Discovery of three loci increasing resistance to charcoal rot caused by *Macrophomina phaseolina* in octoploid strawberry

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Abstract

Charcoal rot caused by *Macrophomina phaseolina* is an increasing economic problem in annualized strawberry production systems around the world. Currently there are no effective postfumigation chemical controls for managing charcoal rot, and no information is available on the genetic architecture of resistance to *M. phaseolina* in strawberry (*Fragaria × ananassa*). In this study, three multiparental discovery populations and two validation populations were inoculated at planting and evaluated for mortality in three consecutive growing seasons. Genome-wide SNP genotyping and pedigree-based analysis with FlexQTL™ software were performed. Two large-effect quantitative trait loci (QTL) increasing charcoal rot resistance were discovered and validated in cultivated germplasm. FaRMp1 was located on linkage group 2A in the interval 20.4 to 24.9 cM, while FaRMp2 was located on linkage group 4B in the interval 41.1 to 61.2 cM. Together these QTLs explained 27% and 17% of the phenotypic variance in two discovery populations consisting of elite breeding germplasm. For both QTLs, the resistant allele showed some evidence of partial dominance, but no significant interaction was detected between the two loci. As the dosage of resistant alleles increased from 0 to 4 across the two QTLs, mortality decreased regardless of the combination of alleles. A third locus, FaRMp3 on 4D, was discovered in FVC 11-58, a reconstituted *F. × ananassa* originating from diverse *F. virginiana* and *F. chiloensis* accessions. This locus accounted for 44% of phenotypic variation in four segregating crosses. These findings will form the basis for DNA-informed breeding for resistance to charcoal rot in cultivated strawberry.

Keywords: fruit breeding; GWAS; haplotype; multiparental population; pedigree-based analysis; QTL

Introduction

The cultivated allo-octoploid strawberry *Fragaria × ananassa* (*2n = 8x = 56*) is one of the most economically valuable crops within the Rosaceae. Strawberry fruit are rich in health-associated compounds including vitamins C and K, fiber, folic acid, manganese, and potassium ([CSC 2015; Lewin 2016]. Strawberry is a hybrid species originating from an accidental cross in Europe between the two wild octoploids, *F. chiloensis* from Chile and *F. virginiana* from eastern North America in the mid-18th century ([Darrow 1966]. The first chromosome-scale genome assembly of cultivated strawberry recently shed light on the most ancient origins of the species, revealing four diploid progenitors ([Edger et al. 2019].

Charcoal rot, caused by the soilborne fungus *M. phaseolina*, causes rapid collapse and death of strawberry plants. Infection by *M. phaseolina* is favored by high temperatures (~30°C), low-soil moisture, and sandy soils ([Peres et al. 2018]. This describes typical strawberry growing conditions in California, Florida, and many other strawberry-producing regions around the globe. The fumigant methyl bromide was previously the industry standard for controlling soilborne pathogens in strawberry production, but with its progressive phase-out ([Montzka et al. 2002; Velders et al. 2007, Holmes et al. 2020] charcoal rot has rapidly become a major disease of strawberry. Alternative fumigants have limited movement through the soil profile leading to decreased effectiveness, and there are no curative fungicides for this disease ([Peres et al. 2018]. Genetic resistance holds promise as a method of control. Differences in resistance in elite germplasm and released cultivars have recently been documented ([Holmes et al. 2020]. Resistance has also been documented in *F. chiloensis* and *F. virginiana* and in reconstituted *F. × ananassa* ([Zurn et al. 2020] generated from diverse sources of both species ([Hancock et al. 2010]. Even moderate reductions in the rate of progression and severity of...
Charcoal rot of strawberry, caused by *Macrophomina phaseolina*, is an increasing economic problem in strawberry production worldwide, with limited means of control besides genetic resistance. Three different genetic loci for resistance were discovered, each significantly slowing the rate of mortality. Together these loci will form a basis for DNA-informed breeding tools against this disease.

