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The characterisation of *Vitis vinifera* 'Refošk' with AFLP and SSR molecular markers and ampelographic traits

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ABSTRACT

The genetic diversity and ampelographic variability of autochthonous red wine cultivar 'Refošk' (*Vitis vinifera* L.) grown in Slovenia were evaluated with AFLP molecular markers and OIV descriptors, respectively. SSR molecular markers were employed to confirm cultivar identity of analysed samples. Eight AFLP primer combinations, one was monomorphic, produced 16 polymorphic markers in 41 out of 113 samples, what classified samples into monomorphic and polymorphic group. Dendrogram constructed with simple matching coefficient and unweighted pair-group method analysis presented genetic diversity within polymorphic group. Refošk biotypes from monomorphic and polymorphic groups were evaluated with 22 OIV descriptors related to bunch, berry and must, but on the basis of ampelographic characterization samples were not differentiated among two major groups obtained with AFLP analysis. Results of genetic analysis indicated that 'Refošk' originated from closely related plants that are phenotypically very similar. With regard to low observed genetic diversity more attention should be dedicated to the selection in order to conserve remaining genetic diversity.

Key words: AFLP, genetic diversity, SSR, cultivar identity, morphological traits, germplasm, grapevine, Refošk, Refosco

IZVLEČEK

KARAKTERIZACIJA ŽLAHTNE VINSKE TRTE (*Vitis vinifera* L.) SORTE 'REFOŠK' Z AFLP IN SSR MOLEKULSKIMI MARKERJI IN AMPELOGRAFSKIMI LASTNOSTMI

Z AFLP molekulskimi markerji in z OIV deskriptorji je bila ovrednotena genetska variabilnost in ampelografska raznolikost avtohtone sorte 'Refošk' (*Vitis vinifera* L.) v Sloveniji. Sortna pristnost analiziranih vzorcev je bila potrjena z mikrosatelitskimi markerji. Pri 41 vzorcih od skupno 113 smo z uporabo osmih parov začetnih selektivnih oligonukleotidov, od katerih je bila ena kombinacija monomorfna, odkrili 16 polimorfnih markerjev. Na podlagi rezultatov AFLP analize smo vzorce razvrstili v dve skupini in sicer v monomorfno in polimorfno skupino. Dendrogram, narejen na podlagi koeficientov enostavnega ujemanja in z metodo netehtanih parnih skupin z aritmetično sredino prikazuje genetsko variabilnost znotraj polimorfne skupine. Trse iz različnih genetskih skupin smo ovrednotili z 22 OIV deskriptorji, ki se nanašajo na grozd, jagode in mošt, vendar se na podlagi ampelografske karakterizacije niso razvrstili v skladu z razvrstitvijo pri AFLP analizi. Rezultati nakazujejo na izvor sorte 'Refošk' iz sorodnih, fenotipsko zelo podobnih starševskih rastlin. Glede na nizko število dobljenih polimorfnih AFLP markerjev bi morali intenzivneje delati na selekciji sorte 'Refošk' z namenom ohranitve obstoječe genetske variabilnosti.

Ključne besede: AFLP, genetska variabilnost, SSR, sortna pristnost, morfološke lastnosti, dednina, vinska trta, Refošk, Refosco

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1 INTRODUCTION

The red wine cultivar 'Refošk' (*Vitis vinifera* L.), in Italy known as Refosco del Carso, Refosco d'Istria or Terrano d'Istria, and in Croatia as Refošk istarski or Teran is a member of the Refosco family. In Slovenia it is cultivated mainly in the Kras and the Slovenska Istra winegrowing districts where it presents 73 % and 45 % of the vineyards area, respectively (MOP, 2011), totalling 1.200 hectares. In the Karst region the produced wine is Karst Teran with high lactic acid and mineral iron contents in comparison with Refošk wine produced from the same cultivar in Slovenian Istria, due to pedoclimatic factors. Content of anthocyanins in Refošk grapes is similar to that in Cabernet Sauvignon (Vrhovesk et al., 2002). 'Refošk' represents one of the earliest cultivated cultivars in this region and due to several biotypes, the ampelographers are still not in agreement on the basic traits of the cultivar. However, it is already known that Italian types of Refosco (e.g. Refosco dal peduncolo rosso, Refoscone, Refosco nostrano, Refosco di Rauscedo) are morphologically and genetically different from 'Refošk' grown in Slovenia (Cipriani et al., 1994; Plahuta and Korosec-Koruza, 2009). In 1989 a collection vineyard in Komen (the Karst district) was established with the aim to choose appropriate clones and to preserve the old local Refošk biotypes. Since only one clone of 'Refošk' is officially certified, there is great need to promote further selection process.

