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ORIGINAL PAPER

Development and test of 21 multiplex PCRs composed of SSRs spanning most of the apple genome

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Abstract A series of 21 multiplex (MP) polymerase chain reactions containing simple sequence repeat (SSR) markers spanning most of the apple genome has been developed. Eighty-eight SSR markers, well distributed over all 17 linkage groups (LGs), have been selected. Eighty-four of them were included in 21 different MPs while four could not be included in any MPs. The 21 MPs were then used to genotype approximately 2,000 DNA samples from the European High-quality Disease-Resistant Apples for a Sustainable agriculture project. Two SSRs (CH01d03 and NZAL08) were discarded at an early stage as they did not produce stable amplifications in the MPs, while the scoring of the multilocus (ML) SSR Hi07d11 and CN44794 was too complex for large-scale genotyping. The testing of the

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remaining 80 SSRs over a large number of different genotypes allowed: (1) a better estimation of their level of polymorphism; as well as of (2) the size range of the alleles amplified; (3) the identification of additional unmapped loci of some ML SSRs; (4) the development of methods to assign alleles to the different loci of ML SSRs and (5) conditions at which an SSR previously described as ML would amplify alleles of a single locus to be determined. These data resulted in the selection of 75 SSRs out of the 80 that are well suited and recommended for large genotyping projects.

Keywords SSR · Multiplex PCR · Genotyping · *Malus*

Introduction

Recently, the interest in developing a set of well-scattered highly polymorphic molecular markers spanning the whole apple genome has increased. Such a set of markers are necessary for the generation of genetic maps to be used for the accurate identification of genes and quantitative trait loci (QTLs) by association studies as well as for "fast breeding" strategies which identify progeny plants with the lowest proportion of undesired genome inherited from a wild apple (Volz et al. 2007). A subset of these selected markers can also be used for the genome scanning approach (GSA; Patocchi and Gessler 2003) or used to fingerprint cultivars and selections.

The development of such a set of markers was necessitated by the European Project High-quality Disease-Resistant Apples for a Sustainable agriculture (HiDRAS), established in 2002 (Gianfranceschi and Soglio 2004), which aimed to identify genetic loci controlling apple fruit quality with the objective of increasing the fruit quality of disease-resistant apples and therefore their acceptability and dissemination, leading to a reduction in the use of fungicides. HiDRAS is based on an innovative approach of association of genotype with phenotype called pedigree-based analysis (PBA). PBA includes the analysis of the segregation of specific chromosomal regions in genetically related cultivars, breeding selections, and small progenies through highly polymorphic codominant markers. This "identity by descent" approach allows the detection of QTLs (Bink et al. 2002; van de Weg et al. 2004).

To increase the efficiency and reduce the cost of this type of genetic study, this set of markers must be polymerase chain reaction (PCR)-based and suitable for organization into multiplexes (MPs). Simple sequence repeat (SSR) markers were our markers of choice as they meet these requirements and because they are also generally highly polymorphic, ideal for map alignments, and highly transferable between laboratories.

More than 300 SSRs have been developed in apple (Guilford et al. 1997; Gianfranceschi et al. 1998; Hokanson et al. 1998; Liebhard et al. 2002; Hemmat et al. 2003; Vinatzer et al. 2004, Silfverberg-Dilworth et al. 2006, Khan et al. 2007, Igarashi et al. 2008; Van Dyk 2008). In addition, the use of pear SSRs can be explored as it has been demonstrated that they can generally be transferred to the apple and vice versa (Yamamoto et al. 2002; Pierantoni et al. 2004; Silfverberg-Dilworth et al. 2006) due to the high level of synteny between the two species. Nearly all apple SSRs have been mapped; the majority of them being mapped in the "reference map of the apple" (Liebhard et al. 2003; Silfverberg-Dilworth et al. 2006). The position within the apple genome of the few that were not mapped in the reference map can be inferred from map alignments using SSR markers in common as references.

Silfverberg-Dilworth et al. (2006) estimated that approximately 100 single-locus markers are required to span the apple genome with an intramarker distance of approximately 15–20 cM and a maximal 10-cM distance from the linkage group ends. The known distribution of the SSRs in the apple genome (reference map) as well as small-scale studies of their level of polymorphism (Liebhard et al. 2002; Silfverberg-Dilworth et al. 2006) allowed the selection of a set of 84 SSRs that covers about 85% of the apple genome (Silfverberg-Dilworth et al. 2006). This set of SSRs was composed of: 75 single-locus or presumed single-locus SSRs, seven multilocus SSRs (CH01d03, CH03g12, CH04c06, CH04g09, CH04h02 Hi07d11, and HB03-SSR) where the position of all the loci is known, and two multilocus SSRs (CN493139 and Hi23g12) where the position of only one locus was known. For one out of the seven multilocus SSRs (CH03g12), both loci (LG1 and LG3) are in interesting positions (i.e., located at extremities of a linkage group). For the other six SSRs, only one of the loci was of main interest as their second locus maps close to a single-locus SSR, which was chosen in preference to the multilocus SSRs to represent their genomic region. For 16 regions of the genome, no SSRs were identified by Silfverberg-Dilworth et al. (2006).

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In this paper, we present an extension of this core set of SSRs spanning most of the apple genome, the development of multiplex PCRs for high throughput application of these SSRs, and finally an evaluation of their performance carried out by screening them over about approximately 2,000 DNA samples of the HiDRAS project.

Materials and methods

Plant material

The HiDRAS plant material genotyped with the selected SSRs is composed of 27 genetically related progenies of variable sizes (on average, 51 individuals with a range of 26 to 98), for a total of 1,373 progeny plants. In addition, another 359 cultivars and breeding lines were analyzed, many of which were represented by replicated DNA samples from different countries (Table S1). These genotypes were part of the pedigrees of the populations analyzed or were so-called common progenitors (genotypes present at different partner sites) or new founders (e.g., heritage cultivars). Including internal controls and repeated samples, 1,997 samples were genotyped.

DNA extraction, quantification, and standardization

DNA was extracted using the Qiagen DNeasy (Qiagen, Hilden, Germany) kit by the owner of the plant material and sent to the Swiss Federal Institute of Technology Zürich. DNA concentration was determined with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Basel, Switzerland), on a SPECTRAFluor Plus microplate reader (Tecan, Männedorf, Switzerland). DNA concentration of each sample was adjusted to 5 ng/µl. DNA samples were reorganized into 96-well plates using a Genesis RSP Liquid-Handling robot (Tecan, Switzerland). On each plate, samples of the genotypes "Prima" and "Fiesta" were added in reference positions H6 and H12. Aliquots were then prepared using a Tecan Genesis RSP Liquid-Handling robot (Tecan, Switzerland) and shipped to the six partners responsible for the genotyping, defined in this paper as "genotypers".

