www.czasopisma.pan.pl PAN www.journals.pan.pl
POLSKA AKADEMIA NAUK

ACTA BIOLOGICA CRACOVIENSIA Series Botanica 61/1: 17–28, 2019
DOI: 10.24425/abcsb.2019.127739

PAN
POLSKA AKADEMIA NAUK
ODDZIAŁ W KRAKOWIE

Genetic Diversity and Differentiation of Coexisting Populations of *Quercus robur* L. and *Q. petraea* (Matt.) Liebl.

Elżbieta Sandurska, Bartosz Ulaszewski and Jarosław Burczyk*

Department of Genetics, Institute of Experimental Biology, Faculty of Natural Sciences, Kazimierz Wielki University, Chodkiewicza 30, 85-064 Bydgoszcz, Poland

Received April 12, 2019; revision accepted May 19, 2019

Pedunculate and sessile oaks (*Quercus robur* L.; *Q. petraea* [Matt] Liebl.) often coexist in mixed forest stands. However, species-specific investigations and forest management actions in such populations require reliable methods of identification of the species status of individuals. We investigated genetic diversity and species differentiation of adult and naturally established seedling cohorts in a mixed forest stand composed of *Q. robur* and *Q. petraea*, located in the Jamy Nature Reserve in north-central Poland. Using nineteen nuclear microsatellite loci and a model-based clustering approach as a tool for species delineation, we efficiently identified 105 and 60 adults, as well as 191 and 456 seedlings of pedunculate and sessile oaks, respectively. While the adult trees of both species were randomly distributed throughout the sample plot, the seedlings demonstrated significant spatial clustering, which was particularly evident for *Q. petraea*. The two oak species exhibited similar levels of genetic diversity in adult and offspring cohorts. Inbreeding was found to be low and significant only at the stage of seedlings. The estimates of effective population size were higher for *Q. robur* than *Q. petraea*, despite the overall greater reproductive success of the later one. There was a significant level of differentiation between the studied oak species, as measured by F_{st} coefficient (0.084 – adults; 0.099 – seedlings). The results on genetic diversity and species differentiation obtained in the studied indigenous near-natural stand of *Q. robur* and *Q. petraea* could be considered as a reference for other population genetic studies of oaks.

Keywords: nature reserve, genetic diversity, genetic differentiation, species identification, effective population size, *Quercus petraea*, *Q. robur*

INTRODUCTION

Genetic variation is the foundation of biodiversity. It allows populations and species to evolve and adapt to changing environmental conditions, increasing their chances of survival (Lindenmayer et al., 2006; Hughes et al., 2008). Genetic diversity of key-stone species important for ecosystem functioning, such as many forest trees, is of particular significance, as it is believed that genetic variation of forest trees is one of the factors shaping the biodiversity and stability of entire forest ecosystems (Whitham et al., 2003; Whitham et al., 2006; Gugerli et al., 2013).

The adaptation ability of populations largely depends on the level of genetic diversity existing within the populations (Booy et al., 2000; Aitken et al., 2008), but also on the mechanisms that determine transmission of this variation from generation to generation. The identification of these mechanisms is fundamental to understand how the level of population genetic variation is shaped and enhances our knowledge on the adaptation of populations and species evolution (Aitken et al., 2008; Sork, 2016). Such knowledge can also have practical applications when creating conservation programs to protect the genetic resources of particular populations and species. In the case of forest trees, it may also have economic implications in the development of appropriate breeding programs that will guarantee the preservation of an appropriate level of genetic variation.

Despite the longevity of most forest trees and their complex age structure, the question arises whether genetic diversity present in adult populations is preserved in young, naturally regenerating generations. Maintaining genetic diversity between generations is considered a major factor in the persistence of forest tree populations

* Corresponding author, email: burczyk@ukw.edu.pl

PL ISSN 0001-5296

(Aitken et al., 2008) and is particularly important in protected areas such as national parks and nature reserves.

Pedunculate oak (*Quercus robur* L.) and sessile oak (*Q. petraea* (Matt.) Liebl.) are among the most important broad-leaved tree species in Central Europe, from an ecological and economic point of view (Eaton et al., 2016). Oaks were the dominant forest species from about 5000 BC, spreading in Europe immediately after the end of the last glaciation, and over the next several millennia the current range of these species has been formed (Huntley, 1988). Human interference in the structure of European forests, like cutting forests for agricultural purposes or replacing them with faster growing conifers, such as spruce, fir or pine, caused decimation of the oak population (Fischer and Fischer, 2012).

Both oak species often grow together in mixed stands, which is possible due to their partially sympatric distribution (Eaton et al., 2016). Morphological differences between *Q. robur* and *Q. petraea* are visible mainly in leaf traits and the length of the acorn peduncle stalk; however, it is hard to indicate one or two distinct morphological features allowing to precisely distinguish these two species from each other. The distinction between the species requires simultaneous analysis of several leaf traits (Kremer et al., 2002). This can be particularly problematic in old stands, where trees are often very high, and the access to well-developed leaves for species identification is difficult. Distinguishing the species based on leaf traits at the stage of young seedlings is even more challenging (Ponton et al., 2004; Boratynski et al., 2008). Additionally, *Q. robur* and *Q. petraea* have the ability to hybridize (Petit et al., 2003; Lepais et al., 2009; Lepais et al., 2013; Chybicki and Burczyk, 2013; Gerber et al., 2014; Lagache et al., 2014), which can result in intermediate leaf phenotypes for some trees in the stand.

The need to distinguish both species was driven mainly by their different ecological requirements (Eaton et al., 2016), that need to be taken into account during artificial reforestation. *Q. robur* prefers more humid areas than *Q. petraea* (Danielewicz, 1995). Additionally, *Q. robur* has a more pioneer character compared to *Q. petraea* and shows a greater capability of seed dispersal with the participation of birds, such as jays. This affects the shape of the local spatial genetic structure, which is different from that observed for seed dispersal only by gravity, which in turn is more frequent in *Q. petraea* (Bossema, 1979; Petit et al., 2003). Recently, newly developed multiplexes of microsatellite loci designed for oaks to facilitate the species delimitation and parentage analyses have become available (Guichoux et al., 2011), providing more power for genetic discrimination between the two species and hybrid detection (Guichoux et al., 2011; Neophytou, 2013; Rellstab et al., 2016).

In our previous study (Sandurska et al., 2017), using 19 nuclear microsatellites, we investigated the genetic diversity of oaks in an old-growth mixed forest stand, composed mainly of beech and oak trees. According to the local forest documentation, the oak population was represented solely by *Q. robur* individuals. Because the plant material for isolation of DNA originated mostly from leaf fragments sampled by shooting leaves with a slingshot, the species status of individuals was not verified, assuming the presence of a single oak species. However, our later and more detailed observations indicated the presence of both oak species, namely *Q. robur* and *Q. petraea*. Here we present a reanalysis of our earlier genetic data and thus correcting the previous results (Sandurska et al., 2017). We first focus on genetic identification of the species status of adult oak trees and seedlings, and then investigate genetic diversity within and between cohorts of the two oak species. Finally, we emphasize the need for proper species identification of oaks and its importance in conservation genetics and forest management of mixed forest stands composed of both oak species.

