Low Genetic Diversity Suggests the Recent Introduction of Dogwood Powdery Mildew to North America

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Abstract

Cornus florida (flowering dogwood) is a popular understory tree endemic to the eastern hardwood forests of the United States. In 1996, dogwood powdery mildew caused by *Erysiphe pulchra*, an obligate biotrophic fungus of large bracted dogwoods, reached epidemic levels throughout the *C. florida* growing region. In the late 1990s, both sexual and asexual stages of *E. pulchra* were regularly observed; thereafter, the sexual stage was found less frequently. We examined the genetic diversity and population structure of 167 *E. pulchra* samples on *C. florida* leaves using 15 microsatellite loci. Samples were organized into two separate collection zone data sets, separated as eight zones and two zones, for the subsequent analysis of microsatellite allele length data. Clone

correction analysis reduced the sample size to 90 multilocus haplotypes. Our study indicated low genetic diversity, a lack of definitive population structure, low genetic distance among multilocus haplotypes, and significant linkage disequilibrium among zones. Evidence of a population bottleneck was also detected. The results of our study indicated a high probability that *E. pulchra* reproduces predominately via asexual conidia and lend support to the hypothesis that *E. pulchra* is an exotic pathogen to North America.

Keywords: asexual reproduction, biotrophic fungus, microsatellite loci, multilocus haplotypes

Cornus florida L., flowering dogwood, is a deciduous understory tree endemic to the eastern United States (Boring et al. 1981; Halls 1977; Johnson 1961). The drupes of *C. florida* are high in fat, calcium, and other nutrients, thus making it a valuable source of food for wildlife (Gill and Healy 1974; Halls 1977; Mitchell et al. 1988). Known for its attractive bracts, drupes, and appealing autumn foliage, *C. florida* is highly valued for its ornamental qualities (Johnson 1961). In 2014, nursery sales of dogwood cultivars and those produced from seed sources surpassed \$27 million in the United States, with Tennessee accounting for approximately 25% of sales (USDA N.A.S.S., 2014).

Until the advent of *Discula destructiva* Redlin, the causal agent of dogwood anthracnose, in the 1970s (Redlin 1991) and *Erysiphe pulchra* Cooke & Peck, dogwood powdery mildew in 1993 and 1994 (Britton 1994; Hagan and Mullen 1997), disease management costs to produce *C. florida* trees were estimated to be approximately \$49/acre/year. Disease management costs (≈\$800/acre/year) increased significantly since the occurrence of these diseases, causing many small nurseries to end *C. florida* production (Li et al. 2009).

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A powdery mildew disease on *C. florida* caused by *Phyllactinia guttata* was first reported in Illinois in 1887 (Burrill and Earle 1887). However, reports of powdery mildew on *C. florida* were rare until 1993 and 1994, when powdery mildew caused by *E. pulchra* occurred statewide in Georgia and Alabama, respectively (Britton 1994; Hagan et al. 1998; Hagan and Mullen 1997; Li et al. 2009). Klein et al. (1998) reported that *P. guttata* does not infect *C. florida*, and that the chasmothecia most likely became associated with *C. florida* after dissemination from *C. anomum*. During subsequent years, powdery mildew caused by *E. pulchra* has been regularly observed throughout the *C. florida* growing region (Daughtrey and Hagan 2001; Hagan and Mullen 1997).

E. pulchra is an obligate biotrophic fungus that causes powdery mildew of three big-bracted dogwoods (*C. kousa*, *C. florida*, and *C. nuttallii*) and can be identified by the characteristic signs of white mycelium, conidiophores, and conidia on the adaxial surface of leaves (Li et al. 2007, 2009; Smith 1999; Windham et al. 2005). Disease symptoms include the impaired growth of trees, necrosis of young leaves, red pigmentation near infected areas in leaves, and decreased flower and subsequent fruit production (Klein et al. 1998; Williamson and Blake 1999; Windham 1996). Although *C. kousa* has been evaluated as very tolerant to powdery mildew (Li et al. 2007, 2009), complete or partial resistance to powdery mildew in seedlings of *C. florida* from open pollinated sources occur at a frequency of 0.001 (Windham and Witte 1998). Breeding for resistance is difficult and time-consuming because the average generation time for flowering dogwood is often 7 years or more. Therefore, cultivars have been selected from seedlings that have demonstrated field resistance and desirable ornamental characteristics (Trigiano et al. 2016; Windham et al. 2005). Resistance in some of these cultivars is attributable to either restricted branching of the primary germ tube without affecting germination of conidia or slower disease progress (longer latent periods) (Li et al. 2005, 2009).

E. pulchra reproduces asexually via conidia and sexually through the development of ascospores within chasmothecia (Daughtrey and Hagan 2001; Hagan and Mullen 1997; Mmbaga 2002). Ascospores were thought to be the primary source of inoculum each year, and air-borne conidia acted as a secondary inoculum source (Mmbaga 2002).

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Both the sexual and asexual stages were observed when *E. pulchra* reached epidemic levels in the eastern United States in 1995 (Li et al. 2009; Mmbaga 2002; Williamson and Blake 1999). However, in recent years, the sexual stage has been observed less frequently (personal observation). It is uncertain whether *E. pulchra* in the eastern United States has become clonal because it reproduces primarily via asexual conidia.