SSRs spanning the whole apple genome

The set of genome-spanning SSRs examined is composed of 88 SSRs developed by different groups: Liebhard et al (2002, 54 SSRs) Silfverberg- Dilworth et al. (2006, 24 SSRs), Yamamoto et al. (2002; NH009 on LG13; NH029 on LG9), Hokanson et al. (1998; GD103 on LG5; GD147 on LG13) Guilford et al. (1997; NZ02b01 on LG15), Vinatzer et al. (2004; CH-Vf1 on LG1), Broggini et al. (submitted manuscript; HB11-SSR on LG1 and HB03-SSR on LG6), Rikkerink (unpublished; NZAL08 on LG3, NZEST67774 on LG4). The primer sequences of the latter two SSRs are: NZAL08-F GGC ACA AGC ACA AGG AAA CA, NZAL08-R GTT TGA GCC AGT CCA TTT TTC CCT AT for SSR NZAL08 and NZEST67774-F CCC GAC TGA CTG AAC CTT TT, NZEST67774-R GTT TCC GTG GAA GTG GAG TGA AG for SSR NZEST67774 (Tm of 60°C).

Assembly of multiplex PCRs for ABI sequencers

The assembly of MP PCRs of the 61 SSRs for genotyping on ABI sequencers was performed at Plant Research International (PRI) on an ABI PRISM® 377 DNA Sequencer (Applied Biosystems). As far as possible, SSRs that mapped to the same LG were assigned to the same MP. Different combinations of primer concentrations were tested until all SSRs of a MP produced similar quantities of amplicons. SSR markers giving unsatisfactory results were moved to other MP PCRs. SSR markers with primers labeled with the same fluorochromes were assigned to the same MP only if the ranges of their allele length were well separated. Further optimization for local conditions was performed at the site of each genotyper, who all used a capillary system. Protocols of the MPs at each genotyping site are presented in Table 1 and Table S1 (full information).

Assembly of multiplex PCRs for LI-COR sequencer

Twenty-seven SSRs were assigned to Julius Kuehn-Institute, the only partner working with an automatic dual-laser DNA sequencer LI-COR 4200 with two different wavelengths (IRD 700–IRD 800). As with the SSRs used on ABI sequencers, the SSRs analyzed on LI-COR were first pooled by linkage group. Protocols for each MP were obtained using the Qiagen Multiplex PCR kit. Due to different intensities of the alleles of different loci as well as to differences in the labeling intensity of the IRD-labeled primers, the primer concentrations had to be specifically adapted for each MP PCR. Protocols for the MP reactions are presented in Table 1.

Nomenclature of the multiplex PCR

The names of the MP PCRs were generated as follows. The first two letters of each MP are "Hi" indicating that the MPs have been developed within the frame of the HiDRAS project. The next number indicates the linkage group represented by the highest number of SSRs in the MP. If two linkage groups are represented by an identical number of SSRs, the number of both linkage groups is indicated separated by a dash. If the resulting name was identical to that of another MP, the letters "a" and "b" were added at the end of the name.

Table 1 PCR protocols of the multiplexes used to screen the HiDRAS germplasm

MP name	SSRs	LG	Fragment analysis	For primer conc (μM)	Rev primer conc (μM)	Labeling	DNA (ng)	Cycling conditions	Vol (μl)
Hi1/6	Hi02c07	1	ABI 3100	0.08	0.08	PET	10	94°C 5′, 10 cycles: 94°C 30″, 58–53°C 45″ (dropping 0.5°C per cycle) and 72°C 1′, 25 cycles: 94°C 30″, 53°C 45″ and 72°C 1′; 72°C 15″	10
	CH05a05	6		0.08	0.08	VIC			
	CH-Vf1	1		0.08	0.08	NED			
	CH03d12	6		0.08	0.08	6FAM			
Hi2[a]	CN493139SSR	2/2/5	ABI 3100	0.033	0.033	NED	10	94°C 2.5′; 33 cycles: 94°C 30″, 55°C 30″ and 72°C 1′; 72°C 5′	10
	CH03d01	2		0.042	0.042	HEX			
	CH02f06	2		0.045	0.045	6-FAM			
	CH05e03	2		0.068	0.068	HEX			
Hi3	CH03g07	3	LI-COR 4200 (dual laser)	0.065	0.175	IRD 800	10	94°C 5′, 38 cycles: 94°C 30″, 60°C 30″ and 72°C 1′, 72°C 10′	10
	Hi03d06	3		0.05	0.15	IRD 700			
	AU223657SSR	3		0.03	0.1	IRD 800			
Hi4a[b]	CH02c02b	4	ABI 310	0.19	0.19	NED	10	94°C 2.5′; 5 cycles: 94°C 30″, 65–61°C for 1′ (dropping 1°C per cycle) and 72°C 1′, 30 cycles: 94°C 30″, 60°C 1′, 72°C 1′; 72°C 10′	15
	CH04e03	5		0.13	0.13	NED			
	CH04e02	4		0.17	0.17	6-FAM			
Hi4b	CH02g01	13	ABI 3100	0.12	0.28	NED	10	Identical to Hi1/6	10
	Hi23g02	4		0.12	0.28	PET			
	NZ02b01	15		0.12	0.28	6FAM			
	CH05d02	4		0.12	0.28	VIC			
Hi4/5	Hi22f12	5	ABI 3100	1.25	1.25	HEX	10	94°C 5′, 10 cycles: 94°C 30″, 65–55°C 30″ (dropping 1°C per cycle) and 72°C 1′, 25 cycles: 94°C 30″, 55°C 30″ and 72°C 1′; 72°C 5′	20
	NZEST67774	4		1	1	NED			
Hi5	Hi04a08	5	LI-COR 4200 (dual laser)	0.02	0.1	IRD 700	10	Identical to Hi3	10
	CH03a09	5		0.015	0.075	IRD 700			
	Hi04d02	5/?		0.03	0.125	IRD 800			
Hi5/10	CH05e06	5	ABI 3100	0.040	0.040	VIC	10	94°C 2.5′, 33 cycles: 94°C 30″, 56°C 30″ and 72°C 1′, 72°C 5′	10
	CH02b12	5		0.090	0.090	PET			
	Hi03a10	7		0.090	0.090	NED			
	MS06g03	10		0.018	0.018	6-FAM			
	CH02b03b	10		0.065	0.065	6-FAM			
Hi7/16	CH04e05	7/?	LI-COR 4200 (dual laser)	0.02	0.125	IRD 800	10	Identical to Hi3	10
	CN444794SSR	7/7		0.15	0.325	IRD 700			
	CH05c06	16		0.035	0.125	IRD 700			
	CH05a04	16		0.4	0.125	IRD 700			
Hi8	CH01c06	8	LI-COR 4200 (dual laser)	0.015	0.1	IRD 800	10	Identical to Hi3	10
	CH01f09	8		0.1	0.225	IRD 700			
	CH01h10	8		0.03	0.125	IRD 800			
	Hi23g12	8/15		0.015	0.1	IRD 700			

