Mating Type Idiomorphs, Heterothallism, and High Genetic Diversity in *Venturia carpophila*, Cause of Peach Scab

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ABSTRACT

Scab (caused by *Venturia carpophila*) is a major disease affecting peach in the eastern United States. The aims of the study were to characterize the mating-type loci in *V. carpophila*, determine whether they are in equilibrium, and assess the population genetic diversity and structure of the pathogen. The mating-type gene *MAT1-1-1* was identified in isolate JP3-5 in an available genome sequence, and the *MAT1-2-1* gene was PCR amplified from isolate PS1-1, thus indicating a heterothallic structure. Mating-type loci structures were consistent with those of other *Venturia* spp. (*V. effusa* and *V. inaequalis*): the mating-type gene is positioned between *APN2* encoding a DNA lyase and a gene encoding a Pleckstrin homology domain. Primers designed to each of the mating-type genes and a reference gene *TUB2* were used as a multiplex PCR to screen a population ($n = 81$) of *V. carpophila* from various locations in the eastern United States. Mating types in five of the nine populations

studied were in equilibrium. Among the 81 isolates, there were 69 multilocus genotypes. A population genetic analysis of the populations with >10 individuals (four populations) showed them to be genetically diverse. Linkage disequilibrium was found in five of nine populations with ≥ 4 isolates. A discriminant analysis of principal components indicated three genetic clusters, although extensive admixture was observed. Mating-type identification in *V. carpophila* provides a basis for understanding reproductive methods of the pathogen and can be a basis for further studies of the genetics of the peach scab pathogen.

Keywords: alpha-box, ascomycete, asexual reproduction, HMG-domain, mating-type gene, MAT equilibrium, MAT locus, mycology, population biology, sexual reproduction

Scab is a major disease affecting peach trees in the eastern United States (Keitt 1917; Scherm and Brannen 2005). Fruit infection by the pathogen *Venturia carpophila* E. E. Fisher results in small, dark surface lesions developing on the peach skin, which leads to downgrading of the fruit quality and concomitant loss in value (Anonymous 2004). *V. carpophila* can infect not only the fruit but also leaves and shoots (Keitt 1917). Overwintering occurs on the previous season's shoots, and it is lesions on these shoots that produce conidia between February and June (late winter through early summer) that infect the subsequent fruit crop (Keitt 1917; Lawrence and Zehr 1982; Scherm et al. 2008). The conidia of the pathogen are predominantly splash dispersed (Lan and Scherm 2003), with most lesions developing on one plane of the fruit (Bock et al. 2011). After infection of fruit, the disease has a prolonged latent period of approximately 40+ days (Keitt 1917), suggesting that it is the earlier infections that are most likely responsible for the

visible symptoms because peaches are harvested by July or August in the eastern United States.

In addition to peach, *V. carpophila* has been identified as the causal agent of scab on apricot and almond (Ogawa et al. 1995; Teviotdale et al. 2002). The sexual stage of the pathogen was first reported on apricot in Victoria, Australia (Fisher 1961). Pseudothecia were described on leaf litter developing toward the close of winter and, after examination and additional studies, the new species *V. carpophila* was named (syn. *Cladosporium carpophilum* (Thüm.), *Fusicladium carpophilum* (Thüm.) Oudem., and *Fusicladosporium carpophilum* (Thüm.) Partr. & Morgan-Jones). The sexual stage of the pathogen has not been described elsewhere, despite the widespread occurrence and importance of scab in the eastern United States and other peach-, almond-, and apricot-growing areas. Furthermore, there is no information on the characteristics of the mating types structure of the fungus, which can provide useful information on and insights into aspects of the population structure, biology, and reproduction of the fungus in areas where scab is a problem for stone fruit or nut production.

Understanding the population biology of a pathogen and the characteristics of the pathogen's reproduction can provide an informed basis for several aspects, including threats to host resistance, development of fungicide insensitivity, and phytosanitary regulation (McDonald 2015; McDonald and Linde 2002; McDonald and Mundt 2016). Based on such information, actionable disease management strategies can be developed for sustainable management of a disease. Although limited information exists on the population biology, population genetics, and reproductive strategies of *V. carpophila*, an earlier study using dominant markers (random amplified polymorphic DNAs and universally primed PCR) and a small but diverse panel of isolates from the eastern United States indicated that the pathogen was polymorphic and

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C. H. Bock and C. A. Young contributed equally to this work.

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exhibited diversity (Chen et al. 2014). Subsequently, a genome of *V. carpophila* was sequenced (Chen et al. 2017) and mined for microsatellites that were screened against a diverse panel of 37 isolates of *V. carpophila* that also indicated substantial diversity (Chen et al. 2018).

In ascomycetous fungi, mating-type genes control sexual reproduction (Turgeon 1998). Fungi that require strains of opposite mating types for a compatible interaction are considered heterothallic (self-sterile), while those species able to undergo sexual reproduction when a single strain is present are termed homothallic (self-fertile, which includes its mitotic descendants). In heterothallic fungi, if a mating type is absent, sexual reproduction does not occur (Chilvers et al. 2014; Wang et al. 2016). The two mating-type genes in many ascomycetes are termed *MAT1-1-1* and *MAT1-2-1* (although additional genes may be present in some fungal groups), are situated at a single locus, and, depending on mating type, are named *MAT1-1* and *MAT1-2* (the *MAT* idiomorphs). The two *MAT* genes *MAT1-1-1* and *MAT1-2-1* encode for a protein with an α -box motif and a DNA-binding domain of the high mobility group (HMG), respectively (Pöggeler 2001; Turgeon 1998; Turgeon and Yoder 2000). Although the mating strategy (homothallism versus heterothallism) of *V. carpophila* is not known, several other *Venturia* spp. have been characterized as heterothallic, including *V. effusa*, *V. inaequalis* and *V. pirina* (Keitt and Palmiter 1937; Langford and Keitt 1942; Young et al. 2018).

Mating types are generally in equilibrium in populations of heterothallic ascomycetes where sexual reproduction is known to occur (Dale et al. 2011; Linde et al. 2002, 2003); in some cases, mating types are in equilibrium yet a sexual stage remains to be identified (Linde et al. 2009; Pearce et al. 2016), providing compelling evidence of a sexual stage in these pathogens. Conversely, it is also possible for clonality to occur in the presence of sexual reproduction in some ascomycetes (Henk et al. 2012). Indeed, the mating types of many plant-pathogenic ascomycetes have now been described (Arie et al. 1997; Groenewald et al. 2006; Linde et al. 2003; Ramirez-Prado et al. 2008; Rau et al. 2005; Waalwijk et al. 2002; Young et al. 2018; Yu et al. 2015), including the mating types of *Venturia* spp. *V. effusa* and *V. inaequalis* that are closely related to *V. carpophila* (Young et al. 2018). Although *V. effusa* has been demonstrated to have mating types in equilibrium at various spatial scales (Bock et al. 2017, 2018; Young et al. 2018), the sexual stage has only been observed in vitro (Charlton et al. 2020). *V. inaequalis*, cause of apple scab, is known to undergo regular sexual reproduction (MacHardy 1996). The apple scab pathogen has population genetic structure that is congruent with this observation, which has allowed an understanding of evolutionary relationships among discernible subpopulations of the pathogen on cultivated and more isolated, native populations of the genus *Malus* in Europe and Asia (Gladieux et al. 2008). Population genetic structure of *V. effusa* on pecan in the United

States similarly supports the contention of sexual recombination (Bock et al. 2017; Charlton et al. 2020; Young et al. 2018). Population genetic characteristics are a product of the reproduction (sexual versus asexual) and dispersal characteristics of a fungus.

The objectives of this study were first, to identify and characterize the mating-type loci in *V. carpophila*; second, to characterize the frequency of the two mating types among the isolates collected from the different populations; and, third, to assess the population genetic diversity and structure among these populations to determine whether there is evidence of sexual recombination occurring in the southeastern United States.

MATERIALS AND METHODS

Sample locations for isolates of *V. carpophila*. In total, 81 isolates of *V. carpophila* were sampled from diseased peach or nectarine (both *Prunus persica*) fruit from orchards representing the eastern United States in Peach County, GA; Decatur County, GA; Alachua County, FL; Greene County, AL; Fayette County, KY; Berks County, PA; Oconee County, SC; Cumberland County, NJ; and Frederick County, VA (Table 1; Fig. 1). Some of these isolates were previously described by Chen et al. (2018). Nectarine orchards were sampled only in South Carolina, and were included to explore the notion that isolates of *V. carpophila* from nectarine may be different to those from peach. In some cases, peach breeding material was sampled; in other cases, samples were collected from blocks of individual cultivars. Up to eight fruit were collected arbitrarily from one to six trees in the individual orchard plantings; in one case, more than a single isolate was taken from a fruit. Different individuals collected fruit in the various orchards and the relative position and distances of fruit in trees was not recorded (they may have come from the same cluster or from different branches). Also, proximity of sample trees to one another within an orchard was not specified for the different orchards.

