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ARTICLE
Development of microsatellite markers based on BAC common bean clones

Eveline Teixeira Caixeta[1*], Aluízio Borém[2], and James D Kelly[3]

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ABSTRACT - *The eukaryotic genome owns simple sequence repeats in tandem, called microsatellites or SSR. The microsatellite-flanking DNA sequences are generally conserved among the plants of a same species, allowing that primers complementary to these regions may be used to amplify SSR-containing fragments. These repetitive regions are therefore used as molecular markers. In the present study, some microsatellite markers were developed using bacterial artificial chromosome (BAC) clones as DNA source. Four BAC clones were sub-cloned, hybridized with three different probes and the positive clones were sequenced. Specific primers were designed and tested in common bean genotypes. Twenty-one pairs of primers amplified single and well-defined bands. One to six alleles per locus were generated, demonstrating the high polymorphism of these markers. This set of developed microsatellite markers together with the other available markers are an important tool in the breeding and genetic study of common bean.*

Key words: SSR, molecular markers, common bean, *Phaseolus vulgaris*.

INTRODUCTION

The eukaryote genomes contain different types of repetitive DNA. The repetitive class denominated microsatellites or SSRs (simple sequence repeats) consist of a short DNA sequences, usually from 1 to 5 bases in length that are tandemly repeated. Preliminary studies showed that the microsatellites are abundant and widely distributed in the plant genome (Morgante and Olivieri 1993, Brunel 1994, Cardle et al. 2000). Morgante and Olivieri (1993) analyzed the EMBL and GenBank data and estimated a frequency of 1 microsatellite, considering di- and trinucleotides, for every 50 kb. Since microsatellites are more commonly found outside of the coding sequences and since the proportion of cDNA clones in plant database is high, the authors suggested that the real frequency could be much higher than the one observed. Cardle et al. (2000) confirmed this possibility, estimating a frequency of 1 SSR for every 6-7 kb.

Microsatellite flanking sequences are conserved among genotypes of the same species, therefore a pair of primers complementary to these regions can be used to amplify DNA fragments containing SSR, making these repetitive regions as a molecular markers. The

[1]BIOAGRO, Universidade Federal de Viçosa (UFV), Viçosa, MG, Brasil, 36.570-000, Brasil. *E-mail: eveline.caixeta@embrapa.br
[2]Departamento de Fitotecnia, UFV
[3]Department of Crop and Soil Science, Michigan State University, E. Lansing, MI, 48824, USA

polymorphism of these markers is mainly a result of the variation in the number of repeat units (Cregan et al. 1999). Tandem-repeated regions such as the SSRs increase the probability to occur unequal crossing-over and DNA polymerase slippage during replication, leading to differences in the number of tandem repeats (Levinson and Gutman 1987, Hammond-Kosack and Jones 1997).

This co-dominant molecular marker detects a high level of allelic variation, which together with the economical and simple PCR procedure, has become an useful tool for studies of eukaryotic genes (Akkaya et al. 1992, Panaud et al. 1996). Besides these features, the reproducibility, high frequency and wide distribution along the genome make them the favored markers for many plant breeding programs (Rallo et al. 2000). SSRs have been used in fingerprinting and variety identification, studies of population dynamics, gene diagnostics, genome mapping, linkage studies and marker-assisted selection (Morgante and Olivieri 1993, Rongwen et al. 1995, Panaud et al. 1996, Chen et al. 1997, McCouch et al. 1997, Stajner et al., 2005).

Molecular markers as RAPD, SCAR and AFLP are already being used to study common bean genetics and applied in breeding programs (Miklas et al. 1993, Haley et al. 1993, 1994, Tohme et al. 1996, Young and Kelly 1997, Kelly and Miklas 1998, Park et al. 1999, Caicedo et al. 1999, Yu et al. 2000a, Alzate-Marin et al. 2001). However, it is necessary to incorporate, in these studies, markers which detect a higher level of polymorphism so that closely related germplasms can be discriminated and crosses between related genotypes analyzed (Panaud et al. 1996). A higher level of polymorphism would also increase the possibility of detecting markers closer to the gene of interest, which would therefore be more effective in breeding programs.

