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Morphological traits and high resolution RAPD markers for the identification of the main strawberry varieties cultivated in Argentina

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Abstract

Eight genotypes of the main *Fragaria* × *ananassa* cultivars grown in Argentina were analysed using the random amplified polymorphic DNA (RAPD) technique combined with electrophoresis in polyacrylamide gels. The high resolution of this procedure allowed the detection, with only 13 random primers, of 37 genotype-specific bands that can be used as markers for verifying the identity of cultivars. By using this approach, three different accessions of the cultivar 'Pájaro' exhibited differences in amplification profiles, confirming the need for DNA analysis to prevent misidentification of cultivars. In addition, RAPD bands and morphological traits were used to assess genetic relatedness among cultivars. Comparison of both dendrograms revealed that there is no correlation between the clustering obtained with molecular and morphological characters.

Key words: *Fragaria* × *ananassa* — genetic similarity — morphological characters — RAPD

The verification of strawberry cultivar identity is essential for farmers and plant breeders, as part of certification programmes to prevent mixtures or misidentification of cultivars and to protect breeders' rights. Currently, morphological characters are used to certify the identity of strawberry cultivars, but these characters often do not yield clear answers concerning discrimination of a plant variety due to ambiguous differences or phenotypic modifications caused by environmental factors.

The molecular approach has proved to be a valuable tool for the identification of plant varieties because it is not only more sensitive but also not susceptible to environmental effects. Random amplified polymorphic DNA (RAPD) is one of the most widely used techniques, since it allows detection of differences at the DNA level using small amounts of genomic DNA, and does not require previous information about the DNA sequence. This technique has been used for the characterization of different strawberry cultivars (Gidoni et al. 1994, Hancock and Callow 1994, Levi et al. 1994, Parent and Pagé 1995, Graham et al. 1996, Degani et al. 1998, Congiu et al. 2000).

Because the RAPD method uses random primers and a low annealing temperature, which allows non-specific binding to the DNA, this technique is by definition non-specific. However, it has been shown by several researchers that the majority of RAPD bands are reproducible if care is taken in producing a standardized protocol that is strictly followed in each reaction (Hedrick 1992, Heun and Helentjaris 1993, Bowditch et al. 1994, Gibbs et al. 1994, Rothuizen and van Wolferen 1994).

Questions have also been raised about the heritability of RAPD fragments (Dowling et al. 1996, Palumbi 1996). Because the exact nature of the variation in RAPD fragments is not known, it is not possible to be sure that they always represent genetically inherited traits. However, RAPD fragments have been found to be heritable in many species, suggesting that RAPD markers can be considered to be Mendelian traits (Scott et al. 1992, Dawson et al. 1993, Heun and Helentjaris 1993, Rothuizen and van Wolferen 1994, Warburton et al. 1996, Stott et al. 1997, Cooper 2000). In strawberry, it has been reported that the majority of the individual bands within an amplification profile segregate as if controlled by a single diploid locus with one or two alleles, even though the commercial strawberry is an octoploid (Hancock and Callow 1994).

In the present work, the main strawberry cultivars grown in Argentina were analysed to detect polymorphic RAPD markers useful for unequivocally distinguishing each cultivar. The RAPD technique combined with polyacrylamide gel electrophoresis and silver-staining procedure was used. RAPD characters were also used to assess genetic relatedness among these cultivars and to evaluate correlations between the similarities observed when using molecular characters and morphological traits recommended by the International Union for the Protection of New Varieties of Plants (UPOV 1995) to characterize these cultivars.

Materials and Methods

Plant materials: The six *Fragaria* × *ananassa* cultivars used in this study were 'Camarosa', 'Sweet Charlie', 'Selva', 'Milsei Tudla', 'Chandler' and 'Pájaro'. The last was represented by three different accessions, named 'Pájaro Mendoza', 'Pájaro Salta' and 'Pájaro Corrientes', according to their provenance. Plants were purchased as dormant crowns from commercial nurseries and grown in a greenhouse. Table 1 shows the list of cultivars studied and their pedigree data. The morphological characters used were the 41 published by the International Union for the Protection of New Varieties (UPOV 1995) and by the Registro Nacional de la Propiedad de Cultivares y Registro Nacional de Cultivares of Argentina.

