Euphytica **122:** 81–89, 2001.

Molecular characterisation of cultivars of apple (*Malus* × *domestica* Borkh.) using microsatellite (SSR and ISSR) markers

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Received 22 February 2000; accepted 5 December 2000

Key words: apple, cultivar identification, Inter-Simple Sequence Repeats, germplasm, marker comparison, phenetic similarity, Simple Sequence Repeats

Summary

In this study, two microsatellite-based methodologies (SSR and ISSR) were evaluated for potential use in fingerprinting and determination of the similarity degree between 41 commercial cultivars of apple previously characterised using RAPD and AFLP markers. A total of 13 SSR primer sets was used and 84 polymorphic alleles were amplified. Seven ISSR primers yielded a total of 252 bands, of which 176 (89.1%) were polymorphic. Except for cultivars obtained from somatic mutations, all cultivars were easily distinguishable employing both methods. The similarity coefficient between cultivars ranged from 0.20 to 0.87 for SSR analysis and from 0.71 to 0.92 using the ISSR methodology. Dendrograms constructed using UPGMA cluster analysis revealed a phenetic classification that emphasises the existence of a narrow genetic base among the cultivars used, with the Portuguese cultivars revealing higher diversity. This study indicates that the results obtained based on the RAPD, AFLP, SSR and ISSR techniques are significantly correlated. The marker index, based on the effective multiplex ratio and expected heterozygosity, was calculated for both analyses (MI = 1.7 for SSR and MI = 8.4 for ISSR assays) and the results obtained were directly compared with previous RAPD and AFLP data from the same material. The SSR and ISSR markers were found to be useful for cultivar identification and assessment of phenetic relationships, revealing advantages, due to higher reproducibility, over other commonly employed PCR-based methods, namely RAPD and AFLP.

Introduction

Apple (*Malus* × *domestica* Borkh.) is economically the most important fruit tree crop of the temperate zones, and a high number of commercial cultivars are available, as the result of open-pollinated seedlings, controlled crosses in breeding programs, and exploitation of naturally or induced somatic mutations in adapted cultivars. An accurate characterisation of the existing cultivars is essential to successful breeding programs, patent protection and nursery control. Molecular markers have been replacing or complementing traditional morphological and agronomic characterisation, since they are virtually unlimited, cover all the genome, are not influenced by the environment and, particularly in the case of fruit trees with long juvenile period, can be less time consuming for the characterisation of new hybrids.

Several molecular markers studies on apple have been published using techniques such as RFLP (Restriction Fragment Length Polymorphism) (Nybom & Schaal, 1990; Watillon et al., 1991), RAPD (Random Amplified Polymorphic DNA) (Koller et al., 1993; Mulcahy et al., 1993; Harada et al., 1993; Dunemann et al., 1994; Gardiner et al., 1996; Goulão et al., 2001), AFLP (Amplified Fragment Length Polymorphism) (Goulão et al., 2001) and SSR (Simple Sequence Repeats) (Guilford et al., 1997; Gianfranceschi et al., 1998).

The increasing development and generalised use of a large number of methodologies during the last years, requires comparative studies in order to choose

the best DNA marker technology to be used in fingerprinting and in diversity studies, considering reproducibility, costs, sensibility and level of polymorphisms detection. Molecular technique comparisons have become important because, depending on the objective of the study, one technique can be more appropriate than another, as well as different techniques being informative at different taxonomic levels. However, the congruence among the different molecular techniques began to be discussed with opposite results among authors. Several works report comparable results among different markers (e.g. de Oliveira et al., 1996; Sensi et al., 1996; Fuentes et al., 1999) while other show considerable differences (e.g. Sánchez de la Hoz et al., 1996; Lanham & Brennan, 1999; Bohn et al., 1999).

A phenetic characterisation of forty-one apple cultivars, comparing RAPD and AFLP markers was reported previously (Goulão et al., 2001). In that study the results obtained were positively correlated, and only small differences were observed between the UPGMA dendrograms originated from both methods. However, these two techniques show some disadvantages regarding reproducibility. In fact, the use of short primers and low annealing temperatures makes RAPD markers extremely sensitive to the reaction conditions and therefore irreproducible among different laboratories. With the AFLP technique, although extremely reproducible in the amplification steps, when most of the widely used DNA extraction protocols are employed, incomplete restriction cuts can occur, resulting in irreproducible band patterns. This fact is particularly important when working with woody species, due to its generally high content in polyphenols, and reinforces the need to develop more reliable markers.

