

# Genetic structure of a natural oak community in central Italy: Evidence of gene flow between three sympatric white oak species (*Quercus*, *Fagaceae*)

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**Abstract.** Incomplete reproductive barriers between species, especially in sympatric areas where several species coexist, may result in hybridization and an increase in genetic diversity. Here we assessed the amount of genetic diversity in a community of three interfertile and sympatric European oaks (*Quercus frainetto* Ten., *Q. petraea* Liebl. Matt. and *Q. pubescens* Willd.) situated in central Italy. We used 11 microsatellite markers derived from Expressed Sequence Tag (EST-SSRs) and we implemented a Bayesian clustering analysis to assign individuals to species or hybrids. All genotyped loci were polymorphic for all the species and three genetic clusters corresponding to each species were detected. Significant differences and a higher level of gene flow were observed between the three oak species. Occurrence of hybrids varied markedly within the studied area: hybrids between *Q. petraea* and *Q. pubescens* were the most frequent, while hybrids between *Q. petraea* and *Q. frainetto* were particularly rare. *Q. pubescens* and *Q. petraea* showed the highest number of alleles compared to *Q. frainetto*, which was characterized by a low number of private, but highly frequent, alleles. However, *Q. frainetto* showed a lower genetic diversity and a stronger reproductive isolation from the other two oak species.

**Keywords** bayesian clustering analysis, interspecific gene flow, Italian Peninsula, hybridization, *Quercus frainetto*, *Q. petraea*, *Q. pubescens*, EST-SSRs

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## Introduction

Hybridization occurs in areas where interfertile taxa coexist and represents an important biological factor in the evolution of plant species (Rieseberg 1997). White oaks (genus *Quercus*, section *Quercus sensu* Nixon 1993) are usually sympatric in different areas of their distribution, a configuration that tends to favour interspecific gene flow (Rushton 1993, Williams et al. 2001, Tovar-Sánchez & Oyama 2004, Lepais et al. 2009). The identification of hybrids in *Quercus* genus can be difficult because of the high morphological variability of hybrids, even when the morphological characters of the parental species are well described. This leads to a high morphological variability and brings confusion in the taxonomy of this group; in the Italian peninsula this is especially true for *Q. pubescens* (Di Pietro et al. 2012). In such a case genetic markers provide an invaluable tool to determine the relative genetic contribution from various parental species and to estimate the incidence of hybridization.

Oak reproductive system, gene flow and hybridization dynamics of European white oaks has been intensively studied in different areas of sympatry (Muir & Schlötterer 2005, Curtu et al. 2007a, Curtu et al. 2007b, Gugerli et al. 2007, Lepais et al. 2009, López de Heredia et al. 2009, Salvini et al. 2009, Viscosi et al. 2009, 2012, Neophytou et al. 2010, Lind & Gailing 2013). On a continental scale, white oaks are characterized by high level of gene flow and high rates of hybridization (Gerber et al. 2014), which could be increased by environmental changes and disturbed environments (Legache et al. 2013).

European white oak species have plastic reproductive barriers characterized by postmaturing prezygotic barriers stronger than postzygotic barriers (Abadie et al. 2012, Lepais et al. 2013). In addition, the frequency and direction of hybridization is affected by several factors such as species abundance, forest management and local environmental conditions (Lepais

et al. 2009) underpinning potential impact of ecological factors on evolutionary processes.

In Europe, evidences of hybridization among sympatric white oaks were reported for many ecological regions and different species using nuclear molecular markers such as microsatellites SSRs. Nuclear SSRs are the markers of choice to estimate the amount of intraspecific and interspecific gene flow because of their high number of alleles and polymorphism level (Guichoux et al. 2011a). Additionally, nuclear SSRs are very valuable because they present variation in different genomic regions including random genomic region (non-genic nSSR) or genic region (EST-SSR) and thus can provide different information about the genomic context of the level of intra- and interspecific variation. Since such markers are highly transferable among related white oak species they represent useful tools to estimate neutral and potentially adaptive genic variation and to detect different pattern of gene flow and local adaptations (Neophytou et al. 2010, Lind & Gailing 2013, Guichoux et al. 2013). Such genetic markers should represent useful tools to estimate neutral and potentially adaptive genic variation to detect different patterns of gene flow and local adaptations (Neophytou et al. 2010, Lind & Gailing 2013, Guichoux et al. 2013). EST-SSRs are advantageous if compared to genomic SSRs because allow to study expressed genomic regions and because they are characterized by a better conservation of primer sites, which are easily transferable across closely related species (Durand et al. 2010).