DNA extraction: Total DNA was extracted from young leaves of each individual of all the accessions studied, using the Nucleon Phytopure kit (Amersham Pharmacia Biotech, UK Ltd., England). DNA

Table 1: Strawberry cultivars used in this study

Cultivar name	Abbreviation	Breeding parents[2]
'Chandler'	Ch	'Douglas' × 'Cal 72-361-105'
'Camarosa'	CAM	'Douglas' × 'Cal 85-218-605'
'Pájaro'[1]	PAJ	'Cal 63-7-101 × 'Sequoia'
'Selva'	S	'Cal 70-3-3-117' × 'Cal 7198-605'
'Sweet Charlie'	SCh	'FL 80-456' × 'Pájaro'
'Milsei Tudla'	MT	'Parker' × 'Chandler'

[1] From the cultivar 'Pájaro' three accessions were analysed: 'Pájaro Mendoza', 'Pájaro Salta' and 'Pájaro Corrientes'.
[2] According to Risser and Navatel 1997.

concentrations were determined by comparison of DNA bands observed in agarose gels (0.7% agarose gel stained with 0.5 μg/ml ethidium bromide) with standard DNA bands of known concentrations (100–1000 ng). Buffer and running conditions were according to Sambrook et al. (1989).

Polymerase chain reaction: DNA was analysed with a set of 20 random sequence 10-mer primers (OPJ series from Operon Technologies, Alameda, CA, USA). Reaction mixtures were prepared in 20 μl containing: 20 ng DNA template, 2 mM $MgCl_2$, 0.1 mM of each dNTP (deoxyribonucleotides), 0.2 μM primer, 1.5 Unit of *Taq* polymerase (Promega Corporation, Madison, WI, USA) and 2 μl of *Taq* buffer (10×). The programme used was 44 cycles of: denaturation, 30 s at 92°C; annealing, 1 min at 35°C; extension, 2 min at 72°C. The reaction was carried out in a MJ Research Thermal Cycler (PTC-100; MJ Research, Inc, Watertown, MA, USA). RAPD experiments were repeated four times to avoid false results and to ensure reproducibility. Negative controls, without DNA, were included in each experiment.

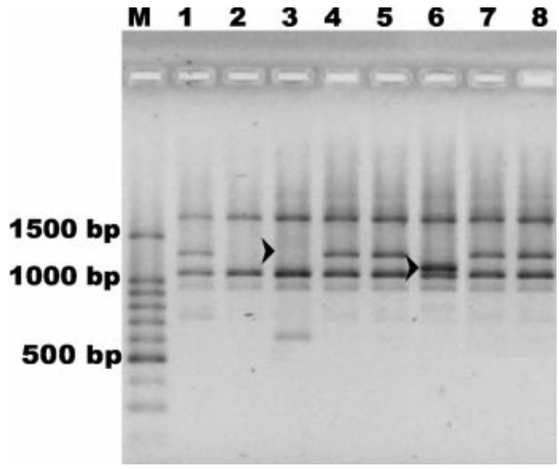

Fig. 1: Agarose gel showing random amplified polymorphic DNA amplification profiles of cultivars analysed obtained with primer OPJ14. M, 100-bp polymerase chain reaction markers; 1, 'Camarosa'; 2, 'Milsei Tudla'; 3, 'Pájaro Corrientes'; 4, 'Pájaro Mendoza'; 5, 'Pájaro Salta'; 6, 'Selva'; 7, 'Chandler'; 8, 'Sweet Charlie'. Arrows indicate differential markers

Electrophoresis: At the end of the polymerase chain reaction (PCR), amplification products were separated by electrophoresis in both agarose and polyacrylamide gels. Electrophoresis in 1.5% agarose gel was performed in TBE (tris-borate-EDTA) running buffer (Sambrook et al. 1989) and gels were stained with ethidium bromide. Molecular weights were estimated using 100 bp PCR markers from Promega.

