Temporal Genetic Differentiation of *Cercospora beticola* Populations in New York Table Beet Fields

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Abstract

Annual epidemics of *Cercospora* leaf spot (CLS), caused by the fungus *Cercospora beticola*, can result in substantial defoliation in table beet fields in New York. High allelic and genotypic diversity have been described within *C. beticola* populations; however, information on the temporal stability of populations is lacking. *C. beticola* isolates were obtained from symptomatic leaves in three table beet fields in successive years. Two of the fields were organic mixed-cropping farms and the third was managed conventionally in a broad-acre cropping system. *C. beticola* isolates ($n = 304$) were genotyped using 12 microsatellite markers. Genotypic diversity (Simpson's complement index = 0.178

to 0.990), allele frequencies, and indices of differentiation between years varied. Pairwise index of differentiation values ranged from 0.02 to 0.25 for clone-corrected data, and indicated significant genetic differentiation at Farm 2. No multilocus genotype was shared between years. The shift in multilocus genotypes between years questions the role of clonally reproducing primary inoculum. Collectively, these results suggest that a dominant inoculum source for initiating annual CLS epidemics is external to the field of interest. These findings have implications for CLS disease management in conventional and organic table beet production.

Cercospora leaf spot (CLS) of table beet (*Beta vulgaris* ssp. *vulgaris* L.), caused by *Cercospora beticola* Sacc., is a destructive foliar disease of table and sugar beet worldwide (Franc 2010; Lartey et al. 2010; McGrath et al. 2007). In the CLS-sugar beet pathosystem, the disease causes reductions in root weight and extractable sucrose (Franc 2010; Shane and Teng 1992; Smith and Martin 1978; Smith and Ruppel 1973). In broad-acre table beet fields, defoliation results in the inability to harvest by top-pulling machinery, which may result in complete crop loss. For fresh market table beet producers, the presence of foliar disease may lead to consumer rejection. In either system, the tolerance for foliar disease is low (Pethybridge et al. 2017b). New York is the second largest producer of table beets in the United States (USDA 2012), where production may be conventional or organic in broad-acre fields or small diversified farms. Broad-acre production of table beet may be for processing or value-added fresh market products. Production in small diversified farms is usually for fresh market sales direct to consumers (Pethybridge et al. 2017b).

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Management recommendations for CLS in table beet are an integrated combination of strategic and tactical practices. The potential to select table beet cultivars that are less susceptible or resistant to CLS is minimal considering that most commercially acceptable cultivars are susceptible or highly susceptible (Pethybridge et al. 2017a). Rotations of at least three years between susceptible crops (i.e., table beet, sugar beet, spinach, mangel-wurzel, and Swiss chard [McKay and Pool 1918; Vestal 1933]) are recommended (Koenick et al. 2017). These guidelines minimize the potential for epidemics to result from local sources originating within the field of interest. Local sources include colonized plant debris and alternative hosts (McKay and Pool 1918; Pool and McKay 1916). To promote degradation of colonized plant debris, burying by plowing or other forms of soil inversion is recommended after harvest (Jacobsen and Franc 2009; Khan et al. 2008). Control of potential alternative hosts is also recommended, as these have been considered green bridges for inoculum between seasons (Jacobsen and Franc 2009; Lartey et al. 2005). However, recent evidence has brought into question the role of the weed lambsquarters (*Chenopodium album*) as a significant source of inoculum. *Cercospora* spp. isolated from CLS lesions found on these plants in a table beet field in New York were identified as *C. chenopodii* (Vaghefi et al. 2017c). Thus, alternative hosts need careful investigation to better understand their role in contributing to CLS epidemics in table beet.

Despite the broad adoption of these management strategies, the high risk of crop loss from CLS epidemics and the polycyclic nature of the epidemics means that fungicides are the dominant control tactic used to reduce the rate of CLS progress (Pethybridge et al. 2017b). In conventional production, the durability of this approach is threatened by the rapid appearance and dominance of resistance to single site mode of action fungicides within *C. beticola* populations. For example, resistance to azoxystrobin in *C. beticola* populations from broad-acre conventional table beet production systems has been documented following field level control failures in New York (Abawi et al. 2014; Vaghefi et al. 2016). Resistance to major classes of fungicides also threatens the sustainability of CLS management in sugar beet. For example, resistance within *C. beticola* populations from sugar beet has been reported for benzimidazoles (Bugbee

1996; Dovas et al. 1976; Georgopoulos and Dovas 1973; Ruppel and Scott 1974; Weiland and Halloin 2001), demethylation inhibitors (Bolton et al. 2012a, b; Karaoglanidis et al. 2000, 2001, 2002), and triphenyl tin hydroxide (Bugbee 1996; Giannopolitis 1978; Giannopolitis and Chrysai-Tokousbalides 1980). The high risk of developing fungicide resistance within *C. beticola* populations is a direct function of the broad genotypic and genetic diversity and the potential for recombination (Bolton et al. 2012c; Groenewald et al. 2007, 2008; Moretti et al. 2006; Vaghefi et al. 2016, 2017a, b, c; Weiland et al. 2001).

