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Microsatellite markers spanning the apple (*Malus* x *domestica* Borkh.) genome

Abstract A new set of 148 apple microsatellite markers has been developed and mapped on the apple reference linkage map Fiesta x Discovery. One-hundred and seventeen markers were developed from genomic libraries enriched with the repeats GA, GT, AAG, AAC and ATC; 31 were developed from EST sequences. Markers derived from sequences containing dinucleotide repeats were generally more polymorphic than sequences containing trinucleotide repeats. Additional eight SSRs from published apple, pear, and *Sorbus torminalis* SSRs, whose position on the apple genome was unknown, have also been mapped. The transferability of SSRs across *Maloideae* species resulted in being efficient with 41% of the markers successfully transferred. For all 156 SSRs, the primer sequences, repeat type, map position, and quality of the amplification products are reported. Also presented are allele sizes, ranges, and number of SSRs found in a set of nine cultivars. All this information and those of the previous CH-SSR series can be searched at the apple SSR database (http://www.hidras.unimi.it) to which updates and comments can be added. A large number of apple ESTs containing SSR repeats are available and should be used for the development of new apple SSRs. The apple SSR database is also meant to become an international platform for coordinating this effort. The increased coverage of the apple genome with SSRs allowed the selection of a set of 86 reliable, highly polymorphic, and overall the apple genome well-scattered SSRs. These SSRs cover about 85% of the genome with an average distance of one marker per 15 cM.

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Introduction

In the last few years, apple genetics has made significant progresses, partly due to the increasing availability of multi-allelic SSR markers. These markers proved to be extremely useful for integrating mapping results from independent studies and in the development of innovative procedures for assessing marker–gene associations. Their high level of transferability, general high level of poly-

morphism, and the relative ease by which they are generated, being PCR-based, makes them the marker of choice for alignments among linkage maps of apple. As a result, recent maps have been based on a backbone of multi-allelic SSR markers embedded in RAPD and/or AFLP markers, which can be produced in large amounts in a relatively short time (Liebhard et al. 2002, 2003b; Kenis and Keulemans 2005). Alignments can be made for the identification and orientation of corresponding homologous linkage groups as well as for relative positions of specific loci.

SSR markers are, therefore, extremely valuable for building integrated genetic maps comprising genes, confidence intervals of QTLs, and other loci gathered from multiple maps. SSR-based maps have been essential for identification and positional comparison of major genes and QTLs for scab, powdery mildew, and fire blight resistance (Durel et al. 2000, 2003; Evans and James 2003; Liebhard et al. 2003c; Calenge et al. 2004; Gygax et al. 2004; James et al. 2004; Patocchi et al. 2004; Tartarini et al. 2004; Vinatzer et al. 2004; Calenge et al. 2005; Calenge and Durel 2005; Khan et al. 2006) as well as morphological or physiological traits (Conner et al. 1998; King et al. 2000; Liebhard et al. 2003a; Costa et al. 2005). Recognition of SSR associations led to the identification of clusters of resistance genes (e.g., Bus et al. 2004) and to the first integrated map of apple that reviews many papers with regard to the location of scab resistance genes (Durel et al. 2004). The recent discovery of many new gene–SSR marker associations, made necessary a new update of this integrated map with recently mapped scab resistance genes (Bus et al. 2005a,b; Patocchi et al. 2005), thus continuously improving our understanding of the organization of the apple genome.

Technically, SSR markers are highly suitable for direct genotyping of any new individual, being easily transferable, multi-allelic, and having a known map position. They are also the most cost effective marker for directed genome-wide genotyping approaches, as with known map positions only a relatively low number of well-selected markers have to be tested to obtain a good coverage. The possibility of multiplexing several SSR markers in the same PCR reaction allows an additional reduction of the costs of genotyping.

A good coverage of the apple genome with SSR markers is the prerequisite for two innovative techniques for assessment of molecular-marker trait associations. Firstly, the Genome Scanning Approach (GSA, Patocchi and Gessler 2003) allows efficient mapping of major genes. This procedure was successfully applied to map the apple scab resistance genes *Vr2*, *Vm* (Patocchi et al. 2004; Patocchi et al. 2005), and *Vb* (Erdin et al. 2006). Secondly, the Pedigree Genotyping concept was developed (Van de Weg et al. 2004), which allows the exploitation of breeding material in the assessment of marker–trait associations and in allele mining by using multiple pedigreed plant populations, which can be any combination of crosses, cultivars, and breeding lines. This concept makes use of directed genotyping and the so-called Identity By Descent

(IBD) concept. It forms the base of the EU-HiDRAS project (Gianfranceschi and Soglio 2004) aimed at a proof of concept for Pedigree Genotyping and at the identification of molecular markers for fruit quality and disease resistance.

The major disadvantage in the use of SSR markers is the considerable initial investment needed to develop and map them. Although around 160 SSRs have been developed for the 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), their distribution within the genome is not homogenous. Almost all linkage groups contain regions with large gaps between two successive SSRs (Liebhard et al. 2003b). The development of new SSRs may solve this problem. Thus far, the most widely used method to produce SSRs is based on the cloning and sequencing of genomic fragments enriched for a repeated sequence and the designing of upstream and downstream primers (Tenzer et al. 1999; Gautschi et al. 2000). Additionally, some new SSR markers have been obtained by selecting the transferable SSR markers from closely related species (Yamamoto et al. 2004).

Recently, apple genomic projects have made thousands of apple EST sequences available, which can now be searched for SSR repeats and used for the development of new SSR markers (Crowhurst et al. 2005; Korban et al. 2005). This approach has several advantages: 1) no enriched genomic library has to be constructed; 2) extensive sequencing is not necessary, thus reducing the cost of the development of the SSR markers; 3) it is possible to develop SSR markers for which it is difficult to construct enriched libraries (e.g., AT repeats), and last but not least, 4) markers are developed from coding sequences.

In this paper, we present a new, extensive set of apple SSRs, developed within the framework of the HiDRAS European project (Gianfranceschi and Soglio 2004), from genomic libraries, publicly available EST sequences, and SSR markers of other species closely related to *Malus*. All these SSRs are also tested for their level of polymorphism and are positioned on a molecular marker linkage map. These SSRs, together with those already published, have been used to select a set of 86 highly polymorph SSRs well-scattered on the apple genome.

Materials and methods

Plant material and DNA extraction

Cultivars Elstar, Golden Delicious, and Florina were used to construct the SSR libraries. A series of nine diploid cultivars (Fiesta, Discovery, Florina, Nova Easygro, TN10-8, Durello di Forlì, Prima, Mondial Gala, and Fuji) was used to estimate the level of polymorphism of the new markers. Forty-four progeny plants of the Fiesta × Discovery cross, which is a subset of the 251 plants used by Liebhard et al. (2003b) to generate "the reference map", were used to map the new SSRs. Three other mapping populations, Discovery × TN10-8 (149 plants),

Durello di Forlì × Fiesta (subset of 60 plants), or Fuji × Mondial Gala (subset of 60 plants), were used to map the SSRs that could not be mapped in the cross Fiesta × Discovery. DNA was extracted according to Koller et al. (2000), gel quantified and diluted to 1 ng/μl.

SSR development

Genomic libraries

SSR-enriched libraries from Elstar were developed at Plant Research International. The procedure for microsatellite enrichment by selective hybridization was modified from Karagyozov et al. (1993) by Van de Wiel et al. (1999) and Van der Schoot et al. (2000). DNA was digested with *Taq*I and size-fractionated by agarose gel electrophoresis. Fragments between 300 and 1,000 bp were recovered by electro-elution, enriched by hybridization to five oligonucleotides (GA, GT, ATC, AAG, ACC), ligated in pGEM-T (Promega) or pCRII-TOPO (Invitrogen) and transformed to competent TOP10 F' (Invitrogen). Colonies were transferred onto Hybond N+ membranes and hybridized with the appropriately labeled oligonucleotides. Positive clones were sequenced with the primers Sp6 and T7 by Greenomics™ (Wageningen, the Netherlands). The enriched libraries of Golden Delicious (ATC) and Florina (AAG and AAC) were developed by Ecogenics GmbH (Zürich, Switzerland) from size-selected digested (*Mbo*I for AAG and AAC libraries and *Tsp*509I for the ATC library) genomic DNA ligated to adaptors and enriched by magnetic bead selection with biotin-labeled corresponding oligonucleotide repeats (Gautschi et al. 2000). DNA fragments were PCR-amplified with the corresponding primer (Table 1). The PCR products of the ATC library were cloned into the vector Topo® (Invitrogen) and transformed in the TOP10 F' competent cells (Invitrogen), while the AAC and AAG libraries were cloned in the vector pDrive (Qiagen) and transformed in the EZ cells (Qiagen). Recombinant cells were spotted over nylon membranes and hybridized with the corresponding SSR repeat. Positive clones were sequenced with the primer M13 reverse by Synergene Biotech GmbH (Zürich, Switzerland).

SSRs from publicly available ESTs

Malus sequences from the NCBI database (September 2003) (http://www.ncbi.nlm.nih.gov/) were examined for microsatellite repeats using the software Tandem Repeat Finder v 3.21 (Benson 1999). From these ESTs, a subset of sequences was selected that contained microsatellite repeats and in which the repeat was sufficiently far from the edge of the sequence to allow design of both forward and reverse PCR primers.

SSRs from the literature

Eight apple SSRs with unknown map position (GD12, -15, -96, -100, -103, -142, -147, and -162; Hokanson et al. 1998) have been tested for polymorphism with and between Fiesta and Discovery as well as with the parents of the other mapping populations available. Polymorphic markers were screened over a segregating mapping population and mapped. In addition, the following 17 SSRs from a map of pear (Yamamoto et al. 2004) were examined: NB102a, NB106a, NB111a, NH020a, NH023a, NH029a, NH025a, NB113a, KA4b, BGT23b, HGA8b, NH002b, NH009b, NH004a, NH015a, NH033b, and MSS6 (Yamamoto et al. 2002a–c; Oddou-Muratorio et al. 2001). This SSRs were selected because they map at positions for which the homologous regions of the apple genome lacked or had only a few SSRs. After verification that they generated amplicons in apple, they were mapped in the Fiesta × Discovery population using a range of annealing temperatures.

Primer design and PCR conditions

Primer pairs flanking the SSR sequence were designed with the program Primer3 (Rozen and Skaletsky 2000) publicly available at http://fokker.wi.mit.edu/primer3/. The ideal annealing temperature (Tm) of the primers was set at 60°C. Some primers were pig-tailed (Brownstein et al. 1996), whereby a variable number of nucleotides was added to the 5′ end of the reverse primer to obtain the sequence GTTT. Primers were synthesized at Microsynth (Balgach, Switzerland). PCR amplification and tests of primers were performed, as described by Gianfranceschi et al. (1998), with the following modifications: the PCR

Table 1 Restriction enzymes, adaptor sequences, and primers used for the construction of the AAG, AAC, and ATC SSR libraries

Library	Restr. enzyme	Adaptor (seq1)	Adaptor seq 2 (rev complementary of seq1)	PCR primer
AAG	MboI	SAULA: 5′GCGGTACCCGGGAAGCTTGG3′	SAULB: 5′GATCCCAAGCTTCCCGGGTACCGC3′	SAULA
AAC	MboI	SAULA: 5′GCGGTACCCGGGAAGCTTGG3′	SAULB: 5′GATCCCAAGCTTCCCGGGTACCGC3′	SAULA
ATC	Tsp509I	TSPAdShort 5′TCGGAATTCTGGACTCAGTGCCAATT3′	TSPAdLong 5′AATTGGCACTGAGTCCAGAATTCCGA3′	TSPAdShort

volume was reduced in some cases from 15 to 10 μl; 0.07 U/μl reaction of *Taq* polymerase (New England BioLabs) was used, and the amplification profile was simplified to an initial denaturation at 94°C for 2 min 30 s followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, both for regular (preliminary test of the primers) and with ^{33}P-labeled forward primers. Before loading, radiolabeled PCR products were denatured by the addition of one volume of denaturing gel-loading buffer (Sambrook et al. 1989) and heating at 94°C for 5 min.

SSR alleles were analyzed by running ^{33}P-labeled PCR products on a 6% denaturing sequencing gel (National Diagnostic, Atlanta, USA) in 1×TBE buffer using a IBI STS-45i DNA sequencing unit. Data scoring was performed, as described by Liebhard et al. (2002), by comparison of the allele size of the nine cultivars with the ^{33}P-labeled size standard 30–330 bp (Invitrogen).

