Fine-Scale Population Genetic Structure and Within-Tree Distribution of Mating Types of *Venturia effusa*, Cause of Pecan Scab in the United States

Clive H. Bock,[†] Carolyn A. Young, Katherine L. Stevenson, and Nikki D. Charlton

First author: U.S. Department of Agriculture–Agricultural Research Service Southeastern Fruit and Tree Nut Research Laboratory, Byron, GA 31008; second and fourth authors: Noble Research Institute, LLC, 2510 Sam Noble Parkway, Ardmore, OK 73401; and third author: Department of Plant Pathology, Coastal Plain Experiment Station, University of Georgia, Tifton 31793.

Accepted for publication 15 May 2018.

ABSTRACT

Scab (caused by *Venturia effusa*) is the major disease of pecan in the southeastern United States. There is no information available on the fine-scale population genetic diversity or the occurrence of clonal types at small spatial scales that provides insight into inoculum sources and dispersal mechanisms, and potential opportunity for sexual reproduction. To investigate fine-scale genetic diversity, four trees of cultivar Wichita (populations) were sampled hierarchically: within each tree canopy, four approximately evenly spaced terminals (subpopulations) were selected and up to six leaflets (sub-subpopulations) were sampled from different compound leaves on each terminal. All lesions ($n = 1$ to 8) on each leaflet were sampled. The isolates were screened against a panel of 29 informative microsatellite markers and the resulting multilocus genotypes (MLG) subject to analysis. Mating type was also determined for each isolate. Of 335 isolates, there were 165 MLG (clonal fraction 49.3%). Nei's unbiased measure of genetic diversity for the clone-corrected data were moderate to high (0.507). An analysis of molecular variance demonstrated differentiation ($P = 0.001$) between populations on leaflets within individual terminals and between terminals within trees in the tree canopies, with 93.8% of variance explained among isolates within leaflet populations. Other analyses (minimum-spanning network, Bayesian, and discriminant

analysis of principal components) all indicated little affinity of isolate for source population. Of the 335 isolates, most unique MLG were found at the stratum of the individual leaflets ($n = 242$), with similar total numbers of unique MLG observed at the strata of the terminal ($n = 170$), tree ($n = 166$), and orchard ($n = 165$). Thus, the vast majority of shared clones existed on individual leaflets on a terminal at the scale of 10s of centimeters or less, indicating a notable component of short-distance dispersal. There was significant linkage disequilibrium ($P < 0.001$), and an analysis of P_{sex} showed that where there were multiple encounters of an MLG, they were most probably the result of asexual reproduction ($P < 0.05$) but there was no evidence that asexual reproduction was involved in single or first encounters of an MLG ($P > 0.05$). Overall, the *MAT1-1-1* and *MAT1-2-1* idiomorphs were at equilibrium (73:92) and in most populations, subpopulations, and sub-subpopulations. Both mating types were frequently observed on the same leaflet. The results provide novel information on the characteristics of populations of *V. effusa* at fine spatial scales, and provide insights into the dispersal of the organism within and between trees. The proximity of both mating idiomorphs on single leaflets is further evidence of opportunity for development of the sexual stage in the field.

Pecan (*Carya illinoensis*) is an important specialty crop in the United States: in 2015, 1.15×10^8 kg of pecan nuts were produced, valued at $\$5.60 \times 10^8$ (NASS 2015). However, diseases can reduce yield of pecan, with pecan scab (causal agent, *Venturia effusa* (G. Winter) Rossman & W. C. Allen) (Rossman et al. 2016) being the single most important yield-limiting disease in the humid southeastern United States (Bock et al. 2016a; Demaree and Cole 1929; Gottwald and Bertrand 1983; Stevenson and Bertrand 2001). The pathogen is spread in rain splash and wind as conidia (Gottwald and Bertrand 1982), which can infect young, expanding foliage

(Gottwald 1985), new developing wood, and developing or mature fruit (Stevenson and Bertrand 2001).

Scab is not easy to manage, in part because the pathogen has developed resistance to several commonly used fungicides (Stevenson 1999; Stevenson et al. 2014) and the pathogen exhibits variability, with specific isolates generally infecting only a small range of cultivars (Conner and Stevenson 2004). This adaptability has resulted in loss of resistance in several previously resistant host cultivars (Goff et al. 1996).

Knowledge of the genetic diversity and population genetic structure of a pathogen can indicate risks posed by particular pathogens to host resistance, and also help guide a range of management strategies (McDonald and Linde 2002; McDonald and Mundt 2016; McDonald et al. 2015; Stukenbrock and McDonald 2008; Stukenbrock et al. 2011). Indeed, characteristics of population genetic structure at various scales have been described for several pathogens of annual (Linde et al. 2002, 2009) and perennial (Everhart and Scherm 2015; Gladieux et al. 2010) crops, and also for pecan scab (Bock et al. 2017). This knowledge of the genetic diversity and population genetic structure can also provide some useful insights into reproduction of the pathogen and spread of the disease, particularly the sources of inoculum and dispersal processes at different spatial scales, including very local scales (Burchhardt et al. 2017; Everhart and Scherm 2015; Hu et al. 2018; Keller et al. 1997; Linde et al. 2002; Sonoda et al. 1991). Clonal occurrence and fitness at the local scale can have important ramifications for opportunities for sexual reproduction and response to selection (for example,

[†]Corresponding author: C. H. Bock; E-mail: clive.bock@ars.usda.gov

Funding: This research was supported by the United States Department of Agriculture–Agricultural Research Service through Current Research Information System project 6606-21220-011-00D.

This article reports the results of research only. Mention of a trademark or proprietary product is solely for the purpose of providing specific information and does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures and three supplementary tables are published online.

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 2018.

fungicide resistance or virulence). The pathosystems referenced here are characterized by moderate to high genetic diversity and generally low clonality, and where that clonality occurs is mostly at small spatial scales due to asexual reproduction; better-adapted clones will be more likely to achieve either sexual or asexual reproduction, maintaining fitness of the species. The sexual stage provides the opportunity for recombination and maintenance of the observed genetic diversity (Keller et al. 1997; Linde et al. 2002) although, in some cases, the sexual stage has not yet been identified, despite the characteristics of the population genetics indicating that it may exist (Linde et al. 2009). The mating type genes of *V. effusa* were recently identified and determined to be at equilibrium (Young et al. 2018) and characteristics of the population genetic structure are indicative of a sexually reproducing species (Bock et al. 2017) but the sexual stage

has not yet been identified. The Bock et al. (2017) study did not investigate characteristics of population genetic structure at the scale below the whole tree, yet information at finer spatial scales can indicate to what degree asexual inoculum plays a role in disease spread (Everhart and Scherm 2015; Keller et al. 1997; Li and Brewer 2016), and also the proximity of clones or compatible mating types, which can indicate opportunities for sexual recombination (Linde et al. 2002).

Studies of *V. inaequalis* (cause of apple scab, and a close relative of *V. effusa*) indicated among samples within-tree variation (73.8% of variance, $P = 0.001$), which was less than expected if there was no subpopulation structure (Xu et al. 2013). Xu et al. (2013) hypothesized that this might be due to a role of conidia as a source of primary inoculum within a tree canopy. Samples within pecan

TABLE 1. Statistics summarizing the genetic variation within populations of *Venturia effusa* at the scale of the orchard, tree, and terminal in a pecan orchard of cultivar Wichita in Byron, GA^a

Tree, terminal ^b	Number of isolates (MLG) ^c	Fraction (%) ^d	Number of genotypes ^e	Number of eMLG ^f	H ^g	G ^h	λ ⁱ	H _{exp} ^j
Tree W1								
T1	32 (17)	53.1	6	10	2.83	17	0.941	0.496
T2	21 (8)	38.1	3	8	2.08	8	0.875	0.514
T3	14 (6)	42.9	3	6	1.79	6	0.833	0.494
T4	19 (8)	42.1	4	8	2.08	8	0.875	0.385
Subtotal	86 (37)	43.0	16	35	3.61	37	0.973	0.507
Tree W2								
T1	27 (11)	40.7	5	10	2.40	11	0.909	0.457
T2	18 (6)	33.3	2	6	1.79	6	0.833	0.368
T3	26 (14)	53.8	6	10	2.64	14	0.929	0.465
T4	18 (10)	55.6	2	10	2.30	10	0.900	0.545
Subtotal	89 (41)	46.1	15	35	3.71	41	0.976	0.487
Tree W3								
T1	20 (17)	85.0	3	10	2.83	17	0.941	0.585
T2	22 (16)	72.7	3	10	2.77	16	0.938	0.458
T3	18 (5)	27.8	3	5	1.61	5	0.800	0.493
T4	25 (16)	64.0	6	10	2.77	16	0.938	0.524
Subtotal	85 (53)	62.4	16	35	3.97	53	0.981	0.520
Tree W4								
T1	19 (7)	36.8	5	7	1.95	7	0.857	0.586
T2	16 (9)	56.3	2	9	2.20	9	0.889	0.403
T3	19 (9)	47.4	4	9	2.20	9	0.889	0.481
T4	21 (11)	52.4	5	10	2.40	11	0.909	0.451
Subtotal	75 (35)	46.7	16	35	3.56	35	0.971	0.496
Total	335 (165)	49.3	84	165	5.10	164	0.994	0.507

^a Clone-corrected data are shown. Analyses were performed using *poppr* (Kamvar et al. 2014).

