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Simple sequence repeats for the genetic analysis of apple

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Abstract The development of highly informative markers, such as simple sequence repeats, for tagging genes controlling agronomic characters is essential for apple breeding. Furthermore the use of these markers is fundamental both for variety identification and for the characterisation and management of genetic resources. We have developed 16 reliable simple sequence repeat (SSR) markers that amplify all alleles from a panel of 19 *Malus x domestica* (Borkh.) cultivars or breeding selections and from *Malus floribunda* 821. Those markers show a high level of genetic polymorphism, with on average 8.2 alleles per locus and an average heterozygosity of 0.78. Due to this high level of polymorphism, it was possible using two selected SSRs to distinguish all cultivars except Starking and Red Delicious. Ten of the markers we developed have been mapped on a RAPD linkage map, proving their Mendelian segregation as well as their random distribution in the apple genome. Finally, we discuss the importance of using co-dominant markers in outbreeding species.

Key words SSR · Microsatellite · Plant breeding · Molecular markers · Germplasm · Polymorphism

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Introduction

Simple sequence repeats (SSRs, also named microsatellites) are becoming the marker of choice in both animal and plant species. SSRs are short stretches of DNA, consisting of tandemly repeated nucleotide units (1–5 nucleotides long). Microsatellites are usually very polymorphic due to the high level of variation in the number of repeats. Weber and May (1989) developed a general method for the detection of polymorphic microsatellites. This method is based on PCR amplification using a unique pair of primers flanking the SSRs. Differences in the number of repeated units can be detected by running the amplification products on high-resolution gels.

SSR markers have several advantages over other molecular markers. They are in fact abundant in most genomes and, generally, are uniformly distributed; they are hypervariable and co-dominant, therefore their information content is very high; SSRs are PCR-based, thus requiring little DNA for the amplification; every SSR locus is defined by a unique pair of primers, so that information exchange between laboratories is easy. The only disadvantages are linked to the need for high-resolution gels to fully exploit SSR information content and to the large initial effort required to clone and sequence microsatellite flanking sequences.

Some SSRs have already been developed in apple (Guilford et al. 1997). The use of multi-allelic, co-dominant markers for the analysis of heterozygous allogamous species is extremely useful since it allows individuals to be uniquely genotyped (Powell et al. 1996). This is particularly important for cultivar identification and to enhance the genetic characterisation of germplasm collections and natural populations, permitting effective conservation of plant genetic resources. Furthermore, the high information content of microsatellites can be fully applied to QTL mapping and in general to apple breeding, enormously

increasing the power of such analysis. In this paper we report the development of 16 new SSR markers and the mapping of ten of them on a RAPD genetic linkage map. We show the high level of polymorphism detected and discuss the importance of SSR markers in future quantitative trait mapping projects.

Materials and methods

Plant material and DNA isolation

Plant material for DNA isolation was kindly provided by Markus Kellerhals at the Swiss Federal Research Station (FAW), Wädenswil, Switzerland, and by Philip Forsline at the National Germplasm Repository for Apple and Grape, NYS Agricultural Experimental Station, Geneva, N.Y., USA. DNA was extracted from the following plants, A679/2, Boskoop, Cox's Orange Pippin, Florina, Golden Delicious, Iduna, James Grieves, Jonafree, Jonagold, Jonathan, Maigold, *Malus floribunda* 821, Nova Easygro, Pinova, Red Delicious, Redfree, Rome Beauty Law, Spartan, Starking and Wagener, according to Dellaporta's protocol (Dellaporta et al. 1983) with modifications after Koller et al. (1994).

Microsatellite enrichment, screening and sequencing

One-hundred micrograms of apple DNA isolated from cultivar Florina was digested to completion with the restriction enzyme *Tsp*509I (New England Biolabs) and then separated on an agarose gel (1 : 1 Agarose : NuSieve; FMC Bioproduct). A 300–600 bp fraction was recovered on a NA-45 DEAE membrane (Schleicher and Schuell) and enriched for (AG)/(CT) repeats through hybridisation and capture with a biotinylated oligonucleotide containing a $(CT)_{13}$ repeat (Morgante, personal comunication). The enriched fraction was cloned into phage Lambda ZapII (Stratagene) according to the manufacturer's instruction.

