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ARTICLE

Estimating the genetic diversity and structure of *Quercus trojana* Webb populations in Italy by SSRs: implications for management and conservation

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Abstract: Studying the genetic diversity and structure of the current forest populations is essential for evaluating the ability to survive future biotic and abiotic changes and planning conservation strategies. *Quercus trojana* Webb is an eastern Mediterranean tree species with a fragmented distribution range, and its westernmost outposts are located in southern Italy. The demand for timber and cropland over the centuries has severely reduced its occurrence in this part of the range. We assessed the genetic diversity and structure of the extant Italian populations of *Q. trojana* and derived conservation guidelines. A total of 322 samples were genotyped with six polymorphic nuclear microsatellite markers. A high genetic diversity in all populations, two main gene pools, and a highly divergent single population were observed. Based on the allelic richness and heterozygosity estimation, we identified populations that can be considered as valuable source material for conservation programs and those requiring adequate measures to reestablish gene flow and reduce fragmentation. Finally, a comparison with a set of eastern Mediterranean samples indicated a relationship between the Italian and the Greek gene pools. The need to protect these marginal, disjunct populations was further reinforced.

Key words: Quercus trojana, genetic diversity, population structure, SSR markers, conservation.

Résumé : L'étude de la diversité génétique et de la structure des populations forestières actuelles est essentielle pour évaluer leur capacité à survivre aux changements biotiques et abiotiques futurs et planifier les stratégies de conservation. *Quercus trojana* Webb est une espèce arborescente de l'est de la région méditerranéenne caractérisée par une aire de distribution naturelle fragmentée. Ses avant-postes les plus à l'ouest sont situés dans le sud de l'Italie. Au cours des siècles, la demande pour le bois et les terres destinées à l'agriculture a sévèrement réduit son occurrence dans cette partie de son aire de distribution. Nous avons évalué la diversité génétique et la structure des populations actuelles de *Q. trojana* en Italie, afin d'en déduire des lignes directrices pour la conservation. Un total de 322 échantillons ont été génotypés pour six marqueurs nucléaires polymorphes de type microsatellite. Les résultats indiquent qu'il y a une grande diversité génétique dans toutes les populations, structurée en deux pools génétiques principaux, ainsi qu'une population unique très divergente. Sur la base des estimations de richesse allélique et d'hétérozygotie, les populations pouvant être considérées comme une source utile de propagules pour les programmes de conservation ont été identifiées, ainsi que les populations nécessitant des mesures adéquates pour restaurer le flux génique et réduire la fragmentation. Enfin, la comparaison avec un jeu d'échantillons provenant de l'est de la région méditerranéenne montre qu'il existe un lien entre le pool génétique de l'Italie et celui de la Grèce. Différentes hypothèses concernant l'origine du pool génétique italien sont abordées dans la discussion. La protection de ces populations marginales disjointes a encore besoin d'être renforcée. [Traduit par la Rédaction]

Mots-clés : Quercus trojana, diversité génétique, structure de populations, marqueurs SSRs, conservation.

Introduction

Forests are considered the most complex terrestrial ecosystems due to their high level of biodiversity in terms of genetic resources, species, and habitat (Geburek and Konrad 2008). However, the degradation and disappearance of natural forests that has occurred in the last centuries have caused serious biodiversity losses. An important indicator of biodiversity is the amount of genetic diversity (Shachak et al. 2008) that is widely recognized as the key component for the long-term survival of a species (Gapare 2014). Genetic diversity is a foundation of sustainability providing raw material for adaptation, evolution, and survival, especially under changing environmental and disease conditions (Reed and Frankham 2003). Hence, studies addressing levels of genetic diversity can help to reduce the risk of loss of biodiversity by identifying the populations and areas that show high values of genetic variability and merit the most attention in terms of conservation priority (Souto et al. 2015). In this context, in situ and ex situ conservation plans must consider the intraspecific genetic variation as a fundamental criterion for developing effective conservation strategies (Eckert et al. 2008). Many studies on tree genetic diversity can provide evidence for the long-term influence of for-

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est management and (or) exploitation on the tree populations; in some cases, a reduction in allelic richness, the number of rare alleles, and heterozygosity was observed (Pautasso 2009). Moreover, habitat fragmentation affects genetic diversity due to the alteration in the landscape features, which leads to reduced gene dispersal (Oddou -Muratorio and Klein 2008). The loss of genetic diversity through genetic drift and increased selfing can cause the local extinction of small populations (Honnay and Jacquemyn 2007). In this context, it is of great importance to assess the genetic diversity and patterns of gene flow of *Quercus trojana* Webb Italian extant populations to understand its current evolutionary and adaptive trends and provide a scientific basis for "conservation in practice".

Quercus trojana is an eastern Mediterranean–Balkan oak belonging to the subgeneric *Quercus* group Cerris (Denk and Grimm 2010; Simeone et al. 2013). This is a Eurasian oak clade with an inferred Miocene origin (Hubert et al. 2014) consisting of evergreen, semievergreen, and deciduous species characterized by toothed or lobed leaves with pointed tips or cusps, long linear or broad, recurved cupule scales (Menitsky 2005). *Quercus trojana* is typically a small, semievergreen tree, easily identified by its subcoriaceous, elongated, glabrous, and regularly dentate leaves and by the acorn enclosed in a characteristic thick and woody dome covered with elongated, pubescent scales (Zielinski et al. 2006). Its distribution range extends from western and southern Anatolia through Turkey, southwest Bulgaria, Greece, and the Aegean region up to the Balkans (Croatia, Serbia, Bosnia, Montenegro, Albania, and Macedonia), with few disjunct outposts in southeastern Italy (Jalas and Suominen 1976; Browicz 1982). *Quercus trojana* is a thermomesophilous species that prefers dry, predominantly limestone neutral to subacid soils. It can, however, adapt to all kinds of edaphic conditions (Menitsky 2005; Giardina et al. 2014) forming pure and mixed supra-Mediterranean, occasionally meso-Mediterranean, forests and sporadically participating to maquis formations under more xerophytic conditions. It can also grow on very dry and extreme habitats (e.g., karst areas), and for this reason, reforestation and afforestation is recommended for extremely poor and degraded sub-Mediterranean habitats (Ballian et al. 2014). European *Q. trojana* woods have been designated as special areas for conservation (Habitat Directive 92/43/EEC, Annex I, Code 9250); their habitats are generally considered in a situation where a change in management or policy is required to return to a favorable status.

