ISSR Analysis of Cultivars of Pear and Suitability of Molecular Markers for Clone Discrimination

Luisa Monte-Corvo,¹ Luis Goulão,¹ and Cristina Oliveira²

Secção de Horticultura, Instituto Superior de Agronomia, 1349-017 Lisboa, Portugal

ADDITIONAL INDEX WORDS. cultivar identification, phenetic relationship, RAPD, AFLP, SSR, SAMPL

ABSTRACT. Inter-simple sequence repeat (ISSR) markers were used for cultivar identification and for determination of the phenetic relationships among 24 pear cultivars (*Pyrus communis* L.). The ability of several molecular marker systems including randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), inter-simple sequence repeats (ISSR), simple sequence repeats (SSR), and selective amplification of microsatellite polymorphic loci (SAMPL) to detect variation among clones of the most significant Portuguese cultivar, Rocha, was also investigated. Each of the eight ISSR primers tested was able to distinguish the 24 pear cultivars. The ISSR primers generated 337 markers, 79.5% of which were polymorphic. The cultivar dendrogram obtained with the ISSR marker data was very similar to that obtained with previous RAPD+AFLP analysis, confirming the genetic divergence of 'Pérola', 'Carvalho' and 'Lawson' from the other cultivars. Eight out of 15 apple [*Malus sylvestris* (L.) Mill. var *domestica* (Borkh.) Mansf.] SSR primers tested also amplified microsatellites in pear. None of the five molecular marker systems analyzed (with a total of 1082 markers) detected reproducible polymorphisms among the nine 'Rocha' clones, in spite of the presence of clear phenotypic differences.

The common pear (*Pyrus communis*) is one of the most widely cultivated temperate fruit crops in the world. More than 5000 cultivars exist today, although only a small percentage of them are cultivated commercially (Bell et al., 1996). The existence of cultivar stability in European pear production is an exceptional phenomenon in tree fruits. The main commercial cultivars of pear were derived by selection among open-pollinated seedlings, especially in France and Belgium, during the golden age of pear improvement from 1750 to 1850 (Bell et al., 1996). The need for pear germplasm collection and preservation has led to establishment of national and international programs for conservation of genetic resources. The preservation of pear germplasm in Portugal has been performed by Estação Nacional de Fruticultura Vieira de Natividade, Alcobaça. This collection includes some European and Asian cultivars, many traditional Portuguese cultivars with unknown pedigrees, and several clones of the main Portuguese cultivar, Rocha.

To be used more efficiently, germplasm collections should be evaluated and well characterized. Molecular markers have been used for studying genetic diversity, relationships, and origins of the cultivars, as well as for cultivar discrimination and fingerprinting of several fruit crops (e.g., Cervera et al., 1998; Dirlewanger et al., 1998; Fang and Roose, 1997; Gianfranceschi et al., 1998; Hokanson et al., 1998; Koller et al., 1993).

Polymerase chain reaction (PCR)-based molecular markers such as randomly amplified polymorphic DNA (RAPD) (Williams et al., 1990) and amplified fragment length polymorphisms (AFLPs) (Vos et al., 1995) have already been used for cultivar identification and taxonomic relationship studies in pears (Botta et al., 1998; Oliveira et al., 1999; Monte-Corvo et al., 2000). Microsatellites, also known as simple sequence repeats (SSR), are composed of tandemly repeated 2 to 5 base pair (bp) DNA sequences. Due to their great

informativeness, SSR markers have become a tool for establishment of unique genetic identities or fingerprints in many different plant species for example in soybean [*Glycine max* (L.) Merrill] (Rongwen et al., 1995). Development of microsatellite markers involves labor-intensive procedures including cloning, sequencing, and design of locus-specific primers. However, flanking regions are sometimes highly conserved in related species. The primer pairs designed for one species can be useful for amplification of the same DNA region in related genomes (Moore et al., 1991). SSR markers have not yet been developed for pears, but a number of SSR primers have been described for apples (*Malus sylvestris* var. *domestica*) (Gianfranceschi et al., 1998; Guilford et al., 1997; Hokanson et al., 1998).

