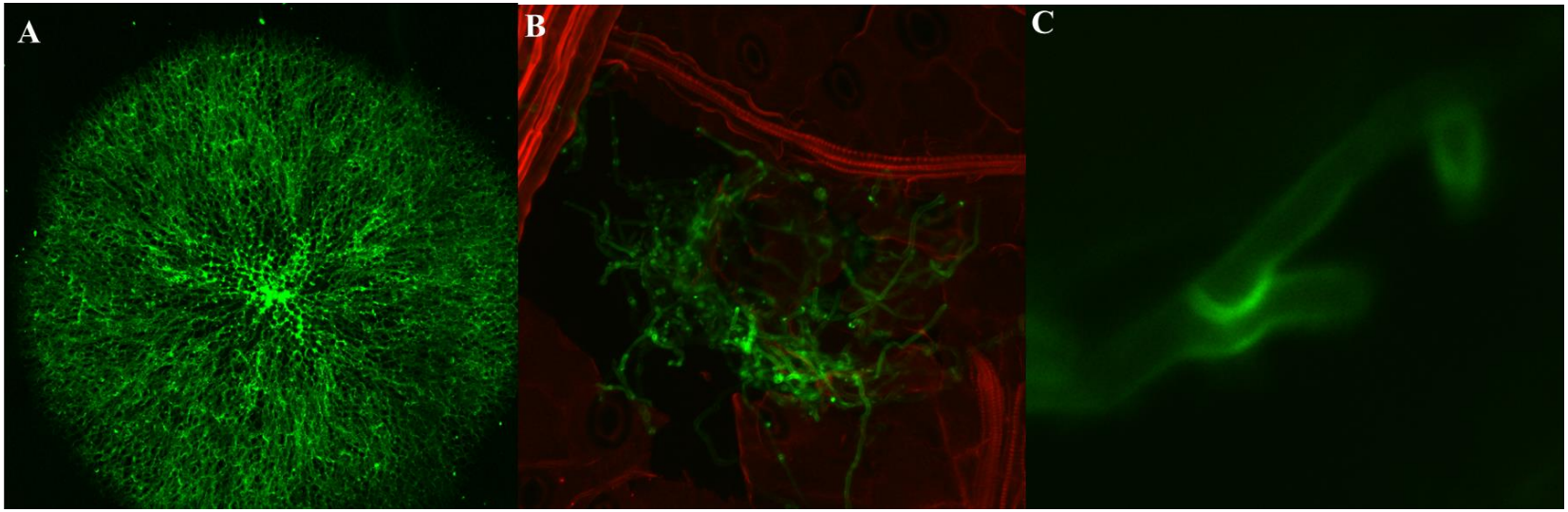

Figure 3.6. Counterstaining of *Exobasidium maculosum* hyphae with wheat-germ agglutinin (green) and plant cell with propidium iodide(red) visualized with a confocal microscope. (A) Leaf spot (200× magnification), (B) disruption of plant cell by hyphae (400× magnification) and (C) apparent branching of hyphae from clamp connection (1000 ×).

CHAPTER 4

PLOIDY AND COPY NUMBER VARIATION AMONG GENOMES OF *EXOBASIDIUM MACULOSUM* CONTRIBUTE TO GENETIC DIVERSITY IN STRUCTURED POPULATIONS

[1]Abrahams, A.D., and Brewer M.T. To be submitted to BMC Genomics

ABSTRACT

Exobasidium maculosum is an important blueberry pathogen, which has the potential to cause significant economic losses. Genomic structural variations have been linked to pathogenicity, adaptation and evolution in many pathogenic fungi and population genomic studies have aided our understanding of the evolutionary histories and epidemiology of these organisms. In this study we assessed ploidy and copy number variation among the genomes of 14 isolates from Georgia, North Carolina and northeast North America. Copy number variation analysis showed an average range of 400 to 500 deletions and 1.25 to 7.25 duplications among populations. Duplications were more common in the northeast North America population which also showed a high level of genome-wide heterozygosity. Phylogenetic analysis revealed that Georgia and North Carolina populations, which had lower levels of heterozygosity, were more closely related than the northeast North America population. Analysis of population differentiation revealed that genes with high F_{ST} values included 4 genes involved in multidrug resistance and 7 genes functioning in oxidation reduction.

INTRODUCTION

Genome plasticity is a common feature in fungal pathogens. The ability to harness genetic variation has profound effects on the pathogenicity of certain fungi that cause damaging human and plant diseases (Li et al. 2012; Selmecki et al. 2010; Tsuge et al. 2016). Although an increase in genetic variation can occur with sexual reproduction and meiosis, there are studies in the literature that highlight other mechanisms (Covo 2020; Taylor et al. 2017). These include mitotic recombination, parasexual recombination, loss of

heterozygosity (LOH), copy number variations (CNVs), intra- and inter-chromosomal rearrangements, polyploidy, aneuploidy, insertions and deletions (indels), translocations, and duplication events (Covo 2020; Kellis et al. 2004; Ohm et al. 2012; Wolfe and Shields 1997). Aberrations in DNA repair mechanisms have also been implicated in hypermutator phenotypes and antifungal resistance in clinically important fungi (Billmyre et al. 2017; Boyce et al. 2017). For example, mutation rates increased in mutants of eight mismatch repair pathway genes in *C. neoformans,* causing rapid evolution and resistance to antifungals (Boyce et al. 2017). These mechanisms are less studied in fungal plant pathogens. However, structural variations and accessory chromosomes that are linked to pathogenicity and host specificity have been described in a few pathosystems (Tsuge et al. 2016; Ma et al. 2010; van Dam et al. 2018). Pathogenicity was directly linked to the presence of accessory chromosome 14 in the plant pathogen *F. oxysporum* f. sp. *lycopersici* as strains without it were unable to cause infection (Vlaardingerbroek et al. 2016). The wheat pathogen *Zymoseptoria tritici* has a high rate of accessory chromosome loss, which sometimes lead to increased virulence in this pathosystem (Moller et al. 2018). The capacity for large-scale genome rearrangements typically provides a fitness advantage for these fungi but is usually associated with genomic instability (Covo 2020). These events and their impact on disease are only beginning to be explored in fungal plant pathogens. With the increase in genomic resequencing and comparative genomics, structural variations and the underlying factors influencing them will likely be observed in more species.

The blueberry pathogen *Exobadium maculosum,* which causes Exobasidium leaf and fruit spot, has a high level of nucleotide and haplotype diversity among strains (Stewart et

al. 2015). Twenty percent of the genes detected in the PacBio reference genome of strain E12A7-4 are segmental duplications and another 12% are determined to be alleles (DOE-JGI 1000 Fungal Genomes Project). Segmental duplications are blocks of DNA that are usually greater than 1 Kb, share more than 90% sequence identity and occur at more than one site in the genome. This type of genomic structure has not been previously observed in the Basidiomycota subphylum Ustilagomycotina, the group which comprises the agriculturally important fungus *Ustilago maydis*, and the skin-inhabiting and dandruff-associated yeasts, *Malassezia* spp. These fungi typically have non-repetitive, haploid genomes (Kämper et al. 2006; Xu et al. 2007). The location of segmental duplications has been associated with chromosomal instability and rearrangement, evolution and disease (Ji et al. 2000; Samonte and Eichler 2002; Armengol et al. 2003; Bailey et al. 2004). Sharp et al. (2005) also showed that regions of segmental duplications are hotspots for chromosomal rearrangements that are likely important in variation and disease facilitation.

We found that *E. maculosum* has ploidy variation among and within strains and the high nucleotide diversity commonly seen in organisms with a high mutation or recombination rate, or frequent chromosomal rearrangements (Chapter 3). Stewart et al. (2015) showed that the high genetic diversity observed among three conserved loci was due to a high pairwise nucleotide diversity and a high level of recombination within and among loci, but genomic-level variations such as CNVs could also be involved. Another study using 90 loci from double digest restriction-site sequencing (ddRAD-seq) revealed that the high level of genetic diversity observed in these three conserved genes is maintained across the entire genome of the pathogen (Stewart et al. 2014). Bayesian cluster analysis using the multilocus genotype data detected little admixture. It was also

found that population genetic structure was based on location with three genetic groups that corresponded to three populations, namely Georgia, North Carolina and northeast North America. However, only 90 SNPs were used, so whole genome-level structure was not assessed.

