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# Kernel weight in maize: genetic control of its physiological and compositional determinants in a dent × flint-caribbean RIL population

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## Abstract

The genetic control of maize kernel weight (KW) determination could be studied through its physiological and/or compositional determinants. Our objective was to dissect the genetic control of maize KW by analyzing its physiological (KGR: kernel growth rate; KFD: kernel filling duration) and compositional (protein, oil, starch) determinants in a dent×flint Caribbean RIL population, which combines a broad genetic background with grains of high added value for industry. An additional objective was to determine the stability of the genetic control under contrasting growing conditions, for which soil nitrogen offer was modified across experiments. Heritability ( $H^2$ ) values were high for KW ( $H^2 = 0.74$ ) and intermediate for the other traits (from 0.62 to 0.42). Kernel weight had a strong correlation with KFD ( $r = 0.69$ ), KGR ( $r = 0.60$ ) and protein concentration ( $r = 0.56$ ). Ten joint QTL with inconsistent effects across years and seven epistatic interactions were detected. Despite changes in effect size, most QTL were significant under both environments. Nine QTL were associated with variations in potential KW ( $KW_p$ ), mean KW, KGR and oil concentration, eight with variations in protein and starch concentration and seven with KFD. Epistatic interactions were related to regions with significant main effects. The most important finding was the existence of a common QTL for  $KW_p$ , KGR and KFD on chromosome 5, for which there was no previous report. Results increased our knowledge on the genetic control of KW through its phenotypic and genetic correlation with KFD, confirming the need to explore different physiological strategies in different genetic backgrounds.

**Keywords:** maize kernel weight, kernel growth rate, kernel filling duration, protein concentration, quantitative trait loci

**Abbreviations:** BLUP, best linear unbiased predictor; CV, coefficient of variation; CNR, cell number regulator;  $CV_M$ , maximum CV;  $CV_S$ , standard CV; E, environment; ENSO, El Niño Southern Oscillation; EST, Expressed Sequence Tags; G: genotype;  $H^2$ , heritability; KFD, kernel filling duration; KGR, kernel growth rate; KW, kernel weight;  $KW_M$ , mean KW;  $KW_p$ , potential KW; LRT, likelihood ratio test; MAS, marker assisted selection; MTME, multi-trait multi-environment; PET, potential evapotranspiration; QTL, quantitative trait loci, RIL, recombinant inbred lines; SSR, Simple sequence repeat; SNP, single nucleotide polymorphic; T, temperature; Tmax, mean daily maximum temperatures; TT, thermal time

## Introduction

Grain yield of cereal crops depends upon the number of harvestable kernels and their individual weight (KW). In maize (*Zea mays* L.), variations in grain yield are primarily explained by variations of the first component (Cirilo and Andrade, 1994; Otegui et al, 1995), which has a large phenotypic plasticity (D'Andrea et al. 2013). Contrary, phenotypic plasticity of KW is usually small (*op. cit.*), evidencing a strong genetic control (Cross, 1975; Hallauer and Miranda Fo, 1988; Alvarez Prado et al, 2014b). Nevertheless, maize KW is very sensitive to reductions in assimilate availability during the active grain-filling phase

(Borrás et al, 2004), with negative impacts on kernel quality. For instance, reductions in relative protein content and increases in relative starch content were reported (Borrás et al, 2002; Cirilo et al, 2011; Mayer et al, 2012). Reductions in kernel quality showed to be independent of the endosperm type (dent, flint and dent × flint). However, the magnitude of the responses differed depending on the tested genotype, suggesting a different genetic control of maize KW and kernel composition depending on the endosperm type and the explored condition.

In spite of mentioned knowledge, efforts for linking phenotypic to genotypic information are still

scarce and largely limited by adequate research on the former (Mifflin, 2000). Most papers published during the last 15 years with focus on genotyping and identification of quantitative trait loci (QTL) included a few phenotypic traits (e.g. plant height, KW, quality properties of grains) with no functional analysis of trait determination at the crop level (e.g., Li et al, 2007, 2009; Gustafson and Leon, 2010; Peng et al, 2011; Peiffer et al, 2014; Zhang et al, 2014). With a few exceptions (Li et al, 2012; Alvarez Prado et al, 2013b), most of them were set in the field in an experimental layout (e.g., one-row plots per genotype) that is incorrect for addressing traits of interest at the crop level, where interaction among plants affects results markedly (Connor et al, 2011; Sadras and Calderini, 2015). Similarly, the large effort set in the number of inbreds and markers included in the analysis has been usually offset by inadequate statistical approaches, which avoid phenotypic correlations between traits and environmental variability leading to overestimate QTL effects and yielding large QTL  $\times$  environment interactions with almost no value for marker assisted selection (MAS).

Recent efforts for dissecting the genetic basis of KW determination focused on the variation of its main physiological determinants (kernel growth rate, kernel filling duration and kernel water relations). A first approach (Alvarez Prado et al, 2013b) was based on the RIL (recombinant inbred lines) population IBM Syn4 (B73  $\times$  Mo17). Inbreds of this family, representative of dent temperate germplasm (Gustafson and Leon, 2010), have a narrow time to anthesis but explore a large range of KWs. Field studies were performed under non-limiting conditions, and authors (Alvarez Prado et al, 2013b) reported (i) a larger genotypic variation in kernel growth rate (KGR) than in kernel filling duration (KFD), (ii) high and medium heritability ( $H^2$ ) for KGR and KFD, respectively, and (iii) transgressive segregation for all evaluated traits. Authors also detected the presence of several QTL with consistent effects across years for KW, KGR and maximum water content. This result is not surprising from the physiological point of view, because a strong phenotypic correlation has been shown to exist among these three traits (Borrás et al, 2003; Gambín et al, 2006) whereas there is little correspondence between KW to KFD (Gambín et al, 2006) among dent temperate germplasm. In a second approach (Alvarez Prado et al, 2014a), authors extended the breadth of the analysis to another two RIL populations (N209  $\times$  Mo17 and R18  $\times$  Mo17), which exhibited a positive response of KW to KFD due to the presence of parental inbreds with different physiological strategies for building KW. For instance, parental line R18 has a very low KW due to a low KGR and a short KFD, while parental line N209 shows a high KW due to a long KFD and an intermediate KGR. Authors concluded that the genetic control of grain filling traits was predominantly influenced by QTL with small additive

effect but intermediate to high background effect. Maximum water content and KFD were the traits that shared the largest proportion of QTL among tested populations (i.e., a reduced background effect). Overall results are highly relevant for MAS, because they demonstrate that trait dissection based on a robust crop physiology background together with multi-trait multi-environment (MTME) analysis are critical to avoid the usual inconsistencies found in this type of studies (Liu et al, 2011; Li et al, 2012). Nevertheless, results are based on RIL populations that shared a common parental line (i.e., Mo17) and a relatively narrow genetic background among inbreds (all temperate dent or dent  $\times$  popcorn genotypes), alerting on the need to validate these findings by using independent genetic backgrounds, particularly those of industrial interest (e.g., specialty corns; Hallauer, 2001). Flint germplasm used for breakfast flakes and snacks belongs to this group of interest, with the additional benefit for farmers of receiving premium prices (Cirilo et al, 2011). This germplasm has been of broad interest in Latin America and chiefly in Argentina, which produces non-transgenic varieties requested by the UE (Greco, 2014). From a physiological point of view, flint germplasm usually shows smaller kernel size and KW than dent germplasm (Tamagno et al, 2015), and a noticeable lower maximum water content (Alvarez Prado et al, 2013a). This leads to a higher kernel density, an important kernel quality aspect for commercialization.