Ampelography is essential in order to obtain information about viticultural performance of cultivars and clones included in selection. This method is based on phenotypic traits that are heavily influenced by different environmental conditions as well as nutritional state and health (Mannini, 2000; Sefc et al., 2001), thus DNA analysis approaches are frequently used in the characterisation of grapevine germplasm (Barth et al., 2009; María Ortiz et al., 2004). Kozjak et al. (2003) tested some selected accessions from the collection vineyard in Komen with 6 microsatellite loci, also known as simple sequence repeats (SSR), and found that two Refošk samples are probably different from cultivar Refošk, showed different patterns, while other accessions revealed identical SSR allelic profiles. The insufficient clone discrimination ability of SSR molecular markers was also stated in other papers (Imazio et al., 2002; Laucou et al., 2011), although microsatellite markers have been widely used for grapevine cultivar identification, defining synonyms and homonyms, and for pedigree reconstruction (Cipriani et al., 2010; Laucou et al., 2011; Rusjan et al., 2012). Molecular markers that have been used on grapevine in several studies to detect intravarietal variability are the inter simple sequence repeats (ISSR) (Regner et al., 2000), amplified fragment length polymorphism (AFLP) (Cervera et al., 1998; Fanizza et al., 2003; Imazio et al., 2002; Konradi et al., 2007; Meneghetti et al., 2012), selective amplification of microsatellite polymorphic loci (SAMPL) (Cretazzo et al., 2010; Meneghetti et al., 2012), microsatellite amplified fragment length polymorphism (M-AFLP) (Cretazzo et al., 2010; Meneghetti et al., 2012) and specific sequence amplified polymorphism (S-SAP) (Carrier et al., 2012; Stajner et al., 2009). Identifying and preserving rare genetic diverse plant material is highly recommended in order to maintain the existing genetic variability within a cultivar to allow a good response to the natural selection pressure (new pests, environmental and management changes, etc.) and to enhance the quality and complexity of wines (Mannini, 2000).

The objectives of this work were to assess the genetic variability of the Refošk cultivar planted in the collection vineyard in Komen and in production vineyards in the Kras and Slovenska Istra winegrowing districts with AFLP markers. Microsatellite markers were employed to confirm the cultivar identity of analysed samples. Ampelographic characters of Refošk biotypes chosen on the basis of AFLP results were describe with OIV described.

2 MATERIAL AND METHODS

2.1 Plant material

Refošk samples were taken from: a collection vineyard established in Komen (N45 48.917 E13 44.692) (biotypes No. 1-35, 37-54, 56, 58-67, 69, 70, 73, 74 and 76, all together 69 samples); thirteen production vineyards randomly chosen in the Kras and Slovenska Istra winegrowing districts (41 samples) and three vines from Merče, Šepulje and Marezige, each more than 150 years old, were also included in analysis (Table 1). A total of 113 samples were included in the analysis.

2.2 DNA isolation

Genomic DNA for SSR and AFLP analysis was extracted from young leaves of shoot tips using the modified cetyltrimethylammonium bromide (CTAB) method described by Kump and Javornik (1996). The DNA was quantified by fluorometric determination using the Quant-iT^{TM} dsDNA Broad-Range (BR) Assay Kit by the QubitFluorometer (Invitrogen, Darmstadt, Germany).

Table 1. List of Refošk samples used for SSR and AFLP analysis from production vineyards, together with old Refošk vines

Origin	Code	Year of planting	Coordinates	Winegrowing district
Križ (a)	1s, 3s, 7s, 8s, 10s	2002	N45 44.566 E13 51.905	Kras
Križ (b)	12s, 13s, 17s, 18s, 19s, 20s	2005	N45 44.566 E13 51.905	Kras
Tomaj	21s, 25s, 26s, 28s, 29s, 30s	1970	N45 45.220 E13 51.077	Kras
Godnje	37s, 38s, 39s, 40s	1990	N45 45.399 E13 50.434	Kras
Dutovlje (a)	45s, 46s, 47s	2002	N45 45.203 E13 49.805	Kras
Dutovlje (b)	54s, 58s	1958	N45 45.204 E13 49.805	Kras
Krajna vas	63s	1999	N45 45.870 E13 48.119	Kras
Šepulje	72s	app. 1780	N45 45.080 E13 52.191	Kras
Merče	73s	app. 1700	N45 42.071 E13 54.047	Kras
Prade	3k, 5k, 10k	1998	N45 32.903 E13 46.909	Slovenska Istra
Pobegi (a)	11k, 12k, 13k, 15k	1980	N45 32.266 E13 47.156	Slovenska Istra
Marezige	18k	app. 1880	N45 30.383 E13 48.143	Slovenska Istra
Truške	19k, 20k, 21k, 22k	2000	N45 29.674 E13 48.960	Slovenska Istra
Boršt	33k	1980	N45 28.647 E13 46.903	Slovenska Istra
Izola	36k	2001	N45 31.745 E13 40.288	Slovenska Istra
Pobegi (b)	41k	2003	N45 32.081 E13 47.396	Slovenska Istra

