Genetic Characterization and Relatedness among Cherry Cultivars in a Germplasm Bank by Randomly Amplified Polymorphic DNA Analysis

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SUMMARY

Random amplified polymorphic DNA (RAPD) analysis was performed on 38 cultivars of cherry (*Prunus avium* L.) grown in the Jerte Valley, Cáceres, Spain. Thirty five selected decamer primers produced 69 reproducible polymorphic amplification products. The degree of polymorphism detected made possible the identification of all the cultivars by combining the RAPD banding patterns of only seven primers: OPK-08, OPQ-14, OPR-09, OPS-19, OPX-02, OPX-15 and OPZ-13. Eleven unique markers allowed identification of nine cultivars while 15 cultivars were identified by unique banding patterns. A similarity matrix derived from the RAPD amplification products generated by all the primers was obtained using the index of similarity of Jaccard. The similarity coefficients among cultivars ranged from 0.27 to 0.81 with an average of 0.50. A dendrogram based on UPGMA clustering method was constructed using the similarity matrix. The dendrogram showed a good correlation between the clustering of cherry cultivars and their geographic origin, especially revealing a stronger genetic proximity between some of the most characteristic cultivars of the Jerte Valley. This result supports the autochthonous origin hypothesis for these cultivars.

KEY WORDS

Prunus avium L.; DNA fingerprinting; collection; RAPD

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INTRODUCTION

Sweet cherry (*Prunus avium* L.) is a deciduous tree originated from around the Caspian and Black Seas. It is a diploid species (2n=16), allogamous, generally self-incompatible, that is cultivated for its wood and edible fruit. A very large number of sweet cherry cultivars have been described, most of them resulting from the opened pollination of old cultivars, with selection of the resulting seedlings being carried out in the production areas. New cultivars from controlled crosses are more recent (Bargioni, 1995).

Spain is currently the sixth world producer, with an annual production of 100.000 t. The main zone of production in Spain is the Jerte Valley, Cáceres, Extremadura, a relative small area (situated in the Centre-West of the country) (Figure 1) that yields around 30 million kilos of cherries. Traditionally the cultivation of cherries of the Jerte Valley has been based on autochthonous or old introduced cultivars that have adapted to the local climatic and agricultural conditions (Herrero, 1964; Alonso, 1967), however, recent decades have seen the massive introduction of cultivars used in others zones. This introduction has induced the genetic erosion of the traditionally cherry germplasm in the Jerte Valley, with the reduction of the area occupied by the local cultivars.

A prospecting survey was initiated in 1992 for establishment of a germplasm bank for old local cherry cultivars in the Jerte Valley that would guarantee conservation of this genetic resource (Moreno and Trujillo, in press). Availability and study of genetic diversity is interesting for plant breeders since a representative reserve of alleles and genotypes may become useful for responding to future changes. In many cases the names of Jerte Valley cherry cultivars refer mainly to some morphological trait (particularly of the fruit), the person who introduced them, or their supposed zone of origin. This has led to the presence of a large number of homonyms and synonyms in local cherry cultivars (Moreno and Trujillo, in press). The diversity of cultivars and the confusion in naming require precise methods of discrimination for cultivar identification of cherry cultivars.

Classical approaches for identification and analysis of genetic variability in cherry cultivars are based on morphological, physiological and agronomic traits (UPOV, 1976; Schmidt et al., 1985). However, these traits have limitations since they are influenced by environment and the need of extensive observations of mature plants. Moreover, the morphological and agronomic traits are limited and do not cover the entire genome which limits their use in assessing genetic diversity. More recently DNA-based markers have been used for genetic diversity and fingerprinting studies such as RAPDs (Gerlach and Stösser, 1998), AFLPs (Struss et al., 2001, 2003; Tavaud et al., 2001) and SSRs (Wünsch and Hormaza, 2002, 2004; Schueler et al., 2003; Struss et al., 2002, 2003).

Figure 1. Location of Jerte Valley in Spain.

