## **Notas Científicas**

# Adaptation of fluorescent technique for genotyping with new microsatellite markers in common bean

Paula Rodrigues Oblessuc<sup>(1)</sup>, Tatiana de Campos<sup>(2)</sup>, Juliana Morini Kupper Cardoso<sup>(1)</sup>, Danilo Augusto Sforça<sup>(2)</sup>, Renata Moro Baroni<sup>(1)</sup>, Anete Pereira de Souza<sup>(2)</sup> and Luciana Lasry Benchimol<sup>(1)</sup>

<sup>(1)</sup>Instituto Agronômico, Centro de Pesquisa e Desenvolvimento de Recursos Genéticos Vegetais, Fazenda Santa Elisa, Caixa Postal 28, CEP 13001-970 Campinas, SP, Brazil. E-mail: parobl@gmail.com, julianamorini@hotmail.com, renatabaroni19@gmail.com, llasry@iac.sp.gov.br <sup>(2)</sup>Universidade Estadual de Campinas, Departamento de Genética e Evolução, Instituto de Biologia, CEP 13083-970 Campinas, SP, Brazil. E-mail: tatyuni@unicamp.br, obelix\_mail@yahoo.com.br, anete@unicamp.br

Abstract – The objectives of this work were to adapt the fluorescent labeling polymerase chain reaction (PCR) technique using M13 universal primer for genotyping purposes, and to present a new set of microsatellite markers for common bean (*Phaseolus vulgaris* L.). A large population (380 common bean lines) was used for microsatellite genotyping. PCR fluorescent labeling method showed to be very efficient for multiplex analysis, providing lower costs and saving time, thus increasing the quality of genotyping analysis. A new set of 50 microsatellites developed from an enriched library derived from cultivar IAC-UNA was presented. This study provides better tools for assisting common bean breeding programs.

Index terms: Phaseolus vulgaris, fluorescence, molecular marker, multiplex analysis.

## Adaptação da técnica de fluorescência para fins de genotipagem com novos marcadores microssatélite em feijoeiro

Resumo – Os objetivos deste trabalho foram adaptar a técnica de marcação fluorescente de produtos da reação em cadeia da polimerase (PCR) com uso do iniciador universal M13, para aplicação em genotipagem, e apresentar novos marcadores microssatélite para o feijoeiro (*Phaseolus vulgaris* L.). Uma população de grande tamanho amostral (380 linhagens) foi utilizada para genotipagem dos microssatélites. O método de PCR marcado por fluorescência demonstrou ser muito eficiente para a análise "multiplex" e proporcionou a redução de custos e ganho de tempo, aumentando a qualidade de análise da genotipagem. Foram apresentados 50 novos locos de microssatélites, desenvolvidos a partir de biblioteca enriquecida a partir da cultivar IAC-UNA. Este estudo fornece ferramentas melhores para assistir aos programas de melhoramento do feijoeiro.

Termos para indexação: Phaseolus vulgaris, fluorescência, marcador molecular, análise multiplex.

Microsatellite genotyping is widely used nowadays for different goals, such as diversity studies and genetic mapping. Microsatellites (Simple Sequence Repeats – SSRs) are molecular markers characterized as small DNA sequences of one to six base pairs tandemly repeated, spread all over the genome of plants and animals (Li et al., 2002; Varshney et al., 2005). They are multiallelic, codominant and have Mendelian inheritance. Moreover, they are easy to assay and are reproducible by polymerase chain reaction (PCR). Currently, the most used technique for SSR genotyping is the 6% polyacrylamide gel electrophoresis stained with silver nitrate (Creste et al., 2001). However, this method is time consuming and costly, especially when working with a large amount of individuals. Due to this fact, microsatellite fluorescencebased detection has been used in many crops in order to reduce general labor and costs. Oetting et al. (1995) were the first group to use the fluorescent method for genotyping purposes and used five primer pairs to perform different multiplex amplification reactions. The technique was based on a fluorescent-labeled universal M13 primer, which allowed automated DNA sequencer analysis and subsequent genotyping with high precision of allele size. In this fluorescent primer strategy report, detection was based on the addition of a sequence to the 5' end of the forward primer of the microsatellite marker with no homology to the target genome. M13 universal primer is a sequence derived from a bacterial vector (David et al., 1993). This primer is fluorescent-labeled for genotyping in high performance sequencing procedures. The forward SSR primer should contain a tail sequence complementary to the M13 in its 5' region, allowing the annealing of the M13 fluorescentlabeled primer to the PCR products generated in each cycle. Consequently, this method confers fluorescence to the final PCR products (Schuelke, 2000). The reverse SSR primer is maintained unaltered.

Alternatively, Missiaggia & Grattapaglia (2006) reported the use of three human sequences of 20 bp without significant complementarities to plant genome for microsatellite genotyping in *Eucalyptus*. These sequences were used as labeled primers. They performed multiplex genotyping by labeling each primer with different fluorophores and were able to differentiate the three SSRs in a single run.

M13 primer has advantages over other techniques because it provides the ability of working with a unique tail sequence and avoids the need for using several different microsatellites. Thus, for multiplex purpose, the only necessary condition is to change the fluorescent colors to label different PCR products of each microsatellite. Moreover, scoring patterns are performed totally by automation using a software that allows the interaction between the generated peak files and the genotyping per se. This technique is indicated when a large amount of data has to be analyzed at the same time.

Until now, there has been no report of the use of fluorescent-labeled microsatellite primers for genetic mapping purposes in common bean (Phaseolus vulgaris L.). In fact, beans are a good model to work with due to their diploid nature (2n = 22) and small genome (Broughton et al., 2003; Schlueter et al., 2008). Microsatellite markers have been developed for beans from published sequences and from microsatellite-enriched libraries (Gepts et al., 2008). A large number of microsatellites for common bean are already available, some of them with known map positions. However, the amount of microsatellite markers is not enough to support neither QTL mapping nor diversity studies, once there are lot gaps on common bean genetic maps to be solved and, consequently, new agronomically important loci could still be found. Likewise, there is the need for developing and releasing additional markers, together

with enhancing the microsatellite genotyping process for large based populations.

The objectives of this work were to describe the adaptation of genotyping processes with increased efficiency for mapping purposes through fluorescentlabeling PCR products using M13 universal primer, and to present a new set of microsatellite markers for common bean.

In order to adapt this approach for common bean, 50 new SSRs (Table 1) were used. These SSRs were developed from an IAC-UNA microsatellite-enriched library according to Benchimol et al. (2007) and have never been published. Each forward SSR primer had an 18 bp tail added (5'-TGT AAA ACG ACG GCC AGT-3') complementary to M13 primer. Two different fluorescences were used to label the M13 primer: 6-FAM (blue) and HEX (green), with a multiplex goal. The amplicons were sequenced with a 3730 DNA sequencer (Applied Biosystems) and analyzed with GeneMapper v.3.7 software.

The first step in a genotyping process with fluorescentlabeled microsatellites is to optimize PCR reactions. In this study, SSR-specific annealing temperatures have already been determined (56 or 60°C). When starting the fluorescent-labeled PCR optimization, it was decided not to alter each primer-specific annealing temperature (SSRs have been previously optimized without the tag) for speeding up the process of genotyping in the high performance sequencer. Since the M13 primer needs a 53°C annealing temperature, eight final cycles were added at the end of PCR cycles to allow the annealing of the M13 labeled with the previously formed amplicons. For that reason, at the end of the PCR, a satisfactory amount of amplicons labeled with fluorescence could be generated, once the reaction is exponential.

Based on the procedures described above, amplification reactions were performed in the following conditions: 94°C (1 min), then 30 cycles at 94°C (1 min)/specific annealing temperature (56 or 60°C) for each SSR (1 min)/72°C (1 min), followed by 8 cycles at 94°C (1 min)/53°C (1 min)/72°C (1 min), and a final extension at 72°C for 10 min.

Different concentrations of each primer were tested (Table 2), since the method is primarily different regarding the three primers in a same reaction (Schuelke, 2000). The reaction showed the best amplification profile with the forward-tail primer at a concentration 25% lower than the other primers (conditions 1 and 2).

This result was expected, since the forward primer cannot compete with the labeled M13, as it is necessary only in the first cycles. Therefore, the amplicons were produced with a smaller concentration of the forward primer in comparison to the labelled M13, so as to guarantee that the PCR products become fluorescent in the sequencer.

This way, in order to guarantee a good amplification of every microsatellite product, it was established that reaction should contain 30 ng DNA, 1U *Taq*-DNA polymerase, 1.5 mmol  $L^{-1}$  magnesium chloride, 0.15 mmol  $L^{-1}$  of each dNTP, 1x buffer, 0.8 pmol  $\mu L^{-1}$  reverse primer and labeled M13 and 0.2 pmol  $\mu L^{-1}$  forward primer, with a final volume of 15  $\mu L$ .