Inter-simple sequence repeat (ISSR) amplification (Zietkiewicz et al., 1994) is another microsatellite-based technique useful for genome studies. The primers used in ISSR amplifications are based on SSR sequences (di-, tri-, tetra-, or penta-nucleotide repeats), anchored to genomic sequences flanking each side of the targeted simple sequence repeats (SSRs) (5' or 3') by using two to four arbitrary, and often degenerate, nucleotides. Unlike SSRs, this technique does not require prior sequence information and generates a high number of polymorphisms. ISSR markers are thought to be particularly useful for study of closely related individuals which exhibit low levels of polymorphism (Zietkiewicz et al., 1994) and have been applied as a very useful alternative to fingerprinting and genetic analysis in fruit crops including citrus (*Citrus* L. sp.) (Fang and Roose, 1997; Fang et al., 1998), grape (*Vitis vinifera* L.) (Moreno et al., 1998), gooseberry (*Ribes* L.) (Lanham and Brennan, 1999) and plum (*Prunus* L. sp.) (Goulão et al., 2001). Another PCR-based DNA technique, selective amplification of microsatellite polymorphic loci (SAMPL), developed by Morgante and Vogel (1994) is a high multiplex ratio marker system which combines the advantages of microsatellites and AFLP markers, and has been used recently in the analysis of conifer genomes (Paglia and Morgante, 1998).

Development of techniques for discrimination of clones, sports, and seedlings obtained by hybridization or selfing is very useful for selection and certification of plant material. Studies related to discrimination of very closely related material have been performed. While Cipriani et al. (1994), Loureiro et al. (1998), Vignani et al. (1996), and Ye et al. (1998) failed to obtain differences among clones of grape using RAPD and SSR markers, Cervera et al. (1998), Moreno et al. (1998), and Sensi et al. (1996) reported discrimination

Received for publication 28 Dec. 2000. Accepted for publication 29 May 2001. This work was supported by Fundação para a Ciência e Tecnologia (FCT), project PRAXIS XXI 3/3.2/HORT/2163/95. The authors thank Rui de Sousa (ENFVN) for providing plant material from 'Rocha' clones, Amado Silva (DRARO), Fernanda Lampreia, and António Moacho (DGPC) for supplying plant material from the rest of pear cultivars, as well as António Monteiro (ISA) for critical reading of the manuscript. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

¹Graduate student.

²Associate professor and corresponding author; e-mail: crismoniz@isa.utl.pt.

Table 1. ISSR primers used, the number of markers, and the level of polymorphism obtained.

Primer ^z	No. of scorable bands	No. of polymorphic bands	Polymorphic bands (%)
(GA) ₈ YG	63	49	77
(GA) ₈ YC	36	27	75
(AG) ₈ YC	49	36	73
(AGC) ₄ YR	38	26	68
VHV(GT) ₇	55	52	95
HVH(TG) ₇	30	24	80
DBD(AC) ₇	28	26	93
HVH(CA) ₇	38	28	74
Mean	42	34	79.5
Total	337	268	

^zB = C or G or T, D = A or G or T, H = A or C or T, V = A or C or G, R = purine, and Y = pyrimidine.

of grape clones using AFLP or ISSR assays. In other fruit crops like apple (Gianfranceschi et al. 1998; Goulão et al., 2001) or almond [*Prunus dulcis* (Mill.) D.A. Webb]. (Bartolozzi et al., 1998) distinction was also not achieved. However, in citrus (Fang and Roose, 1997) and peach [*Prunus persica* (L.) Batsch (Peach Group)] (Dirlewanger et al., 1998), cultivars obtained from somatic mutations could be distinguished with ISSR and AFLP, respectively.

'Rocha' is the most economically important Portuguese cultivar, accounting for 75% of pear production in Portugal. 'Rocha' originated from a chance seedling on a farm located in Sintra, Portugal, between 1830 and 1840 (Matta, 1928). Although this cultivar is not yet well known outside Portugal, it has some very interesting characteristics, such as long storage life and resistance to injury during transport. However, there have not been any breeding efforts to improve 'Rocha'. 'Rocha' is characterized by high within-cultivar phenotypic variability which is probably the result of accumulation of natural mutations (sports).

The following research was conducted with two objectives. The first objective was to use ISSR primers to study phenetic variation among pear cultivars and for cultivar identification, and to compare these results with RAPD and AFLP data, reported previously (Monte-Corvo et al., 2000) and the second objective was to investigate the potential of RAPD, AFLP, ISSR, SSR and SAMPL markers for detecting polymorphisms in mutant clones of the cultivar Rocha.

