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Genetic diversity and clonal variation within the main Sicilian olive cultivars based on morphological traits and microsatellite markers

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ABSTRACT

The richness of *Olea europaea* (L.) genetic resources in Sicily is well documented. In the last 30 years, most of the local cultivars, landraces and ecotypes have been gathered together in a large *ex-situ* collection, containing more than 300 genotypes. In this study, 45 putative clones of the main Sicilian olive cultivars were characterized morphologically using microsatellite markers to unambiguously identify possible superior genotypes. The microsatellites employed were polymorphic (observed heterozygosity = 0.71; polymorphic information content = 0.59), discriminated 52% of the genotypes and enabled the detection of intra-cultivar polymorphism, derived from both somatic mutations, indicating the presence of polyclonal cultivars, or from gametic origin, thus suggesting the presence of cultivar-populations. A high level of genetic variability was detected within the 'Biancolilla', 'Giarraffa' and 'Moresca' genotypes, whereas low variation was found within the 'Cerasuola' and 'Tonda Iblea' genotypes. The combination of UPGMA cluster analysis of data obtained from microsatellite analysis, with canonical discriminant analysis (CDA), based on 18 morphological variables, measured under the same conditions, enabled intra-cultivar diversity, attributable to genetic factors rather than to environmental ones to be identified. The goodness of fit between microsatellite profiles and the CDA analysis was significantly supported by the Mantel test ($r = 0.3$; $p < 0.001$). Genotypes and clonal variants with superior traits (larger fruit size; compact tree habit, apt for high density planting; higher oleic acid content) were identified, suitable for enlarging their area of cultivation.

1. Introduction

Olive (*Olea europaea* L.) is one of the most economically important tree species in the Mediterranean basin, cultivated for its fruit and oil. Italy is the world's second largest olive producer after Spain (Faostat, 2013). Among the Italian regions, Sicily has a significant place in the olive and olive oil production, industry and exportation, being the third largest region of production after Apulia and Calabria (ISTAT, 2013).

Olives have been cultivated in Sicily since antiquity; the Phoenicians brought the olive to Sicily in the sixth century BC and the Romans continued the expansion of olive cultivation throughout the Mediterranean by developing grafting techniques (Zohary and Hopf, 1994; Besnard et al., 2001; Rugini et al., 2011). The genetic richness of Sicilian olive germplasm is well documented (Bottari and Spina, 1952; La Mantia et al., 2005; Caruso et al., 2007; Marra et al., 2013; Besnard et al., 2013) and represents a heritage patrimony of economical and scientific value, particularly for breeding programmes. Parentage studies performed by Marra et al. (2013) demonstrated that both somatic mutations and new genotypes derived through sexual crosses created the rich diversification of the Sicilian olive germplasm.

The first historical investigation on indigenous Sicilian heritage olive was conducted in the second half of the XIX century by Caruso (1883). Sicilian farmers probably selected late ripening cultivars, since long, mild and wet autumns allow for olive oil accumulation and complete ripening. It is likely that vigorous and drought resistant trees, with large-sized fruits were selected by local farmers over the centuries.

Since the 1980s, the Department of Agricultural and Forest Sciences (SAF) of Palermo has worked extensively on the characterization and the conservation of the main local olive cultivars and has studied their efficiency in yield and in oil quality (La Mantia et al., 2005; Caruso et al., 2007; Marra et al., 2013), following the first survey conducted in the middle of the XX century by Bottari

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and Spina (1952). In 2002, a large olive germplasm collection was established in Sicily, which today contains eight well-known and extensively grown cultivars, 17 minor or neglected cultivars and 122 native genotypes.

Currently in the island, olive oil production is based mainly on the cultivars 'Biancolilla', 'Cerasuola', 'Moresca', 'Nocellara del Belice', 'Nocellara Etnea', 'Ogliarola Messinese', 'Santagatese' and 'Tonda Iblea' (Caruso et al., 2007). The table olive industry is also significant (8% of total olive production) and based mainly on the cultivar 'Nocellara del Belice' and, to a minor extent, on 'Nocellara Etnea', 'Ogliarola Messinese' and 'Moresca' producing large-sized fruits of high commercial value (Caruso et al., 2007). As most varieties are often known under different names, according to the areas of cultivation, many cases of homonymy and synonymy occur, complicating cultivar identification. However, a certain amount of morphological variability has been observed within each cultivar, suggesting that the name of a single cultivar could encompass a pool of different genotypes (cultivar populations) and/or populations of clones (mixture of clonal variants). Generations of farmers have played a role in selecting, conserving and genetic improving of the Sicilian olive germplasm. Many of the morphological types of each of the main cultivars, identified thanks to reports of millers, growers and nurserymen, have been collected *ex situ* in a regional repository and observed over the years concerning their canopy architecture, time-course of phenological phases, tree crop efficiency, and fruit, leaf, inflorescence and endocarp traits.

