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PhD Thesis

Determination of microsatellite based fingerprints and pedigree analyses of grapevine varieties

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RESEARCH BACKGROUNDS AND OBJECTIVES

Grapevine is one of the most important crops cultivated worldwide. The world's grapevine area totals some 8 million hectares, 68% of which is found in Europe.

Grapevine and the wine obtained from it have been known and preferred since prehistoric times. However, its introduction into cultivation is the result of a long process.

The cultivation of grapevine was at a high level in the Armenian Empire already as early as around 2000 B.C. It was probably the place where wine grape (*Vitis vinifera* L.) was developed from wild grape (*Vitis sylvestris* GMEL.). There are written records evidencing that grapevine was already grown and cultivated in large gardens around human settlements at that time.

Thousands of years of grapevine cultivation have resulted in the creation of thousands of grapevine cultivars. However, cultivar structure is hard to determine partly because of the large number of existing varieties and partly because of the fact that the cultivars introduced into new geographical areas were often given new names leading, in many cases, to the same cultivar having two separate names. As the old grapevine cultivars have numerous synonyms, it is very difficult to eventually find the original cultivar on the basis of names alone. The works of varietal characterization speeded up at the start of the 20th century along with the development of ampelography, mostly on the basis of geographical location and morphology.

Identification at DNA level was practiced first with the RFLP method and later, when the PCR technique was already available, through the use of chance-based, gene-specific and microsatellite primers.

Microsatellite or SSR (Simple Sequence Repeats) fingerprints have become an efficient tool for the molecular description of grapevine cultivars since 1993, the year when Thomas and Scott identified the first microsatellite sequences suitable for the genotyping of grapevine cultivars.

Microsatellite markers are used not only for cultivar identification but also for the discrimination of clones and for the verification of synonyms and homonyms. Due to their locus specificity and Mendelian codominant inheritance, the microsatellites can be used for the pedigree identification of grapevine cultivars. The parent-progeny relationships may be clearly identified even if the actual or assumed crossing partners are heterozygous in the given microsatellite locus, because the diploid progeny will receive one allele from one parent and the other allele from the other parent.

Microsatellite analysis was used to find out the origin of such well-known international cultivars as ’Cabernet Sauvignon’, ’Chardonnay’ or ’Müller-Thurgau’. Furthermore, SSR analysis was used to identify the origin of several important local cultivars such as ’Posip bijeli’ in Croatia, ’Ansonica’ in Italy and ’Cornalin du Valais’ in Switzerland.

The characterization of grapevine cultivars with microsatellite DNA markers was started in Europe between 1997 and 2002 within the framework of an international cooperation called GENRES 081. For the purpose of cultivar characterization 6 microsatellite primer pairs were identified and proposed.

In Hungary this work was performed in a cooperation between the Institute of Viticulture and Enology and the Institute of Genetics and Biotechnology, producing the microsatellite fingerprints of more than 100 cultivars (Carpathian Basin, international, gene bank and Hungarian cultivars). Within the framework of GrapeGen06, the European cooperation launched as a follow-up to the GENRES 081 project, additional DNA microsatellite markers were involved in the studies. The study of varieties indigenous to foreign countries located in the vicinity of the grapevine genetic center but not part of the European Union yet is also an important part of this program.

The “world cultivars” such as ’Cabernet franc’, ’Cabernet Sauvignon’, ’Chardonnay’, ’Merlot ’ or ’Rajnai rizling’ have been gaining ground to an increasing extent in today’s grape production. Due to the strengthening demand of international markets for well-known cultivars, the varieties of the small regions are gradually ignored. This will inevitably lead to the simplification of the available range of cultivars. Among the countless grapevine varieties cultivated currently, the domestic ‘hungaricum’ cultivars have been losing their importance.

It would be an important task to maintain the indigenous Hungarian cultivars and keep them in cultivation. The collection found in Pécs was established thanks to the classification work started by Márton Németh in 1967 focusing on the search for grapevine cultivars indigenous to the Carpathian Basin and cultivated there for centuries. Today the catalogue of the gene bank kept at the Institute of Viticulture and Enology lists more than 1400 cultivars.

Hungarian cultivars, international cultivars and cultivars cultivated for centuries (autochtonous to) the Carpathian Basin were involved in our study performed with microsatellite markers for the genotyping of grapevine cultivars.

