DENDROBIOLOGY

2018, vol. 79, 1–9

http://dx.doi.org/10.12657/denbio.079.001

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High genetic diversity promotes a common-garden trial of *Quercus robur* as a potential seed source

Received: 2 April 2017; Accepted: 24 July 2017

Abstract: The process of adaptation in forest trees might be facilitated if seeds resulting from crossings among different provenances are used for plantation establishment. This can be accomplished if seeds from existing common-garden trials become available. This paper aims to characterize genetic diversity of a provenance/family common-garden trial of *Quercus robur* which is considered a possible source of highly diverse seed lots. Provenance/family common-garden trial of *Quercus robur* located in Oleszyce, Poland, consisting of 8 to 19 families of six Polish provenances was chosen for the study. With the aid of 16 nuclear microsatellite markers, 1812 trees growing in the trial were genotyped. Standard population genetic parameters were calculated, and genetic variation and inbreeding were compared among provenances. Expected heterozygosity and particularly allelic richness appeared to be high, reaching on average 0.847 and 23.5, respectively. We found no signatures of inbreeding (F_{IS}=0.006) and low, although statistically significant, level of genetic differentiation among provenances (F_{ST}=0.016). On the other hand, we found high allelic differentiation (A_{ST}=0.137) between provenances, though uneven contribution of each provenance to the total allelic richness was noted. Effective population sizes estimated for each provenance based on linkage disequilibrium were highly correlated with the number of families within provenances. We conclude that the studied common-garden trial possesses high genetic diversity and possible mating among different provenances may promote further heterosis effects. Thus the trial may be used in the future as an experimental source of highly diverse seed lots much needed in the context of climate change.

Keywords: Common-garden trials, genetic diversity, microsatellites, seedling seed orchards

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Funding: The study was supported by the Ministry of Science and Higher Education of the Republic of Poland (Grant No: N N309 320136).

The authors declare no conflicts of interests

Introduction

Projected changes in climate and land use in Europe will impact a number of forest tree species (Koskela et al., 2007). The impacts of global change are expected to be acute, yielding large-scale changes in species range, ecosystem functioning and interactions among species (Martin & Henrichs, 2010). Since trees are long-lived and sessile organisms, their options are to disappear, colonize suitable places, or to adapt *in situ* to the on-going climatic changes over a reduced number of generations (Aitken et al., 2008). The extent to which populations can adapt to local conditions depends on within-population genetic diversity and how this diversity is distributed within and among populations. In tree species adaptive capacity appears to rely more on standing genetic variation and recombination than on new mutations (Aitken et al., 2008; Bolte & Degen, 2010).

Genetic diversity allows a species to evolve over time and space, and it plays a key role for both the long-term survival of a species and stability of forest ecosystems (Lindenmayer et al., 2006; Hughes et al., 2008). Therefore, genetic diversity is gaining an increased attention in forest management planning. It is considered an important issue in conservation management, and a particularly important one in long-lived forest trees (Graudal et al., 2014; Koskela et al., 2014). 'Adaptive' forest management should conserve or enhance the evolutionary potential of tree species or populations, by limiting inbreeding and outbreeding depression, reducing population fragmentations, but increasing genetic diversity and effective population sizes.

Increasing genetic diversity within the deployment populations provides a long-term assurance that counteracts the uncertainty of climate prediction or the species/provenances reactions to climate change (Williams & Dumroese, 2013). The problem of selection of seed source to guarantee sustainability of future forest populations facing environmental instability (climate change) has recently gained much attention (Broadhurst et al., 2008; Breed et al., 2012). Specific frameworks and solutions have been proposed to secure sustainability of future forests (Sgrò et al., 2011; Breed et al., 2012; Williams & Dumroese, 2013). However, making decisions on the utilization of local provenances, predicted provenances (provenances suggested based on future climate predictions), composite provenancing or provenance mixtures (Breed et al., 2012) can be done only if information on the distribution of genetic diversity (neutral and adaptive) is already available based on various national and international projects and initiatives. Despite great efforts undertaken in Europe to understand the distribution of forest genetic resources and its utilization in forestry practice (Koskela et al., 2013), the knowledge that is currently available is only to a limited degree transferred to forest management. Certainly, there are no unified recommendations as yet on how to utilize genetic

