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A Multidisciplinary Approach to the Characterisation of Autochthonous Istrian Olive (*Olea europaea* L.) Varieties

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Summary

The Istrian region (Croatia) has a long olive growing and oil producing tradition as well as evident biological diversity in local olive (*Olea europaea* L.) germplasm. The olive oil is one of the most important typical food products in Istria. Considering the current tendency of consumers to select typical regional products, there is a need to define Istrian autochthonous olive varieties and to characterize the specificity of related oils. The aim of this study is to apply a multidisciplinary approach for that purpose. Morphological and molecular descriptions of four varieties (Buža, Buža puntoža, Istarska bjelica and Rosinjola) as well as the results of chemical analyses of their oils are reported. A total of 23 morphological traits, microsatellite profiles on 12 SSR loci, extractability index, olive oil minor compounds, colour and antioxidant activity have been determined and the results are reported in the following paper.

Key words: Olea europaea L., autochthonous varieties, morphology, simple sequence repeats (SSR), monovarietal olive oil

Introduction

Olive (*Olea europaea* L.) is one of the most ancient plants characteristic of the Mediterranean area, nowadays cultivated mostly for obtaining the highly valued oil associated with the benefits of the Mediterranean diet. The olive oil market has recently improved, especially since the consumers pay more attention to both health and nutritional aspects of food. Current tendency of consumers is to select typical products with the specificity of origin. The product such as virgin olive oil obtained from autochthonous olive varieties, with known sensorial, nutritional and health promoting characteristics, is highly valued by the consumers and used for virgin olive oil brand management by the producers. Since olive oil is one of the most important typical food products in Istria (Croatia), there is a need to define and characterize the specificity of olive oils obtained from Istrian autochthonous olive varieties. Despite a long olive growing and oil producing tradition in Istria as well as evident biological diversity in local olive germplasm, the knowledge about their origin, selection and level of molecular variability is still limited.

The first historical records of olive growing in the Istrian region date to the first century BC (*1*). Favourable geographic position of this large Adriatic peninsula has encouraged its long olive growing tradition and this area marks the north eastern border of olive growing. Surrounded by the sea from three sides and by Učka and Ćićarija mountains on the north-east, Istria is a specific olive growing area. Olive trees are spread all along the peninsula (*2*), mostly in the coastal zone, while the distribution of olive trees in the central region of Istria is en-

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abled by the river Mirna canyon entering deeply into the continent allowing the influence of the Mediterranean climate (Fig. 1).

According to the latest official statistical data (*3*), a total of 600 000 olive trees are cultivated in Croatian Istria. Even though introduced, Italian varieties still predominate in new plantations, the importance of old autochthonous varieties has recently increased due to their adaptation to local conditions, high oil quality (*4*) and consumers' preference for typical olive oils with a specific origin. Accordingly, the need and validity for the protection of those oils are arising. One of the crucial prerequisites for its realisation is forming the complete database of identified autochthonous olive varieties with respective oil characteristics. The data currently available are not entirely reliable, due to numerous synonyms and homonyms in designations, labelling mistakes, the presence of varietal clones, and the uncertain identification methods thus far applied. Since recently, only nonsystematic morphological description has been used for identification of local varieties in Istria. Until now, Istrian olive varieties and their oil composition profiles have only been partially investigated when compared to other world olive varieties (*5–10*), but they represent the groundwork for further investigations.

Today, standardized morphological methods (*11*) are usually applied for variety description and identification. Molecular techniques are also widely used for precise genetic characterisation, ascertaining origin and elucidating the dispersal route, owning to their reproducibility, reliability and independence from environmental conditions. Microsatellite markers or simple-sequence repeats (SSRs) have proved to be suitable tools for variety characterisation and a number of loci have already been developed for olive (*12–18*). Also, the composition and quantity of certain olive oil compounds could be used for sorting out oils to a single cultivar or specific geographical area (*19*).

Considering fragmented information concerning Istrian autochthonous olive genofond up to date, the multidisciplinary approach is the most appropriate for establishing an integral database.

