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RAPD and ISSR molecular markers in *Olea europaea* L.: Genetic variability and molecular cultivar identification

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Abstract

Thirty Portuguese and eight foreign olive (*Olea europaea* L.) cultivars were screened using Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) markers. Twenty RAPD primers amplified 301 reproducible bands of which 262 were polymorphic; and 17 ISSR primers amplified 204 bands of which 180 were polymorphic. The percentage of polymorphic bands detected by ISSR and RAPD was similar (88 and 87%, respectively). The genetic variability observed was similar in the Portuguese and foreign olive cultivars. Seven ISSR and 12 RAPD primers were able to distinguish individually all 38 olive cultivars. Twenty specific molecular markers are now available to be converted into Sequence Characterised Amplified Region (SCAR) markers. Relationships among Portuguese and foreign cultivars is discussed.

Introduction

The olive tree, *Olea europaea* L., has been part of the Mediterranean civilisation since before recorded history. Its domestication goes back 6000 years to the East coast of the Mediterranean Sea (Zohary and Spiegel 1975). In the Mediterranean countries olive orchards cover about 7,000,000 ha (Khadari et al. 2003), and olive oil is an important product due to its nutritional and health advantages in comparison to other vegetable oils (Rallo et al. 2000).

Olive tree germplasm was traditionally evaluated by morphological and phenological parameters. Polymerase Chain Reaction (PCR) – based DNA markers are powerful tools for genetic analysis because of their simplicity and ease handling (Kojima et al. 1998) providing an opportunity for direct comparison and identification of olive tree material independently from environment and/or developmental stages. Random amplified polymorphism DNA (RAPD) markers (Williams et al. 1990) are a promising marker system widely used in plant research such as phylogenetic studies, genome mapping, population genetic studies, as well as in cultivar identification and germplasm management (Schnell et al. 1995; Ford et al. 1997; Loureiro et al. 1998; Qian et al. 2001; Bandelj et al. 2002). This technique has several advantages such as: simplicity of use, low

cost and the use of a small amount of plant material (Fritsch and Rieseberg 1996). However, RAPD technology has several limitations including dominance, uncertain locus homology, sensitivity to the reaction conditions, and reliability from lab to lab.

RAPDs have been used in the differentiation of olive cultivars (Cresti et al. 1996; Khadari et al. 2003), to study inter- or intra-cultivar genetic diversity (Wiesman et al. 1998; Mekuria et al. 1999, 2002, Roselli et al. 2002, Belaj et al. 2002, 2003b, c; Gemas et al. 2004), to establish genetic relationships between cultivars (Belaj et al. 2002, 2003b; Besnard et al. 2001a; Khadari et al. 2003), and to study genetic differentiation in the olive complex (Besnard et al. 2001b).

In order to solve some of the problems associated with RAPD, new techniques, such as ISSR were developed (Zietkiewicz et al. 1994). ISSR technique is based on the amplification of regions (100–3,000 bp) between inversely oriented closely spaced microsatellites. ISSRs are PCR products obtained with primers based on dinucleotide, trinucleotide, tetranucleotide and pentanucleotide repeats (Zietkiewicz et al. 1994), and have been used for olive cultivar identification (Hess et al. 2000; Pasqualone et al. 2001; Gemas et al. 2004; Terzopoulos et al. 2005).

The major advantage of ISSR markers is the fact that they do not require the time-consuming and expensive step of genomic or other library construction (Rakoczy et al. 2004), do not need prior knowledge of DNA sequence for primer design, and have similar advantages to RAPDs (Kantety et al. 1995; Yang et al. 1996; Fang and Roese 1997). ISSR amplification has been shown to be much more informative than RAPDs for genetic diversity evaluation in wheat (*Triticum aestivum*), in fruit plants and common bean (*Phaseolus vulgare*) (Nagaoka and Ogihara 1997; Korbin et al. 2002; Galvan et al. 2003). Previous studies have concluded that ISSR markers will be efficient to assess phylogenetic relationships in the *Olea europaea* complex (Hess et al. 2000; Gemas et al. 2004) and to identify drupes from different olive cultivars (Pasqualone et al. 2001).

The main goal of this work was to construct a molecular data-base using RAPD and ISSR markers for *Olea europaea*, to obtain specific molecular markers for individual identification of all the thirty Portuguese 'standard' olive tree cultivars and eight foreign cultivars from eight different Mediterranean countries, to study their genetic variability, and to search for synonymy and homonymy.

Materials and methods

Leaf materials

Leaf samples from 38 olive cultivars were gathered in the field collection of Oliviculture Department of the Portuguese National Breeding Station, Elvas, and frozen in liquid nitrogen and stored at −80°C. Thirty cultivars are standard Portuguese cultivars and the remaining eight from the following Mediterranean countries: France, Greece, Italy, Israel, Morocco, Spain, Tunisia and Turkey (Table 1).

