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ORIGINAL PAPER

Does the genetic diversity among pubescent white oaks in southern Italy, Sicily and Sardinia islands support the current taxonomic classification?

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Abstract

Molecular diversity analysis of deciduous pubescent oaks was conducted for populations from Calabria, Sicily and Sardinia. The aims of this study were twofold. First, to provide data on the genetic diversity of pubescent oaks from an understudied area which currently exhibits one of the highest concentrations of pubescent oak species in Europe. Second, to verify if these groups of oaks are genetically distinct and if their identification is in accordance with the current taxonomic classification. Molecular analyses of leaf material of 480 trees from seventeen populations belonging to putatively different pubescent oak species (*Quercus amplifolia*, *Q. congesta*, *Q. dalechampii*, *Q. ichnusae*, *Q. leptobalanos*, *Q. virgiliana*) were performed. Twelve gene-based Expressed Sequence Tag-Simple Sequence Repeat markers were selected, and genetic diversity and differentiation were calculated. The results showed relatively high values of allelic richness, heterozygosity and number of private alleles for the populations investigated. A weak but positive correlation between geographical and genetic distance was detected. Genetic assignment (STRUCTURE) and principle coordinate analyses exhibited a weak separation into two genetic groups which, however, did not correspond to the taxonomic, chorological and ecological features of the populations investigated. Sardinian populations formed one group which was separated from the Calabrian and Sicilian populations. In light of the results obtained, the taxonomic classification for the pubescent white oaks currently reported in the major Italian floras and checklists for the study area was not confirmed by molecular analyses.

Keywords Biogeography · Bayesian analysis · Genetic variation · Nuclear microsatellites · EST-SSRs · Pubescent oaks · Taxonomy

Introduction

The deciduous oak woods represent the most abundant forest vegetation type in southern Europe (Mucina et al. 2016). On the Italian Peninsula they are dominant throughout the whole Apennine range with an increase in the sclerophyllic evergreen oak component (*Quercus ilex*, *Q. suber* and *Q. coccifera*/*Q. calliprinos*) moving southwards (Blasi and Di Pietro 1998; Blasi et al. 2004; Di Pietro et al. 2010). However, even at the southernmost tip of Italy and Sicily the thermophilous deciduous oak forests cover a wider area than evergreen oak forests (Gianguzzi et al. 2015) whereas the opposite is true for Sardinia where only 15% of the territory is potentially covered by deciduous oaks (Bacchetta et al. 2009). Both taxonomic and phytosociological literature report that the thermophilous broad-leaved forests of southern Italy, Sicily and Sardinia are characterized by different pubescent oak species occurring in sympatry. Pubescent oaks belong to the white oaks (subgenus *Quercus*; section *Quercus*) and are characterized by pubescent leaves and twigs that allow them to be distinguished from other white oak species such as *Q. petraea* and *Q. robur*. The high concentration of putative pubescent white oak species suggests that southern Italy, Sicily and Sardinia acted

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as primary refugia for the oak forest vegetation during the Quaternary cold periods (Sadori and Narcisi 2001; Fineschi and Vendramin 2004). It follows the well-established theory according to which several thermophilous tree species survived the glacial periods in the coastal and hilly belts of the Iberian, Italian and Balkan Peninsulas (Huntley and Birks 1983; Watts et al. 1996; Brewer et al. 2002; Tzedakis et al. 2002). Furthermore, the degree of geographic isolation may have played a non-marginal role in the current degree of phenotypic diversification of the pubescent oaks of the study area. Southern Calabria is a narrow mountainous promontory dividing Tyrrhenian and Ionian Seas, while Sicily and Sardinia are the largest Mediterranean islands that experienced different paleogeographic vicissitudes—as testified by their different type of floristic endemic components (Bacchetta et al. 2005; Médail and Quézel 1997; Brullo et al. 2011; Pignatti 2011; Sciandrello et al. 2015)—which may have affected the evolution of the *Quercus* genetic pools in a different way (see Petit et al. 2002b; Fineschi and Vendramin 2004).

On the basis of the oak classification frameworks reported in the most recently published National floras and in papers on the taxonomy of the *Quercus* genus (e.g., Pignatti 1982; Brullo et al. 1999; Mossa et al. 1999; Pignatti et al. 2018, 2019) seven pubescent oaks are considered as occurring in southern Italy. These are: *Q. amplifolia* Guss., *Q. congesta* C. Presl., *Q. dalechampii* Ten., *Q. ichnusae* Mossa, Bacch. and Brullo, *Q. leptobalanos* Guss., *Q. pubescens* Willd. and *Q. virgiliana* Ten. In the recent checklist of the Italian vascular Flora (Bartolucci et al. 2018) only four of these species are considered as valid names (*Q. pubescens*, *Q. dalechampii*, *Q. congesta* and *Q. ichnusae*) the remaining three being considered as synonyms (Peruzzi et al. 2015, 2019). It is noteworthy that Sicily, Sardinia and southern Calabria are *loci classici* for five of the aforementioned seven pubescent-oak species and that some of these species (e.g., *Q. congesta*, *Q. dalechampii* and *Q. virgiliana*) are considered "good species" not only in Italy but also in several other European countries. In fact, the debate on the taxonomic value of all these pubescent oak species is very heated throughout Europe. Nonetheless there are very few studies that addressed the problem of oak taxonomy using a multidisciplinary approach where molecular analyses are carried out in support of previous morphological analyses (Franjic et al. 2006; Di Pietro et al. 2016, 2020; Musarella et al. 2018). While it is true that cpDNA markers can be useful to establish the conformity of a given material to the populations of its origin and to trace possible routes of migration at a broad geographic scale, cpDNA provides limited taxonomic information on the systematic status of interfertile, sympatric species (see Curtu et al. 2007a, b; Neophytou and Michiels 2013; Blanc-Jolivet and Liesebach 2015). In contrast, co-dominant markers, such as microsatellites, have successfully been tested to study genetic structures and distinguish oak species at regional or local scale (Degen et al. 1999; Gomory 2000; Gugerli et al. 2007; Guicoux et al. 2011b; Hoeltken et al. 2012). Only recently, population genetic studies based on co-dominant nuclear markers have been carried out in white oak populations from restricted areas of southern Italy (Antonecchia et al. 2015; Fortini et al. 2015; Di Pietro et al. 2016, 2020). These studies were based on sampling protocols, which provided a high number of reference samples per population and a high number of populations per unit area.

The aim of this paper is twofold. First, to provide first insights in the genetic diversity from an area that, although being considered as highly important for the European white oaks diversity, has not been characterized at genetic markers. Second, to verify if groups of oak individuals (or populations) are distinguishable on the basis of their genetic features in order to confirm, or support the assumption of the occurrence of different pubescent oak species.

Materials and methods

Study area

This study was carried out in Southern Italy, in mixed deciduous forest habitats which are located in administrative regions of Calabria (the southernmost end of this region included between the Serre Calabre and the Aspromonte massifs), Sicily and Sardinia (41° 18′ N–7° 23′ E; 36° 19′ N–18° 16′ E) (Fig. 1).

Oak tree material

Leaf material of 17 pubescent-oak populations was collected during autumn of 2017 and 2018. The taxa investigated are: *Q. amplifolia* Guss., *Q. congesta* C. Presl., *Q. dalechampii* Ten., *Q. ichnusae* Mossa, Bacch. and Brullo, *Q. leptobalanos* Guss., and *Q. virgiliana* Ten. As already mentioned in the introduction, some authors consider these taxa as valid species while others consider them as morphotypes included in the natural morphological variability of *Q. pubescens* Willd. The morphological characters used to distinguish these taxa are for the most part quantitative characters (Di Pietro et al. 2020) with overlapping values between species in the identification keys. For this reason, and to ensure that both the choice of collection sites and the interpretation of the results of the genetic analysis were subject to the lowest possible degree of subjectivity, we have preferred to maintain a neutral position regarding the name of the species, avoiding to provide our a priori identification. In fact, the six putative species were collected in populations in which these oaks were already identified and published as guide species

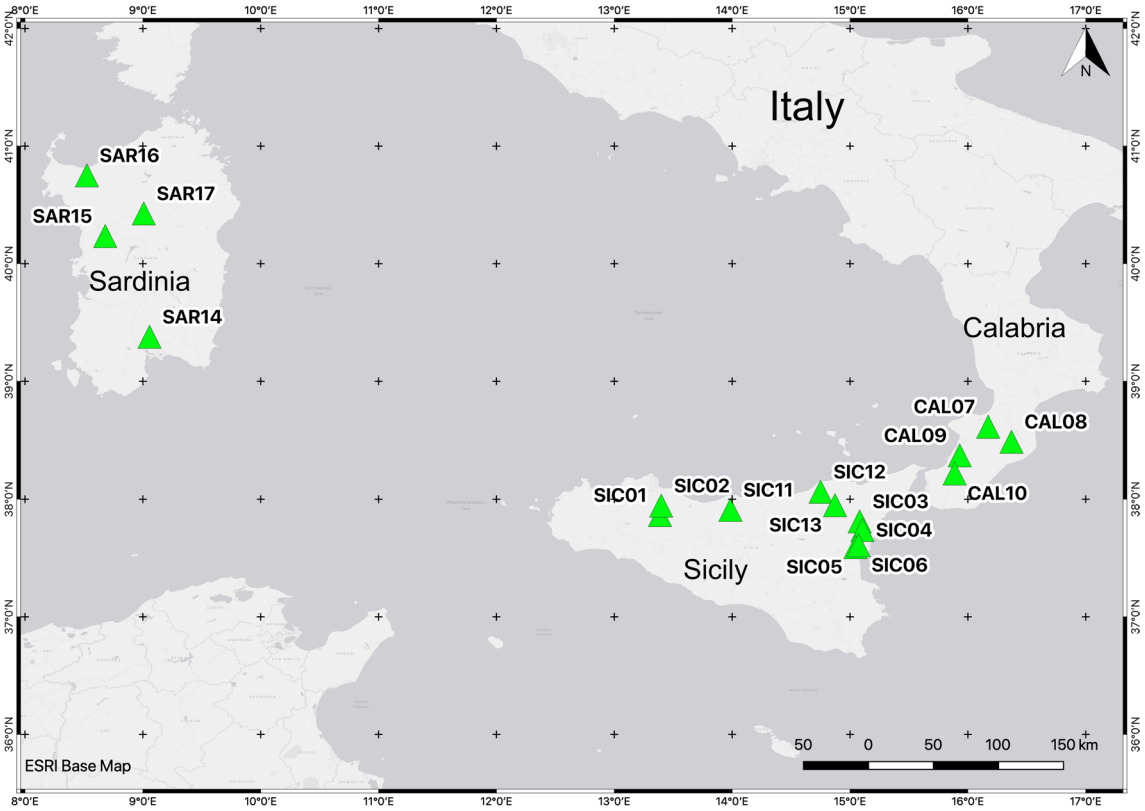

Fig. 1 Study sites in Calabria, Sardinia and Sicily

by other authors expert in the taxonomy and phytosociology of oaks (Table 1) and the abundance indexes of each species were already published in phytosociological tables. These tables were originally assigned to the following associations: *Erico arboreae-Quercetum virgilianae* Brullo et Marcenò 1985, *Festuco heterophyllae-Quercetum congestae* Brullo et Marceno 1985; *Ilici aquifolii-Quercetum leptobalani* Maniscalco et Raimondo 2009; *Lonicero implexae-Quercetum virgilianae* Bacchetta et al. 2004, *Oleo sylvestris-Quercetum virgilianae* Brullo 1984, *Ornithogalo pyrenaici-Quercetum ichnusae* Bacchetta et al. 2004, *Glechomo-Quercetum congestae* Bacchetta et al. 2004, *Arabido turritae-Quercetum congestae* Brullo et Marcenò 1985, *Quercetum leptobalani* Brullo 1984 (Brullo 1984; Brullo and Marcenò 1985; Brullo et al. 2001, 2008; Bacchetta et al. 2004, 2009; Maniscalco and Raimondo 2009). Collection sites SIC03, CAL09, SIC11, SAR15 were located in the proximity of the *loci classici* of *Q. congesta*, *Q. dalechampii*, *Q. leptobalanos* and *Q. ichnusae*, respectively. The other sites were selected from those for which published taxonomic or phytosociological references were available that attest the occurrence of an oak species among those investigated in this research. A total of 480 oak trees were analyzed. In each stand, leaves were collected from a minimum of 15 and a maximum of 34 individuals. The minimum distance between the collected trees was at least 30 m.