MATERIALS AND METHODS

The study was performed in a forest stand being a part of the Jamy Nature Reserve, established in 1968 in the Jamy Forest District in north-central Poland. It functions as a phytocenotic reserve focusing on the conservation of beech forest communities: *Galio odorati-Fagetum* and *Luzulo pilosae-Fagetum*. The central point of the designed round-shaped study plot of approximately 5.5 ha has the coordinates φ 18°56'6.07"E, λ 53°35'9.67"N. The area is relatively flat, with no forest management, at least over the last 50 years. In the canopy of trees, mostly oaks dominate, at the age of over 215 years (based on forest records) and a diameter at breast height often exceeding 100 cm. However, there is also a considerable fraction of beech (*Fagus sylvatica*) of similar age and size with some linden (*Tilia cordata* Mill.) and pine (*Pinus sylvestris* L.) trees. In the understory, there is mainly beech at the age of 40–80 years (DBH: 30–60 m) with an admixture of hornbeam (*Carpinus betulus* L.) and linden. Oaks in the intermediate age class do not occur at the stand. Finally, the layer of the forest floor consists mainly of beech seedlings; however, oak seedlings are also present but at lower densities. For this study, plant material was sampled from two cohorts of oaks: *adults* (165 trees, all individuals sampled); and

seedlings – <30 cm tall (647 seedlings, regardless of the species status, sampled proportionally to the density distribution). All adult individuals were georeferenced using a GPS mapping system Pathfinder® ProXT™ (Trimble, Sunnyvale, USA). The location of seedlings was identified based on the nearest adult trees. The samples of leaves were collected in August of 2013 and 2014.

Total genomic DNA was extracted using GeneMATRIXPlant & Fungi DNA Purification Kit (EURx, Poland). Genotyping was done based on the set of 19 microsatellite markers: *PIE-20*, *PIE-102*, *PIE-215*, *PIE-223*, *PIE-242*, *PIE-243*, *PIE-267*, *ssrQrZAG 7*, *ssrQrZAG 11*, *ssrQrZAG 20*, *ssrQrZAG 96*, *ssrQpZAG 15*, *ssrQpZAG 110* (Guichoux et al., 2011); *ssrQrZAG 30*, *ssrQrZAG 65*, *ssrQrZAG 87*, *ssrQrZAG 101*, *ssrQrZAG 112* (Kampfer et al., 1998); *ssrQpZAG 9* – modified (Steinkellner et al., 1997). PCR products were separated using an ABI PRISM 3130XL sequencer (Applied Biosystems, Foster City, USA). Allele identification was performed with GENESCAN 3.7 and GENOTYPER 3.7 software provided by Applied Biosystems.

In this study, a set of microsatellite loci well-delineating oak species (Guichoux et al., 2011) was used. Therefore, we attempted to identify the species status of individuals by assigning them to one of the two genetic clusters defined with STRUCTURE v.2.3.4 software (Pritchard et al., 2000). Such genetic identification of the species was particularly important in the case of seedlings for which the explicit species recognition is particularly difficult. The analyses were conducted jointly for adults and seedlings, assuming the existence of two groups (K = 2), using 100 000 burn-in periods, 500 000 MCMC repeats and the admixing model, assuming correlations between allele frequencies. Individuals were assigned to a particular genetic cluster according to the value of the admixture coefficient (q). The species status of each of the two clusters was determined based on the leaf morphology of adult trees assigned to particular clusters. Every tree and seedling was assigned to a particular species based on the highest admixture coefficient (q).

Following the species identification, we characterized the spatial distribution of individuals of adults and seedlings. For that purpose, univariate O-ring statistics O(r) (Wiegand and Moloney, 2004) was used with a ring of a constant width of 5 m. The maximal ring was set at 130 m – less than half of the plot width to avoid estimation bias due to edge effects. The significance of the O(r) function was tested with common null model of complete spatial randomness (CSR) and 95% confidence interval was calculated using 25th and 975th of total 1000 randomizations (999 replicates by Monte Carlo simulation plus the observed value) of the function O(r). O(r) values above and below this envelope indicate significant spatial clustering or repulsion (hyper-dispersion), respectively, at radius r.

To characterize the small-scale interaction structure between both oak species we used bivariate analysis with the same parameters as for univariate analyses (ring width = 5 m, maximal ring = 130 m). As a null model, we chose the toroidal shift, where pattern 1 is shifted with a random vector against pattern 2, which is fixed (Wiegand and Moloney, 2014). Moreover, to investigate if the spatial distribution of DBH of the study species was random we conducted bivariate analysis with one quantitative mark. As a function, we chose spatial Moran I correlogram (Legendre and Legendre, 2012) and as a null model – complete spatial randomness (CSR). The main interest in analyzing patterns of this type is to explore the impact of proximity (and mark) of the first pattern on the marking of the second pattern (Wiegand and Moloney, 2014). To test if a given null model fits summary statistic of the observed data over a particular distance interval we used a simulation Goodness-of-Fit test (GoF test) proposed by Loosmore and Ford (2006) (see also Wiegand and Moloney, 2014). All these calculations and simulations were performed using the software PROGRAMITA (Wiegand, 2003).

The parameters of the genetic structure, including the frequencies of *null* alleles and inbreeding coefficients accounting for the presence of *null* alleles and genotyping errors were calculated within each cohort using INEst v.2.0 software (Chybicki and Burczyk, 2009). Allelic richness was estimated using Fstat 2.9.3.2 (Goudet, 1995). Differentiation measures (F_{st}, R_{st}) between different cohorts and between species were calculated using SPAGEDI v.1.5 (Hardy and Vekemans, 2002). Effective population size (Ne) was estimated for individual cohorts based on the linkage disequilibrium method (Waples and Do, 2008) using NeEstimator v2 (Do et al., 2014); however, we screened out alleles with frequencies lower than 0.02 (P_{Crit} = 0.02), as suggested for microsatellite data (Do et al., 2014).

RESULTS

GENETIC IDENTIFICATION OF SPECIES

The STRUCTURE procedure detected a very distinct delineation of oaks into two genetic clusters. Phenotypic analysis of adults' leaves indicated that the resulting cluster 1 was represented by pedunculate oak and the cluster 2 by sessile oak.

Comparisons of species identification based on genetic assignment and leaf morphology revealed that in 154 (93.3%) cases there was full agreement of species identification, while for two (1.21%) individuals there was disagreement between phenotypic and genetic indication. In other cases (9 individuals) the intermediate phenotype was noticed, although among them five individuals were genetically assigned as *Q. robur*, two as *Q. petraea*, and finally in two cases the genetic assignment also indicated an intermediate species status (intermediate *q* values). Finally, the individuals were assigned arbitrarily to one of the two clusters (species) only based on the highest probability of assignment in genetic analyses. Ultimately 105 adult trees were classified as *Q. robur* and 60 as *Q. petraea*, with relatively high probabilities of assignment within each group (0.986 ± 0.047 and 0.965 ± 0.079 for the first and second group, respectively) (Table 1).

