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Genetic diversity among twelve grape cultivars indigenous to the Carpathian Basin revealed by RAPD markers

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Summary

Twelve cultivars (*Vitis vinifera* L.) were subjected to RAPD analysis in order to estimate the genetic diversity among these genotypes and to analyse their genetic relationships. The study was performed using 28 primers that generated 120 polymorphic fragments. There was genetic variation among the cultivars with values of genetic diversity ranging from 0.419 to 0.642 using the Jaccard coefficient. UPGMA analysis of distance matrix resulted in a dendrogram with three clusters. The dendrogram shows that the cultivars of our study can be distinguished to a relatively high degree. Results were compared with the taxonomic classification and with the synonyms of the cultivars. The RAPD technique was useful for identification and discrimination of these grape cultivars.

K e y w o r d s : RAPD, *Vitis vinifera* L., Carpathian-Basin, genetic diversity, cultivar identification.

Introduction

In Hungary more than 100 indigenous grapevine cultivars can be found. The majority of the cultivars grown for centuries in the Carpathian Basin was excluded from production because of their phylloxera sensitivity. Márton Németh collected and rescued these cultivars founding a gene bank at the Research Institute for Viticulture and Enology in Pécs. Native varieties have been analysed previously using morphological data (Csepregi and Zilai 1955, 1960, 1988, Németh 1967, 1970), but the origin of many cultivars and the relationship among them remains uncertain. Classic ampelographic methods using morphological and morphometric characters are very often insufficient to identify grapevine cultivars.

In recent years molecular markers have proven to be a valuable tool for genetic studies and cultivar characterization. Isoenzyme diversity usually reveals little genetic variation (Söylemezoglu *et al.* 2001). RAPD polymorphisms among cultivars were identified neither by ampelography nor by biochemical analyses among Tunisian (Zoghlami *et al.* 2001) or Turkish grapevines (Ergül *et al.* 2002). Three molecular techniques are widely used for grapevine characterization: AFLP (Cervera *et al.* 2000, 2002), microsatellites (Ibánez *et al.* 2003, Hvarleva *et al*. 2004) and RAPD (Stavrakakis *et al*. 1997, Shubhada *et al.* 2001, Leal *et al.* 2004), but the first two techniques are time and labor intensive. The RAPD technique is fast and easy, since it does not require knowledge of the sequences of the markers and can produce abundant polymorphic fragments. RAPD analysis is one of the techniques that has been used successfully to reveal genetic variations among different plant taxa (Skoula *et al.* 1999, Echeverrigaray *et al.* 2001, Viccini *et al.* 2004, Golan-Goldhirsh *et al.* 2004).

The aim of our work was to find the most useful primers for further analysis of the cultivars indigenous to the Carpathian Basin, to identify the grape cultivars and to investigate if RAPD markers could provide systematically meaningful information.

Material and Methods

P l a n t m a t e r i a l : Twelve white grapevine cultivars (*Vitis vinifera* L.) autochthonous in the Carpathian Basin were chosen for identification. Information about their taxonomic classification indicating the supposed origin, and some of their synonyms is given in Tab. 1. According to Németh's (1967, 1970) classification, all are convarietas pontica, subconvarietas balcanica, except Királyleányka (9); this cultivar is subconvarietas georgica and supposedly a hybrid of Kövérszőlő and Leányka. Leaf material was sampled from the collection of the Research Institute for Viticulture and Enology, Pécs, Hungary.

G e n o m i c D N A e x t r a c t i o n : Fresh and young leaves were harvested and ground into a fine powder with liquid nitrogen using a sterile mortar and pestle. DNA was extracted with DNeasy Plant Mini Kit (Quiagen). DNA quality and concentration were checked with lambda DNA standards on agarose gels. A dilution test was carried out to determine the optimal amount of DNA for amplification.