DNA extraction

For all samples from Calabria and Sicily (a total of 393 samples), the DNA was extracted from leaves using the Invisorb® Spin Plant Mini Kit (INVITEK Molecular GmbH, Berlin, Germany) and the work was carried out in the Plant Biology Laboratory of Molise University (Isernia, Italy). For those samples coming from Sardinia (96 samples), DNA was extracted using the Qiagen DNeasy 96 plant kit (Qiagen, Hilden, Germany) and the work was carried out in the Forest Genetics and Forest Tree Breeding Laboratory at the University of Göttingen (Germany).

Twelve gene-based microsatellite markers Expressed Sequence Tag-Simple Sequence Repeats (EST-SSRs) were used: PIE239, PIE227, PIE223, PIE215, PIE020, PIE152, PIE243, PIE242, PIE267, PIE102, PIE258, PIE271 (Durand et al. 2010).

This set of EST-SSR markers (PIE) was chosen according to other recent studies on European white oaks (Lepais et al. 2009; Guichoux et al. 2011a, b; Neophytou et al. 2010; Curtu et al. 2015; Antonecchia et al. 2015; Di Pietro et al. 2020). The primer pairs were combined into three different

Table 1 Geographic features of the 17 oak populations sampled in Calabria, Sardinia and Sicily

Stand ID	No. of samples	Coordinates GMS D° M′ S″	Location	Region	Guide species	Altitude (m a.s.l.)
SIC01	30	37° 51′ 55 98″ N 13° 23′ 13.68″ E	Bosco Ficuzza (Corleone, Palermo)	Sicily	*Quercus leptobalanos* Guss.	919
SIC02	29	37° 56′ 50 59″ N 13° 23′ 43.71″ E	Santuario (Marineo, Palermo)	Sicily	*Quercus virgiliana* (Ten.) Ten.	459
SIC03	27	37° 48′ 40.30″ N 15° 4′ 56.26″ E	Etna, SP Mare-Neve (Chalet delle Ginestre)	Sicily	*Quercus congesta* C. Presl.	1298
SIC04	30	37° 44′ 19.60″ N 15° 6′ 18.64″ E	Etna, SP Mare-Neve (Fornazzo)	Sicily	*Quercus dalechampii* Ten.	900
SIC05	26	37° 35′ 32.67″ N 15° 2′ 42.61″ E	Etna, Monte Ceraulo, Mascalucia (Catania)	Sicily	*Quercus virgiliana* (Ten.) Ten.	538
SIC06	30	37° 36′ 29.87″ N 15° 4′ 17.95″ E	Etna, Trecastagni via P. Togliatti (Catania)	Sicily	*Quercus virgiliana* (Ten.) Ten.	544
CAL07	28	38° 36′ 57.93″ N 16° 10′ 17.16″ E	Serre, Sant' Angelo SS 182 exit SP per Pizzoni (VV)	Calabria	*Quercus dalechampii* Ten.	260
CAL08	32	38° 29′ 16.81″ N 16° 22′ 11.31″ E	Serre, SS 110 exit SP 90 per Nardidipace (VV)	Calabria	*Quercus congesta* C. Presl.	1190
CAL09	29	38° 22′ 24.18″ N 15° 55′ 49.69″ E	Aspromonte, SP Palmi Pontevecchio—Croce Mammone, presso Cirello, Rizziconi (RC)	Calabria	*Quercus dalechampii* Ten.	70
CAL10	30	38° 13′ 9.00″ N 15° 53′ 16.61″ E	Aspromonte SP 3 (ex SS 183) bivio Piani di Carmelia (RC)	Calabria	*Quercus congesta* C. Presl.	980
SIC11	33	37° 54′ 36 56″ N 13° 59′ 06.12″ E	Madonie tra Piano Torre e Piano Zucchi (Collesano, Palermo)	Sicily	*Quercus leptobalanos* Guss.	864
SIC12	28	38° 03′ 51 68″ N 14° 45′ 01.68″ E	Nebrodi, Valle del Fiume Fitalia (Frazzanò, Messina)	Sicily	*Quercus virgiliana* (Ten.) Ten.	246
SIC13	32	37° 56′ 29 75″ N/14° 52′ 34.38″ E 37° 57′ 07 84″ N/14° 52′ 15.22″ E	Nebrodi, Foresta (Bosco del Flascio)	Sicily	*Quercus congesta* C. Presl.	1162–1172
SAR14	30	39° 22′ 38.15″ N 9° 3′ 33.50″ E	M.te Zara, Monastir (Cagliari)	Sardinia	*Quercus amplifolia* Guss.	130–167
SAR15	30	40° 14′ 8.22″ N 8° 40′ 47.49″ E	Monte Sant'Antonio, Macomer (Nuoro)	Sardinia	*Quercus ichnusae* Mossa, Bacch. & Brullo	780
SAR16	21	40° 45′ 5.38″ N 8° 31′ 27.82″ E	Sant'Orsola, Sassari (Sassari)	Sardinia	*Quercus virgiliana* (Ten.) Ten.	138
SAR17	15	40° 25′ 39.90″ N 9° 0′ 24.74″ E	Monte Rasu, catena del Goceano, Bono (Sassari)	Sardinia	*Quercus congesta* C. Presl.	1098–1197

multiplex reactions, called Mu1, Mu2 and Mu3 (Table 2). The dye type and primers are also shown in Table 2. A PCR Mastermix was obtained blending 1 µL DNA, 1.5 µL 10× reaction buffer B (Solis BioDyne, Tartu, Estonia), 1.5 µL $MgCl_2$ (25 mM), 1 µL dNTPs (2.5 mM each dNTP), and 0.2 µL (5 U/µL) HOT FIREPol *Taq* DNA polymerase (Solis BioDyne, Tartu, Estonia). To this admixture, we have added EST-SSR primers. The volume, concentration and dye labels of each primer are shown in Table 2.

The PCR reactions were conducted using a touchdown program as follows: denaturation at 95 °C for 15 min, followed by 10 touchdown cycles of 94 °C for 1 min, 60 °C (−1 °C per cycle) for 1 min, and 72 °C for 1 min. The second step consisted of 25 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, followed by a final extension step of 72 °C for 20 min. PCR reactions were performed in a DNA Biometra Thermocycler TOptical Gradient 96 (Biometra, Goettingen, D, EU). The subsequent separation of fragments was performed using GS 500 ROX (Applied Biosystems, Foster City, USA) as size standard in an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, USA). Allele scoring was done with the GeneMapper 4.0 software (Applied Biosystems, Foster City, CA, USA).

Table 2 Primers combinations and information about the different multiplex (Mu) reactions

	SSR ID	Observed size range (bp)	Motif type	Forward primer (5′–3′)	Reverse primer (5′–3′)	Primer volume (5 pmol/μL)	Expected size (bp)	Dye type
Mu1	PIE239	80–115	$(AT)_{12}$	CAACAAATGGCT CAACAGTG	CCCATTTGGTAG CAAAGAGTC	1 μL	95	6-FAM
	PIE227	140–175	$(TGG)_8$	ACCATGATCTGG GAAGCAAC	AAGGGCTTGGTT GGGTTAGT	0.5 μL	160	6-FAM
	PIE223	180–240	$(GGT)_8$	AGAAGCCCAACA CGGCTAC	AGCAAAACACAA ACGCACAA	1 μL	200	FAM
	PIE215	180–235	$(GAG)_6$	ACGAAATGGAGC TGTTGACC	TCTCCTTCTCTT CTGCCATGA	1 μL	200	HEX
Mu2	PIE020	94–123	$(TA)_{12}$	GCAGAGGCTCTT CTAAATACA GAACT	GGGAGGTTTCTG GGAGAGAT	0.5 μL	180	6-FAM
	PIE152	228–265	$(AG)_{11}$	TGTACCTCTTTC CTCTCTCTA AAACT	GAATTTCTAAAC CACTAGCAT TGAC	0.5 μL	247	HEX
	PIE243	200–236	$(AG)_{15}$	GGGGTCAGTAGG CAAGTCTTC	GAGCTGCATATT TTCCTTAGTCAG	0.5 μL	220	6-FAM
	PIE242	95–129	$(TA)_{10}$	GGAGGGAAAAGA ACAATGC	TTGCAATCCTCC AAATTTAATG	0.5 μL	113	HEX
Mu3	PIE267	85–105	$(AG)_{11}$	CCAACCATCAAG GCCATTAC	GTGCGAACAGAT CCCTTGTC	0.5 μL	100	6-FAM
	PIE102	130–180	$(AG)_{12}$	ACCTTCCATGCT CAAAGATG	GCTGGTGATACA AGTGTTTGG	0.5 μL	160	HEX
	PIE258	120–180	$(TC)_{13}$	TCTCGATCTCAA AACAAAACCA	TTTGATTTGTTT AAGGAAAAT TGGA	0.5 μL	150	6-FAM
	PIE271	181–230	$(TC)_{11}$	CACACTCACCAA CCCTACCC	GTGCGGTTGTAG ACGGAGAT	0.5 μL	190	HEX

Genetic assignment

The main genetic statistics were obtained using GenAlEx software v. 6.5 (Peakall and Smouse 2012). Basic molecular statistics, the mean number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (G_{ST}) per locus and population, and inbreeding coefficient (F_{IS}) per locus were calculated. In addition, pairwise G_{ST} values between populations based on 1000 permutations were calculated. This data set (Pairwise Population Matrix of G_{ST} Values) was also used to perform a Principal Coordinates Analysis (PCoA) based on covariance with data standardisation (using the tri distance matrix). Using GenAlEx software v. 6.5, we also tested for significant correlations between pairwise co-dominant genotypic distance and geographical distance by applying simple Mantel tests with 9999 permutations.

The allelic richness (A_r) was calculated with rarefaction to the lowest sample size using the HP-Rare software v. June-6-2006 (Kalinowski 2005).

FSTAT v. 2.9.4 (Goudet 2001) was used for obtaining the inbreeding coefficient (F_{IS}) per population using 1000 permutations to test for significant differences from "zero". Number of alleles per locus (K), null allele frequencies (F_{null}), polymorphic information content (PIC) and deviations from Hardy–Weinberg Equilibrium (HW) were calculated using Cervus 3.0.7 (Marshall et al. 1998).

Analysis of molecular variance (AMOVA) was performed with Arlequin v. 3.5.2.2 (Excoffier and Lischer 2010).

