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Structure of genetic diversity in *Olea europaea* L. cultivars from central Italy

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Abstract The olive is considered one of the most important fruit crops of the Mediterranean basin where it shows a wide range of variability, with about 2,000 cultivars. Italy, with about 500 cultivars, plays a fundamental role. The ability to discriminate olive cultivars and estimate genetic variability are important factors in better management of genetic resources and in helping to understand how genetic diversity is partitioned among cultivars. The two main objectives of the present investigation were to evaluate the identity of cultivars grown in Abruzzo region, central Italy, and to study their genetic structure. We applied amplified fragment length polymorphism (AFLP) methodology on 84 genotypes belonging to the most relevant and oldest varieties cultivated in Abruzzo and on six unknown genotypes. The information content of data was evaluated using the Marker Ratio index and the Polymorphic Index Content. Moreover, STRUCTURE software was used to investigate the genetic population structure. The analysis enabled us to clearly distinguish eight cultivars within seven clusters. Additionally, one cluster was found to have various minor cultivars and showed a relatively high level of diversity. The partitioning of genetic diversity showed that the largest amount of molecular variance was within groups. Our data suggest that both sexual and clonal propagation have played an important role in the evolution of olive cultivars. In our hypothesis, some ancestral population spread in central Italy with a relevant role of seed propagation, followed by a selection of superior clones from which more traditional varieties originated. In a few cases, hybridization should be taken into consideration to explain the diffusion of recently developed cultivars.

Keywords Olea europaea · Population structure · AFLP · STRUCTURE · Genetic diversity · Olive

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Introduction

The olive (*Olea europaea* L. subsp. *europaea*, $2n = 2x = 46$) is considered one of the most important fruit crops of the Mediterranean basin (de la Rosa

et al. 2003; Gemas et al. 2004) and is characterized by a large number of varieties, mainly used for oil or seasoned fruit production (Mataix and Barbancho 2006). The phylogeny of *O. europaea* has not been completed. As reported by Breton et al. (2008), botanists have attributed the origin of the olive to the Fertile Crescent from subsp. *cuspidata* (Green and Wickens 1989; Riley 2002), whereas geneticists have disagreed: based on molecular data, they have identified the origin of olive in *O. oleaster* (subsp. *europaea*) (Besnard and Bervillè 2000; Besnard et al. 2001a).

The origin of the domesticated olive is also poorly documented (Breton et al. 2008) and still disputed. Breton et al. (2008) report that consumption of olives by humans began 7,000 years ago in the Fertile Crescent and that the first remains that are clearly from domesticated trees date from around 3200 BC. Some evidence suggests that a domestication process could have occurred in west Europe also (Maghreb, Spain and France), where local trees were selected leading to cultivars (Figueral and Terral 2001; Besnard and Bervillè 2000). In 2003 Rotondi and collaborators listed 2,000 olive cultivars in the Mediterranean basin, with Italy playing a primary role with about 500 cultivars. Olive cultivars show a wide range of genetic variability for a large number of agronomic traits, including oil content, fruit size and degrees of adaptation to severe biotic and abiotic stresses (Hatzopoulos et al. 2002; Owen et al. 2005).

The earliest evidence that the Abruzzo region, in central Italy, was one of the most important areas of olive cultivation for both fruit production and oil quality date back to the sixteenth century (Razzi 1574). With a cultivated area of 45,000 ha, and a production of 1,500 t, Abruzzo is still one of the most important area of olive cultivation and oil production in Italy (ISTAT 2008).

Even if the olive has been in cultivation for thousands of years, compatibility between cultivars and the mechanism of self-incompatibility are still unclear. Some cultivars are self-incompatible, which means that the flowers cannot be fertilized by pollen from the same cultivar, and some cultivars are cross-incompatible, where flowers cannot be fertilized by pollen from certain other cultivars (Guerin and Sedgley 2007). Several studies have been carried out to assess the level of self-fertility/sterility and to identify compatible cultivars, but most of them have led to conflicting results (Mookerjee et al. 2005), possibly due to the different environmental conditions under which the studies were conducted or to confusion in cultivar identity (Mekuria et al. 1999). For example, Leccino was mostly found to be self-incompatible (Ugrinovic and Stampar 1996), although Bartoloni and Guerriero (1995) found several selections of Leccino to be self-compatible. The same holds for cultivar Moraiolo, which was identified as self-compatible by Singh and Kar (1980) and as self-incompatible by Bini and Lensi (1981). As an example, Mookerjee et al. (2005) showed that olive trees of cultivar Mission rarely self-fertilized (two cases of self-pollination observed out of the 800 embryos tested). This confirmed a previous report by Griggs et al. (1975) that self-pollination in Mission resulted in very low fruit set. Due to the propagation system and rare occurrence of self-fertilization, olive trees are generally characterized by highly heterozygous genotypes which are favored by selection for their hybrid vigor.

Knowledge of the level and structure of genetic diversity are key factors for the management of genetic resources in successful breeding programs and, as reported by Manel et al. (2003) and Baldoni et al. (2006), analyses at the micro-scale (regional) level are still lacking; this knowledge is also expected to produce crucial insights into the domestication and subsequent crop expansion of the olive.

Since olive traits, olive oil quality and other crop-derived products depend on both the area of cultivation and the genetic constitution of cultivars, germplasm characterization represents a key factor for the traceability of the final product (Consolandi et al. 2008). This is especially important for PDO (Protected Denomination of Origin) products, and the Abruzzo region lists three PDOs for olive oil (*Pretuziano delle colline teramane*, *Aprutino pescarese* and *Colline teatine*).