The samples were prepared for sequencing based on the conditions recommended by Applied Biosystems, with variations regarding the quantity of PCR product and of GeneScan ROX 500 (Applied Biosystems). However, peaks with higher intensity were obtained with 1.5  $\mu$ L of the product from

| Table 1. A set of 50 new microsatellite markers obtained from an enriched library | y from the common bean cultivar IAC-UNA. |
|---|--|
|---|--|

| $\begin{array}{ccccccc} AC-SR289 & AltGGTIGAATIGGCTIGAC & (AC)7 & 60 & 210 \\ ATGGTIAAAGACTCCIGTIGC & & & & & & & & & & & & & & & & & & &$  | Locus/GeneBank accession | Primer sequences $(5' \rightarrow 3')$ | Core motifs           | Ta (°C) | Predicted size (pb) |
|---|--------------------------|--|-----------------------|---------|---------------------|
| $\begin{array}{c c c c c c c c c c c c c c c c c c c $  | AC-SSR289                | ATCGGTTGAATTGGCTTGAC                   | (AC)7                 | 60      | 210                 |
| $\begin{array}{ccccccc} ACS8290 & GGAGAAAGATGCAGGAAGAA & (AC)6 & 60 & 289 \\ TTTTTGGAGTGTTTGGTC & (CA)7 & 60 & 213 \\ CAGCCGTCTTGAGCCTGTGTGC & (CA)7 & 60 & 213 \\ CAGCCGTCTTGAGCCTGTGAGA & (CA)8 & 60 & 189 \\ TGGATTCAAAGTCACCGTG & (CA)6 & 60 & 189 \\ TGGATTGAACTGTAGAACTGTAAGCAAAC & (CA)6 & 60 & 125 \\ CGAAGATGGACGTGGAGTGTTTTC & (CT)3 & GT)2 & 60 & 125 \\ GGAAGATGCACCAACGAAAAG & (AC)6 & (TC)2 & 60 & 125 \\ CGAAGATGCACCAACGAAAAG & (CG)7 & 60 & 127 \\ ACSSR295 & GATTCTTCCCACCCTCACT & (TG)7 and (GT)3 & (GG)3 & 60 & 217 \\ ACSSR296 & TGCTGATTTCCCACCCTCACT & (TG)7 & 60 & 160 \\ GCCCACCCACCACCAACATAAT & (CA)7 & 60 & 160 \\ GCCCACCCCCCCCCCCAGGAAAG & (CA)7 & 60 & 155 \\ GCAGCAGGAACATAAAAGAC & (GT)7 & 60 & 160 \\ GCCCACCCACCCCCCGAGAAGA & (CA)7 & 60 & 155 \\ GCAGCAGGTAGTAAAAGAC & (CA)7 & 60 & 155 \\ GCAGCAGGTAGTAAAAGAC & (CA)7 & 60 & 155 \\ GCAGCAGGGAACATAAAAGAC & (CA)7 & 60 & 155 \\ GCAGCAGGGAGGGAAACATAAAGAC & (CA)7 & 60 & 155 \\ GCAGCAGGGAGGGAAACATAAAGAC & (CA)7 & 60 & 155 \\ GCAGCAGGGAGGGAAACATAAAGAC & (CA)7 & 60 & 150 \\ GGGCGCGGGGAGGGAAACATAAAGAA & (CA)5 & 45 & 197 \\ GGGGCGGGGGGGGGGGAAACAAAAGA & (CA)5 & 45 & 295 \\ TTGTTGTGCTGAGCGAAACCAAACCCA & (CA)7 & 60 & 130 \\ GTCAGCCAGGATAGGAAAACCA & (CA)5 & 45 & 295 \\ TTAGGTTGGGGGGAGGGGAAACCAAACCCA & (CA)7 & 60 & 210 \\ ACSSR301 & GGACAAGGGAATCGAAACCA & (AC)5 & 45 & 240 \\ CCAGGACGGGATGGAAAACCA & (AC)5 & 45 & 240 \\ CCAGGAAGGTTCAATGCCAAACCCA & (CC)5 & 60 & 210 \\ AGGGGGTTCAATGCAATGCAAACCAA & (AC)5 & 45 & 240 \\ CCAAGTCGAAATGCAAACCAAA & (AC)5 & 45 & 240 \\ CCAAGTCGAAATGCAAAGAACAC & (CA)8 & 60 & none \\ ACCSSR305 & ACAGAAAAAGACAAACCAA & (AC)5 & 45 & 240 \\ CCAAGTCGAAATGCAAAGGAAAACC & (GA)8 & 60 & none \\ ACCSSR306 & CCTTCCGCTGTGGGAGGAACGAACCA & (CA)6 & 45 \\ CCAAGCCAATGCAAAGAAGAACAACCA & (CA)6 & 45 \\ CCAAGCCAATGCAAAGAAGAAGAAGAACAA & (CA)6 & 5 \\ CCGGGGGTGTTTGTAGGAAGAAAAGGAAAGAC & (CA)6 & 45 \\ CCASSR301 & TTCAGAGGAAAAGACAAAGCC & (CA)6 & 45 \\ CCASSR301 & CTCCGCAATGCAAAGGAAAAGCA & (GA)8 & 60 & none \\ ACCSSR301 & CTGCACAATGCCAAGGAAAAGACAAAGCC & (CA)6 & 45 \\ CCASSR311 & TAGCCAATGCCAAGGAAAAGCA & (GA)1 & 45 \\ CTTTCAAAGCATTGGAGGAATC$      |                          | ATTGCTTAAAGACTCCTGTTGC                 |                       |         |                     |
| $\begin{array}{c c c c c c c c c c c c c c c c c c c $  | AC-SSR290                | GGAGAAAGATGCAGGAAGAA                   | (AC)6                 | 60      | 289                 |
| $\begin{array}{ccccccccccGGGCAGCCCTGTGTOC (CA)7 60 213 \\ CATGGGGTTGTAGCCCGGGGA \\ ACSSR292 AACAGAAATGAATGAATAAACACAAAC (CA)6 (GA)3 60 237 \\ AGCST22 ACAGAAATGAATGAACACAAAC (CA)6 (GA)3 60 237 \\ AATTIGGACGTGGGAGTGTTIC \\ ACSSR293 ATGCCGTTTTGGATGTGAG (AG)3 GT (GA)3 60 217 \\ ACSSR294 GGAAGATGCACCCACAGAAAAG (AC)6 (TC)2 60 125 \\ GGAAGATGCAACCCCACAGAAAAG (AC)7 and (GT)3 (AG)3 60 217 \\ ACSSR295 GATTCTTCACACCCTCACT (TG)7 and (GT)3 (AG)3 60 217 \\ ACCSSR296 TGCTGATTCACACCTGC (GT)3 G (GT)2 60 192 \\ AGCSSR297 AGGGCATGCAACCTACATGAAAAG (GT)7 60 160 \\ GCCCACCCACCACCAGAAAAG (GT)7 60 160 \\ GCCCACCCACCACCACGTGTG (AC)7 60 155 \\ GCAGCAGGTAAGTAAAAGGAC (GA)7 60 213 \\ ACSSR298 ACACCCACCACCTGTGT (AC)5 (AC)4 60 232 \\ HGTTGTGGTTAAAGTAAAGGAC (GA)4 45 197 \\ GGGCGGGGGGAGAGCTGGGAAACGTAGAAC (CA)4 45 197 \\ GGGCGGGGGGGAGAGCTGGGAAACGAACGC (GA)4 45 197 \\ GGGCGGGGGGGGAGAGCTGGGAAACGACAC (CA)4 45 197 \\ GGGCGGGGGGGGAGAGCTGGGAAAAGA (CA)4 \\ ACSSR300 CATCTGGGGGAAACGTCGGAGAACT (AC)4 45 197 \\ GGGCGGGGGGGGGGGAGGGGAACGCTGGGGAAAGA (CC)4 45 295 \\ TTAGGGACAGGGGAAACGCAGAAAGA (CC)4 45 295 \\ TTAGGACATCAGAAACCCAA \\ ACSSR301 GGACCAGGGAACTCGGAAAAGA (CC)4 45 295 \\ TTAGGGACTAGGGGAAACCTC (GT)2 AT (GT)4 - 000 \\ CTATATCACAAATCACAAACACCA A \\ ACSSR303 TTCATCGCGTTAGGGGTTTC (AC)14 45 295 \\ TTAGGGGGTAAGGGAATCG (CA) ACACCA \\ ACSSR303 TTCATCACAAACACCA (AC)5 45 240 \\ CCAAGTCGAAAAGTCAAACACCA (AC)5 45 240 \\ CCAAGTCGAAAGTCAAACCCA (AC)5 45 240 \\ CCAAGTCGAAAGTCAAACACAA (AC)5 45 240 \\ CCAAGTCGAAAGTCAAACACAA (AC)5 45 240 \\ CCAAGTCGAAAGTCAAACACAA (AC)5 45 240 \\ CCAAGTCGAAATCACAAAGTCAA (AC)5 45 240 \\ CCAAGTCGAAATCACAAAGTCAA (AC)5 45 240 \\ CCAAGTCGAAATCACAAAGTCAAACCC (CA)8 60 0 000 \\ ACSSR305 ACAGAAAAGTCAAACACAA (AC)5 45 240 \\ CCAAGTCGAATTCACAAAGTCAAAGTCA (AC)5 45 240 \\ CCAAGTCGAATTCACAAAGTCAAAGTCA (AC)5 45 200 \\ CCAAGTCGGGGTGAGTAAGGAAAACC (CA) (AC)3 (G)3 45 0000 \\ ACSSR306 CGTCCCTGGAGGAGGG (GA)14 45 0000 \\ AGCSSR307 CTCCCCGAGTTGGAAGGGAACCC (CA)6 45 245 \\ CTTCTCAAAGGAAAAAGAACAACAACC (CA)6 45 245 \\ CTTCTCAAAGGAAAAAGAACAACAACC (CA)6 45 222 \\ TTATGAATGGGGAAAGCAAACCC (CA)6 45 222 \\ TTCTCAAAGGAAAAGAACAAACAC$ |                          | TTTTTTGGATGGTGTTGTC                    |                       |         |                     |
| $\begin{array}{ c c c c c c c c c c c c c c c c c c c$  | AC-SSR291                | CCCCCACGCTCTTGTTGC                     | (CA)7                 | 60      | 213                 |
| $\begin{array}{cccc} AC-S8R292 & AACAGAAATGAATTAACACAAAC (CA)6 & 60 & 189 \\ TGGGATTCAAAGTCACTC \\ AC-S8R293 & ATGCCGTTTTGGATGTGAG (AG)3 & 60 & 237 \\ AATTTGGACGTGGAGTGTTTC \\ AC-S8R294 & TGGAATTGGAACTGTGAAAAC (AC)6 (TC)2 & 60 & 125 \\ GGAAGATGCACCACAGAAAAG & (AC)6 (TC)2 & 60 & 217 \\ AC-S8R295 & GATTCTTCATACCTCT (TG)7 and (GT)3 (AG)3 & 60 & 217 \\ AC-S8R295 & TGTGATTCTCATACACTTC (GT)7 and (GT)2 & 60 & 192 \\ AGACACTGGACAACATAAAT & & & & & & & & & & & & & & & & &$  |                          | CATGCGTCTTGAGCCTGTGA                   |                       |         |                     |