In addition, the software Populations v. 1.2.32 (Langella 1999) was used to calculate phylogenetic trees based on pairwise distances between populations and between individuals using the chord genetic distance of Cavalli-Sforza and Edwards (1967). The phylogenetic tree of populations was obtained with 1000 bootstraps on loci, using MEGA 7.0.26 software (Kumar et al. 2016) to display the trees.

In order to infer molecular clusters and to assign individuals to populations, STRUCTURE v. 2.3.4 based on the Bayesian clustering method was used (Pritchard et al. 2000). We performed genetic analysis with STRUCTURE under the admixture model (Alpha, α) without prior information on the geographical location of populations or their taxonomical classification and applied the correlated allele frequency model (Lambda, λ). Owing to the relatively high number of populations sampled and the unbalanced sampling we have decided to follow Wang (2017) using an Alpha value much smaller than the default. Accordingly, the degree of admixture "Alpha" was set to be inferred for each population and the starting value was set to $1/N$ (where $N = 17$). According to Porras-Hurtado et al. (2013), the effect of the parameter of the distribution of allelic frequencies (λ) is expected to be more important with dense genotyping while some studies using SSRs (e.g., Owusu et al. 2015; Thanou et al. 2017) estimate lambda from the data. In this paper, we have followed the suggestion reported in STRUCTURE manual so that λ value has been set to be inferred for each population (starting from $\lambda = 1$). To assess the number of clusters that best fit the data, a burn-in period of 50,000 and Markov chain Monte Carlo (MCMC) simulations of 100,000 were used, considering values of K from one to ten, with twenty replications for each value of K. STRUCTURE HARVESTER (Earl and VonHoldt 2012) was used to observe the log-likelihoods over different values of K (Evanno et al. 2005) while CLUMPAK software (Kopelman et al. 2015) was used for obtaining graphic representation and summary of STRUCTURE results.

Results

The analysis exhibited a mean of 7.9 different alleles per locus (N_a) for a total of 169 alleles over all populations (Table 3). The locus that exhibited the highest number of alleles (K) was PIE102 with 20 alleles. The mean number of different alleles per locus (N_a) over populations ranged from 2.9 (PIE227) to 11.5 (PIE152), the observed heterozygosity (H_o) ranged from 0.227 (PIE227) to 0.834 (PIE271), and the expected heterozygosity (H_e) ranged from 0.208 (PIE227) to 0.864 (PIE152). PIE227 exhibited the lowest value for N_a, H_o, H_e among all loci. The F_{IS} values were significantly different from zero for four loci and ranged from − 0.005 (PIE020) to 0.398 (PIE239). High and positive F_{IS} values and significant evidence for null alleles were only detected for PIE239 and PIE258.

Mean diversity indices for all populations over all loci are shown in Table 4. The mean number of alleles (N_a) per locus ranged from 6.5 in SAR17 (*Q. congesta* from West Sardinia, only 15 individuals) to 8.8 in both SIC01 and SIC13 (*Q. leptobalanos* and *Q. congesta* from West and East Sicily, respectively) (total mean value 7.9). Mean allelic richness (A_r) ranged from 5.8 for SAR16 (*Q. virgiliana*—NW Sardinia) to 7.2 for SIC01 and SIC02 (*Q. leptobalanos* and *Q. virgiliana* from West Sicily) whereas the mean value across populations was 6.7. The observed heterozygosity (H_o) ranged from 0.589 for SIC12 (*Q. virgiliana*—NE Sicily) to 0.688 for CAL09 (*Q. dalechampii*—SW Calabria) with a total mean value of 0.649. The expected heterozygosity (H_e) ranged from 0.619 for SAR16 (*Q. virgiliana*—NW Sardinia) to 0.727 for SIC13 (*Q. congesta*—NE Sicily) with a total mean value of 0.683. The mean F_{IS} value was 0.066 while the minimum F_{IS} was − 0.013 for SAR15 (*Q. ichnusae*—W Sardinia) and the maximum value was 0.137 for SIC12 (NE Sicily). The total number of private alleles N_p found was 63. The highest number of private alleles (23) was found in SIC13 (*Q. congesta*—Sicily) while the second highest number was found in SIC02 (*Q. virgiliana*—Sicily) with 11

Table 3 Sample size and mean genetic diversity indices over all the 17 populations sampled in Calabria, Sardinia and Sicily

Marker	*N*	*K*	N_a	H_o	H_e	F_{IS}	G_{ST}	PIC	*F* (Null)	HW
PIE020	474	13	5.9	0.516	0.513	− 0.005	0.023*	0.494	0.0137	NS
PIE102	470	20	10.1	0.766	0.750	− 0.021	0.025*	0.760	0.0139	NS
PIE152	469	18	11.5	0.819	0.864	0.052	0.023*	0.893	0.0486	NS
PIE215	471	14	9.4	0.819	0.796	− 0.029	0.010*	0.800	0.0018	NS
PIE223	471	12	8.8	0.813	0.818	0.007	0.030*	0.842	0.0253	NS
PIE227	468	8	2.9	0.227	0.208	− 0.089	0.068*	0.230	0.0031	NS
PIE239	456	14	5.1	0.249	0.414	0.398	0.063*	0.453	0.3013	***
PIE242	471	16	9.2	0.790	0.813	0.028	0.039*	0.845	0.0416	*
PIE243	473	15	6.4	0.612	0.645	0.051	0.034*	0.642	0.0532	NS
PIE258	472	16	10.5	0.603	0.839	0.281	0.026*	0.873	0.1956	***
PIE267	472	9	6.5	0.744	0.727	− 0.023	0.030*	0.728	0.0138	NS
PIE271	472	14	8.2	0.834	0.807	− 0.034	0.029*	0.825	0.0053	NS
Mean	469.9	14.08	7.9	0.649	0.683	0.051	0.030*	0.699	–	–

N number of individuals, *K* total number of alleles at the locus over all populations, N_a mean number of alleles per locus over all populations, H_o observed heterozygosity, H_e expected heterozygosity, F_{IS} inbreeding coefficient, G_{ST} fixation index (*$p < 0.05$), *PIC* polymorphic information content, *F* (null) null allele frequency, *HW* Hardy–Weinberg equilibrium test (significance with Bonferroni correction: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

alleles. No private alleles were found in CAL07 and CAL08 (*Q. dalechampii* and *Q. congesta* from Calabria) and SAR16 (*Q. virgiliana*—NW Sardinia). The values of genetic diversity calculated for each administrative region (Calabria, Sicily and Sardinia) showed that the N_a values increased from Sardinia (7.1) to Calabria (7.9) and Sicily (8.2). A similar pattern was found for allelic richness (A_r) which was 6.3 for Sardinia, 6.7 for Calabria and 6.8 for Sicily. Mean H_e was lowest for Sardinian populations (0.637), while it was very similar for Sicilian (0.698) and Calabrian (0.695) populations. The ANOVA (Table 5) showed that differences in H_e values calculated among administrative regions were statistically significant whereas those in H_o were not. Values for H_e were significantly lower in Sardinian populations as compared to the Calabrian and Sicilian populations. It is possible that H_e data could be influenced by the lower total number of individuals and a lower number of individuals per population in two populations (21 individuals in SAR16 and 15 individuals in SAR17). However, also the H_T values per region showed the lowest value for Sardinia and the highest for Sicily. The F_{IS} value was slightly negative for Sardinia (− 0.017), while it was positive in Calabria and Sicily where it was found to be 0.069 and 0.102, respectively (Table 4).

The genetic distance (G_{ST}) between populations belonging to the same geographical region (Table 4) showed significantly higher values for Sicily (0.084) than for Calabria and Sardinia (0.011 and 0.037, respectively). Pairwise G_{ST} values between geographical regions (Table 6) showed a higher degree of differentiation between Sardinia and Calabria (0.048), and Sardinia and Sicily (0.030) as compared to Sicily and Calabria (0.013).

The highest pairwise G_{ST} value (ESM, Table S1) was observed between SAR16 (*Q. virgiliana*—NW Sardinia) and CAL09 (*Q. dalechampii*—SW Calabria). Only one non-significant G_{ST} value was found in the pairwise matrix, between SIC05 (*Q. virgiliana*—Sicily) and SIC06 (*Q. virgiliana*—Sicily) (*p* value 0.292). These are two oak populations located at similar altitudes at the base of Etna volcano and distant about 4 km from each other as the crow flies.

The PCoA (Fig. 2) showed that all the populations from Sardinia (especially SAR16) segregated in the right part of the diagram, far away from the other populations investigated. Populations from Sicily and Calabria formed a mixed group on the left side of the diagram.

The Mantel test (Fig. 3) showed a positive, although weak, correlation ($R^2 = 0.085$; $p = 0.001$) between genetic distance (Gen by POP GD) and geographic distance (Geographic POP GGD) of populations.

According to the global AMOVA (Table 7), most of the genetic variation (92.05% percentage of variation) was found "within individuals" (*p* value 0.001), followed by that "among individuals within populations" (5.15%) and "among populations" (2.80%).

The Neighbor joining-based tree of populations showed the occurrence of three main clusters (A, B and C) exhibiting a low degree of significance (Fig. 4). Only populations from Sardinia form a distinct cluster with significant bootstrap support (51%). Group A is divided into two main subgroups, one of which (A1) is composed of all the Calabrian populations (CAL07-10) and the other (A2) of two Sicilian populations (SIC05 and SIC06) located very close to each other geographically. Group B comprises two Sicilian populations, one of these (SIC03) referred to a *Q. congesta* population

Table 4 Sample size and mean genetic diversity indices for the 17 populations sampled in Calabria, Sardinia and Sicily

Population	Region	N	N_p	N_a	A_r	H_o	H_e	F_{IS}	G_{ST}	$P(G_{ST})$
SIC01	Sicily	30	5	8.8	7.2	0.632	0.709	0.126		
SIC02	Sicily	29	11	8.6	7.2	0.660	0.721	0.103		
SIC03	Sicily	27	1	7.7	6.7	0.625	0.687	0.110		
SIC04	Sicily	30	3	8.7	6.9	0.606	0.672	0.115		
SIC05	Sicily	26	1	8.2	6.7	0.646	0.683	0.073		
SIC06	Sicily	30	1	7.8	6.7	0.636	0.703	0.112		
SIC11	Sicily	33	2	8.3	6.9	0.676	0.711	0.065		
SIC12	Sicily	28	2	7.3	6.2	0.589	0.669	0.137		
SIC13	Sicily	32	23	8.8	7.0	0.682	0.727	0.077		
Mean		29	5.4	8.2	6.8	0.639	0.698	0.102		
Total		265	49				0.790 (H_T)		0.084	0.001
CAL07	Calabria	28	0	7.8	6.7	0.648	0.684	0.071		
CAL08	Calabria	32	0	7.8	6.5	0.677	0.694	0.040		
CAL09	Calabria	29	1	7.9	6.6	0.688	0.706	0.043		
CAL10	Calabria	30	5	8.1	6.9	0.623	0.696	0.121		
Mean		30	1.5	7.9	6.7	0.659	0.695	0.069		
Total		119	5				0.722 (H_T)		0.011	0.001
SAR14	Sardinia	30	2	6.8	6.1	0.633	0.630	0.011		
SAR15	Sardinia	30	4	8.3	6.8	0.672	0.652	− 0.013		
SAR16	Sardinia	21	0	6.7	5.8	0.681	0.619	− 0.075		
SAR17	Sardinia	15	3	6.5	6.4	0.660	0.645	0.011		
Mean		24	2.3	7.1	6.3	0.662	0.637	− 0.017		
Total		96	9				0.687 (H_T)		0.037	0.001
General Mean		28	3.7	7.9	6.7	0.649	0.683	0.066		
Grand total		480	63	–	–	–	–	–		