Several characteristics (leaf, fruit, pit, and growth habits; Cantini et al. 1999) or methodologies (isozyme analysis; Ouazzani et al. 1993) have been used to evaluate olive diversity and to characterize olive germplasm. However, a major limitation in using these traits as genetic markers is that they are highly dependent on environmental or cultivation factors (Hernandez et al. 2001). Thus, development of cultivar-specific genetic markers, such as those based on molecular markers, would be highly useful

for cultivar identification, variety protection, and oil composition determination. Several papers report the successful assessment of phylogenetic relationships in the *O. europaea* complex using either random amplification of polymorphic DNA (RAPD) (Bogani et al. 1994; Fabbri et al. 1995; Wiesman et al. 1998; Mekuria et al. 1999; Belaj et al. 2001; Besnard et al. 2001b; Sanz-Cortés et al. 2001) or the microsatellite-based technique named inter simple sequence repeat (ISSR) (Hess et al. 2000; Mekuria et al. 2002). Moreover, the multilocus polymerase chain reaction (PCR)-based amplified fragment length polymorphism (AFLP) technique (Angiolillo et al. 1999, Belaj et al. 2003; de la Rosa et al. 2003, Sanz-Cortés et al. 2003; Owen et al. 2005; Baldoni et al. 2006) and the single-locus simple sequence repeat (SSR) analysis (Carriero et al. 2002; Cipriani et al. 2002; Alba et al. 2009; Baldoni et al. 2009; Erre et al. 2009) have been widely used to study genetic relationships in the olive.

The main objectives of the present investigation were to evaluate the distinctiveness of cultivars grown in Abruzzo region and to study their genetic structure, assess the level of diversity and use this information in olive oil traceability as well as in plant breeding. Moreover, the clonal versus sexual origin of these selected cultivars is discussed.

Materials and methods

Plant material

A selection of 84 ancient genotypes were collected from 22 cultivars, together with six unknown genotypes (Table 1 and Electronic Supplementary Material Table 1S). We have used the term cultivar to mean a group of individual trees identified by a common name which are distinguishable from others by a set of morphological traits and because of a specific geographical distribution. Plants were assigned to each cultivar based on information obtained from farmers, on morphological analysis and in accordance with the olive varieties book published by ARSSA Abruzzo (Pietrangeli and Russo 1997). The geographical location of collection sites obtained by GPS data is shown in Fig. 1.

DNA extraction and AFLP analysis

Leaf tissues were collected in situ and immediately transferred into liquid nitrogen. DNA was extracted using the Genelute Plant genomic DNA miniprep kit (Sigma). AFLP analyses were carried out as follows: total DNA (500 ng) was restricted-ligated and pre-amplified according to Vos et al. (1995). Amplifications were performed in a 20-µl reaction mix containing 1/100 of the pre-amplified DNA, 50 ng fluorescent-labeled *Eco*RI+3 oligonucleotide primer, 50 ng of unlabeled *Mse*I primer (Table 2), 2 µl 10× PCR buffer (Invitrogen), 0.2 mM dNTPs, and 0.4 U Taq DNA polymerase (Invitrogen). One microliter of each sample was denatured and run on an ABI 3130xl capillary sequencer (Applied Biosystems). Six randomly chosen samples were analyzed in duplicate and used as positive controls to verify the reproducibility of AFLP analyses.

An AFLP locus was considered to be polymorphic if the amplified band was present in some samples and absent in others, and monomorphic if the band was present in all the evaluated accessions. To avoid underestimation of the genetic similarities, all loci, polymorphic or not, were considered. AFLP fragments were scored as 1 (presence of the band) and 0 (absence of the band), using the Genemapper 4.0 software (Applied Biosystems), and entered into a data matrix.

Statistical data analysis

The informative content of the markers scored was evaluated as MR (Marker Ratio, Powell et al. 1996) and PIC (Polymorphic Index Content, Botstein et al. 1980). For AFLP markers, PIC is equal to $1 - F_{\mathrm{aa}_j}^2 - F_{\mathrm{an}_j}^2$, where F_{aa} is the frequency of the amplified allele and F_{an} is the frequency of the non-amplified allele. The mean PIC value for n loci corresponds to:

$$\mathrm{PIC} = \frac{\sum_{j=1}^{n}\left(1 - F_{\mathrm{aa}_j}^2 - F_{\mathrm{an}_j}^2\right)}{n}$$

where j is the jth locus.

Genetic population structure

STRUCTURE ver. 2.2 software (Pritchard et al. 2000) was used to investigate the genetic population

Table 1 Morphological biometric parameters of cultivars involved in the study

Cultivar	Code	Leaf		Inflorescence			Fruit			Oil yield (%)
		Length (mm)	Wide (mm)	Structure	Number of flowers	Time of flowering	Shape	Max diameter position	Weight (g)	
ASCOLANA TENERA	AS	≤65	>16	Compact	>20	Middle	Elliptical	In the middle	4.0–8.0	12–14
CARBONCELLA	CARB	<50	<10	Rare	16	Middle	Spherical	In the middle	1.59–2.21	18–20
CASTIGLIONESE	CAS	<65	<15	Compact	16	Tardy	Elliptical	In the middle	1.96–2.53	14–16
CROGNALEGNO	CRO	<65	<13	Rare	18	Middle	Elliptical	In the middle	3.04–3.12	16–18
CUCCO	CU	<65	<13	Compact	13	Anticipated	Spherical	In the middle	3.84–4.42	18–20
DRITTA	DR	≤65	>13	Rare	18	Middle	Oval	Apical	2.20–2.70	18–20
FRANTOIO	FRA	≤65	<15	Rare	>20	Tardy	Elliptical	In the middle	2.20–2.50	16–18
GENTILE DELL'AQUILA	GA	≤70	<15	Rare	>18	Middle	Elliptical	In the middle	2.75–3.19	16–18
GENTILE DI CHIETI	GC	>65	>15	Rare	18	Middle	Oval	In the middle	2.2–3.8	16–18
GENTILE FARA SAN MARTINO	GFSM	–	–	–	–	–	–	–	–	–
GHIANDARO	GH	<65	<13	Rare	18	Middle	Elliptical	In the middle	3.04–3.12	16–18
INTOSSO	IN	<65	<15	Compact	16	Anticipated	Elliptical	In the middle	4.29–4.87	12–14
LECCINO	L	<65	>13	Rare	16	Anticipated	Elliptical	In the middle	2,9–3.3	14–16
MONICELLA	MON	<65	<15	Compact	16	Tardy	Oval	In the middle	2.5–2.8	18–22
MORAIOLO	MORA	<65	<15	Compact	16	Anticipated	Spherical	In the middle	2.5–2.8	–
NEBBIO	NB	<65	<13	Compact	>20	Anticipated	Oval	Low	2.10–2,42	20–22
NOSTRANA	NOS	–	–	–	–	–	–	–	–	–
OLIVASTRO	OLI	<70	>15	Rare	<18	Middle	Oval	In the middle	2.5–3.2	17–19
PENDOLINO	PEN	>60	<11	Compact	>20	Middle	Elliptical	Apical	1.59–2.00	–
RUSTICA	RU	<65	>13	Compact	16	Middle	Elliptical	In the middle	2.94–3.47	16–18
TOCCOLANA	TOC	<50	<10	Compact	16	Tardy	Spherical	In the middle	2.0–3.8	18–24
TORTIGLIONE	T	<65	<13	Rare	14	Anticipated	Oval	In the middle	1.30–1.71	17–18
UNKNOWN	IG	–	–	–	–	–	–	–	–	–

structure of olive cultivars. This method uses a Markov Chain Monte Carlo (MCMC) algorithm to cluster individuals into populations on the basis of multilocus genotype data (Pritchard et al. 2000; Falush et al. 2003). In this study the procedure that accounts appropriately for the genotypic ambiguity inherent in dominant markers (Falush et al. 2007) was followed.