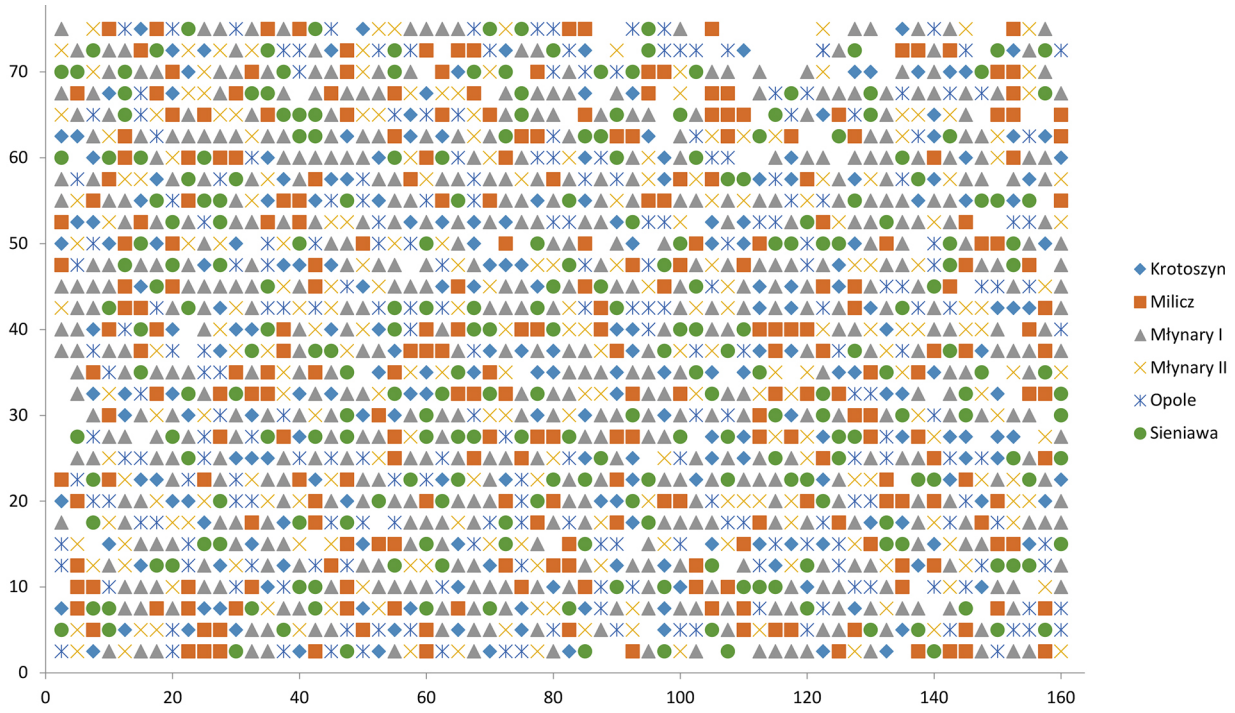

Fig. 1. Distribution of individual trees assigned to one of the six provenances growing in the experimental plot (scales in meters)

diversity that would apply across Europe (Koskela et al., 2014).

Composite provenancing or provenance mixtures (Breed et al., 2012) are interesting ways to increase genetic diversity of planted forests (Broadhurst et al., 2008). It is assumed that subsequent mating among individuals from different provenances may promote genetic diversity in future offspring generations, potentially leading to increased adaptability and heterosis effects (Byrne et al., 2011). However, the possibility of outbreeding depression, resulting from matings between physically distant or genetically extremely different population, should not be ignored (Goto et al., 2011). Nevertheless, the process of adaptation might be facilitated if seeds resulting from crossings among different provenances are used for plantation establishment. This can be done if seeds from existing common-garden trials are available. The concept of using common-garden trials as seed sources is not new (Nanson, 1972; Merlo et al., 2004). The benefits of such approach are multiple and include maintaining a broad genetic basis of the breeding population, relatively simple management, readiness of alterations in the population composition through selective removal of individuals or entire families based on their performance, as well as shortening the time to seed deployment (Nanson, 1972).

Over the past decades, forest geneticists have established a number of experimental trials (provenance/common garden). Initially, those plantations were used for the purpose of tree improvement, but now these populations serve as a source of information about adaptation processes in the context of global climate changes (Koskela et al., 2007). Older trials have already reached the seed production stage and can be used as a seed source.