Among the different DNA-based markers the simplicity of laboratory assays for RAPD (Williams et al., 1990) make them an attractive method for cultivar identification and biodiversity studies. Reproducibility problems with the RAPD can be overcome by replicate analyses, involving independently isolated DNA samples and performed at different times. Caution regarding data analysis (band selection and scoring), seems to be critical for maintaining a high level of accuracy (Weeden el al., 1992).

The objectives of the present paper were to asses the polymorphism of RAPD markers to discriminate cherry cultivars from the Cherry Germplasm Bank of Junta de Extremadura and to study genetic relationships among them.

MATERIAL AND METHODS

Plant material and DNA extraction. Thirty eight cherry cultivars were used in this study (Table 1). Twenty nine were obtained from the Cherry Germplasm Bank of the Junta de Extremadura in Cáceres, Spain, and all of them were collected from prospecting survey at Jerte Valley (Moreno et al., 2001). Nine cultivars were obtained from nurseries including cultivars of different geographic origins. Genomic DNA was extracted from young leaf tissue collected in spring, following the method of Belaj et al., (2001).

Polymerase Chain Reaction. 116 decamer oligonucleotides from kits C, D, J, K, Q, R, S, X, and Z (Operon

Table 1. Cherry cultivars analyzed with their origin: Jerte Valley Bank of Germplasm (BG), nursery (Nur)

Cultivar	Origin	Cultivar	Origin
Ambrunés	BG	Mollar de Cabezuela	BG
Ambrunés Rabo	BG	Mollar Temprana	BG
Burlat	Nur.	Napoleón	Nur
Coloradilla	BG	Navalinda	BG
Corazón de Pichón	BG	Noelia	BG
Cubeto	BG	Pedro Merino	BG
del Cardito	BG	Pico Colorado	BG
del Gordo	BG	Pico Colorado Especial	BG
del Pollo	BG	Pico Limón	BG
Duroni 3	Nur.	Pico Limón Negro	BG
Garrafal del Jerte	BG	Pico Negro	BG
Guardamontes	BG	Pretera	BG
Hedelfingen	Nur.	Ramón Oliva	BG
Jarandilla	BG	Reverchón	Nur.
Lampé	BG	Starking Hardy Giant	Nur.
Lapins	Nur.	Van	Nur.
Lucinio	BG	Venancio	BG
Lucinio de Jerte	BG	Velvet	Nur.
Mollar de Cáceres	BG	Virgo Juliana	BG

Technologies, USA) were screened by PCR. DNA was amplified in 20 μL reaction mixtures containing 20–50 ng of templete DNA, 0.05 U AmpliTaq DNA polymerase Stoffel Fragment (Applied Biosystems, USA), 0.75 mM each dNTP (Roche, Switzerland), 20 μM of the primer, 25 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, (pH 8.3). The reactions were performed in a thermal cycler "Gene Amp PCR System 9600" (Applied Biosystems, USA) programmed for 1 cycle of 1min at 94 °C followed by 40 cycles of 20 s at 94 °C, 20 s at 35 °C and 2 min at 72 °C, for denaturing, primer annealing and extension, respectively. The last cycle was followed by incubation for 6 min at 72°C. All the reactions were repeated three times using DNA of different extractions and different lots of the AmpliTaq DNA polymerase.

Separation and visualization of the amplification products. Agarose gels (AGE) (Seakem, Nusieve, FMC, USA) of 25 x 15 cm (2% w/v) were prepared and run in TBE, pH 8.3, (100 mM Tris-HCl, 89 mM boric acid, and 1 mM Na_2EDTA), buffered at 120 V for 3.3 h, stained with ethidium bromide (0.5 mg.mL^{-1}), and photographed under ultraviolet (UV) light using a DC 120 Digital camera (Kodak, Rochester, N.Y.). Molecular sizes of the amplification products were estimated using a 123-base pair (bp) DNA ladder (Sigma Chem. Co., USA).

