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Characterisation and traceability of extra virgin olive oil and table olive of Azapa variety marketed in Chile using Microsatellite Molecular Markers

[Caracterización y trazabilidad de aceite de oliva virgen extra y aceituna de mesa de la variedad Azapa comercializados en Chile mediante marcadores moleculares microsatélites]

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Abstract: Chile has two certified origin olive products: Extra-Virgin Olive Oil (EVOO) from Huasco valley and the Azapa variety table olive from the Azapa valley. However, efficient methodologies are needed to determine the varieties and raw materials involved in the end products. In this study, we assessed the size of alleles from ten microsatellites in 20 EVOOs and in leaves and fruits of 16 olive varieties cultivated in Chile to authenticate their origins. The identification of varieties relied on specific allele sizes derived from microsatellites markers UDO99-011 and DCA18-M found in leaves and fruit mesocarp. While most Chilean single-variety EVOOs matched the variety declared on the label, inconsistencies were observed in single-variety EVOOs containing multiple varieties. Our findings confirm that microsatellites serve as a valuable as diagnostic tools for ensuring the quality control of Geographical Indication certification for Azapa olives and EVOO with Designation of Origin from Huasco.

Keywords: Azapa variety; Designation of Origin (DO); Extra virgin olive oil from Huasco Valley; Geographic Indication (GI); Microsatellite marker

Resumen: Chile cuenta con dos productos de oliva de origen certificado: El aceite de oliva virgen extra (AOVE) del valle del Huasco y la aceituna de mesa de la variedad Azapa del valle de Azapa. Sin embargo, se necesitan metodologías eficientes para determinar las variedades y materias primas involucradas en los productos finales. En este estudio, evaluamos el tamaño de los alelos de diez microsatélites en 20 AOVEs y en hojas y frutos de 16 variedades de aceituna cultivadas en Chile para autentificar sus orígenes. La identificación de las variedades se basó en los tamaños alélicos específicos derivados de los marcadores microsatélites UDO99-011 y DCA18-M encontrados en las hojas y el mesocarpio de los frutos. Aunque la mayoría de los AOVEs chilenos monovarietales coincidían con la variedad declarada en la etiqueta, se observaron incoherencias en los AOVEs monovarietales que contenían múltiples variedades. Nuestros hallazgos confirman que los microsatélites sirven como valiosas herramientas de diagnóstico para asegurar el control de calidad de la certificación de Indicación Geográfica para aceitunas de Azapa y AOVE con Denominación de Origen de Huasco.

Palabras clave: Variedad Azapa; Denominación de origen (DO); Aceite de oliva extra virgen del Valle del Huasco; Indicación geográfica (IG); Marcador microsatélite.

INTRODUCTION

Extra virgin olive oil (EVOO) offers numerous health benefits and is recommended in diets to improve cardiovascular health, lipoprotein metabolism and alleviate diabetes mellitus symptoms. Studies, such as those by Gaforio *et al*. (2019), have reported reductions in body mass index and blood pressure with the use of virgin olive oils. Additionally, virgin olive oils demonstrated anti-atherosclerotic potential, contribute to the prevention of certain cancers, and exhibit anti-inflammatory and immunomodulatory effects in autoimmune diseases like inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus and sclerosis (Gaforio *et al*., 2019). The significance of olive oil for health, its taste, and culinary trends have stimulated its production. Produced from the fruits of *Olea europea* L., native to the Mediterranean basin, olive oil has been utilized both in cuisine and therapeutically by Mediterranean residents since ancient times. While northern Mediterranean countries such as Spain, Italy, Greece and Portugal dominate the production and consumption of olive oil (68% and 43% respectively, Neves & Pires, 2018), other regions such as South Africa, the USA, South America and Australia have also ventured into olive and olive oil production. In South America, the largest olive tree plantations belong to Argentina, followed by Chile, Peru and Uruguay (Turcato & Mattar, 2014). Notably, Chilean EVOOs have gained recognition for their quality, being 100% extra virgin olive oil (ProChile, 2022), with Chile producing between 16 and 20 thousand tons of olive oil in 2017 (Musquiz, 2018).

Globally, there are over 2600 varieties, some of which serve dual purposes as table olive and for the production of oil, such as Azapa from Chile, Yuaca (Sevillana-Criolla) from Peru and Arauco from Argentina. The Azapa variety, introduced by the Spanish in 1560 (Contreras *et al*., 2018), holds international recognition and Geographical Indication certification (IOC, 2000; INAPI, 2022). This variety was later planted in the Huasco Valley (Atacama Region) (Contreras *et al*., 2018), and local producers believe it has evolved into a different variety, the Sevillana de Huasco. The olives from these plantations are smaller than those from Azapa (Contreras *et al*., 2018), and are mainly used to produce extra virgin olive oil (EVOO).

Automatization, an enhanced production process and an improved EVOO quality from Huasco Valley empowered the local olive growers to establish themselves in the national market. To distinguish their product from larger companies using other varieties, Huasco Valley EVOO producers sought certification. In 2018, they joined the Agricultural Association of the Huasco Province (AGA HUASCO) and secured Designation of Origin certification for Extra Virgin Olive Oil blends mandating at least 10% Sevillana del Huasco variety, accredited by the Chilean National Institute of Industrial Property (INAPI, 2022). These EVOOs have gained national acclaim for their superior organoleptic quality.

To prevent fraud and ensure Designation of Origin compliance, we developed a diagnostic method that can transparently trace and guarantee the quality of the olives used in the EVOOs for an increasingly demanding market. Ensuring high-quality standards in food industries and markets relies on identifying raw materials (Novak *et al*., 2007). The genetic code of the raw material is an effective guarantee of product origin and quality, detect potential adulterations (Novak *et al*., 2007). DNA markers, like Simple Sequence Repeats (SSRs) or microsatellites (short hypervariable DNA tandem repeats of codominant nature), are valuable tools for genetic identification in processed foods. This method, proven in genetic studies of table olives and EVOOs (Pasqualone *et al*., 2016), distinguishes olive varieties and identifies whether an oil is from a single olive variety (single variety) or a blend. This traceability method serves as a quality control technique, verifying the origin of products with Designation of Origin or Geographic Indication certification. Our objectives were (1) assessing the genetic traceability of Sevillana de Huasco in EVOOs from Huasco and Azapa table olives and (2) detecting undeclared varieties in Chilean-marketed EVOO brands.

MATERIALS AND METHODS

Plant material

Fruit and leaf samples were collected, on site, from the center of the canopy of ten Azapa variety trees (Azapa Valley; 18°30'39.2 "S 70°13'26.5 "W) and ten Sevillana del Huasco variety trees (Huasco Valley; 28°27'48.1 "S 71°11'10.4 "W) (Figure No. 1). Additionally, leaf and fruit samples from trees representing the varieties that are used to produce the

most commercialized EVOOs in Chile (i.e. Arbequina, Arbosana, Barnea, Coratina, Empeltre, Frantoio, Koroneiki, Leccino, Liguria and Picual) (Chileoliva, 2019), as well as the varieties Ascolana Tenera, Manzanilla, Gordal Sevillana and Kalamata) were obtained from the INIA Huasco Experimental Station (28 ° 34'44.2 "S 70 ° 47'53.8 "W) located in Vallenar (Huasco Valley). The samples were verified by INIA taxonomists and confirmed by the specialist in olive growth and olive oil technology, Mr. Francisco Tapia.

Figure No. 1
Map showing the locations (Azapa Valley and Huasco Valley) of the samples collected in this study

EVOOs
Five single-variety and blend EVOOs with Designation of Origin certification from the Huasco Valley (Río de Oro, Don Daniel (single-variety Picual)), Don Daniel, Don Daniel Premium and Payantume) were included in the study. Additionally, 15 domestic and imported single-variety and blend EVOOs available in the Chilean market were acquired in 2016 for comparison purposes (Chef, Líder, Oromaule La Española, Talliani, Banquete, Bezma, Las Doscientas, Santiago, Terra Santa, La Raima, Lombardi, RS, Huasco and Oliva).

DNA extraction from olive leaves and fruits
DNA was extracted from leaves and fruits using the method described by Contreras and Tapia, (2016). To assess the integrity of the extracted DNA, 5 µL of the extract mixed with 2.5 µL of bromophenol buffer was run through a 1.2% agarose gel by electrophoresis (Cleaver Scientific). DNA concentration and contamination levels were determined using a COLIBRI microvolume spectrophotometer (Titertek-Berthold, Pforzheim, Germany).

DNA extraction from olive oil
To extract DNA from olive oil, four protocols, described by Ramos-Gómez *et al*. (2014), Consolandi *et al*. (2008), Busconi *et al*. (2003) and Giménez *et al*. (2010), were evaluated. The extraction protocol of Giménez *et al*. (2010), resulted in the best quality, obtaining a higher amount of DNA and lower presence of PCR inhibitors than the other protocols. The protocol of Giménez *et al*. (2010), was further optimized by incorporating elements from the other studies' methodologies. Briefly, 1.8 mL of EVOO was transferred to a 2 mL tube and centrifuged at

14,000 rpm (4°C) for 30 min. 500 µL of the bottom layer was transferred to a new 1.5 mL tube. The oil was mixed with 250 µL of 2X CTAB (0.5% Tween 20 and 50 µM DTT) and 250 µL of hexane and vortexed for 10 sec. This mixture was then incubated for 30 minutes at 60°C in a heat bath, after which 500 µl of chloroform isoamyl alcohol (24:1), previously stored at -20°C) was added and centrifuged for 45 min at 14,000 rpm (4°C). The upper phase (50-300 µL) was carefully recovered and transferred to a new 1.5 mL tube. Equal volumes of isopropanol and 20 µg/mL Linear Acrylamide were added, and the tubes were carefully inverted 30 times until a homogeneous mixture was obtained. This mixture was incubated at -20°C overnight, and centrifuged for 30 min at 14,000 rpm (4°C) the next day, after which the supernatant was removed. The remaining DNA was washed with 500 µL ethanol and centrifuged for 30 min at 14,000 rpm (4°C), after which the supernatant was removed, and the tubes were dried at room T°. When dried, 20 ul of TE was added and the tubes were incubated at 4°C overnight. The next day the concentration of the solution was quantified on a COLIBRI microvolume spectrophotometer (Titertek-Berthold, Pforzheim, Germany).

SSR amplification

In the analysis of the leaf and fruit samples, ten primer pairs were used for PCR-SSR amplification (Table No. 1). However, due to the highly degraded nature of DNA in olive oil, SSR markers producing short amplicons give better results than markers producing larger amplicons (Pasqualone *et al*., 2016), as demonstrated in preliminary tests where SSR markers obtained amplicons up to 140 bp from the oil extracts. Consequently, SSR markers with a smaller PCR product (not exceeding than 140 bp) and high polymorphism (PIC > 0.70) were chosen for this study (Table No. 1). Marker UDO99-011 met all the requirements, being polymorphic (PIC=0.82) and having a size range of 99-127 bp. While markers DCA18 and DCA8 displazed high polymorphism (PIC of 0.79 and 0.74, respectively), their PCR products were too large (165-177 bp and 130-146 bp, respectively). To align with the sizes found in EVOOs, new primers were designed. Primer one starting from accession AJ279867.1 (for marker DCA18): DCA18-M 5'-GTTTTTTTTTTCGTGAG-AGCTCTCTTC-3' (primer 5'FAM-AAGAAAG-AAAGAAAAAGGCAGAATTAAGC-3' was kept from the original pair), and primer two starting from accession AJ279858.1 (for marker DCA8): DCA8-M 5'-ACTGAACTGACGACGACTGAGGAAAGG-3' (primer 5'FAM-ACAATTCAACCTCACCCCCCC-ATACCC-3' was kept from the original pair), which achieved PCR products of 139 bp and 123 bp respectively. The markers DCA3, UDO99-043 and GAPU103 exhibited high level of polymorphisms (PIC > 0.70), prompting the design of new primers flanking the microsatellite. However, the PCR product yields were insufficient, and these primers were excluded from this study.