Using 6 microsatellite markers, we were able to identify the DNA fingerprints of 101 cultivars until 2005, resulting in the proper discrimination of these cultivars from each other.

Objectives

1. Molecular characterization, SSR fingerprinting and origin identification with 6 additional markers in the case of cultivars indigenous to or cultivated for centuries in Hungary kept at the gene bank in Pécs.
2. Identification of the potential parent-progeny relationships and the genetic distance of grape cultivars of unknown pedigree.
3. Verification of the origin of ’Csabagyöngye’ and ’Királyleánka’ and the parent-progeny relationship of ’Mátrai muskotály’ and ’Irsai Olivér’ through the involvement of additional microsatellites for pedigree identification.
4. Identification of homonyms and synonyms.
5. Dendrogram construction to demonstrate the relationship between the studied cultivars.
6. DNA “barcode” construction in order to simplify the comparison of microsatellite allele sizes with those of other laboratories.

MATERIAL AND METHOD

Plant material

The study was carried out with 115 grapevine cultivars obtained from the Institute of Viticulture and Enology.

These cultivars were classified in the following 5 groups:

- Cultivars indigenous to the Carpathian Basin (86)
- Central Asian cultivars (6)
- Gene bank cultivars (5)
- Cultivars bred in Hungary (8)
- International cultivars (10)

Method

DNA extraction

DNA was isolated from young grape leaves with DNeasy® Plant mini kit (Qiagen, Biomarker Kft., Gödöllő) according to the manufacturer’s protocol.

PCR cobnditions and microsatellite analysis

The PCR reaction was performed in a final volume of 25 µl in Bio-Rad iCycler apparatus. As template, the reaction mixture contained the following: 10-20 ng of genomic grape DNA, 10 pM of forward and 10 pM of reverse microsatellite primer, 2.5 µl of 10x buffer, 2 mM of $MgCl_2$, 75 µM of dNTP and 1.2 U of WestTeam *Taq* polymerase (WestTeam BioTech, Pécs). The reaction conditions were as follows: 2 minutes at 94˚C, followed by 40 cycles of 10 seconds at 94˚C, 30 seconds at 57˚C, 90 seconds at 72˚C, followed by a final incubation of 5 minutes at 72˚C. The microsatellite analyses were carried out according to Halász *et al.* (2005). The forward primers used for PCR reactions were labeled with CY-5 fluorescent dye (Metabion, Merck Kft., Budapest).

Molecular markers used for the study

The fluorescent-labeled primers used for microsatellite analyses were as follows: Scu08vv, Scu10vv, ssrVrZAG47, ssrVrZAG62, ssrVrZAG79, ssrVrZAG83, ssrVrZAG112 (Scott *et al.* 2000), VVMD21, VVMD25, VVMD28, VVMD31, VVMD36 (Bowers *et al.* 1996, 1999). The ssrVrZAG62 and ssrVrZAG79 markers and the VVMD28 marker were selected on the basis of proposals from the GENRES 08 project and GrapeGen06 project, respectively, while the other microsatellite markers were chosen in reliance of the primer tests of our own studies.

ALF- Automatic Laser Fluorescent analyses

The PCR products were separated on a 8% denaturing polyacrylamide gel (Reprogel, GE Healthcare Bio Sciences, AP Hungary Kft., Budapest). The allele sizes were determined with ALFexpress II DNA analyzer (Amersham Biosciences, AP Hungary Kft., Budapest) using ALFexpress™ sizer as a molecular weight standard and Alwin Fragment analyzer software.

Evaluation of allele size data

Data analysis with Identity 1.0 software

Identity 1.0 (Wagner and Sefc 1999) software was used to verify the probability of parent-progeny relationships. Identity 1.0 is suitable mostly for the statistical analysis of allele size data. This software prepares a list of the probable parent-progeny relationships, based on codominant inheritance i.e. when the progeny receives one allele from one parent and the other allele from the other parent.

K-means clustering algorithm

The number of clusters is known in advance. First the data are partitioned into "k" number of "not empty" categories (in this case 3 categories for each) and then each observation is assigned to the nearest mean. These steps are repeated until a category contains the cultivars that most resemble each other in terms of the studied loci. This method is suitable for the establishment of cultivar groups characterized with common allele size data in 12, 11, 10 and 9 microsatellite loci.

