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Fingerprinting and genetic diversity of *Olea europaea* L. ssp. *europaea* accessions from the cultivar Galega using RAPD markers

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ABSTRACT

Olea europaea ssp. *europaea* (Oleaceae) grows in Mediterranean countries. The Galega represents one of the most common Portuguese olive tree cultivars, and is used in five out of the six PDO (Protected Designation of Origin) regions. The fruits are used for olive oil and for fruit canning, besides which the cultivar is drought tolerant. The cultivar certification is done through phenotypic characteristics, but environmental influence might blur informativeness, and molecular markers could help in clarifying cultivar accessions certification.

A total of 75 putative Galega accessions considered as belonging to the Galega cultivar, three previously phenotypically identified as GGN (Galega Grada Normal), GGS (Galega Grada de Serpa) and GGE (Galega Grada de Évora), and one as a non-Galega blind control were analyzed. Twenty RAPD primers amplified 144 bands and 32% were polymorphic. A low expected heterozygosity was estimated (10%), as expected for clonal propagated plants.

The Principal Component Analysis (PCA) clearly identified two groups. Group A was comprised of all the putative Galega accessions including GGN, but not individual #502. Group B included NG, GGE, GGS and #502. The analysis of molecular variance confirmed a very high differentiation between the two groups (88%), but also some within groups' diversity (12%). Group A individuals cluster closely to GGN. Assuming that the GGN individual represents a Galega cultivar tree, we might assume that all the A group trees are Galega cultivar accessions. Nevertheless, some within group variability exists, may be due to the accumulation of somatic mutations and/or a putative origin from different, but related genotypes. Finally, the GGS, the GGE, and the #502 plot together with the individual identified as non Galega cultivar tree. Group B trees certainly had a very different genetic origin compared to the group A genotypes, which confirms some differences found in the morphologic characteristics compared to the ordinary Galega cultivar, reflected in the name differences.

The pairwise matrix computed with the Dice similarity index was also used to obtain a dendrogram. Results were similar, but slightly different from the PCA. Three clusters were obtained. This indicates that the B group could be split into two sub-groups. A future study using a broader sampling and higher discriminant molecular markers, such as microsatellites, would help to reveal the phylogeny among the 3 cultivars (GGN, GGS and GGE) and to clarify if the intra-variability of those cultivars is geographically related.

1. Introduction

In the Mediterranean region the olive tree (*Olea europaea* L. ssp. *europaea*) occupies 95% of the world cultivated olive area, and was probably domesticated in the Near-East ca. 6000 years ago (Zohary and Spiegel-Roy, 1975), spanning Westwards with human migration (Breton et al., 2009). The olive is one of the most important crops as a source of oil and in table consumption, with favorable nutritional properties, thus increasing worldwide consumption, and 77% of the olive oil is produced in the Mediterranean countries (Doveri and Baldoni, 2007). The olive tree has been cultivated in Portugal, at least since the Roman domination (Ribeiro, 1991), and this species occupies, presently, around 36% of the cultivated area representing one of the main agricultural crops (INE, 2011).

The cultivated olive is an evergreen, outcrossing, vegetative propagated tree with a very wide genetic background, resulting from both the plant longevity and the scarcity of genotype turnover through centuries of cultivation. A great number of olive cultivars

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(presumed clones) are grown throughout the world and several hundred, supposedly clonal accessions, are described within the Mediterranean region.

During the long history of olive cultivation, different cultivars may have been given the same name (homonymy), while a cultivar may have been named differently (synonymy) in different countries (Besnard et al., 2001). Thus the number of existing cultivars is unclear and the size of the olive germplasm needs to be clarified. Indeed, 1208 cultivars were reported in 52 countries and preserved in 94 collections as referred in the 2005 FAO Olive Germplasm Database (http://apps3.fao.org/wiews/olive/oliv.jsp), but this number is probably underestimated since there is a lack of information about minor local cultivars. The formation of polyclonal cultivars of heterogeneous cultivars (different genotypes) was probably a rule, instead of monoclonal cultivars. Intra-varietal polymorphisms were reported in literature (e.g., Gemas et al., 2004; Lopes et al., 2004; Muzzalupo et al., 2010), in which the observed differences within the same cultivar have been suggested as somatic mutations occurring during vegetative propagation. For olive cultivars no "standard reference variety" was established, and only recently have some papers been trying to achieve a "standard certificate" (Muzzalupo et al., 2010 and references therein).

The olive oil production under specific denominations corresponds to well-defined cultivars. The quality control requires identification of orchard-derived clones, distributed through nurseries. Furthermore, in olive breeding programs it is important to accurately identify the genotypes and to distinguish new cultivars for registration purposes. In addition, for cultivar collection management it is necessary to identify each clone/genotype in order to detect any possible synonyms, mislabeling, and mutants, which have already been detected in olive germplasm collections (e.g., Lopes et al., 2004).