Copy number variations are differences in the number of copies of a gene from one individual to the next which arise from deletions or duplications of gene regions. Single nucleotide polymorphisms on the other hand, are variations in nucleotides at a single position in the genome, and account for most of the genetic variation seen in human and other species (Suh and Vijg 2005). Most population genetic studies have focused on using SNP variation to study evolutionary processes, but with whole genome sequencing becoming less expensive, CNV studies have become more feasible. Though not all CNVs are associated with segmental duplications, they are frequently associated with these regions. Copy number variations can arise in regions of segmental duplications through nonallelic homologous recombination (NAHR) (Yang 2018). This form of homologous recombination generally occurs between regions that are not alleles but have high sequence similarity. When NAHR occurs between regions with low-copy number repeats, changes in copy number can occur (Yang 2018). Nonhomologous end joining (NHEJ), a pathway that repairs double-strand breaks in DNA, can also give rise to CNVs because the process of repair often leaves extra sequence at DNA end junctions (Chang et al. 2017; Yang et al. 2018). Aneuploidy, whether whole or partial, adds or removes copies of DNA sequence and directly affects CNVs. Understanding which CNVs are linked to particular phenotypes can be beneficial. For example, if a particular gene is associated with the virulence of a plant pathogen, CNVs in this gene can cause differences in gene dosage and expression, which

may in turn affect disease management. Loss of heterozygosity (LOH) occurs when there is a change from a heterozygous to homozygous state. LOH can occur with copy number losses where all or a part of a chromosome is deleted or copy number neutral losses where there is either a homologous recombination event or a duplication in the retained chromosome before or after the LOH event, leading to the homozygous state (Ryland et al. 2015). LOH can lead to variation in a population and is linked to certain human diseases such as breast and ovarian cancer, where LOH is associated with loss of the wild-type allele of the BRCA1 gene, leading to a genetic predisposition for these diseases (Merajver et al. 1995).

Understanding the genetic basis for local adaptation of pathogen populations is important for epidemiological studies in plant pathology. It aids our understanding of the evolutionary histories, emergence and lifestyles of these pathogens which can be important for informing disease management. Genes that are involved in adaptation or speciation usually evolve more quickly and genomic approaches can identify these genes (Nolte and Tautz 2010). The fixation index (F_{ST}) is a measure of population differentiation due to genetic structure which compares genetic variability within and between populations (Bhatia et al. 2013; Hudson et al. 1992; Nei 1973; Weir and Cockerham 1984). This measure has been used in many studies of human and other species to infer population structure (Edelaar et al. 2012; Ellstrand and Elam 1993; Hangartner et al. 2012; International HapMap 3 Consortium 2010; Palumbi and Baker 1994; Xu et al. 2009).

Plant pathogens utilize various mechanisms to respond to stress and overcome host defenses and those with a high evolutionary potential tend to develop pesticide resistance more quickly in the field (McDonald and Linde 2002). Genetic architecture is one factor

influencing the evolutionary potential of pathogens. The objective of our study was therefore to explore structural variations in *Exobasidium maculosum*. We used bioinformatics population genomics approaches and assembled the genomes of 14 isolates of *E. maculosum* from three populations, including Georgia (GA), North Carolina (NC) and northeastern North America (NA). Specifically, we aimed to (i) determine if there is ploidy variation among isolates using bioinformatic approaches, (ii) assess copy number variations among isolates, (iii) infer population genetic structure of *E. maculosum* based on whole genome sequencing data, and (iv) identify rapidly evolving regions of the genome that may be involved in adaptation among populations of this plant pathogen.

MATERIALS AND METHODS

Genome sequencing, assembly and quality assessment

The reference strain (E12A7-4) was sequenced using the PacBio sequencing platform, assembled and annotated by the Department of Energy Joint Genome Institute's (DOE-JGI) 1000 Fungal Genomes Project (https://genome.jgi.doe.gov, Project ID: 1150418). Thirteen additional isolates from Georgia (GA), North Carolina (NC) and northeastern North America (NA) were sequenced using the Illumina NextSeq sequencing platform (Table 4.1). DNA extraction was done by using the cetyl trimethylammonium bromide (CTAB) method (Fulton et al. 1995). Briefly, strains were incubated on potato dextrose agar (PDA) plates with sterile cellophane in the dark at 25°C for 7 days. Cells were ground in liquid nitrogen and 500 mg added to 17.5 ml of CTAB lysis buffer (6.5 ml of Buffer A (0.35 M sorbitol; 0.1 M Tris–HCl, pH 9; and 5 mM EDTA, pH 8), 6.5 ml of Buffer B (0.2 M Tris–HCl, pH 9; 50 mM

EDTA, pH 8; 2 M NaCl; 2% CTAB), 2.6 ml of Buffer C (5% Sarkosyl), 1.75 ml PVP (0.1 %), and 1.25 µl Proteinase K). A 2010 Geno/Grinder (SPEX SamplePrep, Metuchen, NJ, USA) was used to homogenize the mixture using 2 5-mm glass beads (VWR Soda Lime, Radnor, PA, USA) at 1750 rpm for 2 min. 5.75 ml 5 M potassium acetate was then added to the tube, inverted ten times, and incubated on ice for 30 min. Tubes were then centrifuged at 14,000 g for 20 min and the supernatant added to one volume of chloroform isoamylalcohol (v/v 24:1). Tubes were again centrifuged at 14,000 g for 10 min, supernatant added to 100 µl RNase A (10 mg/ml) and incubated at 37°C for 120 min. An equal volume of isopropanol and 1/10 volume of sodium acetate was then added. Tubes were then incubated at 25°C for 5 min, centrifuged at 14,000 g for 30 min and the supernatant was discarded. The pellets were then rinsed twice with 70% ethanol, air-dried overnight, and dissolved in 100 µl deionized H_2O. Library preparation and sequencing (Illumina NextSeq PE150 protocol) were done by the Georgia Genomics and Bioinformatics Core (GGBC – Athens, GA, USA).

Paired-end reads were assessed for quality using the default parameters of FASTQC v0.11.8 (Andrews 2010). Low-quality sequences were trimmed with a Phred score threshold of 33 and paired-end adapters removed using TrimGalore 0.4.4. (Krueger 2017). Sequence quality was reassessed using FASTQC v0.11.8 and quality reads were used for de novo assemblies using the SPAdes v3.10.0 genome assembler (Bankevich et al. 2012). The dipSPAdes assembler was used to construct haplotype assemblies for genomes that collapsed using SPAdes. The BUSCO v3.0.2 tool (Simão et al. 2015) was used to assess each genome assembly for completeness with the fungi_odb9 gene set. Assembly quality metrics were also analyzed using QUAST v4.5 (Gurevich et al. 2013).

Ploidy analyses using ploidyNGS

Paired-end reads were mapped to the DOE-JGI reference genome (E12A7-4) using BWA v0.7.15 (Li and Durbin 2009) with the BWA-MEM algorithm. SAMtools v1.10 (Li et al. 2009) was used to sort and index BAM files which were then used as input for ploidyNGS. The ploidyNGS software (Corrêa dos Santos et al. 2017), which uses allele frequencies to predict ploidy, was used to assess each genome. The --guess_ploidy option was used to report the ploidy number most similar to simulated data. Allele frequency graphs produced by the software were visually assessed for confirmation of the ploidy reported by the guess_ploidy function.

SNP and InDel calling, and CNV analyses

Small genomic variants were called from indexed and sorted BAM files using SAMtools v1.6 and BCFtools v1.10.2. Specifically, *samtools mpileup* was used to produce genotype likelihoods and *bcftools call* for SNP and InDel calling. SNPs were filtered using a base quality score ≥ 20. Statistics from variant calls were generated using *bcftools stats*. CNV analysis was performed using the CNVpytor tool (Suvakov et al. 2021), which detects and analyzes CNVs from read depth and allele imbalance in the genome. BAM files were parsed and read depths were calculated and stored in 100 bp intervals. Bin sizes of 1000, 3000, 5000 and 10,000 bp were used to call CNVs. Because there is ploidy variation among isolates, read depth (RD) plots of each scaffold was used to infer the ploidy of the majority of these scaffolds (base ploidy). CNV calls were then merged across strains with the same base ploidy to identify CNVs among strains in each population of the fungus. Genome-wide heterozygosity was calculated using the Perl script vcf2allelePlot (Bensasson Lab, UGA)

using Perl v5.26.1 and R v3.5.0 with VCF files generated from SNP calling as input. It was calculated from the ratio of the number of high-quality point substitutions and high-quality sequence length.

Population genomic analyses

Population genomic analyses were conducted on the 14 isolates to confirm previously inferred population genetic structure (Stewart et al. 2014, 2015) in whole genome data and to identify regions of the genome that may be involved in adaptation of the populations. For phylogenetic analyses indexed VCF files were merged and converted to PHYLIP and NEXUS format using the Python script vcf2phylip.py (Ortiz 2019) and Python v3.8.2. The SplitsTree5 NeigbourNet algorithm (Bryant and Moulton 2004) was used to construct a phylogenetic network using the generated PHYLIP file. The network was constructed using 1,593,859 SNPs.