For electrophoresis in polyacrylamide gels, 8 μl of each denatured sample were loaded onto 6% acrylamide/bisacrylamide (19 : 1), 7 M urea and 0.5× TBE gels. Gels were stained by employing the DNA

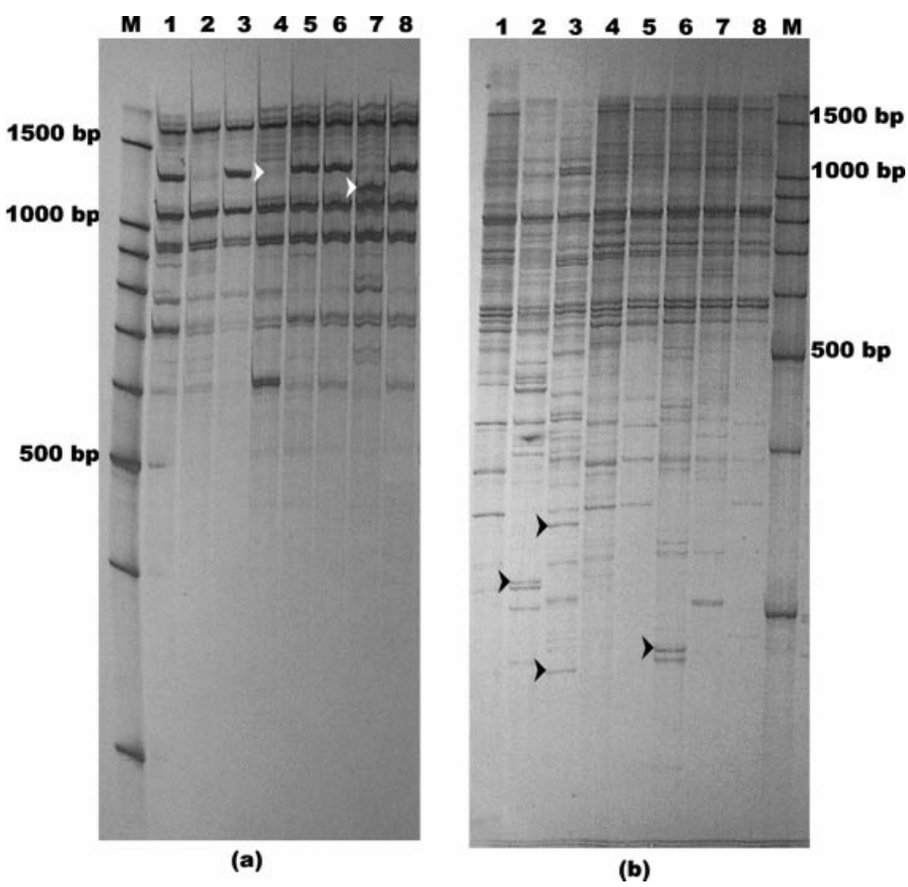

Fig. 2: Polyacrylamide gels showing random amplified polymorphic DNA amplification profiles of cultivars analysed, obtained with primers OPJ14 (a) and OPJ04 (b). a. M, 100-bp polymerase chain reaction (PCR) markers; 1, 'Camarosa'; 2, 'Milsei Tudla'; 3, 'Chandler'; 4, 'Pájaro Corrientes'; 5, 'Pájaro Mendoza'; 6, 'Pájaro Salta'; 7, 'Selva'; 8, 'Sweet Charlie'. b. 1, 'Camarosa'; 2, 'Chandler'; 3, 'Milsei Tudla'; 4, 'Sweet Charlie'; 5, 'Pájaro Corrientes'; 6, 'Pájaro Mendoza'; 7, 'Pájaro Salta'; 8, 'Selva'; M, 100-bp PCR markers. Arrows indicate differential markers

Silver Staining System of Promega; 100-bp ladder PCR markers from Promega were used as molecular weight standards.

Data analysis: Data from morphological characters were scored as present (1) or absent (0) in each of the six cultivars considered. Since the three accessions of 'Pájaro' have the same morphological characters, they were considered as belonging to the same cultivar. RAPD data were also scored by analysing PCR amplified bands. Only bands appearing in three of the four replicates were considered and scored as present in each of the eight samples included in the study. The similarity matrix was built by considering presence (1) or absence (0) of bands in each of the eight genotypes because the three accessions of 'Pájaro' were assumed to be different.

