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RESEARCH PAPER

Discriminating ability of molecular markers and morphological characterization in the establishment of genetic relationships in cultivated genotypes of almond and related wild species

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Abstract: A total 23 morphological traits, 19 AFLP-primer combinations, 80 RAPD primers and 32 SSR primer pair were used to compare the informativeness and efficiency of random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers in establishing genetic relationships among 29 almond cultivars and three related wild species. SSRs presented a high level of polymorphism and greater information content, as assessed by the expected hetrozygosity, compared to AFLPs and RAPDs. The lowest values of expected hetrozygosity were obtained for AFLPs; however AFLPs showed the highest efficiency, owing to their capacity to reveal large numbers of bands per reaction, which led to high values for various types of indices of diversity. All the three techniques discriminated almond genotypes very effectively, except that SSRs failed to discriminate between 'Monagha' and 'Sefied' almond genotypes. The correlation coefficients of similarity were statistically significant for all the three marker systems, but were lower for the SSR data than for RAPDs and AFLPs. For all the markers, high similarity in dendrogram topologies was obtained, although some differences were observed. All the dendrograms, including that obtained by the combined use of all the marker data, reflect relationships for most of cultivars according to their geographic diffusion. AMOVA detected more variation among cultivated and related wild species of almond within each geographic group. Bootstrap analysis revealed that the number of markers used was sufficient for reliable estimation of genetic similarity and for meaningful comparisons of marker types.

Keywords: Amplified Fragment Length Polymorphisms (AFLPs); Random Amplified Polymorphic DNA (RAPDs); Simple-Sequence Repeats (SSRs); germplasm; genetic relationships; breeding; *prunus dulcis*

Introduction

In almond [*Prunus dulcis* (Mill.) D.A.Webb, syn. *Prunus amygdalus* Batsch] traditional methods for cultivar characterization and identification are based on phenotypic observations. These traditional approaches are slow and subject to environmental influences mainly due to the long generation time and the large size of fruit trees (Sorkheh et al. 2007a; 2009; Zeinalabedini et al. 2008). In order to supplement and refine the morphology-based descriptions the isoenzyme markers were used initially to assess genetic variability in almond (Arulsekar et al. 1986; Hauagge et al. 1987; Cerezo et al. 1989; Vezvaei 2003). Later, DNA-based markers provided enhanced opportunities for genetic characterization and biodiversity studies (Wunsch and Hormoza 2002; Shiran et al. 2005; Sorkheh et al. 2007a; 2007b). In recent years, random amplified polymorphic DNA (RAPD) markers have been used in several studies of genetic variability (Bartolozzi et al. 1998; Resta et al. 1998; Woolly et al. 2000; Sorkheh 2006; Zeinalabedini et al. 2008), probably because of their relative advantages of ease over other molecular techniques of that period (1990). Amplified fragment length polymorphism (AFLP) markers, developed by Vos et al. (1995), allowing greater precision, have subsequently been applied to study the genetic relationships among cultivated almonds, wild forms and related species (Sorkheh et al. 2007b).

On the other hand, Simple–sequence repeat (SSR) markers have now become the markers of choice for variability studies in *Prunus* species, because they are transferable, highly polymor-

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phic, multiallelic polymerase PCR-based co-dominant markers, and are relatively simple to interpret (Xu et al. 2004; Mneja et al. 2005; Xie et al. 2006; Martínez-Gómez et al. 2007). Genomic SSR markers have been developed in almost every cultivated fruit species of *Prunus*, including apricot (Lopes et al. 2002; Messina et al. 2004), peach (Cipriani et al. 1999; Testolin et al. 2000, 2004; Sosinski et al. 2000; Aranzana et al. 2002; Bliss et al. 2002; Dirlewanger et al. 2002; Georgi et al. 2002; Wang et al. 2002), sour cherry (Downey and Iezzoni 2000), Japanese plum (Mnejja et al. 2004), and sweet cherry (Clarke and Tobutt 2003). Among these, peach genomic SSR markers have been successfully used for molecular identification and genetic similarity analysis of genotypes within other species of *Prunus* like apricot (Hormaza 2002) and sweet cherry (Dirlewanger et al. 2002; Wunsch and Hormaza 2002; Schueler et al. 2003). Peach genomic SSRs derived from genomic libraries (Martínez-Gómez et al. 2003a; Testolin et al. 2004; Sánchez-Pérez et al. 2006) and SSRs derived from EST (Expressed sequence tags) sequences (Xu et al. 2004) have been assayed in almond.

In general, which technique is most appropriate for any given investigation is not obvious, and it depends on a number of factors, including the purpose of the research, the biology of the species and the resources available (Sorkheh et al. 2009). Comparisons of molecular markers for measuring genetic diversity have been conducted for several plants species (Powell et al. 1996; Milbourne at al. 1997; Pejic et al. 1998; Staub et al. 2000; Zeinalabedeini et al. 2008). In this sense, a better understanding of the various molecular markers is considered to be a high priority step toward almond germplasm characterization and classification, as well as a prerequisite for more effective breeding programs.

Genetic differences between varieties originate from their ultimate basis in differences between DNA sequences. Currently, an absolute measure of genetic difference is not technically feasible by any technique, as arguably this would require a comparison of entire genome sequences and probably an understanding of their incremental impact on the phenotype. Consequently, any methodology used to study variety differences will be a sampling strategy, so that estimations of similarity or dissimilarity often reflect the methodology used as well as the plant material being examined. Potential sampling strategies can involve comparisons based on morphological and/or biochemical characters, and comparisons based on molecular markers. The latter promises to provide a less-noisy reflection of the underlying DNA sequences than the more-traditional morphological characters (Roland-Ruiz et al. 2001). An important issue concerns the properties of the various sampling techniques. In comparisons between synthetic varieties of an out crossing species, the variance of a genetic-distance estimator will be principally affected by both the genetic differences between plants within the varieties (level of heterogeneity) and the precision with which the genome of individual plants is sampled (ignoring technical errors due to imperfections in the measurement processes). Furthermore, when using morphological traits, the developmental steps and genotype by environment noise that is superimposed on the genetic basis needs to be accounted for, plus the various measurement limitations and inaccuracies.

