Doctoral (PhD) thesis

CHARACTERISATION OF APPLE CULTIVARS FROM THE CARPATHIAN BASIN BY MEANS OF POMOLOGICAL ANALYSIS AND MOLECULAR MARKER ANALYSIS BASED ON MICROSATELLITES

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INTRODUCTION

Apples are the fruit species grown in the third greatest quantity in the world after citrus fruits and bananas, but global apple production involves only a few dozen cultivars. Nowadays the role of local cultivars and land races in large-scale production has become completely insignificant. In the case of an ecological catastrophe (climate change, appearance of new pathogen species, etc.) this restricted range of cultivars could endanger the reliability and profitability of apple production.

Old apple cultivars have enormous value from the point of view of biodiversity. Some of these cultivars have good resistance or excellent inner quality, while others satisfy special consumer demands. The choice of fruit available on the market must be broadened if fruit consumption is to become more varied. This could be achieved not only by re-introducing old varieties into cultivation, but also by using them as crossing partners in the development of new cultivars.

Over the last decade surveys have begun in many countries on the diversity available in gene banks, thus making it possible to assess the genetic variety that once existed in the cultivar use of a given region and to determine similarities and differences in the cultivar composition compared with other regions. This work also casts light on accessions of the same cultivar that have been mistakenly documented due to the use of erroneous names. This requires the application of identical analytical methods, which are now being elaborated by ECPGR.

In addition to phenological and morphological descriptions, it is also advisable to carry out genotypic analysis. The determination of the genetic fingerprints of the cultivars is of importance not only for the identification of gene bank accessions, but also to check the trueness-to-type of reproductive materials in nurseries. Knowledge of the characteristic allele composition of individual cultivars also allows their origin to be checked. Well-chosen genetic markers can also be used to detect specific characteristics related to resistance, fertilisation, growth and fruit parameters.

The diversity analysis performed in gene banks makes it possible not only to identify the cultivars, but also to resolve pomological debates on questions such as synonymous cultivar names and the analysis of cultivar groups. Genetic analysis also plays a role in phylogenetic research. The analysis of the cultivars originating from or widespread in a particular region may provide answers to questions involving pedigrees and even origin, if a sufficiently large sample size is available.

The aim of the present work was to provide assistance for gene bank analysis and to achieve better knowledge of cultivar traits, which could be useful both for scientists and growers.

AIMS

Old apple cultivars, either originating from the Carpathian Basin or of foreign origin but grown widely in this region, were examined to provide a characterisation of the cultivars and to determine their genetic background. The aim of the thesis was to observe and evaluate the phenological, morphological and genetic traits of the old apple cultivars using the following methods:

- Observation of flowering phenology traits and the classification of the cultivars into flowering time groups.
- Characterisation of the gene bank collection and the determination of diversity using morphological and molecular markers:
    - preparation of a number-coded characterisation based on the morphological and biological traits laid down in the UPOV guidelines,
    - preparation of genetic fingerprints with the use of 12 SSR markers,
    - determination of the diversity of the collection,
    - evaluation of variability within the cultivar groups and the distinguishing of cultivar variants.
- Analysis of correlations between the results of phenotypic and genotypic analysis.
- Use of SSR markers to check the parent–progeny relationships reported by breeders.
- Evaluation of the applicability of conventional and molecular methods for handling gene bank collections, with special regard to the trueness-to-type of cultivars and the identification of synonyms.

MATERIALS AND METHODS

Cultivars included in the study

Among the old apple cultivars/genotypes found in the Carpathian Basin, phenological observations were carried out on 57, morphological characterisation using UPOV descriptions were prepared for 60 and molecular analyses were performed on 73, not counting the control cultivars. Hungarian land races and variants retrieved from the English National Fruit Collection and collected from orchards in Sub-Carpathia (Ukraine), Transylvania (Romania), the Aggteleki National Park and the Mecsek Hills were also included in the study for the purpose of cultivar identification and characterisation. Pedigree analysis was performed for the cultivars 'Fertődi téli' and 'Budai Domokos'.

Experimental location

The gene bank and cultivar collections serving as the basis for the research are located at the experimental station of the Department of Pomology, Corvinus University of Budapest, in Soroksár. The orchard was established in 2001 on MM 106 rootstocks, with a free spindle crown form. In the larger part of the orchard (the first 10 rows) the trees are arranged in 2 × 2 blocks, i.e. a total of four trees/cultivar in a 5 × 2.5 m space, planted next to each other in two neighbouring rows, while in the remaining part (rows 11–13) three trees per cultivar are planted in blocks within a row, in a space of 3.5 × 1.2 m. The meteorological data were obtained from the online database of the meteorological station located on the Soroksár Experimental Farm. Marker-assisted analysis was performed in the molecular biology laboratory of the Department of Pomology, Corvinus University of Budapest.

Phenotypic analysis

Phenological observations

Observations on flowering phenology were carried out in the years 2007–2011. Data were recorded in the cultivar collection every two days during the flowering period. The beginning of flowering, the peak flowering period and the end of flowering were determined by subjective observations (as laid down in the UPOV guidelines).

The dates recorded according to the Gregorian calendar were converted to Julian days, counted from January 1st, and the flowering order of the cultivars was determined based on the mean rankings over many years. After being ranked in this way, the cultivars were divided into

flowering time groups based on the relative flowering dates of the control cultivars. The cultivars were classified in five flowering time groups, based on both the beginning of flowering and the peak flowering period: very early, early, medium, late, and very late.

The fruit traits linked with phenology are the times of harvest and eating maturity. As required by the UPOV guidelines (2005), the harvest maturity was scored using five categories and the eating maturity using nine, ranging from very early to very late.