While the use of fast and cost effective PCR multiplexing technique was limited in number of loci (Dzialuk et al. 2005, Lepais et al. 2006), two powerful multiplexes were lately developed for white oaks (*Q. petraea* and *Q. robur*). These were a 12-plex of EST-SSRs and an 8-plex of genomic SSRs and showed to work well for other species in the Fagaceae family (Guichoux et al. 2011b). These techniques are usually combined with the use of Bayesian

clustering analysis allowing to study hybridization, to increase understanding of hybridization pattern across the white oak species. Indeed, while *Q. robur* and *Q. petraea* have been extensively studied, the European oak forests often consisted of a species communities composed of other species that usually remain notably understudied. A few exceptions can however be considered, for instance in West-Central Romania, a community of *Q. frainetto*, *Q. petraea*, *Q. pubescens* and *Q. robur* which showed natural hybridization, introgression and different levels of ancient gene flow among species (Curtu et al. 2007a, 2007b). In Spain, the individual admixture rate was assessed in mixed oak forests of *Q. petraea* and *Q. pyrenaica* revealing low levels of introgression between these two species (Valbuena-Carabaña et al. 2007). In Central-Northern Italy a study on mixed forests of *Q. pubescens* and *Q. petraea* indicated the presence of interspecific gene flow and asymmetrical introgression that was probably caused by the relative species abundance (Salvini et al. 2009). In France a study on *Q. petraea*, *Q. pubescens*, *Q. pyrenaica* and *Q. robur* showed evidence of hybridization among species and high variation in the hybridization level between sites (Lepais et al. 2009). In a study of neighboring stands of *Q. petraea* and *Q. robur*, along an ecological gradient from Germany to Balkan were detected effects of gene flow and adaptations on genomic SSRs loci (Neophytou et al. 2010). The Italian peninsula represented an important refuge area during the Quaternary era (Brewer et al. 2002, Petit et al. 2003) and is actually the southern margin of a natural distribution range of several white oaks species (i.e. *Q. frainetto*, *Q. petraea*, *Q. pubescens* and *Q. robur*). In spite of the importance of this area for the European white oaks, there is little information on the genetic differentiation and on the hybridization patterns within such a complex oaks species community in the region. This paper aims to improve the knowledge on the genetic relationships among three

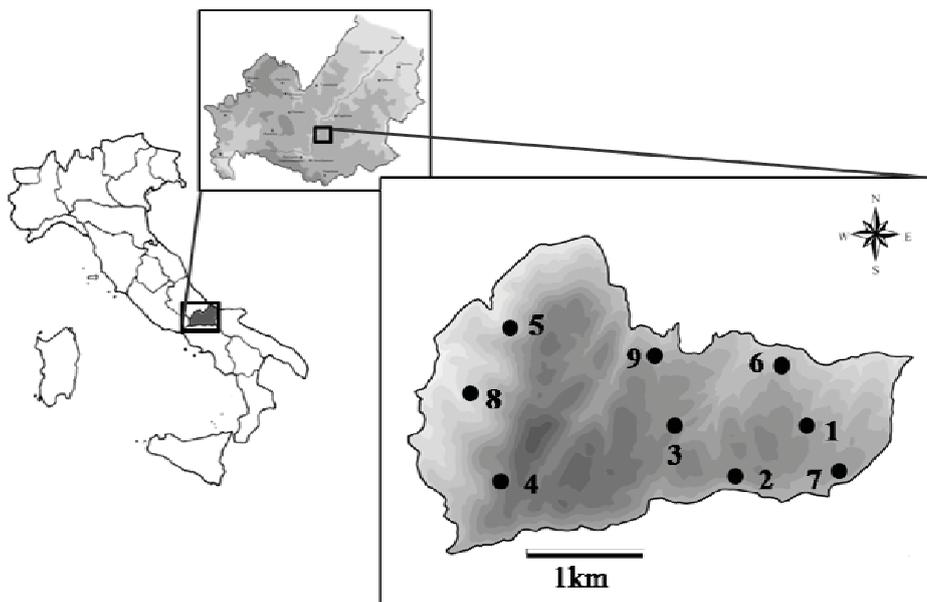
sympatric white oaks (*Quercus frainetto*, *Q. petraea*, *Q. pubescens*) in an Italian area representing both an ancient glacial refuge and the present rear edge of the distribution area of these species. *Quercus pubescens* is widespread across the Italian peninsula owing to its wide ecological requirement and preference for xeric soil condition. *Q. petraea* geographical distribution is scattered in central-southern Italy and this species is usually not a dominant tree within woody communities. Finally *Q. frainetto* has a southern distribution in Italy and is a poorly genetically characterized species among the European white oaks. Indeed only a few papers reported information about its genetic variation (i.e. Curtu et al. 2007a, 2011, 2014, Fortini et al. 2009, Viscosi et al. 2012, Fortini et al. 2013).

