

ABSTRACT

BURCHHARDT, KATHLEEN MARIE. Microsatellite Markers Reveal Population Structure, Genetic Diversity, and the Reproductive Biology of the Blueberry Pathogen *Monilinia vaccinii-corymbosi*. (Under the direction of Dr. Marc A. Cubeta).

The fungus *Monilinia vaccinii-corymbosi* (Mvc) causes disease on the shoots and fruit of blueberry (*Vaccinium* spp.). High disease severity in blueberry fields can lead to substantial fruit yield loss. The overarching goal of this dissertation was to conduct a comprehensive investigation on the population and reproductive biology of Mvc. The objectives were to 1) develop polymorphic microsatellite markers for Mvc for use in subsequent genetic analyses; 2) examine population structure, genetic diversity and gene flow among populations of Mvc in the United States; 3) investigate fine-scale spatial and temporal population dynamics of Mvc within a single blueberry field; and 4) examine the reproductive biology of Mvc by analyzing sexual spores (ascospores) produced from sexual fruiting structures (apothecia).

To address objective 1, 454 pyrosequencing was used to generate sequence reads that were screened to develop 21 polymorphic microsatellite markers. Infected shoots, fruit, and apothecia were collected from 18 blueberry fields in 10 states; one field in GA, MA, ME, MI, MS, NJ, NY, OR, WA, and nine fields in NC. Pure culture isolates of Mvc were obtained from infected plant tissues and single ascospore isolates were generated from apothecia. DNA was extracted either from isolates of Mvc or directly from infected shoots and fruit in cases where a pure culture could not be obtained. The DNA from a total of 1087 samples of Mvc was used to perform PCR with a subset of eight to 10 microsatellite markers. Fragment analysis was performed to determine the multilocus haplotype (MLH) of each sample of

Mvc. Population genetic analyses were conducted based on the MLH of the samples to address objectives 2, 3, and 4.

High genetic diversity and population structure were detected among populations of Mvc from 11 blueberry fields in the Northeast, Northwest, Midwest, and Southeast U.S. In contrast, very low genetic diversity and lack of population structure were detected among seven fields in the Southeast U.S. Results suggest that host specificity may be associated with regional differences in the population biology of Mvc. Based on experimental evidence for restricted gene flow at distances as small as 11 km, infected shoots and fruit were collected and spatially mapped within a 120 x 132 m blueberry plot to examine localized gene flow. Temporal sampling was also conducted by analyzing isolates of Mvc collected at four time points; isolates from infected shoots in 2010, infected fruit in 2011, and infected shoots and fruit in 2012. Spatial autocorrelation analysis suggested unrestricted gene flow at the sampled spatial scale. Little to no genetic differentiation was detected among the four sampling time points, indicating a lack of temporal structuring. Genetic structuring was detected within the plot that was maintained over the three years. Nonrandom mating was suggested by linkage disequilibrium and the repeated detection of two MLHs over the four sampling time points that were significantly unlikely to be the result of random mating.

The presence of multiple genetically different isolates of Mvc within a single fruit and production of genetically different ascospore progeny from apothecia were studied to examine the reproductive biology of Mvc. More than one genetically different isolates of Mvc may colonize a single infected fruit. Single ascospore isolates analyzed from each of eight apothecia had identical MLHs, while at least two genetically distinct MLHs were detected from analyzing isolates from 13 apothecia. These results suggest both outcrossing

and self-fertilizing capabilities exist in *Mvc*. This research represents the first comprehensive examination of the disease ecology and population and reproductive biology of *Mvc*.

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Microsatellite Markers Reveal Population Structure, Genetic Diversity, and the Reproductive
Biology of the Blueberry Pathogen *Monilinia vaccinii-corymbosi*

by
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DEDICATION

For my amazing husband Tom, my family and friends who have supported me along the way.

BIOGRAPHY

Kathleen was born in 1985 in Johnson City, NY and was raised by Mark and Debra Pitcher in DeWitt, NY. Following graduation from the Jamesville-DeWitt Central School District in 2003, Kathleen enrolled at Onondaga Community College in Syracuse, NY. Her interest and enthusiasm for the sciences led to her pursuit of an Associate of Arts degree in Math/Science, which she conferred in 2005. She then completed her Bachelor of Science degree in Biotechnology in 2007 and her Master of Science degree in Environmental and Forest Biology in 2009 at the State University of New York College of Environmental Science and Forestry in Syracuse, NY. Kathleen moved to Raleigh, NC in August 2009 to pursue her Doctorate in Plant Pathology at North Carolina State University. She joined the lab of Dr. Marc Cubeta to conduct research on a fungal pathogen of blueberry, *Monilinia vaccinii-corymbosi*. In July 2010 Kathleen became happily married to Thomas Burchhardt.

Kathleen participated in several extracurricular activities while at NCSU. She was the President and Social Chair of the Plant Pathology Graduate Student Association and the Graduate Student Representative for the Plant Pathology Society of North Carolina. She completed the Certificate of Accomplishment in Teaching program at NCSU. She received several competitive awards including a USDA National Needs Research Fellowship, P.E.O. Scholar Award from the International Chapter of the P.E.O. Sisterhood, NCSU College of Agriculture and Life Sciences Outstanding Graduate Teaching Assistant Award, and travel awards from the Plant Pathology Society of North Carolina, Mycological Society of America, American Phytopathological Society, and Genetics Society of America. In the

summer of 2013 Kathleen participated in an internship at Bayer CropScience in Morrisville, NC. She was recently offered a position at Bayer CropScience as a plant pathologist starting after the completion of her graduate program at NCSU. In her spare time Kathleen loves to skate and is a retired skater and the current Charity Director for the Carolina Rollergirls, a local 501c3 nonprofit roller derby league. She also enjoys spending time with her family, eating at good restaurants, and having a glass of wine with friends.

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CHAPTER I

Microsatellite marker development for the plant pathogenic fungus *Monilinia vaccinii-corymbosi*

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Abstract

We developed 21 polymorphic microsatellite markers to examine the population genetics and biology of *Monilinia vaccinii-corymbosi* (Mvc), a haploid fungus that infects blueberry (*Vaccinium* spp.). Next-generation 454 pyrosequencing was performed with an isolate of Mvc to generate 135,661 sequence reads that were subsequently screened for microsatellite marker development with MSATCOMMANDER. Number of alleles at each locus ranged from two to 12 and mean haploid diversity ranged from zero to 0.379 (55 individuals; six populations). Evidence for significant linkage disequilibrium between markers at one

population and identical multilocus haplotypes of all isolates within two populations supports selfing and/or inbreeding.

Introduction

Mummy berry disease of wild and commercial blueberry (*Vaccinium* spp.) is caused by the haploid fungal pathogen *Monilinia vaccinii-corymbosi* (Mvc). The disease is characterized by fruit infection through the gynoecial pathway leading to fruit desiccation. The life cycle of Mvc involves both asexual and sexual reproductive cycles during a growing season (Batra 1983). Infected fruit (mummies) on the soil surface serve as an inoculum reservoir over the winter and germinate in the spring to produce sexual fruiting bodies (i.e. apothecia) that eject sexual spores to infect emerging blueberry leaves and shoots. Infected tissues become blighted and produce asexual spores that attract bees through pollen mimicry, resulting in fruit infection during pollination (Batra & Batra 1985; Ngugi & Scherm 2004). Our goal is to study intraspecific genetic diversity, population structure and gene flow among populations of Mvc throughout the United States by utilizing microsatellite (i.e. simple sequence repeat) markers. In this study we employed next-generation 454 pyrosequencing to identify 21 polymorphic microsatellite loci in the genome of Mvc.

Materials and Methods

Infected blueberry shoots producing asexual spores were collected from blueberry bushes at several locations within an agricultural field in Oregon (OR), Washington (WA), and

Michigan (MI) and three fields in North Carolina; Ivanhoe (NC-IV), Cullowhee (NC-CW), and West Jefferson (NC-WJ). Samples from OR, WA, and MI were collected from northern highbush (*Vaccinium corymbosum*), NC-IV from southern highbush (*V. darrowii* x *V. corymbosum*), and NC-CW and NC-WJ from rabbiteye (*V. virgatum*) varieties of blueberry. With the exception of isolates from WA and NC-CW, all isolates originated from a single spore per sample that was cultured on either half-strength potato dextrose agar (12 g Difco potato dextrose broth [PDB] and 15 g Difco agar per L [Becton, Dickinson and Company, MD]) or half-strength malt extract agar (16.8 g Difco malt extract broth and 15 g Difco agar per L [Becton, Dickinson and Company, MD]) plates. Due to low spore germination, each isolate from WA and NC-CW originated from a colony of germinated spores per sample. Isolates were transferred to half-strength PDB (12 g Difco PDB per L) and grown at 25°C for 2-3 weeks before being vacuum filtered and squeeze dried. Genomic DNA was extracted from approximately 15 mg of mycelium of an isolate (RL1) from the NC-IV population with the DNeasy Plant Mini Kit (QIAGEN, CA) according to the manufacturer's protocol. All other isolates were extracted according to the cetyltrimethylammonium bromide and chloroform DNA extraction method outlined by Kretzer *et al.* (2000) with the exception that samples were freeze-thawed only once and DNA was precipitated with isopropanol and washed with ethanol before resuspending in 100 µl PCR-grade water.

Approximately 500 ng of purified DNA from isolate RL1 was processed for 454 pyrosequencing on a Roche GS-FLX Titanium (Duke Genome Sequencing & Analysis Core Resource) and yielded 135,661 sequence reads with an average length of 355 bp. Sequence

reads were scanned for di-, tri-, tetra-, and pentanucleotide microsatellite repeats and primer sets were designed targeting 30 microsatellite loci with MSATCOMMANDER (Faircloth 2008; Rozen & Skaletsky 2000). A 'CAG' tail (5'-CAGTCGGGCGTCATCA-3') was added to the 5' end of one of the two primers per set for fluorescent tagging during PCR (Schuelke 2000). Primer sets were tested on 55 isolates; 5 each from OR, WA, MI, NC-CW, and NC-WJ, and 30 isolates from NC-IV. Polymerase chain reaction (PCR) was performed on the isolates with each primer set in 10 µl reaction volumes containing 1x PCR Master Mix (reaction buffer with 0.025 U/µl *Taq* DNA polymerase, 2 mM MgCl₂, and 0.2 mM of each dNTP; Fermentas Life Sciences), 0.05 µM 'CAG'-tailed primer, 0.2 µM untailed primer, 0.2 µM 6-FAM fluorescently-labeled 'CAG' primer ([6-FAM] 5'-CAGTCGGGCGTCATCA-3'), and 0.5 µl of 10⁻¹ diluted DNA extract. Touchdown PCR thermocycle conditions were: 95°C for 3 min, 20 cycles of 95°C for 30 sec, 60°C for 30 sec with a decrease in annealing temperature of 0.5°C each cycle, and 72°C for 30 s followed by 25 cycles of 95°C for 30 s, 50°C for 45 s, and 72°C for 45 s with a final extension of 45 min at 72°C. Fragment analysis was performed with GeneScan 500 LIZ size standard (Applied Biosystems by Life Technologies) on an Applied BioSystems 3730xl DNA Analyzer (Cornell University Life Sciences Core Laboratories Center) and allele size was determined by visually scoring peaks with Peak ScannerTM Software version 1.0 (Applied Biosystems by Life Technologies). Multilocus haplotypes, private alleles, and haploid diversity were determined with GenAlEx version 6.3 (Peakall & Smouse 2006). Arlequin version 3.5 was used to calculate linkage disequilibrium between loci for each population with 10000 Markov chain and

dememorization steps (Excoffier & Lischer 2010). The sequential Bonferroni and Benjamini-Hochberg methods were subsequently applied to correct for multiple comparisons and test for significant linkage disequilibrium between loci (Benjamini & Hochberg 1995; Rice 1989).

Results and Discussion

Of the 30 putative genetic markers designed and tested, we identified 21 polymorphic microsatellite markers (Table 1.1). The markers resolved 45 unique multilocus haplotypes from the 55 Mvc isolates analyzed (Table 1.2). All markers consistently amplified a single fragment, with no null alleles detected at any locus. No identical multilocus haplotypes were identified among the six locations sampled. All five isolates within the NC-CW and OR populations had identical multilocus haplotypes, suggesting self-fertilization, inbreeding, and/or a founder effect within these fields. The number of alleles detected at each locus ranged from two to 12 (mean= 5.33) and mean haploid diversity of the loci ranged from zero to 0.379. Mean haploid diversity was zero at loci Mvc2, 3, 4, 8, and 19, indicating that while diversity was detected among the populations there was no within-population diversity at these loci. Markers Mvc1, 10, 11, 12, 15, 18, 19, 23, 24, 25, 27, and 30 amplified alleles that were not a multiple of their corresponding microsatellite repeat motif. This could be due to imperfections in repeat number and/or indels in the flanking sequence. We designed the markers to avoid amplifying flanking sequence containing mononucleotide repeats as much as possible. However, the reference sequence of loci Mvc1, 10, 11, 12, 18, and 25 indicate

that mononucleotide repeats were amplified and may be responsible for uneven sized alleles. Alleles that were not a multiple of their corresponding microsatellite repeat motif were detected among the populations and not within populations for loci Mvc11, 19, 23, 24, and 30. At the $P < 0.05$ significance level, linkage disequilibrium was supported only within the NC-IV population and between 23 marker pairs; Mvc1-18, 1-22, 1-27, 1-30, 7-9, 7-18, 7-25, 9-23, 9-27, 10-30, 11-30, 12-23, 12-24, 14-30, 18-22, 18-27, 18-30, 22-30, 23-27, 23-28, 23-30, 27-28, and 27-30. After applying sequential Bonferroni correction to control the familywise error rate, only marker pair Mvc1-27 was significant (corrected $P < 0.05$). However, when we corrected with the Benjamini-Hochberg method for false discovery rate control, which is thought to be more statistically powerful (Benjamini & Hochberg 1995), marker pairs Mvc1-27, 12-23, 12-24, 14-30, 18-22, 18-27, and 18-30 were significant (corrected $P < 0.05$). Based on the life history of Mvc and detection of identical multilocus haplotypes, linkage disequilibrium may be due to inbreeding and possibly self-fertilization via homothallic sexual reproduction rather than linkage of the loci. The ability of Mvc to self-fertilize is not known but has been reported in the closely related genus *Sclerotinia* (Ekins *et al.* 2006; Holst-Jensen *et al.* 1997). A total of 76 private alleles were identified across all loci, suggesting high population differentiation and restricted gene flow (Slatkin 1985). The microsatellite markers developed in this study will be deployed in a large scale population genetics study of Mvc throughout blueberry growing regions of the United States. Microsatellite loci were submitted to GenBank (accession numbers JQ358947 to JQ358967).

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Tables

Table 1.1. Polymorphic microsatellite markers developed for *Monilinia vaccinii-corymbosi*. F= forward primer; R= reverse primer; ‘CAG’= 5'-CAGTCGGGCGTCATCA-3' tail added to beginning of primer sequence; Na= total number of alleles detected based on either the NC-IV population or all populations; h= haploid diversity; \bar{h} = mean haploid diversity; S.E.= standard error; Acc No.= GenBank Accession number

Locus	Repeat	Primer sequence (5'-3')	NC-IV			All Populations			Acc No.
			Na	Range (bp)	h	Na	Range (bp)	\bar{h} (S.E)	
Mvc1	(ATC) ₇	F: ‘CAG’CGGGTAAGGAAGGCTCTGG R: GCATGGTGACTTGGTGTCG	2	241-264	0.358	4	241-264	0.06 (0.06)	JQ358947
Mvc2	(ATC) ₈	F: ‘CAG’TACCGATGGGTCGTACAGC R: ACTTACCTGCCCTGCTCAC	1	174	0.0	3	153-180	0.0 (0.0)	JQ358948
Mvc3	(AGC) ₇ (AAC) ₅	F: CCTTTGCCACGTGTTGTCC R: ‘CAG’GGGCGGTTGAGATTCTGG	1	196	0.0	3	193-199	0.0 (0.0)	JQ358949
Mvc4	(GAT) ₆	F: GTTAGGCACTCTTCTTCGGG R: ‘CAG’GCCACAGGATGTCGAACAG	1	172	0.0	3	166-172	0.0 (0.0)	JQ358950
Mvc7	(AAG) ₇	F: ‘CAG’TCTTTATCGTGCGCAACCC R: TCGTACTGTATGTTACAACCTTGCC	3	377-383	0.287	6	359-386	0.128 (0.085)	JQ358951
Mvc8	(ATC) ₉	F: ‘CAG’TTTCGTACAGGACCCGTGG R: ACAGCTCTGGTGAAGGGTG	1	270	0.0	2	264-270	0.0 (0.0)	JQ358952
Mvc9	(GCT) ₈	F: ‘CAG’TTACCAGACCGTAGGGACG R: GCGAAAGTCTGTGCACCTG	3	286-292	0.524	4	280-292	0.167 (0.106)	JQ358953
Mvc10	(GAT) ₁₂	F: ‘CAG’GGCTCTCAGACGATCAATTCG R: CGGAGGCCAATTCCAAGAC	3	240-262	0.380	6	240-269	0.197 (0.09)	JQ358954
Mvc11	(AAC) ₈	F: ‘CAG’TCCATAGTACCTCAGGCAGAC R: CAGCCCTGCCAACGATTTTC	2	207-210	0.278	4	204-210	0.046 (0.046)	JQ358955
Mvc12	(ACA) ₅ (ACT) ₁₅	F: TGGCGCAACCATAACTATTTCAC R: ‘CAG’TGCGAGATTGAAGCTTTGGAG	4	261-293	0.676	10	256-296	0.379 (0.124)	JQ358956
Mvc14	(AGG) ₁₀	F: ‘CAG’GAGTAGACTGCGGTCAATGTTC R: ACGTGGGCCTATCACCAAC	3	168-183	0.287	5	168-183	0.048 (0.048)	JQ358957

Table 1.1 continued

Locus	Repeat	Primer sequence (5'-3')	NC-IV			All Populations			Acc No.
			Na	Range (bp)	h	Na	Range (bp)	h (S.E)	
Mvc15	(ATC) ₄ (ATG) ₇	F: 'CAG'CTACCTGTACCAGCAAGCAC R: AGGTTACAACGGGTCCCAC	1	165	0.0	4	159-168	0.080 (0.080)	JQ358958
Mvc18	(GGTA) ₆	F: 'CAG'GGTCTTTCCACGTATCCGC R: CAACCATGACAGCGCACC	4	245-248	0.638	7	245-254	0.240 (0.115)	JQ358959
Mvc19	(ACAT) ₅	F: TGCAAAGTTTCTCACTGGCG R: 'CAG'TAGCAACGCAGCGATTTGG	1	218	0.0	3	212-218	0.0 (0.0)	JQ358960
Mvc22	(TG) ₁₂	F: 'CAG'CAGTGGGACTTCTGAGCTG R: GACGACTCGCACAGTTAGT	3	127-131	0.438	5	121-135	0.073 (0.073)	JQ358961
Mvc23	(AGC) ₆ CAC (AAC) ₁₃	F: 'CAG'TAGATCCCCTTCTGCCAC R: CATGGTGAGACTGGAAACTG	8	183-219	0.816	12	175-219	0.349 (0.129)	JQ358962
Mvc24	(CAG) ₁₂	F: 'CAG'AGACTGCAATGGACATCAGC R: ATGAAGGGAAGCACCTGCA	2	165-168	0.320	5	162-206	0.053 (0.053)	JQ358963
Mvc25	(AAC) ₁₃	F: 'CAG'TTCTGCTCGGAACTTACGG R: GTGCTTGGGATTGGATGCT	5	180-203	0.244	7	174-203	0.041 (0.041)	JQ358964
Mvc27	(TGAA) ₁₃	F: 'CAG'AGAAGGCGTATCTCATGGTG R: ATCGTCTAGCTGTCCACTCA	3	123-149	0.631	5	123-149	0.159 (0.108)	JQ358965
Mvc28	(AGCC) ₈	F: CGACTTCGTACTIONTCGGACAT R: 'CAG'GAGCACCTGCATAACCTTAC	3	169-189	0.338	4	169-189	0.056 (0.056)	JQ358966
Mvc30	(AGCAG) ₁₂	F: 'CAG'GTTTCGAGGATCTTGGGTCT R: TGTACTCACTCCTTGGGCA	6	220-260	0.771	10	220-260	0.262 (0.131)	JQ358967

Table 1.2. Population statistics based on the 21 microsatellite markers. N= number of tested isolates; MLH= number of multilocus haplotypes resolved; Np= number of private alleles; h= mean haploid diversity of population; S.E.= standard error; No. LD= number of loci pairs in significant linkage disequilibrium (corrected $P < 0.05$) based on sequential Bonferroni (SB) and Benjamini-Hochberg (BH) correction methods; n/a= cannot be calculated based on a single MLH.

