IDENTIFYING INBRED LINES WITH RESISTANCE TO ENDEMIC DISEASES IN EXOTIC MAIZE GERMPLASM

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Abstract

Mal de Rio Cuarto (MRC) and common rust (CR), caused by *Mal de Rio Cuarto virus* (MRCV) and *Puccinia sorghi*, respectively, are endemic diseases affecting maize (*Zea mays* L.) production in Argentina. Exotic maize germplasm is an important source of resistance to these diseases. The aim of this work was to identify maize lines that exhibit MRC and CR resistance. A multi-environment trial was performed to phenotypically assess a diverse panel of inbred lines from the International Maize and Wheat Improvement Center (CIMMYT). The maize lines were evaluated using a disease severity index (DSI) for MRC and CR in the central area of Argentina. A multi-trait mixed linear model was used to identify the lines with the best performance for both diseases and estimate genetic parameters. No correlation of resistance between MRC and CR was found among the tested lines. Additionally, BLUPs of genotypic effects were used as response variable to perform a genome-wide association study (GWAS). The GWAS revealed promising alleles for maize breeding, two associated with MRC and three with CR. Lines with lower DSI for MRC and CR were identified as novel materials for incorporating resistance to the local germplasm.

Keywords: disease severity index, *Mal de Rio Cuarto virus*, *Puccinia sorghi*, Genetic correlation, Multi-environment trial, QTL

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Introduction

Maize (*Zea mays* L.) accounts for 33% of the global cereal crop production, with Argentina ranking fourth in exports (OECD-FAO, 2018). The crop is affected by several pathogens that cause different diseases and reduce production worldwide (Yang et al. 2017). In Argentina, Mal de Rio Cuarto (MRC) and common rust (CR), caused by *Mal de Rio Cuarto virus* (MRCV) and *Puccinia sorghi,* respectively, are endemic diseases that affect maize production, causing severe yield losses with different intensities every year (Gimenez Pecci et al. 2012; Botta & Gonzalez 2015; Guerra et al. 2019). MRCV is classified as a member of the genus *Fijivirus*, family *Reoviridae* (Milne et al. 2005), and is transmitted in a persistent propagative manner by insect vectors, mainly *Delphacodes kuscheli* Fennah (Ornaghi et al. 1993). *Puccinia sorghi* belongs to the Basidiomycota group and is a biotrophic, heteroecious macrocyclic fungus that has been found to have complete occurrence cycles in Argentina, with epidemiological implications (Guerra et al. 2019).

Genetic resistance is the main strategy for reducing production losses caused by MRC in Argentina (Bonamico et al. 2012). It has also been documented as the tool for the control of damage produced by CR in USA (Olukolu et al. 2016). Resistance to MRC behaves as a quantitative trait; molecular markers for this trait have been identified associated with resistance loci in biparental populations evaluated in the area where the disease is endemic (Bonamico et al. 2012; Di Renzo et al. 2004; Rossi et al. 2015). In turn, resistance to CR in maize can be quantitative but also qualitative, and is conferred by dominant genes, with more than 25 major genes called *Rp* having been identified (Delaney et al. 1998; Hooker, 1985; Vanderplank, 1984). Works mapping genomic regions for MRC (Bonamico et al. 2012; Di Renzo et al. 2004; Rossi et al. 2015) and CR (Brown et al. 2001; Lübberstedt et al. 1998) have reported resistance alleles.

The International Maize and Wheat Improvement Center (CIMMYT) is an important source of diverse germplasm for maize breeding programs worldwide. CIMMYT maize lines are carefully selected for their good combining ability and a significant number of value-added traits such as resistance to disease (Wu et al. 2016). Several mixed linear models used to test the effect of a particular genomic region on the phenotype have been proposed with the aim of determining genetic merit and selecting genotypes (Malosetti et al. 2008). Multivariate mixed models allow us to characterize genotypes considering more than one trait and

potential correlations among traits (Covarrubias-Pazaran, 2016). This model might provide information that can help identify materials with resistance to both MRC and CR. Additionally, a genome-wide association study (GWAS) on diverse panels of germplasm with high-density single nucleotide polymorphism (SNP) arrays can offer a valuable first insight into trait architecture for subsequent validation as a breeding tool (Korte and Farlow, 2013). A systematic literature review identified 110 studies that mapped genomic regions for viral or fungal diseases in maize (Rossi et al. 2019). Chromosomes 1, 6 and 10 were reported as linkage groups that contain major-effect genomic regions for resistance to viral disease. In turn, chromosomes 2, 5 and 10 carried major-effect loci for fungal disease. Therefore, there might be regions conferring resistance to both viruses and fungi.