The motivation for this work was to investigate the usefulness of molecular markers as a supplement, a complement and/or an alternative for distinctness testing based on morphological characters. The morphology characters are assessed over a 4-year testing period. The statistical procedure to assess distinctness consists of a t-test in which the 'variety × year' interaction mean square serves as the basis for the standard error of the difference between genotypes of the cultivated almond and the related wild species. In the case of markers, every marker locus represents a discrete variable, where the alleles constitute the possible outcomes. In principle, individual marker loci might be treated as individual morphological characters. However, the power to distinguish between almond genotypes by t-tests on the frequency of bands (or alleles) for specific markers is rather low. Tests for distinctness using DNA-markers should thus combine the information present in a series of markers in order to attain an equivalent power for the distinctness tests. A convenient way to combine information over markers, when comparing pairs of varieties, is to calculate genetic distances that reflect the proportion of bands (or alleles) different between the two varieties. A problem with the use of genetic distances is that their statistical properties are little known. There are few closed expressions for the calculation of the standard errors of genetic distances (Roland-Ruiz et al. 2001).

In this paper the relationships between a number of genotypes of the cultivated almond and the related wild species were explored by using morphological information and molecular markers. Genetic distances obtained by each of these approaches were compared, with special attention given to the coincidences and divergences between the methods. Finally, to compare the discriminating capacity and informativeness of the PCR-based molecular markers RAPD, AFLP and SSR for genotype identification and genetic diversity analyses determining the genetic similarity and genetic relationships among the cultivars and related wild species.

Materials and methods

Plant material and DNA isolation

Twenty-nine almond cultivars and three wild species, widely grown in the some main almond-producing countries (Spain, Italy, France, USA, Russia and Iran) were included in the study (Table 1). All of the samples were obtained from the Agriculture and Natural Resources Research Center of Shahrekord, Iran. Total genomic DNA was isolated from fresh leaf material following the procedure described by Murry & Thompson (1980), as adapted to almond as reported by Shiran et al. (2005) and Sorkheh et al. (2007a).

RAPD analysis

RAPD amplification was performed as described by Williams et al. (1990), with some modification as reported by Shiran et al. (2005). Reaction products were analyzed by electrophoresis on a

1.2% agarose gel and stained with ethidium bromide. A total 80 primers (MWG biotech, Germany and Gene set, France) were surveyed in two cultivars from Iran. Forty-two decamer oligonucleotides showing reproducible, and clearly scorable, polymorphic fragments (present or absent), were used to fingerprint the cultivated and related wild species of almond.

Table 1. Almond cultivars and wild species assayed including the origin, parentage and main agronomic characteristics.

Cultivar	Origin	Pedigree	Shell	Flowering
'Monagha'	Iran	Unknown	Soft	Early
'Sefied'	Iran	Unknown	Soft	Early
'Mamaei'	Iran	Unknown	Hard	Middle
'Rabei'	Iran	Unknown	Hard	Middle
'Shokofeh'	Iran	Ai × Nonpareil (o.p)[a]	Semi-hard	Late
'Azar'	Iran	Ai × Cristomorto	Semi-hard	Late
'Sangi31'	Iran	Unknown	Hard	Early
'Sangi26'	Iran	Unknown	Hard	Early
'Bari'	Italy	Unknown	Hard	Middle
'Filepoceo'	Italy	Unknown	Hard	Late
'Kapareil'	USA	Nonpareil× Eureka (BC)[a]	paper	Middle
'Tuono'	Italy	Unknown	Hard	Late
'Moncayo'	Spain	Unknown	Hard	Late
'Texas'	USA	Unknown	Semi-hard	Late
'IXL'	USA	Unknown	Soft	Middle
'Primorski'	Ukraine	Princesse2077× Nickitsky(BC)	Soft	Very late
'Tardy Nonpareil'	USA	Mutant of Nonpareil	Soft	Very late
'Princesse'	France	Unknown	Semi-hard	Middle
'Genco'	Italy	Unknown	Hard	Late
'Facionello'	Italy	Unknown	Hard	Very early
'Thompson'	USA	Texas(Mission)× Nonpareil	Soft	Late
'Feragness'	France	Cristomorto× Ai	Soft	Late
'Ne plus Ultra'	USA	Unknown	Soft	Middle
'Nonpareil'	USA	Unknown	paper	Middle
'Shahrodi18'	Unknown	Unknown	paper	Middle
'Shahrodi16'	Unknown	Unknown	Soft	Very late
P. scoparia	Iran	Unknown	Hard	Very late
P. communis	Iran	Unknown	Hard	Middle
P. orientalis	Iran	Unknown	Hard	Middle

ao.p – open pollinated, open pollinated parent unknown; BC –backcross

AFLPs analysis

AFLP analysis was performed as described by Vos et al. (1995). Nineteen unlabeled primer combinations with three selective nucleotides were used: 10 *Mse*I primers and 10 *Pst*I primers. The hot amplified products were separated by denaturating via 6% polyacrilamide electrophoresis (PAGE). Reproducibility of the AFLP fingerprints was assessed on three DNA samples by replicating the entire procedure for all the primer combinations. Briefly, total genomic DNA (300 ng) was restricted with 2.5 U of PstI (rare cutter) and 2.5 U of *Mse*I (frequent cutter) (Fermentas), and double stranded adapters were ligated to the fragment ends. The structure of the adapter sequence, pre-amplification, amplification and polyacrylamide-gel electrophoresis conditions were as described by Vos et al. (1995) with some modification and adapted in almond fingerprinting (Sorkheh et al. 2007a; 2007b). Polymorphic amplification products were visualized by silver staining procedures and scored manually. All AFLP polymorphism were scored as dominant markers.

SSR analysis

A set of 32 SSR primer pairs were assayed and synthesized according to the sequences published by Cipriani et al. (1999), Testolin et al. (2000) and Dirlewanger et al. (2002). Primers were synthesized by MWG Biotech, Germany. SSR procedures were described by Cipriani et al. (1999), Testolin et al. (2000) and Dirlewanger et al. (2002) and adapted in almond fingerprinting with some modification by Shiran et al. (2005). The DNA bands were visualized by silver staining, as described by Bassam and Caetano- Anolles (1993). All reactions and electrophoresis were repeated at least twice and each gel was scored independently.

Morphological characterization

For assessing the important agronomic traits and their implication in the cultivar improvement, during the years 2003, 2004, 2005, and 2006, the following traits were studied according with Sorkheh et al. (2007a), Moradi (2005), Dicenta and Garcia (1992), and Sanchez-Perez et al. (2007). The morphological traits studied in 2003, 2004, 2005, and 2006 were: In-shell weight (g), nut length (mm), nut width (mm), nut thickness (mm), nut length/width, nut length/thickness, kernel weight (g), kernel length (mm), kernel width (mm), kernel thickness (mm), kernel length/weight, kernel width/thickness, kernel yield (g), doubles kernels (%) (two deformed kernels in the same nut), kernel bitterness (by tasting some almonds by two or three people, classifying each genotypes as sweet, slightly bitter, and bitter), kernel shriveling (scored 1 smooth, 2 intermediate, 3 wrinkle), percentage kernel (%), productivity (scored between 0 = null and 5 = maximum), ripening date (Julian days when 95% of fruits had their mesocarp opened), shell hardness (scored between 1 = very soft and 5 = very hard, by cracking with a hammer), In-shell / kernel ratio (%), shelling percentage (%), empty nuts (%) (nut without kernels)

Analysis of variance was performed for all traits in order to test the significance of variation among genotypes and related wild species of almond. The standardized mean values of the traits were used to perform principal component analysis using SAS software.