Using newly developed genetic markers, we inferred past gene flow by assigning individuals as purebred or admixed genotypes (putative hybrids) and assessing the genetic differentiation between species in these rear edge populations.

## Materials and methods

### Plant material

A total of 273 oak trees were sampled in a mixed forest community (680 ha) of Central-Southern Italy (Fig. 1) containing three white oak species (*Q. frainetto*, *Q. petraea* and *Q. pubescens*) associated with *Q. cerris* L. and other tree species, as *Acer campestre* L., *Acer opalus* Mill., *Acer cappadocicum* Gled. subsp. *Lobellii* (Ten.) Murray, *Carpinus betulus* L., *Fraxinus excelsior* L., *Fraxinus ornus* L., *Fagus sylvatica* L., *Tilia cordata* Mill. and *Castanea sativa* Mill. The studied natural plant community is located in an important site for the Mediterranean biogeographical region and in these areas mostly occur as high forests, while coppice areas are less common. It represents a community importance site (site code: IT7222295) for



**Figure 1** Localization of the study area in Italian peninsula and Molise region

the Mediterranean biogeographical region and is characterized by a high natural conservation level of a deciduous oak community (habitat code: 91M0).

Specimens were collected in nine 50 by 50 meters plots (Viscosi & Fortini 2011, Viscosi et al. 2012) where at least 30 mature individuals were randomly sampled. Previously, the entire study area has been intensively studied by phytosociological relevés (Mucina et al. 2000); thus, the environmental data, the plant species and their relative covering were recorded. The plots have been identified as representative of homogeneous areas for vegetation and micro-environmental factors (Viscosi & Fortini 2011). In particular, the natural area was characterized by three distinct microclimatic sectors. The plots dominated by *Q. petraea* were located in the northern sector of the studied area and principally characterized by the north-facing hill side; plots located in the southern sector on the studied area were characterized by warmer and dryer microclimatic conditions and by a rare occurrence of *Q. petraea* and a dominance (or co-dominance) of *Q. frainetto* and *Q. pubescens*; finally, a small sector, was characterized by calcareous sub-

strates and dryer microclimatic conditions and dominated by *Q. pubescens*.

Morphological identification was performed following Schwarz (1993), Curtu et al. (2007) and Fortini et al. (2009). In addition, the leaves of all samples were observed using an electron microscope (Fortini et al. 2013). Specimens assigned to *Q. petraea* had glabrous young twigs and stellate trichomes on the abaxial leaf surface; *Q. frainetto* and *Q. pubescens* specimens had pubescent young twigs and fasciculate trichomes. Leaves with more than eight pairs of parallel lateral veins were indicative of *Q. frainetto*; while specimens with fewer than eight pairs of lateral veins were classified as *Q. pubescens*. Individuals with atypical morphology were considered as hybrids.

