Genetic Diversity and Population Structure of *Fusicladium effusum* on Pecan in the United States

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Abstract

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Fusicladium effusum causes pecan scab, which is the most destructive disease of pecan orchards in the United States. Conidia of the pathogen are spread by rain splash and wind. The fungus is pathogenically diverse; yet there is no information on its genetic diversity or population genetics. Universally primed polymerase chain reaction (UP-PCR) was used to investigate the genetic diversity and population structure on a hierarchical sample of 194 isolates collected from 11 orchard locations from Florida to Texas, consisting of three to four isolates from each of five to six trees at each location. Genetic variation was high throughout the region, with all but nine of the multilocus haplotypes being unique. Nei's average gene diversity ranged from 0.083 for a population from Mississippi to 0.160 for a population from Kansas. An analysis of molecular variance of the hierarchically sampled populations found

that the majority of the genetic variability (82.6%) occurred at the scale of the individual tree and only relatively small amounts among populations in trees from an orchard (5.0%) or within groups (i.e., orchard location populations) (12.5%). The results suggest little population differentiation in *F. effusum* in the southeastern United States, although ϕ_{PT} values of genetic distance for pairwise comparisons indicated some populations could be differentiated from others. There was evidence of linkage disequilibrium in certain populations, and the common occurrence of asexual reproduction in *F. effusum* could lead to measurable linkage disequilibrium under certain circumstances. However, the degree of genetic diversity and the scale over which diversity is distributed is evidence that *F. effusum* undergoes regular recombination despite no known sexual stage.

Pecan scab, caused by the fungal pathogen *Fusicladium effusum* (G. Winter), is typically the most destructive disease of pecan (*Carya illinoensis* (Wangenh.) K. Koch). The disease causes considerable economic loss to growers in the southeastern United States and other relatively wet, humid pecan-growing regions in the world. It can substantially reduce both quality and yield of nuts (22,25,56). The pathogen is known only by its asexual phase, with the conidia dispersed by wind and splash (24). When conditions are conducive for dispersal and infection, there can be severe disease throughout the canopy of pecan trees (5).

Pecan scab management is primarily by a combination of fungicide application and host resistance (22). The fungus is resistant to several classes of fungicides (53,55,57), and although novel fungicides continue to become available (4), they too could succumb; thus, durable host resistance to *F. effusum* is an attractive option. But the fungus is pathogenically diverse (12,13,16,27), with genetic adaptation by the pathogen resulting in previously scab-resistant cultivars developing disease (22). Some data suggest that there is physiological specialization with separate pathogenic 'races' on different pecan genotypes (12,13). Breeding to develop and release new cultivars continues, but there is a risk that the pathogen will adapt to the novel resistance developed, which is especially problematic for controlling disease in a tall, long-lived crop like pecan.

Plant pathogens have several ways to maintain and generate genetic diversity (10). Understanding the pathogen population diver-

sity and genetics is fundamental to developing an informed strategy for host resistance breeding that maximizes the probability of generating durable, scab-resistant germplasm. Those plant pathogens possessing a mixed reproductive cycle (asexual and at least one sexual cycle each epidemic cycle) have a high potential for gene flow and thus pose the greatest risk to host resistance genes. Appropriate incorporation of resistance genes into the host and resistance gene deployment in the field are needed to maximize durability (36), and these decisions should be based on the evolutionary potential of the pathogen. Pecan scab is known only by its asexual, conidial phase (24), but lack of an identified sexual stage is by no means evidence of absence. Numerous related fungal plant pathogens, initially known only by an asexual stage, are now known to have a sexual stage that provides a basis for explaining the genetic diversity characteristic of a freely recombining organism (29,40,52,60), and if a sexual stage has not been identified, the mating type loci have been characterized and found to be in near or complete equilibrium, providing compelling evidence for the existence of an as yet unknown sexual stage (28,32,33).