The number of populations (K) was estimated by performing twenty independent runs for each K (from 1 to 12), using 30,000 MCMC repetitions and 30,000 burn-in periods. Any prior information about the population of origin was used, and correlated allele frequencies and admixture were assumed. The average of the log-likelihood estimates for each K was calculated. The ad hoc statistic ΔK (Evanno et al. 2005) was used to set the number of populations (K). The percentage of membership (q) of each accession in each of the inferred K populations was computed by one additional run for 100,000 MCMC repetitions and 100,000 burn-in periods.

STRUCTURE was also used to estimate the F_K parameter for each of the K populations (Falush et al. 2003). This parameter represents the estimated drift from the inferred common ancestor of all of the populations, thus it is similar to F_{ST} but specific for each population and is expected to be proportional to the divergence from a common ancestral population.

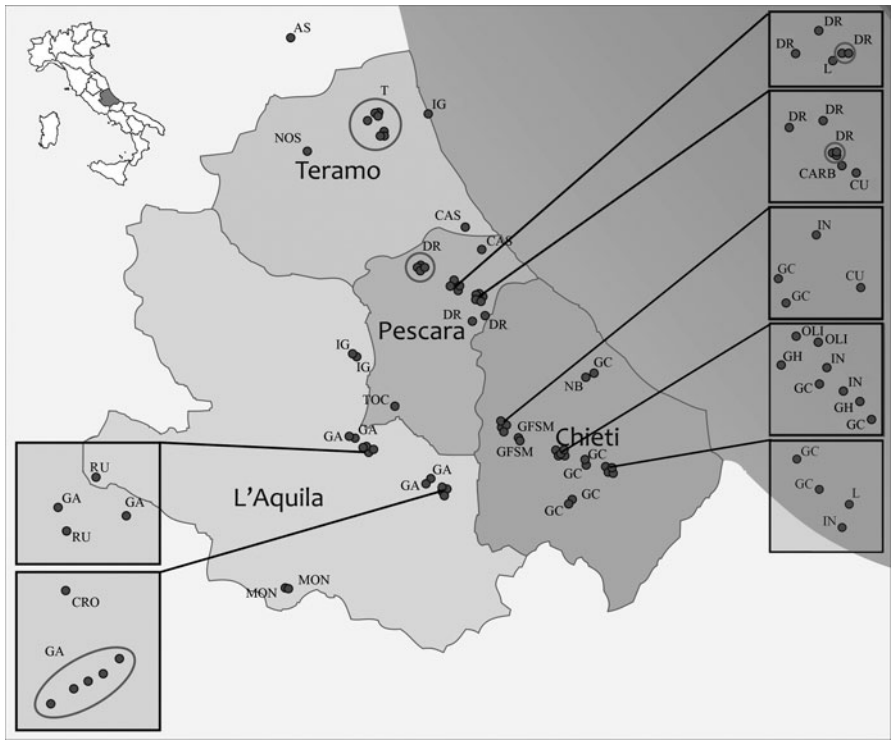

Fig. 1 Geographical location of olive trees collection sites. Each accession is coded with the Cultivar code given in Table 1

A low F_K value indicates little drift away from the ancestral state (Harter et al. 2004).

Genetic diversity and divergence analysis

Not only were the STRUCTURE clusters used for the genetic diversity and divergence analysis but also four groups defined a priori, obtained by grouping the individuals of the main cultivars (Dritta, Gentile di Chieti, Gentile dell'Aquila and Tortiglione). The term 'clusters' was used for STRUCTURE results, whereas 'group' was used to identify the groups defined a priori.

Genetic diversity and divergence analyses were performed excluding monomorphic loci. A Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky 1999) was used to estimate the allele frequencies, using the software AFLP-SURV (Vekemans 2002). This procedure has been shown to produce almost unbiased estimates of allelic frequencies in dominant markers (Krauss 2000). Therefore, allele frequencies were used to compute the gene diversity *Hj* (analogous to unbiased expected heterozygosity *He*; Nei 1978) following the approach of Lynch and Milligan (1994), and the average number of effective alleles per locus, n_e (Kimura and Crow 1964). Only the clusters/groups containing at least nine genotypes were considered for this analysis. The percentage of polymorphic loci for each cluster/group was also calculated. An analysis of molecular variance (AMOVA) was performed in order to investigate the partitioning of the total variance into components for differentiation among and within STRUCTURE clusters and groups defined a priori, quantified with F_{ST} (*F*-statistics, Wright 1951). Allelic frequency estimates were used as input for the AMOVA following the method described in Lynch and Milligan (1994). The clusters/groups described above were used for this analysis, with the exception of the individuals that showed a high level of admixture and for which a well-defined classification was not possible with the STRUCTURE analysis.

The pairwise F_{ST} matrices were computed with AFLP-SURV (Vekemans 2002) for clusters and groups defined a priori.

