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Evidence for Sexual Reproduction: Identification, Frequency, and Spatial Distribution of *Venturia effusa* (Pecan Scab) Mating Type Idiomorphs

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ABSTRACT

Venturia effusa (syn. *Fusicladium effusum*), causal agent of pecan scab, is the most prevalent pathogen of pecan (*Carya illinoinensis*), causing severe yield losses in the southeastern United States. *V. effusa* is currently known only by its asexual (conidial) stage. However, the degree and distribution of genetic diversity observed within and among populations of *V. effusa* are typical of a sexually reproducing fungal pathogen, and comparable with other dothideomycetes with a known sexual stage, including the closely related apple scab pathogen, *V. inaequalis.* Using the mating type (*MAT*) idiomorphs from *V. inaequalis*, we identified a single *MAT* gene, *MAT1-1-1*, in a draft genome of *V. effusa*. The *MAT1-1-1* locus is flanked by two conserved genes encoding a DNA lyase (*APN2*) and a hypothetical protein. The *MAT* locus spanning the flanking genes was amplified and sequenced from a subset of 14 isolates, of which 7 contained *MAT1-1-1* and the remaining samples contained *MAT1-2-1*. A multiplex polymerase chain reaction screen was developed to amplify *MAT1-1-1*, *MAT1-2-1,* and a conserved reference gene encoding β-tubulin, and used to screen 784 monoconidial isolates of *V. effusa* collected from 11 populations of pecan across the southeastern United States. A hierarchical sampling protocol representing region, orchard, and tree allowed for analysis of *MAT* structure at different spatial scales. Analysis of this collection revealed the frequency of the *MAT* idiomorphs is in a 1:1 equilibrium of *MAT1-1*:*MAT1-2*. The apparent equilibrium of the *MAT* idiomorphs provides impetus for a renewed effort to search for the sexual stage of *V. effusa.*

Scab is the most prevalent disease of pecan (*Carya illinoinensis* (Wang.) K. Koch) in the southeastern United States (Demaree 1924). Estimated crop losses in the state of Georgia alone were 15, 10, and 12% in 2013, 2014, and 2015, respectively (Brock and Brenneman 2013, 2014, 2015). The combined cost of the damage and disease control measures was $78.7, $25.7, and $65 million in these 3 years, respectively. The disease is caused by the plant-pathogenic fungus *Venturia effusa* (G. Winter) Rossman & W. C. Allen (Rossman et al. 2016; Schubert et al. 2003; Seyran et al. 2010a), whereby infection by the pathogen can cause serious and even catastrophic yield loss when conditions are favorable for an epidemic (Gottwald and Bertrand 1983; Stevenson and Bertrand 2001).

Venturia effusa was first isolated from mockernut (*C. alba* = *C. tomentosa*) in Illinois (Winter 1885), and is described causing disease on *C. aquatica*, *C. cordiformis* (= *C. amara*), *C. glabra*, and *C. ovata* (Schubert et al. 2003). Along with *C. illinoinensis*, these *Carya* spp. are native to the southeastern United States but pecan is the only cultivated species. The native extent of pecan is thought to be primarily the Mississippi watershed area (Worley 2002), while the remaining *Carya* spp. are of scattered distribution. Since the mid-19th century, pecan has been cultivated mostly as clonal cultivars on rootstocks, although some production of native trees still exists. Much of the acreage in the southeastern United States is planted to susceptible cultivars and, thus, the disease is a major concern in most years.

V. effusa relies on wind and rain splash to spread conidia early in the season (Gottwald and Bertrand 1982). The primary source of inoculum, thought to be conidia, comes from overwintering lesions on shoots and shucks from the previous year (Demaree 1924). In the spring, as conditions become favorable for sporulation, conidia spread to the expanding leaves and cause infection on the foliage. In seasons conducive to the disease, the pathogen can reproduce asexually in as little as 7 to 9 days, and this can happen multiple times throughout the growing season (Latham 1982). As the fruit are produced, they can become infected, and the earliness and severity of infection will dictate the degree of yield loss (Gottwald and Bertrand 1983; Stevenson and Bertrand 2001).

Pecan scab is a challenging disease to manage (Bock et al. 2017a); over time, *V. effusa* has developed resistance to several major classes of fungicides recommended for its management, including both single-site and multisite chemistries (Seyran et al. 2010b). Although scab-resistant pecan cultivars are available, they are often not preferred by either growers or consumers for agronomic or nut quality reasons. Additionally, there is a history of the resistance breaking down as the pathogen population adapts to host resistance (Goff et al. 1989). Furthermore, isolates of *V. effusa* have a race structure based around the pecan cultivar on which they originated and other closely related cultivars; however, the race structure has not been fully examined (Conner and Stevenson 2004; Converse 1960). The degree and distribution of genetic diversity observed within and among populations of *V. effusa* (Bock et al.

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2014a,b, 2016a, 2017b) reflect that of other sexually reproducing fungal pathogens, including the closely related *V. inaequalis*, cause of apple scab (Keitt and Palmiter 1938; Tenzer and Gessler 1999).

The mating type (*MAT*) locus in fungi is known to control sexual reproduction. In ascomycetes, the *MAT* locus consists of two idiomorphs, *MAT1-1* and *MAT1-2*, which can be distinguished by the presence of either a gene that encodes an α-box protein, as found in *MAT1-1* (gene = *MAT1-1-1*), or a high mobility group (HMG) DNA binding protein encoded by *MAT1-2* (gene = *MAT1-2-1*) (Turgeon and Yoder 2000). *MAT* genes have been identified in many species (Pöggeler 2001) using polymerase chain reaction (PCR) with degenerate primers (Arie et al. 1997) and, more recently, they have been readily identified within genome sequences (Pearce et al. 2016; Penselin et al. 2016). Comparative analyses of the gene organization surrounding the *MAT* loci have shown that, although some synteny of the flanking genes can exist, variation occurs across the ascomycetes (Debuchy and Turgeon 2006).

Identification of different *MAT* idiomorphs (*MAT1-1* and *MAT1-2*) in a population and ascertaining whether the two mating types are in equilibrium can provide evidence for a heterothallic mating system, which can influence pathogen population genetics. *MAT* equilibrium has been demonstrated for many other sexually reproducing fungal pathogens in the class Dothideomycetes; for example, *Dothistroma septosporum, Leptosphaeria maculans*, *Zymoseptoria tritici*, *Pyrenophora teres*, and *Phaeosphaeria nodorum* (Dale et al. 2011; Hayden and Howlett 2005; Linde et al. 2002; Rau et al. 2005; Sommerhalder et al. 2006). *Aspergillus flavus* and *Neosartorya fumigata* were once assumed to be asexual species until studies established that populations retained *MAT* idiomorphs that were often in equilibrium, which paved the way to the discovery of the sexual stage and a fuller understanding of the pathogen population biology (Horn et al. 2009; O'Gorman et al. 2009; Ramirez-Prado et al. 2008).

The pecan scab pathogen has demonstrated the ability to adapt to novel host resistance, and the observed genetic diversity across populations suggests a method for generating and maintaining variability. These characteristics are consistent with fungal populations that have the capability to recombine sexually. The recent genome sequence of an isolate of *V. effusa* and the discovery of a potential *MAT* gene (Bock et al. 2016b) has provided the impetus to determine whether this pathogen has the potential for sexual recombination. Here, we describe the identification of *V. effusa MAT* idiomorphs, the development of *MAT* markers, and a screening protocol for isolates of *V. effusa*. We also assess diverse populations previously characterized by simple-sequence repeat (SSR) markers (Bock et al. 2017b) for the ratios of the *MAT* idiomorphs at different spatial scales in the southeastern United States.

