Forensic Botany: Potential Usefulness of Microsatellite-based Genotyping of Croatian Olive (*Olea europaea* L.) in Forensic Casework

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Aim To assess genotyping with microsatellite-based markers of the olive (*Olea europaea* L.) for potential application of olive as legal case evidence, with regard to the degree of variability within the Croatian olive genomic pool and to the effectiveness of the chosen set of microsatellite-based markers in revealing olive divergence.

Methods The total of 44 autochthonous Croatian olive specimens were subjected to genotyping with 16 previously described and developed microsatellite-based markers. According to previous morphological analyses, 44 specimens were classified into 30 cultivars with the exception of an additional, previously unassigned specimen.

Results Genotyping of 44 specimens distinguished a total of 44 different genotype profiles by 16 microsatellite-based loci. Average expected heterozigosity amounted to 0.758, which points to significant diversity of Croatian olives.

Conclusion Croatian olive genotyping showed strong varietal discrimination up to the single tree and considerable potential application of olive as evidence in investigation of crime, accident, and suicide circumstances.

Forensic botany is the study of plants and plant material with the purpose of presenting the plant evidence in court. It includes a number of disciplines, such as plant anatomy and systematics, palynology, plant ecology, limnology, plant chemistry, and plant molecular biology (1). In spite of its high potential in assessing the legal case evidence, only a few cases of plant forensic investigation applying DNA profiling, when a suspect was linked to the crime scene, were described (2,3). Plant DNA profiling serves to identify the origin of detected plant material connected to a crime, suicide, or accident, and hence, it may contribute to identifying the location(s) where the event took place (primary scene), recent location of the body, whether a victim had been transferred or moved (secondary scene), and whether a suspect was present at a crime or accident scene (4,5). DNA profiling is also employed in solving the issues of narcotics and drug enforcement, as well as of unauthorized commercialization of some plants.

Microsatellite-based genotyping, due to its great reproducibility and high degree of certainty in assigning the origin of a biological material that serves as legal case evidence, represents one of the most reliable DNA profiling methods in forensic investigation (6).

Microsatellites, short tandem repeats (STR) or simple sequence repeats (SSR) consist of a number of tandemly repeated short DNA sequences (1-6 base pairs long). They are distributed throughout the eukaryotic genome. In addition, microsatellites are multiallelic due to their high intraspecies variability and are easily amenable to polymerase chain reaction (PCR)-based analysis. Both characteristics make them the DNA markers of choice for human DNA profiling analyses. However, microsatellite-based markers found their way of wider application in different branches of animal and plant sciences.

Olea europaea L. is a diploid, outcrossing species. Cultivated olives have been reproduced mainly by vegetative propagation and sporadically by cross-breeding, which resulted in the creation of a number of varieties due to accidental crosses between cultivated forms or between wild and cultivated forms, but also due to accumulation of mutations, along with local selection of outstanding individuals. Hence, most olive cultivars have a local origin. More than 2000 cultivars have been documented in the Mediterranean region by means of their morphology (7).

At present, microsatellite-based DNA sequences are the most appropriate genetic markers used in olive cultivar characterization and classification. Many microsatellites have been isolated from olives and respective primer pairs have been developed (8-14).

Due to their mainly local origin, specific olive cultivars are indigenous to specific geographical areas. In addition, the same cultivars grown in different environmental conditions have different genotype profiles. Both olive characteristics ensure their relevance in the assessment of the location of origin of the olive sample in question.

Olive trees are abundant in Croatia. In order to assess the application potential of Croatian olive DNA profiling in forensic investigations, we genotyped the total of 44 specimens that comprise 30 cultivars and their 13 varieties, as well as one unassigned olive specimen.

Material and methods

Plant material

Forty-four autochthonous Croatian olive specimens *Olea europaea* subsp. *europaea* var. *europaea* (30 cultivars and their 13 varieties, as well as one unassigned olive specimen) from the south of Croatia, the native area of their cultivation, were selected for this study (Figure 1).

