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Molecular characterization of high performance inbred lines of Brazilian common beans

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ABSTRACT. The identification of germplasm genetic variability in breeding programs of the common bean (*Phaseolus vulgaris*) is essential for determining the potential of each combination of parent plants to obtain superior genotypes. The present study aimed to estimate the extent of genetic diversity in 172 lines and cultivars of the common bean by integrating five tests of value for cultivation and use (VCU) that were conducted over the last eight years by the breeding program of Embrapa Arroz e Feijão in Brazil. Nine multilocus genotyping systems composed of 36 fluorescent microsatellite markers distributed across 11 different chromosomes of the common bean were used, of which 24 were polymorphic. One hundred and eighty-seven alleles were identified, with an average of 7.79 alleles per locus and an average gene diversity of 0.65. The combined probability of identity for all loci was 1.32×10^{-16}. Lines that are more genetically divergent

between the selection cycles were identified, allowing the breeding program to develop a crossbreed between elite genotypes with a low degree of genetic relatedness. H_E values (ranging from 0.31 to 0.63) and the genetic differentiation among the VCU tests (F_{ST} of 0.159) supports for new strategies to increase the genetic base from which the program is conducted. Private alleles (26%) were identified and can be directly incorporated into the gene pool of cultivated germplasm, thereby contributing effectively to the expansion of genetic diversity in this bean-breeding program.

Key words: *Phaseolus vulgaris*; Breeding; Genetic diversity; SSR markers

INTRODUCTION

The common bean (*Phaseolus vulgaris*), that belongs to the family Leguminosae, subfamily Papilionoideae, tribe Phaseoleae, and genus *Phaseolus* is considered the most important grain legume for human consumption in the world (Broughton et al., 2003). The common bean is cultivated in 113 countries and has an estimated worldwide production of 19.51 million tons. The common bean is grown annually on an average of 27 million hectares (ha) (FAO, 2008), and represents 37% of all legumes consumed in the world. Sixty percent of the world's production of the common bean is concentrated in six countries: Brazil, India, China, Myanmar, Mexico, and the United States. Currently, Brazil is the world's largest producer of this legume, representing approximately 20% of global production and occupying the second largest area of cultivation (4.01 million ha) behind India (8.60 million ha). Brazil's production during the 2010/2011 season yielded a total of 3.51 million tons of beans. The yield for the 2020/2021 season is expected to reach 3.82 million tons (0.9% increase), which is linked to a 0.83% projected reduction in planting area and increased productivity (MAPA, 2011). However, both projections for production and consumption in Brazil for the period between 2011 and 2021 suggest a demand for imports between 150,000 and 200,000 tons (CONAB, 2008). Beans are considered an excellent source of protein and provide iron, phosphorus, magnesium, manganese, and, to a lesser extent, zinc, copper, calcium, and B vitamins. In addition, beans have high amounts of fiber and complex carbohydrates (Broughton et al., 2003).

The common bean is cultivated in almost all Brazilian states in various soil and climatic conditions and in different seasons and crop systems. Beans are grown in both subsistence crops, which account for 70% of the country's production, and in highly technical crops (Borém and Carneiro, 2006). Among the producing states, Paraná has the highest production (20%), followed by the States of Minas Gerais (15%), Bahia (10%), São Paulo (10%), Goiás (8%), Santa Catarina (7%), and Rio Grande do Sul (5%), which together represent 75% of the national production (Ferreira et al., 2006). A regional preference for grain type is found among the Brazilian population. For example, black beans are consumed mainly in the State of Minas Gerais, and the demand for the carioca beans, currently considered the most highly consumed bean in Brazil, is extremely high in the States of Rio de Janeiro, Santa Catarina, and Rio Grande do Sul. Thus, common bean breeding programs have focused on the development and indication of new and improved lines due to the constant demand for increasingly productive cultivars with better grain quality, resistance to major diseases and adaptability to local climatic conditions and soil heterogeneity. The cultivation of specialty grains of beans,

such as alubia, cranberry, dark red kidney, and pinto, as alternative products with higher added value in the Brazilian market has increased. However, the cultivation of specialty grains is still considered sparse and incipient when compared to the demand for the carioca grain (Bueno et al., 2011). Brazil exported 17,000 tons of beans in 2011, a 400% increase over the previous year (CONAB, 2008). This increase demonstrates a growing demand for business opportunities for this crop in the international market. Thus, common bean breeding programs should also be synchronized with the supply chain by investing in the research and development of varieties with export potential.

The development of new cultivars through breeding programs is considered a long process with a high cost due to the many steps involved during the course of successive selections and evaluations. A developed cultivar must be distinguished from other existing cultivars by easily identifiable characteristics, and these traits may include morphological, physiological, biochemical, or other characteristics considered sufficient for cultivar identification (MAPA, 2010). In addition, the genetic material must be registered and protected following the instructions provided by Plant Variety Protection Law No. 9456, which has been regulated in Brazil since 1997 to ensure that the breeders will have the intellectual property rights to the cultivars and their derivatives. Many requirements are necessary to register a cultivar, such as testing for distinctness, uniformity, and stability. In addition, breeders should perform value for cultivation and use (VCU) testing to characterize the lines. This test corresponds to the last stage of the breeding program of a new cultivar. Several agronomic traits, such as the cultivar's properties for use in agricultural, industrial, commercial, and consumer activities, are used to evaluate the performance of the lines before its release. The VCU test is conducted according to the National Register of Cultivars/Ministry of Agriculture, Livestock and Supply norms. After the test is performed, the lines that display higher performances than the reference cultivars included in the VCU test are identified. Currently, the public sector is responsible for more than 60% of the registered cultivars in Brazil (Marinho et al., 2011).

The genetic characterization of common bean lines that satisfy the VCU tests has not been reported in the literature. Thus, the use of molecular tools can help breeders to accurately evaluate the level of genetic variability found in the tests and to identify genetic material that may be used for the registration and protection of cultivars. Microsatellite markers have characteristics that bring advantages over other genetic markers, as described by several authors (Gupta et al., 1996). Recently, microsatellite markers have been used for genotyping in systems whereby the simultaneous amplification of several loci is possible using a semi-automated detection system. The development of these systems is not a trivial procedure and requires a detailed prior investigation of the loci that will encompass the genotyping panels. This procedure is performed to adopt criteria for marker selection, such as determining the specificity and size of the amplified products, estimates of locus content, and forensic parameters including the probability of identity, power exclusion, and reduced complementarity between the primers combined in a single panel.

