

Efficiency of RAPD in assessing genetic variation in commercially viable local varieties of blueberry (*Vaccinium*)

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ABSTRACT

The growth in both production and plantation surface area of blueberries in Chile has been positive, with an estimated surface area of 13,057 hectares (16.9% planting surface) and production in the order of 56,000 tons (16.3% mundial production). Of all the different species of blueberry there are several varieties that have advantages with regard to certain conditions; for example, one variety adapts better to the climate of a particular area, while others are better for early or late harvests, they can also be resistant to fungus, bacteria, etc. Companies are very interested in this issue because prior knowledge of such data can bring financial benefits. Blueberries have a significant degree of morphological variation, which enables recognition of different clones with different environmental adaptation characteristics, but it also allows us to discriminate between different levels of fruit quality, which is of commercial interest. However, these morphological characteristics are late in their expression, making it impossible to recognize the clones in the early stage of their development. A more efficient tool to be able to recognize different clones is the use of molecular markers. Of the techniques based the RAPD (randomly amplified polymorphic DNA). They have the advantage that the method has no need

for prior knowledge of the DNA sequence. It is of great interest to the private sector to have prior access to information on the types of clone they possess, in order to then be able to differentiate the varieties, but for this it is necessary to obtain a quick and economical technique. In this study, through the use of RAPD-PCR, it is possible to differentiate between different varieties of *Vaccinium* grown in Chile in order to then optimize blueberry production in terms of time and resources.

Keywords: Blueberry; RAPD; Genetic Variation; Molecular Marker

1. INTRODUCTION

Blueberries are members of the Ericaceae or Heath family, genus *Vaccinium*, subgenus *Cyanococcus*. Genus *Vaccinium* consists of blueberries, cranberries, lingoberrries and many related wild species. The genus is very diverse, containing about 400 species, mostly found in the tropics at high elevation, but also in temperate and boreal regions [1].

In Chile, blueberry plantations are found from the 4th to the 10th regions. The species that are grown are: *Vaccinium corymbosum* (highbush blueberry), *Vaccinium ashei* (rabbit-eye blueberry) and *Vaccinium macrocarpon* (cranberry). National production is basically focused on the international fresh produce market, which has in-

creased in recent years thanks to free trade agreements. The growth in both production and plantation surface area of blueberries in Chile has been positive, with an estimated surface area of 13,057 hectares (16.9% planting surface) and production in the order of 56,000 tons (16.3% mundial production) [2]. The price paid for the blueberries fluctuates according to the month of export, reaching values of US\$ 25 - 35/Kg. The maximum price is achieved due to the location of Chile in the southern hemisphere, which means greater productivity during the October-February period (the spring-summer seasons), a period during which production in the northern hemisphere is at a minimum, as it is the fall-winter season. Of all the different species of blueberry there are several varieties that have advantages with regard to certain conditions; for example, one variety adapts better to the climate of a particular area, while others are better for early or late harvests, they can also be resistant to fungus, bacteria, etc. Companies are very interested in this issue because prior knowledge of such data can bring financial benefits [3].

Phenotypic plasticity in plants is considered an evolutionary and environmental adaptation strategy [4,5]. In this idea, the clone of a species is seen on two recognizable levels of organization: “genets” and “ramets” [6,7]. Evolutionary somatic mutation and genetic variation occur on the level of genes, and studies at this level have provided information on population dynamics (on a genetic level) and have enable monitoring of the evolution of differences generations of clones [8].

The identification of different clones of the same species (such as *Vaccinium*) has mainly been performed through evaluation of [6,9]. Blueberries have a significant degree of morphological variation, which enables recognition of different clones with different environmental adaptation characteristics [10], but it also allows us to discriminate between different levels of fruit quality, which is of commercial interest. However, these morphological characteristics are late in their expression, making it impossible to recognize the clones in the early stage of their development. A more efficient tool to be able to recognize different clones is the use of molecular markers [6,11], examples of which are those based on isoenzymes and DNA. Of the techniques based on DNA, the most notable are AFLP (amplified fragment length polymorphism) [12,13] RFLP (restriction fragment length polymorphism), SSRs (simple sequence repeats) [14], microsatellites and RAPD (randomly amplified polymorphic DNA) [13,15]. The advantage of the latter methodology is that there is no need for prior knowledge of the DNA sequence [16].