Nomenclature for SSR markers

SSR markers developed from enriched genomic libraries were prefixed with 'Hi' (indicating the HiDRAS project, Gianfranceschi and Soglio 2004) followed by a combination of two two-digit numbers, separated by a lower-case letter. The first two-digit number indicates the number of the sequencing plate, the letter and and second two-digit number indicate the well of this plate from which the DNA sequence was obtained. SSR markers developed from EST sequences were designated with the GenBank accession number followed by the subscript "$_{SSR}$". Names of the SSRs from the literature were not changed.

Marker quality

The new SSR markers were divided into four quality classes based on the type and number of additional, non-SSR containing amplicons that appeared on the gels: (1) clean: no extra band, (2) complementary band (s): all additional bands are complementary; this is at a constant distance to (specific) SSR bands, (3) extra bands: non-complementary bands are present that may hamper multiplexing, (4) dirty: many additional, non-complementary bands that hamper scoring of the true SSR bands, not suitable for use in multiplex reactions.

Genetic mapping

The segregation data for the 44 progeny plants genotyped with the new markers were added to the data previously used to develop the "reference maps" of Fiesta and Discovery (Liebhard et al. 2003b). As these reference maps were based on 251 progeny plants, missing data were assigned to the 207 progeny plants not analyzed with the new SSRs. Mapping of the SSRs was performed with JoinMap™ version 2.0 (Stam and Van Ooijen 1995) in

connection with JMDesk 3.6 provided by Dr. B. Koller (Ecogenics GmbH, Switzerland). A LOD score of 5 was used to assign markers to linkage groups. Mapping of the SSRs was considered correct (also third-round maps) if the introduction of the new data did not change, or only slightly changed, distances between markers or orders among the flanking markers. Drawings of the linkage maps were generated with MapChart (Voorrips 2001). Some SSR markers, which showed no polymorphism in the Fiesta × Discovery progeny could be mapped in other crosses. One of the following three crosses was used in such cases: Discovery × TN10-8, Durello di Forlì × Fiesta, or Fuji × Mondial Gala, and the map position was estimated by manual alignment.

Results

One hundred and forty-eight new SSR markers have been developed and mapped, 117 from genomic libraries (65 from dinucleotide repeat libraries, 52 from trinucleotide repeat libraries) and 31 from *Malus* sequences from GenBank (24 containing dinucleotide repeats and seven with larger repeats). Moreover, it was possible to determine the location of previously published SSRs of apple (GD 147), pear (HGA8b, KA4b, NB102a, NH009b, NH029a, and NH033b) and of a *Sorbus torminalis* SSR (MSS6). For all 156 SSRs details of the forward and reverse primers, nucleotides added to the reverse primer to build a pigtail, repeat sequence, repeat type, size-range, number of alleles for the set of nine cultivars (polymorphism level), map position (linkage group), and the quality of the markers is presented (Table 2). For the EST sequences used to develop EST-SSRs, their deduced functions and origins (tissues) have also been indicated (Table 3).

In addition, the allele composition of nine cultivars has been determined to allow the estimation of the level of polymorphism of the marker (Table 4). However, in the presence of only a single allele, it usually remained unclear whether this was due to the presence of an allele at the homozygous state or to the presence of one amplified allele and a null allele (Table 4). These two options could be distinguished for Fiesta and Discovery based on the segregation patterns in the mapping population. For other cultivars, this has still to be clarified and so a single value has been entered (Table 4). For some SSRs, mainly containing trinucleotide repeats, it was not always possible to distinguish SSR amplicons from other PCR products due to lack of stutter bands. In such cases, the size of all amplicons is reported in Table 4. This may have led to an overestimation of the level of polymorphism of these SSRs.

Efficiency of SSR development

The repeat type of the new SSR sequences (2 nt or more) greatly affected the success of development of polymorphic markers that could be mapped. Sixty-one to sixty-three percent of 2-nt repeats and only 33–40% of the SSRs

Table 2 Names, primers, pigtail sequence, repeat type, repeat sequence, range and number of alleles found in nine diploid cultivars, type of marker, map position and quality of the new set of SSR markers developed

SSR name	Forward primer	Reverse primer	Pigtail seq[a]	Repeat type[b]	Repeat seq	Allele range	No. of alleles	Type of marker[c]	LG (s)	Quality[d]
Hi01a03	CGAATGAAAATGTCTAAACAGGC	AAGCTACAGGCTTGTTGATAACG	–	Perf	AAG	168–193	3	SL	10	Clean
Hi01a08	AAGTCCAATCGCACTCACG	CGTAGCTCTCTCCCGATACG	–	Comp	AAG-GA	177–177	2	SL	16	Clean
Hi01b01	GCTACAGGCTTGTTGATAACGC	ACGAATGAAAATGTCTAAACAGGC	–	Perf	AAG	153–189	5	SL	10	Compl. b
Hi01c04	GCTGCCGTTGACGTTAGAG	GTTTGTAGAAGTGGCGTTTGAGG	GTT	Imp	GA	214–232	4	SL	5	Compl. b
Hi01c06	TTAGCCCGTATTTGGACCAG	GTTTCACCTACACACACGCATGG	GTTT	Imp	GT	128–144	3	PML	15	Extra bands
Hi01c09	AAAGGCGAGGGATAAAGAAGC	GTTTGCACATTTGAGCTGTCAAGC	GTTT	Perf	GA	214–218	3	SL	14	Clean
Hi01c11	TTGGGCCACTTCACAACAG	GTTTGAGTTTGATCTCCAACATTAC	GTTT	Imp	GT	138–260	17	ML	8/16	Clean
Hi01d01	CTGAAATGGAAGGCTTGGAG	GTTTACCAATTAGGACTTAAAGCTG	GTTT	Comp	GA-GT	191–222	5	PML	9	Extra bands
Hi01d05	GGTATCCTCTTCATCGCCTG	TTAGATTGACGTTCCGACCC	–	Imp	GA	210–>330	16	PML	7	Extra bands
Hi01d06	GGAGAGTTCCTGGGTTCCAC	AAGTGCACCCACACCCTTAC	–	Imp	GA	115–165	11	ML	11/16	Extra bands
Hi01e10	TGGGCTTGTTTAGTGTGTCAG	GTTTGGCTAGTGATGGTGGAGGTG	GTTT	Perf	GA	126–224	8	SL	4	Compl. b
Hi02a03	GACATGTGGTAGAACTCATCG	GTTTAGTGCGATTCATTTCCAAGG	GTTT	Perf	GA	168–198	9	PML	5	Extra bands
Hi02a07	TTGAAGCTAGCATTTGCCTGT	TAGATTGCCCAAAGACTGGG	–	Imp	GA	254–312	4	SL	2	Extra bands
Hi02a09	ATCTCTAAGGGCAGGCAGAC	CTGACTCTTTGGGAAGGGC	–	Imp	GA	138–158	4	SL	11	Clean
Hi02b07	TGTGAGCCTCTCTATTGGG	TGGCAGTCATCTAACCTCCC	–	Imp	GA	204–216	4	SL	12	Clean
Hi02b10	TGTCTCAAGAACACAGCTATCACC	GTTTCTTGGAGGCAGTAGTGCAG	GTT	Perf	GA	200–254	8	PML	16	Clean
Hi02c06	AGCAAGCGGTTGGAGAGA	GTTTGCAACAGGTGGACTTGCTCT	GTTT	Perf	GA	208–252	8	SL	11	Clean
Hi02c07	AGAGCTACGGGGATCCAAAT	GTTTAAAGCATCCGATTGAAAGG	GTT	Perf	GA	108–150	5	PML	1	Clean
Hi02d02	TTCCTAGGCTACCCGAAATATG	GTTTCTGGCATGGACATTCAACC	GTTT	Comp	GA-GT	152–194	5	SL	15	Clean
Hi02d04	TGCTGAGTTGGCTAGAAGAGC	GTTTAAGTTCGCCAACATCGTCTC	GTTT	Perf	GA	224–250	10	SL	10	Clean
Hi02d05	GAGGGAGAATCGGGTGCATAG	CATCCCTCAGACCCTCATTG	–	Perf	GA	153–205	7	SL	12	Extra bands
Hi02d11	GCAATGTTGTGGGTGACAAG	GTTTGCAGAATCAAAACCAAAGCAAG	GTTT	Imp	GA	198–262	8	SL	14	Extra bands
Hi02f06	TAAATACGAGTGCCTCGGTG	GCAGTTGAAGCTGGGATTG	GTTT	Perf	GA	204–228	6	SL	15	Clean
Hi02f12	ACAATGGCCGAAGACAATGAC	GTTTCAACCTTTATCCCTCCATCTTTC	GTTT	Perf	GA	130–150	6	SL	17	Clean
Hi02g06	AGATAGGTTTCACCGTCTCAGC	GACCTCTTTGGTGCGTCTG	–	Comp	GA-CAC	149–163	4	SL	15	Clean
Hi02h08	GCCACTCATACCCATCGTATTG	GTTTGGCTGGGAATATATGATCAGGTG	GTTT	Comp	GT-GA	170–200	6	SL	16	Clean
Hi03a03	ACACTTCCGGATTTCTGCTC	GTTTGTTGTGTTGGATTATGCC	GTT	Perf	GA	160–228	10	PML	6	Clean
Hi03a06	TGGTGAGAAAGGTGACAGG	GTTTAAGGCCGGGATTATTAGTCG	GTTT	Imp	GA	158–197	5	PML	15	Dirty
Hi03a10	GGACCTGCTTCCCCTTATTC	GTTTCAGGGAACTTGTTTGATGG	GT	Imp	GA	206–290	6	SL	7	Clean
Hi03b03	TGAATTGAGTTTGAGAATGGAATG	GTTTGTCAGGACGGGGTAATCAAGG	GTTT	Perf	GA	196–212	7	SL	12	Clean
Hi03c04	CGTAAATAGCGAATCCGATACC	GTTTCAACATCTGTGGGTCATTGC	GTTT	Perf	GA	169–257	6	SL	10	Clean
Hi03c05	GAAGAGAGAGGCCATGATAC	GTTTAACTGAAACTTCAATCTAGG	GTT	Imp	GA	179–221	8	SL	17	Clean
Hi03d06	TCATGGATCATTTCGGCTAA	GTTTGCCAATTTTATCCAGGTTGC	GTTT	Perf	GA	115–169	8	SL	3	Clean
Hi03e03	ACGGGTGAGACTCCTTGTTG	GTTTAAACAGCGGGAGATCAAGAAC	GTTT	Perf	GA	187–199	6	SL	3	Clean
Hi03e04	CTTCACACCGTTTGGACCTC	GTTTCATATCCCACCACCACAGAAG	GTTT	Imp	GA	132–160	6	SL	13	Clean
Hi03f06	ACGATTTGGTGATCCGATTC	GTTTCGTCGCATTGTGCTTCAC	GTTT	Perf	GA	153–217	9	PML	10	Extra bands
Hi03g06	TGCCAATACTCCCTCATTTACC	GTTTAAAACAGAACTGCACCACATCC	GTTT	Perf	GA	182–204	5	SL	15	Clean
Hi04a02	TTCGTGGAAACCTAATTGCAG	GTTTCCTCGCTCTTCTTCATCTTTGC	GTTT	Perf	GA	82–104	6	SL	13	Clean

Table 2 (continued)