Microsatellite markers are currently the main common markers used to estimate genetic profiles and constitute the international standard for forensic genetic research (Butler, 2006). Automated genotyping systems using microsatellites are available for genetic analyses in many plant species, such as rice (Pessoa-Filho et al., 2007), soybean (Sayama et al., 2011), and the common bean (Masi et al., 2003). These systems have several advantages over conventional genotyping methods that are based on polyacrylamide gels. For example, simultaneous analyses can be performed on a greater number of microsatellite loci, the time

required to genotype a large number of subjects can be reduced, the data collected may be more accurate due to the addition of an internal marker in each sample, and a greater agility and security can be achieved during the data processing.

The present study aimed to establish multiplex genotyping operational systems based on microsatellite markers with higher informative power, followed by the molecular characterization of the genetic variability of common bean lines that were part of five VCU tests performed by Embrapa Arroz e Feijão breeding program between 2003 and 2012.

MATERIAL AND METHODS

Plant material

A total of 172 common bean accessions in bulks of five plants were analyzed, including the lines under evaluation (150) and control cultivars (22) that were part of the five tests for the VCU conducted in the 2003/2004, 2005/2006, 2007/2008, 2009/2010, and 2011/2012 crop years at Embrapa Arroz e Feijao. The 2003/2004 VCU test comprised 62 accessions (54 lines and 8 controls). The 2005/2006 VCU test comprised 37 accessions, of which 25 belonged to the line groups, and the remaing 12 were controls. The 2007/2008, 2009/2010, and 2011/2012 VCU tests comprised 23 (13 carioca and 10 black), 23 (13 carioca and 10 black) and 25 accessions (15 carioca and 10 black), respectively, in addition to the controls analyzed in each group. A detailed description of each VCU test and the controls is displayed in Table 1.

Plant material collection and extraction of genomic DNA

Samples of young trifoliate leaves from each of the common bean accessions evaluated in VCU tests were collected 20 days after planting in the experimental field. The procedure was performed in five steps in accordance with the year and planting period of the test. DNA extraction and quantification were performed as described by Ferreira and Grattapaglia (1998), with some modifications. Plant tissue maceration was performed in 1.5-mL tubes with a porcelain bead using a FastPrep instrument (model FP120 Thermo Electron Corporation). After extraction, the DNA was diluted to final concentration of 3 ng/μL.

Microsatellite genotyping

Nine multilocus genotyping systems based on 36 microsatellite markers labeled with the fluorescent dyes HEX, 6-FAM, and NED were used in the experiments. The selection of markers for co-amplification systems and analysis of forensic performance was performed according to the procedure described by Cardoso (2008). Of the 36 markers used (Table 2), 21 belonged to the BM series (Gaitán-Solís et al., 2002) and 15 belonged to the PvBr series (Buso et al., 2006). The microsatellite amplification reactions were performed using the QIAGEN® 2X Multiplex PCR Kit (Qiagen, CA, USA) in a GeneAmp 9700 Thermal Cycler (Applied Biosystems) with an initial denaturation step at 95°C for 15 min, followed by 30 cycles of denaturation (94°C for 30 s), annealing (57°C for 90 s), and extension (72°C for 1 min), and a final extension step at 72°C for 7 min. The amplified products were diluted in sterile Milli-Q

Table 1. Description of the lines and control cultivars of the common bean based on VCU tests, including names, types of grains and genealogy composition.

Genotype	Type of grain	Genealogy
		VCU 2003/2004
Carioca 11	Carioca	Control variety
Pérola	Carioca	Control variety
CNFC 9435	Carioca	A 769 / 4 / EMP 250 /// A 429 / XAN 252 // V 8025 / G 4449 /// WAF 2 / A55 // GN 31 / XAN 170
CNFC 9458	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 9461	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 9471	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 9484	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 9494	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 9500	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 9504	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 9506	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 9518	Carioca	A 790 / A 767
CNFE 8009	Carioca	PR 17 / ANA 910528
BRS Valente	Black	Control variety
CNFP 7966	Black	CB 733774 / AN 9122526
CNFP 7972	Black	CB 733774 / AN 9122526
CNFP 7994	Black	CB 911863 / AN 9123293
CNFP 8000	Black	CB 911863 / AN 9123293
CNFP 9328	Black	Xamego / TC 1558-1
CNFP 10138	Black	CB 911852 / AN 9123293
TB 94-09	Black	-
TB 97-13	Black	-
BRS Vereda	Color: pink	Control variety
BRS Radiante	Color: striped	Control variety
CNFRJ 10294	Color: striped	-
CNFRJ 10299	Color: striped	-
CNFRX 8035	Color: purple	BP 9 / MA 721340 // FEB 163 / ARA1
CNFRX 10241	Color: purple	XAN 283 / RAB 487 / A 247
BRS Marfim	Mulatinho	Control variety
CNFM 7957	Mulatinho	BC 912052 / AN 9022180
CNFM 7958	Mulatinho	BC 912052 / AN 9022180
CNFM 8057	Mulatinho	ICA COL 10103 / AN 710988 // Milionário 1732 / LA 721477
CNFM 8080	Mulatinho	ICA COL 10103 / AN 710988 // LA 721493 / ICA COL 10103
CNFM 9381	Mulatinho	AN 910518 / A 55
CNFM 9412	Mulatinho	TC 1558-1 / Aporé
CNFM 10375	Mulatinho	LM 95100047 / SC 9029923
CNFM 10385	Mulatinho	LM 95100045 / FEB 156
CNFM 10386	Mulatinho	LM 95100045 / FEB 156
CNFM 10387	Mulatinho	LM 95100045 / FEB 156
CNFM 10390	Mulatinho	LM 95100045 / FEB 156
		VCU 2003/2004 (additional lines)
CNFC 7806	Carioca	Carioca MG // POT 947 / AN910523
CNFC 7813	Carioca	BZ3836 // FEB 166 / AN 910523
CNFC 8075	Carioca	LM 21303 / A 248 // LA 721493 / ICA COL 10103
CNFC 8202	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // Pinto UI 114
CNFP 7726	Black	W22-34 / 84VAN163
CNFP 7762	Black	W22-34 / VAN163
CNFP 7776	Black	AN12567 / México 168
CNFP 8104	Black	Cultivar Corrente submitted to gamma radiation
BRS Timbó	Color: purple	Control variety
CNFRX 7847	Color: purple	VCU/color
CNFRX 7866	Color: purple	FEB 163 / AN512879
ARC 1-30	-	VCU/weevil
ARC 100-4	-	VCU/weevil
ARC 100T-5	-	VCU/weevil
Talismã	Carioca	Control variety
CNFC 9437	Carioca	EMP 250 / 4 / A769 /// A429 / XAN 252 // V8025 / Pinto UI 114
CNFP 7988	Black	LM 202203344 / LM 202203330
CNFP 9346	Black	Xamego / Rudá

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Table 1. Continued.