The Lambda ZapII library was screened by plaque lifting onto nylon membranes (Hybond N, Amersham) and hybridisation to ^{32}P-labelled poly (dA.dG)/(dT.dC) probes as described by Sambrook et al. (1989). The density of the plaques was approximately 500–700 pfu/plate. Pre-hybridisation was performed for 6 h in 1 M NaCl, 50 mM Tris-HCl pH 7.5, 1% SDS, 5% dextran sulphate at 65°C. The overnight hybridisation was at the same temperature. Initial washes were performed at room temperature in 2 × SSPE, 0.1% SDS (Sambrook et al. 1989) followed by two stringent washes of 15 min each at 65°C in 2 × SSPE, 1% SDS. Plaques containing SSRs were identified by autoradiography.

An anchored PCR screening strategy on the purified phage stocks, followed by agarose-gel analysis, was used to determine the approximate size of the insert, as well as the position of the SSR within the clone. The procedure requires that five PCR amplifications per clone are performed. One where the two primers on the vector are used (for example T3 and T7), and the other four PCR reactions each using one of the vector primers in combination with a primer that is 5'-anchored to the repeat. This procedure has been previously described in detail by Rafalski et al. (1995).

Double-stranded pBluescript DNAs, obtained after in vivo excision and plasmid DNA preparation (Wizard Minipreps, Promega Co., Madison, Wis.), were sequenced on an Applied Biosystems 373 A automated sequencer using the ABI PRISM dye terminator cycle sequencing kit.

Microsatellite PCR amplification

Oligonucleotide primer sequences suitable for PCR amplification of unique SSRs were selected using the program Primer version 0.5 available from the Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, USA (*http://www-genome.wi.mit.edu/*). Primer pairs were designed to have similar theoretical melting temperatures of approximately 60°C (see Table 1). Oligonucleotides were synthesised commercially (MWG-Biotech, Ebersburg, Germany). PCR amplifications were performed in a 15 µl vol containing 5 ng of genomic DNA, 10 mM Tris-HCl pH 9.0, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 µM each of forward and reverse primers and 1 U of *Taq* polymerase (Pharmacia Biotech). All SSR amplifications were performed in a GeneAmp PCR System 9600 (Perkin Elmer) under the following conditions: an initial denaturation at 94°C for 2 min 30 s followed by four cycles of 94°C for 30 s, 65°C for 1 min, 72°C for 1 min where the annealing temperature was reduced by 1°C per cycle; the initial cycles were followed by 30 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 1 min. A final 5-min 72°C extension was included. Primer pairs were preliminarily tested by running PCR products on a 1.5% agarose gel in a 0.5 × TBE buffer (Sambrook et al. 1989), stained with ethidium bromide and visualised by UV illumination. Microsatellite alleles were analysed by running radiolabelled PCR products on a 6% denaturing sequencing gel (National Diagnostic, Atlanta, USA) in 1 × TBE buffer using a IBI DNA sequencing unit. Radiolabelled PCR products were obtained by labelling 1/4 of the forward primer with [γ-^{33}P] ATP (1000–3000 Ci/mmol, Amersham Life Science) using a T4 polynucleotide kinase (Pharmacia Biotech). Before loading, radiolabelled PCR products were denatured by the addition of 1 vol of denaturing gel loading buffer (Sambrook et al. 1989) and heating at 94°C for 2–5 min. After the run, gels were transferred onto Whatman 3MM paper, dried at 80°C for 3 h in a gel dryer (Bio Rad) and exposed for 24–48 h to X-ray films (Kodak, X Omat AR) without intensification screens.

Data analysis

The SSR allele composition of each analysed plant was determined and the expected heterozygosity value (H) of each microsatellite was calculated according to the formula:

$$H = 1 - \sum_{i=1}^{n} p_i^2,$$

where p is the frequency of the ith allele of each SSR marker (Nei 1973) and n is the number of alleles.

Segregation analysis and mapping was done on 96 individuals of a Iduna × A679/2 population. The genetic map-position of the SSR markers was assigned in relation to a RAPD linkage map generated in our laboratory (Seglias 1997), using a LOD score threshold of 4.0. All map calculations were done using the software package JoinMap version 2.0 (Stam 1993; Stam and Van Oojien 1995).