Morphological observations

Morphological descriptions were compiled based on the UPOV TG/14/9 guidelines. A total of 53 morphological traits were observed in the dormancy and vegetation periods (Table 1). Data were recorded when the trees were bare, at flowering, at the end of intensive shoot growth and at fruit maturity. In addition to subjective observations, measured data were also recorded, based on the analysis of 20 flowers, leaves and fruit from each cultivar. The samples required for the analyses were collected randomly from three or four trees, collecting no more than one sample from each inflorescence or shoot. The analyses were performed between 2007 and 2011, and observations were made on each cultivar in at least two years. The final number code classification of the cultivars was determined on the basis of several years of observations and measured data. It is important not to average the categories determined over a number of years; the category characteristic of each individual cultivar is taken as that which occurs most frequently in the separate annual classifications.

Table 1. No. of observed and measured traits recorded for individual plant organs

Traits	No. of traits		
	Measured	**Observed**	**Total**
I. Morphological characterisation			
- tree, shoot		9	9
- leaf	3	6	9
- flower	1	3	4
- fruit	2	29	31
II. Phenological observations		3	3
Total			56

Marker analysis using SSR markers

Young, unexpanded leaves measuring 1–2 cm in length were collected in spring and early summer. Genomic DNA was isolated from fresh or frozen leaves using a DNeasy Plant Mini Kit (Qiagen).

The DNA fragments were multiplied by PCR in a DreamTaq™ Green PCR Master Mix (2X) kit (Fermentas) using an Applied Biosystems Thermal Cycler 2720. The genotypes were characterised using 12 SSR markers exhibiting great polymorphism: CH01f02*, CH01h01*, CH02c02a, CH02c09*, CH03g07, CH04e03, CH05d11, CH05e03, CH02c11*, CH02d08*,

CH03a02 and CH05c04 (Gianfranceschi et al., 1998; Liebhard et al., 2002). The five primers marked with an asterisk are among those recommended by ECPGR for cultivar identification. The amplification conditions were those proposed by Galli et al. (2005): 94°C 2'; 94°C 20'', 56°C 30'', 72°C 1', 35×; 72°C 5'.

To determine the exact size of the different fragments, the PCR products amplified by fluorescently labelled forward primers (JOE; FAM) were analysed in an automated sequencer (ABI Prism 3100 Genetic Analyzer; Applied Biosystems) by the Biomi Co. Ltd., Gödöllő. Band scoring was carried out using Genographer 1.6 software.

Statistical analysis

Morphological data

The use of the UPOV number code makes it possible to perform statistical analysis on the data. The frequency of the expression categories for the individual traits, given as a percentage, was analysed using the PASW 18.0 statistical program package.

UPOV considers two cultivars to be distinct if there is a clear difference for at least one trait, indicated by a difference of one category for qualitative (QL) and pseudo-qualitative (PQ) traits and two categories for quantitative (QN) traits.

In the course of the statistical analysis a matrix was compiled in Excel using the number code descriptions of the cultivars, taking the nature of the traits into consideration. During the pairwise comparison of the cultivars, a value of 0 was assigned if a difference of one (QL, PQ) or two (QN) categories was not observed, and a value of 1 if this condition was met. The 0–1 coding values were then added for each cultivar to obtain the similarity matrix used for further statistical analysis.

Hierarchical cluster analysis and dendrogram construction were performed using the Ward method, based on this matrix, using the R program.

SSR fragment lengths

The characteristics of the loci (no. of alleles, allele frequency, expected and observed heterozygosity, null alleles, and the probability that identical genotypes will appear) were determined using the Identity 1.0 program using only the data of diploid cultivars, as this program is unable to handle triploids.

In the hierarchical cluster analysis, each detected allele was scored as present (1) or absent (0). The UPGMA (Unweighted Pair Group Method with Arithmetic mean) algorithm was used to construct a dendrogram based on the Jaccard's similarity coefficients with the PAST software.

Correlation analysis between the morphological and molecular data was performed with Mantel's test using the PASSaGE v2 program. This involved the use of the genetic distance matrix based on the UPOV data and that based on the Jaccard indexes of the binary SSR data.

RESULTS AND DISCUSSION

Phenological traits

Flowering time

In early April 2007 the air temperature gradually increased, followed by a cooler period in the middle of the month, after which the weather warmed up again. As a consequence, the cultivar collection flowered over a period of 20 days (10–30 Apr.). In 2008 rapid efflorescence was followed by a protracted period of deflorescence due to a slight cooling (of around 5°C) at the start of flowering and again a week later, during the flowering peak. The flowering duration in this year was the longest of all the five years, lasting for 26 days from 14 Apr. to 10 May. In 2009 the mean daily temperature remained consistently above 10°C, resulting in rapid flowering (14–28 Apr.). In 2010 the temperature during the first 20 days of April was 2–3°C lower than during the same period in earlier years, so flowering began 7–8 days later on average (21 Apr.–5 May) than in the previous three years. A similar situation was recorded in 2011, when a substantial drop in temperature between 13 and 15 Apr. led to flowering beginning 5–6 days later, though it then proceeded rapidly (18 Apr.–3 May).

According to Soltész (1982), there was only 1–2 days' difference in the start of flowering between cultivars arising from bud mutation, and the same was observed in the present work for cultivars belonging to the Batul group. With regard to the flowering duration, mention should be made of the protracted flowering of 'Zöld batul' and Beregi sóvári 2', which had a very long flowering period (10–15 days) compared with the average of 7–8 days for the collection as a whole.

When making decisions on cultivar association it would be advisable to consider the flowering time groups. Based on this 5-year study, and taking into consideration the reference cultivars recommended by Bodor et al. (2008), the cultivars were divided into five flowering time groups (Table 2).

