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Molecular characterization and similarity relationships among apricot (Prunus armeniaca L.) genotypes using simple sequence repeats

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Abstract A collection of 48 apricot genotypes, originated from diverse geographic areas, have been screened with 37 SSR primer pairs developed in different species of *Prunus* in order to identify and characterize the genotypes and establish their genetic relations. Thirty one of those primer pairs resulted in correct amplifications and 20 produced polymorphic repeatable amplification patterns with the 48 genotypes studied. A total of 82 alleles were detected for the 20 loci. All the genotypes studied could be unequivocally distinguished with the combination of SSRs used. The results obtained evidence for the cross-species transportability of microsatellite sequences, allowing the discrimination among different genotypes of a given fruit-tree species with sequences developed in other species. UPGMA cluster analysis of the similarity data grouped the genotypes studied according to their geographic origin and/or their pedigree information.

Keywords Cultivar characterization · Germplasm management · Molecular markers · Fruit breeding · Microsatellites · PCR

Introduction

The apricot is an edible fruit mainly cultivated in Mediterranean climates. Total world production has reached more than 2.5 million tons, although a few countries (Turkey, Iran, Italy, Pakistan, France, Spain, U.S.A. and Morocco) account for more than 80% of that production (Faostat 1999).

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Under the generic term 'apricot' four different species and one naturally occurring interspecific hybrid are usually included (Mehlenbacher et al. 1990): *Prunus armeniaca* L., the cultivated apricot; *Prunus sibirica* L., the Siberian apricot; *Prunus mandshurica* (Maxim.) Koehne, the Manchurian apricot; *Prunus mume* (Sieb.) Sieb. et Zucc., the Japanese apricot; and *Prunus*×*dasaycarpa* Ehrh., the black or purple apricot. All are interfertile diploid species with eight pairs of chromosomes (2n=16). Most cultivated apricots belong to the species *P. armeniaca* that originated in Central Asia where it has been cultivated for millennia and from where it was later disseminated both eastward and westward. The apricot was introduced into the Mediterranean region from Iran or Armenia around the first century BC (Zohary and Hopf 1993), although more-recently new introductions were made from the Middle East, especially into Southern Europe (Faust et al. 1998). The species can be classified into six main ecogeographical groups (Layne et al. 1996): Central Asian, East Chinese, North Chinese, Dzhungar-Zailij, Irano-Caucasian and European. Nevertheless, this classification is becoming complicated due to the introduction of new cultivars derived from crosses between genotypes of the different groups (Faust et al. 1998). The Central Asian group is the oldest and more diverse; most of the apricots belonging to this group are self-incompatible and show high chilling requirements. The Dzhungar-Zailij group includes mostly self-incompatible small-fruited cultivars. The Irano-Caucasian group includes mostly self-incompatible genotypes with low cold requirements from the Caucasian area, Iran, Iraq, North Africa and some cultivars from Southern Europe. The European group is the most recent and the least variable, comprised by mainly self-compatible genotypes, and includes also the commercial cultivars of America, South Africa and Australia. In spite of its lower variability most of the progress in apricot breeding has been carried out through hybridization and selection within the European group.

In recent years there has been an interest in breeding new apricot cultivars with two main objectives: on the one hand, to develop genotypes that can be cultivated in a broad area since most apricot cultivars are highly specific in their ecological requirements (Layne et al. 1996). Consequently, commercial production is limited to some locations, where usually one or two cultivars account for most of the production. On the other hand, to give a response to the menace of the Plum Pox Virus (PPV) that is causing important damage in most Mediterranean countries where more than 50% of the world production is concentrated (Faostat 1999). This development of new genotypes requires the availability of fast and reliable techniques to study the existing variability in the currently available germplasm and the identification and protection of the new releases.

