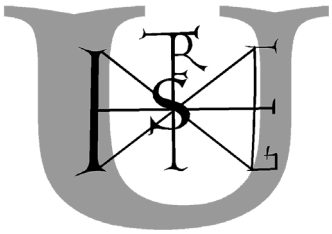

SZENT ISTVÁN UNVERSITY

CHARACTERIZATION AND PEDIGREE ANALYSIS OF GRAPE CULTIVARS, VARIETIES AND BREEDING MATERIALS WITH MOLECULAR MARKERS

Theses of PhD dissertation

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1 HISTORICAL BACKGROUND AND OBJECTIVES

Grapevine cultivation and the consumption of grapes, both as fruit and as wine, have always been closely linked to human culture since ancient times. *Vitis vinifera* L., originating from Eurasia, accounts for more than 80 % of the global wine output and has almost 10 000 known varieties. In grape breeding, it is very important to have a precise description of the cultivars to be crossed and to know their family links and parent-progeny relationships. This is where VIVC (Vitis International Variety Catalogue) (http://www.vivc.de/), created through international cooperation, may be helpful. This database maintains registered items (*Vitis* ssp.) and provides access to their individual descriptions prepared on the basis of their morphological characteristics. However, as the actual development stage and environmental factors have an impact on morphological characteristics, a DNA-based identification system became necessary. In the course of evolution, DNA polymorphisms emerged through various molecular mechanisms such as substitution, insertion, deletion or segmental duplication. Sequencing represents a direct method to detect the genotype differences caused by the resulting DNA sequence variations. However, grape breeders do not need to see all polymorphisms at the same time. Therefore, the use of molecular markers for targeted genomic regions (e.g. SSR, STS, AFLP) offers a quicker, cheaper and less resource-intensive solution. Established under the Genres 081 and GrapeGen 06 projects, the EU-VITIS database (http://www.eu-vitis.de/index.php) contains not only ampelographic data but also the allele sizes (i.e. microsatellite fingerprints) of grape varieties in the relevant nine polymorphic SSR loci (VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZag62 and VrZag79). Efficient and effective microsatellite-based genotyping allows for international data exchange, enabling researchers to clearly identify synonyms and homonyms as well as eventual family relationships between varieties. The EU-VITIS has already been incorporated in the VIVC database, turning this latter into a register of varieties based on molecular and morphological characteristics.

First we studied grape cultivars and *Vitis* seedlings in these 9 microsatellite loci. The co-dominance of microsatellite markers offers a possibility for the verification of parent-progeny relationships as well. However, for the SSR-based reconstruction of parent-progeny relationships it is advisable to increase the number of studied loci to 20 or even 30.

Our red wine culture owes its origin to the acclimatization and cultivation of the ‘Kadarka’ variety in Hungary. Although it is not indigenous to the Carpathian Basin, its cultivation for hundreds of years still makes ‘Kadarka’ an important Hungarian variety. Its molecular analysis was justified by the presence of several ‘Kadarka’ varieties maintained in the Pécs gene bank

(PTE SzBKI) and grown in plantations, and of the cultivar named ‘Kadarka’ or cultivated together with these varieties.

Microsatellite analyses were used to reconstruct the origin and to verify or deny the published pedigree of numerous grape varieties. Pedigree studies based on SSR markers have identified many erroneous documents, leading to the exclusion of mostly the male parent and, to a lesser extent, both registered parents. During our work we prepared SSR fingerprints for *V. vinifera* L. cultivars from Hungarian bred and interspecific hybrids of the PM and DM resistant ‘Seibel’/‘Seyve-Villard’ grapes origin. The allele sizes of the registered parent varieties and 19 French bred ‘Seibel’/‘Seyve-Villard’ hybrids were analyzed together with the allele sizes of Hungarian bred varieties to examine parent-progeny relationships and make comparisons with the registered pedigree.

North-American *Vitis* species play an outstanding role in Hungarian grape breeding. That is why we prepared SSR fingerprints in the recommended 9 loci for the seedling populations of three *Vitis* species of North-American origin (*V. berlandieri* PLANCH.*, V. riparia* MICHX. *and V. rupestris* SCHEELE*)*.

We have also performed marker-based selection with the use of gene-specific markers in order to study the alleles of the *VvMybA1* and *VvMybA2* transcription factor genes associated with berry colour.

Berry colour is an important characteristic which determines the end-product use for both table grapes and wine grapes. Dark berry colour is determined by the volume and composition of the synthesized anthocyanins. In the anthocyanin biosynthetic pathway, the UFGT gene encoding the last key enzyme has been proven to be under the control of two transcription factors (*VvMybA1* and *VvMybA2)*. For both genes it was possible to identify the mutation (insertion of a retrotransposon in the promoter of *VvMybA1* / 2 SNPs in *VvMybA2*) which is associated with a function loss i.e. white berry skin colour in homozygous form. In this thesis, we forecast, on a molecular basis, the expected inheritance pattern of berry colour for the white ‘Nektár’ and the dark ‘Jacquez’ seedling populations.

OBJECTIVES

1. SSR-based molecular analysis of the ‘Kadarka’ varieties maintained at the PTE SzBKI gene bank, the cultivars and their hybrids named ‘Kadarka’ and cultivated together with ‘Kadarka’, and the 2 cultivars of PÁL MÉSZÁROS (‘Mészi kadarka’ and ‘Virághegyi kadarka’), identification of homonyms, synonyms and parent-progeny relationships;
2. Characterization of 20 Hungarian and 4 foreign bred *V. vinifera* L. cultivars in 9 SSR loci and their comparison with the grape varieties registered in their pedigree in order to clarify eventual parent-progeny relationships;
3. Preparation of SSR fingerprints in 9 loci for 23 French and 22 Hungarian bred, actually or presumably PM and DM resistant varieties of ‘Seibel’/‘Seyve-Villard’ origin and for their *V. vinifera* L. parent cultivars in order to study their origin;
4. Verification of the origin of the ‘Eger 2’ cultivar on the basis of the SSR fingerprints of 8 descendants;
5. Microsatellite-based characterization of the open pollinated seedlings of the *V. rupestris* SCHEELE., *V. riparia* MICHX. and *V. berlanieri* PLANCH. genotypes;
6. Berry skin colour selection with molecular markers in the seedling population of ‘Nektár’ x ‘Jacquez’ grapes.

