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Differentiation and identification of grapevine accessions of Ukraine by means of molecular markers

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A molecular-genetic analysis of polymorphism in grapevine varieties, clones and new selections from the collection of the Tairov National Research Centre for Viticulture and Winemaking was conducted by using 9 microsatellite loci. The data were N-coded and analysed with the application of the MEGA 4 programme in order to build a dendrogram of the genetic relationships. The bootstrap test was used to examine fidelity of the phylogenetic trees. The molecular-genetic polymorphism was investigated using chloroplast DNA markers. Three different haplotypes were identified by cpSSR locus ccmp10.
Keywords: grapevine, SSR, genetic relationships, DNA, haplotype.

Differenzierung und Identifizierung ukrainischer Herkünfte von Weinreben mittels molekularer Marker. *Unter Verwendung von 9 Mikrosatelliten-Loci wurde eine molekulargenetische Analyse des Polymorphismus in Sorten, Klonen und neuen Selektionen von Weinreben aus der Sammlung des Tairov National Research Centre for Viticulture and Winemaking durchgeführt. Die Daten wurden N-kodiert und mittels des MEGA 4-Programms analysiert, um ein Dendrogramm der genetischen Verwandtschaftsverhältnisse zu erstellen. Um die Richtigkeit der Dendrogramme zu überprüfen, wurde ein Bootstrap-Test durchgeführt. Weiters wurden mithilfe von DNA-Markern aus Chloroplasten molekulare Verwandtschaftsanalysen durchgeführt. Drei verschiedene Haplotypen wurden durch den cpSSR-Locus ssmp10 identifiziert.*
Schlagwörter: Rebe, SSR, genetische Verwandtschaft, DNA, Haplotyp

La différenciation et l'identification de vignes d'origine ukrainienne à l'aide de marqueurs moléculaires. *Une analyse génétique moléculaire du polymorphisme des variétés, des clones et des nouvelles sélections de vignes provenant de la collection du Tairov National Research Centre for Viticulture and Winemaking a été effectuée sur la base de 9 locus microsatellites. Les données ont fait l'objet d'un codage N et ont été analysées au moyen du logiciel MEGA 4 afin d'obtenir un dendrogramme des liens de parenté génétiques. Un test bootstrap a été effectué pour vérifier la véracité des dendrogrammes. En outre, des analyses de parenté moléculaires ont été effectuées à l'aide de marqueurs ADN issus de chloroplastes. Trois haplotypes différents ont été identifiés à l'aide du locus cpSSR ssmp10.*
Mots clés : vigne, SSR, parenté génétique, ADN, haplotype

A range of Ukrainian varieties was created by means of combining traditional varieties and direct-producer varieties. Crossings were developed to create new genotypes connecting the high quality of traditional vines and resistance of direct producers. The best allochthonous and autochthonous varieties were involved in the hybridization. At present the Ukrainian range of varieties consists of more than one hundred varieties of different use, with complex indispensable agrobiological traits (high capacity of the vine and high

quality of the products, resistance to most common diseases). The total area under vine of these varieties in Ukraine is more than 10 000 hectares. Some international varieties like 'Aligoté' and 'Cabernet Sauvignon' as well as 'Cabernet franc' were introduced. 'Cabernet Sauvignon' was delivered to Russia in 1804. Whereas 'Cabernet franc' is a widely distributed variety, 'Cabernet Sauvignon' rapidly spread over the southern territory of the USSR including Ukraine, Georgia, Azerbaijan, Russia, Kazakhstan, Moldova, and Kyrgyzstan. Increasing interest in grapevine genetics and selection research as well as the introduction of new varieties require new tools for genotype identification. Ampelography and isoenzyme analysis do not completely meet modern requirements, as grapevine traits are characterized by a very high variability (Sanchez-Escribano et al., 1998). Most DNA analyses do not depend on environmental factors, the heterozygous state of a gene or the vegetation period.