No teleomorph has been identified for *C. beticola*, but two mating types have been reported (Groenewald et al. 2006). Mating type frequencies vary between *C. beticola* populations (Bakhshi et al. 2011; Bolton et al. 2012c; Groenewald et al. 2006; Obuya et al. 2011; Vaghefi et al. 2017c). When both mating types are observed in a near 1:1 ratio within a population, the presence of an active sexual cycle is suggested. In contrast, skewed mating type ratios may be reflective of a more clonal population (Milgroom 1996). *C. beticola* populations from table beet fields in New York have exhibited equilibrium and disequilibrium of mating type ratios, while microsatellite loci were in disequilibrium in each population (Vaghefi et al. 2016, 2017c).

C. beticola populations from around the world have been characterized using random amplified polymorphic DNA, amplified fragment length polymorphisms, minisatellites, microsatellites, and single nucleotide polymorphisms (Groenewald et al. 2007; Moretti et al. 2004, 2006; Turgay et al. 2010; Vaghefi et al. 2016, 2017c). These studies have unanimously reported high genotypic diversity, which has been interpreted as further evidence for sexual recombination events (Vaghefi et al. 2017c), although migration, mutation, asexual recombination (nuclear exchange, cytoplasmic exchange, or parasexuality), or mitotic recombination may also be involved (Burdon 1993; Burdon and Silk 1997; Milgroom 1996).

The genetic structure of *C. beticola* populations on table beet in New York was used by Vaghefi et al. (2017c) to test hypotheses regarding major sources of inoculum contributing to CLS epidemics. Genetic homogeneity and low differentiation of *C. beticola* populations among broad-acre fields suggested high levels of gene flow, and thus a potential impact of external inoculum sources. In contrast, high genetic differentiation of *C. beticola* populations between the mixed-cropping farms indicated limited inoculum dispersal between these fields, and was interpreted as evidence that local inoculum sources may play a major role in initiating the annual CLS epidemics in these farms (Vaghefi et al. 2017c). This is also congruent with previous reports that *C. beticola* has the potential to survive as pseudostromata on plant debris for 10 to 22 months (Khan et al. 2008). However, this survival estimate is based on studies conducted in North Dakota, and longevity of *C. beticola* in New York table beet fields is not yet known.

The primary objective of this study was to characterize temporal changes in genetic diversity and differentiation of *C. beticola* populations in table beet fields in New York. Knowledge of these changes, and survival of multilocus genotypes over time, is necessary for the development of hypotheses regarding the relative importance of local and external inoculum sources for initiating CLS epidemics and will inform management recommendations.

Materials and Methods

***C. beticola* sampling and isolation.** Populations of *C. beticola* were collected from CLS lesions on table beet leaves from three table beet fields in New York from late August to early September in each year (Table 1; Fig. 1). Naming of sampling locations followed the convention presented in Vaghefi et al. (2016, 2017c), which used populations from the same locations. Sampling was conducted from a single conventional broad-acre (>0.2 km²) table beet field at Batavia in 2014 and 2016 (Field 2). This field was planted to cv. Ruby Queen (seed sourced from Seneca Foods, Dayton, WA) in both collection years, and rotated with soybean in 2015. The remaining fields were organic mixed-cropping farms in Hector and Phelps (Farms 1 and 2, respectively), and were sampled in 2015 and 2016. The mixed-cropping farms consisted of small-scale vegetable production with plots comprised of three to four 10 m rows of either table beet, Swiss chard, or other vegetables planted on an annual basis in close

proximity. Farm 1 has been producing its own seed (cv. Detroit) since 2013 (seed sourced from Fedco Seeds, Clinton, ME), with this seed planted in both sampling years from the previous year's crop. Farm 2 used seed (cv. Detroit, Touchstone Gold, and Chioggia Guardsmark) from two different commercial suppliers each year (High Mowing Organic Seeds, Wolcott, VT; and Johnny's Selected Seeds, Fairfield, ME).

In each field, diseased leaves were collected following a hierarchical sampling protocol. *C. beticola* populations from 2014 and 2015 were collected in previous studies as described by Vaghefi et al. (2016) and Vaghefi et al. (2017c), respectively. In 2016, *C. beticola* populations were collected as described by Vaghefi et al. (2017c). Briefly, sampling occurred at defined intervals along arbitrarily placed transects at each location. Due to differences in cropping area, the number of leaves and the distance between sampling points varied between the mixed-cropping locations (Farms 1 and 2) and the broad-acre field (Field 2). In Field 2, diseased leaves were sampled from each of five locations at 20 m intervals along two transects. Ten diseased leaves were collected from individual plants within a 5 m radius at each sampling location. Farm 1 and Farm 2 each contained three to four contiguous rows of table beet, with each row sampled at 5 to 10 m intervals.

On each occasion, diseased leaves were stored in plastic bags at 4°C for 24 h before isolation. Lesions with conidia were covered with 50 µl of T-water (Bolton et al. 2012c) and gently abraded with the pipette tip to dislodge conidia. The conidial suspension was spread across 2% (w/v) water agar (Hardy Diagnostics, Santa Maria, CA) (+0.02% ampicillin [Sigma-Aldrich, St. Louis, MO]), and a single germinated conidium was removed after 24 h at room temperature, and transferred to clarified V8 medium agar (Secor and Rivera 2012). Cultures were maintained on clarified V8 medium agar at room temperature for 7 to 14 days. Ten millimeter diameter agar plugs were then removed, dried overnight in a laminar flow cabinet, and kept at -20°C for long-term storage.