Traditional methods to characterize and identify cultivars and rootstocks in fruit tree species are based on phenotypic observations, but this approach is slow and subject to environmental influences mainly due to the long generation time and the large size of the fruit trees. Consequently, new methods based on studies at the DNA level must be incorporated into fruit breeding programs to accelerate and optimize genotype fingerprinting and study the genetic relationships among cultivars (Wünsch and Hormaza submitted). Isozymes were the first molecular markers used in plant breeding (Tanksley and Orton 1983) and they have been extensively applied in several fruit and nut tree species (Torres 1990), including apricot (Byrne and Littleton 1989; Battistini and Sansavini 1991; Badenes et al. 1996). Although they are still useful for some applications, such as studies of interspecific variability or linkage to genes of interest, their use for fingerprinting purposes has been progressively reduced due to the small number of isozyme systems available, the generally low level of polymorphism obtained, and the influence of environmental factors. Consequently, the consensus for fingerprinting has moved towards DNAbased markers that are not influenced by the environment and are able to screen the whole genome, including coding and non-coding regions, revealing a higher degree of polymorphism. Among DNA-based markers, the most-widely used in fruit tree species are RFLPs, RAPDs and SSRs, also called microsatellites. In apricot, RFLP (De Vicente et al. 1998) and RAPD (Takeda et al. 1998; Hormaza 2001) markers have been employed to identify cultivars and to group them according to their genetic similarity.

More recently, microsatellites are becoming the marker of choice for fingerprinting purposes in most living organisms (Goldstein and Pollock 1997; Gupta and Varshney 2000). Microsatellites are based on tandem repeats of short (2–6 bp) DNA fragments scattered throughout the genome that lie between conserved sequences (Litt and Luty 1989; Weber and May 1989) and have been isolated and characterized in a large number of animal and plant species (Tautz 1989; Wang et al. 1994; Powell et al. 1996a; Lefort et al. 1999). Microsatellites show several advantages for genetic fingerprinting such as their high level of polymorphism, the fact that they are mostly codominant single-locus markers, their easy detection through PCR, their even dispersion along the genome, their high reproducibility that allows confident exchange and standardization of protocols among laboratories, their somatic stability, and the possibility of cross-species transportability (Powell et al. 1996b). Only recently have microsatellites been developed in fruit crops, but the current list includes apple (Guilford et al. 1997; Gianfranceschi et al. 1998; Hokanson et al. 1998), avocado (Sharon et al. 1997), cherry (Downey and Iezzoni 2000), citrus (Kijas et al. 1995), grape (Thomas and Scott 1993; Bowers et al. 1996; Loureiro et al. 1998; Sefc et al. 1998; Di Gaspero et al. 2000), kiwifruit (Weising et al. 1996; Huang et al. 1998), olive (Rallo et al. 2000) and peach (Cipriani et al. 1999; Sosinski et al. 2000; Testolin et al. 2000). However, their main drawback is the need for generating primers in every species of interest. The observation that primers developed in one species can amplify microsatellite loci of some genotypes of other species (Moore et al. 1991; Huang et al. 1998; Cipriani et al. 1999; Downey and Iezzoni 2000; Di Gaspero et al. 2000; Sosinski et al. 2000) could overcome that drawback. Consequently, it is necessary to study at a larger scale the cross-transportability of existing primers to closely related species. Thus, in this work, SSRs previously developed in other *Prunus* species, mainly peach, have been used to identify 48 different apricot genotypes obtained from diverse geographical regions and to analyze their genetic similarity.

Material and methods

Plant material

Forty eight different apricot genotypes obtained from diverse geographical areas (Table 1) maintained at the SIA-DGA experimental orchards located at the Campus de Aula Dei in Zaragoza, Spain, were analyzed in this study. The genotypes examined included 19 from Spain, 16 from USA and Canada, eight from France, three from Greece, one from Hungary and one from China.

DNA extraction

Genomic DNA extractions were performed as previously described (Hormaza et al. 1994) with some modifications. Approximately 50 mg of young leaves were ground in a 1.5-ml Eppendorf tube with 200 µl of extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% β-mercaptoethanol, 0.1% NaHSO₃). The samples were incubated at 65° C for 1/2 h, mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 6,000 g for 15 min. The upper aqueous phase was recovered and mixed with 130 µl of cold isopropanol. The nucleic acid precipitate was recovered through centrifugation at 13,000 g for 5 min, washed in 400 µl of 10 mM ammonium acetate in 76% ethanol for 1 hour, dried overnight at room temperature and re-suspended in 200 μ l of modified TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Extracted DNA was quantified spectrophotometrically (GeneQuant II, Pharmacia Biotech, Piscataway, N.J.), diluted to 10 ng/µl with modified TE buffer and used for PCR amplification.