DNA extraction

Total DNA was isolated from young olive leaves and flower buds, following an already published olive DNA isolation method (15), with several

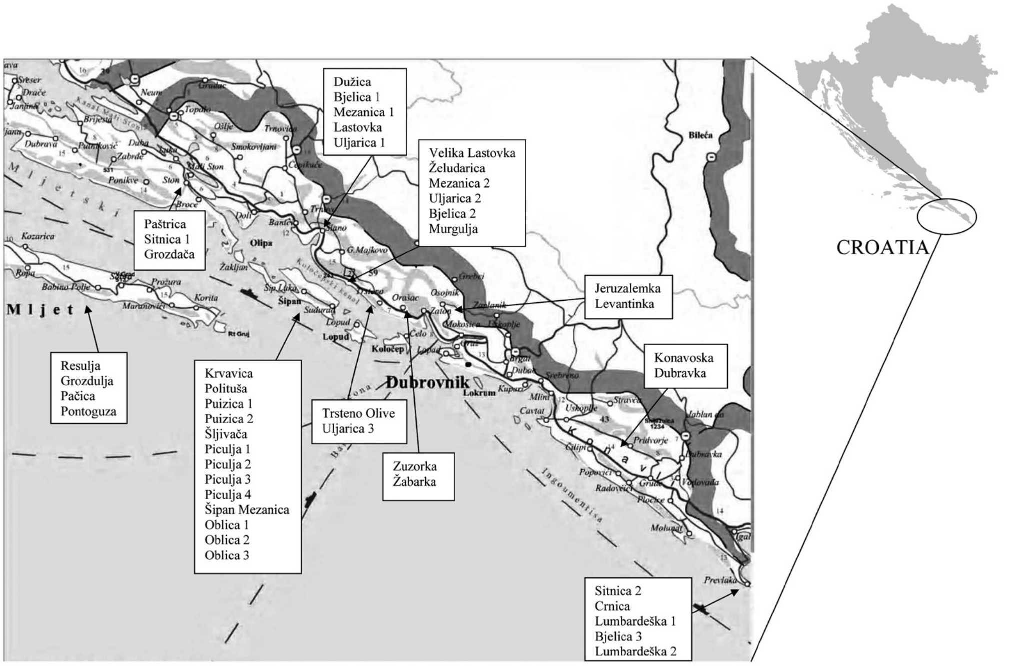

Figure 1. Analyzed olive specimens and native areas of their cultivation. The oval indicates geographical location of olive specimens in Croatia, sampled in this study.

modifications. Young olive leaves and buds were washed by 4% sodium hypochlorite and 0.2 g of plant tissue was ground into liquid nitrogen and incubated in 4 mL of prewarmed CTAB buffer [2%(w/v) CTAB, 100 mM Tris-HCl pH 8, 1.4 M NaCl, 40 mM EDTA pH 8, 0.5% SDS, 6% (w/v) PVP, 0.2%(v/v) 2-mercaptoethanol]. The samples were incubated at 65°C for an hour and a half and mixed gently several times. Equal volume of chloroform-isoamyl alcohol [24:1 (v/v)] was added to the mix and centrifuged twice (incubated for 20 minutes and centrifuged at 8000 rpm for 10 minutes). After the RNase digestion, samples were purified by phenol-chloroform-isoamyl alcohol [25:24:1 (v/v)] and further precipitated by 2 M ammonium-acetate and 2/3 (v/v) isopropanol. DNA was further washed by water using Centricon Centrifugal Filter Devices (with YM-100 MW membrane-Amicon; Millipore, Billerica, MA, USA) in order to remove polyphenols and pigments soluble in water.

Quantification of olive DNA was performed by spectrophotometar (Ultrospec 2000, Pharmacia Biotech (Biochrom) Ltd. Cambridge, UK).

Primers and microsatellite-based marker analysis

Olive specimens were characterized with the following 16 microsatellite-based markers: UDO99-008, UDO99-012, UDO99-019, UDO99-024, UDO99-028, UDO99-031, UDO99-039, UDO99-043 (11), ssrOeUA-DCA3, ssrOeUA-DCA8, ssrOeUA-DCA9, ssrOeUA-DCA10, ssrOeUA-DCA14, ssrOeUA-DCA16 (9), EMO2, and EMO3 (12).