SSR name	Forward primer	Reverse primer	Pigtail seq[a]	Repeat type[b]	Repeat seq	Allele range	No. of alleles	Type of marker[c]	LG (s)	Quality[d]
Hi04a05	GGCAGCAGGGATGTATTCTG	GTTTCATGTCAAATCCGATCATCAC	GTTT	Perf	GA	194–222	8	SL	9	Clean
Hi04a08	TTGAAGGAGTTTCCGGTTTG	GTTTCACTCTGTGCTGGATTATGC	GTT	Perf	GA	211–250	7	SL	5	Clean
Hi04b12	CCCAAACTCCCAACAAAGC	GTTTGAGCAGAGGTTGCTGTTGC	GTTT	Perf	GA	140–156	5	SL	8	Clean
Hi04c05	AGGATGCTCTGCCTGTCTTC	GTTTCTCACTCGCCTGCTCTATCC	GTTT	Perf	GA	179–183	3	SL	15	Clean
Hi04c10	TGCGCATTTGATAGAGAGAGAA	GTTTAACAAAGAACGACCCACCTG	GTTT	Perf	GA	172–238	11	ML	3/4	Dirty
Hi04d02	TTCGTGGCTGAGAAAGGAGT	GTTTGTACGGTGCATTGTGAAAG	GT	Perf	GA	176–238	15	PML	5	Extra bands
Hi04d10	AAATTCCCACTCCTCCCTGT	GTTTGAGACGGATTGGGGGTAG	G	Perf	GA	164–182	4	SL	6	Extra bands
Hi04e04	GACCACGAAGCGCTGTTAAG	GTTTCGGTAATTCCTTCCATCTTG	GTT	Perf	GA	216–246	6	SL	16	Clean
Hi04e05	AAGGGTGTTTGCGGAGTTAG	GGTGCGCTGTCTTCCATAAA	–	Perf	GA	144–144	2	SL	8	Clean
Hi04f08	CGTGAAAACTCTAACTCTCC	GTTTGAAAAGCGCATCAAAGTTCC	GTTT	Perf	GA	218–226	4	SL	10	Clean
Hi04f09	ACTGGGTGGCTTGATTTGAG	GTTTCAACTCACACCCTCTACATGC	GTTT	Imp	GA	222–260	11	PML	13	Compl. b
Hi04g05	CTGAAACAGGAAACCAATGC	GTTTCGTAGAAGCATCGTTGCAG	GTT	Perf	GA	190–258	9	PML	13	Clean
Hi04g11	CAGAGGATTATCAATTGGACGC	AAACTATCTCCAGTTATCCTGCTTC	–	Perf	GA	118–164	5	SL	11	Clean
Hi05b02	GATGCGGTTTGACTTGCTTC	GTTTCTCCAGCTCCCATAGATTGC	GTTT	Perf	GA	120–178	7	PML	10	Clean
Hi05b09	AAACCCAACCAAAGAGTGG	GTTTCTAACGTGCGCCTAACGTG	GTTT	Perf	GA	136–144	3	SL	7	Clean
Hi05c06	TGCGTGTATGGTTGGTTTTG	TGTTTTCTTTGGTTTTAGTTGGTG	–	Comp	GA-GT	136–142	5	PML	17	Dirty
Hi05d10	AATGGGTGGTTTGGGCTTA	GTTTCTTTGGCTATTAGGCCTGC	GTT	Imp	GT	212–212	2	SL	6	Dirty
Hi05e07	CCCAAGTCCCTATCCCTCTC	GTTTATGGTGATGGTGTGAACGTG	GTTT	Perf	GT	214–234	7	SL	9	Extra bands
Hi05f12	TTTGGGTTTGGGTAGGTTAGG	GTTTGTGCAGCGCATGCTAATG	GTTT	Comp	TA-CA	157–177	5	ML	12/3	Dirty
Hi05g12	TCTCTAGCATCCATTGCTTCTG	GTTTGTGTGTTCTCTCATCGGATTC	GTTT	Imp	GT	208–288	10	PML	2	Extra bands
Hi06b06	GGTGGGATTGTGGTTACTGG	GTTTCATCGTCGGCAAGAACTAGAG	GTTT	Imp	GT	236–262	4	SL	11	Extra bands
Hi06f09	AACCAAGGAACCCACATCAG	GTTTCACTTACACGCACACACG	GTTT	Imp	GT	272–288	4	PML	15	Dirty
Hi07b02	ATTTGGGGTTTCAACAATGG	GTTTCGGACATCAAACAAATGTGC	GTTT	Imp	GT	212–218	5	PML	4	Clean
Hi07b06	AGCTGCAGGTAGAGTTCCAAG	GTTTCATTACCATTACACGTACAGC	GTTT	Imp	GT	220–226	4	SL	6	Clean
Hi07d08	TGACATGCTTTTAGAGGTGGAC	GTTTGAGGGGGTGTCCGTACAAG	GT	Perf	CA	222–232	3	SL	1	Extra bands
Hi07d11	CCTTAGGGCCTTTGTGGTAAG	GTTTGAGCCGATTAGGGTTTAGGG	GTT	Imp	GT	200–234	11	ML	11/16	Clean
Hi07d12	GGAATGAGGGAGAAGGAAGTG	GTTTCCTCTTCACGTGGGATGTACC	GTTT	Imp	GT	184–250	8	ML	2/7	Clean
Hi07e08	TTCGTGCTAGGGAGTTGTAGC	GTTTGCCTCCATAGGATTATTTGAC	GTT	Perf	GT	208–241	9	ML	8/3	Clean
Hi07f01	GGAGGGGCTTTAGTTGGGAAC	GTTTGAGCTCCACTTCCAACTCC	GTT	Comp	AT-GT	204–220	5	SL	12	Extra bands
Hi07g10	TATTGGGTTTTGGGTTTGGA	GTTTCAACCCTTTTGGTTGTGAGG	GTTT	Imp	GT	126–128	3	PML	11	Dirty
Hi07h02	CAAATTGGCAACTGGGTCTG	GTTTAGGTGGAGGTGAAGGGATG	GTT	Perf	GT	246–276	10	SL	17	Clean
Hi08a04	TTGTCCTTCTGTGGTTGCAG	GTTTGAAGGTAAGGGCATTGTGG	GTT	Comp	GAA-GT	246–254	4	SL	5	Extra bands
Hi08c05	TCATATAGCCGACCCCACTTAG	GTTTCACACTCCAAGATTGCATACG	GTTT	Perf	AAC	230–240	3	PML	14	Extra bands
Hi08d09	AACGGCTTCTTGTCAACACC	GTTTACTGCATCCCTTACCACCAC	GTTT	Perf	AAC	183–186	2	SL	16	Clean
Hi08e04	GCATGGTGGCCTTTCTAAG	GTTTACCCTGCTGACTCAACCCAAC	GTTT	Perf	AAG	201–234	6	PML	4	Extra bands
Hi08e06	GCAATGGCGTTCTAGGATTC	GTTTGGCTGCTTGGAGATGTG	GT	Perf	AAC	134–138	4	SL	13	Extra bands
Hi08f05	GTGTGGGCGATTCTAACTGC	GTTTCCTTTATTCTAAACATGC CACGTC	GTTT	Perf	AAG	165–165	2	SL	2	Clean

Table 2 (continued)

SSR name	Forward primer	Reverse primer	Pigtail seq[a]	Repeat type[b]	Repeat seq	Allele range	No. of alleles	Type of marker[c]	LG(s)	Quality[d]
Hi08f06	CTTAGAGCATAGATGACCTGCAA	GTTTAGAAATCCAACGGCCAAAG	GTT	Perf	AAG	224–242	5	SL	13	Compl. b
Hi08f12	GGTTTGTAACCCGTCTCTCG	GTTTCGTAGCTCTCTCCGATACG	GTTT	Comp	AAG-GA	116–220	7	SL	16	Extra bands
Hi08g03	ATTCACTTCCACCGCCATAG	GTTTGGAATGATTGCGAGTGAAGC	GTTT	Imp	AAG	104–116	3	PML	6	Clean
Hi08g06	AATCGAACCAGCACAGGAAG	GTTTAGATGGAGGTCGTGGTTACG	GTTT	Imp	AAG	192–198	2	SL	10	Clean
Hi08g12	AGTTCGGTCGGTTCCGTAAT	GTTTAGGGCAAGGGGAAAGAAGT	GTTT	Perf	AAG	188–197	4	SL	2	Compl. b
Hi08h03	GCAATGGCGGTTCTAGGATTC	GGTGGTGAACCCTTAATTGG	–	Imp	AAC	155–158	2	SL	4	Dirty
Hi08h08	TGAACAAATTCCACCACGAA	GTTTGCCAAGGCTACAATTTTCA	GTT	Perf	AAC	236–242	3	SL	5	Clean
Hi08h12	GAAGGAAATCATCATCAAGACG	GTTTCAAGACCATGGAACAACTTGG	GTTT	Perf	AAG	151–203	6	PML	10	Clean
Hi09a01	GAAGCAACCACCAGAAGAGC	GTTTCCCATTCGCTGGTACTTGAG	GTTT	Perf	AAG	183–192	7	SL	11	Clean
Hi09b04	GCGATGACCAATCTCTGAAAC	TGGGCTTGAATTGGTGAATC	–	Imp	AAG	227–278	5	SL	5	Clean
Hi09f01	CACCACCAAAATTCTCCATCTTC	GTTTACCGCCAAATGCTTTGTTAC	GTTT	Imp	AAG	257–266	4	SL	15	Clean
Hi11a01	ACCGCCAAATGCTTTGTTAC	GTTTCCTCCATTAAACTCCTCAGTG	GT	Imp	AAG	214–223	3	SL	15	Extra bands
Hi11a03	GGAATTGGAGCTTGATGCAG	GTTTCATACGGAATGGCAAATCG	GTTT	Perf	GA	141–144	2	SL	5	Compl. b
Hi12a02	GCAAGTCGTAGGGTGAAGCTC	GTTTAGTATGTTCCCTGGTGACG	GTTT	Imp	AAG	249–255	2	SL	16	Clean
Hi12c02	GCAATGGCGGTTCTAGGATTC	GTTTCACCAACAGCTGGGACAAG	GTTT	Imp	AAG	169–190	4	SL	1	Extra bands
Hi12f04	ATTGTCCCCACAAAATTGGA	GGGCACACGAGAGAAAGGATAA	–	Perf	AAC	184–187	2	SL	7	Clean
Hi15a13	TTCTCCCTTCTAAACCAACC	GGTTTCTTGGCGTAACATTG	–	Perf	CT	220–234	3	SL	16	Clean
Hi15b02	TATGGTGGCAACAGTGGAGA	GTTTCGCCACCTCCACTTAACATC	GTTT	Imp	GGT	196–202	4	SL	3	Compl. b
Hi15c07	TCACTTCCCATCATCACTGC	GTTTCAATGTCGAGGCTGGTAATG	GTTT	Perf	ACC	204–210	2	SL	15	Clean
Hi15e04	AAACCTCTGCATTCCGTCTC	CTCATACTCCTCCCCACATTGTC	–	Comp	ATC-GAA	209–212	2	SL	5	Clean
Hi15g11	TGACATGCATAGGGTTACATGC	GTTTGGGTTCGTAATCGTTCTTGTG	GTTT	Perf	ATC	160–163	2	SL	16	Clean
Hi15h12	GAACAAGAAGGACGCGAATC	GTTTGGGCCTCGTTATCACTACCA	GTTT	Perf	ATC	222–228	3	SL	3	Clean
Hi16d02	AACCCAACTGCCTCCTTTTC	GTTTCGACATGATCTGCCTTG	G	Perf	ATC	144–177	5	SL	11	Clean
Hi20b03	AAACTGCAATCCACCAACTGC	GTTTAGTTGCTAATGGCGTGTCG	GTT	Imp	AAC	220–244	4	SL	8	Clean
Hi21c08	TTCTTCTTCCTCCACCACCTC	GTTTGTCACTGAGAAGGCGGTAGC	GTTT	Perf	AAG	227–230	2	SL	5	Clean
Hi21e04	TGGAAACCTGTTGTGGGATT	TGCAGAGCGGATGTAAGTTG	–	Imp	AAG	134–161	8	PML	14	Clean
Hi21f08	GAGAAAACGCAGAAGCATTG	AGTAATGATTTCATCGCGAGTC	–	Imp	GAAAA	234–282	10	PML	10	Clean
Hi21g05	GACGAGCTCAAGAAGCGAAC	GTTTGCTCTTGCCATTTTCTTTCG	GTTT	Imp	AAG	155–164	3	ML	1/15	Clean
Hi22a07	CTCTTCCTTCTCCGCCTCTT	GTTTCACTCAGAATGCCTCACAGC	GTTT	Imp	AAG	153–202	7	PML	5/10	Clean
Hi22d06	CCCGAGCTCTACCTCAAA	CATTATGTTTCCGGTTTTGG	–	Imp	AAC	126–135	4	SL	2	Clean
Hi22f04	TCAATCCTCTGCTCTTCAAGG	GTTTAAATCACCTGCTGCTTG	GTTT	Perf	AAG	135–147	4	SL	10	Clean
Hi22f06	CAATGGCGTCTGTGTCACTC	GTTTACGACGGGTAAGGTGATGTC	GTTT	Perf	AAG	240–246	3	SL	16	Clean
Hi22f12	GGCCTCACCCAGTCTACATT	GTTTGGTGTGATGGGGTACTTTGC	GTTT	Imp	AAG	205–217	4	SL	5	Clean
Hi22g06	GAAAGGACCGAATTTCATGC	GTTTGTCTACAACTGTCTGATGGTAGC	GTT	Imp	AAG	240–249	2	SL	8	Extra bands
Hi23b12	TGAGGCAATGACGTTTTAG	GTTTCAGGCTTTCCCTTCAGTGTC	GTT	Perf	AAC	142–169	3	SL	14	Extra bands
Hi23d02	CCGGCATATCAAAGTCTTCC	GTTTGATGGTCTGAGGCAATGGAG	GTTT	Imp	AAG	157–166	3	SL	11	Extra bands
Hi23d06	TTGAAACCCGTACATTCAACTC	GTTTCAAGAAACCGTGCGAAATG	GTT	Perf	AAC	158–188	5	SL	9	Compl. b
Hi23d11b	GACAGCCAGAAGAACCCAAC	GTTTATTGGTCCATTTCCCAGGAG	GTTT	Perf	CTT	177–184	4	PML	4	Clean

