

## Natural selection for efficiency in the use of nitrogen in common bean identified by analysis of microsatellites and grain yield

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**Abstract** This study was carried out in order to verify whether natural selection acts on segregating common bean populations grown in the presence and absence of N, and to verify that natural selection affects the frequency of microsatellite alleles specifically, and in order to identify those that can assist in selection. Four populations from the crosses Ouro Negro x CI-107 and VC-5 x IAPAR-81 were carried forward in bulk to F<sub>8</sub> at both levels of N. Ouro Negro and VC-5 are considered stress tolerant to nitrogen and IAPAR-81 and CI-107 responsive. One hundred progenies were obtained from each cross and grown in the presence and absence of N. DNA was extracted from 400 progenies, of which 194 were evaluated in the rainy season and 79 in the winter crop for grain yield. These progenies were evaluated in two separate experiments with and without N, in a 14 x 14 lattice design during rainy season and 9 x 9 in the winter season. We selected 35 pairs of polymorphic primers from the parents, with 20 being common from the two crosses. Using DNA of the 400 progenies, it was found that natural selection acted in 33 loci, in at least one environment and cross, and that alleles of the four parents were favoured by natural selection. QTLs were identified for the response rate of N and for grain yield and the linked markers are potential for assisted selection, especially the two most stable; BMD-20, in response to the use of N and PVBR-93 for grain yield.

**Keywords:** adaptation, nitrogen use efficiency, *Phaseolus vulgaris*, SSR markers.

### INTRODUCTION

The common bean plant is very demanding in nitrogen, the nutrient found in abundance in cells and, therefore, of great importance for grain yield. However, most often, it is not available in sufficient quantities in the soils of tropical regions (Ferreira et al. 2004). This problem is due to the fact that the biological fixation of nitrogen is usually insufficient to meet the needs of the plant, even with the huge research effort aimed at improving the efficiency of symbiotic fixation of this nutrient. In order to be economically productive, nitrogen needs to be added.

The vast majority of family farms use virtually no nitrogen fertilizers, because it is a relatively expensive input. Thus, it is important to obtain cultivars whose use this nutrient more efficiently, that is, to develop lines that are productive even in environments with low availability of mineral nitrogen, but are also able to respond to nitrogen fertilization. However, it is difficult to evaluate and select such lines. An alternative to circumvent this problem is through natural selection as to carry forward segregating common bean populations in environments with and without nitrogen fertilization. The assessment of populations carried forward in these environments may allow identification of progenies that are less stringent to the employment of nitrogen fertilizers, as well as those more responsive to nitrogen fertilization, since natural selection tends to select genotypes adapted to each particular environment (Furtini et al. 2009).

The use of molecular markers, such as microsatellites, may contribute to selecting more adaptable alleles (Rodrigues and Santos, 2006; Leite et al. 2011) and some of these alleles may be related to the efficient use of nitrogen in poor soils in this nutrient and also they may contribute to the responsiveness to nitrogen fertilization. Thus, the identification of markers related to more efficient use of N can assist in the selection.

Most studies involving the use of molecular tools to identify genotypes efficient in the use of N were performed with grasses, mainly with corn crops (Hirel et al. 2001; Gallais and Hirel, 2004) and wheat (Quarrie et al. 2005; An et al. 2006; Le Gouis et al. 2008). In the case of common bean, researches in this area are still scarce.

Being this information limited for the bean crop, this study aimed to verify whether natural selection works on segregating common bean populations grown in the presence and absence of N and also to verify if natural selection affects the frequency of microsatellite alleles in a specific way which enables the identification of those alleles that can assist in the selection.

## MATERIALS AND METHODS

Segregating populations derived from the crosses VC-5 x IAPAR-81 and Ouro Negro x CI-107 were used. Three of the four lines used had carioca grain type and the line Ouro Negro had black grains. The lines CI-107 and IAPAR-81 are considered responsive and VC-5 and Ouro Negro tolerant to low nitrogen availability (Furtini et al. 2009). These populations were carried forward by the bulk method in two environments: the first was fertilized with 100 kg N/ha, with 1/3 applied at sowing and 2/3 in coverage and the second, in which nitrogen was not used. This procedure was repeated until the generation F<sub>8</sub> (Furtini et al. 2009). A sample of 2000 seeds was taken from each population and environment in F<sub>2</sub> until F<sub>7</sub>. In F<sub>8</sub> generation progenies were obtained and evaluated in the field for grain yield during rainy season and winter crop.

For this study, 200 progenies descended for each crossing were used, with 100 from the population carried forward in an environment with nitrogen and 100 in nitrogen-free environment. All of these progenies were used with microsatellite markers to assess the effect of natural selection. We performed DNA extraction of 400 progenies following the procedures used by Rodrigues and Santos (2006).

Polymorphic chain reactions (PCR) were carried out with 240 pairs of microsatellite markers whose sequences are available at Wright (2007).

Genotyping of progenies was performed using 35 loci that were polymorphic in the parents, being 20 polymorphic loci in parents of both crosses, twelve polymorphic only for the parents of the cross Ouro Negro x CI-107 and three polymorphic only for the parents of the cross VC-5 x IAPAR-81. For each microsatellite locus the genotypic proportions were compared with the proportions expected by the X<sup>2</sup> test, assuming the absence of selection (Rodrigues and Santos, 2006). It was represented by "A<sup>1</sup>" in each loci, the SSR allele from responsive parent, CI-107 and IAPAR-81 and "A<sup>2</sup>" the allele of the parents stress tolerant to N, Ouro Negro or VC-5.

