Molecular Identification of Commercial Apple Cultivars with Microsatellite Markers

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Additional index words. *Malus ×domestica*, genotyping, somaclonal mutations

Abstract. A collection of 66 commercial apple (*Malus ×domestica* Borkh.) cultivars was screened with six previously described SSR (Simple Sequence Repeat) markers for molecular identification. In total, 55 polymorphic alleles were detected at the 6 SSR loci (average 9.2 alleles per locus) and the polymorphism information content (PIC) averaged 0.72. Successful differentiation of all apple genotypes except for somatic mutants was accomplished by using only four (CH03g07, CH04e03, CH05d11, and CH05e03) SSR markers. Sport mutants proved to be indistinguishable from each other and their progenitors. The cumulative probability of obtaining an identical SSR profile for two randomly chosen apple genotypes was 1.79×10^{-4} , which confirms the high potential of simple sequence repeats (SSRs) for cultivar identification.

The accessibility of reliable genetic markers is essential for variety identification and distinction, for development of efficient breeding methods to create new apple cultivars selected for disease resistance, fruit quality or tree growth characteristics and other traits. The emergence of PCR-based molecular markers has created the opportunity for fine-scale genetic characterizations of germplasm collections, as well. Microsatellites (SSRs) are the most widely used molecular markers in this field. Repetitive nucleotide sequences are ubiquitously found in eukaryotic nuclear genomes and their flanking regions are highly conserved, which make them suitable for amplifying the usually polymorphic intervening repeat loci. Microsatellites show high polymorphism, co-dominant inheritance and good reproducibility. Since all individuals of the same cultivar originated from a single progenitor by vegetative propagation in apple breeding and cultivation, SSR fingerprinting can be used for cultivar differentiation. Up to now, almost 200 SSR markers are available in apple (Guilford et al., 1997; Gianfranceschi et al., 1998; Hokanson et al., 1998; Liebhard et al., 2002). SSR markers have proven to be highly informative and useful for distinguishing genotypes and for determining genetic relationships among *Malus* cultivars and species (Goulão and Oliveira, 2001; Hokanson et al., 2001;

Laurens et al., 2004; Liebhard et al., 2002). In this study six previously described SSR markers (Liebhard et al., 2002) were applied to distinguish 66 different apple cultivars.

Materials and Methods

Young apple leaves used for DNA purification were collected at the Research and Extension Centre for Fruit Growing, Újfehértó, Hungary. Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen) and PCR amplifications were carried out by a Perkin Elmer 9700 thermocycler using Cy-5 labelled SSR primers. The six applied pairs of oligonucleotide primers (CH03g07, CH04e03, CH04g10, CH05c02, CH05d11, and CH05e03) have already been identified and published (Liebhard et al., 2002). The reactions were performed in a final volume of 20 μ L, the reaction mixture consisted of the following components: 50 ng template DNA, 1 $\times$ PCR buffer (50 mM KCl, 10 mM Tris-HCl pH: 8.3, 1.1 mM MgCl₂, 0.01% gelatin), plus 0.9 mM MgCl₂, 0.3 μ M of both primers, 0.2 mM each of dNTPs and 1.2 U of Red-Taq DNA polymerase (Sigma). The cycling profile consisted of an initial denaturation step of 2 min followed by 35 cycles of 20 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C. The amplification process was finished with 5 min at 72 °C. Products were preliminarily tested by running on 1.2% agarose gel, necessarily diluted with TE1 buffer (10 mM Tris, 1 mM EDTA) up to 30 times and microsatellite alleles were visualized and determined in ALFexpress-II DNA analyzer (Amersham BioSciences), following standard protocols. ALF express sizer 50–500 was ap-

plied as internal standard. PCR amplifications using the same extracted DNA as template and allele size determination were repeated at least twice.

Based on the frequencies of observed microsatellite alleles, polymorphic information content (PIC) was calculated by the formula: $PIC = 1 - \sum p_i^2$, where p_i is the frequency of the i th allele of each SSR marker (Anderson et al. 1993). It is essentially the same as the index of heterozygosity. To find the optimal combination and minimum set of markers for identification, the undistinguishable sport mutations were treated as single cultivars so data of 46 different cultivars ($N = 46$) were analyzed according to Tessier et al. (1999). At first, the confusion probability (C) was calculated, i.e. the probability that two randomly chosen individuals from the sample of 46 cultivars have identical banding patterns. The confusion probability is for the i th pattern of the given j th primer present at frequency p_i in this set of cultivars is:

$$c_i = p_i \frac{(Np_i - 1)}{N - 1}$$

For the j th primer, the confusion probability C_j is equal to the sum of the different c_i for all I patterns generated by the primer:

$$C_j = \sum_{i=1}^I c_i$$

The probability of identity (PI) of obtaining same SSR profile for two randomly chosen apple cultivars was calculated by the formula

$$PI = \prod_{j=1}^k C_j$$

The total number of nondifferentiated pairs of cultivars for the j th primer is given by

$$N_j = \frac{N(N-1)}{2} C_j$$

For a given combination of k primers, this number is as follows (Tessier et al., 1999).