Table No. 1
Description of microsatellite primers used in *Olea europaea* L. on 16 samples

Locus and References	DNA Sequences (5' – 3') and Fluorescent Dye	Repeat Motif	Size Range (bp)	T°a	PIC
DCA11 (Sefc et al., 2000)	5'FAM-TGAATCAACCCGTCAATAAGG-3' 5'-TTGTCTCAGTGAACCCTTAAACC-3'	$(GA)_{26}(GGGA)_4$	131-161	50°C	0.64
DCA15 (Sefc et al., 2000)	5'HEX-GATCTTGTCTGTATATCCACAC-3' 5'-TATACCTTTTCCATCTTGACGC-3'	$(CA)_{18}A_6(TAA)_7$	243-263	50°C	0.50
DCA18 (Sefc et al., 2000)	5'FAM-AAGAAAGAAAAAGGCAGAATTAAGC-3' 5'-GTTTTCGTCTCTCTACATAAGTGAC-3'	$(CA)_4CT(CA)_3(GA)_{19}$	165-177	50°C	0.79
DCA3 (Sefc et al., 2000)	5'HEX-CCCAAGCGGAGGTGTATATTGTTAC-3' 5'-TGCTTTTGTCGTGTTTGAGATGTTG-3'	$(GA)_{19}$	230-254	50 °C	0.81
DCA8 (Sefc et al., 2000)	5'FAM-ACAATTCAACCTCACCCCCATACCC-3' 5'-TCACGTCAACTGTGCCACTGAACTG-3'	$(GA)_{18}$	130-146	55°C	0.74
UDO99-011 (Cipriani et al., 2002)	5'FAM-TGACTCCCTTTAAACTCATCAGG-3' 5'-TGCGCATGTAGATGTGAATATG-3'	$(CT)_7(CA)_{10}(CT)_2(CA)_2CT(CA)_2CT(CA)_9$	99-127	53°C	0.82
UDO99-043 (Cipriani et al., 2002)	5'HEX-TCGGCTTTACAACCCATTTC-3' 5'-TGCCAATTATGGGGCTAACT-3'	$(GT)_{12}$	167-217	57°C	0.90

GAPU89 (Carriero et al., 2002)	5'HEX-GATCATTCCACACACGAGAG-3' R5'-AACACATGCCCACAAACTGA-3'	$(AG)_{16}(G)_3(GA)_9$	157-205	57°C	0.70
GAPU103 (Carriero et al., 2002)	5'HEX-TGAATTTAACTTTAAACCCACACA-3' 5'-GCATCGCTCGATTTTTATCC-3'	$(TC)_{26}$	133-185	57°C	0.77
GAPU82 (Carriero et al., 2002)	5'FAM-TGAATCAACCCGTCAATAAGG-3' 5'-TGCTATTTGCACATCATTGTTT-3'	$(AG)_5TC(AG)_3$	182-184	57°C	0.50

For the leaves and fruits, the total PCR mix (24 µL) consisted of 12 µL SapphireAmp Fast PCR Master Mix (Clontech), 3.5 µL of genomic DNA (5 ng/µL) and 1.2 µL (5 µM) of each primer for the DCA8 and UDO99-043 markers and demineralized water to reach the desired volume. The volume of the PCR multiplex reactions was the same as the above mixture, with the same concentration (5 µM), but with a different volume for the primers for each marker: 0.7 µL for the multiplex reactions with the primers for the markers Multiplex Group 1=DAC11-DCA15, Multiplex Group 2=DCA3-DCA18 and Multiplex Group 3=GAPU89-UDO99011; and 0.7 µL and 1.3 µL for the primers for the markers Multiplex Group 4=GAPU103 and GAPU82, respectively. Multiplex PCR reactions were only prepared for the DNA of the leaves and fruits of the different varieties.

The PCR for DNA from EVOOs was prepared for each marker individually (i.e. not multiplex). All PCR reactions (leaves, fruits, and oil) were performed on a Labnet MultiGene OptiMax thermal cycler. For leaves and fruits, we used 30 ng of DNA in total under the following conditions: an initial step of 5 min at 94°C, 45 cycles of 30 s at 94°C, 45 s at T°a (Table No. 1), then 2 min at 72°C, followed by a final extension step of 6 min at 72°C. For oil, we used 30 ng of DNA in total, under the following conditions: an initial step of 3 min at 95°C, 40 cycles of 98°C for 30 s, T°a (Table N°1) of each SSR primer for 30 s, then 30 s at 72°C, followed by a final extension step of 4 min at 72°C. PCR products were analyzed with an ABI3730XL genetic analyzer (Applied Biosystems). One of the SSR primers was labeled with 5 '6-FAM and HEX fluorescence dye, using the size standard Gene Scan™ 400 HD™ Rox (Applied Biosystems), for detection by capillary electrophoresis. Allele sizes were determined using Peak Scanner software (Applied Biosystems, version 1.0). The allele sizes of 16 olive varieties obtained with the ten microsatellite markers were compared with allele data from publications using the same microsatellite markers by Trujillo *et al*. (2014) and Koehmstedt *et al*. (2011).

Statistical analysis

To ensure the repeatability and authenticity of the results, as well as to confirm allele sizes derived from SSR markers, two independent replicates of fragment analysis were conducted for each leaf, fruit and oil PCR product. The determination of the minimum number of loci essential for genetic tagging was done with a Probability Identity multi-locus analysis, executed with GenAlEx v. 6.5 (Peakall & Smouse, 2012). The leaves of all olive varieties were used to estimate the polymorphism information content (PIC) for each marker. The PIC calculations followed the formula $PIC = 1 - \Sigma pi^2$, where *pi* represents the frequency of different alleles detected in the locus (Table No. 1).

RESULTS

Fruits and leaves

Alleles from leaves and fruits of the Azapa variety had precisely the exact sizes, as illustrated in Figure 2 for markers UDO99-011, GAPU 103 and DCA8 (111-113, 133-133 and 136-140 bp respectively), but the fluorescence was slightly lower when using DNA from the fruits, compared to the leaves. Although fruits in brine showed the correct pattern as expected by their variety, the fluorescence intensity (for example, when using the UDO99-043) was below the reference value.

For the Azapa and Sevillana varieties, allele sizes obtained with all 10 SSR markers were identical (Table No. 2), and the same size as in the studies of Koehmstedt *et al*. (2011), except for the three markers used the study of Trujillo *et al*. (2014) (i.e. DCA11, UDO99-011 and GAPU82). The Kalamata variety showed discrepansies in allele sizes of all markers, in comparison with the results reported by Trujillo *et al*. (2014) and Koehmstedt *et al*. (2011),

except for the allele sizes obtained by the DCA8 marker (Table No. 2). The remaining 13 varieties all showed discrepancies in allele sizes obtained with markers UDO99-011 and GAPU82 when comparing our results with those of Trujillo *et al*. (2014). Although Koehmstedt *et al*. (2011), did not use the GAPU82 marker, the allele sizes obtained with marker UDO99-011 in our study are similar to their results (Table No. 2). Discrepancies of more than one nucleotide (shown in brackets) were also observed when we compared the allele sizes obtained by the DCA11 marker with the results of Trujillo *et al*. (2014), for the varieties Arbequina (178 vs 141 bp), Coratina (170 vs 131 bp), Empeltre (178 vs 141 bp), Frantoio (178 vs 131 bp), Leccino (178 vs 131 bp), Picual (176 vs 141 bp), Ascolana Tenera (178 vs 161 bp) and Gordal Sevillana (178 vs 161 bp). Smaller discrepancies were found with the UDO99-043 marker in the Frantoio (214 vs 211 bp), Koroneiki (170 vs 172 and 214 vs 216 bp), Leccino (210 vs 212 bp) and Picual (212 vs 210 bp) varieties. For the rest of the SSR markers (DCA15, DCA18, DCA3, GAPU89, GAPU103 and DCA8), the allele sizes of the 16 varieties used in our study were consistent with those recorded by Trujillo *et al*. (2014) and Koehmstedt *et al*. (2011), (Table No. 2), with the exception for the size of the alleles obtained with markers DCA15 and DCA8 for the Koroneiki variety. Multi-locus probability estimation analysis, performed with the allele sizes of the 16 varieties and 10 SSR markers, showed that the minimum number of SSR markers needed to distinguish all 16 varieties is three. Considering the comparison of our results with the data of Trujillo *et al*. (2014) and Koehmstedt *et al*. (2011), we considered the obtained allele sizes valid reference material for the analysis of the EVOOs when our results coincided with those of Koehmstedt *et al*. (2011).

Table No. 2
Size of alleles, obtained from 16 olive genotypes using 10 SSR primer pairs, compared to alleles recorded in results from the studies of Trujillo *et al*. (2014) and Koehmstedt *et al*. (2011). Differences of more than one nucleotide are highlighted in grey

Variety	References and N° samples	DCA11	DCA11	DCA15	DCA15	DCA18	DCA18	DCA3	DCA3	UDO99-011	UDO99-011	GAPU89	GAPU89	GAPU103	GAPU103	GAPU82	GAPU82	DCA8	DCA8	UDO99-043	UDO99-043
	(1)	140	178	243	254	166	176	237	247	116	119	158	206	133	133	186	188	135	139	172	216
	(2)	141	141					238	248	111	113	158	205					136	140		
Azapa	**n=10**	141	141	243	254	167	177	238	248	111	113	158	205	133	133	184	184	136	140	173	217
Sevillana del Huasco	**n=10**	141	141	243	254	167	177	238	248	111	113	158	205	133	133	184	184	136	140	173	217
	(1)	140	178	243	243	164	174	229	241	116	129	158	158	147	157	186	186	137	137	175	175
Arbequina	(2)	141	141					230	242	111	123	158	158					138	138		
	n=1	141	141	243	243	165	175	230	242	111	123	158	158	147	157	182	182	138	138	175	175
	(1)	134	140	243	243	164	176	229	241	116	119	158	206	157	171	173	186	129	137	175	208
Arbosana	(2)	135	141					230	242	111	113	158	205								
	n=1	135	141	243	243	175	177	230	242	111	113	158	205	157	171	182	182	130	138	175	209
	(1)	140	152	243	243	172	174	229	229	125	125	172	193	174	186	186	186	135	135	166	175
Barnea	(2)																				
	n=1	141	153	243	243	173	175	230	230	119	119	173	193	175	185	182	182	134	134	167	175
	(1)	130	170	243	243	172	176	237	241	114	131	158	174	133	159	186	186	139	139	175	198
Coratina	(2)																				
	n=1	131	131	243	243	173	177	238	242	109	125	158	175	133	159	182	182	140	140	175	199
	(1)	140	178	243	243	166	176	241	243	114	125	156	168	147	171	188	188	129	134	187	216
Empeltre	(2)	141	141					242	244	109	119	156	168					130	134		
	n=1	141	141	243	243	167	177	242	244	109	119	156	168	147	171	184	184	130	134	188	216
	(1)	130	178	243	243	172	174	234	241	114	125	158	193	159	171	186	186	134	139	175	214
Frantoio	(2)	131	131					236	242	109	119	158	194					134	140		
	n=1	131	131	243	243	173	175	236	242	109	119	158	193	159	171	182	182	134	140	175	211
	(1)	140	146	261	263	168	170	237	237	123	125	158	193	147	157	186	186	137	145	170	214
Koroneiki	(2)	141	147					238	238	117	119	158	194					134	136		
	n=1	141	147	243	243	169	171	238	238	117	119	158	193	147	157	182	182	134	136	172	216
	(1)	130	178	254	254	172	172	241	251	114	134	158	206	171	184	186	186	135	135	210	214
Leccino	(2)	131	131					242	252	109	127	158	205					134	136		

	n=1	131	131	254	254	171	173	242	252	109	127	158	205	171	185	182	182	134	136	212	214
Liguria	(1)																				
	(2)	135	135					238	254	119	125	158	158					134	140		
	n=1	135	135	254	254	169	177	238	254	119	125	158	158	159	171	182	182	134	140	174	176
Picual	(1)	140	176	243	254	166	172	237	247	116	119	158	174	133	133	186	186	139	139	208	212
	(2)	141	141					238	248	111	113	158	175					136	140		
	n=1	141	141	243	254	167	173	238	248	111	113	158	175	133	133	182	182	136	140	208	210
Ascolana T.	(1)	160	178	243	243	168	172	229	247	103	119	158	174	133	171	186	186	134	135	175	210
	(2)	161	161					230	248	99	113	158	175					134	136		
	n=1	161	161	243	243	169	173	230	248	99	113	158	175	133	171	182	182	134	136	175	210
Manzanilla	(1)	140	160	254	254	168	176	243	251	119	134	158	206	133	147	173	186	137	137	210	212
	(2)	141	161					244	252	113	127	158	205					136	138		
	n=1	141	161	254	254	169	177	244	252	113	127	158	205	133	147	182	182	136	138	210	212
Gordal S.	(1)	160	178	243	243	172	176	247	251	119	127	174	206	133	184	186	186	135	135	172	212
	(2)																				
	n=1	161	161	243	243	173	177	248	252	113	121	175	205	133	185	182	182	134	136	173	213
Kalamata	**(1)**	134	161	262	263	180	183	229	251	114	119	168	206	159	184	188	188	135	135	208	214
	(2)	135	160					244	252	109	113	168	205					134	136		
	n=1	141	141	243	243	175	177	238	242	113	119	156	205	133	171	184	184	134	136	188	217

Trujillo *et al.* (1), Koehmstedt *et al.* (2); n = number of samples

The allele sizes obtained with markers DCA18 and DCA8 for leaves and fruits of all varieties exhibited strong correlation with Trujillo *et al*. (2014) and Koehmstedt *et al*. (2011). Therefore, we were confident that their modified versions, together with the marker UDO99-11, would result in accurate identification of olive varieties present in the EVOOs. We used leaves to obtain the allele sizes generated by these modified markers (DCA18-M and DCA8-M; Table No. 3). These measured allele sizes served as a reliable reference for the analyses of EVOOs (Figure No. 2).