**Materials and methods**

**QTL discovery populations**

Three multiparental discovery populations were inoculated, planted, and evaluated in three consecutive growing seasons in 2016–2017, 2017–2018, and 2018–2019. Populations in the first 2 years consisted of seedlings obtained from controlled crosses among elite lines within the UF strawberry breeding program. Crosses were chosen to represent the breadth of the elite germplasm as well as to achieve connectedness among parents (Supplementary Figures 1 and 2). A network analysis was performed to visually examine the relationships within discovery populations (Purcell et al. 2007; Neuditschko et al. 2012) (Supplementary Figure 3). The 2016–2017 population consisted of 40 full-sib families obtained from crosses made among 40 parents (Table 1). The 2017–2018 population consisted of 33 full-sib families derived from crosses among 29 parents (Table 1). Although no cross was evaluated across years, seven parents were common between the 2 years providing replication of segregating alleles across years. In 2018–2019, four crosses were evaluated that were derived from FVC 11–58, a reconstituted *F. ×ananassa* of diverse parentage that was previously identified as resistant to charcoal rot (Zurn et al. 2020). This selection was crossed to a single susceptible advanced selection each from the Michigan State University, UF, University of California-Davis and USDA-ARS (Corvallis, OR) breeding programs (Table 2). All seedlings, parents, and checks were clonally multiplied via runners in the UF summer breeding nursery near Malin, Oregon. Four clonal replicates (runners) of each seedling were planted in a randomized complete block design (RCBD) with one runner plant in each of the four blocks (two raised-beds covered with black plastic mulch) at the UF/IFAS Gulf Coast Research and Education Center in Wimauma, Florida. Each plant was inoculated at the time of planting on October 6, 2016, October 6, 2017, and September 27, 2018 into raised beds covered with black plastic mulch. Plants were established with overhead irrigation for 10 days after planting during daylight hours. Drip irrigation was used for the remainder of the growing season. Fertilizer applications and pest control were carried out according to industry standards.

**QTL validation populations**

QTL validation populations were evaluated over two growing seasons. The 2017–2018 population was planted on October 6, 2017 and consisted of 96 individuals including 79 selections, 13 elite selections, and 4 commercial varieties. The 2018–2019 population was planted on October 10, 2018 and consisted of 149 individuals including 121 selections, 13 elite selections, and 4 commercial cultivars. The 2017–2018 population was planted on October 6, 2017 and consisted of 96 individuals including 79 selections, 13 elite selections, and 4 commercial cultivars. Eight clonal replicates were tested, split into two plots of four plants each. The trials were planted as an RCBD, where each block consisted of one raised bed. Validation sets were planted adjacent to the discovery populations tested in the same years and inoculated according to the same protocol.

**Inoculation**

*M. phaseolina* inoculum was produced by reviving three isolates from −80°C storage on September 16, 2016, September 22, 2017, and September 5, 2018. Isolates 01-179, 02-200, and 09-95 were used in year 1, isolates 09-95, 12-467, and 17-29 were used in year 2, and isolates 09-95, 12-467, and 17-29 were used in year 3. Each isolate had been previously collected from strawberry fruiting fields in central Florida by the GCREC small fruit pathology lab. These isolates were chosen as a representative sample of all isolates collected from strawberry in the region with at least an average level of pathogenicity. Isolates were revived on potato dextrose agar and incubated in the dark at 29°C for 3 weeks. For every 10 colonized plates, 400 mL deionized H2O was added and the mixture blended at low speed for 1 minute. This solution was further diluted by adding 1,500 mL of sterilized 0.35% water agar solution in 2016 and 0.25% solution in 2017 and 2018. The total volumes of inoculum were 34, 48, and 32 liters in years 1, 2, and
removed by comparing SNP diplotype of each individual to their respective parental genotypes and siblings. Correct parentage was inferred where incorrect parentage was detected. If the correct parent was not obvious, a “dummy” parent was assigned. In addition, markers with frequent inheritance errors, particularly on linkage groups (LGs) 2A and 4B, were removed from the dataset. These errors were determined in the “mconsistency” and “genomelm” files of FlexQTL™ outputs and re-analyzed in FlexQTL™ multiple times until no errors were observed.