DNA amplification

DNA amplification reactions were carried out in a PTC-100 thermocycler (MJ-Research, Watertown, Mass.). Thirty five primer pairs previously developed in peach, two in sour cherry and one in sweet cherry were used in this study. The amplification program used consisted of one cycle of 2 min at 94°C followed by 35 cycles of 45 s at 92°C, 1 min at 55°C, and 2 min at 72°C. The last cycle was followed by a final incubation for 5 min at 72°C. Amplification reactions were carried out in 20-µl volumes containing $20 \text{ mM Tris HCl, pH } 8.4, 50 \text{ mM KCl, } 4 \text{ mM } MgCl₂, 0.001\% gel$ atin, 100 µM of each dNTP, 0.2 µM of each primer, 0.45 units of *Taq* Polymerase (Life Technologies, Rockville, Md.) and 40 ng of genomic DNA overlaid with a drop of mineral oil. Following amplification the samples were stored at 4°C prior to electrophoresis. Amplified DNA fragments were analyzed by gel electrophoresis in 3% Metaphor agarose in 1×TBE buffer at 5 V/cm, stained with

Table 1 List of the 48 apricot cultivars included in this study

Cultivar	Origin
Bebecou	Greece
Bergeron	France
Blancos	Spain
Búlida	Spain
Canino	Spain
Carrascal	Spain
Castlebrite	U.S.A.
Comédie-Avilor	France
Corbató	Spain
Cristalí	Spain
Currot	Spain
EarlyQueen	U.S.A.
Fantasme-Avikour	France
Ginesta	Spain
Gönci Magyar	Hungary
Goldrich	U.S.A.
Harcot	Canada
Héléna du Roussillon-Aviera	France
Henderson	U.S.A.
Ivresse-Aviklo	France
Jordan	U.S.A.
Katy	U.S.A.
Luizet	France
Merino	Spain
Mitger	Spain
Modesto	U.S.A.
Moniquí	Spain
Moniquí Borde	Spain
Palabras	Spain
Palau	Spain
Peñaflor	Spain
Pepito Rubio	Spain
Pandora	Greece
Paviot	France
Piu Sha Sin	China
Précoce de Tyrinthos	Greece
Queen	U.S.A.
Rojo de Carlet	Spain
Rouge de Rivesaltes	France
RoyalRosa	U.S.A.
SpringGiant	U.S.A.
Stark Earli Orange	U.S.A.
Stella	U.S.A.
SunGlo	U.S.A.
Tadeo	Spain
Tomcot	U.S.A.
Veecot	Canada
Velázquez Tardío	Spain

ethidium bromide, and visualized under UV light with an imageanalysis system (GelDoc, BioRad, Hercules, Calif.).

Data analysis

The amplified fragments were scored as present or absent. A similarity matrix was generated using the equation discussed in Nei and Li (1979) based on the proportion of shared amplification fragments. Genetic relationships among the 48 genotypes included in this study were investigated using UPGMA cluster analysis of the similarity data and depicted in a dendrogram. All the analyses were computed with the NTSYS-pc ver. 2.02 program (Exeter Software, Setauket, N.Y.). Direct-count heterozygosities were calculated as the number of heterozygous genotypes at a given locus divided by the total number of genotypes.

Results

Repeatable amplifications were produced in apricot with 31 of the 37 microsatellites assayed (28 from peach, two from sour cherry and one from sweet cherry) and 20 (19 from peach and one from sweet cherry) produced polymorphic repeatable amplifications with the 48 genotypes studied (Tables 2 and 3). Those primer pairs revealed a total of 82 alleles ranging from two to eight alleles per locus, with a mean value of 4.1 alleles per locus. All the primer pairs except five (pchgms1, UDP96–005, UDP96–018, UDP98–409 and PS12A02) produced a maximum of two bands per genotype.