Microsatellites are regions of short, tandemly repeated DNA sequences of 1 to 6 base pairs, ubiquitous in eukaryotic genomes. Two different marker strategies have been used based on microsatellites: SSR (simple sequence repeats) and ISSR (inter-simple sequence repeats). SSR are highly reproducible codominant markers, in which a single pair of PCR primers that flanks the repeated sequences produces polymorphic patterns among alleles, depending on the number of repeat units. Although these markers are generally highly polymorphic, the initial cost of developing them is relatively high. However, once these primers are determined and its sequences published, they can be shared among groups working with material from the same species or related ones, and the method becomes fast and readily employed. Primer pairs designed for SSRs in apple have been recently published (Guilford et al., 1997; Gianfranceschi et al., 1998) and can be used in further studies.

ISSR (Zietkiewicz et al., 1994) is a different microsatellite-based method, which does not need prior knowledge of the genome, cloning or primer design. While the SSR protocol relies on the amplification of the repeated region using two locus specific primers, in ISSR, a single primer composed of a microsatellite sequence anchored at the 3′ or 5′ end by 2 to 4 arbitrary, often degenerate nucleotides, is used to amplify the DNA between two opposed microsatellites of the same type. Allelic polymorphisms occur whenever one genome is missing the sequence repeated or has a deletion or insertion that modifies the distance between repeats. For 5′ anchored primers, polymorphisms also occur due to differences in the length of the microsatellite. The sequences of repeats and anchored nucleotides are randomly selected. Although ISSR are dominant markers, they have the advantage of analysing multiple loci in a single reaction.

This study has the purpose of investigating possible advantages of the use of microsatellite-based methods, SSR and ISSR, for identification and estimation of phenetic similarities among a set of forty-one apple commercial cultivars, compared with RAPD and AFLP procedures, previously described for the same material (Goulão et al., 2001).

Materials and methods

Plant material and DNA isolation

In this work, the same forty-one cultivars used before for RAPD and AFLP analysis (Goulão et al., 2001) were studied (see dendrograms of Figure 2). The same DNA preparations for these materials, extracted as described by Goulão et al. (2001) were used in microsatellite analyses.

SSR analysis

The thirteen SSR primer sets used in this study are listed in Table 1, and were selected from Guilford et al. (1997) and Gianfranceschi et al. (1998) papers according to the highest number of alleles detected. In each case, reaction mixes and PCR amplification profiles were performed as described by the authors. Following amplification, the fragments were mixed with an equal volume of formamide tracking dye (98% formamide, 10 mM EDTA pH 8.0, 0.05% bromophenol

Table 1. Primers used for SSR and ISSR analysis and number of amplified produts scored per primer set or primer. For SSR primer sequences see [a] Gianfranscheschi et al. (1998) and [b] Guilford et al. (1997)

SSR primer set	No. of alleles detected	SSR primer set	No. of alleles detected
CH02D12[a]	9	CH01H10[a]	5
CH01F02[a]	10	CH01H01[a]	6
CH02B10[a]	7	04H11[b]	5
CH01E12[a]	4	05G8[b]	6
CH02B03b[a]	6	02B1[b]	6
CH02D11[a]	6	01A6[b]	6
CH02C06[a]	8		
ISSR primer	Number of scored bands	Number of polymorphic bands	
(AG)8YT	44	25	
(AGC)4YT	40	24	
(CA)8R	33	22	
(GA)8YG	31	20	
DBD(CA)7	32	27	
VHV(GT)7	32	26	
HVH(TG)7	40	32	

blue 0.05% xylene cyanol). The resulting mixture was heated for 3 min at 94 °C and immediately cooled on ice. Each sample was loaded on pre-warmed denaturing 6% polyacrylamide gels with 7.5M urea, and electrophoresed in 1XTBE buffer, at 50 W constant power until the dye front reached 2/3 of the gels, and silver stained as described in Bassam et al. (1991).