DNA extraction

Genomic DNA was extracted from fresh young olive leaves by a modified CTAB method following the procedure described by Doyle and Doyle (1987). Five grams of leaves were grounded in liquid nitrogen and incubated at 65°C, for 1 h in 15 mL of extraction buffer [100 mM Tris–HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, 2% (w/v) PVP and 1% (v/v) β-mercaptoethanol]. An extraction with an equal volume of chloroform-isoamyl alcohol (24:1 – v/v) and a centrifugation at 10,000 rpm for 50 min was repeated. RNA was removed from the aqueous solution by RNase (100 μg/mL) treatment (Sigma, St. Louis, MO, USA) at 37°C for 1 h. After the isopropanol (0.6 V) precipitations, DNA was recovered and washed with 5 mL of buffer (76% ethanol and 10 mM ammonium acetate), dried and resuspended in 0.2 ml of TE buffer (10 mM Tris–HCl pH 8.0; 1 mM EDTA pH 8.0). DNA concentration was determined by spectrophotometer and was checked for integrity on a 0.7% agarose gel.

RAPD amplification

The effects of *Taq* polymerase concentrations, template DNA concentrations, and different periods of time and temperatures during the annealing

Table 1. List of olive cultivars studied, their geographical region of production, end use, oil content and country of origin.

Code	Cultivar	Geographic region of production	End use	Oil content[a]	Country
PT1	Azeiteira	Alentejo	Table	Low	Portugal
PT2	Blanqueta	Norte Alentejo	Oil	Medium	
PT3	Borrenta	Trás-os-Montes	Oil	Low	
PT4	Carrasquenha	Elvas and Campo Maior (Alentejo)	Oil	High	
PT5	Cobrançosa	Trás-os-Montes, Alentejo, Ribatejo and Beiras	Oil	Medium	
PT6	Conserva de Elvas	Elvas (Alentejo)	Table	Medium	
PT7	Cordovil de Castelo Branco	Beira Interior	Both use	Medium	
PT8	Cordovil de Elvas	Elvas (Alentejo)	Oil	Medium	
PT9	Cordovil de Serpa	Serpa and Moura (Alentejo)	Oil	Medium	
PT10	Cordovil de Trás-os-Montes	Trás-os-Montes	Oil	Medium	
PT11	Cornicabra	Beira Alta	Oil	High	
PT12	Galega	Alentejo, Ribatejo and Beiras	Oil	Medium	
PT13	Galego de Évora	Évora (Alentejo)	Oil	Medium	
PT14	Galego Grado de Serpa	Serpa and Moura (Alentejo)	Oil	Medium	
PT15	Golosinha	Elvas (Alentejo)	Oil	High	
PT16	Leucocarpa	Santarém (Ribatejo)	Oil	Low	
PT17	Maçanilha Carrasquenha	Elvas (Alentejo)	Table	Medium	
PT18	Maçanilha de Elvas	Elvas (Alentejo)	Both use	Medium	
PT19	Maçanilha de Tavira	Tavira (Algarve)	Table	Medium	
PT20	Madural	Trás-os-Montes	Oil	Medium	
PT21	Mora	Elvas (Alentejo)	Oil	Medium	
PT22	Negrinha de Freixo	Trás-os-Montes	Table	Low	
PT23	Negrita	Trás-os-Montes	Table	Low	
PT24	Quinta do Portado	Santarém (Ribatejo)	Oil	Medium	
PT25	Redondal	Trás-os-Montes	Oil	Medium	
PT26	Redondil	Alto Alentejo	Both use	High	
PT27	Tentilheira	Elvas (Alentejo)	Oil	Low	
PT28	Verde Verdelho	Elvas (Alentejo)	Oil	Medium	
PT29	Verdeal de Serpa	Serpa (Alentejo)	Oil	Medium	
PT30	Verdeal de Trás-os-Montes	Trás-os-Montes	Oil	Medium	
Med1	Picual		Oil	High	Spain
Med2	Leccino		Oil	Medium	Italy
Med3	Merhavia		Table	Low	Israel
Med4	Kalamata		Table	Medium	Greece
Med5	Picholine		Both use	Medium	France
Med6	Meski		Table	Low	Tunisia
Med7	Izmir Sofralik		Table	High	Turkey
Med8	Picholine Morocaine		Both use	High	Morocco

[a]*Source*: FAO (2005).

stage of amplification were optimised. A set of 20 oligonucleotide primers, from 107 tested Operon sets (OPA kit-1 to 20, OPC kit, OPE kit, OPO kit, OPX kit, OPQ15, OPQ17, OPS3, OPZ10, OPZ11, OPAA3 and OPAA11), was used to obtain specific molecular markers (Table 2).

The PCR reaction was composed of 1×PCR buffer, 62.5 mM $MgCl_2$, 10 mM of dNTPs, 1.5 U of Taq Polymerase (Fermentas), 50 ng of primer, 70 ng of template DNA and ddH_2O to a final volume of 25 μL.

