ELSEVIER

Scientia Horticulturae 79 (1999) 163–174

SCIENTIA HORTICULTURÆ

Molecular typing of *Pyrus* based on RAPD markers

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Accepted 31 July 1998

Abstract

Molecular characterisation and phenetic similarities between several cultivars of *P. communis* and *P. pyrifolia*, and genotypes of *P. cordata*, *P. bourgaeana* and *P. pyraster* were investigated through RAPD markers. Sixty decamer primers were screened, generating polymorphic patterns of Occidental and Oriental pear genotypes. Twenty-two selected primers originated clear and reproducible patterns, produced a total of 358 bands, 327 of them polymorphic. For 10 of the 12 genotypes analysed it was possible to find genotype-specific RAPDs and fragment patterns which could be used for cultivar identification. The patterns distinguished between genotypes and their analysis established a first approach to phenetic classification within the *Pyrus* genus based on DNA markers, clustering the genotypes according to their geographic origin. RAPD analysis of in vitro and in vivo material of seven cultivars was also performed, resulting in identical patterns for each genotype.

Keywords: Pear; Cultivar identification; Molecular markers; Fingerprinting

1. Introduction

Pears are the third most important fruit produced in temperate regions after grapes and apples (Chevreau and Skirvin, 1992). They belong to the genus *Pyrus*, which comprises at least 22 species of which *P. communis* L., the European pear, and *P. pyrifolia* (Burm.) Nakai (*Pyrus serotina* Rehder), the Asian pear or nashi,

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0304-4238/99/$ – see front matter
PII: S0304-4238(98)00205-2

are the most interesting for fruit production. Both species are diploid ($2x = 34$) and self-incompatible, originating great genetic variability within the species.

The existence of a very large number of rootstocks, cultivars and clones, maintained by vegetative propagation, reinforces the need of a reliable verification of cultivar identity for nurserymen and growers. This represents a very important aspect in the fruit industry, particularly as the sale of fruit trees and planting of orchards represent major investments of time and money. Accurate identification of plants is also desired for patent protection of propagated material. Traditionally, identification of pear cultivars was based on morphological or physiological aspects. More recently, biochemical markers like isozymes provided useful information (Chung and Ko, 1995; Chevreau et al., 1997) but have some disadvantages like the limited number of polymorphisms detected between close genotypes and variations due to the physiological stage. Recently, many papers have described the value of molecular markers like RFLP (restriction fragments length polymorphism) and RAPD (random amplified polymorphic DNA) in taxonomic classification and cultivar-typing in fruit trees. The RAPD assay has the advantages of being readily employed, requiring very small amounts of genomic DNA and eliminating the need for blotting and radioactive detection. For these reasons, RAPD markers have been successfully used for identification and genetic relationships of apple (Koller et al., 1993; Harada et al., 1993; Yae and Ko, 1995), plum (Ortiz et al., 1997), lemon (Deng et al., 1995), peach (Chaparro et al., 1994; Warburton and Bliss, 1996) and grapes (Qu et al., 1996). This suggests that these markers could be used in the genus *Pyrus*, where no significant work has been published in this field. Compared with other fruit trees, the identification and isolation of genes in *Pyrus* are very limited (Bellini and Stefania, 1997) and in the last few years the genetic patrimony of this genus has been eroded through the loss of local cultivars, although some effort in establishing collections, particularly in Italy, has been made.

In this study we used several primers to investigate the potential and limits of the RAPD technique for discriminating among several cultivars and species of *Pyrus* and revealing the relationships between them, based on the observable characteristics, regardless of their ancestral lineage, since most of the commercial cultivars are centenary and their pedigree is unknown. A parallel set was established to compare RAPD patterns of in vitro and in vivo material.