Data analysis

The average number of alleles per locus, the allele frequency, the expected hetrozygosity (*He*), the effective number of alleles per locus, fraction of polymorphic loci (β), and average discriminating power were calculated as reported by Morgante et al. (1994), Powell et al. (1996) and Belaj et al. (2003). The total number of effective alleles (*Ne*) surveyed by RAPD, SSR, and AFLP analyses was calculated by summing the number of effective alleles of all the analyzed loci as $Ne = \Sigma\ ne$ as defined by Pejic et

al. (1998). Assay efficiency index (A_i) was calculated to compare the efficiency among the three methods, where SSRs generally detect multiple alleles and one band per assay, whereas RAPDs and AFLPs detect two alleles and multiple bands per assay. *Ai* combines the effective number of alleles identified per locus and the number of the polymorphic bands detected in each assay as $Ai = Ne/P$, where *Ne* is the total number of effective alleles detected and *P* is the total number of assays performed for their detection.

The Dice coefficient (Dice 1995) was used. The same similarity coefficient was also calculated when all marker data were computed together. The cultivars were grouped by cluster analysis using unweighted pair-group mean method (UPGMA). The computer program used was NTSYS-pc version 2.02 (Rohlf 1998). The cophenetic correlation coefficient was calculated, and the Mantel test (Mantel 1967) was performed to check the goodness of fit a cluster analysis. Analysis of molecular variance (AMOVA, Excoffier 1992) was carried out using the WINAMOVA 1.55 program (Excoffier et al. 1992). The AMOVA variance components were used as estimates of hierarchical molecular diversity, within and among populations. The significance of the estimated values was assessed non-parametrically with 1 000 permutations. Homogeneity of intrapopulation variance (homoscedasticity) was tested by the HOMOVA procedure (Barlett test), also implemented in WINAMOVA (Stewart and Excoffier 1996). Null distributions for Barlett's statistics (Barlett 1937) were obtained using1 000 permutations.

The bootstrap procedure was employed to determine the sampling variance of the genetic similarities calculated from the data sets obtained with the different marker systems. Bootstrap analysis was also used to test if the number of polymorphic loci evaluated was high enough to provide accurate genetic similarities or genetic distance estimates (King et al. 1993; Hallden et al. 1994). All data, irrespective of the diallelic versus multiallelic nature of the marker systems, were scored in the form of binary matrices. For each pair of genotypes, the Dice similarity index (GS) was calculated from 2 000 random sub samples at different sample sizes (10, 50, 100, 150, 200, and using all bands when the total exceeded 200). Bootstrap standard deviation estimates were based on 2 000 samples or permutations. The calculations were performed with the SAS macro "BOOT" (jackknife and bootstrap analysis, SAS Institute Inc.). Finally, the information content of each marker system was calculated for each marker and locus using the polymorphism information content (PIC) (Lynch and Walsh 1998) which provides an estimate of the discriminating power of a locus by taking into account not only the number of alleles that are expressed but also their frequencies. Calculations were made using the following formula: $PIC = 1-\Sigma pi^2$, where p_i the frequency of i^{th} allele.

Results

The 29 almond cultivars and three wild species were surveyed with the three different marker systems. The levels of polymorphism detected with each marker system and index comparing their informativeness are reported in Table 2. The total number of polymorphic bands ranged from 124 for SSRs to 781 for AFLPs (for the RAPD markers, out of 672 initial only 664 well-defined bands were analyzed for the complete set of data). The percentage of polymorphic bands obtained for each assay unit did not correlate with the total number of bands. For instance, the total number of bands scored for RAPDs and AFLPs was 729 with 91% and 813 with 92% of polymorphic bands, respectively, relatively higher than that for SSR markers with hundred percent polymorphic bands. AFLPs had the highest value (0.99) of the discriminating capacity, SSRs for an intermediate value (0.90), while RAPDs showed the lowest value (0.85).

Table 2. Level of polymorphism and comparison of informativeness obtained with RAPD, AFLP and SSR markers in 29 almond cultivars and three related wild species

Marker system	Number of assay units	Number of polymorphic bands	Number of loci	Average discriminating power	Average number of alleles per locus	Fraction of polymorphic loci	Expected hetrozygosity	Effective number of alleles per locus	Assay efficiency index
RAPD[a]	42	672[a] (664)	729	0.85	2	0.91	0.32	1.52	24.03
AFLP	19	781	813	0.99	2	0.96	0.30	1.46	60.01
SSR	18	124	18	0.9	6.88	1	0.42	1.88	1.88

[a]For the RAPD markers, out of a total of 672 polymorphic bands only 664 well-defined bands were included in the data analysis. For H_e, A_i and $n_{u(p)}$ calculation based on the assumption that the whole set of polymorphic bands (672) has the same level of as the 664 polymorphic band- set analysed

Seven out of the set of 32 SSR set primer pairs (BPPCT021, BPPCT015, UDP98-406, UDP96-005, UDP96-003, BPPCT009 and BPPCT008), representing 21.9% of the total SSR primers used, generated multiple products and complex band patterns, probably because of the simultaneous amplification of different loci (Ciprani et al. 2002). An average of 6.83 alleles per locus, ranging from 3 (BPPCT014, BPPCT0279) to 18 (UDP96-019), were observed for these 32 SSRs and the effective number of alleles per locus was 1.88, while for RAPDs and AFLPs the effective values were considerably lower, 1.52 and 1.46, respectively. The very low value of the effective number of alleles per locus for SSR markers in comparison to the average number of allele per locus reflects the presence of many unique or low frequency alleles. The highest assay efficiency and marker index values were observed for AFLPs (60.01 and 41.08, respectively) and the lowest for SSRs (1.88 and 0.42). The values for RAPD markers were intermediate. The very high values of assay efficiency for AFLPs reflects the distinct nature of these markers, namely simultaneous detection of high numbers of polymorphic markers per PCR reaction.