TABLE 1. The numbers of adult trees (N_A) and seedlings (N_S) classified as *Q. robur* or *Q. petraea* based on STRUCTURE software with average individual probability of assignment (P_A, P_S) to a particular species cluster.

	Quercus robur	*Quercus petraea*
N_A	105	60
P_A	0.986 (± 0.047)	0.965 (± 0.079)
N_S	191	456
P_S	0.980 (± 0.059)	0.962 (± 0.080)

Seedlings were assigned to species only based on the highest probabilities of assignment in genetic analyses. In this way – 191 seedlings were identified as *Q. robur*, and the remaining 456 seedlings as *Q. petraea*. The average probabilities of assignment to particular species clusters were very high and equaled 0.980 ± 0.059 and 0.962 ± 0.080 for pedunculate and sessile oak, respectively (Table 1). It should be noticed that in both adults and seedlings the average probability of assignment was higher for cluster 1 (*Q. robur*).

Despite the detection of uneven numbers of adult trees of *Q. robur* and *Q. petraea*, the distributions of the diameter of both species were similar, which was confirmed by the shape of the histograms for both oak species (Fig. 1), as well as not significant *p*-value of Kolmogorov-Smirnov test ($p > 0.05$). This may indicate a similar age structure of both species in the studied area.

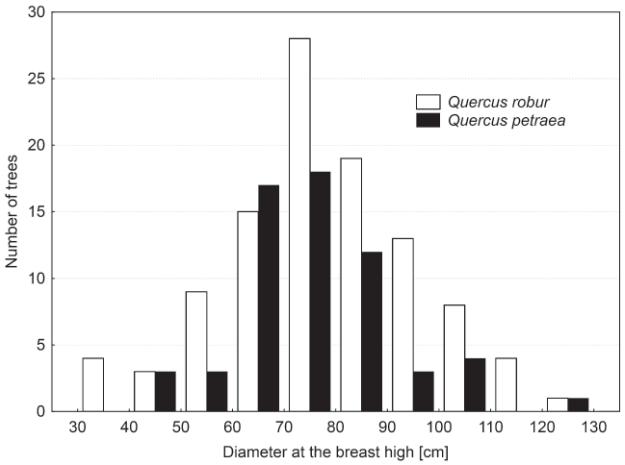

Fig. 1. Distribution of the diameter at breast height (DBH, in cm) among adult trees of *Q. robur* and *Q. petraea*.

SPATIAL DISTRIBUTION OF ADULT TREES AND SEEDLINGS

O-ring analyses showed a random distribution of adult trees of oaks within the population, which was additionally confirmed by the GoF test, giving statistically insignificant values suggesting no deviations from random distribution (Fig. 2), and also the correlogram function of bivariate analysis of both oaks together did not show any spatial grouping of one species against the other (data not shown). Additionally, there was no clear deviation of Moran *I* function from a random distribution of DBH, both for each species separately as well as together in bivariate analysis (data not shown).

In the case of seedlings, the O(*r*) function showed a strong and statistically significant spatial autocorrelation of both species (Fig. 2). In the case of sessile oak, this significance was present for almost all distance classes beyond the furthest ones, while pedunculate oak seedlings were less clustered. The O(*r*) values for *Q. robur* were above the confidence envelope for distances between 0 and 10 m, while for further distances fluctuated close to the upper limit of the confidence interval, sometimes giving statistical significance. Stronger clustering of sessile oak seedlings for close distances is also notable on the diagram of distribution of oak individuals (Fig. 3). Statistically significant deviations from the null hypothesis of random distribution of seedlings were also demonstrated by the GoF test, with *p* values equal to 0.001 for the distance 0–100 m (Fig. 2). The pair correlation function of bivariate analysis for seedling of both oaks together was well within the simulation envelopes, although its values were closer to the upper limit of the confidence interval,

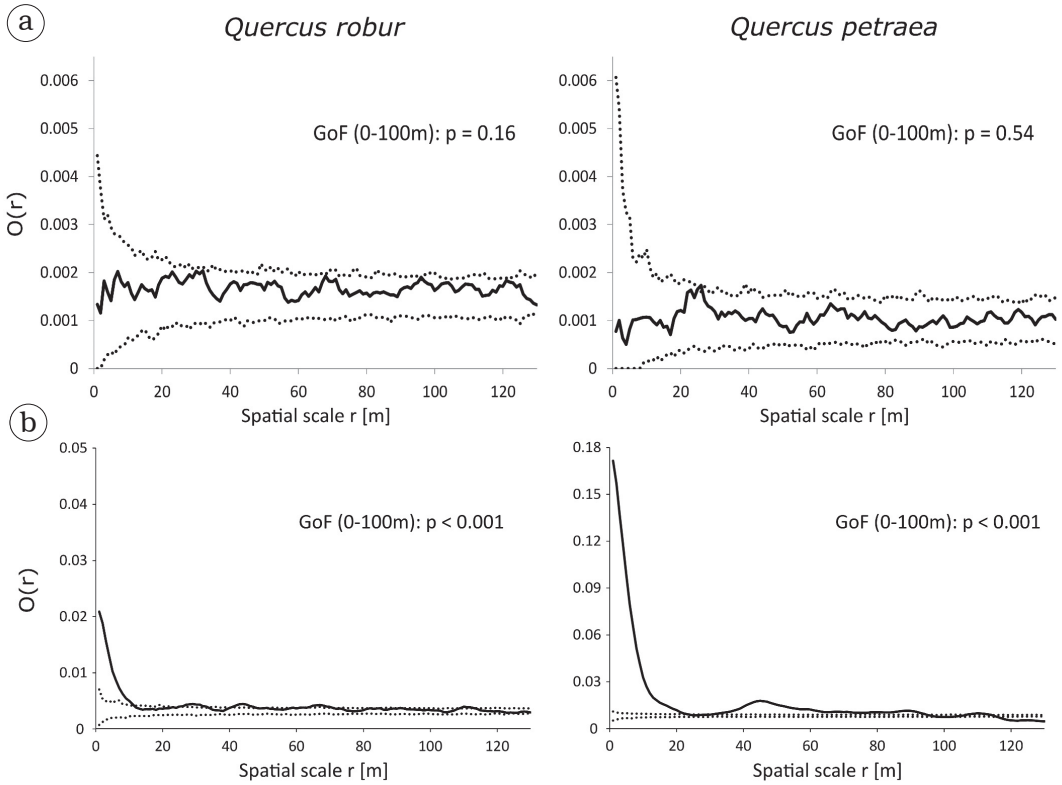

Fig. 2. Spatial demographic structure of (**a**) all adult trees and (**b**) seedlings as measured using the O-ring statistic [O(*r*)]. Dotted lines indicate 95% confidence envelopes about the null hypothesis of random spatial structure. GoF(0-100m) denotes Goodness-of-Fit test results on the given distance. Note that *y* axes show different scales of clarity for adults and seedlings.