Materials and Methods

PLANT MATERIAL. The same 24 *Pyrus communis* cultivars studied by Monte-Corvo et al. (2000) were used for ISSR analysis. The cultivars and their immediate parents where known were 'Conference', 'William's', 'Passe Crassane', 'Beurré Hardy', 'Doyenné du Comice', 'General Leclerc', 'Beurré Bosc', 'Beurré Clairgeau', 'Souvenir du Congrè', 'Alexandrine Douillard', 'Doctor Jules Guyot', 'Delbard Première' ('Akca' x 'D. Jules Guyot'), 'Lawson', 'Clapp's Favourite' ('Flemish Beauty' x 'William's'), 'Highland' ('William's' x 'Doyenné du Comice'), 'D. Joaquina', 'Sete Cotovelos', 'Lambe-os-dedos', 'Carvalho', 'Pérola', 'Carapineira Parda', 'Rocha', 'Packham's Triumph' ('Uvedale St. Germain' x 'William's') and 'Butirra Precoce Morettini' ('Ercolini' x 'William's'). For clone discrimination, nine 'Rocha' clones with clear differences in flower morphology, fruit set, yield, fruit color or fruit size (Sousa et al., 1993) obtained from the germplasm collec-

tion of the Estação Nacional de Fruticultura Vieira de Natividade were analyzed.

DNA EXTRACTION. DNA from the different cultivars and clones was extracted from fresh young, expanded leaves using a hexadecyltrimethylammonium bromide (CTAB) protocol described previously by Oliveira et al. (1999), with additional purification according to Monte-Corvo et al. (2000). Two replicate extractions per tree were performed. For 'Rocha' clone discrimination, material from two trees, vegetatively propagated from the same source tree, was extracted for each clone.

ISSR ANALYSIS OF CULTIVARS. PCR amplifications were performed in 20 µL of reaction mixtures containing the following reagents: 30 ng of template DNA, 1 µM of primer (GIBCO BRL, Paisley, United Kingdom), 1 Unit of *Taq* DNA polymerase (Pharmacia, Biotech, Uppsala, Sweden), 0.25 mM of each dNTP (GIBCO BRL) and 1× reaction buffer supplied with the enzyme (10 mM TRIS pH 9.0, 50 mM KCl, and 1.5 mM MgCl₂). The PCR program described by Zietkiewicz et al. (1994) was used first, but amplification of DNA fragments from the pear cultivars was too weak. Some adjustments in the annealing temperature and changes in the number of amplification cycles were made to the original program to improve the results. PCR reactions were performed in a Biometra UNO II (Biometra, Göttingen, Germany) thermal cycler programmed as follows: an initial heating at 94 °C for 90 s, 30 cycles of denaturing at 94 °C for 30 s, annealing at 48 °C for 45 s, extension at 72 °C for 90 s, and a final extension of 5 min at 72 °C. The capacity of the modified program to generate reliable and reproducible patterns was confirmed.

The ISSR amplification products were mixed with equal volumes of loading dye (98% formamide, 10 mM EDTA, and 0.05% xylene cyanol), denatured at 90 °C for 3 min, immediately cooled in ice, and run for 3.5 h on 6% denaturing polyacrylamide gels with 7.5 M urea at constant 50 W power. A 100-bp ladder (Pharmacia, Biotech) was included to estimate band sizes. Bands were visualized by silver nitrate staining using the method of Bassam et al. (1991). The staining solution was used twice, without any noticeable loss of sensitivity. However, for the second time incubation with staining solution was normally longer. A total of 13 primers were tested in four cultivars, and eight, which gave amplification products on agarose gels, were chosen for further use (Table 1).

ANALYSIS OF ISSR DATA FOR CULTIVARS. For subsequent statistical analysis, clear and well-marked ISSR markers were coded in a binary form, 1 or 0, for the presence or absence in each cultivar, respectively. With the binary matrix, phenetic similarities were estimated among all possible pairs of cultivars using the Dice similarity coefficient: $S_{ij} = 2N_{ij}/(N_i + N_j)$, where N_{ij} is the number of bands in common between cultivars i and j , and N_i and N_j are the number of bands for cultivars i and j , respectively (Nei and Li, 1979). The similarities found with ISSR markers were used to cluster the genotypes and to construct a dendrogram using the unweighted pair group method with arithmetic average (UPGMA) clustering of the NTSYS-PC version 1.8 software (Rohlf, 1993).

RAPD, AFLP, ISSR, AND SAMPL ANALYSIS OF 'ROCHA' CLONES. The 25 primers used for RAPD analysis as well as the RAPD amplification procedure were the same as described by Monte-Corvo et al. (2000). Fragments were separated in 2% agarose gels, stained with ethidium bromide, visualized with ultraviolet light, and photographed. AFLP analysis was performed using the AFLP Analysis System I (Life Technologies Inc., Paisley, United Kingdom), also according to Monte-Corvo et al. (2000). In preliminary experiments, a comparison between radioactive and silver stain detection methods was performed using the amplified DNA produced by three AFLP

Table 2. List of primers belonging to the five marker systems used in the 'Rocha' clones and the number of amplified products detected.

