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Cultivar identification and genetic fingerprinting of temperate fruit tree species using DNA markers

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Summary

In recent years we have witnessed critical advances in the applications of molecular markers for genetic fingerprinting in cultivated plants. Their advantages have been widely recognised but they are even more important in woody perennials due to some particularities of these species such as their long generation time, their large individual size and their vegetative propagation. In this review, the information so far published in molecular fingerprinting of temperate fruit tree species using DNA markers is analysed with the goal of obtaining a common ground that will allow an easier and faster genetic identification that, at the same time, has to be reproducible among laboratories.

Introduction

The production of certified plant material in fruit and nut tree species requires the application of fast and reliable techniques to identify both old and new genotypes. Traditional methods to identify cultivars and rootstocks are based on phenotypic observations; however, this is a slow process due to the long juvenile period of the trees and it is subjected to environmental influences. The incorporation of new methodologies into fruit certification schemes will accelerate and optimise the identification process, by allowing the fingerprinting of each genotype at any stage of development and independently of environmental factors that may influence the phenotype. Furthermore, since most temperate fruit tree species are vegetatively propagated, the identification of genotypes allows a standardizable reference for the identification of any cultivar and to control its propagation.

During the last ten years we have witnessed fast and important advances in the methods used to analyse and study nucleic acids in both animals and plants. These studies have resulted in the development of different kinds of DNA markers that have been successfully applied in plant and fruit tree breeding. Among other applications, DNA markers are a useful tool for selection and gene introgression when they are linked to genes of interest (Hormaza et al., 1994a; Durman & Korban, 1994), for the construction of genetic maps (Rajapakse et al., 1995), and for the identification of cultivars and the study of the similarity and the genetic distance among them (Duneman, 1994).

Almost any kind of DNA markers can be used for fingerprinting fruit tree species. The most widely used have been RAPDs, RFLPs, AFLPs and, more recently, SSRs and ISSRs. In this paper, we review the work carried out using different molecular marker approaches for the genetic fingerprinting of the main temperate fruit tree species, including pome and stone fruits, olive and nuts, and analyse the advantages and disadvantages of the different methods.

RFLPs: Restriction Fragment Length Polymorphisms

RFLPs are DNA fragments obtained from a DNA digestion step followed by a hybridisation step, thus resulting in a specific DNA-restriction enzyme-probe pattern. Following their initial use in human genome studies in the late 70’s (Botstein et al., 1980), RFLPs have been widely used in plant breeding and finger-

printing (Tanksley et al., 1989; De Vicente et al., 1998).

Pome fruits

Initial work with RFLP markers to detect cultivar variation among apple (*Malus* × *domestica* Borkh.) cultivars and clones was carried out using a cDNA library of the micropropagated cultivar McIntosh 'Wijcik' (Watillon et al., 1991). A selected probe revealed enough polymorphism to distinguish among ten apple cultivars and six rootstocks, and it was possible to identify closely related cultivars as 'Golden' and 'Jonagold', but as expected, not the monogenic mutant 'Jonagored' developed from 'Jonagold'. Chloroplast and mitochondrial DNA probes have also been used for RFLP analysis. Thus, Ishikawa et al. (1992) used this technique to analyse eighteen apple cultivars and three rootstocks and observed three different cytoplasmic groups that could imply different genetic origins. On the other hand Nybom et al. (1990) used the minisatellite probe M13 for DNA fingerprinting in several Rosaceae species. They were able to differentiate the four apple cultivars included in the study with any one of the restriction enzymes used, and they detected intracultivar variation in one of them ('Rome Beauty'). However, in a later work (Nybom, 1990), the same approach did not allow to differentiate among fifteen different sports of the apple cultivar 'Red Delicious'.

Stone fruits

Within the stone fruits, the minisatellite probe M13 in combination with four restriction enzymes (Nybom et al., 1990), was used to differentiate four genotypes of black cherry (*Prunus serotina* Ehrh.). Later, 34 peach (*Prunus persica* L.) cultivars were identified with just nine RFLP fragments from six selected genomic clones that were used as probes (Rajapakse et al., 1995). Similarly, 45 different phenotypes from 52 apricot (*Prunus armeniaca* L.) cultivars (De Vicente et al., 1998) were identified using 31 selected probes developed in almond *(P. amygdalus,* Batsch.). The similarity matrix obtained from the molecular data was used to construct a dendrogram that separated the Spanish apricot cultivars from those from Europe and North America.