ISSR analysis

The seven ISSR primers used are listed in Table 1. Amplification reactions were carried out in volumes of 20 μl containing 30 ng template DNA, 1 unit of *Taq* DNA polymerase (Pharmacia, Biotech), 0.25 mM each dNTP (Gibco BRL) and 1 μM primer (Gibco BRL), in 1 × reaction buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris HCl pH 9.0). PCR reactions were performed in a Biometra thermal cycler under the following conditions: 4 min at 94 °C for initial denaturation, 27 cycles of 30 sec at 94 °C (denaturation), 45 sec at 52 °C (annealing) and 120 sec at 72 °C (extension), followed by 7 min at 72 °C for final extension of the single strands. ISSR amplified fragments were mixed with equal volume of dye solution (6 M urea, 0.05% bromophenol blue, 0.05% xylene cyanol), loaded onto pre-warmed non-denaturing 6% polyacrilamide gels with 3M urea, and electrophoresed in 1 × TBE buffer, at 50 W constant power until the dye front reached 2/3 of the gels. The gels were silver stained as described in Bassam et al. (1991).

Data analysis

Clear and well-marked bands were coded in a binary form by '0' and '1', for absence or presence in each cultivar, respectively. For ISSR analysis, only fragments between 100bp and about 2000bp were scored, although a very high number of larger fragments existed. However, the proximity between these markers made them very difficult to score without errors. Even within the scorable range, in regions with a high concentration of bands, the entire row was discarded whenever overlapping or unclear bands occurred. SSR alleles were identified by comparing the patterns from all cultivars with internal length standards: 'Golden Delicious', 'Red Delicious', 'Royal Gala' and 'Granny Smith (cultivars also analysed by Guilford et al., 1997) and 'Florina' (or 'Querina'), 'Golden Delicious', 'Jonagold', 'Red Delicious' and 'Starking' (cultivars also analysed by Gianfranceschi et al., 1998), as suggested by the former authors.

The information content of each marker system was determined according to the indices of Powell et al. (1996): Effective Multiplex Ratio (number of polymorphic products from a single amplification reaction), Expected Heterozygosity ($H = 1\text{-}\Sigma p_i^2$, where p_i is the allele frequency for the i^{th} allele) and Marker Index (the product of Effective Multiplex Ratio and Expected Heterozygosity). These calculations were performed for ISSR and SSR data obtained in this work, and for RAPD and AFLP data obtained previously (Goulão et al., 2001).

Similarity values were estimated based on the fraction of bands common to each pair of cultivars, according to Nei & Li's (1979) coefficient, and cluster analysis was performed to construct dendrograms, using the unweighted pair-group method with arithmetic averages (UPGMA) from the similarity data matrices. The Mantel matrix correspondence test (Mantel, 1967) was used to compare cophenetic with similarity matrices and to estimate the degree of congruence in the results of phenetic relationships for each marker type. The Numerical Taxonomy and Multivariate Analysis System program package for personal computer (NTSYS-pc version 1.8; Rohlf, 1993) was used for this statistical analysis of data. In or-

Table 2. Comparison of the results obtained with the SSR and ISSR methodologies

PCR methodology	SSR	ISSR
Number of primer set/primer used	13	7
Total number of bands scored	84	252
Number of bands per primer set/primer	6.5 ± 1.7	36.0 ± 5.2
Number of polymorphic bands	84 (100%)	176 (89.1%)
Range in DNA diversity (% similarity)[a]	0.20–0.87	0.71–0.92

[a] Excluding cultivars obtained by mutations.

