



AFLP analysis of genetic diversity in determinate and indeterminate snap bean accessions

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ABSTRACT. The present study aimed to estimate and characterize the genetic divergence between determinate and indeterminate snap bean accessions from the Universidade Estadual de Londrina (UEL) germplasm bank based on amplified fragment length polymorphism (AFLP) markers. A total of 40 and 32 accessions with determinate and indeterminate growth habits, respectively, were characterized for this purpose. Seven combinations of primers corresponding to *Eco*R1 and *Mse*I were tested for the AFLP analysis, and the combinations E-AAG/M-CTC, E-ACT/M-CTT and E-ACC/M-CTT were selected. These selective AFLP combinations revealed 485 informative loci in total, and the combination E-ACC/M-CTT detected the greatest number of informative loci (49%). The analysis of dissimilarity frequency distribution showed that the distribution was uniform, ranging from 0.1285 to 0.7310 with a mean of 0.4801, and the accessions with indeterminate growth habits exhibited greater variability than the accessions with determinate growth habits. The clustering UPGMA, PCoA and Bayesian analyses showed the formation of two large clusters, wherein there is a possible association between snap bean growth habit and gene pool. The determinate accessions may be more closely associated with the Andean gene pool, while the indeterminate ones may be associated with the Mesoamerican gene pool. The Bayesian analysis showed accessions intermediate to both groups, suggesting introgression between the Andean and Mesoamerican gene pools.

Keywords: *Phaseolus vulgaris* L., molecular markers, amplified fragment length polymorphism, gene bank.

Análise de AFLP na diversidade genética em acessos de feijão-de-vagem de hábito determinado e indeterminado

RESUMO. O presente trabalho teve como objetivo estimar e caracterizar a divergência genética entre acessos de feijão-de-vagem determinado e indeterminado do banco de germoplasma da UEL com base em marcadores AFLP. Para tanto foram caracterizados 40 e 32 acessos de hábito determinado e indeterminado, respectivamente. Para análise do AFLP, sete combinações de primers *Eco*R1 e *Mse*I foram testadas, sendo selecionadas as combinações E-AAG/M-CTC, E-ACT/M-CTT, E-ACC/M-CTT. Essas combinações de iniciador/enzima revelaram um total de 485 locos informativos, sendo a combinação E-ACC/M-CTT a que detectou o maior número de locos informativos (49%). A análise da distribuição das frequências da dissimilaridade revelou que a distribuição foi uniforme variando de 0.1285 a 0.7310 com média de 0.4801, com os acessos de hábito indeterminado apresentando uma maior variabilidade quando comparado com os de hábito determinado. Pelas análises de agrupamento UPGMA, PCoA e Bayesiana verificou-se a formação de dois grandes grupos, para os quais sugere-se uma possível predominância do hábito de crescimento do feijão-de-vagem quanto ao pool gênico. Os determinados podem estar mais associados ao pool gênico Andino, enquanto o indeterminado ao pool gênico Mesoamericano. Pela análise Bayesiana, verificou-se acessos intermediário aos dois grupos sugerindo-se uma introgressão entre os pool gênicos Andino e Mesoamericano.

Palavras-chave: *Phaseolus vulgaris* L., marcadores moleculares, polimorfismo no comprimento de fragmentos amplificados, banco de germoplasma.

Introduction

The snap bean (*Phaseolus vulgaris* L.) is considered one of the most important vegetable crops worldwide, and it is relatively rich in vitamins, minerals and antioxidants (Abu-Reidah, Arráziz-Román, Lozano-Sánchez, Segura-Carretero,

& Fernández-Gutiérrez, 2013; Blair, Chaves, Tofiño, Claderón, & Palacio, 2010). The estimated production of snap bean worldwide is approximately 21 million tons, and China, Indonesia, India and Turkey are the largest producers, according to data from the Food and Agriculture Organization of the United Nations (FAO, 2014).

In Brazil, data on snap bean production and market relevance are scarce, given the lack of reliable statistics and systematized information. However, this crop has been widely grown by small farmers, and it is considered a good source of income and an alternative by which to diversify production. It is increasingly being used in crop rotation or in the off-season of other vegetable crops, including bell peppers and tomatoes (Francelino, Gravina, Manhães, Cardoso, & Araújo, 2011; Santos et al., 2012).

The snap bean is classified according to three categories: pod type, pod color and growth habit. Pod type may be classified as flat-podded or round-podded. Four basic colors are included in the pod color category (dark green, light green, yellow and purple). Snap bean plants are classified in regards to growth habit as determinate, when the terminal portion of the stem ends in an inflorescence, and indeterminate, when the terminal portion of the stem ends in a vegetative meristem, enabling continued plant growth (Kwak, Toro, Debouck, & Gepts, 2012).