2.3 Microsatellite analysis

To prove cultivar identity, six previously described microsatellite loci were analysed: VVMD5, VVMD7 (Bowers et al., 1996); VVMD27, VVMD32 (Bowers et al., 1999); ssrVrZAG62 and ssrVrZAG79 (Sefc et al., 1999). Amplifications were made with the economic method described by Schuelke (2000) where the loci specific primer was elongated for M13 sequence and four M13 primers fluorescently labelled with dye phosphoramidites (6-FAM, VIC, PET or NED) were used in PCR as well. In a total volume of 15 µl the PCR reaction mixture contained 20 ng of genomic DNA, 1 x Taq Buffer with $(NH_4)_2SO_4$, 0.2 mM of each dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer (Integrated DNA Technologies, Leuven, Belgium), 0.25 µM of M13 fluorescent primer (Applied Biosystems, Cheshire, UK) and 0.375 U of *Taq* DNA polymerase. All chemicals were supplied by Fermentas/Thermo Fisher Scientific, MA, USA. PCR reactions were carried out in a 2720 thermal cycler (Applied Biosystems, Darmstadt, Germany) with a two-step PCR protocol started with initial touchdown cycle: 94 °C for 5 min, followed by five cycles of 30 s at 94 °C, 30 s at 60 °C, which was lowered by 1 °C each cycle, 90 s at 72 °C, followed by 30 cycles with annealing temperature at 55 °C and ending with an 8-min extension step at 72 °C. PCR products were multiplexed as shown in Table 2 and separated by capillary electrophoresis on an Applied Biosystems 3130

Genetic Analyser, using GeneScan™ -500 LIZ® (Applied Biosystemsh, Cheshire, UK) as size standard.

2.4 AFLP analysis

AFLP analysis was performed on 113 samples according to Vos et al. (1995) with the modifications described below. Each 500 ng sample of genomic DNA was digested with *Tru*1I (*Mse*I iso-schizomer) and *Pst*I (3 U each) restriction endonucleases for 120 min at 37 °C (*Pst*I incubation temperature) and 120 min at 65 °C (*Tru*1I incubation temperature) in a 40 µl volume in the presence of 1x Buffer R. After restriction 10 µl of ligation mix, including 50 pmol of *Mse*I adapters, 5 pmol *Pst*I adapters, 2 µl 10 mM ATP, 1µl 10x T4 DNA ligase buffer and 1 U T4 ligase was added to restriction reaction. Adapters were prepared by adding equimolar amounts of both strands (Integrated DNA Technologies, Leuven, Belgium). Ligation was performed at 22 °C for 60 min, followed by the final step at 65 °C for 10 min to inactivate enzymes. The pre-amplification of DNA templates (50 ng) was performed in 50 µl volume with non-selective *Pst*I and *Mse*I primers in a final concentration 0.2 µM, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1x *Taq* Buffer with $(NH_4)_2SO_4$ and 1.25 U *Taq* DNA polymerase.

Selective amplifications were performed in a volume of 10 µl containing the following components: 2 µl 10-times diluted pre-amplification products, 1x *Taq* Buffer with $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM with fluorescent dye (6-FAM, VIC, PET) labelled *Pst*I primer (Applied Biosystems, Cheshire, UK), 0.2 µM unlabelled *Mse*I primer and 0.25 U *Taq* DNA polymerase. Selective amplifications were performed using a total of seven primer combinations with two or three selective nucleotides (Table 3). Primer pairs were chosen based on previous testing of 56 combinations on 8 samples (*Pst*I primers with selective nucleotides: ATA, AAC, AGA and ACA; *Mse*I primers with selective nucleotides: AG, CG, CA, AC, CC, CTT, CAT, CAA, CAG, CAC, CTG, CTA, CTC, ACC) with the aim of obtaining an optimized number of scorable bands for every primer combination (data not shown). PCR protocols were as described by Vos et al. (1995), except preamplification was performed with the initial step of 2 min at 72 °C. PCR products were multiplexed (as shown in Table 3), and separated by capillary electrophoresis with GeneScan™ -500 LIZ® (Applied Biosystemsh, Cheshire, UK) as internal size standard on an Applied Biosystems 3130 Genetic Analyser. All accessions were analysed twice (DNA restriction, pre-amplification and selective amplification) to test the reproducibility of the AFLP profiles.