Genotype	Type of Grain	Genealogy
CNFRJ 10245	Color: striped	CB 91206 3 / AN512717
CNFE 8017	Carioca	PR 9115957 / LR720982CP
CNFM 6911	Mulatinho	BAT 85 x [(A 375 x G 17702) x (A 445 x XAN 112)]
CNFM 7119	Mulatinho	CB511687 / J. PRECOCE
		VCU 2005/2006
BRS Pontal	Carioca	Control variety
IAPAR-81	Carioca	Control variety
Magnífico	Carioca	Control variety
Pérola	Carioca	Control variety
CNFC 10408	Carioca	A 769 / 4 / A 774 /// A 429 / XAN 252 // V 8025 / G 4449 /// WAF 2 / A55 // GN 31 / XAN 170
CNFC 10410	Carioca	EMP 205 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10429	Carioca	EMP 205 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10431	Carioca	EMP 205 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10432	Carioca	EMP 205 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10438	Carioca	EMP 205 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10444	Carioca	EMP 205 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10455	Carioca	EMP 205 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10467	Carioca	AN 51666-0 / LR 720982CP
CNFC 10470	Carioca	PR 9115957 / LR 720982CP
BRS Grafite	Black	Control variety
BRS Valente	Black	Control variety
IPR Uirapuru	Black	Control variety
Soberano	Black	Control variety
CNFP 10035	Black	IAPAR BAC 296 // AN 911113 / POT 51
CNFP 10076	Black	LR 916337 / FE 821732
CNFP 10093	Black	LA 9017179 / FE 821732
CNFP 10103	Black	FT 85-113 / POT 51
CNFP 10104	Black	FT 85-113 / POT 51
CNFP 10109	Black	FT 85-113 / POT 51
CNFP 10120	Black	CB 911846 / AN 91232993
CNFP 10206	Black	RH5-206 / RAI 295 /// Honduras 35 / LA 9017149 // AN 911113 / CB 720160 /4/ AN 911113 / POT 51 / IAPAR BAC 296 /// LA9017149 / CB720160 /// FE 732880
BRS Radiante	Color: striped	Control variety
BRS Timbó	Color: purple	Control variety
Iraí	Color: manteigão	Control variety
Vermelho-2157	Color: red	Control variety
CNFRJ 10559	Color: striped	PR 95105259 / PR 93203382
CNFRJ 10573	Color: striped	PR 95105259 / PR 93201759
CNFRJ 10568	Color: striped	PR 95105259 / PR 93201759
CNFRX 10525	Color: purple	LM 95105713 / LM 95204049
CNFRX 10527	Color: purple	LM 95105713 / LM 95204049
CNFRX 10530	Color: purple	LM 95105713 / LM 95204049
CNFRX 10538	Color: purple	LM 95105713 / LM 95204049
		VCU 2007/2008
BRS Cometa	Carioca	Control variety
BRS Pontal	Carioca	Control variety
IPR Juriti	Carioca	Control variety
Pérola	Carioca	Control variety
CNFC 10703	Carioca	LM 95103381 / 95204151 // AN 9022180 / 95204119 /// A 805
CNFC 10713	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10716	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10721	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10729	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10733	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10742	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10753	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10757	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10758	Carioca	EMP 250 / 4 / A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 10762	Carioca	Pérola / AN 9022180

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Table 1. Continued.

Genotype	Type of Grain	Genealogy
CNFC 10763	Carioca	Pérola / AN 9022180
CNFC 10813	Carioca	MAR 3 /Pérola
BRS Grafite	Black	Control variety
BRS Supremo	Black	Control variety
BRS Valente	Black	Control variety
IPR Uirapuru	Black	Control variety
CNFP 10025	Black	LA 9016920 / FE 821732 // AN 911113
CNFP 10214	Black	AN 730408 / Cornell 49242
CNFP 10221	Black	AN 12567 / LA 720164 // FE 821698 / CB 733795
CNFP 10793	Black	POT 51 /// OAC 88-1 / A 429 // OAC 88 - 1 / RM 35
CNFP 10794	Black	POT 51 /// ICA Pijao / XAN 170 /// BAC 16 / XAN 91
CNFP 10799	Black	FT Tarumã / Xamego
CNFP 10800	Black	FT Tarumã / Xamego
CNFP 10805	Black	Milionário 1732 / Xamego
CNFP 10806	Black	Milionário 1732 / Xamego
CNFP 10807	Black	IAPAR 44 / Macanudo
		VCU 2009/2010
BRS Esplendor	Black	Control variety
BRS Campeiro	Black	Control variety
IPR Uirapuru	Black	Control variety
BRS Supremo	Black	Control variety
CNFP 11973	Black	Pérola / LM 97200605
CNFP 11976	Black	LM 96201531 / TB 9401
CNFP 11978	Black	LM 30630 *3 / AN 9122618
CNFP 11979	Black	LM 30630 *3 / AN 9123293
CNFP 11983	Black	LM 30630 *2 / AN 9122551
CNFP 11984	Black	LM 30630 *2 / AN 9122551
CNFP 11985	Black	LM 30630 *2 / AN 9122618
CNFP 11991	Black	LM 9310606 / TB 94-01
CNFP 11994	Black	LM 9310646 / AN 9021412
CNFP 11995	Black	LM 9310646 / AN 9021412
Pérola	Carioca	Control variety
BRS Cometa	Carioca	Control variety
IPR Juriti	Carioca	Control variety
BRS Estilo	Carioca	Control variety
CNFE 8017	Carioca	-
CNFC 11944	Carioca	AB 136 / Pérola
CNFC 11945	Carioca	AB 136 / Pérola
CNFC 11946	Carioca	AB 136 / Pérola
CNFC 11948(P)	Carioca	LM 96200246 / LP 9632
CNFC 11951	Carioca	MAR 1 / LM 95204101 // FEB 207
CNFC 11952	Carioca	MAR 1 / LM 95204101 // FEB 207
CNFC 11953	Carioca	LM 95102728 /LM 95204115 // H 4 / LM 95204101 /// FEB 207
CNFC 11954(P)	Carioca	Pérola / 8146 // A767
CNFC 11956	Carioca	LM 95102728 /LM 95204115 // H 4 / LM 95204101 /// FEB 207
CNFC 11959	Carioca	EMP 250 /4/ A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 11962	Carioca	EMP 250 /4/ A 769 /// A 429 / XAN 252 // V 8025 / Pinto UI 114
CNFC 11966(P)	Carioca	LM 96201239 / Aporé
		VCU 2011/2012
CNFP 15171	Black	LM 9310639 / LM 93204217
CNFP 15174	Black	LM 9310639 / LM 93204217
CNFP 15177	Black	LM 9310639 / LM 93204217
CNFP 15178	Black	LM 9310639 / LM 93204217
CNFP 15188	Black	TB 94-01 / LM 98203893
CNFP 15193	Black	TB 94-01 / LM 98203893
CNFP 15194	Black	TB 94-01 / LM 98203893
CNFP 15198	Black	TB 94-01 / LM 98203893
CNFP 15207	Black	TB 94-01 / FPGCF058
CNFP 15208	Black	TB 94-01 / FPGCF058
IPR Uirapuri	Black	Control variety

