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DNA Fingerprinting of Olive Varieties by Microsatellite Markers

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Summary

Microsatellites combine several features of an ultimate molecular marker and they are used increasingly in various plant genetic studies and applications. In this work we report on the utilisation of fourteen previously developed olive microsatellite markers for the identification and differentiation of a set of nineteen olive varieties. All analysed microsatellite markers revealed a high level of polymorphism that allowed unique genotyping of the examined varieties. Ninety-six alleles were detected at all 14 loci, which multiplied into a large number of observed genotypes, giving high discrimination value for varietal identification. A minimum number of three microsatellite markers was chosen for the rapid and unambiguous varietal identification of nineteen olive varieties and only two markers were sufficient for differentiation of five local varieties. DNA fingerprints of olive cultivars by means of microsatellites provided meaningful data, which can be extended by additional olive varieties or new microsatellites and used for accurate inter-laboratory comparison. The data obtained can be used for the varietal survey and construction of a database of all olive varieties grown in Slovenia providing also additional genetic information on the agronomic and quality characteristics of the olive varieties.

Key words: olives (*Olea europaea* L.), microsatellites, varietal identification

Introduction

The olive tree has been cultivated for millennia in the Mediterranean basin and olive oil has been an important part of human nutrition in the region. Due to its fatty acid composition and content of other functional food components, interest in olive oil as a healthy food source has also increased outside the Mediterranean region. Since the production of olive oil is much lower than demand, there is a need to improve olive cultivation, both to produce more oil and to enhance its quality, particularly of components beneficial to human health. Major components of polyunsaturated, monounsaturated and saturated fatty acids, and their ratio (0.5:5:1), give olive oil an advantage over most vegetable oils (*1*). Minor components, such as biophenols, tocopheroles, and other biomolecules, are specific and valuable in olive oil (*2*) since they affect its sensorial properties, the shelf life of the oil and its health benefits (*3*,*4*). All these components vary among cultivars (*4–6*), so the varietal structure of olives in a region, together with oil extraction methods that preserve the quality potential of the fruits and oil, significantly contribute to the quality of the oil. Evaluation and characterisation of olive genetic

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resources has therefore been recognised as very important, since both olive productivity and oil quality are traits inherent to a variety (*7*). Varietal surveys have been initiated on national and international levels (*8*) to determine and describe cultivated olive varieties and thus obtain information which can serve for varietal improvement for modern olive growing as well as for distinctive characterisation of varieties specific to different olive growing regions.

In the last twenty years, there has also been a general trend of increased olive oil production in Slovenia and, at present, about 300 thousand olive trees are grown, giving approximately 350 t of olive oil annually. Preliminary surveys based on morphological data of olives grown in Slovenia showed the presence of different olive varieties. The predominant variety is 'Istrska belica', which has been intensively propagated in the past due to its excellent adaptability to the pedoclimatic conditions, especially to low temperatures. Old local varieties can still be found, but they are slowly being replaced by 'Istrska belica' or by introduced Tuscan varieties ('Leccino'). A specific characteristic of oil produced from 'Istrska belica' is a high polyphenol content, which has been reported by many authors (*9–12*), but little is known about the sensorial and constituent characteristics of oil produced from other local cultivars (*13,14*).

One of the important aims of programmes to improve olive growing and olive oil production in Slovenia is to build a database of all varieties grown in the region and of their agronomic and chemical characteristics. Correct varietal identification is crucial, since identification of olive cultivars is complicated by the large number of varietal synonyms and homonyms, the intensive exchange of plant material, the presence of varietal clones, and problems of varietal certification in nurseries. Morphological characterisation by UPOV descriptors has been adopted (*15*) and molecular evaluation has been started, since molecular markers provide a good discriminatory system, independent of environmental conditions. RAPD (Randomly Amplified Polymorphic DNA) markers (*16*) were used at first for identifying and characterising olive varieties kept in an olive collection orchard. The discrimination ability of RAPD markers in olive cultivars has also been successfully applied in several other studies (*17–21*). However, such molecular markers are limited by their low inter-laboratory reproducibility, and the development of microsatellite markers in olives (*22,23*) has provided an improved approach for varietal characterisation. Microsatellites have already been proven to be very suitable markers for cultivar identification and identity typing in several crop species (*24*), allowing precise discrimination even of closely related individuals. Microsatellites combine several features of an ultimate molecular marker: high abundance in eucaryotic genomes, hypervariability, co-dominant nature, high information content, and their reproducibility (*25,26*), and they have thus become very popular in genetic characterisation of varieties.