Population	N	MLH	Np	h (S.E)	No. LD	
					SB	BH
MI	5	5	3	0.084 (0.039)	0	0
OR	5	1	0	0.0 (0.0)	n/a	n/a
WA	5	5	3	0.130 (0.048)	0	0
NC-CW	5	1	18	0.0 (0.0)	n/a	n/a
NC-WJ	5	5	22	0.122 (0.044)	0	0
NC-IV	30	28	30	0.333 (0.059)	1	7

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CHAPTER II

Population dynamics of the blueberry pathogen *Monilinia vaccinii-corymbosi* in the United States

Abstract

The fungus *Monilinia vaccinii-corymbosi* (Mvc) causes disease of blueberry (*Vaccinium* spp.) shoots, flowers, and fruit. The objective of this study was to examine the population biology and genetics of Mvc in the United States. A total of 480 samples of Mvc were collected from 18 blueberry fields in 10 states; one field in GA, MA, ME, MI, MS, NJ, NY, OR, and WA and nine fields in NC. Analysis with 10 microsatellite markers revealed 247 unique multilocus haplotypes (MLHs), with 244 MLHs detected within 11 fields in the Northeast, Northwest, Midwest, and Southeast and three MLHs detected within seven fields in the Southeast US. Genetic similarity and low genetic diversity of Mvc isolates from the seven fields in the Southeast US suggested the presence of an expansive, self-fertile population. Tests for linkage disequilibrium within 10 fields that contained ≥ 12 MLHs supported random mating in six fields and possible inbreeding and/or self-fertilization in four fields. Analysis of molecular variance, discriminate analysis of principal components, and STRUCTURE provided evidence for population structure and restricted gene flow among fields. This research represents the first comprehensive investigation of the genetic diversity and structure of field populations of Mvc.

Introduction

Blueberry production has rapidly expanded in the United States from an approximately \$220 million market in 2003 to over \$781 million in 2012 (USDA NASS). Demand for blueberries has driven the desire for increased yield while minimizing production costs (Strik & Yarborough 2005). The plant pathogenic fungus *Monilinia vaccinii-corymbosi* (Reade) Honey (Mvc) causes mummy berry disease of several blueberry (*Vaccinium*) species throughout North America (Batra 1983, 1991) and has also been reported in Europe (Gosch 2003). The fungus causes shoot blight followed by ovary infection through the gynoecial pathway, leading to mummification of infected fruit (i.e. pseudosclerotia or “mummies”). The three primary marketed species of blueberry, *V. corymbosum* (northern highbush) and associated hybrids (e.g. *V. corymbosum* x *V. darrowii*, southern highbush), *V. virgatum* (rabbiteye), and *V. angustifolium* (lowbush), are susceptible to shoot and fruit infection by Mvc (Batra 1983, 1991). High disease incidence can lead to substantial fruit yield loss (Batra 1983; Hildebrand & Braun 1991; Penman & Annis 2005). While Mvc is an economically significant pathogen on blueberry, virtually nothing is known about the population biology and genetics of this fungus.

The life cycle of Mvc involves production of both asexual and sexual spores (conidia and ascospores, respectively) within a single growing season and aggressive mimicry to successfully infect the ovary of blueberry (Batra 1983; Ngugi & Scherm 2004). Pseudosclerotia from the previous growing season serve as overwintering structures and produce sexual structures (apothecia) and wind-disseminated ascospores that infect blueberry

shoots in early spring (Batra 1983; Cox & Scherm 2001). Infected tissues become blighted and conidia are produced after a latent period of approximately one to two weeks (Batra 1983). Pollinating insects are attracted to infected shoots through production of olfactory cues and also by mimicry as conidia reflect UV light at the same wavelength as blueberry calyxes (Batra & Batra 1985; Shinnars & Olson 1996). Pollinators deposit conidia on the stigma of blueberry flowers, with the conidia then displaying aggressive mimicry to infect the ovary through the gynoecial pathway (Ngugi & Scherm 2004, 2006). The length of time that newly opened blueberry flowers are receptive to conidia is short, with research suggesting a linear decrease in infection success over four days post anthesis (Ngugi *et al.* 2002). Hyphae of *Mvc* colonize locules of infected fruit and cause desiccation resulting in the formation of a hardened pseudosclerotium that detaches from the bush, with continuation of the life cycle the following spring.

An important component of the disease ecology and population biology of plant pathogenic fungi is spore dispersal capability, which contributes to gene flow among populations. Previous research has suggested that dispersal of ascospores and conidia of *Mvc* occurs over short distances. Cox and Scherm (2001) found that 95% of fruit infection occurred within 20 m of a conidial inoculum source, with increased prevalence of disease upwind suggesting the importance of bees as vectors since they are known to forage upwind. These researchers also found that 95% of ascospores were deposited within 30 m of their source. There was also a positive correlation between dispersal distance and wind speed indicating the role of wind in the dissemination of ascospores. However, a long tail in the

dispersal gradient of ascospores suggested that they are capable of travelling further distances. Dispersal of ascospores and/or conidia followed by successful colonization and reproduction on the host contributes to gene flow among fields. Analysis of genetic structuring of populations of Mvc can be used to deduce the extent of gene flow between and among blueberry fields.

Monilinia vaccinii-corymbosi has evolved to synchronize its developmental biology with that of its plant host(s) (Lehman & Oudemans 1997, 2000; Scherm *et al.* 2001). *Vaccinium* spp. require a preset number of hours below a threshold temperature (i.e. chill-hours) for flower bloom (Trehane 2004). *Vaccinium virgatum* cultivars require considerably fewer chill-hours than *V. corymbosum*, restricting their production mainly to the Southeast U.S. while *V. corymbosum* is commonly grown in the Northeast and Northwest U.S (Scherm *et al.* 2001; Trehane 2004). Interestingly, studies examining timing of apothecia formation from pseudosclerotia report that pseudosclerotia have chill-hour requirements that are comparable to their plant host, with pseudosclerotia formed on *V. virgatum* requiring substantially fewer chill-hours than pseudosclerotia formed on *V. corymbosum* to produce apothecia (Lehman & Oudemans 1997, 2000; Scherm *et al.* 2001). Significant variation in timing of apothecia production of pseudosclerotia formed on two cultivars of *V. corymbosum* displaying differences in timing of fruit development has also been reported (Lehman & Oudemans 1997, 2000). The mechanism(s) associated with timing of apothecia production on different *Vaccinium* spp. and cultivars with different developmental phenologies is

unknown, but research suggests that apothecia production may be at least partially under genetic control within populations of *Mvc* (Lehman & Oudemans 2000).

The objective of this study was to conduct a comprehensive investigation of intraspecific genetic diversity, population structuring, and reproductive biology of *Mvc* in blueberry fields throughout the United States. Given the highly specific nature of infection by *Mvc*, variation in developmental biology within populations of *Mvc*, and evidence for limited spore dispersal, it was hypothesized that populations of *Mvc* are genetically structured. Highly polymorphic microsatellite markers that were previously developed for *Mvc* were used to address the objectives (Molecular Ecology Resources Primer Development Consortium *et al.* 2012). This study is the first comprehensive study to report high intraspecific genetic diversity and structuring of populations of *Mvc*.

Materials and methods

Sample collection, isolation of Mvc, and DNA extraction

Samples of infected fruit and/or shoots were collected from 18 blueberry fields in 10 states; one field each in GA, MA, ME, MI, MS, NJ, NY, OR, WA, and nine fields in NC (Fig. 2.1). Blueberry fields were selected with a known history of mummy berry disease. Field sites were selected to capture the geographic range of blueberry production and the three primary blueberry species grown in the continental US; *V. corymbosum* (and *V. corymbosum* x *V. darrowii* hybrids), *V. virgatum*, and *V. angustifolium*. Sampling was conducted between February and August from 2010 to 2012. Depending on the size of the blueberry field and

presence of disease within the field, sampling was conducted either over the entire field or within a region with high incidence of disease. The sampling protocol at each field was designed to systematically collect samples from several rows of planted blueberry bushes and several bushes within rows. Due to differences in size of fields, the number of sampled rows, bushes sampled within each row, and interval between sampled bushes at each field location was modified to best cover the geographic extent of the selected area. Typically five rows were selected for sampling, and within each row at least one infected fruit or shoot would be sampled from a bush near the beginning of the row. Bushes were subsequently systematically sampled in the row at an interval of at least two bushes (approximately 3 m) between sampled bushes. Samples from ME were collected from a wild, unmanaged population of *V. angustifolium* along three transects, with samples collected from bushes spaced 15 m apart along each transect. Samples were stored in Ziploc bags, placed on ice in the field and stored at 4°C until they were processed.

To isolate Mvc from infected shoots, tissues with conidia were lightly tapped over a 60 mm Petri dish containing half-strength potato dextrose agar (PDA) amended to contain 2% agar (12 g Difco potato dextrose broth [PDB] and 20 g Difco agar per L [Becton, Dickinson and Company, MD]) to disperse conidia on the surface of the medium. After incubation for at least 12 h at room temperature (approximately 20°C), an individual germinating conidium was transferred with a scalpel under a dissecting microscope to either half-strength PDA containing 1.5 % agar (15 g Difco agar per L) or half-strength malt extract agar (16.8 g Difco malt extract broth and 15 g Difco agar per L [Becton, Dickinson and

Company, MD]). If a single conidium isolate was not generated due to low germination rates or survival of hyphae, a colony of germinated conidia was transferred to obtain a culture. To isolate Mvc from infected fruit, the outside surface of an individual fruit was disinfested for 30 sec in 10% bleach (NaOCl) followed by 30 sec in 70% ethanol and then rinsed in sterilized water for 30 sec. Infected fruit were cut in half with a sterile scalpel and a piece of fungal mycelium was removed from a single locule and transferred to half-strength PDA containing 1.5% agar.

All isolates were grown at room temperature for approximately 2 weeks before being used to inoculate 250 ml Erlenmeyer flasks containing 50 ml of half-strength PDB (12 g Difco PDB per L). After approximately 2 weeks, mycelium of each isolate was vacuum filtered and squeeze dried. DNA was extracted from approximately 15 mg of mycelium according to the cetyltrimethylammonium bromide (CTAB) and chloroform DNA extraction method outlined by Kretzer *et al.* (2000), with modifications and DNA precipitation as recommended by the authors (10). Due to the inability to obtain a pure culture isolate from several samples in GA, MS, ME, and NJ, DNA was extracted directly from a small piece of tissue from a shoot with conidia or mycelium removed from inside a single infected fruit as described above. The DNA extracts of all samples were diluted either 1:2, 1:5 or 1:10 with PCR grade water and stored at -20°C.

PCR and fragment analysis

PCR was performed on DNA extracts using 10 microsatellite markers (Mvc7, 9, 10, 12, 15, 23, 25, 27, 28, and 30) developed by the authors (Molecular Ecology Resources

Primer Development Consortium *et al.* 2012). The microsatellite markers were chosen based on their ability to distinguish genetically different isolates and high levels of polymorphism. Primer sets for two or three markers were combined (i.e. multiplexed) in PCR reactions, with Mvc7, 9, and 15 (Mvc7-9-15) and Mvc10, 25, and 27 (Mvc10-25-27) amplified in triplexed PCR reactions, and Mvc12 and 23 (Mvc12-23) and Mvc28 and 30 (Mvc28-30) in duplexed reactions. All PCR reactions were setup in 0.3 ml 96-well PCR plates in 10 μ l reaction volumes. PCR products were fluorescently labeled by adding a 'CAG' sequence to the 5' end of one of the primers in each marker set and including an additional 'CAG' primer with either a NED or 6-FAM fluorescent tag attached to the 5' end to the reaction (10). Multiplexed PCR reactions contained 1x PCR Master Mix (reaction buffer with 0.025 U/ μ l *Taq* DNA polymerase, 2 mM MgCl₂, and 0.2 mM of each dNTP; Thermo Scientific), 0.05 μ M of each 'CAG'-tailed primer, 0.2 μ M of each non-tailed primer, either 0.6 μ M of 6-FAM-labeled 'CAG' primer (triplexed reactions) or 0.4 μ M of NED-labeled 'CAG' primer (duplexed reactions) and 1.0 μ l of diluted DNA extract. Touchdown thermocycle PCR reaction conditions and fragment analysis were performed as previously described (Molecular Ecology Resources Primer Development Consortium *et al.* 2012) except that PCR products from reactions Mvc7-9-15 were combined with Mvc12-23 as well as Mvc10-25-27 combined with Mvc28-30 before submission for fragment analysis (Cornell Biotechnology Resource Center).

Descriptive statistics, genetic diversity, and linkage disequilibrium

The length of the allele present at the 10 microsatellite loci was used to determine the multilocus haplotype (MLH) of each sample. The software GenAlEx version 6.5 was used to determine the number of alleles at each locus, MLHs, private alleles (i.e. alleles only detected within a single field), and calculate unbiased haploid diversity within each field (Peakall & Smouse 2006, 2012). The presence of private alleles with a frequency above 0.05 was considered since frequency of private alleles may indicate level of gene flow among fields (Slatkin 1985). GenAlEx was also used to generate a clone corrected dataset by detecting isolates with repeated/identical MLHs within each field and retaining one representative sample per MLH in the dataset. Several subsequent analyses were performed on samples collected from 10 fields containing ≥ 12 MLHs (NC-IVH, NC-IVT, NC-CH, NC-WJ, MI, MA, ME, NJ, NY, and WA) using a clone corrected dataset since several subsequent genetic analyses can be sensitive to low sample size and departure from random mating.

The online implement of Genepop version 4.2 was used to test for significant linkage disequilibrium between markers within the 10 fields containing ≥ 12 MLHs both with and without clone corrected data, using 10,000 dememorizations, 1,000 batches, and 10,000 iterations per batch (Raymond & Rousset 1995; Rousset 2008). Significant linkage disequilibrium between markers indicates that the alleles present at the loci are not randomly associated and therefore may be an indicator of nonrandom mating. The Benjamini-Hochberg method (Benjamini & Hochberg 1995) was implemented to correct the P values for multiple comparisons and test for significant linkage disequilibrium (adjusted $P < 0.05$).

Analyses for population structure

Analysis of molecular variance (AMOVA) was performed with GenAlEx using a clone corrected dataset and the 10 fields containing ≥ 12 MLHs to examine hierarchical partitioning of genetic variation among the fields (Peakall & Smouse 2006, 2012). Results were based on 10,000 data permutations and 10,000 pairwise population permutations. Linearized Φ_{PT} values calculated by AMOVA were plotted against linearized geographic distances between fields to test for isolation by distance using a Mantel test in GenAlEx with 10,000 permutations (Peakall & Smouse 2006, 2012). A Mantel test was performed using results from the 10 fields and separately without field WA since the field was a geographical outlier.

The Bayesian analysis program STRUCTURE version 2.3.4 (Pritchard *et al.* 2000) was used to examine the K number of genetic clusters (i.e. populations) supported using the clone corrected dataset consisting of the 10 fields containing ≥ 12 MLHs. The analysis probabilistically assigns individuals into genetic clusters based on allele frequencies (Pritchard *et al.* 2000). STRUCTURE was run using the admixture model that allows for mixed ancestry with location priors, allele frequencies independent, a burn-in of 20,000, Markov chain Monte Carlo (MCMC) run 500,000 times, and 20 run repeats for each value of K from 1 to 12. The Evanno ΔK method as performed with STRUCTURE HARVESTER web version 0.6.93 (Earl & Vonholdt 2012) was implemented to evaluate K based on the rate of change in the log probability over the 20 successive runs at each value of K (Evanno *et al.* 2005). The K value at which the log likelihood approached stationary and with the highest

log likelihood were also considered when evaluating K (Pritchard *et al.* 2000). Results from STRUCTURE were visualized by first permuting the estimated cluster membership coefficient matrices with CLUMPP version 1.1.2 (Jakobsson & Rosenberg 2007) and running the output in Distruct version 1.1 (Rosenberg 2004). Mean membership probabilities as determined by CLUMPP were also used to examine population assignment. Samples were considered a member of a particular cluster if their membership probability was $Q > 0.70$.

Discriminate analysis of principal components (DAPC), a multivariate analysis, was performed in the R package adegenet version 1.3-6 (Jombart 2008; Jombart *et al.* 2010). Since DAPC does not have evolutionary assumptions built into the analysis, the clone corrected dataset for the 18 fields was used for this analysis. The K-means method was used to determine the K number of clusters of genetically related MLHs in the dataset based on the K value that maximized the variation between clusters (Jombart *et al.* 2010). Ten separate runs of K-means were performed and the mean Bayesian information criterion (BIC) value at each value of K from 1 to 15 was plotted. The K value where the BIC value either decreased by a negligible amount or increased was used to infer the K number of clusters in the dataset (Jombart *et al.* 2010). DAPC was then used to describe clusters of genetically related samples and determine membership probabilities based on the proximity of samples to the different clusters.

Results

Descriptive statistics, genetic diversity, and linkage disequilibrium

Based on analysis of 480 samples with 10 microsatellite markers, 247 unique MLHs were detected (Table 2.1). All markers were polymorphic and number of alleles at each locus ranged from six to 17. Haplotypic diversity was low in fields MS, GA, NC-CW, NC-FR, NC-SM, NC-GR, and NC-WL (see Table 2.1 for abbreviated naming of fields), with a maximum of two MLHs detected within each field and a total of three MLHs detected among the seven fields (herein referred to as MLH1, 2, and 3). MLH1 was detected within fields NC-CW, NC-GR, NC-WL, GA, and MS, MLH2 within NC-CW, NC-FR, NC-SM, and GA, and MLH3 exclusively within NC-FR. Haploid diversity within these seven fields ranged from 0 to 0.054. Genetic diversity was also low in field OR, with only four MLHs detected and a haploid diversity of 0.033. In contrast, each of the other 10 fields (NC-IVH, NC-IVT, NC-CH, NC-WJ, MA, ME, NJ NY, MI, and WA) had higher genetic diversity, with ≥ 12 MLHs detected per site, haploid diversity ranging from 0.205 to 0.582, and each MLH being unique to its field of origin except for two MLHs in WA that were also detected in OR. Private alleles were detected from samples within fields NC-IVH, NC-CH, NC-IVT, NC-WJ, MA, ME, NJ, and NY, with each of the fields containing at least one private allele with a frequency above 0.05. When considering the seven fields in the Southeast with three total MLHs as a single population, four private alleles were detected in the population with frequencies above 0.05. Analysis of linkage disequilibrium within the 10 fields with >12 MLHs using a non-clone corrected dataset supported that one marker pair was in significant

linkage disequilibrium within NC-CH (Mvc23-25), WA (Mvc9-12), and NC-IVH (Mvc9-23), and 14 marker pairs within NY (Mvc10-12, 10-23, 12-23, 7-25, 9-25, 10-25, 12-25, 23-25, 23-28, 9-30, 10-30, 12-30, 23-30, and 25-30) at a Benjamini-Hochberg adjusted $P < 0.05$. However, all marker pairs were in linkage equilibrium within the 10 fields when analyzing a clone corrected dataset (adjusted $P < 0.05$).

Population structure and lack of isolation by distance

Based on AMOVA using a clone corrected dataset consisting of the 10 fields containing ≥ 12 MLHs (NC-IVH, NC-IVT, NC-CH, NC-WJ, MA, ME, NJ NY, MI, and WA), 57% of variance was detected within fields, while 43% was detected among fields. All pairwise field comparisons were statistically significant ($P < 0.0001$) for genetic differentiation, with Φ_{PT} ranging from 0.167 to 0.73 (Table 2.2). The Mantel tests for isolation by distance were not significant when analyzing the 10 fields ($R^2 = 0.0424$, $P = 0.168$) and when field WA was excluded from the analysis ($R^2 = 0.0706$, $P = 0.103$), indicating that geographic distance between fields was not significantly correlated with genetic distance (Fig. 2.2).

Population assignment based on analysis with STRUCTURE varied among the 20 runs at each value of K. When examining runs separately at a given value of K, all samples collected from the same field were typically placed in the same cluster. However, their grouping into the same cluster as samples from another field sometimes varied from run to run. Also, little to no admixture was detected within fields. The Evanno ΔK method, a widely applied method to determine the number of populations in a given dataset, supported $K = 3$

populations (Fig. 2.3A). However, this method was not able to accurately determine K , as two populations that were strongly genetically differentiated based on AMOVA and DAPC (see below), NC-WJ and ME, were placed in the same population. Thus, K was further explored by first plotting the mean $\ln P(K)$ at each value of $K=1-12$ (Fig. 2.3B). The $\ln P(K)$ reached approximately stationary at $K=6$, with the highest mean $\ln P(K)$ at $K=9$. Similar to the approach of Rosenberg *et al.* (2002), population assignment histograms and membership probabilities at each value of K from 3 to 12 were then examined for patterns in population structuring (Fig. 2.4). Due to lack of convergence in population assignment over multiple runs at each value of K , CLUMPP was used to determine the mean membership probabilities (Q) of each individual at each value of K . Samples from fields NC-IVH, NC-CH, MA, and ME were consistently placed into separate populations at $K=6-12$. Samples from fields NC-IVH and NC-CH were placed in separate populations at $Q>0.90$ and samples from MA and ME at $Q>0.80$. There was support that samples from several other fields belonged to separate populations: samples from field NY at $Q>0.75$ for $K=6-12$, NC-WJ at $Q>0.70$ for $K=9-12$, and NC-IVT at $Q>0.80$ for $K=8-12$. Samples from fields NJ and WA were grouped together at $Q>0.70$ for $K=10-12$, with samples having a $Q>0.70$ at $K=8-12$ for WA and $K=10-12$ for NJ. Samples from MI could not be assigned to a population at $Q>0.70$, but were placed in a separate population at $Q>0.60$ at $K=11$ and 12. Overall, results from STRUCTURE support $K=8$ or 9, with samples from fields NC-IVH, NC-CH, NC-IVT, MA, NY, NC-WJ, and ME and from both NJ and WA belonging to separate populations, and limited support for samples from MI belonging to another population.