In Italy, its current distribution is restricted to southern regions (Apulia and Basilicata) and is a remnant of a once more widely spread range that has been severely reduced by intensive human exploitation (Schirone and Spada 1995; Misano and Di Pietro 2007). Deforestation for agricultural purposes and the good technological properties of its wood are the main causes of the decrease in *Q. trojana* occurrence in Italy. The increasing human impact over the last century has led to a highly differentiated and fragmented landscape consisting of the alternation of cultivated fields, patches of forest remains, farms, and urban centers (Bottalico et al. 2006). The consequent habitat loss and deterioration, along with the changes in the accelerating dynamics of global and climatic changes, have resulted in a strong reduction in the size of *Q. trojana* populations size, isolation, and a probable severe loss of their genetic diversity.

We investigated the genetic diversity and structure of the current *Q. trojana* populations in southern Italy by means of microsatellites (SSRs). These markers are widely used to characterize the genetic variation of long-lived species (Vendramin et al. 2004) and many studies highlight the use of them as a tool for identifying populations or areas for conservation (Smulders et al. 2008; Pautasso 2009; Allendorf et al. 2010; van Zonneveld et al. 2012; Lusini et al. 2014; Chiocchini et al. 2016).

The aim of this work was to (1) estimate the main genetic parameters for evaluating population genetic diversity and divergence, (2) identify valuable areas and reservoirs of genetic diversity, and (3) provide guidelines for conservation. Finally, we examined the genetic relationships among the investigated stands and various eastern Mediterranean *Q. trojana* samples to speculate on the origins of the Italian populations.

To the best of our knowledge, this is the first study on the genetic diversity and structure of *Q. trojana* populations in Italy (and across the species' range) based on the use of microsatellite markers. To date, this species has been studied only through purely ecological (Manicone 2007) or historical surveys (Bottalico et al. 2006). Our intention is to contribute to an increase in the knowledge and the preservation of *Q. trojana* in Italy, thereby promoting the sustainable management of the genetic resources of the still poorly known Mediterranean forest ecosystems.

Material and methods

Plant material

A total of 322 georeferenced samples from 17 sites located in south Italy were collected (Fig. 1; Table 1). Sixteen sites were located in two different areas of the Apulia region (National Park of Alta Murgia and Martina Franca), where *Q. trojana* is represented by small groups of trees with a scattered distribution or in mixed forests. The last site was located in Sicily (Riserva Naturale Orientata "Bosco della Ficuzza") and includes a recently identified population of unknown origin (Giardina et al. 2014). The distance between each sampled tree was around 30–50 m. Moreover, to have a preliminary indication of the genetic similarity of the Italian populations with the eastern Mediterranean *Q. trojana* germplasm, we also genotyped 23 samples of *Q. trojana* from Greece and Turkey, kindly provided by T. Denk and G.W. Grimm (Table 2).

DNA extraction and SSR analysis

Total genomic DNA was isolated by grinding 20 mg of fresh leaf tissue and purified using the Dneasy96 Plant Kit (Qiagen) according to the manufacturer's instructions. A set of six nuclear microsatellite primers (QpZAG110, QpZAG7, QrZAG11, CsCAT1, CsCAT2, and CsCAT14) developed in *Quercus petraea* (Matt.) Liebl. and *Quercus robur* L. (Steinkellner et al. 1997) and *Castanea sativa* Mill. (Marinoni et al. 2003) were selected and used for the multiplex PCR analysis. These markers were mapped on two F1 intraspecific crosses of *C. sativa* and *Q. robur* and each selected locus has been shown to belong to a different linkage group (Barreneche et al. 2004; Casasoli et al. 2004). The forward primer of each pair was labelled with a fluorescent dye (6-FAM, NED, or VIC) (Table 3). The PCR multiplex reaction was performed using the Type-it Microsatellite PCR Kit (QIAGEN, Hilden, Germany) in 20 μL total volume containing 20 ng of genomic DNA. Cycling parameters were 5 min at 95 °C, 28 cycles for 30 s at 95 °C, 90 s at 57 °C, 30 s at 72 °C, and a final step of 30 min at 60 °C. Amplification products (1 μL) were added to 9.75 μL of formammide and 0.25 μL of Genescan-500 LIZ and denatured at 95 °C for 5 min. The samples were run on an ABI PRISM 3130 Avant DNA sequencer. The alleles were scored using GeneMapper software (Life Technologies).

Data analysis

Genetic diversity indices

A set of intra- and interpopulation genetic diversity parameters were calculated using the software GeneAlEx 6.4.1 (Peakall and Smouse 2005). The observed (N_a) and effective (N_e) number of alleles, the observed (H_o) and expected (H_e) heterozygosity, and the Shannon diversity index (I) were all calculated at each locus, over all loci, and for each population. For each population were also calculated the H_e weighted on the number of samples and the mean number of private alleles. The fixation index F_{is} (Weir and Cockerham 1984) was computed for each locus across all populations and for each population over all loci using Arlequin 3.11 software (Excoffier et al. 2005). The statistical significance was

Fig. 1. Map illustrating the location of the 17 Italian populations of *Q. trojana* analysed in this study. [Colour online.]