Results

Development of apple SSR markers

A Lambda small-insert genomic library was constructed from the cultivar Florina. The library was screened by plaque hybridisation for the presence of (AG)/(TC) repeats, and clones that gave positive signals were further tested for the presence of the repeat within the cloned insert by means of an anchored PCR assay (Rafalski et al. 1995). This assay minimises the amount of unnecessary sequencing allowing one to determine the location and the distance of the repeat from the cloning sites. Clones containing no repeat, as well as

those in which the repeat is either too far from both cloning sites or to close to one end of the insert, can be immediately discarded. From the initial screening we chose 80 clones which passed both hybridisation and anchored PCR assays. All of them were sequenced from the vector end that is closer to the repeat. In 58 cases it was possible to design unique primer pairs. All primer pairs were pre-screened in a 1.5% agarose gel on eight cultivars. Primer pairs amplifying at least one PCR fragment in every cultivar were further tested on the complete set of cultivars using a denaturing acrylamide gel. Probably due to the high level of polymorphism present in the apple genome, most of the primers failed to amplify all alleles present in the cultivars employed in the pre-screening, although all but five amplified at least one allele from the cultivar Florina from which they originated.

Sixteen SSR markers gave a reproducible banding pattern. These were initially tested on Florina, Iduna, A679/2 and on five individuals of the Iduna × A679/2 population (I × A). Eleven of the sixteen clones contained a perfect type of repeat (according to the definition given by Weber 1990) and the average number of

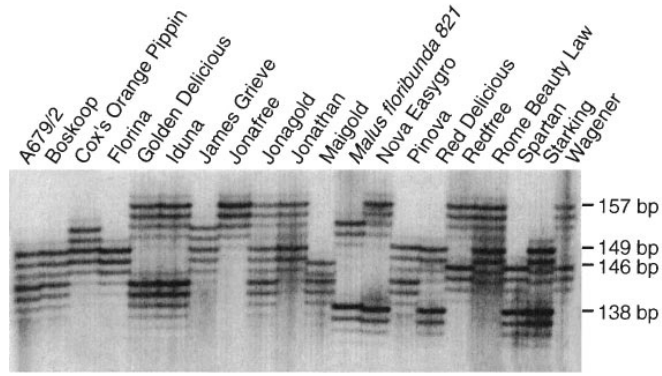


Fig. 1 SSR locus CH02F06 showing alleles amplified from 19 apple cultivars/selections and from *M. floribunda* 821. The fragment size in base pairs (bp) is reported on the right side. In the Jonagold lane it is possible to distinguish the three alleles present in this triploid cultivar

Table 1 SSR primer sequences, repeat type, allele size range, number of alleles and expected heterozygosity found in the cultivars/selections analysed

SSR name		Primer sequence (5′ → 3′)	Repeat type	Allele size range	No. alleles	Exp het
CH01B12	F	CGCATGCTGACATGTTGAAT	Perfect	123–130	5	0.73
	R	CGGTGAGCCCTCTTATGTGA		$(AG)_{20}$		
CH01E01	F	GGTTGGAGGGACCAATCATT	Compound	104–138	7	0.74
	R	CCCACTCTCTGTGCCAGATC		$(GT)_{12}A\ (AG)_{32}$		
CH01E12	F	AAACTGAAGCCATGAGGGC	Perfect	243–248	8	0.76
	R	TTCCAATTCACATGAGGCTG		$(AG)_{32}$		
CH01F02	F	ACCACATTAGAGCAGTTGAGG	Perfect	168–222	11	0.82
	R	CTGGTTTGTTTTCCTCCAGC		$(AG)_{22}$		
CH01F09	F	ATGTACATCAAAGTGTGGATTG	Perfect	112–139	9	0.75
	R	GGCGCTTTCCAACACATC		$(AG)_{22}$		
CH01G12	F	CCCACCAATCAAAAATCACC	Imperfect	107–186	10	0.86
	R	TGAAGTATGGTGGTGCGTTC		$(AG)_{45}$		
CH01H01	F	GAAAGACTTGCAGTGGGAGC	Perfect	107–141	9	0.82
	R	GGAGTGGGTTTGAGAAGGTT		$(AG)_{25.5}$		
CH01H02	F	AGAGCTTCGAGCTTCGTTTG	Imperfect	226–252	7	0.78
	R	ATCTTTTGGTGCTCCCACAC		$(AG)_{17.5}\ (AG)_{8}$		
CH01H10	F	TGCAAAGATAGGTAGATATATGCCA	Perfect	93–119	7	0.72
	R	AGGAGGGATTGTTTGTGCAC		$(AG)_{21}$		
CH02B03b	F	ATAAGGATACAAAAACCCTACACAG	Perfect	77–109	8	0.82
	R	GACATGTTTGGTTGAAAACTTG		$(GA)_{22}$		
CH02B10	F	CAAGGAAATCATCAAAGATTCAAG	Perfect	114–157	12	0.90
	R	CAAGTGGCTTCGGATAGTTG		$(GA)_{19.5}$		
CH02B12	F	GGCAGGCTTTACGATTATGC	Imperfect	124–142	8	0.72
	R	CCCACTAAAAGTTCACAGGC		$(GA)_{26}$		
CH02C06	F	TGACGAAATCCACTACTAATGCA	Perfect	216–254	10	0.83
	R	GATTGCGCGCTTTTTAACAT		$(GA)_{21}\ (GA)_{17}$		
CH02D11	F	AGCGTCCAGAGCAACAGC	Perfect	117–146	7	0.73
	R	AACAAAAGCAGATCCGTTGC		$(AG)_{21}$		
CH02D12	F	AACCAGATTTGCTTGCCATC	Perfect	175–205	9	0.78
	R	GCTGGTGGTAAACGTGGTG		$(GA)_{19}$		
CH02F06	F	CCCTCTTCAGACCTGCATATG	Compound	138–157	7	0.80
	R	ACTGTTTCCAAGCGATCAGG		$(TG)_{10}\ (AG)_{20}$		
COL[a]	F	AGGAGAAAGGCGTTTACCTG	Perfect	213–239	6	0.71
	R	GACTCATTCTTCGTCGTCACT		$(GA)_{17.5}$		