The grouping is given in terms of both the beginning of flowering and the main flowering period, because sources in the literature disagree as to which of these traits should be taken into consideration for cultivar association.

Table 2. Flowering time groups established on the basis of the beginning of flowering and the main flowering period (Soroksár, 2007–2011)

Based on the beginning of flowering period	Cultivar	Based on the main flowering period	Cultivar
Very early	Orbai alma Jászvadóka	**Very early**	Orbai alma Jászvadóka
Early	*Reglindis* Lóci édes almája Fekete tányéralma Édes esocar Kanadai renet 3 Dániel féle renet Hosszúfalusi	**Early**	*Reglindis* Lóci édes almája Fekete tányéralma Édes esocar
Medium	Bánffy Pál Tordai alma *Florina* Széchenyi renet Ízletes zöld Cserepánya Kanadai renet 1 Királyi renet Beregi sóvári 2 Sárga szépvirágú Baumann renet Kanadai renet 2 Nemes szercsika Ontario Szabadkai szercsika Lóci cirmos alma Entz Rozmaring Gyógyi piros Mosolygós batul Szászpap alma Máté Dénes Batul 2 Nemes sóvári Bőrkormos renet Cigány alma Herceg Batthyány Vajki alma Batul 3 Sikulai Kisasszony	**Medium**	Kanadai renet 3 Dániel féle renet Hosszúfalusi Bánffy Pál Tordai alma *Florina* Széchenyi renet Ízletes zöld Cserepánya Kanadai renet 1 Királyi renet Beregi sóvári 2 Sárga szépvirágú Baumann renet Kanadai renet 2 Nemes szercsika Ontario Szabadkai szercsika Lóci cirmos alma Entz Rozmaring Gyógyi piros Mosolygós batul Szászpap alma Máté Dénes Batul 2 Nemes sóvári Bőrkormos renet Cigány alma Herceg Batthyány Vajki alma Batul 3 Beregi sóvári 1 Batul 1 Simonffy piros Csíkos óriás halasi Sándor cár Gegesi piros Tükör alma
Late	*Rewena* Beregi sóvári 1 Batul 1 Simonffy piros Csíkos óriás halasi Sándor cár Tordai piros kálvil Budai Domokos Gegesi piros Masánszki Londoni pepin Tartós Gusztáv Vilmos renet Tükör alma Gomba Károly	**Late**	Sikulai Kisasszony *Rewena* Tordai piros kálvil Budai Domokos Masánszki Londoni pepin Tartós Gusztáv Vilmos renet Gomba Károly Daru sóvári
Very late	Daru sóvári Marosszéki piros Harang alma Sóvári nobil Budai Ignác Zöld batul	**Very late**	Marosszéki piros Harang alma Sóvári nobil Budai Ignác Zöld batul

On the basis of observations on 60 cultivars it can be said that cultivars with very early or very late flowering were placed in the same groups on the basis of both traits, while considerable differences could be observed for the medium and late flowering cultivars. Exact estimates of the stability of the cultivars cannot be given on the basis of this 5-year study, but it is clear that the very early, early and very late flowering cultivars can be regarded as having a stable flowering time, while for most of the medium and late flowering cultivars a longer period of study will be required to make the classification more precise.

Most of the cultivars studied were classified as medium or late flowering. Only the cultivars 'Orbai alma' and 'Jászvadóka' proved to be very early. In all the years the latest flowering cultivars in the collection were 'Daru sóvári', 'Marosszéki piros', 'Harang alma', Sóvári nobil', 'Budai Ignác' and 'Zöld batul', all of which were classified in the very late flowering time group.

The flowering time traits of the cultivars can only be reliably determined after long years of study, so the flowering time groups given in the present work are only of an informative nature.

Characterisation of the cultivars in terms of their number code classification

A complete number code characterisation of 60 cultivars was carried out using the 56 traits proposed by the UPOV TG/14/9 guidelines. Hierarchical cluster analysis was carried out on the basis of the similarity matrix in the R program using the Ward method. The resulting dendrogram is illustrated in Figure 1. Six groups can be clearly distinguished on the dendrogram. The Batul cultivar group and the 'Kanadai renet' genotypes each forms a separate main cluster.

The first main cluster contains all the cultivars in the Batul group with the exception of 'Zöld batul', which was classified in a completely different cluster, at a considerable distance from the other 'Batul' genotypes examined. The second main cluster consists of the 'Kanadai renet' group. No cultivar groups can be identified for the cultivars in the other four clusters.

Rennet cultivars were found in all four groups. Three cultivars in the Sóvári cultivar group ('Nemes sóvári', 'Zöld sóvári', 'Daru sóvári') formed a separate small cluster. In the same main cluster, but at some distance from these, 'Sóvári nobil' could be found in a separate subcluster. Although regarded as being the same cultivar, 'Nemes sóvári' and the 'Sóvári nobil' sample included in the investigations were thus classified in different clusters, so they cannot be called synonyms. The 'Beregi sóvári' genotypes were grouped in a completely different main cluster compared with the other Sóvári cultivars. The two 'Beregi sóvári' genotypes (from UK and Sub-Carpathia) exhibited no similarity based on the number code description, so they are presumably two separate cultivars.

