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DNA Markers for Portuguese Olive Oil Fingerprinting

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The certification of olive oil has led to the definition of Protected Denomination of Origin (PDO) producing regions in European countries. PDO products should be protected, and a solution could be by using DNA fingerprinting. In this work we evaluate the efficiency of RAPD, ISSR, and SSR molecular markers for olive oil varietal identification and their possible use in certification purposes. Twenty-three Portuguese olive oil samples (11 obtained monovarietal and 12 purchased commercial oils) were screened by means of two RAPD, four ISSR, and four SSR markers. The quality of amplified products was used to evaluate the reproducibility and the level of polymorphism. Principal component analysis was performed with DCENTER using unweighted pair group mathematical average (UPGMA) that allowed group formation according to olive oil varietal geographic origin.

KEYWORDS: Olive oil certification; molecular markers; traceability

INTRODUCTION

Olive (*Olea europaea* L. subsp. *europaea* var. *europaea*) is one of the most important tree crops in the Mediterranean basin. Its importance is increasing due to nutritional and health features of the olives and derived oil (*1*−*3*). Several Protected Denomination of Origin (PDO) olive oil regions have been established by legislation to enhance the quality of this product, to ensure both consumers' expectations and producers' profits. Virgin olive oils command a premium in the market, leading to a great temptation to adulterate them with vegetable seed oils (*4*).

Several techniques based on olive oil composition (such as gas chromatography and silica gel column chromatography) have been applied to detect adulterations (*4*−*8*). However, some difficulties have been found in distinguishing olive cultivars based on both drupes and oils of different cultivars because their characteristics are strongly influenced by environmental conditions (*9*). Recently, DNA-based markers, which are independent from environmental conditions, have been successfully applied to overcome this problem.

The use of molecular markers is being investigated as a diagnostic tool for food authenticity and traceability of variety/type composition of complex food matrices in an increasing number of worldwide projects (*10*). For DNA extracted from olive drupes the application of molecular markers has been first achieved using intersimple sequence repeats (ISSR) (*11*).

Significant amounts of DNA are present in olive oil obtained by cold pressing (*12*). However, the filtration process lowers DNA concentrations, which tend to disappear due to nuclease degradation (*9, 13*). In olive oil, once the barrier of DNA extraction has been overcome, several markers have been used to identify olive cultivars that make up a certain olive oil sample. The techniques that have been applied in this context are amplified fragment length polymorphism (AFLP) (*14, 15*), random amplified polymorphic DNA (RAPD) (*13, 14*), sequence characterized amplified region (SCAR) (*14, 16*), microsatellites (*17*−*21*), and, more recently, chloroplast DNA (*22*).

The aim of this study was to use a combination of molecular markers (RAPD, ISSR, and SSR) to establish a relationship between small-scale-produced monovarietal and commercial olive oil samples for certification purposes.

MATERIALS AND METHODS

Olive Oil Samples. Eleven monovarietal olive oil samples, from Portuguese cultivars, were produced using 50 kg of olive drupes, from certified groves, immediately after harvest at the end of November to the beginning of December 2003, using cold centrifugation (**Table 1**). The olive oils were stabilized using paper filtration (Whatman no. 1) and sodium sulfate (25 g/250 mL of olive oil). Twelve commercial olive oils were purchased in 2006 (**Table 1**).

DNA Preparation. Three commercial DNA extraction methods, DNeasy Plant mini kit (Qiagen, Hilden, Germany), Nucleo-Spin Plant, and Nucleo-Spin Food kit (Macherey Nagel, Duren, Germany), and two academic methods, the CTAB method (*23*) and the hexane method (*12*), were previously tested in three olive oil samples. The five methods were compared in terms of starting volume of oil sample, average DNA concentration, and total DNA extraction yield (**Table 2**). DNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

DNA extraction, from the 23 olive oil samples used in this study, was carried out using the method described by Consolandi et al. (*12*). Three DNA preparations for each olive oil lot were performed so different extractions could be compared. Two phases (oily and water) were obtained (*12*) and were used differentiated in PCR amplification reactions.