It is largely accepted that the olive cultivar discrimination based on morphological descriptions is not completely reliable (Belaj et al., 2001) therefore DNA molecular markers, particularly microsatellites (SSRs; simple sequence repeats), are today widely used (Bracci et al., 2011) to complement morphological analyses and to unambiguously identify the accessions held in collections. Genetic variation has been reported among naturally occurring olive clones in literature with molecular markers. Clones were identified with RAPD and ISSR (Gemas et al., 2004; Gomes et al., 2008; Martins-Lopes et al., 2009), with AFLP (Frane et al., 2010), and microsatellites (Lopes et al., 2004; Muzzalupo et al., 2010; Zaher et al., 2011; Albertini et al., 2011; Ipek et al., 2012). Although currently there is intense research to develop reliable techniques for detecting mutations in genes, clone identification is still predominantly based on the study of phenotypic traits, integrated with molecular analyses.

In this study, intra-cultivar variation of the most widely distributed Sicilian olive cultivars, was identified through morphological and molecular characterization, employing a reliable set of SSRs (reviewed in Baldoni et al., 2009), to detect new clonal variants and to confirm the suspected existence of polyclonal cultivars and cultivar-populations. Novel insights on olive genetic diversity were acquired, that will be useful for conservation and breeding purposes, tree nursery genetic certification and oil traceability.

2. Material and methods

2.1. Plant material

A total of 45 putative clones belonging to eight standard cultivars were studied. These comprised: 10 putative clones of 'Biancolilla', six of 'Cerasuola', three of 'Giarraffa', eight of 'Moresca', 11 of 'Nocellara del Belice', two of 'Ogliarola Messinese', four of 'Tonda Iblea' and two genotypes of 'Nocellara Messinese', a minor cultivar the fruits of which could play a role in the further development of the Sicilian table industry. The cultivar 'Nocellara Etnea' was included in the analysis as an internal control. All genotypes were grown at the Sicilian olive regional repository established in 1991 in the 'Azienda Carboj E.S.A.'—Castelvetrano (37.30 Lat N.; Sicily). In that collection, there were nine plants of each putative clone, divided into three replicates of three plants, placed at a distance of 5×7 m and trained to free vase shape.

Sicilian cultivars chosen as standards for genetic and phytosanitary certification purposes (Caruso et al., 2007) were planted in the same farm; they were distributed in the field according to the same experimental scheme adopted for the putative clones, divided into three replicates of three plants, in order enable the comparison with their putative clones and to exclude the influence of environmental factors on the phenotype.

In the text the standard cultivars were indicated as STD.

2.2. Morphological traits and biostatistical analysis

Morphological observations were carried out on all cultivars and their putative clones in 2012–2013. Eighteen morphological variables, interval and quantitative were selected for the characterization of the putative clones among the range of characters defined as the main descriptors of the olive tree (Bottari and Spina, 1952; Barranco et al., 2000; Bartolini et al., 2005; COI, 1997; Caruso et al., 2007). The choice of these traits was guided by previous studies on Italian olive genetic resources (Marra et al., 2013); the interval classification was obtained using the classes reported by Caruso et al. (2007).

Canonical discriminant analysis (CDA) based on the18 morphological variables was performed using the Systat statistical program (SYSTAT Software Inc., Chicago, IL). The power of discrimination of the canonical discriminant functions (CDFs) generated was tested by the "Jackknife misclassification method", a procedure which omits one observation, elaborates the classification function employing all other observations ($n1 + n2 - 1$) and uses the classification function to classify the excluded observation. This process is reiterated for each of the observations (Osuji et al., 2013).

Means of standardized canonical scores of major CDFs were plotted. Two-dimensional CDFs plots were created in order to visualize the groups of putative clones in relation to the cultivar considered as standard. Ellipses marked the 68% confidence level for the analyzed groups.

2.3. DNA extraction and microsatellite evaluation

Genomic DNA was extracted from young leaves according to the protocol developed by Doyle and Doyle (1987). DNA was amplified with nine fluorescently labeled SSR primer pairs, five of which were combined in two multiplexed primer sets as follow DCA: 03, 05, 18 (Sefc et al., 2000); Gapu: 45, 71b (Carriero et al., 2002); and four used in single PCR reactions: DCA13 (Sefc et al., 2000); EMO90 and EMOL (De la Rosa et al., 2002); UDO43 (Cipriani et al., 2002). The number of alleles for each SSR locus, the expected heterozygosity (He), the observed heterozygosity (Ho) and PIC (polymorphic information content) were calculate with PowerMarker v3.25 software (Liu and Muse, 2005). Genotypes that were not distinguishable at the molecular level and/or those showing extra alleles were excluded for the calculation of SSR genetic parameters. A UPGMA dendrogram was also constructed using the PowerMarker V3.25 software employing the coefficient of similarity Nei (1973). A Mantel (1967) test was also computed with PowerMarker (Liu and Muse, 2005) to check the goodness of fit between genetic profiles and CDA analyses, based on morphological traits, of all genotypes analyzed, by using Simple Matching dissimilarity matrixes; p values were also calculated.