$$N_k = \frac{N(N-1)}{2} \prod_{j=1}^k C_j$$

Results and Discussion

Each applied SSR primer-pair generated reliable microsatellite alleles in all 66 apple cultivars. A total of 55 polymorphic alleles were amplified and the number of alleles per marker ranged from 6–13, with an average of 9.2 (Table 1). These markers were chosen by allelic diversity, the rarely visible stuttered bands near the specific product caused by the slippage of Taq polymerase, and considering their reliability, which was verified by repeated tests. The allele sizes determined by the same markers were identical in all replications. Table 2 shows the allelic composition and frequency of each SSR marker based on the results of all 66 cultivars.

Some new allelic forms were detected when comparing our results with those already published by Liebhard et al. (Table 1). This could be explained by the small number of cultivars (seven cultivars and one breeding selection) tested in the above mentioned study. Obviously, this is the reason for the difference of PIC values, as well. Since four cultivars ('Fiesta', 'Florina', 'Prima', and 'Starking')

Received for publication 22 July 2005. Accepted for publication 2 Sept. 2005. Thanks to Eszter Agócs and Tímea Kuttyik for technical assistance. Financial support was provided by the Hungarian Scientific Research Fund (OTKA F63582 and T037251).

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were common in both studies, the reproducibility of SSR markers could be tested. In general, it was adequate, except for the marker CH05e03, for which three base-pair differences were observed probably due to the different size markers used. It supports the perception that the relative size of the alleles is the most important because many factors can modify the migration of fragments through the gel during size determination. Liebhard et al. (2002) used a 10-bp DNA ladder which gave them strikingly consistent, highly accurate results since with the other five SSR markers their allele sizes were the same as ours. It should be noted that alleles of CH05e03 marker in 'Starking' were 186:188 according to Liebhard et al. (2002) while we found the same alleles homozygous 191:191 instead of the expected 189:191, considering the shift of three base pairs for this marker. Since in all somatic mutants of 'Starking' the CH05e03 marker amplified only one 191-bp band in our study (Table 3) we presume that this allele is homozygous and the 186-bp band in the other study could be caused by the slippage of the polymerase enzyme (stutter bands). Another difference was found in 'Prima' where an additional new allele (165 bp) was detected using marker CH03g07.

As can be seen in Table 2 almost every SSR marker had one or two characteristic allele(s) with high occurrence. These alleles can possibly be considered as setpoints of sequence repeats and new alleles might derive from the increase or decrease of the number of repeats. The 135-bp allele at the locus CH04g10 had the highest frequency, and occurred in 60% of

the genotypes. That is why the PIC of marker CH04g10 was the lowest and dropped dramatically when more cultivars were tested (Table 1). Generally, higher number of alleles produces higher PIC values, as well. However, this statement is not always true; the even distribution of allele frequencies is also crucial. For instance, many rare alleles could be determined using the CH05d11 marker causing higher number of alleles but lower PIC than in case of markers CH03g07 or CH05c02. The average frequency of alleles proved to be 11%.

Table 3 shows the allele size results at 6 microsatellite loci obtained for 66 apple cultivars. In cases where only one band was visible, its size is reported twice since the genotype is presumed to be homozygous. To determine whether this is a real homozygous allele or a null allele is involved, access to a segregating population is needed. In practice, the intensity of the specific band usually helps. The peak of the real homozygous allele is much higher. The average amount of heterozygotes is 70% ranging from 41% to 83%. These values are similar to the already published results of Gianfranceschi et al. (1998), Hokanson et al. (1998), and Liebhard et al. (2002) and slightly lower than those obtained by Laurens et al. (2004). The CH04g10 marker resulted in the most homozygote alleles (59%) and has the lowest discriminating power.