In the present study, we report on the morphological and molecular characterisation of four autochthonous Istrian olive varieties: Buža, Buža puntoža, Istarska bjelica and Rosinjola, as well as on some chemical parameters of their oils. To our knowledge, this is the first multidisciplinary approach to characterisation of autochthonous olive germplasm in Istria (Croatia).

Materials and Methods

Plant material

Morphological characterisation was performed on 24 olive trees chosen after accurate field observations in the whole Istrian area as representative of true varieties. Four analyzed varieties (Buža, Buža puntoža, Istarska bjelica and Rosinjola) were represented by nine, six, five and four trees, respectively. Four samples of introduced varieties (Ascolana Tenera, Frantoio, Itrana and Leccino) were obtained from the Institute of Agriculture and Tourism Collection in Poreč, Croatia and included in the study as reference varieties for molecular analyses.

Fig. 1. Distribution of olive trees in Istria (Croatia). Brindled area corresponds to the olive growing territory

For olive oil production, the fruits from three trees of each variety (Buža, Buža puntoža, Istarska bjelica and Rosinjola as representatives of Istrian autochthonous varieties, and Leccino as a reference variety) were obtained from the Institute of Agriculture and Tourism Collection in Poreč, Croatia. Olive trees were grown in the same pedoclimatic conditions and cultivated with the same agrotechnical treatments. From each tree, 10 kg of healthy olive fruits were harvested during November and December 2005, and at the same stage of ripening (70 % of olives just turned dark-coloured and the remaining were still green), except for Istarska bjelica, whose fruits were harvested at an earlier stage of ripening (light green to yellow). This is a late ripening variety, and its harvest traditionally starts when fruits are still not dark-coloured.

Morphological characterisation

Morphological description was performed according to the International Olive Council standards (*11*). In total, 23 characteristics of leaf (2), inflorescence (2), fruit (9) and stones (10) were measured during three years (2003–2005) (Table 1).

DNA extraction and microsatellite genotyping

Total genomic DNA was extracted from young leaflets, following a published procedure (*20*).

Twelve SSR loci were analysed: ssrOeUA-DCA3, ssrOeUA-DCA4, ssrOeUA-DCA7, ssrOeUA-DCA8, ssrOeUA-DCA9, ssrOeUA-DCA11, ssrOeUA-DCA16, ssrOeUA-DCA17, ssrOeUA-DCA18 (*13*), UDO099-019, UDO099-039 and UDO099-043 (*15*). The analyses were performed according to the published procedures (*13*,*15*).

PCR products were checked by agarose-gel electrophoresis and then separated on 5 % denaturing polyacrylamide gels, stained with silver and documented by digital images and photographs. Fragment lengths were determined by comparison with a 10-bp DNA Ladder (Gibco BRL), sequencing reactions of the pGEM-3Zf (+) vector (Promega) and with alleles from reference cultivars.

Olive oil chemical analyses

One year of observation was considered for chemical analyses. Olive fruits were collected separately from each tree, crushed with a hammer mill and malaxed for 30 min. The mixture of oil and vegetable water (oil must) was extracted by pressing the olive paste and the olive oil was further separated in a laboratory centrifuge and filtered through filter paper. The oils obtained were stored at 4 °C in filled up and sealed dark-coloured glass bottles until further analyses.

The total phenols were extracted following the procedure of Gutfinger (*21*) and determined according to the Folin-Ciocalteu colourimetric method, while antioxidant activity was measured following the procedure of Brand-Williams *et al.* (*22*).

Chlorophyll and carotenoid mass fractions were determined following the procedure of Mínguez-Mosquera *et al.* (*23*) and expressed as pheophytin a and lutein, respectively.

Olive oil colour is expressed numerically as chromatic ordinates a^*, b^*, the chroma C and lightness L^* according to the method of Escolar *et al.* (*24*).

All the measurements were performed in triplicate. In order to test the significance of variation among the investigated monovarietal olive oils, analysis of variance (ANOVA) and least significant difference (LSD) comparison test were performed.

Olive oil extractability index

Olive oil extractability index (EI), as a parameter for olive variety characterisation, was calculated according to Beltrán *et al.* (*25*), using the formula:

$$\mathrm{EI} = \frac{Vd}{WF} \times 100 \qquad /1/$$

where V (mL) is the olive oil volume extracted, d (0.915 g/mL) is the mean olive oil density, W (g) is the olive paste mass and F (%) is the fruit oil content (fresh mass) measured by Soxtec apparatus (*26*). The EI values are expressed as average of the results obtained from the three trees for each variety.