A total of 2,500 bootstrapped Simple Matching (SM) distances were generated to construct a neighbor-joining tree using the MEGA 3.1 software (Kumar et al. 2004). Moreover, principal coordinate

Table 2 List of Eco-RI+3/MseI+3 primer combinations used together with Multiplex Ratio (MR), % of polymorphisms and PIC value

Primer combination	N. of bands	MR	% Polymorphisms	PIC
E-CAC/M-ACA	110	77	70.00	0.168
E-CCA/M-ACA	98	73	74.48	0.147
E-CAC/M-ATC	81	53	65.43	0.238
E-AGC/M-CAC	72	45	63.50	0.366
E-CCA/M-AGT	72	29	40.27	0.270
E-AGA/M-CAT	66	22	33.33	0.267
E-ACT/M-CTT	66	56	84.84	0.286
E-AGA/M-CAG	59	36	61.01	0.261
E-CAG/M-ACA	59	38	64.40	0.284
E-AGA/M-CTT	58	26	44.82	0.206
E-AGC/M-CAA	56	30	53.57	0.138
E-AGA/M-CTG	54	41	75.92	0.759
E-AGA/M-CAC	54	36	66.66	0.216
E-ACT/M-CAC	54	32	59.25	0.284
E-AGC/M-CTT	45	23	51.11	0.256
E-AGC/M-CTG	42	24	57.14	0.290
E-AGA/M-CAA	38	14	36.84	0.386
Average	63.76	38.52	58.97	0.283

analysis (PCoA) was performed to estimate genetic similarity between individuals. This analysis, which gives the inter-individual relationship, was carried out with NTSYSpc (Numerical Taxonomy and Multivariate Analysis System) software ver. 2.1 (Rohlf 1998) using the SM coefficient (Sokal and Sneath 1963).

Results

AFLP markers

A total of 90 olive trees (Table 1S) were genotyped using 17 AFLP primer combinations. A total of 1,084 amplification products were scored (Table 2) with a percentage of polymorphism ranging from 33 to 85% depending on primer combination. The molecular weight of the amplification products ranged from 72 bp (E-AGC/M-CAC) to 476 bp (E-AGA/M-CTG). The average number of bands per reaction was 63.76 with a variation from 38 (E-AGA/M-CAA) to 110 (E-CAC/M-ACA) (Table 2). The number of polymorphic products per reaction ranged from 14 (E-AGA/M-CAA) to 77 (E-CAC/M-ACA).

For 11 cultivars (Dritta, Gentile di Chieti, Gentile dell'Aquila, Ghiandaro, Monicella, Nebbio, Pendolino, Nostrana, Carboncella, Tortiglione, Nostrana) and one unknown genotype (IG54), at least one specific band was recorded. Moreover, in three cases the absence of one band separated one cultivar from others and could be considered as informative as a cultivar-specific band (one band missing only in Gentile dell'Aquila genotypes and two bands missing only in Leccino samples). Considering the entire sample set, 58.97% of loci were polymorphic whereas the missing data represent 0.25% of the whole dataset.

Genetic population structure

The plot of the average log-likelihood values over 20 runs for K-values ranging from 1 to 12 and the distribution of ΔK values (Evanno et al. 2005) according to K-values are shown in Fig. 2a and b, respectively. The uppermost hierarchical level of population structure identified using the method based on the estimation of ΔK (Evanno et al. 2005) suggested that our sample was made up of two main genetic groups ($K = 2$). This result shown a clear separation between the Dritta cultivar (cluster 1) and the other cultivars considered (cluster 2). In particular, all the Dritta genotypes were assigned to cluster 1 with a membership value (q_1) higher than 0.90, while the remaining genotypes were assigned with a

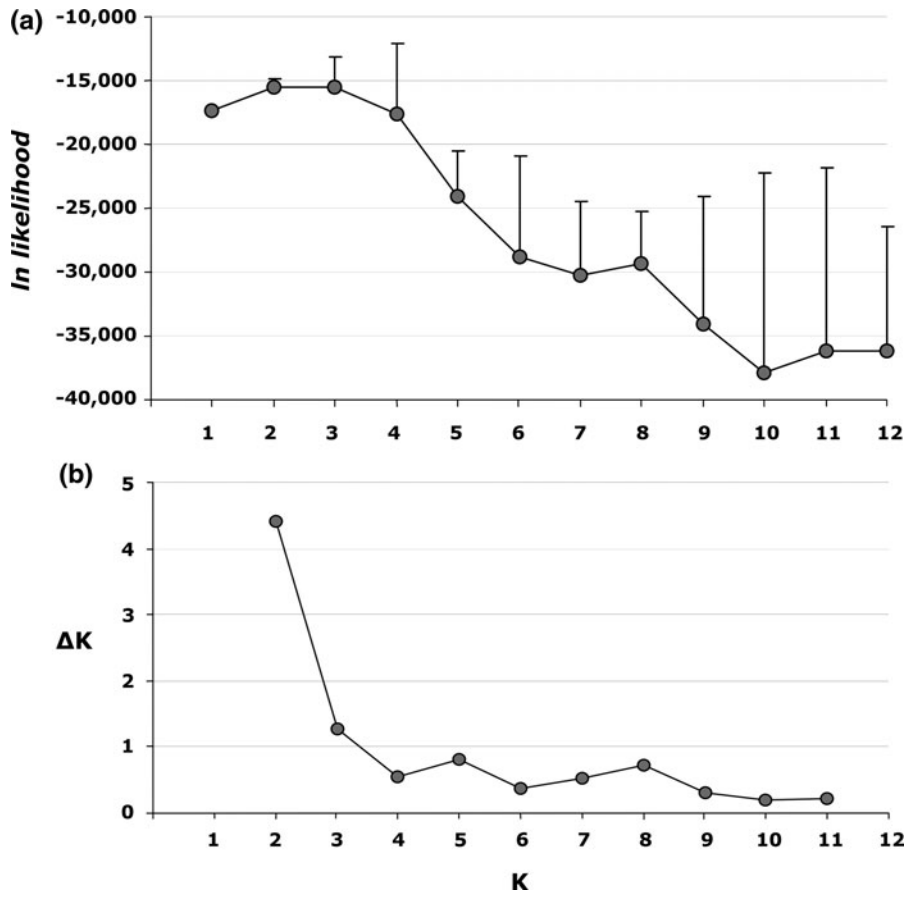

Fig. 2 **a** Average ln likelihood values over 20 runs ± SD for increasing *K*-values, from 1 to 12. **b** ΔK values over 20 runs for increasing *K*-values, from 2 to 11