Other species

In olive (*Olea europaea* L.), RFLPs have also been used to identify five types of chloroplast-DNA patterns in cultivars, old local trees and oleasters (wild olives) from the Mediterranean basin (Amane et al., 1999). The study concluded that one type of chloroplast is mainly found in oleasters and cultivated trees, three are exclusive of oleasters and a fifth is found in both cultivated and oleasters. In walnut, RFLP markers were used to identify and estimate the genetic relationships among 48 accessions of Persian Walnut (*Juglans regia* L.) of different geographical sources grouping the genotypes studied into two main groups according to their origin (Fjellstrom et al., 1994).

Although RFLPs are a strong tool for many plant breeding applications, the main limitation for their utilisation in the identification of genotypes in plants lies in the low relation between the degree of polymorphism generated and the complexity of their use. As a consequence, new strategies mainly based on the Polymerase Chain Reaction (PCR), have been used to obtain molecular markers useful in genetic fingerprinting. Among these, RAPDs and microsatellites have been the preferred markers for fingerprinting fruit and nut tree species.

RAPDs: Randomly Amplified Polymorphic DNA

RAPDs are fragments of genomic DNA amplified through PCR using a decamer primer of random sequence, where polymorphism depends upon the presence or absence of an amplification product. The use of RAPDs in different organisms began in the late 80's (Williams et al., 1990), and due to their simplicity and speed they have become a very valuable tool for cultivar identification and genetic similarity studies in plants. RAPD markers have been used by several laboratories to identify genotypes of different temperate fruit tree species.

Pome fruits

Various examples of the application of RAPDs for fingerprinting and analysis of genetic similarities are available in apple. Thus, Koller et al. (1993) could differentiate eleven cultivars using just two primers, Mulcahy et al. (1993) were able to characterise twenty-five apple accessions representing eight cultivars again with two primers and Tancred et al. (1994) used a single primer to differentiate a new apple genotype from three similar commercial cultivars. Duneman (1994) used RAPD markers to differentiate and study the similarity of 27 apple cultivars and 18 accessions

of different *Malus* species and the results obtained agreed with their known pedigree or phylogenetic information. Similarly, Landry et al. (1994) fingerprinted eight apple rootstocks and the lines derived from them and confirmed the known genetic relationships among them. Although new molecular markers are currently available, RAPD markers are still being used for fingerprinting apple genotypes; thus, Autio et al. (1998) identified 15 rootstocks with just two primers and Oraguzie et al. (2001) have recently identified 155 genotypes representing new and old cultivars with 9 primers.

Contrasting to the work carried out in apple, molecular studies in pear have only recently been initiated. Thus, characterisation of the genus *Pyrus* has been carried out with RAPDs by Oliveira et al. (1999), that fingerprinted and calculated genetic relationships among nine pear *(P. communis* L.) cultivars and 3 wild *Pyrus* species. More recently, Monte-Corvo et al. (2000) also used RAPD markers to fingerprint and study the genetic similarities among 25 common and 4 Japanese pear [*Pyrus pyrifolia* (Burm.) Nak.] cultivars.

Stone fruits

RAPDs have been used extensively to fingerprint genotypes of the most agronomically interesting *Prunus* species, such as peach, almond, plum, cherry or apricot.

In peach, Warburton & Bliss (1996) analysed with RAPDs a set of 136 cultivars from different geographical origins. The genotypes clustered into twelve main groups. Nine of these clusters comprised the Asian cultivars while the European and American cultivars were grouped in three clusters revealing less genetic diversity. Lu et al. (1996) were able to distinguish among eighteen peach rootstocks with RAPDs, obtaining genetic similarity relations that agreed with their putative pedigree. In this sense, genetic diversity of forty-one *Prunus* rootstocks involving different species and interspecific hybrids was analysed with RAPDs by Casas et al. (1999), distinguishing three main clusters in agreement with previously defined groups.

