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ORIGINAL PAPER

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TRIM retrotransposons occur in apple and are polymorphic between varieties but not sports

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Abstract Retrotransposon markers have been demonstrated to be powerful tools for investigating linkage, evolution and genetics diversity in plants. In the present study, we identified and cloned three full-size TRIM (terminal-repeat retrotransposon in miniature) group retrotransposon elements from apple (*Malus domestica*) cv. 'Antonovka', the first from the Rosaceae. To investigate their utility as markers, we designed primers to match the long terminal repeats (LTRs) of the apple TRIM sequences. We found that PCR reactions with even a single primer produced multiple bands, suggesting that the copy number of these TRIM elements is relatively high, and that they may be locally clustered or nested in the genome. Furthermore, the apple TRIM primers employed in IRAP (inter-retrotransposon amplified polymorphism) or REMAP (retrotransposon-microsatellite amplified polymorphism) analyses produced unique, reproducible profiles for 12 standard apple cultivars. On the other hand, all seven of the sport mutations in this study were identical to their mother cultivar. Genetic similarity values calculated from the IRAP/REMAP analyses or the STMS (sequence tagged microsatellite sites) analysis were generally comparable. PAUP cluster analysis based on IRAP and REMAP markers in apple and Japanese quince generated an NJ tree that is in good accordance with both a tree based on SMTS markers and the origin of the studied samples. Our results demonstrate that, although they do not encode the proteins necessary to carry out a life cycle and are thereby non-autonomous, TRIMs are at least as polymorphic in their insertion patterns as conventional complete retrotransposons.

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Introduction

Retrotransposons elements constitute a major component of dispersed repetitious DNA in all eukaryotic genomes. One of the major groups of these elements, the long terminal repeat (LTR) retrotransposons, replicates by cycles of transcription, reverse transcription, and integration of daughter copies back into the genome. These steps require retrotransposon-encoded protein products, available in *cis* or *trans*. Two sub-groups of LTR retrotransposons have been described that lack protein-coding capacity. Members of one, the *la*rge *r*etrotransposon *d*erivatives (LARDs), contain a core domain but encode no protein products (Kalendar et al. 2000). The others, called *t*erminal-*r*epeat retrotransposons *in* *m*iniature (TRIMs), have short LTRs and lack internal domains virtually entirely (Witte et al. 2001). Because they do not encode their own proteins, it is assumed that both the TRIMs and LARDs depend for mobility on *trans*-complementation by the products of autonomous retrotransposons.

Several retrotransposon-based marker systems have been developed that reveal insertional polymorphisms generated by the transposition of the retrotransposons. These have been found useful for linkage, evolution, and genetics studies (reviewed by Schulman et al. 2004). Retrotransposon marker systems generally rely on PCR to generate fingerprints. The marker systems generate fingerprints, or multilocus profiles, for the members of given families of retrotransposons. The inter-retrotransposon

amplified polymorphism (IRAP) technique (Kalendar et al. 1999) generates PCR products from retrotransposons inserted near enough to each other to allow efficient amplification. The IRAP amplification primers are commonly designed to match segments of LTRs conserved within element families. Retrotransposon-microsatellite amplified polymorphism (REMAP) (Kalendar et al. 1999) uses one LTR primer, together with a simple sequence repeat primer with one additional base at the 3′ end of simple sequence repeat (SSR) primer (for example: 5′ $(CA)_9$G-3′).

Because many retroelements, at least in the cereals, have a tendency to insert into regions rich in tandemly repeated DNA (Kalendar et al. 1999; Ramsay et al. 1999), as well as near to, or nested within, each other (SanMiguel et al. 1996; Shirasu et al. 2000), REMAP and IRAP are efficient at detecting insertion events. Whereas retrotransposon insertions are only heritable in sexually propagated plants if the insertion event occurs in a cell that gives rise to a gamete, insertions in somatic tissues can be passed along in vegetatively propagated crops such as apple. Other genetic rearrangements in somatic nuclei that affect the position of priming sites for markers would likewise be detectable.

The aim of this study was: to identify TRIM elements in apple; to develop and apply TRIM elements as molecular markers for cultivar identification and genetic relationships in apple (*Malus domestica*) and a close relative, Japanese quince (*Chaenomeles japonica*); to compare the TRIM markers with the previously well-established STMS (sequence tagged microsatellite sites) analysis.

Materials and methods

Plant material and DNA extraction

Nineteen apple (*Malus domestica* Borkh.) cultivars and one Japanese quince plant, *C. japonica* (Thunb.) Lindl. ex Spach, of unknown origin were used in this study. Leaf samples from apple trees growing at the MTT/Piikkiö research station clone collection were collected from the following cultivars: Atlas and its sport Red Atlas; Melba and its two sports Melba Red Plats and Melba Red Pate; Sävstaholm and its red sport Bergius; two Åkerö sports Tarko and Rajalin; three Cinnamon apple sports Yellow Cinnamon apple, Red Cinnamon apple, and Brown Cinnamon apple; from the Astrakan cultivar group: White Astrakan, Red Astrakan, Gyllenkrok's Astrakan, and Big transparent Astrakan; Antonovka; Transparente Blanche; Golden Delicious. Total genomic DNA from these cultivars, as well as from the Japanese quince, was extracted using the DNeasy ® Plant Mini Kit (Qiagen). From the apple cultivar 'Golden Delicious,' DNA was extracted from fruit skin, using a modification of the CTAB extraction protocol.