Continued on next page

Table 1. Continued.

Genotype	Type of Grain	Genealogy
BRS Campeiro	Black	Control variety
BRS Esplendor	Black	Control variety
CNFC 15001	Carioca	RS*: Aporé, BAT 477, AN 9022180, Corrente, A 806, MAN 48, AND 277, IPA 7, A797, Pinto Villa, EMP 81, V 8025, A 525, A 774, MAR 1 and DOR 500
CNFC 15003	Carioca	RS: Aporé, BAT 477, AN 9022180, Corrente, A 806, MAN 48, AND 277, IPA 7, A797, Pinto Villa, EMP 81, V 8025, A 525, A 774, MAR 1 and DOR 500
CNFC 15010	Carioca	RS: Aporé, BAT 477, AN 9022180, Corrente, A 806, MAN 48, AND 277, IPA 7, A797, Pinto Villa, EMP 81, V 8025, A 525, A 774, MAR 1 and DOR 500
CNFC 15018	Carioca	RS: Aporé, BAT 477, AN 9022180, Corrente, A 806, MAN 48, AND 277, IPA 7, A797, Pinto Villa, EMP 81, V 8025, A 525, A 774, MAR 1 and DOR 500
CNFC 15023	Carioca	RS: Aporé, BAT 477, AN 9022180, Corrente, A 806, MAN 48, AND 277, IPA 7, A797, Pinto Villa, EMP 81, V 8025, A 525, A 774, MAR 1 and DOR 500
CNFC 15025	Carioca	RS: Aporé, BAT 477, AN 9022180, Corrente, A 806, MAN 48, AND 277, IPA 7, A797, Pinto Villa, EMP 81, V 8025, A 525, A 774, MAR 1 and DOR 500
CNFC 15033	Carioca	RS: Aporé, BAT 477, AN 9022180, Corrente, A 806, MAN 48, AND 277, IPA 7, A797, Pinto Villa, EMP 81, V 8025, A 525, A 774, MAR 1 and DOR 500
CNFC 15035	Carioca	RS: Aporé, BAT 477, AN 9022180, Corrente, A 806, MAN 48, AND 277, IPA 7, A797, Pinto Villa, EMP 81, V 8025, A 525, A 774, MAR 1 and DOR 500
CNFC 15038	Carioca	RS: Aporé, BAT 477, AN 9022180, Corrente, A 806, MAN 48, AND 277, IPA 7, A797, Pinto Villa, EMP 81, V 8025, A 525, A 774, MAR 1 and DOR 500
CNFC 15044	Carioca	RS: Aporé, BAT 477, AN 9022180, Corrente, A 806, MAN 48, AND 277, IPA 7, A797, Pinto Villa, EMP 81, V 8025, A 525, A 774, MAR 1 and DOR 500
CNFC 15049	Carioca	RS: Aporé, BAT 477, AN 9022180, Corrente, A 806, MAN 48, AND 277, IPA 7, A797, Pinto Villa, EMP 81, V 8025, A 525, A 774, MAR 1 and DOR 500
CNFC 15070	Carioca	RS: Aporé, BAT 477, AN 9022180, Corrente, A 806, MAN 48, AND 277, IPA 7, A797, Pinto Villa, EMP 81, V 8025, A 525, A 774, MAR 1 and DOR 500
CNFC 15082	Carioca	RS: Aporé, BAT 477, AN 9022180, Corrente, A 806, MAN 48, AND 277, IPA 7, A797, Pinto Villa, EMP 81, V 8025, A 525, A 774, MAR 1 and DOR 500
CNFC 15086	Carioca	RS: Aporé, BAT 477, AN 9022180, Corrente, A 806, MAN 48, AND 277, IPA 7, A797, Pinto Villa, EMP 81, V 8025, A 525, A 774, MAR 1 and DOR 500
CNFC 15097	Carioca	OXI-16
BRS Estilo	Carioca	Control variety
Pérola	Carioca	Control variety

*RS = recurrent selection.

H_2O in proportions ranging from 1:5 to 1:10 depending on the concentration of the amplified products. Electrophoresis was performed using a mixture containing 0.5 µL diluted amplification product, 0.08 µL 500 ROX size standard (Applied Biosystems), and 9.42 µL formamide Hi-Di (Applied Biosystems). The electrophoresis was performed on an ABI3100 platform (Applied Biosystems) using filter "D" for fluorescence reading. The analysis of the fragments was performed using GeneScan Analysis 2.1 (Applied Biosystems), and the sizes of the alleles were obtained using the GeneMapper 3.5 program (Applied Biosystems).