Despite a high polymorphism and a facility of PCR-based markers, microsatellite markers have not been used in common bean, due the limited number of SSR primers developed for this crop. According to Panaud et al. (1996), the development of these primers is expensive and time consuming, but once made available, they are easily incorporated in breeding programs thanks to their simplicity and inexpensive use. On this background, our study aimed at the development of SSR markers for common bean, using BAC (bacterial artificial chromosome) clones as DNA source. Since the inserts sizes of these clones vary from 70 to 120 kb and the SSR frequency in plants is evidently high, the expectation was to identify one or more of this type of markers in these regions.

MATERIAL AND METHODS

Genetic materials and DNA extraction

Ten common bean cultivars were selected to test the developed microsatellite primers. Six from these cultivars belongs to the common bean-breeding program for resistance to angular leaf spot, developed by BIOAGRO-UFV, that is, Rudá, BAT 332, Cornell 49-242, Mexico 54, MAR-2, and AND 277. The other ones, Jalo EEP 558, BAT 93, Black Magic, and Sel 1308 are elite lines from the common bean breeding program at Michigan State University.

The DNA of each genotype was isolated from young leaves and extracted based on the slightly modified methodology of Doyle and Doyle (1990).

Subcloning

Four clones of bacterial artificial chromosomes (BAC clones) were used as DNA source for the construction of a library containing small inserts. The BACs (78K17, 53Eg, 56P12 and gF11) were supplied by Dra. S. MacKenzie, University of Nebraska, to the Commom Bean Breeding laboratory of Michigan State University (MSU Dry Bean Breeding & Genetics Program). These BAC clones were selected from a library derived from the common bean cultivar Sprite, using SAS13 as probe. This probe is associated to locus *co-4* which confers resistance/ susceptibility to the fungus *Colletotrichum lindemuthianum* that cause anthracnose in common bean (Vanhouten and Mackenzie 1999).

An equal amount of each BAC clone (approximately 5 µg of each one) was filled into an Eppendorf™ tube and treated with the restriction enzyme *Not* I (BRL Gibco) for 2 hours at 37 ºC, according to the manufacturer's recommendations. The digestion allowed a separation of the inserts from the vectors, which was confirmed in 1% agarose gel. With the objective of obtaining blunt ends the inserts were treated with Klenow enzyme (Gibco-BRL), precipitated in sodium acetate and 100% ethanol and divided in three aliquots. On each aliquot was added 0.1 mM of BSA (bovine serum albumin) and 20µL of the buffer 10x NEBuffer 4 (New England Biolabs) which contains 20mM Tris-acetate, 10mM magnesium acetate, and 50 mM potassium acetate. One aliquot was digested with the restriction enzymes *Alu* I and *Hpa* I, another with *Rsa* I and *Nae* I and a third with *Hinc* II and *Xmn* I. To all aliquots, 15 units of each enzyme (New England Biolabs) were added and the solution incubated for 18 hours at 37 ºC.

After the digestion with the restriction enzymes, the aliquots were incubated with 15 units of CIP (Alkaline Phosphatase, Calf Intestinal) (New England Biolabs) for 15 minutes at 37 ºC and then at 56 ºC for 15 minutes. Fifteen more units of the enzyme were added and the same two

incubations of 15 minutes at 37 ºC and 56 ºC were realized. The aliquots were then united and the fragments purified with phenol-chloroform-isoamylic alcohol (25:24:1) and precipitated with sodium acetate and 100% ethanol. The precipitate was resuspended in 40 µL water. A total of 1.5 µL of this solution (±0.5 µg of DNA) was used in the ligation reaction with 0.5 µg of the vector pBluescript + KS, previously digested with 10 units of *Sma* I (New England Biolabs). Five units of T4 DNA ligase (Invitrogen) were used, 4.0 µL of the enzyme buffer and 4.5 µL water. The solution was incubated at 16 ºC for 18 hours, followed by the ligase denaturation at 65 ºC for 10 minutes. Competent cells of *Escherichia coli* TOP 10F' (Invitrogen Corp.) were transformed with the vectors linked to the inserts, according to the manufacturer's instructions.