Marker	Primer or primer combination	Total bands
RAPD	OPA-01, OPA-02, OPA-04, OPA-05, OPA-07 OPA-08, OPA-09, OPA-11, OPA-18, OPA-20 OPC-05, OPC-06, OPC-07, OPC-08, OPC-09 OPC-11, OPC-14, OPC-15, OPC-19, OPE-03 OPE-07, OPE-11, OPE-14, OPE-15, OPE-20	178
AFLP ²	E-ACA/M-CTT, E-ACA/M-CAT, E-ACA/M-CTA, E-AAG/M-CAG, E-AAG/M-CTA, E-AAG/M-CAT, E-ACT/M-CTA, E-ACT/M-CAG, E-ACT/M-CTT	407
SAMPL	ISSR (AGC) ₄ YR/M-CAG, ISSR DBD (AC) ₇ /M-CTT, ISSR (GA) ₈ YC/M-CTA, SSR CH01F02R/M-CTT, SSR GD15R/M-CAG, SSR GD15L/M-CAG, SSR CH01F02R/E-ACA, SSR CH01F02L/E-AAG, ISSR DBD (AC) ₇ /E-ACA	272
ISSR	(GA) ₈ YC, VHV(GT) ₇ , (AGC) ₄ YR, (GA) ₈ YC, HVH(TG) ₇ , (AG) ₈ YC, DBD(AC) ₇ , HVH(CA) ₇	
SSR	GD15 ^y , CH01H10 ^x , CH02B10 ^x , CH02D11 ^x , CH01F02 ^x , CH02B03b ^x , CH01E12 ^x , O2B1 ^w	42
Total		1082

²Primer *EcoRI* + 3 selective nucleotides combined with primer *MseI* + 3 selective nucleotides; E = *EcoRI* and M = *MseI*.

^yHokanson et al. (1998).

^xGianfranceschi et al. (1998).

^wGuilford et al. (1997).

primer combinations (E-ACA/M-CAT, E-ACT/M-CTT, and E-AAG/M-CAT). AFLP products from the nine AFLP primer combinations were visualized with silver staining, as described previously for the ISSR analysis of cultivars. The ISSR primers, ISSR-PCR amplification procedure, gel electrophoresis, and silver staining followed the protocols described previously for ISSR assays in pear cultivars. All primers used in these three analyses are listed in Table 2.

The same preamplification products used for AFLP analysis were used for SAMPL amplification. These two techniques differ in the primers used for selective amplification. Paglia and Morgante (1998) used primer combinations of the *MseI* adapter-based primer with two primers containing a dinucleotide microsatellite sequence (ISSR primers). We also tested the following primer combinations: *MseI* adapter-based primers and ISSR primers; *EcoRI* adapter-based primers and ISSR primers; primers flanking apple SSR and *MseI* adapter-based primers, and apple SSR primers and *EcoRI* adapter-based primers (for primer sequences see Table 2). The nine different primer combinations that were analyzed are listed in Table 2. Gel electrophoresis and silver staining were performed as in the AFLP and ISSR assays.

SSR ANALYSIS OF 'ROCHA' CLONES. Fifteen pairs of primers developed for amplification of apple microsatellites by Guilford et al. (1997), Gianfranceschi et al. (1998), and Hokanson et al. (1998) were tested in pear. The PCR amplifications were carried out in 20 µL volumes containing 30 ng of template DNA, 1 µM each of forward and reverse primers (synthesized by GIBCO BRL, according to published sequences), 1 Unit of *Taq* DNA polymerase (Pharmacia, Biotech), 0.25 mM of each dNTP (GIBCO BRL), and 1× reaction buffer supplied with the enzyme. The PCR reactions were performed in a Biometra UNO II thermal cycler using the amplification programs described by Gianfranceschi et al. (1998) or Guilford et al. (1997), according to the primers used. The eight primer combinations that amplified pear sequences are listed in Table 2.

The SSR markers were analyzed by running the PCR products in a 6% denaturing sequencing gel, with detection by silver staining as described for ISSR analysis. A 30-330 bp ladder (Gibco, BRL) was included.

MARKER SCORING. Bands produced by the five marker systems

were scored. When a polymorphic band between clones was observed, the amplification was repeated, first with the same DNA, then with DNA from a different extraction from the same tree. When the difference persisted, another amplification with DNA from different trees of the same clones was performed to verify the polymorphism.