A summary of genetic similarity estimates between pairs of genotypes and species, calculated for each marker system. Microsatellite data gave lower similarity values (0.50) than did RAPDs (0.65) and AFLPs (0.84). The Mantel matrix correspondence tests, used to compare the similarity matrices. The correlation coefficients were statistically significant for all three marker systems. The correlation coefficients among the data similarity matrices showed that the SSR data were least correlated with those obtained from the other two data sets. The cophenetic correlation coefficients between the dendrograms and the original distance matrices for RAPDs, AFLPs and SSRs were all very large, demonstrating strong 'treeness' within each of the data sets (r = 0.96, r = 0.94 and r = 0.91, respectively).

The three markers showed a high degree of similarity in dendrogram topologies (Fig. 1), although there were some differences in the positioning of some genotypes among the main groups. All of the dendrograms reflected the geographic relationships among most cultivars.

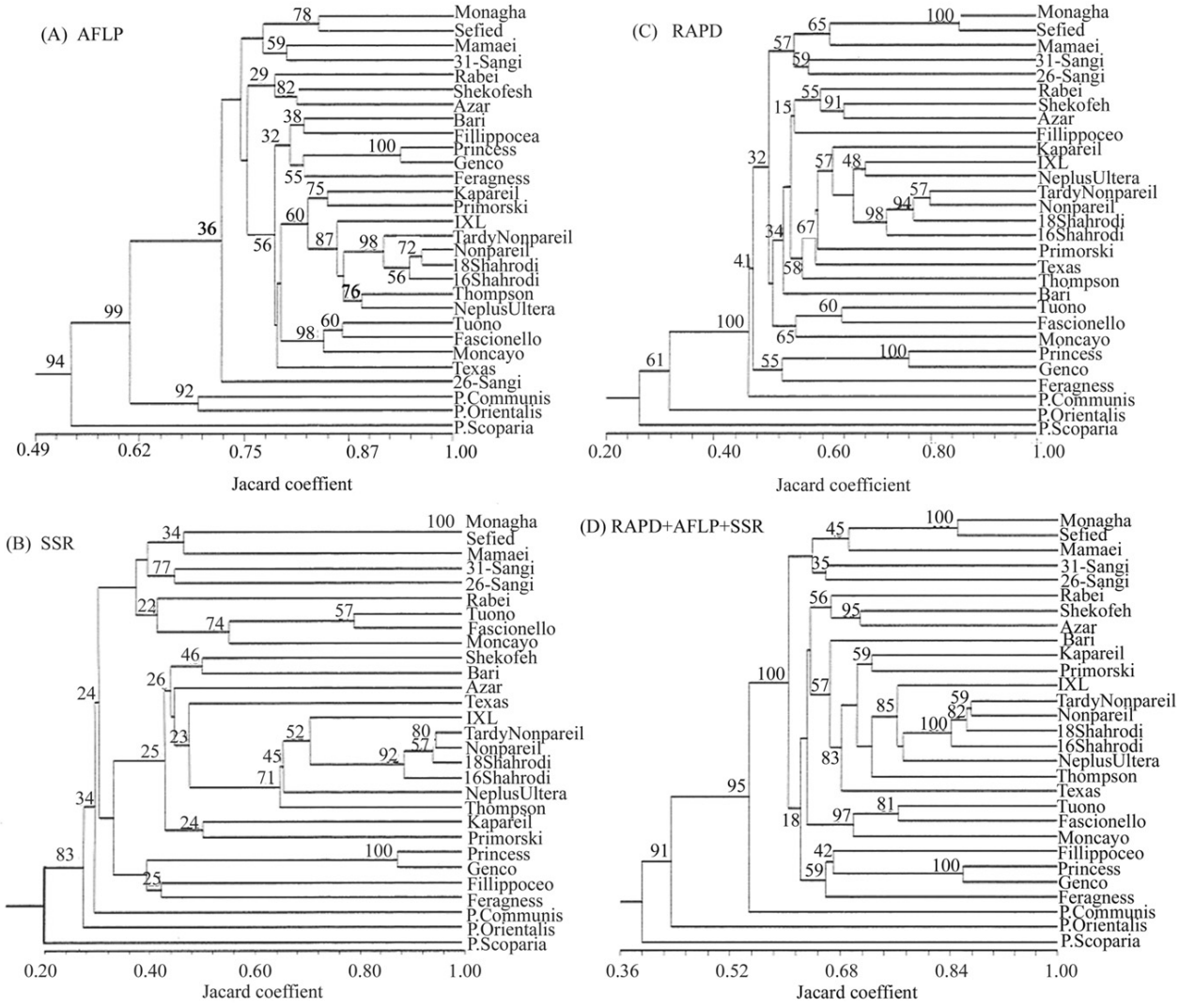

Fig. 1 Dendrograms of 29 almond cultivars and related wild species obtained using AFLP, RAPD and SSR markers separately (A – C) and the entire data set of the three markers (D). The values written on the dendrograms give the stability of nodes, as estimated with a bootstrap procedure (cases with no number label indicate support values of less than 10%).

Notably, three main groups were observed in the AFLP tree (Fig. 1A), Group I, includes 26 cultivars; Group II, involves two related wild Iranian species (*P. communis* and *P. orientalis*); and group III, consists of *P. scoparia* alone. Relationship strengths, as assessed by bootstrapping analysis, varied. In the dendrogram, there is very strong support for clustering of cultivars that were either known to be closely related by pedigree or mislabeled in the group of section Spartioides (Spach), consisting of wild species of *P. scoparia*, which separates from other cultivars with a similarity coefficient of 0.70. *P. orientalis* is not an ancestor of cultivated almonds, belonging to the Euamygdalus group (Kester and Gradzeil 1996), and it differs morphologically from the rest of the taxa in being a shrub and having pubescent leaves (Ladizinsky 1999), although it can be crossed with other almonds. Group II separates from other cultivated almonds with a similarity coefficient of 0.82. This result shows the effectiveness of

AFLPs for separating important groups of almonds. In contrast to the above results, the low similarity indices and quite divergent dendrogram branches points of the wild species of *P. scoparia* place it as an appropriate outgroup. Moreover, the results for the other related species demonstrate great genetic variability of the study material in total. In the group of *Prunus dulcis* (Group I) cultivars, main formed clusters contained Iranian, American or European cultivars only, or mixtures of Iranian and foreign cultivars, indicating a close relation between the three types. The genetic diversity of *P. dulcis* cultivars was high, and it was possible to be discriminative among all cultivars analysed. Foreign and Iranian cultivars were found to associate according to their geographic diffusion. This is a good indication of the robustness of the results that were obtained. The robustness of obtained results are particularly important for breeding purposes for the Iranian cultivars and unknown Shahrodi cultivars, since their genetic diversity, parental relations and origins were unknown, as most mislabeled Iranian cultivars grouped into the same cluster. These analyses of cultivars from Iran have also been confirmed by morphological studies.