SSR-analysis was recommended for genotyping and identification of varieties of grapevine (Thomas and Scott, 1993; Sefc et al., 1999). Simple Sequence Repeats (SSRs) are highly polymorphic loci of DNA containing simple tandem repeat motifs of di-, tri-, tetra- or more nucleotides. Microsatellites are widespread in the eukaryotic genomes (Morgante and Olivieri, 1993; Martínez et al., 2003). Wang et al. (1994) showed that dicotyledons had an average of one microsatellite per 21 200 bp. Dinucleotide repeats have more frequent type $(AT)_n$ tandem repeats. It is shown that 57 % of trinucleotide repeats consist mostly of GC or CG-pairs localized in non-coding regions, while other SSRs are mainly localized in the areas of the genome that are not expressed.

Microsatellite markers are characterized by high reproducibility, locus-specificity, co-dominance and polyallelic structure and high variability.

Hypervariability of microsatellites is rare and a result of frequent changes in the number of the base pair repeats or loci instability (Regner et al., 2006). High level of heterozygosity of the grape genome (69 to 88 %, according to Thomas and Scott, 1993), contributes to the possible combinations of alleles in any SSR-locus, which increases the potential of these markers.

In the last decade research of grape genome has commenced in Ukraine (Lefort et al., 2003). The development of a registration system of genetic resources based on existing DNA-markers, and the construction of a directory of genetic formulas and computer databases compose an important field for research.

The molecular-genetic analysis of the grape genome is common practice in many countries, in Ukraine, however, it has only recently started.

This work is focused on the genotyping of Ukrainian grapevine with the use of ten polymorphic SSR-loci, nine of them recommended as standard markers for identification grape genotypes for The European Vitis Database (www.eu-vitis.de) (This et al., 2004).

Material and methods

Plant material

The eleven accessions from the collection of Tairov National Research Centre for Viticulture and Winemaking were analyzed with the use of SSRs and cpSSR-markers. The analysed grapevine varieties, clones and new selections are: 'Aligoté' 1012 clone, 'Cabernet Sauvignon' 143141 clone, 'Cabernet franc' 243 clone, 'Sukholimanskiy beliy', 'Smena', 'Tairian', 'Zagrey', 'Jantar', 'Aromatniy', 'Odesskiy chorniy', 'Muscat odesskiy' (Table 1).

The studied varieties are promising and widely propagated in Ukraine and other countries as Georgia and other.

DNA extraction

The DNA was extracted from cane samples of varieties and clones of grapevine according to the protocol of Thomas and Scott (1993).

SSR analysis

The samples were analysed with nine SSR-markers and one cpSSR-marker (ccmp10) (Table 2). The following nine microsatellite loci were chosen for genotyping as a standard SSR-locus for characterization of grapevine varieties as recommended previously by This et al. (2004): VVS2 (Thomas and Scott, 1993), VVMD5, VVMD7, VVMD27 (Bowers et al., 1996), VVMD25, VVMD28, VVMD32 (Bowers et al., 1999) and VrZAG62, VrZAG79 (Sefc et al., 1999). The PCR was performed in GeneAmp PCR System 9700 (Biometra, Göttingen, Germany) as follows: 95 °C for 1:30 min, 40 cycles at 94 °C for 45 sec, 50 °C for 1:00 min and 72 °C for 1:00 min, a final extension at 72 °C for 2:00 min and a last step at 8 °C to stop the reaction.

Table 1: The basic descriptive characteristics of 11 Ukrainian grapevine accessions

Varieties	Usage characteristic	Crosses	Utility	Colour of skin	Bunch density	Total disease stability*
Aligoté 1012 clone	Clones selected by Tairov National Research Centre for Viticulture and Winemaking	Pinot noir x Gouais blanc	wine grape	green	very dense	low
Cabernet Sauvignon 143141 clone		Sauvignon blanc x Cabernet franc	wine grape	dark red-violet	dense	low
Cabernet franc 243 clone		-	wine grape	dark red-violet	medium	low
Sukholimanskiy beliy	Varieties bred by Tairov National Research Centre for Viticulture and Winemaking and propagated in Ukraine	Chardonnay x Plavay	wine grape	green	medium	medium**
Odesskiy chorniy		Alicante Bouschet x Cabernet Sauvignon	wine grape	dark red	friable	medium**
Tairian	New promising varieties bred by Tairov National Research Centre for Viticulture and Winemaking	45-35-31 x Vostorg	table grape	yellow red	dense	high
Zagrey		Ovidiopolskiy x Muscat rozoviy	wine grape	yellow	medium	high
Jantar tairovskiy		Zagadka x Vostorg	table grape	yellow	medium	high
Aromatniy		Vertish chilaga x Romulus	wine grape	yellow red	dense	high
Smena		Dattier St. Vallier x Decorativniy	table grape	green	friable	high
Muscat odesskiy		Muscat siniy ranniy x Pierrel	wine grape	green yellow	dense	high