Cloning, sequencing, and primer design

Retrotransposon sequences were cloned from the total genomic DNA of cv. 'Antonovka' using a universal primer method for retrotransposons (R. Kalendar et al., submitted). The method involves amplification between the (-)-strand primer binding sites (PBS) of reverse transcriptase, which are highly conserved in all retrotransposons. Amplified fragments were cloned into the pGEM-5Zf (Promega Corp., USA) plasmid T-vector and sequenced using an ABI3700 (Applied Biosystems, USA) capillary sequencer. Retrotransposon segments within the clones were identified by comparison to known LTRs and internal retrotransposon regions from other elements. Primers (Table 1) were designed by using "FastPCR" software (Kalendar 2005), to match the conserved segments of retrotransposon LTRs in the clones in various orientations.

PCR reactions and detection

IRAP and REMAP

PCR was performed in 20 µl reaction mixtures containing 20–30 ng DNA, 75 mM Tris–HCl (pH 9.0), 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 0.2 µM primer(s), 200µM dNTP, and 1 U Taq polymerase from FIREPol (Solis BioDyne, Tartu, Estonia). Other sources of Taq polymerase, including DyNAzyme™ II (F-501L, Finnzymes Oy, Espoo, Finland), and DNA Polymerase from *Thermus thermophilus* HB27 (product 1001, Biotools S.A., Madrid, Spain) were also tested to determine if the choice of polymerase enzyme has an effect on the products amplified. Amplification was performed in PTC-100 Programmable Thermal Controller (MJ research Inc., Bio-Rad Laboratories, USA) or a Mastercycler Gradient (Eppendorf AG, Germany) in 0.2 ml tubes or 96-well plates. The PCR reaction consisted of a 4 min denaturation at 94°C, followed by 32 cycles of 40 s at 94°C, 40 s at 60°C, 2 min at 72°C, and a 10 min final extension at 72°C. Amplified products were electrophoresed on 2% agarose (RESolute Wide Range Agarose, BIOzym, Netherlands) or LE Agarose (SeaKem) gels and visualized by ethidium bromide staining. Gels were scanned on a FLA-5100 imaging system (Fuji Photo Film (Europe) GmbH., Germany) scanner with resolution of 50 µm or Eagle Eye II (Stratagene, USA). The repeatability of the IRAP markers was tested by performing the same analysis in the authors' two laboratories.

For the *STMS* analyses, nine primer pairs detecting polymorphism, previously described by Liebhard et al. (2002), were selected: CH01d03, CH01h02, CH02c06, CH02c09, CH02c11, CH02d08, CH04c06, CH04e05, and COL. Reactions were performed in a 20 µl reaction mixture containing 20–30 ng DNA, 75 mM Tris–HCl (pH 9.0), 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 0.2 µM of each forward and reverse primer, 200 µM dNTP, and 1 U Taq polymerase (FIRE*P*ol,

Table 1 *Malus* TRIM and ISSR primers used in IRAP and REMAP analysis

Primer	Sequence (5′–3′)
K001	tgatccactcccctgggcgatgtgg
K002	agctcccaaaaggcctcgtgc
K003	tcccaaaaggcctcgtgctaggtag
K004	tcccacatcgcccaggggagtggatc
K005	aggccttttgggagctcactg
K006	tggagcccgggtcaggatgtgac
K007	tagcacgatattgtccgctttgg
K008	gcggacaatatcgtgctacggtg
K009	aaagcggacaatatcgtgctacg
83003	agagagagagagagagagc
8565	gtcaccaccaccaccaccaccac

Solis BioDyne). Amplification was performed in the devices described above, in 0.2 ml 96-well plates. The PCR reactions were performed as described by Gianfranceschi et al. (1998), except for two primer pairs: CH02c06 and COL. For these two primer pairs, a modified PCR protocol was needed to produce the previously described result from standard cultivars (Golden Delicious and Discovery): 4 min denaturation at 94°C, followed by 34 cycles of 40 s at 94°C, 40 s at 58°C, 2 min at 72°C, followed by a 10 min final extension at 72°C. Labelled (Hex, Tet or Fam) amplification products were detected by capillary electrophoresis using MegaBACE^{TM}-500 or MegaBACE^{TM}-1000 DNA sequencers (Amersham Biosciences Ltd., UK), with ET400-R as an internal standard. Stability of the STMS markers was assured by carrying out two consecutive runs of each analysis.

Data analysis

From the IRAP and REMAP profiles, all clearly detectable polymorphic and monomorphic bands were scored for the analysis, and were scored as present (1) or absent (0) in each sample. The STMS markers were scored if they were clearly present in both consecutive runs, and counted as present or absent in each sample as well. The amount of information produced by each marker system was evaluated with several indices. The effective multiplex ratio (the number of polymorphic products from a single amplification reaction) and marker index (MI, the product of the effective multiplex ratio and expected heterozygosity, H_e) as well as the polymorphic information content (PIC) were calculated according to Powell et al. (1996) for all methods. Expected heterozygosity ($H_e = 1-\Sigma p_i^2$, where p_i is the allele frequency for the i^{th} allele) was calculated for the co-dominant STMS markers. For the dominant IRAP and REMAP markers, the same formula was used as for PIC (Tam et al. 2005). For IRAP and REMAP, the frequency of occurrence for a marker band was calculated as representing two alleles at the same locus. The pair-wise similarity (*SM*) value was estimated based on the proportion of common alleles (present and absent bands) from the total number of alleles (present and absent bands) in the samples (Formula 1). The congruence of similarity matrixes obtained with each marker type was assessed with the *t*-test, Wilcoxon signed-ranks test and by correlation coefficient.

$$SM = \frac{100(N_{11} + N_{00})}{(N_{11} + N_{00} + N_{10} + N_{01})}(\%), \quad (1)$$

whereN_{11}: is the number of present bands shared by two samplesN_{00}: is the number of absent bands shared by two samplesN_{10} and N_{01}: are the numbers of bands present in only one sample.