Considering that the natural crossing of beans in the region is approximately T = 0.005 (Marques Junior and Ramalho, 1995), and the corresponding rate of self-fertilization is S = 1-T = 0.995, the genotypic frequencies were estimated for each locus in generation F<sub>8</sub> (n + 1) (Allard, 1999; Rodrigues and Santos, 2006):

$$\begin{aligned} \text{Freq} (A^1A^1) &= f_1^{(n+1)} = S[f_1^{(n)} + 0,25f_2^{(n)}] + T[f_1^{(n)} + 0,5f_2^{(n)}]^2 \\ \text{Freq} (A^1A^2) &= f_2^{(n+1)} = S[0,5f_2^{(n)}] + 2T[f_1^{(n)} + 0,5f_2^{(n)}][f_3^{(n)} + 0,5f_2^{(n)}] \\ \text{Freq} (A^2A^2) &= f_3^{(n+1)} = S[f_3^{(n)} + 0,25f_2^{(n)}] + T[f_3^{(n)} + 0,5f_2^{(n)}]^2 \end{aligned}$$

Given the relative accumulated adaptability coefficients of genotype  $A^1A^1$  as  $\omega_1$ , of genotype  $A^2A^2$  as  $\omega_3$  and of genotype  $A^1A^2$  as  $\omega_2=1$ , the accumulated relative adaptability coefficients of  $F_2$  to  $F_8$  were estimated by means of the expressions (Allard and Hansche, 1964):

$$\omega_1 = \frac{O_1 [(0,5SH + 2T(P + 0,5H))(R + 0,5H)]}{O_2 [S(P + 0,25H) + T(P + 0,5H)^2]}$$

$$\omega_3 = \frac{O_3 [(0,5SH + 2T(P + 0,5H))(R + 0,5H)]}{O_2 [S(R + 0,25H) + T(R + 0,5H)^2]}$$

In which: P and  $O_1$  are the  $A^1A^1$  proportions in the n and n + 1 generations, respectively; H and  $O_2$  are the  $A^1A^2$  proportions in the n and n + 1 generations; R and  $O_3$  are the  $A^2A^2$  proportions in n and n + 1 generations.

The genotypic proportions in generation n are expected to be same as those in  $F_7$  generation if there is no natural selection, considering the rates of 0.005 of crossing and 0.995 self-fertilization.

From the 400 progenies, we selected the most productive and the Carioca grains type from the 2008/2009 rainy season. In which 96 progenies carried forward from an N-free environment and 96 from the environment with N. From the winter crop of 2009 we evaluated 79 progenies, 40 were carried forward from the environment without N and 39 with N which presented the highest and the lowest rates of response to N ( $\alpha$ ). In these two seasons, the progenies were evaluated through the grain yield in environments with and without N. There was used the 14 x 14 triple lattice design in the rainy season, where the four parents were used as controls and 9 x 9 triple lattice design in the winter crop where only the tolerant parents, VC-5 and Ouro Negro were used as controls.

From the average grain yield data, it was estimated the response rate to nitrogen ( $\alpha$ ) through the expression used by Furtini et al. (2009), that is:  $\alpha_i = (N_{1i} - N_{2i})/Q$ , in which  $\alpha_i$ : response rate of nitrogen of the progeny i;  $N_{1i}$ : grain yield of progeny i in the environment with nitrogen application;  $N_{2i}$ : grain yield of progeny i in the environment without nitrogen application. Q: The quantity of nitrogen applied (100 kg/ha).

Variance analyses were performed for the response rate of N in both seasons and for grain yield, for each level of N in each season, and after a joint analysis involving all seasons.

The QTLs were identified by multiple linear regression analysis using the genotyping data, the average grain yield and the values of response rate ( $\alpha$ ) in each season in each environment, and also in the joint analysis considering all seasons.

The multiple linear regression analysis was performed considering the 192 progenies evaluated during the rainy season and 79 assessed in the winter. Assessments were also made considering the origins, using the 96 progenies originated in the absence of N and 96 with N in the rainy season and 40 progenies of origin without N and 39 with N from the winter season. In the joint analysis we only considered the common progenies in both seasons.

Multiple linear regression analyses were performed using SAS version 9.0, using the backward selecting model.

## RESULTS AND DISCUSSION

Among the 240 SSR primers utilized, 35 had polymorphism between the parents. In the Ouro Negro x CI-107 cross the parents were polymorphic in 32 SSR loci and for the IAPAR-81 x VC-5 cross parents

were polymorphic in 23 loci. Out of the 35 SSR primers, 20 were polymorphic in the four parents (Table 1 and Table 2).

Fifteen of the polymorphic primers were mapped on eight different chromosomes (Wright and Kelly, 2011). It should be emphasized that the primer BM210 showed two polymorphic loci according Blair et al. (2003). These loci are mapped on two different chromosomes, B7 and B11. For the other 20 primers, the position in the genome is unknown, or it has not been published yet. This indicates that natural selection has probably exerted its effect throughout the genome and some of these loci may be related to nitrogen use efficiency, since the environmental conditions imposed, particularly the stress of N, would have favoured such an occurrence.

