Characterisation of cv. Refošk (*Vitis vinifera* L.) by SSR markers

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Summary

Fifty-five accessions of cv. Refošk from a clonal selection vineyard in the Karst district (Slovenia) were screened by 6 SSR markers in order to assess their uniformity. Two of the accessions showed different patterns (Clone 7 and Clone 50), while 53 accessions revealed identical SSR allelic profiles. Four of the uniform and the two different accessions were compared to 11 Refošk types from adjacent regions (Slovenia (Koper), Croatia and Italy) using 23 SSR markers. The SSR analysis revealed 7 identical genotypes (4 uniform Karst clones, one Italian and two Koper types), while three Koper (and three Slovenian) and three Italian types, as well as Teran from Croatia, showed genetic polymorphisms on an intra-varietal level. Clone 7, Clone 50 and Sladki Teran (Croatia) showed highly diverse genetic patterns from other types and should be considered different varieties. Comparative analysis allowed reliable construction of the predominant Refošk type grown in Slovenia.

Key words: cv. Refošk, intra-varietal variability, SSR.

Introduction

The cv. Refošk in Slovenia is a member of the large Refosco group, which comprises several different types that are denominated according to the cultivation area and morphological or oenological properties, e.g. Refošk, Teranovka, Teran, Terrano, Refoscone etc. (Hrček and Koršeč-Koruza 1996). It is cultivated mainly in the coastal part of Slovenia (Karst and Koper winegrowing districts), in Croatia (Istria) and in Italy (Friuli-Venezia region). In Slovenia, cv. Refošk is of economic importance as the leading red wine variety and the fourth most frequent variety, following Welschriesling, Chardonnay and Sauvignon blanc. Refošk grapes grown in the Karst district are used to produce the highly appreciated wine Teran, which is denominated by grapes grown in the Karst district (Slovenia) and in Italy (Friuli-Venezia region). In Slovenia, cv. Refošk is of economic importance as the leading red wine variety and the fourth most frequent variety, following Welschriesling, Chardonnay and Sauvignon blanc. Refošk grapes grown in the Karst district are used to produce the highly appreciated wine Teran, which is denominated by traditional appellation of geographic origin.

To assess the genetic constitution of a cultivar, DNA methods provide a complementary tool to well-established ampelographic methods. Today microsatellites (SSR) are among the most frequently used DNA markers for cultivar identification, revealing synonyms and homonyms, geographical origin, studying genetic relationships within large groups of cultivars and for characterising clonal variability (Thomas et al. 1994; Bowers et al. 1996; Bowers and Meredith 1997; Seef et al. 1998; Labra et al. 1999; Seef et al. 2000; Reigner et al. 2000; Crespan and Milan 2001; Fossati et al. 2001). To define intra-varietal variability, a combination of SSR and AFLP molecular markers is often recommended (Labra et al. 2001).

In the present work, we used SSR markers for assessing the clonal variability of selected Refošk accessions and to compare geographically diverse Refošk types. The results show a high genetic uniformity of the analysed clones and a low level of intra-varietal variability within cv. Refošk. The analysis also allowed the separation of some clearly distinctive genotypes and the establishment of SSR allelic profiles for the predominant Refošk genotype grown in Slovenia.

Material and Methods

Plant material and DNA extraction: For the assessment of clonal variability, 55 clones of cv. Refošk were sampled from a clonal selection vineyard established in 1989, with records on morphological descriptors, technological data and sanitary status (collection data, Komen, Karst district, Slovenia). The clones were numbered (1-50, 52-55 and 61). Plant material of other Refošk types was obtained from: (1) a private collection (Koper, Koper district, Slovenia) of old Refošk vines with records on growth and yield data, no longer in production; (2) a private vineyard in Prepotto (Friuli, Italy) from which plant material had been used for propagation; (3) two red varieties Teran and Sladki teran, and a white variety, Beli Teran, to represent the outgroup in SSR analysis (all from a private vineyard in Lesičina, Istria, Croatia), as shown in Tab. 1.

Total genomic DNA was extracted from fresh leaf tissue by CTAB (cetyltrimethylammonium bromide) extraction buffer as described by Kemp et al. (1996), resuspended in TE buffer (10 mM Tris-HCI, 1 mM EDTA, pH 8.0) and stored at 4°C.

