Population Genetics and Fungicide Resistance of *Botrytis cinerea* on *Vitis* and *Prunus* spp. in California

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ABSTRACT

Botrytis cinerea, the causal agent of gray mold, has high genetic diversity and a broad host range. In *Vitis* sp. and *Prunus* spp., *B. cinerea* causes pre- and postharvest diseases, and fungicides are routinely applied to prevent yield loss. In total, 535 isolates of *B. cinerea* collected from *Vitis* sp. and *Prunus* spp. in 2012, 2016, and 2017 were genotyped using 18 microsatellite markers and the transposable elements (TEs) *Boty* and *Flipper*. Only nine of the polymorphic markers and the two TEs were considered informative and retained for the final analyses. Of the 532 isolates, 297 were tested for resistance to seven fungicides representing six Fungicide Resistance Action Committee classes. After clone correction, 295 multilocus genotype groups were retained across the 3 years in 326 individuals, and four genetic subpopulations were detected. High levels of clonality were observed across the dataset. Significant pairwise differentiation was detected among years, locations, and

TE composition. However, most of the diversity observed was within a subpopulation and not among subpopulations. No genetic differentiation was detected among resistant and sensitive isolates for individual fungicide classes. When resistance to the total number of fungicides was compared, regardless of the fungicide class, significant differentiation was detected among isolates that are resistant to two fungicide classes and those resistant to three or four fungicide groups. Fungicide resistance frequencies were stable for most chemistries evaluated with the exception of fluopyram, which increased from 2012 to 2016/2017.

Keywords: ascomycete, *Botrytis cinerea*, disease control and pest management, fungicide resistance, genetic diversity, gray mold, microsatellites, plant pathogen, population biology, *Vitis vinifera*

Botrytis cinerea, the causal agent of bunch rot and gray mold, results in serious loss from pre- and postharvest diseases in >200 economically important crop hosts worldwide and is a serious disease of wine and table grapes, citrus, fig, and stone fruits in California (Ma and Michailides 2005b; Saito and Xiao 2018). Although all parts of the phyllosphere of host plants are susceptible to *B. cinerea*, infection primarily occurs on leaves, buds, and fruit (Wessels et al. 2016; Williamson et al. 2007). In grapes, annual yield losses from gray mold can be 20 to 40%, and fungicide is commonly applied to mitigate disease (Hilton and Molloy 2018). The global annual cost to control *B. cinerea* (or gray mold) exceeds \$1 billion/year (approximately 10% of the world fungicide market), and management heavily relies on the use of synthetic fungicides (Leroux 2004; Yin et al. 2012).

Within *B. cinerea*, two groups (Group I and Group II) have been identified. Originally, the delineation was based on the presence or absence of two transposable elements (TEs), *Boty* (Diolez et al. 1995), and *Flipper* (Levis et al. 1997). It was later shown that *B. cinerea* is a complex of two sibling species (Group I: *Botrytis pseudocinerea* and Group II: *Botrytis cinerea sensu stricto*) or groups living in sympatry, morphologically indistinguishable, and

not delineated by TE absence/presence (Walker et al. 2011). However, TE presence is still more strongly associated with Group II than Group I, and it has been speculated that TE presence can affect fungicide sensitivity (Fournier and Giraud 2008; Fournier et al. 2013; Karchani-Balma et al. 2008; Martinez et al. 2008). Fungicide sensitivity differences exist between Group I and Group II isolates, with Group I isolates having a natural resistance to fenhexamid and a hypersensitivity to fenpropidin (Giraud et al. 1999; Leroux et al. 2010; Martinez et al. 2008).

Few studies have linked genetic diversity and population structure of Group II isolates with fungicide resistance, and limited associations have been observed (Hu et al. 2018; Kretschmer and Hahn 2008; Wessels et al. 2016). In a study of isolates collected from strawberry fields in Germany, researchers found a novel clade (*Botrytis* Group S) within Group II that was strongly correlated with resistance to multiple fungicides (Leroch et al. 2012). This group, which was predominantly observed on strawberries, had a low frequency in German vineyards but was detected in New Zealand vineyards (Johnston et al. 2013; Leroch et al. 2012; Rupp et al. 2016). In the southeastern United States, a study evaluating isolates from strawberry using microsatellites found a significant genetic difference between isolates with resistance to three or four classes of fungicides and those with resistance to five fungicide classes (Hu et al. 2018).

B. cinerea is a “high-risk” pathogen for the development of fungicide resistance owing to its rapid lifecycle, genetic diversity, and high fecundity (Atwell et al. 2015; Hahn 2014). Resistance to Fungicide Resistance Action Committee (FRAC) classes, including demethylation inhibitors, succinate dehydrogenase inhibitors (SDHIs), and quinone outside inhibitors (QoIs), has been reported worldwide (Hahn 2014; Zhang et al. 2016). The increasing prevalence of fungicide resistance in *B. cinerea* has become a serious limitation for effective disease control. In addition, management strategies often focus on *B. cinerea* for a single host

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without considering how surrounding sprays on other hosts might affect the development of fungicide resistance. *B. cinerea* field isolates resistant to one or more fungicide classes have been reported globally in economically important hosts, such as apple, grape, and strawberry (Rupp et al. 2017; Saito et al. 2016b; Weber 2011; Wessels et al. 2016; Yin et al. 2011).