The PopGenome 2.7.5 package (Pfeifer 2020) in R v3.5.0 was used to analyze population differentiation among all three populations. First, VCF files with whole genome SNP data were read into the software using the readData function. The populations of *E. maculosum* were then defined using the set.populations function with strains and populations listed in Table 4.1. The SNP data was then split according to annotation information in the DOE-JGI produced GFF annotation file of strain E12A7-4 using the split_data_into_GFF_attributes function. The site-specific diversity and F_{ST} measurements were calculated using the F_ST.stats and mode="nucleotide" functions. PopGenome uses all pairwise comparisons to get an average nucleotide diversity. The get_gff_info and maxFSTgenes functions were then used to extract information from the GFF file of genes

with an average nucleotide diversity in the top 5% percent of the F_{ST} distribution as they could be involved in adaptation of the pathogen.

RESULTS

Genome assembly and quality metrics

Genome assemblies were initially attempted using the SPAdes assembler, but some isolates had low quality, fragmented assemblies. These genomes were found to have a high level of heterozygosity and included Me1-13S1, 88-9 and 89-5 from the NA population, NCLC1-14 from NC and D1-1 from GA. The dipSPAdes extension of SPAdes which is designed for highly polymorphic diploid genomes successfully assembled genomes of isolates that collapsed using SPAdes. Consensus contigs representing the haplomes for the orthologous regions were produced. Contigs with sequence lengths less than 1000 bp were filtered and removed from the assemblies. When assembled genomes were compared to the 290-BUSCO fungal gene subset to assess genome completeness, > 90% of conserved genes were found among all isolates and > 95% of conserved genes were found in most isolates and in single copies, providing evidence for robust assemblies. Three Genome contiguity was measured using QUAST and 6 of the 14 genomes had N50 values greater than 1 Mb, which means that 50% of the genomes were on contigs equal to or greater than those values. L50 values which were generally low also supports good quality assemblies (Table 4.2). Strains with higher genome-wide heterozygosity had lower quality assemblies. For example, NCLC1-44 which had the highest heterozygosity (0.015) of all the strains in NC, had a low

N50 value of 24525 and a high L50 value of 101. Other genomes with lower quality genomes include strains E12D1-1 and Me1-13S1. The reference genome was assembled by DOE-JGI, but assembly metrics were performed as with other isolates.

Ploidy variation

Ploidy levels and variation among isolates of *E. maculosum* was previously estimated using flow cytometry (Chapter 3). Because the measure of DNA content only provides a broad view of ploidy variation, a bioinformatics approach using NGS data was used to further scrutinize these findings at the whole genome sequence level. The ploidyNGS software uses simulated data to predict the nearest euploid number. Therefore, it did not predict aneuploidies, but most isolates were called as diploids (Table 4.3). For example, strain E12D1-1 which was estimated as aneuploid near diploidy with flow cytometry, was called as diploidy with ploidyNGS. It also predicted that there were diploid and tetraploid strains in the NC population. In the GA population, the software predicted that the reference strain E12A7-4 was haploid as with flow cytometry. It also predicted that 3 of the strains from GA were diploid and 1 was haploid. Ploidy graphs that were produced by the program clearly showed isolates that deviated from standard heterozygous diploid representations although they were called as being closest to diploid. However, two isolates from NA (88-9 and 89-5) were seemingly diploid and heterozygous at a high percentage of loci (Figure 4.2). The main inconsistency between the two methods used for ploidy estimation was that flow cytometry estimated that 9 strains were aneuploid while ploidyNGS produced euploid estimates. It is important to note however that aneuploidies were close to these euploid

estimates in many of these strains. For example, strain E12D1-1 was estimated as aneuploid near diploidy using flow cytometry and ploidyNGS guessed it was diploid.

SNP and InDel calling, and CNV analyses

There was a range of 902,519 total variants in strain E13A1-S in the GA population to 1,733,174 total variants in strain 88-9 in the NA population. SNPs represented most of these small genomic variants. Multiallelic SNP sites represented less than 1% of the total number of SNPs. Transitions and transversions were present in an approximately 2:1 ratio across all isolates.

When CNVs were analyzed using 1000 bp bins, the average number of deletions ranged from 400 in the NA population to 513 in the NC population. Most CNV regions were deletions. Duplications using the same bin size of 1000 bp had an average ranging from 1.25 in GA to 7.25 in NA (Figure 4.3). The NA population had the most duplications and least deletions of all three populations. Large CNV regions were identified using a 3000 bp bin size and filtered for those greater than 10,000 bp (Figure 4.4). Some of these CNV regions contained both duplications and deletions (Figure 4.5). Outside of genes involved in normal cell functions, noteworthy genes found in CNV regions include those involved in DNA repair, oxidation reduction, splicing, heat shock proteins and the cytochrome P450 superfamily, which has well-known links to drug metabolism and resistance (Kelly and Kelly 2013; Zhang et al. 2019). The genome-wide heterozygosity of isolates from NA was much higher than those in the other populations with three of four strains with a value over 0.3 (Table 4.4). Heterozygosity was similar among isolates from GA with a range of 0.003 to 0.021 and NC with a range of 0.005 to 0.015.

Phylogenetic and F_{ST} analyses

A phylogenetic network (NeighborNet) which was inferred based on genome-wide SNP's detected population structure by geographical origin of isolates (Figure 4.6). Although each population was distinct, GA and NC populations were more closely related. The NA population was genetically distant from the other two populations.

Genes in SNP regions with high F_{ST} values and in the top 5% of the distribution included major facilitator superfamily (mfs)-multidrug resistance transporters, synaptic vesicle transporters, which catalyze the efflux of commonly used azoles and 7 genes involved in oxidation reduction (Table 4.5; Figure 4.7)

DISCUSSION

Here, we used a bioinformatics approach to support our findings from flow cytometry that there is ploidy variation among strains of *E. maculosum*. The ploidyNGS software estimates ploidy to the nearest euploid state so it did not predict aneuploidies that were estimated using flow cytometry. However, it did predict the euploid state nearest to the aneuploid state in most of the aneuploid strains. The software also predicted the ploidy of strains that were not haploid in most strains. Both analyses taken together show that there is ploidy variation among isolates, and in some cases within isolates, that the base ploidy of most isolates is diploid, and that aneuploidy is widespread in *E. maculosum*. Many fungal plant pathogens are haploid and reproduce asexually. Chromosome loss is usually lethal in haploid organisms and gene dosage is highly affected by chromosome gain.

Polyploidy is associated with increased rates of aneuploidy and genetic instability (Selmecki et al. 2015; Zhu et al. 2016). Aneuploidy has been reported in oomycetes such as *Phytophtora ramorum* and *Phytophtora infestans* (Kasuga et al. 2016; Van der Lee et al. 2004). In *P. ramorum*, partial aneuploidy was found only in strains that were isolated from oak trees and not in those that were grown in the lab, which suggests that there is some link between the environment and karyotype. Wertheimer et al. (2016) highlighted that exposure to antifungals can lead to LOH and aneuploidy through mitotic defects which first form tetraploids with extra spindles, followed by chromosome mis-segregation, copy number alterations and diversity which can lead to pathogen emergence on new hosts or resistance to antifungals. Fungicides are rarely used on lowbush blueberries (*V. angustifolium*), which are grown in the NA population. Populations in GA and NC are more likely to be exposed to fungicides. In fact, resistance to Pristine has been observed in Georgia (Ingram et al. 2019). Fungicides could potentially play a role in the genomic arrangement of *E. maculosum*. Storchova (2014) suggested that there may be a link between tetraploid-derived aneuploidy and fungicide resistance. Harrison et al. (2014) used time-course flow cytometry and time-lapse fluorescence microscopy to show that *C. albicans* became tetraploid during fluconazole exposure, subsequently had chromosomal instability, and produced aneuploids. The majority of *E. maculosum* strains have aneuploid states near diploidy and tetraploidy which further suggests that fungicides could be involved. It is unclear whether *E. maculosum* aneuploidies are complete or partial because chromosome level assemblies are not available.