q_2 value higher than 0.80, with the exception of two unknown genotypes ($q_2 > 0.70$) and Moraiolo which showed a high level of admixture ($q_2 = 0.54$). However, we identified two other peaks corresponding to $K = 5$ and 8. Thus, we investigated the possibility of a hierarchical genetic structure comparing the percentages of membership of genotypes estimated at $K = 2$, 5, and 8. In order to investigate the sub-structure of our sample, we also computed the percentages of membership for the two different values of K (5 and 8) identified by the Evanno method (Evanno et al. 2005) (Fig. 3). We used a threshold value of $q = 0.80$ to assign individuals to one of the K clusters identified. At $K = 5$ (Fig. 3a), the first cluster (1A) was represented by all the 17 genotypes of the Dritta cultivar, cluster 2A included the Gentile dell'Aquila (10 genotypes), Intosso (4 genotypes), and Rustica (3 genotypes) cultivars, while clusters 3A and 4A were represented by the Gentile di Chieti (14 genotypes) and Tortiglione (9 genotypes) cultivars, respectively. Cluster 5A was characterized by various cultivars (Castiglionese, Ghiandaro, Leccino, Monicella and Nebbio). Moreover, 14 genotypes showed membership values (q) lower than 0.80 and thus were classified as admixed (ADM_A).

The analysis conducted at $K = 8$ allowed us to clearly distinguish six different cultivars in six clusters (Fig. 3b): Dritta (1B), Gentile dell'Aquila (2B, not previously discriminated at $K = 5$), Gentile di Chieti (4B), Tortiglione (5B), Leccino (6B, 3 genotypes), and Ascolana Tenera (8B, 3 genotypes). In addition, cluster 3B included two different cultivars: Intosso (4 genotypes) and Rustica (3 genotypes). Finally, cluster 7B was represented by different cultivars such as Castiglionese, Crognalegno, Ghiandaro, Monicella, and Nebbio. Another cluster (ADM_B) was considered as grouping genotypes that showed a high level of admixture and for which it was not possible to define a classification (Fig. 3b). The Dritta cultivar was clearly separated by all others at all the hierarchical levels ($K = 2$, 5, and 8); however, the

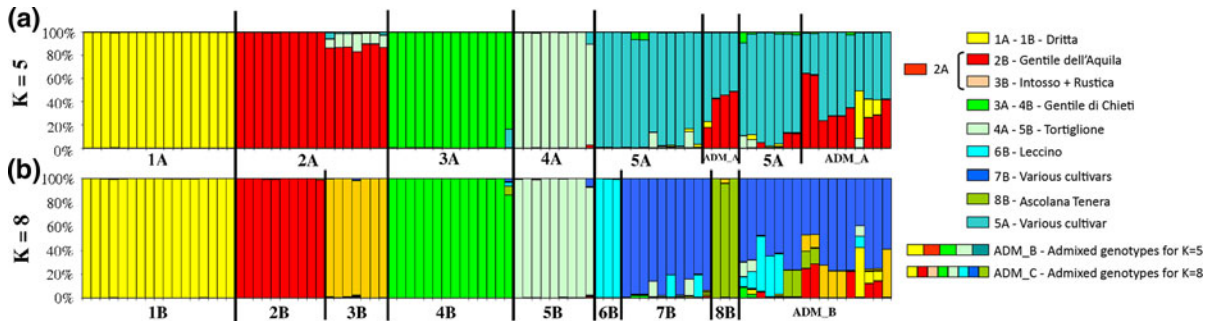

Fig. 3 Percentages of membership of genotypes to clusters as inferred at $K = 5$ (clusters 1A, 2A, 3A, 4A, 5A, and ADM_A) (**a**) and $K = 8$ (clusters 1B, 2B, 3B, 4B, 5B, 6B, 7B, 8B, and ADM_B) (**b**). Each genotype is represented by a *vertical line* divided into coloured segments, the lengths of which indicate the proportions of the genome attributed to the inferred clusters

clustering at $K = 8$ allowed a deeper discrimination of the other analysed genotypes.

The estimated F_K values for each of the inferred clusters at $K = 8$ showed that the lowest F_K value was that of the 7B cluster ($F_{7B} = 0.22$), while the other clusters showed much higher F_K values ranging from 0.86 (5B and 6B) to 0.98 (1B).

Genetic diversity and divergence analysis

Sample size (*S*), the percentage of polymorphic loci, the gene diversity *Hj* (analogous to unbiased expected heterozygosity *He*, Nei 1978) following the approach of Lynch and Milligan (1994), and the average number of effective alleles per locus n_e (Kimura and Crow 1964), for each of the groups defined a priori and each of the clusters defined by STRUCTURE assignment at $K = 8$ were estimated and are reported in Table 3. For clusters containing less than six genotypes (3B, Intosso-Rustica, 6B, Leccino, 8B, Ascolana Tenera), the genetic diversity estimates were not computed.

The lowest genetic diversity ($He = 0.139$ and $n_e = 1.16$) was recorded for Dritta cultivar (cluster 1B). The genetic diversity was slightly higher for the Gentile dell'Aquila (2B; $He = 0.187$ and $n_e = 1.23$) and Gentile di Chieti (4B; $He = 0.161$ and $n_e = 1.19$) cultivars. Among the groups defined a priori the highest genetic diversity estimates ($He = 0.224$ and $n_e = 1.28$) were found for Tortiglione (5B). Cluster 7B, which included various minor and ancient cultivars (Castiglionese, Crognalegno, Ghiandaro, Monicella, and Nebbio), and the ADM_B cluster showed the highest genetic diversity values ($He = 0.312$ and 0.311, respectively, and the same value of $n_e = 1.45$).

The cluster 7B showed, among the defined clusters, the highest percentage of polymorphic loci (63.3%). These values were lower only for the group consisting of accessions that were found to be admixed (ADM_B), which contained 85.7% of polymorphic loci.

The partitioning of genetic diversity (Table 4) showed that, considering all clusters identified by STRUCTURE at $K = 8$, 34.3% of total AFLP variation was between clusters (39.7% for $K = 5$). Thus, the largest amount of molecular variance was within clusters (65.7 and 60.3% for $K = 8$ and $K = 5$, respectively). Performing the same analysis on the four groups defined a priori (Dritta, Tortiglione, Gentile di Chieti and Gentile dell'Aquila), the F_{ST} value was 0.51; thus about half of the total genetic variation was within groups. All the above-mentioned F_{ST} estimates were significantly different from 0 (500 permutations, $P < 0.01$).