2.5 Ampelographic Analysis

Twelve Refošk biotypes, chosen on the basis of AFLP results, grown in the collection vineyard were described with 22 OIV descriptors related to bunch, berry and must (2nd edition of the OIV descriptor list for grape varieties and Vitis species) (O.I.V., 2009). Descriptions were performed on 10 shoots of 3 to 5 vines per biotype. Vines are grafted on rootstock SO4 (*Vitis berlandieri* x *Vitis riparia*), trained as double guyot and cultivated following the instructions of integrated pest management. The vineyard was permanently green covered. Each biotype has 3 to 35 vines planted in the block.

2.6 Data analysis

SSR and AFLP electropherograms were analysed and sized with Gene Mapper software version 4.1 (Applied Biosystems, Chesire, UK). AFLP electropherograms were scored for the presence or absence of bands and expressed in binary data, while microsatellite alleles were presented in the amplification lengths. For AFLP, only reproducible, clear bands falling within the range of 50 - 500 bp were considered for analysis. The total number of fragments and percentage of polymorphic fragments were assessed for every primer combination and in the total set. The genetic similarity among clones was calculated using simple matching (SM) genetic distance. A dendrogram was constructed using the unweighted pair group method average (UPGMA) clustering of the NTSYSpc software package, version 2.02i (Rohlf, 1998). Average gene diversity over loci was calculated based on Nei (1987) formula using the Arlequin program (Excoffier and Lischer, 2010).

The observed mean values of ampelographic characters were transformed to numerical scales according to the OIV descriptors (O.I.V., 2009).

The dendrogram was drawn using UPGMA method and distance (DIST) coefficient for interval measure (quantitative) data. Calculations were performed with NTSYSpc 2.02i software (Rohlf, 1998).

3 RESULTS

3.1 Molecular Analysis

The microsatellite analyses confirmed the cultivar identity of all tested Refošk vines. All 113 samples had the same fingerprint at 6 microsatellite loci (Table 2).

AFLP analysis, conducted on 113 samples, using eight different primer pairs, produced 208 scorable fragments, 16 of which were polymorphic. One combination generated only monomorphic markers, while 7 combinations were informative. Polymorphic fragments and percentage of polymorphism varied from 1 to 5 loci and from 2.3 to 18.8 % per primer combination, respectively (Table 3). The size of polymorphic amplified products ranged from 100 bp to 397 bp. The AFLP analysis was repeated at least twice and all polymorphic bands were reproducible. In general samples included in analysis could be classified into monomorphic group and polymorphic group since 72 samples showed no polymorphisms compared to other 41 samples that showed polymorphisms in terms of gaining new bands compared to the monomorphic group (Figure 1). Average gene diversity over loci for all samples was 0.0294 with standard deviation 0.0155.

Twenty two out of 41 samples had identical fingerprints, while other 19 samples were more diverse, with 78 to 93.8 % genetic similarity compared with the main identical group.

Table 2: SSR allele length (alleles in bp) at 6 microsatellite loci performed on all 113 Refošk samples, fluorescently labelled M13 primer labelled with different dyes for different SSR markers and multiplexing combinations after PCR. Combinations analysed in the same electrophoresis run are marked with the same letters (A and B).

SSR marker	Dye of M13 primer	Electrophoresis multiplex	Genotype
VVMD5	NED	A	241:243
VVMD7	6-FAM	A	262:264
VVMD27	VIC	B	208:208
VVMD32	VIC	A	266:289
VrZAG62	6-FAM	B	210:212
VrZAG79	PET	A	256:268

Table 3: The number of total scorable and polymorphic AFLP markers generated by the selected primer combinations, where "P" and "M" are *Pst*I and *Mse*I primers, respectively. Combinations analysed in the same electrophoresis run are marked with the same letters (A, B and C).

