Genome-Wide Association and Selective Sweep Studies Reveal the Complex Genetic Architecture of DMI Fungicide Resistance in Cercospora beticola

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Abstract

The rapid and widespread evolution of fungicide resistance remains a challenge for crop disease management. The demethylation inhibitor (DMI) class of fungicides is a widely used chemistry for managing disease, but there has been a gradual decline in efficacy in many crop pathosystems. Reliance on DMI fungicides has increased resistance in populations of the plant pathogenic fungus Cercospora beticola worldwide. To better understand the genetic and evolutionary basis for DMI resistance in C. beticola, a genome-wide association study (GWAS) and selective sweep analysis were conducted for the first time in this species. We performed whole-genome resequencing of 190 C. beticola isolates infecting sugar beet (Beta vulgaris ssp. vulgaris). All isolates were phenotyped for sensitivity to the DMI tetraconazole. Intragenic markers on chromosomes 1, 4, and 9 were significantly associated with DMI fungicide resistance, including a polyketide synthase gene and the gene encoding the DMI target CbCYP51. Haplotype analysis of CbCYP51 identified a synonymous mutation (E170) and nonsynonymous mutations (L144F, I387M, and Y464S) associated with DMI resistance. Genome-wide scans of selection showed that several of the GWAS mutations for fungicide resistance resided in regions that have recently undergone a selective sweep. Using radial plate growth on selected media as a fitness proxy, we did not find a trade-off associated with DMI fungicide resistance. Taken together, we show that population genomic data from a crop pathogen can allow the identification of mutations conferring fungicide resistance and inform about their origins in the pathogen population.

Key words: GWAS, CYP51, azole, synonymous mutation, antifungal, selection.
Demethylation inhibitor (DMI) fungicides are critical tools for managing the most important fungal disease of sugar beet worldwide called Cercospora leaf spot, but there has been a sharp increase in incidence of DMI-resistant field isolates in most production areas. We utilized genome sequencing and selective sweep analyses to identify genes that underscore the molecular basis of DMI resistance in this pathogen. This knowledge will aid in the development of rapid detection strategies to manage this disease and make the production of sugar beet sustainable.

Introduction

Demethylation inhibitor (DMI) compounds are effective antifungals in both medicine and agriculture for managing a broad range of fungal pathogens (Becher and Wirsel 2012). The DMIs, or azoles, inhibit fungal growth by interfering with sterol 14α-demethylase (Vanden Bossche et al. 1987), also known as cytochrome P450 monooxygenase family 51 (CYP51). Fungal CYP51 is required for synthesis of ergosterol, a key sterol component of fungal cell membranes required to maintain permeability and fluidity (Daum et al. 1998). DMIs have shown unique durability when compared with other single-site fungicides, with control failures being rare even with widespread and prolonged use (Cools et al. 2013). However, resistance has still emerged in some fungal populations with long-term exposure to DMIs, leading to reduced efficacy of the compounds in use (Price et al. 2015; Fisher et al. 2018; Jørgensen et al. 2021).

DMI resistance is often associated with changes to the molecular target CYP51 (Becher and Wirsel 2012). Amino acid substitutions in CYP51 (Kelly, Lamb, Kelly, et al. 1999; Kelly, Lamb, Loeffler, et al. 1999; Lamb et al. 2000; Snelders et al. 2011) or overexpression of CYP51 (Hamamoto et al. 2000; Ma et al. 2006; Ghosoph et al. 2007; Carter et al. 2014; Villani et al. 2016) can lead to decreased DMI sensitivity. Some filamentous fungi have two or more paralogous CYP51 genes (Liu et al. 2011; Hawkins et al. 2014; Chen et al. 2020), which may result in an inherent reduction in DMI sensitivity and allow these species to overcome some biological costs by restricting acquired resistance to one paralog (Becher and Wirsel 2012; Cools et al. 2013). Gain-of-function mutations in transcription factors (Dunkel et al. 2008; Liu et al. 2015) regulating ergosterol biosynthesis genes have also been linked to reduced DMI efficacy. Non-CYP51 mechanisms of resistance can also be important in fungi. Such mechanisms include enhanced efflux of DMIs (Hahn and Leroch 2015) by plasma membrane-bound transporters in the multifacilitator (MFS) or ATP-binding cassette (ABC) superfamilies (Zwiers et al. 2002; Hayashi et al. 2002a, 2002b; Leroux and Walker 2011; Hellin et al. 2018; de Ramón-Carbonell et al. 2019), calcium signaling regulators (Edlind et al. 2002; Jain et al. 2003; Zhang et al. 2013; Li et al. 2019), the pleiotropic effect of melanization (Lendenmann et al. 2015) and other uncharacterized genes (Ballard et al. 2019).

Genetic variants that confer reduced susceptibility to DMI fungicides may also have a fitness penalty (Hawkins and Fraaije 2018). Mohd-Assaad et al. (2016) found that genetic loci conferring DMI resistance in the barley scald pathogen Rhynchosporium commune negatively impacted in vitro growth rates. However, Pereira et al. (2020) did not find evidence that DMI fungicide resistance was constrained by genetic trade-offs in the wheat pathogen Parastagonospora nodorum. If the benefits of a resistance mutation outweigh the costs, it will increase in frequency in a fungal population that is frequently exposed to DMI fungicides (Milgroom 1989). Signatures of positive selection have previously been detected for variants of CYP51 in Zymoseptoria tritici (Brunner et al. 2016) and ABC transporter genes CDR1 and CDR2 in Candida albicans (Holmes et al. 2008). Mutations, which have recently experienced strong positive selection leave a distinct signature in the genome termed a “selective sweep” that is characterized by a locus deprived of genetic variation and high linkage disequilibrium (LD) in the genomic regions flanking the favorable mutation. This pattern reflects the “hitchhiking” of genetic variants linked to the beneficial mutation, which also increase in frequency (Smith and Haigh 1974). The identification of fungicide resistance loci in selective sweep regions suggests fungicides are a major selective pressure in recent evolution of a fungal pathogen (Hartmann et al. 2020).