Two phenotypic loci have been reported to control the snap bean growth habit. The locus *fin* was the first identified in 1915 and subsequently mapped to chromosome Pv01, and the second locus, without identification, was mapped to chromosome Pv07 (Kolkman & Kelly, 2003; Koinange, Singh, & Gepts, 1996). The locus *fin* accounts for determinacy in most varieties with determinate growth habit, which originate from the Andean gene pool, whereas the second locus accounts for determinacy in some Michigan navy bean cultivars, which resulted from an artificial mutagenesis program (Kelly, 2001). Kwak, Toro, Debouck and Gepts (2012) hypothesize that the determinate growth habit is a domestication phenotype, which aims to decrease the plant biomass and optimize the allocation of photosynthates between vegetative and reproductive growth.

Londrina State University (Universidade Estadual de Londrina, UEL) maintains a snap bean germplasm bank, and most accessions were provided by the International Center for Tropical Agriculture (Ciat), including local varieties, traditional cultivars and lines derived from the Ciat breeding program. Accordingly, the characterization of these accessions is highly important because it may help to identify possible duplicates and provide data to facilitate the organization of accessions in the germplasm bank, thus facilitating their use in breeding programs (Gonçalves et al., 2008a; Moulin, Rodrigues, Gonçalves, Sudré, & Pereira, 2012; Sudré et al., 2010).

The germplasm characterization may be performed considering agronomic, morphological, biochemical, physiological, and molecular characteristics, among others (Gonçalves, Rodrigues, Amaral Júnior, Karasawa, & Sudré, 2008b). However, molecular markers have advantages over the other characteristics because they indicate genetic differences at a higher level of detail and without interference from the environment. Their use also quickly determines the diversity present (Leal et al., 2010).

There are different types of molecular markers, distinguished by their potential to detect differences between individuals, cost, ease of use and the consistency and repeatability of results (Gupta, Rustgi, & Mir, 2013). Amplified fragment length polymorphism (AFLP) markers (Vos et al., 1995) stand out among the types of markers available as a powerful tool by which to characterize germplasm, given their high reproducibility and robustness together with the fact that they generate few artifacts and allow extensive genome coverage (Meudt & Clarke, 2007; Santos, Oliveira, Rodrigues, Ribeiro, & Silva, 2011).

Accordingly, the present study aimed to estimate and characterize the genetic divergence among 72 (determinate and indeterminate) snap bean accessions of the UEL germplasm bank based on AFLP markers.

Material and methods

A total of 72 snap bean accessions belonging to the germplasm bank of the Londrina State University (Universidade Estadual de Londrina - Banco de Germoplasma de Feijão-de-Vagem, UEL - BGFV), including 40 and 32 accessions with determinate and indeterminate growth habits, respectively, were used.

Total DNA was extracted from young leaves of the analyzed accessions using the protocol by Doyle and Doyle (1987). After DNA extraction, the DNA integrity was assessed on a 1.0% agarose gel, which was stained in ethidium bromide solution ($0.5 \mu\text{g mL}^{-1}$) and visualized using a gel imaging system. The DNA content of the samples was quantified using a NanoDrop 2000/2000c spectrophotometer (Thermo Scientific).

AFLP analysis was performed according to Vos et al. (1995), with some modifications. Approximately 700 ng of DNA from each accession was double-digested by the enzymes *EcoR*I and *Mse*I, using 5 U of each, for 18 hours at 37°C. The generated fragments were ligated to the adaptors, 0.5 μM *EcoR*I and 5.0 μM *Mse*I, using the enzyme T4

DNA ligase at 1 U, buffer for T4 DNA ligase at 1 X, 0.05 M sodium chloride (NaCl), 50 ng μL^{-1} bovine serum albumin (BSA) and 0.25 mM Dithiothreitol (DTT), in a final volume of 10 μL . The reaction mixture was incubated at 37°C for 3 hours, 17°C for 30 min. and 70°C for 10 min. a thermocycler and then diluted (1:1) with ultrapure water. Subsequently, the fragments were amplified using a primer pair with one selective base.