[a] The COL sequence was retrieved from the EMBL sequence databank (Accession No. U50187). No. alleles is the number of alleles detected. Exp het is the expected heterozygosity, see Materials and methods section

repeated units present in the cloned alleles was 21.5. Table 1 summarises the primer sequences, the repeat type, the allele size range, the number of alleles and the expected heterozygosity. Nineteen apple cultivars or breeding selections together with *Malus floribunda* 821 were tested using the 16 SSR markers we developed as well as with another one (we named COL) whose primers were designed on a sequence present in the databank, originally obtained by sequencing a RAPD fragment linked to the columnar growth-habit locus, *Co* (Hemmat et al. 1997) (Fig. 1). The number of alleles per SSR ranged from 5 to 12 with an average of 8.2. The average expected heterozygosity of all SSRs was 0.78.

Cultivar or selection identification

Using as few as two SSR markers we were able to differentiate all apple varieties/selections analysed except for Starking and Red Delicious (Table 2). Moreover for the two triploid cultivars included in the screening, Boskoop and Jonagold, it was possible to detect three alleles in nine and ten cases, respectively.

SSR mapping

Ten SSR markers, which showed at least three alleles segregating in the Iduna × A679/2 population, were

Table 2 Allelic composition of the 19 apple cultivars/selections and of *M. floribunda* 821. The numbers indicate the size of each SSR allele (bp). Fragment size was determined by comparison with the T lane of a sequencing reaction of a pBluescript plasmid containing a known microsatellite sequence

Cultivar/selection	CH01B12	CH01E01	CH01E12	CH01F02	CH01F09	CH01G12	CH01H01	CH01H02	CH01H10
A679/2	126:125	110:106	283:245	194:172	129:129	147:134	123:119	248:236	111:105
Boskoop (triploid)	126:125	110:104	283:251:247	204:182:180	139:133:129	184:155:151	133:129:107	248:244	100:93
Cox's Orange Pippin	125:124	108:104	249:249	206:180	139:125	155:151	133:121	248:244	100:100
Florina	126:125	108:108	283:249	206:182	139:133	184:111	133:119	248:236	113:93
Golden Delicious	126:125	110:106	251:245	178:168	129:129	147:107	121:119	250:248	111:93
Iduna	126:123	116:108	251:245	210:180	139:129	151:151	133:119	252:236	105:93
James Grieve	126:125	108:104	251:249	206:204	131:125	155:107	123:121	248:248	100:100
Jonafree	126:123	110:108	283:259	206:206	133:127	184:184	123:115	236:236	100:93
Jonagold (triploid)	126:125	110:108:106	283:251:245	206:178:168	139:129	147:109:107	133:121:119	250:248	111:93
Jonathan	126:124	108:106	283:283	206:206	139:133	184:109	133:117	248:236	100:93
Maigold	126:124	106:106	251:249	206:178	129:123	186:107	119:121	250:250	117:93
M. floribunda 821	130:125	118:138	249:243	178:174	112:112	151:134	141:107	252:226	113:105
Nova Easygro	125:123	108:108	283:249	206:178	139:137	184:111	123:119	252:246	93:93
Pinova	125:124	116:110	251:249	204:168	129:123	151:147	133:121	250:248	111:104
Red Delicious	125:124	108:106	249:249	182:178	139:129	111:111	119:119	246:236	100:93
Redfree	126:123	108:108	249:249	206:178	133:129	151:111	115:115	248:246	100:100
Rome Beauty Law	130:125	110:108	247:277	206:206	129:129	184:151	123:115	236:236	100:100
Spartan	125:124	108:108	251:249	178:172	139:129	134:111	119:117	248:246	100:93
Starking	125:124	108:106	249:249	182:178	139:129	111:111	119:119	246:236	100:93
Wagener	126:125	110:104	283:249	180:168	139:129	141:111	133:119	248:246	100:93