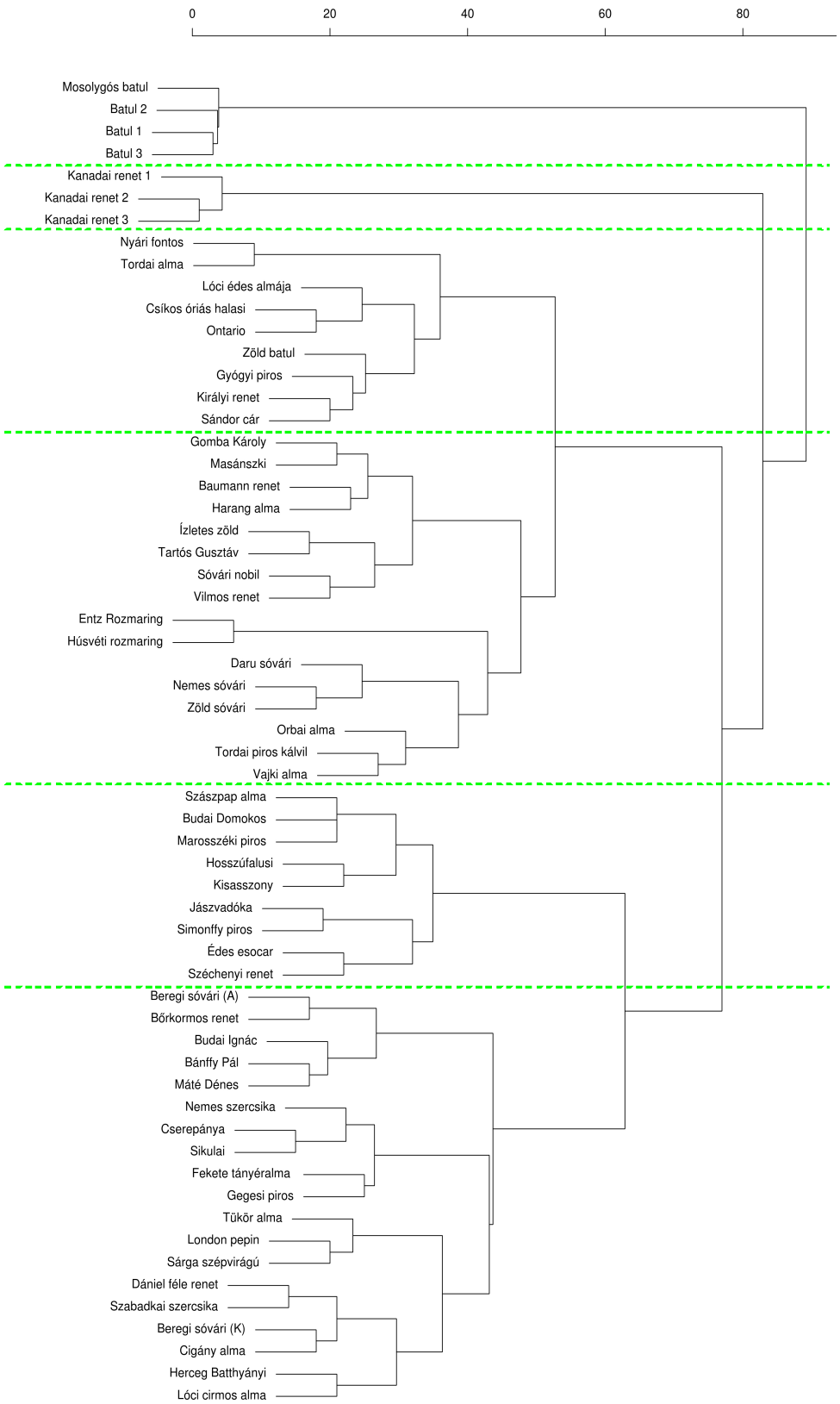

Fig. 1. Genetic relationships between the cultivars on the basis of the similarity matrix compiled from the phenotypic UPOV number codes (A = from the UK, K = from Sub-Carpathia)

Close relationships could be detected between ‘Nyári fontos’ and ‘Tordai alma’ and between ‘Entz rozmaring’ and ‘Húsvéti rozmaring’. On the other hand, the ‘Cigány alma’ and ‘Simonffy piros’ cultivars, often considered to be synonyms, were grouped in two distinct clusters. The number code description also indicated deviations between the two cultivars for almost 50% of the traits.

It is interesting to note, however, that cultivars with a great tendency to exhibit parthenocarpy (‘Orbai alma’, ‘Tordai piros kálvil’, ‘Vajki alma’) formed a common cluster on the dendrogram based on morphology, although there was a great distance between the cultivars.

The genotypes examined included not only cultivars that originated in the Carpathian Basin, but also a number of cultivars that are widespread in Hungary but are probably or certainly not of Hungarian origin, such as ‘Kanadai renet’, ‘Ontario’, Tartós Gusztáv’, ‘Londoni pepin’ and ‘Sárga szépvirágú’. On the dendrogram shown in Figure 1, which is based on morphology, endemic cultivars cannot be clearly distinguished from cultivars of foreign origin, with the exception of ‘Kanadai renet’, which formed a separate group. Little information is available on the origin of the cultivars investigated. The site of origin is known or surmised for approx. ¾ of the cultivars. They originate from all over the Carpathian Basin, but the majority are from Transylvania, while far fewer come from Sub-Carpathia (e.g. the Sóvári cultivars and ‘Vajki alma’), the Carpathian regions of Serbia (e.g. ‘Szabadkai szercsika’), the Great Hungarian Plain (e.g. ‘Jászvadóka’, ‘Simonffy piros’, ‘Harang alma’) or Transdanubia (‘Herceg Batthyány’). The dendrogram does not reflect the regions, so the morphological similarity is not aligned with the information available on origin.

It is also clear from the dendrogram (Fig. 1) that, with the exception of the ‘Batul’ and ‘Kanadai renet’ genotypes, cultivar groups could not be distinguished unambiguously. The distance between the cultivars classified in each subgroup was great, reflecting the great biodiversity of the collection examined.