Bartolozzi et al. (1998) studied the genetic relatedness and possible origins of seventeen California almond varieties using 20 RAPD primers, and they were able to distinguish all the cultivars from each other, except the bud sports mutations, and clustered the genotypes in three groups according to their pedigree.

Ortiz et al. (1997) were able to differentiate among twenty-eight hexaploid and three diploid plum cultivars of different geographical origins with the amplification fragments obtained with three primers, showing a clear separation between the diploid and hexaploid genotypes and a good correlation with their known pedigree. Similarly, Shimada et al. (1999) were able to discriminate among 42 plum cultivars of different ploidies with 20 RAPD markers establishing two major clusters (European and Japanese) with good correlation with previous classifications.

In sweet cherry (*P. avium* L.), molecular characterisation of a set of eighteen cultivars with RAPD markers was carried out by Gerlach & Stösser (1997). They were able to distinguish sixteen of the cultivars with twenty-three RAPD primers but, as expected, they found no differences among sports.

Shimada et al. (1994) studied the genetic relationships among 54 Japanese apricot (*P. mume* Sieb. et Zucc.) cultivars with 95 RAPD primers and classified them in seven groups that reflected their origin. Later, Takeda et al. (1998) investigated the relationships between thirty-three apricot cultivars and two related species (*P. sibirica* L. and *P. brigantina* Vill.) with 18 RAPD primers, clustering the genotypes into two main groups, cultivars originated in the East (eastern China and Japan) and cultivars from the West (Europe, Central Asia and Western China).

Olive

Initial RAPDs studies in olive involved the analysis of a group of seventeen olive cultivars from different origins by Fabbri et al. (1995) with seventeen RAPD primers. The resulting dendrogram clustered the cultivars according to their fruit size-oil content, but not in agreement with their geographic origin. Similar studies have been carried out using RAPD markers, in several sets of olive cultivars from the Mediterranean Basin. Thus, different accessions of six olive cultivars commonly cultivated in Israel and the West Bank were also fingerprinted with RAPDs by Wiesman et al. (1998). Fourteen primers were needed to conclude that the 'Nabali' group contains genetically different genotypes. On the other hand the four 'Souri' variants showed to be genetically identical. The fingerprinting analysis with RAPDs showed a high genetic diversity among the most common cultivated varieties in the area. Mekuria et al. (1999) investigated the genetic variability of twenty-two accessions of three common cultivars ('Manzanillo',

'Kalamata' and 'Verdale'), and seventeen accessions of other eight cultivars that are thought to include synonyms or homonyms. Six primers were used to differentiate among the accessions. A high degree of genetic variability was found among the 'Verdale' accessions while little or no variation at all was detected among the 'Manzanillo', 'Kalamata', 'Nevadillo' and 'Picual' accessions. Twenty-two Spanish olive accessions collected in areas geographically proximal, were identified with RAPD markers by Claros et al. (2000). Similarly, Besnard et al. (2001) distinguished 102 olive cultivars with 45 markers that grouped them according to their geographic origin and to their specific use. Belaj et al. (2001) identified 51 olive accessions obtained in diverse countries with 46 RAPD primers, obtaining a good correlation between similarity results and geographical origin. Finally, Sanz-Cortes et al. (2001) were able to identify 40 olive cultivars with 34 RAPD bands also finding a good correlation of the similarity data with their geographic origin.

Nuts

Initial work on molecular identification of pistachio (*Pistacia vera* L.) was carried out by Hormaza et al. (1994b) and Dollo et al. (1995). They examined fifteen pistachio cultivars with fourteen RAPD primers and clustered them according to their geographical origin distinguishing two major clusters, one comprising cultivars originated in the Mediterranean countries and the other from Iran and the Caspian Sea. In a latter experiment (Hormaza et al., 1998), the number of genotypes closer to the centre of origin analysed by RAPDs was increased, and the results agreed with earlier results since most of the new genotypes fell into the Iranian-Caspian cluster.

Nicese et al. (1998) characterised with eighteen RAPD primers a group of nineteen walnut genotypes that included closely related released cultivars and parents of breeding programs; the cluster analysis separated the genotypes into two groups based in the similarity with their ancestors.