#### DNA extraction and amplification

DNA was extracted from 0.5 g of -80°C stored leaves using spin columns of “Invisor® Spin Plant Mini Kit” and following the protocol manufacturer’s instructions. Twelve nuclear microsatellite loci (EST-SSRs: “PIE020, PIE223, PIE152, PIE242, PIE102, PIE243, PIE239, PIE227, PIE271, PIE267, PIE258, PIE215”)

were genotyped as described elsewhere (Durand et al. 2010, Guichoux et al. 2011b). PCR reactions were performed in a DNA Engine Tetrad (MJ Research Bio-Rad) thermocycler and PCR products were run in an ABI 3730xl capillary sequencer (Applied Biosystems) at the Genome-Transcriptome facility of Bordeaux (Cestas, FR) using Genescan 600 LIZ internal size standard (Applied Biosystems). Alleles were scored using STRand software version 2.3.106 (Toonen & Hughes 2001) and allele binning were performed as described in Guichoux et al. (2011a). We checked the presence of null alleles, stuttering and allele dropout using the Micro-checker v2.2.3 with 1000 randomizations and a 95% confidence interval (Van Oosterhout et al. 2004).

### Statistical analysis

Genetic assignment was performed without prior information on location and morphological classification by means of Bayesian clustering analysis using Structure ver. 2.3.1 (Pritchard et al. 2000; Falush et al. 2003). We used the Admixture model and the Correlated Allele Frequencies model. The ALPHA Dirichlet parameter for degree of admixture has an initial value of 1.0 with a standard deviation of proposal for updating Alpha of 0.025. We assumed a frequency model with different value of  $F_{ST}$  for different populations (prior mean of  $F_{ST}$  for populations of 0.01 and standard deviation of 0.05) using a constant lambda of 1.0. Structure was run from 1 to 9 number of clusters ( $k$ ), with ten repetitions for every  $k$ , 100,000 burn-in periods and 1,000,000 MCMC repeats after burning. Following the  $\Delta k$  method (Evanno et al. 2005), the package CorrSieve (Campana et al. 2011) was used to detect the most likely number of clusters. Individuals were assigned to species or putative hybrid by means of the estimated membership coefficient ( $Q$ ). Following current practices (e.g. Lepais et al. 2009) individuals with probabilities above 0.90 to belong to one clusters ( $Q \geq 0.900$ ) were as-

signed as purebred while individuals with intermediated admixture coefficient ( $0.100 \geq Q \geq 0.900$ ) were assigned as hybrids.

AMOVA (Analysis of Molecular Variance) was performed to analyze the hierarchical partition of genetic variation and to test the degree of genetic differentiation between species. AMOVA was performed, on the pure species, with the software package GenAlEx version 6.4 (Peakall & Smouse 2006) and the significance of  $F_{ST}$  was tested by 9999 random permutations. The following summary statistics were estimated within populations using GenAlex 6.4: average number of alleles per locus [ $N_a$ ], average number of effective alleles per locus [ $Ne$ ], observed heterozygosity [ $H_o$ ], expected heterozygosity [ $H_e$ ] and inbreeding coefficient [ $F_{is}$ ]. In addition, allelic richness [ $R_s$ ], a diversity measure which is “independent of the population size” (Petit et al. 1998), obtained after rarefaction was computed for each population and locus using the program FSTAT v. 2.9.3 (Goudet 2001).

### Results

A total of 268 out of 273 sampled individuals were successfully genotyped. Micro-checker (Van Oosterhout et al. 2004) revealed the presence of a significant amount of null allele at the locus PIE258 for the three species and was thus removed from the subsequent analyses.

All remaining loci were polymorphic with a total of 127 alleles varying from 69 to 260 bp (Table S1) and, in general, fragment sizes matched with the relative SSR reference size, as described in Guichoux et al. (2011b).