We observed copy number variation within and among populations of *E. maculosum*. GA and NC populations mostly had deletions with few duplications while the

NA population had more duplications than GA and NC. Phylogenetic analyses show that the GA and NC populations are also more closely related. Geographic proximity of the GA and NC populations likely plays a role in their genetic similarities, but these populations are associated with similar blueberry hosts, including rabbiteye (*V. virgatum*), southern highbush (*V. corymbosum* × *V. virgatum* or *V. darrowi* hybrids) and sometimes highbush blueberries (*V. corymbosum*) whereas the habitat of the NA population is dominated by lowbush blueberry species (*V. angustifolium*). Copy number changes can be advantageous in conditions of extreme stress. For example, *Saccharomyces cerevisiae* chromosome VIII consistently shows an increase in copy number in copper-rich environments (Whittaker et al. 1988).

E. maculosum aneuploid strains that are near tetraploidy may have had whole genome duplications (WGD). Whole genome duplication plays a significant role in pathogen evolution as it may produce redundant genes, affect gene dosage and expression while providing the basis for complex genetic arrangements that can be used for adaptation to changes in their environment. Diploidization may also be occurring in *E. maculosum* through deletion of genetic material and loss of heterozygosity may be a consequence. It is unclear what other patterns of divergence are responsible for the evolution of *E. maculosum*, but autotetraploidy, genomic allotetraploidy, segmental allotetraploidy, and multiple segmental duplications should be considered for further studies. It is evident from CNV analyses, however, that the genomes have evolved through deletions. There are also segmental duplications which could indicate that segmental allotetraploidy or independent diploidization of different loci may be involved. Polyploid individuals usually maintain a higher level of heterozygosity and have more complex genome organization than diploids.

Studies of genome-wide polymorphisms can help us to identify important traits within and among species. Such traits include genes that are strongly selected in a population and aid our understanding of how different pathogens adapt to their environments. The measure of population differentiation can be used to identify these loci as alleles involved in local adaptation usually diverge from genome-wide average F_{ST} values. For example, Ellison et al. (2011) discovered genes with the highest F_{ST} values in a study of population differentiation among 48 *Neurospora crassa* isolates representing two geographically isolated populations, were involved in temperature response and circadian function. The authors suggested that this is due to adaptation to varying photoperiods and temperatures. In this study, Hudson's F_{ST} (Hudson et al. 1992; Keinan et al. 2007) was used because it produces estimates that are the average F_{ST} values that are independent of sample sizes even when F_{ST} is not identical across populations. In *E. maculosum*, genes with high F_{ST} values included many that were involved in cellular functions such as membrane transport and protein binding. However, other genes that stood out as potentially important for the adaptation of the pathogen were 4 genes which are involved in the transmembrane transport of multiple fungicides (synaptic vesicle transporters (SVOP) and major facilitator superfamily (MFS) and 7 genes which play a role in oxidative stress or oxidation reduction. One such SVOP, Multidrug/Multixenobiotic resistance (MDR1) catalyzes the efflux of commonly used azoles (Mandal et al. 2012) and others have been involved in the transmembrane transport of benomyl, cycloheximide, methotrexate and fluconazole (Basso et al. 2010; Cannon et al. 2009). Gupta et al. (1998) also found that the *Candida albicans* Multi Drug Resistance (CaMDR1) gene when expressed in *S. cerevisiae* confers resistance to several unrelated drugs. Multidrug resistance is now a widespread

issue with serious implications on the acquisition of resistance in clinical, agricultural, and biotechnological environments as these transporters can catalyze the efflux of several cytotoxic compounds (Dos Santos et al. 2014). These findings in *E. maculosum* along with the high rate of aneuploidy that is observed could have implications on the epidemiology of the pathogen and potentially how it interacts with vectors and fungicide resistance that has already been observed.

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Table 4.1. Origin of strains used in genomic studies.

Host (leaf or fruit spot)	Geographic origin	Strain	Collection Year
Vaccinium virgatum 'Climax'(fruit)	Georgia	E12A7-4	2012
Vaccinium virgatum 'Premier'(leaf)	Georgia	E12C1-4	2012
Vaccinium virgatum 'Premier'(leaf)	Georgia	E13A1-S	2013
Vaccinium virgatum 'Powder Blue'(leaf)	Georgia	E12D1-1	2012
Vaccinium virgatum	Georgia	E12-FS2-8S1	2012
Vaccinium angustifolium	Maine	Me1-13S1	2012
Vaccinium angustifolium	Nova Scotia	88-9	1988
Vaccinium angustifolium	Nova Scotia	89-5	1989
Vaccinium angustifolium	Nova Scotia	92-50	1992
Vaccinium corymbosum × *V. darrowi* hybrid 'Legacy' (leaf)	North Carolina	NCLC1-14	2012
Vaccinium virgatum 'Columbus'(leaf)	North Carolina	NCLC1-23	2012
Vaccinium corymbosum × *V. darrowi* hybrid 'Blue Ridge' (leaf)	North Carolina	NCLC1-33	2012
Vaccinium virgatum 'Premier'(leaf)	North Carolina	NCPC1-8	2012
Vaccinium corymbosum 'Duke' (leaf)	North Carolina	NCLC1-44	2012

Table 4.2. Genome assembly and quality metrics of 14 *Exobasidium maculosum* isolates from 3 populations.

Population	Isolate	Genome Size Mbp	Quast N50	Quast L50	Busco % C	Complete	Complete Single Copy	Buscos/290 Duplicated	Missing	Fragmented
Georgia	E12A7-4*	22.3	867357	6	94.1	273	273	0	8	9
(GA)	E13A1S	18.1	614145	10	96.2	279	279	0	9	2
	E12C1-4	17.5	1594186	4	94.5	274	274	0	11	5
	E12-FS2-8S1	19.4	1059710	6	95.2	276	276	0	10	4
	E12D1-1	21.6	176546	29	95.2	276	255	9	14	12
North	NCLC1-14	20.7	552533	12	95.2	276	276	0	11	3
Carolina	NCLC1-23	20.5	1574725	5	95.5	277	277	0	8	5
(NC)	NCPC1-8	20.8	497951	12	96.2	279	279	0	7	4
	NCLC1-44	22.3	24525	101	91.1	264	258	6	15	11
	NCLC1-33	22.5	920603	8	93.8	272	272	0	13	5
NE North	92-50	17.9	1304790	4	96.2	279	279	0	8	3
America	89-5	18.1	707654	16	94.5	274	251	19	12	8
(NA)	Me1-13S1	18.7	250513	22	90.3	261	254	6	21	9
	88-9	19.1	401464	14	93.1	270	251	11	18	10

* PacBio sequenced reference genome (DOE-JGI)

Table 4.3. Ploidy estimation of *Exobasidium maculosum* isolates using ploidyNGS software. Ploidy in parentheses represent the euploid state closest to aneuploid estimates.

Population	Strain	Flow cytometry ploidy estimation	ploidyNGS ploidy estimation
	FB1(*U. maydis*)	haploid	-
Georgia	E12A7-4 (ref)	haploid	haploid
(GA)	E12C1-4	diploid	diploid
	E13A1-S	aneuploid (diploid)	pentaploid
	E12D1-1	aneuploid (diploid)	diploid
	E12-FS2-8S1	aneuploid (diploid)	diploid
NE North	Me1-13S1	aneuploid (tetraploid)	diploid
America	88-9	diploid	diploid
(NA)	89-5	diploid	diploid
	92-50	diploid	pentaploid
North	NCLC1-14	aneuploid	diploid
Carolina	NCLC1-23	aneuploid	diploid
(NC)	NCLC1-33	aneuploid	tetraploid
	NCPC1-8	aneuploid	tetraploid
	NCLC1-44	aneuploid	tetraploid

Table 4.4. Small genomic variants and genome-wide heterozygosity of *Exobasidium maculosum* strains in 3 populations.

Population	Strains	Total Variants	SNP	InDel	Multiallelic SNP sites	Transitions	Transversions	Genome-wide Heterozygosity (H)
Georgia (GA)	E12C1-4	969014	961251	7763	5765	648280	318937	0.005
	E13A1-S	902519	885831	16688	7176	598000	295113	0.003
	E12D1-1	1020096	1004463	15633	8115	676286	336489	0.021
	E12-FS2-8S1	995729	988428	7301	6130	665362	329425	0.004
NE North America (NA)	Me1-13S1	1656096	1639742	16354	16579	1123802	532784	0.382
	88-9	1733174	1727154	6020	9822	1181722	555507	0.384
	89-5	1687112	1680491	6621	9645	1147254	543086	0.343
	92-50	1382172	1377330	4842	9096	927071	459720	0.004
North Carolina (NC)	NCLC1-14	1019235	1012014	7221	5951	681576	336602	0.005
	NCLC1-23	1038025	1028757	9268	6830	692651	343181	0.006
	NCLC1-33	1036153	1030829	5324	6199	693511	343725	0.005
	NCPC1-8	1033444	1027621	5823	6356	691067	343113	0.005
	NCLC1-44	1063065	1057206	5859	7463	711201	353706	0.015

Table 4.5. Annotation and gene ontology of genes with F_{ST} values in top 5% of the distribution.