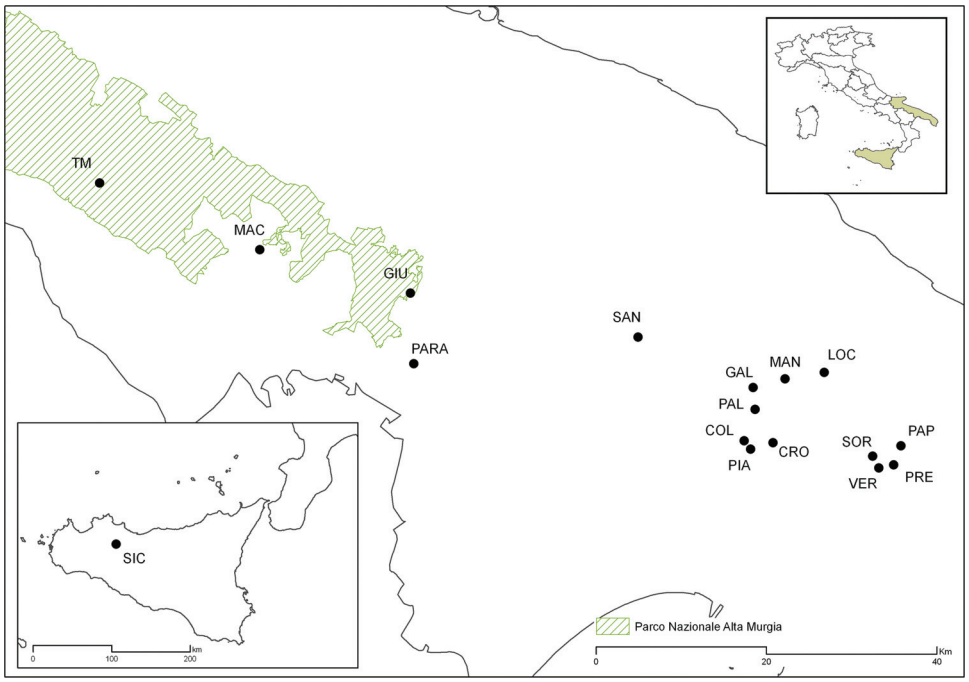


Table 1. Sample ID, number of individuals (*N*), geographical location, and coordinates for 17 *Q. trojana* populations genotyped in this study.

Population	ID	*N*	Location	Latitude	Longitude
Giustino	GIU	14	National Park (Apulia)	40.841	16.744
Lama Corriera	MAC	25	National Park (Apulia)	40.892	16.537
Parata	PARA	13	National Park (Apulia)	40.771	16.748
Trullo di Mezzo	TM	20	National Park (Apulia)	45.358	16.042
Locorotondo	LOC	14	Martina Franca (Apulia)	40.755	17.326
Santuario	SAN	20	Martina Franca (Apulia)	40.798	17.041
Colucci	COL	20	Martina Franca (Apulia)	40.680	17.203
Croce Grande	CRO	19	Martina Franca (Apulia)	40.406	17.148
Galeone	GAL	20	Martina Franca (Apulia)	40.736	17.217
Mangiato	MAN	20	Martina Franca (Apulia)	40.744	17.262
Palazzolo	PAL	20	Martina Franca (Apulia)	40.712	17.223
Papariello	PAP	19	Martina Franca (Apulia)	40.670	17.420
Bosco Pianelle	PIA	19	Martina Franca (Apulia)	40.670	17.216
Presidente	PRE	20	Martina Franca (Apulia)	40.652	17.413
Ciccio la Sorte	SOR	17	Martina Franca (Apulia)	40.661	17.376
Verdurizzo	VER	20	Martina Franca (Apulia)	40.647	17.386
Bosco della Ficuzza	SIC	23	Ficuzza (Sicily)	37.875	13.407

tested with a nonparametric approach described in Excoffier et al. (1992) with 1000 permutations.

Because the presence of null alleles can affect the estimation of population differentiation, null allele frequencies were estimated for each locus and population following the expectation maximization (EM) algorithm of Dempster et al. (1977), implemented in FreeNA software (Chapuis and Estoup 2007). The F_{st} was estimated considering the presence of null alleles (EM-ENA procedure) or excluding this procedure (without EM-ENA procedure).

The estimation of the mean number of alleles per locus as a measure of allelic richness (A_r) can be affected by differences in sample size. For this reason, A_r and the private A_r were calculated by the statistical technique of rarefaction method implemented in HP-Rare 1.1 (Kalinowski 2005). This approach uses the frequencies of alleles at the locus to estimate the expected number of alleles and (or) private alleles in a subsample of *N* individuals selected at random from a sample of *N* individuals in each population.

Population structure analysis

Three different complementary approaches were used to characterise the patterns of genetic structure of Italian *Q. trojana* populations as follows.

(1) Principal coordinates analysis (PCoA) based on a dissimilarity matrix of Nei's genetic distance (Nei 1973) was performed using the software GeneAlEx 6.4.1 (Peakall and Smouse 2005).

(2) Spatial analysis of molecular variance (SAMOVA) was applied to delineate groups using SAMOVA 1.0 software. We tested *K* from 2 to 16 (number of *Quercus* populations minus 1) selecting 100 random starting conditions. We examined patterns of variation among groups (F_{ct}) and within groups (F_{sc}) for each *K* to determine the most appropriate number of population groups. The configuration with the highest F_{ct} was retained as the best partition of *Quercus* populations.

(3) A Bayesian approach implemented in the software STRUCTURE 2.3.4. (Pritchard et al. 2000) was performed. This method attempts to reveal the population structure by placing individuals or pre-

Table 2. Sample ID, geographical origin, and voucher information for 23 specimens of *Q. trojana* from Greece and Turkey genotyped in this study.