Bayesian clustering analysis allowed the identification of three genetic clusters corresponding to the three morphological species: *Q. frainetto*, *Q. petraea* and *Q. pubescens*. Overall, *Q. petraea* was the most frequent species representing 35.82% of the individuals followed by *Q. frainetto* and *Q. pubescens*, which represent 20.90% and 20.52, respective-

ly (Table 1). Hybrids (including backcrossed and F<sub>1</sub> hybrids) varied considerably ranging from 3.33% to 42.86%, between plot 8 (1 hybrid) and 5 (12 hybrids) having the minimum and the maximum numbers of hybrids, respectively. In detail, we identified 29 *petraea* x *pubescens*, 16 *frainetto* x *pubescens*, 7 *petraea* x *frainetto* and 9 trihybrids *petraea* x *pubescens* x *frainetto* (Table 1). It appeared that 15 morphologically intermediate specimens were ge-

netically pure (8 *Q. pubescens*, 5 *Q. frainetto* and 2 *Q. petraea*) while 36 trees morphologically classified as pure species (23 *Q. petraea*, 7 *Q. frainetto* and 6 *Q. pubescens*) were putative hybrids.

Mean allelic richness over loci (Rs) ranged between 6.53 and 9.55 (Table 2) with *Q. pubescens* presenting the higher allelic richness (Table 2). Expected heterozygosity (He) varied from 0.65 to 0.72 in pure species and very

**Table 1** Results of genetic assignment of individuals per plot. For each genotype (pure or mixed) number of individuals (up) and their percentage (down) were reported

Stand	N	<i>fra</i>	<i>pet</i>	<i>pub</i>	<i>fra</i> X <i>pet</i>	<i>fra</i> X <i>pub</i>	<i>pet</i> X <i>pub</i>	<i>pub</i> X <i>pet</i> X <i>fra</i>
1	32	8 (25.00)	18 (56.25)	-	1 (3.13)	-	3 (9.38)	2 (6.25)
2	30	11 (36.67)	1 (3.33)	10 (33.33)	1 (3.33)	4 (13.33)	2 (6.67)	1 (3.33)
3	30	3 (10.00)	20 (66.67)	-	1 (3.33)	-	6 (20.00)	-
4	30	9 (30.00)	2 (6.67)	10 (33.33)	-	7 (23.33)	1 (3.33)	1 (3.33)
5	28	2 (7.14)	11 (39.29)	3 (10.71)	-	3 (10.71)	7 (25.00)	2 (7.14)
6	30	1 (3.33)	23 (76.67)	-	1 (3.33)	-	4 (13.33)	1 (3.33)
7	30	20 (66.67)	3 (10.00)	3 (10.00)	2 (6.67)	2 (6.67)	-	-
8	30	-	-	29 (96.67)	-	-	1 (3.33)	-
9	28	2 (7.14)	18 (64.29)	-	1 (3.57)	-	5 (17.86)	2 (7.14)
Total	268	56 (20.90)	96 (35.82)	55 (20.52)	7 (2.61)	16 (5.97)	29 (10.82)	9 (3.36)

**Table 2** Genetic diversity of species

Species	N	N <sub>a</sub>	N <sub>a</sub> Freq. ≥5%	Ne	I	Private alleles	R <sub>s</sub>	H <sub>e</sub>	H <sub>o</sub>	F <sub>is</sub>
<i>Q. frainetto</i>	56	6.64	4.09	3.28	1.36	0.18	6.53	0.65	0.63	0.04
<i>Q. petraea</i>	96	9.00	4.55	4.10	1.52	0.55	7.90	0.69	0.67	0.01
<i>Q. pubescens</i>	55	9.46	4.91	4.82	1.68	0.55	9.22	0.72	0.72	0.001

Note. Abbreviations: N - number of individuals, N<sub>a</sub> - mean number of alleles per loci, Ne - effective allelic number, I - Shannon's information index, R<sub>s</sub> - mean allelic richness over loci obtained after rarefaction to 46 diploid individuals, H<sub>e</sub> - expected heterozygosity unbiased for sample size, H<sub>o</sub> - observed heterozygosity, F<sub>is</sub> - inbreeding coefficient obtained by 9999 bootstraps of the individuals within species.

similar values between observed heterozygosity ( $H_o$ ) and expected heterozygosity ( $H_e$ ) indicated an Hardy-Weinberg equilibrium. This was confirmed by the low fixation index values (Fis: *Q. frainetto*: 0.035; *Q. petraea*: 0.011; *Q. pubescens*: 0.038) indicating no significant heterozygosity deficit for each species.