Annotation	Gene Ontology	F_{ST}
Nuclear protein export factor	protein export activity	0.63
Predicted E3 ubiquitin ligase	zinc ion binding,protein binding	0.65
Dynein light intermediate chain	unknown function	0.66
Predicted E3 ubiquitin ligase	ATP-dependent peptidase activity	0.53
SWI-SNF chromatin remodeling complex, Snf5 subunit	chromatin remodeling	0.50
Ubiquitin C-terminal hydrolase	protease activity	0.52
Ribosomal protein S36, mitochondrial	mitochondrial translation	0.60
Ca2+/calmodulin-dependent protein kinase kinase beta	protein phosphorylation	0.51
Prolyl-tRNA synthetase	prolyl-tRNA aminoacylation	0.57
DNA helicase, TBP-interacting protein	DNA helicase activity	0.57
Tannase and feruloyl esterase	ferulic and diferulic acids hydrolysis	0.59
ulfatase-modifying factor enzyme 1	posttranslational modification	0.56
ZZ-type Zn-finger	zinc ion binding	0.56
Serine/threonine kinase TIP30/CC3	Kinase activity	0.54
Glutathione S-transferase	protein binding	0.55
Translin family protein	sequence-specific DNA binding	0.52
NA helicase BRR2, DEAD-box superfamily	nucleic acid binding	0.51
Protein tyrosine phosphatase	protein dephosphorylation	0.50
Molecular chaperones GRP170/SIL1, HSP70 superfamily	chaperone activity	0.51
Ubiquitin activating E1 enzyme-like protein	mall protein activating enzyme	0.50
Thyroid hormone receptor-associated coactivator complex	regulation of transcription from	0.59
LysM domain	Peptidoglycan and chitin binding	0.50
Fungal Zn(2)-Cys(6) binuclear cluster domain	sequence-specific DNA binding	0.55
Golgi transport complex COD1 protein	ER function	0.51
Succinyl-CoA synthetase, alpha subunit	ATP citrate synthase activity	0.50
COP9 signalosome, subunit CSN3	cullin-RING regulation	0.58
Transcription mediator complex subunit Med12	transcription regulation	0.53
Fungal Zn(2)-Cys(6) binuclear cluster domain	sequence-specific DNA binding	0.53
Invasion-inducing protein TIAM1/CDC24	guanyl-nucleotide exchange activity	0.57
p21-activated serine/threonine protein kinase	protein kinase activity	0.65
Tscrpt_reg_HTH_APSES-type	DNA binding	0.72
Synaptic vesicle transporter SVOP	transmembrane transport of fungicides	0.61
Phosphatidylinositol-4-phosphate 5-kinase	phosphatidylinositol metabolic process	0.65
Serine/threonine protein kinase	protein kinase activity	0.56
2-enoyl-CoA hydratase	sterol binding	0.66
Yeast mitochondrial distribution and morphology (MDM)	mitochondrion organization	0.65
Transcription-associated recombination protein - Thp1p	protein binding	0.63

Beta-transducin family (WD-40 repeat) protein	protein binding	0.63
Mitochondrial fatty acid anion carrier protein	oxidative stress	0.67
Putative guanine nucleotide exchange factor	Rho guanyl-nucleotide exchange activity	0.67
Preprotein translocase, gamma subunit	protein targeting and transport	0.65
Pyridine nucleotide-disulphide oxidoreductase	oxidoreductase activity	0.61
Guanine nucleotide exchange factor	activation of small GTPases	0.61
Delta 6-fatty acid desaturase/delta-8 sphingolipid desaturase	oxidation-reduction process	0.60
Predicted transporter (major facilitator superfamily)	transmembrane transporter	0.73
Predicted mitochondrial carrier protein	Ca ion binding,transmembrane transport	0.61
Sulfite reductase (ferredoxin)	oxidoreductase activity	0.67
Cutinase	metabolic process, hydrolase activity	0.72
UDP-glucose/GDP-mannose dehydrogenase	oxidoreductase activity	0.63
Nuclear pore complex, Nup160 component	RNA transport	0.62
Ribose 5-phosphate isomerase	ribose-5-phosphate isomerase activity	0.66
Nucleolar GTPase/ATPase p130	GTP binding	0.78
Glucose-repressible alcohol dehydrogenase CCR4	protein binding	0.51
mRNA cleavage and polyadenylation factor II complex	binding	0.58
Peroxisomal membrane anchor protein (peroxin)	membrane protein	0.52
rRNA processing/ribosome biogenesis	rRNA processing	0.52
Anticodon-binding domain	anticodon-binding activity	0.60
Synaptic vesicle transporter SVOP and related transporters	mfs-multidrug-resistance transporter	0.52
Histone acetyltransferase SAGA, TRRAP/TRA1 component	transferase activity	0.65
Putative threonine/serine exporter	transporter activity	0.71
Alanyl-tRNA synthetase	ligase activity	0.66
ATP-dependent RNA helicase A	nucleic acid binding, helicase activity	0.69
Phosphatidylinositol 4-kinase	transferase activity	0.61
Zinc-binding oxidoreductase	zinc ion binding oxidation-reduction	0.62
F-box_dom	protein binding	0.62
Prenyl protein protease	membrane	0.62
Mitochondrial J-type chaperone	protein folding	0.64
Protein phosphatase 2A-associated protein	regulation of signal transduction	0.66
Sensory transduction histidine kinase	phosphorelay sensor kinase activity	0.65
Beta-tubulin folding cofactor C	tubulin folding	0.60
Electron transfer flavoprotein ubiquinone oxidoreductase	oxidation-reduction process	0.68
mRNA splicing factor	RNA processing	0.62
Pattern-formation protein/guanine nucleotide exchange factor	ARF guanyl-nucleotide exchange factor activity	0.63
Beta-fructofuranosidase (invertase)	hydrolase activity	0.62
tRNA methyltransferase	tRNA processing,transferase activity	0.63
Flavin-containing amine oxidase	oxidation reduction	0.68

glucomannan 4-beta-mannosyltransferase	glucomannan 4-beta-mannosyltransferase activity	0.60
Predicted transporter (major facilitator superfamily)	transmembrane transporter activity	0.72
FOG: Zn-finger	metal ion binding	0.71
mRNA-binding protein Encore	nucleic acid binding	0.65
Cell division cycle 37 protein, CDC37	protein kinase binding	0.63
Voltage-gated Ca2+ channels, alpha1 subunits	ion channel activity,	0.61
Protein Mei2	nucleic acid binding	0.63
Dihydrolipoamide acetyltransferase	metabolic process, transferase activity	0.72
The basic amino acid (canavanine sensitivity) transporter	amino acid transmembrane transport	0.65
Glycine cleavage system H protein (lipoate-binding)	glycine cleavage	0.62
protein-L-isoaspartate(D-aspartate) O-methyltransferase	RNA polymerase II transcription activity	0.62
Cutinase	metabolic process,hydrolase activity	0.74
Dihydrolipoamide acetyltransferase	transferase activity	0.72
Transcriptional activator FOSB/c-Fos	transcription factor activity	0.60
Sugar (and other) transporter	transmembrane transporter	0.61
Long-chain acyl-CoA synthetases (AMP-forming)	catalytic activity,metabolic process	0.63
mRNA splicing factor	RNA processing	0.64