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Table 1. Code, Type of Olive Oil, and PDO Region for 11 Small-Scale-Produced Monovarietal Olive Oils and 12 Purchased Commercial Ones

olive oil denomination	code	type of olive oil	PDO region
Azeiteira	AZ	monovarietal[a]	Alentejo
Blanqueta	BL	monovarietal[a]	North Alentejo
Carrasquenha	CAR	monovarietal[a]	Alentejo (Elvas and Campo Maior)
Cobrançosa	COB	monovarietal[a]	Trás-os-Montes, Alentejo, Ribatejo, Beiras
Cordovil de Castelo Branco	CCB	monovarietal[a]	Beira Interior
Cordovil de Serpa	CS	monovarietal[a]	Alentejo (Serpa and Moura)
Galega	GAL	monovarietal[a]	Alentejo, Ribatejo, Beiras
Madural	MAD	monovarietal[a]	Trás-os-Montes
Redondal	REDA	monovarietal[a]	Trás-os-Montes
Redondil	REDI	monovarietal[a]	Alto Alentejo
Verdeal de Serpa	VER	monovarietal[a]	Alentejo (Serpa)
Oliveira da Serra: special (bottle)	Com 1	common	not applicable
Oliveirada Serra: selection (bottle)	Com 2	common	not applicable
Oliveirada Serra: classic (tetra-pack)	Com 3	common	not applicable
Gallo: classic (bottle)	Com 4	common	not applicable
Gallo: classic (can)	Com 5	common	not applicable
Casal da Vilariça (bottle)	Com 6	PDO	Trás-os-Montes
Alfandagh (ac < 0.7%) (bottle)	Com 7	Biologic Agriculture	Trás-os-Montes
Alfandagh (ac < 0.3%) (bottle)	Com 8	Biologic Agriculture	Trás-os-Montes
Quinta do Bispado (bottle)	Com 9	PDO	Trás-os-Montes
Herdade do Esporão-Galega (bottle)	Com 10	common-monovarietal	not applicable
AzeitedeMoura (bottle)	Com 11	PDO	Alentejo
Casa Grande (bottle)	Com 12	PDO	Trás-os-Montes

[a] Small-scale production.

Table 2. Comparison between Five Different DNA Extraction Methods Evaluating Initial Quantity of Oil, Average DNA Concentration, and Total DNA Yield

method	starting vo (mL)	concn (ng/μL)	yield (ng)
Qiagen Plant	1.0	1.75	87
Nucleospin Plant	1.0	13.2	1320
NucleoSpin Food	4.2	23	2300
CTAB (*23*)	6.0	7.75	387
hexane (*12*)	2.0	35.2	3520

Table 3. Total Number of Bands, Polymorphic Bands, and Percentage of Polymorphism Obtained per Each RAPD and ISSR Primer among the 23 Olive Oil Samples

primer[a]	sequence 5′−3′	total no. of bands	polymorphic bands	% polymorphism
UBC 817	$(CA)_8A$	8	8	100
UBC 826	$(AC)_8C$	6	5	83
UBC 846	$(CA)_8RT$	2	2	100
UBC 855	$(AC)_8YT$	2	0	0
total ISSR		18	15	83
OPA13	CAGCACCCAC	3	1	33
OPO4	AAGTCCGCTC	6	6	100
total RAPD		9	7	78

[a] OP, Operon RAPD primers (Operon Technologies, Inc); UBC, ISSR primers from the University of British Columbia.

Random Amplified Polymorphic DNA. RAPD marker amplifications were carried out using the procedure described in Martins-Lopes et al. (*24*), with some modifications with regard to DNA concentrations, using initially 11 RAPD primers (OPA1, OPA4, OPA13, OPC13, OPE1, OPE15, OPO4, OPX3, OPX14, OPX18, OPX19, from Operon Technologies, Alameda, CA) and reducing them to 2 (**Table 3**) on the basis of amplification success (amplification in all DNA samples). The 25 μL reactions were incubated at 94 °C for 3 min, followed by 44 cycles of 94 °C/1 min, 38 °C/1 min, and 72 °C/2 min, and a final step of 72 °C for 10 min. Each reaction contained 62.5 mM $MgCl_2$, 10 mM dNTP, 1.5 units of *Taq* polymerase (MBI Fermentas, Burlington, ON, Canada), 50 ng of primer, and 7 μL of oily phase DNA template in 1× PCR buffer.

Intersimple Sequence Repeat. ISSR amplifications were tested using initially eight primers (UBC809, 810, 817, 823, 826, 846, 855, 856, from the University of British Columbia). On the basis of the number of bands and their reproducibility, four primers were selected (**Table 3**). Each reaction consisted of 3 μL of oily phase of DNA template, 1 μL of primer (5 μM), 10 μL of *Taq*−PCR master mix (Qiagen), and 8 μL of ultrapure distilled water (Qiagen). The cycling profile was 94 °C/5 min, followed by 45 cycles of 94 °C/30 s, 52 °C/45 s, and 72 °C/120 s, with a final step of 72 °C/5 min.