2. Materials and methods

2.1. Plant material

Shoots of *P. cordata* and *P. pyraster* were collected from Serra do Gerês, northwestern Portugal, and *P. bourgaeana* from Serra de Monchique, southern

Portugal. The other genotypes used for molecular analysis were *P. pyrifolia* cultivars 'Kosui' and 'Shinseiki' and *P. communis* cultivars 'Beurré Hardy,' 'Doyenné du Comice,' 'Passe Crassane' (all French); 'Williams Rouge' (an American mutant) and 'Rocha,' Pérola and 'Carapinheira Parda' (Portuguese cultivars), collected at Estação Nacional de Fruticultura Vieira Natividade, Alcobaça, Portugal.

These cultivars have different origins. The Asian cultivar 'Shinseiki' descends from a cross between 'Nijisseiki' × 'Chojuro' and the 'Kosui' from a cross between 'Kikusui' × 'Wasekozo'. 'Beurré Hardy,' 'Passe Crassane' and 'Doyenné du Comice' are of French origin and 'Williams Rouge' results from a somatic mutation of the British 'Williams'. These cultivars had their origin from seedlings in the 19th century. Although Rocha's origin is known, from a seedling in Sintra, in 1850, the genetic background of the other two Portuguese cultivars is not known. In Portugal, *P. cordata* is limited to the northwest; *P. pyraster* is confined to damp places on the northwestern mountains and *P. bourgaeana* is found in dry places of the country (Franco and Afonso, 1965).

In vitro material was obtained from axillary buds, collected from the above-mentioned field-grown trees. Tips (1 mm long) were aseptically cultured in QL medium solidified with 5.5 g l^{-1} purified agar (Merck) for 1 month. Subsequently, they were transferred to a fresh medium, a modified DKW salts (Silva and Dias, 1997), supplemented with 2.25 μM BA, 0.75 μM IBA and 87.6 mM sucrose, for the multiplication phase which lasted 4 months, including two subcultures.

2.2. DNA extraction

DNA was extracted from fresh newly expanded leaves (in vivo material) or shoot tips (in vitro material), using a hexadecyltrimethylammonium bromide (CTAB) protocol adapted from Doyle and Doyle (1990). Briefly, 0.25 g of tissue was ground in liquid nitrogen and subsequently incubated at 65°C for 60 min with 600 μl of isolation buffer (2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA (pH 8.0), 100 mM Tris–HCl (pH 8.0), 1% w/v polyvinylpyrrolidone (PVP-40) and 1% v/v 2-mercaptoethanol). The mixture was allowed to cool to room temperature (RT) and a 1:1 chloroform–isoamyl alcohol (24:1) extraction was performed. The preparation was mixed by inversion to form an emulsion and then centrifuged at RT for 10 min (15 000 *g*) to separate the phases. A RNase digestion was performed (10 μg ml^{-1} RNase A at 37°C during 60 min) followed by a second chloroform–isoamyl alcohol extraction. Subsequently, DNA was precipitated from the aqueous phase by adding 2/3 volume of cold isopropanol. The pellet was washed with 0.2 M sodium acetate and 76% (v/v) ethanol and dried in a vacuum for 20 min. The DNA was redissolved in 50 μl of buffer containing 10 mM Tris–HCl and 1 mM EDTA (TE) pH 8.0. The DNA preparation was diluted in TE pH

8.0 at 50 ng μl^{-1} for RAPD analysis. The concentration and purity of DNA were assessed spectrophotometrically (Gene Quant II, Pharmacia Biotech, Cambridge, UK). The DNA samples had $OD_{260}/OD_{280}\sim1.7$ and $OD_{260}/ OD_{230}\sim1.8–2.0$.