Fenhexamid, a fungicide first registered in 1999, has had resistance reported globally. In *B. cinerea*, fenhexamid-resistant isolates from blueberry, fig, grape, kiwi, pea, and squash have been found at frequencies of 29% in the United States (Ma and Michailides 2005a; Saito et al. 2016b), 50% in French vineyards (Fillinger et al. 2008), and 35 and 45% in Chilean and German small fruit fields, respectively (Esterio et al. 2011; Weber 2011). This variability in fungicide resistance is not uncommon among isolates collected from different hosts, locations, and years (Fernández-Ortuño et al. 2014; Rupp et al. 2016; Yin et al. 2018). These differences in fungicide resistance profiles among locations and crops could be attributed, in part, to regional differences in management strategies for each host, but they may also be the result of limited gene flow among isolates from different crops or regions (Fournier and Giraud 2008).

Previous studies have shown high levels of genetic variation for *Botrytis* spp. among fields and even on a single plant (Fournier and Giraud 2008; Hu et al. 2018). Hu et al. (2018) showed diversity among isolates from the same location and even on the same host plant, with multiple haplotypes and species exhibiting different fungicide resistance profiles existing on the same plant tissue. Very little to no differentiation has been reported in *B. cinerea* populations

at the regional or national scale (Fournier and Giraud 2008; Giraud et al. 1999; Keressies and Bosker-Van Zessen 1997; Ma and Michailides 2005b; Muñoz et al. 2010; Walker et al. 2014; Wessels et al. 2013). In contrast, high levels of differentiation have been reported between populations from different continents collected from Australia and South Asia (Isenegger et al. 2008) as well as Argentina and France (Muñoz et al. 2010). It is important to note that these differences could, in part, be attributed to differences in host plants and molecular markers (Walker et al. 2011).

Understanding adaption to the host is a key issue for “generalist” pathogens, like *B. cinerea*, particularly as it relates to disease management. Although TEs should not be considered a standalone marker for determination of species delimitation, studies have shown that *transposa* strains predominate in populations on grape, strawberries, and tomatoes, whereas *vacuma* strains (isolates without either of two diagnostic TEs present) of *B. cinerea* dominate populations from kiwi and apple (Esterio et al. 2011; Johnston et al. 2013; Martinez et al. 2005; Muñoz et al. 2002). Studies using simple sequence repeat or microsatellite markers further supported these results by recognizing host as the major factor affecting structure of populations at scales greater than geographic location (Fournier and Giraud 2008; Walker et al. 2014). Despite evidence of host as a structuring factor, California *B. cinerea* populations showed no host-related differentiation (Ma and Michailides 2005b). However, in that study, unique microsatellite markers were used, limiting comparisons with other studies, and isolates from many of the hosts were collected from a single field location.

The objectives of this research were to use previously published (Fournier et al. 2002) and novel microsatellite markers to characterize the genetic diversity, fungicide resistance, and population structure of *B. cinerea* populations from California. Specifically, we wanted to determine genetic differentiation and diversity within and among *B. cinerea* populations from (i) different hosts and (ii) years as well as (iii) fungicide resistance frequencies.

MATERIALS AND METHODS

Isolate collection and DNA extraction. In total, 532 isolates of *B. cinerea* from grape and stone fruits (nectarine and apricot) were collected from spatially distinct regions of California from 2012 to 2017 (Fig. 1 and Table 1). Isolates were collected at harvest or postharvest. A minimum of 10 to 20 isolates representing each subpopulation (individual vineyard each year) were collected following practices as described by Grünwald et al. (2017), with the exception of isolates in 2012, which were collected as described by

Fig. 1. Population sampling locations in the San Joaquin Valley, California for isolates of *Botrytis cinerea* on *Prunus* spp. (squares) and *Vitis* sp. (circles). Outlined groupings indicate field locations combined for regional analyses in STRUCTURE.

TABLE 1. Year, location, and number of isolates of *Botrytis cinerea* collected from *Prunus* and *Vitis* hosts in California for population genetic analysis studies

Year	Location	Host	Number of isolates ^a	Number of isolates cc ^b
2012	D1	<i>Vitis</i>	28	25
2012	D2	<i>Vitis</i>	19	18
2012	E1	<i>Vitis</i>	29	19
2012	F1	<i>Vitis</i>	28	18
2016			34	26
2017			155	42
2016	M1	<i>Prunus</i>	13	11
2012	M2	<i>Vitis</i>	36	24
2012	M3	<i>Vitis</i>	22	16
2012	P1	<i>Vitis</i>	33	25
2016	P2	<i>Vitis</i>	33	26
2017			16	6
2012	R1	<i>Vitis</i>	29	16
2016	V1	<i>Prunus</i>	21	19
2012	X1	<i>Vitis</i>	30	24

^a Number of isolates collected.
^b Number of isolates remaining for each location after clone correction.

