CHARACTERIZATION OF GRAPEVINE AND APPLE CULTIVARS WITH MICROSATELLITE MARKERS

PhD Thesis

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BACKGROUND AND OBJECTIVES

Grapevine (*Vitis sp.*) is one of the most important fruit crops cultivated worldwide. By 2010 the global grapevine area is estimated to reach 8.1 million hectares, almost 70% of which is found in Europe. Grapevine plays a major economic role in agriculture and it is also present in many related industries.

The history and traditions of grapevine cultivation started thousands of years ago. In the course of its spreading, grapevine has produced numerous varieties and cultivars. According to certain data sources, there are some 5,000-6,000 cultivars are known globally, while other sources claim that this number may be as high as 20,000 including about 8,000 varieties in cultivation today.

The pioneer of the collection and classification of grapevine cultivars was Márton Németh (1967), who also carried out the classification of the grapevine cultivars indigenous to the Carpathian Basin. Today the collection maintained by the Institute of Viticulture and Enology at the Pécs Science University contains more than 1,200 cultivars (http://www.szbki.pte.hu/menu/19). The number of old cultivars collected from the Carpathian Basin exceeds 100, which were described under the guidance of Pál Kozma (jr.) on the basis of the internationally accepted OIV codes (descriptor system).

Apple (*Malus sp.*) is also among the most important fruits, which is well known for its versatile uses and agricultural importance. According to a FAO report, a total of 91 countries produced 65.9 million tons of apples on some 4.8 million hectares in 2007.

Some 15,000 apple varieties are known in the world but only about 1,200 of them are put into cultivation. The Fruit Production Research and Consulting Station (http://www.ujfehertokutato.hu/index.html) was established after World War II in Újfehértó (Hungary), where Professor Endre Probocskai and the first director Lajos Dániel played a substantial role. One of the Station's tasks was to set up and maintain a cultivar collection. The collection, started with 604 cultivars back then, now consists of more than 950 apple cultivars and 180 apply hybrids.

The issue of which cultivar to use is extremely important for both grapes and apples, and determines everything for years or decades ahead. The task of identifying the genetic bases, collecting the cultivars and varieties, and examining their origin and properties – known as *germplasm management* – is a priority research area in Europe. The use of a simple and clear identification system avoids the errors of vegetative propagation, allows the users to identify the parents of the progeny (origin testing) and permits the setup of the registration framework of new

cultivars. A knowledge of the genetic background as well as the quick and precise identification of the cultivars are important also for the development and implementation of efficient breeding programs.

In addition to identification methods based on ampelographic and pomological features, such molecular genetic methods were also developed in the 20th century that characterize plant cultivars with DNA markers based on the DNA sequence. Cultivar description on the basis of DNA sequence was revolutionized by the PCR technology. Actually, it enables scientists to compare the coding and non-coding genome sections through their amplification. One of such PCR-based genotyping methods is called microsatellite analysis, which is based on the specific amplification of the repeat sequences making up most of the plant genomes. The success of this widely used analysis may be a major contributor to collection and maintenance works and also a useful component of the breeding programs.

Thomas and Scott (1993) were the first to report about the production of grape-specific microsatellite markers and their successful application for genotyping. They proved it through tests that the produced microsatellite markers showed co-dominant Mendelian inheritance and a high level of DNA polymorphism in grapevine. Based on those results, Thomas *et al.* (1994) made a proposal for a microsatellite database to be established under international cooperation to lay down the foundations of a universal cultivar identification system using standardized microsatellite markers.

Guilford *et al.* identified the first microsatellite markers in apple in 1997. The markers showed a high level of polymorphism among the studied cultivars. According to the results, only 6 SSR markers (with an average heterozygosity above 0.7 in the study) are sufficient to identify the vast majority of apple cultivars, though the close relatives may require a higher rate (Guilford *et al.* 1997, This *et al*. 2004). In several grape and apple producing countries the microsatellite markers are used to identify cultivars, distinguish clones, study cultivar collections or identify synonyms/homonyms (Maletic *et al.* 1999, Lopes *et al.* 1999, Laurens *et al.* 2004, Hvarleva *et al*. 2004). Besides, these markers are used also for the purposes of origin testing (Bowers and Meredith 1997, Sefc *et al*. 1998, Dettweiler *et al.* 2000).

During the past few years several online microsatellite databases have been set up for the storage and publishing of microsatellite data, including Greek Vitis Database (Lefort and Roubelakis-Angelakis 2000) and GrapeMicrosatelliteCollection (Grando *et al.* 2002).

Grape genomic research started in 2000 in Hungary. Kiss *et al.* (2003) reported about the testing of RAPD, SCAR and microsatellite markers used for the molecular characterization of grapevine cultivars indigenous to the Carpathian Basin. Pál Kozma *et al.* (2003) used RAPD and

microsatellite markers for the origin testing of ‘Csaba gyöngye’. According to these preliminary results, the crossing of ’Bronnerstraube’ and ’Ottonel muskotály’ could not have produced ’Csaba gyöngye’. By using RAPD primers, Bisztray *et al.* (2003) experienced useful polymorphism suitable for distinguishing cultivars and clones.

Objectives

1. To identify the old *Vitis vinifera* L. cultivars of the Carpathian Basin with microsatellite markers.
2. To use the obtained DNA fingerprints in order to map genetic diversity and parental links and to identify parent-progeny relationships.
3. To verify the origin of ‘Csaba gyöngye’ grape cultivar bred in Hungary and known worldwide as an early cultivar.
4. To study cultivars of known origin, such as Mátrai muskotály’ and ‘Irsai Olivér’, in order to prove the reliability of microsatellite analyses.
5. To establish and publish the microsatellite database of Hungarian grape cultivars.
6. To use microsatellite markers for the determination of the DNA fingerprints of important apple cultivars (*Malus* × *domestica* Borkh.) available also in Hungary and involved in commercial cultivation.
7. To use the obtained data for parent-progeny relationship analysis.
8. The establish and publish the microsatellite database of Hungarian apple cultivars.
9. To characterize the microsatellite markers involved in the analyses, including those applied for apple and primer pairs Scu8 and Scu10 studied for grapevine that were used for the first time by us to study a large collection.

MATERIALS AND METHODS

Plant materials

The study was carried out with 105 grapevine cultivars obtained from the Institute of Viticulture and Enology. These included 97 old cultivars from the Carpathian Basin 5 international cultivars (‘Pinot noir’, ‘Chardonnay’, ‘Ottonel muskotály’, ‘Heunisch weiss’ and ‘Bronnerstraube’), 3 cultivars bred in Hungary: ‘Csaba gyöngye’, ‘Irsai Olivér’ and ‘Mátrai muskotály’ (Annexes: Table 3).

The 66 apple samples (*Malus* × *domestica* Borkh.) were obtained from the collection of the Fruit Production Research Institute of Újfehértó (Annexes: Table 4).

DNA isolation

The DNA of grapevine and apple samples was extracted with DNeasy® Plant Mini kit (Qiagen), according to the manufacturer’s protocol. DNA quality was examined by running on 1.2% agarose gel with ethidium bromide dye and then DNA concentration was measured with a Nanodrop ND-1000 spectrophotometer.