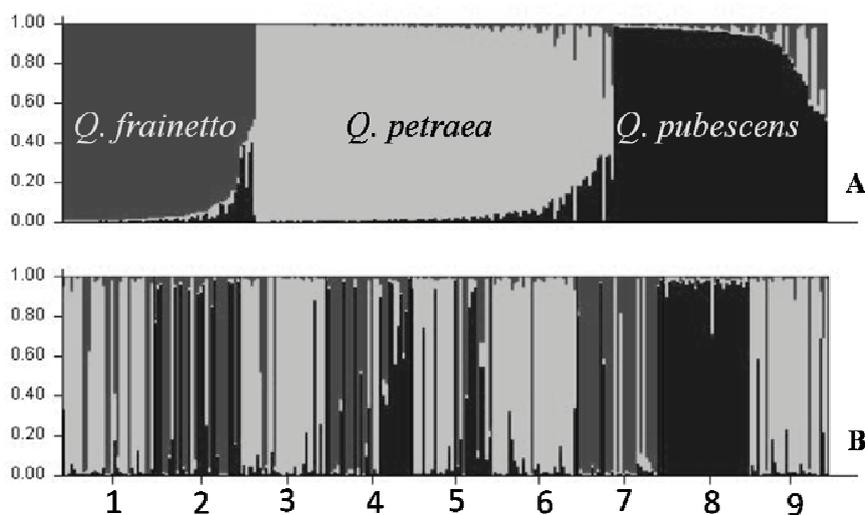
Private alleles were also identified in *Q. pubescens* and in *Q. petraea* while a lower number were found in *Q. frainetto* (Table 2; Table S3). Moreover, 48.03% of all alleles were common in the three species investigated: *Q. petraea* and *Q. pubescens* shared most common frequent alleles (70.97% of common alleles) while *Q. frainetto* shared only 12.90% with *Q. petraea* and 16.13% with *Q. pubescens*.

The AMOVA showed that the percentage of molecular variance is mostly distributed within the species (86% of total variance) while a smaller but significant portion of genetic variability was explained by variance among species (14% of total variance;  $F_{ST} = 0.137$ ;  $P < 0.001$ ). In addition, pairwise AMOVA indicated significant differences between species ( $P < 0.001$ ) with the higher differentiation observed between *Q. frainetto* and *Q. petraea*

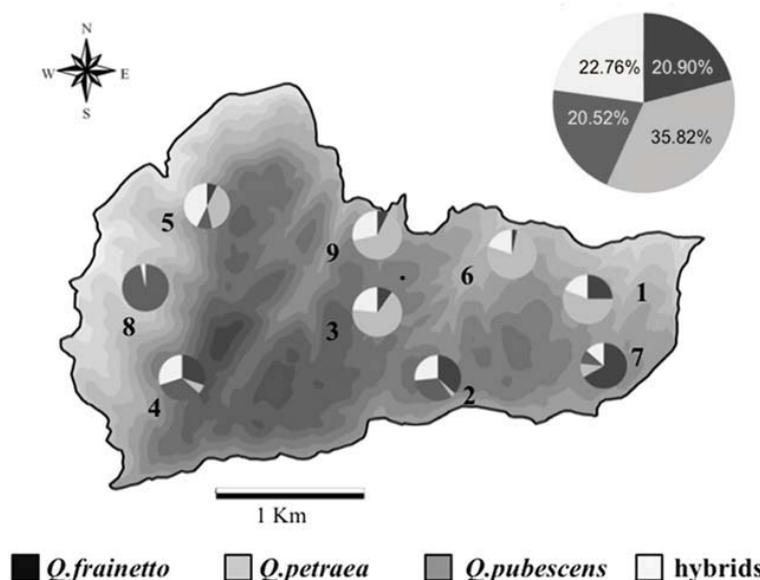
( $F_{ST} = 0.165$ ), followed by the pair *Q. pubescens* and *Q. petraea* ( $F_{ST} = 0.122$ ) and, finally *Q. frainetto* and *Q. pubescens* ( $F_{ST} = 0.115$ ).