Data analysis. RAPD bands were scored from photographs as 1 (present) or 0 (absent) for all markers and for all individuals in the study, and a binary matrix was constructed from the data. A conservative criterion for the selection of bands was used. Only reproducible and well-defined bands for each of the tree replications were considered as potential polymorphic markers. Each RAPD fragment useful for discrimination between genotypes was denoted by the primer used and its approximate size in base pairs (Figure 2). The level of polymorphism of the primer (polymorphic bands per total bands) and its number of banding patterns (different combinations of bands obtained with cultivars) were calculated. Relative frequency of unique markers (number of cultivars with the same unique marker/total number of cultivars) was also calculated. A similarity matrix was generated using Jaccard´s coefficient. A dendrogram was constructed based on the similarity data using unweighted pair group method with arithmetic averages (UPGMA). The computer program used was NTSYS-pc version 2.02 (Rohlf, 1998). Cophenetic correlation coefficient was calculated between the similarity matrix and the cophenetic matrix.

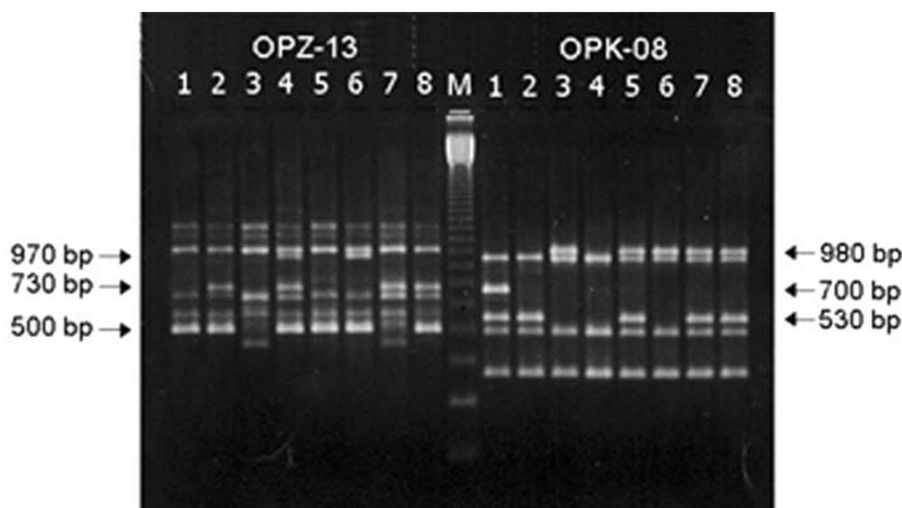

Figure 2.
RAPD bands profiles generated by the Operon primer OPZ-13 and OPK-08 for different cherry genotypes included in this study. 1–Guardamontes, 2–Lapins, 3–Garrafal del Jerte, 4–Ramón Oliva, 5–Lampé, 6–Pedro Merino, 7–Jarandilla, 8–Duroni 3. M–Molecular weight marker 123-bp ladder.

RESULTS

Variability study with RAPD markers. Thirty five, out of 116 primers evaluated, were selected according to polymorphism and reproducibility in the three replications of their amplification products (Table 2). A total of 69 polymorphisms (1.9 polymorphic markers per primer) out of 167 reproducible products (4.6 products per primer) were obtained, corresponding to 41.3 % of the amplification products. The number of bands per primer ranged from one (OPC-15) to eight (OPR-08 and OPS-19). The number of polymorphic bands per primer ranged from one to five. Amplified DNA products ranged in length from 280-bp (OPS-17) to 1170-bp (OPR-04).

The frequency of polymorphic bands in the cultivars studied varied from 0.026 (bands present in only one cultivar of the 38 studied) to 0.974 (bands absent in only one). The selected primers yielded 124 banding patterns (3.5 per primer). The number of banding patterns per primer ranged from two to 10 (Table 2).

Cultivar identification with RAPDs. The variability found allowed identification of the cultivars in various independent ways: unique RAPD markers, unique banding patterns, and combination of the banding patterns provided by different primers.