Similarities were evaluated by estimating the Dice coefficient. This coefficient has been recommended for the evaluation of genetic similarities when using RAPD data (Lamboy 1994).

Cluster analysis was made by using the unweighted pair group method with arithmetic mean (UPGMA). This is the most widely used method in ecology and systematics (Crisci and López-Armengol 1983, van Tongeren 1995). The evaluation of similarity coefficients and cluster analysis were carried out with the NTSYS program (Rohlf 1993).

Results

Of the 20 primers tried in this work, 16 resulted in amplification of DNA fragments. Primers OPJ02, OPJ03, OPJ08 and OPJ13 did not produce amplification profiles. The primers OPJ15 and OPJ20 did not produce polymorphic bands and the primer OPJ19 only showed polymorphisms within the three accessions of the cultivar 'Pájaro' (see below).

Analysis of agarose gels showed that only with the primer OPJ14 could a clear polymorphic band present in the cultivar 'Selva' be detected (Fig. 1). With the same primer, one band absent in the accession 'Pájaro Corrientes' and present in the other two accessions of the same cultivar was observed (Fig. 1). Amplification profiles obtained in agarose gels with the rest of the primers did not allow reproducible and scorable polymorphic fragments, useful for identifying each one of the eight accessions analysed, to be found.

When the same amplification products were separated in polyacrylamide electrophoresis gels a significant increase in resolution was obtained (Fig. 2). The number of bands ranged between 30 and 54, per primer and per accession. In total, 550 bands were scored and 120 of these were polymorphic. From this latter set, 37 bands obtained with 13 primers were selected as markers because they were reproducible and different enough in size to be easily distinguishable from neighbouring fragments and present or absent in only one of the accessions studied (Fig. 2 and Table 2). The results reveal that only with three primers, OPJ04, OPJ05 and OPJ06, could each one of the genotypes analysed in this study be discriminated.

Table 2: Polymorphic RAPD bands that distinguish each of the eight accessions analysed in this study[1]

Primers	Band size (bp)	CAM	Ch	MT	SCh	PC	PM	PS	S
OPJ01	360	−	−	−	+	−	−	−	−
OPJ04[2]	560	−	−	+	−	−	−	−	−
OPJ05[2]	850	−	−	−	+	−	−	−	−
	650	−	−	−	+	−	−	−	−
	400	−	−	−	−	−	−	+	−
OPJ06[2]	800	−	−	−	−	−	−	−	+
	420	−	+	−	−	−	−	−	−
	380	+	−	−	−	−	−	−	−
	370	−	−	−	+	−	−	−	−
	350	−	−	−	−	+	−	−	−
	340	−	+	−	−	−	−	−	−
	310	+	−	−	−	−	−	−	−
OPJ07	530	−	+	−	−	−	−	−	−
	430	−	+	−	−	−	−	−	−
OPJ09	850	−	−	−	−	−	−	−	+
	500	+	+	+	+	+	+	+	−
	490	+	+	+	+	+	+	+	−
	470	+	−	−	−	−	−	−	−
OPJ10	380	−	−	−	−	+	−	−	−
	280	−	−	−	−	+	−	−	−
OPJ11	690	−	+	−	−	−	−	−	−
	360	−	−	−	−	−	−	−	+
	300	−	−	−	−	−	−	−	+
OPJ12	590	−	−	−	+	−	−	−	−
	515	−	−	−	+	−	−	−	−
OPJ14	1200	−	−	−	−	−	−	−	+
OPJ16	790	+	−	−	−	−	−	−	−
	310	+	−	−	−	−	−	−	−
	305	−	−	−	+	−	−	−	−
	230	−	+	−	−	−	−	−	−
OPJ17	610	−	−	−	−	−	−	−	+
	580	−	+	−	−	−	−	−	−
	530	−	+	−	−	−	−	−	−
	480	−	−	−	+	−	−	−	−
	450	−	−	−	−	+	−	−	−
OPJ18	350	+	−	−	−	−	−	−	−
	340	−	−	−	−	+	−	−	−
	270	−	−	−	+	−	−	−	−

[1] +, Presence of band; −, absence of band. CAM, 'Camarosa'; Ch, 'Chandler'; MT, 'Milsei Tudla'; SCh, 'Sweet Charlie'; PC, 'Pájaro Corrientes'; PM, 'Pájaro Mendoza'; PS, 'Pájaro Salta'; S, 'Selva'.
[2] These primers discriminate each of the eight genotypes.