Apart from a comparison of two dominant marker methods (6), there is no information available on the population genetics of *F. effusum* (although limited molecular genetic data has been used to aid classification of this fungus; 3,54). Population genetics and genetic diversity have been studied for several related species. Preliminary studies of genetic diversity in the apple scab pathogen (*Venturia inaequalis*) using dominant markers (random amplified polymorphic DNA [RAPD]) and internal transcribed spacer (ITS) region sequences demonstrated a high degree of genetic diversity (58,59). More recent studies using codominant markers have allowed a more in-depth analysis of populations worldwide (21), providing new insights into the epidemiology and possible management of the disease (64). Overall, the coevolution of *V. inaequalis* and *Malus* species (domestic and natural) is characterized by a high level of genetic diversity and relatively low population differentiation; interpreted as showing the importance of human transport and the existence of sexual spores that are dispersed

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moderate distances (21). A population with this mating and dispersal behavior ensures genetic recombination, gene flow, and genetic equilibrium. Similar genetic studies have been done with several other species including *Pyrenophora teres* (50), *Lepidosphaeria maculans* (17), and *Septoria tritici* (37,68). Studies of the genetic diversity of *F. effusum* are needed to provide a framework for understanding its genetic diversity and as a basis for informed deployment of scab resistance genes in *Carya* germplasm (36).

Bock et al. (6) recently compared RAPDs and universally primed polymerase chain reaction (UP-PCR) as dominant marker systems to investigate the molecular genetic diversity of *F. effusum*. Both systems have the advantages of being inexpensive and relatively straightforward, but the UP-PCR system was found to be more reliable. Dominant markers can provide very useful information on the genetic diversity and population structure of an organism. Although codominant marker methods (such as restriction fragment length polymorphism [RFLP] and simple sequence repeats [SSRs]) are more sophisticated, sensitive, and informative (38), they are also more expensive and time-consuming to develop.

The objective of this study was to use a hierarchical sampling approach (38) and UP-PCR markers to provide insight into the structure and genetic diversity of *F. effusum* in the southeastern United States.

Materials and Methods

Sample locations and isolation of *F. effusum*. A total of 194 isolates of *F. effusum* was obtained from scab-diseased leaves or fruit of pecan from 11 populations across the southeastern United States (Table 1 and Fig. 1). Eighteen diseased leaves or fruit were collected from five to six trees (three to four leaves per tree) in each of the orchards, ensuring that the leaf samples were taken individually from around the canopy of a particular tree, and ensuring that trees were separated by at least four rows or trees on a grid basis, where orchard size allowed. Thus, the sampling was hierarchical, comprising the regional scale (between orchards, 106 to 1,321 km), between trees within an orchard (40 to 400 m), and between leaves on an individual tree (5 to 20 m). The isolates were sampled from both commercially available cultivars in orchards and naturally seeded native-tree groves. The process of isolation for *F. effusum* has been previously described (6). Briefly, conidia of *F. effusum* were scraped from single lesions on the sampled leaf using a scalpel, and a dilute spore solution was prepared in sterile distilled water. Multiple 0.1- μ l aliquots were spread on the surface of replicate water agar plates (WA, amended with lactic acid [0.50 ml/liter], streptomycin [0.20 g/liter], tetracycline [0.05 g/liter], and chloramphenicol [0.05 g/liter]), which were incubated at 27°C for 24 h under fluorescent lights on a 12/12 h day/night cycle. A single germinated spore of *F. effusum* was excised on an agar plug using a scalpel under a microscope, and was transferred to antibiotic-amended potato dextrose agar (PDA, amended as for WA) and incubated for 3 to 5 weeks at 25°C, at which time the DNA was extracted from the sample.

DNA extraction, PCR, and electrophoresis. The fungal mycelium was scraped from the surface of the agar with a scalpel (~200 mg) and the DNA extracted using a ZymoResearch DNA extraction kit (ZymoResearch, Irvine, CA), following a slightly modified protocol for DNA extraction from fungi (54) using a Tissuelyser (Qiagen, Valencia, CA, USA) to lyse the fungal mycelium. Once obtained, the DNA was quantified using a Nanodrop spectrophotometer (Nanodrop Products, Wilmington, DE) and stored in TE buffer at -20°C. The UP-PCR primers (obtained from Eurofins MWG Operon, Huntsville, AL) had been previously tested and found to be reliable and repeatable (6). UP-PCR primers produce dominant markers (9), and have been previously used on pathogens related to *F. effusum* (44). The working primer concentration was 20 μ M for the UP-PCR. A total reaction volume of 50 μ l was used: 6 μ l template DNA (6 ng), 2 μ l primer, 17 μ l dH₂O, and 25 μ l master mix (Promega, Madison, WI). The UP-PCR reaction conditions were as follows (modified from Obanor et al. [44]): denaturation at 94°C for 2 min, 94°C for 50 s, annealing at 56°C for 90 s, and primer extension at 72°C for 1 min all repeated 34 times, with a final extension step of 72°C for 10 min. The PCR products were separated using agarose-gel electrophoresis, stained with gel red (Biotium, Inc., Hayward, CA), and visualized under ultraviolet light. Images were photographed and scored manually by the same individual (to ensure consistency) for the presence (1) or absence (0) of the marker/amplicon. Fragment sizes were estimated based on their migration relative to a known standard (50 to 10,000 bp Ladder; Bionexus, Inc., Oakland, CA). Any amplicon that was sufficiently pronounced to be readily visible was scored as present (6).