MATERIALS AND METHODS

Sample locations and isolation of *V. effusa*. The isolates of *V. effusa* (n = 784 isolates) characterized for *MAT* idiomorphs are based on the collections that have been previously evaluated for population genetic structure using SSR markers (Bock et al. 2017b). Isolation of *V. effusa* was done as previously described by Bock et al. (2014a,b). Isolates of *V. effusa* were obtained from scab-diseased leaves from 11 populations across the southeastern United States (Table 1; Fig. 1). Samples were collected from different cultivars using a hierarchical sampling protocol that was followed at all sites so that the *MAT* structure at different spatial scales could be assessed, with the largest scale being that of the region. There were 11 orchards with six trees sampled in each orchard (except in Kansas, where only three trees were sampled). Up to 19 individual scab lesions were sampled per tree, each from one leaflet on a different compound leaf. Thus, the sampling comprised the regional scale, between orchard locations (106 to 1,321 km), between trees within an orchard (50 to 140 m), and between leaflets within a tree canopy (2 to 20 m). Trees were separated by at least four rows and sampled on a grid basis, where orchard shape and size allowed. The isolates were sampled from both commercially available cultivars in orchards and naturally seeded native-tree groves. A subset of 14 monoconidial isolates was selected, representing different locations and cultivars, to sequence the *MAT* idiomorphs (Table 1).

DNA extraction. DNA was extracted from mycelium scraped off a colony growing on potato dextrose agar using the *Quick*-DNA Fungal/Bacterial Miniprep Kit (ZymoResearch, Irvine, CA), with slight modification (Seyran et al. 2010a). A TissueLyser (Qiagen, Valencia, CA) was used to lyse the fungal mycelium. The DNA was quantified using a Nanodrop spectrophotometer (Nanodrop Products, Wilmington, DE) and stored in Tris-EDTA buffer at –20°C.

Genome sequences and sequence analyses. A draft genome sequence of *V. inaequalis* was generated by Plant and Food Research, New Zealand (Deng et al. 2017) and accessed with permission by Dr. Joanna Bowen (Plant and Food Research, New Zealand) through the Joint Genome Institute Genome Portal (Nordberg et al. 2014) and through GenBank BioProject PRJNA261633. Additional genomes of *V. inaequalis* races were generated by Plant and Food Research, New Zealand and La Trobe University. The GenBank accession numbers for *V. inaequalis MAT1-1* and *MAT1-2* are MG818328 and MG818329, respectively.

The genome sequence of *V. effusa* isolate 3Des10b from the cultivar Desirable is available under GenBank BioProject PRJNA285422 (Bock et al. 2016b). Once we had established the mating type of the subset of 14 isolates, we selected 1 isolate that represented the opposite mating type to 3Des10b to complete the flanking regions of

TABLE 1. Location, elevation, source cultivar, and number of isolates of *Venturia effusa* collected to assess mating type in the southeastern United States[a]

Source population (town, state)	Abbr[b]	*N*[c]	Number of isolates (clone-corrected)[d]	Latitude and longitude of site	Elevation (m)	Pecan cultivar	Year (month)
Fairhope, AL	AL-F	6	93 (92)	30°32.681′ N/87°52.918′ W	62	Desirable	2012 (June)
Lowndesboro, AL[e]	AL-L	6	91 (83)	32°21.236′ N/86°38.393′ W	43	Kiowa	2012 (June)
Byron, GA[e]	GA-B	6	63 (59)	32°65.522′ N/83°65.522′ W	156	Desirable	2010 (August)
Chetopa, KS	KS-C	3	34 (33)	37°01.360′ N/95°02.783′ W	266	Native seedling	2011 (July)
Powhatan, LA[e]	LA-P	6	68 (66)	31°50.211′ N/93°13.328′ W	33	Desirable	2011 (July)
Raymond, MS	MS-R	6	89 (80)	32°11.297′ N/90°29.537′ W	70	Desirable	2012 (June)
Monticello, FL	FL-M	6	52 (50)	30°32.265′ N/83°55.060′ W	69	Desirable	2011 (June)
Burneyville, OK[e]	OK-B	6	103 (100)	33°52.823′ N/97°15.164′ W	244	Western Schley	2012 (June)
Tifton, GA	GA-T	6	45 (44)	31°30.388′ N/83°38.287′ W	112	Desirable	2010 (September)
Bastrop, TX[e]	TX-B	6	93 (86)	30°03.554′ N/97°18.402′ W	123	Wichita	2012 (June)
Carlyle, IL[e]	IL-C	6	53 (48)	38°29.323′ N/89°30.212′ W	143	Native seedling	2012 (June)
Total	…	63	784 (739)	…	…	…	…

[a] Information is reprinted from Bock et al. (2017b).
[b] Abbreviations.
[c] Number of trees sampled.
[d] In all orchards, leaves were sampled arbitrarily around the canopy. Only one lesion was sampled per leaflet from a compound leaf.
[e] Two isolates were chosen at random from each population to sequence the *MAT* locus. In addition, TxPawnee1-1 and TxPawnee1-2 isolated from the fruit of cultivar Pawnee (Brownwood, TX) were also included but these were not tested for clonality by simple-sequence repeat markers.

the *MAT1-2* locus. The draft genome sequence of *V. effusa* isolate TxPawnee1-1 was sequenced at the University of Kentucky, HealthCare Genomics Core Laboratory, using an Illumina MiSeq. The sequencing library was prepared using the Nextera XT DNA library kit (Illumina) producing, in total, 33,616,218 paired-end reads, with an average length of 160 bp. The genome was assembled using CLC Genomics Workbench 8.5.1 and all scaffold sequences at least 500 bp in length were included in the final assembly. The final genome size was 35.5 Mb, represented by 2,469 scaffolds. The N50 is 83.3 kb and the smallest and largest contigs are 2.5 and 456 kb, respectively.

The *V. inaequalis MAT* genes, flanking genes, and a reference gene encoding β-tubulin (*TUB2*) (M97951) were used as queries (gene and encoded protein for each) against the *V. effusa* genomes with custom BLAST searches using SequenceServer (Altschul et al. 1990; Priyam et al. 2015). Once contigs containing these genes were identified, each gene was predicted using FGENESH with *Leptosphaeria* spp. as the training set (Solovyev et al. 2006), with additional manual editing based on the prediction of conserved introns between *V. effusa* and *V. inaequalis*. Each *MAT* locus was annotated using Geneious v10.0.6 (Biomatters Ltd., Auckland, New Zealand) and MacVector v13.5.5 (MacVector, Inc., Apex, NC), and comparisons of each *MAT* idiomorph were performed using Dot Plot in MacVector. Protein domains were identified with InterProScan 5 (Jones et al. 2014). The GenBank accession numbers for *V. effusa MAT1-1*, *MAT1-2*, and *TUB2* sequences are MF167363 to MF167376, MF173107, and MF173108, respectively.