Hybridization with microsatellite probes

Transformed cells were plated on LB agar with 75 µg mL^{-1} ampicillin, 40 µL X-gal (40mg m L^{-1}) and 40 µL IPTG (100 mM) per plate. White colonies containing vector and insert were picked for another Petri plate. Each plate was replicated three times. After 12 hours of growth, the bacterias containing the inserts were lifted onto a positively charged nylon membrane. The membrane was treated with 10% SDS (sodium dodecyl sulfate) for 3 minutes to avoid the diffusion of the plasmid, resulting in a stronger hybridization signal. Later, the cells were denatured in 1.5 M NaCl and 0.5 M NaOH solution for 15 minutes, neutralized in 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) solution for 15 minutes and washed in 0.2 M Tris-HCl (pH 7.5) solution and 2x SSC buffer for 30 seconds. The DNA was linked to the membrane using Stratalinker UV (120 000 µJ of UV) for approximately 30 seconds. The DNA-containing membranes were hybridized with three different probes, $(AT)_{15}$, $(CT)_{15}$, $(ATT)_{10}$, one for each replicate. The probes were previously digoxigenin-marked with a DIG Oligonucleotide Tailing Kit (Roche). The membrane was washed twice in 2x SSC and 0.1% SDS solution for 5 minutes each at room temperature. Thereafter, it was treated twice with 0.5 x SSC and 0.1% SDS solution, 15 minutes each at 62 ºC. Finally it was washed twice in 0.2 SSC and 0.1% SDS solution, 15 minutes each at 62 ºC. After this washing, the membranes were treated with 0.25 mM of CDP-STAR (Roche) and exposed to the X-ray film for an appropriate period of time (5-10 minutes) to give small but clearly defined hybridization signals. The positive clones were picked on the Petri plate and a second hybridization was done. The selected colonies were stored in glycerol at -80 ºC for long term preservation.

The plasmids of all selected clones were isolated using the kit Wizard Plus SV Minipreps – Spin protocol (Promega). To test the presence and determine the insert size of the plasmids they were treated with 5 units of the restriction enzyme *Pvu* II (New England Biolabs) and run on 1% agarose gel. The genomic inserts were sent to Genomics Tecnhnology Support Facility (Michigan State University, East Lansing, MI) where they were sequenced by means of Applied Biosystems cycle sequencing tecnhology. DNA fragments larger than 700 bp were sequenced based on both ends and analyzed with BL2SEQ WorkBench (San Diego Supercomputer Center, University of California, San Diego). The same program was used to determine redundant sequences. SSEARCH (Smith-Waterman Local Alignment), another WorkBench program, was used for detecting microsatellite sequences.

Primer selection and PCR analysis

Specific primers containing 18 to 25 bp complementary to the microsatellite-flanking sequences were synthesized. The primer pairs were designed, using the program PRIMER3 of WorkBench (San Diego Supercomputer Center, University of California, San Diego), to amplify a PCR product with an expected size between 150 and 300 bp and a Tm of 45 to 50 ºC. The use of homogeneous Tm value allowed a PCR amplification of all SSRs to be carried out at the same annealing temperature.

The primers were tested in the BAC clones that the primer sequences were derived and in 10 common bean varieties. PCR reactions were performed in a 20 µl solution containing 50 ηg DNA, 0.8 units of taq DNA polymerase (Perkin Elmer), buffer 1x, 1.5 mM $MgCl_2$, 200 µM of each dNTP and 0.15 µM of each primer. The amplification was carried out in a thermocycler using the touchdown PCR procedure. The procedure consisted in an initial denaturation for 2 minutes at 94 ºC, followed by 15 cycles with a denaturation of 30 seconds at 94 ºC, an annealing of 30 seconds, and an extension of 30 seconds at 72 ºC. The annealing temperature was 62 ºC to 48 ºC, sinking 1 ºC in each cycle. After the 15 cycles, 20 more cycles with denaturation at 94 ºC, annealing at 47ºC and extension at 68 ºC were run for 30 seconds per step. A final extension step was included for 5 minutes at 72 ºC. The PCR products were separated on a 6% denaturing polyacrylamide gel and visualized by silver staining solution.

