GABRIEL BORGES MUNDIM

Genetic diversity, path analysis and association mapping for nitrogen use efficiency in popcorn

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Magister Scientiae*.

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APROVADA: 22 de fevereiro de 2013.

Prof. Roberto Fritsche Neto

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Prof. José Marcelo Soriano Viana
(Orientador)

Aos meus pais, Edilson e Maria Perpétua,
À minha irmã Luísa e aos meus avós,
Ao meu avô TiFio (in memoriam),
À minha namorada Vanessa,
Aos meus demais familiares e amigos.

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BIOGRAFIA

GABRIEL BORGES MUNDIM, filho de Edilson Borges da Silva e Maria Perpétua Mundim Borges, nasceu em 15 de novembro de 1987, em Patos de Minas, Minas Gerais.

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Em março de 2011, iniciou o curso de Mestrado em Genética e Melhoramento pela Universidade Federal de Viçosa, submetendo-se à defesa da dissertação em fevereiro de 2013.

SUMÁRIO

LIST OF MAIN ABBREVIATIONS AND SYMBOLS

NUE	Nitrogen use efficiency
NUpE	Nitrogen uptake efficiency
NUtE	Nitrogen utilization efficiency
SDW	Shoot dry weight
RDW	Root dry weight
TDW	Total plant dry weight
RSR	Root:shoot ratio
DG	Daily growth
RAD	Root average diameter
TRL	Total root length
RSA	Root surface area
RV	Root volume
V_6	Six fully expanded leaves
QTL	Quantitative trait locus
AM	Association mapping
SSR	Simple sequence repeat
PCA	Principal component analysis
df	Degrees of freedom
IL	Inbred line
CV (%)	Coefficient of variation
h^2	Broad-sense heritability

RESUMO

MUNDIM, Gabriel Borges, M. Sc., Universidade Federal de Viçosa, fevereiro de 2013. **Diversidade genética, análise de trilha e mapeamento associativo para eficiência no uso de nitrogênio em milho-pipoca.** Orientador: José Marcelo Soriano Viana. Coorientadores: Fabyano Fonseca e Silva e Glauco Vieira Miranda.

Os objetivos deste estudo foram (i) identificar linhagens de milho-pipoca eficientes no uso de nitrogênio; (ii) avaliar a diversidade genética entre linhagens de milho-pipoca em alto e baixo N; (iii) investigar os efeitos causais de vários caracteres sobre a eficiência no uso de nitrogênio (NUE) e (iv) identificar marcadores SSR associados com caracteres relacionados à NUE. Foram avaliadas 25 linhagens-elite de milho-pipoca pertencentes às populações 'Viçosa' e 'Beija-Flor', em alto e baixo N. Foram mensurados os seguintes caracteres: crescimento diário (DG, cm), massa de parte aérea (SDW, mg), de raiz (RDW, mg), e da planta total seca (TDW, mg), razão parte aérea:raiz seca (RSR), eficiência no uso (NUE, mg mg^{-1}), na absorção (NUpE, mg mg^{-1}) e na utilização (NUtE, mg mg^{-1}) de nitrogênio, diâmetro médio (RAD, mm), comprimento total (TRL, cm), área superficial (RSA, cm^2) e volume (RV, cm^3) de raízes. Foram identificadas linhagens eficientes em cada nível de N. A avaliação da diversidade genética pelo método de agrupamento UPGMA baseado no quadrado da Distância Euclidiana Média resultou em quatro grupos de linhagens para cada nível de N e a análise de componentes principais mostrou que as linhagens poderiam ser agrupadas predominantemente pelos seus caracteres de parte aérea. A eficiência na absorção de N (NUpE) foi a característica mais importante para a NUE em estádios precoces de desenvolvimento da planta em ambos os níveis de N, por apresentar alta correlação e alto efeito direto sobre a variável principal (NUE) na análise de trilha. Em baixo N, a eficiência na utilização de N (NUtE) também apresentou alta correlação e alto efeito direto sobre a variável NUE, mostrando ser uma característica importante para esta condição nesses estádios. Contudo, a seleção direta ainda parece ser o melhor método para aumentar a eficiência de seleção para NUE em estádios precoces. Três marcadores SSR foram validados como associados com os caracteres relacionados à NUE pela análise de mapeamento associativo baseada em ANOVA.

ABSTRACT

MUNDIM, Gabriel Borges, M. Sc., Universidade Federal de Viçosa, February, 2013. **Genetic diversity, path analysis and association mapping for nitrogen use efficiency in popcorn.** Adviser: José Marcelo Soriano Viana. Co-advisers: Fabyano Fonseca e Silva and Glauco Vieira Miranda.

The objectives of this study were to (i) identify efficient inbred lines in nitrogen use; (ii) assess the genetic diversity among popcorn inbred lines under high and low N; (iii) investigate the causal effects of several traits in nitrogen use efficiency (NUE) and (iv) identify SSR markers associated with the traits related to NUE. Twenty-five elite popcorn inbred lines belonging to the 'Viçosa' and 'Beija-Flor' populations were evaluated under high and low N. The following traits were assessed: daily growth (DG, cm), shoot dry weight (SDW, mg), root dry weight (RDW, mg), total plant dry weight (TDW, mg), root:shoot ratio (RSR), nitrogen use efficiency (NUE, mg mg^{-1}), nitrogen uptake efficiency (NUpE, mg mg^{-1}), nitrogen utilization efficiency (NUtE, mg mg^{-1}), root average diameter (RAD, mm), total root length (TRL, cm), root surface area (RSA, cm^2) and root volume (RV, cm^3). Efficient inbred lines were identified under each N level. The genetic diversity assessment using the UPGMA method based on the squared Mean Euclidean distance grouped the inbred lines into four clusters for each N level and the principal component analysis revealed that the inbred lines could be categorized predominantly by their shoot traits. Nitrogen uptake efficiency (NUpE) was the most important trait for NUE in the early stages of plant development under both N levels, due its high correlation with and high direct effect on NUE obtained in the path analysis. Under low N, nitrogen utilization efficiency (NUtE) also showed high correlation with and direct effect on NUE, demonstrating its importance in this N level in these early stages. Notwithstanding, the direct selection still seems to be the best method to increase the selection efficiency for NUE in these early stages. Furthermore, three SSR markers were identified as true associations with the traits related to NUE, through the association mapping analysis based on ANOVA.

1. Introduction

Nitrogen use efficiency (NUE) can be defined as the ratio between grain yield per unit of nitrogen applied to plants and is a function of nitrogen uptake efficiency (NUpE) and nitrogen utilization efficiency (NUtE). Nitrogen uptake efficiency is defined as the ratio between the quantities of nitrogen absorbed and applied, while NUtE corresponds to the ability of a particular genotype to convert the absorbed nitrogen into biomass or grain yield or the ratio between grain yield and nitrogen absorbed (Moll et al. 1982). In studies conducted during the early stages of plant development, NUE is usually estimated based on shoot dry weight (SDW) instead of grain yield. According to Hirel et al. (2007), increased NUE can be obtained by increasing any of its components. Increased NUpE can be achieved through modifying root architecture and morphological traits (Garnett et al. 2009; Liu et al. 2009), while increased NUtE results from improving enzymatic and metabolic processes (Hirel et al. 2001).

Recently, many studies aiming to identify markers related to NUE and nitrogen stress tolerance have been performed in different species (Schnaithmann & Pillen 2013; Wei et al. 2012; Liu et al. 2008). The most important methods used for this purpose are QTL analysis and, more recently, the association mapping (AM), which is described by Liu et al. (2011) as a powerful approach for the dissection of the genetic architecture of quantitative traits. Chen et al. (2011) reported that AM can be useful for marker-assisted selection by enhancing previously known information about quantitative trait loci (QTLs). To investigate responses to abiotic stress, Cai et al. (2012a,b) evaluated a set of 218 maize recombinant inbred lines (RILs) under low nitrogen and low phosphorus supply and identified several QTLs for traits as leaf area, chlorophyll content, plant height, ear height and grain yield. They reported that these QTLs identify chromosomal regions as targets for genetic improvement of low nitrogen and low phosphorus tolerance and may be useful for improving lodging resistance in intensive maize cropping systems with high fertilizer inputs. Given the importance of the root system for nutrient uptake, some studies have also tried to detect QTLs controlling root traits to understand the genetic basis of these traits and their contribution to grain yield (Cai et al. 2012c; Ku et al. 2012).