Molecular fingerprinting of clones of sixteen sweet or European chestnut (*Castanea sativa* Mill.) commercial cultivars from the south of Italy was also carried out with RAPD markers (Galderisi et al., 1998). All the cultivars could be distinguished with the amplification pattern of nineteen primers and some mislabelled clones were uncovered. Oraguzie et al. (1998) compared morphological and RAPD markers of eighteen New Zealand chestnut accessions in an attempt to establish phylogenetic relationships and origin of the genotypes. The results obtained with 10 primers showed to be efficient in grouping the selections according to their geographic origin and to clear some hypothesis about the origin of the accessions.

All these results point out that RAPD markers can be successfully used to establish identification programs for a specific laboratory, but it is difficult to compare or reproduce results among different laboratories. Due to the short length of RAPD primers (10 bp), there can be many complementary sites in the genome, and consequently, the amplification pattern obtained may vary among different assays with the same material (Jones et al., 1997). An alternative technique to generate more reproducible markers from RAPD data could be the use of Sequence Characterized Amplified Regions (SCARs) (Paran & Michelmore, 1991). SCAR markers are obtained from a PCR reaction using longer primers synthesised after the sequencing of selected RAPD amplification fragments, resulting in a more restrictive and robust assay. This technique has been successfully applied for rootstock identification in *Vitis* (Xu & Bakalinsky, 1996) and cultivar identification in raspberry (*Rubus idaeus* L.) (Parent & Pagé, 1998).

AFLPs: Amplified Fragment Length Polymorphisms

Other kind of DNA markers used to a lesser extent in the identification of temperate fruit trees are AFLPs (Vos et al., 1995). These markers combine RFLP and PCR techniques, as they are specific PCR amplified fragments of restriction digests. Their use is more complex than that of RAPDs or SSRs since there are several steps involved besides PCR amplification and marker analysis. They require a genomic restriction digestion, ligation of adapters to the restriction ends and the use of primers that contain the adapter sequence, the enzyme target sequence and selective nucleotides. Although AFLPs are also dominant markers, they reveal a high level of polymorphism and a great amount of markers per assay, resulting in a very high discrimination power for germplasm analysis.

Angliolillo et al. (1999) used AFLP markers to establish genetic relationships in the genus *Olea* by comparing 43 olive cultivars, 30 wild olives and 9 *Olea* species. Five primer combinations produced 121 polymorphic AFLP markers that allowed the genetic analysis of the genotypes. Twenty-five common and

4 Japanese pear cultivars were also analysed with AFLPs by Monte-Corvo et al. (2000) that also studied the genetic similarities among the genotypes studied. They obtained similar results using AFLPs and RAPDs. Recently, Goulao et al. (2001) analysed 24 diploid and 4 hexaploid plum cultivars with AFLPs clearly separating both groups.

Despite the numerous reports on the application of AFLPs for fingerprinting purposes in annual plants, their use in identification of temperate fruit tree genotypes has been scarce, probably due to the availability of other approaches such as microsatellites that are easier to handle.

Microsatellites or SSRs: Simple Sequence Repeats

Besides the development of RAPDs and AFLP markers, newer PCR-based techniques are being increasingly used for fingerprinting purposes. Microsatellite markers or SSRs (Litt & Luly, 1989) are currently becoming the preferred technique for the molecular characterisation of different plant species (Gupta & Varshney, 2000). The presence in the eukaryotic genomes of sequences of repetitive DNA, flanked by specific regions, allows their amplification when the flanking sequences are used as primers in PCR. The polymorphism reflects changes in the number of repeats in each genotype. When there is not enough sequence data available, the process to identify, clone and sequence the flanking regions is cumbersome as it requires the construction and screening of genomic libraries. However, new alternatives are available to avoid the process of library construction and screening. One can be the RAHM (Random Amplified Hibridization Microsatellites) technique (Cifarelli et al., 1995) that hybridises repetitive sequences directly onto RAPD bands. Another alternative is based on recent results that demonstrate that flanking sequences can be transported among related species and genera (Cipriani et al., 1999; Di Gaspero et al., 2000; Hormaza, 2002; Huang et al., 1998; Sosinski et al., 2000; Yamamoto et al., 2001), opening the possibility of using sequences already isolated in a different species for identification purposes.