The objective of current research was to expand the analysis of the genetic control of maize KW by considering its physiological (KGR and KFD) and compositional (protein, oil, starch) determinants in a RIL population of broad genetic background, produced from the cross of B100 (yellow dent of US origin) and LP2 (Caribbean  $\times$  Argentine flint origin). LP2 and B100 have contrasting differences at molecular level, the analysis of the genetic structure grouped these inbreds in different clusters along successive cycles of simulation (Olmos et al, 2014). An additional objective was to determine the stability of the genetic control under contrasting growing conditions, for which soil nitrogen (N) offer was modified across experiments. We hypothesize that the control of final KW is affected to a similar extent by both physiological determinants (KGR and KFD) when a broad genetic background (Caribbean  $\times$  Argentine Flint germplasm) is considered. The expected variation respect to previous studies will partially modify the genomic regions (i.e. QTL) associated with KW determination, and consequently expand the spectrum of candidate genes.

## Materials and Methods

### Plant Material

The plant material evaluated in our research was a RIL population of 181 lines derived from the cross of parental inbreds B100 and LP2. These two inbreds

differ in breeding era, canopy size, grain yield and grain yield components (D'Andrea et al, 2006). They also belong to a different heterotic group of origin; B100 is US semi-dent germplasm and LP2 was derived from Caribbean × Argentine germplasm (Munaro et al, 2011).

#### Crop husbandry and experimental design

Field experiments were conducted at the Pergamino Experimental Station of the National Institute of Agricultural Technology (INTA), Argentina (33°56'S;60°34'W) on a Typic Argiudol soil, during 2009–2010 (Exp 1) and 2011–2012 (Exp 2) growing seasons. Contrasting weather and soil N conditions were explored during both growing seasons, yielding two different environments (E) that were described in D'Andrea et al (2016). Briefly, each growing season corresponded to an extreme phase of the ENSO (El Niño Southern Oscillation) phenomenon, being «El Niño» in 2009 and «La Niña» for 2011 (Climate Prediction Center). About topsoil conditions (0–40 cm), initial soil N availability was high in Exp 1 (72.6 kg N-NO<sub>3</sub> ha<sup>-1</sup>), and very low in Exp 2 (11 kg N-NO<sub>3</sub> ha<sup>-1</sup>). Organic matter level did not differ between experiments (2.06 – 2.08%). No N fertilizer was added to the crops, and phosphorus level was always high (> 30 ppm). Water deficit was always prevented by means of sprinkler irrigation, used to keep the uppermost 1 m of soil near field capacity throughout the cycle. Experiments were kept free of pests, weeds and diseases.

A total of 181 inbreds and their parental lines were distributed in a completely randomized block design with two replicates. Each plot had three rows of 5.5 m length and 0.7 m between the rows. Stand density was always 7 plants m<sup>-2</sup>. Sowing date was October 21 in 2009 (Exp 1). Because of the expected hot mid-summer weather associated to «La Niña» phase of the ENSO in the Pampas region of Argentina during 2011–2012, sowing was delayed for flowering to start at the end of January, when solar radiation and temperature start to decline (Otegui et al, 1996). Thus inbreds were grouped in three categories (early, intermediate and late) based on anthesis dates registered in Exp 1, and were sown on 14 (late), 18 (intermediate) and 23 November 2011 (early) in Exp 2. This strategy helped synchronize the flowering event of the whole experiment (Liu et al, 2011; D'Andrea et al, 2016), and in this way minimized possible confounded effects of differences in weather conditions between early and late inbreds associated to a late sowing date (Otegui et al, 1996). All plots were hand planted at a rate of three seeds per site and thinned to one plant per site at V3 (Ritchie et al, 1992).

In summary, we evaluated genotypes in two contrasting environments: (i) a potential condition in Exp 1, associated with an early sowing date (Otegui et al, 1996) and high initial soil N, and (ii) a non-potential condition in Exp 2, associated with a delayed sowing date and low initial soil N.

#### Measurements

Weather conditions were monitored at the experimental site (Campbell Scientific Inc, Logan, UT), and daily records obtained for mean (T<sub>mean</sub>) and maximum (T<sub>max</sub>) air temperatures (in °C), photosynthetically active radiation (in MJ day<sup>-1</sup>), rainfall (in mm), and potential evapotranspiration (PET, in mm). Mean temperature was calculated as the average of hourly air temperatures. Thermal time (TT, in °Cd) was computed daily as the degrees above a base temperature of 0°C from silking to physiological maturity (Muchow et al, 1990); daily TT values were accumulated for the post-silking period.

Thirteen plants were tagged in each plot, 5 of them at ca. V5 and the rest at the beginning of anthesis. The first group corresponded to consecutive plants located near the middle of the plot, and was used for the (i) non-destructive assessment of anthesis (at least one anther visible in the tassel) and silking dates (at least one silk visible in the apical ear), and (ii) the destructive assessment of mean individual KW (KW<sub>M</sub>, in mg) and kernel composition (as protein, starch, and oil concentration) after physiological maturity of each inbred (black layer in kernels of the mid portion of the ear). Kernel composition (in g kg<sup>-1</sup> of each component on a dry matter basis; Borrás et al, 2002) was established by near-infrared transmittance (Infratec 1227, Tecator, Sweden). Silking date was also recorded for each plant of the second group, and apical ears of these plants were collected on 18, 23, 28, 33, 38, 43, 48, and 53 days after silking. A total of 15 kernels were removed from the same position of each sampled ear (between spikelets ten and fifteen from the bottom of the apical ear) and oven dried at 70°C for at least 96 h. Because KW decreases from proximal (bottommost) to distal (topmost) ear positions (Seebauer et al, 2010), final KW based on ears of this second group was defined as potential (KW<sub>P</sub>, in mg). KW<sub>P</sub> is expected to be larger than KW<sub>M</sub>. Evolution of KW<sub>P</sub> was assessed, and data was used for computing KGR during effective kernel filling (in mg °Cd<sup>-1</sup>) and KFD between silking and maximum KW (in °Cd). For this purpose, a bi-linear model [Eqs (1) and (2)] was fitted to the data set of each plot (Borrás and Otegui, 2001); i.e., to each G × E × replicate combination.

$$KW = a + b \text{ TT for } TT \leq c \quad [1]$$

$$KW = a + b c \text{ for } TT > c \quad [2]$$

where *a* is the y-intercept (in mg), *b* is KGR (in mg °Cd<sup>-1</sup>), and *c* is KFD (in °Cd). The iterative optimization technique of GraphPad Prism version 5.0 ([graphpad.com/scientific-software/prism.htm](http://graphpad.com/scientific-software/prism.htm)) was used for model fitting, and adjusted *r*<sup>2</sup> values ranged from 0.88 to 0.99 in Exp 1 and from 0.82 to 0.99 in Exp 2.

