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Maize Nitrogen Use Efficiency: QTL Mapping in a U.S. Dent x Argentine-Caribbean Flint RILs population

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Abstract

This study was aimed to identify quantitative trait loci (QTL) for nitrogen use efficiency (NUE) and related traits in a maize population derived from a cross between two lines with different genetic background (B100 and LP2). Recombinant inbred lines (181) from this population were evaluated under field conditions during two growing seasons, and significant ($P < 0.01$) phenotypic and genotypic variability was detected for most evaluated traits. Two different mapping methods were applied for detecting QTLs. Firstly, a trait by trait approach was performed on across environments, and 19 QTLs were identified. Secondly, a multi-trait multi-environment analysis detected seven joint QTLs. Almost all joint QTLs had inconsistent additive effects from one environment to another, which would reflect presence of QTL × Environment interaction. Most joint QTLs co-localized with QTLs detected by individual mapping. We detected consistent additive effects for grain yield per plant and NUE, as well as for biomass and nitrogen harvest index in some joint QTLs, especially QTL-1 and QTL-6. These QTLs had positive and stable effects across environments, and presence of some genes within these QTL intervals could be relevant for selecting for both NUE and grain yield simultaneously. Up today, this is a first report on the co-localization of QTLs for enhanced allocation of biomass allocation to grains with NUE, and NUE candidate gene identification. Fine mapping of these regions could allow to detect additional markers more closely linked to these QTLs that could be used for marker assisted selection for NUE.

KeyWords: Candidate Genes, Maize, Nitrogen Use Efficiency (NUE), Quantitative Trait Loci (QTL)

Introduction

Maize (*Zea mays* L.) is one the most important cereals in the world, with a production and consumption of ~1000 million tons (USDA, 2015). Currently, tight balance between world offer and demand causes concerns over long-term food security. Nitrogenous fertilization has been a powerful tool to increase grain yield in cereals. However, the overuse of nitrogen (N) fertilizer implies higher environmental risks associated with non-absorbed N leaching towards underground water and nitrous oxide emissions into the atmosphere, production cost increases, and waste of energy applied to the industrial synthesis of fertilizers (Tilman et al, 2002). Therefore, besides the beneficial effects of N fertilizers on maize yields, to increase N uptake and utilization efficiencies of available soil N is also important for diminishing crop production costs as well as to avoid negative effects on the environment.

In this context, the development of genotypes with improved performance in environments with low soil N content has two purposes: to reduce the cost of crop production and to allow a more sustainable agriculture in areas characterized by N deficiencies. A maize crop with improved N use efficiency (NUE, or grain biomass per unit of N absorbed) could also take advantage of enhanced N supply via fertilizers, making greater grain yield possible.

Several studies have demonstrated the existence of genetic variability for maize grain yield when grown in soils with different amounts of N available (Bänziger et al, 1997). The efficiency in which cereals utilize N content to produce grains depends on multiple processes, including root N uptake, translocation of reduced N to the leaves, and remobilization of N from stalks and leaves to developing grains. Consequently, genetic improvement could allow the increase in NUE of cereal crops as a result of selection for grain yield and for one or more of these processes (Agrama, 2006).

Numerous studies have been carried out to obtain a better understanding about NUE regulation in cereals. In maize, quantitative trait loci (QTLs) for grain yield and its components (kernel number and individual kernel weight) have been reported under different soil N levels. Significant effects under both low and high available N were found for the most of these QTLs; however, some QTLs showed interaction with the environment since they were expressed only under one N condition (Agrama et al, 1999; Ribaut et al, 2007;

Bertin and Gallais, 2001; Hirel et al, 2001). Likewise, Coque et al (2008) demonstrated that QTLs for stay-green in leaves, deep and thin roots, and increased soil N uptake were coincident with loci controlling grain yield under low and high N levels. These associations could be due to pleiotropy or genetic linkage between loci controlling these phenotypic characteristics and NUE. Similarly, the relationship between NUE and the root architecture has been extensively studied. Liu et al (2008) identified QTLs related to features of radical system growing under different N levels, but they were not coincident across environments. One QTL for length of axial roots growing under low N soils co-localized with QTLs previously reported for grain yield and N uptake, highlighting the importance of the root length on N use and overall crop performance. Additionally, Li et al (2015) identified common QTL clusters between NUE and traits related to root architecture, most of them expressed under low as well as high N environments. Similarly, QTLs for grain yield and its components have been detected coincident with QTLs mainly associated with N content in leaves and activity of enzymes related to N metabolism, such as glutamine synthetase (Hirel et al, 2001; Gallais and Hirel, 2004).

In previous studies, inbred lines B100 and LP2 were evaluated phenotypically under contrasting soil N experimental sites, and significant differences were found between them and N treatments for grain yield, number of kernels per plant, NUE and N uptake (D'Andrea et al, 2006, 2009). In addition, a panel of 102 inbred lines, included inbreds B100 and LP2 developed by the temperate maize breeding program of INTA were characterized with Simple Sequence Repeats (SSR) markers and the genetic structure of this panel of lines elucidated (Olmos et al, 2014, 2016). This analysis classified lines B100 and LP2 into different clusters across successive cycles of cluster simulations, giving a molecular support to the phenotypic differences between them previously observed in the field. Moreover, harvest index and NUE of the B100×LP2 single cross were the highest among all tested inbreds and their derived crosses included in a set of complete and incomplete dialelic experiments (Munaro et al, 2011). Accordingly, the B100×LP2 germplasm combination would be appropriate to develop a mapping population for the genetic study of NUE.

The objective of this study was to identify QTLs associated with NUE and related traits in a mapping population of recombinant inbred lines (RILs) of maize derived from the cross of B100 (yellow dent, US genetic background) × LP2 (orange flint, Caribbean×Argentine genetic background), grown under contrasting conditions of soil N offer and climate. Identification of candidate genes into QTLs regions would contribute to a better understanding of the genetic basis of NUE. The availability of the genome sequence of inbred B73 (Schnable et al, 2009) and genetic maps enable a precise location of chromosomal regions, and ultimately of key genes implicated in such an important agronomic trait. The discovery and/or validation of genes that regulate the NUE and associated traits, as well as the development of molecular markers more closely linked to these genes, could contribute to breed new genotypes with enhanced NUE through marker assisted selection (MAS).

Materials and methods

Genetic material

The genetic material evaluated consisted in a population of 181 RILs derived from the single cross B100×LP2. The F_1 was grown in the field at the Pergamino Experimental Station (33° 56´S, 60° 34´O) of the National Institute of Agricultural Technology (INTA), Argentina during the 2001-2002 season. Several F_1 plants were selfed to obtain the F_2 (S_0) generation. Randomly chosen F_2 plants were self-pollinated during successive generations conducted ear to row through single seed descent until the $S_{5:6}$ derived RILs. .

Experimental design

Phenotypic evaluations of the RILs population, the parental lines and a tester line (LP612) were conducted at INTA Pergamino, over a silty clay loam soil (Typic Argiudoll) during 2009-2010 (Exp. 1) and 2011-2012 (Exp. 2) growing seasons, under supplementary irrigation and without N supply as fertilizer. The contrast between these environments was imposed by (i) high initial soil N level (72.6 kg $N\text{-}NO_3$ ha^{-1}), early sowing date (21 October 2009) and '*El Niño*' phase of the ENSO (*El Niño* Southern Oscillation) phenomenon for Exp. 1, and (ii) low initial soil N level (11.0 kg $N\text{-}NO_3$ ha^{-1}), late sowing date (between 14 and 23 November 2011) and '*La Niña*' phase of the ENSO phenomenon for Exp. 2. Soil organic matter was similar across experimental sites (2.06-2.08 %), and phosphorous level was considered high (> 30 ppm). More details of growing conditions can be found in D'Andrea et al (2016).

