Linkage Disequilibrium and Spatial Aggregation of Genotypes in Sexually Reproducing Populations of *Erysiphe necator*

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ABSTRACT

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Random mating and recombination in heterothallic ascomycetes should result in high genotypic diversity, 1:1 mating-type ratios, and random associations of alleles, or linkage equilibrium, at different loci. To test for random mating in populations of the grape powdery mildew fungus *Erysiphe necator*, we sampled isolates from vineyards of *Vitis vinifera* in Burdett, NY (NY09) and Winchester, VA (VA09) at the end of the epidemic in fall 2009. We also sampled isolates from the same Winchester, VA vineyard in spring 2010 at the onset of the next epidemic. Isolates were genotyped for mating type and 11 microsatellite markers. In the spring sample, which originated from ascospore infections, nearly every isolate had a unique genotype. In contrast, fall populations were less diverse. In all, 9 of 45 total genotypes in VA09 were represented by two

or more isolates; 3 of 40 total genotypes in NY09 were represented by two or more isolates, with 1 genotype represented by 20 isolates. After clone correction, mating-type ratios in the three populations did not deviate from 1:1. However, even with clone correction, we detected significant linkage disequilibrium (LD) in all populations. Mantel tests detected positive correlations between genetic and physical distances within vineyards. Spatial autocorrelation showed aggregations up to 42 and 3 m in VA09 and NY09, respectively. Spatial autocorrelation most likely results from short dispersal distances. Overall, these results suggest that spatial genetic aggregation and clonal genotypes that arise during the asexual phase of the epidemic contribute to persistent LD even though populations undergo sexual reproduction annually.

Additional keywords: genetic neighborhood, mating systems, population genetics, selection.

Recombination resulting from sexual reproduction leads to new genotypes that permit organisms to better adapt to changing conditions and purges genomes of the accumulation of deleterious mutations (43). In numerous species of fungi, oomycetes, and plants, and some animals, sexual reproduction results in dormant propagules that permit these organisms to survive adverse conditions, such as drought or extreme temperatures. Many hypotheses have been presented on the advantages of sexual reproduction over asexual reproduction in the evolution of reproductive modes (30). However, there are also costs to sex, such as breaking up favorable allele combinations in stable environments (5), as well as the costs of finding a suitable mate and engaging in mating (2). An enormous diversity of mating systems and reproductive modes are found among fungi (18), yet it is not evident why such diverse reproductive modes are maintained. A mixed mode of reproduction involving regular sexual reproduction combined with multiple cycles of asexual reproduction is common among plant-pathogenic fungi, bringing with it the advantages conferred by sexual reproduction while simultaneously allowing beneficial combinations to persist and increase through asexual reproduction. Populations with mixed reproductive modes may produce diverse genotypes from sexual reproduction but the fittest genotypes can increase in frequency rapidly because of clonal reproduction. This type of evolution, in which the fittest pathogen

clones emerge rapidly in a selective sweep, has been termed an “epidemic” model of population structure (34). Among plant pathogens, mixed modes of reproduction have been hypothesized to lead to rapid evolution to overcome host resistance or develop resistance to fungicides (35).

In a randomly mating, or panmictic, population, all individuals are potential mates. In randomly mating fungal populations, we expect to find all mating types in equal frequencies. Additionally, recombination during meiosis should result in high genotypic diversity and random associations of alleles at different loci, which is referred to as linkage (or gametic) equilibrium (38). Non-random mating occurs due to inbreeding, where selfing is the most extreme form. Inbreeding can occur if genetically differentiated subpopulations are sampled and analyzed as if coming from a single undifferentiated population; this is known as the Wahlund effect (62). A more subtle form of population subdivision, which also results in nonrandom mating, is caused by short-distance dispersal and mating between individuals in small genetic neighborhoods. Short-distance dispersal prevents potential mates from mixing freely over long distances and results in a population structure known as isolation-by-distance, in which nonrandom mating and LD are evident at spatial scales larger than neighborhoods (66).

Although isolation by distance has been studied at relatively large spatial scales (kilometers and larger) for some plant pathogens (39,52,54), spatial genetic structure has been studied at smaller scales (tens of meters) for relatively few species. Spatial autocorrelation of multilocus genotypes has been conducted for several fungal pathogens of trees in forests (17,25,40,61). Spatial analyses of genotypic data have also been conducted for fungal pathogens of agronomic crops within fields ranging from a spatial

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*The e-Xtra logo stands for “electronic extra” and indicates that the online version contains one supplementary table.

scale of hundreds of meters to 1-m rows (6,14,29). The goals in these studies were to better understand dispersal, colonization, dormancy, or reproductive modes of the pathogens.