#### Statistical analyses

##### Phenotypic evaluation

A tester inbred was used to evaluate spatial heterogeneity (Gilmour et al, 2006), and no trend was detected.



Frequency distributions were computed for each trait to evaluate the range and type of variation produced by genotypes (D'Agostino Pearson normality test). The genotypic variation within each experiment was estimated by means of the standard coefficient of variation ( $CV_s$ ; quotient between the standard deviation of the RIL population and its mean) and the maximum coefficient of variation ( $CV_M$ ; quotient between the maximum range detected among inbreds of the RIL population and its mean).

Trait variability was individually evaluated by fitting a linear mixed model (Eq 3). The phenotypic observation of measured trait  $Y_{ijk}$  on genotype  $i$  in replicate  $k$  of environment  $j$  was modeled as in D'Andrea et al (2008):

$$Y_{ijk} = \mu + G_i + E_j + (R/E)_{jk} + (GE)_{ij} + \varepsilon_{ijk} \quad [3]$$

where  $\mu$  is the general mean,  $G$  is the effect of the  $i^{\text{th}}$  genotype,  $E$  is the effect of the  $j^{\text{th}}$  experiment (environment),  $R$  is the effect of the  $k^{\text{th}}$  replicate nested in the environment,  $GE$  is the G×E interaction effect, and  $\varepsilon$  is the residual error. Genotype and G×E interaction effects were treated as random, while  $E$  and  $R$  effects were treated as fixed (Alvarez Prado et al, 2013b).

Broad sense heritability ( $H^2$ ) was estimated from the components of variance as in Eq 4 (Holland et al, 2003).

$$H^2 = \sigma_G^2 / (\sigma_G^2 + \sigma_{GE}^2/j + \sigma_e^2/jk) \quad [4]$$

where  $\sigma_G^2$  is the genetic variance,  $\sigma_{GE}^2$  is G×E interaction variance,  $\sigma_e^2$  is the error variance,  $j$  is the number of environments and  $k$  is the number of replicates.

For QTL analysis, phenotypic data were analyzed considering trait correlation and environmental variability using a mixed model approach following Malosetti et al (2008). Our MTME data set consisted of  $I$  genotypes evaluated in  $J$  environments on  $K$  traits repeated in  $L$  blocks (with  $I = 181$ ,  $J = 2$ ,  $K = 7$ ,  $L = 2$ ). An  $N \times 1$  vector «y» was defined, with  $N = IJKL$  that contains all the observations sorted by trait within environment and within genotype in each block. Genotypes were assumed random and trait × environment (TE) combinations and blocks nested within TE were considered as fixed.

Different variance-covariance models for both matrixes were assumed in order to select the most suitable for our data sets. We tested 7 different models: (i) variance component, (ii) compound symmetry, (iii-v) first order factor analytic «0», «1» and «2», (vi) heterogeneous compound symmetry, and (vii) unstructured model. The choice of the best model was based on a goodness of fit criterion such as the Bayesian Information Criterion (BIC; Schwarz, 1978). The compound symmetry model showed the lowest BIC value of the evaluated structures.

Considering the best variance-covariance structure for our data, the best linear unbiased predictor (BLUP) of each genotype in each environment was estimated for reducing uncontrolled trait variation for QTL mapping (Borevitz et al, 2002; Zalapa et

al, 2007). These analyses were performed with the MIXED procedure of SAS v. 8.2 (SAS Institute, 1999).

Linear regression analysis was applied to the relationships between variables. General trends were established for the correlations between measured traits using R software Version 2.15.2 for Windows (R Development Core Team, 2011).

#### *Genotypic evaluation and genetic map construction*

Leaves were harvested from five random plants of each 181 RIL and both parents at ca. V12. Leaf tissue was lyophilized and DNA extractions were done according to Kleinhofs et al (1993).

Simple sequence repeat (SSR) and single nucleotide polymorphic (SNP) markers were used to genotyping RIL population. Uniformly distributed SSR markers across the ten maize chromosomes were selected. Primer sequences were obtained from Maize Gene Data Base ([www.maizgedb.org](http://www.maizgedb.org)). From a total of 392 run markers, 117 were used for RIL characterization. For the other hand, an Illumina microchip with 96 SNPs associated with genes related to N metabolism and abiotic stress was designed. Eight SNPs polymorphic and with high quality were used for RIL characterization.

Prior to linkage analysis, an FDR test was used to verify 1:1 Mendelian segregation for each molecular marker (Kearsey and Pooni, 1996). Markers showing a distortive segregation were removed from the analysis. Linkage maps were constructed using GQ-Mol version 2008.6.1 (Schuster and Cruz, 2008). Map distances were computed by means of the Kosambi function (Kosambi, 1944). Linkage maps covered a total distance of 1126.2 cM.

#### *Multi-Trait multi-environment QTL analysis*

Phenotypic means (BLUPs) calculated using the mixed model approach were used for QTL mapping. We followed a procedure divided in two main steps: (i) Genome wide scan with tests for joint QTL by considering trait and environment correlations, and (ii) final multi-QTL model by testing the effects and significance of each QTL into a unique model. Our procedure was performed by means of WinQTL Cartographer V2.5 (Wang et al, 2012). The multi-trait mapping procedure, which implements composite interval mapping, was used for the first step. In the composite interval mapping, stepwise regression analysis of Model 6 from WinQTL Cartographer V2.5 (Wang et al, 2012) was performed. A threshold of 0.05 for input and output was established for selecting the putative QTL to be used as cofactors. We used a window size of 10 cM for removing temporarily the marker effects when scanning the chromosome. The threshold for accepting the presence of a significant joint QTL was LOD = 7, with scanning intervals of 2 cM between markers and a putative QTL. Quantitative trait loci positions were assigned to relevant regions at the point of maximum LOD.

Quantitative trait loci positions detected in step 1 were regarded as candidate QTL, and constitut-

**Table 1** - Descriptive statistics, ANOVA and heritability level ( $H^2$ ) of evaluated traits. Data correspond to 181 RIL and their parental inbred lines (B100 and LP2) cropped at two contrasting environments.