DMI fungicides are integral for managing many important crop diseases (Price et al. 2015), including Cercospora leaf spot (CLS) disease of sugar beet (Beta vulgaris spp. vulgaris). CLS remains the most destructive foliar disease of sugar beet worldwide (Rangel et al. 2020). The Red River Valley (RRV) region of North Dakota and Minnesota, United States is the largest sugar beet production area in the United States (NASS 2020) and has historically experienced huge economic losses due to CLS with large reductions in yield and the application of nonefficacious fungicides including the DMIs (Secor et al. 2010; Bolton, Rivera-Varas, et al. 2012). The magnitude of DMI resistance and incidence of resistant isolates in RRV C. beticola field populations has steadily increased since 2006 (Rangel et al. 2020). In C. beticola, overexpression of CbCYP51 has been associated with high levels of DMI resistance in isolates from Greece (Nikou et al. 2009) and the United States (Bolton, Birla, et al. 2012; Bolton et al. 2016). Although it has historically been difficult to clearly associate...
any CbCYP51 haplotype with resistance (Bolton, Birla, et al. 2012; Trkulja et al. 2017), a recent study found amino acid substitutions Y464S, L144F, and I309T (in combination with L144F) to be associated with reduced DMI sensitivity in European C. beticola isolates (Muellender et al. 2021). Evaluating levels of resistance is an important part of CLS fungicide resistance management (Secor et al. 2010) and has been aided by the development of PCR-based mutation detection tools to expedite the process (Birla et al. 2012; Bolton, Birla, et al. 2012; Shrestha et al. 2020). However, molecular methods of resistance detection first require the identification of associated mutations.

Genome-wide association study (GWAS) analysis is a powerful method for identifying genetic variants associated with complex traits (Sanglard 2019). GWAS has been successfully employed to identify loci associated with DMI resistance in several phytopathogenic fungi (Mohd-Assaad et al. 2016; Talas et al. 2016; Pereira et al. 2020). We hypothesized that GWAS would be an ideal strategy to identify genetic determinants underlying DMI resistance in C. beticola, a pathogen that cannot be experimentally crossed but shows considerable genetic variation (Moretti et al. 2004, 2006; Groeneveld et al. 2006, 2008; Bolton et al. 2012; Vaghefi et al. 2016; Rangel et al. 2020; ). In this study, we revealed the genetic architecture of DMI fungicide resistance in C. beticola by performing GWAS in 190 C. beticola isolates. Further, we developed a genome-wide map of selective sweep regions to investigate whether loci significantly associated with DMI fungicide resistance were recently selected in the population. We additionally assessed the effects of CbCYP51 haplotypes on DMI resistance. Finally, using radial plate growth assays as a fitness proxy, we investigated whether fitness penalties exist for DMI resistance in vitro.

Results

Genome Sequencing and Phenotyping of C. beticola Isolates

To generate a C. beticola population for association mapping, we collected unique isolates from two adjacent sugar beet fields in Fargo, North Dakota in 2016 (n = 63) and additional isolates during sugar beet field surveys in Minnesota and North Dakota in 2016 (n = 80) and 2017 (n = 48) and Idaho in 2016 (n = 2) (supplementary table S1, Supplementary Material online). To map the genetic architecture of resistance to DMI fungicides, we performed whole-genome resequencing of all 190 C. beticola isolates and mapped reads of each isolate to the 09-40 reference genome (de Jonge et al. 2018) (NCBI RefSeq assembly GCF_002742065.1). The resulting coverage per genome ranged from 18x to 40x with a mean coverage of 32x (supplementary table S1, Supplementary Material online). After filtering for genotype quality and read depth, 868,218 variants were identified including 732,852 SNPs, corresponding to an average SNP density of ~20 SNPs per kb. A minor allele frequency of 0.05 reduced variants to 424,456, eliminating over half of called variants. Filtering for 10% missing data reduced the total number to 320,530 variants. Mapping power of GWAS was assessed by calculating LD decay for the population. LD decayed to $R^2 < 0.2$ rapidly within $\sim 3.5$ kb (supplementary fig. S1, Supplementary Material online), which is comparable to values found in populations of other closely related filamentous fungal phytopathogens used successfully for GWAS such as Z. tritici (Hartmann et al. 2017) and P. nodorum (Gao et al. 2016; Richards et al. 2019; Pereira et al. 2020). EC$_{50}$ values were calculated for all 190 isolates to tetraconazole, the active ingredient of Eminent fungicide, which is widely used in the RRV region (supplementary fig. S2A, Supplementary Material online).

Population Structure Analyses

We performed a principal component analysis (PCA) to assess population structure amongst the 190 C. beticola isolates. PC1 explained 11% of total variation followed by 3.4% and 3.0% for PCs 2 and 3, respectively. Pairwise plots of the first six PCs from PCA demonstrated that sampling location had little impact on clustering of the C. beticola isolates used in this study (fig. 1A and supplementary fig. S4, Supplementary Material online). Intriguingly, the tight cluster of 66 isolates circled in figure 1A and B was predominantly tetraconazole sensitive (28 isolates are moderately sensitive, 34 isolates are sensitive), whereas the remaining scattered isolates were mainly tetraconazole resistant. Some clustering of sensitive isolates was also visible in additional pairwise plots of the first six PCs from PCA (supplementary fig. S5, Supplementary Material online). Performing PCA on the tight cluster of 66 isolates revealed further separation of isolates, which was also mainly explained by tetraconazole sensitivity when compared with sampling location and year of collection (supplementary fig. S6, Supplementary Material online). Based on this observation, we hypothesized that certain genomic regions encoding fungicide resistance traits may explain more of the variation in the population when compared with other genomic regions, and that this may be visible on a chromosome level. Indeed, chromosome-specific PCAs revealed that chromosome 9 had the highest proportion of variation explained by PC1 at 13% and had the strongest clustering of strains according to tetraconazole sensitivity in pairwise plots of the first two PCs (supplementary fig. S7, Supplementary Material online).

Genetic Architecture of Tetraconazole Sensitivity

To determine the genetic architecture of tetraconazole sensitivity in C. beticola, we performed GWAS using 320,530 genetic variants (SNPs and indels) from all 190 isolates. With a general linear model (GLM) including two principal
components there were 112 significant associations at the Bonferroni-corrected significance threshold of $-\log_{10}(P) = 6.7959$ (fig. 2 and supplementary table S3 and fig. S8A, Supplementary Material online). Of these associated markers, 6 were on chromosome 1, 7 on chromosome 4, and 99 on chromosome 9. A total of 49 markers were within gene coding sequence regions, 21 markers in untranslated regions (5' and 3' UTRs), 9 markers within introns, and 33 markers in intergenic regions. We highlight the most plausible associations on each chromosome based on mutational effect and gene annotation.

**Significantly Associated Markers on Chromosome 9**

There were two main genomic regions significantly associated with tetraconazole sensitivity on chromosome 9: 1332548–1358331 and 1403629–1497163 bp (supplementary table S3, Supplementary Material online). In the first region, we noted the presence of SNP 9_1358331 underlying the amino acid substitution A1770V (alanine to valine) in conidial yellow pigment biosynthesis polyketide synthase CB0940_11350. Cercospora beticola isolates with the A allele at 9_1358331 had significantly higher tetraconazole EC50 values than moderately sensitive isolates with 0.1 μg/ml ≤ EC50 < 1 μg/ml; sensitive = isolates with EC50 < 0.1 μg/ml.
isolates with G at the same site (supplementary fig. S10A, Supplementary Material online, $P < 0.001$). Analysis of LD for markers $\pm 5$ kb showed that two additional markers are in high pairwise LD ($R^2$ = 1) with 9_1358331 within the same gene CB0940_11350 (supplementary fig. S11, Supplementary Material online). The latter region notably contained a synonymous mutation (9_1451478) within the coding region of eburicol 14-alpha demethylase CB0940_11379, otherwise known as the gene encoding DMI fungicide target CbCYP51. Isolates with the T allele at 9_1451478 had significantly higher tetraconazole EC$_{50}$ values than isolates with the C allele at the same site (supplementary fig. S10B, Supplementary Material online, $P < 0.001$). LD analysis for markers $\pm 3$ kb revealed a block of markers down-stream of 9_1451478 in high LD with the marker in question, most located within the gene CB0940_11381 (hypothetical protein) (supplementary fig. S12, Supplementary Material online, $R^2 > 0.8$).