The grape powdery mildew fungus *Erysiphe necator* has a mixed mode of reproduction, with numerous cycles of asexual reproduction throughout the epidemic and regular sexual reproduction annually at the end of the epidemic (22). It is a heterothallic fungus (24,36) and, thus, requires two individuals, each of a different mating type (*MAT1-1* or *MAT1-2*), for sexual reproduction. The products of sexual reproduction are ascospores, which are produced in cleistothecia. Ascospores are an important source of primary inoculum in many regions (13,49) and the only source of primary inoculum in regions with cold winters (49), as in this study. In milder climates, *E. necator* can also overwinter as dormant mycelium inside of buds which, in the spring, give rise to shoots covered with mycelium and conidia, known as flag shoots (50). In Europe and Australia, where *E. necator* has been introduced, populations are structured into two distinct genetic groups that appear to be reproductively isolated (16,42,45,51,60) and, therefore, are not randomly mating. Recent studies of the population genetics of *E. necator* showed that genetic diversity is much greater in the eastern United States than in introduced populations in Europe, Australia, or the west coast of the United States, and that the eastern United States is the likely source for the introductions to these new areas (9,21).

Gene flow of *E. necator* typically occurs by the dispersal of ascospores and conidia, although the fungus can also be dispersed on infected plant material for long-distance introductions. Conidia appear to have a steep dispersal gradient, dispersing mostly on the order of several meters (14). However, less is known about the distance that ascospores of *E. necator* disperse. It is generally assumed that both spore types are dispersed by extreme wind (26) or rain (23); however, whether or not gene flow is limited or long-distance has not been thoroughly investigated. Recent studies

have shown large-scale geographic structure within eastern North America (9,21), which suggests that gene flow is restricted to some extent. Both of these studies also showed evidence of recombination, and Frenkel et al. (21) found a lack of LD across broad geographic scales, albeit with relatively small samples.

We investigated larger samples from local populations of *E. necator* within single vineyards in the eastern United States to test whether random mating was occurring on a local scale, within vineyards. By focusing on this smaller scale (on the order of tens of meters), the genetic differentiation detected over large geographic scales (hundreds of kilometers or more) (9,21) would not be a factor contributing to LD. The objectives of this study were to determine (i) if local populations of *E. necator* are randomly mating as we would expect, given they have an annual sexual cycle with no asexual overwintering in the locations of study; (ii) if genotypes of *E. necator* are spatially aggregated within vineyards; and (iii) if there is temporal or geographic divergence between populations.

MATERIALS AND METHODS

Sampling. We collected isolates of *E. necator* from two vineyards of *Vitis vinifera* ‘Chardonnay’ in fall 2009, at the end of the epidemic: a commercial vineyard in the Finger Lakes Region in Burdett, NY on 21 September 2009 (NY09) and a research vineyard at the Virginia Polytechnic Institute and State University, Alson H. Smith Jr. Agricultural Experiment Station in Winchester, VA on 12 October 2009 (VA09). For NY09, we sampled 100 leaves with powdery mildew colonies from 10 rows at 3-m intervals (10 isolates/row) (Fig. 1). The vineyard in Virginia differed in shape and size; therefore, the same spatial sampling scheme could not be conducted at both vineyards. For VA09, we sampled 100 leaves with powdery mildew colonies from three rows at 3-m intervals (33 or 34 isolates/row). This research vineyard contained

A VA09

B NY09

Fig. 1. Maps representing the sampling locations of multilocus genotypes of *Erysiphe necator* collected in fall 2009 from vineyards of *Vitis vinifera* ‘Chardonnay’ in **A**, Winchester, VA (VA09) and **B**, Burdett, NY (NY09). Sampling schemes differed between locations due to differences in vineyard size and layout. In all, 100 isolates were collected from each vineyard; however, not all samples were genotyped due to losses during isolation and culturing. In both vineyards, rows were 3 m apart and are represented by horizontal lines. Samples were taken at 3-m intervals along each row. Each symbol along a row represents the location of an isolate that was genotyped. Small open diamonds represent unique multilocus genotypes. Large symbols represent the location of isolates with repeated multilocus genotypes that were inferred as being clonal with MLGsim v.2.0 (59). Clones are represented by symbols of the same shape and shade. There were three clonal haplotypes in New York and six clonal haplotypes in Virginia.

several plots of vines treated with different fungicides; however, we sampled from vines within each plot that were not sprayed. Rows were approximately 3 m apart in both vineyards. We recorded the locations of leaf samples within each vineyard for NY09 and VA09.

To understand the diversity of genotypes derived from sexual reproduction prior to multiple cycles of asexual reproduction, we collected isolates at the onset of the epidemic on 12 June 2010 from the Winchester, VA vineyard (VA10). Because flag shoots have not been observed in Virginia (M. Nita, *personal communication*), we assumed that colonies sampled at this time were derived from ascospore infections. Sampling for VA10 was done the same as described for VA09, except that the location of each sample was not recorded. Despite intensive efforts, we could not sample isolates from the New York vineyard (or other vineyards nearby) in spring 2010 because disease incidence was too low early in the season due to unfavorable environmental conditions and fungicide applications. By the time powdery mildew colonies were detectable in July, it was too late in the season for them to be representative of the overwintering sexual population because of asexual reproduction and selection for fungicide-resistant genotypes (65).

