Spatial Distribution of Single-Nucleotide Polymorphisms Related to Fungicide Resistance and Implications for Sampling

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ABSTRACT

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Spatial distribution of single-nucleotide polymorphisms (SNPs) related to fungicide resistance was studied for *Botrytis cinerea* populations in vineyards and for *B. squamosa* populations in onion fields. Heterogeneity in this distribution was characterized by performing geostatistical analyses based on semivariograms and through the fitting of discrete probability distributions. Two SNPs known to be responsible for boscalid resistance (H272R and H272Y), both located on the B subunit of the succinate dehydrogenase gene, and one SNP known to be responsible for dicarboximide resistance (I365S) were chosen for *B. cinerea* in grape. For *B. squamosa* in onion, one SNP responsible for dicarboximide resistance (I365S homologous) was chosen. One onion field was sampled in 2009 and another one was sampled in 2010 for *B. squamosa*, and two vineyards were sampled in 2011 for *B. cinerea*, for a total of four sampled sites. Cluster sampling was carried on a 10-by-10 grid, each of the 100 nodes being the center of a 10-by-10-m quadrat. In each quadrat, 10 samples

were collected and analyzed by restriction fragment length polymorphism polymerase chain reaction (PCR) or allele specific PCR. Mean SNP incidence varied from 16 to 68%, with an overall mean incidence of 43%. In the geostatistical analyses, omnidirectional variograms showed spatial autocorrelation characterized by ranges of 21 to 1 m. Various levels of anisotropy were detected, however, with variograms computed in four directions (at 0°, 45°, 90°, and 135° from the within-row direction used as reference), indicating that spatial autocorrelation was prevalent or characterized by a longer range in one direction. For all eight data sets, the β -binomial distribution was found to fit the data better than the binomial distribution. This indicates local aggregation of fungicide resistance among sampling units, as supported by estimates of the parameter θ of the β -binomial distribution of 0.09 to 0.23 (overall median value = 0.20). On the basis of the observed spatial distribution patterns of SNP incidence, sampling curves were computed for different levels of reliability, emphasizing the importance of sample size for the detection of mutation incidence below the risk threshold for control failure.

Additional keywords: spatial heterogeneity.

The spatial distribution of plant pathogens is the result of multiple, complex interactions between dispersal processes and environmental factors (31). For several decades, the spatial patterns of plant diseases have been of great interest, resulting in the development of a whole range of improved tools to be used by plant disease epidemiologists. This is especially true for the understanding of disease dynamics and the design of sampling strategies in space, time, and space-time (10,12). Thoughtful sampling is essential for sound decision making in plant pathology and plant health management. The development of effective sampling strategies is dependent on the type of data available (binary, categorical, discrete, or continuous) and the spatial aggregation patterns within fields and sampling units. The assessment of disease status is usually based on the evaluation of plant parts, entire plants, or the whole plant population. In plant pathology, the collected data can either be binary (a plant is diseased or not), discrete (counts), or continuous (severity). Thus, because the number of samples is often limited because of spatial, temporal, or economic constraints, count data are commonly pooled and used to measure disease intensity (12).

In the early 1990s, Gareth Hughes and Laurence Madden introduced the use of discrete probability distributions to characterize spatial heterogeneity in plant disease epidemiology (16,17, 26,27,30,32). In this approach, the number of diseased plants among n plants randomly sampled in a quadrat, for example, is assumed to follow a binomial distribution for a completely random spatial pattern and a β -binomial distribution for an aggregated spatial pattern. In fact, one of the parameters (θ) of the β -binomial distribution is a direct measurement of local aggregation. These two discrete probability distributions have been widely used to characterize homogeneity versus heterogeneity of plant disease epidemics for soilborne pathogens and airborne pathogens and viruses. For example, Xiao et al. (52) found that *Verticillium dahliae* microsclerotia in soil were aggregated; in strawberry fields, Turechek et al. (49,50) showed that leaf blight and leaf spot diseases tended to be aggregated; and, for viruses, Dallot et al. (9) recently discovered aggregation in Sharka disease, caused by the *Plum pox virus*. The level of spatial aggregation obtained in these studies was used, in part, to develop effective sampling strategies, which helped determine disease level and improve timing of control measure application.

Even though many integrated management strategies have been developed to delay plant disease epidemics, disease control continues to depend mostly on fungicide applications. An important factor for an efficient fungicide application is the proportion of resistant individuals present in the target population (4). Despite the importance of the spatial distribution of fungicide resistance

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(to be distinguished from the spatial distribution of a plant pathogen), most of the epidemiological studies of fungicide resistance have been targeted on temporal changes, such as the increase in resistance frequency following consecutive fungicide applications (34,35,37). In the late 1990s, Elmer et al. (11) studied the spatial component of dicarboximide resistance in *Monilinia fructicola* populations on stone fruit. The results obtained for stone fruit suggested various levels of heterogeneity in the spatial distribution of dicarboximide-resistant strains of *M. fructicola* populations. Invasion of a resistant strain over a sensitive one mainly follows from the difference in fitness between the two strains as well as the resistance factor associated with the resistant phenotype.