Saito et al. (2016a, 2019). Isolates from 2016 and 2017 were collected from symptomatic fruit by plating a 3 × 3 cm portion of fruit tissue onto a half-strength potato dextrose agar (PDA; Difco Laboratories) plate. Cultures were incubated at 25°C in the laboratory under constant light. Visible *Botrytis* colonies from each isolation were transferred to a fresh PDA plate for monoconidial isolation. After actively growing colonies sporulated, conidia were scraped from the surface of the PDA using a sterile inoculation loop and suspended in 1 ml of sterile double-deionized water (ddH₂O). The conidial suspension (100 µl) was spread onto water agar plates and incubated at 22°C overnight. Germinated conidia were transferred to fresh PDA plates, and resulting monoconidial cultures were used for subsequent analyses. Fungal cultures grown on half-strength PDA plates at 25°C for 10 days were used for DNA extraction following a modified genomic DNA mini-prep protocol (Lee et al. 1988). In brief, cultures were scraped to remove conidia and surface mycelia (0.02 to 0.04 g) using a sterile applicator stick and transferred into 1.5-ml Eppendorf tubes containing 246.9 µl of lysis buffer (150 µl of ddH₂O, 25 µl of 0.5 M EDTA [pH 8], 25 µl of 1.0 M Tris, 43.75 µl of 20% sodium dodecyl sulfate solution, 3.15 µl of 20 mg/liter proteinase K, and 0.0025 g of NaHSO₃). Sample tubes were vortexed for 1 min, incubated at 65°C for 15 min, and centrifuged (13,978 × *g* for 5 min). The precipitates were discarded, and supernatants were transferred to new 1.5-ml Eppendorf tubes. A solution of 50 µl of 7.5 M NH₄OAc was added to each tube, vortexed for 10 s, and chilled on ice for 15 min. Samples were centrifuged (13,978 × *g* for 3 min). Supernatants were again transferred to new 1.5-ml Eppendorf tubes, and 175 µl of isopropanol was added; tubes were mixed and centrifuged (13,978 × *g* for 5 min), and the supernatant was discarded. Final genomic DNA from each isolate was rinsed twice with 500 µl of 70% ethanol solution, dried, resuspended with 50 µl of ddH₂O, and incubated at 30°C for 10 min. DNA concentration was standardized to 50 ng/µl using a Nanodrop 8000 spectrophotometer (Thermo-Fisher Scientific Inc.).

Fungicide resistance frequencies. For in vitro fungicide sensitivity tests, agar media amended with seven technical-grade fungicides were used. To prepare stock solutions, boscalid (an SDHI; FRAC 7, 99% active ingredient [a.i.]; BASF Corporation), cyprodinil (an anilinopyrimidine; FRAC 9, 98% a.i.; Syngenta Crop Protection), fludioxonil (a phenylpyrrole; FRAC 12, 93% a.i.; Syngenta Crop Protection), fluopyram (an SDHI; 97.8% a.i.; Bayer CropScience), pyraclostrobin (a QoI; FRAC 11, 98% a.i.; BASF Corporation), and thiabendazole (a Methyl Benzimidazole Carbamate; FRAC 1, 99% a.i.; Sigma-Aldrich) were dissolved in 100% acetone. Fenhexamid, a KetoReductase Inhibitor (FRAC 17, 95% a.i.; Arysta LifeScience), was dissolved in 100% ethanol. The final concentration of acetone or ethanol in all media was the same, and it never exceeded 0.1% (vol/vol).

In total, 297 isolates from 2016 and 2017 were used in the in vitro fungicide sensitivity tests, and an additional 201 isolates from 2012 previously evaluated for sensitivity to six fungicides were also included in the study to evaluate temporal changes (Saito et al. 2019). Sensitivities of *B. cinerea* isolates to each class of fungicides were tested following the procedures described by Weber and Hahn (2011). Fenhexamid and fludioxonil were tested on 1% malt extract agar (MEA). For pyraclostrobin, the alternative oxidase inhibitor salicyl hydroxamic acid was added to MEA at 100 µg/ml (Mondal et al. 2005; Wise et al. 2008). To exclude amino acids from the cyprodinil assay (Myresiotis et al. 2007), 0.5% sucrose agar was used instead of MEA. For boscalid, 0.5% yeast extract agar was used to avoid the interference of sugars with the assay (Stammler and Speakman 2006). Conidia were harvested by flooding 1- to 2-week-old PDA cultures of *B. cinerea* growing on 9-cm-diameter petri dishes with a sterile scraper and suspended in ddH₂O. The resultant conidial suspension was filtered through autoclaved gauze. The conidial concentration was then quantified microscopically with a hemocytometer and diluted to the desired concentration

described below. Conidial suspensions were prepared immediately prior to inoculation and placed on ice until use.

For each isolate, an aliquot of 10 µl of conidial suspension (approximately 1.0 × 10⁵ conidia per milliliter) was streaked on each agar plate with or without fungicide (control). After 14 to 16 h of incubation at 20°C in darkness, the germ tube growth of 20 representative conidia was measured under a microscope. All experiments were performed twice. The discriminatory concentration of each fungicide and the criteria for the classification of fungicide sensitivity phenotypes were based on Weber and Hahn (2011), with slight modifications (Saito et al. 2016b, 2019). Briefly, except fluopyram, two different concentrations for each class of fungicide used in this study were as follows: for boscalid, 1 and 50 µg/ml; for cyprodinil, 1 and 25 µg/ml; for fenhexamid, 1 and 50 µg/ml; for fludioxonil, 0.1 and 10 µg/ml; and for pyraclostrobin, 0.1 and 10 µg/ml. For boscalid, isolates with a fully grown germ tube at 1 µg/ml and >30% of growth relative to the control were classified as resistant, whereas sensitive isolates showed <50% of growth at 1 µg/ml. For cyprodinil, isolates that showed >50% of growth at 1 µg/ml and almost no growth at 25 µg/ml in relation to those grown on the control were classified as resistant, whereas sensitive isolates grew <50% at 1 µg/ml and showed no growth at all at 25 µg/ml. For fludioxonil, isolates that grew >50% at 0.1 µg/ml and had almost no growth at 10 µg/ml were classified as resistant, whereas sensitive isolates showed almost no growth at 0.1 µg/ml. For fenhexamid, isolates that showed >50% of growth at 0.1 µg/ml and <50% at 50 µg/ml were classified as resistant, whereas sensitive isolates showed <50% of growth at 0.1 µg/ml and little or no growth at 50 µg/ml. For pyraclostrobin, isolates with a fully grown germ tube at 10 µg/ml were classified as resistant, whereas isolates that grew <50% at 0.1 µg/ml were classified as sensitive. For fluopyram, isolates that showed full growth at 0.5 µg/ml were classified as resistant, whereas sensitive isolates showed little or no growth at the same concentration. Associations among fungicide resistance and TE presence/absence were determined by Pearson's correlation using Proc Corr in SAS (V9.3; Statistical Analysis Software).