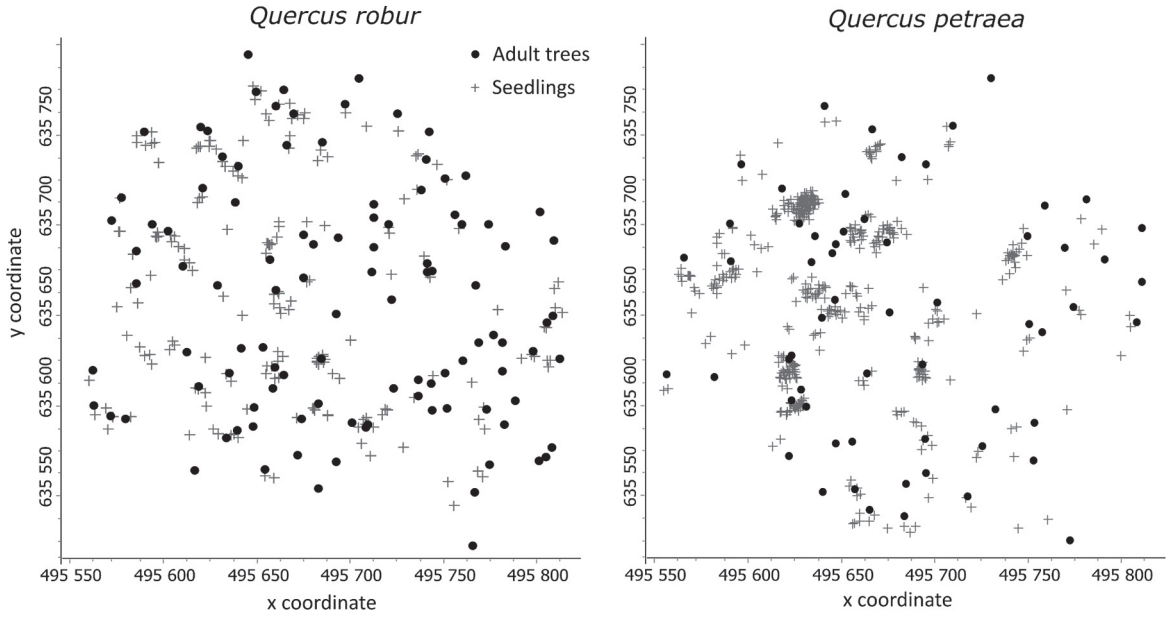

Fig. 3. Distribution of sampled adult trees (circles) and seedlings (crosses) of *Q. robur* and *Q. petraea*.

which resulted in a significant value of GoF test statistic over the distance interval 0–100 m (p = 0.007), indicating a slight grouping of one species against the other.

GENETIC DIVERSITY

The analysis of nineteen microsatellite markers showed very high polymorphism of the loci used in this study (Tables 2 and 3). In the case of *Q. robur* adult trees, the number of alleles per locus ranged from 5 to 30, with an average of 15.158 (± 7.338), and a similar value of mean allelic richness (15.052). In *Q. robur* seedlings, a slightly higher mean number of alleles and mean allelic richness (about 16.7) was recorded. The mean observed heterozygosity was at the same level as the mean expected heterozygosity, both in adults and in seedlings, which resulted in the value of the fixation index (F_{is}) close to zero (Table 2). This corresponded well with nonsignificant deviations from the Hardy-Weinberg expectations. In general, the frequency of *null* alleles was low. In adults, significant levels of *null* alleles were observed only for 2 loci (*QrZag11*, *QrZag65*; Table 2); in seedlings for 5 loci (*PIE242*, *PIE243*,

TABLE 2. Genetic diversity of *Quercus robur* in the reserve Jamy. Details for each locus are presented only for adult population, mean values are given for adults (N = 105) and seedlings (N = 191). ***A*** – mean number of alleles; $\boldsymbol{A_e}$ – effective number of alleles; ***AR*** – allelic richness; $\boldsymbol{H_o}$ – observed heterozygosity; $\boldsymbol{H_e}$ – expected heterozygosity; $\boldsymbol{F_{is}}$ – inbreeding coefficient; ***Null*** – frequency of null alleles; **H–W** – significance of departure from Hardy-Weinberg equilibrium. Standard errors in parentheses.

Locus	*A*	A_e	*AR*	H_o	H_e	F_{is}	*Null*	HW
PIE102	7	2.037	6.998	0.552	0.509	-0.085	0	ns
PIE20	11	4.902	10.903	0.743	0.796	0.067	0.0178	ns
PIE215	6	3.390	6	0.8	0.705	-0.135	0	ns
PIE223	8	5.076	7.998	0.829	0.803	-0.032	0	ns
PIE242	12	5.319	11.855	0.762	0.812	0.062	0.0182	ns
PIE243	5	1.980	4.998	0.505	0.495	-0.019	0	ns
PIE267	13	5.952	12.948	0.857	0.832	-0.03	0	ns
QrZag101	16	9.524	15.912	0.874	0.895	0.024	0.0046	–
QrZag11	28	13.158	27.8	0.865	0.924	0.063	0.0247*	–
QpZag110	16	3.077	15.9	0.667	0.675	0.012	0	ns
QrZag112	17	9.615	16.855	0.905	0.896	-0.01	0	–
QpZag15	9	3.390	8.903	0.705	0.705	0	0	ns
QrZag20	14	6.098	13.851	0.81	0.836	0.032	0	–
QrZag30	30	15.873	29.664	0.962	0.937	-0.026	0	–
QrZag65	28	18.519	28	0.88	0.946	0.069	0.0281*	–
QrZag7	19	13.333	18.883	0.942	0.925	-0.018	0	–
QrZag87	20	10.526	19.805	0.905	0.905	0	0	–
QpZag9	12	7.143	11.905	0.876	0.86	-0.019	0	–
QrZag96	17	2.119	16.807	0.562	0.528	-0.064	0	ns
Mean adults	15.158 (7.388)	7.423 (4.942)	15.052 (7.331)	0.790 (0.136)	0.789 (0.147)	-0.006 (0.053)	0.005 (0.009)	
Mean seedlings	16.737 (8.245)	7.340 (5.030)	16.636 (8.158)	0.779 (0.145)	0.789 (0.144)	0.011 (0.05)	0.0085 (0.011)	

Significance: * p < 0.05; ** p < 0.01; ns – not significant at p < 0.05

QrZag101, *QpZag110*, *QrZag65*). Nevertheless, when accounting for the presence of *null* alleles, a low but significant level of inbreeding was observed only for seedlings (F_{is} = 0.0115; 95% CI: 0.0065–0.0163).