Results and Discussion

Four autochthonous Istrian olive varieties presenting leading Istrian olive assortment were analysed and systematically described in this paper. In the view of a multidisciplinary approach, morphological and molecular variety characterisations as well as chemical analysis of monovarietal oils were conducted.

Variety Buža is one of the most widespread local varieties in Istria (*27*), suitable as a table and oil variety. Its name comes from a dialectal word 'bugio', which means 'hole', because the holes or cavities can be found at the bottom of the trunk. Buža is sensitive to adverse weather conditions during flowering and thereby characterised with an inconstant and alternate bearing (*1*). Variety Buža puntoža yields constantly and more abundantly than Buža and the reason for this can be found in less susceptibility to adverse weather conditions during the flowering period. Istarska bjelica ripens late and is characterised by a good and constant productivity with high mass fraction of oil (*26*). Variety Rosinjola yields constantly and is suitable for oil production. It is resistant to salinity and thrives well in terra rossa, a type of red clay soil, on the calcium rock, characteristic for Istrian region.

Morphological evaluation

Olive samples collected from the whole Istrian area exhibited morphological differences in 13 from 23 analysed characteristics (Table 1). Most of the chosen characteristics are suitable for discriminating between varieties. Some characteristics like inflorescence length, flower number, fruit, stone mass, *etc.* can vary due to exogenous factors (environment, cultivation technology, *etc.*). In the case of uncertainty in category defining, measuring has been repeated on the larger sample and prevailing category was taken into consideration. Variety Istarska bjelica was easily distinguished from other locally grown varieties, because of its specific helicoid

Table 1. Morphological characteristics of four autochthonous Istrian cultivars according to the International Olive Council standards (*11*)

Characteristics	Variety			
	Buža	Buža puntoža	Istarska bjelica	Rosinjola
Leaf: shape	elliptic-lanceolate (2)	elliptic-lanceolate (2)	elliptic-lanceolate (2)	elliptic (1)
Leaf: longitudinal curvature of the blade	flat (2)	flat (2)	helicoid (4)	flat (2)
Inflorescence: length	short (1)	medium (2)	short (1)	medium (2)
Inflorescence: number of flowers	low (1)	low (1)	low (1)	low (1)
Fruit: shape	spherical (1)	ovoid (2)	spherical (1)	ovoid (2)
Fruit: symmetry	symmetric (1)	symmetric (1)	symmetric (1)	symmetric (1)
Fruit: position of maximum transverse diameter	central (2)	central (2)	central (2)	central (2)
Fruit: apex	rounded (2)	pointed (1)	rounded (2)	rounded (2)
Fruit: base	truncated (1)	truncated (1)	truncated (1)	truncated (1)
Fruit: nipple	absent (1)	tenuous (2)	absent (1)	absent (1)
Fruit: presence of lenticels	many (2)	many (2)	many (2)	few (1)
Fruit: size of lenticels	small (1)	small (1)	small (1)	small (1)
Fruit: location of start of colour change	uniformly across the whole epidermis (2)	from the apex (3)	uniformly across the whole epidermis (2)	from the apex (3)
Endocarp: shape	ovoid (2)	elliptic (3)	ovoid (2)	ovoid (2)
Endocarp: symmetry (position A)	slightly asymmetric (2)	symmetric (1)	symmetric (1)	symmetric (1)
Endocarp: symmetry (position B)	symmetric (1)	symmetric (1)	symmetric (1)	symmetric (1)
Endocarp: position of maximum transverse diameter	central (2)	central (2)	central (2)	towards apex (3)
Endocarp: apex	rounded (2)	pointed (1)	rounded (2)	rounded (2)
Endocarp: base	rounded (3)	rounded (3)	rounded (3)	pointed (2)
Endocarp: surface	rugose (2)	scabrous (3)	scabrous (3)	rugose (2)
Endocarp: number of grooves	medium (2)	medium (2)	medium (2)	medium (2)
Endocarp: distribution of grooves	regular (1)	regular (1)	regular (1)	regular (1)
Endocarp: termination of apex	with mucro (2)	with mucro (2)	with mucro (2)	with mucro (2)

Numbers in brackets represent the category code of each characteristic

elliptic-lanceolate leaves (Fig. 2a). Recognizable morphological feature of variety Buža puntoža was a pointed fruit shape with a nipple on the apex (Fig. 2b), wherefrom its name originates (expression 'puntoža' means 'pointed' in local dialect).

