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Genetic Variation for Cold Tolerance in Two Nested Association Mapping Populations

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Abstract: Cold reduces maize (*Zea mays* L.) production and delays sowings. Cold tolerance in maize is very limited, and breeding maize for cold tolerance is still a major challenge. Our objective was to detect QTL for cold tolerance at germination and seedling stages. We evaluated, under cold and control conditions, 919 Dent and 1009 Flint inbred lines from two nested association mapping designs consisting in 24 double-haploid populations, genotyped with 56,110 SNPs. We found a large diversity of maize cold tolerance within these NAM populations. We detected one QTL for plant weight and four for fluorescence under cold conditions, as well as one for plant weight and two for chlorophyll content under control conditions in the Dent-NAM. There were fewer significant QTL under control conditions than under cold conditions, and half of the QTL were for quantum efficiency of photosystem II. Our results supported the large genetic discrepancy between optimal and low temperatures, as the quantity and the position of the QTL were very variable between control and cold conditions. Furthermore, as we have not found alleles with significant effects on these NAM designs, further studies are needed with other experimental designs to find favorable alleles with important effects for improving cold tolerance in maize.

Keywords: cold tolerance; maize; QTL; NAM; RIL

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1. Introduction

Cold stress is an environmental constraint that often threatens maize (*Zea mays* L.) production in temperate and cold areas during early spring, forcing late sowings that shorten the vegetative growth period, reduce yield, and increase the risks of summer drought, heat, pests, and diseases during the reproductive phase [1–3]. However, cold tolerance in maize is very limited based on previous knowledge [2,4–8]. Therefore, breeding maize for cold tolerance is still a major challenge.

In addition to reduced genetic diversity for cold tolerance, the second main handicap for improving cold tolerance is the complex genetic architecture of the trait revealed by quantitative genetics [9], recent QTL studies with bi-parental [10–12] and multi-parental double haploid (DH) populations [5], and inbred panels [6,13–16]. Focusing on the main reports about the genetics of cold tolerance in maize, Strigens et al. [13] studied a panel of 375 European flint and dent maize inbred lines and identified 19 highly significant single nucleotide polymorphism (SNP) for early growth and chlorophyll fluorescence parameters. Revilla et al. [15,17] studied two panels of 306 Dent and 292 European Flint maize inbred lines, respectively, and identified a large number of QTL, particularly in the Flint panel and associated with days to emergence and efficiency of the photosystem II (ΦPSII). This can be explained due to the fact that Flint maize has been adapted to cold environments [18]. Recently, Yi et al. [5] studied a multi-parental advanced generation intercross population (MAGIC) from eight parental inbred lines and found a large number of QTL for cold tolerance at the early seedling stage. In recent work, we carried out a genome-wide association study with a large association panel of 836 inbred lines from temperate origins that represent the genetic diversity available for maize breeding in temperate areas [6]. Though these reports that identified consistent QTL could explain a large proportion of the genetic variance for cold tolerance, we have not found favorable alleles with significant impacts, and the consistency of QTL was not high enough for using them in breeding programs [6]. Mayer et al. [19] demonstrated that mapping haplotype-trait associations with high-resolution DH lines was efficient for making it accessible for elite germplasm improvement. In this last report, the DH were released from maize landraces to capitalize on the diversity for early development traits.

Therefore, we need more conclusive studies for identifying reliable, favorable alleles for improving cold tolerance in maize. Consequently, the objective of this study was to detect QTL for cold tolerance at germination and seedling stages in two nested association mapping designs for deepening in our understanding of the genetic architecture of cold-tolerance regulation and to facilitate genetic improvement of cold tolerance in maize.

2. Materials and Methods

2.1. Plant Materials

We evaluated two Nested Association Mapping (NAM) designs that were described by Bauer et al. [20]. Briefly, the central Flint line UH007 was crossed with 11 Flint founder lines, and the central Dent line F353 with nine Dent founder lines. In addition, each of the central lines was crossed with B73 and with the founder of the reciprocal NAM population, i.e., F353 × UH007 and UH007 × F353, in order to connect the two panels with each other and with the US NAM population [21]. Therefore, we obtained 24 DH populations containing from 35 to 129 DH lines (Figure 1) [20]. The parental inbred lines and the parents represent the diversity of the flint and dent groups adapted to Europe. Altogether, there were 919 DH inbred lines for the Dent, and 1009 for the Flint available for this study.