Statistical analyses

The genetic analysis was conducted based on the molecular profiles generated from the allele frequencies of polymorphic loci, expected heterozygosity (H_E), and observed heterozygosity (H_O), according to Nei (1987). The number of private alleles was determined using the Genetic Data Analysis software (Lewis and Zaykin, 2001). The genetic distances were estimated by the Rogers coefficient modified by Wright using the NTSYS v.2.02 soft-

ware (Rohlf, 1993) and then subjected to cluster analysis using the UPGMA algorithm. The data were plotted on a dendrogram for the visualization of genetic distances. The estimated power of exclusion, probability of identity, and number of identical individuals were calculated using the Identity v.1.0 software (Wagner and Sefc, 1999). Wright F-statistic was calculated with 10,000 bootstrap resamplings using the FSTAT v.2.9.3.2 software (Goudet, 2002). The factorial correspondence analysis (FCA) was obtained using the GENETIX v.4.03 software (Belkhir et al., 2004). Structure analysis was performed using the STRUCTURE v.2.2 software (Pritchard et al., 2000; Falush et al., 2003). The ΔK statistic (Evanno et al., 2005) was used to determine the number of genetically homogeneous groups using the Structure software (Earl and VonHold, 2011).

Table 2. Genotyping panels composed of 36 microsatellite markers used for the molecular characterization of common bean cultivars, including the name of the loci, their respective fluorescence, amplification range in bp, annealing temperature, amplification patterns, chromosomes and references.

Panel	Marker	Fluorescence	Size range (bp)	Amplification pattern	Chromosome	References
1	BM185*	HEX	97-114	Specific	4	Gaitán-Solís et al. (2002)
	BM138	NED	196-202	Specific	7	Gaitán-Solís et al. (2002)
	BM183*	6-FAM	142-156	Specific	4	Gaitán-Solís et al. (2002)
	PvBR163*	6-FAM	210-330	Specific**	1	Grisi et al. (2007)
2	BM143*	HEX	110-168	Specific	9	Gaitán-Solís et al. (2002)
	BM212*	NED	194-210	Specific	8	Gaitán-Solís et al. (2002)
	PvBR025*	6-FAM	153-177	Specific**	9	Buso et al. (2006)
3	PvBR005*	HEX	168-193	Specific	1	Buso et al. (2006)
	BM164	NED	147-182	Specific	9	Gaitán-Solís et al. (2002)
	PvBR35	6-FAM	204-250	Specific	4	Grisi et al. (2007)
4	BM155	HEX	107-111	Specific	7	Gaitán-Solís et al. (2002)
	PvBR168	HEX	180-185	Specific	11	Grisi et al. (2007)
	PvBR087*	NED	150-188	Specific**	5	Grisi et al. (2007)
	BM140	6-FAM	157-175	Specific	10	Gaitán-Solís et al. (2002)
	BM114*	6-FAM	209-261	Specific	11	Gaitán-Solís et al. (2002)
5	BM187*	HEX	162-267	Specific**	1	Gaitán-Solís et al. (2002)
	PvBR113*	NED	74-100	Specific	6	Grisi et al. (2007)
	BM181*	NED	182-190	Specific	5	Gaitán-Solís et al. (2002)
	PvBR053	6-FAM	165-171	Specific	3	Grisi et al. (2007)
6	PvBR012	HEX	188-193	Specific	8	Buso et al. (2006)
	BM068	NED	169-171	Specific	10	Gaitán-Solís et al. (2002)
	BM175	6-FAM	155-188	Specific	7	Gaitán-Solís et al. (2002)
	BM149	6-FAM	245-255	Specific	10	Gaitán-Solís et al. (2002)
7	BM165*	HEX	170-187	Specific	3	Gaitán-Solís et al. (2002)
	PvBR215	NED	220	Specific	6	Grisi et al. (2007)
	BM202*	6-FAM	134-156	Specific**	11	Gaitán-Solís et al. (2002)
	BM154*	6-FAM	160-307	Specific**	11	Gaitán-Solís et al. (2002)
	BM211*	6-FAM	181-242	Specific**	3	Gaitán-Solís et al. (2002)
8	BM201*	NED	96-114	Specific	2	Gaitán-Solís et al. (2002)
	PvBR251	HEX	203-205	Specific	2	Grisi et al. (2007)
9	PvBR011*	HEX	182-190	Specific	9	Buso et al. (2006)
	PvBR169*	HEX	204-212	Specific	6	Grisi et al. (2007)
	BM210*	NED	166-188	Specific**	4	Gaitán-Solís et al. (2002)
	BM189*	NED	102-112	Specific	5	Gaitán-Solís et al. (2002)
	PvBR013*	6-FAM	169-195	Specific	1	Grisi et al. (2007)
	PvBR272*	6-FAM	78-116	Specific**	11	Grisi et al. (2007)

*Polymorphic markers used to derive the genetic parameters for analysis. **Markers that showed interpretable PCR products, although they contained some nonspecific bands.

RESULTS AND DISCUSSION

Characterization of microsatellite markers

Recently, the use of microsatellite markers for individual genotyping has evolved for the development of semi-automated, multilocus genotyping systems due to the increase in automation and efficiency of the reagents for molecular analysis. Over the years, the goal has been to develop universal and robust genetic identification systems to analyze genetic relationships individual identification, population structure, and genetic diversity. The nine multiplex systems used in the present study consisted of 36 microsatellite markers exhibiting no evidence of linkage and were widely distributed across 11 chromosomes in the bean genome according to genetic map information (Grisi et al., 2007; Garcia et al., 2011). The number of marker sets per system ranged from 3 to 6, with an average of 4 markers per panel. The same amplification conditions were used, which allowed for fast, efficient, and simultaneous amplification of the loci. Based on tri-nucleotide repetition, all markers, with the exception of marker BM149, were derived from di-nucleotide repeats with alleles that differ by 2 bp, which is the type of marker currently used for identification purposes in *P. vulgaris*. Despite the inherent limitations of analysis based on di-nucleotides, such as difficulties in interpretation due to stuttering and low detection power for alleles that differ by a few base pairs even when using high-resolution electrophoresis, genotyping based on di-nucleotide repeats still predominates among plants due to their high abundance and ease of identification in the genomes. More recently, because of efforts in genomic sequencing and the availability of expressed sequence tag databanks, a greater number of tri-nucleotide markers are being developed (Blair et al., 2011).

Of the 36 markers used in the VCU analysis, 24 were polymorphic for the five tests. The total number of identified alleles was 187, varying from 3 to 18, with an average of 7.79 alleles per locus. The average H_E identified among loci was 65%, the largest H_E was identified at locus BM154 (88%) and the lowest H_E at the BM212 locus (23%) (Table 3). The H_O, which is a genetic diversity index influenced by the reproductive system of the species, ranged from 0 to 0.045, with an average of 0.013, due to the self-pollination process for obtaining new grains or seeds. Studies on cross-pollination of the common bean involving wild, cultivated (Zizumbo-Villarreal et al., 2005; Ferreira et al., 2007), or transgenic germplasm (Faria et al., 2010) have reported similar results, with outcrossing rates below 2% and slight variations depending on the cultivar and environmental conditions.