For both experiment, genotypes (RILs, parental lines and tester line) were distributed in a randomized complete block design with two replicates. Each plot consisted of three rows of 5.5 m long and 0.7 m apart, with a plant density of 7 plants m^{-2}. Early sowing in Exp. 1 set the flowering period along January. For Exp. 2,

sowing was delayed to avoid the negative effects of probable above-optimum temperatures during January, associated with '*La Niña*' extreme phase of the ENSO phenomenon. In this way, flowering of most inbred lines in Exp. 2 was expected to occur by February when solar radiation and temperature are already declining (Otegui et al, 1996). In order to avoid large variations in the photothermal-environment among genotypes with contrasting phenology, inbreds were grouped in three categories (early, intermediate and late) based on the anthesis date recorded in Exp. 1. Inbred lines sowing dates in Exp. 2 were 14 (late), 18 (intermediate) and 23 November 2011 (early). This strategy helped to synchronize the flowering event (Liu et al, 2011), and thus minimize possible effects of differences in climate conditions between early and late inbreds associated with sowing dates (Otegui et al, 1996). Both experiments were hand-planted at three seeds per hill and thinned to the desired plant population at V_3 (Ritchie et al, 1992).

Weather conditions were monitored daily at the experimental sites (Campbell Scientific Inc., Logan, UT), and records were obtained for mean (Tmean) and maximum (Tmax) air temperatures (in °C), photosynthetically active radiation (in MJ day^{-1}), rainfall (in mm), and potential evapotranspiration (PET, in mm). Accumulated thermal time (in °C day with base temperature of 8 °C) was computed from daily Tmean from sowing onwards (Ritchie and NeSmith, 1991).

Measurements

Nine traits related to grain yield and N metabolism were evaluated. In each plot, phenotypic determinations were done in a group of plants (five plants in Exp. 1 and seven plants in Exp. 2) tagged at the V_3 stage. These traits were evaluated according to the methodology described by D'Andrea et al (2006, 2009). The dates of anthesis (i.e., at least one extruded anther visible) and silking (i.e. at least one extruded silk visible) were registered on all tagged plants. The anthesis-silking interval (ASI) was calculated as the difference in days between silking and anthesis dates of individual plants (Uribelarrea et al, 2002) and averaged for each plot. The ASI was expressed in days. Tagged plants were harvested at physiological maturity in order to obtain total aerial biomass ($BIOM_{PM}$, in g) and plant grain yield (PGY, in g). The harvested plants were dried in oven at 65 °C until constant weight and weighed; distinguishing between grain, ear and rest fractions. Ears of each tagged plant were harvested and threshed separately for computation of PGY. The biomass harvest index (BHI) was calculated as the quotient between PGY and $BIOM_{PM}$. Nitrogen content in vegetative tissues (stem, leaves, husks and cobs) and in grains were estimated for each plant harvested at physiological maturity; in the first case by Micro Kjeldahl method, and in the second by near-infrared transmittance method (NIR; Infratec 1227, Tecator, Sweden) in order to compute the percentage of grain protein (%Protein). Nitrogen uptake at physiological maturity (PN_{UPTAKE}) was calculated as the sum of (i) the product between N concentration in vegetative tissues and non-grain biomass (difference between $BIOM_{PM}$ and PGY), and (ii) the product between N concentration in grain and PGY. Nitrogen use efficiency (NUE, in g of grain per g of absorbed N) was determined as the quotient between PGY and PN_{UPTAKE}. Nitrogen harvest index (NHI) was estimated as the quotient between N content in grains and N content in $BIOM_{PM}$. Nitrogen proportion in plant at physiological maturity ($N/BIOM_{PM}$, in %) was estimated as the ratio between PN_{UPTAKE} and $BIOM_{PM}$.

Statistical analyzes

Descriptive statistics were estimated for the data. For each trait, normality of the dataset distribution was estimated using modified Shapiro-Wilks test. Traits that were not normally distributed were transformed using the appropriate function.

Variability for each trait was tested using a linear mixed model (Eq. 1). The phenotypic observation of measured trait on genotype in replicate and in environment was modeled according to D'Andrea et al (2008). The two growing seasons (Exp. 1 and Exp. 2) were considered as environments.

$$Y_{ijk} = \mu + G_i + E_j + RE_{jk} + GE_{ij} + \varepsilon_{ijk} \quad [1]$$

where is the general mean, G is the effect of the th genotype, E is the effect of the th environment, R is the effect of the th replicate nested in the th environment, GE is the Genotype × Environment interaction effect (G×E) between the th genotype and the th environment, and E is the residual error. Genotype and G×E interaction effects were considered as random, and E and R effects were treated as fixed. The parental line means for all traits were compared with Fisher's least significant difference (LSD). The GLM procedure of SAS V. 8.2 (SAS Institute, 1999) was used. Heritability (h^2) for each trait on a family-mean basis (Holland et al, 2003) was estimated from the components of variance according to Eq. 2 using SAS script (http://www4.ncsu.edu/~jholland/heritability/MultiEnvironRCBDHeritability.sas).

$$h^2 = \sigma_G^2 / (\sigma_G^2 + \sigma_{GE/j}^2 + \sigma_{e/jk}^2) \quad [2]$$

where σ_G^2 is the genetic variance, σ_{GE}^2 is G×E interaction variance, σ_e^2 is the error variance, j is the number of environments and k is the number of replicates.

Likewise, genetic correlations between traits were estimated from variance and covariance data estimated using the MIXED procedure of SAS V. 8.2 (SAS Institute, 1999) according to Eq. 3:

$$r_{GiGj} = \sigma_{GiGj} / \sqrt{\sigma_{Gi}^2 + \sigma_{Gj}^2} \quad [3]$$

where σ_{GiGj} is covariance between the and traits, σ_{Gi}^2 is genetic variance of the trait and σ_{Gj}^2 is genetic variance of the trait.

DNA extraction and molecular markers detection

DNA extraction of each RIL and both parental lines was done from leaves of young maize plants. Samples of five plants of each genotype were selected and bulked at ca. V_6. From each bulk, genomic DNA was extracted according to Kleinhofs et al (1993) method. Genotypic characterization was carried out using SSR and Single Nucleotide Polymorphism (SNP) markers. For the former, the parental lines B100 and LP2 were evaluated with 437 SSR equally distributed across 10 chromosomes. These markers were selected from Maize Genetics and Genomic Database (MaizeGDB; Lawrence et al, 2004). Analyzes indicated that 196 SSR were polymorphic and 131 were finally selected to evaluate the RILs population. The SSR markers were amplified by polymerase chain reaction (PCR); PCR products were resolved in 6% (w/v) denaturing polyacrylamide gel and visualized by silver staining using the conditions described by Olmos et al (2014). For SNP analysis, an Illumina microchip of 96 SNP was designed to be analyzed with the BeadXpress® Reader. For the microchip design, a chip of 1536 SNP described by Yan et al (2009) was used as reference, and 96 SNP were selected. The selected SNP were distributed equally in all chromosomes of maize, giving priority to SNP which were located within genes involved in N metabolism and abiotic stress tolerance. The complete RILs population genotyped with the microchip included control DNA (both parental lines and the reference line B73). Data generated with BeadXpress® Reader were visualized using the GenomaStudio V. 2011.1 software (Illumina, 2011). In order to evaluate SNP quality, three different parameters were used: minor allele frequency (MAF), gene train score (GTS) and cluster sep (CS). Those markers with MAF ≥ 0.1, GTS ≥ 0.7 and CS ≥ 0.2 were considered as high quality SNP. Eight high quality SNP were polymorphic and used to characterize the population.