Information about the genetic diversity of the germplasm available is essential to maize breeders to develop better breeding strategies in any breeding program. Makumbi et al. (2011) evaluated the combining ability, heterosis and genetic diversity of 15 tropical maize inbred lines under drought stress, low N stress and well-watered conditions, aiming to identify breeding strategies for stress and non-stress environments. They assessed the genetic diversity using RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism) and Simple Sequence Repeat (SSR) markers and their results revealed clustering of most lines in accordance with known pedigree and origin, which is consistent with other studies in tropical maize (Betrán et al. 2003; Warburton et al. 2002). In the study of Kumar et al. (2012), the clustering of lines based on root morphology was not consistent with the genetic background and origin of maize lines. Studies of genetic diversity with popcorn germplasm have also been performed. Franzoni et al. (2012) evaluated changes in genetic variability and genetic structure during eight cycles of recurrent selection in the population CMS-43 using SSR markers and concluded that there was no tendency in changes in the allele frequencies, probably, because of the major proportion of the variation in the SSR loci not to be related to selection for grain yield and popping expansion.

Comparisons among maize genotypes under normal and stressed nitrogen conditions allow breeders to develop breeding strategies for each specific condition. Studies try to evaluate the genetic effects involved in NUE, the selection efficiency in each condition and the relationship among traits associated with NUE. DoVale et al. (2012) detected high magnitude correlations between NUpE and NUE and between these efficiencies and shoot dry weight (SDW) under high and low N conditions and identified the additive genetic effects as the most important for the traits associated with NUE. However, these high correlation values indicate no perfect cause and effect relationship among the traits. Badu-Apraku et al. (2012) evaluated ninety extra early maize inbred lines under low N to confirm the reliability of ears per plant and anthesis-silking interval (ASI) for selecting for low N tolerance. Aiming to partition the correlations among several traits into causal and residual effects, Wu et al. (2011) evaluated maize inbred lines under normal and low N conditions and performed the path analysis to investigate the direct and indirect effects of each trait on low-N agronomic efficiency (LNAE).

Considering the foregoing, the objectives of this study were to (i) identify efficient inbred lines in nitrogen use; (ii) assess the genetic diversity among popcorn inbred lines under high and low N; (iii) investigate the causal effects of several traits in NUE and (iv) identify SSR markers associated with the traits related to NUE.

2. Materials and methods

2.1. Plant materials

A total of 25 popcorn inbred lines (S_6) belonging to the popcorn breeding program of the Federal University of Viçosa (UFV), Minas Gerais, Brazil, were used in this study (Table 1). The inbred lines were chosen among the elite inbred lines of the program, which were selected for improving the popping volume, one of the most important traits for popcorn quality. The 25 inbred lines represent a sample of the two populations used as germplasm in the program; with 15 belonging to the 'Viçosa' population and 10 to the 'Beija-Flor' population, which are the base populations of the program. As there is not a definition of heterotic groups of popcorn, the populations cannot be stated as representatives of different heterotic groups. Both populations have similar cycle, vegetative development, shape of grain (pearl), quality and grain yield (Viana et al. 2011).

2.2. Experimental procedures

The contrasting N levels were defined in a preliminary experiment, where two popcorn inbred lines were evaluated under eight N levels (196, 224, 252 and 280 mg L^{-1} - equivalent to the high N levels; and 19.6, 22.4, 25.2 and 28.0 mg L^{-1} - equivalent to the low N levels) with two replications. The results indicated that the contrasting N levels should be 224 and 22.4 mg L^{-1}, for high and low N conditions, respectively, due to a 49% of reduction on SDW of the inbred lines grown under low N when compared with the plants grown under high N.

The experiment was conducted in March 2012 at a greenhouse in the UFV (20º 50' S, 42° 48' W). The seeds were pre-germinated in trays with separate germination cells containing a sand-vermiculite mixture. After emergence, the seedlings were transplanted to 9 dm^3 cylindrical plastic pots (diameter 150 mm and height 50 cm) containing a mixture of 50% sand (0.25-0.5 mm diameter) washed with deionized water for five consecutive days and 50% fine vermiculite (minimum capacity to retain water (m/m) = 60%; cationic exchange capacity = 200 $cmol_c/dm^3$; maximum humidity (m/m) = 25%), as recommended by Walk et al. (2006). Each plot had a single plant in a pot. Plants were harvested in the vegetative stage V_6 (six fully expanded leaves), approximately 30 days after sowing. The plants were cut off at the soil surface and the roots were separated from the sand-vermiculite mixture gently after soaking them in water. The remaining mixture adhering to the roots was then washed away. Then, the shoot was dried in a forced-air oven at 60°C for 72h and the root system was placed in a solution of 70% alcohol for posterior image analysis. The experiment was conducted in a factorial arrangement (25 inbred lines x 2 N levels) in the randomized block design with four replications, resulting in 200 pots.

The nutrient solutions were supplied every two days from the seventh day after transplantation. The solutions contained (in mmol L^{-1}): 2.0 $Ca(NO_3)_2$, 0.75 K_2SO_4, 0.65 $MgSO_4$, 0.1 KCl, 0.25 KH_2PO_4, 1 x 10^{-3} H_3BO_3, 1 x 10^{-3} $MnSO_4$, 1 x 10^{-4} $CuSO_4$, 1 x 10^{-3} $ZnSO_4$, 5 x 10^{-6} $(NH_4)_6Mo_7O_{24}$, and 0.1 Fe-EDTA, at two nitrogen levels (low N - LN; and high N - HN). The LN solution contained 10 times less N (0.2 mmol L^{-1} $Ca(NO_3)_2$) than the HN solution. The low Ca concentration was compensated by the addition of $CaCl_2$ in the LN solution, as described by Chun et al. (2005).

2.3. Trait measurements

The root system was divided into primary, seminal and crown roots, according to Hochholdinger & Tuberosa (2009). Samples of the seminal and crown roots were obtained from each plot. Then, the root samples were floated in a transparent plastic tray (15 x 20 cm) and scanned using the WinRHIZO Pro 2009 software (Regent Instruments, Quebec, Canada) coupled to an EPSON Perfection V700/V750 scanner equipped with additional light (transparency unit) at a resolution of 400 dpi. The following traits were assessed: root average diameter (RAD, mm), total root length (TRL, cm), root surface area (RSA, cm^2) and root volume (RV, cm^3). Then, the total root system was dried in a forced-air oven at 60°C for 72h to obtain the root dry weight (RDW, mg).

At harvest time, the plant height was measured until the insertion node of the last fully expanded leaf. To calculate the daily growth (DG, cm), the plant height was divided by the number of days that each inbred line took to reach the V_6 stage. After drying the shoot and the root system, the shoot dry weight (SDW, mg), the total plant dry weight (TDW, mg) and root:shoot ratio (RSR) were calculated. Furthermore, the genotypes were evaluated for nitrogen use efficiency (NUE = shoot dry weight/N applied, mg mg^{-1}) and its two components using the following concepts (Hirel et al. 2007): nitrogen uptake efficiency (NUpE) = N in plant/N applied (mg mg^{-1}) and nitrogen utilization efficiency (NUtE) = shoot dry weight/N in plant (mg mg^{-1}). The content of N in plant was obtained through samples of 0.2 g from SDW for each plot, following a protocol proposed by Bremner & Mulvaney (1982).

2.4. DNA finger printing

The inbred lines were genotyped for 90 SSR loci chosen from the MaizeGDB database. These markers are related to QTLs for traits associated with abiotic stress (Chen et al. 2009; Chen et al. 2008), popping ability (Babu et al. 2006) and grain yield components (Messmer et al. 2009). Genomic DNA was extracted from young leaf tissue using hexadecyltrimethylammonium bromide (CTAB) following a modified protocol described by Doyle & Doyle (1990). Polymerase chain reactions (PCRs) were performed in a thermal cycler PTC-200 (MJ Research, Watertown, MA) using a touchdown profile and the products were analyzed by gel electrophoresis in 0.8% (w/v) agarose gels. Each PCR mixture contained 1x buffer, 1 mM $MgCl_2$, 0.5 U Taq polymerase, 0.1 µM of each primer, 0.1 µM of each deoxynucleotide (dATP, dTTP, dGTP, and dCTP) and 30 ng of template DNA diluted with water to a total reaction volume of 20 µl. Electrophoresis was conducted in 1x TBE buffer (pH 8.0) at 50 W for 2-3 h in a Sequi-Gen GT (Bio-Rad) apparatus. Bands were visualized using silver nitrate staining.