R A P D a m p l i f i c a t i o n s a n d e l e c t r o p h o r e s i s : Amplification reactions were carried out in a 25 µl volume containing 40 ng of genomic DNA, 10x reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin), 100 µM of each dNTP, 0.5 µM primer, 1 U of Taq DNA polymerase. Reactions were performed in a PTC-200 thermocycler (Perkin Elmer). The cycling program included 2 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 0.5 min at 36 °C and 1 min of 72 °C, followed by a final incubation of 2 min at 72 °C. A 100 Base-Pair DNA Ladder Plus (Fermentas) was added as a molecular ruler. Amplifica-

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Table 1

Grapevine cultivars investigated in this study

Nr.	Hungarian name	Taxonomic classification	Supposed origin	Synonyms
1.	Bajor, kék (blue)	provar. mesocarpa subprovar. banatica	Banat, Serbia	Bajnár, Gohér, Augster blauer, Cerni Moslavez
2.	Bánáti rizling	provar. mesocarpa subprovar. banatica	Banat, Serbia	Kreáca, Kriáca, Creata, Zakkelweiss, Rizling banatsky
3.	Demjén	?	?	Budai gohér
4.	Ezerjó	provar. mesocarpa subprovar. tacica	Upper-Danube	Budai fehér, Hárslevelű, Kolmreifler, Tausendgute, Trummertraube
5.	Furmint, fehér (white)	provar. mesocarpa subprovar. hungarica	Hungary	Demjén, Gemeiner, Görin, Zapfner, Szigeti, Som, Moslavac, Mosler, Tokay
6.	Gohér, fehér (white)	provar. mesocarpa subprovar. hungarica	Hungary	Aranyos sárga, Ágas bajor, Bajor, Cserbajor, Hulló bajor, Guhér, Augster
7.	Hárslevelű	provar. microcarpa subprovar. zemplenica	Zemplen Mountain, Hungary	Lipovina, Lindeblattrige, Feuilles de tilleul, Garsz levelju
8.	Izsáki, fehér (white)	provar. mesocarpa subprovar. macedonica	unknown	Izsáki sárfehér, Fehér kadarka, Fehér dinka, Német dinka, Weiss-Steinschiller
9.	Királyleányka	hybrid of Kövérszőlő and Leányka	Transsylvania	Dánosi leányka, Danesdörfer-Königsast, Feteasca regale, Galbene de Ardeal
10.	Kövidinka	provar. microcarpa subprovar. sirmica	Slavonia, Croatia	Rosentraube, Werschätzer, Steinschiller, Ruzsica, Dinka rossa
11.	Mézes	provar. microcarpa subprova. carpatica	Transdanubia, Hungary	Sárfehér, Mézesfehér, Medovec, Goldtraube, Honigtraube, Bieli medenac
12.	Sárfehér	provar. mesocarpa subprovar. pannonica	Hungary	Alföldi, Ardai, Cseki, Glenovetz, Silberweiss, Sperlin

tion products were analysed on 1.5 % agarose gel electrophoresis in 0.5x TBE buffer, stained with ethidium bromide and photographed under UV light using a BioDoc-It System. From a preliminary study of 40 RAPD primers (Operon Technologies Inc.), we selected 28 primers based on the presence of polymorphism. RAPDs have often been criticized for low reproducibility; in order to avoid this phenomenon we always used highly constant conditions and all reactions were repeated at least twice. Among replicate runs inconsistencies were observed, but faint and non-reproducible bands were excluded from the analysis.

Data analysis: The positions of scorable RAPD bands were transformed into a binary character matrix ('1' for the presence and '0' for the absence of a band at a particular position), which was entered in the RAPDistance computer program (ARMSTRONG *et al*. 1994). These data were used for calculation of pairwise genetic distances among cultivars using the Jaccard coefficient. The computer program calculated the degree of genetic dissimilarity between each pair of the 12 cultivars using the simple equation: JC = 1-a/(a+b+c), where „a" is the number of bands shared by plant „x" and plant „y", „b" is the number of bands in plant „x", and „c" is a number of bands in plant „y". The Jaccard-coefficient ignores absence matches (PODANI 1997). The distance matrix was used for cluster analysis using the unweighted pair-group method with arithmetic averages (UPGMA). The dendrogram was generated using SYN-TAX 5.0 (PODANI 1993).