Primer combination	Total bands	Polymorphic markers	Polymorphism (%)	Electrophoresis multiplex
6-FAM-P-AGA/M-CTT	43	1	2.3	A
VIC-P-AAC/M-CTG	29	2	6.9	A
6-FAM-P-AGA/M-CAT	23	3	13.0	B
VIC-P-AAC/M-AG	34	5	14.7	B
PET-P-ATA/M-CAA	16	3	18.8	B
6-FAM-P-AGA/M-AG	22	1	4.5	C
VIC-P-AAC/M-CTC	16	1	6.25	C
PET-P-ATA/M-CTT	25	/	/	A
Total	208	16	7.7	

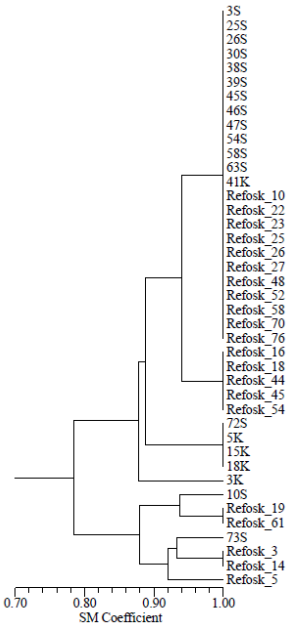

Figure 1: UPGMA-derived dendrogram of genetic similarity based on the SM coefficient among the 41 Refošk samples

3.2 Ampelographic characterization

Twelve Refošk biotypes grown in the collection vineyard were selected on the basis of AFLP analysis, 4 from the monomorphic group and 8 from the polymorphic group, and were evaluated for 22 OIV descriptors (Table 4). Traits, showing variability among Refošk biotypes, were: bunch density (OIV 204) varied from loose to medium; observed length of peduncle of primary bunch (OIV 206) varied from 46.2 to 72.5 mm; peduncle was lignified up to about the middle or more than the middle (OIV 207); the number of wings of the primary bunch (OIV 209) varied from 1 to 5; berry shapes (OIV 223) were either globose or ellipsoid; mean weight of a single bunch (OIV 502) varied from 371 to 696 g; mean weight of 30 typical berries of 5 bunches (OIV 503) varied from 2.77 to 4.30 g; observed anthocyanin coloration of flesh (OIV 231) varied from none to medium; and must traits: sugar (OIV 505) and total acid content expressed as tartaric acid equivalents (OIV 506), varied from 18.2 to 23.3 % and from 10 to 11.8 g l^{-1}, respectively. However, Refošk biotypes did not show any similar distribution with regards to AFLP monomorphic and polymorphic group, since Refošk biotypes 31, 39, 40 and 43, presenting the monomorphic group, were distributed among different clusters (Figure 2).

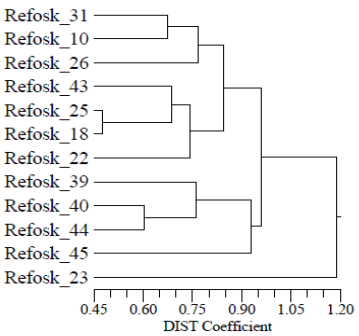

Figure 2: Dendrogram based on ampelographic characterization of the 12 Refošk biotypes from the collection vineyard in Komen (Kras winegrowing district, Slovenia), constructed with UPGMA method and distance (DIST) coefficient

Table 4: Scoring results of 22 OIV codes of the 12 Refošk biotypes from collection vineyard in Komen (Kras winegrowing district, Slovenia). Observations were performed on 10 shoots on 3 to 5 vines per biotype.

OIV code	Characteristic	Refošk biotypes											
		31	39	40	43	10	22	23	18	25	26	44	45
202	Bunch length with peduncle excluded	5	5	5	5	5	5	5	5	5	5	5	5
203	Bunch width	5	5	5	5	5	5	5	5	5	5	5	5
204	Bunch density	5	3	3	5	5	5	5	5	5	5	3	5
206	Length of peduncle of primary bunch	3	3	3	5	5	3	3	5	5	3	3	3
207	Lignification of peduncle	5	5	5	7	7	5	5	5	5	7	5	5
208	Bunch shape	2	2	2	2	2	2	2	2	2	2	2	2
209	Number of wings of the primary bunch	3	2	3	4	2	2	4	2	2	3	3	4
220	Berry length	5	5	5	5	5	5	5	5	5	5	5	5
221	Berry width	5	5	5	5	5	5	5	5	5	5	5	5
222	Uniformity of berry size	2	2	2	2	2	2	2	2	2	2	2	2
223	Berry shape	2	3	3	2	3	2	3	2	3	2	3	2
225	Berry skin colour	6	6	6	6	6	6	6	6	6	6	6	6
226	Uniformity of berry skin colour	6	6	6	6	6	6	6	6	6	6	6	6
231	Intensity of the anthocyanin coloration of flesh	3	1	1	1	3	1	5	1	3	3	1	3
233	Must yield	5	5	5	5	5	5	5	5	5	5	5	5
238	Length of pedicel	3	3	3	3	3	3	3	3	3	3	3	3
240	Ease of detachment from pedicel	3	3	3	3	3	3	3	3	3	3	3	3
502	Weight of a single bunch	5	3	5	5	5	5	3	5	5	7	5	3
503	Single berry weight	3	3	3	3	3	5	3	3	3	5	5	3
505	Sugar content of must	7	7	5	7	7	7	9	7	7	7	5	5
506	Total acid content of must	5	5	5	7	5	7	5	7	7	5	7	7
508	Must specific pH	3	3	3	3	3	3	3	3	3	3	3	3