N number of individuals, N_p number of private alleles, N_a number of alleles, A_r allelic richness rarefacted to the minimum sample size (28 genes), H_o observed heterozygosity, H_e expected heterozygosity, F_{IS} inbreeding coefficient, G_{ST} (analog of F_{ST} adjusted for bias) genetic differentiation among populations, $P(G_{ST})$ statistical significance of G_{ST}, H_T total expected heterozygosity

Table 5 ANOVA for H_o and H_e in Calabria, Sardinia and Sicily

Region	H_o	H_e
Calabria	0.659a	0.695b
Sardinia	0.662a	0.637a
Sicily	0.639a	0.698b
$Pr > F$ (Model)	0.340	0.000
Significant	No	Yes

Tukey–Kramer post hoc test was performed, a and b letters meaning different groups (significant for H_e)

Table 6 Pairwise matrix of G_{ST} values for Calabria, Sardinia and Sicily

	Sicily	Calabria	Sardinia
Sicily	0.000	0.001	0.001
Calabria	0.013	0.000	0.001
Sardinia	0.030	0.048	0.000

G_{ST} values below the diagonal. Probability, based on 999 permutations, is shown above the diagonal

from the montane belt of Etna volcano and the other (SIC12) to a *Q. congesta* population of the lower hilly belt of Nebrodi Mountains. Group C is the most numerous and is composed of a well-distinguishable subgroup (C1), which includes the four Sardinian populations and a set of single Sicilian populations that segregate more or less individually, except for SIC01 and SIC02. Genetic distances, which characterize the three main groups and the four further subgroups, are very low except for the Sardinian subgroup (C1). The generally low bootstrap values indicate low phylogenetic signal, suggesting historically high gene flow among populations.

The Neighbor joining-based tree of individuals (ESM, Fig. S1) is less easily interpretable than that based on populations. However, also in this case the individuals belonging to the Sardinian populations (SAR14, SAR15, SAR16 and SAR17) group together in the same cluster while the individuals of the Sicilian and Calabrian populations tend to mix with each other.

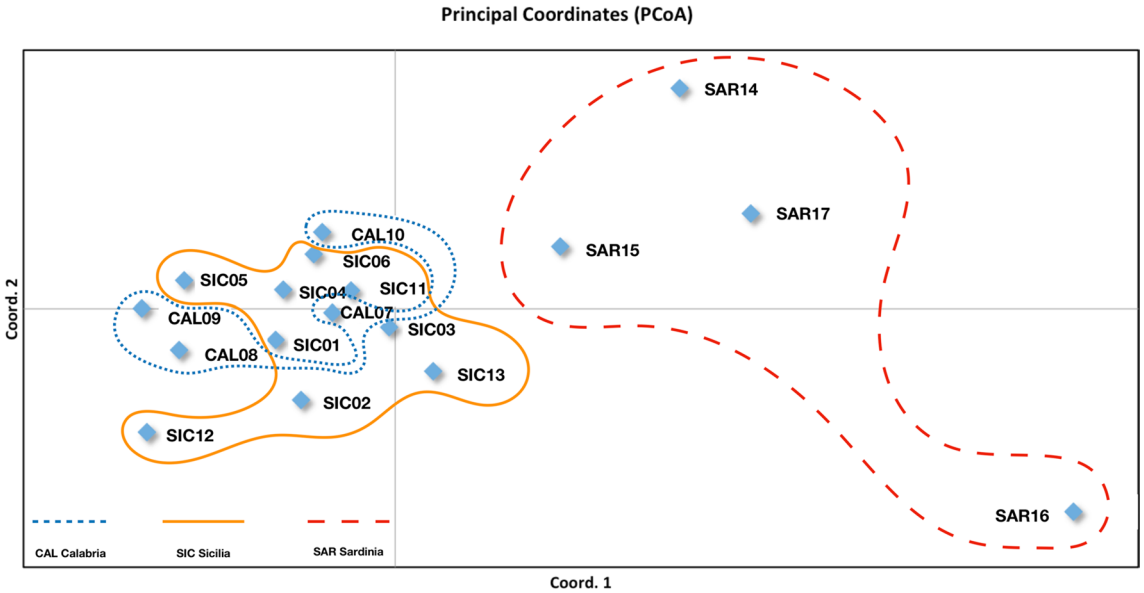

Fig. 2 Principal coordinate analysis (PCoA) of the 17 populations sampled in Calabria, Sardinia and Sicily (coordinate 1 and coordinate 2 explain 33.31% and 15.94% of the variation between populations, respectively)

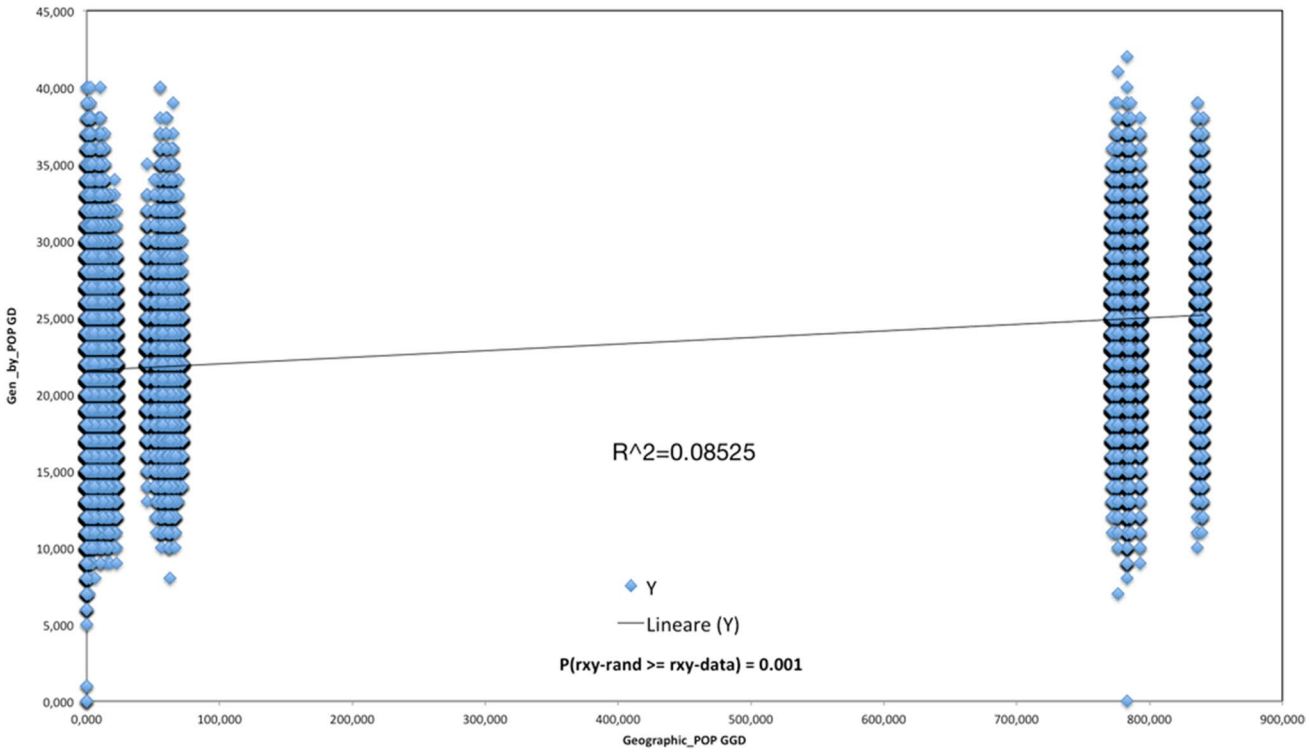

Fig. 3 Isolation-by-distance patterns for individuals, plotting pairwise co-dominant genotypic distance (Gen by POP GD) versus pairwise geographic distances (Geographic POP GGD). The figure includes statistical significance ($p = 0.001$) obtained by simple Mantel tests in GenAlEx, version 6.5. Each point (diamond) represents a pairwise comparison

Table 7 AMOVA results as weighted average over loci for the 17 populations sampled in Calabria, Sardinia and Sicily

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage of variation	Probability
Among populations	16	173.084	0.011644	2.80	0.0001
Among individuals within populations	463	1968.462	0.29870	5.15	0.0001
Within individuals	480	1835.500	3.82396	92.05	0.0001
Total	959	3977.046	4.1541		
Fixation indices	F_{IS} 0.05295	F_{ST} 0.02803	F_{IT} 0.07949		

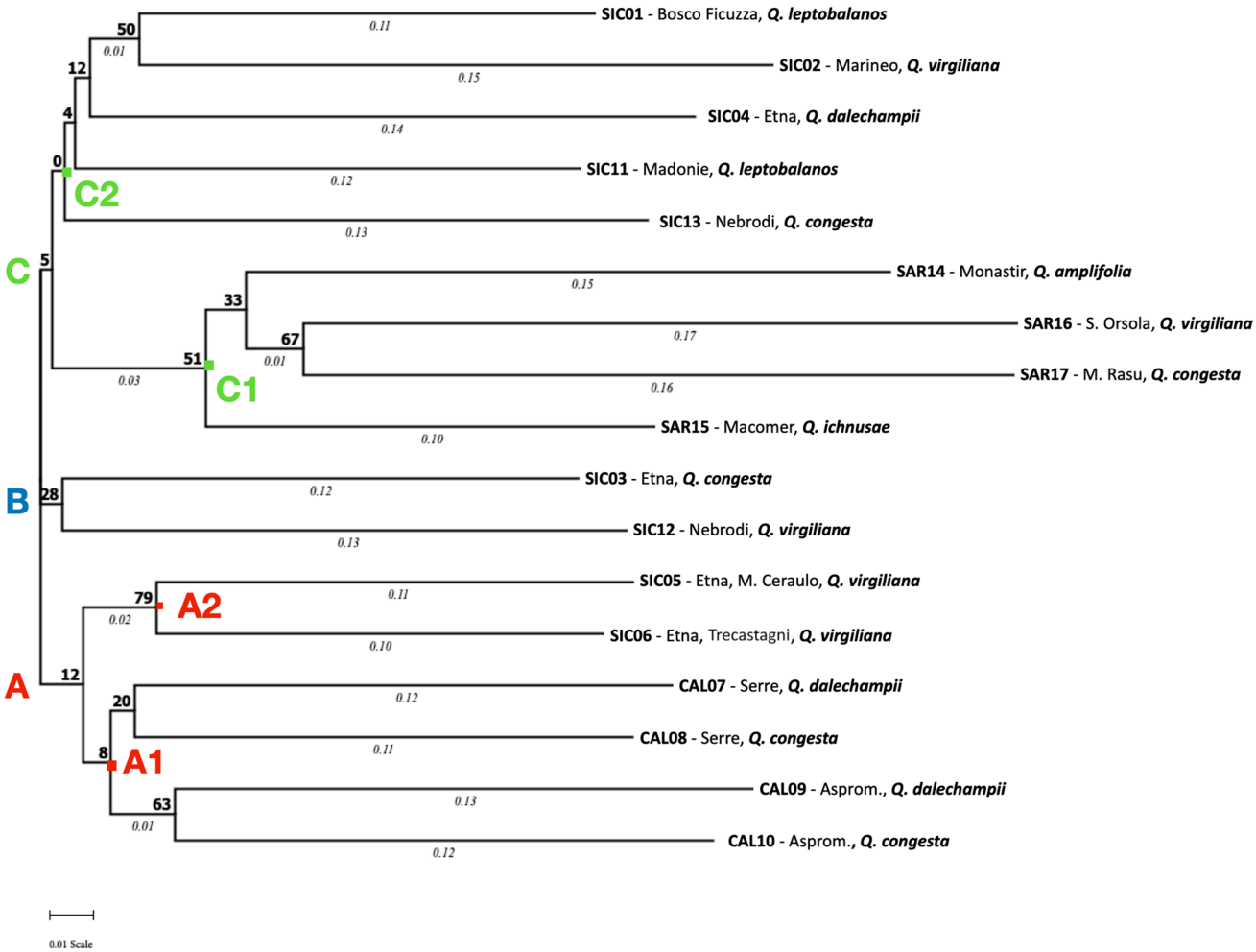

Fig. 4 Neighbor-joining tree (NJT) of the 17 populations sampled in Calabria, Sardinia and Sicily based on the chord genetic distance of Cavalli-Sforza and Edwards (1967)