It is of great interest to the private sector to have prior access to information on the types of clone they possess,

in order to then be able to differentiate the varieties, but for this it is necessary to obtain a quick and economical technique. In this study, through the use of RAPD-PCR, it is possible to differentiate between different varieties of *Vaccinium* grown in Chile in order to then optimize blueberry production in terms of time and resources.

2. MATERIALS AND METHODS

Varieties of *Vaccinium* were obtained from the company, HORTIFRUT. The samples were new leaves of the *V. corymbosum* varieties Misty (V1), Michigan (V2), Elliot (V3 and V6), Bluecrop (V4), Duke (V5), and two unknown varieties (V7 and V8).

DNA Analysis

Total genomic DNA was extracted from the new leaves (approx. 20 days) using the CTAB method [17]. For each 25 mg of leaf, the following was added: 1 ml of 2× CTAB buffer, 1% PVP and 1:1000 2-β-mercaptoethanol, and this was then left to soak for 2 minutes at 4°C. The soaked leaves were then incubated for half an hour at 65°C to completely separate the DNA from the nuclear proteins. During incubation the mixture was carefully stirred by inversion every five minutes. The separation of the genomic DNA from the rest of the cellular components was done by extraction with an organic solvent, for which a quantity of chloroform/isoamyl alcohol (24/1) was added and then mixed by inversion at room temperature. Final separation was achieved by centrifugation are 10,000 rpm at room temperature for 10 minutes.

After centrifugation there were two phases; to minimize sugar co-precipitation the aqueous phase was supplemented with a solution of 5% CTAB in a proportion of 1:10 and this was carefully mixed by inversion. Then 2/3 of the quantity of isopropyl alcohol was added and again mixed by inversion, followed by incubation at room temperature for one hour. This was then centrifuged at 7000 rpm. Finally, the precipitate was re-suspended in an adequate amount of TE buffer solution. In order to avoid contamination with RNA, RNase A was added at a concentration of 730 U/ml with 2 l of the sample. Reaction conditions were set at 37°C for one hour. Once the reaction was completed the samples were stored at -20°C.

In order to amplify the genomic DNA using RAPD, synthetic markers or primers were used (oligonucleotides of around 10 bp, **Table 1**). PCR was carried out in a total volume of 50 µl. The reaction mixture was 47 µl MIX (5 µl 10× amplification dampening solution, 8 µl of a mix of deoxynucleotides 1.25 mmol each, 0.5 µl *Taq* polymerase DNA 5 U/µl and 33.5 µl water), 2 µl of divider at

Table 1. List of characteristics of the chosen primers.

Designated N*	Molecular weight	Sequence
P1	3270	5'-AGA-AGA-AGA-G-3'
P2	3076	5'-CGC-CGC-TCC-T-3'
P3	3085	5'-CCC-ATG-GCC-C-3'
P4	3180	5'-CGG-CGT-TAC-G-3'
P5	3216	5'-TGT-TGT-TGT-G-3'
P6	3269	5'-GGG-CAA-GGT-G-3'
P7	3318	5'-GGA-GGA-GGA-G-3'
P8	3165	5'-GCG-CGG-CAC-T-3'
P9	3300	5'-GTG-GAG-GGG-T-3'
P10	3076	5'-GCC-CCT-CGT-C-3'
P11	3165	5'-CGG-ACC-GCG-T-3'
P12	3158	5'-AAC-GGG-CAC-C-3'
P13	3245	5'-GGG-CGA-GTG-C-3'
P14	3205	5'-GGG-GGC-CTC-A-3'
P15	3045	5'-CCG-CCC-CAC-T-3'
P16	3125	5'-TGC-GCA-GCC-C-3'
P17	3045	5'-CAC-CCC-CTG-C-3'
P18	3165	5'-ACG-GGC-GCT-C-3'
P19	3180	5'-GGT-CGC-AGC-T-3'