Discriminate analysis of principal components supported $K=10$ clusters based on analyzing samples from the 18 fields and plotting the mean BIC values against K (Fig. 2.5). Clustering was visualized by producing scatterplots of discriminate functions (Fig. 2.6) and a histogram of membership probabilities of the samples to the 10 clusters (Fig. 2.7). Herein clusters 1-10 are referred to based on their numbering in Fig. 2.7. Samples collected from field ME and seven fields in the Southeast US (MS, GA, NC-CW, NC-FR, NC-SM, NC-GR, and NC-WL) were assigned to cluster 6 and were genetically distant from all other clusters. The samples from field NC-WJ were assigned to cluster 8 and also clustered distantly from samples from all other fields. The other eight clusters were more closely grouped, indicating that they were more genetically related to each other than to clusters 6 and 8. Two clusters were detected within field NC-IVH, with all but two samples from NC-IVH being placed into either cluster 1 or 2. The majority of samples from a field were assigned to the same cluster: all but two samples from MA were placed in cluster 3, all but one sample in NY in cluster 4, all samples from MI in cluster 5, all from NC-IVT in cluster 7, and all but one sample from NC-CH in cluster 9. Cluster 10 was comprised of all but four samples from NJ and all samples from OR and WA. These results are similar to those from STRUCTURE with the exceptions that MI was considered a separate population and two populations were detected within field NC-IVH with DAPC.

Discussion

While sexual reproduction is required for *Mvc* to complete its life cycle, little information was previously known about the occurrence of sexual recombination within populations of *Mvc*. The presence of numerous MLHs and high haploid diversity within 10 of the sampled fields (NC-IVH, NC-CH, NC-IVT, NC-WJ, MA, ME, MI, NJ, NY, and WA) suggested the presence of sexual recombination within these fields. Random mating was supported within six of the fields (NC-IVT, NC-WJ, MA, MI, ME, and NJ) by linkage equilibrium of microsatellite loci. The possibility of inbreeding and/or self-fertility were suggested in fields NC-CH, NC-IVH, NY, and WA based on the presence of at least one pair of microsatellite markers in linkage disequilibrium when analyzing a non-clone corrected dataset. The presence of several samples with identical MLHs within several fields also provided evidence for self-fertility and/or inbreeding. In particular, 14 loci pairs were in linkage disequilibrium in field NY, and from the 28 samples analyzed from this field only 13 unique MLHs were detected. Linkage disequilibrium was likely not due to physical linkage of the loci during recombination since marker pairs were not consistently in linkage disequilibrium across several fields.

In contrast, very low to no haploid diversity and the presence of only three unique MLHs from 141 samples analyzed from seven fields in the southeastern US (GA, MS, NC-CW, NC-FR, NC-SM, NC-GR, and NC-WL) and only 4 MLHs of 29 analyzed samples from OR suggested inbreeding and/or self-fertility within these fields. Samples from the seven fields in the southeastern US were genetically very similar or identical to each other,

suggesting a large population throughout the sampled geographic region. Several mating systems have been described in fungi, with homothallism (i.e. the ability to self-fertilize) and heterothallism (i.e. obligate outcrossing with a sexually compatible individual) being the predominant mating systems (Billiard *et al.* 2012; Heitman *et al.* 2007). While the mating system of *Mvc* remains unknown, evidence for self-fertility suggests homothallic mating is occurring within the seven fields. However, this does not preclude the presence of heterothallic or another mating system in other populations of *Mvc*. The closely related genus *Sclerotinia* contains species that are homothallic, heterothallic, or display a mixed mating system (Amselem *et al.* 2011; Chitrampalam *et al.* 2013; Ekins *et al.* 2006; Holst-Jensen *et al.* 1997). Another closely related fungus, *Botrytis cinerea*, has a heterothallic mating system based on genome sequencing (Amselem *et al.* 2011; Holst-Jensen *et al.* 1997). Within the genus *Monilinia*, outcrossing and heterothallic mating were supported in *M. fructicola* based on the examination of single ascospore progeny from apothecia (Free *et al.* 1996). High genetic diversity and evidence for sexual recombination and linkage equilibrium in several fields suggests that heterothallic mating may exist in populations of *Mvc*. Furthermore, more than one MLH may be present within a single infected fruit and an apothecium can produce sexually recombinant ascospores (Burchhardt, unpublished, see CHAPTER IV). Overall, the results suggest that populations of *Mvc* may differ in their reproductive biology, but further research is needed to determine the mating system within populations of *Mvc*.

Variability in the reproductive biology and population structure of *Mvc* was detected based on geographic region and possibly host species. Samples collected from seven fields in

the southeastern US were genetically identical or very similar to each other but were highly genetically differentiated from those from other fields except those from ME. The clustering of samples from the seven southeastern fields with those from ME based on DAPC was surprising considering the geographic distance between the fields and differences in their host. Samples from ME were collected from wild lowbush blueberry (*V. angustifolium*), while samples from the seven southeastern fields were collected from rabbiteye blueberry (*V. virgatum*). The range of these two host species only overlaps in North Carolina (USDA, NRCS), and analysis of genetic relatedness of cultivated blueberries supported that cultivars of *V. virgatum* are genetically divergent from *V. angustifolium* as well as northern highbush blueberry (*V. corymbosum*) (Bian 2012). However, it is important to note that while the samples from ME genetically clustered with those from the seven southeastern fields their reproductive biology was different, with high genetic diversity and linkage equilibrium within ME supporting sexual recombination and low genetic diversity in the seven southeast fields supporting self-fertilization. There are several plausible explanations for the grouping of samples from ME with those from the seven sites in the southeast. It is possible that Mvc underwent a geographic and host range expansion that may have been coupled with a shift in its reproductive biology. Another possibility is that Mvc was recently introduced from *V. angustifolium* onto *V. virgatum* in the southeastern US and that low genetic diversity is due to a founder effect. However, this explanation seems unlikely considering the geographic expansiveness of the population in the Southeast. Finally, another explanation is that DAPC was not able to detect underlying population structuring between the seven southeastern

fields and ME. The presence of private alleles in a high frequency within both ME and the Southeast population supports this hypothesis, as the frequency of private alleles may be an indicator of level of gene flow (Slatkin 1985).

Low genetic diversity and strong genetic similarity of Mvc isolates from seven fields in the southeastern US and genetic divergence of these isolates from northern Mvc populations may be explained by several scenarios. One scenario is that a founder effect is responsible for low genetic diversity due to a recent introduction and spread of Mvc throughout the Southeast. This scenario is unlikely based on the large geographic extent of the Southeast population. Considering the strong genetic divergence of the Southeast population from other Mvc populations, another possibility is that cryptic species exist within Mvc that are reproductively isolated, thus preventing genetic exchange. Mating experiments involving sexual crosses are needed along with additional genetic analyses to test for reproductive isolation of genetically divergent isolates of Mvc. It is also possible that host specificity is driving genetic divergence and/or cryptic speciation. Cryptic speciation was detected in the fungus *Botrytis cinerea* based on genetic differentiation of populations on *Vitis vinifera* and *Rubus fruticosus* grown in sympatry and inability to mate (Fournier & Giraud 2008; Walker *et al.* 2011). Interestingly, all isolates of Mvc from the seven fields in the Southeast were collected from infected shoots or fruit of *V. virgatum*. Further support for this scenario is that Mvc samples collected from *V. virgatum* fruit in fields NC-GR and NC-WL were located within approximately 25 km of fields NC-IVH and NC-IVT, where samples were collected from shoots of a *V. corymbosum* x *V. darrowii* hybrid and shoots of

V. virgatum, respectively. A single MLH was detected within fields NC-GR and NC-WL that was also detected in fields MS, GA, and NC-CW and was genetically divergent from Mvc samples from fields NC-IVH and NC-IVT, which contained high genetic diversity and haplotypes that were more genetically similar to Mvc isolates from northern fields. It is possible that the Mvc isolates on *V. virgatum* in the southeast specifically infect the shoots and fruit of *V. virgatum*, while Mvc isolates on *V. corymbosum* and *V. corymbosum* x *V. darrowii* can infect the shoots and fruit of their host of origin as well as the shoots of *V. virgatum*, but not the fruit of *V. virgatum*. Research suggests that cultivars of *V. virgatum* tend to be susceptible to shoot infection (Ehlenfeldt & Stretch 2000), with a history of severe shoot infection in southeastern North Carolina (Milholland 1977). Little to no fruit infection was reported on *V. virgatum* in Southeast North Carolina until 2012 when samples were collected from fields NC-GR and NC-WL (Burchhardt, Cline and Cubeta, unpublished). However, genetic divergence of Mvc isolates originating from different hosts grown in close proximity can be due to other factors than reproductive barriers, such as temporal isolation. Variation in timing of bud break and flower bloom between *V. corymbosum* and *V. virgatum* and timing of apothecia production from pseudosclerotia produced on hosts with different developmental phenologies is known to exist (Lehman & Oudemans 1997, 2000; Scherm *et al.* 2001; Trehane 2004). If asexual and/or sexual spores of Mvc are produced at a time when one plant host species is susceptible while another is not, this could potentially drive temporal isolation and genetic divergence of Mvc populations. In the insect vectored anther smut fungus *Microbotryum violaceum*, genetic differentiation of populations on the plant

hosts *Silene dioica* and *S. latifolia* grown in sympatry was not due to reproductive isolation, but rather other factors such as host phenology and insect vector behavior (Gladieux *et al.* 2011; van Putten *et al.* 2007). Inoculation experiments would be necessary to test the hypothesis of host specificity of isolates of Mvc during fruit infection and also to determine if reproductive isolation and cryptic speciation exists within Mvc.

The lack of isolation by distance found in this study suggests that geographic distance between fields does not necessarily indicate the level of gene flow among fields. Since research suggests that Mvc cannot survive in its host systemically (Batra 1983), methods of gene flow could be through aerial dispersal of ascospores, movement of conidia by insects and other potential vectors, transportation of infected plants, or movement of pseudosclerotia. The *V. corymbosum* breeding program was started in approximately 1908 when plants were selected from the pine barrens in New Jersey for development of cultivated varieties (Trehane 2004). The natural range of *V. corymbosum* covers eastern North America, with cultivars of *V. corymbosum* introduced into the Pacific Northwest (USDA GRIN). Interestingly, DAPC and STRUCTURE supported that isolates of Mvc collected from fields in OR and WA clustered with those from NJ. Lower levels of genetic diversity in OR compared to WA may be the result of a founder effect due to a recent introduction of Mvc into OR. However, further sampling and genetic analyses are needed to examine migration rates into and throughout the Pacific Northwest.

Multiple analytical approaches were applied to examine the presence of population structure of Mvc. Multivariate analysis with DAPC supported further population structure

than Bayesian analysis with STRUCTURE. Research suggests that DAPC is more effective than STRUCTURE at detecting hierarchical structuring (Jombart *et al.* 2010). Another potential benefit of DAPC is that it does not have evolutionary assumptions such as random mating built into the analysis as with STRUCTURE (Jombart *et al.* 2010). Complex hierarchical structuring and possible violations in the assumptions of the model implemented with STRUCTURE may have caused variation in results from multiple runs at a given value of K. While the Evanno method is commonly used in population genetic publications to determine K from STRUCTURE output, the data shows the importance of considering numerous methods and analytical approaches to determine K. The Evanno method supported K=3 and grouped NC-WJ and ME, two fields that were genetically distinct based on DAPC and AMOVA. In this study, further population structuring was supported by considering the $\ln P(K)$ and membership probabilities of the samples to populations. Using this approach, results from DAPC and STRUCTURE were similar, with K=10 supported with DAPC and K=8 or 9 supported for STRUCTURE. A difference between results obtained from DAPC and STRUCTURE was that DAPC placed isolates of Mvc from field MI in a distinct cluster while population assignment of MI was inconclusive with STRUCTURE. Another difference was that two genetically distinct clusters were detected within field NC-IVH with DAPC. Multiple introductions of Mvc into field NC-IVH may explain this observation.

A previous study by Wasilwa *et al.* (2003) used amplified fragment length polymorphism (AFLP) markers and vegetative compatibility tests to examine genetic structuring of 191 isolates of Mvc from several locations in North America. They concluded

that intraspecific genetic diversity of Mvc is limited in North America. In contrast, this study found that polymorphic microsatellite markers were able to detect genetic diversity and structuring of populations of Mvc. The possibility of host specificity needs to be examined further and is of practical significance for plant breeders interested in developing blueberry cultivars with resistance to Mvc. Current breeding programs focus primarily on screening cultivars of *Vaccinium* species for their level of susceptibility to shoot and fruit infection (Ehlenfeldt *et al.* 2010a, b; Ehlenfeldt & Stretch 2000, 2001; Stretch & Ehlenfeldt 2000; Stretch *et al.* 2001). However, the majority of these studies have been conducted in a single location using local sources of inoculum. For results to be reliable in several geographic regions and on different host species it is important to screen plants with isolates of Mvc that broadly represent the phenotypic and genotypic diversity of pathogen populations (Chakraborty *et al.* 2006; Milgroom & Peever 2003; Zhan *et al.* 2008). The extent to which genetic divergence of Mvc populations reflects phenotypic variability in terms of aggressiveness, developmental and reproductive biology of Mvc remains to be determined. The results from this study provide a foundation of knowledge for future studies on the disease ecology and population biology of Mvc.

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Tables and Figures

Table 2.1. Locality and sampling information for the 18 fields where samples of *Monilinia vaccinii-corymbosi* were collected. Location of the 18 fields, year samples were collected, blueberry host cultivar, DNA source of the samples, number of samples (N), multilocus haplotypes (MLH), and private alleles (Np), and unbiased haploid diversity (h) with standard error (S.E.).

Field ID	Location	Year	Cultivar(s) ^a	DNA source ^b	N	MLH	Np ^c	h (S.E.)
NC-IVH	Ivanhoe, NC	2012	Blue Ridge (SHB)	SC	70	65	3 (2)	0.482 (0.074)
NC-IVT	Ivanhoe, NC	2010	Tifblue (RE)	SC	13	12	2 (2)	0.418 (0.086)
NC-CH	Castle Hayne, NC	2012	Abundance, Emerald, Jewel (SHB); Powderblue, Premier, Tifblue (RE)	SC	45	35	3 (1)	0.418 (0.08)
NC-WJ	West Jefferson, NC	2010	Tifblue (RE)	SC	29	17	9 (9)	0.227 (0.077)
NC-CW	Cullowhee, NC	2010	Tifblue (RE)	C	24	2	0	0.008 (0.008)
NC-FR	Franklin, NC	2011	Powderblue, Climax, Tifblue (RE)	SC, C	24	2	0	0.029 (0.029)
NC-GR	Garland, NC	2012	Brightwell (RE)	M	23	1	0	0.0 (0.0)
NC-SM	Scaly Mt., NC	2011	Brightwell, Climax, Premier, Powderblue, Tifblue (RE)	SC, C	23	1	0	0.0 (0.0)
NC-WL	White Lake, NC	2012	Brightwell (RE)	M	15	1	0	0.0 (0.0)
GA	Baxley, GA	2011	Climax, Brightwell (RE)	EC, M	13	2	0	0.054 (0.054)
MS	Perkinston, MS	2011	Tifblue (RE)	C, EC	19	1	0	0.0 (0.0)
MI	West Olive, MI	2010	Bluecrop (NHB)	SC	23	13	0	0.209 (0.08)

Table 2.1. Continued

Field ID	Location	Year	Cultivar(s) ^a	DNA source ^b	N	MLH	Np ^c	<i>h</i> (S.E.)
MA	Franklin, MA	2011	Bluecrop (NHB)	M	33	33	9 (6)	0.582 (0.102)
NJ	Chatsworth, NJ	2011	Berkeley (NHB)	M, EM	19	18	1 (1)	0.407 (0.096)
NY	Stephentown, NY	2011	Blueray, Bluecrop, Nelson, Bluegold, Spartan (NHB)	M	28	13	2 (2)	0.341 (0.064)
OR	Corvallis, OR	2010	Bluetta (NHB)	SC	29	4	0	0.033 (0.022)
WA	Burlington, WA	2010	Reka (NHB)	C	27	13	0	0.205 (0.07)
ME	Columbia, ME	2012	Lowbush clones	M, EM	23	22	10 (7)	0.395 (0.103)

^a Abbreviations for species of the cultivar(s): SHB= *Vaccinium corymbosum* x *V. darrowii* (southern highbush), NHB= *V. corymbosum* (northern highbush), RE= *V. virgatum* (rabbiteye).

^b Abbreviations for source of the DNA of the samples: SC= single conidium isolate, C= isolate derived from several conidia, EC= DNA extracted directly from conidia, M= isolate cultured from mycelium inside a pseudosclerotium, EM= DNA extracted directly from mycelium inside a pseudosclerotium.

^c Number in parentheses represents the number of private alleles with a frequency >0.05 in the field.

Table 2.2. Pairwise Φ_{PT} values as calculated with analysis of molecular variance (below diagonal) and geographic distance in km (above diagonal). Results are based on a clone-corrected dataset using the 10 fields containing >12 MLHs. All Φ_{PT} values were significant at $P < 0.0001$.

NC-CH	NC-IVT	MA	MI	NJ	NY	WA	NC-WJ	ME	NC-IVH	
-	44.417	1024.0	1190.1	664.0	989.3	3965.4	372.3	1423.1	54.2	NC-CH
0.353	-	1020.0	1147.8	656.0	978.0	3920.9	328.0	1421.9	11.0	NC-IVT
0.300	0.238	-	1211.9	369.9	170.2	3978.3	1068.2	407.5	1025.1	MA
0.515	0.474	0.335	-	1033.6	1044.5	2855.5	851.2	1505.5	1140.6	MI
0.359	0.381	0.167	0.449	-	329.3	3879.1	706.5	777.2	660.3	NJ
0.372	0.410	0.281	0.464	0.330	-	3810.4	975.5	54.7	981.4	NY
0.427	0.483	0.276	0.520	0.248	0.426	-	3593.4	4139.1	3911.4	WA
0.618	0.658	0.541	0.713	0.647	0.660	0.730	-	1473.8	318.2	NC-WJ
0.535	0.546	0.442	0.628	0.545	0.533	0.610	0.613	-	1427.6	ME
0.284	0.293	0.222	0.400	0.324	0.327	0.388	0.554	0.486	-	NC-IVH

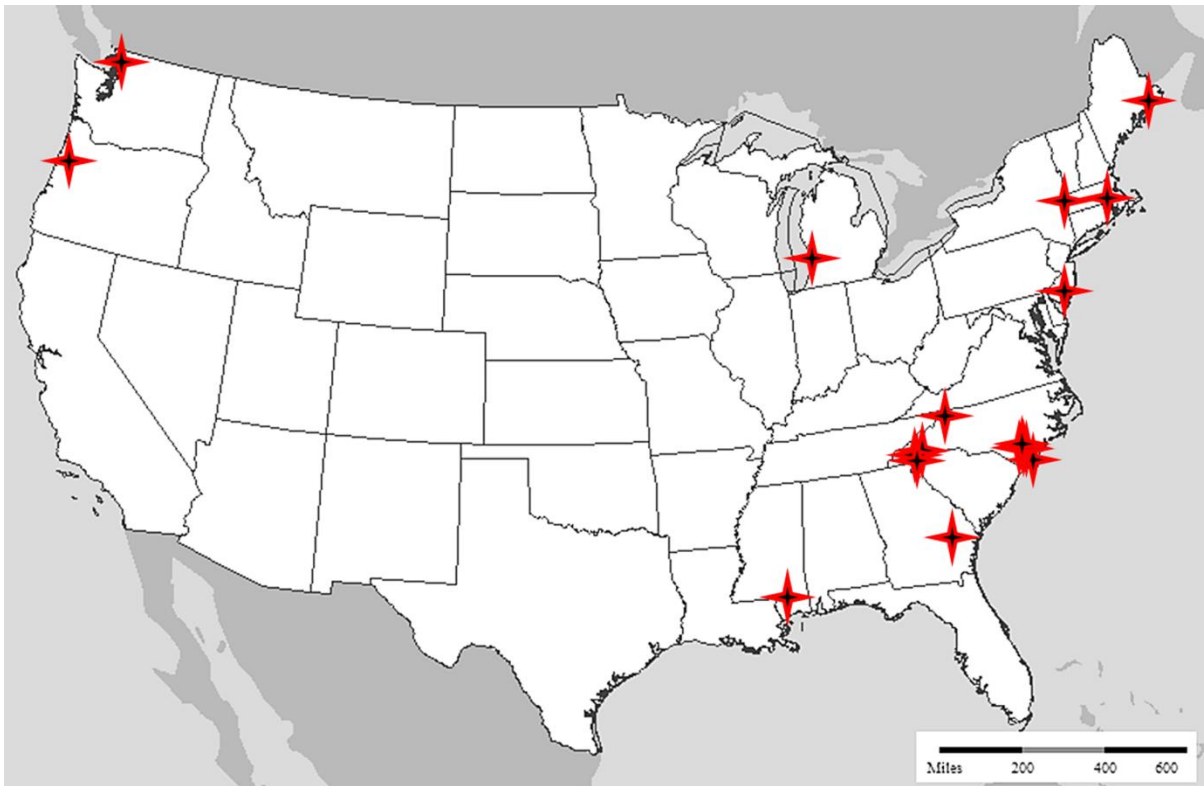


Fig. 2.1. Map of the locations of the 18 blueberry fields where samples of *Monilinia vaccinii-corymbosi* were collected.