Polymerase chain reactions were carried out in a volume of 12.5 µL, containing 1.5 mM $MgCl_2$ for all ssrOeUA markers and EMO2 and EMO3; 2 mM $MgCl_2$ for all UDO99 markers except UDO99-008; and 2.5 mM $MgCl_2$ for UDO99-008 marker; 0.2 mM of each dNTP (Applied Biosystems, Foster City, CA, USA), GeneAmp 10×PCR Buffer II (1.25 µL for ssrOeUA-DCA3, ssrOeUA-DCA14, and ssrOeUA-DCA16; 1.5 µL for ssrOeUA-DCA8, ssrOeUA-DCA9, ssrOeUA-DCA10, EMO2, EMO3, UDO99-019, and UDO99-043; 1.75 μL for UDO99-008, UDO99-012, UDO99-024, UDO99-028, UDO99-031, and UDO99-039 markers; Applied Biosystems), primers, and 0.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR reactions were performed in Applied Biosystems thermocyclers 9600 and 9700 under the following conditions: a step of 10 minutes at 95°C, followed by 35 cycles of 45 seconds at 94°C, 1 minute at the appropriate annealing temperature of the primer, and 1 minute at 72°C, and a final extension at 72°C for 30 minutes.

PCR products were analyzed in an automated sequencer (ABI Prism 310 Genetic Analyzer, Software v3.2, Applied Biosystems) and fragment lengths were determined using Genescan 500 Liz internal size standard (Applied Biosystems).

All PCR reactions were repeated three times if the results were perfectly concordant, and up to six times if there was a discrepancy in the first three amplifications, until obtaining at least three concordant results. Such discrepancies occurred on the average in 25% cases of total amplifications for each microsatellite-based marker, but were resolved in further three amplifications.

Data analysis

The expected heterozygosity (H_E) of each locus was calculated according to the formula $H_E=n(1-\Sigma p_i^2)/(n-1)$, where p_i is the frequency of the i-th allele and n is the number of gene copies in the sample for the given locus (16).

Expected and observed heterozygosities were calculated considering that specimens with only one amplified product for the given locus were homozygotes for that locus. Hence, heterozygosities reported here might have been underestimated in cases when null alleles occurred.

Results

Genotyping analysis was performed on the total of 44 different olive samples, and the total of 163 amplified polymorphic products were obtained after applying 16 previously developed primer pairs used for amplification of microsatellite-based markers (Table 1). The absence of any amplified product occurred only once, ie in genotyping one cultivar applying marker UDO99-028 (Table 2).

Table 1 Composition, size range, and number of alleles detected in 44 olive specimens; the observed (Ho) and expected heterozygosity (H_E)

Locus	Repeat motif	Size range (bp)	No. of polymorphic alleles	Ho	H_E
	(GA)n repeats:				
ssrOeUA-DCA3	$(HB)_{19}$	(229-252)	8	0.932	0.836
ssrOeUA-DCA8	$(HB)_{18}$	(124-154)	9	0.932	0.811
ssrOeUA-DCA9	$(HB)_{23}$	(152-209)	10	0.886	0.829
ssrOeUA-DCA10	$(UB)_{14}(HB)_{17}$	(153-241)	20	0.750	0.910
EMO2	$(AG)_5$-G-$(GA)_{10}$	(184-244)	14	ND*	ND*
	$(CA)_n$ repeats:				
UDO99-008	$(AC)_{13}$	(155-166)	7	0.273	0.792
UDO99-012	$(GT)_{10}$	(155-166)	5	0.659	0.610
UDO99-031	$(TG)_{21}$ $(TATG)_6$	(107-151)	9	0.455	0.583
UDO99-043	$(GT)_{12}$	(171-219)	13	0.886	0.746
ssrOeUA-DCA14	$(DB)_{18}B_6(UBB)_7$	(145-191)	11	ND*	ND*
EMO3	$(CA)_7$	(203-214)	9	0.841	0.841
	$(GA)_n$ and $(CA)_n$ compound repeats:				
ssrOeUA-DCA16	$(HU)_{13}(HB)_{29}$	(124-182)	12	0.886	0.815
	$(CA)_n$ or $(GT)_n$ and $(TA)_n$ compound repeats:				
UDO99-019	$(GT)_{20}(AT)_5$	(99-163)	6	0.636	0.499
UDO99-024	$(CA)_{11}(TA)_2(CA)_4$	(166-195)	9	0.682	0.747
UDO99-028	$(CA)_{23}(TA)_3$	(126-176; 0)	10	0.886	0.831
UDO99-039	$(AT)_5(GT)_{11}$	(106-186)	12	ND*	ND*
Total			164		
Average			10.25	0.747	0.758