10 pmol/μl, and 1 l genomic DNA at 54 ng/μl. This procedure was performed at 4°C. This was followed by complete denaturation of the DNA for 4 minutes at 94°C, then 45 cycles for 1 minute at 94°C, 1 minute at 36°C and 2 minutes at 72°C, and ending with synthesis for 4 minutes at 72°C.

The PCR products from the study of all primers were visualized on a BIO RAD Gel Doc 1000 and the images were then analyzed with the computer program Gel-Pro Analyzer™ version 3.0.

3. RESULTS AND DISCUSSION

RAPD Analysis. Once the quality of the DNA obtained from the 8 varieties of blueberry had been verified, we considered 19 random primers GIBCO®-Life Technologies (**Table 1**), from which it was possible to choose 4 (P1; P5; P10 and P16) which gave individual responses on at least one of the studied varieties (**Figure 1**, other data not shown).

In the case of P1 (**Figure 1(a)**), a band can be seen in lane 3, around 2183 pb, which does not appear in lane 4 or lane 5, which thus differentiates V1 from V2 and V3.

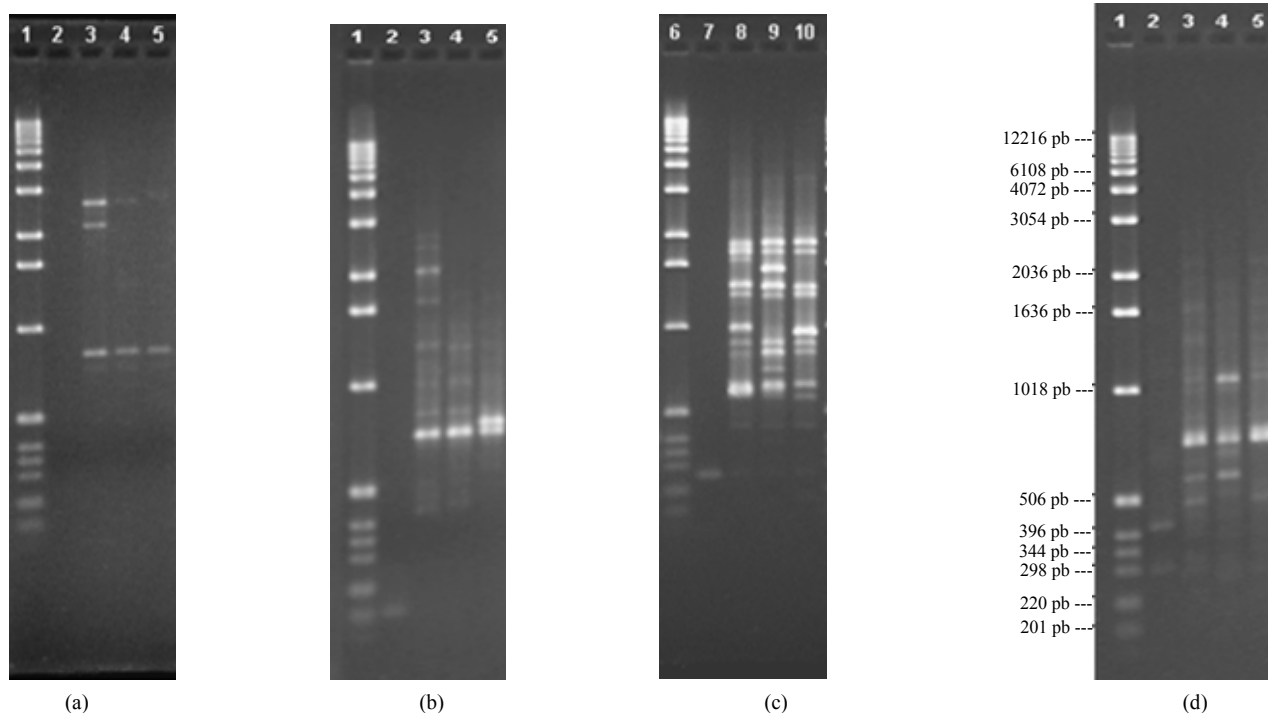


Figure 1. (a) 1.3% agarose gel with DNA amplification of blueberry varieties V1, V2 and V3 using primer P1, lane 1 1 kb marker, lane 2 control with primer only, lane 3 with amplification of variety V1, lane 4 with amplification of variety V2 and lane 5 with amplification of variety V3; (b) 1.3% agarose gel, with DNA amplification of blueberry varieties V1, V2 and V3 using primer P5, lane 1 1 kb marker, lane 2 control with primer only, lane 3 with amplification of variety V1, lane 4 with amplification of variety V2 and lane 5 with amplification of variety V3; (c) 1.3% agarose gel with DNA amplification of blueberry varieties V1, V2 and V3 using primer P10, lane 1 1 kb marker, lane 2 control with primer only, lane 3 with amplification of variety V1, lane 4 with amplification of variety V2 and lane 5 with amplification of variety V3; (d) 1.3% agarose gel with DNA amplification of blueberry varieties V1, V2 and V3 using primer P16, lane 6 1 kb marker, lane 7 control with primer only, lane 8 with amplification of variety V1, lane 9 with amplification of variety V2 and lane 10 with amplification of variety V3.