Population genetics can be used as a vital tool for better utilization of control methods for various plant pathogens (McDonald and Linde 2002; McDonald and McDermott 1993). The study of population genetics has been utilized to locate the probable center of origin for pathogens such as *Phytophthora infestans* (Goss et al. 2014; Grünwald and Flier 2005), *Mycosphaerella graminicola* (Stukenbrock et al. 2006), and *P. ramorum* (Goss et al. 2009, 2011; Kamvar et al. 2015a). Population genetics also supports the understanding of genetic patterns of emergence and primary means of reproduction of a given pathogen (Goss et al. 2009, 2014; Grünwald et al. 2017).

Microsatellite loci or simple sequence repeats (SSR) are tandem repeats of one to six nucleotide base pairs that are flanked by highly conserved regions and distributed throughout most eukaryotic genomes (Jarne and Lagoda 1996; Moges et al. 2016; Selkoe and Toonen 2006); they have become widely used DNA markers in population studies (Benali et al. 2011; Selkoe and Toonen 2006). These regions typically have high mutation rates; therefore, they can potentially be utilized to differentiate and identify individuals in the species of interest (Jarne and Lagoda 1996; Moges et al. 2016; Selkoe and Toonen 2006).

There is currently limited information regarding the genetic diversity and population structure of *E. pulchra* (Mmbaga et al. 2000). Because of the apparent infrequency of sexual reproduction, we hypothesized that *E. pulchra* reproduces almost exclusively via conidia and has become a clonal organism in the eastern United States. To test this hypothesis, species-specific microsatellite loci were developed and utilized to characterize the genetic diversity and population structure of *E. pulchra* in the eastern United States. For the purposes of this study, clonality was considered synonymous with predominant asexual reproduction. If that was the case, then we further hypothesized that *E. pulchra* is clonal in the eastern United States, has low genetic diversity, has few private alleles, has a lack of definitive population structure, and has significant linkage disequilibrium. It should also be noted that not all these conditions are necessary or expected in all clonally reproducing populations.

Materials and Methods

Sample collection and DNA extraction. One hundred eighty-eight samples of *E. pulchra* collected from fresh leaves of *C. florida* growing on forest and landscape trees were used in this study (Supplementary Table S1). Samples were collected along major interstate routes, and only one sample of approximately five leaves was taken

per sampled *C. florida* tree. Infected leaf samples were flash-frozen in liquid nitrogen for 5 min and then homogenized with a Bead Mill 24 (Fisher Scientific Company, Hampton, NH). The tissue homogenization step was repeated twice with a 5-min interval in liquid nitrogen between steps. DNA was extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA) with the following modifications to the manufacturer's protocol: 2% soluble polyvinylpyrrolidone (PVP) was dissolved in the lysis buffer (AP1); the initial incubation time in AP1 at 65°C was increased from 10 to 20 min, with samples vortexed every 2 min; and the elution step was performed two times with 50 µl of elution buffer (AE). Extracted genomic DNA (gDNA) was quantified with the ND1000 Ultraviolet-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and stored at -20°C until it was used for analyses.

Microsatellite development and selection. A library of 237 microsatellite markers was developed by de novo sequencing of DNA from conidia that were isolated from infected leaves using Ion Torrent technology (Wadl et al. 2018). Twenty-eight microsatellite primer pairs were used to screen and identify polymorphisms using 93 of the samples in a 96-well plate. Both a positive control (DNA of *E. pulchra* sample that was amplified with all primer pairs) and a negative control (sterile distilled water substituted for gDNA) were used for all PCR reactions to ensure validity of our data. To eliminate the possibility of amplifying host DNA, all primers were tested against three independent samples of DNA from uninfected dogwood flower buds. None of the primer pairs amplified dogwood DNA. Then, 10-µl PCR reactions were assembled to test microsatellite loci; in subsequent data collections, these contained the following: 10 ng total gDNA isolated from both the host and pathogen, 2.5 µM for each forward and reverse primer, 2X AccuStart II PCR ToughMix (QuantaBio, Beverly, MA), and sterile distilled water. The reaction cycles were completed in a Mastercycler Pro automatic thermal cycler (Eppendorf Biotech Company, Hamburg, Germany) under the following conditions: 3 min of initial denaturation at 94°C followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and a final extension before the final holding temperature at 4°C.

Amplicons were analyzed with a QIAXCEL Capillary Electrophoresis System (Qiagen, Valencia, California) utilizing a 15/600 bp internal alignment marker and scored using a 25-bp DNA size marker to obtain raw allele length data (Dean et al. 2013; Wang et al. 2009). The QIAXCEL capillary electrophoresis system produced both a gel image and electropherogram for each sample. The electropherogram produced peaks for both the alignment markers and alleles that were amplified. Because *E. pulchra* is a haploid, a single peak (amplicon) was expected for each sample. Fifteen of the 28 microsatellite loci tested were polymorphic and used to amplify gDNA from the remaining *E. pulchra* samples in the study (Table 1). All PCR

Table 1. Fifteen microsatellite loci used to examine the genetic diversity and population structure of *Erysiphe pulchra* samples from the eastern United States