2 MATERIAL AND METHOD

2.1 Plant material

- 'Kadarka' varieties, cultivars named 'Kadarka', accompanying varieties grown with 'Kadarka', 'Kadarka' hybrids and their other parent cultivars (30 genotypes)
- 42 Hungarian and 4 foreign bred cultivars studied for pedigree analysis, and 29 varieties belonging to the available documented pedigree study plus another 19 'Seibel' / 'Seyve-Villárd' hybrids
- Open pollinated seedlings of the *V. berlandieri* PLANCH., *V. riparia* MICHX. and *V. rupestris* SCHEELE maternal genotypes (56, 112 and 92 genotypes)
- 49 hybrids from 'Nektár' x 'Jacquez' crossing and their parents

2.2 DNS extraction

The young leaf samples were stored at -70°C before DNA extraction and then pulverized with liquid nitrogen in a mortar. As to the 'Kadarka' variety samples, the 'Seibel'/'Seyve-Villard' hybrids, the varieties produced through crossing and their studied parent partners, the DNA was extracted and purified from 100 mg leaf sample with DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's protocol. As to the 'Nektár' x 'Jacquez' seedling population as well as the *V. berlandieri* PLANCH., *V. riparia* MICHX. and *V. rupestris* SCHEELE samples, the DNA was extracted from 1 g leaf sample with cetyltrimethyl ammonium bromide (CTAB). The DNA stock concentration was measured with NanoDrop spectrophotometer. Then 15 ng/µl dilutions were prepared for a final volume of 100 µl, which were used as templates in the PCR reaction.

2.3 PCR conditions and markers used in this study

For the microsatellite analysis of grape samples we used the 9 SSR markers (VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZag62, VrZag79) recommended in the Genres 081 and then in the GrapeGen 06 project (THOMAS and SCOTT 1993, BOWERS et al. 1996, 1999a, SEFC et al.1999). In our 'Kadarka' cultivar research, we studied a further 11 SSR loci (VrZag25, VrZag47, VrZag64, VrZag67, VrZag83, VrZag112, VVMD6, VVMD21, VVMD31, VVMD36, VVIb32) (SEFC et al. 1999, BOWERS et al. 1999a, MERDINOGLU et al. 2005). For the pedigree analysis of the 'Csillám' cultivar we applied an additional 11 SSR i.e. a total of 31 primer pairs (scu08, scu10, VVS4, VVS5, VVIp60, VVIi51,

VVIv37, VVIp31, VMC6d12, VMC4h6, VMC2h4) (SCOTT et al. 2000 THOMAS and SCOTT 1993, MERDINOGLU et al. 2005, ARRAYO-GARCIA and MARTINEZ-ZAPATER 2004).

For the follow-up of the *VvMybA1* gene we used its allele-specific primers (KOBAYASHI et al., 2004) and the 20D18CB9 CAPS marker linked to the gene (WALKER et al. 2006).

The polymerase chain reactions were performed according to TÓTH-LENCSÉS et al. (2015b) for SSR primers and according to TÓTH-LENCSÉS et al. (2015a) for the CAPS primers.

2.4 Digestion, sequencing, SNaPshot

The fragments amplified by CAPS primers were digested and sequenced according to TÓTH-LENCSÉS et al. (2015a).

The two SNP (*VvMybA2R44/K980, VvMybA2C22)* polymorphisms of the *VvMybA2* gene were studied with the SNaPshot method (ABI PRISM SNaPshot Multiplex kit). A volume of 3 µl of the purified PCR mixture amplified by the *VvMybA2* primer pair was used for the SNaPshot reaction carried out according to the manufacturer's protocol (Applied Biosystems Protocol P/N 4323357) with the ABI PRISM SNaPshot ddNTP Primer Extension Kit (Applied Biosystems).

2.5 Determination of the exact base pair size of microsatellites

ALF-Express II instrument was used on 8% polyacrylamide gel (ReproGelTM High Resolution, GE Healthcare BioSciences) to determine the exact base pair size of SSR alleles according to TÓTH-LENCSÉS et al. (2015b).

2.6 Statistical evaluation of microsatellite data

The parent-progeny relationships were analyzed according to TÓTH-LENCSÉS et al. (2016), while the documented pedigrees were studied according to TÓTH-LENCSÉS et al. (2015b).

3 RESULTS

3.1 SSR-based genotyping

3.1.1 Molecular genetic characterization of the ‘Kadarka’ varieties, the cultivars named ‘Kadarka’, the accompanying cultivars grown with ‘Kadarka’ and the ‘Bíbor kadarka’

The allele sizes of the 30 items associated with ‘Kadarka’ (cultivars, varieties, produced through cross-breeding with ‘Kadarka’), determined in 20 SSR loci, were used to draw a dendogram in order to display genetic distances (Figure 1). We evaluate the microsatellite results and the dendogram in groups.

Group I includes the ‘Kadarka’ varieties. ‘Lúdtalpú kadarka’, ‘Ménesi kadarka’, ‘Kék kadarka’ and ‘Szürke kadarka’ (Werner et al. 2013), which had formerly proven to be identical in 9 microsatellite loci, provided the same DNA fingerprints also in the additional 11 microsatellite loci. Based on the results of twenty SSR loci, the newly involved ‘Kadarka’ varieties such as ‘Fügelevelű kadarka’, ‘Kadarka kék bolondoshím’, ‘Kadarka kék csillagvirágú’, ‘Keresztes levelű kadarka’, ‘Lila keresztes levelű kadarka’, ‘Zöld keresztes levelű kadarka’ and the two clones (‘Fűszeres kadarka’ P.9, ‘Nemes kadarka’ P.8) have demonstrated to have the same genotype as ‘Kék kadarka’. Thus the DNA analysis confirms the old literature statement, according to which the morphologically different plants of the old ‘Kadarka’ plantations of various production regions belong to the same cultivar (ENTZ et al. 1869, TERSÁNCZKY 1869, MOLNÁR 1888, DRUCKER 1906, KOZMA 1963, NÉMETH 1967).