In lane 4 there is an amplified fragment of some 2693 pb, which is repeated in lane 3 but not in lane 5, this then differentiates variety V2 from V3. In the case of lane 5 there is a band approximately 2763 pb in size which is not present in lane 3 or lane 4; this would allow identification of variety V3.

For the case of primer 5 (**Figure 1(b)**), there is a series of bands in lane 3 of 2711; 2435; 2060; 1699; 527; 475 and 431 pb that are not seen in lane 4 or in lane 5, which would differentiate variety V1 from V2 and V3. In lane 4 there is a band of approx. 829 pb in size, which is not in lane 5 but is in lane 3; this differentiates variety V2 from V3, but not from V1 (with this fragment). Finally, in lane 5, we can see a band, of around 763 pb, that is not in lanes 3 and 4, meaning we can differentiate V3 from V2 and V1.

In the case of primer P10 (**Figure 1(c)**), three bands can be seen in lane 8, approx. 1691, 1006 and 604 pb in size, and which are not present in lanes 9 or 10, allowing differentiation of variety V1 from V2 and V3. In lane 9 there are three fragments approximately 1584, 713 and 626 pb in size which are not in lanes 8 and 10, allowing differentiation of variety V2 from V1 and V3. In lane 10, there are three bands, around 971, 634 and 570 pb which cannot be seen in lanes 8 and 9, thus differentiating V3 from V1 and V2.

Finally, for the case of primer P16 (**Figure 1(d)**), a fragment of 632 pb can be seen in lane 3 and not in lanes 4 and 5, differentiating variety V1 from V2 and V3. In lane 4 there are two bands of 658 and 500 pb, which are not present in lane 3 or in lane 5, via which we can differentiate variety V2 from V1 and V3. In lane 5 there is a band of 517 pb which differentiates V3 from V1 and V2.

Once the primers had been selected using the aforementioned criteria, we proceeded to use them on the eight varieties of blueberry in the study. **Figure 2** shows a 1.0% agarose gel, in which the varieties were compared via amplification of primer P1.