Results and Discussion

ISSR ANALYSIS OF CULTIVARS. For cultivar discrimination, a total of 13 ISSR primers were tested; however, the primers (CA)₈R, (AG)₈YT, (TCC)₅RY, (GT)₈YC, and (AT)₈YC did not produce amplification. An example of the patterns obtained is shown in Fig. 1. Using any one of the eight primers listed in Table 1, all 24 pear cultivars could be distinguished. A total of 337 well-defined and scorable markers were obtained, 79.5% of which were polymorphic. The results obtained for each primer are presented in Table 2. The ISSR amplification generated between 28 and 63 markers per primer, with an average of 42. The number of markers obtained per cultivar (multiplex ratio) ranged from 9 to 42. The size of the scorable amplified fragments ranged from 300 to 1400 bp. The primers HVH(TG)₇, DBD(AC)₇, and HVH(CA)₇ generated a large number of markers of high molecular weight (>800 bp), which were very difficult to distinguish by electrophoresis with denaturing gels. Strong background at the upper part of the gels using 5'-anchored ISSR primers was also reported by Sánchez de la Hoz et al. (1996) in barley (*Hordeum vulgare* L.). Most of these bands were not considered in this study, thus affecting the number of scored bands per primer. More scorable markers were produced with 3'-anchored primers (average of 47 bands) than with 5'-anchored primers (average of 38), although the percentage of polymorphisms for the latter was superior to that of the former (74.2% and 86.1%, respectively). Zietkiewicz et al. (1994) and Fang and Roose (1997) observed that primers anchored at the 5' end displayed more specificity than 3'-anchored primers and generated fewer but larger fragments than 3'-anchored primers. Previously, in rice (*Oryza sativa* L.), the dinucleotide repetitive sequence primers were found to be more amenable to ISSR analysis than the tri-, tetra-, and penta-nucleotide primers (Blair et al., 1999). Likewise, in this study, the only trinucleotide ISSR primer (AGC)₄YR, generated the lowest percentage of polymorphic markers (68%).

The dendrogram constructed with the ISSR data (Fig. 2) was

Fig. 1. ISSR profiles obtained from the 24 pear cultivars using the primer (GA)₈YG. 1 = 'Conference', 2 = 'William's', 3 = 'Highland', 4 = 'Packham's Triumph', 5 = 'Butirra Precoce Morettini', 6 = 'Passe Crassane', 7 = 'Beurré Hardy', 8 = 'Doyenné du Comice', 9 = 'General Leclerc', 10 = 'Beurré Bosc', 11 = 'Beurré Clairgeau', 12 = 'Souvenir du Congrès', 13 = 'Alexandrine Douillard', 14 = 'Doctor Jules Guyot', 15 = 'Delbard Première', 16 = 'Lawson', 17 = 'Clapp's Favourite', 18 = 'D. Joaquina', 19 = 'Sete Cotovalos', 20 = 'Lambe-os-dedos', 21 = 'Carvalhal', 22 = 'Pérola', 23 = 'Carapineira Parda', 24 = 'Rocha', and M = molecular marker, 100-bp ladder.

very similar to that obtained previously with RAPD and AFLP markers, confirming genetic divergence of 'Pérola', 'Carvalhal' and 'Lawson' from the other cultivars (Monte-Corvo et al., 2000). The small differences found between the ISSR and RAPD+AFLP dendrograms concerned only phenetic relationships among cultivars belonging to the same cluster. No differences were observed among the two main clusters.

CLONE DISCRIMINATION. None of the five molecular marker

systems used in this study detected reliable polymorphisms among the nine 'Rocha' clones, although a total of 1082 markers were analyzed (Table 2). Some polymorphisms were found, but they were either not confirmed in subsequent analyses performed with the same DNA, with DNA from different extractions of the same tree, or with DNA belonging to different trees from the same clone. The 25 RAPD primers used produced 178 markers, but no reproducible differences among clones were detected. The few false polymorphisms were caused mainly by amplification artifacts (not reproduced between amplifications). The AFLP marker system appeared to be the most powerful technique in terms of producing a large number of molecular markers. Detection by silver staining had fewer reproducibility problems than detection by autoradiography, since many of the weak markers responsible for the false polymorphisms with autoradiography were not detected with silver staining (Fig. 3). Nevertheless, with silver staining, eight (2.2%) irreproducible polymorphisms were observed among the 407 bands produced by the nine primer combinations. The ISSR marker system generated a high number of bands per primer (eight primers generated 183 markers) but no differences in banding patterns between patterns of 'Rocha' clones were observed, demonstrating high reproducibility of this technique, as described previously (Fang and Roose, 1997; Goulão et al., 2001).