Cultivar identification based on phenological and morphological phenotypes from field or nursery observations may not be adequate to assign cultivar identity due to environmental effects on traits (Besnard et al., 2001). Additionally, mislabeling or multicultivar origin is also possible. According to Cordeiro et al. (2008), when twenty nine Portuguese cultivars were characterized using morphological markers (leaves, fruits and stones), five pairs were found to be putative synonyms. Molecular markers may help in sorting out the biodiversity and the cultivar identification in olive. The RAPDs have successfully been used in olive studies including Portuguese cultivars, such as the cultivars' fingerprinting (Cordeiro et al., 2008; Martins-Lopes et al., 2007), and inter or intra-cultivar genetic diversity estimation (Gemas et al., 2000, 2004).

The ancient Galega cultivar represents the majority of the Portuguese olive heritage, and is drought and olive knot resistant, producing fruits with high oil quality, but with medium production content (Cordeiro et al., 2008). This cultivar is used for olive oil production in five out of the six PDO (Protected Designation of Origin) regions (http://www.casadoazeite.pt), and the certification of select cultivars olive trees is mandatory to face consumers' needs and to match PDO requirements. The European Union has developed a PDO designation to olive oils with important regional traditional origins (Council Regulation (EC) 510/2006 20 March 2006). Oil quality is strongly cultivar-dependent, but is also affected by agro-climatic factors and agronomic practices (Doveri and Baldoni, 2007 and references therein).

The specific aims of the present paper, using the RAPD technique, were: (i) to verify if all the studied accessions belonged to the Galega cultivar (hereafter Galega cultivar *sensu stricto*), (ii) to analyze the intra-cultivar genetic relationship among the 79 studied accessions, (iii) to uniquely fingerprint all the accessions. The main purpose of the study was to unambiguously verify if we could include in the Galega cultivar *sensu stricto* the 75 putative Galega genotypes under study, for further deployment and use to produce certified olive oil.

Table 1
Primers used for the RAPD analysis (Pb = polymorphic bands, Nb = number of bands and P = polymorphism).

Primer	Sequence 5′–3′	Pb	Nb	P (%)
OPC-1	TTCGAGCCAG	4	12	33.3
OPC-2	GTGAGGCGTC	6	9	66.6
OPC-3	GGGGGTCTTT	1	5	20.0
OPC-4	CCGCATCTAC	3	7	42.8
OPC-5	GATGACCGCC	3	5	60.0
OPC-6	GAACGGACTC	2	11	18.2
OPC-7	GTCCCGACGA	6	12	50.0
OPC-8	TGGACCGGTG	1	8	12.5
OPC-9	CTCACCGTCC	3	7	42.8
OPC-10	TGTCTGGGTG	4	7	57.1
OPC-11	AAAGCTGCGG	0	2	0.0
OPC-12	TGTCATCCCC	2	7	28.6
OPC-13	AAGCCTCGTC	4	10	40.0
OPC-14	TGCGTGCTTG	1	6	16.7
OPC-15	GACGGATCAG	0	3	0.0
OPC-16	CACACTCCAG	2	5	40.0
OPC-17	TTCCCCCCAG	0	6	0.0
OPC-18	TGAGTGGGTG	0	3	0.0
OPC-19	GTTGCCAGCC	1	7	14.3
OPC-20	ACTTCGCCAC	3	12	25.0
		46[a]	144[a]	31.9[b]

[a] Sum.
[b] Average.

2. Materials and methods

2.1. Plant material

In this study we have used 75 *O. europaea* accessions from a study plot in the Herdade da Quinta do Leão, Estremoz (Portugal). This area is an experimental plot with putative Galega trees collected from around the country. Using morphologic descriptors three trees were identified as Galega *sensu stricto* (GGN = Galega Grada Normal), Galega Grada de Évora = GGE and Galega Grada de Serpa = GGS, and one as non-Galega = NG. According to Martins-Lopes et al. (2007) the different Galega names reflect the different regions of origin, GGN includes trees from Alentejo, Ribatejo and Beiras, GGE from Évora and GGS from Serpa and Moura.

2.2. DNA extraction and amplification

Total DNA was extracted from young leaves with the QIAGEN DNeasy Plant Mini kit and pure genomic DNA was obtained with a high concentration.

The set of the 20 primers used were from the Operon OPC kit (Table 1). The RAPD amplification conditions carried out in a final volume of 25 μl:1 μl of genomic DNA, 1 × Buffer (Bioron), 2.5 mM Mg^{2+} (Bioron), 4 μM of a single decamer primer from KIT C (Operon Technologies – Alameda, CA, USA) (Table 1), 0.3 mM dNTPs (Bioline), 0.5 U Taq polymerase (Bioron), completed with MilliQ sterile water. All the PCR reactions were performed in a thermal cycle (MJ Mini Personal) from BIORAD and the amplification conditions were: an initial denaturation at 94 °C for 1.5 min followed by 35 cycles of 30 s at 94 °C, 45 s at 35 °C and 1.5 min at 72 °C and one last extension step 5 min at 72 °C and hold at 4 °C. Amplifications were performed in a Biorad MJ Mini Personal Thermal Cycler. The amplification products were separated by electrophoresis on a 1.8% agarose gel stained with ethidium bromide, in 1 × TBE buffer during 2 h 30 min at 80 V. The molecular sizes of the fragments were estimated using a 100 bp DNA ladder from Bioron.