Data analysis. The number of markers observed for the 194 isolates with each primer was counted, and the percent polymorphic calculated by population, and by marker. The number of clones (isolates sharing identical multi-locus haplotypes) was calculated. The marker data for all 194 isolates were analyzed using an Un-

Fig. 1. A map of the southeastern United States showing sample sites for populations of *Fusicladium effusum* collected for the population genetic analysis.

Table 1. Location, elevation, source cultivar, and numbers of isolates of *Fusicladium effusum* collected to assess population genetic diversity in the southeastern United States

Source location (population)	Number of isolates	Latitude and longitude of site	Elevation (m)	Pecan cultivar
Fairhope, Alabama	18	30°32.681' N/87°52.918' W	62	Desirable
Lowndesboro, Alabama	18	32°21.236' N/86°38.393' W	43	Kiowa
Byron, Georgia	18	32°65.522' N/83°65.522' W	156	Desirable
Chetopa, Kansas	14	37°01.360' N/95°02.783' W	266	Native seedling
Powhatan, Louisiana	18	31°50.211' N/93°13.328' W	33	Desirable
Raymond, Mississippi	18	32°11.297' N/90°29.537' W	70	Desirable
Monticello, Florida	18	30°32.265' N/83°55.060' W	69	Desirable
Burneyville, Oklahoma	18	33°52.823' N/97°15.164' W	244	Western Schley
Bastrop, Texas	18	30°03.554' N/97°18.402' W	123	Wichita
Tifton, Georgia	18	31°30.388' N/83°38.287' W	112	Desirable
Carlyle, Illinois	18	38°29.323' N/89°30.212' W	143	Native seedling

weighted Paired Group Method of Arithmetic Averages (UPGMA) cluster analysis (SAS V9.3, proc cluster, method=average; SAS Institute, Cary, NC) and a bootstrap analysis (100 samples) performed to generate a consensus phenogram based on the Jaccard coefficient using WinBoot (65). For all calculations, the data were analyzed as dominant markers, with the genetics assumed to be based on a haploid organism, as is typical with asexual forms of other fungi (e.g., 7,51,61).

Two methods were used to explore population genetic diversity: Shannon's information index (31) and Nei's unbiased measure of gene diversity (41) were calculated using GenAIEx V6.5 (46). Pairwise population comparisons were made using Nei's genetic distance, and the parameter ϕ_{pt} (which is analogous to Wright's F-statistics, F_{st} [63] when the data are haploid or binary [45] and is a measure of population differentiation) was used to determine significance in the differentiation between any two populations (using 999 permutations). If the observed ϕ_{pt} value differed significantly from zero, the null hypothesis of no genetic differentiation between populations was rejected.

Linkage disequilibrium was explored using the Index of Association (I_A) (8,35). This tests the importance of clonal versus sexual reproduction within each population and was calculated using Multilocus V1.3 (1,2). The I_A was calculated for each population and compared to the expected value (zero) assuming no linkage disequilibrium, but it is sensitive to the number of loci (1,2). Thus, a second linkage disequilibrium measure, r_d , was calculated which is independent of the number of loci. Significance of r_d was likewise tested by its deviation from the expected value (zero). There were 1,000 randomizations performed for each population.

The hierarchical sampling structure allowed an analysis of molecular variance (AMOVA) to be performed (Arlequin V3.5; 19) assuming haplotypic data with several groups of populations. The

AMOVA partitions the total variance into covariance components at the within population scale (trees), among populations within groups (among trees within orchards), and among groups (orchards) to quantify the diversity accounted for at each spatial scale. The analysis was based on 10,000 permutations, and the percentage of the total variation calculated at each level in the hierarchy (within tree, within orchard, within population, and among populations). To compare populations, a UPGMA analysis of population structure based on Nei's genetic distance (42) was calculated using Popgene V1.31 (66). In addition, digenic linkage disequilibrium was tested among pairs of loci using Arlequin V3.5. To reach a random starting point, 3,000 dememorization steps were performed, and 100,000 alternative permutations were run.