*Oidium, downy mildew, berry rot
** Under conditions of the Northern part of the Black Sea Region

Table 2: Common characteristics of the used microsatellite loci (genetic descriptors of OIV)

Name of locus	Primer sequences (5'-3')	No. of alleles	Size of DNA-fragment, bp	Linkage group	Repeat
VVS2	AAATTCAAAATTCTAATTCAA CAGCCCGTAAATATATCCATC	19	123-161	11	(AG)n
VVMD5	CTAGAGCTACGCCAATCCAA TATACCAAAAATCATATTCCTAAA	8	224	16	(CT)3AT(CT)11ATAG(AT)n
VVMD7	AGAGTTGCGGAGAACAGGAT CGAACCTTCACACGCTTGAT	17	232-266	7	(CT)n
VVMD27	GTACCAGATATGAATACATCCGTAAGT ACGGGTATAGAGCAAACGGTG	23	175-219	5	(CT)n
vrZAG62	GGTGAAATGGGCACCGAACACACG CCATGTCTCTCCTCAGCTTCTCAGC	18	174-220	7	(GA)n
vrZAG79	AGATTGTGGAGGAGGGAACAAACCG TGCCCCCATTTTCAAACTACTCCCTTCC	14	238-264	5	(GA)n
VVMD28	AACAATTCAATGAAAAGAGAGAGAGAGA TCATCAATTTCGTATCTCTATTTGCTG	16	221-279	3	(CT)n
VVMD25	TTCCGTTAAAGCAAAAGAAAAAGG TTGGATTTGAAATTTATTGAGGGG	11	243-275	11	-
VVMD32	TATGATTTTTTAGGGGGGTGAGG GGAAAGATGGGATGACTCGC	11	239-273	4	-
ccmp10	TTTTTTTTTAGTGAACGTGCA TTCGTCGTCGTAGTAAATAG	4	91-300	-	-

The PCR reaction mixture contained: 5 ng total DNA, 2 µl PCR-buffer solution x 10, which consisted of 200 mM Tris-HCl, pH 8.55, 160 mM $(NH_4)2SO_4$, 0.1 % Tween 20, 20 mM $MgCl_2$, 0,1 µl of dNTP solution, and 1.0 µl reverse and 1.0 µl forward primer (0,5 µl of forward primer was labelled with the fluorescent dyes (IR-Dye 700 and IR-Dye 800), and sterile water to 20 µl final volume.
The alleles were detected on a LI-COR Biosciences 4300 DNA Analyzer, LI-COR, Inc., Licor Inc., Lincoln Nebraska US by using the program SAGA Generation 2, Oracle Corporation, Licor Inc., Lincoln Nebraska US.
According to the modified CTAB method and PCR analysis of chloroplast microsatellite locus ccmp10 have been done. The size of amplified fragments was approximately identified by the use of PAGE electrophoresis with size standard of 50 bp ladder (Promega).

Data analysis

According to This et al. (2004) SSR data are comparable with database SSR profiles as soon as they become standardized. Average heterozygosity or polymorphism level (H_e) of each studied SSR-loci given by:
$H_e = 1 - \sum p_i^2$,
where p_i – phenotyping frequency of *i* allele per each SSR-loci (Sivolap, 1998).
The genetic relationships between analysed genotypes were performed with the use of MEGA 4 programme (Tokyo, Japan) in order to build the dendrogrames by UPGMA clustering method (Nei and Li, 1979; Koichiro et al., 2007).