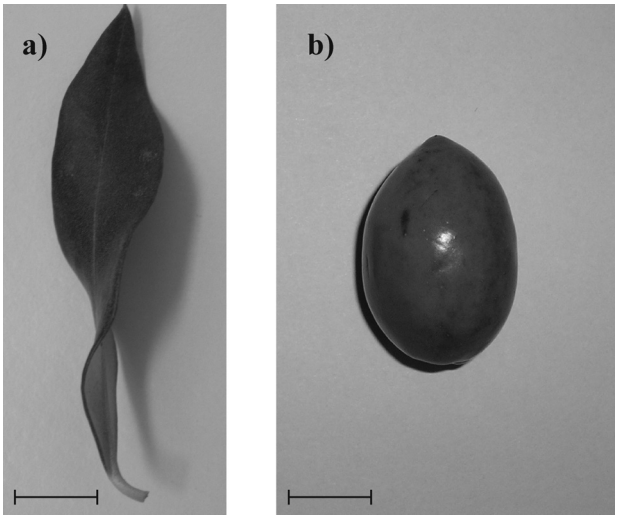

Fig. 2. Recognizable morphological features of some autochthonous varieties: (a) helicoid elliptic-lanceolate leaf of Istarska bjelica, (b) pointed fruit shape of Buža puntoža. Bar=1 cm

Molecular evaluation

DNA fingerprinting of four chosen olive varieties using microsatellite markers was performed in the framework of a wider study (*10*) carried out with the aim of clarifying the genetic relationships of varieties native to Croatian Istria with introduced olive varieties, as well as with varieties in the neighbouring Slovene Istria region.

Amplification was successful with all twelve SSR markers assayed. The choice of microsatellite markers used and protocol optimizations was done based on previous experience and published data (*28*,*29*). To avoid variation in allele sizing, reference genotypes with specific alleles for each locus were used. The most discriminatory set of markers for olive varieties in this region and reference varieties selected earlier (*28*) were chosen for genotyping. Polymorphisms of markers DCA3 and DCA16 were sufficient for discriminating all the examined varieties (*10*). PCR products were separated by standard polyacrylamide sequencing gels and stained with silver, giving a required resolution of 1 bp, between fragments (Fig. 3).

Here, we present for the first time the SSR profiles of four local varieties, together with profiles of four referent varieties (Table 2). All twelve markers used were polymorphic, revealing a total of 71 alleles ranging from four at locus UDO19 to eight alleles at loci DCA9 and DCA16, with an average number of 5.91 alleles per locus in the

