Notas Científicas

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

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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 fluorescence-based 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 fluorescent-labeled 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 fluorescent-labeling 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 fluorescent-labeled microsatellites is to optimize PCR reactions. In this study, SSR-specific annealing temperatures have already been determined (56 or 60ºC). 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 53ºC 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: 94ºC (1 min), then 30 cycles at 94ºC (1 min)/specific annealing temperature (56 or 60ºC) for each SSR (1 min)/72ºC (1 min), followed by 8 cycles at 94ºC (1 min)/53ºC (1 min)/72ºC (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 μL^{-1} reverse primer and labeled M13 and 0.2 pmol μL^{-1} forward primer, with a final volume of 15 µL.

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 µL of the product from

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

Locus/GeneBank accession	Primer sequences (5'→ 3')	Core motifs	Ta (°C)	Predicted size (pb)
AC-SSR289	ATCGGTTGAATTGGCTTGAC ATTGCTTAAAGACTCCTGTTGC	(AC)7	60	210
AC-SSR290	GGAGAAAGATGCAGGAAGAA TTTTTTGGATGGTGTTGTC	(AC)6	60	289
AC-SSR291	CCCCCACGCTCTTGTTGC CATGCGTCTTGAGCCTGTGA	(CA)7	60	213
AC-SSR292	AACAGAAATGAATTAACACAAAC TGGGATTCAAAGTCACCTC	(CA)6	60	189
AC-SSR293	ATGCCGTTTTTGGATGTGAG AATTTGGACGTGGAGTGTTTTC	(AG)3 GT (GA)3	60	237
AC-SSR294	TGGAATTGAAACTTGTAAAAAC GGAAGATGCACCACAGAAAAG	(AC)6 (TC)2	60	125
AC-SSR295	GATTCTTCCCACCCTCACT ACACTTCAAACCCAAACATAAT	(TG)7 and (GT)3 (AG)3	60	217
AC-SSR296	TGCTGATTCTGATGTAACTTTC AGACACTGTGGACATTCAACTG	(GT)3 G (GT)2	60	192
AC-SSR297	AGGGCTAGGAAACATAAAAAGAC GCCCACCCACCAGAAAG	(GT)7	60	160
AC-SSR298	ACACCCAACCTCCCTCTAAAC GCAGCAGGTAGTAAAGATAATGA	(CA)7	60	155
AC-SSR299	AAATAGTCATCAATGCCTGTGT TTGTTGTGCTTGATCCTAAAAAC	(AC)5 A (AC)4	60	232
AC-SSR300	CATGTGGGGGAACGTGAGGACT GGGGCGGGGAGGGAAAAGA	(GA)14	45	197
AC-SSR301	GGACAAGGGACTCGGAAAAGA GTCAGCCAGTAAGAAACACCAA	(CA)5 A (CA)3	60	130
AC-SSR302	TCTCTGCGTTTTGGATTC TTAAGATTAGGAGTAGGGTTTC	(AG)14	45	295
AC-SSR303	TTCATACAAATTCACAAACACTC CTTATATCAGGGAAGAAAAACA	(GT)2 AT (GT)4	–	none
AC-SSR304	TGGTTGTTTTTATTAGGAGTTTA AGGGGGTTCAATATCACTTCT	(CA)8	60	210
AC-SSR305	ACAGAAAAGTCAAACAAAGTCAA CCAAGTCGAAATGGAAATCT	(AC)5	45	240
AC-SSR306	CGTTCCGCATGGTCGTCCT TTCCCGCAATTTTCTCTTCTGT	(CCT)5	60	210
AC-SSR307	GTTGCCATTGGGAGTGTGA CCTCCGTGTGAGTAGTAAGTGAC	(AG)3 (TG)3	45	none
AC-SSR308	GCAACACAAATCACAAGAAGAAC AACCCGTGTACCCTCAAAGA	(GA)8	60	none
AC-SSR309	AAGGAAAAGAAAGAAGGGAGAGG CGGGGGTGGTTTGGAGAG	(GA)14	45	none
AC-SSR310	CTGACCAAGTTTGCGAGATACA GTTTGACTGGGCGAAGAGG	(GT)8	60	184
AC-SSR311	TAGCAAAGGAAAAGACAAACC GTTAAGCCCGTAGTCCAAAGA	(CA)6	45	245
AC-SSR312	TTAATAACCGTAAAAGCAGTCA TCTTCTAAATGAATTGGGTATC	(GA)11	60	222

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each PCR, since the analyses were performed in multiplex, with 9 µL formamide and 0.4 µL 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 µL per sample. Similar to what was done

Table 1. Continuation.