Three bands can be seen in lane 4 in the P1 profiles, of around 2926, 1688 and 1476 pb, and a band that is not at 800 pb that is found in all the other lanes, thus differentiating V4 from V1, V2, V3, V5, V6, V7 and V8. As for lane 5, there is a band of around 644 pb which does not appear in this lane but which is visible in all the others, thus differentiating V1 from V2, V3, V4, V5, V6, V7 and V8. In lane 6 there is one band, of around 1291 pb, that is not in any other lane, thus differentiating V2 from V1, V3, V4, V5, V6, V7 and V8. In lane 7 there are two bands, of around 313 and 2241 pb, that are not in any other lanes, thus differentiating V5 from V1, V2, V3, V4, V6, V7 and V8. In lanes 10 and 11 the same band pattern can be seen, there is a band at around 2745 pb that is not visible in any other lane, thus differentiating V3 and V6

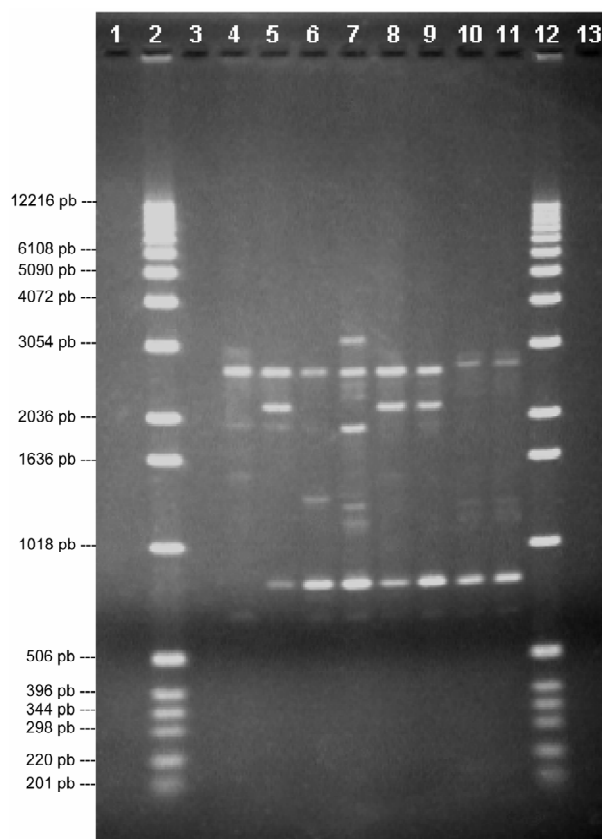


Figure 2. Analysis of chosen primers with all solutions of genomic DNA. Tests were performed with the four chosen primers using the solutions of genomic DNA (V1, V2, V3, V4, V5, V6, V7 and V8), the above are images obtained from electrophoresis with the respective analysis data. 1.0% agarose gel showing the band patterns of the DNAg V1, V2, V3, V4, V5, V6, V7 and V8 with primer 1.

from V1, V2, V4, V5, V7 and V8. Therefore, using this primer we can differentiate the varieties Misty (V1), Michigan (V2), Bluecrop (V4), Duke (V5) and Elliot (V3 and V6), but it is not possible to separate varieties V7 and V8.

When using primer P5 and comparing the 8 varieties (**Figure 3**), it can be seen that there is a band in lane 6, of around 1724 pb, that is not seen in the other lanes, thus differentiating V2 from V1, V3, V4, V5, V6, V7 and V8. In lane 8 there is a band of around 1680 pb that is not seen in the other lanes, thus differentiating V7 from V1, V2, V3, V4, V5, V6 and V8. Thus, with this primer it is only possible to differentiate between Michigan (V2) and V7.

The profiles from primer P10 are shown in **Figure 4**. In lane 4 a band of around 1786 pb can be seen that is not in any other lane, thus differentiating V4 from V1, V2, V3, V5, V6, V7 and V8. In lanes 10 and 11 there are two bands, around 1966 and 227 pb, that are not seen in the other lanes, thus differentiating V3 and V6 from V1,

