

AMPELOGRAPHIC AND RANDOM AMPLIFIED POLYMORPHIC DNA (RAPDS) BASED ANALYSIS OF SIX GRAPEWINE VARIETIES OF RAHOVEC, KOSOVO

Ylber Bajraktari¹, Nadiel Cadri², Stela Papa², Ariola Bacu^{2*}

¹ Department for Viticulture & Winery, Ministry of Agriculture,
Forestry and Rural Development of Kosovo, Xhelal Hajda-Toni, 21000 Rahovec, Kosovo

² Department of Biotechnology, Faculty of Natural Sciences, University of Tirana,
Bulv. Zogu, 25/1, 1001 Tirana, Albania

³ Food and Veterinary Agency of Kosovo, Regional Office,
Sheh Jemini pn., 20000 Prizren, Kosovo

*e-mail: ariolabacu@yahoo.com

Abstract

The Rahovec area located in Kosovo is well known for the production of grapevines of high quality since antiquity. During the last decades more attention is drowned toward the enlargement of the production area as well as to the in depth study of the agronomical and molecular characteristics of the main cultivars, aiming a better judgement on the genetic diversity of grapevines, and the identification of native cultivars. Present study describes the use of morphometric characteristics and Randomly Amplified Polymorphic DNA to verify genetic differences among six cultivars, namely Vranc, Prokup, Rrush Keci, Rrush Melik, Thanz i kuq and Thanz i zi.

The ampelographic data addressed were morphological and agronomical characteristics related to period of flowering, period of ripening, the vegetative growth rate, and biometric characteristics of leaves, considered in levels of estimation according to the descriptor of International Plant Genetic Resources Institute (IPGRI). Molecular data were based on the use of ten decamer primers, applied on template DNA extracted from ten parallel plants from each cultivar. The presence/non presence of the amplified fragments were used to construct a dendrogram of similarity with software NTSYS 2.1 based on Unweighted Pair Group Method with Arithmetic Mean - UPGMA cluster analysis.

Ten RAPD markers produced a total of 76 fragments 50 of each were polymorphic. The dendrogram of similarity grouped cultivars in two clusters, and clarified that they share 0.25 - 0.75% similarity based on the Jaccard's coefficient. There were no polymorphisms detected among parallel plants within each cultivar. The morphometric data displayed a level of similarity among cultivars comparable to that offered by molecular

markers, which clearly differentiate the six cultivars from each-other.

The six main cultivars of Rahovec area differ from each other significantly, and none of them should be considered as homonymous. Phenotypic and genetic differences classify them in a similar way, in two clusters.

Key words: Ampelography, RAPDs, Grapevine cultivars, Dendrogram of similarity.

1. Introduction

Rahovec area located in Kosovo is well known for the production of grapevines of high quality since antiquity. However, during the last decades except the tendency toward the enlargement of the production area, a number of research studies are undertaken for the in depth study of the agronomical and molecular characteristics of main varieties. Among the last the identification of native original cultivars has been a priority.

Worldwide, traditional methods for the identification of grapevine cultivars were based on morphological differences, gathered under the name ampelography, which identifies names and classifies grape cultivars through detailed analysis of the unique characteristics of the plant, morphology and different phases of development. This category of data except their importance present a number of restrictions, as the need to use fully grown plants, the vulnerability of the data from the changing environmental conditions, the need for experts and the huge number of plant material to be analyzed.