The pairwise F_{ST} matrices between both the groups defined a priori and the clusters at $K = 8$ are shown in Table 5. For groups defined a priori, the F_{ST} values ranged from 0.48 (between Gentile dell'Aquila and Gentile di Chieti cultivars) to 0.56 (between Dritta and Gentile di Chieti cultivars). Excluding the admixed cultivars (ADM_C), the pairwise F_{ST} estimates between clusters showed that cluster 7B is less differentiated from all the other clusters/cultivars ($F_{ST} = 0.31$, 0.21, 0.28, 0.27, 0.15, and 0.14 with 1B, 3B, 4B, 5B, 6B, and 8B, respectively). The only exception was cluster 2B which was shown to be closer to 8B ($F_{ST} = 0.22$) rather than to 7B ($F_{ST} = 0.30$). Additionally, the lowest value of F_{ST} was between clusters 7B and 8B. Considering the overall sample set, the lowest F_{ST} value was estimated between 7B and ADM_B.

Table 3 Sample size (*S*), the percentage of polymorphic loci, the gene diversity *Hj* (analogous to unbiased expected heterozygosity, *He*, Nei 1978) and the standard error of *Hj* following the approach of Lynch and Milligan (1994), and the average number of effective alleles per locus, n_e (Kimura and Crow 1964) for each group as defined a priori and by STRUCTURE assignment for $K = 8$

	Sample size	% of polymorphic loci	*Hj*	SE_{Hj}	n_e
Populations defined a priori*					
Dritta	17	1.1	0.139	0.006	1.16
Gentile di Chieti	14	6.3	0.161	0.007	1.19
Gentile dell'Aquila	10	4.6	0.187	0.007	1.23
Tortiglione	9	15.0	0.224	0.008	1.29
Populations identified by STRUCTURE at $K = 8$					
Dritta (1B)	17	1.1	0.139	0.006	1.16
Gentile dell'Aquila (2B)	10	4.6	0.187	0.007	1.23
Intosso-Rustica (3B)**	7	10.5	–	–	–
Gentile di Chieti (4B)	14	6.3	0.161	0.007	1.19
Tortiglione (5B)	9	15.0	0.224	0.008	1.29
Leccino (6B)**	3	7.0	–	–	–
Various Cultivars (7B)	10	63.3	0.312	0.009	1.45
Ascolana Tenera (8B)**	3	4.2	–	–	–
Admixed (ADM_B)	17	85.7	0.311	0.009	1.45

* Only genotypes belonging to the main cultivars were considered

** Genetic diversity estimates were not computed for clusters containing less than 9 genotypes

Table 4 F_{ST} values as computed by AMOVA for different level of population structure

	F_{ST}
Populations identified by STRUCTURE at $K = 5$	0.397
Populations identified by STRUCTURE at $K = 8$	0.343

The neighbor-joining tree and the corresponding percentages of membership for each genotype, estimated by the STRUCTURE analysis for $K = 8$, are shown in Fig. 4. The neighbor-joining tree is highly consistent with results obtained with STRUCTURE; indeed Dritta (1B), Gentile dell'Aquila (2B), Rustica-Intosso (3B), Gentile di Chieti (4B), Tortiglione (5B), Leccino (6B), and Ascolana Tenera (8B) clusters correspond to strongly supported clades (100% bootstrap values). By contrast, cultivars belonging to cluster 7B are scattered all along the dendrogram, and thus not included in a well-defined clade (Fig. 4).

In particular, the neighbor-joining tree is composed of two main clusters: cluster A comprises three sub-clusters supported by very high bootstrap values (100%). The first sub-cluster includes Rustica and Intosso while the second and third include Gentile dell'Aquila and Gentile di Chieti, respectively. Ascolana Tenera and Cucco are grouped together with Gentile dell'Aquila, even though this is supported by a less strong bootstrap value (65%). The second main cluster (B) includes Dritta and Tortiglione which are placed into two well-supported subclusters. Cluster B also includes Leccino, Pendolino, Monicella, Carboncella, Castiglionese, and Frantoio (92% bootstrap value).

Principal coordinate analysis

PCoA of the complete data set was also performed in order to represent the relationships between individuals. The two-dimensional PCoA plot (Fig. 5) shows that the first principal coordinate accounts for 19.87% of total variation and allows to discriminate the Dritta cultivar from the remaining genetic materials. A good discrimination was also shown by the second principal coordinate (16.03% of total variation) which allows to discriminate Gentile di Chieti from Gentile dell'Aquila. Tortiglione is located at the centre of the diagram together with several other cultivars such as Castiglionese, Crognalegno, Carboncella, and Frantoio.

Table 5 The pairwise F_{ST} matrices between, both the groups defined a priori and those obtained by the STRUCTURE analysis at $K = 8$

Populations defined a priori	Dritta	Gentile di Chieti	Gentile dell'Aquila	Tortiglione
Dritta	***			
Gentile di Chieti	0.55	***		
Gentile dell'Aquila	0.56	0.48	***	
Tortiglione	0.51	0.50	0.49	***

Populations identified by STRUCTURE at $K = 8$		1B	2B	3B	4B	5B	6B	7B	8B	ADM_C
Dritta	1B	***								
Gentile dell'Aquila	2B	0.55	***							
Intosso-Rustica	3B	0.47	0.33	***						
Gentile di Chieti	4B	0.56	0.48	0.47	***					
Tortiglione	5B	0.51	0.50	0.38	0.49	***				
Leccino	6B	0.39	0.32	0.27	0.37	0.30	***			
Various Cultivars	7B	0.31	0.30	0.21	0.28	0.27	0.15	***		
Ascolana Tenera	8B	0.38	0.22	0.22	0.31	0.33	0.20	0.14	***	
Admixed	ADM_B	0.31	0.23	0.18	0.28	0.27	0.14	0.04	0.11	***

Discussion

In the present work we have investigated the molecular diversity of 90 olive trees sampled within a relatively restricted region. We compared the molecular diversity with the geographical location, the traditional classification which is mainly based on morphology, and our morphological analysis. The age of most of these trees was over 100 years, and thus the geographical distribution should not be influenced by modern olive nursery techniques and recent introductions. The results shown here are related to the high concordances between traditional classification and molecular analysis. In fact, all individuals classified as belonging to the most important cultivars grown in Abruzzo (Dritta, Gentile di Chieti e Gentile dell'Aquila) along with two minor cultivars (Tortiglione and Ascolana Tenera) and one of the most common Italian cultivars (Leccino) were assigned by STRUCTURE with high q_i values to single clusters which were also assigned to a highly supported clade (100% bootstrap values) in the neighbor-joining tree.