Group II includes cultivars of non ‘Kadarka’ origin which used to be grown together with ‘Kadarka’. The only cultivar named ‘Kadarka’ in this group is ‘Mészi kadarka’. The microsatellite DNA analyses indicated already in 9 loci that ‘Csókaszőlő’ and ‘Cigányszőlő’ were not synonyms, while ‘Rácfekete’ and ‘Cigányszőlő’ were of the same genotype (WERNER et al. 2013). This was confirmed by the results of the additional 11 SSR markers. Figure 1 shows that ‘Csókaszőlő’ and ‘Cigányszőlő’ are distant from each other and from the ‘Kadarka’ varieties. It should be noted that the ‘Csókaszőlő’, ‘Cigányszőlő’, ‘Rácfekete’ and ‘Vad fekete’ cultivars are often referred to in former literature as synonyms. ‘Mészi kadarka’ (cultivar registered as ‘Mészi kadar’ in the National List of Varieties for 2016) is separated both from the ‘Kadarka’ varieties and from the ‘Olasz kadarka’ group, while it shows the closest realationship with ‘Csókaszőlő’ maintained in the Pécs gene bank (Figure 1).

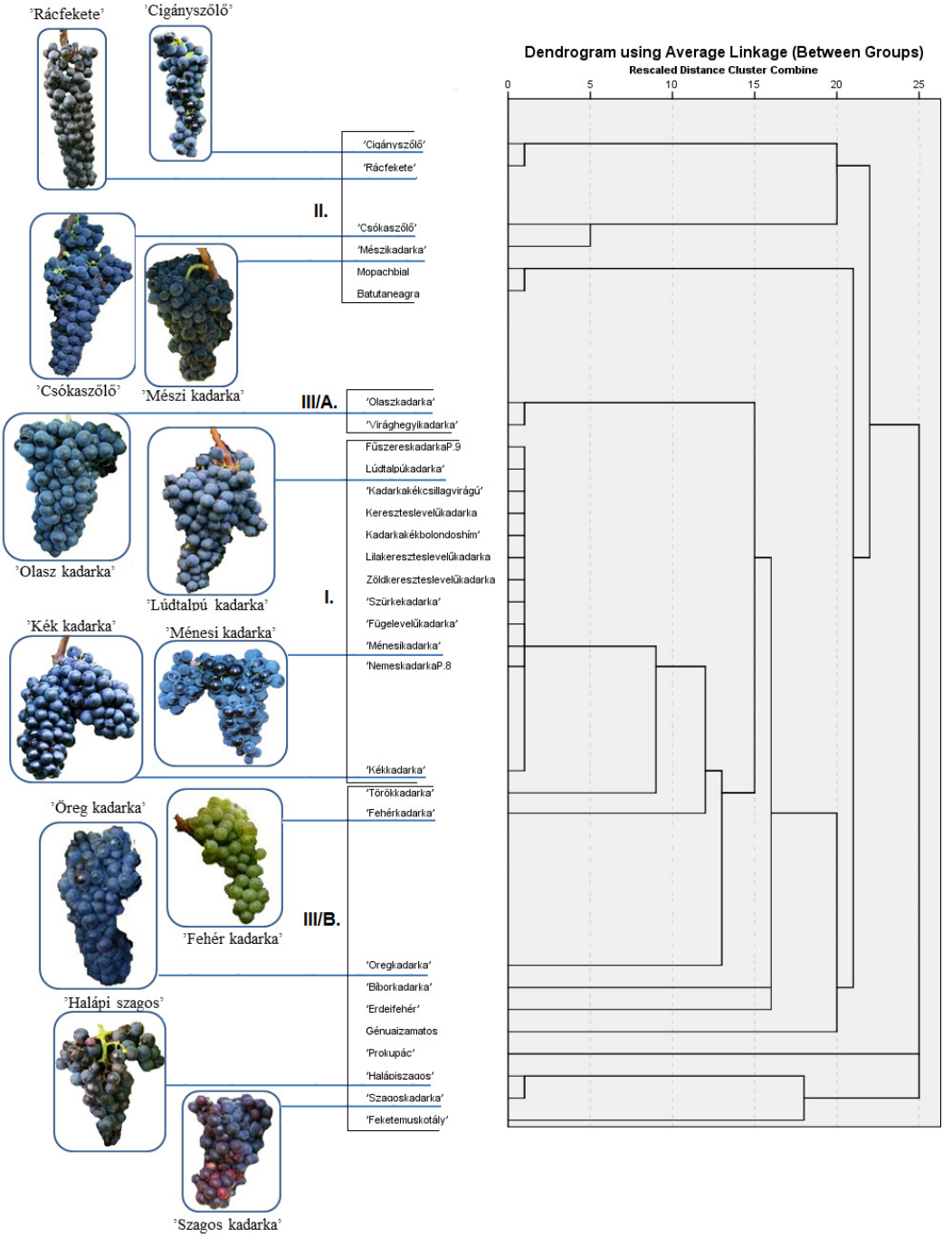

Figure 1 – Genetic distance between our 30 samples on the basis of 20 microsatellite loci. Source of images: http://www.borigo.hu/dunabor/index.php?cmd=cikk&id=00048 and WERNER (2013).

Group III shows 'Kadarka' cultivars formerly cultivated together with 'Kadarka', 'Bíbor kadarka' and the assumed parents. Due to their straightforward morphological differences,

‘Olasz kadarka’ and ‘Kadarka’ are considered separate cultivars by various authors (MOLNÁR 1888, DRUCNER 1906, KOZMA 1963, NÉMETH 1966), and we have confirmed the same through genotyping. The allele sizes of ‘Olasz kadarka’ and those of ‘Virághegyi kadarka’ selected by Pál Mészáros (registered as ‘Virághegyi’ in the National List of Varieties for 2016) were fully identical already in 9 loci (WERNER et al. 2013); then they proved to be the same also in the additional 11 loci, so their identical genotype has been confirmed (Figure 1). The SSR data support the different genotypes of ‘Török kadarka’ and ‘Kadarka’ and thus confirm the findings of MOLNÁR (1888) and DRUCKER (1906). Prior to the phylloxera plague, ‘Öreg kadarka’ was considered as a valuable, well-coloured variety of ‘Kadarka’ by ENTZ et al (1869). However, it is mentioned as a separate cultivar in subsequent studies (DRUCKER 1906, KOZMA 1963, NÉMETH 1966), which is confirmed by previous findings (WERNER et al. 2013) and also by the results of our current extended microsatellite analyses.