^b Tree W1 = tree 1, T1 = terminal 1, and so on. Orchard location and altitude: N 32°65.522', W 83°65.522', 156 m.

^c Number of isolates and number of multilocus genotypes (MLG) in parentheses.

^d Clonal fraction (%).

^e Number of clonal genotypes.

^f Number of expected MLG (eMLG) at the smallest sample size.

^g Shannon-Wiener Index of MLG diversity (H) (Shannon 2001).

^h Stoddart and Taylor's Index of MLG diversity (G) (Stoddart and Taylor 1988).

ⁱ Simpson's Index (λ) (Simpson 1949).

^j Nei's unbiased measure of genetic diversity (H_{exp}) (Nei 1978). Evenness was also measured but = 1 for all populations as clone-corrected (Grünwald et al. 2003).

TABLE 2. Measures of random mating among populations of *Venturia effusa* on four Wichita pecan trees in an orchard in the southeastern United States^a

Tree ^b	Number of MLG ^c	Private alleles (%) ^d	Polymorphic loci (%)	I_A	I_A cc	$\bar{r}_d$	$\bar{r}_d$ cc	Number of N_d pairs (%) ^e
Tree 1	86 (37)	4 (0.020)	100.0	3.1272**	1.8482**	0.1149**	0.0672**	155/406 (38.2)
Tree 2	89 (41)	8 (0.047)	96.6	2.4630**	0.8758**	0.0961**	0.0335**	129/378 (34.1)
Tree 3	85 (53)	29 (0.134)	100.0	1.3732**	1.0200*	0.0516**	0.0378**	84/406 (20.7)
Tree 4	75 (35)	5 (0.018)	100.0	2.4516**	1.3015**	0.0932**	0.0486**	110/406 (27.1)
Total	335 (165)	46 (0.055)	100.0	1.2500**	0.9980**	0.0462**	0.0367**	149/406 (36.7)

^a Mean number of alleles, number of private alleles, gametic disequilibrium measured using the index of association (I_A) (Brown et al. 1980; Smith et al. 1993) and the standardized index of association ($\bar{r}_d$) (Agapow and Burt 2001) for both the full data set and the clone-corrected data (I_A cc and $\bar{r}_d$ cc). The $\bar{r}_d$ is a more robust measure, less sensitive to the number of loci (Agapow and Burt 2001). Statistics were calculated based on 1,000 randomizations. N_d is the number of pairs of loci in disequilibrium. Analyses were performed using *poppr* (Kamvar et al. 2014). Asterisks (**) indicate $P \leq 0.001$.

^b Tree number in orchard.

^c Number of multilocus genotypes (MLG) observed.

^d Number of private alleles (frequency). A private allele is one unique to that population. In total, there were 183 alleles among the 30 loci over all the isolates sampled.

^e Number (and percentage) of N_d pairs of loci not in equilibrium.

trees in orchards showed significant within-tree variation among samples (81%, $P = 0.001$) (Bock et al. 2017). Conidia of *V. inaequalis* are known to overwinter and contribute to epidemics (Holb et al. 2004). Although they have been discussed and described (Demaree 1924; Gottwald and Bertrand 1982), neither the role of conidia in epidemics of pecan scab nor the characteristics of population structures at fine spatial scales have been explored for the pecan-*V. effusa* pathosystem. The overall aim of this study was to gain an understanding of the population genetic structure of *V. effusa* at a fine spatial scale.

Specifically, a previously defined panel of microsatellites for *V. effusa* (Bock et al. 2016b, 2017; Young et al. 2018) were used to screen isolates sampled hierarchically in a pecan orchard. The mating type of each isolate was also determined (Young et al. 2018). The objectives of this study were to (i) determine the population genetic diversity at the spatial scales of the orchard, a tree, a terminal, and a leaflet; (ii) identify any clonality, and the magnitude of clonality at these spatial strata; and (iii) assess the distribution and juxtaposition of mating types.

MATERIALS AND METHODS

Sample location and isolation of *V. effusa*. In mid-June to July 2012, four 28-year old trees in a single pecan orchard consisting of 23 trees (cultivar Wichita) located at the United States Department of Agriculture–Agricultural Research Service South-eastern Fruit and Tree Nut Research Laboratory were selected for sampling. Tree distance was 18 m within rows by 9 m between rows (planted diagonally). Trees (populations) were selected to maximize distance between them, separated by two rows and by three trees within the row. Thus, positions of the four sample trees in the orchard approximated the corners of a square. Within each tree canopy, four evenly spaced terminals (subpopulations) around the

canopy of each tree were arbitrarily selected at a height of approximately 8 m (tree height was approximately 14 m). Up to six leaflets were arbitrarily sampled from different compound leaves on each terminal. All lesions on each leaflet (sub-subpopulations) were sampled (from one to eight lesions). Thus, the scales in the hierarchy were from the orchard (between trees, approximately 27 to 72 m), between terminals within a tree (approximately 3 to 10 m), and between leaflets on an individual terminal (approximately 5 to 25 cm) and lesions on a leaflet (approximately 0.4 to 5 cm). In total, 335 isolates of *V. effusa* were sampled from individual lesions on scab-diseased leaflets using the hierarchical sampling procedure.

The procedure for isolation of *V. effusa* from scabbed leaflets were described previously (Bock et al. 2014a). Using a scalpel, conidia were scraped from the surface of a single lesion, and a dilute spore suspension was prepared in sterile distilled water, of which 100 µl was spread on water agar (WA, amended with lactic acid at 0.50 ml/liter, streptomycin at 0.20 g/liter, tetracycline at 0.05 g/liter, and chloramphenicol at 0.05 g/liter). The conidia were incubated at 25°C for 24 h under fluorescent lights with a 12-h photoperiod. Only a single spore was taken per lesion. Single germinated conidia were transferred under a stereoscopic microscope (at ×50 to ×70) to antibiotic-amended potato dextrose agar (PDA, amended as for WA). The culture was incubated as described for 3 to 5 weeks.

DNA extraction, polymerase chain reaction, and genotyping. Mycelium (approximately 200 mg) was scraped from the surface of the colony growing on PDA and the DNA was extracted using a DNA extraction kit (Zymo Research, Irvine, CA), following the manufacturer’s protocol for DNA extraction. A TissueLyser (Qiagen, Valencia, CA) was used to lyse the fungal mycelium. DNA was quantified using a Nanodrop spectrophotometer (Nanodrop Products, Wilmington, DE) and stored in Tris-EDTA buffer at –20°C.

Fig. 1. Numbers of private alleles detected in populations of *Venturia effusa* across 29 microsatellite loci by **A**, tree and by **B**, mating type. In **B**, 1 = *MAT1-1-I* and 2 = *MAT1-2-I*. Intensity of shading indicates number of private alleles; the higher the number, the lighter the shading.

All isolates were screened against a panel of 30 microsatellite primers (obtained from Eurofins MWG Operon, Huntsville, AL) that were previously characterized and described (Bock et al. 2016b, 2017). Primers were labeled with either FAM, NED, HEX,

or PET dye (some were labeled with M13 tails). The working microsatellite primer concentration was 10 μ M, and the reaction volume was 5 μ l, containing 1.5 μ l of template DNA (1.5 to 2.0 ng), 0.25 μ l of primer, 0.75 μ l of distilled H₂O, and 2.5 μ l of master mix

TABLE 3. Sixty-three clonal genotypes of *Venturia effusa* isolated from four Wichita pecan trees, the number of clones of each, and the estimated probability of a second encounter (P_{sex})^a