	CH02B03b	CH02B10	CH02B12	CH02C06	CH02D11	CH02D12	CH02F06	COL
A679/2	99:97	143:121	140:130	248:230	146:117	199:197	149:143	231:231
Boskoop (triploid)	101:99:97	125:123:121	140:128	250:240:230	146:130:120	193:179	149:143	231:229
Cox's Orange Pippin	99:77	131:119	140:128	240:240	130:119	197:197	153:149	229:229
Florina	101:97	133:131	140:126	252:250	130:130	197:187	149:149	231:217
Golden Delicious	101:81	125:121	140:140	240:236	130:117	197:193	157:143	231:217
Iduna	101:81	129:115	140:126	254:240	130:130	197:193	157:143	231:231
James Grieve	97:77	143:119	140:124	252:240	130:130	197:189	153:149	229:229
Jonafree	99:99	157:157	136:124	240:236	130:119	197:189	157:157	239:229
Jonagold (triploid)	101:97:81	133:125:121	140:124	252:240:236	130:117	197:193:189	157:149:143	231:217
Jonathan	99:97	133:125	126:124	252:236	130:119	197:189	157:149	231:229
Maigold	97:81	137:125	142:140	236:230	117:130	193:189	146:143	231:229
M. floribunda 821	101:95	115:114	138:128	244:216	134:122	203:189	153:138	233:213
Nova Easygro	95:93	157:123	140:128	252:250	130:120	189:175	157:138	239:217
Pinova	99:81	143:121	140:140	240:240	119:117	193:175	149:143	231:229
Red Delicious	109:101	157:131	142:140	250:218	146:130	197:175	149:138	231:217
Redfree	101:99	137:123	140:128	240:230	130:130	197:189	157:146	231:229
Rome Beauty Law	99:81	157:119	142:136	252:240	120:119	197:189	157:149	229:217
Spartan	101:95	129:123	140:140	254:250	122:130	197:175	146:138	239:231
Starking	109:101	131:131	142:140	250:218	146:130	197:175	149:138	231:217
Wagener	101:99	157:123	142:140	252:252	146:119	205:179	157:146	231:229

mapped on a RAPD linkage map previously generated by one of the authors (Seglias 1997). All SSRs except CH01H01 showed a Mendelian type of segregation (Fig. 2).

The RAPD-based linkage analysis led to the production of two maps containing 19 and 17 linkage groups, covering 548 and 689 cM for the Iduna and the A679/2 linkage maps, respectively. The use of SSR markers allowed us to detect six homologous linkage groups and in two cases, where two SSRs per group were present, to align them (Fig. 3). Nine out of ten SSR markers were associated with at least one RAPD marker and appear to be uniformly distributed in the apple genome.