A variation on SSRs are ISSRs or Inter Simple Sequence Repeats (Zietkiewicz et al., 1994). In this system microsatellites are targeted to take advantage of their abundance but without the need of prior sequence knowledge to design the primers. ISSRs are obtained through the amplification of DNA found between microsatellites, by priming the PCR reaction with a repetitive sequence anchored by arbitrary or degenerate nucleotides. As a result, a large number of bands useful for fingerprinting purposes are obtained.

Pome fruits

Isolation and sequencing of microsatellite flanking sequences in the apple genome have been carried out by several laboratories and the obtained polymorphic loci have been used efficiently to determine the identity of various cultivars. Thus, twenty-eight microsatellite primer sequences were obtained from a genomic library of the cultivar 'Royal Gala' enriched with (GA) repeats (Guilford et al., 1997). With fourteen selected pairs of primers, polymorphic loci were observed in most of the twenty-one cultivars analysed, and Mendelian inheritance of those markers was confirmed in the progeny of a cross between 'Royal Gala' and clone A-172-2. Similarly, from an enriched library of the apple cultivar 'Florina', Gianfranceschi et al. (1998) developed sixteen microsatellite primers that were used for the identification of nineteen apple cultivars. Again two cultivars that differ in a few colour mutations ('Starking' and 'Red Delicious') could not be differentiated but it was possible to identify the triploid cultivars due to the presence of three alleles. Hokanson et al. (1998) used eight microsatellite markers developed in the cultivar 'Golden Delicious' to characterise sixty-six apple accessions. Through this screening they were able to determine the genetic diversity among the accessions, although seven pairs of accessions could not be differentiated due to being sport mutations or closely related genotypes. Similarity analysis clustered the accessions in agreement with their putative pedigree. In a recent work (Hokanson et al., 2001) analysed 142 accessions of 23 *Malus* species, hybrids and cultivars with the eight previously developed SSR primers. This set of primers was enough to distinguish all but five pairs of genotypes that were thought to be different, and revealed a high level of variation.

Although molecular studies in pear are scarce, SSRs developed in apple can be useful for pear fingerprinting. Thus Yamamoto et al. (2001) have used that approach to identify 36 pear accessions that included both Japanese pears (*P. pyrifolia*), Chinese pears (*P. bretschneideri Rehder* and *P. ussuriensis* Maxim.), European pears (*P. communis*), a wild species (*P. calleryana* Decne) and interspecific hybrids.

All the genotypes studied except mutants could be distinguished.

Stone fruits

Most SSR sequences in stone fruit species have been developed in peach. Cipriani et al. (1999) cloned and sequenced seventeen microsatellite primer pairs from the cultivar 'Redheaven'; fifteen of those were polymorphic and useful to identify ten peach genotypes and their Mendelian inheritance was demonstrated through analysis of a segregating backcross population. Testolin et al. (2000) used a set of twenty-six microsatellite sequences, including those published by Cipriani et al. (1999), to analyse 50 cultivars. Similarly to other molecular fingerprinting studies, the sport mutants could not be differentiated from the original genotypes. Another set of ten peach microsatellite loci have been reported by Sosinski et al. (2000), who screened 28 peach cultivars.

Microsatellite primer pairs developed mainly in peach have been used for fingerprinting other *Prunus* species. Thus, Downey and Iezzoni (2000) used one primer pair developed in sweet cherry, and 2 developed in sour cherry and peach to fingerprint 66 black cherry (*Prunus serotina*) genotypes; Cantini et al. (2001) used 10 primer pairs from peach, sweet and sour cherry to identify 59 tetraploid sour cherry (*Prunus cerasus* L.) genotypes; Hormaza (2002) identified 48 apricot genotypes with 20 primers pairs from peach grouping the cultivars according to their geographical origin and/or known pedigree information; similarly, Wünsch & Hormaza (submitted) have used nine peach primer pairs to fingerprint 76 sweet cherry genotypes grouping them according to their origin.