Evidence for population genetic structuring was further explored using Structure V2.3.4 (20,49). The 194 individuals were obtained from 11 orchard locations, and thus results for population structuring (K) from $K = 1$ to $K = 11$ were tested. Each estimate of K was based on 20 iterations, each iteration comprising a burn-in of 50,000 and a subsequent Markov Chain Monte Carlo chain of 100,000. An admixture model was used. The relationship between the probability ($\text{Pr}(X/K)$) of the data for a particular K was calculated (along with the standard deviation of the 20 estimates), and was graphed against K (1 to 11). Due to peaks in $\text{Pr}(X/K)$ often failing to reliably detect the true K , Evanno et al. (18) developed an ad hoc quantity (ΔK) based on the second order rate of change of $\text{Pr}(X/K)$ that shows a clear peak at true K . The estimated membership coefficient of each individual in each cluster (Q) was calculated, and represented graphically by showing each isolate as a vertical line partitioned into K colored segments that represent that individual's estimated membership fraction in each of the inferred clusters at $K = 11$.

Table 2. The universally primed polymerase chain reaction primers used in the study, number of markers, and percent polymorphic markers among 194 isolates of *Fusicladium effusum* collected from 11 populations in the southeastern United States

Name ^a	Nucleotide sequence (5'-3')	Reference	Markers per primer (% polymorphic)
AA2M2	CTGCGACCCAGAGCGG	34	6 (66.6)
AS4	TGTGGGCGCTCGACAC	34	13 (100.0)
AS15inv	CATTGCTGGCGAATCGG	15	10 (100.0)
L15	GAGGGTGGCGGTTCT	15	19 (100.0)
L15/AS19	GAGGGTGGCGGCTAG	34	16 (93.8)
Total			64 (92.1)

^a Primers were previously used to study genetic diversity in a related species, *Fusicladium (Spiloea) oleagina* (44).

Table 3. Number of haplotypes, percent polymorphic loci, Shannon's information index, Nei's unbiased measure of gene diversity, and measures of linkage disequilibrium among population of *Fusicladium effusum* from the southeastern United States^a

Population	Haplotypes (genotypic diversity, %)	Percent polymorphic loci (%)	Shannon's information index (SE)	Nei's unbiased measure of gene diversity (SE)	Significant pairwise linkage disequilibrium ($P = 0.05$), percent loci (%) ^b	Indices (I_A , r_d) of multilocus association ^c	
						I_A	r_d
AL(F)	18 (100.0)	34.4	0.146 (0.028)	0.092 (0.019)	21/210 (10.0)	0.119 (0.2)	0.006 (0.2)
AL(L)	17 (94.4)	45.3	0.187 (0.029)	0.117 (0.020)	55/406 (13.5)	0.524 (0.01)	0.019 (0.01)
GA(B)	18 (100.0)	42.2	0.176 (0.030)	0.111 (0.020)	55/351 (15.7)	1.341 (<0.001)	0.053 (<0.001)
KS	14 (100.0)	62.5	0.259 (0.029)	0.160 (0.020)	153/780 (19.6)	4.985 (<0.001)	0.131 (<0.001)
LA	18 (100.0)	29.7	0.141 (0.030)	0.091 (0.020)	19/171 (11.1)	0.156 (0.2)	0.009 (0.2)
MS	18 (100.0)	42.2	0.140 (0.025)	0.083 (0.016)	17/351 (4.8)	1.978 (<0.001)	0.079 (<0.001)
FL	18 (100.0)	36.0	0.154 (0.029)	0.097 (0.019)	17/253 (6.7)	1.420 (<0.001)	0.066 (<0.001)
OK	18 (100.0)	39.1	0.150 (0.027)	0.093 (0.018)	26/300 (8.7)	0.423 (0.04)	0.018 (0.04)
TX	18 (100.0)	40.6	0.167 (0.028)	0.103 (0.019)	30/325 (9.2)	0.262 (0.1)	0.011 (0.1)
GA(T)	18 (100.0)	51.6	0.208 (0.031)	0.131 (0.021)	36/528 (6.8)	0.624 (<0.001)	0.020 (0.002)
IL	18 (100.0)	50.0	0.204 (0.030)	0.128 (0.020)	70/496 (14.1)	0.624 (0.001)	0.020 (0.004)
All pops	185 (94.3)	43.0 (2.75)	0.176 (0.009)	0.110 (0.006)	436/1711 (25.5)	1.250 (<0.001)	0.026 (<0.001)