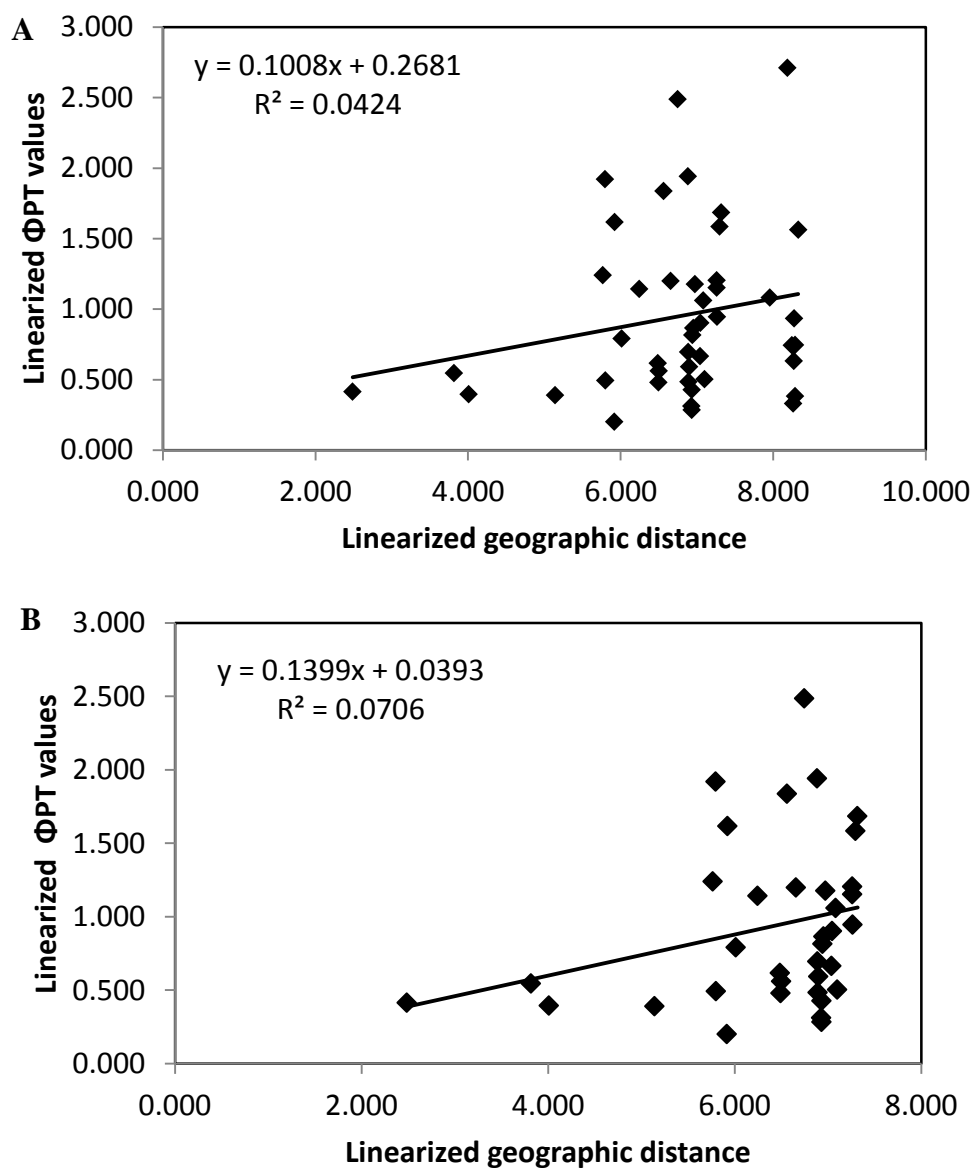


Fig. 2.2. Output from Mantel tests using a matrix of linearized Φ_{PT} values and linearized geographic distances. A= Mantel test based on Φ_{PT} values for the 10 analyzed fields. B= Mantel test excluding field WA.

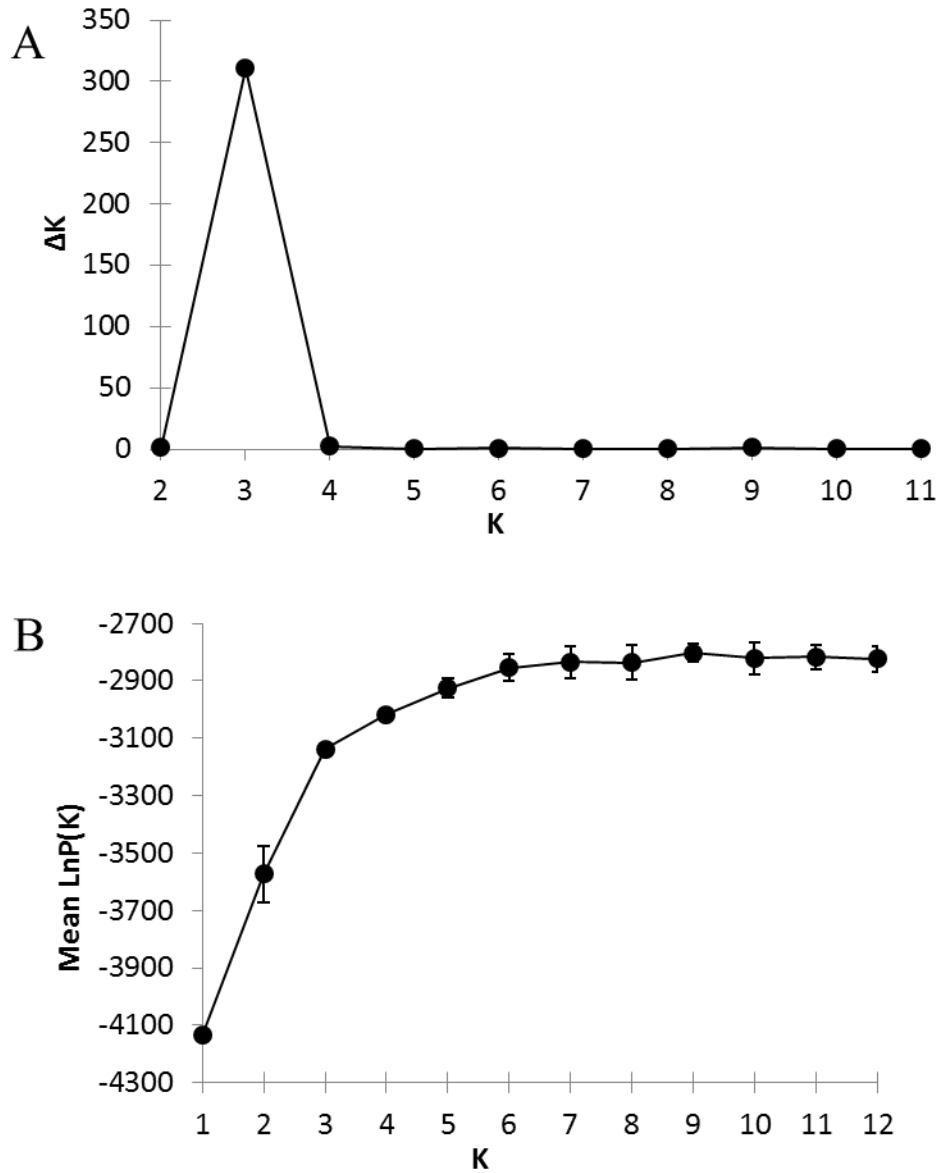


Fig. 2.3. Results from analysis with the program STRUCTURE. A= Evanno method results based on calculating ΔK and plotting against K . B= Mean $\text{LnP}(K)$ plotted against K for 20 runs at each value of K . Error bars indicate standard deviation of the mean.

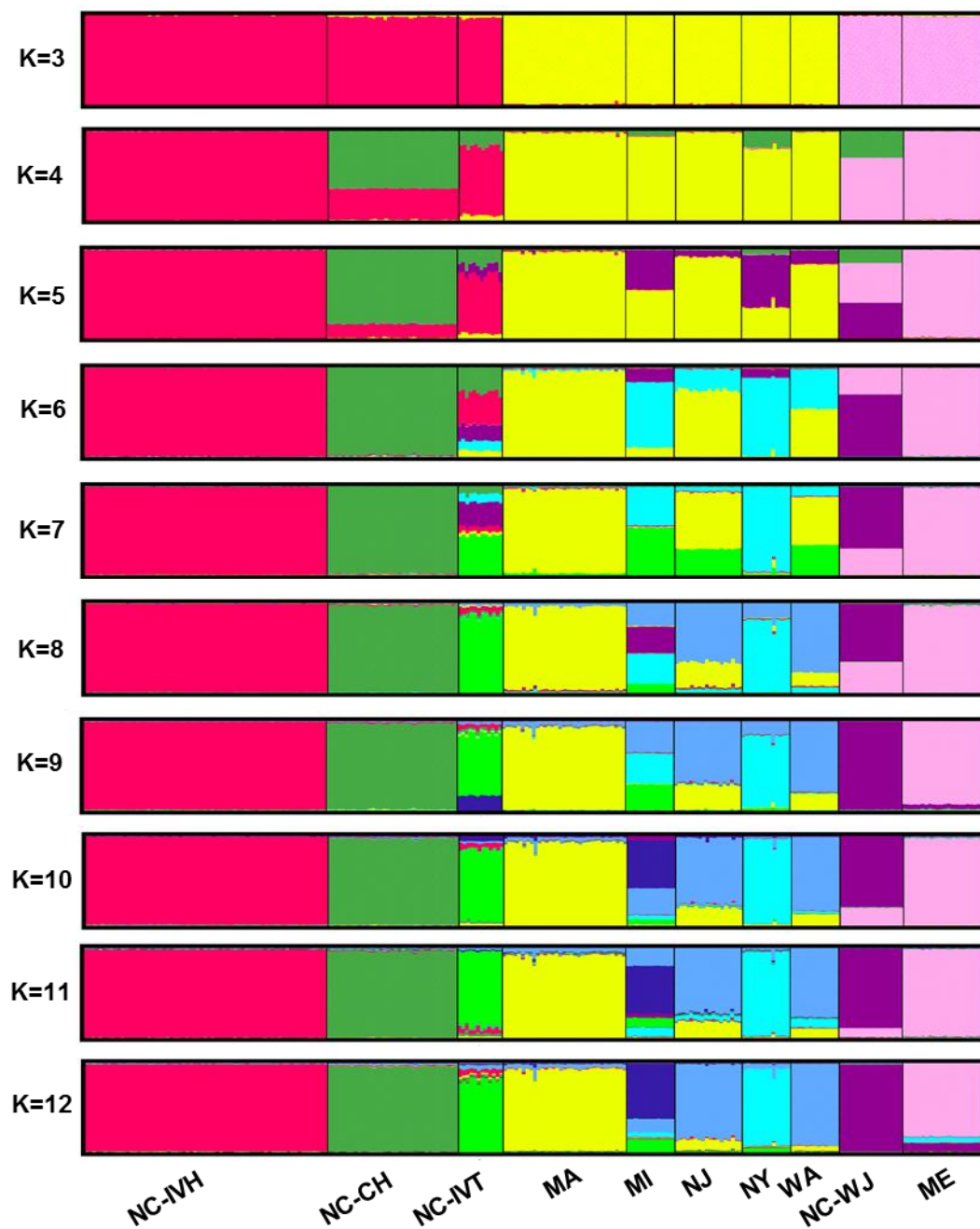


Fig. 2.4. Membership probability histograms from analysis with STRUCTURE at each value of K from 3 to 12. Field abbreviations are based on Table 2.1.

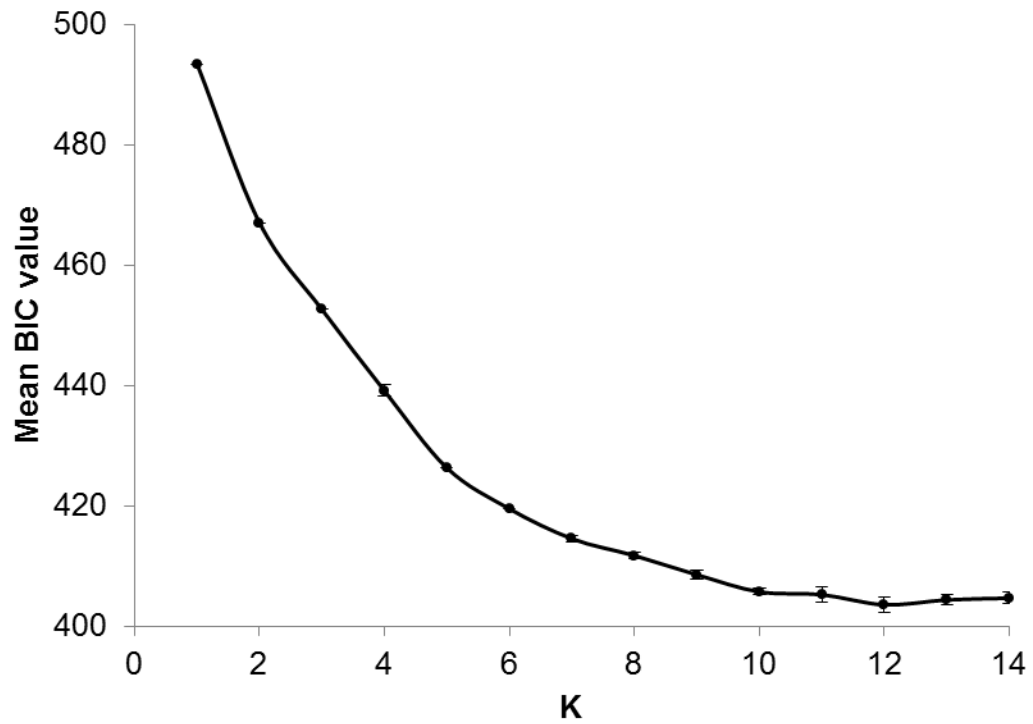


Fig. 2.5. Mean Bayesian information criterion (BIC) plotted against K as calculated using the K-means clustering algorithm implemented in adegenet. Error bars indicate standard deviation of the mean.

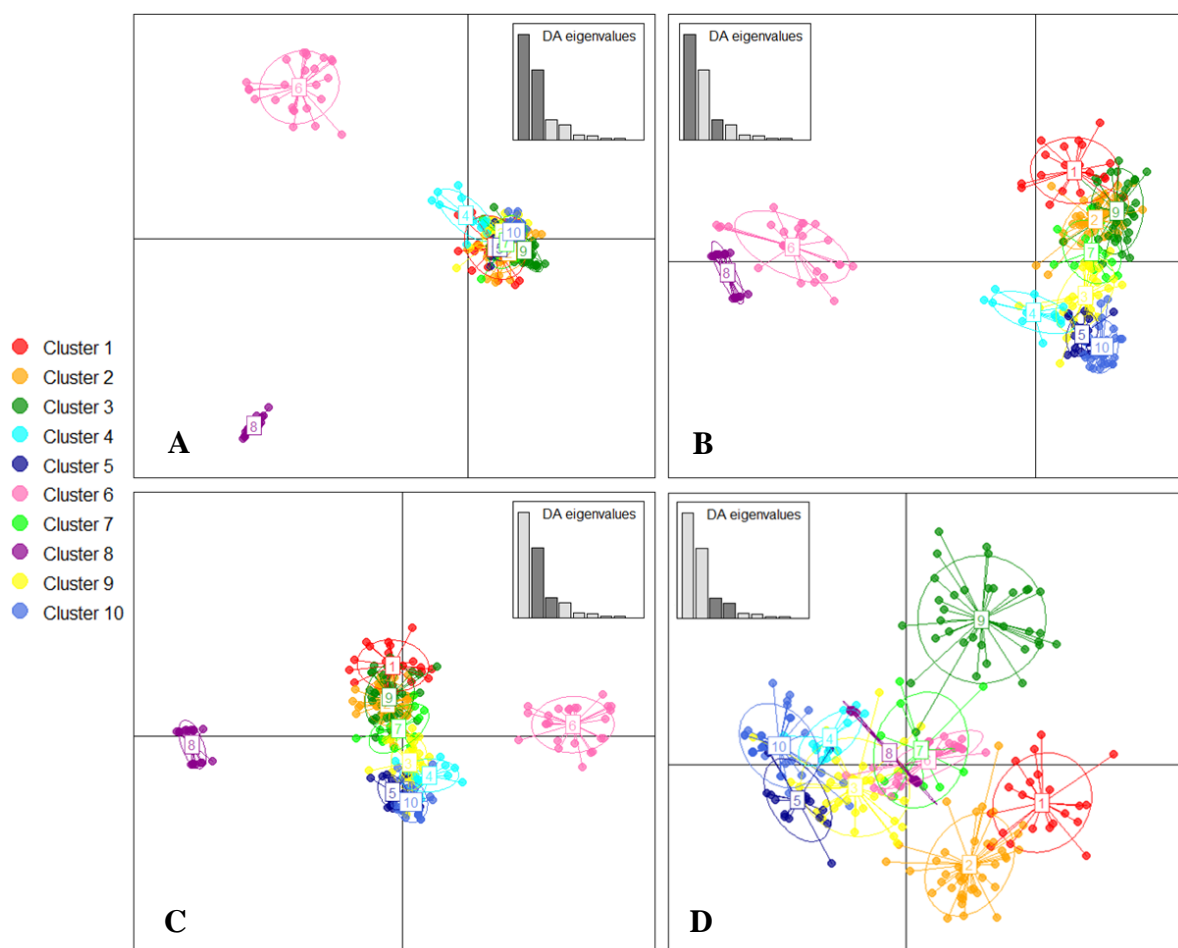


Fig. 2.6. Scatterplots of isolates of Mvc from 18 blueberry fields to 10 clusters based on results from discriminant analysis of principal components. A= scatterplot of axis 1 vs. 2, B= axis 1 vs. 3, C= axis 2 vs. 3, and D= axis 3 vs. 4.

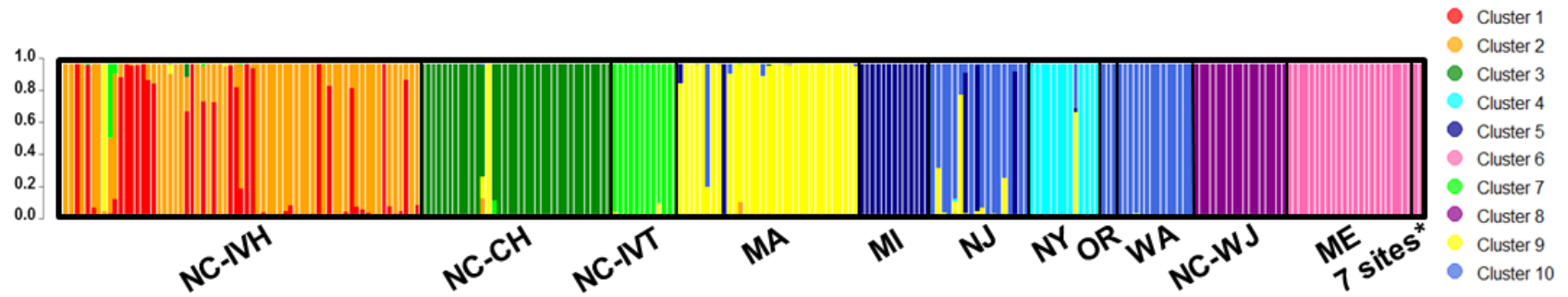


Fig. 2.7. Histogram of membership probabilities of Mvc isolates from 18 fields to 10 clusters based on results from discriminant analysis of principal components. Field abbreviations are based on Table 2.1. * 7 sites indicates the three MLHs detected within fields GA, MS, NC-CW, NC-FR, NC-SM, NC-GR, and NC-WL.

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CHAPTER III

Fine-scale genetic structure of the blueberry pathogen *Monilinia vaccinii-corymbosi*

Abstract

The fungus *Monilinia vaccinii-corymbosi* (Mvc) infects shoots and fruit of blueberry (*Vaccinium* spp.). Localized gene flow and genetic structuring were studied over three years within a 125 m x 132 m blueberry plot. Systematic sampling of shoots and fruit infected by Mvc was conducted at four time points; 56 infected shoots were collected in 2010, 70 infected fruit in 2011, and 132 infected shoots and 137 infected fruit in 2012. DNA was extracted from isolates of Mvc recovered from infected shoots and fruit. Nine polymorphic microsatellite markers were used to identify the multilocus haplotype (MLH) of each isolate. Little to no genetic differentiation was detected among the four sampled time points based on analysis of molecular variance ($\Phi_{PT} = 0.001$ to 0.013 , $P = 0.352$ to 0.017). Nonrandom mating was suggested by linkage disequilibrium and repeated detection of isolates of Mvc with the same MLH that were significantly unlikely to be the result of random mating. Spatial autocorrelation analysis supported unrestricted gene flow at the sampled spatial scale ($\alpha=0.05$). The program STRUCTURE and discriminant analysis of principal components suggested the presence of genetic structuring within the field, with at least $K=3$ genetically distinct clusters maintained over the four sampled time points.

Introduction

The fungus *Monilinia vaccinii-corymbosi* (Reade) Honey (Mvc) causes mummy berry disease of blueberry (*Vaccinium* spp.) that is characterized by shoot blight followed by fruit infection and desiccation (Batra 1983). The fungus overwinters inside desiccated fruit (pseudosclerotia) that lay dormant on the soil surface. In early spring, sexual fruiting structures (apothecia) are formed from pseudosclerotia and produce wind-disseminated sexual spores (ascospores) that infect newly emerging blueberry shoots and cause blighting (Batra 1983; Cox & Scherm 2001). Asexual spores (conidia) are produced on blighted tissues and spread primarily by insects to blueberry stigmas where they exhibit aggressive mimicry to invade the ovary (Batra 1983; Batra & Batra 1985; Cox & Scherm 2001; Ngugi & Scherm 2004). This results in fruit infection and can lead to substantial yield loss in commercial blueberry fields (Batra 1983).