Isolation of *E. necator* and DNA extraction. *E. necator* was isolated from powdery mildew colonies collected in the field onto surface-sterilized leaves of *V. vinifera* 'Cabernet Sauvignon', as described previously (9,19). Young leaves from vines grown in a greenhouse were surface sterilized in 0.6% sodium hypochlorite for 1.5 min, rinsed twice with sterile deionized water, and air dried in a sterile laminar flow hood. Leaves were kept in petri dishes containing 20 ml of 2% water agar. Isolates were obtained by touching a single, isolated mildew colony on a leaf collected in the field to a surface-sterilized leaf. Single conidial chains were transferred to another surface-sterilized leaf after 2 to 3 weeks with a sterile pipet tip. Conidia from colonies arising from single-conidial chains were mass transferred to another surface-sterilized leaf for DNA extraction 2 to 4 weeks later.

Genomic DNA was prepared as described previously (9). Briefly, conidia and hyphae were collected from colonies by touching a 1-cm² piece of office tape (Scotch Tape; 3M) to the colony multiple times until the tape was covered in fungal tissue. The tape was placed in a 1.5-ml microcentrifuge tube with 100 µl of 5% chelex (28,63), vortexed for 30 s, and incubated at 95°C for 20 min. The solution was vortexed again for 5 s and centrifuged briefly, and the supernatant was removed and used as the DNA template for polymerase chain reaction (PCR).

Identification of mating-type. The mating type of each isolate was determined using a PCR-based marker (8). The amplified fragments of the PCR were predicted to be 408 bp for *MAT1-1* and 232 bp for *MAT1-2*. The PCR reaction was conducted in a total volume of 10 µl. Reaction components included 1 µl of 10× PCR buffer (Takara); 1 µl of dNTPs (2.5 mM each); 0.5 µl each of primers EnαF2, EnαR3, EnHMGF1, and EnHMGR1 (10 µM); 0.3 U of ExTaq (Takara); and 1 µl (20 to 300 ng) of DNA template. Cycling conditions included an initial denaturation at 95°C for 2 min followed by 35 cycles with a denaturation step at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; followed by a final extension at 72°C for 5 min. Ten microliters of each PCR product with loading dye was analyzed by electrophoresis through a 1% (wt/vol) agarose/Tris-borate-EDTA gel.

Multilocus genotyping. The genotype of each isolate was determined by using 11 expressed sequence tag (EST) simple-sequence repeats (SSRs) (21). The microsatellite markers used included *EnMS1–EnMS7* and *EnMS9–EnMS11*, and a new marker described here, *EnMS12*. *EnMS12* was identified and developed in the same manner as the other EST-SSRs described by Frenkel et al. (21). The contig that was identified in *E. necator* isolate G14 contained the microsatellite repeat motif (CTT)₈. The PCR

primers for *EnMS12* are *EnMS12F*: 5'-CACGACGTTGTAAA ACGACCGCTCGTGCATCTTTATTGA-3', which includes M13-specific sequence (underlined) at the 5' end for multiplexing with other markers using fluorescent dyes as described previously (55), and *EnMS12R*: 5'-CGTGAAGCCCAAGATAAGC-3'. PCR and fragment analysis were carried out as described previously (21). Briefly, PCR for all primer pairs was carried out in a total volume of 12.5 µl. Reactions included 1.25 µl of 10× PCR buffer (Takara), 1.25 µl of 2.5 mM dNTPs, 0.2 µl of 10 µM forward primer, 0.4 µl of 10 µM reverse primer, 0.5 µl of 10 µM 5'-dye-labeled M13 primer (FAM, VIC, or NED; Applied Biosystems), 0.375 U of ExTaq (Takara), and 1 µl of DNA template (20 to 300 ng). Cycling conditions were the same as described above for mating type. For fragment analysis, PCR products from two or three reactions, each with a different fluorescent dye (FAM, VIC, or NED), were pooled and 1 µl of the pooled reactions was added to 8.5 µl of HIDI formamide (Applied Biosystems), 0.3 µl of double-distilled H₂O, and 0.2 µl of GeneScan 500 LIZ size standard (Applied Biosystems) and heated for 5 min at 95°C. Fragment analyses were conducted at the Cornell University Life Sciences Core Laboratories Center using an Applied Biosystems 3730xl DNA Analyzer. Allele sizes were analyzed using the GeneMapper Software (v3.0; Applied Biosystems). The range of allele sizes for *EnMS12*, including the M13-specific sequence, was 188 to 200 bp. Four alleles with sizes of 188, 191, 197, and 200 bp were observed among the three populations. Allele sizes of the other EST-SSRs were described previously (21).