Among the polymorphic markers from the both crosses, the PvM-21 primer stands out, developed by Hanai et al. (2007). For this locus, the progenies derived from the cross CI-107 x Ouro Negro, grown without N, were affected by natural selection (Figure 1).



**Fig. 1 Pattern of microsatellite bands amplified by PvM-21 primer.** From the left: first lane CI-107 parent; second lane Ouro Negro parent; third to the last lane show the progenies of origin without N from 1 to 31.

Considering the polymorphic loci between parents, since the progeny are descending from a cross, the genotype should be  $A^1A^2$  in the  $F_1$ , followed by Mendelian segregation in the  $F_2$  to  $F_8$ . According to Allard (1999), in the absence of selection, the genotypic proportions expected in  $F_8$ , are 0.4938 for  $A^1A^1$  and  $A^2A^2$ . For  $A^1A^2$ , this proportion is 0.0124. The expression considers the common bean rate of crossing  $T = 0.005$  and inbreeding  $S = 0.995$ . Therefore from the  $X^2$  test we can observe changes in genotypic frequencies for the populations obtained from the two crosses and the two levels of N. Natural selection has acted in 33 of the 35 loci evaluated in at least one environment and in at least one of the crosses. The segregating population Ouro Negro x CI-107, carried forward with N, 27 of the 32 polymorphic loci were affected by natural selection ( $P \leq 0.05$  or  $P \leq 0.01$ ). The same effect was observed in the population carried forward without N in 31 of the 32 polymorphic loci. In this population, the single primer in which the genotypes did not undergo diversion of segregation in the two populations was the BMD-10.

In segregating population IAPAR-81 x VC-5, grown with N, 18 of the 23 polymorphic loci were affected by natural selection ( $P \leq 0.05$  or  $P \leq 0.01$ ), while the population grown without N, 17 of the 23 loci had the same behaviour. Of these loci, four [PVBR 93, BM189, BM205b and PvESTBR\_233] presented changes only in the population with N and three [BMD-33, BM205a and PvESTBR\_72] in the population without N.

Because populations were carried forward in bulk, natural selection must act on all the loci that show significant  $X^2$ , as noted by Rodrigues and Santos (2006) and Leite et al. (2011). These progenies originated in environments with and without N, where selection pressure must have maintained in populations individuals that have alleles with higher adaptive value, especially for the conditions of N stress, and therefore more efficient in using this nutrient. Natural selection may have also acted in keeping individuals in the population with the greatest response to nitrogen fertilization.

Some of the loci that were influenced by natural selection in this study were also subjected to natural selection in the work of Rodrigues and Santos (2006), among them are BM143A, BM156, BM205a, BM210 and PV-atcc003. Others were also targets of natural selection in work carried by Leite et al. (2011), for example, BMD-15, BMD-20, BMD-33, PvM-13, PvM-21 and AZ301561.1. Such loci may be related to adaptive capacity. Among these loci, the BM156 was a QTL with a small effect for grain yield, identified by Pereira et al. (2007), Rodrigues et al. (2007) and Torga et al. (2010). Other markers were also related to some genes of interest such as the primer BM10 that is related to the gene encoding glutamine synthetase. The activity of this enzyme has been proposed as an auxiliary criterion

in the selection of cultivars efficient in nitrogen use, since it is considered a key enzyme in the assimilation of carbon (Hirel et al. 2001; Fontaine et al. 2009). For this locus there was deviation of segregation in the four populations.

Among the primers developed by Blair et al. (2003) that were polymorphic in the parents, the BMD-15 is part of a family of lectin or Phyto-hemagglutinin (PH) coding genes which are glycoproteins present in the cotyledons and endosperm of seeds. For this loci there was deviation of segregation in the two populations obtained from crosses Ouro Negro x CI-107. The BMd20 primer codifies for endokinase. The kinase proteins are related to metabolic processes in the plant cell and also act as regulators through mechanisms of post-translational phosphorylation and dephosphorylation of enzymes such as nitrate reductase (Machado et al. 2001). The genotypes of the four populations had also deviation of segregation for this locus.

The allele frequencies observed in populations of each cross, originating in environments with and without N represented by 100 F<sub>8</sub> progenies are shown in Table 1. As each segregating population is derived from both parents, Ouro Negro x CI-107 and VC-5 x IAPAR-81, each parent contributed with the same allele frequency. Thus, in the absence of natural selection, the frequency of each allele of the marker is expected to be 0.5 even after carried forward the population in bulk, subject to changes due to sampling which certainly were negligible, because populations carried forward in bulk had around 2000 seeds per generation.

It can be seen in Table 1 that natural selection favoured plants carrying alleles of the four parents. For the crossing CI-107 x Ouro Negro, considering the progenies of origin with N, allele frequencies remained practically unchanged with respect to that expected for eight loci. However, in 14 loci the natural selection favoured alleles of the parent CI-107, and for ten loci natural selection favoured alleles of the Ouro Negro parent. Considering the same crossing, for the progenies of origin without N, in six loci allele frequencies did not change, in 16 loci alleles from the parent CI-107 were favoured, and also ten alleles from Ouro Negro were favoured. Therefore, the alleles of the parent CI-107 prevailed in both environments, although this line is considered responsive and the Ouro Negro tolerant line. This result was not expected. It was expected a higher frequency of alleles from the parent Ouro Negro in the population carried forward under N stress, as this parent was considered tolerant.