Amplifications were performed in a Biometra Tgradient thermocycler, with the following PCR cycle: 94°C, 3 min; 94°C, 1 min; 38°C, 1 min; 72°C, 2 min; repeat to step 2, 44 times; 72°C, 10 min.

The amplification products were separated by gel electrophoresis on a 1.5 % agarose gel, in 1×TBE buffer during 2 h at 80 V and stained with ethidium bromide (100 mg/ml).

ISSR amplification

For the ISSR-PCR amplification we tested 100 primers from the set 100/9 (University of British Columbia). Seventeen primers were selected on base of the number of bands and their

Table 2. Primers used for RAPD and ISSR analyses: total number, polymorphic, unique bands and % of polymorphism obtained.

Primer	Sequence 5′–3′	Total number of bands	Polymorphic bands	Unique bands	% Polymorphism
PA1	CAGGCCCTTC	18	17	1	94
OPA13	CAGCACCCAC	19	15	0	79
OPC8	CCCAAGGTCC	22	18	2	81
OPC13	GGTGCGGGAA	14	13	5	93
OPE1	GGTGACTGTG	18	18	1	100
OPE2	TGGACCGGTG	18	16	0	89
OPE16	AAGCCTCGTC	10	8	1	80
OPO3	CTGTTGCTAC	6	5	0	83
OPO4	AAGTCCGCTC	19	18	0	95
OPO5	CCCAGTCACT	15	14	1	93
OPO6	CCACGGGAAG	17	14	0	82
OPO7	CAGCACTGAC	15	14	2	93
OPO10	TCAGAGCGCC	15	14	1	93
OPO12	CAGTCGTGTG	20	18	1	90
OPO13	GTCAGAGTCC	15	14	0	93
OPX3	TGGCGCAGTG	10	8	0	80
OPX14	ACAGGTGCTG	12	9	0	75
OPX15	CAGACAAGCC	11	8	0	73
OPX18	GACTAGGTGG	12	10	0	83
OPX19	TGGCAAGGCA	15	11	0	73
UBC 807	$(AG)_8T$	11	11	0	100
UBC 809	$(AG)_8G$	13	11	0	85
UBC 810	$(GA)_8T$	12	11	2	92
UBC 811	$(GA)_8C$	13	12	0	92
UBC 817	$(CA)_8A$	13	13	0	100
UBC 823	$(TC)_8C$	10	9	0	90
UBC 825	$(AC)_8T$	8	7	0	88
UBC 826	$(AC)_8C$	15	12	1	80
UBC 834	$(AG)_8YT$	9	6	1	67
UBC 841	$(GA)_8YC$	14	13	1	93
UBC 846	$(CA)_8RT$	9	7	0	78
UBC 849	$(GT)_8YA$	14	13	0	93
UBC 850	$(GT)_8TYC$	14	14	0	100
UBC 855	$(AC)_8YT$	12	9	0	75
UBC 856	$(GGAGA)_3$	15	15	0	100
UBC 880	$(GGAGA)_3$	7	6	0	88
UBC 889	$DBD(AC)_7$	15	11	0	73

Y = (CT); R = (AG); D = (AGT); B = (CGT).

reproducibility (Table 2). Each amplification reaction consisted of 1 μL of total genomic DNA (20 ng/μL), 1 μL of primer (5 μM), 10 μL of Taq-PCR master mix (Qiagen) and 8 μL of ultra-pure distilled water (Qiagen).

The amplifications were performed in a Biometra Tgradient thermocycler under the following conditions: 94°C, 5 min; 94°C, 30 s; 52°C, 45 s; 72°C, 2 min; repeat to step 2, 45 times; 72°C, 5 min.

The amplification products were separated by gel electrophoresis on a 1.5% agarose gel, in 1 × TBE buffer during 2 h at 80 V and stained with ethidium bromide (100 mg/ml).

Statistical analysis

The PCR fragments were scored for the presence (1) or absence (0) of equally sized bands and two matrices of the different RAPD and ISSR phenotypes were assembled and used in the statistical analysis. The fragments were only considered when reproducible bands were obtained, meaning that for each primer we repeated the reaction three times with the same result. Cluster analysis was performed to construct dendrograms, with the unweighted pair-group method by arithmetic averages (UPGMA) from the similarity data matrices using Jaccard's coefficient. The Numerical

Taxonomy and Multivariate Analysis System program package for personal computer (NTSYS-PC V.2.02; Rohlf 1998) was used for statistical analysis of the data.

Results

Polymorphism RAPD analysis and phenetic relationships

A total of 301 reproducible bands, ranging from 190 bp (primer OPX19) to 3,010 bp (primer OPO10) were detected using the 20 primers previously screened (Table 2). The number of bands per primer varied from 6 (OPO3) to 22 (OPC8) with an average of 16 bands per primer. Two hundred and sixty two bands (87%) out of the 301 reproducible bands were polymorphic showing an average of 13.1 polymorphic markers per primer, ranging from 5 (OPO3) to 18 (OPC8, OPE1, OPO4, OPO12). The primers that showed the highest polymorphism were OPE1 and OPO4 with 100% and 95%, respectively (Table 2).

