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Comparative study of the discriminating capacity of RAPD, AFLP and SSR markers and of their effectiveness in establishing genetic relationships in olive

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Abstract RAPDs, AFLPs and SSRs were compared in terms of their informativeness and efficiency in a study of genetic diversity and relationships among 32 olive cultivars cultivated in Italy and Spain. SSRs presented a higher level of polymorphism and a greater information content, as assessed by the expected heterozygosity, than AFLPs and RAPDs. The lowest values of expected heterozygosity were obtained for AFLPs, which, nevertheless were the most efficient marker system due to their capacity to reveal the highest number of bands per reaction and because of the high values achieved for a considerable number of indexes. All three techniques discriminated the genotypes very effectively, but only SSRs were able to discriminate the cultivars Frantoio and Cellina. The correlation coefficients of similarity were statistically significant for all three marker systems used but were lower for the SSR data than for RAPDs and AFLPs. For all markers a 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 some relationships for most of the cultivars according to their geographic diffusion. AMOVA analysis detected greater genetic differentiation among cultivars within each country than it did between the two countries.

Keywords AFLPs · RAPDs · SSRs · *Olea europaea* L. · Genetic relationships

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Introduction

The olive tree (*Olea europaea* L.) is a subtropical species typical of the Mediterranean basin where it represents the most important oil-producing crop. It is a diploid, outcrossing species with a very wide genetic patrimony (Bartolini et al. 1998). Most olive varieties have an ancient and local origin (Besnard et al. 2001), with limited diffusion outside their areas of cultivation (Barranco 1997). The considerable diversity in olive and the presence of cases of homonyms and synonyms stress the need for efficient and rapid discriminating methods.

In olive, as in many other species, studies of genetic diversity reflect, to some extent, the history of the development of genetic markers. Morphological data have traditionally been used for variability evaluation (Barranco et al. 2000). In order to supplement and refine the morphology-based descriptions isoenzyme markers were used at first to assess genetic variability in olive (Loukas and Krimbas 1983; Ouazzani et al. 1993, 1996; Trujillo et al. 1995). DNA-based markers provided a new opportunity for genetic characterisation and biodiversity studies in olive. In recent years random amplified polymorphic DNA (RAPD) markers have been extensively used in several studies of genetic variability in olive (Fabbri et al. 1995; Mekuria et al. 1999; Gemas et al. 2000; Gonzalo-Claros et al. 2000; Hess et al. 2000; Belaj et al. 2001, 2002; Besnard et al. 2001; Sanz-Cortés et al. 2001), probably because of their relative advantages over other molecular techniques (Welsh and McClelland 1990; Williams et al. 1990). Amplified fragment length polymorphism (AFLP) markers, developed by Vos et al. (1995), have also been applied to study the genetic

relationships among cultivated olives, wild forms and related species (Angiolillo et al. 1999; Baldoni et al. 2000). Simple-sequence repeat (SSR) markers are becoming the markers of choice for variability studies in olive (Rallo et al. 2000; Sefc et al. 2000; Cipriani et al. 2002) as they are transferable, highly polymorphic, multiallelic polymerase chain reaction (PCR)-based codominant markers, relatively simple to interpret (Rafalski et al. 1996). Such characteristics justify the large initial effort necessary to obtain SSR markers because sequence information is needed (Morgante et al. 1998). Deciding on which technique would be the most appropriate for any given investigation is not obvious and depends on a number of factors including the purpose of the research, the biology of the species and the resources available.

Comparisons of molecular markers for measuring genetic diversity have been carried out in several plant species (Powell et al. 1996; Milbourne et al. 1997; Russell et al. 1997; Pejic et al. 1998; Crouch et al. 2000; Garcia-Mas et al. 2000; Staub et al. 2000) but, to our knowledge, no such studies have yet been reported in olive. A better understanding of the effectiveness of the different molecular markers is considered a priority step toward olive germplasm characterisation and classification, and a prerequisite for more effective breeding programs.

The objectives of this paper are: (1) to compare the discriminating capacity and informativeness of the PCR-based molecular markers RAPD, AFLP and SSR for genotype identification and genetic diversity analyses; (2) to determine the genetic similarity estimates and genetic relationships among the cultivars analysed; (3) to compare the patterns of variability obtained with each marker.