The results of microsatellite based genotyping of nineteen olive varieties, five of which are traditionally grown in Slovene Istria, are presented here. We show that microsatellites can be used for rapid and reliable identification of olive varieties. To our knowledge, this is one of the first genotypings of olive cultivars by means of microsatellites providing meaningful data that can be extended by additional olive varieties and new microsatellites and used for inter-laboratory comparison.

Material and Methods

Plant material

Five local ('Istrska belica', 'Štorta', 'Buga', 'Črnica', 'Unknown 2') and fourteen olive varieties from Italy ('Leccino', 'Nocellara del Belice', 'Frantoio', 'Cipressino', 'Santa Caterina', 'Pendolino', 'Maurino', 'Unknown 1', 'Unknown 3', 'Itrana', 'Ascolana Tenera', 'Leccione'), Spain ('Arbequina') and France ('Picholine') from the olive collection orchard in Slovenia, were used in the study.

DNA isolation and amplification of microsatellites

Genomic DNA was extracted from fresh olive leaves by a modified CTAB method (*27*). Fourteen developed primer pairs for olive microsatellite loci (*22*) were used in the analysis. The loci amplified by these primer pairs were designated as: ssrOeUA-DCA1, ssrOeUA-DCA3, ssrOeUA-DCA4, ssrOeUA-DCA5, ssrOeUA-DCA7, ssrOeUA-DCA8, ssrOeUA-DCA9, ssrOeUA-DCA10, ssrOeUA-DCA11 ssrOeUA-DCA13, ssrOeUA-DCA14, ssrOeUA-DCA15, ssrOeUA-DCA16 and ssrOeUA-DCA17. Amplification of microsatellites was carried out in PCR reactions in a total volume of 10 L, containing 20 ng genomic DNA, 1X supplied PCR buffer (Promega), 0.2 mM of each dNTP (Roche), 0.25 unit of Taq DNA polymerase (Promega) and 0.5 M of each primer. The amplification, with minor modifications for all analysed loci except ssrOeUA-DCA16, was performed according to the published procedure (*22*) in a GeneAmp 9700 thermal cycler (Applied Biosystems) with the following temperature profile: after a first denaturation step at 95 °C for 5 min, the reactions went through 26 or 35 cycles (loci ssrOeUA-DCA5, ssrOeUA-DCA11 and ssrOeUA-DCA14) of 95 °C/20 s, 30 s at the annealing temperature depending on the primer pair (from 50 to 60 °C), 30 s elongation at 72 °C, followed by a final extension step of 7 min at 72 °C. PCR fragments of primer pair ssrOeUA-DCA16 were amplified after denaturation (94 °C/4 min) with 26 cycles (94 °C/45 s, 52 °C/30 s, 72 °C/1 min 30 s).

PCR products were checked by agarose-gel (1.8 %) electrophoresis and then separated on 5 % denaturing polyacrilamide gels, containing 1X TBE buffer and 7.5 M urea. Gels were stained with silver using the Promega Silver Sequence™ protocol with some modifications (*28*). Digital images of gels were made using an A4 scanner. Allele sizes were determined with a 10 bp DNA Ladder (Gibco BRL) and with sequencing reactions of pGEM-3Zf (+) vector (Promega).

Results

Microsatellites were successfully amplified in all nineteen varieties with the fourteen primer pairs used. PCR fragments were separated on sequencing gel at a resolution of 1bp, stained with silver and sized. Banding

Table 1. Allele sizes (bp) detected in analysis of nineteen olive varieties, number of amplified alleles per locus (n) and observed heterozygosity (H_o)