Analysis of genetic diversity in *Q. petraea* adult trees showed slightly lower mean values of population genetic parameters than those observed in *Q. robur* (Table 3). The mean number of alleles per locus was equal to 13.368, and ranged from 6 (*QrZag112*) to 27 (*QrZag65*). The value of the allelic richness coefficient was similar to the mean number of alleles (13.269). In the case of seedlings, these parameters had similar values to *Q. robur*, except for the effective number of alleles, which was the lowest among all studied cohorts. As in the pedunculate oak, the expected heterozygosity values H_e = 0.778 (± 0.128) and the observed H_o = 0.773 (± 0.106) were similar, which resulted in the low value of the fixation index equal to -0.0035. Most of the loci had statistically insignificant levels of *null* alleles, only for 3 loci in adults (*PIE102*, *QrZag20*, *QrZag7*; Table 3) and 5 loci in seedlings (*QpZag15*, *QrZag65*, *QrZag7*, *QrZag87*, *QpZag9*) the level of *null* alleles was significantly different from zero. Again, the level

TABLE 3. Genetic diversity of *Quercus petraea* in the reserve Jamy. Details for each locus are presented only for adult population, mean values are given for adults (N = 60) and seedlings (N = 456). ***A*** – mean number of alleles; A_e – effective number of alleles; ***AR*** – allelic richness; H_o – observed heterozygosity; H_e – expected heterozygosity; F_{is} – inbreeding coefficient; ***Null*** – frequency of null alleles; **H–W** – significance of departure from Hardy-Weinberg equilibrium. Standard errors in parentheses.

Locus	*A*	A_e	*AR*	H_o	H_e	F_{is}	*Null*	HW
PIE102	11	5.076	10.932	0.767	0.803	0.046	0.0342*	ns
PIE20	8	2.591	7.966	0.617	0.614	-0.005	0.0274	ns
PIE215	9	3.650	8.866	0.7	0.726	0.036	0	ns
PIE223	7	1.976	6.966	0.55	0.494	-0.113	0	ns
PIE242	9	6.024	8.967	0.883	0.834	-0.06	0	–
PIE243	7	3.891	7	0.817	0.743	-0.099	0	ns
PIE267	9	4.049	8.966	0.8	0.753	-0.062	0	ns
QrZag101	19	9.804	18.797	0.883	0.898	0.017	0	–
QrZag11	14	3.521	14	0.69	0.716	0.037	0.0103	ns
QpZag110	21	6.329	20.797	0.883	0.842	-0.049	0	ns
QrZag112	6	1.980	5.932	0.6	0.495	-0.213	0	ns
QpZag15	13	5.814	12.932	0.85	0.828	-0.026	0	–
QrZag20	18	7.752	17.831	0.8	0.871	0.081	0.0323*	–
QrZag30	17	3.968	16.832	0.75	0.748	-0.002	0	ns
QrZag65	27	15.152	26.666	0.933	0.934	0	0	–
QrZag7	16	15.152	15.967	0.717	0.934	0.233	0.1086*	–
QrZag87	14	6.096	13.931	0.767	0.836	0.083	0.0218	ns
QpZag9	11	6.135	10.932	0.817	0.837	0.024	0	ns
QrZag96	18	7.7528	17.832	0.867	0.871	0.005	0	–
Mean adults	13.368 (5.639)	6.143 (3.777)	13.269 (5.566)	0.773 (0.106)	0.778 (0.128)	-0.004 (0.092)	0.012 (0.026)	
Mean seedlings	16.526 (7.479)	5.604 (3.220)	16.471 (7.456)	0.751 (0.137)	0.759 (0.138)	0.011 (0.050)	0.009 (0.019)	

Significance: * $p < 0.05$; ** $p < 0.01$; ns – not significant at $p < 0.05$

of inbreeding estimated when accounting for the presence of *null* alleles was found to be statistically significant only for seedlings (F_{is} = 0.0184; 95% C.I.: 0.0115–0.0252).

GENETIC DIFFERENTIATION AMONG COHORTS

Genetic differences between different cohorts within the species were generally low and statistically insignificant (Table 4). The consistency between *F*st and *R*st suggests that genetic drift is the main reason for differentiation between the parent and offspring populations within the species. However, we detected significant levels of genetic differentiation between species. The differences between the *F*st and *R*st parameters for cross-comparisons between species show that the interspecific diversity is also the effect of mutations occurring in the process of differentiation of these species.

TABLE 4. Levels of genetic differences between pairs of parent and offspring (seedlings) populations and between *Q. robur* and *Q. petraea*.

Species	F_{st}	R_{st}
Genetic differentiation between adults and seedlings within species		
Quercus robur	-0.0001 ns	0.0001 ns
Quercus petraea	0.0015 ns	-0.0004 ns
Genetic differentiation between Q. robur and Q. petraea		
Adults	0.0843 *	0.1494 *
Seedlings	0.0989 *	0.1281 *

Significance: * $p < 0.05$; ns – not significant at $p < 0.05$

EFFECTIVE POPULATION SIZE

The estimates of effective population size (*Ne*) appeared to be slightly higher for *Q. robur* than for *Q. petraea* (Table 5); however, considering the ratio of the effective population size to the actual number of individuals (Ne/N), it turned out that this proportion was over 100% in both species. It should be noted that the estimated *Ne* generally refers to the parental population that generated the population under study. The high correspondence between the effective and the actual population sizes in adults indicate that these populations well represent the available gene pools of the studied populations and large numbers of individuals contributed to the generation of these populations.

The effective population sizes of the seedlings correlated relatively well with the actual numbers of adults in the parental population. Interestingly, although the number of sessile oak seedlings was much larger than that of pedunculate oak, the lowest estimates of the effective population size were observed just for sessile oak. This suggests that the population of seedlings of this species was created by a limited number of individuals or their contribution to the offspring generation was largely unbalanced.

TABLE 5. The estimates of effective population sizes obtained based on linkage disequilibrium method (95% CI in parentheses; *N* – sample size).

	Q. robur	*Q. petraea*
Adults	**115.1** (100.8–133.2) *N* = 104	**72.5** (62.8–84.9) *N* = 61
Seedlings	**80.5** (73.6–88.3) *N* = 191	**42.4** (39.5–45.5) *N* = 456

DISCUSSION

Pedunculate and sessile oaks often exist jointly in mixed forest stands (Eaton et al., 2016). Conducting research studies and forest management (e.g., thinning or seed harvesting) of oaks in such populations requires precise species identification of individuals. One way of species determination involves the assessment of leaf morphological traits (Kremer et al., 2002), which is, however, laborious, time-consuming, requires well-developed leaves (preferably from upper parts of the crown), but sometimes is also confusing if intermediate (e.g., resulting from interspecific hybridization) individuals are present. Recently available sets of multiplexed microsatellite loci designed to facilitate discrimination between the two species and hybrid detection (Guichoux et al., 2011) provided an opportunity for genetic species identification of individuals. Some researchers even suggested that for research purposes, genetic methods could be preferred over morphological assessment, given its simplicity and precision (Rellstab et al., 2016). Genetic species identification seems to be appealing particularly for seedlings, where morphological species differentiation is more difficult (Ponton et al., 2004; Boratynski et al., 2008; Truffaut et al., 2017).