Because of the above restrictions, a number of molecular techniques were developed during last twenty years for the genetic discrimination of grapevines. Among the important molecular tools the isoenzymes were among the first, but since the expression of the enzymes, which do not show variation under environmental conditions is limited, the availability of the data taken from them is also limited (Parfit and Arulsekar [1], Walter *et al.* [2], Sefc *et al.* [3], and [4]). These results brought into power the use of DNA for the evaluation of genetic diversity, as a biomolecule which is not prone to environmental fluctuations. Different molecular techniques were applied for this purpose, such as RFLP (Restriction Fragment Length Polymorphisms), RAPDs (Randomly Amplified Polymorphic DNA), both of them with a number of disadvantages, either regarding the amount of DNA required, or the over consuming of time and need for strict experimental conditions (This *et al.* [5], Martin *et al.* [6]). However, RAPDs were used successfully by a number of authors to assess the diversity among grapevine varieties (Stavarakakis *et al.* [7]; Gonzales-Andrez *et al.* [8]; Lefort *et al.* [9]; Aras *et al.* [10]; Zeinali *et al.* [11]; Ercisli *et al.* [12]; Hizarci, *et al.* [13]; Coelho *et al.* [14], etc). With time, microsatellites were seen as a more promising category of molecular markers. The last are repeated simple sequence motifs, which represent a great source of genetic variation (Tauf *et al.* [15]). Among the three subclasses of repetitive DNA the microsatellites show the lowest degree of repetition (from five to one hundred repeats composed of 1 to 6 nucleotides). The establishment of microsatellite markers in an organism is a costly and time-consuming procedure (Sefc *et al.* [4]) involving the construction and screening of genomic libraries and optimization of PCR primers, thus the fact that microsatellites obtained for one species might be used for closely related ones is very important for related plant species as grapes belonging to *Vitis* species. So far, *Vitis* SSR primers have been developed by different groups starting with Bowers *et al.* [16], Sefc *et al.* [17], etc, and their usefulness has been assessed in grapevine varieties in Australia, California and Central Europe. 29 Albanian grapevine cultivars were first studied by microsatellite markers from Ladoukakis *et al.* [18], while there is no report on the molecular discrimination of the cultivars from Kosovo, yet. The morphometric data for main cultivars were verified (Kryeziu *et al.* [19]; Shundi *et al.* [20]; Susaj *et al.* [21], etc.) and interpreted considering the climatic conditions at Dukagjini plane (Koronica *et al.* [22]; Cena *et al.* [23], etc.). In this study were considered cultivars Vranc, Prokup, Rrush Keci, Rrush Melik, Thanz i kuq and Thanz i zi, which are considered from the inhabitants as native based on historical knowledge on their origin.

New data are reported on the period of flowering, period of ripening, the vegetative growth rate, and biometric characteristics of leaves, considered in levels of estimation according to the descriptor of IPGRI (The International Plant Genetic Resources Institute) of CGIAR (Consultativ

Group on International Agricultural Research) based on which, the statistical analysis was completed. Molecular data, reported for the first time, were based on the use of RAPDs decamer primers. The morphometric data displayed a level of similarity among cultivars comparable to that offered by molecular markers, and both manage to differentiate the six cultivars from each-other.

2. Materials and Methods

Main grape cultivars under study were Vranc, Prokup, Rrush Keci, Rrush Melik, Thanz i kuq and Thanz i zi grown in Rahovec, Kosovo. The ampelographic data on these cultivars were morphological and agronomical characteristics, according to the descriptor of IPGRI (The International Plant Genetic Resources Institute) of CGIAR (Consultativ Group on International Agricultural Research) described in details at Tables 1, 2, 3, and 4. Values for each characteristic were used to prepare graphics (Figures 1, 2, 3, 4, and 5) to describe the variability among the six cultivars. The same data were used to prepare a three-dimensional graphic (Figure 5), which elucidated further the variation.

The molecular data were based on RAPDs. Decameric primers used were: OPB8, OPB9, OPB11, OPB12, OPB19, OPB20, OP2, OP6, OPC15, OPB2 from Operon.

The genomic DNA was extracted from ten parallel plants for each cultivar, from fresh leaves, based on CTAB method.

The PCR reaction conditions were: 1UTaq polimeraze, 1X bufer PCR, 1,4 μ l $MgCl_2$, 25mM, 100 μ M from each deoksinucleotide, 100pmole primer, 25ng template ADN. The respective volumes were calculated for a total volume of 10 μ l.

Cycling conditions were: 94 °C for 4 minutes, 36 times the following conditions: 94 °C for 1minute, 94 °C for 10 seconds, 32 °C for 1 minute, 72 °C for 1 minute, 72 °C for 5 minutes.