DNA extraction. A subsample of isolates from each field was chosen randomly for genotyping. Fungal mycelia was harvested from 14-day-old cultures grown in clarified V8 broth (Jeffers 2015) at 25°C. Fifteen to 30 mg of lyophilized mycelium was used for extraction of genomic DNA according to the method described by Vaghefi et al. (2016). The resultant DNA was quantified using a fluorometer (Qubit 2.0; Invitrogen, Inc., Grand Island, NY) for 2014 and 2015 isolates or a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) for 2016 isolates. Each DNA sample was examined for integrity after electrophoresis on a 1% (w/v) agarose gel in Tris-acetate-EDTA containing 0.001% (v/v) GelRed (Biotium, Inc. Hayward, CA).

Mating type identification. To assess mating type ratios in each population, individual isolate mating types were identified using the three and four primer PCR assays described by Vaghefi et al. (2017c). PCR products were visualized after electrophoresis on a 2% (w/v) agarose gel in Tris-acetate-EDTA containing 0.001% (v/v) GelRed (Biotium, Inc., Hayward, CA). Product size was determined against a 100-bp DNA Ladder (Axygen, Inc., Union City, CA).

Genotyping. Genotyping was performed for each *C. beticola* isolate using 12 microsatellite markers (CbSSR1, CbSSR2, CbSSR3, CbSSR6, CbSSR20, CbSSR21, CbSSR22, CbSSR23, CbSSR24, CbSSR25, CbSSR26, and CbSSR27) as described previously (Groenewald et al. 2007; Vaghefi et al. 2017b). Alleles were scored using the Microsatellite Plugin v. 1.4.4 in Geneious v. 10.2.3 (<https://www.geneious.com>, Kearse et al. 2012). To evaluate error, genotyping was replicated for 15% of the samples and error rates, defined as the number of allelic mismatches divided by the number of replicated alleles, were quantified for each locus.

Statistics and data analysis. Microsatellite dataset refinement. Multilocus genotypes (MLGs) for all isolates were determined in the R (R Core Team 2017) package *poppr* v. 2.4.1 (Kamvar et al. 2014, 2015) and the occurrence of MLGs at each location and year was assessed. In order to reduce the effect of genotyping error and missing data on genetic diversity, the data set was filtered in *poppr* using Bruvo's distance (Bruvo et al. 2004) in the farthest neighbor algorithm, with the filtering threshold estimated by the function `cut-off_predictor` (0.02083333). All subsequent analyses were conducted

on the filtered dataset. Clone-corrected datasets were used in analyses as indicated. Population data were clone-corrected to the Location:Year level, i.e., identical MLGs from a particular location in a single year were identified and only one representative isolate from each population was retained for analyses.

Measures of genetic diversity. The number of MLGs, expected number of MLGs after rarefaction, evenness, and Nei's gene diversity (Nei 1978) were estimated in *poppr*. Simpson's complement index of genotypic diversity (Simpson 1949) in *poppr* was used to calculate the probability that two randomly selected genotypes were different, and corrected for sample size by multiplying by $N/(N-1)$. The number of private alleles were estimated in GenAlEx (Peakall and Smouse 2006, 2012). Allelic richness and private allelic richness were estimated with rarefaction in ADZE v. 1.0 (Szpiech et al. 2008). MLG frequencies across populations were obtained using *poppr*. The homogeneity of allele frequencies between years in clone-corrected datasets was assessed at each locus through construction of two way contingency tables and χ^2 tests in POPGENE (Yeh et al. 1997).

Tests of mating type ratios and linkage equilibrium. A χ^2 goodness-of-fit test (Mangiafico 2015) was applied to mating type frequencies to test the significance of deviations from a 1:1 ratio for both clone-corrected and non-clone-corrected population datasets. Chi-square (χ^2) assessments were not performed when mating type sample sizes were less than five (Farm 1 in 2015, and Farms 1 and 2 in 2016 after clone correction).

Multilocus linkage equilibrium was assessed using the index of association (Brown et al. 1980) and standardized index of association (Agapow and Burt 2001), which were estimated after 1,000 permutations in *poppr*, for both clone-corrected and non-clone-corrected datasets of each population.

Detection of recombination. The proportion of compatible pairs of loci (PrCP) (Estabrook and Landrum 1975) for each population was

estimated using MultiLocus (Agapow and Burt 2001). Two loci are considered compatible (PrCP = 1) if all observed genotypes can be accounted for by mutations only in the absence of homoplasy (reversals, parallelisms, or convergences) or recombination. Under the assumption that recurrent, parallel, and reverse mutations are rare, phylogenetic incompatibility (PrCP < 1) provides evidence of genetic exchange, and may be interpreted as evidence for recombination (Milgroom 2015). The PrCP values were assessed for statistical significance by comparing the number of compatible pairs of loci in the observed data set to those from a randomly recombined data set in MultiLocus. The null hypothesis of random mating was rejected if more compatible loci than expected in a randomized population were observed ($P < 0.05$) (Bennett et al. 2005; Xu et al. 2009).