Microsatellite discovery and marker development. Microsatellites were selected from the *B. cinerea* genome (isolate B05.10) publicly available at Ensembl (<http://fungi.ensembl.org/index.html>) using the updated assembly (Amselem et al. 2011; Staats and van Kan 2012). Single copy genes flanked by Microsatellites with trinucleotide repeat motifs or greater were identified using the Microsatellite identification tool, MISA (Thiel et al. 2003). Primers were developed by integrating MISA results with Primer3 (Koressaar and Remm 2007; Untergasser et al. 2012) using Perl scripts modified from MISA (<https://webblast.ipk-gatersleben.de/misa/>). Predicted primer product size was restricted to 100 to 280 bp and an annealing temperature of 60°C.

Eighty-eight primer pairs, including previously published markers (Supplementary Table S1), were initially evaluated on a subset of 15 *B. cinerea* isolates (Supplementary Table S2) representing different years, locations, and hosts to detect potential PCR product band variability for each marker. PCR was carried out in 25-µl reactions with 12.5 µl of 2X GoTaq Green Master Mix (Promega Corp.), 1.25 µl of 10 µM forward primer, 1.25 µl of 10 µM reverse primer (Invitrogen Inc.), 8.0 µl of ddH₂O, and 2.0 µl of approximately 50.0 ng/µl of DNA template. Reaction conditions were 95°C for 2 min followed by 29 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 30 s and then, a final extension at 72°C for 5 min. Amplification of PCR products within the expected size range was confirmed by gel electrophoresis run at 95 V (4.75 V/cm) on a 2% (wt/vol) agarose gel (Cambrex BioScience Rockland, Inc.) for 2.5 h using a 100-bp size standard (New England Biolabs Inc.).

Eighteen primer sets that successfully amplified the 15 *B. cinerea* isolates and showed potential polymorphism were scored on the remaining 517 isolates (Supplementary Table S3). A three-primer

method (Schuelke 2000) was used in the first round of the marker examination on the subset of 15 isolates. The forward primer for each candidate marker had an 18-bp M13 tag (5'-TGTTAAACGA CGGCCAGT-3') attached to the 5' end. The reverse primer contained a "pigtail" sequence (5'-GTTTCTT-3') on the 5' end, which was added to promote adenylation and reduce stuttering (Brownstein et al. 1996). The third primer consisted of the M13 tag labeled with a 6-FAM fluorescent dye (Invitrogen Inc.) on the 5' end.

The PCR volume was 25 µl and contained 12.5 µl of 2× GoTaq Green Master Mix (Promega Corp.), 0.75 µl of 10 µM forward primer, 2.0 µl of 10 µM reverse primer, 1.25 µl of 10 µM 5' 6-FAM-labeled M13 tag primer (Invitrogen Inc.), 6.5 µl of ddH₂O, and 2.0 µl of approximately 50.0 ng/µl of DNA template. Reaction conditions were 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at temperature varied between 50 and 60°C (specific to optimal temperature based on primer design) for 30 s, and extension at 72°C for 30 s and then, a final extension at 72°C for 5 min. One microliter of a 1:10 dilution of PCR product was added to 0.3 µl of GeneScan 500 LIZ-labeled size standard and 9.7 µl of Hi-Di formamide (Applied Biosystems Inc.). Amplicons were denatured by incubation at 95°C for 5 min and immediately placed on ice. Fragment analysis was conducted on an Applied Biosystems 3730xl 96-capillary DNA Analyzer. Geneious v.11.0.3 (Kearse et al. 2012) was used to determine allele sizes based on electropherograms.

TR PCR and analysis. Two previously published primer sets for TEs (*Boty* and *Flipper*) were utilized to assess distribution for each TE among the isolates representing the 295 multilocus genotypes (MLGs) (Martinez et al. 2008). Each isolate was amplified using a single-plex PCR reaction for both primer sets. For each isolate, DNA concentrations standardized to 50 ng/µl were used in a 25-µl PCR reaction with 12.5 µl of 2× GoTaq Green Master Mix (Promega Corp.), 1.50 µl of 10 µM forward primer, 1.50 µl of 10 µM reverse primer (Invitrogen Inc.), 8.0 µl of ssdH₂O, and 1.5 µl of DNA template. Cycling conditions were carried out as previously described, with exception of annealing at 60°C for 30 s. Amplification of PCR products within the expected size ranges (510 and 1,250 bp) was confirmed by gel electrophoresis conditions previously stated.