ID	Origin	Geographical location	Voucher
BAL-17	Greece	Ioannina	Denk, Ruhri & Ruhri 20081013/1-1 S
BAL-18		Ioannina	Denk, Ruhri & Ruhri 20081013/1-2 S
BAL-19		Ioannina	Denk, Ruhri & Ruhri 20081013/1-3 S
BAL-20		Ioannina	Denk, Ruhri & Ruhri 20081013/1-4 S
BAL-21		Ioannina	Denk, Ruhri & Ruhri 20081014/2-1 S
BAL-22		Ioannina	Denk, Ruhri & Ruhri 20081014/2-2 S
BAL-23		Ioannina	Denk, Ruhri & Ruhri 20081014/1-1 S
BAL-44	Northwestern Turkey	Ulubat Gölu	Denk & Grimm 2006365 S
BAL-45		Ulubat Gölu	Denk & Grimm 2006366 S
BAL-05	Central-western Turkey	Yeşildağ	Denk & Grimm 2006278 S
BAL-06		Yukari Gökdere	Denk & Grimm 2006305 S
BAL-07		Siraslı	Denk & Grimm 2006344 S
BAL-36		Yeşildağ	Denk & Grimm 2006284 S
BAL-37		Yukari Gökdere	Denk & Grimm 2006304 S
BAL-39		Sirasi	Denk & Grimm 2006337 S
BAL-42		Yeşildağ	Denk & Grimm 2006279 S
BAL-43		Sirasli	Denk & Grimm 2006338 S
BAL-01	Southwestern Turkey	Ereğli	Denk & Grimm 2006248 S
BAL-03		Ereğli	Denk & Grimm 2006252 S
BAL-04		Madenşehri	Denk & Grimm 2006271 S
BAL-35		Ereğli	Denk & Grimm 2006254 S
BAL-40		Madenşehri	Denk & Grimm 2006269 S
BAL-41		Madenşehri	Denk & Grimm 2006270 S

Table 3. Range of alleles and dye of six microsatellite loci analysed: number of alleles (N_a), number of effective alleles (N_e), Shannon's index (I), expected heterozygosity (H_e), observed heterozygosity (H_o), and within-population inbreeding coefficient (F_{is}).

Locus	Dye	Range (bp)	N_a	N_e	I	H_e	H_o	F_{is}
QpZAG110	FAM	193–235	13.41	8.84	2.34	0.88	0.80	0.09
QpZAG7	VIC	115–153	12.76	7.97	2.26	0.87	0.92	–0.07
QrZAG11	FAM	242–286	2.59	1.78	0.67	0.40	0.17	0.59*
CsCAT14	FAM	100–150	8.65	4.93	1.78	0.78	0.79	–0.02
CsCAT2	FAM	200–250	6.41	2.04	1.05	0.48	0.50	–0.05
CsCAT1	NED	160–199	11.06	6.93	2.11	0.84	0.45	0.47*

Note: Significance of the inbreeding coefficient F_{is} was tested using a nonparametric approach described in Excoffier et al. (1992) with 1000 permutations: *$P < 0.05$.

defined groups in K number of clusters to minimize within-group linkage disequilibrium and deviation from the Hardy–Weinberg equilibrium. The analysis was performed using the admixture model on the whole data set, with no previous population information, and the correlated allele frequencies between population options (Falush et al. 2007). In this study, the range of possible number of clusters (K) tested was from 1 to 19 (the putative number of populations plus 2). Based on the initial results, a series of six independent runs were performed for K between 1 and 6 with a burn-in period of 10 000 steps followed by 10^5 MCMC replicates. The ad hoc statistic ΔK defined by Evanno et al. (2005) was used to detect the most likely number of clusters. This value is based on the rate of change of the second order of $L(K)$ between two successive values of K for six replicates. The six runs from the most probable number of clusters were averaged by applying a FullSearch algorithm provided by CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) and represented graphically with DISTRUCT (Rosenberg 2004).

Hierarchical AMOVA (Excoffier et al. 2005) implemented by the software Arlequin 3.11 was calculated considering the main groups obtained from the STRUCTURE and PCoA analysis. The statistical significance was tested using a nonparametric approach described in Excoffier et al. (1992) with 1000 permutations.

We also tested for isolation by distance performing correlation between genetic and geographic distances using nonparametric pairwise simple and partial Mantel tests (Mantel 1967; Smouse et al. 1986). Assuming a nonlinear distribution of sampling sites, we regressed Slatkin's linearized ($F_{st}/(1 - F_{st})$) pairwise values against the corresponding natural logarithm of geographic distances (straight-line distances in kilometres).

Comparison of Italian and eastern Mediterranean Q. trojana samples

The genetic similarity among the Italian populations and Greek and Turkish samples were tested performing a PCoA based on a dissimilarity matrix of Nei (1973) implemented by the software GeneAlEx 6.4.1 (Peakall and Smouse 2005). The samples from Turkey and Greece were grouped considering their geographic location, as indicated in Table 2.

Results

Genetic diversity

The six SSR loci assayed were all polymorphic and the number of alleles detected for each locus varied between 2.59 (QrZAG11) and 13.41 (QpZAG110) (Table 3). These two loci also showed the lowest (0.40) and the highest (0.88) H_e, respectively. The observed heterozygosity (Ho) ranged from 0.17 (QrZAG11) to 0.92 in QpZAG7. The F_{is} showed positive and significant values in QrZAG11 and CsCAT1 loci (0.59 and 0.47, respectively).

Table 4 shows genetic diversity parameters for each population. The average N_e was 5.41, ranging from 3.69 in the MAC population to 6.91 in the SOR population. The analysis conducted with FREENA software indicated the absence of null alleles (F_{st} using the EM-ENA procedure = 0.034357, F_{st} not using theEM-ENA procedure = 0.036697). The lowest H_o was found in the TM population (0.49) and the highest in the MAN population (0.69) with a mean value of 0.61. The H_e ranged from 0.59 in the TM population to 0.76 in the GIU and LOC populations. These values are comparable with unbiased H_e values indicating no effect due to the imbalance on the sampling size. The F_{is} deviated significantly from zero in four populations (LOC, COL, PAL, and SOR). Private A_r values, calculated with the rarefaction method, ranged from 0.00 (GAL) to 0.45 (PARA), while A_r values ranged from 5.72 (TM) to 9.10 (SOR).

Table 4. Genetic diversity parameters for the seventeen *Q. trojana* populations analysed through six microsatellite loci: mean number of different alleles (N_a), mean number of effective alleles (N_e), Shannon's index (I), observed heterozygosity (H_o), expected heterozygosity (H_e), unbiased expected heterozygosity (UH_e), inbreeding coefficent (F_{is}), mean number of private alleles (NPA), allelic richness (A_r), and private allelic richness (PA_r).