GenBank accession no.	Locus	Forward and reverse primers (5'-3')	Repeat motif	No. of alleles	Allele range	H _{exp} ^z
MG913149	EP027	F:AAAACGCACGACTTACCTACC R:TGGAGTCTTCACCATCAACTC	(CCGCG) ₃	2	177–182	0.48
MG913150	EP030	F:TGCTATCACCTCGTACTTTGC R:CGCTCAACAAAGAAATGAGTC	(GTC) ₄	3	156–161	0.61
MG913151	EP039	F:GCTGTAAGACGGGAGATTTTC R:TCTCTCTACAGTACCGATTGAA	(CGAG) ₃	4	144–154	0.55
MG913152	EP041	F:ATAGGAGGAGCAACCAAAATG R:GCATCCTTTCCACAAGATTT	(ATAA) ₃	4	203–213	0.57
MG913153	EP042	F:TTTGATGAGCTGTGTGTTT R:AAGATGCTGGTACAAGATTGG	(TTA) ₄	2	151–153	0.42
MG913154	EP043	F:GGCAGTGTCTCCATTTATCA R:TTTGAACCAGAATTTGAACCA	(ATTCTA) ₃	3	145–153	0.24
MG913155	EP051	F:ATACTAAAGGATCCCGAAGG R:GCTAGCCCTCTTAATAGTCTCT	(GTAT) ₃	3	148–154	0.52
MG913156	EP052	F:CGCTTAATGCTAGGATTTTG R:AACAAGAGATTTCCTGGCCTA	(AAAT) ₃	3	138–143	0.44
MG913157	EP054	F:GTCTCTTTACAAGGGCTCCAC R:CCCGAGTACCAAGGTAAATGT	(TCA) ₄	3	130–134	0.43
MG913158	EP055	F:AAGAGCCACTGTCTTTCTG R:ATGTTCTTGTCAGTTTGTTTCG	(TTTA) ₃	2	146–149	0.43
MG913159	EP056	F:GCAAGATAGCAGAGAAAGCAA R:TACTGGGATCTGATTCTTCC	(CAAC) ₃	3	151–157	0.51
MG913160	EP058	F:CCTTCGCTTTTACGTTATCC R:GAGCACCTACGGACCCTAC	(AAAT) ₃	3	149–155	0.53
MG913161	EP060	F:TAGAGTCTCTCAATCACCTG R:GGAAGACTTCGGAGTGACAGT	(CAT) ₄	2	148–150	0.49
MG913162	EP064	F:ACAAAACCAAGTGAACAACA R:GGGTGTGTTGATGTCGTTAAG	(ACA) ₄	3	151–157	0.26
MG913163	EP066	F:CTGGGGGAGGACTATTGTCTA R:CGGGTAGAAAGGGTGATTAAG	(TTTC) ₃	2	147–150	0.49

^z H_{exp} = Nei's genotypic diversity.

reactions were completed in 96-well plates containing the aforementioned positive and negative controls. None of the amplified samples exhibited more than a single peak or allele. PCR reactions that did not produce products were repeated at least two times before being considered missing data. Samples with missing data in more than 20% of the microsatellite loci were eliminated from the analyses. Therefore, the original sample size of 188 was decreased to 167 because of missing data.

Genetic diversity. Raw allele length data were binned into statistically similar allelic classes using FLEXIBIN version 2 (Amos et al. 2007). The binned allele data were used for all subsequent analyses. Samples were organized into the following two sets of collection zones: eight arbitrary zones based on relative geographical proximity (Fig. 1) and two zones representing those obtained from north of the Tennessee–Kentucky border (North) and those from south of the Tennessee–Kentucky border (South). The eight-collection zones were grouped arbitrarily based on geographical distribution along the highways where samples were collected. Samples were grouped into five individuals per population (minimum) to satisfy the sample size requirements for the various programs used in subsequent analyses (Kamvar et al. 2014), whereas the north–south dividing line of the two-collection zone analysis was determined based on the assumption that *E. pulchra* conidia are dispersed northward by prevailing winds from the south (Li et al. 2009; Mmbaga 2000). Data were clone-corrected using POPPR v2 (Kamvar et al. 2014, 2015b), which

is a package for R version 3.3.1 (Team 2016), to remove identical multilocus haplotypes (MLH) within each collection zone. The clone correction process used by POPPR v2 keeps samples of an identical MLH if the samples are in different collection zones (Kamvar et al. 2014). To prevent biasing analyses toward clonality, all subsequent analyses were performed with clone-corrected data (McDonald 1997; Tsui et al. 2012).

POPPR v2 was used to compute various genetic diversity indices, including the Shannon–Weiner index of diversity (H), a diversity index that takes in to account both richness and evenness of the given species (Shannon 2001), and Nei’s genotypic diversity, which utilizes the infinite allele model and accounts for varying sample sizes (Nei 1978), across all loci and sample sites for both sets of collection zones. The numbers of private alleles and allelic richness were also calculated using POPPR v2. The standard index of association ($\bar{r}_d$), a measurement of multilocus linkage disequilibrium (Agapow and Burt 2001), was calculated with POPPR v2 using 10,000 permutations for both sets of collection zones. Pairwise Nei’s genetic distance (Nei 1978) of both collection zone data sets were estimated using the R package adegenet v2.1.0 (Jombart 2008). The R package ade4 v1.7–8 (Dray and Dufour 2007) was used to calculate and visualize a Mantel test (Mantel 1967) using 100,000 permutations to compare the relationship between genetic and geographic distances of individuals in both collection zone sets. A heat map was added to visualize the Mantel test utilizing the R package MASS v7.3–50

Fig. 1. Location of the 167 samples of *Erysiphe pulchra* collected from 10 states in the eastern United States. Samples were assigned to eight arbitrary collection zones based on relative geographical proximity. Each collection zone is represented by a symbol of a unique color noted within the key. Because of space constraints of the map, some samples may be overlapped and appear as one sample.