An extensive molecular characterization of CIMMYT maize lines was carried out by Wu et al. (2016), suggesting high genetic diversity. These maize lines have been phenotypically evaluated for resistance to several diseases caused by fungi and viruses in different latitudes (Chen et al. 2015; Ding et al. 2015). The aim of this work was to identify CIMMYT maize lines that exhibit MRC and CR resistance and promising alleles for breeding programs.

Materials and methods

Plant material and field trial

A diverse panel of 165 maize lines from CIMMYT (Supplementary Table 1) was evaluated for resistance to MRC and CR during the 2017-2018 and 2018-2019 crop seasons in three environments (Sampacho 2017-2018 (SA18), Río Cuarto 2017-2018 (RC18), and 2018-2019 (RC19)) of the central area of Argentina. The trials were conducted under natural infection, with trial plots being established adjacent to winter grass crops, the natural reservoirs of MRCV and its vector insect (Rodriguez Pardina et al. 1998). Regarding common rust, since the inoculum is dispersed by wind (Botta and Gonzalez 2015), infestation is highly prevalent in the area. In each environment, a partially replicated (p-rep) design was used (Cullis et al. 2006), with 25% of the genotypes with three replications and the remaining genotypes with one replication. Planting was performed at double density; three weeks after emergence, plants were thinned to 15 plants per plot. Hand weeding was performed as necessary in all plots. Each plot consisted of a single row, 3 m in length and 0.52 m in width. In each environment, maize lines Mo17 and B73 were planted in each block as susceptible checks and BLS14 as a resistant line.

Phenotypic data

All plants in each plot were evaluated and scored for MRC and CR by observing symptoms at flowering stage. For MRC, each plant was classified by the degree of severity, according to the scale proposed by Ornaghi et al. (1999): 0 = no symptoms; 1 = presence of enations; 2 = presence of enations + shortened internode; 3 = maximum development of MRC disease (ears with no kernels). For CR, the ear leaf of each plant and the leaves immediately above and below the ear leaf were classified for severity following the diagrammatic scale proposed by Peterson et al. (1948): 0 = asymptomatic plant, 1 = up to 1% of foliar area affected, 2 = up to 5% foliar area affected, 3 = up to 10% foliar area affected, 4 = up to 20% foliar area affected, and 5 = up to 50% foliar area affected. A disease severity index (DSI) based on disease degree was calculated for each plot and used to rate lines for their resistance to MRC and CR, according to Di Renzo et al. (2002). The DSI ranges between 0 (no diseased plants) and 100 (severely diseased plants).

$$DSI = \frac{\sum degree \times number\ of\ plants\ in\ class}{maximun\ value\ of\ scale \times total\ number\ of\ plants} \times 100$$

Genomic data

The line characterization used for this study was that performed using SNP markers (Wu et al. 2016) available from http://data.cimmyt.org/dvn. Of a total of 362,008 SNPs, 78,543 were selected, which were distributed in the 10 chromosomes. Selection was based on the quality of the marker. In a first step, minor SNP states and minor allele frequency <0.05 were removed. Only markers with low missing data rate (<35%) were kept. The genomic database used in this work is available from https://github.com/PlantbreedingUNRC/GWAS-MRC-CR.

Statistical analysis

Phenotypic data were analyzed using a mixed linear model (MLM), following Malosetti et al. (2008), with the aim of adjusting a multi-trait model. Our multi-trait multi-environment data set consisted of I genotypes, evaluated in J environments with measurements on K traits (I = 165, J = 3, K = 2). Since we are focused on the genetic variation within the population rather than on genotype variation, we assumed random genotypes and genotype-environment (GxE)

interactions. The trait-environment combination was considered as fixed. The MLM for the data was as follows:

$$y = X\beta + Zu + \varepsilon$$

where y is the vector of phenotypic observations, β is a vector of fixed effects due to the environment-trait combination, vector u collects the random genotypic effects per trait by environment combination. Random genetic effects are assumed to be normally distributed, $N(0, \sigma_g^2)$; with G the genetic (co)variance matrix (vcovG). Finally, ε is a vector of non-genetic residuals associated with each observation and normally distributed i.i.d. $N(0, \sigma_e^2)$.