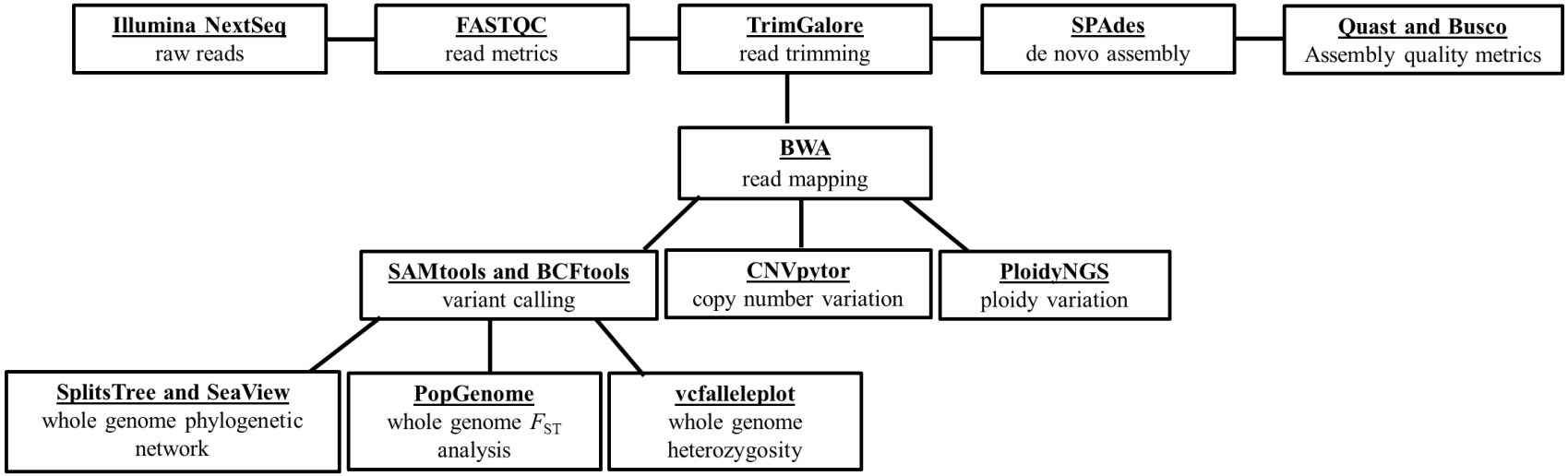


Figure 4.1. Pipeline for genome assembly and comparative analyses

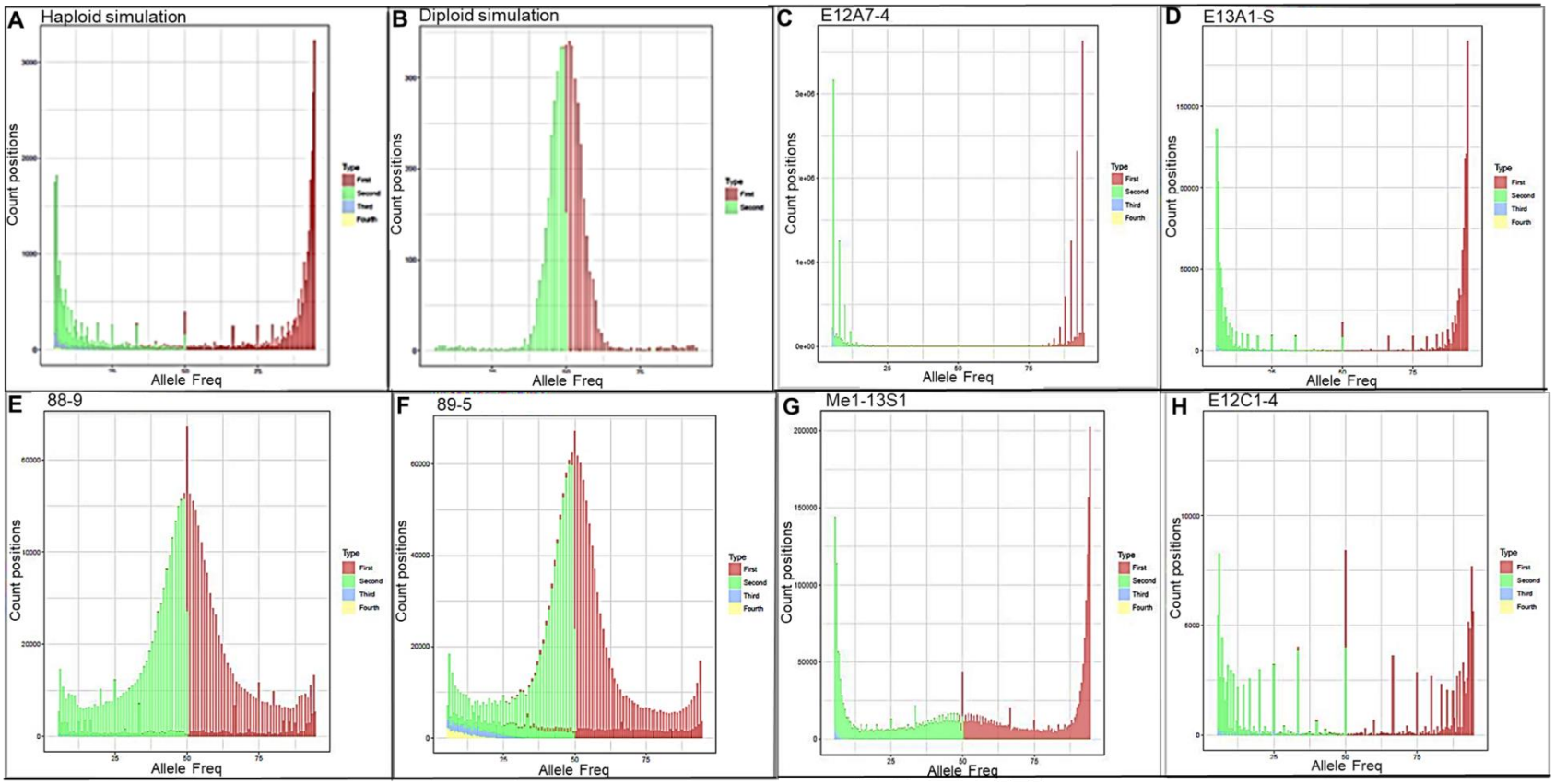


Figure 4.2. Representative allele frequency plots for strains of *Exobasidium maculosum* generated by the ploidyNGS software. Haploid (A) and diploid (B) simulations generated by the software are used for comparison. The reference *E. maculosum* strain E12A7-4 is haploid (C) and two isolates from NA have characteristic heterozygous diploid allele frequencies (E-F), but ploidy was unclear in some isolates (D, G, H). The guess_ploidy function of the software called the nearest ploidy as haploid (C) diploid (E-F) and pentaploid (D)