(Ripley et al. 2012). A genetic distance matrix based on the stepwise mutation model (Bruvo et al. 2004), which was a minimum spanning network based on Bruvo's genetic distance, was generated with the eight-collection zone data set utilizing the R package POPPR (Kamvar et al. 2014) and followed the methods utilized by Kamvar et al. (2014, 2015a).

Population structure. A Bayesian clustering analysis of the eight-collection zone set was completed using STRUCTURE v2.3.4,

Table 2. Genetic diversity indices of eight collection zones of *Erysiphe pulchra* using 15 microsatellite loci²

Collection zone	N	MLG	H	Pa	H _{exp}	A _r	$\bar{r}_d$	$\bar{r}_d$ P-value
1	15	13	2.52	0	0.45	2.84	0.27	<0.001
2	22	14	2.39	1	0.45	2.92	0.26	<0.001
3	42	32	3.28	0	0.41	2.72	0.28	<0.001
4	16	12	2.39	0	0.17	2.34	0.04	NS
5	9	8	2.04	0	0.28	2.55	0.29	<0.001
6	22	17	2.66	0	0.29	2.58	0.25	<0.001
7	17	10	2.12	1	0.21	2.57	0.36	<0.001
8	24	13	2.16	1	0.34	2.82	0.46	<0.001
Total/Average	167	90	2.45	3	0.34	2.67	0.22	

² N = number of samples, MLG = number of multilocus genotypes after clone correction, H = Shannon-Weiner index of diversity, Pa = number of private alleles in each population, H_{exp} = Nei's genotypic diversity, A_r = Allelic richness, $\bar{r}_d$ = standard index of association, NS = not significantly different from 0.

Table 3. Genetic diversity indices of two collection zones of *Erysiphe pulchra* using 15 microsatellite loci²

Collection zone	N	MLG	H	Pa	H _{exp}	A _r	$\bar{r}_d$	$\bar{r}_d$ P-value
South	83	54	3.69	9	0.44	3.57	0.23	<0.001
North	84	47	3.40	2	0.39	3.57	0.24	<0.001
Total/average	167	90	3.54	11	0.42	3.57	0.22	

² N = number of samples, MLG = number of multilocus genotypes after clone correction, H = Shannon-Weiner index of diversity, Pa = number of private alleles in each population, H_{exp} = Nei's genotypic diversity, A_r = allelic richness, $\bar{r}_d$ = standard index of association.

Table 4. Pairwise population matrix of Nei's unbiased genetic distance of *Erysiphe pulchra* utilizing the clone corrected eight-collection zone data set

Collection zone	1	2	3	4	5	6	7	8
1								
2	0.06							
3	0.02	0.04						
4	0.37	0.20	0.33					
5	0.40	0.22	0.37	0.03				
6	0.33	0.19	0.30	0.03	0.02			
7	0.34	0.15	0.31	0.04	0.06	0.04		
8	0.04	0.03	0.03	0.36	0.38	0.32	0.31

Fig. 2. Mantel test of *Erysiphe pulchra* samples from the eastern United States arbitrarily assigned to eight collection zones. The analysis used 100,000 permutations and significance of $P < 0.001$.

which uses a Monte Carlo Markov Chain (MCMC) method to group individuals into definitive population clusters (ΔK) (Pritchard et al. 2000; Tsui et al. 2012). The parameters used in the STRUCTURE analysis were as follows: a burn-in period of 200,000 with 600,000 MCMC repetitions, 20 iterations, and the number of ΔK analyzed (1 to 10). The optimum value of ΔK was estimated with STRUC-TURE HARVESTER web v0.6.94 (Earl and vonHoldt 2012) utilizing Evanno's method (Evanno et al. 2005). POPHELPER (Francis 2017) was used to visualize the STRUCTURE results.

A Bayesian clustering analysis was also performed using InStruct (Gao et al. 2007). The InStruct program does not consider Hardy-Weinberg equilibrium within clusters and is suggested for analyzing highly clonal populations (Gao et al. 2007). The parameters used for the InStruct Bayesian analysis were as follows: 10 MCMC chains for each value of K (1 to 10) with a burn-in period of 500,000 and 20 iterations of thinning intervals (Dean et al. 2015). The optimum value of K was evaluated using the deviance information criterion (DIC) as outlined by Gao et al. (2011). The K-means hierarchical clustering with the R package adegenet was also used to infer genetic clusters by utilizing sequential K-means and model selection (Jombart et al. 2010).

POPVR v2 was used to calculate Bruvo's genetic distance among all individuals, subsequently converted into a Euclidean distance matrix, and visualized through a principal coordinates analysis (PCoA) using the R program adegenet v2.1.0. An AMOVA was performed for both collection zone data sets using POPVR v2, with samples grouped as one hierarchical cluster per instruct result.