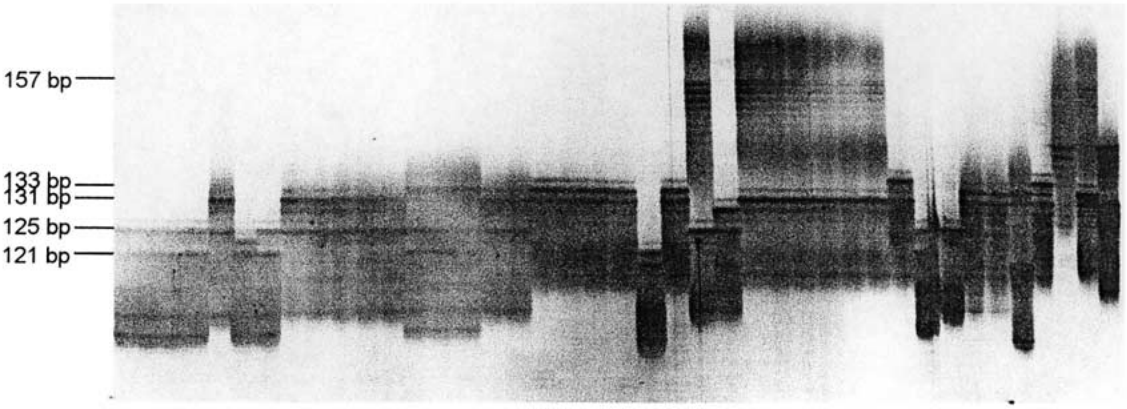

Figure 1. Fingerprints of the forty-one apple cultivars using SSR primer pair CH02B10. The order of the samples corresponds to the order listed in Goulão et al., 2001.

der to validate the structure of relationships amongst cultivars (OTUs) a bootstrapping analysis was carried out considering 100 bootstrapped data sets, using the TREECON for Windows (version 1.3b) software (Van der Peer & De Wachter, 1994). Distance estimation and clustering methods were the same as described previously.

Results

A total of 84 alleles were amplified using the thirteen SSR primer sets listed in Table 1. All alleles detected were polymorphic among the cultivars studied. The number of alleles detected by a single primer set ranged from 4 to 10, with an average of 6.5 (Table 2). In addition to the respective microsatellite sequences, in almost every reaction, slippage of *Taq* polymerase resulted in stuttered bands near the specific product and, in most cases, considerable non-specific amplification was detected on the gels (Figure 1). The allelic composition of the cultivars in common with the work reported by Guilford et al. (1997) and Gianfranceschi et al. (1998) was in accordance with our results.

Two-hundred-and-fifty-two ISSR bands were scored using the seven primers listed in Table 1. A high multiplex ratio (22.8) was obtained, as the average number of amplified bands per primer was 36, ranging from 31 to 44. One hundred and seventy six amplification products were polymorphic among the cultivars, revealing a high percentage of polymorphism (89.1%) (Table 2).

Similarity matrices were calculated independently from SSR and ISSR data and the UPGMA-based dendrograms obtained are shown in Figure 2. The cophenetic matrices computed from tree matrices and compared with the original similarity data showed significant correlations of 88.6% (p =1.00; t = 20.1) and 94.5% (p =1.00; t = 18.8), revealing, in both cases, a good fit of the cluster analyses performed.

The phenetic classification obtained and illustrated in the dendrograms was very similar for both methods, and showed good accordance with the classification previously established using RAPD and AFLP markers (Goulão et al., 2001), separating the cultivars according to the known information about geographical and parental origins. Therefore, for the discussion

