BIOLOGIA PLANTARUM 52 (4): 642-647, 2008

Genetic diversity assessment in Portugal accessions of *Olea europaea* by RAPD markers

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Abstract

Eighty seven olive (*Olea europaea* ssp. *sativa* L.) cultivar accessions from Portugal were characterized by means of randomly amplified polymorphic DNA (RAPD) markers. Of the 11 arbitrary 10-mer primers tested a total of 92 polymorphic bands were obtained, representing 87.6 % of the total amplification products. Twenty nine different genotypes were clearly discriminated. Differences were not found among the amplification profiles from different individuals of the same cultivar. All the genotypes could be identified by the combination of three primers: OPR-1, OPK-14 and OPA-1, seven genotype-specific markers being detected. Genetic relationships were estimated by the unweighted pair-group method with arithmetic averaging (UPGMA). The genetic analysis of the results showed a gradual distance between the various cultivars, making it difficult to identify well-differentiated phylogenetic groups, although two clusters were distinguishable with 35 % similarity, in addition to three independent branches with lower similarity: Galega, Tentilheira and Redondal. The dendrogram reflect some relationships for most of the cultivars according to the use of the fruit and ecological adaptation.

Additional key words: olive cultivars, UPGMA

Introduction

Olive (*Olea europaea* L.) is one of the most important fruit crops in the Mediterranean area, where it is cultivated mainly for oil but also for canned fruits. Olive is a diploid species (2n=46) and more than 2000 cultivars have been described, these exhibiting significant levels of variation in oil content, fruit size, canopy shape and adaptation to the local environmental conditions (Bartolini *et al.* 1998). The origin and the geographical distribution of such high variability in the cultivated olive is still under investigation. Most modern cultivars were derived from the crossing of ancient cultivars, or by their crossing with wild plants, followed by local selection (Angiolillo *et al.* 1999, Besnard and Bervillé 2000, Besnard *et al.* 2001). The ancient cultivar Galega represents 80 % of the Portuguese patrimony and it is apparently resistant to drought and to olive knot. It has a small-medium fruit size with medium oil content, giving high-quality olive oil. The other 20 % of the olive patrimony consists of cultivars from specific regions.

Olive germplasm classification is complicated not only by the richness of its genetic background, but also by the absence of reference and by the confusion on the cultivar names, with numerous cases of homonymy and synonymy (Bartolini *et al.* 1998). For example, Cordeiro (2005), characterized 29 Portuguese cultivars using morphological markers (leaves, fruits and stones) and 5 pairs of presumed synonyms established.

DNA-based markers provide a new opportunity for

Received 12 December 2006, *accepted* 20 September 2007.
Abbreviations: AFLP - amplified fragment length polymorphism; ISSR - intersimple sequence repeat; PCR - polymerase chain reaction; RAPD - randomly amplified polymorphic DNA; RFLP - restriction fragment length polymorphism; SNP - single nucleotide polymorphism; SSR - simple sequence repeat; UPGMA - unweighted paired group method with arithmetic averages.
Acknowledgments: This project was partially funded by the Ministério da Agricultura do Desenvolvimento Rural e das Pescas, Portugal. AIC was granted with a fellowship from the Government of Portugal.
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genetic characterization and biodiversity studies in olive. Molecular analyses have been carried out by means of AFLPs (Angiolillo *et al.* 1999, 2006, Baldoni *et al.* 2000), cpDNA RFLPs (Lumaret *et al.* 2000), mtDNA RFLPs (Khadari *et al.* 2003), SSRs (Rallo *et al.* 2000, Sefc *et al.* 2000, Cipriani *et al.* 2002), ISSRs (Vargas and Kadereit 2001) and SNPs (Reale *et al.* 2006), PCR-RAPD technique (Williams *et al.* 1990, Chakrbarti *et al.* 2006, Narasimhan *et al.* 2006, Padmesh *et al.* 2006, Rout 2006, Saker *et al.* 2006, Dikshit *et al.* 2007). A multilocal origin of olive cultivars based on mitochondrial-DNA polymorphisms has recently been reported (Besnard *et al.* 2001). Belaj *et al*. (2002) reached the same conclusions analysing the main Spanish olive cultivars with RAPDs. Recently, Gemas *et al*. (2004) has analysed the genetic inter-cultivar diversity of 11 olive cultivars and intra-cultivar diversity in cv. Galega.