Mating systems are a key factor in determining the structure of genetic diversity in natural and domesticated populations. Nowadays, olive trees are clonally propagated, a technique used since ancient times; indeed Pliny the Elder (77 AD) had already suggested its use to avoid the degeneration of trees.

However, only a few decades before him, Marcus Terentius Varro (37 BC) was suggesting the use of seed propagation in order to introduce the crop in a new region, inferring that seed propagation was important during the early phases of expansion of olive trees in Italy. Thus, in addition to asexual propagation, sexual reproduction could have had an important role in the evolution of olive cultivars.

Our data suggest that both sexual and clonal propagation played an important role in the evolution of olive cultivars. For instance, the diversity that is present between and within Intosso and Rustica, two cultivars grown in two narrow but distinct eco-geographical areas, are likely due to mutations within a single clonal population. Indeed, they were grouped in two differentiated sub-clusters belonging to the same single and very well-supported clade (100%). This is also supported by their very similar phenotypic characteristics such as leaf shorter than 65 mm and larger than 13 mm, compact inflorescences, and elliptical fruit with a weight of about 4 g (Table 1). Indeed, because olive trees are highly heterozygous, the progeny originated by a cross between two very similar genotypes or even by selfing would show a strong genotypic and phenotypic variation because of segregation and recombination. Including this example, seven out of the eight clusters obtained by STRUCTURE, representing 70% of the whole

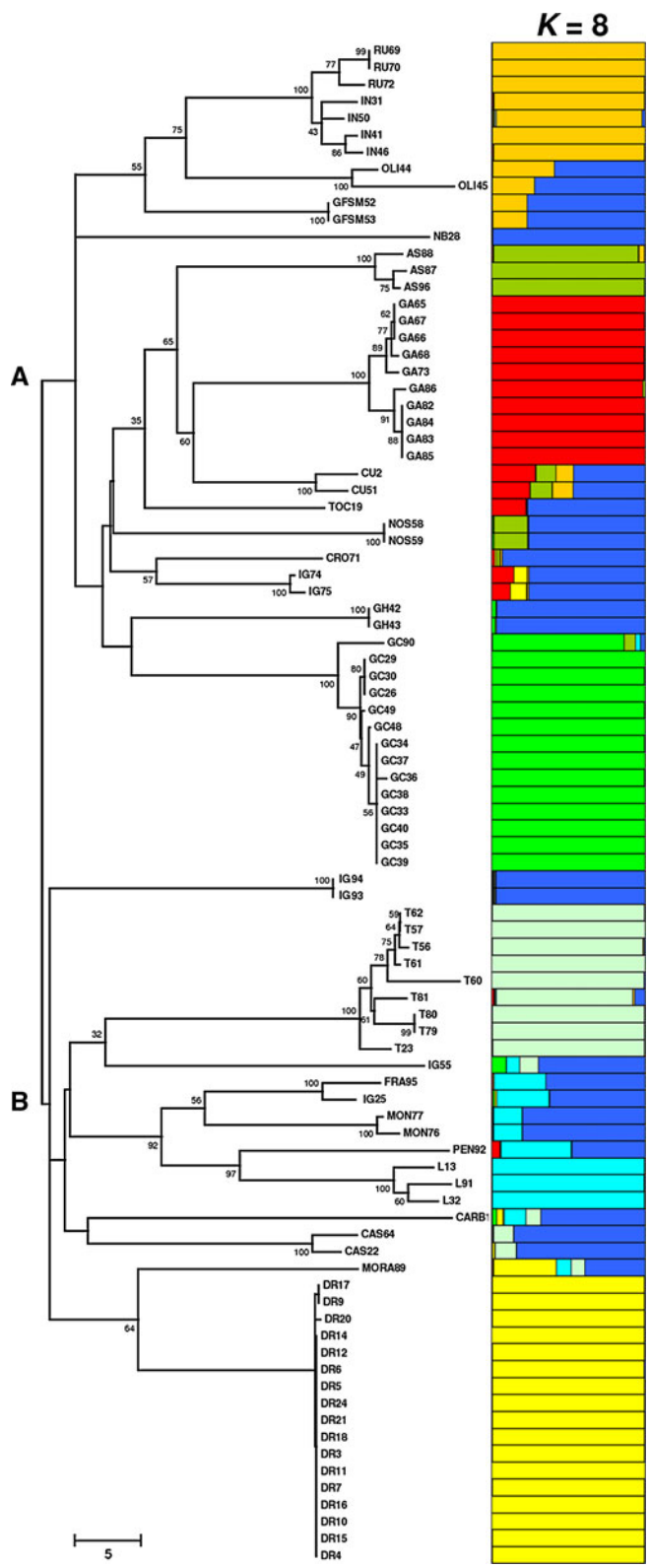

Fig. 4 Neighbor-joining tree (2,500 bootstrap re-samplings; bootstrap values <25% are not showed) compared with the percentage of membership for the 90 genotypes computed considering $K = 8$. Each genotype is represented by an horizontal line divided into K colored segments, the lengths of which indicate the proportions of the genome attributed to the different clusters