Table No. 3
Allele sizes obtained with new primer pairs DCA18-M and DCA8-M and comparison with the original primers DCA18 and DCA8

Variety	DCA18	DCA18	DCA18-M	DCA18-M	DCA8	DCA8	DCA8-M	DCA8-M
Azapa	167	177	127	137	136	140	120	124
Sevillana del Huasco	167	177	127	137	136	140	120	124
Arbequina	165	175	125	135	138	138	122	122
Arbosana	175	177	135	137	130	138	114	122
Barnea	173	175	133	135	134	134	118	118
Coratina	173	177	133	137	140	140	124	124
Empeltre	167	177	127	137	130	134	114	118
Frantoio	173	175	133	135	134	140	118	124
Koroneiki	169	171	129	131	134	136	118	120
Leccino	171	173	131	133	134	136	118	120
Liguria	169	177	129	137	134	140	118	124
Picual	167	173	127	133	136	140	120	124

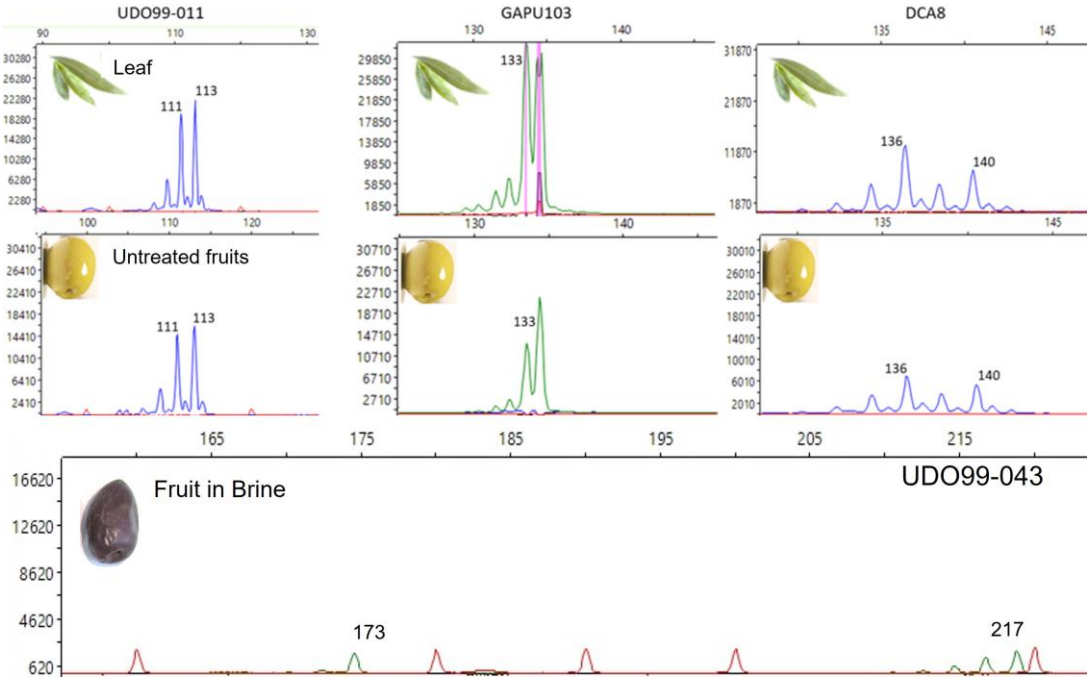

Figure No. 2
The capillary electrophoresis electropherogram shows the PCR products of the UDO99-011, GAPU103 and DCA8 microsatellite markers obtained from leaves and untreated fruits and of the UDO99-43 microsatellite marker obtained from fruits in brine of the Azapa variety. All peaks are scaled in relative fluorescent unit (RFU) on the Y-axis.

EVOOs

We identified the allele sizes of olive DNA in 20 common EVOOs from supermarkets using the original UDO99-011 marker and the two modified markers: DCA18-M and DCA8-M (Table No. 4). The UDO99-011 marker showed high fluorescence intensity for the alleles with sizes 111 and 113 bp in the single-variety EVOOs Chef, Don Daniel, Bezma, Las Doscientas, Don Daniel Premium, Río de Oro and Payantume, corresponding with the olive varieties indicated on the label (Table No. 4). However, all these EVOOs showed lower fluorescence intensity for other alleles. For example, the high fluorescence intensity of the alleles with sizes of 111 and 113 bp in the Chef EVOO coincide with the alleles of the Arbosana variety; however, lower fluorescence intensities were found for alleles with sizes of 125 and 129 bp (Figure No. 3). The same happened with the single-variety Don Daniel EVOO, where the allele with sizes of 111 and 113 bp coincided with the allele sizes of the Picual variety but was accompanied by two "stutter" peaks of 107 and 109 bp (Figure No. 3). In the other single-variety brands even more alleles were amplified: Las Doscientas 6 alleles, Don Daniel Premium 6 alleles, Río de Oro 8 alleles, Bezma 10 alleles and Payantume 10 alleles (Table No. 4; marker UDO99-011) corresponding to other varieties than the one indicated on the label. We also observed that the single-variety Oliva EVOO contained allele sizes of 109 and 113 bp (higher fluorescence intensity); however, alleles with sizes of 111 and 123 bp were absent (even when considering low fluorescence intensity peaks), which excludes the presence of the Arbequina variety that was indicated on their label (Table No. 4). The blend EVOOs Banquete and Santiago did not provide olive variety information on their label but showed high fluorescence intensity for alleles with sizes of 111 and 113 bp, corresponding with the Azapa, Arbosana and/or Picual varieties (Table No. 4). For EVOOs that lacked all information about the type (single-variety vs blend) and variety (Líder, Oromaule, Dan Daniel, La Española, Talliani, Terra Santa, La Raima, Lombardi, RS and Huasco), 4 to 10 alleles were amplified, giving rise to several possible combinations of varieties.

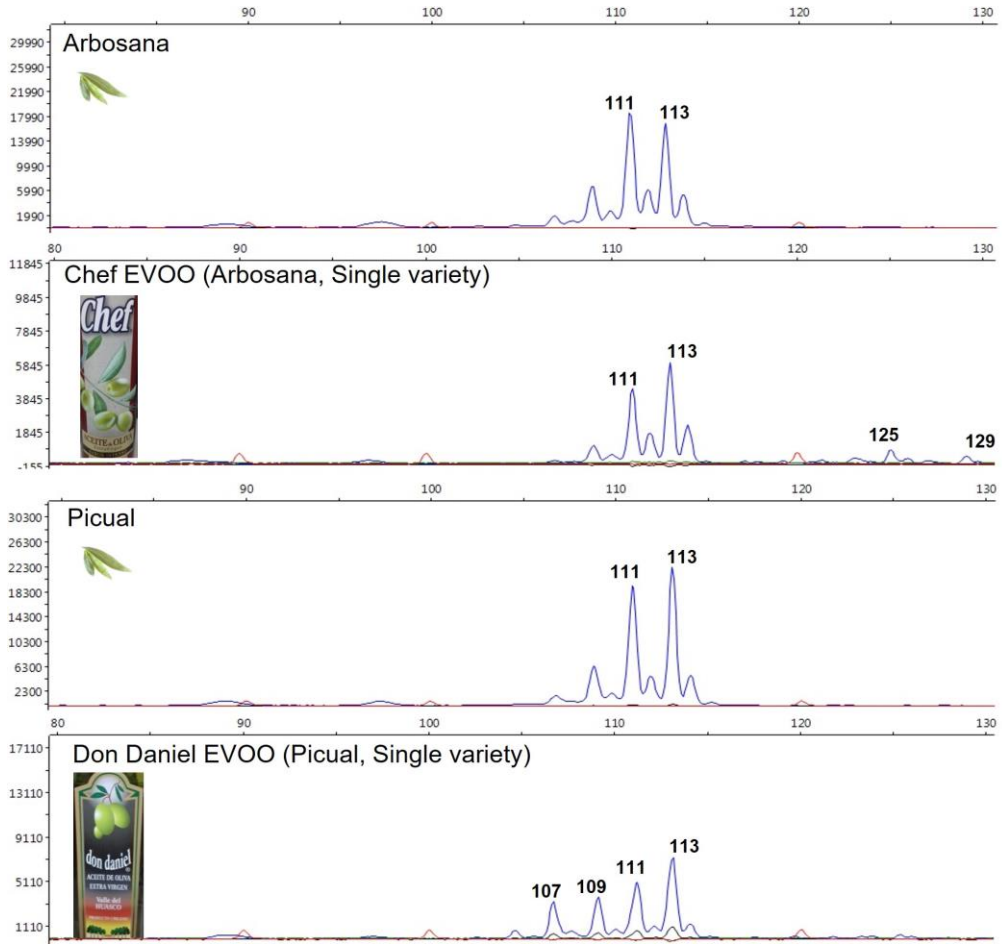

Figure No. 3
Examples of electropherograms resulting from olive leaf Arbosana and Picual cultivars and Chef and Don Daniel EVOO brands using UDO99-011 marker. All peaks are scaled in relative fluorescent unit (RFU) on the Y-axis.

Table No. 4
Allele size for three SSR locus and range of possible varieties detected in 20 different Extra Virgen Olive Oil brands from the Chilean market

SSR marker	Brand EVOO	Type of EVOO	Olive Variety	Allele size (bp) detected in EVOOs[1]	Range of possible varieties[2]	Match
UDO99-011	Bezma+	Single-variety	Azapa	99, 107, 109, **111**, **113**, 115, 117, 121, 123, 125	**Az**, Ab, Pi, Ar, Co, Li	Yes
	Chef+	Single-variety	Arbosana	**111**, **113**, 125, 129	Az, **Ab**, Pi	Yes
	Don Daniel+	Single-variety	Picual	107, 109, **111**, **113**	Az, Ab, **Pi**	Yes
	D. Daniel Premium+	Single-variety	Frantoio	107, 109, **111**, **113**, **115**, 119	Az, Ab, Pi, Br, Em, **Fr**	Yes
	Las Doscientas+	Single-variety	Picual	105, 107, 109, **111**, **113**, 115	Az, Ab, **Pi**	Yes
	Oliva+	Single-variety	Arbequina	105, 107, **109**, **111**, 113, 115	Az, Ab, Pi,	No
	Payantume+	Single-variety	Sev. del Huasco	107, 109, **111**, **113**, 115, 117, 119, 121, 123, 125	**Az**, Ab, Pi, Ar, Br, Co, Em, Fr, Ko, Li	Yes
	Río de Oro+	Single-variety	Sev. del Huasco	107, 109, **111**, **113**, 119, 121, 123, 125	**Az**, Ab, Pi, Ar, Br, Co, Em, Fr, Ko, Li	Yes
	Banquete+	Blend	NVI	107, 109, **111**, **113**	Az, Ab, Pi	-
	Santiago+	Blend	NVI	107, 109, **111**, **113**	Az, Ab, Pi	-
	Don Daniel+	NLI	NVI	107, 109, **111**, **113**, 115, 117, 119, 121, 125	Az, Ab, Pi, Br, Co, Em, Fr, Ko, Li	-
	Huasco+	NLI	NVI	109, **111**, 123, **125**	Co, Ar	-