Results and Discussion

Identity and parentage

All the accessions of the grapevine varieties from the collection of Tairov National Research Centre for Viticulture and Winemaking were firstly genotyped by 9 microsatellite loci (Table 3).

Allele size and frequency

Statistical assay was obtained by allele frequency, quantity of homo- and heterozygous alleles, and homozygosity. The microsatellite analysis of the used SSR-loci showed a high level of polymorphism. The average allele number per locus is higher than 7 (7.11). The average heterozygosity or polymorphism level (H_e) of the nine studied loci was 0.79. The highest level of polymorphism was shown for the loci VRZAG79 (11 alleles) and VVS2 (9 alleles) which is in good accordance to literature (Table 2) (http://www.ncbi.nlm.nih.gov/sites/entrez). The grapevine genome is characterized by a high level of heterozygosis. It was shown that an observed heterozygosis by the SSR-loci of grape varieties varies from 69 % to 90.9 % (Doligez et al., 2002; Martin et al., 2003; Adam-Blondon et al., 2004).
Allele lengths were transformed into the N-coding system proposed by This et al. (2004) (Table 4). This system allowed to compare the data of SSR-analyses that had been obtained in different laboratories using different protocols, and it was useful for identification and differentiation of grapevine genotypes. The authors offered a strategy for comparison of the data by means of standard alleles of widespread varieties. For this reason genetic profiles were developed and should become published in the European Vitis Database.

Inheritance

The data of polymorphism (9 SSR-loci) were coded and analysed using MEGA 4 program for getting dendrogrames (Fig. 1).
In order to improve the reliability of the results of cluster analysis a bootstrap-test was conducted (Fig. 2). The bootstrap method is most often used to test the fidelity of phylogenetic trees. It confirmed the data obtained by clustering and divided samples into two subclusters.
In the dendrogram, involved varieties are divided into two clusters. The first cluster is formed mainly by wine varieties, except table variety 'Tairian'. The second cluster is formed mainly by table varieties, except the technical varieties 'Muscat odesskiy' and 'Aromatniy'. Interestingly a dendrograme obtained by 10 SSR-loci with chloroplasts ccmp10-marker does not show differences to the previous one. But the variety 'Tairian' was closer to the group of variety 'Sukholimanskiy beliy', 'Cabernet Sauvignon' and 'Odesskiy chorniy' (data not shown).
The first cluster is formed by the varieties with mainly close inheritance. The variety 'Sukholimanskiy beliy' is the result of a cross between 'Chardonnay' and 'Plavay' (Moldovan variety of white grape). The variety

Table 3. Ukrainian accessions with molecular profiles at 9 microsatellite loci

Varieties	VVS2	VVMD5	VVMD7	VVMD27	VRZAG62	VRZAG79	VVDM25	VVMD28	VVMD32
Aligote 1012	132 136	226 238	237 237	178 188	192 194	240 242	239 239	227 235	238 238
Cabernet Franc 243	138 146	224 238	237 261	180 188	192 202	244 256	239 255	227 235	238 256
Sukholimanskiy beliy	132 150	226 230	237 255	174 188	186 192	242 244	241 249	233 235	238 254
Smena	134 142	234 236	239 247	180 188	182 186	256 258	255 255	233 243	254 256
Tairjan	134 134	234 238	237 247	178 184	186 192	236 248	239 249	233 257	238 238
Cabernet Sauvignon143141	138 150	230 238	237 237	174 188	186 192	244 244	255 255	233 235	238 238
Zagrey	124 136	226 234	237 245	178 182	192 202	240 252	239 249	226 243	234 234
Jantar	134 148	224 236	245 247	184 192	184 194	252 254	249 255	243 243	238 254
Aromatniy	132 142	224 230	245 247	180 188	202 202	240 258	241 255	233 247	238 272
Odesskiy chorniy	132 150	234 244	237 241	188 192	186 186	244 254	241 249	235 243	238 238
Muscat odesskiy	132 142	234 234	245 247	184 188	- -	250 260	- -	233 267	236 272