Clones (MLG)	P_{sex}	For each sample: Tree_Terminal_Leaflet
2 (885)	4.11×10^{-13}	W1_T1_L1, W1_T1_L4
2 (890)	2.75×10^{-09}	W1_T1_L1, W1_T1_L3
2 (896)	1.84×10^{-08}	W1_T1_L2, W1_T1_L4
2 (931)	6.85×10^{-08}	W1_T2_L4, W1_T2_L5
2 (939)	4.05×10^{-12}	W1_T3_L3, W1_T3_L5
2 (941)	2.10×10^{-08}	W1_T3_L3, W1_T3_L4
2 (951)	1.47×10^{-07}	W1_T4_L1, W1_T4_L5
2 (973)	4.81×10^{-09}	W2_T1_L1, W2_T1_L1
2 (988)	1.75×10^{-14}	W2_T1_L4, W2_T1_L4
2 (993)	3.43×10^{-08}	W2_T1_L5, W2_T1_L5
2 (100)	9.41×10^{-10}	W2_T2_L2, W2_T2_L2
2 (1,015)	9.70×10^{-09}	W2_T3_L1, W2_T3_L2
2 (1,037)	6.17×10^{-14}	W2_T3_L4, W2_T3_L5
2 (1,038)	4.86×10^{-12}	W2_T3_L5, W2_T3_L5
2 (1,046)	1.88×10^{-09}	W2_T4_L3, W2_T4_L3
2 (1,059)	1.49×10^{-08}	W3_T1_L1, W3_T1_L4
2 (1,065)	6.76×10^{-12}	W3_T1_L2, W3_T1_L7
2 (1,075)	1.90×10^{-10}	W3_T1_L7, W3_T1_L7
2 (1,087)	3.77×10^{-08}	W3_T2_L2, W3_T2_L2
2 (1,090)	1.20×10^{-10}	W3_T2_L2, W3_T2_L4
2 (1,094)	4.79×10^{-09}	W3_T2_L3, W3_T4_L3
2 (1,119)	7.35×10^{-13}	W3_T4_L1, W3_T4_L1
2 (1,128)	7.34×10^{-13}	W3_T4_L2, W3_T4_L4
2 (1,129)	2.59×10^{-11}	W3_T4_L3, W3_T4_L3
2 (1,130)	1.44×10^{-17}	W3_T4_L3, W3_T4_L6
2 (1,148)	8.25×10^{-07}	W4_T1_L2, W4_T1_L6
2 (1,149)	8.21×10^{-16}	W4_T1_L2, W4_T1_L5
2 (1,180)	1.51×10^{-11}	W4_T3_L1, W4_T3_L3
2 (1,191)	1.22×10^{-08}	W4_T3_L4, W4_T3_L5
2 (1,199)	7.24×10^{-08}	W4_T4_L1, W4_T4_L5
2 (1,202)	9.96×10^{-09}	W4_T4_L2, W4_T4_L5
2 (1,206)	9.75×10^{-09}	W4_T4_L3, W4_T4_L4
3 (892)	1.68×10^{-06}	W1_T1_L2, W1_T1_L2, W1_T1_L2
3 (956)	2.31×10^{-09}	W1_T4_L2, W1_T4_L3, W1_T4_L3
3 (970)	1.36×10^{-09}	W2_T1_L1, W2_T1_L1, W2_T1_L1
3 (1,028)	4.29×10^{-09}	W2_T3_L3, W2_T3_L3, W2_T3_L4
3 (1,123)	1.28×10^{-08}	W3_T4_L2, W3_T4_L6, W3_T4_L6
3 (1,176)	8.24×10^{-07}	W4_T2_L5, W4_T2_L5, W4_T3_L3
3 (1,184)	1.06×10^{-07}	W4_T3_L2, W4_T3_L2, W4_T3_L5
4 (1,016)	4.69×10^{-14}	W2_T3_L1, W2_T3_L1, W2_T3_L1, W2_T3_L4
4 (1,124)	6.73×10^{-11}	W3_T4_L2, W3_T4_L4, W3_T4_L5, W3_T4_L6
4 (1,150)	6.13×10^{-17}	W4_T1_L2, W4_T1_L3, W4_T1_L5, W4_T1_L5
4 (1,209)	2.99×10^{-11}	W4_T4_L4, W4_T4_L4, W4_T4_L4, W4_T4_L4
5 (886)	1.89×10^{-09}	W1_T1_L1, W1_T1_L1, W1_T1_L3, W1_T1_L3, W1_T1_L3
5 (907)	1.06×10^{-18}	W1_T1_L4, W4_T1_L1, W4_T1_L1, W4_T1_L4, W4_T1_L6
5 (918)	2.06×10^{-17}	W1_T2_L1, W1_T2_L3, W1_T2_L3, W1_T2_L3, W1_T2_L3
5 (952)	2.53×10^{-09}	W1_T4_L1, W1_T4_L1, W1_T4_L1, W1_T4_L5, W1_T4_L5
5 (957)	1.80×10^{-07}	W1_T4_L2, W1_T4_L3, W1_T4_L3, W1_T4_L5, W1_T4_L5
5 (1,022)	3.27×10^{-09}	W2_T3_L2, W2_T3_L2, W2_T3_L2, W2_T3_L2, W2_T3_L3
5 (1,079)	9.77×10^{-10}	W3_T2_L1, W3_T2_L1, W3_T2_L1, W3_T2_L3, W3_T2_L4
5 (1,102)	1.16×10^{-09}	W3_T3_L1, W3_T3_L3, W3_T3_L3, W3_T3_L5, W3_T3_L5
5 (1,111)	2.40×10^{-10}	W3_T3_L4, W3_T3_L4, W3_T3_L4, W3_T3_L4, W3_T3_L4
5 (1,144)	3.23×10^{-10}	W4_T1_L1, W4_T1_L4, W4_T1_L5, W4_T1_L6, W4_T1_L6
5 (1,198)	2.20×10^{-10}	W4_T4_L1, W4_T4_L2, W4_T4_L3, W4_T4_L3, W4_T4_L5
6 (1,101)	7.90×10^{-09}	W3_T3_L1, W3_T3_L1, W3_T3_L2, W3_T3_L2, W3_T3_L3, W3_T3_L3
7 (908)	1.38×10^{-08}	W1_T1_L5, W1_T1_L5, W1_T1_L5, W1_T1_L5, W1_T1_L5, W1_T1_L5, W1_T1_L5
7 (1,166)	3.38×10^{-10}	W4_T2_L2, W4_T2_L2, W4_T2_L3, W4_T2_L3, W4_T2_L4, W4_T2_L4, W4_T2_L4
7 (1,181)	7.21×10^{-09}	W4_T3_L1, W4_T3_L2, W4_T3_L2, W4_T3_L4, W4_T3_L4, W4_T3_L4, W4_T3_L5
8 (888)	5.43×10^{-12}	W1_T1_L1, W1_T3_L2, W1_T3_L2, W1_T3_L3, W1_T3_L4, W1_T3_L4, W1_T3_L5
8 (1,041)	4.02×10^{-09}	W2_T4_L1, W2_T4_L3, W2_T4_L3, W2_T4_L4, W2_T4_L4, W2_T4_L5, W2_T4_L5
10 (906)	2.08×10^{-09}	W1_T1_L4, W1_T2_L1, W1_T2_L1, W1_T2_L1, W1_T2_L1, W1_T2_L2, W1_T2_L4, W1_T2_L5, W1_T2_L5, W1_T2_L5
12 (977)	2.02×10^{-10}	W2_T1_L2, W2_T1_L2, W2_T1_L2, W2_T1_L3, W2_T1_L3, W2_T1_L3, W2_T1_L3, W2_T1_L3, W2_T1_L4, W2_T1_L5, W2_T1_L5
12 (997)	1.82×10^{-06}	W2_T2_L1, W2_T2_L1, W2_T2_L1, W2_T2_L2, W2_T2_L3, W2_T2_L3, W2_T2_L4, W2_T2_L4, W2_T2_L5, W2_T2_L5, W2_T2_L5, W2_T2_L5

^a MLG = multilocus genotypes. If probability of P_{sex} is $P < 0.05$, isolates can be considered members of the same clone whereas, for those with $P > 0.05$, there is no evidence that members belong to the same genotype. All nonclonal isolates and first encounters had $P > 0.05$ (single-encounter MLG not listed).

(Promega Corporation, Madison, WI). The conditions for the polymerase chain reaction (PCR) were 94°C for 1 min, 94°C for 40 s, 58°C for 40 s, and 72°C for 20 s (repeated for 33 cycles), followed by 72°C for 30 min. Amplicons were subsequently denatured at 95°C for 3 min and were processed on a Hitachi 3500 Genetic Analyzer (Thermo-Fisher Scientific, Grand Island, NY) against a Genescan 500LIZ size standard (Applied Biosystems, Foster City, CA). Peaks were scored by base pair size for each marker using GeneMarker (SoftGenetics, State College, PA). A single band at each locus was detected because *V. effusa* is haploid. Multiple reactions were repeated to ensure reliability, and unsuccessful or ambiguous reactions were further repeated to ensure unequivocal results.

Identification of mating types. The multiplex procedure for identification of mating types was previously described (Young et al. 2018). Primers for the *MAT1-1-1* (MTA-F: ATCACACTTGCCGC CAAGCGACC and MTA-R: TTGATGAGAGGGCAGACGAT [242-bp amplicon]) and *MAT1-2-1* (MTB-F1: AAGGTTCTCGCCCAAGC CAATG and MTB-R2: CTAAAGTTGGAAGAGAGGTTGG [775-bp amplicon]) were used; the *TUB2* gene was also amplified to confirm that the reaction worked (tubB-F: AAGGTTCTCGCCCAAGC CAATG and tubB-R: CCGTGTTAGTGTCCTTTGGCCCA [517-bp amplicon]). The PCR was performed in a total volume of 25 µl containing 3 ng of genomic DNA, 1.0 U of GoTaq DNA Polymerase (Promega Corporation), and 1× Green GoTaq reaction buffer containing 1.5 mM MgCl₂, 0.2 mM each dNTP (Promega Corporation), and 1 µM target-specific primers. The cycling parameters were 94°C for 1 min; then, 30 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 45 s; followed by 72°C for 10 min. Reaction products were run using electrophoresis in 1.5% agarose gel in 1× Tris-boric-EDTA buffer and visualized with ethidium bromide by UV transillumination.