Discussion

The most frequently occurring microsatellite dinucleotide repeats in plants are (AT)/(TA), with (AG)/(CT) and (AC)/(GT) as the second and third most frequent (Lagercrantz et al. 1993; Thomas and Scott 1993; Wang et al. 1994). However, due to the difficulties in screening for (AT)/(TA) repeats using hybridisation techniques we decided to develop microsatellite markers containing (AG)/(CT) repeats. Microsatellites are very informative markers and are relatively easy and inexpensive to use; however, costs for their development are high. The latter seems to be especially true for apple where a high level of polymorphism is present in the genome. In fact, although polymorphism is a desirable characteristic enabling the differentiation of closely related plants, it is, on the other hand, a limiting factor for the design of PCR primer pairs able to amplify all alleles present in the apple germplasm. Only 16 out of 58 primer pairs tested amplified all alleles present in the cultivars/selections panel. Since, except in five cases, we could always amplify at least one PCR product from Florina, the cultivar used to produce the genomic library, differences in the primer annealing sequences are probably the reason why PCR failed to amplify some alleles. The presence of polymorphism in the SSR flanking sequences is also supported by the detection of alleles whose sizes did not differ from the others by multiples of the repeat length; such alleles could be explained by single base deletions/insertions (Fig. 1). The occurrence of null alleles was already pointed out by Powell et al. (1996) as a possible problem associated with the use of microsatellites in highly outbred, heterozygous species. The pattern of the PCR products produced by amplification of the genomic DNA was almost always simple. In all cases it was possible to distinguish the main bands containing the repeat from the low-molecular-weight products (most probably PCR artefacts). CH01B12 is a special case since the PCR amplification produces two sets of bands. The first contains the microsatellite repeat, easily recognisable by the presence of secondary bands (stutter bands) but which unfortunately are not amplified from all cultivars; the second differed in size by one nucleotide and therefore most likely do not contain the microsatellite repeat, but nevertheless are highly polymorphic. It is this latter locus that was mapped and used in the cultivars/selections screening.

Using as few as two SSR markers among the most polymorphic, we were able to differentiate all apple varieties/selections except for Starking and Red Delicious. Starking is a somatic mutant of Red Delicious with improved fruit colour rather than a different cultivar obtained by sexual recombination. It is most likely that the two genomes differ only in one or a few small mutations affecting fruit colour. It is therefore not surprising that we were not able to differentiate the cultivars using a restricted number of molecular markers. In most cases SSR markers allowed us to distinguish the two triploid cultivars included in the full test-set (Jonagold and Boskoop) from the diploid ones, due to the presence in the former of three different alleles. In case of Jonagold, derived from a Golden Delicious × Jonathan crossing, when we could detect three alleles it was always possible to distinguish the two alleles of Golden Delicious plus either one or the other allele derived from Jonathan, proving that Jonagold most probably originated from the fertilization of a diploid Golden Delicious ovule, produced by meiotic non-disjunction, with a haploid pollen grain from Jonathan.

Ten of the SSR markers developed were mapped to a RAPD linkage map. Nine of them were linked to at least one RAPD marker. Considering the complete apple linkage map to be 1206 cM (Conner et al. 1997), the Iduna and A679/2 linkage maps represent about 45% and 57% of the apple genome, respectively. Therefore it can be anticipated that few markers fall in genomic regions not