Monilinia vaccinii-corymbosi must undergo both asexual and sexual spore production within a single growing season to complete its life cycle. Research suggests that Mvc cannot survive systemically in blueberry shoots, driving the need for ascospore production for shoot infection (Batra 1983). Furthermore, ascospores are not capable of infecting blueberry ovaries, rendering conidia production necessary for the completion of the life cycle (Batra 1983). Disease dynamics are therefore influenced by both spore types and their infection events on blueberry shoots and flowers. It is not known if allele frequencies of isolates of Mvc from infected shoots and fruit are similar within a growing season. Conidia are the product of asexual reproduction, with all conidia produced from an infected shoot being

genetically identical in the absence of mutation. Therefore, if conidia formed from a single infected shoot are spread to numerous flowers it would result in clonal propagation and possibly influence the allele frequencies within the population. Since ascospores are the product of sexual reproduction it is conceivable that ascospore production contributes to genetic diversity within populations of *Mvc*. However, the mating system of *Mvc* is unknown, and other related fungi commonly form ascospores either through outcrossing or self-fertilization (Amselem *et al.* 2011; Billiard *et al.* 2012; Heitman *et al.* 2007). Previous research suggested the presence of outcrossing and random mating in several populations of *Mvc* in the northeastern U.S. based on high genetic diversity and linkage equilibrium of microsatellite markers (Burchhardt, unpublished, see CHAPTER II). However, the possibility of inbreeding and/or self-fertilization was suggested in several populations based on the repeated detection of isolates of *Mvc* with the same multilocus haplotype and at least one pair of microsatellite markers in significant linkage disequilibrium. Therefore, the population and reproductive biology of *Mvc* needs to be further examined by analyzing allele frequencies and genetic diversity using a large sample size of isolates of *Mvc* from both infected shoots and fruit within a population.

An important component of the population genetics of organisms is gene flow, or the movement of genetic material. In the case of plant pathogenic fungi, gene flow can lead to the movement and introgression of alleles that contribute to disease ecology. Therefore, disease management strategies that restrict gene flow are attractive (McDonald & Linde 2002). Isolates of *Mvc* collected from 18 fields in the United States were recently analyzed to

examine large-scale gene flow and genetic structuring of populations of Mvc (Burchhardt, unpublished, see CHAPTER II). Strong genetic differentiation and structuring were detected between populations of Mvc from blueberry fields in the northeastern U.S., suggesting restricted gene flow. Genetic differentiation between isolates of Mvc from fields spatially separated by as little as 11 km located in southeastern North Carolina further supported locally restricted gene flow. However, the geographic distance at which gene flow becomes locally restricted has yet to be examined.

Spatial autocorrelation analysis can be used to determine the geographic scale at which gene flow is restricted by testing for spatial clustering of genetically similar individuals (Peakall & Smouse 2006, 2012). Localized gene flow has been studied within populations of several fungal species using spatial autocorrelation analysis, primarily in forest ecosystems. For example, spatial autocorrelation analysis was used to detect locally restricted gene flow in the ectomycorrhizal fungi *Cantharellus formosus* (Dunham *et al.* 2006), *Rhizopogon vesiculosus* (Dunham *et al.* 2013), and *Tricholoma scalpturatum* (Carriconde *et al.* 2008) and the tree pathogens *Armillaria mellea* (Travadon *et al.* 2012) and *Cryphonectria parasitica* (Dutech *et al.* 2008). Relatively fewer studies have been conducted on plant pathogenic fungi in agricultural systems. A recent study by Brewer *et al.* (2012) found that gene flow was restricted in populations of the grape (*Vitis* spp.) pathogen *Erysiphe necator* at up to 42 m, with spatial aggregation of clonal genotypes within two populations. Everhart (2012) conducted three-dimensional sampling of *Monilinia fructicola* within peach (*Prunus persica*) canopies to detect significant positive spatial autocorrelation at distances <1

m. More studies are needed to contribute to our knowledge of localized gene flow capabilities of plant pathogenic fungi in agricultural systems.

One component of gene flow is effective spore dispersal since the dissemination of spores followed by successful infection events results in the movement of the organism and genetic material. The dispersal gradients of both ascospores and conidia of *Mvc* were previously examined by Cox and Scherm (2001). Either pseudosclerotia with apothecia (and ascospores) or potted plants with infected shoots producing conidia were placed in rows of uninfected potted blueberry plants. Their results suggested short dispersal gradients for both ascospores and conidia. They found that 95% of blighted shoots were located within 30 m of an ascospore point source and 95% of infected fruit were located within 20 m of a conidial point source. However, the potential for long distance dispersal was suggested by a long tail in their dispersal gradient curves. Short dispersal gradients of both spore types suggested that gene flow may also be locally restricted within blueberry fields.

Gene flow may be restricted due to geographic, reproductive, and/or ecological barriers, leading to genetic structuring. Genetic structuring can be detected by using population genetics-based approaches to test for the presence of genetically differentiated populations. Typically genetic structuring is examined among geographically discrete localities and over large spatial scales. However, it is possible for more than one genetically differentiated population to exist in sympatry. This can occur when barriers to sexual recombination and/or random mating exist, with individuals within an area not having the same probability of mating with each other. Reproductive and ecological barriers can lead to

the maintenance of genetically differentiated populations over time. The detection of more than one population in sympatry can also be due to the recent geographic merging of two populations. However, if the two populations are interfertile and undergo mating, sexual recombination should lead to their genetic admixture and affect allele frequencies over several generations. Temporal sampling within an area can be used to determine if allele frequencies change over time and to test hypotheses for the presence and maintenance of genetic structure.

In this study a comprehensive spatial and temporal sampling was conducted to examine the fine-scale population genetics of *Mvc*. The objectives of this study were to 1) compare allele frequencies of isolates of *Mvc* collected from infected shoots and fruit within a single growing season; 2) perform analyses to test for random mating 3) examine localized gene flow by testing for spatial clustering of genetically similar individuals; and 4) examine temporal dynamics and genetic structuring within the field by analyzing isolates of *Mvc* collected over three consecutive years. This study was conducted in a commercial blueberry field in Ivanhoe, NC that was previously determined to contain a population of *Mvc* with high genetic diversity that was genetically differentiated from other populations of *Mvc* at distances as small as 11 km (Burchhardt, unpublished, see CHAPTER II). The hypothesis for this study was that isolates of *Mvc* collected over three years and from different tissue types (i.e. infected shoots and fruit) would not be significantly genetically differentiated and that nonrandom mating would be detected. Based on previous evidence for restricted gene flow and spore dispersal, it was also hypothesized that locally restricted gene flow would be

detected within the blueberry field. Since previous research suggested that genetic structuring may be present within the field (Burchhardt, unpublished, see CHAPTER II), the hypothesis that genetically differentiated populations of Mvc exist sympatrically was also tested. In this study, the contribution of host cultivar to genetic differentiation was also examined by analyzing isolates of Mvc recovered from two cultivars of southern highbush (*V. corymbosum* x *V. darrowii*) within the established experimental plots within the blueberry field.

Materials and methods

Sampling and isolation of Mvc from infected shoots and fruit

Infected shoots and fruit were sampled from a 50-year-old commercial blueberry field located in Ivanhoe, NC with a history of mummy berry disease. The approximately 200 hectare farm had 85 hectares devoted to commercial blueberry production in 2012. Samples were collected from a 125 m by 132 m plot planted with southern highbush blueberry (*V. corymbosum* x *V. darrowii*). The plot consisted of 23 rows of cultivar (cv.) ‘Legacy’ planted adjacent to 22 rows of cv. ‘Blue Ridge’ established in 2002. Rows were spaced 3 m apart in the plot, with approximately 125 blueberry bushes planted at 1 m intervals in each row. Infected shoots with conidia and/or infected fruit were systematically collected and mapped in 2010, 2011 and 2012. See Fig. 3.1 for a map of the location where each sample was collected in the plot. The systematic sampling scheme was modified in each of the three years, with an increase in sampling intensity and coverage over the three years. Infected

shoots were collected on 6 April 2010 from five rows in each cultivar. Samples were collected from up to six bushes in each row at an interval of at least 3 m between sampled bushes. In 2011, infected fruit were collected on 7 June from 'Blue Ridge' and 16 June from 'Legacy' from four rows in each cultivar. A maximum of 16 samples were collected within each row, and sampled bushes were spaced at an interval of approximately 5 m between sampled bushes. In 2012, extensive sampling of infected shoots on 3 April and infected fruit on 15 May was performed. On both sampling dates infected plant material was collected from 10 rows in each cultivar, with samples collected from approximately eight bushes per row spaced at an interval of approximately 15 m between sampled bushes.

Pure cultures of *Mvc* were obtained by generating single conidium isolations from infected shoots or by culturing isolates from inside infected fruit. Single conidium isolations were performed by scraping conidia off of infected shoots onto Petri dishes containing half-strength potato dextrose agar (PDA) amended to contain 2% agar (12 g Difco potato dextrose broth [PDB] and 20 g Difco agar per L [Becton, Dickinson and Company, MD]). After incubation at room temperature (approximately 20°C) for at least 12 h, individual germinated conidia were transferred to half-strength PDA containing 1.5 % agar (15 g Difco agar per L) using a sterile scalpel under a dissecting microscope. Fungal isolations from infected fruit were performed by surface disinfecting fruit for 30 sec with 10% NaOCl, 30 sec with 70% EtOH and rinsing in sterile H₂O for 30 sec. Infected fruit were cut in half with a sterile scalpel and a piece of internal fungal mycelium was removed from a single locule and transferred to half-strength PDA containing 1.5% agar. Isolates of *Mvc* from conidia and

infected fruit were grown at room temperature for approximately two weeks and then used to inoculate 250 ml Erlenmeyer flasks containing 50 ml of half-strength PDB (12g Difco PDB per L). The mycelium of each isolate was harvested after approximately two weeks by vacuum filtration. Approximately 15 mg of squeeze-dried mycelium was used for DNA extraction.

DNA analysis with nine microsatellite markers

DNA was extracted from each isolate of *Mvc* using the cetyltrimethylammonium bromide (CTAB) and chloroform DNA extraction method outlined by Kretzer *et al.* (2000), with modifications and DNA precipitation as described by Molecular Ecology Resources Primer Development Consortium *et al.* 2012. In instances where a fungal culture from infected fruit was not obtained, DNA was extracted directly from the mycelium located in one locule inside infected fruit using the same protocol. The Polymerase Chain Reaction (PCR) was performed on each DNA extract using nine polymorphic microsatellite markers; *Mvc*7, 9, 10, 12, 23, 25, 27, 28, and 30 (Molecular Ecology Resources Primer Development Consortium *et al.* 2012). Primer sets for multiple markers were combined in PCR reactions (i.e. multiplexed); markers *Mvc*7 and 9 (*Mvc*7-9), *Mvc*12 and 23 (*Mvc*12-23), and *Mvc*28 and 30 (*Mvc*28-30) were setup in duplexed PCR reactions and *Mvc*10, 25, and 27 (*Mvc*10-25-27) in triplexed reactions. To fluorescently label PCR products, a ‘CAG’ sequence was added to the 5’ end of one of the primers in each microsatellite marker set and an additional ‘CAG’ primer with either a NED or 6-FAM fluorescent tag attached to the 5’ end was added to PCR reactions (Molecular Ecology Resources Primer Development Consortium *et al.*

2012). Reactions were setup in 0.3 ml 96-well PCR plates in 10 μ l reaction volumes containing 1x PCR Master Mix (reaction buffer with 0.025 U/ μ l *Taq* DNA polymerase, 2 mM MgCl₂, and 0.2 mM of each dNTP; Thermo Scientific), 0.05 μ M of each ‘CAG’-tailed primer, 0.2 μ M of each untailed primer, either 0.6 μ M (triplexed reactions) or 0.4 μ M (duplexed reactions) fluorescently labeled ‘CAG’ primer, and 1.0 μ l of diluted DNA extract. PCR reactions from *Mvc*7-9 and *Mvc*10-25-27 were fluorescently labeled with 6-FAM and reactions containing *Mvc*12-23 and *Mvc*28-30 were labeled with NED. Thermocycle conditions were as previously described (Molecular Ecology Resources Primer Development Consortium *et al.* 2012). PCR products were then multiplexed by combining product from reactions *Mvc*7-9 with *Mvc*12-23, as well as product from *Mvc*10-25-27 with *Mvc*28-30. Fragment analysis was performed to determine the length of the PCR product at each locus as previously described (Molecular Ecology Resources Primer Development Consortium *et al.* 2012).

The multilocus haplotype (MLH) of each sample was determined based on the length of the PCR product (i.e. allele) present at the nine microsatellite loci. The number of alleles detected at each locus, number of unique MLHs, and unbiased haploid diversity were determined with the software GenAlEx version 6.5 (Peakall & Smouse 2006, 2012). GenAlEx was also used to generate clone corrected datasets by including only one representative isolate of *Mvc* per MLH in the dataset. Clone correction was performed separately for isolates collected from the four sampled time points; isolates from infected shoots collected in 2010, infected fruit in 2011, and infected shoots and fruit in 2012.

Analyses for genetic differentiation among cultivars, infected plant tissue types, and years

Due to slight differences in timing of bloom and fruit set between cv. 'Blue Ridge' and cv. 'Legacy', the hypothesis that allele frequencies were statistically similar when comparing isolates of Mvc sampled from both cultivars was tested. This was done to confirm that isolates from the cultivars could be pooled in subsequent analyses. Analysis of molecular variance (AMOVA) was performed to test for significant genetic differentiation between the two cultivars. The analysis was performed separately for isolates of Mvc from the four sampled time points; isolates from infected shoots collected in 2010, infected fruit in 2011, and infected shoots and fruit in 2012. The analysis was conducted in GenAlEx with 10,000 data permutations and 10,000 pairwise population permutations to calculate Φ_{PT} values as a measure of genetic differentiation and P values for significance. The analysis was performed using clone corrected and non-corrected datasets. This enabled us to examine how repeatedly detected MLHs in the dataset affected measures of genetic differentiation. Since the two cultivars did not significantly differ in their allele frequencies at $\alpha=0.05$ both with and without clone correction, isolates from the cultivars were pooled in subsequent analyses.

Analysis of molecular variance was performed to test the hypothesis that isolates of Mvc collected in different years and from infected shoots and fruit within the same growing season were not significantly genetically differentiated. Genetic differentiation between isolates of Mvc from the four sampled time points (i.e. isolates from infected shoots collected in 2010, infected fruit collected in 2011, and infected shoots and fruit collected in 2012) was

measured based on Φ_{PT} values. The analysis was performed both with and without first clone correcting isolates from each time point as described above.

Linkage disequilibrium and analysis of random mating

The statistic p_{sex} as calculated with the program MLGsim v. 2.0 (<http://www.rug.nl/research/theoretical-biology/downloads>) was used to determine the probability of a MLH being detected a given number of times within the plot (Stenberg *et al.* 2003). The MLHs of isolates from the four sampled time points (2010, 2011, and infected shoots and fruit in 2012) were analyzed separately. P values were calculated based on 1,000 permutations to determine significance at $P < 0.05$. Significant p_{sex} values suggested that isolates associated with a specific MLH were the product of nonrandom mating.

Tests for significant linkage disequilibrium between the nine microsatellite loci were performed using the online implement of Genepop version 4.2, with 10,000 dememorizations, 1,000 batches, and 10,000 iterations per batch (Raymond & Rousset 1995; Rousset 2008). Linkage disequilibrium was calculated for both clone corrected and non-clone corrected datasets. The analysis was performed separately for isolates of Mvc collected at the four sampled time points (2010, 2011, and from infected shoots and fruit in 2012). The method of Benjamini and Hochberg (1995) was used to correct the P values for multiple comparisons and test for significant linkage disequilibrium (adjusted $P < 0.05$).

Spatial autocorrelation analysis

The geographic distance at which gene flow is restricted can be examined by testing for clustering of genetically similar isolates located within a certain distance of each other

(i.e. distance class). Spatial autocorrelation analysis as calculated with GenAlEx was performed to test for a significant correlation between genetic and geographic distances. The analysis calculates correlation coefficients (r) based on the genetic similarity of all pairs of individuals that fall within a particular distance class. Spatial autocorrelation was tested by two methods. The first method uses random permutation to shuffle individuals among the geographic locations and generate a 95% confidence interval of r . The null hypothesis of no spatial autocorrelation is supported when the observed value of r lies outside of the 95% confidence interval of r . The other method uses bootstrap estimates of r to place a confidence interval around the observed estimate of r . From all sets of pairwise comparisons within a distance class, a preset number of sets are randomly selected from the pool to generate a 95% confidence interval. The null hypothesis of no spatial autocorrelation is supported when $r=0$ lies within the bootstrap confidence interval. The analysis was performed separately for isolates sampled from infected fruit in 2011, infected shoots in 2012, and infected fruit in 2012. An additional analysis was performed by combining all isolates from infected shoots and fruit collected in 2012 in the analysis. Isolates of Mvc sampled in 2010 were not analyzed since the sampling scheme was focused near the edges of the plot and therefore not well distributed in the study area. Non-clone corrected datasets were analyzed. Genetic and geographic distance matrices were produced based on pairwise differences between isolates with GenAlEx. The analysis was performed multiple times using different size distance classes, with the smallest distance class set between 5 and 20 m. The 95% confidence intervals of r were calculated based on 1,000 random and bootstrap permutations.

Genetic structuring of Mvc

The program STRUCTURE version 2.3.4 was used to test for genetic structuring within the plot (Pritchard *et al.* 2000). STRUCTURE performs a Bayesian analysis to determine the likelihood of the presence of a certain number of genetically distinct clusters (i.e. populations, K) based on the allele frequencies. Since the model assumes random mating within populations, the clone corrected datasets from 2010, 2011, and from infected shoots and fruit in 2012 were analyzed to examine genetic structuring over the four time points. Analysis was performed at K=1 to 10 using the admixture model with allele frequencies correlated, a burn-in of 20,000 runs, 500,000 Markov chain Monte Carlo simulations, and 20 runs performed at each value of K. The program STRUCTURE HARVESTER (web version 0.6.93) was used to determine the value of K that was supported based on the change in the log likelihood of K (ΔK) using the Evanno method (Earl & Vonholdt 2012; Evanno *et al.* 2005). Histograms of membership probabilities of each sample to a given cluster were generated by first permutating the Q values with the program CLUMPP version 1.1.2 (Jakobsson & Rosenberg 2007) and running the output in Distruct version 1.1 (Rosenberg 2004).

Discriminant analysis of principal components (DAPC) as performed with the R package adegenet was used to further examine genetic structuring within the field using a multivariate analysis approach (Jombart 2008; Jombart *et al.* 2010). The analysis aims to display differences between clusters of genetically distinct individuals while minimizing variation within the clusters. The clone corrected dataset including isolates from 2010, 2011,

and 2012 was analyzed. The K-means cluster algorithm was performed to group samples into clusters and to determine the number of supported clusters based on the Bayesian information criterion (BIC) at each value of K. The value of K at which the BIC begins to either increase or level off can be used to choose K to perform DAPC. Scatterplots and histograms were visualized to examine clustering of the samples and assignment of isolates to the clusters. Results from DAPC were further examined with AMOVA by testing for genetic differentiation between isolates assigned to the K clusters. Each isolate was assigned to one of K clusters based on the cluster they had the highest membership probability to. Genetic differentiation between isolates of Mvc assigned to different clusters was calculated with AMOVA as described above. To further examine genetic differentiation among the clusters, isolates were randomly assigned to clusters and AMOVA was performed to test for significant genetic differentiation among the randomized clusters.

Results

Descriptive statistics and analyses for genetic differentiation with AMOVA

Samples of Mvc were collected from 56 infected shoots in 2010, 70 infected fruit in 2011, and 132 infected shoots and 137 infected fruit in 2012 (Table 3.1). The samples were used to isolate pure cultures of Mvc and extract DNA. Due to inability to obtain a pure culture, DNA was directly extracted from a single locule within 54 infected fruit in 2011 and 25 infected fruit in 2012. The nine microsatellite loci were polymorphic, ranging from four to nine alleles detected at each locus. High haploid diversity was detected over the four sampled time points

(i.e. isolates sampled from infected shoots in 2010, infected fruit in 2011, and infected shoots and fruit in 2012) with a mean of 0.536 (SE \pm 0.033). The majority of samples had unique MLHs, with 45 MLHs detected in 2010, 56 MLHs detected in 2011, and 113 MLHs detected from infected shoots and 121 from infected fruit in 2012. Isolates with repeatedly detected MLHs were identified at the four sampled time points; 20 MLHs were detected at least once at two of the four time points, seven MLHs at three of the four time points, and two MLHs at all four time points.

Isolates of Mvc sampled from cvs. 'Blue Ridge' and 'Legacy' were not significantly genetically differentiated based on AMOVA (see Table 3.1). Therefore, the data from both cultivars were pooled in subsequent analyses. Analysis of allele frequencies with AMOVA supported that isolates of Mvc from infected shoots and fruit sampled in 2012 were not significantly genetically differentiated when analyzing clone corrected ($\Phi_{PT}= 0.005$, $P=0.072$) and non-clone corrected datasets ($\Phi_{PT}=0.002$, $P=0.163$). When the allele frequencies of isolates of Mvc from infected shoots sampled in 2010, infected fruit in 2011, and from infected shoots and fruit in 2012 were compared, little to no genetic differentiation was detected based on AMOVA (Table 3.2). Significant genetic differentiation was observed for the comparison of isolates from infected shoots in 2010 to isolates from infected shoots in 2012 when analyzing the non-clone corrected datasets ($\Phi_{PT}=0.013$, $P=0.017$). However, the comparison was not significant when analyzing clone corrected datasets ($\Phi_{PT}=0.009$, $P=0.071$).