RESULTS AND DISCUSSION

Microsatellite markers for common bean were developed using four BAC clones available in the Commom

Bean Breeding laboratory of Michigan State University. The BAC clones were subcloned and 890 colonies hybridized with three microsatellite probes $(AT)_{15}$, $(CT)_{15}$ and $(ATT)_{10}$. These oligonucleotides were chosen as probes because they are the most frequent microsatellites in the superior plants genome (Morgante and Olivieri 1993, Brunel 1994, McCouch et al. 1997). Yu et al. (1998, 1999), analyzed the common bean microsatellites, and found the same repetitions, AT/TA and CT/AG, as the most common in this species.

From 890 colonies analyzed, 375 were positively hybridized in the first selection. Of these, 72 were positive in the second hybridization, which correspond to 8% of the total analyzed colonies. By using restriction enzyme and agarose gel it was verified that of the potential colonies, 62 had genomic insert. The 62 clones were sequenced and 27 showed microsatellite sequences (Table 1).

Table 1. Result of the sequenced clones

Sequenced clones	**62**
Without insert	6
Contamination with *Escherichia coli* DNA	3
Redundant sequence	4
Problems in sequencing	6
Without microsatellites	16
Sequences with microsatellites	27

Two clones with microsatellite-containing sequences could not be used since the repeat units were very close to the insert end, making the primers design impossible. Some clones had more than one microsatellite. When possible, the primers were obtained so that more than one SSR was amplified. In some cases more than one pair of primers was designed for the same clone, one for each SSR found.

Specific primers were designed flanking 29 microsatellite sequences. To design these primers a Tm between 45 and 50 ºC was used as criterion. This range of temperature was the same chosen for other primers developed for common bean (Yu et al. 1999), which enables the entire set of common bean microsatellite primers to be amplified under the same PCR conditions.

The polymorphism of the developed microsatellites were investigated by amplifying the BAC clones that originated the sequences and 10 common bean cultivars, Rudá, BAT 332, Cornell 49-242, México 54, MAR-2, AND 277, Jalo EEP558, BAT 93, Black Magic, and Sel 1308. Of the primer pairs tested, three did not amplify any product, while five amplified several bands. The amplification of several bands may be, according to Akagi et al. (1996), consequence of inappropriate primer sequences or improper PCR conditions. The lack of amplification of alleles can be ascribed to the divergence in the SSR-flanking sequences, creating a null-allele (Smulders et al. 1997), but in this case the BAC clones that originated the primer, must have PCR product. Another possibility is improper PCR conditions for these primer pairs (Lavi et al. 1994).

Twenty-one primer pairs, which represented 72.4% of the total of primers tested, amplified single and clearly defined bands. Fifteen amplified polymorphic bands and six monomorphic ones (Table 2). The allele numbers per locus ranged from one to six. Some examples of these amplifications are shown in Figure 1. The nomenclature of the developed microsatellites was established according to the criteria proposed by Yu et al. (2000b): PV stands for *Phaseolus vulgaris*, followed by the repeat unit in lower case and an arbitrary number beginning at 001. This number rises as microsatellites with the same repeat unit are being developed.

The primers PV at010 amplified the microsatellite with the largest number of repeat units and amplified PCR products with the greatest allelic variation. According to Weber (1990), the number of alleles in a microsatellite is usually correlated with the number of repeat unit they presents; in general, a higher number of repeats leads to a higher polymorphism. However, some primers observed in this research, such as PV taaa003 and PV taaaa001, had SSRs with only three repeats and amplified two alleles, whereas the pairs PV ag006, PV taa002 and PV att004 with 11, 6 and 5 repeats, respectively, present monomorphic bands. These observations suggest the absence of a correlation between the number of repeat units and the number of detected alleles. This lack of correlation was also mentioned by Panaud et al. (1996), Yu et al. (1999) and Rallo et al. (2000).