Amplicons (RAPD and ISSR) were separated by electrophoresis onto 1.5% (w/v) agarose gels (Seakem agarose) in 1× TBE buffer at 80 V for 150 min, after which the gels were stained in 7 μg mL^{-1} ethidium bromide solution and a digital image was obtained directly under UV light. Each DNA sample was independently amplified at least twice with each primer for each DNA extraction, and only reproducible amplified products were scored.

Microsatellites. The SSR analysis was carried out using four primer pairs [ssrOeUA-DCA1, ssrOeUA-DCA3, ssrOeUA-DCA5, and ssrOeUA-DCA9; see *25*]. The 20 μL reaction volume contained 10 μL of water phase DNA template, 1.5 mM $MgCl_2$, 10 mM dNTP, 10 pmol of each primer, and 1.5 units of *Taq* Hot Start DNA polymerase (Roche, Basel, Switzerland) in 1× PCR buffer. The cycling regimen consisted of 94 °C/4 min, followed by 34 cycles of 94 °C/30 s, 50−55 °C [primer pair dependent, see *25*]/45 s, and 72 °C/60 s, with a final step of 72 °C/10 s. Fragment size was determined after electrophoresis on an automated sequencer (Beckman Coulter Sequencer, Beckman Coulter, Inc., Fullerton, CA) with the help of internal size standards (CEQ DNA Size Standard Kit 400; Beckman Coulter, Inc.).

Statistical Analysis. All PCR amplifications were repeated twice for each DNA sample extracted. The PCR fragments were scored for the presence (1) or absence (0) of equally sized bands, and three matrices of the different RAPD, ISSR, and SSR markers were assembled and used in the statistical analysis. A principal component analysis (PCA) based on the RAPD, ISSR, and SSR genotypic data was performed with double-centering analysis (DCENTER) to reveal patterns of similarity between olive oil samples, relating them with geographical origin. All computations employed the appropriate procedures within NTSYS.pc v2.02 (*26*).

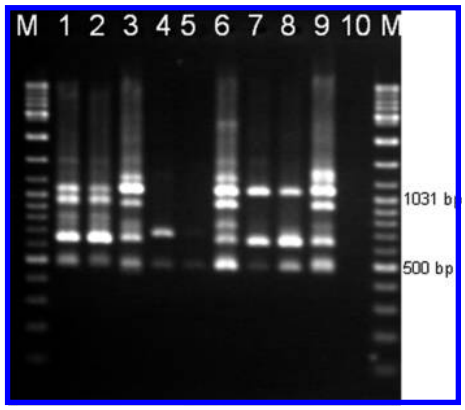

Figure 1. Intersimple sequence repeat profiles obtained in small-scale-produced monovarietal olive oil samples from the first (lanes 1, 4, and 7) and second (lanes 2, 5, and 8) filtrations and in leaf samples (lanes 3, 6, and 9) using three cultivars: Galega (lanes 1−3), Cobrançosa (lanes 4−6), and Azeiteira (lanes 7−9). Lane 10 is blank, and lane M is a DNA ladder (GenRulerTM DNA ladder Mix 10 kbp MBI Fermentas, Burlington, ON, Canada). 404 × 557 mm (96 × 96 DPI).

Table 4. Allele Numbers, Size Range, and Observed Heterozygosity (H_o) in Four SSR (Microsatellite) Loci among the 23 Olive Oil Samples

locus	alleles	size range (bp)	heterozygosity
ssrOeUA-DCA01	6	206–218	0.435
ssrOeUA-DCA03	3	237–250	0.522
ssrOeUA-DCA05	2	205–213	0.043
ssrOeUA-DCA09	11	162–205	0.864

RESULTS AND DISCUSSION

A reliable olive oil DNA extraction method is the basis for any marker-based assessment of olive oil varietal composition. Thus, five DNA extraction methods were tested and compared (**Table 2**). The method described by Consolandi et al. (*12*) was revealed to have the highest yields.

To guarantee that PCR amplifications between different olive oil DNA extractions were reproducible and reliable, we compared PCR profiles, using RAPD and ISSR markers, from different monovarietal olive oil extractions and leaf samples. ISSR profiles are presented in **Figure 1**. PCR profile amplifications were similar between all olive oil DNA extractions. When DNA from olive oil and leaf material was compared, almost all fragments were similar, the exception being the high size fragments observed in leaf samples (**Figure 1**). A comparison was equally made using SSR markers. The same alleles were found between small-scale-produced monovarietal olive oil and leaf samples (data not shown).

