Grapevine clones discriminated using stilbene synthase–chalcone synthase markers

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Abstract: Touriga Nacional is the most popular Portuguese grapevine cultivar. After twenty years of clonal selection, highly recommended qualitative and productive clones have been identified. In the genus Vitis several molecular markers have been used to characterize genetic diversity at the DNA level, namely microsatellites, ISSR (inter simple sequence repeat), AFLP (amplified fragment length polymorphism), and stilbene synthase–chalcone synthase (StSy–CHS) markers.

In this work a novel pure DNA extraction methodology, which occludes the extract in an agarose matrix, thus allowing the removal of the remaining hydrophilic impurities, was used. An optimized and highly reproducible procedure for the detection of differences among Vitis vinifera L clones using the StSy–CHS gene family primer combination is described. With this methodology, 21 clones and a commercial cultivar, ‘Chardonnay’, were evaluated with three StSy–CHS markers. It was possible to obtain 14% discrimination within Touriga Nacional (TN) clones, identifying two different groups. The polyclonal origin of TN clones was previously verified with eight microsatellite markers. The methodology described could be used for clonal selection of grapevine cultivars.

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Keywords: Vitis vinifera; StSy–CHS; molecular markers; amplification conditions

INTRODUCTION

Touriga Nacional (TN) is the most popular Portuguese grapevine cultivar. Taking into account the genetic variability present within this cultivar, a breeding program based on clonal selection was initiated twenty years ago, and clones with highly recommended qualitative and productive characteristics were identified.1 In the genus Vitis, several molecular markers have been used to characterize genetic diversity at the DNA level, exploiting differences in the number and extension of microsatellites and other molecular markers. Microsatellites have been identified for use in fingerprinting and in assisting the selection of grapevine varieties.2–9

Contradictory results have been obtained with the use of microsatellite markers for clonal discrimination. Despite the great stability of these markers Regner et al10 assessed their ability to detect sparse polymorphism among ‘White Riesling’ grapevine clones, and concluded that ISSR were more appropriate markers for distinguishing grapevine clones, due to their high stability and degree of polymorphism. However, Sefc et al11 reported the inability of both microsatellite and ISSR markers to distinguish grapevine clones and indicate the polyclonal origin of cultivars as the cause of some polymorphisms found. More recently, positive results in differentiating grapevine clones have been reported using AFLP markers.12–15 A less complex and more promising method to detect genetic polymorphisms has been described for clonal differentiation. This method is based on the search for polymorphisms on the stilbene synthase (StSy)–chalcone synthase (CHS) 5’ untranslated genomic regions (StSy–CHS markers).

Stilbene synthase synthesizes the backbone of the stilbene phytoalexins, which have antifungal properties and play a role in plant defense against pathogens.16 This enzyme has been reported in peanut,16 pine17 and grapevine.18 In grapevine StSy is thought to be a mutant of the chalcone synthase gene. StSy genes exhibit a high degree of sequence homology with members of the CHS multigene family.19 In

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grapevine, seven different kinds of stilbene synthase have been cloned.\textsuperscript{18,20} Geuna \textit{et al}\textsuperscript{21} found high levels of polymorphism for the classification of grapevine cultivars using \textit{StSy–CHS} coding regions. The same authors\textsuperscript{22} used \textit{StSy–CHS} coding regions in discriminating clones of Chardonnay, Pinot noir, and Sangiovese. Bavaresco \textit{et al}\textsuperscript{23} also refer to the efficiency of this kind of marker to emphasize the differences between two gray mold-resistant clones and susceptible ones.

The objective of this study was to discriminate among 21 TN clones using three \textit{StSy–CHS} markers. In order to detect the possible polyclonal origin of TN clones, a set of nine microsatellite markers was used for preliminary screening.