The overall genetic composition of the sample was heterogeneous (Fig. 2) due to the species composition of the plots (Fig. 3). In fact, five plots consisted in majority of *Q. petraea*. On the other side, three plots were characterized by a greater proportion of *Q. frainetto* while only one plot was mostly composed of *Q. pubescens*. In general, all plots presented more than 70% of purebred individuals at the exception of plot 5 (only 57.14%). Purebred *Q. frainetto* and *Q. petraea* were found in all plots, except in a plot that only contained purebred *Q. pubescens*. Purebred *Q. petraea* dominated most of the plots, while *Q. frainetto* was highly dominant only in a plot (66.67%) and co-dominant with *Q. pubescens* in another plot.

Different mixtures of hybrid genotypes were detected in the plots. Mixed *Q. petraea* x *Q. pubescens* genotypes were widespread and they were detected also for the plots without the two parental species. In contrast, hybrid *Q. frainetto* x *Q. petraea* genotypes were detect-



**Figure 2** Genetic assignment without any a priori information obtained with Structure clustering analysis. (2A) Clustering of individuals to three different genetic clusters with decreasing admixture coefficient  $Q$  (2B) Clustering of individuals of different stands separated by black lines



**Figure 3** Geographical location and genetic structure of the plots

ed in plots where both parental species were found as purebred (Fig. 3). This was observed also for *Q. frainetto* x *Q. pubescens* hybrids that were detected only in plots where purebreds of both parental species were found (Fig. 3). Finally, mixed genotypes characterized by a significant contribution of all three pure species were found in plots that were composed by purebred *Q. frainetto* and *Q. petraea*, while purebred *Q. pubescens* were found only in two plots (Fig. 3).

## Discussion

The analysis of gene flow between sympatric white oak species is important to understand the dynamics of hybridization patterns (Lepais et al. 2009, Lepais & Gerber, 2011). In particular, by using genetic assignment without any a priori information (Pritchard et al. 2000, Duminil et al. 2006) many studies highlighted different levels of hybridization rate among European white oak species (Valbuena et al. 2005, Gugerli et al. 2007, Curtu et al. 2007b, Salvini et al. 2009, Lepais et al. 2009, Viscosi et al. 2012, Guichoux et al. 2013).

Analysis of molecular variance indicated

that the largest percentage of molecular variance explained the differences within species and the pairwise computation highlighted that all the species were significantly differentiated. In particular, the degrees of differentiation ( $F_{ST}$ ) were similarly between *Q. petraea* – *Q. pubescens* ( $F_{ST} = 0.122$ ) and *Q. frainetto* – *Q. pubescens* ( $F_{ST} = 0.115$ ), while *Q. frainetto* – *Q. petraea* ( $F_{ST} = 0.165$ ) showed the greatest differentiation. These results were coherent with similar studies on European oaks but the average ( $F_{ST} = 0.137$ ) and pairwise differentiation among these three species were higher because of the difference in types of sampling and genetic markers. In particular, Curtu et al. (2006) reported a significant  $F_{ST}$  (0.096) among four white oak species; the greatest differentiation was observed between *Q. robur* and *Q. frainetto* ( $F_{ST} = 0.168$ ) while the lowest genetic differences was between *Q. pubescens* with *Q. petraea* (0.045) and with *Q. frainetto* (0.050). In addition, Curtu et al. (2011) reported significant differences ( $F_{ST} = 0.067$ ) between *Q. frainetto* and *Q. pubescens*. In Fortini et al. (2009), the greatest differentiation was observed between *Q. robur* and *Q. frainetto* ( $F_{ST} = 0.242$ ), while *Q. petraea* and *Q. pubescens* ( $F_{ST} = 0.114$ ) were the lowest differentiated;

however, the differentiation of *Q. frainetto* with *Q. petraea* ( $F_{ST} = 0.160$ ) was higher than with *Q. pubescens* ( $F_{ST} = 0.142$ ). Salvini *et al.* (2009) reported a low and significant differentiation ( $F_{ST} = 0.076$ ) between *Q. petraea* and *Q. pubescens*, suggesting that, despite gene flow, the interspecific reproductive barriers contribute to species integrity. Thus, a high differentiation could indicate a greater phylogenetic distance between pair of species; while the low genetic differentiation between species could be a result of higher gene flow rates between species that diverged more recently (Curtu *et al.* 2006).