The pathogen was isolated following the process described by Chen et al. (2018). Briefly, conidia of *V. carpophila* scraped from scab lesions on the fruit surface were suspended in sterile distilled water, and 0.1-ml aliquots of the suspension spread were on the surface of antibiotic-amended 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) in 9.0-cm Petri plates. Cultures were incubated at 25°C for 48 h under fluorescent lights with a 12-h photoperiod. The agar surface was inspected under a microscope at $\times 70$ for single, germinated spores. Germinated spores were carefully excised and transferred to antibiotic-amended potato dextrose agar (PDA) (amended as for WA) and incubated for 3 to 5 weeks on PDA as described for the WA cultures. Isolates were identified as *V. carpophila* based on morphological and cultural characteristics (Fisher 1961; Keitt 1917) and comparison

TABLE 1. Origin and number of isolates of *Venturia carpophila* collected from peach orchards in the southeastern United States and used to compare frequency of the *MAT1-1* and *MAT1-2* mating types in this study

Source location (orchard population)	Host species	Cultivar or genotype (number of isolates)	N ^a	Month and year of collection	Designation in study
Peach County, GA A	Peach	Julyprince (JP) (17)	17	June 2013	GA_PEA
Peach County, GA B	Peach	AP06 (4)	4	May 2013	GA_PEB
Decatur County, GA	Peach	SIO-657-SB-654-GN14 (4), Unknown (1)	5	May 2013	GA_DEC
Alachua County, FL	Peach	415X53 (3), 415XING03-1 (1), 9747KSBC (2)	6	May-June 2013	FL_ALA
Greene County, AL	Peach	Ruston Red (8)	8	July 2013	AL_GRE
Fayette County, KY	Peach	Unknown (1)	1	July 2013	KY_FAY
Berks County, PA	Peach	Sentry (10)	10	July 2013	PA_BER
Oconee County, SC	Nectarine	Red Gold (5)	5	July 2013	SC_OCO
Cumberland County, NJ	Peach	Encore (14)	14	July 2013	NJ_CUM
Frederick County, VA	Peach	Lovell seedling (11)	11	August 2013	VA_FRE
Total	...	...	81	...	...

^a Total number of isolates. Isolates were not collected specifically from different trees or branches. In some cases, fruit might be from the same cluster. In one case, more than one isolate was obtained from a fruit.

with previously identified cultures. All isolates are stored at -20°C on silica at the United States Department of Agriculture–Agricultural Research Service, Byron, GA laboratory, with a duplicate collection similarly housed at the Noble Research Institute, Ardmore, OK laboratory.

DNA extraction. DNA was extracted from mycelium by scraping the surface of a colony growing on PDA using the *Quick-DNA Fungal/Bacterial Miniprep Kit* (ZymoResearch, Irvine, CA, U.S.A.). A *TissueLyser* (Qiagen, Valencia, CA, U.S.A.) was used to lyse the mycelium. The DNA was quantified using a Nanodrop spectrophotometer (Nanodrop Products, Wilmington, DE, U.S.A.) and stored in Tris-EDTA buffer at -20°C . An initial set of eight isolates—one isolate from each Peach County, GA (Julyprince, isolate JP3-5), Alachua County, FL (415X53, isolate 415X53-2), Greene County, AL (Ruston Red, isolate Clanton1-2), Fayette County, KY (unknown cultivar, isolate Kent-2), Berks County, PA (Sentry, isolate PS1-1), Oconee County, SC (nectarine Red Gold, isolate MD1-3), Cumberland County, NJ (Encore, isolate RS2-2), and Frederick County, VA (Lovell seedling, isolate LO1-2) (Table 1)—was selected to evaluate the *MAT* locus by PCR and establish the mating-type multiplex PCR.

Genome sequence analyses. The genome from *V. carpophila* isolate JP3-5 (GenBank accession BioProject PRJNA321389) was used to identify the mating-type idiomorphs (Chen et al. 2017). The *MAT1-2-1* gene and the reference β -tubulin gene (*TUB2*) were identified in scaffold_15 and scaffold_21 in this genome, using

BLAST via a custom sequence server (Priyam et al. 2019), with *V. effusa* sequences MF167364 and MF173107, respectively. A draft genome sequence was developed for *V. carpophila* isolate PS1-1 once it was identified as *MAT1-1-1*. PS1-1 was sequenced at the University of Kentucky, Health-Care Genomics Core Laboratory (Lexington, KY, U.S.A.), using Illumina MiSeq. The sequencing library was prepared using the Nextera XT DNA library kit (Illumina) producing 18,376,086 paired-end reads. The genome was assembled using SPAdes v. 3.10.1 (Bankevich et al. 2012) and all scaffold sequences at least 500 bp in length were included in the final assembly. The final genome size was 33.3 Mb, represented by 1,762 scaffolds with an N_{50} of 60.1 kb, and the largest contig was 242.8 kb. The *MAT1-1-1* and *TUB2* genes were identified in scaffold_341 and scaffold_520 in the PS1-1 genome, using BLAST with *V. effusa* sequences MF167363 and MF173107, respectively.

Mating-type primer development. Primers designed for evaluation of the mating types by PCR are listed in Table 2. To identify and characterize the mating-type sequences of the eight selected isolates, primers (VcaMat_2F and 2R) flanking the mating-type genes were used to amplify a region that spans both mating-type idiomorphs. The PCR and conditions were the same as described below for the multiplex PCR, except the total reaction volume was 40 μl . PCR products were purified with the DNA Clean and Concentrator-5 (ZymoResearch) kit and sequenced using Sanger technology (University of Kentucky, Health-Care Genomics Core Laboratory).

A diagnostic multiplex PCR primer set (Table 2; Supplementary Fig. S1) was established with primers specific to each mating-type gene, used in conjunction with primers specific to *TUB2*, which acted as an internal positive control in all PCR assays. PCR was performed in a total volume of 5 μl containing 0.75 μl (1 ng) of genomic DNA, 2.5 μl of Promega Master Mix (Promega Corporation, Madison, WI, U.S.A.), and 0.125 μM target-specific primers. The cycling parameters were 94°C for 1 min; then, 32 cycles of 94°C for 40 s, 60°C for 40 s, and 72°C for 20 s; followed by 72°C for 10 min. Multiplex and single-target PCR used the same reaction and cycling conditions. The PCR conditions were established using DNA from the eight selected isolates with individual primer sets and subsequently multiplexed with all three-primer sets before testing all 81 isolates. The multiplex amplicons were analyzed by gel electrophoresis using 1.5% agarose gel in 1 $\times$ Tris-boric-EDTA buffer and visualized using GelRed (Biotium, Inc., Fremont, CA, U.S.A.) by UV transillumination. Amplicon size was compared against an All-Purpose Hi-Lo DNA Marker (50 to 10,000 bp; Bionexus, Oakland, CA, U.S.A.).

Analysis of the mating-type loci. Preliminary characterization of an existing genome sequence (Chen et al. 2017) facilitated the identification of the complete mating-type locus and flanking genes. The *MAT1-1* and *MAT1-2* loci were subsequently characterized from two isolates, PS1-1 and JP3-5, representing *MAT1-1* (accession number MN562205) and *MAT1-2* (accession number MN562204), respectively. Using *V. effusa* MF818328 (*MAT1-1-1*) and MF167364 (*MAT1-2-1*) sequences as the reference, the mating-type loci were predicted for each isolate of *V. carpophila* using the autoannotate function in Geneious v10.0.6 (Biomatters Ltd, Auckland, New Zealand) and manually annotated to correctly display features of each gene. Synteny between *V. effusa* and *V. inaequalis* against *V. carpophila* was compared using the Dot Plot function in MacVector v16.0.9 (MacVector, Inc., Apex, NC, U.S.A.) based on a window size of 30 and nucleotide identity of 60%. Geneious v10.0.6 was used for generating pairwise and multiple sequence alignments of nucleotide and protein sequences using the Geneious Alignment option. The amino acid sequence for the α - and HMG-boxes of each mating-type gene were compared among other heterothallic ascomycete species with sequence data available on GenBank, including *V. effusa* (MF167363 and MF167364), *V. inaequalis* (MG818328 and MG818328), *Phyllosticta citricarpa* (KX280782 and KT708823), *Alternaria alternata* (AB009751 and AB009752), *Bipolaris maydis* (AF029913 and

Fig. 1. States and counties within states in the eastern United States where peach orchards were located that were sampled for isolates of *Venturia carpophila*. AL = Alabama, FL = Florida, GA = Georgia, KY = Kentucky, NJ = New Jersey, PA = Pennsylvania, SC = South Carolina, and VA = Virginia.