Results of microsatellite marker analysis

SSR polymorphism

Microsatellite regions were amplified using all the SSR primer pairs for each of the 73 apple cultivars. The SSR data were evaluated using the Identity 1.0 program, based only on the data of the 55 diploid cultivars, as the program is unable to handle triploids.

A total of 160 polymorphic fragments were amplified with the 12 primer pairs for the 55 diploid cultivars. The mean allele number obtained at the 12 SSR loci was 13.33, which is similar to the results reported for many authors investigating the variability of cultivars stored in gene banks

(e.g. Wichmann et al. 2007, Pereira-Lorenzo et al. 2007). Other authors (Guilford et al. 1997, Liebhard et al. 2002) demonstrated a far smaller mean allele number (4–6), but this could be attributed to the fact that they examined fewer, mainly modern commercial cultivars, thus drawing attention to the role of land races in the preservation and expansion of genetic variability.

In the present analysis the CH02c02a marker gave the greatest allele number (19). This marker also resulted in an outstanding number of alleles in the work of other scientists (Urbanovich and Kazlovskaya 2009, van Treuren et al. 2010). The smallest number of alleles was obtained with the CH02c09 marker, which is again in agreement with the findings of other authors (Guarino et al. 2006). Although the markers CHY02c11 (Guarino et al. 2006, Garkava-Gustavsson et al. 2008), CH05e03 and CH05d11 (Farrokhi et al. 2011) were reported to have a double locus, this was not confirmed by the present measurements or by many other authors (e.g. Liebhard et al. 2002, Gasi et al. 2010).

The expected heterozygosity (He) ranged from 0.77 (CH04e03) to 0.91 (CH02c02a) with a mean value of 0.85, which was similar to the values reported by Rouston et al. (2009) and Sikorskaite et al. (2012), while the observed heterozygosity (Ho) ranged from 0.63 to 0.98 (mean: 0.83), which agreed with the data of Sikorskaite et al. (2012) and Pereira-Lorenzo et al. (2007) but was higher than the values reported by Guilford et al. (1998) and Bassil et al. (2009).

At each of the microsatellite loci there were 1–3 alleles that occurred at much greater frequency than the other alleles at the given locus. On the basis of allele frequency 49 (31%) rare alleles (with a frequency of 0.05–0.01) and 37 (23%) unique alleles were identified among the total of 160 alleles. These values were similar to those reported in the literature (Gasi et al. 2010, Potts et al. 2012). The large number of rare and unique alleles detected in the course of marker analysis and the large distances observed between the cultivars on the dendrogram (Fig. 2) confirmed the enormous value of old cultivars for genetic diversity. Many authors have drawn attention to the fact that the limited number of cultivars grown could lead to an ecological catastrophe. To avoid this, the old cultivars should be exploited as gene sources (Kellerhals et al. 2012, Tóth et al. 2013).

The primers used for the analysis led to a large number of alleles (10–19) and high heterozygosity values, so they appear to be suitable for assessing the genetic variability of cultivar collections.

Trueness-to-type and the evaluation of cultivar groups on the basis of allele composition and cluster analysis

In the course of the work microsatellite fingerprints were determined for 78 apple genotypes (five of them control genotypes), including 47 old cultivars from the Carpathian Basin, 7 of

unknown but probably Hungarian origin, 16 old foreign cultivars widely grown in the Carpathian Basin, 5 international control cultivars and 3 genotypes of uncertain (Hungarian or foreign) origin. In a few cases several genotypes of the same cultivar were examined to establish trueness-to-type or due to the existence of numerous variants or debatable origin.

Of the 78 genotypes 55 were diploids, 17 were probably triploids (29%) and three ('Vajki alma', 'Cserepánya' and 'Csíkos óriás halasi') appeared to be tetraploid judging from the allele composition. These ratios are similar to those reported for the analysis of other cultivar collections (Pereira-Lorenzo et al. 2007: 28%, Gasi et al. 2010: 21%).

A total of 68 different genetic profiles were obtained. The three samples of 'Kanadai renet', and two samples from different locations for each of the cultivars 'Téli arany parmen', 'Honti alma', 'Fertődi téli' and 'Török Bálint' exhibited 100% allele identity, so these samples could all be regarded as true-to-type. The genetic fingerprints of 'Nyári fontos' and 'Tordai alma' and those of 'Húsvéti rozmaring' and 'Entz rozmaring' were also 100% identical, as were those of 'Batul 2', 'Batul 3' and 'Mosolygós batul'.

On the dendrogram constructed using the fragment lengths revealed by marker analysis, based on the genetic distance matrix calculated from Jaccard indexes (Fig. 2), nine major groups can be identified. As in the case of the dendrogram constructed on the basis of morphological data, the cultivars classified in the individual main clusters did not exhibit identity in terms of origin.

As also reported by Laurens et al. (2004) and Potts et al. (2012) on the basis of SSR analysis, no correlation was found between the geographical origin of the cultivars and the clusters obtained. This was true both of old and modern foreign cultivars and of cultivars originating from the Carpathian Basin, all of which exhibited mixed distribution. In the work of Gasi et al. (2010) the international cultivars could be clearly distinguished from traditional cultivars on the basis of molecular data, but this was more ambiguous in the case of morphological data. It was interesting to note, however, that 'Tartós Gusztáv', 'Édes escoar' and 'Marosszéki piros' each formed a separate main cluster.

Two surprising cases of uniformity were observed when analysing the SSR data. The cultivar pairs 'Gegesi piros'/'Sikulai' and 'Csikos óriás halasi'/'Vajki alma' exhibited 100% conformity for all the loci. This agreement was not confirmed, however, by the morphological analysis. In connection with the tetraploid cultivar 'Vajki alma', Halász et al. (2011) suggested that duplicated genomes (polyploid cultivars) were instable. The cultivars 'Cserepánya' and 'Csikos óriás halasi' were also shown by flow cytometry to be tetraploid (Nagyistván 2012).