Fifteen out of the 262 markers were cultivar-specific (Table 3). Eleven absent bands, present in all cultivars except one, were observed (Table 3). A total of seventeen cultivars could be distinguished from the others using RAPD primers. Independently, the primers OPC13, OPA1, OPO7 and OPC8 were able to distinguish three cultivars.

Genetic distances were obtained with UPGMA algorithm using Jaccard's coefficient (Figure 1). The genetic similarities ranged from 0.54 ('Cordovil de Serpa' – 'Izmir') to 0.79 ('Golosinha' – 'Picholine'). The cultivars were grouped into two major clusters and five independent branches. Two out of the five branches belonged to foreign cultivars ('Izmir' and 'Leccino') and the other three belonged to Portuguese cultivars ('Cordovil de Serpa', 'Madural' and 'Leucocarpa').

Polymorphism ISSR analysis and phenetic relationships

A total of 204 reproducible ISSR bands were observed, of which 180 were polymorphic, accounting for a high percentage (88%) of the observed polymorphism (Table 2), which ranged from 280 to 3,000 bp. All primers produced polymorphic bands, with an average of 12 ISSR markers per primer being scored, where the largest number (15) were obtained with primers UBC826, UBC856 and UBC889, and the lowest number (7) were obtained with primer UBC880.

Five out of the 180 markers were cultivar-specific and nine absent bands, present in all cultivars except one, were also observed (Table 3). A total of eleven cultivars could be distinguished from the others using only ISSR markers. Primer UBC810 allowed the discrimination of four different cultivars. The remaining primers were only able to distinguish one of the cultivars studied.

The dendrogram of the ISSR markers of 38 olive cultivars is shown in Figure 2. The genetic similarities ranged from 0.55 ('Leucocarpa' - 'Madural') to 0.84 ('Cordovil Castelo Branco' – 'Redondil'). The cultivars were grouped into 3 clusters and 4 independent branches, curiously, belonging to Portuguese cultivars ('Galega', 'Negrinha de Freixo', 'Madural' and 'Leucocarpa'). The last two also formed independent branches when RAPD markers were analysed (Figure 1).

Combined phenetic relationship

In order to have an overview of the genetic similarities/distance between the olive cultivars under study, a combined UPGMA analysis was performed, using Jaccard's coefficient (Figure 3). The genetic similarities ranged from 0.54 ('Leucocarpa' – 'Cordovil de Serpa') to 0.79 ('Blanqueta' – 'Kalamata'). Four major clusters and five independent branches were obtained. Four of these branches belonged to Portuguese cultivars ('Negrinha de Freixo', 'Madural', 'Cordovil de Serpa' and 'Leucocarpa') and only one fit in a foreign cultivar ('Izmir').

Cluster I contained 16 Portuguese and 3 foreign olive cultivars. In cluster II, 9 Portuguese and 1 foreign cultivar from Morocco were present. Cluster III included one foreign cultivar 'Kalamata' and the Portuguese cultivar 'Blanqueta'. In cluster IV two foreign cultivars were present (see Figure 3).

Several close relationships between cultivars were constant in all the analyses performed: 'Kalamata' and 'Blanqueta'; 'Cordovil de Castelo Branco' and 'Redondil'; 'Golosinha' and 'Picholine'; and 'Cornicabra' and 'Merhavia', whereas,

Table 3. Combination of different molecular markers used to identify and discriminate the 30 Portuguese and 8 foreign olive cultivars, referring the primer and band of each marker, as well as the specific markers for some olive cultivars.