Regarding ISSRs, Goulao et al. (2001) have compared the results obtained analysing 24 diploid and 4 hexaploid plum cultivars with AFLPs and ISSRs. All the genotypes could be identified and the genotypes separated into two groups according to their ploidy. They reported higher reproducibility for ISSRs than for AFLPs.

Olive

Thirteen microsatellite loci have been identified in olive from a genomic enriched library of the cultivar 'Arbequina' (Rallo et al., 2000). This set of SSR primers were used to screen a set of 46 olive cultivars, five of them revealed polymorphic patterns of the expected size, and were useful to differentiate 95% of the genotypes analysed. A segregating population of 96 genotypes from the cross of 'Leccino' and 'Dolce Agogia' was also screened and Mendelian segregation was confirmed for those loci that were polymorphic between the parental genotypes.

Conclusions

The different works reviewed in this paper reveal that the studies to identify temperate fruit tree species with different molecular DNA markers, have succeeded in distinguishing among accessions, clarifying synonyms, identifying mislabelled cultivars, establishing genetic similarities or geographical origins and giving hints about the process of domestication. In fact, molecular identification is especially useful in fruit tree species, generally characterised by a high level of polymorphism among cultivars and no variation within cultivars due to vegetative propagation. DNA fingerprinting is even more important in the case of rootstocks since often there are little morphological differences among them, and once they have been grafted, their identification results extremely difficult.

However, most of the results reported deal with research carried out in unrelated laboratories and the cultivars studied vary among the different experiments, a situation that makes difficult the comparison of results among laboratories or among the different approaches used. Therefore, it is becoming necessary the establishment of standard protocols for molecular identification of these species; in fact, this standardisation is indispensable in order to be used in any official certification system.

Each type of marker system has advantages and disadvantages and it is necessary to evaluate the usefulness of each marker before its application. Among the DNA markers developed in the last two decades, probably microsatellites are currently the marker of choice for fingerprinting purposes in fruit tree species. One of the advantages is the high level of polymorphism they reveal due to the high mutation rates associated to the repetitive sequences. Another advantage is the fact that they are mostly codominant markers which allows to distinguish between heterozygous and homozygous individuals, at the same time they are inherited in a Mendelian fashion which permits to carry out paternity analyses. Furthermore the high reproducibility of microsatellites compared to other markers (McGregor et al., 2000) allows confident exchange and standardisation of protocols among laboratories. The main disadvantage of microsatellite

markers is the large amount of effort that has to be dedicated to isolate them. Nevertheless the possibility to transport microsatellite loci among species and genera, makes a whole set of isolated microsatellites readily available for germplasm characterisation. In the case of fruit tree species, microsatellites developed in apple and peach can be useful for other pome and stone species respectively. None of the other kind of currently widespread markers used for fingerprinting purposes (RAPDs, RFLPs, ISSRs or AFLPs) meet those advantages at the same level than microsatellites.

New DNA technologies are constantly being developed in human genomic research. Among those, SNPs (Single Nucleotide Polymorphisms) (Wang et al., 1998) seem to be of growing interest due to their high frequency. Although SNPs are just beginning to be used in plants (Coryell et al., 1999) and their application into fruit breeding schemes will not take place immediately, fruit tree identification will surely benefit from them in the future.

On the other hand, we have always to keep in mind that in several fruit species, such as apple, some of the main commercial cultivars are somatic mutations of different traits, 'sports', that constitute an improvement over the original genotype. However, since most 'sports' are single or few gene mutations, the original and the derived cultivar are identical at the genetic level except for the mutation responsible for the difference. As evidenced in some of the works described here the distinction between the original and the derived genotype is difficult using just the currently implemented molecular approaches. However, since most of these mutations are identifiable phenotypically (fruit colour, fruit shape, tree size, tree shape, branching habit, etc.), phenotypic observations must still complement the results obtained using molecular markers to identify clones that differ in one o few genes at least until new molecular methods become available. In fact, molecular identification is just another tool that will be added to the battery of approaches used to identify fruit tree cultivars and rootstocks and both phenotypic and genotypic observations must be mutually complementary for cultivar fingerprinting.

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