4 DISCUSSION

The genetic diversity of cultivar and the presence of several biotypes are of great importance because of their adaptation to different climate conditions as this can contribute to typical characteristics of vine. In the present study, the cultivar identity was confirmed with SSR markers and genetic diversity of Refošk was assessed with AFLP molecular markers. The amplification of six SSR loci revealed the same allele patterns in all 113 accessions and confirmed the genetic identity of the cultivar. Kozjak et al. (2003) distinguished samples labelled with number 7 and 50 from other Refošk biotypes with microsatellite analysis, while all other analysed samples had the same microsatellite fingerprint in our research. The discrepancies that were observed between the Refošk samples 7 and 50 by Kozjak et al. (2003) and our results, were probably due to mistakes at planting or collecting stage. No other previous information is available on genetic diversity within cultivar Refošk grown in Slovenia.

The *Pst*I-*Mse*I primer combinations used reveal 16 reproducible polymorphisms out of 208 scorable markers and thus allowed to differentiate analysed samples in polymorphic and monomorphic group. Overall mean value of gene diversity was lower than published for example for Pinot Noir clones and similar as observed for Pinot gris clones (Blaich et al., 2007; Konradi et al., 2007).

The dendrogram presented genetic variability within the polymorphic group (Figure 1). When analysing clonal diversity of grapevine cultivars in other studies, a wide range of obtained AFLP polymorphisms and power of discrimination have been reported. For example, Fanizza et al. (2003) did not manage to differentiate 4 clones of the table grapevine cultivar Italia, although 3880 markers had been produced with 49 primer combinations; Filippetti et al. (2005) discriminated only 3 polymorphic clones out of 26 using 9 primer combinations; Konradi et al. (2007) revealed 72 polymorphic markers of total 375 among 32 Pinot clones exhibiting up to 5 % dissimilarity, on the other hand Anhalt et al. (2011) obtained 135 polymorphic markers out of 305 with 10 primer combinations when studying 86 Riesling clones, but most clones showed none, one or two mutations over all primer combinations

Discrimination of samples in monomorphic and polymorphic groups could indicate that Refošk grown in Slovenia originated from different, but genetically and morphologically very similar plant material. A possible explanation for this phenomenon is provided by Filippetti et al. (1999) who demonstrated that seedlings from a single self-pollinated vines were morphologically similar, but at the DNA level could be differentiated. The results show that genetically different plant material is equally represented in production vineyards, indicating that vine nurseries propagate genetically mixed material. Due to low detected variability (only 10 different AFLP fingerprints) it is necessary to continue with the analysis to determine as much genetic variability as possible for efficient and proper conservation.

Results of ampelographic description showed that Refošk biotypes differ in several traits. However, correlation between ampelographic characters and either monomorphic or polymorphic group according to AFLP results were not observed. Since traits including berry weight, number of wings of the primary bunch, lignification of the peduncle, and the must traits of sugar and total acid content could vary from year to year, due to different crop level and other biotic and abiotic factors, multiple years of ampelographic observations should be considering for comparison.

5 CONCLUSION

Genetic diversity is needed for efficient adaptation of cultivars to environmental changes and to be more resilient to environmental shocks. ‘Refošk’ is cultivated across a relatively large region but AFLP genetic analysis showed that very little genetic diversity exists within cultivar, which subsequently presents higher production risks. In recent years Refošk was recognized as very susceptible to Grapevine yellows and few vineyards were already grubbed-up. When

selecting morphological appropriate grapevine, genetic analysis should complement ampelography what allows to prevent diminishing of genetic variability. Using AFLP markers we were able to detect greater variability compared to microsatellite molecular markers, where no polymorphisms were discovered and thus provided valuable information for further selection and conservation processes.