Despite the similar variety name, we have not found any genotype identical with ‘Kadarka’ in this group. However, the polymorphic pattern of the 20 SSR loci enabled us to determine parent-progeny relationships. Accordingly, we have proven that there is a parent-progeny relationship between ‘Olasz kadarka’, ‘Öreg kadarka’ and ‘Kadarka’ (LACOMBE et al. 2013), and that ‘Török kadarka’ also originates from the ‘Kadarka’ cultivar. The long history of ‘Kadarka’ grapevine culture gave a chance for spontaneous hybridization and enabled these seedlings to become a separate cultivar. Both ‘Csókaszőlő’ and ‘Mészi kadarka’ carry a common allele in 20 loci, which is a proof for their parent-progeny relationship. The identification of the origin of ‘Mészi kadarka’ is especially important because it is confirmed to be the progeny of a Hungarian red wine variety known already in the Middle Ages (‘Csókaszőlő’ is an old Hungarian variety of unknown origin). Pál Mészáros (responsible for selecting and maintaining the variety) describes ‘Mészi kadarka’ as “a scruffy variety with small berries and small bunch of grapes” (TOMPA és BÁNYAI 2008); this description is also valid for ‘Csókaszőlőre’.

The alleles determined in 20 SSR loci confirm or reject the registered parent-progeny relationships also in the case of ‘Kadarka’ hybrids. Based on results obtained from 20 microsatellite loci, ‘Kadarka’ can be proven to be one parent of ‘Fehér kadarka’ from the Pécs collection, although the ‘Erdei fehér’ variety is excluded by SSR data. It also means that ‘Fehér kadarka’, the old southern variety maintained in the Pécs collection and involved in our study, is not identical with ‘Fehér kadarka’ created by PÁL KOCSIS with the crossing of ‘Kadarka’ x ‘Erdei fehér’ (‘Bakarka’). In 9 SSR loci, ‘Szagos kadarka’ has proven to be identical with ‘Halápi szagos’, maintained in the collections, and the relevant data fail to confirm (WERNER et al. 2013) that ‘Kadarka’ is one of the parents referred to in literature (NÉMETH 1966).

According to our current extended molecular genetic analysis, 3 out of 20 loci exclude the potential parentage of 'Fekete muskotály' listed in the pedigree of 'Szagos kadarka'; accordingly, it has been concluded that none of the presumed parents took part in the creation of 'Szagos kadarka'. Results from the additional 11 loci also confirm the identical genotypes of 'Szagos kadarka' and 'Halápi szagos'.

'Bíbor kadarka', obtained through cross-breeding, has been confirmed to originate from the 'Kadarka' parent referred to in literature. Further analysis will be required concerning the other parent.

3.1.2 Pedigree analysis for cultivars obtained through crossing within the *V. vinifera* L. species

We examined 20 Hungarian and 4 foreign hybrids obtained through crossing within the *V. vinifera* L. species. Using Identity 1.0 to analyze the allele sizes of a total of 44 genotypes detected in 9 loci, we have obtained the groups shown in Table 1. The DNA of both parent cultivars was available for hybrids in Group 1, 3 and 5. However, we examined only one parent for hybrids in Group 2 and 4.

The SSR data confirm the registered pedigree of the Hungarian bred 'Attila', 'Boglárka', 'Generosa', 'Kármin', 'Kozma Pálné muskotály', 'Nektár', 'Pannónia kincse', 'Rozália', 'Zenit' and 'Zengő' varieties and the foreign bred 'Hamburgi muskotály', 'Itália' and 'Kerner' varieties in Group 1 (Table 1). GYÖRFFYNÉ et al. (2011) published similar data for the 'Zenit' and 'Zengő' cultivars. In that study 3 of the 6 SSR primers were the same as ours, which means that data obtained from a total of 12 microsatellite loci confirm the pedigree of 'Zenit' and 'Zengő'.

As to Group 2 including 'Bíbor kadarka', 'Cegléd szépe', 'Favorit' and 'Mathiasz Jánosné muskotály', we have confirmed the available parent (Table 1). According to the SSR data of the EU-VITIS database, 'Muscat Bouschet', the other parent of 'Bíbor kadarka', is excluded by 5 out of 9 loci.

Group 3 includes the Hungarian bred 'Cserszegi fűszeres', 'Korona' and 'Pátria' varieties and the foreign bred 'Müller-Thurgau' variety (Table 1). In this group each pollinating parent was excluded; thus in the case of the 'Müller-Thurgau' variety used as control we have confirmed the origin theory of Thomas et al. (1994), Sefc et al. (1997) and Dettweiler et al. (2000).