Hi9	CH01f03b	9	LI-COR 4200	0.02	0.1	IRD 700	10	Identical to Hi3	10
	Hi05e07	9	(dual laser)	0.015	0.075	IRD 800			
	CH01h02	9		0.03	0.1	IRD 700			
	CN444542SSR	9		0.015	0.075	IRD 800			
Hi10[c]	CH03d11	10	ABI 3100	1	1	NED	10	Identical to Hi5/10	20
	CH02b07	10		0.875	0.875	6-FAM			
	CH02c11	10		1.75	1.75	HEX			
	CH04g09	10/5		1.5	1.5	HEX			
Hi11	CH04h02	11	ABI 3100	0.2	0.2	TAMRA	10	95°C 15′, 30 cycles: 94°C 30″, 60°C 1′30″ and 72°C 1′, 60°C 15′, 72°C 15′	10
	Hi07d11	11/?		0.2	0.2	HEX			
	CH02d08	11		0.2	0.2	FLUO			
	CH04a12	11		0.2	0.2	FLUO			
	Hi16d02	11		0.2	0.2	HEX			
Hi12a	CH04c06	10/17	ABI 3100	0.093	0.093	NED	10	94°C 5′; 37 cycles: 94°C 30″, 60°C 30″ and 72°C 45″; 72°C 8′	10
	CH05d04	12		0.240	0.240	HEX			
	CH01d03	4/12		0.200	0.200	HEX			
	CH04d02	12		0.067	0.067	NED			
	CH04g04	12		0.187	0.187	6FAM			
	CH01h01	17		0.120	0.120	6FAM			
Hi12b	CH01g12	12	LI-COR 4200 (dual laser)	0.03	0.125	IRD 800	10	Identical to Hi3	10
	CH03c02	12		0.02	0.125	IRD 700			
	Hi07f01	12		0.02	0.125	IRD 700			
Hi13a	Hi04g05	13	ABI 3100	0.17	0.17	VIC	10	Identical to Hi1/6	15
	CH03e03	3		0.17	0.17	NED			
	CH05h05	13		0.17	0.17	6FAM			
	GD147	13		0.17	0.17	PET			
	HB03-SSR[d]	6		0.05	0.05	6FAM	10	Identical to Hi5/10	10
Hi13b	CH05f04	13	ABI 3100	0.12	0.28	NED	10	Identical to Hi1/6	10
	GD103	5		0.12	0.28	VIC			
	NH009b	13		0.12	0.28	6FAM			
	CH05g08	1		0.12	0.28	PET			
Hi14	CH01g05	14	LI-COR 4200 (dual laser)	0.04	0.1	IRD 700	10	Identical to Hi3	10
	CH04c07	14		0.03	0.075	IRD 800			
	MDAJ761SSR	14		0.015	0.075	IRD 800			
Hi15	CH02c09	15	ABI 3100	0.15	0.15	HEX	5	94°C 2.5′, 33 cycles: 94°C 30″, 65°C 30″, 72°C 1′; 72°C 5′	20
	CH01d08	15		0.15	0.15	6-FAM			
	CH02d11	15		0.12	0.12	NED			
	Hi03g06	15		0.12	0.12	NED			
Hi16	CH02a03	16	LI-COR 4200 (dual laser)	0.015	0.1	IRD 700	10	Identical to Hi3	10
	Hi04e04	16		0.02	0.1	IRD 700			
	CH04f10	16		0.04	0.15	IRD 800			
Hi17	AT000174SSR	17	ABI 3100	0.2	0.2	FLUO	10	Identical to Hi11	10
	Hi03c05	17		0.2	0.2	HEX			
	Hi02f12	17		0.2	0.2	FLUO			

Table 1 (continued)

MP name	SSRs	LG	Fragment analysis	For primer conc (μM)	Rev primer conc (μM)	Labeling	DNA (ng)	Cycling conditions	Vol (μl)
	CH05d08	9/17		0.2	0.2	HEX			
	Hi07h02	17		0.2	0.2	FLUO			
	CH04g07	11		0.2	0.2	TAMRA			

[a] CH02g01 worked in this multiplex at PRI, CH02g01 moved to MP Hi4b for germplasm screen

[b] CH05d02 and Hi23g02 worked in this multiplex at PRI; both moved to MP Hi4b for germplasm screen

[c] PCRs performed separately for CH04g09

[d] PCRs performed separately. Two microliters HB03AT loaded with 1 μl MPHi13

? Unknown map position of the second locus of the presumed multilocus SSR, *MP name* name of the multiplex PCR, *SSRs* SSR markers present in the multiplex, *LG* linkage group on which the SSRs have been mapped, *Fragment analysis* indicates the instrument used to separate the amplicons, *For and Rev primer conc (μM)* concentration of the different primers in the multiplex PCRs, *Labeling* fluorochrome used to label the primer, *DNA (ng)* amount of DNA used in the PCR reaction, *Cycling conditions* PCR profile used for the specific multiplex, *Vol* final volume of the PCR reaction

Division of multiplexes

Multiplexes were divided over the six genotyper sites, so that each multiplex was applied at a single site. In this way, we avoided inconsistencies in allele sizing that easily occur when data for the same SSR come from different laboratories. ABI capillary platforms were used at five of the six sites, while a LI-COR was used at the sixth site.