In the last decade, breakthroughs at the individual level have been made in the identification of molecular mechanisms related to fungicide resistance. For the model organism *Botrytis cinerea*, responsible for diseases of >225 hosts, single-nucleotide polymorphisms (SNPs) have been identified in both field and lab mutants and related to resistant phenotypes for at least six fungicide groups (1,2,7,8,13,14,24,25,38,39,46,47,54). Ranges of resistance factor and impact on pathogen fitness were also determined for each of these SNPs. In addition, the results of Van der Heyden et al. (unpublished data) suggest that patterns of spatial co-existence are also possible at the SNP scale and that a genetic niche occupied by a specific SNP might either prevent invasion by or co-existence with other SNPs within a given population.

Therefore, breaking down the phenotypic resistance into genotypic factors (i.e., SNPs) is of great practical importance for monitoring fungicide resistance and foreseeing shifts in resistant populations. Considering the value of knowing the frequency of resistant strains carrying a particular SNP in a given population, the question of sampling naturally arises in this approach. Few studies on fungicide resistance have focused on spatial aspects, and we could not find any studies in which the spatial distribution patterns of SNPs related to fungicide resistance were investigated (11). Hence, knowing the level of local aggregation is essential to making accurate inference and determining appropriate sample size. Accordingly, by using the geostatistical and distributional approaches, the objectives of this study were to (i) formally characterize the spatial distribution pattern of three SNPs related to fungicide resistance to boscalid and iprodione within *B. cinerea* populations in vineyards, (ii) do the same characterization for one SNP related to fungicide resistance to iprodione within *B. squamosa* populations in onion fields, and (iii) compute sampling curves relative to the estimation of SNP frequency for various levels of spatial aggregation.

MATERIALS AND METHODS

Sampling protocols. Sampling for *B. cinerea* was conducted during fall 2011 in two commercial vineyards (of 9 and 4.5 ha, respectively), each one planted with 'Seyval' grape cultivar and located in the Eastern Townships region of the Province of Québec (Canada). In both vineyards, sampling was organized following a 100-by-100-m grid installed in the middle of the field and divided in 100 10-by-10-m quadrats. At the center of each quadrat, spores from 10 single-sporulating colonies were individually sampled in duplicate, using a dry BBL culture swab (Fisher Scientific, Ottawa, ON, Canada). Once in the lab, culture swabs were placed into a 2-ml skirted screw-cap micro-test tube, containing 380 µl of isopropanol (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Each sample was placed at -20°C until upstream polymerase chain reaction (PCR) analysis.

Sampling for *B. squamosa* was conducted in commercial onion fields in a muck land area near Montréal (Québec, Canada) during fall 2009 and 2010. In 2009, 'Red Bull' onion was seeded on 23 April 23 at the rate of 30 seeds m⁻¹, with row spacing of 0.45 m in a 6-ha field; in 2010, 'Hamlet' onion was seeded on 26 April at

the same rate and row spacing in a 12-ha field. Sites were different in the 2 years but, at each site, sampling was organized in the middle of the field following a 100-by-100-m grid divided in 100 10-by-10-m quadrats. At the center of each quadrat, 10 infected leaves were randomly collected on different onion plants and kept on ice, prior to being brought to the lab by the end of the day. Each leaf was cut into 2-cm pieces and placed in a 100-by-15-mm petri dish with a sheet of 90-mm Whatman filter paper amended with 500 µl of milliQ water (Fisher Scientific). The petri dishes (10 per quadrat) were placed at room temperature (22 to 25°C) for 2 to 5 days. In each petri dish, spores from single-sporulating lesions were harvested using a cotton swab drenched with isopropanol (Sigma-Aldrich Canada Ltd.) and placed into a 2-ml skirted screw-cap micro-test tube containing 300 µl of isopropanol (Sigma-Aldrich Canada Ltd.). All samples were kept at -20°C before DNA extraction and PCR analysis.

DNA extraction and restriction fragment length polymorphism PCR. To obtain total DNA, 80 µl of spore suspension was transferred to a sterile 2-ml screw-cap micro-test tube containing 100 mg of glass beads (425 to 600 µm) (Sigma-Aldrich Canada Ltd.). Tubes were shaken in a FastPrep-24 instrument (MP Biomedicals, Irvine, CA) at 4 m/s for 20 s to fragilize the conidia cell wall and promote membrane disruption. Conidia were precipitated by centrifugation for 5 s at 10,000 × g, and isopropanol was evaporated under vacuum using a SpeedVac (Savant Instruments, Inc., Holbrook, NY) instrument for 20 min at 55°C. Conidia were resuspended in 300 µl of DNA extraction buffer, consisting of nuclease-free water (Integrated DNA Technologies, Inc., Coralville, IA) and 5% chelex-100 molecular-biology grade resin (Bio-Rad Laboratories, Hercules, CA). Following resuspension, tubes were placed in a dry bath at 105°C for 15 min to complete cell disruption and DNA extraction. Tubes were finally agitated for 5 s, using a table vortex first and then a centrifugation at 15,000 × g for 5 min at 4°C. Supernatant containing genomic DNA was placed at 4°C until upstream PCR analysis for a maximum of 2 h or placed at -20°C until future use.