The aim of the present paper is to evaluate (using the RAPD technique) the inter-cultivar diversity of 29 olive cultivars grown in Portugal, and the genetic relationships among the cultivars analysed.

Material and methods

A total of 87 accessions were used in this study representing 29 different Portuguese olive (*Olea europea* L.) tree cultivars (Table 1). Plant material was obtained from the Estação Nacional de Melhoramento de Plantas (ENMP), belonging to the Instituto Nacional de Investigação Agrária (INIA), Elvas (Portugal). Total DNA was extracted from young leaves from these cultivars as described in Belaj *et al*. (2001).

After decamer oligonucleotides from kits A, C, K, R, S, X and Z from *Operon Technologies* (Alameda, CA, USA) were screened by PCR amplification, DNA was amplified using the reaction mixtures described by Belaj *et al*. (2001). The PCR reactions were performed in a thermal cycler (*GeneAmp PCR System 9600*) from *Applied Biosystems* (Foster City, CA, USA) programmed for 1 cycle of 1 min at 94 ºC followed by 40 cycles of 30 s at 94 ºC, 30 s at 35 ºC and 2 min at 72 ºC, for denaturing, primer annealing, and extension, respectively. The last cycle was followed by incubation for 7 min at 72 ºC. All the reactions were performed three times using DNA of different extractions and different lots of the AmpliTaq DNA polymerase (*Amersham Pharmacia Biotech*, Foster City, CA, USA).

RAPD bands were scored as 1 (present) or 0 (absent) in a binary matrix. A conservative criterion for the selection of bands, and reproducible and well-defined bands in each of the three replications were considered for the analysis. Jaccard's similarity coefficient (Jaccard 1908) was calculated. 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)**.** Cophenetic correlation coefficient was calculated, and Mantel test (Mantel 1967) was performed to check the goodness of fit of a cluster analysis to the matrix on which was based. Allele phenotypes were analysed with *Correspondence analysis* (CORRESP) (Greenarce 1984), available also in the programme *NTSYS-pc*.

Table 1. Number of accessions analysed in cultivars used in this study and their localization in Portugal (C - centre, N - north, S - south).

No.	Cultivar	Accession number	Localization
1	Azeiteira	4	C
2	Bico de Corvo de Serpa	3	C-S
3	Blanqueta de Elvas	3	C
4	Borrenta	3	N
5	Carrasqueña	3	C
6	Cobrançosa	3	C-N
7	Conserva das Barrancas	3	C
8	Conserva de Elvas	3	C
9	Cordovil de Castelo Branco	3	C
10	Cordovil de Elvas	3	C
11	Cordovil de Serpa	3	S
12	Cordovil Tras-os-Montes	3	N
13	Galega	3	C
14	Galego Evora	3	C
15	Galego Grado Serpa	3	C-S
16	Golosinha	3	C
17	Maçanilha de Elvas	3	C
18	Maçanilha de Tavira	3	C-S
19	Madural	3	N
20	Negrinha	4	C-N
21	Planalto	2	C
22	Quinta do Portado	2	C
23	Redondal	3	N
24	Redondil	3	C
25	Tentilheira	3	C-N
26	Verdeal de Serpa	3	C-S
27	Verdeal Tras-os-Montes	3	N
28	Verdeal Elvas	3	C
29	Verde Verdelho	3	C

Results and discussion

The selection of the 11 primers, based on the number and quality of the markers obtained, proved to be an appropriate strategy that will facilitate future studies and make the whole process more efficient in further analyses. Of a total of 105 reproducible amplified bands, 92 were polymorphic (87.6 %), constituting a large

number of polymorphisms (Table 2). The number of bands per primer ranged from 2 (OPS-3) to 17 (OPX-3), while the number of polymorphic fragments varied per marker from 1 (OPS-3) to 16 (OPX-3). In addition, no differences were found between the amplification profiles for the different individuals of the same cultivar.

Table 2. Sequences of the primers used and level of polymorphism found by the RAPD analysis. TNB - total number of bands, NPB - number of polymorphic bands, P - percentage of polymorphic band.

