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Molecular characterization of olive (*Olea europaea* L.) Sicilian cultivars using SSR markers

Giuseppina Las Casas [1], Francesco Scollo [1], Gaetano Distefano, Alberto Continella, Alessandra Gentile, Stefano La Malfa*

Dipartimento di Scienze delle Produzioni Agrarie e Alimentari, University of Catania, Via Valdisavoia 5, 95123 Catania, Italy

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ABSTRACT

Olive (*Olea europaea* L.) is one of the economically most important fruit crops for the Mediterranean area, with production being mainly destined to oil extraction. In Sicily, olive has been cultivated since ancient times and its germplasm is characterized by a wide genetic diversity that could be related to its domestication and spread in ancient times, and to some reproductive biological peculiarities as self-incompatibility. This analysis was conducted on 65 genotypes with the purpose of characterizing a large collection of Sicilian accessions (47 genotypes) and to compare them with varieties coming from Southern Italy and from the most important countries of the Mediterranean basin. With this aim we used 8 simple sequence repeat (SSR) markers, which detected a total of 74 alleles and identified an average of 19.5 genotypes in the population investigated. A larger variability than expected was found in the analyzed genotypes, some synonymies already reported in literature were confirmed, but also some cultivars considered as identical were discriminated such as in the case of Castriciana, Ogliarola messinese and Passalunara. The whole study revealed a wide intraspecific variability within the gene pool examined, independently from the geographical origin.

1. Introduction

Olive (*Olea europaea* L. subsp. *europaea*) is one of the most economically important and widespread fruit crops of the Mediterranean area and is characterized by a prevalent allogamy and great longevity which have determined high levels of heterozygosis (Rallo et al., 2000) as well as the accumulation of a number of mutations (Lopes et al., 2004; Baali-Cherif and Besnard, 2005).

The lack of replacement of traditional and well-adapted cultivars and its vegetative propagation, gave rise to very rich germplasm with a large number of cultivars present in the main olive oil producing countries (Baldoni and Belaj, 2009).

However, this rich biodiversity often implies difficulties in identification of plant material due to the frequency of synonyms and homonyms (Bartolini, 2007). Among the olive growing countries, Italy has about 600 different accessions, representing a large part of the known world olive germplasm (Bartolini et al., 2005). In Sicily, olive is widely cultivated since ancient times and wild olives are important components of the Mediterranean scrublands (Zohary and Hopf, 1994). Sicily

* Corresponding author.
E-mail address: stefano.lamalfa@unict.it (S. La Malfa).
[1] These authors contributed equally to this work.

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offers a number of favorable growing conditions for olive due to different climatical and edaphic characteristics and therefore a wide genetic patrimony has been accumulated (Bottari and Spina, 1952). Furthermore, the presence of intra-cultivar variation represented by plants with the same phenotype which display small genetic differences has been reported (Lopes et al., 2004; Muzzalupo et al., 2011; Ipek et al., 2012).

Biochemical and molecular markers offer a wide choice of analytical systems to analyze plant germplasm; in olive AFLPs (Angiolillo et al., 2006), SCARs (Hernandez et al., 2001; Bautista et al., 2003), ISSRs (Gomes et al., 2009) and SNPs (Muleo et al., 2009; Hakim et al., 2010) have been used for the characterization. In the last years, SSRs markers, widely used in the genetic identification and discrimination of cultivars, have been isolated from olive germplasm (Sefc et al., 2000; Carriero et al., 2002; Cipriani et al., 2002). Microsatellites represent a powerful tool for varietal identification and have also been used to evaluate inter and intra-cultivar variability (Belaj et al., 2008; Roubos et al., 2010; Ercisli et al., 2011).

In the current report, the genetic polymorphism level and the relationships among 47 Sicilian olive accessions and 18 varieties coming from other Italian regions and Mediterranean countries was investigated by microsatellite markers in order to provide a reliable and unambiguous tool for the characterization of genetic resources especially in germplasm collection farms.