Significantly Associated Markers on Chromosome 1

There were two main genomic regions significantly associated with tetraconazole sensitivity on chromosome 1: 2402041–2415450 and 315698 bp (supplementary table S3, Supplementary Material online). Most notably, the SNP 1_2402041 encoded amino acid substitution E170K (glutamic acid to lysine) in hypothetical protein CB0940_00893, which lacked conserved domains. C. beticola isolate with a G allele at 1_2402041 had significantly higher tetraconazole EC$_{50}$ values than isolates carrying an A allele (supplementary fig. S10C, Supplementary Material online, $P < 0.001$). LD analysis of markers $\pm 3$ kb revealed additional SNPs in high LD with 1_2402041 ($R^2 > 0.8$) within genes CB0940_00893 and CB0940_00894 (supplementary fig. S13, Supplementary Material online).

Significantly Associated Markers on Chromosome 4

There were two main genomic regions significantly associated with tetraconazole sensitivity on chromosome 4: 455568–455695 and 788989–849731 bp (supplementary table S3, Supplementary Material online). Most notably, the SNPs 4_849506 and 4_849507 encoded amino acid substitution N241T (asparagine to threonine) in hypothetical protein CB0940_04131. This protein had no conserved domains and significant BLASTp hits were only found within the Cercospora genus. Cercospora beticola isolates harboring either the A or C allele at 4_849506 were not significantly different in tetraconazole EC$_{50}$ values (supplementary fig. S10D, Supplementary Material online). LD analysis showed that additional markers within $\pm 3$ kb are in high LD with 4_849506 ($R^2 \approx 1$, supplementary fig. S14, Supplementary Material online).

Significant Associations Vary with Different Ranges of Tetraconazole Sensitivity

Because the tetraconazole sensitivity phenotype in C. beticola was highly quantitative, we decided to analyze different subsets of the phenotypic values to see if, and how, significantly associated markers varied. In theory, a less quantitative phenotype will have reduced genetic complexity, allowing for easier detection of associated markers in GWAS. GLMs with two principal components were run for two different ranges of phenotypic values: “extreme” tetraconazole EC$_{50}$ values without intermediate values (0–0.1; 30–100 $\mu$g/ml), and a lower range of tetraconazole EC$_{50}$ values (0–10 $\mu$g/ml) (supplementary figs. S8 and S9 and B, Supplementary Material online). The model with the “extreme” phenotypic values did not yield significant associations at the Bonferroni-corrected threshold ($\alpha = 0.05$) but there were 572 significant associations at the false discovery rate threshold of $\alpha = 0.05$, and these overlapped with 95.5% of the significant markers (107/112) from the initial GWAS (supplementary fig. S9A and tables S3 and S4, Supplementary Material online). All of these associations were unique when compared with the significant associations from the initial GWAS (supplementary tables S3 and S5, Supplementary Material online). The most significant marker was SNP 9_1452111, 124-bp upstream of the start codon of CbCYP51 (CB0940_11379). The GWAS with the lower tetraconazole EC$_{50}$ values had ten significant associations at the Bonferroni significance threshold ($\alpha = 0.05$) (supplementary fig. S9B and table S5, Supplementary Material online). All of these associations were unique when compared with the significant associations from the initial GWAS (supplementary tables S3 and S5, Supplementary Material online). The most significant marker was a SNP in the 5’ UTR of gene CBE0940_02172 encoding tripeptidyl-peptidase Sed2-like (supplementary table S5, Supplementary Material online).

Synonymous and Nonsynonymous Mutations in CbCYP51 Are Associated with DMI Fungicide Resistance

Genome-wide association analyses of tetraconazole sensitivity suggested the involvement of the CbCYP51 locus with significantly associated SNP 9_1451478 within the coding region of the gene (fig. 2). This SNP gives rise to a synonymous change at E170, changing the 170th codon from GAG to GAA, both of which encode glutamic acid. In C. beticola, CbCYP51 is a single-copy intron-free gene of 1,632 bp (NCBI XP_023450255.1, CB0940_11379) (Nikou et al. 2009; Bolton, Birla, et al. 2012). No insertions or retrotransposons were found immediately upstream of CbCYP51 although several SNPs were identified within 3 kb on either side of the gene (supplementary fig. S10, Supplementary Material online).

We also investigated the presence of target site mutations in CbCYP51 that may influence DMI sensitivity. We found 11 different CbCYP51 gene coding sequences (haplotypes) in our set of 190 RRV region isolates. There were three different “DMI-sensitive” haplotypes; the most common harbored by
Isolates (haplotype 3), a highly divergent haplotype (haplotype 1) found in three isolates that harbored 113 SNPs and an 82-bp deletion and a third (haplotype 2) represented by a single sensitive isolate harboring a silent mutation at I122 and the amino acid substitution V467A (fig. 3). When comparing the remaining haplotypes to the most common sensitive haplotype, there were five different nonsynonymous mutations (L144F, I309T, I387M, Y464S, and V467A; fig. 3). The presence of amino acid substitutions L144F, I387M, or Y464S gave higher tetraconazole EC50 values when compared with sensitive haplotype #2 (fig. 3).

The most common CbCYP51 haplotype associated with resistance (56 isolates) had the single silent mutation at E170 (fig. 3). Presence of the E170 mutation in strains was associated with a significant increase in tetraconazole EC50 value (P < 0.001, supplementary fig. S15, Supplementary Material online). Because synonymous codons at position 170 were associated with differential tetraconazole resistance, we questioned whether codon bias might help explain this phenomenon. Consequently, we calculated genome-wide codon usage for C. beticola (supplementary table S6, Supplementary Material online). We assessed codon frequencies for glutamic acid (E) and found that the GAG codon was used slightly more often (56%) than the GAA codon (44%).