Electrophoresis of the PCR products was conducted in 1.5% agarose gels in 1x TAE-Tris Acetate EDTA. The markers used to estimate the fragment's dimensions were 20bp, 100bp and 1kbp and photos were taken under UV light.

The statistical analysis of the results was initiated through the preparation of a binary matrix, which was further processed via the software NTSYS, 2.1 to create a dendrogram of genetic similarity among the six cultivars.

3. Results and Discussion

Ampelographic data on Vranc, Prokup, Rrush Keci, Rrush Melik, Thanz i kuq and Thanz i zi cultivars for the Rahovec area are presented in Tables 1, 2, 3, and 4. Morphometric data related to different levels of estimation were measured for four main characteristics,

respectively for the: Period of flowering, period of ripening, vegetative growth rate, and mean values for biometric characteristics of leaves. Values presented at Table 1 and Figure 1, are describing flowering time for the six cultivars. Differences in flowering time among the cultivars are a few days, while cultivars named Thanz i zi and Thanz i kuq have the same period of flowering. **Evaluation of the flowering time** (Code O-302 of IPGRI) for each cultivar is completed based on the mean flowering period at four levels:

1. Very early, when flowering starts 4 - 5 days earlier than medium period.
2. Medium, when flowering starts 2 - 3 days earlier or after medium period.
3. Late, when starts 1 week after the medium period.
4. Too late, when starts 7 - 8 days later than medium period.

Table 1. Flowering time for the cultivars of Rahovec, Kosovo

Nr	Cultivar	Start of flowering	End of flowering	Period of flowering	Evaluation of period (Code O-302)
1	Prokup	04/06	11/06	7	2
2	Melik	03/06	11/06	8	2
3	Vranac	09/06	17/06	8	2
4	Thanzi i zi	29/05	07/06	9	1
5	Thanzi i kuq	28/05	06/06	9	1
6	Pllovdin (Keci)	30/05	07/06	8	1

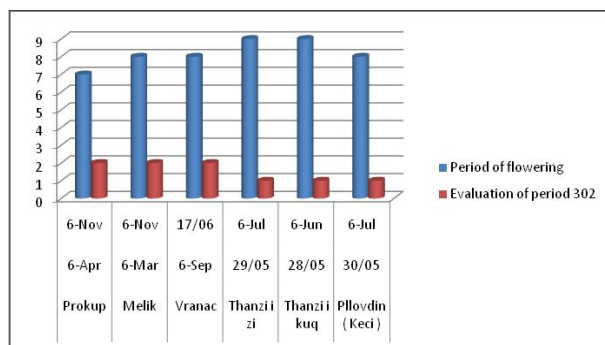


Figure 1. Flowering time for the cultivars of Rahovec, Kosovo

Period of ripening is another characteristic of importance evaluated according to the levels described by the descriptor of IPGRI as Ripening (Code O-304). Grapes are considered ripped when the sugar content reaches the maximal values. For this, days needed until that point are calculated. There are five levels (1-5):

1. Very early, 105 - 135 days are needed.
2. Early, 136 - 155 days are needed.
3. Middle, 156 - 165 days are needed.
4. Late, 166 - 180 days are needed.
5. Very late, more than 180 days are needed.

Table 2. Period of Ripening

Nr	Cultivar	Start of flowering	End of flowering	Period of flowering	Evaluation of period (Code O-304)
1	Prokup	07/08	02/10	167	7
2	Melik	20/07	18/09	152	5
3	Vranac	03/08	28/09	162	6
4	Thanzi i zi	15/07	10/09	144	3
5	Thanzi i kuq	16/07	14/09	148	3
6	Pllovdin (Keci)	24/07	12/09	146	3

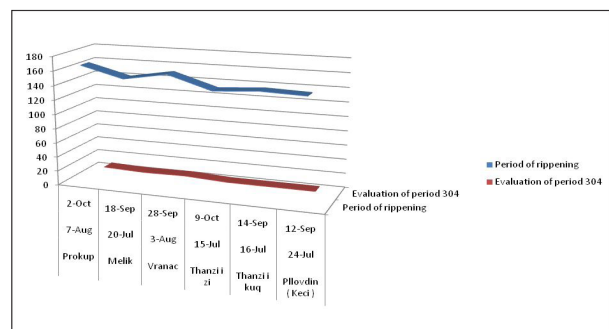


Figure 2. Period of Ripening for six cultivars of Rahovec

The data in Table 2 show that these cultivars have an early ripening (2), medium (3) and late ripening (1). As a result, they are not endangered from the rainfalls and putrefaction during the autumn months, since the temperatures fall down and rainfalls become heavier after the middle of October, time when harvesting and transport have finished.