2.5. Statistical analysis

The analysis of variance under each N level, the joint analysis and the estimation of genetic parameters for all the traits were performed using the restricted maximum likelihood/best linear unbiased prediction (REML/BLUP) method (Patterson & Thompson 1971; Henderson 1974). In matrix terms, assuming the inbred lines as a random factor, and blocks and N levels as fixed effects, the models are

$y = X\beta + Z_1u_1 + \varepsilon$, for the individual analysis and

$y = X\beta + Z_1u_1 + Z_2u_2 + \varepsilon$, for the joint analysis

where y is the vector of phenotypic values; X is the incidence matrix of the fixed effects; β is the vector of fixed effects; Z_1 and Z_2 are the incidence matrices of the random effects; u_1 is the vector of genotypic values of the inbred lines, where $u_1 \sim N(0, G_1)$, being $G_1 = A\sigma_g^2$; u_2 is the vector of genotype x N levels interaction, where $u_2 \sim N(0, G_2)$, being $G_2 = I\sigma_{ge}^2$ and ε is the residuals vector, where $\varepsilon \sim N(0, R)$, being $R = I\sigma_e^2$.

In this study, the coefficient of coancestry (A) was not considered and, consequently, the matrix G_1 was assumed as an identity matrix. Thus, σ_g^2 corresponds to the genetic variance among the inbred lines and the vector $\tilde{u}_1$ corresponds to the predictions of the genotypic values of the inbred lines. Based on the predicted genotypic values, the genotypic correlation matrix among the traits studied was estimated using the Pearson's coefficient. The probability values of the significance tests were obtained from the standardized normal distribution (Littell et al. 2006). The analyses were performed using the MIXED procedure of the SAS System v.9.2 (SAS Institute 2007). All other analyses were performed for each N level.

The phenotypic dataset was analyzed using the NTSYSpc v.2.2 software (Rohlf 2009) to assess the genetic diversity among the inbred lines. The data were standardized to minimize problems relative to units of each measure. Then, the squared Mean Euclidean distance was calculated. The distance matrix was used to perform a principal component analysis (PCA) to identify the major traits accounting for most of the variation among the studied inbred lines. The inbred lines were also grouped using the Unweighted Pair-Group Method using Arithmetic Averages (UPGMA). Furthermore, the genotypic correlation matrix was used to perform a path analysis in order to partition the genotypic correlations into causal and residual effects, and the causal effects into direct and indirect effects. This analysis was performed using the procedures CORR and IML of the SAS System v.9.2 (SAS Institute 2007).

The association mapping (AM) analysis was performed using the single-locus F-test procedure in the PowerMarker software (Liu & Muse 2005). In this test, each marker is regarded as a factor in a one-way ANOVA layout, as each genotype stands for a different level, and an F-test is then performed for each marker. The set of inbred lines was subdivided into two subsets, each one belonging to its origin population. Ten markers described in chromosome 5 and close to the gene "nnr2 - *nitrate reductase2*" (Long et al. 1992) were considered. The analysis was performed for the following traits: SDW, RDW, NUpE, NUtE, NUE and TRL. A p-value < 0.05 was considered to identify the markers associated with each trait.

3. Results and discussion

3.1. Inbred lines performance

At harvest time of the experiment, the plants grown under high N were better nourished than the plants grown under low N. These latter presented the lower leaves yellow, a typical symptom of N deficiency. The root system was composed of the embryonic primary root; from one to four seminal roots depending on the genotype; the first postembryonic crown roots and lateral roots.

The genotypes presented differential behavior for most traits under the two contrasting N levels, which can be evidenced by the significant effect of the G x N interaction (Table 2). These results demonstrate the differential expression of genes under each N level and the need for evaluation under each environment separately. For the traits DG, NUpE and RAD, the selection could be realized in any N level or when considering the means of the inbred lines across the two N levels, as evidenced by the absence of significance for the G x N interaction (Table 2). Rare studies (Gardner et al. 1990; DoVale et al. 2012) concluded that there was no G x N interaction. Clearly, these results depend on the set of genotypes studied and it is mainly evidenced when hybrids are evaluated.

Another way to show G x N interaction is to consider the correlation between the two N levels $\left(r_{g(HN,LN)}\right)$. These correlations also allow us to infer about the nature of the G x N interaction. The correlation among the inbred lines under high and low N levels ranged from 0.04 to 0.97 and indicates the simple G x N interaction for most traits, except for NUtE (Table 2). Bertin & Gallais (2000) and Presterl et al. (2003) found an average correlation coefficient of 0.75. According to Robertson (1959), the simple G x N interaction is provided only by the differences in variability among genotypes in the two environments and it is not a problem for the selection of the superior genotypes.

Regardless of the N level, the analyses showed significant genetic variability among the inbred lines for most traits (Tables 3 and 4), except for RAD under low N (Table 4) and for NUtE under high N (Table 3). These results demonstrate the possibility of obtaining genetic gains in the selection of efficient inbred lines in nitrogen use. Many studies have shown a genetic variability for NUE or at least one of its two components under a given level of N (DoVale et al. 2012; Presterl et al. 2002; Worku et al. 2007; Bertin & Gallais 2000). Regarding the NUtE, the genetic variability among the inbred lines under high N may have been suppressed over time due to the advance of selfing generations and selection of superior inbred lines was always conducted under optimal conditions of N supply. Moreover, the NUtE tends to be less pronounced in the early stages of plant development (Caixeta 2012).

The coefficients of variation (CVs) ranged from 9.3 to 36.0% under high N (Table 3) and from 10.9 to 43.0% under low N (Table 4). The CVs were high for several traits; however, they are in the range observed in other studies of abiotic stresses (DoVale et al. 2012; Maia et al. 2011; Souza et al. 2008) and indicate satisfactory experimental precision and high reliability of the results, since the CVs need to be classified according to each trait evaluated (Fritsche-Neto et al. 2012). Regarding the broad-sense heritabilities, the estimates ranged from 0.25 to 0.83 under high N (Table 3) and from 0.22 to 0.79 under low N (Table 4). The estimates of heritability were generally lower under low N and in accordance with other studies (Abdel-Ghani et al. 2013; Wu et al. 2011). According to Coque & Gallais (2006), the genetic variability and the heritabilities are reduced under conditions of low N due to an increase in environmental error and because the genotypes tend to present a similar performance, which hampers the selection.

The SDW of the plants grown under low N (Table 4) decreased 14.6% compared with the plants grown under high N (Table 3). The RDW remained almost constant across the two N levels (Tables 3 and 4), while the TRL of the plants grown under low N (Table 4) increased 15.5% compared with the TRL of the plants grown under high N (Table 3). These results suggest a better allocation of resources in the root growth under N deficiency, as reported by other authors (Liu et al. 2008; Chun et al. 2005).

To achieve gains in NUE, breeders should select those genotypes that are more efficient in terms of nitrogen uptake and utilization (Hirel et al. 2001). The predicted genotypic values of the inbred lines for NUpE and NUtE under high and low N are shown in the Figures 1 and 2, respectively. Through this representation, it is possible to identify the desired genotypes regarding the two components of nitrogen use efficiency. Some inbred lines had predicted genotypic values very contrasting according to the N level, such as the IL 05-383, IL 05-391-1 and 03-583-2, as well as shown in the evaluation of maize inbred lines under contrasting N and P levels performed by DoVale et al. (2013). Under high N, the inbred line 03-658-7 was the most efficient in nitrogen use due to its highest genotypic value for NUpE (Figure 1). Under low N, the IL 05-402-1 was the most efficient due to its high genotypic values for NUpE and NUtE (Figure 2). This inbred line also had the second highest genotypic value for NUpE under high N, revealing its genetic potential for NUE under the two N levels.

3.2. Genetic diversity

The results obtained with the principal component analysis (PCA) are shown in Table 5. The scores of the inbred lines under high (Figure 3) and low N (Figure 4) were plotted in a plane defined by the two principal components. Under high N, the first two principal components explained about 85.8% of the total variation among the inbred lines (Table 5). The relative magnitude of eigenvectors for the first component was 64.6%, explained mainly by NUE, SDW and NUpE, as can be seen through the correlation coefficients higher than 0.90 among these traits and the first component (Table 5). From the second principal component, which contributed with 21.2% of the total variation, the most predominant trait was DG. Under low N, the first component explained 73.2% of the total variation with contributions from NUE, SDW and RDW. Once again, DG was the highest contributor for the second component, which explained 15.0% of the total variation. The popcorn inbred lines could be categorized predominantly by their shoot traits, such as NUE and SDW under the two N levels. Only one trait of the root system had significant contribution for the PCA. The RDW was responsible for a small portion of the total phenotypic variation under low N.