AF037687), *Didymella tanacetii* (KU030827 and KU342705), *Leptosphaeria maculans* (AY174048 and AY174049), *Parastagonospora nodorum* (AY212018 and AY212019), *Dothiostroma pini* (DQ915449 and DQ915451), *Zymoseptoria tritici* (AF440399 and AF440398), *Epichloë festucae* (FJ171711 and HQ680590), *Neurospora crassa* (NEUAMTR and NEUMTA1A), and *Cryphonectria parasitica* (AF380365 and AF380364).

Phylogenetic analysis of peach scab isolates using the *TUB2* gene sequence. The *TUB2* gene was predicted using *V. effusa TUB2* (MF173107) sequence as the reference, using the autoannotate function in Geneious v10.0.6 and manually annotated to correctly display features of each gene. A phylogenetic tree of the partial *TUB2* sequences was inferred by maximum likelihood (ML). *TUB2* sequences of available *Venturia* spp., including unique sequences from *V. carpophila* isolates JP3-5, Clanton1-2, and PS1-1 from peach and MD1-3 from nectarine (accession numbers MN577971, MN562199, MN548432, and MN562198, respectively), were aligned with MUSCLE 3.8.1 (Edgar 2004) with Gblocks curation via the Phylogeny.fr website (<http://www.phylogeny.fr>). ML analyses were performed with PhyML 3.1/3.0 (Guindon et al. 2010), with branch support estimated by the approximate likelihood-ratio test; the likelihood substitution model was HKY85 with a likelihood value of $-\ln L$ 1365.66 (Dereeper et al. 2008, 2010).

Genotyping of isolates. All 81 isolates of *V. carpophila* were individually genotyped using 16 microsatellite markers (Table 3) (Chen et al. 2018). The primers and conditions used for the reactions were as follows. The reaction volume (5 µl) contained 1.5 µl of template DNA (1.5 to 2.0 ng), 0.5 µM each primer, 0.75 µl of

distilled H₂O, and 2.5 µl of PCR master mix (Promega Corporation). The cycling parameters were 94°C for 3 min; followed by 10 cycles of denaturation at 94°C for 30 s, a primer extension at 60°C for 45 s (with a -0.5°C ramp/cycle), and a final extension step at 72°C for 30 s; and a further 29 cycles of denaturation at 94°C for 30 s, a primer extension at 55°C for 45 s, and a final extension step at 72°C for 30 s; ending with a final extension of 72°C for 30 min. Primers were labeled with either FAM, NED, HEX, or PET dye (all with M13 tails). All marker primer combinations were run on a Hitachi 3500 Genetic Analyzer (Thermo-Fisher Scientific, Grand Island, NY, U.S.A.). The resulting peaks were analyzed and scored by base pair size for each marker using GeneMarker (SoftGenetics, State College, PA, U.S.A.). *V. carpophila* is a haploid organism and, thus, only a single band was observed at each locus for each isolate. To ensure repeatability of the PCR assays, 25% of the reactions were repeated and all resulted in identical marker patterns for the marker–isolate combinations tested.

Data analysis. *Analysis of mating-type frequencies.* Mating-type frequencies were calculated for isolates from each orchard sample. Additionally, the isolates were pooled into a single overall population to determine whether isolates of each mating type were in equilibrium. The analyses were performed on both the original data and a clone-corrected data set. Clone correction was performed based on the haploid multilocus genotypes (MLGs) identified by combining the alleles for each microsatellite. Analyses were performed using SAS V9.4 (SAS Institute, Cary, NC, U.S.A.). The proportion of *MAT1-1* and *MAT1-2* isolates present was tested for equilibrium (the null hypothesis of a 1:1 ratio) using an exact

TABLE 2. Primers used in PCR to detect the mating-type idiomorph loci *MAT1-1* and *MAT1-2* in isolates of *Venturia carpophila* collected from peach orchards in the southeastern United States

Target gene	Primer name	Sequence (5'–3')	Product size (bp)
<i>MAT1-1-1</i> ^a	VcaMat_2F	GTGTCCACGGCTCCTACAAA	2,204
	VcaMat_2R	AGGCATACACATGGTCGCAA	...
<i>MAT1-1-1</i> ^b	VcaMat_8F	TGGATCCATCCTTCATCCTC	212
	VcaMat_8R	CCTAGCTGGCTGGACTTTTG	...
<i>MAT1-2-1</i> ^b	VcaMat_5F	ATGTCTTCCCAACTCCGCAGCT	585
	VcaMat_5R	AATAGTCCGGGTGCTGCAGCAAGT	...
<i>TUB2</i> ^c	Vcatub_F2	CAGATATCGTACAAAGCCTCGTTG	380
	Vcatub_R2	TTCCGTCCAGATAACTTCGTCTTC	...
<i>TUB2</i> ^d	Vctub2-F	CAACCTCACTCCGTAACATG	1,753
	Vctub2-R1	CGTTGTGGGAATCCATTCGAC	...

^a Primers used to amplify complete *MAT1-1-1* and *MAT1-2-1* genes.

^b Primers used for multiplex PCR to differentiate isolate mating type.

^c Housekeeping gene primer set used to confirm PCR in multiplex PCR.

^d Primer set used for *TUB2* phylogeny.

TABLE 3. Microsatellite markers used for assessing population genetic diversity of *Venturia carpophila*^a

Primer name ^b	Scaffold	Forward primer sequence	Reverse primer sequence	Product size range (bp)
Vc007	2	ACGTTATGAGCAGTAGGTAGGGAG	AGGAAGTCGGTCATGAGAAAGAAC	280–296
Vc012	3	AAAGATATTGGGTTACATGCTCT	AAAAGTGAGAGATGGAAGGATGAG	289–319
Vc021	6	GTAAGAAGCCTTACCACTGTCTT	GCTTGAAGAATAGCAGTGTTGTGT	241–260
Vc038	14	ATAGTCGGAGCAAATCTAGTGTC	GACCTAGTCCAGAACACCAATGAT	316–319
Vc046	21	TTATTCTTTATAGCTAGCCGTCCG	AGTGATTGATTCATTCTTGTTGA	242–255
Vc055	33	ATAGAGGCTTATTCCTTTCCATCC	TCAAAATGTCCCTTTTCCTTTATC	295–498
Vc056	33	CAATCTCTACTACGATGACGGCT	AACAGTAGCTTAGTTGTCCCTTGG	290–317
Vc060	35	ACGTTTCGTAATAATAGGGAGCGTA	AACCTACGAAGGTACGAGTTTACC	257–450
Vc077	44	CCCACCTAGGACTGCTTAGTACAT	AAGAGATCGATCAAGGAAATACG	279–314
Vc085	48	GGTAGGTAGGTACATGAAAGCCAC	GAAATGTGAAGTGTGTCTCCATC	153–167
Vc089	61	GTATCCTTTCTCTTACCAATGCT	ACTTGAGAGTTCCTTGCTTCTTGT	293–300
Vc097	90	CATGATATTCTTGTCTGCGATACC	AGAAGTTTCTGTCCCTGCTCTTC	308–316
Vc098	92	GCGTATTTTCGTAATGCTCTATCC	TTTCAAGTTGTTATGAGTGACAGA	281–306
Vc109	116	GTTATACCCAGATGGAATCCAGAC	GAAGAGAATGGAGGACAAGGG	270–276
Vc113	119	AGTAAGAGCTGGGTGGTGTGAT	CCTTCGTTTCTGACCTACCATAT	128–132
Vc118	124	ATCGTTTTTCAGATCGGGGT	AATCCAAGTACCAAAACCACCTAAA	296–308

^a These microsatellites were previously demonstrated to be polymorphic and reliable (Chen et al. 2018).

^b Each primer name is designated with the species two-letter initials and a serial number.

binomial test (two-tailed) for goodness-of-fit to determine whether the observed mating-type frequency deviated from the 1:1 ratio.