	La Española*	NLI	NVI	107, 109, **111**, **113**, 115, 117, 119, 121, 125	Az, Ab, Pi, Br, Co, Em, Fr, Ko, Li	-
	La Raima+	NLI	NVI	107, 109, **111**, **112**	Az, Ab, Pi	-
	Líder+	NLI	NVI	107, 109, **111**, **113**	Az, Ab, Pi	-
	Lombardi+	NLI	NVI	**117**, **119**	Ko, Br	-
	Oromaule+	NLI	NVI	105, 107, 109, **111**, **113**, 117, 119, 121, 123, 125	Az, Ab, Pi, Ar, Br, Co, Em, Fr, Ko, Li	-
	RS*	NLI	NVI	109, 111, **113**, 117, **119**	Az, Ab, Pi, Br, Em, Fr, Ko	-
	Talliani+	NLI	NVI	107, 109, **111**, **113**, 121, 123	Az, Ab, Pi, Ar	-
	Terra Santa*	NLI	NVI	107, 109, **111**, **113**, 117, 119, 123, 125	Az, Ab, Pi, Ar, Br, Co, Em, Fr, Ko, Li	-
DCA18-M	Bezma+	Single-variety	Azapa	125, **127**, 135, **137**, 139	**Az**, Ab, Ar, Em	Yes
(Modified)	Chef+	Single-variety	Arbusana	123, **125**, **127**, 129, 133, 135, 137	Az, **Ab**, Pi, Ar, Br, Co, Em, Fr, Li	Yes
	Don Daniel+	Single-variety	Picual	123, 125, **127**, **129**, 131, 133, 135, 137, 139	Az, Ab, **Pi**, Ar, Br, Co, Em, Le, Ko, Fr, Li	Yes
	D. Daniel Premium+	Single-variety	Frantoio	123, **125**, **127**, 129, 131, 133, 135, 137	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, **Fr**, Li	Yes
	Las Doscientas+	Single-variety	Picual	123, **125**, **127**, 129, 131, 133, 135, 137	Az, Ab, **Pi**, Ar, Br, Co, Em, Le, Ko, Fr, Li	Yes
	Oliva+	Single-variety	Arbequina	123, **125**, **127**, 133, 135, 137	Az, Ab, Pi, **Ar**, Br, Co, Em, Fr	Yes
	Payantume+	Single-variety	Sev. del Huasco	123, **125**, **127**, 129, 131, 133, 135, 137	**Az**, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	Yes
	Río de Oro+	Single-variety	Sev. del Huasco	**127**, 133, **135**, 137	**Az**, Em	Yes
	Banquete+	Blend	NVI	123, **125**, **127**, 131, 133, 135, 137, 139	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	-
	Santiago+	Blend	NVI	123, **125**, **127**, 133, 135, 137	Az, Ab, Pi, Ar, Br, Co, Em, Fr	-
	Don Daniel+	NLI	NVI	123, **125**, **127**, 129, 131, 133, 135, 137	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	-
	Huasco+	NLI	NVI	131, 133, **135**, **137**	Ar, Br	-
	La Española*	NLI	NVI	123, **125**, **127**, 131, 133	Pi, Le	-
	La Raima+	NLI	NVI	123, **125**, 127, 129, 131, 133, **135**, 137	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	-
	Líder+	NLI	NVI	**123**, 125, **127**, 129, 131, 133, 135, 137	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	-
	Lombardi+	NLI	NVI	123, **125**, **127**, 137	Az, Em	-
	Oromaule+	NLI	NVI	123, **125**, **127**, 129, 133, 135	Pi, Ar, Br, Fr	-
	RS*	NLI	NVI	123, **125**, **127**, 129, 131, 133, 135, 137	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	-
	Talliani+	NLI	NVI	123, **125**, **127**, 129, 131, 133, 135, 137, 139	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	-
	Terra Santa*	NLI	NVI	123, **125**, **127**, 133, 135, 137	Az, Ab, Pi, Ar, Br, Co, Em, Fr	-
DCA8-M	Bezma+	Single-variety	Azapa	114, 116, **118**, **120**, 122, 124	all varieties	
(Modified)	Chef+	Single-variety	Arbusana	114, 116, **118**, **120**, 122, 124	all varieties	
	Don Daniel+	Single-variety	Picual	114, 116, **118**, **120**, 122, 124	all varieties	
	D. Daniel Premium+	Single-variety	Frantoio	114, 116, **118**, **120**, 122, 124	all varieties	
	Las Doscientas+	Single-variety	Picual	114, 116, **118**, **120**, 122, 124	all varieties	
	Oliva+	Single-variety	Arbequina	114, 116, **118**, **120**, 122, 124	all varieties	
	Payantume+	Single-variety	Sev. del Huasco	114, 116, **118**, **120**, 122, 124	all varieties	
	Río de Oro+	Single-variety	Sev. del Huasco	114, 116, **118**, **120**, 122, 124	all varieties	
	Banquete+	Blend	NVI	114, 116, **118**, **120**, 122, 124	all varieties	
	Santiago+	Blend	NVI	114, 116, **118**, **120**, 122, 124	all varieties	
	Don Daniel+	NLI	NVI	114, 116, **118**, **120**, 122, 124	all varieties	
	Huasco+	NLI	NVI	114, 116, **118**, **120**, 122, 124	all varieties	
	La Raima+	NLI	NVI	114, 116, **118**, **120**, 122, 124	all varieties	
	La Española*	NLI	NVI	114, 116, **118**, **120**, 122, 124	all varieties	
	Líder+	NLI	NVI	114, 116, **118**, **120**, 122, 124	all varieties	
	Lombardi+	NLI	NVI	114, 116, **118**, **120**, 122, 124	all varieties	
	Oromaule+	NLI	NVI	114, 116, **118**, **120**, 122, 124	all varieties	
	RS*	NLI	NVI	114, 116, **118**, **120**, 122, 124	all varieties	
	Talliani+	NLI	NVI	114, 116, **118**, **120**, 122, 124	all varieties	
	Terra Santa*	NLI	NVI	114, 116, **118**, **120**, 122, 124	all varieties	

No Label Information = NLI; No variety indicated =NVI; Imported = *; Chilean product = +; 1 Allele pairs with the highest fluorescence intensity were highlighted with bold type letters; 2 According to the alleles size of the olive tree varieties registered in Table No. 2, or Table No. 3. Azapa (= Sevillana del Huasco) (Az), Arbequina (Ar), Arbosana (Ab), Barnea (Br), Coratina (Co), Empeltre (Em), Frantoio (Fr), Koroneiki (Ko), Leccino (Le), Liguria (Li), Picual (Pi)

The DCA18-M marker showed that the single-variety EVOOs Chef, Don Daniel, Bezma, Las Doscientas, Don Daniel Premium, Río de Oro, Oliva and Payantume contained alleles with sizes of 125, 127, 129, 135 or 137 bp (Table No. 4), corresponding with the allele sizes of the variety indicated on the product label. However, some alleles that match the variety showed low fluorescence intensity. For example, the allele sizes of the Sevillana del Huasco variety match well with the allele sizes found in the Rio de Oro (127 and 137 bp) and Bezma (127 and 137 bp) EVOOs, but other alleles with sizes of 133 and 137, and 135 and 139 bp, were present as well. The EVOO of the Huasco brand contained alleles with sizes of 131, 133, 135 and 137 bp but lacked the allele of 127 bp to match the Sevillana del Huasco variety (Figure No. 4). The DCA18M marker amplified four to nine alleles for the oils that did not declare type and variety on their label (Líder, Oromaule, Dan Daniel, La Española, Talliani, Terra Santa, La Raima, Lombardi, RS and Huasco), corresponding to the results obtained by the UDO99-11 marker.

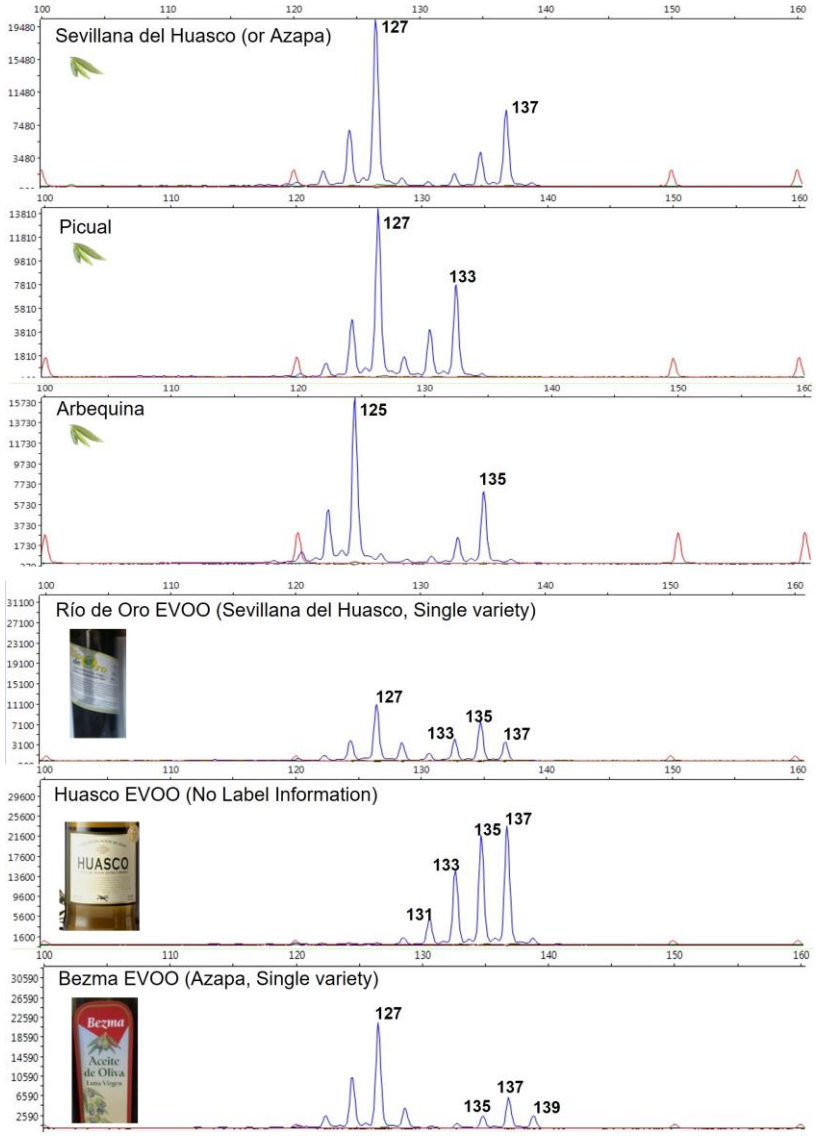

Figure No. 4

Example of electropherograms resulting from olive leaf Sevillana del Huasco (or Azapa), Picual and Arbequina cultivars, and Río de Oro, Huasco and Bezma EVOO brands using DCA18-M marker. All peaks are scaled in relative fluorescent unit (RFU) on the Y-axis

The DCA8-M marker showed high fluorescence intensity for alleles of 118 and 120 bp and lower fluorescence intensity for alleles of 114, 116, 122 and 124 bp with, for all the EVOOs analyzed independent of their type (single-variety or blend) (Table No. 4). As the marker matched all the varieties, it did not allow us to identify which varieties were used in the EVOOs (see Figure No. 5 for an example), as the electropherogram patterns of all EVOOs are similar. This marker was therefore excluded from further analysis.

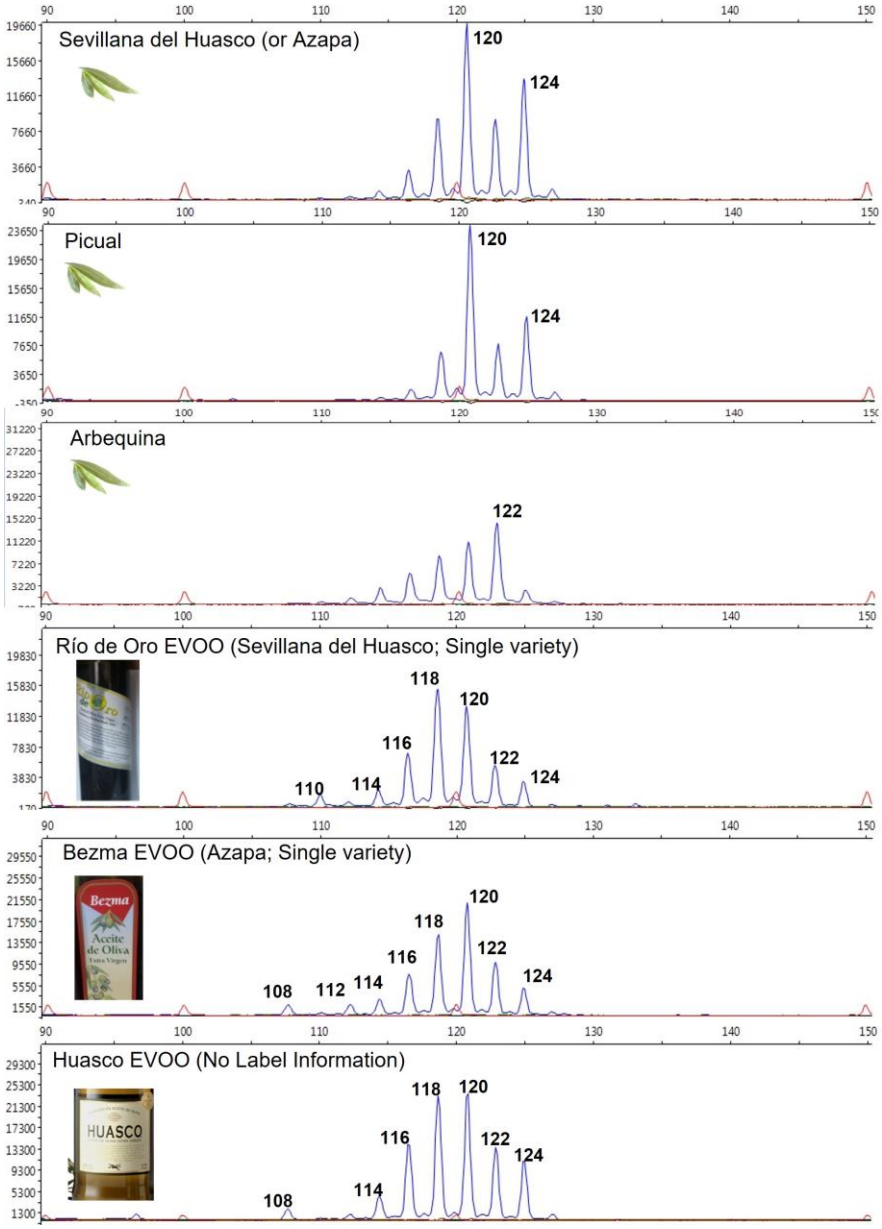

Figure No. 5

Example of electropherograms resulting from olive leaf Sevillana del Huasco (or Azapa), Picual and Arbequina cultivars, and Río de Oro, Huasco and Bezma EVOO brands using DCA8-M marker. All peaks are scaled in relative fluorescent unit (RFU) on the Y-axis