The data also allowed for a comparison of results between laboratories based on common markers previously characterized in diverse sources of germplasm accessions, such as all the markers belonging to the "BM" series and some markers belonging to the "PvBr" (PvBr35, PvBr87, PvBr163, PvBr243) series. Overall, the estimates of genetic diversity based on microsatellite markers described in other studies have been small compared to the set of markers used in the present study. Benchimol et al. (2007) characterized varieties of the common bean with a set of 87 di-nucleotide SSRs in two of the major gene pools (Andean and Mesoamerican). These authors observed an average number of alleles of 2.82 and an average PIC of 0.45, significantly lower than those found in the present study (7.79 and 0.65, respectively). Similarly, Métais et al. (2002) reported an average PIC of 0.44 for 15 markers based on the analysis of 45 common bean lines representing nine classes of

grains. Recently, studies by Blair et al. (2009) using the cultivated and wild germplasms of Andean and Mesoamerican origins revealed close PIC estimates for di- (0.48) and tri-nucleotides (0.40).

Table 3. Genetic descriptors of 24 microsatellite loci of the common beans analyzed, including the number of alleles per locus (N_A), expected heterozygosity (H_E), observed heterozygosity (H_O), probability of exclusion (PE) and probability of identity (PI).

Locus	Size range (bp)	N_A	H_E	H_O	PE	PI
BM114	209-261	7	0.583	0.022	0.353	0.303
BM143	110-168	9	0.819	0.006	0.636	0.109
BM154	160-307	17	0.876	0.037	0.752	0.051
BM165	170-187	5	0.465	0.017	0.212	0.514
BM181	182-190	3	0.306	0.000	0.141	0.589
BM183	142-156	6	0.575	0.006	0.352	0.300
BM185	97-114	7	0.764	0.034	0.550	0.161
BM187	162-267	11	0.745	0.026	0.533	0.167
BM189	102-112	5	0.610	0.006	0.334	0.378
BM201	96-114	7	0.756	0.045	0.537	0.172
BM202	134-156	5	0.605	0.013	0.313	0.410
BM210	168-188	9	0.635	0.017	0.367	0.342
BM212	194-210	3	0.230	0.000	0.108	0.655
PvBr005	168-193	8	0.814	0.044	0.626	0.115
PvBr011	182-190	3	0.478	0.000	0.198	0.569
PvBr013	169-195	7	0.815	0.028	0.626	0.115
PvBr025	153-177	11	0.771	0.011	0.574	0.145
PvBr035	204-250	5	0.582	0.006	0.290	0.446
PvBr087	150-188	8	0.636	0.000	0.389	0.295
PvBr113	74-100	8	0.724	0.022	0.498	0.201
PvBr163	210-330	18	0.839	0.025	0.701	0.066
PvBr169	204-212	3	0.406	0.035	0.170	0.573
PvBr243	221-255	10	0.832	0.018	0.674	0.085
PvBr272	78-116	12	0.873	0.006	0.739	0.058
Average	-	7.79	0.656	0.013	-	-
Total		187	-	-	0.999	1.32×10^{-16}

In the present study, the markers selected for the genotyping panels also had high discriminatory power, which was confirmed by the high power of exclusion (0.999) and low values of combined probability of identity (1.32×10^{-16}). Thus, the probability of identifying two individuals with the same genotype in a population using this set of markers is extremely low (Table 3). However, even with the high power of discrimination among accessions by microsatellite markers used in the present study and blind experiments for genetic analysis, where the ratio of genetic relationships among the samples was not known, the results indicated the presence of identical genotypes within and between VCU tests. This result was due to the selection of similar lines for testing. The presence of identical genotypes could be avoided by tighter control of the pedigree or by using the microsatellite characterization results as a criterion to choose the accessions that will be part of the VCU tests.

To identify the genetically indistinguishable lines, new genotyping was performed using bulk decomposition and the criterion of at least two genetic differences to declare two materials as distinct from each other with high reliability. The identical genotypes included the carioca lines CNFC 9500 and CNFC 9494 from VCU 2003/2004 and the line CNFC 10444 from VCU 2004/2005, all of which were derived from the same cross (i.e., EMP 250 / 4 / A 769 /// A 429 / 252 XAN // V 8025 V / Pinto UI 114). The same result was observed for the black grain lines, CNFP 10805 and CNFP 10806, which were derived from the Millionaire 1732/Xamego cross.

The largest number of lines sharing the same molecular profile was observed in the 2011/2012 VCU test, where 12 lines exhibited total genetic identity with at least one line within the test itself. Similarly, the analysis allowed for the identification of genetically divergent lines between the selection cycles, which can drive the development of populations with wider genetic basis, thereby increasing the possibility of new allele combinations from accessions selected by breeders for the characteristics considered important.

Molecular characterization of VCU tests

A total of 150 lines integrating the five VCU tests were characterized. In addition, the estimates of genetic parameters per test and estimates based on the analysis of 24 common microsatellite loci were described (Table 4). A total of 145 alleles were identified in the 2003/2004 VCU test, and the average H_E among accessions was 0.50. The highest H_E was identified for the color group (0.63), and the lowest was observed in the weevil group (0.24). The highest estimates of genetic diversity obtained for the four remaining VCU tests were due to the more diverse composition of common bean accessions, because they are composed of five distinct grain classes. Similarly, the 2005/2006 VCU test, which had the second highest estimate of H_E (0.49), also included a test called "VCU Colors", which comprises three different types of beans (striped, purple, and pinkish).

Table 4. Genetic diversity estimators of cultivated germplasms including advanced lines of the common bean organized by value for cultivation and use.