Table 2 (continued)

SSR name	Forward primer	Reverse primer	Pigtail seq[a]	Repeat type[b]	Repeat seq	Allele range	No. of alleles	Type of marker[c]	LG (s)	Quality[d]
Hi23g02	TTTTCCAGGATATACTACCCTTCC	GTTTCTTCGAGGTCAGGGTTTG	GT	Perf	AAC	230–257	6	SL	4	Compl. b
Hi23g08	AGCCGTTTCCCTCCGTTT	GTTTGTTGGATGAGAAGCACAGTCA	GTT	Perf	CTT	211–220	3	SL	4	Clean
Hi23g12	CCCTTCCCTACCAAATGGAC	GTTTAAAGGGGCCCACAAAGTG	GTTT	Comp	CAA-AGC-CCA	223–241	4	PML	8	Clean
Hi24f04	CCGACGGCTCAAAGACAAC	TGAAAAGTGAAGGGAATGGAAG	–	Imp	AAG	144–153	5	SL	2	Clean
AF057134ssr	ACTACCCAATGCCCACAAAG	TATCCTCGCCCAAAAGACTG	–	Perf	GA	202–224	7	SL	10	Compl. b
AF527800ssr	TGGAAAGGGTTGATTGACCT	AACAGCGGGTGGTAAATCTC	–	Perf	GA	168–194	5	SL	17	Compl. b
AJ000761assr[e]	CTGGGTGGATGCTTTGACTT	TCAATGACATTAATTCAACTTACAAAA	–	Imp	TA	260–272	5	SL	14	Clean
AJ000761bssr	CCCTAAACACACAGCCTCCT	GTTTCAGCATCGCAGAGAACTGAG	GTTT	Perf	GA	210–208	8	SL	14	Clean
AJ001681ssr	CCTGAGGTTATTGACCCAAAA	CACTCAGTTGGAAAACCCTACA	–	Perf	GA	169–195	6	SL	17	Clean
AJ251116ssr	GATCAGAAAATTGCTAGGAAAAAGG	AGAGAACGGTGAGCTCCTGA	–	Perf	GA	165–167	2	SL	2	Compl. b
AJ320188ssr	AACGATGCTTGAGGAAGAACA	GCTTAACAGAAACATCGCTGA	–	Perf	GA	191–245	8	SL	9	Clean
AT000174ssr	CGGAGGCCGCTATAATTAGG	CCTGGAAAGAAAGTAAAAGGACA	–	Comp	TA-GTA-GT	178–200	6	PML	17	Extra bands
AT000400ssr	CTCCCTTTGCTCCCTCTT	AGGATGTCAGGGTTGTACGG	–	Imp	CAG	198–232	7	SL	2	Extra bands
AT000420ssr	TTGGACCAATTATCTCTGCTATT	GATGTGGTCAGGGAGGAGAG	–	Imp	GA	189–209	5	SL	4	Extra bands
AU223486ssr	TGACTCCATGGTTTCAGACG	AGCAATTCCTCCTCCTCCTC	–	Comp	GAA-GA	205–217	3	SL	13	Extra bands
AU223548ssr	ACCACCACTGCAGAGACTCA	GACGCACCCATTCATCTTTT	–	Perf	GGA	262–278	4	SL	10	Extra bands
AU223657ssr	TTCTCCGTCCCCTTCAACTA	CACCTTGAGGCCTCTGTAGC	–	Imp	GA	219–233	6	SL	3	Clean
AU223670ssr	GGACTCAATGCCTTTTCTGG	AGGATGGCAGCAATCTTGAA	–	Perf	ACC	194–202	3	PML	5	Extra bands
AU301431ssr	TCTTCCTCCCTCCTCCCTCCTC	TCTTTTTCTTCTTGGGGTCTTGG	–	Perf	AAG	213–216	2	SL	16	Extra bands
AY187627ssr	GAGGACTGAATTGGTTGAGGTC	GTTTCTCACCCGTATATAGGCCAAC	GTTT	Comp	GT-TA	300–300	2	SL	17	Clean
CN444542ssr	ATAAGCCAGGCCACCAAATC	GTTTGCAGTGGATTGATGTTCC	GT	Perf	GA	110–156	8	PML	9	Clean
CN444636ssr	CACCACTTGAGTAATCGTAAGAGC	GTTTGCCAGTTAAGGACCACAAGG	GTTT	Comp	AT-GT	239–243	3	SL	2	Clean
CN444794ssr	CATGGCAGGTGCTAAACTTG	GTTTGCAACTCACACAATGCAAC	GTT	Perf	TA	230–306	8	PML	7	Extra bands
CN45290ssr	TCTCAGTTGCTCTGGCTTTG	GTTTGCAATCAATGCCACTCTTC	GTT	Perf	GA	230–242	3	SL	6	Clean
CN45599ssr	TCAAATGGGTTCGATCTTCAC	GTTTGCCTGGCTGTAACTGTTTGG	GTTT	Perf	TA	130–176	11	PML	5	Extra bands
CN491050ssr	CGCTGATGCGATAATCAATG	GTTTCACCCACAGAATCACCAGA	GTT	Perf	GA	>330->330	3	SL	11	Clean
CN493139ssr	CACGACCTCCAAACCTATGC	GTTTATGAAAGTACGGCACCCATC	GTTT	Perf	TA	124–162	10	PML	2	Clean
CN496002ssr	TCAGAATCTCAAGCAAGATCCTC	GTTTGATTGATCGTGGCGATATG	GTT	Comp	TA-CTT	243–261	4	SL	5	Clean
CN496913ssr	TGCCTTTGAGAATCGAAATG	TGTTTGTCAATTTCTTGGAACTC	–	Perf	TA	236–278	5	SL	12	Clean
CN581493ssr	GCTTTTCATGGTGGAAAAACTG	GTTTGACTCTCCGCTCTGATGGAC	GTTT	Perf	TA	184–228	8	SL	2	Clean
U78948ssr	GATCGTCCGCCACCTTAAT	AGGGTTTTCATCATGCACATT	–	Imp	TA	178–190	7	SL	14	Extra bands
U78949ssr	TTTGTCTACCTCTGATCTTAACCAA	CAGCATCGCAGAGAACTGAG	–	Perf	GA	172–225	12	ML	6/14	Extra bands
Z38126ssr	AAGAGGGTGTTCCCAGATCC	TGTTCGATGTGACTTCAATGC	–	Perf	TA	214–240	3	SL	7	Clean
Z71980ssr	TCTTTTCTGAAGCTCTCATCTTTC	GGACATGGATGAAGAATTGGA	–	Imp	TA	170–172	2	SL	8	Clean
Z71981ssr	GCACTTACCTTTGTTGGGTCA	CCGGCATTCCAAATGTAACT	–	Perf	AAG	212–232	5	SL	15	Clean
GD147	TCCCGCCATTTCTTCTGC	GTTTAAACCGCTGCGTGCTGAAC	GTTT	u	GA	135–155	6	SL	13	Dirty

Table 2 (continued)

SSR name	Forward primer	Reverse primer	Pigtail seq[a]	Repeat type[b]	Repeat seq	Allele range	No. of alleles	Type of marker[c]	LG (s)	Quality[d]
HGA8b	AACAAGCAAAGGCAGAACAA	CATAGAGAAAGCAAAGCAAA (Tm 55°C)	–	Comp	GA-GCTTT	133–164	6	ML	3/11	Dirty
KA4b	AAAGGTCTCTCTCACTGTCT	CCTCAGCCCAACTCAAAGCC (Tm 55°C)	–	Imp	GT	137–141	3	SL	1	Compl. b
NB102a	TGTTATCACCTGAGCTACTGCC	CTTCCTCTTTATTTGCCGTCTT	–	Perf	GA	181–183	3	PML	16	Extra bands
NH009b	CCGAGCACTACCATTGA	CGTCTGTTTACCGCTTCT	–	Perf	GA	138–162	6	SL	13	Extra bands
NH029a	GAAGAAAACCAGAGCAGGGCA	CCTCCCGTCTCCCACCATATTAG (Tm 55°C)	–	Perf	GA	91–99	5	SL	9	Compl. b
NH033b	GTCTGAAACAAAAAGCATCGCAA	CTGCCTCGTCTTCCTCCTTATCTCC	–	Perf	GA	163–189	7	SL	2	Clean
MSS6	CGAAACTCAAAAACGAAATCAA	ACGGGAGAGAAACTCAAGACC	–	u	GT	273–279	3	SL	4	Extra bands

[a] Nucleotides added to the primer to create a pigtail (Brownstein et al. 1996)

[b] Perfect (Perf), imperfect (Imp), compound (Comp) (Weber 1990)

[c] Single locus (SL): for each sample, a maximum of only two alleles is amplified and the marker has been mapped to a single locus; multilocus (ML): more than two alleles for each sample is amplified and the alleles amplified with the marker have been mapped to two different loci; presumed multilocus (PML): more than two alleles present or presence of other bands similar to SSR alleles, the second locus was not mapped (not polymorph),

[d] Clean: no extra bands; complementary band(s) (Compl. b.): the only extra band(s) visible are at a constant distance from the SSR allele; Extra bands: the SSR alleles are clearly visible but other amplicons (few or many) are present; Dirty: many non SSR-like amplicons visible, scoring of the SSR difficult; multiplexing not advised

[e] For this EST, two pairs of primers have been developed, AJ000761a and b

with 3-nt repeats (or more) could be mapped (Table 5). The origin of the sequence (enriched library or GenBank accession) did not affect the success rate (Table 5). The repeat type similarly affected the observed allelic diversity of the markers, being highest for the 2-nt repeats (Table 5). However, if the average number of repeats found in the sequence used for the development of the SSRs is considered, it can be affirmed that the higher allelic variation observed for the 2-nt containing SSRs is probably due to the higher number of repeats. In fact, sequences from 2-nt repeats genomic libraries and ESTs contain on average 28 and 14 repeats, respectively, while 3-nt repeat genomic libraries and ESTs contain nine and six repeats, respectively (Table 5).

A relatively high number of SSRs (41%) developed for pear and *Sorbus torminalis* were transferred to the apple. Surprisingly, only one out of eight previously published apple SSRs (GD series) could be mapped (Table 5).

To be efficiently used in genotyping projects, SSRs need to be sufficiently polymorphic and easy to score. If the threshold to declare a single-locus SSR as sufficiently polymorphic within our current set of nine cultivars is set at five alleles, 62% of the 2-nt repeat SSRs and 24% ≥3-nt repeat SSRs can be considered (Table 6). The two most polymorphic single-locus SSRs were Hi02d04 and Hi07h02, both being 2-nt repeat SSR with ten alleles in the set of nine cultivars.

Approximately 58 and 10% of the new SSRs have been classified as being "clean" or showing "complementary bands" (amplification of amplicons at a constant distance from the SSR allele), respectively. On the other hand, 26 and 6% of the new markers have been classified as "extra bands" or "dirty", respectively. Under our PCR conditions, these SSRs amplify several additional non-SSR amplification products, which make them unsuitable for high-throughput genotyping. The SSR amplicons of the markers classified as "extra bands" are clearly visible, while those of class "dirty" may be difficult to recognize. Improvement of these SSRs by the design and testing of alternative primers was not pursued unless they are shown to be located in regions of high interest in which no other, high quality SSRs are located.