\textbf{MATERIALS AND METHODS}

\textbf{Plant material and DNA extraction}

Twenty-one clones obtained from two geographically distinct germplasm collections (Table 1) were used in this study, as well as a commercial Chardonnay cultivar. Quinta da Leda belongs to the Douro Superior sub-region and Quinta N S Lurdes to the Baixo-Corgo sub-region. Based on the results of twenty years of clonal selection in the Douro Superior sub-region, the ten most, and ten least, productive clones were chosen for this study to increase the probability of finding polymorphism among them. In addition, one clone of TN from Baixo-Corgo, and Chardonnay as a control, were added to the study.

Clean young leaves were harvested and immediately stored in 15 ml sterile polypropylene tubes containing at least 15 times the leaf weight of humidity indicator silica gel (Merck, Darmstadt, Germany), according to Wang \textit{et al}.

DNA was extracted directly from the dried leaves using the method described by Faria \textit{et al}\textsuperscript{25} based on a methodology developed by Wang \textit{et al}.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Sample No\textsuperscript{a} & Plant material & Origin & Yield (kg ha\textsuperscript{−1}) & Microsatellite study & \textit{StSy–CHS} study \\
\hline
1 & TN 3811 & Douro superior & 1.410 & x & x \\
2 & TN 4707 & Douro superior & 0.547 & x & x \\
3 & TN 0103 & Douro superior & 1.381 & x & x \\
4 & TN 4506 & Douro superior & 0.539 & x & x \\
5 & TN 4106 & Douro superior & 1.332 & x & x \\
6 & TN 4301 & Douro superior & 0.521 & x & x \\
7 & TN 5107 & Douro superior & 1.299 & x & x \\
8 & TN 1024 & Douro superior & 0.499 & x & x \\
9 & TN 4502 & Douro superior & 1.289 & x & x \\
10 & TN 4505 & Douro superior & 0.484 & x & x \\
11 & TN 4227 & Douro superior & 1.273 & x & x \\
12 & TN 1027 & Douro superior & 0.457 & x & x \\
13 & TN 0906 & Douro superior & 1.251 & x & x \\
14 & TN 5316 & Douro superior & 0.470 & x & x \\
15 & TN 4124 & Douro superior & 1.245 & x & x \\
16 & TN 2503 & Douro superior & 0.451 & x & x \\
17 & TN 1610 & Douro superior & 1.244 & x & x \\
18 & TN 5108 & Douro superior & 0.445 & x & x \\
19 & TN 3817 & Douro superior & 1.242 & x & x \\
20 & TN 5217 & Douro superior & 0.357 & x & x \\
21 & TN 3932 & Baixo Corgo & — & — & x \\
22 & Chardonnay\textsuperscript{b} & Commercial & — & — & x \\
\hline
\end{tabular}
\caption{Description of samples (origin and yield of plant material) used in SSR and \textit{StSy–CHS} studies}
\end{table}

\textsuperscript{a} For each plant material, two different DNA extractions were performed.

\textsuperscript{b} Obtained from Quinta de S\textsuperscript{3} B\textsuperscript{á}bara at Centro de Estudos Vitivinícolas do Douro, Régua, Portugal.
Molecular markers
Two studies were conducted using two different kinds of molecular markers.

Polyclonal evaluation study
The microsatellites used were VVMD8, VVMD21, VVMD27, VVMD28, VVMD29, VVMD31, VVMD32, VVMD36 and VVMD37.

SySt–CHS study
Two primer combinations of SySt–CHS untranslated genomic regions were used: SS1/SS10 and SS1/SS13.

Amplification conditions
PCR amplifications were performed in a Biometra T3 thermal cycler (Whatman/Biometra, Gottingen, Germany) in 200 µl PCR tubes each containing 4 µl DNA extract, 0.02 nmol each of the forward and reverse primer, 4 µmol each of the four deoxynucleotide triphosphates (dNTP), 0.5 U Taq DNA polymerase (EcoTAQ, Ecogen, Barcelona, Spain), 2 µl 10 × PCR buffer (provided with the polymerase) and 0.03 µmol MgCl2 made up to a final volume of 20 µl with ultrapure water, giving a concentration of 1.5 mM for MgCl2. The amplification protocols were optimized for the analyzed loci. VVMD8: 2 min at 94°C, 5 cycles of (30 s at 92°C, 30 s at 54°C, 1 min at 72°C), and 35 cycles of (30 s at 80°C, 30 s at 54°C, 1 min at 72°C), and 7 min at 72°C. For the remaining primers, amplification conditions consisted of 2 min at 94°C followed by 40 cycles of (30 s at 92°C, 30 s at 55°C and 2 min at 72°C), and 7 min at 72°C.