2.2. Genotypic Data

We used the genotypic data of the 1928 DH inbred lines and the 23 founder inbred lines for the 56,110 SNPs contained in the Illumina MaizeSNP50 BeadChip cf. [22] and the consensus maps for both NAM designs cf. [23]. In each NAM design, markers with missing data for any parental inbred were removed, as well as those with redundant information (located at the same genomic positions), resulting in 7921 and 8702 markers for QTL analyses of the Dent and Flint-NAM designs, respectively. The construction process of the linkage map followed Giraud et al. [23], and the two consensus maps obtained are available at Maize GDB (http://maizegdb.org/cgi-bin/displayrefrecord.cgi?id=9024747, data available on 29 December 2022).

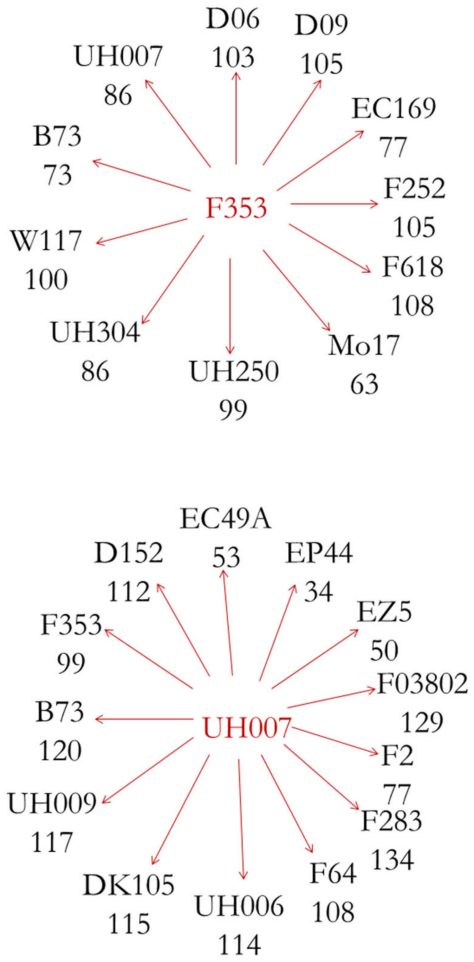

Figure 1. Nested Association Mapping populations with 11 inbred parents and the double haploids (DH) obtained from each cross with the central Dent parent F353 (**above**), and 13 inbred parents and the DH obtained from each cross with the central Flint parent UH007 (**below**).

2.3. Growth Chamber Trial

We evaluated 1928 DH inbred lines and the 23 parent inbred lines for cold tolerance in a growth chamber, following Revilla et al. [15], under cold and control conditions. Cold and control experiments were carried out in turns separately. Temperature and light conditions were set at 25 °C/14 h with light and 20 °C/10 h in the dark for the control experiment, and 14 °C/14 h with light and 10 °C/10 h in the dark for the cold experiment. Above each shelf (0.5 m high), 7 very high-output fluorescent lamps were used for producing cool light with a photosynthetic photon flux of 228 µmol m^{-2} s^{-1}. The details of sterilized peat, multi-pot trays, and irrigation followed Revilla et al. [15].

The phenotypic traits were measured under control and cold conditions separately: (1) relative leaf chlorophyll content (SPAD units; Chlo) in the second leaf, using a hand-held CCM-200 Chlorophyll Content Meter (Opti-Sciences, Tyngsboro, MA, USA); (2) maximum quantum efficiency of photosystem II calculated from minimum fluorescence (F_o) and maximum fluorescence (F_m) as F_v/F_m, where $F_v = F_m - F_o$, recorded in the second leaf by using a portable OS-30p Chlorophyll Fluorometer (Opti-Sciences) after at least 20 min dark treatment; and (3) dry weight of the plants above ground after drying them in an oven at 80 °C for 5 days (DW). Other common traits recorded were number of days from sowing to emergence, number of days from sowing to the second leaf with ligule, and early vigor by using a scale from 1 = weak to 9 = vigorous. The harvest was 15 and 37 days after sowing under control and cold conditions, respectively.