Demography. Both Sign and Wilcoxon signed-rank significance tests were applied in the BOTTLENECK v1.2.02 program (Cornuet and Luikart 1996) to assess if a recent expansion or bottleneck in the population had occurred (Piry et al. 1999). The Sign test has a null hypothesis of equal probability of positive and negative differences between observed and expected heterozygosity (Cornuet and Luikart 1996), whereas the Wilcoxon signed-rank test is a one-tailed test with a null hypothesis of no significant excess of heterozygosity (Luikart et al. 1998; Piry et al. 1999). The two-collection zone dataset was utilized for this test because of the BOTTLENECK program requires large sample sizes. The two-collection zone data were coded as diploid for this analysis according to Tsui et al. (2012) because haploid data cannot be input in BOTTLENECK (Mantooth et al. 2017). The following three models were used to determine if the 15 loci used remained in mutation-drift equilibrium: stepwise mutation model, infinite allele model, and two-phase model (Piry et al. 1999).

Results

Genetic diversity. Five of the 28 loci evaluated were monomorphic; therefore, they were removed from further analyses. Fifteen primers used in this study amplified at least 80% of the samples (Table 1). Genetic diversity of the 15 loci ranged from 0.24 (EP054) to 0.61 (EP030) (Table 1). The number of alleles identified after allelic binning varied between two (EP027, EP042, EP055, EP060, and EP066) and four (EP039 and EP041) (Table 1). The average allelic richness was calculated using POPVR; it was 2.7 for the eight-collection zone set (Table 2) and 3.6 for the two-collection zone set (Table 3).

Data were analyzed using the following two different approaches when grouping samples into collection zones: eight arbitrary zones based on relative geographical proximity and two zones representing samples from north and south of the Tennessee-Kentucky border. Within the eight-collection zone data set, 48 individuals were removed after clone correction, which reduced the original sample size from 167 to 119 (Table 2). Clone correction of the two-collection zone data set reduced the original sample size from 167 to 101 samples. Clone correction through POPVR allows no more than one member of a specific MLH to be present within each zone. Ninety MLH were observed in both collection zone data sets after clone correction; a reduced sample size after clone correction included multiple samples of a given MLH if found in different collection zones (Tables 2 and 3).

The Shannon-Weiner index of diversity (H) of the eight-collection zones ranged from 2.04 to 3.28 (Table 2). Within the two-collection

zone data set, H was 3.40 for the north collection zone and 3.69 for the south collection zone (Table 3). The eight-collection zone data set contained three private alleles (P_a), found within three of the zones (Table 2), whereas the two-collection zone set contained 11 P_a (Table 3). The genetic diversity (H_{exp}) of the eight-collection zone data set was 0.34, and it ranged from 0.17 to 0.45 (Table 2). The average H_{exp} was 0.42 in the two-collection zone dataset (Table 3).

The total $\bar{r}_d$ of both the eight-collection and two-collection zone data sets differed significantly from 0, indicating a lack of sexual recombination (Tables 3 and 4). One of the zones within the eight-collection zone data set (zone 4), however, did not significantly differ from 0, suggesting possible sexual reproduction in this zone (Table 2).

The pairwise population matrix of Nei's genetic distance for the eight-collection zone data set estimated the highest genetic distance between zone 1 and zone 5 at 0.397, whereas the lowest genetic distance of 0.021 was calculated between zone 1 and zone 3 (Table 4). Nei's genetic distance for the two-collection zone data set was 0.097. The Mantel test was used to determine correlation between genetic distance and geographical distance. Significant positive correlation ($r = 0.128$) between genetic and geographical distance was also calculated ($P < 0.0001$) (Fig. 2). The minimum spanning network of the 90 MLH utilizing the eight-collection zone dataset indicated that the genetic distance among the majority of MLHs was 0.033 (Fig. 3). Two subgroups of MLHs were separated by a genetic distance of 0.107. The majority of MLHs were found in only a single zone, whereas one MLH was found in six of the eight collection zones.

Fig. 3. Minimum spanning network (MSN) of *Erysiphe pulchra* based on Bruvo's genetic distance for 15 microsatellite loci. The nodes of the MSN represent individual multilocus haplotypes (MLH), with the size and color representing population membership size and designated collection zone, respectively. To avoid overlapping nodes, the size was scaled to $\log_{1.75} n$, where n equals the node sample size. Lines between nodes represent genetic distance between MLH, as determined by Prim's algorithm. Subgroups in which more than 3 MLHs are separated by a genetic distance no more than 0.033 are highlighted in various colors.