Fragment analysis

For fragment analysis performed on the ABI PRISM® 3100 or 310 DNA capillary sequencer (Applied Biosystems), 1 μl of PCR product was mixed with 10 μl deionized formamide and 0.2-μl 500-LIZ or 500-ROX ladder (Applied Biosystems). Chromatographs were generated using Genescan 3.7 software and SSR fragment lengths were scored with Genotyper 3.6 (Applied Biosystems).

For fragment analysis performed on the LI-COR sequencer, 2 μl of the multiplex PCR reactions was diluted with 15-μl formamide loading buffer and denatured for 3 min at 95°C. A 0.5-μl diluted reaction was loaded on the gel. As a sizing standard, a 50–350 DNA ladder composed of 14 IRD-labeled fragments (LI-COR) was loaded separately in every ninth lane (five lanes per gel total). The detection of alleles of SSRs analyzed on LI-COR 4200 was performed using the fragment analysis software SAGA (LI-COR). Analysis was initially performed automatically; however, for a large part of the data, a manual scoring was necessary.

Verification of the consistencies of allele sizing

Following verification of nonsignificant shift of the allele sizes of the genotypes of reference "Prima" and "Fiesta" between plates, the allele sizes were rounded to the most appropriate integer according to the length of the repeated motif (2 or 3nt repeat). Rounded values were verified for consistency of the data within and among pedigrees as described in Patocchi et al. (2007). In brief, the procedure consisted of firstly checking if a progeny of a cross inherited an allowed combination of alleles from its two parents using an excel macro called "Gob validator" (D. Gobbin, unpublished). Then, consistency among all the connected HiDRAS pedigrees was verified using the statistical software FlexQTL™ (www.flexqtl.nl). Then, the parts of the pedigrees generating inconsistencies were visualized using the software Pedimap (Voorrips 2007). Finally, the electropherograms of the plants involved in the inconsistencies were checked, and, if justified, the data scores were corrected. If scores were changed, another check with the software FlexQTL™ was performed. This cycle of the validation procedure was performed until no improvement of data was possible.

Results

Modification of the set of SSRs spanning the apple genome

A core set of 84 SSRs spanning about 85% of the apple genome was already proposed by Silfverberg-Dilworth et al. (2006). One SSR in this set (CH03b06, LG15) showed difficulties in multiplexing. As NZ02b01 (SSR of the core set of Silfverberg-Dilworth et al. 2006) mapped at only 3 cM from CH03b06, this SSR was not replaced. Five SSRs were added as they mapped on unrepresented regions: CH02b12 (LG5, Liebhard et al. 2002), GD103 (LG5) and GD147 (LG13; Hokanson et al. 1998), and NZAL08 (LG3) and NZEST67774 (LG4; Rikkerink, unpublished).

The current core set is now composed of 88 SSRs representing 89 regions (Fig. 1). For 13 regions, no SSR has yet been identified. As 101 single-locus SSRs are necessary to span the whole apple genome (to meet the criteria explained in the "Introduction"), the present coverage of the genome is approximately 89%.