Primer	Sequence (3'-5')	TNB	NPB	P [%]
OPA 01	CAGGCCCTTC	9	8	88.8
OPC 15	GACGGATCAG	11	8	72.7
OPK 14	CCCGCTACAC	9	8	88.8
OPR 01	TGCGGGTCCT	14	14	100.0
OPR 06	GTCTACGGCA	7	6	85.7
OPR 07	ACTGGCCTGA	8	7	87.5
OPS 03	CAGAGGTCCC	2	1	50.0
OPS 14	AAAGGGGTCC	11	10	90.9
OPX 01	CTGGGCACGA	12	11	91.6
OPX 03	TGGCGCAGTG	17	16	94.1
OPZ 19	GTGCGAGCAA	5	3	60.0
Total		105	92	87.6

Table 3. Seven genopype-specific RAPDs markers.

No.	Cultivar	RAPD marker
4	Borrenta	OPK-14-3a
8	Conserva de Elvas	OPA-1-5
9	Cordovil de Castelo Branco	OPZ-19-3
13	Galega	OPR-7-4
23	Redondal	OPR-1-1a
26	Verdeal de Serpa	OPX-3-2
27	Verdeal Tras-os-Montes	OPK-14-4

Of the 92 polymorphic bands, some were specific to a given cultivar. The cultivars Galega, Redondal, Cordovil de Castelo Branco, Borrenta, Verdeal Tras-os-Montes, Verdeal de Serpa, and Cordovil de Elvas were univocally identified by a single marker (Table 3). The marker OPR-7-4 was absent exclusively in the cv. Galega while the markers OPR-1-1a, OPZ-19-3, OPK-14-3a, OPK-14-4, OPS-3-2 and OPA-1-5 appeared only in the cvs. Redondal, Cordovil de Castelo Branco, Borrenta, Verdeal Tras-os-Montes, Verdeal de Serpa, and Cordovil de Elvas. Therefore, 7 single polymorphic bands were found, these representing genopype-specific markers (Table 3). High variability was found in the frequency of the polymorphic bands (0.028 to 0.972) for the cultivars studied (data not shown). In general, the mean frequency of the aforementioned bands was high (0.50). The cultivars were identified by different combinations of the band patterns found for the 11 primers. For the identification of the cultivars that did not present single markers, a combination of 2 or 3 markers was needed. Only with the combination of 3 primers (OPR-1, OPK-14 and OPA-1) was it possible to identify all the cultivars studied.

In general, low similarity values were found between the cultivars studied (data not shown), from 0.13 to 0.75, confirming the hypothesis of a high degree of diversity among the olive cultivars. The lowest similarity values were found for the cultivars Redondal and Cordovil de Castelo Branco (0.13), Redondal and Cordovil Tras-os-Montes (0.13), Galega and Tentilheira (0.20), Redondal and Cordovil de Elvas (0.21), and Galega and Redondal (0.24), while the highest values were between Cordovil de Serpa and Cobrancosa (0.75), and Negrinha and Azeiteira (0.68). These values indicate that the RAPD markers effectively differentiated the cultivar studied, even the Negrinha and Azeiteira, which have been considered synonyms using morphological markers (Cordeiro 2005). Our results confirm that these are different cultivars that have genetic differences.

In our study, pairs of cultivars Maçanilha de Tavira and Maçanilha de Elvas, Verdeal Tras-os-Montes and Verdeal de Serpa, Conserva de Elvas and Conserva das Barrancas and three cvs. Cordovil de Elvas, Cordovil de Castelo Branco and Cordovil de Serpa were studied. The similarity values in the case of the Maçanilha, Verdeal, Conserva and Cordovil cvs. were 0.54, 0.43, 0.34, and 0.64 -0.39, respectively. These genetic-similarity values can be considered as medium to low, indicating that a shared denomination among certain cultivars due to a common characteristic does not signify high genetic similarity. The relatedness of the accessions studied was efficiently established through the use of RAPD markers. In the dendrogram obtained by the UPGMA method (Fig. 1), the differences among all the cultivars clearly appear, with the similarity coefficients between all possible pairs of genotypes ranging from 0.30 to 0.75. The genetic analysis of the results showed a gradual distance between the various cultivars, making it difficult to identify well-differentiated phylogenetic groups, although two clusters (I and II) were distinguishable with 35 % similarity, in addition to 3 independent branches with lower similarity: Galega, Tentilheira and Redondal (Fig. 1). These cultivars were the most differentiated from the others and showing a greater number of different markers.