Population structure. STRUCTURE, which uses a Bayesian clustering analysis, estimated an optimum ΔK of 2, which indicated two clusters within the eight-collection zone set (Fig. 4A). Zones 1, 2, 3, and 8 were highly admixed between the two clusters, whereas zones 4, 5, 6, and 7 clustered together with low admixture (Fig. 5). In the two-collection zone dataset, both zones were admixed (Fig. 5). The InStruct Bayesian clustering analysis of the eight-collection zone set calculated the most probable ΔK to be 1, and it indicated a single population cluster of the pathogen (Fig. 4B). InStruct results of one cluster was confirmed utilizing the K-mean test of the R package adegenet. One continuous cluster without definite structure was indicated by the PCoA of both collection zone datasets, which supported the InStruct results (Fig. 6). Both Bayesian clustering analyses

Fig. 4. Bayesian clustering probabilities for *Erysiphe pulchra* samples in eight arbitrary collection zones in the eastern United States utilizing InStruct and STRUCTURE version 2.3.4 (optimum value of K estimated using STRUCTURE HARVESTER web version 0.6.95). **A**, Using the deviance information criterion calculations, InStruct indicated that the highest probability of K is 1. **B**, Evanno's method, performed with STRUCTURE, indicated that the optimum value of K is 2.

Fig. 5. Structure bar graph indicating two genetic clusters ($\Delta K=2$) of *Erysiphe pulchra* samples from the eastern United States organized as eight and two arbitrary collection zones (represented on the x-axis) and analyzed utilizing 15 microsatellite loci. Each bar represents the probability of an individual sample belonging to a designated cluster (represented by different colors).

were used for the two-collection zone dataset with the same outcomes (InStruct data not shown).

The AMOVA of the eight-collection zone dataset indicated that the majority of genetic variation was found between individuals within collection zones (82.33%), with only 17.67% of variation identified among collection zones (Table 5). The AMOVA of the two-collection zone dataset estimated variations between individuals within zones as 90.94% and among the two-collection zones as 9.06% (Table 5). Differentiation among zones in both datasets were moderate to low ($\Phi_{st} = 0.177$ and $\Phi_{st} = 0.09$) (Table 5).

Demography. An analysis the two-collection zone dataset with BOTTLENECK v1.2.02 using the Sign and Wilcoxon tests indicated a significant excess of genetic diversity or heterozygosity in relation to the number of alleles present in the grouped data. The evidence suggested that a bottleneck had recently occurred (Table 6).

Discussion

With sexual reproduction less commonly observed during the past decade, our hypothesis for the present study was that *E. pulchra* has become clonal because of predominant reproduction via asexually produced conidia in the eastern United States. For the purposes of this study, clonality was considered synonymous with predominant asexual reproduction. If *E. pulchra* is clonal in the eastern United States, then we expect to find low genetic diversity, few private alleles, lack of a definitive population structure, and significant linkage disequilibrium. The results of our study supported our hypothesis of low genetic diversity, absence of population structure, significant linkage disequilibrium, and a significant correlation between geographical and genetic distances among sampled *E. pulchra* individuals.

Similar to the observations of other clonally reproducing phytopathogens (Kamvar et al. 2015a; Lehner et al. 2015), genetic diversity among the arbitrarily based collection zones of *E. pulchra* was low, lending support to the hypothesis that *E. pulchra* is a clonal

pathogen. Nei's genotypic diversity (H_{exp}) of all eight collection zones was also low; however, it was lowest in northern zones, with the exception of New Jersey (zone 8). The limited genetic diversity of the northern *E. pulchra* collection zones may suggest that conidia, dispersed by prevailing winds in the spring from the south toward the north, are the predominant means of inoculum in these zones, similar to *Peronospora tabacina* (Aylor 1999; Aylor et al. 1982; Spring et al. 2018) and *Puccinia triticina* (Hamilton and Stakman 1967; Kolmer 2005).

Powdery mildew fungal pathogens produce conidia that can be wind-dispersed over long distances (McDonald and Linde 2002). Barley powdery mildew (*E. graminis*) conidia are wind-dispersed from the United Kingdom to Denmark, a distance of approximately 500 miles or 800 km (Hermansen et al. 1978; Parks et al. 2009). *E. pulchra* conidia have a latent period of 7 days (Li et al. 2005), which allows for rapid dispersion of the pathogen locally and to more distant geographic ranges during a single season (Aylor 2003). The pairwise population matrix of Nei's genetic diversity indicated high gene flow between all zones, conceivably as a consequence of wind dispersal over a large geographical range (Aylor et al. 1982; Barrès et al. 2008). The higher diversity observed in the New Jersey samples may be explained by the introduction of infected *C. kousa* or *C. florida*, presumably imported on breeding materials, from either the southern states or other locations.

The standard index of association ($\bar{r}_d$), a diversity index commonly used to determine linkage disequilibrium and sexual recombination of plant pathogens (Kamvar 2016; Kamvar et al. 2014, 2015b; Mantooth et al. 2017; Talas and McDonald 2015), revealed significant linkage disequilibrium in both collection zone datasets and supported the hypothesis of predominantly asexual reproduction. Within the eight-collection zone dataset, however, one zone indicated linkage disequilibrium was not significantly different from 0, thus suggesting random mating. However, that should be interpreted with caution because the apparent indication of sexual

Fig. 6. Discriminant analysis of principal components (DAPC) of *Erysiphe pulchra* samples in **A**, eight and **B**, two arbitrary collection zones from the eastern United States using 15 microsatellite loci. **C**, Axes 1 and 2 explain 29% variation observed in **A**.

recombination may be an artifact caused by small sample sizes and missing data that affects the $\bar{r}_d$ of a collection zone (Kamvar 2016). Sexual recombination in this zone, however, cannot be ruled out. The zone in which this occurred had more than 50% missing data for 3 of the 15 loci. The criteria used to form the eight-collection zones, arbitrarily based on geographic proximity, may also have had an effect on the accuracy of the results because of the subjective nature of the collection zone organization. When clone-corrected and analyzed as a two-collection zone dataset (with larger sample sizes), significant linkage disequilibrium was evident in both zones.