The most common amino acid substitution found was L144F (41 isolates) and could be achieved by either a T or C mutation in the third position of the 144th codon (fig. 3 and supplementary figs. S10 and S11, Supplementary Material online). Both TTT and TTC versions of L144F were associated with increased tetraconazole EC50 values (P < 0.01 and P < 0.001, respectively), but the TTC codon had a significantly higher mean EC50 value than the TTT codon (P < 0.001) (supplementary fig. S10, Supplementary Material online). Because codon usage again could underscore DMRI resistance, we assessed codon frequencies for phenylalanine (F) (supplementary table S6, Supplementary Material online). The phenylalanine codon TTC was found in the coding sequence 70% of the time compared with TTT, which was found in the remaining 30%. This is the largest difference in codon usage for any amino acid found in C. beticola.

To further investigate the involvement of these mutations in DMRI fungicide resistance, we sequenced CbCYP51 in 52 additional C. beticola isolates collected in 2019 from commercial fields in the RRV of North Dakota and Minnesota. The results corroborated the haplotype analyses of the 2016 and 2017 GWAS isolates. As before, the most common haplotype associated with resistance had the silent mutation E170 (supplementary fig. S16, Supplementary Material online). We found that the amino acid substitutions L144F and Y464S were again associated with increased tetraconazole EC50 values (supplementary fig. S16, Supplementary Material online). The amino acid mutation H306R was also found alongside L144F in a single 2019 isolate (supplementary fig. S16, Supplementary Material online).
Genome-Wide Scan for Recent Selection in the North American C. beticola Population

We next addressed if DMI fungicide application had left signals of recent selection in the C. beticola population. To this end, we focused on the population of 89 isolates exhibiting DMI fungicide resistance to conduct a genome-wide screen for selective sweeps. To account for the effect of demographic events on the distribution of genetic variation along the genome, we first inferred the recent population history of the C. beticola population using a simulation approach based on the unfolded site frequency spectrum (SFS) using the software FastsimCOAL (Excoffier et al. 2013). We assessed the likelihood of four demographic scenarios: 1) a recent population expansion, 2) a recent population bottleneck, 3) a bottleneck followed by a population expansion, and 4) a population bottleneck followed by a second recent bottleneck (supplementary fig. S17, Supplementary Material online). Comparing the Akaike Information Criterion (AIC) values of the four models, we found evidence for a demographic scenario involving one single recent bottleneck (supplementary table S7 and fig. S18, Supplementary Material online). The model including an ancient bottleneck and a recent expansion also showed a high likelihood, whereas the model with population expansion was the less likely demographic scenario. We further compared the observed SFS to the expected SFS for each model. One hundred replicate runs of each of 100,000 simulations were created to infer parameter values (supplementary fig. S19, Supplementary Material online). Using this strategy, we also found that the residuals between the expected and the observed SFS were minimized under the single bottleneck model and therefore provided further support for this scenario. We have used the parameter values obtained from the best inferred demographic scenario to simulate data under the neutrality using the software ms (Hudson 2002). For the specific parameters used for the simulations under neutrality (see the Materials and Methods).

Two methods with different sensitivity to genomic signals along genome alignments were used to detect selective sweeps in the C. beticola subpopulation that shows increased fungicide tolerance. OmegaPlus and RAiSD, identified 583 and 322 selective sweep regions, respectively, distributed across the C. beticola chromosomes (fig. 4 and supplementary tables S9 and S10, Supplementary Material online). These regions show significantly higher values of the statistics $\omega$ and $\mu$ when compared with the highest statistic values obtained from the 10,000 simulations under the neutral demographic scenario. The selective sweep regions ranged in length from 0.73 to 104.53 kb for OmegaPlus and from 1.02 to 672.21 kb for RAiSD. The number of variants included in these regions varied from 84 to 707 SNPs for OmegaPlus and from 52 to 6,208 for RAiSD. Furthermore, the number of predicted coding regions within the inferred selective sweeps were from zero to 79 for OmegaPlus and from zero to 59 for RAiSD. We further compared the output of the two independent approaches of OmegaPlus and RAiSD. Although these analyses detect different signatures in the genome data, some selective sweep regions were overlapping (fig. 4). In total, we identified 198 overlapping regions of the two selective sweep maps (using a minimum overlap size of 150 bp) spanning from 157 to 7.7 kb (supplementary table S11, Supplementary Material online) that represented ~2.94% of the genome. As many as 172 of the 198 overlapping selective sweeps are including coding sequences (from zero to 44 coding regions per overlapping region) revealing a set of functional traits that have experienced recent selection (supplementary table S11, Supplementary Material online).

Signatures of Recent Selective Sweeps Colocalize with DMI Fungicide Resistance Candidates in the C. beticola Population

Two of the selective sweep regions on chromosome 9 identified by RAiSD contained variants identified in the GWAS analysis that correlate with DMI fungicide resistance (supplementary table S14, Supplementary Material online). The two RAiSD regions spanned the positions 1402834–1412304 and 1495335–1503162 bp of chromosome 9 (supplementary table S10, Supplementary Material online). The first region encompassed intergenic markers 9_1404771 and 9_1405148, and 9_1403629 within the coding region of CB0940_11365, which encoded an aldehyde reductase (supplementary table S12, Supplementary Material online). The latter region contained markers 9_1496540 and 9_1497163 within the coding region of CB0940_11398, which encoded a hypothetical protein. To evaluate the significance of overlap between GWAS candidates and selective sweeps, we performed a randomization test. When randomizing both sets of features (GWAS candidates and selective sweeps), we report a significant deficit of overlap that can be explained by the fact that the GWAS candidates are nonrandomly distributed along the genome. Because we observed several GWAS candidates overlapping with the two sweeps on chromosome 9, we further asked how likely it is to observe such a number of SNPs in these regions by chance. By randomizing the GWAS candidates on the corresponding chromosome, we found the observed numbers to be higher than expected by chance ($P$ value = 0.0095 for the first sweep region, $P$ value = 0.0508 for the second sweep region, and $P$ value = 0.0011 when the two sweeps are considered together, computed with 100,000 randomizations). Coordinates of the predicted genomic features in the selective sweep regions on chromosome 9 are summarized in supplementary table S13, Supplementary Material online.
Assessing Fitness Penalties for DMI Fungicide Resistance Loci

To investigate whether there was a fitness penalty associated with DMI fungicide resistance, we measured the radial growth rates of all 190 *C. beticola* strains as a proxy for fitness (supplementary fig. S2B and table S2, Supplementary Material online). We performed association analyses for radial growth rate and obtained allelic effect estimates for each marker. There was a very weak positive correlation between allelic effects of DMI fungicide resistance (tetraconazole EC$_{50}$ values) and radial growth rate (Pearson correlation coefficient = 0.041, $P < 2.2e-16$) (fig. 5A). The most significant markers associated with tetraconazole resistance also appeared to have a negligible effect on growth rate (supplementary table S14 and fig. 5A, Supplementary Material online). We also

Fig. 4.—Genomic scans for selective sweeps using RAiSD and OmegaPlus (A) Selective sweep map obtained by RAiSD; the $\mu$-statistic values were calculated and plotted along the genome. Significant outlier loci are shown in red. (B) Selective sweep map obtained by OmegaPlus; $\alpha$-statistic values were plotted across the genome. Significant outlier loci are shown in red. The significance thresholds of the $\mu$ and $\alpha$ statistics were determined with demographic simulations (see Materials and Methods). Blue lines indicate selective sweeps longer than 150 bp detected with both methods.
performed association analyses for radial growth of isolates under salt stress (1 M NaCl) ( supplementary fig. S2C, Supplementary Material online). There was a slight negative correlation between the allelic effects of DMI fungicide resistance (tetraconazole EC50 values) and radial growth rate under salt stress (Pearson correlation coefficient $r = -0.051$, $P < 2.2e-16$) (fig. 5B). Again, the most significant markers associated with tetraconazole resistance did not appear to have meaningful impact on growth rates under salt stress (supplementary table S14, Supplementary Material online).