Microsatellite markers used for the study

Grapevine

For general cultivar identification the following six microsatellite markers were used: Scu08, Scu10 (Scott et al. 2000), VVMD21, VVMD36 (Bowers *et al.* 1996, 1999), ssrVrZAG64, ssrVrZag79 (Sefc *et al.* 1999). The primer pairs were selected partly upon the proposal of the GENRES#81 EU project (Dettweiler and This 2000) and partly on the basis of our preliminary test results (Kiss *et al.* 2003). We were the first to use “Scu” markers for large-scale sample analysis, while many literature results are available for the other markers.

The following 21 SSR markers were involved for pedigree analysis: VVIb23, VVIh54, VVIi51, VVIm11, VVIt14 (Merdinoglu *et al.* 2005), VMC4c6, VMC4g6 (Di Gaspero *et al.* 2000), VMC6d12 (Arroyo-Garcia and Martinez-Zapater 2004), ssrVrZAG21, ssrVrZag25 ssrVrZag47, ssrVrZag62, ssrVrZag83, ssrVrZag112 (Sefc *et al.* 1999), VVMD5, VVMD7 (Bowers *et al.* 1996), VVMD25, VVMD28, VVMD31, VVMD32 (Bowers *et al.* 1999), VVS2 (Thomas and Scott 1993).

Apple

The six applied pairs of oligonucleotide primers (CH03g07, CH04e03, CH04g10, CH05c02, CH05d11 and CH05e03) have been identified by Liebhard *et al.* (2002).

PCR conditions

Grapevine

The polymerase chain reactions were performed in GeneAmp 9700 thermal cycler (ABI Perkin-Elmer) and Bio-Rad iCycler apparatus. Final volume of reaction mixture: 25 µl. Composition of reaction mixture: 20 ng template DNA, 1µM of both primers, 75 µM/dNTP (dATP, dCTP, dGTP, dTTP), 2 mM $MgCl_2$, 1 x PCR buffer (Promega) and 1 U *Taq*-polymerase (Promega).

Reaction conditions: pre-denaturation for 4 minutes at 95°C followed by 36 cycles of denaturation for 20 seconds each at 95°C; primer annealing for 30 seconds at 56°C; elongation and chain extension for 1 minute at 72°C. The amplification process was closed with a cycle for 5 minutes at 72°C.

Apple

The PCR reactions were performed in Perkin-Elmer GeneAmp 9700 and Bio-Rad iCycler apparatus. Final volume of reaction mixture: 20 µl. Composition of reaction mixture: 50 ng template DNA, 1 x reaction buffer (Sigma), 0.9 mM $MgCl_2$ (in addition to that present in the buffer), 200 µM/dNTP, 0.3 µM of both primers and 1.2 U Red-Taq DNA polymerase (Sigma).

The cycling profile of the reaction was as follows: pre-denaturation for 2 minutes followed by 35 cycles of denaturation for 20 seconds each at 94°C; primer annealing for 30 seconds at 56°C and chain extension for 60 seconds at 72°C. The amplification process was closed with a cycle for 5 minutes at 72°C.

Detection of PCR products

The forward primers of both grapevine and apple were labeled with Cy-5 fluorescent dye (IDT, Inc./ BioSciences). The PCR products were separated on a 8% denaturing polyacrylamide gel (Reprogel High Resolution, Amersham Bioscience, Uppsala, Sweden). The PCR products were detected with Automatic Laser Fluorometer (ALFexpress II DNA analyser, Amersham Biosciences). The allele sizes were determined with ALFexpress™ sizer™ 50-500 (Amersham Biosciences) and with our own standards using the ALFwin Fragment Analyser 1.03 software (Amersham Biosciences).

Statistical evaluation

In the case of both grapevine and apple the IDENTITY 1.0 software (Wagner és Sefc 1999) was used to perform the statistical evaluation of the microsatellite markers and the population. This software was used to calculate the probability ratios in terms of parent-progeny relationships and the PIC values for marker comparisons (Anderson 1993) assisted by the PowerMarker 3.5 software (Liu és Muse 2005).

The minimum number and combination of markers required for separation were determined on the basis of the calculations of Tessier *et al.* (1999). For such purpose the somatic mutants of the same microsatellite fingerprint were treated as a single genotype. Date were handled with the use of Microsoft Excel.

Cluster and discriminant analysis

For the cluster analysis the data were converted into binary codes. The analysis was performed with the UPGMA method based on Jaccard's similarity coefficients (Jaccard 1908). A dendrogram was constructed to show the results. The calculations were made with the SPSS 11.0 (Microsoft)® software where "Between group linkage" corresponds to the UPGMA method. The relationship between the microsatellite fingerprints of the cultivars and their belonging to different cultivar groups was studied through discriminant analysis, also with the help of the SPSS 11.0 software.

Creation of databases

The databases were created with the Microsoft® Access database management system. Macromedia Dreamweaver 8.0 software was used for the web design work.

RESULTS

Microsatellite analysis of grapevine cultivars

The 105 cultivars under study were genotyped with the Scu8vv, Scu10vv, VVMD21, VVMD36, ssrVrZag64 and ssrVrZag79 primer pairs. The amplified fragment sizes determined with ALF Express II are shown in Annexes: Table 3.

By using the international cultivars under study ('Pinot noir', 'Heunisch weiss' 'Chardonnay') as a reference, we were able to compare our data with the results of other research groups. The allele size differences deriving from the different methods and laboratory conditions can be avoided with uniform data coding (This *et al.* 2004).

The applied SSR markers showed high polymorphism in the studied cultivars. A total of 44 polymorph fragments were amplified with the 6 primer pairs, from 3 to 11 per locus. The average number of alleles was 7.33/marker. The observed heterozygosity averaged 0.69. The microsatellite fingerprints show a high genetic diversity of the genotypes. The probability of obtaining the same genotype for two randomly selected individuals is as follows for all markers: $PI_{total} = 7.62x10^{-5}$ (Paetkau *et al.* 1995) and $PI=4.9x10^{-4}$ (Tessier *et al.* 1999). It means that the applied markers are able to distinguish a large number of cultivars.

As to information content, the order of the applied microsatellite markers is as follows: VVMD36ssr ~ VrZag79> ssrVrZag64 > VVMD21 > Scu10vv > Scu8vv. Being the least informative, Scu8vv has a very low heterozygosity value (Table 1) and very poor separating ability. The reason is that this primer pair amplified only 3 fragments of different sizes, one of which (185 bp) has an approximate presence of 88% in the cultivars under study. This marker is not suitable for the purpose of further *Vitis vinifera* L. cultivar identification studies. However, 'Halápi' displayed an individual fragment size, which may ensure quick cultivar identification. Scu10 (which was also used for the first time for large population study) performed averagely but it may be a useful supplementary marker in the study of parent-progeny relationships.

Table 1: Statistical data obtained with 6 microsatellite markers in genotyped grapevine population.