In this paper we study genetic diversity of the provenance/family common-garden trial established for *Quercus robur* in 1996. Despite the scientific importance of this plantation in quantitative genetic research, it might be considered that this population will be converted into an experimental seed source. The trial is currently almost 20 years old and has reached the stage at which thinning and other management interventions are required. The knowledge on the distribution of genetic diversity might be helpful in taking decisions on the selection of particular provenances or families, in complement with quantitative trait measures. Our specific objectives were to assess the overall level of genetic diversity of the plantation and the degree of inter-provenance genetic differentiation. We were also interested to see if the spatial distribution of genetic diversity within the experimental site minimizes the chance for mating between related individuals, thus increasing genetic diversity of offspring generations. Such information, complemented with measures of adaptive traits (e.g. phenology), growth performance and survival of provenances/families, will help to design the strategies for management and utilization of common garden trials as potential seed sources.

Materials and methods

Family/provenance experiment

The common garden experiment of *Quercus robur* selected for this study was established by Forest Research Institute (Warsaw) in Forest District Oleszyce, in the south-eastern part of Poland (N50°10'52"; E22°58'01"). Oak acorns were collected from qualified seed collection stands in the autumn of 1995 and represented 8 to 19 families of six Polish provenances (Table 1). Seedlings were planted at 2 × 2 m spacing, based on a single-tree plot and completely randomized block designs. Initially 1920 seedlings were planted, but finally 1812 were sampled for leaves in 2009.

Laboratory methods

Total genomic DNA was isolated from 50 mg of dried leaf tissue. The tissue was ground in a Mixer

Table 1. Provenances of *Quercus robur* used to establish common garden trial in Oleszyce, Poland

Provenance name	Forest district	Geographic coordinates	Number of families	Effective number of families	Number of individuals
Krotoszyn	Smoszew	N51°39'34" E17°30'20"	8	8.22	242
Milicz	Walkowo	N51°30'11" E17°19'28"	9	9.26	275
Młynary I	Kisielewo	N54°02'57" E19°48'03"	19	19.57	570
Młynary II	Stołbity	N54°10'52" E19°49'39"	8	8.23	239
Opole	Narok	N50°45'32" E17°46'57"	8	8.22	242
Sieniawa	Przyjemek	N50°12'37" E22°47'02"	8	8.23	244

Table 2. Details about the optimized multiplex-PCR reaction mixtures and cycling conditions

Multiplex 1	μl	Multiplex 2	μl	Multiplex 3	μl
ssrQrZAG11 (FAM)	0.15	ssrQrZAG4 (FAM)	0.20	MSQ4 (PET)	0.15
ssrQrZAG25 (PET)	0.25	ssrQrZAG15 (VIC)	0.20	ssrQrZAG7 (VIC)	0.20
ssrQrZAG39 (VIC)	0.10	ssrQrZAG90 (NED)	0.20	ssrQpZAG9 (NED)	0.20
ssrQrZAG65 (PET)	0.35	ssrQrZAG102 (PET)	0.30	ssrQrZAG20 (FAM)	0.20
ssrQrZAG101 (NED)	0.25	ssrQrZAG103 (NED)	0.45	ssrQpZAG110 (VIC)	0.15
ssrQrZAG112 (FAM)	0.30	–		–	
MMM	5	MMM	5	MMM	5
H_2O	1.6	H_2O	1.65	H_2O	2
DNA	2	DNA	2	DNA	2

Note: MMM – Qiagen Multiplex Master Mix (2x); DNA – 10 ng/μl; all primers had concentration of 5uM.
Multiplex 1: cycling of 95°C for 15 min followed by: 10 cycles of 94°C for 30 s, 60°C for 90 s with a reduction in temperatures of 1°C per cycle, 72°C for 60 s; then 25 cycles of 94°C for 30 s, 55°C for 90 s, 72°C for 60 s; and a final cycle of 72°C for 10 min.
Multiplex 2: cycling of 95°C for 15 min followed by: 6 cycles of 94°C for 30 s, 53°C for 90 s with a reduction in temperatures of 1°C per cycle, 72 °C for 60 s; then 25 cycles of 94°C for 30 s, 48°C for 90 s, 72°C for 60 s; and a final cycle of 72°C for 10 min.
Multiplex 3: cycling of 94°C for 5 min followed by: 31 cycles of 94°C for 60 s, 50°C for 30 s, 72°C for 60 s; and a final cycle of 72°C for 10 min.

Mill MM301 (Retsch, Haan, Germany) and DNA was isolated following a CTAB protocol (Doyle, 1990). The amount of DNA was adjusted to a concentration of 10 ng/μl using DNA calculator (Biophotometer, Eppendorf).