^a Population abbreviations: Fairhope, Alabama (AL(F)), Lowndesboro, Alabama (AL(L)), Byron, Georgia (GA(B)), Chetopa, Kansas (KA), Powhatan, Louisiana (LA), Raymond, Mississippi (MS), Monticello, Florida (FL), Burneyville, Oklahoma (OK), Bastrop, Texas (TX), Tifton, Georgia (GA(T)), Carlyle, Illinois (IL).

^b Based on Fischer's exact test extended to a contingency table of arbitrary size (Arlequin V3.5, 19). To reach a random starting point 3,000 dememorization steps were performed, and 100,000 alternative permutations were subsequently explored.

^c The indices of multilocus association (I_A , r_d) were calculated by measuring the distance between all pairs of loci, and comparing the actual value to the expected value (zero) assuming there was no linkage disequilibrium (Multilocus V2.1, 2). Number of randomizations to estimate I_A and r_d was 1,000.

A Mantel test was used to compare the relationship between Log genetic distance (Nei's genetic distance) and Log physical distance between population locations (km).

Results

There were a total of 64 scorable UP-PCR markers, and the majority scored (92.1%) were polymorphic among the 194 isolates (Table 2), confirming that they had utility in detecting genetic variability in this pathogen. Within each population, the vast majority of isolates had a unique haplotype (Table 3), and between 29.7 (LA) and 62.5% (KS) of the loci were polymorphic within each population, with a total of 43% polymorphic over all populations. The number of isolates (194) precluded presenting the consensus dendrogram, but the bootstrapped node values of the first two branches were 87% (2 and 192 isolates in separate clades) and 52% (1 and 191 isolates in separate clades), respectively. The third branch had a node value of only 29% (14 and 177 isolates in separate clades), and bootstrap values at branching below that level were 1 to 99%, depending on the node. Isolates did not form into clades related to source location.

Both Nei's unbiased measure of gene diversity and Shannon's information index (Table 3) suggested essentially similar and mod-

erate to high gene diversity in all 11 populations of *F. effusum* sampled throughout the region (Table 3) (Nei's measure of gene diversity = 0.083 [MS] to 0.160 [KS], Shannon's information index = 0.140 [MS] to 0.259 [KS]). Both measures were highly correlated ($r = 0.99$, $P < 0.0001$). The proportion of loci with significant pairwise linkage disequilibrium within each population was most often relatively low and ranged from 6.8% (GA(T)) to 19.6% (KS). The composite sample (all populations taken as a single unit) had 25.5% of loci with significant pairwise linkage disequilibrium. The observed indices of multilocus association (I_A and r_d) were significantly different from zero for 8 (AL(L), GA(B), KS, MS, FL, OK, GA(T), and IL) of the 11 populations, suggesting that in these populations a hypothesis of solely random mating was questionable, but in three of the populations there was evidence for random mating. Overall, the number of shared or clonal haplotypes was very low (Table 4), with only a single population (AL(L)) having two isolates with a common haplotype, and eight other haplotypes being shared between two to three populations; thus the majority of isolates were unique multilocus haplotypes, with an average region-wide genotype diversity of 0.94 (or 94% of the maximum possible). The number of markers was similar among populations (Fig. 2), and although a very few private markers were evident in some populations, these were very rare, although some populations had certain markers that were locally common (KS, GA(T), and IL).

The pairwise comparisons of populations (Table 5) based on Nei's genetic distance showed for the most part the distance between populations was relatively small (Nei's genetic distance = 0.0113 to 0.0473), although the ϕ_{PT} values indicated there were significant differences between many pairs of populations. The UPGMA analysis of the populations based on Nei's genetic distance showed the relative divergence, with certain populations (for example, KS) being more divergent than others (Fig. 3). The AMOVA showed the majority of the genetic variability (82.6%) occurred at the scale of the individual tree (Table 6), with only a relatively small amount being explained among populations in trees in an orchard (5.0%) or within groups (i.e., orchard populations) (12.5%), which suggests that there is very little population differentiation in *F. effusum* in the southeastern United States based on data from UP-PCR markers.