The Bayesian analysis revealed $K = 2$ as the most probable number of genetic clusters obtained with the ad hoc statistic ΔK (ESM, Fig. S2 and Tables S2, S3). A total of 177, out of 480 samples analyzed exhibited Q values > 0.90 of which 74 belonging to cluster 1 and 103 to cluster 2. All the $Q > 90$ samples coming from the four Sardinia populations belong to cluster 2 whereas almost all the Sicilian and Calabrian populations were found to be composed of samples belonging to both clusters (Fig. 5 and ESM, Fig. S3). The only exceptions were populations SIC04 and CAL08 which presented $Q > 90$ individuals

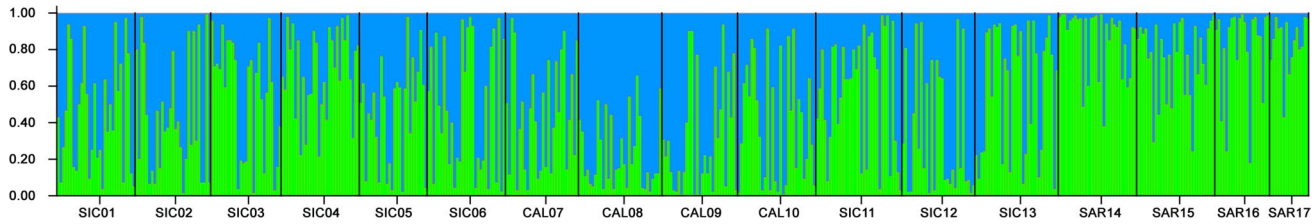

Fig. 5 STRUCTURE analysis ($K=2$) for all the 17 populations sampled in Calabria Sardinia and Sicily

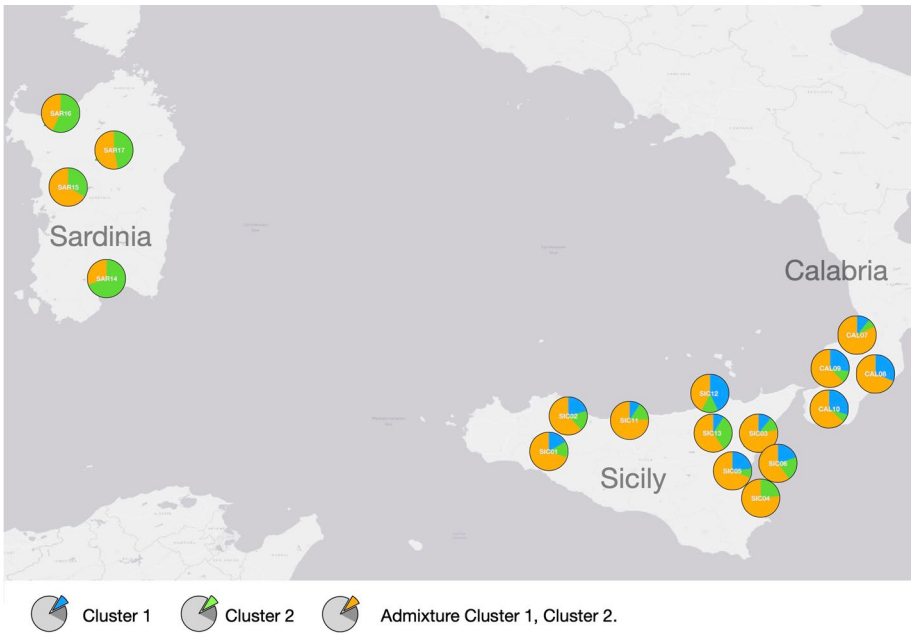

Fig. 6 Distribution of individuals with $Q>90$ for each cluster in all 17 populations sampled in Calabria Sardinia and Sicily on the geographical base map

all belonging to a single cluster (cluster 2 and cluster 1, respectively).

Plotting the STRUCTURE (Fig. 6) results on the geographic map shows that individuals not clearly assigned to cluster 1 or 2 prevail for most of the investigated populations. Individuals assigned to cluster 1 are absent from Sardinia, while cluster 2 individuals are more frequent and mixed individuals are less frequent in Sardinia than in Calabria and Sicily.

Discussion

In the last 25 years we have witnessed an increase in molecular studies in Europe aimed at identifying possible distinctive characteristics within white oaks (Bacilieri et al. 1995; Bruschi et al. 2000, 2003; Csaikl et al. 2002; Curtu et al. 2007a, b; Fortini et al. 2009; Lepais et al. 2009; Lepais and Gerber 2011; Enescu et al. 2013; Yücedag and Gailing 2013; Fortini et al. 2015). However, no references were available about in-detail inter-population genetic studies undertaken for the southernmost part of Italy, Sicily and Sardinia even though all these areas are unanimously considered of great importance for the evolution and phenotypic differentiation of white oaks. In fact, some interesting phylogeographic studies based on cpDNA diversity (Fineschi and Vendramin 2004) had already shown that some of the haplotypes observed in central and northern Europe originated from Italy and in particular from the southern and island regions as result of postglacial recolonization (Fineschi et al. 2002, 2004; Petit et al. 2002a).

The present study fills a gap in the genetic knowledge of the genus *Quercus* in Italy and provides a better understanding of phenotypic and taxonomic diversity among pubescent white oaks in southern Italy which is considered a center of diversity for pubescent oaks in Europe (see Tutin et al. 1993; Pignatti et al. 2018, 2019). However, there is still a very lively debate, especially among the taxonomists of southern Europe, about the possibility of keeping all these pubescent-oak taxa at the species or subspecies ranks or whether to consider the phenotypic diversity observed as included in

the morphological variability pattern of a single widely distributed species (e.g., a pan-European *Q. pubescens* Willd.). Accordingly, in addition to shedding light on genetic diversity of white oaks in an area still without detailed molecular studies, the aim of this work was to evaluate whether this high phenotypic and taxonomic diversity corresponded to an equally significant level of genetic diversity or biogeographical identity.

The populations investigated show a fair level of genetic polymorphism. Taking into account individual loci, we have found a rather high average number of alleles per locus (14.08) ranging between 8 (PIE227) and 20 (PIE102). The average number of alleles per population and locus was found to range between 2.9 (PIE227) and 11.5 (PIE152). For H_o and H_e we found values of 0.649 and 0.683, respectively. All these values appear to be quite high when compared with those obtained in a similar study performed in the Apulian Peninsula in the south-easternmost sector of Italy (Di Pietro et al. 2020, Table 2). These results were unexpected, especially considering that Sicily, Sardinia and southern Calabria exhibit a higher geographical isolation when compared to that of the Apulian Peninsula. In fact, the degree of gene polymorphism for the study area was expected to be lower than that from continental areas where, at least in theory, it is conceivable that there may be a greater possibility of gene flow among populations. It is possible that the rugged geomorphology of southern Calabria, Sicily and Sardinia when compared with that of Apulia played a major role in determining such differences in the genetic diversity of these two areas. The Apulian Peninsula is composed of carbonate plateaus (1116 m the highest culmination) separated from the rest of the Italian Peninsula by a vast cultivated plain where the forest stands are scattered in a general matrix of olive groves, vineyards and wheat fields or separated from each other by mosaics of Mediterranean maquis and steppe-like grasslands (Di Pietro and Misano 2009; Biondi et al. 2010; Terzi et al. 2010). On the other hand, Sicily, Sardinia and southern Calabria are all characterized by remarkable mountainous systems whose highest peaks are all ranging between 1600 and 2000 m (see Aspromonte, Nebrodi, Madonie, Gennargentu, Supramonte massifs) with Etna volcano main culminations well above 3000 m. The presence of these mountains has probably made available a greater variety of habitats (and consequently of refuge sites) for the forest vegetation during the climatic oscillations of the Quaternary that allowed a greater territorial contiguity for the surviving oak woods. Three loci (PIE020, PIE239 and PIE242) showed an excess of homozygosity (deviation from Hardy–Weinberg equilibrium $p < 0.05$ for the first two loci and $p < 0.01$ for the third). For PIE020, PIE239 null alleles were also detected and therefore possible non-random distribution of genotypes and distorted values of heterozygosity (Brown et al. 2005).

The level of intra-population genetic diversity in the study area shows an average value of alleles per population of 7.9 with an average allelic richness of 6.7. H_o and H_e values are also quite high (0.64 and 0.68, respectively) and do not show substantial differences if we consider the three study areas (Sicily, Southern Calabria and Sardinia) separately (Table 5). Instead, G_{ST} values between populations are very different if the three study areas compared. It is possible, however, that the high values in Sicily when compared to Calabria and Sardinia are influenced by the number of populations analyzed which for Sicily are more than double those of the other two regions. In general, the genetic diversity indices that emerge from this study were found to be significantly higher than those exhibited by the pubescent oak populations of the Apulian Peninsula but lower if compared with those found for a pubescent oak population from the southern/central Apennines (Mount Vairano) which were analyzed at the same markers (ESM, Table S5). It is possible that the higher indices found in the Mount Vairano *Q. pubescens* forests are due to the co-occurrence and potential hybridization with other white oak species (e.g., *Q. frainetto* and *Q. petraea*) and to the spatial contiguity of the *Q. robur* stands occurring in the foothills.

Genetic assignment, Principal Coordinate Analysis and the Neighbor-Joining tree separated Sardinian populations from Sicilian and Calabrian populations. This separation into two groups can probably be addressed to the greater insularity of Sardinia as compared to Sicily, the latter being separated from southern Calabria by only a narrow stretch of sea (3 km). Despite the close geographical proximity between Calabria and Sicily, however, the interactions between the oak populations of these two territories may have been less than one might expect. In a study on the non-coding regions of chloroplast DNA of Italian populations of deciduous oaks, Fineschi and Vendramin (2004) hypothesized that the missing seed migration from Calabria to Sicily of an eastern haplotype was related to the depth of the Ionian Sea which prevented its freezing even during the phases of maximum glacial extension and prevented the establishment of a land corridor between the two regions. However, not all authors agree on this point. According to some paleontologists during the Quaternary period territorial connections were established between Calabria and Sicily through which several mammalian taxa from continental areas dispersed into Sicily (Bonfiglio et al. 2002).

The correlation between genetic and geographical distance among populations revealed by the Mantel test was found to be positive and statistically significant, however very low. In fact, most of the genetic diversity found is observed within single individuals (92.05%) followed by genetic diversity among individuals within the same populations (5.15%) and among different populations (2.80%).

On the basis of Bayesian cluster analysis (STRUCTURE), the most probable number of clusters considering all individuals from all the populations is two ($K=2$).