The molecular marker system, SAMPL, generated many markers (272 bands with nine primer combinations), but reproducibility was poor. None of the 16% polymorphisms detected with this technique were confirmed. The original protocol (Paglia and Morgante, 1998) combined ISSR primers with AFLP primers that included the *MseI* adapter. We tried to improve reproducibility of this technique by using other combinations, specifically apple SSR primers combined with

AFLP-*MseI* primers or with AFLP-*EcoRI* primers (since *EcoRI* is a rare cut restriction enzyme), and ISSR primers combined with AFLP-*EcoRI* primers. In this way, we reasoned that the complexity of the banding patterns should decrease, improving reproducibility of the technique. Amplification with AFLP-*EcoRI* primers resulted in fewer markers, but with the same lack of reproducibility. Use of SSR primers also did not improve reliability of the amplifications. The SAMPL marker system proved to have more reproducibility problems than AFLP, perhaps due to the fact that the experimental conditions were adapted from AFLP markers, and thus were not optimum for this technique. Eight of the 15 apple SSR primers resulted in amplification with the 'Rocha' clones. Some of the primers generated complex patterns, with the number of bands ranging from 2 to 12. Examples of the SSR patterns are presented in Fig. 4. The large number of bands (42) obtained with SSR primers was probably related to 'polymerase slippage' of the *Taq* polymerase and/or to some artifacts difficult to distinguish from actual

Fig. 2. Dendrogram of the pear cultivars based on the genetic similarity matrix from ISSR data.

alleles. For discrimination among clones, all the bands with molecular weights between 50 and 300 bp were considered independently from the microsatellite loci, since detection of differences was, in some cases, impossible. No differences among clones were found with the eight primers tested.

The false polymorphisms found with some of the techniques that we used indicate that great care must be taken when performing analysis of clonal variation. In studies with closely related genotypes, duplicate DNA extractions from the same tree and from different trees belonging to the same clone are recommended to identify reproducible polymorphisms. Similar recommendations have been made by Loureiro et al. (1998) for grape clones.

Selection of the best 'Rocha' clones is of great importance to improve productivity and fruit quality, as well as to increase export of fruit. Detection of markers that distinguish clones could provide a valuable tool for management of clonal selection and, later, for certification of identity. RAPD, AFLP, SAMPL, and ISSR marker systems all detect polymorphisms resulting from mutations in primer annealing sites, or from insertions or deletions in the sequences between primer sites, and explore only a small fraction of the genome. These nine 'Rocha' clones clearly show differences in phenotypic traits such as flower morphology, fruit set, yield, fruit color or fruit size (Sousa et al., 1993), probably as the result of somatic variation. The results obtained in this study indicate that these mutations are restricted to a small part of the genome. The PCR products generated from different individuals may differ in sequence, but not in length or sequence differences may be so rare that, chances are, they would not be detected with these techniques, but could be identified by other techniques like single-strand conformation polymorphism (SSCP),

temperature gradient gel electrophoresis (TGGE), or denaturation gradient gel electrophoresis (DGGE). The usefulness of these and other techniques is currently under investigation.

CROSS-SPECIES TRANSPORTABILITY OF SSR PRIMERS. The same eight apple SSR primers tested for clone discrimination were also applied to other pear cultivars (data not presented). With the exception of the primer pair GD15, all of the apple SSR primers amplified polymorphic patterns with fragments of different lengths. As in 'Rocha' clones, the presence of some confusing products was observed. The 'stuttered' patterns and some nonspecific amplification products made identification of true alleles and discrimination among pear cultivars difficult. Examination of a segregating population of pear cultivars and the inclusion of the apple DNA controls on the gel could also facilitate discrimination of true alleles. The transportability of microsatellites between different related species

has been already demonstrated in several taxa, including fruit crops such as grape (Thomas and Scott 1993), citrus (Kijas et al., 1995), kiwi (*Actinidia chinensis* Planch.) (Huang et al., 1998), and plum (Cipriani et al., 1999; Downey and Iezzoni, 2000). Successful cross-species amplification indicates high level of sequence conservation which SSR flanking regions (Kijas et al., 1995) and depends mainly on the evolutionary distance between the species from which the SSR primers were developed and the tested species (Downey and Iezzoni, 2000). Cipriani et al. (1999) found that only 18% of the peach SSR primers they used could also amplify microsatellites in apples. We demonstrated that more conserved microsatellite flanking regions (more or less 50%) exists between apples and pears, although more work is necessary to optimize the PCR conditions specific for each primer and to provide better specific amplification.

Fig. 3. Comparison of AFLP fingerprints (E-ACT/M-CTT) from the nine 'Rocha' clones, using *Eco*RI primer end-labelled with [γ^{32}] d-ATP and detected by (A) autoradiography or (B) silver staining. Arrows indicate irreproducible fragments in the autoradiography gels.

Fig. 4. Identical amplification patterns in the nine 'Rocha' clones using apple SSR primers (A) O2B1 and (B) CH01F02.