2. Materials and methods

2.1. Plant material and DNA extraction

In this work, 47 Sicilian cultivars were analyzed and compared with 10 of the most representative Italian cultivars and 8 representatives from other Mediterranean countries (Supplementary Table S1). Twenty-four Sicilian cultivars were sampled from the Experimental Farm of Catania University (lat. 37°24′41″N; long. 15°03′15″E) in which most of the accessions described by Bottari and Spina (1952) are conserved. All the other accessions (41) were collected from the Collection Field of Olive Germplasm of "CNR-ISAFOM" and "Provincia Regionale di Enna" (lat. 37°30′52″N; long. 14°17′46″E). Young leaves were sampled, 100 mg of fresh tissue were ground using Qiagen Tissuelyser II (Qiagen, Hilden, Germany) and genomic DNA was extracted using the Plant DNA Mini Kit (Bioline, London, UK). Quantity and quality of extracted DNA were checked by spectrophotometry using a Nanodrop 2000 system (Thermo Scientific, Waltham, Massachusetts, USA).

2.2. SSR analysis

Eight SSR loci, out of ten originally investigated, were selected for the analysis: ssrOeUA-DCA3, ssrOeUA-DCA9, ssrOeUA-DCA16, GAPU59, GAPU101, UDO99-008, UDO99-012, UDO99-024 (Table 1). The choice was made based on the degree of polymorphism, as well as on clearness and reproducibility of amplified DNA fragments. The amplifications were carried out in 15 μl volumes containing about 30 ng genomic DNA, 1X NH_4 Reaction Buffer (Bioline), 0.2 mM dNTPs (Bioline), 1 mM $MgCl_2$, 0.167 nM of forward and reverse primers, 0.13 nM of M13F labeled with a fluorescent dye (6-FAM, NED and PET; MWG Operon, Ebersberg, Germany) and 1 U of Taq Polymerase (Bioline). Amplification was performed in two thermal cyclers (GeneAmp PCR 9700 and 2700 systems from Applied Biosystems, Foster City, USA) with an initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, annealing temperature 53 °C for 30 s and 72 °C for 45 s, and a final extension at 72 °C for 15 min. An aliquot of 0.4–0.6 μl of PCR product (depending on the performance of amplification of each primer pair) was mixed with 13 μl of formamide and 0.3 μl of LIZ-500 size standard and denatured at 95 °C for 5 min. Up to three PCR products labeled with FAM, PET, or NED were pooled before separation in the ABI 310 Genetic Analyzer (Applied Biosystems)

Table 1
Primer sequences, repeat motif, range (bp) of amplified fragments used for olive germplasm evaluation.

Source	Locus	Primer sequence (5–3)	Repeat motif	Size range (bp)
Sefc et al. (2000)	(B) ssrOeUA-DCA3*	F: CCCAAGCGGAGGTGTATATTGTTAC R: TGCTTTTGTCGTGTTTGAGATGTTG	$(GT)_{19}$	227–255
	(Y) ssrOeUA-DCA9*	F: AATCAAAGTCTTCCTTCTCATTTCG R: GATCCTTCCAAAAGTATAACCTCTC	$(GT)_{23}$	162–212
	(B) ssrOeUA-DCA16*	F: TTAGGTGGGATTCTGTAGATGGTTG R: TTTTAGGTGAGTTCATAGAATTAGC	$(GT)_{13}(GA)_{29}$	121–231
Carriero et al. (2002)	(R) GAPU59	F: CCCTGCTTTGGTCTTGCTAA R: CAAAGGTGCACTTTCTCTCG	$(CT)_9$	201–219
	(Y) GAPU101*	F: CATGAAAGGAGGGGGACATA R: GGCACTTGTTGTGCAGATTG	$(GA)_8(GA)_3(AG)_3$	182–219
Cipriani et al. (2002)	(B) UDO99-008	F: AAAAACACAACCCGTGCAAT R: AAATTCCTCCAAGCCGATCT	$(AC)_{13}$	155–169
	(R) UDO99-012	F: TCACCATTCTTAACTTCACACCA R: TCAAGCAATTCCACGCTATG	$(GT)_{10}$	155–167
	(Y) UDO99-024	F: GGATTTATTAAAAGCAAAACATACAAA R: CAATAACAAATGAGCATGATAAGACA	$(CA)_{11}(TA)_2(CA)_4$	165–199

M13F sequence (CACGACGTTGTAAAACGAC) was added at 5 end of forward primers; forward primers fluorescently labeled: *B*, blue dye (6-FAM); *Y*, yellow dye (NED); *R*, red dye (PET).