Before assessing the genetic diversity among the inbred lines, an analysis to compute the relative contribution of each trait to the diversity was performed using a procedure based on canonical variables. According to this evaluation, the TDW and RSA traits were eliminated, as they were considered to be less variant among genotypes and therefore redundant. Then, the squared Mean Euclidean distance matrix was computed based on the 10 remaining traits to perform the UPGMA cluster analysis. For each N level, a dendrogram was created (Figures 5 and 6), in which the presence of four clusters of inbred lines can be noticed. Just as in the principal component analysis, the efficient inbred lines were grouped closely, as were the inefficient lines. Sharma et al. (2010) also found four major clusters through phenotypic and molecular characterization of a set of 48 selected maize landrace accessions. The majority of the populations from the same geographical origin were grouped together, due its similarity for the nine morphoagronomic traits evaluated. On the other hand, the maize inbred lines used in the study of Kumar et al. (2012) did not cluster according to their genetic background and origin, indicating that a high level of variability for root architecture traits is still present among and within the different genetic backgrounds.

3.3. Path analysis

The path analysis involved the effects of NUpE, NUtE, SDW and RDW on NUE (Table 6) under each one of the two N levels. These traits are the main explanatory variables of NUE, regardless of the N level, which can be confirmed by comparing the determination coefficients of the diagrams ($R^2 = 0.99$, for both N levels) with the residual effects (0.10 and 0.09), which represents the effect of the other variables not included in the diagrams. The shoot and root dry weight had high correlations with NUE under both N levels, ranging from 0.77 to 0.94 (Table 6). Similar results were found in the study of DoVale et al. (2012), where the correlations between SDW and the root traits with NUE ranged from 0.83 to 1.00. These results are in accordance with those obtained by Pereira (2011), which identified the SDW as the essential trait for early and indirect selection of maize genotypes with high NUE. However, it is possible that there is no clear evidence of cause and effect relationship between SDW and RDW with NUE, due its low direct effects, which ranged from -0.02 to 0.19 (Table 6). Thus, only these two traits may not be adequate to perform indirect selection of superior inbred lines for NUE.

The highest contributions of the shoot and root dry weight for NUE happened by the indirect effects through NUpE under both N levels (from 0.39 to 0.69). These results suggest that NUpE has to be considered in the selection for NUE, regardless of the N level. Moreover, NUpE had the highest correlations (0.95 and 0.89) and direct effects (0.82 and 0.60) on NUE under both N levels (Table 6). Popcorn plants uptake more nutrients than using them in the early stages of development, as well as maize plants. Thus, the NUpE becomes the most important trait for NUE in these early stages of development. However, in the course of plant development, the NUtE component has its importance increased in detriment of the NUpE (Caixeta 2012). DoVale et al. (2012) also demonstrated that the genetic differences for NUE in the early stages of plant development were due to NUpE, regardless of the N level. The NUtE also had a high correlation with and a direct effect on NUE (0.84 and 0.50, respectively) under low N (Table 6). Gallais & Hirel (2004) reported that the genetic variability for NUE occurs due to differences in NUpE at high N and is a function of NUtE under low N, as well as described by Gallais & Coque (2005). From the physiological point of view, it is interesting to note that the nitrogen utilization is less pronounced under high N than under low N level. As the nitrogen from soil is not limiting under high N, it can be absorbed at any time and in greater quantities. Then, the need for remobilization of the nitrogen absorbed is less pronounced. In contrast, as the nitrogen availability is limiting under low N, the processes of uptake and utilization have to be complementary.

The use of physiological traits to help in the selection will be efficient only if the traits used for indirect selection have the following qualities: high heritability, greater than the primary trait (NUE); high genetic correlation with the primary trait (NUE); easy to measure on a great number of genotypes; measurable in young stage and highly correlated with grain yield in the end of plant development (Gallais 1983). The only trait that presented all these qualities was the SDW. However, the direct effects of SDW on NUE were reduced in the two N levels (Table 6). Thereby, the results from the path analysis and the heritabilities estimated suggest that the direct selection in each specific condition of N availability still seems to be the best method to increase the selection efficiency for NUE.

3.4. Association mapping

Considering the subset of inbred lines belonging to the 'Viçosa' population, only one marker (umc1110, bin 5.03) was identified as associated with SDW under high N and with SDW, RDW, TRL and NUtE under low N (Table 7). When considering the inbred lines belonging to the 'Beija-Flor' population, three different markers (phi085, umc1153 and umc1447) were identified as associated with SDW, RDW, TRL and NUE under low N and two markers (bnlg161 and umc1792) were identified as associated with SDW, NUtE and NUE under high N. Among these markers associated with these traits related to NUE, the loci phi085, umc1792 and umc1153 can be considered as true associations with the gene "nnr2 - *nitrate reductase2*" (bin 5.07, 211.2 cM), responsible for the regulation of maize root nitrate reductase mRNA levels, as described by Long et al. (1992). The reason why only these associations were validated is that these markers are located very close to the gene, with a correspondent distance less than 6.0 cM (Table 7). These markers explained from 57.6 to 80.8% of the total variation in the traits associated with NUE in the early stages of plant development when considered the inbred lines belonging to the 'Beija-Flor' population (Table 7).

Under normal field conditions, nitrate (NO_3^-) is the most common form of nitrogen available to plants. Nitrate taken up by the plant may be reduced in the roots, stored in the vacuoles or transferred to the shoots before being processed. The first enzyme involved in the reduction of nitrate within the plant is the nitrate reductase (Forde 2000; Forde & Clarkson 1999; Miller & Smith 1996). Therefore, the detection of markers very close to the gene "nnr2 - *nitrate reductase2*" (Long et al. 1992) can help maize and popcorn breeders in the identification of superior genotypes in nitrogen use through marker-assisted selection.

4. Conclusions

Twenty-five popcorn inbred lines were evaluated under high and low N. Efficient inbred lines in nitrogen use were identified under each N level. The genetic diversity assessment grouped the inbred lines into four clusters for each N level and the principal component analysis revealed that the inbred lines could be categorized predominantly by their shoot traits. Nitrogen uptake efficiency (NUpE) was the most important trait for nitrogen use efficiency (NUE) in the early stages of plant development under both N levels. Notwithstanding, the direct selection still seems to be the best method to increase the selection efficiency for NUE in these early stages. Furthermore, three SSR markers were identified as true associations with the traits related to NUE.

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Table 1 List of the 25 popcorn inbred lines with their origin populations.

Inbred Lines	Origin population
IL 05-351-8	Viçosa
IL 05-356-6	Viçosa
IL 05-381-1	Viçosa
IL 05-383	Viçosa
IL 05-391-1	Viçosa
IL 05-470-2	Viçosa
IL 05-284-7	Viçosa
IL 05-394-2	Viçosa
IL 05-389-4	Viçosa
IL 03-583-2	Beija-Flor
IL 05-274-3	Viçosa
IL 03-658-7	Beija-Flor
IL 03-657-4	Beija-Flor
IL 03-687-1	Beija-Flor
IL 05-343-2	Viçosa
IL 03-591-7	Beija-Flor
IL 05-402-1	Viçosa
IL 03-610-7	Beija-Flor
IL 03-655-3	Beija-Flor
IL 03-566-5	Beija-Flor
IL 03-677-5	Beija-Flor
IL 03-689-4	Beija-Flor
IL 05-345-1	Viçosa
IL 05-388	Viçosa
IL 05-425-1	Viçosa

Table 2 Genetic parameters for traits associated with nitrogen use efficiency evaluated in 25 popcorn inbred lines under contrasting N levels.