Genetic mapping
An in-house genetic map “14.95” (Verma et al. unpublished) was used to assign SNP markers to 28 LGs of octoploid strawberry. The 14.95 map was constructed from 165 progeny derived from a cross between "FL 08-10" and "FL 12.115-10" using JoinMap 4.1 software (Van Ooijen 2006, 2011). The marker order and subgenome assignments followed the SSR reference map of Van Dijk et al. (2014). Fraction values of observed (oDR) and expected (eDR) double recombinant singletons were identified using FlexQTL™ software and markers with oDR—eDR ≥ 0.05 were eliminated from the map. A total of 14,332 SNP marker loci were genetically mapped. To reduce computation time in FlexQTL™, marker density was decreased by eliminating markers at identical map positions with the lowest minor allele frequency (MAF). A small percentage of markers with MAF below 0.1 were retained to capture unique map positions. To further curate the map, graphical genotyping was performed following the procedure of Bassil et al. (2015) for the LGs that showed a QTL. A final set of 9,670 markers that were highly informative for both the 2016–2017 and 2017–2018 discovery populations was used for QTL analysis. For 2018–2019 discovery population, ~6,000 common SNP markers between IStraw35 and FanaSNP arrays were scrutinized. Of these, 3,402 SNPs were already included in the 14.95 genetic map and were used for the QTL analysis.

QTL analysis and GWAS
QTL analysis was performed using FlexQTL™, which simulates MCMC-based Bayesian analysis using an additive model and incorporated the infinitesimal model (TIM) to account for polygenic effects, detailed in Bink et al. (2014). The maximum number of QTL was set to 15, the prior number of QTL was set to either 2 or 3 (Bink et al. 2002, 2014), and genome-wide analyses were performed three times for each prior. To save computational time, the final map was further curated by keeping every fourth marker, equating to 25% of mapped markers, with the exception

Table 1: Summary statistics of QTL discovery and validation populations evaluated for resistance to charcoal rot caused by *Macrophomina phaseolina*. Parents and seedlings were clonally propagated via runners for field evaluations

| Family  | Mother          | Father | n  |
|---------|-----------------|--------|----|
| 18 41   | FL 13.42-5      | FVC 11–58 | 55 |
| 18 68   | 0SC109F002      | FVC 11–58 | 16 |
| ORUS 3792 | FVC 11–58 | ORUS 2780-1 | 31 |
| ORUS 3801 | MSU61      | FVC 11–58 | 26 |

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Disease ratings
Ratings for the discovery populations began with the collapse of the first plants in the trial on October 28, 2016, November 1, 2017, and October 22, 2018 and continued until 75% of the entire plant population collapsed. Plant collapse was recorded weekly with a 0 assigned to surviving plants, and a 1 assigned to collapsed plants. Plants were considered collapsed when 75% or more of the total leaf canopy, considering leaves from all developed crowns, of each plant had wilted and collapsed onto the bed surface. Re-isolations from necrotic portions of crowns of randomly chosen collapsed plants were performed multiple times each season and confirmed *M. phaseolina* infection. Collapse over time was used to calculate the area under the disease progress curve (AUDPC) for each individual. Rating of the validation population began on October 28, 2016 and concluded on March 14, 2018 when mortality reached 66%. Rating of the validation set in 2018–2019 began on October 29, 2018 and concluded on January 9, 2019 when plant collapse reached 46%, since after that date mortality from other pathogens began to be detected in re-isolations. A single AUDPC value per individual was generated for QTL analysis by considering mortality over time of the multiple clonal replicates for each individual.

Genotyping
DNA extractions for all populations were carried out according to Mangandi et al. (2017). Genotyping was performed using the Affymetrix Axiom® IStraw35 SNP array (Verma et al. 2017) in 2016–2017 and 2018–2019 and the FanaSNP 50K array (Hardigan et al. 2020) in 2018–2019. Genotyping errors were detected and
of the two LGs where QTL were initially located. In all, there were 2,608 markers used in each iteration of the genome-wide QTL analyses. Following the genome-wide analyses, three iterations were run with only the LGs where the QTL were detected (2A and 4B) for comparison. For all analyses, different starting seeds were used in order to create independence between runs, using simulation chain lengths of 100,000, 150,000, and 200,000 iterations with thinning values of 100, 150, and 200, respectively. The effective sample size was set to 101 to ensure convergence with effective chain sizes of at least 100. Each iteration converged with effective chain samples or ECS ≥ 100 for each of the parameters—mean (mean1), variance of the error (vERR1 1), variance of the polygenic model (vTIM1 1), number of QTL (nQTL), and the variance for the number of QTL (vQTL1 1) as recommended by Bink et al. (2014). The Bayes factor parameter (2lnBF10) was interpreted as giving nonsignificant (0–2), positive (2–5), strong (5–10), or decisive (>10) evidence for presence of QTL (Kass and Raftery 1995). VisualFlexQTL™ (www.flexqtl.nl) software was used to visualize FlexQTL™ outputs including traces of convergence of QTL models, positions, and QTL genotypes. The mode value (identified in the Flex.log file) was used as the most probable location for each QTL. Complete FlexQTL™ parameters and their explanations are provided in Mangandi et al. (2017).