The ability of the IRAP and REMAP markers and STMS markers to reveal genetic relationships among apple cultivars and between apple and Japanese quince was evaluated using the neighbour-joining (NJ) method for phylogenetic analysis of the accessions. Analyses were performed with PAUP 4.0 using distance methods (Swofford 2003). Owing to the small number of REMAP markers, the data was pooled together with IRAP data to represent the retrotransposon markers in comparisons of the pair-wise similarity matrixes and NJ groupings.

Results

The structure of *Malus* TRIMs

Three full-size TRIM retrotransposons were cloned from apple (*M. domestica*) cv. 'Antonovka' (Fig. 1). Nucleotide sequences of the apple TRIMs can be found in the NCBI GenBank database under accession numbers AY603366-AY603368 (*M. domestica*). The apple elements have LTRs of about 306 bp that flank a short internal domain. The ends of both LTRs contain a 10-nucleotide terminal inverted repeat (TIR) with the sequence 5′-TGTAACATCCC—GGGATGTGACA-3′. The 71–nucleotide internal domain contains a PBS for (-)-strand cDNA synthesis, located three nucleotides (AAA or ATT) downstream of the 5′LTR, that is complementary to the methionine tRNA. The polypurine tract (PPT), 5′-AAGGGGGCTAGAT, is also

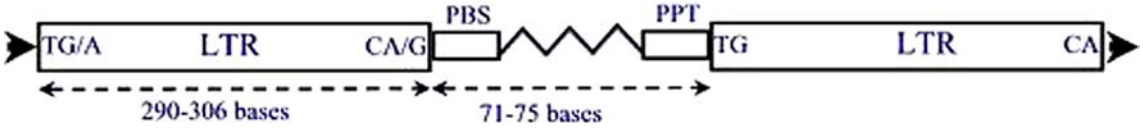

Fig. 1 Schematic diagram of the general structure of apple TRIM elements

highly conserved in the three apple TRIMs and is located immediately upstream of the 3′LTR. The apple elements are flanked by 5 bp repeats, which were generated by retrotransposons following insertion.

Genetic identities in IRAP, REMAP, and STMS analysis

All nine of the apple TRIM primers produced polymorphic DNA fingerprint patterns, even when they were used singly in IRAP reactions (Fig. 2). One primer produced from 6 to 15 informative fragments, yielding effective multiplex ratios between 1.8 and 5.8 (Table 2). Most IRAP bands from the apple samples were not shared with Japanese quince. Furthermore, combining the results of the nine IRAP primers, all standard apple cultivars had unique profiles, whereas all sport mutations of a single cultivar gave identical patterns. The results obtained from analyses carried out in two geographically distant laboratories were identical. All polymerases tested produced similar profiles. Some differences were found in the intensities of the detected bands. The Biotools DNA Polymerase (from *T. thermophilus*) clearly favoured amplification of larger products than did the Taq polymerases.

Apple TRIM primers were also tested in combination with anchored microsatellite (SSR) primers in REMAP analysis with the same set of samples as in IRAP analysis (19 apple cultivars and one Japanese quince). The REMAP analysis, combining the information of three primer pairs, produced informative DNA profiles (Fig. 3), which could clearly distinguish the two species and all cultivars. Similarly to IRAP, the sport mutations were identical to the cultivars from whence they were derived. There were from 7 to 27 informative fragments in the REMAP profiles, and the effective multiplex ratios of the three tested REMAP primer pairs were between 2.5 and 6.3 (Table 3).

Analyses of the STMS data from nine primer pairs produced relationships between the samples very similar to those derived from the retrotransposon markers. The