Analyses for random mating. Data were clone corrected for some analyses to eliminate the effects of asexual reproduction on tests for random mating. The same multilocus genotypes can arise more than once by recombination, depending on the number of markers used and the diversity of alleles at each locus. Therefore, to determine which repeated multilocus genotypes could be inferred as clonal and should be clone corrected, we estimated the probability that each multilocus genotype that was sampled more than once could have arisen by sex (p_{sex}). These estimates are based on two probabilities (4,46). First, the probability that each multilocus genotype can arise by chance in a randomly mating population, p_{gen} , is estimated as the product of the observed allele frequencies within each vineyard at each locus genotyped because *E. necator* is haploid. Second, the probability of encountering a specific multilocus genotype x times in a sample of size N is a binomial probability,

$$\Pr\{X = x\} = \binom{N}{x} p_{\text{gen}}^x (1 - p_{\text{gen}})^{N-x}$$

where p_{gen} is the probability of the multilocus genotype occurring independently. Probability p_{sex} was estimated using the program MLGsim v.2.0 (59). The P value for testing the significance of p_{sex} for each repeated multilocus genotype was estimated by simulating 1,000 randomly mating populations of size N using observed allele frequencies to generate a null distribution of p_{sex} against which the actual value of p_{sex} could be compared.

Mating-type distributions of uncorrected and clone-corrected samples were tested for deviation from expected ratios of 1:1 using chi-square goodness-of-fit tests. Genotypic diversity ($\hat{G}$), the probability that two individuals taken at random have unique multilocus genotypes, was estimated for each population. $\hat{G}$ is estimated as $[N/(N-1)](1 - \sum p_i^2)$, where p_i is the frequency of the i th multilocus genotype and N is the sample size for each population. Multilocus LD was determined for each uncorrected and clone-corrected sample using the index of association (I_A) (10,34) and $\bar{r}_d^2$, estimated with MultiLocus v.1.3b (1): $I_A = s_k^2 / \sigma_k^2 - 1$, where k is the number of loci at which pairs of individuals have different alleles, s_k^2 is the observed variance of k , and σ_k^2 is the expected variance of k under linkage equilibrium. I_A is not significantly different from zero when the population is in linkage equilibrium. P values were estimated by 1,000 random permu-

tations of the data. The magnitude of I_A can increase with the number of loci studied; therefore, we used $\bar{r}_d$, which is a standardized measure of I_A for comparing LD among populations that vary by the number of polymorphic loci analyzed. Significance testing for LD between pairs of polymorphic loci was done with GENEPOP v.4.0 (53). Bonferroni correction was applied to account for multiple comparisons. Significance of linkage disequilibrium for each pair was evaluated at the overall type I error rate, $\beta = \alpha/n$, where α is the chosen level of significance for a single comparison and n is the total number of comparisons.

Spatial genetic analyses. Mantel tests for matrix correspondence (33,56) were conducted in GenAlEx v.6.41 (47) to determine if a significant relationship existed between genetic and physical distances of isolates in vineyard populations of *E. necator* (samples NY09 and VA09). Genetic distances between isolates were calculated as the sum of differences between genotypes where the same allele at a locus yields a value of 0 and a different allele yields a value of 1. A Mantel test provides a correlation coefficient (R_{xy}) for the two data matrices of pairwise genetic and physical distances between isolates. P values were determined by comparing the frequency distribution of 1,000 random permutations with the original data.

To determine whether clusters of similar genotypes of *E. necator* existed within vineyards, spatial autocorrelation based on the methods of Smouse and Peakall (57) was performed in GenAlEx v.6.41 on the NY09 and VA09 samples. The autocorrelation coefficient, r , is a measure of the genetic similarity of pairs of isolates within specified distance classes. If the observed r is greater or less than the 95% confidence interval about the null hypothesis of no spatial genetic structure, determined by the distribution of 1,000 random permutations of the data, it indicates local spatial genetic structure within the distance class. To account for spatial organization without the influence of repeated multilocus genotypes, the central spatial coordinate of each repeated multilocus genotype was used for autocorrelation analyses in the program GenClone v.1 (3). Autocorrelation with and without repeated multilocus genotypes was measured as the kinship coefficient, F_{ij} , proposed by Loiselle (32), which is similar to r and is a measure of the genetic similarity of pairs of isolates within specified distance classes. P values were estimated by randomly permuting genotypes to sampling locations.

Population structure analyses. Nei's genetic identity (I) (44) and genetic differentiation (Φ_{ST} , an analog of F_{ST} measured via analysis of molecular variance) (48) were determined among NY09, VA09, and VA10 samples using GenAlEx v.6.41 on full data sets. Significant differentiation between populations was determined by comparing observed values to the distribution of 1,000 randomizations of the data. Principal coordinates analysis was conducted with GenAlEx v.6.41 on pairwise genetic distances between all genotypes in order to visualize major patterns of variation within and among populations.

RESULTS

Genotyping and clonal composition of vineyard populations.

Of 11 EST-SSR markers, 10 were used for analyses in each

of the populations. Allele frequencies and unbiased gene diversity (h) for loci in each of the three populations are listed in the Supplementary Table. The marker *EnMSI* was excluded from analyses of the NY09 population because we found two different size ranges among 10 alleles; therefore, it was considered hyper-variable and unreliable for interpreting that all fragments came from a single locus and that alleles of the same size were identical by descent (21). However, only four alleles within a small size range were detected at *EnMSI* for the VA09 and VA10 populations; therefore, it was not excluded from these analyses. The marker *EnMS4*, however, was excluded from the VA09 and VA10 analyses due to inconsistent PCR amplification.