Cluster I included the cultivars Cordovil de Elvas and Planalto and was the more homogeneous of the two others. This cluster presented two subgroups. Subgroup A was comprised of cultivars with a greater degree of similarity (60 %) including Cordovil de Elvas and Maçanilha de Tavira, cultivated in the southern and central zones of the country (Algarve, Alentejo and Beira interior). Subgroup B had 50 % similarity between cultivar Verdeal Tras-os-Montes and Planalto (Fig. 1). In cluster II, the genotypes between Redondil and Quinta do Portado presented certain variability (Fig. 1). In this subgroup, the cultivars are grown in the central and northern zone of the country (Tras os Montes, Alentejo and Beira interior) as in the case of Cordovil Tras-os-

Montes, Verdeal Tras-os-Montes, Madural and Borrenta, while cv. Galega, widely distributed throughout the country, appears distant from the others.

Of the 29 olive cultivars in Portugal 9 cultivars represent about 90 % of the total number of grown olive trees (Cordeiro 2005): Carrasquenha, Conserva das Barrancas, Galega, Golosinha, Madural, Maçanilha de Elvas, Maçaninha Tavira, Negrinha, and Redondal. Genetic relationships among the 9 cultivars were established by correspondence analysis (Greenarce 1984) (Fig. 2). The first (Co1) and second (Co2) coordinates accounted for 28.36 and 29.90 % of the total variation, respectively. The third coordinate accounted for a further 13.23 % of the variation, bringing the total explained

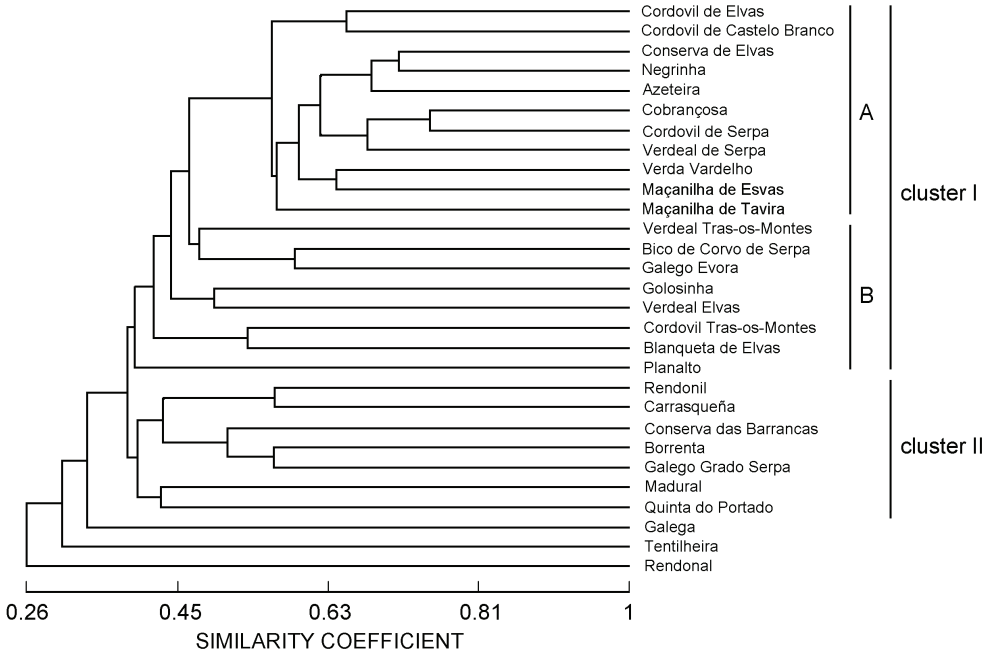

Fig. 1. Dendrogram of the Portuguese cultivars using UPGMA clustering methods and Jaccard's similarity index.

the available information, main groups can be identified in the diagram (Fig. 2) highlighted by different shapes. Therefore, we can consider that Coordinate 1 establishes a adaptation scale of the cultivars, responsible for 28.36 % of the variation observed, segregating most wild cv.Galega at the left side of diagram and the rest in a close scope in the middle (Fig. 2). Coordinate 2 (Co2) reveals a further 29.90 % variation based on the use diversity among different cultivars, depending on the use of the fruit (oil/canning) (Fig. 2). Thus, cultivars spread across Co2 indicate that the upper ones are used for table and the bottom ones for oil.