Genetic mapping

One hundred and forty-eight SSRs out of 156 have been mapped on the reference map derived from the cross Fiesta × Discovery (Liebhard et al. 2003b). The remaining eight were mapped in other crosses: four in Discovery × TN10-8 (Hi23b12, Hi01c09, Hi09f01, Hi08f05), three in Durello di Forlì × Fiesta (Hi03c05, Hi08d09, Z71980$_{SSR}$) and one in Fuji × Mondial Gala (Hi11a01) (Fig. 1).

The 156 new SSR primer pairs enriched the reference map with 168 new loci (12 primer pairs amplified two loci that could both be mapped). The linkage groups (LGs) with highest increase in loci are LG 16 with 16 loci and LGs 10 and 5 with 14 loci each, followed by LGs 2, 11, and 15 with 13 loci each. The LG with the lowest increase of loci is LG

Table 3 Description and putative functions of the mapped ESTs as found at NCBI GenBank

SSR name[a]	Definition
AF057134-SSR	*Malus domestica* NADP-dependent sorbitol 6-phosphate dehydrogenase (S6PDH) gene, complete cds
AF527800-SSR	*Malus* x *domestica* expansin 3 (EXP03) mRNA, complete cds
AJ000761a,b-SSR	*Malus domestica* mRNA for MADS-box protein, MADS7
AJ001681-SSR	*Malus domestica* mRNA for MADS box protein MdMADS8
AJ251116-SSR	*Malus domestica* mRNA for B-type MADS box protein (mads13 gene)
AJ320188-SSR	*Malus domestica* mRNA for MADS box protein (MADS12A gene)
AT000174-SSR	AT000174 Apple young fruit cDNA library *Malus* x *domestica* cDNA clone af180, mRNA sequence
AT000400-SSR	AT000400 Apple peel cDNA library *Malus* x *domestica* cDNA clone ap189, mRNA sequence
AT000420-SSR	AT000420 Apple peel cDNA library *Malus* x *domestica* cDNA clone ap212, mRNA sequence
AU223486-SSR	AU223486 Apple shoot cDNA library *Malus* x *domestica* cDNA clone S0016, mRNA sequence
AU223548-SSR	AU223548 Apple shoot cDNA library *Malus* x *domestica* cDNA clone S0279, mRNA sequence
AU223657-SSR	AU223657 Apple shoot cDNA library *Malus* x *domestica* cDNA clone S0159, mRNA sequence
AU223670-SSR	AU223670 Apple shoot cDNA library *Malus* x *domestica* cDNA clone S0086, mRNA sequence
AU301431-SSR	AU301431 Apple shoot cDNA library *Malus* x *domestica* cDNA clone S1069, mRNA sequence
AY187627-SSR	*Malus* x *domestica* S-RNase (S) gene, S9 allele, partial cds
CN444542-SSR	Mdfw2003g22.x1 Mdfw *Malus* x *domestica* cDNA clone Mdfw2003g22 3-similar to TR:Q9SSL1 Q9SSL1 F15H11.6 Hypothetical protein At1g70810
CN444636-SSR	Mdfw2003l01.x1 Mdfw *Malus* x *domestica* cDNA clone Mdfw2003l01 3-similar to TR:O81077 O81077 PUTATIVE CYTOCHROME P450, mRNA sequence
CN444794-SSR	Mdfw2001i05.y1 Mdfw *Malus* x *domestica* cDNA clone Mdfw2001i05 5-, mRNA sequence
CN445290-SSR	Mdfw2002h21.y1 Mdfw *Malus* x *domestica* cDNA clone Mdfw2002h21 5-, mRNA sequence
CN445599-SSR	Mdfw2003f11.y1 Mdfw *Malus* x *domestica* cDNA clone Mdfw2003f11 5-similar to TR:O81062 O81062 T18E12.21 Hypothetical protein At2g03120
CN491050-SSR	Mdfw2008p11.y1 Mdfw *Malus* x *domestica* cDNA clone Mdfw2008p11 5-, mRNA sequence
CN493139-SSR	Mdfw2012f06.y1 Mdfw *Malus* x *domestica* cDNA clone Mdfw2012f06 5-similar to TR:O81808 O81808 HYPOTHETICAL 62.6 KD PROTEIN, mRNA sequence
CN496002-SSR	Mdfw2021d09.y1 Mdfw *Malus* x *domestica* cDNA clone Mdfw2021d09 5-similar to TR:O23131 O23131 HYPOTHETICAL 37.1 KD PROTEIN, mRNA sequence
CN496913-SSR	Mdfw2023a24.y1 Mdfw *Malus* x *domestica* cDNA clone Mdfw2023a24 5-, mRNA sequence
CN581493-SSR	Mdfw2039o14.y1 Mdfw *Malus* x *domestica* cDNA clone Mdfw2039o14 5-similar to TR:Q9ZQF5 Q9ZQF5 PUTATIVE RING-H2 FINGER PROTEIN, mRNA sequence
U78948-SSR	*Malus domestica* MADS-box protein 2 mRNA, complete cds
U78949-SSR	*Malus domestica* MADS-box protein 3 mRNA, complete cds
Z38126-SSR	*M. domestica* gene for calmodulin-binding protein kinase
Z71980-SSR	*M. domestica* mRNA for knotted1-like homeobox protein
Z71981-SSR	*M. domestica* partial gene for kn1-like protein

[a]The first part of the SSR name corresponds to sequence accession number

1 with only five new loci. The largest distances between two flanking SSRs are on Discovery 6 (36.2 cM) and Fiesta 7 (37.5 cM). The maps of Fiesta and Discovery have been enriched by 99 and 115 loci, respectively (54 loci in common). The maps now span a total of 1,145.3 cM (Fiesta) and 1,417.1 cM (Discovery). This corresponds to an increase of 1.5 cM for the map of Fiesta and a decrease of 37.5 cM for the map of Discovery compared with the maps of Liebhard et al. (2003b). Most of the reduction of the total length of the map of Discovery is due to the splitting of its LG3 (reduction of 10 cM). Also, the average chromosome lengths of Fiesta and Discovery did not change substantially, being 67.4 cM (previously 67.4 cM) and 83.35 cM (previously 85.6 cM), respectively. The fact that no substantial changes in the total length of the parental maps are observed is an indication that the genome coverage is close to completion.

Selection of a genome covering set of apple SSRs

To facilitate efficient genome-wide mapping approaches, we aimed at developing a set of 100 SSRs that cover the entire apple genome. The high number of mapped SSRs in Fiesta × Discovery as well as in various other available mapping populations (data not shown) allowed the first design of such a set. Eighty-six SSRs were selected that span around 85% of the apple genome and that have an average distance between markers of 15 cM (Fig. 2). Regrettably, SSRs for 16 regions are not yet available. Out

Table 4 Allele composition of nine diploid cultivars

SSR name	Fiesta	Discovery	Florina	Nova Easaygro	TN10-8	Durello di Forli	Prima	Mondial Gala	Fuji
Hi01a03	168:193	178:193	168	nd	193 nd	193 nd	nd	193	nd
Hi01a08	null	177:null	null	null	null	177	null	177	null
Hi01b01	165:189	174:189	165	162 153	153 189	162 189	153 162	189	nd
Hi01c04	218:222	222:232	218	214 232	214 218	214 218 222	218	nd	nd
Hi01c06	142:142	128:142	nd	128	142	128 142	144	142	142
Hi01c09	216:218	216:218	216 218	216	216	214 216	216	216 218	216 218
Hi01c11	216:220[x] 260:260[a] 138 200	null:null[a] 234:240[y] 138 204	138 152 202 206 220	138 146 152 206 220	138 144 146 204 234 240	138 148 200 204 240	138 148 176 220	138 144 152 216	138 148 152 218
Hi01d01	199:220	195:220	199 220	195	191 195	195 222	191	195 199	nd
Hi01d05	320:>330 a 210 242	320:null 210 222 244 300 >330 b	210 222 > 330 a/b	212 226 242 >330 a/b	210 >330 b	210 242 318	210 222 242	212 222 242 300 320 >330 c/d	210 242 322 >330 a/b
Hi01d06	125:133	124:131	155 163	135 165	137	137	137 131 124	115 155	129 155
Hi01e10	220:220	214:126	220 224	224	204 208	182 208	204 212	224	nd
Hi02a03	176:186	178:186	170 176	168	170 198	184 188	176	174 186	176 186
Hi02a07	254:282	280:280	312	312	254 312	280	280	280	282
Hi02a09	138	138:158	158	138	138 158	138 152	138	148	nd
Hi02b07	214:null	null	216	216	204 216	nd	204 216	204	nd
Hi02b10	228:228[a] 202:202[a]	226:null 200:200[a]	200 226	200 224	200 226	226 240	200 202 226	224 226 254	202 224 226
Hi02c06	230:244	230:242	252	208 242	242 252	232	227 242	212 242	nd
Hi02c07	116:150	150:150	116 118	114 118	116 118	116	108 118	116	nd
Hi02d02	null:null	152:null	nd	154 194	156 194	152	194	nd	nd
Hi02d04	218:250	226:246	234	230 240	234 250	224 232	240 244	224	nd
Hi02d05	153:197	153:205	153 205	153 175	153 175	153 173	153 175	153 191	195 205
Hi02d11	234:244	244:198	234 258	254 258	262	244 198	254 198	246 258	248 262
Hi02f06	216:224	208:214	216 228	208 214	204 228	214 216	208 228	216 228	nd
Hi02f12	130:132	140:140	134 150	132	130 150	138 150	nd	132 150	nd
Hi02g06	161:161	161:163	161	161	161 163	149 155 161	161 163	161	161 163
Hi02h08	172:172	170:178	172	172	172	174 200	172 180	172	172
Hi03a03	188:192 224	172:196 224 228	160 228	160 224 228	160 172 228	186 194 218 228	186 194 218 228	nd	nd
Hi03a06	158:160 178 197	158:197	160 178 197	160 178 197	158 160 197	nd	160 178 183 197 197	158 197	nd
Hi03a10	216:240	216:284	216 240	216 290	nd	206 226	nd	nd	nd
Hi03b03	210:212	204:210	206 210	200 206	200 210	196 208	200 210	212	210
Hi03c04	169:null	null	169 201	201	175 201	257	201	201	169 201
Hi03c05	205:221	205:221	179 195	191 193	215	209	nd	nd	nd
Hi03d06	117:117	143:169	133 143	143	117 145	115 147	117	117	nd
Hi03e03	193:199	193:197	199	189 199	193 199	189 198	187 199	199	nd
Hi03e04	148:160	132:148	132	132 150	146	146 160	134 160	150 160	132

Table 4 (continued)

SSR name	Fiesta	Discovery	Florina	Nova Easaygro	TN10-8	Durello di Forlì	Prima	Mondial Gala	Fuji
Hi03f06	153:177	153:179	153 161 179	153 211	153 217	153 176 205 211	153 211	153 177 212	nd
Hi03g06	198:200	182:198	182 196	182 196	182	nd	204	196 204	196
Hi04a02	82:102	90:104	102	102	88	100 102	88 102	82 102	102
Hi04a05	194:204	194:198	194 204	204 222	198 208	194 202 210 222	194 204 216	nd	nd
Hi04a08	226:246	216:250	216 218	212 216	212 216	212 216	212 216	216 226	nd
Hi04b12	150:156	140:156	156	142 156	150 156	146 150	156	142 156	142 156
Hi04c05	181:181	183:181	181	181	181	181 183	179 183	179 183	181
Hi04c10	230:null[x] 206:206[y] 176	228:238[x] 200:206[y]	206 234	206 234	204 234	172	172 202 234	178 202 234	172 202 234
Hi04d02	208:222	238:null 180 194	176 182 204	182 190 204	190 198 222	186 204 214 218	190 204 214	176 192 222	186 204 218 222
Hi04d10	164:164	null	164 182	180	164	164	182	nd	164 180
Hi04e04	226:246	222:228	228 246	216 246	216 222	230	226 246	228 246	216 226
Hi04e05	144:null	null	null	null	nd	null	null	144	null
Hi04f08	202:218	218:226	226	226	220	218	220 226	226	218
Hi04f09	222 244 254	230[b] 224 244	222 224 254 260	224 226 252 258	224 256	224,254	224 226 244 260	222 224 244 254	nd
Hi04g05	null:null 230	190:194 230	194 258	232 258	240 254	230	230	230	232 248
Hi04g11	null:null 166	118:null	118	158	158	140	158	164	118
Hi05b02	126:126	120:122	126 178	122 178	134 162	120 144 162 178	122 126	126 178	126 134
Hi05b09	140:144	136:136	136	136 144	136 140	136	140 144	nd	nd
Hi05c06	139:138	136:136	138 141	138	138 141	141 142	138	138 139	136 138
Hi05d10	212:null	null	212	nd	212	212	nd	212	212
Hi05e07	212:214	216:230	214 230	null	214 234	null	214 230	214	228
Hi05f12	173: null[x] 169:169[y]	173:177[X] 157:169[y]	157 173	157 171	157 173	157 173	157 173	157 173 177	157 173
Hi05g12	230[b] 208 216 238	216	216 238	216.192	216 238	222 242	208 216 230 238	208 216 220 228	216
Hi06b06	242:262	236:262	260	260	260	236 260	260	262	242 260
Hi06f09	280:288	272:274	280 288	272 288	272 288	274 288	288	272 280	272 288
Hi07b02	214:218	214:null	212 216	214 216	nd	212	nd	216	216
Hi07b06	210:220	220:222	226	226	226	226	222 226	222 226	222 226
Hi07d08	222:232	222:226	nd	nd	nd	nd	nd	nd	nd
Hi07d11	214:218[x] 222:234[y]	216:226[x] 234:null[y] 200:200	214 220	220	216 232	228	200 218 226	200 220	212 220
Hi07d12	246:null[x] 184:null[y]	194:218[x] 246 250	248	248	198 246	198	246	184 206	250
Hi07e08	208:208[x] 233:235[y]	208:212[x] 231:241[y]	208 222 231 233	208 233	208 233 234	208 233 234	210 222 231	nd	nd
Hi07f01	204:214	204:210	206 220	210	210	210 220	210 220	210	206 210
Hi07g10	126:null	128:null	nd	nd	nd	nd	nd	nd	nd