Detection of the I365S allele in *B. cinerea* was performed using a PCR restriction fragment length polymorphism (RFLP) assay, as described by Oshima et al. (38), and that of the H272R and H272Y alleles was completed with the procedure described by Yin et al. (53). Similarly, identification of the I365S allele in *B. squamosa* was performed in a PCR-RFLP assay. The primer pair BsQdxF (5'-CAGATATTCTCGAGTGAGGTCTCC-3') and BsQdxR (5'-CTCTGCCTGAACCTTCTGTGTCAA-3') was used for amplification of a 560-bp fragment. The PCR reactions were conducted in a 25-µl reaction volume containing 3 µl of DNA extraction, 2.5 µl of PCR buffer (10×), 2 µl of bovine serum albumen (10 mg ml⁻¹), 0.25 µl of dNTPs (20 mM), 0.38 µl of each primer (10 µM), and 0.5 µl of Titanium Taq polymerase (Clontech Laboratories, Mountain View, CA). The amplification was carried in a SureCycler 8800 (Agilent, La Jolla, CA), with conditions set as follows: first, 95°C for 5 min; then, 35 cycles of 30 s at 95°C, 45 s at 62°C, and 30 s at 72°C; and, finally, 10 min at 72°C.

The PCR products amplified by the primer pairs BcOS10R/BcOS5a and BsQdxF/R were digested with the enzyme TaqαI, as recommended by the manufacturer. Digested products were separated by electrophoresis in 1.7% agarose gel in 1× Tris-acetate EDTA buffer and visualized after ethidium bromide staining under UV light.

Geostatistical analyses. Even though geostatistics were originally developed for continuous quantitative data, the semivariance was computed here on discrete quantitative data and was considered to be a reasonable measure of spatial autocorrelation in these conditions. Initially, maps of SNP incidence were produced from the number (from 0 to 10) of samples tested positive for the presence of a given SNP at the 100 spatially referenced quadrats on a 100-by-100-m sampling grid; these count data were assigned to the centers of quadrats. Then, the first phase of the method of

coregionalization analysis with a drift (CRAD) was performed to detect and remove any possible drift (i.e., large-scale component of the spatial pattern) (44). In field phytopathological studies, it is expected that a drift includes the spatial variation that is not attributed to the intrinsic spatial behavior of the studied pathogen; for example, the presence of a slope would be incorporated into a drift. In this initial geostatistical analysis, the count data collected on a grid for a given SNP were considered as a partial realization of a random function (Z), and their spatial pattern was decomposed into an estimated drift ($\hat{d}$) plus a residual component ($\hat{\epsilon}$):

$$Z(u_i) = (\hat{d})(u_i) + (\hat{\epsilon})(u_i) \quad (i = 1, \dots, 100) \quad (1)$$

where u_i represents quadrat i . The drift was estimated with the estimated generalized least squares (EGLS) method, performed in a local procedure (L_1), including the selection of an optimal moving window size. Our CRAD analyses were carried out with the freeware available at <http://environmetricslab.mcgill.ca/Programs.html>.

Second, to assess the presence of spatial autocorrelation in the count data, omnidirectional and directional experimental variograms were computed on residuals ($\hat{\epsilon}_i$) and analyzed using PROC VARIOGRAM of SAS (version 9.3; SAS Institute, Cary, NC). Eight equi-frequent distance classes were used to compute the variograms; their boundaries were 10, 14.14, 20, 22.36, 30.36, 36.06, 41.16, and 44.72 m. There were at least 150 pairs of observations in each distance class, which assured a good level of precision in the estimation of semivariance values. Matheron's classical semivariance estimator (33) was used, which for omnidirectional variograms is given by:

$$\hat{\gamma}(h) = \frac{1}{2A(h)} \sum_{(i,i')} (\hat{\epsilon}(u_i) - \hat{\epsilon}(u_{i'}))^2 \quad (2)$$

where h refers to a certain distance class, u_i and $u_{i'}$ denote any two quadrats whose centers are separated by a distance belonging to the distance class considered, and $A(h)$ is the number of pairs of such quadrats. In addition, the semivariance was also estimated as a function of distance in four directions: at 0° , 45° , 90° , and 135° from the within-row direction, with 90° representing the across-row direction. In this case, only the pairs of observations (i.e., CRAD first-phase residuals) in the direction considered were included in the computation of the semivariance estimator. Variogram ordinates that start low and increase with increasing distance lag are a sign of the presence of spatial autocorrelation. The distance lag at which the semivariance reaches a plateau (i.e., a constant value) is called "range", and indicates the distance beyond which observations are no longer spatially correlated. When the plateau can only be reached asymptotically, the range is said to be "practical", and then corresponds to the distance at which the semivariance is equal to 95% of the plateau. Other important variogram model parameters are the nugget effect (i.e., the variogram ordinate at infinitely small distance) and the partial sill (measured as the difference between the height of the plateau and the nugget effect).