Table 1 – Literature pedigree and SSR-based origin analysis of the 24 *V. vinifera* L. cultivars. (The verified parent-progeny relationships are highlighted)

	Pedigree			SSR-based origin analysis	
Group	Progeny	Parent 1	Parent 2	Number of identical alleles: Parent 1	Number of identical alleles :Parent 2
1	'Attila'	'Rosa menna'	'Mathias Jánosné muskotály'	9/9	9/9
	'Boglárka'	'Génuai zamatos'	'Pannónia kincse'	9/9	9/9
	'Generosa'	'Ezerjó'	'Piros tramini'	9/9	9/9
	'Hamburgi muskotály'	'Alexandriai muskotály'	'Kék trollinger'	9/9	9/9
	'Italia'	'Bicane'	'Hamburgi muskotály'	9/9	9/9
	'Kármin'	'Kadarka'	'Petit boushet'	9/9	9/9
	'Kerner'	'Kék trollinger'	'Rajnai rizling'	9/9	9/9
	'Kozma Pálné muskotály'	'Itália'	'Irsai olivér'	9/9	9/9
	'Nektár'	'Judit'	'Cserszegi fűszeres'	9/9	9/9
	'Pannónia kincse'	'Szőlőskertek királynője muskotály'	'Cegléd szépe'	9/9	9/9
	'Rozália'	'Olaszrizlig'	'Tramini'	9/9	9/9
	'Zenit'	'Ezerjó'	'Bouvier'	9/9	9/9
	'Zengő'	'Ezerjó'	'Bouvier'	9/9	9/9
2	'Bíbor kadarka'	'Kadarka'	'Muscat Bouschet'**	9/9	na.
	'Cegléd szépe'	'Chasselas blanc croquant'*	'Chasselas rouge royal'	n.a.	9/9
	'Favorit'	'Chasselas Queen Victoria white'*	'Szőlőskertek királynője muskotály'	n.a.	9/9
	'Mathiasz Jánosné muskotály'	'Chasselas rouge de foncee'*	'Ottonel muskotály'	n.a.	9/9
3	'Müller-Thurgau'	'Rajnai rizling'	'Zöld szilváni'	9/9	9/2
	'Cserszegi fűszeres'	'Irsai olivér'	'Tramini'	9/9	9/4
	'Korona'	'Juhfark'	'Irsai olivér'	9/9	9/4
	'Pátria'	'Olaszrizling'	'Tramini'	9/8	9/9
4	'Narancsízű'	'Chasselas Queen Victoria white'*	'Szőlőskertek királynője muskotály'	n.a.	9/8
	'Szőlőskertek királynője muskotály'	'Erzsébet királyné emléke'*	'Csabagyöngye'	n.a.	9/7
5	'Zefír'	'Hárslevelű'	'Leányka'	9/5	9/5

No DNA was available for cultivars marked with *. The allele sizes in 9 SSR loci for cultivars marked with ** are included in the EU-VITIS database.
n.a.: not available

The female parent was not examined but the male parent was excluded for 'Narancsízű' and 'Szőlőskertek királynője muskotály' listed in Group 4. The exclusion of the pollinating parent may often be the result of a pollination defect caused by the lack of proper isolation (Lacombe et al. 2013).

As to the Hungarian bred 'Zefír' white wine cultivar in Group 5, the allele sizes exclude both parents from the list of potential crossing partners, in line with the former findings of Halász et al. (2005). It is probably caused by an administrative error.

3.1.3 Pedigree analysis for North-American PM resistant hybrids

Among the hybrids created by Hungarian resistance breeders, 22 cultivars of 'Seibel'/'Seyve-Villard' origin were examined. The exact allele sizes were determined in 9 microsatellite loci for the 22 cultivars and the *V. vinifera* L. parents. We used Identity 1.0 to compare the obtained allele sizes with those of 23 French bred 'Seibel'/'Seyve-Villard' cultivars detected in the same 9 SSR loci. During the pedigree analysis the 22 Hungarian bred cultivars were classified into 4 groups as shown in Table 2.

Group 1 includes 'Chardonnay', the pedigree of which was verified during an SSR-based study (Bowers et al. 1999b). This group also includes the 'Bianca', 'Göcseji zamatos', 'Medina', 'Nero', 'Palatina', 'Reflex', 'Reform', 'Refrén', 'Suzy', 'Teréz' and 'Vértes csillaga' varieties, where the parent-progeny relationships described by Hajdu and Ésik (2001) in the 9 SSR loci cannot be excluded (Table 2).

No male parent was available for the pedigree analysis of 'Eszter', 'Flóra' and 'Lidi' in Group 2; however, based on allele data, the female parents are in line with literature findings.

As to 'Dunagyöngye', 'Fanny', 'Viktor' and 'Zalagyöngye' listed in Group 3, one parent registered in the pedigree is excluded by 9 SSR marker data.

Group 4 includes 'Csillám', 'Pölöskei muskotály', 'Sarolta' and 'Viktória gyöngye'. As to these varieties, none of the parents corresponded to the documented crossing work. Other researchers dealing with parent-progeny relationships have already published similar mismatching results of their molecular analyses with 'Csabagyöngye' (Hillebrand et al. 1972, Bauer et al. 2002, Kozma et al. 2003, Kiss et al. 2006, Lacombe et al 2013). According to Lacombe et al. (2013), published pedigrees not verifiable through DNA analysis represent a recurring issue. It is independent of countries or centuries and caused either by improper pollination or by the fact that, due to homonyms, the pollinator genotype differs from the one described by the breeder. It may also happen that breeders want to keep the pedigree of their excellent cultivar in secret.

Table 2 – Pedigree of the 22 Hungarian bred cultivars (HAJDU and ÉSIK 2001), and parent-progeny allele matching with 9 SSR primers in the 4 groups. (The verified parent-progeny relationships are highlighted).