Locus	Allele number	Probability of identity (PI)	Expected heterozygosity (He)	Observed heterozygosity (Ho)	Probability of parent exclusion	Estimated frequency of null alleles	PIC
Scu8vv	3	0.67	0.22	0.23	0.10	-0.007	0.19
Scu10vv	6	0.25	0.69	0.76	0.43	-0.044	0.63
VVMD21	5	0.23	0.69	0.64	0.45	0.028	0.64
VVMD36	11	0.11	0.80	0.83	0.62	-0.016	0.78
ssrVrZag64	9	0.13	0.79	0.85	0.58	-0.036	0.75
ssrVrZag79	10	0.11	0.80	0.83	0.62	-0.018	0.77

Cumulative probability of identity) (PI_{tot}): 7.62e-005

Separation of grapevine cultivars

97 of the 105 cultivars produced individual fingerprints, while the applied method was not able to distinguish 4 pairs (berry color versions) –'Gohér fehér'-'Gohér piros', 'Lisztes fehér'-'Lisztes piros', 'Piros bakator'-' Tüdőszínű bakator', 'Furmint'-'Furmint piros'. The literature cites several examples of the failure to distinguish color versions or clones with SSR markers (Bowers *et al*. 1996, Gribaudo *et al*. 2006).

The old Hungarian cultivars are cited at such synonym designations in former literature, ampelographies (Németh 1967, 1970; Hajdu 2003) and the international VIVC database (www.vivc.bafz.de) which are listed also as independent cultivar names: 'Aprófehér': 'Bálint', 'Sárfehér'; 'Bakator piros': 'Rózsaszőlő'; 'Balafánt': 'Kéknyelű', 'Pikolit'; 'Bálint': 'Sárfehér'; 'Beregi': 'Rózsaszőlő'; 'Ezerjó': 'Hárslevelű'; 'Csókaszőlő': 'Kadarka (kék)'; 'Furmint': 'Demjén', 'Kéknyelű', 'Fehér gohér'; 'Fehér gohér': 'Bajor', 'Kolontár', 'Kozma'; 'Hosszúnyelű': 'Budai', 'Izsáki': 'Szőkeszőlő', 'Vékonyhéjú'; 'Juhfark': 'Hosszúnyelű', 'Sárfehér'; 'Királyszőlő': 'Lágylevelű'; 'Leányka': 'Leányszőlő'; 'Mézes (fehér)': 'Sárfehér'; 'Rakszőlő': 'Vékonyhéjú'; 'Vékonyhéjú': 'Polyhos'; 'Izsáki': 'Sárfehér'; 'Juhfark': 'Vékonyhéjú'; 'Kövidinka': 'Vörös dinka'; 'Szeredi': 'Sárpiros'; 'Tulipiros': 'Rózsaszőlő'.

The following cultivars have been studied also by other authors: 'Bajor kék', 'Gohér fehér', 'Furmint', 'Demjén', 'Ezerjó', 'Hárslevelű', 'Mézes', 'Sárfehér' (Kocsis *et al.* 2005), 'Gohér', 'Bajor', 'Kozma', 'Demjén', 'Furmint' (Bodor *et al.* 2008). Furthermore, straightforward conclusions can be made in view of the results of Bisztray *et al.* (2005), Jahnke *et al.* (2009), Varga (2009).

According to our data, the samples under study can be distinguished on the basis of their unique SSR fingerprints. This supports the fact that there were different cultivars and confirms the results of other research groups.

Cluster and discriminant analyses of grapevine cultivars

The data obtained with the 6 microsatellite markers were made subject to a cluster analysis, the results of which are shown on a dendrogram (Figure 1). It displays the structure of genetic variability and allows the study of parental relationships. Genetic similarity was found first in the case of those cultivars and varieties which could not be distinguished with the applied SSR markers. In the case of the 'Bakator' cultivars our results confirm that 'Tüdőszínű bakator' is a berry color version of 'Piros bakator' (Németh 1970) but 'Kék bakator' is genetically remote from these two cultivars. Actually, 'Sárfehér' and 'Sárpiros' are not the same cultivars but judged

from their SSR fingerprints, they may be close relatives (Németh 1970). Kocsis *et al.* (2005) applied RAPD analysis to verify the family relationship, assumed on the basis of their morphological similarity, between 'Bajor' 'Gohér' cultivars. This is supported by our results obtained with the 6 SSR markers.

The dendrogram fails to clearly show the parent-progeny relationships. Actually, only the 'Ottonel muskotály' – 'Mátrai muskotály' link and the 'Csaba gyöngye' - 'Irsai Olivér' link can be seen from it. However, it is an interesting coincidence that this group includes 'Csíkos muskotály', which means that there is a genetic relationship between the muskotály (muscatel) cultivars.

The result of the cluster analysis does not show any correlation with the natural classification of the cultivars. Thus a discriminant analysis was performed to see whether there is any link between the fingerprints and the classification into the geographical/ecological cultivar groups of the natural system. The cultivars were put into four groups for the analysis: 1: *Vitis vinifera* L. convarietas **occidentalis**, 2: *Vitis vinifera* L. convarietas **pontica**, 3: *Vitis vinifera* L. convarietas **orientalis** (according to Németh 1967), 4: the fourth group included the cultivars for which no classification information was available. The analysis generated two functions: the first and second account for 76.4% and 23.6% of the variance (100%), respectively. Based on the elements of the weighted coefficient vector associated with the functions, it was found that the strongest discriminating variables were the alleles (242, 258) of ssrVrZag79 in the case of both functions. However, this alone is not sufficient for determining which cultivar belongs to which group.

The function-based classification was the same (100%) as the original classification. This proves that, similarly to former results (Györffyné Jahnke 2006), the separation of geographical/ecological cultivar groups may have a genetic background. Thee non-classified cultivars may also be grouped by these discriminant functions. However, it should be treated with care as the discriminant functions were generated based on the genotype data of the classified cultivars under study, and it is true that oriental and occidental cultivar groups are represented by a low number of cultivars.

