GENOMIC DEOXYRIBONUCLEIC ACID (DNA) OF THE DISTANT HYBRIDS OF VINE (VITIS VINIFERA L. x MUSCADINIA ROTUNDIFOLIA MICHX.)

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Abstract

For the isolation of the genomic deoxyribonucleic acid (DNA) from vine leaves of distant hybrids (Vitis vinifera L. x Muscadinia rotundifolia Michx.), the DNA isolation protocol based on CTAB method was used. The grouping of distant hybrids in the obtained dendrogram shows that at the DNA level there are some differences between them, differences sometimes unnoticeable at the level of ampelographic characterization. As a result, in the characterization of varieties and hybrids of vines, the ampelographic analysis needs to be supplemented by an analysis at the molecular level, based on DNA amplification techniques. As a result of distant hybrids grouping based on the size of alleles, it was found that there are two distinct main groups denoted by A and B, each having secondary branches. The hybrid F4 BC3 DRX-M5 541 1 is closely akin to the variety Chasellas doré. Also, the two samples of Vitis sylvestris Gmel. have been found to be genetically different, being placed in different subgroups. The hybrid F4 BC3 DRX-M5 536 is genetically close to the male specimen of Vitis sylvestris Gmel. The distant hybrid DRX-M5 660, which proved to have larger differences at the molecular level, isn’t grouped in a cluster with any other hybrid.

Keywords: alleles, distant hybrids, DNA, leaves, primers

INTRODUCTION

SSR (Simple Sequence Repeats) genetic fingerprinting technique can be used successfully in the determination of phylogeny relationships in the biological material analyzed. The representation of the number and size of alleles using the barcode technique gives a clear view of the molecular similarities and differences that occur between hybrids and reference varieties analyzed.

MATERIALS AND METHODS

The distant hybrids of vine (Vitis vinifera L. x Muscadinia rotundifolia Michx.): BC1 – DRX-55 (prob. 1); BC4 – DRX-M4-536 (prob. 2), DRX-M4-578 (prob. 3), DRX-M4-545 (prob. 4), DRX-M4-604 (prob. 5), DRX-M4-508 (prob. 6), DRX-M4-660 (prob. 7); BC2 – DRX-M3-3-1 (prob. 8); Vitis sylvestris Gmel. (♀) (prob. 9); Vitis sylvestris Gmel. (♂) (prob. 10); BC3 – DRX-M4-580 (prob. 11), DRX-M4-541 (prob. 12), DRX-M4-507 (prob. 13), DRX-M4-537 (prob. 14) served as study material [1, 2, 3,15].

For the isolation of genomic deoxyribonucleic acid (DNA) from vine leaves of distant hybrids (Vitis vinifera L. x Muscadinia rotundifolia Michx.), the specimens of Vitis sylvestris Gmel. and the two international varieties taken as reference, it was used the DNA isolation protocol, based on CTAB method (the protocol of Lodhi et al., 1997, modified by Rodica Pop et al., 2003). The quantification of the quality and quantity of deoxyribonucleic acid (DNA) was performed using Nanodrop ND-1000 Spectrophotometer (Thermo Scientific) [4, 5, 6, 7, 8, 10]. Each sample has been subjected to three readings using Nanodrop with the aim of obtaining an average value used for the dilutions required for PCR amplification. It was used a concentration of DNA of 20 ng/μL. [11, 12, 13, 14, 15, 16] PCR amplification was performed in thermocycler type Palm Cycler (Corbett Research) under the conditions of touch down. The primers used were VVS2, MD5, MD7, MD27, ZAG 62 and ZAG 79.
synthesized by the company IDT (USA). The selection of primers was done taking into consideration the recommendations of the gene bank European Vitis Database [17, 18]. The characteristics of the used primers are shown in Table 1.