Results and Discussion

Twelve cultivars autochtonous in the Carpathian Basin were studied by RAPD markers in order to evaluate the degree of genetic diversity. As an initial step, a total of 40 arbitrary 10-mer primers were first screened on 6 cultivars, under the above mentioned amplification conditions. Only 28 informative primers were retained, due to their ability to produce polymorphic, unambiguous and stable RAPD markers (Tab. 2). Various banding patterns were revealed by different primers, we used only polymorphic fragments of high intensity and moderate size between 100 and 3000 bp. A minimum of 1 (OPB-08, OPG-18) and a maximum of 8 (OPA-18, OPG-06, OPG-14, OPN-05) unambiguously amplified bands were generated, with a total of 120 polymorphic bands, and with an average of 4.3 bands per primer. Examples of the banding patterns obtained are shown in Fig. 1. No single primer permitted the differentiation of all 12 cultivars.

The ability to differentiate the tested cultivars by RAPD bands suggested that this technique may provide a rapid and inexpensive method for the identification of cultivars indigenous in the Carpathian Basin, even between phenotypically similar grape cultivars (YE *et al*. 1998). The RAPD method can solve one of the major problems of varietal identification in grapevines, the existence of homonyms and synonyms, particularly with regard to varieties that have been cultivated for centuries and are widely distributed (BORREGO *et al.* 2002).

T a b l e 2

Primers used, their sequences and the number of polymorphic bands

Primer code	Nucleotid sequence (5' to 3')	Number of polymorphic bands
OPA-01	CAGGCCCTTC	3
OPA-04	AATCGGGCTG	4
OPA-05	AGGGGTCTTG	3
OPA-09	GGGTAACGCC	5
OPA-11	CAATCGCCGT	6
OPA-12	TCGGCGATAG	2
OPA-18	AGGTGACCGT	8
OPB-06	TGCTCTGCCC	2
OPB-08	GTCCACACGG	1
OPG-02	GGCACTGAGG	6
OPG-03	GAGCCCTCCA	2
OPG-06	GTGCCTAACC	8
OPG-08	TCACGTCCAC	4
OPG-10	AGGGCCGTCT	4
OPG-11	TGCCCGTCGT	3
OPG-12	CAGCTCACGA	2
OPG-14	GGATGAGACC	8
OPG-15	ACTGGGACTC	5
OPG-17	ACGACCGACA	6
OPG-18	GGCTCATGTG	1
OPN-05	ACTGAACGCC	8
OPN-11	TCGCCGCAAA	6
OPN-13	AGCGTCACTC	2
OPN-16	AAGCGACCTG	6
OPN-20	GGTGCTCCGT	4
OPO-05	CCCAGTCACT	6
OPT-20	ACACACGCTG	2
OPW-08	GACTGCCTCT	3

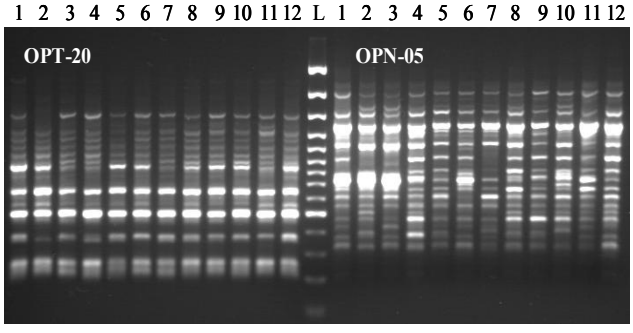

Fig. 1: Results of the 12 samples obtained with primers OPT-20 and OPN-05. Numbers indicate cultivars as listed in Tab. 1. (L=100 bp DNA Ladder Plus).