With the 20 primer pairs utilized it was possible to unambiguously discriminate all the accessions studied. Genotypes showing a single amplified fragment were considered as homozygous for that particular locus since segregation analysis is needed to detect the presence of putative null alleles (Callen et al. 1993).

Direct-count heterozygosities ranged between 0.08 for UDP98–405 to 0.94 for UDP98–021, with an average value of 0.51 for all the loci studied. There were several rare alleles detected in only one or few cultivars; two were present and one absent in 'Stella'; two were specific for 'Piu Sha Sin', one for 'Katy', and one was only present in 'Harcot' and 'Piu Sha Sin'.

The dendrogram generated from the UPGMA cluster analysis based on the Nei and Li similarity index classified all the 48 genotypes included in this study into two main groups depicted in Fig. 1. The first one includes most of the cultivars of European origin and can be divided into two subgroups; one comprises all the cultivars of Spanish origin and 'Bebecou' and 'Précoce of Tyrinthos' from Greece, 'Sunglo' and 'Royal Rosa' from the U.S.A., and 'Paviot' from France; the other subgroup is composed by European cultivars of non-Spanish origin. The second group includes cultivars of American origin and 'Pandora' from Greece and 'Piu-Sha-Sin' from China.

Fig. 1 Dendrogram of 48 apricot cultivars based on UPGMA analysis using the similarity matrix generated by the Nei and Li coefficient after amplification with 20 pairs of microsatellite primers

Table 2 List of microsatellites that produced polymorphic repeatable amplification patterns among the cultivars studied

Discussion

The results obtained in this work show that microsatellites can be effectively used for fingerprinting purposes in apricot. Actually, amplification was produced with 31

of the 37 microsatellite primer pairs tested, developed in other species, and 20 were revealed as useful in apricot. From those 20 primer pairs, 19 have been developed in peach and one in sweet cherry. The mean value of 4.1 alleles per locus obtained with the microsatellites that pro-

Table 3 List of microsatellites that did not result in scorable polymorphic repeatable amplification patterns among the cultivars studied

Locus name	Origin	Size range (bp)
pchcms1 pchcms2 pchcms3 UDP96-013 UDP96-015 UDP97-401 UDP97-403 UDP98-022 UDP98-024 UDP98-025 UDP98-407 UDP98-408 UDP98-410 UDP98-414 UDP98-416 PceGA34	Sosinski et al. 2000 Sosinski et al. 2000 Sosinski et al. 2000 Cipriani et al. 1999 Cipriani et al. 1999 Cipriani et al. 1999 Cipriani et al. 1999 Testolin et al. 2000 Testolin et al. 2000 Testolin et al. 2000 Cipriani et al. 1999 Cipriani et al. 1999 Testolin et al. 2000 Testolin et al. 2000 Testolin et al. 2000 Downey and Iezzoni, 2000	$130 - 150$ n.a. ^a $220 - 240$ 200 n.a. $80 - 120$ 120 120 $90 - 100$ 120 n.a. n.a. n.a. n.a. $90 - 100$ 150
[AB]	Downey and Iezzoni, 2000	210

^a n.a.: no or weak amplification

duced polymorphic amplification patterns is similar to the values reported in peach by Sosinski et al. (2000) and Testolin et al. (2000) who developed in that species most of the primer pairs used in this study. In this work, agarose-gel electrophoresis has been used for the screening of the microsatellites since, compared to polypolyacrylamide-gel electrophoresis or automated analysis, this is the most-appropriate technology for routine analysis of these kinds of markers especially for the transfer of the protocols to the productive sector such as nurseries or control agencies. However, it is possible that an automated detection system would be able to resolve allelic variation at a finer scale than gel-electrophoresis analysis and, consequently, the number of alleles obtained would even be higher than that reported in this work.