| $\begin{array}{ c c c c c c c c c c c c c c c c c c c$  | AC-SSR292                | AACAGAAATGAATTAACACAAAC                | (CA)6                 | 60      | 189                 |
| $\begin{array}{cccc} AC-SSR293 & ATGCCGTTTTTGGAGG (AG)3 GT (GA)3 & 60 & 237 \\ AATTGGAGTGGAGGGGGTGTTTC \\ AC-SSR294 & TGGAATTGAAACTGTAAAAAC (AC)6 (TC)2 & 60 & 125 \\ GGAAGATGCACCACAGAAAAAC (AC)6 (TC)2 & 60 & 125 \\ AC-SSR295 & GATTCTTCCCACCCTCACT (TG)7 and (GT)3 (AG)3 & 60 & 217 \\ AC-SSR296 & TGCTGAACTTCCAATGAACTTC (GT)3 G (GT)2 & 60 & 192 \\ AGGACACTGTGGAACATCAACTG (GT)7 & 60 & 160 \\ GCCCACCCACCAGAAAAG (GT)7 & 60 & 155 \\ GCAGCAGGTAGTAAAAAGAC (CA)7 & 60 & 155 \\ GCAGCAGGTAGTAAGAATAAAAAGAC (CA)7 & 60 & 155 \\ GCAGCAGGGAACGTGAGGAAACTGCAGAAC (CA)7 & 60 & 155 \\ GCAGCAGGGAAGGTAGTAAAAAAGAC (CA)7 & 60 & 126 \\ TTGTGTGGCTGATCCAATGCCTGTGT (AC)5 A (AC)4 & 60 & 232 \\ TGTGTGGGGGGAAGGAACAGA (CA)5 A (CA)3 & 60 & 130 \\ GTCAGCCAGTGAAAAAAGA (CA)5 A (CA)3 & 60 & 130 \\ GTCAGCCAGTAAGAAAAACACA (CA)5 A (CA)3 & 60 & 130 \\ GTCAGCCAGTAAGAAAACACA (CA)5 A (CA)3 & 60 & 130 \\ GTCAGCCAGTAAGAAAACACA (CA)5 A (CA)3 & 60 & 130 \\ GTCAGCCAGTAAGAAAAACCA (CA)5 A (CA)3 & 60 & 210 \\ AC-SSR302 & TCTCTGCGTTTGGGTTC (AC)5 A (CA)3 & 60 & 210 \\ AC-SSR303 & TCCAAAATCACAAACCACTC (GT)2 AT (GT)4 & - & & & & & & & & & & & & & & & & & $  |                          | TGGGATTCAAAGTCACCTC                    |                       |         |                     |
| $\begin{array}{ c c c c c c c c c c c c c c c c c c c$  | AC-SSR293                | ATGCCGTTTTTGGATGTGAG                   | (AG)3 GT (GA)3        | 60      | 237                 |
| $\begin{array}{cccc} AC-SSR294 & TGGAATTGAAAACTTGTAAAAAC (AC)6 (TC)2 & 60 & 125 \\ \hline GGAAGAGTGCACCACGAAAAAG & & & & \\ AC-SSR295 & GATTCTTCCCACCCTCACT (TG)7 and (GT)3 (AG)3 & 60 & 217 \\ \hline ACACTTCAAACCCAAACATAAT & & & & & \\ ACACTTCAAACCCAAACCTATAT & & & & & & \\ ACACTTCAAACCCAAACCTATAAT & & & & & & \\ AGACACTGTGGACATTCAACTG & & & & & & \\ AGACACTGTGGACATTCAACTG & & & & & & \\ AGACCACTGTGGACATTCAACTG & & & & & & \\ AGACCACTGTGGACATTCAACTG & & & & & & \\ AC-SSR297 & AGGGCTAGGAAACATAAAAAGAC (GT)7 & 60 & 160 \\ \hline GCCCACCCAACCTCCTCTTAAAC & & & & & & \\ GCAGCAGGTAGTAAAATAGA & & & & & & \\ CACSSR298 & ACACCCACCCACCGAAAAG & & & & & & \\ AC-SSR299 & AAATAGTCATCCATGCCTGTGT & & & & & & & \\ AC-SSR300 & CATGTGGGGGAACGGACT & & & & & & & \\ GGGCGGGGGGGGGAGGAAAGA & & & & & & \\ GGGCCGGGGGGGGGGGAGAAAGA & & & & & & \\ & & & & & & & & \\ GGGCCGGGGGGGGGG$  |                          | AATTTGGACGTGGAGTGTTTTC                 |                       |         |                     |
| GGAAGATGCACCACAGAAAAG      AC-SSR295    GATTCTTCCCACCCCTCACT    (TG)7 and (GT)3 (AG)3    60    217      AC-SSR296    TGCTGATTCTGATGTAACTTC    (GT)3 G (GT)2    60    192      AGACACACTGTGGACATTCAACTG    AGACACACTGTGGACATTCAACTG    (GT)7    60    160      AC-SSR297    AGGGCTAGGAAACATAAAAAGAC    (GT)7    60    155      GCCACCCACCCACCCACCACGAAGG    (CA)7    60    155      GCCAGCAGGTAGTAAAGATAAGA    (AC)5    4(C)5 A (AC)4    60    232      TTGTTGTGTGCTGAAGGTAGGGAACT    (AATAGTAGCAATGAAAAGA    (AC)5    45    197      GGGGCGGGGAGGGAAAGAA    (CA)5 A (CA)3    60    130    10      AC-SSR300    CAGTAGGGAAACGTACCAGAAAGA    (CA)5 A (CA)3    60    130      GGACAAGGGACCGGAAAAGA    (CA)14    45    295      TTCATGCGGTTTGGAGAACCCA    (AC)5 A (CA)3    60    210      AC-SSR303    TTCATGCAAAAGTCAAACACTC    (GT)2 AT (GT)4    -    none      CTTATATCAGGGAAAGTAGAAAACA    (CC)5    60    210    AC-SSR304    CTGTGTGTATACAC  | AC-SSR294                | TGGAATTGAAACTTGTAAAAAC                 | (AC)6 (TC)2           | 60      | 125                 |
| AC-SSR295    GATTCTTCCCACCCTCACT<br>ACACTTCAAACCCAAACATAAT    (TG)7 and (GT)3 (AG)3    60    217      AC-SSR296    TGCTGATTCTGATGTAACTTAACTG    (GT)3 G (GT)2    60    192      AGACACTGTGGACATTCAACTG    (GT)3 G (GT)2    60    192      AC-SSR297    AGGCCTAGGAAACATAAAAAGAC    (GT)7    60    160      AC-SSR298    ACACCCACCCACCAGAAAG    (CA)7    60    155      GCAGCAGGTAAAAGATAAAAAGA    (AC)5 A (AC)4    60    232      TTGTTGTGTGTGATGATCAATGCCTGTGT    (AC)5 A (AC)4    60    232      TTGTTGGTGTGGAGAGCTGGGAAAAGA    (GA)14    45    197      GGGGCGGGGAGGGGAAAGA    (GA)14    45    295      AC-SSR300    CATGTGGGAAAGGAGACTCGGAAAAGA    (CA)3    60    130      AC-SSR301    GGACAAGGAGATTC    (AG)14    45    295      AC-SSR302    TTCATACAAATTACAACTC    (GT)2 AT (GT)4    -    none      CTATACAAAGAGAAACACACA    (CA)8    60    210      AC-SSR303    TTCATACAAGAGAAAACA    (AC)5    45    240      CCASS  |                          | GGAAGATGCACCACAGAAAAG                  |                       |         |                     |
| $\begin{array}{c c c c c c c c c c c c c c c c c c c $  | AC-SSR295                | GATTCTTCCCACCCTCACT                    | (TG)7 and (GT)3 (AG)3 | 60      | 217                 |
| $\begin{array}{c c c c c c c c c c c c c c c c c c c $  |                          | ACACTTCAAACCCAAACATAAT                 |                       |         |                     |
| $\begin{array}{ c c c c c c c c c c c c c c c c c c c$  | AC-SSR296                | TGCTGATTCTGATGTAACTTTC                 | (GT)3 G (GT)2         | 60      | 192                 |
| AC-SSR297  AGGCCTAGGAAACATAAAAAGAC  (GT)7  60  160    AC-SSR298  ACACCCAACCTCCCTCTAAAC  (CA)7  60  155    AC-SSR299  AAATAGTCATCAAAGCTTGCTGT  (AC)5 A (AC)4  60  232    TTGTTGTGTGCTTGATCTAAAAAC  (GA)14  45  197    AC-SSR300  CATGTGGGGGAACGTGAGAAAAGA  (CA)5 A (CA)3  60  130    AC-SSR301  GGACAAGGGACTGGAAAAGA  (CA)5 A (CA)3  60  130    AC-SSR302  TCTCTGCCTTTTGGATTC  (AG)14  45  295    TTAAGATAGGAGTAAGAGATTC  (GT)2 AT (GT)4  -  none    AC-SSR303  TTCATACAAATTCACAAACCCAA  (CA)8  60  210    AC-SSR304  TGGTTGTTTTATAGGAGTTA  (CA)8  60  210    AC-SSR305  ACAAGAAAACAACT  (CT)5  60  210    AC-SSR306  CGTTCGCATGGGAAGAAACACT  (CT)5  60  210    AC-SSR306  CGTCGCCGTGGTGCTCT  (CCT)5  60  210    AC-SSR306  CGTGTCGCATGGGAAGAACAC  (AG)3 (TG)3  45  none    AC-SSR307  GTTGCCCGTGGGGAGGAAGAAGAC  (GA)8   |                          | AGACACTGTGGACATTCAACTG                 |                       |         |                     |