In case of five cultivars, three distinguishable alleles were determined with at least one primer pair, indicating triploidy; although only three of them ('Charden', 'Jonagold', and 'Sir Prize') are truly triploids. In cultivars 'Akane' and 'Pinova', the additional bands may indicate

the presence of another locus. Presumably these are not amplification artifacts since the obtained patterns were the same in all replications. The possible amplification of more than one locus was also observed by Guilford et al. (1997) and Liebhard et al. (2002). Surprisingly, the popular triploid cultivar 'Mutsu' produced only one or two alleles with all six primer pairs, indicates the abundance of homozygous and/or null alleles. Or else, it was just a coincidence, since in other study (Kitahara et al. 2005), among 10 applied SSR markers, 6 produced 3 different alleles in 'Mutsu'.

The accuracy of the obtained SSR data was confirmed by checking the presumed or known parentage of the genotypes in one hybrid ('Jonagold' = 'Golden Delicious' × 'Jonathan') for which both parents were also analyzed. For many of the other cultivars tested, however, only one of the parents was present among the tested cultivars. Checking these combinations, all data seemed to be consistent, i.e., one of the alleles always originated from the proper parent. For example, the cultivar 'Golden Delicious' was used as one of the parents for developing new hybrid combinations in case of 13 subsequent cultivars ('Angold', 'Charden', 'Elstar', 'Gala', 'Greensleeves', 'Jonagold', 'Mutsu', 'Ozark Gold', 'Pink Lady', 'Pinova', 'Sampion', 'Sir Prize', and 'Snygold'). In their allelic composition, the presence of one allele from 'Golden Delicious' is always evident. The same situation could be observed with five cultivars ('Akane', 'Florina', 'Idared', 'Jonager', and 'Jonagold') in which 'Jonathan' was used as one of the partners to generate new hybrids with improved features.

Table 1. Microsatellite primer sequences, number and size range of alleles found by Liebhard et al. (2002) and in the present study, and the polymorphism information content (PIC) values of simple-sequence repeat (SSR) markers based on the results of 8 (Liebhard et al. 2002) and 66 (present study) analyzed genotypes. The confusion probabilities (C) were used to find the optimal combination of SSR markers for identification.

SSR name	Forward primer sequence reverse primer sequence 5'–3'	Size	Alleles	PIC	Size	Alleles	PIC	C
		range	(no.)		range	(no.)		
		Liebhard et al. 2002			Present study			
CH03g07	aat aag cat tca aag caa tcc g ttt ttc caa atc gag ttt cgt t	119–181	5	0.77	119–179	7	0.74	0.235
CH04e03	ttg aag atg ttt ggc tgt gc tgc atg tct gtc tcc tcc at	179–222	11	0.88	178–222	12	0.77	0.206
CH04g10	caa aga tgt ggt gtg aag agg a gga ggc aaa aag agt gaa cct	127–168	5	0.83	127–168	6	0.60	0.377
CH05c02	tta aac tgt cac caa atc cac a gcg aag ctt tag aga gac atc c	168–200	4	0.60	160–200	7	0.70	0.256
CH05d11	cac aac ctg ata tcc ggg ac gag aag gtc gta cat tcc tca a	171–211	5	0.69	169–211	10	0.67	0.299
CH05e03	cga ata ttt tca ctc tga ctg gg caa gtt gtt gta ctg ctc cga c	158–190	10	0.87	160–193	13	0.83	0.128

Table 2. Allele sizes in base-pair and frequencies of 6 SSR loci distributed on 66 tested apple cultivars.

CH03g07		CH04e03		CH04g10		CH05c02		CH05d11		CH05e03	
Size	Frequency	Size	Frequency	Size	Frequency	Size	Frequency	Size	Frequency	Size	Frequency
119	25.6%	178	2.5%	127	4.4%	160	5.3%	169	13.7%	160	0.8%
123	17.6%	184	4.2%	135	60.0%	168	50.0%	171	5.1%	163	16.4%
127	9.6%	186	10.1%	137	14.4%	170	14.0%	173	53.0%	164	4.1%
129	38.4%	190	6.7%	139	1.1%	172	3.5%	175	10.3%	168	3.3%
153	0.8%	196	20.2%	143	8.9%	174	11.4%	181	0.9%	172	3.3%
165	4.0%	198	41.2%	168	11.1%	176	4.4%	187	2.6%	173	11.5%
179	4.0%	202	5.9%			200	11.4%	195	0.9%	175	0.8%
		204	3.4%					197	12.0%	176	0.8%
		208	2.5%					205	0.9%	179	15.6%
		210	1.7%					211	0.9%	181	0.8%
		216	0.8%							185	29.5%
		222	0.8%							191	9.8%
										193	3.3%