Table 1 Olive cultivars analysed, countries (Spain and Italy) and areas (within these countries) where those cultivars are widely grown, Register Number (R.N.) in the World Olive Germplasm Bank of Cordoba (Spain)

Cultivar	Country	Area of diffusion	R.N.
Alfafara	Spain	East	605
Arbequina	"	East	231
Bical	"	South-West	387
Blanqueta	"	East	11
Castellana	"	Center	576
Changlot Real	"	East	15
Cornicabra	"	Center	10
Empeltre	"	East	13
Farga	"	East	12
Gordal Sevillana	"	South-West	234
Hojiblanca	"	South-West	2
Lechín de Granada	"	South-West	54
Lechín de Sevilla	"	South-West	5
Manzanilla Cacereña	"	Center	430
Manzanilla de Sevilla	"	South-West	21
Morrut	"	East	224
Picual	"	South-West	9
Picudo	"	South-West	3
Sevillenca	"	East	227
Verdial de Badajoz	"	Center	988
Verdial de Huevar	"	South-West	6
Villalonga	"	East	364
Ascolana Tenera	Italy	Center	62
Carolea	"	South-West	736
Cellina	"	South-East	179
Coratina	"	South-East	79
Frantoio	"	Center-North	80
Itrana	"	Center	68
Leccino	"	Center	82
Leccio del Corno	"	Center	83
Moraiolo	"	Center	78
Rosciola	"	Center	88

Materials and methods

Plant material and DNA isolation

Thirty-two olive cultivars, widely grown in the two main olive-producing countries, Spain and Italy, were included in the study (Table 1). All of the samples were obtained from the World Olive Germplasm Bank of the Centro de Investigación y Formación Agraria (CIFA) Alameda del Obispo in Cordoba (Spain) and collected from one single tree per cultivar, assuming that all of them have a clonal origin.

For the RAPD and SSR analyses, total genomic DNA was isolated from fresh leaf material following the procedure previously described by Belaj et al. (2001). For the AFLP analysis, the method used for DNA extraction was that described by Angiolillo et al. (1999).

Marker analyses

RAPD

RAPD amplifications were performed as described by Belaj et al. (2001). All reactions were conducted three times using DNA of various extractions and different lots of the AmpliTaq DNA polymerase. The amplification products were separated on polyacrylamide gels containing 10% acrylamide, 0.126% piperazine diacrylamide crosslinker in 375 m*M* Tris-HCl, pH 8.8, using Tris-glycine buffer (25 m*M* Tris, and 192 m*M* glycine), and were visualized by silver staining as described by Bassam et al. (1991). Twenty-one primers from kits A, F, I, J, K, P, Q, X, and Z (Operon Technologies, Alameda, Calif.) were used in the study. RAPD bands were selected following a highly conservative criterion.

AFLP

AFLP analysis was performed as described by Angiolillo et al. (1999). Five primer combinations with three selective nucleotides were used: four *Mse*I primers (M-CAC, M-CAA, M-CTG and M-CTT) and three *Eco*RI primers (E-AGC, E-ACT and E-AAC). *Eco*RI primers were end-labelled with γ-[^{33}P]-ATP. The hot-amplified products were separated by denaturing 6% polyacrylamide electrophoresis (PAGE). Reproducibility of the AFLP fingerprints was assessed on three DNA samples by replicating the entire procedure for all the primer combinations.

SSR

SSR assays were performed as described by Cipriani et al. (2002) using eight primers from the 30 sets described by these authors. They were: UDO99-008, UDO99-009, UDO99-011, UDO99-014, UDO99-024, UDO99-031, UDO99-039 and UDO99-043. One of the two primers was labelled with γ-[^{33}P]-ATP, and the PCR products were separated on 6% denaturing polyacrylamide gel (Long Ranger, FMC BioProducts, USA). Allele scoring and sizing was done visually using the pUC18 plasmid sequence lanes as a length reference. The cultivar Frantoio, from which the microsatellites had been originally isolated and sequenced, was also used as a reference lane in all gels as recommended by Cipriani et al. (2002).

All PCR reactions and electrophoreses were repeated at least twice and each gel was scored independently.