| AC-SSR298  ACCCACCCACCAGAAAG    AC-SSR298  ACACCCAACCTCCTCTAAAC  (CA)7  60  155    AC-SSR299  AAATAGTCATCAATGCCTGTGT  (AC)5 A (AC)4  60  232    AC-SSR300  CATGTGGGGGAACGTGAGAAAAAC   | AC-SSR297                | AGGGCTAGGAAACATAAAAAGAC                | (GT)7                 | 60      | 160                 |
| AC-SSR298  ACACCCAACCTCCCTCTAAAC  (CA)7  60  155    AC-SSR299  AAATAGTCAATAGCAATGGA  (AC)5 A (AC)4  60  232    AC-SSR300  CATGTGGGGGAACGTGAGGAACT  (GA)14  45  197    GGGGCGGGGGGGGGGGGGAGGAAAAGA  (CA)5 A (CA)3  60  130    AC-SSR301  GGACAAGGGACTCGGAAAAGA  (CA)5 A (CA)3  60  130    AC-SSR302  TCTCTGCGTTTTGGATTC  (AG)14  45  295    AC-SSR303  TTCATACAAGTAAGAAACACCAA  (CA)7  60  210    AC-SSR303  TTCAGGGGGAGGAAAAACCA  (CA)14  45  295    AC-SSR303  TTCATACAAATCACACTC  (GT)2 AT (GT)4  -  none    AC-SSR304  TGGTTGTTTTATAGGAGTTA  (CA)8  60  210    AC-SSR305  ACAGAAAAGTCAAACAAGTCAA  (AC)5  45  240    AC-SSR306  CGTCCGCATGGTGGTCCT  (CCT)5  60  210    AC-SSR307  GTGCCGTGGGGGGGGGGGGGGGGGC  (AC)5  45  none    AC-SSR308  GCAACACAAATCACAAGTGAAAC  (GA)8  60  none    AC-SSR309  AAGGAAAAGAAGGAGAGGG   |                          | GCCCACCCACCAGAAAG                      |                       |         |                     |
| GCAGCAGGTAGTAAAAGATAATGA    AC-SSR299  AAATAGTCATCAATGCCTGTGT  (AC)5 A (AC)4  60  232    TIGTGTGGTCTGATCCTAAAAAC  AC-SSR300  CATGTGGGGGGAACGTGAGGACT  (GA)14  45  197    AC-SSR301  GGGACAGGGAAAGA  (CA)5 A (CA)3  60  130    AC-SSR302  TCTCTCGCGTTTGGATCC  (AG)14  45  295    AC-SSR303  TTCATCAGAAAGAAACACCAA  -  none    AC-SSR303  TTCATACAAATTCACAAACACTC  (GT)2 AT (GT)4  -  none    AC-SSR304  TGGTGTTTTATTAGGAGTATGAGATTAA  (CA)8  60  210    AC-SSR305  ACAGAAAAGTCAACAAAGTCAA  (AC)5  45  240    AC-SSR306  CGTTCCGCATGGTAGTCAA  (AC)5  45  240    AC-SSR306  CGTTCCGCATGGTAGTCAA  (AC)5  45  none    AC-SSR307  GTTGGCAGTGGGAAGAAAACCA  (AG)3 (TG)3  45  none    AC-SSR308  GCAACACAAATCACAAAGTGAA  (AG)3 (TG)3  45  none    AC-SSR309  AAGAGAAAAGAAGAAGAAGAAGAAGAAGAAGAAGA  GTTACCGGGGAGAGG  GA)14  45  none    AC-SSR309  AAGAGAAAAGAAAGAAG   | AC-SSR298                | ACACCCAACCTCCCTCTAAAC                  | (CA)7                 | 60      | 155                 |
| AC-SSR299  AAATAGTCATCAATGCCTGTGT  (AC)S A (AC)4  60  232    AC-SSR300  CATGTGGGGGAACGTGAGGACT  (GA)14  45  197    AC-SSR301  GGACAAGGGAACTGGAAGACT  (GA)14  45  197    AC-SSR302  GGACAAGGGAACTGGAAAAGA  (CA)5 A (CA)3  60  130    AC-SSR302  TCTCTGCGTTTTGGATTC  (AG)14  45  295    AC-SSR303  TTCATCAAAATTCACAAACACCAA  -  none    AC-SSR303  TTCATACAAATTCACAAACACCAA  -  none    AC-SSR303  TTCATCAAAATTCACAAACACCAA  -  -  none    AC-SSR304  TGGTTGTTTTTAGGAGTATTA  (CA)8  60  210    AC-SSR305  ACAGAAAAGTCAAACACAA  (AC)5  45  240    AC-SSR306  CCAAGTCGAATGGAAATCT  (CCT)5  60  210    AC-SSR306  CCTCGGTGTCT  (CCT)5  60  210    AC-SSR307  GTGCCATTGGGAGTGTGA  (AG)3 (TG)3  45  none    AC-SSR308  GCAACACAAAATCACAAGAAAAGAGAACA  (GA)8  60  none    AC-SSR309  AAGGAAAAGGAAGAAGAAGGAAGAC  (GA)8   |                          | GCAGCAGGTAGTAAAGATAATGA                |                       |         |                     |
| TTGTTGTGCTTGAGAAACAC-SSR300CATGTGGGGGAACGTGAGGACT(GA)1445197GGGCCGGGGGGGGGGGGGAAGGAAAGA(CA)5 A (CA)360130AC-SSR301GGACAAGGGACTCGGAAAAGA(CA)5 A (CA)360130AC-SSR302TCTCTGCGCTTTTGGATTC(AG)1445295TTAAGATTAGGAGTAGGGTTC(GT)2 AT (GT)4-noneAC-SSR303TTCATACAAATTCACAAACACTC(GT)2 AT (GT)4-noneAC-SSR304TGGTTGTTTTTATTAGGAGTTA(CA)860210AC-SSR305ACAGAAAGTCAAACAAGTCAA(AC)545240AC-SSR306CGTTCCGCATGGTCGTCCT(CCT)560210CCAAGTCGAAATGGAAATCAAC-SSR307GTTCCCGCATGGTCGTCCT(CCT)560210CCACGTGTAGTAGTAGTAAGTAAGTAA(AG)3 (TG)345noneAC-SSR308GCAACAAAATCACAAGAAGAAC(AG)3 (TG)345noneAC-SSR309AAGGAAAAGTAAAGAAGGAAGG(GA)1445noneAC-SSR310CTGACCAAGTTTGCGAGTAGCAAGG(GT)860184AC-SSR311TAGCAAAGGAAAAGCAAACC(CA)645245AC-SSR312TTAATACCCGTAAAAGAAGCACAAACC(CA)645245AC-SSR312TTAATGCCAAATGGAAAGGAACA(GA)1160222   | AC-SSR299                | AAATAGTCATCAATGCCTGTGT                 | (AC)5 A (AC)4         | 60      | 232                 |
| AC-SSR300  CATGTGGGGGAACGTGAGGACT  (GA)14  45  197    AC-SSR301  GGACAAGGGACTGGAAAGA  (CA)5 A (CA)3  60  130    GTCAGCCAGTAAGAAACACCAA  (CA)5 A (CA)3  60  130    AC-SSR302  TCTCTGCGTTTTGGATTC  (AG)14  45  295    AC-SSR303  TTCAACAAATTCACAAACACTC  (GT)2 AT (GT)4  -  none    CTTATATCAGGGAATAGGAAAACAC  AC-SSR303  TTCAACAAATTCACAAACACTC  (GC)2 AT (GT)4  -  210    AC-SSR304  TGGTTGTTTTTATTAGGAGTTTA  (CA)8  60  210  240    AC-SSR305  ACAGAAAAGTCAAACAAAGTCAA  (AC)5  45  240    AC-SSR306  CGTTCCGCATGGTAGTCGTCAT  (CCT)5  60  210    AC-SSR307  GTTGCCATTGGGAGTGTGA  (AG)3 (TG)3  45  none    AC-SSR308  GCAACACAAAATCACAAGAAGAAC  (GA)8  60  none    AC-SSR309  AAGGAAAAGAAAGAAAGAAGAGAAGGGAGG  (GA)14  45  none    CGGGGGTGTTGGAAGGAGAGG  (GA)14  45  none  20    CGGGGGTGTTGGAAGGAGAGG  (GA)14  45  none  20  20<  |                          | TTGTTGTGCTTGATCCTAAAAAC                |                       |         |                     |
| GGGGCGGGAGGGAGGGAAAAGAAC-SSR301GGACAAGGGACTCGGAAAAGAC(CA)5 A (CA)360130AC-SSR302TCTCTGCGTTTTGGATTC(AG)1445295TTAAGATTAGGAGTAGGGTTC(GT)2 AT (GT)4-noneAC-SSR303TTCATACAAATTCACAAACACTC(GT)2 AT (GT)4-noneCTTATATCAGGGAAGAAAAACAnoneAC-SSR304TGGTTGTTTTTATTAGGAGTTA(CA)860210AC-SSR305ACAGAAAAGCAAAACAACACTCAC-SSR306CGTTCCGCATGGTCGTCA(CCT)560210CCAAGTCGAAATGGAAATCTAC-SSR306CGTTCCGCATGGTCGTCCT(CCT)560210CCTCCGTGTGAGTAGTGGA(AG)3 (TG)345noneCCTCCGTGTGAGTAGTAAGTGAA(GA)860noneAC-SSR308GCAACACAAATCACAAGGAGAC(GA)860noneAC-SSR309AAGGAAAAGAAAGAAGGAGAGG(GA)1445noneCGGGGGTGGTTTGGAGAGAGAGGGTTGACCAAGTTGGGAGATACA(GT)860184GTTGACCAAGTTGCGAAGAGGAAAGAC(GA)1445245245AC-SSR310CTGACCAAGGAAAAGAAAGAAAGAAAGCA(GA)1160222AC-SSR312TTAATAAGGAATCGA(GA)1160222TCTTCTAAATGAATGGGTATC222  | AC-SSR300                | CATGTGGGGGGAACGTGAGGACT                | (GA)14                | 45      | 197                 |