**Discussion**

We used whole-genome resequencing combined with genome-wide association studies and selective sweep identification to investigate the evolution of DMI fungicide resistance in field populations of the sugar beet pathogen *C. beticola*. Our results revealed a multilocus architecture of fungicide resistance including mutations in the fungicide target CbCYP51, as well as additional previously unreported proteins. We did not find a correlation between in vitro growth rate of isolates and DMI fungicide sensitivity, suggesting that DMI fungicide resistance loci can persist in the absence of fungicide.

Strikingly, we observed minor underlying population structure due to DMI fungicide resistance in these North American isolates. Tetraconazole-sensitive strains were clustered with more similar genetic backgrounds whilst tetraconazole-resistant strains were generally more distantly related. This could be attributed to strong selection pressure exerted on North American *C. beticola* populations due to widespread and repeated use of DMI fungicides, enabling the survival and proliferation of DMI-resistant isolates, indiscriminate of genetic background. It was important to consider that the underlying population structure explained by tetraconazole sensitivity could be confounding in downstream association mapping analyses, leading to false positive associations. Therefore, we attempted to correct for this stratification by using sufficient principal components from a PCA in the GLM. Despite the use of statistical significance thresholds, it is still possible that some significant associations were false positives. Consequently, functional genomics studies must be performed to verify the individual effects of associated loci.

Similar to previous genome-wide association studies for pesticide resistance (Mohd-Assaad et al. 2016; Hartmann et al. 2020; Yean et al. 2021), we found that additional proteins are likely involved in addition to the target protein. One significant GWAS marker was within polyketide synthase (PKS) gene CB0940_11350 which was part of a nonreducing PKS cluster previously induced in response to tetraconazole in a DMI-resistant isolate of *C. beticola* and repressed in a DMI-sensitive isolate (Bolton et al. 2016). We also note that unlike previous studies in fungi (Talas et al. 2016; Hartmann et al. 2020; Pereira et al. 2020), CbCYP51 was significantly associated with DMI resistance in genome-wide association. This was likely due to the high allele frequency of the E170 variant within our population (0.29), whereas other fungal populations, such as in *Z. tritici* (Hartmann et al. 2020), and *P. nodo-
rum (Pereira et al. 2017), have exhibited lower frequencies of CYP51 alleles associated with DMI resistance.

GWAS using different subsets of the phenotype suggested that the CYP51 locus is associated with DMI resistance for higher EC50 values (10–100 μg/ml), because CYP51 markers were only significantly associated with the presence of isolates when the analysis included isolates with EC50 values over 10 μg/ml. Removing these highly resistant DMI isolates gave completely different significant associations, emphasizing the highly quantitative nature of this trait. Previous genomic studies in fungal phytopathogens have investigated the presence of fitness penalties associated with fungicide resistance loci with varying results (Mohd-Assaad et al. 2016; Delmas et al. 2017; Pereira et al. 2020). Similar to P. nodorum (Pereira et al. 2020), we did not find evidence of fitness penalties for DMI resistance in C. beticola, using fungal growth rate as a fitness proxy. We demonstrated that the resistance loci identified through GWAS had little effect on radial growth rate, with or without salt stress. This was corroborated by finding no clear correlation between genome-wide allelic effects on tetraconazole EC50 values and radial growth rates on unamended media. Competition assays performed on sugar beet in the field between sensitive and resistant isolates resulted in a significant reduction in frequency of resistant isolates (Karaoglanidis et al. 2001). It is possible that a trade-off exists for DMI fungicide resistance in C. beticola, but we have not observed it with the proxy phenotype and conditions tested in this study.

Strong selective sweeps, in which a beneficial mutation is swept to fixation in a population alongside hitchhiking flanking regions (McVean 2007), have previously been shown to overlap with pesticide resistance loci in insects (Weedall et al. 2020), weeds (Kreiner et al. 2021), and fungi (Hartmann et al. 2020). Using DMI-resistant C. beticola isolates identified in this study, we also show that genome-wide selective sweeps overlap with some loci associated with DMI fungicide resistance, suggesting that application of these fungicides has been a recent selection pressure for the North American population. GWAS loci within the hypothetical protein CB0940_11398 and aldehyde reductase CB0940_11365 were within putative selective sweep regions and therefore alleles at these loci may have conferred a strong fitness advantage to C. beticola. However, functional and genetic approaches are necessary to confirm their selective advantage. Fungicide resistance loci identified in GWAS also overlapped with selective sweep regions in Zymoseptoria tritici, indicating recent positive selection (Hartmann et al. 2020). Similar to Z. tritici (Hartmann et al. 2020), CbCYP51 mutations in the present study did not lie within selective sweep regions.

In fungal human and plant pathogens, the ability of CYP51 to accept structural changes and maintain function has often led to the accumulation of amino acid changes and selection of haplotypes that reduce DMI binding and inhibition (Becher and Wirsel 2012). The amino acid substitutions L144F, I387M, and Y464S were previously reported in C. beticola strains from Serbia and, as in our study, were individually associated with DMI resistance (Trkulja et al. 2017). We found that L144F was the most common CbCYP51 amino acid change in RRV C. beticola isolates from 2017, 2018, and 2019. Using the CYP51 labeling convention proposed by Mair et al. (2016), L144F or I387M do not appear to have orthologous sites in other fungal species that have been associated with DMI resistance (Mair et al. 2016). However, the Y464S mutation appears to be analogous to Y461S/G/H that have been associated with DMI resistance in Z. tritici (Cools and Fraaije 2012; Mair et al. 2016). Additionally, alterations in equivalent residues in Y459 to Y461 have been found in A. fumigatus (Howard et al. 2006), C. albicans (Perea et al. 2001) and Mycosphaerella fijiensis (Canas-Gutierrez et al. 2009), all of which were associated with increased resistance to DMIs. Expression of ZtCYP51 encoding Y461H in S. cerevisiae conferred decreased sensitivity to all DMIs (Cools et al. 2010). Molecular modeling predicted this residue to be integral to the CYP51 active site with alterations directly impacting DMI binding (Mullins et al. 2011). Despite the widespread association of residues Y459 to Y461 to DMI resistance in fungal species, the Y464S amino acid exchange was not common in our study with only two isolates harboring this mutation. To the best of our knowledge, we also present three novel CbCYP51 amino acid substitutions in C. beticola, H306R, I309T, and V467A but the impact of these relatively rare mutations is still unclear.