Although most of the primer pairs produced a maximum of two bands per genotype, five primer pairs produced more bands than expected according to the diploid constitution of this species, probably implying the duplication of some loci in apricot; in fact, the nuclear DNA content of apricot can be estimated to be about 10% higher than that of peach (Arumuganathan and Earle 1991; Bennet et al. 1998). This, however, cannot be considered a problem for fingerprinting purposes. On the other hand, the size range of most of the amplification fragments obtained in this work were similar to those reported for the same microsatellites in peach by Cipriani et al. (1999), Sosinski et al. (2000) and Testolin et al. (2000), and in sweet cherry by Downy and Iezzoni (2000).

All the genotypes included in this study could be differentiated with the combination of primer pairs used. This high discrimination occurs even between closely related genotypes. Thus, 'Castlebrite', 'Modesto', 'Harcot', 'Goldrich', 'Tomcot' and 'Veecot' have the cultivar 'Perfection' in their pedigree (Brooks and Olmo, 1997) and the French cultivars Fantasme, Ivresse, Hélena du

Roussillon and Comédie have Bergeron in their pedigree (Lichou 1998). This indicates that the approach used in this work could be valid to distinguish other accessions of this species. In this sense, they are currently being used (Hormaza and Herrero, unpublished data) to discriminate among putative clones of the same cultivar, 'Moniquí'.

The dendrogram generated from the UPGMA cluster analysis produced several significant groups related to the pedigree and/or the geographical origin of the genotypes. Thus, we can classify the genotypes into two large groups. The first one includes all the European cultivars except the Greek cultivar 'Pandora'; this group is also divided into two subgroups; one comprises all the cultivars of Spanish origin and 'Paviot' and 'Rouge de Rivesaltes' from France, 'Bebecou' and 'Précoce of Tyrinthos' from Greece, and 'Sunglo' and 'Royal Rosa' from U.S.A.; the other, includes the rest of the French cultivars and 'Gönci-Magyar' from Hungary. These results corroborate previous studies carried out using RFLPs (De Vicente et al. 1998) and RAPDs (Hormaza 2001) where 'Bebecou' also clustered in the group of Spanish cultivars. However, using isozymes Badenes et al. (1996) found Spanish cultivars in all of the subgroups generated by their study, probably due to the lower discrimination power of isozymes compared to DNA-based markers. The presence of other European cultivars in this subgroup would support the hypothesis that most of the Spanish cultivars have originated by hybridization between genotypes of the European group and genotypes from the Irano-Caucasian group introduced by the Arabs (Faust et al. 1998). However, we should expect a high level of variability among the Spanish cultivars, and the results obtained in this work suggest that they probably share a common genetic background and show a low degree of polymorphism compared to the other groups. This contradiction between an expected high variability due to hybridization in the Spanish cultivars and the results obtained could be explained (De Vicente et al. 1998) by an erosion of the variability present in the Spanish cultivars due to the small geographical area where apricot is cultivated and to the predominance of few cultivars such as 'Búlida', 'Canino' or 'Moniquí' that could be ancestors of most of the cultivars currently available in Spain. The presence of 'SunGlo' and 'Royal Rosa' in this group can be explained by the fact that all the American cultivars carry European germplasm in their pedigree, although combined to a higher or lesser extent with Asian germplasm. The second subgroup comprises the rest of the French cultivars and 'Gönci-Magyar' from Hungary. Interestingly, 'Luizet' and 'Gönci-Magyar' cluster together and it has been suggested (Faust et al. 1998) that the presence of the central-European cultivar 'Hungarian Best' occurs in the pedigree of 'Luizet'.

The other group includes the rest of the American genotypes, 'Piu Sha Sin' from China and 'Pandora' from Greece. 'Piu Sha Sin' and several other cultivars of American origin show most of the rare alleles found in

this study. The explanation for this observation could lie in the fact that most American cultivars have not only European germplasm in their pedigree but they have also been enriched with germplasm of Asian origin, thus increasing their level of polymorphism compared with the European cultivars. Unfortunately, the parentage of some of the earliest American cultivars is unknown.