Genetic map construction and QTL detection

The molecular markers segregation was verified using the false discovery rate (FDR) test (Benjamini and Hochberg, 1995). Only markers which fitted the expected Mendelian segregation (1:1) were used to made linkage maps. Recombination frequencies were transformed to map units using following Kosambi (1944). The best order of markers in the map was estimated by the maximum-likelihood method. The segregation of markers and linkage maps construction were carried out using GQMol V. 2008.6.1 software (Cruz and Schuster, 2008), and the maps were charted using MapChart 2.2 software (Voorrips, 2002). Linkage map of the RILs population was constructed using 112 markers with 1:1 segregation (105 SSR and 7 SNP). The maps consisted of 94 markers (18 non-linked) covering a total distance of 1129.5 cM with an average interval between markers of 11.8 cM.

Two mapping strategies were used to detect QTLs. Firstly, QTL analysis was done for each individual trait in each environment using the composite interval mapping (CIM) method. Model 6 of the Zmapqtl procedure of WinQTL Cartographer V. 2.5 software (Wang et al, 2012) was utilized with a walk speed of 1 cM, a window size of 10 cM and five default markers included as cofactors. Threshold LOD scores were estimated using the 1000 permutation test (Churchill and Doerge, 1994). Secondly, a multi-trait multi-environment (MTME) QTL analysis was done according to Alvarez Prado et al (2013). The multiple trait analysis is appropriate when traits are genetically correlated. Causes of genetic correlation among different traits could be pleiotropy or linkage disequilibrium. For MTME QTL mapping, the phenotypic data were analyzed considering correlations between traits and variability between environments using the mixed model approach followed by Malosetti et al (2008). Only traits with genetic correlations ≥ 0.50 (with at least one trait) were used for the analysis. The model MTME consisted of I genotypes evaluated in J environments on K traits repeated in L blocks (with I=181, J=2, K=9, L=2). Defining an N×1 vector "y" with N=IJKL that contains all the observations sorted by trait in each environment for each genotype and in each block. Genotype effects were assumed as random, while trait-environment combinations (TE) and blocks nested within TE were considered as fixed effects. Seven different variance-covariance models for both matrixes were tested in order to select the most suitable for the analysis of data sets. Tested models were: (i) variance component, (ii) compound symmetry, (iii-v) factor analytic "0", "1" and "2", (vi) heterogeneous compound symmetry, and (vii) unstructured. The choice of the best model was based

on a goodness of fit criterion such as the Bayesian Information Criterion (BIC; Schwarz, 1978). A minor value of BIC implies a better model. The best model of variance-covariance structure for this data set was the compound symmetry model.

Once chosen the variance-covariance structure, the best linear unbiased predictor (BLUP) of each genotype in each environment was estimated to reduce uncontrolled trait variation for QTL mapping (Borevitz et al, 2002; Zalapa et al, 2007). These analyzes were performed using the MIXED procedure of SAS V. 8.2 (SAS Institute, 1999). BLUPs estimated by the mixed model were used for QTL mapping. However, QTL analysis was done using a mixture model (Jiang and Zeng, 1995) according to Alvarez Prado et al (2013). Mixture model has greater precision than mixed model to detect QTLs in unsaturated maps. For this approach, a two main step procedure was used. In the first step, joint QTLs were identified using the JZmapqtl procedure of WinQTL Cartographer V. 2.5 software (Wang et al, 2012), which can analyze multiple traits simultaneously. Composite interval mapping was implemented using the forward regression method of model 6 with a window size of 10 cM and five markers as cofactors. A LOD threshold of 7.0 was used to declare a joint QTL as significant. In the second step, a final multi-QTL model was constructed to evaluate the effects and significance of each QTL into a unique model. For that, QTLs detected in the first step were used as candidate QTLs to construct the initial multi-QTL model by means of multi trait multi interval mapping procedure (MT-MIM) of WinQTL Cartographer V. 2.5 (Wang et al, 2012.). For this initial model, parameters were estimated, positions of detected QTLs were optimized, new QTLs and epistatic interaction were searched, and the genetic variance explained by the model was calculated (Basten et al, 2004). The Eq. 4 was used to compare the significance of each optimized model with initial model likelihood ratio test (LRT):

$$LRT = 2(logL1 - logL0) \qquad [4]$$

where L1 and L0 were the likelihood under the refined and the initial models, respectively. The difference between log-likelihoods was multiplied by a factor 2, so it distributes as the statistic. Then statistical significance of LRT was assessed using standard 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 models.

For both mapping approaches, QTL positions were assigned in the maximum LOD score (LODmax), and confidence intervals were calculated subtracting one unit of LOD to each side of the LODmax position.

Identification of candidate genes

Potential candidate genes were identified within the QTL intervals detected by MTME. Firstly, physical coordinates (in bp) of flanking markers were established using option Locus Lookup in the MaizeGDB database (Andorf et al, 2010). Once physical position of intervals were known, candidate genes in QTL regions were identified based on B73 RefGen_v3 genome sequence, using BioMart software in the Gramene database www.gramene.org (last revision 14-07-2016). The function of each gene was confirmed by their homology to orthologous genes in *Arabidopsis thaliana* or *Oryza sativa*. Identified genes were classified according to their putative functions into six main categories based on the criteria established by Munich Information Center for Protein Sequences (MIPS; Ruepp et al, 2004).

RESULTS

Weather conditions

Weather conditions were different between years, and consistent with the contrasting phases of the ENSO phenomenon. While Tmean was similar between both experiments (≈ 22.0 °C), Tmax in Exp. 2 (29.8 °C, average of daily Tmax values) was higher than in Exp. 1 (28.2 °C). Furthermore, Exp. 1 presented only one day with Tmax ≥ 35 °C, whereas for Exp. 2 there were 20 days above this threshold. However, this condition occurred before flowering in Exp. 2. As expected from the ENSO phenomenon, total rainfall in Exp. 1 (ca. 900 mm) was higher than in Exp. 2 (ca. 563mm), which caused an opposite trend in PET, with daily values of 4.8 and 5.8 mm respectively. However, rainfall during the flowering period was not markedly different between experiments (ca. 240 mm). Delayed flowering in Exp. 2 exposed the crop to low levels of incident solar radiation, and consequently to lower PET values (4.7 mm d^{-1}) than those registered in Exp. 1 (5.7 mm d^{-1}).

Descriptive statistics and mixed model analysis of measured traits

Parental inbreds (B100 and LP2) were compared for all measured traits (Table 1) and significant differences were found between them for ASI, %Protein and NUE. Parental line LP2 exhibited a larger ASI than B100 ($P < 0.05$) in both experiments. Differences ($P < 0.05$) between parental inbreds for NUE (LP2 > B100) and %Protein (LP2 < B100) were observed only in Exp. 2. The RILs population showed transgressive segregation for all traits, at least on one side of the frequency distribution. For most traits, there were recombinant inbred lines with smaller records than those observed for the inferior parental.

Results revealed significant genetic variability among lines ($P < 0.01$) for most evaluated traits (Table 1), except PN_{UPTAKE}. There were significant differences between experimental environments for almost all traits (Table 1) and a significant G×E interaction was always observed ($P < 0.001$). In Exp. 2 RILs population showed a significant ($P < 0.05$) reduction in PGY, $BIOM_{PM}$, BHI, PN_{UPTAKE} and %Protein (24%, 21%, 5%, 36% and 14%, respectively), and an increase in NUE (14%). Narrow sense heritabilities were estimated for all traits and varied from 0.37 for $BIOM_{PM}$ to 0.74 for ASI. The PN_{UPTAKE} showed null heritability (Table 1).