Genotypes of sampled individuals were determined based on the set of 16 nuclear microsatellite markers: *ssrQrZAG7, ssrQrZAG25, ssrQpZAG110, ssrQpZAG102* (Steinkellner et al., 1997), *ssrQpZAG9 ssrQrZAG11*, *ssrQrZAG15*, *ssrQrZAG20*, *ssrQrZAG39, ssrQrZAG65, ssrQrZAG90*, *ssrQrZAG101, ssrQrZAG103, ssrQrZAG112, ssrQrZAG4* (Kampfer et al., 1998) and *MSQ4* (Dow et al., 1995). The markers were divided into three sets and subjected to PCR-multiplex protocols (Table 2). Multiplexing was facilitated with the aid of Multiplex Master Mix (Qiagen). The amplification products were sized using an ABI 3130XL sequencer (Applied Biosystems, USA). The identification of alleles based on their size was determined using GeneMapper software v. 4.0 (Applied Biosystems).

Statistical methods

In order to characterize the contribution of particular provenances to the plantation, effective family numbers were estimated based on the distribution of individual families. For this purpose, we used the unbiased estimator by Nielsen et al. (2003). Per locus and per provenance a genetic structure was described with a list of parameters, including number of alleles (A), allelic richness (AR), observed (H_o) and expected heterozygosity (H_e) and inbreeding coefficient ($F_{IS} = 1 - H_o/H_e$). Analyses were conducted using FSTAT software (Goudet, 1995). Using the Friedman rank test (see Chybicki et al., 2012), we verified whether provenances represented a similar level of genetic variation and inbreeding. Furthermore, we estimated the contribution of each provenance to the total allelic diversity using the method of Caballero and Rodríguez-Ramilo (2010). In this approach, allelic diversity is partitioned into within-deme (A_S) and between-deme (D_A) components. Then, the contribution of each subsample is quantified as gain or loss of allelic diversity after removal of this subsample. Based on A_S and D_A, the allelic differentiation index (A_{ST}) was also computed. In order to characterize partitioning of genetic variation, we performed AMOVA using Arlequin 3.5 (Excoffier & Lischer, 2010). 95% confidence bounds around the F-statistics were approximated with 20,000 bootstraps. Genetic effective size was characterized using two approaches. Firstly, under the assumption that a number of males per female is large, we estimated the effective number of breeders (N_b) as 4 × effective number of families. Secondly, using genetic data and LDNe software (Waples & Do, 2010), we computed the effective population size (N_e). Then, using 1000 bootstrap samples, we computed (harmonic) mean N_e together with the 95% confidence interval. Applying the Bonferroni correction for multiple comparisons, we verified whether individual N_es deviate from the overall mean. The N_b and N_e were estimated for each provenance and for the mixture. Finally, we tested whether alleles are randomly distributed within the site. For this purpose, we performed spatial autocorrelation analysis with Nason's kinship coefficient (Loiselle et al., 1995) as a correlation-like statistics. The method produces a plot (correlogram) which shows the relationship between genetic similarity (kinship) between individuals and the distance between them. To verify the null hypothesis of no spatial genetic structure, the observed correlogram was compared with the null distribution obtained after randomizing positions of genotypes 20,000 times. The analysis was conducted using INEST 2.0 (Chybicki & Burczyk, 2009).

Results

As indicated by the effective number of families, the contribution of particular provenances to the plantation was fairly uniform, except for Młynary I provenance (Table 1). The harmonic mean of effective number of families equaled 9.3. For the mixture of provenances, the number of alleles ranged from 15 to 48 (27.4 on average) (Table 3). The expected heterozygosity was high and ranged across loci from 66.9% to 95.3%, with the average equal to 84.7%. Similarly, the observed heterozygosity varied from 59.4% to 97.6%, with the average of 83.6%. We observed slight and insignificant deficiency of heterozygous genotypes, as the mean inbreeding coefficient was almost zero. Nonetheless, significant deficiency and excess of heterozygotes was observed at 6 and 1 loci, respectively. Thus, 56% loci did not deviate significantly from $F_{IS} = 0$.