In this study, we used model-based approach implemented in STRUCTURE software to identify species status based on genetic markers, which was successfully applied for the same purpose also in other oak studies (Lepais et al., 2009; Neophytou et al., 2010). For example, Neophytou et al. (2010),

using 14 microsatellite loci, demonstrated clear delineation of *Q. robur* and *Q. petraea* individuals, with relatively high probabilities of assignment of individuals to a particular cluster, reaching 0.982, on average. In our study, STRUCTURE grouping appeared to be particularly efficient, resulting in the high probability of individual assignment to the species clusters, which was equal to 0.986 for *Q. robur* and 0.965 for *Q. petraea*. Slightly lower values of the assignment probability for *Q. petraea* may be caused by the presence of interspecific asymmetric introgression, which was observed between these two species (Petit et al., 2003; Lepais et al., 2009; Chybicki and Burczyk, 2013; Lepais et al., 2013; Lagache et al., 2014). The precise identification of the species status of individual trees and seedlings enabled subsequent demographic and population genetic analyses.

The trees belonging to the two oak species were largely spatially intermixed with no apparent clustering of any species. Although *Q. robur* predominated in the generation of adult trees (63.3%), most seedlings were classified as *Q. petraea* (70.5%). This discrepancy of reproductive success between the species could be incidental, although it was previously reported in other Polish stands (Boratynski et al., 2010); however, several site-specific factors are expected to influence the difference between the species reproductive output (Annighofer et al., 2015). The sessile oak is known to tolerate denser and more shaded condition than *Q. robur*, therefore site specific conditions in an old-growth forest stand, as the studied one, may favor regeneration of *Q. petraea*.

The spatial structure of forest stands, especially natural populations, is a frequent and attractive subject of research in forest ecosystems (Szwagrzyk, 1990; Szwagrzyk and Czerwczak, 1993; Szwagrzyk et al., 2001). Such studies are of particular interest when they concern multispecies stands, where apart from the distribution pattern of individuals of particular species, it is possible to study inter-specific relationships of spatial arrangement (Strimbu et al., 2017).

In this study, we used the O(r) function which examines the degree of aggregation of individuals in a given class of distance, and unlike the Ripley's K function is not cumulative (Wiegand and Moloney, 2004). Therefore, the function O(r) seems to be more appropriate to investigate the relationship between the spatial structure of the population and its spatial genetic structure, and has often been used for this purpose (Chung et al., 2011; Lara-Romero et al., 2016). The recommended ring width of 5 m has been used in this study (Wiegand, 2003). Nevertheless, it has been reported that the width of the ring may influence the interpretation of the results (Strimbu et al., 2017).

We found a relatively regular distribution of adult trees of both oak species (Fig. 2a) with no noticeable groups of any species, which along with the observed normal distribution of DBH of trees (Fig. 1), may suggest their long-term coexistence in the study area. However, we noticed a highly concentrated distribution of seedlings, especially those of sessile oak (Figs. 2b, 3). Different clustering intensities of seedlings of both oak species seedlings may be due to the differences related to the predominant modes of seed dispersal. Pedunculate oak acorns are preferred by jays, which spread them over further distances and shape the long seed dispersal of this species (Bossema, 1979; Ducousso et al., 1993; Petit et al., 2003). The seed dispersal in sessile oak is shaped mainly by gravity, and therefore is more limited, so this clustering (in low distance classes) can result from the natural distribution of seedlings.

The parameters of the genetic structure, obtained in this study in the Jamy Reserve, were similar to those observed by other authors (Streiff et al., 1998; Cottrell et al., 2003; Neophytou et al., 2010; Curtu et al., 2015; Burczyk et al., 2018). However, an interesting aspect of population genetic studies is how the genetic variation is transferred from adult to offspring generations. This is particularly important in populations where natural regeneration occurs. This type of research has been recently conducted in oaks (Dering and Chybicki, 2012; Vranckx et al., 2014). Vranckx et al. (2014) investigated various parameters of the genetic structure in *Q. robur* stands and did not find any significant differences between adult and seedlings cohorts. However, these results were based on a relatively small numbers of adults and seedlings, which makes statistically significant differences difficult to be detected. In the other study, Dering and Chybicki (2012) compared the genetic variation of the parent and offspring generations of mixed *Q. robur* and *Q. petraea* stands (natural regeneration and plantations), and observed a decrease in expected heterozygosity in seedling populations compared to relevant adult populations, which is consistent with our findings. However, it should be noted that the decrease in heterozygosity was smaller in populations of naturally regenerating seedlings than in planted ones. The authors also noticed the differences in the estimates of effective population size of the naturally established seedlings (Ne from 49 to 237), which correlated well with the level of genetic variation between generations, expressed by F_{st} coefficient.

We found no inbreeding in the adult oak populations. However, low but significant levels of inbreeding were noticed among seedlings (*Q. robur*: 0.0115, *Q. petraea*: 0.0184). This contrasts with

results from some other studies, where significant levels of inbreeding were found also in adult populations (e.g., F_{is} = 0.070–0.082 for *Q. petraea*; F_{is} = 0.100-0.117 for *Q. robur*; Neophytou et al., 2010). Nevertheless, the elevated levels of inbreeding coefficients reported in the literature are likely to be the effect of the presence of *null* alleles which biased inbreeding estimates.

The level of genetic differentiation (*F*st) between the two oak species was considerable and reached 0.084 for adults and 0.099 for seedlings. These estimates were very similar to earlier reports including the same pair of species based on microsatellites (0.096 – Curtu et al., 2007; 0.101 – Neophytou et al., 2010) or even based on AFLP markers (0.0733 – Coart et al., 2002).

CONCLUSIONS

Unpredictable consequences of forest management and climate changes on genetic diversity of forest trees in the long term call for population genetic studies of natural populations that are not influenced by human activities. Such populations could be considered as indigenous reference populations. The results on genetic diversity and species differentiation in a near-natural stand of *Q. robur* and *Q. petraea* obtained in this study can thus be used as a reference point for comparisons of the genetic status of oak populations artificially created and managed by humans.

Relatively high estimates of genetic diversity parameters, considerable effective population sizes but low levels of genetic differentiation between adults and offspring indicate that the gene pool of the studied adult generations is well represented in the seedling cohorts in both oak species. The uniformly high level of genetic diversity parameters at different life stages suggests that the studied oak populations probably reached the maximum available level of genetic diversity, taking into account the set of genetic markers used in this study.

AUTHORS' CONTRIBUTIONS

ES, BU and JB designed the research and wrote the article. ES and BU collected samples, genotyped plant material and contributed to statistical analyses. JB obtained funding.