PGY was positively correlated with NUE, NHI, PN_{UPTAKE}, $BIOM_{PM}$ and BHI, and negatively correlated with ASI, %Protein and $N/BIOM_{PM}$ (Table 2). Traits that presented high correlations with PGY were NUE, NHI and BHI. Likewise, some traits had a strong correlation between them. For instance, NUE was positively correlated with NHI (0.90) and with BHI (0.85). By the contrary, % Protein had high and negative correlation coefficients with NUE (-0.73) and with BHI (-0.80).

QTL analysis

Nineteen QTLs were identified associated with PGY, BHI, NUE, NHI, ASI, $N/BIOM_{PM}$ and %Protein across the two experiments by CIM (Table 3). The QTLs were mapped in the most of chromosomes, except 3, 6, 7, and 10 (Figure 1). Percentage of phenotypic variance explained (PVE) by QTLs ranged from 6.0 to 13.3 %. Four QTLs were identified for BHI (BHI1a, BHI1b, BHI1c and BHI2). The QTL BHI1a in Exp. 1 and BHI2 in Exp. 2 were both located on chromosome 1, on the same markers interval (phi339017 and phi095), and showed positive additive effects. Three QTLs were detected for NHI, two of them located on chromosomes 8 (NHI1a) and 9 (NHI1b) in Exp. 1, and one located on chromosome 1 (NHI2) in Exp. 2. The QTLs NHI1b and NHI2 showed positive additive effects whereas the NHI1a showed negative additive effects. Likewise, three QTLs were identified for %Protein, two of them in Exp. 1 (%Protein1a, %Protein1b) and one in Exp. 2 (%Protein2). Only %Protein1b on chromosome 5 showed positive additive effect. QTLs %Protein1a

Table 1 Descriptive statistics, variance components, year effect and heritability (h^2) values of traits measured in 181 RILs and their parental lines (B100 and LP2) measured in two contrasting environments (Exp. 1 and Exp. 2)

		PGY (g)	$BIOM_{PM}$ (g)	BHI	PN_{UPTAKE} (g)	NUE (g grain g N^{-1})	NHI	$N/BIOM_{PM}$ (%)	%Protein	ASI (days)
Exp. 1	B100[a]	62.2	150	0.42	1.6	38.7	0.64	10.8	10.3	-1.50†
	LP2	58.4	138	0.43	1.51	40.2	0.62	10.8	9.67	3.50†
	RILs	55.1	151	0.37	1.6	35.1	0.57	10.6	10.4	1.51
	Rangeb	11.4-87.5	85.0-264	0.08-0.50	0.98-3.58	11.4-50.9	0.20-0.72	8.44-14.1	8.03-13.2	(-2.00)-7.00
	CV (%)	25.9	18.5	20.6	22.5	22.9	18.6	9.67	9.41	110
	P valuec	0.33	<0.001	<0.001	<0.001	<0.001	<0.001	0.07	0.48	0.006
Exp. 2	B100[a]	49	135	0.35	1.23	38.5†	0.58	9.15	9.59†	-2.00†
	LP2	53.7	130	0.41	1	56.0†	0.67	7.65	7.44†	3.00†
	RILs	41.9	120	0.35	1.03	40.7	0.56	8.62	8.91	1.74
	Range	6.57-70.0	75.9-168	0.08-0.47	0.68-1.63	9.01-60.8	0.12-0.72	6.54-10.5	6.70-11.8	(-2.75)-10.5
	CV (%)	30	15.5	24.3	16.5	27	21.4	9.49	12.1	124
	P value	0.02	0.11	<0.001	0.43	<0.001	<0.001	0.32	0.01	<0.001
Variance Components ± s.e.	G	58.1 ± 15.1*	131 ± 46.3*	0.003 ± 0.0006*	0.007 ± 0.006	35.6 ± 7.90*	0.006 ± 0.001*	0.26 ± 0.07*	0.47 ± 0.09*	2.34 ± 0.34*
	G × E	55.8 ± 14.6*	183 ± 51.3*	0.002 ± 0.0004*	0.034 ± 0.008*	33.1 ± 6.40*	0.003 ± 0.0008*	0.30 ± 0.07*	0.32 ± 0.07*	0.67 ± 0.17*
	Residual	132 ± 10.1	473 ± 36.9	0.003 ± 0.0002	0.074 ± 0.006	43.3 ± 3.32	0.006 ± 0.0005	0.60 ± 0.05	0.53 ± 0.04	1.56 ± 0.12
	Year effect	<.001	<.001	<.001	<.001	<.001	0.09	<.001	<.001	0.05
	h^2 ± s.e.	0.46 ± 0.08	0.37 ± 0.10	0.63 ± 0.06	0.16 ± 0.12	0.56 ± 0.07	0.63 ± 0.07	0.44 ± 0.08	0.59 ± 0.06	0.74 ± 0.04

*a Mean value of B100, LP2 and RILs; b minimum and maximum values of RILs population; c Shapiro-Wilk normality test: significantly different from normal P < 0.01; † indicates a significant difference (P < 0.05) between B100 and LP2 (LSD Test); *Indicates significant difference (P < 0.01); ASI: anthesis-silking interval; BHI: biomass harvest index; $BIOM_{PM}$: total plant biomass at physiological maturity; Exp. n: experiment n; $N/BIOM_{PM}$: N proportion in plant biomass at physiological maturity; NHI: N harvest index; NUE: N use efficiency for grain production; %Protein: percent grain protein; PGY: plant grain yield; PN_{UPTAKE}: plant total N uptake at physiological maturity, s.e.: standard error.*

and %Protein2 were located on chromosome 1 sharing at least part of intervals between markers. For NUE, three QTLs were detected, two located on chromosomes 1 (NUE1a) and 9 (NUE1b) in Exp. 1, and one on chromosome 1 (NUE2) in Exp. 2. All QTLs presented positive additive effects. For N/BIOM$_{PM}$ three QTLs were identified, one on chromosome 8 (N/BIOM$_{PM}$1) in Exp. 1, and two in Exp. 2, one of them located on chromosome 4 (N/BIOM$_{PM}$2a) and the other on chromosome 8 (N/BIOM$_{PM}$2b). For PGY, only one QTL was detected (PGY2), placed on chromosome 1 between phi339017 and phi095 markers in Exp.2.