Population structure and differentiation. Analysis of molecular variance (AMOVA) was performed on non-clone-corrected and clone-corrected datasets in GenAlEx (Peakall and Smouse 2006, 2012) to examine the genetic diversity among the six populations (Φ_{PT}), with significance tested using 999 permutations. Genotypic diversities were assessed for significant differences between sampling years in GenoDive (Meirmans and Van Tienderen 2004) using 999 bootstrap replicates.

The relationships between populations were assessed using a dendrogram based on Nei's genetic distance calculated using the unweighted pair group method with arithmetic mean (UPGMA) method in *poppr*. Discriminant analysis of principal components (DAPC) (Jombart et al. 2010) among the six a priori clone-corrected populations was conducted in the R package *adegenet* v.2.0.1 (Jombart 2008).

Analyses of population differentiation between years were performed on a priori populations (defined as individuals from the same location:year) using both non-clone-corrected and clone-corrected datasets. Pairwise Φ_{PT} (Excoffier et al. 2005), an analog of F_{ST}

Table 1. Metadata associated with the origin of *Cercospora beticola* populations sampled from table beet in New York

Location	County	Year	Production type	Host cultivar	N ^a
Field 2	Genesee	2014	Conventional broad-acre	Ruby Queen	44
Field 2	Genesee	2016	Conventional broad-acre	Ruby Queen	38
Farm 1	Schuyler	2015	Organic mixed-cropping	Detroit	34
Farm 1	Schuyler	2016	Organic mixed-cropping	Detroit	43
Farm 2	Ontario	2015	Organic mixed-cropping	Detroit (62) / Touchstone Gold (52)	114
Farm 2	Ontario	2016	Organic mixed-cropping	Chioggia Guardsmark (24) / Touchstone Gold (7)	31
Total					304

^a Number of isolates within each population.

Fig. 1. Sampling locations for *Cercospora beticola* populations from table beet fields in New York. These images were modified in Microsoft PowerPoint 2016 from https://commons.wikimedia.org/wiki/File:Map_of_New_York_County_Outlines.svg.

assuming a stepwise mutation model, was calculated with 999 randomizations in GenAlEx (Peakall and Smouse 2006, 2012). Jost's measure of population differentiation, where 0 and 1 indicate none and complete differentiation of populations, respectively (Jost 2008), and Hedrick's measure of genetic differentiation, (0–1; Hedrick 2005) were estimated using the package *mmod* (Winter 2012) in R with 95% confidence intervals estimated after 1,000 bootstrap simulations. For Hedrick's measure of genetic differentiation, the magnitude of the statistic is the proportion of the maximum differentiation possible for the level of subpopulation homozygosity observed (Hedrick 2005).

A stepwise mutation model based on Bruvo's distance (Bruvo et al. 2004) was used to calculate the genetic distance between isolates in *poppr*. These distances formed the basis of a minimum spanning network for both years at each location. The minimum spanning networks were viewed using the *imsn*() tool.

Results

***C. beticola* populations.** Three hundred and four *C. beticola* isolates obtained from table beet leaves collected from two mixed-cropping farms (Farms 1 and 2) in 2015 and 2016, and one broad-acre table beet field (Field 2) in 2014 and 2016 (Table 1; Fig. 1) were genotyped. The genotyping error rate in the 2015 populations was 0.01 for primer pair CbSSR3. No genotyping error was detected for the 2014 and 2016 populations.

Multilocus genotypes. In the 304 *C. beticola* isolates, 131 unique MLGs were detected, resulting in 57% clonality of the combined populations (Table 2). Clonality varied between populations and between years for geographic origin. Field 2 populations exhibited the lowest clonality (29 and 16% in 2014 and 2016, respectively). Populations at Farm 2 had higher levels of clonality (58 and 71% in 2015 and 2016, respectively), while the highest clonal proportions were found in populations collected from Farm 1 (79 and 91% in 2015 and 2016, respectively).

Indices of genetic diversity. The number of alleles across the combined *C. beticola* populations ranged from two (CbSSR2) to 28 (CbSSR3) with an average of 6.75 alleles per locus (Table 2). Nei's unbiased gene diversity (H_e) per locus ranged from 0.045 (CbSSR1) to 0.89 (CbSSR3). Noninformative or monomorphic loci were present in each population (Table 2). Locus CbSSR1 was monomorphic in all populations except Farm 1 in 2015, while six loci were monomorphic at Farm 1 in 2016.

Genotypic diversity, described by Simpson's complement index, ranged from 0.178 to 0.990 (Table 2). Field 2 in both years and Farm 2 in 2015 had genotypic diversity values greater than 0.9. No MLG was shared between populations. There was a significant reduction in genotypic diversity between successive years at Farm 1 (0.496 to 0.178; $P = 0.001$) and Farm 2 (0.966 to 0.656; $P = 0.005$). A significant increase in genotypic diversity was observed in Field 2 between years (0.978 to 0.990; $P = 0.015$).