The dendrogram obtained with RAPD markers (Fig. 1B) was general similar to the AFLP tree, but with some exceptions. For instance, *Prunus communis* was clustered in Group I instead of in Group II as it did with AFLPs, while some cultivars such as 'Monagha', 'Sefied', 'Shekofeh', 'Azar', 'Genco', 'Princess', 'Tardy Nonpareil', 'Nonpareil', '18-Shahrodi', '16-Shahrodi', 'Touno', 'Fasscionello' and 'Moncayo' were clustered together into Group I. *P. orentalis* was clustered as its own main group, separating from the other two main groups. At the subgroup level, some associations were maintained in both the AFLP and RAPD dendrograms. This was the case for the cultivated varieties 'Monagha', 'Sefied', 'Shekofeh', 'Azar', 'Touno', 'Fasscionello', 'Moncayo', 'Tardy Nonpareil', '18-Shahrodi' and '16-Shahrodi'.

The similarity (especially at the subgroup level) between dendrograms obtained with AFLP markers and with SSR markers (Fig. 1C) was less than that between RAPDs tree and AFLP tree. The following differences were observed: cultivated varieties 31-Sangi, 26-Sangi, Fillipoceo and Feragness were clustered together in Group I with SSR markers, but in the AFLP dendrogram were clustered separately within Group I. Ten cultivars ('Monagha', 'Sefied', 'Princess', 'Genco', 'Kapareil', 'Primorski', 'Tardy Nonpareil', 'Nonpareil', '18-Shahrodi' and '16-Shahrodi') from Group I in the AFLP dendrogram were clustered in Group I with SSRs. Furthermore, the cultivated varieties 31-Sangi, Bari, Texas, Thompson did not cluster together as shown in dendrograms with AFLP and RAPD markers, although they were part of Group I. Some interesting shared associations were observed in the case of RAPD and SSR. For instance, cultivated varieties 'Monagha', 'Sefied', 'Touno', 'Fasscionello', 'Moncayo', 'Tardy Nonpareil', 'Nonpareil', '18 Shahrodi', '16 Shahrodi', 'Princess' and 'Genco' all were clustered together at the same subgroup for both markers sets, and other cultivars were clustered with almost the same cultivars in both cases. The RAPD dendrogram showed a great similarity among cultivars from the same or nearby cultivation areas, as the case for the AFLP and SSR dendrograms.

The dendrogram constructed by using the combined data of the three sets of molecular markers (Fig. 1D) was very similar to those obtained separately with each marker. However, there were some differences, which led to a better representation of the relationships for most cultivars, again according to their geographic area of diffusion. Three main groups in the dendrogram with the combined markers were observed as obtained in the other dendrograms with separated marker (Fig. 1 A-C), and a clustering of the majority of the cultivars from Iran, Spain, Italy, USA, and France together was observed in Group I. Cultivated varieties 'Monagha', 'Sefied', '31-Sangi', '26-Sangi', 'Tardy Nonpareil', 'Nonpareil', '18-Shahrodi', '16-Shahrodi', 'Fasscionello', 'Princess' and 'Genco' were also clustered together into Group I. Hierarchical analysis of phenotypic diversity was performed by using AMOVA, to allocate variation for each marker system, partitioning variation of cultivars from Iran, Italy, Spain, Russia, USA and France into components within and among populations (Table 3). Although most of the genetic diversity was attributable to differences among cultivars within each population (86.49%, 84.63% and 80.72% for RAPDs, AFLPs and SSRs, respectively), significant ϕ-values between populations ($P \leq 0.001$) were found for all the markers used in the study. This suggested the existence of some differentiation. Corresponding HOMOVA analyses revealed that molecular variances were homogenous across populations in the case of RAPD and SSR analyses but heterogeneous in the case of AFLP (Bp= 1.48, P=0.093).

Table 3. AMOVA analysis for the partitioning of RAPD, AFLP and SSR variation of almond varieties and wild species among and within populations

Source of variation (Groups based on geographical origin [b])	*df*	Variance components	Percentage total variance	ϕ-statistics	*p*-value[a]
RAPDs among population	2	10.98	13.51	0.135	$p<0.001$
RAPDs within population	26	70.35	86.49		
AFLPs among population	2	7.41	15.37	0.154	$p<0.001$
AFLPs within population	26	40.81	84.63		
SSRs among population	2	2.68	19.28	0.193	$p<0.001$
SSRs within population	26	11.22	80.72		

[a] Significance of variance component was expressed as the probability of obtaining a more extreme random value, and computed from nonparametric procedures (1 000 data permutation); b Three groups consisted of cultivars and related wild species that obtained from different geographical origin.

The value of SSR polymorphism information content (PIC) was higher than those values of RAPD and AFLP (Fig. 2). Differences in distribution profiles also occurred between dominant and co-dominant markers, with dominant markers having higher standard deviations than co-dominant markers. The differences between minimum and maximum PIC value were lower for AFLP and RAPD than for SSR. The SSR markers gave the highest mean PIC value for all loci combined (PIC = 0.96) and

the RAPD markers the secondary (PIC = 0.77), with the dominant markers, RAPD and AFLP (PIC = 0.77, 0.68, respectively) having mean PIC values of almost the same magnitude.