When provenances were treated separately, the average number of alleles (A) per locus ranged from 21.4 to 24.3, with the mean of 22.2 (Table 4). Despite tiny differences, the number of alleles appeared to differ significantly between provenances, as revealed by the Friedman test (p-value = 0.005). However, after rarefaction (AR), differences in the polymorphism level deflated substantially so that provenances no longer differed from each other (the Friedman test; p-value = 0.593). Similarly, provenances did not differ significantly in H_e, H_o and F_{IS}. When considered separately, provenances were characterized by slightly lower genetic diversity (H_e) compared to the mixture. On the other hand, 4 out of 6 provenances showed negative inbreeding coefficient and thus a slight excess of heterozygotes as compared with the Hardy-Weinberg proportions. At the level of individual loci (data not shown), 71.9% loci did not deviate significantly from $F_{IS} = 0$, while 18.8% and 9.4% loci showed heterozygosity deficiency and excess, respectively. Thus, at the level of provenances slightly more loci were in agreement with the HW proportions as compared to the mixture. However, the difference was not significant, as revealed by the chi-square test (p-value = 0.237).

Except for Młynary I and Młynary II, provenances contributed positively to the between-provenances allelic diversity component (Fig. 2). On the other hand, unlike the others, Młynary I contributed to the within-provenances diversity component. Sieniawa had the greatest overall contribution to the allelic diversity, while Młynary II had the smallest. A_{ST} equaled 0.137, reflecting the contribution of many unique alleles at low frequencies by different provenances (Caballero & Rodriguez-Ramilo, 2010).

Table 3. Genetic structure parameters computed for the mixture of provenances (A – number of alleles, AR – allelic richness, H_o – observed heterozygosity, H_e – expected heterozygosity, F_{IS} – Wright's fixation index)

Locus	A	AR	H_o	H_e	F_{IS}
ZAG11	29	26.3	0.873	0.926	0.057*
ZAG25	48	43.8	0.867	0.942	0.080*
ZAG39	39	34.0	0.917	0.904	–0.014
ZAG65	35	33.2	0.917	0.953	0.038*
ZAG101	20	17.0	0.854	0.860	0.007
ZAG112	29	25.5	0.918	0.904	–0.016
ZAG15	43	31.8	0.868	0.882	0.016
ZAG90	35	33.9	0.827	0.946	0.126*
ZAG102	16	12.2	0.679	0.697	0.026
ZAG103	20	14.4	0.976	0.671	–0.455*
ZAG4	26	17.4	0.594	0.689	0.138*
ZAG110	26	21.0	0.652	0.669	0.026
ZAG20	15	14.1	0.789	0.837	0.057*
ZAG7	21	19.5	0.917	0.915	–0.002
ZAG9	17	16.9	0.904	0.911	0.007
MSQ4	20	15.3	0.831	0.838	0.008
Mean	27.4	23.5	0.836	0.847	0.006
SE	2.52	2.341	0.108	0.104	0.131

* significantly deviates from zero (after Bonferroni correction).

Table 4. Summary of genetic structure statistics per provenance (for each parameter only a mean across loci is shown; standard errors are given in parentheses)

Provenance	A	AR	H_o	H_e	F_{IS}
Krotoszyn	22.0 (2.343)	21.9 (2.328)	0.838 (0.024)	0.856 (0.031)	–0.023 (0.033)
Milicz	21.7 (2.209)	21.2 (2.187)	0.822 (0.029)	0.831 (0.033)	–0.015 (0.035)
Młynary I	24.3 (2.471)	22.2 (2.343)	0.838 (0.028)	0.839 (0.030)	–0.006 (0.033)
Młynary II	21.6 (2.125)	21.6 (2.119)	0.841 (0.024)	0.860 (0.023)	–0.031 (0.034)
Opole	21.4 (2.173)	21.3 (2.167)	0.846 (0.023)	0.825 (0.027)	0.018 (0.038)
Sieniawa	22.3 (2.294)	22.1 (2.277)	0.828 (0.028)	0.805 (0.030)	0.021 (0.036)
Mean	22.2	21.7	0.836	0.836	–0.006