Tests for random mating, Mating-type ratios. For the non-clone-corrected data set, mating-type frequencies significantly deviated from a 1:1 ratio expected under random mating for Field 2 in 2014, Farm 2 in 2016, and Farm 1 in both years (Table 3). After clone correction, mating type frequencies were significantly different from a 1:1 ratio for Field 2 in 2014.

Linkage disequilibrium of microsatellite loci. Significant linkage of microsatellite loci was detected in all populations with and without clone correction of datasets, rejecting the null hypothesis of random mating (Table 3).

Population structure. AMOVA of the combined non-clone-corrected populations found 52% of the observed genetic diversity was partitioned among the populations and 48% among individuals within populations ($\Phi_{PT} = 0.52$; $P = 0.001$). After clone correction, and subsequent removal of the population collected from Farm 1 in 2016 due to a small sample size ($n = 4$), 30% of the observed genetic diversity was partitioned among the populations and 70% among individuals within populations ($\Phi_{PT} = 0.30$; $P = 0.001$).

The UPGMA dendrogram, based on Nei's distance, and the DAPC analysis, using location:year as predefined populations, demonstrated that the population from Farm 2 in 2015 clustered separately from the other populations (Figs. 2 and 3).

Temporal population differentiation. The indices of differentiation quantified the genetic variation of populations between years (Table 4). Non-clone-corrected *C. beticola* populations at Field 2 ($\Phi_{PT} = 0.07$; $P = 0.019$) and Farm 1 ($\Phi_{PT} = 0.15$; $P = 0.004$) exhibited low but significant differentiation. In contrast, populations from Farm 2 ($\Phi_{PT} = 0.49$; $P = 0.001$) exhibited the highest level of population differentiation between years. After clone correction, Farm 2 ($\Phi_{PT} = 0.25$; $P = 0.001$) was the only location to exhibit significant population differentiation between years. χ^2 tests found significant differences ($P < 0.05$) in allele frequencies between years at seven, four, and nine loci for Field 2, Farm 1, and Farm 2, respectively (Table 5; Supplementary Table S1).

Table 2. Statistics and indices of genetic diversity for *Cercospora beticola* populations collected from table beet fields in New York

Location	Year	N ^a	Na ^b	Ra ^c	Pa ^d	Prare ^e	%P ^f	NI loci ^g	He ^h	MLG ⁱ	eMLG ^j	λ^k	E5 ^l	CF ^m	PrCP ⁿ	
															Obs.	P value
Field 2	2014	44	4.33	1.44	9	0.36	91.7	1 ^o	0.436	31	23.61	0.978	0.83	0.29	0.59	<0.001
Field 2	2016	38	3.83	1.39	5	0.25	91.7	1 ^o	0.387	32	26.90	0.990	0.92	0.16	0.48	0.076
Farm 1	2015	34	2.50	1.28	4	0.24	91.7	1 ^o	0.278	7	6.64	0.496	0.47	0.79	1.00	<0.001
Farm 1	2016	43	1.75	1.04	2	0.18	50.0	6 ^o	0.045	4	3.37	0.178	0.42	0.91	1.00	0.331
Farm 2	2015	114	3.50	1.35	1	0.66	75.0	3 ^o	0.354	48	20.92	0.966	0.70	0.58	0.48	<0.001
Farm 2	2016	31	3.58	1.33	2	0.30	91.7	1 ^o	0.335	9	9.00	0.656	0.50	0.71	0.86	0.002
Total	-	304	6.75	-	-	-	100	-	0.566	131	23.25	0.969	0.43	0.57	0.11	0.014

^a Population size before clone correction.

^b Mean number of alleles per locus.

^c Allelic richness after rarefaction to the smallest sample size.

^d Number of private alleles.

^e Private allelic richness: number of private alleles after rarefaction to the smallest sample size.

^f Percent polymorphic loci defined as loci with a frequency of at least 0.01.

^g Number of monomorphic or noninformative (minor allele frequency <0.01) loci.

^h Nei's index of gene diversity defined as the probability that two randomly chosen alleles are different.

ⁱ Number of multilocus genotypes (MLGs).

^j Expected number of MLGs after rarefaction.

^k Simpson's complement index defined as the probability that two genotypes randomly chosen from the population are different.

^l Population evenness defined as an estimation of the uniformity in distribution of the MLGs.

^m Clonal fraction.

ⁿ Proportion of phylogenetically compatible (PrCP) pairs of loci, where Obs. denotes the observed value.

^o Loci with minor allele frequency <0.01 in each population. Field 2 - 2014: CbSSR1; Field 2 - 2016: CbSSR1; Farm 1 - 2015: CbSSR24; Farm 1 - 2016: CbSSR1, CbSSR2, CbSSR22, CbSSR23, CbSSR24, and CbSSR27; Farm 2 - 2015: CbSSR1, CbSSR2 and CbSSR27; Farm 2 - 2016: CbSSR1.