In our study, polymorphisms were detected in microsatellites with a small number of repeat unit such as $(TAAA)_3$. There is no definition of the number of repeats required to consider a sequence as microsatellite (Yu et al. 1999). Petes et al. (1997), however, suggested a size varying from 15 to 50 base pairs. The data obtained by Yu et al. (1999) demonstrated that microsatellites with small repeats number are also important sources for the development of polymorphic SSR markers in common bean, confirming what we observed in the present work.

The polymorphisms revealed by some primer pairs showed that a length difference between alleles is not always multiple of the nucleotide number of the repeat

Table 2. Description of the developed microsatellites and their allelic variation in the 10 tested common bean genotypes

Nome*	SSR	Primer sequence	Band size (bp)	Nr. of alleles	Tm
PV attt001	$(ATTT)_5$	caaaaagaacataatcagaa	165	3	48
		cttagttgtattgatttgga			48
PV catt001	$(CATT)_3$	agggtgtcatagattagatag	193	1	48
		gagatccttatattgtaatgtg			48
PV ct001	$(CT)_2$ $(CAT)_2$	gatactatcaagacacgattac	248	2	48
	$(CT)_4$	gtatatacactgaagcaataagt			48
PV ag006	$(CA)_5$ n $(AG)_6$	atataaatactaggacgatgac	210	1	47
		atattgacttatgagtatctgag			47
PV att001	$(ATT)_4$ n	gtttctataattttctcacttaac	295	2	49
	$(TTTA)_3$	gtatcatttatgttgtattcct			48
PV aaat002	$(AAAT)_4$	aggaatacaacataaatgatac	238	2	48
		gatactcaaagtaaaattggt			48
PV at010	$(AT)_{16}$	agactcacgtttcttatttt	171	6	48
		gacttaatcttatatgggacat			50
PV gtat001	$(GTAT)_3$	acgtgtcctatagtttgttac	272	1	49
		cttttactcaggttctcttta			49
PV taa002	$(TG)_2(TAA)_4$	aagataatcctaagaggtga	300	1	48
		gttataatccgatcatgttt			49
PV taaa003	$(TAAA)_3$	gtcaatataaattattgtaagc	221	2	46
		gatttagtttgatttattgtgt			48
PV ttc001	$(TTC)_4$ n	atatttagtggactgtcaaa	192	3	47
	$(GT)_2(TG)_3$	gttatcaaatgtaaagggat			48
PV ttc002	$(TTC)_4$ CC	atatcttacagccattacattc	203	2	51
	$(TCGG)_3$	ctcatcacccagtcacct			54
PV aat002	$(AAT)_4$ n	tcctaattatttatggtaagag	121	2	48
	$(AATT)_3$	caattgtatatagagtcaactaaa			48
PV ct002	$(CT)_5$	ttagactttcaaacattcac	122	2	48
	$(TGTT)_3$	gatactacttaaatgaggaaca			48
PV taaaa001	$(TAAAA)_3$	gtcaatataaattattgtaagca	221	2	49
		gatttagtttgatttattgtgt			48
PV at011	$(AT)_6$	tatacaacgggagatattta	293	2	48
		tggttattaagaagctaagac			49
PV att003	$(ATT)_7$	aatcctacaaattatggc	237	2	47
	$(AGT)_2$	tcactaaagagatatgaactaac			48
PV taca001	$(TACA)_3$	ctttccttgagtattaagaag	220	1	48
		gactattctaaaatcttctcct			48
PV ag007	$(AG)_8$ n $(GA)_4$	agatgataactggtctgagt	187	2	48
		tttcttaaacactgatgatg			48
PV att004	$(ATT)_5$	tactgtcctttctttttctc	298	1	49
		catacggagatatttactcata			49
PV tgtt001	$(TTA)_2$	atattaacagtcttaccttgg	244	2	48
	$(TGTT)_3$	tttggtaaataagtgatgtc			48