Allele*	Locus													
	DCA1	DCA3	DCA4	DCA5	DCA7	DCA8	DCA9	DCA10	DCA11	DCA13	DCA14	DCA15	DCA16	DCA17
A	208	232	132	195	135	129	163	156	131	120	173	250	124	107
B	216	236	134	199	145	135	173	158	135	124	179	260	128	109
C	218	238	152	203	149	139	183	160	143	126	181	270	148	115
D	258	240	162	207	151	141	185	162	147	140	183		152	117
E	268	242	164	209	153	143	187	166	163		187		156	119
F		248	166		169	145	193	176			189		158	145
G		252	186			151	195	180			191		166	173
H							205	196					174	179
I							207	222					176	181
J								224						203
K								240						
L								244						
n	5	7	7	5	6	7	9	12	5	4	7	3	9	10
H_o	0.631	0.947	0.684	0.631	0.842	0.947	1.000	1.000	0.263	0.263	1.000	0.722	1.000	0.842

*The letters indicate alleles at each locus
Note: Loci ssrOeUA-DCAno are designated DCAno

Table 2. Microsatellite identification key of 19 olive varieties: L-Leccino, IB-Istrska belica, B-Buga, Č-Črnica, Š-Štorta, C-Cipressino, NB-Nocellara del Belice, F-Frantoio, SC-Santa Caterina, P-Pendolino, A-Arbequina, U2-Unknown 2, Pi-Picholine, M-Maurino, U1-Unknown 1, At-Ascolana Tenera, I-Itrana, Le-Leccione, U3-Unknown 3

Locus/allele	L	IB	B	Č	Š	C	NB	F	SC	P	A	U2	Pi	M	U1	At	I	Le	U3
DCA10/156						+													
DCA10/158	+		+		+				+	+		+		+	+		+	+	+
DCA10/160						+													
DCA10/162				+	+							+				+			
DCA10/166											+								
DCA10/176																	+		
DCA10/180	+							+		+				+	+				
DCA10/196		+		+			+	+					+					+	+
DCA10/222		+							+				+			+			
DCA10/224							+												
DCA10/240			+																
DCA10/244											+								
DCA3/232											+	+	+			+			
DCA3/236				+	+			+						+					
DCA3/238		+	+														+		
DCA3/242	+					+	+	+		+	+				+			+	+
DCA3/248		+	+		+		+		+							+	+		
DCA3/252	+			+					+	+		+	+	+	+				+
DCA16/148											+		+		+			+	
DCA16/152	+		+			+	+	+		+				+				+	+
DCA16/158				+	+			+							+				
DCA16/174		+								+				+					

Note: Loci ssrOeUA-DCAno are designated DCAno

patterns generated by primer pair ssrOeUA-DCA3 in 14 olive cultivars are shown in Fig. 1. Based on previous results (*22*), primer pairs will be referred to as loci and DNA bands as alleles. All fourteen microsatellite markers were polymorphic, revealing a total of 96 alleles with an average number of 6.8 alleles per locus in the nineteen cultivars examined. At most loci, except for ssrOeUA-DCA11 and ssrOeUA-DCA13, at least 63 % of the varieties were heterozygous (Table 1). Among 96 alleles detected, twenty-five were specific to different olive varieties. One specific allele was detected in varieties 'Leccino', 'Maurino', 'Buga' and 'Picholine', two in varieties 'Unknown 2', 'Itrana', and 'Nocellara del Belice'. Three specific alleles were characteristic of 'Istrska belica' and the highest number of variety specific alleles was found in 'Arbequina' (five) and 'Cipressino' (seven).

The allelic polymorphisms allowed the discrimination of all analysed cultivars. A minimum number of

Table 3. Identification of five local varieties by two microsatellite markers: ssrOeUA-DCA10 and ssrOeUA-DCA3

Locus/allele	**Istrska belica**	**Buga**	**Črnica**	**Štorta**	**Unkn. 2**
DCA10/158	–	+	–	+	+
DCA10/162	–	–	+	+	+
DCA10/196	**+**	–	+	–	–
DCA10/222	**+**	–	–	–	–
DCA10/240	–	+	–	–	–
DCA3/232	–	–	–	–	+
DCA3/236	–	–	+	+	–
DCA3/238	+	+	–	–	–
DCA3/248	+	+	–	+	–
DCA3/252	–	–	–	–	+