After all isolates were amplified using both primer sets, inspection of gel images for amplicons was assessed based on four distinct presence or absence patterns. Isolates observed with a single amplicon size respective of the primer set were categorized as either *Flipper* or *Boty* depending on the primer set used. Isolates with amplicons from both PCR reactions were considered *transpos* type, and isolates producing no products for either primer set were considered to be *vacua* type (Diolez et al. 1995; Fournier and Giraud 2008; Levis et al. 1997; Walker et al. 2011).

Multiplex PCR and analysis. All 18 primer sets consistently produced peaks within the expected size range for the 15 isolates, and they were tested using multiplex and single-plex PCR on the remaining 517 isolates. Five multiplex reactions (multiplex 1: Bc_5, Bc_24, Bc_37, and Bc_75; multiplex 2: Bc_3, Bc_26, Bc_30, and Bc_54; multiplex 3: Bc_4, Bc_32, and Bc_74; multiplex 4: Bc_27 and Bc_29; and multiplex 5: Bc_10 and Bc_18) were used to genotype the panel of isolates. Forward primers of the Microsatellite

markers selected for multiplex PCR were labeled at the 5' end with one of three fluorescent dyes from the DS-33 dye set: 6-FAM, VIC, PET, or NED (Invitrogen Inc.). Multiplex reactions were optimized so that loci with alleles of similar size were labeled with different dyes. Product size ranges were identified based on the PCR reactions established from the original panel of 15 isolates during marker testing. All 532 isolates were genotyped with the 15 markers tested using the multiplex reactions and 3 markers (Bc_6, Bc_8, and Bc_67) utilizing single-plex reactions. Multiplex PCR was conducted using the Type-it Microsatellite PCR kit (Qiagen) in 10-µl reactions with 5 µl of 2× Type-it Master Mix buffer, 1 µl of 10× primer mix (2 µM each primer in the multiplex), 1 µl of sterile distilled H₂O, 1 µl of Q-Solution, and 2 µl of approximately 50.0 ng/µl of DNA template. Reaction conditions were 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30s and then, a final extension at 72°C for 5 min. The PCR products were prepared as described above, and fragment analysis was conducted on an Applied Biosystems 3730xl 96-capillary DNA Analyzer.

Analyses used and described are based on 326 individuals retained in the clone-corrected data. Genetic population indices were calculated using *Poppr* (Kamvar et al. 2014) within R (v3.4.4) statistical software (R Core Team 2019), including number of observed and expected MLG (genotypic richness), number of clone-corrected MLG, rarefaction statistics, genotypic evenness (*E*₅), genotypic abundance of MLG diversity (Shannon–Wiener Index [*H*]) (Shannon 2001), Stoddart and Taylor's index of MLG diversity (*G*) (Stoddart and Taylor 1988), Simpson's index (*λ*) (Simpson 1949), Nei's unbiased gene diversity (*H*_{exp}) (Nei 1978), and the linkage disequilibrium (index of association [*I*_A]) (Brown et al. 1980). Analysis of molecular variance (AMOVA) was performed using *Poppr*. Genetic diversity was determined based on MLGs and allele frequency in the populations based on the nine microsatellite markers and two TE markers shown to be informative using *Poppr* (Kamvar et al. 2014).

Using the software GenAIE 6.05 (Peakall and Smouse 2012), pairwise population genetic identity among and between populations based on location, year, host, and fungicide resistance frequencies was calculated, and significance was determined (based on 999 permutations). Population differentiation was tested for statistical significance (based on the mean and 95% confidence interval). Genetic differentiation (*F*_{ST}) among subpopulations was classified as little, moderate, great, or very great as described by Wright (1978) and implemented by Hartl and Clark (2007). Subpopulations with <11 individuals were not evaluated except as part of a larger group (e.g., year or host level).

Population structure was estimated using STRUCTURE v2.3 (Pritchard et al. 2000) and *k* (number of subpopulations present) determined by the log likelihood difference as described by Evanno et al. (2005) using STRUCTURE Harvester (Earl and vonHoldt 2012). In brief, a single population was used to determine *λ* for subsequent testing. A total of 30 *k* values were tested in STRUCTURE using the admixed model with correlated allele frequencies (Falush et al. 2003). The admixed model with correlated allele frequencies was chosen based on results from linkage disequilibrium testing within *Poppr* to determine the subpopulation number. Each *k* value was tested with a burn in of 10,000 Markov Chain Monte Carlo

TABLE 2. Number of isolates of *Botrytis cinerea* evaluated for sensitivity to seven different fungicides from *Prunus* and *Vitis* hosts in California

	Fungicide						
Phenotype ^a	Boscalid	Cyprodinil	Fenhexamid	Fluopyram	Fludioxonil	Pyraclostrobin	Thiabendazole
S	57	203	264	278	296	28	32
R	233	87	33	12	1	269	61
Total ^b	290	290	297	290	297	297	93

^a Fungicide sensitivity is designated as sensitive (S) or resistant (R).

^b Total number of isolates evaluated for sensitivity to each fungicide.

(MCMC), 50,000 replicate MCMC, and a λ of 0.55527. The best k was based on results from STRUCTURE Harvester (Earl and vonHoldt 2012) (Supplementary Fig. S1). Clusters were visualized by category using a custom R script (Jarrod Morrice), which sorted individual members of the population based on predefined categories within R statistical software (v3.1.1).