Therefore, the combination of the UDO99-011 and DCA18-M markers was used to determine the olive varieties in 20 EVOO brands (Table No. 5). The brands Chef, Don Daniel, Líder, Banquete, Las Doscientas, Santiago, La Raima and Oliva all matched with the Azapa, Arbosana and/or Picual varieties (Table No. 5). The EVOO La Española matched with the Picual variety and Huasco matched with the Arbequina variety, while the Río de Oro brand matched with two, the Azapa, Arbosana and/or Picual and the Empeltre variety. Others such as Oromaule, Talliani and Bezma matched with three to four varieties, while Don Daniel, Terra Santa, Don Daniel Premium, RS and Payantume brands matched with six to ten varieties. When combining the information from the two SSR markers, the EVOO brand Lombardi was the only one that did not match with any variety.

Table No. 5
Match of possible varieties based on UDO99-011 and DCA18-M marker information for 20 different types of Extra Virgen Olive Oil brands

Brand EVOO	Type of EVOO	Olive Variety	Possible varieties using UDO99-011	Possible varieties using DCA18-M	Match
Bezma+	Single-variety	Azapa	Az, Ab, Pi, Ar, Co, Li	Az, Ab, Ar, Em	Az, Ab, Ar
Chef+	Single-variety	Arbosana	Az, Ab, Pi	Az, Ab, Pi, Ar, Br, Co, Em, Fr, Li	Az, Ab, Pi
Don Daniel+	Single-variety	Picual	Az, Ab, Pi	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	Az, Ab, Pi
D. Daniel Prem.+	Single-variety	Frantoio	Az, Ab, Pi, Br, Em, Fr	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	Az, Ab, Pi, Br, Em, Fr
Las Doscientas+	Single-variety	Picual	Az, Ab, Pi	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	Az, Ab, Pi
Oliva+	Single-variety	Arbequina	Az, Ab, Pi,	Az, Ab, Pi, Ar, Br, Co, Em, Fr	Az, Ab, Pi
Payantume+	Single-variety	Sev. del H.	Az, Ab, Pi, Ar, Br, Co, Em, Fr, Ko, Li	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	Az, Ab, Pi, Ar, Br, Co, Em, Fr, Ko, Li
Río de Oro+	Single-variety	Sev. del H.	Az, Ab, Pi, Ar, Br, Co, Em, Fr, Ko, Li	Az, Em	Az, Em
Banquete+	Blend	NVI	Az, Ab, Pi	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	Az, Ab, Pi
Santiago+	Blend	NVI	Az, Ab, Pi	Az, Ab, Pi, Ar, Br, Co, Em, Fr	Az, Ab, Pi
Don Daniel+	NLI	NVI	Az, Ab, Pi, Br, Co, Em, Fr, Ko, Li	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	Az, Ab, Pi, Br, Co, Em, Fr, Ko, Li
Huasco+	NLI	NVI	Co, Ar	Ar, Br	Ar
La Española*	NLI	NVI	Az, Ab, Pi, Br, Co, Em, Fr, Ko, Li	Pi, Le	Pi
La Raima+	NLI	NVI	Az, Ab, Pi	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	Az, Ab, Pi
Líder+	NLI	NVI	Az, Ab, Pi	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	Az, Ab, Pi
Lombardi+	NLI	NVI	Ko, Br	Az, Em	-
Oromaule+	NLI	NVI	Az, Ab, Pi, Ar, Br, Co, Em, Fr, Ko, Li	Pi, Ar, Br, Fr	Pi, Ar, Br, Fr
RS*	NLI	NVI	Az, Ab, Pi, Br, Em, Fr, Ko	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	Az, Ab, Pi, Br, Em, Fr, Ko
Talliani+	NLI	NVI	Az, Ab, Pi, Ar	Az, Ab, Pi, Ar, Br, Co, Em, Le, Ko, Fr, Li	Az, Ab, Pi, Ar
Terra Santa*	NLI	NVI	Az, Ab, Pi, Ar, Br, Co, Em, Fr, Ko, Li	Az, Ab, Pi, Ar, Br, Co, Em, Fr	Az, Ab, Pi, Ar, Br, Co, Em, Fr

No Label Information = NLI; No variety indicated =NVI; Imported = *; Chilean product = +; Azapa (= Sevillana del Huasco) (Az), Arbequina (Ar), Arbosana (Ab), Barnea (Br), Coratina (Co), Empeltre (Em), Frantoio (Fr), Koroneiki (Ko), Leccino (Le), Liguria (Li), Picual (Pi).

DISCUSSION

The authentication of two exclusive Chilean products, Azapa olives from the Azapa Valley (with Geographical Indication certification) and EVOOs from Huasco (with Denomination of Origin certification) posed the need for robust quality control methodologies. Our most significant finding is that our results reveal identical allele sizes for the Azapa and the Sevillana del Huasco varieties, challenging the belief in a distinct genotype for Sevillana del Huasco among olive growers in the Huasco Valley. This poses challenges for quality control analysis in both the Designation of Origin certification for Huasco olive oil and the Geographical Indication for table olives from the Azapa Valley. Notably, despite their genetic similarities, practical distinctions remain - the Azapa olives, larger in size, are predominantly used as table olives, while the smaller-sized olives from Huasco are mainly used to produce EVOO (Contreras *et al*., 2018).

Fruits and leaves

Although several genetic studies on specific SSR markers for olives have been published (Sefc *et al*., 2000; Carriero *et al*., 2002; Cipriani *et al*., 2002), few studies have focused on recording the allele sizes across different varieties obtained by several markers (Koehmstedt *et al*., 2011; Trujillo *et al*., 2014). The allele sizes of all varieties obtained in this study were validated as they aligned with the findings of Koehmstedt *et al*., (2011) using the WEO-NCGR-USDA collection of USA, except for the Kalamata variety. The above demonstrates that our analysis and results are reliable. The results further demonstrate that the collection of olive trees from the INIA

Huasco Experimental Center is suitable for genetic analyses of olive (oil). However, the authenticity of the Kalamata variety should be verified as it shows discrepancies with the allele sizes recorded in the other two studies (Koehmstedt *et al*., 2011; Trujillo *et al.*, 2014).

We observed discrepancies in 15 other varieties that were analyzed, when comparing our results to the results of Trujillo *et al*. (2014), while using the same SSR markers. Although it is expected to find differences of one nucleotide in allele size between studies, the differences were more prominent when we compared our results with those of Trujillo *et al*. (2014). Discrepancies in the assignment of allele sizes of different varieties when using the same SSR markers are not uncommon (Carvalho *et al*., 2021; Yadav *et al*., 2021), and may arise from varying DNA quality, different equipment or PCR reagents variations, highlighting the necessity for careful SSR marker selection (Carvalho *et al*., 2021). Recognizing this, international olive organizations and researchers have stressed the need to establish a list of accepted alleles and SSR markers to accurately identify olive varieties stored in gene banks or collections (Yadav *et al*., 2021).

The allele size of treated and untreated fruits was precisely the same as those from the leaves; however, the fluorescence intensity was lower. This aligns with previous studies that have shown that olive fruits can be successfully identified using leaf tissue and SSR, AFLP, ISSR and RAPD markers (Ipek *et al*., 2015; Contreras & Tapia, 2016; Contreras *et al*., 2018; Crawford *et al*., 2020).

Our study shows that microsatellites are a good methodological approach for genetic identification and quality control of Azapa olive products within the Chilean domestic market. Nonetheless, international authentication faces challenges due to shared genotypes with other American varieties. In detail, the Azapa variety has exactly the same allele sizes as the Mission Leiva (Colombia), Yuaca (synonymous to Mostazal and Sevillana-Criolla; Peru) (Koehmstedt *et al*., 2011) and Arauco (Argentina) varieties (Trujillo *et al*., 2014). Belaj *et al*. (2022), confirmed that the Azapa variety is synonymous with accessions from Argentina. However, our verification of this similarity, using three microsatellites and imported Arauco fruits (from the World Olive Germplasm Bank of Cordoba (WOGBC) collection from Spain), reveals a discrepancy in allele sizes obtained with markers DCA11, UDO99-011 and GAPU89 when comparing the Arauco to the Azapa variety.

EVOOs

Although the 16 cultivars used in this study could be identified using any combination of three SSR markers of the 10 SSR markers tested, only UDO99-011, DCA18 and DCA8, showed reliable PCR amplification results for EVOOs. To optimize these markers for oil analysis, modifications, including new primer pairs to reduce the amplicon size of the markers DCA18 and DCA8, were applied, aligning with a previous study by Vietina *et al*. (2011), of different markers. The modified primer pairs DCA18-M and DCA8-M demonstrated accurate amplification and allele sizes, when tested on olive leaf and fruit samples.

The SSR markers gave a more consistent and robust electropherogram pattern for leaves and fruits than for olive oil. Only in a few cases did the electropherogram pattern of single-variety EVOO matrix show the same pattern as the leaf reference, even though the three SSR markers (UDO99-011, DCA18-M and DCA8-M) showed the correct amplification for all the oil matrices. However, the electropherogram patterns for EVOOs presented challenges, showing more peaks than expected for most of the single-variety EVOOs and blends, commonly called "stuttering". This phenomenon is common when using dinucleotide microsatellite markers, especially when dealing with DNA mixtures from multiple individuals (Sardina *et al*., 2015). Stutter bands can occur as a result of enzyme slippage during amplification (Pasqualone *et al*., 2016) and complicate distinguishing minor peaks due to "stuttering" from peaks of true alleles (Sardina *et al*., 2015). Another reason why multiple peaks might occur in the electropherograms is that the increase in the number of cycles in the PCR program or the higher concentration of primers in the PCR reaction facilitates a higher production of peaks and stutter bands (Carvalho *et al*., 2021). Despite the theoretical possibility of additional alleles from paternal DNA in seed embryos (Doveri *et al*., 2006), it was demonstrated that DNA contamination from the seed embryo doesn't occur in single-variety EVOOs (Muzzalupo *et al*., 2007). The markers UDO99-011 and DCA18-M (which has an interrupted compound microsatellite motif, $(CT)_7(CA)_{10}(CT)_2(CA)_2CT(CA)_2$

$CT(CA)_2CT(CA)_9$ and $(CA)_4CT(CA)_3(GA)_{19}$; (Table No. 1) consistently provided discriminating patterns for EVOOs, while the DCA8-M marker (which has a pure dinucleotide motif, $(GA)_{18}$) had discrimination problems in all EVOOs. This is likely due to the fact that dinucleotide repeats (DCA8-M in this study) produce more than one major peak per allele, whereas trinucleotide repeats (UDO99-011 and DCA18-M in this study) typically show a very clear major peak (Flores-Rentería & Krohn, 2013).

The fluorescence intensity of the peaks of the electropherograms of the EVOOs in our study exceeded levels reported in other studies. For example, Piarulli *et al*. (2019), recorded peaks with an intensity lower than the size standard (size standard = Relative Fluorescence Unit (RFU) between 400 to 500), while we obtained peaks with > 5,000 RFU for most of the samples, ensuring reliable allele size values. Pafundo *et al*. (2005), proposed a threshold of 2,000 RFU for identifying olive varieties in olive oil arrays when using AFLP markers. The high RFU levels obtained in most of our electropherograms suggest that the combination of different protocols with the method of Giménez *et al*. (2010), resulted in a high-quality and reliable oil DNA extraction method.