VCU	Type	Accessions	Alleles	H_E	H_O	F_{ST}
VCU 2003/04	Carioca	17	103	0.571	0.020	0.122
	Black	14	85	0.499	0.018	95%CI = 0.083-0.163
	Colors	07	78	0.630	0.079	
	Mulatinho	13	86	0.576	0.008	
	Weevil	3	35	0.236	0.014	
		54	145	0.502	0.028	
VCU 2005/06	Carioca	10	62	0.402	0.083	0.259
	Black	8	64	0.495	0.000	95%CI = 0.184-0.336
	Colors	7	67	0.580	0.080	
		25	122	0.492	0.029	
VCU 2007/08	Carioca	13	64	0.466	0.000	0.286
	Black	10	66	0.443	0.000	95%CI = 0.194-0.380
		23	93	0.455	0.000	
VCU 2009/10	Carioca	13	84	0.497	0.024	0.190
	Black	10	64	0.408	0.041	95%CI = 0.123-0.256
		23	102	0.453	0.033	
VCU 2011/12	Carioca	15	62	0.314	0.000	0.415
	Black	10	54	0.394	0.000	95%CI = 0.328-0.501
		25	83	0.354	0.000	
Average		-	-	0.451	0.018	0.159*
						95%CI = 0.100-0.227
Total		150	189	-	-	

*VCU that is significantly different from zero ($P < 0.05$). H_E = expected heterozygosity; H_O = observed heterozygosity; F_{ST} = average inbreeding coefficient; 95%CI = 95% confidence interval.

Considering only the estimates derived from the classes of grains, such as black and carioca, the 2003/2004 VCU test exhibited the highest H_E value (0.53), followed by the 2005/2006, 2007/2008, and 2009/2010 VCU tests, with estimates close to 0.45; the lowest value was observed in the 2011/2012 test, with an H_E value of 0.35. Because the 2003/2004

and 2011/2012 VCU tests were performed with a similar number of lines (31 and 25, respectively), the reduction in H_E could have been attributed to the loss of genetic diversity caused by the selection of lines that were more similar, which was evidenced in the most recent VCU test (2011/2012). Particularly for tests performed with the carioca grain, the total H_E was reduced by approximately 50% and ranged from 0.57 to 0.31 for the 2003/2004 and 2011/2012 VCU tests, respectively. Similarly, the average number of alleles was reduced from 7.8 (2002/2003 VCU) to 4.13 (2011/2012 VCU). The diversity reduction in breeding programs indicates a narrowing of the genetic base upon which the lines are selected. This reduction may be due to the use of a limited number of parents, genetically related parents, and/or a frequent occurrence of the same ancestors. These results can drastically reduce the short-term genetic gain for agronomically important traits such as yield. The economic impact will be greater for cultivars of carioca grain, which currently represents 80% of the national preference, since only the variability enables the imposition of the selection process (Ramalho et al., 2004).

Several authors working in major research institutions in Brazil have reported estimates of genetic genetic gain for the culture of the common bean, especially for cultivars such as carioca. In general, the relative average progress is approximately 1.21 to 1.90% per year, representing absolute gains of approximately 14-30 kg/ha/year (Abreu et al., 2004). Recently, Matos (2005) reported gains of only 0.6% per year after evaluating the period from 1974 to 2004. According to Chiorato (2008), since 1997, when the use of recommended controls registered in MAPA (2011) in the VCU tests became mandatory, the relative productivity gains tended to be lower due to the difficulty of developing more productive genotypes than controls, even though new attributes, such as grain quality and disease resistance, are used as differentiators.

Based on the estimates of allele frequency, 16.6% of the alleles displayed a frequency of ≤5% and only 0.5% had a frequency of ≤1%. Of the 189 alleles identified using 24 microsatellites, 48 (26%) corresponded to private alleles (exclusive alleles), and the vast majority (65%) occurred at frequencies below 5%. A total of 15 lines (10% of the total) displayed exclusive alleles. The groups of grains with the highest number of private alleles were black (7 alleles) and carioca (6 alleles), which occurred in 12 of the 24 loci used in the analysis. These data are relevant because they allow for the selection of lines that have more divergent alleles to incorporate agronomically rare alleles and contribute to the expansion of genetic diversity in the pool of cultivated germplasm.

The estimates of genetic distance obtained from the VCU tests and the total pool of genotypes characterized are both important findings of the data analysis (Figure 1, Panels A and B). Among the five trials, the average genetic distance was 0.34. The smallest distance was that from the 2003/2004 and 2005/2006 VCUs (0.23) and the highest was that from the 2003/2004 and 2011/2012 VCUs (0.44) , with a cophenetic correlation coefficient (r) of 0.97 ($P \leq 0.031$). The more recent VCUs (2009/2010 and 2011/12) also displayed a low genetic distance (0.26), which is a troubling indication that the lines comprising each cycle of VCU are derived from genetically close groups. Based on the line and grain type, the smallest genetic distances were obtained between the carioca and black grains (0.32) and the highest among the black and weevil (0.53) groups, with an average of 0.41 and an index cophenetic correlation (r) of 0.98 ($P \leq 0.031$).

The distribution of the groups through spatial structure can be best visualized by the FCA illustrated in Figure 2. The establishment of a pre-breeding program, in which the cultivated germplasm serves as a source of genetic variability, is reported as a strategy to increase

the genetic base of cultivars (Rangel et al., 1996). A molecular characterization of 50 cultivars of *P. vulgaris* performed at different institutions (Cardoso, 2008) revealed an average genetic distance of 0.72 (r = 0.96), indicating that there is satisfactory genetic differentiation between materials. Thus, the recurrent use of these divergent genotypes with adequate characteristics can contribute effectively to increase the genetic base of the breeding program.

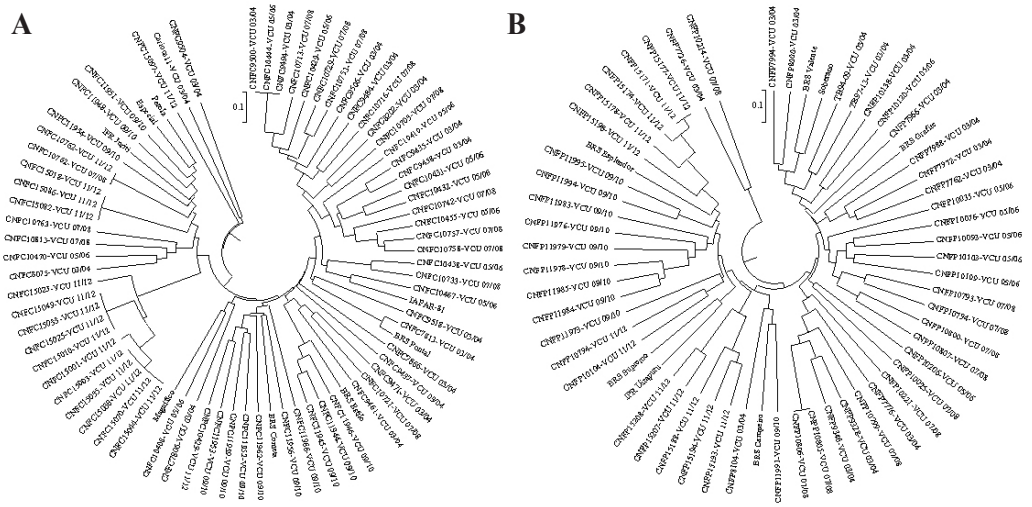

Figure 1. Dendrogram displaying the genetic distances between the carioca (Panel A) and black grain (Panel B) lines and controls based on the average genetic distance of Rogers' coefficient modified by Wright (P = 0.002; 1000 permutations).