2.4. Experimental Design

The DH lines of the Flint and Dent-NAM designs were arranged following a randomized complete block design experiment with 6 replicates under each treatment, cold or control conditions. Bi-parental populations were assigned to the main plots and DH inbred lines to sub-plots. Best linear unbiased estimates (BLUEs) were calculated for the DH lines under each condition according to the following model:

$$\text{R: yij} = \mu + \tau\text{i} + \beta\text{j} + \text{uij i} = 1, 2, \cdots, \text{number of DH; j} = 1, 2, \cdots, 6$$

where yij is the random variable that represents the observation (i)-th of the block (j)-th; μ is a constant effect that measures the average level of response for all the units, called the global mean; τi is the effect produced by the i-th level of DH line; βj is the effect produced by the j-th level of block factor; and uij is the experimental error.

2.5. Statistical Analyses

QTL analyses were performed separately for each trait on the Dent and Flint-NAM designs, using conventional multifamily connected models and their corresponding consensus maps. For each NAM (Dent or Flint), a multi-loci model based on linkage analysis was used in which the significance of each QTL was tested, conditional on the inclusion of other QTL positions used as cofactors. This model is a conventional multi-family connected model that considers the connections between families through the sharing of the central line, assuming that each parental line had a different allele for each locus and that the effect of each allele was independent of the family, following Giraud et al. [23]:

$$y = J.\mu + X_q.a_q + \Sigma_{(c \neq q)}\, X_c.a_c + e,$$

where y was the vector (N × 1) of the adjusted phenotypic means of the N individuals of the dataset; J was a (N × P) matrix of 0 and 1 that linked each individual to the family it belonged to with P being the total number of families; m was the column vector (P × 1) of family means; and Xq and Xc were (N × K) matrices with K being the number of parents. Each element (ranging from 0 to 2) of these matrices corresponded to the expected number of alleles of the parent k at QTL q and cofactor c for each individual, according to the genotyping information at the position of q and c when this information was available (i.e., when these positions correspond to markers polymorphic in the population the individual belongs to) or at flanking markers otherwise. Then, aq and ac were the column vectors (K × 1) of the additive intrafamily effects associated with QTL q and cofactor c, respectively. Additionally, e was a column vector (N × 1) of the residuals of the model.

The significance of each QTL was probed using other QTL positions as cofactors. The interface of the software MCQTL [24] was used to detect QTLs using an iterative composite interval QTL mapping method (iQTLm). At each tested position, the presence of a QTL was tested using a Fisher test under an additive connected model. The thresholds of $-\log10$ p-value for considering a QTL as significant were computed by the genome-wide method for each trait and each NAM set using 1000 permutations of the phenotypes across DH populations and assuming an experiment-wide error equal to 0.1. In the iQTLm approach, an iterative forward selection method was used to automatically select markers to operate as cofactors using a threshold of 80% of QTL detection threshold. The search of QTL or cofactors was omitted in a 5 cM window to the left and to the right of each QTL or cofactor, respectively. The QTL confidence intervals at 0.95 were computed on the basis of a 1 LOD unit drop. Variances were estimated by using the Proc Mixed model of SAS with the method REML, and heritability was calculated following Holland et al. [25].

3. Results

The genetic diversity for cold tolerance was not consistent across DH populations, germplasm groups or environments, and, consequently, the significance of variances and QTL was not homogeneously distributed across genetic groups or growth conditions.

Furthermore, the −log10 *p*-value thresholds to declare a QTL significant varied among traits and between the Dent and the Flint-NAM without clear patterns of variation (Table 1). In general, threshold values were higher in the Flint than in the Dent-NAM, possibly due to higher heterogeneity of within-family variation across flint DH populations (Table 2).

Table 1. Thresholds for −log10 *p*-values to declare a QTL as significant in the Dent and Flint-NAM sets under cold and control conditions.