Although CI-107 line was considered responsive in previous works, we cannot rule out the possibility that line alleles display tolerance to stress, which could increase their frequency in conditions of stress or even natural selection may have affected some alleles not related to the effectiveness of using N. Other relevant information is that during the experiment, we selected only the carioca grain type, and according to Gepts and Debouck (1993) there are more than 18 genes that are involved in coat colour. Since progenies from the CI-107 x Ouro Negro were selected for seed colour, this certainly skewed the results in favour of retaining CI-107 alleles as stated. This may have contributed in reducing the frequency of alleles of the parent Ouro Negro.

Considering the cross IAPAR-81 x VC-5 for the progenies that originated in an environment with N, seven loci showed virtually no change in allele frequencies relative to expected. However, for nine loci, we can see that natural selection favoured the alleles from the parent IAPAR-81 and for seven loci, natural selection favoured alleles from the parent VC-5. Considering the progenies that grown under stress of N, for ten loci the allele's frequency did not change with respect to what was expected and for four loci the allele's frequency from the parent IAPAR-81 was favoured by natural selection from the parent VC-5, natural selection favoured nine alleles.

For the environment with N, natural selection favoured a higher number of alleles from the responsive parent (IAPAR-81), which was expected, since in this environment those families may have inherited alleles of the parent that also allow positive response to nitrogen fertilization. A higher proportion of alleles of the parent VC-5, which is tolerant to N stress, were observed as expected. Natural selection must have acted to increase the proportion of alleles of stress tolerance in order to provide greater adaptation of genotypes to the environment without nitrogen fertilizer.

The relative accumulated adaptability coefficients were estimated for all homozygote genotypes for each primer pair selected in each crossing and each level of N studied. Considering the alleles A<sup>1</sup> from responsive parents CI-107 and IAPAR-81, and alleles A<sup>2</sup> from tolerant parents Ouro Negro and VC-5,

we estimated  $\omega_1$  for genotypes  $A^1A^1$  and  $\omega_3$  for parents  $A^2A^2$ . The coefficient of relative adaptability to heterozygote  $A^1A^2$ ,  $\omega_2$  was considered equal to 1.0 (Allard and Hänsche, 1964). Estimates of  $\omega_1$  and  $\omega_3$  equal to 1.0 indicate that there was no natural selection of genotypes, that is, they had the same reproductive efficiency as the heterozygote (Allard and Hänsche, 1964). For values of  $\omega_1$  and  $\omega_3$  lower than 1.0 natural selection acted by reducing the frequency of this genotype compared to heterozygote and for  $\omega_1$  and  $\omega_3$  values greater than 1.0 natural selection increased the frequency of homozygote in relation to the heterozygote, which in this case is the lowest adaptability (Hedrick, 2011).

The estimates of the relative accumulated adaptability coefficients ( $\omega_1$  and  $\omega_3$ ) for the cross IAPAR-81 x VC-5 ranged from 0.036 to 0.404 for  $\omega_1$  and 0.071 to 0.615 for  $\omega_3$ , considering the progenies originating from nitrogen-free environment. As for the same cross, the progenies originated with N showed values of  $\omega_1$  ranging from 0.020 to 1.049 and the values of  $\omega_3$  ranged from 0.048 to 1.466. Considering the estimates of the relative accumulated adaptability coefficients ( $\omega_1$  and  $\omega_3$ ) for the progenies from the cross CI-107 x Ouro Negro and originated in N free environment,  $\omega_1$  values ranged from 0.023 to 0.740 and for  $\omega_3$  the values ranged from 0.030 to 0.834. As for the same cross, the progenies originated in an environment with nitrogen had  $\omega_1$  values ranging from 0.045 to 1.845 and  $\omega_3$  values ranging from 0.017 to 1.34 (Table 2).

It is important to remember that these accumulated coefficients refer to the effect of natural selection on individuals with homozygote loci in the  $F_2$  through  $F_7$  generation, for six generations. The values of adaptability varied greatly from locus to locus, indicating that the intensity of selection for each locus was also particular. The average values of the relative accumulated adaptability coefficient for the environment with N were higher than those obtained in the N-free environment, regardless of the cross, which indicates that the action of natural selection was more intense in the environment without N (Table 2). We also observe that for environments with nitrogen, the adaptability values reached the upper limits and surpassed them, considering both  $\omega_1$  and  $\omega_3$ , with coefficients larger than 1 still appeared which means that for some loci, such as PV 93, PvESTBR\_6 and PvM-21 the adaptability of the homozygote was higher than the heterozygote.

The adaptability of the homozygote was unique for each locus. It was possible to observe genotypes with alleles from both responsive and tolerant parents with high values of the relative accumulated adaptability coefficients. Considering the progenies without N for the cross IAPAR-81 x VC-5, the averages of the coefficients were higher for tolerant genotypes similar to VC-5. However, for the cross CI-107 x Ouro Negro no significant difference among the averages of the coefficients was seen. Considering the progenies with N, there was a higher average for tolerant genotypes similar to VC-5 for the cross IAPAR-81 x VC-5, while for progenies of the cross CI-107 x Ouro Negro, from the same origin the average for responsive genotypes, similar to the parent CI-107 was superior. These results indicate that the intensity of natural selection varied from locus to locus and from cross to cross (Table 2).