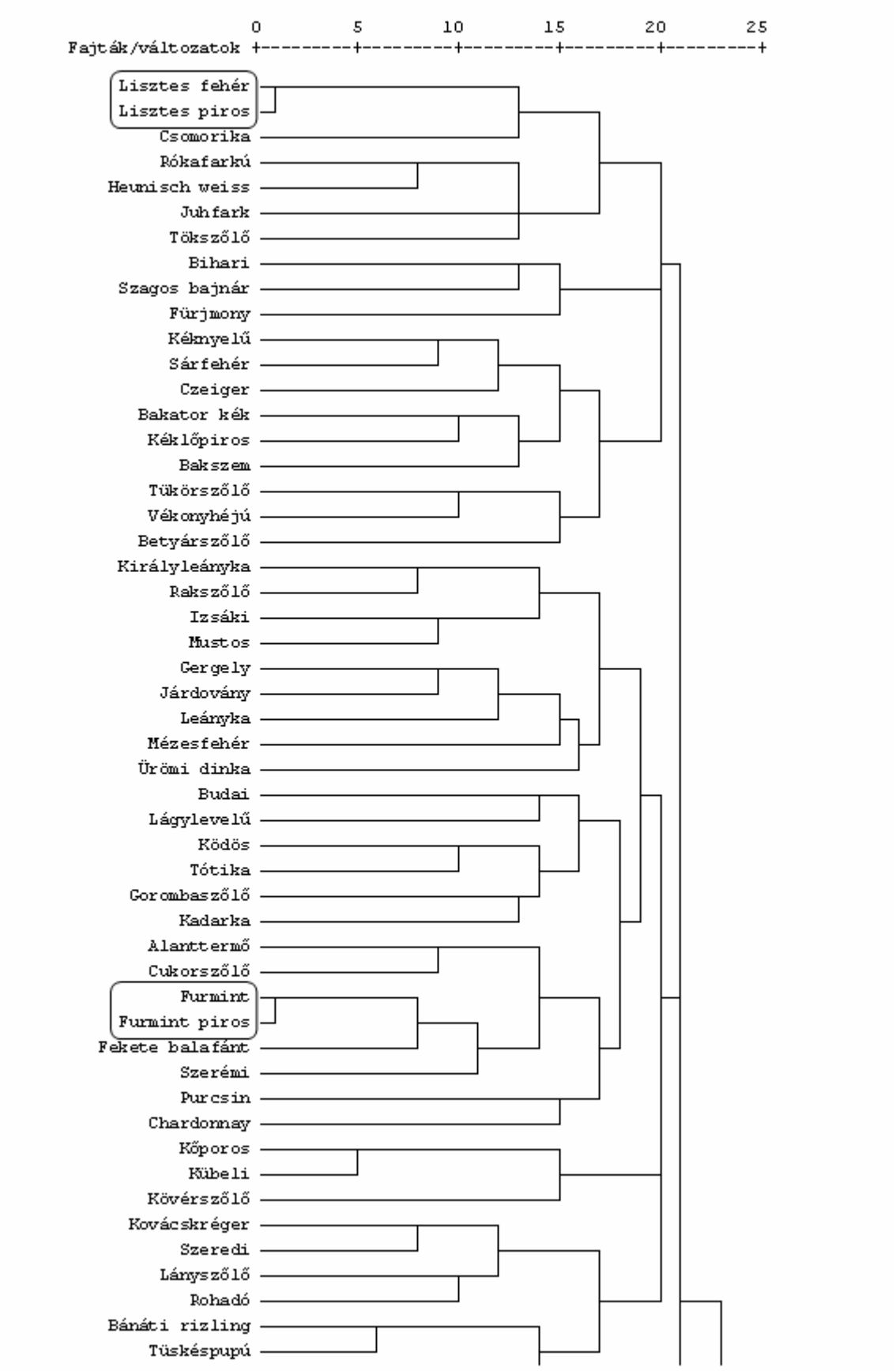

Figure 1: Cluster analysis results shown on dendrogram. 105 cultivars and 6 SSR markers (from similarity matrix based on Jaccard's coefficient).

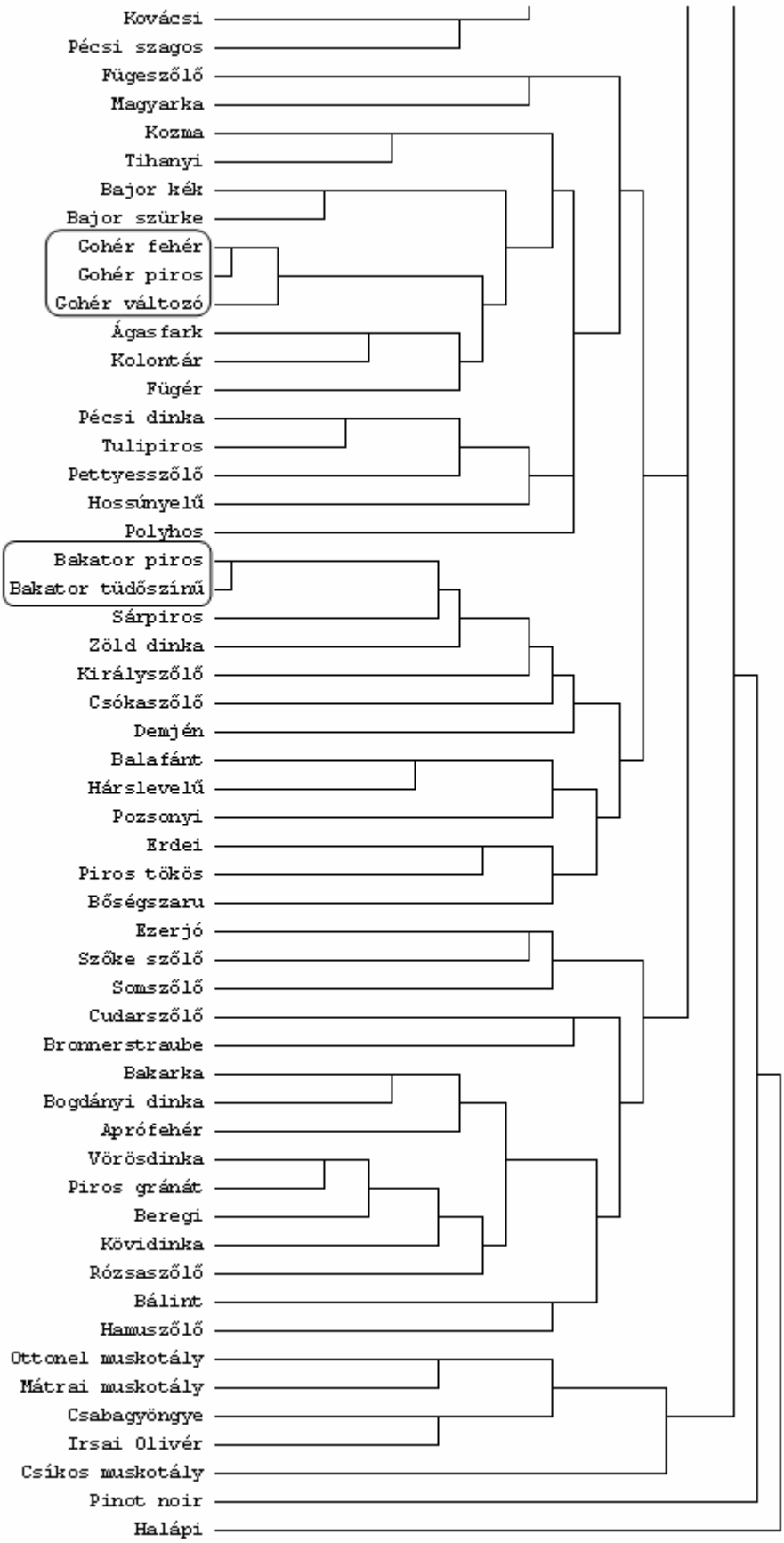

Figure 1 continued: Cluster analysis results shown on dendrogram. 105 cultivars and 6 SSR markers (from similarity matrix based on Jaccard's coefficient).

Pedigree analysis of selected grapevine cultivars

The study included reference cultivars of known pedigree for which breeding data are available. Mátrai muskotály' was produced by Pál Kozma in 1952 by crossing 'Izsáki' with 'Ottonel muskotály', while 'Irsai Olivér' was created by Pál Kocsis in 1930 by crossing 'Pozsonyi fehér' with 'Csaba gyöngye' (Csepregi and Zilai 1988, Hajdu 2003, Bényei and Lőrincz 2005). The tests with the 6 SSR markers produced several options for possible parent-progeny relationships in both cases, which means that 6 SSR microsatellite markers are not sufficient for the verification of parent-progeny relationships. Thus another 21 microsatellite markers were subsequently involved in the pedigree analysis of the two cultivars. The results support the breeding data and the literature information: 'Irsai Olivér' = 'Pozsonyi' x 'Csaba gyöngye', 'Mátrai muskotály' = 'Izsáki' x 'Ottonel muskotály'.