Trait	**Dent-NAM**		**Flint-NAM**	
	Control	Cold	Control	Cold
Dry weight (g/plant)	5.21575	9.28918	6.46503	5.87618
F_v/F_m [1]	10.8409	5.71184	23.4355	12.0236
Chlorophyll [2]	4.30574	5.67056	5.64056	5.12795

[1] F_v/F_m: quantum efficiency of photosystem II, recorded using an OS-30p Chlorophyll Fluorometer (Opti-Sciences). [2] Relative leaf chlorophyll content (SPAD) using a hand-held CCM-200 Chlorophyll Content Meter (Opti-Science).

Estimates of genetic variances (σ^2_g) within families for F_v/F_m were significantly different from zero for more DH families under control conditions than under cold conditions for the Dent-NAM, while the opposite was true for the Flint-NAM; not all DH populations had significant σ^2_g under both control and cold conditions (Supplementary Table S1). Likewise, the distribution of significant σ^2_g values did not follow any common pattern for the other traits. Regarding chlorophyll content, six families (CFD01, CFD04, CFD06, CFD07, CFD10, and CFD12) of the Dent-NAM showed significant σ^2_g under both conditions, while one family (CFD03) had no variability in any condition. Seven families (CFF01, CFF02, CFF03, CFF07, CFF12, CFF013, and CFF15) of the Flint-NAM showed variability in both conditions, and two families (CFF09 and CFF10) showed no significant σ^2_g in any conditions. The Flint-NAM showed greater diversity than the Dent-NAM under cold and control conditions for dry weight (12 Flint vs 6 Dent DH populations under control and 10 Flint vs 8 Dent under cold conditions). Within family, σ^2_g was higher under control than under cold conditions except for fluorescence. In addition, average within family variation for fluorescence was four times lower in the Dent-NAM than in the Flint-NAM (Supplementary Table S1). The highest heritability was for Fv/Fm but no clear patterns of variation were observed for Dent and Flint DH. Heritability ranged between 0.29 for chlorophyll in the Flint and 0.63 for Fv/Fm in the Flint DH.

No significant QTL was detected for number of days from sowing to emergence, number of days from sowing to the second leaf with ligule, or early vigor in either NAM. Furthermore, significant QTL for the traits under study were not found in the Flint-NAM. We detected one QTL for plant weight and four for fluorescence under cold conditions and one for plant weight and two for chlorophyll content under control conditions in the Dent-NAM (Table 2). The number of significant QTL was lower under control (3) than under cold (5) conditions, and half of the QTL were for F_v/F_m.

The distribution of significant allelic effects for all QTL was not homogeneous among the 11 DH populations produced from the respective inbred parents and from the common parent of the Dent-NAM (Table 3). Variability across inbred lines of the Dent-NAM set goes from being restricted to the parents of a single DH population for the QTL for chlorophyll content on chromosome 2 at bin 2.02 to being significant in all DH populations for the QTL on chromosome 3 at bin 3.05 for the same trait.

Table 2. QTL detected in the Dent-NAM set for dry weight (plant without the root), chlorophyll content (SPAD) and F_v/F_m under control and cold conditions. Genetic positions and 95% confidence intervals (CI) of QTLs are shown, as well as names and physical positions of the closest markers to the limits of the CI (on the Maize B73 RefGen_v3), the percentage of the phenotypic variance explained by each QTL and by all QTL included in the model for a particular trait, and the −log10 *p*-value of the Fisher test for each QTL.

				QTL Potion						R^2	
Trait	Treatment	Chromosome	Bin	Genetic Position (cM)	CI [1]	Marker interval	Marker interval position (bp)	−log10 *p*	QTL	Model
Dry weight	Cold	9	9.07	90.7	89.85–91.80	SYN27145-SYN39040	147612352-149283765	11.08	0.07	0.07
Dry weight	Control	7	7.04	84.7	76.28–92.61	PZE-107084200-PZE-107118905	139561428-165357869	5.30	0.05	0.05
Chlorophyll	Control	2	2.02	16.1	-	-	-	4.77	0.03	0.11
Chlorophyll	Control	3	3.05	56.5	56.44–56.52	PZE-103083897-PZE-103084731	138902786-140208059	6.32	0.09	
F_v/F_m	Cold	1	1.09	134.9	131.70–137.01	PZE-101213812-PZE-101222240	245333971-253563972	8.09	0.05	0.22
F_v/F_m	Cold	4	4.09	121.2	117.79-121.20	SYN29114-PZE-104146082	231865672-234967370	6.32	0.04	
F_v/F_m	Cold	5	5.03	59.8	59.80–63.01	PZE-105064695-PUT-163a-71766852-3520	65453204-81988171	14.52	0.11	
F_v/F_m	Cold	7	7.05	117.6	117.58–117.62	PZE-107132474-PZE-107132516	172770620-172774835	7.06	0.06	