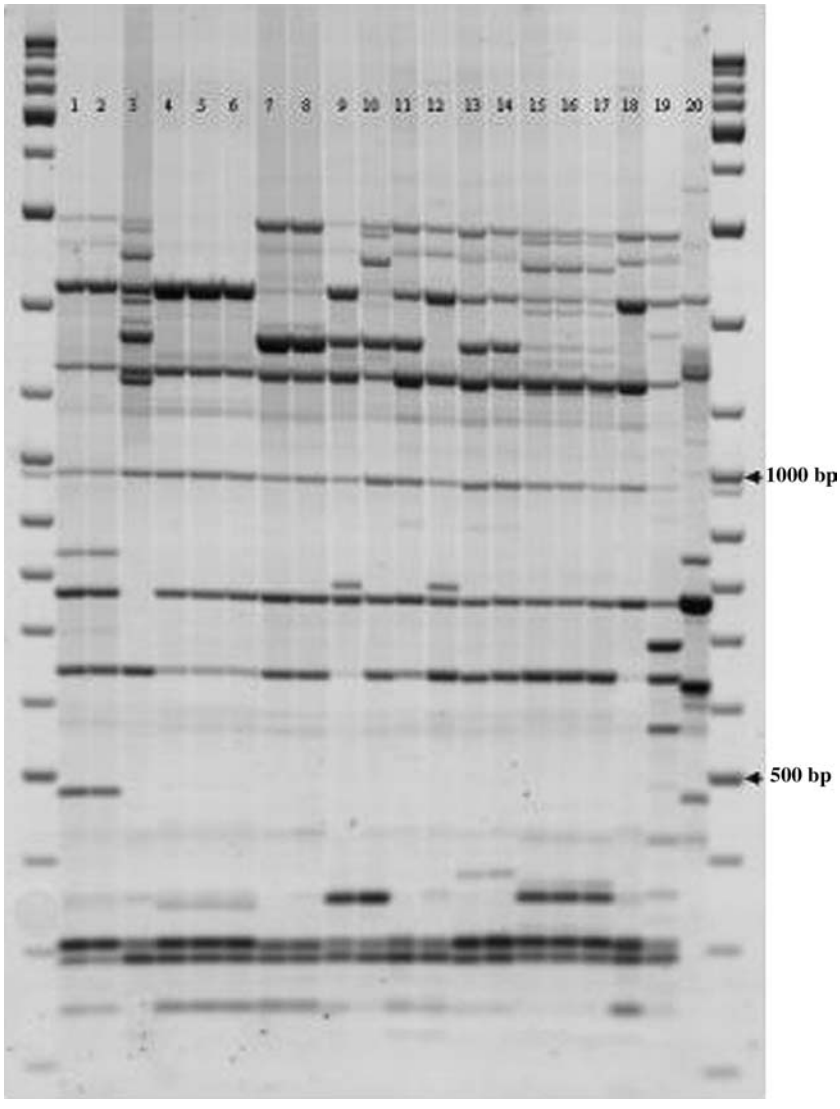

Fig. 2 IRAP analysis of apple and Japanese quince, using apple TRIM primer K008. DNA-samples: *1* Atlas; *2* Atlas, red; *3* Antonovka; *4* Melba; *5* Melba, red Plats; *6* Melba, red Pate; *7* Bergius; *8* Sävstaholm; *9* White Astrakan; *10* Red Astrakan; *11* Gyllenkrok's Astrakan; *12* Big transparent Astrakan'; *13* Åkerö, Tarko; *14* Åkerö, Rajalin; *15* Yellow Cinnamon apple; *16* Red Cinnamon apple; *17* Brown Cinnamon apple; *18* Transparente Blanche'; *19* Golden Delicious; *20* *Chaenomeles japonica*

Table 2 IRAP analysis of 19 apple cultivars and one Japanese quince using single apple TRIM primers

Primer no.	Total number of clearly detectable bands	Bands polymorphic in *Malus domestica*	Bands monomorphic in *Malus domestica*	Bands specific to *Chaenomeles japonica*	Effective multiplex ratio in *Malus domestica*
K001	12	10	1	1	5.8
K002	14	8	1	5	4.5
K003	7	5	1	1	2.6
K004	6	2	2	2	1.8
K005	8	5	1	2	2.7
K006	10	6	1	3	3.5
K007	11	7	2	2	4.8
K008	13	8	3	2	4.2
K009	15	10	1	4	3.2

primer pairs selected for this study detected 2–4 alleles per locus. Six primer pairs, CH01h02, CH02c09, CH02c11, CH02d08, CH04c06, and CH04e05, also produced bands from the Japanese quince sample. These bands all differed in size from the apple alleles of the same loci. Only alleles that were present in both replicate experiments were scored. All cultivars could be distinguished and all but one of the sport mutations was identical to the parent from which it was derived. Atlas and its sport Atlas Red have a four base length