We fitted models that assume the effects of genotypes to be independent (without genetic correlation) and models with correlated effects of genotypes expressed by a realized additive relationship matrix, which was estimated from SNPs, as proposed by Endelman (2011). The GxE effects were assumed to be normally distributed with zero mean and different variance-covariance matrix structures (homogeneous variance model and heterogeneous variance model) (Covarrubias-Pazaran, 2018). The G and G×E random effects were assumed to be independent. The likelihood ratio test was used to determine the most suitable variance-covariance matrix structure model. Then, the Akaike information criterion (AIC) was used to select between models with and without genetic correlation. The MLMs were fitted using 'mmer' function in "sommer" package (Covarrubias-Pazaran, 2016), software R (R Core Team, 2016). The variance components (REML estimates) were used to calculate mean-basis heritability, as proposed by Hallauer and Miranda (1988).

In each environment:

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \left(\sigma_e^2/p\right)}$$

Across environments:

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \left(\sigma_{ge}^2/e\right) + \left(\sigma_e^2/p\right)}$$

Where σ_g^2 is the genotypic variance, σ_{ge}^2 is the variance of G×E interaction, σ_e^2 is the error variance, e is the number of environments, and p is a weighted mean of the number of replications per genotype in each environment and across environments. The weighting used was that proposed by Holland et al. (2003).

After fitting the MLM for the phenotypic values, the BLUPs of genotypic effects were extracted to be used in the GWAS analysis as response variables that do not carry environment effects. Association tests for the 78,543 SNPs were performed using the software TASSEL 5.2.59 (Bradbury et al. 2007). Another MLM was used for GWAS using PCA + K model (Zhao et al. 2007) to control bias due to potential genetic structure in the panel of lines. For this model, the first five PCs and the kinship matrix obtained with a centered identity by state (IBS) coefficient were used. The multiple test correction for the GWAS was conducted following the methodology proposed by Li and Ji (2005). First, we determined the effective number of tests from the eigenvalue decomposition of the molecular data matrix using the "eigen" function in R software (R Core team, 2016). Then, this effective number (almost half a total number of markers) was used to determine the significant level following the Bonferroni-type of adjustment. The putative candidate genes adjacent to each genomic region were identified in MaizeGDB (http://www.maizegdb.org).

Results

The frequency of the DSI values in both diseases showed a continuous distribution in each environment (Figure 1). The histograms show a normal distribution for DSI-MRC and a skewed distribution for DSI-CR. Logarithmic transformation was applied, but no differences were observed. Therefore, for simplicity, the analyses were carried out with the original scale. Mixed models for DSI data that included correlation among lines, measured through a matrix of additive genetic relationships estimated from SNPs, yielded better fits than the models that assumed lines to be independent (Table 1). The model with heterogeneous variance-covariance matrix for the G×E effects and genetic correlation between G effects was the most suitable for explaining variability of DSI-MRC and DSI-CR (Table 1).

In all environments, the joint analysis of DSI-MRC and DSI-CR, from the selected model (HeVgc), revealed that average DSI in each environment was higher for CR than for MRC. The mean-basis heritability was similar for both traits in the RC-17-18 environment, whereas in RC-18-19 it was greater for MRC. Non-significant correlation was observed between DSI-

MRC and DSI-CR. In the SA-17-18 environment, the susceptible lines showed a relatively low DSI level and the CIMMYT lines varied slightly (Table 2). Therefore, SA-17-18 was not included in further analyses. With the aim of obtaining a precise estimation of BLUPs of genotypes, a new linear mixed model was fitted with variance-covariance structure for the homogeneous G×E interaction, including only the environments RC-17-18 and RC-18-19. The mean-basis heritability estimate across environments was 0.54 for MRC and 0.58 for CR.

Of the 165 lines evaluated, eight lines for MRC and eight lines for CR were selected, using the 5th percentile of the BLUPs of genetic effects, as best performing lines across environments (Table 3). Lines derived from African mid-altitude/subtropical, lowland and subtropical breeding programs were the best among the lines selected for CR resistance. For MRC resistance, in addition to the above mentioned groups, South America LT breeding program is included. The lines CML509, belonging to the CIMMYT heterotic group A, and CML321, belonging to the CIMMYT heterotic group A, were selected among the best lines for both diseases (Table 3).