ACKNOWLEDGEMENTS

The study was supported by the National Science Center (Grant No: 2012/04/A/NZ9/00500). Plant material was sampled based on the permission WPN.6205.42.2013.KLD issued by the Regional Directorate for Environmental Protection in Bydgoszcz, Poland. The authors are thankful to Katarzyna Meyza and Ewa Sztupecka for laboratory work and field assistance.

REFERENCES

Aitken SN, Yeaman S, Holliday JA, Wang T, and Curtis-McLane S. 2008. Adaptation, migration or extirpation: climate change outcomes for tree populations. *Evolutionary Applications* 1(1): 95–111.

Annighofer P, Beckschafer P, Vor T, and Ammer C. 2015. Regeneration patterns of European oak species (*Quercus petraea* (Matt.) Liebl., *Quercus robur* L.) in dependence of environment and neighborhood. *PloS ONE* 10: e0134935.

Booy G, Hendriks RJJ, Smulders MJM, Groenendael JV, and Vosman B. 2000. Genetic diversity and the survival of populations. *Plant Biology* 2(4): 379–395.

Boratynski A, Marcysiak K, Lewandowska A, Jasinska A, Iszkulo G, and Burczyk J. 2008. Differences in leaf morphology between *Quercus petraea* and *Q. robur* adult and young individuals. *Silva Fennica* 42(1): 115.

Boratynski A, Marcysiak K, Lewandowska A, Jasinska A, and Iszkulo G. 2010. Interrelations among con-generic and co-occurring tree species: Asymmetric hybridization and the high success of *Quercus petraea* (Matt.) Liebl. regeneration in mixed *Q. petraea*/*Q. robur* L. stands. *Polish Journal of Ecology* 58: 273–283.

Bossema I. 1979. Jays and oaks: an eco-ethological study of a symbiosis. *Behaviour* 70(1): 1–116.

Burczyk, J, Chybicki IJ, and Trojankiewicz M. 2018. High genetic diversity promotes a common-garden trial of *Quercus robur* as a potential seed source. *Dendrobiology* 79: 1–9.

Chung MY, Nason JD, and Chung MG. 2011. Significant demographic and fine-scale genetic structure in expanding and senescing populations of the terrestrial orchid *Cymbidium goeringii* (Orchidaceae). *American Journal of Botany* 98: 2027–2039.

Chybicki IJ, and Burczyk J. 2009. Simultaneous estimation of *null* alleles and inbreeding coefficients. *Journal of Heredity* 100: 106–113.

Chybicki IJ, and Burczyk J. 2013. Seeing the forest through the trees: comprehensive inference on individual mating patterns in a mixed stand of *Quercus robur* and *Q. petraea*. *Annals of Botany* 112: 561–574.

Coart E, Lamote V, De Loose M, Van Bockstaele E, Lootens P, and Roldàn-Ruiz I. 2002. AFLP markers demonstrate local genetic differentiation between two indigenous oak species [*Quercus robur* L. and *Quercus petraea* (Matt.) Liebl.] in Flemish populations. *Theoretical and Applied Genetics* 105(2–3): 431–439.

Cottrell JE, Munro RC, Tabbener HE, Milner AD, Forrest GI, and Lowe AJ. 2003. Comparison of fine-scale genetic structure using nuclear microsatellites within two British oakwoods differing in population history. *Forest Ecology and Management* 176: 287–303.

Curtu AL, Gailing O, and Finkeldey R. 2007. Evidence for hybridization and introgression within a species-rich oak (*Quercus* spp.) community. *BMC Evolutionary Biology* 7(1): 218.

Curtu A, Craciunesc I, Enescu C, Vidalis A, and Sofletea N. 2015. Fine-scale spatial genetic structure in a multi-oak-species (*Quercus* spp.) forest. *iForest – Biogeosciences and Forestry* 8: 324–332.

Danielewicz W. 1995. Dęby w lasach Polski [Oaks in the forests of Poland]. Ośrodek Kultury Leśnej, Głuchów, pp. 3–9.

Dering M, and Chybicki I. 2012. Assessment of genetic diversity in two-species oak seed stands and their progeny populations. *Scandinavian Journal of Forest Research* 27: 2–9.

Do C, Waples RS, Peel D, Macbeth GM, Tillett BJ, and Ovenden JR. 2014. NeEstimator v2: reimplementation of software for the estimation of contemporary effective population size (Ne) from genetic data. *Molecular Ecology Resources* 14(1): 209–214.

Ducousso A, Michaud H, and Lumaret R. 1993. Mating system and gene flow in oak species. *Annales des Sciences Forestières*, Vol. 50, pp. 91–106.

Eaton E, Caudullo G, Oliveira S, and De Rigo D. 2016. *Quercus robur* and *Quercus petraea* in Europe: distribution, habitat, usage and threats. In: San-Miguel-Ayanz J, de Rigo D, Caudullo G, Houston Durrant T, Mauri A (eds.). *European Atlas of Forest Tree Species*. *Publication Office of the European Union*, Luxembourg, pp. 160–163.

Fischer A, and Fischer H. 2012 Restoration of Temperate Forests: An European Approach. In: van Andel J, Aronson J. (eds.). *Restoration ecology: The new frontier*. Second edition. John Wiley & Sons, Ltd, Chichester, UK.

Gerber S, Chadoeuf J, Gugerli F, Lascoux M, Buiteveld J, Cottrell J, Dounavi A, Fineschi S, Forrest LL, Fogelqvist J, Goicoechea PG, Jensen JS, Salvini D, Vendramin GG, and Kremer A. 2014. High rates of gene flow by pollen and seed in oak populations across Europe. *PLoS ONE* 9(1): e85130.

Goudet J. 1995. FSTAT (version 1.2): a computer program to calculate F-statistics. *Journal of Heredity* 86(6): 485–486.

Gugerli F, Brandl R, Castagneyrol B, Franc A, Jactel H, Koelewijn HP, Martin F, Peter M, Pritsch K, Schroder H, Smulders MJ, Kremer A, Ziegenhagen B, and Evoltree JC. 2013. Community genetics in the time of next-generation molecular technologies. *Molecular Ecology* 22: 3198–3207.

Guichoux E, Lagache L, Wagner S, Leger P, and Petit RJ. 2011. Two highly validated multiplexes (12-plex and 8-plex) for species delimitation and parentage analysis in oaks (*Quercus* spp.). *Molecular Ecology Resources* 11: 578–585.

Hardy OJ, and Vekemans X. 2002. SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes* 2: 618–620.

Hughes AR, Inouye BD, Johnson MT, Underwood N, and Vellend M. 2008. Ecological consequences of genetic diversity. *Ecology Letters* 11(6): 609–623.

Huntley B. 1988. European post-glacial vegetation history: a new perspective. *Acta XIX Congressus Internationalis Ornithologici* 1: 1061–1077.

Kampfer S, Lexer C, Glössl J, and Steinkellner H. 1998. Characterization of (GA)*n* microsatellite loci from *Quercus robur*. *Hereditas* 129(2): 183–186.