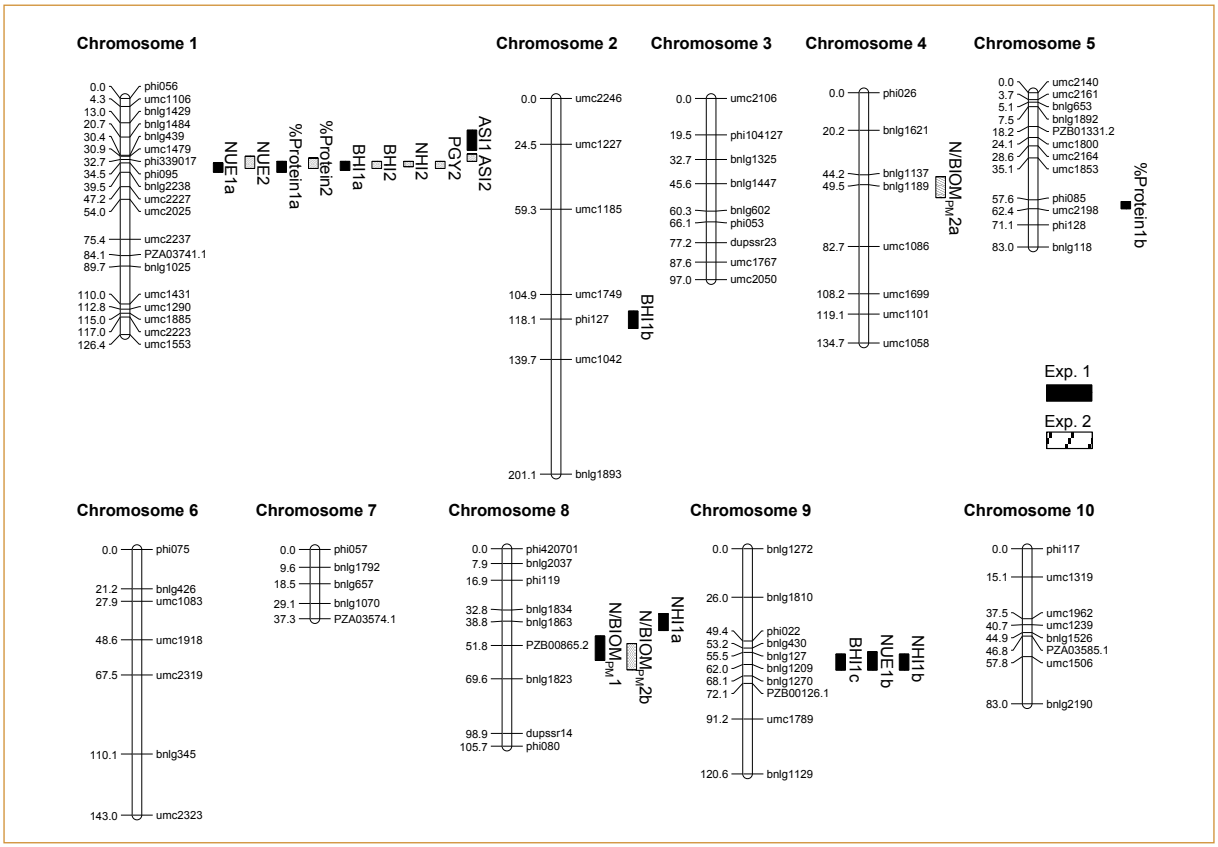

Figure 1 Chromosomal location of the QTLs detected by CIM. Bar lengths are proportional to confidence interval lengths (cM). ASI: anthesis-silking interval; BHI: biomass harvest index; N/BIOM$_{PM}$: N proportion in plant biomass at physiological maturity; NHI: N harvest index; NUE: N use efficiency for grain production; %Protein: percent grain protein; PGY: plant grain yield

Table 2 Genetic correlation of measured traits

	PGY	BIOM$_{PM}$	BHI	PN$_{UPTAKE}$	NUE	NHI	N/BIOMPM	%Protein	ASI
PGY (g)	1	0.45	0.79	0.25	0.91	0.87	-0.26	-0.51	-0.34
BIOM$_{PM}$ (g)		1	0.14	0.90	0.08	0.21	-0.30	-0.02	0.03
BHI			1	-0.15	0.85	0.95	-0.06	-0.80	-0.32
PN$_{UPTAKE}$ (g)				1	-0.26	-0.16	0.26	0.36	0.04
NUE (g grain g N^{-1})					1	0.90	-0.47	-0.73	-0.30
NHI						1	-0.27	-0.45	-0.45
N/BIOM$_{PM}$ (%)							1	0.62	-0.001
%Protein								1	0.06
ASI (days)									1

ASI: antesis-silking interval; BHI: biomass harvest index; BIOM$_{PM}$: plant biomass at physiological maturity; G: genotype; E: environment; N/BIOM$_{PM}$: N proportion in plant biomass at physiological maturity; NHI: N harvest index; NUE: N use efficiency for grain production; %Protein: percent grain protein; PGY: plant grain yield; PN$_{UPTAKE}$: plant total N uptake at physiological maturity.

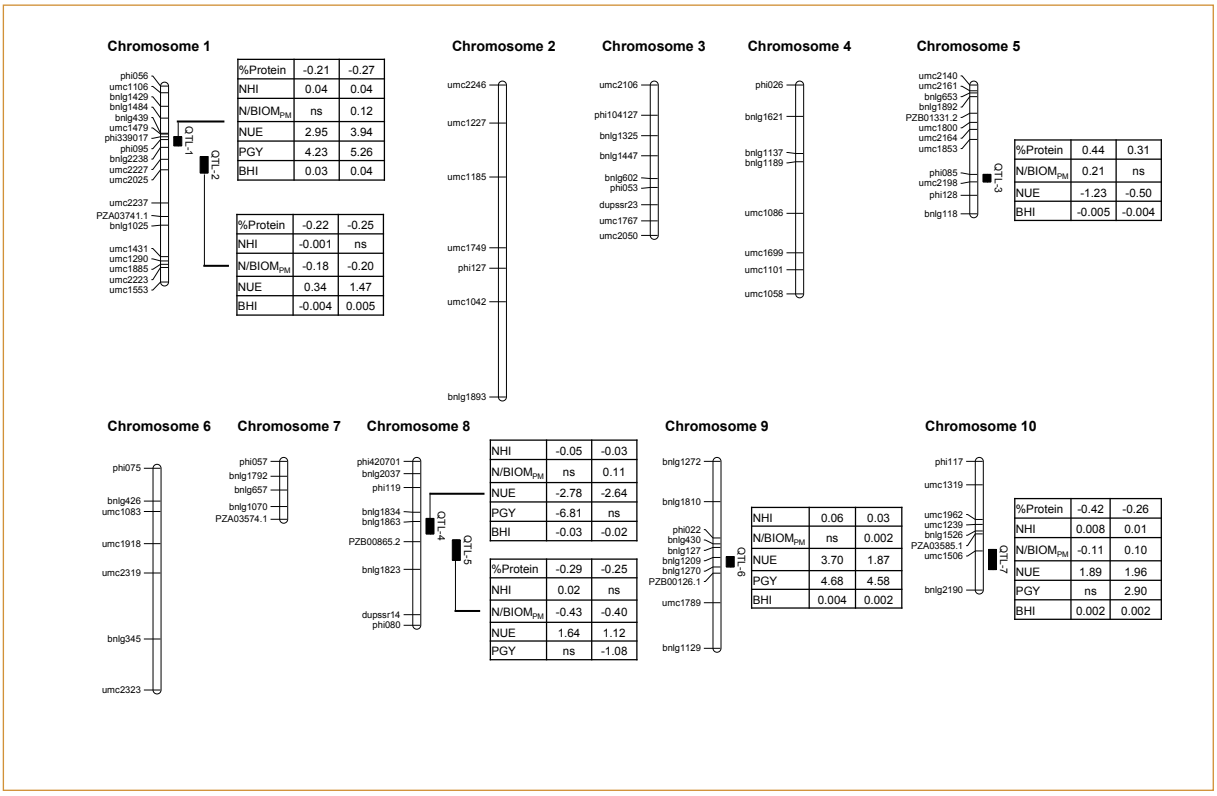

Figure 2 Chromosomal location of the QTLs detected by MTME. Bar lengths are proportional to confidence interval lengths (cM) BHI: biomass harvest index; NUE: N use efficiency for grain production; NHI: N harvest index; N/BIOM$_{PM}$: N proportion in plant biomass at physiological maturity; %Protein: percent grain protein; PGY: plant grain yield. Additive effect corresponding to the LP2 parental allele

This QTL showed a PVE of 12.6 % and positive additive effect. Finally two QTLs were identified for ASI, one with effect in each experiment, both on chromosome 1, but located on different marker intervals. These QTLs showed negative additive effects.