ID	N_a	N_e	I	H_o	H_e	UH_e	NPA	A_r	PA_r	F_{is}
GIU	8.33	5.53	1.75	0.58	0.76	0.79	0.17	7.60	0.19	0.07
LOC	8.00	5.65	1.75	0.63	0.76	0.79	0.33	7.44	0.32	0.10*
MAC	8.17	3.69	1.47	0.62	0.65	0.67	0.67	6.02	0.39	−0.14
PARA	9.17	5.71	1.79	0.64	0.74	0.77	0.50	8.49	0.45	0.02
SAN	10.50	5.99	1.82	0.60	0.74	0.76	0.50	8.15	0.30	0.04
TM	6.83	3.91	1.34	0.49	0.59	0.61	0.17	5.72	0.22	0.01
COL	9.83	5.56	1.72	0.56	0.69	0.71	0.33	7.86	0.34	0.10*
CRO	9.00	5.79	1.66	0.56	0.68	0.70	0.00	7.35	0.05	0.04
GAL	8.50	4.75	1.56	0.65	0.66	0.68	0.00	6.80	0.00	−0.04
MAN	10.00	5.67	1.79	0.69	0.75	0.77	0.50	7.75	0.29	−0.09
PAL	10.67	6.27	1.88	0.65	0.75	0.77	0.17	8.51	0.22	0.08*
PAP	9.67	5.60	1.75	0.59	0.72	0.74	0.17	7.82	0.13	0.02
PIA	8.50	5.77	1.59	0.64	0.65	0.67	0.00	7.16	0.02	−0.08
PRE	9.33	5.21	1.72	0.58	0.70	0.72	0.33	7.71	0.23	0.04
SOR	10.67	6.91	1.91	0.56	0.75	0.77	0.17	9.10	0.25	0.09*
VER	9.50	5.74	1.77	0.66	0.73	0.75	0.17	7.68	0.13	0.03
SIC	8.83	4.27	1.60	0.61	0.70	0.71	0.33	6.74	0.19	−0.03
Mean	9.15	5.41	1.70	0.61	0.71	0.73	0.27	7.52	0.22	−0.02

Note: Significance of inbreeding coefficient F_{is} was tested using a nonparametric approach described in Excoffier et al. (1992) with 1000 permutations: *$P < 0.05$.

Population structure

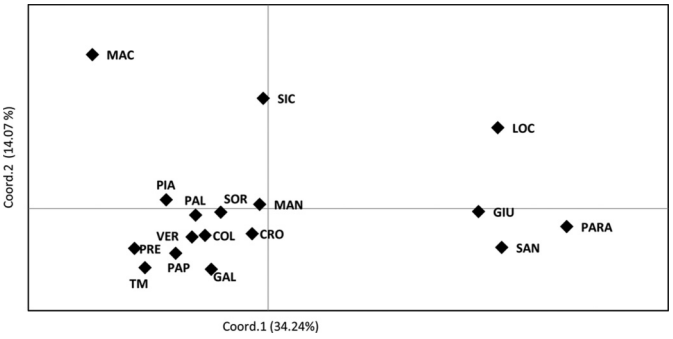
The PCoA (Fig. 2) based on Nei's unbiased genetic distance matrix (Table 5) suggested the presence of a nonrandom association of populations. The combined first two axes explained 48.31% of the variation. Although the genetic distance was not high, two main groups could be distinguished. The first group includes populations sampled in the Martina Franca area (COL, CRO, GAL, MAN, PAL, PAP, PIA, PRE, SOR, and VER) as well as population TM, located inside the National Park of Alta Murgia. A low genetic distance was found between these populations (Table 5). The second group (with respect to the second axis) includes two populations bordering the National Park of Alta Murgia (PARA and GIU), a population sampled in the Martina Franca area (LOC), and a population bordering the Martina Franca area, located between this area and the National Park (SAN). Finally, the MAC population, located in the inner part of the National Park, was genetically distant from all of the other populations from Apulia. The Sicilian population (SIC) was not included in either of the two main groups.

SAMOVA results indicated $K = 2$ as the most appropriate number of populations groups ($F_{sc} = 0.02197$, $F_{ct} = 0.05543$, $P < 0.001$; see Supplementary Table S1)[1]. Considering the grouping based on $K = 2$, all of the populations except MAC were included in the same gene pool.

The subsequent STRUCTURE analysis corroborate the SAMOVA and PCoA results and provided additional information on the level of genomic admixture among populations (Fig. 3).

The most probable division with the strongest support in terms of log-likelihood values was detected at $K = 4$ (Fig. 3A). The MAC population was assigned to cluster I (blue) as well as SIC; cluster II (red) grouped together the neighboring populations of the National Park of Alta Murgia (GIU, PARA, SAN, and LOC), which had already been identified as a distinct group in the analysis of principal coordinates. The populations in Martina Franca area displayed a high degree of admixture of the four clusters with the prevalence of clusters III and IV (green and yellow) (Figs. 3B and 3C).

Fig. 2. Principal coordinate analysis of 17 *Q. trojana* populations from southern Italy (Apulia and Sicily).



The hierarchical AMOVA was carried out according to the three main gene pools obtained with the PCoA and STRUCTURE analysis. All of the populations from the Martina Franca area were grouped together, while the other two groups correspond to STRUCTURE cluster I and cluster II, respectively. The molecular variance among groups inferred was 2.24% ($P < 0.01$). The majority of molecular variance was partitioned within individuals (94.96%, $P < 0.01$) (Table 6).

The pairwise linearized genetic differentiation values ($F_{st}/(1 - F_{st})$) and the natural logarithm of geographic distances among sampling sites were not significantly correlated (Mantel test, $r = 0.128$, $P = 0.113$).