Analysis of loci. Locus-based statistics were explored for the 16 microsatellite markers used. Most analyses were performed using R V3.5.0 (R Core Team 2018) and the package *poppr* (Kamvar et al. 2014). The data sets were checked for missing data and were coded accordingly as missing values in the analyses. Number of alleles per locus and effective number of alleles per locus were calculated. The polymorphic information content (PIC) value (Botstein et al. 1980) was calculated for each of the selected microsatellite markers as

$PIC = 1 - \sum (P_i)^2$ (where P_i is the proportion of samples carrying the i th allele of a particular locus). Shannon's information index (H) for each locus based on the marker frequency data was calculated (Shannon and Weaver 1949), and Nei's unbiased gene diversity (H_{exp}) was calculated for each locus (Nei 1978).

Analysis of population genetic data. Four populations with sufficient sample sizes (≥ 10 individuals, which included Berks County, PA, Frederick County, VA, Peach County B, GA, and Cummings County, NJ) were included in a rarefaction analysis. For these four populations, genotypic and gene diversity was analyzed

Fig. 2. Organization of *Venturia carpophila* mating-type genes and synteny between *Venturia* mating-type loci. **A**, Mating-type idiomorphs from *V. carpophila* isolate PS1-1 *MAT1-1* and isolate JB3-5 *MAT1-2*. Each gene is represented by an arrow showing the direction of transcription over boxes that represent exons of the coding sequence. Primer locations are indicated with lines with primer names above or below the sequence line. Locus structure is compared with that of two closely related species, *V. effusa* and *V. inaequalis*. Synteny comparison of *Venturia* mating-type loci: **B**, *V. carpophila* *MAT1-1* and *MAT1-2* loci; **C**, *V. carpophila* and *V. effusa* *MAT1-1*; **D**, *V. carpophila* and *V. effusa* *MAT1-2*; **E**, *V. carpophila* and *V. inaequalis* *MAT1-1*; and **F**, *V. carpophila* and *V. inaequalis* *MAT1-2*. Dot plots show conserved regions based on a window size of 30 nucleotides with 60% identity. Percent similarity of the encoded proteins are indicated at the gene transects in panels B to F.

for the original data and for the clone-corrected data (clone correction was performed at the appropriate level in the population hierarchy for the analysis). Analyses were based on the haploid MLGs and microsatellite allele presence and frequency, as described previously (Bock et al. 2017), and followed recommended approaches (Grünwald et al. 2017). Analyses were performed using R V3.5.0 packages *poppr* and *adgenet* (Jombert et al. 2018). The number of MLGs observed and the clone-corrected number of MLGs were calculated; the genotypic diversity, which measures both genotypic richness and abundance, was measured using the Shannon-Wiener index of MLG diversity (H) (Shannon 2001), Stoddart and Taylor's index of MLG diversity (G) (Stoddart and Taylor 1988), and Simpson's index (λ) (Simpson 1949). Genotype evenness was measured as $E0.5$ (Grünwald et al. 2003; Ludwig and Reynolds 1988; Pielou 1975), and Nei's unbiased gene diversity was calculated (H_{exp}) (Nei 1978). For all populations, the number of private alleles was calculated. For the nine populations with ≥ 4 individuals, linkage disequilibrium was assessed 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). Significance of I_A and $\bar{r}_d$ was tested by its deviation from the expected value (zero) based on 1,000 randomizations.

For the four populations (Peach County B, GA; Green County, AL; Cumberland County, NJ; and Frederick County, VA) where there were sufficient numbers of isolates ($n \geq 10$), further analyses of population genetic diversity and structure were conducted. An analysis of molecular variance (AMOVA) was performed on both the original and clone-corrected data. To test whether there were differences between populations, 1,000 randomizations were calculated based on the sample matrices, with no population differentiation assumed if the observed Φ (the population differentiation statistic) value fell within the distribution expected from the randomizations. A discriminant analysis of principal components (DAPC) was performed on the clone-corrected data. The appropriate number of clusters (K) for the DAPC was also identified based on the Bayesian information criterion in *adgenet*. A minimum-spanning tree based on the (squared) distances between populations was calculated to indicate actual proximities between populations. Finally, to optimize the DAPC, the a-score was calculated, which is the difference between the proportion of successful reassignments of the analysis (observed discrimination) and values obtained using random groups (random discrimination). Based on the analysis, membership probabilities were calculated to describe the admixture within the isolates. A minimum-spanning network (MSN) was calculated for the original data from the same four populations using Bruvo's distance.

Genotypic, gene diversity, and genetic diversity and structure were also analyzed based on mating type. Thus, the numbers of

isolates of *MAT1-1* and *MAT1-2* were presented and clone corrected to provide number of MLGs. The Shannon-Wiener index, Stoddart and Taylor's index, Simpson's index, genotype evenness, Nei's unbiased gene diversity, and number of private alleles were calculated for both the original data and the clone-corrected data, as described previously for the population data. Also as described above, linkage disequilibrium was assessed using I_A and $\bar{r}_d$. To explore any structure associated with mating type, AMOVA was performed, and DAPC and MSN analyses were performed as described for the orchard population level data, except analyses were based on mating type.

RESULTS

Identification and structural arrangement of the MAT loci. The mating type of *V. carpophila* isolate JP3-5 was identified as *MAT1-2* using BLAST with the *V. effusa* mating-type genes against the available genome sequence (Fig. 2A). PCR performed on a subset of isolates with the VcaMat_5F/5R primer set specific for the *MAT1-2-1* gene identified three isolates (JP3-5, MD1-2, and Clanton1-2) of the eight screened that amplified, which suggested that the remaining five were of the opposite mating type. The mating-type genes were sequenced from PCR products of five *MAT1-1* isolates (415X53-2, Kent-2, LO1-2, PS1-1, and RS2-2) and three *MAT1-2* isolates (JP3-5, MD1-2, and Clanton1-2) amplified with primers (VcaMat_2F and -2R) designed to conserved regions flanking both mating-type genes. The complete mating-type locus from isolate PS1-1 (accession number MN562205) was later made available from a genome sequence. Each mating-type gene was flanked by two previously identified genes, *APN2* encoding a DNA lyase and a gene with similarity to genes encoding a protein with a Pleckstrin homology domain. Synteny between the mating-type loci from *V. carpophila*, *V. effusa*, and *V. inaequalis* using the Dot Plot function in MacVector revealed sequence homology >60% of the two putative genes *unk2* and *unk3* (Fig. 2B to F). BLASTx analyses with the unknown gene sequences identified sequence similarity to *unk3* in some other ascomycetes, whereas *unk2* appears unique to *Venturia* spp. and was annotated with unknown function in *V. inaequalis* and *V. nashicola* genomes. The putative function of the genes encoded by *unk1*, *unk2*, and *unk3* remain unknown, as well as whether a putative role is associated with mating in these fungi.

Comparison of the mating-type genes to other species and a phylogeny based on the TUB2 gene. The *V. carpophila* isolate PS1-1 *MAT1-1* gene is 1,069 bases in length, encoding a predicted protein of 335 amino acids. The single intron of 61 bases

Fig. 3. Alignment of the deduced amino acid sequence of the regions containing the *MAT1-1-1* α -box and the *MAT1-2-1* high-mobility group box (HMG) from *Venturia carpophila* compared with those from other heterothallic ascomycetes species. Sequence accession numbers from GenBank are indicated.

was not associated with the α -box region. No sequence variation was observed within the five sequenced *MAT1-1-1* genes from isolates 415X53-2, Kent-2, LO1-2, PS1-1, and RS2-2. The *V. carpophila* isolate JP3-5 *MAT1-2-1* gene was 1,211 bases in length, encoding a predicted protein of 386 amino acids. The single intron of 50 bases was found within the HMG-box region with

conserved placement across *Venturia* and other Dothideomycete species. The *MAT1-2-1* gene from MD1-2, a nectarine isolate, had five single-nucleotide differences resulting in three nonsynonymous amino acid changes when compared with the gene sequences from isolates JP3-5 and Clanton1-2. The structure of *MAT1-1-1* and *MAT1-2-1* confirmed the presence of the α - and HMG-box,

Fig. 4. Phylogenetic tree based on the partial β -tubulin gene sequences of representative *Venturia* spp. Numbers on branches indicate approximate likelihood-ratio test (maximum likelihood) support where they are >50%. The likelihood substitution model was HKY85 with a likelihood value of $-\ln L$ 1365.66. Species is followed by isolate (accession number) and, where known, the host. Isolates from peach, nectarine, and almond all cluster in a single, well-supported clade, *Venturia carpophila*.

respectively, as described for several other closely related ascomycetes (Fig. 3). The alignments show close similarity between *V. carpophila*, *V. effusa*, and *V. inaequalis* amino acid sequences within the region of the α - and HMG-boxes but showed greatest identity to *V. effusa* (85.7 and 82.9% identity to *MAT1-1-1* and *MAT1-2-1*, respectively), followed by *V. inaequalis* (83.3 and 69.5% identity to *MAT1-1-1* and *MAT1-2-1*, respectively).