We obtained 62, 78, and 69 isolates for NY09, VA09, and VA10, respectively (Table 1; Fig. 1), rather than the 100 originally sampled because colonies were not viable or isolates were lost prior to DNA extraction. Genotypic diversity was 0.897 to 0.996. A large proportion of the isolates sampled from the vineyards in the fall (NY09 and VA09) were clones (Tables 1 and 2). All individuals in NY09 and VA10 with identical multilocus microsatellite genotypes had the same mating type; however, individuals with two of the multilocus microsatellite genotypes identified more than once in VA09 were represented by both mating types and were considered different genotypes overall. A single clone, comprising 20 of the 62 isolates sampled in NY09, dominated the population. We found nine genotypes with two or more isolates in VA09. The four most common clones were repre-

TABLE 2. Probabilities of multilocus genotypes (MLGs) of *Erysiphe necator* (p_{gen}) and their reoccurrence resulting from distinct sexual events (p_{sex}) within vineyards in Burdett, NY sampled in fall 2009 (NY09) and Winchester, VA sampled in fall 2009 (VA09) and spring 2010 (VA10)

Population, MLG ^a	Number of isolates ^b	p_{gen}^c	p_{sex}^d	P value for p_{sex}
NY09				
1	20	5.1×10^{-3}	3.1×10^{-15}	<0.001
2	3	1.2×10^{-6}	7.0×10^{-14}	<0.001
3	2	1.3×10^{-5}	3.0×10^{-7}	0.006
VA09				
1	12	5.6×10^{-4}	4.0×10^{-15}	<0.001
2	9	2.6×10^{-3}	7.8×10^{-13}	<0.001
3	6	2.4×10^{-5}	2.2×10^{-16}	<0.001
4	5	1.6×10^{-3}	2.1×10^{-7}	0.004
5	2	7.2×10^{-6}	1.6×10^{-7}	0.004
6	2	3.7×10^{-5}	4.0×10^{-6}	0.014
7	2	3.5×10^{-4}	3.5×10^{-4}	0.210
8	2	3.2×10^{-4}	3.1×10^{-4}	0.118
9	2	2.9×10^{-4}	2.5×10^{-4}	0.168
VA10				
1	2	2.8×10^{-6}	1.8×10^{-8}	<0.001
2	2	9.8×10^{-5}	2.3×10^{-5}	0.203

^a Population and repeated MLGs. Each unique genotype was determined with 10 microsatellite markers and mating type.

^b Number of isolates in each repeated genotype.

^c Probability of the MLG occurring in the population and is calculated as the product of the allele frequencies.

^d Probability of obtaining the observed number of identical genotypes in the population as a result of random mating (4,46). p_{sex} and its significance were estimated using the program MLGsim v.2.0 (59).

TABLE 1. Clonal composition of populations of *Erysiphe necator* in vineyards in Burdett, NY sampled in fall 2009 (NY09) and Winchester, VA sampled in fall 2009 (VA09) and spring 2010 (VA10)

Population	N	g^a	$\hat{G}^b$	Repeated fraction ^c	g_2^d	Number of isolates in each repeated genotype
NY09	62	40	0.897	0.36	3	20, 3, 2
VA09	78	45	0.923	0.42	9	12, 9, 6, 5, 2, 2, 2, 2, 2
VA10	69	67	0.996	0.03	2	2, 2

^a Number of multilocus genotypes.

^b Genotypic diversity ($\hat{G}$) = $[N/(N-1)](1 - \sum p_i^2)$, where p_i is the frequency of the i th genotype and N is the sample size.

^c Repeated fraction = $1 - (g/N)$.

^d Number of genotypes represented by two or more isolates.

sented by 12, 9, 6, and 5 isolates among a total of 78 isolates; five additional repeated genotypes in VA09 each had 2 isolates. Based on estimates and significance of p_{sex} , we could not reject the null hypothesis that three of these genotypes with two isolates arose independently by recombination (Table 2). In the spring sample (VA10), we found 67 multilocus genotypes among 69 isolates, with 2 genotypes each with 2 isolates. One of the repeated genotypes likely arose from sexual reproduction (Table 2), whereas the other is likely to have resulted from clonal reproduction.

Analyses of random mating. Mating-type ratios did not differ significantly from 1:1 ($P > 0.05$), except for the NY09 uncorrected sample (Table 3). A clone that occurred at high frequency in this population was *MAT1-2*, which skewed the mating-type ratio. After clone correction, the NY09 population did not deviate from 1:1, although sample size and statistical power to detect deviations were reduced accordingly.