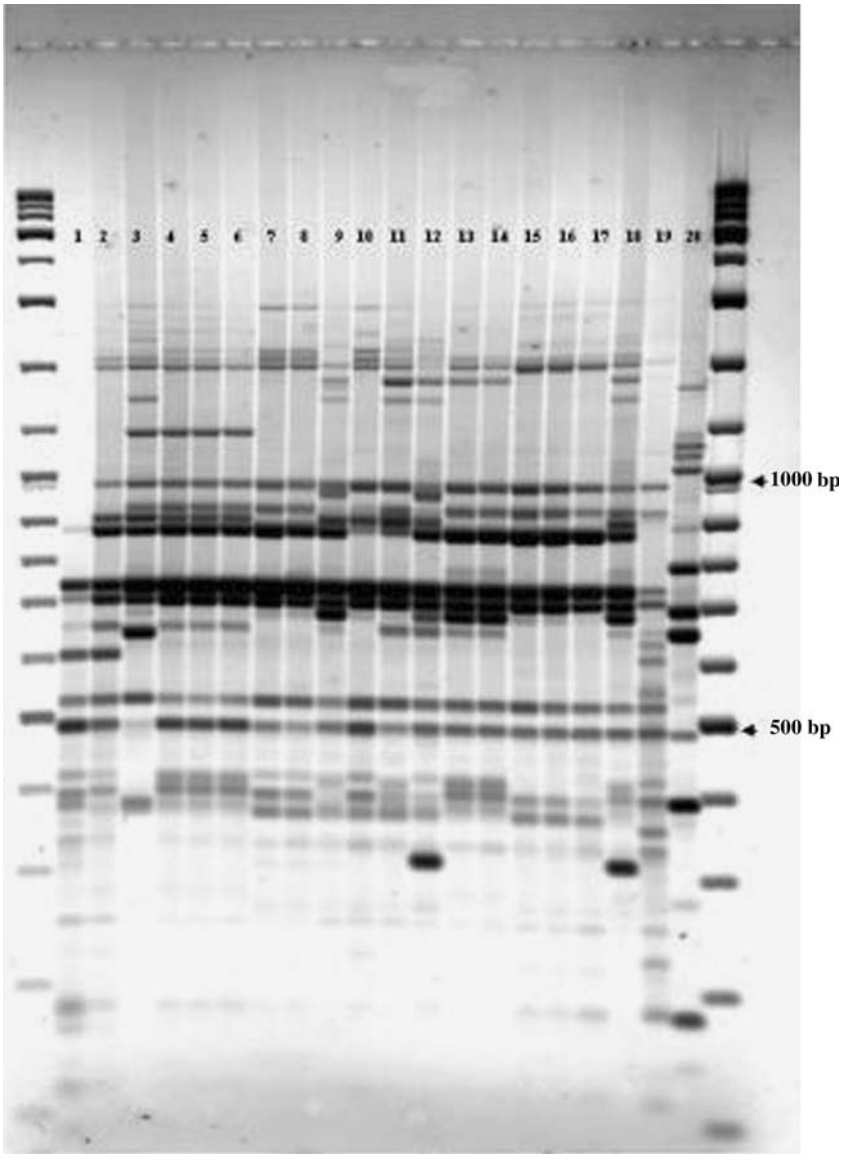

Fig. 3 REMAP analysis of apple and Japanese quince, using apple TRIM primer K008 + ISSR 83003. DNA-samples: *1* Atlas; *2* Atlas, red; *3* Antonovka; *4* Melba; *5* Melba, red Plats; *6* Melba, red Pate; *7* Bergius; *8* Sävstaholm; *9* White Astrakan; *10* Red Astrakan; *11* Gyllenkrok's Astrakan; *12* Big transparent Astrakan'; *13* Åkerö, Tarko; *14* Åkerö, Rajalin; *15* Yellow Cinnamon apple; *16* Red Cinnamon apple; *17* Brown Cinnamon apple; *18* Transparente Blanche'; *19* Golden Delicious; *20* *Chaenomeles japonica*

Table 3 REMAP analysis of 19 apple cultivars and one Japanese quince using TRIM primers in combination with SSR primers

Primer combination	Total number of clearly detectable bands	Bands polymorphic in *M. domestica*	Bands monomorphic in *M. domestica*	Bands specific to *C. japonica*	Effective multiplex× ratio in *M. domestica*
K001 + 3003	15	8	2	5	3.2
K007 + 8565	7	4	1	2	2.5
K008 + 83003	27	15	6	6	6.3

difference in one of the alleles produced by primer pair CH02c11. This difference was stably repeatable, even using two different polymerase enzymes. Comparisons of marker indices and genetic variation values calculated from the IRAP, REMAP, and STMS analyses are presented in Table. 4 and 5.

Genetic relationships

Genetic similarities between pairs of apple cultivars were generally comparable between the marker methods; however, for 147 out of 171 pairwise comparisons of IRAP with STMS data, and in 150 out of 171 comparisons of combined IRAP and REMAP data, retrotransposon markers gave higher similarity values than STMS markers (Table 6). These differences were found both in comparisons of very closely related cultivars, such as the Astrakan group, as well as for more distantly related ones, including Golden Delicious and Antonovka. The average similarity calculated from the IRAP data alone was 73.5, from combined IRAP and REMAP data 72.9, and from the STMS data 67.5. The correlation between the combined IRAP and REMAP data and STMS data similarity matrices was 0.775. In pair-wise comparisons of the same two datasets ($n = 171$), the *t*-test gave a statistic of 19.3, the chi-square 12.09, and the Wilcoxon signed–ranks test a value of 128, all indicating that there is no statistically significant difference between these two similarity matrixes.

Cluster analysis yielded similar trees from the combined retrotransposon data and from the STMS data (Figs. 4, 5). *Chaenomeles* was distinctly separated from all *Malus* samples. The apples formed two clades: (1) North American cultivars, Atlas and Melba and their sports, together with the three Cinnamon apple sports as one group; (2) European cultivars Sävstaholm and its sport Bergius, two Åkerö sports, four Astrakan cultivars and Transparente Blanche. The placement of Antonovka and Golden Delicious depended on the marker method. In the SMTS-based tree, Antonovka was grouped together with the North American cultivars, whereas in the IRAP–REMAP tree it was left outside of both groups. Golden Delicious, on the other hand, was placed in the North American group by the IRAP–REMAP data and completely alone in its own group by the STMS data.

Discussion

A new group of retrotransposons in apple

Three full-length retrotransposons were cloned in this study. A few other retrotransposons and DNA transposons have been characterized in apple (Shi et al. 2002; Tignon et al. 2001; Wakasa et al. 2003). None of these elements is related to the retrotransposons found in this study. Our *Malus* sequences are structurally similar to the TRIM retrotransposons reported by Witte et al.