Note: Loci ssrOeUA-DCAno are designated DCAno

three microsatellite markers was chosen for rapid varietal identification of 19 olive varieties. Specific allele profiles at locus ssrOeUA-DCA10 were first assigned to ten varieties: 'Leccino', 'Buga', 'Črnica', 'Cipressino', 'Nocellara del Belice', 'Frantoio', Santa Caterina', 'Arbequina', Ascolana Tenera' and 'Itrana', the next seven varieties, 'Istrska belica', 'Štorta', 'Unknown 2', 'Picholine', 'Maurino', 'Leccione' and 'Unknown 3', were differentiated by ssrOeUA-DCA3, and the remaining two varieties, 'Pendolino' and 'Unknown 1', were additionally genotyped by ssrOeUA-DCA16. The identification key for the 19 olive varieties is presented in Table 2. All local varieties were identified with only two markers, ssrOeUA-DCA10, ssrOeUA-DCA3 (Table 3).

The number of observed genotypes per locus ranged from 3 (ssrOeUA-DCA13) to 14 (ssrOeUA-

Table 4. Genotypes of the olive varieties at fourteen microsatellite loci (allele sizes in bp)

Cultivar	**DCA1**	DCA3	**DCA4**	**DCA5**	**DCA7**	**DCA8**	**DCA9**
Leccino	208:258	242:252	132:134	199:207	145:151	139:141	163:207
Istrska belica	208:216	238:248	134:186	195:207	135:153	135:141	191:195
Buga	208:208	238:248	134:164	207:209	135:151	129:141	195:207
Črnica	208:268	236:252	132:164	199:207	145:151	129:145	183:195
Štorta	208:208	236:248	132:164	207:207	145:151	141:145	195:205
Cipressino	208:208	240:242	134:164	203:207	153:169	141:143	163:187
Nocellara del B.	208:216	242:248	186:186	207:207	169:169	141:143	163:173
Frantoio	208:268	236:242	132:134	199:207	145:151	139:145	183:207
Santa Caterina	208:208	248:252	164:164	207:207	135:169	141:141	163:195
Pendolino	208:268	242:252	132:132	207:207	145:151	139:141	163:207
Arbequina	208:218	232:242	134:162	203:207	149:149	141:143	185:207
Unknown2	208:208	232:252	132:152	207:209	151:169	139:141	163:205
Picholine	208:268	232:252	162:162	203:207	153:169	141:143	193:195
Maurino	216:268	236:252	132:134	207:207	151:153	145:151	205:207
Unknown1	208:208	242:252	132:134	207:207	145:151	141:145	163:207
Ascolana Tenera	208:208	232:248	134:164	207:209	169:169	139:141	195:207
Itrana	208:218	238:248	132:166	199:207	135:151	141:145	183:195
Leccione	216:268	242:242	132:132	207:207	153:169	141:145	163:183
Unknown3	208:268	242:252	132:132	199:207	145:151	141:145	163:183
Cultivar	**DCA10**	**DCA11**	**DCA13**	**DCA14**	**DCA15**	**DCA16**	**DCA17**
Leccino	158:180	135:135	120:120	181:183	260:270	152:176	109:119
Istrska belica	196:222	143:163	120:120	181:187	260:270	128:174	115:115
Buga	158:240	143:143	120:120	189:191	250:250	152:176	115:117
Črnica	162:196	135:143	120:120	183:191	250:250	158:176	119:179
Štorta	158:162	143:143	120:120	181:191	250:250	128:158	117:179
Cipressino	156:160	131:143	120:126	173:183	250:270	152:166	117:203
Nocellara del B.	196:224	147:147	124:140	179:191	250:250	152:176	117:145
Frantoio	180:196	135:135	120:120	183:191	250:270	152:158	119:145
Santa Caterina	158:222	163:163	124:140	NA*	250:270	128:176	117:117
Pendolino	158:180	143:143	120:120	173:191	260:270	152:174	109:145
Arbequina	166:244	143:143	120:126	189:191	250:270	124:148	115:181
Unknown2	158:162	143:143	120:120	189:191	250:270	128:156	117:173
Picholine	196:222	135:135	120:120	189:191	NA*	148:176	107:117
Maurino	158:180	135:143	120:120	173:183	260:270	152:174	109:145
Unknown1	158:180	143:143	120:120	173:191	250:260	148:158	109:119
Ascolana Tenera	162:222	163:163	120:120	181:191	250:270	128:156	115:117
Itrana	158:176	147:147	120:126	179:191	250:250	124:128	117:117
Leccione	158:196	135:135	120:120	181:191	250:270	148:152	117:145
Unknown3	158:196	135:143	120:120	173:183	250:260	152:176	109:145