Table 4 (continued)

SSR name	Fiesta	Discovery	Florina	Nova Easaygro	TN10-8	Durello di Forli	Prima	Mondial Gala	Fuji
Hi07h02	256:264	270:276	246 256	276	256 268	266	248 274	248 264	246 260
Hi08a04	246:254	246:250	246 248	246	248 250	246 254	246 250	246 254	246 254
Hi08c05	231:231	231:240	231 240	231 234 240	231 240	231 234	231 234	231 234 240	231 234 240
Hi08d09	183	183	186	183	183	183 186	nd	nd	183 186
Hi08e04	207:216	201:null	216	207	201 216	216 225	216	207 234	207
Hi08e06	134:138	134:null	134	134	134	134 137	134	134 138	134
Hi08f05	165	165	165	nd	165 157	165	nd	nd	nd
Hi08f06	227:230	224:242	230	230	224 227	230 233	227 230	227 230	230
Hi08f12	131:218	116:131	116 220	116 218	206	131 206	158 218	218 220	129 220
Hi08g03	113:116 104	116:116 104	104 113 116	nd	nd	104 113	104 113	104 113 116	104 113 116
Hi08g06	192:198	192:192	192 198	192 198	192	192	192 198	nd	192
Hi08g12	188:188	188:194	188 194	191 194	188 194	188 197	188	188 191	188 191
Hi08h03	155:155	155:158	155	155	155	nd	nd	155	nd
Hi08h08	236:236	236:239	236 242	236	236 242	236 239	236 239	236 239	236
Hi08h12	157:203	163:169	169 203	151 203	157 163	151	169 203	151 172	157 172
Hi09a01	183,186	186:192	183 186	180,183	183,186	183,192	183,186	186	183,192
Hi09b04	230:227	278:278	242	227 269	227 242	227 242	230 242	242 269	230 242
Hi09f01	257:260	257:266	260	260	257 260	257 266	257 266	260	260
Hi11a01	214:217	214:217	217	217	217	214 217	214 223	214 223	217
Hi11a03	141:141	141:144	141	141	141 144	141 144	141	141	141
Hi12a02	249:255	255:255	249 255	249 255	249 255	249 255	255	255	255
Hi12c02	190:178	169:null	178	169	178	169 178	169	169	190 178
Hi12f04	184:184	184:187	187	184	184	184	184	184 187	184
Hi15a13	220:232	220:220	232	234	232	220	220	232	232
Hi15b02	196:199	199:199	202	null	202	202	199	199	202
Hi15c07	210:210	204:210	204 210	210	210	204 210	210	204 210	204 210
Hi15e04	209:209	209:212	209	209 212	209	209	209 212	209	209 212
Hi15g11	160:163	160:160	163	163	nd	160	160 163	160 163	163
Hi15h12	222:222	222:225	222	222	222	222 228	222 225	222 225	222
Hi16d02	144:144	144:147	144 165	144	144 165	144 177	144	144	144 153
Hi20b03	220:238	220:229	nd	nd	nd	220 244	229	220 244	nd
Hi21c08	230:230	230:227	230 227	230 227	230 227	230 227	230	230 227	nd
Hi21e04	134:134 149 156 158 161	134:136 149 156 158 161	134 136 149 158	136 149 156 158 161	134 149 158 161	134 138 148 149 158 161	134 136 149 158 161	136 138 149 156 161	134 138 149 156 161
Hi21f08	242:246 280 282	242:242 234 244 250 272 282	242 248 280 282	242 248 266 280	242 250 280	242 250 282	250 266 272 280	244 248 272 280 282	244 248 282
Hi21g05	158:164	155:158:164	155 158	155	155 158 164	158	155 158 164	155 158 164	155 158
Hi22a07	192:198[x] 153:189[y]:	196:196[a] null:null[a]	192 202	153 196 202	153 189 202	153 202	189 192 196	189 198 202	189 198
Hi22d06	129:129	126:129	132	129 135	129 135	132	126 129	129 135	129 135

Table 4 (continued)

SSR name	Fiesta	Discovery	Florina	Nova Easaygro	TN10-8	Durello di Forli	Prima	Mondial Gala	Fuji
Hi22f04	138:147	135:138	141 147	141	138	138	138 141	138 141	138
Hi22f06	240:246	243:246	240 246	240 246	246	243	246	240 246	240 246
Hi22f12	211:null	211:217	214 217	205	217	211 217	205	211	217
Hi22g06	240:249	240:240	240	240	240	240	240	249	240
Hi23b12	154:169	154:169	154 169	142 169	142 169	169	142 154	154 169	142
Hi23d02	160:166	160:160	157 160	160	157 160	160	160	160	157 160
Hi23d06	161:161	158:161 170 173	161 170	161	161	161	161 170	161 188	158 188
Hi23d11b	181:184 177	178:184 177	177 184	177 184	177 178 184	177 178 181	177 184	177 178 184	177 184
Hi23g02	248:257	254:257	239	239 255	239 248	255	239	255	230 248
Hi23g08	220:220	214:220 211	211 220	220	211 220	211 220	214 220	220	211 220
Hi23g12	223:223 241	223:226 241	226 241	226 235 241	223 226 235 241	223 226 235 241	223 226 235 241	223 235 241	223 235 241
Hi24f04	144:150	147:153	153	153	150 153	153	150 153	144 150	152 153
AF057134$_{SSR}$	210:216	208:224	208	216	216	214 220	216	208 216	202 216
AF527800$_{SSR}$	178:null	168:184	168	168	168	168	168 194	168 178	nd
AJ000761a$_{SSR}$	262:262	262:266	262 264	260 262	262 264	260 266	264 272	nd	nd
AJ000761b$_{SSR}$	248:248	210:248	244 248	210 252	246 248	208 226	210 250	226 244	244 248
AJ001681$_{SSR}$	169:191	189:191	169 187	169 187	183	189 195	191	169	189
AJ251116$_{SSR}$	165:167	167:167	167	167	167	165 nd	nd	167	nd
AJ320188$_{SSR}$	213:null	null:null 199	199 213	203 207	219	191 245	199	199	199
AT000174$_{SSR}$	186:194	186:192	178 186	178 186	186 188	178 nd	178 200	188 194	178
AT000400$_{SSR}$	198:216 226	216:216	210 224 232	210 224 232	198 210 216 226 232	216 224	210 216 232	216 224 232	214 224
AT000420$_{SSR}$	201:201	203:205	201 205	201	203 209	201 189	201 205	201	201 205
AU223486$_{SSR}$	205:205	205:217	205 208	208	205 217	205 208	205 208	205 208	208
AU223548$_{SSR}$	270:278	270:270	262 278	262 270	274	270 278	262 270	262 270	262 278
AU223657$_{SSR}$	225:225	221:225	225 231	219 225	231	233 223	231	231	nd
AU223670$_{SSR}$	194:202	194:196 202	194 196 202	194 196 202	194 202	194 202	194 196 202	nd	nd
AU301431$_{SSR}$	213:216	216:216	213 216	213 216	213 216	216	213 216	216	213 216
AY187627$_{SSR}$	300:null	null:null	300	300	300	300	null	null	null
CN444542$_{SSR}$	136:146 110:110	136:156 110:110 128	110 124 136	110 124 136	110 126 146	110 124 140	110 136	110 136 146	110 124 136
CN444636$_{SSR}$	241:241	239:243	nd	nd	239 241	nd	239 241	241	239
CN444794$_{SSR}$	260:306	230:298	270	260	230	254 270	256 272	254 270	256
CN445290$_{SSR}$	230:230	230:236	230	230	230	236	230 242	242	236 242
CN445599$_{SSR}$	154:164 131	153:176 130 132	130 146 176	131 136	131 132 150 154	130 131 152	131 136 154	130 132 146 154	130 131 146
CN491050$_{SSR}$	>330 c:c	>330 a:c	nd	nd	>330 c	>330 a	>330 b	>330 b c	>330 b c

Table 4 (continued)

SSR name	Fiesta	Discovery	Florina	Nova Easaygro	TN10-8	Durello di Forti	Prima	Mondial Gala	Fuji
CN493139$_{SSR}$	144:156 124	136:150 124 142	126 140 142 162	124 126 140 162	124 142 146	124 142	124 126 142 144	140 144 162	124 140 156 162
CN496002$_{SSR}$	243:257	243:259	257 261	nd	243 261	243 257	243 257	243 259	243 257
CN496913$_{SSR}$	236:236	236:242	236	236	236	236 278	236 244	236 252	236 244
CN581493$_{SSR}$	194:194	184:194	228	194 218	192 220	194 228	194 226	194 218	194 218
U78948$_{SSR}$	188:190	180:186	nd	178 182	nd	184 188	180 186	nd	nd
U78949$_{SSR}$ [b]	215^x 176 209 225	221^x 174^y 209 225	176 205 219	174 219	176 207 219	172 176 190 211 221	174 176 190 211 174 215	205	190 205
Z38126$_{SSR}$	216:216	214	214 216	214	214	216 240	214	214	214 240
Z71980$_{SSR}$	170	170	172	nd	nd	172	nd	170	nd
Z71981$_{SSR}$	222:224	224:232	212 224	222 224	224	222	nd	216 222	222 232
GD147	147:151	141:153	141 135	155 151	147	141 153	135 153	141 153	141 153
HGA8b	160:164^x 135:151^y	156:164^x 151:151^y 135 160	156 160 164	133 156 160	151 156 160 164	156 164	135 156 160 164	151 156 160 164	151 156 160 164
KA4b	141:137	139:141	141	137	141	137 141	141	137	137 141
NB102a	181:null	181:183	null	183	183	null	181	null	183
NH009b	148:162	148	144 148	144 158	138 148	144 152	144 148 158 162	158 162	148 162
NH029a	91:91	91:93	91	99	97	95	93 95	nd	93 95
NH033b	189:189	175:183	163 177	163 177	175 183	179 183	183 189	177 189	177 187
MSS6	279:279	277:273	279	279	273	273	279	279	279

For the alleles of Fiesta and Discovery, the numbers separated by ":" indicate the estimated size of the alleles of the same locus, while the other numbers indicate the size of undefined loci. For multiloci SSRs, ^{x} and ^{y} indicate the alleles assigned to locus x and y, respectively

nd allele size not determined

[a] Amplicons that could be alleles of the locus x as well as of locus y. For the other seven cultivars, the size of the amplicons are indicated; single alleles can indicate homozygosity or the presence of a null allele

[b] Due to the complex pattern of alleles amplified of this SSR instead of pair of alleles only an allele per locus was identified and is presented in the table (allele that has been mapped as a dominant marker)

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Table 5 Statistics on SSR development

SSR library/origin of the sequence	Sequenced clones[a]	Sequences used for primer design[a]	Markers mapped[b]	Average no. of alleles per origin of the SSRs	Average no. of repeats per origin of the SSRs[c]
GA/GT libraries	571	103	65 (63%)	6.6	28
AAG[d]/AAC/ATC[d] libraries	587	131	52 (40%)	4.0	9
GenBank 2nt		39	24 (61%)	5.8	14
≥3nt		21	7 (33%)	4.3	6
Pear and *S. torminalis*[e]		17	7 (41%)	4.7	na
Apple SSRs from literature[f]		8	1 (13%)	13[g]	na
TOTAL	1,158	319	157 (49%)		

na: not analyzable; sequences not available

[a]The difference between the number of sequenced clones and sequences used for primer design is due to redundant sequences, absence of a SSR repeat, or a too-short-sequence stretch before or after the SSR repeat for primer design

[b]In Fiesta × Discovery, or, alternatively in Durello di Forlì × Fiesta, Discovery × TN10-8, or Fuji × Mondial Gala

[c]Found in the sequence used for primer designing. Compound SSRs with repeats of different length (e.g., GT-CAA) were not considered. Compound SSR with different repeats but with the same number of nucleotides composing the repeat (e.g., GT-TA) were considered and the length of the two repeats was summed

[d]Positive clones of these two libraries were screened by PCR for the presence of highly redundant fragments. The number of clones sequenced after this check are reported

[e]Yamamoto et al. (2002a,b); Oddou-Muratorio et al. (2001)

[f]Hokanson et al. (1998)

[g]The number of alleles may be overestimated due to low quality of the amplifications

of these 86 SSRs, 24 (28%) were developed during this study. Some of the selected markers showed a low level of polymorphism in our set of reference cultivars. They were, nevertheless, included lacking more polymorphic alternatives for these specific genomic regions.