Nonrandom mating detected based on p_{sex} and linkage disequilibrium

When isolates of Mvc sampled in 2010, 2011, and infected shoots and fruit in 2012 were analyzed separately, two MLHs had significant p_{sex} values in 2010, three MLHs in 2011, and one MLH from infected shoots and five MLHs for fruit in 2012. The two MLHs in 2010 (herein MLH1 and MLH2) had highly significant p_{sex} values ($P < 1e-5$), with MLH1 represented by four isolates and MLH2 by six isolates. Isolates of Mvc with MLH1 and MLH2 were also detected multiple times within the other three time points. Seven isolates with MLH1 were detected in 2011, as well as four isolates from infected shoots and nine isolates from infected fruit in 2012. The p_{sex} values for MLH1 were highly significant ($P < 1e-5$) with the exception of isolates of Mvc with MLH1 recovered from infected shoots in 2012 ($P = 0.146$). MLH2 was detected four times in 2011 and six times from infected shoots and twice from infected fruit in 2012. All p_{sex} values were highly significant ($P < 1e-5$) except for isolates from infected fruit in 2012 ($P = 0.159$). One MLH that was represented by two isolates in 2011 was also significant ($P = 0.026$). The four other MLHs that had significant p_{sex} values for isolates from fruit in 2012 were represented by two ($P = 0.032$), two ($P = 1.23e-6$), two ($P = 1e-8$), and three ($P = 2.8e-6$) isolates, respectively.

Based on analysis of non-clone corrected datasets, significant linkage disequilibrium was detected between 15 pairs of microsatellite markers in 2010 (Mvc9-10, 9-12, 9-23, 10-23, 12-23, 12-27, 23-27, 12-28, 23-28, 7-30, 9-30, 10-30, 12-30, 23-30, and 27-30), 14 marker pairs in 2011 (Mvc 7-9, 9-12, 10-12, 9-23, 12-23, 12-27, 23-27, 12-28, 27-28, 10-30, 12-30, 23-30, 27-30, and 28-30), and six pairs for infected shoots (Mvc 9-12, 12-23, 12-27,

23-27, 12-28, and 12-30) and 16 pairs for infected fruit in 2012 (Mvc7-9, 9-12, 10-12, 7-23, 9-23, 12-23, 7-27, 9-27, 12-27, 23-27, 9-30, 10-30, 12-30, 23-30, 27-30, and 27-28). Once the datasets were clone corrected, three marker pairs were in significant linkage disequilibrium in 2010 (Mvc12-23, 23-27, and 23-30), two in 2011 (Mvc12-23, 27-30), and none for infected shoots and seven for infected fruit in 2012 (Mvc9-12, 7-27, 12-27, 23-27, 27-28, 9-30, and 10-30). None of the marker pairs were consistently in linkage disequilibrium among the four time points, suggesting that linkage disequilibrium was not likely due to physical linkage of the loci.

Lack of positive spatial autocorrelation suggests unrestricted gene flow

Spatial autocorrelation analysis was performed to test for spatial clustering of genetically similar isolates of Mvc. Separate analyses of isolates of Mvc sampled from infected fruit in 2011, infected shoots in 2012, infected fruit in 2012, and both infected shoots and fruit in 2012 were performed. Regardless of distance class sizes set in the analyses, no positive spatial autocorrelation was detected. Examples of correlograms from spatial autocorrelation analysis are presented in Fig. 3.2.

Presence of genetic structuring within the plot

Analysis with the program STRUCTURE indicated the presence of more than one genetically distinct population of Mvc within the plot. The Evanno method supported K=3 genetic clusters (Fig. 3.3). However, additional structure may have been present based on an increase in the mean LnP(K) at K=5 (Fig. 3.4). The three populations were detected in each of the four time points and there was no discernible pattern in the geographic arrangement of

isolates belonging to the clusters. Admixture was suggested since several of the isolates could not be assigned to one cluster with a high membership probability, but rather were evenly assigned to at least one other cluster (Fig. 3.5). However, even membership assignment to more than one population may have been due to the inability of the model to assign an isolate to a particular population rather than genetic admixture.

Discriminant analysis of principal components also suggested that at least three genetically distinct clusters were present within the plot. The exact number of clusters could not be reliably determined as the BIC value continued to decline from $K=1$ to 10 (Fig. 3.6). Therefore, results from several independent runs of K-means clustering and DAPC at each value of K were individually examined to detect the minimum amount of clustering present. Membership assignment of isolates to clusters at $K>3$ was inconsistent when separate runs of K-means clustering were performed. However, when multiple runs of K-means were performed at $K=3$ the isolates were consistently assigned to the same clusters. Clustering was most visually apparent at $K=3$ (Fig. 3.7). Although there were some discrepancies, assignment of isolates to the three clusters was similar when comparing results from STRUCTURE and DAPC (see Fig. 3.5). When the isolates were separated into populations using their cluster assignment with DAPC, the three clusters were highly significantly genetically differentiated from each other based on AMOVA ($\Phi_{PT} \geq 0.202$, $P \leq 0.0001$). No significant genetic differentiation was detected when isolates were randomly assigned to one of three clusters and AMOVA was performed ($\Phi_{PT} \leq 0.001$, $P \geq 0.353$).

Discussion

The most important result of this study was the detection of several genetically differentiated populations of Mvc within a 125 x 132 m blueberry plot, with maintenance of genetic differentiation over three consecutive years. At least $K=3$ genetically distinct populations were detected within the plot by analysis with STRUCTURE and DAPC. Significant genetic differentiation between isolates of Mvc assigned to different genetic clusters with DAPC as determined by AMOVA provided additional support for the presence of multiple genetically distinct populations. Furthermore, when isolates were randomly assigned to clusters and AMOVA was performed no significant genetic differentiation was detected. The large-scale population genetics of Mvc in 18 fields in the US was previously studied (Burchhardt, unpublished, see CHAPTER II). The 70 isolates of Mvc sampled from shoots of cv. 'Blue Ridge' in 2012 were included in that study, with DAPC suggesting the existence of at least two genetically distinct populations within the plot. Further genetic structuring was detected in this study by analyzing 395 isolates of Mvc collected from infected shoots and fruit within the plot in 2010, 2011 and 2012.

Temporal maintenance of genetic structure was suggested as isolates with high membership probabilities to the three genetically differentiated populations were detected in all three years. Furthermore, AMOVA indicated little to no genetic differentiation among the three years despite differences in the sampling scheme used in each year. This suggested that allele frequencies were similar over the three years and barriers to gene flow were maintained among the populations. However, significant genetic differentiation was detected between

isolates sampled from infected shoots in 2010 and 2012 when analyzing non-clone corrected datasets. Therefore, the hypothesis that genetic differentiation among samples of Mvc collected in different years would not be detected was rejected. The lack of significant genetic differentiation once datasets were clone corrected demonstrated the impact of clonality on measures of genetic differentiation.

The presence and temporal maintenance of several genetically differentiated populations of Mvc within the plot may be explained by reproductive and ecological barriers to gene flow. Reproductive barriers may prevent mating between isolates of certain haplotypes. One genetic mechanism that prevents mating of isolates is heterothallic mating in which only isolates with the opposite mating type allele at their mating locus can undergo sex (Billiard *et al.* 2012; Heitman *et al.* 2007). Therefore, isolates of the same mating type are reproductively isolated. Heterothallic mating has been reported in the closely related fungus *Botrytis cinerea* (Amselem *et al.* 2011; Holst-Jensen *et al.* 1997). However, the ascospore progeny of a heterothallic mating event are expected to be sexually recombinant, which should rearrange alleles and prevent genetic differentiation of isolates with different mating types. It is possible that genetic mechanisms other than the mating locus influence sexual reproduction and recombination in Mvc, preventing genetic exchange between certain haplotypes. However, the mating system of Mvc is not known. The haplotype of single ascospore progeny from a single apothecium of Mvc was recently analyzed (see CHAPTER IV). The results indicated that apothecia can produce sexually recombinant ascospores with unique multilocus haplotypes, while others may produce ascospores with identical multilocus

haplotypes. The production of genetically identical ascospores does not support heterothallism, but rather suggests homothallic (i.e. self-fertility) capabilities in *Mvc*. Some fungi are known to have mixed mating systems that involve both outcrossing and self-fertilization. A mixed mating system has been reported in the closely related genus *Sclerotinia* (Ekins *et al.* 2006; Uhm & Fujii 1983). The results suggest that *Mvc* may also have a complex mating system that permits self-fertilization and outcrossing. The genetic mechanisms that control mating in *Mvc* and how they contribute to genetic structure need to be examined further.

Another ecological force that may be driving the maintenance of sympatric genetic differentiation is competition. Conidia of different haplotypes may be more or less aggressive during stigma penetration and ovary infection, with particular haplotypes outcompeting others during ovary colonization. If an isolate of *Mvc* outcompetes all others and is the sole haplotype within an infected fruit and the isolate is capable of self-fertilization, then selection would favor the maintenance of the haplotype. Recently, isolates of *Mvc* from up to five locules within a single infected fruit were cultured. Results suggested that isolates that are genetically different can exist within a single infected fruit, with some harboring more than one isolate with different haplotypes while others contained only a single haplotype (Burchhardt, unpublished, see CHAPTER IV). Variation in aggressiveness of isolates has been described in several species of plant pathogenic fungi such as *Fusarium graminearum* (Chakraborty *et al.* 2006) and *Botrytis cinerea* (Reino *et al.* 2004). Flower inoculation

experiments would need to be performed to study aggressiveness and competition of isolates of Mvc with different haplotypes.

Variation in timing of ascospore and conidia production also may enforce sympatric genetic differentiation. Research from previous investigations suggests that pseudosclerotia can vary in timing of apothecia production on different species of blueberry and different cultivars within species (Lehman & Oudemans 1997, 2000; Scherm *et al.* 2001). Furthermore, apothecia production from pseudosclerotia collected underneath bushes of a single blueberry cultivar can occur over several weeks (Lehman & Oudemans 1997), and a single apothecium may produce ascospores over several days (Lehman & Oudemans 2000). It is possible that isolates of Mvc with different haplotypes produce apothecia and ascospores at different times, resulting in differences in timing of shoot infection. Variation in the incubation and latent periods following ascospore infection and production of conidia has not been studied. If differences in timing of ascospore production results in differences in timing of conidia production, then haplotypes that produce conidia within the same timeframe should be more likely to co-inoculate a flower, allowing them to undergo sexual recombination. Timing of conidia production is particularly critical because flowers are most receptive to infection on the day of anthesis, with decreasing susceptibility over time (Ngugi *et al.* 2002). Batra (1983) reported that conidia produced from a single infected shoot are only viable for up to six days and conidia production can occur within a field over a month. Further research to examine how the reproductive biology and ecology of Mvc influence its genetic structure is warranted.

Since infected shoots and fruit are the result of infection by asexual and sexual spore types (conidia and ascospores, respectively), the hypothesis that allele frequencies will be similar when analyzing isolates of Mvc collected from infected shoots and fruit within a single growing season was tested. Analysis of molecular variance suggested that isolates from infected shoots and fruit were not genetically differentiated regardless of the presence of repeatedly detected MLHs. Therefore, the hypothesis that allele frequencies are similar between isolates of Mvc collected from infected shoots and fruit within the same growing season was accepted. Everhart (2012) also found that isolates of *Monilinia fructicola* recovered from blighted peach blossoms and infected fruit were not significantly genetically differentiated.

Nonrandom association of alleles at unlinked, neutral loci can be caused by deviation from random mating and can be detected through analysis of linkage disequilibrium between loci. While haplotypic diversity was high each year, nonrandom mating was suggested based on the presence of linkage disequilibrium and repeatedly recovered MLHs over three years of sampling that were statistically unlikely to be the product of random mating. Interestingly, two MLHs (MLH1 and MLH2) were detected numerous times within each year of sampling, had highly significant p_{sex} values and were always assigned to different clusters with STRUCTURE and DAPC, suggesting their genetic dissimilarity (*data not shown*). STRUCTURE also suggested that isolates with MLH1 or MLH2 were not genetically admixed, but had high membership probabilities to a single cluster. Therefore, these results suggest clonal propagation of the two MLHs.

No restricted gene flow was detected within the examined 125 x 132 m plot. This was unexpected as previous research suggested that asexual and sexual spore dispersal occurs primarily within 30 m of a source (Cox & Scherm 2001). There are several plausible explanations for the absence of positive spatial autocorrelation within the plot. First, although the sampling area was chosen based on previous research on spore dispersal capabilities (Cox & Scherm 2001), the sampling area may not have been large enough to detect restricted gene flow. To test this hypothesis the sampling area would need to be enlarged to a scale at which restricted gene flow could be detected. It is also possible that the presence of several genetically differentiated populations of *Mvc* within the plot influenced the results from spatial autocorrelation analysis. This may have occurred if genetically similar isolates that belonged to the same population were in a distance class that also contained isolates that were strongly genetically divergent and belonged to a different population. Genetic structuring that is caused by factors other than geography needs to be examined further. Finally, the sampling scheme within the plot may not have been sufficient for detecting restricted gene flow in *Mvc*. Although samples of infected shoots and fruit were systematically collected, the sampling scheme did not account for the possible presence of hierarchical structuring. At each sampled locality within the plot, *Mvc* was isolated from a single infected shoot or fruit. Genetic diversity of isolates of *Mvc* in the canopy of a single blueberry bush should be examined. Everhart (2012) reported high genetic diversity within the canopy of a single peach tree. The study was also the first to use three dimensional mapping to detect positive spatial autocorrelation at <1 m in canopies, demonstrating the

importance of hierarchical sampling. Furthermore, Mvc was arbitrarily isolated from a single locule of an infected fruit to obtain pure cultures or extract DNA. It was recently determined that several genetically unique isolates of Mvc can be isolated from different locules in a single infected fruit (Burchhardt, unpublished, see CHAPTER IV). Genetic diversity of isolates of Mvc recovered from a single fruit and from an individual bush should be examined.

Results from this study suggest that the population biology of Mvc is complex and warrants further examination. It was determined that genetic structuring existed within a 125 x 132 m plot and that structure was maintained over three years. Linkage disequilibrium and repeatedly detected MLHs with significant p_{sex} values supported nonrandom mating within the plot. Spatial autocorrelation analysis supported unrestricted gene flow. Research is needed to determine the mating system of Mvc and examine hierarchical structuring within a plot.

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Tables and Figures

Table 3.1. Descriptive statistics based on analysis of isolates of *Monilinia vaccinii-corymbosi* (Mvc) collected over three years with nine microsatellite markers. N= number of analyzed isolates of Mvc from cultivars ‘Blue Ridge’ and ‘Legacy’; $\Phi_{PT} CC$ = results from analysis of molecular variance when comparing isolates collected from ‘Blue Ridge’ to those from ‘Legacy’, with clone correction of multilocus haplotypes before performing the analysis and P values in parentheses; $\Phi_{PT} NC$ = results from analysis of molecular variance without clone correcting isolates collected from ‘Blue Ridge’ to those from ‘Legacy’, with P values in parentheses; MLH= number of multilocus haplotypes detected; h = mean unbiased haploid diversity; S.E.= standard error of the mean, and LD=number of pairs of microsatellite markers in significant linkage disequilibrium.

Year	Sampled tissue	Cultivar	N [∞]	$\Phi_{PT} CC$	$\Phi_{PT} NC$	MLH	h (S.E.)	LD*
2010	Infected shoots	Blue Ridge	27 (22)	0.006 (0.295)	0.007 (0.259)	45	0.551 (0.070)	15/3
		Legacy	29 (25)					
2011	Infected fruit	Blue Ridge	26 (22)	0 (0.630)	0.019 (0.080)	56	0.507 (0.079)	14/2
		Legacy	44 (37)					
2012	Infected shoots	Blue Ridge	70 (65)	0 (0.446)	0.009 (0.065)	113	0.531 (0.065)	6/0
		Legacy	62 (55)					
2012	Infected fruit	Blue Ridge	66 (65)	0.002 (0.337)	0.004 (0.208)	121	0.555 (0.064)	16/7
		Legacy	71 (61)					

[∞] Number of unique multilocus haplotypes (MLHs) are indicated within parentheses.

*Number of marker pairs in significant linkage disequilibrium before clone correcting / number of marker pairs in significant linkage disequilibrium after clone correcting.

Table 3.2. Results from analysis of molecular variance based on isolates of *Monilinia vaccinii-corymbosi* sampled from infected shoots in 2010, infected fruit in 2011, and from infected shoots and fruit in 2012 (2012 S and 2012 F, respectively). Pairwise Φ_{PT} values based on analyzing clone corrected datasets are reported below the diagonal and Φ_{PT} values using non-clone corrected datasets above the diagonal, with P values in parentheses.

Φ_{PT}	2010	2011	2012 S	2012 F
2010	–	0.003 (0.290)	0.013 (0.017)	0.004 (0.184)
2011	0.000 (0.425)	–	0.006 (0.077)	0.001 (0.352)
2012 S	0.009 (0.071)	0.009 (0.050)	–	0.002 (0.163)
2012 F	0.000 (0.436)	0.000 (0.436)	0.005 (0.072)	–

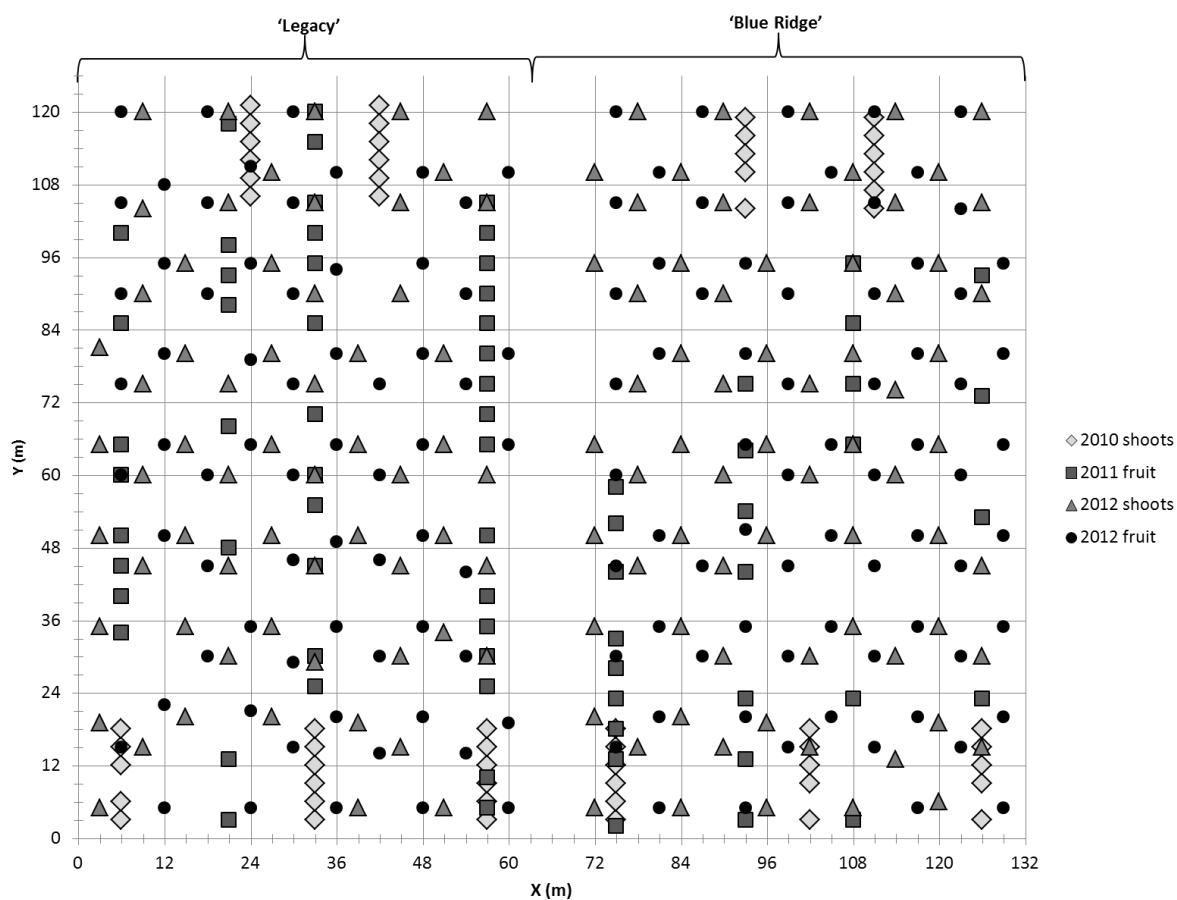


Fig. 3.1. Map of the location where shoots and fruit infected by *Monilinia vaccinii-corymbosi* were collected in 2010, 2011 and 2012 in Ivanhoe, North Carolina. Sampling in rows from 0 to 62 m were from cultivar ‘Legacy’ and 65 to 132 m from cultivar ‘Blue Ridge’. The X axis represents rows of blueberry planted at 3 m between rows and the Y axis plants within rows spaced at 1 m between plants.