<table>
<thead>
<tr>
<th>No.</th>
<th>Name of the primer</th>
<th>Nucleotide sequence</th>
<th>Fm (melting T°)</th>
<th>Type of fluorescence marking</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>vvs2 forward</td>
<td>5'- CAGCTCTGAAATGATTCCATC-3'</td>
<td>58.3</td>
<td>F. Well Red D2</td>
</tr>
<tr>
<td>2</td>
<td>vvs2 reverse</td>
<td>5'-AAAAGTTAACCTAATTGACCTG-3'</td>
<td>48.9</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>MD5 forward</td>
<td>5'- CAGCTCTGAAATGATTCCATC-3'</td>
<td>58.9</td>
<td>F. Well Red D3</td>
</tr>
<tr>
<td>4</td>
<td>MD5 reverse</td>
<td>5'- TAYACAAAAAGATTTGCAAA-3'</td>
<td>50.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>MD7 forward</td>
<td>5'- AGTGTCAGAAAGACGAT-3'</td>
<td>56</td>
<td>F. Well Red D4</td>
</tr>
<tr>
<td>6</td>
<td>MD7 reverse</td>
<td>5'- CCACATCCAGACTGTTATG-3'</td>
<td>56.6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>MD9 forward</td>
<td>5'- CCGCAGATGTTGAAACCAAT-3'</td>
<td>58.8</td>
<td>F. Well Red D4</td>
</tr>
<tr>
<td>8</td>
<td>MD9 reverse</td>
<td>5'- ACCTGTTATAGCAGCGATG-3'</td>
<td>60.3</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>ZAG 34 forward</td>
<td>5'- AGCTGTCAGAAAGACGAT-3'</td>
<td>64.1</td>
<td>F. Well Red D4</td>
</tr>
<tr>
<td>10</td>
<td>ZAG 34 reverse</td>
<td>5'- CGGTGTTACGCTATGTTATG-3'</td>
<td>77.7</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>ZAG 79 forward</td>
<td>5'- AGATTTTGAGGAAAGCTAC-3'</td>
<td>80.8</td>
<td>F. Well Red D2</td>
</tr>
<tr>
<td>12</td>
<td>ZAG 79 reverse</td>
<td>5'- TTGCTATCCAGACCGCTCC-3'</td>
<td>80.0</td>
<td></td>
</tr>
</tbody>
</table>

Improving the amplification protocol consisted in using Touchdown PCR amplification so that the truthfulness of the final results was consistent with the specialized literature. It is worth mentioning that after the optimization of all amplification protocols, all the used primers generated amplification products, which were studied with the help of the genetic analyzer CEQ 8800™ capillary DNA analysis system (Beckman Coulter, Fullerton, CA, USA) in the next stage of experimentation, in order to determine the number of alleles and their size.

In order to identify the optimum temperature of attaching primers, there was performed a heat shock that exceeded by about five degrees Celsius the melting temperature of the forward primer, then the temperature gradually decreased with about one degree Celsius at each amplification cycle until it was reached the temperature at which primers attachment could be more specific.

The optimization of the amplification protocol is important because it helps to avoid obtaining non-specific amplification products. It was also found that the attachment optimum temperature depends on the melting temperature of the most unstable primer, from thermal point of view, of the primer pair.

In Table 2 there are presented the PCR amplification programs which were optimized and used in order to study the migration of the reaction products in the genetic analyzer.

Table 2. Amplification protocol of vine samples analyzed with the primers vvs2, MD5, MD7, MD27, ZAG 62, ZAG 79

<table>
<thead>
<tr>
<th>No.</th>
<th>Name of the primer</th>
<th>PCR condition</th>
<th>The composition and the volume (µL) of the PCR reaction mixture</th>
<th>DNA quantity (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>vvs2</td>
<td>1. 35°C: 0.5 min (1 cycle of amplification)</td>
<td>H2O: 4 MgCl2: 1.2 dNTP mix: 0.6</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>MD5</td>
<td>1. 35°C: 0.5 min (1 cycle of amplification)</td>
<td>H2O: 4 MgCl2: 1.2 dNTP mix: 0.6</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>MD7</td>
<td>1. 35°C: 0.5 min (1 cycle of amplification)</td>
<td>H2O: 4 MgCl2: 1.2 dNTP mix: 0.6</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>ZAG 62</td>
<td>1. 35°C: 0.5 min (1 cycle of amplification)</td>
<td>H2O: 4 MgCl2: 1.2 dNTP mix: 0.6</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>ZAG 79</td>
<td>1. 35°C: 0.5 min (1 cycle of amplification)</td>
<td>H2O: 4 MgCl2: 1.2 dNTP mix: 0.6</td>
<td>3</td>
</tr>
</tbody>
</table>

PCR amplification products obtained after using the 6 SSR primers mentioned above were verified by migration in agarose gel 1.4 % (1.4 g agarose LE Analytical Grade, Promega in 100 ml solution TAE). In Figure 1 there are shown the PCR amplification products obtained with primers pair MD5 and migrated in agarose gel and the ladder of 100 bp used [19, 20, 21].

