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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 (Slovenia) 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.

K e y w o r d s : 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 Korošec-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 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; Sefc *et al.* 1998; Labra *et al.* 1999; Sefc *et al.* 2000; Regner *et al.* 2000; Crespan and Milani 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

P l a n t m a t e r i a l a n d D N A e x t r a c t i o n : 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 Lesišćina, 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 Kump *et al.* (1996), resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4 ºC.

M i c r o s a t e l l i t e a n a l y s i s : 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 (Sefc *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 $MgCl_2$, 10 mM Tris-HCl,

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Table 1

Plant material used for SSR analysis

No.	Cultivar: Label	Origin
1	Refošk: Clone 6	Slovenia
2	Refošk: Clone 7	(Karst district, clonal
3	Refošk: Clone 10	selection vineyard)
4	Refošk: Clone 23	
5	Refošk: Clone 50	
6	Refošk: Clone 61	
7	Teran	Croatia
8	Sladki teran: ST	(Istria, Lesi√æina)
9	Beli teran: BT	
10	Refošk: Koper5	Slovenia
11	Refošk: Koper10	(Koper district)
12	Refošk: Koper18	
13	Refošk: Koper22	
14	Refošk: Koper27	
15	Refošk: Italy3/5	Italy
16	Refošk: Italy5	(Prepotto)
17	Refošk: Italy10	
18	Refošk: Italy12	

pH 9.0 and 0.1 % Triton X-100). One of each of the primer pairs was labelled with fluorescent Cy-5 dye. PCR conditions were 5 min at 95 ºC followed by 26–40 cycles (depending on the primer pair used) of: denaturation 94 ºC for 30 s; annealing at 50–58 ºC (depending on the primer pair used) for 30 s; extension at 72 ºC for 90 s. PCR products were mixed with loading buffer (Dextran blue 5 mg ml^{-1} in 100 % formamid) and 2 µl of 4 fmol Cy-5 labelled size markers ranging from 50-500 bp according to the expected allele sizes (Pharmacia Biotech, Vienna, Austria). Samples were denatured for 4 min at 95 ºC and analysed on a sequencing gel (6 % acrylamid gel, 1 x TBE buffer, 7 M urea) on an ALFexpress sequencing apparatus (Pharmacia Biotech, Vienna, Austria). Allele sizes of microsatellite fragments were determined by internal and external size markers using the software program AlleleLocator 1.03 (Pharmacia Biotech, Vienna, Austria).

Data analysis: Genetic similarities among Refošk types based on SSR data were calculated using Jaccard coefficients of similarity (J = a /(n-d); a and d represent the presence or absence of a band and n the total sample size). A dendrogram was constructed using an unweighted pair group method (UPGMA) for clustering, in the NTSYS-PC software package (ROHLF 1998). Cv. Beli teran was excluded from data analysis and was used only for the construction of the dendrogram.

Results and Discussion

Clonal variability: Fifty-five Karst-Refošk accessions, which represent the main propagation material of Refošk 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 (KOZJAK *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 Refošk 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 Refošk accessions from adjacent regions (5 Refošk types from Koper, Slovenia; 4 Refošk types from Italy; Teran and Sladki teran from Croatia) using 23 SSR markers.

SSR analysis of 17 Refošk types revealed a total of 72 alleles over 23 microsatellite *loci*. The highest polymorphism was detected at *loci* VrZAG112, VrZAG67, VVMD14 and VVMD24, although none of them was able to discriminate all accessions. Two monomorphic *loci* were detected (VVS5 and VVMD36). Most of the variable alleles were characteristic of three Refošk types: two Karst accessions (Clone 7 and Clone 50) and Sladki teran. A dendrogram (Figure) 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 Refošk selection vineyard flourishing 30 years ago, may represent disappearing genotypes of the cv. Refošk 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

Table 2

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

Locus	Identical genotypes of 53 Karst clones	Predominant genotypes	Different genotypes		
VVS1		183:190	180:183 [8]		
VVS2	134:154	134:154	132:154 [2,5]	152:152 [8]	
VVS4	167:172	167:172	167:174 [2,5,8]		
VVS5	98:98	98:98			
VVMD6	199:207	199:207	199:209 [5]	189:199 [8]	
VVMD7		248:248	240:248 [2,5]		
VVMD8		137:153	137:137 [8]		
VVMD14		222:241	222:235 [5]	222:239 [16]	233:241 [8]
VVMD17	222:222	222:222	212:222 [2,5]		
VVMD24		214:219	210:219 [5]	212:217 [12,13]	210:214 [8]
VVMD25		251:267	249:267 [10]	251:269 [13]	
VVMD27		191:191	183:191 [2,5]		
VVMD31		210:210	212:216 [5]		
VVMD32		255:275	255:263 [5]		
VVMD36		254:254			
VrZAG21	192:202	192:202	208:208 [7]	202:208 [8]	
VrZAG47		167:167	159:167 [2,5]		
VrZAG62		195:195	197:207 [8]		
VrZAG64		151:163	143:163 [5]		
VrZAG67		132:153	130:148 [5]	150:150 [8]	
VrZAG79		238:250	238:256 [8]		
VrZAG83		190:196	196:202 [5]	190:190 [8]	
VrZAG112		237:245	239:247 [12]	235:243 [17,18]	245:245 [8]

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

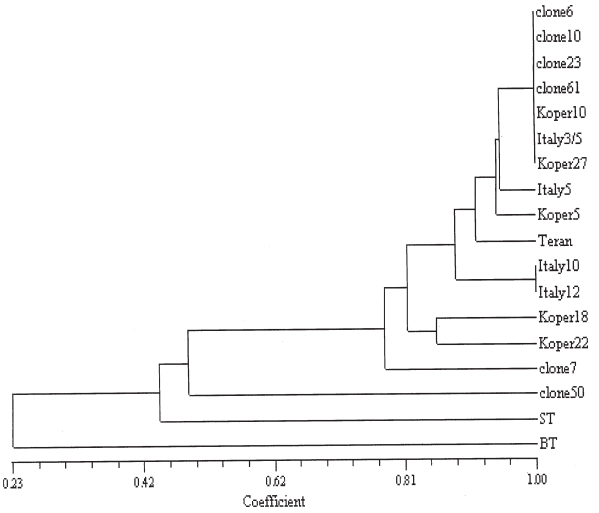

Figure: Dendrogram obtained from SSRs data at 23 *loci* constructed using Jaccard coefficients as genetic similarities.

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: 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

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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