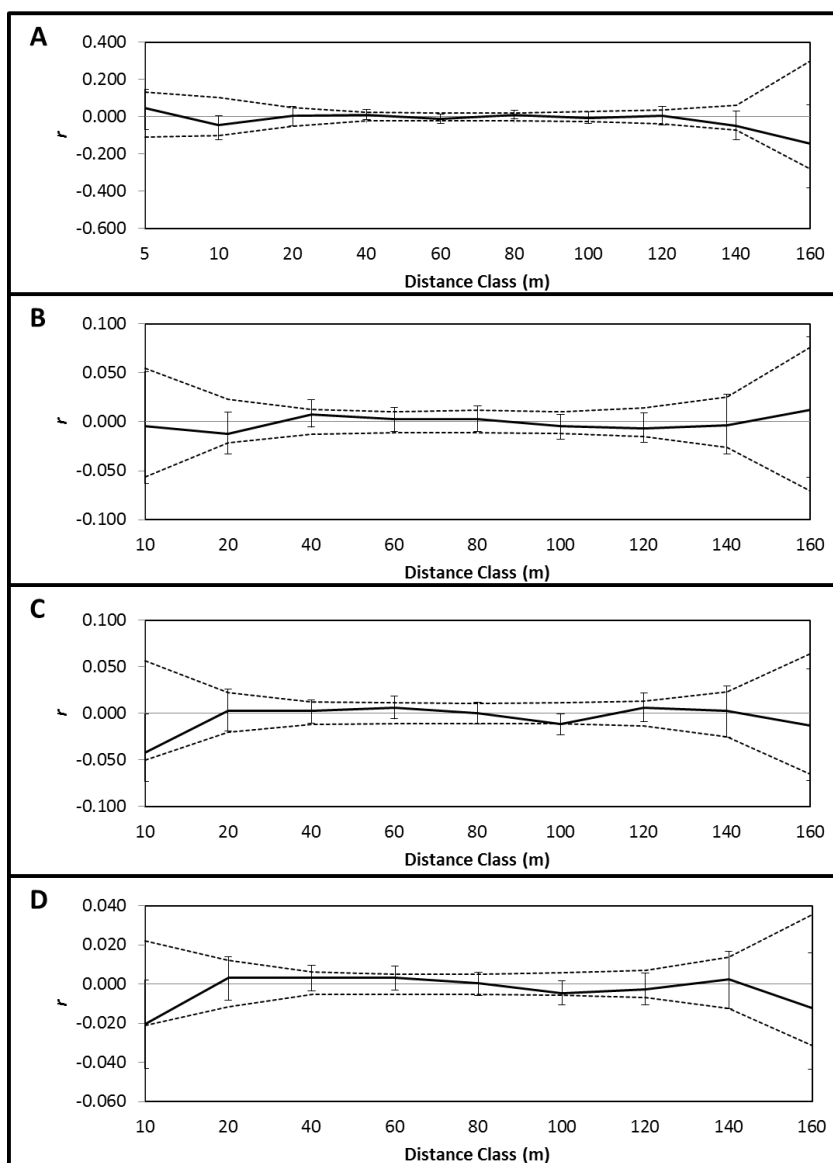


Fig. 3.2. Correlograms generated by spatial autocorrelation analysis. The thick line in each correlogram represents the r correlation coefficient at each distance class. Dotted lines denote the 95% confidence interval based on random permutation. Error bars indicate 95% confidence intervals generated through bootstrap resampling. A= isolates of *Monilinia vaccinii-corymbosi* (Mvc) from infected fruit collected in 2011. B= analysis with isolates of Mvc from infected shoots collected in 2012. C= analysis with isolates of Mvc from infected fruit collected in 2012. D= all isolates of Mvc collected in 2012 analyzed together.

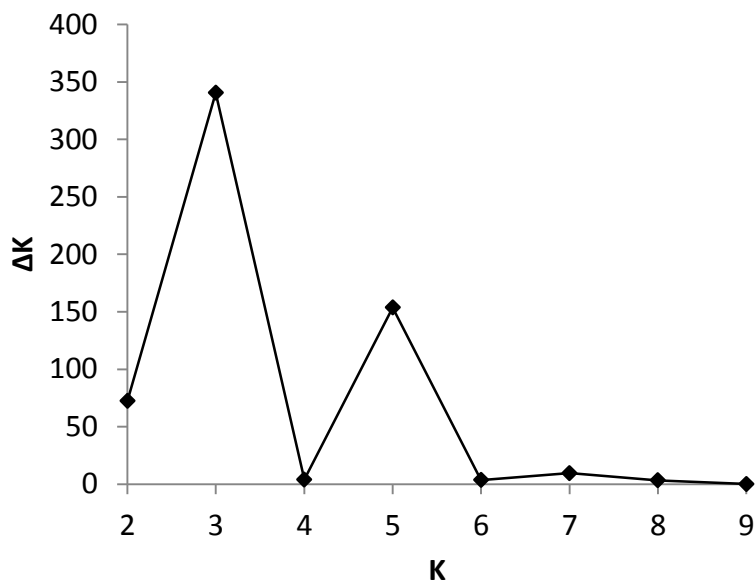


Fig. 3.3. Calculated values of ΔK based on the change in $\text{LnP}(K)$ over 20 runs at each value of K using STRUCTURE and the Evanno method.

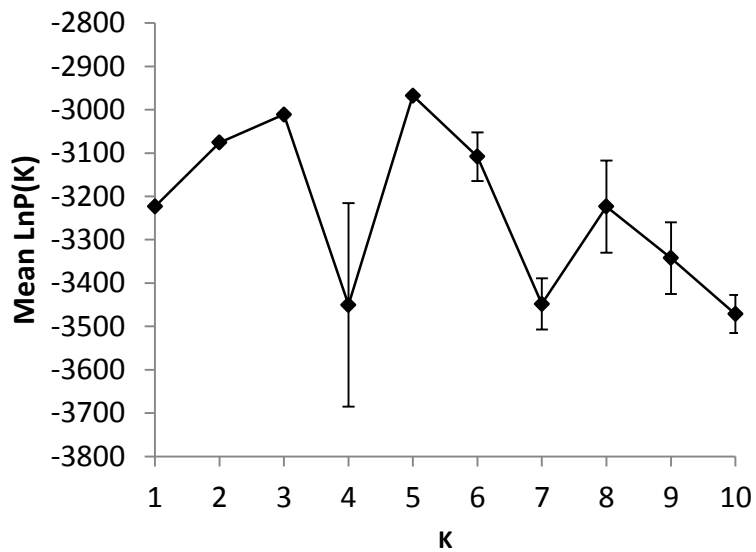


Fig. 3.4. Results from analysis with STRUCTURE, with 20 runs at each value of K used to determine the mean $\text{LnP}(K)$. Error bars represent standard deviation of the mean.

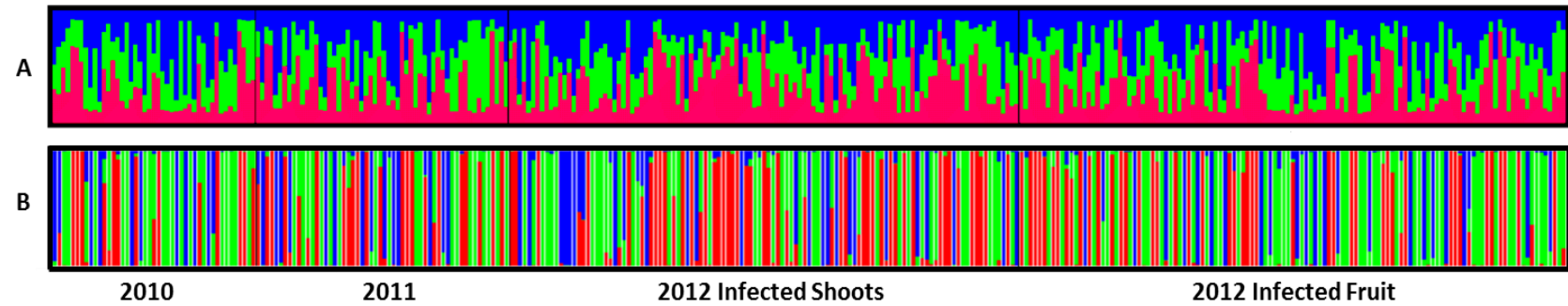


Fig. 3.5. Membership probabilities of each isolate to $K=3$ clusters based on analysis with STRUCTURE (A) and discriminant analysis of principal components (B).

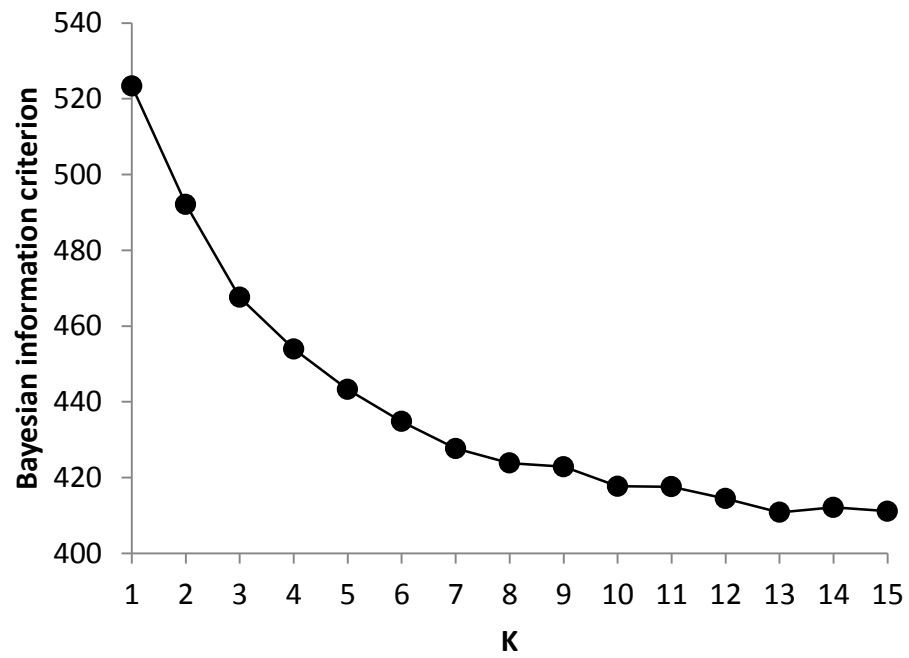


Fig. 3.6. Bayesian information criterion as calculated with the K-means algorithm implemented in adegenet at each value of K from 1 to 15.

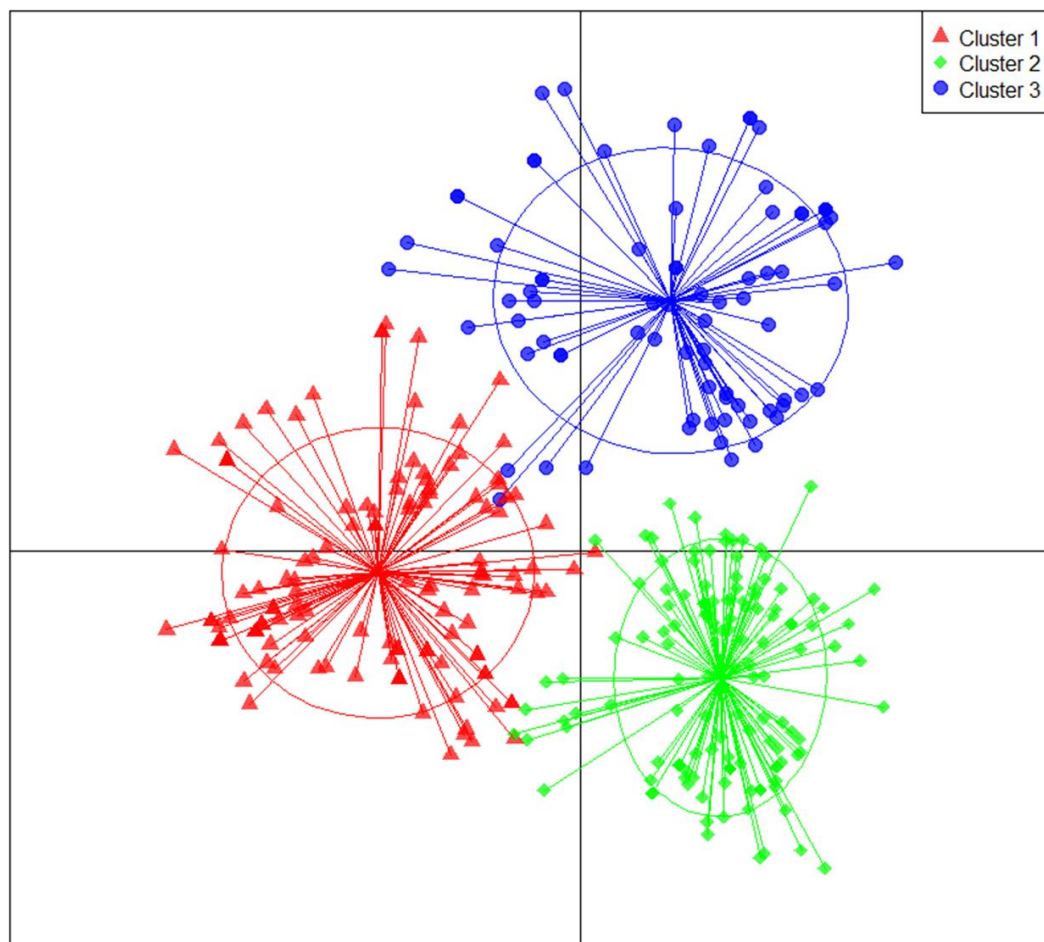


Fig. 3.7. Scatterplot from discriminant analysis of principal components based on $K=3$ clusters.

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CHAPTER IV

Single ascospore progeny support outcrossing and self-fertility within a population of *Monilinia vaccinii-corymbosi*

Abstract

The life history of *Monilinia vaccinii-corymbosi* (Mvc), a fungus that infects the shoots and fruit of blueberry (*Vaccinium* spp.), involves both asexual and sexual reproduction. Infected fruit are desiccated to form pseudosclerotia that serve as overwintering structures. Sexually-derived reproductive structures called apothecia are produced on pseudosclerotia. Apothecia forcibly eject sexual spores (ascospores) that are dispersed by wind to infect newly emerging blueberry shoots. Asexual spores (conidia) are produced on infected shoots and spread by insects to flowers where they invade the ovary through the gynoecial pathway. Previous research has reported high genetic diversity within populations of Mvc. In this study, the genetic diversity of 92 isolates of Mvc within the locules of 24 infected blueberry fruit of southern highbush (*V. corymbosum* x *V. darrowii*) cultivar 'Blue Ridge' and 190 single ascospore isolates of Mvc from 21 apothecia was examined. DNA was extracted from each isolate and analyzed with eight polymorphic microsatellite markers to determine their multilocus haplotype (MLH). One or two genetically unique isolates of Mvc were detected within individual fruits arbitrarily sampled from eight blueberry bushes. Single ascospore isolates analyzed from each of eight apothecia had identical MLHs, while at least two genetically distinct MLHs were detected from analyzing isolates from 13 apothecia. Tests for

linkage disequilibrium suggested that genetically diverse ascospore progeny were the product of sexual recombination. This study is the first to suggest both outcrossing and self-fertilizing capabilities in Mvc and the presence of genetic diversity within infected blueberry fruit.

Introduction

Mummy berry disease on blueberry (*Vaccinium* spp.) is caused by the fungus *Monilinia vaccinii-corymbosi* (Reade) Honey (Mvc). Both asexual and sexual reproduction are necessary within a growing season for the fungus to complete its life cycle (Batra 1983). The fungus overwinters inside of desiccated infected fruit (pseudosclerotia) on the soil surface. After environmental conditioning sexual structures (apothecia) are produced from pseudosclerotia and forcibly discharge sexual spores (ascospores) that infect newly emerging blueberry shoots (Batra 1983; Lehman & Oudemans 1997; Milholland 1974). Infected shoots become blighted and asexual spores (conidia) are produced that attract insects through olfactory and visual cues (Batra & Batra 1985; Shinnars & Olson 1996). Pollinating insects transport conidia to blueberry flowers, with Mvc using aggressive mimicry to infect the ovary through the gynoecial pathway (Cox & Scherm 2001; Ngugi & Scherm 2004). Infected fruit eventually desiccate and detach from the bush, serving as a means of pathogen survival.

The ovary of a blueberry flower contains approximately 65 ovules, all of which need to be fertilized by pollen tubes to form seeds that are contained within five compartments called locules (Trehane 2004). Germination of pollen on the stigma and directional growth of pollen tubes through the style is controlled by chemical signals in the gynoecial pathway

(Lord & Russell 2002; Ngugi & Scherm 2004). Research suggests directional growth of hyphal germ tubes of *Mvc* through the style by mimicking pollen tubes and exploiting the plant-based signals (Ngugi & Scherm 2004). Hyphae have the greatest rate of growth through the style on day of flower anthesis and can reach the ovary within four days post inoculation (Ngugi *et al.* 2002). Hyphal growth rate and infection success decreases with an increase in flower age, and fruit disease incidence also decreases when flowers are pollinated at least one day prior to inoculation (Ngugi *et al.* 2002). However, the gynoecial pathway does not rapidly shut down after pollination, allowing for the ingress of pollen tubes and hyphal germ tubes over several days (Ngugi *et al.* 2002). Once reaching the ovary, immature seeds are colonized by hyphae and become necrotic (Shinners & Olson 1996). Some or all locules inside a fruit may be colonized by *Mvc* (Lehman *et al.* 2007).

Recently, a comprehensive examination of genetic structure, gene flow and genetic diversity of *Mvc* within a 120 x 132 m blueberry plot in Ivanhoe, NC was conducted (see CHAPTER III). Results supported high genetic diversity and at least three genetically differentiated populations of *Mvc* within the plot. Also, spatial autocorrelation analysis suggested that gene flow was unrestricted at the sampled spatial scale. However, hierarchical partitioning of genetic variation was not examined in the study. Genetically diverse isolates of *Mvc* may exist on a single blueberry bush. Based on the phenology of flowers and their receptiveness to infection over several days post anthesis, it is also possible that several genetically unique isolates of *Mvc* may colonize a single fruit. Hierarchical partitioning of

genetic variation within a field should be considered as it may influence the population dynamics and disease ecology of *Mvc*.

Fungi in the phylum Ascomycota typically produce ascospores by either outcrossing or self-fertilization (Billiard *et al.* 2012; Heitman *et al.* 2007). Previous research in the blueberry plot in Ivanhoe, NC supported the presence of linkage disequilibrium and the repeated recovery of the same multilocus haplotypes (MLHs) over three years of sampling within a single commercial field of blueberry (see CHAPTER III), suggesting inbreeding and/or self-fertility (Billiard *et al.* 2012; Heitman *et al.* 2007; Milgroom 1996). However, high genetic diversity within the field may be an indicator of outcrossing (Milgroom 1996). The presence of several genetically different isolates of *Mvc* within a fruit may promote outcrossing and generation of genetic diversity via sexual recombination. A mating event between genetically different isolates should result in ascospore progeny that are genetically recombinant. Since *Mvc* is haploid, the ascospores also should have a single allele at each locus derived from either the maternal or paternal fungal strain as a result of crossing over. Neutral genetic markers can be used to determine if ascospores are genetically recombinant.

Polymorphic microsatellite markers were previously developed for *Mvc* and used to study the large and fine-scale population genetics of *Mvc* (Molecular Ecology Resources Primer Development Consortium *et al.* 2012, see CHAPTERS II and III). The objectives of this study were to use the markers to 1) examine genetic diversity of isolates of *Mvc* within the locules of infected fruit and 2) determine if single ascospore progeny from the same apothecium are genetically identical or recombinant. A preliminary examination of genetic

diversity and similarity of isolates of Mvc recovered from the same blueberry bush was also conducted.

Materials and methods

Sample collection

This study was conducted in a commercial blueberry field located in Ivanhoe, NC. Sampling was conducted within a plot of southern highbush blueberry (*V. corymbosum* x *V. darrowii*) cultivar 'Blue Ridge' that had a history of disease. The plot consisted of 22 rows spaced 3 m apart and with approximately 120 bushes planted at 1 m intervals within the plot. Pseudosclerotia with apothecial initials were arbitrarily collected within the plot on 3 March 2011 and 21 February 2013. Typically pseudosclerotia with apothecial initials were found in moist areas of soil within the plot directly underneath bushes. Pseudosclerotia were placed in a plastic fruit clamshell and stored at 4°C until use. Infected fruit that were still attached to bushes were also sampled on 29 May 2013. At this time, the skin of infected fruit had begun to turn a salmon pink color while healthy fruit remained green, allowing us to differentiate and sample infected fruit. Three infected fruit were removed from eight bushes spaced in the plot, with samples collected from the 10th and 110th bushes in rows 3, 11 and 19 and samples collected from the 60th bush in rows 7 and 19. Infected fruit were placed in Ziploc bags and stored on ice in the field until they could be stored at 4°C in the lab.

Apothecia production, ascospore germination, and isolation of Mvc from infected fruit

A layer of sand approximately 1 cm thick was added to rectangular glass containers (10.0 x 6.0 x 6.5 cm) and distilled water was added until saturation. Pseudosclerotia were transferred to the containers with forceps and placed on the saturated sand with the stipes of apothecial initials placed upright. The pseudosclerotia were spaced at least 1 cm apart in the boxes, with approximately 12 pseudosclerotia per container. Additional distilled water was added to each pseudosclerotium to moisten their surface, and glass lids were placed on top of the containers. The containers were placed under a 40-W fluorescent light with a photoperiod of 14 h at room temperature (approximately 20°C) for up to three weeks to allow for the development of mature apothecia.

Mature, fully expanded apothecia were excised from pseudosclerotia and affixed to lids of Petri dishes containing half strength Potato dextrose agar (PDA) amended to 2% agar (12 g Difco potato dextrose broth [PDB] and 20 g Difco agar per L [Becton, Dickinson and Company, MD]) with Vaseline. The pseudosclerotium that the apothecium was removed from was recorded to examine several apothecia produced from the same pseudosclerotium. The apothecia were attached near the edge of the Petri dish lid and were positioned to release their ascospores towards the agar surface. The lid was rotated 90° every hour for 3 h to eject ascospores on the agar surface. After incubating at room temperature for at least 12 h, single germinated ascospores were transferred to half strength PDA plates containing 1.5% agar (15g agar per L) with a sterile scalpel under a dissecting microscope. Up to 14 germinated ascospores were transferred per apothecium.