Table 4 Comparison of indices calculated form IRAP, REMAP, and STMS apple datasets

PCR methodology	IRAP	REMAP	STMS
Number of primer set/primers used	9	3	9
Number of bands scored	74	36	67
Number (average) of polymorphic bands per set/primer	6.8	9.0	7.3
Total number of polymorphic bands	61 (82%)	27 (75%)	67 (100%)
Effective multiplex ratio	3.6	4.0	2.1

Table 5 Genetic variation values for IRAP, REMAP, and STMS apple datasets

	Expected heterozygosity (H_e)	Polymorphic index content (PIC)	Marker index (MI)	Range in genetic similarity (excluding sports) (%)
IRAP		0.25	0.9	56.8–81.1
IRAP + REMAP		0.30	1.1	49.1–80.9
SMTS	0.78		1.6	55.2–88.1

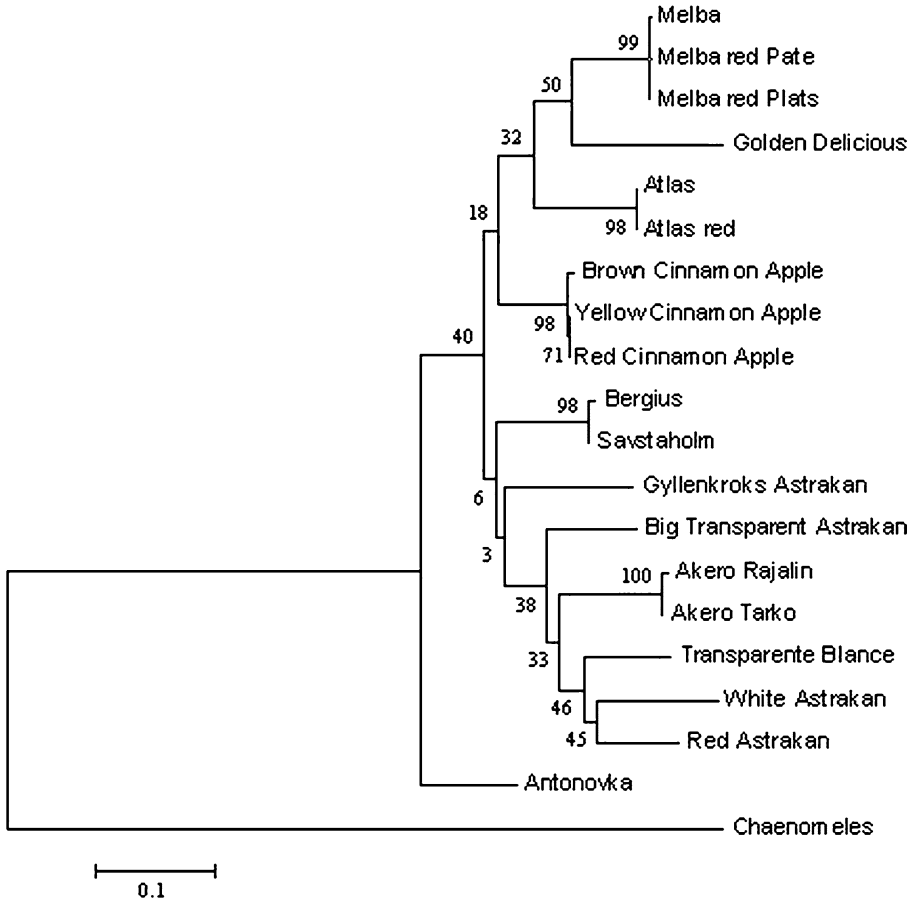

Fig. 4 PAUP dengrogram of 19 apple cultivars and one Japanese quince generated by the neighbour-joining method from the IRAP–REMAP similarity matrix shown in Table 6