As a consequence, microsatellite markers can also be used for identification of ancestries in unknown hybrid combinations and crossings (Cabe et al., 2005; Kitahara et al., 2005). In special cases, the pollen and ovule donor parents

can be identified, as well. For instance, besides the triploidy of 'Jonagold' (presence of three different alleles) it was possible to determine which parent provided the ovule (two 198-bp alleles derived from 'Golden Delicious') and

which one the pollen grain (one 186-bp allele present from 'Jonathan') with the CH04e03 SSR marker. The 196-bp allele of 'Jonathan' did not take part in the generated cultivar 'Jonagold'. Surprisingly, for all other markers

Table 3. Allelic composition of 66 apple cultivars analyzed by 6 SSR markers.^z

Cultivar	CH03g07	CH04e03	CH04g10	CH05c02	CH05d11	CH05e03
Akane	123:179	196:210:216	135:143	170:200	173:173	185:185
Angold	119:129	198:198	135:135	168:170	171:173	173:185
Braeburn	127:129	198:202	168:168	168:168	171:173	191:191
Charden	119:129:129	198:198:198	135:135:135	168:174:200	169:173:175	175:179:185
<u>Elstar</u>	119:119	190:198	135:135	168:170	173:187	164:179
Red Elstar	119:119	190:198	135:135	168:170	173:187	164:179
Fiesta	119:123	186:196	135:135	168:168	173:197	164:185
Florina	123:127	196:198	135:168	168:200	173:197	163:191
Freedom	129:129	198:198	135:135	168:168	173:187	179:191
Fuji	119:127	196:198	143:168	168:168	173:197	163:191
<u>Gala</u>	119:129	196:198	135:135	168:170	173:173	173:185
Galaxy	119:129	196:198	135:135	168:170	173:173	173:185
Imperial Gala	119:129	196:198	135:135	168:170	173:173	173:185
Regal Prince (Gala Must)	119:129	196:198	135:135	168:170	173:173	173:185
Royal Gala	119:129	196:198	135:135	168:170	173:173	173:185
Gloster	123:129	198:198	127:137	160:168	195:197	160:191
<u>Golden Delicious</u>	119:129	198:198	135:135	168:174	169:173	179:185
Goldenir (Lysgolden)	119:129	198:198	135:135	168:174	169:173	179:185
Golden Reinders	119:129	198:198	135:135	168:174	169:173	179:185
Golden Spur	119:129	198:198	135:135	168:174	169:173	179:185
Gibson Golden Delicious	119:129	198:198	135:135	168:174	169:173	179:185
Goldstar	119:129	190:198	135:143	168:170	173:173	185:193
Granny Smith	129:153	196:198	127:137	160:172	171:173	168:181
Greensleeves	129:129	190:198	135:143	170:174	171:173	173:185
<u>Idared</u>	123:129	186:198	135:135	168:200	173:197	172:185
Red Idared	123:129	186:198	135:135	168:200	173:197	172:185
Jerseymac	165:165	184:198	135:137	160:176	173:197	163:173
Jonager	123:127	196:196	135:135	170:200	173:175	168:185
Jonagold	119:123:129	186:198:198	135:135:135	168:174:200	169:173:175	163:179:185
<u>Jonathan</u>	119:123	186:196	135:135	168:200	173:175	163:185
Jonathan M41	119:123	186:196	135:135	168:200	173:175	163:185
Jonathan Csányi1	119:123	186:196	135:135	168:200	173:175	163:185
Red Jonathan	119:123	186:196	135:135	168:200	173:175	163:185
Szatmárcsekei Jonathan	119:123	186:196	135:135	168:200	173:175	163:185
Watson Jonathan	119:123	186:196	135:135	168:200	173:175	163:185
Judeline	123:129	196:198	135:135	168:174	173:173	163:185
Julyred	165:165	184:198	135:143	160:176	175:197	163:163
Liberty	123:129	178:198	137:137	168:168	173:211	163:176
McIntosh	129:165	184:198	139:143	168:168	173:175	163:163
Mizsei	123:129	186:208	135:135	160:168	173:173	163:185
Mutsu	129:129:179	198:198:198	135:135:135	168:168:168	173:173:173	173:173:179
Ozark Gold	119:129	196:198	135:168	168:172	173:173	168:179
Pilot	129:179	196:196	135:135	168:168	173:173	164:185
Pink Lady	129:129	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	135:135	168:176	173:175	173:173
Poiana	129:129	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	168:168	173:181	173:185
Red Rome Van Well	123:129	186:198	135:135	168:168	173:173	163:163
Red Stayman	127:129	196:204	127:135	168:172	169:173	163:191
Reglindis	129:179	198:198	135:135	168:168	169:173	173:173
Relinda	123:129	196:208	135:143	168:172	173:173	172:185
Remo	119:123	178:190	135:135	168:170	173:173	163:172
Rewena	119:123	178:210	135:135	168:168	173:173	164:185
Rubinola	129:129	184:198	135:135	168:176	173:173	179:193
Sampion	119:129	190:198	135:135	170:174	169:173	179:193
Sir Prize	119:123:129	198:198:204	135:135:135	168:168:168	169:169:173	173:179:179
Snygold	129:129	198:198	135:135	170:174	173:205	173:179
<u>Starking Delicious</u>	127:129	198:202	137:168	168:168	173:197	191:191
Starkrimson Delicious	127:129	198:202	137:168	168:168	173:197	191:191
Redchief Delicious	127:129	198:202	137:168	168:168	173:197	191:191
Redspur Delicious	127:129	198:202	137:168	168:168	173:197	191:191
Topred Delicious	127:129	198:202	137:168	168:168	173:197	191:191
Wellspur Delicious	127:129	198:202	137:168	168:168	173:197	191:191
Topaz	119:129	190:198	135:135	168:168	169:173	185:193