Because the spherical variogram model provides a good compromise between the Gaussian and exponential models in terms of rate of decrease of spatial autocorrelation with distance (10), the spherical model was chosen for fitting:

$$\gamma(h) = c + b \left(1.5 \frac{h}{a} - 0.5 \frac{h^3}{a^3} \right) \text{ for } 0 < h < a \quad (3)$$

where c represents the nugget effect, b is the sill associated with the spherical variogram model, and a denotes the range of spatial autocorrelation. The fitting of spherical variogram models to experimental variograms was performed by EGLS (43). Anisotropy was assessed by comparing the variogram model parameter estimates (i.e., range, nugget effect, and partial sill) obtained in

the four directions and with the corresponding omnidirectional variogram.

Distributional analyses. The index of dispersion, D , was used to assess departure from complete randomness on each count data set. This index is a ratio of two variances: the observed variance of SNP incidence in the numerator and the variance of SNP incidence, estimated under the assumption of a binomial distribution, in the denominator:

$$D = \frac{s_y^2}{\hat{p}(1-\hat{p})/n} \quad (4)$$

where $\hat{p}$ is the estimated probability (empirical frequency) of presence of a SNP. The general rule of interpretation is that a value of $D = 1.0$ (i.e., the two variances are equal) indicates a completely random pattern within a sampling unit (i.e., a quadrat). A value of $D > 1.0$ is indicative of a departure from a completely random pattern and is suggestive of heterogeneity. Under the assumption of a completely random spatial distribution, $(N-1)D$ follows a χ^2 distribution with $N-1$ degrees of freedom, where N is the sample size (i.e., number of quadrats) (31).

Binomial and β -binomial distributions were fitted to the frequency distributions of each count data set, using a SAS code developed by L. V. Madden and obtained from <http://www.oardc.ohio-state.edu/pp702/Downloads.htm>. When the location of an isolate carrying a given SNP is independent of the location of another isolate carrying the same SNP, the probability p of an isolate carrying the SNP is constant over the field and the number X of isolates carrying the SNP, out of n isolates randomly sampled, follows a binomial distribution characterized by the probability function:

$$P(X=x) = \frac{n!}{x!(n-x)!} p^x (1-p)^{n-x} \quad x = 0, 1, 2, \dots, n \quad (5)$$

The theoretical mean and variance of X are equal to np and $np(1-p)$, respectively. Compared with the binomial distribution (X), the β -binomial distribution (Y) has a supplementary parameter, θ , used as a measure of local aggregation in space, and its probability function changes for:

$$P(Y=y) = \frac{n!}{y!(n-y)!} \frac{\prod_{j=0}^{y-1} (p+j\theta) \prod_{j=0}^{n-y-1} (1-p+j\theta)}{\prod_{j=0}^{n-1} (1+j\theta)} \quad (6)$$

It follows that the theoretical mean and variance of Y are np and $np(1-p)(1+n\theta)/(1+\theta)$, respectively. Thus, the theoretical β -binomial variance can be seen as the product of the theoretical binomial variance [$np(1-p)$] and a heterogeneity component [$(1+n\theta)/(1+\theta)$]. Accordingly, when local aggregation increases, the theoretical β -binomial variance is inflated with increasing θ value; in contrast, as θ approaches 0, the β -binomial distribution approaches the binomial distribution (16,26,27,31). Therefore, a good fit of the β -binomial distribution (i.e., the θ estimate is far from zero) indicates a departure from a completely random pattern, which means local aggregation of isolates carrying the same SNP within a quadrat in our study.

A likelihood-ratio test was performed to determine whether the β -binomial distribution provided a better fit to the observed frequencies of count data than the binomial distribution. The χ^2 test statistics of goodness-of-fit were computed for the β -binomial and binomial distributions. Finally, a $C(\alpha)$ test based on the z statistic was also used to determine whether the aggregation in the spatial distribution of each SNP was better described by a β -binomial distribution (26). The null hypothesis of our χ^2 tests of goodness-of-fit was randomness, with aggregation as the alterna-

tive hypothesis. The $C(\alpha)$ test is more specialized than the χ^2 test because, even if the null hypothesis is the same, the alternative hypothesis of the former is more specific (i.e., that the β -binomial is appropriate) (48).