Because the markers on the FanuSNP array are anchored to the “Camarosa” genome (Edger et al. 2019; Hardigan et al. 2020), a GWAS of the 2018–2019 discovery population was performed using the physical positions of 49,331 SNPs using mixed linear models (MLMs) in the Genome Association and Prediction Integrated Tool in R (GAPIT version 3) (Wang and Zhang 2020). We did not perform SNP filtering prior to GWAS analysis and used default parameters for the MLM.

**Genetic parameters estimates**

The phenotypic variance explained (PVE) by each QTL was estimated using outputs derived from FlexQTL™ software. Narrow-sense heritability ($h^2$) was calculated using the formula:

$$h^2 = \frac{VP - VE}{VP}$$

Where VP is the total phenotypic variance of the trait explained by all genetic factors included in the genetic model and VE is the residual error variance (Bink et al. 2014). The proportion of PVE by each QTL was calculated using the formula:

$$PVE = \left(\frac{AVt}{VP}\right) \times 100$$

where AVt is the additive variance of the trait being evaluated and VP is the total phenotypic variance of the trait as determined by the QTL model.

Because the phenotype inputs for FlexQTL™ analyses were a single AUDPC value per individual, generated from the collapse over time of the multiple clonal replicates of that individual, variability among replicates was not directly considered when calculating genetic parameters in FlexQTL™. To take individual replicates into account in calculating genetic parameters, variance components were calculated for years 1 and 2, first separately, then together, using ASReml (Gilmour et al. 2009). Incidence of plant collapse (0 or 1 for each plant) at the last evaluation date for each season was used. Univariate analyses for plant collapse were based on the binary scale (0 to 1) and analyzed using general linear mixed models (GLMMs). Variance components were estimated following Mangandi et al. (2017). All analyses were based on a parental model with a pedigree tracing back two generations. Replication was treated as a fixed effect, while bed, family, and genotype were considered as random effects. Broad sense ($H^2$) and narrow sense ($h^2$) heritability for each variable were estimated as follows:

$$H^2 = \frac{\sigma^2_r}{2\sigma^2_g + \sigma^2_s + \sigma^2_e + \sigma^2_r}$$

$$h^2 = \frac{4\sigma^2_r}{2\sigma^2_g + \sigma^2_s + \sigma^2_e + \sigma^2_r}$$

The variance components $\sigma^2_G$, $\sigma^2_G$, $\sigma^2_s$, $\sigma^2_e$, and $\sigma^2_r$ were derived similarly to the methods of Osorio et al. (2014). The error variance ($\sigma^2_e$) of the estimates for plant collapse caused by M. phaseolina was fixed within GLMM to a value of 3.29 (Gilmour et al. 2009).

**Haplotyping and allele effects estimation**

PediHaploTyper software (Voorrips et al. 2016) was utilized to identify haploblock alleles within two QTLs regions (Voorrips et al. 2016; Verma et al. 2019). Haplotypes were initially constructed for the 2016–2017 and 2017–2018 discovery populations using 28 SNP markers spanning 21 to 24 cm on LG 2A and 16 SNP markers spanning 41 to 50 cm on LG 4B. Two SNPs for each QTL, representing the full haplotype diversity in the populations with the fewest possible markers, were used for manual curation of the haplotypes (Table 3). Imputation of the missing marker information was carried out using the pedigree-based imputation method in Fimpute software developed by Sargolzaei et al. (2014) (test dataset accuracy of 97%).

Using one-way ANOVA in R software (R Core Team 2019), statistical significance of the phenotypic effects was determined for each haplotype on LGs 2A and 4B separately. The general linear hypothesis was adjusted for multiple comparisons using false discovery rate. Diploype effects were evaluated using the same method except that the general linear hypothesis was adjusted using the Bonferroni test for multiple comparisons. Haplotypes and diploypes were used to represent and characterize QTL allele and genotype effects, respectively.