It is interesting to note that about one third of the individuals (177) had a value of $Q>90$ so they could be considered as genetically "pure". It therefore seems plausible to hypothesize the existence of two distinguishable ancestral populations for the pubescent oaks in the investigated area which for the most part are mixed (in variable proportions) in single individuals. However, genetic clusters do not represent different taxonomic units, but rather differentiation, between Sardinian and Calabrian/Sicilian populations, and this genetic differentiation does not correspond with taxonomic descriptions in the published taxonomic-phytosociological reports. For example, all the Sardinian populations belong to a single genetic cluster (cluster 2) so the records for *Q. congesta*, *Q. amplifolia*, *Q. ichnusae*, *Q. virgiliana* reported in the taxonomic and phytosociological literature for Sardinia should be summarized to one single taxon (Fig. 6). Furthermore, since pure individuals belonging to cluster 2 were also found in Sicilian and Calabrian populations, the presence of an endemic Sardinian species could not be confirmed (e.g., *Q. ichnusae*). Also, for Sicilian and Calabrian populations genetic differentiation patterns do not suggest the presence of more than one taxon displaying inconsistencies that are difficult to interpret in a taxonomic key. For example, population CAL08 that was described as *Q. congesta* population (Nebrodi mountains montane belt) is characterized by unadmixed genetically pure individuals all belonging to cluster 1 whereas population SIC13 that should be another *Q. congesta* stand is characterized by a large dominance of unadmixed individuals assigned to cluster 2.

According to the phytosociological literature, the four Sardinian populations belong to at least three different species. Overall, the dendrogram does not cluster populations according to putative species, but shows a weak phylogeographic pattern with the Sardinian populations separated and populations collected in neighboring sites grouping together (Fig. 4, see subgroups A1 and C2). Both the first and the second level of clustering, bring together different pubescent-oak (putative) species which, at least based on their original diagnosis and current coenological knowledge (Brullo and Marcenò 1985; Brullo et al. 1999; Mossa et al. 1999; Bacchetta et al. 2009) would have a very different ecology from each other. In the group of Sardinian populations of *Q. congesta*, *Q. virgiliana* and *Q. ichnusae* group together. The Calabrian group includes *Q. congesta*, and *Q. dalechampii*. However, the two further subgroups of which the main Calabrian subgroup is composed of (CAL09-CAL10 and CAL07-CAL08) are both composed of a population of *Q. congesta* and one of *Q. dalechampii*. In particular, CAL09 was described as a *Q. dalechampii* population of the Mesothermo Mediterranean bioclimate of the Gioia Tauro plain less than 100 meters a.s.l. while CAL10 was described as a *Q. congesta* population of the lower mountain belt of the Aspromonte massif at about 1000 m a.s.l. Only the subgroup SIC05-SIC06 clusters two populations belonging to the same putative species (*Q. virgiliana*).

Taking into account all the results of the work, the most plausible interpretation of the results is that all the oak populations sampled belong to a single oak taxon which is characterized by a large ecological and morphological amplitude. Although there is still no scientific certainty, the morphological and molecular pattern among pubescent white oaks evidenced in this paper, and those already shown in previous papers for other pubescent-oak populations from the central Mediterranean area (Franjic et al. 2006; Viscosi et al. 2009, 2012; Ballian et al. 2010; Di Pietro et al. 2016, 2020), increasingly reinforce the idea that this "single highly variable pubescent oak taxon" could be the result of repeated events of hybridisation and introgression between an ancient pubescent white oak species (which for simplicity we could here name *Q. pubescens*) and other European white oak species (e.g., *Q. petraea*, *Q. frainetto*, *Q. robur*). These events would have taken place continuously since the Tertiary and may have even intensified during the Pleistocene following the drastic paleogeographic and paleoclimatic events that characterized this Era. Such a consideration, if translated into a taxonomic key, would exclude a too divisive classification within the collective group of *Q. pubescens*, and indeed would support the "minimalist" view considering just a single pubescent oak taxon at the rank of species. The Bayesian analysis suggests that there are two main genetic clusters among the pubescent oaks of southern Italy and major islands. Further studies involving other white oak species could provide information on the origin and genetic diversity of the clusters identified. We did not find any correlation between these two genetic groups and the current taxonomic classification of the pubescent oaks in southern Italy. Furthermore, this paper shows a genetic diversification among the Sardinian populations as result of geographical isolation. This result was not completely unexpected if we consider that Fineschi and Vendramin (2004) identified a Sardinian-Corsican endemic haplotype for oaks restricted to these two islands. Actually, population SAR16 (a relic population composed of individuals currently identified as *Q. virgiliana* from a northwestern Sardinian plain) exhibits comparatively low allelic diversity (see Table 4) as reflected in the complete absence of private alleles and the about lowest values for allelic richness and number of alleles among all populations. The comparatively low allelic diversity could be the result of geographic and topographic isolation. This population appears to be composed of few individuals very similar to each other, surrounded by several very young juvenile

trees. This suggests that SAR16 population may have experienced selective removal of trees aimed at favoring particular phenotypes that in addition to changing physiognomy and structure could have also influenced the genetic diversity of the forest ecosystem. A narrow selection of seed-producing trees may in fact lead to a lower variability in forest stands (Dostálek et al. 2011) so that it could be assumed that the centuries-old individuals scattered in the SAR16 population (or at least a part of them) are progeny of few progenitor oaks. The hypothesis that SAR16 stand originates from just a few progenitors is in agreement with what reported in Lawson et al. (2018), as the relatively strong genetic drift experienced by these trees would cause them to appear as a discrete cluster.

Conclusion

As a first study on the genetic diversity of the southern Italy and major islands pubescent-oak populations, this paper displayed relatively high values for all parameters of genetic diversity although more than two thirds of the study area was made up of island territory. We hypothesize that the rugged morphology and wide altitudinal amplitude may have played a role in preserving the spatial contiguity between oak woods in the study area during the Quaternary climatic oscillations and therefore in preserving also high levels of gene flow.

A genetic confirmation for a taxonomical classification providing up to seven pubescent oak species as occurring in the study area did not emerge from this study, despite reported by the most recent floras and checklists and by phytosociological papers as well. Such a result is in accordance with morphological and molecular analyses carried out on pubescent oak populations in south-eastern Italy (Di Pietro et al. 2016, 2020) where it was demonstrated that neither morphological nor molecular results supported the occurrence of more than one pubescent oak species whereas four species were reported by previous phytosociological studies (Biondi et al. 2004, 2010). The oak material analyzed in our study did not show a degree of molecular diversity, within and among populations, sufficient to support this wide taxonomical splitting. However, Bayesian analysis, multivariate statistics and the NJ dendrogram separated Sardinian populations from Calabrian and Sicilian populations mirroring the geographic separation of populations. Our results suggest that all populations investigated belong to a single taxon characterized by a wide range of intra-individual and intraspecific genotypic and phenotypic diversity as result of ecological pressures to which particular groups of oak species are subjected (Kremer and Hipp 2019). The comparatively high genetic diversity highlighted among these S-Italy populations may suggest innumerable events of hybridization and introgression that could have happened between an ancestral pubescent oak (which for simplicity we will call here *Q. pubescens* s.l.) and other sympatric thermophilous white oaks over the ages. Thanks to the favorable geographical location of southern Italy, in Sicily and Sardinia these events could have occurred without significant interruptions even during the coldest periods of the Quaternary where the different oak species (*Q. pubescens* s.l., *Q. petraea*, *Q. robur* and *Q. frainetto*) were forced to live in very restricted areas. Possible hints of a process of speciation in progress for the Sardinian populations related to the highlighted (weak) correspondence between genetic and geographic distance and to the geographical isolation of this island are premature and will require further and more detailed studies.

Beyond the phylogenetic or taxonomic relevance, the results have implications for forest economy (timber certification) or nature conservation, if we consider that some of the oak names in issue occur in the list of diagnostic species for European Habitats included in the 92/43/EC Directive (European Commission 2013).

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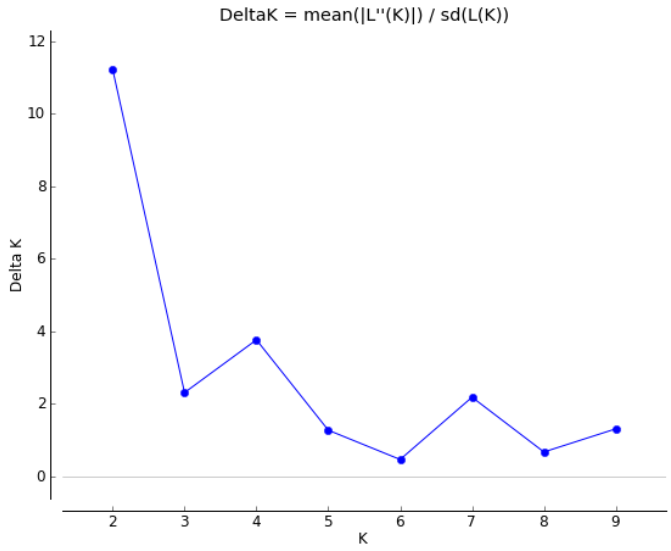
Supplementary Table S1 Pairwise population matrix of G_{ST} values

	SIC01	SIC02	SIC03	SIC04	SIC05	SIC06	CAL07	CAL08	CAL09	CAL10	SIC11	SIC12	SIC13	SAR14	SAR15	SAR16	SAR17
SIC01		0.048	0.007	0.004	0.005	0.014	0.013	0.010	0.001	0.001	0.033	0.004	0.057	0.001	0.001	0.001	0.001
SIC02	**0.004**		0.003	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.004	0.001	0.001	0.001	0.001	0.001
SIC03	**0.007**	**0.007**		0.002	0.001	0.004	0.001	0.001	0.001	0.002	0.002	0.002	0.059	0.001	0.001	0.001	0.001
SIC04	**0.006**	**0.011**	**0.008**		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
SIC05	**0.007**	**0.012**	**0.010**	**0.014**		0.292	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
SIC06	**0.005**	**0.010**	**0.007**	**0.011**	0.001		0.023	0.001	0.001	0.009	0.002	0.001	0.003	0.001	0.001	0.001	0.001
CAL07	**0.006**	**0.010**	**0.008**	**0.013**	**0.010**	**0.004**		0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
CAL08	**0.005**	**0.007**	**0.012**	**0.016**	**0.010**	**0.008**	**0.007**		0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
CAL09	**0.010**	**0.010**	**0.011**	**0.018**	**0.008**	**0.008**	**0.008**	**0.006**		0.019	0.001	0.001	0.001	0.001	0.001	0.001	0.001
CAL10	**0.011**	**0.012**	**0.008**	**0.011**	**0.013**	**0.005**	**0.012**	**0.009**	**0.005**		0.001	0.001	0.001	0.001	0.001	0.001	0.001
SIC11	**0.004**	**0.012**	**0.008**	**0.012**	**0.008**	**0.007**	**0.010**	**0.014**	**0.011**	**0.014**		0.001	0.010	0.001	0.001	0.001	0.001
SIC12	**0.008**	**0.008**	**0.010**	**0.017**	**0.015**	**0.013**	**0.013**	**0.012**	**0.011**	**0.017**	**0.018**		0.001	0.001	0.001	0.001	0.001
SIC13	0.003	**0.009**	0.003	**0.010**	**0.013**	**0.006**	**0.009**	**0.012**	**0.011**	**0.011**	**0.005**	**0.011**		0.001	0.001	0.001	0.001
SAR14	**0.021**	**0.027**	**0.023**	**0.025**	**0.029**	**0.019**	**0.023**	**0.029**	**0.029**	**0.020**	**0.022**	**0.037**	**0.022**		0.001	0.001	0.001
SAR15	**0.010**	**0.014**	**0.007**	**0.016**	**0.015**	**0.011**	**0.008**	**0.017**	**0.017**	**0.016**	**0.009**	**0.020**	**0.011**	**0.013**		0.001	0.001
SAR16	**0.037**	**0.035**	**0.033**	**0.045**	**0.048**	**0.038**	**0.036**	**0.045**	**0.049**	**0.042**	**0.038**	**0.048**	**0.027**	**0.039**	**0.030**		0.001
SAR17	**0.023**	**0.023**	**0.016**	**0.029**	**0.026**	**0.015**	**0.021**	**0.029**	**0.030**	**0.017**	**0.024**	**0.034**	**0.018**	**0.023**	**0.015**	**0.029**	

G_{ST} values below the diagonal; significant values are in bold. Probability, based on 999 permutations, is shown above diagonal.
G_{ST} = analog of F_{ST} adjusted for bias: $G_{ST} = (cH_t - cH_s)/cH_t$.