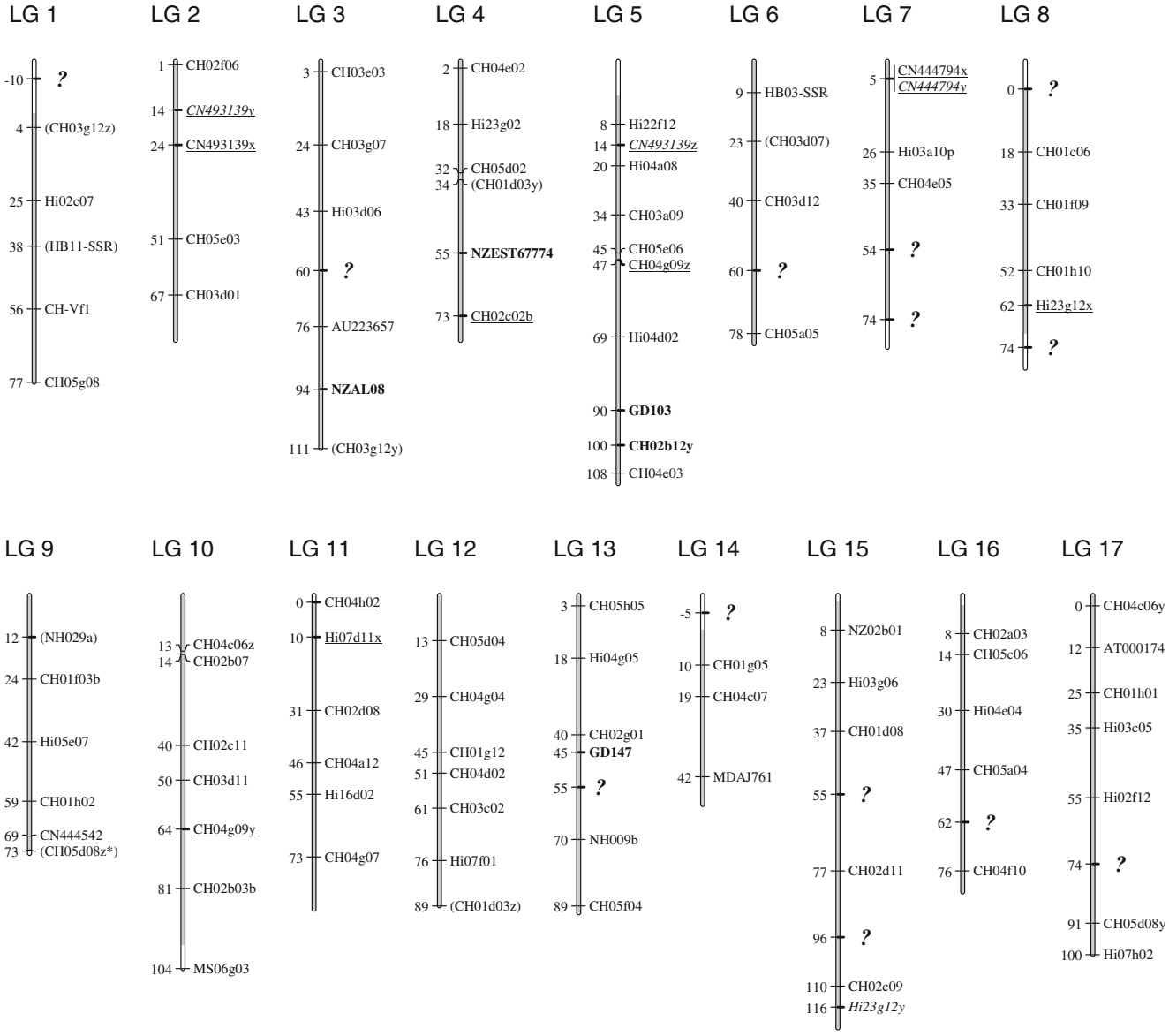

Fig. 1 Coverage of the apple genome with the selected set of SSRs. Five SSRs (in *bold*) have been added to the set selected by Silfverberg-Dilworth et al. (2006); one SSR (CH03b06, LG15) has been removed. Regions for which no SSR is currently available are indicated by *question marks*. SSRs of the set which could not be used to screen the germplasm set (see text) are indicated in *brackets*. Loci of the five SSRs for which scoring was judged too complex for large-scale applications (CN493139, CH04g09, CH04h02, Hi07d11, and CN444794) or loci judged not sufficiently polymorphic (CH02c02b and Hi23g12) are *underlined*. These SSRs need to be replaced as soon as alternatives are available. Additional loci of the SSRs CN493139 (LG2 and 5) and Hi23g12 (LG15) identified in this study are indicated with *italics*. *Asterisks*, alleles of locus z (LG9) of multilocus SSR CH05d08 (LG9/17) were not amplified under the conditions used in this study (see Table 2)

Multiplex PCRs

Twenty-one MP PCRs containing in total 82 out of the 88 selected SSR markers have been developed. Thirteen MP PCRs, containing in total 56 SSR markers, were developed and tested on ABI sequencers (ABI-MPs), while eight MP PCRs, containing 27 SSRs, were developed and tested with a LI-COR sequencer (Li-MPs; Table 1 and Table S2). Four SSRs, HB11-SSR, CH03g12, CH03d07, and NH029 did not perform well in any MP and have not been further tested. SSR NZAL08 performed well under the conditions at PRI but failed under the conditions at the site of the genotyper (therefore, also considered not included in a MP). In addition, SSR CH01d03 (MP Hi12a) did not give stable amplifications and has not been further considered.

The highest number of SSR markers (six) placed in MPs was achieved by MPs Hi12a and Hi17. In two MPs, Hi10 and Hi13a, one SSR could not be placed directly in the MP (CH04g09 in Hi10 and HB06-SSR in Hi13); however, following an independent PCR, the products could be added to the MP for fragment analysis.

MPs Hi13b and Hi4b (four SSRs each) allowed the highest efficiency as their amplicons could frequently be combined for fragment analysis. Their SSRs generally amplified alleles in well-separated allele size ranges. For the screening of the segregating populations with these two MPs, it was first ascertained whether the alleles of the parents overlapped and, if this was not the case, the PCR products of both MPs were mixed together before performing fragment analysis. Therefore, within a single run, alleles of eight loci could be separated.

Three out of the eight LI-COR-MPs contained four SSRs, while the other five MPs each contained three SSRs. Using the Qiagen Multiplex PCR kit, it was possible to establish a robust protocol for each multiplex. None of the LI-COR SSR markers had to be amplified on its own.

Multilocus SSRs and assignment of alleles to the different loci

Although single-locus SSRs were preferred to multilocus SSRs (ML) in the selection of the set, for some genomic regions, the only available SSRs were multilocus (CH04c06, CN493139, CH04g09, HB03-SSR, GD103, CH04h02 Hi07d11, and Hi23g12) or were presumed to be multilocus (CH02b12, CH05d08, Hi04d02, CH04e05, CN444794). Therefore, it was necessary to assign the alleles to the different loci in order for these SSRs to be used for the "identity by descent" approach.

SSRs CH02b12, CH04e05, Hi04d02 (all presumed ML), and CH05d08 (previously proven to be ML) only amplified one locus in the conditions used in this study and therefore no splitting of the data was necessary for these SSRs. For the other SSRs, CH04c06, HB03-SSR, GD103, and Hi23g12, a simple procedure was developed to assign the alleles to the different loci (Table 2) and this was applicable also for large-scale genotyping efforts. For SSRs CN493139, CH04g09, CH04h02, CN444794, and Hi07d11, adequate

Table 2 Description of the procedure applied to identify the alleles of the different loci amplified by multilocus and presumed multilocus SSRs

SSR	MP	LGs	Procedure
CH02b12	Hi5/10	5/?	Under the conditions used only LG5 alleles amplified
CH04e05	Hi7/16	7/?	Under the conditions used only LG7 alleles amplified
CH05d08	Hi17	9/17	Under the conditions used only LG17 alleles amplified
Hi04d02	Hi5	5/?	Under the conditions used only LG5 alleles amplified
GD103	Hi13b	5/10	Simple, alleles >90 bp belong to locus z (LG5), alleles <90 bp to locus y (LG10)
HB03-SSR	Hi13a	6/16	Simple, alleles >350 bp belong to locus y (LG6), alleles <350 bp to locus x (LG16)
Hi23g12	Hi8	8/15	Simple, the alleles 221 and 224 bp belong to locus x (LG 8), alleles >233 bp belong to locus y (LG15)
CH04c06	Hi12	10/17	Simple, intensity difference, only two alleles found for locus z on LG10 (157 and 171 bp); however, allele ranges overlap; therefore, this SSR is not fully adequate for high throughput genotyping
CH04g09	Hi10	10/5	Complicated, overlapping size ranges; only the z locus can be scored and distinguished from the y locus due to great differences in amplification efficiency
CH04h02	Hi11	11/11	Allele assignment is relatively easy, but adequate scoring is difficult due to a combination of overlapping size ranges and large differences in amplification efficiency; use possible only in some segregating populations and using flanking markers (mapping)
CN444794	Hi7/16	7/7	Complicated, use possible only in some segregating populations
CN493139	Hi2	2/2/5	Complicated, use possible only in some segregating populations
Hi07d11	Hi11	11/?	Complicated, too many unspecific amplicons for use

? Presumed multilocus SSR (position of the second locus unknown), *MP* name of the multiplex, *LGs* linkage groups carrying the loci amplified by the multilocus SSR, *Procedure* method used to assign the alleles to the different loci

scoring and assignment of alleles to the corresponding loci was too complex for large-scale genotyping efforts due to a combination of overlapping allele ranges and differences in amplification efficiency between the loci. For the first three SSRs, the splitting of the data was only possible in certain crosses and circumstances, and this was considered too time-consuming for CN444794 and Hi07d11 and therefore not undertaken in this study. Data could therefore be produced for 80 SSRs (85 loci).