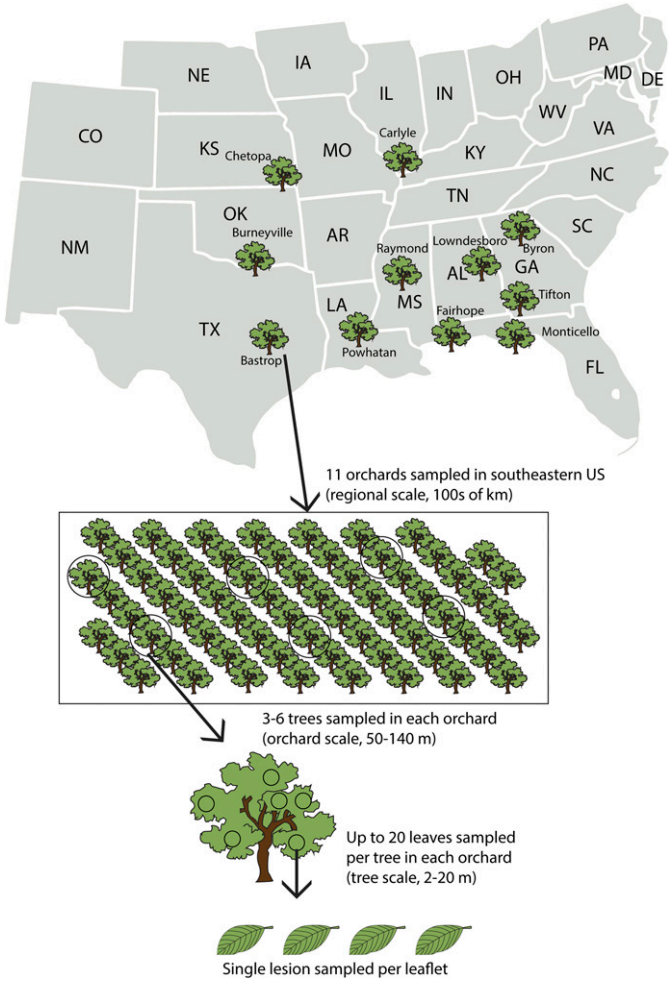

Fig. 1. Sampling strategy for isolates of *Venturia effusa* from pecan trees in orchards in the southeastern United States. Reprinted from Bock et al. (2017b).

PCR of the *MAT* region. PCR conditions with individual primer sets for *MAT* or flanking genes (Table 2) were initially established using DNA from a subset of 14 isolates representing six states and six cultivars. The multiplex PCR was established with primer sets for *MAT1-1-1*, *MAT1-2-1*, and *TUB2* (Table 2) with the same samples prior to screening the hierarchical collection of 784 isolates. PCR was performed in a total volume of 25 µl containing 3 ng of genomic DNA, 1.0 U of GoTaq DNA Polymerase (Promega Corp., Madison, WI), 1× Green GoTaq Reaction Buffer containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP (Promega Corp.), and 1 µM target-specific primers (Table 2). 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. Multiplex and single-target PCR used the same reaction and cycling conditions.

The region (7.8 kb) spanning the *MAT* locus between the flanking genes was amplified using Promega GoTaq Long PCR master mix (Promega Corp.). PCR was performed in a total volume of 50 µl containing 100 ng of DNA, GoTaq Long DNA Polymerase (Promega Corp.), and 1 µM target-specific primers (dnaL-F1 and PHdom-R) (Table 2). The cycling parameters were 94°C for 2 min; followed by 30 cycles of 94°C for 15 s, 56°C for 30 s, and 72°C for 8 min; followed by 72°C for 10 min. Idiomorph-specific primers (Supplementary Table S1) were designed to complete sequencing across the entire 8-kb amplicon from each isolate.

All amplicons were analyzed by gel electrophoresis using either 1.5 or 0.8% agarose gel in 1× Tris-borate-EDTA buffer and visualized with ethidium bromide by UV transillumination. When necessary, PCR products were purified with the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) and sequenced with Big Dye Terminator Chemistry (version 3.1; Applied Biosystems, Foster City, CA) using a 3730 DNA analyzer (Thermo-Fisher Scientific, Grand Island, NY). *MAT* loci sequence data generated from each *V. effusa* isolate by Sanger sequencing was assembled using Sequencher DNA sequence analysis software (v5.1; Gene Codes Corporation, Ann Arbor, MI) and evaluated with Geneious and MacVector.

SSR genotyping. Clonality of each isolate (apart from TxPawnee1-1 and TxPawnee1-2) was previously determined by Bock et al. (2017b) using 30 microsatellite markers known to be reliable for genotyping *V. effusa* (Bock et al. 2016a). The primers and conditions used for the reactions were described by Bock et al. (2016a, 2017b). Briefly, the reaction volume (5 µl) contained 1.5 µl of template DNA (1.5 to 2.0 ng), 0.25 µl of primer, 0.75 µl of distilled H_2O, and 2.5 µl of PCR Master Mix (2×) (Promega Corp.). The cycling conditions consisted of an initial denaturation at 94°C for 1 min; followed by 33 cycles of 94°C for 40 s, 58°C for 40 s, and 72°C for 20 s; and a final primer extension

TABLE 2. Primer combinations used to detect the mating type (*MAT*) loci of *Venturia effusa*[a]

Target	Primer name	Sequence 5′→3′	Product size (bp)
MAT1-1-1[b]	MTA-F	ATCACACTTGCCGCCAAGCGACC	242
	MTA-R	TTGATGAGAGGGCAGACGAT	…
MAT1-2-1[b]	MTB-F1	AAGGTTCCTCGCCCAGCCAATG	775
	MTB-R2	CTAAAGTTGGAAGAGAGGTTGG	…
TUB2[b]	tubB-F	TCCATCTTCAGACTGGCCAATG	517
	tubB-R	CGGTGTAGTGTCCTTTGGCCCA	…
APN2[c]	dnaL-F	GAGCAGAAGATCAATGCGAGAC	365
	dnaL-R	AACATGTCTTTGATACTCCTTC	…
hypo[c]	PHdom-F	ATGGTGACTTTCACCGCTTTCCT	387
	PHdom-R	GAAGATGCTCCTGTACATGCTCTG	…
MAT locus	dnaL-F1	GAAGAATGGACCAAGCTCTTCAC	7,886
	PHdom-R	GAAGATGCTCCTGTACATGCTCTG	…

[a] Primers were designed to coding regions of *V. effusa* genes (GenBank accession numbers MF167363 to MF167376 and MF173107) identified in this study and tested against 14 different isolates.
[b] Primers used for multiplex polymerase chain reaction to differentiate isolate mating type.
[c] Genes flanking the *MAT* genes.

step at 72°C for 30 min. Forward primers were labeled with either FAM, NED, HEX, or PET dye. All marker primer combinations were run on a Hitachi 3500 Genetic Analyzer (Thermo-Fisher Scientific). The resulting peaks were analyzed and scored by base pair size for each marker using GeneMarker (SoftGenetics, State College, PA). *V. effusa* is a haploid organism and, thus, only a single band was observed at each locus for each isolate.