Data analysis

To compare the efficiency of the three markers (RAPD, SSR, AFLP) in varietal identification, diversity and differentiation, we estimated the following for each assay unit (U) (the product of PCR amplification obtained with one set of primers) used:

1) Number of polymorphic bands (n_p);
2) Number of non polymorphic bands (n_{np});
3) Average number of polymorphic bands per assay unit (n_p/U);
4) Number of loci (L): in the case of RAPD and AFLP markers the theoretical maximum number of loci is equal to total number of bands ($n_p + n_{np}$) obtained for each marker type;
5) Number of loci per assay unit: $n_u = \frac{L}{U}$;
6) Number of banding patterns for each molecular marker (T_p);
7) Average number of patterns per assay unit (I);
8) Confusion probability (C_j) of the j-th assay unit (Tessier et al. 1999): $C_j = \sum_{i=1}^{I} p_i \frac{(Np_i-1)}{N-1}$ where p_i is the frequency of the ith pattern; N, sample size; I, total number of patterns generated by the jth assay unit;
9) Discriminating power (D_j) of the jth assay unit as reported by Tessier et al. (1999): $D_j = 1 - C_j = 1 - \sum_{i=1}^{I} p_i \frac{(Np_i-1)}{N-1}$;
10) Limit of D_j as N tends toward infinity: $D_L = \lim(D_j) = 1 - \sum_{i=1}^{I} p_i^2$;
11) Effective number of patterns per assay unit: $P = \frac{1}{1-D_L}$;
12) Average number of alleles per locus (n_{av}). For SSRs the average number of alleles per locus is calculated according to the formula: $n_{av} = \frac{n_p}{L}$. For RAPDs and AFLPs two alleles per assay are considered ($n_{av} = 2$);
13) Expected heterozygosity (H_{ep}) of the polymorphic loci for a genetic marker: $H_e = 1 - \sum p_i^2$ where p_i is the allele frequency for the ith allele and the arithmetic mean of the expected heterozygosity of the polymorphic loci: $H_{ep} = \frac{\sum H_{np}}{n_p}$, where n is the number of markers analysed;
14) Fraction of polymorphic loci (β) according to Powell et al. (1996): $\beta = \frac{n_p}{n_p+n_{np}}$;
15) Expected heterozygosity (H_e) as reported by Powell et al. (1996): $H_e = \beta \frac{\sum H_{np}}{n_p}$;
16) Effective number of alleles per locus (n_e) according to Morgante et al. (1994): $n_e = \frac{1}{\sum p_i^2}$ where p is the frequency of the ith allele;
17) Total number of effective alleles (N_e) as defined by Pejic et al. (1998): $N_e = \sum n_e$;
18) Assay efficiency index (A_i) according to Pejic et al. (1998): $A_i = \frac{N_e}{U}$;
19) Effective multiplex ratio (E) according to Powell et al. (1996): $E = n_u\beta$;
20) Marker index (MI) as defined by Powell et al. (1996): $MI = EH_p$, $MI = nH_{ep}$, $MI = n\beta H_p$,where n is a number of bands ($n = n_p + n_{np}$).

For AFLP, RAPD and SSR analysis, Dice's coefficient (Dice 1945) was used. The same similarity coefficient was also calculated when all marker data were computed together.

The cultivars were grouped by cluster analysis using the unweighted pair-group method (UPGMA). The computer program used was NTSYS-PC version 2.02 (Rohlf 1998). The cophenetic correlation coefficient was calculated, and Mantel's test (Mantel 1967) was performed to check the goodness of fit of a cluster analysis to the matrix on which it was based.

The analyses of molecular variance (AMOVA, Excoffier et al. 1992) were carried out on the RAPDs, AFLPs and SSRs data using the WINAMOVA 1.55 program (Excoffier 1992). The AMOVA variance components were used as estimates of molecular diversity at each hierarchical level, between and within countries (Spain and Italy), for each marker system. The significance of ϕ values was tested non-parametrically after 1,000 permutations.

Homogeneity of intrapopulation variances (homoscedasticity) was tested by the HOMOVA procedure (Barlett's test), also implemented in WINAMOVA (Stewart and Excoffier 1996). Barlett's statistics (Barlett 1937) null distributions were obtained after 1000 permutations.

Results

Levels of polymorphism and discriminating capacity

All three markers proved to be highly effective in discriminating the 32 cultivars analysed. The results obtained are summarised in Table 2.