The pre-selective amplification was performed in a final volume of 10 μL , using 3.5 μL of the GoTaq[®] Green Master Mix kit (Promega), 4.75 μM pre-selective primer and 3.0 μL from the diluted restriction/ligation reaction. The thermocycler program consisted of 2 minutes at 72°C, followed by 20 1-s cycles at 94°C, 30 s at 56°C and 2 min. at 72°C and, lastly, 30 min. at 60°C. The selective amplification reactions consisted of 0.54 μL of each selective primer, 5 μM *MseI* and 1 μM *EcoRI*, 3.5 μL GoTaq[®] Green Master Mix (Promega) and 3 μL of the pre-amplified DNA diluted (1:8) with ultrapure water, in a final volume of 10 μL . Seven combinations of primers corresponding to *EcoRI* and *MseI* (E-AAG/M-CTC, E-ACT/M-CTT, E-ACC/M-CTT, E-ACG/M-CTT, E-ACA/M-CTT, E-AAC/M-CTT, E-ACT/M-CTA), with three selective nucleotides, were tested for the selective amplification. The reactions with selective nucleotides were performed in a thermocycler as follows: initial cycle of 2 min. at 94°C; 30 s at 65°C and 2 min. at 72°C; 8 1-s cycles at 94°C, 30 s at 64°C and 2 min. at 72°C, decreasing 1°C at each cycle; 23 1-s cycles at 94°C, 30 s at 56°C and 2 min. at 72°C and, finally, 30 min. at 60°C. Three of the seven tested combinations were selected (E-AAG/M-CTC, E-ACT/M-CTT, E-ACC/M-CTT), wherein the primers for *EcoRI* were labeled with fluorophores: blue (FAM), green (HEX) and yellow (NED), respectively.

The products of selective reactions were added to 0.2 μL of ROX-500 size standard (Applied Biosystems) and 8.8 μL of highly deionized (Hi-Di) formamide. Then, the products were denatured and subjected to capillary electrophoresis using the automated system 3500 xL (Applied Biosystems). All amplifications were performed in a thermocycler GeneAmp PCR System 9700 (Applied Biosystems).

For the AFLP analysis, the data were interpreted according to the presence and absence of bands, generating a binary matrix. The Jaccard similarity coefficient complement was used to estimate the genetic distances between the (determinate and indeterminate) snap bean accessions. The simplified representation of genetic distances between accessions was obtained using the hierarchical

clustering method Unweighted Pair-Cluster Method Using Arithmetic Average (UPGMA) and principal coordinates analysis (PCoA). Pseudo F (Calinski & Harabasz, 1974), Pseudo T² (Duda & Hart, 1973) and Cubic Clustering Criterion (Sarle, 1983) statistics were used to assess the optimal number of clusters by UPGMA clustering, while the clustering reliability was assessed by Bootstrap analysis with 1000 replicates. The distance matrix estimate and PCoA and UPGMA clusters were performed using the software R (<http://www.r-project.org>).

The software STRUCTURE version 2.3.3 (Hubisz, Falush, Stephens, & Pritchard, 2009) was used to identify the number of similar accessions clusters (*K*). The analysis of the number of clusters was performed using the admixture model with a burn-in and run lengths of 10,000 and 100,000 interactions, respectively. The number of clusters was determined following the guidelines of Pritchard and Wen (2004) and Evano, Regnaut and Goudet (2005), in the online software Structure Harvester (Earl & Vonholdt, 2012).

Results and discussion

The three combinations of AFLP selective primers used (E-AAG/M-CTC, E-ACT/M-CTT, E-ACC/M-CTT) revealed a total of 485 loci. The combination E-ACC/M-CTT detected the greatest number of informative loci (237), while the combinations E-AAG/M-CTC and E-ACT/M-CTT detected 105 and 143 informative loci, respectively. Maciel, Echeverrigaray, Gerald and Grazziotin (2003) and Blair, Chaves, Tofiño, Claderón and Palacio (2010) also noted more polymorphisms for the combination E-ACC/M-CTT when evaluating the genetic variation and the relationship between common bean and snap bean accessions using AFLP markers.

The analysis of the dissimilarity frequency distribution of 2,556 combinations of the 72 snap bean accessions showed that the distribution was uniform, with genetic distance ranging from 0.1285 to 0.7310 with a mean value of 0.4801 (± 0.1327 ; Figure 1). The highest frequency was found in the interval between 0.40 and 0.60, with 51.4%. When evaluating per group, the genetic distance of the accessions with determinate growth habits ranged from 0.1285 (GB-SB-31 x GB-SB-32) to 0.6446 (GB-SB-31 x GB-SB-06), with a mean value of 0.3683 (± 0.1111), while the accessions with indeterminate growth habits ranged from 0.1523 (GB-SB-76 x GB-SB-83) to 0.7011 (GB-SB-65 x GB-SB-84), with a mean of 0.4402 (± 0.1097 ;

Figure 1). Both accession groups (determinate and indeterminate) were mostly derived from the CIAT germplasm bank.