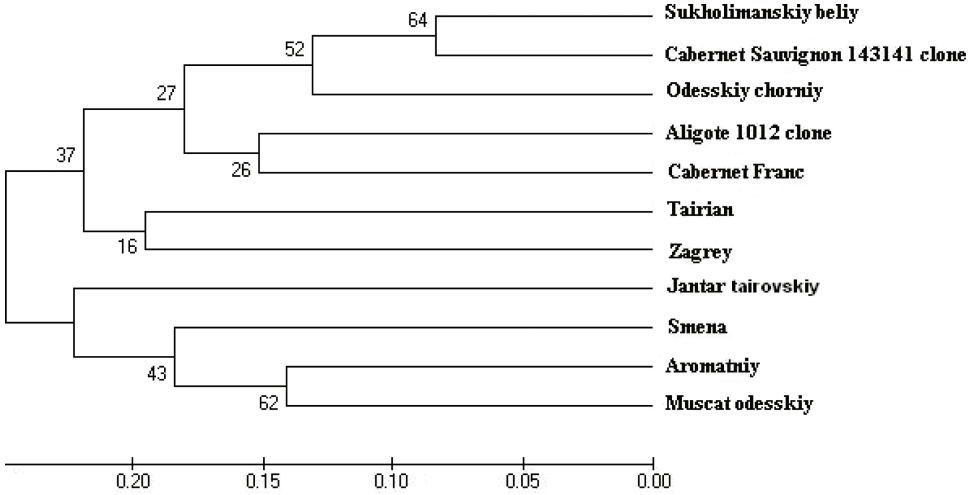

Fig. 1: Dendrogram of genetic relationships between investigated grape varieties constructed according to genetic distances that have been calculated on the database of SSR-analysis by using UPGMA method (figures near the bifurcation branches - the authenticity of the selection of the cluster)