These results also indicate that in an N-free environment natural selection has favoured some loci with higher stress tolerance to N. Whereas the environment with N favoured some loci more responsive to N. However, considering the average of the loci, the parent IAPAR-81 concentrates fewer N tolerant alleles than the VC-5 as expected, the Ouro Negro and CI-107 had similar proportions. In the environment with N, both populations exhibited similar proportions of loci responsive to N. Note also that in both environments the loci more tolerant and responsive identified in a population did not coincide with those of other population, this indicate the possibility of selection of superior recombinants from new crosses involving the four parents.

Due to the absence of heterozygote individuals, we could not estimate  $\omega_1$  and  $\omega_3$  for some primers in both crosses and N levels, since the relative accumulated adaptability coefficient is estimated in comparison to the heterozygote, whose coefficient ( $\omega_2$ ) is considered as one. Although no heterozygote was detected for some loci, the expected frequency of this genotype in the absence of natural selection is 0.0124; this frequency is due to the reproduction method of the common bean which is a typically autogamous species. Therefore, the frequency of loci where heterozygote was observed in a sample of 100 plants for each cross and at each level was very high, showing the adaptive superiority of the heterozygote. The maintenance of the heterozygote in the population by natural selection is a way to retain genetic variability (Allard, 1999). It can be inferred that the high number of heterozygote microsatellite loci in advanced generations of crosses, refers to genomic regions that contribute to better adaptation and, in this particular case, the adaptive alleles to N stress

and also responsive. As for treating heterozygosis, in principle, it can be related to genomic regions whose genetic dominance effects are important for adaptation.

Analyses of variance carried out showed that the effects of levels and seasons were highly significant. The difference in levels indicates that the experiment with application of N had higher grain yield than that without the application of N. On average, the experiment with nitrogen fertilization during the rainy season was 8% more productive than the experiment under nitrogen stress and 86% higher in the winter crop.

The progenies differed in grain yield ( $P \leq 0.01$ ) regardless of their origins, with or without N. There were also interaction progenies by levels of N ( $P \leq 0.01$ ) regardless of the origin of the progenies. This was expected given that progenies differ in terms of efficiency and response to nitrogen, which leads to different behaviour on both levels. These results show that it is possible to have a positive result through selection.

There was also variability for the rate of response to N among the progenies ( $h^2 = 30.2\%$ ) and also differences between seasons. Even so the common bean showed a positive response to nitrogen fertilization similar to that observed in other experiments (Vieira, 2006; Binotti et al. 2007; Furtini et al. 2009). However, comparison of the magnitude of response in the experiments is difficult, since the response to N fertilization depends on several environmental factors such as humidity, temperature and soil organic matter, as well as genotypic factors as the cultivars evaluated.

Response rates showed a range of variation from 15.27 with upper and lower limits of -0.5 and 14.77. The progeny with -0.5 index showed superior performance in the experiment without N. As for the progeny with a 14.77 index, it produced about 50% more in the experiment with N. Most progenies with high response rate, 57%, derived from environment with N, that is, they produced well in soil with nitrogen. And most of the progenies that produced well in the absence of N, 61%, derived from the environment without N. These results show the effect of natural selection as an auxiliary form of selection for efficiency in the use of N.

The variability of the progenies and their performance in two different levels permitted to identify DNA markers (SSR) that explained part of the phenotypic variation. Through multiple linear regressions using the "backward" method, QTLs were identified for each season for N response rate. We considered the 192 progenies evaluated in the field during the 2008/2009 rainy season and 79 progenies in the winter of 2009. In joint analysis, we considered the common progenies of the two crops (Table 3).

The determination coefficient of the main markers was lower in the rainy season ( $R^2 = 20.37\%$ ) than in the winter crop, when the progenies exhibited a much higher response to N application (Table 3). Among the markers, BMD-20 and PVBR-59 were stable in the two seasons. However, two others were important in specific crops, and this is expected due to the interaction progenies by seasons. The marker PvESTBR-233, belonging to the B01 linkage group (Wright and Kelly, 2011), can be specifically associated with increased responsiveness to N. These markers also showed higher frequency of the allele from responsive parents in both crosses and also a higher frequency and relative adaptability coefficients to genotypes from the same parents (Table 1, Table 2, Table 3 and Table 4). Therefore, these loci are potential in selection for response to nitrogen fertilization. These results also showed a tendency of natural selection to select genotypes with high response to fertilization. One must consider that these alleles were selected by natural selection in progenies from the same environment under N stress.

It should be noted that the BMD-20 marker, belonging to the B05 linkage group, was constructed from cDNA of a gene coding for an endokinase. Kinases act as regulators through mechanisms of post-translational phosphorylation and dephosphorylation of nitrate reductase activity and the activity of this enzyme has been proposed as an auxiliary criterion in the selection of cultivars efficient in the use of N (Machado et al. 2001). Therefore, this marker could assist in selection for efficiency in the use of N.

The multiple regression analysis for response rate were also performed to see whether the loci identified for all progenies regardless of their origin were also identified when only the progenies of origin without N were considered. Note that several markers explained the rate of response to with N emphasis on the BMD-20 that was identified again in both seasons and in the joint analysis (Table 4).

Another marker that deserves mention is the BM189, belonging to the B08 linkage group (Wright and Kelly, 2011). It was common in both seasons. In this locus, natural selection acted on keeping higher allele frequencies from responsive parent (CI-107 and IAPAR-81) (Table 3), therefore, became it another potential marker for response to fertilization.