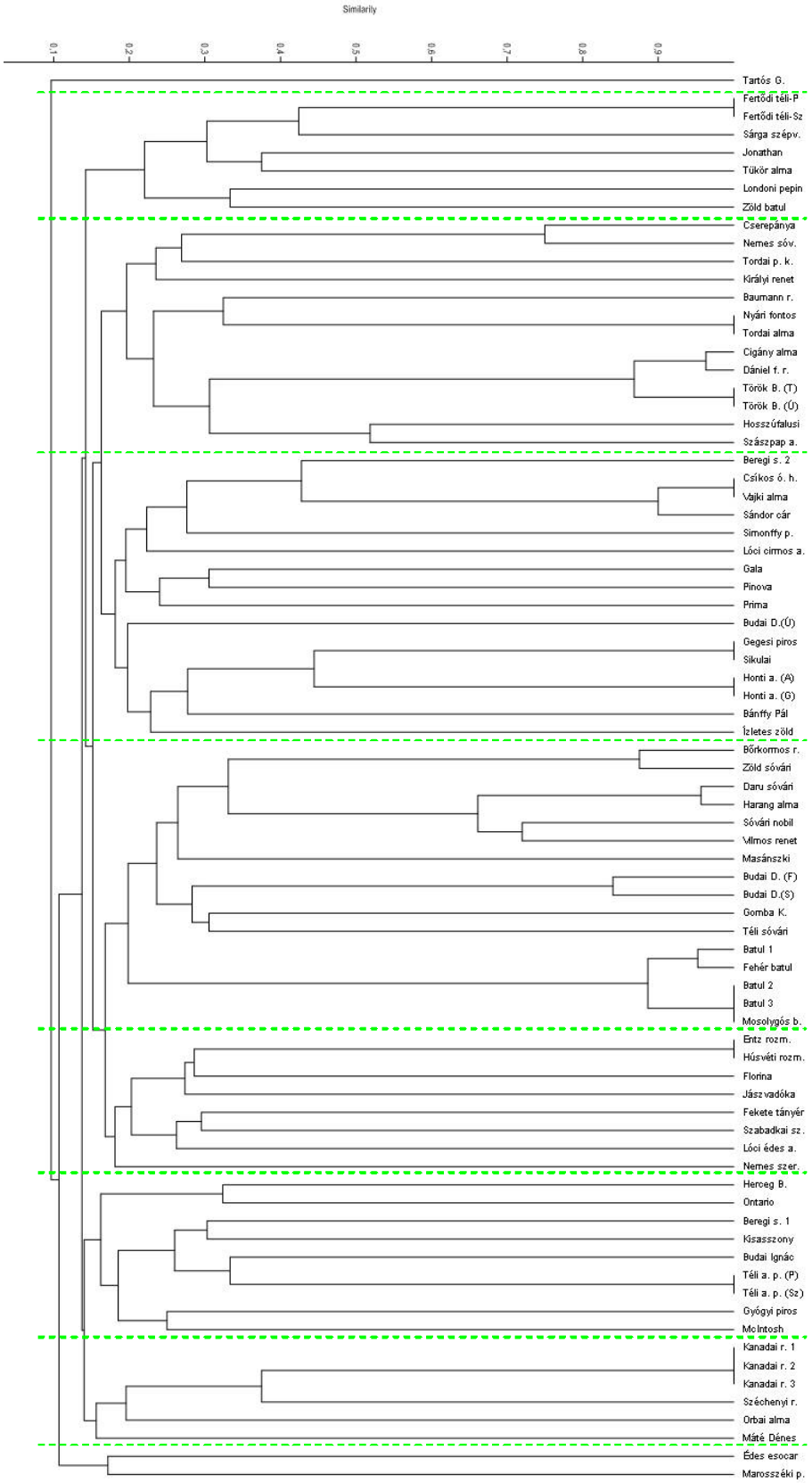

Fig. 1. Genetic relationships between the cultivars based on the genetic distance matrix for Jaccard indexes calculated from the SSR fragments

The microsatellite fingerprints revealed great genetic diversity between the genotypes. It is also clear from the dendrogram that, with the exception of the identical cultivars and the "Batul' cultivars, all the cultivars were at a considerable distance from each other. More than 80% similarity was only found in a few cases. The apple microsatellite primers employed proved to be suitable for determining the individual DNA profiles of the cultivars, with the exception of cultivars derived from bud mutation. Nevertheless, interesting cases of conformity were also detected.

Identification of parents on the basis of SSR

Very little information is available in the literature on the origin of old varieties, mainly because they often grew from seed, rather than being developed by crossing.

The cultivar 'Fertődi téli' was reported to be developed by Aladár Porpáczy Sr from the crossing of 'Jonathan' × 'Török Bálint'. The present analysis revealed that 'Jonathan' could have been one of the parents (allele similarity: 100%), whereas 'Török Bálint' could not possibly have been a parental cultivar. The agreement between samples of 'Török Bálint' and 'Fertődi téli' collected and tested from two locations excluded the possibility of cultivar mixing. The seedling that resulted in the 'Fertődi téli' cultivar either originated from open pollination, or the wrong pollen was used for the cross, so in the future the male parent must be treated as unknown.