	Pedigree			SSR-based origin analysis	
Group	Progeny	Parent 1	Parent 2	Number of identical alleles: Parent 1	Number of identical alleles: Parent 2
1	'Chardonnay'	'Pinot noir'	'Heunisch weiss'	9/9	9/9
	'Bianca'	'Seyve-Villard 12375'/'Eger2'	'Bouvier'	9/9	9/9
	'Göcseji zamatos'	'Seyve-Villard 12286'/'Eger1'	'Medoc noir'	9/9	9/9
	'Medina'	'Seyve-Villard 12286'/'Eger1'	'Medoc noir'	9/9	9/9
	'Nero'	'Seyve-Villard12375'/'Eger2'	'Medoc noir' x 'Csabagyöngye'	9/9	9/9
	'Palatina'	'Seyve-Villard 12375'	'Szőlőskertek királynője'	9/9	9/9
	'Reflex'	'Pannónia kincse'	'Seibel 5279'	9/9	9/9
	'Refrén'	'Glória Hungariae'	'Seibel 5279'	9/9	9/9
	'Reform'	'Csabagyöngye'	'Seibel 5279'	9/9	9/9
	'Suzy'	'Seyve-Villard 12375'/'Eger2'	'Pannónia kincse'	9/9	9/9
	'Teréz'	'Seyve-Villard 12375'/'Eger2'	Olimpia'	9/9	9/9
	'Vértes csillaga'	'Seyve-Villard 12286'/'Eger1'	'Medoc noir'	9/9	9/9
2	'Eszter'	'Seyve-Villard 12375'/'Eger2'	'Magaracsi csemege I.'	9/9	na.
	'Lidi'	'Seyve-Villard 12375'/'Eger2'	'Magaracsi csemege III.'	9/9	na.
	'Flóra'	'Seyve-Villard 12375'/'Eger2'	'Magaracsi csemege III.'	9/9	na.
3	'Dunagyöngye'	'Seibel 4986'	'Csabagyöngye'	9/9	9/4
	'Fanny'	'Seyve-Villard 12375'/'Eger2'	Téli muskotály x Olimpia	9/9	9/7
	'Viktor'	'Zalagyöngye'	'Kadarka'	9/9	9/1
	'Zalagyöngye'	'Seyve-Villard 12375'/'Eger2'	'Csabagyöngye'	9/6	9/9
4	'Csillám'	'Seyve-Villard 12375'	'Csabagyöngye'	9/4	9/2
	'Pölöskei muskotály'	'Zalagyöngye'	'Gloria Hungaria' x 'Erzsébet királyné'	9/3	9/2-n.a.
	'Sarolta'	'Zalagyöngye'	('Gloriae Hungariae' x 'Szőlőskertek királynője') x 'Téli muskotály'	9/6	9/8
	'Viktória gyöngye'	'Seyve-Villard 12375'	'Csabagyöngye'	9/1	9/5
n.a.: not available					

3.1.4 The origin of 'Eger 2'

There are several Hungarian bred North-American resistant hybrids which were produced by crossing 'Seyve-Villard 12-375' or 'Eger 2' and some *V. vinifera* L. cultivar. Az Literature data are contradictory regarding the origin of 'Eger 2'. The authors consider 'Eger 2' as the descendant (CSEPREGI and ZILAI 1988) or selected clone of 'Seyve-Villard 12-375' (CSEPREGI and ZILAI 1988, HAJDU and ÉSIK 2001), and the hybrid of 'Seyve-Villard 12-375' x selective = 'Eger 2' / 5375 / (BÁNYAI 2012) or that of 'Seyve-Villard 12-375' x unknown *V. vinifera* L. (PHYTOWELT 2002).

Nevertheless, breeders and vine-growers generally agree – including PÁL KOZMA, senior research fellow at PTE SzBKI – that the 'Eger 2' variety is identical with the 'Seyve-Villard 12-375' hybrid.

As in our former studies we reported about the SSR-based parent-progeny relationship analysis of Hungarian bred hybrids – 9 of which originate from 'Seyve-Villard 12-375' / 'Eger 2' ['Bianca', 'Eszter', 'Fanny', 'Flóra', 'Lidi', 'Nero', 'Suzy', 'Teréz', 'Zalagyöngye' (TÓTH-LENCSÉS et al. 2015b)] –, we decided to check the 'Eger 2' pedigree via these hybrids. Among the hybrids we excluded 'Zalagyöngye' from the potential descendants of 'Seyve-Villard 12-375' / 'Eger 2' (TÓTH-LENCSÉS et al. 2015b), and continued our work with 8 descendants.

The origin analysis of 'Eger 2' was based on the fact that 'Seyve-Villard 12-375' is heterozygous in 9 SSR loci, which makes it suitable for origin verification on the basis of the allele composition of its 8 descendants.

As both its small and large alleles were detected in all the 9 SSR loci of the 8 descendants, we have concluded, accepting literature data, that 'Eger 2' is either (1) a variety produced from 'Seyve-Villard 12-375' through selection or (2) a seedling with heterozygous genotype in the 9 loci produced from the crossing of 'Seyve-Villard 12-375'. Therefore it can be concluded that 'Eger 2' is not the open pollinated seedling of 'Seyve-Villard 12-375' (PHYTOWELT 2002) i.e. it is not a hybrid from a crossing with unknown *V. vinifera* L. because in that particular case the 8 descendants would display other alleles in addition to the alleles of 'Seyve-Villard 12-375' and the other parent. However, when the general opinion of breeders is compared with the results of our SSR-based study, we conclude that 'Eger 2' is identical with 'Seyve-Villard 12-375'.

3.1.5 The origin of 'Csillám'

According to literature information, 'Csillám' was obtained from the crossing of 'Seyve-Villard 12-375' x 'Csabagyöngye', which can be denied right on the basis of the alleles of the 9 SSR loci (Figure 2). Using Identity 1.0, we applied the data of 383 genotypes (included in the study) detected in 9 loci in order to identify parent-progeny combinations. The following combination was revealed: 'Csillám' = 'Kékfrankos' x 'Seibel 4643'. In order to clarify and confirm the origin, 11 additional SSR markers were used to study 'Csillám' as well as its registered and assumed parent pairs. The resulting data were combined with the data of 20 varieties named 'Kadarka' or associated with 'Kadarka'. According to the findings obtained by running Identity 1.0 on the resulting 25 genotypes, the probability of 'Csillám' being the hybrid of 'Kékfrankos' x 'Seibel 4643' is 7.03×10^{23} higher than the probability of 'Csillám' being the hybrid of two cultivars randomly selected from the population. In the case of grapevine the verification of parent-progeny relationships based on SSR allele sizes requires the checking of 20 loci (Sefc et al. 1999). However, according to certain authors, the verification of suggested or assumed parent-progeny relationships requires the involvement of even more SSR loci

(Vouillamoz et al. 2003). Therefore we relied on the results of another 11 i.e. a total of 31 (9+11+11) SSR loci to prove that 'Csillám' is the result of crossing 'Kékfrankos' with 'Seibel 4643'.