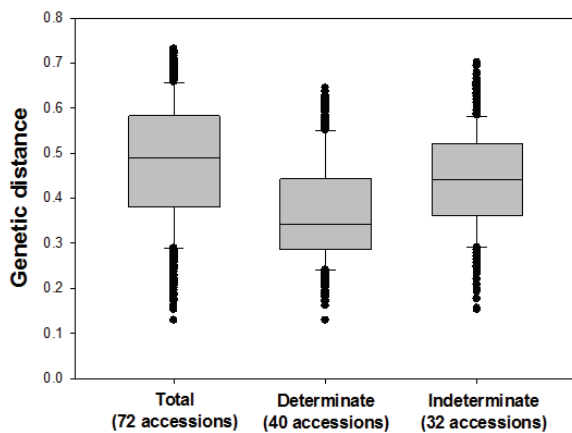


Figure 1. Box plot of the dissimilarity based on AFLP markers between 72 snap bean accessions (determinate and indeterminate).

UPGMA hierarchical clustering analysis showed the formation of two large clusters. The first cluster was exclusively formed by accessions of determinate growth habit, while the second cluster consisted of accessions with indeterminate growth habit and six accessions of determinate growth habit (Figure 2).

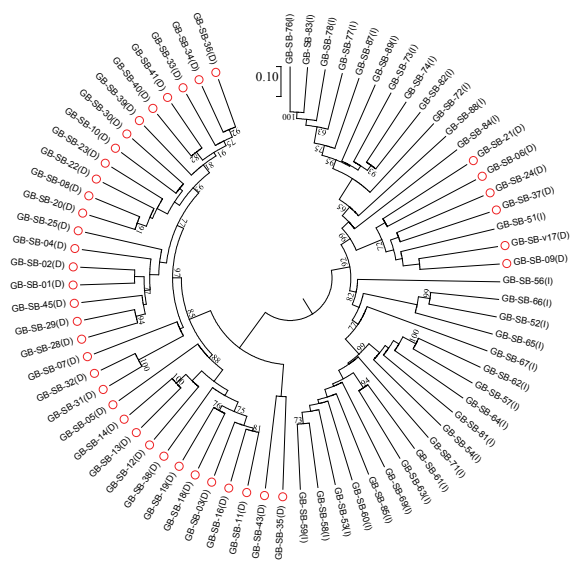


Figure 2. UPGMA dendrogram of the genetic relationships among 72 snap bean (determinate and indeterminate) accessions based on AFLP. Bootstrap values are given at the corresponding node for each cluster.

This result was corroborated by the Bayesian statistics, which showed that the highest value of ΔK was reached upon the formation of two large clusters. Cluster I (in red) was formed by most accessions with indeterminate growth habits, while

cluster II (in green) was formed by accessions with determinate growth habits. In addition, some accessions showed an intermediate classification pattern, as shown in the bar plot (Figure 3).

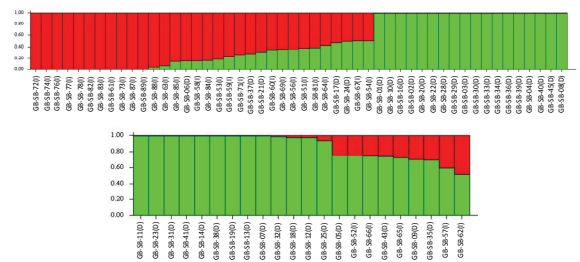


Figure 3. Bar plot structure based on three AFLP combinations of primer/enzyme (E-AAG/M-CTC, E-ACT/M-CTT, and E-ACC/M-CTT) for the 72 snap bean accessions (determinate and indeterminate), showing the distributions of both groups.

The PCoA also showed the separation of both groups of snap bean (Figure 4). The compact growth habit is related to legume domestication, which manifests as a number of characteristics, including reduced branching, shorter internodes, smaller number of nodes and reduced twining. In some cases, the terminal portion of the stem ends in an inflorescence, according to Smartt (1990). All wild species of *Phaseolus* are indeterminate, as are some domesticated types, while all determinate accessions are domesticated. Kwak et al. (2012) noted that *PvTFL1y* (a gene associated with determinacy) originated in both Andean and Mesoamerican gene pools when studying the multiple origins of the determinate growth habit in common bean plants.