2.3. DNA amplification

The PCR reaction mixtures had a total volume of 25 µl. The mixture contained 0.75 units of Taq DNA polymerase (Promega), 0.4 µM primer, 0.2 mM of each dNTP (Promega), 2.5 mM $MgCl_2$, 1 × appropriate reaction buffer (50 mM, Tris–HCl pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween®20, 0.5% Nonidet® P 40) and 50 ng template DNA. Reactions were performed in a PTC 200 thermal cycler, (MJ Research, MA) programmed as follows: 1.5 min at 94°C for initial denaturation, 40 cycles of 30 s at 94°C (denaturation), 1 min at 36°C (annealing), and 1 min at 72°C (extension). A final extension step at 72°C for 10 min followed. A total of sixty 10-mer primers, of arbitrary sequence (Kit A, C, E from Operon Technologies, Alameda, CA) were tested for PCR amplification. The amplification products were visualised on 1.5% agarose gels stained with ethidium bromide, using standard methods (Sambrook et al., 1989). Three replications of each reaction for each selected primer were carried out, using DNA from in vivo material. RAPDs of in vivo and in vitro material were compared using four selected primers and seven genotypes.

2.4. Data analysis

Only the bands that were present in the three replications were considered and scored for each genotype and primer. For phenetic similarities, amplified products were analysed by a comparison of the genotypes based on the percentage of common fragments and similarity matrix (Nei and Li, 1979). A matrix was constructed and the NTsys-pc software (Numerical Taxonomy System) (Rohlf, 1989) was used for cluster and principal coordinate analysis. The specific bands useful for identifying genotypes were named with the primer code followed by the approximate number of base pairs of the amplified fragment.

3. Results

Twenty-two primers provided clear, non-monomorphic patterns (Table 1). For the genotypes tested, between 12 and 22 bands were obtained for each primer and of a total of 358 clear and reproducible bands, 327 were polymorphic (RAPDs). The amplified DNA fragments normally ranged from 250 to 2000 bp. An example of RAPD pattern, obtained with primer OPA09, is shown in Fig. 1. The gels were also screened for primers revealing RAPDs unique to particular

Table 1
List of primers selected from Kit A, C, E (Operon Technologies, Alameda, CA)

Primer	Sequence (5′ to 3′)	No. of bands	No. of patterns	No. of polymorphic bands
OPA-01	CAGGCCCTTC	12	11	11
OPA-02	TGCCGAGCTG	12	10	9
OPA-04	AATCGGGCTG	19	12	18
OPA-05	AGGGGTCTTG	12	12	12
OPA-07	GAAACGGGTG	15	12	14
OPA-08	GTGACGTAGG	16	10	16
OPA-09	GGGTAACGCC	20	10	20
OPA-11	CAATCGCCGT	12	11	12
OPA-13	CAGCACCCAC	18	12	14
OPA-18	AGGTGACCGT	22	11	20
OPA-20	GTTGCGATCC	22	11	21
OPC-02	GTGAGGCGTC	20	11	19
OPC-05	GATGACCGCC	16	11	13
OPC-11	AAAGCTGCGG	18	10	16
OPC-19	GTTGCCAGCC	16	12	14
OPE-03	CCAGATGCAC	14	11	12
OPE-04	GTGACATGCC	13	10	12
OPE-07	AGATGCAGCC	20	12	19
OPE-11	GAGTCTCAGG	13	10	12
OPE-14	TGCGGCTGAG	16	11	15
OPE-15	ACGCACAACC	14	12	12
OPE-20	AACGGTGACC	18	12	16

genotypes (Table 2). For 10 of the 12 genotypes, it was possible to find at least two such primers. Some primers also allowed the distinction of Oriental from Occidental genotypes. Primers OPA04, OPA05, OPA07, OPA08, OPA09, OPA11, OPA13, OPA18, OPA20, OPC05, OPC11, OPE03, OPE04 and OPE20 revealed specific RAPDs for the two Oriental cultivars studied. The Occidental genotypes showed specific bands with OPA01, OPA05, OPA09, OPA18, OPE04 and OPE11.

Similarity coefficients originated the following dendrogram (Fig. 2), using UPGMA as the clustering method. The cophenetic matrix computed from the tree matrix showed a significant correlation of 96.8% with the original similarity matrix.