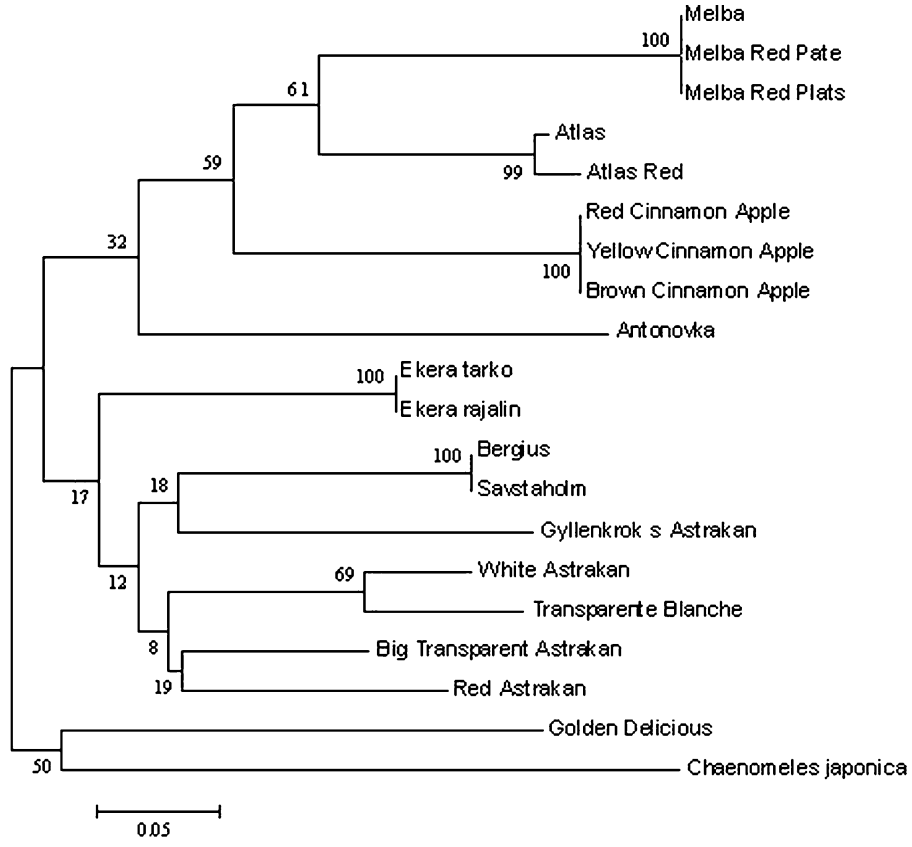

Fig. 5 PAUP dengrogram of 19 apple cultivars and one Japanese quince generated by the neighbour-joining method from STMS similarity matrix shown in Table 6

Table 6 Similarity matrix of 19 apple cultivars based on combined IRAP–REMAP and STMS data, respectively

IRAP–REMAP/STMS	Atlas red	Antonovka	Melba	Melba red Plats	Melba red Pate	Bergius	Savstaholm	White Astrakan	Gyllenkroks Astrakan
Atlas	100.0/97.0	61.8/61.2	79.1/76.1	79.1/76.1	79.1/76.1	72.7/70.1	72.7/70.1	64.5/59.7	74.5/67.2
Atlas red		61.8/61.2	79.1/73.1	79.1/73.1	79.1/73.1	72.7/67.2	72.7/67.2	64.5/59.7	74.5/64.2
Antonovka			66.4/61.2	66.4/61.2	66.4/61.2	74.5/55.2	74.5/55.2	64.5/65.7	72.7/64.2
Melba				100.0/100.0	100.0/100.0	71.8/61.2	71.8/61.2	60.0/53.7	71.8/61.2
Melba red Plats					100.0/100.0	71.8/61.2	71.8/61.2	60.0/53.7	71.8/61.2
Melba red Pate						71.8/61.2	71.8/61.2	60.0/53.7	71.8/61.2
Bergius							100.0/100.0	75.5/74.6	78.2/73.1
Savstaholm								75.5/74.6	78.2/73.1
White Astrakan									68.2/68.7
Gyllenkroks Astrakan									
Big Transparent Astrakan									
Red Astrakan									
Akero Rajalin									
Akero Tarko									
Yellow Cinnamon Apple									
Red Cinnamon Apple									
Brown Cinnamon Apple									
Transparente Blance									
Golden Delicious									

(2001), and we therefore classify them as TRIMs. However, the TRIM sequences cloned from *Malus* in this study form a specific but more widespread subgroup that will be more fully described elsewhere (manuscript submitted).

Apple TRIM elements are useful for assessing genetic identities and relationships

All the primers that were designed to match *Malus* TRIM elements produced multiple bands from apple genomic DNA even when used individually in IRAP. The IRAP with one primer requires that elements of the same family be in a head-to-head or tail-to-tail orientation sufficiently close to produce amplification products. Therefore, the data suggest either that the copy number of these elements is high, or that they are clustered in the genome, or both. Preliminary in situ hybridizations (data not shown here) demonstrate that the TRIM elements are evenly distributed in all the apple chromosomes. Such characteristics make them very promising as sources of molecular markers.

The utility of the TRIMs was shown in the analysis of the 19 apple cultivars used in this study. The DNA fingerprints produced with either only the TRIM primers (IRAP method, Fig. 2) or in combination with selected anchored microsatellite primers (REMAP, Fig. 3) resulted in polymorphic patterns that are able to distinguish even closely related cultivars. For example, White Astrakan and Big Transparent Astrakan, which were reported as identical in a recent study with RAPD markers (Garkava-Gustavsson and Nybom 2003), turned out to be clearly different in our IRAP and REMAP analyses.

The genetic relationships derived from the *Malus* TRIM data are in good accordance with those based on microsatellite polymorphisms. Similarity (*Sim*) values from IRAP and REMAP analyses are generally somewhat higher. This is likely due to our scoring of not only polymorphic but also clearly detectable monomorphic bands for the analysis of IRAP and REMAP profiles. The STMS primers, however, selected for this study were previously known to be highly polymorphic. Indeed, in our study material, excluding sports, there were no monomorphic STMS bands at all. Scoring of monomorphic bands is in keeping with UPOV rules for determining genetic relatedness for crops such as lettuce (http://www.worldseed.org). It has been applied, as well, in establishing genetic distance in rose, like apple, a member of the Rosaceae (Vosman et al. 2004). Hence,