The  $R^2$  values were high indicating that the progenies derived from populations carried forward in an environment without N were more affected by natural selection, including the SSR loci explaining high percentage of phenotypic variation. Remember, however, that small populations such as the winter crop may have overestimated the  $R^2$  values (Bernardo, 2008).

Considering the progenies derived from environments fertilized with nitrogen, two markers that were identified in the progenies of origin without N, specifically the BMD-33, belonging to the B11 linkage group (Wright and Kelly, 2011). That marker is important in both the winter and rainy season, has stood out (Table 5). At this locus, the alleles from the N stress tolerant parents (Ouro Negro and VC-5) were more frequent, the same way the genotype frequencies and the relative accumulated adaptability coefficients of genotypes similar to these parents. Thus, alleles from the tolerant parents may be associated with the response to nitrogen fertilization. In the winter season again highlights the marker PvESTBR233 probably directly associated with response to greater N level as already seen in Table 3.

The gain with selection assisted by molecular markers will be greater when the proportion of variation explained by the marker is greater than the heritability of the character (Bernardo, 2002). Given the heritability of response rate to N equal to 30.2%, note that coefficient values of determination were greater than the heritability in some cases as in the winter season, considering all the progenies and in the two seasons considering only the progenies derived from the environment without N. This indicates that assisted selection should be effective in these cases. Moreover, given the ease of selection via markers, a greater segregating population carried forward in an environment without N can be pre-selected and only the most promising progenies may be evaluated in the field.

Being that the main point of interest of common bean fertilized with N is grain yield, we also identified potential QTL markers of this trait in the presence and absence of N. Considering the 192 progenies in the rainy season and 79 in winter; we noted that the coefficients of determination ranged from 12.15% to 40.14% (Table 6). We observed common loci in both seasons, such as BM143A. This locus, belonging to the B02 linkage group (Wright and Kelly, 2011), was significant in experiments with nitrogen fertilization in the two seasons and in the joint analysis, but most of the markers were identified in only one season and this may be due to the QTL x environment interaction. Studies such as that of Fontaine et al. (2009), on efficiency of N use in wheat, showed that this interaction is common and of the eighteen QTLs identified for glutamate dehydrogenase enzyme activity by the authors, only one appeared in the three environments evaluated and three were identified in two environments, the other ten were identified in only one environment. It must be reminded that in multiple regression through the backward method, markers that are considered redundant are excluded from the final analysis.

It should be noted that if the objective is to obtain cultivars that produce well under conditions of N stress, markers of greater importance are those obtained for the same environment. It allows selecting more productive lines within this environment. However, it is also interesting to obtain more productive lines under nitrogen fertilization, and to identify QTLs for such an environment, as it would allow recommendations of cultivars for wider use as it has been usually performed.

Quarrie et al. (2005), found that, for grain yield, QTLs - detected under low N fertilization conditions - can be used to improve the stability of production by a combination of QTLs related to production that are expressed in environments with low N.

The progenies originated in an N-free environment, which were affected by natural selection, should be more adapted to this environment, and may have greater reproductive capacity than those that originated in an environment with nitrogen fertilization. Despite have being affected by natural selection, these progenies have different alleles for adaptation and reproductive capacity, therefore having sufficient variability to distinguish these progenies. The identification of QTLs for these progenies is interesting and allows us to infer whether the QTLs useful for such progenies are the same for all progenies regardless of origin.



Considering only the 96 progenies of origin without N in the rainy season and the 40 of the same origin in the winter, the  $R^2$  values can be noted ranging from 18.06 to 49.87 (Table 7). Most of the identified markers were not common in both seasons due to QTL x environment interaction as was observed considering all progenies regardless of origin by Fontaine et al. (2009). However, it can also see common markers in both seasons and nitrogen levels as with PvM21, which was also identified in the analysis of these progenies without N in the winter crop for response to nitrogen fertilization. This locus has undergone natural selection in the evaluation of progenies without N in both crosses, and may have been selected because of selection pressure due to the absence of N. The highlight was the increased frequency of the allele of the parent Ouro Negro that is tolerant and had a higher relative accumulated adaptability coefficient in this environment (Table 1 and Table 4). It can be observed that this locus was identified in both experiments, with and without N.

Knowing that obtaining more productive cultivars in growing conditions with nitrogen fertilization is also of interest to farmers, we identified QTLs for grain yield for progenies originated in growing conditions in which there is nitrogen, that is, without exposure to the stress of N. In multiple regression analysis, it can be noted that the marker BM205b was very stable for progenies with N, and explained part of the variation in both seasons and in the two N levels, with and without N, and therefore, it is a potential locus for selection of productive plants (Table 8). This locus, belonging to the B11 linkage group (Wright and Kelly, 2011), was also affected by natural selection and responsive parent alleles were favoured both in the absence or presence of nitrogen (Table 3). It is important to mention that this same marker was identified by Blair et al. (2006) as a QTL for weight of 100 seeds, and is thus directly related to production. Other loci also explained the phenotypic variation such as the BM143B, which appeared in the experiments with N for both seasons, and others were present in only one experiment, showing action of QTL x environment interaction.

## CONCLUDING REMARKS

1. Natural selection acts on several microsatellite loci that can be associated with specific genomic regions for more efficient use of N.
2. Several markers were identified as potential for assisted selection, aiming at the efficient use of N, especially the BMD-20 and PVBR-93 that were more stable.