All olive oil samples were equally successful in terms of PCR amplification, indicating that the Consolandi et al. (*12*) extraction method gives satisfactory amplification rates for both commercial and small-scale-produced olive oils. The success was equal for both extra virgin PDO commercial olive oils and common commercial brands. Monovarietal olive oils stored for three years produced amplification rates and fragment sizes similar to those obtained in different time scales, even after being submitted to a filtration and stabilizing process (**Figure 1**), giving evidence that DNA present in this type of olive oil is stable and suitable for PCR for long periods of time. The stability of DNA is critical if amplified fragments are to serve as references for olive oil certification, to determine varietal composition of blended oils, as is suggested by Testolin and Lain (*18*).

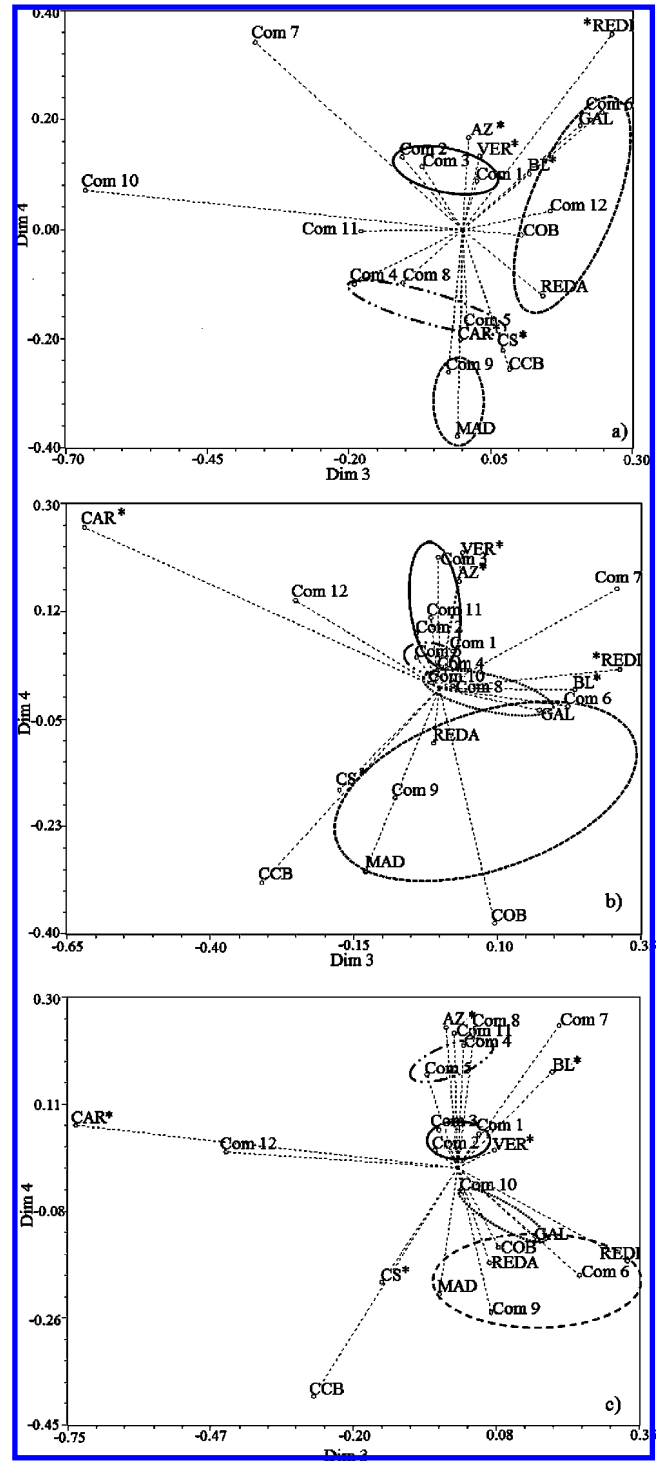

Figure 2. Principal component analyses performed with DCENTER among 11 monovarietal and 12 commercial olive oil samples (for codes see **Table 1**) with different DNA samples analyzed with different markers. When a combined (ISSR, RAPD, and SSR) matrix is factored, using the EIGEN program, the elements of the eigenvectors corresponding to positive eigenvalues are interpreted as the coordinates of each point in a Cartesian space (dotted lines represent EIGEN-vectors): **(a)** RAPD extraction 2 + ISSR extraction 1 + SSR; **(b)** RAPD extraction 2 + ISSR extraction 2 + SSR; **(c)** RAPD extraction 3 + ISSR extraction 2 + SSR. (∗) Small-scale-produced monovarietal olive oil samples from PDO Alentejo region; (—) three samples from commercial brand Oliveira da Serra; (–· · –) two samples from commercial brand Gallo; (---) PDO olive oil samples from Trás-os-Montes; (· · ·) commercial and small scale produced Galega monovarietal olive oil samples.