		Potential kernel weight (mg)	Kernel growth rate (mg °Cd <sup>-1</sup> )	Kernel filling duration (°Cd)	Mean kernel weight (mg)	Protein (%)	Oil (%)	Starch (%)
Experiment 1	B100†	253	0.32	1071	186	10.3	4.8	70
	LP2	231	0.39	896	172	9.67	4.13	71.1
	RIL	240	0.35	990	204	10.4	4.68	70.1
	Range‡	122-355	0.24-0.47	675-1187	106-292	8.03-13.2	3.57-5.64	68.2-72.5
	CVS-M§ (%)	15.3-97.1	12.0-65.7	8.4-51.7	16.9-91.2	9.4-49.7	8.1-44.2	1.2-6.1
	Normality¶	ns	ns	-0.83/2.32	ns	ns	ns	ns
Experiment 2	B100	228	0.36	969	154	9.59	5.91	66.8
	LP2	208	0.38	848	153	7.44	5.47	68.2
	RIL	249	0.37	982	172	8.91	5.52	67.5
	Range	170-337	0.25-0.51	795-1161	99-230	6.7-11.8	4.04-6.49	65.1-71
	CVS-M (%)	12.2-67.1	13.0-70.3	6.7-37.3	16.0-76.2	12.1-57.2	7.7-44.4	1.6-8.7
	Normality	ns	ns	ns	ns	ns	ns	0.61/0.69
Source of variation	G*	669 ± 100 <sup>††</sup>	0.00081 ± 0.00018	1567 ± 446	587 ± 87	0.47 ± 0.09	0.085 ± 0.016	0.37 ± 0.08
	G × E <sup>‡‡</sup>	252 ± 53.5	0.00038 ± 0.0002	1414 ± 486	169 ± 45.9	0.32 ± 0.07	0.053 ± 0.011	0.073 ± 0.08
	Residual	413 ± 31.6	0.00167 ± 0.0001	5248 ± 402	429 ± 32.8	0.53 ± 0.04	0.083 ± 0.006	1.08 ± 0.08
	Year effect	<0.001§§	<0.001	ns	<0.001	<0.001	<0.001	<0.001
$H^2$		0.73 ± 0.04	0.56 ± 0.07	0.42 ± 0.09	0.74 ± 0.04	0.59 ± 0.06	0.62 ± 0.06	0.53 ± 0.07

†Mean values of parental inbreds (B100 and LP2) and the RIL population; ‡Minimum and maximum values of the RIL population; §Coefficients of variation (standard-maximum); ¶D'Agostino Pearson normality test. Values represent skew/kurtosis coefficients when  $P \leq 0.05$ ; ns indicates not significantly different from normal ( $P > 0.05$ ); \*Significant for all traits at  $P < 0.001$ ; ††Expected mean square  $\pm$  s.e. of random effects; ‡‡Significant for all traits at  $P < 0.01$ , except for starch concentration; §§P level of significance for the fixed effect of years; ns: not significant.

ed the initial model for step 2. In this final step, we constructed a multi-QTL model by using the multi-trait multiple interval mapping procedure. By giving an initial model, the procedure estimated the model parameters, refined the estimates of QTL positions within intervals, tested the significance of all parameters by using a LOD = 3 for individual traits, searched for more QTL and finally calculated the genetic variance explained by the model (Basten et al, 2004). We used the likelihood ratio test (LRT) to compare the significance of each model refinement:

$$\text{LRT} = 2 (\log L_1 - \log L_0) \quad [5]$$

where  $L_1$  and  $L_0$  were the likelihood under the refined and the initial model, respectively. The difference between log-likelihoods was multiplied by a factor 2, so it distributes as the  $\chi^2$  statistic. This was then assessed for statistical significance using standard  $\chi^2$  significance levels. The degrees of freedom for the test were equal to the difference in the number of parameters between the refined and the initial model.

Digenic epistasis of molecular markers was estimated using a linear regression model (Li et al, 2008) with the QTL IciMapping software (available from [www.isbreeding.net](http://www.isbreeding.net)). A LOD score of 3.5 and a step size of 10 cM for improving detection accuracy were used (Zhang et al, 2012).

#### Candidate genes

Candidate genes were localized in the regions delimited by flanking markers to each detected QTL. In a first step, the coordinates of those markers were established in the reference genome of maize (B73 RefGen.v2 sequence) using the «Locus Pair lookup» option of the Maize Gene Data Base ([www.maizegdb.org](http://www.maizegdb.org)) or the «Marker Search» option of the Panzea

Base ([www.panzea.org](http://www.panzea.org)). Genes in those regions were searched in the «Filtered Gene Set» and the «Working Gene Set» ([maizesequence.org](http://maizesequence.org)) data bases, and their sequences were systematically searched by means of Blastn for EST (Expressed Sequence Tags) with a 0.01 threshold. We considered candidate genes those with an E-value  $\leq e^{-20}$  for *Zea mays*. Gene products were searched in the NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) data base, and were identified based on homology ( $\geq 70\%$ ) with candidate genes for *Zea mays*, *Oryza sativa* or *Arabidopsis thaliana*.

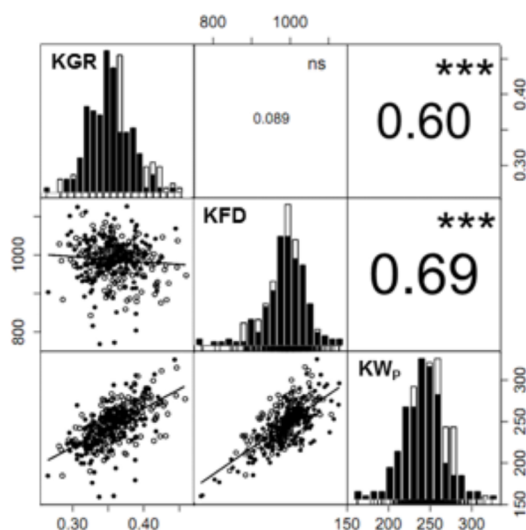
## Results

### Weather conditions

Growing seasons differed markedly in overall weather conditions. The El Niño phase of the ENSO registered during Exp 1 was accompanied by increased rainfall (886 mm) and reduced Tmax (27.8°C) as compared to Exp 2 (563 mm and 29°C, respectively). Nevertheless, mean rainfall (244 mm and 249 mm, respectively) and Tmean (23.2°C and 23.0°C, respectively) during the flowering period did not differ markedly between experiments, and delayed flowering of Exp 2 exposed the crop to already declining levels of PET values (5.7 mm day<sup>-1</sup> for Exp 1 and 4.7 mm day<sup>-1</sup> for Exp 2).

### Phenotypic variability for KW determination and composition traits

For all traits, the G component of variance was larger than the G × E interaction component (Table 1). The G effect accounted for the highest portion of the treatment variation (excluding residual) for starch, KW<sub>M</sub>, KW<sub>P</sub> and KGR (84%, 78%, 73%, and 68%, respectively), while for KFD, protein and oil concentra-



**Figure 1** - Phenotypic variability and correlation for kernel growth rate (KGR), kernel filling duration (KFD) and potential kernel weight ( $KW_p$ ). Frequency distributions are in the diagonal, Pearson correlation values above the diagonal and regression analysis plots below the diagonal. Data correspond to best linear unbiased predictors of 181 RIL and its parental inbred lines (B100 and LP2) cropped at two contrasting environments (black symbols for Exp 1 and white symbols for Exp 2). ns, not significant, \*\*\* for  $P < 0.001$ .

tions the G×E component showed the highest variation (47%, 41%, and 39 %, respectively). There were differences ( $P < 0.001$ ) between experimental years in all evaluated traits except for starch concentration (mean values of 70.1% in Exp 1 and 67.5% in Exp 2). Nevertheless, the environment had (i) minor effects ( $< 5\%$ ) on  $KW_p$ , KGR, and KFD (Table 1), and (ii) major effects ( $> 5\%$ ) on  $KW_M$ , protein and oil concentration (Table 1).