Sampling curves. Sampling curves were developed from the results obtained with the β -binomial distribution, so that the mean SNP incidence could be estimated with the “coefficient of variation of the sample mean” (31), denoted CV hereafter:

$$CV = \frac{\sqrt{\hat{p}(1-\hat{p})(1+\hat{p}(n-1))/nN}}{\hat{p}} \quad (7)$$

where $\hat{p} = \hat{\theta}/(\hat{\theta} + 1)$, n is the number of subsamples (isolates) taken in each sampling unit (quadrat), and N is the number of sampling units (number of quadrats). This equation can be rearranged to extract the sample size N :

$$N = \frac{(1 - \hat{p})(1 + \hat{p}(n - 1))}{n\hat{p}CV^2} \quad (8)$$

The minimum number N of sampling units needed to estimate p with a given level of reliability was calculated from sampling curves developed for CV values of 10, 20, and 30%. Sampling curves were calculated for three values of θ : the maximum,

minimum, and median of the estimated values of θ for the β -binomial distributions fitted with our eight data sets (28,31).

RESULTS

SNP incidence. The raw count data collected in this study are mapped in Figure 1 (see legend for the sample means and standard errors of the sample means). SNP incidence in *B. cinerea* populations was not consistently higher for one of the two vineyards, because the mean incidence is higher in the first vineyard (OR) for the H272R allele but higher in the second vineyard (TE) for the I365S and H272Y substitution. The mean incidence for the I365S substitution in *B. squamosa* populations was greater (in a ratio of 2.72) in 2010 than in 2009 (Fig. 1).

Geostatistical analyses. A drift (i.e., large-scale spatial component) was detected with the CRAD method in four of the eight data sets, accounting for 10.4 to 30.0% of the total variation (Table 1). After the removal of estimated drifts, spatial autocorrelation was found in six of the eight omnidirectional variograms of residual components. The estimated range of spatial autocorrelation varied from 11.9 to 28.7 m, the mean being 19.9 m (Table 1). The nugget value tended to increase with increasing mean SNP incidence, whereas the sill of the spherical variogram model

TABLE 1. Results of coregionalization analysis with a drift (CRAD) analysis and variogram model parameter estimates (range, nugget effect, and sill of spherical structure) for single-nucleotide polymorphism (SNP) distributions within *Botrytis cinerea* and *B. squamosa* populations in two vineyards and two onion fields sampled in 2009 to 2011

Site	Year	SNP	Drift ^a	Direction (°) ^b	Partial sill ^c	Nugget effect	Range of spatial autocorrelation (m)
OR	2011	H272R	0	Omni	3.387	1.214	13.36
				0	...	4.734	...
				45	0.702	3.940	23.9
				90	...	4.200	...
				135	...	4.478	...
OR	2011	H272Y	0	Omni	0.969	2.406	22.96
				0	1.308	1.989	21.1
				45	0.579	2.743	21
				90	1.292	2.030	21.5
				135	1.161	2.045	20
OR	2011	I365S	0.253	Omni	1.349	2.863	21.52
				0	1.837	2.258	21
				45	...	4.000	...
				90	0.962	3.065	21.5
				135	1.417	2.667	21.1
DE	2009	I365S	0	Omni	4.089	0	12.88
				0	...	4.073	...
				45	...	3.914	...
				90	...	4.108	...
				135	...	3.864	...
TE	2011	H272R	0.301	Omni	1.601	4.041	27.76
				0	...	5.441	...
				45	1.093	4.273	20.8
				90	3.146	2.469	21.3
				135	0.960	4.379	20.2
TE	2011	H272Y	0.23	Omni	2.668	2.349	28.72
				0	2.517	2.482	33.3
				45	3.494	1.368	20.7
				90	...	4.854	...
				135	2.150	2.444	20.4
TE	2011	I365S	0	Omni	3.941	2.275	11.92
				0	...	6.420	...
				45	0.355	6.152	31.3
				90	0.470	5.928	21.5
				135	0.426	6.053	21.1
VH	2010	I365S	0.104	Omni	0	4.763	...
				0	0.445	4.699	56.6
				45	...	4.531	...
				90	0.032	4.6	30.2
				135	...	4.739	...

^a Drift estimated by estimated generalized least squares in first phase of CRAD (44).

^b Direction used as reference for the computation of directional experimental variograms, expressed in degrees, where 0° refers to the within-row direction; Omni means that the experimental variograms were computed in all directions (i.e., they are omnidirectional).

^c The sill (i.e., variance component) of the spherical structure used in variogram modeling.

remained relatively small in general, implying that a moderate portion of the variance was explained by a spatially autocorrelated structure. Furthermore, directional variograms showed a great variety of spatial dependency, from acceptable isotropy in spatial autocorrelation (i.e., H272Y for OR in 2011) to various patterns of anisotropy, including the presence of spatial autocorrelation in only one direction (i.e., H272R for OR in 2011) and a large difference in the estimated range with direction (i.e., I365S for VH in 2010) (Table 1).