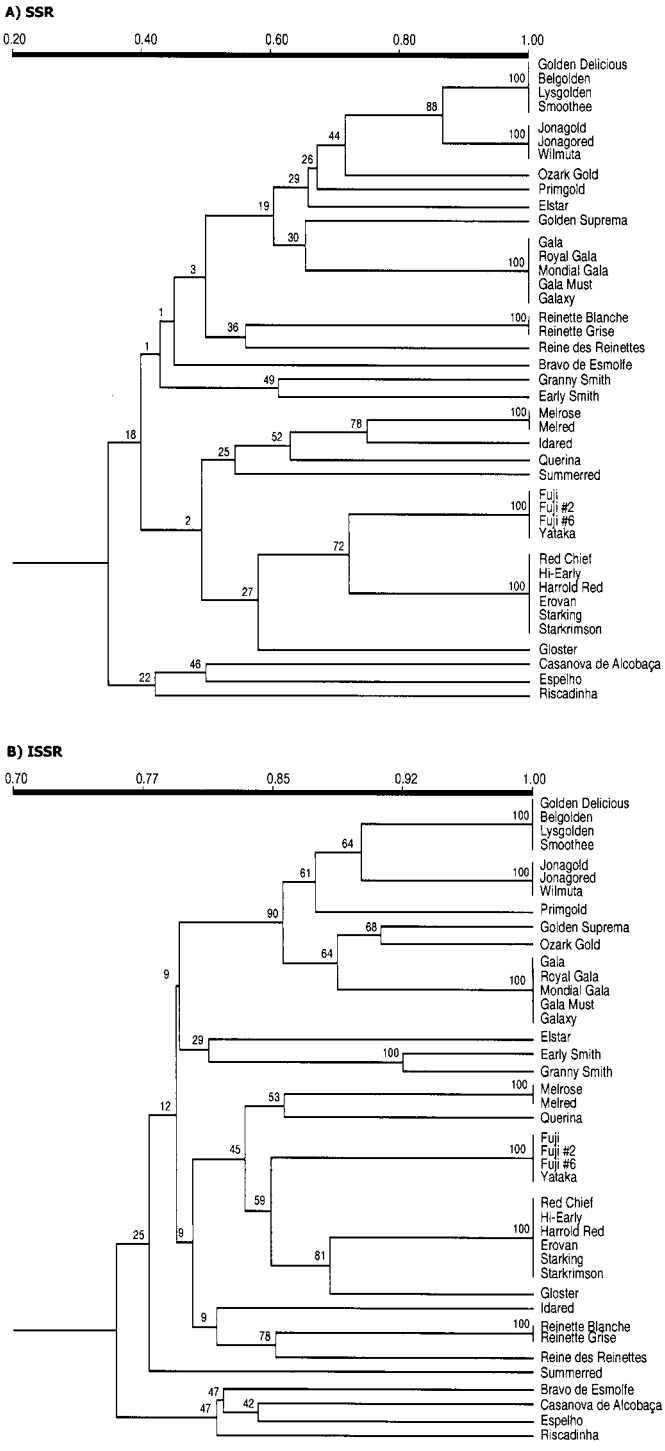

Figure 2. UPGMA dendrograms based on Nei & Li's (1979) similarity index, representing phenetic relationships among the forty-one apple cultivars analysed by (A) SSR and (B) ISSR markers. Numbers indicate the percentage of bootstraps in which the branch was observed.

Table 3. Comparison of the marker information obtained with RAPD, AFLP, SSR and ISSR methodologies in apple

	Effective multiplex ratio	Expected heterozygosity	Marker index	Reference
RAPD	3.4	0.59	2.0	Goulão et al. (2001)
AFLP	16.0	0.58	9.3	Goulão et al. (2001)
SSR	1.9	0.86	1.7	This work
ISSR	12.0	0.70	8.4	This work

Table 4. Correlations among Nei & Li's (1979) similarity matrices based on RAPD, AFLP, SSR and ISSR data. A significant correlation (p =1.00) was obtained among matrices generated by all the four assays

	Similarity based on			
	RAPD[a]	AFLP[a]	SSR[b]	ISSR[b]
RAPD	–			
AFLP	r = 0.737; t = 6.52	–		
SSR	r = 0.601; t = 6.37	r = 0.615; t = 6.48	–	
ISSR	r = 0.735; t = 8.92	r = 0.669; t = 8.08	r = 0.558; t = 7.40	–

[a] Goulão et al. (2001) unpublished data; [b] This work.

of parentage see Goulão et al. (2001). Again, the four Portuguese cultivars ('Bravo de Esmolfe', 'Casanova de Alcobaça', 'Riscadinha' and 'Espelho'), which represent a material geographically distinct from and not included in international breeding programs, were clearly separated. In the SSR dendrogram, however, 'Bravo de Esmolfe', did not appeared in the same cluster that groups the other Portuguese cultivars. However, bootstrapping analysis revealed that the position of 'Bravo de Esmolfe' in the dendrogram is not supported. Bootstrapping analysis suggest that this result is probably due more to the lower number of bands scored for SSR markers, leading to a bias of the graphical representation, rather than to differences in the way that polymorphisms were detected.

Nei & Li's (1979) similarity coefficient between paired cultivars of the 41 apple cultivars ranged from 0.20 using SSRs (among 'Golden Suprema' and 'Riscadinha'), or 0.71 using ISSRs (among 'Golden Suprema' and 'Bravo de Esmolfe') to 1, for mutation derived clones. When excluding mutations, the highest similarity coefficient was found among 'Golden Delicious' and 'Jonagold' (s = 0.87) for SSR, and among 'Early Smith' and 'Granny Smith' (s = 0.92) for ISSR analysis, respectively.