Microsatellite analysis: To evaluate clonal variability, the 55 Karst Refošk clones were screened at 6 SSR loci (VVS2, VVS4, VVS5, VVMD6, VVMD17, VrzAG21) and for further analysis of the 17 Refošk types and Beli teran, 23 SSR loci were used: VVS1, VVS2, VVS4, VVS5 (Thomas and Scott 1993), VVMD6, VVMD7, VVMD8 (Bowers et al. 1996), VVMD14, VVMD17, VVMD24, VVMD25, VVMD27, VVMD31, VVMD32, VVMD36 (Bowers et al. 1999), VrzAG21, VrzAG47, VrzAG62, VrzAG64, VrzAG67, VrzAG79, VrzAG83 and VrzAG112 (Seef et al. 1999).

PCR was performed in 10 μl of a mixture containing 20 ng DNA, 0.25 U Taq DNA polymerase (Roche, Mannheim, Germany), 10 μM of each primer and 200 μM of each dNTP and reaction buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCI,
Results and Discussion

Clonal variability: Fifty-five Karst-Refosk accessions, which represent the main propagation material of Refosk in Slovenia, were examined at 6 SSR loci in order to assess their genetic uniformity on the varietal level. All accessions except for two (Clone 7 and Clone 50) showed identical SSR allelic profiles. Clone 50 was distinguished from the group of identical genotypes at 4 loci (VVS2, VVS4, VVMD6 and VVMD17) and Clone 7 at three loci (VVS2, VVS4 and VVMD17). The allelic profiles of clones are listed in Tab. 2. These analyses confirmed the overall genetic uniformity of the selected clones, while data on morphology, growth and yield (collection data) show differences among them, which can be partly explained by the sanitary status of the plants or by clonal diversity within the variety. The differentiation of Clone 50 from other clones was previously indicated by phylometric analysis using 30 leaf parameters, though a clear distinction of Clone 7 was not found by this analysis (Koznik et al. 2001). The clonal diversity within vineyards can be interpreted by propagation of the plant material mostly on the basis of technological and oenological properties rather than morphological characters and without knowledge of the genetic background of the mother plants.

The significant differentiation of accessions, such as Clone 7 and Clone 50, confirms the usefulness of SSR molecular analysis in revealing misidentified plants, which could result from a mistake at planting or inability to identify these plants by visual inspection in the vineyard.

Analysis of Refosk types: Four uniform Karst clones (6, 10, 23 and 61) and the two distinctive accessions (7 and 50) were chosen to be compared to 11 Refosk accessions from adjacent regions (6 Refosk types from Italy; Teran and Sladki teran) using 23 SSR markers. Accessions (7 and 50) and Sladki teran. A dendrogram (Fig.) constructed on pairwise Jaccard coefficients of similarity best shows the genetic relationships among the analysed types. Four clones from the Karst, two types from Koper and one Italian type revealed identical profiles, to which one Koper and three Italian types, as well as Croatian Teran, clustered very closely. Their genetic similarities were >0.90 which can be considered to be intra-varietal variability according to Cervera et al. (1998). The presence of identical genotypes in adjacent regions (Slovenia-Koper, Karst and Italy) may be due to the exchange of plant material in the past, especially in the pre-phylloxera era.

Two Koper types (18 and 22) showed higher distance, and clustered to the other clones at a GS value >0.8. These two Koper accessions, which were part of a Refosk selection vineyard flourishing 30 years ago, may represent disappearing genotypes of the cv. Refosk group. Identification of genetically different vines that are no longer in commercial use, such as Koper types, is important for establishing a gene reservoir for possible future application in breeding. The smallest GS values were found for Clone 50 (GS 0.50) and Sladki teran (GS 0.46), as well as Clone 7, which were clearly separated from other types in the dendrogram. Clone 7
SSR markers for cultivar characterisation

and Clone 50 are genetically different to a degree suggesting a variety other than Refošk. It would be worth investigating their identity, since they have valuable oenological properties and stable productivity. Sladki teran (Sweet teran) was included in the analysis in order to discover whether the name is a synonym for less productive and sweeter grapes of the Refošk variety or a case of a similar name being used for another variety. SSR analyses confirmed the latter.