Statistics. The isolates of *V. effusa* were pooled depending on the hierarchy of spatial scales to determine *MAT* distribution on leaflets in trees, trees within orchards, and throughout the region. Due to clonality potentially biasing the analysis, the analyses were performed on both clone-corrected and non-clone-corrected data sets (Bock et al. 2017b). The clonal correction was adjusted specifically for the hierarchical scale being studied, such that all populations analyzed contained only a single copy of each genotype based on the microsatellite multilocus haplotype. The clones were identified using GenAlex (Peakall and Smouse 2006, 2012), and all analyses were performed using SAS (v9.4; SAS Institute, Cary,

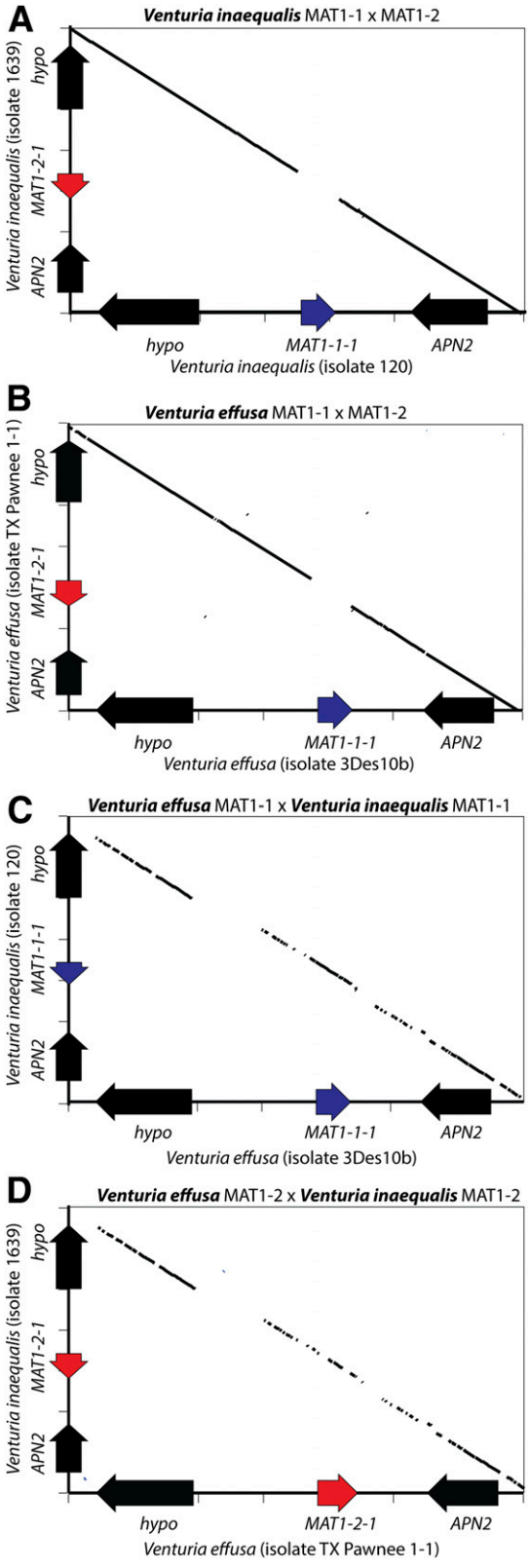

Fig. 2. Synteny across the mating type (*MAT*) locus between isolates of *Venturia effusa* and *V. inaequalis*. Comparison of **A,** *V. inaequalis MAT1-1* and *MAT1-2*; **B,** *V. effusa MAT1-1* and *MAT1-2*; **C,** *V. effusa MAT1-1* and *V. inaequalis MAT1-1*; and **D,** *V. effusa MAT1-2* and *V. inaequalis MAT1-2*. Dot plots show conserved regions based on a window size of 30 nucleotides with 60% identity.

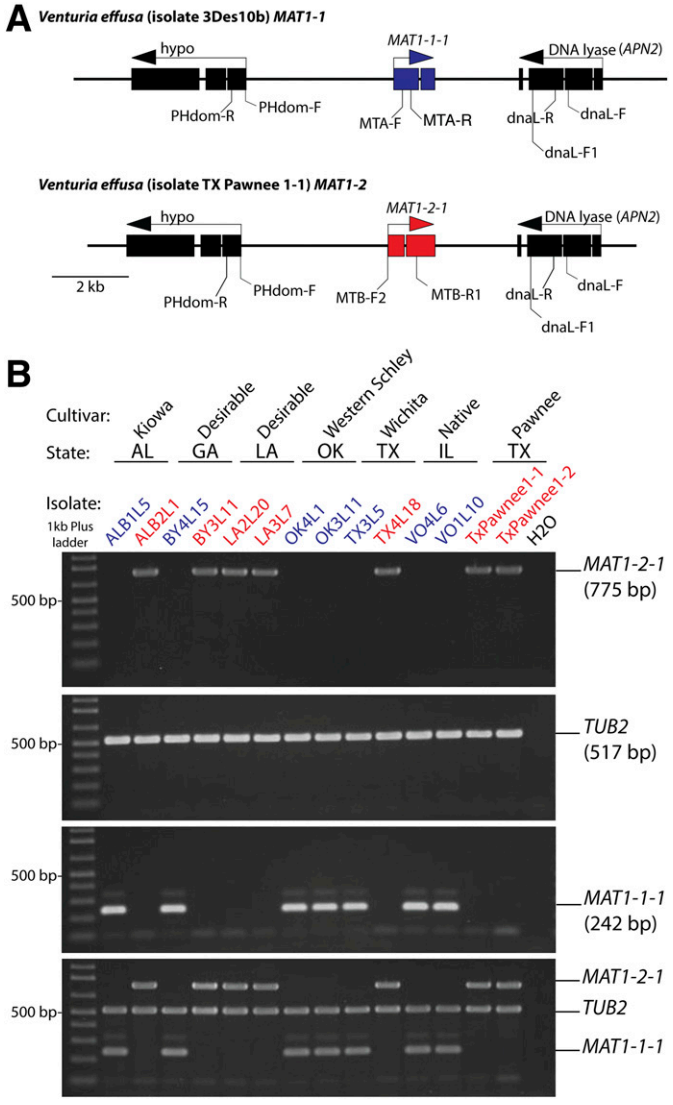

Fig. 3. *Venturia effusa* mating type (*MAT*) locus and multiplex polymerase chain reaction (PCR) screen. **A,** Gene organization of each *MAT* locus of *V. effusa*. Each gene is represented by an arrow showing the direction of transcription over boxes that represent exons of the coding sequence. Primer locations are shown as lines below the sequence. PHdom-R and dnaL-F1 were used to amplify the complete *MAT* locus from the subset of 14 isolates. Each *MAT* locus is drawn to scale. **B,** Amplicons resulting from a PCR screen to determine the mating type of *V. effusa* isolates. The top three gels show PCR amplification products using single primer sets for *MAT1-2-1* (primers MTB-F1 and MTB-R2), *TUB2* (primers tubB-F and tubB-R), and *MAT1-1-1* (primers MTA-F and MTA-R). The bottom gel shows the results of the multiplex PCR when all three primer sets are combined. Faint nonspecific shadow bands are seen with the *MAT1-1-1* primer set.

NC). The proportion of *MAT1-1* and *MAT1-2* isolates present in populations at different spatial scales (the region, within orchards, and within individual trees) was tested for equilibrium (the null hypothesis of a 1:1 ratio). For each analysis, a χ^2 test was used to determine whether the proportions departed significantly from the 1:1 ratio (Everitt 1992). Furthermore, additional contingency table χ^2 analyses were performed at the spatial scales of the region and orchard to determine whether individual subpopulations (orchards or trees) within the larger spatial scale (region or orchard, respectively) differed in *MAT* 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 *MAT* frequencies within populations deviated from the 1:1 ratio.