Dendrogram construction with SPSS 11.0 for Windows software

First the data were converted into binary codes and then inserted into the table of the SPSS 11.0 for Windows software in order to construct the dendrogram.

Calculation of the probability (p-value) of family relationships with SPSS 11.0 for Windows software

Probability values were calculated to support the family relationships between cultivars grouped into pairs through k-means analysis. The p-value ranges from 0 to 1. If it is 0, there is no correlation between the two cultivars. If it is 1, there is a full correlation. A p-value above 0.05 justifies the acceptance of the H_0 hypothesis, which means that there is a correlation between two cultivars grouped into pairs.

RESULTS

Microsatellite allele patterns of grapevine cultivars

In 106 cases the analysis of 115 cultivars resulted in unique and discriminative microsatellite fingerprints (Table 1 and Table 2), which means that the cultivars can be safely characterized on the basis of SSR allele structure. Only the berry color variants of 'Piros muskotály', 'Sárga muskotály', 'Bakator' (piros-tüdőszín), 'Gohér' (piros-fehér-változó) and 'Lisztes' (piros-fehér) cultivars matched the allele patterns of the selected 12 primer pairs. Similar results were reported by Sefc *et al.* (2000) regarding the study of 100 cultivars with 10 SSR primers, where the color variants could not be discriminated.

The same allele sizes were found also for 'Fodroslevelű' and 'Betyárszőlő'. In the case of other genotypes these cultivars could not be discriminated even with polymorphic microsatellite primers. This may be due to some record-keeping error.

In all 12 loci the allele sizes of the Central Asian cultivars were in the same range as those of the Carpathian Basin cultivars. No family relationships were found between the 6 cultivars involved in the study. As to their allele size data, they showed a great variability and no cultivar of any other group showed matches in 9-12 loci.

Analysis of parent-progeny relationships with Identity 1.0 software

The study used reference cultivars of known pedigree, where the parent-progeny relationships were supported either by breeding data ('Irsai Olivér', 'Mátrai muskotály') or by microsatellite analyses ('Chardonnay'). Applied to microsatellite allele size data, the Identity 1.0 software identified 32 potential parent-progeny combinations.

1. Betyárszőlő = Fodroslevelű x Kéklöpiros
2. Betyárszőlő = Fodroslevelű x Tökszőlő
3. Bihari = Ágasfark x Fürjmony
4. Bihari = Fürjmony x Vékonyhéjú
5. Furmint = Ágasfark x Balafánt
6. Furmint = Balafánt x Gorombaszőlő
7. Furmint = Balafánt x Kovácskréger
8. Gorombaszőlő = Kadarka x Ködös
9. Kadarka = Gorombaszőlő x Tótika
10. Kövérszőlő = Királyszőlő x Heunisch weiss
11. Lányszőlő = Bánáti rizling x Furmint
12. Lányszőlő = Bánáti rizling x Kadarka
13. Lányszőlő = Bánáti rizling x Szerémi
14. Lisztes fehér = Csomorika x Rókafarkú
15. Lisztes fehér = Juhfark x Szagosbajnár
16. Lisztes piros = Csomorika x Rókafarkú
17. Lisztes piros = Juhfark x Szagosbajnár
18. Piros muskotály = Pécsi szagos x Sárga muskotály
19. Piros muskotály = Sárga muskotály x Alexandriai muskotály
20. Rakszőlő = Járdovány x Vörösdinka
21. Tökszőlő = Betyárszőlő x Lisztes fehér
22. Tökszőlő = Betyárszőlő x Lisztes piros
23. Tökszőlő = Fodroslevelű x Lisztes fehér
24. Tökszőlő = Fodroslevelű x Lisztes piros

25. Tükörszőlő = Ágasfark x Heunisch weiss

26. Ürömidinka = Bakarka x Izsáki

27. Vékonyhéjú = Ágasfark x Heunisch weiss

28. Vékonyhéjú = Bihari x Heunisch weiss

29. Mátrai muskotály = Izsáki x Ottonel muskotály

30. Irsai Olivér = Pozsonyi x Csabagyöngye

31. Chardonnay = Pinot noir x Heunisch weiss

32. Csabagyöngye = Szőlőskertek királynője x Madeleine Angevine

As the applied 12 microsatellite markers did not discriminate the berry color variants and the 'Fodroslevelű' and 'Betyárszőlő' cultivars (Galbács *et al.* 2009), the members of these pairs are listed in the same combinations. The combinations representing a verified pedigree are highlighted in the list.