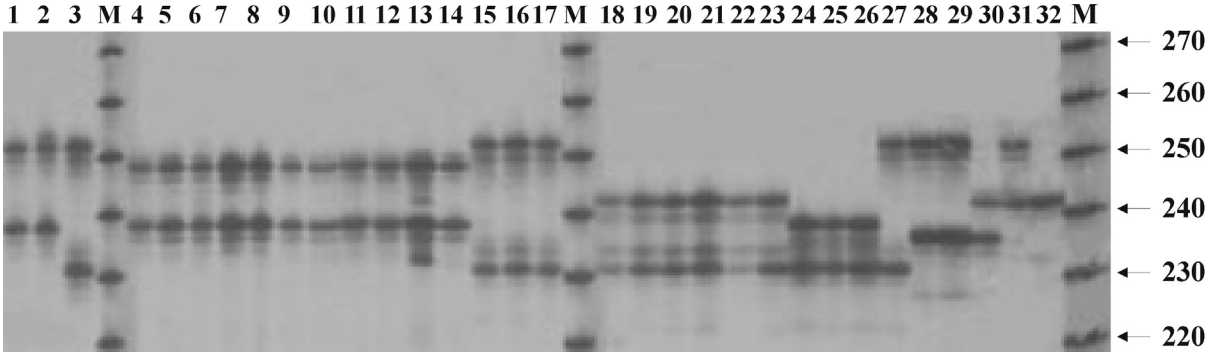

Fig. 3. Microsatellite alleles of DCA3 locus in 11 olive varieties: 1, 2 – Buža; 3 – Buža puntoža; 4–11 – Istarska bjelica; 12–14 – Itrana; 15–17 – Buža puntoža; 18–23 – Rosinjola; 24–26 – Bilica; 27 – Picholine; 28,29 – Črnica; 30 – Frantoio; 31 – Leccino; 32 – Leccione; M – size marker (30–330 bp)

Table 2. Genotypes of four autochthonous and four referent olive varieties at twelve microsatellite loci (allele sizes in bp)

Cultivar	Locus											
	DCA3	DCA4	DCA7	DCA8	DCA9	DCA11	DCA16	DCA17	DCA18	UDO19	UDO39	UDO43
Buža	238:252	132:132	135:151	129:141	195:205	143:163	152:176	117:179	174:178	133:133	178:178	179:179
Buža puntoža	232:252	134:164	135:151	129:141	163:205	143:183	152:176	115:117	174:178	133:147	178:178	179:215
Istarska bjelica	238:248	134:186	135:153	135:141	193:195	143:163	128:174	115:115	174:178	103:133	178:190	177:217
Rosinjola	232:242	134:166	153:153	129:139	173:205	135:143	146:156	129:179	159:178	133:147	178:180	179:221
Leccino	242:252	132:134	145:151	139:141	163:207	135:183	152:176	109:119	178:178	103:169	172:176	215:219
Ascolana Tenera	232:248	134:164	169:169	139:141	195:209	163:179	128:156	115:117	174:178	133:133	178:178	179:213
Frantoio	236:242	132:134	145:151	139:145	183:207	135:183	152:158	119:145	178:180	133:169	172:172	179:219
Itrana	238:248	132:164	135:151	141:145	183:195	147:183	126:128	115:117	174:182	133:169	NA	177:179

NA=not amplified

eight examined varieties. At all loci, except for UDO39, at least 75 % of the varieties were heterozygous.

Reported molecular data represent one of the first attempts at detailed genotyping of local olive germplasm by means of microsatellites.

Chemical evaluation

Minor compounds (chlorophyll, carotenoids, phenols), colour and antioxidant activity of monovarietal virgin olive oils obtained from four autochthonous and one introduced variety (Leccino) were determined.

Influence of variety on total phenolic mass fraction, antioxidant activity, and chlorophyll and carotenoid mass fractions can be observed in Table 3. Considering the total phenolic mass fraction, Rosinjola emerged as a variety with the highest measured amount ($p \leq 0.05$), even higher than Istarska bjelica, which is known as a variety with high total phenolic mass fraction (*5*,*7*,*30*). Statistically significant differences in total phenolic mass fraction and in antioxidant activity among the investigated varieties were found. The correlation of total phenolic mass fraction and antioxidant activity was positive and highly significant (R^2=0.8564).

Monovarietal olive oil samples included in this investigation were processed by the same extraction procedure and in the same stage of ripeness to avoid variability caused by those factors (*31*–*33*) except for Istarska bjelica variety, which is known to be a variety that reaches dark colouration very late and it is usually harvested at an earlier ripening stage (light green to yellow). Therefore, the highest amounts of chlorophyll and carotenoid pigments were observed in Istarska bjelica oils, which are also distinguished as statistically different from all the other oil samples at $p \leq 0.05$. Generally, no statistically significant differences were found between other oil samples, probably because the influence of the stage of ripeness on the chlorophyll and carotenoid pigment content is greater than the influence of the variety.

The colour of olive oil samples was evaluated from the chromatic coordinates a^* corresponding to the green zone, b^* corresponding to the yellow zone, lightness, L^*, and chroma, C, of the absorption spectrum (Table 3).