Table 4. Inter-population and intra-population shared haplotypes^a of *Fusicladium effusum* in the southeastern United States

Population with shared haplotype	Shared haplotypes
Alabama (Lowndesboro)	1
Alabama (Lowndesboro) × Florida (Monticello)	1
Louisiana (Powhatan) × Oklahoma (Burneyville) × Illinois (Carlyle)	1
Mississippi (Raymond) × Oklahoma (Burneyville)	1
Florida (Monticello) × Oklahoma (Burneyville)	2
Oklahoma (Burneyville) × Texas (Bastrop)	1
Texas (Bastrop) × Louisiana (Powhatan)	1
Texas (Bastrop) × Illinois (Carlyle)	1
Total shared haplotypes	9

^a Shared haplotypes occur either within or between two or more populations.

Fig. 2. Total number of markers, numbers of markers with frequency >5%, number of private markers, and numbers of locally common markers with frequency ≤25 and ≤50% in each population, respectively. Nei's unbiased measure of genetic diversity is also plotted for each population. Population abbreviations: Fairhope, Alabama (AL(F)), Lowndesboro, Alabama (AL(L)), Byron, Georgia (GA(B)), Chetopa, Kansas (KA), Powhatan, Louisiana (LA), Raymond, Mississippi (MS), Monticello, Florida (FL), Burneyville, Oklahoma (OK), Bastrop, Texas (TX), Tifton, Georgia (GA(T)), Carlyle, Illinois (IL).

Structure analysis supported this contention. The plot of $\text{Pr}(X/K)$ against $K = 1$ to 11 failed to show a clear mode for true K (lower graph, Fig. 4A). However, the plot of ΔK against $K = 1$ to 11 (upper chart, Fig. 4A) showed a peak at $K = 1$, which suggested that there was no meaningful sub-structuring in these data, and the isolates could be considered a single population. The Q plot (Fig. 4B) was based on $K = 11$ rather than $K = 1$. By presenting $K = 11$, the relatively uniform admixture of haplotypes in each population is demonstrated, providing compelling evidence for little population differentiation in *F. effusum*. The alpha plots (not shown) were stabilized within the burn-in period for the simulation. Thus, these data provide support for the AMOVA analysis that the majority of the genetic admixture resides within populations.

The Mantel test to explore any association between physical distance and genetic distance showed no evidence of a relationship ($r = 0.0694$, $P = 0.6$).

Discussion

These data suggest that *F. effusum* is a genetically diverse organism, confirming an earlier report demonstrating it was polymorphic (6). The dominant UP-PCR markers successfully differentiated the vast majority of isolates showing their utility in quantifying genetic diversity for *F. effusum*. Out of a total of 194 isolates, only 9 were clonal (4.6%).

The gene diversity observed in this study (Nei's measure of gene diversity = 0.083 to 0.160) using UP-PCR is comparable with that observed among other related fungal pathogens (for example, *Pyrenophora*, *Gremmeniella*, *Venturia*, *Mycosphaerella* [Stagonospora], and *Rhynchosporium* spp.) using either isozymes (23) or other dominant marker systems (47,61), including from a study based on RAPDs with the closely related *V. inaequalis* (58,59). The ability of marker systems to give different estimates of gene diversity has been commented on before, and codominant marker systems such as RFLPs and SSRs may be more sensitive and provide more realistic and greater measures of gene diversity (38). Nonetheless, our results confirm that considerable genetic variability exists within populations of *F. effusum*.

Among the multilocus haplotypes, the associations among the different alleles at different loci can provide evidence for clonality. The linkage disequilibrium detected by UP-PCR within the 11 populations in this study was low, ranging from 6.8% (GA(T)) to 19.6% (KS), which is comparable to levels of linkage disequilibrium found in populations of related fungal species that experience regular sexual recombination (11,32,47,50). The index of association (I_A and the equivalent statistic r_d) was devised to help distinguish the null hypothesis of recombination from clonality (35), and in three of the 11 populations there was no evidence of clonality (observed I_A not significantly different from zero), but in the remaining eight populations, the observed value of I_A was significantly different from zero; which is not unexpected as *F. effusum* is

known to reproduce very effectively through its asexual phase, allowing departure from panmixis. Previous studies have found a proportion of populations of known sexually reproducing fungi that have an I_A significantly different from zero using both dominant and codominant marker systems (11,17,32,50). It is worth noting that factors other than clonal reproduction, including decay of historic disequilibrium, population admixture, and genetic drift, might result in I_A values different from zero (39).