Determination of the vegetative growth rate was conducted three times each month, from the 10th of May until the 30th of August. It results, that after the 10th of August the vegetative growth is not considerable, and that for different cultivars values are shown at table 3. Growth rate (Code O-351) is evaluated in 5 levels:

1. Very short growth, annual growth is < 100 cm.
2. Short, annual growth is 101-150 cm.
3. Middle, annual growth is 151-300 cm.
4. Long, annual growth is 301-450 cm.
5. Very long, annual growth is > 451 cm.

Table 3. The vegetative growth rate in (cm)

Nr	Cultivar	Date					Evaluation (Code O-351)
		30/5	30/6	30/7	30/8	30/9	
1	Prokup	46	132	212	242	242	5
2	Melik	41	112	161	167	168	5
3	Vranac	44	179	224	350	354	5
4	Thanzi i zi	68	280	305	341	346	7
5	Thanzi i kuq	71	267	320	336	341	7
6	Keci	52	196	270	291	296	5
Sum		323	1166	1492	1727	1747	
Mean value		52	194	248	287	291	

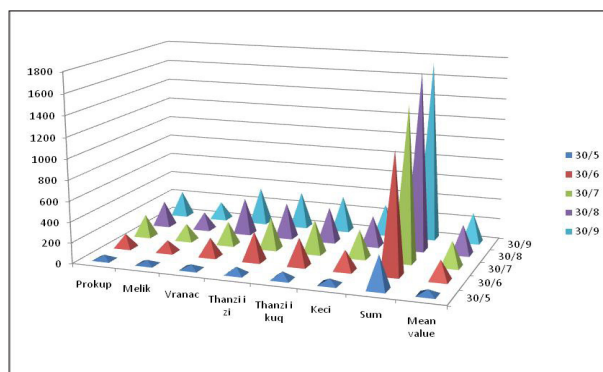


Figure 3. Comparison among the vegetative growth rate in (cm) of the six cultivars

For determination of the mean values of the biometric characteristics of leaves 10 leaves were taken from each plant at different parts. The parameters were evaluated as a mean value for each plant and then was calculated the mean value for each cultivar. These data show many differences among phenotypic characteristics of the cultivars.

The results in Table 4 are showing that cultivars develop healthy and big leaves, which prove that they are grown under favorable conditions. The dimensions and health of leaves is closely linked also to the quantity and quality of production at grapevines.

Figure 5 describes the variability of the ampelographic data, showing that the six cultivars appear different, even though values are close to each-other.

Molecular data on genetic variability of grapevine cultivars of Rahovec, based on RAPDs were used for preparation of binary matrix and to build a dendrogram of similarity among the six cultivars.

Table 4. Mean values for the biometric characteristics of the leaves (mm)

Nr	Cultivars	Length of tail of leaves	Length of leaves N ₁	Upper sinus	Tooth		Angle of leaf opening
					N ₂	N ₄	
1	Prokup	85	133	52	13	11	97
2	Melik	78	115	70	7	8	95
3	Vranac	110	151	62	19	17	92
4	Thanzi i zi	143	162	65	17	12	103
5	Thanzi i kuq	141	138	47	13	13	110
6	Plovdin (Keci)	95	142	48	14	10	98