Supplementary Fig. S1 Neighbor-joining tree (NJT) of all the pubescent oak individuals sampled in the three Regions (Calabria, Sardinia and Sicily) based on the chord genetic distance of Cavalli-Sforza and Edwards (1967) using software Populations 1.2.32. For graphic processing MEGA 7.0.26 software (Kumar et al. 2016) was used. The Sardinian individuals (marked in yellow) are mostly concentrated in a single cluster of the NJT while those from Sicily and Calabria (not marked with a color) show a wider distribution along the entire Tree.
ST01=SIC01; ST02=SIC02; ST03=SIC03; ST04=SIC04; ST05=SIC05; ST06=SIC06; ST07=CAL07; ST08=CAL08; ST09=CAL09; ST10=CAL10; ST11=SIC11; ST12=SIC12; ST13=SIC13; ST14=SAR14; ST15=SAR15; ST16=SAR16; ST17=SAR17.



Supplementary Fig. S2 DeltaK graph plot by CLUMPACK. Optimal K by Evanno method is: 2.

Supplementary Table S2 Raw STRUCTURE output from Structure Harvester.

File name	Run #	K	Est. Ln prob. of data	Mean value of Ln likelihood	Variance of Ln likelihood
run_19_f	19	1	-19934.3	-19897.3	74.1
run_16_f	16	1	-19935.3	-19897.4	75.7
run_17_f	17	1	-19935.7	-19897.4	76.7
run_12_f	12	1	-19935.6	-19897.4	76.3
run_7_f	7	1	-21049.7	-19907.3	2284.8
run_2_f	2	1	-19943.2	-19897.3	91.8
run_4_f	4	1	-20630.8	-19903.6	1454.4
run_6_f	6	1	-20164.5	-20127.3	74.4
run_15_f	15	1	-21025.4	-19907.2	2236.6
run_3_f	3	1	-19938.7	-19897.5	82.3
run_5_f	5	1	-19975.4	-19897.9	155.1
run_18_f	18	1	-19934.3	-19897.4	73.8
run_8_f	8	1	-19935.2	-19897.4	75.6
run_11_f	11	1	-19940.5	-19897.5	86.0
run_20_f	20	1	-20398.9	-19901.5	994.8
run_10_f	10	1	-19936.6	-19897.6	77.9
run_14_f	14	1	-19940.3	-19897.4	85.8
run_9_f	9	1	-19934.2	-19897.3	73.9

run_1_f	1	1	-19936.3	-19897.5	77.8
run_13_f	13	1	-20590.2	-19903.3	1373.9
run_27_f	27	2	-19809.1	-19565.3	487.6
run_21_f	21	2	-19826.5	-19567.5	518.0
run_35_f	35	2	-19871.8	-19585.5	572.6
run_25_f	25	2	-19838.2	-19576.9	522.5
run_36_f	36	2	-19859.5	-19582.9	553.1
run_30_f	30	2	-19824.3	-19573.0	502.7
run_38_f	38	2	-19821.5	-19575.7	491.6
run_40_f	40	2	-19843.0	-19570.1	545.8
run_31_f	31	2	-19832.9	-19574.6	516.5
run_39_f	39	2	-19839.0	-19568.5	541.2
run_34_f	34	2	-19829.4	-19567.4	524.0
run_32_f	32	2	-19879.6	-19574.7	609.8
run_22_f	22	2	-19842.4	-19572.4	539.8
run_23_f	23	2	-19823.0	-19569.3	507.4
run_24_f	24	2	-19887.7	-19584.0	607.4
run_26_f	26	2	-19849.7	-19569.6	560.2
run_29_f	29	2	-19819.6	-19568.7	501.8
run_28_f	28	2	-19835.6	-19571.1	529.1
run_37_f	37	2	-19812.7	-19573.7	478.1
run_33_f	33	2	-19813.6	-19569.8	487.6
run_56_f	56	3	-19711.6	-19273.8	875.5
run_47_f	47	3	-19764.5	-19286.4	956.1
run_43_f	43	3	-19769.2	-19287.4	963.7
run_48_f	48	3	-19753.9	-19312.9	882.1
run_50_f	50	3	-19823.8	-19292.1	1063.3
run_60_f	60	3	-19843.4	-19301.6	1083.5
run_46_f	46	3	-19768.4	-19282.2	972.3
run_53_f	53	3	-19847.0	-19290.5	1113.0
run_51_f	51	3	-19715.4	-19276.3	878.2
run_45_f	45	3	-19798.7	-19290.8	1015.8
run_52_f	52	3	-19721.2	-19276.8	888.7
run_59_f	59	3	-19785.0	-19283.1	1003.8
run_42_f	42	3	-19780.0	-19288.6	982.9
run_54_f	54	3	-19749.2	-19284.1	930.3
run_55_f	55	3	-19821.3	-19300.3	1041.9
run_57_f	57	3	-19776.3	-19291.3	970.2
run_44_f	44	3	-19744.6	-19279.3	930.7

run_58_f	58	3	-19770.2	-19285.2	970.0
run_49_f	49	3	-19728.9	-19279.3	899.0
run_41_f	41	3	-19803.6	-19286.7	1033.7
run_79_f	79	4	-19582.1	-19051.0	1062.2
run_67_f	67	4	-19671.9	-19055.7	1232.5
run_72_f	72	4	-19600.2	-19051.9	1096.5
run_73_f	73	4	-19626.8	-19058.2	1137.2
run_65_f	65	4	-19645.7	-19053.2	1185.1
run_66_f	66	4	-19598.3	-19050.0	1096.6
run_76_f	76	4	-19579.3	-19051.3	1056.1
run_80_f	80	4	-19625.4	-19052.7	1145.4
run_61_f	61	4	-19580.3	-19054.0	1052.5
run_69_f	69	4	-19598.3	-19050.4	1095.7
run_70_f	70	4	-19607.0	-19054.3	1105.4
run_78_f	78	4	-19632.8	-19054.7	1156.3
run_62_f	62	4	-19629.5	-19053.1	1152.9
run_77_f	77	4	-19583.6	-19055.3	1056.6
run_71_f	71	4	-19616.8	-19058.7	1116.1
run_74_f	74	4	-19586.8	-19052.4	1068.9
run_64_f	64	4	-19623.9	-19050.9	1146.0
run_75_f	75	4	-19663.8	-19058.9	1209.9
run_63_f	63	4	-19614.0	-19048.5	1131.0
run_68_f	68	4	-19675.1	-19055.3	1239.6
run_94_f	94	5	-19569.6	-18874.6	1390.0
run_83_f	83	5	-19593.7	-18877.1	1433.0
run_98_f	98	5	-19595.5	-18862.3	1466.3
run_85_f	85	5	-19555.8	-18879.0	1353.6
run_90_f	90	5	-19609.0	-18877.1	1463.7
run_97_f	97	5	-19528.8	-18877.5	1302.5
run_81_f	81	5	-19501.0	-18869.9	1262.1
run_89_f	89	5	-19579.7	-18878.8	1401.9
run_95_f	95	5	-19549.6	-18868.6	1362.0
run_100_f	100	5	-19598.3	-18880.9	1434.8
run_88_f	88	5	-19527.0	-18867.9	1318.3
run_92_f	92	5	-19561.6	-18872.0	1379.3
run_96_f	96	5	-19637.3	-18880.6	1513.5
run_84_f	84	5	-19641.9	-18886.1	1511.7
run_91_f	91	5	-19531.4	-18879.2	1304.4
run_93_f	93	5	-19669.8	-18881.2	1577.2

run_99_f	99	5	-19559.9	-18865.8	1388.3
run_82_f	82	5	-19538.5	-18875.7	1325.6
run_87_f	87	5	-19578.5	-18872.9	1411.1
run_86_f	86	5	-19538.2	-18867.6	1341.3
run_120_f	120	6	-19634.1	-18713.3	1841.5
run_108_f	108	6	-19587.3	-18717.6	1739.4
run_118_f	118	6	-19500.2	-18716.5	1567.2
run_107_f	107	6	-19540.0	-18720.2	1639.5
run_112_f	112	6	-19813.4	-18720.8	2185.1
run_103_f	103	6	-19552.0	-18711.0	1681.9
run_101_f	101	6	-20093.9	-18729.8	2728.4
run_102_f	102	6	-19538.6	-18714.7	1647.8
run_110_f	110	6	-19470.8	-18706.9	1527.9
run_114_f	114	6	-19541.9	-18710.4	1663.0
run_106_f	106	6	-19551.3	-18718.6	1665.4
run_119_f	119	6	-19540.9	-18714.3	1653.2
run_105_f	105	6	-19512.3	-18705.1	1614.5
run_104_f	104	6	-19603.4	-18721.4	1764.0
run_117_f	117	6	-19570.8	-18718.2	1705.2
run_113_f	113	6	-19510.9	-18704.2	1613.2
run_111_f	111	6	-19485.1	-18708.6	1552.9
run_115_f	115	6	-19488.1	-18708.2	1559.9
run_116_f	116	6	-19701.2	-18722.4	1957.5
run_109_f	109	6	-19449.3	-18705.9	1486.7
run_125_f	125	7	-19398.2	-18549.2	1698.1
run_136_f	136	7	-19564.6	-18561.3	2006.6
run_122_f	122	7	-19428.5	-18558.7	1739.6
run_133_f	133	7	-19762.4	-18577.0	2370.8
run_130_f	130	7	-19529.0	-18567.6	1922.8
run_131_f	131	7	-19480.4	-18558.3	1844.2
run_123_f	123	7	-19518.9	-18557.6	1922.7
run_137_f	137	7	-19609.8	-18573.9	2071.7
run_124_f	124	7	-19564.8	-18558.9	2011.9
run_134_f	134	7	-19573.3	-18575.6	1995.4
run_126_f	126	7	-19411.9	-18558.8	1706.2
run_140_f	140	7	-19522.3	-18569.0	1906.5
run_129_f	129	7	-19608.8	-18563.9	2089.9
run_128_f	128	7	-19575.1	-18581.5	1987.1
run_127_f	127	7	-19502.8	-18568.2	1869.3