Cultivars	Unique bands	Unique missing bands	Discriminating markers
Azeiteira	–	UBC823-670	OPC8-2005, UBC889-910
Blanqueta	–	UBC856-705	OPE16-987
Borrenta	–	–	OPC8-2005
Carrasquenha	OPC13-1092	–	OPX18-700, UBC826-1910
Cobrançosa	–	–	UBC 826-1910
Conserva de Elvas	–	–	OPO3-465
Cordovil de C. B	OPE16-480	–	OPO3-465, OPO10-820
Cordovil de Elvas	–	OPA1-1050, UBC810-2100	UBC889-490
Cordovil de Serpa	UBC810-470	OPE16-610, OPA1-350	–
Cordovil de T.M.	OPC13-1282	–	–
Cornicabra	–	–	OPX18-700
Galega	–	OPO4-736, UBC846-1100	OPO13-700, OPO12-1046
Galego de Évora	–	–	UBC850-610
Galego G. S	UBC826-680, UBC841-510	UBC810-630	OPO7-111
Golosinha	–	–	OPO13-700, OPX18-700, UBC850-610
Leucocarpa	UBC810-1320	OPA13-532, OPC8-888, OPO7-748, UBC810-1475	OPA1-769, OPA13-1787, OPO10-820, UBC889-910, UBC810-1320, OPC8-888
Maçanilha C.	–	–	OPO12-1046
Maçanilha de E.	–	–	OPO7-111, UBC889-910
Maçanilha de T.	OPO10-820	–	OPO10-510, OPO13-700
Madural	OPO7-1116	–	OPO7-111, UBC850-610, UBC889-910
Mora	–	–	OPA13-1787
Negrinha de Freixo	OPC13-334; 1400; 1528, UBC834-800	OPC13-986	OPE16-987, OPO12-496
Negrita	OPA1-2108, OPE1-976	–	–
Quinta do Portado	–	–	UBC889-490
Redondal	–	–	OPO12-496, OPO12-1046
Redondil	–	–	OPO3-465, OPO10-510, UBC889-910
Tentilheira	OPO7-1757	OPO4-675	UBC850-610
Verde Verdelho	OPC8-748	–	OPO4-1311
Verdeal de Serpa	OPO12-617	–	–
Verdeal de T. M.	–	–	OPA1-2108
Picual	OPC8-566	–	–
Leccino	–	UBC880-490	OPX18-700, OPO12-1046
Merhavia	–	–	UBC889-910, UBC889-490
Kalamata	–	–	OPE16-987
Picholine	–	UBC825-810	UBC826-1910, UBC823-670
Meski	OPO5-306	UBC889-887	OPX18-700, UBC850-610, OPO12-1046
Izmir Sofralik	–	OPO6-2286, OPX18-550	UBC889-490
Picholine Morocaine	–	–	OPA1-769, OPO4-1311

the Portuguese cultivars 'Madural' and 'Leucocarpa' always presented independent branches in all three analyses. 'Negrinha de Freixo' was an independent branch in the ISSR and in combined analyses (Figures 2 and 3), but was integrated in cluster II of the RAPD analysis. Nevertheless, it was the first to derivate from that cluster. 'Cordovil de Serpa' had a similar performance as 'Negrinha de Freixo', because it was an independent branch in the RAPD and combined analyses (Figures 1 and 3), although it was integrated in cluster I of the ISSR dendrogram (Figure 2).

Most of the foreign cultivars were not clustered independently from the Portuguese cultivars in general, once they were dispersed in all clusters obtained in the different analyses. Only the cultivar 'Izmir' was an independent branch in both RAPD and combined dendrograms (Figures 1 and 3), but in the ISSR analysis it belonged to cluster III (Figure 2).

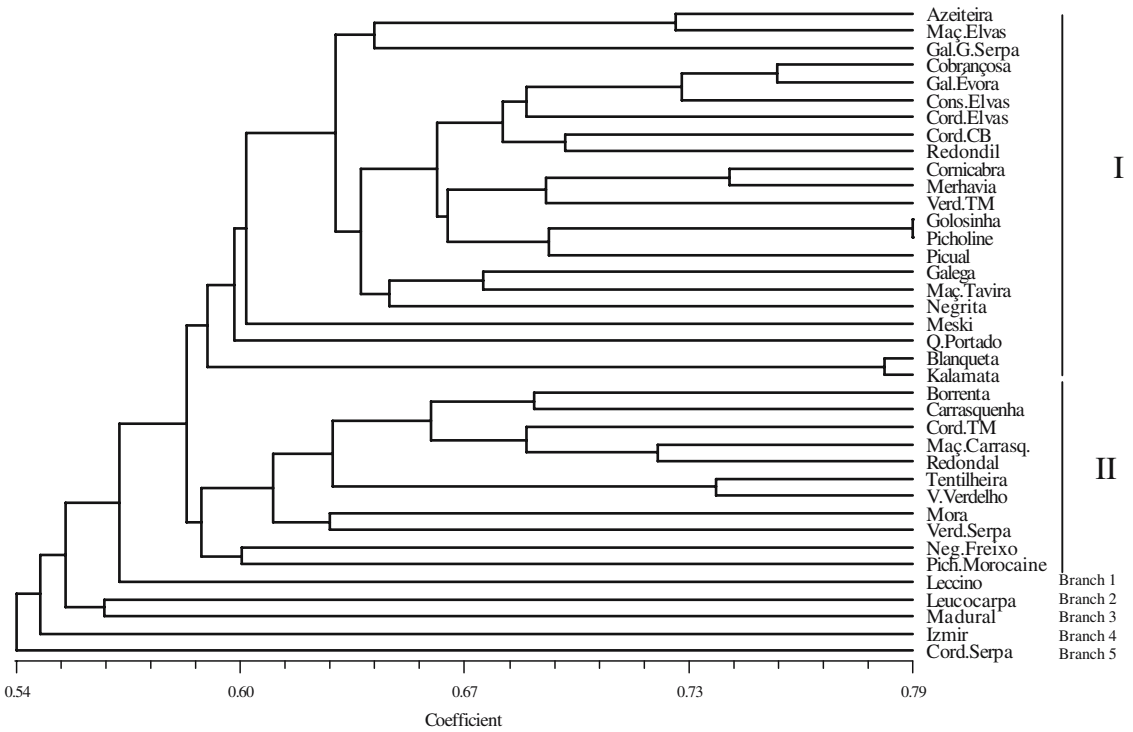

Figure 1. UPGMA dendrogram based on Jaccard's coefficient illustrating the genetic similarities and distance among olive cultivars obtained by RAPD data, generated by the UPGMA cluster analysis (NTSYS).