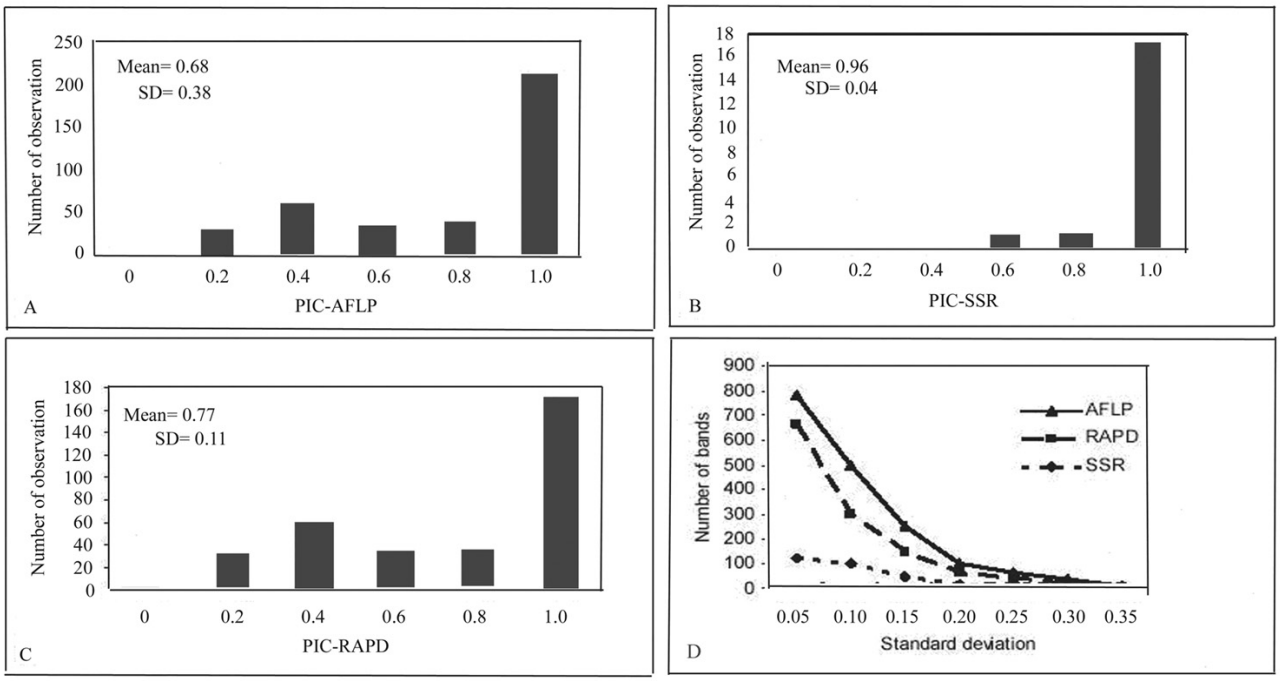

Fig. 2 Distribution of polymorphism information content (PIC) for data was obtained using amplified fragment length polymorphism (AFLP, Fig. 2A), simple sequence repeat (SSR, Fig. 2B) markers, random amplified polymorphic DNA (RAPD, Fig. 2C), and the variation was assessed by bootstrap sampling of genetic similarity between cultivated and related wild species of almond across different marker systems with decreasing numbers of markers (bands, Fig. 2D). SD = standard deviation.

Table 4. Summary statistics of the agro-morphological traits measured in 29 cultivated genotypes and related wild species

Year	Nut length	Nut width	Nut thickness	Nut length / width	Nut length / thickness	Kernel weight	Kernel length	Kernel width	Kernel thickness	Kernel length / weight
2003	22a	19b	14.3a	0.9a	1.1a	1.0b	16.3a	13.1b	6.3a	1.0a
2004	31b	18.9b	12.7b	1.0a	2.0b	1.1b	20.1b	14.1b	10.2b	1.9b
2005	30b	21.2b	18.1b	1.0a	1.0a	3.4a	26.0c	16.2b	11.0b	2.0b
2006	26ab	24.3ab	20.2c	0.8b	2.3ab	4.0a	29.1d	14.5ab	12.2b	1.3a
Mean	27.3	20.6	16.3	0.9	1.6	2.4	22.9	14.5	10.0	1.6
Minimum	22.7	18.3	13.2	0.8	1.0	0.9	16.0	11.2	6.0	1.1
Maximum	45.7	28.1	22.0	1.9	3.2	5.3	30.1	19.3	10.2	2.0

Year	Kernel width / thickness	Kernel yield	Double kernels	Kernel shrivelling	Productivity	Ripening date	In-shell weight	Shell hardness	Shelling percentage (%)	In-shell / kernel ratio	Empty nuts
2003	3.0b	0.2a	9.0a	1.8a	0.5d	205d	3.2a	3.4b	32b	33a	1.0a
2004	2.2c	3.0b	2.0b	1.5b	1.2c	216c	3.3a	3.4b	33b	34a	1.0a
2005	2.1c	4.2b	3.0b	1.1b	2.0a	224b	3.3a	3.6ab	36ab	37a	1.0a
2006	3.4d	4.5b	4.0b	1.8a	1.9b	226a	3.2ab	3.7a	37b	3.6a	1.0a
Mean	2.7	3.0	4.5b	1.6c	1.4	217.8	3.3	3.5	34.5	35.0	1.0
Minimum	1.3	0.2	0.9	1.0	1.0	200	1.1	1.2	20.0	18.2	0.0
Maximum	5.3	4.8	60.0	2.5	3.0	244	5.3	5.0	58.2	63.0	22.0

Values with different letters showed statistically significant differences between years at the 5% level according to the Duncan test. Each trait value is the mean value for each year; mean, minimum, and maximum for the four years

The values of the double kernels for the four years largely varied from the minimum (0.9) to the maximum (60%) (Table 4). Relatively, the shelling percentage had less variable values between the four years, but with genotypes of very hard (20%) or soft (58.2%) shells. The values of mean for nut and kernel weight were standard as were those of length, width and thickness. In other hands, all of other traits related to nut and kernel shape (from spherical form to pronounced elongated and flatted form) evidenced less variable.

Principal component analysis was used to provide a reduced dimension model that would indicate measured differences among groups. The standardized principal components of morphological traits were shown in Table 5. The principal components, PC1, PC2 and PC3 accounted for 54%, 18% and 10% of the total variation (82%) among groups, respectively.

Bootstrap analysis with decreasing numbers of subsampled

markers was used to determine the sampling variance of genetic similarities that were calculated from different molecular marker sets. The relationships between number of bands and sampling variance of genetic similarity among all pairs of genotypes and related wild species of almond for each method are presented in Fig. 2. The results indicated that above 150 bands there was a diminishing return in the precision gained by adding additional bands. As the number of bands decreases below this threshold value the standard deviation increases (and precision decreases) at greater rates.

The principal component coefficients show that double kernels is a major discriminating coefficient among clusters with kernel weight and kernel yield making smaller contribution. The second principal component is dominated by Nut traits. The third principal component reveals that shelling percentage (0.70) and productivity (0.72) play a much larger role in separating the clusters (Table 5).