The genetic distance of each cultivar to all others is shown in Tab. 3. In case of presence-absence data, the similarity ratio can be estimated using the Jaccard coefficient (Podani 1997). The values of genetic distance ranged from 0.419 for the most closely related cultivars (Demjén (3) - Gohér (6)) to 0.642 for the most distant related cultivars (Ezerjó (4) - Kövidinka (10)). Relationships among the 12 cultivars based on their genetic distances were clustered using UPGMA analysis in a dendrogram shown in Fig. 2. The cultivars were grouped into three subclusters. The first subcluster includes Bajor (1), Demjén (3), Gohér (6), Bánáti rizling (2) and Kövidinka (10). The first three cultivars have synonyms indicating of a relationship: Bajor (1) has the name Gohér, and Demjén (3) is Budai gohér. Based on the RAPD results they are very similar in their DNA fingerprints. According to Németh (1967) Bajor (1), Demjén (3) and Gohér (6) appear rather closely related, according to their morphological similarity. Bánáti rizling (2) and Kövidinka (10) are separate from this group and have a 0.482 genetic distance value. The second subcluster includes: Furmint (5), Izsáki (8), Királyleányka (9), Mézes (11) and Sárfehér (12). In this group, too, we note similar synonyms and morphology among the cultivars. On the one hand, Furmint (5) and Királyleányka (9) are similar in many morphological characters, *e. g.* trichomes of the abaxial leaf surface, on the other hand, Furmint (5) and Kövérszőlő (parents of Királyleányka (9)) are closely related to each other (Csepregi and Zilai 1988). Izsáki (8) has the synonym Izsáki sárfehér, but Németh (1970) emphasized, that Izsáki (8) and Sárfehér (12) are not identical. The wing of Királyleányka (9) and Mézes (11) shows similar morphology. Among the synonyms of Mézes (11) names like Sárfehér can be found but they are different cultivars with a distance value of 0.455. The third subcluster includes two cultivars: Ezerjó (4) and Hárslevelű (7). Their distance value is 0.512, Ezerjó (4) having the synonym Hárslevelű.

Some contradictions can be found between clustering and synonyms; the ampelographic descriptors used the name of Demjén (3) as a synonym of Furmint (5). In our analysis these two cultivars are in different subclusters and have a genetic distance value of 0.604. According to Csepregi and Zilai (1955, 1960), Izsáki (8) and Kövidinka (10) are closely related as indicated by the synonyms of Izsáki, Fehér dinka or Német dinka. In this study they are in different subclusters but have a genetic distance value of 0.489.

The genetic distance indicates that *e.g.* the mostly related cultivars (Demjén (3) - Gohér (6)) are 42 % different or 58 % identical, and the most distant cultivars (Ezerjó (4) - Kövidinka (10)) are 64 % different and 36 % identical. According to Novy *et al*. (1994), varieties having a 100 % similarity are either identical or genetically very close. Thus, varieties with similarity percentages of more than 90 % were considered genetically close and those with less than 40 % similarity as genetically distant. Seven pairs of cultivars were similar, with similarity degrees less than 40 % similarity, 16 pairs are similar in the range 50-60 %, the others have 40-50 % similarity in their RAPD bands.

According to This *et al*. (1997), some primers seem to be more efficient than others in producing stable and reproducible DNA fingerprints. Primers yielding faint bands ought to be excluded from the analysis (Ortiz *et al*. 1997, Luo *et al*. 2001). Guirao *et al*. (1995) reported that about 45 RAPD markers should be sufficient for the establishment of genetic relationships. The selection of primers and the total number of polymorphic bands are essential for discrimina-

T a b l e 3

Genetic distance values of 12 grape cultivars

	Bajor, kék	Bánáti rizling	Demjén	Ezerjó	Furmint, fehér	Gohér, fehér	Hárs-levelű	Izsáki, fehér	Király-leányka	Kövi-dinka	Mézes	Sár-fehér
Bajor, kék	-											
Bánáti rizling	0.511	-										
Demjén	0.475	0.505	-									
Ezerjó	0.559	0.630	0.602	-								
Furmint, fehér	0.525	0.552	0.604	0.548	-							
Gohér, fehér	0.463	0.544	0.419	0.573	0.450	-						
Hárslevelű	0.543	0.552	0.536	0.512	0.512	0.523	-					
Izsáki, fehér	0.527	0.536	0.612	0.483	0.465	0.556	0.533	-				
Királyleányka	0.444	0.560	0.528	0.604	0.523	0.516	0.558	0.426	-			
Kövidinka	0.545	0.488	0.539	0.642	0.534	0.454	0.551	0.489	0.527	-		
Mézes	0.533	0.602	0.543	0.538	0.555	0.500	0.571	0.525	0.449	0.510	-	
Sárfehér	0.505	0.580	0.582	0.593	0.511	0.522	0.563	0.483	0.471	0.465	0.455	-