In this study we have found eleven unique markers (presence or absence of one band as compared to the rest of the cultivars) that allow the identification of nine cultivars (Table 3). Regarding the unique banding patterns, a total of 25 have been found that allow the identification of 15 cultivars (Table 4). Primers differ significantly in their ability to differentiate, depending on the number of polymorphic bands and their frequencies. Using a combination of several primers greatly enhances the ability to discriminate, thus a combination of seven primers (OPK-08, OPQ-14, OPR-09, OPS-19, OPX-02, OPX-15 and OPZ-13) allowed identification of all the cultivars included in this study. Other combinations of primers can provide other possibilities for identification of all the cultivars.

All homonyms considered in this study 'Lucinio' and 'Lucinio de Jerte', 'Mollar de Cáceres' and 'Mollar de Cabezuela', could all be discriminated by different primers.

Genetic relationships among cherry cultivars. A relatively high range of similarity values among genotypes (data not shown) was observed, with similarity measures ranging between 0.27 and 0.81 and an average of 0.50. The greatest similarity were obtained between the cultivars 'Navalinda' and 'Pico Limón Negro' and the smallest similarity were obtained between the cultivars 'Guardamontes' and 'Pico Negro'.

The dendrogram resulting from the UPGMA cluster analysis is shown in Figure 3. The value of the cophe-

Table 2. Selected primers. Their sequences, the number of polymorphic (P), the total number of bands (T), and the number of bands patterns obtained among the 38 cultivars studied

Primer	Sequence 5´ to 3´	P/T	BP
OPC-03	GGGGGTCTTT	1/2	2
OPC-06	GAACGGACTC	2/3	3
OPC-11	AAAGCTGCGG	2/5	3
OPC-15	GACGGATCAG	1/1	2
OPC-20	ACTTCGCCAC	3/4	4
OPD-02	GGACCCAACC	1/5	2
OPD-03	GTCGCCGTCA	4/6	8
OPD-07	TTGGCACGGG	2/6	3
OPD-08	GTGTGCCCCA	3/5	5
OPD-13	GGGGTGACGA	2/6	3
OPD-16	AGGGCGTAAG	2/5	4
OPD-20	ACCCGGTCAC	2/6	4
OPJ-11	ACTCCTGCGA	1/4	2
OPJ-20	AAGCGGCCTC	1/5	2
OPK-08	GAACACTGGG	3/5	5
OPQ-05	CCGCGTCTTG	2/4	4
OPQ-14	GGACGCTTCA	1/3	2
OPQ-15	GGGTAACGTG	1/6	2
OPQ-17	GAAGCCCTTG	3/4	7
OPR-03	ACACAGAGGG	2/4	3
OPR-04	CCCGTAGCAC	1/2	2
OPR-05	GACCTAGTGG	1/4	2
OPR-08	CCCGTTGCCT	1/8	2
OPR-09	TGAGCACGAG	5/7	10
OPR-12	ACAGGTGCGT	2/7	3
OPR-20	ACGGCAAGGA	1/6	2
OPS-03	CAGAGGTCCC	1/2	2
OPS-07	TCCGATGCTG	1/5	2
OPS-17	TGGGGACCAC	1/6	2
OPS-19	GAGTCAGCAG	2/8	4
OPX-02	TTCCGCCACC	3/3	4
OPX-15	CAGACAAGCC	3/6	5
OPZ-07	CCAGGAGGAC	3/6	4
OPZ-09	CACCCCAGTC	2/2	4
OPZ-13	GACTAAGCCC	3/6	6
Total		69/167	124

Table 3. Cultivars identified by the use of unique markers, and criteria used to score them

Cultivar identified	Unique markers	Identification criteria
Colorodilla	OPR-12.430	Presence
del Cardito	OPC-11.500	Presence
Guardamontes	OPD-08.920	Absence
Guardamontes	OPD-13.890	Presence
Guardamontes	OPK-08.700	Presence
Hedelfingen	OPC-06.370	Presence
Lampé	OPD-07.630	Presence
Lucinio	OPZ-07.1090	Presence
Starking Hardy Giant	OPX-02.490	Absence
Velvet	OPR-03.900	Presence
Venancio	OPX-02.690	Presence