No relationship to geographic origin and end-use in Portuguese cultivars was found (figure not shown).

Discussion

In our work, the olive cultivars under study formed different groups when RAPD (Figure 1) and ISSR (Figure 2) primers were used independently. This can be explained by the fact that ISSR primers target specific genome regions, while a RAPD primer amplify arbitrary regions.

Combined phenetic relationship

RAPD and ISSR techniques revealed a high level of polymorphism. There were no significant differences between the genetic variability obtained in the group of Portuguese olive cultivars and in the foreign cultivars. In RAPDs the percentage of polymorphism observed was 50.8 and 47.75%, for Portuguese and foreign cultivars, respectively. Slightly higher values were registered for ISSR (57.4% for Portuguese cultivars and 58.75% for foreign ones) what confirmed that the ISSR marker system is more polymorphic than RAPD in many plant species (Nagaoka and Ogihara 1997; Korbin et al. 2002; Galvan et al. 2003). These values were at the same level as the polymorphism reported within 12 clones of 3 Italian olive tree cultivars by AFLP (Sensi et al. 2003). The high polymorphism for RAPDs observed in comparison with other studies (Nagaoka and Ogihara 1997, Korbin et al. 2002, Galvan et al. 2003) may also be due to the high selection pressure of the primers, 20 most informative ones out of 107 primers initially used.

The high level of polymorphism observed in this study and in other reports of Portuguese (Gemas et al. 2004; Lopes et al. 2004) and foreign cultivars (Fabbri et al. 1995; Weisman et al. 1998; Barranco et al. 2000; Belaj et al. 2003a, b; Terzopoulos et al. 2005) indicated that olive is a highly polymorphic species. The high diversity found between olive cultivars is probably due to a diverse germplasm origin, that presumably results from crosses

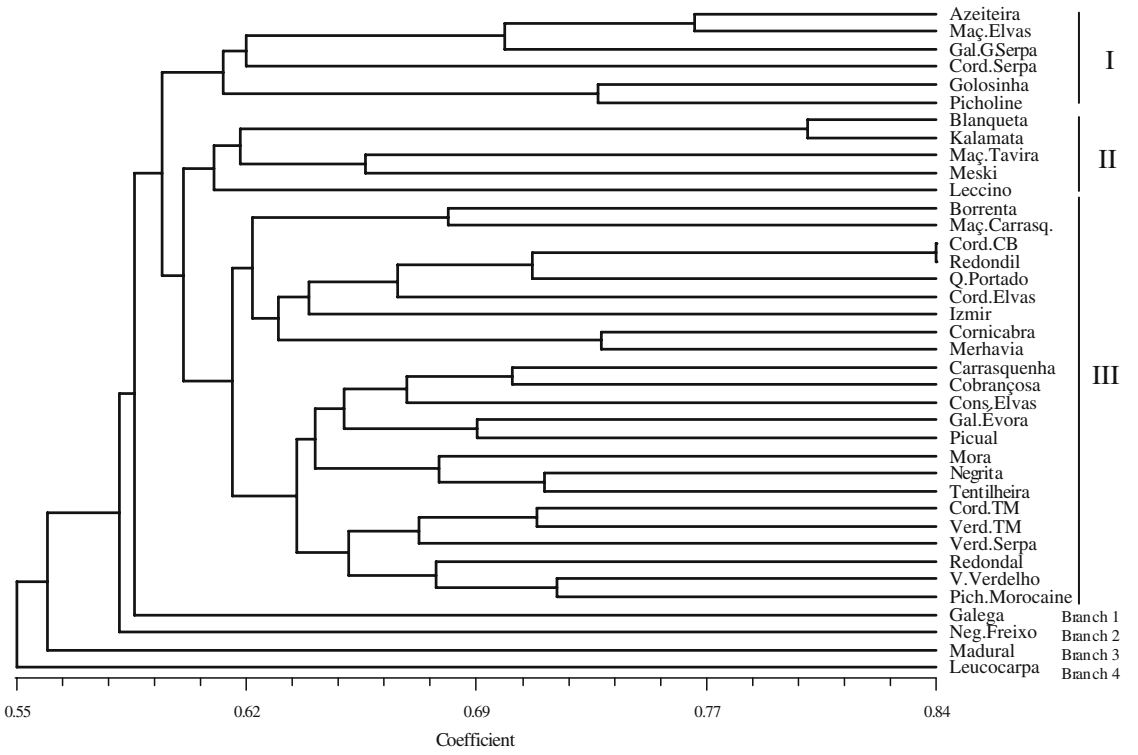

Figure 2. UPGMA dendrogram based on Jaccard's coefficient illustrating the genetic similarities and distance among olive cultivars obtained by ISSR data, generated by the UPGMA cluster analysis (NTSYS).

between wild and cultivated olives resulting in new cultivars in different parts of the Mediterranean, and low breeding pressures (Besnard et al. 2001a; Contento et al. 2002; Belaj et al. 2003c).