Locus/GeneBank accession	Primer sequences (5'→3')	Core motifs	Ta (°C)	Predicted size (pb)
AC-SSR313	ATGCTTGCTGATATGTCTGCT ACGATTTGCCCTGTTTC	(GT)9	45	291
AC-SSR314	TGCACTTCGACGCTCAA CCAGGGATTAGGGAAGATTT	(TA)2 TT (TG)3	45	153
AC-SSR315	AAAGTCTCCCTTCTTCTAATAAA GAAAATACCTCCCCGAAACT	(CA)6	–	none
AC-SSR316	GAAGATCCCAACAGAAAATGAA TTGCTCTACGCTCTGCTATGTC	(AG)10 and (GA)5	–	none
AC-SSR317	TGTATTGCAGCTTTCACTTTTG TGGTTGGGCATAGGTAATCA	(TC)22	45	250
AC-SSR318	ATATAGGTTGTTCTGTCTTGATG GGGGTTTCAGTCGTGTTC	(GT)7	50	128
AC-SSR319	TGAAGGATGAGAAGGAGGACTAA AAGGAAATAACCCCCGATGAA	(TC)16	–	none
AC-SSR320	ATGGTGGCCTGTCGTTCA TGTCCTCTGGATTCTGTGTCTAC	(TG)6	56	220
AC-SSR321	GTCCCTAATCTGATCGTAAGTT CTACCATGAAATAGTAATGTAAAATA	(AC)7	51.4	163
AC-SSR322	TCGGATGCGTGAGTGTG ATGTAATTTGGTGGGTGGTTT	(TG)8	51.4	262
AC-SSR323	TCTGTCCGATGCGTGTCA CACCTGGCTCAGTCATTCTTAG	(CA)5	45	183
AC-SSR324	AATATGGGGTGCCTGAAGAGAC GGCTGGTATCCGACAATGG	(CA)6	60	258
AC-SSR325	TGGGATTCAAAGTCACCTC CAACCGATTGTTTATGGAAG	(GT)5	60	208
AC-SSR326	TGGCCTTGCATCAGTTG AGGTGTTGCCATTGCTTAT	(CA)6	60	230
AC-SSR327	AACAAAGTCATGCATAAATACAA AGCATCCAGGAGAACCAG	(GT)7	60	256
AC-SSR328	ACGCTGCGCTCGGTGTCA TACGGGGGCCCTTATGGTG	(GA)2 (CT)3	60	266
AC-SSR329	CTGACCAAGTTTGCGAGATACA GTTTGACTGGGCGAAGAGG	(CT)8	60	184
AC-SSR330	TAGCAAAGGAAAAGACAAACC GTTAAGCCCGTAGTCCAAAGA	(CA)6	45	245
AC-SSR331	CAAACCTAGAAAGCAGCACAG ACTATTTTGGGGATTCTCTTGA	(CT)19	60	152
AC-SSR332	GCAGGGCGCGTGATTCTTG GACTGGATGCTCGTGGGTGATG	(AT)3 GG (AT)3	60	237
AC-SSR333	GCTTGCCCGTGCTTGAGG TCTGTCCACCCACGTATCTGAG	(AC)6 (AT)2 (AC)2	60	141
AC-SSR334	CCTAGAAAGCTACTGGCAAAGAG CGTGGTAGTCGGTGGGATTC	(CT)9	60	182
AC-SSR335	GAAAGAAGAAGAGGGAAGGA CACAGCATGATGGAAAAGTAA	(GA)7	45	380
AC-SSR336	CCCAGAAGACCTAGCCACCAG CAATTTCAACACCCCCACTACC	(GA)7	60	300
AC-SSR337	TATTTTATTAGTGGCATTGTCC TGAGAGATCCTGTAGAAGTAGTAGT	(GA)9	60	187
AC-SSR338	GCTGCAACCTACTACCTGG TCTCTTCTCCTCCGCTCTTT	(AG)26	45	190