On chromosome 1, 10 QTLs (NUE1a, NUE2, %Protein1a, %Protein2, BHI1a, BHI2, ASI1, ASI2, PGY2 and NHI2) were identified between markers bnlg1429 and umc2238. Most of them shared at least part of their confidence intervals. Whereas the QTLs for BHI, NUE, NHI and PGY presented positive additive effects, those for %Protein and ASI showed negative additive effects. Thus, alleles from LP2 would increase mean values of NUE, BHI, NHI and PGY, and would decrease mean values of %Protein and ASI. Similarly, the NUE1b, NHI1b and BHI1c located on chromosome 9, between markers bnlg430 and bnlg1270, showed positive additive effects, indicating that the alleles from LP2 would increase the mean values of mentioned traits. Likewise, the QTLs N/BIOM$_{PM}$1 and N/BIOM$_{PM}$2b were located on the same region on chromosome 8 (between markers bnlg1863 and PZA00865.2) and showed negative additive effects.

Most of the detected QTLs were expressed in only one of the two experiments. Only the QTLs for BHI (BHI1a and BHI2) and %Protein (%Protein1a and %Protein2) on chromosome 1, and the QTLs for N/BIOM$_{PM}$ (N/BIOM$_{PM}$1 and N/BIOM$_{PM}$2b) on chromosome 8 were mapped in both environments. The confidence intervals of QTLs ranged from 2.8 to 14.0 cM, being larger intervals corresponding to N/BIOM$_{PM}$.

MTME mapping was carried out considering only the traits with significant genetic variability ($P < 0.05$; Table 1) and with genetic correlation coefficients greater than 0.50 (Table 2). In the first step, seven joint QTLs were identified on chromosomes 1, 5, 8, 9, and 10. For each QTL, the interval between flanking markers in cM was determined as LODmax ± 1 LOD unit, and the physical intervals (in Mbp) were estimated from flanking markers coordinates (Table 4). Physical intervals were highly variable from 2.2 Mbp for QTL-1 to 81.0 Mbp for QTL-6. All QTLs detected in this step were considered candidate QTLs and incorporated in the initial model. No candidate QTL was excluded, and any new QTL was added to the final model. Epistatic interactions between QTLs were not detected. Genetic map location for all joint QTLs and additive effects corresponding to the LP2 allele for each trait and in each environment are showed in the Figure 2.

All joint QTLs showed inconsistent additive effects across environments at least for one trait, which would be attributed to G×E interaction effects. The trait with the highest inconsistency between environments was $N/BIOM_{PM}$. Likewise, the QTL-1, QTL-4, QTL-6 and QTL-7 presented additive effects of the same sign for NHI, BHI, NUE and PGY, and in most cases their effects resulted consistent across environments. Additive effects were positive for QTL-1, QTL-6, and QTL-7, whereas these effects were negative for the QTL-4.

The PVE by the multi-QTL model ranged from 10.8 to 36.7% across traits and environments. On average, traits with higher PVE were %Protein, BHI, NUE and NHI (28.1, 23.9, 21.0 and 20.4%, respectively), whereas $N/BIOM_{PM}$ had the lowest PVE (5.5%).

Table 3 Chromosomal location and effects of the QTLs detected by CIM

QTL	Exp[a]	LOD[b]	QTL POSITION					LODmax	Genetic effect	
			Chr	Bin	cM[c]	Interval (cM)	Marker Interval		R^2	A
			1	1.03	34.5	33.7-37.5	phi339017-phi095	5.9	12.6	4.51
BHI1a	Exp. 1	2.7	1	1.03	36.5	33.7-38.5	phi339017-phi095	4.9	10.4	0.02
BHI1b			2	2.06-2.08	118	114-123	umc1749-phi127	3.4	6.1	-0.02
BHI1c			9	9.03-9.04	61.5	56.5-65.0	bnlg127-bnlg1209	5.4	10.3	0.02
BHI2	Exp. 2	2.7	1	1.03	34.5	33.7-37.5	phi339017-phi095	6.2	13.3	0.03
NUE1a	Exp. 1	3.0	1	1.03-1.04	37.5	34.5-39.5	phi095-bnlg2238	4.8	12.3	2.78
NUE1b			9	9.04	62.0	55.2-65.0	bnlg430-bnlg1209	4.7	10.3	2.54
NUE2	Exp. 2	2.9	1	1.03	34.5	30.9-37.5	umc1479-phi095	3.8	8.4	3.24
NHI1a	Exp. 1	2.7	8	8.03	39.8	34.8-43.8	bnlg1834-bnlg1863	3.0	6.6	-0.03
NHI1b			9	9.03-9.04	62.0	56.5-65.0	bnlg127-bnlg1209	6.0	12.5	0.04
NHI2	Exp. 2	3.1	1	1.03	34.5	33.7-36.5	phi339017-phi095	4.5	9.7	0.04
$N/BIOM_{PM}1$	Exp. 1	2.8	8	8.03	51.8	46.8-59.8	bnlg1863-PZB00865.2	4.6	10.9	-0.37
$N/BIOM_{PM}2a$	Exp. 2	2.9	4	4.06-4.07	49.5	45.2-56.5	bnlg1137-bnlg1189	3.2	6.9	-0.24
$N/BIOM_{PM}2b$			8	8.03	55.8	50.8-64.8	bnlg1863-PZB00865.2	3.5	12.7	-0.33
%Protein1a	Exp. 1	2.8	1	1.03-1.04	37.5	33.7-39.5	phi339017-bnlg2238	5.1	10.5	-0.32
%Protein1b			5	5.06-5.07	61.6	58.6-62.4	phi085-umc2198	6.4	13.1	0.37
%Protein2	Exp. 2	3.6	1	1.03	33.7	31.9-37.5	umc1479-phi095	4.7	10.8	-0.35
ASI1	Exp. 1	2.9	1	1.02-1.03	20.7	17.0-27.7	bnlg1429-bnlg1484	2.9	6.0	-0.39
ASI2	Exp. 2	3.0	1	1.03	30.4	29.7-33.7	bnlg1484-phi339017	3.2	6.4	-0.52

a Only those with data; b LOD threshold obtain by permutation test; c QTL position in cM (corresponding to LODmax value); R2: total proportion of phenotypic variance explained for each QTL; A: additive effect corresponding to the LP2 parental allele; ASI: anthesis-silking interval; BHI: biomass harvest index; Chr: chromosome; LODmax: maximum LOD value; N/BIOMPM: N proportion in plant biomass at physiological maturity; NHI: N harvest index; NUE: N use efficiency for grain production; %Protein: percent grain protein; PGY: plant grain yield.

Table 4 Chromosomal location of the QTLs detected by MTME analysis

QTL	POSITION				LODmax
	Chr	Marker interval	Genetic interval (cM)	Physical interval (Mbp)	
QTL-1	1	phi339017-phi095	32.7-38.5	45.9-48.1	9.0
QTL-2	1	bnlg2238-umc2025	45.5-56.0	55.1-91.3	8.6
QTL-3	5	phi085-umc2198	57.6-62.4	203-217	9.4
QTL-4	8	bnlg1834-bnlg1863	36.8-46.8	63.5-91.6	9.5
QTL-5	8	bnlg1863-PZB00865.2	50.8-63.8	91.6-125	9.3
QTL-6	9	bnlg127-bnlg1209	61.5-68.0	26.9-107	13.5
QTL-7	10	PZA03585.1-umc1506	56.8-69.8	105-133	7.8

Chr: chromosome; LODmax: maximum LOD value

Identification of candidate genes

The analysis identified a total of 3961 genes within the intervals corresponding to the seven detected joint QTLs, which were classified according to their putative function (S1_table). A complete list of genes is showed in the S2_table. The 71.2% of identified genes (2819 genes) corresponded to uncharacterized protein, whereas the remaining 28.8% (1142 genes) belonged to proteins with putative functions.