Spatial statistics. The value of the index of dispersion, D , was 1.71 to 2.97, indicating a departure of medium-to-large magnitude

of the frequency distribution of counts from a binomial distribution (Table 2). For all eight data sets, the maximum likelihood estimation procedure converged and the likelihood-ratio test showed that the β -binomial distribution provided a better fit to count data than the binomial distribution (Table 2). The z -value of the $C(\alpha)$ -test statistic was 5.16 to 14.44 with P values of 0.0001 (Table 2), meaning there was local aggregation within quadrats, and this is well described with the β -binomial distribution (Fig. 2). The estimates of the β -binomial distribution parameters varied from 0.16 to 0.68 (average: 0.43) for $\hat{p}$ and from 0.09 to 0.23 (median: 0.20) for $\hat{\theta}$. The aggregation index values ($\hat{\theta}$) for

Fig. 1. Raw count data maps for *Botrytis cinerea* in vineyard OR for **A**, H272R (observed mean incidence, 0.68 ± 0.023 , standard error); **B**, H272Y (0.21 ± 0.021); and **C**, I365S (0.55 ± 0.024); and in vineyard TE for **E**, H272R (0.61 ± 0.021); **F**, H272Y (0.20 ± 0.018); and **G**, I365S (0.59 ± 0.020); and for *B. squamosa* for I365S homologous in **D**, 2009 (0.16 ± 0.020) and **H**, 2010 (0.42 ± 0.025).

H272R, H272Y, and I365S in *B. cinerea* populations were all lower in vineyard OR than in vineyard TE (Table 2). In contrast, the difference between θ estimates for I365S in the *B. squamosa* populations of 2009 and 2010 was small (Table 2).

Sampling curves. The developed sampling curves are presented in Figure 3. For a lower CV (i.e., higher precision), the minimum sample size N required that the estimate of the mean SNP incidence should be larger. For $\hat{\theta} = 0.09$ (i.e., smallest θ estimate in our study), ≈ 153 , 38, and 17 quadrats should be sampled, with 10 isolates from each, to estimate a mean SNP incidence of 0.1 with CV values of 10, 20, and 30%, respectively; for $\hat{\theta} = 0.20$ (median estimated value), the three numbers of quadrats increase to 223, 56, and 25; and, for $\hat{\theta} = 0.23$ (greatest estimated value), to 243, 61, and 27 (Fig. 3). By comparison, for a mean SNP incidence of 0.4 (i.e., fourfold >0.1), the required number of sampling quadrats is much smaller: ≈ 26 , 7, and 3 with $\hat{\theta} = 0.09$ and 37, 8, and 5 with $\hat{\theta} = 0.20$ for CV values of 10, 20, and 30%, respectively (Fig. 3).

DISCUSSION

Management strategies, including fungicide applications, exist either to delay disease onset or to level off disease progress. Among the limitations regarding fungicide efficacy, the development of resistance by target organisms is becoming a priority for growers, crop specialists, and manufacturers. Despite the progress made toward understanding fungicide resistance at the individual level, establishing the relationship between theoretical and practical resistance is tedious and our knowledge remains limited. Recent research on resistance mechanisms, discovery of SNPs related to fungicide resistance, and development of molecular detection tools enables the breakdown of global phenotypic resistance into genotypic resistance. When moving from classical phenotypic to genotypic resistance, several hypotheses regarding practical aspects of resistance can be tested readily. For numerous aspects of fungicide resistance epidemiology, acquiring knowledge about spatial distribution patterns is essential, especially for sampling. Consequently, the data from two vineyards and two onion fields sampled in our study in 2011 and 2009 to 2010, respectively, were used to characterize the spatial distribution patterns of SNPs related to fungicide resistance in *B. cinerea* and *B. squamosa* populations.

In both systems, sampling was carried out toward the end of the growing season, after numerous secondary disease cycles; hence, diseased onion leaves or grape berries were found in each of the quadrats (5). However, within an onion field or a vineyard, the results of our geostatistical analyses suggest that quadrat counts