* Not amplified

Note: Loci ssrOeUA-DCAno are designated DCAno

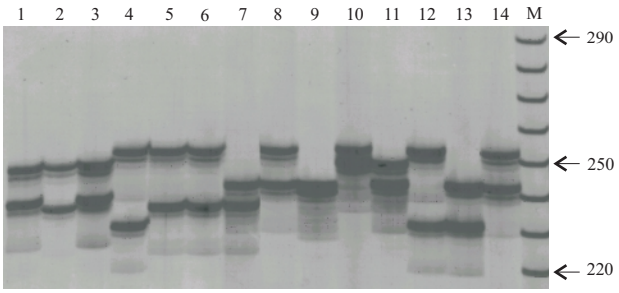

Fig. 1. Polymorphism of fourteen olive cultivars at locus ssrOeUA-DCA3: 1-Istrska belica, 2-Štorta, 3-Buga, 4-Unknown 2, 5-Črnica, 6-Maurino, 7-Frantoio, 8-Pendolino, 9-Cipressino, 10-Santa Caterina, 11-Nocellara del Belice, 12-Picholine, 13-Arbequina, 14-Unknown 1, M- size marker

-DCA16), with a total of 127 different genotypes, revealing 30.5 % of all possible genotypes (417). The genotypes of analysed cultivars are presented in Table 4. Genotype frequencies were relatively low, except for genotype [AA(120:120)] at locus ssrOeUA-DCA13 (0.74), which was present in fourteen varieties. Genotypes ssrOeUA-DCA1 [AA(208:208)], ssrOeUA-DCA5 [DD(207:207)], ssrOeUA-DCA7 [BD(145:151)] and ssrOeUA-DCA15 [AC(250:270)] were also relatively frequent, and were characteristic of different sets of cultivars. Altogether sixty-eight unique genotypes were observed, mostly at loci ssrOeUA-DCA3, ssrOeUA--DCA10, ssrOeUA-DCA16. Their distribution is presented in Fig. 2.

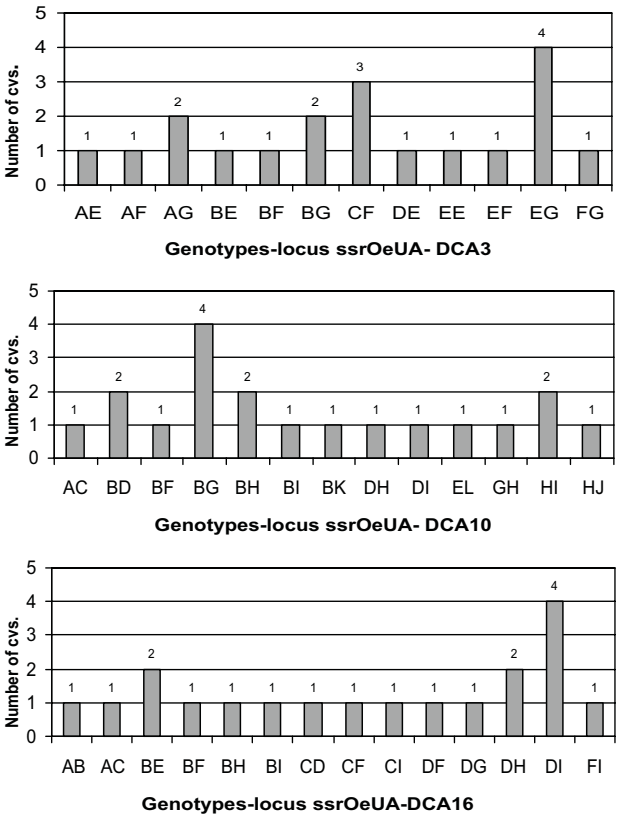

Fig. 2. Number of observed genotypes in nineteen olive varieties at loci ssrOeUA-DCA3, ssrOeUA-DCA10 and ssrOeUA--DCA16; for genotype designation see Table 1