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

The best optimization condition for fluorescent-labeled 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^{-1}	0.16 pmol μL^{-1}	0.08 pmol μL^{-1}	0.8 pmol μL^{-1}
Reverse primer	0.8 pmol μL^{-1}	0.16 pmol μL^{-1}	0.8 pmol μL^{-1}	0.8 pmol μL^{-1}
Forward-tail primer	0.2 pmol μL^{-1}	0.04 pmol μL^{-1}	0.8 pmol μL^{-1}	0.8 pmol μL^{-1}

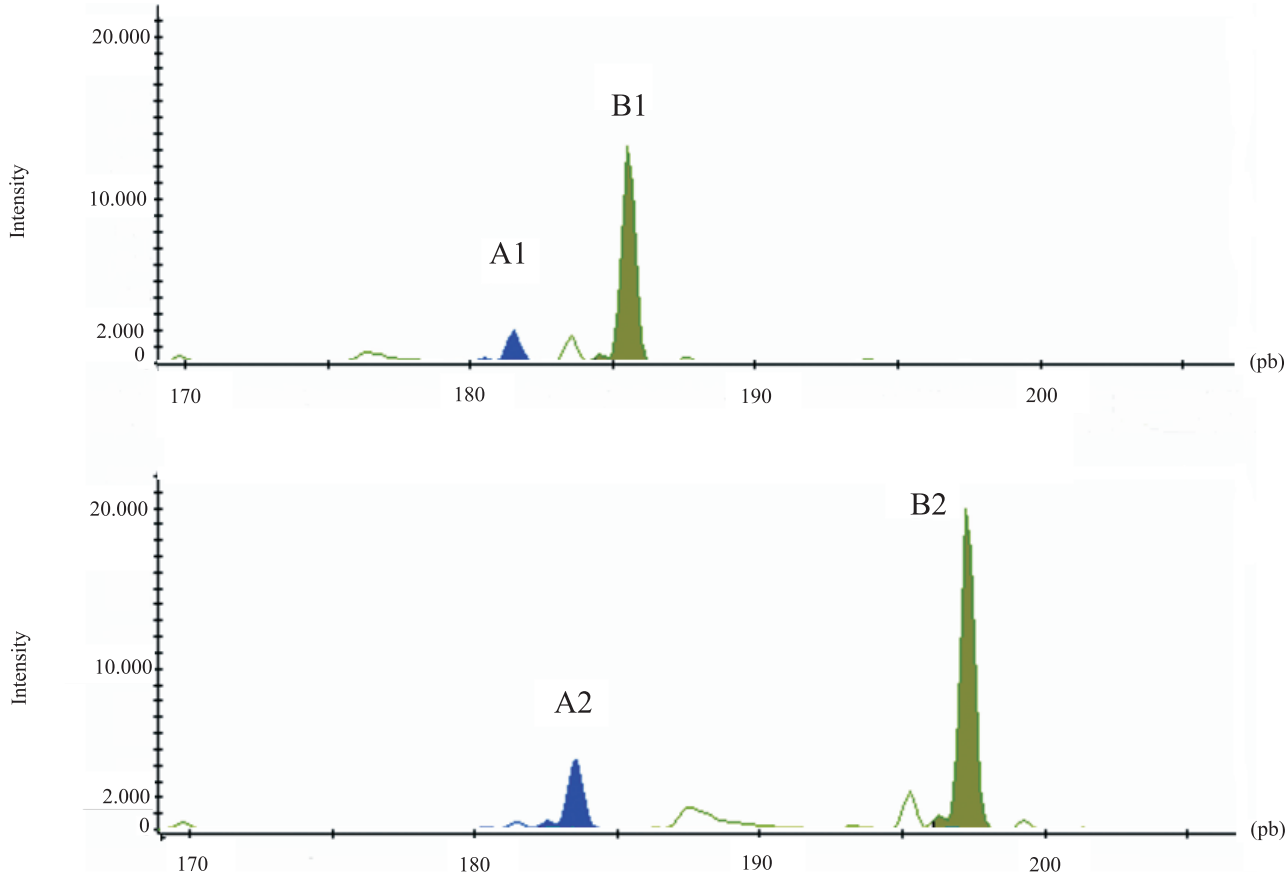

Figure 1. Optimization results showing allele peaks from two differentially-labeled microsatellites. A1 and A2 are alleles from IAC-SSR334 labeled with 6-FAM (blue). B1 and B2 are alleles from IAC-SSR300 labeled with HEX (green). The different intensity of the blue and green peaks resulted from the specific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