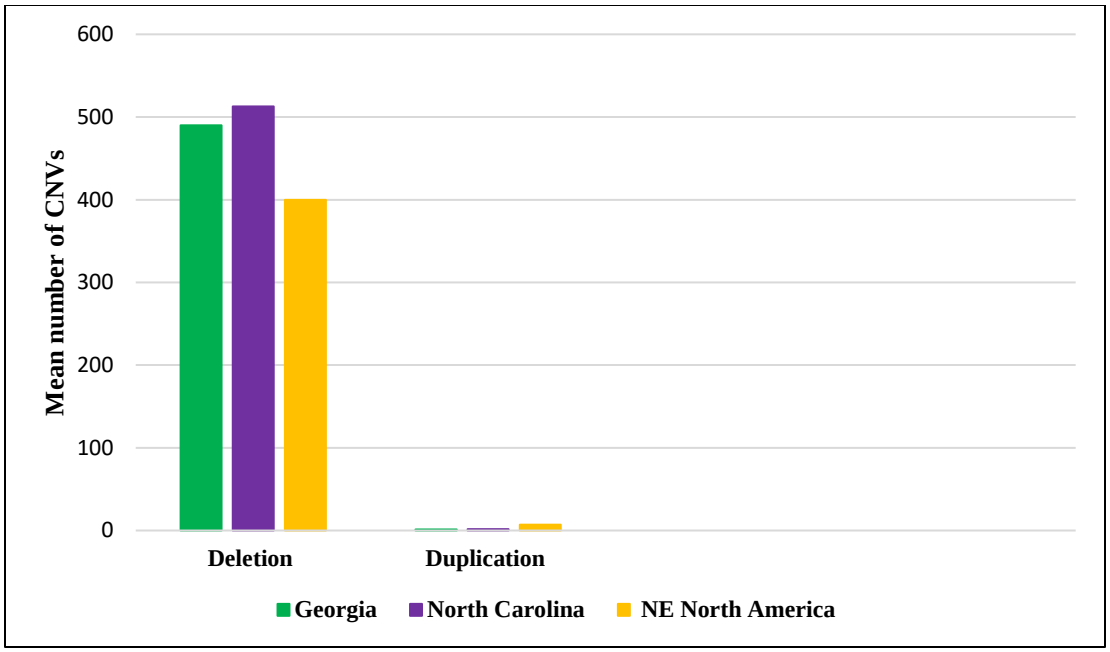
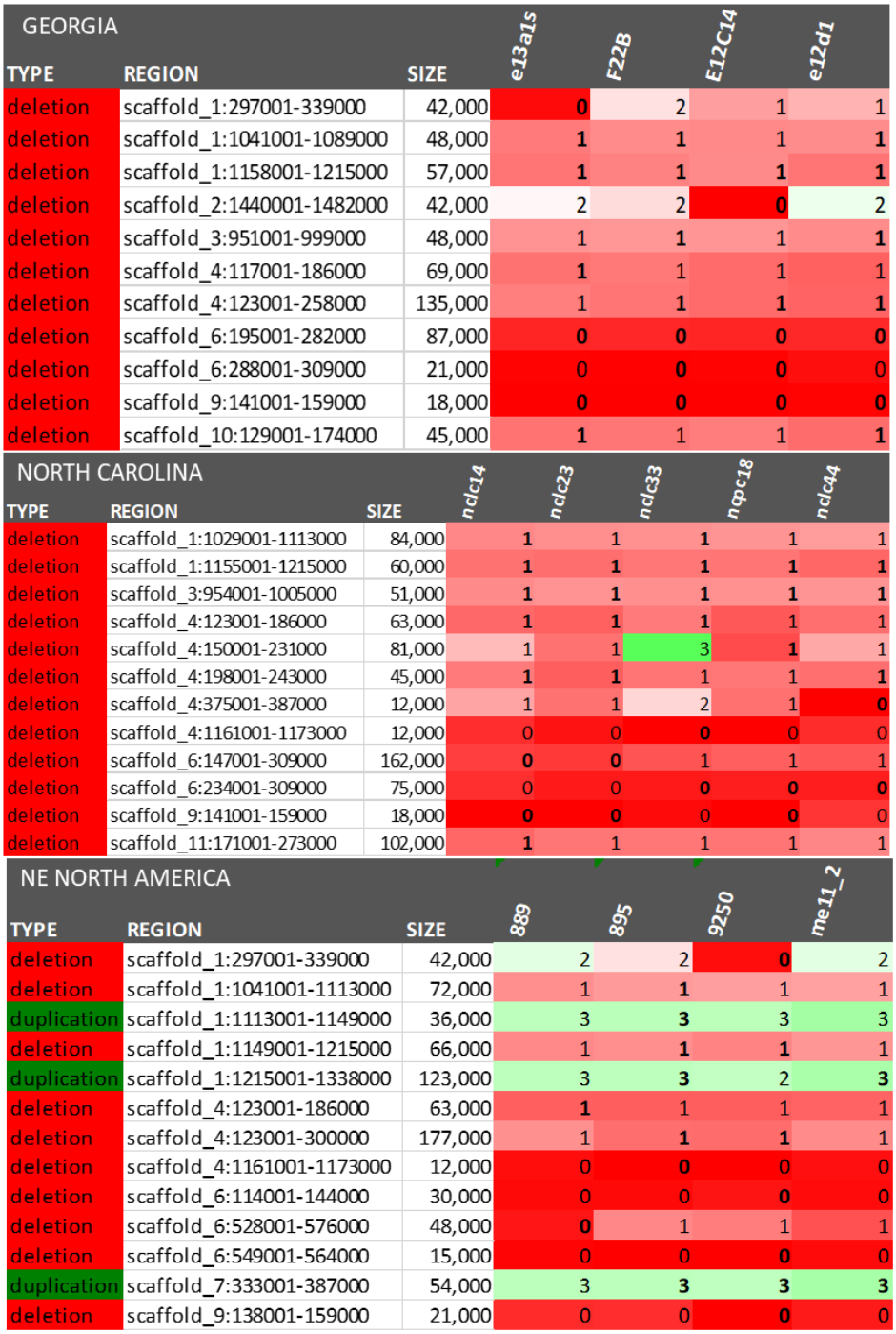


Figure 4.3. Distribution of CNVs across populations of *Exobasidium maculosum* using a bin size of 1000 bp. The mean number of deletions in each population was 100-fold the number of duplications. NE North America had a greater number of duplications than Georgia and North Carolina.



GEORGIA

TYPE	REGION	SIZE	e13a1s	F22B	E12C14	e12d1
deletion	scaffold_1:297001-339000	42,000	0	2	1	1
deletion	scaffold_1:1041001-1089000	48,000	1	1	1	1
deletion	scaffold_1:1158001-1215000	57,000	1	1	1	1
deletion	scaffold_2:1440001-1482000	42,000	2	2	0	2
deletion	scaffold_3:951001-999000	48,000	1	1	1	1
deletion	scaffold_4:117001-186000	69,000	1	1	1	1
deletion	scaffold_4:123001-258000	135,000	1	1	1	1
deletion	scaffold_6:195001-282000	87,000	0	0	0	0
deletion	scaffold_6:288001-309000	21,000	0	0	0	0
deletion	scaffold_9:141001-159000	18,000	0	0	0	0
deletion	scaffold_10:129001-174000	45,000	1	1	1	1

NORTH CAROLINA

TYPE	REGION	SIZE	ncc14	ncc23	ncc33	ngpc18	ncc44
deletion	scaffold_1:1029001-1113000	84,000	1	1	1	1	1
deletion	scaffold_1:1155001-1215000	60,000	1	1	1	1	1
deletion	scaffold_3:954001-1005000	51,000	1	1	1	1	1
deletion	scaffold_4:123001-186000	63,000	1	1	1	1	1
deletion	scaffold_4:150001-231000	81,000	1	1	3	1	1
deletion	scaffold_4:198001-243000	45,000	1	1	1	1	1
deletion	scaffold_4:375001-387000	12,000	1	1	2	1	0
deletion	scaffold_4:1161001-1173000	12,000	0	0	0	0	0
deletion	scaffold_6:147001-309000	162,000	0	0	1	1	1
deletion	scaffold_6:234001-309000	75,000	0	0	0	0	0
deletion	scaffold_9:141001-159000	18,000	0	0	0	0	0
deletion	scaffold_11:171001-273000	102,000	1	1	1	1	1

NE NORTH AMERICA

TYPE	REGION	SIZE	889	895	9250	me11_2
deletion	scaffold_1:297001-339000	42,000	2	2	0	2
deletion	scaffold_1:1041001-1113000	72,000	1	1	1	1
duplication	scaffold_1:1113001-1149000	36,000	3	3	3	3
deletion	scaffold_1:1149001-1215000	66,000	1	1	1	1
duplication	scaffold_1:1215001-1338000	123,000	3	3	2	3
deletion	scaffold_4:123001-186000	63,000	1	1	1	1
deletion	scaffold_4:123001-300000	177,000	1	1	1	1
deletion	scaffold_4:1161001-1173000	12,000	0	0	0	0
deletion	scaffold_6:114001-144000	30,000	0	0	0	0
deletion	scaffold_6:528001-576000	48,000	0	1	1	1
deletion	scaffold_6:549001-564000	15,000	0	0	0	0
duplication	scaffold_7:333001-387000	54,000	3	3	3	3
deletion	scaffold_9:138001-159000	21,000	0	0	0	0

Figure 4.4. Regions with large copy number variations within 3 populations of *Exobasidium maculosum* using a bin size of 3000 bp and filtered for regions greater than 10,000 bp. There were only a few mixed CNV regions containing both deletions and duplications