Discussion

Utilisation of fourteen microsatellite markers in the analysis of olive varieties revealed a high level of genetic polymorphism, consistent with the high heterozygosity of the olive tree. The high polymorphism allowed unique genotyping of all analysed varieties and only three markers were sufficient for unambiguous identification of the nineteen olive varieties. In the set of nineteen cultivars, 96 alleles were detected which multiplied into a large number of observed genotypes at each locus, giving high discrimination value for varietal identification. Most of the unique genotypes used for varietal identification were found at loci ssrOeUA-DCA10, ssrOeUA-DCA3 and ssrOeUA-DCA16. There are no reports yet on the application of the microsatellites used in this work in olive varietal genotyping (*22*). Another group (*23*) developed a different set of five microsatellite markers in olives, and reported on their discriminatory value. They were able to identify 95 % of 46 varieties analysed. However, they detected three unique alleles, which is a relatively small number in comparison with our 25 unique alleles and indicates a high informative value of the microsatellite markers used in our study.

The detection method used showed that silver staining is a reliable alternative to other methods for sizing microsatellites, and less expensive than automated fluorescence detection. The allele sizes determined were within the range of 1-2 bp and the degree of error typical of allele size scores based on different methods can be overcome by comparison with reference alleles of specific varieties. Allele sizes differ by multiples of 2 bp, as is to be expected from the dinucleotide repeat motifs of the microsatellites used, and the sizes of our amplified alleles varied in a similar range to that reported (*22*). Our results represent one of the first attempts at detailed olive genotyping by means of microsatellites and thus provide meaningful data that can be enlarged by additional olive varieties and new microsatellites, or used for varietal survey as a powerful discrimination system. Microsatellites are not very demanding technically, and a particularly important advantage is that microsatellite data can be easily compared among laboratories and are suitable for computer databases, which is not always the case with other markers, such as RAPD. These results can be of practical use in olive varietal identification, for verifying the origin of vegetatively propagated plants and of seedling material, or for distinguishing closely related varieties and thus contributing to the knowledge of the olive varietal structure in a region.

Our predominant local variety, 'Istrska belica', was easily distinguished from other locally grown varieties, since it has eight unique genotypes and three specific alleles. Other local varieties showed more similarities with Italian varieties. For example, 'Črnica' shared eight identical genotypes, mostly with varieties from Tuscany. Similarly, 'Buga', 'Štorta' and 'Unknown 2' were identical at several loci to genotypes of Italian olive cultivars. It has already been suggested (*29*) that morphological similarity can be found among Italian and some Istrian

varieties as well as similarities in varietal names. In the authors' opinion, 'Buga' belongs to the population of the Tuscan variety 'Frantoio'. Our results also showed a degree of similarity between Tuscan and Slovene varieties, which suggests that the analysed old Slovene olive varieties were probably derived from Italian cultivars, with local selection on a regional level in the past.

In conclusion, the present study confirmed the usefulness of microsatellite markers as a powerful tool for olive varietal identification. The data obtained can be used for varietal survey and the construction of a database of all olive varieties grown in Slovenia, providing also additional genetic information on the agronomic and quality characteristics of olive varieties.

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Određivanje DNA otiska različitih sorta masline pomoću mikrosatelitskih markera

Sažetak

U različitim genetičkim istraživanjima biljaka sve se više koriste mikrosateliti kao najpogodniji molekularni marker. U četrnaest analiziranih lokusa otkriveno je ukupno 96 alela, što omogućava vrlo veliku razlikovnost pri identifikaciji pojedinih sorta. Tipiziranjem genoma različitih sorta masline pomoću mikrosatelita dobivene su vrlo korisne informacije koje se mogu dopuniti uporabom dodatnih markera. Tom se metodologijom mogu karakterizirati i nove sorte maslina, a i obavljati pouzdane međulaboratorijske usporedbe rezultata. Dobiveni podaci mogu se koristiti pri izradbi baze podataka za identifikaciju pojedinih sorta i njihovu rasprostranjenost na području Slovenije. Oni također omogućavaju da se agronomske značajke i kakvoća različitih vrsta maslina dopune dodatnim genetičkim informacijama.