Our research revealed that cv. Refošk comprises several different genotypes with the majority of analysed accessions showing high genetic relatedness (Italy 5, Koper 5, Teran, Italy 10, Italy 12, Koper 18 and Koper 22). The detected intra-varietal diversity among cv. Refošk accessions might be explained by polyclonal origin. According to RIVES (1961) different genotypes can be generated either by accumulation of bud mutations or from seedlings of self- or cross-pollinated siblings or parents of progenitors. The analysis carried out by FILIPPETTI et al. (1999) demonstrated that self-pollination can generate morphologically indistinguishable seedlings while they can be differentiated at DNA level. Cultivars constituted of different, yet genetically closely related and phenotypically similar genotypes, are assigned as polyclonal cultivars, as was shown for cv. Fortana (SILVESTRONI et al. 1997). According to our results, cv. Refošk can also be considered to be a polyclonal cultivar.

**Predominant Refošk genotypes at 23 SSR loci**

The genetic variability determined within Refošk provided data for assigning the characteristic genotype at 23 SSR loci of cv. Refošk grown in Slovenia (Tab. 2), taking into account the most frequent allelic pattern. The proposed

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**Table 2**

Genotypes at 23 SSR loci of cv. Refošk clones and accessions (alleles in bp)

<table>
<thead>
<tr>
<th>Locus</th>
<th>Identical genotypes of 53 Karst clones</th>
<th>Predominant genotypes</th>
<th>Different genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>VVS1</td>
<td>183:190</td>
<td>180:183</td>
<td></td>
</tr>
<tr>
<td>VVS2</td>
<td>134:154</td>
<td>132:154</td>
<td>152:152</td>
</tr>
<tr>
<td>VVS4</td>
<td>167:172</td>
<td>167:174</td>
<td></td>
</tr>
<tr>
<td>VVS5</td>
<td>98:98</td>
<td>98:98</td>
<td></td>
</tr>
<tr>
<td>VVMD6</td>
<td>199:207</td>
<td>199:209</td>
<td>189:199</td>
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<tr>
<td>VVMD7</td>
<td>248:248</td>
<td>240:248</td>
<td></td>
</tr>
<tr>
<td>VVMD8</td>
<td>137:153</td>
<td>137:137</td>
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<tr>
<td>VVMD14</td>
<td>222:241</td>
<td>222:235</td>
<td>222:239</td>
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<tr>
<td>VVMD17</td>
<td>222:222</td>
<td>212:222</td>
<td>233:241</td>
</tr>
<tr>
<td>VVMD24</td>
<td>214:219</td>
<td>210:219</td>
<td>210:214</td>
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<tr>
<td>VVMD25</td>
<td>251:267</td>
<td>249:267</td>
<td>251:269</td>
</tr>
<tr>
<td>VVMD27</td>
<td>191:191</td>
<td>183:191</td>
<td></td>
</tr>
<tr>
<td>VVMD31</td>
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<td>212:216</td>
<td></td>
</tr>
<tr>
<td>VVMD32</td>
<td>255:275</td>
<td>255:263</td>
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</tr>
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<td>VVMD36</td>
<td>254:254</td>
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<tr>
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<td>202:208</td>
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<tr>
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<td>159:167</td>
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<tr>
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<td>195:195</td>
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<td>VrZAG112</td>
<td>237:245</td>
<td>239:247</td>
<td>245:245</td>
</tr>
</tbody>
</table>

Superscript numbers refer to the accession numbers as listed in Tab. 1.

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Figure: Dendrogram obtained from SSRs data at 23 loci constructed using Jaccard coefficients as genetic similarities.
genotypes can serve as reference profiles for variety identification and the relationship of Refošk to other grapevine varieties.

This work presents the characterisation of Refošk grown in Slovenia, using molecular markers. A low level of genetic variability among the different Refošk accessions was found for the majority of the clones. However, molecular analysis allowed the detection of three highly distinctive genotypes (Clone 7, Clone 50 and Sladki teran), which are more likely to be different from cv. Refošk. An analysis established the characteristic genotype at 23 SSR loci of cv. Refošk grown in Slovenia.

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