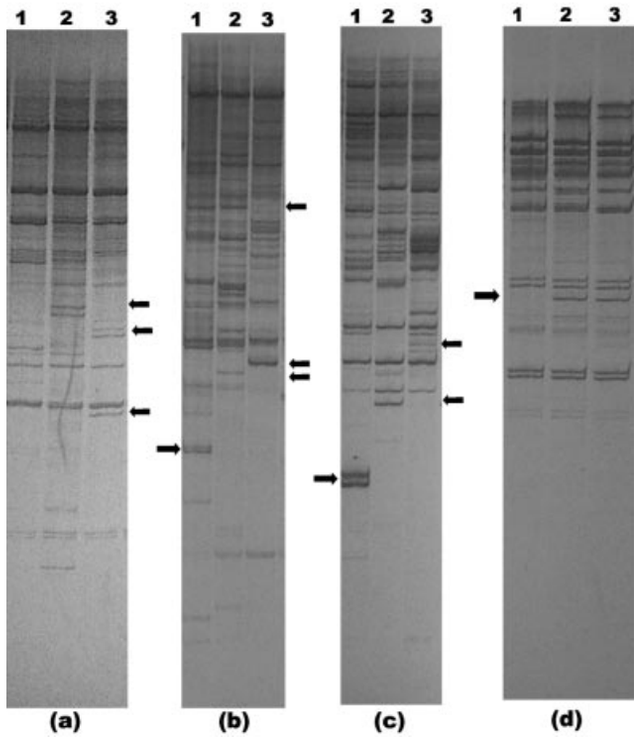

Fig. 3: Polyacrylamide gels showing random amplified polymorphic DNA (RAPD) amplification profiles of the three accessions of the cultivar 'Pájaro' obtained with primers OPJ18 (a), OPJ06 (b), OPJ07 (c) and OPJ09 (d). Lanes: 1, 'Pájaro Corrientes'; 2, 'Pájaro Mendoza'; 3, 'Pájaro Salta'. Arrows indicate differential markers

The results show that the three accessions of the cultivar 'Pájaro' are not identical. Differences among the three accessions of this cultivar were detected with primers OPJ18, OPJ06, OPJ07, OPJ09 (Fig. 3) and also with primers OPJ05, OPJ10, OPJ14, OPJ16 and OPJ19.

Figure 4 shows the dendrogram generated using all RAPD characters, both common and non-common. Common bands were those present in all accessions analysed. The range of similarities of cultivars was between 0.65 and 0.78, indicating that they are closely related. It is notable that 'Camarosa' and 'Chandler' group in distinct clusters, despite a common parent (Table 1). In contrast, 'Sweet Charlie' groups closer to 'Pájaro', as it is one of its parents (Table 1).

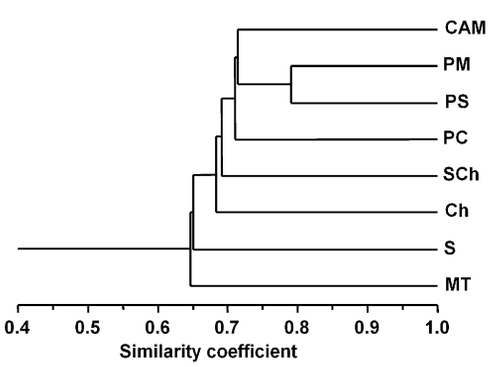

Fig. 4: Dendrogram generated with all (550) random amplified polymorphic DNA characters. CAM, 'Camarosa'; Ch, 'Chandler'; MT, 'Milsei Tudla'; SCh, 'Sweet Charlie'; PC, 'Pájaro Corrientes'; PM, 'Pájaro Mendoza'; PS, 'Pájaro Salta'; S, 'Selva'