Table 5. Eigenvalues, percent of variation, cumulative variation, and principal component (PC) after assessing morphological traits in 29 cultivated genotypes of almond and related wild species of almond

Principle Component	Nut length	Nut width	Nut thickness	Nut length / width	Nut length / thickness	Kernel weight	Kernel length	Kernel width	Kernel thickness	Kernel length / weight	Kernel width / thickness
PC1	0.01	0.15	0.24	-0.04	-0.12	0.26	0.05	0.1	0.2	-0.15	0.25
PC2	0.6	0.8	0.53	0.38	0.42	-0.06	0.23	-0.21	-0.52	0.39	0.41
PC3	-0.22	-0.14	-0.02	-0.13	-0.14	-0.02	0.03	-0.14	-0.06	-0.3	-0.03

Principle Component	Kernel yield	Doubles kernels	Kernel flavour	Kernel shrivelling	Percentage kernel	Productivity	Ripening date	In-shell weight	Shell hardness	Shelling percentage	In-shell / kernel ratio	Empty nuts
PC1	0.2	0.95	0.21	0.18	0.26	0.3	0.33	0.18	0.1	0.24	0.1	0.13
PC2	0.01	-0.1	0.11	-0.29	0	-0.22	-0.3	-0.04	-0.01	-0.27	-0.26	0.01
PC3	0.03	-0.06	0.18	-0.23	-0.12	0.72	-0.01	-0.11	-0.16	0.70	0.15	0.18

Discussion

The first and second sections in Discussion mainly focus on the high-level polymorphism obtained by SSR method and the related comparison between your results and others. But the repeated mean obviously occurred in current two sections. Therefore, combined, concise new section is suggested to be given for replaced the original two sections.

In this paper we have shown that the number of alleles detectable in almond cultivated and related wild species by SSRs is higher in comparison to that by other methods. This high level of polymorphism is to be expected because of the unique mechanism responsible for generating SSR allelic diversity by replication slippage (Tautz et al. 1986). It is also known that when SSRs have been compared to other marker systems they have revealed the highest level of polymorphism (Wu and Tanskly 1993; Morgante et al. 1994; Powell et al. 1996). The high-level polymorphism observed in this study for all three marker systems is consistent with results from previous studies conducted on almond cultivars by means of different molecular markers (Martínez-Gómez et al. 2003b; Martins et al. 2003; MirAli et al. 2003; Shiran et al. 2005; Sorkheh et al. 2007a). This result confirms the presence of great diversity within cultivated almond germplasm (Browicz and Zohary 1996; Zeinalabedini et al. 2008).

The higher level of polymorphism detected in almond cultivars by SSR markers than with RAPDs and AFLPs highlights the discriminating capacity of the former. This result is in accordance with previous studies where SSRs were compared to other marker systems (Powell et al. 1996; Pejic et al. 1998; Downey et al. 2000; Shiran et al. 2005; Belaj et al. 2003; Zeinalabedini et al. 2008). In addition, the hypervariability observed at SSR loci was expected because of the unique mechanism by which this variation is generated replication slippage is thought to occur more frequently than single nucleotide mutations and insertion/deletion events that generate polymorphism detected by AFLP and RAPD analyses (Powell et al. 1996; Milbourne et al. 1997). In addition, the co-dominant nature of SSR markers permits the detection of a higher number of alleles per locus and contributes to higher levels of expected hetrozygosity being reached than for RAPDs and AFLPs. However, this difference among markers does not always hold true. For example, in barley (McGregor et al. 2000), AFLPs scored a higher level of expected hetrozygosity than did SSRs and RAPDs.

The number of loci required for a reliable estimate of genetic similarity has been shown to range from 15 RFLP probes, giving 56 bands in *Brassica* sp. (Dos Santos et al. 1994), to 100 RFLP clone-enzyme combinations (Messmer et al. 1993). Similarly, Tivang et al. (1994), investigating the sampling variance of a RFLP dataset in maize, found that the number of bands required for a coefficient of variation (CV) of 10% was 388, 150 and 38 for closely, intermediately, and distantly related breeds, respectively. Our results using the bootstrap procedure suggest that 150 bands are sufficient for reliable estimates of genetic similarity. Accordingly, the average number of assays that could have been used in this study to attain such a precision were 30–40 primers for RAPDs, 10–18 primers for SSRs, and 4–5 enzyme combinations for AFLPs. Based on estimates, the disagreement of the RAPD results in comparison to the other types of markers might be explained by the sufficient number of primers used.

The results of this study indicate that, with the exception of RAPDs, the other two kinds of DNA markers provide consistent information for germplasm identification and pedigree validation. We have shown that SSR and AFLP profiling technologies can be good candidates to replace RFLP and RAPD markers in determining genetic similarities and variety description, and that they have comparable accuracy in grouping genotypes of almond. They are generally much simpler to apply and more sensitive

than traditional morphological and biochemical methods. A major advantage of the SSR and AFLP methods is that they can be automated. While SSRs with the characteristics of the multi-allelism and co-dominant, appear to be suited for the analysis of outcrossing heterozygous individuals, AFLPs, with their high multiplex ratio, offer a distinctive advantage when genome coverage is a major issue due to the presence of linkage disequilibrium (Sorkheh et al. 2008).

We found that the estimates of polymorphism information content (PIC) based on SSR markers were the largest and had the lowest standard deviations. As expected, the PIC distribution revealed that, in terms of genetic distances, dominant markers had lower levels of polymorphism information as compared to codominant markers. However, we also found that the SSRs markers had a more heterogeneous distribution for individual PIC values than did the other two markers, although this might have been due to the lower number of polymorphic loci evaluated for SSRs (Barbosa et al. 2003). Although the AFLP markers gave the lowest mean PIC value, it was only slightly smaller than that of the RAPD markers, and hence the two marker types provided similar degrees of polymorphism information content, in agreement with the results of Becker et al. (1995), and Pejic et al. (1998).

Comparisons of the genetic distances/ genetic similarities generated by different molecular markers in diversity studies, as reported by several authors (Hahn et al. 1995), have revealed only moderate difference between genetic distances estimates using RFLP and RAPD markers. In contrast, Pejic et al. (1998) compared different molecular markers to assess the genetic similarities between maize inbred lines and found great differences in the RAPD similarity clustering patterns. The results obtained in our study showed high agreement in the estimates of AFLP and RAPD genetic distances, such estimates having also been highly correlated in other studies (Belaj et al. 2003). Indeed, we found that the RAPD and AFLP markers produced sufficient numbers of polymorphic bands to produce reliable genetic distance estimates, evidenced by high correlations between these two marker systems. The similarity between the results may be explained by the fact that both marker types are similar techniques based on restriction site changes. Thomann et al. (1994) reported that the number of bands required to obtain a mean CV of 10% was 327 for RAPDs and 294 for genomic RFLPs, with respect to estimates of genetic relationships within and between cruciferous species.

Our results indicate that, apart from the RAPD markers, the other DNA marker systems provided consistent values in populations and produced genetic distance estimates with good agreement. The SSR markers were promising in terms of the polymorphism and information content revealed, but may involve some additional initial costs associated with primer development. The results also suggest that the number of SSR loci evaluated should be increased. In addition, our results suggest that AFLP markers are the best choice for evaluation of diversity and for assessing the genetic relationships between genotypes of almond with high accuracy. The AFLP system presents good levels of precision in genetic estimates and single cross predications. An AFLP result also correlated highly with those obtained using the RAPD system and is a fast and reliable system capable of supporting a multiplex approach not requiring previous knowledge of DNA sequence information.