on SNPs related to fungicide resistance are spatially autocorrelated, the estimated range of spatial autocorrelation varying between 21.0 and 31.7 m in omnidirectional analyses. The spatial autocorrelation measured was largely dependent on quadrat size and the organisms concerned in plant diseases. For example, Larkin et al. (22) found an average range of spatial autocorrelation of ≈ 15 m for *Phytophthora capsici* in bell pepper in soil and soil water. For almond leaf scorch disease, the estimated ranges of spatial autocorrelation averaged at 28 m (15) and, for *Venturia inaequalis*, the range of autocorrelation for potential ascospore dose was 40 m on average (6). In addition to relatively short ranges of spatial autocorrelation, our results indicated the presence of some anisotropy, which occurred in particular in the onion field sampled in 2010 and was then characterized by spatial autocorrelation restricted to the within-row direction and the direction perpendicular to it and by a much longer range of spatial autocorrelation in the former direction. These results support those of Elmer et al. (11), who also found spatial autocorrelation in the within-row direction in dicarboximide-resistant isolates from *M. fructicola* populations in stone fruit. In the two vineyards sampled in our study, the vines were planted 0.9 m apart on rows separated by 3 m, thus forming a compact hedge. The presence of such hedges could explain the presence of a directional bias, which was moderate here. In the onion field with an anisotropic spatial autocorrelation in quadrat counts on SNPs related to fungicide resistance in 2010, the canopy height was lower and the crop density was much higher. It is unclear why the other onion field sampled the year before did not show a similar anisotropy, and actually did not show any spatial autocorrelation at all in quadrat counts on SNPs related to fungicide resistance. It is true, however, that the high values of the nugget effect relative to the sills of spherical variogram models fitted for the 2010 onion data set indicate that only a small part of the total variance could be explained by a spatially autocorrelated component. In addition, a short range of spatial autocorrelation is a prerequisite to the fitting of a β -binomial distribution.

Both *B. cinerea* isolates carrying the H272R, H272Y, and I365S mutations in the vineyards sampled and the *B. squamosa* isolates carrying the I365S homologous mutation in the onion fields sampled were heterogeneously distributed among sampling quadrats. The median estimated value of the parameter θ of the β -binomial distributions fitted to the eight-count data sets (Table 2) was 0.20, indicating a relatively high level of small-scale spatial (i.e., local) aggregation. In fact, the β -binomial distribution provides an appropriate description of the frequency distribution of the number of mutations related to fungicide resistance per 10 isolates over sampling quadrats, the resistance status of a *Botrytis*

TABLE 2. Observed values of the index of dispersion and parameter estimates for the β -binomial and binomial distributions

Site	Year	SNP	MI ^a	D^b	C(α) test ^c		Parameter estimates ^d		LRT ^e	$\chi^2_{\text{BDD}}^f$	$P(\chi^2_{\text{BDD}})^f$	$\chi^2_{\text{BD}}^g$	$P(\chi^2_{\text{BD}})^g$
					z	$P(z)$	$\hat{p}$ (SE)	$\hat{\theta}$ (SE)					
OR	2011	H272R	0.607	1.87*	6.37	0.0001	0.6076 (0.02103)	0.1107 (0.03048)	28.72***	5.30	0.380	15.66	0.030
OR	2011	H272Y	0.197	1.96*	7.01	0.0001	0.1969 (0.01713)	0.1185 (0.03390)	31.06***	2.08	0.555	25.11	0.0001
OR	2011	I365S	0.593	1.71*	5.16	0.0001	0.5936 (0.02020)	0.0851 (0.02709)	19.69***	1.05	0.975	17.51	0.002
TE	2011	H272R	0.684	2.49*	10.94	0.0001	0.6843 (0.02306)	0.2056 (0.04645)	68.89***	8.55	0.200	30.40	0.0001
TE	2011	H272Y	0.211	2.64*	12.06	0.0001	0.2135 (0.01976)	0.2067 (0.04766)	69.95***	2.06	0.725	39.14	0.0001
TE	2011	I365S	0.550	2.42*	10.40	0.0001	0.5494 (0.02434)	0.1934 (0.04145)	65.00***	3.39	0.690	18.22	0.001
VH	2010	I365S	0.422	2.51*	11.10	0.0001	0.4238 (0.02451)	0.1979 (0.04369)	70.05***	4.54	0.604	24.45	0.0001
DE	2009	I365S	0.155	2.97*	14.44	0.0001	0.1592 (0.01760)	0.2335 (0.05449)	79.85***	4.41	0.219	39.65	0.0001

^a Mean incidence.

^b Index of dispersion; * indicates significant at 0.05 level.

^c Observed values of the z statistic and corresponding probabilities of significance.

^d Together with n = the number of sampling quadrats here, θ = the local aggregation index, and p = the mean single-nucleotide polymorphism (SNP) incidence are the three parameters of the β -binomial distribution; the binomial distribution has only two parameters, n and p . Standard errors (SEs) are reported in parentheses.

^e Values of the likelihood ratio test (LRT) statistic, obtained by using the binomial distribution as the “null hypothesis” ($\theta = 0.0$) and the β -binomial distribution as the “alternative hypothesis” ($\theta > 0.0$). The critical value used was 10.83, for $\alpha = 0.001$ (*, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively).

^f Observed values of the goodness-of-fit χ^2 test statistic for the β -binomial distribution (BDD) and corresponding probabilities of significance.