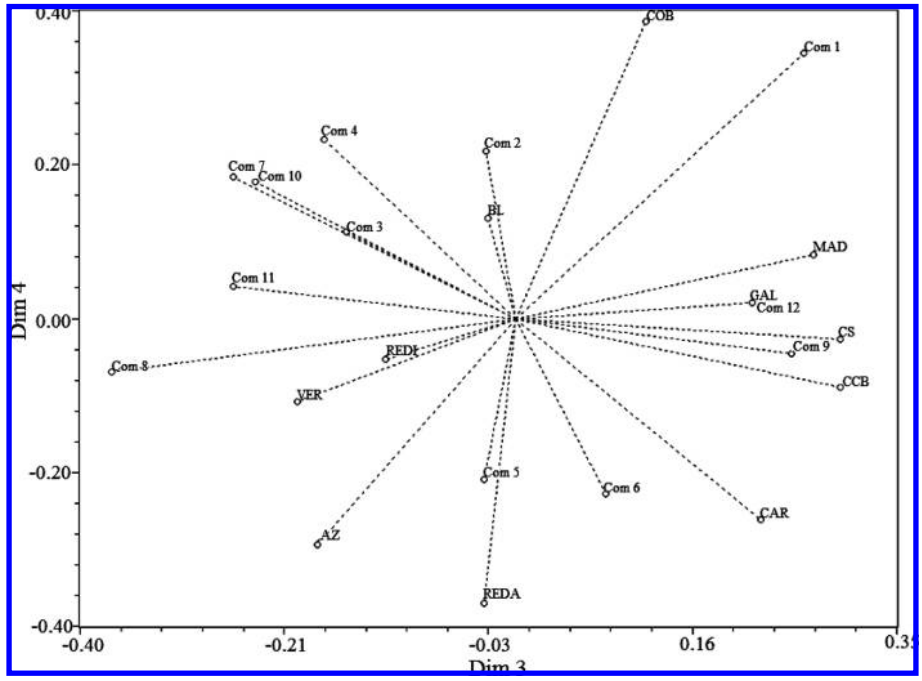

Figure 3. Principal component analyses performed with DCENTER among 11 monovarietal and 12 commercial olive oil samples (for codes see **Table 1**). When a SSR matrix is factored, using the EIGEN program, the elements of the eigenvectors corresponding to positive eigenvalues are interpreted as the coordinates of each point in a Cartesian space (dotted lines represent EIGEN-vectors).

DNA extracted from all 23 olive oil samples was amplified using RAPD, ISSR, and SSR. Eleven RAPD primers were tested, but only two produced reproducible bands in all olive oil samples. Among RAPD markers, seven of the nine bands were polymorphic (**Table 3**). The highest percentage of polymorphic products per RAPD profile (100%) was generated by primer OPO4, and the mean level of polymorphism was 78%. A total of 18 reproducible ISSR fragments were scored, of which 15 were polymorphic (**Table 3**). Primers UBC817 and UBC846 were the most informative of the ISSRs; when all of the products were polymorphic, the mean proportion across all ISSRs primers was 83%. Thus, overall, the ISSR marker system in olive oil is more informative than RAPD, as has been described when leaf material was used in other species (*27, 28*).

SSR amplification was satisfactory only when water phase DNA was used in the reaction. The SSR markers identified 22 alleles (5.5 alleles per locus) (**Table 4**). The shortest SSR allele was 162 bp (ssrOeUA-DCA09) and the longest, 250 bp (ssrOeUA-DCA03) (**Table 4**). Observed heterozygosity values indicated that ssrOeUa-DCA09 is the most informative and ssrOeUA-DCA05 the least informative of the SSR assays (**Table 4**).

Three combined PCAs are presented in **Figure 2**. Some groups are consistent between the three PCA analyses, where different extractions and amplification reactions were used. The Alentejo small-scale-produced monovarietal olive oil samples were spread, in all analyses, throughout the four quadrants (marked with ∗, **Figure 2**). Some particular cultivars from this region maintained always the same clustering (Azeiteira, Blanqueta, and Verdeal).