**Results**

Wide variability in plant collapse due to M. phaseolina infection was observed in all populations tested. The AUDPC values in the discovery populations ranged from 0 to 90 in 2016–2017, 0–130 in 2017–2018, and 0–70 in 2018–2019. In all populations/years, both the resistant and susceptible extremes of the range were well-populated (Figure 1). In the validation populations, the ranges were similar with 0–130 in 2017–2018 and 0–60 in 2018–2019, but the distributions were weighted more heavily toward lower AUDPC values (resistance) (Figure 2). Two large-effect QTLs were detected with decisive evidence in UF breeding germplasm in both years (Figure 3). The first was located on LG 2A with an interval spanning 20.4 to 24.9 cm (FaRmp1). The second was located on LG 4B spanning 41.1 cm to 61.2 cm (FaRmp2). LG 2A corresponds to chromosome (Chr) 2-2 of the “Camarosa” reference genome (Edger et al. 2019), and LG 4B corresponds to Chr4-3 (Hardigan et al. 2020). Naming of the two QTL followed the convention Fragaria resistance to M. phaseolina locus/gene 1&2. For the discovery populations in 2018–2019 derived from FVC 11-58,
there was an increase in resistance (decrease in AUDPC) when the number of resistant alleles increased from 0 to 4, regardless of the specific combination of alleles across the two loci. Generalizing across the three populations/years, an increase from 0 to 2 resistant alleles resulted in a 30%–60% reduction in AUDPC, while an increase from 0 to 4 resistant alleles resulted in a 73%–79% reduction in AUDPC (Figure 6).

**Discussion**

To our knowledge this is the first report of loci contributing resistance to charcoal rot caused by *M. phaseolina* in strawberry. The discovery of *FaRMp1* on LG 2A and *FaRMp2* on LG 4B in cultivated germplasm was carried out in two different complex population sets, tested in two separate years. These populations were representative samples of the crosses made in the elite strawberry breeding population at UF. The two populations were highly related, even sharing multiple direct parents. Thus, alleles at both loci were well-replicated across populations and years. Furthermore, the effects of *FaRMp1* and *FaRMp2* on resistance were validated in two separate sets of cultivars and advanced selections across two seasons via the observation of consistent and significant marker haplotype (QTL allele) effects.

Average estimates of phenotypic effects of the two loci together from FlexQTL™ outputs were 27% in the first discovery population and 17% in the second discovery population consisting of UF breeding material. Comparing these estimates with heritability estimates from the same analyses (Table 3) suggests that one-third to two-third of the genetic variability for resistance was accounted for by the two loci. Such estimates are, of course, strongly influenced by allele frequencies. Because these elite populations were not made for the purpose of studying the inheritance of resistance, some crosses in both seasons did not segregate for either QTL. A more direct way to assess the potential utility of these loci in breeding is to measure the relative gains in resistance from selecting resistant alleles. This question was examined in the two discovery populations and the larger validation population in which the effects of QTL alleles and their interactions were quantified (Figure 6). Increasing from 0 to 2 resistance alleles (rrrr to RRrr, rrRR, or RrRr) would reduce the area under the disease progress curve by 30%–60%, while
increasing from 0 to 4 resistance alleles (rrrr to RRRR) would reduce disease progress by 73%–79%. Thus, selecting for more resistant alleles at both loci should be an effective strategy for increasing resistance in the population and in future cultivars.

While we have demonstrated that loci for charcoal rot resistance exist within commercial germplasm, it is important to discover and characterize additional novel loci and alleles in order to increase the strength of resistance and its long-term durability. FVC 11–58 is a reconstituted F. ×ananassa of diverse parentage that was previously identified as resistant to charcoal rot (Zurm et al. 2020). This individual was crossed with susceptible selections from four different U.S. breeding programs and the four full-sib families screened for resistance, resulting in the discovery of FaRMp3. Because this population was genotyped with a next-generation array representing more complete chromosome coverage (Hardigan et al. 2020) and with SNP probes anchored to the “Camarosa” genome, a GWAS analysis was performed that confirmed the presence of a single locus on Chr4-2. The estimate of PVE by this locus was nearly 80%, which was not consistent with a narrow sense heritability estimate of 0.44. The reason for the lack of agreement between these two estimates was not clear but may relate to the high frequency of the resistant allele in the population. Regardless, the resistant effect of FaRMp3 may be stronger than for the loci in commercial germplasm. Most of the FVC 11–58 progeny displayed greater resistance than commercial selections with a single dose of either FaRMp1 or FaRMp2. The susceptible parents crossed to FVC 11–58 did not have resistant alleles at FaRMp1 or FaRMp2. Therefore, we are not yet able to shed light on potential interactions among these loci and FaRMp3. Additional crosses have been made to examine the effects of pyramiding all three loci in future studies.