The *TUB2* gene was identified from the sequenced genome of isolate JP3-5 and was successfully amplified using primers Vctub2-F and Vctub2-R1 from the eight selected isolates. Phylogenetic analysis of *TUB2* based on maximum likelihood separated the isolates of *V. carpophila* from peach, nectarine, and almond into a single well-supported clade (96%), most closely related to *V. effusa* (Fig. 4). The *TUB2* sequences for *V. carpophila* isolates 415X53-2, Kent-2, and RS2-2 were identical to JP3-5, and LO1-2 was identical to Clanton1-2. These identical sequences were not included in the phylogeny.

Genetic diversity and structure. Missing data were minimal and, thus, not a hindrance to analysis. Overall, there was 0.23% missing data (with a maximum of 16.7% at one locus–isolate combination). Depending on the locus, there were two to eight alleles among the 81 isolates sampled (Supplementary Table S1). The loci were all informative (PIC value ≥ 0.44) and all had an effective allele number > 1.79 . Similarly, measures of allele diversity (Simpson's index, 0.44 to 0.79; H_{exp} , 0.44 to 0.80) demonstrated all loci to be informative. Thus, all 16 microsatellites were retained for the population genetic analyses. Specific allele frequencies varied substantially among populations (Supplementary Table S2). Some rare alleles were absent in most populations or only present at relatively low frequency. Frequencies of specific alleles in the population of *V. carpophila* from nectarine (Oconee County, SC) was comparable with other populations from peach. Considering all 16 loci, clone correction had only a marginal effect on the Simpson's index (≤ 0.035) (Supplementary Fig. S2).

TABLE 4. Statistics summarizing the genetic diversity (genotypic and gene) within nine populations of *Venturia carpophila*^a

Analysis, population ^b	N ^c	MLG ^d	eMLG (SE) ^e	Clonal fraction	H ^f	G ^g	λ^h	E.5 ⁱ	H _{exp} ^j
Original data									
PA_BER	10	9	9.00 (0.000)	0.10	2.16	8.33	0.880	0.952	0.542
VA_FRE	11	10	9.18 (0.386)	0.09	2.27	9.31	0.893	0.955	0.528
GA_PEB	17	15	9.34 (0.633)	0.12	2.67	13.76	0.927	0.949	0.489
NJ_CUM	14	9	7.36 (0.788)	0.36	2.11	7.54	0.867	0.906	0.442
Clone-corrected data									
PA_BER	9	9	–	–	2.20	9	0.889	–	0.569
VA_FRE	10	10	–	–	2.30	10	0.900	–	0.553
GA_PEB	15	15	–	–	2.71	15	0.933	–	0.496
NJ_CUM	9	9	–	–	2.20	9	0.889	–	0.479

^a Both original and clone-corrected data are presented. Analyses were performed using *poppr* (Kamvar et al. 2014); – indicates that result was meaningless for clone-corrected data.

^b Population: PA_BER = Berks County, PA; VA_FRE = Frederick County, VA; GA_PEB = Peach County B, GA; and NJ_CUM = Cummings County, NJ.

^c Number of individuals in the population.

^d Number of multilocus genotypes (MLGs) in that population.

^e eMLG is an approximation of the number of genotypes that would be expected at the smallest, shared sample size. Standard error (SE) based on eMLG is shown in parentheses.

^f Shannon-Wiener index of MLG diversity (Shannon 2001).

^g Stoddart and Taylor's measure of genotypic diversity (Stoddart and Taylor 1988).

^h Simpson's index (λ), which is one minus the sum of squared genotype frequencies (Simpson 1949). The index provides an estimation of the probability that two randomly selected genotypes are different (from 0 = no genotypes are different to 1 = all genotypes are different).

ⁱ Evenness (E.5) is a measure of the distribution of genotype abundances. In a population with equally abundant genotypes E.5 = 1 and a population dominated by a single genotype E.5 = approximately 0 (thus, for clone-corrected population, E.5 = 0) (Grünwald et al. 2003). For the clone-corrected data, E.5 = 1.0 for all populations.

^j Nei's unbiased estimate of average heterozygosity (or gene diversity) (Nei 1978).

TABLE 5. Measures of random mating among populations of *Venturia carpophila* from 10 peach orchards in the eastern United States

Population ^b	N (MLG) ^c	Alleles ^d	Original data ^a		Clone-corrected data ^a	
			I_A (P value)	$\bar{r}_d$ (P value)	I_A (P value)	$\bar{r}_d$ (P value)
GA_PEA	4 (4)	1	0.907 (0.109)	0.091 (0.109)	0.907 (0.117)	0.091 (0.117)
FL_ALA	6 (6)	0	3.752 (0.001)	0.315 (0.001)	3.752 (0.001)	0.315 (0.001)
GA_DEC	5 (4)	1	2.265 (0.004)	0.163 (0.004)	0.123 (0.369)	0.009 (0.369)
AL_GRE	8 (7)	2	2.451 (0.001)	0.165 (0.001)	1.051 (0.006)	0.071 (0.006)
PA_BER	10 (9)	5	1.809 (0.001)	0.121 (0.001)	1.597 (0.001)	0.107 (0.001)
SC_OCO	5 (5)	1	–0.273 (0.729)	–0.02 (0.729)	–0.273 (0.724)	–0.02 (0.724)
VA_FRE	11 (10)	2	1.103 (0.001)	0.079 (0.001)	0.805 (0.003)	0.058 (0.003)
GA_PEB	17 (15)	1	0.379 (0.008)	0.029 (0.008)	0.145 (0.155)	0.011 (0.155)
NJ_CUM	14 (9)	2	2.839 (0.001)	0.191 (0.001)	1.577 (0.002)	0.106 (0.002)
KY_FAY ^e	1 (1)	0	NS	NS	NS	NS

^a Gametic disequilibrium was measured using the index of association (I_A) and the standardized index of association ($\bar{r}_d$). I_A is a measure of linkage disequilibrium. The $\bar{r}_d$ is a more robust measure, less sensitive to the number of loci (Agapow and Burt 2001; Brown et al. 1980; Smith et al. 1993). Statistics were calculated based on 1,000 randomizations for both the original data and the clone-corrected data. Analyses were performed using *poppr* (Kamvar et al. 2014). Data in bold indicate populations with loci that deviate significantly from random recombination.

^b Population: GA_PEA = Peach County A, GA; FL_ALA = Alachua County, FL; GA_DEC = Decatur County, GA; AL_GRE = Greene County, AL; PA_BER = Berks County, PA; SC_OCO = Oconee County, SC; VA_FRE = Frederick County, VA; GA_PEB = Peach County B, GA; NJ_CUM = Cummings County, GA; and KY_FAY = Fayette County, KY.

^c N = number of individuals and multilocus genotypes (MLG).

^d Number of private alleles. A private allele is one unique to that population. There were 73 alleles among the 16 loci over all 81 isolates sampled.

^e Only one isolate was sampled from the Kentucky population. Thus, no statistics (NS) could be calculated.

Four populations with sufficient sample sizes ($n \geq 10$ individuals, which included Berks County, PA; Frederick County, VA; Peach County B, GA; and Cummings County, NJ) were included in the rarefaction analysis and, at the smallest shared sample size of 10, the populations had similar numbers of MLGs, although the population from Cummings County, NJ tended to have fewer MLGs compared with the other three populations (Supplementary Fig. S3). The population genetic analysis of the isolates from the four different orchards from the eastern United States showed them to be a genetically diverse group (Table 4). There were 43 MLGs. Clone correction did not greatly alter the different measures of diversity, which consistently indicated substantial genotypic and gene diversity. Thus, for the clone-corrected data, the Shannon-Weiner index of MLG diversity ranged from 2.20 to 2.71, depending on

population. Nei's unbiased measure of gene diversity ranged from 0.479 to 0.569, depending on population. Even the nonclone-corrected data set showed unique MLGs to be abundant in all populations with ≥ 10 isolates sampled ($E.5$ was 0.906 to 0.955, depending on population). Despite the presence of some clones in some populations (particularly the Cummings County, NJ population), the MLGs were distributed fairly evenly among the four populations with ≥ 10 isolates of *V. carpophila* (Supplementary Fig. S4). Across all nine populations, there was an association between measures of genotypic diversity but not gene diversity and the sample size in a population (Supplementary Table S3). Associations were stronger based on MLGs (i.e., clone-corrected data). The population of *V. carpophila* from nectarine (Oconee County, SC) had genotypic and gene diversity similar to that of other populations

TABLE 6. Hierarchical analysis of molecular variance (AMOVA) of the population structure of *Venturia carpophila* collected from peach fruit in four different orchards in the eastern United States based on both original and clone-corrected data^a

Analysis, source	df	Sum of squares	Mean squares	σ	Variation (%)	ϕ (P value)
Original data						
Between orchard	3	86.45	28.82	1.62	16.90	0.17 (0.001)
Within orchard	48	381.98	7.96	7.96	83.10	...
Total	51	468.43	9.19	9.58	100	...
Clone-corrected data						
Between orchard	3	62.25	20.75	1.14	12.02	0.12 (0.001)
Within orchard	40	335.04	8.38	8.38	87.98	...
Total	43	397.29	9.24	9.52	100	...