| AC-SSR301  GGACAAGGGACTCGGAAAAGA  (CA)5 A (CA)3  60  130    AC-SSR302  TCTCTGGGTTTGGATTC  (AG)14  45  295    TTAAGATTAGGAGTAGGATTC  (GT)2 AT (GT)4  -  none    AC-SSR303  TTCATACAAATTCACAAACACCC  (GT)2 AT (GT)4  -  none    AC-SSR304  TGGTTGTTTTTATTAGGAGTTTA  (CA)8  60  210    AC-SSR305  ACAGAAAAGTCAAACAAAGTCAA  (AC)5  45  240    AC-SSR306  CGTTCGCGATGGGTGTCCT  (CCT)5  60  210    AC-SSR306  CGTTCCGCATGGGAGTGTGA  (AC)5  45  240    AC-SSR307  GTTGCCATTGGGAGTGTGA  (AG)3 (TG)3  45  none    AC-SSR308  GCAAACAAAAGAAAAGAAGAAC  (GA)8  60  none    AC-SSR308  GCAAACAAAAGAAAGAAGAAC  (GA)8  60  none    AC-SSR310  CGAGGAAAAGAAAGAAGAAGAAGAAC  (GA)14  45  none    CGGGGGTGGTTTGGAGAA  GTTTGACAGGAAAAGAAAGAAGAAGAAGAA  GTTTGACCGTAAGTCA  (GT)8  60  184    GTTTGACCGTAGGCGCAAGGG  GAACAGAAAGGAAAGAAAAGAAAAGAAAAGAAAAGAAA  |                          | GGGGCGGGGGAGGGAAAAGA                   |                       |         |                     |
| GTCAGCCAGTAAGAACACCAAAC-SSR302TCTCTGCGCTTTTGGATTC<br>TTAAGATTAGGAGTAGGGTTTC(AG)1445295AC-SSR303TTCATACAAATTCACAAACACTC<br>CTTATATCAGGGAAGAAAAACA(GT)2 AT (GT)4-noneAC-SSR304TGGTTGTTTTTATTAGGAGTTTA<br>AGGGGGTTCAATATCACTTCT(CA)860210AC-SSR305ACAGAAAAGCAAAACAAAGTCAA<br>  | AC-SSR301                | GGACAAGGGACTCGGAAAAGA                  | (CA)5 A (CA)3         | 60      | 130                 |
| AC-SSR302  TCTCTGCGTTTTGGATTC  (AG)14  45  295    TTAAGATTAGGAGTAGGGTTTC  TCAAGATTAGGAGTAGGGTTTC  (GT)2 AT (GT)4  -  none    AC-SSR303  TTCATACAAATCACAAACACTC  (GT)2 AT (GT)4  -  none    AC-SSR304  TGGTTGTTTTTATAGGAGTTTA  (CA)8  60  210    AGGGGGTTCAATATCACTTCT  -  -  -  -    AC-SSR305  ACAGAAAAGTCAAAACAAAGTCAA  (AC)5  45  240    CCAAGTCGAAATGGAAATCA  CCT)5  60  210    AC-SSR306  CGTTCCGCATGGTCGTCCT  (CCT)5  60  210    AC-SSR307  GTTGCCATTGGGAGTGTGA  (AG)3 (TG)3  45  none    CCTCCGTGTGAGTAGTAAGTAGAAC  (GA)8  60  none  -    AC-SSR308  GCAACACAAATCACAAGAAGAAC  (GA)8  60  none    AC-SSR310  CTGACAAGTAAGAAGAAGGAAGGG  (GA)14  45  none    CGGGGGGGGTTGGGAAGAGG  (GA)14  45  245    GTTTGACAAGGAAAAGAAAGAAGGAAGGG  (GA)14  45  245    AC-SSR310  CTGACAAGGAAAAGAAGAAGGAAGAG  (GT)8  60  1   |                          | GTCAGCCAGTAAGAAACACCAA                 |                       |         |                     |
| TTAAGATTAGGAGTAGGGTTTCAC-SSR303TTCATACAAATTCACAAACACACC(GT)2 AT (GT)4-noneCTTATATCAGGGAAGAAAACACACGTTCATACAAATTCACAACAACACA(CA)860210AC-SSR304TGGTTGTTTTATAGGAGTTTA(CA)860210AC-SSR305ACAGAAAAGTCAAACAAAGTCAA(AC)545240CCAAGTCGAAATGGAAATCTCCT)560210AC-SSR306CGTTCCGCATGGTCGTCCT(CCT)560210TTCCCGCAATTTTCTTTTCTGTTTCCCGCAATTGTGGAGTGTGA(AG)3 (TG)345noneAC-SSR307GTTGCCATTGGGAGTGTGA(AG)3 (TG)345noneCCTCCGTGTGAGATAGTAAGTGACGA)860noneAACCSSR308GCAACACAAAATCACAAGAAGAAC(GA)860noneAC-SSR309AAGGAAAAGAAAGAAAGGAAGAGG(GA)1445noneCGGGGGTGGTTTGCGAGAG45245AC-SSR310CTGACCAAGTTTGCGAGATACA(GT)860184GTTTAGACTGGGCGAAAGAGA45245AC-SSR312TTAATAACCGTAAAGCAAAACC(GA)1160222222100   | AC-SSR302                | TCTCTGCGTTTTGGATTC                     | (AG)14                | 45      | 295                 |
| AC-SSR303  TTCATACAAATTCACAAACACTC  (GT)2 AT (GT)4  -  none    AC-SSR304  TGGTTGTTTTATTAGGAGATTA  (CA)8  60  210    AGGGGGTCAATATCACATTCT  .  .  .  .    AC-SSR305  ACAGAAAAGTCAAACAAAGTCAA  (AC)5  45  240    .  .  .  .  .  .    AC-SSR305  ACAGAAAAGTCAAACAAAGTCAA  (AC)5  45  240    .  .  .  .  .  .  .    AC-SSR306  .  .  .  .  .  .  .    AC-SSR307  . <td></td> <td>TTAAGATTAGGAGTAGGGTTTC</td> <td></td> <td></td> <td></td>   |                          | TTAAGATTAGGAGTAGGGTTTC                 |                       |         |                     |
| CTTATATCAGGGAAGAAAAACAAC-SSR304TGGTTGTTTTATTAGGAGTTTA<br>AGGGGGTTCAATATCACTTCT(CA)860210AC-SSR305ACAGAAAAGTCAAACAAAGTCAA<br>CCAAGTCGAAATGGAAATCT(AC)545240AC-SSR306CGTTCCGCATGGTCGTCCT<br>TTCCCGCAATTTTCTCTTCTGT(CCT)560210AC-SSR307GTTGCCATTGGGAGTGTGA<br>CCTCCGTGTGAGTAGTAGTAAGTGAC(AG)3 (TG)345noneAC-SSR308GCAACACAAATCACAAGAAGAAC<br>ACCCGTGTGACTACCCTCAAAGA(GA)860noneAC-SSR309AAGGAAAAGAAGAAGAGGAGAGG<br>CGGGGGTGGTTTGGAGTAGTAAGTGAGGA(GA)1445noneAC-SSR310CTGACCAAGTTTGCGAGATACA<br>GTTTGACTGGGCGAAGAGG(GT)860184GTTTGACTGGGCGAAGAGAG<br>GTTAAGCCCGTAAAAGAAGAAACC<br>AC-SSR3121TAAGCAAAGGAAAAGACAGACA<br>CAAAGGAAAAGAAGAAGACAGACA(GA)1160222AC-SSR312TTAATAACCGTAAAAGACGTCA<br>TCTTCTAAATGAATTGGGTATC(GA)1160222  | AC-SSR303                | TTCATACAAATTCACAAACACTC                | (GT)2 AT (GT)4        | -       | none                |
| AC-SSR304TGGTTGTTTTATTAGGAGTTTA<br>AGGGGGTTCAATATCACTTCT(CA)860210AC-SSR305ACAGAAAAGTCAAACAAAGTCAA<br>CCAAGTCGAAATGGAAATCT(AC)545240AC-SSR306CGTTCCGCATGGTCGTCCT<br>TTCCCGCAATTTTCTCTTCTGT(CCT)560210AC-SSR307GTTGCCATTGGGAGTGTGA<br>CCTCCGTGTGAGTAGTAAGTAAGTGAC(AG)3 (TG)345noneAC-SSR308GCAACACAAAATCACAAGAAGAAC<br>ACCCGTGTACCCTCAAAGA(GA)1445noneAC-SSR309AAGGAAAAGAAAGAAGGGAGAGG<br>GGGGGTGGTTTGGAGGAGTTGGAGAGG(GA)1445noneAC-SSR310CTGACCAAGTTTGCGAGATACA<br>GTTTGACTGGGCGAAGAGG(GT)860184GTTAGCCCGTAGTCGAAAAACC<br>GTTAACCCGTAAAAGCAAACC(CA)645245AC-SSR312TTAATAACCGTAAAAGCAGTCA<br>GTTAAGCCCGTAAATGGGTATC(GA)1160222   |                          | CTTATATCAGGGAAGAAAAACA                 |                       |         |                     |
| AGGGGGTTCAATATCACTTCTAC-SSR305ACAGAAAAGTCAAACAAAGTCAA(AC)545240CCAAGTCGAAATGGAAATCGAAATCGAAATCT(CCT)560210AC-SSR306CGTTCCGCATGTCTCTTCTTCTGT(CCT)560210AC-SSR307GTTGCCATTGGGAGTGTGA(AG)3 (TG)345noneCCTCCGTGTGAGTAGTAGTAGTGAC(AG)860noneAC-SSR308GCAACACAAATCACAAGAAGAAC(GA)860noneAC-SSR309AAGGAAAAGAAAGAAGGGAGAGG(GA)1445noneCGGGGGTGGTTTGGAGAGACAA(GT)860184GTTTGACTGGGCGAAGAGG(CA)645245AC-SSR310CTGACCAAGTTGCGAAGAAACC(CA)645245AC-SSR311TAGCAAAGGAAAAGAAAGAAACC(CA)645245AC-SSR312TTAATAACCGTAAAAGCAGTCA(GA)1160222TCTTCTAAATGAATTGGGTATCCGA)1160222   | AC-SSR304                | TGGTTGTTTTTATTAGGAGTTTA                | (CA)8                 | 60      | 210                 |
| AC-SSR305ACAGAAAAGTCAAACAAAGTCAA<br>CCAAGTCGAAATGGAAATCT(AC)545240AC-SSR306CGTTCCGCATGGTCGTCCT<br>TTCCCGCAATTTTCTCTTCTGT(CCT)560210AC-SSR307GTTGCCATTGGGAGTGTGA<br>CCTCCGTGTGAGATAGTGAC(AG)3 (TG)345noneAC-SSR308GCAACACAAATCACAAGAAGAAC<br>ACCCGTGTACCCTCAAAGA(GA)860noneAC-SSR309AAGGAAAAGAAAGAAGAAGGAGG<br>CGGGGGTGGTTTGGAGGAG(GA)1445noneAC-SSR310CTGACCAAGTTTGCGAGAACA<br>GTTTGACTGGGCGAAGAGG(GT)860184AC-SSR311TAGCAAAGGAAAAGAAAGACAAACC<br>GTTAAGCCCGTAGTCCAAAGA(CA)645245AC-SSR312TTAATAACCGTAAAAGCAGTCA<br>TCTTCTAAATGAATTGGGTATC(GA)1160222   |                          | AGGGGGTTCAATATCACTTCT                  |                       |         |                     |