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## Tables

**Table 1. Allele frequencies for the populations of two crosses, Ouro Negro x CI-107 and IAPAR -81 x VC -5, for the environment with and without.**

PRIMERS	With N				Without N			
	CI-107x Ouro Negro		IAPAR-81 x VC-5		CI-107x Ouro Negro		IAPAR-81 x VC-5	
	A <sup>1</sup>	A <sup>2</sup>	A <sup>1</sup>	A <sup>2</sup>	A <sup>1</sup>	A <sup>2</sup>	A <sup>1</sup>	A <sup>2</sup>
BM143A	0.55	0.45	0.21	0.79	0.59	0.41	0.27	0.73
BM210	0.82	0.18	0.74	0.26	0.73	0.27	0.85	0.15
BM184	0.3	0.7	0.18	0.82	0.35	0.65	0.14	0.86
BMd-33	0.23	0.77	0.48	0.52	0.33	0.67	0.38	0.62
BMd-20	0.67	0.33	0.86	0.14	0.86	0.14	0.74	0.26
BMd-15	0.38	0.62	----	----	0.24	0.76	----	----
PV-atcc003	----	----	0.51	0.49	----	----	0.53	0.47
PVBR 93F	0.48	0.52	0.84	0.16	0.58	0.42	0.56	0.44
PvESTBR_230	0.39	0.61	0.38	0.62	0.29	0.71	0.39	0.61
PvESTBR_6	0.42	0.58	0.58	0.42	0.38	0.62	0.47	0.53
BM143B	0.30	0.70	0.20	0.80	0.47	0.53	0.31	0.69
BM189	0.63	0.37	0.64	0.36	0.72	0.28	0.47	0.53
BM205a	0.26	0.74	0.43	0.57	0.26	0.74	0.34	0.66
BM205b	0.56	0.44	0.6	0.4	0.86	0.14	0.47	0.53
PvESTBR_233	0.51	0.49	0.63	0.37	0.73	0.27	0.52	0.48
PvESTBR_76	----	----	0.3	0.7	----	----	0.25	0.75
PVBR67	0.88	0.12	----	----	0.9	0.1	----	----
PVBR59	0.7	0.3	0.62	0.38	0.78	0.22	0.62	0.38
PvESTBR_10	0.92	0.08	----	----	0.86	0.14	----	----
PvM21	0.46	0.54	0.54	0.46	0.78	0.22	0.52	0.48
PvESTBR_176	0.64	0.36	----	----	0.64	0.36	----	----
BM156	0.40	0.60	0.06	0.94	0.2	0.8	0.16	0.84
BM151	0.825	0.175	----	----	0.28	0.72	----	----
PVBR31	0.34	0.66	0.52	0.48	0.64	0.36	0.55	0.45
PvM13A	0.75	0.25	----	----	0.62	0.38	----	----
BMd-10	0.53	0.47	----	----	0.42	0.57	----	----
PVBR-145	0.84	0.16	----	----	0.78	0.22	----	----
PVESTBR-72	0.30	0.70	0.49	0.51	0.38	0.62	0.43	0.57
AZ30156-1	0.72	0.28	----	----	0.73	0.27	----	----
PVESTBR-98	0.74	0.26	----	----	0.58	0.42	----	----
PvM13B	0.73	0.27	----	----	0.85	0.15	----	----
PVBR218	0.4	0.6	----	----	0.27	0.73	----	----
PVESTBR-279	0.50	0.50	0.72	0.28	0.78	0.2	0.76	0.24
PVBR-45	0.68	0.32	0.76	0.24	0.61	0.39	0.5	0.5
PVESTBR-71	----	----	0.22	0.78	----	----	0.29	0.71

A<sup>1</sup>-allele from the parent CI-107 or IAPAR -81, A<sup>2</sup>-allele from the parent Ouro Negro or VC-5; -- absence of polymorphism for the crossing in question.

**Table 2. Estimates of the relative accumulated adaptability coefficients of populations originating from the two crosses and two levels of N.**

Primer	Progenies grown without N				Progenies grown with N			
	IAPAR 81 x VC-5		Ouro Negro x CI 107		IAPAR 81 x VC-5		Ouro Negro x CI 107	
	$\omega_1$	$\omega_3$	$\omega_1$	$\omega_3$	$\omega_1$	$\omega_3$	$\omega_1$	$\omega_3$
BM143A	----	----	0.112	0.074	----	----	0.272	0.335
BMd-33	0.067	0.118	0.404	0.834	0.396	0.421	0.045	0.182
BMd-15	----	----	0.185	0.632	----	----	0.123	0.213
PV BR93	----	----	----	----	1.049	0.190	0.396	0.421
PvESTBR_230	0.183	0.234	----	----	----	----	----	----
PvESTBR_6	----	----	----	----	1.036	1.466	0.518	0.720
BM143B	0.183	0.423	0.136	0.155	0.152	0.666	0.244	0.573
BM189	----	----	----	----	----	----	0.385	0.221
BM205a	----	----	----	----	----	----	1.845	0.657
PvESTBR_76	0.202	0.615	----	----	0.114	0.282	----	----
PVBR59	0.379	0.227	0.316	0.080	0.379	0.227	0.430	0.177
PvM21	0.152	0.139	0.640	0.177	0.113	0.092	1.163	1.340
PvESTBR_176	----	----	0.065	0.030	----	----	0.094	0.049
BM156	0.066	0.415	0.023	0.132	0.020	0.460	0.036	0.059
PVBR31	0.133	0.071	0.117	0.071	0.135	0.121	0.067	0.138
PVBR145	----	----	0.138	0.031	----	----	0.152	0.017
PVESTBR-72	0.259	0.348	0.468	0.771	0.607	0.632	0.136	0.344
AZ30156-1	----	----	0.295	0.101	----	----	0.154	0.051
PVESTBR-98	----	----	0.074	0.049	----	----	0.113	0.030
PVBR218	0.036	0.119	0.036	0.119	----	----	0.072	0.114
PVESTBR-279	----	----	----	----	0.152	0.053	0.166	0.170
PVBR45	0.093	0.093	0.758	0.480	0.179	0.048	0.202	0.088
PVESTBR-71	0.045	0.124	----	----	0.065	0.271	----	----
BM151	0.404	0.076	----	----	----	----	----	----
$\omega$ average	0.169	0.231	0.251	0.249	0.338	0.379	0.334	0.292