Although a limited number of accessions for each species was considered, there was a clear separation between Occidental and Oriental genotypes in the study. Within the Occidental genotypes, the wild species *P. bourgaeana*, *P. cordata* and *P. pyraster* were separated from cultivated *P. communis* (61% similarity). Among the cultivated varieties of *P. communis*, the 'Williams Rouge' showed greater proximity to the Portuguese cultivar 'Rocha,' and 'Carapinheira

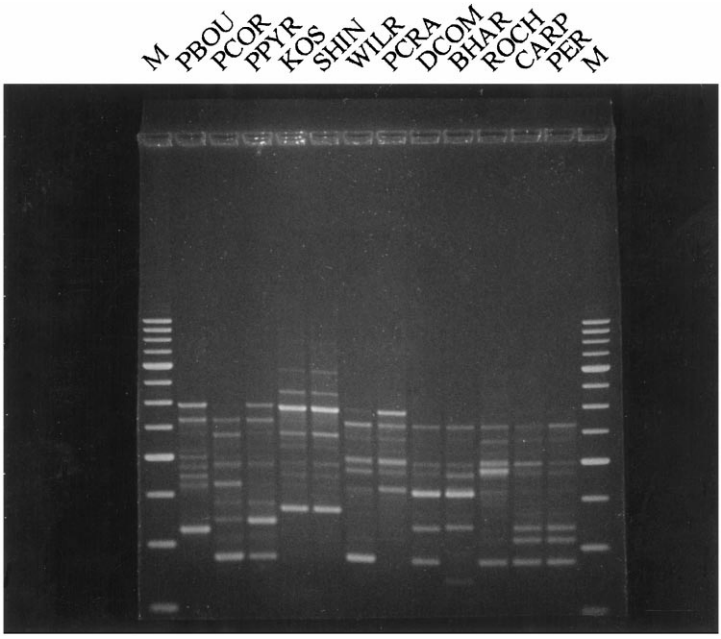

Fig. 1. RAPD patterns of 12 genotypes within the genus *Pyrus* obtained with primer OPA09. M: DNA size markers (DNA Molecular Weight Marker XVI, Boehringer Manheim, 250 bp ladder). See Table 2 for cultivar abbreviations.

Table 2
Specific RAPD fragments among the 12 genotypes

Accession	Code	Primers revealing specific RAPDs (no. of base pairs of a band)
P. bourgaeana	PBOU	A01 (1429), A02 (1222), A04 (427), A09 (563), C02 (977), E04 (396), E20 (727), E20 (1292)
P. pyraster	PPYR	A04 (292), A20 (817), C05 (1200), C19 (634), C19 (1650)
P. cordata	PCOR	A20 (682), E11 (1340)
P. communis		
Williams Rouge	WILR	A20 (2625), E11 (1125)
Rocha	ROCH	A04 (1643), C11 (1857), E07 (1000), E20 (983)
Carapinheira Parda	CARP	—
Pérola	PER	A08 (724), A18 (1600), E03 (1042)
Passe Crassane	PCRA	A05 (708), A07 (720), A18 (806), A20 (867), C05 (821), E20 (411)
Doyenné du Comice	DCOM	—
Beurré Hardy	BHAR	A01 (617), A09 (325), A13 (553), A20 (983), C02 (533)
P. pyrifolia		
Kosui	KOS	A05 (1250), E15 (513)
Shinseiki	SHIN	A07 (700), C05 (1075), C11 (2042), C11 (1750), C11 (1450)