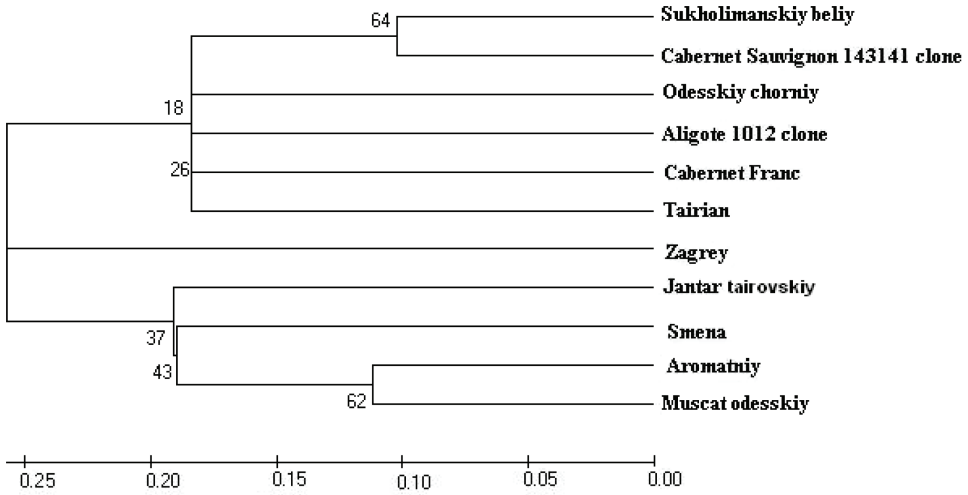

Fig. 2: Tree illustrating genetic distance obtained according to bootstrap test

Table 4: Transformed data of SSR-analysis to N-code

Sample	VVS2	VVMD5	VVMD7	VVMD25	VVMD28	VVMD32	VVMD27	VRZAG62	VRZAG79
Aligote 1012	N+10 N+14	N+4 N+16	N+6	N	N+10 N+18	N+2	N+2 N+12	N+16 N+18	N N+2
Cabernet franc 243	N+16 N+24	N+2 N+16	N+6 N+30	N N+16	N+10 N+18	N+2 N+20	N+4 N+12	N+16 N+26	N+4 N+16
Sukholimanskiy b.	N+10 N+28	N+4 N+8	N+6 N+24	N+2 N+10	N+16 N+18	N+2 N+18	N-2 N+12	N+10 N+16	N N+4
Smena	N+12 N+20	N+12 N+14	N+8 N+16	N+16	N+16 N+26	N+18 N+20	N+4 N+12	N+6 N+10	N+16 N+18
Tairian	N+12	N+12 N+16	N+6 N+16	N N+10	N+16 N+40	N+2	N+2 N+8	N+10 N+16	N-4 N+8
Cab. Sauv. 143141	N+16 N+28	N+8 N+16	N+6	N+16	N+16 N+18	N+2	N-2 N+12	N+10 N+16	N+4
Zagrey	N+2 N+14	N+4 N+12	N+6 N+14	N N+10	N+8 N+26	N-2	N+2 N+10	N+16 N+26	N N+12
Jantar	N+12 N+26	N+2 N+14	N+14 N+16	N+10 N+16	N+26	N+2 N+18	N+8 N+16	N+8 N+18	N+12 N+14
Aromatniy	N+10 N+20	N+2 N+8	N+14 N+16	N+2 N+16	N+16 N+30	N+2 N+36	N+4 N+12	N+26	N N+18
Odesskiy chorniy	N+10 N+28	N+12 N+22	N+6 N+20	N+2 N+10	N+18 N+26	N+2	N+12 N+16	N+10	N+4 N+14
Muscat odesskiy	N+10 N+20	N+12	N+14 N+16	- -	N+16 N+50	N N+36	N+8 N+12	- -	N+10 N+20

'Aligoté' was obtained from a crossing of 'Pinot noir' and 'Gouais blanc' like the variety 'Chardonnay', that was the result of a cross between the 'Pinot noir' and 'Gouais blanc' ('Heunisch') (Table 1). So the genetic distance between 'Sukholimanskiy beliy' and 'Aligoté' 1012 is 0.1801.

The variety 'Odesskiy chorniy' (Synonym: 'Alibernet') was bred in 1950 in the Tairov institute from crossing of 'Alicante Bouschet' x 'Cabernet Sauvignon'. That can explain the low genetic distance between variety 'Odesskiy chorniy' and 'Cabernet Sauvignon' 143141 (0.1313).

The variety 'Cabernet Sauvignon' was a result of crossing 'Cabernet franc' x 'Sauvignon blanc', and in this case 'Cabernet Sauvignon' 143141 is close to 'Cabernet franc' 243 on the dendrogram with the genetic distance 0.1801.

Polymorphism of chloroplasts DNA

Simple sequence repeats of the chloroplast genome are often used for defining genetic relationships between different grapevines. The rarity of mutations in chloroplast genomes allows using this character as a strong phylogenetic marker. The chlorotype polymorphism is based in many aspects on evolutionary population biology, as organelle genomes are typically non-recombinant, uniparentally inherited and effectively haploid (Grassi et al., 2002).

Allelic variation by a polymorphic cpSSR locus was used to characterize haplotype diversity between new selection lines from our collection. A molecular-genetic analysis of polymorphism of eleven accessions involved in breeding programs was performed.

A genetic polymorphism of this chloroplast marker has been revealed among investigated accessions. Three polymorphic chlorotypes were observed: 1) 113 bp – 'Aligoté'; 2) 114 bp – 'Sukholimanskiy beliy', 'Tairian', 'Cabernet Sauvignon', 'Zagrey', 'Jantar', 'Aromatniy', 'Odesskiy chorniy'; 3) 115 bp – 'Cabernet franc', 'Smena', 'Muscat odesskiy'.

The 'Cabernet Sauvignon' 143141 does not have the same chlorotype with the parental variety 'Cabernet franc'. 'Cabernet Sauvignon' inherited chloroplasts from the mother variety 'Sauvignon blanc'. In fact it is shown that the chloroplast markers are useful for differentiation of varieties and identification of mother plants.

The obtained results show the possibility of using SSR markers for identification and differentiation of grape genotypes by alleles of 9 SSR-loci of the nuclear genome and one SSR locus of the chloroplast genome.

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