The high level of polymorphism found in this study agrees with results of previous studies using morphological markers on Portuguese olive cultivars (Martins *et al.* 1997, Cordeiro 2005). This high degree of variability is probably due to the great diversity of olive cultivars (Bartolini *et al.* 1998).,

In present study, this polymorphism found was adequate to characterize all the Portuguese cultivars studied and the identification of all the cultivars using a small number of primers (3) evidences the high discrimination capacity of RAPD markers. The ability to distinguish unambiguously between cultivars and to clarify synonyms and homonyms is of major importance for solving problems concerning the management of olive germplasm. However, due to the problems of consistency of the PCR-RAPD technique, there is a need for the replication of analyses that involve independently

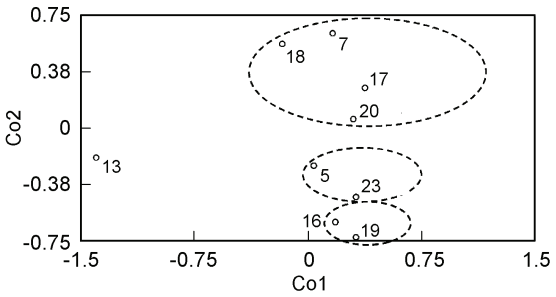

Fig. 2. Genetic relationships among the 9 cultivars and the breeding line were established by correspondence analysis (Greenarce 1984). Genotypes numbers as in Table 1. Three groups were defined: cultivars used for oil (Madural and Golosinha), cultivars used for canning (Conserva das Barrancas, Maçanilha de Tavira, Maçanilha de Elvas, and Negrinha) and cultivars that serve for both purposes (Carrasquenha and Redondal). The first (Co1) and second (Co2) coordinates accounted for 28.36 and 29.90 % of the total variation, respectively. The third coordinate accounted for a further 13.23 % of the variation, bringing the total explained variation up to 62.53 % (data not shown).

variation up to 62.53 % (data not shown). Based on isolated DNA samples and that are performed at different times with a conservative criterion of band selection and scoring (Belaj *et al.* 2003). One major application of RAPD markers in the olive is the identification between cultivars. Therefore, the potential of each primer to yield different genotypes for as many cultivars as possible, with a minimum risk of confusion, is of great interest, and the selection of the most informative primers reduces the cost of analysis for reliable cultivar distinction. In addition, genotype-specific markers developed in the present study would be of immense significance for any further studies related to the genetic resource characterization.

RAPD molecular markers have revealed two types of evidence: one indicates that the olive cultivars have no special relationship (Belaj *et al.* 2001), while the other, which arises with the selection of cultivars with a certain geographic relationship, show that the differentiation of the olive cultivars follows a criterion strongly influenced by edaphoclimatic characteristics (Wiesman *et al.* 1998, Gonzalo-Claros *et al.* 2000). In our study, the distances between the accessions enable the grouping of the Portuguese cultivars that lack a clear correlation with their geographic origin (Fig. 1). This result agrees with the hypothesis of the autochthonous origin of most olive cultivars and their limited spread beyond their original cultivation zone. In the present study, the different position of the cv. Galega in comparison to the other 28 cultivars, similar to that reported by other authors (Gemas *et al.* 2004, Lopes *et al*. 2004), suggest that it has a different origin. Moreover, Belaj *et al.* (2002), studying 6 of these 29 Portuguese cultivars, found the same grouping among them as in the present work, and Galega also was independent. However, it is noteworthy that the intra-cultivar diversity detected by Gemas *et al*. (2004) was not found in the 3 analyzed accessions of Galega.

In the cultivated olive, the risk of the disappearance of genetic resources is lower than in other species, due to the presence of traditional olive orchards and the longevity of the species (Barranco and Rallo 2000). Therefore, the conservation and study of the genetic resources are necessary to perform studies of cultivar improvement and selection.

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