The cultivar 'Budai Domokos' was reported to originate from a cross between 'Téli arany parmen' and 'Jászvadóka' (Angyal 1926). When the specimen of 'Budai Domokos' found in the Soroksár collection was analysed it became obvious that neither the male nor the female parent given by the breeder could have been involved in the cross, as the extent of allele similarity was 41 and 16%, respectively. The sample of 'Téli arany parmen' obtained from Pölöske had an SSR fingerprint 100% identical to that of the sample used earlier, so it was regarded as true to cultivar. Unfortunately no other sample of 'Jászvadóka' could be obtained, so it was not possible to check the cultivar identity. However, on the basis of the cultivar description in the literature (Bereczki 1882) it would appear to be a true-to-type cultivar. The possibility of errors arising due to using a cultivar that was not true-to-type as crossing partner was excluded in the case of 'Téli arany parmen', and the same can be assumed for 'Jászvadóka', so it became necessary to check the trueness to cultivar for the 'Budai Domokos' cultivar.

The 'Budai Domokos' sample available in the Újfehértó Gene Bank could not be regarded as identical on the basis of fruit morphology or SSR fingerprints, and the allele similarity of this sample with the reported parental cultivars was 75% and 25%, respectively, so it was again impossible to confirm the pedigree given by the breeder.

Cultivar groups, synonyms and trueness to cultivar on the basis of morphological and molecular analyses

The evaluation of cultivar groups and the identification of synonyms were performed with the joint consideration of the results of morphological and molecular analyses, as a more realistic picture and clearer answers to the relationships between the cultivars could be obtained in this way.

Both dendrograms (Figs. 1 and 2) revealed great genetic variability between the cultivars, as both the molecular and morphological similarity indexes indicated great distances between all the cultivars except for those obtained by bud mutation and for cultivar groups. No correlations between the cultivars were found within the larger clusters, while cultivar groups could be distinguished within the smaller clusters, as also reported by Bassil et al. (2009).

Relationships between cultivar groups

Batul cultivar group

The Batul cultivar group formed a separate main cluster on the dendrograms. On the basis of both morphological and molecular data, all the 'Batul' genotypes except 'Zöld batul' were found in the same small cluster (Figs. 1 and 2). The cultivars 'Batul' and 'Mosolygós batul' could not be distinguished on the basis of the results. Many authors have drawn attention to the similarity of the cultivars comprising the Batul cultivar group and the difficulties faced when trying to distinguish between them. Nagy-Tóth (1998), Bereczki (1899) and Bordeianu et al. (1964) all noted that 'Mosolygós batul' resembled 'Batul' for a number of traits, and could be a seedling of this cultivar.

On the basis of both morphological and molecular analyses, 'Zöld batul' was placed in a completely different cluster, at a great distance from the other 'Batul' genotypes examined. Judging by the results of these two analytical methods and by differences in the flowering time, 'Zöld batul' does not exhibit any direct relationship with the 'Batul' cultivar, and is probably a distant descendant of the basic cultivar. In the future it should thus be registered as a separate cultivar.

Renet cultivar group

The cultivars in this group could be clearly distinguished from each other on the basis of phenotypic traits and genetic fingerprints. The multiplicity of the rennet cultivars is clear from the dendrograms, where almost all of them were placed in different clusters, while even those found in the same main cluster were distant from each other, in separate subclusters.

Pomologists classify the Renet cultivars in the same group on the basis of fruit traits, primarily their taste. Renets are characterised by dense, tender flesh and a characteristic spicy taste (Rapaics 1937). It is one of the largest cultivar groups.

Sóvári cultivar group

The Sóvári cultivars are grouped together on the grounds that they appear to be of common origin. Most sources in the literature speak of them as variants of the cultivar 'Közönséges sóvári'. On the dendrogram compiled using phenotypic data, three members of the Sóvári cultivar group ('Nemes sóvári', 'Zöld sóvári', 'Daru sóvári') formed a small cluster, and 'Sóvári nobil' was placed in the same main cluster, while the 'Beregi sóvári' genotypes were to be found in a completely different main cluster. Based on their positions on the SSR dendrogram, 'Sóvári nobil', 'Daru sóvári', 'Téli sóvári' and 'Zöld sóvári' were located in the same main cluster, but at great distances from each other. 'Nemes sóvári' and the two 'Beregi sóvári' genotypes were placed in two other main clusters.

Although 'Nemes sóvári' and the cultivar 'Sóvári nobil', maintained in the English National Fruit Collection, are considered to be synonyms, the results of morphological and molecular analyses indicated that they are not the same cultivar, so they cannot be regarded as synonyms. This was confirmed by the analysis of the *S*-genotype, performed by Halász et al. (2011), which revealed different *S*-allele compositions for the two genotypes.

The complex analysis showed that the two 'Beregi sóvári' genotypes (from the UK and Sub-Carpathia) could not be regarded as the same cultivar. The latter was found to be identical with the cultivar described by Máté Bereczki, so the specimen of 'Beregi sóvári' maintained in the English National Fruit Collection (Morgan and Richards 1993) should be registered as an unknown genotype.

Identification of synonyms

Simonffy piros – Cigány alma – Roter Stettiner – Török Bálint

The genotypes 'Cigány alma' and 'Simonffy piros', which are frequently mentioned as synonyms, proved on the basis of morphological and molecular analysis to be clearly distinct from each other. They were also placed in two separate clusters on the dendrograms. Three alleles were found at several loci for 'Cigány alma', while 'Simonffy piros' proved to be diploid not only in the present work, but also in the analyses of Wichman et al. (2007, 2010) and Halász et al. (2011).

In the course of marker analysis, Holler et al. (2012) observed that the genetic fingerprint of 'Cigány alma' was identical with that of the 'Roter Stettiner' cultivar widely grown in Germany.