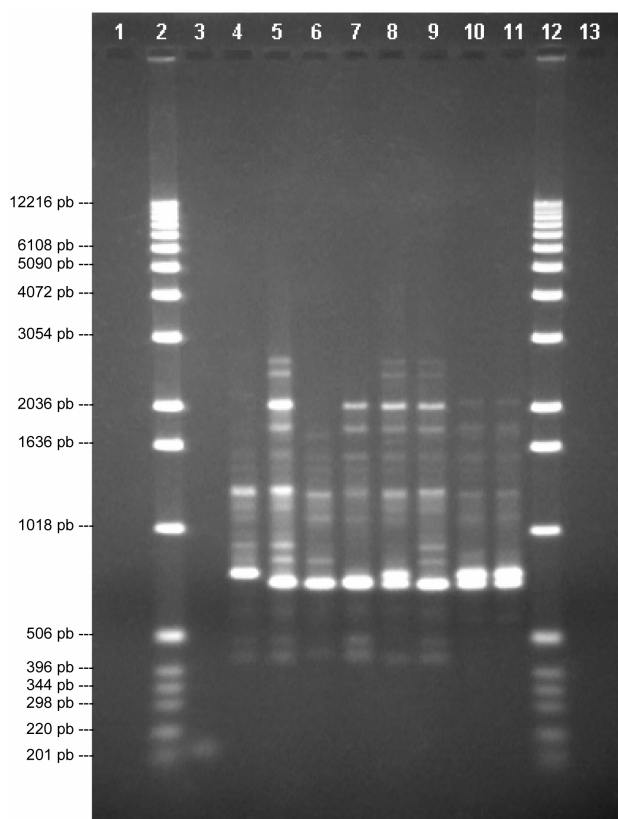


Figure 3. Analysis of chosen primers with all solutions of genomic DNA. Tests were performed with the four chosen primers using the solutions of genomic DNA (V1, V2, V3, V4, V5, V6, V7 and V8), the above are images obtained from electrophoresis with the respective analysis data. 1.0% agarose gel showing the band patterns of the DNAg V1, V2, V3, V4, V5, V6, V7 and V8 with primer 5.

V2, V4, V5, V7 and V8. Thus, with this divider it is only possible to differentiate Bluecrop (V4) from Elliot (V3 and V6).

Finally, with primer P16 (**Figure 5**) we can see a band on lane 7, of around 879 pb, that is not found in any other lane and a band that is not in this lane and is in all the others, around 2125 pb, thus differentiating V5 from V1, V2, V3, V4, V6, V7 and V8. In lane 8 there is a band, around 758 pb, that is not seen in the other lanes, thus differentiating V7 from V1, V2, V3, V4, V5, V6 and V8. Hence, this divider differentiates between Duke (V5) and V7.

Based on two extracts of DNAg (V3 and V6) from the same variety (Elliot) it can be seen that they followed the same band profile with all four of the chosen primers (P1, P5, P10 and P16).

The varieties that could not be determined are V7 and V8, of which V7 could be differentiated from the rest, meaning that it is a variety that is different from the other six mentioned above. Variety V8, on the other hand, could not be differentiated from all other varieties using