Individuals assigned to pure species were the most frequent (77.24%) while the admixed genotypes represented a low percentage. Different types of hybrids were detected: hybrids between *Q. petraea* and *Q. pubescens* were the most frequent while the hybrids between *Q. petraea* and *Q. frainetto* were particularly rare. Besides, the low number of hybrids detected in the area could be explained by the hypothesis that pure individuals should be more adapted to local environmental conditions (Lepais *et al.* 2009). In addition, such observation could be due to the existence of strong reproductive barriers among species (Abadie *et al.* 2012, Lepais *et al.* 2013). In fact, previous studies of different oak species revealed that interspecific gene flow was relatively common but often asymmetric and intermediate forms and hybrids were mostly located at the environmental edges of the species respective ecological niches (Lind & Gailing 2013). This supports the hypothesis that white oaks are not totally reproductively isolated and that genes exchange producing different hybridization patterns may have potential evolutionary consequence. Differences in hybridization rates among sympatric species at different sites could result from several factors, such as species abundance, environmental factors, ecological species preferences, species geographic distribution and anthropic activities (Lepais *et al.* 2009, Gerber *et al.* 2014). In addition, perturbing the spatial

structure of communities and decreasing the density of conspecifics increase the hybridization rates in natural oak communities (Legache *et al.* 2013). Differences in rates of hybridization among the oak communities indicates the presence of both pre- and postzygotic barriers that can be variable in relation to diverse ecological conditions and also during oaks recolonization after the glaciations (Abadie *et al.* 2012). Prezygotic and postzygotic barriers contribute differently to reproductive isolation in oak species and the strongest contribution of prezygotic isolation probably results from the reinforcement phenomena, due to selection against hybrid formation (Lepais *et al.* 2013).

In addition, the within species genetic diversity and its potential relationship with interspecific gene flow were studied. *Q. pubescens* and *Q. petraea* had the highest number of alleles compared to *Q. frainetto* and the latter species was also characterized by a low number of private, but highly frequent, alleles. This was in sharp contrast with the higher number of private alleles found in *Q. petraea* and *Q. pubescens*, which also shown similar levels of genetic differentiation when compared to *Q. frainetto*. A likely explanation for this pattern may be that the higher number of common alleles shared between *Q. petraea* and *Q. pubescens* indicates a more extensive and long-term gene flow between these two species.

In contrast, the low genetic diversity found in *Q. frainetto* and a low rate of interspecific mating between this species and *Q. petraea* or *Q. pubescens* could be temptingly explained by a stronger reproductive barrier between *Q. frainetto* and the other species (Curtu *et al.* 2011). However differences in rates of interspecific gene flow for this species may also be related to prezygotic barriers such as differences in flowering times and pollen viability (Salvini *et al.* 2009).

## Conclusions

A higher proportion of *Q. pubescens* alleles were detected in hybrids, even in plots where *Q. pubescens* individuals were absent. *Q. pubescens* seems to hybridize more frequently with *Q. petraea* than with *Q. frainetto*. Putative hybrids of *Q. frainetto* and *Q. petraea* were extremely rare and a low rate of interspecific mating was found between *Q. frainetto* and *Q. pubescens*. The rarity of hybrids between *Q. frainetto* and *Q. pubescens* could be due to the spatial distributions of species and their relative abundance. The high allelic richness found in *Q. pubescens* may be explained by the propensity of this species to hybridize with other species, in particular considering the extensive gene flow recorded with *Q. petraea* in several areas of their distribution.

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### Supporting Information

The online version of the article includes the Supp. Info.

**Table S1:** Alleles, in bp, detected for all EST-SSR in the data set.

**Table S2:** Characteristics of alleles.

**Table S3:** Summary of private alleles by species.