The total number of polymorphic bands ranged from 61 for SSRs to 261 for AFLPs (of these only 98 well-defined bands were analysed in the whole set of data). The percentage of polymorphic bands obtained for each assay unit did not correlate to the total number of bands. For instance, the total number of bands scored for RAPDs and AFLPs was relatively high, 135 and 319, respectively, with 81% and 82% of them being polymorphic. In

Table 2 Levels of polymorphism and comparison of the discriminating capacity of RAPD, AFLP and SSR markers in 32 olive cultivars

Indexes with their abbreviations		Marker system		
		RAPD	AFLP	SSR
Number of assay units	U	21	5	8
Number of polymorphic bands	n_p	109	261[a] (98)	61
Number of monomorphic bands	n_{np}	26	58	0
Average number of polymorphic bands/assay unit	n_p/U	5.19	19.60	7.63
Number of loci	L	135	319	8
Number of loci/assay unit	n_u	6.43	63.80	1.00
Number of banding patterns	T_p	201	146	105
Average number of patterns/assay unit	I	9.57	29.20	13.13
Average confusion probability	C	0.15	0.01	0.10
Average discriminating power	D	0.85	0.99	0.90
Average limit of discriminating power	D_L	0.82	0.96	0.87
Effective number of patterns/assay unit	P	5.39	27.63	7.58

[a] For the AFLP markers, from a total of 261 polymorphic bands, only 98 well-defined bands were included in the data analysis

Table 3 Comparison of informativeness obtained with RAPD, AFLP and SSR markers in 32 olive cultivars

Indexes with their abbreviations		Marker system		
		RAPD	AFLP[a]	SSR[b]
Average number of alleles per locus	n_{av}	2.00	2.00	7.60
Expected heterozygosity of the polymorphic loci	H_{ep}	0.35	0.31	0.42
Fraction of polymorphic loci	β	0.81	0.82	1.00
Expected heterozygosity	H_e	0.28	0.25	0.42
Effective number of alleles per locus	n_e	1.59	1.52	1.88
Total number of effective alleles	N_e	173.02	396.67	9.38
Assay efficiency index	A_i	8.24	79.33	1.88
Effective multiplex ratio	E	5.19	52.20	1.00
Marker index	MI	1.79	16.26	0.42

[a] For AFLP markers, H_{ep} and n_e calculations were based on 98 randomly chosen polymorphic bands; for H_e, N_e, A_i, n_u, and MI, calculations were based on the assumption that the whole set of polymorphic bands (261) has the same level of H_{ep} and n_e as the 98 polymorphic band-set analysed
[b] Only five SSR single locus primer sets were used for the comparison of informativeness

contrast, the lowest number of total bands was obtained for SSR markers, but all of these were polymorphic.

Also, the number of banding patterns per assay unit for each marker type was not consistent with the total number of banding patterns. While the latter ranged from 105 for the SSR markers to 201 for RAPDs, with an intermediate value of 146 for AFLPs, the number of banding patterns per assay unit for SSRs (13.13) was somewhere between the values found for RAPDs (9.57) and AFLPs (29.20).

Low values of average confusion probability were obtained for the three markers, especially for the AFLPs and SSRs. The discriminating capacity, negatively correlated to the confusion probability, showed the highest value for AFLPs (0.99), an intermediate value for SSRs (0.90), while RAPDs showed the lowest value (0.85). D_L values, estimated for the three markers, were very close to the actual discriminating power of each of them, respectively.

The effective number of patterns indicates the size of an ideal population in which, given the frequencies of the patterns obtained with a marker system, all of the individuals can be distinguished. This means that with one RAPD primer almost six patterns can be obtained when the population size tends to infinity – i.e up to six varieties can be distinguished with the same primer – while, with only one AFLP primer combination up to 28 varieties can be distinguished and with one SSR primer set about eight cultivars can be discriminated.

Comparison of informativeness obtained with RAPD, AFLP and SSR markers

Three out of eight SSR primer pairs, UDO99-009, UDO99-011 and UDO99-014, which represent 37.5% of the total SSR primers used, generated multiple bands, probably because of the simultaneous amplification of different loci (Cipriani et al. 2002). Each amplification product (allele) was easily identified, but the allele attribution at the respective locus was not ascertainable. For that reason, the informativeness of the SSR primers was compared only on the other five single-locus SSR primer pairs (Table 3).