SySt–CHS study
In order to evaluate the repeatability of the PCR amplifications, two sets of plant material were extracted separately from each clone, and two different thermal cyclers were used, a Biometra T3 and a PTC-100 thermal cycler (MJ Research, Watertown, Massachusetts, USA). In a preliminary study conducted using the PCR conditions established by Geuna et al., the annealing temperature of 50°C was not successful in obtaining well-defined and polymorphic bands. Attempting to improve the resolution and the polymorphic level of the amplified fragments, six annealing temperatures and three MgCl2 concentrations were tried (Table 2) using the commercial variety, Chardonnay, as a control. The general amplification conditions were as follows: 2 min at 94°C followed by 39 cycles of (30 sec at 94°C, 30 sec annealing, 1 min at 72°C) and 5 min at 72°C. Amplification reaction mixtures consisted of 4 µl DNA extract, 0.02 nmol each of the forward and reverse primers, 4 µmol each of the four dNTPs, 1 unit Taq DNA polymerase, 2 µl 10 × PCR buffer and MgCl2 at the concentrations shown in Table 2, after making up to the final volume of 20 µl with ultrapure water.

Gel electrophoresis
Amplification was visualized by running 5 µl of the PCR reaction mixture on 20 gl−1 agarose gel stained with ethidium bromide and the gels were photographed in UV light using a Kodak EDAS 120 electrophoresis documentation system. Fifty microlitres of denaturing dye solution (950 gl−1 formamide, 5 gl−1 bromophenol blue, 5 gl−1 xylene cyanol) was added to the remaining 15 µl of the sample. Six microlitres of this mixture was then denatured at 95°C for 5 min and analyzed on 52 cm 40 gl−1 denaturing polyacrylamide gel containing 7.5 M urea. Electrophoresis was carried out at 35 mA for 3 h in TBE (Tris-borate-EDTA) buffer.

The product sizes of the separated compounds were estimated by comparison with a 100 bp DNA ladder (Promega, Madison, USA). The gel was silver stained according to the protocol described by Bassam et al.

Statistical analysis
The coefficients of genetic similarity (GS) between pairs of clones were calculated according to Dice and Nei and Li, where GS is the similarity coefficient between clones i and j. Ni,j reflects the number of common bands for i and j, Ni and Nj reflect the total number of bands detected in clones i and j, respectively. The identity of two clones will result in a GS value of one, while totally unrelated clones will give rise to a GS value of 0.

RESULTS AND DISCUSSION
DNA extraction
Non-degraded DNA was successfully extracted from dried frozen leaves at concentrations ranging from 3.5 to 9.2 µg ml−1.
Microsatellite study
No differences among the TN clones were detected with the nine microsatellites evaluated (Fig 1) which is in agreement with Sefc et al\textsuperscript{11} who refer to the inability of these markers to detect polymorphisms among different clones. Notwithstanding, these results may be helpful for the elimination of any possible polyclonal origin.

StSy–CHS study
The StSy–CHS markers showed high repeatability in both thermocyclers used (data not shown), as well as in both samples extracted from each of the plant materials.