*Ho and H_E were not determined since some specimens yielded three different amplified products.

Table 2 The lengths of DNA sequences (bp) encompassing 16 microsatellites as a results of genotyping 44 different Croatian olive specimens*

	Markers															
Cultivars	U-008	U-012	U-019	U-024	U-028	U-031	U-039	U-043	DCA3	DCA14	DCA16	DCA8	DCA9	DCA10	EMO2	EMO3
Resulja	165	155/157	129/143	172/186	134/151	108/137	177/180	175/177	236/250	170/178	152	136/154	164/188	208/217	184/210/225	203/212
Grozdulja	164	155/160	129/154	179/184	172	108/146	156/177	181	229/246	187	152	124/134	205/207	153/194	219/225/230	210
Pačica	165	155	129	172/186	151/172	148	180	175/177	236/246	170/187	157/161	136/138	205/207	163/213	219/225/230	211/212
Pontoguza	160/165	155/164	129/143	172/186	151/172	108	177/180	177/215	229/246	187	127/157	134/136	164/207	215/239	220/225	211/212
Paštrica	160/165	155/164	129/143	172/186	151/172	108	177/180	177/215	229/246	187	127/157	134/136	164/207	215/239	219/225	208/212
Sitnica 1	160	155/164	129/143	172/186	134/172	108	178	179/215	229/236	184/187	152/157	124/134	205/207	159/239	219/225	203/212
Sitnica 2	162	155/164	129/143	172/186	134/172	108	178	179/215	229/236	187	152/157	124/134	207/209	159/239	219/225	203/212
Grozdača	166	155	129/143	184/186	134/151	110	179/184	177/179	229/246	178/187	152/179	134/136	164/205	155/239	225	203/210
Dužica	166	155	129	166/186	NA	108	106	177/179	246/250	178	157/182	136	164/184	217	224/225	208
Bjelica 1	163/165	155/164	129	184	132/153	108/151	106/177	177/179	229/240	153/175/187	152/157	130/134	205/207	153/237	219/223/230	210/211
Bjelica 2	163/165	155/164	129	184	132/153	108/151	106/179	179	229/240	153/175/187	152/157	130/134	205/207	153/237	219/225/230	211
Bjelica 3	163/165	155/164	129	184	132/153	108/151	106/177	179/181	229/240	153/175/187	152/157	130/134	197/207	153/237	220/225/230	210
Mezanica 1	160/166	155/166	129	186	134/151	110	177/180	177/179	240/250	178/191	124/126	136/138	174/196	155/237	202/225/230	208/211
Mezanica 2	160/166	155/166	129	186	134/151	110	177/180	177/179	240/250	178/187	124/126	136/138	174/196	155/239	202/224/228	208/211
Lastovka	155/164	157/164	99/129	184/188	134	108	106/179	171/219	229	187	152/157	130/138	188/196	153/195	211/219/244	210/213
Uljarica 1	164	155/164	129/163	184	134/141	108/151	106/177	179/217	229/240	180/187	152/157	126/134	174	239	202/210/223	211
Uljarica 2	164	155/164	129/163	184	134/141	108/151	106/177	179/217	229/240	180/187	152/157	126/134	174	239	201/210/223	211/212
Uljarica 3	164	155/164	129/163	184	134/141	108/151	106/177	179/217	229/240	178/187	152/157	126/134	152/174	158/239	202/210/223	211/212
Vel. Lastovka	165	155/166	129/143	172/186	126/134	108	178/180	177/215	229/250	187/189	157/177	134/136	164/205	157/239	202/225/230	208/212
Želudarica	165	155	129	166/186	126/153	108	106	177/179	242/250	178/187	124/127	130/136	164/205	155	225/230	210/211
Murgulja	165	155/164	129/143	186	126/172	108	106/177	179/213	236/246	178/187	152	124/136	164/207	159/217	220/225	203/208