The study involved 'Leányka' and 'Kövérszőlő' cultivars which, according to literature information (Németh 1970, Csepregi and Zilai 1988, Hajdu 2003) are the presumed parents of 'Királyleányka'. 4 of the 6 SSR markers 4 (Scu10, VrZag79, VVMD21, VVMD36) excluded the parent-progeny combination of 'Leányka' x 'Kövérszőlő' = 'Királyleányka'.

'Csaba gyöngye' is a Hungarian cultivar with disputed origin and producer. According to most Hungarian authors, it was created by János Mathiasz by crossing 'Bronnerstraube' with 'Ottonel muskotály' (Németh 1975, Csepregi and Zilai 1988, Hajdu 2003, Bényei and Lőrincz 2005). However, our results excluded this option.

Microsatellite analysis of apple cultivars

In the case of the 66 apple cultivars under study 6 apple SSR primer pairs were used to amplify the microsatellite regions and to measure exact fragment sizes. A total of 55 polymorph fragments were amplified, from 6 to 13 per marker. The average number of alleles was 9.2 (Table 2).

Table 2: Statistical data of markers used for the apple cultivars. The allele number per marker refers to the 66 apple cultivars but for technical reasons the 'Akane', 'Pinova' and triploid cultivars were ignored for the calculation of other data.

Locus	Allele number	Probability of identity (PI)	Expected heterozygosity (He)	Observed heterozygosity (Ho)	Probability of parent exclusion	Estimated frequency of null alleles	PIC
CH03g07	7	0.18	0.73	0,83	0.50	-0.056	0.69
CH04e03	12	0.15	0.72	0.79	0.53	-0.039	0.70
CH04g10	6	0.35	0.47	0.35	0.28	0.082	0.45
CH05c02	7	0.21	0.62	0.69	0.42	-0.044	0.59
CH05d11	10	0.22	0.61	0.74	0.41	-0.081	0.58
CH05e03	13	0.07	0.84	0.79	0.68	0.024	0.82

Cumulative probability of identity) (PI_{tot}): 4.02e-005

The observed heterozygosity averaged 0.70. The probability of obtaining the same SSR fingerprint for two different and randomly selected cultivars at each locus under study is as follows: PI = 1.79 x 10^{-4} (Tessier *et al*. 1999) and 4.02 x 10^{-5} (Paetkau *et al.* 1995). It means that the selected six markers are able to distinguish a large number of apple cultivars. As to information content, the order is as follows: CH05e03 > CH04e03 > CH03g07 > CH05c02 > CH05d11 > CH04g10.

In the case of five cultivars at least one marker produced three clearly distinguishable allele forms on the SSR fingerprint. It means either multiple loci or triploidy. However, only 5 of the 66 cultivars are real triploids ('Charden', 'Jonagold', 'Mutsu', 'Red Stayman' and 'SirPrize'). As to 'Akane' and 'Pinova', the presence of a third allele may be explained by another locus. Presumably, these are not unexpected artifacts since the observed patterns were the same in all replications. The possibility of amplification from multiple loci was also observed by Guilford *et al.* (1997) and Liebhard *et al.* (2002).

Separation of apple cultivars, cluster analysis, parental relationships

Except for cultivars born through bud mutation, we were able to distinguish the cultivars. The somatic mutants originating from the same cultivar could not be distinguished from each other and their "parents". All SSR data were used for the cluster analysis and a dendrogram was constructed on the basis of the similarity matrix (Figure 2). The dendrogram show some coincidences. The cultivars where one of the parents is 'Golden Delicious' were gathered in the same group (cluster). The triploid 'Jonagold' was listed between 'Jonatán' and 'Golden Delicious' but was closer to the latter (it may have received two alleles from the latter). Close parental relationship is visible also between 'Idared' and 'Jonathan'. The Hungarian 'Mizsei' of unknown origin shows the greatest similarity with 'Red Rome Van Well'. However, no far-reaching conclusions can be drawn as the applied six markers are not sufficient for exact origin analyses.

As to the studied cultivars, there is only one case where a cultivar and both its parents are included in the study since 'Jonagold' is the hybrid of 'Golden Delicious' and 'Jonathan'. It enables us to support the exactness of the obtained data. In this particular case it produced the expected result i.e. the alleles of 'Jonagold' are present, in the corresponding combination, also in the parents.

In special cases even the male and the female parents can be indentified. It can be determined which of the two parents gave two alleles to triploid 'Jonagold' since it may have received the two 198-bp alleles at the locus CH04e03 only from 'Golden Delicious', while it inherited only one 186-bp allele from 'Jonathan', presumably with this latter cultivar's pollen.