^a AMOVA was performed using *poppr* (Kamvar et al. 2014). Abbreviations and symbols: df = degrees of freedom, σ = the variance for each hierarchical level, Variation (%) = the proportion of σ , ϕ = between-orchard population differentiation, and P = probability of ϕ based on a randomization test with 1,000 permutations to determine whether populations were significantly different (i.e., significant population differentiation given that the observed ϕ does not fall within the distribution expected from the permutation).

Fig. 5. Results of **A**, discriminant analysis of principal components based on an analysis of the 43 isolates in the clone-corrected data set characterized by the 16 microsatellite markers from four populations (Berks County and Frederick County, VA; Peach County B, GA; and Cummings County, NJ). A minimum-spanning tree based on the squared distances between the populations is indicated with the center of each group identified by a cross. PCA = principal component analysis. **B**, Membership probability chart for each of the 43 unique multilocus genotypes from the four populations based on the discriminant analysis of principal components.

sampled, despite having only five isolates (genotypic and gene diversity data not shown for populations with <10 individuals). Each isolate from the nectarine population was a unique MLG.

Private alleles were observed in most populations (not in the Alachua County, FL or Fayette County, KY populations, which had

six and one isolates, respectively) (Table 5; Supplementary Fig. S5). The Berks County, PA population had most private alleles (five private alleles in 10 isolates). All other populations had one to two private alleles. The population of *V. carpophila* from nectarine (Oconee County, SC) had a single private allele. All other alleles

Fig. 6. Minimum spanning network (MSN) based on the matrix of pairwise Bruvo genetic distances showing the relatedness among multilocus genotypes from populations of *Venturia carpophila* in four peach orchards in the eastern United States. The MSN was calculated in R using the *poppr* package (Kamvar et al. 2014). Populations: BER = Berks County, PA; FRE = Frederick County, VA; PEB = Peach County B, GA; and CUM = Cummings County, NJ. Small circles = single isolate, medium circles = two identical isolates (clone), and large circles = three identical isolates (clones). Only two populations shared a clone (FRE and BER). All other clones were restricted to the same population.

were shared with other populations of *V. carpophila* sampled from peach fruit. Of the total of 73 alleles in all populations, 15 (20.5%) were private alleles. Linkage disequilibrium was observed in both original data and the clone-corrected data using both I_A and $\bar{r}_d$. With the nonclone-corrected data, seven of the nine populations displayed linkage disequilibrium. With clone correction, five of nine populations showed linkage disequilibrium, demonstrating an effect of clonality. The Oconee, SC population was one of only two populations sampled that showed no linkage disequilibrium based on I_A or $\bar{r}_d$ (the other was the Peach County A, GA population). Furthermore, for the four populations with most isolates ($n \geq 10$ isolates: Berks County, PA; Frederick County, VA; Peach County B, GA; and, Cummings County, NJ), linkage disequilibrium ($\bar{r}_d$) ranged from 0.011 to 0.107 ($P = 0.001$

TABLE 7. Frequencies of mating types (*MAT1-1*, *MAT1-2*) of isolates of *Venturia carpophila* sampled from each of 10 peach orchards in different states and counties in the eastern United States^a

Analysis, sample	Sample size	<i>MAT1-1</i> : <i>MAT1-2</i>	<i>P</i> ^b
Original data			
Peach County, GA (A)	4	1:3	0.6
Peach County, GA (B)	17	4:13	0.05
Decatur County, GA	5	3:2	1.0
Alachua County, FL	6	4:2	0.7
Greene County, AL	8	0:8	0.008
Fayette County, KY	1	1:0	NS
Berks County, PA	10	7:3	0.3
Oconee County, SC	5	3:2	1.0
Cumberland County, NJ	14	12:2	0.01
Frederick County, VA	11	10:1	0.01
Total	81	45:36	0.2
Clone-corrected data			
Peach County, GA (A)	4	1:3	0.6
Peach County, GA (B)	15	3:12	0.04
Decatur County, GA	4	2:2	1.0
Alachua County, FL	6	4:2	0.7
Greene County, AL	7	0:7	0.02
Fayette County, KY	1	1:0	NS
Berks County, PA	9	6:3	0.5
Oconee County, SC	5	3:2	1.0
Cumberland County, NJ	9	8:1	0.04
Frederick County, VA	10	9:1	0.02
Total	69	36:33	0.8

^a Original data and clone-corrected data are presented.
^b Probability from an exact binomial analysis (two-tailed) to test whether mating-type frequencies deviate significantly from a 1:1 ratio. *P* values in bold indicate populations with loci that deviate significantly from random recombination.

to 0.155). Most loci in these populations had no or only moderate evidence of linkage disequilibrium ($\bar{r}_d > 0.25$) (Supplementary Fig. S6).

Based on the four populations with >10 isolates, the AMOVA indicated that most variance could be found within orchards for both original (83.10%) and clone-corrected (87.98%) data sets (Table 6). However, in both cases, population structure could be detected ($\phi P = 0.001$). Based on the clone-corrected data, the DAPC analysis of the isolates in the four populations indicated that they clustered in three distinct groups (Fig. 5A). Also, estimating cluster number based on the Bayesian information criterion indicated three clusters (Supplementary Fig. S7). The probability of group membership indicated that most isolates had $\geq 20\%$ probability of membership in two or three of the source populations (Fig. 5B), although a few isolates had almost 100% membership probability in one population (particularly some isolates from the Berks County, NJ and Peach County B, GA populations). The MSN based on Bruvo's distance indicated that, although there was a slight tendency for individuals from the same population to group (Fig. 6), there were individuals in all populations that had closely related MLGs from the other three populations. Isolate numbers in these four populations were still limited; therefore, the results are considered only suggestive of some possible underlying population structure.

Mating-type frequency and genetic structure. The frequency of *MAT1-1* and *MAT1-2* mating types varied among orchards but sample sizes in all cases were small ($n \leq 14$) (Table 7). Clonality existed in six of the orchard samples (Peach County B, GA; Green County, AL; Cumberland County, NJ; and Frederick County, VA) but in no case did clone correction change the outcome of the exact binomial test. In all orchards with at least two isolates, both mating types were identified, except the one in Greene County, AL. In Greene County all eight isolates were *MAT1-2*. In all orchards, except those in Peach County, the number of *MAT1-1* isolates was greater than *MAT1-2* isolates. The frequency of mating types of isolates of *V. carpophila* from nectarine (Oconee County, SC) were in equilibrium when analyzed based on original data or the clone-corrected data. Overall, there was no evidence that the mating types were not in equilibrium for either all original location data combined (*MAT1-1*/*MAT1-2*, 45:36), or the clone-corrected data (*MAT1-1*/*MAT1-2*, 36:33). For the clone-corrected data, the exact binomial test ($P = 0.8$) of mating-type frequencies in this widely sourced selection of isolates from the eastern United States demonstrated mating types to be in equilibrium at the regional scale.