Gomes *et al*. (2018), successfully identified different varieties in EVOOs with Denomination of Origin certification, using DCA18 UDO99-011 markers. Therefore, we recommend using UDO99-011 and DCA18-M markers for EVOO analysis, particularly those containing the Azapa variety, given the consistent patterns observed in our study.

While many studies effectively used SSR markers successfully for identifying olive varieties in single-variety EVOOs (Vietina *et al*., 2011; Gomes *et al*., 2018), very few studies have analyzed EVOO blends (Gomes *et al*., 2018) due to the complexity of detecting varieties in mixed products. Fingerprinting applications for blend EVOOs do not necessarily require the identification of each variety, as it is enough to detect the absence of expected alleles based on the declaration on the product label (Pasqualone *et al*., 2016) or detect alleles of undeclared varieties to prove fraud (Pasqualone *et al*., 2016). The product label plays a crucial role in conveying accurate information to consumers. However, in our study, over half of the 20 EVOO brands (3 imported from Spain and 17 of Chilean production) lacked essential label information, highlighting the need for improved labeling practices. Testing several brands of single-variety and blend EVOOs with UDO99011 and DCA18-M markers revealed content consistencies and inconsistencies compared to label declarations.

None of the three imported olive oil products provided information on whether it is a single-variety or blended oil, nor did they specify the variety/varieties used. Instead, they claimed "this product contains all types of varieties", complicating the verification process. The analysis of imported EVOOs using the UDO99011 and DCA18-M markers revealed that the alleles in "La Española" (Spain) align with those of the Picual variety, suggesting that this EVOO may be a single-variety type. "Terra Santa" (Spain) and "RS" (Spain) exhibited matches with at least five varieties (including Arbosana, Picual, Barnea, Empeltre and Frantoio) indicating that these olive oils are blends of multiple varieties.

The lack of information on the label extended to the seven Chilean single-variety EVOOs and two blends. The brands "Líder, Santiago, La Raima and Banquete" contained Azapa, Arbosana and/or Picual varieties, and could be classified as blends due to the observed coincidence of the three varieties. However, discriminating between Picual, Azapa and Arbosana remains challenging, as they share identical allele sizes when amplified with the UDO99-011 marker. Therefore, whether these EVOOs are, single-varieties or a blend remains uncertain. Meanwhile "Oromaule and Talliani" contained four varieties (among which Picual and Arbequina), and "Don Daniel" exhibited a diverse blend of up to nine varieties (including Arbequina, Arbosana, Frantoio, Picual, Koroneiki, Azapa and Coratina). "Huasco" seemed to be a single-variety EVOO of the Arbequina variety. "Lombardi" while lacking allele coincidence with any variety, might be a single-variety EVOO, possibly containing the Azapa. This inference is based on the DCA18-M marker and its production in the Azapa-dominated Arica-Parinacota Region of Chile.

The combination of the two SSR markers (UDO99011 and DCA18-M) showed that the alleles detected in most of the Chilean single-variety EVOOs align with the variety declared on the label (Chef/Arbosana, Don Daniel/Picual, Bezma/Azapa, Las Doscientas/Picual, Don Daniel Premium/Frantoio, Río de Oro/Sevillana del Huasco and Payantume/Sevillana del Huasco). However, the

alleles found in "Oliva" did not coincide with the claimed Arbequina variety when using both markers, although it was picked up by the DCA18-M marker when used alone. The EVOOs Chef (Arbosana) and Las Doscientas (Picual) are produced in the south of the central zone of Chile, where the Arbosana and Picual varieties are grown. Although we can therefore discard the presence of the Azapa variety, we cannot distinguish between Picual and Arbosana varieties due to identical allele sizes. Although we observed the varieties that were declared on the labels in most of the single-variety EVOOs, many of these EVOOs contained between two to ten varieties, resulting in incomplete product disclosure. The addition of olives of other varieties is likely to meet demands, considering limited supply, especially in regions like the Arica-Parinacota Region of Chile.

Less than 2% of Chile's approximately 25,000 hectares of olive plantations grow Sevillana del Huasco (Azapa). Only EVOO produced with these olives from the Huasco Valley qualifies for Designation of Origin certification, leading to a limited national market presence. Extra virgin olive oil from Huasco must be made with at least 10% of the Sevillana del Huasco variety. However, they can be blended with other varieties such as Manzanilla, Liguria, Empeltre, Frantoio and Arbequina, as outlined by the National Institute of Industrial Property (INAPI, 2022).

Single-variety EVOO brands Río de Oro (Sevillana del Huasco) and Payantume (Sevillana del Huasco), produced in the Huasco Valley (Atacama Region) with the correct olive variety meet the requisites for a Designation of Origin certification. In contrast, the single-variety EVOO Bezma (Azapa), although produced with the correct variety, cannot obtain this certificate as it is produced in the Azapa Valley (Arica-Parinacota Region). Despite using the same olive variety, physiochemical and organoleptic differences exist in these EVOOs. Azapa olives from the Azapa Valley, being larger (both in length and width) are favored as table olives, while the olives from the Huasco Valley are more suitable for oil production (Contreras *et al*., 2018). Ensuring the quality control or the Designation of Origin-certified EVOO from Huasco and Geographical Indication-certified olives from Azapa would therefore benefit from verifying the location of production.

The microsatellite allele detection technique has a high sensitivity and discrimination power (Pasqualone *et al*., 2016). The detection limit for SSR-High-resolution melting (HRM) assays is even more sensitive and ranges from 5-15% of DNA contribution in olive oil mixtures. single nucleotide polymorphism SNP-HRM assays offer even greater sensitivity with a 5% detection limit (Chedid *et al.*, 2020). To enhance robustness in future analyses, we therefore recommend complementing microsatellite fragment analysis with other efficient techniques such as SSR-High-resolution melting (HRM) and single nucleotide polymorphism (SNP)-HRM. These methods, proven to be suitable and reliable for identifying single-variety and blend EVOOs (Gomes *et al*., 2018; Carvalho *et al*., 2021), can establish an effective quality control method that will support Sevillana del Huasco olive producers in the Huasco Valley to market their exclusive product.

CONCLUSIONS

These results confirm the value of microsatellite markers in identifying olive varieties from leaves and fruits. The microsatellites indicated that most of the samples of Chilean single-variety EVOOs contained the declared variety, but most of them also contain other varieties. The microsatellites serve as a diagnostic tool for quality control of the Geographical Indication certification of Azapa olives and the Designation of Origin certification of EVOOs from Huasco, facilitating their commercialization within the Chilean domestic market. Using the microsatellites, we confirmed the similarities between the Sevillana del Huasco and Azapa varieties. We therefore suggest that quality control for Designation of Origin EVOOs from Huasco, should include microsatellite fragment analysis in combination with other efficient techniques, such as SSR-High-resolution melting (HRM) and single nucleotide polymorphism (SNP)-HRM. Additionally, we propose genetic analysis with microsatellites on whole olive fruits before crushing at the oil mill.

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Supplementary materials

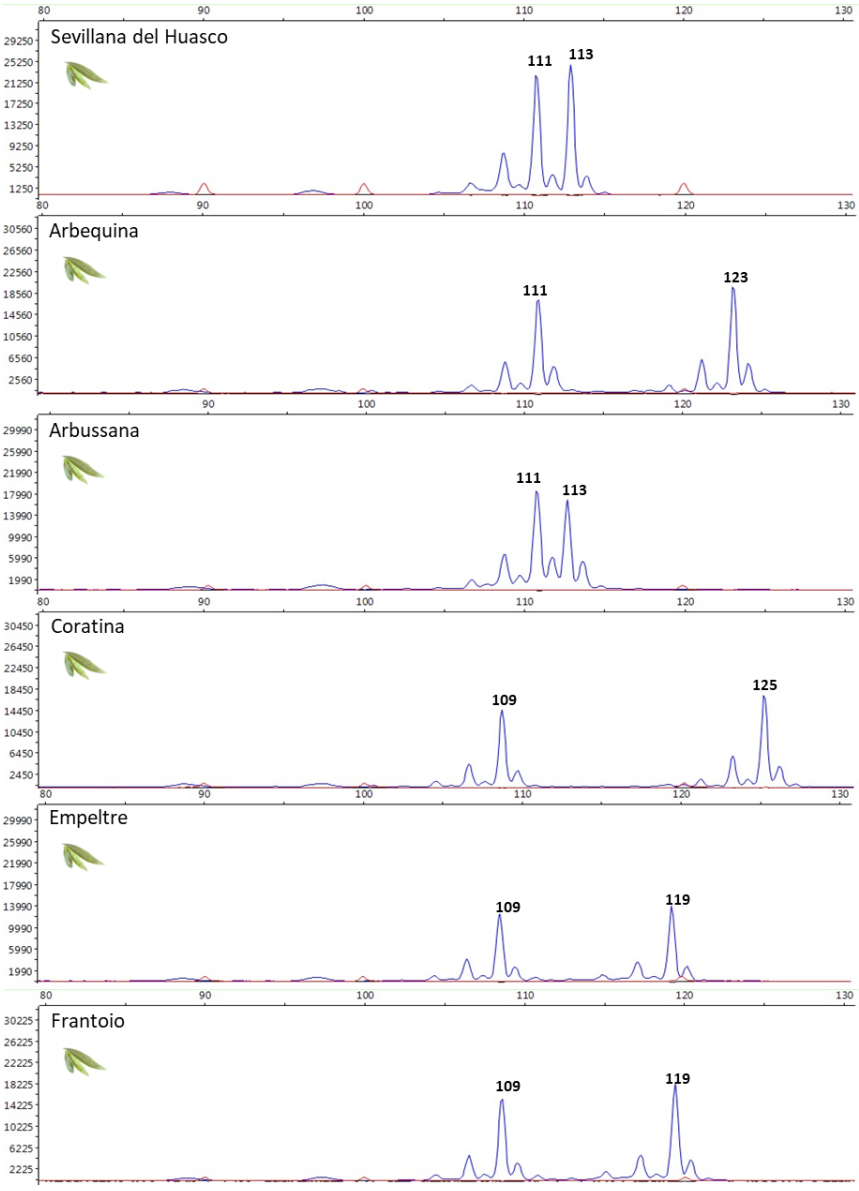
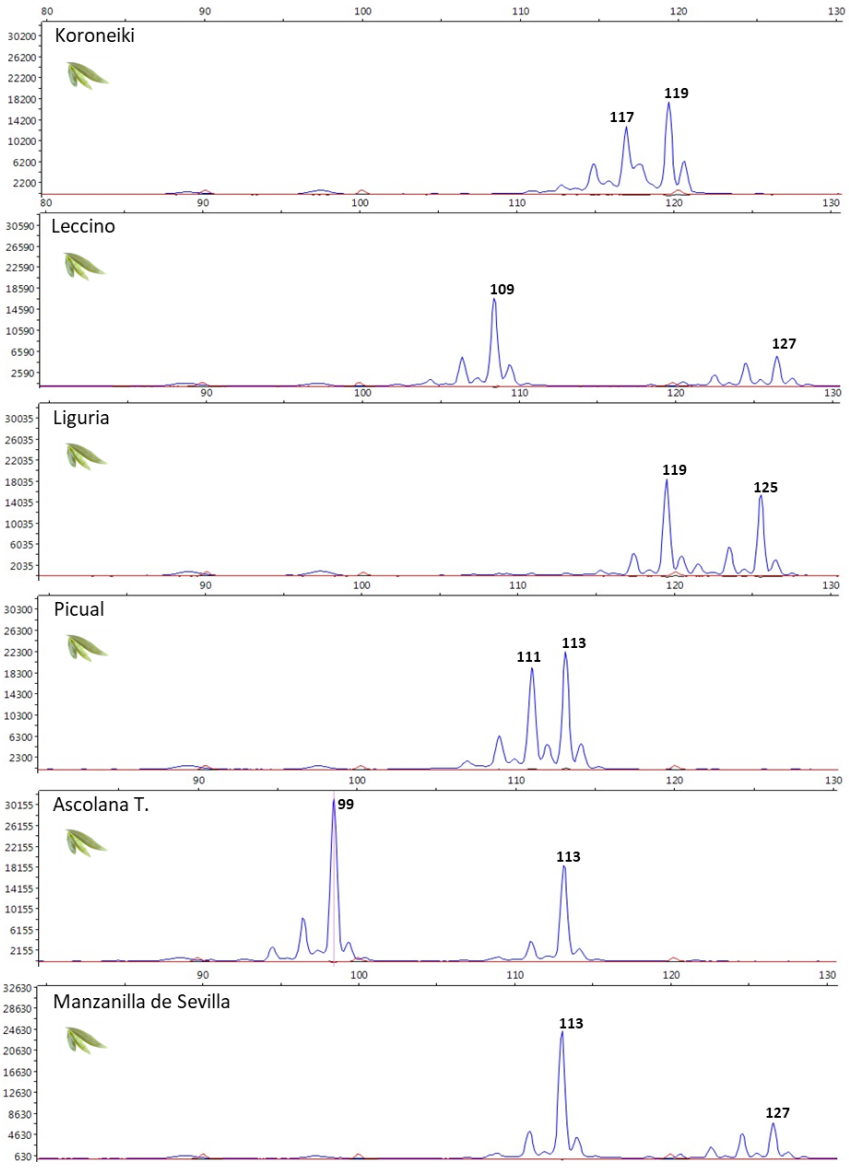