Population genetic and gene diversity was analyzed for each *MAT* component (*MAT1-1* and *MAT1-2*) of the population at the region and orchard scales. Analyses were based on the haploid multilocus genotypes (MLG) and microsatellite allele presence and frequency in the populations described previously (Bock et al. 2017b). Analyses were performed using the R (v3.4.1) (R Core Team, 2012) package *poppr* (Kamvar et al. 2014) for all isolates and the clone-corrected data. The number of MLG observed and the clone-corrected number of MLG were calculated; expected MLG (eMLG), which approximates the number of genotypes (genotypic richness) that would be expected at the largest, shared sample size based on rarefaction (Grünwald et al. 2003), was also calculated. Genotypic diversity, which measures both genotypic richness and abundance, was measured 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 (λ) (Simpson 1949). Genotype evenness was measured as *E*0.5 (Grünwald et al. 2003; Ludwig and Reynolds 1988; Pielou 1975), and Nei's unbiased gene diversity was calculated (H_{exp}) (Nei 1978).

RESULTS

Identification and arrangement of the *MAT* loci. The *MAT* idiomorphs from two races of the closely related species *V. inaequalis* (Fig. 2A) were used as query sequences to search the available genome of *V. effusa*. Each *MAT* locus of *V. inaequalis* contained either a *MAT1-1-1* or *MAT1-2-1* gene that was flanked by conserved genes encoding a DNA lyase (*APN2*) and a hypothetical protein with sequence similarity to a Pleckstrin homology (PH) domain-containing protein (Fig. 2A). BLASTn and tBLASTn searches using these four gene and protein sequences of *V. inaequalis*, respectively, matched a region on a single contig (contig 00032) in the genome sequence *of V. effusa* (isolate 3Des10b) that contained *MAT1-1-1* and the two flanking genes (Fig. 3A). Pairwise comparison of the 14-kb region encompassing the *MAT1-1* idiomorph from *V. effusa* 3Des10b and *V. inaequalis* confirmed synteny across the *MAT* locus, which showed that *V. inaequalis* and *V. effusa* have the same gene organization (Fig. 2). The *MAT1-1-1* gene is predicted to contain a 61-bp intron and encode a 335-amino-acid protein that shares 74% identity (84% similarity) with the *V. inaequalis* ortholog.

PCR used to test for the presence of *MAT1-1-1* (primers MTA-F and MTA-R) from a subset of 14 isolates of *V. effusa* representing different locations and cultivars resulted in amplification of a specific band in seven of these isolates (Fig. 3B). However, we were able to amplify the two flanking genes from all 14 isolates using primer sets dnaL-F/dnaLR and PHdom-F/PHdom-R (data not shown). This result indicated that the remaining isolates likely represented the opposite mating type. Long-range PCR was used to amplify each *MAT* locus with primers, dnaL-F1 and PHdom-R, designed to the conserved flanking genes, which resulted in the amplification of a 7.8-kb fragment from each isolate. Sequence analyses using BLASTn and tBLASTn with the *MAT* genes and protein sequences of *V. inaequalis*, respectively, confirmed that of the 14 isolates, seven contained a sequence homologous to *MAT1-1-1* and the remaining seven showed similarity to *MAT1-2-1*.

The *MAT1-2-1* gene of *V. effusa* is predicted to encode a 390-amino-acid protein. Similar to other *MAT1-2-1* genes is the placement of an intron located in the region encoding the HMG domain (Debuchy and Turgeon 2006). The predicted protein of the *MAT1-2-1* gene of *V. effusa* shares 59% identity and 71% similarity to the *MAT1-2-1* gene of *V. inaequalis*, with the greatest identity across the region containing the HMG domain. A draft genome generated from *V. effusa* TxPawnee1-1 using NextGen sequencing was used to complete the gene sequences flanking *MAT1-2-1* (Fig. 3A). The *MAT1-2* locus was located on a 121.3-kb contig. Pairwise comparisons of *V. effusa MAT1-2* show gene organization similar to *V. inaequalis MAT1-2* and only varied at the location of each *MAT* gene with *V. effusa* isolate 3Des10b (Fig. 2).

TABLE 3. Mating type (*MAT*) frequencies of *Venturia effusa* in populations from pecan orchards in different locations in the southeastern United States

	Clone corrected				Nonclone corrected			
Pop[a]	*MAT1-1*/*MAT1-2* (*n*)	χ^2 (*P*)[b]	*P*[c]	χ^2 (*P*)[d]	*MAT1-1*/*MAT1-2* (*n*)	χ^2 (*P*)[b]	*P*[c]	χ^2 (*P*)[d]
AL-F	47:45	0.1 (0.8)	0.9	$0.6\ (1.0)_5$	48:45	0.1 (0.8)	0.8	$0.7\ (0.9)_5$
AL-L	41:42	0.0 (0.9)	1	$4.2\ (0.5)_5$	46:45	0.01 (0.9)	1	$5.8\ (0.3)_5$
GA-B	19:40	7.5 (0.006)**	0.009**	$7.7\ (0.2)_5$	19:44	9.9 (0.002)**	0.002**	$8.7\ (0.1)_5$
KS-C	14:19	0.8 (0.4)	0.5	$0.2\ (0.9)_3$	14:20	1.1 (0.3)	0.4	$0.1\ (1.0)_2$
LA-P	28:38	1.5 (0.2)	0.3	$3.3\ (0.7)_5$	28:40	2.1 (0.2)	0.2	$3.6\ (0.6)_5$
MS-R	43:37	0.5 (0.5)	0.6	$8.1\ (0.2)_5$	47:42	0.3 (0.6)	0.7	$7.9\ (0.2)_5$
FL-M	29:21	1.3 (0.3)	0.3	$9.1\ (0.1)_5$	30:22	1.2 (0.3)	0.3	$9.4\ (0.09)_5$
OK-B	45:55	1.0 (0.3)	0.4	$4.1\ (0.5)_5$	46:57	1.2 (0.3)	0.3	$3.6\ (0.6)_5$
GA-T	28:16	3.3 (0.07)	0.1	$4.7\ (0.5)_5$	29:16	3.8 (0.05)*	0.07	$5.1\ (0.4)_5$
TX-B	35:51	3.0 (0.09)	0.1	$9.3\ (0.1)_5$	38:55	3.1 (0.08)	0.1	$9.7\ (0.09)_5$
IL-C	26:22	0.3 (0.6)	0.7	$8.2\ (0.2)_5$	29:24	0.5 (0.5)	0.6	$9.2\ (0.1)_5$
Total	355:384	1.1 (0.3)	0.3	$17.9\ (0.06)_{10}$	374:410	1.65 (0.2)	0.2	$21.6\ (0.02)_{10}$*

[a] Populations (Pop) AL-F, AL-L, GA-B, KS-C, LA-P, MS-R, FL-M, OK-B, GA-T, TX-B, and IL-C from Fairhope, AL; Lowndesboro, AL; Byron, GA; Chetopa, KS; Powhatan, LA; Raymond, MS; Monticello, FL; Burneyville, OK; Tifton, GA; Bastrop, TX; and Carlyle, IL, respectively.

[b] The χ^2 value is based on a contingency χ^2 between clone-corrected *MAT* 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 and an asterisk (*) indicates that the *MAT* frequencies were significantly different from 1:1 (** indicates $P < 0.01$).