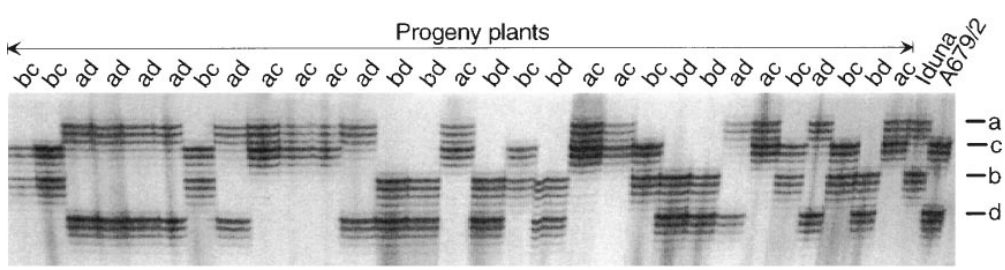
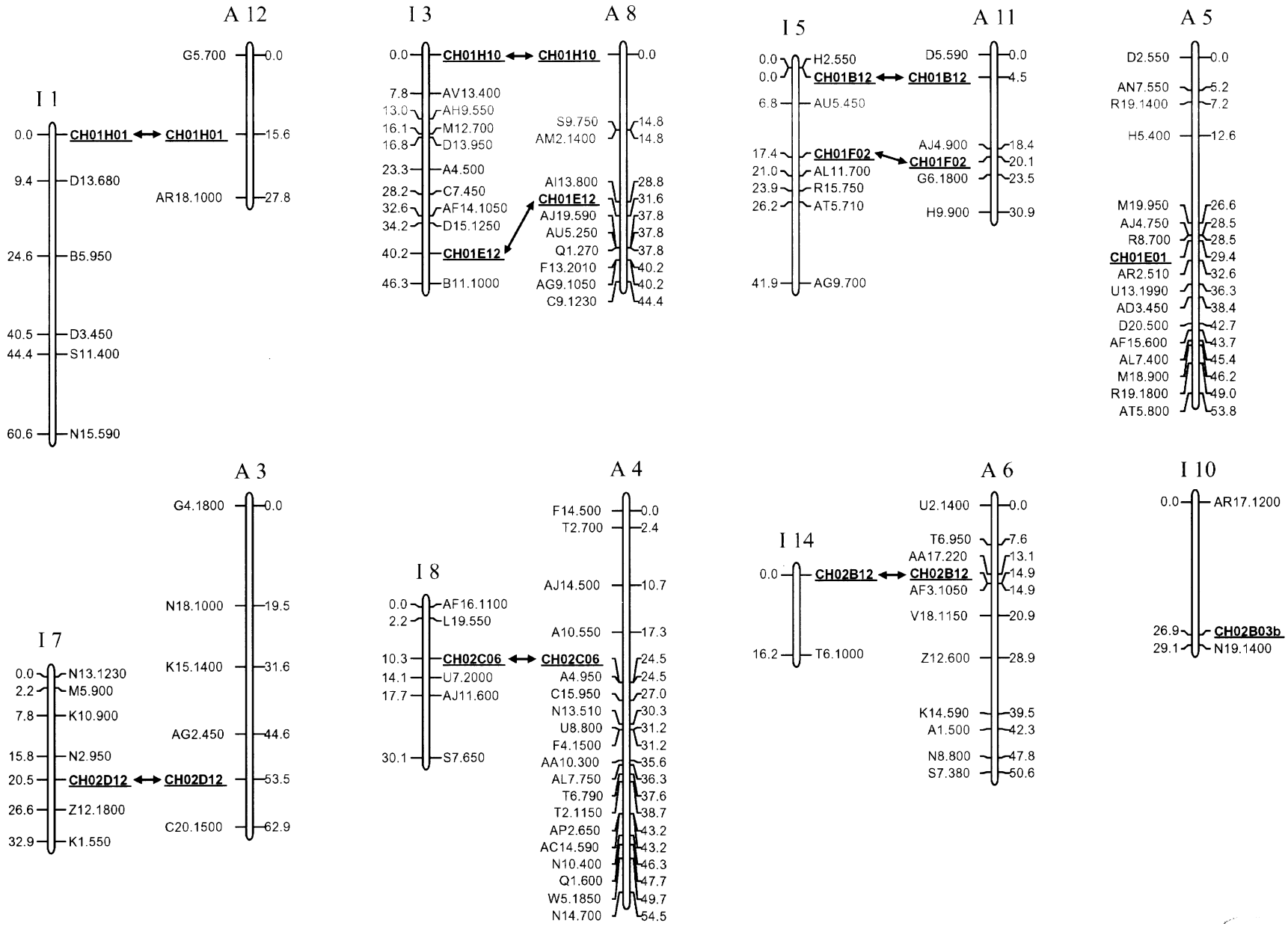


Fig. 2 Example of Mendelian segregation of SSR CH02C06 in progeny plants of the Iduna × A679/2 population. All four alleles (indicated with the letters *a*, *b*, *c*, *d*) could be differentiated