Estimates	DG (cm)	SDW (mg)	RDW (mg)	TDW (mg)	RSR	NUE (mg mg^{-1})
$\hat{\sigma}^2_g$	0.0040 ***	45488.8 **	11287.7 **	96882.2 **	0.0830 **	1.8072 ^{ns}
$\hat{\sigma}^2_{ge}$	0.0001 ^{ns}	23232.6 **	7198.3 **	52386.8 **	0.0569 *	5.6230 **
$\hat{\sigma}^2_e$	0.0041	70476.6	24593.0	161495.4	0.2506	11.5122
$r_{g(HN,LN)}$	0.97	0.66	0.61	0.65	0.59	0.24
$\hat{h}^2_g$	0.87	0.69	0.63	0.68	0.58	0.30
Mean	0.49	864.0	467.6	1331.6	1.96	9.46
CV (%)	13.3	30.7	33.5	30.2	25.5	35.9
Estimates	NUpE (mg mg^{-1})	NUtE (mg mg^{-1})	RAD (mm)	TRL (cm)	RSA (cm²)	RV (cm³)
$\hat{\sigma}^2_g$	0.0011 **	0.5608 ^{ns}	0.0001 *	228003.1 *	1638.9 ^{ns}	0.0782 ^{ns}
$\hat{\sigma}^2_{ga}$	0.0002 ^{ns}	11.9291 **	0.0001 ^{ns}	301002.6 *	3676.0 **	0.2787 **
$\hat{\sigma}^2_e$	0.0051	52.7211	0.0011	1221879.4	11403.6	0.8051
$r_{g(HN,LN)}$	0.83	0.04	0.63	0.43	0.31	0.22
$\hat{h}^2_g$	0.59	0.04	0.43	0.43	0.33	0.25
Mean	0.22	37.15	0.326	2899.4	292.63	2.38
CV (%)	32.0	19.5	10.1	38.1	36.5	37.7

DG, daily growth; SDW, shoot dry weight; RDW, root dry weight; TDW, total plant dry weight; RSR, root: shoot ratio; NUE, nitrogen use efficiency; NUpE, nitrogen uptake efficiency; NUtE, nitrogen utilization efficiency; RAD, root average diameter; TRL, total root length; RSA, root surface area; RV, root volume.

^{ns} non-significant; *, ** and *** significant at 0.10, 0.05 and 0.01 probability, respectively.

Table 3 Genetic parameters for traits associated with nitrogen use efficiency evaluated in 25 popcorn inbred lines under high N.

Estimates	DG (cm)	SDW (mg)	RDW (mg)	TDW (mg)	RSR	NUE (mg mg^{-1})
$\hat{\sigma}^2_g$	0.0057 **	86123.5 **	16749.8 **	167134.3 **	0.2222 **	0.4587 **
$\hat{\sigma}^2_e$	0.0046	87103.2	21944.6	177726.7	0.2178	0.4902
$\hat{h}^2_g$	0.83	0.80	0.75	0.79	0.80	0.79
Mean	0.53	931.9	448.9	1380.8	2.18	2.24
CV (%)	12.8	31.7	33.0	30.5	21.4	31.3
Estimates	NUpE (mg mg^{-1})	NUtE (mg mg^{-1})	RAD (mm)	TRL (cm)	RSA (cm²)	RV (cm³)
$\hat{\sigma}^2_g$	0.0006 **	2.2933 ^{ns}	0.0003 *	433842.6 **	3943.0 *	0.2488 *
$\hat{\sigma}^2_e$	0.0007	28.0512	0.0010	659597.3	8453.4	0.7796
$\hat{h}^2_g$	0.78	0.25	0.57	0.73	0.65	0.56
Mean	0.08	29.18	0.341	2690.8	286.75	2.45
CV (%)	33.3	18.2	9.3	30.2	32.1	36.0

DG, daily growth; SDW, shoot dry weight; RDW, root dry weight; TDW, total plant dry weight; RSR, root: shoot ratio; NUE, nitrogen use efficiency; NUpE, nitrogen uptake efficiency; NUtE, nitrogen utilization efficiency; RAD, root average diameter; TRL, total root length; RSA, root surface area; RV, root volume.

^{ns} non-significant; *, ** significant at 0.05 and 0.01 probability, respectively.

Table 4 Genetic parameters for traits associated with nitrogen use efficiency evaluated in 25 popcorn inbred lines under low N.

Estimates	DG (cm)	SDW (mg)	RDW (mg)	TDW (mg)	RSR	NUE (mg mg^{-1})
$\hat{\sigma}^2_g$	0.0025^{***}	51323.4^{***}	20223.4^{***}	131412.1^{***}	0.0575^{*}	14.3995^{***}
$\hat{\sigma}^2_e$	0.0037	53846.3	27240.2	145256.3	0.2835	22.5347
$\hat{h}^2_g$	0.73	0.79	0.75	0.78	0.45	0.72
Mean	0.44	796.1	486.4	1282.5	1.75	16.68
CV (%)	13.8	29.2	33.9	29.7	30.5	28.5

Estimates	NUpE (mg mg^{-1})	NUtE (mg mg^{-1})	RAD (mm)	TRL (cm)	RSA (cm^2)	RV (cm^3)
$\hat{\sigma}^2_g$	0.0020^{*}	22.6284^{**}	0.0001^{ns}	624144.7^{**}	6685.4^{**}	0.4646^{***}
$\hat{\sigma}^2_e$	0.0095	77.4056	0.0011	1784141.0	14354.0	0.8306
$\hat{h}^2_g$	0.45	0.54	0.22	0.58	0.65	0.69
Mean	0.37	45.13	0.310	3107.9	298.51	2.31
CV (%)	26.5	19.5	10.9	43.0	40.1	39.5

DG, daily growth; SDW, shoot dry weight; RDW, root dry weight; TDW, total plant dry weight; RSR, root: shoot ratio; NUE, nitrogen use efficiency; NUpE, nitrogen uptake efficiency; NUtE, nitrogen utilization efficiency; RAD, root average diameter; TRL, total root length; RSA, root surface area; RV, root volume.

^{ns} non-significant; *,** and *** significant at 0.10, 0.05 and 0.01 probability, respectively.

Table 5 Correlation coefficients among traits related to NUE and the first two principal components (PC) in popcorn inbred lines under high and low N conditions.

	High N		Low N	
Trait	PC1	PC2	PC1	PC2
Daily growth (DG)	0.58	0.63	0.43	0.87
Shoot dry weight (SDW)	0.94	-0.20	0.95	-0.18
Root dry weight (RDW)	0.87	-0.32	0.93	-0.26
Nitrogen uptake efficiency (NUpE)	0.93	0.28	0.81	0.22
Nitrogen utilization efficiency (NUtE)	-0.10	-0.88	0.89	0.02
Nitrogen use efficiency (NUE)	0.97	0.02	0.97	0.16
Total root length (TRL)	0.86	-0.31	0.88	-0.35
Percentage of total variance	64.6	21.2	73.2	15.0
Cumulative % of total variance	85.8		88.2	

Table 6 Estimates of direct and indirect effects obtained from the path analysis involving the dependent variable nitrogen use efficiency (NUE, mg mg^{-1}) and the independent variables nitrogen uptake efficiency (NUpE, mg mg^{-1}), nitrogen utilization efficiency (NUtE, mg mg^{-1}), shoot dry weight (SDW, mg) and root dry weight (RDW, mg) evaluated in 25 popcorn inbred lines under each N level.

Trait	Association effect	Estimates	
		High N	Low N
NUpE	Direct in NUE	0.82	0.60
	Indirect through NUtE	-0.06	0.26
	Indirect through SDW	0.16	0.04
	Indirect through RDW	0.03	-0.01
	Total	0.95	0.89
NUtE	Direct in NUE	0.18	0.50
	Indirect through NUpE	-0.26	0.31
	Indirect through SDW	0.02	0.05
	Indirect through RDW	0.01	-0.01
	Total	-0.05	0.84
SDW	Direct in NUE	0.19	0.06
	Indirect through NUpE	0.69	0.44
	Indirect through NUtE	0.02	0.42
	Indirect through RDW	0.04	-0.01
	Total	0.94	0.90
RDW	Direct in NUE	0.04	-0.02
	Indirect through NUpE	0.55	0.39
	Indirect through NUtE	0.02	0.41
	Indirect through SDW	0.16	0.05
	Total	0.77	0.83
Determination coefficient (R^2)		0.99	0.99
Residual effect		0.10	0.09

Table 7 Markers associated (*p-value < 0.05)* with traits related to NUE evaluated in popcorn inbred lines from two origin populations under contrasting N levels.