The two mating-type populations were genetically diverse groups, with very similar measures for both genotypic and gene

TABLE 8. Statistics summarizing the genetic variation (genotypic and genic) within the two mating type populations of *Venturia carpophila*^a

Analysis, mating type	<i>N</i> ^b	MLG ^c	eMLG (SE) ^d	<i>H</i> ^e	<i>G</i> ^f	λ ^g	<i>E</i> .5 ^h	<i>H</i> _{exp} ⁱ
Original data								
<i>MAT1-1</i>	45	36	30.0 (1.20)	3.51	30.2	0.967	0.904	0.592
<i>MAT1-2</i>	36	33	33.0 (0.00)	3.47	30.9	0.968	0.961	0.581
Clone-corrected data								
<i>MAT1-1</i>	36	36	–	3.58	36	0.972	–	0.600
<i>MAT1-2</i>	33	33	–	3.50	33	0.970	–	0.580

^a Both original and clone-corrected data are presented. All populations (81 isolates for the original data and 69 for the clone-corrected) were included in the analysis. Analyses were performed using *poppr* (Kamvar et al. 2014); – indicates that result was meaningless for clone-corrected data.
^b Number of individuals in the population.
^c Number of multilocus genotypes (MLGs) in that population.
^d eMLG is an approximation of the number of genotypes that would be expected at the smallest, shared sample size. Standard error (SE) based on eMLG is shown in parentheses.
^e Shannon-Wiener Index of MLG diversity (Shannon 2001).
^f Stoddart and Taylor's measure of genotypic diversity (Stoddart and Taylor 1988).
^g Simpson's index (λ), which is one minus the sum of squared genotype frequencies (Simpson 1949). The index provides an estimation of the probability that two randomly selected genotypes are different (from 0 = no genotypes are different to 1 = all genotypes are different).
^h Evenness (*E*.5) is a measure of the distribution of genotype abundances. In a population with equally abundant genotypes *E*.5 = 1 and a population dominated by a single genotype *E*.5 = approximately 0 (thus, for clone-corrected population, *E*.5 = 0) (Grünwald et al. 2003). For the clone-corrected data, *E*.5 = 1.0 for all populations.
ⁱ Nei's unbiased estimate of average heterozygosity (or gene diversity) (Nei 1978).

diversity whether the data were original or clone corrected (Table 8). The Shannon-Wiener index of MLG diversity was 3.51 and 3.47 for *MAT1-1* and *MAT1-2* for the original data and 3.58 and 3.50 for the clone-corrected data, respectively. Other measures of genotypic diversity (G or λ) were similar. Gene diversity was 0.592 and 0.581 for *MAT1-1* and *MAT1-2* for the original data and 0.600 and 0.580 for the clone-corrected data, respectively. *MAT1-2* had slightly more equally abundant genotypes (0.961) compared with *MAT1-1* (0.904), due to the greater number of clones in *MAT1-1* (10).

There were private alleles within both mating-type groups (Table 9; Supplementary Fig. S8). *MAT1-1* had 11 private alleles, while *MAT1-2* had 9 private alleles. Sample sizes were limited, which could explain the presence of private alleles within the sampled populations. Linkage disequilibrium measured as I_A and $\bar{r}_d$ was prevalent in both mating types in both the original and clone-corrected data sets. As with the population-level data, only a few pairwise comparisons of loci had low to moderate linkage disequilibrium ($\bar{r}_d > 0.1$) (Supplementary Fig. S9).

Based on the two mating types, the AMOVA indicated that almost all variance could be accounted for within the mating types for both original (98.56%) and clone-corrected (99.69%) data sets (Table 10). In the case of the original data, population structure between mating types could be detected ($\phi P = 0.04$). However, when clone corrected, there was no evidence of any population structure between mating types ($\phi P = 0.3$). Based on the clone-corrected data, and as for the population level results, the DAPC analysis indicated that they clustered in three distinct groups (Fig. 7A). The probability of group membership indicated that most isolates had an approximately 50% probability of membership to either mating type, although slightly more isolates tended to show greater membership to *MAT1-2* (Fig. 7B). The MSN based on Bruvo's distance indicated little tendency for individuals from either mating type to group (Fig. 8), and there were individuals from both mating types distributed throughout the network. Although the

sample size and sampling structure did not allow for analyzing genetic characteristics at the suborchard level, the data do confirm that different mating types exist within the same peach tree canopy (Supplementary Table S4).

DISCUSSION

This study is the first to define and characterize the mating types of *V. carpophila* and the genetic diversity within the species based on previously developed microsatellites (Chen et al. 2018). *V. carpophila* was identified as a heterothallic species, a mode of sexual reproduction that it shares with several other *Venturia* spp. (Keitt and Palmiter 1937; Langford and Keitt 1942; Young et al. 2018). The structure of the mating-type loci in *V. carpophila* is conserved with that of other *Venturia* spp. (*V. effusa* and *V. inaequalis*). The structure of the two mating-type idiomorphs confirmed the presence of the α -box and the HMG-box, described for several other closely related ascomycetes. Interestingly, although the *MAT1-1-1* gene contained an intron, it was not associated with the α -box as has been found with other fungal species. The sequence alignments of the mating-type genes showed greatest identity to *V. effusa*, followed by *V. inaequalis*. This was consistent with the phylogenetic analysis of *TUB2*, which indicated that *V. carpophila* was most closely related to *V. effusa*. This result lends weight to the phylogeny described from earlier observations by Schnabel et al. (1999), who used sequence from the ITS1-5.8S-ITS2 region of *Venturia* and *Cladosporium* spp. These results also support the taxonomic positions as defined by Schubert et al. (2003) for *Fusicladium carpophilum* (syn. *V. carpophila*).

A sexual stage has been described for *V. carpophila* (Fisher 1961). However, this was described from apricot freckle fungus in Victoria, Australia. The pathogen is also reported to infect almond (Teviotdale et al. 2002). Based on the *TUB2* phylogenetic analysis, an isolate from almond (MN548431) grouped with the isolates of *V. carpophila* from peach and nectarine. We found no evidence that

TABLE 9. Measures of random mating between the two mating types (*MAT1-1* and *MAT1-2*) of *Venturia carpophila* from 10 peach orchards in the eastern United States

Type ^c	Number of individuals ^a		Alleles ^d	Original data ^b		Clone-corrected data ^b	
	Orig	Clone		I_A (P value)	$\bar{r}_d$ (P value)	I_A (P value)	$\bar{r}_d$ (P value)
<i>MAT1-1</i>	45	36	11	0.842 (0.001)	0.056 (0.001)	0.460 (0.001)	0.031 (0.001)
<i>MAT1-2</i>	36	33	9	0.263 (0.003)	0.018 (0.003)	0.179 (0.035)	0.012 (0.035)

^a Mean number of alleles. Orig = original data and Clone = clone-corrected data.

^b Gametic disequilibrium measured using the index of association (I_A) and the standardized index of association ($\bar{r}_d$), for both the original data and the clone-corrected data. I_A is a measure of linkage disequilibrium. The $\bar{r}_d$ is a more robust measure, less sensitive to the number of loci (Agapow and Burt 2001; Brown et al. 1980; Smith et al. 1993). Statistics were calculated based on 1,000 randomizations. Analyses were performed using *poppr* (Kamvar et al. 2014). Data in bold indicate populations with loci that deviate significantly from random recombination.

^c Mating type. All populations (81 isolates for the original data and 69 for the clone-corrected) were included in the analysis.

^d Number of private alleles. A private allele is one unique to that population. There were 73 alleles among the 16 loci over all 81 isolates sampled.

TABLE 10. Analysis of molecular variance (AMOVA) of the population structure by mating type of *Venturia carpophila* collected from peach fruit in 10 different orchards in the eastern United States based on both original and clone-corrected data^a

Analysis, source	df	Sum of squares	Mean squares	σ	Variation (%)	ϕ (P value)
Original data						
Between mating types	1	14.94	14.94	0.14	1.44	0.01 (0.04)
Within mating types	79	744.31	9.42	9.42	98.56	...
Total	80	759.25	9.49	9.56	100.0	...
Clone-corrected data						
Between mating types	1	10.50	10.50	0.03	0.31	0.003 (0.3)
Within mating types	67	635.41	9.48	9.48	99.69	...
Total	68	645.91	9.50	9.51	100.0	...

^a AMOVA was performed using *poppr* (Kamvar et al. 2014). Abbreviations and symbols: df = degrees of freedom, σ = the variance for each hierarchical level, Variation (%) = the proportion of σ , ϕ = between-mating-type population differentiation, and P = probability of ϕ based on a randomization test with 1,000 permutations to determine whether mating-type populations were significantly different (i.e., with the clone-corrected data, no significant population differentiation given that the observed ϕ does not fall within the distribution expected from the permutation).

the isolates sampled from nectarine (Oconee County, SC) were genetically different from those sampled from peach elsewhere. Measures of genotypic and gene diversity, and the observation that all but one allele was shared with other peach populations at comparable frequencies, is indicative that there is transfer of genetic material among the populations infecting peach and nectarine. This may not be surprising because nectarine is considered a smooth-skinned peach (*P. persica*). To the best of our knowledge, host range studies have not been performed with isolates collected from the three hosts peach, apricot, and almond; population genetic studies need to be performed to ascertain whether the populations from the hosts are freely recombining. There may be areas of the world where the populations of *V. carpophila* on these three hosts coexist sympatrically. However, in many areas (such as Peach County, GA in the southeastern United States), only one host species is cultivated. In California, peach and almond are both cultivated but it is not known whether the pathogen populations on these crops are freely mixing.