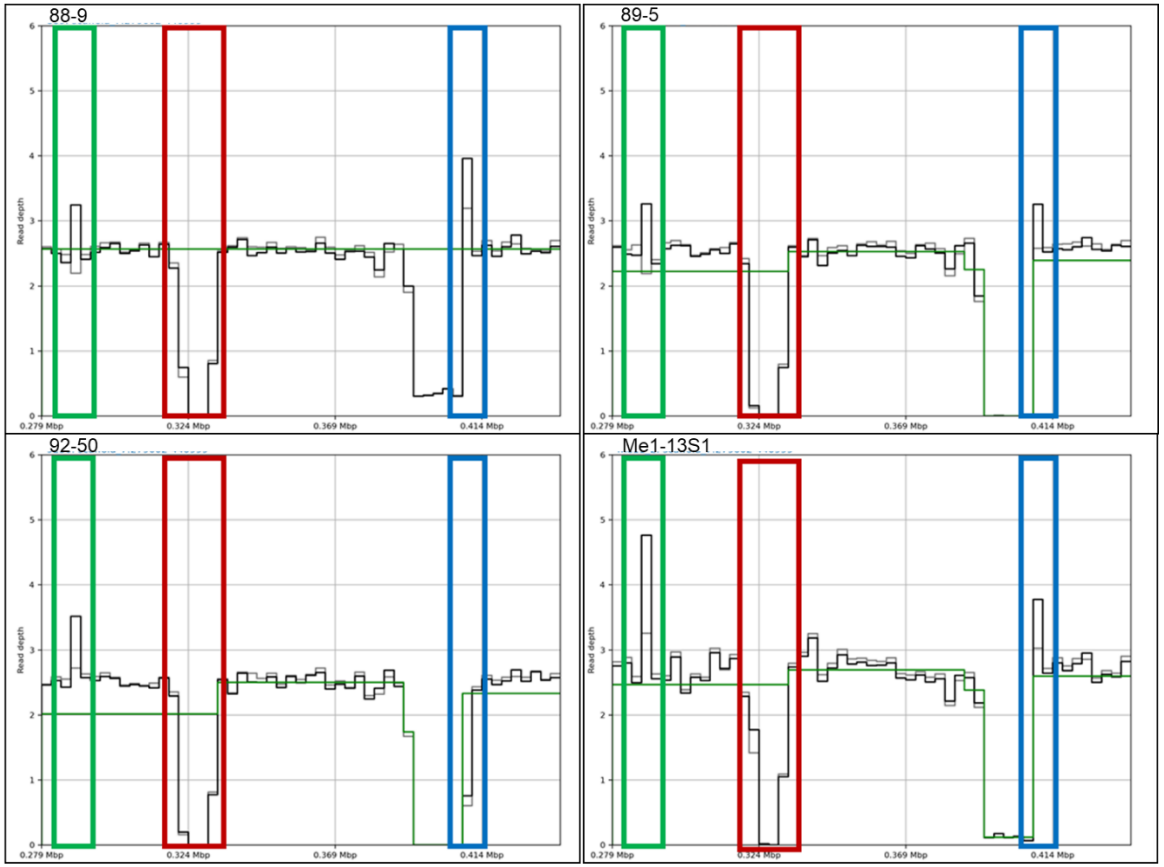


Figure 4.5. Read depth plots of four strains of *Exobasidium maculosum* from northeast North America showing a mixed CNV region. The region highlighted in red indicates deletions in all four strains at that locus. The region highlighted in green indicates duplications in all four strains at that locus and the region highlighted in blue shows duplications in strains 88-9, 89-5 and Me1-13S1 and a deletion in 92-50 at that locus.

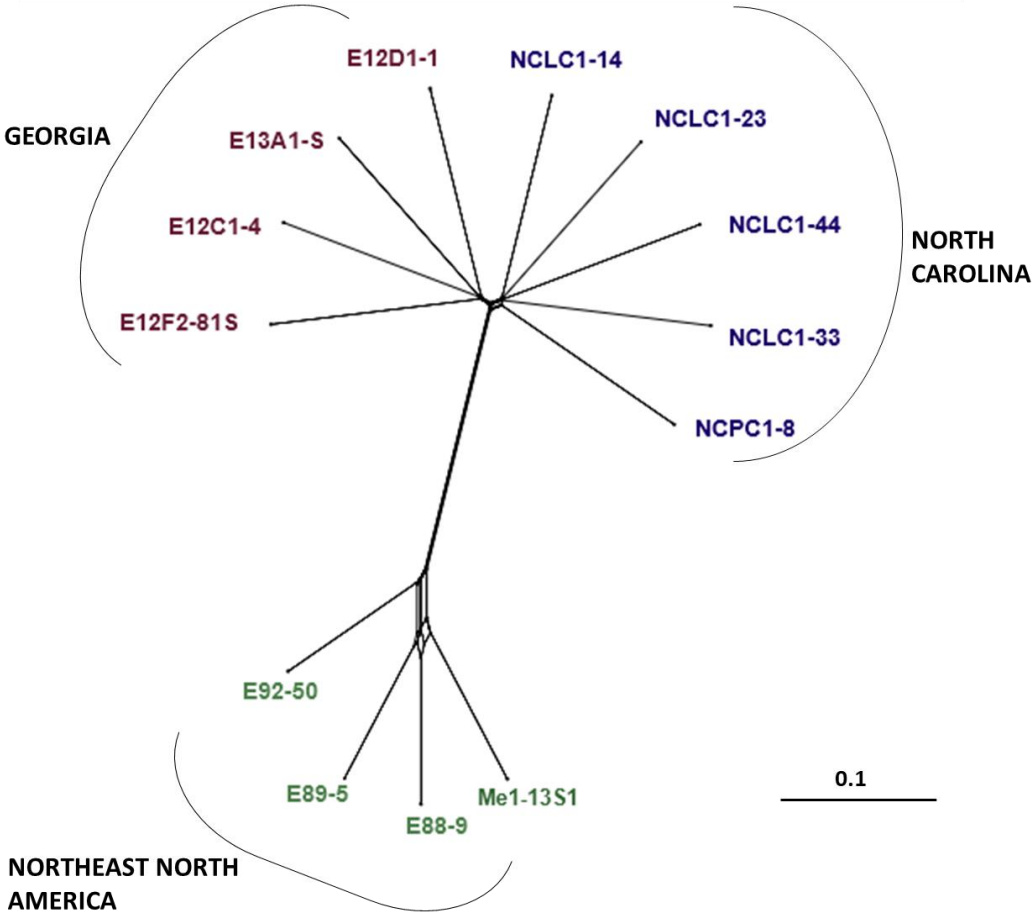


Figure 4.6. Neighbor network detected population structure based on the geographical origin of isolates. The North Carolina and Georgia populations are more genetically similar to each other than the NE North America population.

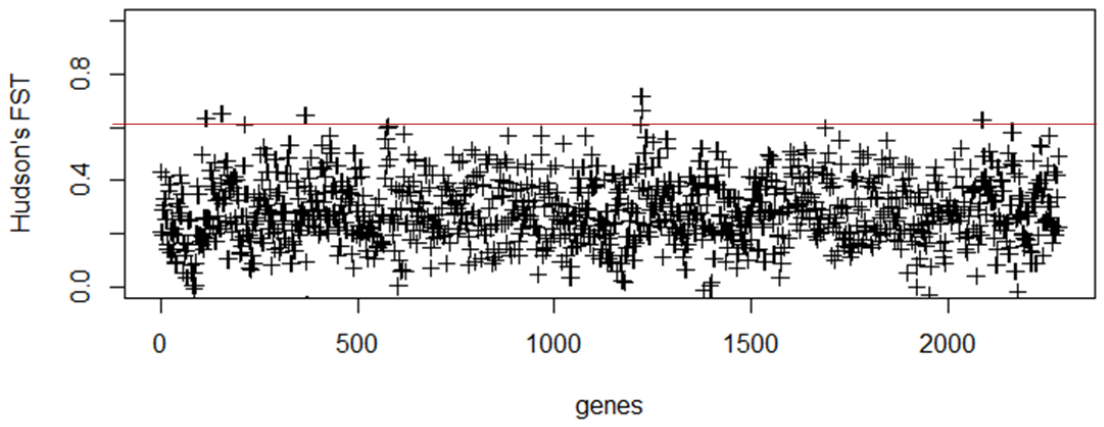


Figure 4.7. Hudson's F_{ST} value for genes across scaffold 1 among three populations of *Exobasidium maculosum*. The site specific pairwise nucleotide diversity is calculated, and average nucleotide diversity calculated for each site. All SNP sites with a F_{ST} value within the top 5% of the distribution (red line) are then overlayed with annotation information from the GFF file and the gene in the region of each SNP is given.

CHAPTER 5

CONCLUSIONS

Exobasidium maculosum has a complicated lifestyle that may involve vector transmission. Although we did not conclusively identify specific vectors in our study (Chapter 2), these new dynamics maybe importantin disease management. This is especially important because genes involved in the efflux of commonly used pesticides and the development of pesticide resistance in clinical and agricultural settings were found to be fast evolving among populations of the fungus (Chapter 4). They can therefore be involved in adaptation of the pathogen to different agricultural environments. The development of resistance to Pristine in Georgia further supports the notion that the fungus may be deploying these genes as an adaptive strategy.

E. maculosum also has a high level of genetic diversity which seems to be driven by a variety of factors such as ploidy variation, a high incidence of aneuploidy and a high spontaneous mutation rate (Chapter 3). There are clinically important fungi that adapt to different environments or stress by switching ploidy. While aneuploidy can decrease fitness, it is also known to play a role in pathogenicity and adaptation. The high rate of aneuploidy in *E. maculosum* could mean that there is some level of genetic instability that can cause increased sequence diversity and rapid evolution. Moreover, the pathogen has a high spontaneous mutation rate which potentially increases genetic instability. The

variations in gene copy number observed among populations (Chapter 4) can influence gene expression levels and may be specific for the lifestyle of the pathogen in these environments. There is also evidence that *E. maculosum* has variation in the number of nuclei per cell in its hyphal stage (Chapter 3). If the preliminary findings are supported in future studies and these nuclei are genetically different, an increased level of heterozygosity that is transferable to all stages of the lifecycle can be expected.

Taken together, these findings indicate that *E. maculosum* has a more complex lifestyle than previously thought. They also suggest that the pathogen has a high evolutionary potential and that methods of disease management may not be durable.