Variety Istarska bjelica showed statistically significant differences from the other investigated varieties considering the measured colour parameters. It had the highest a^*, b^* and C values in a similar way to the values observed for chlorophyll and carotenoid mass fractions, while the luminosity of Istarska bjelica oil correlated negatively with the pigment mass fraction. This is probably the consequence of the harvest at an earlier stage of ripeness compared to the other investigated varieties. It is known that with increased ripening, the values of these ordinates decrease similarly to those of pigment mass fraction, which is in agreement with the loss of colour intensity in the corresponding oils (*23*).

Since only one year of observation of olive oil samples was considered for chemical analyses, the reported results are indicative, but a more complete database of chemical characteristics based on several years of observation is in course of setting up.

Extractability index

In our previous work on the same varieties, the theoretical oil mass fraction during two consecutive harvests, 2004 and 2005, was determined (*26*). This investigation showed that fruits from Istarska bjelica variety had the highest mass fraction of oil (44.75–48.45 % oil on dry mass basis), while Leccino and Buža puntoža fruits had the lowest. The oil mass fraction is not completely reliable as a variety characteristic, since it also depends on the growing area and climatic conditions, ripening degree, cultural practices and other minor factors. However, if the influence of these factors is minimised, the oil content monitored in a single year reflects mostly the influence of the variety. These data were used for the estimation of extractability index, which is considered as a parameter for olive variety characterisation (*25*). This parameter is influenced by olive fruit characteristics, extraction conditions and variety, and also shows important changes during fruit ripening. The extractability index determined for each investigated variety ranged from 0.40 (Rosinjola) to 0.56 (Istarska bjelica), which is in agreement with EI values of leading Spanish cultivars (*25*).

Table 3. Total phenolic mass fraction, antioxidant activity, chlorophyll and carotenoid mass fractions, and the colour (expressed as chromatic coordinates a^*, b^*, lightness L^* and chroma C) of virgin olive oils obtained from four Istrian autochthonous varieties and one introduced (Leccino)

Variety*	w(total phenolics as caffeic acid) / mg/kg	Antioxidant activity as Trolox equivalent / mmol/kg	w(chlorophyll as pheophytin a) / mg/kg	w(carotenoids as lutein) / mg/kg	a^*	b^*	L^*	C
Istarska bjelica	206.9±63.0^{b}	1.32±0.33^{b}	3.64±0.32^{a}	2.39±0.21^{a}	–11.11±0.27^{d}	104.81±4.33^{a}	92.50±0.17^{c}	106.64±4.38^{a}
Rosinjola	347.1±15.2^{a}	1.98±0.10^{a}	2.39±0.30^{b}	1.51±0.16^{b}	–12.50±0.04^{bc}	78.59±1.04^{bc}	94.78±0.08^{b}	80.19±1.05^{bc}
Buža puntoža	77.8±5.7^{cd}	2.00±1.42^{bc}	1.67±0.01^{bc}	1.26±0.23^{b}	–12.89±0.23^{ab}	67.03±7.21^{cd}	96.47±0.61^{a}	68.57±7.24^{cd}
Buža	124.6±30.4^{c}	1.27±0.08^{b}	1.26±0.88^{c}	0.96±0.51^{b}	–13.22±0.38^{a}	56.69±12.22^{d}	97.03±0.93^{a}	58.19±12.26^{d}
Leccino	35.2±10.3^{d}	0.96±0.03^{c}	1.72±0.39^{bc}	1.40±0.11^{b}	–12.27±0.04^{c}	83.76±1.02^{b}	94.90±0.31^{b}	85.39±1.03^{b}

Significant differences between varieties are presented with different superscript ($p \leq 0.05$)
*Each variety is represented with the mean±standard deviation of the results of the parameters measured for the olive oil samples (harvest 2005)

Conclusions

A multidisciplinary approach used in this work, combining genetic methods and morphological measurements supplemented with chemical analyses, represents the starting point for further systematic description of Istrian autochthonous olive varieties and respective oils. In order to obtain a comprehensive database with accurate variety descriptions and defined composition profiles of respective oils, further systematic sampling and analyses are needed. Information gathered in such database could be used for protecting and controlling olive oils with declared nutritional and sensory characteristics, and geographical and varietal origin, recognisable on the global market.

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