[1] Confidence intervals based on 1 LOD unit fall are not presented when the most probable position for the QTL based on the lowest −log10 *p*-value of the Fisher test lies outside that confidence interval.

Table 3. Additive effects of QTL alleles carried by each parental inbred line for chlorophyll content (Chlorophyll) dry weight (Weight) and F_v/F_m (Fluorescence) under control and cold conditions.

QTL	Treatment	F353	UH007	B73	D06	D09	EC169	F252	F618	Mo17	UH250	UH304	W117
Chlorophyll-chr2	Control	0.7897							−0.7897				
Chlorophyll -chr3	Control	0.3786	2.3930	−0.4784	1.1829	3.485	1.388	−0.185	0.247	0.9714	−0.1514	2.4745	−6.7565
Weight-chr7	Control	0.001						0.013	−0.003				−0.012
Weight-chr9	Cold	0.0069				0.056			−0.013				
Fluorescence-chr1	Cold	−0.0134			0.1866		−0.0953				−0.0779		
Fluorescence-chr4	Cold	−0.0436		0.0365			0.0071						
Fluorescence-chr5	Cold	−0.0298	−0.0499		0.0102	0.0495		0.0137		−0.0254		−0.0105	0.0422
Fluorescence-chr7	Cold	0.012		0.0268	−0.0415	−0.0685		0.0094	0.019	0.0096		−0.0025	0.036

The inbred parent with the most significant additive effects was F353, having positive effects for all chlorophyll and dry weight QTL, and for the QTL in bin 7.05 of F_v/F_m, while the effects were negative for the other three QTL of F_v/F_m. The German inbred lines D09, UH007, and UH304 had the highest positive effects on chlorophyll content under control conditions while W117 had the highest negative effect. For dry weight, besides F353, F252 had positive effects under control conditions and D09 under cold conditions, while negative effects were found for F628 under both control and cold conditions, and W117 under control conditions. Finally, for F_v/F_m under cold conditions, the highest positive effects were provided by D06 while EC169 had the highest negative effects, followed by UH250.

4. Discussion

Neither the observed genetic diversity nor the detected QTL for cold tolerance were consistent across DH populations, germplasm groups or environments, in agreement with previous results [15] (2018). The diversity was higher within the Flint than in the Dent-NAM, which suggests that breeding programs for improving cold tolerance could

be more successful with Flint than with Dent germplasm, as previously proposed by Hölker et al. [18]. However, marker-assisted selection could not be carried out in these Flint populations as significant QTL were not detected for the traits under study in the Flint-NAM. Possible explanations are that the contribution of rare alleles with small effects is large in the Flint-NAM, and also the large experimental errors associated with the wide phenotypic diversity, shown within the Flint populations.

Our study confirmed large genotypic and phenotypic diversity for maize cold tolerance within these two NAM derived from 23 parent inbred lines, in agreement with previous results with diverse materials [3,6,12,15,18].

We detected one QTL for plant weight and four for fluorescence under cold conditions, and one for plant weight and two for chlorophyll content at control conditions in the Dent-NAM. The number of significant QTL identified with these two NAM designs is much lower than in previous reports with bi-parental [10–12], multi-parental [5], and inbred panels [6,13–16]. The low number of QTL detected in this study, compared with the previous ones, could be due to the election of the materials, as in this case the criteria for choosing the parents of the NAM were adaptation to European conditions and genetic diversity, while parents of populations used in other QTL studies were chosen based on their contrasting performance for the traits under study.