There was some genetic differentiation between certain pairs of populations of *F. effusum*, but not others, and the UPGMA analysis of all 194 isolates failed to identify the source populations, suggesting that they were genetically relatively homogeneous and could be considered a fairly uniform metapopulation. The analysis of molecular variance showed that the majority of genetic diversity (82.6%) was present at the scale of the individual tree. The remainder was explained among orchards and among trees within orchards. Thus, the majority of diversity occurred at the smallest spatial scale sampled. Analysis from Structure also suggested that

Fig. 3. Unweighted paired group method of arithmetic averages (UPGMA) separation of populations of *Fusarium effusum* based on universally primed polymerase chain reaction (UP-PCR) using the multiple populations option (Popgene V1.31, 67) and based on Nei's measure of genetic distance (42). Population abbreviations: Fairhope, Alabama (AL(F)), Lowndesboro, Alabama (AL(L)), Byron, Georgia (GA(B)), Chetopa, Kansas (KA), Powhatan, Louisiana (LA), Raymond, Mississippi (MS), Monticello, Florida (FL), Burneyville, Oklahoma (OK), Bastrop, Texas (TX), Tifton, Georgia (GA(T)), Carlyle, Illinois (IL). Scale shows Euclidean distance.

Table 5. Nei's unbiased measures of genetic distance^a (42) (below diagonal) and ϕ_{PT} values^b (above the diagonal)^c

Population	AL(F)	AL(L)	GA(B)	KS	LA	MS	FL	OK	TX	GA(T)	IL
AL(F)		0.125*	0.193*	0.221*	0.234*	0.169*	0.219*	0.191*	0.140*	0.152*	0.150*
AL(L)	0.0213		0.098*	0.122*	0.187*	0.145*	0.159*	0.128*	0.093*	0.123*	<u>0.078</u>
GA(B)	0.0327	0.0189		0.097*	<u>0.062</u>	0.224*	<u>0.050</u>	0.124*	0.112*	<u>0.061</u>	<u>0.085</u>
KS	0.0473	0.0288	0.0227		0.164*	0.234*	0.112*	0.140*	0.159*	0.108*	<u>0.068</u>
LA	0.0365	0.0324	0.0113	0.0335		0.232*	<u>0.076</u>	0.126*	0.153*	0.070*	0.120*
MS	0.0239	0.0233	0.0366	0.0480	0.0340		0.271*	0.164*	0.131*	0.144*	0.144*
FL	0.0350	0.0279	0.0100	0.0236	0.0124	0.0429		0.134*	0.137*	<u>0.006</u>	0.101*
OK	0.0290	0.0219	0.0206	0.0285	0.0187	0.0232	0.0206		0.083*	0.101*	0.074*
TX	0.0223	0.0173	0.0198	0.0349	0.0242	0.0197	0.0224	0.0138		<u>0.074</u>	0.085*
GA(T)	0.0279	0.0257	0.0137	0.0278	0.0136	0.0249	0.0045	0.0188	0.0152		<u>0.065</u>
IL	0.0271	0.0168	0.0175	0.0187	0.0215	0.0246	0.0190	0.0141	0.0168	0.0156	

^a For Nei's measure of genetic distance, bold indicates all pairwise comparisons <0.0180 .

^b For ϕ_{PT} , italics and asterisks indicates ϕ_{PT} ($P \leq 0.001$) showing significant genetic differentiation between pairs of populations, and underscores represent nonsignificant comparisons (based on 999 permutations).