| CCAAGTCGAAATGGAAATCTAC-SSR306CGTTCCGCATGGTCGTCCT<br>TTCCCGCAATTTTCTCTTCTGT(CCT)560210AC-SSR307GTTGCCATTGGGAGTGTGA<br>CCTCCGTGTGAGTAGTAAGTGAC(AG)3 (TG)345noneAC-SSR308GCAACACAAATCACAAGAAGAAC<br>AACCGTGTACCCTCAAAGA(GA)860noneAC-SSR309AAGGAAAAGAAGAAGAGGGAGAGG<br>CGGGGGTGGTTTGGAGAGAGAG(GA)1445noneAC-SSR310CTGACCAAGTTTGCGAGATACA<br>GTTTGACTGGGCGAAGAGG(GT)860184AC-SSR311TAGCAAAGGAAAAGACAAACC<br>GTTAAGCCCGTAGTCCAAAGA(CA)645245AC-SSR312TTAATAACCGTAAAAGCAGTCA<br>TCTTCTAAATGAATTGGGTATC(GA)1160222   | AC-SSR305                | ACAGAAAAGTCAAACAAAGTCAA                | (AC)5                 | 45      | 240                 |
| AC-SSR306CGTTCCGCATGGTCGTCCT<br>TTCCCGCAATTTTCTCTTCTGT(CCT)560210AC-SSR307GTTGCCATTGGGAGTGGA<br>CCTCCGTGTGAGTAGTAAGTGAC(AG)3 (TG)345noneAC-SSR308GCAACACAAATCACAAGAAGAAC<br>AACCGTGTACCCTCAAAGA(GA)860noneAC-SSR309AAGGAAAAGAAGAAGGAGGG<br>CGGGGGTGGTTTGGAGAAGAGA(GA)1445noneAC-SSR310CTGACCAAGTTGCGAGATACA<br>GTTTGACTGGGCGAAGAGG(GT)860184AC-SSR311TAGCAAAGGAAAAGACAAACC<br>GTTAAGCCCGTAGTCCAAAGA(CA)645245AC-SSR312TTAATAACCGTAAAAGCAGTCA<br>TCTTCTAAATGAATTGGGTATC(GA)1160222   |                          | CCAAGTCGAAATGGAAATCT                   |                       |         |                     |
| TTCCCGCAATTTTCTCTTCTGTAC-SSR307GTTGCCATTGGGAGTGGAG<br>CCTCCGTGTGAGTAGTAAGTGAC(AG)3 (TG)345noneAC-SSR308GCAACACAAAATCACAAGAAGAAC<br>AACCCGTGTACCCTCAAAGA(GA)860noneAC-SSR309AAGGAAAAGAAGAAGAGGAGAGGG<br>CGGGGGTGGTTTGGAGAGAG(GA)1445noneAC-SSR310CTGACCAAGTTGCGAGAAGAG<br>GTTTGACTGGGCGAAGAGG(GT)860184AC-SSR311TAGCAAAGGAAAAGACAAACC<br>GTTAAGCCCGTAGTCCAAAGA(CA)645245AC-SSR312TTAATAACCGTAAAAGCAGTCA<br>TCTTCTAAATGAATTGGGTATC(GA)1160222   | AC-SSR306                | CGTTCCGCATGGTCGTCCT                    | (CCT)5                | 60      | 210                 |
| AC-SSR307  GTTGCCATTGGGAGTGTGA<br>CCTCCGTGTGAGTAGTAAGTGAC  (AG)3 (TG)3  45  none    AC-SSR308  GCAACACAAATCACAAGAAGAAC  (GA)8  60  none    AC-SSR309  AAGGAAAAGAAAGAAGGAGAGG  (GA)14  45  none    CGGGGGTGGTTTGGAGAGAG  (GT)8  60  184    AC-SSR310  CTGACCAAGTTGCGAGAGAGG  (GT)8  60  184    AC-SSR311  TAGCAAAGGAAAGACAAACC  (CA)6  45  245    AC-SSR312  TTAATAACCGTAAAAGCAGTCA  (GA)11  60  222   |                          | TTCCCGCAATTTTCTCTTCTGT                 |                       |         |                     |
| CCTCCGTGTGAGTAGTAAGTGACAC-SSR308GCAACACAAATCACAAGAAGAAC(GA)860noneAACCCGTGTACCCTCAAAGAAAGGAAAAGAAAGAAGAAGGAGAGGG(GA)1445noneCGGGGGTGGTTTGGAGAGGCTGACCAAGTTTGCGAGAGAGG(GT)860184AC-SSR310CTGACCAAGTTTGCGAGAGAGG(GT)860184GTTTGACTGGGCGAAGAGGCA)11TAGCAAAGGAAAAGACAAACC(CA)645245AC-SSR312TTAATAACCGTAAAAGCAGTCA(GA)1160222TCTTCTAAATGAATTGGGTATCCA)1160222   | AC-SSR307                | GTTGCCATTGGGAGTGTGA                    | (AG)3 (TG)3           | 45      | none                |
| AC-SSR308GCAACACAAATCACAAGAAGAAC<br>AACCCGTGTACCCTCAAAGA(GA)860noneAC-SSR309AAGGAAAAGAAGAAGAAGGGAGAGG<br>CGGGGGTGGTTTGGAGAG(GA)1445noneAC-SSR310CTGACCAAGTTTGCGAGATACA<br>GTTTGACTGGGCGAAGAGG(GT)860184AC-SSR311TAGCAAAGGAAAAGACAAACC<br>   |                          | CCTCCGTGTGAGTAGTAAGTGAC                |                       |         |                     |
| AACCCGTGTACCCTCAAAGAAC-SSR309AAGGAAAAGAAGAAGGGAGAGG<br>CGGGGGTGGTTTGGAGAG(GA)1445noneCGGGGGTGGTTTGGAGAG(GT)860184AC-SSR310CTGACCAAGTTTGCGAGATACA<br>GTTTGACTGGGCGAAGAGG(CA)645245AC-SSR311TAGCAAAGGAAAAGACAAACC<br>GTTAAGCCCGTAGTCCAAAGA(CA)645245AC-SSR312TTAATAACCGTAAAAGCAGTCA<br>TCTTCTAAATGAATTGGGTATC(GA)1160222  | AC-SSR308                | GCAACACAAATCACAAGAAGAAC                | (GA)8                 | 60      | none                |
| AC-SSR309AAGGAAAAGAAGAAGAAGGGAGAGG<br>CGGGGGTGGTTTGGAGAG(GA)1445noneAC-SSR310CTGACCAAGTTTGCGAGATACA<br>GTTTGACTGGGCGAAGAGG(GT)860184AC-SSR311TAGCAAAGGAAAAGACAAACC<br>GTTAAGCCCGTAGTCCAAAGA(CA)645245AC-SSR312TTAATAACCGTAAAAGCAGTCA<br>TCTTCTAAATGAATTGGGTATC(GA)1160222   |                          | AACCCGTGTACCCTCAAAGA                   |                       |         |                     |
| CGGGGGTGGTTTGGAGAGAC-SSR310CTGACCAAGTTTGCGAGATACA<br>GTTTGACTGGGCGAAGAGG(GT)860184AC-SSR311TAGCAAAGGAAAAGACAAACC<br>GTTAAGCCCGTAGTCCAAAGA(CA)645245AC-SSR312TTAATAACCGTAAAAGCAGTCA<br>TCTTCTAAATGAATTGGGTATC(GA)1160222   | AC-SSR309                | AAGGAAAAGAAAGAAGGGAGAGG                | (GA)14                | 45      | none                |
| AC-SSR310CTGACCAAGTTTGCGAGATACA<br>GTTTGACTGGGCGAAGAGG(GT)860184AC-SSR311TAGCAAAGGAAAAGACAAACC<br>GTTAAGCCCGTAGTCCAAAGA(CA)645245AC-SSR312TTAATAACCGTAAAAGCAGTCA<br>TCTTCTAAATGAATTGGGTATC(GA)1160222   |                          | CGGGGGTGGTTTGGAGAG                     |                       |         |                     |
| GTTTGACTGGGCGAAGAGGAC-SSR311TAGCAAAGGAAAAGACAAACC<br>GTTAAGCCCGTAGTCCAAAGA(CA)645245AC-SSR312TTAATAACCGTAAAAGCAGTCA<br>TCTTCTAAATGAATTGGGTATC(GA)1160222  | AC-SSR310                | CTGACCAAGTTTGCGAGATACA                 | (GT)8                 | 60      | 184                 |
| AC-SSR311 TAGCAAAGGAAAAGACAAACC (CA)6 45 245<br>GTTAAGCCCGTAGTCCAAAGA<br>AC-SSR312 TTAATAACCGTAAAAGCAGTCA (GA)11 60 222<br>TCTTCTAAATGAATTGGGTATC   |                          | GTTTGACTGGGCGAAGAGG                    |                       |         |                     |
| GTTAAGCCCGTAGTCCAAAGAAC-SSR312TTAATAACCGTAAAAGCAGTCA<br>TCTTCTAAATGAATTGGGTATC60222   | AC-SSR311                | TAGCAAAGGAAAAGACAAACC                  | (CA)6                 | 45      | 245                 |
| AC-SSR312 TTAATAACCGTAAAAGCAGTCA (GA)11 60 222<br>TCTTCTAAATGAATTGGGTATC  |                          | GTTAAGCCCGTAGTCCAAAGA                  |                       |         |                     |
| TCTTCTAAATGAATTGGGTATC  | AC-SSR312                | TTAATAACCGTAAAAGCAGTCA                 | (GA)11                | 60      | 222                 |
|   |                          | TCTTCTAAATGAATTGGGTATC                 |                       |         |                     |