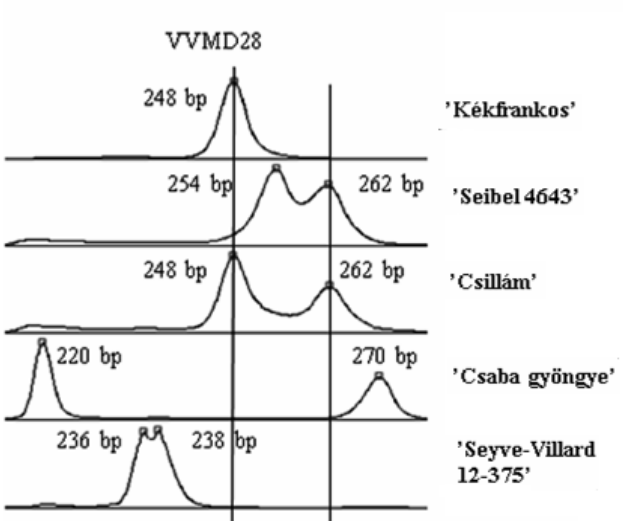

Figure 2 – Exact allele sizes of 'Csillám' determined in the VVMD28 SSR locus for its registered parents ('Csabagyöngye', 'Seyve-Villard 12-375') and for the "new" parents ('Kékfrankos', 'Seibel 4643') identified by us (ALFexpress II).

3.1.6 SSR-based analysis of the seedlings of North-American *Vitis* species (*V. berlandieri* PLANCH.*, V.riparia* MICHX. *and V. rupestris* SCHEELE*)*

Based on allele sizes detected in 9 SSR loci for the open pollinated seedlings of North-American *Vitis* species, we have identified the actual alleles carried by *V. berlandieri* PLANCH.*, V. riparia* MICHX. and *V. rupestris* SCHEELE at each SSR locus. Its importance is that if any of these is involved in a breeding program, breeders will already have genotyping data at their disposal.

Determining the allele polymorphism of the *VvMybA1* gene with gene specific and linked markers

3.1.7 Using molecular markers to select berry skin colour in 'Nektár' x 'Jacquez' progeny

The purpose of the studies was to use the 20D18CB9 CAPS marker linked with the *VvMybA1* gene to forecast the berry colour of the 49 hybrids obtained from the crossing of blue-berried 'Jacquez' (*V. Bourquina =V. aestivalis* MICHX. *x V. vinifera* L.) with white-berried 'Nektár' (*V. vinifera* L.). In the case of pure *V. vinifera* reference cultivars, in 'Nektár' and 17 hybrids the 20D18CB9 CAPS marker produced a fragment of 577 bp.. The *V. aestivalis* MICHX. sample displayed a fragment of 543 bp. In the case of 'Jacquez' and 24 hybrids both allele sizes were detectable. Therefore, we did not even need restriction digestion to discriminate *V. aestivalis* MICHX. or the presumably blue-berried hybrids including 'Jacquez'. However, except for the latter, no phenotype data were available to us. The 20B18CB9 CAPS primer linked with the *VvMybA1* gene was able to detect a deletion of 34 bp of *V. aestivalis* MICHX. origin in 'Jacquez' and in the presumably red-berried descendants without any need for digestion.

Use of the CAPS marker linked with the *VvMybA1* gene proves in the case of 'Nektár' x 'Jacquez' hybrids that this marker is suitable for detecting the *V. aestivalis* MICHX. component in the 'Jacquez' parent cultivar. In other words, it can be used as a simple locus-specific PCR marker.

3.1.8 Proving the origin of 'Csillám'

The two cultivars identified as parents have blue berry colour, while 'Csillám' has white berry colour; therefore both the parents and the hybrid were checked for the alleles encoding the *VvMybA* transcription factors that regulate the biosynthesis of anthocyanin. In most grape cultivars, white berry colour is genetically caused by the insertion of the *Gret1* retrotransposon in the promoter of *VvMybA1* (Kobayashi et al. 2004) or by point mutations in *VvMybA2* (Walker et al. 2007, Carrasco et al. 2015).

The presence or absence of *Gret1* was detected with the CAPS marker, which proved that the 'Kékfrankos' cultivar is a coloured heterozygous genotype, while the also blue-berried 'Seibel 4643' showed a white homozygous genotype. We analyzed the allele composition of the *VvMybA2* transcription factor gene with the SNaPshot method and concluded in view of the reference samples that 'Seibel 4643' carries a functional SNP in heterozygous form at both of the studied SNP positions. Therefore anthocyanin is synthesized in the berry skin even if both alleles of *VvMybA1* become dysfunctional as a result of *Gret1* insertion. The results prove that 'Csillám'

has inherited from both parents the “white” alleles carrying the *VvMybA1+Gret1* and *VvMybA2* SNP mutations.

3.1.9 Examining the berry skin of ‘Kék kadarka’ and ‘Szürke kadarka’

The results of our extended analysis with 20 SSR markers has confirmed that ‘Szürke kadarka’ is a colour variant of ‘Kadarka’. The polymorphism of the gene encoding the *VvMybA1* transcription factor that regulates the biosynthesis of anthocyanin was also studied with the purpose of discriminating ‘Kék kadarka’ from ‘Szürke kadarka’ of the same genotype.

While in the former two cases the allele polymorphism of the *VvMybA1* gene was analyzed with success, the same method was unable to distinguish ‘Kék kadarka’ from ‘Szürke kadarka’. Further studies will be carried out in this direction, in reliance of point mutations already identified in the *VvMybA2* gene.