Data analysis. Some basic population genetic statistics were obtained from the raw data but most subsequent analyses were based on clone-corrected data. Clone-corrected data were used because censoring for clones prevents bias in the statistical analysis of the genetic structure of populations due to multiple representatives of the same multilocus genotypes (MLG) in a population (Lee and Neate 2007), as well as providing more conservative measures for rejecting the null hypothesis of random mating (Smith et al. 1993). Analyses were performed using the package *poppr* V2.4.1 (Kamvar et al. 2014) in R V3.4.1 (R Core Team 2012) for all isolates and the clone-corrected data, unless otherwise stated. The number of MLG observed, the clone-corrected number of MLG, the expected MLG (eMLG, which measures genotypic richness) at the smallest sample size based on rarefaction, the clonal fraction, genotypic diversity (which measures both genotypic richness and abundance using the Shannon-Wiener index of MLG diversity) (Shannon 2001), Stoddart and Taylor's index of MLG diversity (Stoddart and Taylor 1988), and Simpson's index (Simpson 1949) were calculated. Evenness (*E0.5*) (Grünwald et al. 2003; Ludwig and Reynolds 1988; Pielou 1975) and Nei's unbiased genetic diversity (*H_{exp}*) (Nei 1978) were also calculated.

A Bayesian model was used to determine clustering (*K*) of the isolates and their relatedness among populations using Structure V2.3.4 (Falush et al. 2007; Pritchard et al. 2000). Individuals were assigned to preselected clusters (the source populations). In total, 20 independent runs each of *K* = 1 to 4 were performed. The analysis was run using default settings with a burn-in of 5,000 iterations followed by 50,000 Monte Carlo Markov chain repetitions. Using the second-order rate of change of the variable *Pr(X/K)* (i.e., ΔK), population cluster assignment was determined (Evanno et al. 2005). The result was represented graphically by showing each isolate as a vertical line partitioned into *K* colored segments that represent an individual's estimated membership fraction in each of the inferred clusters. Based on all 335 isolates, a minimum-spanning network was also generated based on Bruvo's distance. Assuming no prior knowledge of group membership, and using the clone-corrected data, a discriminant analysis of principal components (DAPC) was performed to visualize patterns among MLG from the four tree populations. Genetic structure was further explored using analysis of molecular variance

(AMOVA; based on 1,000 permutations) for both raw and clone-corrected data, which was conducted at four spatial scales (diversity at the level of trees in the orchard, terminals within trees, among leaflets within terminals, and populations of lesions on leaflets).

Linkage disequilibrium was assessed using the index of association (*I_A*) (Brown et al. 1980) and the standardized index of association ($\bar{r}_d$) (Agapow and Burt 2001). Significance of *I_A* and $\bar{r}_d$ was tested by its deviation from the expected value (zero) based on 1,000 randomizations. The numbers and frequencies of private alleles were calculated. The probability that isolates were likely to arise from independent formation of identical sexual spores or as a result of clonal propagation was explored using *P_{sex}*. If the *P* value for the second or subsequent encounters of that isolate is less than the α value (in this case, α = 0.05), isolates can be considered members of the same clone, whereas those with *P* > 0.05 indicates that there is no evidence that the isolate has arisen asexually. These metrics were analyzed using various functions in *poppr*.

The proportions of the *MAT1-1-1* and *MAT1-2-1* mating types in all isolates sampled in the orchard and on individual trees, terminals, and leaflets were calculated based on the raw and clone-corrected data. The clone correction was performed so as to be appropriate at each spatial scale of population (thus, only a single copy of each MLG was represented in a population unit at that scale). For each population or subpopulation unit at each spatial scale, the proportion of *MAT1-1-1* and *MAT1-2-1* isolates was tested for equilibrium (the null hypothesis being 1:1 ratio). A χ^2 test was used to determine whether the proportions departed significantly from the 1:1 ratio (Everitt 1992). A contingency table χ^2 analyses was performed at each spatial scale to determine whether individual subpopulations (trees and terminals) within the larger spatial scale (orchard and tree, respectively) differed in mating type frequencies. Due to the limitations of the χ^2 test when analyzing small sample sizes (generally populations with expected values < 5), an exact binomial test (two-tailed) for goodness-of-fit was used to determine whether observed mating type frequencies within populations deviated from the 1:1 ratio. Due to the low number of isolates collected from individual leaflets, the leaflet sub-subpopulations were not subject to analyses of equilibrium. Analyses of mating type data were performed using SAS V9.4 (SAS Institute, Cary, NC).

RESULTS

Genetic diversity. All loci produced products of expected amplicon sizes. Only three isolates had missing marker data (total of four loci) due to null alleles, and the mean total missing data were 0.04%. No isolate had >7% missing data. Thus, no data were excluded from the analysis. However, of the 30 microsatellites screened, 1 (Fe232_GA6) was found to be uninformative and was not included in the analysis (statistics associated with the remaining 29 are presented in Supplementary Table S1). On a per tree basis, the observed number of alleles per locus ranged from 1 to 14, the mean effective number of alleles per locus ranged from 2.3 to 2.5, and *H_{exp}* per locus from 0.0000 to 0.8466. A genotype accumulation curve showed that the 29 primers adequately separated the 165 MLG sampled in this study (Supplementary Fig. S1). There were 335 isolates collected from the four trees comprising the 165 MLG (Table 1) (results from nonclone-corrected data shown in Supplementary Table S2). The number of isolates collected from each of the four trees ranged from 75 (tree W4) to 89 (tree W2), with the number of MLG ranging from 35 to 53. The number of isolates from each terminal ranged from 14 to 32, and the number of MLG ranged from 5 to 17. Overall, at the scale of the orchard, the clonal fraction was 49.3%, more variable at the scale of the tree (43.0 to 62.4%), and most variable at the scale of the terminal (27.8 to 85.0%). The number of eMLG at the smallest sample size for trees was 35 for all trees, and for terminals ranged from 5 to 10. Of the 165 MLG sampled, 63 (38.2%) were clonal, with the remaining 102 MLG being represented only once. The clonal genotypes were isolated between 2 and 12 times, depending on MLG.

The Shannon-Weiner Index of diversity not surprisingly depended on spatial scale of sampling, being greatest at the scale of the orchard (5.10), followed by trees (3.56 to 3.97), and least on terminals (1.61 to 2.83) (Table 1). Stoddart and Taylor's index of

MLG diversity followed a similar pattern, as did Simpson's index of diversity. However, H_{exp} did not depend on spatial level. The overall H_{exp} was indicative of moderate to high diversity (0.507) and ranged from 0.487 to 0.520 among trees and 0.368 to 0.586

TABLE 4. Hierarchical analysis of molecular variance (AMOVA) of the population structure of *Venturia effusa* collected from pecan orchards and trees within orchards in the southeastern United States using both raw and clone-corrected data^a

Data set, source ^b	Df	SS	MS	Est. var.	%	φ	P ^c
All data							
Between trees	3	101.4	33.8	−0.03	−0.5	0.31	0.6
Between terminals within trees	12	426.6	35.6	1.26	17.3	0.17	0.001
Between leaflets within terminals	65	592.8	9.1	1.02	14.0	0.17	0.001
Within leaflets	254	1,275.5	5.0	5.02	69.2	−0.01	0.001
Total	334	2,396.3	7.1	7.26	100.0	...	...
Clone-corrected							
Between trees	3	29.8	9.9	−0.05	−0.2	0.06	0.5
Between terminals within trees	12	120.9	10.1	0.26	3.5	0.03	0.001
Between leaflets within terminals	57	419.3	7.4	0.22	2.9	0.04	0.08
Within leaflets	93	642.0	6.9	6.90	93.8	−0.002	0.001
Total	165	1,211.9	7.4	7.36	100.0	...	...

^a AMOVA was performed using *poppr* (Kamvar et al. 2014). Df = degrees of freedom, SS = sum of squares, Est. var. = estimated variance, % = percentage of total variance, φ = differentiation between-trees, between-terminals within trees, between leaflets within terminals, and among isolates on leaflets.
^b Trees = populations, terminals = subpopulations, and leaflets = sub-subpopulations.
^c P = probability. A randomization test with 1,000 permutations was used to determine whether populations are significantly different at each stratum.

Fig. 2. Minimum-spanning network based on Bruvo's genetic distance for 335 isolates of *Venturia effusa* (cause of pecan scab) collected from four Wichita pecan trees in an orchard in Georgia. Node size (and number in node) indicates clones, and the edges of the branches indicate the genetic distance between isolates. Data are not clone-corrected. Node shading represents population membership; node size indicates number of clones (number in node).

among terminals. $E0.5$ on terminals for the raw data ranged from 0.519 to 0.944, and is a measure of the distribution of genotype abundance.