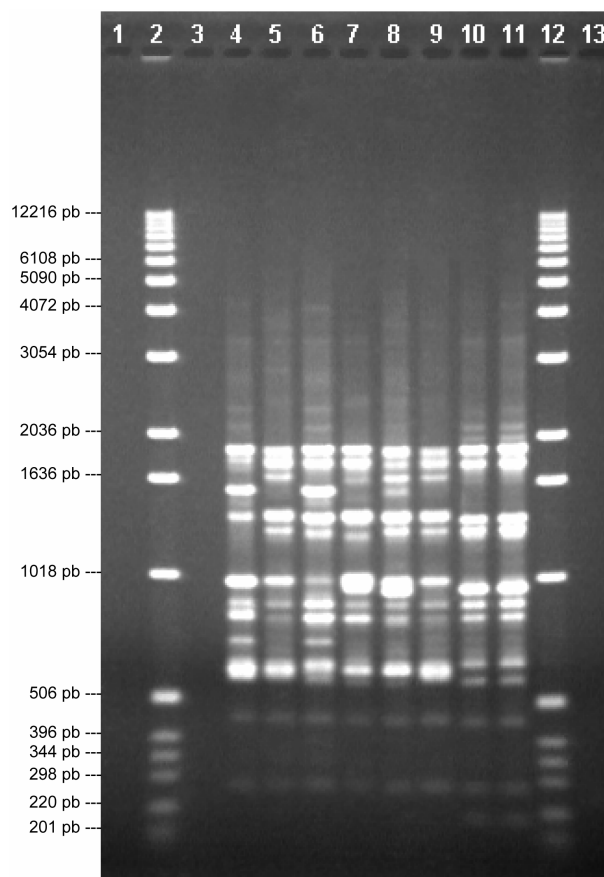


Figure 4. Analysis of chosen primers with all solutions of genomic DNA. Tests were performed with the four chosen primers using the solutions of genomic DNA (V1, V2, V3, V4, V5, V6, V7 and V8), the above are images obtained from electrophoresis with the respective analysis data. 1.0% agarose gel showing the band patterns of the DNAg V1, V2, V3, V4, V5, V6, V7 and V8 with primer 10.

the chosen primers, but there were very similar patterns with between V8 and V1 (Misty). Comparing these it can be seen that the band patterns of V1 with primers 10 and 16 are exactly the same as those of V8 with the same primers. With primer 5, V1 also has the same pattern as V8 with this primer, though the latter has lower band intensity. The only difference found was with the pattern given by primer 1: with V8 a band can be seen in the profile of around 644 pb that is not seen in the pattern given with V1. This may mean that V8 is a sub-variety of V1.

4. CONCLUSION

With the results, it can be conclude that it is possible to establish banding patterns, obtained by RAP-Marker technique, to determine the varieties of blueberry *Vaccinium corimbosum* (nursery commercial interests). It's of great interest to the productive sector: a quick and economical access to information of clones, to optimiz-

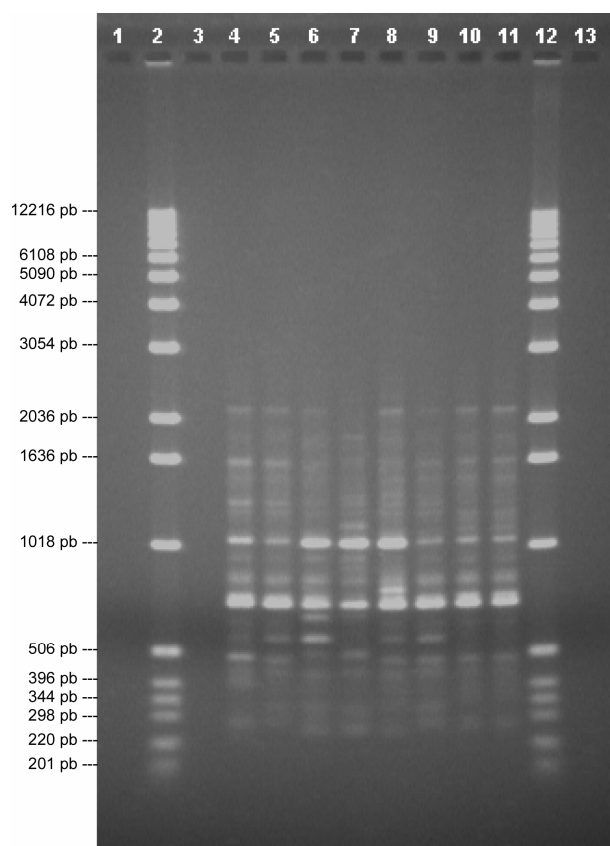


Figure 5. Analysis of chosen primers with all solutions of genomic DNA. Tests were performed with the four chosen primers using the solutions of genomic DNA (V1, V2, V3, V4, V5, V6, V7 and V8), the above are images obtained from electrophoresis with the respective analysis data. 1.0% agarose gel showing the band patterns of the DNAg V1, V2, V3, V4, V5, V6, V7 and V8 with primer 16.

ing the production and profitability of the sector.

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