* The microsatellites were named according to a nomenclature proposed by Yu et al. (2000b)

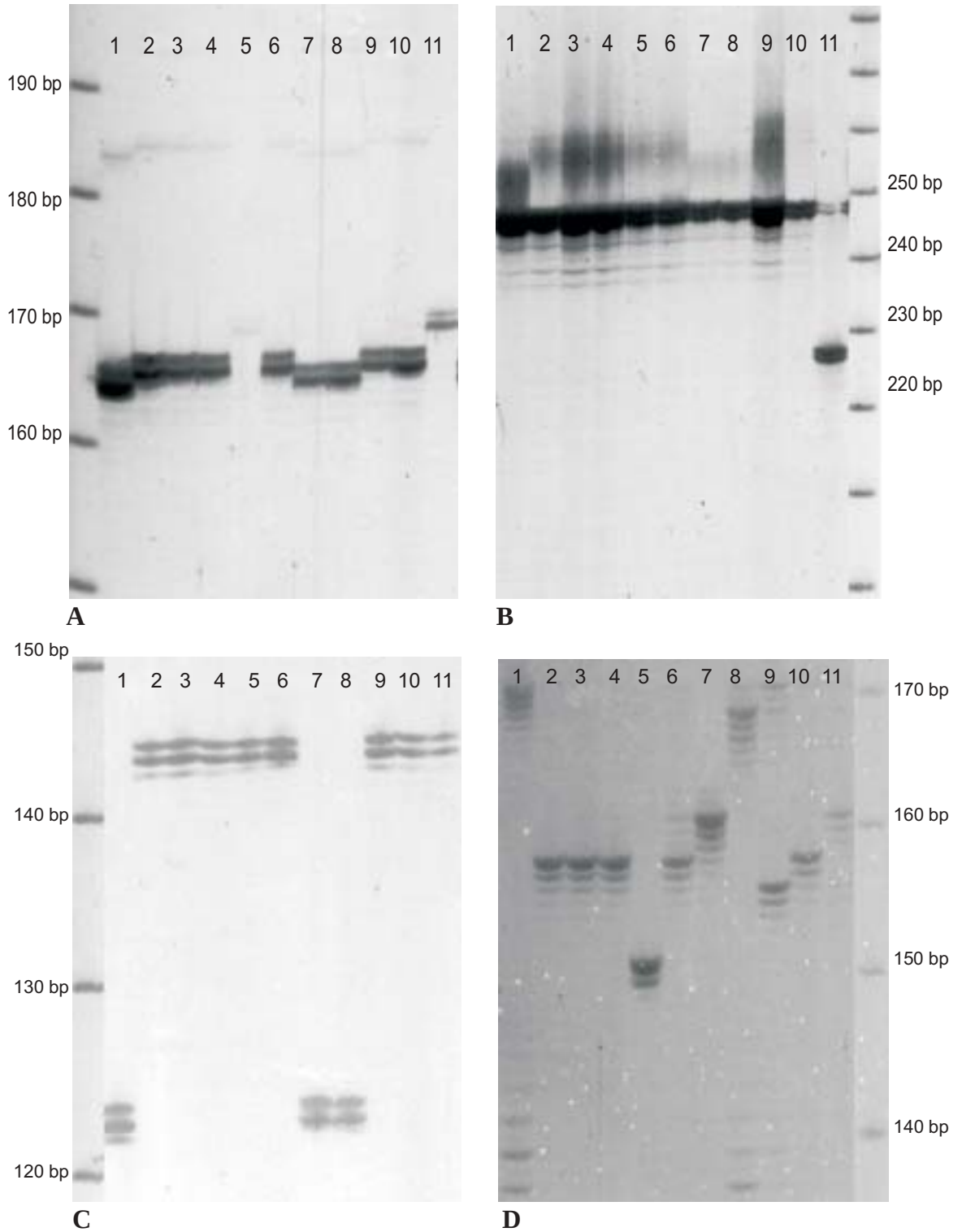

Figure 1. Electrophoretic analysis of the products amplified with some SSR primers developed in the present work. Line 1 represents the BAC clones; 2 - Rudá; 3 - BAT 332; 4 - Cornell 49-242; 5 - México 54; 6 - MAR-2; 7 - AND 277; 8 - Jalo EEP558; 9 - BAT 93; 10 - Black Magic; and 11 - Sel 1308. 1A: Amplification of microsatellite PV attt001; 1B: microsatellite PV ct001; 1C: microsatellite PV ct002; and 1D: microsatellite PV at010