3.2 New scientific results

1. Based on 20 loci, we have prepared SSR fingerprints for 30 ‘Kadarka’ varieties, cultivars named ‘Kadarka’ and genotypes linked to ‘Kadarka’. Based on 9 loci, we have determined SSR fingerprints for an additional 93 cultivars, varieties and hybrids as well as for 260 open pollinated seedlings originating from *V. berlandieri* PLANCH., *V. riparia* MICHX. and *V. rupestris* SCHEELE.
2. We have verified synonyms on the basis of allele sizes (in 20 loci): ‘Halápi szagos’ - ’Szagos kadarka’, ‘Cigányszőlő’ - ’Rácfekete’, ‘Virághegyi kadarka’ - ’Olasz kadarka’, ‘Batuta neagra’ - ’Mopach bial’.
3. We have confirmed that the studied eight ‘Kadarka’ varieties are of the same genotype as ‘Kék kadarka’ (in 20 loci).
4. We have proven that ‘Kadarka’ is one of the parents of the ‘Olasz kadarka’, ‘Virághegyi kadarka’, ‘Öreg kadarka’ and ‘Török kadarka’ cultivars (in 20 loci), while ‘Mészi kadarka’ is a progeny of ‘Csókaszőlő’ (in 20 loci).
5. We have confirmed or clarified the assumed origin of an additional 44 varieties through microsatellite analysis (in 9 loci).
6. While studying the origin of the Hungrian bred ‘Eger 2’ variety, we have concluded that this variety is identical with the ‘Seyve-Villard 12-375’hybrid.
7. We have determined in 31 microsatellite loci the SSR fingerprints of the parent cultivars of ‘Csillám’ as well as the parent cultivars in the documented and in our “new” pedigree. We have proven for the first time that the white wine grape cultivar ‘Csillám’ is the hybrid of the blue-berried ‘Kékfrankos’ and ‘Seibel 4643’. We have further supported this finding through a study of the allele polymorphism of the *VvMYBA1* and *VvMYBA2* genes.
8. We have developed a method for the selection of white and coloured berry skin genotypes in the ‘Nektár x ‘Jacquez’ seedling populations.

4 SUMMARY

This thesis presents our results obtained with molecular markers used for various purposes. The morphological characterization of grapevine cultivars must be supplemented with a molecular analysis in order to safely identify a particular cultivar. Thus we have used DNA fingerprinting to characterize (1) 'Kadarka' cultivars and varieties, (2) *Vitis vinifera* L. genotypes, (3) hybrids of 'Seibel' / 'Seyve Villard' origin, and (4) the seedlings of three North-American *Vitis* species.

We have identified parent-progeny relationships by increasing the number of SSR loci. Furthermore, we have used gene-specific primers to perform marker-based selection in a segregating population generated by crossing a white-berried aromatic wine grape cultivar ('Nektár') with a PM resistant cultivar ('Jacquez'). We have used this marker system to confirm the origin in the case of our reconstructed 'Csillám' pedigree.

We have used our results to prove that the ampelographically different 'Kadarka' varieties ('Lúdtalpú kadarka', 'Ménesi kadarka', 'Fügelevelű kadarka', 'Kadarka kék bolondoshím', 'Kadarka kék csillagvirágú', 'Keresztes levelű kadarka', 'Lila keresztes levelű kadarka', 'Zöld keresztes levelű kadarka') are of the same genotype as the basic 'Kadarka' cultivar in 20 SSR loci. 'Olasz kadarka' and 'Virághegyi kadarka' are the same but they are not identical with 'Kék kadarka'. This fact also supports the need to supplement the morphological characterization of grapevine cultivars with a molecular analysis. This statement is further confirmed by the fact that we identified additional synonyms during our study of a total of 30 genotypes that can be associated with the 'Kadarka' cultivar: 'Halápi szagos' - 'Szagos kadarka'; 'Cigányszőlő' - 'Rácfekete'; 'Batuta neagra' - 'Mopach bial'. Our pedigree reconstruction work has revealed that 'Kadarka' is one of the parents of the 'Olasz kadarka', 'Virághegyi kadarka', 'Öreg kadarka' and 'Török kadarka' cultivars, while 'Mészi kadarka' is the progeny of 'Csókaszőlő'. Regardless of the 20 SSR loci used, we could not distinguish 'Szürke kadarka' from 'Kék kadarka'.

As to the microsatellite analysis of the registered pedigrees of 46 cross-bred grapevine cultivars, (1) (when examining both parents) we have confirmed one of the parents for 24 cultivars and excluded one of the parents for 8 cultivars and both parents for 5 cultivars; (2) (when examining one of the parents) we have confirmed the registered cultivars in 7 cases and excluded them in 2 cases. As suggested by other authors, we also think that at least 20 loci should be examined for the microsatellite-based study of registered pedigrees. By examining 31 SSR loci in the case of 'Csillám', we have not only excluded the registered origin of 'Seyve-Villard 12-375' x 'Csabagyöngye' but established that the cultivar is the hybrid of 'Kékfrankos'

x ’Seiben 4643’. For the origin analysis of the Hungarian bred ‘Eger 2’ cultivar we have relied on the microsatellite results of its 8 hybrids and assumed parent (‘Seyve-Villard 12-375’). We have proven that except for ‘Seyve-Villard 12-375’ no other *V. vinifera* L. cultivar was involved in the pedigree.

Furthermore, we have performed marker-based selection with the use of gene-specific markers: in particular, we have studied the *VvMybA1* and *VvMybA2* transcription factor genes that play a role in the determination of berry colour.

As the 20D18CB9 CAPS primer pair used to examine the mutation of the *VvMybA1* gene responsible for berry colour detected a deletion of 43 bp in *V. aestivalis* MICHX. (or an insertion into this sequence of the *V. vinifera* L. species), a simple PCR carried out with the CAPS primer pair is sufficient to forecast the berry color of seedlings at the time of examining the progeny population created by crossing the dark-berried ‘Jacquez’ (originating from *V. aestivalis* MICHX.) with the white ‘Nektár’ cultivar.

Neither SSR nor *VvMybA1* gene analyses were sufficient to distinguish ‘Szürke kadarka’ from ‘Kék kadarka’, we suggest further studies in reliance of *VvMybA2* gene mutations.

In addition to SSR analysis (31 loci), we have studied *VvMybA1* and *VvMybA2* gene mutations in order to confirm the pedigree of ‘Csillám’. Through the application of berry colour markers, we have proven, on a molecular basis, that ‘Csillám’ – created by crossing two dark-berried cultivars – is white because it inherited from both parents the “white” alleles carrying the *VvMybA1+Gret1* and *VvMybA2* SNP mutations.

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