Our results clearly confirm the origin of 'Mátrai muskotály' and 'Irsai Olivér'. Accordingly, 'Mátrai muskotály' originates from the crossing of 'Ottonel muskotály' x 'Izsáki' crossing (Hajdu 2003, Kiss *et al.* 2005), while 'Irsai Olivér' is the product of 'Pozsonyi fehér' x 'Csabagyöngye' (Hajdu 2003, Kiss *et al.* 2005). The origin of 'Chardonnay' was also proved through SSR analysis (Bowers *et al.* 1999). Our results are also in accordance with the 'Gouais blanc' x 'Pinot noir' pedigree, where 'Gouais blanc' is identical with a cultivar known as 'Heunisch weiss' in Hungary.

However, there are also counter-examples, as in some cases the microsatellite data contradict the assumed parent-progeny relationships. One of such counter-examples is 'Királyleányka' which is assumed to be a spontaneous hybrid of 'Kövérszőlő' and 'Leányka' (Csepregi and Zilai 1988). Our statement is confirmed also by Bisztray *et al.* (2005) and Jahnke *et al.* (2007) who exclude the parent-progeny relationship of 'Kövérszőlő' x 'Leányka' = 'Királyleányka'. Our SSR data did not confirm the assumed pedigree of 'Csabagyöngye' either, according to which this early cultivar originates from the 'Bronnerstraube' x 'Ottonel muskotály' crossing (Csepregi and Zilai 1988, Hajdu 2003). This option is not included at all on the list of potential origins obtained with the Identity 1.0 software. The combination of 'Csabagyöngye' = 'Szőlőskertek királynője muskotály' x 'Madeleine Angevine' shown in Section 32 indicates a relationship between these cultivars. 'Szőlőskertek királynője' was created by János Mathiász in 1916 with the crossing of 'Erzsébet királyné emléke' and 'Csabagyöngye' (Csepregi and Zilai 1988, Kozma 1972) and, according to several authors, 'Madeleine Angevine' may be one of the parents of 'Csabagyöngye'. This assumption is also confirmed by the microsatellite results. The list results derive from the similarity grouping of

alleles, as the Identity 1.0 software assigns to each cultivar only the two most similar partners that are characterized with the same allele size data in all 12 loci. The combinations obtained with the software require further analyses as a pedigree can be safely identified with the involvement of at least 30 loci, instead of the 12 loci used in our study. In the case of 'Irsai Olivér' and 'Mátrai muskotály' we have identified the data of more than 30 loci.

Assessment of parent and half-sibling relationships with SPSS k-means analysis

Efforts were made to reveal more family relationships through further data analyses. We used the k-means cluster analysis of SPSS 11.0 for Windows, classified the studied cultivars into groups and narrowed down the groups to create associations of the cultivars that showed the highest level of similarity in the studied 12 loci.

Full match in all 12 loci was found in a total of 29 cases. The relationships were also verified with probability calculations. The applicability and reliability of the method is confirmed by the first ten cultivar pairs as their parent-progeny relationships are also supported by literature data:

1. **Chardonnay - Heunisch weiss** **p=0.581**
2. **Chardonnay - Pinot noir** **p=0.502**
3. **Csabagyöngye - Madeleine Angevine** **p=0.582**
4. **Irsai Olivér - Csabagyöngye** **p=0.702**
5. **Irsai Olivér - Pozsonyi** **p=0.523**
6. **Kossuth - Madeleine Angevine** **p=0.491**
7. **Leányka - Királyleányka** **p=0.502**
8. **Mátrai muskotály - Izsáki** **p=0.579**
9. **Mátrai muskotály - Ottonel muskotály** **p=0,662**
10. **Szőlőskertek királynője - Csabagyöngye** **p=0.582**

The p-values calculated for the various cultivar pairs also confirmed the correlation between the cultivars in each case. Being always greater than 0.05, the p-values provided a basis also for the acceptance of the H_0 hypothesis.

With regard to the 115 cultivars, the Identity 1.0 software revealed parent-progeny relationships in 32 cases, while the K-means cluster analysis showed a match between the "cultivar pairs" for all 12 loci in 29 cases, 11 loci in 20 cases, 10 loci in 40 cases and 9 loci in 42 cases. Although such level of similarity between the allele size data may represent family relationships, both the probability of the pedigrees obtained with the Identity 1.0 software and

the reliability of the statistical results regarding half-sibling relationships require further studies and technical considerations.