Table 4. Cultivars identified by unique banding patterns

Cultivar	Primer
Coloradilla	OPR-12
Corazón de Pichón	OPD-03
del Cardito	OPC-11, OPR-09
del Gordo	OPR-09
Garrafal del Jerte	OPZ-13
Guardamontes	OPD-08, OPD-13, OPK-08, OPQ-17
Hedelfingen	OPC-06, OPQ-17, OPS-19
Lampé	OPD-03, OPD-07, OPQ-17
Lucinio	OPZ-07
Pedro Merino	OPQ-17, OPZ-13
Pretera	OPR-09
Starking Hardy Giant	OPX-02
Velvet	OPQ-17, OPR-03
Venancio	OPX-02
Virgo Juliana	OPR-09

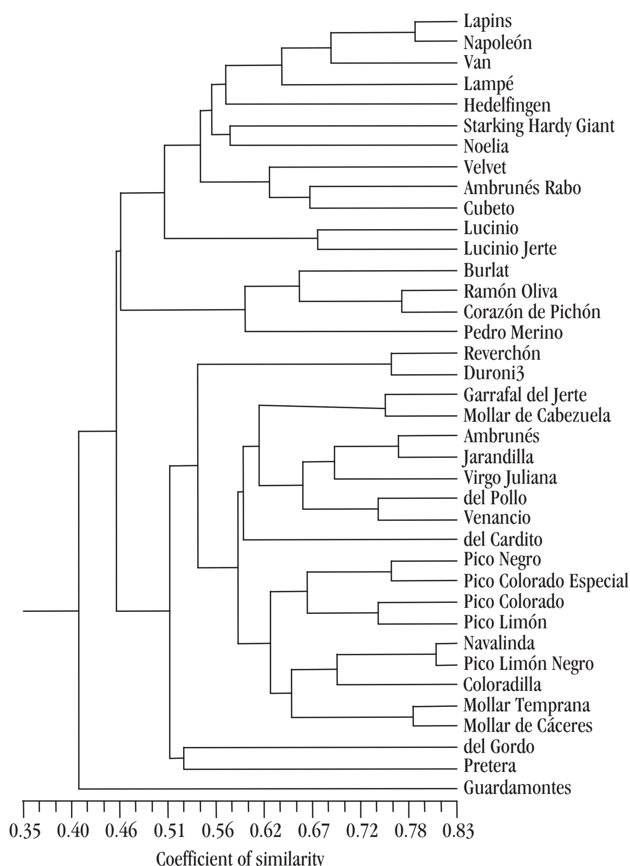

Figure 3.
An UPGMA dendrogram based on tha Jacccard similarity index among 38 cherry cultivars for 69 RAPD polymorphic fragments.

netic correlation coefficient between the dendrogram and the original distance matrix was 0.76. Most of the cultivars could be classified into three major groups. Evidence of relationships for most of the cultivars according to their geographic origin was found.

Group 1 is constituted by ten cultivars: four cultivars obtained from breeding programs in North America 'Van', 'Starking Hardy Giant', and 'Velvet' (Brooks and Olmo, 1952) and 'Lapins' (Lane and Schmid, 1984), two old German cultivars 'Napoleón' (Bargioni, 1995) and 'Hedelfingen' (Lichou et al., 1995), and four cultivars of uncertain origin 'Lampé' described and cultivated in Spain (Cambra, 1974) and 'Ambrunés Rabo', 'Cubeto' and 'Noelia' which not were previously described and were found in the Jerte Valley.

The second group includes four cultivars of possible French origin: 'Burlat' and 'Corazón de Pichón' ('Coeur de Pigeon') (Societé Pomologique de France, 1947), 'Pedro Merino' (a synonymous of 'Garrafal Tigré' (Cambra, 1974)) and 'Ramón Oliva' (Hedrick, 1915). These cultivars have two unique marker bands in common (OPD-03.610 and OPZ-13.1010).