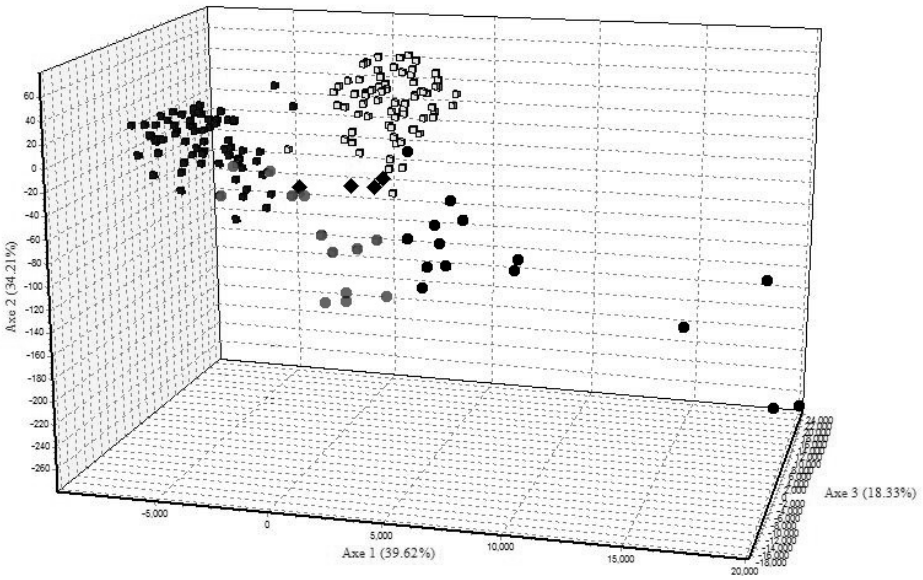

Figure 2. FCA and distribution pattern of genetic variability among the different line groups that are part of the VCU analysis: carioca group (white squares), black group (black squares), mulatinho group (gray circles), weevil group (diamonds), and VCU/color (black circles).

Applying Wright F-statistic to estimate genetic structure (VCU test) (Table 4), the inbreeding coefficient (F_{ST}) values indicated a high degree of differentiation in the tests evaluated, with indices ranging from 0.122 (VCU 2003/2004) to 0.415 (VCU 2011/2012). However, the inbreeding coefficient values decreased between tests of the same grain type due to the frequent occurrence of the same ancestors in the genealogy of the studied lines (Table 1). The average F_{ST} value estimated between the five VCU tests was 0.159, indicating genetic differentiation among the line groups tested. This differentiation could be attributed to the selection of differentiated progenitors that represent the specific tests for each type of grain. This result occurs because the parents that are used for crossing and/or backcrossing (obtaining genetic variability for exploitation in the breeding program) are selected based on Brazilian regions targeted by the program and for characteristics such as productivity, adaptation, and resistance/tolerance to pests and diseases, as well as other specific characteristics. The largest structure was observed (F_{ST} = 0.415) between the groups of black and carioca grains in the 2011/2012 VCU test. These estimates are consistent with those obtained using the method for obtaining lines. The black grain lines were derived from bi-parental crosses composed of only five parents, and the carioca grain lines were obtained by recurrent selection consisting of 14 parents, resulting in higher differentiation between groups.

In addition, Bayesian clustering analysis performed using the STRUCTURE software resulted in the formation of two distinct groups (K = 2). This division can be attributed to the genotypes that comprise the population base of the breeding program. The lines of the three VCUs conducted from 2003 to 2008 share part of the genealogy of the different groups of grains characterized, and the same result is found between the last two tests (2009-2012). This structure is explained when the pedigrees of the materials are verified, corresponding to line members of the VCU tests over the different periods. The blocks of crosses performed resulted in a large number of lines with characteristics that are interesting for the breeding programs, and, over the years, these lines were evaluated for agronomic performance using VCU tests. Lines derived from the same initial crossing block can constitute consecutive VCU tests due to the program pressure to maintain target characteristics over a number of years. When lines share the same ancestry and the potential to evaluate the VCUs is not available, lines derived from new crosses are developed and integrated into subsequent VCUs. Thus, VCUs conducted in consecutive years tend to group according to their genealogy, and therefore, sharing of the same genetic structure is expected.

CONCLUSIONS

Genetic diversity analysis from VCU testing using molecular markers generated relevant data for the common bean breeding program, such as determining and monitoring the level of genetic variation between groups of lines within and between tests and the identification of genotypes that are genetically indistinguishable. Consequently, these genetically indistinguishable lines can be eliminated from the tests. These findings lead to direct cost reductions and to the identification of the molecular profile of each strain, thereby constituting an innovative tool for genetic diversity analysis of the cultivar of the common bean for the process of varietal protection. The set of di-nucleotide loci used in the present

study resulted in the establishment of a genetic analysis system for the common bean with high resolving power. This system currently integrates and complements the routine activities of the program, thus constituting effective molecular tools to characterize and distinguish bean lines. The fact that the carioca and black bean commercial lines have reduced genetic variability indicates the need for the inclusion of parents with a larger genetic base in the blocks of the initial crosses to allow for the emergence of new allelic combinations in the bean line selection program. The data generated from the molecular analysis proved to be effective, providing a real scenario of genetic diversity contained in the gene pool integrated by lines used in the VCU tests of the common bean. This result allows for new strategies to be planned to increase the genetic base from which the program is conducted and, therefore, to increase the genetic gain per selection cycle to minimize the risk of vulnerability to biotic and abiotic stresses and the occurrence of low levels of productivity.

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