The assignment of alleles to the different loci allowed the mapping of additional loci from three ML SSRs (CN493139, Hi23g12, and CN444794) that were not identified by Silfverberg-Dilworth et al. (2006; Fig. 1). SSR CN493139 amplified three loci; the first additional locus (y) is on LG2 at 10 cM proximal from the known locus x of CN493139, while the second additional locus (z) was identified on LG5 between SSR markers Hi22f12 and Hi04a08. The second locus of SSR Hi23g12 has been mapped at the distal end of LG15 at approximately 6 cM from marker CH02c09. The second locus of CN444794 is a copy of the first locus at exactly the same position (top of LG7; Fig. 1).

Degree of polymorphism and size ranges of alleles of the tested SSRs

Screening the 80 SSRs over a much wider germplasm collection than the eight to nine cultivars tested during the development of the SSRs allowed a better estimation of their degree of polymorphism (Table 3). The average number of alleles per locus (85 loci) was 13.3. The most polymorphic single-locus SSRs were CH03d12 (24 alleles), CH04f10 (22 alleles), and Hi05e07 (20 alleles). The single-locus SSR with the lowest level of polymorphism was CH02c02b (four alleles). The ML SSR Hi23g12 was selected for its position on LG8. Regrettably, this locus proved to have a low level of polymorphism (three alleles), which was not evident in Silfververg-Dilworth et al. (2006).

The large-scale testing of the selected SSRs also allowed a better estimation of the allele size range that can be expected from each specific SSR (or each locus in case of ML SSR; Table 3). Direct comparisons between our results and those of Liebhard et al. (2002) and Silfverberg-Dilworth et al. (2006) are feasible, as all their reference cultivars except for "Nova Easygro" were tested by us. Such comparisons showed various differences in relative allele sizes, e.g., a difference of 10 nt between two alleles observed by us was previously reported as a 12-nt difference. As such differences can easily occur due to different fragment analysis and sizing methods and as they have little substantial impact on the estimation of the degree of polymorphism and size ranges of alleles, we did not elaborate on this.

Discussion

Set of SSRs

Out of the initial 88 SSRs that had been chosen for the core set of genome-covering SSR, 82 were extensively analyzed. This analysis allowed the identification of the 75 SSR markers that are well suited for large genotyping projects. These SSRs are well distributed within the apple genome, give robust amplifications in the MPs, and are sufficiently polymorphic and, for the few ML SSRs, the identification of the alleles belonging to the different loci is simple. We suggest that seven of the 82 tested SSRs need to be replaced as soon as better options become available (CH02c02b, Hi23g12, CN493139, CH04g09, CH04h02, Hi07d11, and CN444794). SSRs Hi23g12 (locus on LG8) and CH02c02b were discovered to have an extremely low level of polymorphism. Hi23g12 was included in the set because of its strategic position at the distal part of LG8 where no other SSRs were available, even though the presumed ML SSR Hi23g12 was already suspected to have low polymorphism (five alleles in total for two loci identified in nine genotypes, Silfverberg-Dilworth et al. 2006). The fact that the single-locus SSR CH02c02b amplifies only four different alleles in this study is somewhat surprising because the same SSR was reported to amplify five different alleles from as few as eight cultivars (Liebhard et al. 2002). This difference is probably caused by a combination of suboptimal PCR conditions and underloading of the capillaries in the current study, resulting in inability to detect the larger alleles.

SSRs CN493139, CN444794, CH04g09, CH04h02, and Hi07d11 are all ML SSRs with alleles of the different loci having similar sizes, making the assignment of alleles to the loci labor-intensive and, moreover, making the scoring of individual alleles prone to errors. All these SSRs were chosen because of their strategic position and lack of better SSRs in terms of quality of the amplifications and expected degree of polymorphism. The use of these five ML SSRs in large genotyping projects is not advised.

For full coverage of the apple genome, around 101 single-locus SSRs are required. Currently, no SSRs are available for 13 loci (Fig. 1). Including the five ML SSRs found unsuitable within this project, new SSRs for 18 loci have to be found (Fig. 1). A new series of apple SSRs have been recently developed and mapped (Igarashi et al. 2008; Van Dyk 2008) some of which are located in the current gaps (e.g., LG15). New pear SSRs have also been developed (Fernández-Fernández et al. 2006; Inoue et al. 2007) and others are in development (Yamamoto T. personal communication). Once these SSRs have been mapped, making use of the synteny between apple and pear, it should be ascertained whether any of these SSRs could fill the gaps.

Table 3 SSR allele number and size identified within HiDRAS plant material, separated by locus