Minimum spanning networks indicated the relationships of MLGs within and between years for each location (Fig. 4). Field 2 had MLGs from both years intermixed in the network. Farm 1, with less overall diversity, had a small genetic distance separating the two dominant MLGs (Bruvo's measure of genetic distance = 0.073; three mutational steps, 314 to 320 bp, at CbSSR3) between years. At Farm 2, the MLGs were most closely linked within each year.

Discussion

This study quantified temporal shifts in genetic structure of *C. beticola* populations and provided evidence of significant annual changes in genotypic diversity and allele frequencies within table beet fields in New York. Temporal population dynamics are affected by several processes, acting in combination, including selection, mutation, random genetic drift, migration, and sexual and asexual recombination (i.e., nuclear exchange, cytoplasmic exchange, or parasexuality) (Burdon 1993; Milgroom 2015; Pontecorvo 1956; Tinline and MacNeill 1969). Each of these processes has been reported or hypothesized for *C. beticola* populations (Bolton et al. 2012c; Groenewald et al. 2008; Turgay et al. 2010), and may vary in importance across environments.

C. beticola is reported to survive as pseudostromata on leaf debris, which is the primary inoculum in the subsequent season, and may persist for 10 months to 3 years in the field, depending on

environmental conditions (Khan et al. 2008; Solel 1970). However, the role of survival on leaf debris in New York has not been assessed. The lack of recurring MLGs within the *C. beticola* populations sampled from the same field in subsequent years, particularly in the populations from Farm 1 containing dominant MLGs, suggests that there is limited clonal survival between years. In a temporal study on *Puccinia striiformis* f. sp. *tritici*, Ali et al. (2014) reported that limited resampling of the same MLGs over time may result from high genetic diversity or sexual recombination, and that it further suggests limited survival through clonality.

External sources of inoculum may also play a role in establishing populations, and would partly explain the observed genetic relationships of populations between years. Movement of *C. beticola* has been described on colonized host debris, seeds, and contaminated machinery, and conidia may be further dispersed locally via wind, insects, and water (McKay and Pool 1918). Alternative hosts, such as Swiss chard, may also exhibit CLS, with *C. beticola* populations not being differentiated from those of table beet (Vaghefi et al. 2017c), indicating the potential for further dispersal via alternative host plants or seed in mixed-cropping systems. The only example of significant genetic differentiation between years originated in the organic, small-scale mixed-cropping system at Farm 2, where commercial seed from two different sources was used to establish table beet crops each year. Although commercial seed was also grown at Field 2, the same

Table 3. Tests of linkage equilibrium of mating-type frequencies and microsatellite loci for *Cercospora beticola* populations collected from table beet fields in New York

Location	Year	Non-clone-corrected			Clone-corrected			Non-clone-corrected		Clone-corrected	
		MAT1-1	MAT1-2	χ^2 ^a	MAT1-1	MAT1-2	χ^2	$\bar{r}_d$ ^b	I_a ^c	$\bar{r}_d$	I_a
Field 2	2014	36	8	17.82	24	7	9.32	0.0655	0.689	0.027	0.285
Field 2	2016	21	17	0.42	18	14	0.5	0.0631	0.608	0.0514	0.498
Farm 1	2015	7	27	11.77	3	4	.. ^d	0.7062	6.983	0.4359	4.219
Farm 1	2016	2	41	35.37	2	2	.. ^d	0.7334	3.482	0.4026	1.59
Farm 2	2015	54	60	0.32	20	28	1.33	0.0805	0.709	0.0749	0.696
Farm 2	2016	27	4	17.1	7	2	.. ^d	0.5215	5.084	0.1286	1.214
Total	-	147	157	0.33	74	57	2	0.2218	2.341	0.1395	1.473

^a Chi-square (χ^2) value based on 1:1 ratio and one degree of freedom. Mating-type frequencies with significant deviation from one ($P < 0.05$) are indicated in bold.

^b Standardized index of association ($\bar{r}_d$) scaled for the number of loci, with significant deviations from zero ($P < 0.05$) indicated in bold.

^c Index of association (I_a), with significant deviations from zero ($P < 0.05$) indicated in bold.

^d Not applicable or not calculated due to small sample sizes.

Fig. 2. Dendrogram of the relationships among **A**, non-clone-corrected and **B**, clone-corrected *Cercospora beticola* populations sampled from each of three table beet fields in successive years in New York. The dendrogram was constructed using the unweighted pair group method with arithmetic mean based on Nei's genetic distance. Numbers at branch points indicate the percent occurrence of the cluster to the right of the branch in 1,000 bootstrapped dendrograms.

supplier was used each year. Thus, the maintenance of diversity and low differentiation between years at Field 2 would be supported by a recurring diverse founder population. This implication requires that seedborne inoculum is present on the purchased seed and has the

potential to start a genetically distinct population. Evidence for seedborne inoculum, or infections originating from seed, is limited (McKay and Pool 1918; Vereijssen et al. 2004). Further examination of the potential role of seedborne inoculum in New York is required.

Fig. 3. Discriminant analysis of principal components for clone-corrected *Cercospora beticola* populations collected from table beet fields (Field 2 and Farms 1 and 2) in New York in 2014, 2015, and 2016. The frequency distributions of the eigenvalues (insets) of the discriminant analysis (DA) and the principal component analysis (PCA) showed that the first two principal components adequately accounted for the genetic structure of the populations.