Group 3 includes 19 cultivars and is composed of two clusters. The first cluster includes two cultivars of Italian origin: 'Duroni 3' (Lichou et al., 1995) and 'Reverchón' (Societe Pomologiqué de France, 1947), they show a high level of similarity of 0.76 and has a unique marker band (OPJ-11.500) in common. The second cluster includes 17 cultivars collected from exploration survey at Jerte Valley, and can distinguish two subclusters, the first includes the cultivars: 'Garrafal del Jerte', 'Mollar de Cabezuela', 'Ambrunés', 'Jarandilla', 'Virgo Juliana', 'del Pollo', 'Venancio' and 'del Cardito' and the second includes the cultivars 'Pico Negro', 'Pico Colorado Especial', 'Pico Colorado', 'Pico Limón', 'Navalinda', 'Pico Limón Negro', 'Coloradilla', 'Mollar Temprana' and 'Mollar de Cáceres', these subclusters appear to belong to two populations obtained by local propagation in two separate zones of Jerte Valley, around the villages of Cabezuela del Valle and Navaconcejo, respectively.

The dendrogram includes five cultivars, from Jerte Valley, relatively unrelated to the main groups. This was the case of the pairs 'Del Gordo' and 'Pretera', and 'Lucinio' and 'Lucinio Jerte', and the cultivar 'Guardamontes'.

DISCUSSION

The level of polymorphism observed with the 35 selected primers was 41.3%. This is higher than that obtained by Gerlach and Stösser (1998) in cherry (27.8%) with 18 cultivars. These differences could be explained by differences in the primer sets used, criteria for selecting markers and the number and origin of the cultivars analyzed. The polymorphism observed is lower than that reported by Resta (1998) in almond (57.7%) with 17 cultivars, but higher than that reported by Warburton and Bliss (1996) in peach (39%) with 136 cultivars. There is a correlation between the variability found in a species and its mating system: it is low in the self-pollinating (self-compatible) species as peach, and high in the allogamous and self-incompatible species as almond,

the same results have been obtained with isozymes in *Prunus* (Byrne and Littleton, 1989).

Identification of the 38 cherry cultivars has been possible using a combination of seven primers (out of 35). Furthermore a high number of cultivars (15) could be identified with just only one primer, due to the presence of unique RAPD markers or unique banding patterns. A high discriminating ability of RAPD markers applied to cherry was also found by Gerlach and Stösser (1998).

The ability to identify a cultivar using just one primer or a small number of them demonstrate that RAPD markers are suitable for sure, easy, quick and inexpensive identification cherry cultivars. For management of a germplasm bank, the discrimination of homonymus and identification of synonyms between cherry denominations is particularly useful. The presence of genetic differences between varieties with the same name, agrees with previous morphological studies (Moreno and Trujillo, in press), which demostrates that generic names such as “Mollar” (soft) include different cultivars.

The data obtained in the present study show correlation between the grouping of cherry cultivars and their geographic origin. The fact that cultivars obtained from breeding programs in North America clustered together with old German cultivars could be explained by the fact that first cultivars which were introduced by the colonist to the New World had their origin from North Europe (Faust and Surányi, 1997). For the cultivars from Jerte Valley: ‘Noelia’, ‘Ambrunés Rabo’, and ‘Cubeto’ which branched to this group two hypotheses are proposed. First is that they are unidentified varieties originating in other zones of production, and second is that they are the result of crosses of local Jerte Valley varieties with varieties introduced from other areas.

Local selection of the best individual from seedling could explain the clustering together of tha majority of the cultivars from Jerte Valley. Besides, there is a great variability of morphological and agronomic characters. For instance, the fruit skin color range from vermilion (‘Mollar’) to black (‘Pico Negro’), the firm flesh includes very firm (‘Ambrunés’) and tender (‘Coloradilla’) cherries and the season of maturity for picking ranges from early (‘Navalinda’) to extremely late (‘Pico Colorado’) (Moreno and Trujillo, in press).

In summary, RAPD markers provided a good tool for identification of cherry cultivars. On the other hand, RAPD markers were able to group cherry cultivars according to their local origin and determine genetic similarities between cultivars. The higher level of similarity observed among old cultivars from the same or nearby geographic origins agrees with the hypothesis of autochthonous origin.

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