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7 REFERENCES

Anhalt U.C.M., Crespo Martínez S., Rühl E., Forneck A. 2011. Dynamic grapevine clones—an AFLP-marker study of the *Vitis vinifera* cultivar Riesling comprising 86 clones. Tree Genetics & Genomes, 7, 4: 739-746, DOI: 10.1007/s11295-011-0370-x.

Barth S., Forneck A., Verzeletti F., Blaich R., Schumann F. 2009. Genotypes and phenotypes of an ex situ Vitis vinifera ssp. sylvestris (Gmel.) Beger germplasm collection from the Upper Rhine Valley. Genetic Resources and Crop Evolution, 56, 8: 1171-1181, DOI: 10.1007/s10722-009-9443-1.

Blaich R., Konradi J., Ruhl E., Forneck A. 2007. Assessing Genetic Variation among Pinot noir (*Vitis vinifera* L.) Clones with AFLP Markers. American Journal of Enology and Viticulture, 58, 4: 526-529

Bowers J.E., Dangl G.S., Meredith C.P. 1999. Development and Characterization of Additional Microsatellite DNA Markers for Grape. American Journal of Enology and Viticulture, 50, 3: 243-246

Bowers J.E., Dangl G.S., Vignani R., Meredith C.P. 1996. Isolation and characterization of new polymorphic simple sequence repeat loci in grape (*Vitis vinifera* L.). Genome, 39, 4: 628-633, DOI: 10.1139/g96-080.

Carrier G., Le Cunff L., Dereeper A., Legrand D., Sabot F., Bouchez O., Audeguin L., Boursiquot J.-M., This P. 2012. Transposable Elements Are a Major Cause of Somatic Polymorphism in *Vitis vinifera* L. PLoS ONE, 7, 3: e32973

Cervera M.T., Cabezas J.A., Sancha J.C., Martínez de Toda F., Martínez-Zapater J.M. 1998. Application of AFLPs to the characterization of grapevine *Vitis vinifera* L. genetic resources. A case study with accessions from Rioja (Spain). TAG Theoretical and Applied Genetics, 97, 1-2: 51-59

Cipriani G., Frazza G., Peterlunger E., Testolin R. 1994. Grapevine fingerprinting using microsatellite repeats. Vitis, 33: 211-215

Cipriani G., Spadotto A., Jurman I., Di Gaspero G., Crespan M., Meneghetti S., Frare E., Vignani R., Cresti M., Morgante M., Pezzotti M., Pe E., Policriti A., Testolin R. 2010. The SSR-based molecular profile of 1005 grapevine (*Vitis vinifera* L.) accessions uncovers new synonymy and parentages, and reveals a large admixture amongst varieties of different geographic origin. TAG Theoretical and Applied Genetics, 121, 8: 1569-1585, DOI: 10.1007/s00122-010-1411-9.

Cretazzo E., Meneghetti S., De Andrés M.T., Gaforio L., Frare E., Cifre J. 2010. Clone differentiation and varietal identification by means of SSR, AFLP, SAMPL and M-AFLP in order to assess the clonal selection of grapevine: the case study of Manto Negro, Callet and Moll, autochthonous cultivars of Majorca. Annals of applied biology, 157, 2: 213-227, DOI: 10.1111/j.1744-7348.2010.00420.x.

Excoffier L., Lischer. H.E.L. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources, 10: 564-567, DOI: 10.1111/j.1755-0998.2010.02847.x.

Fanizza G., Chaabane R., Ricciardi L., Resta P. 2003. Analysis of a spontaneous mutant and selected clones of cv. Italia (*Vitis vinifera*) by AFLP markers. Vitis, 42, 1: 27-30

Filippetti I., Intrieri C., Cemtinari M., Bucchetti B., Pastore C. 2005. Molecular characterization of officially registered Sangiovese clones and of other Sangiovese-like biotypes in Tuscany, Corsica and Emilia-romagna. Vitis, 44, 4: 167-172

Filippetti I., Silvestroni O., Thomas M.R., Intrieri C. 1999. Diversity assessment of seedlings from self-

pollinated Sangiovese grapevines by ampelography and microsatellite DNA analysis. Vitis, 38, 2: 67-71

Imazio S., Labra M., Grassi F., Winfield M., Bardini M., Scienza A. 2002. Molecular tools for clone identification: the case of the grapevine cultivar 'Traminer'. Plant Breeding, 121, 6: 531-535, DOI: 10.1046/j.1439-0523.2002.00762.x.