Although the sexual stage has not been observed as frequently in recent years, there is currently no official census of chasmothecia throughout the *C. florida* growing range. The lack of genetic diversity and sexual recombination in the eastern United States population may also be the result of the introduction of a limited number of genetically dissimilar individuals (Barrès et al. 2008; Goss et al. 2009). The minimum spanning network of the 90 MLHs indicated a short genetic distance between all MLHs, and the greatest distance dividing the two subgroups of MLHs was also short. If few genetically novel individuals were introduced to the eastern United States, then sexual reproduction would do little to increase the genetic diversity of *E. pulchra*, as has been seen with other exotic plant pathogens (Barrès et al. 2008; Giraud 2004; Goss et al. 2009; Raboin et al. 2007).

To determine whether there was a correlation between the geographic and genetic distances of *E. pulchra* in the eastern United States, the Mantel test was utilized. Our Mantel results indicated a significant positive correlation between genetic and geographic distances. This positive correlation is indicative of isolation by distance (Barrès et al. 2008). The correlation of genetic and geographical distances suggested a natural dispersal of the pathogen spores, presumably by long distance wind dispersal of conidia, similar to other powdery mildew pathogens (Brown 1994; Brown and Hovmöller 2002; Brown et al. 1991; Parks et al. 2009), as opposed to isolated long-distance dispersal of infected material to each geographic area.

The correlation between geographic and genetic distances may also suggest a limit to where pathogen can overwinter. Overwintering of *E. pulchra* in the eastern United States may occur in southern states, resulting in an inoculum source for epidemics in northern states annually. If *E. pulchra* were to overwinter in all collection zones, then the correlation between genetic and geographical distances would be limited because of long-distance dispersal; over time, divergence based on the rates and distance of pathogen spread would decrease and the genetic structure would be more pronounced (Barrès et al. 2008; Kamvar et al. 2015a). If this were the case, then the population structure according to location would be expected to be similar to that of *E. graminis* f. sp. *hordei* (Brown 1994; Brown et al. 1991) and *Blumeria graminis* f. sp. *tritici* (Parks et al. 2009); however, this was not evident in our results. It may be advantageous to perform a census of *E. pulchra* chasmothecia throughout the

C. florida native range to obtain a better determination of whether this is the case.

Our results indicated a lack of defined genetic structure of the *E. pulchra* population in the eastern United States. InStruct, a Bayesian clustering program that excludes Hardy-Weinberg equilibrium assumptions (Gao et al. 2007; Hartl 1988), was utilized in the present study and indicated one cluster as the most probable population structure of *E. pulchra*. When the population structure was analyzed with STRUCTURE utilizing Evanno's method, a ΔK of 2 was indicated to be the most probable structure. However, Evanno's method utilized by STRUCTURE Harvester for determining the optimum value of ΔK cannot be used to mathematically calculate a ΔK of 1 (Evanno et al. 2005). The PCoA results of the eight-collection zone data set indicated a lack of grouping, and the K-means test of both collection zone data sets indicated one cluster as the most probable structure. Both of these results added support to the InStruct results.

Many other primarily asexually reproducing plant pathogens, such as *P. ramorum* (Ivors et al. 2006; Kamvar et al. 2015a), *Sclerotinia sclerotiorum* (Kamvar et al. 2017; Lehner et al. 2015), and *Discula destructiva* (Mantooth et al. 2017), have exhibited a defined population structure, whereas in the eastern United States, the *E. pulchra* population lacks a definitive structure. This difference in structure may be indicative of how *E. pulchra* has been dispersed and/or introduced in the United States as compared with the aforementioned pathogens. The population structure of *D. destructiva*, for example, resulted from multiple introductions of the pathogen to both the eastern and western United States (Mantooth et al. 2017). The lack of population structure found in our results may suggest a narrow introduction of the pathogen with limited genetic diversity to the eastern United States (Barrès et al. 2008; Goss et al. 2009).

In an effort to genetically differentiate *E. pulchra* from *P. guttata*, the powdery mildew pathogen of *Cornus amomum*, Mmbaga et al. (2004) amplified the ITS regions of *E. pulchra* samples in Tennessee; they found a 99% match between their samples (GenBank accession

Table 6. Bottleneck analyses for *Erysiphe pulchra* samples utilizing the two-collection zone data set separated as two populations (north and south) using 15 microsatellite loci^a

Genetic cluster	Mutation model (excess/deficit) ^y			Mode shift ^z	P-value
	IAM	TPM	SMM		
South	15/0	15/0	15/0	Shifted	<0.001
North	15/0	15/0	15/0	Shifted	<0.001

^a IAM = infinite allele model, TPM = two-phase mutation model, SMM = stepwise mutation model.

^y Excess/deficit designate the number of loci exhibiting either excess or deficit of gene diversity under mutation-drift equilibrium.

^z A shifted mode is expected in collection zones that had a recent bottleneck.