This may also explain why in the dendrograms no distinction was observed between foreign and Portuguese cultivars. The same was observed between French and foreign cultivars, and in sample of Mediterranean cultivars (Besnard et al. 2001a). No relationship to geographic origin between Portuguese and foreign cultivars was found in our work. Khadari et al. (2003) using French and foreign olive cultivars and Besnard et al. (2001a) analysing 102 genotypes from several Mediterranean countries have reported similar results. However, in some cases a clustering of cultivars of the same region was observed (Besnard et al. 2001a; Bandelj et al. 2004).

In terms of olive end-use (oil, table and both use) we could not find any clear clustering, oppositely to studies of Besnard et al. (2001a) and Gemas et al. (2004). This could be explained by the fact that the number of cultivars and primers used in the previous studies were different. Gemas et al. (2004) analysed only 11 of the 30 Portuguese cultivars used in our study. They did not use ISSR in their study, which may not be covering the entire range of variability available in the genoma.

In the dendrogram obtained from our data several cultivars were consistently very far apart from the rest of the cultivars studied. Surprisingly, four of these cultivars were Portuguese ('Negrinha de Freixo', 'Madural', 'Leucocarpa' and 'Cordovil de Serpa'). 'Leucocarpa' was already suspected to be an outsider, due to its morphological characteristics (very small white fruit), commonly known as an albino wild *Oleaster*.

The distinct phenetic position of 'Madural' was previously reported with RAPD markers (Gemas et al. 2004) and SSR markers (Lopes et al. 2004). Belaj et al. (2002) also reported a distant phenetic position of 'Cordovil de Serpa', in relation to the other cultivars. 'Negrinha' was reported to be linked with 'Cobrançosa', 'Azeiteira' and 'Negrita' when SSR markers are used (Lopes et al. 2004),

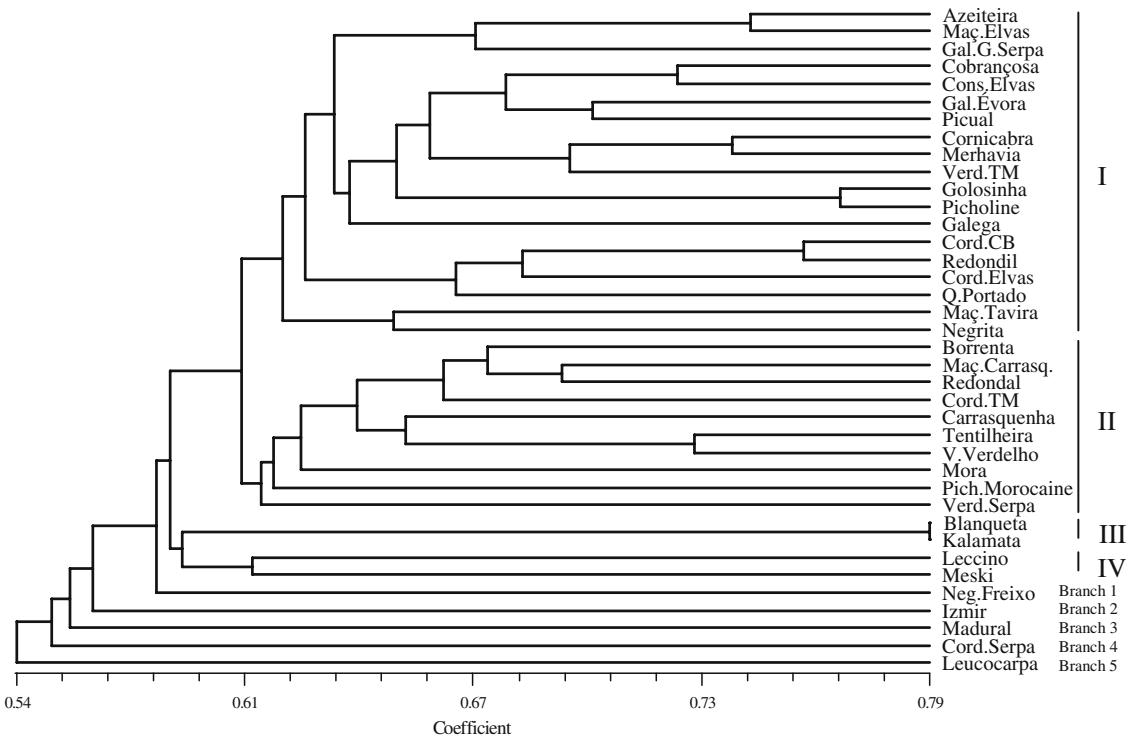

Figure 3. UPGMA dendrogram based on Jaccard's coefficient illustrating the genetic similarities and distance among olive cultivars obtained by RAPD and ISSR data, generated by the UPGMA cluster analysis (NTSYS).

and with 'Azeiteira' when RAPD markers are used (Gemas et al. 2004). However, in our study 'Negrinha de Freixo is an independent branch. This supports that 'Negrinha' used in previous studies is not the same cultivar as 'Negrinha de Freixo'.