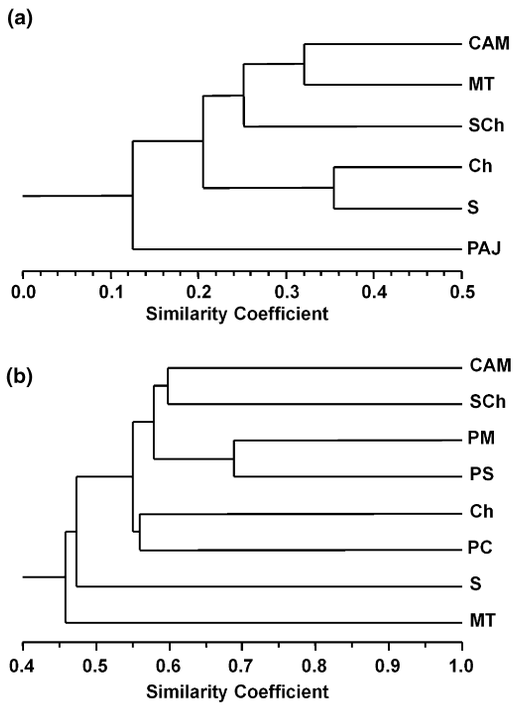

Fig. 5: Dendrograms generated with morphological characters (a) and non-common RAPD characters (b). CAM, 'Camarosa'; Ch, 'Chandler'; MT, 'Milsei Tudla'; SCh, 'Sweet Charlie'; PAJ, 'Pájaro'; PC, 'Pájaro Corrientes'; PM, 'Pájaro Mendoza'; PS, 'Pájaro Salta'; S, 'Selva'

In order to evaluate if relatedness among cultivars based on molecular markers correlates with relatedness based on the morphological differential traits employed to identify cultivars, dendrograms based only on non-common RAPD bands and on differential morphological characters are shown in Fig. 5. Significant differences in the clustering pattern indicate that morphological data are not in agreement with molecular data. Moreover, the dendrogram generated with the non-common RAPD bands is topologically different from the dendrogram based on all RAPD bands (Fig. 4 and Fig. 5b).

Discussion

By combining the RAPD technique with the high resolution of polyacrylamide gel electrophoresis, cultivar-specific markers could be identified with a small number of primers. The advantage of detecting these fragments is that they can be cloned, sequenced and used to develop specific 'designed' sequence characterized amplified region (SCAR) primers for diagnostic markers. SCAR primers are longer than RAPD primers and highly stringent annealing temperatures can be employed, avoiding mismatching in the priming site during DNA amplification (Paran and Michelmore 1993). The use of SCAR markers for the identification, certification and protection of commercial strawberry cultivars would prevent some problems derived from the RAPD technique, which require repeated amplifications to avoid artefacts and obtain reliable results. Although RAPD markers have been reported to be suitable for forensic purposes to clarify the identity of unauthorizedly commercialized varieties (Congiu et al. 2000), having a set of variety specific SCAR markers will simplify the identification procedure in the case of forensic applications. These markers can also be very useful in marker-assisted

breeding programmes to track the genetic background of new hybrids.

The results obtained by RAPD analysis of the three accessions of 'Pájaro' show clearly that morphological traits alone could lead to misidentification of cultivars. The differences found at molecular level in the 'Pájaro' accessions could be explained by the fact that, in Argentina, this old cultivar is no longer protected and has been vegetatively reproduced by runnering and also by micropropagation without using the founding clones. Consequently, one may speculate that repeated micropropagation events might have generated genetic changes that would account for the differences observed in this molecular analysis.

The disagreement between results obtained for the three accessions of 'Pájaro' when molecular and morphological characters are considered is also observed for the other genotypes, as revealed by differences in topology of the dendrograms presented in Fig. 5. These differences reveal that with the RAPD technique DNA regions were examined that are not expressed in the phenotypic characters considered by UPOV as variety-discriminating traits. But up to now the RAPD characters cannot be used to release new varieties. These new results suggest that the morphological traits suggested by the UPOV may not be good characters to evaluate the genetic relatedness among cultivars. There are other morphological and anatomical characters that can be considered diagnostic traits because they are less susceptible to environmental influences and correlate with RAPD characters when evaluating the genetic relatedness among *F.* × *ananassa* and its wild related species (Ontivero et al. 2000).

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