unit. One example is the primer pair PV att001 that amplified the microsatellite $(ATT)_4n(TTTA)_3$, and, therefore has a repeat unit with 3 bp and another with 4 bp. This pair amplifies two bands with a difference of only 1 bp. This fact had already been observed by Rallo et al. (2000). According to these authors, the polymorphism of microsatellites may not only be based on the number of repeats, but also found in SSR-flanking regions. Deletions and insertions of a single base or even of long DNA fragments in flanking regions as source of variation of SSRs were mentioned in other studies (Gianfranceschi et al. 1998, Buteler et al. 1999). In the present work, we further observed that the primers pair PV ttc002 amplified a band of 203 bp in the BAC clone that originated it. This fragment contains the microsatellite TTC with four repeats and the TCGG with three repeats according to sequenced. However, these primers amplify a fragment of 153 bp in another genotype. This fragment with 50 bp less than the obtained in the BAC clone indicates that there was a much higher deletion in this genotype than the SSR. These data confirm that the variation of the microsatellite markers may be a result of deletions/insertions outside of the repeat units.

Microsatellites are effective markers for mapping as much as for assisted selection in breeding programs since they are co-dominant markers, PCR based and highly polymorphic. Their development, however, is work, time and cost-consuming (Rallo et al. 2000). In an attempt to solve these problems, in the present study, microsatellite markers were developed based on BAC clones. The adopted procedure showed to be efficient for the development fo microsatellite markers development. The strategy of usage available BAC clones, as there was no need of constructing a large DNA library, was relatively fast and involved lower cost and labor. However, only a small region of the genome can be analyzed with the developed primers, the one contained into the BAC clones. The future identification of these markers involving other libraries should be performed, so that the whole genome can be mapped with these markers. The utility of the microsatellite markers for breeding programs increases as a larger number of primers is being developed (Panaud et al. 1996).

The microsatellite markers developed in this work, combined with those developed earlier by Yu et al. (1999, 2000b) and together with RFLP, AFLP, RAPD and SCAR markers, provide an important and efficient tool for use in breeding and genetic studies of common bean. Additionally, these markers will be highly useful to saturate the specific area; the one containing the BAC clones, of the common bean linkage map.

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Desenvolvimento de marcadores microssatélites baseado em clones BAC de feijoeiro

RESUMO - *O genoma eucarioto possui seqüências simples repetidas em tandem, chamadas de microssatélites ou SSR. As seqüências de DNA que flanqueiam os microssatélites são geralmente conservadas entre os indivíduos de uma mesma espécie, permitindo que* primers *complementares a essas regiões sejam usados para amplificar fragmentos contendo os SSRs. Estas regiões repetitivas são, portanto, utilizadas como marcadores moleculares. No presente trabalho, foram desenvolvidos alguns marcadores microssatélites utilizando clones de cromossomos artificiais de bactérias (BAC) como fonte de DNA. Quatro clones BAC foram sub-clonados, hibridizados com três sondas diferentes e os clones positivos foram seqüenciados.* Primers *específicos foram desenhados e testados em genótipos de feijoeiro. Vinte e um pares de* primers *amplificaram bandas únicas e bem definidas. Foram gerados de um a seis alelos por loco, demonstrando o alto polimorfismo desses marcadores. Esse conjunto de marcadores microssatélites desenvolvidos combinados com os demais marcadores disponíveis são uma importante ferramenta para uso no melhoramento e no estudo genético do feijoeiro.*

Palavras-chave: SSR, marcadores moleculares, feijão, *Phaseolus vulgaris*.

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