Gemas et al. (2004) reported that 'Galega' and 'Blanqueta' are also two independent branches. We could confirm this fact for 'Galega' when the ISSR data were considered, but not in the RAPD and in combined analysis (Figures 2 and 3). The differences found between these studies might be due to the different number of primers analysed and to different marker systems used. In our analysis, 'Blanqueta' was associated with 'Kalamata', which is quit interesting because the phenotype analysis of these two cultivars revealed that both have very long leaves. Although the similarities in leaf length and genetic distance, 'Kalamata' and 'Blanqueta' could not be considered as synonymies once they differ in other important phenotypical characteristics, like fruit shape, and end-use.

In our study, the cultivars 'Golosinha' and 'Picholine' revealed to be related to each other. However, they are distinctive cultivars not only because their origin, end-use and oil content are different (see Table 1), but also due to different morphological specificities such as different fruit size and leaf shape and colour.

Cultivars synonymous and homonymous

The ability to distinguish among cultivars and to clarify synonymous and homonymous is of major importance for solving problems like olive germplasm management and nursery mislabelling.

Similar Portuguese cultivar designations may induce the hypothesis that those cultivars were genetically related. On the basis of genetic similarity showed in Figure 3, the analyses of all the three 'Galega' cultivars ('Galega', 'Galego de Évora', and 'Galego Grado de Serpa') confirmed that they were closely related. However, all 'Cordovil' cultivars ('Cordovil de Castelo Branco',

'Cordovil de Elvas', 'Cordovil de Serpa' and 'Cordovil de Trás-os-Montes') appeared to be genetically different. The same was observed in the case of the two 'Verdeal' cultivars ('Verdeal de Serpa' and 'Verdeal de Trás-os-Montes'). These results could be explained probably by their geographical distances. We could relate two of the three 'Maçanilha' cultivars ('Maçanilha de Elvas' and 'Maçanilha de Tavira') but the third one, 'Maçanilha Carrasquenha', was closer to the other homonymous, 'Carrasquenha'. All the results presented here are consistent with those reported by Lopes et al. (2004) using SSR markers. These studies may help olive germplasm databases in organising the files relating to each olive cultivar.

In terms of synonymous, although in FAO's database for olive germplasm (www.fao.org) 'Madural' and 'Cornicabra' are considered as the same cultivar, this was not confirmed by our results (Figure 3), or by Lopes et al. (2004). As a matter of fact they have distinct morphological characters ('Madural' has an oval average fruit and short and wide leaves with light green colour, while 'Cornicabra' shows a long oval fruit with longer leaves and medium green colour). Instead, we found that 'Cornicabra' was closely linked with 'Merhavia', an Israeli cultivar, although they differ from leaf size.

Differential discrimination of the olive cultivars by RAPD and ISSR molecular markers

The molecular polymorphism observed among the olive genotypes was appropriate to differentiate cultivars. All the Portuguese olive tree cultivars, plus the eight from foreign countries, spread in the Mediterranean basin, revealed RAPD and ISSR molecular markers that allowed identification of each one of the studied cultivars. As far as we know, it is the first time that RAPD and ISSR markers were able to identify the complete Portuguese standard cultivars collection of *Olea europaea* L. Other markers (AFLP, mtDNA RFLP, SSR, tandem repeated DNA sequences) were also used in several European olive cultivars studied for establishing its genetic variability, genetic relationships and genotyping (Pasqualone et al. 2001; Contento et al. 2002; Belaj et al. 2003b; Sensi et al. 2003; Khadari et al. 2003; Bandelj et al. 2004).

In our work, five ISSR and 15 RAPD primers allowed the screening of 20 molecular markers specific to different cultivars, which can be converted into SCAR markers for individual cultivar identification analysis (Hernández et al. 2001; Bautista et al. 2002).

Conclusions

The diversified origin of olive germplasm in the Mediterranean basin could be established. The genetic variability among the thirty Portuguese cultivars was at the same level as the one observed in the group of the foreign cultivars studied. The genetic affinities between Portuguese cultivars could be found in some cases but not in others.

Our data also showed the relevance of molecular studies for management and olive genetic resources conservation. The results of this study indicated that RAPD and ISSR techniques constitute a useful tool to find new specific molecular markers that allowed us to identify individually all the 38 olive cultivars studied.

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