Big Transparent Astrakan	Red Astrakan	Akero Rajalin	Akero Tarko	Yellow Cinnamon apple	Red Cinnamon apple	Brown Cinnamon apple	Transparente Blance	Golden Delicious
67.3//64.2	72.7/65.7	69.1/64.2	69.1/64.2	76.4/74.6	76.4/74.6	76.4/74.6	72.7/62.7	59.1/55.2
67.3/61.2	72.7/65.7	69.1/64.2	69.1/64.2	76.4/74.6	76.4/74.6	76.4/74.6	72.7/62.7	59.1/55.2
76.4/61.2	63.6/56.7	65.5/64.2	65.5/64.2	74.5/65.7	74.5/65.7	74.5/65.7	67.3/71.6	49.1/55.2
68.2/58.2	66.4/56.7	71.8/67.2	71.8/67.2	79.1/65.7	79.1/65.7	79.1/65.7	70.0/59.7	66.4/55.2
68.2/58.2	66.4/56.7	71.8/67.2	71.8/67.2	79.1/65.7	79.1/65.7	79.1/65.7	70.0/59.7	66.4/55.2
68.2/58.2	66.4/56.7	71.8/67.2	71.8/67.2	79.1//65.7	79.1/65.7	79.1/65.7	70.0/59.7	66.4/55.2
76.4/79.1	69.1/74.6	78.2/73.1	78.2/73.1	80.0/62.7	80.0/62.7	80.0/62.7	74.5/65.7	59.1/58.2
76.4/79.1	69.1/74.6	78.2/73.1	78.2/73.1	80.0/62.7	80.0/62.7	80.0/62.7	74.5/65.7	59.1/58.2
75.5/83.6	80.9/79.1	73.6/68.7	73.6/68.7	68.2/64.2	68.2/64.2	68.2/64.2	75.5/88.1	53.6/62.7
78.2/73.1	69.1/68.7	69.1/70.1	69.1/70.1	72.7/56.7	72.7/56.7	72.7/56.7	69.1/71.6	50.0/64.2
	76.4/80.6	78.2/76.1	78.2/76.1	76.4/62.7	76.4/62.7	76.4/62.7	72.7/71.6	52.7/64.2
		74.5/74.6	74.5/74.6	72.7/67.2	72.7/67.2	72.7/67.2	78.2/70.1	52.7/65.7
			100.0/100.0	74.5/65.7	74.5/65.7	74.5/65.7	78.2/71.6	54.5/64.2
				74.5/65.7	74.5/65.7	74.5/65.7	78.2/71.6	54.5/64.2
					100.0/100.0	100.0/100.0	74.5/64.2	64.5/56.7
						100.0/100.0	74.5/64.2	64.5/56.7
							74.5/64.2	64.5/56.7
								53.6/56.7

we believe that the IRAP and REMAP data may be less biased in their representation of genetic relatedness.

IRAP and REMAP analyses with *Malus* TRIMs do not detect sport mutations

DNA transposon insertions have been shown to cause mutations in the apple genome (Sunako et al. 1999; Yao et al. 2001). Although segments of a retroelement were earlier identified in apple, no polymorphism has been attributed to it (Tignon et al. 2001). The activity, however, of high-copy number retrotransposons in somatic tissues (Vicient et al. 2001) suggested that retrotransposons could provide a useful marker system for vegetatively propagated crops such as apple. Indeed, they do in at least one *Citrus* species, a member of the *Rutaceae*, which is in a different phylogenetic order than for *Malus* (Bretó et al. 2001). Here, we used a new group of retrotransposons from *Malus* to try to distinguish clonal mutations, or sports.

Within each of the four pairs of sports and their original varieties, the IRAP and REMAP analyses produced identical profiles, even though one STMS primer pair detected one stably repeatable difference between Atlas and its Red sport. There can be several explanations to this result. The sport mutations may be SNPs (single nucleotide polymorphisms) or small insertions or deletions and thus not detected by IRAP or REMAP analysis. Second, the mutation may not have been associated with a member of the TRIM group we assayed, or caused by an insertion too distant from other TRIMs to produce an IRAP or REMAP band. Alternatively, an entirely different family of transposable elements may have been involved in generating the sport mutations. This was found for cultivars of another *Citrus*. *Copia*-like retrotransposons that revealed high rates of polymorphism in cultivars with a common vegetative origin, but primers anchored in *gypsy*-like retroelements did not (Bernet et al. 2004). Lastly, if nested insertions of retrotransposons are as common in apple as in the cereals (Shirasu et al.2000; Ma et al. 2004), the IRAP and REMAP primers may not detect new insertions. This is because the primers are located at the edge of the retrotransposon LTR and oriented away from the element. The amplification efficiency of IRAP in apple lends some support for this last suggestion. Primers can, however, be designed to detect specifically nested insertions. These considerations illustrate a special property of transposable element markers: it is possible to choose a system that resolves accessions at the phylogenetic level of choice. The system applied here would indicate

that sports are essentially derived from their background cultivars.

Conclusions

We have isolated a new group of transposable elements from apple, and shown that these TRIM retrotransposon elements are informative as molecular markers. Their informativeness is consistent with the transpositional activity of the TRIM elements in spite of their not encoding the proteins required for transposition. Applied in IRAP or REMAP analyses, they accurately detect genetic recombination and predict the genetic relationships expected. The IRAP and REMAP methods proved to be relatively robust and repeatable even in different laboratories. The TRIMs did not distinguish sport mutations in the present study, but could be useful even for that purpose with modifications to the protocols. The very recent work by Venturi et al. (2005), published subsequent to the acceptance of our work here, is consistent with our findings. Although several novel marker bands S-SAP, generated with the retrotransposon display technique, appeared in two sport varieties, the reliance of this method on restriction digestion and PCR means that the novel bands, as with our STMS primer pair, are not necessarily derived from a new retrotransposon insertion. The best method for this purpose remains RBIP (Flavell et al. 1998), which uses primers flanking the insertion site to unequivocally establish the presence or absence of an element at a particular locus.

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