An average of 7.6 alleles per locus, ranging from 5 (UDO99-008, UDO99-039) to 12 (UDO99-043), were observed for these SSRs. For the same markers, the effective number of alleles per locus was 1.88, while for RAPDs and AFLPs these values were slightly lower, 1.59 and 1.52, respectively. This was reflected in lower values of the expected heterozygosity for both RAPD and AFLP markers. The very low value of the effective number of alleles per locus for SSR markers in comparison to the average number of alleles per locus may suggest the presence of many unique or less frequent alleles. The highest assay efficiency and marker index values were observed for AFLPs (79.33 and 16.26, respectively) and the lowest for SSRs (1.88 and 0.42). The values for RAPD markers were intermediate between those of AFLPs and SSRs. The high value of the marker index for AFLPs is the result of a very high multiplex ratio component (E = 52.20). The very high values of assay efficiency and marker index for AFLPs highlights the distinctive nature of these markers. This is due to the simultaneous detection of several polymorphic markers per single reaction.

Genetic similarities and relationships

A summary of the genetic similarity estimates between pairs of cultivars, calculated for each marker system, is shown in Table 4. Microsatellite data gave lower similarity values (0.36) than did RAPDs (0.56) and AFLPs (0.68).

The Mantel matrix correspondence test was used to compare the similarity matrices. The correlation coefficients (Table 5) were statistically significant for all three marker systems. The correlation coefficients of similarity matrices, resulting from the SSR data were, however, lower than those obtained with the other two markers. The cophenetic correlation coefficients between the dendrogram and the original distance matrix for RAPDs, AFLPs

Table 4 Average, minimal and maximal values of Dice similarity coefficients (RAPDs, AFLPs, and SSRs) among 32 olive varieties (496 pairwise comparisons)

Parameters	Marker system		
	RAPDs	AFLPs	SSRs
Average	0.56	0.68	0.36
Minimum	0.28	0.48	0.00
Maximum	1.00	1.00	0.93

Table 5 Cophenetic correlations[a] among matrices

Marker system	Marker system		
	RAPDs	AFLPs	SSRs
RAPDs	**0.67*** **	0.23**	0.42***
AFLPs	0.40***	**0.66*** **	0.12^{ns}
SSRs	0.39***	0.33***	**0.76*** **

*** Significant at $P < 0.001$, ** significant at $P < 0.01$, * significant at $P < 0.05$, ns, non-significant $P > 0.05$ (Mantel's test significance)
[a] Below diagonal, original similarity matrix comparison; diagonal (in bold), goodness of fit of a cluster analysis to the similarity matrix on which it was based; above diagonal, cophenetic value matrix (matrix of ultrametric values) comparison (after UPGMA clustering procedure)

and SSRs were significant but relatively low ($r = 0.67$, $r = 0.66$ and $r = 0.76$, respectively).

All three markers showed a high degree of similarity in dendrogram topologies (Fig. 1), though with some differences in the positioning of some cultivars at the main groups. All the dendrograms reflect relationships among most of the cultivars, depending upon their area of diffusion.

In the AFLP tree (Fig. 1A), two main groups were observed: Group I, including 18 cultivars and Group II, 12, while the cvs. Bical and Castellana clustered separately at a distance lower than 0.65. Of the 18 cultivars in Group I, 13 were from Spain (69% from south west and central) and only five from Italy. Four cultivars, Alfafara, Blanqueta, Changlot Real and Farga from eastern Spain, as well as five Italian cultivars also clustered at this group in different subgroups according to their geographic diffusion.

Group II included five eastern Spanish cultivars (Arbequina, Empeltre, Morrut, Sevillenca and Villalonga) and cvs. Lechín de Sevilla and Picual, both of the latter from the southern part of the country, together with Italian cvs. Coratina, Frantoio, Cellina, Leccino and Leccio del Corno.