Figure S1

Electropherogram of leaf DNA amplicons from different olive varieties with the UDO99-011 marker



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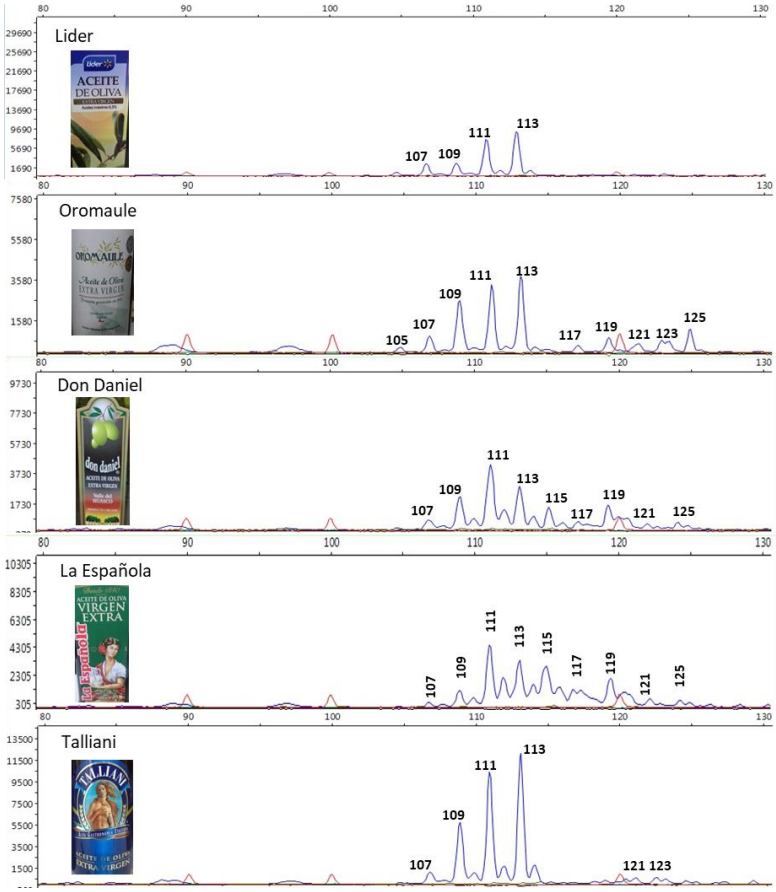
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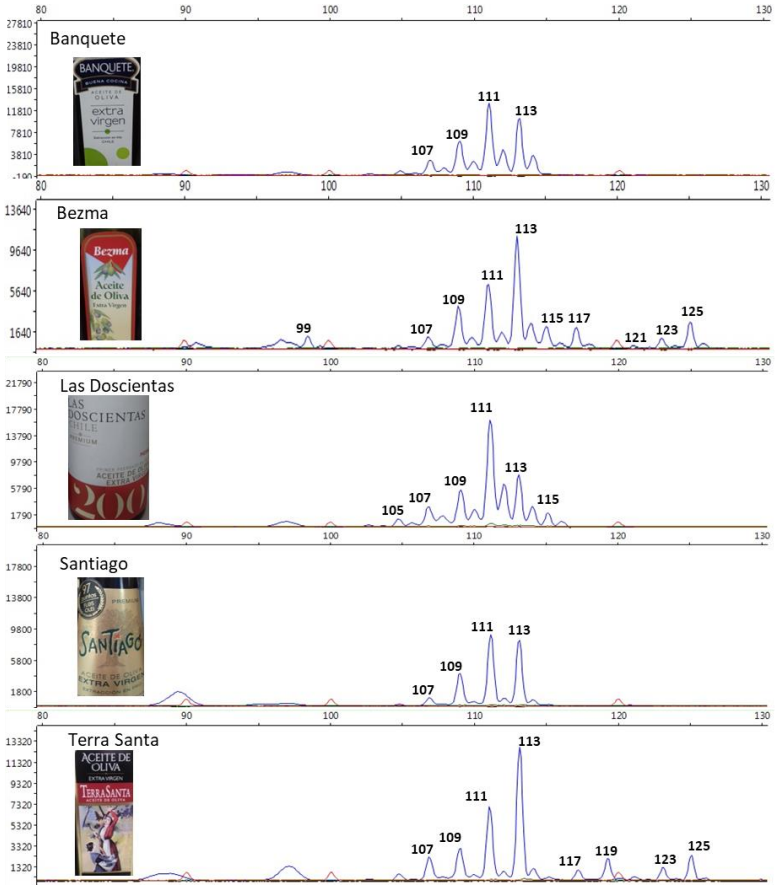
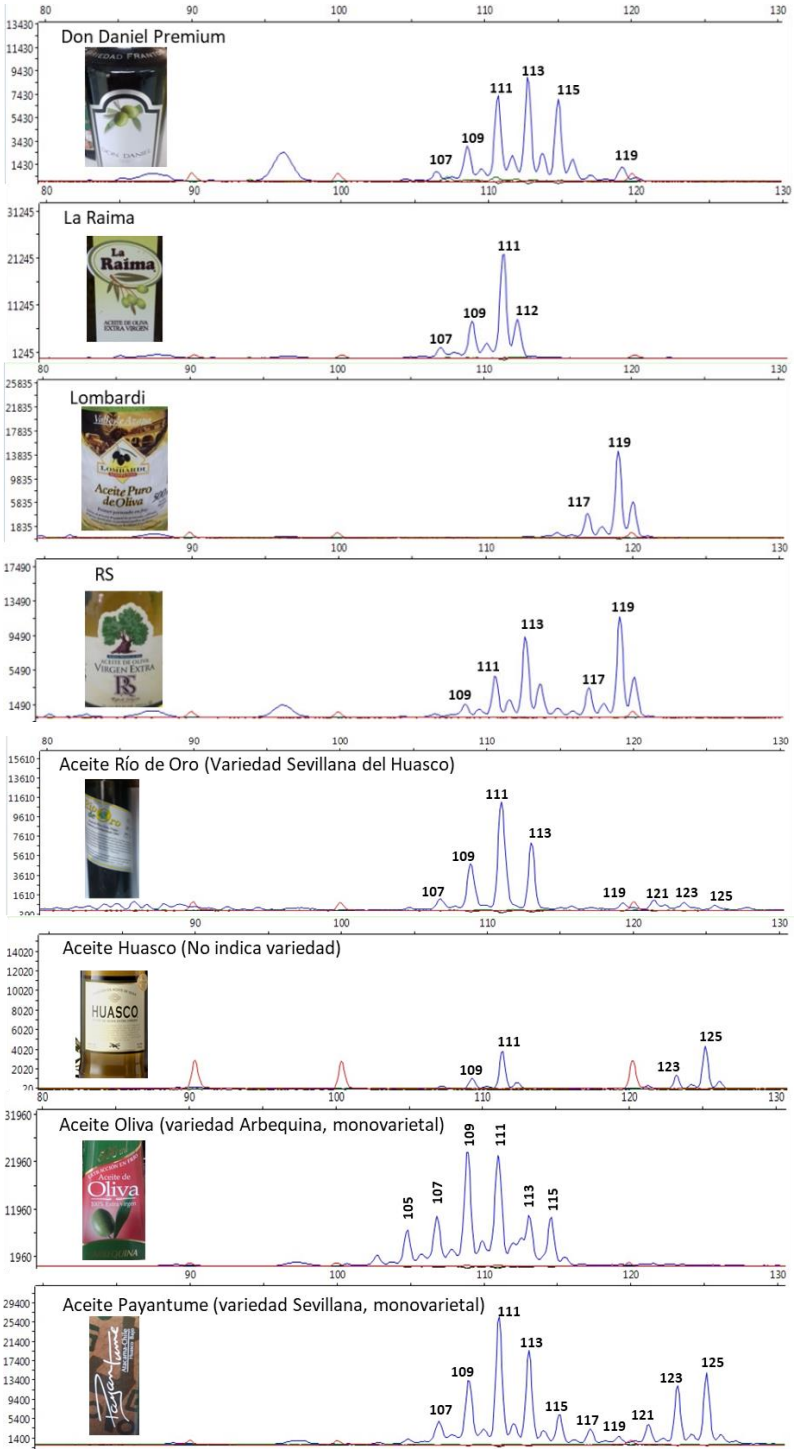