I 1
A 12
0.0 CH01H01 CH01H01 15.6
9.4 D13.680
AR18.1000 27.8
24.6 B5.950
40.5 D3.450
44.4 S11.400
60.6 N15.590
G5.700 0.0
I 3
A 8
0.0 CH01H10 CH01H10 0.0
7.8 AV13.400
13.0 AH9.550
16.1 M12.700
16.8 D13.950
23.3 A4.500
28.2 C7.450
32.6 AF14.1050
34.2 D15.1250
40.2 CH01E12 CH01E12 28.8
46.3 B11.1000
S9.750 14.8
AM2.1400 14.8
AI13.800 31.6
AJ19.590 37.8
AU5.250 37.8
Q1.270 37.8
F13.2010 40.2
AG9.1050 40.2
C9.1230 44.4
I 5
A 11
0.0 H2.550
0.0 CH01B12 CH01B12 4.5
6.8 AU5.450
17.4 CH01F02 CH01F02 18.4
21.0 AL11.700 20.1
23.9 R15.750 23.5
26.2 AT5.710 30.9
41.9 AG9.700
D5.590 0.0
AJ4.900
G6.1800
H9.900
A 5
D2.550 0.0
AN7.550 5.2
R19.1400 7.2
H5.400 12.6
M19.950 26.6
AJ4.750 28.5
R8.700 28.5
CH01E01 29.4
AR2.510 32.6
U13.1990 36.3
AD3.450 38.4
D20.500 42.7
AF15.600 43.7
AL7.400 45.4
M18.900 46.2
R19.1800 49.0
AT5.800 53.8
A 3
G4.1800 0.0
N18.1000 19.5
K15.1400 31.6
AG2.450 44.6
C20.1500 62.9
I 7
0.0 N13.1230
2.2 M5.900
7.8 K10.900
15.8 N2.950
20.5 CH02D12 CH02D12 53.5
26.6 Z12.1800
32.9 K1.550
A 4
I 8
0.0 AF16.1100
2.2 L19.550
10.3 CH02C06 CH02C06 24.5
14.1 U7.2000
17.7 AJ11.600
30.1 S7.650
F14.500 0.0
T2.700 2.4
AJ14.500 10.7
A10.550 17.3
A4.950 24.5
C15.950 27.0
N13.510 30.3
U8.800 31.2
F4.1500 31.2
AA10.300 35.6
AL7.750 36.3
T6.790 37.6
T2.1150 38.7
AP2.650 43.2
AC14.590 43.2
N10.400 46.3
Q1.600 47.7
W5.1850 49.7
N14.700 54.5
A 6
I 14
0.0 CH02B12 CH02B12 14.9
16.2 T6.1000
U2.1400 0.0
T6.950 7.6
AA17.220 13.1
AF3.1050 14.9
V18.1150 20.9
Z12.600 28.9
K14.590 39.5
A1.500 42.3
N8.800 47.8
S7.380 50.6
I 10
0.0 AR17.1200
26.9 CH02B03b
29.1 N19.1400

covered by the RAPD map. The exclusive use of dominant markers, such as RAPDs, leads to the construction of two linkage maps including markers which are heterozygous only in Iduna or in A679/2, respectively. Only the use of more informative markers allows: (1) the identification of homologous linkage groups; (2) the alignment of linkage groups in cases where at least two informative markers per group are present, and (3) a comparison of the frequency of cross-over events occurring in a specific genomic region in the two parents. All SSRs segregate as expected except for CH01H01. We do not know the cause of this distorted segregation. Valid explanations could be either the presence of a gametophytic factor influencing pollen competitivity and/or fertility, or linkage with a mutation reducing plant viability. However, since none of the RAPD markers flanking CH01H01 show distorted segregation, it is more likely that the bias is due to a random sampling error.

The multi-allelic nature of SSR markers can be fully exploited in QTL mapping projects on outbreeding species. It is in fact very important to be able to follow the segregation of most of the alleles involved in order to detect QTLs and to establish the relative importance of the QTL alleles in controlling the quantitative trait in question.

Although RFLP cDNA probes have been developed, they did not prove to be as informative as expected. In fact, due to the polyploid origin of apple most of the random cDNA probes detect duplicated loci, rendering more difficult the interpretation of the hybridisation pattern (Van Heusden et al. 1997). Therefore it is important to have informative and easy to score markers in order to obtain consistent and reproducible results over different laboratories. In this respect the SSRs we developed fulfil these needs.

Even though initial costs for developing SSR markers are high, SSRs remain the marker of choice for apple identification and breeding. They will, most probably, become landmarks for the comparison of linkage maps produced in different laboratories and will help in the production of a readily available consensus apple linkage map. It has been shown that SSR analysis could be easily automated using fluorescence-based DNA detection systems and multiplex PCR techniques (Kijas et al. 1995; Mitchell et al. 1997). The routine application of these techniques will enable fast and accurate genotyping of large populations, speeding up the selection process. Since apple has a long juvenile period (3–5 years) it is very important to recognise plants having the desired genotype as early as possible in order to reduce field space and labour, while at the same time increasing the efficiency of selection. SSRs can also be considered as a major tool for the management of plant genetic resources in order to maintain a broad genetic diversity and to develop an effective conservation strategy.

Fig. 3 Schematic representation of the homologous linkage groups of Iduna and A679/2 containing SSR markers. The letter *I* or *A* before the linkage group number indicates that the group includes RAPD markers polymorphic in Iduna and A679/2, respectively. *Numbers* along linkage groups indicate the distance in centi-Morgan (cM) of the marker from the top of the linkage group. In six cases SSRs allowed us to identify homologous linkage groups and in two cases (I3–A8 and I5–A11) to align them

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