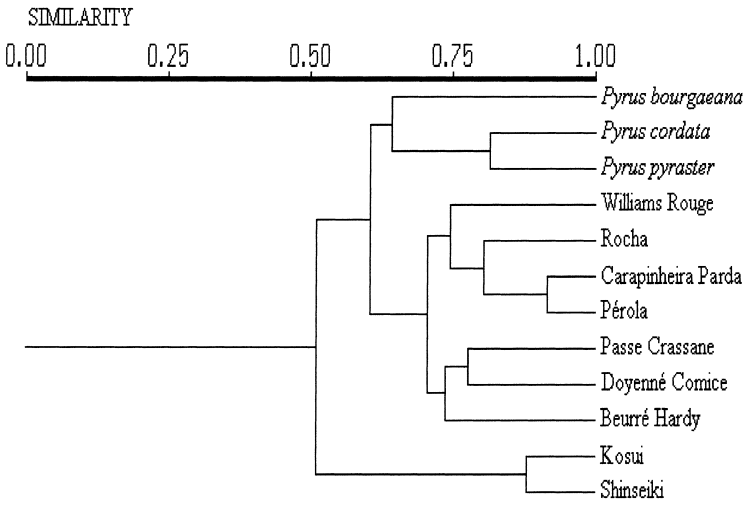

Fig. 2. Dendogram of 9 pear cultivars and 3 wild species based on Nei and Li's (1979) similarity coefficients using UPGMA as the clustering method.

Parda' and 'Pérola' were closely related. The French cultivars 'Passe Crassane,' 'Doyenné du Comice' and 'Beurré Hardy' formed a subcluster within the Occidental cultivars.

The Principal Coordinate Analysis projection obtained (Fig. 3) provided similar information to the dendrogram although with small differences among the *P. communis* group, derived from the calculation method, which implies a reduction of 63% in the global variation. This situation occurred with 'Williams Rouge,' separating more from Portuguese cultivars and moving closer to French ones, and with 'Rocha' moving closer to 'Carapinheira Parda' than shown in the dendrogram.

The amplification experiment compared RAPD analysis of in vivo and in vitro leaf material and gave identical patterns for each of the seven genotypes and four primers, as shown in Fig. 4.

4. Discussion

In this study RAPDs detected a high degree of polymorphism among several species and cultivars of the genus *Pyrus*, providing useful information for classification within this genus. However, it is necessary to remember the important limitations of this method, when interpreting the results. Above all, we have to consider the mentioned lack of reproducibility of the technique. In this experiment 93% of the bands were present in the three replications and therefore