The correct interpretation of the gels using the annealing temperature of 50°C recommended by Geuna et al\textsuperscript{21} was difficult, due to smearing of the bands in both StSy–CHS marker combinations (Fig 2: gels 1 and 2). Improved resolution and a decrease in smearing was noticed with an increase of the annealing temperature from 50 to 58°C (Fig 2: gels 1 to 6). This could be explained by the more stringent conditions, which improved amplification selectivity. For the majority of samples, deficient amplification

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{PAGE of the amplification products of VVMD31 on ten clones of Touriga Nacional. Lane number corresponds to sample number in Table 1.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Eight agarose gels showing the 12 treatment combinations\textsuperscript{1} in both StSy–CHS markers of four samples: 21, 17, 4 and 22. Each treatment has 8 lanes, corresponding to 4 samples repeated twice. The sample order in each treatment is as follows: sample 21 — lanes 1 and 2; sample 17 — lanes 3 and 4; sample 4 — lanes 5 and 6; sample 22 — lanes 7 and 8. L: Hyperladder I. \textsuperscript{1} See Table 2.}
\end{figure}
was noted when the annealing temperature was raised from 58 to 60°C (Fig 2: gels 7 and 8). As a result the annealing temperature of 58°C was selected (Fig 2: gels 5 and 6) since more specific and well-defined bands were obtained.

In general, the effect of the increase on Mg$^{2+}$ concentration on resolution was not as evident as that of the annealing temperature. However, a slight difference in amplification efficiency could be seen on PAGE gels. PAGE gel separations were performed on material from the annealing temperature of 58°C for both StSy–CHS markers and three MgCl$_2$ concentrations (Fig 3). It was found that the optimal amplification conditions for the SS1/SS10 marker combination were achieved with 1.5 mM MgCl$_2$, and for SS1/SS13 with 2.0 mM MgCl$_2$.

All the samples were analysed using these optimized amplification conditions. This allowed the identification of two groups of clones (Figs 4 and 5). One group consisted of samples 4, 17 and 21 and the other of the remaining 18 samples. A total of 56 bands was identified for the SS1/SS10 marker, of which seven were polymorphic, ranging from 100 to 300 bp. Six of the polymorphic bands originated from samples 4, 17 and 21, and one from the remaining samples (Table 3). A total of 50 bands was identified for the SS1/SS13 marker, of which three were polymorphic, ranging from 100 to 250 bp, and originated from samples 4, 17 and 21. The coefficient of genetic similarity between the two groups was 0.93 and 0.97 for SS1/SS10 and SS1/SS13, respectively (Table 3).

**CONCLUSIONS**

The occlusion of the extract in an agarose matrix and elimination of the remaining hydrophilic impurities
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Figure 4. Two PAGE of the amplification products of StSy–CHS marker combinations obtained with the optimized amplification conditions: SS1/SS10, 58 °C annealing temperature, and 1.5 mM Mg²⁺; SS1/SS13, 58 °C annealing temperature, and 2.0 mM Mg²⁺. The sample order for each treatment is as follows: sample 4 — lanes 1 and 2; sample 17 — lanes 3 and 4; sample 18 — lanes 5 and 6; sample 19 — lanes 7 and 8; sample 21 — lanes 9 and 10. Samples 18 and 19 represent the group of the remained identical samples. All polymorphic bands between 250 and 300bps are shown. On the left-hand side of each gel, black dashed lines indicate polymorphic bands, and gray ones indicate non-polymorphic bands. L: 100 bp DNA ladder.

Figure 5. Dendogram of the 21 studied TN clones using Euclidian distances and the UPGMA method.

<table>
<thead>
<tr>
<th>Type of band</th>
<th>SS1/SS10</th>
<th>SS1/SS13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymorphic⁵</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Polymorphic⁶</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Non-polymorphic</td>
<td>49</td>
<td>47</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>50</td>
</tr>
<tr>
<td>GS</td>
<td>0.93</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Table 3. Number of bands obtained with an annealing temperature of 58 °C and two StSy primer combinations (SS1/SS10 and SS1/SS13) on the samples studied

was needed for the extraction of a pure clone DNA from the cultivars studied.

The polyclonal origin was not detected among the 21 clones studied, using nine microsatellite markers. With respect to the StSy–CHS study, we have developed an optimized and highly reproducible procedure for detection of differences among Vitis vinifera L TN clones, using the StSy–CHS gene family primer combination. With this methodology, 14% discrimination within Touriga Nacional clones was possible. The described methodology could be used for clonal selection of grapevine cultivars.

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