[c] Probability from an exact binomial analysis (two-tailed) to test whether *MAT* frequencies deviate significantly from a 1:1 ratio (more appropriate for comparing small populations than a χ^2 test).

[d] 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). Subscript indicates degrees of freedom; the *P* value in parentheses indicates probability that the *MAT* frequencies differ significantly from 1:1.

Six single-nucleotide polymorphisms (SNP) were identified within the *MAT1-1-1* gene across the seven *MAT1-1* isolates, of which three SNP resulted in nonsynonymous changes to the encoded sequence. Four SNP were identified within the *MAT1-2-1* gene across the seven *MAT1-2* isolates, which resulted in three nonsynonymous changes to the encoded sequence. Pairwise comparison of each *V. effusa MAT* locus within a single mating type revealed nucleotide sequence identity ranging from 94.7 to 97.1% for all the *MAT1-1* isolates and 93.7 to 100% for the *MAT1-2* isolates. Only the two TxPawnee isolates, isolated from the same fruit, had identical *MAT* loci. Sequence similarity of the *MAT* locus between *V. effusa MAT1-1* and *MAT1-2* isolates, excluding the *MAT* gene, ranged from 90.4 to 96.6%.

Development of a multiplex PCR screen to detect mating type. A PCR screen was developed to identify mating type and determine whether the *MAT* genes were present in equilibrium within populations of *V. effusa*. Primers specific for *MAT1-1-1*, *MAT1-2-1*, and *TUB2* were established as a multiplex PCR. The amplicons for each mating type were differentiated based on size (the amplicon sizes for *MAT1-1-1* and *MAT1-2-1*are 242 and 775 bp, respectively), and the presence of the reference gene (*TUB2*, a 517-bp amplicon) provided confirmation of the genomic DNA quality (Fig. 3B). The primer combinations were tested on 14 isolates as individual primer sets and within a multiplex PCR, which resulted in amplification that was consistent with the *MAT* sequence data (Fig. 3B).

The hierarchical collection of isolates of *V. effusa* ($n = 784$) was tested for mating type using the multiplex PCR. Nearly all samples amplified a specific single *MAT* gene. However, a very low percentage of samples (1.5%, $n = 12$ isolates) initially amplified both *MAT* bands, although this was resolved when DNA was reisolated from a monoconidial culture. In total, we obtained confirmation of mating type for all 784 isolates. The total number of isolates included in this study after clone correction was reduced to 739 (Table 1).

Mating type frequencies at different spatial scales. Clone correction had a small effect on isolate counts in the different populations (Table 3). The clone-corrected data showed that, at the largest spatial scale (the whole southeastern region population), there was no detectable disequilibrium between the two mating types (*MAT1-1-1*/*MAT1-2-1* ratio = 48:52%, $\chi^2 = 1.1$ [$P = 0.3$]; exact binomial test, $P = 0.3$) (Table 3). However, at the orchard scale, there was 1 population (GA-B, Desirable cultivar) out of the 11 that departed from equilibrium (32:68% ratio, $\chi^2 = 7.5$ [$P = 0.006$]; exact binomial test, $P = 0.009$) (Table 3). In contrast, the orchard-scale data that were not clone-corrected had two populations (GA-B and GA-T) that deviated from a 1:1 *MAT* ratio based on the χ^2 test (Table 3), demonstrating the impact of clones on the analysis. However, the exact binomial test showed only one of these populations (GA-B) to have mating types present at frequencies different from 1:1. The contingency χ^2 test comparing *MAT* ratios among orchards based on the clone-corrected data in the southeast indicated no differences ($\chi^2 = 17.9$ [$P = 0.06$]) (Table 3). The contingency χ^2 test comparing *MAT* ratios on trees within each of the orchards also detected no difference in the *MAT* frequencies among individual trees within orchards ($\chi^2 = 0.2$ to 9.3 [$P = 0.1$ to 0.9]) (Table 3). There was also no apparent effect of host cultivar on the frequency of mating types. The 11 populations are represented by four cultivars: Desirable (6 populations), Kiowa (1 population), Wichita (1 population), and Western Schley (1 population), plus two

TABLE 4. Mating type (*MAT*) frequencies of *Venturia effusa* in populations from individual pecan trees that deviated from 1:1 equilibrium in orchards in different locations in the southeastern United States[a]

	Clone corrected			Nonclone corrected		
Pop, tree[b]	*MAT1-1*: *MAT1-2* (*n*)	χ^2 (*P*)[c]	*P*[d]	*MAT1-1*: *MAT1-2* (*n*)	χ^2 (*P*)[c]	*P*[d]
GA-B						
1	**4:12**	**4.0 (0.05)***	**0.08**	**4:12**	**4.0 (0.05)***	**0.08**
2	5:7	0.3 (0.6)	0.8	5:7	0.3 (0.6)	0.8
3	**1:10**	**7.4 (0.007)****	**0.01****	**1:12**	**9.3 (0.002)****	**0.003****
4	3:4	0.1 (0.7)	1	3:4	0.1 (0.7)	1
5	4:2	0.7 (0.4)	0.7	4:2	0.7 (0.4)	0.7
6	2:7	2.8 (0.1)	0.2	2:7	2.8 (0.1)	0.2
MS-R						
1	6:8	0.3 (0.6)	0.8	6:9	0.6 (0.4)	0.6
2	5:6	0.1 (0.8)	1	7:7	0.0 (1.0)	1
3	**12:4**	**4.0 (0.05)***	**0.08**	**12:4**	**4.0 (0.05)***	**0.08**
4	4:7	0.8 (0.4)	0.6	5:7	0.3 (0.6)	0.8
5	10:4	2.6 (0.1)	0.2	11:5	2.3 (0.1)	0.2
6	6:9	0.6 (0.4)	0.6	6:10	1.0 (0.3)	0.5
FL-M						
1	4:5	0.1 (0.7)	1	4:5	0.1 (0.7)	1
2	4:7	0.8 (0.4)	0.6	4:7	0.8 (0.4)	0.6
3	6:5	0.1 (0.8)	1	6:6	0.0 (1.0)	1
4	4:3	0.1 (0.7)	1	5:3	0.5 (0.5)	0.7
5	**8:0**		**0.008***	**8:0**		**0.008****
6	3:1	1.0 (0.3)	0.6	3:1	1.0 (0.3)	0.6
TX-B						
1	12:6	2.0 (0.2)	0.2	12:6	2.0 (0.2)	0.2
2	**3:13**	**6.3 (0.01)****	**0.02***	**3:13**	**6.3 (0.01)****	**0.02***
3	5:8	0.7 (0.4)	0.6	6:9	0.6 (0.4)	0.6
4	3:8	2.3 (0.1)	0.2	4:11	3.3 (0.07)	0.1
5	7:8	0.1 (0.8)	1	7:8	0.1 (0.8)	1
6	5:8	0.7 (0.4)	0.6	6:8	0.3 (0.6)	0.8

[a] Data in bold indicate tree populations that deviate from equilibrium of mating types.
[b] Populations (Pop) GA-B, MS-R, FL-M, and TX-B from Byron, GA; Raymond, MS; Monticello, FL; and Bastrop, TX, respectively.
[c] The χ^2 value is based on a contingency χ^2 between clone-corrected *MAT* 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 and the asterisk (*) indicates *MAT* frequencies significantly different from 1:1 (* and ** indicate $P < 0.05$ and 0.01, respectively).
[d] Probability from an exact binomial analysis (two-tailed) to test whether *MAT* frequencies deviate significantly from a 1:1 ratio (more appropriate for comparing small populations than a χ^2 test).

populations of native trees. The only population that departed from equilibrium was a Desirable population from GA-B (Table 3).