The percentage of polymorphic loci was consistently high (100% for all isolates and 96.6 to 100% among tree populations) (Table 2). Private alleles were found in all trees and ranged in number from 4 to 29. However, the frequency was consistently low (0.020 to 0.130%) and, although 21 of the loci had private alleles in different trees, most often it was a single allele, and only one locus (Fe050_CA6) had six alleles unique to a tree (W3) (Fig. 1A). Similarly, among the two mating types, private alleles were observed at 19 of the loci but the number of private alleles per locus was most often 1, although two loci had five private alleles (Fe050_CA6 and Fe179_CTT6) (Fig. 1B).

Linkage disequilibrium. Both measures of multilocus linkage disequilibrium, I_A and $\bar{r}_d$, showed that none of the tree populations had loci at equilibrium with one another (Table 2). Overall, there were 36.7% of locus pairs not at equilibrium and, among trees, this ranged from 20.7 to 38.2%. Thus, the majority of pairs of loci appeared to be at equilibrium in these populations. There were 63 different MLG that were clonal (Table 3). Based on estimates of P_{sex} , these isolates were all the result of asexual, conidial propagation ($P < 0.05$ in all cases of the second encounter). Furthermore, the analysis indicated that, for the first encounters and the remaining

102 isolates, there was no evidence against sexual reproduction ($P > 0.05$). These data are presented graphically in Supplementary Figure S2.

Population genetic structure. With both the raw data and the clone-corrected data, the AMOVA demonstrated significant differentiation at various spatial scales (Table 4). With the clone-corrected data, there was differentiation ($P = 0.001$) between populations on leaflets within individual terminals and between terminals within trees in the tree canopies. With the raw data, the highest percentage of overall variance explained among isolates within leaflet populations was moderate (69.2%) but, with the clone-corrected data, this was 93.8%. With the clone-corrected data, only 3.5% of variance was found between terminals within trees. There was no detectable variance (0%) between trees within the orchard and only 2.9% between leaflets within a terminal (it should be noted that negative variance values in the AMOVA can occur when there is no detectable genetic structure due to random chance). Genetic admixture was confirmed by the minimum-spanning network analysis comparing all isolates, which showed that source tree had little impact on genetic affiliation of the isolate (Fig. 2). Isolates did not cluster by source tree. The discriminant analysis also demonstrated the overlap among isolates from the different trees (Fig. 3). Furthermore, the Bayesian analysis failed to determine a subpopulation structure

Fig. 3. Discriminant analysis (DA) of principal components showing a high degree of overlap among isolates from populations of *Venturia effusa* from different leaflets of four terminals in each of four Wichita pecan trees in an orchard in Georgia. Inset indicates the number and contribution of axes from the principal components analysis retained in the DA. Data are not clone-corrected.

Fig. 4. Q plot of 166 isolates of *Venturia effusa* sampled from four trees in an orchard (cultivar Wichita) in Georgia. Based on Bayesian analysis using the program Structure (Pritchard et al. 2000) at $K = 4$, there is a high degree of admixture among populations (assumed population identity). Indeed, using the second-order rate of change of $\Pr(X/K)$ (i.e., ΔK), population cluster assignment was determined to be < 2 (Evanno et al. 2005): there was no detectable population differentiation. Data are clone-corrected.

TABLE 5. Mating type frequencies of *Venturia effusa* in populations on different terminals in Wichita pecan trees in Georgia^a

Tree, terminal ^c	MAT1-1-I: MAT1-2-I ^b		χ^2 (P) ^d	P ^e	χ^2 (P) ^f
	Number	Percent			
W1					
T1	3:14	18:82	7.1 (0.008)	0.01	14.9 (0.005)
T2	4:4	50:50	0.0 (1.0)	1.0	5.8 (0.2)
T3	3:3	50:50	0.0 (1.0)	1.0	2.9 (0.4)
T4	5:3	63:37	0.5 (0.5)	0.7	3.1 (0.4)
Subtotal	15:22	41:59	1.3 (0.3)	0.3	5.9 (0.1)
W2					
T1	8:3	73:27	2.3 (0.1)	0.2	2.1 (0.7)
T2	4:2	67:33	0.7 (0.4)	0.7	2.2 (0.7)
T3	6:8	43:57	0.3 (0.6)	0.8	2.4 (0.7)
T4	2:8	20:80	3.6 (0.06)	0.1	1.5 (0.8)
Subtotal	20:21	49:51	0.02 (0.9)	1.0	6.8 (0.08)
W3					
T1	9:8	53:47	0.06 (0.8)	1.0	7.9 (0.2)
T2	5:11	31:69	2.3 (0.1)	0.2	7.2 (0.9)
T3	2:3	40:60	0.2 (0.7)	1.0	6.1 (0.2)
T4	7:9	44:56	0.3 (0.6)	0.8	4.2 (0.5)
Subtotal	23:30	43:57	0.9 (0.3)	0.4	1.6 (0.7)
W4					
T1	3:4	43:57	0.1 (0.7)	1.0	3.6 (0.6)
T2	1:8	11:89	5.4 (0.02)	0.04	5.0 (0.3)
T3	5:4	56:44	0.1 (0.7)	1.0	2.6 (0.6)
T4	6:5	53:45	0.09 (0.8)	1.0	5.9 (0.2)
Subtotal	15:20	43:57	0.7 (0.4)	0.5	4.9 (0.2)
Total	73:92	44:56	2.2 (0.1)	0.2	0.6 (0.9)

^a Data are clone-corrected.
^b The clone-corrected number of each of the two mating types and the percentage of the total.
^c Tree W1 = tree 1, T1 = terminal 1, and so on.
^d The χ^2 value based on a contingency χ^2 between clone-corrected mating type ratios with 1 degree of freedom. The χ^2 value is based on a 1:1 ratio with 1 degree of freedom for the clone-corrected sample; the P value in parentheses indicates if the mating type frequencies were significantly different from 1:1 (significant if $P \leq 0.05$).
^e Probability from an exact binomial analysis (two-tailed) to test whether mating type frequencies deviate significantly from a 1:1 ratio (more appropriate for comparing small populations than a χ^2 test).
^f The χ^2 value is based on a contingency χ^2 analyses of trees within orchard populations (except for the cell in row labeled Total that represents a contingency χ^2 analyses among orchard populations). The P value in parentheses indicates probability that the mating type frequencies differ significantly from 1:1 (significant if $P \leq 0.05$).

(ΔK peaked at $K \leq 2$). The Q-plot for $K = 4$ shows the high degree of population admixture in isolates among tree populations (Fig. 4).

Mating type frequencies. Overall, the mating type frequency (*MAT1-1-1*:*MAT1-2-1* = 73:92) of the clone-corrected sample was not significantly different from equilibrium when analyzed using a χ^2 test ($P = 0.1$) or based on an exact binomial analysis ($P = 0.2$) (Table 5). The mating types were at equilibrium in each tree (χ^2 test: $0.3 \leq P \leq 0.9$; exact binomial analysis: $0.3 \leq P \leq 1.0$). However, at the scale of the terminal, based on the χ^2 test, 3 of 16 (18.8%) had mating types that were not at equilibrium; however, based on the exact binomial analysis, it was only 2 of 16 (12.5%). Mating type frequencies for the non-clone-corrected data is presented in Supplementary Table S3.

The mating type frequency in individual subpopulations at the scale of the trees was not significantly different (χ^2 test: $0.08 \leq P \leq 0.7$) (Table 5). However, at the scale of the terminal, according to the χ^2 test, one population (6.3%) was not at equilibrium (W1T1, $P = 0.005$).

TABLE 6. Mating type frequencies of *Venturia effusa* in populations on different leaflets of four terminals in each of four Wichita pecan trees in an orchard in Georgia^a

Tree and terminal ^c	Leaflet ^d	<i>MAT1-1-1</i> : <i>MAT1-2-1</i> ^b	
		Raw number (%)	CC number (%)
W1T1	1	0:8 (0:100)	0:7 (0:100)
	2	5:1 (83:17)	3:1 (75:25)
	3	0:5 (0:100)	0:3 (0:100)
	4	0:5 (0:100)	0:5 (0:100)
	5	0:8 (0:100)	0:2 (0:100)
	NC	NC	NC
W1T2	1	1:4 (20:80)	1:1 (50:50)
	2	0:2 (0:100)	0:2 (0:100)
	3	5:0 (100:0)	2:0 (100:0)
	4	2:3 (40:60)	2:3 (40:60)
	5	0:4 (0:100)	0:2 (0:100)
W1T3	2	0:2 (0:100)	0:1 (0:100)
	3	3:1 (75:25)	3:1 (75:25)
	4	1:3 (25:75)	1:1 (50:50)
	5	1:3 (25:75)	1:3 (25:75)
	NC	NC	NC
W1T4	1	4:0 (100:0)	2:0 (100:0)
	2	3:1 (75:25)	3:1 (75:25)
	3	5:0 (100:0)	3:0 (100:0)
	5	4:2 (67:33)	2:2 (50:50)
	NC	NC	NC
W2T1	1	4:2 (67:33)	2:1 (67:33)
	2	5:0 (100:0)	2:0 (100:0)
	3	6:1 (86:14)	2:1 (67:33)
	4	3:1 (75:25)	2:1 (67:33)
	5	5:0 (100:0)	0:3 (100:0)
	NC	NC	NC
W2T2	1	3:0 (100:0)	1:0 (100:0)
	2	3:2 (60:40)	3:1 (75:25)
	3	2:1 (67:33)	1:1 (50:50)
	4	2:0 (100:0)	1:0 (100:0)
	5	5:0 (100:0)	2:0 (100:0)
W2T3	1	2:5 (29:71)	2:3 (40:60)
	2	1:5 (17:83)	1:2 (33:67)
	3	3:4 (43:57)	3:3 (50:50)
	4	0:3 (0:100)	0:3 (0:100)
	5	2:1 (67:33)	1:1 (50:50)

(Continued)

^a Both the raw data and the clone-corrected (CC) data are presented. NC = no isolates were collected from some leaflets.