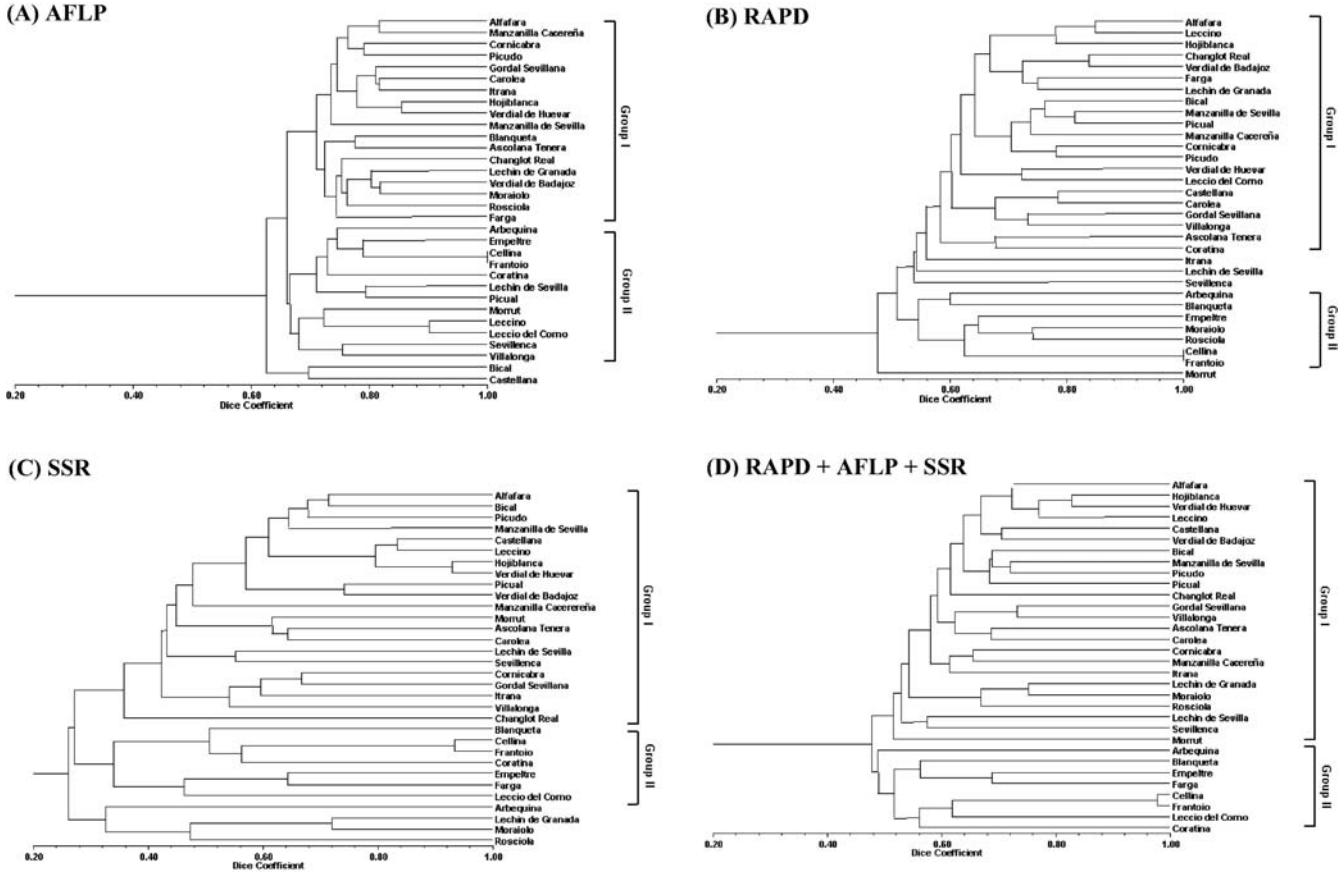

Fig. 1 Dendrograms of 32 olive cultivars obtained using AFLP, RAPD and SSR markers separately (**A–C**) and the whole data set of the three markers (**D**)

Table 6 AMOVA and HOMOVA analysis for the partitioning of RAPD, AFLP and SSR variation of olive varieties among and within countries

Source of variation	*df*	Variance components	Percentage total variance	ϕ-statistics	*P*-value	Bartlett's index	*P*-value
RAPDs among countries	1	0.02	7.35	0.074	<0.001	0.008	0.580
RAPDs within countries	30	0.21	92.65				
AFLPs among countries	1	0.02	9.21	0.092	<0.001	0.051	0.092
AFLPs within countries	30	0.15	90.79				
SSRs among countries	1	0.02	7.19	0.072	<0.001	0.0257	0.338
SSRs within countries	30	0.31	92.81				

The dendrogram obtained with RAPD markers (Fig. 1B) showed a similar topology with some exceptions. For instance, cv. Blanqueta clustered in Group II instead of Group I as it did with AFLPs, while some cultivars, such as Picual, Coratina, Leccino, Leccio del Corno and Villalonga clustered together in Group I instead of the Group II of AFLPs. Cultivars Itrana, Sevillenca and Lechín de Sevilla clustered separately from the two main groups of the dendrogram, while, Bical and Castellana were, in this case, included in Group I.

At the subgroup level, some associations were maintained in both the AFLP and RAPD dendrograms. This was the case of cvs. Changlot Real, Lechín de Granada, Verdial de Badajoz, Moraiolo, Rosciola and Farga.