RESULTS

Fungicide resistance. Fungicide sensitivity varied greatly among the 297 isolates (Table 2). All of the fungicides evaluated were labeled for both crops, with the exception of fludioxonil. Most isolates (91.6%) were resistant to one or more of the fungicides evaluated, with only eight isolates being sensitive to all seven fungicides tested. When years were combined, isolates with resistance to two fungicides were the most common (38.7%) followed by resistance to three (27.6%), four (12.1%), one (10.1%), and five (0.03%) fungicides. The remaining isolates were sensitive to the fungicides evaluated, but those isolates were not evaluated against all seven fungicides. Fungicide resistance frequencies

TABLE 3. Fungicide resistance frequency among isolates of *Botrytis cinerea* compared across years (isolates were collected from *Prunus* and *Vitis* hosts in California)

Year	N ^a	Fungicide, %						
		Bos ^b	Cyp	Fen	Flu	Flud	Pyr	Thia
2012	201	84.58	23.38	12.94	0.50	0.00	94.03	ND ^c
2016	75	58.67	50.67	9.33	12.00	1.33	80.00	54.67
2017	19	100.00	10.53	0.00	10.53	0.00	100.00	94.74

^a Total number of isolates evaluated for sensitivity to each fungicide.
^b Fungicides evaluated included boscalid (Bos), cyprodinil (Cyp), fenhexamid (Fen), fluopyram (Flu), fludioxonil (Flud), pyraclostrobin (Pyr), and thiabendazole (Thia).
^c No data are available on Thia resistance from 2012.

compared across years showed differences among years, with a notable increase in detection of isolates resistant to fluopyram and fludioxonil in 2016 and 2017 (Table 3). A single isolate of *B. cinerea* from grape with resistance to fludioxonil was detected in 2016; this was the first report of fludioxonil resistance in grape in California. Resistance to multiple fungicides varied from 2012 to 2017, with resistance to two fungicides being most common in 2012. When isolates from 2016 and 2017 were combined, resistance to three fungicides was most common.

Genotypic diversity and population structure. The nine polymorphic microsatellites and two TEs readily differentiated most isolates, with the number of alleles detected per marker ranging from 7 to 15 and having a mean number of 8 alleles per marker (Supplementary Table S4). Based on all clone-corrected populations, 296 different MLGs were identified, with 323 identified when separated by year (Table 4). Genotypic richness, measured as the expected number of MLGs after rarefaction, was similar across years. Indices of genotypic diversity (genotypic richness and abundance) were high across populations when grouped by year for Shannon–Weiner Index (H), Stoddart and Taylor’s index (G), Simpson’s index (λ), and Nei’s unbiased measure of gene diversity (H_{exp}) (Table 4). Evenness (E) was uniform across all three populations (average = 0.942). The index association (I_A) indicated a high level of clonality (0.601) across years. The standardized index value ($\bar{r}_d$) was significant ($P = 0.001$) and low (0.0605), also indicating linkage among markers, which suggests clonality. AMOVA indicated generally low differentiation among populations based on year (4.13%), field (2.52%), TE presence (17.8%), and host (1.99%). Only year and TE were significant at $P = 0.05$ (Table 5).

STRUCTURE assigned the 319 isolates retained after clone correction with full passport information (host, year, field, and TE status) into a proportion of membership within four subpopulations (Supplementary Table S5). Individual isolates were classified as admixed (belonging to no single cluster) or assigned to an

TABLE 4. Population genetic statistics summarizing characteristics of genetic variation within populations of *Botrytis cinerea* based on year of collection using microsatellite and transposable element markers (isolates were collected from *Prunus* and *Vitis* hosts in California, and statistics are based on clone-corrected data)

Population	N ^a	MLG ^b	eMLG ^c	SE ^d	H ^e	G ^f	λ ^g	E ^h	H _{exp} ⁱ	I _A ^j	$\bar{r}_d$ ^k	P $\bar{r}_d$ ^l
2012	186	186	49	0.000	5.23	186	0.995	1.000	0.632	0.501	0.0505	0.001
2016	91	87	49	0.883	4.45	83.6	0.988	1.000	0.620	0.631	0.0639	0.001
2017	49	49	49	0.000	3.89	49	0.980	1.000	0.503	1.130	0.1142	0.001
Total ^m	326	295	48.2	0.863	5.65	267	0.996	0.942	0.626	0.601	0.0605	0.001

^a Number of isolates.
^b Number of multilocus genotypes (MLGs) observed.
^c Number of expected multilocus genotypes (eMLGs) at the smallest sample size.
^d Standard error (SE) based on eMLG (Simpson 1949).
^e Shannon–Wiener Index of MLG diversity (Shannon 2001).
^f Stoddart and Taylor’s index of MLG diversity (Stoddart and Taylor 1988).
^g Simpson’s index (Simpson 1949).
^h Genotypic evenness.
ⁱ Nei’s unbiased measure of gene diversity (Nei 1978).
^j Index of association (Brown et al. 1980).
^k Standardized index of association.
^l P value for $\bar{r}_d$.
^m Total or average.

TABLE 5. Analysis of molecular variance of populations of *Botrytis cinerea* isolates collected from *Prunus* and *Vitis* hosts in different geographic regions of California and stratified by year of collection (2012, 2016, and 2017)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variation, % ^a	P value ^b
Between years ^c	2	70.40	35.20	4.13	0.001
Within years ^d	323	2,258.77	6.99	95.87	
Total	325	2,329.17	7.167	100	

^a Percentage of variation of the total when stratified by year.
^b Degree of statistical significance based on 999 permutations.
^c Variation between years.
^d Variation within samples across years.

individual cluster based on a minimum of 60% membership within a single cluster. Individuals from Cluster 1 were the least common with only 26 isolates. Cluster 2 was the dominant subpopulation with 105 isolates, whereas Clusters 3 and 4 had 89 and 70 isolates, respectively. Only 29 isolates were admixed (having <60% identity with a single cluster) (Supplementary Table S5).