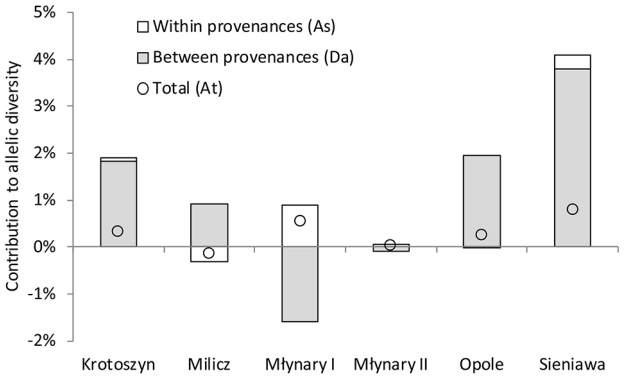

Fig. 2. The contribution of provenances to different components (As, Da) of the total allelic diversity (At). Note that the contribution is quantified as the loss (positive values) or gain (negative values) of allelic diversity after removal of a given provenance

Table 5. Results of AMOVA together with the *F*-statistics

AMOVA			
Source of variation	SS	Variance components	Percentage variation
Among populations	340.9	0.106	1.556
Among individuals within populations	12008.2	–0.005	–0.073
Within individuals	12069.0	6.691	98.517
F-statistics			
Parameter	Estimate	95% Confidence interval	
F_{IS}	–0.001	–0.046, 0.056	
F_{ST}	0.016	0.014, 0.017	
F_{IT}	0.015	–0.061, 0.041	

The AMOVA revealed that the majority of genetic variation (98.5%) is captured within provenances (Table 5). Nonetheless, F_{ST} index (0.016) was significantly different from zero. On the other hand, F_{IS} and F_{IT} were insignificant.

The effective number of breeders (N_b) computed per provenance ranged between 32.9 and 78.3, with the harmonic mean of 37.2 (Fig. 3). N_b computed for the mixture of provenances equaled 247.6. The effective population size was highly correlated with N_b. Nevertheless, all N_e values were significantly larger as compared with N_b. When compared to the harmonic mean N_e, only values for Krotoszyn and Młynary I provenances differed significantly from the mean. It is worth noting that the sum of N_e for separate provenances (332.3) was significantly larger than N_e estimated for the mixture of provenances (271.6).

Generally, the analysis showed that the plantation was characterized by nearly random spatial genetic structure (Fig. 4). We observed only a slight excess of genetic correlation between trees separated by distance of 57.7–67.5 meters. This might be due to the presence of trees from the same maternal family.

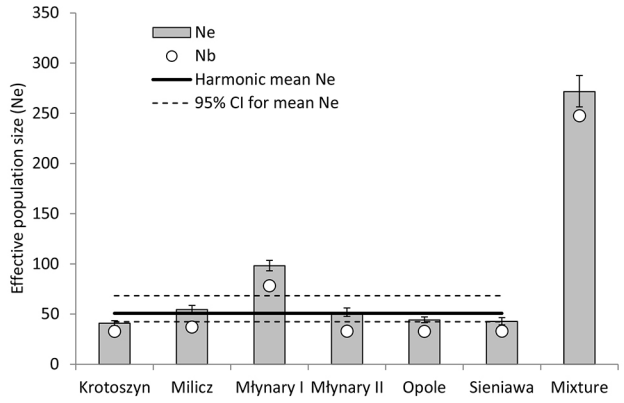

Fig. 3. Effective number of breeders (N_b) and effective population size (N_e) estimated for each provenance and the mixture of provenances. N_b was computed based on the distribution of families. N_e was estimated using genetic data (linkage disequilibrium)

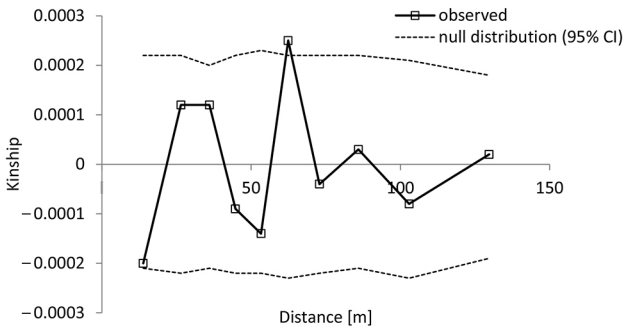

Fig. 4. Observed spatial genetic structure estimated based on genetic data together with 95% envelope for the null distribution computed based on permutations

Discussion

Analyses of neutral genetic diversity of common-garden trials provide important information on the distribution of genetic diversity within and among provenances or families (Zelener et al., 2005; Zhang et al., 2016), facilitate the interpretation of quantitative trait analyses (João Gaspar et al., 2009; Gauzere et al., 2013) and may be useful for pedigree monitoring in tree improvement programs (Bell et al., 2004).