The dendrogram obtained with SSR markers (Fig. 1C) was to some extent less similar (at the subgroup level) to that obtained with AFLP markers than the dendrogram resulting from RAPDs. The following differences were observed: cvs. Blanqueta and Farga clustered at Group II instead of Group I, six cultivars (Lechín de Sevilla, Picual, Morrut, Leccino, Sevillenca and Villalonga) from Group II in the AFLP dendrogram clustered at Group I with SSRs. Cultivars Frantoio and Cellina, discriminated by two primer pairs, UDO99-039 and UDO99-014, always clustered at Group II. Cultivars Lechín de Granada, Moraiolo and Rosciola formed a separate group with cvs Arbequina yet maintained the same reciprocal relationships as with AFLP and RAPD markers. Furthermore, cvs. Bical and Castellana did not cluster together, as they did with AFLP markers, but were part of Group I.

Some interesting common associations of cultivars were observed in the case of RAPDs and SSRs. For instance, cvs. Hojiblanca and Leccino clustered together at the same subgroup for both markers, and cv. Blanqueta clustered with almost the same cultivars in both cases. The RAPD dendrogram showed a greater similarity among cultivars from the same or nearby cultivation areas than did the AFLP and SSR dendrograms.

The general dendrogram (Fig. 1D), constructed using the combined data of the three sets of molecular markers, 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 of the cultivars, according to their geographic area of diffusion. Two main groups were observed: as in the other dendrograms (Fig. 1A–C), a clustering of the majority of the cultivars from southern and central Spain together with some Italian varieties was observed in Group I. The number of cultivars from southern Spain in this group was higher than in the AFLP dendrogram due to the presence of cvs. Lechín de Sevilla, Picual and Bical. Five Italian cultivars, Moraiolo, Rosciola, Carolea, Ascolana Tenera and Itrana, also clustered together in Group I as well as five cultivars from eastern Spain: Alfafara, Villalonga Changlot Real, Sevillenca and Morrut. Group II included four eastern Spanish cultivars (Arbequina, Blanqueta, Empeltre, Farga), together with cvs. Coratina, Frantoio, Cellina, Leccino and Leccio del Corno, similarly to what was observed in the separated dendrograms for each marker.

Hierarchical analysis of phenotypic diversity using AMOVA was performed to analyse the partition of each marker system variation in Spanish and Italian varieties between and within countries (Table 6). Although most of the genetic diversity was attributable to differences among cultivars within each country (92.65%, 90.79% and 92.81% for RAPDs, AFLPs and SSRs, respectively), significant ϕ-values between countries ($P < 0.001$) for all the markers used in the study suggested the existence of phenotypic differentiation. Corresponding HOMOVA analyses revealed that molecular variances were homogeneous between countries in the case of RAPD and SSR analysis but heterogeneous in the case of AFLP (B_p = 0.051, $P = 0.092$).

Discussion

The high level of polymorphism observed in this study for all three marker systems is consistent with results from previous studies carried out on olive cultivars by means of different molecular markers (Fabbri et al. 1995; Wiesman et al. 1998; Angiolillo et al. 1999; Baldoni et al. 2000; Rallo et al. 2000; Belaj et al. 2001; Besnard et al. 2001; Sanz-Cortés et al. 2001), thereby confirming the great diversity within the cultivated olive germplasm (Bartolini et al. 1998).

The higher level of polymorphism detected in olive 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; Russell et al. 1997; Pejic et al. 1998).

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, which generate the polymorphisms detectable by AFLP and RAPD analyses (Powell et al. 1996; Milbourne et al. 1997). The codominant nature of these markers permits the detection of a high number of alleles per locus and contributes to higher levels of expected heterozygosity being reached than would be possible with RAPDs and AFLPs. However, this result also depends on the species under study. In barley (Russell et al. 1997) and in tetraploid potato (McGregor et al. 2000), for example, AFLPs scored a higher level of expected heterozygosity (also called diversity index) than SSRs and RAPDs.

The very similar levels of polymorphism and expected heterozygosity observed in olive with AFLP and RAPD analyses are consistent with results obtained in other plant species (Powell et al. 1996; Milbourne et al. 1997; Garcia-Mas et al. 2000) and is probably due to how variation is sampled. However, in rice, Fuentes et al. (1999) found that AFLPs detected higher levels of polymorphism than RAPDs, while in barley (Russell et al. 1997), AFLPs scored the lowest polymorphism when compared to other markers.