^b The CC number of each of the two mating types and the percentage of the total. Neither a χ^2 analysis nor an exact binomial analysis was performed to test whether mating type frequencies deviate significantly from a 1:1 ratio due to the small size of populations on individual leaflets.

^c Tree W1 = tree 1, T1 = terminal 1, and so on.

^d Leaflet number on terminal.

Sample sizes on individual leaflets were not sufficient to be subject to a χ^2 test or an exact binomial analysis (Table 6). However, of the total of 81 leaflets sampled, 36 had only a single mating type represented in the lesions sampled on the leaflet and, of those 36 single mating types, 8 had only a single clone (the remainder had multiple genotypes of the same mating type).

Spatial dependency of clones. A comparison of the total numbers of MLG at each of the spatial scales of the whole orchard, trees within the orchard, terminals, and on the leaflets (Table 1 and Figure 5) shows how sampling stratum, as might be expected, affected total number of clones on each unit at that stratum. Of the total isolates including clones ($n = 335$), most unique MLG were found at the stratum of the individual leaflets ($n = 242$), with similar total numbers of unique MLG observed at the strata of the terminal ($n = 170$), tree ($n = 166$), and orchard ($n = 165$). Thus, very few isolates were clones at the strata of the terminals or trees compared with the stratum of the leaflet. The dependency on spatial scale is readily seen in Figure 5A and B, which shows that, as a proportion of the total isolates, it is between the terminal and leaflet scale that

TABLE 6. (Continued)

Tree and terminal ^c	Leaflet ^d	<i>MAT1-1-1</i> : <i>MAT1-2-1</i> ^b	
		Raw number (%)	CC number (%)
W2T4	1	0:1 (0:100)	0:1 (0:100)
	2	1:3 (25:75)	1:3 (25:75)
	3	0:4 (0:100)	0:2 (0:100)
	4	1:4 (20:80)	1:3 (25:75)
	5	0:4 (0:100)	0:2 (0:100)
W3T1	1	1:4 (20:80)	1:4 (20:80)
	2	2:0 (100:0)	2:0 (100:0)
	3	2:1 (67:33)	2:1 (67:33)
	4	0:2 (0:100)	0:2 (0:100)
	5	1:1 (50:50)	1:1 (50:50)
W3T2	7	5:1 (83:17)	4:1 (80:20)
	1	2:6 (25:75)	2:4 (33:67)
	2	1:4 (20:80)	1:3 (25:75)
	3	2:3 (40:60)	2:3 (40:60)
	4	1:2 (33:67)	1:2 (33:67)
W3T3	5	0:1 (0:100)	0:1 (0:100)
	1	0:3 (0:100)	0:2 (0:100)
	2	0:3 (0:100)	0:2 (0:100)
	3	0:4 (0:100)	0:2 (0:100)
	4	5:0 (100:0)	1:0 (100:0)
W3T4	5	1:2 (33:67)	1:1 (50:50)
	1	2:2 (50:50)	1:2 (33:67)
	2	2:4 (33:67)	2:4 (33:67)
	3	2:3 (40:60)	1:3 (25:75)
	4	2:1 (67:33)	2:1 (67:33)
W4T1	5	2:0 (100:0)	2:0 (100:0)
	6	2:3 (40:60)	2:2 (50:50)
	1	1:3 (25:75)	1:2 (33:67)
	2	2:1 (67:33)	2:1 (67:33)
	3	0:1 (0:100)	0:1 (0:100)
W4T2	4	0:3 (0:100)	0:3 (0:100)
	5	1:3 (25:75)	1:2 (33:67)
	6	1:3 (25:75)	1:2 (33:67)
	1	0:3 (0:100)	0:3 (0:100)
	2	0:2 (0:100)	0:1 (0:100)
W4T3	3	0:4 (0:100)	0:3 (0:100)
	4	0:4 (0:100)	0:2 (0:100)
	5	1:2 (33:67)	1:1 (50:50)
	1	2:1 (67:33)	2:1 (67:33)
	2	4:0 (100:0)	2:0 (100:0)
W4T4	3	1:2 (33:67)	1:2 (33:67)
	4	4:1 (80:20)	2:1 (67:33)
	5	3:1 (75:25)	3:1 (75:25)
	1	2:1 (67:33)	2:1 (67:33)
	2	2:0 (100:0)	2:0 (100:0)
	3	5:0 (100:0)	4:0 (100:0)
	4	1:5 (17:83)	1:2 (33:67)
	5	2:3 (40:60)	2:3 (40:60)

clone numbers change most dramatically. Thus, the majority of the clones occurred among and on the leaflets at the scale of the terminal (10s of centimeters), indicating a notable component of short-distance dispersal.

DISCUSSION

The orchard population of *V. effusa* exhibited relatively high genetic diversity, as has been previously noted for this pathogen (Bock et al. 2017) and also for many characterized sexually and asexually reproducing ascomycetes (Everhart and Scherm 2015; Gladieux et al. 2010; Keller et al. 1997; Linde et al. 2002). Pathogens with sexual and asexual life cycles often have an “epidemic” model population structure, typified by high genetic diversity yet occurrence of some clonality due to the relative fitness of specific genotypes during the asexual “epidemic” phase (Milgroom 1996; Smith et al. 1993). Despite no described sexual stage, *V. effusa* maintains genetic diversity ($H_{\text{exp}} = 0.479$, not clone-corrected) that is evenly distributed ($E0.5 = 0.704$), comparable with that described for *Monilinia fructicola* on peach (Everhart and Scherm 2015) and other ascomycetes. Furthermore, the mating type

idiomorphs of *V. effusa* (*MAT1-1-1* and *MAT1-2-1*) were recently described and found to be in equilibrium at the scale of the southeastern United States, the orchard, and the tree (Young et al. 2018), providing further indirect evidence for the occurrence of the sexual stage in field populations of the fungus. The results from this study also found mating-type idiomorphs in equilibrium at smaller spatial scales. Populations of other ascomycetes with similar population structure and genetic diversity characteristics have been found with mating types in equilibrium, yet lacking an identified sexual stage (for example, *Rhynchosporium commune*). In this case, the sexual stage was postulated based on the observed population genetic characteristics (Linde et al. 2009). We assert that this is likely the case with *V. effusa*.

Furthermore, results of analyses of the clone-corrected data demonstrate that the majority of the genetic variance (93.8%) could be explained at the stratum of the isolates sampled from lesions on leaflets, which indicates that the populations of *V. effusa* at the finest spatial scale sampled were genetically diverse. Even for the nonclone-corrected data, there was compelling evidence that the majority of variance (69.2%) could be found among the isolates on single leaflets. The AMOVA also suggested that there was little variance explained at the scale of the leaflets on a terminal (2.9%) or between terminals on trees (3.5%, also with significant differentiation) but little to no variance attributable between trees (approximately 0%). Slightly negative values, as we observed for estimates of the variance at the scale of the tree, can occur with an AMOVA and indicate an absence of genetic structure at that stratum. The fact that there was no detectable difference in scab populations among trees in this orchard is not surprising. In a previous study of *V. effusa* in pecan trees in 11 orchards, significant ($P = 0.001$) differentiation among trees within orchards was found but the variance attributable was just 3%, yet 81% (differentiation, $P = 0.001$) of variance was found at the scale of isolates collected within the canopy of the tree (Bock et al. 2017).