^zThe numbers indicate the sizes of the alleles in bp. Indistinguishable sport mutations are in bold and their progenitors are underlined. In case of triploid cultivars where only two distinguishable alleles were found, the more intensive band was assumed to contain two alleles. This hypothetical third allele was indicated in italics since there is ambiguity if a null allele is involved.

Table 4. Selection of the most efficient minimum set of SSR markers for the identification of the 46 different cultivars.

Marker combination	Indistinguishable pairs (no.)	
	Observed	Expected
CH05e03	32	132.5
CH05e03 + CH04e03	5	27.3
CH05e03 + CH04e03 + CH03g07	2	6.4
CH05e03 + CH04e03 + CH03g07 + CH05d11	0	1.9

Comparison of the experimentally observed and theoretical efficient (X_e) marker combinations according to Tessier et al. 1999. Somatic mutants were treated as one single cultivar in this analysis.

at least one allele size was common in both parent cultivars. Similarly, it looks very likely that another triploid cultivar 'Charden' derives from the fertilization of a diploid 'Golden Delicious' ovule produced by meiotic non-disjunction with a haploid pollen grain from 'Clochard Renet', as revealed by CH05c02, CH05d11 and CH05e03 markers. This can be determined without *a priori* knowledge of the allelic composition of 'Clochard Renet' (pers. comm.). 'Sir Prize' is the third triploid cultivar of which one parent (also 'Golden Delicious') was present in our analysis. However, in this case, 'Golden Delicious' used for crossing was tetraploid (Williams et al. 1975) whereby the random disjunction of doubled homologue chromosomes during meiosis can produce homozygous ovules more frequently. Since the other parent ('PRI 14-152') is diploid, 'Golden Delicious' still could be determined as the ovule donor.

Successful differentiation of all apple genotypes except for somatic mutants was accomplished by using only four (CH03g07, CH04e03, CH05d11, and CH05e03) of the SSR markers (Table 4). Sport mutants proved to be indistinguishable from each other and their progenitors. This is not surprising since the genomes of somatic mutants differ only in one or a few small regions, which affect economically important characteristics, such as improved fruit colour, size or flavor. The chance is thus very small to find SSR marker(s) allowing us

to differentiate somatic mutants.

Using the three most polymorphic SSR markers (CH05e03, CH04e03, and CH03g07) only two genotype-pairs ('Topaz'-'Goldstar' and 'Starking'-'Braeburn') were indistinguishable (Table 4). The CH05c02 is the fourth most polymorphic marker but still unable to differentiate the genotype pair 'Starking'-'Braeburn', which was achieved by CH05d11 marker. That is why this marker was the fourth one selected for molecular identification despite of its slightly lower PIC value (Table 1).

The probability that two different and unrelated cultivars displayed the same genotype at all investigated loci (PI) was calculated as 1.79×10^{-4} , which confirms the high potential of SSRs for differentiation of apple genotypes and that other types of molecular markers could be necessary only in case of sport mutations. Developed SSR profiles can also be used in practice, e.g., for selection of distant parents to obtain higher genetic variation in progenies, to identify outcrosses or self-pollinated individuals, which do not belong to the progeny of the applied cross, for nursery control and protection of breeders' right.

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