Oak populations usually exhibit high levels of genetic diversity as assessed based on nuclear microsatellites (Streiff et al., 1998; Degen et al., 1999; Mariette et al., 2002; Cottrell et al., 2003; Curtu et al., 2007; Dering & Chybicki, 2012). However, natural regeneration of oak stands or establishing plantations based on seeds originating from a single stand do not guarantee the increase or even the maintenance of genetic diversity levels in offspring generations (Dering & Chybicki, 2012). Therefore mixing seeds from different provenances or promoting matings between different provenances might be an interesting alternative to increase genetic diversity of seeds used for reforestation (Breed et al., 2012). The studied common garden trial considered as a set of 1812 individuals appeared to reveal high genetic diversity. Additionally, because at the level of provenances we found no signatures of inbreeding, the slight excess of homozygotes observed for the mixture is attributable solely to the Wahlund effect.

We identified large numbers of alleles within loci, but this should not be surprising as our sample size was large. Four out of six provenances contributed positively to the total allelic diversity of the mixture. Given that adaptive genetic variation is characterized by higher differentiation between populations (e.g. Dillon et al., 2014), we believe that the contribution of different provenances to allelic richness of the synthetic population can be even more pronounced at the level of functional genes.

The level of genetic differentiation among oak populations is usually low (Gömöry et al., 2001; Mariette et al., 2002; Muir et al., 2004; Alberto et al., 2010). The provenances used for establishing of the studied common-garden trial originated from different parts of Poland. The two Młynary provenances originate from the northern parts of the country, Sieniawa provenance is located 480 km due south-east. On the other hand, the three remaining provenances (Krotoszyn, Milicz, Opole) originate from south-western-central parts of Poland (at the approximate distance of 300–400 km from Młynary and 350–420 km from Sieniawa). Despite dispersed origin within the country (still low as compared to the large species distribution range), the degree of genetic differentiation among provenances was low, although statistically significant. Low neutral genetic differentiation among provenances suggests that outbreeding depression in offspring generations is rather unlikely. However, direct observations are needed once seeds become available.

Although most of population genetic parameters indicated high similarity among provenances, the number of alleles (Table 4) and the effective population size (Fig. 3) were distinctly larger for Młynary I provenance. However, this discrepancy resulted from the disproportionally large representation of this provenance in the total sample (twice as many families and individuals as in other provenances). Note that the estimates of effective population size within provenance were highly correlated with the number of breeders (Fig. 3).

All of these population genetic parameters indicate that the studied common-garden trial has the potential to provide seed crops of high genetic diversity, if used in the future as a seed source. On the other hand, high genetic diversity and fairly uniform effective population sizes across provenances, along with the lack of genetic differentiation (Table 4), indicate, that all provenances are comparable in terms of neutral diversity and potential differences among provenances in quantitative traits should not be attributed to their variable demographic backgrounds (João Gaspar et al., 2009; Gauzere et al., 2013).

Because the studied plantation is composed of half-sib families originating from several provenances, one concern is the bi-parental inbreeding of the next generation offspring of the plantation arising from matings among individuals belonging to the same family (Muir et al., 2007; João Gaspar et al., 2009). However, because the number of half-sib families in this experiment is relatively large (60), and the distribution of individuals from the same family within the experiment is random, the probability of inbreeding should be insignificant. As expected, we found no signs of clustering of related genotypes within the plantation (Fig. 4).

Further studies are needed to investigate if individuals originating from different populations are synchronous in flowering. Although the plantation is still in the juvenile stage, the assessment of budburst phenology could be helpful in determining the potential for synchronous flowering (Franjić et al., 2011).

The studied common-garden experiment, although composed of individuals originating from only six provenances, represents high genetic diversity as assessed with the aid of nuclear microsatellite markers. We found indications of low genetic differentiation among provenances. However, the concept of converting older common-garden experiments into experimental seed sources needs further attention. If seeds become available, testing the performance of progenies from such plantations should provide the ultimate answer on their utility in reforestation efforts.

Acknowledgements

The study was supported by the Ministry of Science and Higher Education of the Republic of Poland (Grant No: N N309 320136). The authors are thankful to Ewa Sztupecka and Katarzyna Meyza for laboratory assistance, and Elżbieta Sandurska, Jan Kowalczyk, Piotr Markiewicz and the personnel of State Forests of Poland for their help in collecting oak samples in the field. The authors wish to thank Magdalena Lewandowska for proof-reading and English correction of the manuscript.

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