High genotypic diversity is typical of sexually reproducing species and unlikely to be supported by parasexuality (Linde et al. 2009). The substantial genotypic diversity observed for *V. effusa* and the limited or lack of structure at both the tree and terminal spatial scales was further confirmed in the cluster analysis of the populations at both the tree and terminal levels, and by the minimum-spanning network analysis of all isolates. Furthermore, the Bayesian analysis failed to determine a subpopulation structure and confirmed the similarity among isolates from different tree populations (as did the DAPC). Taken together, these various methods indicate little differentiation among the tree subpopulations or among the terminal sub-subpopulations, with most genotypic diversity being similar among populations of isolates on leaflets on terminals. Nonetheless, there was a nonrandom association between 36.7% of pairs of loci, and linkage disequilibrium was observed in all four tree populations, even for the clone-corrected data. This is consistent with the previous study of populations of *V. effusa* in 11 orchards, where 6.21 to 58.16% pairs were not in equilibrium (Bock et al. 2017); yet, in that study, one population was at equilibrium. Nonetheless, analysis of P_{sex} demonstrated that, although there was very strong evidence that the isolates with common MLG were clones resulting from asexual reproduction (virtually all found at small spatial scales), it also provided evidence that, for the remaining isolates, there was little or no evidence of asexual reproduction. Linkage disequilibrium was also observed in most populations of *M. fructicola* on peach in the southeastern United States (Everhart and Scherm 2015) and globally for *R. commune* (Linde et al. 2009). Furthermore, private alleles were observed at moderate frequencies in the Linde et al. (2009) study in populations from different regions. In that study only 14 markers were employed, compared with 30 in the current study, which found private alleles at a much smaller spatial scale both between trees in the same orchard and between the two mating types in those trees. The sexual stage is characterized for *M. fructicola* but not for *R. commune*. Private alleles and nonrandom

Fig. 5. A, Proportion of clones (both mating types combined) of *Venturia effusa* for isolates from populations of *Venturia effusa* at spatial scales on the leaflets, terminals, trees, and the whole orchard (cultivar Wichita) in Georgia, demonstrating the scale at which most clones existed (the stratum of the leaflet on the terminal shows the decline in proportion) and B, frequency of different isolate numbers (both mating types combined) sampled from leaflets and the clone-corrected counts demonstrating that many clones occurred at the level of the leaflet.

associations among alleles and loci could be due to several different factors, including population isolation, nonrandom mating, linkage (perhaps due to locus proximity), asexual reproduction, or genetic drift (Crow and Kimura 1970; Milgroom 1996; Taylor et al. 1999), all of which can lead to linkage disequilibrium.

Despite the observed genetic diversity and demonstrated spatial population structure, clonality was a major component of the populations of *V. effusa* at the smallest spatial scale (the isolates on leaflets on terminals). Overall, 49.3% of isolates were clonal. A component of clonality at small spatial scales is characterized in several ascomycete pathogens with contributions from both sexual and asexual reproductive stages (Everhart and Scherm 2015; Keller et al. 1997; Li and Brewer 2016; Linde et al. 2002). Thus, populations of *V. effusa* with a genetic structure at a fine scale on leaflets in terminals of canopies of pecan trees typified by clonality might not be unexpected. Indeed, it can be hypothesized that asexually produced conidia might be locally dispersed in rain splash or wind up to distances of a few 10s of centimeters, and sexually produced ascospores might become entrained in air currents, dispersed greater distances, and result in the observed distribution of the genetic diversity, following the epidemic model of Smith et al. (1993). The fact that so few clones were found at scales greater than a few 10s of centimeters suggests that this might be the case. Indeed, although aerial dispersal of pecan scab conidia occurs (Gottwald and Bertrand 1982), conidia are also found in substantial quantities in splash droplets collected in tree canopies (Gottwald and Bertrand 1982). The relative contribution of aerially or splash-dispersed conidia to disease development is not known. Our data indicate that short-distance events occur, more likely typical of splash dispersal. Lesions on overwintered terminal shoots are thought to be important as sources of primary inoculum early in the spring (Demaree 1924) but our data do not indicate that there are a few widely dispersed clones within or between tree canopies; rather, they are highly localized on individual leaflets on a terminal. Leaves do develop resistance as they become fully expanded (Turechek and Stevenson 1998); therefore, they will only be subject to infection for those few weeks while they are being produced and expanding. Thus, potential later infection may not be represented after most of the leaves on a terminal have matured by late June. Fruit, which are not present early in the season but remain susceptible throughout their development, may provide further insight into the presence and spread of clones in the mid and late season.

As noted, the mating type idiomorphs *MAT1-1-1* and *MAT1-2-1* were found to be at equilibrium, providing further evidence for the occurrence of the sexual stage. It was interesting to note that both mating types were present on 55.6% of the leaflets sampled. This would likely be higher but, inevitably, some isolations from a few individual lesions failed, thus precluding confirmation of all isolate mating types on that leaflet. Furthermore, in many cases, leaflets in the field may have many more lesions than those selected in this study. Indeed, leaflets with counts of 90 or more scab lesions per leaflet are not uncommon (Latham 1979), particularly on very susceptible cultivars (C. H. Bock, personal observation). The opportunity for opposite mating types to be closely associated or even in conjoined lesions is very possible, as has been noted on individual leaves of wheat for the wheat pathogen *Zymoseptoria tritici* (Linde et al. 2002). Such situations may provide an opportunity for the fungal hyphae to interact, for heterokaryon formation, and for eventual development of the sexual stage. Similar diversity of mating types of *V. effusa* would be expected on the fruit shucks as observed on leaflets but this has not yet been explored. Considering the number of lesions that can develop on pecan leaflets and fruit, there should be ample opportunity for such associations among mating types. Assuming the sexual stage occurs in the field, it is unknown where or when it might occur, or what role ascospores would have in the epidemic.

The timing of sample collection relative to the contribution of sexual and asexual components of the epidemic may affect results of

a population genetic analysis, as has been discussed for *V. inaequalis*, cause of apple scab (Xu et al. 2013). The samples of *V. effusa* in this study were collected in mid-June to July 2012. Generally, most terminals on a pecan tree have produced the full complement of compound leaves on the terminals by mid-June, and the leaves will be fully expanded (some indeterminate or secondary growth of terminals may occur). Fully expanded leaves are effectively resistant to infection with scab (Gottwald 1985; Turechek and Stevenson 1998). Thus, overwintering lesions on twigs and the earliest infections on the oldest leaves on the pecan terminal are likely to be local sources of conidia for infection, resulting in clonal lineages being more likely to develop on individual terminals as the season progresses (scab is a polycyclic disease). The fact that the samples of leaflets were collected at random from all the leaves on the terminal and also at the end of foliage development makes it representative of the overall population on the foliage. Although we can conclude that the clonal component of the population is asexual, it remains unknown what proportion of the remainder of the population would be from sexual versus asexual spores.

The results from this study provide novel information on the characteristics of populations of *V. effusa* at fine spatial scales, and provide insights into the dispersal of the organism within and between trees. Genetic diversity is high at small spatial scales, and there is evidence for local asexual spread. Additional studies are warranted to better understand the population genetic characteristics on leaves of different ages on a terminal and also on fruit during the season, so as to gain insight into the progression of clonal reproduction during a season. Furthermore, sampling of multiple terminals within a small area both vertically and horizontally will be informative, as will sampling at various time points during the season to monitor any changes in the population characteristics. As concluded elsewhere (Everhart and Scherm 2015), tree architecture may impact the development of epidemics and should be considered in the pecan-*V. effusa* pathosystem. There is a risk of a super race of a pathogen developing in a tree canopy (Passey et al. 2016) but further temporal studies will indicate how stable populations of pecan scab are from year to year. Finally, the proximity of *MAT1-1-1* and *MAT1-2-1* mating types on a single leaflet provides ample evidence that the sexual stage has potential to exist in the field.

ACKNOWLEDGMENTS

We thank M. Zhang, W. Evans, S. de Vos, B. Rawls, G. Moreland, T. Endalew, and K. Venkatesan for input and technical help in the collecting and processing of isolates; and B. McDonald, ETH-Zurich, for providing valuable comment and discussion.

LITERATURE CITED

- Agapow, P.-M., and Burt, A. 2001. Indices of multilocus linkage disequilibrium. *Mol. Ecol.* 1:101-102.
- Bock, C. H., Brennenman, T. B., Wood, B. W., and Stevenson, K. L. 2016a. Challenges of managing disease in tall orchard trees—Pecan scab, a case study. *CAB Rev.* 12, 008:1-18. <https://www.cabi.org/cabreviews/>
- Bock, C. H., Chen, C., Yu, F., Stevenson, K. L., Arias, R. S., and Wood, B. W. 2016b. Characterization of microsatellites in *Fusicladium effusum*, cause of pecan scab. *For. Pathol.* 46:600-609.
- Bock, C. H., Endalew, T. T., Biswas, B. K., Yadav, A. K., Sither, V., Hotchkiss, M. W., Stevenson, K. L., and Wood, B. W. 2014a. A comparison of UP-PCR and RAPD markers to study genetic diversity of the pecan scab fungus (*Fusicladium effusum* G. Winter). *Forest Pathol.* 44:266-275.
- Bock, C. H., Hotchkiss, M. W., Young, C. A., Charlton, N. D., Chakradhar, M., Stevenson, K. L., and Wood, B. W. 2017. Population genetic structure of *Venturia effusa*, cause of pecan scab, in the southeastern United States. *Phytopathology* 107:607-619.
- Brown, A. H. D., Feldman, M. W., and Nevo, E. 1980. Multilocus structure of natural populations of *Hordeum spontaneum*. *Genetics* 96:523-536.
- Burchhardt, K. M., Miller, M. E., Cline, W. O., and Cubeta, M. A. 2017. Fine-scale genetic structure and reproductive biology of the blueberry pathogen *Monilinia vacciniae-corymbosi*. *Phytopathology* 107:231-239.