To study the potential of each marker type to detect polymorphism among the apple cultivars, the effective multiplex ratio, expected heterozygosity and marker index are listed in Table 3. RAPD and AFLP results were also included, allowing a comparison of the PCR-based molecular marker systems more currently used. These data are the result of work reported previously (Goulão et al., 2001) although these indexes were unpublished. The average expected heterozygosity values were higher for microsatellite-based markers than for RAPD and AFLP. Marker Index values were low for RAPD (2.0) and SSR (1.7), but high for AFLP (9.3) and ISSR (8.4). AFLP was the most informative marker, mainly due to its higher effective multiplex ratio. It should be noticed that these results are dependent upon the set of cultivars used and should not be used as absolute values, although they allow comparison of methods.

The correlation of similarity matrices obtained with the different markers is shown in Table 4. The results suggest that all the techniques are related. Only one cultivar from somatic mutation groups was included in these calculations in order to exclude overweight of the markers detected in these groups.

Reproducibility tests were performed, by amplification of two samples at different times and identical patterns were always generated for both techniques. The identical patterns obtained among all cultivars derived from somatic mutation of the same cultivar also

confirm the high degree of reproducibility achieved with the use of microsatellite-based methods.

Discussion

Assessment of the genetic variability within a cultivated crop has important consequences in plant breeding and the conservation of genetic resources. It is particularly useful in the characterisation of individual accessions and cultivars, in detecting duplications of genetic material in germplasm collections, and as a general guide in the choice of parents for breeding hybrids (Dávila et al., 1998). Several molecular markers have been widely used for genetic variability assessment and cultivar identification in a large number of species. In this work we report the use of microsatellite-based markers, SSR and ISSR, for molecular characterisation of apple cultivars. For comparative purposes, the data here reported were compared directly to the RAPD and AFLP data of Goulão et al. (2001), in order to investigate marker congruence and to discuss advantages and drawbacks of each of the four more widely used PCR-based markers.

Our study demonstrates that all methods are positively correlated and only slight differences are detected in some of the closer branches of the dendrograms constructed by cluster analysis. Although still significantly correlated, the lower correlation values (r) were obtained among SSR similarity values and the other markers studied. This fact can be attributed more to the significantly lower number of alleles scored in this technique than to differences in the way that polymorphisms are detected. With the 84 SSR alleles analysed, in the dendrogram obtained some clusters not well supported by bootstrapping analysis, particularly in the cases of 'Bravo de Esmolfe', the group formed by the three Reinette cultivars and 'Granny Smith' and 'Early Smith'. These were the cultivars that were not always clustered in the same branches, when a comparison is made with the other analyses. However, most of the clusters revealed to be stable enough. In a previous work using RAPDs, below 100–150 bands, the dendrograms resulting from cluster analysis were completely rearranged (Fanizza et al., 1999). SSR markers, due to its co-dominant nature do not amplify non-parental bands, so a lower number of bands could be enough for diversity studies comparing with RAPDs. Furthermore, the identity of SSR alleles is a guarantee since same size products have the same sequence. SSR analysis showed expected levels of heterozygosity, since apple is outbreeding and strongly heterozygous. This aspect is revealed by the SSR multiplex ratio observed (1.93), which reveals that two alleles were amplified in almost all of the diploid cultivars. Only three triploid cultivars were included in this study: 'Jonagold', 'Reinnete Grise' and 'Espelho' and, in these cases, it was possible to detect three alleles for some primer pairs (see Figure 1) (sports were not considered for the calculation of this index).