Percentages assigned to each category were established for the 1142 genes with predicted function. A 32.7% corresponded to proteins involved in basal metabolism. Proteins related to synthesis and degradation of amino acids, nucleotides, carbohydrates, lipids and other secondary metabolites fall in this category, as well as those implicated in processes related to energy production such as glycolysis, respiration, fermentation, photosynthesis, oxidation of fatty acids, etc. On the one hand, 43.5% of genes corresponded to proteins involved in information pathways, such as DNA processing (synthesis, replication, recombination, repairing, etc.), cell cycle, transcription (synthesis, processing and modification of rRNA, mRNA and tRNA), protein synthesis (ribosomal proteins and proteins involved in translation), protein fate (protein folding and modification, assembly of protein complexes and protein degradation). Genes encoding proteins involved in the ubiquitin (Ub)-proteasome pathway of protein degradation (ubiquitin-protein ligase, ubiquitin fusion degradation UFD1 family protein, proteasome maturation factor UMP1 family protein, 26S protease regulatory subunit 4, putative ubiquitin-conjugating enzyme family, etc.) and genes encoding Clp proteases were identified in all QTL intervals. On the other hand, 6.0% of genes corresponded to protein implicated in cellular transport. Among them, amino acid transporters genes were identified on intervals of the QTL-5, QTL-6 and QTL-7. A 13.4% of genes were associated with perception and response to stimulus such as cellular communication and signal transduction, cellular defense to biotic and abiotic stress, cellular detoxification and environmental interaction. Some QTL intervals included genes that encode proteins involved in cellular detoxification, as metallothionin, glutathione-S-transferase, superoxide dismutase and peroxidase. Finally, 4.4% of genes were associated with processes related to plant development such as biogenesis of cellular components, cell type differentiation (organs, tissues and cell), sexual determination and senescence.

Discussion

Variability of the RILs population and their parental lines (B100 and LP2) for NUE and related traits was evaluated across two contrasting environments. The combined effect of low initial soil N and warm conditions during Exp. 2 determined unfavorable growth conditions, causing reductions in N absorption (36%),biomass production (21%) and PGY (24%). The population had a broad genetic variation in PGY and all traits of interest, except PN_{UPTAKE}. A significant G×E interaction was also detected for all traits, which highlighted a differential performance among genotypes when exposed to different environments as well as the importance of phenotypic evaluation in different environments (Crossa, 1990). Parental inbreds differed markedly for NUE only when grown under low soil N level and higher temperatures (Exp. 2). In this environment, LP2 had a higher NUE than B100, indicating that LP2 would be more efficient for using N when this nutrient is scarce and/ or when growing under warmer environments.

Genotypic characterization of the RILs population was mainly done using SSR markers. A high percentage of the tested SSR (almost 50%) were polymorphic between parental lines, which is in agree with the results obtained by Olmos et al (2014). The high level of polymorphism allows us to deduce that the RILs population derived from B100 and LP2 lines was appropriate for QTL mapping. Although the number of useful SNP was reduced, it allowed an increase of 6% in total marker number. Moreover, 19% of evaluated markers had distortive segregation. Numerous studies have detected deviation of marker segregation in maize (Gardiner et al, 1993; Pereira and Lee, 1995; Sibov et al, 2003). Segregation distortion can be attributed to chromosomal regions called segregation-distortion loci (SDL). Markers close to these loci can be affected by distortion (Vogl and Xu, 2000). In current study, two distortive segments were detected that coincide with regions previously described as SDL by Lu et al (2002): one on chromosome 2 (bin 2.04), close to the *gams1* (gametophytic male sterile1) gene, and other on chromosome 5 (bin 5.02-5.04). Distortive segregation affects recombination frequency, and hence precludes precise QTL mapping. In this work, distortive markers were excluded from genetic map construction and only those markers with 1:1 segregation were used. Linkage maps covered 1129.5 cM with an average marker interval of 11.8 cM. However, some genomic regions presented marker intervals up to 30 cM. For example, map corresponding to chromosome 2 showed two extensive genomic regions, whereas map corresponding to chromosome 6 presented one large region between umc2319 and bnlg345. In some case, many of the tested SSR in these

regions resulted monomorphic between the parental lines, so were not used for mapping; moreover, markers that were polymorphic showed distortive segregation. Thereby, most chromosome maps presented low coverage regions that coincided with larger bins. Linkage map corresponding to chromosome 1 was the most covered, whereas the less covered map was that of chromosome 7.

When current genetic map is compared with genetic maps in the MaizeGDB database, we observed that most published maps derive from the B73×Mo17 cross which are, in addition, more saturated. Inbreds of this family are representative of dent temperate germplasm which had been extensively studied (Gustafson and de Leon, 2010), whereas the B100×LP2 (US yellow dent × Caribbean-Argentine orange flint) population combines a more complex genetic background than the B73×Mo17 population, that has not been deeply elucidated. For instance, LP2 is a flint inbred line from the Argentine × Caribbean Derived Stocks (Olmos et al, 2014, 2016) which shares high added value for industry (Greco, 2014) whereas B100 is a semi-dent US inbred line but with a distant genetic relationship with B73 and Mo17 (Romay et al, 2013). This implies that the cross B100 x LP2 and its derived RILs would serve as novel sources of undiscovered alleles for genetic studies and breeding purposes. Thus, in spite of the low genetic map saturation, QTL detection for NUE and related traits was achieved and many of these QTLs were stable across evaluated environments. However, some QTL could have not revealed due to low genome representation.

Nineteen QTLs were identified associated with NUE and related traits across two environments using CIM. In many cases, intervals of a QTL detected for a trait showed partial or complete overlap with intervals of other QTL identified for other trait. Moreover, most of detected the joint QTLs co-localized, at least in part, with several QTLs identified by individual mapping. The markers interval corresponding to the QTL-1 (phi339017 and phi095) showed total overlapping with the interval of the QTLs BHI1a, BHI2, PGY2 and NHI2, and partial overlapping with the QTLs NUE2, %Protein1a and %Protein2. In addition, the interval of the QTL-6 (bnlg127 and bnlg1209) presented total overlapping with the QTLs NHI1b and BHI1c, and partial overlapping with NUE1b. Likewise, overlapping between intervals of QTL detected by both approaches was evidenced for the QTL-3, QTL-4, and QTL-5 but fewer traits were significant for both methods. Several researchers have found that results from individual QTL mapping cannot be compared across environments. However, when MTME approach is used, a higher stability in QTL expression across environment is observed (Malosetti et al, 2008; Messmer et al, 2009; Liu et al, 2014). In general, the joint QTL confidence intervals were narrower than QTL confidence intervals detected by individual mapping. When an individual trait is analyzed, the statistical power of the test is reduced, and the variance of the estimation increases; moreover, most of biological processes involve multiple traits that can be genetically correlated due to pleiotropy or close linkage (Jiang and Zeng, 1995). Thus, MTME analysis is a more powerful and realistic approach than QTL mapping for individual traits across environments.