Comparison of Italian and eastern Mediterranean *Q. trojana* samples

Figure 4 shows the PCoA performed to test the genetic similarity of the Italian in relation to the Greek and Turkish samples. The combined first two axes explained 63.84% of the variation. This analysis confirmed the population subdivision of the Italian germplasm recorded in Fig. 3 and further highlighted a genetic similarity among the populations of the Martina Franca area and the samples from Greece. The SAN, PARA, GIU, and LOC populations and the MAC and SIC populations formed gradually less related groups, whereas the Anatolian samples (NW-TK, CW-CK, and SW-TK) displayed a higher genetic distance from all of the other (Italian and Greek) *Q. trojana* populations.

Discussion

Genetic diversity

To the best of our knowledge, this is the first report on the genetic diversity and structure of *Q. trojana* populations in Italy as well as all across the species' current range. Our results thus provide the first insight into the potential of this species to adapt to environmental changes and could serve as a benchmark for future management and conservation policies on genetic resources.

Our data set exhibited high levels of genetic diversity at the SSR loci examined. However, two SSR markers (QrZAG11 and CsCAT1) showed a positive and significant F_{is}. A possible interpretation for the allelic frequencies that deviate from equilibria involves hitchhiking effects between SSR loci and various adaptive traits. The allele fixation might have been favored by selection and variability reductions in neighboring genome areas as a well-recognized result of directional selection for a specific adaptive trait (Andolfatto 2001). Allele fixation can also be the result of genetic drift. However, diversity reductions due to genetic drift would affect the genome rather uniformly (Alberto et al. 2010), which is not the case with

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjfr-2016-0311.

Table 5. Unbiased Nei's genetic distance matrix for 17 *Q. trojana* populations.

GIU	LOC	MAC	PARA	SAN	SIC	TM	COL	CRO	GAL	MAN	PAL	PAP	PIA	PRE	SOR	VER	
0.000																	GIUS
0.193	0.000																LOC
0.420	0.416	0.000															MAC
0.166	0.228	0.526	0.000														PARA
0.240	0.167	0.498	0.183	0.000													SAN
0.295	0.195	0.211	0.323	0.298	0.000												SIC
0.295	0.368	0.242	0.387	0.304	0.231	0.000											TM
0.251	0.266	0.236	0.295	0.258	0.157	0.093	0.000										COL
0.203	0.208	0.276	0.272	0.222	0.140	0.134	0.081	0.000									CRO
0.233	0.269	0.277	0.306	0.250	0.189	0.087	0.072	0.071	0.000								GAL
0.246	0.267	0.263	0.245	0.324	0.210	0.218	0.158	0.122	0.140	0.000							MAN
0.260	0.275	0.194	0.305	0.258	0.169	0.114	0.100	0.107	0.107	0.133	0.000						PAL
0.263	0.303	0.231	0.327	0.265	0.184	0.095	0.089	0.090	0.065	0.138	0.076	0.000					PAP
0.289	0.262	0.201	0.384	0.285	0.146	0.115	0.077	0.064	0.094	0.164	0.120	0.094	0.000				PIA
0.317	0.341	0.235	0.395	0.317	0.203	0.098	0.113	0.096	0.105	0.127	0.084	0.074	0.122	0.000			PRE
0.230	0.226	0.211	0.289	0.237	0.147	0.138	0.085	0.058	0.075	0.108	0.090	0.083	0.066	0.095	0.000		SOR
0.242	0.274	0.212	0.303	0.256	0.166	0.099	0.068	0.079	0.064	0.151	0.080	0.063	0.086	0.087	0.059	0.000	VER

Fig. 3. Population structure inferred for 17 *Q. trojana* populations using the STRUCTURE software (Pritchard et al. 2000). (A) Second order of change of the log-likelihood of data (Δ*K*) as a function of *K* calculated over six replicates. (B) Individuals' estimated membership percentage in *K* clusters (*Q* values); each individual is represented by a vertical line and the different populations are separated by a vertical black line. (C) Map representation of the populations' membership percentage (Q_i) in the inferred *K* = 4 clusters. [Colour online.]