Continue...

each PCR, since the analyses were performed in multiplex, with 9  $\mu$ L formamide and 0.4  $\mu$ L ROX 500 standard (Figure 1). It was possible to obtain great differentiation of analyzed SSRs in the multiplex process, even for those amplicons with similar sizes, since the software enables independent visualization of different fluorescences for allele identification with a precision of 1 bp.

In order to optimize allele identification, PCR sample purification was also tested before being submitted to the sequencer. The hypothesis was that this step could possibly eliminate salt contamination present in the PCR and optimize the results. Purification was performed using isopropanol. After drying, samples were resuspended in pure water, re-establishing the final volume of 15  $\mu$ L per sample. Similar to what was done

| Table 1. Continuation |
|-----------------------|
|-----------------------|

| Locus/GeneBank accession | Primer sequences $(5^{\prime} \rightarrow 3^{\prime})$ | Core motifs                        | Ta (°C) | Predicted size (pb) |
|--------------------------|--|------------------------------------|---------|---------------------|
| AC-SSR313                | ATGCTTGCTGATATGTCTGCT                                  | (GT)9                              | 45      | 291                 |
|                          | ACGATTTGCCCTGTTTC                                      | (01)                               |         |                     |
| AC-SSR314                | TGCACTTCGACGCTCAA                                      | (TA)2 TT (TG)3                     | 45      | 153                 |
|                          | CCAGGGATTAGGGAAGATTT                                   |                                    |         |                     |
| AC-SSR315                | AAAGTCTCCCTTCTTCTAATAAA                                | (CA)6                              | -       | none                |
|                          | GAAAATACCTCCCCGAAACT                                   |                                    |         |                     |
| AC-SSR316                | GAAGATCCCAACAGAAAATGAA                                 | (AG)10 and (GA)5                   | _       | none                |
|                          | TTGCTCTACGCTCTGCTATGTC                                 |                                    |         |                     |
| AC-SSR317                | TGTATTGCAGCTTTCACTTTTG                                 | (TC)22                             | 45      | 250                 |
|                          | TGGTTGGGCATAGGTAATCA                                   |                                    |         |                     |
| AC-SSR318                | ATATAGGTTGTTCTGTCTTGATG                                | (GT)7                              | 50      | 128                 |
|                          | GGGGTTTCAGTCGTGTTC                                     |                                    |         |                     |
| AC-SSR319                | TGAAGGATGAGAAGGAGGACTAA                                | (TC)16                             | -       | none                |
|                          | AAGGAAATAACCCCCGATGAA                                  |                                    |         |                     |
| AC-SSR320                | ATGGTGGCCTGTCGTTCA                                     | (TG)6                              | 56      | 220                 |
|                          | TGTCCTCTGGATTCTGTGTCTAC                                |                                    |         |                     |
| AC-SSR321                | GTCCCTAATCTGATCGTAAGTT                                 | (AC)7                              | 51.4    | 163                 |
|                          | CTACCATGAAATAGTAATGTAAAATA                             |                                    |         |                     |
| AC-SSR322                | TCGGATGCGTGAGTGTG                                      | (TG)8                              | 51.4    | 262                 |
|                          | ATGTAATTTGGTGGGTGGTTT                                  |                                    |         |                     |
| AC-SSR323                | TCTGTCCGATGCGTGTCA                                     | (CA)5                              | 45      | 183                 |
|                          | CACCTGGCTCAGTCATTCTTAG                                 |                                    |         |                     |
| AC-SSR324                | AATATGGGGTGCCTGAAGAGAC                                 | (CA)6                              | 60      | 258                 |
|                          | GGCTGGTATCCGACAATGG                                    |                                    |         |                     |
| AC-SSR325                | TGGGATTCAAAGTCACCTC                                    | (GT)5                              | 60      | 208                 |
|                          | CAACCGATTGTTTATGGAAG                                   |                                    |         |                     |
| AC-SSR326                | TGGCCTTGCATCAGTTG                                      | (CA)6                              | 60      | 230                 |
|                          | AGGTGTTGCCATTGCTTAT                                    |                                    |         |                     |
| AC-SSR327                | AACAAAGTCATGCATAAATACAA                                | (GT)7                              | 60      | 256                 |
|                          | AGCATCCAGGAGAACCAG                                     |                                    |         |                     |
| AC-SSR328                | ACGCTGCGCTCGGTGTCA                                     | (GA)2 (CT)3                        | 60      | 266                 |
|                          | TACGGGGGCCCTTATGGTG                                    |                                    |         |                     |
| AC-SSR329                | CTGACCAAGTTTGCGAGATACA                                 | (CT)8                              | 60      | 184                 |
|                          | GTTTGACTGGGCGAAGAGG                                    | (21)                               |         |                     |
| AC-SSR330                | TAGCAAAGGAAAAGACAAACC                                  | (CA)6                              | 45      | 245                 |
|                          | GITAAGCCCGTAGTCCAAAGA                                  | (OTT) 4.0                          | <u></u> | 1.50                |
| AC-SSR331                | CAAACCIAGAAAGCAGCACAG                                  | (C1)19                             | 60      | 152                 |
| 4 C C C C D 2 2 2        | ACIAITIIGGGGAITCICIIGA                                 |                                    | (0)     | 227                 |
| AC-SSR332                | GCAGGGCGCGTGATTCTTG                                    | (AI)3 GG (AI)3                     | 60      | 237                 |
| A C C C C D 222          | GAUIGGAIGUIUGIGGUGIGAIG                                |                                    | (0)     | 1.4.1               |
| AC-88K333                |  | (AC)6 (AT)2 (AC)2                  | 60      | 141                 |
| AC 55D224                |  | (CT)9                              | 60      | 100                 |
| AC-55K554                | CCTCCTAGTCCCCTCCCATTC                                  | (C1)9                              | 00      | 182                 |
| AC SSD225                |  | $(C \Lambda)$ 7                    | 15      | 200                 |
| AC-55K555                |  | (GA)/                              | 43      | 380                 |
| AC SSD226                |  | (CA)7                              | 60      | 200                 |
| AC-33K330                |  | (UA)/                              | 00      | 500                 |
| AC-SSR337                | TATTTATTAGTGGCATTGTCC                                  | (GA)9                              | 60      | 187                 |
| 10.001001                | TGAGAGATCCTGTAGAAGTAGTAGT                              | $(\mathbf{U}\mathbf{A})\mathbf{z}$ | 00      | 10/                 |
| AC-88R338                | GCTGCAACCTACTACCTGG                                    | (AG)26                             | 45      | 190                 |
| 10 001000                | TCTCTTCTCCTCCGCTCTTT                                   | (10)20                             | 70      | 170                 |
|                          |  |                                    |         |                     |