Multilocus LD (I_A and $\bar{r}_d$) was significantly greater than zero within each population ($P < 0.001$). Estimates of I_A and $\bar{r}_d$ were higher in fall populations with a high frequency of clones (NY09 and VA09) than in the spring population (VA10). Multilocus LD was reduced by clone correction in these populations; however, values remained significant ($P < 0.001$). Of the 45 pairs of loci in each population, there was significant ($P \leq 0.05$) pairwise LD between 8 (18%), 35 (78%), and 37 (82%) pairs of loci in the clone-corrected samples of the NY09, VA09, and VA10 populations, respectively. With Bonferroni correction ($\beta = \alpha/n = 0.001$, for $\alpha = 0.05$, $n = 45$), there was no significant pairwise disequilibrium detected in the NY09 population, but 9 (20%) and 17 (38%) pairs of loci showed significant ($P < 0.001$) LD in the clone-corrected VA09 and VA10 samples respectively.

Spatial autocorrelation of genotypes. Spatial autocorrelation analyses indicated that spatial genetic structure exists within vineyard populations of *E. necator*. Mantel tests showed significant correlations between genetic distance and geographic distance in NY09 ($R_{xy} = 0.082$; $P = 0.027$) and VA09 ($R_{xy} = 0.282$; $P = 0.001$). Isolates were more genetically similar to each other than expected at random in the smaller distance classes (Fig. 2). With the entire VA09 dataset, isolates in distance classes of ≤ 24 or ≤ 39 m based on r or F_{ij} , respectively, were more similar to each other than expected at random ($P \leq 0.05$). Conversely, isolates in distance classes of 40 to 87 m or distance classes 57 m based on r or F_{ij} , respectively, were less similar to each other than expected at random (Fig. 2A). With the entire NY09 dataset, only isolates in the smallest distance class (3 m) were more similar than expected at random in both analyses (Fig. 2B). We still detected significant spatial autocorrelation of genetic similarities in distance classes up to 42 m in VA09 when clones were accounted for by using the central coordinate of repeated multilocus genotypes (Fig. 2A). However, no spatial autocorrelation was detected in NY09 when replicate genotypes were removed.

The slope of the regression of the correlation between genetic distance and geographic distance for VA09 was -0.019 ($P < 0.001$) for both the entire data set and the analysis without the repeated multilocus genotypes. The slopes for NY09 were -0.003 ($P = 0.11$) and -0.004 ($P = 0.14$) for the entire data set and without repeated multilocus genotypes, respectively.

Population structure. The loci *EnMS1* and *EnMS4* were removed from analyses comparing the NY09 and VA09 or VA10 populations because they were only genotyped in one or the other

Fig. 2. Spatial autocorrelation analyses of vineyard populations of *Erysiphe necator* shown as correlograms for **A**, Winchester, VA (VA09) and **B**, Burdett, NY (NY09) sampled in fall 2009. The autocorrelation coefficient r , indicated by the heavy solid line with error bars, is a measure of the standardized pairwise genetic similarity of isolates within the corresponding distance class. The 95% confidence intervals for the null hypotheses of random spatial genetic structure based on 1,000 permutations of the data are represented by dashed lines. Observed r values outside of the 95% confidence interval indicate significant spatial structure for the corresponding distance class. Kinship coefficients (F_{ij}) are depicted by a thin solid line or a dotted line for values with and without repeated multilocus genotypes, respectively. Open circles or triangles along the lines indicate significant F_{ij} values that are more or less similar than expected at random ($P < 0.05$) for analyses with and without repeated multilocus genotypes, respectively.

TABLE 3. Mating-type distributions and multilocus linkage disequilibrium for populations of *Erysiphe necator* in vineyards in Burdett, NY sampled in fall 2009 (NY09) and Winchester, VA sampled in the fall 2009 (VA09) and spring 2010 (VA10)

Population ^a	N	Mating-type distributions ^b		Multilocus linkage disequilibrium ^c		
		<i>MAT1-1</i>	<i>MAT1-2</i>	χ^2	I_A	$\bar{r}_d$
NY09						
Uncorrected sample	62	17	45	12.64**	1.49	0.17
Clone-corrected sample	40	17	23	0.90	0.40	0.05
VA09						
Uncorrected sample	78	37	41	0.20	1.70	0.19
Clone-corrected sample	48	23	25	0.08	1.05	0.12
VA10						
Uncorrected sample	69	28	41	2.45	0.60	0.07
Clone-corrected sample	68	27	41	2.88	0.57	0.07

^a Clone-corrected samples include a single representative of each multilocus genotype. Exceptions are for three multilocus genotypes from VA09 and one multilocus genotype from VA10 that were each repeated twice and were not inferred as clonal genotypes.

^b Asterisks (**) indicate that mating-type ratios deviated from 1:1 ($P \leq 0.001$). P values for all other samples are >0.05 ($\chi^2 < 3.84$).