Pairwise comparisons (F_{ST}) indicated significant differentiation among years, location, and fungicide sensitivity. The 2017 population was differentiated ($P \leq 0.001$) from both the 2012 (0.2595) and 2016 (0.2402) populations (Fig. 2A). The 2016 and 2017 populations were not significantly differentiated from each other; this was consistent with the results of the AMOVA in which greater variation existed within compared with among years (Table 5). Cluster 1 individuals were found at a higher frequency in 2017 than 2016 and 2012, whereas Cluster 4 individuals were not detected in 2017.

Fig. 2. STRUCTURE results for *Botrytis cinerea* grouped by year of isolate collection. Each vertical line represents a single isolate, and the colors represent different genetic clusters. Proportion of membership within a cluster is based on a 0 to 1 scale, where 0 indicates no representation and 1 equals 100% representation. Cluster 1 (purple), Cluster 2 (yellow), Cluster 3 (light blue), and Cluster 4 (steel blue) are grouped by **A**, year, **B**, location, and **C**, presence or absence of transposable elements. Isolates of *B. cinerea* were collected from *Prunus* and *Vitis* hosts from different locations in different geographic regions in California.

For isolate source host, no significant differentiation was seen between the genera *Prunus* and *Vitis* when all locations and years were combined. All clusters were represented by both hosts, although Cluster 4 was represented at a lower proportion in the genus *Prunus* than in the genus *Vitis*. Because host and geography were linked, regional- and field-level variability were also examined. Field to field comparisons showed high levels of differentiation among isolates collected in Visalia (V1), Exeter (X1), and field 2 in Madera (M2) from most other sites (Table 6). Isolates from V1 and M2 were collected from *Prunus* spp.; however, X1 isolates were from grape. When isolates were compared at a more regional level (Fig. 1), Fresno isolates were significantly differentiated from those from most other regions, including Madera, Delano, Exeter, Parlier, and Pixley (Fig. 2B), and Madera was significantly differentiated from Pixley, Parlier, and Fresno. However, the genetic differentiation was relatively low ($F_{ST} < 0.05$) among regional groups.

TE presence was compared among isolates. Most isolates (61.4%) of the 319 MLGs retained were considered *transposon*, with both *Boty* and *Flipper* present. The remaining isolates grouped into categories of only *Boty* (27.2%), only *Flipper* (0.04%), or neither TE (*vacuna*; 0.08%). Little differentiation ($F_{ST} < 0.05$) was detected among these groups, with most cluster variation present within each TE class (Fig. 2C and Table 7).

Genetic differentiation in sensitivity to the fungicides thiabendazole, fenhexamid, and cyprodinil was compared. Sample sizes were too disparate among resistant and sensitive isolates for the remaining fungicides to be evaluated (Table 2). None of the fungicides evaluated showed significant differentiation between resistant and sensitive isolates based on microsatellite markers. Resistance to multiple fungicides was compared among isolates based on the total number of fungicides to which an isolate exhibited resistance. Significant differentiation was detected between resistance to multiple fungicides group 2 and all other resistance to multiple fungicides groups (0, 1, and 3 to 5). This differentiation was limited but significant (<0.02 , $P > 0.05$). When resistance to multiple fungicides groups were compared for association with TE presence (*transposon*), absence (*vacuna*), and composition (*Boty* or *Flipper* only), no significant correlation was observed (data not shown).

TABLE 7. Pairwise differentiation (F_{ST}) among transposable elements in *Botrytis cinerea* using microsatellite markers (isolates of *B. cinerea* were collected from *Prunus* and *Vitis* hosts from different locations in different geographic regions and years in California)

Transposable element	<i>Flipper</i>	<i>transposon</i>	<i>vacuna</i>
<i>transposon</i>	-0.001	–	–
<i>vacuna</i>	0.007	0.014 ^a	–
<i>Boty</i>	-0.008	0.005 ^a	0.001

^a Degree of statistical significance (P value ≤ 0.05) based on 999 permutations.

TABLE 6. Pairwise differentiation (F_{ST}) of locations from which isolates of *Botrytis cinerea* were collected based on microsatellite and transposable element markers (isolates were collected from *Prunus* and *Vitis* hosts in different geographic regions and years in California)

Field	F1 ^a	V1	M1	P2	D2	D1	P1	E1	M2	M3
V1	0.173 ^b									
M1	0.113	0.142								
P2	0.054	0.245 ^b	0.101							
D2	0.001	0.206 ^b	0.124	0.050						
D1	0.054	0.098	0.154	0.123	0.047					
P1	0.010	0.161 ^b	0.117	0.081	0.012	0.024				
E1	0.066 ^b	0.229 ^b	0.188 ^b	0.037	0.043	0.133 ^b	0.105 ^b			
M2	0.123 ^c	0.282 ^b	0.187 ^b	0.089	0.078	0.198 ^b	0.144 ^b	0.049		
M3	0.067	0.194 ^b	0.131	0.113	0.051	0.075	0.039	0.159 ^b	0.187 ^b	
R1	0.012	0.213 ^b	0.090	0.014	0.025	0.090	0.018	0.0232	0.048	0.044

^a Indicates the location where isolates were collected. Designations are as parented in Figure 1.