Discussion

The aim of our research was to obtain a set of highly polymorphic SSR markers that cover the entire apple genome, to enable directed genotyping approaches. Directed genotyping is a target-directed, cost- and time-efficient approach for the genome-wide genotyping of new crosses, cultivars, and breeding lines. By facilitating this method of genotyping, assessments of new molecular marker–trait associations, allele mining and validation of candidate genes will also be enhanced. This paper reports the generation and mapping of a large new set of apple SSR markers. These SSRs have allowed the enrichment of the reference map of the apple with 168 new loci. This almost doubled the number of mapped SSR markers.

Efficiency of SSR development

The newly mapped SSRs have been obtained from different sources: genomic libraries, publicly available EST sequences, the literature, and from SSRs developed for other *Maloideae* species. The efficiency by which each of these sources gave new SSR markers is evaluated in Table 5. Only about 20% of the library sequences were unique (not redundant), contained a microsatellite repeat, and were suitable for designing compatible primers in the regions flanking the repeat. When exploiting published EST sequences, there is no need to generate enriched libraries, or to sequence, and considerable amounts of money and time can be saved.

Table 6 Frequency distribution (numbers, percentages, and percentage cumulative) of the number of alleles assessed in a set of nine diploid cultivars of single-locus (SL) SSRs divided by the length of the SSR repeat (two nucleotides or more than two nucleotide repeats)

| Absolute no. of SL SSRs		No. of alleles									
		2	3	4	5	6	7	8	9	10	Total
No. of nt repeats	2	6	11	8	10	13	6	9	0	2	65
	≥3	13	9	13	7	1	3				46
%											
No. of nt repeats	2	9	17	12	15	20	9	14	0	3	100
	≥3	28	20	28	15	2	7	0	0	0	100
Cumulative %											
No. of nt repeats	2	9	26	38	54	74	83	96	96	100	
	≥3	28	48	76	91	93	100	100	100	100	
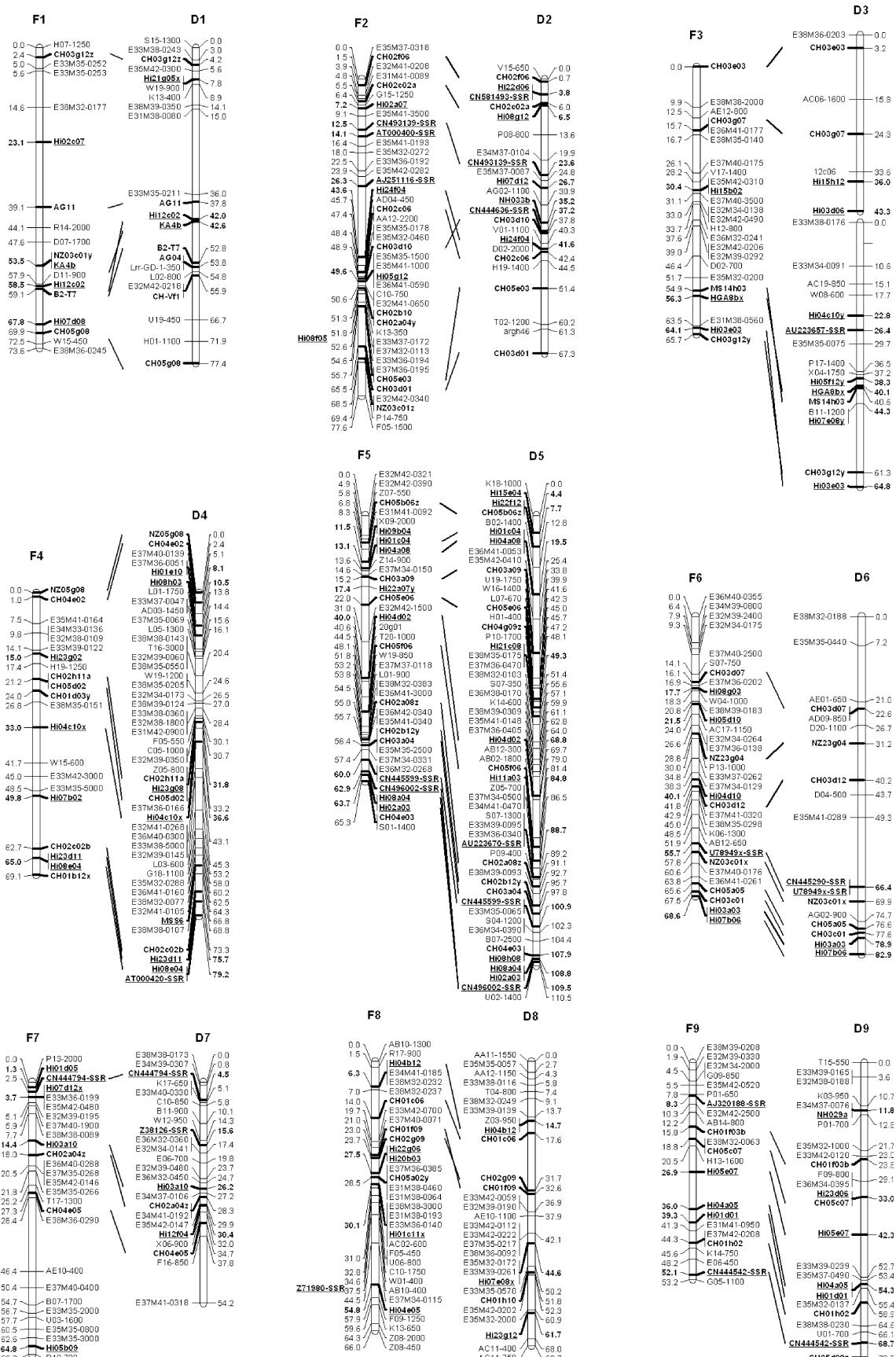
Fig. 1 Genetic map of Fiesta (*F*) and Discovery (*D*). Numbering of the linkage groups is according to Maliepaard et al. (1998), and consistent with Liebhard et al. 2002, 2003b. SSRs are indicated in *bold*. SSRs of the new set have been additionally *underlined* and their map position is also in *bold*

Fig. 1 (continued)
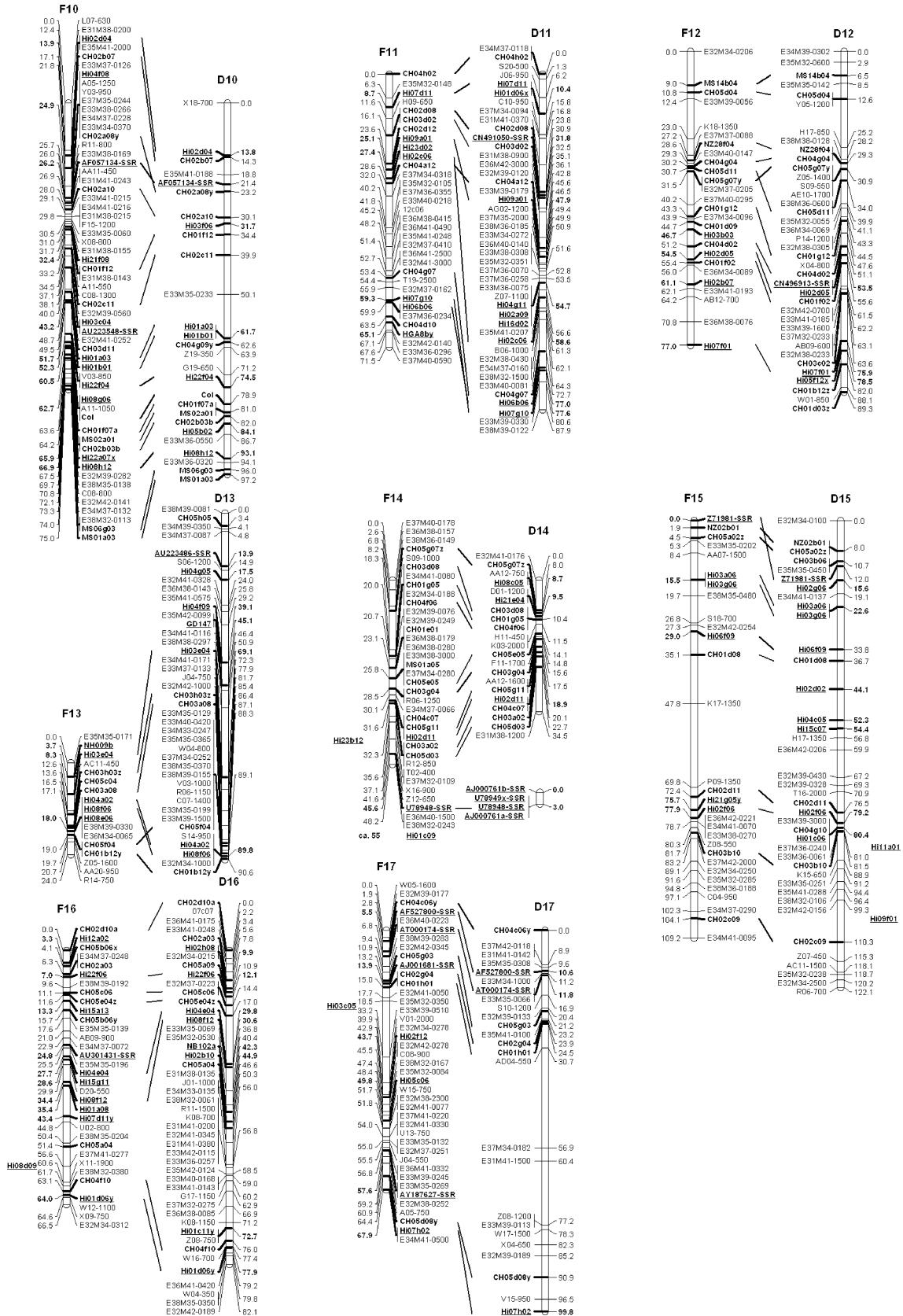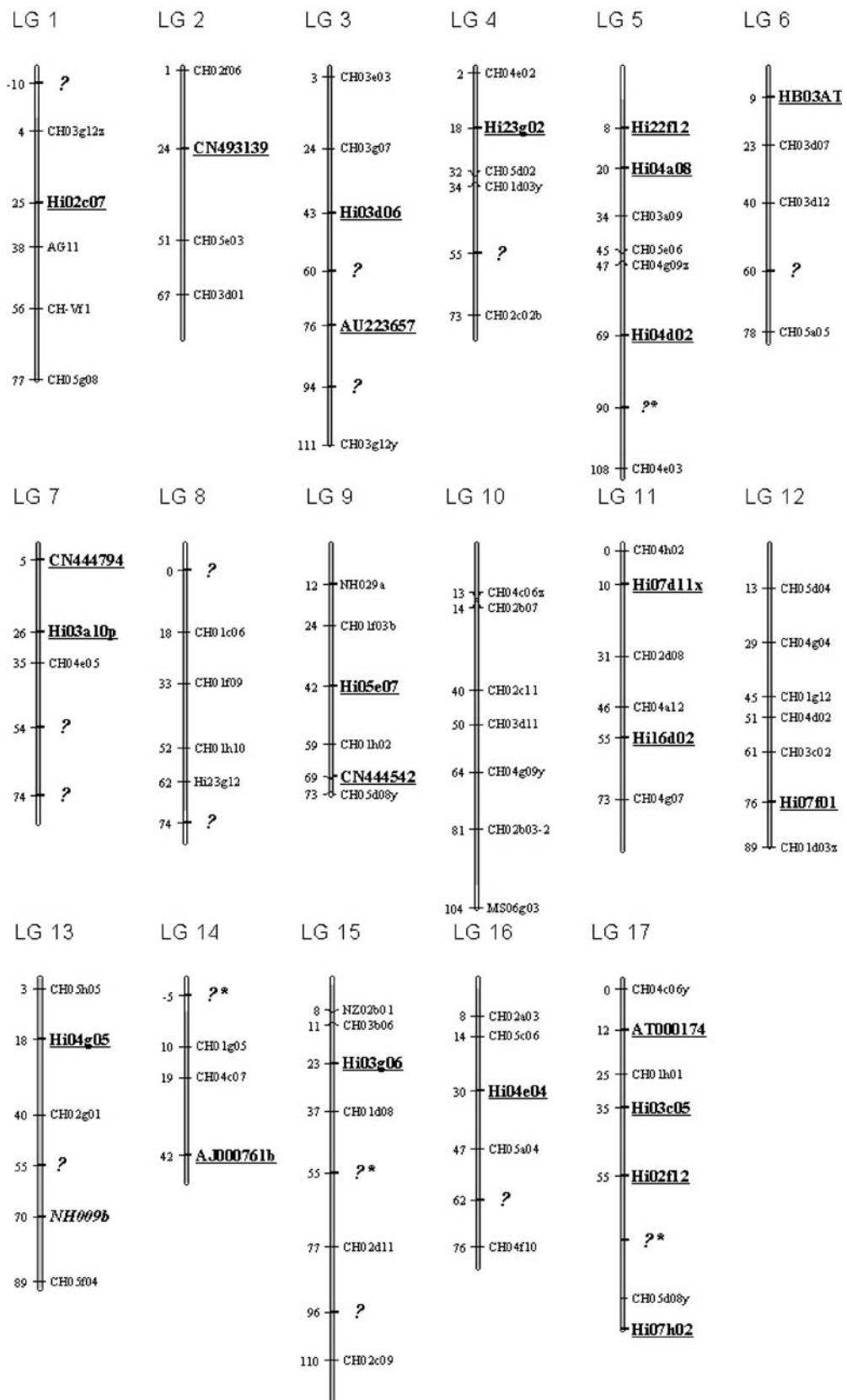
Considering the relative high percentage of ESTs containing SSR repeats (about 5%, data not shown), the efficiency by which SSR containing EST sequences lead to SSR markers and the continuous increase of publicly available EST sequences (Korban et al. 2005; Crowhurst et al. 2005), it is clear that the next SSR makers should be