Locus name	LG	No of alleles[a]	No of alleles[b]	Range (bp)[b]	Allele size[b,c]																									
AT000174-SSR	17	6	14	181–211	null	*181*	185	187	**189**	191	193	195	**197**	199	201	*203*	205	211												
AU223657-SSR	3	6	8	219–233	null	219	221	223	**225**	229	*231*	233																		
CH01c06	8	6	15	150–190	150	152	154	*156*	158	***160***	162	164	168	**170**	172	178	186	188	190											
CH01d08	15	6	16	244–299	244	246	248	250	254	256	***259***	261	265	273	***277***	279	285	287	295	299										
CH01f03b	9	7	17	137–193	137	*139*	141	143	145	151	157	***159***	161	163	169	**171**	177	179	181	183	193									
CH01f09	8	7	16	113–162	113	115	121	123	**125**	127	129	131	133	*135*	137	**141**	143	153	*160*	162										
CH01g05	14	6	19	138–186	null	138	*140*	142	**144**	146	150	152	154	*156*	158	160	164	166	168	172	**174**	184	186							
CH01g12	12	6	18	106–186	106	108	***110***	112	132	134	136	138	140	146	*150*	152	**154**	156	178	182	184	186								
CH01h01	17	6	13	110–145	110	118	120	122	124	126	128	130	132	134	136	138	145													
CH01h02	9	6	13	226–254	null	226	230	232	***236***	238	242	**244**	*246*	248	250	252	254													
CH01h10	8	5	15	92–221	*92*	98	***100***	102	104	106	110	112	114	116	118	120	134	194	221											
CH02a03	16	7	15	100–170	100	126	136	140	144	146	148	***150***	152	**154**	156	164	166	168	*170*											
CH02b03b	10	8	13	75–107	null	**75**	79	81	89	*91*	93	***95***	97	99	101	103	107													
CH02b07	10	7	15	98–140	98	100	102	**104**	*106*	108	110	112	**116**	118	120	122	*124*	126	140											
CH02b12y	5	13	11	118–146	null	118	128	**130**	***132***	134	138	140	142	*144*	146															
CH02c02b	4	5	4	112–122	null	***112***	***116***	122																						
CH02c09	15	6	7	238–262	***238***	244	246	*248*	250	**254**	260																			
CH02c11	10	7	14	202–233	202	208	210	**212**	214	216	218	222	223	***225***	227	*229*	231	233												
CH02d08	11	7	15	217–269	null	217	221	223	225	227	229	**231**	233	235	253	255	257	***261***	269											
CH02d11	15	7	14	118–156	118	*122*	**124**	126	128	130	132	134	***136***	138	140	152	154	156												
CH02f06	2	7	13	137–164	null	137	139	143	145	149	152	154	**156**	158	160	162	***164***													
CH02g01	13	5	15	184–236	null	184	194	196	200	202	204	212	214	216	218	222	***224***	230	236											
CH03a09	5	6	12	121–145	121	**125**	127	129	***131***	133	*135*	137	139	141	143	145														
CH03c02	12	5	12	106–162	106	114	120	122	124	***126***	128	132	134	138	142	162														
CH03d01	2	7	11	94–118	null	94	98	**104**	106	108	110	112	*114*	***116***	118															
CH03d11	10	6	12	108–176	null	108	110	114	***116***	118	**120**	122	124	128	174	176														
CH03d12	6	7	24	96–171	null	96	**102**	104	106	108	110	112	114	116	*118*	120	126	128	**130**	138	140	142	*146*	148	150	152	154	171		
CH03e03	3	6	11	188–218	null	*188*	191	198	**200**	202	204	206	**208**	214	218															
CH03g07	3	5	14	119–179	null	**119**	121	**123**	125	127	*129*	137	*165*	167	171	175	177	179												
CH04a12	11	8	13	165–197	null	**165**	171	*173*	175	*177*	179	181	**185**	187	189	195	197													
CH04c06y	17	8	12	167–194	167	*175*	177	*179*	**181**	184	186	187	**188**	190	192	194														
CH04c06z	10		2	158–171	*158*	***171***																								
CH04c07	14	8	16	96–134	null	96	98	100	*106*	***108***	110	112	**114**	116	118	120	122	130	132	134										
CH04d02	12	3	7	123–151	null	**123**	***125***	131	135	137	151																			
CH04e02	4	6	9	141–168	null	141	143	145	**152**	154	156	***160***	*168*																	
CH04e03	5	11	19	182–236	null	182	*187*	**189**	191	193	195	197	**199**	201	203	205	*207*	209	210	212	214	224	236							
CH04e05	7	8	17	175–227	*175*	189	197	**199**	201	203	205	207	*209*	211	215	217	219	221	223	225	**227**									
CH04f10	16	9	22	166–316	null	166	*172*	174	186	188	194	224	226	**230**	234	*236*	238	240	**242**	244	246	248	250	252	264	316				
CH04g04	12	5	14	155–211	155	161	163	173	175	**177**	*179*	181	183	***187***	191	193	195	211												
CH04g07	11	9	15	153–214	153	***155***	164	166	168	170	172	174	*176*	178	**180**	182	185	197	214											
CH04g09y	10	11	6	138–154	null	138	**143**	145	*152*	154																				
CH04g09z	5		16	135–178	null	135	137	144	146	***148***	150	152	155	161	167	*169*	172	174	176	178										