Trsteno Olive	163	157/166	99/129	181/184	176	108/137	175/177	189/219	242/252	145/153/175	124/163	130/140	188/207	153/175	210/225/230	212/214
Zuzorka	160	155/164	129/143	172/186	134/172	108	178	179/215	229/236	187	152/157	124/134	205/207	159/241	219/225/230	203/211
Žabarka	165	155	129	186/195	134/172	108	144/177	175/179	229/250	153/176/187	177	134/140	164/188	220	220/225/240	208
Levantinka	166	155	129	186	151/153	110/151	177/186	179/183	236	178/187	152/176	124/134	164/207	177/215	225/230	208/211
Jeruzalemka	165	155	99/129	172/186	172	108/137	123/138	175/179	229/246	145/178	152/157	136/140	184/207	159/217	220	208/212
Crnica	166	155/164	129/143	186	151/172	108	106/179	177/179	236/246	187	152/176	124/136	196/207	155/239	202/219/230	203/208
Lumbardeška 1	165	155	129/143	172/186	134/151	108	177/180	177/179	236/250	178/187	152/177	134/136	164/205	217	225/230	208/212
Lumbardeška 2	165	155	129/143	171/186	134/151	108	177/180	177/179	236/250	178/187	152/177	134/136	164/205	217	225	208/212
Konavoska	166	155/164	129/143	186	134/151	108	106/177	177/179	236/250	187	157/177	136	196/205	155/159	202/225/230	210
Dubravka	165	155	129	166/186	126/153	108	106	177/179	242/250	178/187	124/127	130/136	164/205	155	225/230	210/211
Krvavica	166	155/164	129/143	186	126/172	108	106/177	179/211	236/248	178/187	127/152	124/136	164/207	159/215	220/225	203/208
Polituša	166	155	113/129	172/186	151/172	107/148	180	175/177	236/246	170/187	157/161	136/138	205/207	163/215	220/225/230	211/212
Puizica 1	164	157/166	129	166/186	134/162	124/151	177	179	240/252	170/187	146/152	134/148	174	169/237	202/224	206/212
Puizica 2	164	157/166	129	166/186	134/162	124/151	177	179	240/252	170/187	152	134/148	174	169/237	202/223	206/212
Šljivača	166	155	113/129	172/186	151/172	107/148	180	179	236/246	170/187	157/161	136/138	205/207	163/215	219/225/230	210/211
Piculja 1	155/164	157/166	129	166/181	153/172	108/144	106/123/138	179/185	229/242	170/187	124/146	124/130	174/205	153/212	202/225/230	206/209
Piculja 2	155/164	157/166	129	166/181	153/172	108/151	106/123/138	179/185	229/242	170/187	124/146	124/130	174/205	153/215	202/225/230	206/209
Piculja 3	155/164	157/166	129	166/181	153/172	108/151	106/123/138	179/185	229/242	170/187	124/146	124/130	174/205	153/212	202/225/230	206/209
Piculja 4	155/164	157/166	113/129	166/181	153/172	108/151	106/123/138	179/185	229/242	170/187	124/146	124/130	174/197	153/212	202/225/230	206/209
Šipan Mezanica	164	155/164	129/163	184	134/141	108/151	106/177	179/217	229/240	180/187	152/157	126/134	174	158/239	202/210/223	211/212
Oblica 1	166	155	129/143	172/186	134/151	108	177/180	177/179	236/250	178/187	152/176	134/136	164/205	217	225/230	208/211
Oblica 2	166	155	129/143	172/186	134/151	108	177/180	177/179	250	178/187	152/176	134/136	164/205	217	225	208/212
Oblica 3	166	155	129/143	172/186	134/151	108	177/180	177/179	236/250	178/187	152/176	134	164/205	217	225/230	208/212

*Abbreviations: U-No. refer to UDO99-No.; DCANo. refer to ssrOeUA-DCANo.; NA – no amplified product.