The genetic dissection of PGY into their determinants $BIOM_{PM}$ and BHI, or PN_{UPTAKE} and NUE, revealed that variation in PGY was strongly associated with BHI and NUE, and weakly associated with PN_{UPTAKE} and $BIOM_{PM}$. Also, h^2 values computed for BHI, NUE and most traits of interest were larger than for PGY, except for PN_{UPTAKE} and $BIOM_{PM}$. This result supports the claim that dissection of complex trait as PGY might not always result in improved genetic prediction (Lee et al, 2005; Alvarez Prado et al, 2013; Mandolino et al, 2016). The highly significant correlations among PGY, BHI, NUE and NHI were consistent with additive effects detected for all these traits in QTL-1, QTL-4, QTL-6 and QTL-7. This result proposed a common genetic control (e.g., linkage or pleiotropic effects) among these attributes for the current RILs population. As documented in previous studies, a tight link exists between BHI and NHI (Ciampitti and Vyn, 2012; Fageria, 2014), indicative of an enhanced N partitioning from stover to grains depending upon an enhanced allocation of plant biomass to grains. As proposed by Ciampitti and Vyn (2012), the challenge will be to optimize BHI, NHI and NUE simultaneously. In this sense, it is necessary to be cautious about the negative association between remobilized N and postsilking N uptake (Coque and Gallais, 2007; Gallais et al, 2007). Fortunately, the consistent effects for BHI, NHI and NUE observed in the QTL-1, QTL-4, QTL-6 and QTL-7 suggest that an effective simultaneous selection of these traits could be feasible. This is an important finding that has not been reported previously. Moreover, the QTLs in chromosomes 1 (QTL-1 and QTL-2), 8 (QTL-5) and 10 (QTL-7) with additive effect <0 for %Protein and additive effect >0 for PGY, BHI or NUE are consistent with the negative relationships detected for these traits. These results support the existence of a trade-off between PGY, NUE and %Protein among grain crops (Dudley et al, 1977; Feil et al, 1990; Lemaire and Gastal, 2009; Ciampitti and Vyn, 2013; Gastal et al, 2015), which has affected breeding efforts for the simultaneous increase

of grain yield and grain protein concentration. Finally, a negative correlation was established between PGY and ASI. This trend, related to the negative correlation between ASI and kernel number, has been observed in other studies (Gallais and Hirel, 2004; Lafitte and Edmeades, 1995). In our study, however, only two QTLs linked to the ASI were detected on chromosome 1 using CIM (ASI1 and ASI2); in addition, this trait was not taken into account for MTME approach because of low genetic correlation (<0.50) with NUE and related attributes. Such apparent discrepancy with previous reports may be linked to the proportionally larger effect of water deficit than of N shortage on ASI values, particularly among inbreds (Munaro et al, 2011).

Most of the QTLs detected in this study shared regions with previously reported QTLs. There was a relevant coincidence between the QTL-3 on chromosome 5 (associated with %Protein, NUE, $N/BIOM_{PM}$ and BHI) and a QTL for nitrate content (NO_3^-), PGY, and activities of nitrate reductase and glutamine synthetase (GS) identified by Hirel et al (2001), as well as another one for PGY detected by Bertin and Gallais (2001). Such coincidences are consistent with the positive correlation of GS activity with NUE (Hirel et al, 2001; Masclaux et al, 2001) and grain yield (Masclaux et al, 2001; Gallais and Hirel, 2004). These co-localizations support at molecular level the consensus that the enzymatic activity in the leaf cytosol is one of the major steps controlling biomass and N allocation from leaves to grains (Hirel et al, 2001) and its manipulation could potentially raise NUE through a more efficient internal recycling of N from old to new leaves (Foulkes et al, 2009). Likewise, the QTL-1 on the chromosome 1 coincides with a QTL for grain protein concentration (Wassom et al, 2008) and a QTL for longer axial root detected by Liu et al (2008) under conditions of low and high N availability. Similarly, the QTL-5 location on chromosome 8 was consistent with QTLs previously detected for grain protein concentration (Li et al, 2009), N remobilization (Coque et al, 2008) and plant N content (Gallais and Hirel, 2004). Additionally, the QTL-6 on chromosome 9 associated with NUE, NHI, BHI, PGY and $N/BIOM_{PM}$ coincides with QTLs detected for NUE (Bertin and Gallais, 2001), PGY under high N condition (Liu et al, 2010) and N remobilization (Coque et al, 2008). This fact confirms the importance of N remobilization on NUE and PGY determination (Coque and Gallais, 2007; Ciampitti and Vyn, 2013). Finally, the QTL-7 on chromosome 10 coincides with a joint QTL for protein and starch concentration (Li et al, 2009).

Some candidate genes were found to be of value for control of the physiological process underlying NUE. One of the most interesting genes identified was *rth3* gene on the QTL-1. This gene encodes a monocot-specific COBRA-like protein involved in cellular expansion and wall cellular biosynthesis. Defects in the *rth3* gene have been found to affect hair root elongation (Hochholdinger et al, 2008). Likewise, the product of the *rth3* gene has been highly associated with grain yield under conditions of high N content, and with seedling root traits under contrasting soil N (Abdel-Ghani et al, 2015). Extension of root hairs increase surface of root so enhances water and nutrients (as N) uptake. In current research, however, no genotypic effect was detected for PN_{UPTAKE}, and this trait had no link with NUE. Consequently, no inference can be made about genotypic differences in root development and root architecture for the evaluated germplasm (Hirel et al, 2007).

About the glutamine synthetase4 (*gln4*) and the glutamate-oxaloacetate transaminase 2 (*got2*) genes identified on the QTL-3, both are involved in N flow between organic compounds. The *gln4* gene plays a role in ammonium assimilation from glutamate (glutamine biosynthesis), whereas the *got2* gene is involved in N transfer from glutamate to aspartate. Finally, within the QTL-6 interval the asparagine synthetase 1 (*asn1*) gene and two possible amino acid transporters (amino acid permease 1 and amino acid transporter-like protein) were identified. The *asn1* gene is involved in asparagine synthesis from glutamine, it is essential to N assimilation, distribution and remobilization in plants through phloem pathway (Chevalier et al, 1996). Amino acid transporters could play an important role in distributions of organic N and therefore would regulate the fate of this resource into the plant.

Conclusions

One the most important result of our study was the consistent positive effects for BHI, NHI, PGY and NUE observed in some joint QTLs, which could allow an efficient simultaneous selection for these traits. Therefore these QTLs seem to be the most interesting for breeding purpose. Special focus should be given to the QTL-1 and QTL-6 due to their positive additive effects, the stability across the contrasting environments explored, and because of there are genes that could be relevant for NUE and yield within these QTL intervals.

The detection of significant associations between traits related to NUE and molecular markers will allow marker assisted selection for NUE. The advantage of this approach is that presence of these markers can be used as selection criterion in maize breeding in early development stage, increasing parental control, and consequently greater selection responses.

Occasionally, evaluation and indirect selection of genotypes based on their molecular profile could be convenient when it is compared with evaluation and direct selection for physiological traits. These are often difficult to screen under field conditions when dealing with a large number of genotypes. Simulation studies showed that a distance of 5 cM between QTL and marker would be appropriated to marker assisted selection (Boopathi, 2013). However, considering that intervals of the detected QTLs are still quite large, fine mapping will be convenient to identify markers closely linked to these QTLs with focus on the desired genes. Likewise, increasing markers density in low coverage regions would be allow to detect additional QTL associated with NUE and related traits.

Candidate genes identification, despite extension of intervals, was important because it allowed us to select regions of interest within the intervals. Detection of new markers within or closely to these genes associated with NUE and related traits could be used for marker assisted selection in breeding programs.

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