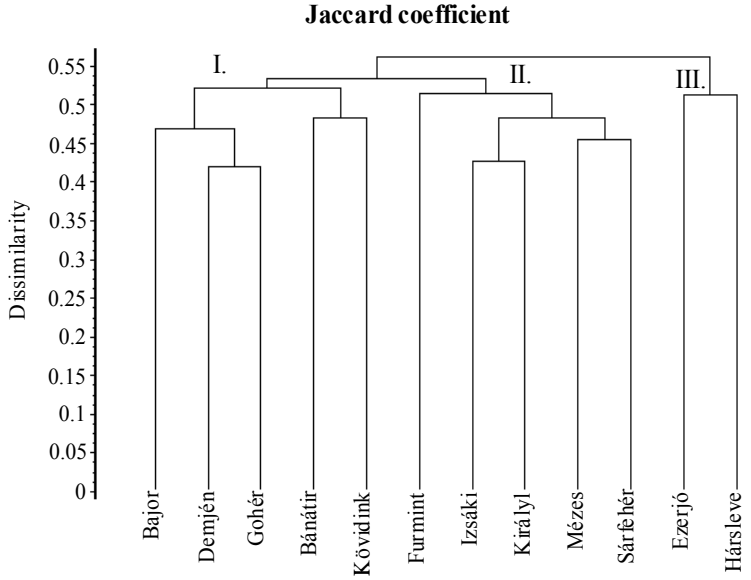

Fig. 2: Dendrogram of grape cultivars based on 120 RAPD amplification products illustrating the genetic relationship among the analysed cultivars.

tion analyses. STAVRAKAKIS *et al.* (1998) used 11 RAPD primers resulting in 115 polymorphic bands to identify and discriminate 14 Muscat grape cultivars. ERGÜL *et al.* (2002) used 110 polymorphic bands from 22 RAPD markers to identify 17 Turkish grape cultivars. Obviously, the more bands are scored and the more plants are studied the higher will be the statistical significance of the calculation. About 100 bands should be enough to obtain statistically significant results.

NÉMETH's (1967, 1970) taxonomic classification indicates the supposed origin of the cultivars (Tab. 1). About 6 cultivars originate from Hungary, Ezerjó (4) and Hárslevelű (7) from the northern part of Hungary, Mézes (11) from Transdanubia. Three of the cultivars (Bajor (1), Bánáti rizling (2) and Kövidinka (10)) originate from "south" (Serbia, Croatia), and Királyleányka (9) from "east" (Transsylvania). The taxonomic classification of Hárslevelű (7), Kövidinka (10) and Mézes (11) is provar. microcarpa, but they are in different subclucters of the dendrogram. Bajor (1) and Bánáti rizling (2) are in the same taxonomic category (subprovar. banatica), Furmint (5) and Gohér (6) are in subprovar. hungarica, but neither pair can be found in the same subcluster. We note that there is no relation between taxonomic categories or supposed origin and the dendrogram obtained by our results. We note only that the cultivars examined represent a diverse grouping of Carpathian basin cultivars.

Consistent with other results, our RAPD analysis allowed discrimination among grape cultivars (YE *et al.* 1998, VIDAL *et al.* 1999). On the basis of the RAPD profiles, the resulting distance values and the dendrogram, it can be concluded that all the cultivars of our analysis are different to a relatively high degree. We shall continue working on other

cultivars with primers having the most polymorphic patterns and with microsatellite analysis. The preservation of these grapevines is important for germplasm and breeding, for social-economic reasons and for the local cultural tradition.

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