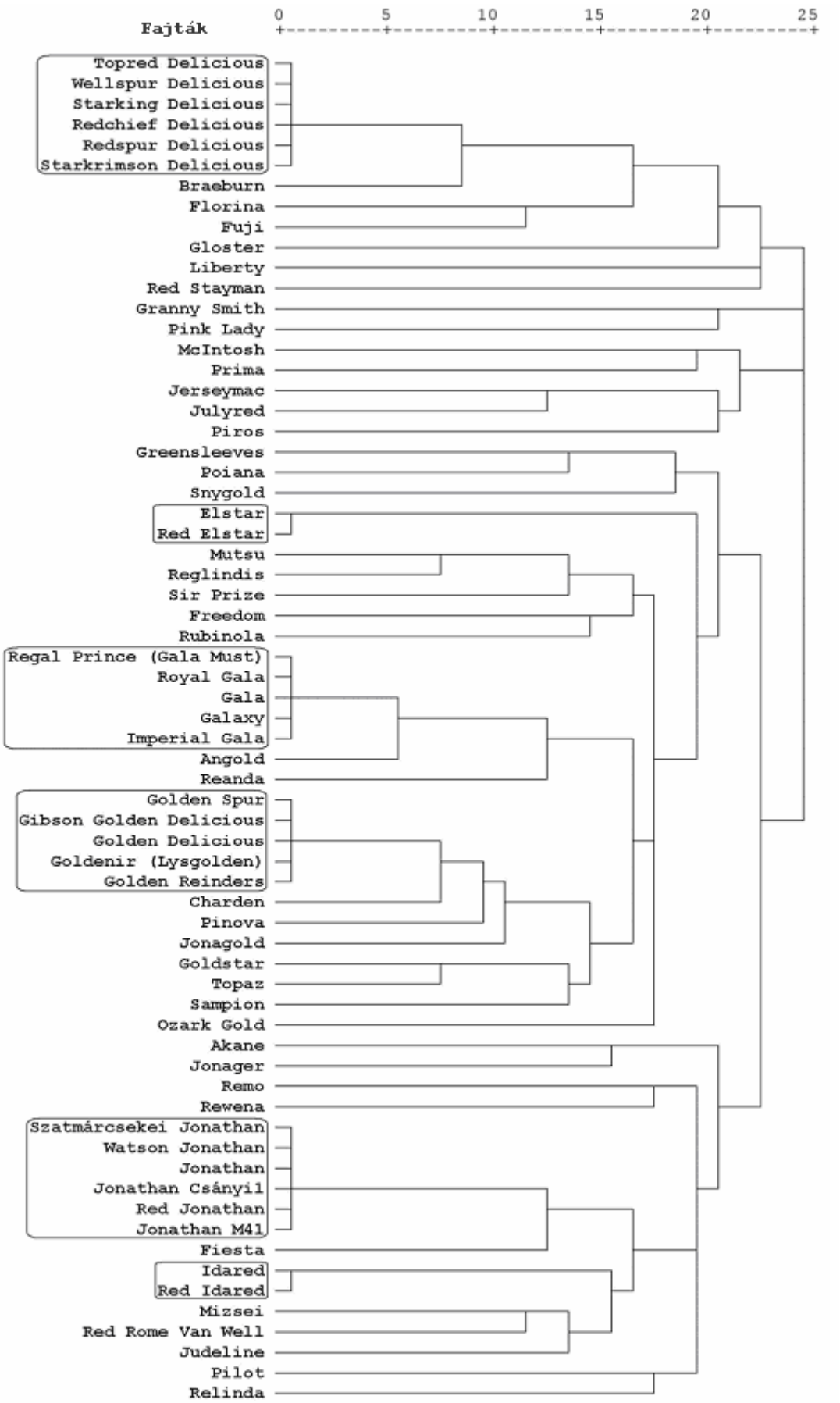

Figure 2: Cluster analysis results of apple cultivars shown on dendrogram.

Creation of databases

A large data volume has been generated as a result of the large number of markers, cultivars and varieties. For an easier data handling, we have created a database for internal use. Data to this database can be uploaded either through forms or the traditional design method. After that the data can be queried in any combination with the help of fields and records.

In order to facilitate their access and use, we have posted the public microsatellite data on the internet: (http://www.mkk.szie.hu/dep/gent/HVSDProject/HVSD.html, http://www.mkk.szie.hu/dep/gent/Alma%20mikroszatellit/HASD.html) (Figure 3 and 4).

Figure 3: Start page of the grapevine microsatellite database. (http://www.mkk.szie.hu/dep/gent/HVSDProject/HVSD.html)

Figure 4: Start page of the apple microsatellite database (http://www.mkk.szie.hu/dep/gent/Alma%20mikroszatellit/HASD.html)

The information may be queried on the basis of marker names or alphabetically arranged cultivar names. This online access enables the user to query the entire cultivar list if required.

New scientific results

1. We were the first to determine the DNA fingerprints of 97 old Carpathian Basin cultivars and ’Irsai Olivér’, ’Mátrai muskotály’, ’Bronnerstraube’ on 6 microsatellite loci.
2. We have proved that the selected microsatellite markers are able to distinguish and identify the cultivars that are indigenous to or have been cultivated for a long time in the Carpathian Basin (except for berry color versions).
3. With the use of only 6 microsatellite markers we have proved that ‘Csaba gyöngye’ cannot be the direct progeny of ‘Bronnerstraube’ and ‘Ottonel muskotály’.
4. Through the results of only 6 SSR markers we have proved that ‘Királyleánya’ cannot originate from the crossing of ‘Leánya’ x ‘Kövérszőlő’, although our results did not rule out ‘Leánya’ as a possible parent.
5. With the use of molecular markers (27 SSR) we have confirmed that ‘Irsai Olivér’ originates, in line with literature data, from the crossing of ‘Pozsonyi fehér’ and ‘Csaba gyöngye’, while ‘Mátrai muskotály’ is the result of the combination of ‘Izsáki’ x ‘Ottonel muskotály’.
6. We have posted our grapevine microsatellite data in a web-based database to create the first online Hungarian Vitis Microsatellite Database.
7. We have determined the microsatellite fingerprints of 66 important apple cultivars in commercial cultivation on 6 SSR loci.
8. We have proved that it is sufficient to use 6 carefully selected microsatellite markers to separate apple cultivars or cultivar groups. However, even such 6 markers are unable to distinguish the bud mutants within the cultivar groups.
9. We have posted our apple microsatellite data in a web-based database to create the first online Hungarian Apple Microsatellite Database).

DISCUSSION AND SUGGESTIONS

Our results clearly prove that microsatellite markers can be efficiently used to perform the molecular separation and comparison of the cultivars and to identify homonyms and synonyms. However, the markers are unable to distinguish the close relatives (bud mutants).

We have not found any redundancy or synonym/homonym in the range of cultivars under study. In fact, the individual fingerprints have enabled us to distinguish the cultivars (except for berry color versions and bud mutants).

The microsatellite markers showing a low polymorphism among the cultivars (in this case Scu8) are not suitable for further studies. The information content of the microsatellite markers greatly depends also on the composition of the population under study. Except for Scu8, the markers have proved to be suitable for our studies.

In line with the findings of Sefc *et al.* (2001), our results obtained with apple and grapevine have proved that 5-6 SSR markers are sufficient to distinguish the cultivars. However, pedigree analyses require the use of at least 25 markers.

The DNA fingerprints obtained through microsatellite markers excluded the birth of ‘Királyleánya’ as a result of the spontaneous crossing of ‘Kövérszőlő’ and ‘Leánya’. As to all three cultivars, we have examined the samples maintained at the Institute of Viticulture and Enology in Pécs and collected at the Tarcal station of the Ministry’s Institute of Viticulture and Enology, and we have obtained the same results for each cultivar. However, the cultivar collection of the Corvinus University contains two individuals with different allele patterns but under the same name of ‘Királyleánya’ (Bisztray *et al*. 2005). In view of that it would be important, for further origin testing and cultivar maintenance works, to perform an overall and uniform study and comparison of other collections, with the use of both molecular and morphological markers, kept in Hungary and the Carpathian Basin in order to arrive at reliable conclusion.

The pedigree analysis of ‘Csaba gyöngye’ has excluded the direct parent link of ‘Bronnerstraube’ and ‘Ottonel muskotály’. Further studies should be made with the involvement of cultivars referred to in various theories as well as other cultivars of the Carpathian Basin and the former vineyard of Adolf Stark so that the origin of ‘Csaba gyöngye’ can be clarified.

In the case of ‘Királyleánya’ and ‘Csaba gyöngye’ 6 microsatellite markers were enough to exclude the presumed parent-progeny relationship. However, with regard to the entire cultivar list and, in particular, to old Hungarian and Carpathian Basine cultivars, there are still several unconfirmed options, the clarification of which would require the use of more SSR primer pairs.

Nevertheless, using a large number of microsatellite markers (27), the pedigree analysis of ‘Irsai Olivér’ bred in 1930 and ‘Mátrai muskotály’ created in 1952 has confirmed the origin of such cultivars also at DNA level. It means that the method is clearly suitable for mapping parent-progeny relationships and, as a result, the pedigrees assumed or available in the case of ‘Királyleánynka’ and ‘Csaba gyöngye’ are invalid.