run_138_f	138	7	-19484.2	-18576.7	1815.1
run_121_f	121	7	-19535.3	-18576.7	1917.2
run_135_f	135	7	-19510.2	-18576.1	1868.3
run_132_f	132	7	-19424.1	-18555.0	1738.2
run_139_f	139	7	-19569.3	-18581.0	1976.6
run_153_f	153	8	-19526.8	-18432.8	2188.0
run_157_f	157	8	-19931.8	-18459.7	2944.2
run_147_f	147	8	-19524.2	-18428.3	2191.8
run_156_f	156	8	-19511.2	-18428.4	2165.7
run_144_f	144	8	-19395.8	-18422.9	1945.8
run_149_f	149	8	-19591.0	-18430.6	2320.9
run_151_f	151	8	-20288.0	-18446.9	3682.2
run_141_f	141	8	-19445.8	-18420.8	2049.9
run_145_f	145	8	-19404.0	-18425.0	1958.0
run_143_f	143	8	-19603.5	-18436.4	2334.1
run_142_f	142	8	-19453.9	-18424.5	2058.9
run_148_f	148	8	-20077.6	-18484.8	3185.6
run_159_f	159	8	-19385.4	-18412.8	1945.1
run_160_f	160	8	-19540.6	-18437.5	2206.3
run_155_f	155	8	-19499.2	-18428.5	2141.4
run_152_f	152	8	-19834.3	-18447.7	2773.1
run_146_f	146	8	-19442.7	-18429.9	2025.6
run_154_f	154	8	-20535.3	-18492.1	4086.4
run_150_f	150	8	-19586.8	-18432.5	2308.8
run_158_f	158	8	-19527.9	-18426.1	2203.5
run_164_f	164	9	-19456.9	-18295.6	2322.7
run_178_f	178	9	-19794.5	-18318.1	2952.8
run_179_f	179	9	-19459.2	-18297.7	2323.2
run_169_f	169	9	-19480.9	-18291.3	2379.1
run_172_f	172	9	-19352.7	-18276.8	2151.9
run_173_f	173	9	-19491.4	-18284.2	2414.5
run_162_f	162	9	-19599.5	-18300.2	2598.5
run_166_f	166	9	-19707.1	-18317.7	2778.7
run_163_f	163	9	-19591.6	-18308.9	2565.3
run_171_f	171	9	-19652.2	-18305.2	2694.0
run_177_f	177	9	-19507.1	-18300.9	2412.2
run_165_f	165	9	-19577.1	-18294.5	2565.2
run_170_f	170	9	-19287.3	-18282.1	2010.3
run_161_f	161	9	-19666.3	-18283.6	2765.5

run_175_f	175	9	-19462.4	-18284.4	2356.0
run_174_f	174	9	-19556.1	-18296.4	2519.5
run_180_f	180	9	-20194.4	-18312.5	3763.9
run_168_f	168	9	-19666.3	-18294.8	2743.0
run_167_f	167	9	-19354.2	-18281.8	2144.7
run_176_f	176	9	-19570.7	-18296.2	2548.9
run_192_f	192	10	-19536.4	-18176.9	2718.8
run_196_f	196	10	-19495.9	-18160.2	2671.4
run_186_f	186	10	-19702.0	-18187.4	3029.2
run_183_f	183	10	-19703.4	-18184.0	3038.8
run_187_f	187	10	-19623.6	-18164.7	2917.7
run_194_f	194	10	-19942.6	-18204.4	3476.4
run_199_f	199	10	-19545.6	-18165.7	2759.9
run_182_f	182	10	-19803.2	-18174.8	3256.7
run_188_f	188	10	-19499.8	-18165.8	2667.9
run_198_f	198	10	-19547.9	-18169.4	2757.0
run_195_f	195	10	-19974.5	-18193.2	3562.5
run_191_f	191	10	-20017.6	-18174.4	3686.3
run_184_f	184	10	-19458.8	-18163.5	2590.7
run_189_f	189	10	-20775.8	-18225.0	5101.6
run_185_f	185	10	-19810.8	-18182.3	3257.0
run_200_f	200	10	-19902.4	-18188.9	3427.1
run_197_f	197	10	-19643.5	-18179.0	2929.0
run_193_f	193	10	-19783.9	-18196.9	3174.0
run_190_f	190	10	-19641.4	-18180.8	2921.2
run_181_f	181	10	-19390.9	-18164.9	2452.0

Supplementary Table S3 Evanno table output from Structure Harvester

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	20	-20150.755000	375.821925	—	—	—
2	20	-19837.955000	22.147507	312.800000	248.655000	11.227223
3	20	-19773.810000	40.198572	64.145000	92.585000	2.303191
4	20	-19617.080000	30.024527	156.730000	112.905000	3.760426
5	20	-19573.255000	43.370272	43.825000	54.845000	1.264576
6	20	-19584.275000	146.285942	-11.020000	66.600000	0.455273
7	20	-19528.695000	83.713782	55.580000	182.175000	2.176165
8	20	-19655.290000	317.411990	-126.595000	210.490000	0.663144
9	20	-19571.395000	193.899818	83.895000	252.500000	1.302219
10	20	-19740.000000	303.750835	-168.605000	—	—

Supplementary Table S4 Sample size and mean genetic diversity indices for the *Quercus pubescens* s.l. stands in Apulia (APU; Di Pietro et al. 2020), M. Vairano, Molise (MOL; Antonecchia G. unpublished data), Calabria (CAL), Sardinia (SAR), and Sicily (SIC)

Locus	Region	N	K	H_o	H_e	F_{IS}	Locus	Region	N	K	H_o	H_e	F_{IS}
PIE020	APU	312	7	0.583	0.585	-0.067	PIE239	APU	302	9	0.248	0.435	0.399*
	MOL	55	7	0.527	0.467	-0.129°		MOL	54	13	0.741	0.800	0.074°
	CAL	118	8	0.473	0.467	-0.012		CAL	115	11	0.575	0.645	0.109
	SAR	95	9	0.635	0.573	-0.107		SAR	86	3	0.239	0.207	-0.153
	SIC	261	13	0.490	0.467	-0.049		SIC	255	11	0.501	0.632	0.206
PIE102	APU	321	13	0.523	0.750	0.279*	PIE242	APU	318	14	0.748	0.841	0.093*
	MOL	55	13	0.782	0.815	0.041°		MOL	55	11	0.891	0.851	-0.047°
	CAL	117	15	0.859	0.797	-0.077		CAL	118	11	0.798	0.787	-0.014
	SAR	95	15	0.749	0.722	-0.037		SAR	95	13	0.844	0.808	-0.044
	SIC	258	18	0.719	0.735	0.021		SIC	258	15	0.791	0.830	0.047
PIE152	APU	314	17	0.834	0.890	0.043*	PIE243	APU	282	10	0.688	0.683	-0.038
	MOL	53	14	0.868	0.893	0.028°		MOL	52	7	0.827	0.730	-0.133°
	CAL	117	13	0.828	0.860	0.037		CAL	118	8	0.599	0.606	0.012
	SAR	94	13	0.902	0.834	-0.081		SAR	95	10	0.676	0.705	0.042
	SIC	257	18	0.829	0.877	0.055		SIC	260	12	0.596	0.636	0.062
PIE215	APU	281	12	0.384	0.707	0.417*	PIE258	APU	-	-	-	-	-
	MOL	55	11	0.745	0.755	0.013°		MOL	-	-	-	-	-
	CAL	113	12	0.837	0.822	-0.018		CAL	118	13	0.666	0.850	0.216
	SAR	95	11	0.863	0.810	-0.064		SAR	95	14	0.810	0.827	0.020
	SIC	263	12	0.654	0.748	0.126		SIC	259	16	0.528	0.849	0.378
PIE223	APU	318	11	0.811	0.822	-0.002	PIE267	APU	313	12	0.425	0.667	0.334*
	MOL	55	9	0.818	0.819	0.001°		MOL	54	6	0.722	0.759	0.048°
	CAL	113	11	0.803	0.823	0.025		CAL	118	8	0.738	0.764	0.035
	SAR	95	11	0.857	0.833	-0.030		SAR	95	9	0.810	0.696	-0.163
	SIC	263	12	0.687	0.766	0.103		SIC	259	9	0.733	0.737	0.006

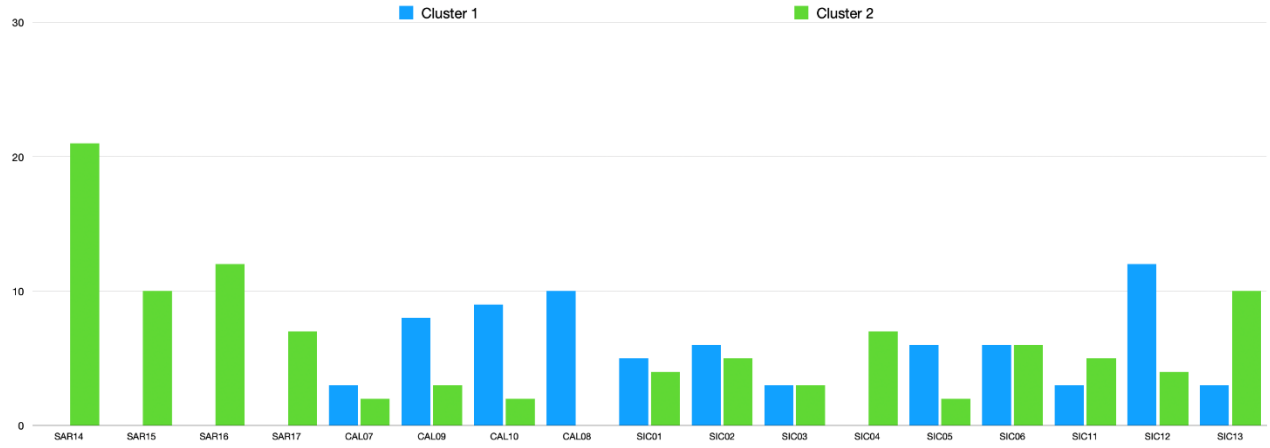
PIE227	APU	323	4	0.223	0.251	0.095*	PIE271	APU	311	12	0.949	0.843	-0.153
	MOL	55	4	0.182	0.201	0.096°		MOL	55	9	0.818	0.831	0.016°
	CAL	115	4	0.230	0.204	-0.128		CAL	118	11	0.848	0.836	-0.015
	SAR	88	1	0.011	0.011	-0.023		SAR	95	10	0.857	0.774	-0.107
	SIC	265	8	0.383	0.447	0.143		SIC	259	12	0.843	0.808	-0.043

N number of individuals, *K* number of alleles at the locus, H_o observed heterozygosity, H_e expected heterozygosity, F_{IS} Fixation index (* = P < 0.05), ° significance not available

Supplementary Table S5 Sample size and mean value of the genetic diversity parameters for the *Quercus pubescens* s.l. stands in Apulia, M. Vairano (Molise), Calabria, Sardinia, and Sicily

Region	**M**	**N_t**	**N_a**	**A_r**	**H_o**	**H_e**	**F_{IS}**
Apulia	15	312	5.8	4.4	0.583	0.629	0.039
Molise	55	55	9.4	9.2	0.720	0.720	0.001
Calabria	30	119	8.2	6.8	0.639	0.698	0.102
Sardinia	24	96	7.1	6.3	0.662	0.637	-0.017
Sicily	29	265	7.9	6.7	0.659	0.695	0.069

M mean number of samples per population, N_t total number of samples for all population investigated, N_a number of alleles, A_r allelic richness, H_o observed heterozygosity, H_e expected heterozygosity, F_{IS} inbreeding coefficient



Supplementary Fig. S3 Distribution of individuals with Q>90 for each cluster in all 17 population sampled in the study area (CAL=Calabria; SAR=Sardinia; SIC=Sicily).