Optimal dilutions of PCR products were obtained by probing and we found that satisfactory results concerning the migration...
conditions were recorded at the following dilutions:
- PCR products amplified with the primer ss2 were diluted at a ratio of 1:5 and then a volume of 1μL was used for migration;
- PCR products amplified with the primer md5 were diluted at a ratio of 1:20 and then a volume of 1μL was used for migration;
- PCR products amplified with the primer md7 were diluted at a ratio of 1:40 and then a volume of 1μL was used for migration;
- PCR products amplified with the primer md27 were diluted at a ratio of 1:40 and then a volume of 1μL was used for migration;
- PCR products amplified with the primer ZAG 62 were diluted at a ratio of 1:40 and then a volume of 1μL was used for migration;
- PCR products amplified with the primer ZAG 79 were diluted at a ratio of 1:5 and then a volume of 1μL was used for migration;

The PCR products obtained with the help of the six primers were migrated in the genetic analyzer Ceq TM 8800 from Beckman Coulter, using a volume of 0.25 μL standard 400 bp from Beckman Coulter and solution for migration -38.3 μL SLS (sample loading solution).

In order to analyze more accurately the results, there were used for comparison two international vine varieties, Sauvignon Blanc and Chasselas Dóre, whose size and number of alleles are given in the literature.

The determination of the number and size of alleles at the analyzed varieties was performed automatically with the help of the software used for data interpretation included in the genetic analyzer CEQ 8800 from Beckman Coulter Company.

The dendrogram on the way of grouping of hybrids was done with the help of the programs PAST and FIG TREE using the EUCLIDEAN method.

RESULTS AND DISCUSSIONS

Results on DNA isolation
The amount of DNA (ng/μL) and its purity (expressed through the values of the ratio 260/280) obtained from the analyzed vine samples are shown in the images from below:

After quantification of the samples, DNA dilutions were made so that all the samples used for migration to have a concentration of 20 ng/μL. In Table 4 there are shown the average values of the samples of DNA, and the values of the dilution factor and the volumes of DNA stock and those of sterile double-distilled
The results obtained concerning the number and size of alleles and are shown in Table 5:

Table 4. The analyzed number and size of the obtained alleles of the local and newly created varieties (the red colour indicates the international varieties used as reference in this study)

Data grouping was done using the program “Excel” (Table 5), establishing the time of identification of alleles’ size so that it could include all the values obtained after the migration of the samples analyzed in the genetic analyzer Beckman Coulter Ceq 8800 TM. The migration of PCR products was performed in the genetic Analyser Beckman Coulter Ceq 8800 TM in order to identify the number and size of alleles of vine varieties using the SSR technique. In the figures 4 and 5 are shown some migrated samples so that the heterozygous (at the same locus, allele, Figure 4.) or homozygous state (Fig. 5, sample 2 - F3 BC2 - DRX-M3-3-1) may be highlighted.

Table 5. The representation of the number and size of alleles of the distant hybrids analyzed by DNA barcode

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size (bp)</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>120-140</td>
<td>12</td>
</tr>
<tr>
<td>Sample 2</td>
<td>140-160</td>
<td>10</td>
</tr>
<tr>
<td>Sample 3</td>
<td>160-180</td>
<td>8</td>
</tr>
<tr>
<td>Sample 4</td>
<td>180-200</td>
<td>6</td>
</tr>
</tbody>
</table>

Fig. 5. Migration in the genetic Analyzer Beckman Coulter Ceq 8800 TM in order to identify the number and size of alleles of vine varieties using the technique SSR with the primer VVS2.

Grouping distant hybrids based on the size of the alleles identified using SSR technique was performed in order to determine their type of genetic similarity/difference (Fig. 6). Thus, it can be seen that there were formed two different main groups denoted by A and B, each of them having some secondary ramifications. It is worth mentioning the fact that the hybrid F4 BC3 DRX-M4-541 is very akin to the variety Chasellas dóre and it is possible that the latter may have contributed to the formation of the hybrid mentioned...
above.
Also, the two samples of *Vitis sylvestris* Gmel. were found to be genetically different, being placed in separate subgroups. The hybrid F4 BC3 DRX-M4-536 is genetically close to the male specimen of *Vitis sylvestris* Gmel., and it may have contributed to the formation of the hybrid.
Among the hybrids F4 BC3, DRX-M4-660 stands out, because it has proved to have more differences at the molecular level, being unable to be grouped in a cluster with any other hybrid.

![Fig. 6. The dendrogram, drawn up according to the Euclidean method, on the backcross hybrids and the reference varieties analyzed.](image)

### CONCLUSIONS

1. SSR (Simple Sequence Repeats) genetic fingerprinting technique can be used successfully in the determination of phylogeny relationships in the biological material analyzed.
2. The representation of the number and size of alleles using the barcode technique gives a clear view of the molecular similarities and differences that occur between the hybrids and the reference varieties analyzed.
3. The grouping of hybrids in the generated dendrogram shows that there are some differences between them at DNA level, differences sometimes unnoticeable at the level of ampelographic characterization. As a result, the characterization of varieties and hybrids of vine requires the ampelographic analysis to be completed by an analysis at the molecular level, based on DNA amplification techniques.

### REFERENCES