Unexpectedly, we discovered a potential codon usage effect for the L144F substitution in CbCYP51. We observed that strains with L144F encoded by the TTC codon had a significantly lower EC50 value than strains with L144F encoded by the TTG codon. We did not find another mutation within or close to CbCYP51 (± 1 kb) in LD with the codon difference. In C. beticola, the phenylalanine codon TTG is used just 30% of the time in coding sequence when compared with the codon TTC at 70%, representing the biggest difference in codon usage for a single amino acid in C. beticola. The model fungus N. crassa exhibits a similar codon bias for phenylalanine with TTC used in ~67% of cases (Kazusa codon usage database). The use of rare versus optimal codons in N. crassa has been shown to impact transcript levels (Zhou et al. 2016, 2018), protein abundance (Zhou et al. 2015) and co-translational folding of proteins (Yu et al. 2015). Functional studies will be necessary to confirm these hypotheses.

Intriguingly, we identified a silent mutation (E170) associated with DMI resistance in our study. Obuya et al. (2015) also associated this mutation with DMI resistance using RRV isolates, and it was also previously associated with resistance in C. beticola in isolates from Greece (Nikou et al. 2009) and Serbia (Trkulja et al. 2017). Obuya et al. (2015) heterologously expressed a C. beticola CYP51 haplotype harboring E170 in S. cerevisiae strain R1 lacking multidrug resistance transporter Pdr5 (ΔPdr-5) and found no change in DMI sensitivity.
However, it is possible that this mutation has a C. beticola-specific influence on DMI sensitivity through codon usage, and thus functional studies in alternative hosts may not be conclusive. For glutamic acid (E), the GAG codon seen in more DMI-sensitive strains is used slightly more often (56%) than the GAA codon (44%). It is possible that codon usage in this context leads to differential co-translational CbCYP51 folding, protein structure, and DMI binding as suggested above for L144F. Because the GAA codon found in resistant strains is the nonoptimal codon, it seems unlikely that it would increase the translation rate and CbCYP51 protein levels. Another possibility is that the synonymous change influences DMI resistance via CbCYP51 expression levels, for example, via promotion of premature transcription termination (Zhou et al. 2018), chromatin structure (Zhou et al. 2016), mRNA stability (Duan and Anteza 2003), or even small-RNA-based gene regulation (Lee et al. 2010). Alternatively, the E170 mutation in RRV strains is in high LD with another mutation, which could affect CbCYP51 gene expression and be directly involved in DMI resistance. However, because isolates from disparate locations including the RRV (Obuya et al. 2015), Greece (Nikou et al. 2009), and Serbia (Trkulja et al. 2017) have identified an association between E170 and DMI resistance, it is tempting to speculate a direct involvement between this mutation and DMI resistance. Functional studies will be necessary to confirm the involvement of E170 with DMI resistance.

To conclude, association mapping and selective sweep analyses were used together for the first time in a Cercospora species. Future studies should establish if the mutations identified are directly involved in DMI fungicide resistance and clarify the role of CbCYP51 overexpression. Overall, we have demonstrated that GWAS was useful even for local populations of C. beticola. The identification of markers associated with DMI resistance has allowed for the development of methodologies to identify resistant strains (Shrestha et al. 2020), which was a major goal for this study. Moreover, the available isolate genotyping data and selective sweep maps can be used in future studies to establish the genetic architecture and evolutionary origins of other important traits, including virulence on the sugar beet host.

Materials and Methods

Field Sampling of C. beticola

The 190 C. beticola isolates were collected from sugar beet leaves harvested from naturally infected commercial fields in the RRV region of Minnesota and North Dakota, and Idaho (n = 2), in 2016 (n = 142) and 2017 (n = 48) (supplementary table S2, Supplementary Material online). Conidia were liberated from sugar beet lesions as described by Secor and Rivera (2012). Of the 142 isolates collected in 2016, 62 were collected from two adjacent fields. Random representative sampling of strains was performed in these two fields, as outlined by McDonald (1997), by walking diagonally across the field and collecting a leaf every meter from a corner to the center of the field. Prior to fungicide application, 60 diseased leaves were harvested from each field and 100 leaves were harvested from each field post-application of tetraconazole (Eminent fungicide). All isolates collected from these two adjacent Fargo fields were genotyped using eight microsatellite markers to remove any potential clones, as described by Vaghefi et al. (2016), which led to the selection of 62 as part of the final population (n = 190) (supplementary table S2, Supplementary Material online). The remaining isolates collected in 2016 (n = 80) and 2017 (n = 48) were obtained as part of annual C. beticola fungicide resistance surveys in the RRV region, where growers send infected sugar beet leaves to the Secor lab at North Dakota State University for fungal isolation and sensitivity testing. Isolates were later confirmed to be C. beticola, and not C. apiicola, by analyzing CbCAL (CB0940_08426) gene haplotypes (Groenewald et al. 2005; Knight and Pethybridge 2020).

Phenotyping for DMI Fungicide Sensitivity

To measure sensitivity to the DMI fungicide active ingredient tetraconazole, EC50 values were calculated from radial growth of the C. beticola isolates on amended media, as described by Secor and Rivera (2012). The single spore subcultures for all 190 isolates were transferred to clarified V8 (CV8) medium plates (10% v/v clarified V8 juice [Campbell’s Soup Co.], 0.5% w/v CaCO3, 1.5% w/v agar [Sigma-Aldrich]) and incubated at 20 °C for 15 days in a continuous light regime. An agar plug of 4 mm in diameter was excised from the growing edge of the colony and placed in the center of a set of CV8 plates: one nonamended control plate and the rest amended with serial 10-fold dilutions of technical grade tetraconazole (active ingredient of Eminent 125SL [Sipcam Agro]) from 0.001 to 100 μg/ml. All plates were incubated in the dark at 20 °C for 15 days after which two perpendicular measurements were made across the colonies and the diameter averaged. The percentage reduction in growth compared with the nonamended media was calculated for each tetraconazole concentration. The EC50 value for each isolate was calculated by plotting the percentage reduction in growth against logarithmic tetraconazole concentration and using regression curve fitting to find the tetraconazole concentration that reduced growth by 50%. Statistical analysis was performed in RStudio (RStudio Team 2020) and was comprised of one-way ANOVA followed by a post-hoc Tukey test to identify significant differences between groups.