Identification of homonyms and synonyms

According to the available data, 'Leánynka' and 'Leányszőlő' represent two different genotypes, which means that they are not synonyms (Csepregi and Zilai 1988). However, 'Betyárszőlő' and 'Fodroslevelű' seem to be synonyms of each other despite the fact that they have been referred to in literature as two distinct cultivars since the late 13th century (Csoma 1994). Further studies will be required to determine whether they are in fact synonyms or they were only thought to be synonyms due to some record-keeping error.

As to the cultivar 'Bakator kék', the name 'Bakator' is a homonym since 'Kék bakator' is clearly distinct from 'Piros bakator' and 'Tüdőszínű bakator' that have fully identical SSR patterns.

Description of family relationships with dendrogram

In addition to the high level of polymorphism and the reliability of analysis, the advantages of the widespread use of SSR markers include a possibility for the quantification of the exact fragment sizes. For the 115 cultivars the 12 SSR primer pairs produced a total of 2760 allele sizes. Although these objectively characterize the genotypes and are really suitable for varietal discrimination, they require in-depth study as only a pair-by-pair comparison is able to find out whether the allele sizes match or differ in the various loci.

The dendrogram constructed for the description of family relationships proves that the 12 microsatellite markers can be efficiently used for varietal discrimination in most of the cases. At the same time, the dendrogram visibly displays the similarities and also the indiscriminability of certain genotypes. However, the "visibility" of parent-progeny relationships is limited in the dendrogram. 'Irsai Olivér' was grouped in the same subcluster only with 'Csabagyöngye', while 'Chardonnay' only with 'Heunisch weiss'. Nevertheless, the family relationship between 'Madeleine Angevine' and 'Csabagyöngye' can be confirmed by means of the dendogram. The dendrogram reflects the parent-progeny relationship, confirmed by breeder notes and evidenced with SSR data, only in the case of '**Mátrai muskotály**' = '**Izsáki** ' **x** '**Ottonel muskotály**' where all three cultivars (parents and progeny) were grouped in the same cluster. The dendrogram classified the 115 grapevine cultivars in three large groups. The berry color variants of 'Piros muskotály', 'Sárga muskotály', 'Bakator' (piros-tüdőszín), 'Gohér' (piros-fehér-változó) and 'Lisztes' (piros-fehér) cannot be discriminated in the dendrogram. As to the cultivar 'Bakator kék', the name 'Bakator' is a homonym.

One of the greatest advantages of SSR allele size data is that they can be easily digitized. The data were used for the construction of cultivar-specific barcodes allowing us to see at first sight that the microsatellite markers do really produce the unique DNA fingerprints of the genotypes.

Each bar on the DNA barcode corresponds to a certain allele size. A lower index (2) was used on the barcode to indicate the overlaps i.e. where the same value was found in two different loci. In most of the cases such overlaps were detected between VrZag62 and VrZag83.

The display of microsatellite allele sizes in this form represents an easily understandable and manageable solution for the protection and identification of cultivars, which may be quickly and reliably used in eventual commercial disputes. Figure 2. summarizes in a flowchart how we can obtain a DNA analysis based barcode for the grapevine varieties.