The three techniques have discriminated most genotypes very effectively, but only SSR markers were able to discriminate cvs. Frantoio and Cellina.

The fact that all three marker systems showed very low levels of confusing probability support their utility in identification studies. The values of average discriminating power followed the pattern AFLP > SSR > RAPD, as a direct consequence of their confusion probability values. Similarly, in grape Tessier et al. (1999) obtained higher values of D_L for SSRs than for RAPDs. Therefore, AFLPs and SSRs should probably be preferred to RAPDs for olive variety identification and plant certification.

The three SSR primer pairs which amplified two different loci showed high values of *D*. The same primer pairs gave multiband loci when applied to 12 olive cultivars (Cipriani et al. 2002). A high frequency of microsatellites amplifying multiple loci has also been reported in olive by Rallo et al. (2000). This phenomenon is relatively common in species with an allopolyploid origin, although this has not been clearly demonstrated in olive (Minelli et al. 2000) and may be due to genome fusion and chromosome duplication events during evolution (Buteler et al. 1999).

The relatively high values of the effective number of patterns per assay units (*P*) for all the markers used give evidence of their discrimination capacity when handling a large number of samples. This is very important for the management of germplasm banks where numerous cultivars need to be accurately characterised and identified. AFLPs showed the highest value of *P*, probably due to the high number of loci (or bands) simultaneously analysed in each experiment. The very conservative criteria that were applied for the selection of polymorphism may have reduced, to some extent, the values of *P* obtained for RAPDs.

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 distinctive value of marker index (*MI*) for AFLP data is related to the effective multiplex ratio (*E*) value. In other words, it depends more on the high number of alleles (polymorphic bands) obtained in each profile than on the allelic heterozygosity found among cultivars. Both RAPDs and AFLPs have higher multiplex ratios than SSRs in the cultivar set studied. In soybean, however, SSRs scored higher values of *MI* than did RAPDs (Powell et al. 1996). These results reinforce the need for specific studies of marker comparisons for each plant species. Similarly to the *MI* values, the information measured as the assay efficiency index (A_i), which correlates with the number of effective alleles per assay, was greater for AFLPs than for the other markers (AFLPs > RAPDs > SSRs). Very high values of A_i detected by AFLPs, as against 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 observed in the present study for SSRs might be due to their codominant nature. The type of genetic polymorphism detected by the three markers and the number of primers used may also affect the correlations among different markers.

The finding of a slightly higher resolution of genetic similarities by RAPDs and AFLPs, compared to SSRs, may be due to the high polymorphism of SSRs which may render them less suitable for determining genetic relationships among cultivars (Staub et al. 2000).

No differentiation of the cultivars was observed between countries, probably as a consequence of an interchange of genetic material between Italy and Spain, but most of the cultivars from any given area clustered 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 olive (Besnard et al. 2001), a limited diffusion of olive cultivars out of their areas of cultivation (Sanz-Cortés et al. 2001) and a possible exchange of plant material among the different regions and countries along the history of olive cultivation (Angiolillo et al. 1999).

Genetic differentiation among cultivars, as detected by the three molecular markers, was higher within each country (Italy and Spain) than between them. This is consistent with the general observation that woody perennial outbreeding species, as olive, maintain most of their variation within each country (Lamboy et al. 1996; Bartish et al. 2000; Gauer and Cavalli-Molina 2000; Oraguzie et al. 2001).

Our study has demonstrated that the three marker systems may have different applications in olive, accord-

ing to their characteristics: SSRs had the highest polymorphism and expected heterozigosity (H_e) and intermediate values of discriminating capacity (D) and effective number of patterns (P); AFLPs were characterised by the highest *MI, D* and *P* values but the lowest H_e; finally, RAPDs had an intermediate value of *MI* but the lowest values of H_e, D and P. 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 three techniques may provide useful information on the level of polymorphism and diversity in olive, showing their utility in the characterisation 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 reliable RAPD data can be generated following a standard protocol, replication of amplification reactions and a conservative criterion of bands selection. The higher informativeness of SSRs and AFLPs, together with the above-mentioned problems for RAPDs, will limit their use in DNA fingerprinting. However, they will remain useful where financial investment is limited.

Both, RAPDs and AFLPs, were highly efficient in detecting genetic similarities in olive, while the codominant nature of SSRs will make them the marker of choice for segregation studies and genome mapping in olive.

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