Although isolate numbers were small in several cases, only 4 tree populations in three orchards (GA-B tree 1 and 3, MS-R tree 3, and TX-B tree 2) of the 63 tree populations sampled deviated from *MAT* equilibrium based on the χ^2 test (6.3% of trees) (Table 4; Supplementary Table S2). When based on the exact test of equilibrium, only three tree populations in three orchards (GA-B tree 3, FL-M tree 5, and TX-B tree 2) deviated from a 1:1 ratio (4.8%), indicating that the populations in individual trees were generally in equilibrium. Analysis of the non-clone-corrected data suggested little effect of clone correction at the spatial scale of the tree (Table 4).

Genetic and gene diversity in relation to mating type at different spatial scales. The number of MLG ranged from 14 (KS-C, *MAT1-1*) and 19 (KS-C, *MAT1-2*) to 45 (OK-B, *MAT1-1*) and 55 (OK-B, *MAT1-2*). However, the eMLG value indicated that both mating types in all populations had the same expected number of genotypes (14) at the largest, shared sample size based on rarefaction (Table 5). Shannon-Weiner's (2.64 to 3.93), Stoddart and Taylor's (14 to 55), and Simpson's (0.929 to 0.982) indices of diversity all demonstrated that genetic diversity was similar regardless of whether they were *MAT1-1* or *MAT1-2* in all orchard populations (Table 5). Not unexpectedly, the magnitude of the indices showed some relationship to sample size (for example, the GA-B *MAT1-1* population had only 19 isolates and Shannon-Weiner's index was 2.94, compared with 3.74 for *MAT1-1*, which comprised 42 isolates) (Grünwald et al. 2003). Other populations where there was either a small sample size or a difference in sample size for isolates of *MAT1-1* and *MAT1-2* included orchards KS-C and GA-T. Otherwise, the populations of the two *MAT* idiomorphs had similar magnitudes of genetic diversity. Due to the number of isolates, measures of diversity for both mating types were greatest (yet similar to each other) at the scale of the region (Shannon-Weiner's index = 5.85 and 5.92 for *MAT1-1* and *MAT1-2*, respectively). Evenness was very high in all populations, based on both all data and the clone-corrected data, regardless of mating type (clonality was low). Nei's unbiased measure of gene diversity was similar for each population regardless of *MAT* idiomorph, and was also highest at the regional scale.

DISCUSSION

The recent genome sequencing efforts invested in *V. effusa* (Bock et al. 2016b) and the closely related heterothallic sexual species, *V. inaequalis* (Deng et al. 2017), provided an opportunity to compare *MAT* loci across each of these pathogens. Two *MAT* idiomorphs were identified within the genome sequences, with each isolate containing a single idiomorph. The sequence information enabled us to develop a PCR assay to screen a large collection of isolates, where we determined that the *MAT* idiomorphs were present in equilibrium in populations of *V. effusa*, a characteristic often typical of sexually recombining heterothallic species. Until now, *V. effusa* has been considered strictly an asexual pathogen that can rapidly spread to epidemic levels on pecan throughout the growing season due to its

TABLE 5. Statistics summarizing the genetic diversity of populations of *Venturia effusa* in relation to mating type (*MAT*) idiomorph in the southeastern United States

				Clone corrected				Nonclone corrected				
Pop[a]	MAT	MLG[b]	eMLG[c]	SW[d]	ST[e]	SI[f]	Nei[g]	SW[d]	ST[e]	SI[f]	E.5[h]	Nei[g]
AL-F	*MAT1-1*	47	14	3.85	47	0.979	0.58	3.84	46.1	0.978	0.988	0.579
	MAT1-2	45	14	3.81	45	0.978	0.609	3.81	45	0.978	1	0.609
AL-L	*MAT1-1*	41	14	3.71	41	0.976	0.619	3.67	36.5	0.973	0.931	0.605
	MAT1-2	42	14	3.74	42	0.976	0.651	3.71	39.7	0.975	0.967	0.65
GA-B	*MAT1-1*	19	14	2.94	19	0.947	0.578	2.94	19	0.947	1	0.578
	MAT1-2	40	14	3.69	40	0.975	0.549	3.63	33.4	0.97	0.885	0.547
KS-C	*MAT1-1*	14	14	2.64	14	0.929	0.722	2.64	14	0.929	1	0.722
	MAT1-2	19	14	2.94	19	0.947	0.712	2.93	18.2	0.945	0.973	0.702
LA-P	*MAT1-1*	28	14	3.33	28	0.964	0.516	3.33	28	0.964	1	0.516
	MAT1-2	38	14	3.64	38	0.974	0.505	3.62	36.4	0.973	0.974	0.512
MS-R	*MAT1-1*	43	14	3.76	43	0.977	0.598	3.72	38.8	0.974	0.937	0.593
	MAT1-2	37	14	3.61	37	0.973	0.581	3.61	35.3	0.972	0.957	0.582
FL-M	*MAT1-1*	29	14	3.37	29	0.966	0.58	3.35	28.1	0.964	0.981	0.578
	MAT1-2	21	14	3.04	21	0.952	0.57	3.03	20.2	0.95	0.975	0.557
OK-B	*MAT1-1*	45	14	3.81	45	0.978	0.686	3.8	44.1	0.977	0.987	0.685
	MAT1-2	55	14	4.01	55	0.982	0.664	3.99	53.3	0.981	0.981	0.661
GA-T	*MAT1-1*	28	14	3.33	28	0.964	0.563	3.32	27.1	0.963	0.981	0.557
	MAT1-2	16	14	2.77	16	0.938	0.576	2.77	16	0.938	1	0.576
TX-B	*MAT1-1*	35	14	3.56	35	0.971	0.637	3.53	32.8	0.97	0.962	0.637
	MAT1-2	51	14	3.93	51	0.98	0.606	3.88	43.8	0.977	0.902	0.604
IL-C	*MAT1-1*	26	14	3.26	26	0.962	0.673	3.21	22.7	0.956	0.918	0.676
	MAT1-2	22	14	3.09	22	0.955	0.688	3.06	20.6	0.951	0.96	0.684
Total	*MAT1-1*	355	355	5.87	355	0.997	0.719	5.85	335	0.997	0.964	0.718
	MAT1-2	384	353	5.95	384	0.997	0.717	5.92	355	0.997	0.949	0.718
Grand total	…	739	363	6.61	739	0.999	0.718	6.58	688	0.999	0.955	0.719

[a] Populations (Pop) AL-F, AL-L, GA-B, KS-C, LA-P, MS-R, FL-M, OK-B, GA-T, TX-B, and IL-C from Fairhope, AL; Lowndesboro, AL; Byron, GA; Chetopa, KS; Powhatan, LA; Raymond, MS; Monticello, FL; Burneyville, OK; Tifton, GA; Bastrop, TX; and Carlyle, IL, respectively.

[b] Multilocus genotypes (MLG) = genotypic richness.

[c] Expected multilocus genotypes (eMLG) = approximates the number of genotypes that would be expected at the largest, shared sample size based on rarefaction (Grünwald et al. 2003).

[d] The Shannon-Weiner index (SW) (Shannon 2001) described the genetic diversity in the populations, with higher numbers indicating greater diversity (it is a measure of both richness and evenness).