The results of this study demonstrate that both mating types can coexist in the same orchards and the same tree in the southeastern United States and, thus, the potential for sexual recombination is available. Insufficient samples were taken from individual fruit to ascertain whether both coexisted on a single fruit (only one fruit was sampled for multiple isolates). This has ramifications for the epidemiology of the disease, because more than one spore type may play a role in the disease cycle. Currently, the epidemiology of peach scab in the eastern United States is based on a model defining the importance of the splash-dispersed asexually produced conidia (Keitt 1917; Lan and Scherm 2003; Scherm et al. 2008), yet a sexually produced ascospore could play a significant role in disease spread. With the apple scab pathogen, although ascospores are

known to be a major inoculum source (MacHardy 1996), locally asexually produced and locally dispersed conidia may also be important in the epidemic at small spatial scales (Xu et al. 2013).

Primers to *MAT1-1-1* and *MAT1-2-1* genes were developed that effectively amplified parts of the gene in the presence of a primer for a housekeeping gene (*TUB2*), with the reaction being multiplexed to facilitate efficiency and economy of identification in large numbers of isolates. Although preliminary, due to sample sizes, both nonclone-corrected and clone-corrected data show that the two mating types of *V. carpophila* exist in equilibrium in over half the populations and overall. In several cases, both mating types were observed in the same tree canopy. However, in individual orchards, the numbers were small, as has been the case in some other studies such as with *Rhynchosporium secalis* (Linde et al. 2003) and *D. septosporum* (Dale et al. 2011). The suggestion of mating-type equilibrium we observed in some populations indicates that the fungus is undergoing sexual reproduction at sufficient frequency to ensure equivalent frequency of mating types. Mating-type frequencies of many other sexually reproducing filamentous ascomycetes are in equilibrium in most populations, especially when sample size is sufficient (Gout et al. 2006; Sommerhalder et al. 2006). Mating types have also been found in equilibrium in some species of filamentous ascomycetes for which a sexual stage has not yet been identified (Linde et al. 2003). As noted, both mating types were found in the same peach tree canopies, indicating that they may be spatially close. Sampling at a small spatial scale will reveal whether the two mating types are found on the same fruit.

Although structural organization of the two mating-type loci and regions of the α - and HMG-box sequences may have similarity, they are characteristic for individual species (Turgeon 1998). It is important to identify and characterize the mating-type genes

Fig. 7. Results of **A**, discriminant analysis of principal components based on an analysis of the 69 isolates in the clone-corrected data set characterized by the 16 microsatellite markers. Populations defined by mating type (*MAT1-1* and *MAT1-2*). A minimum-spanning tree based on the squared distances between the populations is indicated with the center of each group identified by a cross. PCA = principal component analysis. **B**, Membership probability chart for each of the 69 unique multilocus genotypes from the two mating types based on the discriminant analysis of principal components.

because they can be indicators of the reproductive strategy, which can directly affect diversity. These results indicate that sexual reproduction is likely a component of the lifecycle of *V. carpophila* in the eastern United States. Indeed, the population genetic characteristics and balance of mating types of the closely related *V. effusa* were initially indicative of a sexual stage (Bock et al. 2017; Young et al. 2018), which was only very recently characterized for

that species (Charlton et al. 2020). The sexual stage of *V. carpophila* has been described in Australia (Fisher 1961) but its likely presence and possible role in the United States has remained uncharacterized prior to this study.

The populations were genetically diverse (both genotypic and gene diversity). Most isolates of *V. carpophila* were unique MLGs and, although some clones (12 isolates overall) were found, this

Fig. 8. Minimum spanning network (MSN) based on the matrix of pairwise Bruvo genetic distances showing the relatedness among multilocus genotypes from populations of *Venturia carpophila* from the two mating types (*MAT1-1* and *MAT1-2*) from 10 peach orchards in the eastern United States. The MSN was calculated in R using the *poppr* package (Kamvar et al. 2014). Small circles = single isolate, medium circles = two identical isolates (clone), and large circles = three identical isolates (clones).

might be expected when considering that a small-scale, splash-dispersed component is described for *V. carpophila* (Keitt 1917; Lan and Scherm 2003; Scherm et al. 2008), especially because all but one of the clones were from trees in the same orchard (that might have been adjacent) or from fruit in the same canopy (and those fruit might have been adjacent). Such proximity would increase the chance of the clones being from the same inoculum source. The genotypic and genetic diversity observed among these 81 isolates of *V. carpophila* is suggestive of a sexually reproducing pathogen; however, there was evidence of linkage disequilibrium in most populations. Linkage disequilibrium might be due to spatially restricted epidemics, close physical association among loci, selection processes, gene flow and drift (Milgroom 1996), or a clonal component due to the reproduction of the pathogen (McDonald 2004) or, possibly, the sampling. In most cases, only some of the pairwise loci comparisons demonstrated severe disequilibrium; thus, it was likely biased by these pairs. However, the loci were carefully selected based on even coverage of the entire genome, thus avoiding risk of linkage caused by physical proximity (Chen et al. 2018). In other studies, linkage disequilibrium was observed in populations of both the closely related *V. effusa* on pecan (Bock et al. 2017) and *V. inaequalis* on apple (Xu et al. 2013), both of which have a sexual stage. Private alleles were observed both within individual populations and between mating types but did not appear to be associated with the size of the population. Nonetheless, private alleles may indicate some degree of population differentiation. Sampling using hierarchical approaches and larger sample sizes will provide more definitive results on these aspects of populations of *V. carpophila*.

For the four population with sufficient numbers of isolates (≥ 10 isolates), there was evidence of population structure. The AMOVA indicated that, although most variance was explicable at the level of the population, a smaller proportion (12.02% for the clone-corrected data) was accountable between orchards. The DAPC and MSN both provided additional evidence of genetic clustering, although all analyses indicated considerable admixture and relatedness among many individual isolates from most populations as well. The AMOVA based on mating type using clone-corrected data showed no evidence of population structure (99.69% of the variance was explicable within the mating-type populations) but the DAPC of these isolates did identify three distinct genetic clusters, confirming the result from the population-level DAPC. The membership probabilities based on mating type tended to show a reasonably even admixture for most isolates. The MSN of these data bore out this result. Isolates from the two mating types did not show any marked evidence of clustering associated with mating type. These data are also indicative that sexual reproduction is likely occurring among populations of *V. carpophila* in the eastern United States. However, as noted above, larger samples of isolates from several trees in several orchards are needed to confirm what these data indicate and define more clearly the genetic diversity, reproduction, and population structure.

Phytosanitary regulation may be affected by the existence of single mating types of a fungus in some areas; for example, black spot of citrus (Wang et al. 2016) and Dothistroma needle blight of pine (Groenewald et al. 2006). It is not known whether both mating types of *V. carpophila* exist in all other peach-, apricot-, and almond-producing areas but, clearly, this should be ascertained because these stone fruit are introduced crops in most areas where they are cultivated. Cooccurrence of both mating types provides the opportunity for sexual reproduction, which is the vehicle for recombination and reassortment of existing and novel genetic variability. Such variability is an important basis for adaptability, and this can affect any endeavors to manage the disease through resistance breeding, as well as affect development and propagation of fungicide resistance (McDonald 2015; McDonald and Linde 2002; McDonald and Mundt 2016). Both mating types of *V. carpophila* exist in the eastern United States and were demonstrated to exist in the

southeast of Australia because the sexual cycle was observed (Fisher 1961) but their existence is uncharted in the western United States, South America, parts of Africa, and other locations where these stone fruit are produced and peach scab occurs (Keitt 1917). Knowledge of the occurrence of mating types on stone fruit in other areas would be useful.

The ability to identify mating types of *V. carpophila* provides a basis for characterizing populations in different stone fruit production areas where scab is an issue, which can provide insights into reproductive methods and is a basis for studying the genetics of the fungus through in vitro crosses of opposite mating types. It may also provide a basis for an improved understanding of the epidemiology of the disease, and possible management opportunities to achieve better scab control.

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