Genome-wide association study

Linkage disequilibrium (LD) for the entire panel and within chromosomes was measured (Figure 2). LD decay varied across the 10 chromosomes, as well as across different genetic regions within chromosomes. The average LD decay distance over all 10 chromosomes in the entire panel with $r^2 = 0.1$ was 20 kb. The rapid LD breakdown proves that the panel studied was truly diverse. A Manhattan plot was obtained for each disease using the selected 78,543 SNPs for GWAS (Figure 3). A total of two genomic regions for DSI-MRC and three for DSI-CR were significantly associated at a threshold of -log10 (P-value) > 4 (P-value < 0.0001) (Table 4). The genomic regions of resistance to MRC were located in chromosomes 2 and 3. For CR, regions were identified in chromosomes 2, 8 and 9. Each selected genomic region explained 13–20% of the total genotypic variance, whereas together they explained 24% of the total proportion of genotypic variance for resistance to MRC and 19% for CR. Most of lines mentioned in Table 3 carry the resistant allele for genomic regions associated with DSI of both diseases.

Discussion

Mal de Río Cuarto disease is transmitted by insects; hence, natural disease pressure varies between environments (Presello, 1991). In CR, pathogen development largely depends on

temperature and air humidity conditions, which are characteristic of each environment (Botta & Gonzalez, 2015). This fact might explain the observed heterogeneous variance-covariance structure in the G×E interaction. Such structure suggests that each environment has its own genetic variance and that there is no genetic correlation between environments. Plant breeders routinely deal with data involving collections of genotypes evaluated for multiple traits across multiple environments. Mixed models offer a suitable framework to jointly analyze such data without imposing unrealistic assumptions, like zero genetic correlations between environments and traits, and constant variance across environments (Malosetti et al. 2008). In our study, the correlation between DSI-MRC and DSI-CR was non-significant. However, the multi-trait model that obtains BLUPs of genotypes considering correlation between traits, such as the one used in this study, would be a powerful tool in cases of high genetic correlation between traits.

In this work, a diverse population of maize lines was used, covering the different breeding programs and environments of origin present in the CIMMYT germplasm. The results indicate the wide genotypic variability in the mapped population. Heritability estimated for DSI-MRC was higher than that observed in previous studies that evaluated biparental populations (Bonamico et al. 2012; Di Renzo at al. 2004; Rossi et al. 2015). For the DSI- CR, the estimated heritability values were lower than those observed by Lübberstedt et al. (1998) and Olukolu et al. (2016), who evaluated four biparental populations and a diverse panel of maize lines, respectively. The values for the estimated mean-basis heritability across two environments reveal predominance of additive control of responses of maize inbred lines to both diseases and favor the power of QTL detection, as suggested by Yu et al. (2008). Precise phenotypic evaluation is very important to identify promising genotypes and increase the power to detect genomic regions of interest. Therefore, we estimated BLUPs of genotypes considering only two environments, where natural pressure of both diseases allowed us to observe the high genetic variability present in the germplasm and discriminate genotypes by their reaction to both diseases. The 5^{th} percentile of the BLUPs of genetic effects allowed us to select lines with the best performance for MRC and CR. The incorporation of these maize lines into local maize breeding programs will contribute to the increase of resistance to both diseases.

Molecular characterization of CIMMYT maize lines was performed by Wu et al. (2016). These authors indicate that three major environmental adaptation groups were clearly present

in the collection of CIMMYT maize lines (CML). To consider this population structure in the GWAS, we used a mixed model with population structure inferred by principal component analysis, as fixed effect, and pairwise kinship coefficients matrix as random effect (Zhao et al. 2007). The lines selected by using the 5th percentile of the BLUPs of genetic effects belong to Lowland Tropical and Subtropical/Mid-altitude subgroups.

Using GWAS, we identified one region in bin 2.02 for DSI-MRC that coincides with previously reported virus resistance QTL. Redinbaugh et al. (2018) reported QTL cluster for virus-borne diseases in this bin. Specifically, the cluster reported in bin 2.02 contains a major QTL resistant to Mal de Río Cuarto virus and/or *Maize Rough Dwarf virus*, which was patented by Martin et al. (2010). In CR, specific resistance prevents pathogen dispersal in the plant via a hypersensitivity reaction, whereas general or quantitative resistance reduces the pathogen development rate in mature leaves and, therefore, is more durable (Vanderplank, 1984). In our work, some of the identified genomic regions for resistance to CR were located in positions close to QTL identified by Lubberstedt et al. (1998), who evaluated four biparental populations of European flint maize.