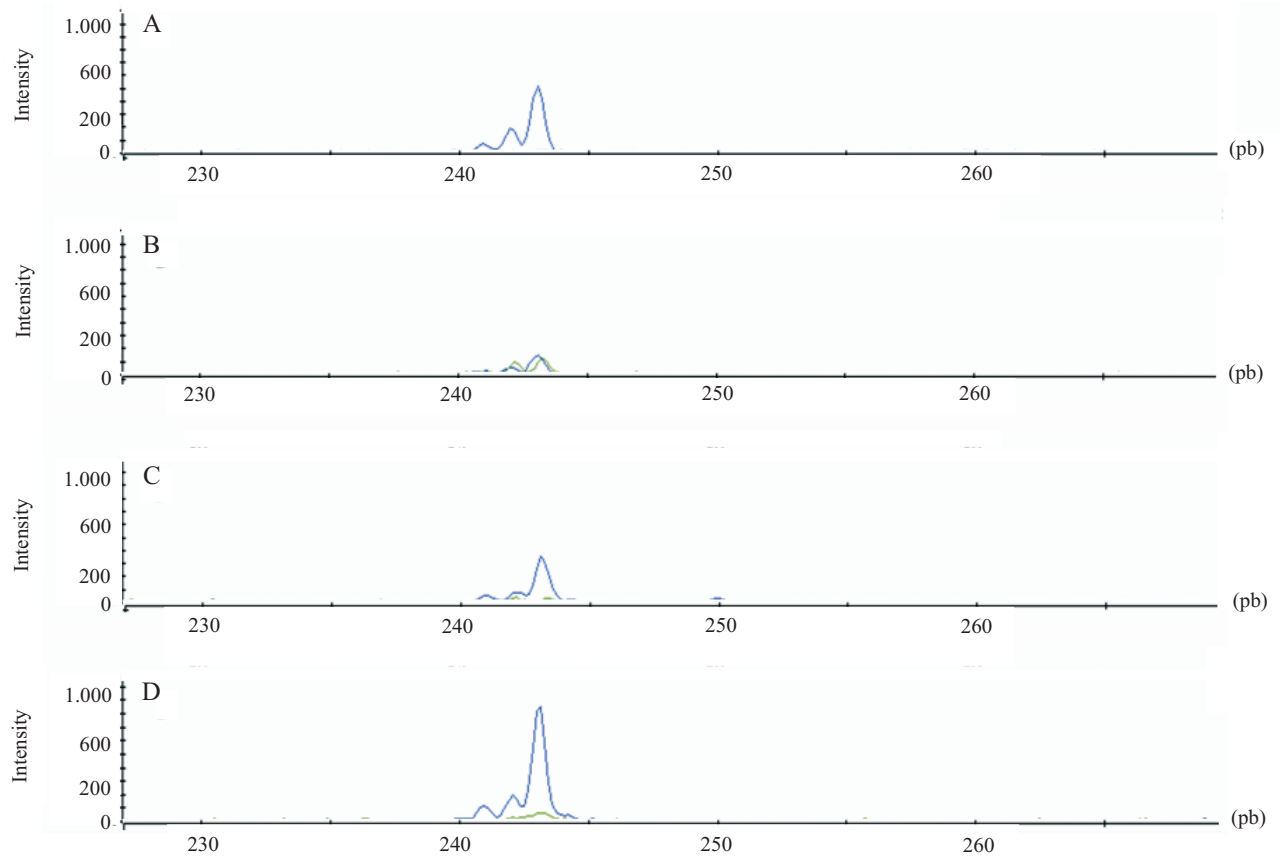

Figure 2. Peak intensity of different treatments are shown using polymerase chain reaction (PCR) purification. All peaks resulted from IAC-SSR293 labeled with 6-FAM. A: control with no purification; B, C and D: tests with 0.5 µL, 1 µL and 1.5 µL purified DNA, respectively. The highest intensity peak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

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