Radial Growth Assays

All 190 isolates were grown on CV8 plates for 15 days at 20 °C in a continuous light regime, as described above. An agar plug of 4 mm in diameter was taken from the leading edge of...
these cultures and transferred to a new CV8 plate. Three unamended CV8 plates were initiated per isolate, and these were grown at 23 °C under continuous light. The radius of each culture was measured after 2, 6, 9, 13 and 16 days and a mean value was calculated for each day. Three CV8 plates amended with 1 M NaCl were also initiated per isolate and grown under the same conditions. The radius was measured for these cultures after 6, 9, 12, 16, 20 and 23 days and a mean value was calculated for each day. Linear regression of radius (mm) versus time (days) was performed using SAS software to establish the rate of radial growth in mm/day for both unamended and salt stress conditions.

DNA Extraction and Whole-Genome Resequencing

High-quality genomic DNA was extracted for library preparation from liquid cultures of C. beticola. A single 6-mm agar plug excised from the source PDA plate was sliced into small pieces and used to inoculate 100 ml Difco potato dextrose broth (PDB). Cultures were grown at 25 °C for 7 days, shaking at 150 rpm. The mycelia were filtered through Miracloth, flash frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle. The method of Zhang et al. (1996) was followed for large-scale isolation of genomic DNA but replacing the chloroform: isoamyl (24:1) with phenol: chloroform: isopropanol (25:24:1). The resultant DNA was then cleaned up further using the Qiagen DNeasy Plant Mini Kit (cat. no. 69106) according to the manufacturer’s instructions. DNA samples were sent to Beijing Genome Institute (BGI) for library preparation (400 bp inserts) and 100- or 150-bp paired-end whole-genome resequencing using the Illumina HiSeq 4000 platform to achieve approximately 25× genome coverage per isolate. All sequencing reads were deposited in the NCBI short-read archive under BioProject PRJNA673877.

Variant Calling

Sequencing read quality was analyzed using FastQC (Andrews 2011) and Trimomatic (Bolger et al. 2014) was subsequently used to trim reads (HEADCROP:10) and remove unpaired reads. The trimmed reads were aligned to the reference C. beticola 09-40 genome (NCBI RefSeq assembly GCF_002742065.1) (de Jonge et al. 2018) using BWA-MEM. Duplicate reads (PCR and optical) were output sam files to sorted, indexed bam files and to index the reference genome. Duplicate reads (PCR and optical) were removed from bam files using Picard MarkDuplicates (Institute B 2016). Genome Analysis Tool Kit (GATK) V4.0.8.1 HaploTypeCaller (McKenna et al. 2010) was used to identify SNPs and small indels between each isolate and the 09-40 reference sequence. We used the default diploid ploidy level, instead of -ploidy 1 option in our haploid fungus, to allow us to filter out variants in any poorly aligned regions that resulted in heterozygous calls. GATK CombineGVCFs was used to combine all HaplotypeCaller gVCFs into a multisample gVCF, which was genotyped by GATK GenotypeGVCFs to produce the final VCF. Vcftools (Danecek et al. 2011) was then used to filter variants for genotyping quality (–minGQ 10) and sequencing depth (–minDP 3).

Population Structure and LD Decay Analyses

Before performing PCA, the VCF from above was filtered using Vcftools to retain SNPs only (–remove-indels). Plink (Chang et al. 2015) was used to prune the SNPs for LD, with option –indep-pairwise 50 10 0.1, to analyze pairwise association between SNPs (R²) in chromosomal windows of 50 SNPs at a time and removing pairs with R² > 0.1 before shifting the window 10 bp. PCA was performed using the SMARTPCA function as part of the EIGENSOFT package (Patterson et al. 2013) and plots of PCs were created using PCAviz (Novembre et al. 2018).

Association Mapping

Association mapping was performed using GAPIT v3.0 (Wang and Zhang 2018). The imported genotyping VCF was first filtered in TASSEL v5.0 (Bradbury et al. 2007) to convert heterozygous calls to missing data and to establish a minor allele frequency of 0.05 and minimum SNP count of <10% missing. As the tetraconazole EC50 phenotype had highly positive skewing (not normally distributed), all values were log10 transformed prior to association mapping. A GLM was run as a naive model and as a model incorporating the optimal number of components derived from PCA as fixed effects to correct for population structure (we aimed to account for a minimum of 30% background variation). A mixed linear model was also ran incorporating a kinship matrix (K, calculated using the default VanRaden algorithm) as a random effect. The most appropriate model for a trait association was selected through visualization of the quantile–quantile (Q–Q) plots to achieve significant associations whilst not over-inflating P values. We also looked for the most significantly associated markers to be consistently appearing throughout multiple models. The R package qqman (Turner 2011) was used to generate Manhattan and Q–Q plots. Allelic effect estimates for phenotypes were derived from association mapping in GAPIT. R v.4.0.2 was used for the Pearson’s product–moment correlation test.

Evaluation of Associated Loci

To assess LD at significantly associated loci, LDheatmap (Shin et al. 2006) was used to plot color-coded values of pairwise LD (R²) between markers in the filtered VCF surrounding the significantly associated marker. SNPEff (Cingolani et al. 2012) was used to predict the effects of associated mutations within genes.
Inference of Demographic History

Prior to the scan of selective sweeps along the C. beticola genome, we computed the site frequency spectrum (SFS) to infer the demographic history of the population of isolates showing DMI fungicide resistance. Our analysis was based on the fit of four demographic models (supplementary fig. S12, Supplementary Material online) to the observed frequency spectrum of derived alleles (Unfolded or derived Allele Frequency Spectrum [DAFS]). We extracted the DAFS from the VCF file obtained from the population genomic data set and filtered the data set to include only SNPs with at least 1-kb distance to predicted coding sequences and 0.15-kb distance from each other to minimize the effects of selection and LD. We used the software ART (Huang et al. 2012) for in silico sequencing of the latest Cercospora cf. flagellaris assembly available on NCBI under the project PRJNA503907. Subsequently, we mapped the reads produced by ART to the C. beticola reference genome. The SNP ancestral states were assumed to be those present in the C. cf. flagellaris outgroup. The site frequency spectrum (SFS) was computed based on a total of 47,865 biallelic SNPs. To infer the demographic history of the C. beticola population, we used FastSIMCOAL2 (Excoffier et al. 2013). FastSIMCOAL2 performs coalescent simulations to approximate the likelihood of the data given a certain demographic model and specific parameter values. Maximization of the likelihood was achieved using several Expectation Maximization iterations. To this end we generated: 1) 100,000 simulations to approximate the likelihood with high precision, 2) 40 cycles of the expectation maximization algorithm to ensure that the maximum was reached, and 3) several independent replicate estimates to ensure that the global maximum likelihood was found.