Infected fruit were surface sterilized for 30 sec in 10% NaOCl followed by 30 sec in 70% EtOH and rinsed in sterilized water for 30 sec. Infected fruit were cut in half with a sterile scalpel and the number of locules colonized by Mvc was recorded. A piece of mycelium was removed from each infected locule and transferred to half-strength PDA containing 1.5% agar using a scalpel that was sterilized between transfers.

DNA extraction and PCR with microsatellite markers

Isolates of Mvc derived from single ascospores or from the locules of infected fruit were incubated at room temperature for approximately 14 d. Each isolate was subsequently grown in liquid culture and the mycelium was used for DNA extraction with the cetyltrimethylammonium bromide (CTAB) and chloroform extraction method and isopropanol precipitation as previously described (Molecular Ecology Resources Primer Development Consortium *et al.* 2012) . The DNA extracts were used to perform PCR using eight polymorphic microsatellite markers; Mvc 7, 9, 10, 12, 23, 27, 28, and 30 (Molecular Ecology Resources Primer Development Consortium *et al.* 2012). The PCR reactions were performed with two markers duplexed in the reactions; Mvc7 with Mvc9, Mvc10 with Mvc27, Mvc12 with Mvc23, and Mvc28 with Mvc30. Duplexed PCR reaction setup and thermocycle conditions were as previously described (see CHAPTER II), with duplexed reactions Mvc7-9 and Mvc10-27 labeled with fluorescent tag 6-FAM and Mvc12-23 and Mvc28-30 labeled with NED. PCR products were multiplexed before submission for fragment analysis as well, with PCR products from Mvc7-9 combined with Mvc12-23 and Mvc10-27 with Mvc28-30. Fragment analysis was performed to determine the length of the

PCR product (i.e. allele) at the microsatellite loci as previously described (Molecular Ecology Resources Primer Development Consortium *et al.* 2012). The multilocus allelic profile of the isolates (i.e. multilocus haplotype, MLH) was subsequently analyzed.

Analysis of isolates from infected fruit

The software GenAIEx version 6.5 was used to detect isolates of *Mvc* with genetically unique or identical MLHs within a single infected fruit based on their allelic profiles (Peakall & Smouse 2006, 2012). The presence of isolates of *Mvc* with the same MLH in different fruit was also examined. Mean unbiased haploid diversity was calculated with GenAIEx based on all isolates from the three fruit per bush. The genetic similarity of all isolates from the eight bushes was examined by principal coordinates analysis (PCoA) with GenAIEx. This was done to visually assess if isolates from the same bush were more genetically similar to each other than to isolates recovered from the other bushes. A genetic distance matrix based on pairwise differences in alleles between isolates was first generated for input into PCoA.

Analysis of single ascospore isolates

GenAIEx was used to determine the number of ascospores with unique MLHs produced by a single apothecium. Mean unbiased haploid diversity was also calculated with GenAIEx for apothecia with a sample size of ≥ 8 single ascospore isolates. Two analyses for linkage disequilibrium were performed to test for sexual recombination. First, tests for significant linkage disequilibrium between pairs of the microsatellite markers were performed using the online implement of Genepop version 4.2 with 10,000 dememorizations,

1,000 batches and 10,000 iterations per batch (Raymond & Rousset 1995; Rousset 2008). The method of Benjamini and Hochberg (1995) was applied to correct the P values for multiple comparisons and identify markers in significant linkage disequilibrium (adjusted $P < 0.05$). The index of association (I_A) was also calculated as a measure of linkage disequilibrium with the R package poppr using 10,000 permutations to generate P values and test for significance at $P < 0.05$ (Kamvar *et al.* 2013). Both analyses were conducted separately for each apothecium that had a sample size of ≥ 8 single ascospore isolates and produced ascospores with at least two unique MLHs.

Results

Genetically unique isolates of Mvc may colonize a single fruit

From 24 infected blueberry fruit examined, three locules were infected within three fruit, four locules within seven fruit, and five locules within 14 fruit (Fig. 4.1). A total of 92 pure cultures of Mvc from the 24 infected fruit were successfully generated and analyzed. An isolate of Mvc was successfully obtained from all infected locules within 16 fruit. An isolate of Mvc was not recovered from at least one infected locule in the remaining eight fruit, which was carefully considered in data interpretation. Either one or two genetically distinct MLHs were detected within each infected fruit. Nine fruit contained at least two genetically unique MLHs. In the nine fruit one of the MLHs was detected in only a single locule, while the second MLH was detected in all other analyzed locules. Four MLHs (herein MLH1, 2, 3, and 4) were detected in more than one infected fruit; MLH1 was detected within four fruit

from three bushes, MLH2 and MLH3 were each detected within two fruit on different bushes, and MLH4 within two fruit on the same bush. Based on a previous study, MLH1 was also repeatedly detected in the plot from 2010 to 2012 (see CHAPTER III). Two to four genetically unique MLHs were detected when analyzing all isolates from the three fruit on the same bush. Haploid diversity when analyzing all isolates from the same bush ranged from 0.333 to 0.490, with a mean of 0.419 (S.E. \pm 0.019). Principal coordinates analysis indicated that genetically unique isolates recovered from the same bush were not necessarily genetically similar as clustering of isolates from the same bush was not visually apparent (Fig. 4.2). In the analysis axis one explained 30.8% and axis two 21.9% of genetic variation among MLHs.

Single ascospore isolates suggest self-fertilization and outcrossing

A total of 190 single ascospore isolates were obtained from 21 apothecia produced by 16 pseudosclerotia. A minimum of two and maximum of 14 single ascospore isolates were generated from each apothecium (mean =9.05, S.E. =0.874). When analyzing single ascospore isolates from each apothecium separately, the isolates recovered from eight apothecia had the same MLH, while 13 apothecia produced ascospores with more than one genetically unique MLH (Fig. 4.3). One or two alleles were detected at each microsatellite locus when analyzing isolates from the same apothecium. More than one apothecium was analyzed from four pseudosclerotia. One pseudosclerotium produced three apothecia that were used to generate 36 single ascospore isolates, all of which had the same MLH. Another pseudosclerotium produced two apothecia that were used to generate 17 isolates. Sixteen

unique MLHs were detected from the 17 isolates, with two or three alleles detected at each of the eight microsatellite loci. Two apothecia and 26 isolates were recovered from another pseudosclerotium. While 13 MLHs were detected from the 26 isolates, one of the MLHs was represented by isolates from both apothecia. Similarly, a pseudosclerotium collected in 2011 that produced two apothecia and 15 isolates consisting of nine MLHs, with two of the MLHs being detected in both apothecia sampled. Furthermore, one of the MLHs detected in both apothecia was also represented by an isolate from an apothecium sampled in 2013. At least eight single ascospore isolates were obtained from 13 apothecia. Tests for linkage disequilibrium could not be conducted on seven of the 13 apothecia since they produced single ascospore isolates that had the same MLH. Index of association was not significant for the six apothecia with at least two MLHs detected ($\alpha = 0.05$, Table 4.1). No microsatellite marker pairs were in significant linkage disequilibrium (adjusted $\alpha = 0.05$). The mean haploid diversity for isolates from the six apothecia ranged from 0.187 to 0.536, with an overall mean of 0.336 (S.E = 0.046).

Discussion

This study is the first to report the occurrence of more than one genetically different isolates of Mvc in the locules of a single blueberry fruit. Evidence that Mvc is capable of both outcrossing and self-fertilization was also found. Results from this study support those from a previous study within the same plot in Ivanhoe, NC (see CHAPTER III). Previous research indicated that several microsatellite marker pairs were in significant linkage disequilibrium

when analyzing isolates collected in the plot over three years. Furthermore, several MLHs were repeatedly detected within the plot over three years that were statistically not likely to be the product of random mating, supporting inbreeding and/or self-fertilization. However, high genetic diversity was detected within the plot, suggesting the presence of sexual recombination. The present study provides additional evidence for self-fertilizing and outcrossing capabilities in *Mvc* based on the analysis of ascospore progeny from the same apothecium.

Single ascospore isolates have been studied in other fungi to examine sexual recombination. Gladieux *et al.* (2011) used microsatellite markers to determine that pseudothecia of *Venturia inaequalis* can produce genetically different ascospore progeny. In contrast, the fungus *Teratosphaeria nubilosa* produced pseudothecia with genetically identical ascospore progeny based on analysis with microsatellite markers (Perez *et al.* 2010). A mixed mating system with both self-fertilization and outcrossing was suggested in several populations of the chestnut blight fungus *Cryphonectria parasitica* by analyzing ascospores from perithecia (Marra *et al.* 2004; Milgroom *et al.* 1993). This study further demonstrates that single ascospore isolates are useful at elucidating the reproductive biology of fungi.

Outcrossing was detected as 13 apothecia produced single ascospore isolates with at least two different MLHs. Since polymorphisms were commonly detected at more than one locus it is unlikely that genetic differences were due to mutation alone. Furthermore, analyses for linkage disequilibrium suggested that the ascospores were the result of sexual recombination. Eight apothecia were inferred to be the product of self-fertilization since they

produced single ascospore isolates with same MLH. However, mating between closely related isolates could also result in ascospore isolates with identical MLHs. This explanation seems unlikely based on high genetic diversity and polymorphisms at the eight microsatellite markers examined. Further support for self-fertilization in *Mvc* was the detection of a MLH (MLH1) within four infected fruit that was previously detected multiple times over the prior three years of sampling (see CHAPTER III). Previous research determined that this repeatedly detected MLH was highly unlikely to be the product of random mating (see CHAPTER III). Additional experiments are needed to better confirm that isolates with repeatedly detected MLHs are capable of self-fertilizing.

In the phylum Ascomycota the most commonly reported mating systems are heterothallic and homothallic mating (Billiard *et al.* 2012; Heitman *et al.* 2007). Heterothallic fungi are self-sterile and mate with a genetically compatible isolate to produce ascospores. Since evidence for self-fertilizing capabilities in *Mvc* was found, it is unlikely that the mating system is predominantly heterothallic. In contrast, homothallic fungi are able to self-fertilize, giving rise to genetically identical ascospore progeny. However, outcrossing is not precluded in homothallic fungi as genetically different isolates can still undergo sex. It is therefore probable that the mating system of *Mvc* more closely reflects homothallism. The closely related fungus *Sclerotinia sclerotiorum* is homothallic (Amselem *et al.* 2011; Holst-Jensen *et al.* 1997). To verify that *Mvc* is homothallic, flowers would need to be inoculated with a single isolate of *Mvc* and the resulting pseudosclerotia would need to be examined to

determine whether they give rise to apothecia and fertile ascospores that exhibit the same MLH as the isolate used for the initial inoculation.

While homothallic and heterothallic mating systems are well documented in fungi in the Ascomycota, other mating systems have been reported. For example, the mating system of *Sclerotinia trifoliorum* has been classified as “bipolar heterothallic” and allows for self-fertilization as well as outcrossing (Ekins *et al.* 2006; Uhm & Fujii 1983a, b). The eight ascospores within an ascus of *S. trifoliorum* are dimorphic, with four ascospores being smaller than the other four. The larger ascospores are self-fertile, while the smaller ascospores are self-sterile and can only be fertilized by spermatia (microconidia) of larger ascospore isolates (Uhm & Fujii 1983a, b). Interestingly, Batra (1983) previously reported three types of ascospores produced by Mvc based on their morphology. The first spore type produces granules, one or two germ tubes, and has a normal cytoplasm. The second spore type does not have granules, has an alveolate cytoplasm, may have abortive germ tubes, and produces microconidia. The third type of ascospore is smaller, does not have granules, has a scant cytoplasm, and does not produce germ tubes or microconidia. Research is needed to determine if ascospores with different morphologies influence mating in Mvc.

More than one genetically unique isolate of Mvc was detected within nine of 24 infected fruit. Several genetically dissimilar isolates of Mvc may also exist on a single blueberry bush. This suggests the presence of hierarchical genetic variation that needs to be examined further. A limitation of this investigation of isolates within locules of the same fruit was sample size. While an isolate of Mvc was cultured from all infected locules within 16

fruit, a culture of Mvc could not be obtained from all locules within eight fruit, limiting the conclusions. Also, only isolates within three infected fruit per bush were analyzed. While this allowed us to gain preliminary evidence that isolates recovered from the same bush are not necessarily more genetically similar than those from other bushes, this study should be repeated with a larger sample size. For example, Everhart (2012) studied genetic diversity and spatial structuring of *Monilinia fructicola* within peach tree canopies. A large sample size and spatial mapping of isolates within the canopies allowed for the detection of high genetic diversity and positive spatial autocorrelation at distances <1m within canopies. Previous research supported the lack of positive spatial autocorrelation within the 120 x 132 m plot, possibly because the sampling scheme did not consider genetic diversity and structure within individual blueberry canopies and fruit (see CHAPTER III).

It is important to consider the reproductive biology of plant pathogenic fungi to develop appropriate disease management strategies (McDonald & Linde 2002). Sexual recombination can aid in creating progeny capable of surviving in the presence of management strategies such as fungicides and genes for resistance (R genes). Selfing via homothallic reproduction and asexual reproduction can propagate and maintain isolates with advantageous combinations of alleles. Thus, fungi with mixed mating systems such as Mvc have been hypothesized to possess a greater evolutionary advantage and are therefore more challenging to manage (McDonald & Linde 2002).

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Tables and Figures

Table 4.1. Descriptive statistics for 13 apothecia with single ascospore isolate sample sizes greater than eight. Sample ID lists the apothecium (A) and pseudosclerotium (P) number corresponding to Fig. 4.3. N= number of single ascospore isolates, MLHs= number of unique multilocus haplotypes detected, I_A = index of association with P value in parentheses, uh = haploid diversity with standard error in parentheses. Dashes indicate that the analysis could not be performed due to a MLH sample size of one.

Sample ID	N	MLHs	I_A	uh
A1 P1	13	1	-	-
A2 P1	11	1	-	-
A3 P1	12	1	-	-
A4 P2	14	1	-	-
A5 P3	13	1	-	-
A7 P5	10	1	-	-
A8 P6	9	1	-	-
A9 P7	14	5	0.2503 (0.0772)	0.187 (0.092)
A11 P9	8	5	-0.0592 (0.667)	0.272 (0.103)
A12 P7	12	9	0.0623 (0.3604)	0.267 (0.101)
A14 P10	14	12	0.0446 (0.3673)	0.378 (0.083)
A15 P11	11	10	0.2565 (0.0986)	0.536 (0.006)
A17 P12	13	13	-0.2967 (0.9991)	0.378 (0.083)

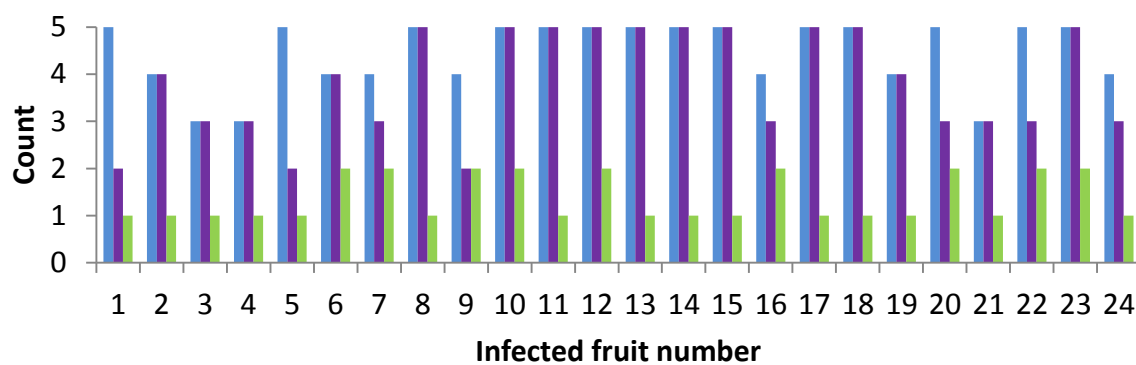


Fig. 4.1. Number of infected locules (blue bars), isolates of *Monilinia vaccinii-corymbosi* successfully cultured from locules (purple bars), and unique multilocus haplotypes recovered for each of 24 infected fruit.

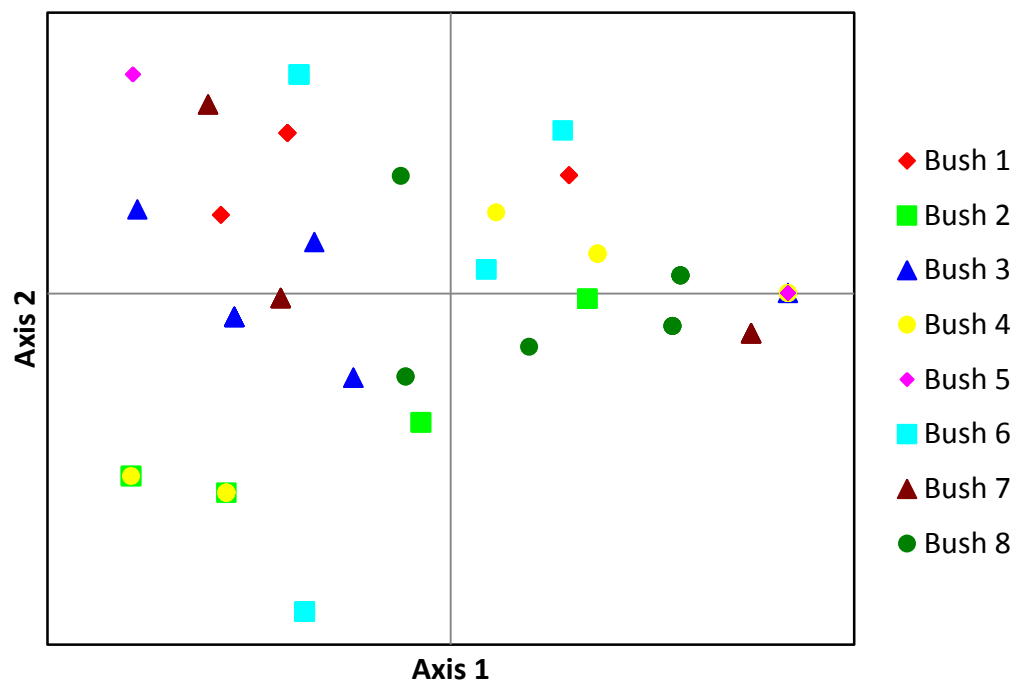


Fig. 4.2. Principal coordinates analysis based on genetic similarity of isolates of *Monilinia vaccinii-corymbosi* with different multilocus haplotypes detected within the locules of three fruit from eight southern highbush blueberry bushes.

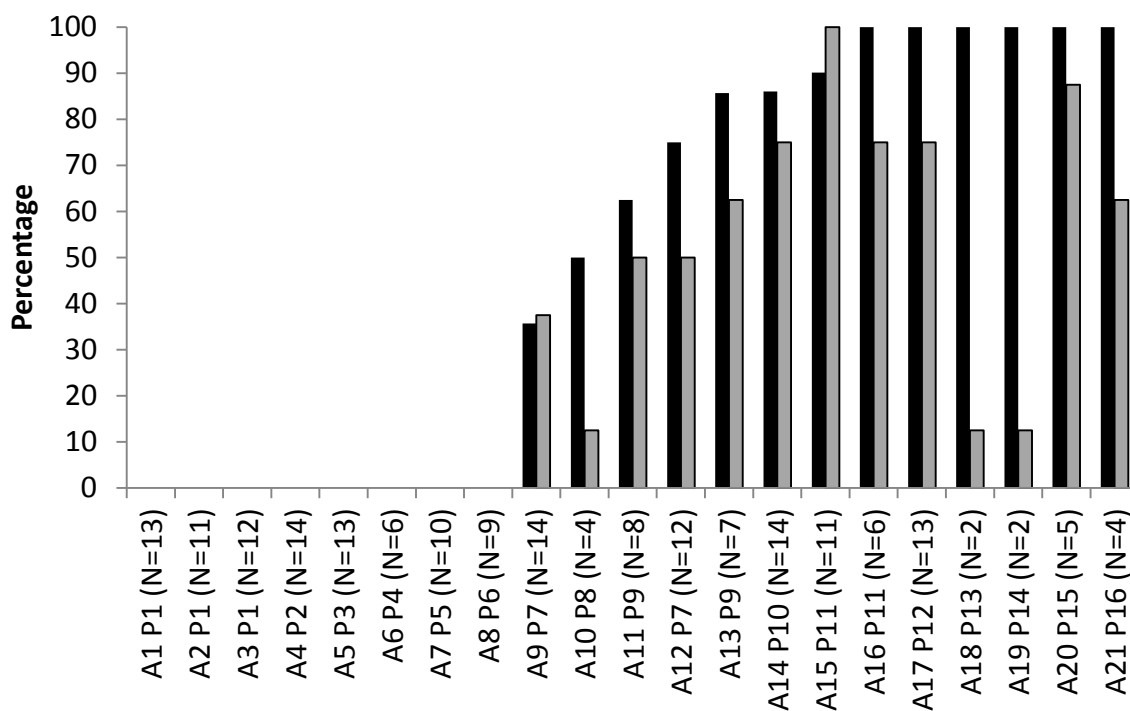


Fig. 4.3. Analysis of single ascospore isolates of *Monilinia vaccinii-corymbosi* from 21 apothecia. A= apothecium number, P= pseudosclerotium number that the apothecium was derived from, and N= number of single ascospore isolates analyzed. Black bars indicate percent genetically unique single ascospore isolates and grey bars denote percentage of polymorphic loci.

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