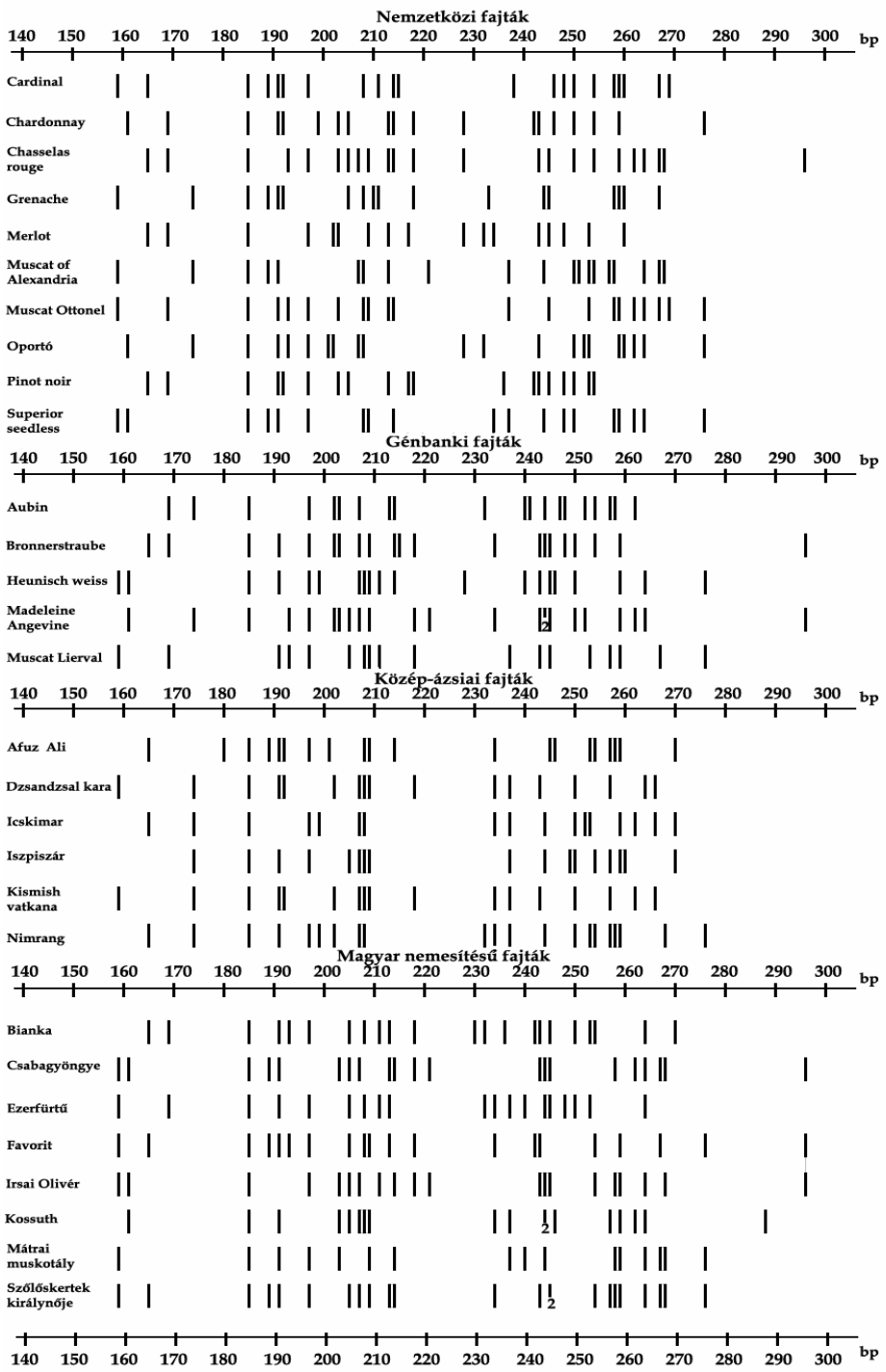
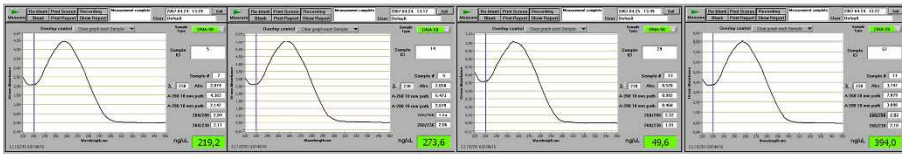


Figure 1: DNA barcodes of the studied grapevine cultivars (Central Asian, gene bank, international and bred in Hungary)

Izsáki | Ottonel muskotály | Mátrai muskotály | Kismis vatkana

1. DNA isolation

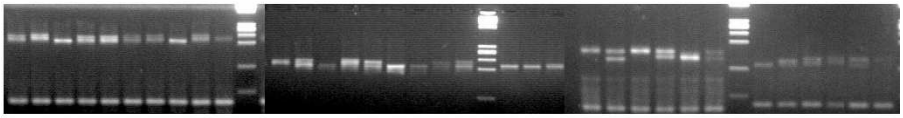
2. Qualitative and quantitative analysis of DNA with Nano Drop spectrophotometer