^g Observed values of the goodness-of-fit χ^2 test statistic for the binomial distribution (BD) and corresponding probabilities of significance.

isolate in a sampling quadrat being dependent on the resistance status of another *Botrytis* isolate in the same sampling quadrat. Using Lloyd's patchiness index, Elmer et al. (11) found that dicarboximide-resistant strains of *M. fructicola* in stone fruit were aggregated in all blocks and all years. Because very few studies have been conducted on the spatial distribution of resistant

phenotypes or genotypes, the quantitative results obtained in our study are difficult to compare. However, the spatial aggregation characteristics of white mold epidemics caused by *Sclerotinia* spp. in bean ($\hat{\theta}_{\text{median}} = 0.10$) and pyrethrum ($\hat{\theta}_{\text{median}} = 0.16$), of leaf spot in strawberry ($\hat{\theta}_{\text{median}} = 0.20$), and of grape downy mildew (0.02 to 0.21) were lower or similar (19,29,45,49). For *B.*

Fig. 2. Frequency distribution of the observed number of *Botrytis* isolates carrying a given single-nucleotide polymorphism (SNP) per quadrat (empty bars), together with the expected numbers of *Botrytis* isolates carrying the SNP per quadrat under the β -binomial distribution model (filled bars) and the binomial distribution model (dashed bars), for *B. cinerea* in vineyard OR for **A**, H272R; **B**, H272Y; and **C**, I365S; in vineyard TE for **E**, H272R; **F**, H272Y; and **G**, I365S; and for *B. squamosa* for I365S in **D**, 2009 and **H**, 2010.

squamosa, the heterogeneity in the spatial distribution was well described with Iwao's patchiness regression technique (3). In that study, the authors concluded that the basic components of the *Botrytis* leaf blight lesion population were aggregates, contagiously distributed in the onion fields (3). The results reported here are suggestive of a small-scale dispersal pattern of genetic variants (i.e., the individuals carrying, or not, SNPs related to resistance), by opposition to the dispersal of a well-dispersed pathogen characterized by a completely random spatial distribution pattern (51). Spatial aggregation of plant diseases is predominantly determined by spore formation and dispersal. Consequently, if resistance to a given fungicide is associated with reduced sporulation, spore survival, or infection ability, the spatial distribution of resistant isolates is expected to be aggregated. However, recent studies by Parnell et al. (41,42) have demonstrated that invasion is not the only possible outcome and various levels of co-existence between resistant and sensitive strains can be observed over a prolonged period of time in a mixed fungal population. Small-scale distribution can also be explained, in part, by the presence of autoinfection mechanisms, which are known to result in local clustering (36). The results of this study suggest that *Botrytis* strains carrying one or a series of SNPs may be considered as a subpopulation with different spatial behaviors.

Using the cluster sampling methodology described by Hughes et al. (18), we calculated the minimum sample sizes required to estimate mean SNP incidence with given levels of statistical precision and spatial aggregation. It was found (Fig. 3) that a larger sample size is required when the SNP follows an aggregated spatial distribution, compared with a completely random one. In other studies aimed to survey fungicide resistance using classical phenotypic assays, the sample size often comprised 10 to 100 samples per sampling site (20,21,23,40). Sampling for fungicide resistance may not or should not require the same numbers of samples needed if resistance is tested by classical phenotypic assays or by genotypic assays, because the classical method encompasses all the genotypic variations. In fact, sampling for SNPs related to fungicide resistance requires a high level of precision. It might also be more extensive. For example, sampling for SNPs could be used for different purposes, such as day-to-day monitoring and surveillance of a population shift. Thus, sample size and level of reliability could vary depending on the objectives of the survey.

Because of the clonal reproduction and haploid dispersion of *Botrytis* spp., the expected range of spatial autocorrelation might be longer than the ranges estimated in our study. These were obtained from count data, after removal of a drift when present, for a given size of sampling quadrat. Nevertheless, our results strongly suggest aggregation in the spatial distribution patterns of SNPs related to fungicide resistance, which has important implications for questions of sample size. The large number of samples required when high precision matters represents a possible limitation toward sampling capacities. New detection methods belonging to different approaches allowing the analysis of bulked samples, such as pyrosequencing-based techniques or spore sampling devices for the quantification of mixed populations, should then be considered. Finally, thresholds for the proportion of resistant isolates above which fungicide control is inadequate still need to be determined.

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Fig. 3. Sampling curves calculated under the assumption of a β -binomial distribution, using low, intermediate, and high values of parameter θ that correspond to the minimum, median, and maximum values of θ estimates obtained in our study, for three levels of precision given by coefficient of variation of the sample mean (CV) values of 10, 20, and 30%. Continuous, dotted, and dashed lines represent the minimum sample size required to estimate mean single-nucleotide polymorphism (SNP) incidence with a CV of 10, 20, and 30%, respectively.

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