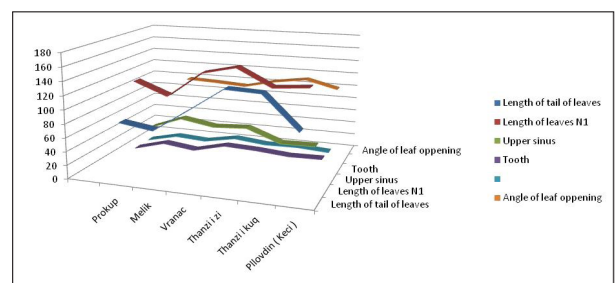


Figure 4. Mean values for the biometric characteristics of the leaves (mm) for the six cultivars

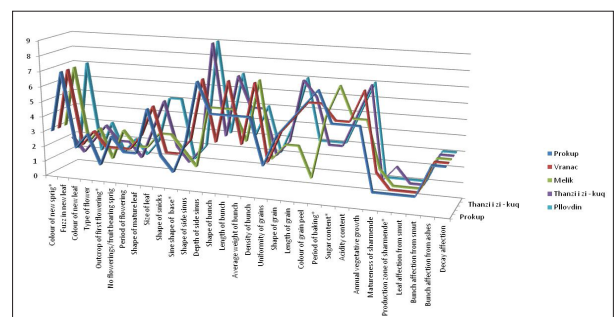


Figure 5. Variability of the ampelographic characteristics among grapevine cultivars of Kosovo based on 31 parameters according to the descriptor of IPGRI of CGIAR (Consultativ Group on International Agricultural Research)

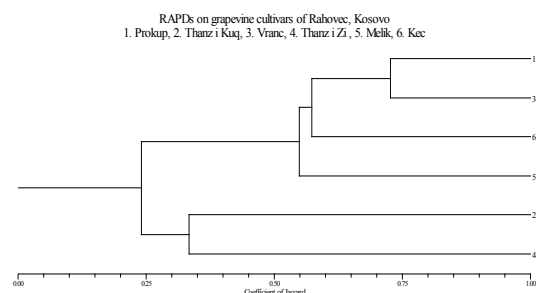


Figure 6. Dendrogram of similarity among six cultivars built from the results of the RAPDs using UPGMA clustering method of NTSYS2.1 program

The UPGMA method based dendrogram groups the six cultivars in two clusters, Prokup, Vranc, Kec and Melik first, and Thanz i kuq and Thanz i zi the second group. Based on Jaccard's coefficient they share similarities which vary from 0.25 to 0.75%, demonstrating a considerable level of variability. Table 5 describes the level of polymorphism for each of the primers used. The total number of bands produced were 76 from each 50 were polymorphic (65%), the lowest number of bands per primer were 5 and the highest 11.

Table 5. Primers, total number and polymorphic bands

No.	Primers	Total number of bands	No of polymorphic bands
1	OPB8	7	3
2	OPB9	6	2
3	OPB11	5	4
4	OPA12	10	6
5	OPB19	8	6
6	OPB20	8	6
7	P2	11	10
8	P6	9	8
9	OPB2	6	2
10	OPC15	6	3
	Total	76	50

There were no polymorphisms detected among parallel plants within each cultivar. The morphometric data displayed a level of similarity among cultivars comparable to that offered by molecular markers, which clearly differentiate the six cultivars from each other. The six main cultivars of Rahovec area differ from each other significantly, and none of them should be considered as homonymous.

4. Conclusions

- Six cultivars from Rahovec area of Kosovo were investigated, and morphological and molecular data were used to verify their uniqueness and genetic relatedness.

- Results from both morphometric and molecular analysis clarify that the six cultivars named Thanz i kuq, Thanz i zi, Melik, Kec (Pllodvin), Prokup and Vranc are not homonymous.

- Clustering of cultivars based on molecular data divides them in two main clusters, which share 25% similarity, grouping respectively Thanz i kuq and Thanz i zi in one cluster, and the rest at the second cluster.

- Based on molecular and morphometric data within clusters, cultivars Prokup and Vranc group together leaving Melik as separate branch.

- This is the first report in molecular level on the above six cultivars of Kosovo. This work will continue with other cultivars of Kosovo in order to establish genetic relatedness among those believed to be native cultivars with already known cultivars of foreign origin.

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