Although extremely reproducible, due to 'slippage', SSR gels were difficult to score. Slippage during PCR and slow moving dinucleotide-containing strand were also previously observed (Olsen & Eckstein, 1989; Wu & Tanksley, 1993; Brady et al., 1996). According to Schlotter & Tautz (1992), variation in the copy number of the basic repeat unit, which is the basis of observed polymorphism, is possibly caused by the same polymerase slippage phenomenon during replication, combined with point mutations, unequal crossing-over, and recombination events. It has been shown that the amplification of loci containing tri-, tetra- or penta-nucleotide repeats reduce or even eliminate this 'stuttering' (Todd et al., 1990; Edwards et al., 1992; Rongwen et al., 1995). However, while di- and tri-nucleotide simple repeats seem to have a random distribution (Bell & Ecker, 1994), four-nucleotide repeats seems to be distributed in preferential locations on the chromosomes (Arens et al., 1995), and thus are less suitable for diversity studies. The presence of stutter patterns on the gels can be a major drawback for locus detection and identification, employing manual or computer scoring electrophoresis gels, or employing capillary-length electrophoresis (Garland et al., 1999). Also, the presence of non-specific products was evident in most of the primer sets used. This can be a consequence of arbitrary amplification of some regions of the genome, other than the SSR, or due to non-specific annealing, as the result of bad primer design, in which considerable differences in the melting temperatures of the two primers is evident. The use of internal size standards is, therefore, desirable.

Once primer sequences flanking the SSRs are available, this technique is, per reaction, less labour and time intensive than AFLP, and comparable to ISSR. However, the higher multiplex ratio associated with the two latter analyses makes them more efficient. The RAPD methodology is easier than SSR due to the use of agarose gels and ethydium bromide staining. However, the sensitivity of the RAPD assay to the reaction conditions, leads to reproducibility problems. Furthermore, DAF (DNA Amplification Fingerprint-

ing), a variant of the RAPD technique (Caetano-Anollés et al., 1991) has been used by several authors as a way to increase the number of fragments detected (e.g. Prabhu & Gresshoff, 1994; Guohao & Prakash, 1997; Caetano-Anollés et al., 1999). This technique employs polycrylamide gel electrophoresis and silver staining and, therefore, is comparable to SSR in time and labour per analysis. In addition, for phenetic similarity calculations, RAPD may require the identification of more bands than SSR, due to its dominant character, as already discussed.

The AFLP approach was the most efficient method in detecting DNA polymorphic bands per reaction due to its higher multiplex ratio. However, the ISSR assay showed a very similar Marker Index (Table 3). The latter methodology has advantages over the former, since, as observed by others (Tohme et al., 1996; Hartl & Seefelder, 1997; Donini et al., 1997), the AFLP procedure is, in some material like apple (Goulão et al., 2001), more demanding in its template DNA quality, as a way to assure reproducibility in the restriction cut step of the protocol. The high reproducibility, simplicity and low cost of the ISSR experimental procedure, should be, therefore, considered when a large number of samples have to be processed.

Both microsatellite-based techniques, SSR and ISSR, showed higher expected heterozygosity than RAPD and AFLP (Table 3). This indicates a higher percentage of band sharing in RAPD and AFLP markers among the samples and makes the latter techniques more suitable for estimation of pedigree relationships. However, when considering dominant markers (RAPD, AFLP and ISSR), same size products on the gels do not necessarily have the same sequence. This aspect limits its use in estimation of diversity at the interspecific level.

For identification purposes (fingerprinting), microsatellites (both SSR and ISSR markers) are more appropriate since they provide a higher level of polymorphism. The results obtained in this study indicate that microsatellites provide a powerful tool for cultivar identification and diversity studies in apple, exhibiting advantages over RAPD and AFLP analyses.

If the objective is assessment of phenetic similarities, high multiplex ratio markers are the more reasonable choice. Although the highest Marker Index was obtained with AFLPs, ISSR analysis seems to be a best marker due to its simplicity and higher reproducibility in apple. High multiplex ratio gels (AFLP and ISSR) have regions with many bands in the gels, which can make difficult its use for straightforward cultivar identification by gel to gel comparisons, unless a control sample is analysed in both gels. Therefore, markers generating simple patterns as RAPDs or SSRs can be desirable. In this case, SSR is advantageous over RAPD analysis due to its higher percentage of polymorphism. Furthermore, an easy and reliable transfer of information across laboratories is possible.

As expected, all these techniques allowed an easily differentiation of all cultivars except for those obtained by somatic mutations (sports). This result is in accordance with other works in apple (Nybom, 1990; Mulcahy et al., 1993; Harada et al., 1993; Gianfranceschi et al., 1998; Pancaldi et al., 1999).

Acknowledgements

The authors gratefully acknowledge A. Silva (DRARO) for supplying most of the plant material used. This study was supported by PRAXIS XXI – project 3/3.2/Hort/2163/95.

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