Origin population	Trait	Low N					High N				
		Marker	*p-value*	bin [a]	Position [a] (cM)	R^2 (%)	Marker	*p-value*	bin	Position (cM)	R^2 (%)
'Viçosa'	SDW	umc1110	0.0012	5.03	82.5	86.1	umc1110	0.0164	5.03	82.5	74.2
	RDW	umc1110	0.0069	5.03	82.5	79.1					
	TRL	umc1110	0.0253	5.03	82.5	71.3					
	NUtE	umc1110	0.0036	5.03	82.5	82.1					
'Beija-Flor'	SDW	phi085	0.0113	5.06	205.2	77.6	bnlg161	0.0495	5.05	171.5	70.0
		umc1153	0.0059	5.09	211.8	76.9					
		umc1447	0.0336	5.03	33.8	67.7					
	RDW	phi085	0.0131	5.06	205.2	76.5					
		umc1153	0.0109	5.09	211.8	72.5					
	TRL	phi085	0.0471	5.06	205.2	63.9					
		umc1153	0.0495	5.09	211.8	57.6					
	NUtE						umc1792	0.0368	5.08	211.6	80.8
	NUE	umc1153	0.0395	5.09	211.8	60.3	bnlg161	0.0250	5.05	171.5	77.1

SDW, shoot dry weight; RDW, root dry weight; TRL, total root length; NUtE, nitrogen utilization efficiency; NUE, nitrogen use efficiency

[a] Chromosome bins of the marker and position taken from MaizeGDB database.

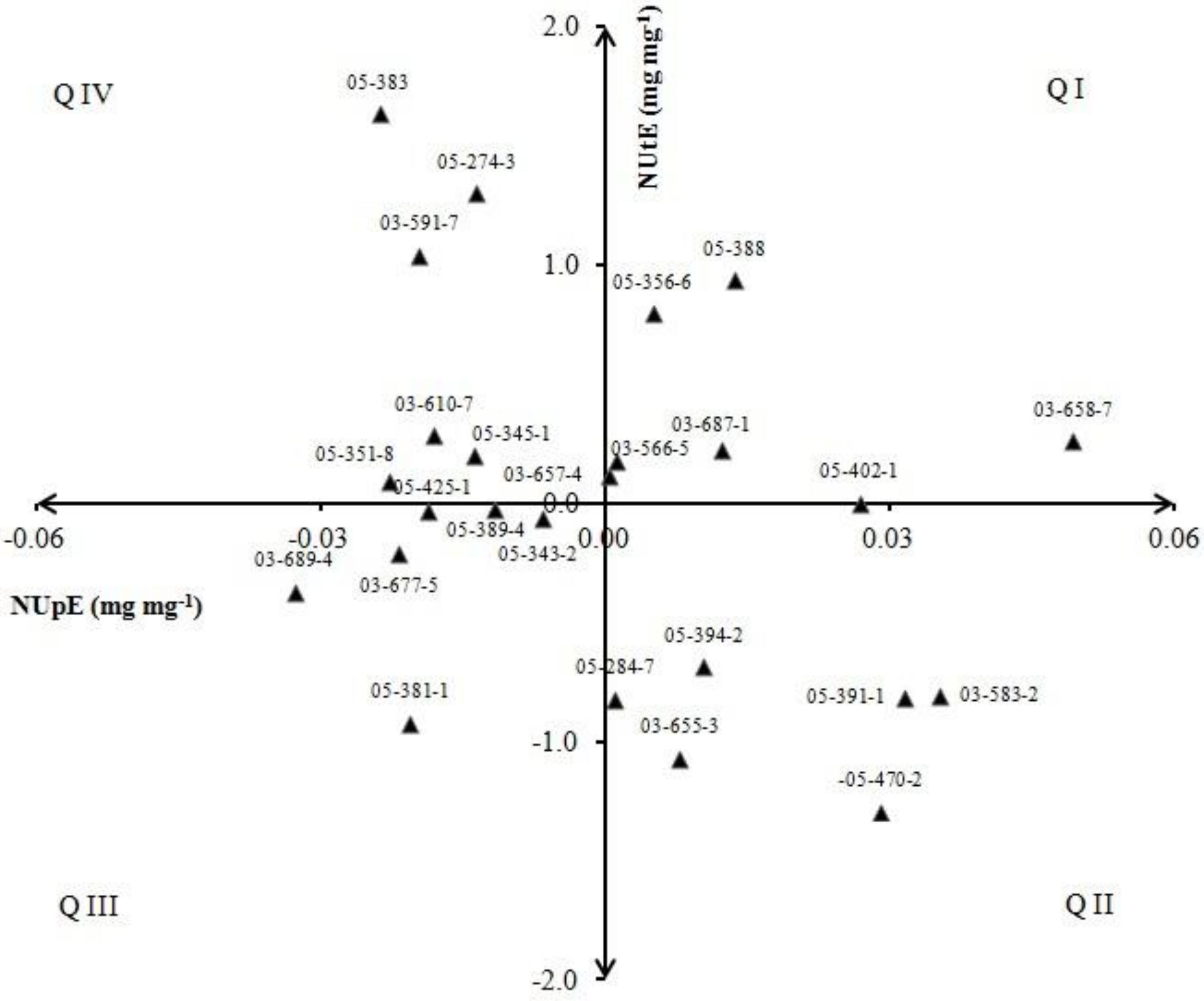

Figure 1 Genotypic values for nitrogen uptake efficiency (NUpE) and nitrogen utilization efficiency (NUtE) obtained from 25 popcorn inbred lines under high N.

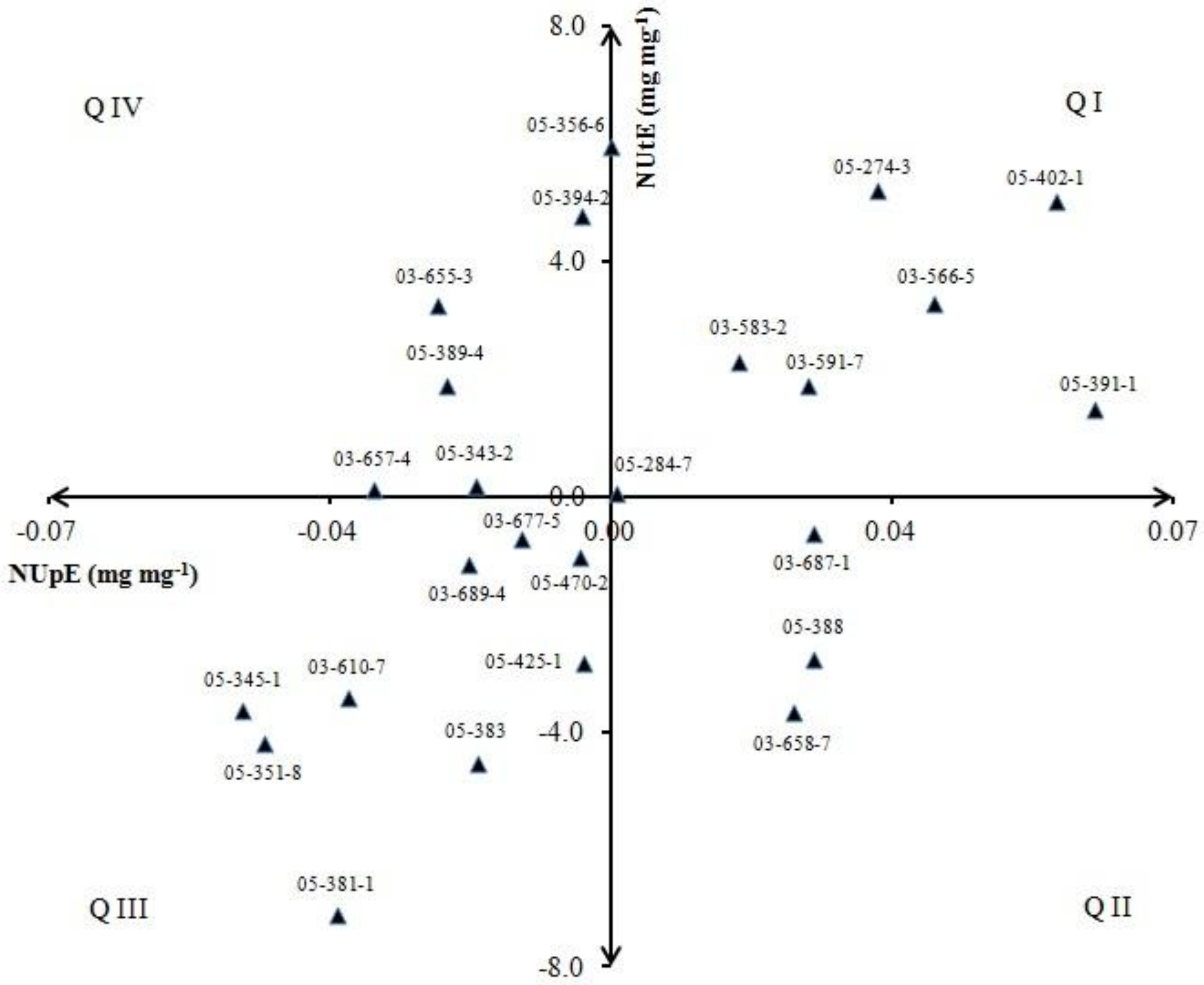

Figure 2 Genotypic values for nitrogen uptake efficiency (NUpE) and nitrogen utilization efficiency (NUtE) obtained from 25 popcorn inbred lines under low N.