CH04h02	11	14	31	161–298	161	171	175	179	181	183	185	189	191	195	197	199	203	204	206	207	209	210	214	221	233	246	249	254
					258	261	269	272	278	294	298																	
CH05a04	16	8	16	151–215	null	151	159	161	*165*	**166**	167	169	181	*183*	185	**190**	195	197	213	215								
CH05a05	6	6	13	202–257	null	*202*	206	210	212	214	216	220	**222**	*224*	228	**235**	257											
CH05c06	16	8	16	84–130	84	90	***102***	104	106	*108*	110	112	114	**116**	118	120	122	124	126	130								
CH05d02	4	9	13	194–223	null	194	200	203	205	207	209	211	**213**	**217**	219	*221*	223											
CH05d04	12	7	18	173–223	null	173	181	***183***	185	187	189	*193*	195	197	199	201	203	205	207	209	211	**223**						
CH05d08y	17	10	10	122–146	null	122	***124***	**126**	132	136	138	140	142	146														
CH05e03	2	10	17	166–197	null	166	168	169	**170**	173	174	176	178	179	180	181	182	*185*	187	***191***	197							
CH05e06	5	8	15	120–162	null	120	124	126	128	134	136	*138*	142	144	***152***	154	156	158	162									
CH05f04	13	6	8	158–170	null	158	*160*	162	***164***	166	**168**	170																
CH05g08	1	5	12	148–205	null	148	160	*164*	170	177	***179***	**182**	184	197	199	205												
CH05h05	13	4	16	149–185	null	149	155	158	*165*	167	169	**171**	173	175	177	179	*181*	183	185									
CH-Vf1	1	n.a	12	128–188	null	128	138	***140***	142	154	*160*	162	164	168	174	188												
CN444542-SSR	9	8	16	112–150	112	118	120	122	124	126	128	***130***	132	134	**140**	142	144	146	148	150								
CN493139-SSRx	2	10	12	136–164	null	136	138	*140*	***142***	148	151	153	**155**	157	161	164												
CN493139-SSRy	2		5	134–149	***null***	134	138	144	149																			
CN493139-SSRz	5		3	121–122	null	***121***	*122*																					
GD103	5	n.a	15	103–153	*null*	103	105	107	**109**	111	113	115	**117**	119	121	129	131	133	153									
GD147	13	6	17	123–172	123	129	*131*	133	135	137	139	141	142	143	**145**	147	***150***	152	154	162	172							
HB03-SSRy	6	n.a	16	353–422	null	***353***	355	357	361	365	367	**376**	378	382	408	412	416	418	420	422								
Hi02c07	1	5	12	106–150	null	106	*110*	112	114	**116**	*118*	120	124	136	148	**150**												
Hi02f12	17	6	9	129–149	null	**129**	**131**	133	137	139	143	147	*149*															
Hi03a10	7	6	10	201–295	null	201	205	***216***	219	227	256	285	291	295														
Hi03c05	17	8	10	176–219	null	176	194	197	199	**203**	*207*	213	*217*	**219**														
Hi03d06	3	8	18	115–169	null	115	***117***	119	121	123	127	131	133	139	141	143	145	147	149	151	153	*169*						
Hi03g06	15	5	16	175–215	null	175	181	185	187	189	191	193	195	**197**	**199**	201	*203*	205	213	215								
Hi04a08	5	7	15	206–250	null	206	*210*	211	212	*214*	216	218	**224**	230	**240**	244	246	248	250									
Hi04d02	5	3	16	176–240	null	*176*	182	188	202	206	**208**	212	*214*	218	220	**222**	224	228	238	240								
Hi04e04	16	6	13	214–246	214	216	218	220	222	224	*226*	**228**	230	232	236	240	***246***											
Hi04g05	13	8	16	180–256	180	182	187	191	220	*222*	224	***226***	228	235	241	245	249	251	254	256								
Hi05e07	9	7	20	186–232	186	188	190	192	194	196	198	200	202	204	208	**210**	***212***	214	220	222	226	*228*	230	232				
Hi07f01	12	5	13	180–218	180	184	196	198	200	202	**204**	*206*	208	210	**212**	216	*218*											
Hi07h02	17	10	15	223–279	null	223	239	243	245	*247*	**255**	259	**263**	265	267	269	*273*	275	279									
Hi16d02	11	5	8	126–167	null	126	***142***	144	152	162	164	167																
Hi22f12	5	4	6	197–213	null	197	*200*	**207**	210	213																		
Hi23g02	4	6	12	220–263	null	220	229	231	235	*237*	239	**247**	252	**255**	257	263												
Hi23g12y	15	5	5	231–245	231	*233*	237	***239***	245																			
Hi23g12z	8		3	221–224	null	***221***	*224*																					
MDAJ761-SSR	14	8	18	204–254	204	*206*	208	212	214	220	222	224	226	236	238	240	**242**	*244*	246	250	252	254						
MS06g03	10	9	13	155–192	null	155	157	159	161	*168*	170	178	**182**	184	**186**	188	*192*											
NH009b	13	6	13	133–164	133	*139*	141	**143**	145	148	152	154	156	158	***160***	162	164											
NZ02b01	15	n.a	10	212–236	212	***214***	216	220	222	*226*	228	232	**234**	236														
NZEST6774	4	n.a	11	220–260	220	234	236	**238**	240	246	248	*250*	252	***254***	260													

[a] Liebhard et al (2002, 8 different genotypes tested) or Silfverberg- Dilworth et al. (2006, 9 different genotypes tested)
[b] Data of this study
[c] "Prima" alleles are in italics, "Fiesta" alleles in bold
n.a. Not available

Multiplex PCRs

Very high levels of efficiency in genotyping have been obtained by developing PCR multiplexes. As 1,997 DNA samples were genotyped with 82 SSRs organized in 21 MPs, 121,817 PCRs and fragment analyses were saved from a total of 163,754. This allowed a considerable (approximately 75%) saving on labor, time, and consumables. The efficiency of the current MP can still be further increased. Where the current ABI-MP were based on a three-dye system, now up to four different dyes can be used. The increased knowledge about size ranges also allows the number of SSRs within MPs to be increased, as SSRs with well-distinct allele ranges can be included using the same dye. This approach has already systematically successfully applied with the LI-COR-MPs where only two different dyes can be used, as well as with some ABI-MPs.

ABI-platforms proved to be much more useful for high through SSR-genotyping than the LI-COR system, both in terms of number of SSR per MP as in consistency in allele sizing and scoring. The information on compatibility of SSRs from the LI-COR system could be used to develop ABI-MPs for these SSRs, for laboratories with the ABI system only.

As far as possible, SSRs mapping on the same LG have been placed in the same or in a maximum of two MPs. This approach was generally successful, as only the SSRs mapping on LG4, LG5, and LG13 had to be placed in more than two MPs. SSRs organized in this way are very useful in view of data validation during the genotyping process as well as for some applications, e.g., to add an SSR backbone to a specific LG of a genetic map based on poorly transferable (AFLP) markers where a QTL has been identified or for genome scanning approaches aiming at the identification of markers associated with a specific trait.

Multilocus SSRs in the set

For more than half of the ML or presumed ML SSRs, either no second locus was amplified (four cases) or it was possible to find a simple way to distinguish the alleles belonging to the different loci (four cases, Table 2). This demonstrates that ML and presumed ML SSRs need not be excluded *a priori* from large genotyping projects; however, as large genotyping projects by definition require high throughput, the splitting of the data must be as robust as possible. If this is not the case, those SSRs should be substituted as soon as alternative single-locus markers are available.

Degree of polymorphism of the tested SSRs

Coart et al. (2003) found a rough correlation between the number of alleles identified in a small group of accessions and the total number of alleles present in a wide germplasm collection. Liebhard et al. (2002) and Silfverberg-Dilworth et al. (2006) estimated the level of polymorphism of their SSRs on the basis of eight and nine quite unrelated cultivars, respectively. Enlarging the test to more than 350 genotypes showed that all the 34 single-locus SSRs amplifying at least seven different alleles in a small set of accessions amplified at least ten different alleles in the larger screen (Table 3). The average number of alleles amplified by these 34 SSRs is 15.5. The average number of alleles of the 36 single-locus SSRs amplifying less than seven alleles in the small set is 12.3, indicating that, among these SSRs, highly polymorphic SSRs (SSRs with at least ten alleles) are also present. Indeed, the probability of finding them would decrease only slightly, namely from 100% to about 72% (26 out of 36 SSRs). It may thus be wise to include such putatively low polymorphic SSRs in directed genotyping approaches if no alternative markers are available.

Conclusions

A large set of highly variable, easy-to-score SSRs has been identified that are well distributed over the whole apple genome. The SSRs have been organized in multiplex PCRs and used to generate, in a fast and cost-efficient way, solid genotyping data of around 2,000 apple accessions. These data will now be combined with different parameters of fruit quality (e.g., firmness, acidity) in a PBA to identify QTL for these traits.

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