Pesq. agropec. bras., Brasília, v.44, n.6, p.638-644, jun. 2009

previously, different volumes of PCR products were tested along with size standard, and the best result was found for approximately 1.5  $\mu$ L of each PCR product, 9  $\mu$ L formamide and 0.4  $\mu$ L size standard (Figure 2).

The best optimization condition for fluorescentlabeled microsatellite PCR (Table 2 -condition 1) was used for microsatellite genotyping of 380 common bean lines for genetic mapping purposes towards reducing general costs and time expenses. In fact, all 50 microsatellites presented a satisfactory signal on DNA sequencer in multiplex analyses, allowing the genotyping of all lines quickly and accurately. Even so, same amplicons showed better signals then others.

Fluorescence PCR-labeled technique using the M13 universal primer is an easy method to assay and very interesting to speed up data scoring, producing high-

**Table 2.** Four polymerase chain reaction (PCR) conditions tested for genotyping by fluorescent-labeled microsatellite in common bean. Each amplification condition involves only different concentrations of the three primers involved in PCR (M13 primer, reverse and forward-tail). Other reagents were kept in fixed concentrations.

| Primers             | Condition 1                           | Condition 2                | Condition 3                    | Condition 4                          |
|---------------------|---------------------------------------|----------------------------|--------------------------------|--------------------------------------|
| M13 primer          | 0.8 pmol μL <sup>-1</sup>             | 0.16 pmol µL <sup>-1</sup> | 0.08 pmol μL <sup>-1</sup>     | 0.8 pmol $\mu$ L <sup>-1</sup>       |
| Reverse primer      | 0.8 pmol μL <sup>-1</sup>             | 0.16 pmol μL <sup>-1</sup> | 0.8 pmol $\mu$ L <sup>-1</sup> | $0.8 \text{ pmol } \mu\text{L}^{-1}$ |
| Forward-tail primer | $0.2 \text{ pmol } \mu \text{L}^{-1}$ | 0.04 pmol μL <sup>-1</sup> | 0.8 pmol μL <sup>-1</sup>      | 0.8 pmol μL <sup>-1</sup>            |



**Figure 1.** Optimization results showing allele peaks from two differentially-labeled microsatellites. A1 and A2 are alleles from IAC-SSR334 labeled with 6-FAM (blue). B1 and B2 are alleles from IAC-SSR300 labeled with HEX (green). The different intensity of the blue and green peaks resulted from the specific dynamics of each PCR, depending exclusively on the primer pairs assayed. Both fluorescences showed similar results with great peak intensity.



**Figure 2.** Peak intensity of different treatments are shown using polymerase chain reaction (PCR) purification. All peaks resulted from IAC-SSR293 labeled with 6-FAM. A: control with no purification; B, C and D: tests with 0.5  $\mu$ L, 1  $\mu$ L and 1.5  $\mu$ L purified DNA, respectively. The highest intensity peak was found for 1.5  $\mu$ L PCR-purified product (D).

throughput genotyping, especially for the purpose of mapping large segregant populations with a huge number of markers. This fluorescent-based genotyping showed to be faster than the silver staining genotyping method with polyacrylamide gels. It was also more practical to use universal M13 primers than labeling each microsatellite primer with a specific fluorescence. These results reinforce the high analysis capacity of this method, reducing time, costs and labor.

#### Acknowledgements

To Dr. Marcos Antônio Machado and Dr. Marcos Takita at Centro de Citricultura Sylvio Moreira, Instituto Agronômico, Cordeirópolis, SP, Brazil, who kindly provided the 3730 DNA analyzer (Applied Biosystems); to Fundação de Amparo à Pesquisa do Estado de São Paulo, for the financial support and the scholarship granted.

### References

BENCHIMOL, L.L.; CAMPOS, T. de; CARBONELL, S.A.M.; COLOMBO, C.A.; CHIORATTO, A.F.; FORMIGHIERI, E.F.; GOUVÊA, L.R.L.; SOUZA, A.P. de. Structure of genetic diversity among common bean (*Phaseolus vulgaris* L.) varieties of Mesoamerican and Andean origins using new developed microsatellite markers. **Genetic Resources and Crop Evolution**, v.54, p.1747-1762, 2007.

BROUGHTON, W.J.; HERNÁNDEZ, G.; BLAIR, M.; BEEBE, S.; GEPTS, P.; VANDERLEYDEN, J. Beans (*Phaseolus* spp.) - model food legumes. **Plant and Soil**, v.252, p.55-128, 2003.

CRESTE, S.; TULMANN NETO, A.; FIGUEIRA, A. Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. **Plant Molecular Biology Reporter**, v.19, p.299-306, 2001.

DAVID, L.S.; SCOTT, L.S.; STEPHEN, C.R. An alternate universal forward primer for improved automated DNA sequencing of M13. **BioTechniques**, v.15, p.580-582, 1993.

GEPTS, P.; ARAGÃO, F.J.L.; BARROS, E. de; BLAIR, M.W.; BRONDANI, R.; BROUGHTON, W.; GALASSO, I.; HERNÁNDEZ, G.; KAMI, J.; LARIGUET, P.; MCCLEAN, P.; MELOTTO, M.; MIKLAS, P.; PAULS, P.; PEDROSA- HARAND, A.; PORCH, T.; SÁNCHEZ, F.; SPARVOLI, F.; YU, K. Genomics of *Phaseolus* beans, a major source of dietary protein and micronutrients in the tropics. In: **Genomics of Tropical Crop Plants**. New York: Springer Press, 2008. v.1. p.113-143.

LI, Y.C.; KOROL, A.B.; FAHIMA, T.; BEILES, A.; NEVO, E. Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. **Molecular Ecology**, v.11, p.2453-2465, 2002.

MISSIAGGIA, A.; GRATTAPAGLIA, D. Plant microsatellite genotyping with 4-color fluorescent detection using multiple-tailed primers. **Genetics and Molecular Research**, v.5, p.72-78, 2006.

OETTING, W.S.; LEE, H.K.; FLANDERS, D.J.; WIESNER, G.L.; SELLERS, T.A.; KING, R.A. Linkage analysis with multiplexed

short tandem repeat polymorphisms using infrared fluorescence and M13 tailed primers. **Genomics**, v.30, p.450-458, 1995.

SCHLUETER, J.A.; GOICOECHEA, J.L.; COLLURA, K.; GILL, N.; LIN, J.Y.; YU, Y.; KUDRNA, D.; ZUCCOLO, A.; VALLEJOS, C.E.; MUÑOZ-TORRES, M.; BLAIR, M.W.; TOHME, J.; TOMKINS, J.; MCCLEAN, P.; WING, R.A.; JACKSON, S.A. BAC-end sequence analysis and a draft physical map of the common bean (*Phaseolus vulgaris* L.) genome. **Tropical Plant Biology**, v.1, p.40-48, 2008.

SCHUELKE, M. An economic method for the fluorescent labeling of PCR fragments. **Nature Biotechnology**, v.18, p.233-234, 2000.

VARSHNEY, R.K.; GRANER, A.; SORRELLS, M.E. Genic microsatellite markers in plants: features and applications. **Trends in Biotechnology**, v.23, p.48-55, 2005.

Received on February 10, 2009 and accepted on May 25, 2009