Kremer A, Dupouey JL, Deans JD, Cottrell J, Csaikl U, Finkeldey R, Espinel S, Jensen J, Kleinschmit J, Van Dam B, Ducousso A, Forrest I, Lopez de Heredia U, Lowe AJ, Tutkova M, Munro RC, Steinhoff S, and Badeau V. 2002. Leaf morphological differentiation between *Quercus robur* and *Quercus petraea* is stable across western European mixed oak stands. *Annals of Forest Science* 59: 777–787.

Lagache L, Klein EK, Ducousso A, and Petit RJ. 2014. Distinct male reproductive strategies in two closely related oak species. *Molecular Ecology* 23: 4331–4343.

Lara-Romero C, Garcia-Fernandez A, Robledo-Arnuncio JJ, Roumet M, Morente-López J, López-Gil A, and Iriondo JM. 2016. Individual spatial aggregation correlates with between-population variation in fine-scale genetic structure of *Silene ciliata* (Caryophyllaceae). *Heredity* 116(5): 417–423.

Legendre P, and Legendre LF. 2012. *Numerical Ecology* (vol. 24). Elsevier.

Lepais O, Petit RJ, Guichoux E, Lavabre JE, Alberto F, Kremer A, and Gerber S. 2009. Species relative abundance and direction of introgression in oaks. *Molecular Ecology* 18: 2228–2242.

Lepais O, Roussel G, Hubert F, Kremer A, and Gerber S. 2013. Strength and variability of postmating reproductive isolating barriers between four European white oak species. *Tree Genetics and Genomes* 9(3): 841–853.

Lindenmayer DB, Franklin JF, and Fischer J. 2006. General management principles and a checklist of strategies to guide forest biodiversity conservation. *Biological Conservation* 131(3): 433–445.

Loosmore NB, and Ford ED. 2006. Statistical inference using the G or K point pattern spatial statistics. *Ecology* 87(8): 1925–1931.

Neophytou C, Aravanopoulos FA, Fink S, and Dounavi A. 2010. Detecting interspecific and geographic differentiation patterns in two interfertile oak species (*Quercus petraea* (Matt.) Liebl. and *Q. robur* L.) using small sets of microsatellite markers. *Forest Ecology and Management* 259: 2026–2035.

Neophytou C. 2013. Bayesian clustering analyses for genetic assignment and study of hybridization in oaks: effects of asymmetric phylogenies and asymmetric sampling schemes. *Tree Genetics and Genomes* 10: 273–285.

Petit RJ, Bodénès C, Ducousso A, Roussel G, and Kremer A. 2003. Hybridization as a mechanism of invasion in oaks. *New Phytologist* 161: 151–164.

Ponton S, Dupouey JL, and Dreyer E. 2004. Leaf morphology as species indicator in seedlings of *Quercus robur* L. and *Q. petraea* (Matt.) Liebl.: modulation by irradiance and growth flush. *Annals of Forest Science* 61: 73–80.

Pritchard JK, Stephens M, and Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155(2): 945–959.

Rellstab C, Bühler A, Graf R, Folly C, and Gugerli F. 2016. Using joint multivariate analyses of leaf morphology and molecular-genetic markers for taxon identification in three hybridizing European white oak species (*Quercus* spp.). *Annals of Forest Science* 73: 669–679.

Sandurska E, Ulaszewski B, and Burczyk J. 2017. Genetic insights into ecological succession from oak-(*Quercus robur* L.) to beech-(*Fagus sylvatica* L.) dominated forest

stands. *Acta Biologica Cracoviensia Series Botanica* 59(1): 23–33.

Sork VL. 2016. Gene flow and natural selection shape spatial patterns of genes in tree populations: implications for evolutionary processes and applications. *Evolutionary Applications* 9(1): 291–310.

Steinkellner H, Fluch S, Turetschek E, Lexer C, Streiff R, Kremer A, Burg K, and Glössl J. 1997. Identification and characterization of (GA/CT)*n*-microsatellite loci from *Quercus petraea*. *Plant Molecular Biology* 33(6): 1093–1096.

Streiff R, Labbe T, Bacilieri R, Steinkellner H, Glossl J, and Kremer A. 1998. Within-population genetic structure in *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. assessed with isozymes and microsatellites. *Molecular Ecology* 7: 317–328.

Strimbu BM, Petritan IC, Montes C, and Biris IA. 2017. An assessment of the O-ring methodology using virgin stands of mixed European beech – sessile oak. *Forest Ecology and Management* 384: 378–388.

Szwagrzyk J. 1990. Natural regeneration of forest related to the spatial structure of trees: A study of two forest communities in Western Carpathians, southern Poland. *Vegetatio* 89: 11–22.

Szwagrzyk J, and Czerwczak M. 1993. Spatial patterns of trees in natural forests of east-central-Europe. *Journal of Vegetation Science* 4: 469–476.

Szwagrzyk J, Szewczyk J, and Bodziarczyk J. 2001. Dynamics of seedling banks in beech forest: results of a 10-year study on germination, growth and survival. *Forest Ecology and Management* 141: 237–250.

Truffaut L, Chancerel E, Ducousso A, Dupouey JL, Badeau V, Ehrenmann F, and Kremer A. 2017. Fine-scale species distribution changes in a mixed oak stand over two successive generations. *New Phytologist* 215(1): 126–139.

Vranckx G, Jacquemyn H, Mergeay J, Cox K, Kint V, Muys B, and Honnay O. 2014. Transmission of genetic variation from the adult generation to naturally established seedling cohorts in small forest stands of pedunculate oak (*Quercus robur* L.). *Forest Ecology and Management* 312: 19–27.

Waples RS, and Do C. 2008. LDNE: a program for estimating effective population size from data on linkage disequilibrium. *Molecular Ecology Resources* 8: 753–756.

Whitham TG, Young WP, Martinsen GD, Gehring CA, Schweitzer JA, Shuster SM, Wimp GM, Fischer DG, Bailey JK, Lindroth RL, Woolbright S, and Kuske CR. 2003. Community and ecosystem genetics: A consequence of the extended phenotype. *Ecology* 84: 559–573.

Whitham TG, Bailey JK, Schweitzer JA, Shuster SM, Bangert RK, LeRoy CJ, Lonsdorf EV, Allan GJ, DiFazio SP, Potts BM, Fischer DG, Gehring CA, Lindroth RL, Marks JC, Hart SC, Wimp GM, and Wooley SC. 2006. A framework for community and ecosystem genetics: from genes to ecosystems. *Nature Review Genetics* 7: 510–523.

Wiegand T. 2003. PROGRAMITA, a software to perform point pattern analysis with Ripley's L and O-ring statistic. (http://www.thorsten-wiegand.de/towi_download_manual.html).

Wiegand T, and Moloney KA. 2004. Rings, circles, and null-models for point pattern analysis in ecology. *Oikos* 104(2): 209–229.

Wiegand T, and Moloney KA. 2014. *A handbook of spatial point pattern analysis in ecology*. Chapman and Hall/CRC Press, Boca Raton, FL.