Microsatellites are becoming the marker of choice for fingerprinting and genetic diversity studies in a wide range of living organisms. The approach described in this paper shows that microsatellite analysis is a powerful tool also for the characterization of apricot cultivars. However, the widespread practical use of microsatellite technology, or of any PCR-based marker technology that targets specific sequences, is hampered by the generation of primers that involve the construction of a genomic library and includes obtaining sequence data, primer design and synthesis as well as the testing of the selected fragments. The fact that none of the primers used in this study have been developed in apricot, demonstrates the possibility of cross-species transportability of a high number of SSRs and, consequently, the availability of a number of primers developed in one species of *Prunus* will most likely be useful to characterize other species of this genus. Thus, it would not be required to develop new microsatellite primers in every *Prunus* species. Moreover, since some primer pairs revealed even more alleles in apricot than in the species where they were originally developed, probably the performance of a given primer pair is more dependent on the primer itself than on the species where it originated. This possibility of using primers developed in one species to correctly amplify microsatellite loci in other species was already demonstrated in mammals at the beginning of the 90s (Moore et al. 1991) and has been later shown in a variety of species including fruit trees (Huang et al. 1998; Cipriani et al. 1999; Di Gaspero et al. 2000; Downey and Iezzoni 2000; Sosinski et al. 2000), but this work represents a confirmation at a much larger scale in a single species. It has been suggested that usually a greater genetic distance implies a decrease both in the ability to amplify the loci and in the amount of polymorphism detected (Steinkellner et al. 1997), as well as a shortening of the length of the microsatellites (Ellegren et al. 1997) probably due to the process of microsatellite development where the sequences selected usually are biased in favor of longer repeat regions (Perry and Bousquet 1998). The decrease in the amount of polymorphism detected has been confirmed in this study since several primer pairs did not produce polymorphic amplification patterns, but the number of available microsatellite primer pairs in *Prunus* suggests that this cannot be considered as a problem. On the other hand, a decrease in the ability to amplify the microsatellite loci has not been observed in this work. The genus *Prunus* is usually divided into five subgenera (Watkins 1976), although the cultivated species appear in three of them (Westwood 1993): Amygdalus (that includes peach and almond), Prunophora (plum and apricot) and Cerasus (sour and sweet cherry). In this work, primers developed in species of the subgenera Amygdalus and Cerasus have been successfully used in Prunophora. Consequently, it is likely that most of the primers developed in any *Prunus* species can work in other species of the genus, and the wider the taxonomic range where primers can be used the more economically interesting microsatellite development becomes. In this sense, most of the primer pairs used in this study can be successfully used for the molecular characterization of sweet cherry cultivars (Wünsch and Hormaza, submitted) and different *Prunus* species and interspecific hybrids used as rootstocks (Hormaza, in preparation). These results suggest that the genetic distance among species of *Prunus* should not be very high compared to other genera where microsatellites have been developed. In fact, nuclear DNA content estimates in the diploid species of the genus suggest that the small nuclear genome of *Prunus* is relatively stable, and probably this is also related to the frequent examples of interspecific hybrids or compatible graft combinations found between species of the genus (Baird et al. 1994).

The use of molecular markers for fingerprinting, germplasm management or breeding purposes can be even more advantageous in fruit tree species than in herbaceous species since cultivation of fruit trees has involved a change from sexual to vegetative propagation. Thus, cultivars of most fruit tree species are maintained by vegetative reproduction and selection has operated only during a limited number of generations; consequently, the current cultivars should not have diverged markedly from their progenitors whereas in most herbaceous crop species selection has operated continuously during centuries (Zohary and Hopf 1993). The results obtained from this study indicate that SSR sequences are highly conserved in *Prunus* and the currently available sequences in different species of *Prunus* will be useful not only for the management of apricot or *Prunus* genetic resources with the aim of establishing core collections and allowing the detection of homonymies and synonymies, but also for their use in plant breeding programs to design crosses that maximize genetic variability with the objective of obtaining new genotypes that could be cultivated in a wide geographical area as well as to study different hypotheses concerning germplasm movement in apricot. Work is under way to characterize and study other species of the genus including a more extensive germplasm repertory.

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