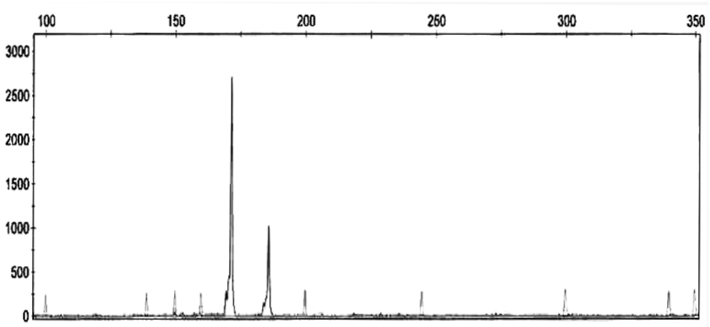

Figure 2. The electropherogram of olive DNA obtained in this study by applying UDO99-024 microsatellite-based marker (internal size standard – LIZ 500). X-axis indicates fragment lenght (bp), while Y-axis indicates relative flourescent units (RFU) that are proportional to the amount of PCR product.

The allele size ranges and the total number of alleles, as well as the expected and observed heterozygosities for each locus are presented in Table 1. The average expected heterozygosity amounted to 0.758.

An electropherogram of microsatellite-based olive DNA, serving as an illustration of the amplified products obtained in this study, is demonstrated in the Figure 2.

Three microsatellite-based primer pairs (UDO99-039, ssrOeUA-DCA14, and EMO2) amplified three different products for some cultivars (UDO99-039 for all four Piculja varieties, ssrOeUA-DCA14 for all three Bjelica varieties, as well as for two cultivars – Trsteno Olive and Žabarka, and EMO2 for 25 out of 44 specimens), thus increasing their power of discrimination of genotyped olive specimens.

Discussion

By genotyping more than one different specimen of the same olive cultivar denominations we have demonstrated that it is possible to distinguish among different intracultivar varieties by means of a set of 16 microsatellite-based markers.

In our study, 164 polymorphic alleles were characterized using 16 microsatellite-based markers over 44 different specimens and 30 denominations defined previously by agronomic and morphological means (17,18). In comparison, in the study employing 14 microsatellite-based markers over 130 specimens comprising 67 different denominations, 135 alleles were detected (19), while in the study employing 12 microsatellite-based markers over 50 specimens comprising 34 different cultivars, 119 alleles were detected (20). Furthermore, in the study applying 15 microsatellite-based markers over 47 cultivars, 124 alleles were detected (9), while in the study applying 7 microsatellite-based markers over 23 cultivars, 45 polymorphic alleles were detected (12). Finally, in our study the average expected heterozygosity amounted to 0.758, while in other studies it amounted to 0.679 (19), 0.760 (20), 0.693 (9), 0.648 (12), and 0.681 (13). Hence, comparable levels of genetic variability were observed in other studies as well. In addition, we showed that the applied set of microsatellite-based markers efficiently resolved all the cases of intracultivar variability, which points to a high potential of 16 chosen microsatellite-based markers in revealing olive genetic diversity.

The mechanism of the occurrence of three different microsatellite-based alleles amplified by one primer pair is not elucidated, but might be ascribed to chromosome rearrangements (21), genome fusions (22,23), or "chimerism" (24).

Microsatellite-based olive genotyping application is justified in solving criminal and civil cases by its ability to assign olive DNA to an individual tree and at the same time by its capability of yielding the same molecular profile for the same tree due to the olive microsatellite somatic stability.

General reproducibility of microsatellite-based genotyping data among laboratories should result in a comprehensive olive genotyping database that might be searched in case of a need to assign the origin of the found plant material connected to crime or accident or suicide scene.

In conclusion, we demonstrated that Croatian olive cultivar genotyping using 16 microsatellite-based markers may provide the possibility of olive specimen identification up to the individual tree and may open the possibility of their successful application in forensic investigations.

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