Literature Cited

- Bartolozzi, F., M.L. Warburton, S. Arusekar, and T.M. Gradziel. 1998. Genetic characterization and relatedness among California almond cultivars and breeding lines detected by randomly amplified polymorphic DNA (RAPD) analysis. *J. Amer. Soc. Hort. Sci.* 123:381–387.
- Bassam, B.J., G. Caetano-Anolles, and P.M. Gresshoff. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.* 196:81–88.
- Bell, R.L., H.A. Quamme, R.E.C. Layne, and R.M. Skirvin. 1996. Pears, p. 441–514. In: J. Janick and J.N. Moore (eds.). *Fruit breeding, vol I. Tree and tropical fruits*. Wiley, New York.
- Blair, M.W., O. Panaud, and S.R. McCouch. 1999. Inter-simple sequence repeat (ISSR) amplification for analysis of microsatellite motif frequency and fingerprinting in rice (*Oryza sativa* L.). *Theor. Appl. Genet.* 98:780–792.
- Botta, R., A. Akkarak, G. Me, L. Radicati, and V. Casavecchia. 1998. Identification of pear cultivars by molecular markers. *Acta Hort.* 457:63–70.
- Cervera, M.T., J.A. Cabezas, J.C. Sancha, F. Martinez-de-Toda, and J.M. Martinez-Zapater. 1998. Application of AFLPs to the characterization of grapevine *Vitis vinifera* L. genetic resources: A case study with accessions from Rioja (Spain). *Theor. Appl. Genet.* 97:51–59.
- Cipriani, G., G. Frazza, E. Peterlunger, and R. Testolin. 1994. Grapevine fingerprinting using microsatellite repeats. *Vitis* 33:211–215.
- Cipriani, G., G. Lot, W.G. Huang, M.T. Marrazzo, E. Peterlunger, and R. Testolin. 1999. AC/GT and AG/CT microsatellite repeats in peach [*Prunus persica* (L.Batsch)]: Isolation, characterisation and cross-species amplification in *Prunus*. *Theor. Appl. Genet.* 99:65–72.
- Dirlwanger, E., S. Duha, M.A. Viruel, R. Saunier, and R. Monet. 1998. Identification of peach varieties using molecular markers. *Acta Hort.* 465:69–77.
- Downey, S.L. and A. Iezzoni. 2000. Polymorphic DNA markers in black cherry (*Prunus serotina*) are identified using sequences from sweet cherry, peach and sour cherry. *J. Amer. Soc. Hort. Sci.* 125:76–80.
- Fang, D.Q., R.R. Krueger, and M.L. Roose. 1998. Phylogenetic relationships among selected *Citrus* germplasm accessions revealed by inter-simple sequence repeat (ISSR) markers. *J. Amer. Soc. Hort. Sci.* 123:612–617.
- Fang, D.Q. and M.L. Roose. 1997. Identification of closely related citrus cultivars with inter-simple sequence repeat markers. *Theor. Appl. Genet.* 95:408–417.
- Gianfranceschi, L., N. Seglias, R. Tarchini, M. Komjanc, and C. Gessler. 1998. Simple sequence repeats for the genetic analysis of apple. *Theor. Appl. Genet.* 96:1069–1076.
- Goulão, L., L. Cabrita, C.M. Oliveira, and J.M. Leitão. 2001. Comparing RAPD and AFLP™ analysis in discrimination and estimation of genetic similarities among apple (*Malus domestica* Borkh.) cultivars. *Euphytica* (in press).
- Goulão, L., L. Monte-Corvo, and C.M. Oliveira. 2001. Phenetic characterisation of cultivars of plum (*Prunus* sp.) by high multiplex ratio markers: Amplified fragment length polymorphisms and inter-simple sequence repeats. *J. Amer. Soc. Hort. Sci.* 126:72–77.
- Guilford, P., S. Prakash, J.M. Zhu, E. Rikkerink, S. Gardiner, H. Bassett, and R. Forster. 1997. Microsatellites in *Malus × domestica* (apple): Abundance, polymorphism and cultivar identification. *Theor. Appl. Genet.* 94:249–254.
- Hokanson, S.C., A.K. Szewc-McFadden, W.F. Lamboy, and J.R. McPerson. 1998. Microsatellite (SSR) markers reveal genetic identities, genetic diversity and relationships in *Malus × domestica* Borkh. core subset collection. *Theor. Appl. Genet.* 97:671–683.
- Huang, W.G., G. Cipriani, M. Morgante, and R. Testolin. 1998. Microsatellite DNA in *Actinidia chinensis*: Isolation, characterisation, and homology in related species. *Theor. Appl. Genet.* 97:1269–1278.