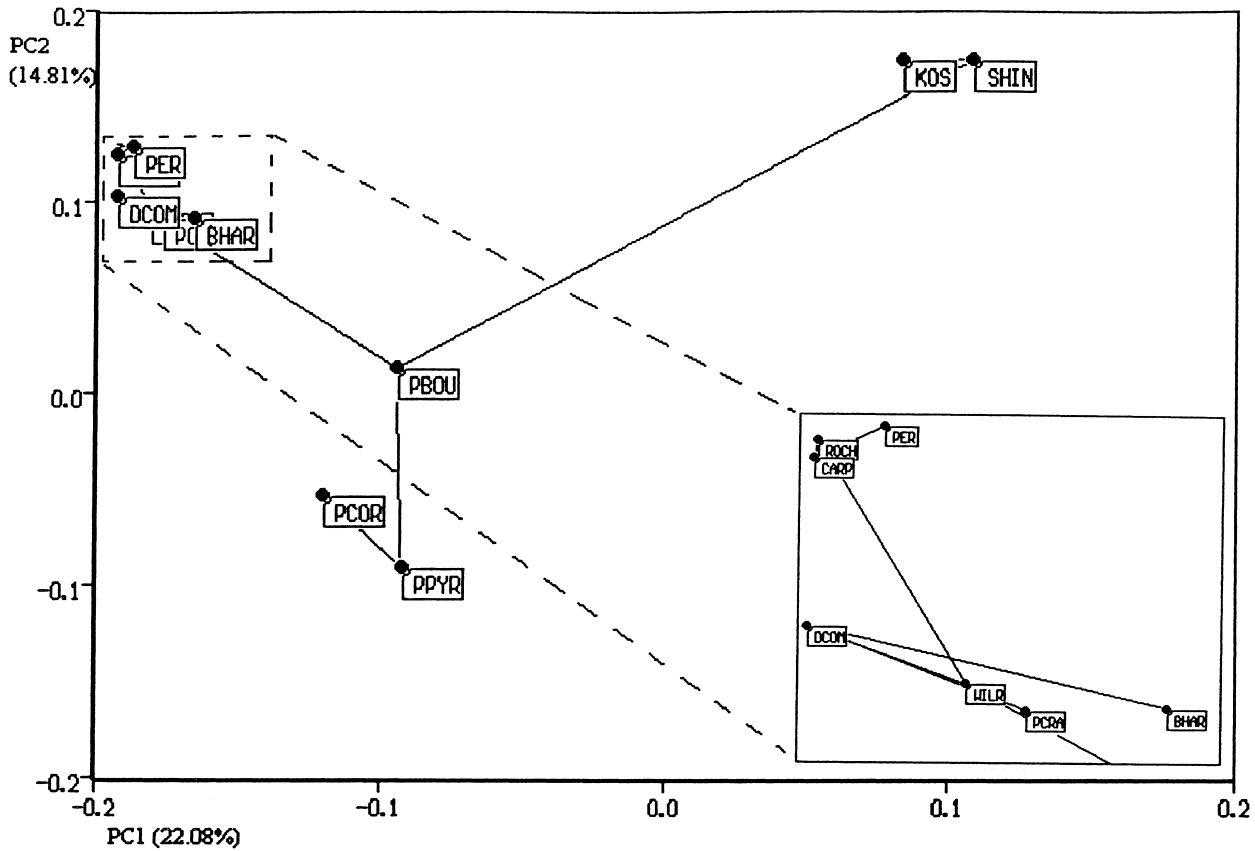

Fig. 3. A two-dimensional association and Minimum Spanning Tree among the 12 genotypes revealed by Principal Coordinate Analysis based on Nei and Li's (1979) similarity coefficients.