The parent-progeny relationships were confirmed by the SSR data also in the case of apple. Actually, the microsatellite markers may enable researchers to verify the origin of hybrids of unknown origin (Cabe *et al*. 2005; Kitahara *et al*. 2005). The relationship of ‘Jonagold’ = ‘Jonathan’ x ‘Golden Delicious’ was proved by the results obtained on the 6 microsatellite loci under study. In special cases even the male and female parents can be identified.

Cluster analysis is able to display the parent-progeny relationships only to a limited extent and, in the case of grapevine, it does not show any correlation with the natural classification of the cultivars. Nevertheless, the discriminant analysis has shown a relationship between the genotypes verified through microsatellite markers and the geographical/ecological cultivar groups. It is an evidence that the separation of cultivar groups may have a genetic background.

Through the involvement of international reference cultivars we have proved that our results are reliable and that the obtained data are suitable for comparison with the findings of other laboratories. The absolute allele sizes are not suitable but the data compared to reference cultivars are suitable for such examinations.

The microsatellite markers are unable to distinguish the bud mutants from each other or from the base cultivar in either apple or grapevine. The average heterozygosity and the PIC values are almost the same and cumulative PI is almost similar for the two species, indicating their similar genetic diversity (Table 1 and 2). However, it is important to note that these values greatly depend on the applied markers and the composition of the population under study.

The genotyping of grapevine and apple cultivars with molecular markers is extremely important both in breeding programs and for maintaining the diversity of genetic resources. The microsatellite analysis results described above are suitable for performing a genotype identification or distinction and pedigree verification for both species in an objective manner.

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ANNEXES

Table 3: Fragment sizes detected with 6 SSR markers in 105 grapevine cultivars (the old Hungarian cultivars involved in commercial cultivation are underlined).

Cultivar	Scu8	Scu10	Vvmd21	Vvmd36	ssrVrzag64	ssrVrzag79
Alanttermő	185	202:208	250:259	254:276	161:165	254:260
Aprófehér	185	208:214	250	264:266	141:145	246:254
Ágasfark	185:192	202	244:250	254:264	145:165	252:262
Bajor kék	185:192	202:208	250:257	252	145:165	252:262
Bajor szürke	185:192	202:208	250:257	254	145:165	252:262
Bakarka	185	214	244:250	264:266	141:145	254
Bakator kék	185	202:208	250	264	141:165	252:262
Bakator piros	185	202:208	244:257	266:288	145:165	254
Bakator, tüdőszínű	185	202:208	244:257	266:288	145:165	254
Bakszem	185:192	202:208	250	252:264	141:165	240:262
Balafánt	185:192	202	244:259	276:288	145:165	240:254
Balafánt, fekete	185:192	202	250	254:276	161:165	252
Bálint	185:192	208:214	250:259	264:276	141:145	252:254
Bánáti rizling	185	208:211	250:257	254:288	161	254:262
Beregi	185	208:214	244:250	254:288	139:145	254:262
Betyárszőlő	185	202:214	250:257	264:266	139:165	262
Bihari	185	202:205	250	264	141:161	250:262
Bogdányi dinka	185	214	244:250	264:266	139:145	254:262
Bőségszaru	185	202:205	244:250	276:296	145:165	248:252
Bronnerstraube	185	202:214	250	244:296	141:145	248:254
Budai	185:192	208:214	250	254	141:165	252
Chardonnay	185:192	205:214	250	254:276	161:165	246:248
Cudarszőlő	185	208:214	250	244:254	145	242:254
Cukorszőlő	185	202:208	250:259	254:276	141:161	254:262
Csaba gyöngye	185	205:214	244:267	264:296	161	258:262
Csíkos muskotály	185	208:217	250	244:264	143:161	254:258
Csókaszőlő	185	202:208	257	288	161:165	240:254
Csomorika	185	208:211	257	288	141:145	240:262
Czeiger	185	202:208	250	264:288	139:165	254
Demjén	185	202	244:257	254:288	141:165	254:262
Erdei	185	202:214	244:250	264	145:165	246:254
Ezerjó	185	202	244:250	258:276	139	240:254
Furmint	185:192	202:208	250:259	254:276	161:165	240:252
Furmint, piros	185:192	202:208	250:259	254:276	161:165	240:252
Fügér	185:192	208	244	254:264	141:145	252
Fügeszőlő	185:192	208	244	264:288	145	240:252
Fürjmony	185:192	205:208	250:257	254:264	141:161	250:254
Gergely	185	208:214	244:250	266:276	159:165	240:254
Gohér fehér	185:192	202:208	244:257	254:288	141:145	252:262
Gohér piros	185:192	202:208	244:257	254:288	141:145	252:262
Gohér változó	185:192	202:208	244:257	254:288	145	252:262
Gorombaszőlő	185	208:214	250:259	254:266	139:145	252
Halápi	188	208:217	244:267	244:254	141:157	252:258
Hamuszőlő	185	208	250	264:276	139:141	248:254
Hárslevelű	185	202:208	244:259	264:276	145:165	240:254
Heunisch weiss	185	208:214	250	264:276	161	240:246
Hosszúnyelű	185	208:214	244:257	254:288	141:145	240:254
Irsai Olivér	185	205:214	244	264:296	139:161	254:258
Izsáki	185	208:214	244:250	254:276	139:161	240:246
Járdovány	185	208:214	244:250	266:276	141:161	240:254
Juhfark	185	208	250:257	264:276	141:165	240:252

Table 3 continued: Fragment sizes detected with 6 SSR markers in 105 grapevine cultivars (the old Hungarian cultivars involved in commercial cultivation are underlined).