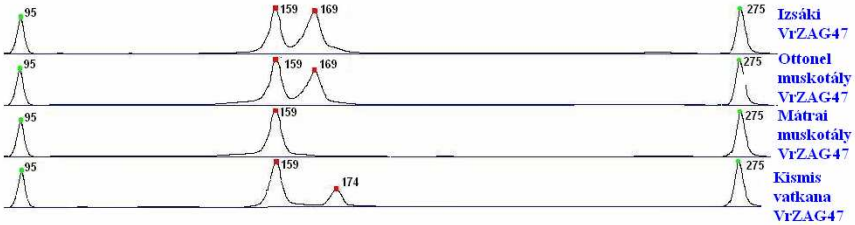
Absorption spectra of DNA samples displayed by the NanoDrop spectrophotometer

3. PCR with fluorescently labelled primers

4. Separation of the PCR products in agarose gel



5. Separation of the PCR products in polyacrylamide gel and determination of their sizes with ALFwin Fragment Analyzer programme



6. Allele size data compiled in a table

	Scu8vv	Scu10vv	VrZAG 47	VrZAG 62	VrZAG 79	VrZAG 83	VrZAG 112	VVMD21	VVMD25	VVMD28	VVMD31	VVMD36
Izsáki	185:185	208:214	159:169	191:207	240:246	197:203	237:245	244:250	245:259	236:258	209:211	254:276
Ottonel muskotály	185:185	208:214	159:169	191:197	258:262	193:203	237:245	267:267	253:259	258:268	209:213	264:276
Mátrai muskotály	185:185	214:214	159:159	191:191	240:258	197:203	237:237	244:267	259:259	258:268	209:209	264:276
Kismis vatkana	185:192	202:208	159:174	191:207	250:262	191:208	237:266	250:257	243:243	218:234	209:209	250:250

7. Bar codes based on allele size data

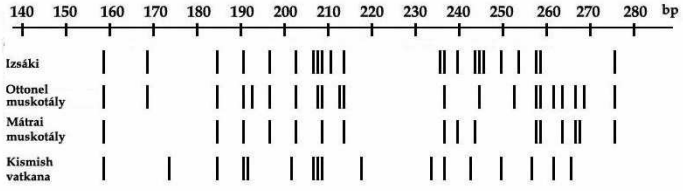


Figure 2 Flowchart of barcode construction: from plant to bar code

NEW SCIENTIFIC RESULTS

1. Microsatellite allele sizes of 115 grape cultivars were determined at seven and twelve loci:
    a. We detemined with 12 SSR primer pairs the allele-combination of the following varieties: 6 varieties indigenous the Carpathian Basin, 5 cultivars from the Hungarian breeding programs, 6 Central Asian cultivars and 4 gene bank accessions. For validation of the allele sizes we involved 8 international cultivars in the examinations.
    b. We characterised with 7 SSR marker 80 Carpathian Basin cultivars, 3 cultivars from the Hungarian breeding programs and 1 gene bank accession. W:e proved the reliability of the analyses with SSR data of 2 international cultivars.
2. We supported the documented pedigree of ’Irsai Olivér’ and ’Mátrai muskotály’ with microsatellite data
    a. We proved that ’Madeleine Angevine’ is really one of the parents of ’Kossuth’ as it can be read in Adolf Stark ’s „cathalogus”.
    b. We justified the parent-progeny relationship between ’Csabagyöngye’ and ’Szőlőskertek királynője’.
3. We concluded that our SSR data contradict to the following putative combinations: ’Csabagyöngye’ = ’Bronnerstraube’ x ’Ottonel muskotály’, and ’Királyleánynyka’ = ’Leánynyka’ x ’Kövérszőlő’. .
    a. We manifested that ’Madeleine angevine’and ’Leánynyka’ are one of the parents of ’Csabagyöngye’ and’ Királyleánynyka, respectively..
4. We identified cultivars, sharing identical alleles in 12, 11, 10 and 9 loci, this way genetic relationships can be assumed between them, providing the bases of further pedigree studies. The results also revealed that, in the case of cultivar ’Bakator kék’, the name Bakator is a homonym.
5. We justified with 115 grapevine varieties, that the dendrogram constructed on the basis of 2,760 SSR data, is suitable for visual representation of genetic distances between the varieties, and the demonstration of the identities and the differences.
6. We converted the SSR results to DNA barcodes by uncoupling the allele size and the corresponding SSR locus information and then sorting the allele size data from lowest to highest. The resulting barcode system is a visual representation of the data, allowing easy detection of genotypic differences.

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