Table 5. Analysis of molecular variance (AMOVA) for *Erysiphe pulchra* across 15 microsatellite loci for the eight-collection zone data set grouped as one hierarchical group and two-collection zone data set grouped as one hierarchical cluster

Eight Collection Zones					
Source of variation	df	Sum of squares	Variance components ^y	Variation %	P value
Among collection zones	7	80.27	0.60 V a	17.67	<0.001
Within collection zones	118	310.96	2.80 V b	82.33	
Total	125	391.23	3.32	100	
$\Phi_{st}^Z = 0.18$					
Two collection zones					
Source of variation	df	Sum of squares	Variance components ^y	Variation %	P value
Between collection zones	1	19.32	0.32 V a	9.06	<0.001
Within collection zones	99	318.60	3.22 V b	90.94	
Total	100	337.92	354	100	
$\Phi_{st}^Z = 0.09$					

^y Variance components followed by different letters are significantly different at $\alpha = 0.05$.

^z Φ_{st} = genotypic differentiation index, or the variance among collection zones in relation to the total variance.

no. AY224136) and an *E. pulchra* sample from *C. kousa* in Japan (GenBank accession no. AB015935) (Mmbaga et al. 2004; Takamatsu et al. 1999). This result led Mmbaga et al. (2004) to the hypothesis that the rapid spread of *E. pulchra* in the eastern United States was possibly caused by an introduction of *E. pulchra* on infected *C. kousa* imported to breed cultivars of dogwood resistant to *D. destructiva* (Mmbaga et al. 2004). *C. kousa* trees are generally resistant to *E. pulchra*, but the disease may develop on seedlings (Li et al. 2007, 2009; Windham 1996) or on shoots from epicormic buds (personal observation). *E. pulchra* was first reported in China in 2012, infecting *C. kousa* subsp. *Chinensis*; it was identified by sequencing the ITS region (GenBank accession no. KF601334), which matched the same sample of *C. kousa* from Japan (Bai et al. 2014; Takamatsu et al. 1999). The low genetic diversity, lack of population structure, and bottleneck found in the present study add support to the hypothesis by Mmbaga et al. (2004) that *E. pulchra* is an exotic plant pathogen (Goss et al. 2009) that was most likely introduced to the eastern United States from Japan or other locations in Asia where *C. kousa* is grown. Further research regarding the possible spread of *E. pulchra* from Asia to the eastern United States is warranted and should include more *E. pulchra* samples from *C. florida* and *C. nuttallii* from the western United States (windham et al. 2005) and Italy (Garibaldi et al. 2009).

The results of our study indicated low genetic diversity, significant linkage disequilibrium, short genetic distance between MLHs, and few private alleles, thereby suggesting that *E. pulchra* had essentially become clonal in the eastern United States because of predominantly asexual reproduction. However, the low genetic diversity and lack of defined population structure may also be an indicator of a limited introduction of *E. pulchra* with little genetic diversity to the eastern United States (Barrès et al. 2008; Goss et al. 2009). With the *E. pulchra* chasmothecia and ascospores becoming less common in recent years, it may be advantageous to focus research on why this may be the case. Currently, the only study of the winter survival of *E. pulchra* was completed by Mmbaga et al. (2000), who found that chasmothecia were counted over a 3-year period, and its abundance was limited by the formation of chasmothecia later in the season. Their study suggested that environmental conditions may affect either the host or the pathogen, thus possibly restricting chasmothecia development (Mmbaga 2002). *Erysiphe pulchra* may be heterothallic, and the formation of chasmothecia and ascospores is dependent on the presence of two mating types (Debuchy et al. 2010; Mmbaga 2002). Assuming that *E. pulchra* is heterothallic, three possibilities exist regarding why sexual reproductions appears to be less common now compared with that during the late 1990s. First, during the original introduction of *E. pulchra* to the eastern United States, one of the mating types was less common than the other. Second, one of the mating types was less fit for the new environment. Third, a combination of the two aforementioned possibilities is possible. The second possibility of less fitness is probably more likely because extensive sexual reproduction was common in the 1990s, indicating that both mating types were present. However, we cannot eliminate the possibility that the current environment has become less supportive of one of the mating types compared with the environment in the 1990s. The notion of changes caused by environment would be similar to the lack of *P. infestans* oospores in the United States (Goodwin et al. 1995). To determine if this is the case, focusing on the development of microsatellites specific to different mating types of *E. pulchra* and possible homologous regions of DNA within other powdery mildews may be necessary.

The present study of the genetic characterization of *E. pulchra* in the eastern United States is one of a few population analyses of a powdery mildew pathogen (Bardin et al. 1999; Brown et al. 1991; Caffier et al. 1999; McDermott et al. 1994; Brewer and Milgroom 2010; Parks et al. 2009). The low genetic diversity, lack of population structure, and presence of linkage disequilibrium support our hypothesis that *E. pulchra* in the eastern United States has become clonal in nature because it predominantly reproduces asexually. The study strongly infers that *E. pulchra* is an exotic pathogen to *C. florida*; however, more research is needed to conclude the origin of the pathogen and epidemiological changes with chasmothecia development.

The results of our study also indicated that *E. pulchra* most likely overwinters in the south, conceivably with long-distance wind dispersal to the north by prevailing winds.

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