Small-scale-produced monovarietal olive oil samples that included Cordovil (CS and CCB, see **Table 1**) in their denomination clustered in the PCAs (**Figure 2**). These results are surprising because they are not similar when DNA from leaves was used (*24*). However, the analyses were different in terms of number and marker technology used.

With regard to common appellation, the three olive oil samples from the commercial brand "Oliveira da Serra" all grouped close together [group represented with a solid line (—) in **Figure 2**]. The same was observed for the commercial brand "Gallo" [group represented with two dots and a line (—••—) in **Figure 2**]. In both cases, although different containers were used (see **Table 1**), it did not interfere with DNA extraction and amplification. Both of these commercial brands seem to be produced in the Alentejo region because they group close to some small-scale-produced monovarietal olive oil samples (Azeiteira, Blanqueta, and Verdeal) that belong to this particular region (**Table 1**).

The commercial PDO olive oil from Alentejo region (Moura, Com 11) was in two of the three PCAs near monovarietal olive oil Azeiteira, which is typical from this particular region (**Figure 2**). Nevertheless, Com 11 is always far from the small-scale-produced monovarietal olive oils from the Trás-os-Montes region.

PDO commercial olive oils from Trás-os-Montes (Com 6, 9, and 12) appeared to be linked to the typical small-scale monovarietal olive oils of the region [group represented with a broken line (- - -) in **Figure 2**], which belong to the PDO's appellation. Com 9 clustered near monovarietal olive oil Madural, whereas Com 12 was more related to Cobrançosa and Redondal (**Figure 2a**). In **Figure 2b**, Com 6 and 9 are located in the same quadrant as the monovarietal olive oils of the region (Madural, Cobrançosa, and Redondal). Only Com 12 was removed from this quadrant due to some amplification problems of the sample that belong to this extraction. A similar distribution was observed among the olive oil samples from this specific region in **Figure 2c**.

Galega's small-scale-produced monovarietal (GAL) oil showed to be closely related to its commercial version [Com 10: group represented with dots (••) in **Figure 2**], except when the DNA extraction presented some amplification problems (**Figure 2a**). The two samples (GAL and Com 10) do not have a coincident

position in the PCA, which could be explained either due to differences between clones of Galega cultivar (*29*) or due to differences found among pollenizers (*19, 20*).

The two olive oil samples derived from agricultural farming (Com 7 and 8, **Table 1**) were closely positioned only in **Figure 2c**. In all PCAs performed these two oil samples behaved strangely, because they were always positioned closer to small-scale-produced monovarietal olive oils from the Alentejo (marked with ∗ in **Figure 2**) instead of Trás-os-Montes, where the oils are originated. This could be related to the fact that other cultivars are included in their production, once they are not certified PDO olive oils.

Most of the studies on olive oil traceability are based on SSR markers (*17*−*21*). Although this type of marker is advantageous for DNA identification of PDO oils (*21*), it may not always be as efficient as expected. When the SSR data were converted into a matrix and PCA was performed (**Figure 3**), no correlation was found among common appellations. This is notable between the olive oil samples from the commercial brands (Oliveira da Serra Com 1−3 and Gallo Com 4 and 5) because they were positioned in different PCA quadrants. The same was observed between PDO olive oil samples from Trás-os-Montes (Com 6, 9, and 12) that were not closely clustered within themselves or with the small-scale monovarietal olive oil samples from which they are produced (REDA, MAD, and COB). This reflects the importance of using extra marker technology to more accurately identify each sample with its appellation, mainly when it is composed of several cultivars and a small number of markers are used.

Although it is known that the DNA content of olive oil has a contribution of alleles from the stone, which have some contribution of the male parent (*19, 20*), what is used in PDO olive oil production are whole fruits. This makes it necessary to identify olive oils from these types of mixtures, instead of the ones derived from olive flesh.

The PCA with different marker technology could indicate in a simple way the origin of the appellation of olive oils and to which region they belong. This could be used as a first approach because it gives a wider view of the cultivar composition and is less expensive. Cultivar identification and quantification should be looked into afterward using more expensive marker technologies such as real time PCR and LDR−universal array analysis (*12*).

ABBEVIATIONS USED

PDO, Protected Denomination of Origin; SSR, Simple Sequence Repeat; RAPD, random amplified polymorphic DNA; ISSR, intersimple sequence repeat; PCA, principal component analysis.

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