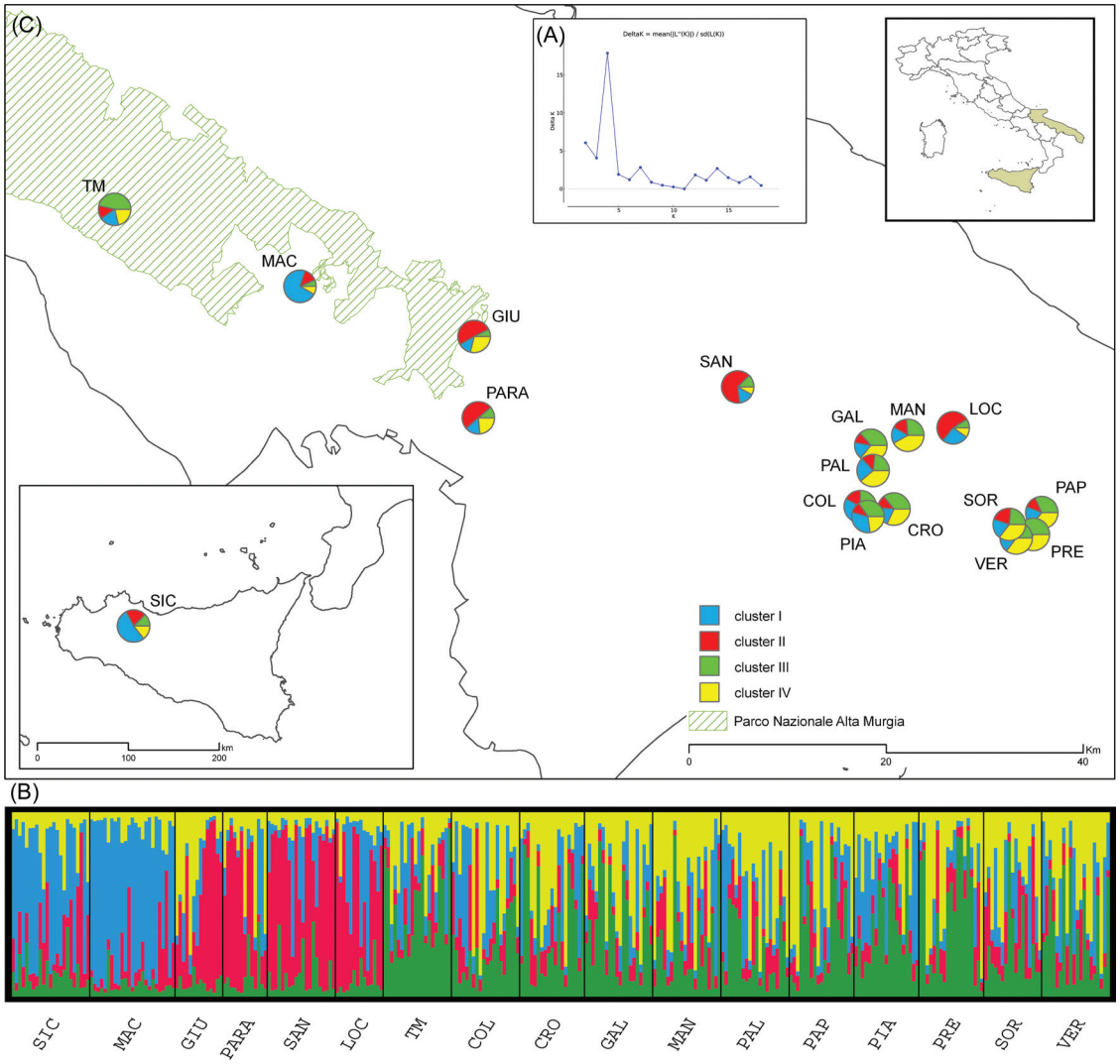
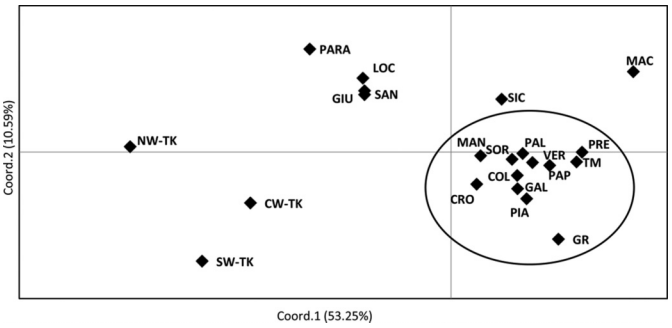


Table 6. Hierarchical AMOVA (Excoffier et al. 2005) and *F*-statistics analysis calculated considering the main gene pools obtained with PCoA and structure analysis.

Source of variation	df	Variance components	% of variation	*F*-statistic
Among groups	2	0.03563 Va	2.24	F_{ct} 0.02241*
Among populationwithin groups	14	0.02865 Vb	1.80	F_{sc} 0.01844*
Among individuals within populations	305	0.01538 Vc	1.00	F_{is} 0.01038*
Within individuals	322	1.50932 Vd	94.96	F_{it} 0.05040*

*$P < 0.001$.

Fig. 4. Principal coordinate analysis of *Q. trojana* Italian populations and eastern Mediterranean samples (Greece and Turkey). GR, Greece; NW, northwestern Turkey; CW, central-western Turkey; SW, southwestern Turkey. The circle includes populations from the Martina Franca area and Greek samples.



the other SSR loci examined. Indeed, more extended SSR investigations are required to clarify this point and to assign a selective response of these two loci. High values of A_r, of heterozygosity, and of the Shannon index were generally observed in our data set. These results are in agreement with those observed in other *Quercus* species in Italy (Antonecchia et al. 2015), France (Alberto et al. 2010), Germany, Greece, and Turkey (Alberto et al. 2013), thus highlighting the overall good conditions of the Italian populations of *Q. trojana* in terms of gene diversity. The F_{is} values obtained for the populations were significantly positive only for the LOC, COL, PAL, and SOR populations. This indicates that the Italian populations are largely outbreeding, with only minor levels of inbreeding. *Quercus* species are generally wind-pollinated. Outcrossing rates are expected to be high for wind-pollinated tree species, whereas the positive and significant inbreeding coefficient detected in some populations revealed instances of biparental inbreeding (i.e., inbreeding among genetically related trees). In contrast, the MAC population showed a highly negative value. Although statistically not supported, this result could be due to different causes, such as the fusion of formerly isolated populations or adaptive advantage of heterozygote individuals.

Population structure

The PCoA, SAMOVA, and STRUCTURE analyses enable us to group the 17 natural populations into three main different gene pools. A little genetic differentiation among populations was observed, while the greatest percentage of molecular variation was found within individuals. These results are in accordance with findings in other *Quercus* Italian populations (Bruschi et al. 2003). The formation of these different gene pools may be the result of the increasing fragmentation and isolation of the populations of *Q. trojana* in Apulia due to the massive land use changes over time. This may have altered the gene flow among populations that previously formed a more homogeneous gene pool and led to the selection of characteristic gene pools within the stands. In agreement with this, the populations in the Martina Franca area, located within continuous forests, appear to have no barriers to gene flow. Further explanations for the different gene pools include the selective dispersal of seeds from different sources, which could have originated naturally or by humans. The effect of human pressure on current population structure could be confirmed by the lack of significant correlation observed between geographical and genetic distances. This study showed that the population SIC is genetically similar to the Apulian populations, especially to MAC. We can therefore assume that the *Q. trojana* population recently (and unexpectedly) discovered in the "Bosco della Ficuzza" Natural Reserve may have a common origin with the Apulian populations. The species could have been introduced into Sicily quite recently, most probably in the last century. However, in agreement with Giardina et al. (2014), we cannot totally exclude the natural origins of this population due to the occurrence in the same area of many other deciduous trees relating to chorotype southeastern European *sensu latu* (*Celtis tournefortii* Lam, *Fraxinus ornus* L., *Ostrya carpinifolia* Scop, *Quercus cerris* L., *Quercus dalechampii* Ten, and *Sorbus aria* L.).