We compared a set of models with different population size change scenarios. The four demographic scenarios that we compared were: 1) a recent population expansion, 2) a recent population bottleneck, 3) a bottleneck followed by a population expansion, and 4) a population bottleneck followed by a second recent bottleneck (supplementary fig. S12, Supplementary Material online). FastSIMCOAL2 expects the effective population sizes (Ne) to be given in number of haploid individuals, an estimation for the neutral mutation rate and the recombination rate of the species. To define the search range for the current Ne, we estimated the present-day effective population size of the C. beticola population based on Watterson’s θ and the pairwise nucleotide differences (π) both obtained with the software Arlequin v. 3.5.2.2 (Excoffier and Lischer 2010), and a range of realistic neutral mutation rates (5 × 10^{-7}, 5 × 10^{-8}, 3 × 10^{-8}, 1 × 10^{-8} mutations per site per generation). Subsequently, the lowest and the highest Ne estimations were used as the lowest and the highest value of the range. As the neutral mutation rate of Cercospora spp. is unknown we additionally performed 25 replicated runs of 100,000 simulations with 40 cycles of the expectation maximization for each of the combinations of all four demographic scenarios and four different mutation rates (5 × 10^{-7}, 5 × 10^{-8}, 3 × 10^{-8}, 1 × 10^{-8} mutation per site per generation) in 25 replicated runs per specified mutation rate. We have compared the 16 models using the AIC and choose the neutral mutation rate that showed the lowest AIC value for our final simulations (supplementary table S7, Supplementary Material online). Regarding the recombination rate, the literature is very limited for C. beticola. We have used estimations published for the fungal plant pathogen Microbotryum lycnids-dioicae (Badouin et al. 2015).

We used the estimates of the present-day Ne, the best inferred neutral mutation rate and the recombination rate estimation to simulate the four demographic models. For each demographic model, we performed 100,000 simulations, 40 cycles of the expectation maximization, and 50 replicate runs from different random starting values. We recorded the maximum-likelihood parameter estimates that were obtained across replicate runs. Finally, we calculated the AIC and selected the model with the lowest AIC as the demographic model that best fitted the data. Parameter values were inferred in a second step by performing 100,000 simulations, 40 iterations of the expectation maximization and 100 replicate runs from different random starting values. Wrong polarization of the SNPs for the calculation of the derived SFS can introduce bias in the demographic history inference. We followed the same methods described above to further infer the demographic history of the population using the folded SFS and compared the models inferred using the folded (supplementary fig. S18, Supplementary Material online) and unfolded SFS (summarized in supplementary text, Supplementary Material online).

Genome Scans for Selective Sweeps

Genomic scans for selective sweeps were performed by two approaches with the programs 1) OmegaPlus v. 3.0.3 (Alachiotis et al. 2012) and 2) RaISD v 2.9 (Alachiotis and Pavlidis 2018). OmegaPlus is a scalable implementation of the ω statistic (Nielsen et al. 2005) that can be applied to whole-genome data. It utilizes information on the LD between SNPs. The selective sweep analysis by OmegaPlus was performed for each chromosome separately. The grid size (the number of positions for which the ω statistic is calculated) was equal to the number of variants that each chromosome contained (28,698–77,617 points). The minimum and maximum window sizes were set to 1,000 and 100,000 bp, respectively. RaISD computes the μ statistic, a composite evaluation test that scores genomic regions by quantifying changes in the SFS, the levels of LD, and the amount of genetic diversity along the chromosome.
Scripts for the selective sweep analyses are provided in the randomization test using 100,000 randomizations in R. All of GWAS candidates and selective sweeps, we performed a left position of the windows were used to define the selective sweeps, under the best inferred demographic scenario using the software SFS_CODE (Hernandez 2008). The genomes were simulated as 37 fragments of 1 Mb to resemble our genome data. A population fitness parameter ($\gamma$) was given as the product of the effective population size ($Ne$) and the selection coefficient ($S$). In the simulations we used a set of different values for the parameter $\gamma$ ($NeS$): 50, 75, 100, and 200. For these simulations, we used the scaled mutation rate and recombination rate as inferred for the best fitting model from the FastSIMCOAL2 simulations (see “Inference of Demographic History” section). Subsequently, 1,000 simulations under the best neutral demographic model was used to estimate the top 5% cut-off. In the simulated data, outlier loci were only caused by background selection and not positive selection. This allowed us to determine a cut off of the FPR (Alachiotis and Pavlidis 2018).

To determine the significance of the identified selective sweeps, we computed the $\omega$ and $\mu$ statistics on 10,000 data sets simulated under the best neutral demographic scenario using the program ms (Hudson 2002). Full genomes were simulated as 37 1-Mb fragments. The inferred scaled mutation rate ($\theta = 2Ne\mu$) and recombination rate ($\rho = 2Ne\nu$) of the inferred demographic model described under the section “Inference of Demographic History” were used for these simulations. Setting a significance threshold for the deviation of the $\omega$ and $\mu$ statistics based on simulated data sets under the best neutral demographic model allowed us to control for the effect of demographic history of the population on the SFS, LD, and genetic diversity along the genome (Nielsen et al. 2005; Pavlidis et al. 2013). For both OmegaPlus and RAiSD, we have merged the overlapping consecutive windows that showed significant $\omega$ and $\mu$ values. The most right and most left position of the windows were used to define the selective sweep regions. To assess statistical significance of the overlap of GWAS candidates and selective sweeps, we performed a randomization test using 100,000 randomizations in R. All scripts for the selective sweep analyses are provided in the supplementary material, Supplementary Material online.

**CbCYP51 Gene Sequencing**

*Cercospora beticola* isolates were sampled and single spored from the RRV region of North Dakota and Minnesota in 2019 ($n = 52$), as described above. DNA was extracted directly from PDA cultures using a quick sodium dodecyl sulfate (SDS) lysis prep (Fran Lopez Ruiz, personal communication). The entire *CbCYP51* gene sequence (NCBI XP_023450255.1) was amplified in PCR with primers 530 and 532 from Bolton, Birla, et al. (2012), using standard conditions. Sanger sequencing of the entire PCR product was carried out using external primers 530 and 532, and internal primers 426, 349, and 566 from Bolton, Birla, et al. (2012).

**Codon Usage Assessment**

Predicted coding sequences for the 09-40 *C. beticola* reference were downloaded from NCBI (RefSeq assembly accession GCF_002742065.1) and entered into the Codon Usage tool in the Sequence Manipulation Suite (Stothard 2000) in order to calculate number and frequency of each codon type.

**Supplementary Material**

Supplementary data are available at Genome Biology and Evolution online.

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**Data Availability**

Supplementary material is available at https://doi.org/10.6084/m9.figshare.16543590.v1. Raw reads for all 190 *Cercospora beticola* isolates are available in the NCBI short-read archive under BioProject PRJNA673877. All scripts employed in this study can be found at https://github.com/Jimi92/Cerco-DMI-resistance (last accessed September 15, 2021).

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