The QTL for dry plant weight in bin 9.07 (R^2 = 0.07) overlapped with QTL for F_v/F_m under cold conditions reported by Yi et al. [6]. The QTL for dry plant weight under control condition in bin 7.04 was consistent with the QTL for early vigor reported by Revilla et al. [15] and co-localized with the QTL for days to emergence reported by Yi et al. [5]). This second QTL explains the slightly lower proportion of variation (R^2 = 0.05) than the previously mentioned. Presterl et al. [12] found several QTL for plant weight, including one in chromosome 7 but none in chromosome 9. Mayer et al. [19] reported 37 haplotypes associated with early vigor, including two that explained 45 and 50% of the variation, respectively, in a GWAS study made with 899 DH lines derived from maize landraces. These authors also reported 55 haplotype-trait associations for early plant height, including three that explained 41, 53, and 42% of the variation, respectively. Therefore, the use of maize landraces by Mayer et al. [19] was more efficient than the analyses of NAM populations reported here because they found a large number of significant haplotype-trait associations for early vigor or early plant height.

Although, under cold conditions, there was no significant QTL for chlorophyll content in the NAM populations, some QTLs for this trait were found under control conditions. Specifically, there was a significant QTL under control conditions in bin 2.02 that was close to a QTL for chlorophyll content previously reported by Yi et al. [5], indicating that that region of the genome could have a consistent association with chlorophyll content.

The largest number of significant QTL was detected for F_v/F_m under cold conditions: first, a QTL in bin 1.09 close to previously reported QTLs under cold conditions, one for early vigor and two for F_v/F_m [6]. Other QTL for F_v/F_m under cold conditions were detected in bins 4.09 and 5.03, overlapping with two and three QTLs, respectively, reported for F_v/F_m under cold conditions by Yi et al. [6]. Finally, the QTL in bin 7.05 for F_v/F_m under cold conditions was close to a QTL for early vigor under cold conditions that was previously reported by Yi et al. [5]. The higher number of QTL for F_v/F_m under cold conditions than for any other trait is consistent with previous reports [5,6]. Our current results show that most of the QTL reported by Yi et al. [6] in a large panel, and by Yi et al. [5] in a MAGIC population, were not identified in the current study with two NAM populations. NAM mapping populations have been able to detect fewer QTL than multi-parental populations and panels, likely due to the difficulty of detecting rare alleles using a NAM approaches with few founders [5,6]. Nevertheless, these results encourage the use of photosystem II (F_v/F_m) as selection criterion for improving cold tolerance.

None of the 11 parental lines of the Dent-NAM or the 12 parental lines of the Flint-NAM was among those of the 292 Flint and 306 Dent European panels, respectively, with the highest cold tolerance when evaluated per se in cold conditions in a growth chamber [17].

However, Dent inbreds, D06, Mo17, and UH304, and Flint inbreds, UH007, D152, and UH006, were among inbred lines with moderate cold tolerance in the European panels [15]. It is worthwhile to note, the central parent of the Dent-NAM (F353) was cold sensitive while the central parent of the Flint-NAM (UH007) was cold tolerant, and both were crossed to inbred lines with diverse degrees of cold tolerance. In addition, variability for cold tolerance among Flint inbreds was higher, and rare alleles had a higher contribution to variability compared to Dent inbreds [15]. Therefore, reduced QTL detection power in the Flint compared to the Dent NAM could be a consequence of selecting a common founder for the Flint-NAM that resulted in cold tolerance, along with differences for genetic variability between Flint and Dent inbreds. Contrarily, the Dent-NAM was a suitable instrument to uncover QTL for cold tolerance that was not detected in the whole dent inbred panel [15]. Consistently, in that last report, the number of QTL detected was low for chlorophyll content in the Dent panel (2) and for F_v/F_m (2) in the Flint panel.

Our results also supported the large genetic discrepancy between optimal and low temperatures, as the quantity and the position of the QTL were very variable between control and cold conditions [6,9]. However, as we have not found alleles with great effects in these NAM designs, further studies are needed with other experimental designs for finding favorable alleles with important effects for improving cold tolerance in maize. Finally, the current NAM populations were indeed very helpful and efficient for detecting QTL for hybrid performance [23,26] but might not be ideal for cold tolerance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13010195/s1. Supplementary Table S1. Within family variance estimations in the Dent and Flint-NAM sets under control and cold conditions.

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Data Availability Statement: Data are available from the authors upon request.

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