^c Population abbreviations: Fairhope, Alabama (AL(F)), Lowndesboro, Alabama (AL(L)), Byron, Georgia (GA(B)), Chetopa, Kansas (KA), Powhatan, Louisiana (LA), Raymond, Mississippi (MS), Monticello, Florida (FL), Burneyville, Oklahoma (OK), Bastrop, Texas (TX), Tifton, Georgia (GA(T)), Carlyle, Illinois (IL).

genetic diversity was for the most part uniformly distributed throughout the region, with a single cluster describing the distribution of the genetic admixture. Several other studies on related fungal pathogens (*Venturia*, *Mycosphaerella* [*Stagonospora*], *Alternaria*, *Leptosphaeria*, and *Rhynchosporium* spp.) have similarly found that a greater proportion of the genetic diversity can be found at a small spatial scale, including on a single plant or in a plot compared to larger spatial scales (26,30,32,38,64). These pathogens either have, or are believed to have, a sexual stage that provides the basis for regular recombination, and combined with dispersal through the sexual and/or asexual stage this allows gene flow over distance (32,50). It should be noted that the asexual stages may play an important role in the epidemic in some cases (64), resulting in locally genetically similar populations. No sexual stage has been identified for *F. effusum*, so there is no known basis for it to undergo sexual recombination. However, lack of an identi-

fied sexual stage is not evidence for its non-existence, and compelling evidence for sexual stages can be found through studying the frequency of mating type genes (28,32,33,50). If the mating type loci are in equilibrium, there is likely to be an undiscovered sexual stage (28,33). In the absence of a sexual stage of *F. effusum*, it would be valuable to locate and study the mating type loci. In the present study, there was no detectable effect of physical distance on genetic relatedness between isolates. Effect of distance has been previously reported with related pathogens (38), although it was often measured over considerably larger geographic distances. Also, a slight effect of physical distance was observed for *F. effusum* in a much smaller study (6). Thus, it is possible that UP-PCR markers were insufficiently sensitive to discern a distance effect, or perhaps the area over which the sample was taken is too small to readily discern distance effects with a pathogen metapopulation so genetically homogeneous.

Table 6. Analysis of molecular variance^a of 11 populations of *Fusicladium effusum* in the southeastern United States

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among groups (orchards)	10	134.5	0.53	12.5
Among populations within groups (among trees within orchards)	54	222.7	0.21	5.0
Within populations (trees)	129	451.2	3.50	82.6
Total	193	808.36	4.24	

^a Analysis of molecular variance for the populations of *F. effusum* was based on 10,000 permutations using Arlequin V3.5 (19). Data were analyzed as haplotypic data, with several groups of populations.

Fig. 4. Results of the Structure analysis of the universally primed polymerase chain reaction (UP-PCR) marker data for $K = 1$ to 11 populations of *Fusicladium effusum* sampled throughout the southeastern United States. Average log likelihood $\text{LnPr}(X/K)$ ($\pm$ SD) and ΔK indicating true K (number of population groups based on genetic partitioning) (**A**), and Q plot of $K = 11$ demonstrating the high degree of admixture among populations precluding population differentiation (**B**).

Other explanations might be made for the distribution of genetic diversity. At least one study found a similar hierarchy of genetic diversity, but attribution of causation was postulated to be due to recent multiple introductions as responsible in the absence of a sexual stage (44). In that case, it was a related pathogen (*F. oleaginea*), infecting olive; the pathogen is an exotic to New Zealand, and no sexual stage is reported for the fungus in New Zealand or elsewhere (53). In contrast, *F. effusum* is native to the southeastern United States and was first described on *Carya tomentosa* (= *C. alba*) in 1885 (62), making it unlikely that multiple introductions of founder populations would result in this pattern of diversity. There are other possible explanations, including parasexuality through the Pontecorvo cycle (48), but this has been confirmed for very few fungal pathogens as a vehicle driving the pattern of genetic diversity (14,43,67). Sexual stages of fungal pathogens can be cryptic and restricted by exacting environmental requirements both temporally and spatially, and if a sexual stage should exist for *F. effusum*, this will most likely be the case.

In conclusion, *F. effusum* appears to be a genetically variable pathogen, and that diversity appears to be well distributed throughout the pecan-growing region of the southeastern United States. This suggests that the methods of recombination and/or dispersal of propagules allow a genetically reasonably uniform metapopulation to be maintained. With most genetic diversity at the scale of individual trees, pecan breeders will need to consider strategies that ensure durable resistance (36). However, if a sexual stage exists, it too may become a target of management strategies to reduce its efficiency in maintaining diversity over the whole region, which will contribute to durability of host resistance (12,13), and in addition, minimize the risk of the development and spread of fungicide resistance, which is also an issue in *F. effusum* (55). Considering the destructive nature of this disease on pecan, and the need for a fuller understanding of the pathogen diversity, population structure, and gene flow, codominant markers should be developed to further explore the population genetic characteristics observed in the current study using UP-PCR.

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