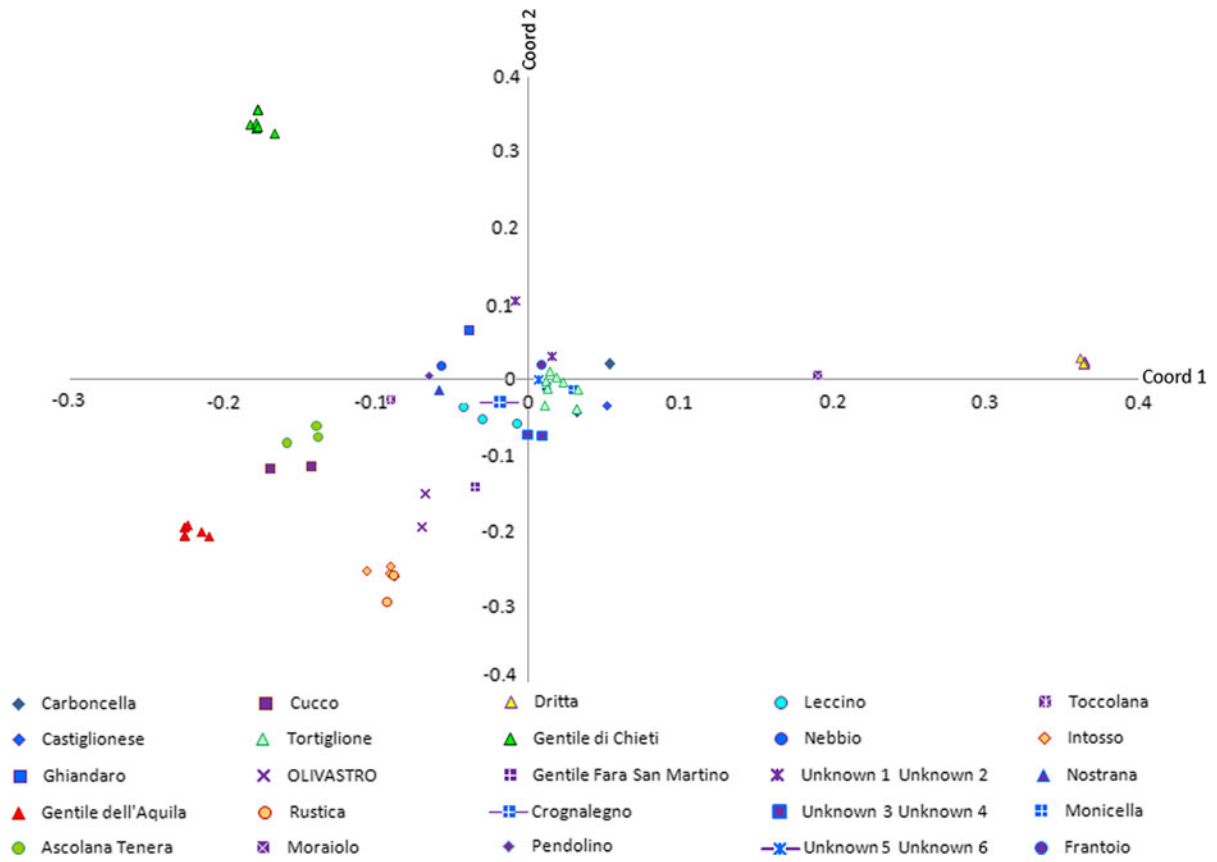

Fig. 5 Plot of the principal co-ordinate analysis obtained with all AFLP data for the 90 genotypes. To each genotype a color resembling that of STRUCTURE cluster assignment and a variety-specific form was assigned

sample, were found to belong to populations where, most likely, the molecular variation originates mainly by mutation within a clonal population. Indeed, these clusters showed a low level of polymorphism (less than 15% of polymorphic fragments). In particular, apart from Tortiglione and Intosso-Rustica, in the remaining five groups, less than 7% of loci were polymorphic with the exception of the extreme case of Dritta which showed only 1.1% of polymorphisms. Thus, six of the clusters defined by STRUCTURE can be considered as six clonal populations. On the other hand, STRUCTURE identified an additional cluster (7B) that included various minor traditional cultivars which are very old and characteristic of a restricted area of cultivation. This group is spread throughout the Abruzzo region. In contrast to all the other groups defined by STRUCTURE, cluster 7B shows a much higher level of polymorphism (63.3%) and does not group as a single clade in the neighbor-joining tree. Additionally, cluster 7B is less differentiated from other cluster/cultivars. Interestingly, genotypes classified as admixed showed, in all cases, the predominant membership coefficient of cluster 7B which ranged from 0.39 (Moraiolo) to 0.72 (Gentile di Fara San Martino) with an average of 0.65. This pattern suggests that the 7B cluster may represent an ancestral group of genotypes typical of Abruzzo and that sexual reproduction played a relevant role in shaping the genetic diversity of olive varieties in the early stages of olive tree spread. It is interesting to consider that some of the genotypes classified as admixed belong to some of the most important and widespread Italian modern varieties such as Moraiolo, Frantoio, and Pendolino. In particular, the STRUCTURE analysis may suggest that Pendolino and Frantoio derived from hybridization between a putative ancestral genotype and Leccino, while Moraiolo derived from hybridization between a

putative ancestral genotype and Dritta. The hypothesis that the genotypes included in cluster 7B represent an ancestral population is also supported by its very low F_K estimate, about four to five fold lower than those of all the other clusters. However, this last result should be considered with caution because it could be largely due to the specific population history and structure.

Our results suggest a scenario where an initial ancestral population spread in Abruzzo and probably in the whole central Italy. A relevant role in this was played by seed propagation and selection of superior genotypes that were subsequently clonally propagated. In the following stages, hybridization and seed propagation probably led also to the development of traditional varieties as well as to modern cultivars.

The important role of propagation through seed is also supported when considering the analysis of population structure (Table 4) where the component of genetic variance due to differences between individuals within the cluster/cultivars varies from 58 to 66%. Indeed, these results appear too high if assuming only asexual reproduction. Additionally, the level of population differentiation is largely due to the presence of Dritta cultivar, which seems to originate from a different gene pool compared to the other cultivars as showed by PCoA analysis. Indeed, when Dritta is removed from the AMOVA, the level of the within-population component of genetic diversity shows a large increase.

It is worth noting that we have found no homonyms in the Abruzzo germplasm analyzed. The only case was genotype IG25 (Unknown1), which was disputed. In fact, while the owner of the farm where the tree is grown persists in classifying this tree as Dritta, ARSSA technicians are sure that this classification is not based on phenotypic characteristics. Our data demonstrate that the genotype does not belong to Dritta and, in the best case, it is the only example of homonym we have found.

The low level of polymorphisms found within Gentile di Chieti and Gentile dell'Aquila could be ascribed to somatic mutations that occur in long-lived trees reproduced through vegetative propagation, as also reported by several authors (Gemas et al. 2000; Cipriani et al. 2002; Belaj et al. 2004; Bracci et al. 2009).

The results of this work are useful in view of the genetic and sanitary certification process of nursery materials. The presence of varieties that can be clearly characterized and identified will allow farmers and industry from this area to produce olive oils characterized by each unique set of flavors. Traceability of these oils as well as those already classified as Abruzzo PDOs will be the main objectives of the follow-up to this characterization project.

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