In our work, no genomic region reported for resistance to MRC disease is located on the same genomic regions as those with CR disease. The meta-analysis used by Rossi et al. (2019) to evaluate the consensus among QTL findings in the literature partially showed the same results. The low correlation between the DSI-MRC and DSI-CR traits and the absence of common regions to both traits is consistent with findings reported by Lisec et al. (2008), who indicated that the chance of sharing at least one QTL between traits increases with stronger correlations, and overall the correlation increases with the number of shared QTL.

Several genes are probably involved in natural variation for plant disease resistance (Kump et al. 2011). We identified genes immediately adjacent to the five genomic regions here identified, which may function in known plant disease-resistance pathways. The putative candidate gene Zm00001d006775 codes for Protein PELPK, which is responsive to biotic factors, elicitors, and defense hormones (Rashid, 2016). The predicted function of candidate gene Zm00001d011628 is receptor-like protein kinase (RLK). Morris and Walker (2003) reported that the RLK gene family is involved in pathogen recognition, among other functions. Plant cytochrome P450 is also a predicted function of candidate gene: it participates in many modifications of plant molecules among which phytoalexin production and other defense responses could be found (Zhou et al. 1999).

Our results suggest that the studied population of maize lines have high genetic variability and reveal novel sources of resistance to MRC and CR diseases. Additionally, genomic regions of resistance to MRC and CR were identified. Further research is necessary to validate the effects of the identified candidate genes and confirm that they confer resistance to MRC and CR in maize. The incorporation of this exotic germplasm into local maize breeding programs will contribute to the formation of hybrids whose heterosis shows high degree of resistance to both diseases.

Author contribution statement. EAR and NCB—requested the plant material from CIMMYT; EAR, MR, NCB and MGB—designed the experiment; EAR, MR and NCB— conducted the field evaluations; EAR and MR—carried out the statistical analysis of phenotypic data and GWAS analysis; EAR, MR, NCB and MGB—interpreted the results and drafted the manuscript.

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Figure 1. Frequency distribution of disease severity index for Mal de Rio Cuarto (left) and common rust (right) calculated in Rio Cuarto 2017-2018 (A), Sampacho 2017-2018 (B) and Rio Cuarto 2018-2019 (C).

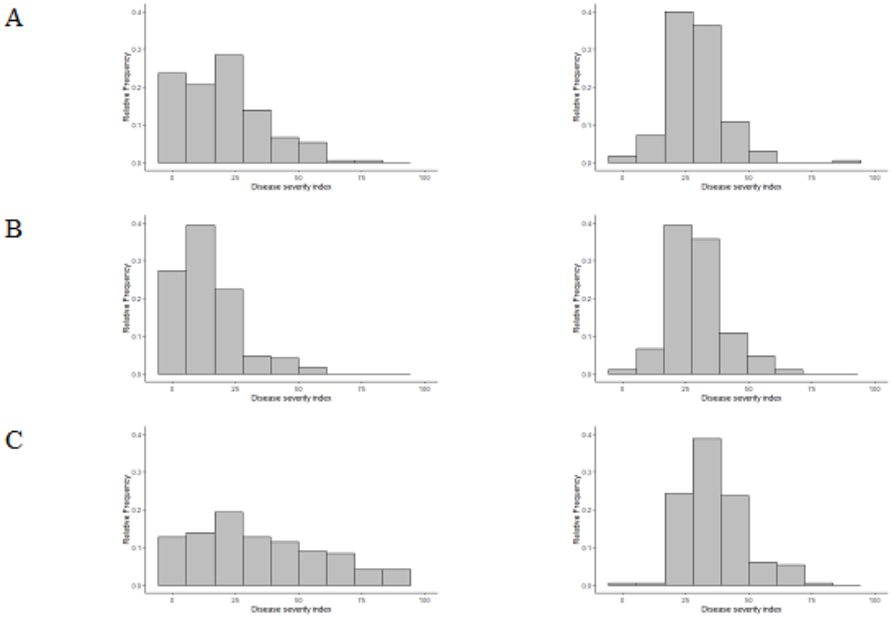

Figure 2. Linkage disequilibrium across the 10 maize chromosomes measured with 78,543 SNPs.