Table 4. Indices of temporal differentiation for *Cercospora beticola* populations collected in each of two years from three table beet fields in New York. Probability values (*P*) and 95% confidence intervals (CI) were estimated after 999 randomizations.

Location	Index of differentiation		
	Φ_{PT} (<i>P</i> value)	Jost's D (95% CI)	Hedrick's G' _{ST} (95% CI) ^a
<i>Non-clone-corrected</i>			
Field 2 (<i>n</i> = 82)	0.07 (0.019)	0.08 (0.047 to 0.113)	0.18 (0.114 to 0.239)
Farm 1 (<i>n</i> = 77)	0.15 (0.004)	0.09 (0.069 to 0.106)	0.37 (0.323 to 0.427)
Farm 2 (<i>n</i> = 145)	0.49 (0.001)	0.57 (0.528 to 0.614)	0.80 (0.760 to 0.832)
<i>Clone-corrected</i>			
Field 2 (<i>n</i> = 63)	0.02 (0.198)	0.07 (0.031 to 0.103)	0.15 (0.076 to 0.218)
Farm 1 (<i>n</i> = 11)	0.14 (0.193)	0.11 (0.000 to 0.225)	0.24 (0.076 to 0.404)
Farm 2 (<i>n</i> = 57)	0.25 (0.001)	0.30 (0.201 to 0.409)	0.47 (0.353 to 0.596)

^a Confidence interval of non-clone-corrected dataset calculated by subtracting the difference between the point estimate of the statistic and the mean of the bootstrap distribution from the extremes of the interval. Confidence interval of clone-corrected dataset utilized lower.normal and upper.normal centered on the observed value of the statistic and standard deviation across replicates to generate limits (also called the normal-method of obtaining a confidence interval: the intervals are the observed value statistic $\pm 1.96 \times$ the standard error of the bootstrap sample) (Winter et al. 2017).

In contrast to Farm 2 and Field 2, Farm 1 established crops annually from seed produced at the same location. The low diversity, along with the more stable allele frequencies, at Farm 1 suggested a significant effect of the farm-based seed production, potentially by limiting introduction of external inoculum. Farm 1 had a unique dominant MLG in each year, and while the genetic distance between these MLGs was relatively small (three mutational steps), it does indicate a complete shift in the dominant MLG between years. The mutation producing this shift occurred at the highly polymorphic dinucleotide simple sequence repeat locus, CbSSR3 (Groenewald et al. 2007). The high frequency of polymorphisms at this locus could be impacted by an increased error rate (Vaghefi et al. 2017c); however, as no error was detected for these isolates, a high mutation rate at this locus is the most likely source of variation. While a mutation could create the genetic difference in the MLGs between years, it does not account for the absence of the dominant 2015 MLG in the subsequent year. This finding suggests that the MLG composition of the population in the previous year is unlikely to contribute to subsequent CLS epidemics, again discounting the dominance of survival through local clonal inoculum.

The role of mutation in the differentiation of populations between years may explain some of the minor genetic variation and infrequent

MLGs; however, mutation is unlikely to explain all of the genetic diversity observed in *C. beticola* populations (Bolton et al. 2012c). Mutation rates of microsatellites vary between species, but are generally appreciable (10^{-3} to 10^{-6} per generation) and have been suggested to be an important factor in the creation and maintenance of genetic variation (Ellegren 2000; Karaoglu et al. 2004; Schug et al. 1997; Weber and Wong 1993; Xu et al. 2000). Few studies of mutation rates in fungal plant pathogens are available (Karaoglu et al. 2004), but the multiple generations of *C. beticola* produced during polycyclic CLS epidemics provides a conducive system for the occurrence of mutations in microsatellite regions.

A study of the pathogen *Sclerotinia sclerotiorum* demonstrated that with a sublethal fungicide selection pressure, estimated mutation rates in six microsatellite markers were 1.7- to 60-fold higher than expected under neutral conditions (Amaradasa and Everhart 2016). Field 2 was maintained using conventional practices (fungicide-treated seed, in-furrow fungicide applications for pocket rot control, and two to three foliar fungicides applications [Reiners et al. 2017]) and it may be expected that a subset of the field population would be exposed to sublethal fungicide levels. Therefore, mutation due to exposure with a sublethal fungicide selection pressure, in a population with high diversity, may allow for the consistently high allelic and genotypic diversity in populations surviving as local inoculum. Alternatively, high genotypic diversity may also be consistent with an active sexual morph.