Figure S2
Electropherogram of DNA amplicons from EVOOs with the UDO99-011 marker



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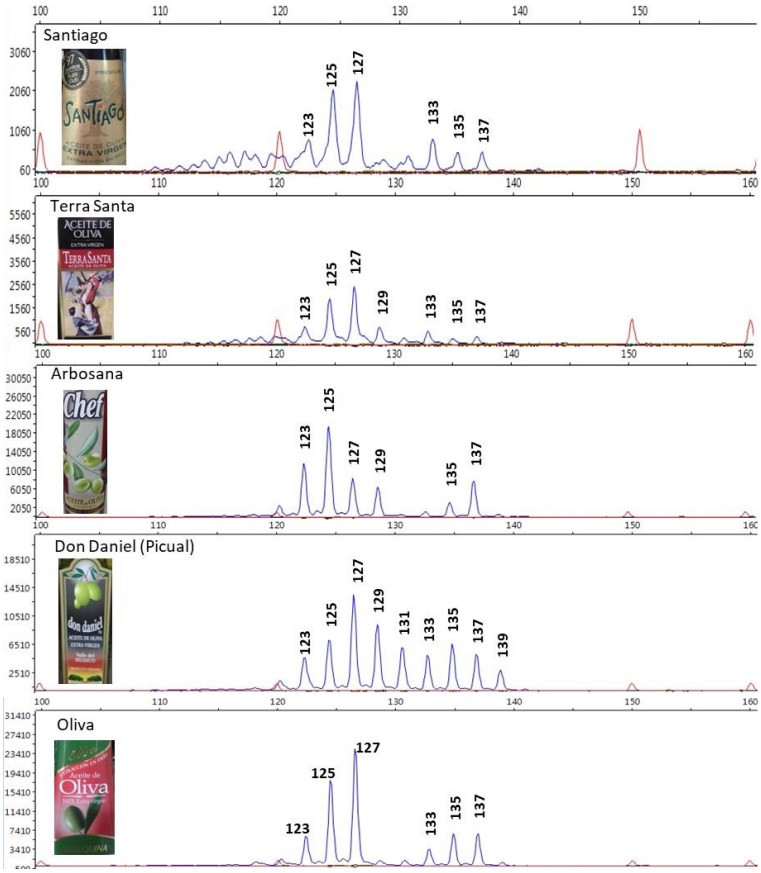
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Don Daniel (Picual)
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Oliva
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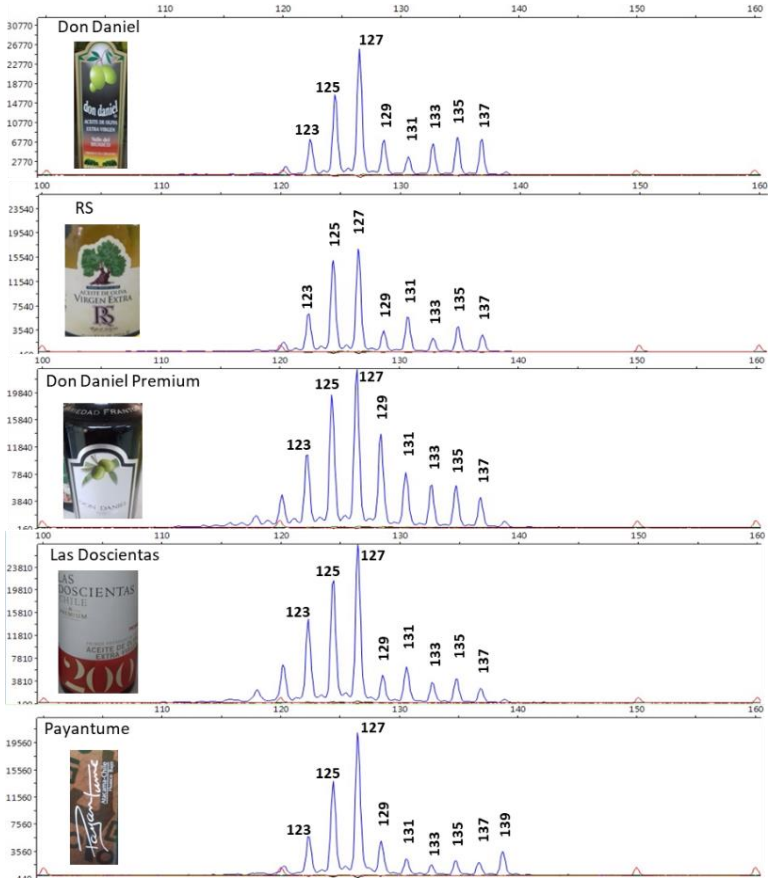
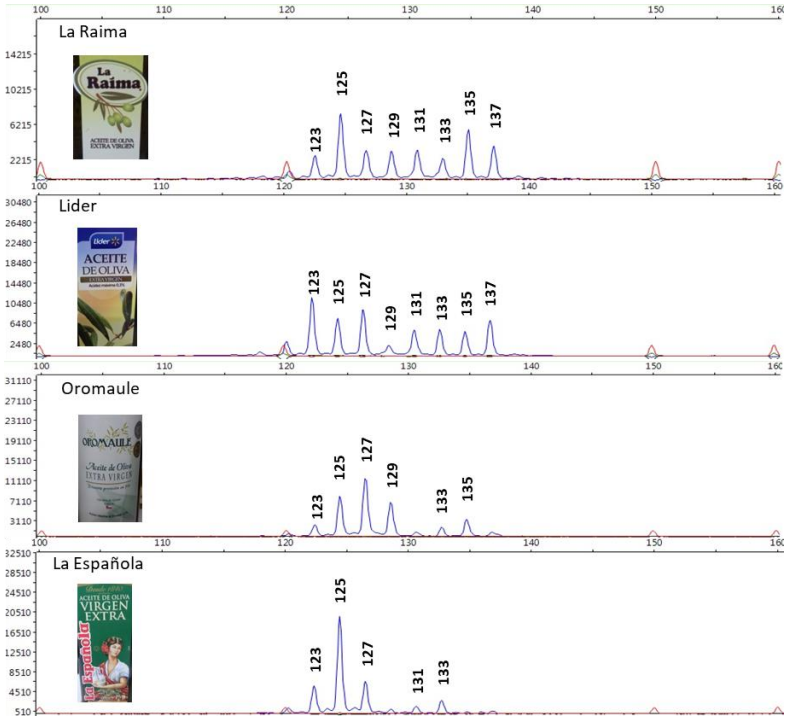
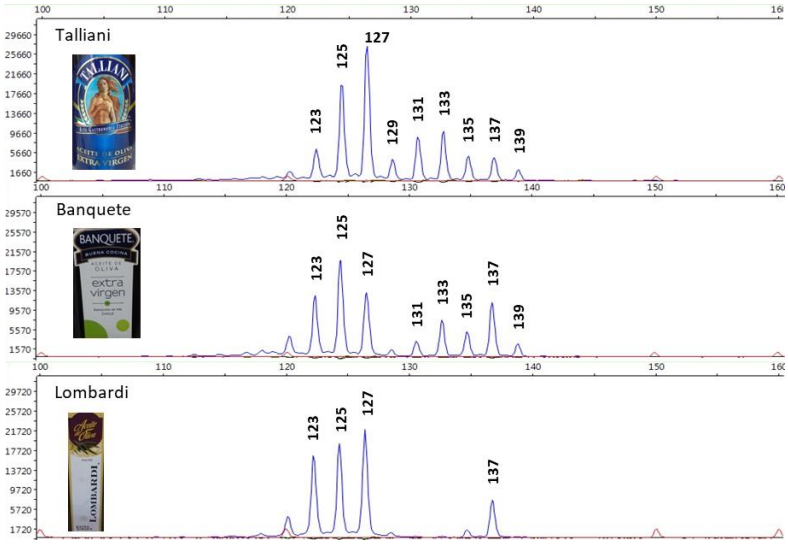


Figure S3
Electropherogram of DNA amplicons from EVOOs with the DCA18-M marker.





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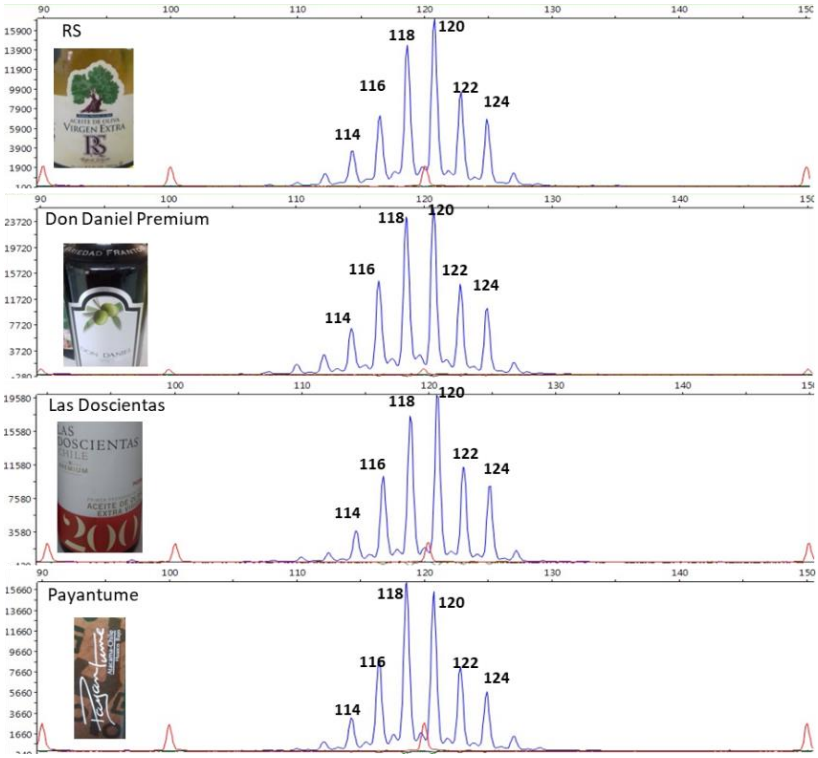


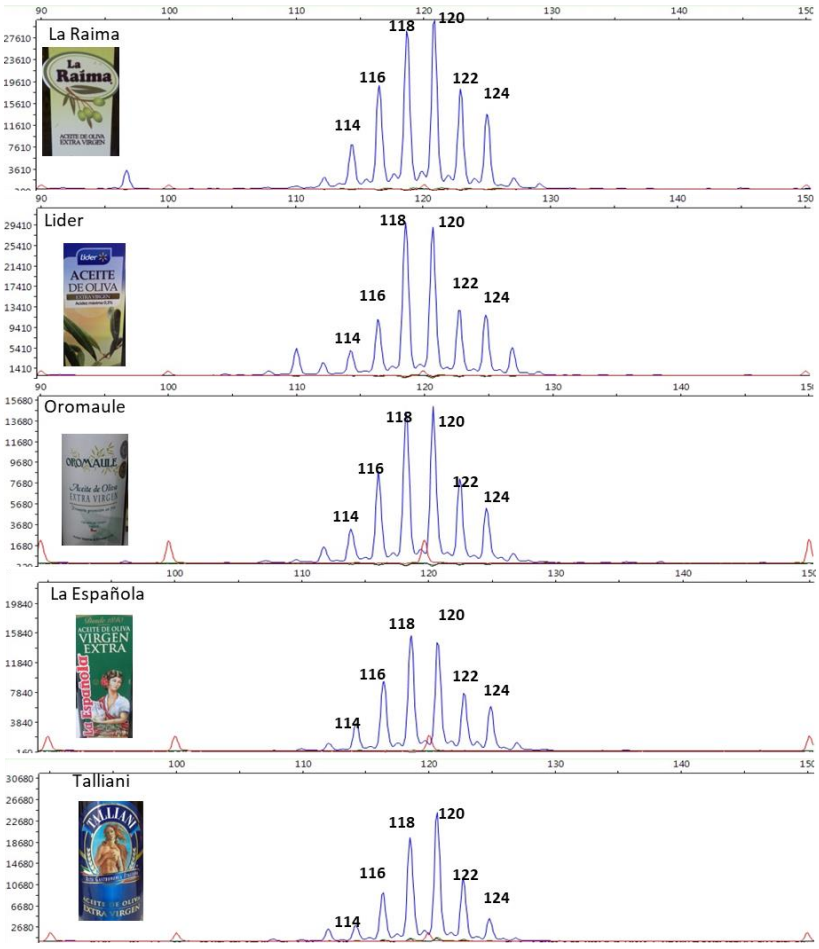
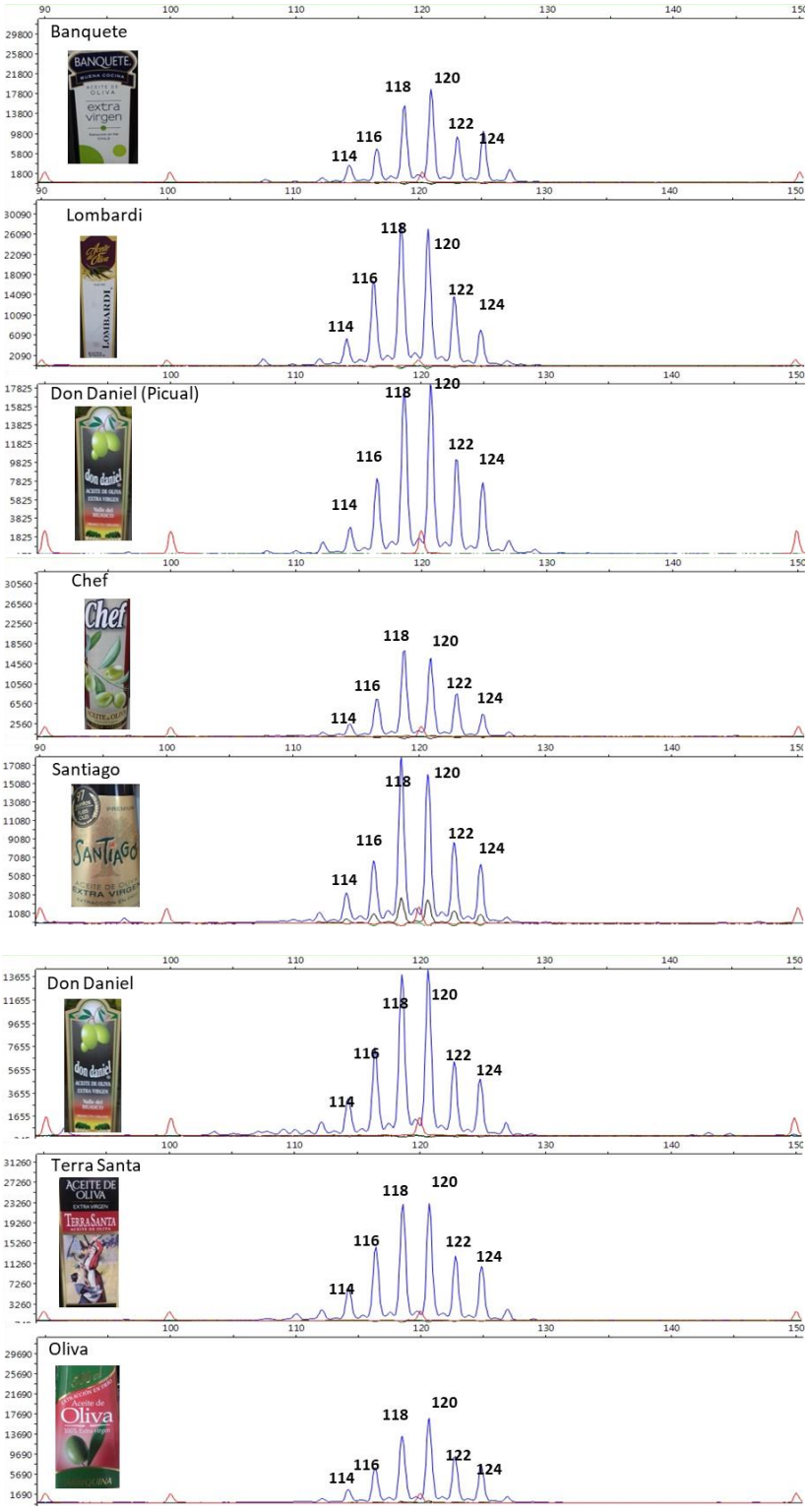


Figure S4. Electropherogram of DNA amplicons from EVOOs with the DCA8-M marker**.**



Continuation Figure S4.