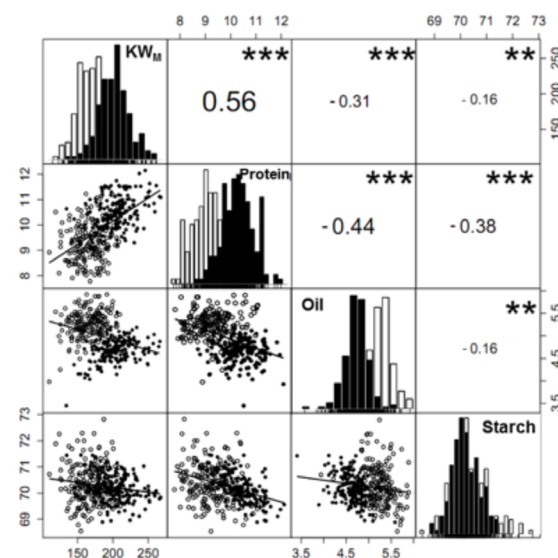
Potential and mean KW, and KGR had a normal frequency distribution ( $P > 0.05$ ) in both years (Table 1, Figure 1). Contrary, a significant ( $P < 0.001$ ) negative skew was detected for KFD in Exp 1 (Table 1, Figure 1) due to the presence of six lines with  $KFD \leq 800^\circ\text{C day}$ . Differences in frequency distribution were also detected among kernel composition traits; protein and oil concentrations had a normal frequency distribution in both years (Table 1, Figure 2), whereas starch concentration presented a significant ( $P < 0.001$ ) positive skew in Exp 2 due to a set of inbred lines with high starch values ( $> 72\%$ ). The wide range of variation registered for all measured traits in the RIL population was accompanied by a significant ( $P < 0.05$ ) transgressive segregation (Table 1); i.e., presence of inbreds of the RIL population that exceeded parental phenotypic values in either a negative or positive direction.

As expected, dent parental inbred (B100) always had larger  $KW_p$  ( $\sim 9.5\%$ ) and  $KW_M$  (0.7-8 %) values

than the flint parental inbred (LP2; Table 1), but both inbreds reached the highest levels of these traits during Exp 1. The trend described for parental inbreds was also observed for mean  $KW_M$  of the RIL family, as commented above. However, no difference in  $KW_p$  was observed between experiments (Table 1). Genotypic variation for  $KW_M$  and  $KW_p$  was much larger under the favorable environmental conditions of Exp 1 than under the poor environment of Exp 2 ( $CV_M$  Exp 1  $> CV_M$  Exp 2).

For both parental inbreds, the environmental effect was (i) similar for KFD, though this trait was always larger for B100 than for LP2 (Table 1), and (ii) less clear for KGR, though this trait was always larger for LP2 than for B100 (Table 1). Mean values of the physiological determinants of KW did not differ markedly between years for the RIL family, but important crossovers (i.e., change in ranking) were detected among inbreds (G×E effect, Table 1). For instance, KFD of inbred 7534 was larger than that of inbred 7663 in Exp 1 ( $963^\circ\text{Cd}$  and  $684^\circ\text{Cd}$ , respectively), and the opposite trend was observed in Exp 2 ( $822^\circ\text{Cd}$  and  $912^\circ\text{Cd}$ , respectively). The genotypic variation computed for KGR and KFD (Table 1) tended to be smaller than that registered for KWs, as well as the effect of the environment on this variation ( $CV_M$  values; Table 1).

Regarding kernel composition, parental inbreds did not differ markedly for these traits under favorable conditions of Exp 1 (Table 1), but protein concentra-



**Figure 2** - Phenotypic variability and correlation for mean kernel weight ( $KW_M$ ), protein, oil and starch concentrations. Frequency distributions are in the diagonal, Pearson correlation values are above the diagonal, and regression analysis plots are below the diagonal. Data correspond to best linear unbiased predictors of 181 RIL and its parental inbred lines (B100 and LP2) cropped at two contrasting environments (black symbols for Exp 1 and white symbols for Exp 2). \*\* for  $P < 0.01$ ; \*\*\* for  $P < 0.001$ .

tion of LP2 decreased markedly under less favorable conditions of Exp 2 (Table 1). For these inbreds, protein and starch concentrations were higher in Exp 1, whereas oil concentration was higher in Exp 2. For the RIL family, mean values of these traits followed the same trend described for parental inbreds, with increments for protein (+16.7%) and starch (+3.4%) during Exp 1 and an increase for oil (+17.9%) during Exp 2. Genotypic variation among inbreds for these traits was much larger for protein and oil than for starch (Table 1). In spite of the large genotypic effect on starch concentration, this trait exhibited the highest residual variation, which reduces the genotypic variation and the environmental effect (Table 1).

#### Phenotypic correlations and broad sense heritability

Based on  $KW_p$ , both physiological determinants were related to KW determination (Figure 1), showing a stronger association with KFD ( $r = 0.69$ ,  $P < 0.001$ ) than with KGR ( $r = 0.60$ ,  $P < 0.01$ ), while no

association was detected between KFD and KGR ( $r = 0.09$ ,  $P > 0.05$ ). Based on  $KW_M$ , a positive relationship was observed with protein concentration ( $r = 0.56$ ,  $P < 0.001$ ) and a negative one with oil ( $r = -0.31$ ,  $P < 0.001$ ) and starch ( $r = -0.16$ ,  $P < 0.01$ ) concentrations (Figure 2). Additionally, the association between kernel composition traits was always negative; protein was moderately related to oil ( $r = -0.44$ ,  $P < 0.001$ ) and starch ( $r = -0.38$ ,  $P < 0.001$ ), and oil was slightly related to starch ( $r = -0.16$ ,  $P < 0.01$ ) (Figure 2).

Heritability values (Table 1) were (i) highest for  $KW_M$  (0.74) and  $KW_p$  (0.73), (ii) intermediate for oil concentration (0.62), protein concentration (0.59), KGR (0.56) and starch concentration (0.53), and (iii) low for KFD (0.42).