The very similar levels of polymorphism and expected hetrozygosity observed in almond with AFLP and RAPD analyses are consistent with results obtained on other plant species (Powell et al. 1996; Milbourne et al. 1997), probably due to how variation is sampled. However, in rice, Fuentes et al. (1999) found that AFLPs detected higher levels of polymorphism than RAPDs.

All the three techniques have discriminated the genotypes of almond cultivated and related wild species very effectively, with the exception that SSR markers did not separate the cultivated varieties Monagha and Sefied. The fact that all the three marker systems showed very low levels of confusing probability supports their utility in identification studies. The values of average discriminating power followed the pattern AFLPs>SSR>RAPD, as a direct consequence of their confusion probability values. Generally, AFLPs and SSRs should be preferred to RAPDs for almond variety identification and plant certification.

For five SSR primer pairs no amplification was observed. The same primer pairs gave multiband loci when applied to almond/ peach cultivars (Cipriani et al. 1999). A high frequency of microsatellite PCR amplifying multiple loci has also been reported in almond by different authors (Martínez-Gómez et al. 2003a; 2003b; Sánchez-Pérez et al. 2006). This phenomenon is relatively common in species that have allopolyploid origin, although this has not been clearly demonstrated in almond (Minelli et al. 2000), and alternatively multiband amplification might be due to genome fusion and chromosome duplication events during evolution (Buteler et al. 1999).

The utility of a given marker is a balance between the level of polymorphism it can detect (information content) and its capacity to identify multiple polymorphisms (Powell et al. 1996). The information measured as the assay efficiency index (Ai), which correlates with the number of effective alleles per assay, was greater for AFLPs than for the other markers (AFLPs>RAPDs>SSRs). Very high value of Ai detected by AFLPs, as opposed to RAPDs, SSRs and RFLPs, were reported in maize inbreed lines by Pejic et al. (1998).

The main reason for the limited, yet significant, correlation similarities for SSRs observed in the present study might be due to their codominant nature. The various types of genetic polymorphism detected by the three markers and the number of primers used may also affect the correlations among different markers. In addition, the finding of slightly higher resolution of genetic similarities by RAPDs and AFLPs compared to SSRs may be due to the polymorphism of SSRs which may render them less suitable for determining genetic relationships among cultivars (Staub et al. 2000).

No differentiation of the sampled cultivars was observed between countries, probably as a consequence of an interchange of genetic relationships between countries and second, most California almond cultivars originated from the Languedoc area of southern France (Kester and Gradzeil 1996). This finding is fur-

ther confirmed by the presence of S_5 incompatibility allele in Primorski as well as American cultivars such as, Thompson, Texas and Neplus Ultera (Chanaatapipat et al. 2003). But most of the countries from any given area are grouped together in the three dendrograms obtained. This structure of the genetic diversity compared with the geographic origin of cultivars most likely reflects a process of multilocal selection in almond (Besnard et al. 2001), and possible exchange of plant material during the history of almond cultivation (Scorza et al. 1996).

Genetic differentiation among characters, as detected by the three types of molecular markers, was higher in internal populations than inter-populations. This result is consistent with the general observation that woody perennial outbreeding species, such as olive, maintain most of their variation within each country of origin (Lamboy et al. 1996; Oraguzie et al. 2001). Our study has demonstrated that the three marker systems may have different applications in almond, according to their characteristics: SSRs had the highest polymorphism and expected hetrozygosity (H_e) and an intermediate value of discriminating capacity (D); AFLPs were characterized by the highest D but the lowest H_e; finally, RAPDs had the lowest values of H_e and D. Such properties, together with other considerations of practical and economical nature, must be taken into account when choosing a marker system for specific applications.

All the three techniques may provide useful information on the level of polymorphism and diversity in almond, and thus each may have utility in the characterization of germplasm accessions. For RAPD analysis, the problems of reliability and transferability among laboratories should be considered (Jones et al. 1997). We have found that RAPD data can be reliable through replication of amplification reactions and by using a conservative criterion for band selection. Nonetheless, the higher informativness of SSRs and AFLPs, together with the above- mentioned problems for RAPDs will limit the use of in DNA fingerprinting, although they will remain useful where financial investment is limited. Finally, both RAPDs and AFLPs were highly efficient in detecting general similarities in almond, while the codominant nature of SSRs make them (microsatelite) the marker of choice for several suggested studies in almond including genome mapping.

To provide an objective comparison, we examined correlations between distance matrices calculated on the basis of AFLP, SSR and morphological data using a Mantel test (data not shown). The correlation between SSR and morphological data was higher than that between AFLP and morphological data. The results suggest that SSR markers may be a better choice for marker-traits association genetic studies of cross -pollinated almond genotypes than AFLP markers. Working with 16 ryegrass varieties, Roldan-Ruiz et al. (2001) reported a correlation value of r = -0.06 between AFLP and 15 morphological characters. The correlation between the two molecular markers was higher than the morphology (data not shown). Therefore, when compared with DNA fingerprinting techniques, morphological traits are relatively less reliable and efficient for precise discrimination of closely related accessions and analysis of their genetic relationships. Despite this limitation, morphological traits are useful for preliminary evaluation because it is fast, simple, and can be used as a general approach for assessing genetic diversity among morphologically distinguishable accessions (Beyene et al. 2005).

Conclusions

All the three molecular techniques (RAPD, SSR and AFLP) effectively discriminated the almond genotypes studied and the related wild species. However in the case of SSR markers the discrimination level was lower and failed to discriminate between ‘Monagha’ and ‘Sefied’ almond genotypes. In the molecular studies RAPD and SSR markers showed a better application to routine than AFLP. Overall SSR was the best markers with the exception of the lower discrimination level in some cases whereas RAPD marker only showed some deficiencies from the point of view of the repeatability of results. In addition, the correlation coefficients of similarity were statistically significant for all three marker systems, but were lower for the SSR data than for RAPDs and AFLPs.

On the other hand, for all markers, high similarity in dendrogram topologies was obtained, although some differences were observed. All the dendrograms, including that obtained by the combined use of all marker data, reflect relationships for most of cultivars according to their geographic diffusion. AMOVA detected more variation among cultivated and related wild species of almond within each geographic group. Bootstrap analysis revealed that the number of molecular markers used in the case of RAPD, SSR and AFLP was sufficient for reliable estimation of genetic similarity and for meaningful comparisons of marker types.

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