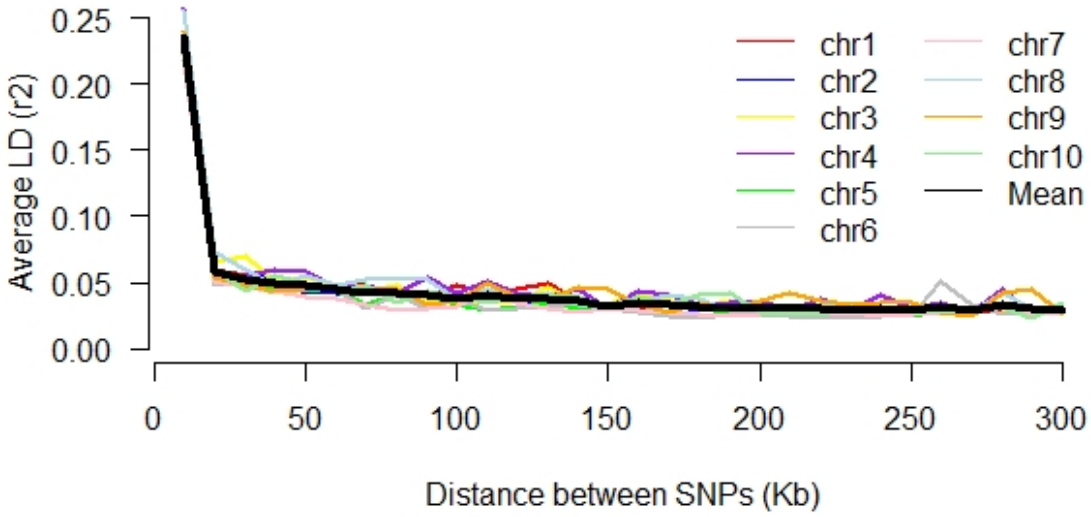

Figure 3. Manhattan plots of GWAS results for resistance to Mal de Rio Cuarto (A) and common rust (B) across environments.

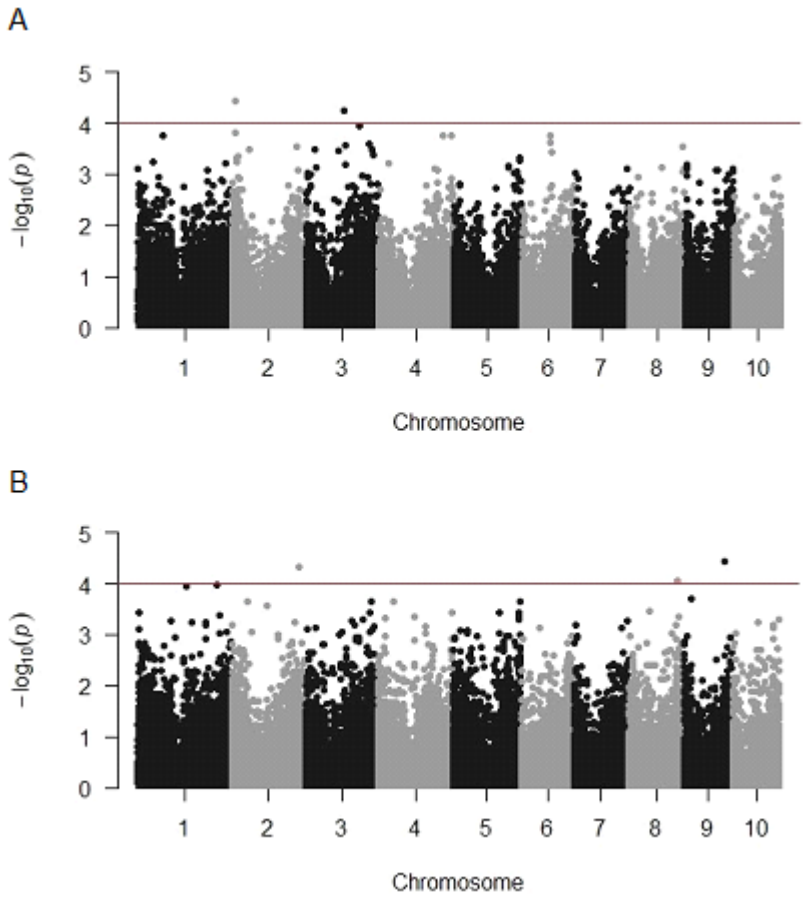

Table 1. Comparison of models for disease severity index of Mal de Rio Cuarto and Common rust