- Conner, P. J., and Stevenson, K. L. 2004. Pathogenic variation of *Cladosporium caryigenum* isolates and corresponding differential resistance in pecan. *HortScience* 39:553-557.
- Crow, J. F., and Kimura, M. 1970. An Introduction to Population Genetics Theory. Harper and Row, New York.
- Demaree, J. B. 1924. Pecan scab with special reference to sources of the early spring infections. *J. Agric. Res.* 28:321-333.
- Demaree, J. B., and Cole, J. R. 1929. Behavior of *Cladosporium effusum* (Wint.) Demaree on some varieties of pecan. *J. Agric. Res.* 18:363-370.
- Evanno, G., Regnaut, S., and Goudet, J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.* 14:2611-2620.
- Everhart, S. E., and Scherm, H. 2015. Fine-scale genetic structure of *Monilinia fructicola* during brown rot epidemics within individual peach tree canopies. *Phytopathology* 105:542-549.
- Everitt, B. S. 1992. The Analysis of Contingency Tables, 2nd ed. Chapman & Hall, London.
- Falush, D., Stephens, M., and Pritchard, J. K. 2007. Inference of population structure using multilocus genotype data: Dominant markers and null alleles. *Mol. Ecol.* 7:574-578.
- Gladieux, P., Zhang, X.-G., Roldan-Ruiz, I., Caffier, V., Leroy, T., Devaux, M., van Glabeke, S., Coart, E., and Le Cam, B. 2010. Evolution of the population structure of *Venturia inaequalis*, the apple scab fungus, associated with the domestication of its host. *Mol. Ecol.* 19:658-674.
- Goff, W. D., McVay, J. R., and Gazaway, W. S. 1996. Pecan Production in the Southeast. Coop. Ext. Syst. Circ. ANR-459. University of Alabama, Auburn.
- Gottwald, T. R. 1985. Influence of temperature, leaf wetness period, leaf age, and spore concentration on infection of pecan leaves by conidia of *Cladosporium caryigenum*. *Phytopathology* 75:190-194.
- Gottwald, T. R., and Bertrand, P. F. 1982. Patterns of diurnal and seasonal airborne spore concentrations of *Fusicladium effusum* and its impact on a pecan scab epidemic. *Phytopathology* 72:330-335.
- Gottwald, T. R., and Bertrand, P. F. 1983. Effect of time of inoculation with *Cladosporium caryigenum* on pecan scab development and nut quality. *Phytopathology* 73:714-718.
- Grünwald, N. J., Goodwin, S. B., Milgroom, M. G., and Fry, W. E. 2003. Analysis of genotypic diversity data for populations of microorganisms. *Phytopathology* 93:738-746.
- Holb, I. J., Heijne, B., and Jeger, M. J. 2004. Overwintering of conidia of *Venturia inaequalis* and the contribution to early epidemics of apple scab. *Plant Dis.* 88:751-757.
- Hu, M.-J., Dowling, M. E., and Schnabel, G. 2018. Genotypic and phenotypic variations in *Botrytis* spp. isolates from single strawberry flowers. *Plant Dis.* 102:179-184.
- Kamvar, Z. N., Tabima, J. F., and Grünwald, N. J. 2014. Poppr: An R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ* 2:e281.
- Keller, S. M., McDermott, J. M., Pettway, R. E., Wolfe, M. S., and McDonald, B. A. 1997. Gene flow and nodular reproduction in the wheat glume blotch pathogen *Phaeosphaeria nodorum* (anamorph *Stagonospora nodorum*). *Phytopathology* 87:353-358.
- Latham, A. J. 1979. Some factors influencing pecan scab development. *Highlights Agric. Res. Stn. Auburn Univ.* 26:7.
- Lee, S. H., and Neate, S. M. 2007. Population genetic structure of *Septoria passerinii* in Northern Great Plains barley. *Phytopathol* 97:938-944.
- Li, H.-X., and Brewer, M. T. 2016. Spatial genetic structure and population dynamics of gummy stem blight fungi within and among watermelon fields in the southeastern United States. *Phytopathology* 106:900-908.
- Linde, C. C., Zala, M., and McDonald, B. A. 2009. Molecular evidence for recent founder populations and human-mediated migration in the barley scald pathogen *Rhynchosporium secalis*. *Mol. Phylogenet. Evol.* 51:454-464.
- Linde, C. C., Zhan, J., and McDonald, B. A. 2002. Population structure of *Mycosphaerella graminicola*: From lesions to continents. *Phytopathology* 92:946-955.
- Ludwig, J. A., and Reynolds, J. F. 1988. Statistical Ecology: A Primer in Methods and Computing. Wiley, New York.
- McDonald, B. A., and Linde, C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annu. Rev. Phytopathol.* 40:349-379.
- McDonald, B. A., and Mundt, C. C. 2016. How knowledge of pathogen population biology informs management of *Septoria tritici* blotch. *Phytopathology* 106:948-955.
- McDonald, M. C., McDonald, B. A., and Solomon, P. S. 2015. Recent advances in the *Zymoseptoria tritici*-wheat interaction: Insights from pathogenomics. *Front. Plant Sci.* 6:102.
- Milgroom, M. G. 1996. Recombination and the multilocus structure of fungal populations. *Annu. Rev. Phytopathol.* 34:457-477.
- NASS. 2015. Pecan production statistics for 2015. Online publication. National Agricultural Statistics Service. <https://quickstats.nass.usda.gov/results/29594F91-5ED0-3A4E-8F5E-4C26CC0071D8>
- Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89:583-590.
- Passey, T. A. J., Shaw, M. W., and Xu, X.-M. 2016. Differentiation in populations of the apple scab fungus *Venturia inaequalis* on cultivars in a mixed orchard remain over time. *Plant Pathol.* 65:1133-1141.
- Pielou, E. C. 1975. Ecological Diversity. Wiley, New York.
- Pritchard, J. K., Stephens, M., and Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155:945-959.
- R Core Team. 2012. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna.
- Rossman, A. Y., Allen, W. C., and Castlebury, L. A. 2016. New combinations of plant associated fungi resulting from the change to one name for fungi. *IMA Fungus* 7:1-7 <http://www.imafungus.org/Issue/71/11.pdf>
- Shannon, C. E. 2001. A mathematical theory of communication. *ACM SIGMOBILE Mob. Comput. Commun. Rev.* 5:3-55.
- Simpson, E. 1949. Measurement of diversity. *Nature* 163:688.
- Smith, J. M., Smith, N., O'Rourke, M., and Spratt, B. G. 1993. How clonal are bacteria? *Proc. Natl. Acad. Sci. USA* 90:4384-4388.
- Sonoda, R. M., Ogawa, J. M., and Manji, B. T. 1991. Population structure of *Monilinia fructicola* in *Prunus persica* var. *nucipersica* tree canopies. *Mycol. Res.* 95:893-895.
- Stevenson, K. L. 1999. Fungicide resistance management in pecans. *Southeast. Pecan Grow. Assoc.* 92:58-64.
- Stevenson, K. L., and Bertrand, P. F. 2001. Within-season dynamics of yield loss due to pecan scab fruit infections. (Abstr.) *Phytopathology* 91:S85.
- Stevenson, K. L., Breneman, T. B., and Brock, J. 2014. Results of the 2014 Pecan Scab Fungicide Sensitivity monitoring program. *Ga. Pecan Grow.* 26:16-23.
- Stoddart, J. A., and Taylor, J. F. 1988. Genotypic diversity: Estimation and prediction in samples. *Genetics* 118:705-711.
- Stukenbrock, E. H., Bataillon, T., Dutheil, J. Y., Hansen, T. T., Li, R., Zala, M., McDonald, B. A., Wang, J., and Schierup, M. H. 2011. The making of a new pathogen: Insights from comparative population genomics of the domesticated wheat pathogen *Mycosphaerella graminicola* and its wild sister species. *Genome Res.* 21:2157-2166.
- Stukenbrock, E. H., and McDonald, B. A. 2008. The origins of plant pathogens in agro-ecosystems. *Annu. Rev. Phytopathol.* 46:75-100.
- Taylor, J., Jacobson, D., and Fisher, M. 1999. The evolution of asexual fungi: Reproduction, speciation and classification. *Annu. Rev. Phytopathol.* 37:197-246.
- Turechek, W. W., and Stevenson, K. L. 1998. Effects of host resistance, temperature, leaf wetness, and leaf age on infection and lesion development of pecan scab. *Phytopathology* 88:1294-1301.
- Xu, X., Harvey, N., Roberts, A., and Barbara, D. 2013. Population variation of apple scab (*Venturia inaequalis*) within mixed orchards in the UK. *Eur. J. Plant Pathol.* 135:97-104.
- Young, C. A., Bock, C. H., Charlton, N. D., Chakradhar, M., Krom, N., Bowen, J. K., Templeton, M., Plummer, K. M., and Wood, B. W. 2018. Evidence for sexual reproduction: Identification, frequency and spatial distribution of *Venturia effusa* (pecan scab) mating type idiomorphs. *Phytopathology* 108:837-846.