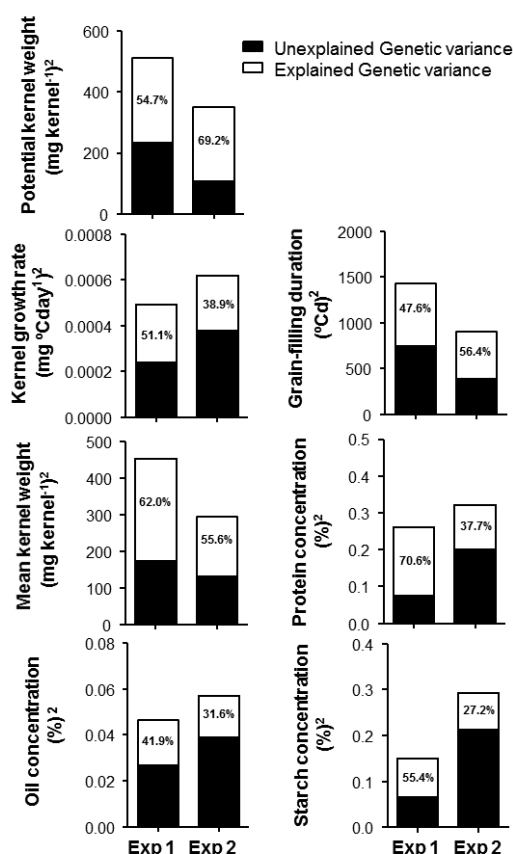
#### QTL detection

The proportion of the total genetic variance explained by the multi-QTL model ranged from approximately 27 to 71% across traits and environments (Figure 3). On average,  $KW_p$ ,  $KW_M$ , protein concentration and KFD were the traits with the highest proportion of explained genetic variance (62%, 59%, 54%, and 52%, respectively), followed by KGR, starch concentration and oil concentration (45%, 41%, and 38%, respectively). The heterogeneity of variance across environments is associated with the existence of G×E interaction effects (Table 1).

Based on initial MTME analysis, ten putative joint QTL were detected for KW determination and composition traits (Figure 4 and Supplementary Table 1). All ten putative QTL detected by the joint analysis were regarded as candidate QTL, and included in the initial model of the multi-QTL model (i.e., no candidate QTL was eliminated from the analysis). Nine QTL were associated with variations in  $KW_p$ ,  $KW_M$ , KGR and oil concentration, eight QTL with variations in protein and starch concentration, and seven QTL affected KFD (Figure 4).

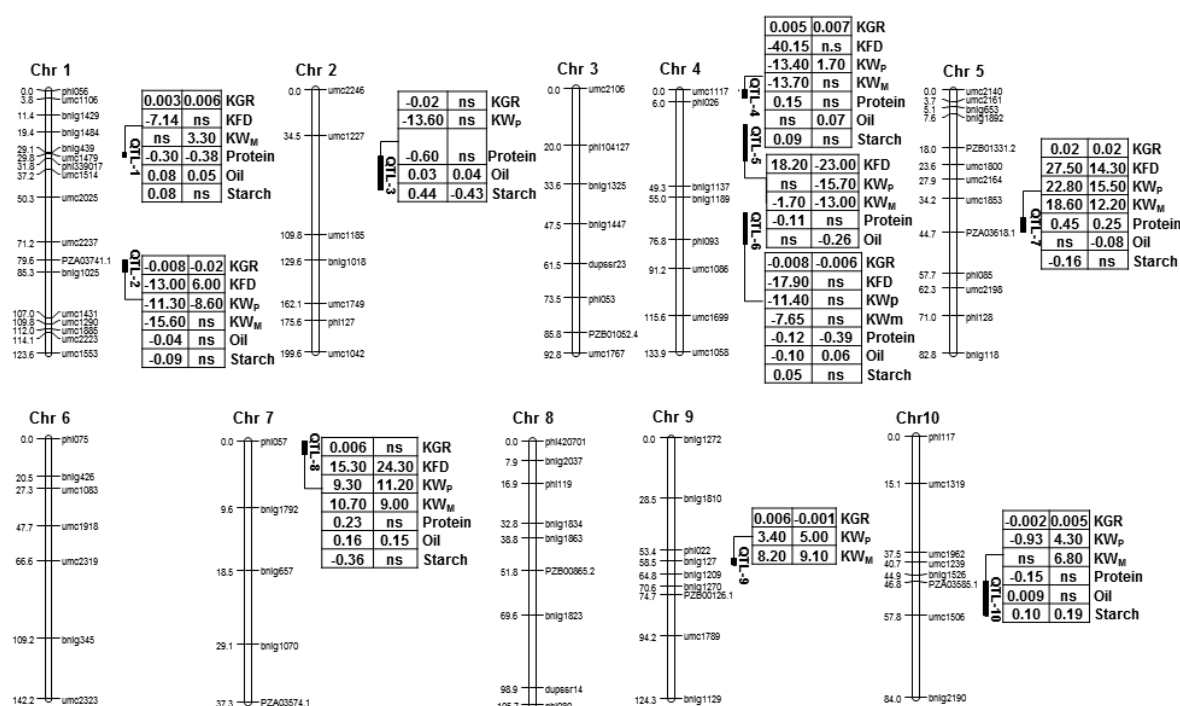
Detected QTL generally showed inconsistent effects across environments, which is in line with the heterogeneity of variance across environments. Inconsistent effects were the product of different types of G×E effects. For example, QTL for  $KW_p$ , KGR, KFD, oil and starch concentrations had changes in effect size and sign between experiments, i.e. cross-over interactions. For all traits there were QTL with significant effects in one of the two explored environments. In spite of mentioned changes in effect size, most QTL were significant and of consistent action (i.e. sign) in both environments (Figure 4). Among these QTL, we identified those with remarkable effect (e.g. larger than 1% based on the trait mean) that are summarized in Supplementary Figure 1. The QTL 7 on chromosome 5 exceeded from all the rest due to its large effects on most traits of interest, except on oil and starch contents.

The positions and effects of the different QTL can help to understand the causes of genetic correlations between traits. Potential kernel weight had a strong



**Figure 3** - Total genetic variance for measured traits in a family of 181 RIL and its parental inbred lines (B100 and LP2) cropped at two contrasting environments (Exp 1 on the left and Exp 2 on the right). The proportion of explained variance by the final multi-QTL model is indicated in the white column with its respective value indicated in %.





**Figure 4** - Chromosomal location of QTL detected for potential and mean kernel weights (KW<sub>p</sub> and KW<sub>m</sub>, respectively), kernel growth rate (KGR), kernel filling duration (KFD), and protein, oil and starch concentrations. QTL are represented by bars with a connector to the corresponding position on the chromosome. The two columns represent the additive effects of the QTL in each environment (Exp 1 on the left and Exp 2 on the right column), in units of the corresponding trait (KW<sub>p</sub> and KW<sub>m</sub>: in mg; KGR: in mg °Cd<sup>-1</sup>; KFD: in °Cd; Protein, Oil, and Starch concentrations: in %). Additive effects correspond to the LP2 parental allele.

positive response ( $r^2 \geq 0.60$ ) to KFD and KGR (Figure 1), and consistent genetic correlations supported these phenotypic trends. For instance, its correlation with KFD was evident in QTL located on (i) chromosomes 5 and 7 in both experiments, and (ii) chromosomes 1 (QTL 2) and 4 (all QTL) under one of them (Figure 4). Its correlation with KGR was supported by QTL located on (i) chromosomes 1 (QTL 2), 5, and 10 in both experiments, and (ii) chromosomes 2, 4 (QTL 4 and QTL 6), 7 and 9 under one of them (Figure 4). In agreement with the lack of phenotypic correlation between KGR and KFD (Figure 1), the majority of the genetic correlations were inconsistent across environments for both traits, except for that found on chromosome 5. The phenotypic correlation of KW<sub>m</sub> with its components (Figure 2) was moderate and positive for protein, and low and negative for oil and starch. For protein concentration, a genetic correlation with KW<sub>m</sub> was established on chromosome 5 that held across environments (Figure 4) and was consistent with that of KW<sub>p</sub> and its physiological determinants (KGR and KFD). Additional genetic correlations between protein concentration and KW<sub>m</sub> were detected on chromosomes 4 (QTL 5 and QTL 6) and 7 under one of the two explored environments. Oil and starch concentration QTL showed almost no co-localization with any KW<sub>m</sub> related QTL. However, several QTL for protein and oil concentration co-localized with QTL

for KGR and KFD, respectively; both showing similar effect sign (Figure 4).