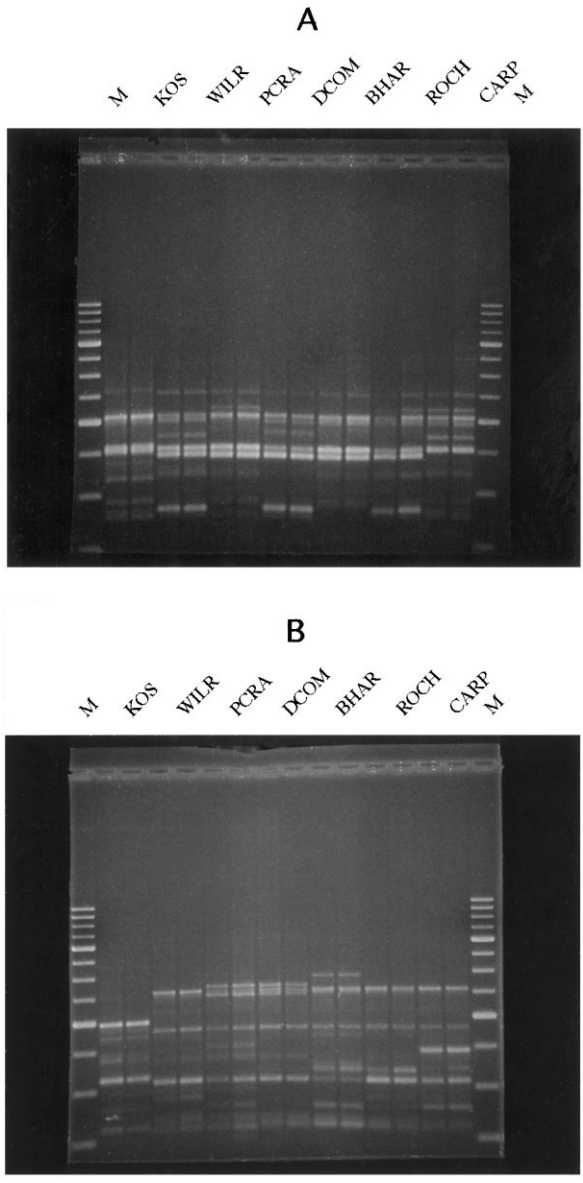

Fig. 4. Comparison of RAPD patterns between in vivo (even lanes) and in vitro (uneven lanes) leaf material, obtained with primers OPA02 (A) and OPA18 (B). Lanes 1 and 15: M, DNA size markers (DNA Molecular Weight Marker XVI, Boehringer Manheim, 250 bp ladder). See Table 2 for cultivar abbreviations.

this was not a real issue. Also, different amplification products of the same size in different samples may be indistinguishable on the electrophoresis gel (Williams et al., 1990). Nevertheless, it is generally agreed that, when using close

genotypes, apparently identical bands indeed represent the same product (Tingey and Tufo, 1993), although the identity of the products should be verified. In order to assure reproducibility it is necessary to select with care the concentration of genomic DNA, the temperature profile of the amplification program, the magnesium concentration and the thermostable polymerase used. Therefore, it is very important to establish the conditions of the analysis, particularly the amount of DNA used. The addition of too much genomic DNA results in smeary patterns, while the use of too little leads to irreproducibility. The intensity of the products on the gel depends strongly on the number of amplification cycles and on the amount of DNA template. Analysis of the results is very dependent on the electrophoresis run, and it is of great importance that the separation of the bands in the gel provides a clear pattern. This fact is even more important when the purpose is choosing primers that provide a clear distinction between cultivars. In order to increase the reliability of the results, the amplifications should be performed three times and only reproducible and well-marked bands should be considered for numerical analysis. The similarity coefficient used (Nei and Li, 1979) is adequate for dominant markers, as is the case with RAPD markers, where only presence or absence of a band is scored.

The similarities established among the different genotypes of *P. communis* and between different species were close to those usually accepted. These similarities were related to the geographic or to the phenotypic proximity of the genotypes. French, Portuguese and Asiatic cultivars, formed distinct clusters.

Similar results were obtained when reducing the number of RAPDs considered in the statistical analysis. Significant correlations between similarity matrices, $r \geq 95\%$, were obtained when decreasing randomly the number of rows introduced from 350 up to 150 rows, supporting the idea that RAPD markers can be of great value as a fast method for taxonomic studies, in spite of the disadvantages caused by the lack of easy reproducibility. Within sets of similar cultivars, the distinction through RAPD markers was more difficult, as RAPD analysis provides only a medium level of polymorphism (multiplex ratio between three and twelve, agreeing with Rafalski et al., 1996), being less suitable to distinguish very close genomes like different clones from the same cultivar or siblings, where very similar patterns are expected. This aspect was tested in our work and identical patterns with six primers for nine clones of the Portuguese cultivar 'Rocha' were found (data not shown). In these cases, where precise identification demands very rigorous distinction between very close genomes, markers like VNTRs (variable number of tandem repeats) or restriction fragments obtained by the AFLP technique might be more suitable. The latter method which involves selective amplification of an arbitrary subset of restriction fragments, generated by total digestion of the genome with single or double-enzyme combinations, has been tested for clonal distinction; however, its higher cost compared with RAPD does not justify its use for cultivar identification.

Acknowledgements

The authors thank the Director of ENFVN for continuous support and facilities and Dr. J. Leitão (UCTA-UAlg) for scientific advice. CO thanks Dr. K. Tobutt (HRI, UK) for critical reading and correcting the manuscript. This work was financed by PIDDAC-project 810 and PRAXIS XXI – project 3/3.2/HORT/2163/95.

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