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Intra-varietal variability and genetic relationships among the homonymic East Adriatic olive (*Olea europaea* L.) varieties

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ABSTRACT

The intensive exchange and diffusion of plant material over the whole Mediterranean Basin including the East Adriatic region, has contributed to the confusion in denomination of olive varieties, and formation of a large number of synonyms and homonyms. In the present study, we report on clonal diversity and genetic relationships among a total of 190 olive trees sampled along the East Adriatic coast in Slovenia (SVN), Croatia (HRV) and Montenegro (MNE). Samples belonged to the six East Adriatic olive varieties with homonymic denomination: 'Istrska belica' (SVN), 'Bjelica' (HRV), 'Žutica' (MNE), 'Črnica' (SVN), 'Crnica' (HRV), and 'Crnica' (MNE). Results showed polymorphism in all twelve microsatellite markers (DCA3, DCA9, DCA11, DCA14, DCA16, DCA18, EMO03, EMO90, GAPU101, GAPU103A, GAPU71B and UDO99-019), revealing a total of 83 alleles. A total of 36 different genotypes in the panel of 190 samples were found. Genetic profiling of six homonymic East Adriatic olive varieties revealed four different varieties: 1) 'Istrska belica' (SVN), 2) 'Bjelica' (HRV)/'Žutica' (MNE), 3) 'Crnica' (HRV)/'Crnica' (MNE) and 4) 'Črnica' (SVN). Detailed clonal and genetic relationship analyses demonstrated genetic uniformity of 'Istrska belica' (SVN), negligible low diversity of 'Črnica' (SVN), and moderate diversity of 'Crnica' (HRV)/'Crnica' (MNE). Polyclonal property was assigned to only 'Bjelica' (HRV)/'Žutica' (MNE) variety. High genetic similarity between 'Bjelica' (HRV)/'Žutica' (MNE), and 'Crnica' (HRV)/'Crnica' (MNE), with different distribution of clones within them, reflects the past selection and propagation processes in Croatia and Montenegro. For the first time, international synonymy of 'Žutica' (MNE) and 'Bjelica' (HRV), and homonymy of 'Istrska belica' (SVN) and 'Bjelica' (HRV)/'Žutica' (MNE), as well as 'Črnica' (SVN) and 'Crnica' (MNE, HRV) were reliably confirmed with DNA markers.

1. Introduction

Olive genetic patrimony is characterized by a large number of varieties. There are more than 1250 olive varieties under cultivation (Bartolini, 2008), which originated from selections made by growers over many centuries (Rugini and Baldoni, 2005). The intensive exchange and diffusion of plant material over the whole Mediterranean Basin and introduction of olives in continents with similar climate conditions, contributed to the confusion in denomination of olive varieties, and formation of a large number of synonyms and homonyms. Numerous studies by DNA marker systems have been conducted to overcome olive denomination problems (Belaj et al., 2016; Bracci et al., 2011), and some studies have demonstrated that erroneous denominations occur more frequently among local germplasm than in common varieties (Rao et al., 2009). Regionally selected varieties represent the base for production of locally typical olive oils and their maintenance and research is of great importance for adaptation of olive cultivation to changing ecological conditions. Due to traditional cultivation, with distinct selection and propagation processes, intra-varietal diversity is often reported for these varieties. Characterization of the main olive varieties in olive producing countries has been widely reported, while for most of the traditional and local varieties information is still unavailable (Baldoni and Belaj, 2009; Belaj et al., 2016).

In the East Adriatic region, olive growing represents a significant

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source of agricultural income for local communities, and is an important constituent of the healthy Mediterranean diet. Olive cultivation extends along the East Adriatic coast and nearby islands (2137 km long, referring to the coasts of Slovenia, Croatia, Bosnia and Hercegovina and Montenegro) where numerous varieties have developed during the last thousands of years. Over the centuries, different civilizations and administrations have dominated throughout this area - Phoenicians, Greeks, and Romans in ancient times, followed by the rule of the Republic of Venice, Ottoman Empire, and Austro-Hungarian Empire until the end of World War I, which had very significant impacts on olive growing, including varietal structure. Croatia, Montenegro and Slovenia (among others) were part of the common country, Yugoslavia, which disintegrated in the 1990s. In Slovenia, Croatia and Montenegro, a total of 24,368 ha of olives are planted, comprising 18,500 ha in Croatia (EUROSTAT, 2017), 2068 ha in Slovenia (MKGP, 2018) and 3200 ha in Montenegro (Lazović and Adakalić, 2012) and the annual production in these three countries is estimated to be 43,408 tons of olives (FAOSTAT, 2017). According to surveys based on morphological and molecular evaluation, numerous olive varieties are still under cultivation in the East Adriatic region (Bakarić, 2002; Bandelj et al., 2004; Lazović, 2001; Miranović, 2006; Strikić et al., 2010).

The northernmost part of the region is Slovenian and Croatian Istria where, until the frost of 1956, the traditional local variety 'Črnica' (Slovenian 'črno' = Croatian/Montenegrin 'crno' = black; named according to the black colour of the mature fruit) was the most common (Hugues, 1999), whereas nowadays it can be found only in abandoned olive groves and overgrown terraces. In the southernmost part of Croatia, in the area of Konavle, the homonymous variety 'Crnica' has been cultivated through the centuries and is considered to be a Croatian autochthonous variety used for oil production (Bakarić, 2002). This variety occupies 44 ha and represents 8.5% of total olive orchards in southern Croatia. Crossing the border, in the north of Montenegro, the homonymous variety 'Crnica' is found in the area of Boka Kotorska where it constitutes 29% (Miranović, 1978) and in Budva and Bar subarea with about 15% of total olive trees.