On the basis of the descriptions published by Lucas (1875a) and Bereczki (1887) it is possible that the cultivar in the Soroksár collection, described by Bereczki as 'Cigány alma', may be identical with 'Roter Stettiner'.

According to Stoll (1888) and Bereczki (1877) 'Török Bálint' is a synonym of 'Roter Stettiner'. 'Cigány alma' and 'Török Bálint' were placed close to each other on the dendrogram compiled from the SSR data. A comparison of the allele composition of the 'Cigány alma' and 'Török Bálint' trees examined in the present work showed that 100% of the alleles in 'Török Bálint' were also present in the 'Cigány alma' genotype. However, on the basis of phenotype (external fruit traits) and ploidy level, the two cultivars cannot possibly be identical. The two cultivars appear to be in a parent–progeny relationship with each other. It is probable that 'Cigány alma' arose from 'Török Bálint' as the result of anomalous gamete formation or self-fertilisation.

Entz rozmaring – Húsvéti rozmaring – Honti alma

These three cultivars are considered by Angyal (1926), Herszényi (1934) and Brózik and Régius (1957) to be synonyms. In the present work, only 'Húsvéti rozmaring' and 'Entz rozmaring' were included in the morphological analysis, while all three cultivars were examined in the marker-assisted analysis. The genetic fingerprints of 'Húsvéti rozmaring' and 'Entz rozmaring', which have very similar morphological traits, exhibited 100% agreement. The two rozmaring cultivars could not be distinguished using SSR markers, but due to the slight morphological deviation, it would be worth applying other types of markers to determine whether they really are the same cultivar, or possibly cultivars originating from bud mutation. One allele at each of 10 loci in 'Honti alma' was identical with those of the other two cultivars, so there appears to be a parent–progeny relationship. In other words, 'Honti alma' is not a synonym of either of the rozmaring cultivars. This is confirmed by the observations of Tóth et al. (2007), who found that the fruit of 'Honti alma' was smaller and less elongated than that of 'Húsvéti rozmaring', while the tree had a more spreading crown.

Correlations between the data of morphological and molecular analysis

In order to prove correlations between the matrix compiled from morphological (UPOV) data and the genetic distance matrix based on Jaccard indexes calculated from SSR data, the Mantel test was performed. This test revealed a significant, moderately strong correlation (r=0.33343, p=0.00000) between the morphological and molecular data matrixes, which was similar to the results obtained by Giancola et al. (2002) for soy beans (r=0.353). By contrast, no correlation between the morphological (UPOV) and molecular (SSR) data was detected with the Mantel test by Guarino et

al. (2006) or Gasi et al. (2010) in apple (r=0.095, p=0.05) or by Tommasini et al. (2003) for rape varieties (r=0.098, p=0.283). As correlations were not always reported in the literature between the morphological and molecular distance matrixes, it is recommended that, for the purposes of cultivar description and genetic studies, phenotypic characterisation should be combined with genotypic analysis involving a small number (~10) of SSR markers exhibiting a high level of polymorphism.

NOVEL SCIENTIFIC RESULTS

1. The flowering times were determined for 57 old apple cultivars and genotypes, which were then divided into flowering time groups on the basis of both the beginning of flowering and of the peak flowering period.
2. Microsatellite fingerprints were compiled for 73 old apple cultivars or genotypes widespread in the Carpathian Basin, and 60 cultivars were characterised using the UPOV TG/14/9 number code.
3. Parallel phenotypic and genotypic analyses were performed to determine the genetic variability of apple cultivars in the Carpathian Basin. It was found that:
    a) The genetic variability of the old cultivars was greater than that of commercial cultivars (based on the heterozygosity values found in the literature).
    b) Great diversity was observed within the Sóvári and Rennet cultivar groups.
    c) The dendrograms compiled from molecular and morphological data matrixes did not reflect geographical origin.
4. Based on the results of phenotypic and SSR analyses performed for the purposes of cultivar identification, it was established that:
    a) ‘Zöld batul’ did not exhibit any direct relationship with the ‘Batul’ cultivar, and can probably be considered as a distant descendant of the basic cultivar. It should thus be registered as a separate cultivar in future.
    b) The ‘Beregi sóvári’ cultivars obtained from Sub-Carpathia and from the English National Fruit Collection were not identical. The Sub-Carpathian sample proved to be the same as that described by Máté Bereczki, so the tree maintained in the English National Fruit Collection should be regarded as an unknown genotype.
    c) Despite being considered as synonyms, the cultivar ‘Nemes sóvári’ and the ‘Sóvári nobil’ sample maintained in the English National Fruit Collection were not identical.

5. On the basis of allele composition, the existence of parent–progeny relationships was confirmed or confuted for three cultivars.
    a) The male parent of ‘Fertődi téli’ cannot be ‘Török Bálint’, as reported by the breeder. The pedigree should thus be given correctly as: ‘Fertődi téli’ = ‘Jonathan’ × unknown.
    b) The parental cultivars reported by the breeder for the cultivar ‘Budai Domokos’ (‘Téli arany parmen’ and ‘Jászvadóka’) did not exhibit 100% allele identity with the supposed progeny, so the origin of this cultivar should be considered to be unknown.
    c) A parent–progeny relationship was found between ‘Cigány alma’ and ‘Török Bálint’. The probable origin is: ‘Cigány alma’ = ‘Török Bálint’ × unknown, or ‘Cigány alma’ = unknown × ‘Török Bálint’. It is likely that ‘Cigány alma’ arose from the self-pollination of ‘Török Bálint’.
6. ‘Csikos óriás halasi’ and ‘Vajki alma’ exhibited 100% identity at 12 SSR loci, but on the basis of phenotype they cannot be the same cultivar. This confirms the instability of duplicated genomes (polyploid cultivars).

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