Fig. 2 Set of 102 SSR primer pairs for global coverage of the apple genome. Map positions (in cM) are aligned to the Discovery maps of Fig. 1. *Gray filled bar segments* indicate the linkage group segments covered by the Fiesta and Discovery maps of Fig. 1. *Open bar segments* indicate linkage group segments not covered by the maps of Fig. 1, but which were revealed by other, unpublished linkage maps. CH markers mapped by visual alignment that were initially mapped in other mapping populations are: CH04c06z [mapped in Prima × Fiesta and Jonathan × Prima (J×P)] and CH02g01 (Discovery × TN10-8 and J×P). For 16 loci, indicated with the symbol *?*, no primer pairs are publicly available yet. The symbol *?** marks positions of unpublished SSR markers, which are expected to become available in the near future. *Underlined* SSRs have been developed in the present work

developed from ESTs. Sequences containing long dinucleotide repeats are preferable because of their higher level of polymorphism (Table 5), making them more valuable in directed genotyping approaches. Other new SSRs may be located from the mapping of candidate genes that have SSRs in their non-coding region (e.g., Gao et al. 2005a,b; Costa et al., in preparation), or by the sequencing of previously mapped markers (Gao and Van de Weg 2006).

The approach of transferring SSRs developed in other *Maloideae* species was efficient (41%). The high syntheny between apple and pear was used (Yamamoto et al. 2004; Dondini et al. 2004) to select SSRs, which, from the map position in pear, could fill the gaps between SSRs mapped in apple or could enrich regions with few SSRs. This strategy proved to be successful. One of the few new SSR markers mapped on LG1 is from pear (KA4b) and some regions (e.g., LG2, LG9, LG16) of the apple genome, which, at the beginning of the project did not contain a high density of SSRs, were enriched.

The efficiency of mapping previously developed apple GD-SSRs is probably underestimated due to our stringent PCR conditions (see later). Indeed, Hemmat et al. (2003) were able to map all 18 published GD-SSR markers except GD15, which showed very low polymorphism (Hokanson et al. 1998). Ten of these markers could be aligned to our map (data not shown), while seven (those tested in this work) could not be unequivocally aligned due to a low number of markers in common.

Level of polymorphism

The screening of a newly developed SSR over a restricted number of cultivars (nine in our case) allows an estimation of its true level of polymorphism. Although the number of alleles identified is limited compared to what is present in the apple germplasm, more extensive tests support a rough correlation between the allele numbers identified in a small set of cultivars and the number in germplasm collections (Coart et al. 2003). This information is, thus, useful for a first selection of markers for genotyping projects as well as for genetic diversity studies.

New SSRs designed to meet high-throughput criteria

During the development of new SSRs, variable annealing temperature (Tm) could be used to improve the amplification profile of individual markers. As our aim was to develop SSR markers suitable for high-throughput testing, we, therefore, decided to design all primers with a single Tm (60°C), which should enable multiplex PCRs. The relatively high Tm used was set to improve specificity, avoiding amplification of additional, non-SSR 'bands' that could hamper both scoring and multiplexing. On the other hand, some SSR-containing sequences may not have delivered an SSR marker, although primers for lower Tm might have been feasible. Tm other than 60°C were accepted only for SSR markers developed and mapped in other *Maloideae* and that had a good chance of filling gaps in the SSR linkage map.

Differences between our standard PCR conditions and profiles may be the reason why most GD–SSRs could not be mapped by us because of very complex band patterns that rendered impossible the recognition of the SSR alleles, despite giving good results in other studies (Hokanson et al. 1998, 2001; Hemmat et al. 2003; Van de Weg, unpublished).

Determination of allele sizes

Problems occurring in assessing the size of the amplified amplicons of a cultivar, using ^{33}P-labeled primers and polyacrylamide sequencing gel electrophoresis, have been already discussed by Liebhard et al. (2002). The authors stated that 1) the absolute fragment size could be determined only with an accuracy of ±1 base, 2) differences in size estimation between replications may occur, but relative size differences among amplicons of tested cultivars are constant. These statements were confirmed in this study. Marker assessments with other technology platforms (fluorescently labeled primers, automated sequencers) will also lead to different absolute values, though size differences should remain constant (This et al. 2004). To allow comparisons among studies, we propose to include two or three universal reference cultivars, for which we propose Fiesta, Discovery, and Prima. We find these cultivars suitable because they have been tested with most apple SSR markers and have been involved in many genetic studies, being parental cultivars of various mapping populations in Europe.

Accuracy of map positions

Although the mapping of the new SSRs is based on the analysis of 44 progeny plants, their map position can be considered to be sufficiently accurate for our purposes. The order of the SSRs was usually identical for both the parental Fiesta and Discovery, thus confirming the validity of their relative position (Fig. 1). In only few cases is the

order of flanking SSRs inverted. In all these cases, the SSRs involved maps very close together. Relative positions of tightly linked markers are usually uncertain due to the effects of missing values and to differences in segregation information among markers (Maliepaard et al. 1997). The data do allow assessment of approximate map positions of the new SSRs, which is sufficient for our current purpose to fill in the gaps in previous maps. In only one case, $Z71981_{SSR}$ (LG15), is the approximate map position still to be determined. Some SSRs of the Hi set have already been mapped in other crosses than Fiesta × Discovery, and their map position relative to other SSRs was confirmed (Patocchi et al. 2005; Gardiner et al. 2006; Erdin et al. 2006; Durel, unpublished).

Genome covering set of apple SSRs

Currently, around 300 SSRs are mapped on the apple genome, mostly in Fiesta × Discovery (Fig. 1). Soon there will be sufficient SSR markers to enable an initial genome-wide genotyping with a set of 100 SSR markers that have an average inter-marker distance of 15 cM (Fig. 2). Our goal is to develop such a set applying the following criteria: (1) distance between successive markers generally not larger than 20 cM, though occasionally allowed to be up to 25 cM; (2) most proximal and most distal marker of a linkage group preferably within 10 cM from the linkage group ends; (3) cleanness of the amplifications to allow easy scoring and multiplexing; (4) high level of polymorphism; (5) (un)suitability for multiplexing; (6) range of the allele sizes in view of multiplexing markers from the same LG; (7) preference of CH- over Hi-SSR markers because of the generally wider experience with the former; and (8) preference of single-locus markers over multi-locus markers for ease of data interpretation.

Applying these criteria, we came up with a set of 86 primer pairs, 24 of which (28%) were developed in the current research. This set still lacks markers for 16 chromosome segments (Fig. 2); however, in four segments, new SSRs have already been identified in other projects (unpublished data). To fill the remaining gaps, RAPD or AFLP markers previously mapped in these regions could be transformed into SCAR markers, which could be used as probes to screen apple BAC libraries. From the positive BACs, sequences containing SSR repeats can be obtained and used for the development of SSR markers. PCR-based methods for the "extraction" of sequences containing SSR repeats from BAC clones are available (Vinatzer et al. 2004).

The current set of 86 SSR markers covers around 85% of the genome. This set will be used to genotype 350 cultivars and breeding selections as well as 1,400 descendants of 24 crosses within the framework of the HiDRAS project (Gianfranceschi and Soglio 2004). This work will supply additional information on the level of polymorphism of these markers and their compatibility in multiplexed PCRs.

SSR database online

Information on the currently available apple SSR markers is scattered over various publications, and published information remains limited to the initial experiences with these markers. In the course of their use internationally, new information concerning SSR markers will arise with regard to level of polymorphism, range of allele sizes, number of loci, suitability for multiplexing, etc. Worldwide, various groups are also developing new SSR markers from the continuously increasing amount of EST data. For efficient genotyping of cultivars and breeding lines, and for an efficient generation of maps from new crosses, markers should be sorted according to their map position, polymorphism, and/or quality. To facilitate searches for and updates of SSR information, and to concentrate worldwide efforts in the development of new SSR markers, an on-line apple SSR database was constructed (http://www.hidras.unimi.it). This includes information on all SSR markers of the CH (Liebhard et al. 2002), Hi (this paper), and NZ (Guilford et al. 1997; Liebhard et al. 2002) series, and, once available, will include information about the SSRs of the GD series (Hokanson et al. 1998; Hemmat et al. 2003).

This database also allows advanced searches, e.g., a list of SSRs mapped on a specific linkage group, among which SSRs of a certain quality can be selected. For SSR markers with amplification profiles of insufficient quality, new primers can be designed and tested as the sequences of the clones from which the SSR markers were derived can be downloaded for all CH and Hi markers. Updates and comments can be added to each of the SSRs. Researchers are encouraged to share their experiences, especially in the reporting of improvements on problematic markers.

Although more than 300 apple SSRs have been mapped, there are regions of the genome not sufficiently covered with SSRs (Fig. 2). Saturation of these regions is required to allow the construction of maps based solely on SSRs. In addition to the method previously proposed, this could be achieved by the further mapping of EST sequences containing SSR repeats, as hundreds of such sequences are available. Such a work could be performed most efficiently by coordinating action among groups working with apple genetic maps all over the world. The current database is the first step towards the creation of such a worldwide platform. A form is available for announcing that certain EST sequences are under investigation, so that duplication of work could be avoided. Contact addresses are also available so that different research groups can get in touch and, if desired, exchange information.

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