- Kijas, J.M.H., J.C.S. Fowler, and M.R. Thomas. 1995. An evaluation of sequence-tagged microsatellite site markers for genetic analysis within *Citrus* and related species. *Genome* 38:349–355.
- Koller, B., A. Lehmann, J.M. McDermott, and C. Gessler. 1993. Identification of apple cultivars using RAPD markers. *Theor. Appl. Genet.* 85:901–904.
- Lanham, P.G. and R.M. Brennan. 1999. Genetic characterization of gooseberry (*Ribes grossularia* subgenus *Grossularia*) germplasm using RAPD, ISSR and AFLP markers. *J. Hort. Sci. Biotechnol.* 74:361–366.
- Loureiro, M.D., M.C. Martínez, J.M. Boursiquot, and P. This. 1998. Molecular marker analysis of *Vitis vinifera* 'Albariño' and some similar grapevine cultivars. *J. Amer. Soc. Hort. Sci.* 123:842–848.
- Matta, G.J. 1928. A Pereira 'Rocha'. *Estação Agrária Nacional, Ministério da Agricultura, Lisboa, Portugal*.
- Monte-Corvo, L., L. Cabrita, C.M. Oliveira, and J. Leitão. 2000. Assessment of *Pyrus* cultivars by RAPD and AFLP markers. *Gen. Resources Crop Evol.* 47:257–265.
- Moore, S.S., L.L. Sargeant, T.J. King, J.S. Mattick, M. Georges, and D.J.S. Hetzel. 1991. The conservation of dinucleotide microsatellites among mammalian genomes allows the use of heterologous PCR primer pairs in closely related species. *Genomics* 10:654–660.
- Moreno, S., J.P. Martín, and J.M. Ortiz. 1998. Inter-simple sequence repeats PCR for characterization of closely related grapevine germplasm. *Euphytica* 101:117–125.
- Morgante, M. and J. Vogel. 1994. Compound microsatellite primers for the detection of genetic polymorphisms. *U.S. Patent Appl* 08/326456.
- Nei, M. and W.H. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* 76:269–273.
- Oliveira, C., M. Mota, L. Monte-Corvo, L. Goulão, and D. Silva. 1999. Molecular typing of *Pyrus* based on RAPD markers. *Scientia Hort.* 79:163–174.
- Paglia, G. and M. Morgante. 1998. PCR-based multiplex DNA fingerprinting techniques for the analysis of conifer genomes. *Mol. Breeding* 4:173–177.
- Rohlf, F.J. 1993. NTSYS-PC. Numerical taxonomy and multivariate analysis system. Version 1.8. Exeter Publications, Setauket, N.Y.
- Rongwen, J., M.S. Akkaya, A.A. Bhagwat, U. Lavi, and P.B. Cregan. 1995. The use of microsatellite DNA markers for soybean genotypes identification. *Theor. Appl. Genet.* 90:43–48.
- Sánchez de la Hoz, M.P., J.A. Dávila, Y. Loarce, and E. Ferrer. 1996. Simple sequence repeats primers used in polymerase chain reaction amplifications to study genetic diversity in barley. *Genome* 39:112–117.
- Sensi, E., R. Vignani, W. Rohde, and S. Biricolti. 1996. Characterization of genetic biodiversity with *Vitis vinifera* L. Sangiovese and Colorino genotypes by AFLP and ISTR DNA marker technology. *Vitis* 35:183–188.
- Sousa, R.M., J.T. Ferreira, and M. Durão. 1993. Seleção clonal em pereira 'Rocha'. II Congresso Ibérico de Ciências Hortícolas. *Actas de Hort.* 9:96–103.
- Thomas, M.R. and N.S. Scott. 1993. Microsatellite repeats in grapevine reveal DNA polymorphisms when analyzed as sequence-tagged sites (STSs). *Theor. Appl. Genet.* 86: 985–990.
- Vignani, R., J.E. Bowers, and C.P. Meredith. 1996. Microsatellite DNA polymorphism analysis of clones of *Vitis vinifera* 'Sangiovese'. *Sci. Hort.* 65:163–169.
- Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res.* 23:4407–4414.
- Williams, J.G.K., A.R. Kubelik, K.J. Livak, A. Rafalski, and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18:6531–6535.
- Ye, G.N., G. Soylemezoglu, N.F. Weedon, W.F. Lamboy, R.M. Poll, and B.I. Reisch. 1998. Analysis of the relationship between grapevine cultivars, sports and clones via DNA fingerprinting. *Vitis* 37:33–38.
- Zietkiewicz, E., A. Rafalski, and D. Labuda. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20:176–183.