Finally, our data provide preliminary insights regarding the origin of the Apulian populations of *Q. trojana*. Although the number of east Mediterranean samples used for the comparison was limited, the PCoA analysis showed a clear separation between the Anatolian and the Italian samples. Conversely, a lower genetic distance between the Greek samples and the Italian data set was evident. In particular, the Greek samples clustered together with the populations of the Martina Franca area. Such a strong genetic similarity could suggest a common origin for the Greek and the Italian gene pools, and Martina Franca appears to be the area that is most directly linked to the Greek germ plasm. Human-mediated seed exchanges might have eventually occurred during the Greek settlement in southern Italy in the eighth century BC. In this case, populations of Martina Franca would likely have descended from the original Greek seed stock and acted as sources for the rest of the Apulian range. If this was the case, all other Apulian populations probably originated from seed lots harvested in a way that altered the original allele frequencies or from slightly different Greek seed sources. Nevertheless, it is now widely acknowledged that the occurrence of *Q. trojana* in Apulia is a part of a framework of several plant taxa belonging to an east Mediterranean vegetation type (e.g., *Quercus ithaburensis* subsp. *macrolepis* (Kotschy) Hedge and Yalt, *Periploca graeca* L., *Salvia triloba* L., and *Phlomis fruticosa* L.), all recognized as the "Apulian paleo-Aegean stock" (Francini Corti 1966). In addition, a close phylogeographic relationship between the two regions on both sides of the Adriatic Sea has been detected in other oak species, such as *Quercus frainetto* (Fineschi et al. 2002), *Quercus ilex* (Lumaret et al. 2002), *Quercus coccifera* (de Heredia et al. 2007), and *Q. cerris* (Bagnoli et al. 2016), and herbaceous taxa (e.g., Musacchio et al. 2006; Hilpold et al. 2014). In fact, land connections during the Messinian salinity crisis and (or) eustatic sea-level shifts creating land bridges during the

Pleistocene glaciations allowed biotic exchanges between the Balkans and southeastern Italy (Nieto Feliner 2014). Our data would therefore be consistent with the Apulian populations of *Q. trojana* as the remnants either of a once continuous ancestral range or of a colonization wave that moved westward from the Balkan range in more recent times. Clearly, additional data from the nearby west Balkan region (e.g., Croatia, Albania, and Montenegro) and from the cradle of the Cerris group (the Aegean area) (Denk and Grimm 2010) will help in precisely defining the origin of this important oak in Italy.

Implications for conservation

The analysis of genetic variation within and between populations of a species can help in highlighting the historical processes behind the genetic diversity (Dumolin-Lapegue et al. 1997) and providing useful information to establish adequate programs for the conservation of genetic resources. High levels of genetic variation are expected to increase the potential of the species to respond to selective pressure (Kalinowski 2004). It is essential, therefore, to identify the populations and areas that show high values of genetic diversity and divergence so as to identify which populations merit the most attention in terms of conservation priority (Petit et al. 1997; Ollivier and Foulley 2013). Populations showing characteristic gene pools are also considered as valuable source material for genetic conservation programs; thus, the populations' structure analysis provides complementary indications as to the intrapopulation genetic diversity.

The highest allelic richness and genetic diversity were scored by the GIU, LOC, PARA, MAN, PAL, SAN, and SOR populations. However, the high fixation indexes evidenced by LOC, PAL, and SOR would suggest the better use of GIU, PARA, MAN, and SAN for an efficient conservation of the species gene diversity and as germ plasm reservoir for afforestation and reforestation programs in Apulia. The GIU population is included in the National Park, thus benefitting from all the related management regimes (e.g., sustainable silviculture). PARA, MAN, and SAN showed a high number of private alleles, indicating some kind of isolation, which is confirmed by the large amount of cultivated lands surrounding these two populations. These populations also span most of the distribution of *Q. trojana* in Apulia, connecting the two main areas of the species (National Park of Alta Murgia and Martina Franca area). A reduction in their isolation and a reestablishment of the gene flow via ecological connections with the nearby forest patches (e.g., with tree plantations in abandoned open spaces and private farms) would greatly contribute to maintaining high values of A_r and H_e for the whole species in the region. However, the high inbreeding coefficient displayed by SOR, PAL, and LOC needs to be investigated. The possible causes of inbreeding in these populations should be addressed by evaluating both demographic factors (age of the trees and silviculture regime, which both affect the number of reproductive individuals) and ecological factors (biotic and abiotic disturbances). Inter- and intrapopulation gene flow should then be restored, and the next generations should be evaluated relative to their F_{is} and H_e. To preserve all the genetic diversity identified in this study, the MAC population in Apulia certainly deserves further attention. This population showing a highly negative F_{is} and the highest mean number of private alleles resulted as the most divergent. This might be indicative of an origin from different seed sources and (or) adaptive advantage of the heterozygote individuals.

The TM population located in the inner part of the National Park showed the lowest gene diversity values, suggesting that germ plasm belonging to the same genetic cluster (e.g., MAN) should be reintroduced into this population. The current management regimes should be maintained to preserve the Sicilian population (SIC) but more extensive research should be conducted to clarify its origin (La Mantia and Pasta 2005).

Concluding remarks

This study can be taken as an example of how to apply marker-based genetic tools in conservation programs for a marginal Mediterranean forest species with highly fragmented distribution.

The evaluation of genetic diversity, genetic structure, and gene flow of Italian *Q. trojana* populations allowed (1) identification of priorities for *Q. trojana* conservation in southern Italy and (2) proposal of possible measures to counteract stand fragmentation, isolation, and inbreeding.

Besides the genetic inputs provided, complementary actions can be suggested such as the preservation of the natural habitat, the ecological connection among residual forest patches, and the control of related biotic stress factors (pests, pathogens, and alien species). In addition, appropriate conservation programs for Italian *Q. trojana* could greatly benefit from a more extensive knowledge of the species genetic resources available along the whole distribution range (Balkan region).

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