A total of 42 pairs of markers with significant interaction effects that were stable across environments were detected (Supplementary Table 2). Except for starch, all traits showed between one and two epistatic interactions that involved one main effect marker. Interaction effects were negative for all traits with the exception of KFD, which showed both positive and negative effects. Remaining epistatic interactions involved loci that were individually non-significant. Considering all traits, individual epistatic interactions explained from 3.0% to 5.3% of the phenotypic variation (Supplementary Table 2).

#### Candidate genes associated with QTL

Genes were identified for the intervals of the ten detected QTL and classified depending on their predicted function (Table 2). Most intervals between flanking markers of mentioned QTL ranged between 2 and 16 Mbp. The QTL 5, 8, and 9 had intervals larger than 70 Mbp and gene number within flanking markers too large for a precise identification of candidate genes and consequently were discarded.

A total of 4002 genes were identified across the ten QTL, but this number dropped to 1338 when QTL 5, 8 and 9 were removed from the analysis (Table 2). Most of the latter (68%) had unknown functions; however, 422 genes could be associated with differ-

ent functions: 25% were related to basal metabolism, 1.9% to protein metabolism, 1.6% to hormone or signal transduction, and 3.4% to other processes (carbohydrate or lipid metabolism, cell division, plant defense and stress response).

Among genes involved in cell division and expansion, those that regulate cytoskeleton organization (actin depolymerizing factor and kinesin) and the metabolism of cytokinins (Cis-Zeantin O-Glucosyltransferase 1 and Cytokinin O-Glucosyltransferase 2) are located within the intervals of QTL 1, 3, 6, 7, and 10. Similarly, cell number regulator factors 1 (CNR1) are within the interval of QTL 6 (chromosome 4), the auxin regulated gene *ZAR1* involved in organ size corresponds to QTL 10 (chromosome 10), the *alpha-expansin 13* gene to QTL 2 (chromosome 2), and the *beta-expansin 3* gene to QTL 7 (chromosome 5).

Genes involved in sugars, proteins and lipids metabolism could be documented for most detected QTL. For proteins there are genes regulating amino acids synthesis (asparagine synthetase, tryptophan synthase, acetylglutamate synthase, threonine synthase, homoserine dehydrogenase) as well as genes regulating proteins synthesis (elongation factor Tu and translation initiation factor). Moreover, genes that promote zeins synthesis (Z1A alpha zein protein and zein-alpha 19A2), which dominate the group of reserves proteins in maize kernels, are in the interval of QTL 4. Some genes involved in protein degradation by the ubiquitin-proteasome proteolytic pathway (Proteasome subunit  $\alpha$  type, RING-type protein, Ubiquitin-protein ligase, ARM repeat-containing protein containing family protein and F-box domain) were detected in most of QTL intervals of interest.

Within the intervals of QTL 4 we identified genes involved in sugar transport, as Glucose-6-phosphate/phosphate translocator 2 and Monosaccharide transport protein 2.

## Discussion

The evaluated RIL family had a broad variation in KW, its physiological determinants and kernel composition. The transgressive bi-directional segregation detected for all measured traits stemmed from the recombination of QTL with antagonist effects (Rieseberg et al, 2003), which yields extreme phenotypes. The described variability allowed the identification of inbreds with similar  $KW_p$  obtained from the combination of contrasting KGR and KFD (data not shown), a result that had been previously documented for this RIL population (Piedra et al, 2010; Piedra, 2011).

Based on  $H^2$  values computed for KW and its components, a curious result rise where  $H^2$  computed for KW was larger than for its physiological and compositional determinants. This fact does not support the contention of an improved genetic prediction based on trait dissection, in agreement with Alvarez Prado et al (2013b) and Lee et al (2005).

The variation observed in  $KW_p$  was strongly as-

sociated with both physiological determinants of KW (i.e., KGR and KFD). This result deserves further analysis, because it is (i) partially different to previous evidence obtained with commercial maize hybrids (Borrás and Otegui, 2001; Gambín et al, 2006) and with the mapping population derived from B73×Mo17 (Alvarez Prado et al, 2013b), which found no link between KW and KFD, and (ii) in agreement with other studies based on inbred lines (Borrás et al, 2009) as well as on other mapping populations derived from Mo17 (Alvarez Prado et al, 2014a). This could be explained by the epistatic interactions detected between loci with significant main effects and loci without significant main effects. The QTL effects that exhibit interactions with unlinked genes may be altered dramatically when they are incorporated into a genetic background different from the one in which they were mapped (Holland et al, 1997). Contrary, we confirmed the lack of phenotypic correlation between KGR and KFD (Borrás et al, 2009; Borrás and Gambín, 2010; Alvarez Prado et al, 2013b; Alvarez Prado et al, 2014a), which was supported by the lack of common QTL for these traits. These results also support the idea of their independent genetic control, and consequently the possibility of their independent selection. Individual QTL co-localization between  $KW_p$  and its physiological components demonstrate that no single trait is an exclusive determinant of KW (Figure 4). In other words, selection in breeding programs can focus in both physiological determinants of KW with no negative trade-off effects. The existence of a co-localizing QTL for KW and its physiological determinants, however, may be irrelevant (or even negative) under environments with a short growing season, as those at high latitudes and/or altitudes. In these environments, where exploitable cycle duration for adequate crop fitness is reduced, KW is expected to increase due to enhanced KGR and no variation in KFD. Consequently, a co-localizing QTL for both traits is an unwanted alternative. The opposite is true when selecting germplasm for environments with a long growing season, like those of intermediate latitudes and the tropical ones.