^c Index of association (I_A) and $\bar{r}_d$ were calculated with MultiLocus v.1.3b (1). All P values are <0.001 and were determined by 1,000 randomizations.

population, as described above. No significant genetic differentiation (Φ_{PT}) was detected between the New York and Virginia populations (Table 4). No differentiation was detected between the Virginia fall (VA09) and spring (VA10) populations. VA09 and VA10 were more similar to each other, as measured by Nei's genetic identity, than NY09 was to either VA09 or VA10. Principal coordinates analysis revealed considerable overlap among multilocus genotypes from all three populations (Fig. 3). The first two coordinates explained 55.9% of the variation. Overall, the genotypes formed a continuous group and no distinct patterns were evident. There was more overlap among genotypes from the fall (VA09) and spring (VA10) populations from Virginia than with genotypes from the NY09 population.

DISCUSSION

We tested the hypothesis that populations of *E. necator* are randomly mating within vineyards. As predicted for random mating, genotypic diversity in the three populations was high and mating-type ratios on clone-corrected data did not deviate significantly from 1:1. These results are consistent with the biology of *E. necator* because sexual reproduction occurs regularly and ascospores are the source of primary inoculum each season, especially in cold climates, as in this study, where ascospores are the only form of overwintering inoculum (49). In contrast to predictions under the hypothesis of random mating, however, significant LD was detected in all three populations. LD is not

TABLE 4. Geographic and temporal genetic differentiation, measured by Φ_{PT} (above the diagonal) and Nei's genetic identity (below the diagonal) between vineyard populations of *Erysiphe necator* in Burdett, NY sampled in fall 2009 (NY09) and Winchester, VA sampled in fall 2009 (VA09) and spring 2010 (VA10)^a

Population	Population		
	VA09	VA10	NY09
VA09	...	0.001 (0.448)	0.02 (0.087)
VA10	0.98	...	0.001 (0.357)
NY09	0.80	0.78	...

^a *P* values for Φ_{PT} are in parentheses; Φ_{PT} , an analog of F_{ST} measured via analysis of molecular variance (48), was determined using GenAlEx v.6.41 (47).

expected among neutral markers in populations that undergo regular sexual reproduction because recombination breaks down LD. The LD observed in these populations is most likely the result of asexual reproduction; *E. necator* typically has ≥ 10 asexual generations per year. Clones were prevalent in populations at the end of epidemics in both vineyards after multiple asexual generations but were rare in the one vineyard sampled early in the epidemic. Variation in frequencies among clonal genotypes, caused either by differential fitness or random colonization events, could result in LD, as explained further below.

For pathogens with mixed modes of reproduction (i.e., both sexual and asexual generations occur on regular cycles), clone correction is a way of inferring the underlying genetic structure caused by sexual reproduction (34,41). Clone correction is valid as long as the probability of the same genotype occurring more than once due to an independent sexual reproduction event (p_{sex}) is small. In all populations of *E. necator* in this study, p_{sex} was significant for the majority of genotypes sampled more than once, indicating a significant contribution by clonal reproduction. Yet, not all fungi with a mixed mode of reproduction show LD within populations, including *Gibberella zeae* (67). For some fungal plant pathogens, clone correction reduces LD to insignificant levels; for example, *Cryphonectria parasitica* (41) and *Mycosphaerella graminicola* (68). LD after clone correction has been detected in other plant-pathogenic ascomycetes with a mixed mode of reproduction (7,11,15). The observation that clone correction did not eliminate LD in populations of *E. necator* in this study suggests that LD is caused by factors other than asexual reproduction alone.

LD may be caused by a variety of processes besides non-random mating (including asexual reproduction), such as epistatic selection (20), hitch-hiking selection, admixture of genetically different populations, or physical linkage among markers (27). Epistatic selection, which favors combinations of alleles at different loci that are advantageous, can cause LD in randomly mating populations if selection is strong compared with the recombination breaking up associations among alleles. Epistatic selection has been suggested as a possible cause of LD in the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei*, where the use of resistant cultivars and selective fungicides favored specific combinations of avirulence and fungicide resistance alleles (11,64). Selection for avirulence or fungicide resis-

Fig. 3. Principal coordinates analysis conducted with GenAlEx v. 6.41 (47) on pairwise genetic distances between genotypes of 209 isolates of *Erysiphe necator* from three populations. VA09 and VA10 were collected from the same vineyard of *Vitis vinifera* 'Chardonnay' in Winchester, VA in fall 2009 and spring 2010, respectively. NY09 isolates were collected from a vineyard of *V. vinifera* Chardonnay in Burdett, NY in fall 2009.

tance alleles during the asexual phase also results in selection of clones, some of which might increase markedly in frequency. Future studies would be needed to address whether dominant clones of *E. necator* at the end of the epidemic have greater fitness due to fungicide resistance or other traits that affect fitness, such as adaptation to environmental conditions or better dispersal abilities. Alternatively, dominant clones could be due to genetic drift resulting from chance colonization events of ascospores early in the epidemic. Ascospores of *E. necator* are released over the course of many weeks, between bud break and anthesis, when environmental conditions are favorable (22). Genotypes of colonies derived from ascospores that are established early will have more generations of asexual reproduction than genotypes that are established later.