^b Degree of statistical significance (P value ≤ 0.05) based on 999 permutations.

^c Degree of statistical significance (P value ≤ 0.001) based on 999 permutations.

DISCUSSION

B. cinerea is a devastating plant pathogen with a broad host range and high genetic diversity. This genetic diversity has contributed to the rapid development of resistance to multiple fungicides and the overwhelming of host defenses, making disease management challenging for many crops. In isolates collected from grape in 2012, Saito et al. (2019) demonstrated that 100% of isolates evaluated were sensitive to fluopyram and fludioxonil. Six years later, our data show that resistance to both fungicides was detected in grape vineyards in California. The populations sampled showed 10 to 12% of isolates resistant to fluopyram, which had no history of use on grapes in California before 2015. Resistance to fludioxonil seems to be rare because only one isolate with resistance was detected, and that isolate was sensitive to all other fungicides tested. This is similar to a 2019 study on strawberry in which fludioxonil resistance was found in only 2% of the isolates tested (Cosseboom et al. 2019). Some of the differences in fungicide resistance among years (2012 versus 2016/2017) and with other chemistries could be, in part, because of the inclusion of *Prunus* spp. in this study. Host differences and fungicide usage patterns on different crops might contribute to fungicide resistance among isolates because not all classes of fungicides have been registered for as long on both *Prunus* and *Vitis* hosts. However, resistance to multiple fungicides was common across years and hosts. Among the 2012 isolates, resistance to two fungicides was most common, but in 2016/2017, resistance to three fungicides was most common. These data suggest that pathogen populations could continue to gain multiple fungicide resistances as new fungicides are rotated into management programs and that resistance to many of these classes of fungicide may not have a significant fitness cost. This is similar to results from Germany and the southeastern United States, where the frequency of isolates with resistance to multiple fungicides increased over time in horticultural crops (Hu et al. 2018; Rupp et al. 2016). When comparing multiple fungicide sensitivity, resistance to two fungicides was the most common phenotype. When populations with resistance to three or more fungicides were compared, population structure changed little, supporting the theory that genetic diversity may not significantly decrease as fungicide resistance is gained.

Although it has been postulated that TE composition could affect fungicide sensitivity (Leroux et al. 2010; Martinez et al. 2008), it is unclear if composition could influence development of resistance to multiple fungicides. Based on our results, the presence or absence of TEs seems to have no association with the frequency of multiple fungicide resistance and only a modest relationship with population structure. *B. cinerea* isolates from California were previously found to contain primarily the TE *Boty* or both *Boty* and *Flipper*, with only one isolate described as *vacua* (Ma and Michailides 2005a, b). Our data showed that most isolates (61.4%) had both TEs present or just *Boty* (27.2%), with only a small portion having neither TE present (0.08%) or only *Flipper* (0.03%). This was a smaller proportion of isolates (61.4%) exhibiting the TE *transposa* compared with in the previous study (83%), even when only comparing isolates collected from the Parlier/Reedley regions. These changes could be owing to the differences in sampling strategies (host versus regions) between the two studies or changes that have occurred in *B. cinerea* populations across the past decade. In our study, clonality was high when compared across years, indicating that these genotypes are relatively stable across years and locations in California, despite having high genetic diversity. This was further evident in subpopulation structure, where few individuals (9%) had admixed lineages across years and locations.

Previous studies have shown that genetic diversity within *Botrytis* subpopulations is greater than genetic diversity among subpopulations. In this study, differentiation could be seen among subpopulations from individual fields, but it was mostly masked at higher-level (e.g., regional and host) comparisons. This could be because of limited sampling in some of the regions or temporal

differences in the year that isolates were collected. For example, the Visalia location, which consisted of isolates collected from the genus *Prunus*, was differentiated from most other locations when grouped across years, with the exception of Madera (also *Prunus* isolates) and Dinuba. Yet, when compared by host, the genus *Prunus*-derived isolates (31) were not differentiated from grape-derived isolates (286). Large differences in sample numbers and the temporal/geographic differences in isolate collection could mask subtle differentiation between these two hosts. In 2005, Ma and Michailides (2005a, b) looked at the genetic structure of *B. cinerea* populations within the Reedley/Parlier and Kern County regions based on different hosts of origin, including grape. That study found that location and not host was the primary differentiator among subpopulations, and differences among region were quite small ($F_{ST} < 0.05$). In our study, although some fields did exhibit high differentiation ($F_{ST} > 0.20$), when a more regional scale was evaluated, F_{ST} values were much lower. Isolates collected across 3 years at Fresno from grape were the most diverse, but also, they had the greatest number of individuals representing that region. This suggests that greater sampling for any given region could reveal a fairly homogenous mix of MLG groups across central California, and more replicated and structured sampling for both hosts and locations is needed.

The Central Valley of California produces many agricultural crops susceptible to *B. cinerea*, which are often grown in close proximity. Regional management strategies should consider the diversity of susceptible hosts and adjust fungicide programs to develop more sustainable solutions in regard to development of fungicide resistance. This study provides a broad evaluation on the prevalence of fungicide resistance and population structure in California isolates of *B. cinerea* from two perennial crops. However, to better understand the genetic diversity and development of fungicide resistance in multihost environments, future work should sample more isolates from more hosts to gain more insight into these relationships.

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