About compositional traits, a marked variation was observed in response to contrasting growing conditions explored in current research. The variation was larger in protein and oil concentrations than in starch concentration. There was, however, a positive phenotypic correlation between  $KW_M$  and protein concentration, which was supported by a co-localizing QTL for both traits in chromosome 5 (QTL 7). This result proposed a common genetic control (e.g., linkage or pleiotropic effects) between these attributes for the current RIL population. Similarly, a co-localizing QTL in chromosome 1 (QTL 1) with additive effect  $>0$  for oil concentration and additive effect  $<0$  for protein concentration is consistent with the negative relationship detected for these traits. This finding supports the reported trade-off found between these

traits for some oil crops (Triboi and Triboi-Blondel, 2002). Nevertheless, other co-localizing QTL highlight the possibility of a positive simultaneous selection for oil and protein concentrations. For example, the QTL 8 in chromosome 7 for oil concentration and  $KW_M$  together with the QTL 7 in chromosome 5 for protein concentration and  $KW_M$ , all with additive effect  $>0$ . These considerations deserve further analysis.

Registered G×E interaction effects modified the number of significant QTL between experiments, which was smaller in the environment with reduced initial soil N (Exp 2) than in the environment with high N (Exp 1). In this context, gene expression may vary depending upon the environment (i.e. QTL×E effect). Moreover, estimated QTL position may vary depending upon mentioned G×E effect as well as upon the genetic background and the experimental error (Wassom et al, 2008). Nevertheless, an important number of QTL were consistent across evaluated environments, which is a remarkable result that adds robustness to detected QTL. In addition, low  $CV_s$  values give confidence on the high precision of the performed phenotyping, reducing the error in QTL detection. The fitted QTL model explained a considerable proportion of the genetic variance ( $> 50\%$  for most traits), larger than in previous research based on a similar approach (Malosetti et al, 2008; Alvarez Prado et al, 2013b).

Interestingly, almost all QTL identified in current multi-QTL model matched previously reported QTL. Among those with largest magnitude, as QTL 7 in chromosome 5 and QTL 8 in chromosome 7, the former matched QTL registered for (i) KW and KGR in a dent × popcorn Chinese family (Li et al, 2012), (ii)  $KW_M$  in a Flint × iodent French family (Hirel et al, 2001), and (iii) starch in a high oil × dent family (Zhang et al, 2008). Similarly, there was a relevant coincidence in QTL 8 for KW and KGR (Li et al, 2012; Alvarez Prado et al, 2013b) as well as for protein content (Zhang et al, 2008). Additionally, the QTL for protein (Schön et al, 1994; Zhang et al, 2008), starch (Zhang et al, 2008), KW (Li et al, 2012) and KGR (Li et al, 2012; Alvarez Prado et al, 2014a) detected on chromosome 1 co-localized with the consistent QTL 1 for protein, oil and KGR detected in current study. A reported QTL for KW (Schön et al, 1994) and protein (Séne et al, 2001) matched with our QTL 3. On chromosome 4, several QTL were reported for quality traits and KW physiological components, which co-localized with our findings: (i) for oil concentration on QTL 4 (Zhang et al, 2008), QTL 5 (Séne et al, 2001; Zhang et al, 2008) and QTL 6 (Séne et al, 2001), (ii) for starch concentration on QTL 4 (Wassom et al, 2008; Zhang et al, 2008) and QTL 5 (Zhang et al, 2008), (iii) for protein concentration on QTL 5 (Wassom et al, 2008), and (iv) for  $KW_p$  and KGR on QTL 4 (Alvarez Prado et al, 2013b). Also, a QTL for KW on chromosome 9 was reported (Séne et al, 2001), in agreement with our consistent QTL 9 for this trait. Finally, Li et al

(2007, 2012) detected a QTL for KW, Alvarez Prado et al (2014a) for KGR and KW, and Li et al (2009) for protein and starch concentration in the same region that our QTL 10. There was, however, no coincidence with previous reports for QTL representative of KFD. For this trait, current research detected two QTL (one on chromosome 5 and the other on chromosome 7) linked to KW, whereas previous studies found only one QTL that co-localized for KW and KFD, on chromosome 5 for the B73 × Mo17 RIL population (Alvarez Prado et al, 2013b) and on chromosome 1 for the N209 × Mo17 RIL population (Alvarez Prado et al, 2014a).

The precise phenotyping mentioned before, the consistency of detected QTL across tested environments, their coincidence with QTL reported in previous research and the high proportion of genetic variance explained by the fitted QTL model gave high reliability to our findings. Map saturation with markers within the intervals of QTL 5, 8, and 9 would optimize their position, adding precision to the map and improving the identification of candidate genes (Collard et al, 2005; Boopathi, 2013). Independently of this consideration, some candidate genes were found to be of value in the control of the physiological process underlying kernel growth and its composition. Among the 18 putative genes that regulate  $KW_p$  (cell division/cell growth), the most important are those related to *alpha-expansin 13*, *CNR1*, *CNR2*, and *ZAR1* on chromosomes 2, 4, and 10, respectively. During growth, expansin proteins produced by plants affect cell elongation and a broad range of processes that demand cell wall modifications (Sampedro and Cosgrove, 2005). We identified the *alpha-expansin 13* gene on chromosome 2 and the *beta-expansin 3* gene on chromosome 5, the latter associated with a consistent QTL for  $KW_p$ . Apparently, gene *CNR1* has a negative regulation of cell number and organ expansion, in agreement with negative additive effects (or lack of effect) found for  $KW_p$  in QTL 6. Within the interval of QTL 10, the auxin regulated gene *ZAR1* is a putative orthologous to the ARGOS gene in Arabidopsis, which is related to kernel size as well as to enhanced grain yield and drought tolerance (Guo et al, 2014). The expression of *ZAR1* had a marked interaction with the environment, increasing grain yield in a drought temperate conditions and reducing it in a humid temperate one. Similarly, the additive effect detected in current research for  $KW_p$  and KGR in QTL 10 was  $< 0$  in Exp 1 and  $> 0$  in Exp 2 (i.e. QTL×E interaction).

Finally, candidate genes involved in protein and carbohydrate metabolisms were numerous and distributed in almost all detected QTL. Among these genes, there are several that regulate protein degradation through the ubiquitin-proteasome proteolytic pathway, as reported by Alvarez Prado et al (2013b); i.e., genes encoding the RING-type protein in QTL 3, 6 and 10 and related with the F-box domain in QTL

1, 4 and 6 contribute markedly to grain development because this protein affects a broad range of processes that include embryogenesis, hormone signaling and senescence (Moon et al, 2004).

### Conclusions

In current research we phenotyped KW together with its main physiological determinants (KGR and KFD) and kernel compositional traits (starch, protein and oil concentrations) in a dent × flint Caribbean maize RIL family. The inclusion of a flint inbred in the study of KW determination increased the variability of the explored mechanisms allowing the quantification of a broad genotypic variation as well as an intermediate to high heritability. In comparison to previous research, the fitted QTL model explained a higher proportion of the genetic variance for the evaluated traits (>50% for most traits). Detected QTL for KW and related traits were grouped in agreement with observed phenotypic correlations and with QTL observed in different genetic backgrounds. An important finding was the existence of a co-localizing QTL for KW<sub>p</sub>, KGR and KFD on chromosome 5, for which there is no previous report. This finding might suggest that a simultaneous effect of KGR and KFD may explain an important part of the genotypic variations observed in KW, which would help breeders dealing with this type of germplasm.

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