Another potential cause of LD is population admixture; for example, sampling genetically different subpopulations and analyzing them as one population. Limited dispersal of *E. necator* within a vineyard could contribute to LD because of admixture. If random mating occurs only within clusters representing spatially restricted genetic neighborhoods, then population admixture will give the effect of inbreeding and lead to LD when assessed across the entire population (9,38,66). We found spatial aggregation of genotypes in both vineyards at the end of the epidemics, although spatial structure was no longer detected in the New York population when clonal aggregation was eliminated from the analyses. Mantel correlations between physical and genetic distances were significant for both populations; isolates sampled from relatively short distances apart were more similar to each other than expected at random. Spatial autocorrelation is indicative of some degree of isolation by distance or subdivision into genetic neighborhoods on a small scale within vineyards. This pattern most likely arose because of restricted dispersal distances; longer-distance dispersal would be expected to lead to random mixing of genotypes and no detectable spatial structure. In the VA09 population, genotypes were more similar to each other than by chance up to 42 m apart, whereas NY09 significant aggregation occurred only up to 3 m. These differences in cluster sizes could be due to differences in sampling patterns. The Virginia vineyard was rectangular in shape, with three rows approximately 100 m long, whereas the New York vineyard was sampled earlier in a square pattern, approximately 30 by 30 m (Fig. 1). Other factors that could contribute to the differences in cluster sizes are that the Virginia vineyard is a small research vineyard with few other grapevines nearby, whereas powdery mildew samples from the New York vineyard came from part of a large commercial vineyard in the Finger Lakes Region, which is a large grape production area. Additionally, the presence of a single dominant clone in New York versus several clones represented by fewer individuals in Virginia may have contributed to the differences in cluster sizes detected. Despite the many differences in details between New York and Virginia samples, the important result is finding significant spatial autocorrelation at a small scale, within vineyards.

Unlike other studies that investigated the large-scale geographic structure of *E. necator* in the eastern United States (9,21), we did not detect population structure between vineyards in New York and Virginia. Similarly, we found no differences in allele frequencies in the fall and spring populations from the same vineyard (VA09 and VA10), suggesting that the overwintering bottleneck is not small enough to affect this population. Although the allele frequencies were not different between VA09 and VA10, we did not find the same genotypes between years because initial inoculum each year is from ascospores produced by sexual reproduction; similar inferences were made for other plant pathogens when the same genotypes were not found between years (12,31). Studying the population structure of *E. necator* representative of the ascospore population prior to the contribution of asexual reproduction was challenging because of the difficulty of

finding powdery mildew colonies in vineyards in the spring. Many commercial vineyards are managed aggressively with fungicides early in the spring so that there are almost no colonies present at ascospore release, preventing an analysis of all potential combinations in offspring. We attempted to sample individuals derived from ascospores by placing unsprayed trap plants in the New York vineyard when ascospores were being released; however, no colonies were observed (data not shown). Research vineyards, like the one we sampled in Virginia, may be an alternative because they usually have nonsprayed control plots. However, research plots that have been inoculated with *E. necator* are not suitable because population structure could potentially be biased by the influx of a few inoculated genotypes.

We are fairly confident that the LD detected in this study is not due to close linkage of markers on the same chromosomes because very few pairs of these markers were in LD at the broad geographic scale (21). Additionally, the markers were obtained haphazardly from transcriptome sequence and are not likely to be physically linked by chance in a large genome; the three sequenced powdery mildew genomes are estimated to be 120 to 160 MB in size (58). Ideally, sexual crosses would be constructed to determine whether markers are genetically linked. However, despite repeated attempts, we have not been able to obtain enough viable progeny from crosses of *E. necator* to evaluate recombination and segregation of alleles at these EST-SSR loci.

We further hypothesize that a mixed reproductive mode combined with short-distance dispersal is likely contributing to LD in *E. necator*. We speculate that LD persists in populations of *E. necator* because the increase in LD caused by an increase in clonal genotypes during the asexual phase is not completely broken down by an annual generation of sexual reproduction. LD between unlinked loci can only decay by a maximum of 50% with recombination in one sexual generation; therefore, many of the alleles in the dominant clones are likely to remain nonrandomly associated. Inbreeding, which is directly calculated as a decrease in observed relative to expected heterozygosity (27), is difficult to measure in haploid fungi. An alternative would be to genotype the diploid phase as the composite genotype of the progeny from each cleistothecium (37). We tried to genotype ascospores from single cleistothecia from both vineyards but were not able to get reliable or consistent results, even with whole-genome amplification. We cannot rule out inbreeding as a contributor to repeated multilocus genotypes; however, the abundance of repeated genotypes in the fall samples versus the spring sample suggests that repeated multilocus genotypes are the result of asexual reproduction.

Modeling of recombination, clonal selection, drift resulting from chance colonization events, and limited dispersal in *E. necator* and other plant-pathogenic fungi may provide support for the hypothesis that LD can persist in sexual populations. Understanding how LD affects the population structure and evolution of *E. necator* is important for understanding the biology of this economically significant plant pathogen (22) and determining whether or not association mapping can be done in this fungus.

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