Cultivar	Scu8	Scu10	Vvmd21	Vvmd36	ssrVrzag64	ssrVrzag79
Kadarka	185	208:214	250	266:276	145:165	252
Kéklőpiros	185	202:208	250:257	264:270	159:165	252:262
Kéknyelű	185	202:208	244:250	252:264	159:165	252:254
Királyleányka	185	208:214	244:250	254:266	161	252:254
Királyszőlő	185	202:208	250:259	266:288	145:165	254:262
Kolontár	185:192	202:208	244:250	254:264	141:145	252:262
Kovácsi	185:192	208	257	264:288	161	254
Kovácskréger	185	202:211	250:257	254	145:161	252:254
Kozma	185	202:208	257:267	254:264	141:145	262
Ködös	185	208	257:259	254:276	145:165	252
Kőporos	185	208:214	257:259	264:266	145:165	254:260
Kövérszőlő	185	208	250:259	264:266	145:161	240:254
Kövidinka	185	208:214	244:250	264	139:141	254:262
Kübeli	185	208:214	257:259	264:266	161:165	254:260
Lányszőlő	185	208:211	250:257	254:276	161	252:254
Lágylevelű	185	202:214	250	254	165	252:254
Leányka	185	202:208	250	266:276	161:165	240:254
Lisztes fehér	185	208	250:257	276:288	141:161	240:262
Lisztes piros	185	208	250:257	276:288	141:161	240:262
Magyarka	185:192	208	244:250	264:288	145:165	248:254
Mátrai muskotály	185	214	244:267	264:276	139:161	240:258
Mézesfehér	185:192	208:214	250:257	266:276	141:165	254:262
Mustos	185	208:214	244:250	254:276	145:161	246:252
Ottonel muskotály	185	208:214	267	264:276	139:161	258:262
Pettyesszőlő	185	202:208	244	254:288	145:165	250:252
Pécsi dinka	185	202:208	244	254:288	141:145	252:254
Pécsi szagos	185	208:211	257:267	264:288	161	254:258
Pinot noir	185:192	205:217	250	254	141:165	242:248
Piros gránát	185	208:214	244:250	254:264	139:145	250:254
Piros tökös	185	202:214	244:250	276:288	145:165	252:254
Polyhos	185	202	244:259	254:288	145:161	252:262
Pozsonyi	185:192	202:214	244:259	264	139:145	254
Purcsin	185	208:214	250	254:276	161:165	250:258
Rakszőlő	185	208:214	244	254:266	139:161	254
Rókafarkú	185	208:214	250	264:276	141:165	240:246
Rohadó	185	208	250:257	264:276	145:161	252:254
Rózsaszőlő	185	208	244:250	264	139:145	246:254
Sárfehér	185:192	202:208	244:250	264	139:165	252:254
Sárpiros	185	202:208	244	264:288	145:165	254:260
Somszőlő	185	202:214	244:250	252:276	139:153	252:254
Szagos bajnár	185	205:208	250	264:288	139:161	250:262
Szeredi	185	202	250:257	254:276	145:161	252
Szerémi	185	202:208	250	276	161:165	252:258
Szőke szőlő	185	202:208	244:250	272:276	139:145	254:260
Tihanyi	185	208	257:267	254:264	145	252:262
Tótika	185	208:214	250:257	254:276	145:165	252:254
Tökszőlő	185	208:214	257	264:276	161:165	240:262
Tulipiros	185	208	244	254:288	145	252:254
Tükörszőlő	185	202:214	250	254:264	161:165	246:262
Tüskéspúpú	185	208:211	257	254:288	145:161	254:262
Ürömi dinka	185	214	250	266:276	145:161	246:254
Vékonyhéjú	185	202:208	250	264:276	161:165	246:262
Vörösdinka	185	208:214	244:250	254:264	139:145	254:262
Zöld dinka	185	202:208	244:257	264	145	254

Table 4: 66 apple cultivars under study and their base pair allele sizes obtained with six microsatellite markers.

Cultivar	CH03g07	CH04e03	CH04g10	CH05c02	CH05d11	CH05e03
Akane	123:179	196:210:216	135:143	170:200	173	185
Angold	119:129	198	135	168:170	171:173	173:185
Braeburn	127:129	198:202	168	168	171:173	191
Charden	119:129	198	135	168:174:200	169:173:175	175:179:185
Elstar	119	190:198	135	168:170	173:187	164:179
Red Elstar	119	190:198	135	168:170	173:187	164:179
Fiesta	119:123	186:196	135	168	173:197	164:185
Florina	123:127	196:198	135:168	168:200	173:197	163:191
Freedom	129	198	135	168	173:187	179:191
Fuji	119:127	196:198	143:168	168	173:197	163:191
Gala	119:129	196:198	135	168:170	173	173:185
Galaxy	119:129	196:198	135	168:170	173	173:185
Imperial Gala	119:129	196:198	135	168:170	173	173:185
Regal Prince (Gala Must)	119:129	196:198	135	168:170	173	173:185
Royal Gala	119:129	196:198	135	168:170	173	173:185
Gloster	123:129	198	127:137	160:168	195:197	160:191
Golden Delicious	119:129	198	135	168:174	169:173	179:185
Goldenir (Lysgolden)	119:129	198	135	168:174	169:173	179:185
Golden Reinders	119:129	198	135	168:174	169:173	179:185
Golden Spur	119:129	198	135	168:174	169:173	179:185
Gibson Golden Delicious (Smoothee)	119:129	198	135	168:174	169:173	179:185
Goldstar	119:129	190:198	135:143	168:170	173	185:193
Granny Smith	129:153	196:198	127:137	160:172	171:173	168:181
Greensleeves	129	190:198	135:143	170:174	171:173	173:185
Idared	123:129	186:198	135	168:200	173:197	172:185
Red Idared	123:129	186:198	135	168:200	173:197	172:185
Jerseymac	165	184:198	135:137	160:176	173:197	163:173
Jonager	123:127	196	135	170:200	173:175	168:185
Jonagold	119:123:129	186:198	135	168:174:200	169:173:175	163:179:185
Jonathan	119:123	186:196	135	168:200	173:175	163:185
Jonathan M41	119:123	186:196	135	168:200	173:175	163:185
Jonathan Csányi1	119:123	186:196	135	168:200	173:175	163:185
Red Jonathan	119:123	186:196	135	168:200	173:175	163:185
Szatmárcsekei Jonathan	119:123	186:196	135	168:200	173:175	163:185
Watson Jonathan	119:123	186:196	135	168:200	173:175	163:185
Judeline	123:129	196:198	135	168:174	173	163:185
Julyred	165	184:198	135:143	160:176	175:197	163
Liberty	123:129	178:198	137	168	173:211	163:176
McIntosh	129:165	184:198	139:143	168	173:175	163
Mizsei	123:129	186:208	135	160:168	173	163:185
Mutsu	129:179	198	135	168	173	173:179
Ozark Gold	119:129	196:198	135:168	168:172	173	168:179
Pilot	129:179	196	135	168	173	164:185
Pink Lady	129	198:204	135:137	160:168	169:171	168:179
Pinova	119:127:129	198:222	127:135	168:174	169:173	163:179:185
Piros	165:179	196:198	135	168:176	173:175	173
Poiana	129	190:198	135:137	170:174	169:171	163:185
Prima	129:165	184:204	135:143	168:176	169:173	179:185
Reanda	119:129	196:208	135:137	168	173:181	173:185
Red Rome Van Well	123:129	186:198	135	168	173	163
Red Stayman	127:129	196:204	127:135	168:172	169:173	163:191
Reglindis	129:179	198	135	168	169:173	173
Relinda	123:129	196:208	135:143	168:172	173	172:185
Remo	119:123	178:190	135	168:170	173	163:172
Rewena	119:123	178:210	135	168	173	164:185
Rubinola	129	184:198	135	168:176	173	179:193

Table 4 continued: 66 apple cultivars under study and their base pair allele sizes obtained with six microsatellite markers.

Cultivar	CH03g07	CH04e03	CH04g10	CH05c02	CH05d11	CH05e03
Sampion	119:129	190:198	135	170:174	169:173	179:193
Sir Prize	119:123:129	198:204	135	168	169:173	173:179
Snygold	129	198	135	170:174	173:205	173:179
Starking Delicious	127:129	198:202	137:168	168	173:197	191
Starkrimson Delicious	127:129	198:202	137:168	168	173:197	191
Redchief Delicious	127:129	198:202	137:168	168	173:197	191
Redspur Delicious	127:129	198:202	137:168	168	173:197	191
Topred Delicious	127:129	198:202	137:168	168	173:197	191
Wellspur Delicious	127:129	198:202	137:168	168	173:197	191
Topaz	119:129	190:198	135	168	169:173	185:193

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