Interestingly, varieties whose names mean the opposite of black (i.e. white or yellowish), are the most widespread varieties in Slovenia, in the northernmost and southernmost part of Croatia and in Montenegro. Thus, the most common variety in the Slovenian and Croatian peninsula of Istria is 'Istrska belica' (Slovenian) or 'Istarska bjelica' (Croatian) (Slovenian 'belo' = Croatian 'bijelo' = white) (Bandelj et al., 2012). Similarly, in the south of the East Adriatic region (southernmost Croatia and Montenegro) the cultivation of two olive varieties overlaps. The first is 'Bjelica' (named according to the whitish colour of the leaf backside), a variety in the south of Croatia representing 18% of olive trees in the area of Dubrovnik (Bakarić, 2002). 'Bjelica' is used as a synonym for variety 'Žutica' (Croatian/Montenegrin 'žuto' = yellow), the name that corresponds to the Montenegrin principal variety, characterized with a yellow colour of the fruits before maturation. This variety in Montenegro occupies about 65%, while in some southern regions it is almost monovarietal with 95–98% of 'Žutica' trees.

Nowadays, molecular markers are routinely used for management of plant genetic resources and are a particularly efficient tool for identifying varieties and clones of cultivated plants. Among the available DNA marker systems, microsatellites combine several properties of an ideal marker such as their highly polymorphic nature and information content, codominance, abundance in the genome, availability, easy and fast assay, amenability to automation, high reproducibility, and easy exchange of data between laboratories (Kumar et al., 2009). Development of microsatellite markers in olives (Carriero et al., 2002; Cipriani et al., 2002; De la Rosa et al., 2002; Gil et al., 2006; Rallo et al., 2000; Sefc et al., 2000) has enabled successful introduction of these markers into numerous olive germplasm characterization studies (Belaj et al., 2016; Bracci et al., 2011; Delgado-Martinez et al., 2012), and core collection development (Belaj et al., 2012; Haouane et al., 2011). Microsatellites proved to be a suitable tool even for differentiating closely related olive varieties and clonal identification within olive varieties (Fernández i Martì et al., 2015; Lopes et al., 2004; Muzzalupo et al., 2010; Muzzalupo et al., 2014; Rony et al., 2009; Rotondi et al., 2003; Trujillo et al., 2014; Zaher et al., 2011).

In the present study, we report on molecular characterization, clonal diversity and genetic relationships among six olive varieties with homonymic denomination from the East Adriatic coast. Molecular evaluation was performed with twelve microsatellite markers. The research of national olive genetic patrimony with DNA markers and implementation of results into international frameworks contribute significantly to our knowledge of the diversity of leading olive varieties in Slovenia, Croatia and Montenegro and provides information on their historical diffusion and propagation in the east Adriatic region.

2. Materials and methods

2.1. Plant material

In total, 190 olive trees sampled along the East Adriatic coast in Slovenia, Croatia and Montenegro were analyzed in this study: 49 samples of variety 'Istrska belica' (Slovenia - SVN), 24 'Bjelica' (Croatia - HRV), 49 'Žutica' (Montenegro - MNE), 24 'Črnica' (SVN), 22 'Crnica' (HRV), and 21 samples of 'Crnica' (MNE). An olive tree from Kaštel Štafalić (HRV), also known as 'Perišićeva mastrinka' (belonging to the Perišić family), was also included in the analysis. The age of this tree is estimated to be about 1500 years, which is considered as an ancient, most likely wild (or feral) example of olive situated in the middle of the research area. All samples were collected based on previous morphological evaluation. Samples were collected in the oldest plantations and from the oldest trees. Collection sites are presented in Fig. 1 and basic descriptive characteristics of varieties and their synonyms are shown in Table 1.

2.2. DNA extraction and microsatellite analysis

Genomic DNA of samples was extracted from fresh olive leaves by a modified CTAB method following the procedure described by Kump and Javornik (1996). Twelve primer pairs from different sets of microsatellites were used for genotyping: DCA3, DCA9, DCA11, DCA14, DCA16, DCA18 (Sefc et al., 2000), EMO03, EMO90 (De la Rosa et al., 2002), GAPU101, GAPU103A, GAPU71B (Carriero et al., 2002), and UDO99-019 (Cipriani et al., 2002). Microsatellites for olive genotyping were chosen on the basis of previously reported genetic characteristics of loci. Nine (DCA3, DCA9, DCA14, DCA16, DCA18, EMO90, GAPU101, GAPU103A, GAPU71B) of the 12 loci were selected from a recommended list of microsatellites for olive genotyping procedures, which was established to make genotyping data comparable among different research groups and to establish an universal database of olive accessions (Baldoni et al., 2009). The other three loci (DCA11, EMO03, UDO99-019) were chosen according to our previous experience with olive genotyping, considering the criteria peak intensity, specific amplification, number of amplified alleles per locus, observed heterozygosity, and polymorphic information content.

One primer of each pair was elongated for M13(-21) 18 bp sequence (5′-TGTAAAACGACGGCCAGT-3′) for economic fluorescent labelling (Schuelke, 2000). All SSR primers were synthesized by Integrated DNA Technologies (IDT), four fluorescently labelled universal M13(-21) primers (6-FAM, VIC, NED and PET) were synthesized by ABI (Applied Biosystems®).

PCR amplifications were carried out in a total volume of 15 µl containing 20 ng of DNA, 1x Flexi buffer GoTaq (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each locus specific primer, 0.25 µM of M13(-21) primer labelled with 6-FAM (DCA9, DCA14, GAPU103A), VIC (DCA16, EMO03, GAPU71B), PET (DCA18, EMO90, GAPU101) or NED (DCA3, DCA11, UDO99-019) (Applied Biosystems®), and 0.375 U of Taq polymerase (Promega). PCR reactions were