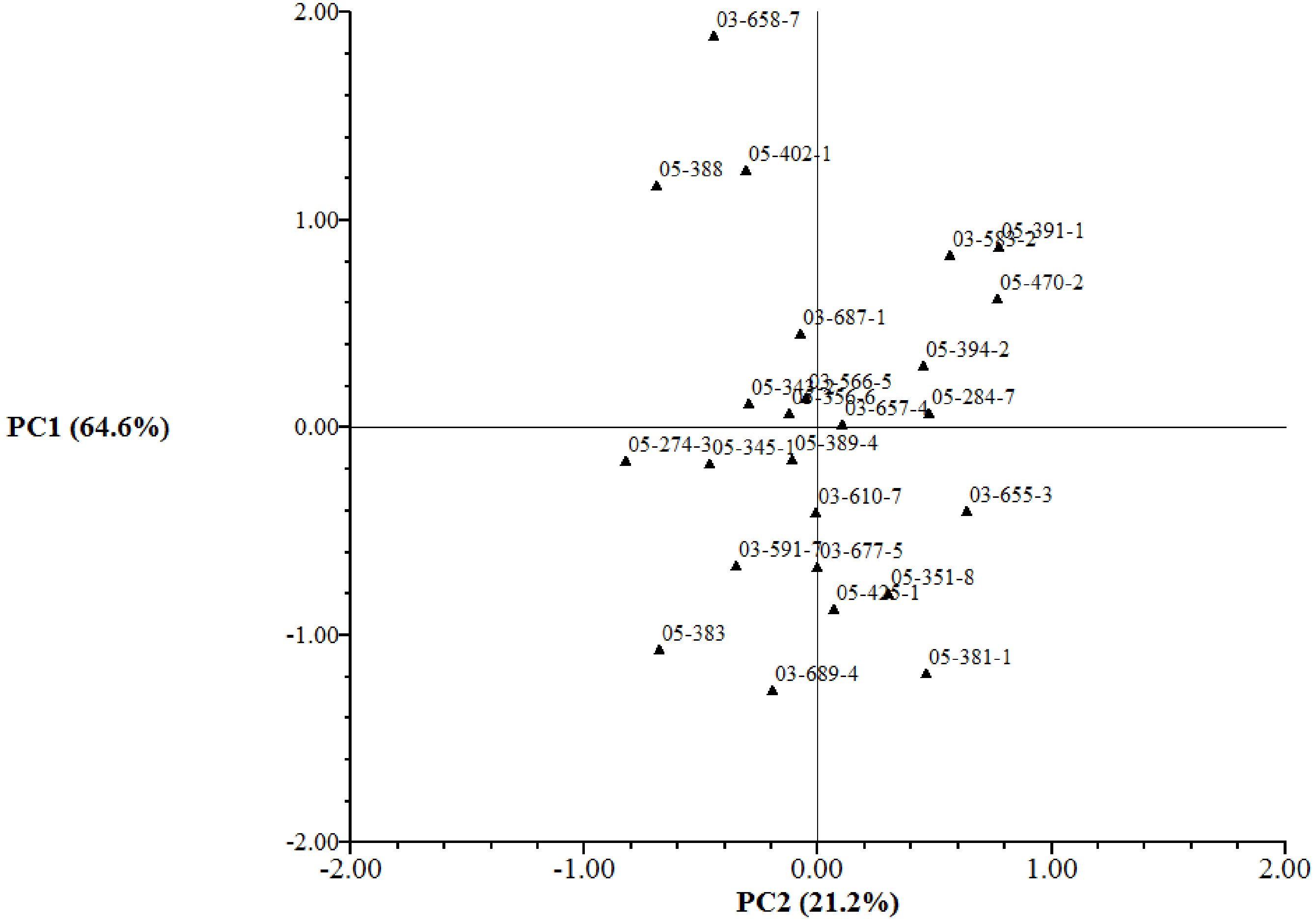

Figure 3 Principal component analysis of traits related to NUE evaluated in 25 popcorn inbred lines under high N.

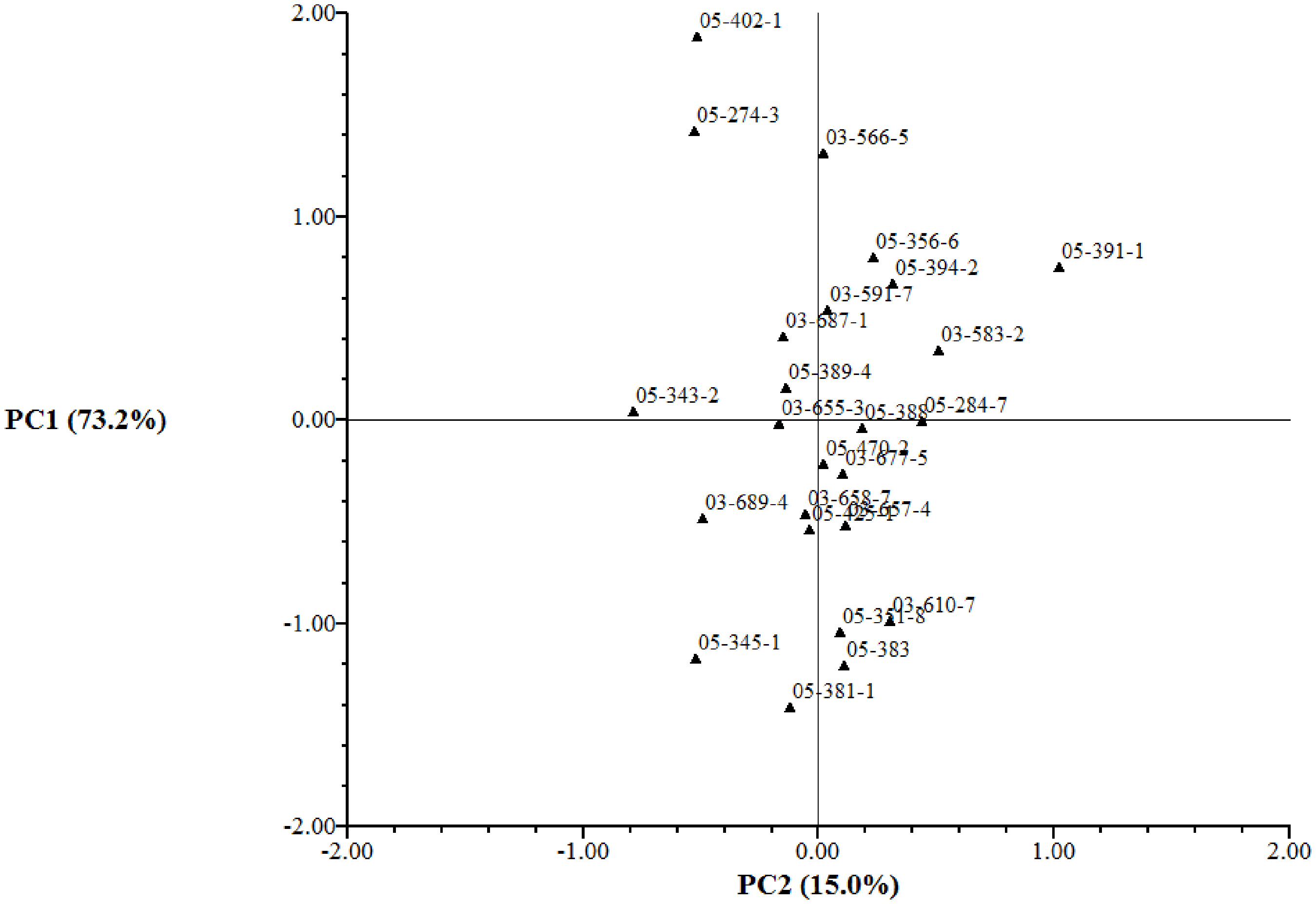

Figure 4 Principal component analysis of traits related to NUE evaluated in 25 popcorn inbred lines under low N.