Indications of sexual recombination have been described for *C. beticola* populations based on the presence of both mating types in equilibrium, high genotypic diversity, and gametic equilibrium, but no teleomorph has been identified (Groenewald et al. 2008; Vaghefi et al. 2016, 2017a, c). Each population in this study contained both mating types but only two of the six populations exhibited equilibrium, which is an indication of random mating (Milgroom 1996). For example, equilibrium was detected at Farm 2 in 2015 but not in the subsequent year, reflecting the differentiation of populations. However, at Field 2, the 2016 population moved into mating type equilibrium. A change in allele frequencies, including mating types, may be the result of genetic drift caused by bottleneck effects or selection events in the founder population (Chen et al. 1994). The disequilibrium of microsatellite loci has been similarly described by Vaghefi et al. (2017c), with the significant disequilibrium not being of suggestive of random mating (Milgroom 1996). These results, however, are not conclusive evidence of the lack of sexual recombination (Vaghefi et al. 2017c). Studies of New York *C. beticola* populations have suggested the presence of a mixed mode of reproduction

Table 5. Comparison of allele frequencies at 12 microsatellite loci between years for clone-corrected *Cercospora beticola* populations at each table beet field using a chi-squared (χ^2) analysis. Probability values (*P*) were estimated after 999 randomizations

Locus	Field 2			Farm 1			Farm 2		
	χ^2	df ^a	<i>P</i> value	χ^2	df	<i>P</i> value	χ^2	df	<i>P</i> value
CbSSR1	2.10	1	0.15	4.71	1	0.03	0.00	0	1.00 ^b
CbSSR2	13.04	1	<0.001	4.71	1	0.03	33.99	1	<0.001
CbSSR3	94.26	20	<0.001	22.00	9	0.01	80.16	10	<0.001
CbSSR6	41.30	7	<0.001	3.99	2	0.14	51.04	5	<0.001
CbSSR20	2.69	2	0.26	7.18	3	0.07	56.28	3	<0.001
CbSSR21	10.38	4	0.03	4.71	2	0.09	40.05	5	<0.001
CbSSR22	6.52	3	0.09	2.79	1	0.09	36.59	2	<0.001
CbSSR23	2.40	2	0.30	2.79	1	0.09	20.34	4	<0.001
CbSSR24	22.15	4	<0.001	0.00	0	1.00 ^b	0.65	3	0.88
CbSSR25	23.96	4	<0.001	10.48	3	0.01	52.45	5	<0.001
CbSSR26	7.65	2	0.02	0.39	1	0.53	3.58	1	0.06
CbSSR27	4.13	2	0.13	2.79	1	0.09	46.02	1	<0.001

^a df = degrees of freedom.

^b Monomorphic loci in both years.

Fig. 4. Minimum spanning networks for *Cercospora beticola* multilocus genotypes (MLGs) based on Bruvo's measure of genetic distance from **A**, Field 2 in 2014 and 2016, **B**, Farm 1 in 2015 and 2016, and **C**, Farm 2 in 2015 and 2016. Each node represents an MLG and node sizes are proportional to the frequency of the MLGs. Edge width and color are proportional to the genetic distance between MLGs.

(Vaghefi et al. 2016), with potential for infrequent recombination events.

Assessment of clonality was performed by testing the presence of phylogenetic incompatibility between pairs of loci. This test has an assumption that recurrent, parallel, and reverse mutations are highly improbable and phylogenetic incompatibility of loci may only arise by recombination (Milgroom 2015). Fourteen to 52% of pairs of loci were incompatible for Field 2 and Farm 2 in both years, while no pairs of loci were incompatible at Farm 1 in both years. This suggests recombination may have occurred in the Field 2 and Farm 2 populations. The high diversity at Field 2 also suggests recombination. Populations at Farm 2 possessed some features reflective of recombination, but the significant differentiation and change in proportion of phylogenetically compatible pairs suggests a dominant role of external inoculum. Vaghefi et al. (2017c) suggested that epidemics in mixed-cropping farms may originate from local inoculum sources but a role for infested seed as an inoculum source was also identified. Based on similarities of populations between broad-acre fields, Vaghefi et al. (2017c) also suggested that external inoculum sources are contributing to CLS epidemics in these fields. The lack of differentiation at Field 2 supports the role of a recurrent external inoculum source, or founder population, at this location. Further investigation of temporal changes in diversity of *C. beticola* populations, coinciding with more intensive sampling, assessment of potential inoculum sources, and surveys for sexual stages, are therefore essential.

A significant factor for consideration during temporal comparisons is the effect of sampling strategy. In a highly diverse population, intensive sampling is required to identify the same low frequency MLG between sampling times. Therefore, a lack of recurring MLGs between years would be possible due to sampling effects, such as in Field 2. However, in a less diverse population, such as Farm 1, it would be more likely that a high frequency MLG would be resampled. As no MLG was resampled between years at any location, the roles of recombination, selection, genetic drift, and inoculum sources must be examined.

The differentiation, nonrecurrent MLGs, and changes in genotypic diversity and allele frequency observed in this study demonstrate potential for annual genetic change in *C. beticola* populations. This has important implications for disease management strategies, particularly an increased risk of fungicide resistance development. A high frequency of *C. beticola* isolates resistant to azoxystrobin have been reported in New York (Abawi et al. 2014). High diversity and rapid genetic change coinciding with the application of fungicides increases the potential for a shift in the frequency of resistance genes, which highlights the need to adhere to best management guidelines for fungicides in conventional table beet production.

To improve our knowledge on the role of primary inoculum in CLS epidemics, further mechanistic research is required. As the three locations in this study exhibited distinctly different temporal relationships, the potential for genetic shifts in *C. beticola* populations between seasons also needs to be accommodated in future studies.

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