[e] Stoddart and Taylor's index of diversity (ST) (Stoddart and Taylor 1988) measures genotypic diversity.

[f] Simpson's index (SI) (Simpson 1949) is based on the probability of two randomly selected individuals being the same (a number close to '1' indicates a low probability that two individuals will be the same).

[g] Nei's unbiased gene diversity (Nei) (Nei 1978) is based on the allele frequencies. All analyses were performed using the R package *poppr* (Kamvar et al. 2014) for all isolates and the clone-corrected data.

[h] Evenness (E.5) (Grünwald et al. 2003; Ludwig and Reynolds 1988; Pielou 1975) measures the distribution of genotype abundance (on a scale 0 to 1), where a population with equally abundant genotypes has a value equal to 1.

polycyclic nature. Although a sexual cycle of *V. effusa* has never been identified, the presence of opposite mating types and the genetic diversity observed in populations of *V. effusa* (Bock et al. 2017b) is indicative of a sexually active, heterothallic pathogen.

The genetic architecture associated with gene organization and content of the *MAT* loci can vary but many heterothallic ascomycetes have *APN2* encoding a DNA lyase and *SLA2* encoding a cytoskeletal protein associated near or flanking the *MAT* genes (Debuchy and Turgeon 2006). The gene organization surrounding the *MAT* idiomorphs of Dothideomycetes most commonly have *APN2* gene present but more variation is seen with the other flanking gene, because *SLA2*, *GAP* (encodes GTPase-activating protein), *PPO* (encodes pyridoxamine phosphate oxidase), and a gene encoding a protein containing a PH domain have been identified (Chilvers et al. 2014; Cozijnsen and Howlett 2003; Pearce et al. 2016; Wang et al. 2016). The *MAT* idiomorphs of *V. effusa* and *V. inaequalis* have a similar gene organization, where each *MAT* gene is flanked by *APN2* encoding a DNA lyase and a gene encoding a hypothetical protein with sequence similarity to proteins containing a PH domain. However, an InterProScan of the hypothetical protein of *V. effusa* did not reveal a PH domain (IPR011993). The dothideomycete *Phyllosticta citricarpa* (family Botryosphaeriaceae), cause of citrus black spot, also has mating type organization similar to that described for *Venturia* spp. (Wang et al. 2016) but varies with the orientation of the *MAT* genes with respect to the flanking genes.

The frequency of the *MAT* genes was established at multiple spatial scales in the southeastern region of the United States (784 isolates). The clone-corrected data showed that *MAT* frequencies were in equilibrium at the regional scale. This is despite one orchard (GA-B) within which trees had populations of *V. effusa* at frequencies of the *MAT* idiomorphs that significantly deviated from 1:1 (based on both the clone-corrected and all data). Therefore, no compelling evidence is present to reject the null hypothesis of equilibrium overall, which is consistent with frequency-dependent selection (May et al. 1999). The disequilibrium of mating types in occasional populations has been noted before in other plant pathosystems (Linde et al. 2003; Sommerhalder et al. 2006). Thus, the approximately 1:1 ratio of *MAT1-1* and *MAT1-2* idiomorphs is indicative of random mating in *V. effusa*. This result is consistent with many other heterothallic ascomycete fungi where mating types have been characterized and random mating is known to occur (Chen and McDonald 1996; Dale et al. 2011; Hayden and Howlett 2005; Keller et al. 1997; Linde et al. 2002; Rau et al. 2005; Tenzer and Gessler 1999). Furthermore, populations of *V. effusa* are known to have characteristics of a sexually recombining fungus; it is genetically diverse and much of the population genetic diversity is at a small spatial scale and, although linkage disequilibrium exists, many pairs of loci are at equilibrium (Bock et al. 2014a,b, 2016a, 2017b). Also, the populations of *V. effusa* based on mating type in this study were deemed to be similarly diverse and had the same eMLG (n = 14) after rarefaction, indicating the same genotypic richness regardless of population or mating type. However, some fungal pathogens exhibit a skew in mating type in some populations, even when the pathogen is known to have a sexual cycle, whereby the incidence of a sexual stage may occur at a frequency or scale that can result in clonality and linkage disequilibrium, yet present substantial genetic diversity (Henk et al. 2012; Horn et al. 2016; Moore et al. 2013). Reproductive mechanisms occur in these populations to ensure that deleterious mutations do not build up in an irreversible manner. With *V. effusa*, there remains a possibility that other processes such as mitotic recombination might explain the observed characteristics of genetic diversity (Pontecorvo 1956); however, the *MAT* ratio described in this study is indicative of an unidentified sexual stage that occurs frequently enough to maintain equilibrium. The majority of the data, both clone and nonclone corrected, at the region, orchard, and tree scales, support equilibrium of mating type. These results are in agreement with similar observations on other sexually reproducing pathogens where clonality has been observed at small spatial scales (Chen and McDonald 1996; Douhan et al. 2002; Linde et al. 2002; Sommerhalder et al. 2006). This has been hypothesized to occur due to local dispersal from sites of earlier infections, and is consistent with what is known regarding local dispersal of conidia of *V. effusa*.

The fact that no sexual stage has yet been found for *V. effusa* could be due to the sexual cycle occurring on different hosts. The pear scab pathogen (*V. pirina*) has been demonstrated to have the capacity to undergo its sexual cycle on senescing and dead leaves of apple (Stehmann et al. 2001). It is possible that *V. effusa* may undergo the sexual cycle on related, widely distributed species of *Carya*, some of which are described as hosts (Schubert et al. 2003; Winter 1885). Furthermore, most pecan orchards are clonal but wild stands and native seedling orchards will have a diversity of genotypes, and it is not inconceivable that certain genotypes might more readily support the sexual stage. However, in all orchard populations, genetic diversity was similar regardless of source (cultivar) population (Bock et al. 2017b) or whether they were from the *MAT1-1* or *MAT1-2* subpopulations, and in the region as a whole, which provided further evidence that the two subpopulations share and undergo assortment of genetic material. Not unexpectedly, there were observable effects of sample size, and measures of diversity increased for both *MAT* subpopulations with the scale of the population (Grünwald et al. 2003).

Some related fungal pathogens such as *Z. tritici* that were initially thought to go through a sexual stage during a saprobic phase in the lifecycle have since been shown to undergo the sexual cycle throughout the entire year (Zhan et al. 1998). However, others appear to have a more seasonal cycle. The closely related *V. inaequalis* on apple (Bock et al. 2016a; Schubert et al. 2003; Seyran et al. 2010a) produces ascospores during the spring that develop slowly over winter on colonized leaf litter (MacHardy 1996). *Oculimacula* spp. on cereals produce ascospores from stubble during the spring (Dyer et al. 2001). Pecan scab develops not only on leaflets but also on the rachis, shoots, and fruit (Demaree 1924). Leaf material generally breaks down fairly rapidly; however, the fruit shucks, rachis, and shoots do not break down as rapidly as the leaves, and would provide a basis for a saprobic phase of *V. effusa*. The apparent equilibrium of the *MAT* idiomorphs provides impetus to a renewed effort to search for a sexual stage of *V. effusa* in both the field and lab. Once a sexual stage is identified and understood, it may provide further opportunity to manage the disease, and will allow the genetics of the fungus to be explored in paired crosses, opening a new dimension of study in relation to the host.

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