**Table 3. Summary of multiple linear regression analysis using the backward selection method, to the response rate for N in each season, for the 192 progenies of the rainy season, the 79 progenies in the winter crop, and the joint analyses considering common progenies of the two seasons of the two crossings independent of the origin.**

Seasons	Markers	F	Pr > F	R <sup>2</sup>
Rainy	BM184, BMd-20, PVBR59	7.14	0.0002	20.37%
Winter	BMd-20+PvESTBR-233+PVBR59	12.03	<.0001	35.36%
Both	BMd-20+PVBR59+BM205a	7.61	0.0002	25.69%

**Table 4. Summary of the multiple regression analysis through the backward method, to find the rate of response to N in each season of the 96 progenies in the rainy season and the 40 progenies in the winter crop of the two crosses without N.**

Seasons	Markers	F	Pr > F	R <sup>2</sup>
Rainy	BM143A+BM210+BM184+BMd-33+ BMd-20+BM189+BM205a+PVBR59+ PvESTBR_10	8.38	0.0001	43.12
Winter	BMd-20+BM189+PvM21	7.35	0.0011	46.88
Both	BM143A+BM210+BM184+BMd-33+ BMd-20+PVBR 93F+BM205a	13.25	<.0001	89.55

**Table 5. Summary of the multiple regression analysis through the backward method, to the rate of response to N in each season of the 96 progenies in the rainy season and the 39 progenies in the winter crop of the two crosses with N.**

Seasons	Marker	F	Pr > F	R <sup>2</sup>
Rainy	BMd-33+BM143B	8.32	0.0005	19.91
Winter	BMd-33, PvESTBR_233	4.45	0.0187	19.82
Both	BM143B, PvESTBR_233	3.45	0.0426	16.08

**Table 6. Summary of multiple linear regression analysis, through the backward selection method for grain yield, considering the 192 progenies of the rainy season and 79 progenies in the winter crop of two crosses regardless of the origin in experiments with and without N.**

Seasons	Levels	Markers	F	Pr > F	R <sup>2</sup> (%)
Rainy	N-free	BM184+BM205a+ PVBR-45+PvM21	9.12	0.0016	17.38
Rainy	N	BM210+PvM21+ PVBR-45+BM143A	7.03	0.0096	12.15
Winter	N-free	PVBR93	12.6	0.0007	15.63
Winter	N	BM143A+PvESTBR-230+ PvESTBR-233+PvESTBR-10	10.91	<.0001	40.17
Both	N-free	BM184+PVBR93+ BM189	6.78	0.0005	23.55
Both	N	BM143A+BM210+ BM189+BM205a+ PvESTBR-233+PvESTBR-10	4.41	0.0009	29.58

**Table 7. Summary of multiple linear regression analysis, through the backward selection method, for grain yield, considering the 96 progenies of the rainy season and 40 progenies in the winter crop of the two crosses without N in experiments with and without N.**

Seasons	Levels	Marker	F	Pr > F	R <sup>2</sup>
Rainy	N-free	BM184 O+PvM21+ PV-atcc003	12.09	0.0015	25.92
Rainy	N	BM184+PVBR 93+BM205a+ PVBR59+PvM21	9.34	0.0037	41.22
Winter	N-free	PVBR93+PvESTBR_6+ BM205b	6.47	0.0009	49.87
Winter	N	PvM21	11.47	0.0021	28.33
Both	N-free	BM184+PVBR 93+BM205b	6.93	0.0013	43.5
Both	N	BM205a	6.39	0.0172	18.06

**Table 8. Summary of multiple linear regression analysis, through the backward selection method for grain yield, considering the 96 progenies of the rainy season and 39 progenies in the winter crop of the two crosses with N in experiments with and without N.**

Seasons	Levels	Markers	F	Pr > F	R <sup>2</sup>
Rainy	N-free	BM205b+PvESTBR233+ LBM156+PVBR-45	12.82	0.0006	24.97
Rainy	N	BM143B+BM205b	4.45	0.039	14.91
Winter	N-free	BM205b	4.26	0.046	10.32
Winter	N	BM143A+BMd-33+BM143B, BM205b+PvESTBR-233+PVBR59	8.98	<.0001	66.96
Both	N-free	BM205b	5.53	0.024	13.01
Both	N	Bmd-20+PVBR59+BM205b	7.01	0.0027	28.03