The FaRMp2 (Chr 4-3) and FaRMp3 (Chr 4-2) loci were located to different subgenomes of chromosome group four. Preliminary
Figure 3 Trace plots and posterior probability graphs from Visual FlexQTL™ outputs showing the position of two QTL conferring resistance to plant collapse caused by Macrophomina phaseolina. SNP probes mapped to these locations on LGs 2A and 4B are shown. No other QTL with significant probabilities were observed. SNP probes in bold were used for haplotyping.

Figure 4 Manhattan plot from GWAS of the 2018–2019 discovery population derived from FVC 11–58. Chr4-2 corresponds to LG 4D. Chromosomes are named as per Edger et al. (2019) and Hardigan et al. (2020).

Table 4 Haplotypes at the FaRMp1 and FaRMp2 loci described by their phased SNP marker alleles

| Haplotype | Allele 1 | Allele 2 | Haplotype | Allele 1 | Allele 2 |
|-----------|---------|---------|-----------|---------|---------|
| Mp1H1     | T       | A       | Mp2H1     | A       | G       |
| Mp1H2     | T       | C       | Mp2H2     | G       | T       |
| Mp1H3     | G       | C       | Mp2H3     | A       | T       |
| Mp1H4     | G       | A       | Mp2H4     | G       | G       |
| SNP Probe | AX-89781417 | AX-89819829 | SNP Probe | AX-89790211 | AX-89830947 |
| Position (cM) | 22.9 | 24.1 | Position (cM) | 48.6 | 50.6 |
analysis of the two QTLs intervals suggests that FaRMp2 and FaRMp3 could reside in homoeologous regions, as they are both centered at physical positions of roughly 7–8 Mb on their respective subgenomes. The intervals will need to be narrowed considerably in order to further investigate the question of homoeology. We should note that a small number of significant markers were identified in the GWAS for the FVC 11–58 populations on Chr 4-3 and 4-4 in addition to 4-2 (Figure 4). AsFaRMp2 was not segregating in the FVC 11–58 populations, a possible explanation is that these markers were assigned to the wrong subgenomes and instead reside on 4-2.

In our field trials, plants of even the most resistant individuals eventually collapse with enough time under warm conditions. However, delaying plant collapse is still of great benefit to strawberry growers, allowing them to produce greater marketable yields. This is especially important since early-season yields are usually more profitable than late-season yields in most production regions of the world. Marker-assisted selection at FaRMp1 and FaRMp2 can be applied in the short term to reliably increase charcoal rot resistance in cultivars. Meanwhile, FaRMp3 will be introgressed into cultivated germplasm. It will also be beneficial to characterize the genomic regions represented by these loci toward understanding the genes and causal polymorphisms involved. Given that genetic resistance is still the only effective post-fumigation control for charcoal rot of strawberry, the search for novel sources of resistance should continue in order to increase and diversify the genetic arsenal available to breeders.

Data availability statement
A “Supplementary Figures” file contains six supplementary figures cited in this article and their captions. All marker genotypes, phenotypes, and pedigrees are available in additional supplementary data files. File S6, “Whitaker_pedigree_data policy documentation” contains detailed descriptions of Files S1–S5. File S1 contains genotypic, pedigree, phenotypic, and genetic map data for the 2016–2017 QTL discovery population set. File S2 contains genotypic, pedigree, phenotypic, and genetic map data for the 2017–2018 QTL discovery population set. File S3 contains genotypic data, physical map, and phenotypic data used for discovery population 2018–2019 GWAS analysis. File S4 has genotypic and phenotypic data used for the validation population 2017–2018. File S5 has genotypic and phenotypic data used for the validation population 2018–2019. Supplementary Material available at figshare: https://doi.org/10.25387/g3.13003700.

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Conflict of interest: The authors declare that they have no conflicts of interest.

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