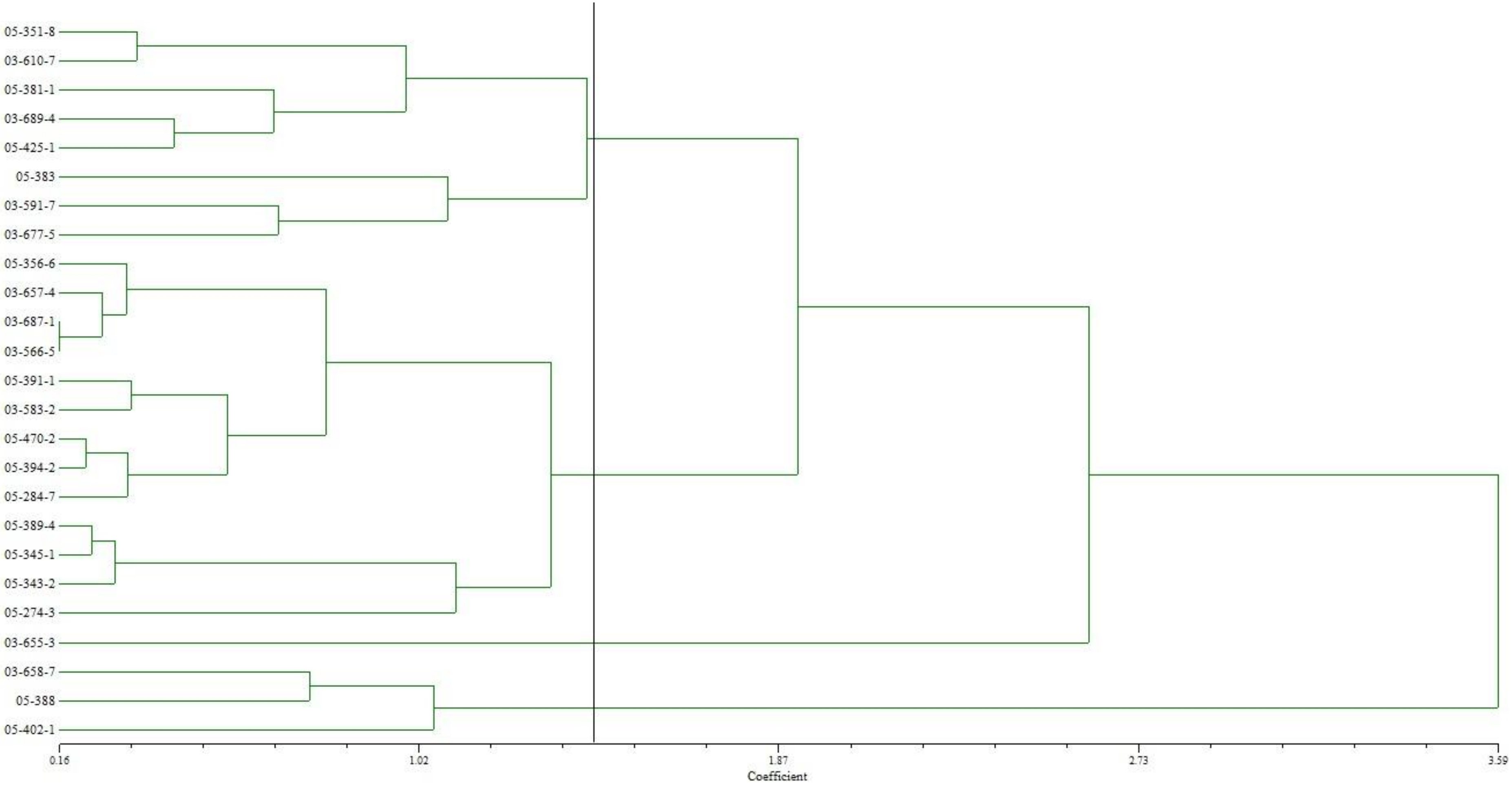

Figure 5 Dendrogram of 25 popcorn inbred lines evaluated under high N.

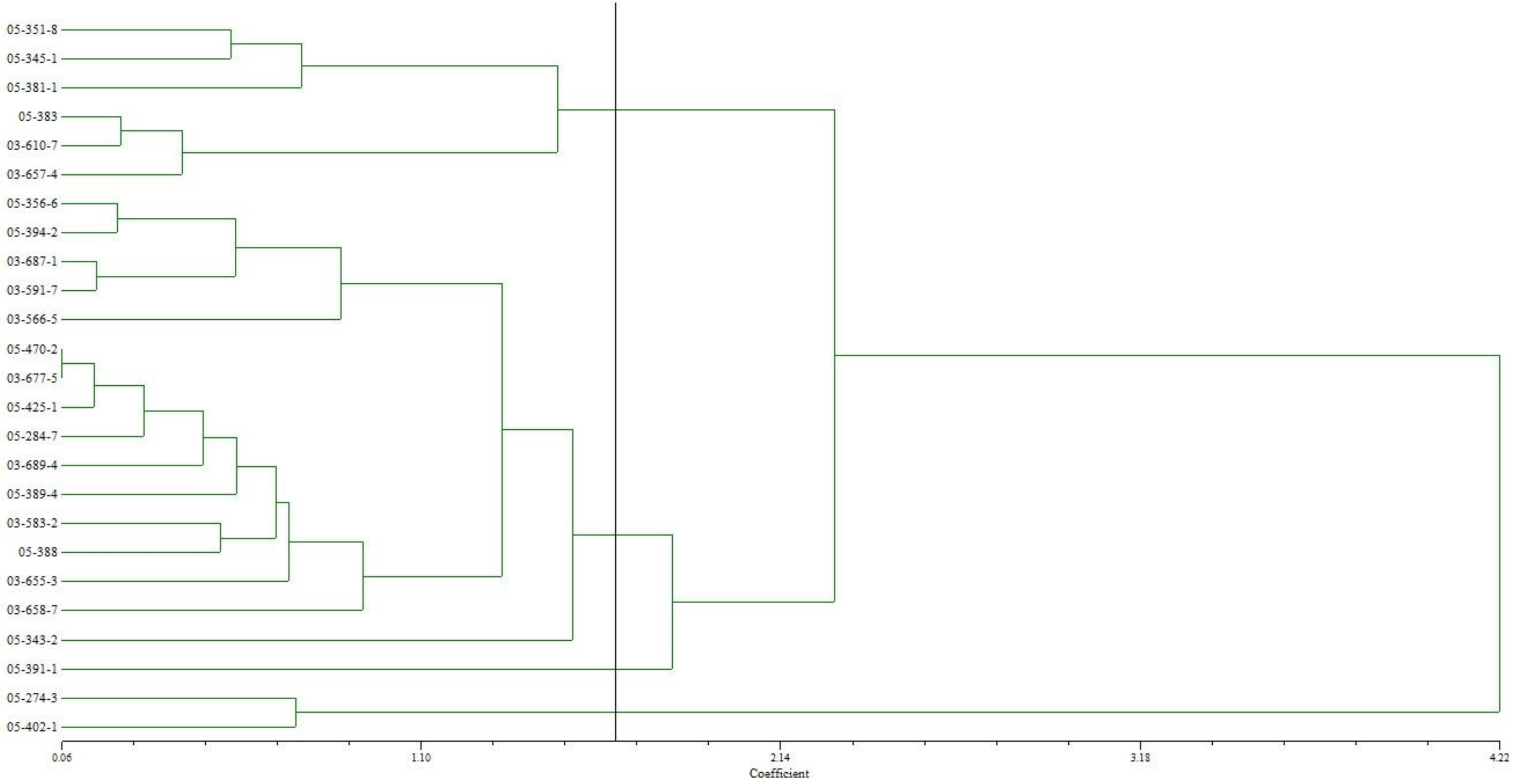

Figure 6 Dendrogram of 25 popcorn inbred lines evaluated under low N.