Konradi J., Blaich R., Forneck A. 2007. Genetic Variation among Clones and Sports of 'Pinot noir' (*Vitis vinifera* L.). European Journal of Horticultural Science, 72, 6: 275-279

Kozjak P., Korosec-Koruza Z., Javornik B. 2003. Characterisation of cv. Refošk (*Vitis vinifera* L.) by SSR markers. Vitis, 42, 2: 83-86

Kump B., Javornik B. 1996. Evaluation of genetic variability among common buckwheat (*Fagopyrum esculentum* Moench) populations by RAPD markers. Plant Science, 114, 2: 149-158, DOI: 10.1016/0168-9452(95)04321-7.

Laucou V., Lacombe T., Dechesne F., Siret R., Bruno J.P., Dessup M., Dessup T., Ortigosa P., Parra P., Roux C., Santoni S., Varès D., Péros J.P., Boursiquot J.M., This P. 2011. High throughput analysis of grape genetic diversity as a tool for germplasm collection management. TAG Theoretical and Applied Genetics, 122, 6: 1233-1245, DOI: 10.1007/s00122-010-1527-y.

Mannini F. 2000. Clonal selection in grapevine: interactions between genetic and sanitary strategies to improve propagation material. Acta Horticulturae, 528: 703-712

María Ortiz J., Pedro Martín J., Borrego J., Chávez J., Rodríguez I., Muñoz G., Cabello F. 2004. Molecular and morphological characterization of a Vitis gene bank for the establishment of a base collection. Genetic Resources and Crop Evolution, 51, 4: 403-409, DOI: 10.1023/B:GRES.0000023451.09382.45.

Meneghetti S., Costacurta A., Morreale G., Calò A. 2012. Study of Intra-Varietal Genetic Variability in Grapevine Cultivars by PCR-Derived Molecular Markers and Correlations with the Geographic Origins. Molecular Biotechnology, 50, 1: 72-58, DOI: 10.1007/s12033-011-9403-9.

MOP. 2011. Registry of wine and grape growers. Ljubljana, Slovenia, Ministry of the Environment and Spatial Planning

Nei M. 1987. Molecular Evolutionary Genetics. New York, Columbia University Press: pp. 512

O.I.V. 2009. OIV descriptor list for grape varieties and *Vitis* species. 2nd ed. Paris, International Organisation of Vine and Wine

Plahuta P., Korosec-Koruza Z. 2009. 2 x sto vinskih trt na Slovenskem. Ljubljana, Prešernova družba: pp. 367

Regner F., Wiedeck E., Stadlbauer A. 2000. Differentiation and identification of White Riesling clones by genetic markers. Vitis, 39, 3: 103-107

Rohlf F.J. 1998. NTSYSpc. Numerical taxonomy and multivariate analysis system, version 2.02i. New York, Exeter Software

Rusjan D., Pipan B., Pelengic R., Meglic V. 2012. Genotypic and Phenotypic Discrimination of Grapevine (Vitis vinifera L.) Varieties of the 'Vitovska' and 'Garganja' Denominations. European Journal of Horticultural Science, 77, 2: 84-94

Schuelke M. 2000. An economic method for the fluorescent labeling of PCR fragments. Nature Biotechnology, 18, 2: 233-234, DOI: 10.1038/72708.

Sefc K.M., Lefort F., Grando M., Scott K.D., Steinkellner H., Thomas M.R. 2001. Microsatellite markers for grapevine: A state of the art. In: Molecular Biology and Biotechnology of the Grapevine Roubelakis-Angelakis K.A. (ed). Dordrecht, Kluwer Academic Publishers: 433-456

Sefc K.M., Regner F., Turetschek E., Glössl J., Steinkellner H. 1999. Identification of microsatellite sequences in *Vitis riparia* and their applicability for genotyping of different *Vitis* species. Genome, 42, 3: 367-373, DOI: 10.1139/gen-42-3-367.

Stajner N., Jakse J., Javornik B., Masuelli R.W., Martinez L.E. 2009. Highly variable AFLP and S-SAP markers for the identification of 'Malbec' and 'Syrah' clones. Vitis, 48, 3: 145-150

Vos P., Hogers R., Bleeker M., Reijans M., Lee T.v.d., Hornes M., Friters A., Pot J., Paleman J., Kuiper M., Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research, 23, 21: 4407-4414, DOI: 10.1093/nar/23.21.4407.

Vrhovesk U., Vanzo A., Koruza B., Korosec Koruza Z. 2002. Polyphenolic potential of Slovenian red grapes. In: Vinogradi in vina za tretje tisočletje? Nova Gorica, Grafika Soča: 359-367