Models	AIC	BIC	log likelihood	LRT
HoV	752.76	783.84	-370.38	reference model
HoVgc	725.63	756.70	-356.81	
HeV	635.94	667.02	-311.97	Statistically different from reference model
HeVgc	610.90	641.98	-299.45	

HoV: Homogeneous variance model without genetic correlation; HoVgc: Homogeneous variance model with genetic correlation; HeV: Heterogeneous variance model without genetic correlation; HeVgc: Heterogeneous variance model with genetic correlation.
Genetic correlation was estimated using 78,543 SNPs and introduced to the models through realized additive relationship matrix.
LRT: likelihood ratio test. LRT compares the maximized log-likelihoods of the reference model and the alternative models; if the associated p-value is lower than the predefined threshold (0.05), it means that the alternative model fits the data significantly better than the reference model.
The optimal fitted model, with or without genetic correlation, is identified by the minimum value of AIC: Akaike Information Criterion and BIC: Bayesian Information Criterion.

Table 2. Means, genotypic variance components (σ_g^2) and mean-basis heritability (H^2) of Disease severity index (DSI) for Mal de Rio Cuarto (DSI-MRC) and common rust (DSI-CR) estimated from 165 maize lines in a single environment and across environments of central Argentina.

Environment	DSI-MRC			DSI-CR			
	Mean[Ł]	σ_g^2	H^2	Mean[Ł]	σ_g^2	H^2	r_g
SA-17-18	13.45±1.42	6.00	0.04	29.85±0.86	7.54	0.16	---
RC-17-18	19.25±1.87	162.97**	0.43	28.77±0.77	14.47**	0.46	0.10
RC-18-19	35.56±2.24	255.73**	0.50	36.46±1.16	34.99**	0.32	-0.22

Ł Mean±SE of DSI on a 0-100 scale.

*Significant at P < 0.01

r_g genetic correlation

Table 3. Selected lines with lower disease severity index to Mal de Rio Cuarto (DSI-MRC) and common rust (DSI-CR).

Genotype	DSI-MRC	Heterotic group	Seed color	Breeding program	Genotype	DSI-CR	Heterotic group	Seed color	Breeding program
CML429	6.32	--	Yellow	Asia Lowland	CML509	22.67	A	White	Africa MA/ST
CML391	7.86	A	White	Africa MA/ST	CML275	22.85	--	Yellow	Lowland
CML509	7.98	A	White	Africa MA/ST	CML507	22.98	B	White	Africa MA/ST
CML494	8.32	AB	White	Lowland	CML536	23.08	A	White	Africa MA/ST

CML428	8.46	--	Yellow	Asia Lowland	CML179	23.31	--	White	Subtropical
CML520	9.04	A	White	Africa MA/ST	CML321	23.64	B	White	Subtropical
CML531	9.04	--	White	South America LT	CML515	23.72	A	White	Lowland
CML321	10.39	B	White	Subtropical	CML383	23.79	B	White	Subtropical

Table 4. Details of SNP markers associated with resistance to Mal de Rio Cuarto (MRC) and common rust (CR) identified in this study.

Region	Trait	Marker	Chromosome	Bin	Alleles	p-value	R^2	Putative candidate gene	Predicted function of candidate gene
1	MRC	S2_124411430	2	2.02	A/C	$3.56x10^{-5}$	0.20	Zm00001d002416	Putative RING zinc finger domain superfamily protein
2	CR	S2_216557276	2	2.08	A/G	$4.84x10^{-5}$	0.16	Zm00001d006775	Protein PELPK
3	MRC	S3_121560333	3	3.04	T/C	$5.61x10^{-5}$	0.18	Zm00001d041458	Uncharacterized protein
4	CR	S8_156256057	8	8.06	C/A	$8.93x10^{-5}$	0.15	Zm00001d011628	Receptor-like protein kinase
5	CR	S9_130526232	9	9.05	C/G	$3.65x10^{-5}$	0.13	Zm00001d047452	Cytochrome P450

The exact physical position of the SNP can be inferred from marker's name, for example, S9_130526232: chromosome 9; 130,526,232 bp. R^2: phenotypic variance explained.