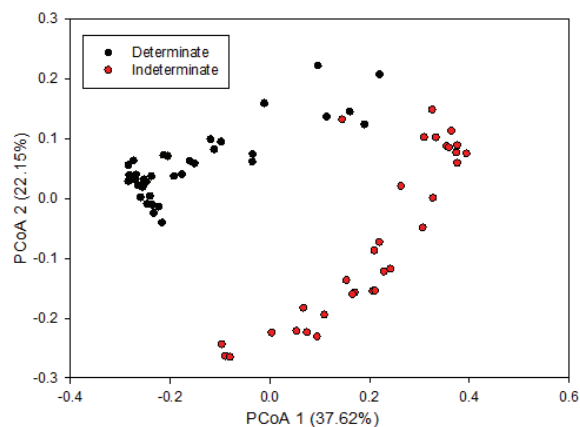


Figure 4. PCoA of 72 snap bean (determinate and indeterminate) accessions based on AFLP.

Similarly to the other types of beans, the primary center of diversity for snap bean is believed to be the Americas. However, this crop has a wide distribution and is quite diverse in several regions of the world, especially Europe and Asia (Myers &

Baggett, 1999). According to Blair et al. (2010), there is a misconception among several authors that all or most snap bean genotypes were derived from the Andean gene pool because the initial analysis showed that the determinate snap bean usually shows patterns of Andean-type phaseolin proteins ('T', 'C', or 'H'; (Brown, Mcferson, Bliss, & Hall, 1982; Gepts, Osborn, Rashka, & Bliss, 1986). However, Blair et al. (2010) noted that the accessions belonged to both gene pools, with a predominance of the Mesoamerican gene pool over the Andean, when evaluating the genetic diversity of 120 accessions of indeterminate snap bean using AFLP molecular markers and microsatellites.

Accordingly, a possible predominance of snap bean growth habit in relation to the gene pool is suggested based on the results of the present study, wherein the determinate accessions may be more closely associated with the Andean gene pool, while the indeterminate ones may be more closely associated with the Mesoamerican gene pool. That fact is corroborated by the fact that some indeterminate snap bean accessions used in the present study were derived from the Blue Lake (GB-SB-51, GB-SB-52, GB-SB-53, GB-SB-54, GB-SB-60, GB-SB-73 and GB-SB-87), Osu 4852 (GB-SB-61 and GB-SB-63), Lago Azul (GB-SB-64), G15300-Zambia (GB-SB-69) and G15801-Zambia (GB-SB-77) varieties, which were classified as Mesoamerican by Blair et al. (2010).

Regarding the diversity within the snap bean accession groups (growth habits), Pseudo T² and CCC statistics showed the formation of six subclusters of indeterminate growth habit accessions in the UPGMA clustering, while five subclusters were formed in the clustering of determinate accessions. The pseudo F parameter precluded an assessment of the optimal number of clusters because the calculated F increased with the number of clusters formed, indicating the lack of a 'natural' data partition.

In the indeterminate accessions, subcluster I consisted of 12 accessions, with all accessions clustered with the same pattern as suggested by the Bayesian analysis and represented by the bar plot. Single accessions were allocated to subclusters II, III and V (GB-SB-51(I), GB-SB-56(I) and GB-SB-67(I), respectively), while subclusters IV and VI were formed by three and 14 accessions, respectively. The accessions from those subclusters (II, III, IV, V and VI) showed a two-color pattern (red and green) in the bar plot (Figure 3), indicating introgression in the gene pool. This introgression is most evident in accessions GB-SB-52(I), GB-SB-66(I), GB-SB-SB65(I), GB-SB-57(I) and GB-SB-62(I), wherein an intermediate pattern of the gene pools is noted.

In the determinate growth habit accessions, subclusters I, II, III, IV and V consisted of 6, 2, 10, 3 and 19 accessions, respectively. Subcluster I (GB-SB-21(D), GB-SB-06(D), GB-SB-24(D), GB-SB-37(D), GB-SB-17(D) and GB-SB-09(D)) was the most divergent from the other clusters, as those accessions showed a broad effect of the gene pool, as shown in the bar plot. Accessions GB-SB-43(D) and GB-SB-35(D) from subcluster II and accessions GB-SB-05(D) from subcluster III also showed that same effect. The other accessions showed a single color pattern in the bar plot. The introgression of Andean and Mesoamerican gene pools was also shown in other studies (Blair et al., 2010; Cunha, Hintz, & Griffiths, 2004; Metais, Hamon, Jalouzot, & Peltier, 2002). According to Blair et al. (2010), the introgression between the gene pools in snap beans may be more likely than in dry beans because seed size and color constraints are different in snap beans than in dry beans.

Conclusion

The results showed a good level of genetic variability in the germplasm bank of snap beans of Universidade Estadual de Londrina. This on formation will be useful in future breeding programs. UPGMA, PCoA and Bayesian analysis showed the formation of two large groups, suggesting a possible relationship between the bean growth habitat and the gene pool.

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